High-Efficiency Loading, Transfection, and Fusion of Cells by Electroporation in Two-Phase Polymer Systems

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ABSTRACT A method to concentrate drugs, DNA, or other materials with target cells in two-phase polymer systems for high-efficiency electroloading is described. The two-phase polymer system is utilized for cell and loading material selection, as well as for cell aggregation before electrofusion. The phase mixing of several water-soluble polymers is characterized, and the polyethylene glycol-Dextran (PEG m.w. 8,000 + Dextran m.w. 71,000) mixture is selected to illustrate the advantage of the two-phase systems. Fluorescently labeled Dextran or DNA is loaded into Chinese hamster ovary (CHO) and JTL cells, using electroporation in either the two-phase polymer system or the conventional single-phase suspension. The loading efficiency is 4 to 30 times higher for the two-phase system, with the best advantage at lower applied field range. Transfections of CHO, COS, Melan C, and JTL lymphoid cells using pSV-β-galactosidase (for CHO and COS), pBK-RSV-tyrosinase, and pCP4-fucosidase plasmids, respectively, by electroporation in the two-phase polymer system and the conventional single-phase electroporation method, are compared. The former method is far superior to the latter in terms of efficiency. The threshold and optimal field strengths for the former are significantly lower than those for the latter method, so the former method is more favorable in terms of equipment requirement and safety. Electrofusion efficiency in the two-phase system is comparable to that in polyethylene glycol suspension alone and is a significant improvement from the conventional electrofusion method with dielectrophoresis. The two-phase polymer method is, therefore, a valuable technique for gene delivery to a limited cell source, as in ex vivo gene therapy.

INTRODUCTION

Electroporation has gained popularity as a technique for introducing genetic and other foreign materials into cells. In many cases electroporation has been reported to be more efficient than chemical and viral methods. The principle of electroporation is the application of one or several short and sufficiently strong electric pulses to a cell suspension or monolayer, such that some parts of the cell membrane are broken down temporarily to form minute pores. The surrounding chemicals, such as DNA or other materials, then diffuse or are driven into cells during the time when the cell membrane remains permeable. Many cells survive the electroporation process and recover, although a large proportion of cells may die during the process, depending on the strength and the duration of the electric pulses. The efficiency of loading and transfection depends on electric field parameters, pulsing and recovery media, and cell mortality (see, for instance, Chang et al., 1992). The best loading and transfecting effect usually occurs when the viability is about 50%.

The advantage of electroloading is that the process is applicable to almost all cell types, ranging from bacteria to human tissue cells. Many cells not transfectable by calcium phosphate, DEAE-Dextran, or cationic liposomes can be transfected by electroporation. Unlike methods employing chemical and viral vectors, the parameters in electroporation can be controlled easily and independently to optimize the yield (Hui, 1995; Kubiniec et al., 1990).

A common problem in electroloading (as in most other methods) is that the amount of material inserted into cells is minuscule compared to what is used in the process. Another problem is low viability rate. These problems pose obstacles in the application of conventional transfection methods for gene delivery to a limited supply of target cells, because under such circumstance, the tremendous waste of cells and gene materials cannot be tolerated.

In this paper we report a technique for utilizing two-phase polymer systems to concentrate both the target cells and loading materials (plasmid DNA, drugs, and other chemicals) in one of the two phases, so that the rate of insertion of materials by electroloading is much higher. The two-phase polymer method has been applied to separate cells, proteins, and minerals (Walter et al., 1985, 1992; Moore and Moore, 1989). The principle is that the partition of particles (including macromolecules) into different polymer phases depends on the interfacial energy of the particles and the polymer solutions. By varying the interfacial energy governed by the polymer and salt concentrations, selected particles (cells, macromolecules) can be driven into a given phase, confining the loading materials and target cells to restricted volumes to minimize the waste of cells and loading materials, achieving high-efficiency electroloading.

MATERIALS AND METHODS

Polymers used in the two-phase polymer systems, namely, poly(ethylene glycol) (PEG), poly(propylene glycerol) (PPG), poly(vinyl alcohol) (PVA),

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Dextran (Dx), Ficoll (Fi), and methyl cellulose, were purchased from Sigma (St. Louis, MO). Chinese hamster ovary (CHO) cells were purchased from the American Type Cell Collection (Rockville, MD). COS and melan-C cells were supplied by Dr. Richard Swank, and human B-lymphoid JTL cells were given to us by Dr. Richard Diciocio.

Plasmids coding for pSV-β-galactosidase were extracted from transfected *Escherichia coli*. The plasmid contains a 6821-bp segment composed of a 3737-bp β-galactosidase gene and a 3084 bp pSV promoter, as verified by excision by BAM-H14151 and Hind-H1414 restriction enzymes. The stock solution was diluted to 20 μg/ml before use. Plasmids coding for pBK-RSV-tyrosinase (pBK-RSV 4447 bp, total 6407 bp) were purchased from Stratagene (La Jolla, CA). Plasmids coding for pCP4-fucosidase (pCP4 10.4 kbp, total 12.4 kbp) were provided by Dr. Richard Diciocio. T7 phage DNA was purchased from Sigma and labeled with propidium iodide for measurements by fluorescence microscopy. The DNA concentrations used were 100 μg/ml and 4 μg/ml, respectively, for loading in Hanks' balanced salt solution (HBSS) and two-phase systems.

The polymer systems used in experiments were chosen for optimal partitioning of cells and chemicals (see criteria for optimal conditions later). All polymers used, including FITC-Dextran of two molecular weights (147,000 and 3,000), were purchased from Sigma. In each experiment, cells and chemicals were mixed in respective phases in the initial step. Typical steps are described for the PEG8000/Dx72000 system. In this case, the loading materials (drugs, plasmids, enzymes, and other materials) were mixed in the Dextran phase. Cells may be suspended in either the Dextran or the PEG phase, but the partition is more favorable if the cells are initially placed in the Dextran phase.

