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The lipid composition determines the kinetics of adhesion and spreading of liposomes on mercury electrodes

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ABSTRACT

The dependence of membrane properties on their composition was studied by following the adhesion and spreading of unilamellar and multilamellar liposomes on static mercury electrodes with the help of chronoamperometry. The analysis of the peak-shaped signals allows determining the kinetic parameters of the three-step adhesion-spreading process. The presence of cholesterol in the membrane stabilizes the bilayer in the liquid-crystalline phase, and destabilizes the gel phase. The kinetic parameters also show the effect of superlattice formation in the DMPC–cholesterol system. The detergent triton X-100 is only incorporated in the liquid-crystalline DMPC membranes, and it is expelled to the solution when the membrane is transformed to the gel phase. In the liquid-crystalline membrane, it enhances the adhesion-spreading of liposomes on mercury. The lytic peptides mastoparan X and melittin affect the adhesion-spreading in a similar manner. For the rupture-spreading step, their effect is explained by pore formation. The results obtained with lecithins of different length suggest that the bilayer opening process has much in common with flip-flop translocations. For this process the activation energies were found to be independent of the chain length of the lecithin molecules, while the preexponential factor in the Arrhenius equation decreases drastically for longer chains.

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1. Introduction

Liposomes are highly interesting and useful models for biological cell membranes. In previous studies [1–3] we could show that chronoamperometry can be used to monitor the adhesion and spreading of liposomes on a mercury electrode. These measurements are helpful to investigate the properties of liposome membranes. In the present work, we have expanded these studies to liposomes with deliberately modified membranes, especially those in which cholesterol and DPPC have been incorporated in DMPC-based liposomes. Additionally, the effect of triton X-100, mastoparan X, and melittin which were dissolved in the aqueous phase, were elucidated with respect to the liposome adhesion and spreading behavior.

Cholesterol is an extraordinarily important constituent of biological membranes. Occasionally, in mammalian cells, it can reach concentrations as high as 30–50 mol%. In phospholipid bilayers it acts as a *crystal breaker,* disturbing the translational order of the lipid molecules in the gel state [4,5] and thus making the membrane fluid even at low temperatures. On the other hand, in the liquid-like phases, cholesterol causes a straightening of the disordered phospholipid acyl chains and reduces the mean headgroup area. This property is known as the *stabilizing effect* of cholesterol [4,6]. Cholesterol embedded in lipid bilayers can form different phase domains (rafts) in which the gel or the liquid-crystalline phases (depending on the temperature) coexists with the liquid ordered phase [7]. The geometry of the vesicles containing such domains can be theoretically modeled by minimizing an energy functional that includes the bending resistance, lateral tensions, line tension and normal pressure difference [8,9]. Interestingly, cholesterol has been shown to affect also lipid monolayers [10], in which case critical upper miscibility points appear.

Many studies have been focused on elucidating the role played by cholesterol in membranes, e.g., its effect under the action of osmotic stress [4], the effects on the uptake of drugs [11,12], etc. The formation of superlattices in lipid–cholesterol bilayers has been demonstrated to occur at certain molar fractions of cholesterol (e.g., at 0.154, 0.20, 0.25, 0.333, 0.40) [13–17].

Like cholesterol, also the presence of other lipids in the membrane can prompt the formation of phase domains. Thus, in certain temperature and composition ranges, the presence of DPPC in DMPC membranes can act in a similar way [18]. This led us to perform also experiments with mixed DMPC–DPPC liposomes.

2. Experimental

2.1. Outline of the chronoamperometric measurements and the applied data analysis

The adhesion and spreading of liposomes on a static mercury electrode causes capacitive spikes (peaks) in chronoamperometry

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because the capacity of the double layer changes due to the adsorption of lecithin on the electrode surface. By counting the number of peaks within a given time interval, i.e., their frequency, at different temperatures, it is possible to calculate the activation energy of the overall process. Integrating the *individual* peaks, i.e., integrating the current over time curves of each peak, yields charge (Q) transients for the single adhesion-spreading events. These transients can be fitted by the empirical equation

$$Q_{\mathrm{lip}} = Q_0 + Q_1\left(1-e^{t/\tau_1}\right) + Q_2\left(1-e^{t/\tau_2}\right) \quad (1)$$

where t is the time, Q_0, Q_1, and Q_2 are amplitudes of the three respective normal modes, and τ_1 and τ_2 are the time constants of the two time-resolvable kinetic modes. The first term on the right side stands for the very fast "docking" of the liposome on the mercury surface, the second term reflects the rearrangement of the molecules in initial contact with the electrode (called the "opening" process and controlled by the turning around of the lecithin molecules in the membrane), and the third term results from the spreading of the lecithin island on the mercury surface (controlled by the formation of pores in the liposome membrane to release the inner solution) (Fig. 1). A more detailed elaboration of the adhesion-spreading model led us to suggest more precise terms for the different steps: the first step will now be called "interaction-docking", the second and third steps will be described by the terms "bilayer opening", and "rupture-spreading".

The goodness of fit of the charge transients by Eq. (1) was estimated by the chi-square-based probability as described in [19]. Data series from liposomes in the liquid-crystalline phase were taken. Since the liposomes differ in size, the data series were normalized with respect to their global maximum. The average relative standard deviation of the series was evaluated. The product from the relative average standard deviation and the global maximum of any of the data series should be an estimate for the average standard deviation of a single point within a series, i.e., the standard deviation that would be observed for that point if the adhesion-spreading of that same liposome could be recorded several times. The Q-probabilities estimated from these standard deviations and from the chi-square values of the nonlinear regression are in any case larger than 0.001, and in many cases larger than 0.9, showing that the fitting of the model to the data is reasonable.

