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Lipid Transfer between Charged Supported Lipid Bilayers and Oppositely Charged Vesicles

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The bidirectional transfer of phospholipids between a charged, supported lipid bilayer (SLB) on SiO_2 and oppositely charged, unilamellar vesicles was studied by means of quartz crystal microbalance with dissipation (QCM-D) and optical reflectometry techniques. SLBs and vesicles were prepared from binary mixtures of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) mixed with different fractions of either 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine] (POPS) (negatively charged) or 1-palmitoyl-2-oleoyl-*sn*-glycero-3-ethylphosphocholine (POEPC) (positively charged). The interaction process consists of an attachment−transfer−detachment (ATD) sequence, where added vesicles first attach to and interact with the SLB, after which they detach, leaving behind a compositionally modified SLB and ditto vesicles. When the process is complete, there is no net addition or reduction of total lipid mass in the SLB, but lipid exchange has occurred. The time scale of the process varies from a few to many tens of minutes depending on the type of charged lipid molecule and the relative concentration of charged lipids in the two membranes. Electrostatically symmetric cases, where only the charge sign (but not the fraction of charged lipid) was reversed between the SLB and the vesicles, produce qualitatively similar but quantitatively different kinetics. The time scale of the interaction varies significantly between the two cases, which is attributed to a combination of the differences in the molecular structure of the lipid headgroup for the positively and the negatively charged lipids used, and to nonsymmetric distribution of charged lipids in the lipid membranes. The maximum amounts of attached vesicles during the ATD process were estimated to be 25−40% of a full monolayer of vesicles, with the precise amount depending on the actual charge fractions in the vesicles and the SLB. Interrupted vesicle exposure experiments, and experiments where the bulk concentration of vesicles was varied, show that vesicles in some cases may be trapped irreversibly on the SLB, when only partial transfer of lipid molecules has occurred. Additional supply of vesicles and further transfer induces detachment, when a sufficient amount of oppositely charged lipids has been transferred to the SLB, so that the latter becomes repulsive to the attached vesicles. Possible mechanistic scenarios, including monomer insertion and hemifusion models, are discussed. The observed phenomena and the actual SLB preparation process form a platform both for studies of various intermembrane molecular transfer processes and for modifying the composition of SLBs in a controlled way, for example, for biosensor and cell culture applications.

Introduction

The transfer/exchange of molecular components between biomembranes plays a pivotal role in biological systems. For example, it is central in energy supply to and communication between cells and for the function of a large fraction of drugs. Such processes are studied at many different complexity levels ranging from whole organisms to cell cultures to simpler model systems. Among the latter, lipid vesicles (liposomes) and supported lipid bilayers (SLBs) constitute attractive model systems to explore various aspects of the membrane processes, including membrane−membrane interactions and the transfer of molecules between them.[1] These model systems allow various systematic variations of, for instance, the lipid composition and the fraction of charged versus noncharged lipid molecules in both the SLB and vesicles. Through such studies, the role of electrostatic and other nonspecific and specific interactions in biological membrane processes can be elucidated. The possibility to induce (uni- or bidirectional) lipid transfer[1] between vesicles and preformed SLBs also open up new ways of modifying the lipid content of supported membranes, which is of interest both for biosensing[2,3] and cell studies[4,5] involving SLBs.

Compared to bulk phase measurements, usually involving vesicle−vesicle interactions, the use of vesicles interacting with SLBs offer several advantages. First, the use of surface sensitive techniques can be employed, and the interaction and observation area is stationary and confined to the SLB. Real time measurements of vesicle interaction with SLBs, with appropriate time resolution (of the order of 1 s or less), can be performed using techniques based on, for example, quartz crystal microbalance with dissipation (QCM-D), reflectometry, ellipsometry, surface plasmon resonance (SPR), fluorescence, or IR spectroscopy techniques. All these, except fluorescence-based techniques, are label-free techniques. Furthermore the lipid transfer between SLBs and vesicles offer more variability and flexibility compared to bulk phase studies, for example, with regard to interruption (transient) experiments (flushing out the vesicles while keeping the SLB under continuous observation, or pulsed experiments) or sequential experiments with a single SLB using different vesicles of different composition, in sequence.

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The focus of the present work is membrane−membrane interaction and specifically bidirectional transfer of lipid material between two membranes in contact, where one membrane is surface-supported, that is, an SLB,[6−8] and the other one is in the form of unilamellar vesicles in the bulk phase. Following up recent studies of our group,[9,10] we are particularly interested in how such transfer is influenced by the presence of varying amounts of charged lipids in the two interacting membranes. The process of SLB formation on solid materials, which is the first step in the type of experiments described below, has been studied in our and many other groups in much detail.[6,11−14] By different combinations of solid supports (e.g., silica,[11,15−17] mica,[15,17] or titania[11,16,18]) and vesicle lipid composition, a range of different SLBs are possible. However, not all lipid compositions in the SLBs can be obtained. Transfer of lipid molecules between vesicles in the bulk phase and preformed SLBs will offer a possibility to extend the range and variability in SLB composition by modification of the preformed SLBs *in situ*. Similar approaches also provide ways to incorporate other membrane components in the preformed SLB, and they might in principle also be extended to cells.

Prior basic work on lipid transfer has primarily been performed by observing transfer between vesicles in the bulk phase. Using fluorescence microscopy, NMR, light scattering, free-flow electrophoresis, and electron microscopy, lipid transfer has been observed between oppositely charged vesicles.[19−22] It has been shown that the rate of lipid transfer between oppositely charged vesicles depends on the fraction of charged lipids[22] and on the phospholipid polar headgroup (in particular, the hydration of the headgroup is believed to be important).[19] Furthermore, it has been demonstrated that the transfer of lipid molecules is mediated or controlled by direct contact of the oppositely charged vesicles, and not by lipid transfer via diffusion of molecules in the bulk solution.[23,24] When studying lipid transfer between lipoproteins, a dependency of the transfer rate on the type of lipid headgroup and hydrocarbon chain was identified.[25,26] It was found that lipid transfer is enhanced by shorter carbon chains and higher number of double bonds. An example of the influence of the properties of the headgroup is that the transfer of negatively charged phosphatidyl serine lipids was found to be faster than the transfer of the neutral (zwitterionic) phosphatidyl choline lipids. It has also been shown that anionic lipids mixed into POPC vesicles destabilize them toward rupture, which might indicate that they are also more apt to transfer of lipids.[27]

In summary, previous work in the bulk phase on lipid exchange/transfer between vesicles and lipoproteins indicates influences on the process by both the molecular structure of the lipid molecules and by the electrostatic interaction between charged vesicles.

The first study, to the best of our knowledge, of lipid transfer between SLBs and vesicles was reported by Reinl and Bayerl.[28] Using IR spectroscopy, NMR, and differential scanning calorimetry, they demonstrated lipid transfer between neutral SLBs and charged unilamellar vesicles. The main result was that lipids are transferred from vesicles to an SLB and that charged lipids are transferred about 1 order of magnitude faster than neutral lipids. Furthermore, they concluded that the two types of studied charged lipids were transferred to both leaflets of the neutral SLB and distributed in a symmetrical fashion. Additional work on the transfer between SLBs and vesicles was performed by Sapuri et al.,[29] who reported lipid transfer between charged SLBs and oppositely charged membrane-coated microbeads (diameter ca. 5 μm), or vesicles, employing fluorescence microscopy. They showed that electrostatically mediated lipid transfer can be used to selectively transfer lipid molecules to an array of supported membrane corrals with different net charges. In recent studies in our group, electrostatically driven lipid transfer between charged SLBs and oppositely charged vesicles was observed, employing the QCM-D method, optical reflectometry, fluorescence measurements, and time-of-flight secondary ion mass spectroscopy (TOF-SIMS).[9,10,30] It was observed that the charged vesicles in the beginning of the exposure attach to the oppositely charged bilayer, due to electrostatic interaction. While attached to the SLB, the vesicles exchange lipid material with the SLB (via bidirectional lipid transfer), as evidenced both by transfer of fluorescently labeled lipids and by sequential experiments with differently charged vesicles. The conclusion was that there is a transfer of lipids between the vesicles and the SLB, which counterbalances the original charge disequilibrium between the vesicles and the bilayer, until the electrostatic attraction is reduced enough, or reversed, so that the vesicles detach from the surface. In the following, we refer to this process as an attachment−transfer−detachment (ATD) process. After completion of the ATD process, new vesicles no longer attach to the SLB. It is worth noting that after the ATD process has completed, the composition of the SLB (as well as of detaching vesicles) has changed from originally binary lipid mixtures (neutral and positive or neutral and negative) to ternary mixtures containing both neutral, positive and negative lipids.

The purpose of the present work was to study the interaction between vesicles and SLBs of opposite charge in much

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more detail, and with much larger lipid composition variations compared to our first studies,[9,10,30] in order to scrutinize the kinetics and shine light on the mechanism(s) of lipid transfer. The combination of reflectometry with QCM-D makes it possible to discriminate between lipid mass alone and lipid plus coupled water mass,[31] which in turn made quantification possible of the amount of adsorbed vesicles on the SLB during the ATD process.

