

# Kinetics of Calcium-Induced Fusion of Cell-Size Liposomes with Monolayers in Solutions of Different Osmolarity

Natalia Stoicheva, Iana Tsoneva

Central Laboratory of Biophysics, Bulgarian Academy of Sciences, Sofia 1113, Bulgaria

Dimitar S. Dimitrov\*

Department of Chemical Engineering, Carnegie-Mellon University, Pittsburgh, PA 15213, USA

and Ivan Panaiotov

Department of Physical Chemistry, University of Sofia, Sofia 1126, Bulgaria

Z. Naturforsch. **40c**, 92–96 (1985); received July 16, 1984

Membrane Fusion, Giant Liposomes, Phospholipid Monolayers

The effects of osmolarity, calcium concentration and cell-size liposomes in the subphase on the surface tension of phospholipid monolayers were investigated. The monolayers were spread from chloroform solutions of phosphatidic acid at air/water solution interface. The liposomes (of average diameter 3  $\mu\text{m}$ ) were formed from phosphatidic acid/egg lecithin (1:2) mixtures in water or 0.1 M water solutions of sucrose.

For this system there were critical concentrations of calcium ions to produce a large reduction of the monolayer surface tension. The threshold calcium concentrations depended upon the sucrose concentration in the subphase. Without sucrose the threshold calcium concentration was 8 mM, while for isoosmotic sucrose solutions (0.1/0.1 M in/out of liposome) it was 14 mM. It sharply increased to 28 mM  $\text{CaCl}_2$  at sucrose concentration difference across the liposome membrane 0.02 M and decreased to 26 mM, 19 mM, and 18 mM with further increase of that difference to 0.04 M, 0.06 M, and 0.08 M, respectively.

The rate of monolayer surface tension decrease was measured as a function of time at 30 mM  $\text{CaCl}_2$  and different sucrose concentrations in the subphase solution. The initial rates at first decreased with increasing the osmotic pressure and after that they increased. The minimum occurred at sucrose concentration gradient across the liposome membrane 0.02 M, *i.e.*, at the point of maximum threshold calcium concentration required for large decrease of the monolayer surface tension. These facts may be explained by recent theories of dynamics of adhesion, instability and fusion of membranes modeled as thin films.

## Introduction

Membrane fusion is a fascinating phenomenon, involved in basic biological process [1–3]. Investigations on model systems, such as monolayer, bilayer or multilayer lipid membranes, have given much of our knowledge on membrane fusion. Fusion, induced by calcium or other divalent cations, is one of the phenomena, which has been thoroughly studied, both for its relevance to biological systems and for understanding the underlying physicochemical mechanisms [4–9].

Recently, an approach, based on the physical chemistry of thin films and surfaces, has been developed to describe the kinetics of membrane adhesion, instability and fusion [10–17]. One of the

basic results was that the rate of membrane approach and the occurrence of membrane instability (in particular membrane rupture and rupture of the film between membranes) strongly depends on the membrane tension.

From experimental point of view fusion of liposomes with monolayers has a basic advantage that the rate of the fusion process can be easily monitored by measuring the monolayer surface tension [8, 9]. Ohki and Duzgunes [8] studied in detail divalent cation-induced interaction of small phospholipid vesicles with monolayers. They found that for several lipid systems (*e.g.*, phosphatidylserine/phosphatidylcholine vesicles and phosphatidylserine monolayer) there are critical concentrations of divalent cations to produce a large reduction in surface tension of the monolayers. In a previous work [9] we found similar phenomena for cell-size liposomes from egg lecithin/phosphatidic acid (2:1) and monolayers from phosphatidic acid at air-water interface in water and sucrose solution subphase.

\* *Permanent address:* Central Laboratory of Biophysics, Bulgarian Academy of Sciences, Sofia 1113, Bulgaria.

Reprint requests to N. Stoicheva.

0341-0382/85/0100-0092 \$ 01.30/0



It was concluded in both works [8, 9] that the large reduction in surface tension can be due to fusion of liposomes with monolayers.

The basic goal of this work is to investigate the effect of osmotic pressure gradients across the liposome membrane on the occurrence and rate of fusion of cell-size liposomes with monolayers. The osmotic pressure gradients are induced by changing the osmolarity (the concentration of sucrose) of the medium surrounding the liposomes. They result in an increase of the membrane tension, which should affect the rate of membrane adhesion and fusion [10, 14]. These experiments have shown that this is the case and quantitatively accounted for that effect, providing a basis for future theoretical computations.

