ELSEVIER

Biochimica et Biophysica Acta 1195 (1994) 31–38

BBA Biochimica et Biophysica Acta

Electrofusion of cell-size liposomes

Natalia G. Stoicheva [1], Sek Wen Hui *

Biophysics Department, Roswell Park Cancer Institute, 666 Elm Street, Buffalo, NY 14263, USA

Received 13 December 1993; revised 19 April 1994

Abstract

Cell size liposomes of egg phosphatidylcholine (PC), *trans*-acylated egg phosphatidylethanolamine (PE), bovine brain phosphatidylserine (PS) and egg phosphatidylglycerol, suspended in 2.5% of polyethylene glycol (M_r 8000), Ficoll (M_r 400 000) or Dextran (M_r 71 200) were aligned by dielectrophoresis and fused by applying a 1.7 kV/cm pulse of varying duration. Because the internal and external media of liposome suspension can be controlled, and the surface charge is known, the results can be described mathematically. The fusion yields (FY) at different pulse length were measured by microscopy. The FY curves were sigmoidal, with a common minimally required pulse length of 19 μs, and shifted to longer pulse length with increasing external media conductivity or vesicle surface charge. The types of polymer in solutions had little effect. The minimal required pulse length was interpreted to be the minimum rise time for the smallest vesicle capable of reaching the bilayer breakdown potential induced by the pulse, and the sigmoidal shape FY curves represented the cumulative vesicle size distribution curve. The shifting of FY curves upon changing media conductivity or vesicle surface charge were quantitatively accounted for by the balance of pulse-induced dipole-dipole attraction and electrostatic repulsion. Deviation from sigmoidal shape FY curves in the cases of charged liposomes was explained by increasing electrostatic repulsion due to vesicle deformation under pulse-induced dipole pressure. The data confirm the hypothesis that membrane potential breakdown is a pre-requisite or minimum requirement for electrofusion, and support our earlier proposition that pulse-induced dipole force plays an important role in the electrofusion process, and that electrostatic repulsion posts additional barrier to electrofusion.

Keywords: Electrofusion; Liposome; Phospholipid; Fusion; Dielectrophoresis; Membrane potential

1. Introduction

Electrofusion is a new tool in cell biology and cell genetics. Compared with other cell-fusion techniques, electrofusion is simple, rapid and highly efficient. The molecular mechanism of this technique has been a subject of intensive studies. A key question in the fusion mechanism is the force involved in the fusion process. While the intermembrane force between fusing cell partners is difficult to assess because many factors are involved, forces between lipid bilayers are relatively well defined. Electrofusion between cell size liposomes offers a simpler model to study membrane factors influencing electrofusion efficiency.

Cell-size liposomes have been used as a model to study different phenomena like aggregation, bilayer domains [1] and other morphological changes [2], Ca-induced fusion of cell-size liposomes with monolayers [3], as well as gross shape changes [4,5]. However, very few studies in electrofusion of cell size liposomes have been reported. An early work of Buschl et al. [6] used polymerized liposomes as a model in electrofusion. More recently, large liposomes were used to study the influence of electric field on the membrane integrity and its permeabilized state [7]. No studies on the effects of the lipid composition or the forces between lipid bilayers in electrofusion have been reported.

The goals of this work are to investigate the effect of different lipid compositions, hence their different interacting forces on liposome electrofusion. We also investigated the influence of contribution of pulse-in-

* Corresponding author. Fax: +1 (716) 8458683. E-mail: roswhui-@ubvms.cc.buffalo.edu

[1] Permanent address: Central Laboratory of Biophysics, Bulgarian Academy of Sciences, Acad. G. Bonchev Str. 21, 1113 Sofia, Bulgaria.

SSDI 0005-2736(94)00177-4

duced dipole force in electrofusion, and the low concentrations of water soluble polymers such as PEG, as compared to Ficoll and Dextran, on electrically induced liposome fusion.

2. Materials and methods

2.1. Chemicals

Egg phosphatidylcholine (PC), *trans*-acylated egg phosphatidylethanolamine (PE), and bovine brain phosphatidylserine (PS) in chloroform were obtained from Avanti Polar Lipids. (Alabaster, AL). Egg phosphatidylglycerol (PG) in chloroform was obtained from Sigma (St. Louis, MO). Ficoll Type 400 (M_r 400 000), dextran (M_r 71 200), and polyethylene glycol (PEG M_r 8000) were obtained from Sigma. Sucrose, chloroform and sodium chloride were obtained from J.T. Baker (Phillipsburgh, NJ). All chemicals were of analytical grade. All water used was doubly-distilled in an all-glass apparatus.

