

Material Property Characteristics for Lipid Bilayers Containing Lysolipid

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ABSTRACT The apparent area expansion modulus and tensile strength of egg phosphatidylcholine (EPC) membranes are measured in the presence of monooleoylphosphatidylcholine (MOPC). The apparent area expansion modulus decreases from 171 mN m^{-1} for pure EPC membrane to 82 mN m^{-1} for a membrane containing 30 mol % MOPC. This significant decrease of the apparent area expansion modulus is attributed to the change of the membrane area due to the tension-dependent exchange of MOPC between the bathing solution and the membrane. Similar to the apparent area expansion modulus, the tensile strength of the membrane decreases with the increase of the molar concentration of MOPC in the membrane. The tensile strength of pure EPC membrane is 9.4 mN m^{-1} whereas that for a membrane containing 30 mol % MOPC is only 1.8 mN m^{-1} , and for a membrane containing 50 mol % MOPC it is even smaller, on the order of 0.07 mN m^{-1} . The decrease of the tensile strength is coupled with a decrease of the work for membrane breakdown, which changes from $4.3 \times 10^{-2} \text{ kT}$ for pure EPC membrane to $2 \times 10^{-6} \text{ kT}$ for a membrane with 50 mol % MOPC. Overall, these results show that the decrease of the apparent area expansion modulus in the presence of exchangeable molecules is a fundamental property for all membranes and depends on the area occupied by these molecules. The method presented here provides a unique tool for measuring the area occupied by an exchangeable molecule in the bilayer membrane.

INTRODUCTION

The strength of natural cell membranes is determined primarily by their lipid composition; however, it also depends on the presence of other molecules. For example, the presence of cholesterol increases the membrane strength (Needham and Nunn, 1990), whereas the presence of micelle-forming molecules, such as the bile acids released from the gall bladder, decreases it (Evans et al., 1994). This decrease of membrane strength in the presence of micelle-forming species has an important role in digestion and is also used by some parasites, such as *Schistosoma mansoni*, which produce lysolipids as a means to attack target cells (Golan et al., 1986).

Membrane strength is characterized by the maximal tension that can be supported by the membrane before breakdown (this maximal tension is also called the critical membrane tension). Although the exact mechanism of membrane failure is unknown, it is widely observed that membrane strength is closely related to the membrane elasticity, characterized by the area expansion modulus. The measurement of both mechanical characteristics shows that they change simultaneously for a wide range of membrane compositions (Evans and Needham, 1987; Needham, 1995), which establishes their dependence on the membrane structure. This dependence is confirmed by the comparison of the change of the ordering in the membrane hydrophobic region in the presence of cholesterol measured by x-ray diffraction

(McIntosh, 1978; McIntosh et al., 1989; Simon et al., 1992) and the area expansion modulus and the tensile strength (Needham and Nunn, 1990). The addition of cholesterol to the membrane increases membrane ordering and at the same time increases both the area expansion modulus and the tensile strength.

The primary goal of the present work is to establish the composition-property relation for the area expansion modulus of a bilayer membrane in the presence of a well characterized exchangeable species, the lysolipid monooleoylphosphatidylcholine (MOPC). The secondary goal is to measure the coupling between the area expansion modulus and membrane strength for membranes with different MOPC concentrations and to characterize the work for membrane breakdown. MOPC is chosen as a test molecule because of its similarity to egg phosphatidylcholine (EPC). The two molecules are similar except in their hydrocarbon chain regions; EPC is a diacyl lipid and has two hydrocarbon chains, whereas MOPC is a lysolipid and has one chain. Having a single hydrocarbon chain, the lysolipid has higher solubility in aqueous solution than the diacyl lipid and therefore has a faster rate of exchange with the bathing solution. The rate of exchange of MOPC with EPC membranes is on the order of 0.3 s^{-1} (Needham and Zhelev, 1995; Zhelev, 1996; Needham et al., 1997), whereas the diacyl lipid is practically nonexchangeable. Structurally, MOPC is very compatible with the bilayer and is positioned such that its headgroup is in the same plane as the headgroups of the diacyl lipid (McIntosh et al., 1995). This leads to an apparent increase of the species density in the headgroup region in the presence of MOPC (McIntosh et al., 1995). The apparent area expansion modulus of this two-component membrane is modeled in a similar way as the area expansion modulus of the stearyl-glycerophosphatidylcholine (SOPC):bile acid salt membrane (Evans et al.,

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1994). Here, the area expansion modulus is considered to have three components: one component related to the intrinsic area expansion modulus of EPC, another component related to the intrinsic area expansion modulus of MOPC, and a third component related to the number of exchanged MOPC molecules.

MATERIALS AND METHODS

Vesicle formation

Giant unilamellar vesicles (5–40 μm in diameter) were made from either EPC (Avanti Polar Lipids, Alabaster, AL) or EPC containing different concentrations of monooleoylphosphatidylcholine (MOPC) (Avanti Polar Lipids). The amount of MOPC for all preparations was 0.52 mg. The desired molar ratio of EPC to MOPC was obtained by the addition of different amounts of EPC. The lipids were dissolved in chloroform, the chloroform was then evaporated under nitrogen, and the formed lipid layers were swollen overnight in 2 ml of 168 mOsm sucrose. The lipid preparation contained giant unilamellar vesicles as well as multilamellar structures. Only unilamellar vesicles were chosen for the experiments.

Because MOPC is exchanged between the membrane and the bathing solution (Needham and Zhelev, 1995; Zhelev, 1996; Needham et al., 1997), it was necessary for all suspending solutions to have the same concentration of MOPC. Therefore, the experimental chamber was filled with glucose solution containing MOPC (chamber solution). This solution was prepared as described below. The same amounts of MOPC and EPC were used for preparing the chamber solution as in the preparation of vesicles. The chloroform was evaporated under nitrogen, and the formed lipid layers were swollen overnight in 2 ml of 171 mOsm glucose. The swollen lipid was centrifuged, and the supernatant was collected and used to fill the experimental chamber. This procedure for vesicle and chamber solution preparation provided similar concentrations of MOPC for both the vesicle suspension and the solution used to fill the experimental chamber. Therefore, the transfer of the vesicles to the experimental chamber did not induce change of the partitioning of MOPC into or out of the bilayer membrane.

Micromanipulation

In the micromanipulation experiments, a single vesicle was manipulated with a micropipet. To minimize the interaction of the lipid membrane with the pipet, the solutions used in the vesicle experiments usually contained albumin. However, because albumin binds strongly to lysolipids, there was a concern that the addition of albumin to the bathing solution would change the bulk MOPC concentration. To minimize possible concentration changes, the albumin was used to coat the pipet surface before the experiments while the suspending solutions were albumin-free. Before the experiment, the pipet was incubated for 1 h in 171 mOsm glucose solution that contained 1% bovine serum albumin (Sigma Chemical Co., St. Louis, MO). After the incubation, the pipet was immersed in the experimental chamber and MOPC solution from the chamber was aspirated for 10 min. This procedure was repeated for every pipet to wash the unbound albumin from the pipet surface and to saturate the denatured bound albumin with MOPC. After the washing of the pipet, the vesicles were added to the chamber and the mechanical tests were performed. The solution in the chamber, as well as the pipet, were changed every hour.

