Soft Matter

Cite this: *Soft Matter*, 2011, **7**, 8582

www.rsc.org/softmatter

PAPER

Ion-mediated changes of supported lipid bilayers and their coupling to the substrate. A case of bilayer slip?

Angelika Kunze,* Fang Zhao, Anna-Kristina Marel, Sofia Svedhem and Bengt Kasemo

Received 13th May 2011, Accepted 4th July 2011
DOI: 10.1039/c1sm05886j

Ion-mediated (Ca^{2+}) changes in viscoelastic, structural and optical properties of negatively charged solid supported lipid bilayers (SLBs) on SiO_2 surfaces were studied by means of quartz crystal microbalance with dissipation (QCM-D) monitoring and optical reflectometry. Despite the sensitivity of QCM-D to viscoeleastic/structural variations, it has not often been used to probe such changes for SLBs. SLBs were prepared from binary phospholipid mixtures of 1-palmitoyl-2-oleoyl-sn-*glycero*-3-phosphocholine (POPC, neutral) and 1-palmitoyl-2-oleoyl-sn-*glycero*-3-phospho-(1′-rac-glycerol) (POPG, negatively charged) on SiO_2 sensor surfaces in a Ca^{2+}-containing buffer. Interestingly, for bilayers containing POPG fractions above 35%, large QCM-D dissipation shifts occurred, when Ca^{2+} was removed from buffer in contact with the SLB (while maintaining 100 mM NaCl). The accompanying frequency changes were small. These Ca^{2+} mediated QCM-D responses are reversible, and a signal for considerable changes in the viscoelastic and structural properties of the SLB. Variation of Ca^{2+}-concentration revealed a threshold concentration of around 0.4 mM for the changes in the SLB to occur. Below this value, at >35% POPG concentration in the SLB, the SLB appears to become more weakly attached to the SiO_2 substrate, which is partly attributed to a weakening of the POPG-substrate interaction in the absence of Ca^{2+}. A consequence of this is an oscillation-amplitude dependent dissipation, which we attribute to slip of the bilayer at higher oscillation amplitudes. Complementary experiments using a combined QCM-D/reflectometry instrument showed that the Ca^{2+}-induced changes in the viscoelastic/structural properties of the SLB are accompanied by changes in the optical properties. We discuss different scenarios to explain the observed reversible effect of Ca^{2+}-ions on the dissipative and optical properties of the mixed SLBs. Based on our results we propose the observed phenomenon to be a combination of geometric changes, internal structural changes, changes in the interfacial water layer, and a slip mechanism, *i.e.* friction between the SLB and the substrate.

Introduction

The cell membrane is the most vital interface in living cells, where many important life processes are taking place. It is involved in cell metabolism, signal transduction, cell migration, and in the interplay of various membrane components, such as lipids, proteins or steroids. Furthermore, the cell membrane is an effective diffusion barrier, upholding concentration gradients of ions and molecules across the membrane.

The complexity of the natural cell membrane makes controlled studies of single processes difficult. Thus, there has been, and is, a demand for model membranes mimicking various aspects of the native cell membrane, and of suitable methods to study them. One of the most frequent, and maybe also the most successful model system for various membrane interactions is the solid supported lipid bilayer (SLB), where the distal leaflet is exposed to the bulk solution and the proximal leaflet is facing a solid substrate, *e.g.* a sensor surface, allowing the application of surface sensitive techniques like surface plasmon resonance spectroscopy (SPR),[1–3] reflectometry,[4,5] impedance spectroscopy,[6–8] waveguide spectroscopy,[9] atomic force microscopy (AFM)[3,10–13] and the quartz crystal microbalance with dissipation monitoring technique (QCM-D).[1,3,11,14,15]

Extensive efforts have been invested in controlling the preparation of SLBs and their subsequent modifications, to allow for studies of membrane interactions with peptides and proteins,[6,7,16–18] virus like particles,[19] liposomes,[20,21] and cells.[22,23] All of these studies depend on good quality SLBs and, even more importantly, the knowledge about the properties of the bilayer, in order to allow for correct interpretations of the achieved results.

A common protocol for the preparation of good quality SLBs has been developed based on the liposome adsorption method originally reported by Brian and McConnell.[24] In this process, liposomes prepared from a lipid mixture in the liquid phase are

Dept. of Applied Physics, Chalmers University of Technology, Göteborg, Sweden. E-mail: angelika.kunze@gu.se

allowed to adsorb to a suitable surface, *e.g.* SiO_2, where the interaction with the surface leads to formation of a SLB. As detailed in later work this occurs in a process called the adsorption-rupture-fusion process where the primary liposome adsorption and surface interaction induces the rupture and fusion of the vesicles on the surface and, eventually, the formation of a coherent planar bilayer (SLB). This process and the characteristics of the resulting bilayer, as recorded *via* their optical, viscoelastic and structural properties, have been shown to depend on lipid composition,[9,13,15,25] substrate material,[15,26] and the surrounding solution, in particular the presence of divalent cations like Ca^{2+} which are routinely used to form negatively charged SLBs on negatively charged surfaces.

Several studies has pointed out that Ca^{2+} can bind to the phosphate and/or to the carbonyl of phospholipid polar head groups.[12,27–31] Binding of Ca^{2+}-ions to the lipid headgroups commonly involve structural changes which alter the overall properties of phospholipid structures. For example, changes in nanomechanical properties of dimyristoyl phosphocholine (DMPC) SLBs in the presence of Ca^{2+}-ions have been probed by atomic force microscopy (AFM).[12] In particular, an increasing force was needed to puncture/penetrate the lipid bilayers in the presence of Ca^{2+}-ions, indicating stabilization of the SLB. In other studies, based on bulk measurements on liposomes, Ca^{2+}-ions have been shown to change lipid membrane phase behavior, typically inducing a stabilization of the more rigid gel phase.[29,30] Ca^{2+}-ions may furthermore cause phase separation in mixed lipid membranes.[31] Besides the interaction with the lipid molecules, Ca^{2+}-ions also interact with the negatively charged SiO_2-surface leading to a change of the (effective) surface charge (zeta potential).[32] Such an interaction competes with the Ca^{2+}-ion interaction with the lipid headgroup. A consequence of this competition can in principle also be a bridge bond between the negative lipid headgroup and the negative surface sites, as suggested by several authors.[12,33,34] For example, it has been shown by means of QCM-D that several divalent cations promote vesicle-to-bilayer formation, when compared to monovalent cations.[34] This effect was attributed to a strengthened vesicle-surface interaction (*i.e.* a stronger interaction with SiO_2 of the part of the vesicles that faces the SiO_2) and an accompanying higher deformation of vesicles.

In summary, Ca^{2+}-ions are known to bind to lipid structures at their headgroups, to change their structural and stability properties, and furthermore, to act as bridging molecules between lipid headgroups and a silica surface. Based on this knowledge we assume that removal of Ca^{2+}-ions from a SLB that required Ca^{2+} for bilayer formation may have substantial effects on the properties of the SLB. An ideal tool to study such a phenomenon is the QCM-D technique due to its unique feature to sense dissipation, *i.e.* energy losses, in thin layers at the sensor surface, which in turn can be related to changes in viscoelastic/structural properties. A shift of the resonance frequency (Δf) of the quartz crystal resonator is related to how much mass is (dynamically) coupled to the substrate, whereas the damping of the periodic oscillation of the sensor, measured as a dissipation shift (ΔD), gives information about energy losses that occur during the periodic oscillation of the sensor, which in turn gives information about the rigidity/viscoelastic properties of the adsorbed film (shear viscosity). In principal, energy losses can also be caused by friction/slip between the adlayer and the substrate, although this is rarely seen and confirmed. By taking into account the dissipation due to viscoelastic behavior of the adlayer, the QCM-D technique has very significantly improved the mechanistic understanding of bilayer formation.[1,3,11,15,35] Using QCM-D with a 5 MHz fundamental frequency sensor, a "typical" frequency shift in the range $\Delta f = -24$ to -27 Hz (independent of overtone when normalized to the fundamental frequency, depending on lipid composition) and a "typical" dissipation shift $\Delta D < 0.5 \times 10^{-6}$ were introduced by our group,[35] and such values have generally been considered to be indicatives of good quality SLBs. Especially the dissipation shift will be higher if there is a coexistence of adsorbed intact vesicles and SLB on the surface. However, this categorization may not always be adequate for all types of SLBs and complementary measurements and analyses are then required. Therefore, a combination of QCM-D with other techniques, often an optical technique, has commonly been used to strengthen and refine the interpretation of QCM-D data (and *vice versa*). This combination makes it possible (i) to obtain optical and viscoelastic properties of the bilayer and (ii) to discriminate between lipid mass alone and lipid plus coupled water mass,[4,5] which in turn makes the interpretation of the results less ambiguous and more detailed.