The empirical Eq. (1) has the same structure as the following equation

$$Q_{\mathrm{lip}} = Q_0 + Q_1\left(1-e^{-t/\frac{1+K_1}{k_1K_1}}\right) + Q_2\left(1-e^{-t/\frac{1+K_2}{k_2K_2}}\right) \quad (2)$$

which can be theoretically derived based on the following scheme of reaction steps of the adhesion-spreading process [see also references [3,4]]:

$$\mathrm{L} \underset{\rightarrow}{\overset{\leftarrow}{K_0}} \mathrm{L}' \xrightarrow{k_0} \mathrm{L_D} \underset{\rightarrow}{\overset{\leftarrow}{K_1}} \mathrm{L_{D*}} \xrightarrow{k_1} \mathrm{L_O} \underset{\rightarrow}{\overset{\leftarrow}{K_2}} \mathrm{L_{O*}} \xrightarrow{k_2} \mathrm{L_E} \underset{\rightarrow}{\overset{\leftarrow}{K_3}} \mathrm{L_A} \quad (\mathrm{I})$$

In this scheme the symbols have the following meaning: L: the free liposome, L′: the liposome in contact with the mercury surface, L_D: the docked liposome, L_{D*}: the adsorbed docked liposome in a deformed state, L_O: the opened liposome, L_{O*}: the adsorbed opened liposome, L_E: the "deconvoluted" liposome i.e., lecithin island that is not yet adsorbed, and L_A: the island of adsorbed lecithin molecules. K_1, K_2 and K_3 are adsorption equilibrium constants, and k_0, k_1 and k_2 are the rate constants of the interaction-docking, the bilayer opening and the rupture-spreading steps respectively. K_0 represents the equilibrium between liposomes in the bulk and intact liposomes on the mercury surface, and does not generate any charge response. Solving the appropriate kinetic equations starting from L_D (as the interaction-docking reaction is too fast to be detected), considering the fast adsorption equilibria following each step, and expressing each of the species in terms of the response factor (charge), Eq. (2) has been derived previously [2]. The above described model has been commented by Žutić et al. [20], and the misunderstandings of these authors have been explained in our response [21]. Our previously proposed model (Scheme I, and the corresponding Eqs. (1) and (2)) allow a consistent interpretation of experimental results [1–3], and the kinetic model is similar to those proposed by other authors to explain the interaction of liposomes with various interfaces [22,23], and it is also similar to models of liposome fusion [24,25]. Here, we shall report new experimental results which are in accordance with our previously developed model.

Chronopotentiometric measurements of liposome adhesion events with concomitant oxygen reduction have been used recently to study the potential dependence of liposome adhesion in air saturated suspensions, the reduction of oxygen helping to improve the signals at potentials near the potential of zero charge (pzc) [26]. Such experiments are useful to characterize the suspension in terms of concentration, polydispersity and potential range of adhesion, but do not provide any deeper insight into the kinetics and the actual mechanism of the adhesion-spreading of vesicles at the electrode surface, which is needed in order to extract information about the properties of the membrane.

2.2. Chemicals and electrochemical measurements

High-purity 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) were donated by Lipoid GmbH, Ludwigshafen, Germany, and they were used without further purification. The electrolyte potassium chloride (Suprapur®) was obtained from Merck (Darmstadt, Germany). Cholesterol was purchased from Fluka (Deisenhofen, Germany). Mastoparan X and melittin were purchased from Bachem (Bubendorf, Switzerland) and triton X-100 was a product of Sigma-Aldrich (Steinheim, Germany). The water used was prepared from deionised water by distilling it once with an addition of alkaline permanganate solution to oxidize traces of organic compounds, followed by a second distillation. Before measuring, the suspensions were deaerated for 20 min with high-purity nitrogen. Electrochemical measurements were performed with an AUTOLAB PGSTAT 12 (Eco Chemie, Utrecht, Netherlands) interfaced to a P4 PC in conjunction with an electrode stand VA 663 (Metrohm, Herisau, Switzerland). A multimode mercury electrode was used as working electrode, a platinum rod served as auxiliary electrode and an Ag|AgCl (3 M KCl, E=0.208 V vs. SHE) electrode was used as reference electrode. The surface area of the mercury drop was 0.48 mm^2, as determined by weighting 50 drops. The chronoamperometric measurements were performed at −0.9 V vs.

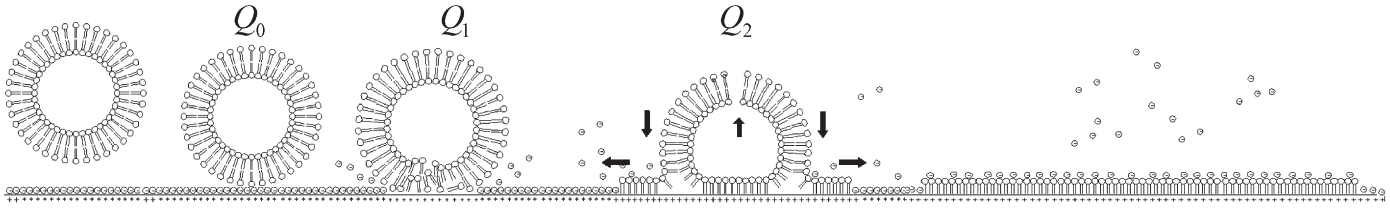

Fig. 1. Mechanism of adhesion of a single liposome on a mercury electrode, according to Scheme I. Q_1 and Q_2 are functions of time, as depicted in Eq. (1).

