Evidence for the "Cold Fusion" of Bilayer Membranes

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Received: July 10, 1998; In Final Form: October 14, 1998

Polymerization of the lipid headgroups inhibits calcium-induced fusion of small unilamellar vesicles of the lipid di-*n*-dodecyloxypropyl *beta*-nitrostyryl phosphate but does not influence vesicle aggregation. Addition of a copolymer of lauryl methacrylate and acrylamide (LMPAM) provides the vesicles with a steric shield that prevents both fusion and aggregation. Accurate microcalorimetric determination of the enthalpies of vesicle aggregation and fusion was possible by comparison of titrations of vesicles into $CaCl_2$ in the absence and presence of LMPAM, both before and after polymerization of the lipids in the vesicles. Whereas calcium-induced aggregation is associated with an enthalpy of $+2.6 \pm 0.1$ kJ/mol of lipid, fusion occurs with a minimal endothermic heat effect. We contend that the driving force of membrane fusion is of entropic origin.

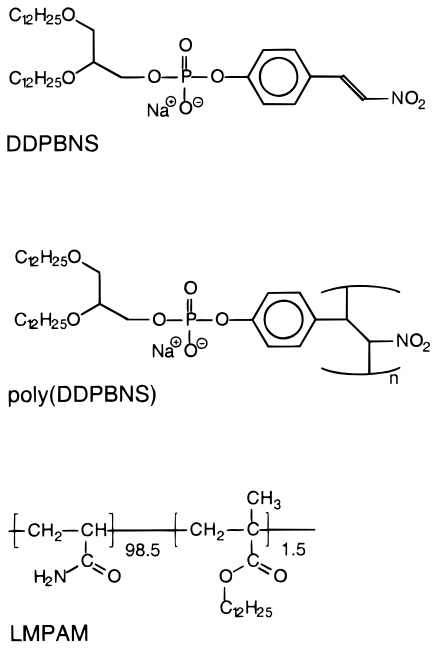
Introduction

A better understanding of membrane fusion poses a challenge to bioscientists, because of both the fundamental importance of membrane fusion as a primordial biological event and its relevance in the development of trans-membrane transport vehicles for medical purposes, such as gene therapy. A consistent picture of the molecular rearrangements that occur during fusion of lipid bilayers is slowly emerging.[1] Analogous models for calcium-induced membrane fusion have been proposed.[2] The bulk of the research on membrane fusion currently is focused on biological events such as exo- and endocytosis and viral infection and the role of the fusion protein machinery that is involved.[3]

A fundamental perspective of membrane fusion that clearly has remained underdeveloped both theoretically and experimentally is a thermodynamic consideration of the process. This is perhaps even more surprising in view of the extensive thermodynamic descriptions of shape transformations of bilayers, which have been framed in the spontaneous curvature model of Helfrich[4] and the bilayer-coupling model proposed by Svetina and Zeks.[5] The stalk−pore model of membrane fusion is partly based on calculations of the Gibbs energy of formation of hypothetical fusion intermediates from planar lipid bilayers.[1b] Thus, the model provides some insight into the activation parameters of membrane fusion, albeit in vacuo, but it does not elucidate the thermodynamic driving force(s) of membrane fusion. Papahadjopoulos and co-workers[6] reported the experimental enthalpy of the reaction between phosphatidylserine liposomes and the divalent calcium ion, but the heat effect is a combined result of calcium ion−lipid binding, phase transitions in the bilayer, and liposome aggregation and fusion. Recently, a microcalorimetric analysis of the fusion between liposomes and the influenza virus indicated a rather strongly endothermic enthalpy of membrane fusion.[7] Unfortunately, the authors did not elaborate on the potential entropic driving force for the endothermic process or on the role of specific protein−lipid interactions.

