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Planar lipid bilayers on solid supports from liposomes – factors of importance for kinetics and stability

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Abstract

One method to create planar lipid bilayers on solid substrates involves the transfer of lipids from liposomes to the support. We have varied the composition of liposomes systematically using factorial experimental designs and analyzed the adsorption behaviour of lipids from these liposomes onto solid supports. The hydrophilic supports were either used plain or modified with a monolayer of a lipid mixture, exposing hydrophobic groups. The monolayer-covered supports were used to identify factors important for adhesion and stability. Lipid adsorption kinetics was primarily studied on plain silicon supports in an ellipsometric cell or on a silicon nitride surface in a resonant mirror system (IAsys), using the systematic approach. Saturated phospholipids were essential for the required stability. Mixtures of dipalmitoylphosphatidylcholine, dipalmitoylphosphatidylglycerol, dipalmitoylphosphatidylethanolamine and cholesterol in combination with proteins were investigated in further detail as regards kinetics. The propensity to form a supported planar bilayer could be manipulated by the presence of calcium ions.

Keywords: Ellipsometry; Factorial experimental design; Liposome fusion; Phospholipid; Resonant mirror

1. Introduction

Planar lipid bilayers on solid supports could be a useful tool in biomembrane research as complements to liposomes and black lipid membranes and are also of interest for biosensor applications [1]. Several methods to form these structures have been reported during the last decade. The classical Langmuir–Blodgett technique has many drawbacks. Still, it has recently been used successfully for transferring lipids to surfaces modified with polymers, which provide a hydrophilic space between the support and the membrane [2,3]. A membrane vesicle (liposome) fusion technique described by Brian and McConnell [4] has been frequently used, on a wide range of unmodified or modified surfaces [5–11]. A hybrid method of the two mentioned above is to transfer a lipid monolayer by LB-technique and then deposit a second leaflet from vesicles [12–14]. Possible mechanisms for fusion and formation of a planar bilayer on hydrophilic and hydrophobic surfaces have been suggested and discussed [1,14,15].

The ultimate use of the supported membrane will determine the quality demands required and thus which preparation method is preferred. Our goal is to develop a membrane-based biosensor and stability is one of the most important quality criteria. Our definition of stability is that the supported membrane should

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remain bound to the support upon storage and upon transfer between different media by passing the water/air interface. The method must permit the introduction of membrane proteins with retained activities. We have shown that lipid composition is one of several important factors for obtaining a good adhesion of a lipid Langmuir–Blodgett monolayer to various supports [16–19]. Such supported lipid monolayers, as well as plain supports, were used for fusion with liposomes and proteoliposomes. Stable lipid bilayers could be obtained and the incorporated proteins retained their activities, in some preparations for weeks [20,21]. The lipid composition used for preparing liposomes in these studies was not optimal and this prompted us to perform the present study. The aim was thus to find lipid compositions for liposomes that produced a bilayer-like structure on both Pt and Si/SiO_2 surfaces. These structures should withstand harsh treatments such as passing the water/air interface, short drying procedures and storage in buffer. Additionally, the lipid composition should be similar to what is found in biological membranes, permit the introduction of membrane proteins and reproducibly produce liposomes with good recovery. Finally, we aimed at finding optimal compositions and conditions for rapid planar bilayer formation and/or ways to increase the adsorption rate. Factorial experimental designs were used throughout the investigation.

2. Materials and methods

2.1. Materials

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) and cholesterol (Chol) were from Avanti Polar Lipids (Alabaster, AL, USA), while 1,2-dipalmitoyl-*sn*-glycero-3-glycerol (DPPG), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphate (DPPA) and cardiolipin (CL) from bovine heart were from Sigma (St. Louis, MO, USA). Purity was checked by e.g. thin layer chromatography. Octylglycoside was obtained from Boehringer Mannheim GmbH (Germany). Water was taken from a Milli-Q® Plus 185 (Millipore, Molsheim, France) ultrapure water system with a resistivity > 18 MΩ cm. A cuvette-based resonant mirror system (IAsys) was from Affinity Sensors, Cambridge, UK. The single wavelength, null ellipsometer was a modified Rudolph model 43603.

20 mM Tris–HCl, pH 7.4, 100 mM NaCl was used as buffer.

2.2. Ellipsometry

The optical parameters Δ and Ψ were used for calculating apparent thickness of deposited material on supports, setting the refractive index $N = 1.45$ for both condensed lipid and proteolipid films. Average values from 5 measurements on Pt or Si/SiO_2 support served as reference for the subsequent film thickness calculations, with 3 measurements on each sample.

An equal angle, triangular-shaped quartz cuvette, with the three walls having a length of 3 cm and a height of 4 cm, was used for liquid measurements. The cuvette contents were continuously mixed with a magnetic stirrer in a special cuvette holder. After submerging a substrate into buffer and measuring at several locations, the laser beam was fixed at a central point, where all subsequent measurements were taken at 5 min intervals.

2.3. Membrane protein preparation

A membrane protein mixture was prepared from bovine brain. The tissue was homogenized in 1 M NaCl in 10 mM phosphate buffer pH 7.4, the homogenate was centrifuged and the pellet extracted with 1% Triton X-100 in phosphate buffer. After centrifugation, the supernatant was dialyzed against buffer given under 2.1. to reduce the detergent concentration 20-fold. The protein content was 6 mg/ml, as determined with Coomassie Brilliant Blue G-250 and bovine serum albumin as standard [22].

2.4. Supports and LB-deposition of lipid monolayer

Polished 100-oriented, boron-doped silicon wafers (Aurel GmbH, Landsberg, Germany) were cut into 35×9 mm pieces and cleaned in NH_4OH:H_2O_2:water (1:1:5) at 85°C for 5 min, rinsed with water and cleaned for 5 min in HCl:H_2O_2:water

(1:1:6) at 85°C. They were then rinsed in running water for a minimum of 30 min and stored in water for no more than 2 h before use. The slides were highly hydrophilic, with a contact angle of about 5 degrees. Platinum supports were prepared by thermal evaporation onto cleaned silicon slides to a thickness of 60 nm [20]. They were cleaned in 2% Hellmanex® for 20 min, followed by rinsing in running water for 30 min and drying in a stream of nitrogen before use. These slides were slightly less hydrophilic than the silicon slides, with contact angles of approximately 10°.

