Characterization of PEG-Mediated Electrofusion of Human Erythrocytes

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ABSTRACT Polyethylene glycol (PEG) and electrofusion were applied together in a simple and highly efficient cell fusion method. PEG (8000 M_r) was used to bring human erythrocytes into contact, and a single 4.4 kV/cm, 80 μs duration pulse was applied to cell suspensions. The fusion yield (FY) is PEG concentration-dependent. A maximum FY (50%) was found at about 10% PEG. Higher PEG concentrations (>10%) suppressed FY caused by colloid osmotic shrinkage. Morphological changes, such as colloidal osmotic swelling and shrinking, and the expanding and contraction of fusion lumen, when suspension media were changed from PBS to isotonic 15% dextran solutions, was examined by microscopy. FY was found to depend on both simple osmotic and colloidal-osmotic swelling. From the swelling behavior, we propose two types of electropores: the pre-fusion sites between cell pairs, and electropores on each individual cell connecting intracellular and extracellular space. The latter type is responsible for the colloidal osmotic swelling and shrinking of cell which, together with simple osmotic swelling, is responsible for expanding the pre-fusion sites into fusion lumens. Resealing of electropores resulted in reducing FY, but the FY can be restored by simple osmotic shock. Apparently, PEG plays two opposite roles in this fusion method; one is to promote pre-pulse and post-pulse cell-cell contact, protecting pre-fusion sites, and the other suppresses FY by colloid osmotic shrinkage of cells after pulsing, especially when high PEG concentration is used. 10% PEG 8000 represents the optimal combination of these properties.

INTRODUCTION

Electrofusion is an efficient artificial method to induce cell fusion (Zimmerman, 1982). The essential steps in electrofusion are the formation of good cell-cell contacts, the application of one or several fusogenic pulses, and the development of pre-fusion sites into fusion lumens (Abidor and Sowers, 1992; Stenger et al., 1991, 1986; Sowers, 1984). In addition to dielectrophoresis, two modified methods have been applied to bring cells into contact before the application of fusogenic pulses: by growing cells into confluent monolayers (Teissie et al., 1982), or by using centrifugation force to form cell pellets (Abidor et al., 1993). Because of the ease of changing post-pulse medium in these methods, the post-pulse swelling effect and the influence of macromolecules on cell swelling have been examined using these systems to gain information about fusion mechanisms (Abidor et al., 1994a).

The commonly used chemical fusogen polyethylene glycol (PEG) induces aggregation of most cell types (Hui et al., 1985; Knutton and Pasternak, 1979; Power et al., 1978). This property may be explored to make good cell contacts for electrofusion (Zhelev and Dimitrov, 1989; Weber et al., 1981). In addition to forming cell contacts, high concentration of PEG induces membrane destabilization. The rapid osmotic swelling from sudden dilution of PEG is believed to expand the pre-fusion sites caused by membrane destabilization (Boni and Hui, 1987; Huang and Hui, 1986). These steps are in common with electrofusion. A combination of these two methods may be advantageous.

This paper shows a simple and efficient electrofusion method combining the advantages of both techniques. We found that electrofusion mediated by a low concentration of PEG offers a simpler and equally efficient alternative to other electrofusion methods. From this study, we achieve a better understanding of the formation and resealing of electropores, and the colloidal osmotic effect of macromolecules such as PEG, dextran, and hemoglobin on FY.

MATERIALS AND METHODS

Human blood from healthy donors was washed 3 times in PBS (150 mM NaCl + 5 mM NaPi, pH 7.4) to remove plasma proteins, platelets, and white cells. All suspension media were made to 300 mOsm/kg including 5 mM of sodium phosphates (NaPi) at pH 7.4, unless specifically mentioned. PEG (8000 M_r, Sigma Chemical Co., St. Louis, MO)-containing media were prepared by dissolving various weights of PEG in isotonic PBS. Dextran (8800 M_r, Sigma)-containing medium was prepared by dissolving dextran in certain concentrations of PBS to a final 300 mOsm/kg. PEG and dextran concentrations were expressed in w/w × 100%.

