J. Membrane Biol. 140, 177–182 (1994)

The Journal of
Membrane
Biology

Electrically Induced Fusion of Mammalian Cells in the Presence of Polyethylene Glycol

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Received: 11 January 1994/Revised: 4 April 1994

Abstract. Chinese Hamster Ovary (CHO) cells were fused by subjecting cell suspensions to an exponentially decaying electric pulse in the presence of polyethylene glycol (PEG), Dextran or Ficoll. PEG (MW 1,000, 3,350, 8,000, 10,000 and 18,500), Dextran (MW 71,200) and Ficoll (MW 400,000) were added to the pulsing medium. A single exponential electric pulse with peak field strength of 4 kV/cm, and a half-time of 0.72 msec was used. The combination of two techniques, PEG-induced fusion and electrofusion, resulted in highly efficient fusion of CHO cells. Fusion yields (FY) at different concentrations of these polymers were measured using phase-contrast microscopy. FY was highly dependent on the concentration of PEG in media, while the presence of Dextran and Ficoll had no influence on fusion yield. PEG with MW 8,000 was found to be the most effective in causing cell aggregation, and to give the highest FY (40%). An optimal concentration for fusion was found for PEG of each molecular weight. Diluting cells suspended in higher concentrations of PEG to these optimal concentrations after the pulse application regained the optimal FY. It was concluded that PEG-induced prepulse aggregation and moderate cell swelling immediately after the pulse were important factors in achieving high fusion yields.

Key words: CHO cells — Electrofusion — Polyethylene glycol

Introduction

Artificially induced cell fusion is a basic method in cell biology. Different techniques were developed to achieve membrane fusion. These techniques include polyethylene-glycol-induced fusion, virus-induced fusion and electrofusion. Each of these techniques has its advantages and disadvantages. A variety of agents have been used as fusogens, particularly in studies involving mammalian somatic cell fusion. At present, polyethylene glycol (PEG), which has been used to induce fusion of both plant and animal cells, is largely the agent of choice for somatic cell fusion, due to its efficacy, reproducibility and ease of preparation and use.

PEG causes cell shrinking and aggregation, and after removal of PEG, cell fusion takes place (Robinson et al., 1979; Krahling, 1981; Hui et al., 1985). A direct interaction of PEG with membrane lipids is assumed to cause a decrease of the membrane fluidity (Ohno et al., 1981). An indirect influence of PEG on the membrane structure might be due to the physicochemical properties of the aqueous PEG solution (Arnold, Pratsch & Gawrisch, 1983). The molecular structure of PEG results in a high binding capacity for water. At concentrations higher than 40 wt.%, no free water exists (Tilcock & Fisher, 1979). Even at a low (10%) concentration, the depletion of PEG from cell and vesicle surface causes cells and vesicles to aggregate (Boni et al. 1981; Arnold et al., 1990). Moreover, there are additional factors in PEG-induced membrane fusion. Membrane areas devoid of intermembranous particles (IMP) have been found in regions of cell-cell contact, i.e., potential fusion sites (Knutton, 1979; Robinson et al., 1979; Hui et al., 1985; Huang & Hui, 1986). Osmotic swelling is also a very important step (Zimmerberg, Cohen & Finkelstein, 1980). The importance of these factors in PEG-induced membrane fusion has been examined by Hui et al. (1985).