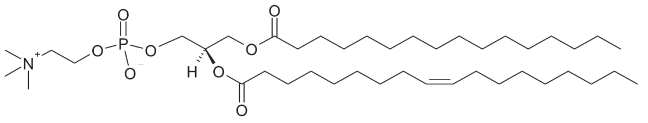
Materials and Methods

Chemicals and Vesicle Prepartion. Unless otherwise stated, chemicals were obtained from commercial sources and used without further purification. Water was deionized (resistivity > 18 MΩ/cm) and purified using a MilliQ unit (MilliQ plus, Millipore, France). Buffers were filtered and degassed before use. The molecular structure of the lipids used are shown in Figure 1. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine] (POPS), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-ethylphosphocholine (POEPC) (Avanti Polar Lipids Inc., AL) were dissolved in chloroform to prepare films of the desired composition. Unilamellar vesicles were prepared based on the protocol by Hope et al.[32] In short, the lipids were placed at the desired ratio in a round-bottom flask and dried, first under a gentle stream of nitrogen, to form a thin lipid film, and second under vacuum for 3 h. The lipid films were hydrated in TRIS-buffer (10 mM TRIS, 100 mM NaCl, pH 8). After vortexing, the solutions were extruded first 11 times through a 100 nm polycarbonate membrane and then another 11 times through a 30 nm membrane. The resulting vesicle solutions were refrigerated under N_2.

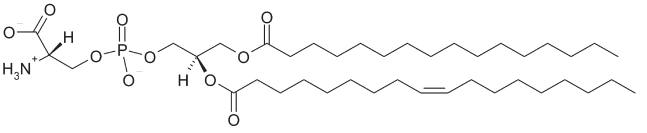
Dynamic Light Scattering: Characterization of Vesicle Size and Zeta Potential. Vesicle solutions were characterized using dynamic light scattering (Malvern Zetasizer Nano ZS, Malvern Instruments, U.K.) with respect to size and zeta potential. Cuvettes were rinsed with water and dried under N_2. Vesicles were diluted to a final concentration of 0.4 mg/mL. The light scattering data were evaluated by using Dispersion Technology software (Malvern Instruments, U.K.). The measurements were carried out at 22 °C. The zeta potentials of the different vesicles, extruded as described above, are listed in Table 1. While the zeta potential varies with the fraction of charged lipid used, the mean size of the different vesicles is about the same, 89 ± 4 nm. POPC vesicles are slightly negative charged, having a zeta potential of about −5.0 mV. The zeta potential of negatively charged vesicles containing 25% POPS is about −27.3 mV, while vesicles containing 50% POPS have a potential of about −38.6 mV. For the solutions containing positively charged vesicles, the zeta potential of vesicles containing 25% POEPC was +27.1 mV, and +38.1 mV for vesicles containing 50% POEPC.

QCM-D. The QCM-D measurements were performed in flow mode using a Q-Sense E4 instrument (Q-Sense AB, Sweden). AT-cut quartz crystals with a fundamental frequency of 5 MHz, coated with Au, were purchased from Q-Sense AB. On the top electrode, a 100 nm thick layer of SiO_2 was evaporated. Prior to the experiment, the crystals were cleaned in a 10 mM sodium dodecyl sulfate solution (overnight), rinsed thoroughly with water, dried under N_2, and treated in an UV-ozone chamber for 2 × 15 min with rinsing and drying in between (ozone removes organic carbon contamination). The measurements were carried out at 22 °C using a flow rate of 50 μL/min. After stabilization, buffer solution was exchanged by vesicle solution (0.1 mg/mL) and bilayer formation was monitored. For the bilayer formation, positively charged vesicles were diluted in TRIS-buffer, while negatively charged vesicles were diluted in TRIS-buffer containing 10 mM $CaCl_2$ (to promote SLB formation). After SLB formation was completed, the bilayers were rinsed with TRIS-buffer containing 10 mM EDTA, followed by rinsing for several minutes with TRIS-buffer. After its completion, the bilayer was exposed to oppositely charged vesicles (concentrations are given in the corresponding experiments). Frequency and dissipation shifts were measured at the ninth overtone and normalized to the fundamental frequency by dividing the values by 9.

1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine (POPC)



1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-[Phospho-L-Serine] (POPS)



1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Ethylphosphocholine (POEPC)

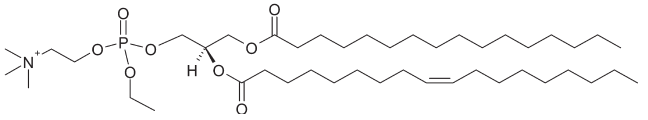


Figure 1. Structure of phospholipids used in this study.

Table 1. Zeta Potentials of Vesicles Prepared from Different Lipid Mixtures[a]

composition	zeta potential/mV
POPC	−5.0 ± 1.2
POPC/POEPC 75:25	27.1 ± 2.1
POPC/POEPC 50:50	38.1 ± 0.9
POPC/POPS 75:25	−27.3 ± 1.7
POPC/POPS 50:50	−38.6 ± 1.4

[a] The vesicle size was 89 ± 4 nm for all lipid compositions.

Combined QCM-D and Reflectometry. Combined QCM-D/reflectometry measurements were carried out with a new prototype instrument from Q-Sense AB.[31] Signals of the QCM-D and the reflectometry were taken simultaneously using the same sensor surface, an AT-cut quartz crystal coated with SiO_2. The same procedure as described above for the regular QCM-D experiments was used, with the exception that the flow rate was set to 100 μL/min (instead of 50 μL/min) to compensate for the larger flow cell volume.

Results

The interaction between SLBs and vesicles was studied with QCM-D as the main technique, which allows label-free, real time monitoring of all steps from the preparation of the initial SLB to the subsequent vesicle−SLB interaction. Complementary results were obtained with a new combined QCM-D/reflectometry setup, simultaneously measuring with reflectometry and QCM-D on the same QCM-D sensor surface.[31] With this combined technique, one obtains a direct discrimination between (dry, optical) lipid mass and wet, acoustically coupled lipid mass,[30,31] which is facilitating interpretation and quantification. All vesicles used for these experiments were obtained by extrusion, having the same size in all experiments (a diameter of 89 ± 4 nm), that is, there was no attempt to explore vesicle size effects.

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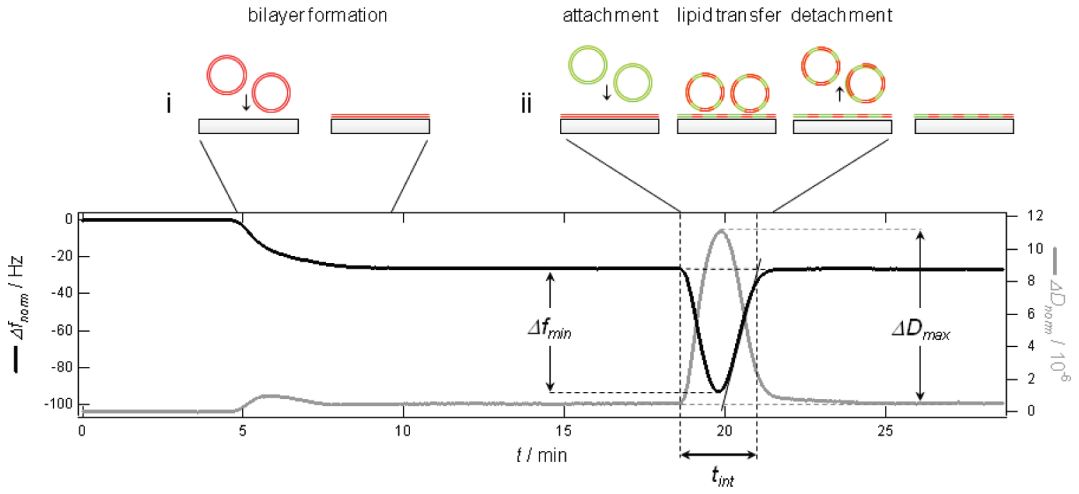

Figure 2. Typical time curves of frequency shift (black curve) and dissipation shift (gray curve) for a lipid transfer measurement with the QCM-D. Corresponding to the time curve, the process is schematically depicted including bilayer formation of a charged SLB (75% POPC and 25% POEPC) and the interaction with negatively charged vesicles (50% POPC and 50% POPS) which is divided in three subprocesses: attachment, lipid transfer, and detachment.

Scheme of the QCM-D Measurements. Figure 2 shows QCM-D versus time curves for the frequency (Δf) and dissipation shifts (ΔD) in a typical experiment. In the first step, marked by (i), a positively (red) charged SLB is formed on the negatively charged SiO_2 surface by adsorption and rupturing of positively charged vesicles. There is in this case an immediate (on the time scale of the experiment) vesicle rupture and concerted fusion of the rupture fragments to a supported bilayer.[11−13] Following the correct protocol, high quality bilayers are formed over the whole sensor surface, leading to characteristic values for the final frequency and dissipation shifts of $\Delta f \sim -25$ Hz (normalized to the fundamental frequency) and $\Delta D < 0.5 \times 10^{-6}$, respectively.[6] The frequency shift is a measure of how much mass has been accumulated on the surface, whereas the dissipation shift gives information about its rigidity (shear viscosity). In particular, the dissipation value is an indicator of the bilayer quality (should be $< 0.5 \times 10^{-6}$), because the bilayer itself causes very low dissipation, while other lipid structures, such as trapped, intact vesicles, cause higher dissipation.[6,12]

In the second step, marked by (ii), the positively charged SLB is exposed to vesicles of opposite charge, using a constant flow of vesicles through the cell. In the beginning, a decrease of the resonant frequency (increase in mass) and an increase of the dissipation are observed, which is signaling adsorption of vesicles on the SLB. The frequency and dissipation shifts eventually reach extreme values Δf_{min} and ΔD_{max}. The magnitudes of these values, especially the high dissipation, suggest that the adsorbed vesicles stay intact, rather than rupture (see further below). They also suggest accumulation of significant amounts of vesicles on the surface (but less than a full monolayer, see below). After some time, Δf and ΔD begin to return from their extreme values, until eventually the values for the initial bilayer are regained. In other words, when the experiment is completed, there is no, or very little, sign of net added mass or changed dissipation, in spite of clear evidence for substantial SLB−vesicle interaction in the preceding interaction period.