The adsorption of albumin to the pipet surface was characterized in a separate experiment. In this case, a pipet was incubated with rhodamine-labeled albumin (Sigma) under the same conditions as the ones used in the experiments. After incubation, the pipet was washed with solution containing MOPC for more than 1 h and the fluorescence from the rhodamine-labeled albumin was measured. The time of illumination for every measurement was 10 s to minimize probe bleaching. Fig. 1 A shows the measured intensity profile of a micropipet covered with a layer of rhodamine-labeled albumin. The intensity profile has four maxima that correspond

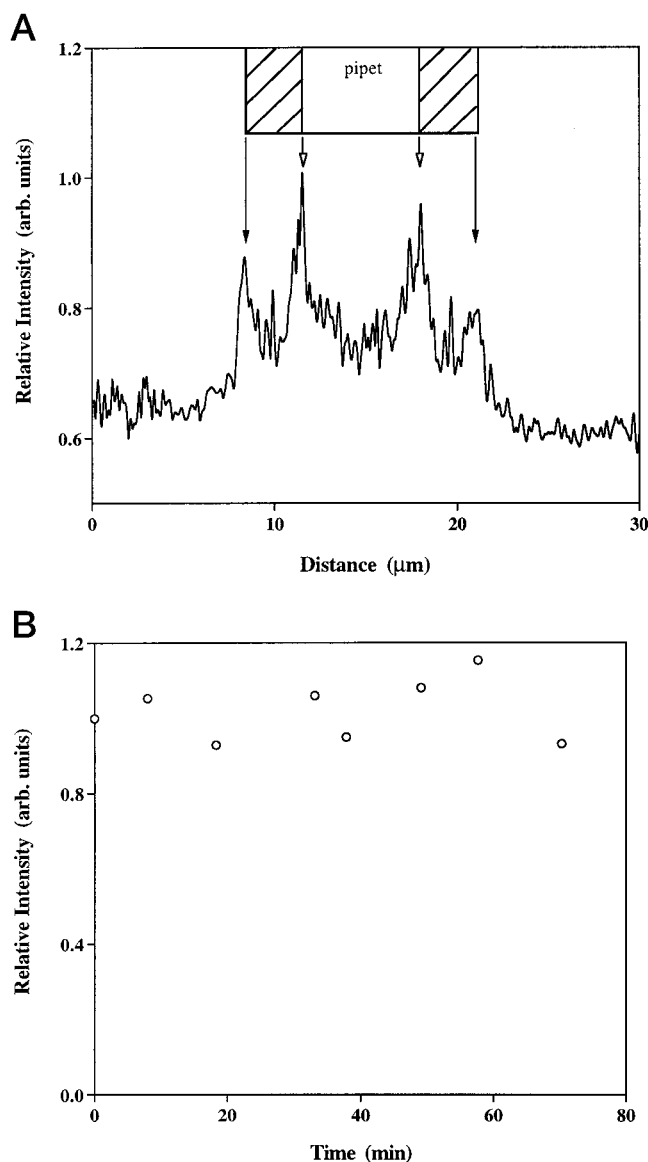


FIGURE 1 (A) Fluorescence intensity profile of rhodamine-labeled albumin adsorbed on the surface of a glass pipet. The open arrows point to the internal surface of the pipet and the closed ones point to its outer surface. The pipet fluorescence is obtained by averaging the intensity from the four peaks. (B) Average relative fluorescence intensity versus time.

to the inside and the outside surfaces of the pipet. The time dependence of the measured fluorescence intensity is shown in Fig. 1 B. It is seen that for the solutions used and for the duration of one set of measurements (equal to 1 h), the fluorescence from the adsorbed albumin does not change. This suggests that the albumin remains on the pipet surface throughout the duration of the experiment.

The manipulation of vesicles has been described elsewhere (Zhelev et al., 1994). Briefly, the vesicles were observed either in bright field using an inverted Leitz microscope with a 100 \times oil immersion objective or by a Hofmann modulation using an inverted Nikon microscope with a 30 \times long-distance objective. The microscope images were recorded using a Hamamatsu CCD camera (for the bright-field images) and a Hamamatsu SIT camera (for the fluorescence images). The experimental chamber was 3 mm thick and open at both sides for micromanipulation. A temperature-controlled chamber was used in which there was a flow of water between two glass slides on the top of the chamber. In all experiments, the

temperature of the chamber was kept equal to 14°C, which was the dew point. Micropipets were made of glass capillary tubing (0.75 mm outside diameter) pulled to a fine point with a vertical pipet puller and cut to the desired tip diameter using a microforge. The pipet was filled with the same solution as the one used in the chamber. The pipet was connected to a manometer system that measured the pipet-chamber pressure using a transducer (DP15-24, Validyne, Northridge, CA) and also allowed this pressure to be changed from 1 Pa to 4000 Pa. The measured pressures were multiplexed onto the recorded images with a multiplexer (model 401, Vista Electronics, La Mesa, CA).

Measuring the area expansion modulus and critical membrane tension

To determine the area expansion modulus it is necessary to apply a well defined membrane tension and to measure the corresponding change of the membrane area. The applied membrane tension τ_α was calculated using the law of Laplace (Evans and Skalak, 1980):

$$\Delta P = 2\tau_\alpha \left(\frac{1}{R_{\text{pip}}} - \frac{1}{R_{\text{out}}} \right) \quad (1)$$

where ΔP was the applied suction pressure, R_{pip} was the pipet radius, and R_{out} was the radius of the outside spherical portion of the vesicle.

The change in membrane area was calculated from the geometric characteristics of the vesicle: the vesicle projection length inside the pipet (L_p), the radius of the outside part of the vesicle (R_{out}), and the pipet radius (R_{pip}).

In micropipet experiments, the measured apparent relative area change had two major contributions (Evans and Rawicz, 1994). One related to the increase of the vesicle area due to the decreased amplitudes of membrane thermal fluctuations after the application of membrane tension and another related to the increase of the area per molecule in the membrane. Thermal fluctuations had a measurable effect on the experimentally determined relative area changes for tensions on the order of 1.5×10^{-5} mN m⁻¹ (Duwe et al., 1990; Faucon et al., 1989), up to $2-3 \times 10^{-3}$ mN m⁻¹ (Evans and Rawicz, 1990; Zhelev et al., 1994). Above this threshold tension the measured area changes essentially corresponded to the change of the area per molecule in the membrane. In this range of tensions the dependence of the relative area change on the applied tension was linear (Evans and Rawicz, 1990; Zhelev et al., 1994). In this case, the relationship between the relative area change and the applied tension τ_α was given by (Kwok and Evans, 1981)

$$\alpha = \frac{\tau_\alpha - \tau_0}{K_{\text{ap}}} \quad (2)$$

where τ_0 was the initial tension, K_{ap} was the apparent area expansion modulus, and $\alpha = \Delta A/A_0$ was the relative membrane area change, where ΔA was the area increase and A_0 was the extrapolated vesicle area at zero membrane tension (see Zhelev et al., 1994).

