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Adhesion and rupture of liposomes mediated by electrostatic interaction monitored by thickness shear mode resonators

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Abstract Adhesion and spreading of negatively charged unilamellar vesicles composed of POPG/POPC and DPPG/DPPC on positively charged self-assembly monolayers of 11-amino-1-undecanethiol were monitored by means of thickness shear mode (TSM) resonators with a fundamental frequency of 5 MHz. Changes of frequency and motional resistance upon vesicle adsorption were recorded as a function of surface charge density and lyotropic phase state of the lipids. From the readout of the TSM resonator, changes of the shape of the vesicles as well as the formation of supported lipid bilayers can be inferred in a quantitative manner. Increasing surface charge densities on the vesicles, which are tunable by the POPG content, led to decreasing frequency and resistance changes. At very high PG content, a lower limit of 3–12 Hz was found, indicative of the formation of planar bilayers due to vesicle rupture induced by the strong electrostatic interaction forces. Vesicles composed of DPPG/DPPC were less susceptible to deformation and rupture, a fact that can be attributed to the higher bending rigidity of DPPG/DPPC liposomes. More than 70 mol% of DPPG were needed to induce adhesion-controlled rupture of surface-attached vesicles, while only 30–50% of POPG were sufficient to form planar lipid bilayers on the quartz.

Keywords 1,2-Dipalmitoylphosphatidylglycerol · Liposomes · Quartz crystal microbalance · 1-Palmitoyl-2-oleoylphosphatidylcholine · Thickness shear mode resonator

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Introduction

The attachment and spreading of cells on a surface in vitro is a complex and versatile process involving a large variety of molecules and interaction forces. The importance of the research originates from a broad range of biomedical applications, such as the development of new implant materials that are based on cells immobilized on solid substrates, creating a biologically active interface. The complex nature of the adhesion process, however, prevents a systematic study of the individual molecular contributions and their importance for cell adhesion. As a consequence, suitable model systems were developed based on unilamellar vesicles of variable size (25 nm–100 μm) equipped with functional units such as lipids bearing receptor molecules, charged headgroups, and proteins attached to functionalized self-assembly layers on flat surfaces (Sackmann 1996; Kloboucek et al. 1999; Sackmann and Bruinsma 2002).

Understanding the process of adhesion and rupture of vesicles on artificial surfaces is also attractive with respect to the development of solid-supported lipid bilayers for sensor applications. Solid-supported lipid bilayers are the ideal matrix to study the specific interaction of lipids with peptides and proteins. However, it remains to be elucidated how formation of solid-supported membranes can be controlled by vesicle composition and in particular how many anchor lipids are needed to achieve a high surface coverage with lipid bilayers (Steinem et al. 1996; Hubbard et al. 1998).

Pioneering work on this issue was done by Tamm and co-workers, employing total internal reflection fluorescence spectroscopy, and Galla and co-workers, using impedance spectroscopy (Kalb et al. 1992; Steinem et al. 1996). Thickness shear mode (TSM) resonators offer the possibility to study the process of vesicle spreading in great detail and to distinguish between deformed and ruptured liposomes. Höök and co-workers as well as Lüthgens et al. recently reviewed the applicability of the quartz crystal microbalance (QCM),

with emphasis on the impact of surface functionalization on vesicle adsorption (Lüthgens et al. 2003; Reimhult et al. 2003).