The fusogenic electrical pulse treatment is the same as that described elsewhere (Abidor et al., 1994b). Briefly, 5×10^8 cells (concentration determined by a hemacytometer) were mixed with PEG-containing media. 90 μl of such mixture was introduced into a cylindrical shaped chamber with one stainless steel electrode on each end, and a quasi-square electric pulse was applied across the chamber at room temperature with cells in suspension. A digital storage oscilloscope (Model OS-6121, Iwasu, Japan) was used to monitor the pulse strength and duration. One pulse with 4.4 kv/cm, 80 μs duration was used in all cases.

After each sample was pulsed, the cells were divided into 5–6 aliquots of 15 μl each. These were transferred to Eppendorf tubes, and 1 ml of PBS or dextran-containing medium was added to dilute the PEG after different incubation periods. After 80 s (unless otherwise mentioned), the sample was centrifuged (Eppendorf Centrifuge 5414, Brinkmann Instruments, Inc.) and resuspended after changing supernatant twice by PBS, and the FY was

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Abbreviations used: PEG, Poly(ethylene glycol); PBS, phosphate buffer solution; FY, fusion yield; NaPi, sodium phosphates.

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determined as the ratio of the number of fused cells to the number of total cells, as counted under the microscope. Because the size of erythrocytes is fairly uniform and the final product of electrofusion is distinct morphologically, fused cells were distinguished by their shape and size. The same fused cells were also counted and compared by changing supernatant to 100 mOsm/kg PBS in a flow chamber (see next paragraph). In their swollen state in 100 mOsm/kg PBS, fused cell may be further discerned under the phase microscope by their fusion lumen. The FY determined by both methods are within 5%. All data were repeated at least 3 times, and the SEs were within 8%.

To study the cell shape change under the microscope, a special flow chamber was fabricated. Briefly, a rectangular hole longer than the cover glass was cut out from a threefold stack of parafilm. The parafilm was mounted on a glass slide with the cover glass on top, leaving the trough open on both ends. This sandwich was heated, and the parafilm was melted so that the trough was sealed all around. Cells were introduced from one opening to the chamber. After the cells have settled on the glass slide, some of them will not move even with fluid flow during washing. A piece of absorbing material was placed on the other opening to absorb the surplus medium. In this way, we can investigate the shape change of the fusion tube of the same cells in different medium under the microscope. Micrographs were taken with an inverted phase microscope (Olympus Model IMT-2).

The osmolarity was measured by a vapor pressure osmometer, (Model 5100B, Wescor, Inc.). All chemicals were of research grade.

RESULTS

The pulse induced FY is very sensitive to the concentration of PEG. The effect of PEG concentration on FY is shown in Fig. 1. A maximum FY of about 50% was found at 10% PEG concentration. It is interesting to note that FY decreases when PEG concentration increases beyond 10%. When PEG concentration is 30% or higher, FY is insignificant.

To assess the importance of colloidal osmotic swelling (or shrinking) of cells in the development of pre-fusion sites into fusion lumens, we used media containing different dextran concentrations (final osmolarity was always 300 mOsm/kg) to dilute samples immediately after the pulse application. The dependence of FY on the concentration of dextran in the post-pulse dilution medium is shown in Fig. 2. It was found that FY decreases as dextran concentration increases. When the dextran concentration reaches 10% or above, no significant fusion is detected.

