# Effect of Cationic Lipids in the Formation of Asymmetries in Supported Bilayers

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ABSTRACT We have studied the formation of a supported bilayer containing both cationic and zwitterionic lipids by fusion of small unilamellar vesicles (SUV) onto the solid surface at low salt conditions using a combination of attenuated total reflection infrared (ATR-IR) and deuterium NMR spectroscopy with microcalorimetry. The data suggest that a significant cationic lipid asymmetry between the outer (distal) and the inner (proximal) monolayer of a supported bilayer results under conditions of prolonged incubation times of the solid support with the SUV coating solution. For a SUV composition of DPPC/DHDAB (4:1) we observed an enrichment of the cationic component in the proximal monolayer of up to 200% compared to the distal monolayer after 12 h incubation. It is suggested that the electrostatic potential arising from the solid surface is the driving force for the creation of this asymmetry by means of directed flip-flop between the monolayers and/or by temporary fusion between SUV from the bulk with the supported bilayer.

## INTRODUCTION

The coating of a solid surface with a bilayer in order to obtain a supported bilayer (Bayerl and Bloom, 1990; Tamm and McConnell, 1985) provides not only a highly biocompatible surface but also can be used to control the coupling of water-soluble proteins to the bilayer via its thermodynamic phase state (Loidl-Stahlhofen et al., 1996b). The coupling becomes highly charge-selective and dominated by Coulomb interactions when charged lipids (anionic or cationic) are used as minor constituents of the supported bilayer (Loidl-Stahlhofen et al., 1996a). However, due to the (mostly negative) charge of the solid surface, a complex Coulomb interaction pattern between bilayer and surface exists (Reinl and Bayerl, 1994) which can interfere with the protein coupling to the surface. The complexity of these interactions, particularly in the limit of short distances, hampers efforts to understand these protein-supported bilayer interactions on a molecular scale. One crucial question is whether the charged lipid constituents of a supported bilayer are symmetrically distributed between its two leaflets or if the solid surface charge gives rise to static or dynamic lipid asymmetries. Another question is about the technique used for the preparation of such bilayers and the possible creation of asymmetries. Owing to a lack of experimental techniques for detecting and quantifying the extent of lipid asymmetries in supported bilayers, none of the above questions has been satisfactorily addressed yet.

Very recently, we showed that solid-state NMR can clearly distinguish between the two monolayers of a supported bilayer and can measure lipid diffusivity in each monolayer separately (Hetzer et al., 1998). Combined with attenuated total reflection infrared (ATR-IR) spectroscopy to measure the total amount of each lipid in a supported bilayer (Reinl and Bayerl, 1994), NMR can be used to answer the above-mentioned questions.

Cationic amphiphiles with close structural resemblance to typical membrane-forming lipids, often also termed cationic lipids, are used for the functionalization of surfaces (Rädler and Sackmann, 1992), for wetting purposes (Rädler et al., 1995) and are crucial constituents of aggregates used for shuttling genes into cells (Monk and Cullis, 1997). In all these applications the cationic headgroup is essential for these functions. Since the surfaces of cells and plasma membranes as well as many solid surfaces used for functionalizations or wetting are mostly negatively charged, an attractive Coulomb potential dominates their interactions with cationic lipids. A number of cationic lipids have been studied with respect to their phase behavior in mixtures with zwitterionic synthetic lecithins in bilayers (Linseisen et al., 1996) and monolayers (Lee et al., 1990), their interactions with DNA (Mitrakos and MacDonald, 1996; Rädler et al., 1997), and with bilayers of other lipids and their wetting behavior on negatively charged solid surfaces (Rädler et al., 1995).

However, the question about possible solid surface-induced asymmetries in the cationic lipid content between the two monolayers of a supported bilayer is still open. The existence of such imbalances would not only affect the Coulomb interaction properties of the supported bilayer but would also create asymmetries in the lipid mixing properties between the monolayers, and thus change phase transition behavior of the supported bilayer. There is a biologically relevant background to this problem: since mixtures containing cationic lipids are often used for encapsulating DNA, the question arises whether the negatively charged DNA might induce asymmetries in its coating layer in a similar way as a charged solid surface may do. A similar situation may arise in cell membranes by the electrostatic interaction of cytoskeleton components with the bilayer lipids.

