Chitosan-conjugated lipid microbubble combined with ultrasound for efficient gene transfection

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ABSTRACT
Non-viral vectors, as gene carriers, have advantages of biological safety and cost-efficiency over viral vectors. However, low transfection efficiency hampers their further clinical application. In this study, we developed a novel chitosan-conjugated lipid microbubble (CMB), which has good biocompatibility and high gene loading capacity. By ultrasound-targeted microbubble destruction (UTMD) technology, plasmid DNA could be efficiently delivered into HEK293T cells. The parameters for UTMD including acoustic intensity (AI), duty cycle (DC), exposure time (ET) and microbubble concentration were systematically optimised. Under the optimal conditions (AI, 1.0 W/cm²; DC, 10%; ET, 60 s; 10% CMBs), the gene transfection efficiency was significantly enhanced, without obvious impairment of the cell viability (over 80%). This study described a novel gene transfection strategy that combines CMBs with ultrasound to facilitate safe and efficient gene transfection in vitro with the potential for in vivo targeted gene delivery.

Introduction
In recent years, ultrasound is increasingly employed as an efficient, simple and safe gene delivery tool, showing great potential for targeted gene therapy for a variety of diseases [1]. Ultrasound-targeted microbubble destruction (UTMD) is a kind of gene transfection technique which takes advantage of the interaction of ultrasound and microbubbles to induce a series of biological effects, such as cavitation and sonoporation to promote gene delivery in vitro or in vivo [2,3]. By UTMD, the permeability of the cell membrane could be strengthened because of the transient holes generated on the surface of the cells [4,5]. Consequently, more genes could enter the cells [6]. However, despite of its great potential for gene therapy, the transfection efficiency by UTMD is still not satisfactory.

A lot of work has been done to promote the gene transfection efficiency by the combination of microbubbles and ultrasound [7–9]. The shell of microbubbles is generally composed of chitosan, lipid, albumin or polymer and the core is filled with air or perfluoropropane [10]. Chitosan is biodegradable and positively charged, making chitosan-based microbubble a promising gene carrier [11]. Ultrasound parameters including acoustic intensity (AI), duty cycle (DC) and exposure time (ET) also play an important role in gene transfection. These parameters should be tuned deliberately to realise the highest transfection efficiency without obvious mechanical damage to the cells [12,13].

In the present study, we developed a novel chitosan-conjugated lipid microbubble (CMB). CMB was used to transfer a plasmid which encodes enhanced green fluorescent protein (eGFP) into HEK293T cells. AI, DC, ET and CMB concentration were optimised to promote gene transfection. The transfection efficiency of different groups (chitosan, US+MB and US+CMB) was evaluated by fluorescence imaging and flow cytometry.

Materials and methods

Materials
Biotinylated chitosan was purchased from Creative PEG-Works (USA). DSPE-PEG 2000, DSPC and DSPE-PEG (2000)-Biotin were from Avanti (USA). FITC-Streptavidin was from Invitrogen (USA). Dulbecco’s modified Eagle’s medium (DMEM) was from Hyclone (Logan, UT, USA). Fetal bovine serum (10%), trypsin, penicillin (100 U/mL) and streptomycin (100 U/mL) were from Life (Carlsbad, CA, USA). The HEK293T cell line was from ATCC (USA).

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The cell counting kit (CCK-8) was from Dojindo (Japan). The plasmid encoding eGFP is kept in our laboratory.

**Chitosan–DNA complex preparation and characterisation**

The biotinylated chitosan was diluted to 1% at 45 °C in acetic acid (pH = 5.5). The solution was further diluted to 0.05% by 5 mmol/L acetic acid and filtered using a 0.22 μm filter. Then, 10 μg plasmids were dissolved into 50 μL sodium sulphate solution (4.3 mmol/L). The biotinylated chitosan and the plasmids were individually incubated at 55 °C for 20 min. Subsequently, the chitosan was added into the plasmid solution. The mixture was vigorously vortexed for 1 min and incubated at room temperature for 20 min. The particle size and the zeta-potential of the chitosan–DNA complexes were determined by Malvern Zetasizer Nano ZS90.

**Synthesis and characterisation of microbubbles**

DSPC, DSPE-PEG2000 and DSPE-PEG2000-biotin were mixed in chloroform at a molar ratio of 9: 0.5: 0.5. The solution was vortexed in a 65 °C water bath. The chloroform was evaporated by nitrogen injection. The residual solvent was further eliminated by rotary evaporation in vacuum (about 1 h). The dried phospholipid mixture was hydrated in Tris buffer (Tris: glycerol: propanediol = 8: 1: 1 by volume) in a vial. Subsequently, the gas in the vial was replaced by perfluoropropane. The mixture was vortexed for 45 s to form microbubbles. The lipid microbubbles were suspended in phosphate buffered saline (PBS) and purified by centrifugation (670 g, 45 s); this step was repeated twice.