In the present study, we show that the viscoelastic properties (dissipation shift) of a bilayer containing a significant fraction of negatively charged POPG is highly sensitive to Ca^{2+}-ions in bulk solution. This change in viscoelastic properties is reversible upon addition and removal of Ca^{2+}-ions. Complementary experiments were performed using a combined QCM-D/reflectometry and a dual-frequency QCM-D instrument to facilitate the interpretation of the results. Possible mechanistic scenarios for the Ca^{2+}-induced change in the bilayer properties are discussed. We consider structural changes of the bilayer, its coupling to the substrate, and slip/friction between the bilayer and the substrate on the shear oscillating sensor.

Materials and methods

Materials

All chemicals were obtained from commercial sources and used without further purification. 1-Palmitoyl-2-oleoyl-sn-*glycero*-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-sn-*glycero*-3-phospho-(1′-rac-glycerol) (POPG) were from Avanti Polar

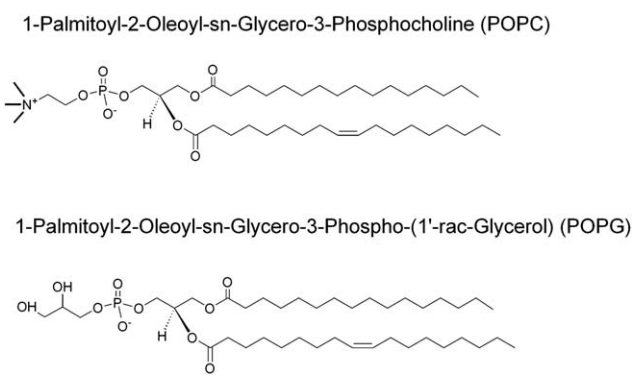

Fig. 1 Chemical structure of phospholipids used in this study.

Lipids Inc., AL (Fig. 1). Water was deionized (resistivity > 18 MΩ/cm) and purified using a MilliQ plus unit (Millipore, France). TRIS buffer (pH 8) contained 10 mM TRIS and 100 mM NaCl. TRIS-$CaCl_2$ buffer was TRIS buffer with additionally 10 mM $CaCl_2$. Buffers were filtered and degassed before use.

Vesicle preparation and characterization

POPC and POPG were dissolved in chloroform to prepare thin lipid films of the desired composition on the wall of a round-bottom flask. The solvent was removed, first under a gentle stream of N_2 to form the film, and secondly under vacuum for 3 h. The lipid films were hydrated in TRIS-buffer. After vortexing, the solutions were extruded 11 times each through a 100 nm and a 30 nm polycarbonate membrane. The resulting vesicle solutions were refrigerated under N_2.

Vesicle solutions (diluted to 0.01 mg/ml in TRIS buffer) were characterized using dynamic light scattering (Malvern Zetasizer Nano ZS together with the Dispersion Technology software, Malvern Instr., UK) with respect to size and zeta potential at 22 °C.

Size and zeta potential of the different vesicles used in the present study are listed in Table 1. While the zeta potential of POPG vesicles varied with the fraction of charged lipid and chosen buffer, the mean size of the different vesicles was about the same, 90 ± 7 nm.

QCM-D

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 SiO_2 were purchased from Q-Sense AB. Prior to the experiment the crystals were cleaned in a 10 mM sodium dodecyl sulfate aqueous solution (overnight), rinsed thoroughly with water, dried under N_2, and treated with UV/ozone for 3 × 15 min with rinsing and drying in between. The measurements were carried out at 22 °C using a flow rate of 100 μL/min. Frequency and dissipation shifts were measured at the ninth overtone. Frequency shifts were normalized to the fundamental frequency by dividing the values by 9.

The dissipation of the QCM-D signal is defined as:

$$D = \frac{E_{dissipated}}{2\pi E_{stored}} \quad (1)$$

Table 1 Zeta potentials of vesicles prepared from different lipid mixtures of POPC and POPG in Ca^{2+}-free and Ca^{2+}-containing TRIS buffer (10 mM TRIS, 100 mM NaCl, 10 mM $CaCl_2$, pH 8)[a]

	Zeta Potential/mV	
Fraction POPG	0 mM $CaCl_2$	10 mM $CaCl_2$
0%	−1 ± 1	4 ± 1
10%	−15 ± 1	−5 ± 1
25%	−28 ± 2	−10 ± 2
35%	−31 ± 1	−9 ± 1
50%	−38 ± 1	−13 ± 1
75%	−40 ± 2	−15 ± 3
100%	−47 ± 2	−21 ± 1

[a] The vesicle size was 90 ± 7 nm for all lipid compositions.

where D is the dissipation, $E_{dissipated}$ is the energy dissipated from the sample, E_{stored} is the entire energy contained in the crystal's movement. This means that D tells how large fraction of the total energy stored in the oscillator that is dissipated in one cycle of oscillation.

Combined QCM-D/reflectometry

Combined QCM-D/reflectometry measurements were carried out with a new prototype instrument.[4] QCM-D and reflectometry signals were recorded simultaneously using the same sensor surface. Gold-coated QCM-D sensor crystals from Q-Sense AB, Sweden were modified to meet the requirement for sensitive reflectometry measurements by deposition of a 100 nm layer of Ti followed by a 110 nm layer of SiO_2 (thermal evaporation at a pressure < 5×10^{-6} mbar) (HVC600, AVAC) onto the sensor surface. Prior to mounting in the vacuum chamber, the sensors were cleaned in an ultrasonic bath (5 min in 2-propanol and 5 min in water, followed by blow-drying with N_2), in a microwave plasma system (250 W, 2 min, Plasma Strip TePla 300PC (TePla AG, Germany)), and, finally, by rinsing with water and blow-drying with N_2. The same procedure as described above for the regular QCM-D experiments was used.

The principle of optical reflectometry is that the reflectivity of a solid surface changes when material adsorbs onto it. If a material having the refractive index n that is different from the refractive index n_0 is adsorbed on the sensor surface a change in the reflectometry signal ΔR is observed. This change, *i.e.* the optical response, ΔR, is related to the thickness d and the refractive index n of the material adsorbed to the sensor surface *via*:[36]

$$\Delta R = d(n - n_0)A \quad (2)$$

where A is the sensitivity factor[37] dependent on the optical setup. In the present study A is determined to be $A = 0.015$.

Dual-frequency QCM-D/varying oscillation amplitude

In the present study it became interesting to perform QCM-D measurements with a variation of the oscillation amplitude of the sensor. For this purpose we used a dual-frequency setup described earlier by Edvardsson *et al.*[38] In short, an additional frequency generator was added to the QCM-D setup for continuous excitation of the QCM-D sensor at variable driving amplitudes, while performing QCM-D, *i.e.* combined frequency and dissipation, measurements. Experiments were carried out on the same crystals as used for normal QCM-D measurement, using the same cleaning treatment as described above. The QCM-D measurements were performed at 35 Hz (7th overtone) with variable driving amplitude (0–6 V) at 5 MHz (1st overtone). The experiments were carried out at 22 °C using a flow rate of 100 μL/min.