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We have selected the cationic lipids DMTAP and DHDAB in binary mixtures with zwitterionic synthetic lecithins (DMPC and DPPC) to study the formation of asymmetries in supported bilayers prepared by the widely used vesicle fusion technique (Tamm and McConnell, 1985). We were particularly interested in the effect of the incubation time during supported bilayer preparation on the formation and the extent of asymmetries. The combination of infrared and NMR techniques allowed us to measure cationic lipid asymmetry after both very short (10 min) and very long (12 h) incubations of the solid surface with the coating solution, which was made from small unilamellar vesicles having a given ratio of cationic and zwitterionic lipids.

## MATERIALS AND METHODS

### Materials

Selectively deuterated 1,2-dipalmitoyl-sn-glycero-3-phosphocholine-$d_8$ (DPPC-$d_8$), chain perdeuterated 1,2-dimyristoyl-sn-glycero-3-phosphocholine-$d_{54}$ (DMPC-$d_{54}$), and 1,2-dimyristoyl-3-trimethylammonium-propane (DMTAP) were obtained from Avanti Polar Lipids (Alabaster, AL), and dihexadecyl-dimethyl-ammonium-bromide (DHDAB) was purchased from Fluka (Buchs, Switzerland). Monodisperse nonporous silica beads were specially synthesized by Degussa AG, Department of Anorganic Chemistry (Hanau, Germany). Using electron microscopy, the beads were shown to be spherical with a diameter of 620 ± 40 nm. Deuterium-depleted water used in $^2$H-NMR experiments was from Isotec Inc. (Miamisburg, OH). The water used for all other experiments and preparations was purified with a Milli-Q System (Millipore Corp., Bedford, MA).

All lipid mixtures were prepared by dissolving appropriate amounts of dry phospholipids (DPPC-$d_8$/DHDAB and DMPC-$d_{54}$/DMTAP) in chloroform and evaporating the solvent by overnight vacuum desiccation. Multilamellar vesicles (MLV) were obtained by swelling the lipid mixtures in buffer A (20 mM HEPES, 20 mM NaCl, 0.5 mM EDTA; pH 7.0) and incubating them under gentle vortexing for 30 min at temperatures well above their phase transition temperature ($T_m$). Small unilamellar vesicles (SUV) were obtained by treatment of the MLV suspension with a titanium rod sonifier in a thermostatted water bath at temperatures 10°C above the $T_m$ of the mixture. Sonication was done at continuous power mode until the SUV solution appeared optically transparent in white light.

Spherical supported bilayers (SSB) and supported planar bilayers (SPB) were prepared by condensation of SUV onto the support surface. For SSB, silica beads of 640 nm diameter were washed in p.a. grade methanol, dried, and then resuspended in excess buffer A and degassed before the coating with the SUV solution. All other details of the SSB preparation were described previously (Köchy and Bayerl, 1993; Naumann et al., 1992). For NMR experiments, the water in the final SSB dispersion was substituted by deuterium-depleted water by a succession of three washing steps.

For SPB preparation, the two large faces of a germanium ATR plate (52 × 30 × 2 mm, 45° aperture mounted inside a homebuilt cell of ≈1.5 ml liquid volume (cf. Reinl and Bayerl, 1994 for details) were used as the solid support. Before the bilayer coating, the plate was hydrophilized by immersion in 2% SDS solution for 2 days followed by washing in purified water. For SPB formation, the SUV solution (2 ml volume, lipid concentration 2 mg/ml) was flushed into the cell while the temperature was maintained at 41°C, which was well above the $T_m$ of the SUV solution.

