

Association of a Fluorescent Amphiphile with Lipid Bilayer Vesicles in Regions of Solid–Liquid-Disordered Phase Coexistence

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ABSTRACT The effects of solid-fluid phase separations on the kinetics of association of a single-chain fluorescent amphiphile were investigated in two different systems: pure DMPC (dimyristoylphosphatidylcholine) and a 1:1 mixture of DMPC and DSPC (distearoylphosphatidylcholine). In pure DMPC vesicles, solid (s) and fluid (ℓ_d) phases coexist at the phase transition temperature, T_m , whereas a 1:1 mixture of DMPC and DSPC shows a stable s - ℓ_d phase separation over a large temperature interval. We found that in single-component bilayers, within the main phase transition, the experimental kinetics of association are clearly not single-exponential, the deviation from that function becoming maximal at the T_m . This observation can be accounted for by a rate of desorption that is slower than desorption from either fluid or solid phases, leaving the rates of insertion unchanged, but a treatment in terms of stable fluid and solid domains may not be adequate for the analysis of the association of an amphiphile with pure DMPC vesicles at the T_m . In DMPC/DSPC mixtures with solid-fluid phase coexistence, association occurs overall faster than expected based on phase composition. The observed kinetics can be described by an increase in the rate of insertion, leaving the desorption rates unchanged. The fast kinetics of insertion of the amphiphile into two-phase bilayers in two-component vesicles is attributed to a more rapid insertion into defect-rich regions, which are most likely phase boundaries between solid and fluid domains. A two-component mixture of lipids that shows a stable phase separation between ℓ_d - s phases over a large temperature interval thus behaves very differently from a single-component bilayer at the T_m , with respect to insertion of amphiphiles.

INTRODUCTION

An intriguing aspect of biological membranes is their chemical heterogeneity. Over the past years, evidence has accumulated indicating that heterogeneity may be an important requirement for normal cell functioning (Vaz, 1994–1996; Brown and Rose, 1992; Melo et al., 1992; Lisanti et al., 1994; Welti and Glaser, 1994; Mayor and Maxfield, 1995; Thompson et al., 1995; Edidin, 1997; Hwang et al., 1998). The possibility that lipid phase separations participate in the control of membrane processes raises many questions regarding their specific functions (Vaz and Almeida, 1993). A number of important cell processes, such as signal transduction and the insertion of membrane proteins, involve the interaction of amphipathic substances with cellular membranes. The central question is whether phase heterogeneity (or domain coexistence) plays any role in the process. For instance, are proteins preferentially inserted at phase-domain boundaries? Do domains influence the activity of membrane-bound regulatory proteins? Several studies have indicated that clustering of membrane-associated proteins in lipid domains may serve to target them to specific sites within the cell (Simons and Ikonen, 1997; Muniz and Riezman, 2000). Signal transduction across cellular membranes may involve the formation of lipid domains (Hinderliter et al., 1998), which must then represent part of the signaling pathway, to be recognized by the next element in a sequence

of events. How are different signals generated and how are they read and propagated? Furthermore, as in any process where the response of a system to a signal depends on the time scale of the initial stimulus, the time scale on which substances interact with membranes will determine their mode of action. Therefore, a more detailed knowledge of the kinetics of interaction of amphiphiles with lipid bilayers is essential for the understanding of the membrane response to these processes.

Membrane heterogeneity may be of various types. Several lipid lamellar phases that may also exist in biological membranes have been identified and characterized: lipid bilayers can exist as highly ordered solid (gel) phases (s phase), highly disordered fluid phases (ℓ_d phase), or relatively ordered fluid phases, which are often rich in cholesterol (ℓ_o phase). Under the appropriate conditions a lipid bilayer can exist as either one of these phases or as a mixture of coexisting phases. In a previous paper (Pokorny et al., 2000) we investigated the influence of phase separations involving the ℓ_o phase on the association of a fluorescent amphiphile. The ℓ_o phase is of particular interest because model systems containing cholesterol in amounts that are typical for biological membranes show the formation of an ℓ_o phase. The ℓ_o phase is thus presumed to exist in a variety of cellular membranes (Ahmed et al., 1997), possibly in coexistence with an ℓ_d phase. We found that in a system with phase separation between ℓ_o and ℓ_d phases, the association of a small amphiphile is not influenced by the existence of phase boundaries. In the case of ℓ_o - s phase coexistence, however, we found lower binding constants and slower kinetics of insertion than into either pure ℓ_o or pure s phases. This finding suggested that bilayers with ℓ_o - s

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phase separations are effectively sealed against leaks or boundary-related incorporation of amphiphiles. The next question that arises is whether this is a characteristic of cholesterol-containing systems or of liquid-solid phase separations in general.

Phase separations of the solid-fluid type exist in single-component bilayers within the main phase transition temperature, T_m , as well as in two-component bilayers without cholesterol, such as in mixtures of phosphatidylcholines of different acyl-chain lengths. Systems of this kind have been studied with respect to their permeability to polar solutes and an increased permeability was found both in single-component bilayers around the T_m and in two-component, two-phase lipid bilayers (Papahadjopoulos et al., 1973; Marsh et al., 1976; Cruzeiro-Hansson and Mouritsen, 1988; Clerc and Thompson, 1995). In both cases the observed increased permeability was attributed to the existence of defects caused by acyl-chain packing mismatch between domains of s and ℓ_d phases. Studies of this kind, however, mask to a large extent the time domain over which defect structures exist: short-lived defect structures will be more easily detectable in experiments that measure efflux from vesicles loaded with a high concentration of solute than in experiments that involve the association of an amphiphile in dilute solution with lipid vesicles. In this article we investigate the consequences of s - ℓ_d phase separations on the kinetics of insertion of a single-chain fluorescent amphiphile. We studied pure dimyristoylphosphatidylcholine (DMPC) and a 1:1 mixture of DMPC and distearoylphosphatidylcholine (DSPC). In pure DMPC vesicles s and ℓ_d phases coexist at the T_m , whereas a 1:1 mixture of DMPC and DSPC shows a stable s - ℓ_d phase separation over a large temperature interval (Fig. 1). We found that in single-component bilayers within the main phase transition the rate of desorption of the amphiphile is decreased in comparison with desorption from either fluid or solid phases, leaving the respective rates of insertion unchanged. In mixtures of DMPC and DSPC exhibiting coexistence of solid and fluid phases, association occurs considerably faster overall than expected based on phase composition. We can account for this observation by assuming an increased rate of insertion with no detectable change in the rates of desorption.