Results

The influence of Ca^{2+}-ions on negatively charged SLBs was studied with QCM-D as the main technique, and with reflectometry to obtain complementary optical data. The lipid mixtures used for vesicles and SLBs were composed of

zwitterionic POPC and negatively charged POPG lipids (Fig. 1). Pure POPC vesicles/SLBs were used for reference measurements.

Scheme of the QCM-D measurements

QCM-D *versus* time curves for the measured frequency (Δf) and dissipation shifts (ΔD) in a typical experiment are shown in Fig. 2. In the first step, marked by (i), a negatively charged bilayer composed of (in this case) 50% POPC and 50% POPG is formed on the negatively charged SiO_2 sensor surface (pH 8) by adsorption and rupturing of lipid vesicles in a Ca^{2+}-containing buffer.† The bilayer formation follows a typical behavior as reported many times before.[15,35,39] After the injection of vesicles, marked by (i), Δf decreases (mass uptake) while ΔD increases, indicating attachment of intact vesicles at the sensor surface. After a certain time Δf increases (mass loss) and ΔD decreases again, signaling rupture of adsorbed vesicles, where the frequency rise is caused by loss of trapped water and the dissipation decrease is caused by the higher rigidity of the SLB compared to adsorbed vesicles. Frequency and dissipation eventually stabilize at Δf_1 and ΔD_1 when a high quality bilayer has been formed over the whole sensor surface (characterized by $\Delta f \sim -27$ Hz and $\Delta D < 0.5 \times 10^{-6}$).

In the second step, marked by (ii), the Ca^{2+}-containing buffer was replaced by Ca^{2+}-free buffer. An initial transient drop in frequency followed by a slower increase is observed after which the frequency shift stabilizes at $\Delta f_2 \leq \Delta f_1$ (Fig. 2), *i.e.* the acoustically coupled mass has decreased slightly due to removal of Ca^{2+} in the buffer. Such small bulk shifts are often observed upon changing buffers in contact with SLBs. However, quite unexpectedly, there was in this case a simultaneous large increase in dissipation, *i.e.* $\Delta D_2 \gg \Delta D_1$, indicating that upon removal of Ca^{2+}-ions from the buffer, the SLB undergoes a considerable structural change. This is a new behavior, not observed before. The increase in ΔD upon the removal of Ca^{2+}-ions appears to cause formation of a structure that is acoustically more weakly coupled to the surface, causing an increase in ΔD. Possible explanations for this behavior are discussed below. The dissipation change is reversible, since when changing back to Ca^{2+}-containing buffer in the next step, marked by (iii), we find that the frequency and dissipation shifts return back to the values of the initial SLB ($\Delta f_3 \sim \Delta f_1$ and $\Delta D_3 \sim \Delta D_1$), indicating that the lipid mass at the surface stays constant during the removal/addition of Ca^{2+}-ions, thus ruling out mass loss as the cause of $\Delta f_2 \leq \Delta f_1$. Repeated buffer changes show that the observed changes in frequency and dissipation are repeatedly reversible ($\Delta f_4 \sim \Delta f_2$ and $\Delta D_4 \sim \Delta D_2$, $\Delta f_5 \sim \Delta f_1$ and $\Delta D_5 \sim \Delta D_1$).

These observations show that there is a reversible change in the properties of the SLB, and/or in the way the SLB is attached to the support, caused by the presence or absence of Ca^{2+}-ions in the buffer. The effect is mainly seen in the high dissipation shifts, while the changes in the frequency shifts are very small or negligible. To obtain further information about this phenomenon, which is not observed for a pure POPC bilayer, experiments were performed where the *fraction of charged* lipids in the SLB or the *concentration of $CaCl_2$* in buffer was varied, and by employing reflectometry.

† Note that neither EDTA nor EGTA was used. Thus there may be traces of irrevesible bound Ca^{2+}-ions after buffer exchange to Ca^{2+}-free buffer. However it is clear from the present data that there is a substantial fraction of Ca^{2+}-ions that can be reversibly removed leading to the observed effect.

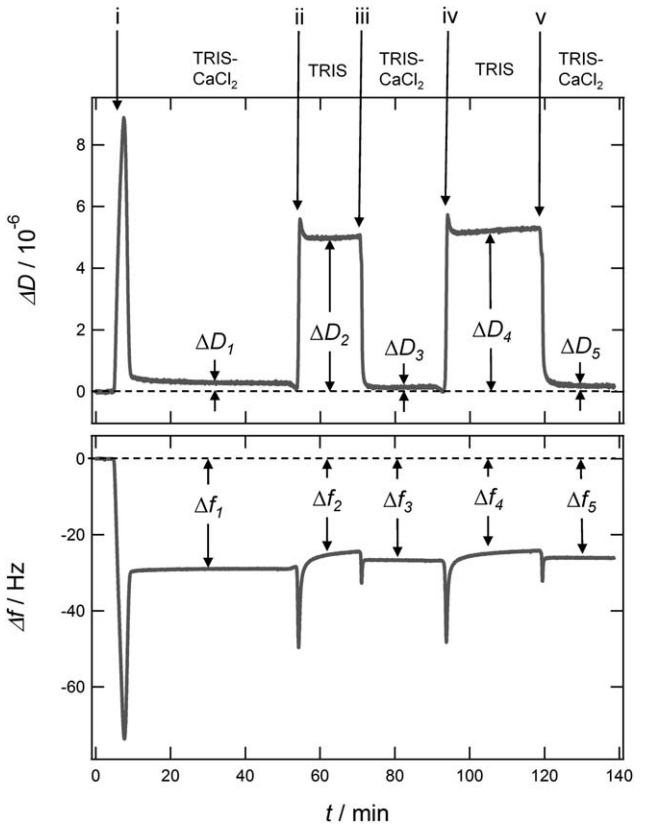

Fig. 2 Typical signal *versus* time curves of the dissipation, ΔD, and frequency, Δf, shifts (normalized to the fundamental frequency) obtained by QCM-D during initial bilayer formation and subsequent sequential removal and addition of Ca^{2+}-ions. (i) A SLB was formed by adsorption and spontaneous rupture of vesicles containing 50% POPG and 50% POPC on SiO_2 in TRIS-$CaCl_2$ (10 mM TRIS, 100 mM NaCl, 10 mM $CaCl_2$, pH 8) followed by sequential buffer changes to (ii) TRIS (10 mM TRIS, 100 mM NaCl, pH 8), (iii) TRIS-$CaCl_2$, (iv) TRIS, and (v) TRIS-$CaCl_2$.