In most experiments, the initial volume of the Dextran phase containing cells and loading materials is usually set at one-eighth of the total volume. The rest is PEG phase. The two-phase solution is then vortexed for a second to create an emulsion. Under the optical microscope, the Dextran phase containing cells and loading materials are encapsulated in small droplets suspended in the surrounding PEG solution (Fig. 1 *A*). For most loading materials in media of various ionic strengths, including HBSS or B + K medium (Baker and Knight, 1983), the partition favors the Dextran phase. A typical encapsulation of human B-lymphoid JTL cells within the Dextran-phase droplets suspended in the surrounding PEG solution is shown in Fig. 1 *B*. Fine partition adjustments may be made by changing the ionic strength in the polymer solutions. HBSS contains 125 mM NaCl, 5 mM KCl, 4 mM $CaCl_2 \cdot 2\ H_20$, 2.5 mM $MgCl_2 \cdot 6\ H_20$, and 5 mM Tris·HCl. B + K medium contains 125 mM KCl, 15 mM NaCl, 1.2 mM $MgCl_2$, 3 mM glucose, and 25 mM HEPES.

The mixture is now ready to be exposed to the pulsing electric field. Electroporation is performed using one to three exponential electric pulses (field strength 0.5–2 kV/cm, half-time 300–500 μs) generated by any exponential pulse generator. The samples are typically in a standard narrow cuvette chamber with a volume capacity of 600 μl. Two gold-plated electrodes are inserted within the cuvette chamber. The distance between the two parallel electrodes in our case is 2.5 mm. The number of pulses and the geometry of the pulse chamber are not critical, as long as the field time integral is kept approximately constant (Hui, 1995).

Immediately after electroporation, the suspension may be diluted directly from suspension with buffer solutions. Upon dilution, the system is converted into a single phase. In transfection experiments, the treated cell suspension is transferred to the recovery medium (B + K medium containing 33% bovine serum) and incubated at 37° for 20 min in a CO_2 incubator. After that time, cells are washed and transferred to cell culture medium for further growth. The transfection efficiency is measured by the number of transfected cells, as indicated by X-gal staining in the total cell population at 24 h after transfection. In cell loading experiments, the loaded cells are collected by centrifugation. The loading efficiency is based either on the number of loaded (fluorescent) cells in the total population, as counted in randomly chosen microscopic fields, or on fluorescence-activated cell sorting (FACS) or fluorimetry. Samples of cells are removed from time to time for viability testing by Trypan blue exclusion.

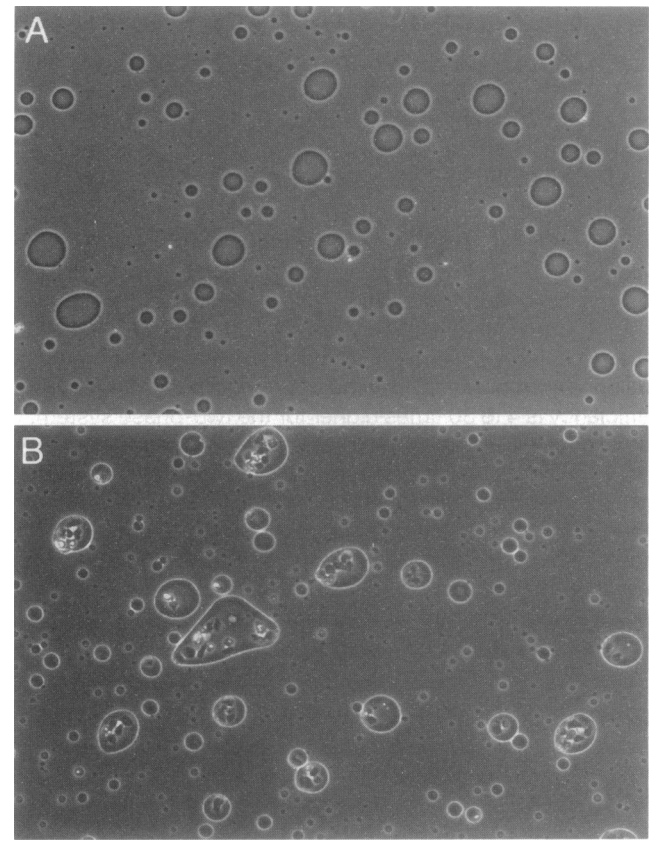

FIGURE 1 Phase contrast micrographs of (*A*) microemulsion of phase separated polymers mixtures of PEG 8000 + Dx 71000 immediately after vortexing, and (*B*) the same microemulsion but with JTL lymphoid cells added to the PEG phase before vortexing. Cells in small droplets of Dextran phase are visible.

RESULTS AND ANALYSES

Characterization of two-phase polymer systems

Our aim is to find two-phase polymer systems that are suitable for concentrating both target cells and loading materials in the same phase, and to reduce the volume of this phase by osmotic control, so that cells and loading materials are encapsulated in this phase in a concentrated form during electroporation. The loading materials are driven into cells during electroporation and by the subsequent colloidal-osmotic swelling of cells after electroporation. The balance of polymer concentrations is such that the post-pulse colloidal osmotic swelling is limited, to reduce cell lysis, but is sufficient to encourage material influx. The two-phase polymer system could also be optimized for cell-cell and cell-liposome attachment to facilitate electrofusion as a means of introducing materials into cells or onto cell surfaces (Mouneimne et al., 1990).