We report experiments with the purpose of determining the enthalpy of calcium-induced fusion in a model system composed of unilamellar vesicles of a polymerizable lipid (di-*n*-dodecyloxypropyl *beta*-nitrostyryl phosphate, DDPBNS),[8] in either the absence or presence of a copolymer of lauryl methacrylate and acrylamide (LMPAM) that efficiently anchors into the hydrophobic interior of the bilayer[9] (Chart 1). In this model system, the complexities of the biological fusion event have been reduced to three distinguishable processes: calcium ion binding, vesicle aggregation, and bilayer fusion. Admittedly, this is a gross simplification of in vivo membrane fusion, but we contend that the results of our conceptually simple approach are of general significance. Membrane mimetic systems have proven extremely useful in this respect.[10]

CHART 1



Small unilamellar vesicles (SUVs) of DDPBNS were prepared by sonication. The main phase transition (T_m) of the DDPBNS bilayer occurs at −1 °C (DSC). Photopolymerization of DDPBNS is achieved by brief UV irradiation and yields oligo-

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10.1021/jp9829748 CCC: $15.00
Published on Web 11/26/1998

(styrene) derivatives.[8] The size, morphology, and integrity of the vesicles are not affected by the polymerization.[8,11] The main phase transition becomes less cooperative, but T_m is essentially unaffected.[8] The vesicles become less permeable as a result of polymerization.[11] The inhibitory effect of lipid oligomerization on calcium-induced fusion of lipid vesicles of comparable composition has been studied in detail.[11] Also, the interaction of LMPAM and small vesicles of negatively charged lipids has been described.[9] We note that at the low concentrations used in this study (<0.25% w/v) depletion interaction due to the polymer can be ignored.

The present study provides experimental evidence that calcium-induced vesicle fusion can be dissected into successive steps of calcium ion binding, vesicle aggregation, and bilayer fusion by means of lipid photopolymerization and the coating of the vesicles with a hydrophobically modified polymer. The enthalpy of each of the successive steps of the fusion process was measured by ultrasensitive titration microcalorimetry and provides insight into the thermodynamics of calcium-induced vesicle fusion.

Experimental Section

Materials and Vesicle Preparation. DDPBNS, poly(acrylamide) (PAM) and LMPAM were synthesized as described.[8,12] The molecular weight of LMPAM and PAM is estimated as 18 200 (viscosimetry), and LMPAM carries an average of four lauryl substituents per molecule (^{1}H NMR). Small unilamellar vesicles of DDPBNS were prepared by sonication of thin lipid films in 5 mM HEPES/NaAc buffered solutions, and polymerization of DDPBNS vesicles was induced by UV irradiation.[8,11] Coating of the DDPBNS vesicles with LMPAM was achieved by a 40/1 DDPBNS/polymer molar ratio mixing of appropriate solutions of vesicles and polymer (a small aliquot of a concentrated aqueous polymer solution was added to a solution of vesicles in buffer).

***n*-Octadecyl Rhodamine Assay of Lipid Mixing**. The *n*-octadecyl rhodamine (R18) assay of lipid mixing[13] was performed as described.[11] DDPBNS vesicles without fluorescent label (20 μM) were added to 5 μM of DDPBNS vesicles with 2 mol % *n*-octadecyl rhodamine in 2 mL of 5 mM HEPES/NaAc buffer (pH 7.4, 140 mM NaCl added) at 25 °C. No spontaneous probe exchange was observed. LMPAM or PAM was added at a DDPBNS/polymer ratio of 40/1. The addition of LMPAM resulted in a small instantaneous increase of fluorescence (3−5%), presumably reflecting the insertion of the hydrophobic anchor into the vesicle bilayers; PAM had no effect on R18 fluorescence. The addition of either polymer did not result in spontaneous exchange of the R18 probe. Fusion was induced by the addition of 1−10 mM of $CaCl_2$, and after the fluorescence increase had stopped, calcium ion was removed by addition of 4 equiv of EDTA. Fluorescence before the addition of $CaCl_2$ was taken as 0% fusion (F_0), and the extent of mixing (F_{obs}) was measured after the addition of EDTA. The value for complete fusion (F_{100}, complete lipid mixing, i.e., 1/5 surface dilution of R18) was calculated from the fluorescence at infinite dilution (F_{inf}) upon the addition of 0.5% w/v of Triton X-100. F_{obs} and F_{inf} were corrected for volume changes. The extent of fusion for each experiment was calculated as extent (%) = $(F_{obs} - F_0)/0.8(F_{inf} - F_0) \times 100\%$. The initial rate of fusion was calculated from the tangent of the increase of fluorescence versus time and was expressed in percentage of fusion per second. Fluorescence was recorded in stirred 2-mL cells using an SLM Aminco SPF-500C spectrophotometer with 560-nm excitation and 590-nm emission wavelengths.