2.2. Cell-size liposome preparation

Cell-size liposomes were prepared following a method in [8] with some modifications. Briefly, 3 mg of lipid was dissolved in 2 ml chloroform. The solvent was then evaporated under vacuum. 20 ml of 0.5 mM NaCl solution was added to swell the dried lipid for 4 h in a 70°C water bath. Gentle shaking of the glass flask for a few seconds resulted in the formation of liposomes of diameters up to 50 μm. It should be noted, however, that the average diameters of liposomes formed from different lipids were slightly different. At these conditions the percentage of 'unilamellar' and bigger-size liposomes (see Results) were higher for charge lipids, compared to uncharged ones. In order to create different conductivity outside and inside the liposomes, they were washed and collected by centrifugation at 12 000 $\times g$ for 20 min and resuspended in 1 mM sucrose or other polymers as stated.

2.3. Conductivity measurement

All conductivity measurements of the external media were performed on an Electro-Mark Analyzer (Markson, Del Mar, CA). The values generally fell between (0.860 ± 0.005) μS/cm and (20.0 ± 0.5) μS/cm. The conductivity inside the liposomes was (58.5 ± 0.5) μS/cm, the conductivity of 0.5 mM NaCl solution. According to the conductivity measurements, the liposomes were stable (no leak) within 30 min of vesicle suspension, i.e., there was no changes in outside conductivity.

2.4. pH measurement

pH measurements were performed on a Beckman 040 pH Meter. Since all the electrofusion experiments were made in low conductivity medium without buffer, it was necessary to know the changes in pH during the experiment. It was found that in the case of unbuffered solution, pH was changed after 20 min. The change for solutions of 100 μl and 20 ml volume at room temperature was ΔpH = 0.07 and ΔpH = 0.05, respectively.

2.5. Osmolarity effects

In order to ensure that the liposomes were not under the osmotic stress during experiments we measured the osmolarity of media used. The osmolarity was measured by a Wescor (Logan, UT) 5100B osmometer. According to these measurements there was no differential osmolarity effects if we use 2.5% solutions of Ficoll 400 000 Dextran 71 200, PEG 8000, or 1 mM sucrose in the external media. The osmotic effects on liposome morphology were visible at more then 5% of the polymer solutions.

2.6. Negative staining electron microscopy

A drop of the specimen was placed on a Formvar-carbon coated grid (treated with 0.01% Bacitracin). The bulk of the sample was removed with filter paper while applying a 2% ammonium molybdate stain for 10 s. The grids were air dried and examined in a Hitachi H-600 Electron Microscope at a magnification of 60 000.

2.7. Electrofusion protocol

Electrofusion measurements were made in an Interdigitated chamber [9]. The contact between liposomes was established by application of an alternating electric field with a frequency of 200 kHz and amplitude from 160 to 320 V/cm for different experiments. This a.c. field induced dielectrophoretic motion and pearl-chain formation of cell-size liposomes.

A high voltage pulse generator (Model 345, Velonex, Santa Clara, CA) was used to supply the rectangular high voltage pulses. Single electric pulse with strength 1.7 kV/cm and duration varying from 19 μs to 240 μs was used for each experiment. The applied ac and dc fields across the fusion chamber were monitored on a digital storage oscilloscope (Iwatzu OS-6121). All fusion experiments were visualized by phase microscopy (Olympus IMT-2). The volume of the fusion chamber was 100 μl. For electrofusion measurements, 50 μl of liposome suspension plus 50 μl of 5% Ficoll, 5% Dextran, or 5% PEG solutions were used.

Control electrofusion measurements were done in order to estimate if there is a difference in fusion yield, using two different electrofusion protocols. (1) Fresh liposome suspension was used in each trial, i.e., liposomes have not been subject to shorter pulses before. (2) the liposome suspension was not changed after each trial with shorter, non-fusing pulses. Fusion yield was calculated for each given liposome suspension and pulse duration. It was found that there was no significant difference (i.e., within the experimental error) in fusion yield (FY) between these two protocols. Therefore all subsequent experiments were done by the second protocol (i.e., vesicle may or may not have been subjected to previous, shorter non-fusing pulses prior to a single, fusing pulse).

2.8. Calculation of fusion yield

The fusion yield was calculated the following way. During the application of ac field, the liposomes were arranged in dielectrophoretic (DEP) chains. Before applying the rectangular pulses, the number of liposome/liposome contacts in a given area were counted. After pulsation, the remaining unfused contacts within the same area were counted again. The fusion yield was given by the following formula:

$$FY\% = (1 - N_{uf}/N_o) \times 100 \quad (1)$$

where: FY, fusion yield; N_{uf}, number of contacts after the pulse; N_o, number of all contacts before the pulse.

For all experiments we used the same liposome concentration. This concentration was chosen to satisfy the condition, such that DEP chains were not excessively long. To be more consistent, we counted N_{uf} and N_o only within the distance 70 μm from the electrodes.