The most commonly used two-phase polymer system is polyethylene glycol (PEG; m.w. 8,000) and Dextran (Dx; m.w. 9,000, 71,000, and 249,000). These polymers are selected because of their nontoxic nature. For these polymers to be used in a two-phase system, the phase separation

regions of these polymers must be determined. Table 1 shows the phase diagrams of three PEG/Dx mixtures. Within practical PEG and Dextran concentrations, there are usable phase separation regions. The PEG (m.w. 8000) + Dextran (m.w. 9000) combination is not a good candidate, because the phase separation range occurs at polymer concentrations that are too high for cell vitality. PEG (m.w. 8,000) + Dextran (m.w. 249,000) and PEG (m.w. 8000) + Dextran (m.w. 71,000) combinations are well separated systems in the concentration ranges of 0% to 25%. We chose to use the PEG 8000 + Dx 71000 system because of better osmotic conditions compared to the PEG 8000 + Dextran 249000 mixture. The PEG 8000 + Dx 71000 mixture is hereafter referred to simply as PEG-Dx.

Because the osmolarity of PEG and Dx varies with their concentrations, their volumes after mixing will readjust to osmotic equilibrium if the PEG-Dextran mixture is phase separated. This osmotic reequilibrium may be exploited to concentrate solutes in one of the phases. PEG 8000 has a higher osmolarity than Dextran 71000 at comparable concentration, therefore the Dextran phase tends to shrink after mixing. This so-called dextran shrinkage (the PEG phase removing water from the dextran phase) depends on both the PEG and dextran concentrations and the initial PEG/Dx volume ratio. To approximate the shrinkage, we assume that the osmolarity is a linear function of the concentration of polymers (which is not exactly true), and that at the same concentrations, the osmolarity of PEG is M times that of Dextran (M is constant only if the linearity relationship for concentration/osmolarity for both polymers holds). In a two-phase mixture, the osmotic equilibrium is reached through the volume change v from one phase to the other,

TABLE 1 Phase diagrams of PEG/Dextran mixtures

	PEG conc (%)					
Dx conc (%)	0	5	10	15	20	25
Dx m.w. 71000						
0	—	—	—	—	—	—
5	—	M	M	M	S	S
10	—	M	M	S	S	S
15	—	M	S	S	S	S
20	—	M	S	S	S	S
25	—	M	S	S	S	S
Dx m.w. 249000						
0	—	—	—	—	—	—
5	—	M	M	S	S	S
10	—	M	S	S	S	S
15	—	M	S	S	S	S
20	—	M	S	S	S	S
25	—	M	S	S	S	S
Dx m.w. 9000						
0	—	—	—	—	—	—
5	—	M	M	M	M	M
10	—	M	M	M	M	M
15	—	M	M	M	M	M
20	—	M	M	M	M	S
25	—	M	M	M	M	S

$$C_pV_p(V_d - v) = MC_dV_d(V_p + v), \quad (1)$$

where C and V, with subscripts p and d, are the concentrations and the initial volumes of PEG and Dextran, respectively. The dextran shrinkage v/V_d can then be expressed as

$$v/V_d = (C_p - MC_d)/\{C_p + MC_d(V_d/V_p)\}. \quad (2)$$

For each mixture of given initial concentrations of PEG and Dextran, the shrinkage is more pronounced at low V_d/V_p ratios. Because the linear relationship between concentration and osmolarity holds for neither dextran nor PEG, both Eqs. 1 and 2 are inexact, and the value of M is not constant. An empirical curve must be determined for each mixture, as shown in Fig. 2. It is possible to find mixtures such that the empirical $M \gg 1$, and at the limit of $V_p \gg V_d$, a high shrinkage ratio is obtained. Such mixtures are found in 10% PEG/15% Dx and 10% PEG/20% Dx. At V_d/V_p ratios of 1:2 to 1:5, the curves do not show appreciable shrinkage, but for ratios lower than 1:5, shrinkage goes up dramatically. It is also seen from the graph that for different PEG-Dx concentrations, the percentage of shrinkage is different. The 20% PEG-15% Dx and 20% PEG-20% Dx combinations show different behaviors, inasmuch as the values of M for these mixtures are different. It is possible to achieve a desired shrinkage for concentrating materials in the Dextran phase with a prior knowledge of the system.

Cell viability in a two-phase polymer system

The selection of PEG 8000 is based on our previous experience that PEG 8000 is the best choice for cell viability. Results of CHO cell viability tests in various polymer solutions are shown in Fig. 3. Cell viability in 10% or 20% PEG alone for more than 1 h is very high. Viabilities in 15% and 20% Dextran are similar and lower than that in PEG solutions. In 25% Dextran, the viability is poor. The cell viability in 10% PEG/20% Dx is about the same as in 10% PEG alone.

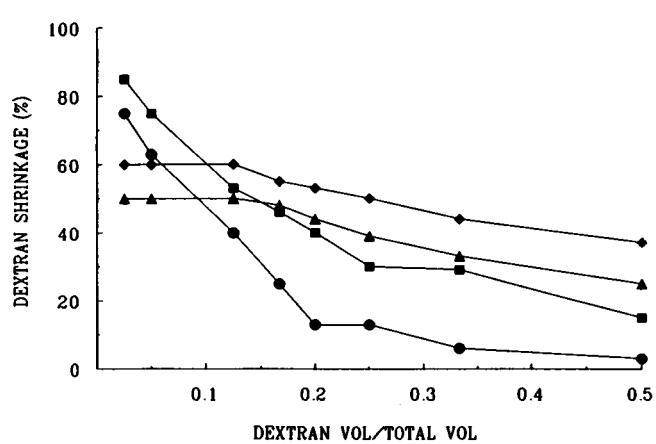

FIGURE 2 Dependence of dextran shrinkage on the dextran volume/total volume fraction. ●, 10% PEG-20% Dx phase; ■, 10% PEG-15% Dx phase; ▲, 20% PEG-20% Dx phase; and ◆, 20% PEG-15% Dx phase.