Turbidity Assay of Vesicle Aggregation. Aggregation of small unilamellar vesicles of DDPBNS was monitored as an increase in turbidity upon the addition of 5−10 mM of $CaCl_2$. The concentration of DDPBNS was 0.1 mM in 5 mM HEPES/NaAc (pH 7.4), and the molar ratio of DDPBNS to polymer was 40/1. Turbidities were measured under stirring at 25 °C as the absorbance at 500 nm in 2-mL cells in a Philips PU 8740 UV/vis spectrophotometer.

Electron Microscopy. Samples of vesicle solutions in 5 mM HEPES/NaAc buffer (pH 7.4, [DDPBNS] = 0.5 mM) were stained with 1% uranyl acetate and examined in a Philips EM 300 electron microscope operating at 80 kV.

Isothermal Titration Microcalorimetry. Titration microcalorimetry was performed by injecting 10-μL aliquots of the vesicle solution into the sample cell containing a $CaCl_2$ solution (1.37 mL). From 10 to 12 injections were done at 5-min intervals. The sample cell was stirred at 350 rpm. Integration of the heat flow vs time yields the enthalpy for each injection. The concentration of DDPBNS in the syringe was 5.0 mM in 5 mM HEPES/NaAc (pH 7.4), and the concentration of $CaCl_2$ in the cell was 5.0 mM in 5 mM HEPES/NaAc (pH 7.4). Polymers were added to the contents of the syringe as well as to the contents of the cell at a 40/1 DDPBNS/polymer molar ratio before the titration experiment. The instrument was an Omega isothermal titration microcalorimeter (Microcal Inc.; Northampton, MA) and was operated at 30 °C. Before the titration experiments, the sonicated DDPBNS solutions were stored at room temperature for about 10 days. The solutions were briefly vortexed directly before use.

Results and Discussion

Separation of Calcium Ion Binding, Vesicle Aggregation, and Bilayer Fusion. The fusion of SUVs of DDPBNS was monitored using the R18 assay for lipid mixing.[13] In view of the observation that the R18 assay disagrees with other fusion assays under certain conditions, we refer to the Experimental Section for further details on this assay and to previous work[11] which warrants its use. As shown in Figure 1, the vesicles fuse readily upon the addition of 3−10 mM $CaCl_2$. After polymerization of the lipids, fusion is largely inhibited. The effect is particularly striking at low calcium ion concentrations.[11] In the presence of a low concentration of LMPAM, no calcium-induced lipid mixing is observed at all. The minimal concentration of LMPAM required to inhibit lipid mixing corresponds to a molar ratio of DDPBNS to LMPAM of 40/1. In contrast, PAM of comparable molecular weight does not affect fusion at these concentrations.

Aggregation of the SUVs was measured as an increase of turbidity upon the addition of $CaCl_2$. The rates of aggregation of the vesicles before and after polymerization of DDPBNS are equal. Whereas aggregation is not influenced by the nonanchoring PAM, aggregation is completely inhibited if LMPAM is added to the vesicles in a 40/1 DDPBNS/polymer ratio (Figure 2).

