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● *Original Contribution*

INVESTIGATION OF OPTIMIZED TREATMENT CONDITIONS FOR ACOUSTIC-TRANSFECTION TECHNIQUE FOR INTRACELLULAR DELIVERY OF MACROMOLECULES

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Abstract—Manipulation of cellular functions and structures by introduction of genetic materials inside cells has been one of the most prominent research areas in biomedicine. High-frequency ultrasound acoustic-transfection has recently been developed and confirmed by intracellular delivery of small molecules into HeLa cells at the single-cell level with high cell viability. After we proved the concept underlying the acoustic-transfection technique, treatment conditions for different human cancer cell lines have been intensively investigated to further develop acoustic-transfection as a versatile and adaptable transfection method by satisfying the requirements of high-delivery efficiency and cell membrane permeability with minimal membrane disruption. To determine optimal treatment conditions for different cell lines, we developed a quantitative intracellular delivery score based on delivery efficiency, cell membrane permeability and cell viability after 4 and 20 h of treatment. The intracellular delivery of macromolecules and the simultaneous intracellular delivery of two molecules under optimal treatment conditions were successfully achieved. We found that DNA plasmid was delivered by acoustic-transfection technique into epiblast stem cells, which expressed transient mCherry fluorescence. (E-mail: sangpil@usc.edu) **© 2017 World Federation for Ultrasound in Medicine & Biology. All rights reserved.**

Key Words: **Acoustic-transfection with high-frequency ultrasound, Intracellular delivery of macromolecules, Single-cell application, Optimized treatment conditions, Epiblast stem cell.**

INTRODUCTION

The intracellular delivery of cell membrane-impermeable molecules into the cytoplasm of cells has been one of the most attractive areas of research in biomedicine (Chen 2010; Glover et al. 2005; Kim et al. 2009; Leader et al. 2008; Shi et al. 2015). Controlling cellular functions and structures by delivering exogenous therapeutic or genetic materials into cells enables the exploration for visualization of specific cellular structures (Herce et al. 2013), analysis of cell mechanisms (Weill et al. 2008) and treatment of genetic diseases (Nabel et al. 1990). In these applications, investigations into unique expression profiles of individual cells at the single-cell level have increasingly emphasized elucidation of potentially undetected particular cellular functions and structures of individual cells and cell-to-cell interaction within a cell population (Chattopadhyay et al. 2014; Hoppe et al. 2014; Wang et al. 2009). A range of techniques for delivering foreign molecules into cells have been developed, such as virus-mediated transfection (Tanaka et al. 1997), lipid-mediated transfection (Felgner et al. 1987), microinjection (Meacham et al. 2014; Mehier-Humbert and Guy 2005), electroporation (Escoffre et al. 2007; Mehier-Humbert and Guy 2005; Palanker et al. 2006) and optical transfection (Mehier-Humbert and Guy 2005; Watanabe et al. 2004; Zeira et al. 2007), Virus-mediated transfection is highly efficient, whereas viral vectors possess limited packaging capacity and sometimes cannot be specifically integrated into the target cell. Lipid-mediated transfection has relatively low toxicity; however, transfection efficiency relies largely on cell types and culture conditions. Microinjection is straightforward and efficient, whereas this technique requires direct perforation of cell membranes, which may result in physical damage to the cells. Electroporation and optical transfection have relatively high transfection efficiency; however, these methods may cause irreversible membrane damage under the influence of electrical fields and shorter wavelengths of laser radiation, respectively.

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Therefore, there is still a need for the development of approaches that are capable of simultaneously satisfying the requirements of high transfection efficiency, minimal cytotoxicity and single-cell selectivity independent of cell types and transfer molecules (Antkowiak et al. 2013; Kawamura et al. 2009; Kim and Eberwine 2010; Kollmannsperger et al. 2016; Nishikawa and Goldstein 2008; Sharei et al. 2013).