Electrically induced cell fusion has recently become a powerful technique in cell biology (Teissie et al., 1982; Zimmermann, 1982; Zimmermann & Vienken, 1982; Stenger & Hui, 1986). Compared with other cell-fusion techniques, electrofusion is simple, rapid

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and highly efficient, and usually non-cell-specific. A short, high-voltage electric pulse causes temporary and reversible electric breakdown of the cell membrane. If cells are closely packed during the pulse application, dielectrophoretic compression and simultaneous breakdown of adjacent cell membranes result in the formation of transient fusion sites, which may expand during subsequent cell swelling (Stenger et al., 1988; Stenger, Kaler & Hui, 1991; Song et al., 1993). Zhelev and Dimitrov (1989) found that if the cells (pea protoplasts) are brought into contact by DEP, the presence of PEG (up to 50% MW 3,000) does not influence fusion yield. This is reasonable because dielectrophoretic compression is dominant and PEG influence is negligible (Zhelev & Dimitrov, 1989). Weber et al. (1981) found stimulation of yeast protoplast fusion by electric field when the protoplasts were treated with PEG in the presence of calcium chloride. They used 40% PEG MW 6,000 to achieve aggregation. Broadly speaking, several steps in the electrofusion process are in common with the PEG-induced fusion process.

In this study we combined these two basic fusion techniques (PEG-induced fusion and electrofusion) to introduce highly efficient CHO cell fusion. It is known that PEG causes aggregation, but membrane destabilization by PEG is inefficient. Applying electric pulses causes temporary pore formation, but aggregation must be achieved by dielectrophoresis or by centrifugation. By combining both techniques, we are able to obtain, with only one electric pulse, more than twice the efficiency of CHO cell fusion than with PEG-induced fusion alone. So the combination is a valuable strategy. We found that the electrofusion efficiency depends on both the PEG concentration and PEG molecular weight to induce proper aggregation. Also, we found that cell swelling, immediately after pulsing in the presence of PEG, increases fusion yield. It seems that both aggregation and swelling induced by PEG improves CHO cell fusion in electrofusion experiments.

Materials and Methods

Chemicals

PEG MW 1,000, 3,350, 8,000, 10,000 and 18,500, Ficoll Type 400 (MW 400,000), Dextran (MW 71,200) were obtained from Sigma (St. Louis, MO). PSN (penicillin, streptomycin and neomycin) antibiotic mixture, NCS (newborn calf serum), F10 nutrient mixture with L-glutamine and trypsin 0.25% were obtained from GIBCO Laboratories (Grand Island, NY). All chemicals were of analytical grade.

Cell Preparation

CHO cells grown in petri dishes in monolayer were used for fusion. After treating with trypsin 0.25% (5 min), CHO cells were suspended in full medium containing 84% F10 medium, 15% NCS and 1% PNS. Just before the electrofusion experiments, CHO cells were washed and resuspended in BSS (balanced salt solution containing 125 mM NaCl, 5 mM KCl, 4 mM $CaCl_2 \cdot 2\ H_2O$, 2.5 mM $MgCl_2 \cdot 6\ H_2O$ and 5 mM TRIS.HCl) buffer at pH 7.2.

Fusion Protocol

Electrofusion measurements were made in a cuvette chamber with two parallel electrodes with a distance between them of 2.5 mm and volume 400 μl. A high power pulse generator (Model Permeator EC-102, Electrocell, NY) was used to supply the exponential high-voltage pulses. A single exponential electric pulse with amplitude 1,000 V peak (strength 4 kV/cm) and half-time duration of 0.72 msec was used for each experiment.

All fusion experiments were done in a fusion mixture consisting of 50 μl CHO cells (cell concentration 2×10^6 cells/ml), 150 μl BSS buffer and 200 μl of PEG of different molecular weight, or 200 μl Dextran or Ficoll of different concentrations. For the fusion experiments, PEG 8,000 at concentrations from 5 to 40%, Dextran 70,000 from 5 to 20% and Ficoll 400,000 from 5 to 20% were used. After the pulse, equal volumes of BSS buffer were added immediately in some experiments, and cells were left in the fusion chamber for 3 min. Samples were taken, and the fusion yield was calculated by using phase-contrast microscope (Model IMT-2 Olympus Inverted Research Microscope). Fusion yield was determined as a ratio between the number of fused cells *vs.* the total number of cells. Fused cells were determined by both shape and the number of nuclei. Calculation of fusion yield by using these two ways showed up to a 3% difference. In this study, fusion yield was calculated by shape.