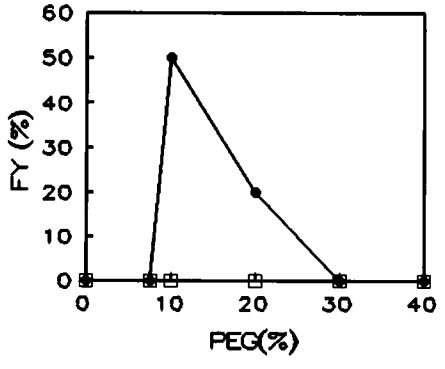

FIGURE 1 The effect of PEG concentration on FY. The solid circles represent the FY with pulse (4.4 kv/cm, 80 μs). The open squares represent the FY of control, i.e., under the same condition except without pulse treatment. After pulse, cells were diluted immediately with PBS, left in PBS suspension for 80 s, and centrifuged and washed twice. The FY was counted under the phase microscope.

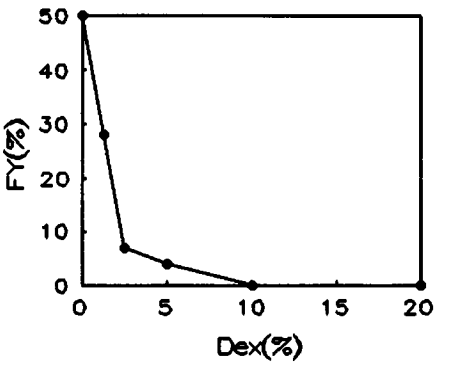

FIGURE 2 The dependence of FY on dilution media with different concentrations of dextran. After cells were pulsed in 10% PEG, they were diluted immediately with media containing different dextran concentrations; then the FY was assayed.

The relative importance of osmotic and colloid-osmotic effect in PEG solution on FY is illustrated in Fig. 3. After the pulse application, the samples were diluted with PBS immediately. The results show that pulsing media with lower osmolarity gives higher FY (comparing 20% in column 1 to 13% in column 2). However, for column 2 and 3, which have almost the same osmolarity (1300 and 1100 mOsm/kg, respectively), but with different PEG:ion ratios (hence, with different colloidal osmotic pressure exerting on cells after pulse application), the FYs are significantly different. For column 3, there is practically no fusion, whereas for column 2, a distinct FY of 13% could be detected.

The colloidal osmotic effect of macromolecules in the medium on the morphological change of the fusion lumen is shown in Fig. 4. The same cell pair was photographed in time

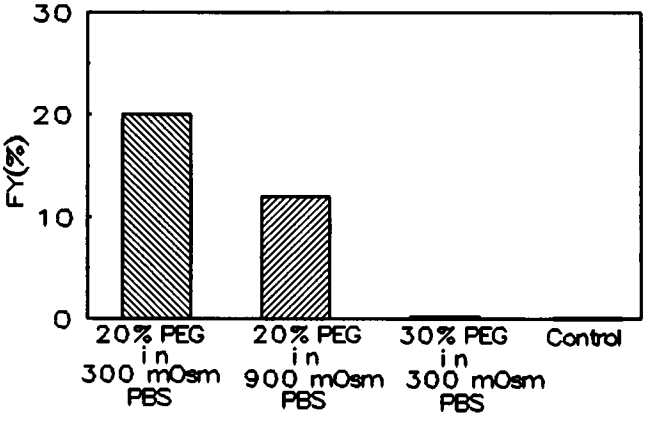

FIGURE 3 The effect of high PEG concentration on FY. After pulse application, cells were diluted immediately by PBS. Column 1 shows the FY when the pulsing medium contained 20% PEG in 300 mOsm/kg PBS (final osmolarity was 700 mOsm/kg); Column 2 represents the FY when cells were in pulsing medium containing 20% PEG in a 900 mOsm/kg concentrated PBS (final osmolarity was 1300 mOsm/kg); Column 3 represents the FY of cells in the pulsing buffer containing 30% PEG in a 300 mOsm/kg concentrated PBS (final osmolarity was 1100 mOsm/kg); and the column 4 is the control to which no pulse was applied.

