Biochimica et Biophysica Acta, 688 (1982) 275–278
Elsevier Biomedical Press

BBA Report

BBA 70037

THE RATE OF FUSION OF PHOSPHOLIPID VESICLES AND THE ROLE OF BILAYER CURVATURE

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(Received December 7th, 1981)

Key words: Fusion rate; Phospholipid; Bilayer curvature

Kinetics of Ca^{2+}-induced fusion of phosphatidylserine vesicles is studied for lipid concentrations varying from 1 μM to 100 μM. Fusion is monitored by mixing of aqueous vesicle contents and by explicitly accounting for leakage. The analysis provides separately rates of aggregation and fusion. The rate of fusion per se decreases steeply with vesicle size.

In the study of the molecular mechanisms of membrane fusion, phospholipid vesicles, as relatively simple model systems, are being used extensively [1,2]. Recently several fusion assays have been developed which monitor the mixing of vesicle contents within the encapsulated aqueous volume of the fusing vesicles [3,4]. The kinetics of fusion of phospholipid vesicles has been followed [3,5] over a time range of seconds to minutes, to examine the initial stages, e.g. dimer formation, which are of most interest from the biological point of view.

Fusion of phospholipid vesicles consists of at least two distinct but kinetically coupled stages. First, vesicle aggregation, resulting in a close apposition of their surfaces and second, fusion of the membranes, involving local bilayer destabilization and resulting in the mixing of the encapsulated aqueous contents.

Recently, a theoretical mass action kinetic model has been developed which describes the time course of aggregation of colloidal particles in general and of phospholipid vesicles in particular [6–8]. The analysis [6] of Ca^{2+}-induced fusion of small unilamellar vesicles of phosphatidylserine (PS) provided the rates of the overall fusion process. It was concluded [6] that under certain conditions, the overall rate of Ca^{2+}-induced fusion of PS vesicles is limited by the rate of aggregation, and not by the fusion step per se. A similar conclusion was reached [5] by studying the initial rates as a function of lipid concentration.

Here results of Ca^{2+}-induced fusion of PS vesicles are presented for a wide range of vesicle concentrations. The analysis of these data in terms of the mass action kinetic model provides for the first time the rates of fusion per se and indicates their dependence on vesicle size.

The kinetics of fusion is followed with an assay for mixing of aqueous vesicle contents [3,5]. $Tb(citrate)_3^{6-}$ is encapsulated in one population of vesicles and a 10- or 20-fold molar excess of the sodium salt of dipicolinic acid in another. Fusion of the vesicles results in the fast formation of the fluorescent $Tb(dipicolinic\ acid)_3^{3-}$ complex, which can be monitored continuously. EDTA (0.1 mM) and Ca^{2+} (> 1 mM) prevent the formation of the above complex in the external medium. The experiments employed both PS sonicated (250 Å diameter) and large unilamellar vesicles (diameter

1000 Å) [9,10]. Vesicles were prepared from bovine brain PS, as described previously [5]. Fusion was initiated by addition of 100 mM $CaCl_2$ directly to the cuvette to a final concentration of 5.0 mM and 2 mM in suspensions containing large and small vesicles, respectively.

Due to leakage of vesicle contents during the fusion a small fraction of the Tb complex may be released to the external medium. As described previously [6], the measured Tb fluorescence can be corrected for this release by independently measuring the kinetics of the release of carboxyfluorescein [3,5]. The leakage is initially quite small [5] and for large vesicles under the conditions treated here it never amounts to more than 10% of the encapsulated material. The Tb fluorescence, corrected for this leakage, is denoted $\%Tb^{corr}$. If all of the vesicles were to fuse to doublets then $\%Tb^{corr}$ would be 50% since only one-half of these doublets would contain both Tb and dipicolinic acid. The other half would contain only one component, e.g., Tb, and would not contribute to the observed fluorescence.

The mass action model we employ contains all reactions of the form

$$V_1 + V_1 \xrightarrow{C_{11}} V_2 \xrightarrow{f_{11}} F_2 \tag{1.1}$$

$$\begin{array}{l} V_1 + V_2 \xrightarrow{C_{12}} V_3 \\ \qquad\qquad\quad \Big\downarrow f_{11} \\ V_1 + F_2 \xrightarrow{C'_{12}} V_1F_2 \xrightarrow{f_{12}} F_3 \end{array} \tag{1.2}$$

and so on for aggregate-fusion products composed of four, five and larger numbers of vesicles. Here V_1 denotes the original vesicle monomer and V_i denotes an aggregate of i unfused vesicles. F_2 denotes a fused doublet and F_i denotes the fusion product of i vesicles. Likewise, V_1F_2 denotes an intermediate product of a monomer adhering to a fused doublet. Although our primary interest is the initial dimerization reaction (1.1), we had to consider higher orders as well [6–8]. The time development of the distribution of fused vesicles is given by the concentration of fused doublets (F_2, V_1F_2), triplets (F_3, V_1F_3), etc. [6]. Numerical integration of the mass action kinetic equations given by reactions (1.1), (1.2) and higher orders provides these concentrations. C_{11} is the rate constant for the initial dimerization reaction and f_{11} is the rate constant for the fusion of the preformed dimer. In order to minimize the number of adjustable parameters, we have set the higher order aggregation rate constants (C_{ij} and C'_{ij}) equal to C_{11} and the higher order fusion rate constants (f_{ij}) equal to f_{11}. This simplification is justified since we are only analyzing the initial stage of the process where the fluorescence is primarily due to fused doublets, the higher fusion orders contributing less than 10%. We have also assumed that the reverse reactions of dissociation are negligible, which is justified by both our earlier findings [6] and the evident agreement with the experimental data which cover a range of two orders of magnitude in lipid concentration.