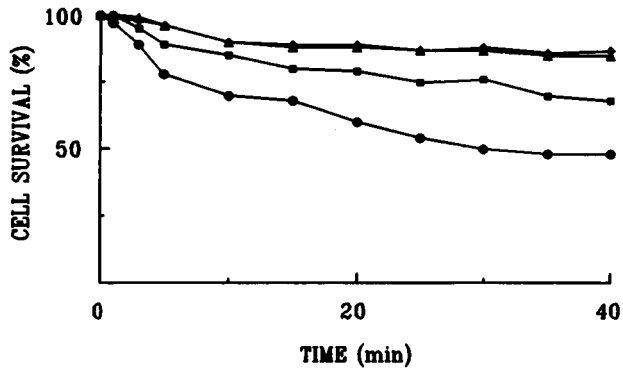

FIGURE 3 CHO cell survival curves with time at different media. ●, 25% Dextran 71000; ■, 20% Dextran 71000; ▲, 10% PEG 8000; ♦, 10% PEG-20% Dextran.

CHO cell survival in two-phase polymer systems after electroporation was tested (Fig. 4). The survival rates in 10% PEG/20% Dx and 20% PEG/20% Dx mixtures are comparable to that in HBSS. Comparing viability curves of 10% PEG/20% Dx in Figs. 3 and 4, one can see that electroporation only reduces the viability sightly in this polymer mixture.

Loading neutral macromolecules of different molecular weights (FITC Dextran) into CHO cells

Different molecular weights of Dextrans labeled with fluorescein isothiocyanate (FITC) are introduced into CHO cells by the two-phase polymer method. We used a high-molecular-weight loading material (FITC-Dextran 147000) and a low-molecular-weight one (FITC-Dextran 3000). We used 2×10^6 cells/ml and 30 μg/ml FITC-dextran. Electroporation was carried out in the conventional suspension in HBSS or in a two-phase system (PEG-Dextran), using the same amount of FITC-Dextran in both samples. Three exponential electric field pulses were applied. The loading efficiency was assayed by the amount of fluorescence in cells as determined by FACS.

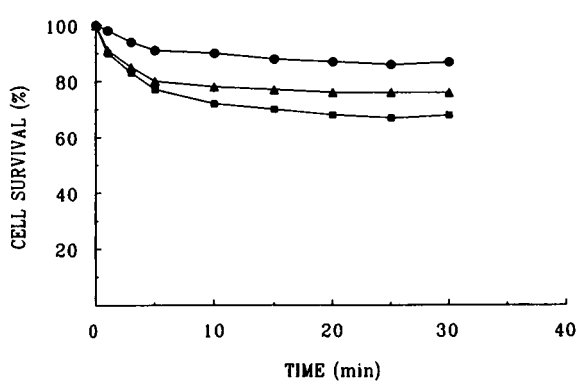

FIGURE 4 CHO cell survival curves with time in one- and two-phase systems after being subjected to three 1.7-kV/cm, 400-μs electric pulses. ●, 10% PEG-20% Dx; ■, 20% PEG-20% Dx; ▲, HBSS medium.

Both the one-phase (in HBSS) and the two-phase samples were pulsed using the same setting of the pulse generator. The HBSS sample has lower resistance than the two-phase polymer sample, therefore the pulse half-time (RC) is shorter. A pair of typical FACS histograms is shown in Fig. 5. In the case of high-molecular-mass (147 kDa) material and at all field strengths, the best loading by the two-phase system is up to 6 times that by the conventional system (Table 2 *A*). The most effective two-phase combination is 10% PEG/20% Dx (or 10% PEG/15% DX). To differentiate the effects due to a shorter pulse (lower resistance) from that due to the two-polymer phase system, a separate experiment was carried out using identical pulse lengths in both cases. The result is shown in Table 2 *A*. Apparently the higher loading is due to the use of the two-phase system.

For the low-molecular-weight (FITC-Dextran 3000) material and at high field strength (2 kV/cm), loading by the conventional method is not uniform. There are two peaks in the fluorescence histogram, indicating two populations of cells—one with very low fluorescence (about 40% of the population) and one with high fluorescence (about 60%). Comparison of the loading with the best obtained using a two-phase system shows that loading by the two-phase method is quite uniform, and the mean fluorescence is slightly less than that of the high loading population by the conventional method. The loading is higher when 10% (instead of 20%) PEG and 20% dextran are used. At a lower field strength (500 V/cm), both the conventional and two-phase methods give uniform loading; the two-phase method is 2.5 times more effective than the conventional method (Table 2 *B*).

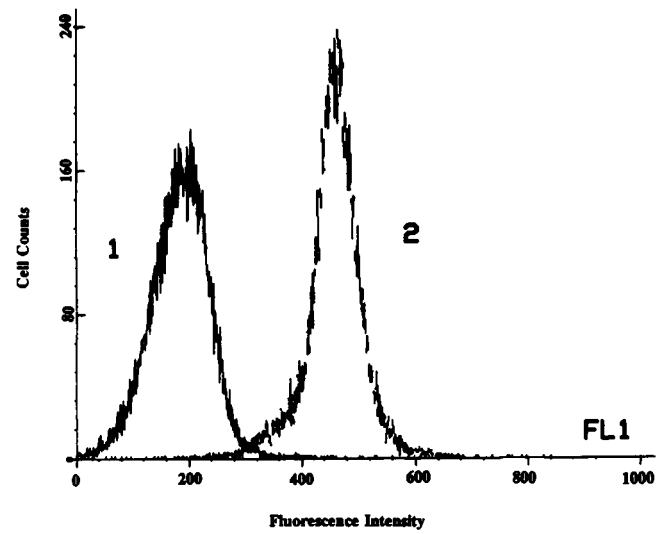

FIGURE 5 FACS histograms of CHO cells electroloaded with FITC-Dextran 147,000 by pulsing in HBSS with three 1.7-kV/cm, 300-μs pulses (*curve 1*), and in 10% PEG-20% Dx with three 1.7-kV/cm, 400-μs pulses (*curve 2*). The same number of cells were counted in each sample, and their relative fluorescence intensity at 530 nm is given along the *x* axis.