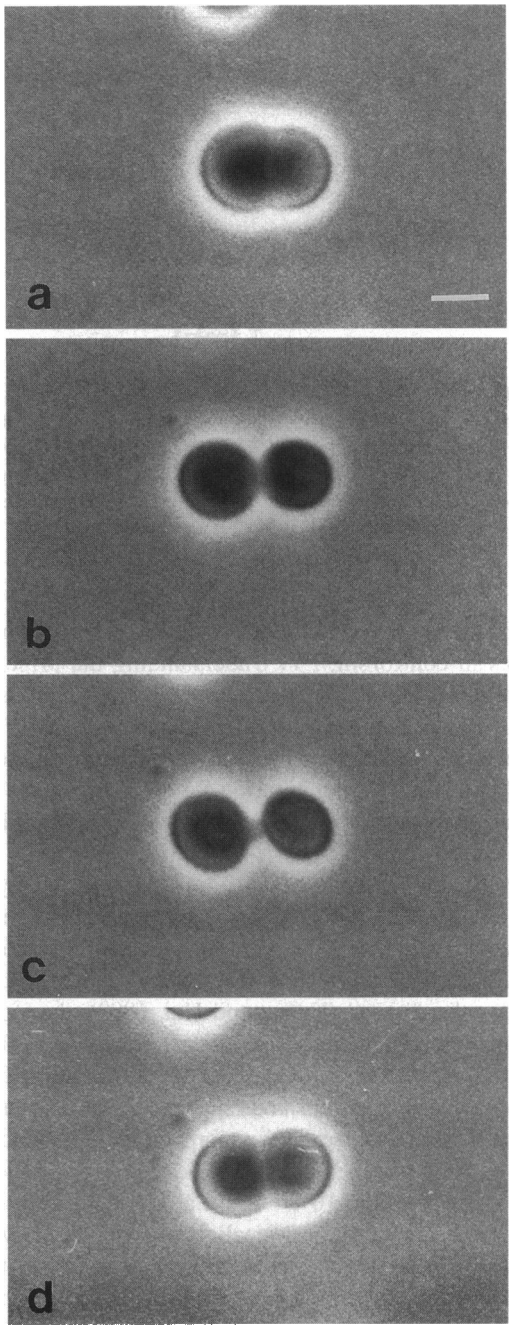

FIGURE 4 Morphologic change of fusion lumen caused by macromolecules. Cells were pulsed in 10% PEG and diluted immediately with PBS. The four micrographs of the same cell pair are shown in time sequence. *a* shows the fused cell pair; *b* and *c* show the shrinkage of the cells, revealing fusion lumen between them after PBS was replaced by 15% dextran; *d* shows the widening of the fusion lumen when cells were returned to PBS. Bar = 10 μm.

sequence. After cells in 10% PEG were pulsed and immediately diluted by PBS, apparent cell fusion was observed (Fig. 4 *a*). The cell pair seems to be connected by a fusion lumen. By replacing the PBS medium with 15% dextran solution (300 mOsm/kg), the same pair of cells were seen with an adjoined narrowed fusion lumen (Fig. 4, *b* and *c*), the dynamic change of the fusion lumen can be seen by comparing Fig. 4 *a* with Fig. 4, *b* and *c*. After returning the cells to PBS from 15% dextran medium, the fusion lumen again returned to its former shape (Fig. 4 *d*) as in Fig. 4 *a*. It should be noted that, at all times, the total osmolarity inside and outside cells are equal and unchanged. The cell swelling and shrinking after the pulse application is controlled mainly by the colloid-osmotic effect of macromolecules both inside (hemoglobin) and outside (dextran) cells. With added dextran, the swelling is reversed.