In Figs. 1 and 2 the experimental data for $\%Tb^{corr}$ are shown (by symbols) for several PS concentrations and the corresponding theoretically predicted curves are given. The rate of aggregation C_{11} was determined by the fluorescence data for 1 μM PS, where the aggregation is rate limiting [6]. The value for f_{11} was then determined by choosing the value which gave the best fit to the data for the higher vesicle concentrations. Since the data can vary (between experiments) by ± 1 unit in $\%Tb^{corr}$, we find that $f_{11} = (8 \pm 4) \cdot 10^{-2}\ s^{-1}$ and $C_{11} = (6.5 \pm 1.0) \cdot 10^7\ M^{-1} \cdot s^{-1}$ for large vesicles in 5 mM Ca^{2+}. For fusion of small vesicles induced by 2 mM Ca^{2+} in 100 mM Na^+ the value of C_{11} is $4.5 \cdot 10^7\ M^{-1} \cdot s^{-1}$, while f_{11} is $5 \pm 3\ s^{-1}$. The predicted curves track the experimental points quite well.

Since for times of interest the aggregation process is second order in vesicle concentration whereas the fusion is first order, it can be anticipated that in very dilute vesicle concentrations the overall fusion rate is limited by the rate of aggregation. With an increase in vesicle concentration, X_0, the rate of aggregation increases more rapidly than the overall rate of fusion. A detailed analysis indicates that the quantity which determines whether aggregation or fusion will be rate limiting is $K = f_{11}/C_{11}X_0$. When $K \gg 1$ the aggregation is rate limiting. For instance, for 1 μM of lipid, $X_0 = 1.2 \cdot 10^{-11}$ M (large vesicles), so that $K = 100$. In

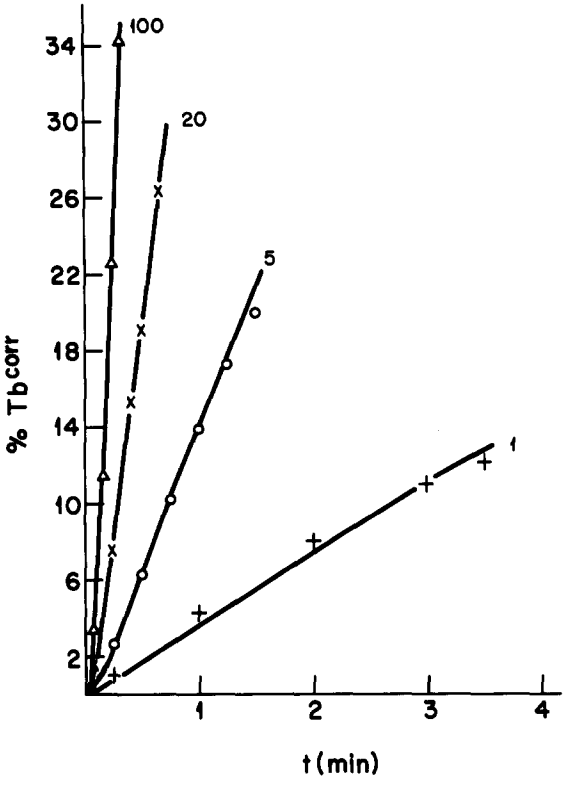

Fig. 1. Fusion of large PS vesicles at various concentrations induced by 5 mM Ca^{2+}. The theoretical curves (solid lines) are shown for the time development of the Tb fluorescence. Corresponding experimental results corrected for the release of vesicle contents as described before [6] are shown as data points. X_0, the molar concentration of vesicles, was calculated from the lipid concentration using a number of 80000 lipid molecules per vesicle. With each curve PS concentration (μM) is shown. The data for 2, 10 and 50 μM PS were predicted equally well by the theory, but were omitted for clarity. The vesicles were made in either (a) 2.5 mM $TbCl_3$/50 mM sodium citrate (b) 50 mM dipicolinic acid (sodium salt)/20 mM NaCl or (c) 50 mM carboxyfluorescein (sodium salt). In addition all media contained 2 mM L-histidine and 2 mM *N*-tris (hydroxymethyl)methyl-2-aminoethanesulfonic acid (Tes), adjusted to a final pH of 7.4. Vesicles were separateted from non-encapsulated material by gel filtration on Sephadex G-75 and assayed for lipid phosphorus content [5]. Measurements were carried out, as described before [5], with an SLM-4000 fluorimeter in a final volume of 1.0 ml 100 mM NaCl, 2 mM L-histidine, 2 mM Tes, 0.1 mM EDTA, pH 7.4. The temperature of the sample holder was maintained at 25°C and the solution in the cuvette was stirred magnetically. Tb- and dipicolinic acid-containing vesicles were present in a 1 : 1 ratio based on lipid phosphorus. In all cases, the fluorescence scale was calibrated to the total amount of Tb present [5].

