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KINETIC AND THERMODYNAMIC STUDIES OF THE FUSION OF SMALL UNILAMELLAR PHOSPHOLIPID VESICLES

BRUCE P. GABER and JAMES P. SHERIDAN

Bio/Molecular Optics Section, Optical Probes Branch, Code 6510 Naval Research Laboratory, Washington, DC 20375 (U.S.A.)

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Small phospholipid vesicles, prepared so as to minimize impurities, fuse relatively slowly resulting in the time-dependent development of a characteristic endotherm in differential scanning calorimetry and corresponding changes in the Raman spectrum. The stability of small vesicles towards fusion increases with increasing acyl chain length for the series C-14 through 18. Within the protocols of these experiments, the fusion rate remains unchanged whether the vesicles are held at 10°C below T_m or at T_m itself. We have determined enthalpies of transition for small vesicles and fusion product for C-14 through C-18. In each case ΔH for small vesicles is lower than that of the corresponding multilamellar vesicles, while the fusion product ΔH is intermediate between small and multilamellar vesicles. The apparent lack of concensus in the literature as to the nature of the fusion process is ascribed to the variety of protocols used as well as the presence or absence of fusion-inducing impurities.

Introduction

One of the drawbacks of the use of small unilamellar phospholipid vesicles is their propensity to undergo conversion, below T_m, to larger, less-well defined liposomes. The factors governing this process have been a topic of considerable interest and controversy [1–7]. In an effort to determine which parameters serve to stabilize small vesicles against fusion, we have examined the effect that acyl chain length exerts in enhancing small unilamellar vesicle stability. We have employed a calorimetric procedure which permits the description of the kinetics of small unilamellar vesicle fusion in terms of appearance of the fusion product. This technique is based on that originally described by Suurkuusk et al. [1] to measure fusion of DPPC vesicles. The experiments reported here differ in that the calorimeter used may be equilibrated very rapidly. Thus it has been possible to determine the relative distribution of small unilamellar vesicle and fusion product at times close to the initiation of the fusion process. Similarly, we have been able to determine enthalpies of transition for preparations of small unilamellar vesicle which are virtually free of fusion product. The data suggest that increasing chain length results in small unilamellar vesicle of increasing kinetic and thermodynamic stability. Parallel experiments using Raman spectroscopy confirm the kinetic observations made by calorimetry, and provide additional physical insight into the stabilizing effect of chain length.

Methods and Materials

Dimyristoyl-, dipalmitoyl- and distearoylphosphatidylcholine were obtained from both

Abbreviations: DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine.

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Sigma Chemical Co. and Avanti Biochemicals (Birmingham, AL). Lipids from both sources were free of detectable impurities when subjected to thin-layer chromatography ($CHCl_3/CH_3OH/H_2O$, 65:25:4, v/v). However longer small unilamellar vesicle half lives and significantly lower Raman backgrounds were achieved using lipids which had been acetone-precipitated by the latter supplier. Phosphate was determined by the method of Bartlett [8]. For multiple samples, the standard deviation in the assay was $\pm 1\%$.

Small unilamellar vesicles were prepared by sonication and centrifugation above T_m [9]. A well-polished microprobe or the Heat Systems Cup Horn accessory was used. While the latter technique yielded samples with lower Raman background, it was slower and, in our hands, less reproducible than the probe procedure. Barrow and Lentz [10] recently described optimal conditions for use of the Cup Horn accessory.

Our small unilamellar vesicle preparations are more stable when subjected to two stages of sonication with an intervening short centrifugation. In practice the first period of sonication is sufficiently long to result in a transparent blue-tinged solution. This material may be centrifuged immediately at $6900 \times g$ or held overnight above T_m for centrifugation the next morning. In either case, only a short period of sonication of the supernatant (the pellet is discarded) is required to bring the solution to near clarity. Final ultracentrifugation was conducted at $189000 \times g$ for 2.5 h in a swinging-bucket rotor (SW 50.1) which had been heated above the T_m of the lipid used. The result is a clear solution of highly stable small vesicles at a concentration comparable to that of the initial dispersion. All sample transfers were done in an oven, using prewarmed labware. In this way, samples were not taken below T_m until the actual start of a measurement.