TABLE 2 FITC-Dextran loading into CHO cells as measured by FACS

Media	Relative mean fluorescence: Population 1	Relative mean fluorescence: Population 2	Pulse parameters
A. FITC-Dextran 147,000			
BSS	50		0.5 kV/cm; 500 μs
10% PEG-15% Dx	25		0.5 kV/cm; 500 μs
10% PEG-20% Dx	60		0.5 kV/cm; 500 μs
BSS	17		2 kV/cm; 300 μs
10% PEG-15% Dx	100		2 kV/cm; 500 μs
10% PEG-20% Dx	85		2 kV/cm; 500 μs
10% PEG-25% Dx	60		2 kV/cm; 500 μs
20% PEG-10% Dx	28		2 kV/cm; 500 μs
20% PEG-20% Dx	19		2 kV/cm; 500 μs
BSS	12		2 kV/cm; 300 μs
10% PEG-20% Dx	88		2 kV/cm; 300 μs
B. FITC-Dextran 3,000			
BSS	22.5		0.5 kV/cm; 300 μs
10% PEG-20% Dx	50		0.5 kV/cm; 500 μs
BSS	20	118	2 kV/cm; 300 μs
10% PEG-20% Dx	90		2 kV/cm; 500 μs
10% PEG-25% Dx	70		2 kV/cm; 500 μs
20% PEG-10% Dx	40		2 kV/cm; 500 μs
20% PEG-20% Dx	70		2 kV/cm; 500 μs

TABLE 3 Relative efficiencies of the two methods

Medium	% Loading	Electric field parameters
A. Propidium iodide-labeled T7 phage DNA loading into CHO cells measured by fluorescence microscopy		
BSS	0	0.4 kV/cm; 300 μs
10% PEG-20% Dx	88	0.4 kV/cm; 500 μs
BSS	3.3	1.8 kV/cm; 300 μs
10% PEG-20% Dx	93	2 kV/cm; 500 μs
B. Fluorescently labeled pCP4-fucosidase plasmid DNA loading into JTL cells measured by fluorimetry		
Calcium phosphate	0	
Electroporation		
B + K medium	48	0.5 kV/cm; 300 μs
10% PEG-20% Dx	1500	0.5 kV/cm; 500 μs
B + K medium	160	0.8 kV/cm; 200 μs
10% PEG-20% Dx	1350	0.8 kV/cm; 400 μs
B + K medium	430	2 kV/cm; 200 μs
10% PEG-20% Dx	1600	2 kV/cm; 400 μs

Loading fluorescently labeled DNA into CHO and JTL cells

For experiments on CHO cells, we used DNA type T7 Coliphage (m.w. 25×10^6) purchased from Sigma. DNA was labeled with propidium iodide (PI). Twenty micrograms of labeled DNA was used per electroporation run. Cell suspensions were placed into the cuvette chamber and pulsed with three exponential electric field pulses (2 kV/cm and 300-μs halftime). The results from DNA loading are shown in Table 3 *A*. Both high-field-strength (2 kV/cm) and low-field-strength (400 V/cm) pulses were used. In all experiments cell concentration and DNA concentration were the same. Data in Table 3 *A* show that in both types of experiments, DNA loading in the two-phase system is almost 100%, whereas the conventional electroporation method gives no more then 3%. In the two-phase system we used, the most favorable polyethylene glycol-dextran combination is 10% PEG-20% DX. In control, one-phase experiments, when 20 μg labeled DNA was added after the pulses and incubated for 5 min at room temperature, no fluorescent cells were detected.

The conditions for loading the plasmid pCP4-fucosidase DNA into human B-lymphoid JTL cells are tested by fluorimetry, using fluorescently labeled plasmids (Table 3 *B*). The use of calcium phosphate results in no detectable loading. In the comparison between the two-phase system and conventional one-phase electroporations, although the former usually experiences about twice the pulse length because of its higher electric resistance, the loading is 4 to 30 times higher, with better relative efficiency at the lower field strengths. The improvement in efficiency exceeds that expected from the increase in pulse length alone (Hui, 1995), especially at the lower field range.

Transfection

Electrotransfection of CHO cells is done with trypsinized cells in the electroporation cuvette chamber. In transfection experiments 10^6 cells/ml and 5–20 μg of pSV-β-galactosidase plasmid DNA are used per run. The first experiment is to test the transfection efficiency of electroporation in a conventional (in B + K medium) and in a two-phase polymer system. The transfection data shown in Table 4 have typical errors of less than 5% due to counting error as well as variations among repeating experiments. Although electric parameters for paired experiments in a conventional (in B + K medium) and in a two-phase polymer system are not identical (usually the field strength and pulse duration is slightly higher in the latter case, because of higher electric resistance of the samples), the improvement in transfection efficiency for the two-phase method far exceeds that expected from these slight variations in electric parameter. The transfection efficiency of the two-phase method is measured as functions of plasmid DNA concentration, applied field strength, and pulse length. The results are given in Fig. 6, *A–C*, respectively. Apparently the peak field strength is at 1 kV/cm, much lower than the traditional electroporation threshold of 2 kV/cm. The advantage of the two-phase method is optimal at a pulse field strength at which the conventional electroporation method does not work well. A pulse duration longer than 300 μs brings little additional effect. The two-phase method produces about