In our experiments, the linear region of the above dependence was observed only for membranes containing 30 mol % MOPC or less; therefore, we restricted our measurements to these MOPC concentrations.

There was a limitation for the use of Eq. 2 to determine the apparent area expansion modulus of membranes containing MOPC. Because MOPC was easily exchangeable between the membrane and the bathing solution, the vesicle area changed with time (Evans et al., 1994). This apparent relaxation of the membrane occurred with a characteristic time on the order of tens of seconds, which was consistent with the rate of MOPC desorption on the order of 0.3 s^{-1} measured earlier (Needham and Zhelev, 1995; Zhelev, 1996; Needham et al., 1997). Therefore, the vesicle area used to calculate the apparent area expansion modulus was measured at least 1 min after the application of the next tension.

Membrane breakdown is a time-dependent process (Chernomordik et al., 1985). Therefore, it was possible for the measured critical membrane tensions to be time dependent. To explore this possibility, the critical

membrane tension was measured in three different sets of experiments, where the applied tension was increased stepwise every 10, 30, and 120 s. The vesicles used in this experiment contained 10 mol % MOPC. After exploring the time dependence of the critical membrane tension, the critical tension was measured for 0, 10, 20, 30, and 50 mol % MOPC. The concentration dependence of the critical membrane tension was measured by increasing the applied tension in a stepwise manner every 30 s, and the critical membrane tension was recorded for the vesicles that broke 10 s after the last increase of the membrane tension. This procedure was chosen to minimize the effect of waiting time on the measured data.

RESULTS

Apparent area expansion modulus

The procedure of measuring the area expansion modulus is illustrated in Fig. 2. The pipet suction pressure is increased in a stepwise manner every 2 min. Immediately after the increase of the suction pressure the vesicle projection length increases. After the instantaneous initial increase, the vesicle projection length continues to increase in a slow process that takes tens of seconds before it eventually reaches a stationary position. The initial fast increase of the vesicle projection length and the subsequent slow increase are observed after every increase of the suction pressure. The initial area increase is determined mostly by the increase of the area per molecule immediately after increasing the membrane tension, whereas the following slow area increase is determined by the rate of MOPC transport from the

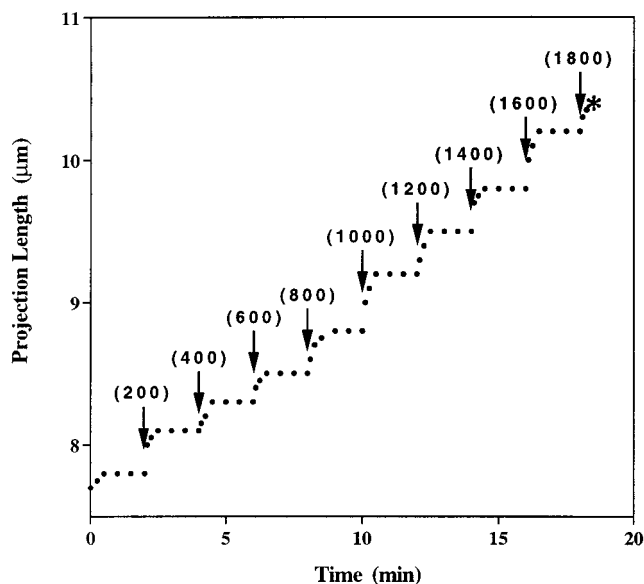


FIGURE 2 Measuring the vesicle projection length for different suction pressures of the holding pipet. The suction pressures are increased stepwise every 2 min. The projection length of the vesicle increases immediately after the increase of the suction pressure and then continues to increase for tens of seconds. Approximately 1 min after the increase of the suction pressure, the projection length reaches a stationary position. This projection length is used to calculate the apparent area expansion modulus. The maximal measured projection length is denoted by a star. This is the projection length at which the vesicle broke down. The outside radius of the vesicle was 8.9 μm and the radius of the pipet was 2.6 μm.

bathing solution. The vesicle area at stationary projection length is measured and used to determine the apparent area change. The applied membrane tension is then plotted against the apparent vesicle area change (Fig. 3). The apparent area expansion modulus is determined from the slope of the linear region of the dependence of the applied tension on the apparent area change (see Eq. 2).

The values of the apparent area expansion modulus for different MOPC concentrations are shown in Fig. 4. It is seen from these data that the apparent area expansion modulus decreases with increasing MOPC concentration. For a membrane containing 30 mol % MOPC the apparent area expansion modulus is approximately one-half of the area expansion modulus of pure EPC membrane.

Model for the apparent area expansion modulus

The apparent area expansion modulus for an EPC membrane containing MOPC is modeled in a similar way (see Appendix II) to the apparent area expansion modulus of membranes containing bile salts (Evans et al., 1994). The apparent area expansion modulus K_{ap} is found by combining Eqs. AII.2 and AII.3:

$$\frac{1}{K_{ap}} = \left(\frac{a^{b,o}}{a^{ap,o}} \right) \frac{(1-n)}{K^b} + \left(\frac{a^{l,o}}{a^{ap,o}} \right) \frac{n}{K^l} + \left(\frac{a^{l,o}}{a^{ap,o}} \right) \frac{n}{\tau} \left(\exp\left(\frac{a^{l,o}(\tau/2)}{kT} \right) - 1 \right) \quad (3)$$

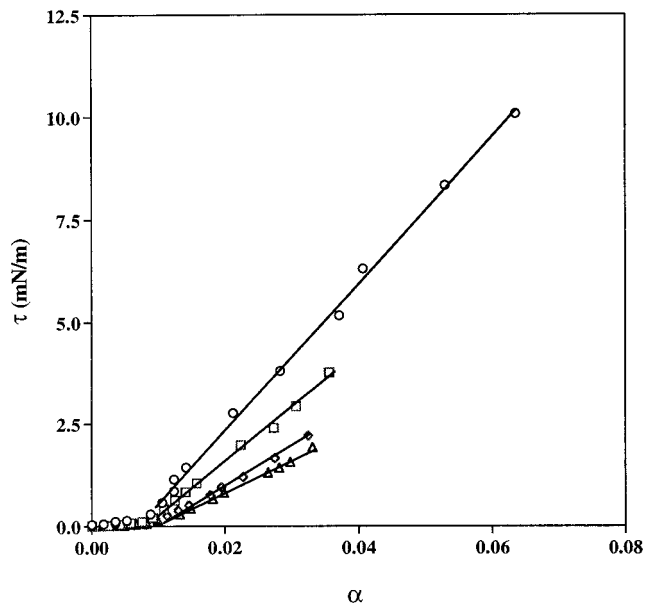


FIGURE 3 Membrane tension τ versus relative area change α of individual vesicles made of EPC and 0 mol % MOPC (\circ), 10 mol % MOPC (\square), 20 mol % MOPC (\diamond), and 30 mol % MOPC (\triangle). For calculation of the area expansion modulus, data are used with τ equal to 0.5 mN m^{-1} or larger. In this case, the dependence of the relative area increase on the applied tension is linear (the data in this region are approximated by lines), and the relative area increase represents the increase of the area per molecule in the membrane (Evans and Rawicz, 1990). The apparent area expansion modulus is equal to the slope of the linear relationship between τ and α as defined by Eq. 2.