Variation of the fraction of charged lipid in the SLB

The experiment with POPC/POPG SLBs described above was extended by varying systematically the fraction of POPG in POPC/POPG vesicles used for the SLB formation between 0 and 100% of POPG. Table 2 summarizes the frequency and dissipation shifts observed for the initial bilayer formation (Ca^{2+}- containing buffer) and upon buffer changes between Ca^{2+}-containing and Ca^{2+}-free buffer for the different lipid compositions used. Formation of a high quality SLB was observed only up to a fraction of 50% POPG in the liposomes ($\Delta f \sim -27$ Hz and $\Delta D \leq 0.5 \times 10^{-6}$). For higher POPG fractions the frequency and dissipation shifts were higher. For example, the results for a fraction of 75% POPG in the lipid vesicles were $\Delta f \sim -35$ Hz and $\Delta D = 2.1 \times 10^{-6}$, which indicate that a heterogeneous system is formed, *i.e.* that there is a coexistence of adsorbed intact vesicles and SLB on the sensor surface. For 100% POPG vesicles, very high frequency and dissipation shifts

Table 2 Mean values of the frequency and dissipation shifts and the corresponding standard deviations for the set of experiments with varying fraction of POPG: (I) after the SLB is formed in Ca^{2+}-containing TRIS buffer (step (i), Fig. 2), (II) after exchange to Ca^{2+}-free TRIS buffer (step (ii), Fig. 2), and (III) exchange to Ca^{2+}-containing TRIS buffer (step (iii), Fig. 2)

		(I)10 mM $CaCl_2$		(II) 0 mM $CaCl_2$		(III) 10 mM $CaCl_2$	
Fraction POPG		Δf_1/Hz	$\Delta D_1/10^{-6}$	Δf_2/Hz	$\Delta D_2/10^{-6}$	Δf_3/Hz	$\Delta D_3/10^{-6}$
0%	SLB	27.3 ± 0.2	0.5 ± 0.1	26.1 ± 0.5	0.4 ± 0.1	26.5 ± 0.5	0.3 ± 0.1
10%	SLB	27.1 ± 0.2	0.5 ± 0.1	25.6 ± 0.5	0.2 ± 0.2	26.1 ± 0.2	0.3 ± 0.1
25%	SLB	27.7 ± 0.7	0.3 ± 0.1	29.5 ± 0.5	0.4 ± 0.1	27.0 ± 1.2	0.2 ± 0.1
35%	SLB	27.9 ± 0.6	0.2 ± 0.1	29.2 ± 3.3	5.2 ± 0.5	25.3 ± 1.5	0.3 ± 0.2
50%	SLB	28.9 ± 0.2	0.3 ± 0.2	24.2 ± 0.4	5.1 ± 0.3	26.5 ± 0.5	0.1 ± 0.2
75%	SLB+vesicles	35.4 ± 1.5	2.1 ± 0.4	20.4 ± 0.9	4.1 ± 0.2	22.8 ± 1.2	1.0 ± 0.1
100%	vesicles	156.5 ± 2.8	25.3 ± 3.7	vesicles are removed			

indicate the adsorption of solely or primarily intact vesicles to the sensor surface. In the following we focus on the fractions of POPG, where a good quality bilayer is formed.

For these bilayer compositions (*i.e.* POPG fractions ≤ 50%), different behaviors were observed upon exchange from Ca^{2+}-containing to Ca^{2+}-free buffer for different POPG fractions. In Fig. 3 the average frequency and dissipation shifts obtained upon buffer exchange ($\Delta f_{2-1} = \Delta f_2 - \Delta f_1$ and $\Delta D_{2-1} = \Delta D_2 - \Delta D_1$) are plotted *versus* the fraction of POPG in POPC/POPG vesicles. The frequency shift $\Delta f_{2-1} < 5$ is small compared to the frequency shift corresponding to the bilayer formation ($\Delta f_{SLB} \sim 27$ Hz) for all fractions of POPG displayed in Fig. 3. Up to a fraction of 25%

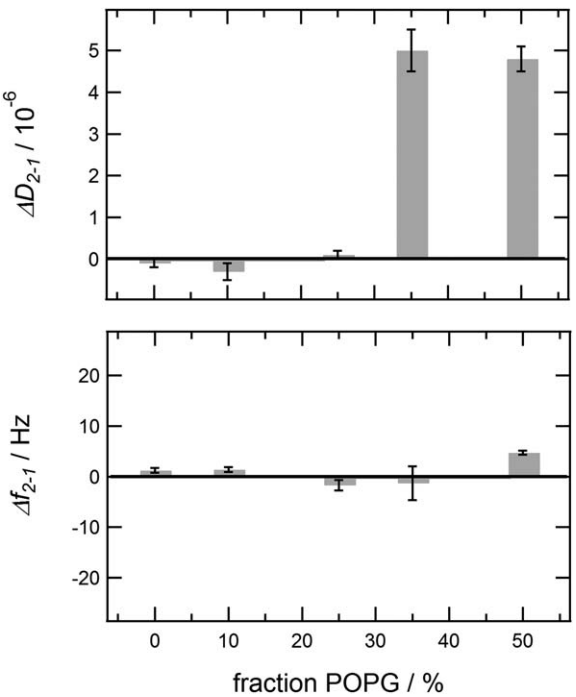

Fig. 3 Average dissipation shift $\Delta D_{2\text{-}1} = \Delta D_2 - \Delta D_1$ and frequency shift $\Delta f_{2-1} = \Delta f_2 - \Delta f_1$ (normalized to the fundamental frequency) upon buffer exchange from Ca^{2+}-containing to Ca^{2+}-free TRIS buffer (step (ii) in Fig. 2) *versus* the fraction of POPG in the SLB. The data show that the presence or absence of Ca^{2+} has small effects on the signals for small POPG fractions, while it has a large effect on the dissipation signal for POPG fractions above 30%.

of POPG in the SLB also very small dissipation shifts $\Delta D_{2-1} < 0.5 \times 10^{-6}$ are found upon buffer exchange, indicating small changes in the SLB upon Ca^{2+}-removal/addition. In other words, in this range the SLB behaves nearly the same as a pure POPC bilayer. However, above 35% of POPG, a large dissipation shift $\Delta D_{2\text{-}1} > 4 \times 10^{-6}$ is observed when Ca^{2+}-containing buffer is exchanged by Ca^{2+}-free buffer. Changing back from Ca^{2+}-free to Ca^{2+}-containing buffer, the dissipation shifts turn back to the initial value, *i.e.* $\Delta D_3 \sim \Delta D_1$ (Table 2), similar to what was seen already in Fig. 2. Taken together these results show that there is a critical fraction of POPG in the mixed SLB, above which the system is very sensitive to the presence or absence of Ca^{2+}-ions in the buffer.

In order to explore the headgroup specificity, the lipid molecule POPG was replaced by POPS, a phosphatidylserine lipid that has the same charge as POPG but with a different chemical structure in the headgroup. Using POPC/POPS vesicles and Ca^{2+}-containing buffer defect-free SLBs could unfortunately only be formed on the sensor surface for POPC concentrations below 25% (data not shown). For those bilayers no effect on buffer exchange was observed. Since the large changes in behaviour upon Ca^{2+} addition/removal occurred only above 25% POPG this experiment provided no further insight. We thus do not exclude that a similar effect as for POPG may occur for higher fractions of POPS, if such bilayers could be formed.

Variation of Ca^{2+}-concentration in the buffer

Based on our results above, especially the lower dissipation shifts in the presence of Ca^{2+}-ions, indicating a lipid assembly which is more firmly attached to the surface, it is likely that the Ca^{2+}-ions form bridge bonds between the negatively charged lipid headgroup and the negative sites on the SiO_2 surface. In order to estimate how strongly Ca^{2+} is associated with the SLB, experiments were performed where the relation between the Ca^{2+}-concentration and the QCM-D signals were investigated. In Fig. 4 the dissipation shifts ΔD_{2-1} are shown as a function of Ca^{2+}-concentration for a mixed bilayer containing 50% POPC and 50% POPG. In other words, the dissipation shift ΔD_{2-1} after buffer exchange from TRIS buffer containing 10 mM $CaCl_2$ to TRIS buffer containing x.x mM $CaCl_2$ (step (ii) Fig. 2 but varying the $CaCl_2$ concentration) is shown. This presentation has been chosen to estimate the dissociation constant for Ca^{2+}-ions in the bridging site between the SLB and the SiO_2 surface. In Fig. 4