Samples of SUVs of DDPBNS in the absence and presence of LMPAM and PAM before and after the addition of $CaCl_2$ were examined in the electron microscope (Figure 3). In the absence of polymer, extensive vesicle fusion was evident from an up to 10-fold increase in the average vesicle diameter upon the addition of $CaCl_2$. Similar observations were made in the presence of nonanchoring PAM. However, fusion is blocked in the presence of LMPAM (40/1 DDPBNS/polymer). Aggregation but no fusion is observed upon the addition of $CaCl_2$ to vesicles of polymerized DDPBNS.[11]

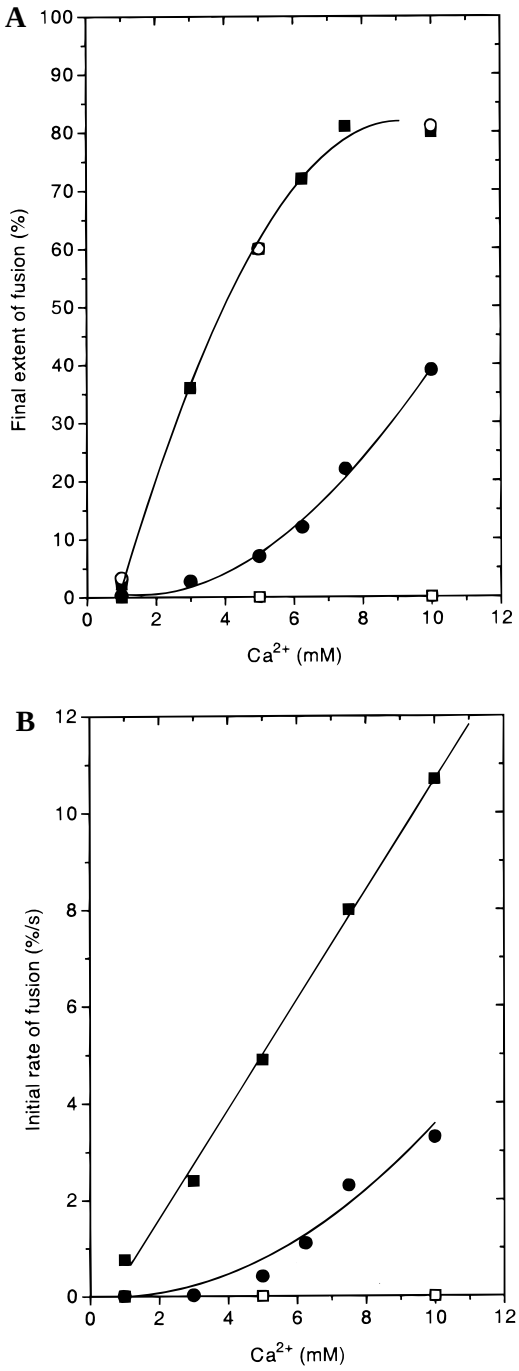

Figure 1. *n*-Octadecyl rhodamine assay of lipid mixing during calcium-induced fusion of small unilamellar vesicles of DDPBNS. The extent of lipid mixing is shown in part A; the initial rate of mixing is shown in part B: solid squares, DDPBNS; solid circles, poly(DDPBNS); open circles, DDPBNS + PAM; and open squares, DDPBNS + LMPAM.

