Ultrasonics Sonochemistry 21 (2014) 833–839

Contents lists available at ScienceDirect

Ultrasonics Sonochemistry

journal homepage: www.elsevier.com/locate/ultson

Stabilizing *in vitro* ultrasound-mediated gene transfection by regulating cavitation

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ARTICLE INFO

Article history:
Received 25 June 2013
Received in revised form 11 October 2013
Accepted 17 October 2013
Available online 29 October 2013

Keywords:
Ultrasound
Gene transfection
Sonoporation
Cavitation

ABSTRACT

It is well known that acoustic cavitation can facilitate the inward transport of genetic materials across cell membranes (sonoporation). However, partially due to the unstationary behavior of the initiation and leveling of cavitation, the sonoporation effect is usually unstable, especially in low intensity conditions. A system which is able to regulate the cavitation level during sonication by modulating the applied acoustic intensity with a feedback loop is implemented and its effect on *in vitro* gene transfection is tested. The regulated system provided better time stability and reproducibility of the cavitation levels than the unregulated conditions. Cultured hepatoma cells (BNL) mixed with 10 μg luciferase plasmids are exposed to 1-MHz pulsed ultrasound with or without cavitation regulation, and the gene transfection efficiency and cell viability are subsequently assessed. Experimental results show that for all exposure intensities (low, medium, and high), stable and intensity dependent, although not higher, gene expression could be achieved in the regulated cavitation system than the unregulated conditions. The cavitation regulation system provides a better control of cavitation and its bioeffect which are crucial important for clinical applications of ultrasound-mediated gene transfection.

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1. Introduction

Gene therapy is becoming increasingly important in the treatment of certain inherited or acquired disorders, such as congenital immunodeficiency, atherosclerosis, and cancer. However, the results of clinical studies have not always been satisfactory, partially because of difficulties in gene delivery, and hence the development of effective carriers for delivering genes into cells has attracted a great deal of attention. Viral vectors often exert cytopathic effects, induce oncogenic effects, or trigger unexpected immune responses, which can be circumvented by using nonviral vectors. Several physical methods such as ultrasound (US), electroporation, and gene gun have been used to improve the transfection efficiency. Comparing with others, US has the advantage of being able to focus and induce desired bioeffects on a small area [1–3]. Moreover, at the energy level required for gene transfer, US is considered less invasive to human body and will not induce immune response [4]. Therefore, US-assisted gene delivery could be a promising method for site-specific gene therapy in future clinical applications.

It is believed that ultrasonic mechanical waves or other subsequent interacting mechanisms can alter the cell membrane permeability and might thus increase the uptake of macromolecules such as plasmid DNA [5–7]. The uptake of macromolecules has been found to be correlated with the stimulus acoustic energy, frequency, pulsing strategy, and microbubble concentration [8–10]. It has also been suggested that the US-facilitated cell membrane permeation are attributable to the amount of acoustic cavitation, which can be determined by the abovementioned acoustic parameters [11,12]. The main problem using acoustic cavitation for transfection is the inherent random behavior of the cavitation dynamics, leading to a poor reproducibility of the biological effects on cells.

Therefore, the control of inertial cavitation could be crucially important in the induction of membrane permeation, and also to avoid unwanted cell toxicity. In our previous study, we have developed an ultrasound cavitation regulation system based on real-time monitoring of the inertial cavitation signals [13,14]. The regulation process leads to a better reproducibility and

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http://dx.doi.org/10.1016/j.ultsonch.2013.10.017

stability of cavitation activity especially for low acoustic intensity conditions. In this study, we employed the cavitation regulation system to test if it has beneficial effect on *in vitro* gene transfection compared to traditional unregulated conditions.

2. Material and methods

2.1. Ultrasound apparatus and setup

The US setup used in the experiments is shown in Fig. 1. All experiments are performed in a tank containing degassed water at 35–37 °C (O_2 saturation in water less than 3 ppm). The US field is generated by a 1-MHz plane piezoelectric transducer with an active diameter of 3.8 cm (A392S, Panametrics, Waltham, MA, USA). A 24-well plate is placed 5.4 cm above the transducer, and only one well at a time is exposed to US by aligning the center of the exposed well with the center of the transmitting transducer. The 5.4 cm distance is chosen to ensure that the culture well is within the −3 dB pressure distribution in the near field of the transducer. The plate is covered with its original cover to avoid contamination with the outside environment.

A program written by using Labview (v.11.0, National Instruments, Austin, TX, USA) subroutines is implemented to control a Field-Programmable Gate Array (FPGA) based device (National Instruments, Austin, TX, USA) which allows the control of US exposure time, duty cycle, and output voltage supplied to the power amplifier (1040L, Entrepreneurship & Innovation, Rochester, NY, USA) which is monitored by a power meter (Model 4421, BIRD Electronic Corporation, Solon, OH, USA).