TABLE 4 Transfection efficiency

Cell and medium	% cell transfected	Electric field parameters
A. CHO cells (pSV-β-gal)		
Electroporation		
B + K medium	9.5	2.1 kV/cm; 260 μs
10% PEG-20% Dx	43.0	2.3 kV/cm; 340 μs
B. COS cells (pSV-β-gal)		
DEAD-Dextran	4.6	
Electroporation		
B + K medium	3.7	2 kV/cm; 300 μs
10% PEG-20% Dx	13.9	2 kV/cm; 500 μs
C. Melan C cells (pSV-β-gal + pbk-RSV-tyr)		
DEAE-Dextran	0	
Electroporation		
B + K medium	0	0.5 kV/cm; 300 μs
10% PEG-20% Dx	6.0	0.5 kV/cm; 500 μs
B + K medium	0	2 kV/cm; 300 μs
10% PEG-20% Dx	5.5	2 kV/cm; 500 μs
D. JTL B-lymphoid cells (pCPr-fuc)		
Relative fucosidase activity		
Electroporation		
B + K medium	—	0.4 kV/cm; 246 μs
10% PEG-20% Dx	6.4	0.4 kV/cm; 264 μs
B + K medium	1.6	1.0 kV/cm; 246 μs
10% PEG-20% Dx	22.4	1.0 kV/cm; 258 μs
B + K medium	26.4	2.0 kV/cm; 246 μs
10% PEG-20% Dx	69.6	2.0 kV/cm; 260 μs

two-thirds of the optimal transfection, even at 5 μg of plasmid DNA per run, a concentration at which conventional electroporation produces no transfection.

COS cells were transfected with the plasmid pSV-β-galactosidase. The results are given in Table 4 *B*. DEAD-Dextran and the conventional electroporation methods give the same transfection efficiency, whereas the two-phase polymer method is three- to fourfold more effective. This cell line is easy to transfect, and the advantage of the two-phase method is not fully appreciated.

Melan C immortalized melanocytes are very difficult to transfect by the conventional DEAD-Dextran method. The cells are isolated from the epidermis of albino mice. The DNA vectors are pBK-RSV-tyrosinase and pSV-β-galactosidase. The transfection results are given in Table 4 *C*. Two field strengths (2 kV/cm and 0.5 kV/cm) are used. At either electrical setting, no significant transfection is detected by the conventional electroporation. Only the two-phase method gives useful transfection.

Human B-lymphoid JTL cells from periphery blood are transfected by the plasmid pCP4-fucosidase. Again, transfection with calcium phosphate and lipofectin is unsuccessful. The transfection results from electroporation in B + K medium and in the two-phase polymer system are compared in Table 4 *D*. The results shown in Table 4 *D* indicate that