Acoustic-transfection with high-frequency ultrasound was developed in our laboratory as a new transfection method for delivering membrane-impermeable molecules into the cytoplasm of cells at ultrasound frequencies higher than 150 MHz (Yoon et al. 2015, 2016a, 2016b, 2017). A tightly focused high-frequency ultrasound beam physically stretches the cell lipid bilayer on plasma membranes, which generates transient and reversible holes in the cell membranes. The focused ultrasound beam is on for only a single ultrasound pulse of a few microseconds within an area approximately 10 μm in diameter, which might result in the formation of a physical pathway for introducing membrane-impermeable molecules into cell cytoplasm and passive diffusion driven by the concentration gradient across transiently generated holes. We have also developed an impedance matching network to optimize the excitation frequency of transducers (Kim et al. 2016). The acoustic-transfection technique was confirmed by live-cell fluorescence imaging of the time-based intensity changes in delivered Ca^{2+} and propidium iodide (PI) molecules and 3-kDa dextran labeled with Alexa 488 delivered into HeLa cells at the single-cell level. In addition, acoustic-transfection targets cells remotely with minimum cell toxicity (Yoon et al. 2016a, 2016b).

Additional experiments were necessary to determine the optimal acoustic-transfection conditions that enable the acoustic-transfection technique to be a versatile and viable transfection tool. Optimal treatment conditions for the acoustic-transfection technique across different cell types and molecules were needed to achieve high delivery efficiency and high cell membrane permeability with minimal membrane disruption.

In this article, we give a detailed description of how we determined the optimal treatment conditions at which to achieve high delivery efficiency with low cytotoxicity. Optimal treatment conditions for acoustic-transfection using high-frequency ultrasound were investigated in four human cancer cell lines: human cervical cancer cell (HeLa), Michigan Cancer Foundation-10A (MCF-10A), Michigan Cancer Foundation-7 (MCF-7) and M. D. Anderson metastatic breast 231 (MDA-MB231). To determine the optimal treatment conditions, a criterion termed the *intracellular delivery score* (IDS) was proposed and calculated from results—obtained on four human cancer cell lines—for (i) delivery efficiency; (ii) cell membrane permeability study, which was measured as the fluorescence intensity of propidium iodide (PI) after treatment on target cells; and (iii) a cell viability study after 4 and 20 h of treatment under different treatment conditions and a control condition. Adjustable parameters for acoustic-transfection were peak-to-peak voltage (V_{pp}), treatment time (T_t) and number of cycles used: six different V_{pp} for six different T_t with 1 cycle. After live-cell fluorescence imaging of treated cells, the fluorescence intensity changes in PI inside treated cells under different treatment conditions were compared with those of background fluorescence intensity to quantify and calculate delivery efficiency and cell membrane permeability based on IDS. The cell viability study, the results of which were needed to compute IDS, was performed with a LIVE/DEAD Cell Imaging kit after 4 and 20 h of treatment. The IDS was plotted with respect to six values of V_{pp} at each of the six values of T_t on an intracellular delivery graph (IDG) for easier viewing. We chose optimal treatment conditions for IDSs >9 points on the IDG, which indicates high delivery efficiency and high cell membrane permeability with minimal effects on cells. We conducted experiments on the intracellular delivery of 70-kDa dextran labeled with Oregon Green and simultaneous intracellular delivery of two molecules such as 70-kDa dextran and PI into the four human cancer cell lines under optimal treatment conditions. By use of one of the optimized treatment conditions, mCherry-expressing DNA plasmid was delivered into epiblast stem cells to further confirm the performance of acoustic-transfection technique.

METHODS

High-frequency ultrasonic transducer and impedance matching network

A single-element lithium niobate ($LiNbO_3$) high-frequency ultrasonic transducer was designed and fabricated using a previously reported approach (Lam et al. 2013). The aperture size and *f*-number of the fabricated ultrasonic transducer were 1 mm and 1, respectively. To optimize power transfer and efficiency by minimizing reflections between the ultrasonic transducer and the excitation source, a custom-built impedance matching network (IMN) was developed. After the suitable topology and component values of IMN were determined using the Smith chart, IMN was optimized with a topology (series-added capacitor/shunt-added inductor) and component values (22 pF/22 nH), and thus it was integrated with the ultrasonic transducer. A detailed description of the IMN optimization process for high-frequency ultrasonic transducers is provided by Kim et al. (2016). Figure 1a shows the pulse-echo waveform and echo spectrum obtained from the ultrasonic transducer with IMN; Figure 1b shows a B-mode wire target image along with the axial and lateral brightness profiles measured by scanning a 2.5-μm-diameter tungsten wire target with a pulser/receiver generating energy