Langmuir–Blodgett films were prepared at room temperature from a lipid mixture of 32 mol% DPPA, 30 mol% DPPE and 19 mol% each of DPPC and cholesterol, compressed to 45 mN/m [18]. The subphase was 0.1 mM $CaCl_2$ and the equipment was a KSV LB 5000 system (Helsinki) with a 15×54 cm trough. The substrates were immersed through the monolayer to cover 2 cm^2, with a dipping rate of 5 mm/min. Transfer ratios were 1.2 ± 0.1. They were stored in a desiccator at 100% humidity until used for fusion experiments later the same day. Ellipsometry was performed on each slide before and after LB-transfer.

The resonant mirror cuvettes were cleaned as the silicon slides, but limiting the washing procedure to 2 min at room temperature.

2.5. Liposomes

Unilamellar liposomes with estimated diameter of 200–300 nm were prepared from 16 μmol lipid mixtures by a detergent depletion technique [23]. The lipids were dissolved in chloroform and deposited on the wall of a 100 ml round bottom flask in a rotatory evaporator under a stream of nitrogen gas. Traces of chloroform were removed by vacuum for three hours. The vessel was warmed in a waterbath at 40°C and a few glassbeads were added. A 1.4 ml volume of prewarmed standard buffer with 50 mg octylglycoside (110 mM) was added to dissolve the lipids and form mixed micelles during 30 min. When proteins were to be incorporated, they were introduced with the detergent–buffer solution. The detergent was removed on a Sephadex G-50 column (44×1.6 cm) and a flow rate of 45 ml/h. Liposome-containing fractions were identified by light scattering and the four fractions (in total 8 ml) with the highest values were pooled. Phospholipid concentration was quantified [19]. Sodium azide was added as preservative and storage was at 4°C.

2.6. Fusion of liposomes to supported monolayer. End-point analysis

After measuring the initial, apparent thicknesses ellipsometrically, the lipid-covered supports were transferred to a horizontal fusion cell with four chambers, each having separate inlet and outlet. Each chamber and connecting tubing had been filled with 3 ml of the liposome preparation under study, diluted with Tris–HCl/NaCl to a phospholipid concentration of 0.05 mM. The dispersion was recirculated at a flow rate of 1.3 ml/min over the supports for about 18 h at room temperature. The substrates were then withdrawn from the chamber, washed several times with water applied by a pasteur pipette and dried under a stream of nitrogen before ellipsometry.

The difference in apparent thickness (after fusion – before fusion) was taken as a measure of the ability of the liposomes to fuse on the monolayer and the deposited material to remain bound to the support in the first washing cycle. For supports showing positive thickness difference values a second stability test was performed by storing them in cuvettes with 150 mM NaCl for 4–5 h and then repeat the washing cycle before ellipsometry.

2.7. Factorial experimental design

The experiments were in accordance with the principles of factorial design [24]. In such designs, all the variables of interest are changed in a systematic way for each experimental run, and all observations can be used for gaining information on each of the main effects and sometimes also interaction effects. The number of experiments are limited and dependent on the number of factors and of levels for each factor.

A commercial PC-based programme (Modde®, Umetri, Umeå) was used for designing and analysing the experiments. The variables ('factors') in this study concerned compositions of and ways to prepare liposomes. The results ('responses') in ellipsometric measurements are given as apparent thickness (Å) and in resonant mirror measurements as arc seconds.

For the latter, a correlation factor to thickness could be approximated from experiments with DOPC. Rate constants given as responses in the tables were determined by semilog plots, $\ln(\text{extent}_\infty - \text{extent}_t)$ vs. time.

Table 1
Screening for evaluating the composition of lipids in liposomes for adsorption to solid supports

Factors			Responses	
PC type	Negatively charged PL	DPPE/ Chol	Platinum Δ thickness, Å	Silicon Δ thickness, Å
DPPC	DPPA	0.15	−2	0
DPPC	DPPG	3.325	32	5
DPPC	CL	6.5	5	5
POPC	DPPA	3.325	−5	−2
POPC	DPPG	6.5	5	9
POPC	CL	0.15	−7	−5
DOPC	DPPA	6.5	−8	−2
DOPC	DPPG	0.15	1	−3
DOPC	CL	3.325	−3	−5

Liposomes were prepared at 40°C. Lipid composition was varied in a systematic way, with two qualitative and one quantitative factors, each at 3 levels resulting in 9 preparations.
Phosphatidylcholine content was 50 mol%, the negatively charged phospholipid 20 mol% and remaining 30 mol% were shared between DPPE and cholesterol (Chol). The lowest ratio DPPE/Chol corresponds to 4 mol% DPPE, the highest to 26 mol%.
The responses are given as change in apparent thickness of the lipid film, measured ellipsometrically, after circulating 0.05 mM liposome dispersion over lipid monolayer-coated supports for 18 h followed by a wash procedure. Mean values of two experiments which were used in the statistical analysis of data for evaluating significance of factors.

3. Results

3.1. Lipid composition and protein content in liposomes: Influence on ability to form supported lipid structures determined by end-point analysis

3.1.1. Comparison of phosphatidylcholines and of negatively charged phospholipids

Screening is usually the first step in a systematic investigation, with the aim to identify important factors and eliminate less important ones. In the first fractional factorial design we studied three variables regarding lipid composition. Two of them were qualitative and studied at three levels: type of phosphatidylcholine (DPPC, POPC or DOPC at a fixed molar fraction of 50 mol%) and type of negatively charged phospholipid (DPPA, DPPG or CL at 20 mol%). The third was a quantitative factor, varying the ratio DPPE/ cholesterol, the total amount of the two corresponding to 30 mol%. Liposomes were prepared at 42°C and the recovery of lipids was good, as evident from phosphate analysis. These liposomes were tested in the end-point analysis. The experimental design and the results are shown in Table 1.