All calorimetry was conducted using a Perkin-Elmer DSC-2 differential scanning calorimeter. When used in conjunction with a substantial heat sink (Neslab RTE 8 refrigerated bath), the calorimeter is at thermal equilibrium within 3 min after placing the sample in the instrument. Sample pans consisted of a three part (pan, cover, o-ring) hermetically sealed stainless steel assembly (Perkin-Elmer). Initial experiments showed that 40 μl was the maximum amount of sample and 2.5 K/min the maximum scan rate at which a constant integrated peak area could be maintained. The calorimeter was calibrated for temperature and peak area using a sample of indium in a stainless steel pan. Peak areas were determined using a precision planimeter. In cases in which significant overlap of peaks occurred, the less obscured half of the endotherm was measured and the area doubled.

The protocol for a kinetic measurement was as follows: A measured volume of sample (35 μl for small, or 20 μl for multilamellar vesicles was added to warm preweighed sample pans. Samples of small vesicles were taken from the top of the centrifuge tube. Three such samples were taken, interspersed with three 20-μl aliquots taken for assay. A sample pan was immediately transferred to the calorimeter which had been equilibrated at the holding temperature desired (usually $T_m - 10°C$). A flat isothermal baseline was taken to indicate that the calorimeter had reached equilibrium at which time a scan was initiated. At the conclusion of a scan, the calorimeter was rapidly (40 K/min) returned to the start temperature. The time used to define the kinetic data is the total time the sample was held at the starting or holding temperature; not the total time below T_m. Infinite time data were taken from the same samples after at least a week in the refrigerator. For small vesicles of chain lengths 16 and 18, fusion was sufficiently slow relative to the calorimeter equilibration time that enthalpy measurement could be made directly on populations of pure small vesicles. For DMPC a small endotherm corresponding to fusion product was invariably encountered even at very early times. Consequently DMPC enthalpies were determined by plotting the endotherm areas of the small vesicles and fusion product at various times and extrapolating to zero time (small vesicle area) and infinite time (fusion product area) [1].

Raman spectra were collected on a computer-controlled Spex 14018 double monochromator as previously described [11]. Samples held in melting point capillaries were excited at 5145 Å at an average power of 500 mW.

Results

The endotherm for a freshly prepared sample of pure small vesicles of DPPC is compared with that for the corresponding multilamellar preparation in Fig. 1. The small vesicle endotherm is, as is well known, much broader and occurs at a lower T_m than that for the multilamellar material. What is notable in this scan (taken after less than 5 min at 31°C) is the complete absence of a peak attributable to multilamellar vesicles or fusion product.

When a sample of small vesicles is held below T_m for more than a few minutes, a second endotherm appears at higher temperature which corresponds to the product of vesicle fusion. For DPPC small vesicles (Fig. 2) a fusion peak is evident after only 20 min at 10°C below T_m. It is significant that the sample of small vesicles used for Fig. 1 had been held above T_m for 48 h prior to the calorimetric scan and yet showed no evidence of a fusion product endotherm. With time below T_m the fusion product endotherm grows at the expense of the small vesicle peak, which in turn appears to gradually shift to higher temperature.

Our measurements on pure preparations of DPPC small vesicles yield 6.1 kcal/mol, in good

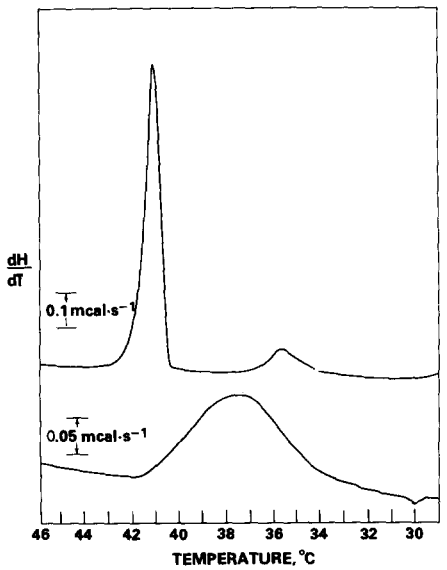

Fig. 1. Endotherms for freshly prepared small unilamellar vesicles of DPPC (lower trace) and the multilamellar dispersion (upper trace) from which the vesicles were prepared. Scan rate 2.5 K/min, sensitivity as indicated in figure.