Ag|AgCl within 1 s with sampling each 50 μs or within 40 ms with sampling each microsecond for high resolution measurements. Previous experiments have shown that the chronoamperometric peak-like signals obtained at this potential are not different from those obtained at potentials near to the pzc [3], meaning that under the studied conditions the adsorbed monolayers are stable and, as far as from chronoamperometry can be judged, are also of similar structure. Of course, it cannot be ruled out that, depending on the potential, the adsorbed lipid layer contains defects as has been described by other authors [see, e.g., [27–30]].

The program "Signal Counter" [31] was used to determine the frequency of peaks (number of adhesion-spreading peaks per time interval). The solutions were thermostated with an accuracy of ±0.1 K. The distribution of liposome diameters was controlled by light-scattering measurements with a Zetasizer 3000 HS (Malvern Instruments, Herrenberg, Germany).

In the cases where the changes in the kinetic parameters are reported as significant, such claim is supported by a *t*-test, with a confidence level of at least 95% for all cases and higher than 99.99% for certain data sets. In the cases where it is claimed that no significant change of the kinetic parameters is observed, the statement is supported also by the *t*-test with confidence levels of at least 95%.

2.3. Giant unilamellar vesicles (GUV)

The technique of preparation of GUVs reported by Moscho et al. [32] was slightly modified: 3 mg of lecithin (DMPC, DPPC or a mixture of both) were dissolved in 2.2 mL of a 1:10 mixture of methanol: chloroform. Then 30 mL of 0.1 M KCl solution were added carefully by pouring along the flask walls and the organic solvent was rapidly removed with the help of a rotary evaporator (Laborota 4000, Heidolph, Nürnberg, Germany) using a Rotavac control pump (Heidolph, Nürnberg, Germany) at 40 °C (45 °C for pure DPPC liposomes) and a final pressure of 10 mbar. The rotation speed was 30 rpm. That procedure allowed obtaining a clear suspension containing a high yield of GUVs. Formation of GUVs was confirmed by comparing the size distribution derived from light-scattering measurements with the size distribution of the chronoamperometric peaks, which changes according to the lamellarity. In case of MLVs (multilamellar vesicles) and GUVs of the same size, the MLVs give larger signals of adhesion as they contain a significantly larger amount of lecithin molecules than the GUVs [3,4]. To study of the influence of mastoparan X and melittin on the adhesion-spreading kinetics of liposomes, the peptides were added from a stock solution to the electrochemical cell. The concentrations of mastoparan X and melittin in the stock solutions were determined by means of the optical absorption of tryptophan (absorption coefficient: 5570 L mol^{-1} at the wavelength of 280 nm). The liposome suspensions with the addition of mastoparan X and melittin were stirred for 30 min to ensure that the peptides can interact with the liposome membrane. In the experiments with triton X-100, a concentrated solution was prepared and for each experiment the amount necessary for a final concentration of 0.15 μM was added to the suspension. Temperature variation experiments (with 1 K increments) were performed by slowly cooling down the freshly prepared liposomes. After reaching the phase transition temperature, the suspension was cooled to 2 °C and the temperature was then slowly risen in 1 K increments and the measurements of adhesion events were performed at each temperature. This temperature program had the objective to minimize shape changes [33,34], esp. when the liposomes were in the liquid-crystalline phase.

2.4. Multilamellar vesicles containing cholesterol

The above described technique does not allow preparing unilamellar vesicles with cholesterol, as this molecule is too lipophilic with its very small hydrophilic end and it thus will remain completely dissolved in the organic phase until that is completely evaporated. Therefore, measurements had to be performed with multilamellar vesicles. Multilamellar DMPC vesicles with different cholesterol contents (from 1 to 40%) were prepared by the thin film hydration method. DMPC and cholesterol were separately dissolved in chloroform. For each composition both components were mixed in such way as to ensure a total lipid concentration of 0.1 g L^{-1}. The mixed solution of DMPC and cholesterol was diluted with chloroform in a 50 mL round bottom flask and the solvent was then removed with a Laborota 4000 (Heidolph, Nürnberg, Germany) using a Rotavac control pump (Heidolph, Nürnberg, Germany) at 45 °C and a final pressure 440 mbar. Rotation speed was 180 rpm. After solvent evaporation, 50 mL of 0.1 M KCl solution in bidistillated water were added, as well as small glass pearls, and the flask was shaken vigorously until all lipids were suspended. The suspension was then extruded through a 450 nm filter to obtain MLVs.