One of these preparations resulted in good fusion to the supported monolayer. The others resulted in poor fusion or in some cases partially peeled off the predeposited monolayer. The statistical analysis of the data for platinum supports showed that the only significant factor was DPPG, with a positive effect on fusion. For silicon supports, DPPC, DPPG and a high DPPE/Chol ratio had positive and significant effects,

Table 2
The effect of PC type in a lipid mixture and temperature during liposome preparation for liposome fusion

Factors		Responses		
PC type	Temperature, °C	Phospholipid concentration, mM	Platinum Δ thickness, Å	Silicon Δ thickness, Å
DPPC	5	0.62	20	6
POPC	5	0.53	−5	−2
DPPC	20	1.04	25	4
POPC	20	0.89	10	−1
DPPC	40	1.28	32	5
POPC	40	1.38	−6	−8

A full factorial design, with two variables at three levels. PC content was 50 mol%, DPPE 23 mol%, DPPG 20 mol% and Chol 7 mol%. Thickness responses as for Table 1. A third response is phospholipid concentration in the liposome preparation, i.e. recovery of lipids. Data represent the mean values from two experiments and these were used in the statistical evaluation.

while DOPC had a significant and negative effect. The statistical analysis provided information on the direction of subsequent experiments. Consequently, DPPG was chosen as the negatively charged phospholipid and DOPC was not further used in lipid mixtures.

3.1.2. Effect of temperature during liposome formation combined with varying phosphatidylcholine type

Next, we tested if the liposome preparation temperature had any influence on the ability of liposomes to form lipidic structures on supports. There were two reasons for these experiments. Firstly, it might be advantageous to prepare proteoliposomes at lower temperatures to prevent protein denaturation. Secondly, we have speculated that non-perfect liposomes may be more favourable for planar lipid membrane formation [20]. One way to introduce stress could be to prepare liposomes at temperatures below the transition temperature of the phospholipids.

A mixed full factorial design with two variables, type of phosphatidylcholine (a qualitative factor at two levels) and temperature (a quantitative factor) at three levels was used (Table 2). DPPG at 20 mol%, DPPE at 23 mol% and cholesterol at 7 mol% were kept constant. The saturated DPPC has a high transition temperature (41°C), while POPC has a much lower value (−2°C), DPPG 41°C and DPPE 63°C. The test performed was again the end point analysis, using monolayer-covered Pt and Si/SiO_2 as supports.

There was a distinct effect of temperature but not of PC type on the recovery of lipids in liposomal form as evident from Table 2. For fusion, the reverse pattern was equally evident. DPPC when compared to POPC resulted in a significantly better fusion on both types of support, while temperature was without effect, an observation which speaks against the above-mentioned hypothesis. The thickness increase on Pt supports corresponded to a monolayer.

3.1.3. Introducing proteins into the liposomes and varying DPPE / DPPG ratio

Two experimental series were designed to evaluate the effect of membrane proteins in the liposomes and to further study the effects from varying amounts of DPPG and DPPE. The series comprised 14 liposome compositions with DPPC and cholesterol at constant levels of 50 and 7 mol%. DPPG levels were 0, 10, 20 and 40 mol% and consequently DPPE was varied between 3 and 43 mol%. The levels of protein was 0, 0.5, 2 and 4 mg per 16 μmol lipid. Liposomes were prepared at 5°C and evaluated in the end-point analysis. In many cases, we found increases in film thickness that corresponded to a lipid monolayer. Only two preparations resulted in negative values. Deposited material remained on the supports during stability tests. The results thus confirmed that the

Table 3
Evaluation of liposome compositions in adsorption kinetics on plain silicon supports

Factors		Responses					
Number	DPPE/Chol	Protein mg	Initial rate Å/min	k_{on} min^{-1}	Extent 1 Å	Extent 2 Å	Extent 3 Å
N1	0.3	0	0.0	0	0	39	76
N2	14	0	0.2	0.034	14	164	71
N3	0.3	2	1.6	0.044	61	101	74
N4	14	2	3.0	0.068	76	185	57
N5	2	1	0.8	0.028	40	135	81
N6	2	1	0.6	0.038	23	118	n.d.
N7	2	1	0.2	n.d.	16	115	38

Liposomes were prepared at 40°C and contained 50 mol% DPPC, 20 mol% DPPG and 30 mol% DPPE + Chol, in proportions given in the design. Indicated amount of membrane protein was added to 16 μmol lipid. Responses were collected by ellipsometric measurements. The concentration of liposomes corresponded to 0.07 mM phosphate. The initial rate was determined over the first 15 min and the rate constant was calculated from semilog plot. Extent 1 is plateau value after liposome addition. Extent 2 is total apparent thickness after adding 20 mM $CaCl_2$ and extent 3 is after washing with buffer. The responses are given as mean values. The statistical evaluation was based on individual data.

conclusions drawn from the first two experimental series were justified.

A statistical analysis of all these responses was however considered to be meaningless, as many of the liposome preparations showed an unacceptably low lipid recovery (less than 50%). The true lipid composition was thus uncertain. Still, some general conclusions could be drawn and used as information for the final design. We could confirm an earlier observation [20] that liposomes containing a moderate amount of proteins more readily form stable lipidic structures on solid supports than pure liposomes. Additionally, the presence of proteins and a high DPPG/DPPE ratio promoted liposome formation at temperatures otherwise unfavourable.

3.2. Influence of lipid composition and protein content on the kinetics of planar lipid membrane formation

The time-dependent adsorption was monitored by two optical systems, ellipsometer and resonant mirror. Clean unmodified surfaces were used in these experiments, as it is impossible to transfer a lipid monolayer by LB-technique to the IAsys cuvette. The washing procedure was the same for the silicon nitride cuvettes and the silicon slides.

In the final experimental design (Table 3), protein content was again varied (0, 1 and 2 mg to 16 μmol lipid). DPPC was kept at 50 mol% and DPPG at 20 mol%, as suggested from screening data above. Remaining 30 mol% lipid was used for studying the effects of DPPE and cholesterol, similar to the first screening experiment but with different domains. Liposomes were prepared at 40°C to ascertain successful formation of liposomes, which was confirmed with phosphate analysis. Experiments were also performed on liposomes containing only DOPC.

3.2.1. The ellipsometric analysis

The adsorption behaviour for some of the liposome preparations in Table 3, followed by ellipsometry, is illustrated in Fig. 1. The increases in apparent thickness with time differed considerably between the preparations, proteoliposomes generally giving a faster incline. The addition of a high concentration of calcium chloride had profound effects in all the experiments. Deposited material remained on the support after washing twice with calcium-free buffer, a procedure that implied an exposure of the slide to air–water interface four times. The final apparent thickness was the same for all the liposome preparations and corresponded approximately to the thickness of a lipid bilayer.