### Methods

Infrared spectra were obtained with a Perkin-Elmer System 2000 infrared spectrometer equipped with a TGS detector, a KRS-5 wire grid polarizer, and a vertical attenuated total reflection (ATR) unit to accommodate the above-mentioned cell. Typically, 16 scans were collected at polarizations parallel and perpendicular to the plate normal with a resolution of 2 $cm^{-1}$. ATR spectra of pure buffer recorded under identical conditions were used as a reference. The spectra were baseline-corrected and analyzed with Grams 3.01B software (supplied with the spectrometer). ATR cell temperature control by an external water bath was within ±0.2°C.

The ATR-cell temperature was 41°C to ensure that all the lipids in both SPB and SUV were in the fluid phase over the 12 h incubation time of the measurement. To distinguish between the signals from each lipid, we used chain perdeuterated DMPC-$d_{54}$ and took into account that the signal arising from the nondeuterated DMTAP (symmetric $CH_2$-stretching at 2852.5 $cm^{-1}$, asymmetric at 2923.0 $cm^{-1}$ for $T = 41$°C) chains exhibits a factor of 2.9 higher intensity than that from the same amount of deuterated DMPC-$d_{54}$ chains (symmetric $CD_2$-stretching at 2092.5 $cm^{-1}$, asymmetric at 2195.5 $cm^{-1}$ for $T = 41$°C) (Reinl and Bayerl, 1994). Polarization measurements allowed us to ensure that the detected signal resulted from SPB and not from bulk- or SPB-associated SUV. This is because the dichroic ratio differs for planar geometry significantly from that of spherical geometry. This was confirmed by checking the (unchanged) dichroic ratio of the SPB in the presence and absence of bulk vesicles (i.e., after flushing the ATR cell with buffer A solution to remove all SUV).

DSC measurements were performed using a Microcal MC-2 (Microcal Inc., North Hampton, MA). Both ascending and descending temperature modes were done at a scan rate of 30°C/h. To ensure that the system was in equilibrium, all samples were measured twice in succession and the second heating scan was used after baseline correction.

Deuterium NMR experiments were performed using a Bruker AMX-500 spectrometer equipped with a 10-mm solid-state probe at a temperature of 60°C. The spectra were obtained by applying a quadrupolar echo (QE) pulse sequence with two 90° pulses of 5.5 μs duration, a pulse separation of $\tau = 35$ μs, and a CYCLOPS phase cycling sequence. The repetition time was 150 ms and 4096 complex data points were collected in quadrature with a dwell time of 2 μs. The number of scans was 250,000 and the spectra were obtained by a one-dimensional Fourier transform starting at the top of the echo. Spectral deconvolution was achieved using the de-Pake-ing algorithm (Sternin et al., 1983).

## RESULTS

### Infrared spectroscopy

In a first set of experiments the time evolution of the lipid ratio of cationic DMTAP and zwitterionic DMPC-$d_{54}$ in a supported planar bilayer (SPB) was examined over an incubation time range of 12 h after the start of SUV fusion on the ATR crystal. We were interested in learning about the equilibrium ratio between the two lipid species in the SPB in comparison to the ratio in the SUV. This was done by flushing an excess amount of the SUV solution of known (binary) lipid composition into the ATR cell at $t = 0$ and measuring the methylene stretching vibration intensities of both lipids simultaneously as a function of incubation time.

From the data obtained at different times $t > 0$ the fraction of cationic DMTAP in the SPB formed was calculated (cf. Materials and Methods). The results are shown for the case of a SUV lipid ratio of 1:4 (DMTAP/DMPC-$d_{54}$) in Fig. 1. The time course clearly indicates an increase of the cationic DMTAP fraction in the SPB from the initial value of 20%, which was the same as in the SUVs to ~26% for both symmetric and asymmetric vibration modes. Over the same time, an increase of ~10% of the total lipid coverage of the ATR crystal was observed by monitoring the simultaneous increase of both the $CH_2$- and $CD_2$-stretching vibration intensities. This increase over the same time period