A needle hydrophone (SPEH-S-0500, ONDA, Sunnyvale, CA, USA) is located in the vicinity of the sonicated well and used to listen to the cavitation noise produced in the exposed medium. The received signal is amplified (20 dB, NF Electronic Instruments BX31), digitized (acquisition card NI-5781R, 14 bit resolution, 100 MHz sampling frequency, National Instruments), transferred to a computer and analyzed by the Labview program. The cavitation activity is quantified using an inertial cavitation index (CI) [14]. This index is calculated by estimating the broadband noise enhancement during the sonication of the medium. The broadband noise is calculated from the mean arithmetic value of the overall frequency dB magnitudes of the hydrophone signal spectra. Note that the relative weight of harmonics, sub-harmonics and ultra-harmonics due to non-inertial cavitation is minimized using a logarithmic scale and their contributions are negligible in the studied CI range. The designed cavitation regulation system is based on the instantaneous calculation of the inertial cavitation level. When setting up the fixed intensity sonication mode, the voltage supplied to the transducer is fixed to a constant value, and therefore the inertial cavitation level is calculated for every feedback loop as a monitoring of the cavitation activity. When setting up the fixed cavitation sonication mode, the instantaneous inertial cavitation level is calculated and compared to a target CI value. If different, the voltage supplied to the transducer is modulated in real-time to reach the target CI value. It is worth noting that in both sonication modes, the calculation of the CI value and its regulation (in fixed cavitation mode only) are completed within a feedback loop of nearly 300 μs. Further details of the acquisition device, the calculation of CI, and the feedback control system can be found in our previous reports [13,14].

2.2. Exposure parameters

As a first step, CI obtained by varying the applied acoustic intensity (range 0–0.8 W/cm^2, measured by a UPM-DT-10 ultrasound calibrator, Ohmic Instruments, USA), volume of water inside the exposure well (1 or 2 mL), and duty cycle (10%, 15%, 20%, 25% and CW), are evaluated to determine the CI range available with the system, and the optimal exposure parameters. The pulse length is fixed at 27.5 ms for exposure conditions [10].

To evaluate the efficiency of the regulated cavitation system on sonoporation, the transfection efficiencies of reporter genes are measured and compared after US exposure, either at fixed voltage and fixed CI conditions. Four target CI values, namely 2, 7, 10 and 16, are selected. These values, selected within the available CI range, allow exploring a wide range of cavitational phenomena, from non-cavitational state (CI = 2) to large inertial cavitation state (CI = 16). The averages over the US exposure time of the voltage supplied to the transducer during each measurement in fixed CI conditions define the fixed voltage conditions: 0.042, 0.21, 0.31 and 0.41 V. The averaged voltage varies substantially between tests and is thus measured before each set of experiments. Four sets of experiments are performed. They are (1) inertial cavitation levels measured by hydroxyterephthalic acid; (2) intra-tests and between tests variability of measured CI; (3) levels of gene transfection; and (4) levels of cytotoxicity.

2.3. Quantification of inertial cavitation using HTA

To confirm that the proposed CI value is a good indicator of inertial cavitation, the process of inertial cavitation at the origin of water sonolysis is evaluated. Several chemical (iodide dosimeter [15] of Fricke dosimeter [16]) or physical techniques (spin trapping method by paramagnetic resonance [17]) have been proposed to dose the quantity of hydroxyl radicals generated by inertial cavitation. The terephthalate dosimeter is known to be more sensitive [18,19] than the previous methods as being fast reacting with the hydroxyl radicals in order to generate hydroxyterephthalic acid (HTA), and is quantified through fluorescent measurements. The fluorescent level of hydroxyterephthalic acid (HTA) formed by the reaction of TA with OH, reflecting the quantity of hydroxyl radicals produced, is used as an indicator of the amount of inertial cavitation. A 20 mM terephthalic acid (TA) stock solution (Sigma–Aldrich, St. Louis, MO, USA) is prepared according to the method of Mason et al. [19] and stored at 4 °C in the dark for several weeks. Prior to use, its pH (7.3 ± 0.1) is systematically checked. We use 2 mL of 2 mM TA solution in the 24-well plate placed above a US probe. After US exposure, aliquots (200 μL) of the solution are transferred to 96-well plates and fluorescence (HTA) measurements are conducted using the microplate spectrophotometer system (Infinite M200, Tecan, Austria). The maximum excitation and emission wavelengths are, respectively, λex = 323 nm and λem = 424 nm. The residual fluorescence of a control solution (2 mM TA) is set to 0.

2.4. Cell culture, plasmid preparation and cell sonication

BNL 1MEA.7R.1 (chemically-transformed liver cells) are purchased from American Type Culture Collection (Manassas, VA, USA). These cells are grown in Dulbecco's modified Eagle's medium containing 4.5 g/L glucose (DMEM, High Glucose, Gibco, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (Gibco), and a 1% mixture of penicillin G, streptomycin and amphotericin B (Gibco) at 37 °C in 5% CO_2. Firefly luciferase cDNA (luc) is subcloned into the pCI-neo Vector, resulting in the construct pCI-neo-luc. Plasmid pCI-neo-luc is transformed into competent *Escherichia coli* DH5α, and endotoxin-free plasmid DNA is purified using the Qiagen EndoFree Plasmid Max kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

BNL cells are seeded in a well of a 24-well plate at an initial density of 5×10^4 in 1 mL of growth medium and incubated for 24 h prior to US exposure. The 24-well plate is placed above a US probe with a thin layer of US gel in between. In each well, 2.0 mL of culture medium containing 10 μg of luciferase plasmid and 5 μL

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