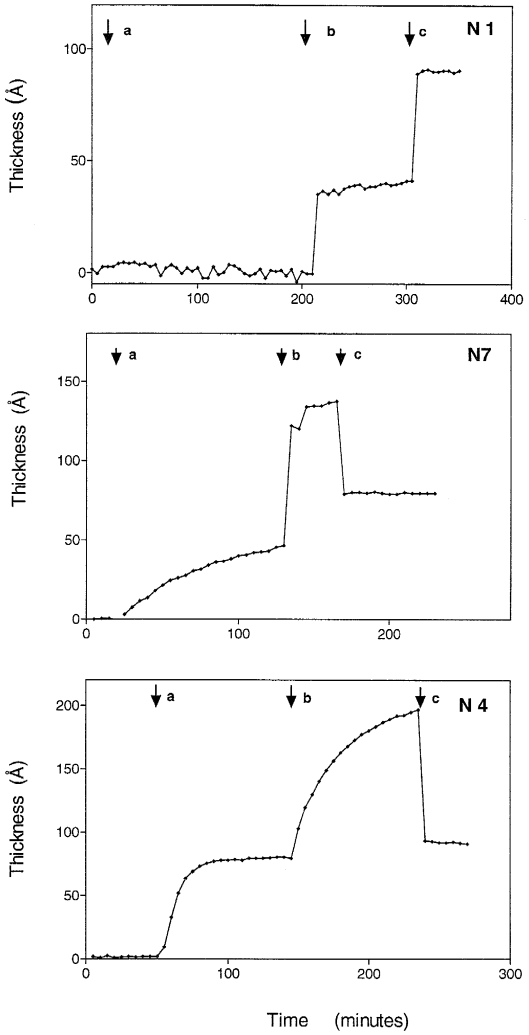

Fig. 1. Ellipsometrically measured increase in apparent thickness on plain silicon supports upon exposure to different liposomes, preparations N1, N7 and N4 from Table 3. The experiments were run at room temperature, in 20 mM Tris–HCl pH 7.4, 100 mM NaCl. Arrow a denotes the addition of liposomes to a concentration corresponding to 0.07 mM phosphate. Arrow b denotes addition of calcium chloride to a final concentration of 20 mM. Arrow c denotes when the solution containing liposomes and calcium chloride was replaced with the starting buffer. Ellipsometric measurements were collected every 5 min.

Five responses for this experimental design were

Table 4
Statistical evaluation of responses from Table 3 on factors in liposome composition of importance for fusion to silicon surfaces

Response	Factor	Coefficient	p
Initial rate (Å/min)	Constant	1.44	3×10^{-6}
	DPPE/Chol	0.48	0.010
	Protein	1.03	6×10^{-5}
k_{on} (min^{-1})	Constant	0.043	1×10^{-7}
	DPPE/Chol	0.013	2×10^{-3}
	Protein	0.016	4×10^{-4}
Extent 1 (Å)	Constant	46.3	2×10^{-7}
	DPPE/Chol	8.0	0.042
	Protein	25.4	4×10^{-5}
Extent 2 (Å)	Constant	130.0	2×10^{-12}
	DPPE/Chol	42.7	6×10^{-8}
	Protein	13.9	5×10^{-4}
Extent 3 (Å)	Constant	66.6	2×10^{-6}
	DPPE/Chol	−4.1	0.54
	Protein	−4.0	0.55

selected (Table 3). Initial rate of apparent thickness increase after addition of liposomes was calculated from the first three measurements (15 min). The rate constant, k_{on}, was estimated from semilog plots. The remaining responses were plateau value for the first part of the curve (Extent 1), and after addition of calcium ions (Extent 2) and the final apparent thickness after washing (Extent 3). The statistical evaluation was based on individual data from each of the 13 experiments and provided clear results on the effect of the factors (Table 4). Both protein and a high ratio of DPPE to cholesterol contributed positively and significantly to the values of the first four responses. No factor was significant for the last response and the average value (67 ± 14 Å) for all the experiments is at a reasonable level corresponding to the thickness of a lipid bilayer with proteins.

Two control experiments on the effect of Ca^{2+} were performed. The first one was to add calcium ions to a lipid monolayer covered silicon slide in buffer in the absence of liposomes. Ellipsometric parameters were continuously collected for more than one hour and were unchanged. The second control was to add calcium chloride after the final wash with calcium-free buffer at the end of an experiment with liposomes. Again, the ellipsometric parameters remained unchanged.

Two of the preparations (N1 and N3 in Table 3) were tested also with the end-point analysis. The liposomes were circulated in calcium-free buffer for 4 h over lipid monolayer-covered Pt supports, followed by the usual wash procedures. Ellipsometric measurements in air indicated thicknesses of 35 Å for the protein-free preparation and 51 Å for the support exposed to proteoliposomes.

Three of the preparations in Table 3 (N1, N2 and N7) were studied as regards kinetics on lipid monolayer-covered platinum. All three showed monophasic adhesion behaviour similar to the example in Fig. 2, with initial rates of 1–2 Å/min, rate constants of 0.05–0.1 min^{-1} and extents corresponding to deposition of at least an extra monolayer to the predeposited LB-monolayer, reached within 30 min. Lipid-covered platinum was thus better than plain silicon for bilayer formation. This difference is not remarkable after considering that the optimisation of lipid composition was done for such hydrophobic surfaces.

Liposomes prepared from DOPC showed an adsorption behaviour to plain Si/SiO_2 distinct from any of the lipid mixture liposomes tested (Fig. 3). A thickness corresponding to a bilayer was observed at the first measurement, 5 min after the addition of liposomes. The rate of this process prevented any kinetic analysis. No further material was deposited upon addition of Ca^{2+} and all the material was removed during the washing procedure.

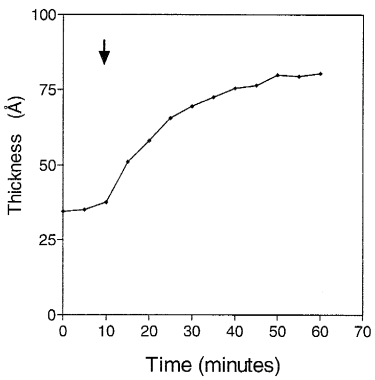

Fig. 2. Kinetics for lipid transfer from liposomes to a lipid monolayer-covered platinum surface, measured ellipsometrically. The starting value represents the apparent thickness of the pre-deposited lipid monolayer. Protein-free liposomes (N2 in Table 3) were added at the arrow. Experimental conditions were the same as for the first part of Fig. 1.

