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● *Original Contribution*

SHOCK WAVES AND DNA-CATIONIC LIPID ASSEMBLIES: A SYNERGISTIC APPROACH TO EXPRESS EXOGENOUS GENES IN HUMAN CELLS

Blanca Millán-Chiu,* Giselle Camacho,* Alfredo Varela-Echavarría,† Elisa Tamariz,†§ Francisco Fernández,* Luz M. López-Marín,* and Achim M. Loske*

*Centro de Física Aplicada y Tecnología Avanzada, Universidad Nacional Autónoma de México, Campus Juriquilla, Querétaro, México; and †Instituto de Neurobiología, Universidad Nacional Autónoma de México, Campus Juriquilla, Querétaro, México

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Abstract—Cationic lipid/DNA complexes (lipoplexes) represent a powerful tool for cell transfection; however, their use is still limited by important concerns, including toxicity and poor internalization into deep tissues. In this work, we investigated the use of shock wave-induced acoustic cavitation *in vitro* for the transfection of lipoplexes in human embryo kidney 293 cells. We selected shock waves with the ability to internalize 10-kDa fluorescein isothiocyanate-dextran into cells while maintaining survival rates above 50%. Cell transfection was tested using the green fluorescent protein-encoding plasmid *pCX::GFPGPI2*. Confocal microscopy and fluorescence-assisted cell sorting analyses revealed successful transfection after treatments ranging from 1 to 3 min using 60 to 180 shock waves at peak amplitudes of 12.3 ± 1.5 MPa. Interestingly, the combination of shock waves and lipoplexes induced a 3.1- and 3.8-fold increase in the expression of the reporter gene compared with the use of lipoplexes or shock waves alone, respectively. These results indicate that cationic DNA assembly and shock waves act in a synergistic manner to promote transfection of human cells, revealing a potential approach for non-invasive site-specific gene therapy.
(E-mail: lmlm@unam.mx)

Key Words: **Cell transfection, Shock waves, Lipoplexes, Acoustic cavitation, HEK 293 cells.**

INTRODUCTION

In concert with the genomic revolution, a wide set of medical innovations are expected to be launched in the near future. In particular, the ability to reprogram cellular function through the delivery of nucleic acids, referred to as gene therapy, is emerging as a powerful tool for treating otherwise incurable diseases. Nevertheless, the use of viral vectors, which are the most common tools for nucleic acid delivery, is currently limited to life-threatening cases because they can trigger difficult-to-control, fatal side effects (Wang et al. 2013). To address this problem, chemical and physical methods for gene transfer have been increasingly explored in recent years. One of the most important chemical tools for cell transfection is the use of cationic lipids. However, although cationic lipids represent powerful tools for *in vitro* cell transfection, their *in vivo* use poses several important concerns, including toxicity associated with the doses required for systemic delivery (Morille et al. 2008) and poor internalization into deep tissues (Kostarelos et al. 2005). In this regard, methods that are intended to permeabilize cells, thereby promoting the transient destabilization of cell membranes, have the potential to enhance nucleic acid delivery through cationic vectors. The most studied physical methods for cell membrane permeabilization are electroporation, that is, cell permeabilization through the application of an electrical field (Mir 2009), and sonoporation, a method based on the application of high-frequency (approximately 0.2 to 2 MHz) ultrasound (Liu et al. 2012). In addition to these methods, cells can be permeabilized by underwater shock waves, that is, sharp discontinuities involving a sudden change in pressure and density. The mechanisms associated with cell membrane permeabilization by shock waves can be explained by acoustic cavitation, a phenomenon in which microbubbles within a cell suspension or tissue are compressed by the positive pressure pulse of each shock wave. After the shock waves pass, these microbubbles expand, significantly increasing their volume in only 50 to 100 μs, until

Address correspondence to: Luz M. López-Marín, Centro de Física Aplicada y Tecnología Avanzada, Universidad Nacional Autónoma de México, Boulevard Juriquilla 3001, Querétaro, Qro. 76230, México. E-mail: lmlm@unam.mx
§Present address: Instituto de Ciencias de la Salud, Universidad Veracruzana, Av. Castelazo Ayala s/n, 91190 Xalapa, Ver., Mexico.

they collapse violently after approximately 250 to 600 μs. In most cases, the pressure difference outside each bubble creates a microjet of fluid that burrows through the bubble, exiting it at a high speed (up to 400 m/s) and acting as a microsyringe. According to Ohl and Ikink (2003), the amount of liquid that can be injected into a cell by a microjet is approximately $0.1R^3$, where R is the bubble radius before arrival of the shock wave. Furthermore, the collision between the inward-moving wall of the bubble and the microjet produces a strong secondary shock wave, which represents an additional mechanism for membrane permeabilization.