## Materials and Methods

Phosphatidic acid (Koch-Light Lab. Ltd.) showed a single spot on silica gel thin-layer chromatographic plates. L- $\alpha$ -lecithin was also from Koch-Light Lab. Ltd. The calcium salt ( $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ) was Merck purest quality. The conductivity of the double-distilled water was  $2 \times 10^{-6} \Omega^{-1} \text{cm}^{-1}$ , pH-6. Sucrose (Riedel) was used without further purification.

Monolayers were formed from phosphatidic acid at air/water solution interface of fixed area ( $63.6 \text{ cm}^2$ ) in a glass dish. The monolayer was spread from spectroscopically pure chloroform (Merck) until the surface tension reached the value  $67 \text{ dyn/cm}$ . This corresponds to approximately  $0.95 \text{ nm}^2$  area per molecule. The surface tension was measured with an electronic microbalance (Beckman) with Teflon plate ( $10 \times 20 \times 1 \text{ mm}$ ) as a Wilhelmy plate. Without vesicles comparative measurements of the surface tension were also performed with glass plates. The value measured by the Teflon plate is proportional to that measured by the glass plate. For pure water those values are  $22 \text{ dyn/cm}$  and  $72.5 \text{ dyn/cm}$ , respectively. Unless stated specifically, the values further presented are those measured by the Teflon plate and are proportional to the surface tension, but not equal. The use of Teflon plate was necessary to avoid glass/vesicle interactions which lead to erotic output of the microbalance.

Cell-size liposomes were prepared following a recently developed method [18] and its modification

[19]. 1 mg phosphatidic acid and 2 mg L- $\alpha$ -lecithin were dissolved in 1 ml chloroform. The solvent is then evaporated under vacuum. Double-distilled water or 0.1 M sucrose solution was added to swell the dried lipid for 3 h in a  $65^\circ \text{C}$  water bath. Gentle shaking of the glass flask for a few seconds resulted to formation of liposomes of diameters from 1 to  $10 \mu\text{m}$ . Figure 1 shows the size distribution of giant liposomes obtained by this procedure in 0.1 M sucrose dilution. Similar hystogrammes were also found for liposomes prepared in water and in sucrose solutions of different osmolarity. It should be pointed, however, that the average diameter of liposomes formed in water is larger and the size distribution wider than for those formed in sucrose [9].

The experimental protocol included the following steps:

1. Measurement of the surface tension with glass plate and Teflon plate before adding liposomes and spreading the monolayer.
2. Liposome suspension is added and stirred.
3. The monolayer is spread.
4. After establishment of an equilibrium surface tension calcium salt of increasing concentrations is injected.
5. After establishment of the final equilibrium surface tension EDTA is added.

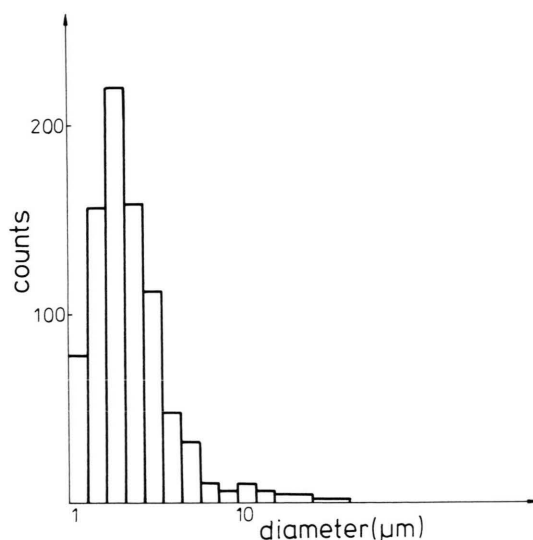


Fig. 1. Size distribution of liposomes in 0.1 M sucrose. The most probable diameter is  $3 \mu\text{m}$ .

The surface tension is recorded continuously during all these steps. The experiments were performed at 20 °C.

## Results and Discussion

Figure 2 shows typical experimental data for the case of 0.1 M sucrose solutions in and out of the liposomes, *i.e.*, for isoosmotic conditions. It is seen that the surface tension remains almost constant after adding liposome suspension and waiting for 25 min. The spreading of the monolayer causes a decrease of the surface tension. After that it remains constant until the concentration of the added calcium salt reaches a critical value of 14 mM. The surface tension did not change if only monolayer or only liposomes were present at any calcium concentration up to 30 mM. No changes of the surface tension were found in the liposome/monolayer system without calcium. Adding EDTA after the large change of the surface tension had occurred had no effect, *i.e.*, the process was irreversible.