3.2.2. Resonant mirror analysis

IAsys is a cuvette-based resonant mirror system, with a cuvette volume of 0.2 ml. The adsorption of material onto the horizontal sensor surface with the outermost part being silicon nitride is followed by resonance angle shifts, provided the adsorbed material has a refractive index different from the one of the bulk solution. These shifts are measured in arc seconds. The cuvette system has several advantages but also drawbacks. Firstly, the geometry of the cuvette is unsuitable for Langmuir–Blodgett depositions. Secondly, the price of a cuvette precluded the possibility to use a new surface for each experiment and a washing scheme was developed. A short exposure to acidic ethanol (1.2 M HCl in ethanol) was adequate to regenerate a surface exposed to liposomes containing lipids only. An etching and washing procedure similar to the one used for the silicon slides was included as a standard before the start and at the end of a series of experiments and between experiments involving proteins. Monitoring resonance angles shifts and inspecting the resonance scan, in which deviations from a sharp and symmetric peak are indicative of inhomogeneities, were used to decide when the surface was ready for the next experiment and when the cuvette should be replaced.

3.2.2.1. Kinetics for liposomes in Table 3. An adsorption curve typical for liposomes of Table 3 is shown in Fig. 4. Liposomes were added after establishing a stable baseline. Data were collected at five per second initially and one per second in the later part. The progress of adsorption was much faster than in ellipsometric experiments. The resonance scan was checked frequently, and if irregularities were observed, the data was not used for determining the kinetic constants. After reaching a plateau value the liposome dispersion was pumped out and the surface was washed with buffer 2–3 times. This usually had no effect on the signal. The final treatment was a procedure to mimic the last step in the end-point analysis by temporarily stopping the experiment, washing the cuvette extensively with water, drying it under a stream of nitrogen gas and finally adding buffer and restarting. This step resulted in an increased signal for all liposomes in this series, and reliability was checked by resonance scan.

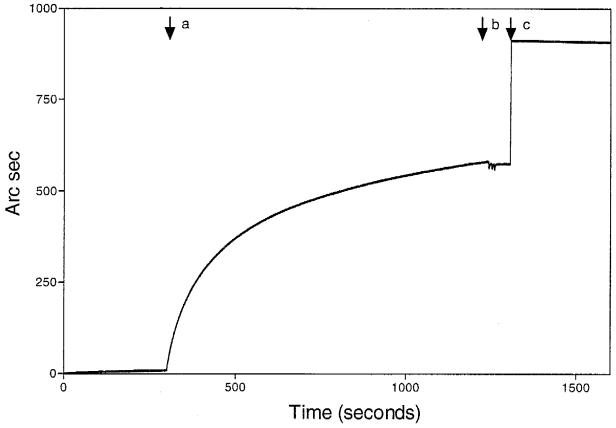

Fig. 4. Adsorption of lipids from liposomes to a plain silicon nitride surface, measured in a resonant mirror system (IAsys). The cuvette contained 142 μl 20 mM Tris HCl pH 7.4, 100 mM NaCl, maintained at 20.0°C. At arrow a, 8 μl of liposomes (N4 from Table 3) was added, to a final phospholipid concentration of 0.05 mM. The liposome-containing buffer was changed to buffer at arrow b, followed by two extra washes. Arrow c marks temporarily pausing in measurements, while the cuvette was removed from the instrument, washed with water and dried with a stream of nitrogen gas. Fresh buffer was added and the instrument was restarted.

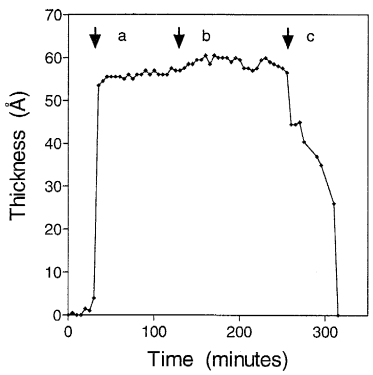

Fig. 3. Adsorption of lipids from DOPC liposomes to a plain silicon surface measured by ellipsometry. Additions, indicated by the arrows, and experimental conditions were the same as in Fig. 1.

The IAsys contains software for evaluating binding events, including a test of monophasic or biphasic binding. The rate constant, k_{on}, is obtained from semilog plots. For this part of the study, a biphasic fit was more appropriate than a monophasic one but not perfect. A limited number of experiments were evaluated also by derivative plots. One example is shown

in Fig. 4, clearly indicating the complex adsorption behaviour.

Values obtained from a simplified, monophasic adsorption behaviour are shown in Table 5. Initial rates were obtained as resonance angle shifts during the first 5–10 s and k_{on} from semilog plots. Extent 1 is plateau value and extent 2 angle shift after washing and drying. The statistical evaluation was based on individual data from 25 experiments and showed significance only for one factor in one response: increasing the amount of protein resulted in a lower rate constant.

3.2.2.2. Kinetics for liposomes in the presence of Ca^{2+}. The presence of 20 mM calcium chloride in the buffer throughout the experiment had profound effects on the adsorption kinetics, as is evident when comparing initial rates and extents in Table 5. Furthermore, the progress of adsorption followed a simpler pattern, approaching monophasic binding curves (Fig. 5). A complete analysis could only be performed for the proteoliposomes (N3–N7). The initial rate was the only parameter that could be determined reliably for protein-free N2 liposomes. Resonance scans indicated irregularities on the surface for the later parts of the binding curves and persisted after the wash and dry procedure (extent 2). Same problems were observed with N1-liposomes. However, extent 2 could usually be estimated, indicating that irregularities on the surface were washed away or smoothed out during the wash/dry treatment.