3. Results

According to the procedure used for the preparation of unilamellar cell-size liposomes, it was also possible to obtain multilamellar cell-size liposomes with more than 4–5 bilayers. We observed by electron microscopy these liposomes in order to differentiate 'unilamellar' and 'multilamellar' vesicles. We found that most liposomes with diameter up to 10–15 μm had no more than 3–4 bilayers. Those liposomes with less than 3–4 bilayers were difficult to discern under phase contrast microscope, and were only visible by adjusting the refractive index of the suspension medium. These vesicles were regarded as 'unilamellar'. They were less stable in the fusion process.

In all experiments, 1 mM sucrose, 5% of PEG (M_r 8000), Ficoll (M_r 400 000) or Dextran (M_r 71 200) were added to the external medium, to adjust the osmolarity to that of the internal content of the liposomes, and to increase the contrast for microscopic observation. The difference in refractive indices between the internal content of the liposomes (salt solution) and the external dilute solution of the polymers was sufficient to view 'unilamellar' vesicles which were difficult to distinguish from the background even with phase contrast (Fig. 1). It was important to obtain good microscopic images on which all fusion data were based.

To align PC or PE liposomes by DEP force, we used an ac field at a frequency of 200 kHz and amplitude

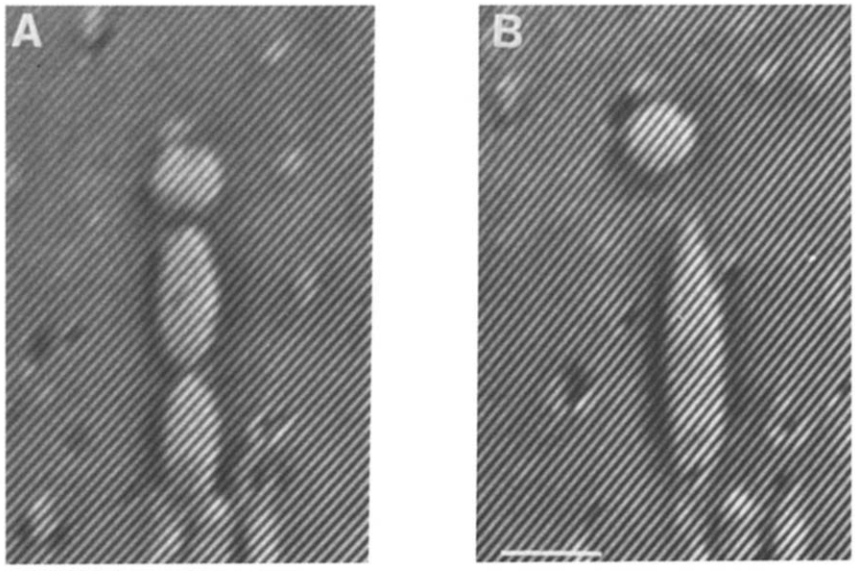

Fig. 1. Phase-contrast micrographs of cell-size PS liposomes in 2.5% Ficoll 400 000. (A) Liposomes are aligned using an a.c. field of amplitude 240 V_{pp}/cm and frequency 200 kHz. (B) Same liposomes are fused using a pulse field strength 1.7 kV/cm and duration 70 μs. (Bar = 10 μm).

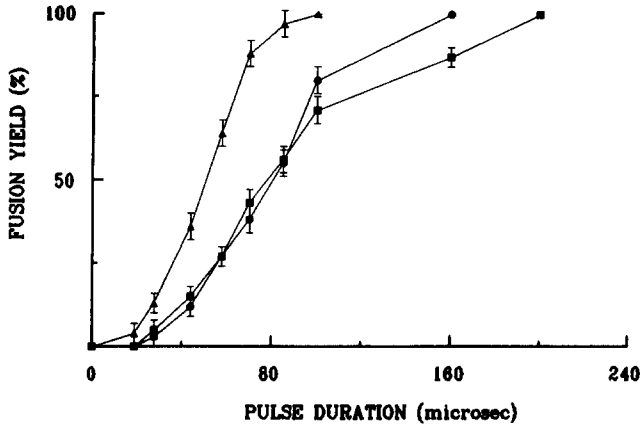

Fig. 2. Dependence of fusion yield (FY) on pulse duration for egg PC cell-size liposomes in three different media: 2.5% Ficoll 400000 (●——●), 2.5% Dextran 71200 (■——■) and 2.5% PEG 8000 (▲——▲). Error bars show the standard deviation from three experiments.