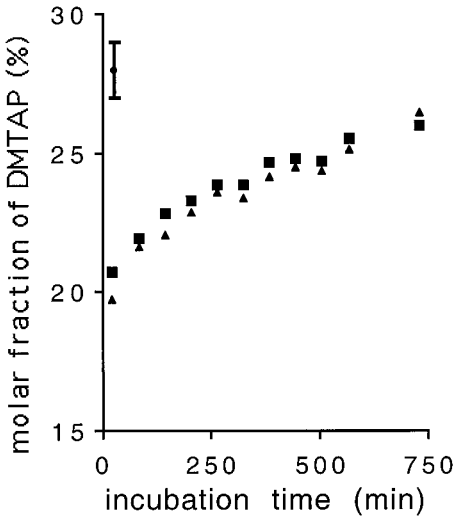


FIGURE 1 Molar fraction of cationic DMTAP in an SPB formed by SUV fusion onto the ATR plate as a function of the SUV incubation time, calculated from the measured band intensities of both symmetric (*squares*) and asymmetric (*triangles*) $CH_2$-stretching modes.

is similar to that observed when coating a pure DMPC-SPB, i.e., a bilayer without any charged lipids, and may be indicative of a slow annealing of the SPB.

Cooling the SPB below its phase transition temperature and reheating to 41°C in the presence of the SUV after 12 h of measurements, and finally flushing the ATR cell with buffer A to remove all SUV, caused a further 4% increase of the DMTAP fraction in the SPB to a total of 30%. Qualitatively similar results were observed using a SUV lipid composition of 1:9 (DMTAP/DMPC-$d_{54}$) for the SPB formation. Here we determined a final lipid composition in the SPB of 1:4 (DMTAP/DMPC-$d_{54}$) after the same treatment as described above, i.e., an increase from 10% initially to 20% DMTAP over 12 h incubation.

## $^2$H-NMR

Although the IR results clearly indicated an increase in the fraction of cationic species in the SPB with increasing SUV incubation time, there is no way to learn from these data about the distribution of the cationic species between the two monolayers of the SPB (lipid asymmetry). Deuterium ($^2$H)-NMR is uniquely suited to provide this information for the case of SSB, where the spherical support consists of a silica bead that exhibits similar anionic surface charge properties to the surface of the ATR crystal (Hetzer et al., 1998). For technical reasons, we had to use cationic DHDAB and zwitterionic DPPC-d8 (selectively chain-deuterated at the C-8 and C-9 position of the acyl chain) for these experiments. Hence, both lipids were again of equal chain length, as was the case in the IR experiments, but this length was now two methylene groups longer. Accordingly, to ensure that we did the experiment at the same reduced temperature, all NMR measurements were performed at 60°C, where both lipids are fluid.

We compared two SSB samples which were both prepared by SUV condensation onto the silica beads at 60°C using a SUV lipid composition of 1:4 (DHDAB/DPPC-$d_8$): one sample (sample 2) was incubated with the SUVs for only 10 min, while the other sample (sample 3) had a 12-h incubation time with the same amount of SUV. To establish an equilibrium distribution of pure zwitterionic DPPC between the two monolayers of the SSB, we measured a pure DPPC-$d_8$ SSB sample as a reference (sample 1).

Fig. 2 shows the $^2$H-NMR spectra of the SSB samples 1 and 3. Both spectra exhibit the well-known Pake powder pattern lineshapes characteristic for a spherical distribution of molecular director axes. This spherical distribution is enforced by the presence of the spherical silica support. However, the support causes additionally a splitting of the DPPC-$d_8$ signals into two separate but superimposed Pake doublets. This splitting is not observed for a multilamellar dispersion (MLV) of this lipid mixture or for pure DPPC-$d_8$, i.e., without the solid support (data not shown). As we have recently demonstrated (Hetzer et al., 1998), this splitting into a signal A ($\Delta\nu_Q$ = 26.0 and 30.0 kHz for samples 1 and 3) and a signal B ($\Delta\nu_Q$ = 22.0 and 24.8 kHz for samples 1 and 3) arises from a different molecular order in the two monolayers of the supported bilayer and thus each signal reflects the amount of DPPC-$d_8$ in the corresponding monolayer (Hetzer et al., 1998). It was also shown that signal A can be assigned to the proximal monolayer facing the silica surface while signal B comes from DPPC-$d_8$ in the distal monolayer facing the bulk.