The statistical analysis was hampered by the lack of data points, 9 out of 23 experiments being incomplete. As the reproducibility between runs with one and the same liposome preparation was much better than in experiments without calcium ions, a fact that is understandable in view of the simpler adsorption pattern, the analysis still came out with significant factors. One is obvious as seen from Table 5: protein contributed negatively to initial rate. As was found in corresponding experiments with the ellipsometric set-up, no factor contributed significantly to the final

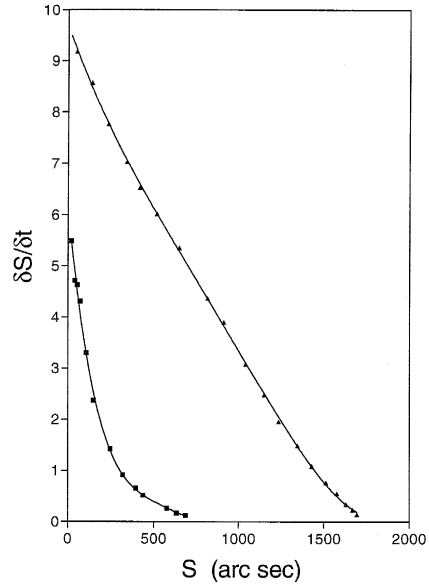

Fig. 5. Derivative plots for kinetics of lipid adsorption from liposomes in IAsys. The experiments were run with the same liposome preparation as described for Fig. 4, in the absence (■) and presence (▲) of 20 mM $CaCl_2$.

Table 5
Responses in adsorption kinetics for liposomes in Table 3 on plain silicon nitride, measured with resonant mirror

Liposome designation in Table 3	Without Ca^{2+}				With 20 mM Ca^{2+}			
	Initial rate arc s/s	k_{on} ($\times 10^3$) s^{-1}	Extent 1 arc s	Extent 2 arc s	Initial rate arc s/s	k_{on} ($\times 10^3$) s^{-1}	Extent 1 arc s	Extent 2 arc s
N1	5.6	24.6	132	611	25.8	*	*	1216
N2	3.4	6.2	269	531	21.1	*	*	*
N3	4.3	4.7	211	505	7.9	8.1	627	965
N4	3.3	4.0	384	709	8.0	5.3	1316	1674
N5	1.2	10.0	147	435	4.5	2.4	1039	1983
N6	1.1	2.4	109	203	5.1	2.3	1469	1330
N7	7.7	11.0	400	n d	4.7	2.8	1324	1130

The concentration of liposomes corresponded to 0.05 mM phosphate. Initial rate was calculated from the first 5–10 s, rate constant from semilog plot, extent 1 is plateau value and extent 2 after a wash and dry cycle. * denotes missing value because of unreliable signal (asymmetric resonance scan peak). Data shown are mean values from a maximum of 7 experiments.

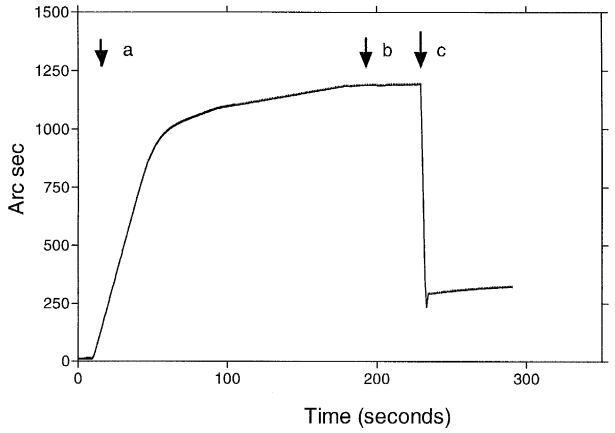

Fig. 6. Adsorption behaviour for DOPC liposomes on silicon nitride, measured by resonant mirror. Experimental conditions and symbols as for Fig. 4.

extent. All preparations ended up in about the same amount of deposited material (1372 ± 185 arc s, $n = 18$, N2 not included).

3.2.2.3. Kinetics for DOPC liposomes. Rapid adsorption of material was observed for DOPC liposomes, as was the case also in the ellipsometric system (Fig. 6). Most of the process took place at a constant rate, 26.7 ± 2.6 arc s/s ($n = 4$). The extent was 1053 ± 238 arc s. The deposited material remained bound upon exchange to buffer but not when exposed to the harsher treatment with water followed by drying. The same experiment performed in presence of 20 mM $CaCl_2$ made no difference in any of the parameters.

The extent value obtained for these liposomes was used as reference value for a homogeneous lipid bilayer on the silicon nitride surface. It corresponds well to calculations based on the amount of bound protein and expected angle shifts given in the manuals for IAsys, taking into account the same refractive index for the two types of biomolecules.

4. Discussion

The success and ease in forming a bilayer structure from liposomes on a solid support is dependent on several factors. Type, structure (including roughness) and cleaning of the support, composition and size of the liposomes, composition of the surrounding medium and the geometry of and flow dynamics in the fusion cell are all factors to be taken into consideration. The large number of combinations makes a systematic approach, using the principle of factorial design attractive [24]. A prerequisite for such studies to be meaningful is that one identifies the properties one is aiming at, and that these properties can be reliably quantified (the responses). Such properties were listed in the Introduction, and we correlate them without any quality tests with amount of material on the supports, measured by optical techniques.

Lipid monolayer covered Pt and Si/SiO_2, as well as bare Si/SiO_2 and Si_3N_4 were used as substrates but not included as factors in the designs. Earlier studies suggest firstly that Pt is superior to Si/SiO_2 as solid support regarding ease of membrane transfer as well as stability, and secondly that liposome fusion to bare and lipid-coated Pt results in membranes with similar characteristics [20,21]. The factorial designs included lipid composition, protein content and an environmental factor, temperature for liposome preparation. Liposomes prepared from unsaturated phosphatidylcholines such as POPC or DOPC are most frequently used for forming supported planar bilayer structures, as they are in the liquid crystalline state at room temperature, facilitating rapid spreading and formation of a fluid membrane on different types of support. Therefore, DOPC liposomes were used as reference liposomes in the present work, showing a rapid transfer of lipids to both Si/SiO_2 and Si_3N_4. These supported membranes were stable as long as they were kept in buffer but removed when subjected to the stability tests. Inferior adhesion appears to be general for phosphatidylcholines, independent on fatty acid composition, as shown by a number of Langmuir–Blodgett studies [19,25].