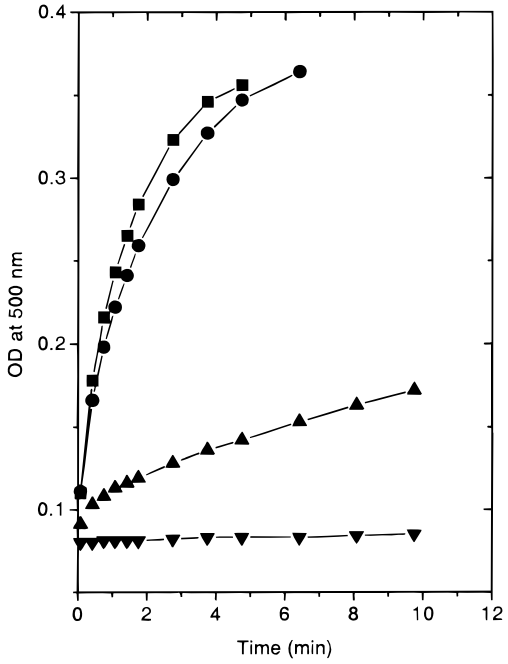

Figure 2. Aggregation of small unilamellar vesicles of DDPBNS upon the addition of $CaCl_2$ (10 mM) monitored as an increase in turbidity: solid squares, DDPBNS; solid circles, poly(DDPBNS); solid up triangles, DDPBNS with 5.0 mM $CaCl_2$, and solid down triangles, LMPAM-coated DDPBNS and LMPAM-coated poly(DDPBNS).

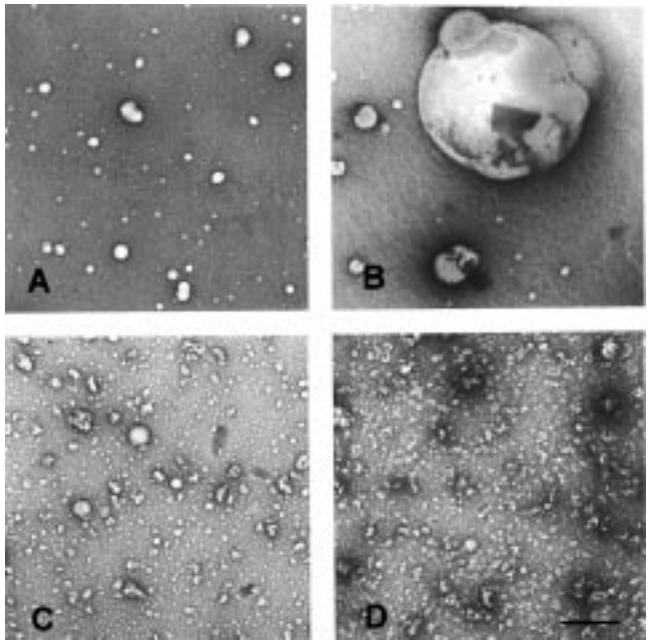

Figure 3. Micrographs of small unilamellar vesicles of DDPBNS in 5 mM HEPES/NaAc buffer (pH 7.4, [DDPBNS] = 0.5 mM); (A) Small vesicles of DDPBNS. (B) Large vesicles of DDPBNS that result from calcium-induced fusion of small vesicles. (C) Small vesicles of DDPBNS in the presence of LMPAM (DDPBNS/LMPAM = 40/1). (D) After addition of LMPAM, calcium-induced aggregation and fusion of the vesicles are completely inhibited. Bar represents 250 nm.

As reported previously,[11] lateral diffusion of the polymerized lipid molecules over the bilayer surface is slow relative to diffusion of the monomers, and fusion is strongly inhibited. In addition, unfavorable bilayer curvature effects upon lipid polymerization may play a role.[11] However, calcium-induced aggregation of the vesicles is not affected by polymerization. LMPAM provides the vesicles with a steric shield that prevents both aggregation and fusion, even at high calcium ion concentrations. Similar observations have been described for liposomes containing a small percentage of poly(ethylene glycol)-derived lipids, as well as for lipids substituted with various other water-soluble polymers.[14] PAM has no hydrophobic moiety and is unable to anchor into the vesicle bilayer.[9] Consequently, it lacks the inhibitory effect of LMPAM on aggregation and fusion.