Fig. 2 shows quite clearly from the intensities of signals A and B that a significant difference in the amount of DPPC-$d_8$ present in the two monolayers exists for sample 3 in comparison to that of the pure DPPC-$d_8$ sample 1. To precisely determine these intensities, in particular the differences for 10-min (sample 2) and 12-h (sample 3) incubation, we have numerically deconvoluted the spectra of all three samples using a so-called de-Pake-ing algorithm (Sternin et al., 1983).

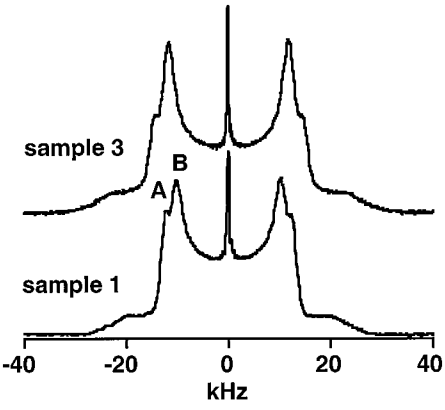


FIGURE 2 $^2$H-NMR spectra of spherical supported bilayers (SSB) prepared by SUV fusion onto silica beads for SUV lipid compositions of pure DPPC-$d_8$ (sample 1) and of 1:4 DHDAB/DPPC-$d_8$ after 12 h SUV incubation (sample 3).

The resulting half-spectra of both cationic SSBs (sample 2 and 3; for sample 2 we got $\Delta\nu_Q$ = 29.2 and 23.7 kHz for signals A and B) are shown in Fig. 3 along with that obtained from the pure DPPC-$d_8$ SSB (sample 1). Ratioing the intensities gives a DPPC-$d_8$ ratio $R_{p/d}$ between proximal and distal monolayers of $R_{p/d}$ = 1.3 for pure DPPC-$d_8$-SSB and of $R_{p/d}$ = 1.0 (sample 2) and $R_{p/d}$ = 0.7 (sample 3). A correction for the different transverse relaxation times of the two signals A and B due to different lateral diffusion in both monolayers yields $R_{p/d,corr.}$ = 1 (pure DPPC-$d_8$ bilayer), $R_{p/d,corr.}$ = 0.8 (sample 2) and $R_{p/d,corr.}$ = 0.5 (sample 3). The relaxation correction takes into account that the quadrupolar echo transverse relaxation time of the DPPC-$d_8$ in the proximal monolayer is 310 $\mu$s, compared to 260 $\mu$s in the distal layer (Hetzer et al., 1998).

Hence, after 12 h incubation, the proximal monolayer contains only 50% of the amount of DPPC-$d_8$ present in the distal monolayer. This is 30% less DPPC-$d_8$ in the proximal monolayer than measured after only 10-min incubation. It is straightforward to assume that the cationic lipid replaced the DPPC-$d_8$ in the proximal monolayer by about the same amount. Otherwise, the molecular order of the distal monolayer of sample 3 and thus its quadrupolar splitting should have decreased drastically, which was clearly not the case.

Assuming that the PC-proportion of the distal monolayer is similar to that in the SUV coating solution (80%), the NMR results provide via the $R_{p/d}$ value an estimate of the total lipid proportion in each monolayer. Furthermore, the sum over both monolayers can then be compared with the IR results, which provide the total ratio of DMTAP/DMPC-$d_{54}$ in the supported bilayer. The above assumption is certainly justified during the whole incubation time ($t_{inc}$) while there is a reservoir of SUV in the bulk. However, after $t_{inc}$ the SUVs are gone and the supported bilayer system has to adjust to a new equilibrium. This uncertainty is met by allowing the PC concentration of the distal monolayer to be 80–90%. In order to compare both IR and calculated NMR results, we have to assume 1) that the behavior of the (fluid state) lipid mixtures used for the IR and the NMR experiments is similar with respect to the creation of lipid asymmetries, and that 2) there are no significant differences between the asymmetries in planar and curved bilayers. While 1) seems acceptable considering that we have done the experiments at the same reduced temperatures of the mixtures, assumption 2) is not generally valid. However, our SSB samples have diameters of 640 nm, which is well below the critical curvature above which we have observed curvature-induced lipid asymmetries in a previous study (Brumm et al., 1996).