3. Results

3.1. Lipid mixtures: MLVs containing cholesterol

Fig. 2 depicts a plot of activation energies of the steps of liposome adhesion-spreading as a function of cholesterol content for both the gel and the liquid-crystalline phases. At low temperatures (Fig. 2-a), it can be seen that the effect of cholesterol is stronger for the microscopical processes, whose activation energies decrease with increasing cholesterol content. That observation is related to the well known *crystal breaker* property of cholesterol and to the increasing fluidity of the membrane with increasing cholesterol content at temperatures below the phase transition temperature (PTT) [16,35]. When the cholesterol content is increased, the activation energies of all processes follow a similar trend like the elastic properties of the membrane [35–37]. This is pointing to a relationship between elastic properties and adhesion-spreading kinetics. As stated before, the microscopical processes involve a deformation and a rupture of the membrane, thus making understandable such relationship. The overall activation energy increases steadily with increasing cholesterol content, with the exception of vesicles with 25% cholesterol, where the energy barrier of the process is unusually high; and for 40% cholesterol, where it decreases to a value near that of pure DMPC MLVs. At concentrations above $x_c = 0.333$ (x_c is the molar ratio of cholesterol), the membrane forms a liquid ordered phase in the entire temperature range, and the phase transition disappears. This phenomenon is caused by the rigid nature of cholesterol, which prevents the cooperative ordering of the long hydrocarbon chains of the lipids [38]. At 40% cholesterol it is therefore understandable that the behavior is different as the membrane of the MLVs is liquid.

On the other hand, at high temperatures it can be clearly seen that the activation energies of all processes increase with cholesterol concentration, with the exception of the overall activation energy at 30% cholesterol and the energy barriers of the microscopical process at 40% cholesterol, which are lower than expected according to the observed tendency. The overall activation energy at 20% cholesterol is too high and also outside the trend observed for the rest of the MLVs. Contrary to what happens in the gel phase, above the PTT, cholesterol embedded in membranes contracts the membrane laterally and causes an increase in the compressibility and bending modulus, i.e., increases the membrane rigidity and diminishes its fluidity [35,36]. Here again, we observed a correlation between kinetic and elastic parameters.

As discussed above, Fig. 2 shows that the overall activation energies for $x_c = 0.20$ and 0.25 do not follow the trend observed for other cholesterol concentrations. At high temperatures, the vesicles with 20% cholesterol behave differently than the others, while at low temperatures the same can be said about MLVs with 25% cholesterol. For vesicles with $x_c = 0.4$, at high temperatures, the sudden decrease of the activation energy of the

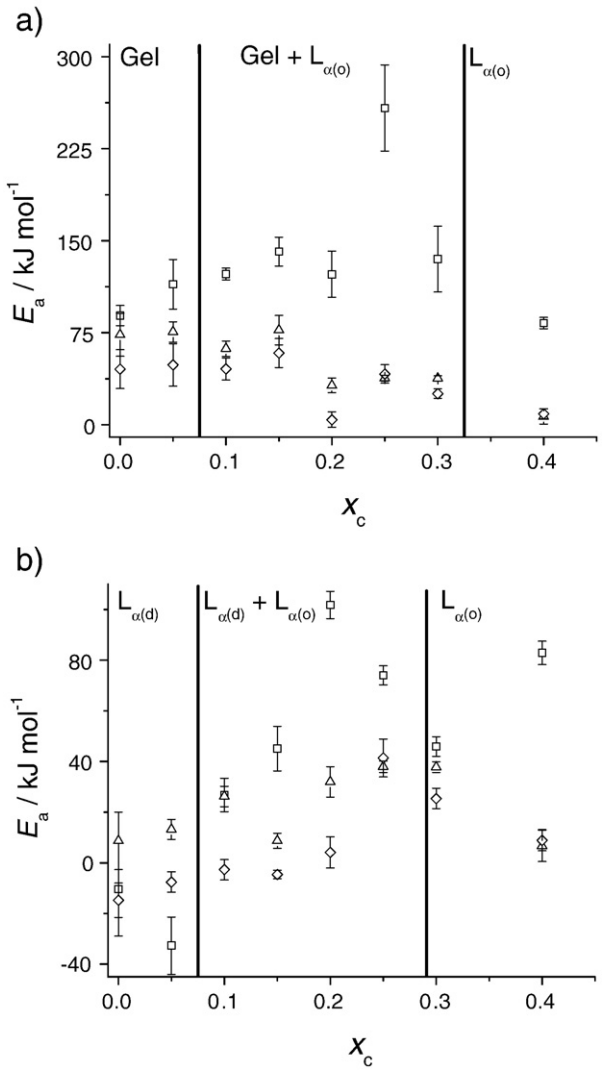

Fig. 2. Activation energies of the adhesion-spreading step as function of the molar fraction x_c of cholesterol in multilamellar DMPC liposomes a) at low (2–23 °C), and b) at high temperatures (24–45 °C): □ overall process, △ bilayer opening process, ◇ rupture-spreading process. The vertical bars indicate the standard deviations of measurements.

microscopic process is also outside the trend. For these compositions, the formation of superlattices has been reported [13,14,16,17]. At these compositions the different phase domains disappear and the membrane acquires a homogeneous composition with highly ordered lecithin molecules surrounding the cholesterol molecules. This is the reason for the observed differences in behavior. Remarkably, the slope of the Arrhenius plot for the superlattice compositions in the phase coexistence range (x_c=0.20 and 0.25) changes near the pretransition temperature (T^{-1}=0.0035317 K^{-1}) of pure DMPC (Fig. 3) and not at the main phase transition at T^{-1}=0.003371 K^{-1}. For pure lecithin liposomes, the phase above the pretransition temperature has already the characteristics of a superlattice, i.e., a high degree of local order. The fact that *no* main phase transition is observable in our measurements (see the straight line between T^{-1}=0.003193 K^{-1} and T^{-1}=0.0035193 K^{-1}) indicates that once a DMPC–cholesterol superlattice is formed, it is stable even at temperatures above the PTT of pure DMPC. Interestingly, the overall activation energy for the liposome adhesion-spreading process for this superlattice is positive, whereas that of pure DMPC liposomes in the pretransition state is negative. That means that the adsorption equilibrium at the beginning of the liposome adhesion is still favored in spite of the increasing temperature.