Two control experiments were done before each fusion experiment: (i) estimation of the percentage of innate polynuclear CHO cells, was 2–3% and (ii) electrofusion in control fusion medium, consisting of 50 μl CHO cells and 350 μl BSS buffer without PEG.

Osmolarity Measurements

The osmolarity of PEG, Dextran and Ficoll at various concentrations was measured at room temperature with a vapor pressure osmometer (Model 5100B, Wescor). The osmolarity of BSS buffer is 300 mOsm/kg.

Results

Figure 1 shows the dependence of fusion yield on PEG 8,000 concentration (%w/v). We did two sets of experiments: (i) CHO cells were fused with PEG only (curve with circles) and (ii) CHO cells were fused with PEG and one exponential electric pulse of 4 kV/cm, half-time 0.72 msec (curve with triangles). The highest fusion yield, 45 and 20% respectively, was at 10% PEG 8,000 with and without pulse. Fusion yield in cells pulsed in BSS buffer without PEG was about 10%. In 15% PEG 8,000, fusion yield began to decrease, and in 25% PEG 8,000, it almost reached the control level. All estimations of fusion yield were made 3 min after pulsing.

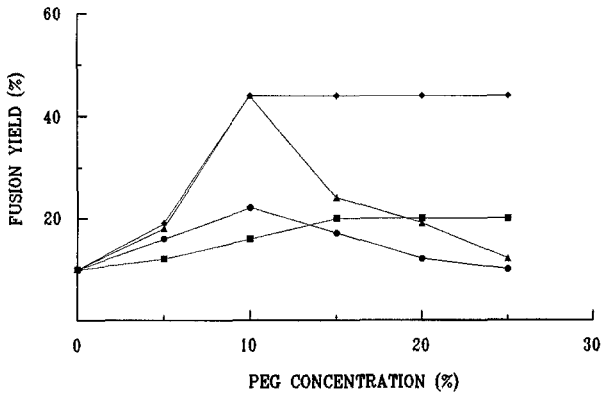

Fig. 1. Dependence of CHO cell fusion yield (FY) on PEG 8,000 concentrations. Fusion yield in different PEG concentrations: without pulse (circle); without pulse and immediate dilution (square); with one exponential electric pulse (triangle); and with one pulse and immediate dilution (diamond). Standard deviation is 2–3%.

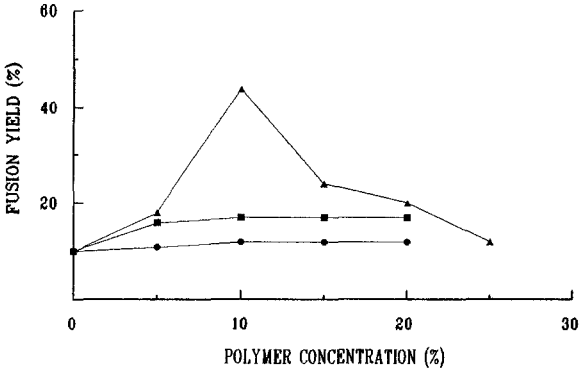

Fig. 2. Dependence of fusion yield (FY) on polymer concentrations. Three different polymers were used: Dextran 71,200 (circles), Ficoll 400,000 (squares) and PEG 8,000 (triangles). Standard deviation is up to 3%.

To determine if fusion yield was dependent on cell swelling, we did another series of experiments. Immediately after the pulse we diluted the pulsing medium. Dilution was done by adding BSS buffer. In all experiments the cell suspension volume was 400 μl. To reach a final PEG concentration of 10%, we added 200, 400 and 600 μl of BSS buffer to the samples containing 15, 20 and 25% of PEG, respectively. Dilution experiments showed that the fusion yield at higher PEG concentrations remained as high as the values at 10% PEG. So the highest fusion yield in 15, 20 and 25% PEG, in experiments with and without pulse, was about 45 and 20%, respectively (curves with diamond and square symbols, respectively). By diluting the medium immediately after pulsing, we created the electrofusion conditions that were equivalent to pulsing cells in 10% PEG 8,000).

