Gene packaging with lipids, peptides and viruses inhibits transfection by electroporation in vitro

Arlena L. Coulberson\textsuperscript{a}, Nicholas V. Hud\textsuperscript{b}, Joseph M. LeDoux\textsuperscript{c}, Igor D. Vilfan\textsuperscript{b}, Mark R. Prausnitz\textsuperscript{a,c,*}

\textsuperscript{a}School of Chemical Engineering, Georgia Institute of Technology, Atlanta, GA 30332-0100, USA
\textsuperscript{b}School of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, GA 30332-0400, USA
\textsuperscript{c}The Wallace H. Coulter Department of Biomedical Engineering at Georgia Tech and Emory University, Atlanta, GA 30332-0535, USA

Received 7 August 2002; accepted 1 November 2002

Abstract

To develop improved methods of gene delivery, packaging DNA in chemical or viral vectors could increase electroporation-mediated transfection. To test this hypothesis, electroporation was applied to DU145 prostate cancer cells incubated with green fluorescent protein-encoded DNA plasmid either naked or packaged with cationic lipid (Lipofectin), polycationic peptide (salmon protamine) or retroviral vectors (Moloney murine leukemia viruses) and then assayed for gene expression and cell viability. Cationic lipid or electroporation alone each significantly increased transfection, but their combination was less effective. Addition of protamine peptide during electroporation was also less effective than electroporation alone. The combination of retroviral vectors and electroporation transfected fewer cells than retrovirus alone. We conclude that the combination of electroporation with chemical or viral vectors does not improve gene transfection in vitro.

\textsuperscript{*}Corresponding author. School of Chemical Engineering, Georgia Institute of Technology, 778 Atlantic Drive, Atlanta, GA 30332-0100, USA. Tel.: +1-404-894-5135; fax: +1-404-894-2866. E-mail address: mark.prausnitz@che.gatech.edu (M.R. Prausnitz).

Keywords: Electroporation; Gene delivery; DNA transfection; Salmon protamine; Lipofectin cationic lipid; Retrovirus vector

1. Introduction

The promise of gene therapy has been significantly limited by the need for safe and effective methods of gene delivery [1,2]. Most clinical studies have packaged genes in viral vectors, which provide relatively efficient gene delivery, but have raised safety concerns [3]. Although enhancement of gene uptake by chemical methods (e.g., cationic lipids, polycationic peptides) is widely used in vitro, efficacy in vivo has been less successful [4,5]. Electroporation is also in common use for in vitro transfection and an increasing number of animal trials have demonstrated effective gene delivery in vivo [6,7]. Each of these methods affects DNA or RNA and
cell membranes to promote transport of genes into cells. Typically, effective gene packaging requires (i) condensation of genes into small particles and (ii) surrounding of genes with compounds that promote cell membrane interactions that lead to translocation into the cytosol.

1.1. Viral, chemical and electrical methods of gene delivery

Viruses package and deliver genes using receptor-mediated binding, membrane fusion and intracellular entry of the virus containing tightly condensed genetic material [8,9]. Retroviruses, which were the first and remain a commonly used viral vector in clinical gene therapy, are RNA viruses that introduce genes permanently into cells by integration into chromosomal DNA [10]. Retroviral particles contain two copies of identical RNA genomes that are wrapped in a protein coat and encapsulated by a lipid bilayer membrane. To gain entry into cells, these viruses attach to specific cell surface receptors through surface proteins that protrude from the viral membrane and, upon internalization, the viral genome is released into the cytoplasm. These RNA genomes are reverse transcribed from RNA to DNA, transported into the nucleus, and integrated into the cell’s genome.

Cationic lipids are believed to complex with DNA in a manner that reduces charge and increases lipophilicity, which in turn facilitates DNA transport across cell membranes [4,11]. This approach is the most widely explored method for nonviral gene therapy. Cationic lipids have been designed to interact with the negatively charged phosphate groups of DNA, thereby reducing their net negative charge. This minimizes charge–charge repulsion at the surface of cell membranes, which facilitates DNA plasmid access to cells. The lipophilic nature of the lipid–DNA package further promotes interaction with cell membranes, which leads to cellular uptake by active transport mechanisms, probably endocytosis, or possibly membrane fusion [12].