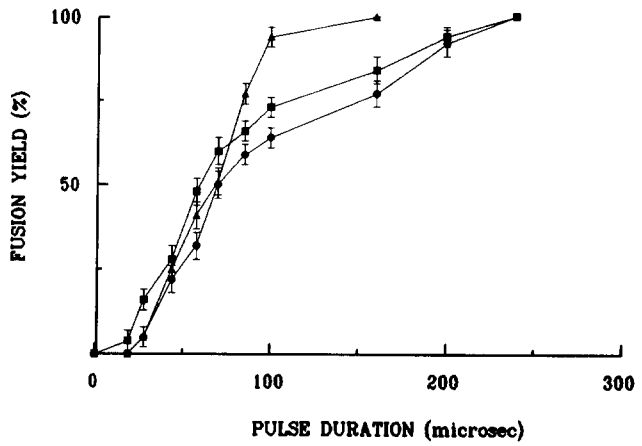

Fig. 3. Dependence of fusion yield (FY) on pulse duration for brain PS cell-size liposomes in three different media: 2.5% Ficoll 400000 (●——●), 2.5% Dextran 712000 (■——■) and 2.5% PEG 8000 (▲——▲). Error bars show the standard deviation from three experiments.

480 V_{pp}/cm. Fig. 2. shows the dependence of fusion yield on pulse duration for cell-size egg PC liposomes. For all the experiments the dc pulse strength was 1.7 kV/cm. Liposomes in 1 mM sucrose (data not shown) showed exactly the same results as those in 2.5% Ficoll. For the entire range of fusion yield (from 0% to 100%) shorter pulses were necessary to induce the same percentage of fusion in medium containing PEG than in media containing Ficoll and Dextran. To obtain 100% FY we had to use dc pulse with duration of 100 μs for PEG medium, 160 μs for Ficoll, and 200 μs for Dextran-containing medium. The results for media containing Ficoll and Dextran were almost the same except that some 30% of liposomes seemed to be more resistant to fusion in Dextran than in Ficoll. It seemed that PEG facilitated electrofusion more than did Ficoll and Dextran. Results for PE liposomes (not shown) were almost identical to those of PC.

Fig. 3. shows FY dependence on dc pulse duration for bovine PS cell-size liposomes. Because the DEP threshold for charged liposomes were lower (see accompanying paper), we used an ac field with frequency 200 kHz and amplitude 320 V_{pp}/cm. Unlike PC and PE liposomes, PS liposomes showed significant difference in fusion yield between different media only at fusion yield above 60%. It indicated that only about 40% of liposome population were sensitive to the external media. There was a slight difference between Ficoll and Dextran media. Results for PG liposomes (not shown) were similar to those of PS liposomes.

Although PS and PG liposomes aligned by DEP at a lower ac field threshold, they required a longer pulse than those for PC and PE liposomes to fuse. The results are summarized in Fig. 4 for FY in 2.5% PEG. The liposomes were brought into a contact by using ac fields with frequency 200 kHz and amplitude 440, 480, 320 and 320 V_{pp}/cm, respectively for PE, PC, PG and PS liposomes. It is seen from the figure that for all ranges of fusion yield (from 0% to 100%) there is a difference in charged versus uncharged liposome fusion. Electrofusion of charged PS and PG liposomes required longer pulses. For PE and PC liposomes, 50% fusion yield was achieved at pulse duration 44 μs, while for PS and PG liposomes a 70 μs pulse was needed. Same tendency is for 100% fusion yield: for PC and PE liposomes, it was necessary to use pulses of 100 and 80 μs, while for PS and PG liposomes pulses of 160 μs and 200 μs, respectively were necessary. A similar trend applies also to the long pulse ranges with samples in Dextran or Ficoll media (results not shown).

We found that fusion medium could play a certain role in electrofusion of liposomes. Media containing

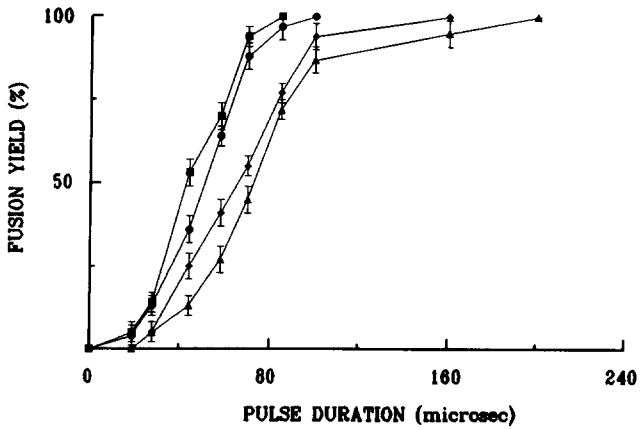

Fig. 4. Dependence of fusion yield (FY) on pulse duration for egg PE (■——■), egg PC (●——●), egg PG (▲——▲) and brain PS (◆——◆) cell-size liposomes in a low conductivity medium (2.5% PEG 8000 solution with conductivity = 9 μS/cm). Error bars show the standard deviation from three experiments.

PEG favored the fusion process in some way more than did Ficoll and Dextran. We also performed the same experiments with 3.5% and 4.5% of PEG, Ficoll and Dextran solutions, and obtained similar results (not shown) as in 2.5%. Higher than 5% concentrations of these solutions caused liposomes to leak due to osmotic imbalance.