The morphological behavior of the fused cell pair may be understood by assuming that there are two types of membrane pores created by the permeating pulse. One type is represented by the pre-fusion sites between cell pairs, which may develop into fusion lumens upon cell swelling. The other is the electropores over the remaining cell surface (referred simply as electropores hereafter), connecting intracellular and extracellular space, and being permeable to small molecules but not to macromolecules. Ionic equilibrium is reached soon after the permeating pulse. With hemoglobin inside cells but without dextran in the outside medium, colloidal osmotic effect causes swelling of cells and facilitates fusion site development. These two types of pores were predicted also from a fluorescence leakage and content mixing experiment (Stenger and Hui, 1988).

The time course for fusion is shown in Fig. 5. This figure illustrates the FY at various time points after the samples were diluted from 10% PEG with PBS immediately after pulse application. Cell fusion was observed almost immediately after samples were diluted and incubated. FY increases quickly within the first 1 min or so, and reaches a constant value of 50% after 80 s.

Because the FY depends on the colloidal osmotic swelling of the cells and the subsequent expansion of pre-fusion sites into fusion lumens, both the life time of the electropores, which mediate colloidal osmotic swelling, and the life time of the pre-fusion sites, which are the precursors of the fusion lumens, set the time course of fusion. The effect of the life time of the electropores on FY is demonstrated in Fig. 6. In this experiment, the osmolarity was kept constant, and the swelling was controlled entirely by the colloidal osmotic effect. The resealing of electropores depends upon incubation

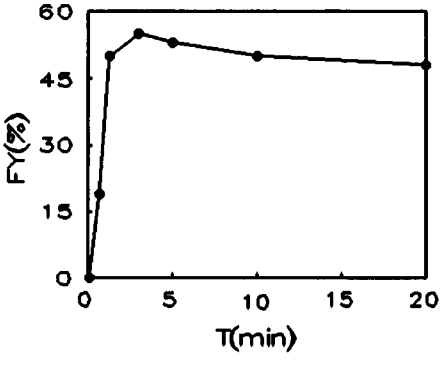

FIGURE 5 The time course for cell fusion. After pulse application, the sample was immediately diluted with PBS. 10% PEG was used as the pulsing medium.

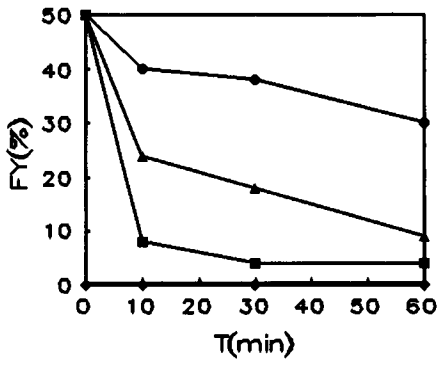

FIGURE 6 The dependence of FY on the delay time between pulse application and dilution at different temperatures. After delayed and incubated at different temperatures for given times in the same pulsing medium of 10% PEG, cells were diluted and assayed for FY. Circles, triangles, and squares correspond to cells incubated in 10% PEG at 4, 20, or 37°C, respectively. Diamonds represent the control, i.e., without pulse treatment.

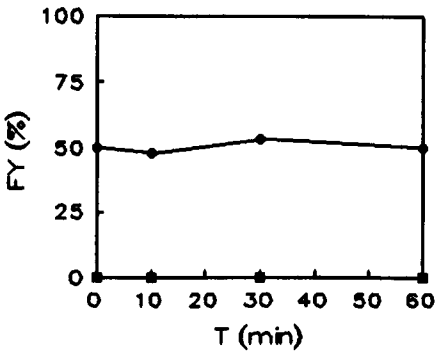

FIGURE 7 Live time of pre-fusion sites. Cells were diluted after certain delay time and incubated at 37°C in the same pulsing medium of 10% PEG. FY was measured after diluting cells from 10% PEG to 100 mOsm/kg PBS. Solid circles represent FY of cells pulsed and diluted with 100 mOsm/kg PBS after some delay time; solid squares represent the control, i.e., under the same conditions except without pulse application.

temperatures. After being pulsed in 10% PEG, the cells were left in the same 10% PEG medium at different temperatures (4, 20, and 37°C) for a specified time for electropores to reseal before isotonic dilution, and then FY was measured. FY decreases with the increase of delay time between pulse and dilution. The longer the delay, the lower the FY. FY also depend on incubation temperatures: the higher the incubation temperature, the lower the FY.