Polycationic peptides can package DNA by ionic complexation and condensation into small, dense particles that facilitate cellular uptake by an absorptive mechanism [13]. Protamines are a class of polycationic peptides found naturally in the sperm of many vertebrates that package DNA into condensed toroidal structures by binding their arginine-rich domains to the phosphodiester backbone of DNA in a base sequence-independent fashion. This unique packaging has been shown to increase resistance of DNA to degradation by nucleases and promote DNA transfection [13,14].

In contrast to chemical and viral methods, electroporation does not package genes for delivery, but is believed to increase transfection by creating short-lived pores in cell membranes through which DNA can enter cells [7,15]. Electroporation is able to deliver molecules into almost any cell type by exposing cells to an electric pulse that generates a transmembrane voltage of a few hundred millivolts for microseconds to milliseconds. This is believed to create transmembrane pores of 1–10 nm with lifetimes of milliseconds to minutes. Exogenous molecules, such as DNA, can enter cells through these pores by diffusion and/or electrically driven transport.

1.2. Motivation for this study

Because each of these gene delivery methods is believed to act by different mechanisms, this study was designed to determine if genes packaged using chemical or viral vectors might be more effectively delivered in combination with electroporation. We specifically hypothesized that (i) membrane destabilization by electroporation can facilitate membrane–lipid interactions that lead to uptake and expression of DNA–lipid constructs, (ii) reduction in DNA particle size by condensation with polycationic peptides can facilitate DNA uptake and expression in cells by electroporation and (iii) membrane destabilization by electroporation can facilitate virus invasion into cells and thereby increase RNA uptake, transcription and expression.

To test these hypotheses, we initially optimized electroporation conditions for a model human prostate cancer cell line. We then combined electroporation with a model cationic lipid (Lipofectin), a model polycationic peptide (salmon protamine) and model retroviral vectors (Moloney murine leukemia viruses) and measured levels of transfection with a DNA plasmid encoding for green fluorescent protein or
viral RNA encoding for β-galactosidase. To facilitate interpretations, we also measured cell viability.

2. Materials and methods

Experiments were carried out using suspensions of DU145 prostate cancer cells exposed to electroporation pulses in the presence of DNA packaged with cationic lipids or protamine peptides or RNA packaged in retroviral vectors. Expression of green fluorescent protein was assayed by fluorescence microscopy and cell viability was estimated using a Coulter counter.

2.1. Preparation of cell suspensions

DU145 prostate cells (American Type Culture Collection, Rockville, MD, USA) were grown in complete growth media consisting of 90% RPMI-1640, 10% heat inactivated fetal bovine serum, and 100 μg/ml penicillin–streptomycin antibiotic in a 5% CO₂, 37 °C environment (Model 3110 incubator, Forma Scientific, Marietta, OH, USA). To make cell suspensions, cells were harvested at 30% confluency using trypsin (2.5%, without Ca/Mg) diluted 10-fold in Dulbecco’s phosphate-buffered saline (PBS). For experiments using viruses, cells were harvested using 0.02% EDTA in PBS. All media were obtained from Cellgro (Herndon, VA, USA). After harvest, cell concentration was determined using a hemacytometer (Hausser Scientific, Horsham, PA, USA). Cells were then spun down (1000 g, 6 min, GS-15R centrifuge, Beckman, Palo Alto, CA, USA) and re-suspended to a predetermined concentration between 1 × 10⁶ and 1 × 10⁷ cells/ml in RPMI-1640 containing 25 mM HEPES buffer (RPMI/HEPES, Sigma, St. Louis, MO, USA).

2.2. Addition of DNA / RNA and packaging agents

To assess gene transfection, a DNA plasmid encoding for green fluorescent protein (pEGFP-N1) was purchased from Clontech (Palo Alto, CA, USA), amplified by expression in Escherichia coli (JM109) [16] and purified using a Qiagen giga kit (Valencia, CA, USA). For experiments without packaging agents, a 1.42 mg/ml stock solution of pEGFP-N1 DNA plasmid was diluted to a final concentration of 20 μg/ml (unless otherwise specified) into a suspension of 1 × 10⁶ cells/ml.

