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Ultrasound-mediated intracellular delivery of fluorescent dyes and DNA into microalgal cells☆

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ABSTRACT

Microalgae cells gained much attention as resources for food, drug, and biofuels. For last few decades, researchers have made efforts to increase the productivity in utilizing microalgae through genetic improvement by introducing plasmids or genes. However, the barriers of the cell wall and membrane hindered the efficient intracellular delivery and required the development of more effective strategy. Herein, we introduce the ultrasound-mediated transfer as a noninvasive and non-viral strategy and demonstrate its effectiveness in delivering fluorescent dyes and DNA into *Chlamydomonas reinhardtii* and *Cholera vulgaris* at high-frequency low-intensity (HFLI) (600 kHz-15 W) and low-frequency high-intensity (LFHI) (40 kHz-161 W) conditions. The HFLI ultrasound showed superior intracellular delivery of calcein, quantum dots, and cyano fluorescent protein (CFP) compared to the LFHI. The efficiency of the transient CFP expression by the HFLI was nearly two times greater than that of the conventional electroporation. PCR results also confirmed successful transfection of hygromycin phosphotransferase (*hpt*). The HFLI-induced observed enhancement in transfection presumably attributes to the mechanical resonance under stable cavitation oscillation, enhancing cell membrane permeability. To the best of our knowledge, this is the first report to show the HFLI ultrasound as an effective and viable route for intracellular delivery into microalgal cells.

1. Introduction

Microalgae are the important primary producers in the aquatic ecosystem that have major ecological and economic significance. Ecologically, they are the basis of the aquatic food web and contribute to the carbon fixation, having a massive impact on the global carbon cycle. Economically, diverse microalgae are used for expensive auxiliary products such as cosmetic compounds, nano-materials, recombinant proteins, drugs, vaccines, antibodies, and food [1]. Furthermore, the main storage chemical compounds of microalgae, lipids (40–60%), are considered as one of the most suitable and alternative feedstock for biofuel production. A number of different approaches are being made to improve the efficiency of photosynthesis and/or lipid production of algal cells by making genetic modifications for better secretion of essential compounds from live algal cells [2].

For genetic improvement, the efficient delivery of materials of interests into the algal cells would be critical [3,4]. Unlike the animal cells that are covered with soft lipid membrane, the plant, algal, and bacterial cells feature rigid cell walls made of complex carbohydrates. For easy penetration through the barriers of the cell wall as well as plasma membrane and nuclear membrane, a conventional chemical treatment with transfection reagents would not be sufficient, which led to utilization of more direct strategies such as glass beads or silicon carbide whiskers, particle bombardment or electroporation [5].

Alternatively, the use of ultrasound has been shown to enhance the permeability of cell membranes, allowing larger molecules to pass through the plasma membrane via the phenomenon known as "sonoporation" [6,7,8]. The mechanism of the sonoporation, which generates transient holes in the plasma membrane, primarily attributes to the formation of momentary microperforations induced by cavitation effects. However, the collapse of these transient cavitation bubbles generated at low frequencies can induce extreme local pressures of ~10^5 atm and temperature of ~5000 K [9]; under such severe conditions, cell viability would significantly decrease. For instance, Azencott et al. [10] reported the reduction in cell viability to 25% when low frequency (~24 kHz) ultrasound was applied on *Chlamydomonas reinhardtii* to deliver calcein. Nevertheless, the sonoporation technique still has benefits such as affordability and simplicity in the experimental procedures. Therefore, the main motivation behind this research was to determine a condition in which the intracellular delivery into microalgal cells was improved while maintaining viability using the ultrasonic stimulation. For that, we tested two

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experimental conditions of the high-frequency low-intensity (HFLI) (600 kHz-15 W) and the low-frequency high-intensity (LFHI) (40 kHz-161 W) to identify more suitable stimulation for better uptake efficiency of fluorescent calcein and quantum dots (QDs).

Two types of microalgal cell species, *C. reinhardtii and Chlorella vulgaris*, were chosen as representative models of algal organisms and potential biofuel crops to examine the intracellular delivery efficiency of fluorescent dyes and DNA under two different ultrasonic conditions. In particular, the transfection of PtCrCFP gene into *C. reinhardtii* and *C. vulgaris* was also tested at the HFLI mode, and the efficiency of transient gene expression was compared with that of the conventional electroporation.

2. Materials and methods

2.1. Microalgal cells and sample preparation

C. reinhardtii (CC125, cell diameter 6–8 μm) wild-type strain was originally obtained from the Chlamydomonas Genetics Center at Duke University. *C. vulgaris* strain (UTEX 2714, cell diameter 2.8–4.6 μm) was purchased from The Culture Collection of Algae at the University of Texas at Austin. Microalgal cells were cultured in Tris-Acetate-Phosphate (TAP) medium under continuous light at 25 °C in a plant growth chamber (JSR, JSPC-200C, Korea) [11]. Mid-log phase cells were harvested by centrifugation at 5000 rpm for 5 min and re-suspended in TAP medium to achieve final cell concentration of 1.0×10^8 cells/ml [5,12].

Calcein (C0875, molecular mass 623 Da, 0.6 nm diameter, Sigma), a negatively charged and biologically inert green fluorescent molecule at a concentration of 6.23 mg/L (made from stock solution) was used as an indicator of intracellular uptake and drug delivery test [10]. To test the intracellular QDs delivery, the luminescent carbon dots of an average diameter of about 5 nm were synthesized [14] and 100 μL of phosphate-buffered saline (PBS) solution containing QDs (10 mg/L) was used to test intracellular uptake. For DNA transformation, plasmid pPtCrCFP with hygromycin phosphotransferase (*hpt*) was used [13]. Typically, 5 μg of DNA (approximate number of DNA copies were 6.85×10^{11}) was used in all experiments. The ratio of DNA molecules/cell concentration was fixed as 5 μg/10^8 cells.