This type of behavior was first reported by Wikström et al. recently.[9] The charged vesicles attach to the bilayer of opposite charge, reside there for some time, and finally detach again. During the interaction period, lipid transfer between the vesicles and the SLB occurs, as demonstrated both previously[9,10,30] and below. The detachment (desorption) is attributed to weakening or reversal of the electrostatic interaction between the charged SLB and the oppositely charged vesicles, as a consequence of transfer of lipid material between the planar bilayer in the SLB and the bilayer in the vesicles. In addition, sequential experiments where differently charged vesicles were injected over the surfaces one after the other to probe the charge state of the SLB, and fluorescently labeled lipids have also been used to follow the transfer process. Here, we extend these studies to explore in much more detail the ATD process by addressing the question of how the kinetics of lipid transfer is influenced by the type and amount of charged lipids in the SLB and vesicles. We also vary vesicle supply rates and perform interrupted experiments. Based on these data, we also discuss in more detail possible mechanistic scenarios and rate limiting steps.

Based on our experimental findings with different combinations of charge in the SLB and the vesicles, we find it convenient to introduce an "interaction time" t_{int}. The latter is defined in Figure 2 as the time from the moment when the frequency shift begins to drop and the dissipation shift begins to increase (i.e., when vesicles begin to attach to the SLB), to the moment when both values have regained equilibrium at or close to the initial bilayer values, that is, when no further changes are seen in Δf and ΔD. In the following, we explore how t_{int} depends on the combination of charged lipids, of opposite sign, in the SLBs and vesicles. Two other observables of interest in this context are the amplitudes of Δf_{min} and ΔD_{max} for the different combinations of SLB and vesicles charge fractions; these values reflect the maximum amount of accumulated vesicles on the SLB surface. In general, one would expect very slow transfer kinetics (long t_{int}) to lead to a large mass of accumulated vesicles on the surface before the interaction is completed, and vice versa. For some situations, depending on what the rate limiting steps are, one might also expect an influence of the bulk concentration of vesicles on t_{int}, Δf_{min}, and ΔD_{max}. This is articulated at the beginning of the Discussion section.

In the following, we will first focus on how the lipid transfer kinetics is influenced by different fractions of

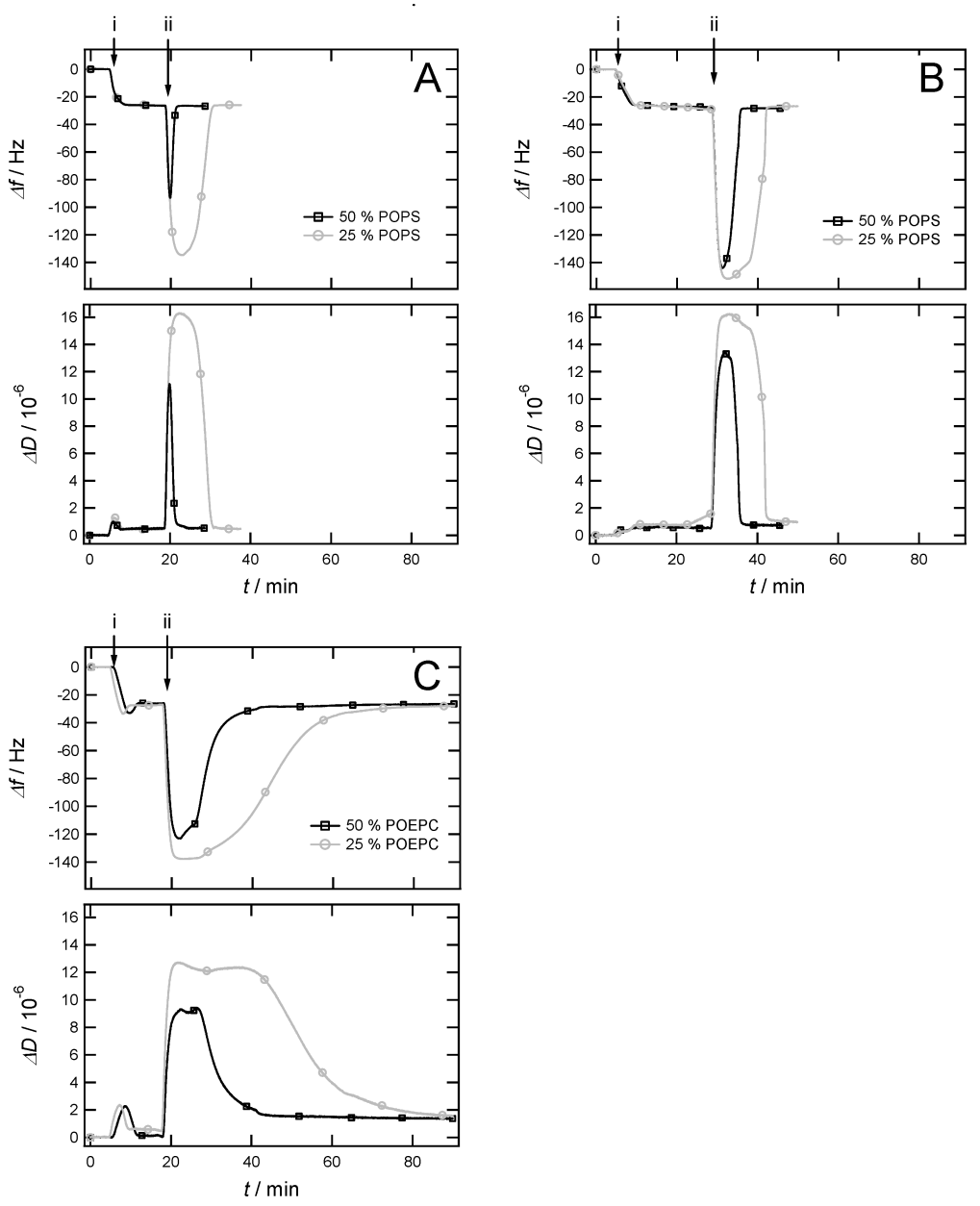

Figure 3. Time curves of the frequency shift (normalized to the fundamental frequency) and the dissipation shift obtained by QCM-D where (i) charged SLBs were formed and (ii) exposed to oppositely charged vesicles. In A and B, a SLB was formed by spontaneous rupture of positively charged vesicles containing (A) 75% POPC and 25% POEPC or (B) 50% POPC and 50% POEPC on SiO_2, followed by exposure to oppositely charged vesicles: (black, squares) POPC/POPS 50:50 and (gray, circles) POPC/POPS 75:25. The SLB in (C) was formed on SiO_2 from negatively charged vesicles (POPC/POPS 75:25) in Ca^{2+}-containing buffer (10 mM TRIS, 100 mM NaCl, 10 mM $CaCl_2$, pH 8). After rinsing with Ca^{2+}-free buffer, positively charged vesicles were injected: (black, squares) POPC/POEPC 50:50 and (gray, circles) POPC/POEPC 75:25. Lipid concentration: (i) 0.1 mg/mL and (ii) 0.4 mg/mL. Buffer: 10 mM TRIS, 100 mM NaCl, pH 8.

charged lipid in the SLB and in the vesicles (Figure 3). In these experiments, the vesicle supply was interrupted a little while after Δf_{min} and ΔD_{max} had been passed, since it made no difference if the exposure was then continued or not (see Figure 6 and the discussion in connection with this figure). For selected parts of the data, we then present combined QCM-D and reflectometry measurements (Figure 4), in order to quantify the amounts of "wet" and "dry" lipid mass involved in the studied process, and the vesicle coverage during the ATD process. Finally, we present measurements where the bulk concentration of lipids was varied (Figure 5), and furthermore "interruption experiments" (Figure 6) where the vesicle exposure was interrupted at different times before the lipid transfer process had gone to completion. The latter two experiments elucidate the origin(s) of t_{int} and some other mechanistic aspects.