In the case of x_c=0.30 and high temperatures, the determined activation energies are also outside the general trend described above. These liposomes almost exactly correspond to the line in the phase diagram dividing the coexistence region and the pure liquid ordered region. The phase in which such vesicles are found is then unclear, and small changes in the temperature may give rise not only to changes in the kinetic parameters of adhesion-spreading, but also to the phase composition of the vesicles. Therefore, to be on the safe side, for the analysis of the results, these data were not considered.

The time constants of the microscopical (bilayer opening and rupture-spreading) processes are depicted in Fig. 4 as a function of the cholesterol content. An important observation is that the rupture-spreading process is much slower for the liquid ordered phase (L$_{\alpha(o)}$) than for the phase coexistence range (Gel+L$_{\alpha(o)}$). The large time constant of rupture-spreading at x_c=0.25 is certainly caused by the existence of a superlattice structure at that cholesterol content. According to Vanderlick and coworkers [39], the critical tension of the membrane decreases, compared to the liquid ordered phase, when there are phase domains; meaning that the membrane is more easily ruptured when phase domains are present in the membrane. For our model [4] it has been assumed that the rupture-spreading rate is controlled by the formation of pores which allow the inner solution to flow out. This idea is supported by experimental findings which show that a destabilizing of the membrane, i.e., in the (Gel+L$_{\alpha(o)}$)-range; leads to a faster rupture-spreading. This can also be deduced from the experiments performed at 30 °C, i.e., for the phase coexistence range (L$_{\alpha(d)}$+L$_{\alpha(o)}$) in comparison to the liquid ordered phase range L$_{\alpha(o)}$.

3.2. Lipid mixtures: DMPC–DPPC GUVs

Fig. 5 shows how the DMPC–DPPC membrane composition affects the time constants of the rupture-spreading step in case of GUVs. For liposomes in the gel phase, large experimental errors happen to occur, most likely because of the known size dependence of the rate constant of the rupture-spreading step [3]. Despite the rather large scattering of data, the average time constants are smallest for liposomes in the phase coexistence region, i.e., for that region the rupture-spreading step is faster than for liposomes with membranes made up of the pure gel and fluid phases. This is in accordance with reports of Bizotto and Nelson who have found that liposomes rupture more easily when the membrane is in the gel-fluid phase coexistence region than for fluid

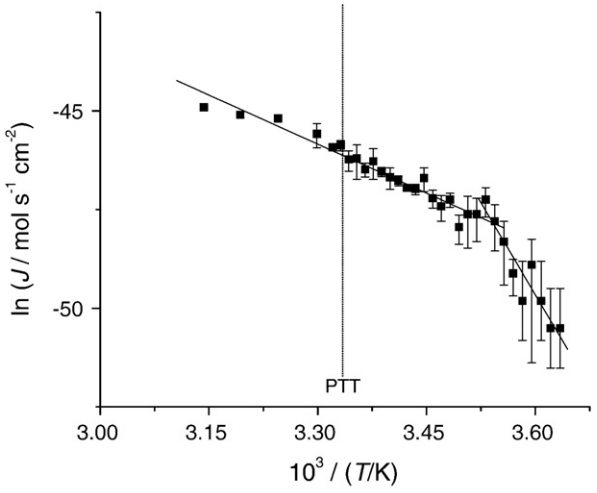

Fig. 3. Arrhenius plot for multilamellar DMPC liposomes containing 25 mol% of cholesterol. The natural logarithm of J, the number (in mol) of adhesion-spreading events per second and per square centimeter, is plotted versus the reciprocal temperature. Error bars were calculated from the standard deviation of the peak frequency.