MATERIALS AND METHODS

Chemicals

DMPC was purchased in powder form from Avanti Polar Lipids, Inc. (Alabaster, AL) and cholesterol from Serva Fine Biochemicals (as supplied by Boehringer-Ingelheim, Heidelberg, Germany). U-6 (4-(*N,N*-dimethyl-*N*-tetradecylammonium)methyl-(7-hydroxycoumarin)chloride) was obtained from Molecular Probes Europe, B.V. (Leiden, the Netherlands). Chloroform p.A. was from Merck (Lisbon, Portugal) and all other chemicals from Sigma Chemical Co. (Sigma-Aldrich Quimica Madrid, Spain). Lipids and other chemicals were used without further purification.

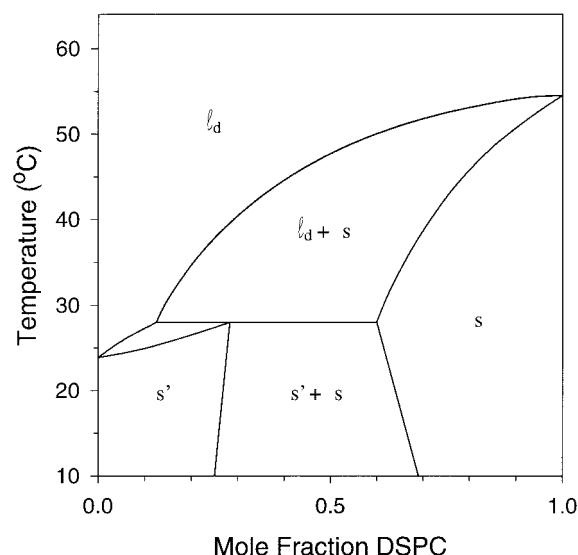


FIGURE 1 DMPC/DSPC phase diagram. s , solid phase (s' and s refer to different types of solid phases); ℓ_d , liquid-disordered phase. After Mabrey and Sturtevant, 1976; Knoll et al., 1981.

Preparation of large unilamellar vesicles (LUV)

DMPC and DSPC were separately dissolved in chloroform to give a final concentration of 10 mM each. Appropriate amounts of these chloroform solutions were mixed in a round-bottomed flask and rapidly dried using a rotary evaporator (Heidolph VV2000) at 60–70°C to ensure mixing of the components. After evaporation of solvent the lipid film was hydrated by addition of buffer, pre-heated to at least 10°C above the transition to the ℓ_d phase, to give a final lipid concentration of 10 mM. Swirling of the flask yielded a turbid suspension of multilamellar vesicles that was subsequently extruded ~ 10 times through two stacked Nuclepore polycarbonate filters of 0.1 μm pore size. Extrusion was performed well above the melting temperature of the component with the largest T_m using a water-jacketed high-pressure extruder from Lipex Biomembranes Inc. The LUV suspension obtained in this fashion was subjected to several cooling and heating cycles to ensure annealing of the lipid. The suspension was diluted in buffer to the desired concentration and used for fluorescence measurements. The buffer used in all experiments was 10 mM boric acid, pH 8.5, 10 mM KCl, 0.1 mM EGTA. Lipid concentrations were assayed through a modified version of the Bartlett phosphate assay (Bartlett, 1959).

Fluorescence experiments

U-6 was added to the buffer from a 0.50 mM stock solution in ethanol. Kinetic traces were obtained using a stopped-flow fluorometer (Hi-Tech model SF-61). Equal volumes of a U-6 solution (5.0 μM) and a lipid suspension were rapidly mixed and the time course of fluorescence was monitored. The sample was excited through a monochromator at 392 nm and fluorescence emission monitored through a long-pass filter. Data were acquired using commercially available software supplied by Hi-Tech. The fluorescent probe U-6 (Kraayenhof et al., 1993) is a single-chain amphiphile with a critical micelle concentration of $\sim 20 \mu\text{M}$ (data not shown). In the ground state the hydroxycoumarin undergoes an acid-base equilibrium with a pK_a of ≈ 7.3 in aqueous solution at 25°C. The basic form absorbs maximally at $\approx 395 \text{ nm}$, and the protonated one at 340 nm. In phosphatidylcholine vesicles the pK_a of the ground state is shifted to ~ 8.3 . The excited state has a pK_a well below 2, so that at a pH > 4 emission occurs exclusively from the deprotonated state with a maximum at 480 nm. We took advantage of the pK_a -shift of the ground state absorption

upon insertion into vesicles to obtain a difference signal that was used to monitor incorporation.

Analysis of experimental data

Molecular rates and binding constants were obtained from an analysis of the kinetic data as described previously (Pokorny et al., 2000). Briefly, we can describe the association of a fluorescent probe, P , with lipid vesicles, V , composed of a single phase by the following reaction scheme with the rate constants k_{on}^* and k_{off} for the on- and off-rate, respectively.



Expressing the vesicle concentration in terms of lipid concentration, $[L] = [V] \times 10^5$, we obtain for the time course of fluorescence

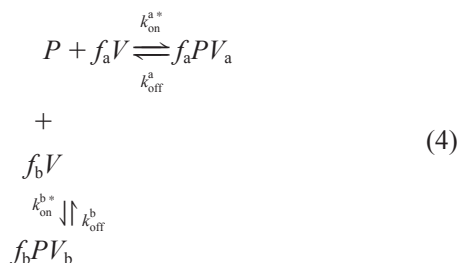
$$F(t) = F_o(Ae^{-k_{obs}t} + B) \quad (2)$$

where

$$k_{obs} = k_{on}[L] + k_{off}, \quad (3)$$

and $k_{on} = k_{on}^* \times 10^{-5}$. A and B are amplitude factors, which are explicit functions of the association equilibrium constant ($K_a = k_{on}/k_{off}$), initial probe concentration, probe fluorescence yields, and lipid concentration (see Pokorny et al., 2000, for the exact expressions of A and B). A plot of k_{obs} against $[L]$ yields a straight line with slope k_{on} . The off-rate, k_{off} , can then be determined either through the y -intercept or directly from each kinetic trace.

For probe molecules, P , inserting into vesicles composed of two phases, a and b , with fractions f_a and f_b , we can construct the following reaction scheme: 4



In this case, the vesicle concentration is multiplied by the fractional amounts of each phase present, f_a or f_b , and the probe can interact with either phase a , forming the bound state PV_a or with phase b , forming PV_b . The time course of fluorescence is characterized by two apparent rate constants, $-\lambda_1$ and $-\lambda_2$:

$$F(t) = A' + B'e^{\lambda_1 t} + C'e^{\lambda_2 t}, \quad (5)$$

where A' , B' , and C' are amplitude factors, which are *not* freely adjustable parameters, but are explicit functions of all the molecular rate constants, the lipid concentration, the fraction of phases present, and the initial conditions $P(0)$, $PV_a(0)$, $PV_b(0)$. We omit the exact expressions to avoid unnecessary repetition, since they are given in Pokorny et al. (2000). The two apparent rates are given by:

$$\begin{aligned} \lambda_1 = -1/2(k_{off}^b + k_{off}^a f_a - k_{on}^b L f_a \\ + k_{on}^a L f_a + k_{on}^b L - k_{off}^b f_a - \omega) \end{aligned} \quad (6)$$

$$\begin{aligned} \lambda_2 = -1/2(k_{off}^b + k_{off}^a f_a - k_{on}^b L f_a \\ + k_{on}^a L f_a + k_{on}^b L - k_{off}^b f_a + \omega) \end{aligned}$$

where

$$\begin{aligned} \omega = [(1 - f_a)^2(k_{on}^b L + k_{off}^b)^2 + f_a^2(k_{on}^a L + k_{off}^a)^2 + k_{on}^{b2} L^2 \\ + 2f_a(1 - f_a)(k_{on}^a k_{on}^b L^2 - k_{on}^a k_{off}^b L - k_{on}^b k_{off}^a L - k_{off}^a k_{off}^b)]^{(1/2)} \end{aligned}$$