Figure 2 illustrates the dependence of fusion yield on different polymers. In our experiments we used Dextran and Ficoll to compare with PEG. We used Dextran MW 71,200 and Ficoll MW 400,000 because they provide equivalent osmolarity to PEG in the pulsing medium. Electrofusion of CHO cells in the presence of Dextran 70,000 MW, Ficoll 400,000 MW and PEG 8,000 MW was done by applying only one exponential electric pulse. Results are shown on curves with circle, square and triangle symbols, respectively. Fusion yield in the presence of Dextran was the same as that of the control, while the fusion yield in the presence of Ficoll was slightly higher—about 17% more than the control. It should be mentioned that with Dextran and Ficoll, concentrations higher than 20% caused cell damage.

When the fusion yields in the presence of Dextran and Ficoll were compared to that with polyethylene glycol, it was clear that, among polymer solutions of similar osmolarity, PEG had distinct influence in cell electrofusion. The other polymers did not play any significant role in cell electrofusion. No optimal concentration was found for Dextran or Ficoll solutions in electrofusion experiments. The data reproducibility was very good in all experiments. Standard deviation for all experiments was up to 3%.

To explain the fusion results, we did an estimation of the rate of CHO cell aggregation in the presence of different polymers. Cell aggregation was determined as a ratio between the number of aggregated cells *vs.* the total number of cells. For comparison, we chose PEG MW 8,000 because it happened to be the most effective fusogen among PEG of different molecular weights. Figure 3 (*A–D*) shows aggregation kinetics of CHO cells in the presence of Ficoll MW 400,000, Dextran MW 70,000, PEG MW 8,000 (with BSS as control), and PEG 3,350, respectively. Within 1 min after the cells were introduced in fusion media, most of the cell aggregation took place. A slow increase of cell aggregation with the time after 1 min was observed, but the increase was not significant. This was true for all polymers used and for the control which had no PEG but still spontaneously re-aggregated after trypsinization. The maximal aggregation percentages were about 22% for control and all Ficoll and Dextran samples. Aggregation in PEG was concentration dependent. For PEG 8,000 and 3,350, maximum aggregations of 45 and 28% were attained at 10 and 25%, respectively.

We observed broken cells with increasing incubation time for a certain polymer's concentrations (e.g., 15% PEG, Dextran, or Ficoll). Therefore, to expose CHO cells in these fusogenic media for more than 5–6 min is not advisable. Consequently, we did all our fusion experiments within 3 min, and our cell viability test showed that, at the end of experiments, 85–90% of cells survived.

Figure 4 illustrates the dependence of fusion yield on different molecular weight PEG concentrations. We