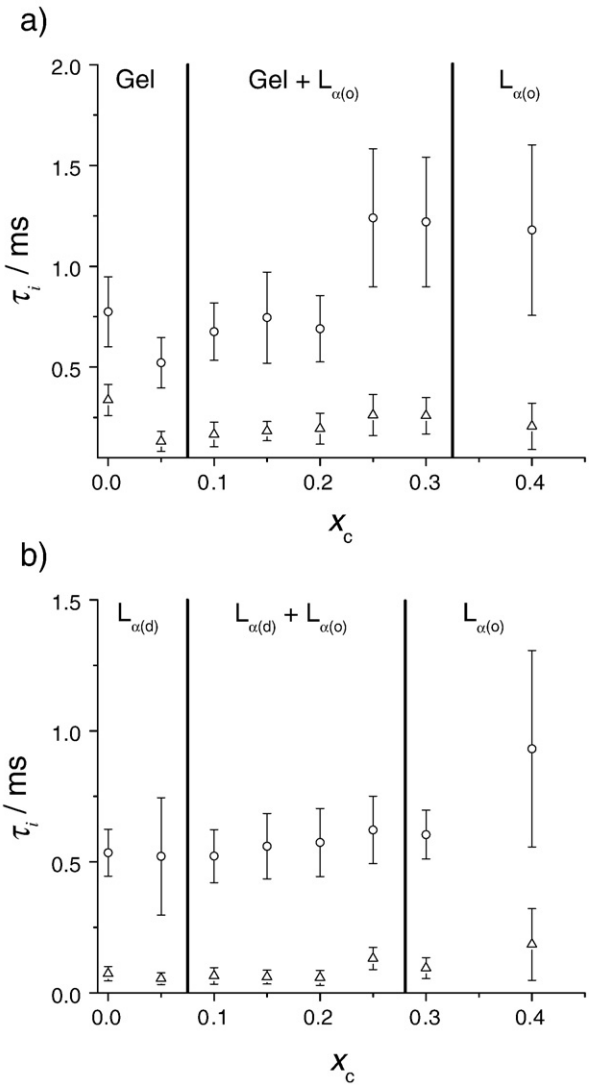

Fig. 4. Time constants at a) 288 K and b) 303 K as a function of the cholesterol content of multilamellar DMPC liposomes: △ bilayer opening (i=1), ◯ rupture-spreading (i=2). The vertical bars indicate the standard deviations of measurements.

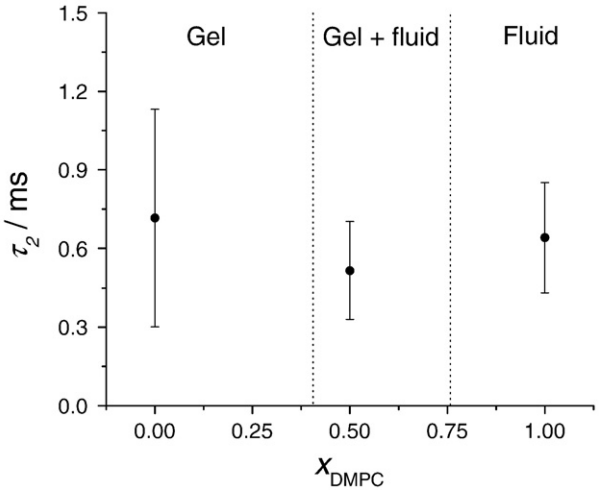

Fig. 5. Time constant of the rupture-spreading step of unilamellar liposomes at 306 K as a function of the DMPC fraction (x_{DMPC}) on the total lipid content (DMPC+DPPC) and the phase composition.

membrane vesicles [39]. The results shown in Fig. 5 are in agreement with our assumption that pore formation is the limiting step of the rupture-spreading step. Determining the time constants at different temperatures within the range where the phase coexistence at X_{DMPC}=0.5 is observed (30–35 °C), the activation energy of the rupture-spreading step of vesicles with phase domains is found to have an apparent value of −37.4±9.6 kJ mol^{-1} which is much more negative than the −9±4.9 kJ mol^{-1} determined for pure DMPC vesicles in the liquid-crystalline phase, showing that the apparent energy barrier of the rupture-spreading decreases when there are coexisting phases. For the case of bilayer opening, Fig. 6 (T=45 °C; i.e., conditions where only the liquid-crystalline phase exists) shows that the bilayer opening is always faster when DMPC is present in the DPPC membrane. The decrease of τ_1 occurs already at rather small DMPC concentrations and remains almost constant above x_{DMPC}=0.3. In Table 1 the activation energies and preexponential factors are listed for the bilayer opening process and different vesicle compositions. Obviously, the effect of the composition on the activation energy is rather small, especially when considering the error margins and the fact that the liquid-crystalline phase of pure DPPC vesicles is found in a different temperature range than that of DMPC. However, the preexponential factor decreases by four orders of magnitude when comparing DMPC with DPPC, meaning that the bilayer opening is much slower for the latter. For mixed DMPC–DPPC vesicles (molar ratio 1:1), the value of the preexponential factor is intermediate to those of the individual components. According to our model, the bilayer opening step involves the turning around of lecithin molecules. Indeed, from the experimental results it can be concluded that this is easier for shorter molecules like DMPC compared to DPPC. A similar process controls also the flip-flop translocation of lipids in membranes, as they have to turn around before crossing the transmembrane space [40]. Liu and Conboy [41] demonstrated that the rate constant of that process depends on the chain length of the lipids. That dependence results from changes in the preexponential factor, which decreases by several orders of magnitude with increasing chain length, while the activation energy of the process remains constant since the polar headgroups are the same. The rate constant of the bilayer opening step as determined by our measurements follows the same trend, and it is therefore tempting to assume that the turning around of molecules controls the bilayer opening process. Furthermore, previously [3] we have shown for vesicles in the gel phase that the time constant of bilayer opening can be extrapolated to the turning around of a single lecithin molecule in a lipid membrane. For DMPC the activation energy of bilayer opening of such system, i.e., the

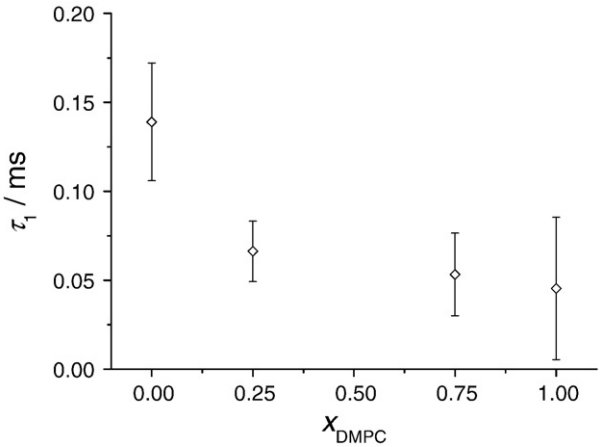

Fig. 6. Time constant of the bilayer opening step of unilamellar liposomes at 318 K as a function of the DMPC fraction on the total lecithin content (DMPC+DPPC) in liposomes with just one phase. The vertical bars indicate the standard deviations of measurements.