RESULTS

In the analysis of the experimental data the strategy was the following: first, obtain the kinetic molecular rate constants corresponding to each phase (s or ℓ_d) from a fit of the theoretical, single-exponential function (Eqs. 2 and 3) to the kinetic data for association with that phase, at each temperature; second, in the two-phase regions ($\ell_d + s$), calculate the kinetics that would result if association with a two-phase region were a simple combination of parallel associations with each single phase, weighted by the amounts of each phase present from the phase diagram, at a given temperature, as given by Eqs. 5 and 6; third, compare the calculated kinetic curve with that obtained experimentally and establish whether or not the simple description holds.

DMPC at the phase transition temperature

The kinetics of insertion of U-6 into pure DMPC in the solid or the fluid phase are described by a single exponential with an apparent rate that is an explicit function of the molecular rates ($k_{obs} = k_{on}[L] + k_{off}$), as predicted by Eqs. 2 and 3. Those molecular rates were obtained by measuring k_{obs} as a function of vesicle concentration between 14°C and 36°C. Plots of k_{obs} as a function of the lipid concentration according to Eq. 3 (Fig. 2) were used to determine the on- and off-rates from the slope and the y -intercept, respectively. The values thus obtained, separately, for the molecular on- and off-rates for pure ℓ_d and s DMPC phases as a function of temperature were extrapolated to the T_m and used in the description of a two-phase system according to Eqs. 5 and 6. We assumed 50% ℓ_d phase to indicate that the probability of finding any given lipid in a solid or fluid state is equal to 1/2 at the T_m . With those rates the theoretical curve at the T_m is essentially a single exponential in the time frame of the experiment because the second exponential contributes only with a very small amplitude. Fig. 3 *A* shows such a theoretical curve (Eqs. 5 and 6) superimposed onto an experimental trace at 23°C (see also Table 1). Evidently, at the phase transition temperature this curve fails to describe the time course of insertion. Allowing the fraction of fluid phase to deviate from 50% (which could arise because of an uncertainty in the temperature) does not improve the fit. The predicted curve lacks the distinct, slow, second kinetic phase that is present in the experimental trace. To describe this experimental result at the T_m we must assume an off-rate from the solid phase that is much smaller than determined from an all-gel vesicle (Fig. 3 *B* and Table 1). The details of the analysis leading to this conclusion are

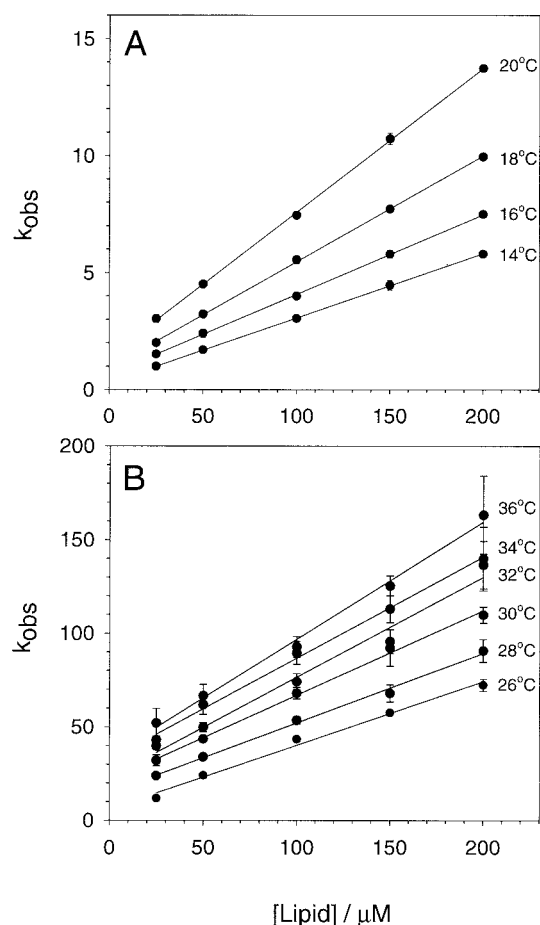


FIGURE 2 The apparent rate of association, k_{obs} , of probe with lipid vesicles as a function of vesicle concentration. (A) s phase DMPC vesicles. (B) ℓ_d phase DMPC vesicles. The solid lines represent a linear least-squares fit to the experimental data. The error bars indicate the standard deviation for a minimum of three experiments.

presented in the Appendix. The failure of the simple two-phase model to describe the kinetics of association with pure DMPC at T_m is even more clearly shown in Fig. 4, where the deviations from a single exponential fit are plotted against temperature. A pronounced maximum is observed at T_m .

Regions of phase coexistence in DMPC/DSPC mixtures

The kinetics of amphiphile association with two-component, two-phase vesicles were studied in a 1:1 mixture of DMPC and DSPC, which shows phase separation between s and ℓ_d phases over a wide temperature interval (Fig. 1). The molecular on- and off-rates characteristic of the fluid (ℓ_d) phase as a function of temperature were obtained from the analysis of single-phase vesicles of pure DMPC; for the solid (s) phase a 2:8 mixture of DMPC/DSPC was used

(Fig. 5). In both cases a single-exponential function, given by Eqs. 2 and 3, was expected and observed. Again, at each given temperature, if the two-phase system behaved as a simple combination of the two phases involved, the kinetics should be described by Eqs. 5 and 6, where the molecular rates are those obtained from the analysis of the separate kinetics of association with s or ℓ_d phases, at the same temperature, and the fractions of phases present are taken from the phase diagram (Fig. 1). Fig. 6 A shows the experimental kinetics of amphiphile association with two-phase vesicles at 34°C and the theoretical curve calculated from the model (Eqs. 5 and 6). The experimental kinetics appear largely monoexponential. This was expected, on the time scale of the experiment, despite the fact that Eq. 5 is a two-exponential, because the amplitude associated with the smaller eigenvalue is not expected to be noticeable. However, the kinetics are considerably faster than predicted based on the rates determined from pure s and pure ℓ_d phases. In fact, they are systematically faster in the entire temperature region between 30 and 44°C (Fig. 6 B). The theoretical equation can be made to fit the data by adjusting the on-rate into the ℓ_d phase (Fig. 7 A and Table 2, Model 2) or the fraction of ℓ_d phase present (Fig. 7 B, Table 2, Model 3). The rationale for adjusting the amount of fluid phase will be discussed shortly, and stems from the possibility that the *effective* amount of fluid phase in the system might appear larger than predicted by the lever-rule from the phase diagram. However, adjusting the amount of fluid phase leads to a larger, if still small, amplitude of the slow component. The curve thus calculated does not fit the experimental trace very well (Fig. 7 B). Above 44°C the experimental traces actually become slower than the model predicts (Fig. 6 B) because association with a DSPC-rich fluid is slower (see Discussion).