Influence of the Fraction of Charged Lipids, in the SLB and Vesicles, on the Lipid Transfer Kinetics. If the lipid transfer observed between a charged SLB and oppositely charged vesicles is governed by electrostatic interaction,[28,29] it should be reflected in how the interaction kinetics depends on the vesicle and SLB charge. In order to address this question, experiments were performed where the fractions of charged lipid (negatively charged POPS or positively charged

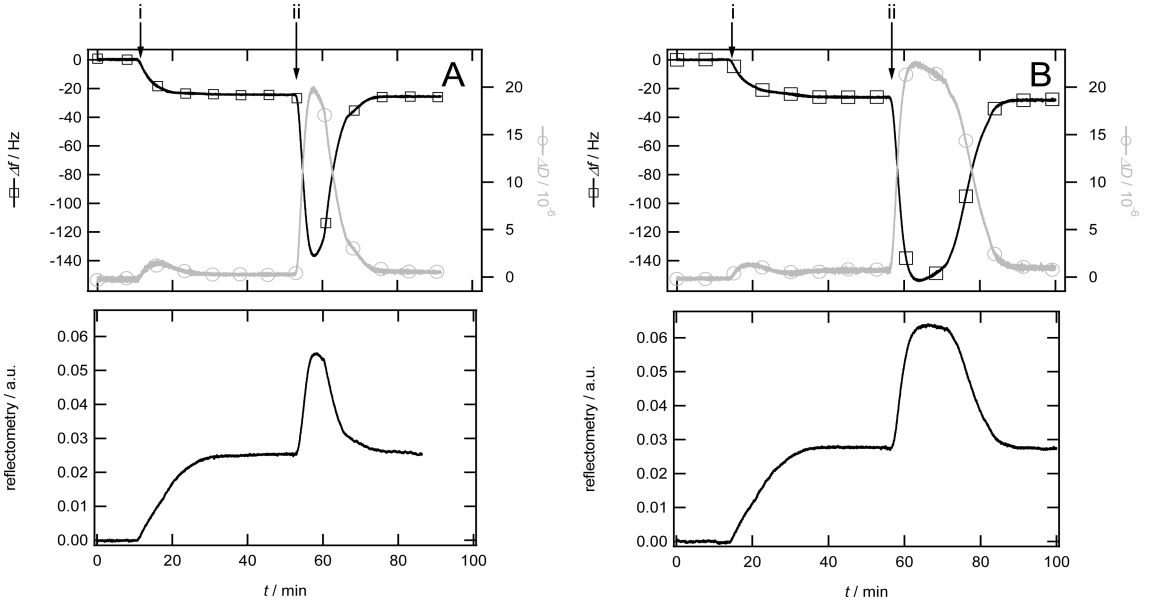

Figure 4. Time curves of the frequency shift (normalized to the fundamental frequency) and the dissipation shift (top), and reflectometry signal (bottom) obtained by combined QCM-D/reflectometry. First, (i) a positively charged SLB (POPC/POEPC 75:25) is formed and then (ii) exposed to negatively charged vesicles (A) POPC/POPS 50:50 and (B) POPC/POPS 75:25. Lipid concentration: (i) 0.1 mg/mL and (ii) 0.4 mg/mL. Buffer: 10 mM TRIS, 100 mM NaCl, pH 8.

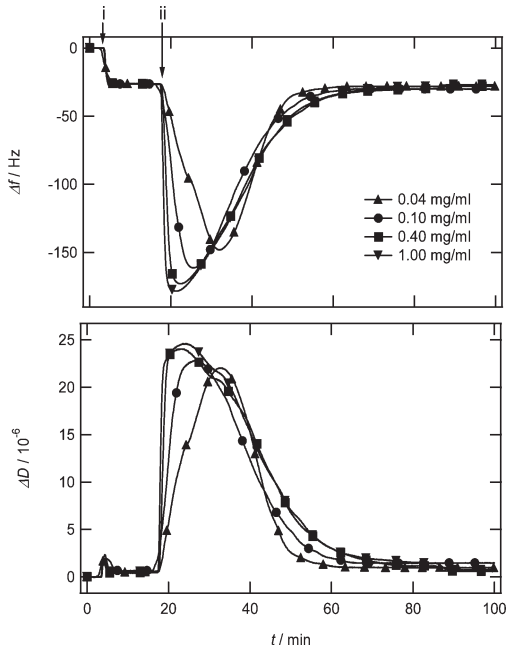

Figure 5. Time curves of the frequency shift (normalized to the fundamental frequency) and the dissipation shift obtained by QCM-D where (i) positively charged SLBs (POPC/POEPC 75:25, concentration 0.1 mg/mL) were formed on SiO_2 and (ii) exposed to negatively charged vesicles (POPC/POPS 50:50) of different concentrations. Buffer: 10 mM TRIS, 100 mM NaCl, pH 8.

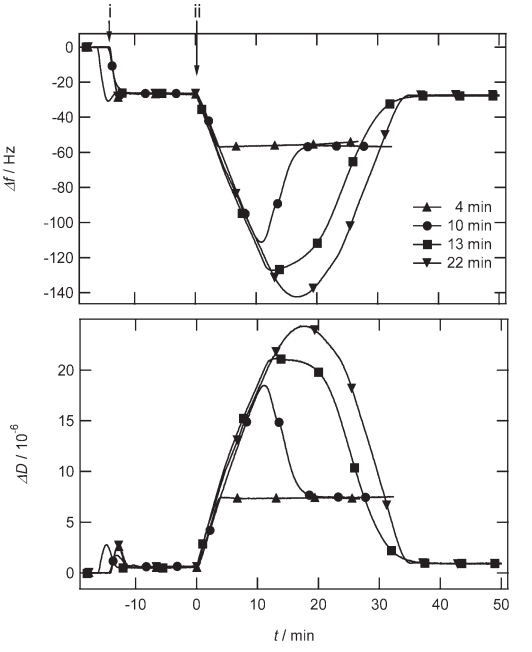

Figure 6. QCM-D data for interruption experiments. (i) Positively charged SLBs (POPC/POEPC 75:25) were formed on SiO_2 and (ii) exposed to negatively charged vesicles (POPC/POPS 50:50). The vesicle supply was interrupted at different times. Lipid concentration: (i) 0.1 mg/mL and (ii) 0.04 mg/mL. Buffer: 10 mM TRIS, 100 mM NaCl, pH 8. The frequency shift is normalized to the fundamental frequency.

POEPC, in binary mixtures with the zwitterionic POPC) in the vesicles and in the SLB were varied systematically. Figure 3 and Table 2 show the frequency and dissipation shifts for the different lipid compositions used. SLBs containing different fractions of charged lipids (three different cases, A−C) were formed on SiO_2 and then exposed to vesicles with different fractions of opposite charge.

A. Positively Charged SLB (25% POEPC) and Negatively Charged Vesicles. In Figure 3A, a positively charged SLB, composed of 75% POPC and 25% positively charged POEPC, was exposed to vesicles composed of POPC and the negatively charged lipid POPS at two different fractions, namely, 50% (black curve, squares) and 25% POPS (gray curve, circles). The same qualitative kinetics behavior is observed for the two cases: an initial regime dominated by adsorption of vesicles on the SLB (decreasing frequency shift and increasing dissipation shift) followed by a regime dominated by detachment (increasing frequency shift and decreasing dissipation shift). However, quantitatively the kinetics for the different vesicle compositions have quite different

Table 2. Mean Values and the Corresponding Standard Deviations for the Set of Experiments Shown in Figure 3

	SLB	vesicle	t_{int}/min	Δf_{min}/Hz	$\Delta D_{max}/10^{-6}$
A	POPC/POEPC 75:25	POPC/POPS 50:50	3 ± 1	80 ± 8	12 ± 1
		POPC/POPS 75:25	13 ± 3	108 ± 5	13 ± 2
B	POPC/POEPC 50:50	POPC/POPS 50:50	8 ± 2	113 ± 10	13 ± 1
		POPC/POPS 75:25	13 ± 3	123 ± 5	15 ± 1
C	POPC/POPS 75:25	POPC/POEPC 50:50	15 ± 2	98 ± 5	11 ± 1

time scales; with more charge (50% versus 25% POPS) in the vesicles, the kinetics is faster. There are also differences in the Δf_{min} and ΔD_{max} values; the amplitudes are smaller for the faster kinetics (i.e., for larger vesicle charge). For the interaction between the positively charged SLB and the negatively charged vesicles containing 50% POPS (black curve, squares), the entire process of attachment, lipid transfer, and detachment takes about $t_{int} \sim 3$ min and the extreme values for the resonant frequency and dissipation shifts are $\Delta f_{min} \sim -80$ Hz and $\Delta D_{max} \sim 12 \times 10^{-6}$, respectively. A smaller fraction of charged lipids in the vesicles prolongs the interaction time and leads to increased Δf_{min} and ΔD_{max} values. By halving the fraction of negatively charged POPS in the vesicles to 25%, the interaction time increases by almost a factor of 4 to $t_{int} \sim 12$ min (gray curve, circles), and the maximum frequency and dissipation shifts increase by almost a fourth to $\Delta f_{min} \sim -108$ Hz and $\Delta D_{max} \sim 13 \times 10^{-6}$, respectively. These values indicate, at this stage, that the interaction time becomes shorter when the charged lipid content is larger in the vesicles, and that as a consequence of the faster kinetics fewer vesicles accumulate on the surface before they start to detach.

B. Positively Charged SLB (50% POEPC) and Negatively Charged Vesicles. In the next experiment, the fraction of positively charged POEPC lipid in the SLB was increased (doubled) to 50% and experiments with the same fractions of charged lipids in the vesicles as above were carried out (Figure 3B). The same qualitative trend as in Figure 3A is observed; that is, with an increasing fraction of negatively charged POPS in the vesicles, the interaction time t_{int} and the magnitudes of the extreme values Δf_{min} and ΔD_{max} decrease. However, in contrast to when the charge fraction was increased in the vesicles, an increase of the charge fraction in the SLB leads to a longer interaction time. Quantitatively, when vesicles with 50% POPS were allowed to interact with a 50% positively charged SLB (i.e., with twice as much POEPC as above, Figure 3A), we found (Figure 3B) a *longer* interaction time and *larger* extreme frequency and dissipation shifts (the values for 25% POEPC in the SLB (Figure 3A) are given in brackets); $t_{int} \sim 8$ min [3 min], $\Delta f_{min} \sim -113$ Hz [80 Hz], and $\Delta D_{max} \sim 13 \times 10^{-6}$ [12×10^{-6}] (black curve, squares) (see also Table 2). Halving, for the same SLB composition, the fraction of POPS in the vesicles to 25% leads to an increase of the interaction time to $t_{int} \sim 13$ min [12 min], and the extreme values increase as well to $\Delta f_{min} \sim -123$ Hz [-108 Hz] and $\Delta D_{max} \sim 15 \times 10^{-6}$ [13×10^{-6}] (gray curve, circles).