The life time of the pre-fusion sites was measured by imposing osmotic swelling on cells after resting pulsed cells in 10% PEG for various times. In the experiments, after cells were pulsed in 10% PEG, the cells remained in the same 10% PEG medium at 37°C for a specified time, then 100 mOsm/kg PBS medium was used to impose an osmotic shock, and FY was assayed. Fig. 7 shows that up to 1 h delay at 37°C, no significant decrease in FY was detected. The constant FY indicates that pre-fusion sites are long lasting.

DISCUSSION

Although PEG was used in combination with dielectrophoresis to enhance electrofusion yield of protoplasts (Zhelev and Dimitrov, 1989), or in combination with $CaCl_2$ to increase hybrid formation of yeast protoplasts by electrical pulse (Weber et al., 1981), the principle of PEG effects in facilitating cell electrofusion is not clear. In this paper, by combining electrofusion and PEG fusion procedures, we have not only simplified the electrofusion method and obtained high FY, but also determined the basic mechanism of PEG-facilitated electrofusion. PEG can bring cells into good contact by both the depletion effect (Arnold et al., 1990) and by producing deformable cell membranes in osmotically shrunken cells (Zhelev and Dimitrov, 1989). Furthermore, because the optimum FY requires only 10% PEG (Fig. 1), which has little detrimental effect on cells (Boni and Hui, 1987), there is a great potential for the application of this method to cell fusion. Throughout this study, we have asked the questions: why higher concentrations of PEG suppresses FY, what is the role of dilution, and what is the role of electropores in controlling FY. Understanding the principle of the combined PEG and electrofusion method allows us to know the limit and to improve further the method.

Before we discuss the fusion mechanism further, we should point out that the criterion for cell fusion used in this paper is based on morphology rather than on commonly used fluorescence methods. Those two approaches differ in that membrane continuity at pre-fusion sites detected by fluorescence dequenching or energy exchange may or may not be observed morphologically. However, within a short lifetime, fluorescent probe diffusion across a narrow contact point is rather limited (Huang and Hui, 1990); therefore, the difference between fluorescence and morphology methods is not a serious concern. On the other hand, the possibility of fluorescent probe exchange artifacts through close but discontinuous contacts is not a problem in the morphological approach. The results by these two methods should be parallel if not identical.

The optimal FY at 10% but not at higher concentrations of PEG is based on the physical chemistry of cell swelling in polymer solutions. The macromolecules played a vital role in this system. As shown in Fig. 4, by changing the suspending medium from isotonic PBS (300 mOsm/kg) to 15% dextran (300 mOsm/kg), or vise versa, one can modify the fusion lumen size by shrinking it (Fig. 4, *b* and *c*) or expanding it (Fig. 4 *d*), as long as the cells remain permeated by the applied pulse. It is known that dextran can balance intracellular hemoglobin, which causes colloid-osmotic swelling (Abidor et al., 1994b). The phenomenon presented in Fig. 4 is the result of the balanced effect of colloid-osmotic pressure exerted on cells by intra- or extra-cellular macromolecules. The swelling and shrinking of the cell pair in PBS and dextran indicate that there are two types of pores on the membranes after pulse; one is the pores connecting the intracellular and extracellular spaces, through which the colloid-osmotic effect is experienced by cells, and the other type of pores is represented by the pre-fusion sites connecting the cell pair, which can be modified by cell swelling.