DISCUSSION

In the present study we investigated the effect of ℓ_d - s phase coexistence on the insertion of a simple fluorescent amphiphile into lipid vesicles. Phase separations of that type exist immediately around the T_m in one-component vesicles or over a larger temperature interval in some two-component lipid mixtures, such as in DMPC/DSPC mixtures. Domain boundaries are often thought of as sites for the preferential insertion of proteins or for increased ion permeability (Cruzeiro-Hansson and Mouritsen, 1988; Clerc and Thompson, 1995). In a previous study (Pokorny et al., 2000) we showed that in regions of phase separation in PC/cholesterol systems the kinetics of association with two-phase vesicles are either described by a simple sum of the parts (ℓ_d - ℓ_o phase coexistence) or even slower (ℓ_o - s phase coexistence) than for single-phase vesicles. In the present study, two additional questions are investigated. First is the type of phase separation observed within the phase transition of pure lipid vesicles equivalent, with respect to the insertion of amphiphiles, to the fluid-solid phase separation that ex-

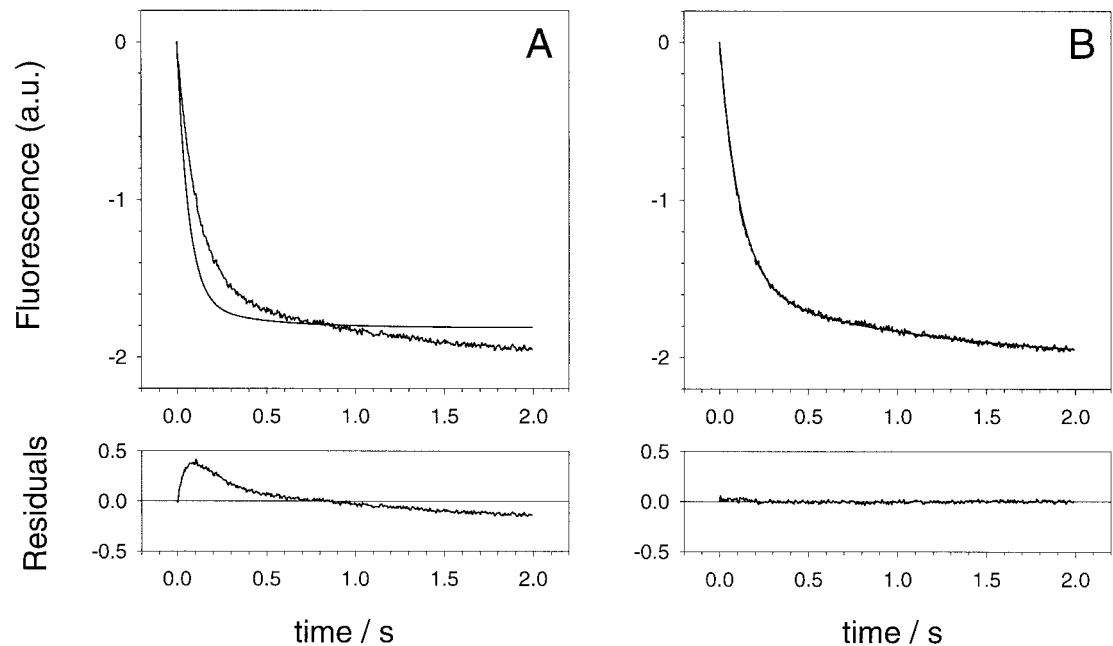


FIGURE 3 Kinetics of association of U-6 with pure DMPC vesicles at 23°C. (A) The thick solid line represents the kinetics of association as predicted by the two-phase model, assuming on- and off-rates for s and ℓ_d phases that have been extrapolated to the T_m (see Table 1). (B) The thick solid line represents the kinetics of association again as predicted by the two-phase model, but with the molecular rates into the putative s and ℓ_d phases adjusted to give a good description of the experimental data (see Table 1).

ists in two-component vesicles? Second, are the slow kinetics of association observed in ℓ_o - s phase separated systems characteristic of fluid-solid phase separations in general or of cholesterol-containing systems only?

Our approach was to study the kinetics of association with solid and fluid phases separately, which can be rigorously analyzed. The theoretical expression for this process gives the time dependence of the amphiphile fluorescence as a function of the molecular rate constants for insertion and desorption. Next, we asked the question of whether the two-phase system composed of those solid and fluid phases behaves as a simple combination of its parts. To answer this question, we calculated the predicted kinetics of association for that case, at each temperature, using the theoretical expressions (Eqs. 5 and 6). A comparison of this prediction with the experimental kinetics in the solid-fluid regions provides a clear answer to the problem considered. If the calculated curve does not describe the data, then either interfaces between solid and fluid phases do play a role, enhancing

or slowing down the overall rate of the association process, or the two-phase regions cannot be described by a stable coexistence of solid and fluid phases. A subtle point is the following. In a two-phase system, the theoretical equation for the kinetics of association contains two exponentials. However, with the values obtained for the molecular rates (k_{on} , k_{off}), separately, for the solid and fluid phases (single-phase vesicles), the calculated kinetic curves in the solid-fluid regions of DMPC/DSPC or pure DMPC at T_m would appear monoexponential, on the time scale of the experiment, because the smaller eigenvalue (λ_1) is associated with a very small amplitude, which is close to the experimental noise.

DMPC at the T_m

In pure DMPC at the T_m , solid and fluid states coexist in the same vesicle (Sugár et al., 1994, 1999), and the average amount of each state is 50%. The experimental traces of the

TABLE 1 Molecular rate constants, apparent rates, and associated amplitudes for pure DMPC at T_m ; the fractions of solid and fluid phase were set to 0.50

	k_{on}^{solid} ($s^{-1} M^{-1}$)	k_{off}^{solid} (s^{-1})	k_{on}^{fluid} ($s^{-1} M^{-1}$)	k_{off}^{fluid} (s^{-1})	$-\lambda_1$ (s^{-1})	Relative amplitude of slow phase ($-\lambda_1$)	$-\lambda_2$ (s^{-1})
Model 1*	1.3×10^5	3.6	3.2×10^5	10.8	2.6	0.087	15.8
Model 2†	1.1×10^5	0.015	1.6×10^5	5.4	0.87	0.23	8.7

*Molecular rates for insertion and desorption for each phase at 23°C were obtained by extrapolation of the results from solid and fluid pure DMPC vesicles.
†Molecular rates from a fit of the two-phase model (Eq. 5) to the experimental data at 23°C.