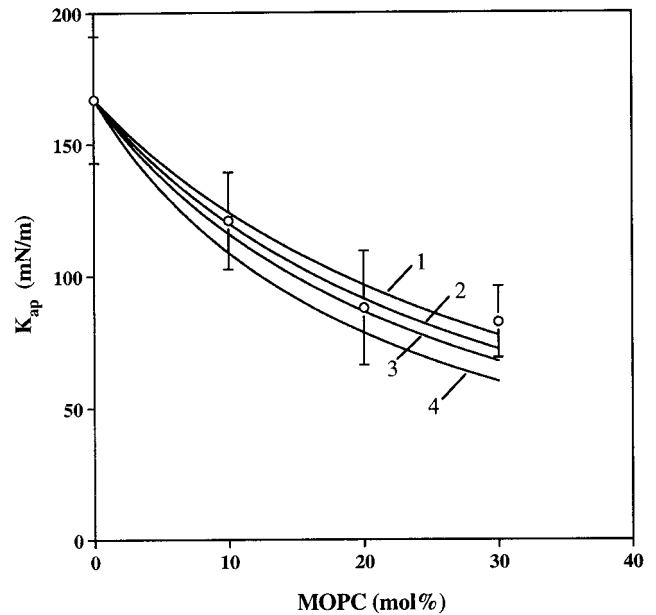


FIGURE 4 Apparent area expansion modulus of EPC membranes containing different amounts of MOPC. The experimental data are fitted using Eq. 4 assuming that the only unknown parameter is the intrinsic area expansion modulus of MOPC K^l . The values of the known parameters are as follows: area expansion modulus of EPC = 170 mN m^{-1} ; area per molecule of EPC = 65 \AA^2 ; area per molecule of MOPC = 35 \AA^2 ; temperature $T = 14^\circ\text{C}$. The values of the intrinsic area expansion modulus of MOPC are varied as shown: 1) K^l is equal to infinity, 2) $K^l = 200 \text{ mN m}^{-1}$, 3) $K^l = 100 \text{ mN m}^{-1}$, and 4) $K^l = 50 \text{ mN m}^{-1}$.

where n is the molar concentration of MOPC in the membrane at zero membrane tension, τ is the membrane tension ($a^{ap,o} = (1-n)a^{b,o} + na^{l,o}$, where $a^{b,o}$ and $a^{l,o}$ are the area per EPC and MOPC molecule, respectively), and K^b and K^l are the intrinsic area expansion moduli of EPC and MOPC, respectively.

In Eq. 3 the apparent area expansion modulus is considered to have three major components: one related to the intrinsic area expansion modulus of EPC, another related to the intrinsic area expansion modulus of MOPC, and the third component related to the exchange of MOPC with the membrane. The last term is of particular interest because it represents the softening of the membrane due to MOPC intercalation. The mechanical energy of this intercalation is given by $a^{l,o}(\tau/2)$. When this energy is small compared with the thermal energy kT , the exponent in Eq. 3 can be replaced by the first two terms of its Taylor expansion. In this case, the contribution of MOPC exchange to the apparent area expansion modulus becomes independent of the applied tension. This allows the two terms related to MOPC (namely, the intrinsic area expansion modulus of MOPC and the exchange term) to be combined into an apparent area expansion modulus for MOPC:

$$\frac{1}{K_{ap}} = \left(\frac{a^{b,o}}{a^{ap,o}} \right) \frac{(1-n)}{K^b} + \left(\frac{a^{l,o}}{a^{ap,o}} \right) n \left(\frac{1}{K^l} + \frac{a^{l,o}}{2kT} \right) \quad (4)$$

where the first term gives the contribution of EPC and the second term represents the apparent area expansion modulus of MOPC.

Equation 4 or 3 can be used to calculate the apparent area expansion modulus when all other membrane characteristics are known or to find one of the unknown characteristics from the measured apparent area expansion modulus. The second possibility is compelling, because for the bilayer membranes considered here, all characteristics are measurable except the area expansion modulus of MOPC. However, because of the large standard deviation of the experimental data and the large contribution of the exchange term (which is seen later) to the measured decrease of the apparent area expansion modulus, the exact determination of the area expansion modulus of MOPC is not possible. Therefore, it is instructive to analyze the contribution of MOPC exchange to the membrane softening assuming that the intrinsic modulus of MOPC is an unknown. In this case, all characteristics except K^1 are known from independent measurements and the intrinsic modulus of MOPC is varied from zero to infinity.

The values of the known characteristics are 170 mN m^{-1} for the intrinsic area expansion modulus of EPC, 65 \AA^2 for the area per molecule of EPC (McIntosh and Simon, 1986), and 35 \AA^2 for the area per molecule of MOPC (see Appendix I). (Note that the area per molecule is chosen equal to the area of the exposed hydrocarbon region for both EPC and MOPC. This choice is made because during mechanical deformation the membrane is considered incompressible (see Appendix I) and because the hydrocarbon region, being "water free," is considered to represent the incompressible region of the membrane.) The maximal applied monolayer tensions are equal to or less than one-half of the critical membrane tensions (see Fig. 6). Therefore, they are from 5 mN m^{-1} for the pure EPC membrane to 0.04 mN m^{-1} for the membrane containing 50 mol % MOPC. The mechanical energies, corresponding to these tensions are from $1.7 \times 10^{-21} \text{ J}$ to $1.4 \times 10^{-23} \text{ J}$. These values are smaller than the thermal energy, which for the temperature used here is on the order of $4.1 \times 10^{-21} \text{ J}$. Therefore, Eq. 4 is used to model the data in Fig. 4. Curves 1 to 4 represent the values of the apparent area expansion modulus for different MOPC concentrations when the intrinsic modulus of MOPC is equal to infinity (curve 1), 200 mN m^{-1} (curve 2), 100 mN m^{-1} (curve 3), and 50 mN m^{-1} (curve 4). When the area expansion modulus of MOPC is infinity, the apparent area change is due only to the molecular exchange. The comparison of the experimental data with the model curves shows that the curve for the infinite area expansion modulus of MOPC is very close to the experimental data. This suggests that the exchange of MOPC with the membrane is the major factor for the decrease of the apparent area expansion modulus.