Lipofectin (Invitrogen, Carlsbad, CA, USA) was used as a model cationic lipid packaging agent. Samples were prepared by first diluting a stock solution of pEGFP-N1 plasmid into 100 μl of RPMI/HEPES to a final concentration of 20 μg/ml and then diluting 25 μl of 1 mg/ml Lipofectin reagent into 100 μl of RPMI/HEPES and incubating at room temperature for 30–45 min. These two solutions were mixed at the desired ratio, incubated at room temperature for 10–15 min and diluted into a suspension of 1 × 10⁶ cells/ml to achieve a final concentration of 2–40 μg/ml Lipofectin and 20 μg/ml DNA plasmid.

Salmon protamine (salmine) was used as a model polycationic peptide packaging agent. Protamine sulfate (Grade X, Sigma) was extensively dialyzed against 10 mM HCl to convert it from the sulfate form to the chloride form. Samples were then prepared by combining equal amounts of 17 μM protamine in deionized water and 120 μg/ml PEGFP-N1 in 2 mM Tris buffer, incubating the mixture at room temperature for 3 min and then diluting into a suspension of 1 × 10⁶ or 1 × 10⁷ cells/ml to achieve a final concentration of 2.35 μM protamine and 20 μg/ml DNA plasmid. These concentrations provide an approximately 1:1 charge ratio between the protamine and DNA plasmid, which facilitates DNA condensation [17].

Two Moloney murine leukemia viruses containing RNA encoding for β-galactosidase were used as model viral vectors: an amphotropic variant (produced by the Ψ⁻ CRIP packaging cell line, a kind gift of Jeffrey R. Morgan, Shriners Burns Hospital, Boston, MA, USA) that infects human cells and an ecotropic variant (produced by the BAG CRE BAG 2 packaging cell line, ATCC CRL 1858, American Type Culture Collection) that infects only mouse cells. Virus stock solutions were prepared by growing viruses in DMEM medium (Cellgro) with 10% bovine calf serum (HyClone, Logan, UT, USA), 1% penicillin–streptomycin (HyClone) and 1% sodium pyruvate (HyClone). Experimental samples were prepared by spinning down DU145 cells and resuspending them at a concentration of 1 × 10⁶ cells/ml in virus stock solution of approximately 1 × 10⁵
cfu/ml. Polybrene (Sigma) was added at a final concentration of 8 µg/ml to facilitate association between viruses and cells. The cell/virus suspension was transferred to 12-well plates (Becton Dickinson, Franklin Lakes, NJ, USA), spun down (1000 g, 4 °C, 1 h, Allegra 6R Centrifuge, Beckman) and re-suspended in RPMI/HEPES to a final concentration of 1×10^6 cells/ml.

2.3. Electroporation of cells

To perform electroporation, 400 µl of cell suspension was transferred to a 2-mm gap cuvette with parallel-plate aluminum electrodes (BTX, Genetronics, San Diego, CA, USA). Three exponential decay pulses were applied at room temperature (22 °C) at a field strength of 500 V/cm (unless otherwise specified) with 20 ms time constant and 20 s inter-pulse spacing. After “recovery” at 37 °C for 10 min in a water bath (Isotemp 210, Fisher Scientific, Pittsburgh, PA, USA), the cell suspension was transferred to a six-well or 12-well plate (Becton Dickinson) and the cuvette was rinsed into the well plate using 400 µl of growth medium. An additional 1–2 ml of growth medium was added to the well and the cells were incubated for 2–3 days in 5% CO₂ at 37 °C.

Electroporation was carried out using an exponential-decay pulser (ECM 600, BTX) coupled to an oscilloscope (54603B, Agilent, Palo Alto, CA, USA) and current monitor (Model 411, Pearson Electronics, Palo Alto, CA, USA) used to determine the applied voltage, current and pulse length. The electrical resistance offered by RPMI/HEPES solution in a cuvette was determined to be 9.7 Ω by dividing electrode spacing (0.22 cm) by the product of cell suspension conductivity (12.44 mS/cm; Model 32, YSI; Yellow Springs, OH, USA) and electrode area (1.82 cm²) [18]. The field strength was calculated by multiplying resistance and current and then dividing by electrode spacing. For the conditions used in this study, the actual field strength was approximately 90% of the field strength that is commonly and incorrectly determined by dividing applied voltage by electrode spacing. Our method of calculation accounts for voltage drops outside the cell suspension, such as at the electrode–solution interface [19].