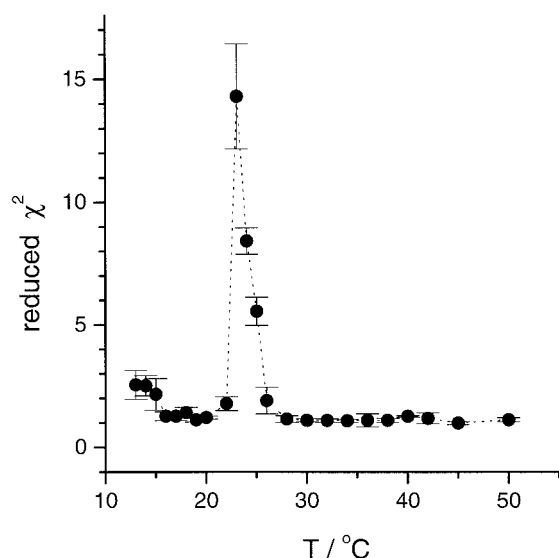


FIGURE 4 Goodness-of-fit of a single-exponential function (reduced χ^2) to the experimental kinetics as a function of temperature. The error bars indicate the standard deviation for a minimum of three experiments.

kinetics of association obtained at the T_m of DMPC display two clearly discernible exponentials, with the deviation from a single-exponential function being maximal at the T_m (Fig. 4). The system clearly does not behave as a simple combination of solid and fluid (Fig. 3 A). We had noted the pronounced deviation from monoexponential kinetics at the T_m of DMPC already in the first article of this series (Pokorny et al., 2000). At the time we did not analyze the kinetics around the T_m in detail because this was not the question under investigation. A detailed analysis of the

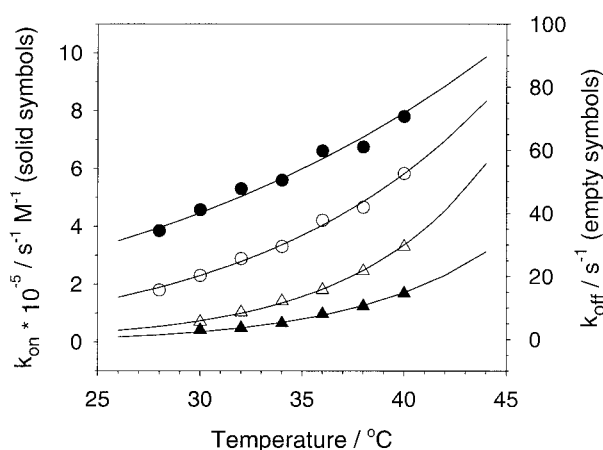


FIGURE 5 Molecular on- and off-rates for the liquid-disordered (ℓ_d) and solid (s) phases. The values for the ℓ_d and s phases are obtained from pure DMPC and 2:8 DMPC/DSPC vesicles, respectively. Circles, ℓ_d phase; triangles, s phase. Open symbols, off-rates; solid symbols, on-rates. The lines are fits of the Arrhenius equation ($A_0 \exp(-E_a/kT)$) to the data points, where E_a is the activation energy, and are used here as a means to extrapolate the data to a larger temperature interval.

kinetics of association at the T_m presented here shows that the second, slow phase can only be accounted for by assuming a slowing down of the rates of desorption of the probe from the bilayer, as shown in Fig. 3 B and Table 1 (see also Appendix for a more detailed treatment). Within the scope of the two coexisting phases model, the only way to account for the experimental results is to assume that the smallest off-rate is even smaller than determined from pure solid-phase vesicles. We may understand this apparent slowing down of desorption if the off-rate determined for the pure solid phase ($k_{\text{off}}^{\text{solid}} \approx 1 \text{ s}^{-1}$) were an overestimate. This could occur because an all-solid vesicle has many packing defects, which are probably absent in the solid-fluid coexistence region because the phase domains are smaller. Thus, the smaller estimate $k_{\text{off}}^{\text{solid}} \approx 10^{-2} \text{ s}^{-1}$ would be the correct value for a well-packed solid. Another possibility would be that the addition of an impurity (the fluorescent probe) is correlated with the fluctuations in the solid, basically suppressing them and leading to a smaller off-rate. However, it is probable that a different model, dominated by fluctuations, may be more appropriate for a description of the kinetics of association with a system at the phase transition. In fact, the main transition of phosphatidylcholines is not first-order (Sugár et al., 1994; Jerala et al., 1996).

The s - ℓ_d phase coexistence in DMPC/DSPC mixtures

Consider now a DMPC/DSPC mixture in the middle of the solid-fluid coexistence region. The nature of this stable phase coexistence is different from that in pure DMPC at T_m . When the relative amounts of solid and fluid phases are similar (at 34°C, the system has $\approx 65\%$ s phase, Fig. 1), the experimental kinetics for insertion and desorption of probe appear mainly monoexponential on the time scale of the experiment (Fig. 7 and Table 2).

However, over a large temperature interval (between 30 and 44°C), these experimental kinetics are consistently faster than predicted using the rates found for each separated phase in Eqs. 5 and 6, which basically consists of a simple sum of parallel processes for two coexisting phases, weighted by their respective mass fractions. A legitimate criticism is that the amount of fluid phase may effectively appear to be larger than that given by the phase diagram if the probe molecule were able to sample a certain surface area of the vesicle before inserting. Thus, on the one hand, if the probe, upon collision with the vesicle, first encounters a fluid domain, the insertion process is very fast, close to being activationless ($\Delta G^{\ddagger} \approx 1 \text{ kcal/mol}$ at 24°C). If, on the other hand, the collision occurs on a solid domain, insertion will be slower and the probe will be able to sample a region of vesicle surface before inserting or going back into solution: “a diffusing molecule that has bumped against the surface of a cell is by that very circumstance destined to wander around in that vicinity for a time, most likely hitting