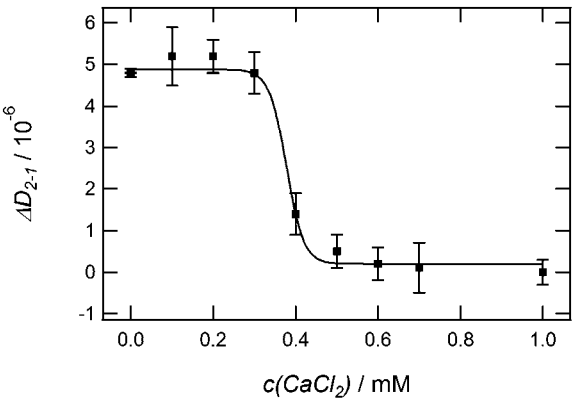

Fig. 4 As shown in Fig. 3 addition/removal of Ca^{2+}-ions from solution has a large effect on the dissipation shift ΔD_{2-1} for a bilayer composed of 50% POPC and 50% POPG. This graph shows how this change of dissipation shift ΔD_{2-1} depends on the actual Ca^{2+}-concentration. Average dissipation shift ΔD_{2-1} *versus* the fraction of concentration of $CaCl_2$ after buffer exchange from TRIS buffer containing 10 mM $CaCl_2$ to TRIS buffer containing x.x mM $CaCl_2$ (step (ii) Fig. 2 but varying the $CaCl_2$ concentration) for SLBs composed of 50% POPC and 50% POPG.

we can clearly observe a dependency of ΔD_{2-1} on the Ca^{2+}-concentration following a sigmoidal behavior, indicating a critical concentration of Ca^{2+}-ions for the observed phenomenon to occur. Analysis of these data revealed an estimate of the dissociation equilibrium constant, $K_d = 0.38$ mM, for the interaction between Ca^{2+} and the SLB. Using Gibbs free energy $dG = R \cdot T \cdot lnK_d$, with $R = 8.31$ J mol^{-1} and $T = 297$ K, the average binding energy for Ca^{2+}-ions to the bridging binding site is ~0.2 eV which corresponds to a time constant of the order of 0.1–1 ns, assuming a preexponential factor for unbinding of the order of 10^{-13} s. This time constant, or exchange rate, is much faster than the time resolution of our experiments.

These results strongly support our assumption that the observed phenomenon is related to Ca^{2+}-ions in buffer solution affecting the SLB structure and/or its interaction with the substrate and/or other properties (see Discussion).

Combined QCM-D/reflectometry

The above results clearly show that the viscoelastic properties of a POPC/POPG SLB depend on both the fraction of POPG in the SLB and on the concentration of Ca^{2+}-ions in buffer solution. As already pointed out, due to the reversibility of the QCM-D signals caused by addition/removal of Ca^{2+}-ions, there is no net loss of lipids, but rather a reversible structural rearrangement of the SLB, causing a change in the acoustically coupled mass and in the dissipative properties of the SLB. As previous studies have shown the employment of optical techniques[4] is a very useful approach to shine more light on such situations, and we therefore included measurements with a combined QCM-D/reflectometry instrument, where the QCM-D and reflectometry signals are measured on the same sensor surface simultaneously. The reflectometry signal ΔR obtained is not only sensitive to the refractive index n of the adsorbed material but also to the thickness d of the adsorbed layer (eqn (2)).

Fig. 5 shows QCM-D and reflectometry *versus* time curves for the frequency and dissipation shifts as well as for the reflectometry signal. Fig. 5A depicts the response of a POPC/POPG (50% POPC and 50% POPG) SLB upon buffer exchange, while Fig. 5B, for comparison, shows the behavior of a POPC SLB. The QCM-D curves in Fig. 5A reproduce the observations described above in Fig. 2. In the first step (i), the SLB is formed, represented by the characteristic behavior of the frequency and dissipation shifts. During bilayer formation, the reflectometry signal increases monotonically and eventually stabilizes at 0.011. In the second step (ii) Ca^{2+}-containing buffer is replaced by Ca^{2+}-free buffer, leading to an increasing and high dissipation shift as described above. The change in the reflectometry signal is sizable and negative, down to 0.008 (around 30% decrease) upon buffer exchange in this case. When Ca^{2+}-free buffer is replaced by Ca^{2+}-containing buffer again (step (iii)) the reflectometry signal turns back to close to the initial value of the bilayer 0.010. This behavior is surprising because, as we noted above, there is no mass change since the Δf and ΔD signals are reversible upon buffer exchange. In contrast no/very small shifts are observed in the dissipation and frequency and reflectometry signals in the case of the pure POPC SLB (Fig. 5B), when Ca^{2+}-ions are removed/added back. The latter shows that the change in the reflectometry signal in Fig. 5A is not an effect caused by optical changes of the bulk solution, but due to a change in the optical, and associated (based on the dissipation signal) structural properties of the POPC/POPG SLB.

Discussion

The main finding of the present study is the reversible effect of Ca^{2+}-ions on the dissipative (indicating structural and/or frictional changes of the SLB) and optical properties of a mixed lipid bilayer composed of POPC and POPG. We found that this effect depends on both the concentration of Ca^{2+}-ions in buffer and on the fraction of POPG in the SLB. Specifically, the changes occur when a mixed POPC/POPG bilayer, formed in Ca^{2+}-containing buffer, is exposed to the same buffer but without Ca^{2+}-ions, and when the POPG fraction in the bilayer is 35–50%. At higher POPG concentrations mixed SLB and intact vesicles were formed, making them less interesting for the present type of study. At POPG concentrations of 25% or below, addition/removal of Ca^{2+} causes no changes and the SLB behaves similarly to a plain POPC bilayer. Furthermore, at sufficiently high Ca^{2+}-concentrations the SLB is also not significantly affected by the buffer change, as judged by the QCM-D responses.

These results lead us to propose that the observed effect is related to an interaction between the negatively charged lipid headgroup, the positively charged Ca^{2+}-ions and the negatively charged SiO_2 support. The question then arises what the more detailed nature of this interaction is and if/how the observed reversible changes in the ΔD and reflectometry signals can be explained.

Mechanistic scenarios

Based on the known ability of Ca^{2+}-ions to modify both the properties of a lipid membrane itself, and its interactions with a substrate (SiO_2 in this case) (see Introduction) we can identify several possible mechanistic scenarios to explain the QCM-D and reflectometry signals observed in the present work. We consider the following possible mechanisms, which to some