Recently, we studied the influence of molecular recognition units such as biotinylated lipids on the response of a functionalized thickness shear mode resonator with respect to the shape of the liposomes and rupture probability. We found that up to a concentration of 30 mol% biotinylated lipids the vesicles remain intact but deform considerably. This was corroborated by in situ scanning force microscopy. A higher biotin content leads to fast rupture and formation of supported planar lipid bilayers (Pignataro et al. 2000; Reiss et al. 2003). Here we report on the influence of charged lipids on the adhesion and spreading of large unilamellar vesicles (diameter = 1 μm) composed of either 1,2-dipalmitoylphosphatidylglycerol (DPPG) and 1,2-dipalmitoylphosphatidylcholine (DPPC) in the gel phase or 1-palmitoyl-2-oleoylphosphatidylglycerol (POPG) and 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) in the liquid crystalline phase. We could find distinct differences in the spreading and deformation properties of the vesicles depending on the mixture and nature of the lipids. Mixtures of gel phase lipids withstand the spreading to a higher extent than those in the liquid crystalline state, i.e. higher charge-densities are necessary to induce rupture when DPPG/DPPC vesicles are used instead of liposomes composed of POPG/POPC.

Materials and methods

Materials

Lipids were obtained from Avanti Polar Lipids (Alabasta, Ala., USA); 11-amino-1-undecanethiol was from Probior (Munich, Germany). The 5 MHz overtone polished AT-cut quartz crystals (diameter 14 mm, plano-plano, polished) were purchased from KVG (Neckarbischofsheim, Germany). Gold (99.99% purity) used for the gold electrodes on the surface of the quartz crystals was from Goodfellow (Bad Nauheim, Germany); chromium was purchased from Bal-Tec (Balzers, Liechtenstein).

Preparation of liposomes and surface chemistry

Phospholipids were used as purchased and dissolved in chloroform/methanol (1:3, v/v) to prepare films exhibiting different ratios of POPG/POPC and DPPG/DPPC, respectively. After removal of the solvent under a stream of nitrogen above the main phase transition temperature of the lipids, the films were dried under vacuum for 3 h at 60 °C. Large unilamellar vesicles (LUVs) were prepared from the films by the extrusion method (1000 nm nominal pore diameter) using a LiposoFast system from Avestin (Mannheim, Germany), as described elsewhere (MacDonald et al. 1991). The preparation was performed well above the main phase transition temperature of the highest melting lipid. The buffer solution was made of ultrapure water, 5 mM sodium citrate and 0.5 mM EDTA and adjusted to pH 5.0 by titration with diluted hydrochloric acid. The same buffer was used in the succeeding QCM and impedance spectroscopy experiments.

Surface functionalization was achieved via self-assembly of 11-amino-1-undecanethiol. After cleaning the gold electrodes in an argon plasma, 1 mM 11-amino-1-undecanethiol dissolved in ethanol was added and incubated for at least 30 min. Directly before use, the surface was rinsed extensively first with ethanol and subsequently with buffer solution to remove remaining ethanol and thiols.

Experimental setup

To examine the adhesion process of phospholipid vesicles on the functionalized gold surface, we made use of the QCM technique. The experimental setup for the measurements used in the present study is schematically depicted in Fig. 1. The setup basically consists of the quartz resonator, a flow system, and a phase-lock oscillator (PLO-10) from Maxtek (Santa Fe, Calif., USA) that allows monitoring frequency and resistance changes simultaneously. The 5 MHz quartz crystals were equipped on both sides with circular gold electrodes (diameter 5 mm). The quartz slides were first coated with 5 nm of chromium to provide an adhesive layer for the subsequent preparation of gold (100 nm). A detailed description is given elsewhere (Steinem et al. 1996).

The flow system exhibits a total volume of 2 mL pumped with a flow rate of 500 μL/min through the measuring cell. The cell is designed in stagnation flow-point geometry as shown in the inset of Fig. 1. To provide a constant temperature, the cell was placed in a climatic cabinet, which also served as a Faraday cage. All experiments were carried out at 25 °C. The phase-lock oscillator provides the resonance frequency and the resistance of the oscillator. The signals were read out by a frequency counter (Agilent 53181A) and a multimeter (Agilent 34401A) from Agilent Technologies (Palo Alto, Calif., USA) and were transferred via GPIB to a computer.