In summary, the trends we observe are as follows: (i) the higher the fraction of charged lipids is in the vesicles, the faster is the kinetics and the smaller are the extreme values of the frequency and dissipation shifts, and (ii) the higher the charged fraction in the SLB, the longer is the interaction time (slower kinetics) and the larger are the extreme values of the frequency and the dissipation shifts.

C. Negatively Charged SLB (25% POPS) and Positively Charged Vesicles. The experiments were then "mirrored"; that is, instead of exposing a positively charged SLB to negatively charged vesicles, we investigated the interaction between negatively charged bilayers and positively charged vesicles (Figure 3C). In terms of charge fractions, this is a real "mirror", or symmetric, case. However, one should keep in mind that the local atomic/molecular arrangements and compositions in the respective headgroups are quite different and are also likely to affect the kinetics, and therefore, complete symmetry in the kinetics of the transfer of a charged lipid molecule from the SLB to the vesicles or vice versa might not be expected. Another potential difference making the nominally symmetric cases asymmetric is that the distribution of charged lipids between the two leaflets of the SLB might be asymmetric due to the surface interaction, or that the distribution between the two leaflets in the vesicles might be asymmetric due to polarization effects.[33]

Negatively charged bilayers were formed by adsorption/fusion of vesicles containing 25% POPS, using a $CaCl_2$ buffer.[34] In order to exclude an effect of different cations on the SLB−vesicle interaction, the bilayers were then rinsed with Ca^{2+}-free buffer after preparation, before oppositely charged vesicles were injected (the residence times of ions at the headgroups are expected to be orders of magnitude shorter than the experimental times, so the rinsing should remove Ca^{2+} ions effectively[35]).

The interaction times for the "mirrored" case were, for similar charge fraction combinations, much longer compared to the interaction between a positively charged bilayer and negatively charged vesicles. For example, for the interaction between a 25% negatively charged SLB and vesicles containing 50% POEPC (black curve, squares; compare Figure 3C with A), the interaction time was $t_{int} \sim 15$ min (the extreme values of the frequency and dissipation shifts were $\Delta f_{min} \sim -98$ Hz and $\Delta D_{max} \sim 11 \times 10^{-6}$). For the mirror case (see above), with the same fraction of positive charge in the SLB and the same negative fraction in the vesicle, the corresponding numbers were $t_{int} \sim 3$ min (about 5 times shorter) (the frequency and dissipation shifts were similar $\Delta f_{min} \sim -80$ Hz, $\Delta D_{max} \sim 12 \times 10^{-6}$). Similarly to the case with the positive SLB, we find also here that a decreasing fraction of the charged POEPC lipid in the vesicles leads to an increase of the interaction time and also that the extreme values of the frequency and dissipation shifts then increase. Thus, for vesicles containing 25% POEPC, the interaction time was $t_{int} \sim 40$ min and the extreme values were $\Delta f_{min} \sim -121$ Hz and $\Delta D_{max} \sim 12 \times 10^{-6}$ (gray curve, circles). The

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(35) Sachs, J.; Nanda, H.; Petrache, H.; Woolf, T. B. *Biophys. J.* **2004**, *86*, 3772–3782.

corresponding mirror case values were $t_{int} \sim 12$ min (3 times shorter), $\Delta f_{min} \sim -108$ Hz, and $\Delta D_{max} \sim 13 \times 10^{-6}$.

Combined QCM-D and Reflectometry Measurements. In QCM-D measurements, the measured mass uptake includes both the biomolecular mass and water coupled to it.[6,11,14] For SLBs, the dissipation values are very small, close to that of the naked sensor surface, and the amount of trapped water is also small.[14,30] However, for adsorbed vesicles, the amount of trapped water inside the vesicles and between them is large and the dissipation is high.[14,30] That means that the measured Δf values are composed of the sum of the lipid mass and the solvent associated with it. Therefore, in order to quantify, or at least estimate, the amount of lipid mass and trapped water mass during the course of the ATD process on the SLB, a separate experimental method was needed. Additional experiments were therefore carried out using a new experimental setup combining QCM-D and optical reflectometry.[31] This setup allows simultaneous detection of the QCM-D and reflectometry signals on one and the same sensor surface and gives complementary information about the uptake of biomolecular mass including coupled water (from QCM-D, "wet mass") and the uptake of biomolecular mass excluding coupled water (from reflectometry, "dry mass").[30,31]

In Figure 4, the results of such combined QCM-D and reflectometry measurements are shown. Figure 4A depicts the interaction between a positively charged SLB (containing 25% POEPC) and negatively charged vesicles containing 50% POPS, while Figure 4B shows the interaction of the same bilayer with vesicles (composed of POPC and POPS) containing 25% POPS. The QCM-D curves reproduce the observations described above in Figure 3A. In the first step (i), a positively charged bilayer is formed, represented by the characteristic behavior of the frequency and dissipation shifts. During the bilayer formation, the reflectometry signal increases monotonically and eventually stabilizes at ~0.028. This is a very similar behavior as in previous studies by combined QCM-D and reflectometry[9,30] as well as by combined QCM-D and SPR measurements (performed in a common flow cell, but on different surfaces).[36] In the second step (ii), the positively charged SLB is exposed to oppositely charged vesicles. The frequency and dissipation shifts show the same behavior as described above (Figures 2 and 3), namely, an initial decrease of the frequency (i.e., mass increase) and increase of the dissipation (due to adsorbed vesicles), followed by an increase of the frequency (i.e., mass loss) and a decrease of the dissipation until both values return to and stabilize at the values they had before the SLB was exposed to oppositely charged vesicles. The reflectometry signal qualitatively follows the QCM-D signals; that is, it initially increases and then decreases again. This behavior shows that the "wet" and "dry" masses correlate well in this case (in contrast to the SLB formation kinetics discussed in detail elsewhere).[14,30] In the case shown in Figure 4A, the reflectometry signal reaches a maximum at ~0.055, while in the case shown in Figure 4B it reaches a maximum at ~0.064 during lipid transfer. Having access to both the "wet" and "dry" masses from this experiment allows us to estimate the amount of vesicles attached to the SLB during the course of the lipid transfer occurring during the ATD process (see Discussion).

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Influence of Vesicle Supply (Bulk Concentration). The supply rate (bulk concentration) of oppositely charged vesicles to a preformed charged SLB was varied in order to probe its influence on the lipid transfer process. The basic idea was to acquire some information about the transport component of the interaction time t_{int}. The supply rate was modified by varying the concentration of vesicles in bulk or by interrupting the vesicle supply at different times in the course of the transfer kinetics.

A. Influence of Vesicle Bulk Concentration on the ATD Process. Figure 5 depicts how a positively charged SLB, composed of 75% POPC and 25% positively charged POEPC, was exposed to negatively charged vesicles, composed of 50% POPC and 50% negatively charged POPS, at different vesicle bulk concentration (0.04, 0.10, 0.40 (the same as above), and 1.0 mg/mL). For the initial parts of these curves, before the extreme points, different time dependences are observed for the different concentrations of vesicles in the bulk. With increasing concentration, the initial slopes of the frequency and dissipation versus time curves become steeper, the extreme values Δf_{min} and ΔD_{max} are reached faster, and their magnitudes increase. Here, the process is obviously strongly influenced by the vesicle supply rate. After the extreme values, Δf and ΔD return back from the extreme values, until the initial values of a bilayer are regained, at approximately the same time for all of the different concentrations. In comparison to the broad variation of the concentration by over a factor of 25, the differences in the interaction time t_{int} for the different concentrations is insignificant. There is thus a striking difference before and after the extreme points with respect to the dependence of the Δf and ΔD kinetics on concentration. This reflects different origins of the time dependences in these two regions; at the initial stage, the kinetics is dominated by the supply function, that is, delivery by diffusion[13] of vesicles to the bilayer from the bulk phase through the stagnation layer, while after the extreme points the process is dominated by the surface kinetics, that is, by the lipid transfer processes in the coupled SLB-attached vesicle system and the eventual vesicle detachment. This is further illustrated by the next experiment and articulated in the Discussion.

B. Interruption of the Vesicle Supply after Different Supply Times. Following the protocol used for a typical lipid transfer experiment, all vesicles have detached from the bilayer after lipid transfer is completed (Figure 2 and also Figure 3). However, one piece of information that we are lacking so far is what happens if we interrupt the vesicle supply before the transfer process has reached completion. Several scenarios can be envisaged: (i) For sufficiently few vesicles (early interruption), the ratio between the number of lipids coming from vesicles and from the preformed SLB is small. The SLB is then the main source of lipids. (ii) For a somewhat larger number of vesicles, the same ratio is of order unity. (iii) After a sufficiently large exposure time, the dominant number of lipid molecules has come from vesicles, that is, we have the reverse case to (i).