2.4. Measurement of transfection/transduction and viability

The number of cells expressing green fluorescent protein in each well was imaged by fluorescence microscopy (Model IX70, Olympus, Osaka, Japan) and counted manually. To detect β-galactosidase expression, each well was rinsed with 2 ml of PBS, fixed in 2 ml of 0.5% glutaraldehyde (Sigma) for 10 min, washed twice with 2 ml of 1 mM MgCl₂ (Sigma) for 5 min and incubated overnight in 5% CO₂ at 37 °C with 1 ml of X-gal solution (ICN, Aurora, OH, USA) to stain cells expressing β-galactosidase blue. Colonies of two, four or eight blue cells were counted with the aid of a dissecting microscope with the assumption that each colony arose from a single infectious event.

Cell viability was estimated by removing adherent (i.e., assumed to be viable) cells from each well, suspending them in 500 µl of growth media, diluting 20 µl of the resulting cell suspension into 20 ml of PBS and counting the total number of cells in a Coulter counter (Multisizer II Analyser, Beckman). The number of cells in each sample divided by the number in control samples yielded the percent cell viability.

2.5. Statistical analysis

Replicate experimental data points are reported as the mean and standard error of the mean. A Student’s t-test was used to compare two experimental conditions. One-way analysis of variance (ANOVA, α=0.05) was performed to compare three or more experimental conditions to a single factor. In all cases, a value of P<0.10 was considered statistically significant.

3. Results and discussion

3.1. Preliminary experiments to optimize electroporation conditions for transfection

Before studying the combined effects of electroporation and packaging agents, we first wanted to optimize conditions for electroporation of DNA plasmid alone. In these initial experiments, DU145
prostate cancer cells were mixed with DNA plasmid encoding green fluorescent protein (GFP) and exposed to electroporation pulses to drive the plasmid into cells and facilitate expression. Previous studies suggest that DNA plasmid concentration and pulse field strength, length and number all affect expression.

To optimize DNA plasmid concentration, we electroporated cells with different concentrations of plasmid. As shown in Fig. 1, transfection increased with increasing DNA plasmid concentration between 0 and 20 mg/ml (ANOVA, P<0.10), but then plateaued at 20–40 mg/ml (ANOVA, P>0.10). This saturation of effect at high DNA plasmid concentration may be explained by previous observations that high levels of intracellular DNA can be toxic to cells [20]. Additional experiments showed that in the absence of electroporation, cells exposed to DNA plasmid over this range of concentrations exhibited almost no transfection (i.e., <<10 cells per sample) (data not shown). We conclude that transfection was optimized for DNA plasmid concentrations greater than or equal to 20 μg/ml.

To optimize field strength, Fig. 2A shows that transfection depended strongly on field strength.
(ANOVA, $P<0.01$), with an apparent maximum at 500 V/cm. This observation can be better understood by examining cell viability (Fig. 2B). For these same cell samples, viability did not significantly change for field strengths up to 500 V/cm (ANOVA, $P>0.10$), but decreased dramatically at higher field strengths (ANOVA, $P<0.001$). We interpret this to mean that transfection initially increased with field strength due to increased DNA uptake into cells without significant loss of cell viability, but at higher field strengths the possible increase in DNA uptake was counteracted by large losses in cell viability that reduced overall transfection.

To optimize pulse length and number, we used information from the literature. Previous studies have shown that transfection is most effective with multiple, long pulses, where optimal pulse length and number probably depend only weakly on DNA plasmid and cell type [21]. Following literature recommendations and extensive experience electroporating DU145 cells in our laboratory [22], we selected three pulses of 20 ms duration as optimal electroporation conditions.

### 3.2. DNA packaged in Lipofectin cationic lipid

To address the first question of this study, we examined the effect of packaging DNA in cationic lipids on electroporation-mediated transfection. We were guided by the hypothesis that membrane destabilization by electroporation can facilitate membrane–lipid interactions that lead to uptake and expression of DNA–lipid constructs. Fig. 3 provides information to answer that question using Lipofectin as a model cationic lipid.