An aliquot of streptavidin was added into the biotinylated chitosan and incubated for 15 min at room temperature. The free streptavidin was eliminated by PBS washing three times. Subsequently, the complexes were incubated with lipid microbubbles at room temperature for 15 min. The free microbubbles were eliminated by PBS washing. The pure chitosan-conjugated lipid microbubbles (CMBs) were stored at 4 °C for use.

The structure of MBs and CMBs was observed under an optical microscope (OLYMPUS CKX41) or a fluorescence microscope (Nikon Eclipse TS100-F). The particle size and zeta-potential were determined by Malvern Zetasizer Nano ZS90.

**DNA-binding analysis**

The microbubble/DNA solution was centrifuged at 1.12 × 10⁴ g for 30 min. The concentration of the DNA in the supernatant was determined by Nanodrop2000 spectrophotometer. The DNA binding efficiency was calculated according to the following formula:

\[
\text{Gene loading efficiency (\%)} = \left( \frac{C_{\text{total DNA}} - C_{\text{free DNA}}}{C_{\text{total DNA}}} \right) \times 100
\]

**Cell viability analysis**

For the cytotoxicity assay, 1 × 10⁴ cells/well were seeded in a 96-well plate; 24 h later, 10 μL CCK-8 reagent per well was added and the cells were further incubated in a 37 °C incubator for 2 h. The absorbance at 450 nm was measured by a microplate reader. Cell viability (%) was calculated according to the following equation:

\[
\text{Cell viability (\%)} = \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}} \times 100
\]

Each experiment was performed independently three times.

**Gene transfection by UTMD**

HEK293T cells were divided into four groups: cells without any treatment as control; cells transfected by chitosan–plasmid complexes; cells transfected by plasmid + MBs + US; cells transfected by plasmid + CMBs + US. A total of 1.5 × 10⁵ cells were seeded in each well in a 24-well plate. The concentration of the microbubbles was 10%. The plasmids and the microbubbles were mixed in 0.5 mL Opti-MEM medium and added into the cells. The cells were treated by ultrasound under different conditions. Parameters including acoustic intensity (AI), duty cycle (DC) and exposure time (ET) were systematically optimised. Four hours later, the medium was exchanged by DMEM medium supplemented with 10% fetal calf serum. The cells were extensively incubated in a 37 °C incubator for 72 h.

**Assessment of transfection efficiency**

The cells were observed under a microscope to evaluate the fluorescence intensity. Then the transfection efficiency was determined by flow cytometry. Briefly, the cells were washed twice in PBS and harvested by trypsinisation. The cells were collected by centrifugation at 1.12 × 10³ g for 5 min. The pellet was re-suspended in PBS. The percentage of eGFP expressing cells was determined by a flow cytometer equipped with BD Accuri C6 (Becton Dickinson, San Jose, CA).

**Statistical analysis**

Statistical analysis was performed using the SPSS 13.0 software. All the data were expressed as mean values.
with standard deviation (±SD). The whole experiment was performed independently three times. Group comparisons were performed using Student’s t-test. $P < 0.05$ was considered to indicate a statistically significant difference.

**Results and discussion**

**Physicochemical properties of chitosan, MBs and CMBs**

The chitosan–DNA complex solution appeared light blue to the naked eye. The size and the zeta-potential of the chitosan–DNA complexes were determined as $(345.15 ± 12.65)$ nm and $(41.69 ± 1.26)$ mV, respectively. Under the optical microscope, the MBs were spherical in shape, with an intact and smooth surface (Figure 1(A)). The chitosan showed bright rings surrounding CMBs (Figure 1(B)). The FITC-labelled chitosan appeared green surrounding MBs under the fluorescence microscope, suggesting that the chitosan had successfully bound to MBs (Figure 1(C)). There was no obvious change in the size of CMBs. The size of CMBs was $(7.15 ± 0.05)$ μm, compared with $(6.65 ± 0.09)$ μm for MBs. The zeta-potential of CMBs was $(16.95 ± 3.04)$ mV after chitosan decoration, which is in favour of DNA loading. After modification, the gene-loading efficiency increased significantly from 5.20% (MBs) to 29.29% (CMBs). The properties of the vectors are summarised in Table 1.