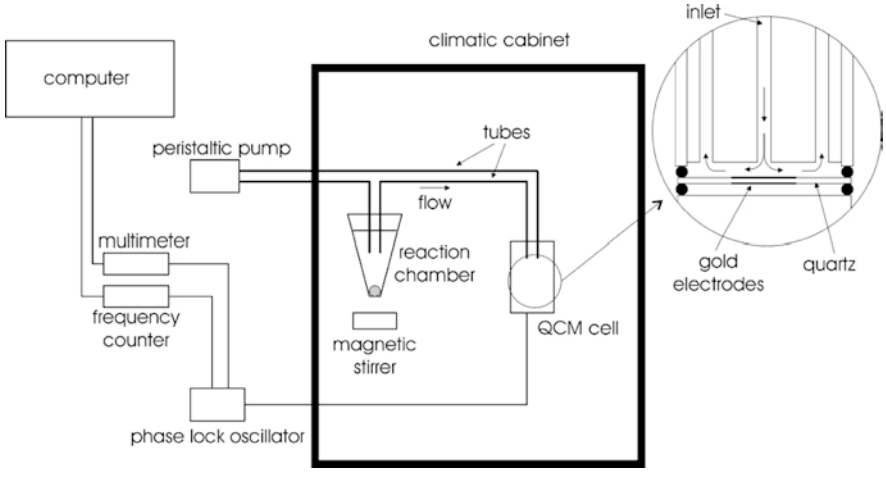

Fig. 1 Experimental setup of the quartz crystal microbalance (QCM). The magnification depicts the stagnation flow-point geometry of the QCM cell

Impedance spectroscopy

AC impedance spectroscopy was performed by a continuous wave impedance gain/phase analyzer SI 1260 from Solartron Instruments (Farnborough, UK). The magnitude of the impedance $|Z(f)|$ and the phase angle $\Phi(f)$ between current and voltage were recorded in the frequency range of 10^{-1}–10^{6} Hz with an AC amplitude of 30 mV and 0 mV DC offset. Sixty-one data points were sampled at logarithmic spacing by a personal computer. The electrochemical cell consists of the quartz crystal with one gold electrode serving as the working electrode and a reference electrode (DRI-REF 2) from World Precision Instruments (Sarasota, Fla., USA). Quantitative analysis of the spectra was performed by fitting the parameters of the assumed electrical model to the data ($|Z(f)|$) by means of a non-linear least-squares fit according to the Levenberg–Marquardt algorithm (Bevington 1969). Two different equivalent circuits were used for the fitting procedure (see Fig. 4A).

General measurement procedure

The experiments were performed first by functionalization of the gold surface with 11-amino-1-undecanethiol. The quality of the self-assembly monolayer was verified by impedance spectroscopy. Then, LUVs composed of POPG/POPC or DPPG/DPPC of varying composition were prepared and injected into the reaction chamber, resulting in an overall concentration of 50 μg/mL. Finally, the course of the resonance frequency and the motional resistance was monitored by the QCM as described above.

Results and discussion

Our main objective was to provide a well-controlled system that allows study of the adhesion and rupture of unilamellar vesicles on charged surfaces. This was achieved by using self-assembled monolayers consisting of 11-amino-1-undecanethiol chemisorbed on gold electrodes that were deposited on a thickness shear mode resonator exhibiting a fundamental frequency of 5 MHz. 11-Amino-1-undecanethiol was preferred over short-chain cysteamine because it enabled us to monitor monolayer formation by means of impedance spectroscopy, owing to its smaller capacitance, to ensure that the coverage of the surface is well over 90%.

Vesicles composed of a binary mixture of neutral lipids such as POPC or DPPC and phospholipids bearing negative charges, i.e. POPG or DPPG, were employed to study the impact of charge density and bending modulus on the deformation and spreading of adhered liposomes.