An estimate of the intrinsic area expansion modulus of MOPC is found from the data in Fig. 4. It is seen that the best fit for the experimental data is found for K^1 from 200 mN m^{-1} to infinity. Another estimate of the same area expansion modulus is found from the Van der Waals gas

models of the bilayer membrane (see Appendix III). The calculated intrinsic area expansion modulus of MOPC from these models is on the order of 150 mN m^{-1} . Therefore, the area expansion modulus of MOPC is considered to be on the same order as the area expansion modulus of EPC.

Critical membrane tension and work for membrane breakdown

Membrane strength is characterized by the critical membrane tension that is measured at membrane breakdown. Because membrane breakdown is a statistical process, it is possible for the critical membrane tension to depend on the time of application of membrane stress. This dependence has been studied for black lipid membranes (Chernomordik et al., 1985), and it has been shown that the lifetime of black lipid membranes decreases exponentially with increasing applied stress. Because the considered dependence is exponential, it is possible for the critical membrane tensions to show stronger dependence on the membrane composition than on time. As the composition of the membrane for different vesicles in the same liposome preparation may vary, it is possible for the membrane tensions of the different vesicles to be substantially different. To explore these possibilities, the critical membrane tension was measured for membranes containing 10 mol % MOPC and the applied tension was increased in a stepwise manner using the procedure illustrated in Fig. 2. Three different experiments were performed with three different waiting times: 1) waiting time of 10 s, 2) waiting time of 30 s, and 3) waiting time of 120 s. The increment of the membrane tension in each step was on the order of 0.4 mN m^{-1} . The results from these measurements are shown in Fig. 5. It is seen that the average critical membrane tension decreases with the increase of the waiting time. However, the decrease is small compared with the difference of the critical membrane tension between different vesicles in the same preparation (characterized by the standard deviations). For the time intervals used in this study, the difference between the critical membrane tension of the different vesicles is larger than the decrease of the average critical membrane tension with the waiting time. Therefore, the critical membrane tension is considered independent of the time of membrane exposure to stress. (In making this conclusion it is recognized that membranes that break at large membrane tensions have been exposed to low tensions much longer than the vesicles that break at these low tensions.) The weak dependence of the critical membrane tension on the time of exposure to stress suggests that the measured critical membrane tension represents adequately the strength of the membrane.

The presence of MOPC reduces membrane strength, which is seen from the data in Fig. 6. As has been mentioned earlier, the critical membrane tensions represent the maximal applied tensions before membrane breakdown. Therefore, the critical membrane tensions can be used to determine the maximal work before membrane breakdown

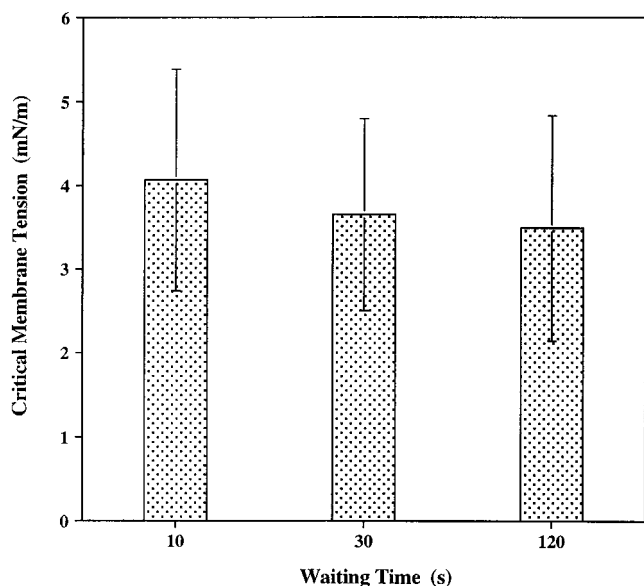


FIGURE 5 Critical membrane tension versus waiting time. The critical membrane tension is measured for different waiting times before the suction pressure in the holding pipet is increased in a stepwise manner (see Fig. 2). The increase of the membrane tension corresponding to each pressure increase is on the order of 0.4 mN m^{-1} .

(or the work for membrane breakdown). This work is equal to the maximal work per unit membrane area w :

$$w = \alpha \tau_c \quad (5)$$

where α is the relative area change and τ_c is the critical membrane tension.

Usually, the critical membrane tension τ_c is much larger than the initial tension τ_0 in Eq. 2, and the initial tension can

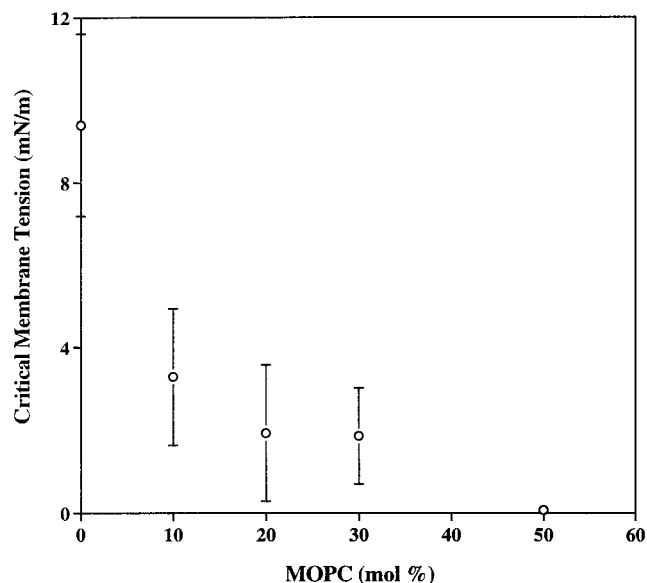


FIGURE 6 Critical membrane tension (or tension for membrane breakdown) of EPC membranes containing different molar concentrations of MOPC.

be neglected. Then, the work for membrane breakdown is found after combining Eqs. 2 and 5 and, assuming that there are N_m lipid molecules per unit area of a membrane monolayer,

$$w_o = \frac{w}{2N_m} = \frac{\tau_c^2}{2K_{ap}N_m} \quad (6)$$

where τ_c is the critical membrane tension and K_{ap} is the apparent area expansion modulus.

The critical membrane tension and the apparent area expansion modulus are measured experimentally (see Figs. 4 and 6). The number of molecules per unit membrane area is calculated from the molar concentration of MOPC and the areas per molecule of EPC and MOPC, respectively (see Appendix I). The calculated work for membrane breakdown in kT units is shown in Fig. 7. It is seen that with increasing MOPC concentration the work for breakdown decreases. The data in Fig. 7 are fitted with a logarithmic curve, suggesting that the work for membrane breakdown has a power law dependence on the MOPC concentration.