Shock waves have been extensively used in clinical medicine since the early 1980s, when they were applied for extracorporeal lithotripsy (Chaussy et al. 1980). Shock waves have proven to be a non-invasive tolerable method for calculus pulverization and various orthopedic/regenerative treatments (Loske 2007). In particular, a set of studies addressed the use of shock waves for cell membrane permeabilization, indicating the great potential of this physical method for gene transfection (Bao et al. 1998; Bekeredjian et al. 2007; Lauer et al. 1997; Michel et al. 2004; Murata et al. 2007; Tschoep et al. 2001). Here we report the use of shock waves to permeabilize human cells and promote transfection with both cationic lipid-assembled and naked DNA.

METHODS

Plasmid DNA

Enhanced green fluorescent protein was used as a reporter in a plasmid vector. The vector consists of a 6-kb fusion construct containing enhanced green fluorescent protein, an N-terminal signal sequence from the sperm secretory protein acrosin and a C-terminal GPI (glycophosphatidylinositol) anchor sequence under the regulation of a CAG promoter (Rhee et al. 2006). The plasmid was propagated in XL1-blue subcloning-grade ultracompetent cells (Stratagene, La Jolla, CA, USA), and purified using an endotoxin-free plasmid DNA purification kit (MaxiKit, Qiagen, Valencia, CA, USA). The purified plasmid was quantified by UV spectrophotometry using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) to determine the absorbance ratio at 260/280 nm. Intact and restriction endonuclease-digested samples were subjected to gel electrophoresis in 0.8% agarose to verify the identity and purity of the plasmid. After development, the gels were stained with SYBR Safe DNA gel stain (Molecular Probes, Eugene, OR, USA) and analyzed using a Gel Doc EZ Imager (Bio-Rad Laboratories, Philadelphia, PA, USA).

Cell culture

Human embryonic kidney (HEK) 293 cells were selected for this study because they are amenable to most classic gene transfection methods, minimizing the intrinsic transfection limitations of a given model. Cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cultures were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen Life Science Technologies, Grand Island, NY, USA), 100 IU/mL penicillin and 100 μg/mL streptomycin (Sigma Aldrich, St. Louis, MO, USA). Cells at 75%–80% confluence were gently detached from tissue culture plates (10 cm in diameter) by 5 min treatment with 2 mL trypsin-EDTA (Invitrogen Life Science Technologies). Proteolysis was blocked by the addition of 4 mL complete DMEM, and the cells were washed and suspended at a density of 2×10^6 cells/mL in culture medium in the presence of fluorescent dye, plasmid or plasmid/cationic lipids (see below). Cells were counted in a Neubauer chamber and seeded on culture plates with supplemented DMEM. The plates were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 48 h. Cell survival was determined in duplicate for each experimental condition using the trypan blue exclusion method in a Neubauer chamber. Only wells containing ≥95% viable cells were used for shock wave experiments.

Shock wave treatments

For fluorescent dye internalization, a 400 μM solution of either calcein or neutral fluorescein isothiocyanate (FITC)-dextran (FD-10, molecular weight = 10,000 Da, Sigma) in phosphate-buffered saline (PBS) was mixed with a volume of HEK 293 cells to achieve a final concentration of 200 μM dye and 2×10^6 cells/mL. Immediately after shock wave treatment, the cells were transferred to a centrifuge tube, washed with 5 mL PBS, spun at 1500 rpm for 5 min and resuspended in PBS. After additional washes with PBS, the cells were fixed with 3.5% paraformaldehyde and conserved at 0°C–4°C until analysis.

For transfection, the cell suspension was prepared at 2×10^6 cells in 0.9 mL. Naked plasmid was dissolved in endotoxin-free purified water at 3.33 mg/mL, and 3 μL of plasmid solution with 97 μL of DMEM was added to the cell suspension immediately before treatment (*i.e.,* 10 μg plasmid/vial). Alternatively, 100 μL of self-assembled DNA-cationic lipid complexes was used at an equivalent DNA concentration (10 μg plasmid/vial). Complexes were prepared by incubating the plasmid DNA with a mixture of DMEM and commercially available cationic lipids (FuGene 6, Roche Applied Science, Penzberg, Germany) for 20 min. The mixture was prepared so that each 100 μL contained 15 μL cationic lipid solution and 10 μg plasmid.

Cell suspensions were prepared in sterile conditions with fluorescent dye or plasmid immediately before treatment, and 1-mL aliquots were placed into sterile