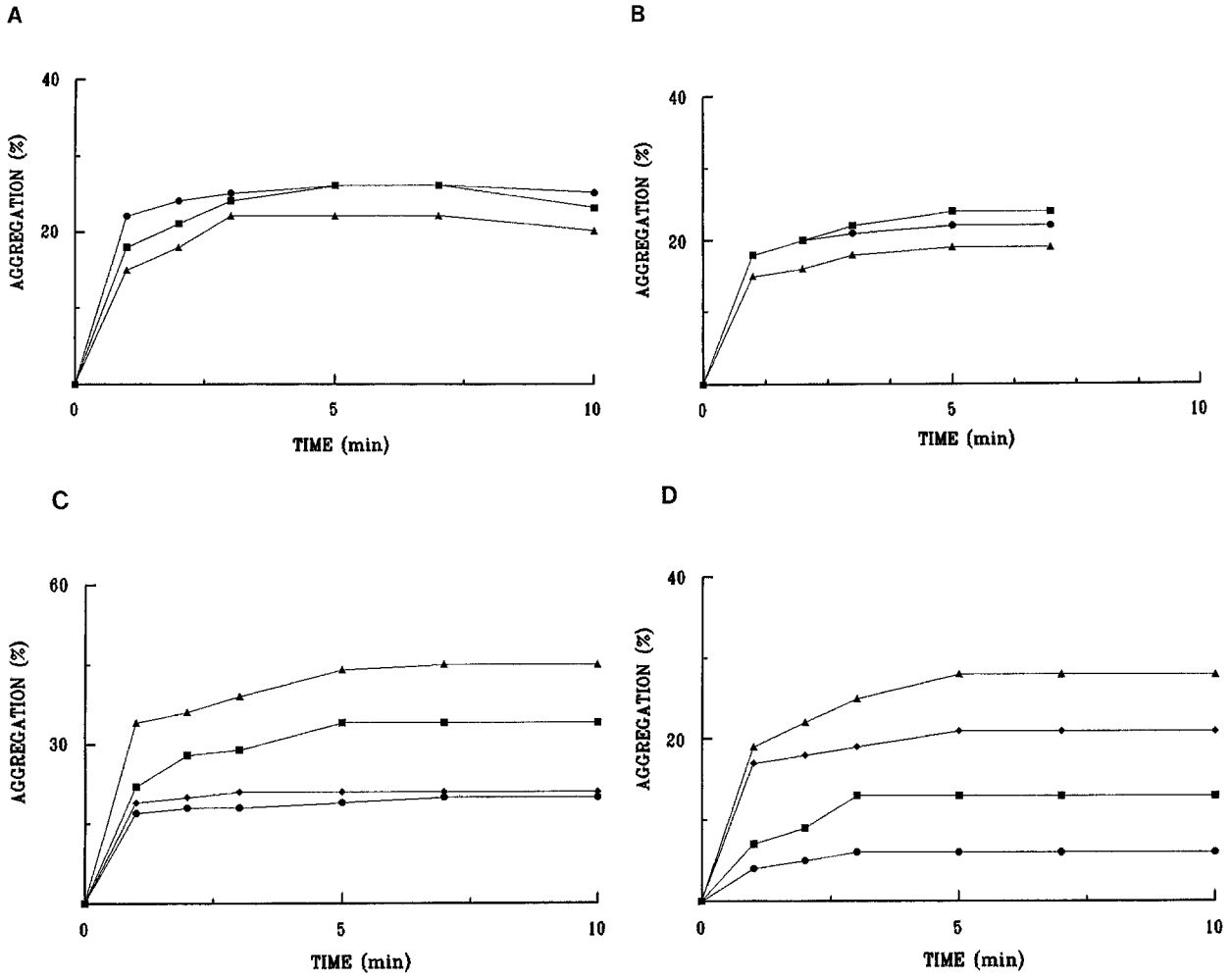

Fig. 3.(*A*) Aggregation kinetics of CHO cells in the presence of Ficoll 400,000. Circle, square and triangle symbols are for 5%, 10% and 15% Ficoll concentration, respectively. (*B*) Aggregation kinetics of CHO cells in the presence of Dextran 70,000. Circle, square and triangle symbols are for 5%, 10% and 15% Dextran concentrations, respectively. (*C*) Aggregation kinetics of CHO cells in the presence of PEG 8,000. Circle, square, triangle and diamond symbols are for control (BSS buffer), 5, 10 and 15% PEG concentrations, respectively. (*D*) Aggregation kinetics of CHO cells in the presence of PEG 3,350. Circle, square, triangle and diamond symbols are for 15, 20, 25 and 30% PEG concentrations. Aggregation kinetics at 5 and 10% PEG concentrations are the same as that for 15% (*data not shown*). Standard deviation is up to 3%.

estimated five different molecular weight PEG—1,000, 3,350, 8,000, 10,000 and 18,500. The most effective fusogen was PEG with molecular weight 8,000, and the most effective concentration was 10%. Each of the PEG had its own most efficient concentration. For PEG MW of 1,000, 3,350, 8,000, 10,000 and 18,500, the optimal concentrations of PEG were 30, 25, 10, 10 and 10%, respectively, and their fusion yields were 28, 28, 45, 28 and 20%, respectively. The least-effective fusogen was PEG 18,500, while PEG 1,000, 3,350 and 10,000 at optimal concentrations had approximately the same efficiency.