**Effects of UTMD on cell viability**

CMBs at a concentration of 10% showed no obvious cytotoxicity to the cells: the cell viability was > 80%. However, the cell viability decreased significantly when the concentration of CMBs reached above 15% (cell viability < 70%) (Figure 2(A)). The effects of ultrasound parameters including AI, DC, and ET on the cell viability were investigated. When DC was set at 5%, ET 30 s and the concentration of CMBs 10% by volume, the viability of the cells decreased with increasing AI (between 0.6 W/cm² to 1.4 W/cm²) (Figure 2(B)). The cell viability was the lowest at 1.4 W/cm² (about 70%), whereas at 0.6 W/cm² and 1 W/cm², there was no obvious damage to the cells (>90%). Under the condition of 10% CMBs and 5% DC, ET (between 30 to 120 s), the cell viability was not influenced considerably by ET (Figure 2(C)). The cell viability in the 20% DC group (76.16% ± 3.96%) was significantly lower than that in the 5% and 10% groups (95.06% ± 2.94% and 89.28% ± 3.93%) (Figure 2(D)). Under the optimised UTMD condition (AI, 1.0; DC, 10%; ET, 60 s; 10% CMBs), the cell viability was above 80%.

**Gene transfection by chitosan, MBs and CMBs combined with ultrasound**

Under the optimised ultrasound conditions (AI, 1.0; DC, 10%; ET, 60 s), MBs and CMBs were tested and compared for gene transfection efficiency. The ratio of the cells expressing eGFP was significantly elevated in the US + CMBs group compared with other groups (Figure 3). The gene transfection efficiency was quantitatively evaluated by flow cytometry. The efficiency by US + CMB was $(13.26 ± 1.43)$% (Figure 4), which was significantly higher than that of the CS group (3.15 ± 0.78)% and US + MB group (4.09 ± 1.02)%.

**Final remarks**

With the development of molecular biology, gene therapy has emerged as an increasingly important means for the treatment of diseases such as tumour and inherited diseases. For gene therapy, it is essential to deliver therapeutic genes into the targeted cells efficiently. However, the lack of efficient and safe gene carriers hampers its extensive clinical application [14].

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### Table 1. Size, zeta-potential and gene loading efficiency of CS, MBs and CMBs.

| Material type | Zeta-potential | Size       | Gene-loading efficiency |
|--------------|----------------|------------|-------------------------|
| CS           | 41.69 ± 1.26 mV| 345.15 ± 12.65 nm | —                        |
| MBs          | -25.35 ± 1.34 mV| 6.65 ± 0.09 μm  | 5.20%                   |
| CMBs         | 16.95 ± 3.04 mV| 7.15 ± 0.05 μm  | 29.29%                  |

Note: Data are mean values ± SD; n = 3.
Figure 2. Effect of CMB concentration (A), Al (B), ET (C) and DC (D) on HEK293T cell viability.
Note: Data are mean values ± SD; n = 3.

Figure 3. Fluorescence imaging of negative control (A) and eGFP-expressing cells transfected by plasmid + chitosan (B), plasmid + MBs + US (C) and plasmid + CMBs + US (D).
Note: Scale bar = 10 µm.
Currently, viral and non-viral vectors are commonly-used gene carriers. Although viral vectors can achieve high gene transfection efficiency, the potential risks of mutagenesis and immunogenicity restrict their clinical use. Non-viral vectors provide a promising tool for gene therapy for their biocompatibility and ease of modification [15]. However, the majority of non-viral vectors are less efficient for gene transfection, compared with viral vectors. In recent years, ultrasonic medicine has expanded its application from disease diagnosis to targeted therapy; for example, UTMD is utilised to promote drug/gene delivery into targeted cells. Under ultrasonic irradiation, microbubbles expand and contract regularly. When the acoustic pressure reaches a certain threshold, the microbubbles explode, causing a series of biological effects such as cavitation and sonoporation. In this course, transient pores form on the surface of the cells. Consequently, the cell membrane increased its permeability to the therapeutic materials. UTMD, as a noninvasive, targeted and efficient technique, holds great promise for in vitro or in vivo gene delivery in both basic and clinical studies.

Lipids are one of the most commonly-used materials for microbubble construction. Lipid microbubbles are usually filled with gas or perfluorocarbon. Perfluorocarbon is used to preserve the stability of the microbubbles in many studies [16]. To improve the DNA-loading efficiency, one strategy is to modify the microbubbles with other materials with higher gene-binding capacity. Chitosan, which has strong positive charge and good biocompatibility, is an ideal candidate for this purpose [17].

UTMD parameters including AI, DC, ET and CMB concentration are systematically analysed to find the safe conditions. Naturally, the optimal parameters may vary in different laboratories because of different conditions used such as ultrasound generators. In our laboratory, the ultrasound machine used for UTMD was devised by us.

Conclusions
The present study provides a novel chitosan-conjugated lipid microbubble (CMB) and combines it with ultrasound to promote gene transfection in vitro. By optimisation of the UTMD parameters including AI, DC, ET and the microbubble concentration, genes could be efficiently and safely delivered into HEK293T cells by UTMD. Further study will be directed to the in vivo studies.

Declaration of interest
The authors have no conflict of interest.

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