DISCUSSION

Area expansion modulus

The use of Eq. 4 for interpreting the data in Fig. 4 needs discussion. The exchange of MOPC with the membrane can occur in monomer, oligomer, and micelle form (Needham et al., 1997). Also, there is no experimental evidence that MOPC in the membrane is only in a monomer form. Furthermore, the rate of exchange of the different MOPC species with the membrane is different (Zhelev, 1996; Needham et al., 1997), therefore, the exchange of MOPC with the membrane generates a shift in the balance between

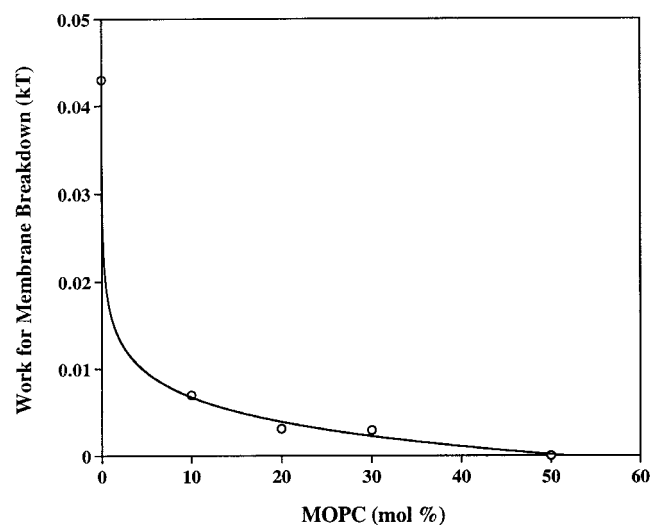


FIGURE 7 Work for membrane breakdown calculated from Eq. 6. The values for the critical membrane tension are the same as the ones shown in Fig. 6. The values for the apparent area expansion moduli are calculated from Eq. 4 assuming that K^1 is equal to 200 mN m^{-1} (see text).

the different species in the bathing solution and in the membrane. This shift of the balance between the different species will cause an exchange of MOPC between the existing pools of MOPC. Therefore, the complete balance of MOPC between all pools of lysolipid needs to include all chemical potentials. Such a complete model will have many unknown parameters, which will compromise its use to interpret experimental data. Therefore, a simplified model is used here, where there is only one chemical potential for the bathing solution and one chemical potential for the membrane. In addition to the unknowns related to the partitioning of MOPC, there is also a concern about the equilibrium of the system. In general, a true equilibrium is never reached for a liposome system. However, because of the very low rate of molecular desorption for the bilayer lipids (e.g., palmitoylcholine, which has a similar energy of association with the membrane as EPC, has a rate of desorption on the order of 10^{-6} s^{-1} (Marsh, 1990)) compared with the time necessary to measure the area expansion modulus (usually on the order of minutes), pure bilayer membranes are considered quasi-equilibrium systems. When fast exchangeable molecules, such as MOPC, are present, the equilibrium of the system changes during the experiment. With this understanding, the measurement of the vesicle area corresponding to a given applied tension is performed when experimental stationary conditions are reached. Therefore, in the results presented here, it is assumed that semi-stationary conditions are reached that are close to the ones for true thermodynamic equilibrium.

Another concern in using Eq. 4 is the concentration of MOPC in the membrane. There is no direct method for measuring this concentration for a single vesicle. During the calculation of the concentration of MOPC in the membrane it is assumed that the lysolipid partitions both in the bathing solution and in the membranes of all liposomes. In the bathing solution it partitions as a monomer, an oligomer, and a micelle, and in the membranes it partitions equally among all vesicles. The amount of MOPC in the membrane is determined from equilibrium measurements and from measuring MOPC exchange. The maximal amount of MOPC in EPC membrane is determined from equilibrium lipid mixtures (Van Echteld et al., 1981) and is on the order of 50 mol %. The study of the exchange of MOPC with EPC membranes (Needham and Zhelev, 1995) shows that the uptake of MOPC from 5 μM MOPC solution is on the order of 25 mol %. This value for the lysolipid molar concentration in the membrane is close to the solubilization limit for MOPC. Therefore, it is expected that the bulk MOPC saturation concentration, corresponding to 50 mol % MOPC in the membrane, is in the micromolar range. The amount of MOPC (MW 521) used for solution preparation is 0.52 mg, and this amount is dissolved in 2 ml of a water solution. This gives an apparent concentration of MOPC on the order of 0.5 M. This concentration is well above the micromolar concentration required for maximal loading of the vesicle membranes. Therefore, it is assumed that most of the lyso-

lipid is partitioning in the membranes, and the amount of lysolipid lost in the bathing solution is neglected.

The measured values of the apparent area expansion modulus of EPC:MOPC membranes are well described using Eq. 4. The experimental data show that the major factor for the decrease of the apparent area expansion modulus is the exchange of MOPC. The replacement of some of the EPC molecules by MOPC in a unit membrane area has only a minor effect. Similar results are obtained by Evans et al. (1994) for membranes containing readily exchangeable bile salts (Evans et al., 1994). This suggests that the reduction of the area expansion modulus in the presence of exchangeable molecules is a fundamental property for all membranes. Overall, the data presented here show that the apparent area expansion modulus depends strongly on the molar concentration of exchangeable molecules in the membrane and on the area that these molecules occupy in the membrane. Therefore, the measurement of the apparent area expansion modulus provides a unique tool for determining the area occupied in the membrane by exchangeable molecules.

Work for membrane breakdown

It is instructive to discuss in more detail the work for membrane breakdown. This work is calculated from Eq. 6 and is proportional to the product of the square of the critical membrane tension and the inverse apparent area expansion modulus. It is seen from Eq. 4 that the inverse apparent area expansion modulus has two major components: one related to the intrinsic moduli of EPC and MOPC and another related to the exchange of MOPC. The work for breakdown depends on the inverse area expansion modulus and, therefore, can also be divided into two components: an intrinsic component and an exchange component. The intrinsic component gives the maximal work for increasing the area per molecule in the membrane, whereas the exchange component gives the work for creating vacancies for the exchangeable molecules. Fitting the data in Fig. 4 suggests that the intrinsic moduli of EPC and MOPC are similar. Therefore, it is expected that the intrinsic term of the work for membrane breakdown will be independent of MOPC concentration. In this case, the exchange component of the work for breakdown will increase with the increase of MOPC concentration, providing the membrane tension is kept constant. In addition to the dependence of the work for breakdown on MOPC concentration, it also depends on the critical membrane tension. This dependence is the strongest and determines the overall trend of decrease of the work for breakdown with increasing MOPC concentration. Therefore, the dependence of the critical membrane tension on MOPC concentration is the major factor determining the tendency of the overall work for breakdown to decrease with the increase of MOPC concentration (Fig. 7).