The adhesion of the vesicles on the charged surface at low ionic strength is mainly governed by coulombic interaction. Increasing the adhesive forces inevitably leads to stronger deformation of the vesicles on the surface and as a consequence generates an increasing edge stress (Seifert and Lipowsky 1990). It is expected that at a certain molar ratio (χ_{PG}) this stress eventually induces rupture of the adsorbed vesicles, causing the formation of a planar lipid bilayer.

Figure 2A exemplarily shows changes of the resonance frequency Δf and the motional resistance ΔR versus time for POPG/POPC vesicles with $\chi_{POPG} = 0.3$.

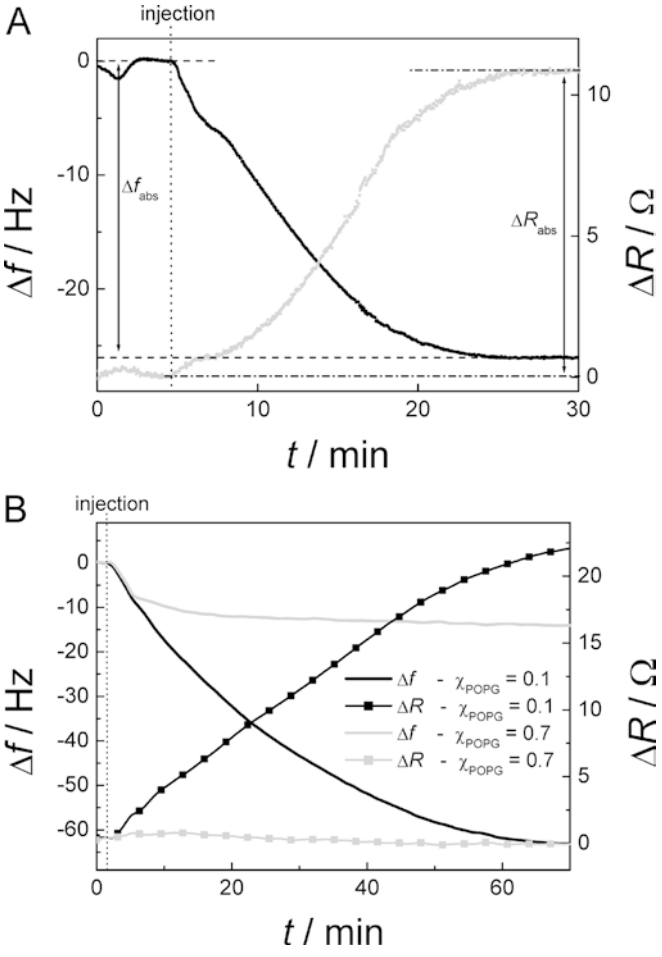

Fig. 2 **A** Course of the resonance frequency and the motional resistance after injection of POPG/POPC (3:7) vesicles. The frequency shift Δf_{abs} and the change in motional resistance ΔR_{abs} are extracted graphically from the raw data. **B** Comparison of the frequency shift and the change of the resistance for two different molar ratios of POPG in POPG/POPC vesicles. Note that high molar ratios of POPG lead to small frequency and resistance shifts

The absolute frequency shift Δf_{abs} and the change in resistance ΔR_{abs} are derived graphically from the diagram, since an adequate analytical scheme describing vesicle adhesion and spreading is still lacking. In Fig. 2B, two extreme situations are displayed. While at low PG content the frequency change is considerably large, we found a very small shift in resonance frequency if the molar ratio χ_{POPG} in POPG/POPC vesicles is increased above a certain level. Simultaneously, the resistance shows a significant change at low χ_{POPG}, whereas at high χ_{POPG} virtually no resistance change is found.