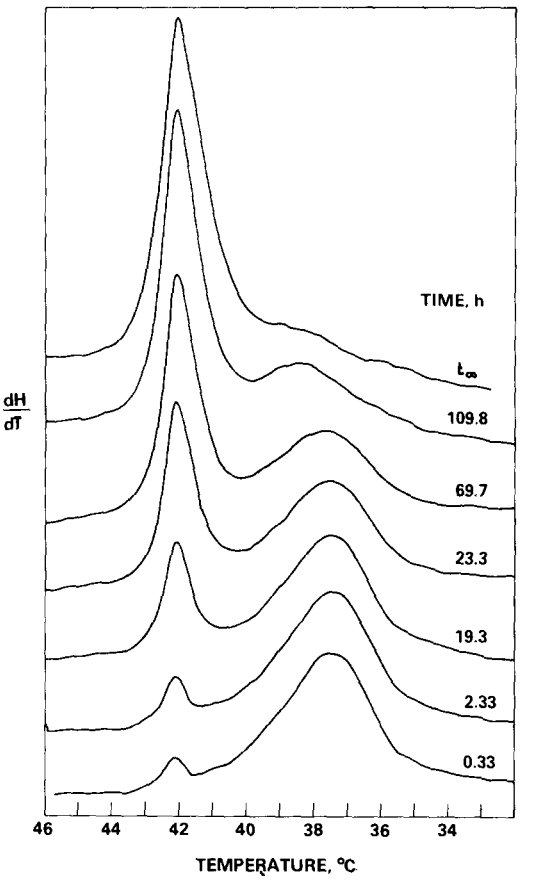

Fig. 2. Multiple endotherms for small unilamellar vesicles of DPPC as a function of time (hours) held at 10°C below T_m. This particular sample has been maintained above T_m for two days prior to the measurement; only a small fusion product endotherm is seen after 20 min at 10°C below T_m.

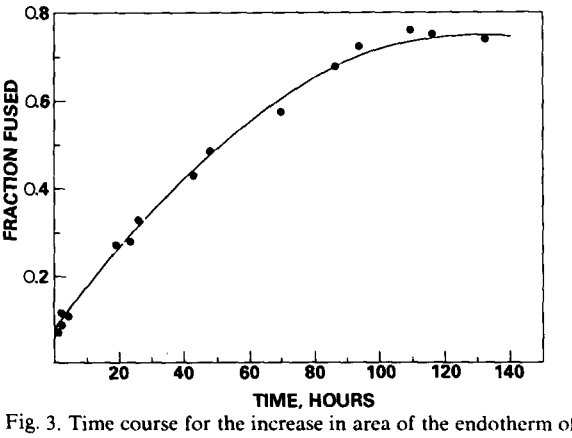

Fig. 3. Time course for the increase in area of the endotherm of the product formed from fusion of DPPC relative to the area obtained for the same sample (t_∞) held for 1 week at 4°C. Time is the period during which the sample was held at 10°C below T_m.

TABLE I

THERMODYNAMIC DATA FOR THE MAIN PHASE TRANSITION OF DMPC, DPPC AND DSPC

SUV, small unilamellar vesicle; LMV, large multilamellar vesicle.

	Temperature			Enthalpy (kcal·mol^{-1})			Entropy (cal·mol^{-1}·deg^{-1})		
	SUV	Product	LMV	SUV	Product	LMV	SUV	Product	LMV
DMPC	20.8	24.4	23.9	3.7	4.56	5.57	12.5	15.3	18.7
DPPC	37.5	41.1	41.4	6.10	7.15	8.68	19.6	22.7	27.5
DSPC	51	54.9	54.9	8.96	8.98	10.67	27.6	27.4	32.5

agreement with the value of 6.3 kcal/mol first determined by Suurkusk et al. [1], who use a procedure in which the peak areas for small vesicle and fusion product are plotted and the data extrapolated to 100% small vesicles. Thermodynamic data for small vesicles, fusion product and multilamellar vesicles as a function of chain length are reported in Table I. To our knowledge this is the first complete set of such data for the series of small vesicles C-14 through C-16. Van Dijck et al. [12] have reported calorimetric data for DPPC and DMPC, however their enthalpy values for both small and multilamellar vesicles are significantly larger than those reported here, while our values for the multilamellar vesicles agree well with those obtained by Mabrey and Sturtevant [13].