With the above assumptions in mind, we calculated the composition of the proximal monolayer of samples 2 and 3 as given in Table 1 using $R_{p/d,corr.}$ obtained from NMR. Taking into account an error of 10% for the quantification of the de-Paked spectra in addition to an error of 5% for the IR results, both measurements agree quite well.

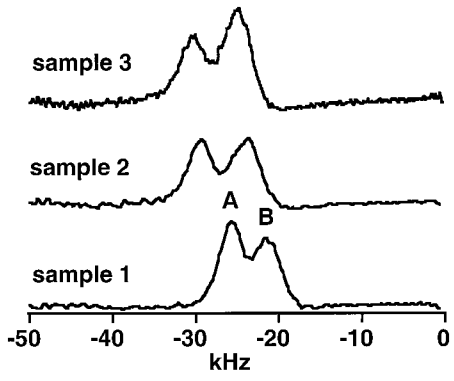


FIGURE 3 Numerically deconvoluted $^2$H-NMR (half)-spectra of spherical supported bilayers (SSB) prepared by SUV fusion onto silica beads for SUV lipid compositions of pure DPPC-$d_8$ (sample 1) and of 1:4 (mol) DHDAB/DPPC-$d_8$ incubated for 10 min (sample 2) and for 12 h (sample 3).

## DSC

The samples used for the NMR experiments were studied by DSC before and after the NMR measurements and the results did not indicate changes in the phase transition behavior of the samples during the NMR measurements. However, we observed significant differences between the DSC endotherms of samples 2 and 3, which differed only by the incubation time of the solid support with the SUV solution of DHDAB/DPPC-$d_8$ (1:4). This is shown in Fig. 4 where the endotherm obtained for a multilamellar dispersion (MLV) of the mixture is compared with those obtained for samples 2 and 3. The MLVs exhibit a slightly asymmetric but otherwise rather cooperative transition endotherm with a peak at $T_m$ = 48°C and no indications of significant demixing. The rather high $T_m$ of the MLVs is in agreement with previous findings of DPPC mixtures with cationic lipid analogs (Linseisen et al., 1996).

In contrast, the SSB samples showed endotherms consisting of two superimposed peaks (sample 2, 10-min incubation) and of two nearly separate peaks (sample 3, 12-h incubation) and are generally broader than the MLV endotherm. The transition for sample 2 is still rather cooperative at 44°C with a superimposed component at 45–46°C and its reduction in $T_m$ compared to the MLVs is compatible with

**TABLE 1 Summary of the ATR-IR and NMR results regarding the PC proportion in the bilayer and in both monolayers**

| ATR-IR/NMR Results | Sample 1 | Sample 2 | Sample 3 |
|---|---|---|---|
| Original PC-conc. (%) in SUV | 100 | 80 | 80 |
| Incubation time ($t_{inc}$) | — | 10 min | 12 h |
| Resulting overall PC (%) (ATR-IR) | — | 80 | 70 |
| Intensity ratio $R_{p/d}$ of signal A:B (NMR) | 1.3 | 1.0 | 0.7 |
| Relaxation time-corrected $R_{p/d,corr.}$ (NMR) | 1.0 | 0.8 | 0.5 |
| PC of proximal monolayer (%) (NMR)* | — | 64–72 | 40–45 |
| Resulting overall PC (%) (NMR) | — | 72–76 | 60–62.5 |

*Calculated assuming that distal layer contains 80–90% PC.