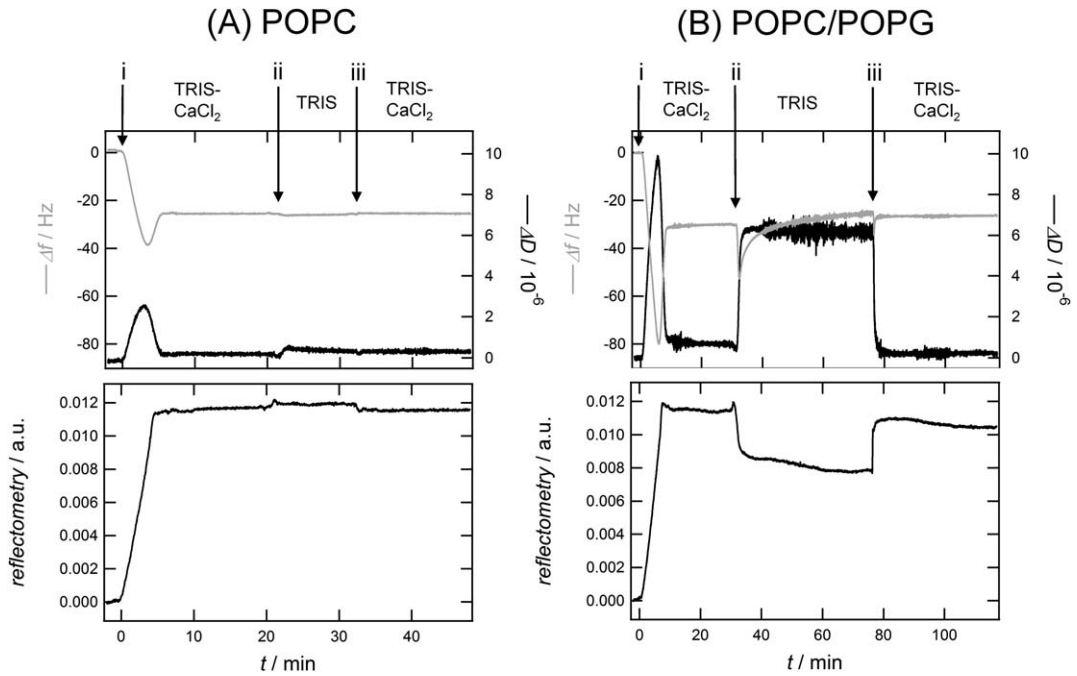

Fig. 5 The upper panels show, for two lipid compositions, dissipation and frequency shifts (the latter normalized to the fundamental frequency) as functions of time during bilayer formation and subsequent removal and addition of Ca^{2+}-ions in solution. The lower panels show the corresponding reflectometry signals. The measurements were made simultaneously on the same surface in a combined QCM-D/reflectometry instrument. (i) First a SLB was formed by vesicles containing (A) POPC and (B) 50% POPG and 50% POPC in TRIS-$CaCl_2$ (10 mM TRIS, 100 mM NaCl, 10 mM $CaCl_2$, pH 8) followed by buffer change to (ii) TRIS (10 mM TRIS, 100 mM NaCl, pH 8) and (iii) TRIS-$CaCl_2$. Note that for the pure POPC bilayer there is no change in any of the signals upon the removal or addition of Ca^{2+}-ions. In contrast there are large changes in the dissipation and reflectometry signals upon the removal or addition of Ca^{2+}-ions.

extent are connected and can be combined to explain the overall observed behavior (Fig. 6 illustrates some aspects of these mechanisms):

I. Removal of Ca^{2+}-ions causes a geometric change of the SLB in the sense that it abandons a flat SLB appearance, and takes a more undulated or other complex shape. This would by necessity involve a larger surface area, and either more lipid mass (supported by neither the QCM-D frequency shift nor the reflectometry signal) or a thinning of the bilayer, compensating for the area increase.

II. Addition/removal of Ca^{2+}-ions causes an internal structural change of the SLB itself, still with maintained flatness, but making it more dissipative in the absence of Ca^{2+}-ions.

III. A change primarily in the interfacial water layer between the SLB and the substrate.

IV. A mechanism involving I, and/or II and/or III which leads to slip, and associated interfacial friction between the SLB and the SiO_2 surface, in the absence of Ca^{2+}-ions.

In the formulation and discussion of these mechanisms the underlying assumption is that Ca^{2+}-ions are involved primarily *via* their bridging ion action (I, III, IV), and/or *via* their effects on the structure and stability of the SLB itself (II). In the discussion we first analyze to what extent those mechanisms, or combinations of them, may explain the observed frequency, dissipation and reflectometry signals. We then make a synthesis to identify the most likely mechanistic scenario.

I. Geometric changes. SLBs on a planar substrate are in general regarded to be flat or planar. However, undulations of

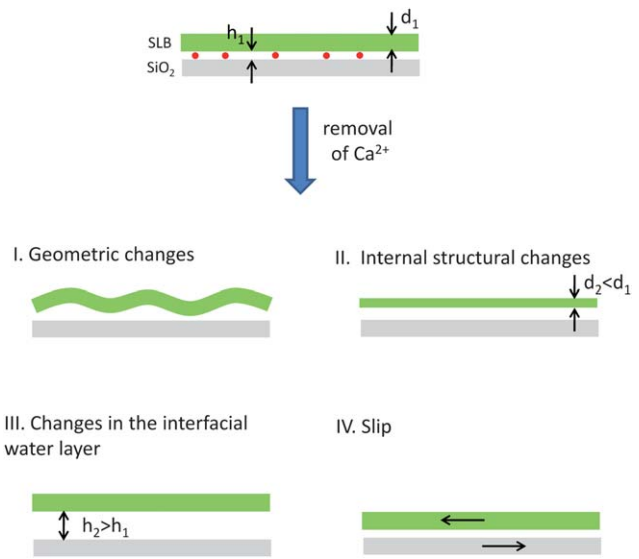

Fig. 6 Schematic drawing of the possible scenarios discussed in the text. The upper panel illustrates the bilayer in the presence of sufficient concentration of Ca^{2+}-ions (red dots), namely a well attached and planar bilayer. The four lower panels illustrate four possible mechanisms contributing to the measured signals when Ca^{2+}-ions are removed/added. *I.* Undulation of the bilayer upon the removal of Ca^{2+}-ions causing weakened interaction between the bilayer and the SiO_2 substrate. *II.* Internal structural changes in the bilayer. *III.* Changes in the amount and structure of the interfacial water layer. *IV.* Slip between the substrate and the bilayer.

SLBs on planar substrates have been reported.[40] An interplay of different forces between lipid headgroups and the substrate may lead to such undulations. (Note that our specific case deals with an SLB composed of two different kinds of lipids, whose headgroups interact differently with the SiO_2 substrate.) Recently, the formation of supported bacterial lipid membranes on TiO_2 was reported by Merz *et al.* using QCM-D, fluorescence recovery after photobleaching (FRAP) and optical waveguide light mode spectroscopy (OWLS).[41] Interestingly, the QCM-D signal of the E. coli SLB was rather atypical, *i.e.* $\Delta f < -50$Hz (compared to the "normal" value of 27 Hz) and $\Delta D > 2.5 \times 10^{-6}$, pointing to the possible existence of a heterogeneous system of adsorbed vesicles and SLB. However, complementary measurements using FRAP and OWLS suggested that a complete E. coli SLB was formed on TiO_2. The group proposed the observed high frequency and dissipation shifts of the QCM-D signal to be due to undulations of the E. coli SLB. Note that, in the present study, we observe a high dissipation shift accompanied by no/low frequency shift making undulation, with an accompanying increase in mass per unit area, less likely to be the main cause for the observed QCM-D signals. Furthermore the question arise where the additional bilayer surface comes from, the surface of an undulating bilayer being larger than the surface of a flat SLB. It is possible that only a central area is probed on the sensor and that additional material is accessible from the peripheral regions of the sensor which are not probed. However, additional lipid material is not necessarily required for the undulating SLB. A thinning of the SLB (see below), *i.e.* an increase of the distance of the lipid molecules would lead to an increase of the surface of the SLB.

We assume undulation less likely to be the main cause for the observed QCM-D signal, however undulation combined with some other effect, like internal structural changes or slip (see below) cannot be ruled out.

II. Internal structural changes. In a recent study Mashaghi *et al.*[9] showed by means of dual polarization interferometry (DPI) that the refractive index and the thickness of a SLB depends on the lipid composition and on Ca^{2+}-ions in solution. In the case of lipid bilayers composed of POPC and POPS (ratio 8 : 2) only small effects of Ca^{2+}-ions (2 mM $CaCl_2$) on the refractive index and the thickness were observed, whereas strong effects were observed when dioleoyl-*glycero*-phospho-lipids (two unsaturated alkyl chains) were used instead of palmitoyl-oleyl-*glycero*-phospho-lipids (one unsaturated and one saturated alkyl chain). Both thickness and refractive index of DOPC bilayers were higher in Ca^{2+}-buffer than in Ca^{2+}-free buffer. In the case of a mixed bilayer, *i.e.* 80% DOPC and 20% DOPS, made in Ca^{2+}-buffer, thickness and refractive index were highest compared to DOPC bilayers.