In the end-point studies we started with a pre-deposited lipid monolayer which is very resistant in stability tests. The liposomes prepared according to the designs were intended to form the outer lipid layer, therefore they must make contact with the alkyl chains of the deposited monolayer. It is obvious from Tables 1 and 2 that the underlying solid support is important for the process, with Pt generally resulting in better transfers than Si/SiO_2. From the tables, we can also observe a good correlation between the responses on the two surfaces for the different liposome preparations. Several of these experiments resulted in a net transfer from the surface rather than

the intended transfer to the surface. The statistical analysis of the designs in Tables 1 and 2 shows that unsaturated phosphatidylcholines are inferior to DPPC for obtaining membranes with the demanded qualities. High fluidity in the hydrocarbon chains per se appears not to be the reason for this effect, since cardiolipin is polyunsaturated but had no significant (although slightly negative) effect on the responses. A mismatch due to the differences in area occupied per molecule in the densely packed monolayer and the much greater area occupied by each unsaturated phosphatidylcholine in the liposomes might be a reason for an inferior hydrophobic interaction, with consequences for the transfer.

A schematic representation of different models for the process of lipid (and protein) transfer from (proteo)liposomes to hydrophilic and hydrophobic surfaces is given in Fig. 7. The most common model (Fig. 7A) is suggested by among others Rädler et al. [15] and Nollert et al. [10]. The model for transfer to hydrophobic surfaces (Fig. 7B) is by Kalb et al. [14], while the third alternative was suggested recently by Salafsky et al. [26]. The optical methods used here are based on changes in refractive index. Since the refractive index of intact liposomes is the same as for the surrounding medium, the optical signal obtained from step 1 in Fig. 7A can be very small. It is due only to the attachment area of the liposome, which is small when compared to the total area occupied by one liposome [27]. If the attached liposomes become flattened (step 2 in Fig. 7A), as suggested by Nollert et al. [10], the signal should increase. A complex adsorption behaviour to plain surfaces observed for several of the liposome preparations is thus not surprising, due to several, tentative steps as well as to the indiscriminate optical measurement principle.

However, DOPC liposomes formed bilayer structures within 5 min in the ellipsometer and 20–30 s in the IAsys. We interpret these results as instantaneous attachment and rupture (steps 1 and 3 in Fig. 7A), followed by a rapid spreading by bilayer sliding [15]. For the mixed lipid (proteo)liposomes, where the lipids should be less fluid, the process was slower and, for some compositions, hampered at an early step resulting in incomplete coverage. An example was given in Fig. 1A, where no apparent thickness increase was registered after two hours exposure to liposomes, which could be interpreted as hampering at the step of liposome attachment. In absence of Ca^{2+} only the protein-richest preparations resulted in an apparent thickness corresponding to a complete bilayer coverage (Extent 1 in Table 3). The IAsys experiments also indicated hampering at one of the steps and incomplete coverage. The wash/dry procedure which should promote the rupture of attached liposomes resulted in a greater signal, indicating that the material was spread out but not sufficiently for full coverage (Table 5, compare extents 1 and 2, absence of Ca^{2+}). These effects should mainly be due to electrostatic repulsion, as the content of DPPG

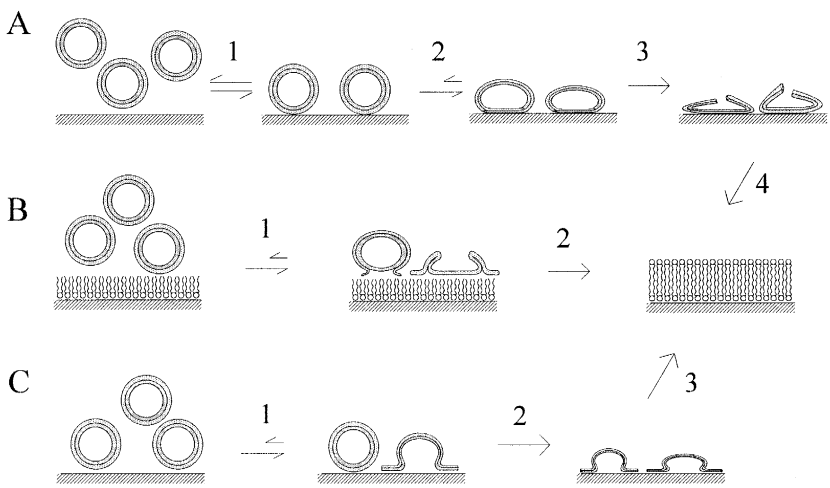

Fig. 7. Schematic representation of possible mechanisms for planar bilayer formation from liposomes on hydrophilic and hydrophobic surfaces. The drawings are out of scale, with a lipid molecule on the support enlarged approximately 50-fold compared to a liposome.

is high. Increasing the concentration of liposomes affected the rate of adsorption but not surface coverage.

Simpler adsorption patterns were observed in two cases: on hydrophobic surfaces (lipid monolayer covered platinum) and on plain surfaces in presence of calcium ions. In the first case, we suggest a simpler scheme (Fig. 7B). Attachment is a consequence of rupture, not the other way around as for plain supports. Another distinction is that the break of the liposomes takes place towards the surface. Rupture is followed by spreading, driven by the hydrophobic interactions between alkyl chains, which is the step the ellipsometer monitors.

Adding a high concentration of calcium ions promoted lipid transfer from the liposomes to both types of plain supports and simplified the adsorption pattern. The final structures after washing had thicknesses expected for (protein-containing) lipid bilayers. We suggest that Ca^{2+} had many effects on several of the steps in Fig. 7A. First, the ion electroneutralises DPPG, which may alter the density of adsorbed particles in steps 1 and 2. In the ellipsometric system, we also noticed stacking, which is another indicator of electroneutralisation. Second, the ions may contribute to the bonding strength to the support, acting as a bridge between oxidised silicon and DPPG. This hypothesis implies that dissociation should be more pronounced in the absence of Ca^{2+} ions, but no support for this was found in the experiments.