In sum, lipid mixing assays, turbidity measurements, and electron microscopy (as well as contents leakage assays and quasi-elastic light scattering)[11] provide consistent evidence for a three-step calcium-induced fusion of small vesicles of DDPBNS, that can be stopped at any of the three successive stages. At room temperature, vesicles of DDPBNS fuse ef-

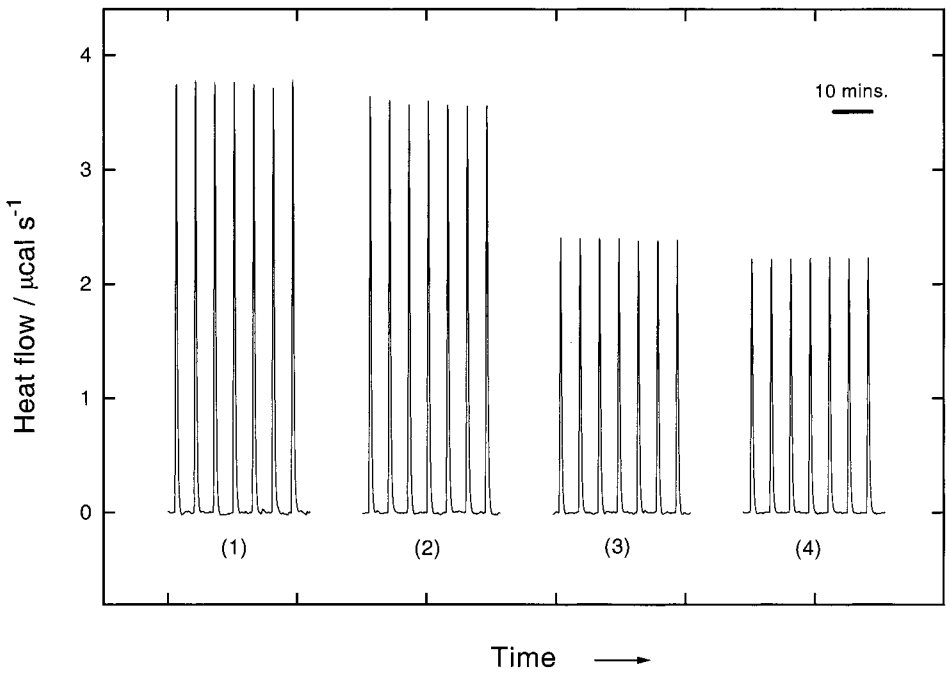

Figure 4. Enthalpograms corresponding to the titrations of small unilamellar vesicles of DDPBNS (5 mM lipid) into 5 mM $CaCl_2$; (1) Unpolymerized vesicles. (2) Polymerized vesicles. (3) Unpolymerized vesicles coated with LMPAM. (4) Polymerized vesicles coated with LMPAM.

ficiently at a total lipid concentration of 25−200 μM and a calcium ion concentration of 5.0 mM. After polymerization of the lipid headgroups in the vesicles, the vesicles bind calcium ions and aggregate, but they do not fuse. If the vesicles are coated with LMPAM, the vesicles bind calcium ions but do not aggregate or fuse.

Titration Microcalorimetry of Calcium-Induced Vesicle Fusion. To assess the thermodynamics of bilayer aggregation and fusion, we carried out titration experiments in a highly sensitive titration microcalorimeter. In these titration experiments, 10-μL aliquots of a sonicated dispersion of SUVs (5.0 mM DDPBNS) were injected into 1.37 mL of a 5.0 mM aqueous $CaCl_2$ solution at 30 °C, and the traces of heat flow vs time were integrated to obtain the corresponding enthalpies of reaction. It is important to note that under these conditions aggregation and fusion are rapid processes (Figures 1 and 2), and indeed, in all cases, the baseline was reached before the next aliquot was injected (Figure 4). The time scale of the observed heat effect matches the kinetics of vesicle aggregation and bilayer fusion. No systematic dependence of the observed enthalpy per injection on the injection number was found (Figure 4). As a result, each experiment affords an accurate heat of reaction which was taken as the average for 12 injections. A 10-day maturing period of the vesicles after sonication is critical to obtain reproducible results. No spontaneous polymerization or degradation of DDPBNS occurs during this period, and provided the vesicle solution is briefly vortexed before the fusion experiments, the extent and rate of aggregation and fusion do not change.