Because of the essentially equal molar masses of POPC and POPG, the large differences in Δf_{abs} and ΔR_{abs} have to be explained in terms of different adhesion forces of the vesicles as a function of surface charge density, accompanied by substantial deformation of the soft shells. Moreover, lipid vesicles as opposed to colloidal particles may rupture to form planar lipid bilayers, which are firmly attached to the solid support if the

adhesion becomes strong enough to laterally stretch the bilayers beyond a critical value. The energetics of vesicle spreading has been elucidated in great detail by Seifert (1997). From a mechanical point of view, the resulting planar lipid bilayers can be treated as a thin rigid film. This is the reason that two extreme situations can be distinguished in TSM measurements. On the one hand, vesicles are viscoelastic bodies, i.e. soft shells that generate a large frequency and resistance response if adhesion is moderate; and on the other hand, solid-supported lipid bilayers are best described as a thin organic film producing merely a little shift in resonance frequency and almost no shift in resistance, owing to a negligible loss of energy in the bilayer.

In general, the frequency response is a function of the thickness of the deposited material. A thicker layer with the same shear modulus will always result in larger shifts in frequency and resistance. Deviation from a simple linear relationship between frequency shift and changes in film thickness arises when viscoelastic bodies are involved. This has been extensively investigated by several groups and will not be reviewed here (Benes 1984; Reed et al. 1990; Granstaff and Martin 1994; Johannsmann 1999; Voinova et al. 2002; Lucklum and Hauptmann 2003). Moreover, the amount of deposited vesicles depends on the lateral dimensions, which in turn depend on the grade of deformation. For instance, roughly half as many vesicles can adsorb on a surface if the deformation (in the z-direction) is 90%.

Despite a missing simple analytical framework, the trend towards smaller shifts in resonance frequency and motional resistance with increasing adhesion forces should be visible. A thorough study of the TSM response as a function of electrostatic forces between the self-assembly layer and the vesicles should therefore provide a means to infer vesicle topology from the shear displacement measurements.

Figure 3A shows the average frequency shift versus the molar ratio of POPG in POPG/POPC vesicles. As mentioned before, the frequency shift becomes smaller with increasing POPG content. Up to a molar ration of $\chi_{POPG} = 0.3$, small changes in the vesicle composition already produce relatively large changes in Δf_{abs}. The average change in frequency drops from 176 Hz for neat POPC vesicles to 26 Hz for vesicles with $\chi_{POPG} = 0.3$. A further raise of the POPG content only slightly affects the frequency shift. The frequency shift ranges between 3 Hz and 12 Hz for $\chi_{POPG} > 0.5$. This finding is indicative of the formation of a planar lipid bilayer, whereas high-frequency shifts are due to the adhesion of intact vesicles to the surface displaying the deformation of liposomes as a function of negative charges. It can be assumed that there exists a transition regime around $\chi_{POPG} = 0.3$ (grey box in Fig. 3) where vesicles and bilayer coexist on the surface. This is supported on the one hand by earlier studies using scanning force microscopy (Kumar and Hoh 2000; Pignataro et al. 2000; Lüthgens et al. 2003). On the other hand, our AC impedance analysis reveals that, with increasing charge density, insulating bilayer patches can be detected.

The interpretation can be reasoned in terms of Sauerbrey's equation (Sauerbrey 1959), which is solely valid for thin rigid films:

$$\Delta f = -S_f \frac{\Delta m}{A} \tag{1}$$

where Δm denotes the mass uptake, A the electrode area, and S_f the Sauerbrey constant, which in our case is 0.057 Hz cm^2 ng^{-1}. Considering the average molar weight of the lipids as $M = 750$ g/mol and a surface headgroup area of 70 Å^2 per molecule (Bong et al. 2000), the theoretical frequency decrease that a defect-free lipid bilayer produces on a 5 MHz resonator is