In order to explain media influence on electrofusion of cell-size liposomes, we examined the conductivity of the media. We found that media containing 2.5% Ficoll and 2.5% Dextran had higher conductivity than that of 2.5% PEG, i.e., 2.5% PEG solution had conductivity 9 μS/cm, whereas 2.5% Ficoll or Dextran solution had conductivity 20 or 22 μS/cm, respectively. If the difference in conductivity was solely responsible for the difference in fusion yield, adjusting the conductivity (by adding NaCl) of PEG solution to that of Ficoll or Dextran solution would reduce the fusion yield in PEG solution to the same level as those in Dextran or Ficoll solution. Fig. 5. shows the FY dependence on dc pulse duration for egg PC liposomes in 2.5% PEG adjusted conductivity medium, 2.5% Ficoll and 2.5% PEG media. It is apparent from Fig. 5. that there is no difference in fusion yield between liposomes in 2.5% Ficoll medium and 2.5% PEG medium with the same conductivity, for the entire FY range. There was significant difference between fusion yields for PC (egg) liposomes in 2.5% PEG at conductivities of 9 and 20 μS/cm. To obtain 100% fusion yield in 2.5% PEG medium (conductivity 9 μS/cm), pulses with duration 100 μs were necessary, while in 2.5% PEG or in 2.5% Ficoll medium (both had conductivity 20 μS/cm) pulses with duration 200 μs or 160 μs, respectively were necessary. These results confirm our suspicion that solution conductivity played a more significant role than the nature of polymer itself. Results

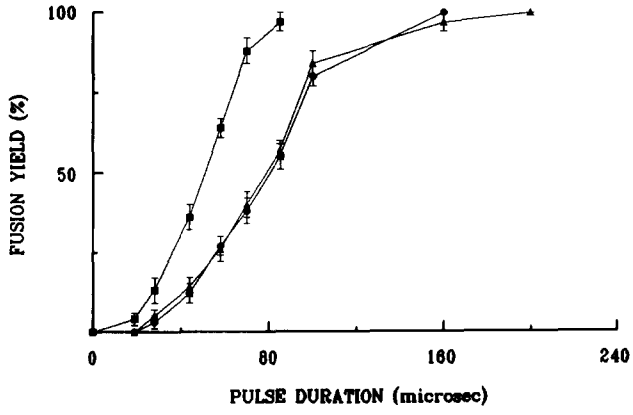

Fig. 5. Dependence of fusion yield (FY) on pulse duration for egg PC liposomes in 2.5% PEG 8000 (■ ——— ■) (σ = 9 μS/cm), 2.5% PEG 8000 adjusted conductivity (▲ ——— ▲) and 2.5% Ficoll 400000 (both with σ = 20 μS/cm) (● ——— ●). Error bars show the standard deviation from three experiments.

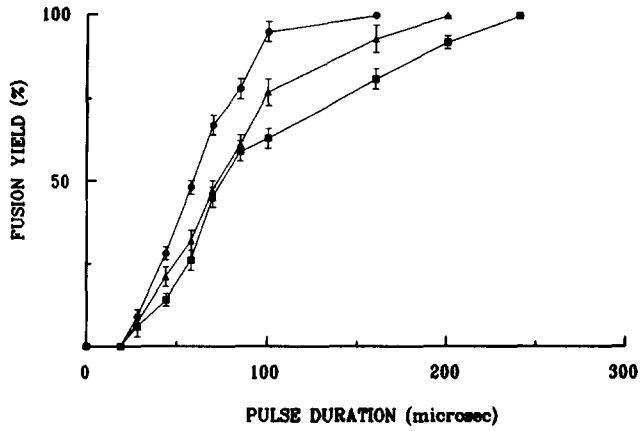

Fig. 6. Dependence of fusion yield (FY) on pulse duration for egg PG cell-size liposomes in 2.5% PEG 8000 (● ——— ●) (σ = 9 μS/cm), 2.5% PEG 8000 adjusted conductivity (▲ ——— ▲) and 2.5% Ficoll (both with σ = 20 μS/cm) (■ ——— ■). Error bars show the standard deviation from three experiments.

for PE liposomes (not shown) were similar to those of PC.

Fig. 6. shows fusion yield dependence on dc pulse duration for egg PG cell-size liposomes in 2.5% PEG or in 2.5% Ficoll at conductivity 20 μS/cm and 2.5% PEG media at conductivity 9 μS/cm. There was significant difference in fusion yield in 2.5% PEG of different conductivities, for the entire range of fusion yield. To obtain 100 percent fusion yield of egg PG cell-size liposomes in 2.5% Ficoll and 2.5% PEG at adjusted conductivity of 20 μS/cm, dc pulses with duration 240 μs and 200 μs, respectively were necessary. For the same liposomes in 2.5% PEG at a conductivity of 9 μS/cm, only 160 μS pulses were needed. Results for PS liposomes (not shown) were similar to those of PG.