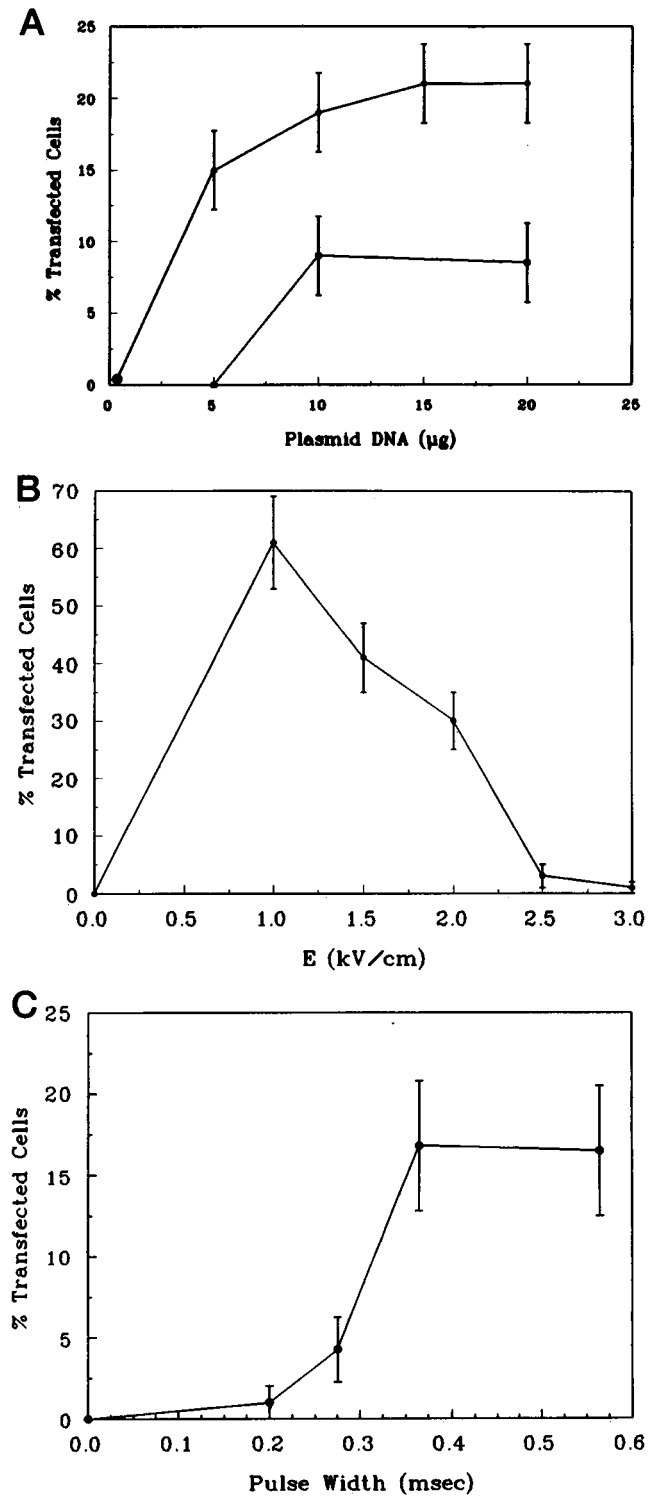

FIGURE 6 The transfection efficiency of CHO cells with the plasmid pSV-β-gal, by electroporation in 10% PEG-20% Dx, is measured as functions of (*A*) plasmid DNA concentration, using three 320-μs pulses at 2.2 kV/cm (the lower curve represents results of electroporating in B + K medium); (*B*) applied field strength, using three 300-μs pulses and 10 μg of plasmid DNA; and (*C*) pulse length, using three 1-kV/cm pulses and 10 μg of plasmid DNA.

the two-phase system has an advantage over conventional electroporation in B + K medium, especially at the low field range. For most other electric parameters tested, the two-phase polymer method is about 3 times more efficient than the conventional electroporation methods carried out in B + K medium.

Fusion

The two-phase polymer method can also be used to facilitate electrofusion. The current practice of electrofusion of cells is to use dielectrophoresis to form "pearl chains" of cells, so that cells are in contact when the fusing pulse is applied. We have experimented with a number of alternative methods to form cell-cell contacts before the application of fusing pulses. Centrifugation pellets (Abidor et al., 1994) as well as PEG-induced aggregation (Stoicheva and Hui, 1994; Li and Hui, 1994) have been successfully tested. These methods have the advantages of being simpler and more effective, eliminating the use of the AC field, thereby cutting the complexity of equipment. The maximum fusion yield of CHO cells using these methods is about 45% viable fusion (Stoicheva and Hui, 1994).

Electrofusion of CHO cells in 10% PEG-20% Dx is measured as a function of the initial Dextran/PEG volume ratio (V_d/V_p). Cells are placed initially in either the Dextran or PEG phase and suspended in a vortexed emulsion. A single 4-kV/cm and 0.72-ms half-time exponential electric pulse is used. Fig. 7 shows the fusion yield of both samples as a function of the Dextran volume fraction. Fusion yield is much higher if cells are first placed in the Dextran phase, in accordance with the expected cell partition behavior (Walter et al., 1992). A peak in fusion yield (60%) is found at the Dextran volume fraction of 0.13. At a low ratio of 0.08, the fusion yield (45%) is equivalent to that in 10% PEG solution alone (Stoicheva and Hui, 1994), indicating that a maximum cell-cell contact that is equivalent to that in 10% PEG is reached. Lower yield at a lower Dextran volume fraction ratio may imply adverse effects of Dextran at the extreme shrinkage condition. Dextran volume ratios higher than 0.13 give lower fusion yield, indicating that the Dextran volume shrinkage is not sufficient to induce good cell-cell contact.

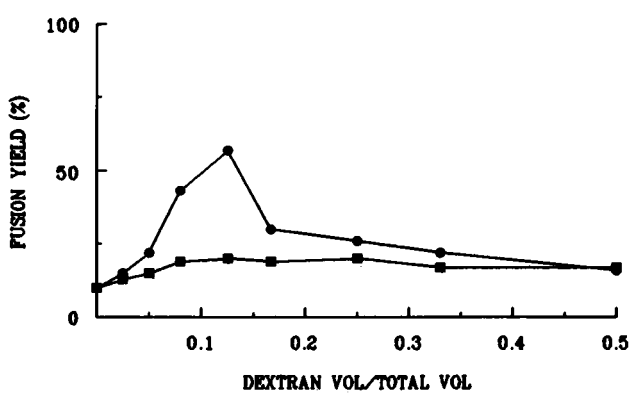

FIGURE 7 Electrofusion yield of CHO cells in 10% PEG-20% Dextran suspension is measured as functions of the initial Dextran volume fraction of the mixture. A single 4-kV/cm and 0.72-ms half-time exponential electric pulse was applied to the suspension to induce fusion. The percentage of fusion was obtained by microscopic counting of syncytia. ●, Samples of cells initially placed in Dextran phase; ■, samples of cells initially placed in PEG phase.

The fact that the electrofusion yield in a two-phase system is higher than that in pure PEG solution signifies a fusion-promotive and/or lysis-protective effect of the phase boundary. This mechanism is currently under investigation. The high fusion field and versatility of this two-phase aggregation method are applicable to cell-membrane-vesicle and cell-liposome fusion. This opens up a new possibility of receptor insertion (Mouneimne et al., 1990) onto cell surfaces, as well as drug and gene delivery into cytoplasm with high efficiency.

Electroporation of *E. coli*

We have also applied the two-phase polymer method to introduce labeled plasmid DNA into bacteria. YOYO-labeled pCMV-βgal plasmid was used to test the introduction of DNA into *E. coli* by the two-phase method as against the conventional single-phase suspension. In the two-phase method, *E. coli* was added to the 10% PEG phase, whereas labeled plasmids were added to the Dextran phase. In the single-phase method, both *E. coli* and DNA, at the same concentrations as in the two-phase method, were added to isotonic sucrose solution. A series of pulse field strength and duration was used. The fluorescence intensity of the sample treated by the two-phase method is about five times that by the conventional single-phase method. The best loading occurs at 4.5 kV/cm, with three pulses of 7 ms each. This condition is much milder than conventional electroporation methods, which call for 10 kV/cm pulse fields for bacteria loading. Preliminary results from the transfection of *E. coli* by pSV-pen-gal plasmids, by both the two-phase and one-phase methods, indicate that the two-phase method produces many more survival colonies of transfected penicillin-resistant cells than the single-phase method (results not shown). The simplicity, high loading and transfection efficiency, and low voltage requirement indicate the two-phase polymer method to be a favorable technique for bacterial transfection as well.