Table 1
Preexponential factors A and activation energies E_a of the bilayer opening step of liquid-crystalline GUVs of different lecithin compositions

	$A_{(opening)}/s^{-1}$	$E_{a(opening)}$/kJ mol^{-1}
DMPC	1.1×10^{10}[a]	34.7±5
DMPC:DPPC 1:1	1.2×10^{8}[b]	25.5±7.3
DPPC	4.7×10^{6}[c]	17.2±2.7

[a] $\ln A_{(opening)} = 23.1 \pm 2$.
[b] $\ln A_{(opening)} = 18.6 \pm 2.7$.
[c] $\ln A_{(opening)} = 15.4 \pm 1$.

energy barrier for turning around one DMPC molecule, was found to be 93±19 kJ mol^{-1}. Applying the same extrapolation to the data obtained for DPPC GUVs in the gel phase, the activation energy is 67.1±31.1 kJ mol^{-1}, which is clearly in the same range. Both values are in agreement with the flip-flop activation energy of 81 kJ mol^{-1} determined by Kornberg and McConnell [42] for the flip-flop of molecules in liposomes composed of DPPC and egg PC and also with the 85 kJ mol^{-1} determined by Abreu et al. [43] for the flip-flop of labelled DMPE in a POPC matrix. The preexponential factors determined in our experiments are 7.1×10^{20} s^{-1} and 2.6×10^{15} s^{-1} for DMPC and DPPC respectively, a five orders of magnitude decrease that corresponds well to the behavior reported in the literature. Thus, the analysis of chronoamperometric peaks caused by liposome adhesion-spreading on a mercury electrode seems to be a useful tool also for determining the activation energy of flip-flop translocation.

3.3. Effect of triton X-100

To asses the effect caused by the detergent triton X-100 on liposome adhesion-spreading, the rather low concentration of 0.15 μmol L^{-1} of triton X-100 has been used because higher concentrations cause the immediate and complete disintegration of the liposomes before measurements. The data in Table 2 show that an effect of triton X-100 on the time constant of the two microscopical processes can be observed when the membrane is in the gel phase. Triton X-100 was added to the liposome suspension at 35 °C, i.e., above the PTT, and after about 30 min the temperature was slowly decreased below the PTT (23.5 °C). This means that the triton molecules had the chance to dissolve in the liposome membrane. However, we observed that the capacitive background current at the beginning of the life time of the static mercury drops was remarkably decreased when the liposomes were in the gel phase. This is a strong indication of the presence of triton molecules in the aqueous phase surrounding the gel phase liposomes. Triton is known to strongly adsorb on mercury and thus it decreases the capacitive background current. Very interestingly, when the liposomes are kept above the PTT, i.e., when they are in the liquid-crystalline phase, the background current does not change in the presence of triton. This shows that the triton molecules are no longer dissolved in the aqueous phase, but they are mainly present in the liposome membrane. The concentration of free triton in the aqueous phase must be very small so that it does not affect the capacitive background. This understanding of the experimental results is also in agreement with the observation that triton affects the time constants of the bilayer opening and rupture-spreading steps under these conditions: Both the bilayer opening and the rupture-spreading processes are faster when the membrane is in the liquid-crystalline phase and triton X-100 is present. Takiguchi et al. [44] have shown earlier that triton X-100 causes the formation of large holes in zwitterionic membranes. These holes appear and disappear in cycles in which the membrane changes from a tense to a quaking structure. Takiguchi et al. used triton X-100 concentrations above the critical micellar concentration (CMC). Our results show that the same effect seems to be operative also at concentrations below the CMC; however, not accompanied by the disintegration of the vesicle. Triton X-100 is incorporated in the liquid-crystalline phase membranes, and it is obviously expelled when the temperature drops below the PTT. At these lower temperatures the triton is in the aqueous phase, and it is obviously quickly adsorbed on the mercury electrode. However, the adhesion and spreading of the gel phase liposomes on the triton loaded mercury electrode is not altered. These findings are of great interest, as the interaction mechanism between membranes and different detergents and surfactants is still a matter of debate, and our results show that triton X-100 is not soluble in the gel phase of DMPC, or only to a very small extend.

Table 2
Comparison of the time constants of the bilayer opening and the rupture-spreading steps of pure unilamellar DMPC liposomes with unilamellar liposomes treated with 0.15 μM of triton X-100

	T/K	τ_1/ms		τ_2/ms	
		Only DMPC	With 0.15 μM triton X-100	Only DMPC	With 0.15 μM triton X-100
Gel phase (average values)	284	0.23±0.082	0.18±0.1	0.91±0.45	0.75±0.14
	287	0.19±0.06	0.14±0.08	0.78±0.23	0.59±0.25
	290	0.15±0.054	0.12±0.023	0.68±0.11	0.46±0.13
Liquid crystalline	297	0.088±0.043	0.075±0.022	0.56±0.17	0.27±0.06
	300	0.081±0.028	0.067±0.02	0.56±0.14	0.26±0.027
	303	0.075±0.017	0.059±0.016	0.56±0.11	0.25±0.085