For each chain length the transition temperature for small vesicles falls several degrees below that for the multilamellar vesicles; similarily so for the enthalpies. It has been reported [1] that the enthalpy of the DPPC fusion product is identical to that for multilamellar vesicles. To the contrary we find that for all chain lengths the fusion product enthalpies are intermediate between small and multilamellar vesicles. Furthermore for DSPC the enthalpies for small vesicles and fusion product are identical, but lower than the corresponding multilamellar value by approx. 1.7 kcal.

The kinetics generated from the calorimetric experiment are typified by those for DPPC (Fig. 3). The data are characterized by a fairly rapid initial burst followed by a slower monotonic increase in fusion product. A half-life may be estimated from the slow portion of the curve by determining the area of the fusion product endotherm at $t = \infty$. Repeating these experiments for small vesicles chain lengths 14 through 18 results in the following half-lives: DMPC, 13 h; DPPC, 45 h; DSPC, 132 h.

If, as has been suggested [2] fusion is enhanced

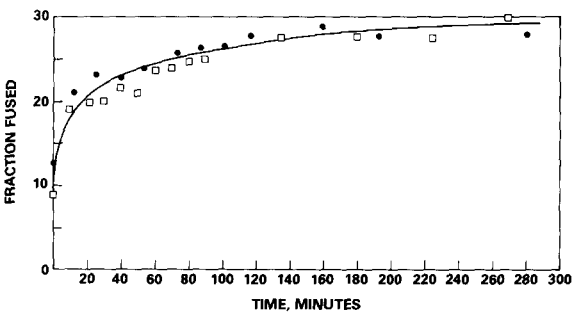

Fig. 4. Time course for the increase in area of the endotherm of the product formed by fusion of DMPC small unilamellar vesicle relative to the area obtained for the same sample t_∞ held for 1 week at 4°C. Solid circles represent a sample held at T_m for the period indicated; open squares are for a sample held at 10°C below T_m.

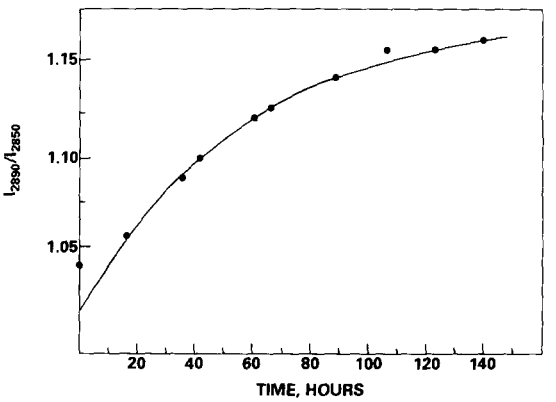

Fig. 5. Increase with time of the ratio of the heights of the Raman peaks at 2890 cm^{-1} and 2850 cm^{-1} for DPPC small unilamellar vesicles held continuously at 10°C below T_m.

TABLE II

LATERAL ORDER PARAMETER FOR SUV AND LMV OF CHAIN LENGTHS 14–20

SUV, small unilamellar vesicle; LMV, large multilamellar vesicle; DAPC, diarchidoylphosphatidylcholine.

Lipid	Chain length	S_{Lat}	
		SUV	LMV
DMPC	14	0.11	0.41
DPPC	16	0.19	0.38
DSPC	18	0.24	0.35
DAPC	20	0.30	0.33

at or above T_m, then the calorimetric determination of kinetic data may lead to spurious results due to multiple passage through T_m. (In fact it has been suggested [12] that repeated cycling through T_m speeds fusion.) We have tested this possibility in two ways. Shown in Fig. 4 are data from two experiments on DMPC; the first taken by holding the sample at 10°C below T_m for the indicated time and the second in which the sample is maintained at T_m prior to scanning. The rate of appearance of fusion product is identical in each case. Note that the half-life for this particular sample is shorter than in our best samples due, we believe, to slight impurities. In a related experiment (data not shown) the time interval between calorimetric scans was varied, thereby altering the number of passes through T_m; again the rate of fusion remained the same.