The minimal pulse duration for fusion of cell-size liposomes, using a 1.7 kV/cm pulse was 19 μs for all types of liposomes (from PE, PC, PG and PS).

4. Discussion

4.1. Membrane breakdown potential

Mechanisms by which the electric field induces fusion – liposome or cell – are not yet fully understood. It is generally assumed that the fusion is caused by an electrical breakdown of the membrane [10]. If the applied field is a sinusoidal electric field, the maximum amplitude of the induced membrane potential is [11]:

$$V_{max} = frE/\left(1 + \omega^2\tau_m^2\right)^{1/2} \quad (2)$$

where ω is the angular frequency and τ_m is the membrane relaxation time, r is the cell or liposome radius, E is electric field strength (1.7 kV/cm in our case), f is a shape factor, which is 1.5 for a sphere. The

membrane relaxation time τ_m is given by the formula:

$$\tau_m = rC_m(1/\sigma_i + (f-1)/\sigma_e) \quad (3)$$

where C_m is the membrane capacitance per unit area (in our case $0.7 \cdot 10^{-2}$ F/m^2, σ_i and σ_e are internal and external conductivities, respectively.

According to the above analysis, the only effect from the external suspending media on the electric breakdown of membranes is the conductivity. Indeed, the different pulse length requirements we observed from liposomes suspended in PEG, Ficoll and Dextran seem to depend entirely on their conductivity, because liposomes in PEG with adjusted conductivity to those of Ficoll and Dextran behave exactly as those in the latter media. The dielectric constants of these polymers in 2.5% concentration are very similar, hence the selection of polymer is not critical. The conductivity is a much more sensitive parameter.

Electrofusion is a result of simultaneous breakdown of apposing bilayers of adjacent vesicles, and the joining of the broken down areas across the interbilayer space. Therefore, the minimum time requirement for fusion to occur is the time needed to reach the membrane breakdown potential and the time needed to bring the apposing bilayers across the interbilayer gap. In the case of uncharged cell-size liposomes (PE and PC) placed in low conductivity media, the membrane relaxation time τ_m is calculated (by Eq. (3)) to be 25 μs for liposomes with a mean radius of 5 μm, using an inside conductivity of 58 μS/cm and an outside conductivity of 9 μS/cm. For liposomes with mean radius of 8 μm, τ_m becomes 40 μs. The relaxation time sets the rising time of the membrane potential due to the square applied pulse. Pulses shorter than τ_m cannot induce the maximum attainable membrane potential (V_{max} at the poles of liposomes). According to Eq. (2), V_{max} is a function of both r and τ_m, which itself is also a function of r. Combining Eqs. (2) and (3) gives:

$$w^2 = \left(f^2 r^2 E^2 - V_{max}^2\right) / \left(rC_m(1/\sigma_i + (1-f)/\sigma_e)V_{max}\right)^2 \quad (4)$$

For a given pulse field strength E, the maximal attainable membrane potential is frE for an externally imposed field of any real frequency (or Fourier components of any imposed pulses). For our experimental pulse of 1.7 kV/cm, to reach a membrane breakdown potential, which is approx. 1 V [11], liposomes must have a radius of 4 μm and greater. The rise time for a 4 μm liposome is 20 μs under the above condition. This calculation explains the universal minimal fusion pulse length requirement of 19 μs in our experiment. This minimal pulse length is required to cause the breakdown of the bilayer walls of the smallest vesicles capable of attaining membrane breakdown voltage in our heterogeneous size liposome population. Larger liposomes have correspondingly longer rise time in the same medium. For liposomes with radii r much greater than V_{max}/fE, V_{max} becomes independent of r, and the time to reach the breakdown membrane potential of 1 V is about 125 μs. This corresponds to our experimental pulse length (about 100 μs) to achieve 100% fusion in neutral PC and PE liposomes (Fig. 4). The above analysis indicates that the time needed for fusion of these liposomes in low conductivity media is that required for the simultaneous breakdown of apposing bilayers. This time must also be sufficient for the pulse-induced dipole force to bring the two bilayers together within a fusion distance. The sigmoidal FY curves thus reflect the Gaussian cumulative distributions of liposome sizes.

The membrane relaxation or potential rise/fall time depends on the external conductivity. Higher external or internal conductivity leads to shorter relaxation time. When the external conductivity of 2.5% PEG was adjusted from 9 μS/cm to 20 μS/cm, the value of τ_m for a liposome of 5 μm radius was calculated to decrease from 25 μs to 15 μs. One would then expect the FY curve to shift towards a shorter pulse duration. In fact the shift was just the opposite, as seen in Fig. 5. In our experiment, higher external conductivity invariably led to longer required pulse duration. It indicates that longer time may be needed to close the interbilayer gap for vesicles in a higher external conductivity medium.