This threshold calcium concentration was found to depend upon the concentration of the sucrose solution in the subphase. We kept the sucrose con-

centration in the liposomes constant and equal to 0.1 M, while decreasing it in the medium surrounding the liposomes by 0.02, 0.04, 0.06, and 0.08 M. For those values of the sucrose concentration difference across the liposome membranes the calcium threshold concentrations were 28 mM, 26 mM, 19 mM, and 18 mM, respectively. Another experiment was performed, where double-distilled water was in and out of the liposomes. The threshold calcium concentration was 8 mM. This may be due to the larger diameter and wider size distribution of liposomes in pure water than in sucrose solutions [9].

The very interesting result is that at first the calcium threshold concentration increases with increasing the imposed osmotic pressure gradients, respectively increasing the membrane tension. At sucrose concentration difference 0.02 it has a maximum value. Further increase of the osmotic pressure gradients (here assumed to be proportional to the sucrose concentration difference and the membrane tension), however, leads to a decrease of the calcium threshold concentration.

Similar phenomenon was observed for the kinetics of surface tension change after the threshold point. Figures 3 through 4 show the rate of surface tension

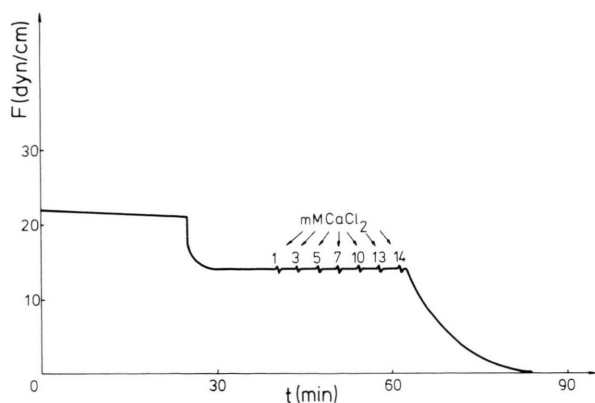


Fig. 2. Dependence of the upward force (which is proportional to the surface tension), exerted on a Teflon plate by a phosphatidic acid monolayer in presence of phosphatidic acid/L- $\alpha$ -lecithin vesicles and calcium in 0.1 M sucrose solutions, on time. At time zero the vesicles were added. After 25 min the monolayer was spread from chloroform solutions. Within another 15 min the injection of calcium chloride began at 1 mM concentration. The calcium concentration was increased until a threshold level of 14 mM was reached after which a large reduction of the surface tension occurred in approximately 15 min.

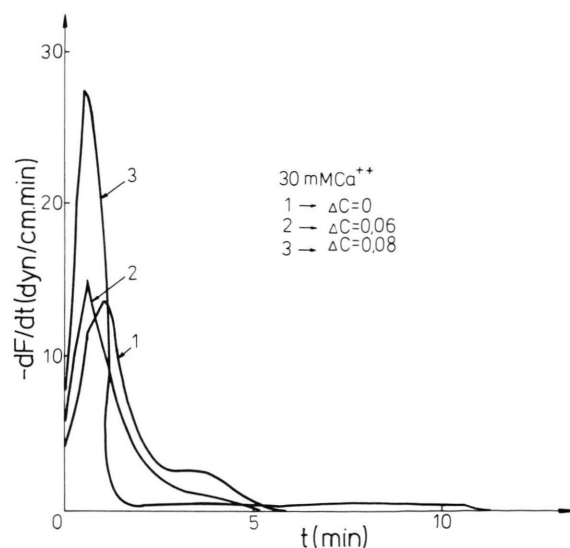


Fig. 3. Dependence of the rate of upward force change upon time at 30 mM calcium salt concentration and isoosmotic conditions in a 0.1 M sucrose solution. Rate of upward force change vs. time for sucrose concentration difference across the liposome membrane  $\Delta C = 0.06$  M. Rate of upward force change vs. time for sucrose concentration difference across the liposome membrane  $\Delta C = 0.08$  M.

