Biomolecule delivery into canola protoplasts by centrifuging cells with microbubbles

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**Abstract**

We have successfully delivered FITC and FITC-Dextran (70, 250 kDa) into canola protoplasts by centrifuging cells with different amounts of microbubbles at variable centrifuge speed. The efficiency is around 90%, while cell viability remains high. Confocal microscopy images show that both FITC and FITC-Dextran are scattered inside the cytoplasm and the cell nucleus. Pores are observed on canola protoplast cell membranes and cell walls when centrifuged with microbubbles, while the membrane of cells centrifuged alone remain intact and smooth. We hypothesize that the collision between the microbubbles and cells or the bursting of microbubbles are the main reasons for the formation of these pores. Biomaterials can diffuse into the cells once the pathway is created.

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1. Introduction

Although numerous studies have been performed on the delivery of biomolecules into mammalian cells, normal chemical-based methods usually do not achieve desired results on plant cells [1]. The main reason is, unlike mammalian cells, plant cells have a solid cell wall made up of cellulose and hemicellulose. This stiff structure is very hard to penetrate. In addition, the cell wall also restricts the plant cells' endocytotic ability [2], and thus the probability for the cells automatically uptaking the biomolecules is very low when biomolecule carriers, like polymers or lipids, approach the cell surface. The biolistic or particle bombardment system has a long history of being used in plant transformation [3–5]. In this method, biomolecules along with gold bullets enter the target cells due to air pressure acceleration [6–7]. Other methods have also been used including electroporation and sonoporation. For instance, Blackhall has achieved the delivery of FITC-Dextran (150 kDa) into plant protoplasts by electroporation [8], while Harold investigated the effect of electroporation transmembrane potential, acoustic energy exposure, uptake molecule size and the presence of a cell wall on intra-cellular uptake and cell viability [9]. These methods can achieve high delivery efficiency, but cell viability is usually low as a trade-off.

In this article, we developed a new method of delivering biomolecules such as fluorescein isothiocyanate (FITC) and FITC-Dextran into canola protoplasts using the microbubble assisted centrifuge process. FITC had been widely used as a cell labeling tool for a long time [10–11], and FITC-Dextran is usually considered a membrane impermeable molecule [12–13]. In this experiment, both FITC and FITC-Dextran of molecule weight 70 and 250 kDa, respectively were used for a delivery study. As Fig. 1 shows, the canola protoplasts are first extracted from the original canola cell clusters. The protoplasts were then centrifuged with a mixture of microbubbles and biomolecules. The role of microbubbles is similar to their application in sonoporation: the bursting of microbubbles in a high pressure environment induces cavitation or liquid streaming resulting in pores on the cell membrane [14–20]. In this system, microbubbles were broken possibly due to the centrifuge field or collisions with the canola cells. Flow cytometry (FACS) histograms and confocal microscopy images proved that the biomolecules chosen in our experiment entered the protoplasts after the treatment. The delivery efficiency was around 90%, and the cell viability was around 100% based on cell counts after staining by fluorescein diacetate (FDA). Overall, FITC and FITC-Dextran with molecule weight around 70 and 250 kDa, respectively were...
efficiently delivered into the protoplasts with no compromise in cell viability. The pores formed on the protoplast as shown in the SEM image confirm the delivery mechanism of this method.

2. Materials and methods

2.1. Microbubble preparation and measurement

Twenty milligram 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) (850365P, Avanti Polar Lipids), 2 mg Tween-80 (P1754, Sigma–Aldrich) and 6 ml chloroform (288306, Sigma–Aldrich) were mixed together in a round-bottom flask (30 ml, Kontes) and then evaporated on a rotatory evaporator (LABOROTA 4000, Heidolph). The dried lipid film was stirred with 4 ml PBS solution (14190–250, Gibco) to a final concentration of 5 mg/ml at 60°C for 2.5 h. Finally, 4 ml Glycerol (G5516, Sigma) was added to the previously prepared solution, and the mixture was then stirred at 60°C for another 1.5 h to form the microbubble solution. Vialmix™, equipment which was used to activate the commercialized ultrasound contrast agent Definity®, was applied to integrate air into the lipid-coated monolayer through a high-speed agitation process at 4530 oscillations per minute for 45 s. To accurately measure the size of the microbubbles, 100 µl activated microbubble solution was diluted into a 50 ml beaker, and the microbubble solution was passed through a 20 µm aperture to obtain size distribution information with a Multisizer™ 3 Coulter Counter™.

