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A Low-Cost Intracellular Delivery System Based on Microbubble and High Gravity Field

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Abstract — **In this paper, we developed a low-cost intracellular delivery system based on microbubble and high gravity field. We successfully delivered FITC-Dextran (40kD) into hard-to-deliver THP-1 cells. The results showed that our method achieved high delivery efficiency up to 80%. It was found that the delivery efficiency and cell viability were closely related to the centrifuge speed. We speculated that the burst of microbubbles causes transient pore opening thus increasing the chance of biomolecules entering cells. This fast, low-cost and easy-to-operate protocol is very promising for delivering therapeutic genes and drugs into any cells which do not actively take up extracellular materials. This method is most effective for *in-vitro* delivery, but after delivery, treated cells might be injected back to human for in-vivo imaging.**

I. INTRODUCTION

Physical-based gene delivery method has been intensively investigated in the scientific world. Recently, the most two widely used techniques include electroporation and sonoporation [1-9]. For example, the electroporation based device Nucleofector® has claimed itself to be stable and efficient even to difficult-to-transfect cell types [10]. While sonoporation based Sonidel SP100® seems to be feasible in animal test [11]. Compared to chemical-based gene delivery method, the advantages of physical-based gene delivery method is that it is more universal to different cell types. Due to the nature of physical force, it doesn't require much endocytosis ability of the cells. It is also more useful when there is a requirement on target delivery. Yet, the high cost of the devices seriously impeded the spread of this new technology. Thus, a new low-cost physical-based delivery method is in high demand both in research and clinical areas.

In this paper, we report a new low-cost physical-based delivery system. By mixing our in-house designed microbubbles into live cell medium, the high gravity field created by high speed centrifugation enables us to efficiently deliver extracellular materials directly into the cell nucleus. For a proof-of-concept study, we have chosen FITC-Dextran (40KD) as a fluorescent label which is usually considered impermeable to cell membranes [12]. To explore the potential of this method, we chose human acute monocytic leukemia cells (THP-1) which is a notoriously hard-to-deliver cell-line due to its small size and low endocytic activity [13]. The results show that up to 80% delivery efficiency could be achieved. The confocal microscopy results indicate this method could directly deliver material into the cell nucleus. Considering the promising result and no requirement on special device in the experiment, we consider this method to be one potential candidate of low-cost intracellular delivery methods. The cells delivered with fluorescent materials might be injected back to human body for tracking [14].

II. MATERIALS AND METHODS

A. Microbubble preparation

1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC) (850365P, Avanti Polar Lipids) and Tween-80 (P1754, Sigma-Aldrich) were dissolved in chloroform (288306, Sigma-Aldrich) at a ratio of 10:1. The solution was then processed in a rotary evaporator (LABOROTA 4000, Heidolph) to form a thin film. PBS solution (14190-250, Gibco) was added to the vial afterwards to hydrate the thin film. The solution was stirred at 60°C which is above the main phase transition temperature of the phospholipid (~55°C for DSPC) for 2.5hrs. The resulting solution could then be stored at -20°C or be processed further. In the final step, glycerol (G5516, Sigma) was added to the solution with a ratio of 1:1, the mixture was then stirred at 60°C for another 1.5hrs to form the microbubble solution. The solution could be activated to generate microbubbles or stored at -20°C. To activate the microbubbles, a certain amount of the final microbubble solution was transferred into a 1.5mL centrifuge vial. The vial was then activated in Vialmix™ for 45 seconds. As a result, the microbubbles were filled with air. In order to obtain fluorescent microbubbles, 20μL FITC solution (46950, Fluka) was added into the microbubble solution before activation.

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B. *Microbubble detection*

The fluorescent microbubble image was observed using a CW225A fluorescent microscope (Leica) with a DXM1200 digital camera (Nikon). To accurately measure the size of the microbubbles, 100μL activated microbubble solution was diluted into a 50mL beaker, and the microbubble solution was passed through a 20μm aperture to obtain size distribution information with a Multisizer™3 Coulter Counter®.

C. *Cell culture*

THP-1 cells were cultured in suspension in RPMI1640 (11875-093, Gibco) medium with 10% fetal bovine serum (FBS) and 1% Penicillin and streptomycin (P/S) in 750mL flasks in a cell culture incubator at 37°C containing 5% CO2. Before the experiment, THP-1 cells were harvested and resuspended in PBS solution. Thereafter, 60x104 cells were transferred to each 1.5mL centrifuge tube.

D. *Centrifugation experiment*

15μL of FITC-Dextran (5mg/mL) solution (FD40S, Sigma-Aldrich) was added to the cell solution followed by certain amount of activated microbubble solution. The cell mixture was then transferred to an Allegra™ 25R centrifuge (Beckman Coulter) and centrifuged at 4°C for designed parameters. To evaluate the relationship between delivery efficiency and cell viability, different centrifugation speed were used.