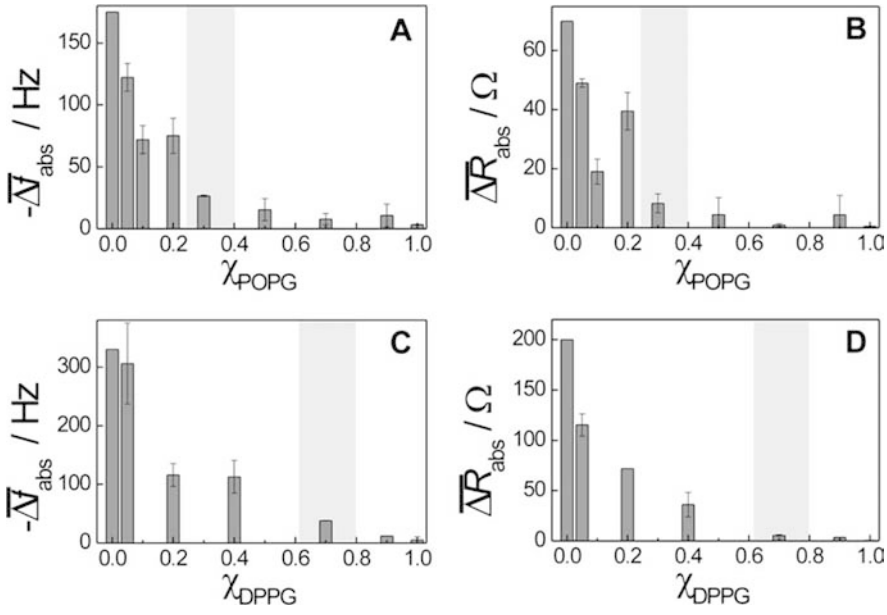

Fig. 3A–D Results of the QCM measurements. **A** Average frequency shift versus the molar ratio χ_{POPG} after adhesion of POPG/POPC vesicles. **B** Average change of the motional resistance for POPG/POPC vesicles. **C** Average frequency shift as a function of the molar ratio χ_{DPPG} in liposomes consisting of DPPG and DPPC. **D** Average change of the motional resistance after attachment of DPPG/DPPC vesicles to the surface

11.5 Hz. Frequency shifts of less than the calculated 11.5 Hz for a bilayer are probably due to incomplete coverage of the surface.

As the molar weights of POPC and POPG are only marginally different, large changes in Δf_{abs} cannot be explained by changes in the mass uptake. Therefore the average height of the vesicle layer determines the frequency shift. Less electrostatic interaction reduces the deformation and simultaneously increases the height of the vesicle layer. This explains the increasing frequency shift with diminishing POPG content. The trend in the change of the motional resistance supports this interpretation (Fig. 3B). Although the frequency response of planar lipid bilayers can be evaluated in a straightforward manner, it is far more difficult to describe the viscoelastic behavior of the soft shells on the surface. Voinova et al. (2002) as well as Höök and co-workers (Reimhult et al. 2003) used a Voight element to describe the viscoelastic behavior of liposomes, extending the theory of Reed et al. (1990), who described the response of a TSM resonator to viscoelastic load in terms of a one-dimensional model. As described by Lucklum and Hauptmann (2003), depending on the load situation, an "extra mass" or "missing mass" effect arises, which explains the deviation from Sauerbrey's law due to the unique combination of the shear-storage modulus G' and the shear-loss modulus G''. Voinova et al. (2002) claim that vesicles display a small "missing mass" effect, which is in accordance with our observations showing smaller frequency shifts as expected when assuming the vesicles behave as hard particles with zero G'' and large G'.

As a consequence, liposomes with higher bending rigidity ($\kappa_{DPPC} = 10.5\times10^{-19}$ J, $\kappa_{POPC} = 1.43\times10^{-19}$ J) (Frisken et al. 2000; Lee et al. 2001), such as DPPC and DPPG, are expected to be less easily deformed by adhesion forces and should therefore be distinguishable from the POPG/POPC system.