Another interesting observation was found using PEG MW 3,350. In fusion media containing PEG 3,350 of concentrations below 20%, fusion yield was even lower than that of the control. It seems that at these concentrations, PEG 3,350 acts like a fusion inhibitor. We did some estimations of cell aggregation kinetics at these concentrations and found that the extent of aggregation is less than 6% (Fig. 3*D*), compared to that of control cells (22%, Fig. 3*C*).

From the results shown in Fig. 4, it is seen that the maximal combined PEG and electrofusion yield are very much dependent on the PEG concentration as well as on the polymer molecular weight.

Discussion

Artificially induced cell fusion was first made possible by the use of chemical agents such as PEG. Recently, electrofusion was shown to be an effective and promis-

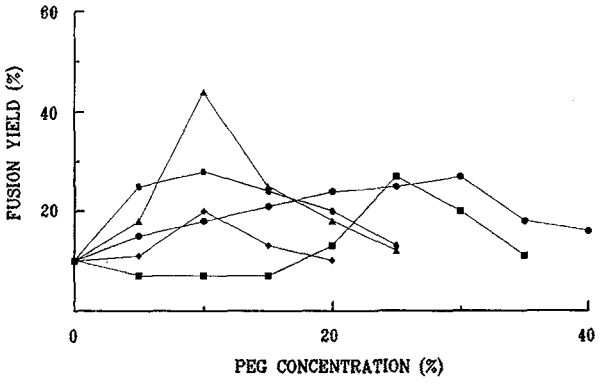

Fig. 4. Fusion yield dependence on PEG concentrations. Different PEG molecular weights were used. Circle, square, triangle, hexagonal and diamond symbols are for PEG of MW 1,000, 3,350, 8,000, 10,000, and 18,500, respectively. Standard deviation is 3%.

ing technique. In this study, we provide a rationale for using a combination of these techniques. Our results show that by combining these fusion techniques we can increase fusion efficiency considerably, and the cell viability remains high. Furthermore, the combined protocol is an easy and quick experimental procedure. Fusion was achieved by using only one pulse, although by using three pulses (with the same electric field parameters) we were able to obtain more than 70% fusion yield (*data not shown*). It is possible to achieve an even higher fusion yield by optimizing electric pulse parameters (pulse strength and duration) and the number of pulses according to the special experimental needs.

As far as the choice of polymer is concerned, we found that the most effective fusogen is PEG with molecular weight 8,000 at 10% concentrations. From the experimental data, it is clear that higher molecular weight PEG is detrimental for CHO cell fusion. For PEG 3,350 we found that, at concentrations up to 20%, CHO cell fusion yield was very low, even lower than that of the control. Only at 25% concentrations were we able to reach fusion yield comparable to that of the other experiments.