2.2. Ultrasonic apparatus and sonoporation

Ultrasound produces cavitation bubbles, either stable ones that are oscillating or transient ones that collapse. These cavitation bubbles give rise to diverse physicochemical effects in stimulated cells and among the effects, the generation of reactive free radical has been pinpointed as a critical process, especially in transient collapsing bubbles. Pure mechanical force from stable cavitation is likely to generate a low level of hydroxyl radicals, to induce the cell permeability and maintain the cell viability.

Two types of ultrasound systems consisting of two different piezoelectric transducers were used to generate frequencies of 40 and 600 kHz, respectively. The 600 kHz ultrasonic device (Hanmei Co. Ltd., China, 1 cm diameter of a transducer) with an electrical power output of 15 W was used to generate a representative higher-frequency ultrasound with low intensity (HFLI). The bottom of a cover glass confocal dish (SPL Lifesciences, Korea) was tightly fastened to the outer surface of the transducers for free passage of vibration energy and cavitation of algal suspension (Fig. S1). On the other hand, the standard ultrasonic cleaning bath (WUC-A02H, Daihan Scientific, Korea, and $150 \times 135 \times 100$ mm) with a maximal electrical energy output power of 161 W was used to produce 40 kHz ultrasound as a typical low-frequency high-intensity (LFHI). To measure the transient cavitational activity, potassium iodide (KI) dosimetry was performed using 0.1 M concentration of potassium iodide (KI, 80,370–1201, Kyoto, Japan) with a solution of 10 wt.% acetonitrile-water solvents. To enhance oxidation of iodide, a few drops of carbon tetrachloride were added to 150 μL of KI solution in the reaction system [15]. The absorbance of I_3^- was measured at 355 nm using a UV–Visible spectrophotometer (Thermo Scientific, US) at 0, 5, 10 and 30 s ultrasonic treatment.

For fluorescence dye delivery, calcein (6.23 mg/L) was added into 200 μL of standard suspensions of *C. reinhardtii* and *C. vulgaris* were sonicated up to 30 s in addition to 0, 5, 10 s at a given frequency of LFHI and HFLI ultrasound, respectively. The temperature was monitored continuously using a digital thermometer and was maintained below 30 °C. Cells after sonoporation treatment were thoroughly washed three times by centrifugation (3500 rpm, 3 min) and re-suspended in a final volume of 0.5 mL of fresh growth medium [10]. We then let the samples recover from the transient damage for 15 min at room temperature before flow cytometry analysis. The cell numbers were counted using a haemocytometer, and results were reported as the average value of cell remaining, calculated using the equation below:

$$\%\text{cell remaining} = (C_{final}/C_{initial}) \times 100\%$$

For QDs delivery, the fluorescence probe (10 mg/L) was added into *C. reinhardtii* or *C. vulgaris* suspension and the mixtures were sonicated up to 30 s in addition to 0, 5, 10 s at a given frequency of LFHI and HFLI ultrasound, respectively. The cells were washed following the procedure described for calcein delivery. For plasmid DNA delivery into microalgal cells, the mixtures of *C. reinhardtii* or *C. vulgaris* and pPtCrCFP plasmids were sonicated using HFLI ultrasound for 10 s. The cells were added to a 0.5 mL TAP medium and incubated for 24 h. Fluorescent assay using flow cytometry and confocal microscopy were performed after cell incubation and/or recovery [11]. All experiments were performed in triplicate.

2.3. Calcein, quantum dots, and cyan fluorescent protein fluorescent assay

Intracellular calcein uptake and cell viability were assessed by the flow cytometry following Azencott's work [10]. Cytometric analysis was performed using a BD FACS Canto II Flow Cytometer (BD, San Jose, USA) to analyze approximately 50,000 cells from each sample, where cells were distinguished from debris on the basis of forward and side scatter. After ultrasonic treatment, samples were recovered for 15 min at room temperature before adding propidium iodide (PI) at a final concentration of 0.1 mg/mL [10]. For calcein delivery, from the flow cytometry, we can then differentiate the dead versus live cells based on their red (488 nm argon laser excitation and a 630 nm emission filter) versus green (488 nm argon laser excitation and a 515 to 560 nm band-pass filter) emission [16]. Non-fluorescent, green, and red would each indicate live cells with unsuccessful delivery of calcein, live cells with successful delivery of calcein, and dead cells, respectively. Similarly, the intracellular delivery of QDs was detected using 488/30 bandpass filter in the flow cytometry. CFP protein fluorescence (405 nm and the emission was collected through 425 to 525 emission filter) was interpreted as a CFP gene expression in transformed cells, and red fluorescent cells were interpreted as non-viable cells labeled with propidium iodide [17]. Fluorescence-activated cell sorting (FACS) data was analyzed using BD FACSDiva 6.1 software. Live cell imaging was also performed using a LSM 710 laser scanning confocal microscope system (Zeiss, Germany) coupled to an XL-LSM 710 S incubator and equipped with a 63× oil immersion objective. The auto-chlorophyll fluorescence was excited at 633 nm and the emission was collected through long-band pass filter at 654 nm. The acquired images were processed by ZEN 2012 software [11].

2.4. Plate and PCR assay

To examine the integration of the hygromycin gene in the *C. reinhardtii* and *C. vulgaris*, the cell plate and PCR assays were conducted. *C. reinhardtii* and *C. vulgaris* were grown in TAP medium supplemented with hygromycin (50 μg/ml) and incubated under