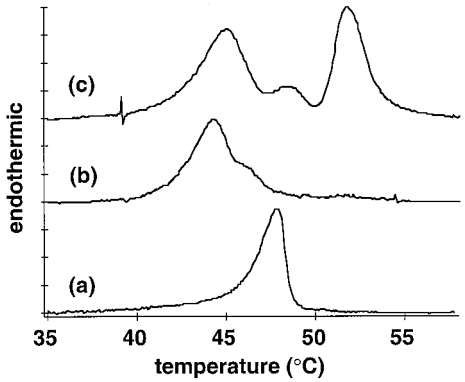


FIGURE 4 DSC endotherms of 1:4 DHDAB/DPPC-$d_8$ MLV (*a*), and of SSB prepared by SUVs of the same lipid composition (*b*) after 10 min (sample 2) and (*c*) for 12 h (sample 3) SUV incubation time.

the established $T_m$ reduction due to the presence of the spherical support (Naumann et al., 1992). However, sample 3 showed two additional peaks at higher temperatures, a small one at the position of the MLV peak (48°C) and a large one at even higher temperature (52°C), giving the whole endotherm a more symmetrical shape centered around the MLV transition at 48°C. It should be pointed out that the small center peak of sample 3 is unlikely to arise from the presence of SUV or MLV in the sample since repeated washing steps of the SSB sample in excess buffer did not change the endotherm.

## DISCUSSION

The results obtained by three independent methods provide strong indications for the evolution of a cationic lipid asymmetry in single supported bilayers prepared by SUV fusion under conditions of prolonged incubation times. 1) IR measurements indicated that the cationic component of the SUV mixture used for the formation of the supported bilayer became enriched in the SPB with increasing incubation time. 2) Deuterium NMR indicated that the cationic component was slightly asymmetrically distributed between the two monolayers of the SSB for short incubation times (10 min) but became substantially enriched in the monolayer facing the solid support after longer incubation (12 h). 3) DSC results indicated that the phase transition of the SSB was rather cooperative for short incubation time but became segmented and very broad for long incubation, very likely reflecting different mixing in both monolayers through the evolving asymmetry of the cationic component.

Since such evolving lipid asymmetries were not observed for MLVs nor for SUVs, the presence of the solid surface must provide the primary driving force for this process. Silica exhibits a negative surface charge at neutral pH owing to the presence of silanoyl groups at the surface (1–5 silanoyl groups per $nm^2$) (Iler, 1979). The Grahame equation allows us to calculate the surface potential according to

$$\psi_0 = \frac{2kT}{e} \operatorname{arcsin} h\left(\frac{\sigma}{\sqrt{8\varepsilon\varepsilon_0 kT}\sqrt{[\mathrm{NaCl}]}}\right). \qquad (1)$$

Assuming only two negative charges per $nm^2$ on the silica surface and a 20 mM NaCl electrolyte at 40°C, this equation yields a surface potential $\psi_0$ of $-195$ meV.

The separation between the bilayer and the silica surface is $<20$ Å. This value was measured for pure zwitterionic lecithin bilayers (Johnson et al., 1991) while the presence of cationic lipids can only decrease it even further. Moreover, our experiments were done at a NaCl concentration of 20 mM in the buffer solution, so the Debye screening length is $1/\kappa \approx 2.2$ nm, indicating that there is a non-negligible electrostatic potential at the position of the bilayer. As the trans-bilayer separation of cationic headgroup charges is ≈4 nm and the electrostatic potential can be assumed to follow the Gouy-Chapman theory, as (Israelachvili, 1992)

$$\psi_x \approx \frac{4kT}{e}\gamma e^{-\kappa x} \quad \text{where} \quad \gamma = \tanh(e\psi_0/4kT), \qquad (2)$$

the cationic lipids in the proximal and in the distal monolayer experience a considerable potential difference. For our case this results in an electrostatic energy difference between the two monolayer headgroups of

$$e\Delta\psi \approx e|\psi_{x=2\mathrm{nm}} - \psi_{x=6\mathrm{nm}}| \approx 34 \text{ meV}$$

which is larger than the thermal energy ($1\ kT = 27$ meV at 40°C) and thus may represent the driving force for the formation of asymmetry. It should be emphasized that this energy difference is a lower limit, as we assumed in our calculation only two electronic charges per $nm^2$ on the silica surface and the maximum value of 20 Å for the bilayer-silica surface separation.