The higher bending modulus should lead to higher values of Δf_{abs} and ΔR_{abs} because vesicles are less deformed at a given charge density and therefore provide a larger film thickness. In addition, the vesicles remain intact on the surface at higher DPPG content than POPG/POPC liposomes. Figure 3C shows the TSM resonator's response due to the addition of DPPG/DPPC vesicles to a monolayer of 11-amino-1-undecanethiol. Mostly intact vesicles can be found on the surface up to $\chi_{DPPG} = 0.4$, the transition regime that is dominated by the coexistence of bilayer and vesicles reaches from $\chi_{DPPG} = 0.6$ to 0.8, and solely bilayer formation ($\Delta f_{abs} \leq 12$ Hz) appears not until $\chi_{DPPG} \geq 0.9$.

This is also obvious from the change in resistance (Fig. 3D). Comparing the lipids in different phase states (Fig. 3B and Fig. 3D) shows the expected behavior that gel phase lipids provide higher resistance shifts due to their higher bending modulus.

From the experiments it can be deduced that the TSM resonator is an excellent tool to probe vesicle deformation and to determine the critical adhesion energy that is needed to induce rupture. Vesicles containing lipids that are in the gel state and thus exhibit a larger bending modulus withstand deformation considerably better than vesicles from lipids in the liquid crystalline state. From these experiments, clear directions can be derived on how to obtain solid-supported membranes for a given liposome system. As a rule of thumb, vesicles rupture if at least 30% of lipids that interact strongly with the surface are present. This corresponds well to our earlier findings using biotinylated lipids (Pignataro et al. 2000) or lipids bearing a thiolated headgroup (Steinem 1997; Drexler and Steinem 2003). Rupture occurs if at least 30 mol% biotin-X-DHPE or 50 mol% DPPTE is present.

In order to provide additional evidence that solid-supported lipid bilayers were formed when the charge density is higher than the threshold value for liposome rupture, we employed impedance spectroscopy (IS). IS is particularly suited to find small defects in a dielectric layer on top of an electrode. Impedance spectra using the resonator's electrode as the working electrode and an additional reference electrode were taken before and after the QCM measurements, i.e. liposome addition. While the frequency-dependent impedance in the absence of a bilayer is best described by a simple serial combination of an ohmic resistance and a capacitance representing the self-assembled monolayer, we had to extend the circuit by a parallel *RC* network to account for the membrane resistance and capacitance if bilayer formation occurs. If the PG content is too small to induce vesicle rupture, no significant changes of the impedance spectra were found. Only if planar bilayers were formed we could detect the presence of the lipids in the spectra. Figure 4A shows the results of the impedance analysis in which the bilayer shows a decrease in capacitance from 3.31 to 1.21 $\mu F/cm^2$ and a membrane resistance of 35 $k\Omega\ cm^2$. The process of bilayer formation can also be time resolved by monitoring the change of the capacitance during the adhesion process. The time-dependent surface coverage $\Theta_M(t)$ can then be calculated from:

$$C_M(t) = \Theta_M(t) C_{M,theo} + \big(1 - \Theta_M(t)\big) C_G \qquad (2)$$

where $C_M(t)$ denotes the time-dependent membrane capacitance, C_G the capacitance of the 11-amino-1-undecanethiol monolayer, and $C_{M,theo}$ the membrane capacitance in the case of complete surface coverage (Sabatani et al. 1987). Assuming $C_{M,theo} = 0.6\ \mu F/cm^2$ (Steinem 1997), we observed maximal coverage of 80% for POPG/POPC and 90% for DPPG/DPPC (Fig. 4B).