Here for the first time, we dissect the observed heat effect into three well-defined processes: calcium ion binding, vesicle aggregation, and the ultimate merging of the bilayers. In comparison, the thermodynamic analysis of virus−protein-induced membrane fusion is much more complicated.[7] The enthalpy accompanying the titration of vesicles of unpolymerized lipid into a $CaCl_2$ solution reflects not only aggregation and fusion but also calcium ion binding to the phosphate headgroups and any concomitant ion dehydration effects. Similarly, the titration of vesicles of polymerized lipid into a $CaCl_2$ solution yields a reaction enthalpy which solely includes aggregation and calcium ion binding to the polymerized phosphate headgroups. However, we do not anticipate that calcium ion binding to polymerized and unpolymerized bilayers is thermodynamically equivalent, and the simple subtraction of the two heat effects is not likely to afford the true enthalpy of vesicle fusion.

This problem can be solved by employing the macromolecular hydrophobic anchor LMPAM, which allows for independent measurements of the calcium ion binding effects. In the presence of this polymer, aggregation and fusion are fully blocked for both polymerized and unpolymerized lipids, and any differences in reaction enthalpies reflect differences in calcium ion binding only. Through the use of this approach, a reliable value for the enthalpy of fusion can be calculated. This value does not include enthalpic effects caused by the dilution of the polymer or calcium−LMPAM interactions because the polymer has been added to the contents of both the syringe and the sample cell. The enthalpy of aggregation is obtained by subtracting the heat effects accompanying the titration of LMPAM-coated polymerized vesicles into a $CaCl_2$ solution *(binding only)* from the enthalpy for the titration of polymerized vesicles into a $CaCl_2$ solution of the same molarity *(aggregation + binding).* This approach is illustrated in Figure 5, and Table 1 summarizes the results.

Thermodynamics of Calcium Ion Binding, Vesicle Aggregation, and Bilayer Fusion. The data show that calcium ion binding to the phosphate headgroups is endothermic by 7.0 kJ/mol of DDPBNS before polymerization and 6.5 kJ/mol of DDPBNS after polymerization. This value is lower than the heat effect accompanying binding of calcium ions to phosphate ions in aqueous solutions which is endothermic by, on average, 11.5−13 kJ/mol.[15] Calcium−phosphate ion binding is driven by the release of hydration water,[16] and the difference can be explained by the facts that the phosphate headgroups are less accessible to bind calcium ion at the vesicle surface and that they are already partially dehydrated. It is likely that binding of calcium ion to polymerized DDPBNS phosphate headgroups is slightly less endothermic than binding to monomer DDPBNS

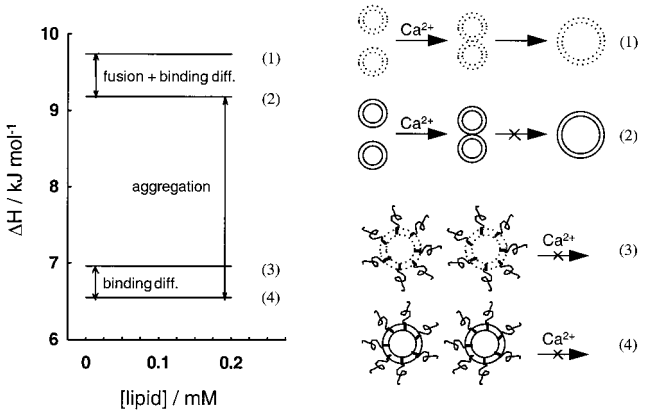

Figure 5. Dissecting enthalpies of aggregation and fusion from calcium ion binding effects: a schematic representation. Unpolymerized bilayers are represented by dotted lines, and polymerized bilayers are represented by solid lines. Numbering corresponds to Table 1 and Figure 4.