In a first set of control experiments, cells were exposed to DNA–lipid mixtures without electroporation (white bars in Fig. 3A). As expected, the addition of lipid increased transfection levels, but at concentrations of 10 µg/ml or greater, transfection was independent of lipid concentration ($P>0.10$). As a second control experiment, cells were electroporated without lipid, which also increased transfection (black bar on left of Fig. 3A). Comparing these two control experiments, electroporation and cationic lipid were each by themselves equally effective at achieving transfection ($P>0.10$ for lipid concentrations of 10 µg/ml or greater).

Considering the combination of electroporation and cationic lipids, adding increasing amounts of lipid appears to decrease levels of transfection (black bars in Fig. 3A), indicating that electroporation alone is superior to the combination of electroporation and lipid. Comparing to the use of lipid alone (white vs. black bars at each lipid concentration), the addition of electroporation reduced transfection levels, especially at lipid concentrations greater than 10 µg/ml (Student’s $t$-test, $P<0.10$). Combined, this suggests

![Fig. 3. Effect of Lipofectin cationic lipid concentration on (A) transfection and (B) cell viability either with (black bars) or without (white bars) electroporation (mean±S.E., $n=3–7$). These results suggest that DNA–lipid packaging inhibits DNA delivery into cells by electroporation and, at high lipid concentrations, electroporation enhances lipid toxicity.](image-url)
that the use of either electroporation alone or cationic lipid alone is superior to their use in combination.

Examining cell viability in Fig. 3B shows that increasing concentration of lipid in the absence of electroporation (white bars) decreases viability (ANOVA, $P<0.10$), indicating that lipid is toxic. Application of electroporation without lipid (black bar on left) also kills cells. Adding low levels of lipid ($\leq 10 \, \mu g/ml$) during electroporation does not significantly reduce viability relative to electroporation alone (Student’s $t$-test, $P>0.10$), but at higher lipid concentrations the combination kills more cells (Student’s $t$-test, $P<0.01$). Additional experiments combining electroporation and lipid with no DNA plasmid present gave similar results (data not shown), suggesting that the plasmid did not contribute to the observed toxicity.

Together these results suggest that at low lipid concentrations the presence of lipid does not further reduce viability, but does reduce transfection probably because DNA–lipid packaging inhibits DNA delivery into cells by electroporation. This may be due to difficulty transporting large DNA–lipid agglomerates through small electropores, which suggests that the proposed hypothesis of electroporation facilitating lipid-mediated DNA uptake is incorrect. At high lipid concentrations, transfection is reduced due to the combination of reduced viability and reduced intracellular DNA delivery. This suggests that electroporation may enhance lipid toxicity by facilitating intracellular delivery of excess lipid unassociated with DNA. Note that transfection did not increase with addition of lipid beyond 10 $\mu g/ml$ (Fig. 3A), which suggests that at higher concentrations DNA may be fully coated with lipid, which could lead to increasing amounts of free lipid.

3.3. DNA packaged by salmon protamine

The second question of this study concerned the effect of packaging DNA with polycationic peptides on electroporation-mediated transfection. In these experiments, we were guided by the hypothesis that reduction in DNA particle size by condensation with polycations can facilitate DNA uptake and expression in cells by electroporation. Fig. 4 addresses this question using salmon protamine as a model polycationic peptide.

In the absence of electroporation, the addition of protamine modestly increased transfection (Student’s $t$-test, $P<0.01$) (Fig. 4A). Electroporation at low voltage (195 V/cm) did not increase transfection either with or without protamine, but electroporation at high voltage (500 V/cm) increased transfection in both cases (Student’s $t$-test, $P<0.01$). High-voltage electroporation alone appeared to yield greater trans-
fection than when combined with protamine. Thus, in the absence of electroporation, addition of protamine enhances transfection, but in the presence of high-voltage electroporation, addition of protamine is detrimental.

Considering cell viability in Fig. 4B, application of electroporation, either with or without protamine, decreased cell viability with increasing voltage (ANOVA, \( P<0.10 \)). The addition of protamine, either with or without electroporation, generally did not affect viability (Student’s \( t \)-test, \( P>0.10 \); except for 195 V/cm).