E. *Methods for evaluating delivery efficiency and cell viability*

To wash down the FITC-Dextran remaining on cell membrane, the cell mixture was resuspended by vortexing and washed three times by centrifuging in clean PBS for 10 minutes at 4°C. The delivery efficiency was directly evaluated using a FACSCalibur (Becton-Dickinson) right after the washing step. For cell viability assessment, cell samples were continued to be cultured up to 48 hours. The cell viability was evaluated by MTS assay at three time points, which is 0 (directly after experiment), 24 and 48 hours after the experiment. At a certain time point, the cell viability number was calculated through dividing the absorbance of cell centrifuged with microbubble by the absorbance of cell centrifuged only. Different time points had their own cell only control samples. Both of the absorbance was read by the 96-plates reader.

F. *Methods for taking confocal microscopy image*

To check the FITC-Dextran location inside the THP-1 cells under a confocal microscope, THP-1 cells were centrifuged at 9000RPM with 45μL activated microbubble solution. After the experiment, THP-1 cells were first attached to the cover slip by poly-L-lysine (P4832, Sigma). Then 1mL 2% paraformadelne (158127, Sigma-Aldrich) was employed to fix the cells for 2 hours. After that, the cell membrane was stained with rhodamine phalloidin (Molecular Probes®, Invitrogen) for 40 minutes. Finally, the cell sample was flipped on the slide with the Vectashield® mounting medium with DAPI (H-1200, Vector). Confocal images were taken using a Leica TCS SP5 II confocal microscope.

III. RESULTS

A. *Microbubble morphology and size distribution*

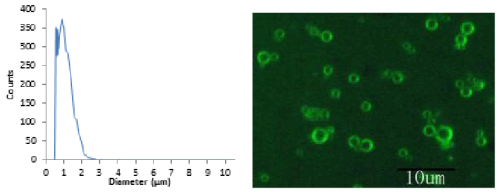

Figure 1. left: microbubble size distribution measured by Multisizer™3; right: fluorescent microbubble observed under fluorescent microscope

Accurate microbubble size distribution result obtained by Multisizer™ 3 is shown in the left of Figure 1. The horizontal axis denotes the particle size, while the vertical axis conveys the particle number under a particular size. Since the measurement was performed under a 20μm aperture which had a proper measurement range from 0.4μm-12μm. The microbubble size distribution curve was cut-off at around 500nm. The curve clearly showed that the microbubble size distribution peak was around 1μm. The microbubbles were also checked under the fluorescent field. In the right of Figure 1, it is very obvious to see the core-shell structure of the microbubbles under the fluorescent field. It was also observed that most of the microbubbles were broken after centrifugation at the speed as low as 1500RPM.

B. *Delivery efficiency and cell viability of THP-1 cell in microbubble coupled high gravity field system*

The delivery efficiency and cell viability of THP-1 cells in microbubble coupled high gravity field are shown in Figure 2. For delivery efficiency shown in top of Figure 2, all the samples of cell only present very low background compared to the samples treated with microbubble and FITC-Dextran. When treated with 1500RPM, the delivery efficiency is around 23%, while 3000RPM is chosen, the efficiency increased slightly to 37% ($p<0.05$). However, when treated with 5000RPM, the efficiency increased significantly to 71% ($p<0.05$). The efficiency reached the maximum value of 80% with 9000RPM. The result of cell viability was shown in the bottom of Figure 2. It should be mentioned that all the materials chosen to make microbubbles were biocompatible. The final concentration of DSPC, Tween80 and glycerol led to minimal effect on cells based on the literature study [15-17]. The solid line represents the cell viability after 0 hours as a function of

centrifugation speed. Similarly, the dashed line shows the result after 24 hours, while the dotted line shows the result after 48 hours. Generally speaking, the viability dropped as culture time increased. The exceptions were at 1500RPM and 3000RPM, the cell viability after 48 hours were higher than the result after 24 hours. 5000RPM was one special point because the differences in cell viability across different time points were the smallest. But the absorbance read by 96-plate reader for cells centrifuged with microbubbles actually did not change much at different time points. In the opposite, the absorbance for THP-1 cells centrifuged alone slightly grew as culture time increased. The absorbance was directly related to viable cell number. The difference in absorbance growing trend indicated the existence of proliferation difference between the samples which could be explained by a normal cell growth curve. Generally speaking, cells would proliferate faster if the base cell number is larger. Since there were more viable cells left when centrifuged alone, the proliferation rate will be faster compared to the cells treated with microbubble. As a result, the cell number gap between sample centrifuged with microbubble and without microbubble would be larger as the culture time increased which was reflected in the cell viability as calculated.