DISCUSSION AND CONCLUSION

Exploiting the selection and osmotic properties of water-soluble, nontoxic, two-phase polymer systems, we have improved the efficiency of electroporation as a delivery vector for foreign chemicals and genes into cultured mammalian cells. We have found a number of advantages in electroloading, electrotransfection, and electrofusion of cells in a two-phase polymer system. The advantages are believed to be based on the concentrating and protecting

effects of the second phase (Dextran) solution in the first (PEG) environment. The advantages vanish if experiments are performed using Dextran and PEG mixtures in the single-phase region of the phase diagram (Table 1); therefore, they are not merely the results of altering the physical properties of the pulsing media by these polymers. To fully utilize the advantages of the two-phase system, experimental characterization of the two-phase system used should be carried out, to select an optimal concentration and volume ratio of the polymers. We have tried a number of concentrations combinations, as well as other polymer systems, including PPG, PVA, Fi, and methyl cellulose. Certain pairs of combinations of these polymers do show phase separation and have preferential partitioning of cells and labeled macromolecules. For instance, cells suspended in PPG, PAA, and Fi are morphologically similar to those in PEG and Dx, and are recoverable. When suspended in the above polymer pairs, cells and most labeled macromolecules, with the exception of some synthetic peptides, partition favorably to the sugar polymer phase. However, the applicable concentration ranges, as limited by phase separation, cytotoxicity, and osmolarity, are narrower than the PEG 8000 + Dx 71000 system we used. Even so, it does not preclude these and other polymer systems from being used for particular cell and chemical combinations.

A significant finding is that the loading efficiency of the two-phase method for both the CHO and the JTL cells, as well as their corresponding transfection efficiency, remains high at lower pulse field strength, as compared to the single-phase method (Table 3). The difference in electric conductance of polymer solutions is probably responsible for lowering the threshold and optimal applied field strengths, by concentrating the potential difference against the confined second-phase droplets of lower electric conductance. As a result of favorable cell survival, the transfection efficiency reaches a maximum at lower field strengths, but the level is much higher than that of the single-phase method (Table 4). This finding enables us to reduce the field strength needed for transfection. With reduced threshold voltages, we can render the electroloading method safer and reduce the cost of equipment, as well as extend the present low-voltage equipment application to bacteria transfection, which usually demands higher voltages.

With increasing demand for high-efficiency nonviral vectors for gene delivery, especially in ex vivo condition, the two-phase method provides a means of concentrating a limited number of target cells and a small amount of specific genetic materials in a confined space to optimize delivery. The method also utilizes the high osmotic pressure against the confined droplet environment to protect cells against post-pulse colloidal-osmotic lysis, and possibly enhances uptake by allowing limited cell swelling. The simplicity of sample preparation, small sample quantity per run, lower and safer power requirement, and stability of polymers make this method easy to scale up once a suitable polymer system is found, and conditions are optimal for a certain application.

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REFERENCES

Abidor, I. G., L.-H. Li, and S. W. Hui. 1994. Studies of cell pellets. II. Osmotic properties, electroporation and related phenomena; membrane interactions. *Biophys. J.* 67:427–435.

Baker, P. F., and D. E. Knight. 1983. High voltage techniques for gaining access to the interior of cells: application to the study of exocytosis and membrane turnover. *Methods Enzymol.* 98:23–37.

Chang, D. C., B. M. Chassy, J. A. Saunders, and A. E. Sowers, editors. 1992. Guide to Electroporation and Electrofusion. Academic Press, San Diego.

Hui, S. W. 1995. The effect of pulse length, and pulse field strength in electrofusion. *In* Methods in Molecular Biology, Vol. 48, Animal Cell Electroporation and Electrofusion Protocols. J. A. Nickoloff, editor. Humana Press, Totowa, NJ. 29–40.

Kubiniec, R. T., H. Liang, and S. W. Hui. 1990. Effects of pulse length and pulse strength on transfection by electroporation. *BioTechniques.* 8:1–3.

Li, L-H., and S. W. Hui. 1994. Characterization of PEG-mediated electrofusion of human erythrocytes. *Biophys. J.* 67:2361–2366.

Morre, D. J., and D. M. Morre. 1989. Cell membrane Techniques. Part II. Preparation of mammalian plasma membranes by aqueous two-phase partition. *Biotechniques.* 7:946–958.

Mouneimne, Y., P.-F. Tosi, Y. Gazitt, and C. Nicolau. 1990. Electroinsertion of full length recombinant CD4 into red blood cell membrane. *Biochim. Biophys. Acta.* 1027:53–58.

Stoicheva, N. G., and S. W. Hui. 1994. Electrically induced fusion of mammalian cells in the presence of polyethylene glycol. *J. Membr. Biol.* 140:177–182.

Walter, H., D. E. Brooks, and D. Fisher, editors. 1985. Partitioning in Aqueous Two-Phase Systems. Theory, Methods, Uses, and Applications to Biotechnology. Academic Press, Orlando.

Walter, H., T. J. Webber, and E. J. Krob. 1992. Effect of cell exposure to top or bottom phase prior to cell partitioning in dextran-poly(ethylene glycol) aqueous phase systems: erythrocytes as a model. *Biochim. Biophys. Acta.* 1105:221–229.