The following experiment, shown in Figure 6, demonstrates the case when a positively charged SLB (composed of 75% POPC and 25% POEPC) was exposed at $t = 0$ min to negatively charged vesicles (composed of 50% POPC and 50% POPS), and where the exposure to vesicles was interrupted after different exposure times. For exposure times 22 and 13 min, the curves after interruption, although slightly

different in shape, both bend upward (mass decrease and decreasing dissipation) immediately after the exposure was terminated, and the Δf and ΔD values return to asymptotic values corresponding to a single SLB on the surface; that is, the process proceeds as if the exposure had not been interrupted. This corresponds to case (iii) above and illustrates a situation where vesicles from the bulk phase play no or minor role. For these exposure times, it thus appears that the lipid transfer process results in complete detachment of all lipid mass exceeding a bilayer. However, for shorter exposure times, that is, earlier interruption (Figure 6), this is not the case. For interruption at 10 min with the same vesicle concentration, the curves also bend upward, signaling detachment of lipid mass; however, they flatten out at $\Delta f = -55$ Hz and $\Delta D = 7.5 \times 10^{-6}$, which are values corresponding to much larger net mass uptake and total dissipation, compared to a SLB, thus indicating significant larger amounts of lipid mass on the surface, most likely adsorbed vesicles, than those for a single SLB. We interpret this as an incomplete transfer of charged lipids between bulk lipids and the preformed SLB at the point of interruption, so that some vesicles remain electrostatically locked to the SLB surface. We believe this corresponds to an intermediate situation between cases (ii) and (iii) above; neither the bulk lipid mass nor the SLB is the dominating lipid source but the former is slightly larger.

If the supply is interrupted already at 4 min with a low bulk concentration of vesicles of about 0.04 mg/mL, the frequency and the dissipation shifts do not change at all after the vesicle exposure is terminated but flatten out immediately at $\Delta f = -55$ Hz and $\Delta D = 7.5 \times 10^{-6}$, indicating that all attached vesicles remain on the SLB. Also, here our picture is electrostatic "locking". Whether this is closer to case (i) or (ii) above is not clear. However, mechanistic considerations (see Discussion) suggest case (ii).

Discussion

The purpose of the present work was to experimentally study lipid transfer between two lipid membranes, one SLB and the other the membrane of unilamellar vesicles, and to explore how the transfer is influenced by the amount of charge, of opposite sign, in the two membranes. As described in the Introduction, lipid transfer has been studied by several groups before, primarily between vesicles in the bulk phase but also for SLBs. Detailed systematic measurements of the transfer kinetics for different charge combinations in the interacting membranes were, however, lacking before the present work, and the mechanistic picture is still unclear.

We emphasize that the main outcome of the present work is the experimental results presented above. They illustrate the important role of electrostatic interactions between membranes containing charged lipids, and they provide a picture of the trends that can be expected for different charge lipid fractions in the SLB and vesicles. They constitute a platform for further experiments, and they also provide a guideline for how to use this platform to modify and prepare, *in situ*, lipid bilayers of different composition.[10] Finally, they may elucidate some important intermembrane processes in real biological systems. In the latter case, the charge may come from lipids or from other charged molecular components such as peptides or proteins. The data also allow a discussion of different mechanistic aspects and scenarios. Before we discuss the data in this respect, we make some general comments and considerations about the possible mechanisms of lipid transfer for the studied systems and, in the light of these possible mechanisms, about the experimentally observed time scales.

General Comments and Considerations. *Models of Lipid Transfer/Exchange*. There are several models proposed in the literature attempting to explain exchange/transfer of lipid molecules between lipid bilayers (Figure 7): (i) a monomer transfer model,[1] (ii) a transient collision model,[1,37,38] (iii) a hemifusion model,[1,39] and (iv) an insertion model,[40] which is a variant of (ii). In (i), lipid molecules are transferred by desorption from a donor membrane, followed by diffusion through the bulk aqueous phase, and eventually incorporation into an acceptor membrane. In the transient collision model,[37,38] lipid molecules are transferred between two lipid membranes that are brought into close contact (approximately 1.5 nm) in a transient collision. The transfer is enhanced by the proximity of the hydrated donor and acceptor membranes; that is, the monomer does not have to completely, or at all, leave the donor membrane as in (i) before insertion into the acceptor membrane. As a consequence, the activation energy for the transient collision process is likely to be lower compared to (i). In the hemifusion model, the donor and acceptor membranes of the facing leaflets of the two membranes fuse, and lipid molecules are transferred by lateral diffusion within the common leaflet. Here, the activation energy for the process is most likely associated with the initial formation of the hemifused structure, since once it is formed, the diffusion in a leaflet is very fast. Alternatively, for the hemifusion case, the rate limiting step could be the reverse process, namely, the retraction of the hemifused structure into two separated membranes making vesicle detachment possible. In the insertion model (iv), which resembles (ii), lipid molecules are transferred directly from the outer leaflet of the vesicle to the lower leaflet of the SLB by lipid monomer insertion steps; that is, they are guided directly through the SLB to the surface without change of orientation (i.e., without flip-flop, which would be required in (ii)). In cases (i), (ii), and (iv), the two membranes retain their integrity throughout the whole transfer process, in contrast to (iii). A more detailed presentation of these models is given by Brown[1] (monomer transfer, transient collisions, and hemifusion model) and Zhdanov and Kasemo[40] (insertion model). In Arrhenius type language, reaction rates are determined by the product of the pre-exponential factor and the activation energy factor of the Arrhenius rate expression, for the rate limiting step(s). Since each of the mechanisms (i)−(iv) above are likely to have different activation energies and different pre-exponential factors, which are all poorly known, it is difficult to even estimate the ranking of the corresponding transfer rates.

The experimental data tell us that the lipid transfer occurs through a vesicle attachment−transfer−detachment (ATD) process, which has a characteristic time, t_{int}, that ranges from 3 to 40 min depending on the composition of the SLB and vesicles (see Figure 3). Since attachment is necessary, as evidenced by the relatively large mass of attached vesicles in most of our studied cases (QCM-D and reflectometry results, Figures 3−6), before they start to detach, we rule out the monomer diffusion model (i) as a major mechanism. The

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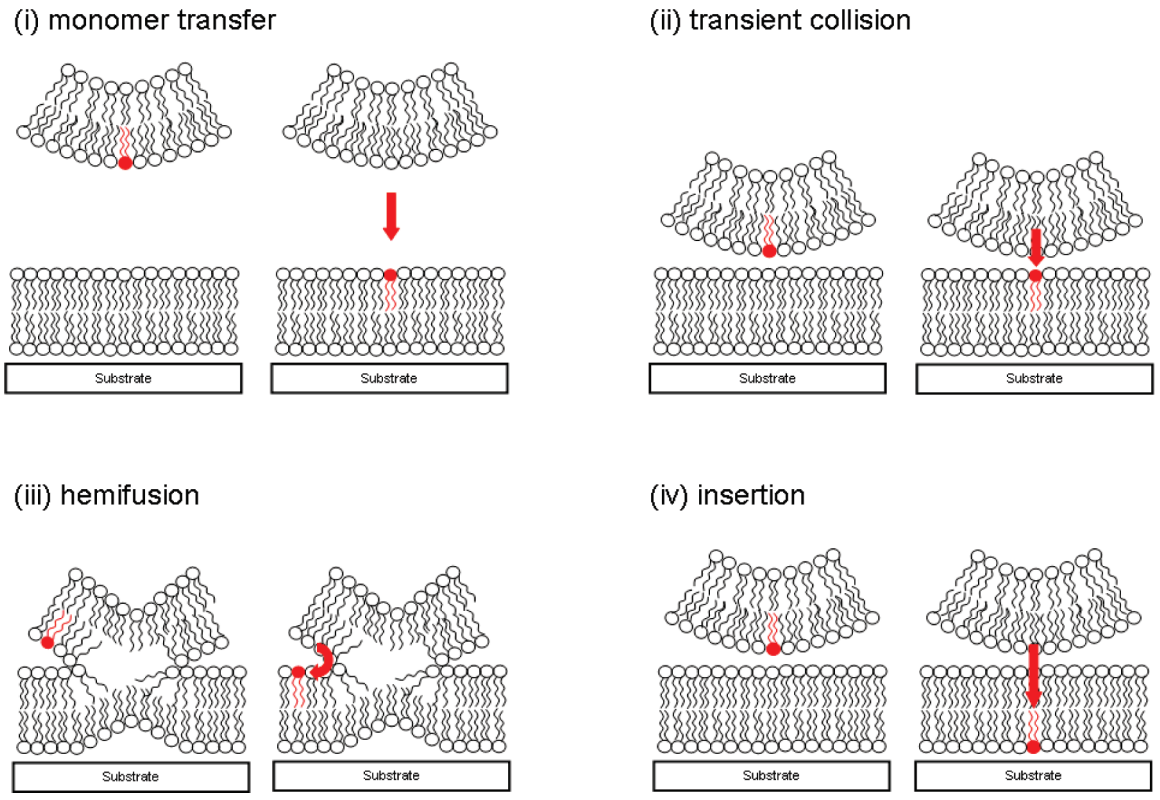

Figure 7. Models for lipid transfer between a lipid vesicle and a SLB.