TABLE 1: Enthalpies of Calcium-Induced DDPBNS Vesicle Aggregation and Fusion[a]

vesicle	description	enthalpy, kJ/mol of DDPBNS
1	fusion, aggregation, binding to mono(DDPBNS)	9.74 ± 0.11
2	aggregation, binding to poly(DDPBNS)	9.18 ± 0.10
3	binding to mono(DDPBNS)	6.96 ± 0.13
4	binding to poly(DDPBNS)	6.55 ± 0.13
2−4	aggregation	2.6 ± 0.1
(1−2) − (3−4)	fusion	0.15 ± 0.1

[a] Numbers correspond to Figure 5.

headgroups, because the polymerized headgroups pack more closely and are slightly more dehydrated relative to the monomer headgroups.[11] Endothermic, entropy-driven binding of calcium ions to phosphatidylcholine/phosphatidylglycerol liposomes has been reported.[17]

Vesicle aggregation is endothermic by 2.6 ± 0.1 kJ/mol of DDPBNS (Table 1). Vesicle aggregation is probably the result of the endothermic but entropically favorable release of hydration water upon close approach of adjacent bilayers. Increased counterion binding of the lipid headgroups most likely will contribute to this effect.[18] Because calcium-induced aggregation is a spontaneous process, the driving force is entropic in origin.

Vesicle fusion is associated with an enthalpy of only 0.15 ± 0.1 kJ/mol of DDPBNS (Table 1).[19] It is known that reduction of bilayer curvature promotes formation of larger vesicles from small ones: vesicle fusion proceeds much more readily if the "reactant" vesicle size remains well below 100 nm.[20] Assuming that bilayer curvature becomes negligible, the Gibbs energy of fusion can be estimated to be −225 kJ/mol of vesicle,[21] i.e., approximately −0.0225 kJ/mol of lipid for bilayer vesicles of 10 000 negatively charged lipids. We note that vesicle fusion is exergonic, in accord with a spontaneous process. Because we find that bilayer fusion per se is associated with a minimal endothermic enthalpy effect ("cold fusion"), *entropy* should provide the driving force for fusion of bilayer membranes. A key question now is how the relief of curvature strain is expressed at a molecular level. The enthalpy associated with the gel-to-liquid crystalline bilayer phase transition is known to become more endothermic as the vesicle size increases, indicating a concomitant increase in lateral packing efficiency of lipid molecules.[22] There is further calorimetric evidence that relief of bilayer curvature is exothermic.[23] If this were the dominant factor in vesicle fusion, the formation of large vesicles from smaller ones would be enthalpically favorable. This is not consistent with the present results. Therefore, we contend that the entropic driving force for fusion is provided by (1) the increase of the number of (translational, rotational, and undulational) modes of freedom of the lipid molecules in going from aggregated clusters of small vesicles to conformationally less restricted, larger fusion products and (2) a release of hydration water molecules from the lipid headgroups as the bilayer curvature is relieved and the headgroup packing efficiency increases. Finally, it should be noted that the enthalpy loss upon fusion of pure lipid membranes is an order of magnitude smaller than that observed for protein-mediated virus−liposome fusion (2.5−3.0 kJ/mol of viral lipid).[7] This suggests that the enthalpy of mixing of protein-rich virus and pure lipid membrane[24] dominates the thermodynamics of virus−liposome fusion whereas lipid rearrangements most likely contribute marginally to the overall enthalpy of fusion.

Acknowledgment. We are grateful to Prof. A. D. R. Brisson and Mr. J. F. L. van Breemen for their hospitality in the Laboratory of Electron Microscopy.

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