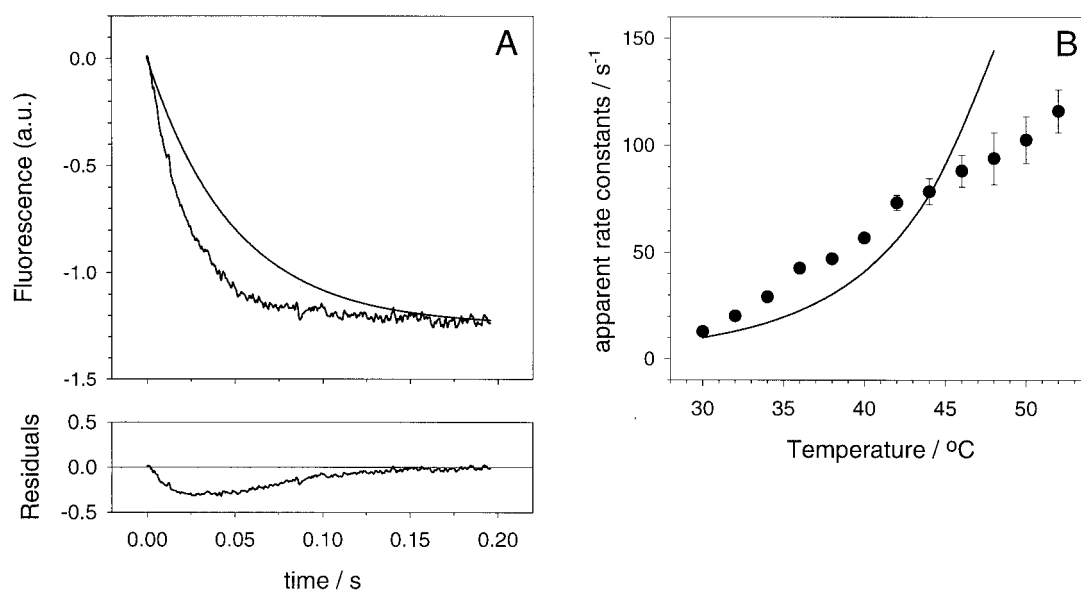


FIGURE 6 Two-phase, two-component vesicles. (A) Experimental kinetics of association of U-6 with two-phase vesicles composed of a 1:1 mixture of DMPC and DSPC at 36°C. The experimental trace represents the average of six shots. The smooth curve represents the kinetics of association as predicted by the two-phase model. Fraction of solid phase, $f_s = 0.61$, $k_{\text{on}}^s = 9.2 \times 10^4 \text{ s}^{-1} \text{ M}^{-1}$, $k_{\text{off}}^s = 15.9 \text{ s}^{-1}$, $k_{\text{on}}^{\ell} = 6.3 \times 10^5 \text{ s}^{-1} \text{ M}^{-1}$, $k_{\text{off}}^{\ell} = 36.2 \text{ s}^{-1}$. (B) The circles are the observed apparent rates, $-\lambda_2$, as a function of temperature. The line is the calculated apparent rate, $-\lambda_2$, according to the two-phase model, assuming on- and off-rates into each phase as determined from the analysis of pure DMPC vesicles (ℓ_d phase) and 2:8 DMPC/DSPC vesicles (s phase). The fraction of phases present at each temperature was determined from the phase diagram in Fig. 1.

the cell many times before it wanders away for good” (Berg and Purcell, 1977). The area sampled can be estimated as that covered by a random walk on the surface with a diffusion coefficient $D \approx 10^{-6} \text{ cm}^2/\text{s}$ for the probe in water

and a time for insertion into the solid phase $\tau \approx 10^{-5} \text{ s}$ (Table 2). From the mean-square displacement, $\langle r^2 \rangle \sim D\tau$, we obtain a linear distance of $r \approx 50 \text{ nm}$ on the vesicle surface. If the phase domains were significantly smaller

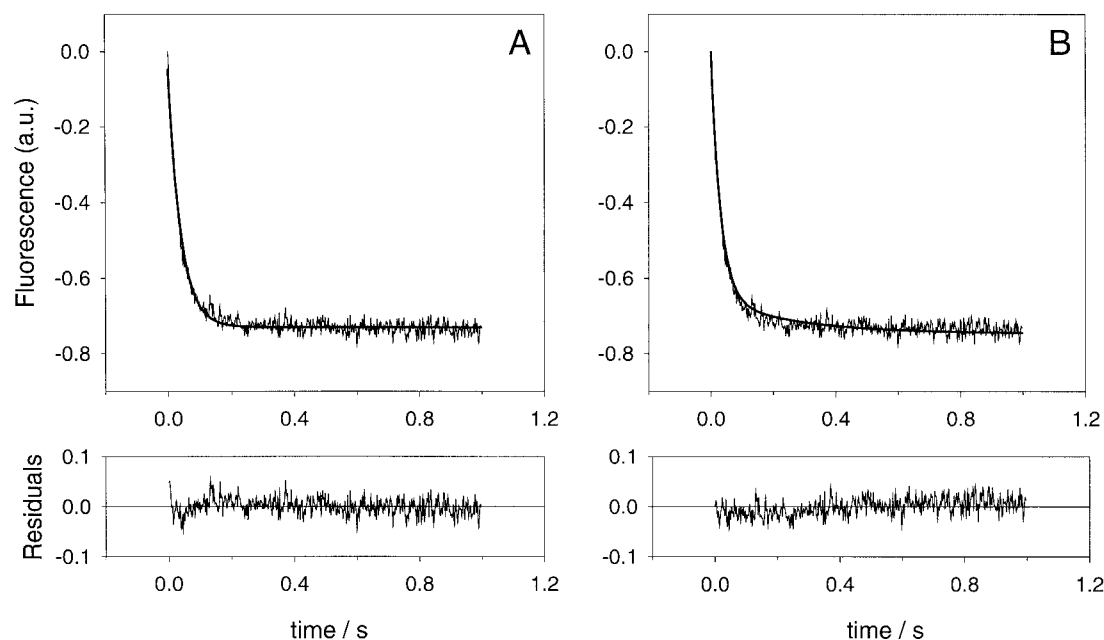


FIGURE 7 Kinetics of association of U-6 with DMPC/DSPC 1:1 at 34°C. (A) Varying the on-rate into the fluid phase, k_{on}^{ℓ} , to describe the experimental data. (B) Varying the fraction of fluid phase to fit the data. Values are listed in Table 2.

TABLE 2 Molecular rate constants, apparent rates, and associated amplitudes for two-phase, DMPC/DSPC vesicles at 34°C (65 mol% solid)

	f_s	$k_{\text{on}}^{\text{solid}}$ ($\text{s}^{-1} \text{M}^{-1}$)	$k_{\text{off}}^{\text{solid}}$ (s^{-1})	$k_{\text{on}}^{\text{fluid}}$ ($\text{s}^{-1} \text{M}^{-1}$)	$k_{\text{off}}^{\text{fluid}}$ (s^{-1})	$-\lambda_1$ (s^{-1})	Relative amplitude of slow phase ($-\lambda_1$)	$-\lambda_2$ (s^{-1})
Model 1*	0.65	6.7×10^4	11.5	5.7×10^5	29.9	7.9	0.030	17.1
Model 2†	0.65	6.7×10^4	11.5	1.4×10^6	29.9	7.7	0.0067	25.5
Model 3‡	0.31	6.7×10^4	11.5	5.7×10^5	29.9	3.9	0.13	32.7

*Molecular rates for insertion and desorption for the fluid phase were obtained from fluid DMPC vesicles and for the solid phase from solid 2:8 DMPC/DSPC vesicles. The fraction of solid phase, f_s , is taken from the phase diagram.