CHO cells have a natural tendency to aggregate after being trypsinized and suspended (about 20%, *see* control curve in Fig. 3*C*). In polymer solutions, the aggregation rate may be impeded by the viscosity, or may be enhanced by the differential osmotic pressure due to polymer depletion layers on the cell surfaces (Arnold et al., 1990; Guo, Alderfer & Hui, 1992). The balance of these two effects for different polymers at different concentrations determines if cell aggregation is promoted or inhibited. In the case of cells in PEG 8,000, increasing concentration increases the differential osmotic pressure, which presses the adjacent membranes of aggregated cells together. The application to aggregated cells of a pulse electric field greater than the membrane breakdown potential will trigger fusion. With the addition of a dehydration-destabilization effect at higher PEG concentrations, membrane fusion is also initiated if the cells remain together, as in the case of PEG-induced fusion in cell pellets. Otherwise, with increasing dehydration, cells shrink and become de-aggregated rather than fused. This explains the lower aggregation effect at higher PEG concentrations. In the case of PEG 3,350, the depletion effect is expected to be weaker. At concentrations up to 20% of PEG 3,350, cell aggregation is still very low (about 12%, Fig. 3*D*), much below that of control cells. The differential osmotic pressure is still weak at a high PEG concentration, when the dehydration effect is significant. As a result, cells shrink, and the aggregation percentage is low. Weakly depleted or nondepleted PEG 3,350 also increases the medium viscosity between cells and impedes cell-cell aggregation, thereby further reducing fusion yield to values even below the control (with no PEG in the medium).

Our experimental results substantiate that osmotic effects play an important role in mammalian cell electrofusion. It has already been reported from several authors that osmotic stress is one of the key steps in fusion process for both cells and vesicles (Hui et al., 1985; Vienken & Zimmermann, 1985; Stenger et al., 1988; Song et al., 1993). They observed that osmotically induced cell swelling following membrane breakdown is important for the giant cell formation (especially for erythrocytes). The formation of giant cells after fusion was prevented when osmotic swelling was inhibited by hypertonic media (Knutton & Pasternak, 1979). Our observations also show that, following electrically induced cell fusion in the presence of PEG, CHO cells round-up into giant cells faster if the medium causes cell swelling.

The fusion yield from pulsed cells in PEG is significantly higher than from cells in PEG alone without being pulsed. Our hypothesis is that electric pulses create pores (probably coaxial) and temporary (or committed) fusion sites between cells. Cell swelling enlarges these fusion sites which then become irreversible, while cell shrinking disconnects them. To prove this point, we gently diluted the samples immediately after the pulse. This procedure is supposed to enlarge fusion sites because of cell swelling. The result is higher fusion yield. The osmolarities of pulsing media containing 10, 15, 20 and 25% PEG were 220, 300, 340 and 440 mOsm/kg, respectively. We deliberately created the same post-pulse osmotic conditions by diluting the samples containing 15, 20 and 25% PEG to that of 10% PEG. As a result, we obtained high fusion yield identical to that in 10% PEG. Further dilution caused significant cell damage. The data indicate that the number of fusion sites induced by the same electric pulse conditions are the same for all aggregated cell samples.

By suspending the post-pulse cells in a slightly hypotonic medium with the same osmolarity of 10% PEG 8,000, the same fusion yields were attained. Thus, the reason for low fusion yield at higher PEG content is that cells are shrunk under hypertonic conditions. PEG (10%) seems to give the optimal osmotic pressure for post-pulse swelling. Furthermore, swelling before or during pulses has no bearing on fusion yield.

For comparison, we measured electrofusion of CHO cells in the presence of Dextran 71,200 and Ficoll 400,000. Our measurement showed that these two polymers did not have any influence on the fusion (Fig. 2). A likely reason is that the fusion yield is limited by the low aggregation potential of these polymers. Ficoll has a slightly higher aggregation potential (Fig. 3*A*) and a lower viscosity than Dextran, both favoring a slightly higher fusion yield. PEG 8,000, the most popular choice of polymer fusogen, seems to have the most favorable combination of aggregation potential, osmolarity and dehydration threshold for use as chemical fusogen alone (at $> 35\%$) and as an auxiliary agent in electrofusion (at 10%). These empirical choices now have a physicochemical basis.

This work is supported by a grant GM-30969 from the National Institutes of Health. Traveling fellowship to N.G.S. was supported from Foundation "Cyrill and Methodius" and grant N-189 from MCES of Bulgaria.

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