Nevertheless, our calculation is only a crude estimate since it is not exactly known whether the $Na^+$ and $Cl^-$ ions in the narrow water gap between bilayer and surface behave like ions in the bulk solution. However, the experimentally established asymmetry indicates that the 20 mM $Na^+$ ions present in the buffer do not provide complete shielding of the silica surface charge to prevent electrostatic interaction with cationic lipids in the supported bilayer.

The validity of our assumption about the electrostatic energy difference between the two monolayers as being the driving force for lipid asymmetry is supported by an infrared ATR experiment performed under the same conditions but at a salt concentration of 0.5 M NaCl. Under these conditions, $1/\kappa = 0.4$ nm and thus the screening is nearly complete at the position of the SPB. As expected, this experiment did not show any indication for an enrichment of the cationic lipids in the SPB over 12 h SUV incubation.

It has been demonstrated previously that the most likely for the enrichment of cationic lipids in a SPB in the presence of SUV is temporary (partial) fusion between SUV and SPB (Reinl and Bayerl, 1994). However, this process cannot account for the imbalance in the distribution of the cationic lipids between the monolayers. Hence, there must be some flip-flop between the monolayers. Furthermore, inhomogeneities in the SPB-like temporal pores may allow lateral diffusion of cationic lipids from the distal to the

proximal monolayer through the surface of such pores. In both cases the driving force might be the above-mentioned electrostatic energy difference.

NMR shows quite clearly that the DHDAB enrichment in the proximal monolayer can be as high as ≈200% compared to the distal layer. Quasi-equilibrium is (theoretically) reached when the cationic lipids in the proximal layer effectively screen the silica surface, thus reducing the flip-flop driving energy below $kT$. Assuming a DHDAB molecular area of 0.5 $nm^2$ and comparing this with the silica charge density (1–4 silanoyl groups per $nm^2$) shows that the DHDAB in the proximal layer alone is insufficient for complete screening even at highest measured asymmetry (sample 3). It is likely that the sodium ions in the gap provide the remaining shielding.

It is interesting to compare the imbalance between the two monolayers as seen by NMR with that detected by DSC. For the latter method we observe an endotherm for sample 3, which consists of three almost separate peaks, while that for sample 2 exhibits one rather broad peak at lower temperature. Considering the NMR results and the phase diagram of this binary mixture (Linseisen et al., 1996) we can now assign the peak at highest temperature (sample 3) to a DHDAB-enriched region and thus to the proximal monolayer, while the peak at lowest temperature must arise from a region where the DHDAB concentration was rather unchanged compared to sample 2 (similar peak temperatures), i.e., from the distal monolayer.

Both transitions appear to be coupled by the superimposed small center peak at 48°C, at the position of the MLV transition temperature of this mixture. Since the presence of MLVs in the SSB samples is very unlikely (cf. Methods) it may arise from regions having a DHDAB content between the two extremes. One explanation could be that the enrichment of DHDAB in the proximal monolayer is accompanied by a significant demixing of the two lipids in the distal monolayer when the system is cooled down to the gel phase (note that the DSC endotherms are heating scans).

## CONCLUSIONS

The preparation of supported bilayers containing cationic lipids by the vesicle fusion technique under low salt conditions is prone to an increase of the cationic lipid content of the supported bilayer as a whole. This is due to cationic lipid enrichment in the inner (proximal) monolayer, driven by the incompletely shielded electrostatic potential of the solid support surface, while the concentration of cationic lipids in the distal monolayer remains unchanged compared to that of the SUV in the bulk. We cannot identify from our data whether the molecular mechanism leading to this imbalance is flip-flop, temporary fusion of the SPB with SUV, or a combination of both, but its driving force is very likely the electrostatic potential arising from the solid surface. The use of high-electrolyte (NaCl) concentrations can prevent the formation of asymmetries.

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