†Fit of the two-phase model (Eq. 5) to the experimental data at 34°C, allowing only for $k_{\text{on}}^{\text{fluid}}$ to vary relative to Model 1 (value changed in bold).

‡Fit of the two-phase model (Eq. 5) to the experimental data at 34°C, allowing only for f_s to vary relative to Model 1 (value changed in bold).

than this value, the probe would encounter, during its search, a fluid area into which it would insert preferentially. As a consequence, the probability of the probe molecule encountering an area of fluid phase would be much higher than predicted based on the amount of fluid phase from the phase diagram. However, estimates of the sizes of solid-phase domains in 1:1 mixtures of DMPC and DSPC yield values between 300 and 3000 nm. Sugár et al. (1999) estimated a value of ~ 30 nm from a combination of experimental heat capacity curves and Monte Carlo simulations for a small system (40×40 lattice). Scaling arguments (Stauffer and Aharony, 1994) suggest that for a lattice of the size of a real LUV, these domains should be ~ 10 times larger, giving a lower bound of 300 nm. The upper bound is an estimate by Coelho et al. (1997) based on Monte Carlo simulations of FRAP experiments, or using an analytical approximation given by Almeida et al. (1993). With solid domain sizes this large the probe would not be able to encounter the limits of a solid domain before either inserting into it or diffusing off the vesicle surface. This speaks against the above interpretation to account for the faster-than-expected kinetics of association. Furthermore, increasing the fraction of ℓ_d phase in our kinetic model causes the amplitude of the second, slow exponential to increase (Fig. 7 B and Table 2). Its contribution, on the time scale of the experiment, is only on the order of 10% and cannot be easily detected due to experimental noise, but the experimental traces appear monoexponential even at a time $t = 30 \times \tau$ (Fig. 7). The fit using a second slow phase is clearly worse, as can be seen by the trend in the residuals (Fig. 7 B).

Another way to account for the faster-than-expected kinetics of amphiphile-vesicle association between 30 and 44°C is to assume an increase in the on-rate into the fluid phase (Fig. 7 A and Table 2). Our model only takes two phases into account, but any additional process that is not included in the model with an on-rate that is faster than into either ℓ_d or s phase would determine the magnitude of the largest eigenvalue and thus lead to a faster overall kinetics of association. The experimental curves can be described if we use in Eqs. 5 and 6 an on-rate into the ℓ_d phase larger than what was determined here. A plausible interpretation of this fact is that faster insertion occurs at the interface be-

tween solid and fluid domains. An increased permeability of two-component vesicles in a region of phase separation has been observed in phosphatidylcholine mixtures and has been correlated with the amount of interface present. (Cruzeiro-Hansson and Mouritsen, 1988; Corvera et al., 1992; Clerc and Thompson, 1995; Xiang and Anderson, 1998). The lifetime of these putative packing defects at the solid-fluid interface must be on the order of microseconds or longer to be detected under our experimental conditions, because the low probe concentration we use restricts the time resolution to the detection of relatively long-lived defects (Pokorny et al., 2000). The faster-than-predicted kinetics of association should then be correlated with the amount of interface present and thus play less of a role at temperatures where there is either little fluid or little solid phase. This is observed experimentally here.

Above 44°C the experimental kinetics of association become slower than predicted by the model (Fig. 6 B, circles). This is because the molecular rates used to calculate the association kinetics for the ℓ_d phase were obtained from pure DMPC vesicles (Fig. 6 B, solid curve). Below 44°C this is a good approximation, but above that temperature a 1:1 DMPC/DSPC mixture contains $\geq 40\%$ DSPC, and insertion into a fluid phase with a high content of DSPC is slower (the last 3 points in Fig. 6 B are on or above the *liquidus* line in the phase diagram). We point out that, in the two-phase region (30–44°C), if values for a fluid phase containing DSPC were used in the model, the discrepancy between prediction and experiment would be even larger, strengthening our conclusion that this two-phase region cannot be described by a simple sum of solid plus fluid.

CONCLUSION

With respect to insertion of amphiphiles, the ℓ_d - s phase separation that exists at the T_m of pure DMPC behaves very differently from a two-component mixture that shows a stable phase separation over a large temperature interval (DMPC/DSPC). Information on phase coexistence and its effects on membrane properties, such as permeability, obtained from the study of pure lipid systems at the T_m are thus