ATD process could occur through any of the three other mechanisms. However, we think the ATD expression is more appropriate than "transient collision", since the latter implies a fast process (collision times $\ll$1 s) and no real settling down of the vesicles on the surface at the time scales observed in our experiments. Our data show much longer residence times (a few to many minutes), compared to the time a simple vesicle−SLB collision would take, which suggests that a very close proximity is established between the two membranes for a significant time (it is actually proven by the interruption experiments in Figure 6). The SLB and the vesicles are likely to (as a time average) be separated by at most one or a few water monolayers (hydration shells), that is, a separation of $<$1 nm, during the interaction/attachment time. The picture we tentatively suggest is that lipid molecules are transferred between the two closely connected bilayers, with or without flip-flop, but with each membrane keeping its bilayer integrity (mechanisms (ii) or (iv)), or that a hemifusion structure is created, where lipid transfer occurs rapidly once the hemifusion structure has formed (mechanism (iii)). In the former case, the monomer lipid transfer rate or place exchange should be rate limiting for the overall process (if the bulk vesicle concentration is not too low; in the latter, case the supply may be the global rate limiting factor at all times), while the formation (or retraction) of the hemifused structure is most likely to be rate limiting for the latter case, in view of the very fast intraleaflet diffusion.[41−43]

Time Scale of Lipid Transfer. Some additional comments on the time scale of the lipid transfer are appropriate. The time scale, t_{int}, for the experiments can have at least two origins. If the lipid transfer from the donor to the acceptor membrane is slow compared to the arrival of vesicles from the bulk phase (which might occur at sufficiently high bulk concentration), many vesicles will attach and essentially saturate the SLB surface before significant transfer has occurred, with a resulting high vesicle coverage, before detachment of vesicles begins. In this case t_{int}, is determined by the lipid transfer rate at the interface between the two bilayers. In the opposite case (sufficiently low bulk concentration), t_{int} will be determined by the supply rate from the bulk phase. In this case, attached vesicles should have time to equilibrate their lipid content with the SLB before the next vesicle arrives. One could then, at first thought, expect low vesicle coverage. However, it is not necessarily so, which the interruption experiments show. How much coverage that builds up on the surface in this case depends also on how much total transfer of charged lipids that must occur, before vesicle detachment can occur. A single or a few vesicles from the bulk phase are not sufficient to modify the charge state of the whole SLB enough to make it repulsive to the vesicles (or sufficiently weakly interacting to make the residence time on the SLB surface very short). In other words, it is possible, or even likely, that the bulk vesicles must act as a sink for the supply of lipids of opposite charge to the SLB and that significant mass of attached vesicles must build up before the original charge of the SLB has been altered such that detachment begins. However, contrary to this picture, one might argue that a single first vesicle on the SLB should always be fully charge-reversed with lipids from the SLB and therefore should always detach. At larger number of vesicles, the situation becomes more complex, since both the SLB and vesicle contain significant fractions of lipids of both charge signs. In the latter case, due to the fact that opposite charges attract each other, one may have some "locking" of vesicles to the SLB, where the locking mechanism is that the lipids of

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(42) Karakatsanis, P.; Bayerl, T. *Phys. Rev. E.* **1996**, *54*(2), 1785–1790.
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one charge type in the SLB attracts lipids of opposite charge in the vesicle, and where there is not enough dominance of either charge type to separate the vesicle from the SLB. This picture is at least partly supported by our observations made for different bulk concentrations (see Figure 5) and by the interruption experiments (see Figure 6). The former shows that, at low concentrations of vesicles in the bulk, the detachment begins at a much later time than that for high concentrations, illustrating that for low concentrations it takes longer time to get a sufficient amount of vesicles attached to the SLB before detachment begins. Furthermore, our observations made by the interruption experiments (see Figure 6) directly show that attachment of a minimum amount of vesicles is needed before detachment occurs. We note that the exchange of lipid monomers between attached vesicles and the underlying SLB may also be affected by vesicle−vesicle interactions on the surface. For example, when significant amounts of charge have been transferred from attached vesicles to the SLB and the vesicles have acquired close to the same net charge as the initial SLB, and are ready to soon detach, they may interact attractively with vesicles from the bulk and exchange lipids with them in the same way as the vesicle−SLB complex exchanges lipids. Although there is no evidence in the data for this, we cannot exclude that such SLB−vesicle−vesicle complexes are formed locally and transiently.

Deformation of Attached Vesicles. At a more detailed level, there is one additional factor that we believe is important for the kinetics, namely, the deformation of the attached vesicles. For a large fraction of charge (of opposite signs) in both the SLB and the attached vesicle, the interaction should intuitively be stronger than that for low fractions. As a consequence, the vesicles should get in closer proximity of the SLB (which might increase the lipid monomer transfer rate, see discussion above) and the deformation (flattening) of the attached vesicles should be highest in the case of a high charge fraction in the vesicles. The QCM-D data shown in Figure 3 support this picture, as shorter interaction times also lead to smaller frequency and dissipation shifts, indicative of a larger degree of deformation.

Such an electrostatically mediated deformation of vesicles on a charged surface has been studied by Faiss et al. using QCM.[44] They observed that deformation of vesicles attached on thiol-functionalized gold electrodes increases with increasing fraction of charged lipid in the vesicles. Deformation of vesicles has also been seen by atomic force microscopy for POPC vesicles on SiO_2 and mica surfaces.[14,15] Based on our and others' investigations of lipid bilayer formation from POPC vesicles on SiO_2 and other surfaces,[11,14,15] and Monte Carlo modeling in our group,[45−47] we are quite convinced that the deformation of the vesicles, and especially the point of minimum bending radius, where an attached vesicle is departing from the surface, is a critical point for, for example, vesicle rupture. It seems very reasonable that this part of the vesicle, with a highly strained/deformed/curved membrane, is also a point where lipid transfer may have a reduced activation barrier and thus enhanced rate, or it may be a point where hemifusion is facilitated. A recent paper actually indicates that a sufficiently low radius of curvature of lipid membranes on spherical nanoparticles induces pore formation.[48] If the point of minimum curvature is the point where the local transfer rate is highest, we have an even more complex situation, compared to when the transfer is dominated by the interface region between two flat bilayers, since due to the continuous change in charge state of the SLB during the ATD process, also the interaction strength and thus the minimum curvature of attached vesicles will change.

Charge Polarization in the SLB and in the Vesicle. Yet another point that is likely to influence the details of the kinetics is electrostatic polarization effects (briefly mentioned above) in the SLB induced by attached vesicles: Because of the attraction of unlike charges and the fast diffusion of lipid monomers within one leaflet, a vesicle of one charge may, upon approach to the SLB, transiently induce a lateral flow of lipid monomers of opposite charge, primarily in the outer leaflet of the SLB, to the proximal area of the SLB, even if the latter, due to previous transfer, has mixed positive and negative lipids. Thus, even when the SLB has reversed its average charge compared to the initial one, via lipid exchange with the vesicles, it might still locally have/assemble enough of original charged lipids to form electrostatically attached vesicles. This possibility is supported by a recent report by Khan.[33] Using dual polarization interferometry, Khan showed that lipid redistribution occurs in adsorbing vesicles containing PS to titania. Such polarization effects were also illustrated in recent Monte Carlo Simulations of charged vesicles approaching a silica surface.[47]

Discussion of the Specific Experiments. *Effect of the Fractions of Charged Lipids in the SLB and Vesicles*. The qualitative trends in the measured vesicle−SLB interactions (see Figure 3) are the same for the different studied cases: the higher the concentration is of charged lipids in the vesicles, the shorter is the interaction time, while, in contrast, an increasing fraction of charged lipids in the SLB makes t_{int} longer. The fact that an increased fraction of charged lipids in the vesicles is reducing the completion time of the ATD process is perhaps at first sight counterintuitive, as more charged lipid material then needs to be transferred from the SLB to the vesicles before the latter desorb. However, the results clearly show that it is rather the amount of transfer of lipids *from* the vesicles to the SLB that is rate limiting, and then a higher fraction of charged lipids in the vesicles, at constant bulk concentration of vesicles, accelerates the supply rate of lipids of opposite charge to the SLB. In other words, the conversion of the SLB from a vesicle-attracting to a vesicle-repelling surface is sped up by an increasing concentration of oppositely charged lipids in the vesicles. (Please note that the ATD process is rather complex as discussed above and below. Under the chosen conditions used in the present study, transfer of lipids from the vesicles to the SLB is rate limiting. Under other conditions, other factors (i.e., the structure of the lipid headgroup or the influence of the solid support) might affect the kinetics more strongly.)

Vesicle Coverage on the SLB during the ATD Process. Additional insight into the ATD process was provided by the reflectometry results (see Figure 4), where we analyzed the interaction between a positively charged SLB (containing 25% POEPC) and vesicles containing 50% and 25%

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negatively charged lipids. The maximum amount of lipid mass attached during the ATD process was about 0.027 in the first case (reflectometry signal; Δf_{min} = 80 Hz) (the higher fraction of charged lipids) and about 0.036 in the latter case (Δf_{min} = 108 Hz). Thus, with decreasing fraction of charged lipid in the vesicles, the amount of adsorbed mass (at the extreme value) is increasing, in agreement with the QCM-D results and with the general picture discussed above. (Note that the ratio of these two reflectometry signals is nearly the same as the corresponding ratio for the Δf_{min} signals, as one would expect).