The suppression of FY by high PEG concentration (>10%) may be attributed to the shrinkage of pre-fusion sites because of colloid-osmotic effect, in addition to the traditional explanation of high osmolarity causing cell shrinkage and FY decrease. As shown in Fig. 3, cells suspended in 700 mOsm/kg medium (column 1) had higher FY than those suspended in 1300 mOsm/kg medium (column 2). This result is caused by the difference in conventional osmotic effect and has been reported elsewhere (Stenger et al., 1988; Song et al., 1993; Perkins et al., 1991). However, comparing column 2 with column 3, one can see that the media had approximately the same osmolarity (~1200 ± 100 mOsm/kg), but those cells represented in column 3 had higher colloid-osmotic pressure exerting on them (30% PEG ~ 800 mOsm/kg) than those in column 2 (20% PEG ~ 400 mOsm/kg); hence, the shrinkage would be much more severe than those in column 2 after pulse application. The more severe shrinkage could disconnect most pre-fusion sites, thereby suppressing FY. In 20% PEG, the colloidal osmotic shrinkage is limited, and some pre-fusion sites may survive to give limited FY upon dilution.

For the fusion process to complete, the pre-fusion sites have to be expanded into lumens. This process takes time, as shown in Fig. 5. The colloid osmotic swelling as an additional force in transforming the weak pre-fusion sites into fusion lumens is further illustrated in Fig. 2. Dextran is a well known molecule that can prevent pulse-induced cell swelling (Abidor et al., 1994a; Schwister and Deuticke, 1985). Here pulsed cells in 10% PEG were diluted by media with different concentrations of dextran (300 mOsm/kg). The FY decreases dramatically, even to zero, when the dextran concentration reaches 10% or above. On the other hand, diluting the samples with 450 mOsm/kg PBS did not decrease the FY (data not shown). The retardation and inhibition of colloidal osmotic swelling by dextran keep the pre-fusion sites from expanding. Eventually, these pre-fusion sites become disrupted and disconnected by the mechanical agitation of the fluid, and the fusion process is aborted.

The assumption that pre-fusion sites need an extra force, i.e., swelling, to expand them into fusion lumen may be further tested experimentally. After pulse application, if we reseal electropores connecting the intracellular and extracellular space by incubating cells at different temperatures before dilution, we can reduce the colloidal osmotic swelling and decrease the FY. The results shown in Fig. 6 are indeed as predicted, i.e., the longer the incubation time and closer the incubation temperature to 37°C, the more quickly electropores reseal (Zimmermann, 1982), and the lower the FY. After electropores reseal, even replacing PEG-containing media with 300 mOsm/kg PBS did not provide sufficient swelling to restore the FY. However, if 100 mOsm/kg PBS instead of 300 mOsm/kg PBS was used to dilute the pulsed cells, full FY could be regained even if cell dilution was delayed and cells were incubated at 37°C for 1 h (Fig. 7). Apparently, simple osmotic swelling can also achieve the same goal as colloid osmotic swelling in expanding the pre-fusion sites to become fusion lumen, if pre-fusion sites exist. In our case, because of cell aggregation and inhibition of colloid osmotic swelling in 10% PEG, the pre-fusion sites do not reseal, and they exist for a long time.

The optimum FY in Fig. 1 can now be understood to be caused by the balance of two opposite effects caused by PEG: the ability of bringing cells into good contact and the ability of shrinking and disconnecting the pre-fusion sites after pulse application. In 10% PEG, the depletion effect (Arnold et al., 1990) is enough to cause aggregation to bring about cell contacts to form pre-fusion sites upon electric pulse, and to save these sites against mechanical disruption. Yet the colloid-osmotic pressure is not high enough to disconnect pre-fusion sites. This is in contrast to dextran 8000, which does not cause cell aggregation but does cause colloidal osmotic shrinking. No fusion was observed by using dextran 8000 instead of PEG 8000 (results not shown). At higher PEG concentrations, colloidal osmotic shrinkage is too severe to sustain pre-fusion sites. The fusion process is interrupted.

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