The forces involved in the electrofusion of cells (or liposomes) in a pearl chain has been analyzed by Stenger et al. [12]. They found that the predominant force in electrofusion is the force between induced dipoles in aligned cells during the pulse, rather than the potential breakdown of individual cell membranes. The dipole-dipole attraction increases both difference between the external and internal conductivity, according to Maxwell-Wagner polarization. Experimentally, we found that the measured pulse durations needed to attain the same fusion percentage of PC or PE vesicles in high conductivity ($\sigma_e = 20$ μS/cm) medium was 1.5-times that for those in low conductivity ($\sigma_e = 9$ μS/cm) medium (Fig. 5). The finding supports the idea that the pulse-induced dipole force is likely to be the primary force to close the inter-membrane gap. Time-dependent electron microscopy studies of electrofusion also showed that the intercellular gap was significantly reduced during the pulse [13]. In this context, it is not surprising to find that PE requires a slightly shorter pulse to achieve the same FY as PC (Fig. 4), since the interbilayer repulsion distance is shorter in PE [14].

4.2. Charged vesicles

In the case of charged liposomes (PG and PS) in low conductivity media, the conditions are more compli-

cated. In spite of the observation that PS and PG vesicles are more responsive to dielectrophoresis (accompanying paper), these charged vesicles require longer pulse duration to attain the same FY of the neutral PC and PE vesicles (Fig. 4). The surface charge creates a electric double layer. This electric double layer lowers the effective induced membrane potential on the one hand [15], but the increased surface conductance also increases the effective internal conductivity, thereby increase the effective dipole moment and the associated dipole force. Furthermore, the shielding of vesicle surface charge is not very effective in a low conductivity medium, and the electrostatic repulsion between charged bilayers must be taken into consideration.

The effect of surface charge (and the resulting surface current) on the membrane potential has been formulated by Grosse and Schwan [15]. For a charged sphere having a surface conductance σ_s and a equivalent surface conduction layer of thickness d, the maximum potential given by Eq. (2) is modified to be:

$$V_{max} = frE/(1 + d\sigma_s/r\sigma_e + jwrC_m(1/\sigma_i + 1/2\sigma_e + d\sigma_s/r\sigma_i\sigma_e)) \quad (5)$$

At low frequencies, the maximum voltage is modified mainly by the factor $d\sigma_s/r\sigma_e$. For charged PS or PG vesicles, σ_s may be calculated from the ion density in the double layer. This is in turn determined by the membrane potential ϕ_0, which is given by the Gouy equation [16]:

$$\exp(e\phi_0/kT) = \delta^2/2\epsilon_e CkT \quad (6)$$

where C is the density of ions of the external bulk medium (set to correspond to $\sigma_e = 20\mu S/cm$ in our case), and δ is the charge density on the vesicle surface. Using an area of 60 Å^2 per PS or PG molecule, the surface potential is calculated by the above equation to be about 250 mV, which agrees with the value given by McLaughlin [16] under similar conditions. The average ion density within the Debye shell, as calculated from the Boltzmann's equation, gives a surface conductivity of 46 mS/cm. Using this value and a Debye length of 10 nm corresponding to our value of σ_e, we found that the value for V_{max} was significantly reduced by 5-fold, and is very sensitive to a change in external medium conductivity. Then, according to Eq. (4), the minimal vesicle size that could reach the breakdown potential of 1 V would be 5-times as large. The charging time for these vesicles is 5-times as long, according to Eq. (3), and is expected to be sensitive to σ_e also. However, the results shown in Figs. 4 and 6 showed only a 50% increase in the required pulse length for FY of PS and PG as compared to that of PC and PE (Fig. 4), and the FY of PS and PG was not as sensitive to σ_e as did PC and PE (Fig. 6). Therefore the membrane potential breakdown calculation by the above method seems to be an over-estimation.

The proposed surface current also modifies the pulse-induced dipole force by modifying the complex permitivity. The detail formulation is given in the accompanied paper and will not be repeated here. The calculation based on the above mentioned values resulted in an *increase* of attractive force, leading to a *decrease* of required pulse length from 1.4 to 2.0 times those in the absence of surface current, for σ_e of 9 and 20 μS/cm respectively. This is in contrast to the results depicted in Figures 4 and 6, which showed an *increased* pulse duration for PS and PG, and the FY for the first 60% was insensitive to σ_e. The surface current is, therefore, not likely to be a significant factor in determining the FY of charged vesicles.