We can use these results to estimate the total amount of attached lipids to the SLB during the ATD process and to say something about their structural state. The maximum change of the reflectometry signal during lipid transfer for the case shown in Figure 4A is about the same value as that obtained after the formation of the initial SLB (~0.028), while for the case shown in Figure 4B the signal is ~30% higher. The relative change of the reflectometry response during lipid transfer is, when compared with the SLB signal, much smaller than the corresponding frequency and dissipation shifts obtained by QCM-D. This tells that there is a large amount of solvent associated with the surface-attached lipid structures,[14] which supports the picture that intact vesicles are attached to the SLB. Furthermore, by comparing the reflectometry signal for the formation of the SLB with the maximum signal during lipid transfer, we can conclude that the SLB is not fully covered by vesicles during the lipid transfer process, but rather is at about 25−35% of a full monolayer vesicle coverage for the two cases studied by reflectometry. Using a linear extrapolation of these numbers for the case with the highest Δf_{min} = 123 (see Table 2), we estimate the maximum vesicle coverage in that case to be around 40% of a full monolayer. We have in these estimates assumed that the attached vesicles retain their original size, and have not considered different shapes for different cases. We also assume that there is no vesicle−vesicle fusion process occurring on the SLB during the lipid transfer process, which cannot be totally excluded.

Dependence on Vesicle Bulk Concentration and Interruption Experiments. Both these types of experiments clearly show that the rate determining steps for the ATD kinetics are different for the initial and later stages of the vesicle−SLB interaction. Initially, the rate of supply, that is, the bulk vesicle concentration, is determining the kinetics. A minimum amount of lipid matter seems to be needed on the SLB surface in order to induce enough transfer of lipids to the SLB, to make it repulsive to (or very weakly interacting with) the vesicles, before the latter begin to detach. This is shown both by the much slower initial kinetics of the lowest concentration curve in Figure 5 (0.04 mg/mL), before the minima in ΔD and Δf, and by the interruption experiments in Figure 6, where too early interruption leads either to no detachment of vesicles at all (interruption after 4 min, after which all vesicles stay on the SLB surface) or to incomplete lipid transfer, leaving some vesicles attached to the surface (10 min interruption), with the latter evidenced by the larger ΔD and Δf signals than those for a SLB. (We make the reservation here that a much smaller number of exposed vesicles, i.e., much lower exposures, than we have investigated might lead to the ATD process, as discussed above, and that the irreversible attachment for some exposures is already in the intermediate exposure regime where there are comparable amounts of lipids of opposite sign in the SLB.)

A short while after the minima in ΔD and Δf have been passed, the kinetics is no longer determined by the bulk supply of vesicles, but rather by the lipid transfer process itself, demonstrated by the fact that here a continued bulk supply, or not, has no influence on the kinetics. Compare, for example, the interruption curves for 13 and 22 min in Figure 6. They exhibit nearly the same kinetics and both terminate asymptotically at the same final ΔD and Δf values corresponding to a SLB.

Asymmetry in the Kinetics between the "Mirrored" Experiments. From a purely electrostatic point of view, the quite different kinetics time scales observed in Figure 3A and C, which are electrostatically symmetric, seems surprising. In the first case, a positively charged bilayer was exposed to negatively charged vesicles, while in the second case a negatively charged bilayer was exposed to positively charged vesicles, with the same charge fractions in the SLB and vesicles in both cases. The different kinetics show that, although the overall electrostatics are important, it is by no means the only factor. This leads us to discuss possible explanations for the observed asymmetry.

(i) Influence of the Molecular Structure of Different Lipid Head Groups. The present study was designed such that all lipids had similar molecular structure in the lipid tails, but the headgroups were by necessity different to achieve the different charge states (see Figure 1). Inevitably, several physical properties are changed by changing the headgroup molecular structure. One example is the phase transition temperature T_m. It is likely that T_m plays a role for the interleaflet transfer (T_m affects the intraleaflet mobility). Thus, it is likely that the rate constants for the insertion/removal of individual lipid molecules to/from membranes of different composition vary with the molecular headgroup structure, since the latter defines the activation barrier for insertion. This assumption is supported by the work of De Cuyper and Joniau; they showed that intervesicular transfer of anionic phospholipids is influenced by the nature of the polar headgroup.[19] They discuss this observation in terms of differences in headgroup hydration. A further interesting observation, similar to ours, was made by Reinl and Bayerl;[28] they found that the kinetics of lipid transfer between charged vesicles and an oppositely charged SLB depends on the composition of the SLB on silicon.[28] In a first step, negatively charged dimyristoylphosphatidylglycerol (DMPG) or positively charged dioctadecyl diammonium bromide (DODAB) was transferred from vesicles (containing 100% of the charged lipid) to a preformed neutral SLB (containing 100% dimyristoylphosphatidyl choline (DMPC)). In both cases, they observed an equal amount of charged lipid being transferred (after lipid transfer, the resulting SLBs contained about 85% of the charged lipid and 15% of the zwitterionic PC). In the next step, the obtained mixed SLBs were exposed to oppositely charged vesicles (containing 100% of the charged lipid). Here, they noticed different kinetics for the two different cases; the cationic DODAB was transferred faster than the anionic DMPG. Reinl and Bayerl interpreted this as an effect of an asymmetric lipid distribution. At first glance, this result is in contrast to our observation that the interaction time, in the case of a positively charged SLB exposed to negatively charged vesicles, is shorter than that in the opposite case, but it is important to point out that different substrates and different lipids were used in the two studies. Comparing the results of our study with the work of Reinl and Bayerl leads

us to the conclusion that, in addition to the overall electrostatics and charge state of the interacting lipid membranes, (i) the structure of the lipid molecules plays a pivotal role and that (ii) the different kinetics in the mirrored cases might be due to an asymmetric distribution of the lipids in the SLB. Actually, the headgroups are molecular structures with different chemical properties, different spatial extents, different hydrophobic/hydrophilic properties, different hydration shells, and so on, and these properties must play some roles superimposed on the electrostatic interactions.

(ii) Asymmetric Distribution of Lipids in the Leaflets of the Initial SLB. The interaction between an SLB, containing a mixture of lipids, and the support may create an asymmetry in the relative lipid concentrations between the upper and lower leaflets, because the supporting surface has a net charge. With a net positive SLB, there is likely a strong interaction with the negative surface charges of the SiO_2 support (at the actual pH), which could reduce the amount of positive charge in the upper leaflet and increase it in the lower one. For a negative SLB, the opposite effect might be at hand. Thus, although the "mirror" experiments were designed to be symmetric, they may not be so, because of the interaction with the support. Indeed, recent reports[17,41] indicate that the lipid monomer distribution in the two leaflets depends strongly on the combination of solid support, lipid mixture, and chosen buffer. There are several reports about asymmetric distribution of polystyrene (PS) lipids. Rossetti et al. reported an asymmetric distribution of PS molecules in SLBs on TiO_2 supports in the presence of Ca^{2+} ions while such a behavior was not observed on SiO_2.[41] A similar observation was reported by Richter et al.[17] They found that PS is asymmetrically distributed in SLBs on mica, though they were unable to draw a clear conclusion of the distribution on a SiO_2 support. To our knowledge, there are no reports about asymmetric distribution of cationic lipids besides the study of Reinl and Bayerl.[28] As described above, they interpret their results as an effect of an asymmetric distribution of lipids in a ternary SLB.

(iii) Vesicle Curvature. The curvature of the attached vesicles is likely to influence the transfer of lipids between the vesicle and the SLB as already discussed above. Presumably, the transfer rate is highest at the point of highest curvature. The curvature of the attached vesicle depends on interaction strength between head−head contact as well as on the bending rigidity of the vesicles.[49] It is likely that vesicles of different lipid compositions have different bending rigidities and consequently different curvatures. Thus, a positively charged vesicle attached to an oppositely charged SLB is likely to have another curvature than a negatively charged vesicle attached to a positively charged SLB. Consequently the transfer of lipid molecules would then be different for the "mirror" experiments, if the dominant transfer occurs at the curved part of the vesicle−SLB interface.

We conclude, from this section, that an influence of the substrate causing an asymmetric distribution of lipids in the SLB is possible, and that the difference in curvature for negatively and positively charged vesicles as well as differences in headgroup molecular structure for negative and positive lipids may contribute to nonsymmetric kinetics for the electrostatically symmetric cases. By our experimental results, it is not possible to discriminate between these factors. We intend to elucidate this question by further experiments and Monte Carlo simulations.[50]

Conclusion

We have measured lipid transfer between charged SLBs and oppositely charged vesicles and determined the dependency of this process on the fractions of charged lipids in the bilayer and the vesicles. From the general behavior, we conclude that lipid transfer occurs through an attachment−transfer−detachment (ATD) process. The time constant for the ATD process increases with increasing amount of charge lipids in the SLB and decreases with increasing amount of charged lipids in the vesicles. Furthermore, it appears that a minimum amount of charged vesicles must adsorb on the SLB before detachment of vesicles begins (a very early stage with detachment might have been missed in the experiment). The latter two observations are consistent with a picture, where an important condition for vesicle detachment is that a certain minimum amount of lipids must be transferred to the SLB, probably leading to charge reversal in the latter. Different kinetics observed in electrostatically symmetric cases are likely caused by a combination of molecular headgroup asymmetry and an asymmetric charge distribution in the SLB, due to its interaction with the negatively charged SiO_2 support. Different rigidities of oppositely charged vesicles, combined with dominant lipid transfer rates at the curved part of the vesicle−SLB interface, are also possible origins of the asymmetric kinetics. Mechanistically, we suggest that the transfer occurs via monomer transfer in both directions between the two bilayers, but we cannot exclude hemifusion structures or other concerted place exchange. The type of data obtained here and the developed procedures, and their extensions in the future, will provide new insight into how lipid transfer between lipid membranes takes place, and maybe also about fusion, endocytosis, and other molecular transfer processes at membrane surfaces and membrane−membrane interfaces. They also provide a new platform for modifying and controlling SLBs that can be used in biosensing technology and cell culturing involving SLBs.

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