Since the reflectometry signal is sensitive to both, the refractive index and the thickness of the adsorbed material/layer (eqn (2)) such changes in thickness and refractive index, as described by Mashaghi *et al.* may explain the observed changes in the reflectometry signal for a POPC/POPG SLB upon buffer exchange. Using eqn (2), assuming the sensitivity factor A to be constant, our analysis shows that the observed change in the reflectometry signal would correspond to a change of the refractive index from 1.49 (10 mM $CaCl_2$) to 1.45 (0 mM $CaCl_2$) if the thickness of the SLB is constant. On the other hand, assuming the refractive index to be constant, the observed change would correspond to a change of the thickness of the SLB from 4.5 nm (10 mM $CaCl_2$) to 3.3 nm (0 mM $CaCl_2$). The thickness of SLBs commonly varies between 3.8 and 5.8 nm depending on lipid composition and bulk solution.[5,9,42] Thus, thinning of the SLB to 3.3 nm seems too large to be a reasonable explanation. However, a combination of a more reasonable thinning of the bilayer and a lower refractive index in Ca^{2+}-free buffer could lead to the observed change of the reflectometry signal.

III. Changes in the interfacial water layer. Between a solid supported lipid bilayer and the underlying substrate a complex interplay exists including van der Waals, electrostatic, hydrophobic and steric interactions. As a result a multimolecular thin layer of water (often quoted as 1–2 nm) is trapped between the bilayer and the substrate.[43–45] It is reasonable to assume that that the distance between the SLB and the substrate depends on both the composition of the lipid bilayer, the substrate, and bulk solution, in particular on the concentration and type (monovalent and/or multivalent) of cations. As described above Ca^{2+}-ions are known to act as bridging ions between a negatively charged lipid headgroup and a negatively charged surface, *i.e.* the bilayer is linked to the substrate *via* Ca^{2+}-ions. Removal of Ca^{2+}-ions will result in a removal, or rather weakening (since monovalent ions and other interaction forces remain), of the linkage. As a result decreased electrostatic attractive forces between the negatively charged bilayer and the negatively charged SiO_2 sensor surface may lead to an increase in the thickness of the water layer. Such a difference or change of the thickness of the water layer, or in other words of the amount of trapped water, should be reflected in the QCM-D signal as an increase in frequency and dissipation shifts. We should note here that such a repulsive interaction must be stronger for the POPG lipids compared to the POPC lipids, a factor that could even lead to phase separation between the two types, which in turn could be one factor causing undulation of the bilayer. However, we have no evidence for such phase separation.

IV. Slip. An alternative, not very common mechanism that could explain the large dissipation increase upon Ca^{2+}-ion removal, is the appearance of friction between the SLB and the substrate, in the absence of Ca^{2+}-ions, *i.e.*, a slip mechanism, where the SLB upon the shear motion of the substrate, does not follow the substrate perfectly but slips on it, and thereby causes frictional losses, appearing in the dissipation signal. This mechanism would have to be associated with the POPG lipids (no effect was seen for pure POPC). It would be consistent with a weak, or even net repulsive, coupling between POPG and SiO_2 in the absence of Ca^{2+}-ions, and with critical concentrations of both POPG and Ca^{2+}-ion concentration.

Proposed Scenario

For all reported QCM-D measurements on SLBs hitherto the reported dissipation has been small if a typical frequency shift (27 Hz) was observed. Our finding of a high dissipation accompanied by a typical frequency shift for an SLB is thus a rather remarkable situation. However, we also note that a very special situation is at hand; the observed phenomenon only occurs for

a mixed bilayer for specific range of POPG fractions in the SLB (the remaining part being POPC) and for Ca^{2+}-ion fractions below a critical value in the buffer. Specifically we suggest that the large dissipation shift occurs when there is a sufficiently high fraction of POPG headgroups in the SLB and a sufficiently low concentration of Ca^{2+}-ions in the buffer to reduce the number of Ca^{2+}-bridge bonds to a low number, causing decoupling of POPG head groups from surface. In this situation only the POPC head groups keep the SLB in place on the surface. If intact liposomes containing POPG only are adsorbed to the sensor surface in Ca^{2+}-containing buffer, those liposomes consequently detach upon removal of Ca^{2+}-ions (Table 2).

There might even be a phase separation between POPC and POPG lipids in the leaflet facing the substrate, and/or an asymmetric distribution of POPC and POPG lipids between the leaflet facing the substrate and the leaflet facing bulk, but for this we have no proof, and it is not necessary for the proposed mechanism. Internal structural changes, and associated dielectric changes, of the lipid bilayer caused by removal of Ca^{2+}-ions, are likely to be the main reason for the observed reflectometry signal, but do not provide an explanation for the observed high dissipation shift of the QCM-D signal. The tentative mechanism that we propose is, that a sufficiently large number of POPG lipids in the SLB, and a sufficiently low Ca^{2+}-ion concentration, leads to a large fraction of the bilayer decoupled from the surface, which in turn causes the whole bilayer to slip relative to the substrate, as the latter moves back and forth in its oscillatory motion.

To our knowledge there are only a few studies reporting slip at a solid-liquid interface[46–49] measured with QCM-D and no studies reporting slip of a lipid bilayer. Recently, Vittorias *et al.* studied the mechanical microcontacts of fine particles with QCM.[46] Using a modified QCM set up varying the driving amplitude of the oscillation of the QCM sensor the group identified slip of the micro particles shown in a dependency of the frequency and bandwidth (which corresponds to dissipation) on the driving amplitude. If the driving amplitude is changed, changes in the frequency and dissipation shifts are observed in case of slip/friction. In the case of a viscoelastic contact (Voight element), the frequency shift and dissipation are amplitude independent, *i.e.* constant if the driving amplitude is changed.

The type of experiments described by Vittorias *et al.* seemed promising to directly prove slip of the lipid bilayer in the present study. Thus the experiment in Fig. 2, was repeated for different driving amplitudes using a dual-frequency QCM-D setup as described previously by Edvardsson *et al.*[38] Fig. 7 shows frequency and dissipation shifts monitored at the 7th overtone *versus* the driving amplitude varied at the fundamental frequency between 50 mV and 6 V for a POPC/POPG bilayer in Ca^{2+}-containing (Fig. 7A) and in Ca^{2+}-free (Fig. 7B) buffer. In Ca^{2+}-containing buffer frequency and dissipation shifts are constant over the amplitude range of 50 mV to 6 V, *i.e.* the SLB can be described by a Voight model. However, in a Ca^{2+}-free buffer the frequency shift increases and the dissipation shift decreases when the driving amplitude is raised indicating slip/friction of the SLB. This result compared to studies of Vittorias *et al.* supports our assumption that the POPC/POPG bilayer slips in Ca^{2+}-free buffer.

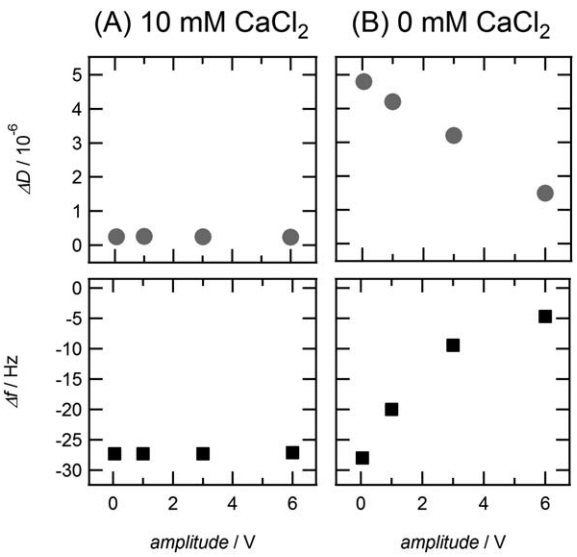

Fig. 7 Dissipations and frequency shifts for a lipid bilayer measured as functions of the oscillation amplitude of the QCM-D sensor surface. The bilayer was composed of 50% POPC and 50% POPG. The left panels (A) are for a Ca^{2+}-containing buffer. The right panels (B) are for a Ca^{2+} free buffer. The dissipation shifts (top) and frequency shifts (bottom) were monitored at a fixed small oscillation amplitude at the 7th overtone (normalized to the fundamental frequency). The oscillation amplitude was varied at the fundamental frequency.