Together, these data suggest that protamine alone is able to modestly increase transfection without affecting viability. However, the addition of protamine during electroporation inhibits transfection, probably due to difficulty transporting DNA–protamine condensates through small electropores. It is believed that stable electropores are as small as 1 nm and that DNA slithers through them as a long, linear molecule [23]. Although DNA–protamine condensates are of smaller volume than free DNA, they are condensed into toroidal structures with radii much greater than 1 nm [24]. This suggests that the proposed hypothesis of protamine condensation facilitating transport through electropores is incorrect.

3.4. RNA packaged in retroviral vectors

The third question of this study concerned the effect of packaging RNA in retroviral vectors on electroporation-mediated transfection. In these experiments, we were guided by the hypothesis that membrane destabilization by electroporation can facilitate virus invasion into cells and thereby increase RNA uptake, transcription and expression. Fig. 5 addresses this question using Moloney murine leukemia viruses as model retroviral vectors.

As a positive control experiment, cells were first exposed to an amphotropic virus that normally infects human cells [25]. This resulted in significant transduction of our human prostate cancer cells (Fig. 5). Following established practice, polybrene was added in this experiment to facilitate intimate association between cells and virus particles [26]. When electroporation was added to the protocol, transduction was significantly reduced (Student’s \( t \)-test, \( P<0.10 \)), indicating that electroporation did not facilitate virus invasion into cells. When electroporation was performed without the presence of polybrene, transfection was still further reduced (Student’s \( t \)-test, \( P<0.01 \)), suggesting that electroporation was not capable of replacing polybrene as a means to facilitate cell–virus association. Finally, a negative control experiment involving electroporation with polybrene, but without virus particles, yielded no transduction.

In a second set of experiments, cells were exposed to an ecotropic virus that infects mouse cells but does not infect human cells [27]. No transduction of human prostate cancer cells was observed (data not shown). Use of a broad range of different electroporation conditions—including field strengths of 0–1100 V/cm, pulse lengths of 0–40 ms and 0–10 pulses—also yielded no transduction (data not shown). This suggests that the association of viral surface proteins with murine cell receptors required for infection could not be replaced by electroporation-mediated membrane destabilization.

Together, these data suggest that the hypothesis that membrane destabilization by electroporation can facilitate virus invasion into cells is incorrect. Al-
though viability data could not be obtained using the viral transduction assay, electroporation probably reduced cell viability without enhancing viral entry into cells and therefore reduced transduction.

4. Conclusions

This study tested the hypotheses that (i) membrane destabilization by electroporation can facilitate membrane–lipid interactions that lead to uptake and expression of DNA–lipid constructs, (ii) reduction in DNA particle size by condensation with polycationic peptides can facilitate DNA uptake and expression in cells by electroporation and (iii) membrane destabilization by electroporation can facilitate virus invasion into cells and thereby increase RNA uptake, transcription and expression. Our data suggest that each of these hypotheses is incorrect. The combination of electroporation and cationic lipid (Lipofectin) reduced transfection, probably because electropores were too small to permit entry of large DNA–lipid constructs, but were large enough for entry of toxic, free lipid molecules. DNA condensation using polycationic peptides (salmon protamine) did not affect cell viability, but probably reduced transfection by creating DNA–peptide condensates of dimensions too large to fit through electropores. Electroporation did not increase transduction using RNA packaged in retroviral vectors (Moloney murine leukemia viruses), probably because electroporation did not facilitate virus–cell interactions that led to infection, but did kill cells. In conclusion, electroporation, as well as cationic lipids, polycationic peptides and retroviral vectors, each individually enhanced gene transfection, but the combination of electroporation with these chemical and viral methods was detrimental.

Acknowledgements

We thank Esi Ghartey-Tagoe, Delfi Krishna and Robyn Schlicher for helpful discussions. This work was supported in part by the National Science Foundation (A.L.C., M.R.P.), National Institutes of Health (N.V.H., I.V.) and a GEM fellowship (A.L.C.).

References

[19] U. Pliquett, E.A. Gift, J.C. Weaver, Determination of the electric field and anomalous heating caused by exponential