A third factor to be considered is the electrostatic repulsion between charged vesicles. The magnitude of repulsion between charged spheres may be calculated as follows. The liposomes suspended in the external medium are represented by charged hard spheres with surface charge density δ, and the Debye length $1/\kappa$. Within a gap distance of less than the Debye length, and providing $r\kappa >> 1$, the force may be approximated by [17]:

$$F_{eh} = (1/2)\epsilon_e \kappa r \phi_0^2 \ln(1 + \exp(-\kappa l)) \quad (7)$$

Using the values in the previous paragraph, we calculated the force between the spheres at a distance l less than the Debye length of 10 nm. Using again a surface charge density of one electron charge per 60 Å^2, the area per fluid phase PS molecule, the repulsion force is calculated to be 10^{-8} Newton. However, if the spheres are deformable, and the apposing surface is flatten to an area A, the force should be modified by the flat plate approximation [18]:

$$F_{es} = (1/2)\epsilon_e \kappa^2 \phi_0^2 A \quad (8)$$

For a flatten area of 1 μm^2, the force is calculated to be 10^{-7} Newton, much larger than that for hard spheres. The pulse-induced dipole attraction force, as calculated from Eq. (5), using the modified ϵ_i values by the surface current, is $2 \cdot 10^{-7}$ Newton on an apposing area of 1 μm^2, for $\sigma_e = 9$ μS/cm. These estimated forces are comparable, such that much of the dipole-dipole attraction due to the pulse is reduced by the electrostatic repulsion of the charged vesicles. This repulsion force could be responsible for the 50% longer pulse duration required for PS and PG vesicles to attain the same FY as those of PC and PE vesicles. The larger is the flattened area, the more significant is the reduction, because F_{es} is proportional to A while F_D is proportional to $1/A$. Flattened area is expected to be larger for larger vesicles, because of geometric consideration as well as their stronger dipole-dipole attraction. The observed instantaneous compression of

the pearl chains during the pulse application supports this hypothesis. The effect of electrostatic is therefore more pronounced in larger vesicles, leading to the more significant departure of the FY curves from the sigmoidal shape at the high FY ends of the curves (Figs. 3 and 6). Furthermore, F_{es} increases with σ_e through the increase of κ^2, while the F_{eh} dependence on σ_e is weak due to both the κ and the $\exp(-\kappa l)$ factors. This causes the deviated portions at the upper ends of the FY curves attributable to large deformable vesicles, to be more sensitive to σ_e (Figs. 3 and 6).

In summary, we have demonstrated that cell-size liposomes can be induced to fuse, and have established the conditions for electrofusion of these vesicles. The electrofusion process for liposomes is precisely governed by physical principle. The outcome of this study would help us to understand and to design better protocols for electrofusion of more complicated systems, including the use of liposomes as delivery vehicles to cells and tissues.

Acknowledgement

This work is supported by a grant GM-30969 from the National Institutes of Health. Travelling fellowship to NGS was supported from Foundation in 'Cyrill and Methodius' and grant N-189 from MCES of Bulgaria.

References

[1] Gaub, H., Sackmann, E., Buschl, R. and Ringsdorf, H. (1984) Biophys. J. 45, 725–731.
[2] Decher, G., Kuchinka, E., Ringsdorf, H., Venzmer, J., Bitter-Suermann, D. and Weisgerberg, C. (1989) Angew. Makromol. Chem. 166–167, 71–80.
[3] Stoicheva, N., Tsoneva, I., Dimitrov, D.S. and Panaiotov, I. (1985) Z. Naturforsch. 40c, 92–96.
[4] Farge, E. and Devaux, P. (1992) Biophys. J. 61, 347–357.
[5] Lipowsky, R. (1991) Nature 349, 475–481.
[6] Buschl, R., Ringsdorf, H. and Zimmermann, U. (1982) FEBS Lett. 150, 38–42.
[7] Needham, D. and Hochmunt, R.M. (1989) Biophys. J. 55, 1001–1009.
[8] Hub, H.H., Zimmermann, U. and Ringsdorf, H. (1982) FEBS Lett. 140, 254–256.
[9] Stenger, D.A., Kubiniec, R.T., Purucker, W.J., Liang, H. and Hui, S.W. (1988) Hybridoma 7, 505–517.
[10] Zimmermann, U. and Vienken, J. (1982) J. Membr. Biol. 67, 165–182.
[11] Holzapfel, C., Vienken, J. and Zimmermann, U. (1982) J. Membr. Biol. 67, 13–26.
[12] Stenger, D.A., Kaler, K.V.I.S. and Hui, S.W. (1991) Biophys. J. 59, 1074–1084.
[13] Stenger, D.A. and Hui, S.W. (1986) J. Membr. Biol. 93, 43–53.
[14] Lis, L.J., McAlister, M., Fuller, N., Rand, R.P. and Parsegian, V.A. (1982) Biophys. J. 37, 657–666.
[15] Grosse, C. and Schwan, H.P. (1992) Biophys. J. 63, 1632–1642.
[16] McLaughlin, S. (1977) Curr. Topics Membr. Transport 9, 71–143.
[17] Verway, E.J.W. and Overbeek, J.Th.G. (1948) Theory of the Stability of Lyophobic Colloids in the Interaction of Sol Particles having an Electric Double Layer, Elsevier, New York.
[18] Wangsness R.K. (1979) Electromagnetic Fields, John Wiley and Sons, New York.