An independent confirmation of these results was obtained by measuring the change in relative peak heights of the Raman bands arising from the symmetric (2850 cm^{-1}) and asymmetric (2890 cm^{-1}) CH_2 stretching vibrations of the phospholipid acyl chains. These bands are thought to reflect the extent of lateral chain order and known to have significantly different intensity ratios for small and multilamellar vesicles [14]. Throughout the Raman measurements, which were conducted in parallel with the DSC runs, samples were maintained at 10°C below T_m and never cycled through T_m. The time course for the increase in I_{2850}/I_{2890} (Fig. 5) is very nearly the same as that derived from the DSC experiment. The half-life for DPPC small unilamellar vesicle estimated from the Raman data is $\simeq 38$ h compared to 45 h based on DSC. It is noteworthy that I_{2850}/I_{2890} for the 'infinite time' point for the fusion product is lower than that for multilamellar vesicles (1.15 vs. 1.24).

Closer examination of the Raman data permits some physical insight into the influence of chain length on small vesicle stabilization. A parameter S_{Lat}) has been devised [14] which permits normalization of the Raman data on a scale such that n-alkane liquids have $S_{Lat} = 0$ and crystalline alkanes, $S_{Lat} = 1$. Viewed in this way, it has previously been shown [14] that S_{Lat} for DPPC small vesicles differs substantially from the corresponding multilamellar vesicles; thereby demonstrating a difference in chain packing order for the two preparations. These comparisons have been extended to include DMPC and DSPC and the 20 carbon chain length lipid diarchidoylphosphatidylcholine. In each instance the data (Table II) were collected at 10°C below the relevant T_m. The trend with increasing chain length is for the multilamellar values to decrease ~20% while the small vesicle values increase almost 300%. The decreasing multilamellar values are attributable to the higher temperatures at which the longer chain lipids are measured. To correct for the effect of temperature, (which is assumed to be the same for small and multilamellar vesicles) the ratio of S_{Lat} for each was determined. Plotted versus chain length (Fig. 6), the ratio changes monotonically; approaching zero for a chain length of approx.

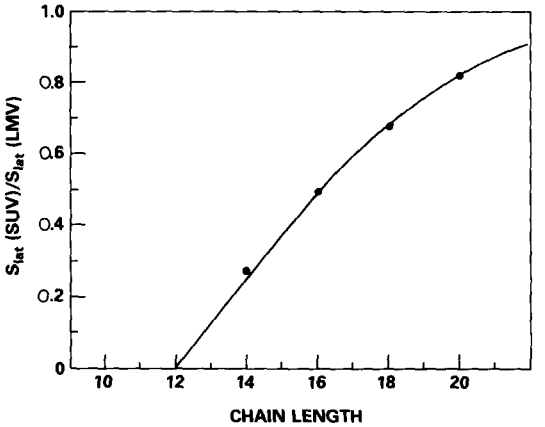

Fig. 6. Chain length dependence of the ratio of the Raman order parameter S_{lat} for small unilamellar vesicle (SUV) and large multilamellar vesicle (LMV); values measured at 10°C below the T_m for each lipid.

11–12 and tending toward 1.0 for chain lengths greater than 20.

Discussion

There is no question that small phosphatidylcholine vesicles formed from saturated fatty acids convert to larger species. What remains to be understood is the nature of the conversion process. The change in the Raman intensity ratio and the appearance of a high temperature endotherm in the DSC confirm that an actual change in bilayer structure occurs; thus the final product formed is unlikely to be simply an aggregate of small vesicles. Although the experiments described here cannot discriminate true fusion with retention of internal contents from a mechanism involving diffusion of single phospholipids, we believe it is reasonable to describe the conversion of small vesicles to product as fusion. A fusion process is suggested by the dye trapping measurements of Schullery et al. [7]. Furthermore, evidence based on photon correlation spectroscopy [15] indicates that the product is a reasonably narrow distribution of particles with a mean size of $\simeq 700$ Å, consistent with recent electron microscopic observations [7].

The product of small vesicle fusion bears superficial resemblance to multilamellar vesicles, but on closer examination it is clearly different. As shown previously by Suurkuusk et al. [1], for DPPC and confirmed and extended here for DMPC and DSPC, the product is characterized by a gel-liquid crystal transition altered slightly from that of multilamellar vesicles both in terms of T_m and transition enthalpy which fall between values for small and multilamellar vesicles, consistent with the view that the gel state of the product species is more stable than that of small vesicles. Further, the Raman lateral order parameter (S_{Lat}) for the fusion product is lower than that for multilamellar vesicles indicating a state of chain packing intermediate between small and multilamellar vesicles.