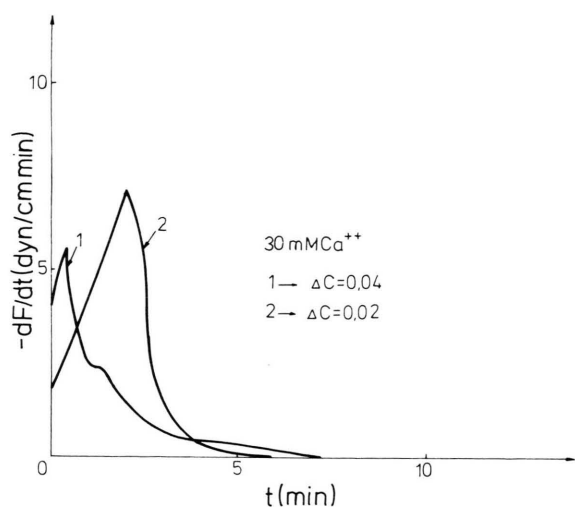


Fig. 4. Rate of upward force change vs. time for sucrose concentration difference across the liposome membrane  $\Delta C = 0.04$  M. Rate of upward force change vs. time for sucrose concentration difference across the liposome membrane  $\Delta C = 0.02$  M.

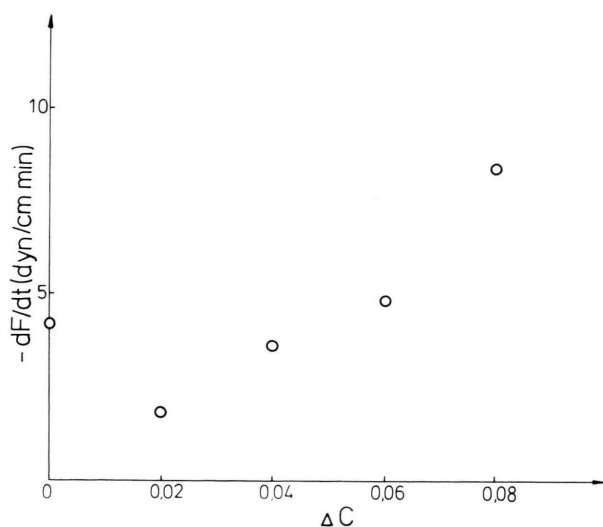


Fig. 5. Dependence of the initial rates of upward force change upon the sucrose concentration difference. It is seen the sharp minimum at  $\Delta C = 0.02$  M.

change as a function of time at different (increasing) osmotic pressure gradients, 0, 0.02, 0.04, 0.06, and 0.08, respectively, and calcium concentration 30 mM. Figure 5 shows the initial rates of surface tension change as a function of the sucrose concentration

difference  $\Delta C$ . The most interesting result is the minimum of the initial rate at  $\Delta C = 0.02$  M.

The maximum of the threshold calcium concentration and the minimum of the initial rate of surface tension change are at the same sucrose concentration difference  $\Delta C = 0.02$ . This fact can mean that the operating mechanism for both processes (threshold-like occurrence of the surface tension change and the initial stages of liposome/monolayer interaction) is the same.

The existence of extremum may indicate at least two effects, which play significant role in the liposome/monolayer interaction (suppose it is fusion). We may hypothesize that at zero membrane tension ( $\Delta C = 0$ ) the contact area between the liposome and the monolayer is large and favor the development of the instability of the membrane system due to attractive long-range intermolecular forces. The increase of the membrane tension leads to increase resistance to membrane deformation. The liposome tends to keep its spherical shape, which leads to smaller value of the attractive long-range intermolecular forces than in the case of zero membran tension. (It should be pointed, however, that the hydrodynamic resistance when a sphere approaches a flat surface is smaller than for the case of mutual approach of plane surfaces [10].) So larger calcium concentration are necessary to induce membrane fusion. This statement seems also supported by the fact that the larger liposomes in distilled water need smaller threshold calcium concentrations. In addition, larger contact area may lead to larger fluctuations of membrane shape, resulting in destabilization at smaller calcium concentrations. Further increase of the osmotic pressure (membrane tension, respectively), however, leads to destabilization of the membranes themselves. This favors membrane fusion at lower calcium concentrations. The same mechanism may operate for the initial rate of membrane fusion. Destabilization of the liposome membrane may also be responsible for the observation that osmotic swelling of liposomes causes them to fuse with a planar bilayer membrane [20].

It is interesting to point out that the maximum rate of surface tension decrease depends upon the sucrose concentration difference and has a minimum value at  $\Delta C = 0.04$  M. This fact may indicate that during fusion other effects begin to manifest themselves.

The above hypothesis needs serious verifications. Currently, in progress are our theoretical computations, based on the thin film model of membrane systems [10–17], with the basic goal to describe our experimental data and those of others [8, 20–22] on related phenomena.

#### *Acknowledgements*

Useful discussions with Dr. N. Duzgunes, Dr. J. Bentz, Dr. D. Papahadjopoulos, S. Taneva, D. Zhelev, R. Mutafchieva, and M. Angelova are highly appreciated.

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