We suggest that the most important effect of Ca^{2+} in our systems is related to the well-known fact that this ion is a strong fusogenic agent, promoting the fusion of cells and liposomes [28,29]. By light scattering we found that this was the case also for liposomes in this study. The direct effect of liposome fusion is negative for the transfer rate of lipids from the liposomes to the support, as the number of particles decreases, but this is totally overshadowed by opposing effects. One of these is that Ca^{2+} may reduce the repulsive hydration force and promote hydrophobic interactions by dehydrating the phospholipids of the outer leaflet of the vesicles. This affects both liposomes in the solution and for liposomes attached to the surface, leading to a closer contact. Furthermore, intermediate structural changes occurring after the aggregation of liposomes, such as inverted micelles or regions of hexagonal II phases within the bilayer structure, i e an enhancement of the hydrophobic forces [30,31], may be the driving force behind the rupture and spreading steps in the scheme. Evidence for this hypothesis is found in the identification of significant factors for the fusion process. We found that 'intrinsic' fusogenic agents such as protein and a high DPPE/cholesterol ratio often promoted the transfer of lipids to the solid surfaces.

The orientation of proteins is an important issue for applications of supported bilayers. The scheme in Fig. 7A suggests that the outer leaflet of liposomes forms the inner leaflet of the supported membrane. Provided that the proteins have an outside-out orientation in the liposomes, the fusion mechanism of Fig. 7A will result in an outside-in orientation. However, both Salafsky et al. [26] and Contino et al. [32] have recently shown that the orientation of the leaflets was preserved upon fusion of proteoliposomes to plain glass, a finding that was the basis for model 7C by the former authors. This model is similar to what occurs on hydrophobic surfaces, although the spreading may be more restricted. We have also found that the activities of some proteins in supported bilayers were similar when formed from proteoliposomes on lipid monolayer-covered and on plain platinum, respectively [20]. Current research is to determine which scheme is correct for liposomes with compositions similar to those described in this paper.

In conclusion, we have shown that stable lipid bilayers can be formed on various solid supports from mixed liposomes. The composition of the liposomes is important for the ease of planar bilayer formation. Some instability present or inducible in the liposomes promotes the transfer of material from the liposomes to the support. The outer and inner leaflet can contain different lipid compositions by fusion on a lipid monolayer-covered support.

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References

[1] E. Sackmann, Science 271 (1996) 43–48.
[2] M. Kühner, R. Tampé, E. Sackmann, Biophys. J. 67 (1994) 217–226.

[3] D. Beyer, G. Elender, W. Knoll, M. Kühner, S. Maus, H. Ringsdorf, E. Sackmann, Angew. Chem. Int. Ed. Engl. 35 (1996) 1682–1685.
[4] A.A. Brian, H.M. McConnell, Proc. Natl. Acad. Sci. 81 (1984) 6159–6163.
[5] J. Spinke, J. Yang, H. Wolf, M. Liley, H. Ringsdorf, W. Knoll, Biophys. J. 63 (1992) 1667–1671.
[6] M. Stelzle, G. Weissmüller, E. Sackmann, J. Phys. Chem. 97 (1993) 2974–2981.
[7] C. Duschl, M. Liley, G. Corradin, H. Vogel, Biophys. J. 67 (1994) 1229–1237.
[8] H.M. Reinl, T.M. Bayerl, Biochemistry 33 (1994) 14091–14099.
[9] S. Heyse, H. Vogel, M. Sänger, H. Sigrist, Protein Sci. 4 (1995) 2532–2544.
[10] P. Nollert, H. Kiefer, F. Jähnig, Biophys. J. 69 (1995) 1447–1455.
[11] C. Steinem, A. Janshoff, W.-P. Ulrich, M. Sieber, H.-J. Galla, Biochim. Biophys. Acta 1279 (1996) 169–180.
[12] U.P. Fringeli, H.-J. Apell, M. Fringeli, P. Läuger, Biochim. Biophys. Acta 984 (1989) 301–312.
[13] P. Wenzl, M. Fringeli, J. Goette, U.P. Fringeli, Langmuir 10 (1994) 4253–4264.
[14] E. Kalb, S. Frey, L.K. Tamm, Biochim. Biophys. Acta 1103 (1992) 307–316.
[15] J. Rädler, H. Strey, E. Sackmann, Langmuir 11 (1995) 4539–4548.
[16] G. Puu, I. Gustafson, P.-Å. Ohlsson, G. Olofsson, Å. Sellström, in: Progress in Membrane Biotechnology, J. Gómez-Fernández, D. Chapman, L. Packer (Eds.), Birkhäuser Verlag, Basel, 1991, pp. 279–290.
[17] Å. Sellström, I. Gustafson, P.-Å. Ohlsson, G. Olofsson, G. Puu, Colloids Surf. 64 (1992) 275–287.
[18] Å. Sellström, I. Gustafson, P.-Å. Ohlsson, G. Olofsson, G. Puu, Colloids Surf. 64 (1992) 289–298.
[19] P.-Å. Ohlsson, G. Puu, Å. Sellström, Colloids Surf. B, Biointerfaces 3 (1994) 39–48.
[20] G. Puu, I. Gustafson, E. Artursson, P.-Å. Ohlsson, Biosensors Bioelectron. 10 (1995) 463–476.
[21] P.-Å. Ohlsson, T. Tjärnhage, E. Herbai, S. Löfås, G. Puu, Bioelectrochem. Bioenerg. 38 (1995) 137–148.
[22] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.
[23] L.T. Mimms, G. Zampighi, Y. Nozaki, C. Tanford, J.A. Reynolds, Biochemistry 20 (1981) 833–840.
[24] G.E.P. Box, W.G. Hunter, J.S. Hunter, Statistics for Experimenters. An Introduction to Design, Data Analysis and Model Building, Wiley, New York, 1978.
[25] J.B. Peng, M. Prakash, R. MacDonald, P. Dutta, J.B. Ketterson, Langmuir 3 (1987) 1096–1097.
[26] J. Salafsky, J.T. Groves, S.G. Boxer, Biochemistry 35 (1996) 14773–14781.
[27] T. Tjärnhage, G. Puu, Colloids Surf. B Biointerfaces 8 (1996) 39–50.
[28] J. Wilschut, D. Hoekstra, Trends Biochem. Sci. 9 (1984) 479–483.
[29] D.E. Leckband, C.A. Helm, J. Israelachvili, Biochemistry 32 (1993) 1127–1140.
[30] C.A. Helm, J.N. Israelachvili, P.M. McGuiggan, Biochemistry 31 (1992) 1794–1805.
[31] P.R. Cullis, B. de Kruijff, Biochim. Biophys. Acta 559 (1979) 399–420.
[32] P.B. Contino, C.A. Hasselbacher, J.B.A. Ross, Y. Nemerson, Biophys. J. 67 (1994) 1113–1116.