The magnitude of the work for breakdown for pure EPC membrane is on the order of 0.045 kT (see Fig. 7). This

value is ~ 20 times smaller than kT , which suggests that the process of membrane breakdown is not a single-molecule event but involves many molecules (see also the Discussion in Needham and Nunn, 1990). In this regard, membrane breakdown is considered a first-order chemical reaction that involves many molecules. First-order chemical reactions are characterized by a change in the Gibbs energy in the transition from the initial state to the activated state (often called barrier energy). The experiments discussed here cannot measure this total change of the Gibbs energy; however, the work for breakdown gives the change of the Gibbs energy of the initial state before breakdown. The data in Fig. 6 are for similar membrane lifetimes; therefore, it can be assumed that the change of the Gibbs energy during the transition from the stressed initial state to the activated pore state is similar for all membranes. In this context, the work for breakdown gives the difference of the barrier energies for membranes containing different amounts of MOPC. Therefore, the data from Fig. 7 indicate that the increase of MOPC concentration in the membrane decreases the activation energy for membrane breakdown. The fact that the work for breakdown becomes equal to zero for MOPC concentrations above 50 mol % suggests that the barrier energy for defect formation becomes very small and the thermal fluctuations have enough energy to initiate membrane breakdown. Therefore, an increase in the MOPC concentration above 50 mol % will result in an apparent disappearance of the spontaneously formed bilayer structures and their replacement by micellar structures, which is observed experimentally (Van Echteld et al., 1981).

CONCLUSION

In conclusion, the addition of MOPC to vesicle membranes reduces their apparent area expansion modulus. In a similar manner, the tensile strength of the membrane decreases with increasing MOPC concentration. This decrease in membrane strength determines the substantial decrease in the work for membrane breakdown in the presence of MOPC. The data for the apparent area expansion modulus are well fitted with the proposed model, where the apparent area expansion modulus depends on the intrinsic elasticities of EPC and MOPC and the exchange of MOPC. The exchange of MOPC is the major contributor to the decrease of the measured membrane area expansion modulus. This decrease depends on the molar concentration of MOPC and the area occupied by a single MOPC molecule in the membrane. Thus, the model and the procedures described here provide a unique method for measuring the area of an exchangeable molecule in the membrane.

APPENDIX I

Apparent area per MOPC molecule

McIntosh et al. (1995) have shown that the thickness of pure EPC membranes is 2 Å less than that of (1:1) EPC:MOPC membranes. The electron

density profiles from the same study show that the addition of MOPC causes a minor disordering of the central region of the bilayer. The authors have suggested that the headgroup of MOPC is in the same plane as the headgroups of the host bilayer and that its hydrocarbon chain is oriented in the same direction as the chains of the host lipid. Therefore, as a first approximation, the hydrocarbon chain of MOPC in EPC membranes is considered to occupy a cylinder with a volume equal to the volume of the hydrocarbon chain and a length equal to one-half the thickness of the membrane hydrocarbon region. The area per lipid molecule is defined as the area of its acyl chains in the hydrocarbon region of the membrane. The area per molecule is then calculated by dividing the volume of the hydrocarbon chain (or chains) by one-half of the thickness of the membrane's hydrocarbon region.

In the above calculation (to be completed below) of the area per molecule, it is assumed that the lipid hydrocarbon chains are incompressible. The incompressibility of the hydrophobic region of the bilayer membrane is suggested by a number of experiments where the membrane is subjected to a mechanical stress (Evans and Simon, 1975; Parsegian et al., 1979). Zhelev et al. (1988) and Needham and Hochmuth (1989) have shown that the membrane tension and the force of electrocompression are complementary factors for membrane stability. Their results show that, when the work for creating a membrane defect is considered to be proportional to the applied force, the same result is obtained regardless of the direction of the applied stress (either by applying a membrane tension in the plane of the membrane or by applying an electrocompression in the transverse direction). This result also suggests that the membrane hydrocarbon region is incompressible. Another result suggesting that the hydrocarbon region of the bilayer membrane is incompressible is the comparison between the area expansion modulus and the area compression modulus. The area expansion modulus of EPC membranes measured with micropipets is on the order of 131–167 mN m⁻¹ (Kwok and Evans, 1981; McIntosh et al., 1995), and the area compression modulus calculated from the x-ray data of Parsegian et al. (1979) is on the order of 110 mN m⁻¹.

The thickness of the hydrocarbon region then is found from the membrane thicknesses measured by x-ray diffraction. McIntosh and Simon (1986) measured 37.8 Å for the distance between the phosphates at the two sides of EPC membrane. Simon and McIntosh (1986) show that the apparent interface of the membrane is near the carbonyl groups of the membrane lipids. Therefore, the distance of the phosphate to the apparent interface of the hydrocarbon region is $4-5 \times 1.25$ Å. This gives 26.6 Å for the thicknesses of the hydrocarbon region. The length of the hydrocarbon region of a single EPC molecule is equal to one-half of this thickness. From the value of the length of EPC chain and its area per molecule (taken to be equal to 65 Å² (McIntosh and Simon, 1986)) the calculated volume of the chain of EPC is 865 Å³. This value is close to the value of 870 Å³, which is the volume of the hydrocarbon chain of DOPC (Tardieu et al., 1973), which has approximately the same number of hydrocarbons as EPC. The thickness of the hydrocarbon region of the MOPC:EPC membrane depends on the molar concentration of the lysolipid. McIntosh et al. (1995) have found that the thickness of a membrane containing 50 mol % MOPC decreases by 2 Å. This corresponds to a decrease of the thickness of the hydrocarbon region of one monolayer of 1 Å. The area of MOPC in EPC membranes is calculated from the measured membrane thickness of (1:1) EPC:MOPC membranes and by assuming that the volume of the hydrocarbon region of MOPC is one-half of the same volume of EPC. The calculated area per MOPC molecule is 35 Å².

The area per molecule of EPC in a pure EPC membrane and the area of MOPC in a (1:1) EPC:MOPC membrane can be estimated also from the surface pressure of the molecules in the membrane surface. The surface pressure in a membrane monolayer at zero membrane tension is (Evans and Skalak, 1980)

$$\pi_o = \frac{ckT}{(A_o - A_v)} \quad (\text{A1.1})$$

where π_o is the interfacial tension of the acyl chain in water, A_o is the area per molecule in the membrane, A_v is the excluded area equal to the

cross-sectional area of the acyl chains, k is Boltzmann constant, T is temperature, and c is the number of acyl chains.

π_0 is calculated from the activation energy of transfer of a single hydrocarbon group from the membrane into water. This activation energy is on the order of $0.68 \text{ kcal mol}^{-1}$ (Israelachvili, 1991). The cross-sectional area of one acyl chain is 20 \AA^2 (Ruocco and Shipley, 1982), and the length of one hydrocarbon group is $\sim 0.9 \text{ \AA}$. This gives a value of 33 mN m^{-1} for the apparent interfacial tension. For EPC, c is equal to 2 and A_v is equal to 40 \AA^2 (which is twice the value of the cross-sectional area of a single acyl chain). Then, the calculated area of the EPC molecule is 64 \AA^2 . This value is close to the value of 65 \AA^2 determined from x-ray data (McIntosh and Simon, 1986). For MOPC, the corresponding parameters are as follows: c is equal to 1 and A_v is equal to 20 \AA^2 . Then, the area per MOPC molecule is 32 \AA^2 . This value is also close to the area of MOPC molecule calculated from x-ray data.