3.4. Effect of pore forming peptides

Previously [4] we have shown that the activation energy of the rupture-spreading step decreased remarkably when a small amount of mastoparan X, a peptide component of the wasp venom of *Vespa xanthoptera*, was added to the liposome suspension. Mastoparan X is known to form pores in membranes or at least to diminish their resistance for pore formation [45–47]. Here we can show that increasing the concentration of mastoparan X remarkably decreases the activation energy of the rupture-spreading step (Fig. 7), confirming that pore formation is the limiting factor during the rupture-spreading step. The addition of melittin (the major protein component of the venom of the honey bee *Apis mellifera*), which is also known to create pores in the membrane [48–51], also accelerates the rupture-spreading step (cf. Table 3). A very interesting observation is that for a given concentration of either peptide,

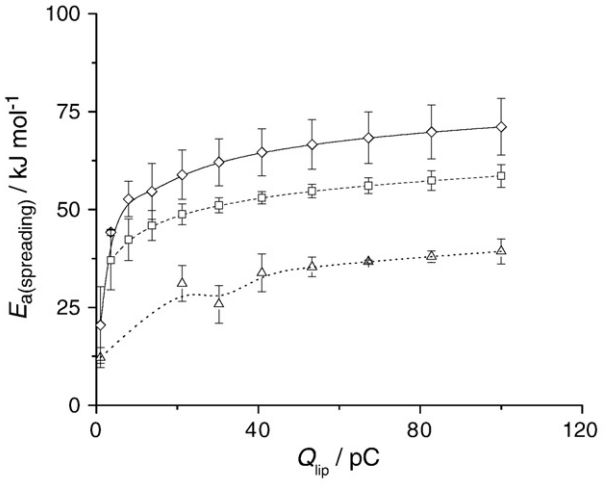

Fig. 7. Activation energy of the rupture-spreading step as a function of the total charge displaced by the unilamellar DMPC liposomes and of the concentration of mastoparan X added to the suspension. Solid line: only DMPC, dashed line (squares): 0.01 μM mastoparan X, punctured line (triangles): 0.1 μM mastoparan X.

Table 3
Time constants τ_1 and τ_2 for the adhesion-spreading of unilamellar liposomes on a mercury electrode when lytic peptides (mastoparan X or melittin) are added to the suspension

Parameter	Pure DMPC	Mastoparan X			Melittin		
		0.01 μM	0.1 μM	0.5 μM	0.01 μM	0.1 μM	0.5 μM
τ_1/ms at 290 K[a]	0.15±0.05	0.14±0.04	0.10±0.02	0.084±0.013	0.19±0.07	0.11±0.04	0.095±0.023
τ_2/ms at 290 K[a]	0.68±0.11	0.48±0.11	0.43±0.10	0.36±0.06	0.73±0.18	0.41±0.14	0.36±0.07
τ_1/ms at 303 K	0.075±0.017	0.13±0.03	0.068±0.026	0.057±0.022	0.13±0.04	0.066±0.022	0.065±0.027
τ_2/ms at 303 K	0.56±0.11	0.41±0.16	0.28±0.09	0.27±0.09	0.49±0.16	0.25±0.06	0.26±0.04

[a] Average values.

the time constants are very similar, independent of the type of peptide. In the liquid-crystalline phase this effect is even more pronounced. These results suggest that the action of melittin is very similar to that of mastoparan X.

4. Conclusions

Chronoamperometry proved to be a valuable tool to study membranes of liposomes: The mechanism of adhesion and spreading of liposomes on a mercury electrode can be described as a three step process. Each of the steps is clearly identified and the activation parameters can be determined for the bilayer opening and rupture-spreading steps. The experiments allow studying the effect of foreign molecules which are present in the membrane on the membrane properties. As expected, the presence of cholesterol was found to have a strong effect on the activation energy of the overall macroscopical process. The *stabilizing effect* at temperatures *above* the PTT, as well as the role of cholesterol as *crystal breaker below* the PTT was clearly detectable in the kinetic parameters of liposome adhesion and spreading. Further, the formation of superlattices clearly affects the kinetic parameters of the adhesion-spreading process. Thus chronoamperometric measurements can be easily used for an accurate determination of the temperature of superlattice formation, without using complex microscopic techniques.

The results obtained with mastoparan X, melittin, and triton X-100, which obviously interact with the membrane, give new insight into the way in which such substances affect the membrane. The obtained kinetic parameters of the adhesion and spreading suggest that triton X-100 is only incorporated in the membrane when the lipids are in the liquid-crystalline phase. The results obtained with the pore forming peptides (melittin and mastoparan X) point to a very similar effect of both peptides in the membrane, as a certain amount of melittin has the same effect as the same molar amount of mastoparan X even though the primary structures of both peptides are quite different.

The chronoamperometric technique also allows to access information on the different steps of liposome adhesion. The dependence of the rupture-spreading rate on the pore forming capabilities of the membrane could be demonstrated as well as the relationship between the bilayer opening step and the ability of lecithin molecules to turn around within the lipid membrane. The energy barrier of flip-flop translocation is related to the latter process and is accessible by extrapolating the kinetic data determined for the bilayer opening of liposomes in the gel phase to the case of the turning around of a single lecithin molecule. The fact that these processes, i.e., bilayer opening and rupture-spreading steps are analogous to those involved in cell fusion (hemifusion stalk=bilayer opening, fusion pore=rupture-spreading), will help in understanding better the role played by several molecules in the process of cell fusion.

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