With regard to the kinetics of fusion, the model -dependent half-life of 5 h estimated by Suurkuusk et al. [1], seems to be an underestimate resulting from assuming the reversibility of the fusion process. Approximately one day may be inferred from dye-trapping data [7] a time more nearly consistent with the approx. 45 h reported here by DSC. For DPPC we have confirmed the DSC-derived half-life by Raman. Using photon correlation spectroscopy [15] to examine a more dilute solution the value obtained is about 67 h. Thus the half-lives reported here appear to be the longest yet observed.

Increased chain length clearly results in a significant enhancement of small vesicle stability toward fusion. A 10-fold increase in small vesicle half life is seen in progressing from DPMC to DSPC; acyl chain length clearly plays a significant role in determining small vesicle fusion kinetics. The enhanced stability against fusion is, of course, not the result of the relief from curvature-induced strain; the increase in small vesicle radius due to addition of four carbons is at most approx. 10%.

The Raman data (Fig. 6) show that increased chain-chain interaction accompanies increasing chain length. For chains greater than 20 carbons the ratio of the values of S_{Lat} approaches unity. In other words the extent of chain-chain packing interaction is nearly the same for small and multilamellar vesicles at long chain lengths. With decreasing length, the ratio extrapolates to zero at 12 carbons, indicating a chain order for small vesicles comparable to that of a liquid hydrocarbon. Thus small vesicles of longer chain length are demonstrably more stable.

Two factors, in addition to chain length, appear to be important in stabilizing small vesicles against fusion: lipid purity and maintaining the sample above T_m. Several studies [2,3,5] have examined the effect of putative fusogens. It has been suggested [15] that micellar by-products are produced by sonication which are not readily separated from small vesicles by centrifugation. In our experience, lipid samples for which the multilamellar vesicles yield slightly broadened endotherms or do not exhibit a well-defined pretransition invariably fuse rapidly. The preparative protocol we have adopted, requiring two sonications and an intervening low speed centrifugation does seem to play a role in vesicle stability. Although we have not attempted to vigorously validate the idea, we believe the enhanced stability may result from sequestering of low levels of fusogenic material by fusion product formed during the first sonication.

Occasionally, preparations of small vesicles are encountered which are anomolous. Some prepara-

tions of DSPC have shown endotherms with multiple peaks and even transitions above the T_m for the multilamellar vesicles. With DMPC the anolomy results in an extremely broad endotherm and curiously stable vesicles. These factors indicate that the population consists of vesicles of intermediate size rendering them more stable against fusion and producing the appearance of a broadened endotherm. A broad (17°C) DPPC endotherm has been reported for small vesicles in which electron microscopy indicates a polydisperse population with sizes extending well beyond 1000 Å [2]. These observations may explain reports [17] of purported DMPC small vesicles which do not exhibit a lowered T_m. Recalling Fig. 2, we note that the course of fusion is marked not only by the development of the narrow endotherm of the ultimate fusion product, but by an upward shift in the broad peak characteristic of the small vesicles. It is reasonable to suppose that the shift results from formation of intermediate species which lie along the path to the final fusion product.

In our experience properly prepared small vesicles retain their integrity for at least two days when maintained above T_m. Neither passage through T_m nor holding at T_m seems to change the kinetics as measured by both DSC and Raman. On the other hand, it has been reported [3] that acidic phospholipids become sensitive to Ca^{2+}-induced fusion above T_m. Evidently the factors governing fusion of neutral lipids differ, in some respects from those involved in Ca^{2+} acidic lipid systems. Once exposed to temperatures below T_m, small vesicles begin fusion, the rate of which is slowed but not terminated by holding above T_m. It would appear that fusion must be nucleated; a process favored below T_m. There is growing evidence that aggregation proceeds [15] and this could be the initiating step.

In summary we find that with careful attention to sample purity and preparative protocol, the fusion rates of small vesicles range from hours to days depending on chain length. Increasing acyl chain length results in vesicles of greater stability toward fusion. A possible physical basis for this stabilization may be the increased effectiveness of chain packing demonstrated by Raman spectroscopy. The product of fusion is different calorimetrically and in terms of its Raman properties from both small and multilamellar vesicles.

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