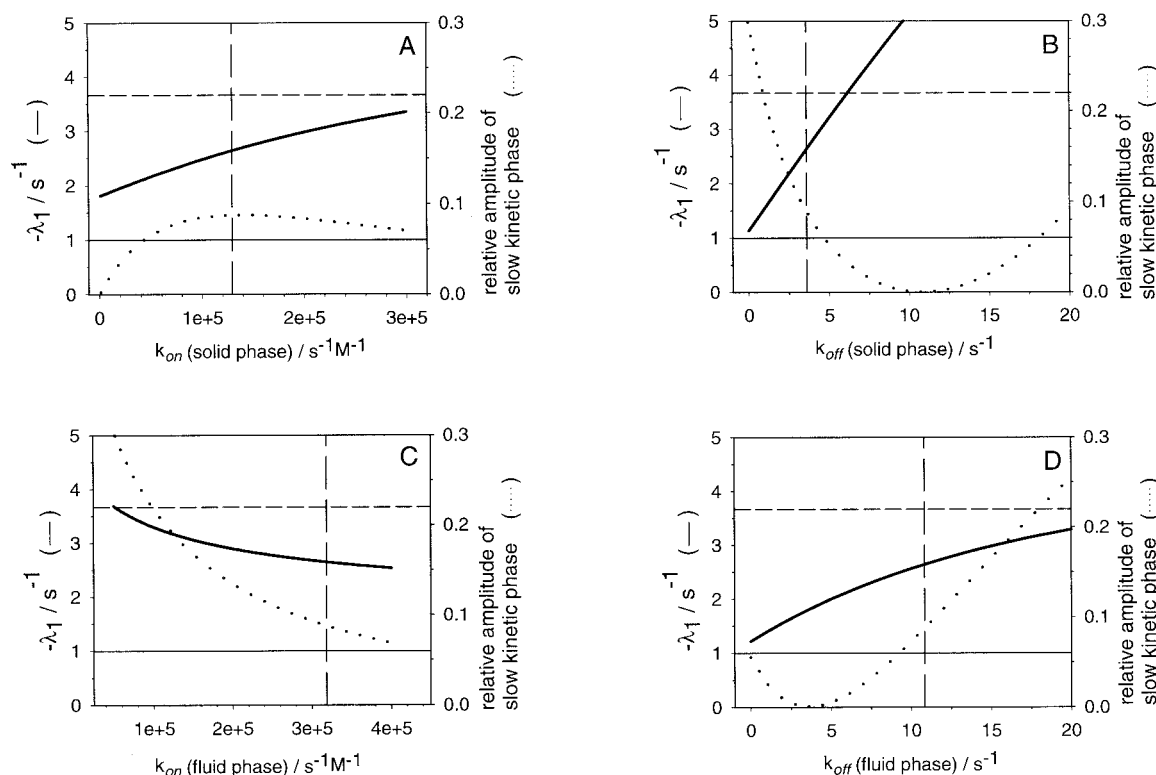


FIGURE 8 Pure DMPC vesicles at the T_m . Dependence of the magnitude of the apparent rate of the slow kinetic component, $-\lambda_1$ (solid lines) and its amplitude (dotted lines) on the molecular rates for insertion and desorption for each phase as predicted by the two-phase model. In each graph one molecular rate is varied, keeping the other three molecular rates fixed at the values that have been determined from pure solid- and pure fluid-phase vesicles and extrapolated to 23°C. The fraction of s phase in the model is 50%. (A) Dependence of $-\lambda_1$ and its amplitude on k_{on}^s . (B) Dependence of $-\lambda_1$ and its amplitude on k_{off}^s . (C) Dependence of $-\lambda_1$ and its amplitude on $k_{on}^{\ell_d}$. (D) Dependence of $-\lambda_1$ and its amplitude on $k_{off}^{\ell_d}$. The two thin horizontal lines in each graph refer to the magnitude of the slow rate (solid line) and its amplitude (short-dashed line) that have been determined experimentally for the association of U-6 with DMPC vesicles at 23°C. The vertical long-dashed line in each graph refers to the value of the molecular rate under consideration that has been determined for 23°C by extrapolation from pure s and pure ℓ_d phase DMPC vesicles. Its intersection with the thick solid line reflects the value for $-\lambda_1$ and its intersection with the thick dotted line gives the amplitude of this phase, as predicted by the model (see model prediction 1 in Table 1). If we want to find out how we would have to change a particular molecular rate to better describe the experimental data we need to look for the intersection of the two solid lines and the intersection of the dotted and short-dashed lines. If they do not intersect (as in (A)) no change in this molecular rate will result in a good description of the experimental data. If only the dotted and the short-dashed lines intersect, as in (C), we can change the amplitude of the slow phase to match the experimentally observed one, but not the rate. To describe the experimental data, the intersections of the dotted with short-dashed and two solid lines have to fall, in principle, on the same x -coordinate (= molecular rate). Of course, changing one molecular rate will in turn influence the dependence of $-\lambda_1$ on all of the other rates, and will also change the value of $-\lambda_2$. Therefore, these plots only serve as a general guideline as to how the small eigenvalue, λ_1 , changes with each rate. To adequately fit the experimental data all rates have to be changed iteratively, also taking into account their correlation. The values of the molecular rates that result in an adequate description of the experimental data are listed in Table 1.

not easily transferable to a two-component mixture. In the solid-fluid coexistence region of DMPC/DSPC, between 30 and 44°C, the faster kinetics of association can most likely be attributed to a more rapid insertion into defect-rich regions, which could be phase boundaries between solid and fluid domains. These putative packing defects at the phase boundaries must have a lifetime of the order of microseconds, at least, because shorter-lived defects would not be detectable in our experiment. In the DMPC/cholesterol system, which we studied previously (Pokorny et al., 2000), association of the same amphiphile probe with vesicles was never faster than predicted by the same model. In the fluid-fluid, ℓ_o - ℓ_d coexistence region of DMPC/cholesterol the probe inserted exactly as predicted if the system be-

haved as a sum of its parts. In the solid-fluid, $s + \ell_o$ coexistence region the probe inserted much slower than predicted. In view of the current study, that indicates that the presence of cholesterol in phase-separated systems seals the membrane against uncontrolled leaks or insertion processes.

In summary, taking together our previous work and the present one, we have examined four different types of phase coexistence regions: a pure phospholipid at the phase transition ($s + \ell_d$), a stable phase coexistence region ($s + \ell_d$) of a non-ideal mixture of two phospholipids, a solid-fluid mixture in a phospholipid/cholesterol mixture ($s + \ell_o$), and a fluid-fluid mixture in a phospholipid/cholesterol mixture ($\ell_o + \ell_d$). In the last type, which is probably the most relevant for biological systems, the phase domain interfaces

do not lead to any change in the rate of amphiphile insertion. Systems of coexistence of $s + \ell_d$ or $s + \ell_o$ phases behave very differently from each other. In the former, amphiphile insertion is accelerated; in the latter, it is severely slowed down. The difference is that the second system contains cholesterol, which seems to seal any misfits at the interface. Finally, the first type, a pure phospholipid at the T_m , behaves in an altogether different way, exhibiting more complex amphiphile association kinetics.

APPENDIX

Here we analyze the effects of changing the various molecular rates on the curve that describes association with DMPC vesicles at the main transition temperature. The experimental kinetic data are correctly described by a two-exponential curve (Fig. 3 B). The two-coexisting-phases model (Eqs. 5 and 6) also predicts two exponentials, the slower of which, however, is still faster than the experimentally observed second kinetic phase. Furthermore, this second kinetic phase would be expected to contribute only with a small amplitude ($<10\%$), making the curve appear largely monoexponential (Fig. 3 A, *smooth curve*). In general, the two eigenvalues, λ_1 and λ_2 , that determine the time course of the theoretical curve are a complex combination of all molecular rates involved (Eq. 6). The larger of those two eigenvalues (λ_2) is determined by the largest rates and the smaller one (λ_1) is determined by the smaller rates. However, exactly which are the smaller molecular rates, which contribute to the slower processes, ceases to be intuitive. This depends on the exact values involved because the two on-rates appear in the expressions for the two λ s multiplied by the vesicle concentration and the fractions of phases present; those products can be of the same order of magnitude as the off-rates. Thus, in order to understand the system better, we calculated (from Eq. 6) the dependence of the smaller apparent rate ($-\lambda_1$) and its associated amplitude on the four molecular rates involved (Fig. 8, A–D). Inspection of Fig. 8 shows that the only way to simultaneously decrease the value of $-\lambda_1$ and increase the amplitude associated with it is to *decrease* the off-rates. Fig. 3 B shows a theoretical trace that was adjusted in an iterative process varying the molecular rates to yield an adequate description of the experimental curve. The parameters required to describe the experimental curve are listed in Table 1.

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