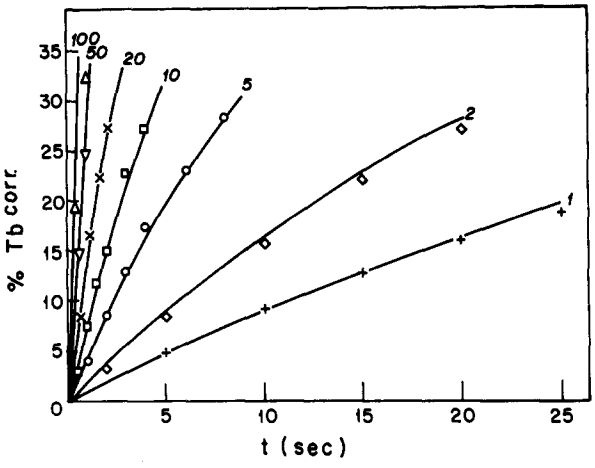

Fig. 2. Fusion of small PS vesicles at various concentrations induced by 2 mM Ca^{2+}. Experimental data are taken from Ref. 5. With each curve PS concentration (μM) is shown.

this case aggregation is rate limiting. However, when the lipid concentration is 100 μM then $K = 1$ and the fusion per se limits the rate of increase of $\%Tb^{corr}$. In fact, the Tb fluorescence develops over 3-times more slowly than would be anticipated if aggregation were still limiting the overall reaction.

The reverse deaggregation reaction whose rate constant is D_{11} can be neglected whenever (i) $D_{11} \ll C_{11} X_0$ which means that dissociation is (initially) much slower than association, or (ii) $D_{11} \ll f_{11}$, which means that a dimer fuses before it has a chance to dissociate. For the small vesicles previous Na^+-induced aggregation studies [7,8] give a range of values of 0.2 to 0.02 s^{-1} for D_{11}, which means that $D_{11} \ll f_{11}$. The value of D_{11} for the large vesicles in 5 mM Ca^{2+} can be at least two orders of magnitude smaller than for the small vesicles in 2 mM Ca^{2+} [7]. Hence, the neglect of deaggregation reaction is fully justified in this study, but it will have to be considered in other cases such as neutral phospholipid vesicles, or PS at low Ca^{2+} concentrations.

In order to clarify how the measured fusion rate constant relates to the time course of the fusion event per se, let us examine the situation wherein the vesicles are preaggregated into dimers. When the fusion starts from this initial state, then the time rate of change of the concentration of un-

fused dimers is initially

$$\frac{d[V_2]}{dt} = -f_{11}[V_2] \quad (2)$$

while that of the fused doublets is

$$\frac{d[F_2]}{dt} = f_{11}[V_2] \quad (3)$$

When $f_{11}t \ll 1$ these kinetic equations can be solved to yield

$$[F_2] \simeq [V_2(0)]\, f_{11} t \quad (4)$$

where $[V_2[0)]$ is the initial concentration of dimers. Consider the small PS vesicles in 2 mM Ca^{2+} where $f_{11} \approx 5\ s^{-1}$. 1% of the dimers will have fused within only 2 ms, i.e. $t = f_{11}^{-1}[F_2]/[V_2(0)] = (0.2\ s) \cdot (0.01) = 2$ ms. The time required for 1/1000 of the dimers to fuse is 200 μs. Clearly we can anticipate that the values of the fusion rates f_{11} will increase with higher Ca^{2+} concentrations. It was already pointed out [11,12] that membrane instability increases with the amount of Ca^{2+} or Mg^{2+} bound to PS.

Earlier work showed that smaller vesicles have an intrinsically greater capacity to fuse than larger vesicles [5] and the curvature effect on vesicle fusion has been previously noted in other vesicles [13,14]. With the rigorous fusion model applied in this study, it becomes possible to relate this effect of bilayer curvature to the actual fusion rate constants. We believe that the bilayer destabilization [1] is the key step in vesicle fusion and that the 'strain' imposed on the strongly curved bilayer of a small vesicle [15] facilitates this destabilization. This curvature effect is clearly indicated for PS vesicles in Mg^{2+}. The large vesicles aggregate extensively, but do not fuse (i.e. $f_{11} = 0$), whereas the small ones do fuse under these conditions [16].

J.B. and S.N. acknowledge the support of NIH Grants GM-23850 and CA-16056. The experimental work presented in this paper was carried out by J.W. in the laboratory of Dr. D. Papahadjopoulos, UCSF, and supported by NIH Grant GM-28117 and the Netherlands Organization for the Advancement of Pure Research (Z.W.O.). We wish to thank Mrs. Karin van Wijk for expert technical help.

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