The area of the MOPC molecule in EPC membranes calculated above is smaller than the same area measured for pure MOPC membranes in the L_β tilted interdigitated phase. The latter area is $44\text{--}45.5 \text{ \AA}^2$ (Mattai and Shipley, 1986; Hui and Huang, 1986) and is considered to represent the area of the phosphatidylcholine headgroup. Thus, MOPC in EPC membranes partially covers the hydrocarbon region of its diacyl neighbors, as suggested by McIntosh et al. (1995). In the calculation of the apparent area expansion modulus, the area per MOPC is considered equal to the exposed hydrocarbon area (35 \AA^2 as calculated above), because the hydrocarbon region represented the incompressible region of the membrane.

APPENDIX II

Area expansion modulus of EPC:MOPC membrane

The area expansion modulus calculated from Eq. 2 is an apparent characteristic, defined as the ratio of the change of the membrane tension to the fractional area change. The fractional area change depends on the deformability of the molecules in the membrane as well as on the number of molecules that are exchanged with the bathing solutions. A two-component membrane is considered where only one of the components is exchangeable (in this case MOPC). For a fixed bathing solution concentration, the exchange of MOPC is triggered by a change in the membrane tension.

The number of exchanged molecules is determined from the dependence of the Gibbs energy on the applied membrane tension. It is assumed that, when there is a membrane tension, only the molecules in the membrane experience a change of their Gibbs energy. At equilibrium, the Gibbs energies of the exchangeable molecules in the bathing solution and in the membrane are equal:

$$\mu_w^0 + kT \ln(n_w) = \mu_m^0 + kT \ln(n_\tau) - a^{1,o} \left(\frac{\tau}{2} \right) \quad (\text{AII.1})$$

where n_w is the molar concentration of MOPC in the bathing solution, μ_w^0 and μ_m^0 are the standard chemical potentials of MOPC in the bathing solution and in the membrane, respectively, $a^{1,o}$ is the area of MOPC molecule in the membrane, $\tau/2$ is the tension applied to one of the membrane monolayers, and n_τ is the membrane concentration of MOPC at tension τ .

When the monolayer tension changes from zero to $\tau/2$, the Gibbs energy remains constant because the left side of Eq. AII.1 is independent of the applied stress. Thus, Eq. AII.1 gives the dependence of the change Δn_τ of MOPC in the membrane on the membrane tension:

$$\Delta n_\tau = n \left(\exp \left(\frac{a^{1,o}(\tau/2)}{kT} \right) - 1 \right) \quad (\text{AII.2})$$

where n is the membrane concentration of MOPC at zero membrane tension.

As has already been mentioned, the fractional area change in Eq. 2 has three major components: 1) fractional area change due to the increase in the

area per EPC molecule, 2) fractional area change due to the increase in the area per MOPC molecule, and 3) fractional area change due to the exchange of MOPC. Taking into account these three components of the fractional area change, the apparent area expansion modulus K_{ap} is

$$\frac{1}{K_{ap}} = \left(\frac{a^{b,o}}{a^{ap,o}} \right) \frac{(1-n)}{K^b} + \left(\frac{a^{l,o}}{a^{ap,o}} \right) \frac{n}{K^l} + \left(\frac{a^{l,o}}{a^{ap,o}} \right) \frac{\Delta n_\tau}{\tau} \quad (\text{AII.3})$$

where K^b and K^l are the area expansion moduli of EPC and MOPC, respectively, $a^{b,o}$ is the area per EPC molecule, $a^{ap,o}$ is the average area per molecule of EPC:MOPC membrane defined by $a^{ap,o} = (1-n)a^{b,o} + na^{l,o}$, and n is the concentration of MOPC in the membrane at zero membrane tension.

The formulae for calculating the apparent area expansion modulus is found by substituting Δn_τ from Eq. AII.2 into Eq. AII.3.

APPENDIX III

Calculating the intrinsic area expansion modulus of MOPC

The intrinsic area expansion modulus of MOPC is calculated using the Van der Waals gas model of Evans and Skalak (1980) and that of MacDonald and Simon (1987). According to the model of Evans and Skalak (1980), the area expansion modulus K_o of a lipid molecule in the membrane is

$$K_o = 2\pi_0 \frac{A_o}{A_o - A_v}, \quad (\text{AIII.1})$$

where π_0 is the surface pressure at zero membrane tension (Evans and Skalak (1980) take for the value of π_0 the interfacial tension of a lipid acyl chain in water), A_o is the area of a given membrane segment, and A_v is the excluded area of the lipid chains in this segment. The difference ($A_o - A_v$) gives the free space in the plane of the membrane, where molecular fluctuations take place.

This model is first applied for pure EPC membrane in order to check its validity. π_0 is calculated in Appendix I and has a value of 33 mN m^{-1} . The area A_o is chosen equal to the area occupied by a single EPC molecule, which is on the order of 65 \AA^2 (McIntosh and Simon, 1986). The exclusion area is on the order of 40 \AA^2 (Ruocco and Shipley, 1982), which is twice the cross-sectional area of a single acyl chain. The substitution of the above values in Eq. AIII.1 gives a value of the area expansion modulus on the order of 170 mN m^{-1} . This value is in very good agreement with the measured value of 167 mN m^{-1} (McIntosh et al., 1995) for the area expansion modulus of pure EPC membrane. The area expansion modulus of MOPC is calculated using the same value for π_0 as in the case of EPC membrane. The area occupied by a single MOPC molecule A_o is equal to 35 \AA^2 (see Appendix I) and the excluding area A_v is 20 \AA^2 . Then, the calculated intrinsic area expansion modulus for MOPC is 150 mN m^{-1} .

In the model of MacDonald and Simon (1987), the Brownian motion of individual hydrocarbon groups is considered, as opposed to the Brownian motion of the whole chain considered in the model of Evans and Skalak (1980). According to the model of MacDonald and Simon (1987), the apparent surface pressure π of one of the membrane monolayers is given by

$$\pi = \frac{mkT}{A_{ch}}, \quad (\text{AIII.2})$$

where m is the number of hydrocarbons per acyl chain and A_{ch} is the area occupied by the chain.

Similar to Eq. AIII.1, the surface pressure is proportional to the area expansion modulus (Evans and Skalak, 1980). Thus, from Eq. AIII.2 the ratio of the intrinsic area expansion moduli of EPC and MOPC can be calculated.

$$\frac{K^l}{K^b} = \frac{\pi^l}{\pi^b} = \frac{m^l A_{ch}^b}{m^b A_{ch}^l}. \quad (\text{AIII.3})$$

Because the number of hydrocarbon groups in the acyl chains of EPC and MOPC is similar, the ratio of the intrinsic area expansion moduli is inversely proportional to the ratio of the areas per acyl chain. The area per acyl chain of EPC is 32.5 \AA^2 and that of the MOPC chain is 35 \AA^2 . This gives for the ratio of the intrinsic area expansion moduli a value on the order of 0.92. From this ratio, the calculated intrinsic area expansion modulus for MOPC is 153 mN m^{-1} . It is seen from both models that the intrinsic area expansion modulus of MOPC is on the order of 150 mN m^{-1} .

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