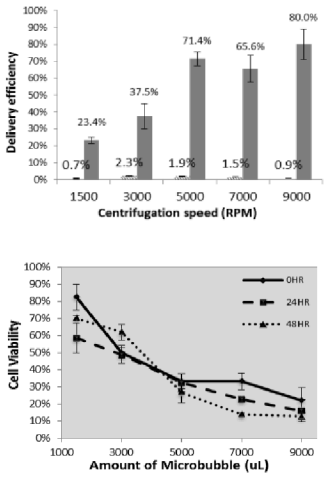

Figure 2 Top: Delivery efficiency of THP-1 cells at different centrifugation speeds; Bottom: Cell viability of THP-1 cells at different centrifugation speeds.

C. Confocal microscopy image of THP-1 cells in microbubble coupled high gravity field

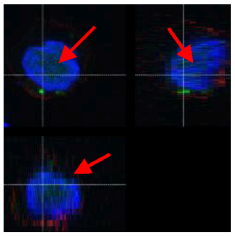

Figure 3. Confocal microscopy image of THP-1 cells in microbubble coupled high gravity field

The confocal microscopy images of THP-1 cells in microbubble coupled high gravity field are presented in Figure 3. Images taken from different layers were also stacked to form a dissection-view. The FITC-Dextran existed in the cytoplasm and cell nucleus. All these information indicate that when centrifuged with microbubble solution, FITC-Dextran could be delivered into the cell plasma and nucleus as pointed out by the red arrows.

IV. Discussion

A. Delivery efficiency and cell viability of THP-1 cells in microbubble coupled high gravity field

It is well known that cell membrane is mainly composed of a lipid bilayer. The flexibility of the cell membrane is largely due to this structure. In a high gravity field, everything will under a centrifugal force. It is reasonable to assume that when the cell morphology is elongated due to a high centrifugal force, thus the lipids between each other will no longer be as firmly connected. The higher the centrifugation speed, the looser the cell membrane structure. Although this cell membrane loosening effect is not lethal, it helps make the cell membrane become more easily penetrated, but by centrifugation alone cannot efficiently open pores on the cell membrane. For sonoporation, the mechanisms of opening pores on cell membrane are always related with cavitation effect or acoustic radiation force. Here in our method, cavitation effect could also be realized by the bursting of microbubbles in the high gravity fields. Thus, we propose that the effectiveness of our method depends on two factors - the degree of cell membrane loosening and the strength of microbubble bursts. Increasing the centrifugation speed means further elongate the cells and lead to a more permeable cell membrane barrier. When a certain level of microbubble is bursting, the more loosening the cell membrane, the more fragile the cell is. The experimental results show the delivery efficiency is inversely proportional to cell viability. The above analyses could well explain the trends observed in Figure 2. Unlike sonoporation, where the force only impact on cell membrane, the centrifugal force effects on every component of the cell. Therefore, the above speculations could also be applied to cell nucleus whose outside layer is similar to cell membrane. There is no doubt that most of the cells will eventually die if the centrifugation speed is too high or too much microbubble solution is used. However, higher level of cell damage also means higher delivery efficiency. From a practical point of view, people more care about real delivery, instead of cell viability. Because to get the desired functional cells, only one successfully transfected cell is needed in order to keep the purity.

B. Method overview

Compared to electroporation and sonoporation, our developed delivery system has the following advantages: (1) No special equipment is required. The only equipment used is a centrifuge which exists in almost every laboratory. (2) Easy to scale up. For sonoporation, the experiment is usually performed in a petri dish or tube. To perform large-scale sonoporation, special containers are required. Moreover the ultrasound transducer allocation and power present cost issues. With a centrifuge, it is very common to cope with several tubes simultaneously. One could easily accommodate one's needs by changing to an appropriate rotator. (3) Till now, electroporation and sonoporation could only treat one cell-line at a time. For our method, it is very easy to treat different cell-lines at the same time just by allocating them to different tubes. Overall, our method could easily spread and has no special costs needed.

V. CONCLUSION

In this paper, we proposed an innovative delivery system based on microbubble and high gravity field. We have successfully delivered FITC-Dextran into hard-to-deliver THP-1 cells with efficiency close to 80%. We speculated the mechanism of this method is a result of synergistic effects of transient cell membrane opening due to microbubble bursting. Using FITC-Dextran has its unique advantage because the fluorescent material could be visualized directly in order to determine its localization inside cells. Our method is a new physical-based intracellular delivery method which is achieved by microbubble and high gravity field. The method is low cost, and no special equipment is required. Most importantly, microbubble is made of biocompatible materials and is biodegradable after delivery.

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