Conclusions

We have demonstrated that the viscoelastic and optical properties of SLBs containing POPG are sensitive to Ca^{2+}-ions in bulk solution. In particular a dependency of the observed effect on fraction of POPG in the bilayer and on Ca^{2+}-concentration was observed. We propose the observed phenomenon to be a combination of different effects like geometric changes, internal structural changes, changes in the interfacial water layer and a slip mechanism. The later mechanism is supported by our findings obtained with dual-frequency QCM-D.

Acknowledgements

Financial support provided by Swedish Research Council (Linneus program SUPRA) is gratefully acknowledged.

References

1 C. A. Keller, K. Glasmastar, V. P. Zhdanov and B. Kasemo, *Phys. Rev. Lett.*, 2000, **84**, 5443–5446.
2 R. Naumann, S. M. Schiller, F. Giess, B. Grohe, K. B. Hartman, I. Karcher, I. Koper, J. Lubben, K. Vasilev and W. Knoll, *Langmuir*, 2003, **19**, 5435–5443.
3 E. Reimhult, M. Zäch, F. Höök and B. Kasemo, *Langmuir*, 2006, **22**, 3313–3319.
4 G. Wang, M. Rodahl, M. Edvardsson, S. Svedhem, G. Ohlsson, F. Höök and B. Kasemo, *Rev. Sci. Instrum.*, 2008, **79**, 075107.
5 M. Edvardsson, S. Svedhem, G. Wang, R. Richter, M. Rodahl and B. Kasemo, *Anal. Chem.*, 2009, **81**, 349–361.
6 E. Briand, M. Zach, S. Svedhem, B. Kasemo and S. Petronis, *Analyst*, 2010, **135**, 343–350.
7 A. Janshoff and C. Steinem, *Anal. Bioanal. Chem.*, 2006, **385**, 433–451.
8 C. Steinem, A. Janshoff, W. P. Ulrich, M. Sieber and H. J. Galla, *Biochim. Biophys. Acta, Biomembr.*, 1996, **1279**, 169–180.

9 A. Mashaghi, M. Swann, J. Popplewell, M. Textor and E. Reimhult, *Anal. Chem.*, 2008, **80**, 3666–3676.
10 J. Jass, T. Tjarnhage and G. Puu, *Biophys. J.*, 2000, **79**, 3153–3163.
11 R. Richter, A. Mukhopadhyay and A. R. Brisson, *Biophys. J.*, 2003, **85**, 3085–3047.
12 S. Garcia-Manyes, G. Oncins and F. Sanz, *Electrochim. Acta*, 2006, **51**, 5029–5036.
13 S. Garcia-Manyes, L. Redondo-Morata, G. Oncins and F. Sanz, *J. Am. Chem. Soc.*, 2010, **132**, 12874–12886.
14 I. Reviakine and A. R. Brisson, *Langmuir*, 2000, **16**, 1806–1815.
15 R. Richter, B.R. and A. R. Brisson, *Langmuir*, 2006, **22**, 3497–3505.
16 A. Janshoff, C. Steinem, M. Sieber and H.-J. Galla, *Eur. Biophys. J.*, 1996, **25**, 105–113.
17 K. Glasmästar, C. Larsson, F. Höök and B. Kasemo, *J. Colloid Interface Sci.*, 2002, **246**, 40–47.
18 R. Richter, J. L. K. Him, B. Tessier, C. Tessier and A. R. Brisson, *Biophys. J.*, 2005, **89**, 3372.
19 G. E. Rydell, A. B. Dahlin, F. Höök and G. Larson, *Glycobiology*, 2009, **19**, 1176–1184.
20 A. Wikström, S. Svedhem, M. Sivignon and B. Kasemo, *J. Phys. Chem. B*, 2008, **112**, 14069–14074.
21 A. Kunze, S. Svedhem and B. Kasemo, *Langmuir*, 2009, **25**, 5146–5158.
22 A.-S. Andersson, K. Glasmästar, D. Sutherland, U. Lidberg and B. Kasemo, *J. Biomed. Mater. Res.*, 2003, **64**, 622–629.
23 S. Svedhem, D. Dahlborg, J. Ekeroth, J. Kelly, F. Höök and J. Gold, *Langmuir*, 2003, **19**, 6730–6736.
24 A. A. Brian and H. M. McConnell, *Proc. Natl. Acad. Sci. U. S. A.*, 1984, **81**, 6159–6163.
25 M. Sundh, S. Svedhem and D. S. Sutherland, *Phys. Chem. Chem. Phys.*, 2010, **12**, 453–460.
26 F. F. Rossetti, M. Textor and I. Reviakine, *Langmuir*, 2006, **22**, 3467–3473.
27 A. McLaughlin, C. Grathwohl and S. McLaughlin, *Biochim. Biophys. Acta, Biomembr.*, 1978, **513**, 338–356.
28 R. A. Böckmann and H. Grubmüller, *Angew. Chem., Int. Ed.*, 2004, **43**, 1021–1024.
29 H. Binder and O. Zschörnig, *ChemPhysLipids*, 2002, **115**, 39–61.
30 M. P. Nieh, T. A. Harroun, V. A. Raghunathan, C. J. Glinka and J. Katsaras, *Biophys. J.*, 2004, **86**, 2615–2629.
31 W. Tamura-Lis, E. J. Reber, B. A. Cunningham, J. M. Collins and L. J. Lis, *ChemPhysLipids*, 1986, **39**, 119–124.
32 P. M. Dove and C. M. Craven, *Geochim. Cosmochim. Acta*, 2005, **69**, 4963–4970.
33 A. Berquand, D. Levy, F. Gubellini, C. Le Grimellec and P. E. Milhiet, *Ultramicroscopy*, 2007, **107**, 928–933.
34 B. Seantier and B. Kasemo, *Langmuir*, 2009, **25**, 5767–5772.
35 C. A. Keller and B. Kasemo, *Biophys. J.*, 1998, **75**, 1397–1402.
36 J. C. Dijt, M. A. Cohen Stuart and G. Fleer, *Adv. Colloid Interface Sci.*, 1994, **50**, 79–101.
37 T. Roques-Carmes, F. Membrey, C. Filiâtre and A. Foisy, *J. Colloid Interface Sci.*, 2002, **245**, 257–266.
38 M. Edvardsson, M. Rodahl, B. Kasemo and F. Hook, *Anal. Chem.*, 2005, **77**, 4918–4926.
39 E. Reimhult, F. Höök and B. Kasemo, *Langmuir*, 2003, **19**, 1681–1691.
40 R. Hirn, T. M. Bayer, J. O. Radler and E. Sackmann, *Faraday Discuss.*, 1999, **111**, 17–30.
41 C. Merz, W. Knoll, M. Textor and E. Reimhult, *Biointerphases*, 2008, **3**, 41–50.
42 Z. Salamon, G. Lindblom and G. Tollin, *Biophys. J.*, 2003, **84**, 1796–1807.
43 C. M. Ajo-Franklin, C. Yoshina-Ishii and S. G. Boxer, *Langmuir*, 2005, **21**, 4976–4983.
44 P. Fromherz, V. Kiessling, K. Kottig and G. Zeck, *Appl. Phys. A: Mater. Sci. Process.*, 1999, **69**, 571–576.
45 V. Kiessling and L. K. Tamm, *Biophys. J.*, 2003, **84**, 408–418.
46 E. Vittorias, M. Kappl, H. J. Butt and D. Johannsmann, *Powder Technol.*, 2010, **203**, 489–502.
47 D. Johannsmann, I. Reviakine and R. Richter, *Anal. Chem.*, 2009, **81**, 8167–8176.
48 M. Berglin, A. Olsson and H. Elwing, *Macromol. Biosci.*, 2008, **8**, 410–416.
49 H. Zhuang, P. Lu, S. P. Lim and H. P. Lee, *Anal. Chem.*, 2008, **80**, 7347–7353.