Both frequency response of the TSM resonator and impedance analysis of bilayer formation confirm that vesicle spreading occurs at a high surface charge. Rupture of vesicles not only affects the maximal frequency shift but also influences the kinetics of the system. Saturation is reached faster if vesicles attach, deform and eventually spread to occupy a larger area as if they adsorb in a hard particle fashion. This is important,

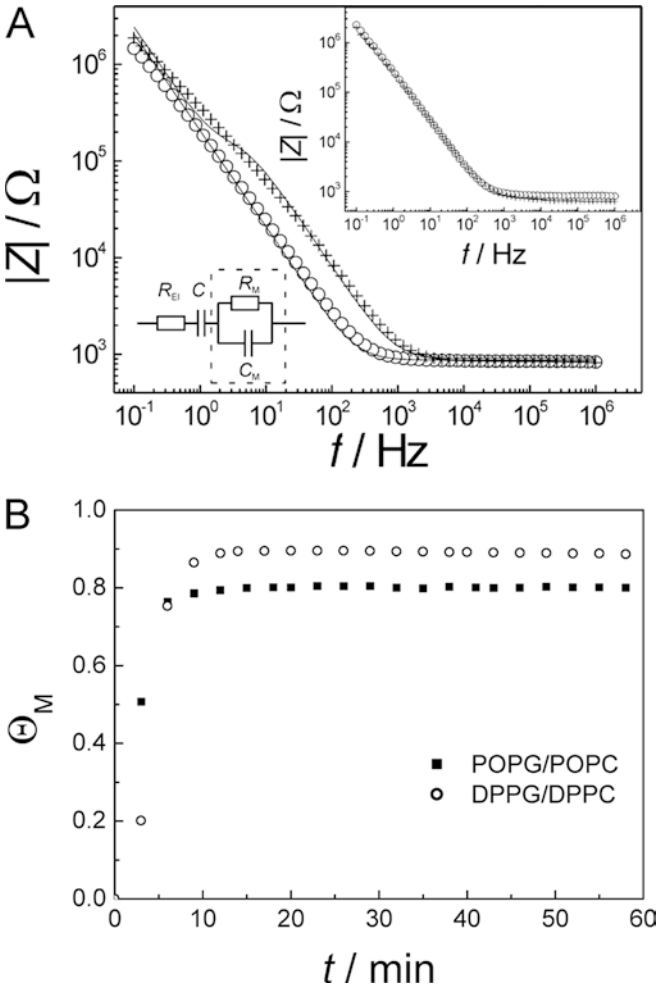

Fig. 4 **A** Impedance spectra of the self-assembled 11-amino-1-undecanethiol monolayer (*open circles*) and an additional POPG/POPC (9:1) bilayer (*plus signs*) formed due to vesicle rupture. The obtained parameters were $C = 3.3$ μF/cm^2 and $R_{El} = 871$ Ω for the monolayer and $C = 2.8$ μF/cm^2, $R_{El} = 862$ Ω, $C_M = 1.2$ μF/cm^2 and $R_M = 34750$ Ω cm^2 after formation of the bilayer (the index M marks the parameters of the membrane). The *inset* shows the impedance spectra for the self-assembled monolayer (*open circles*) and a layer of POPG/POPC (5:95) vesicles (*plus signs*) where virtually no change in membrane capacitance or resistance is visible. **B** Time-dependent coverage Θ_M of the functionalized surface with lipid bilayer during the incubation with POPG/POPC and DPPG/DPPC vesicles

especially if kinetic data are extracted from the transition regime, where intact vesicles and planar bilayers coexist.

Conclusions

TSM resonators can be used to determine the regime when vesicles rupture to form planar lipid bilayers. They can be used to tune the surface properties in order to provide a specific situation where liposomes are still intact but immobilized or are spread to planar lipid bilayers. We found that owing to the larger bending modulus of gel phase lipids such as DPPG/DPPC, deformation is reduced, as indicated by a larger frequency shift than in the case of POPG/POPC. However, it is possible to obtain planar bilayers from both systems.

The study shows that TSM resonators in conjunction with impedance spectroscopy provide a powerful tool to investigate the kinetics of adhesion and rupture of vesicles on functionalized surfaces. This renders the QCM an invaluable tool to study soft matter beyond pure detection of its presence on the surface.

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