2.2. Cell culture and protoplast preparation

Canola cell suspension was maintained on a rotary shaker (160 RPM) at 20°C in NLN media (pH 6.0, containing 6.5% sucrose, 30 mg/l glutathione, 800 mg/l glutamine, 100 mg/l l-serine, 0.5 mg/l a-naphthaleneacetic acid (NAA), 0.05 mg/l 6-benzylaminopurine (BA) and 0.5 mg/l 2,4-D). To extract the protoplasts, plant cells were first incubated with CPW13M solution, consisting of 27.2 mg/l KH2PO4 (P5655, Sigma–Aldrich), 101 mg/l KNO3 (P8291, Sigma–Aldrich), 1480 mg/l CaCl2·2H2O (C7902, Sigma–Aldrich), 246 mg/l MgSO4·7H2O (63138, Sigma–Aldrich), 0.16 mg/l KI (60399, Sigma–Aldrich), 0.025 mg/l CuSO4·5H2O (C3036, Sigma–Aldrich), and 130 g/l Mannitol (M1902), pH 5.8 for 1 h at room temperature. The solution was then replaced with a digestion solution, consisting of MS salts, 0.06% 2-(N-Morpholino) ethanesulfonic acid (MES), 13% mannitol, 0.025% Pectolyase Y23 (Kanematsu-Gosho, Tokyo, Japan) and 0.4% Cellulase Onozuka R-10 (Yakult Honsha Co., Japan), pH 5.8. The solution was then incubated for 5 h at 25°C in the dark. The digestion mixture was then filtered through a sterile nylon cell strainer (40 µm, BD Falcon, USA) to remove the large cell clusters, and centrifuged (40×g) for 8 min afterwards. The resulting pellet was resuspended in CPW13M solution.

2.3. Microbubble-assisted centrifuge process

1 × 10^6 Canola protoplasts were transferred to a 1.5 ml centrifuge tube for the microbubble-assisted centrifuge experiments. A small amount of CPW13M solution was left to submerge the small canola protoplast pellets in the bottom of the centrifuge tube. Five microlitre FITC solution (0.01 mg/ml, dissolved in CPW13M) was added into the centrifuge tube and mixed with the microbubble solution. The solution was then centrifuged at different speeds (expressed in relative centrifugal force (RCF)/centrifuge speeds) (1 × g/100 RPM, 28 × g/500 RPM, 112 × g/1000 RPM, 252 × g/1500 RPM and 447 × g/2000 RPM) in an Allegra™ 25R centrifuge (Beckman Coulter) for 2 min. The process was repeated three times. The final amount of FITC solution added was 15 µl, and the final amount of microbubble solution tested was 5, 15, 25, 45 and 65 µl. Finally, the canola protoplasts were centrifuged at 252 × g (1500 RPM) for an additional 4 min. Before checking the delivery results, 400 µl
CPW13M solution was used to finally wash the sample at 252×g (1500 RPM) for 4 min.

To deliver FITC-Dextran, the experiment procedures were the same as the delivery of FITC. In short, each time, 10 μl 100 μM FITC-Dextran (70 or 250 kDa, dissolved in CPW13M) was mixed with 5 μl microbubble solution and centrifuged at 252×g (1500 RPM) for 4 min. Overall, the final amount of FITC-Dextran solution added was 30 μl, and the final amount of microbubble solution was 15 μl. After that, the canola protoplast solution was centrifuged at 252×g (1500 RPM) for 4 min. FITC-Dextran is sticky. To wash down the FITC-Dextran remaining on protoplasts’ membranes, the canola protoplasts were washed in 400 μl CPW13M at 252×g (1500 RPM) for 4 min.

Comparison experiments were performed in the similar way as the delivery experiments. Except that, after 1×10^6 protoplasts were submerged in CPW13M solution which just covered the cell pellets’ surfaces, 15 μl FITC (0.01 mg/ml, dissolved in CPW13M) or 30 μl 100 μM FITC-Dextran (FD70 kDa or FD250 kDa, dissolved in CPW13M) were directly added in the protoplast solution and left for 6 min. The solution was replaced with 300 μl CPW13M solution and washed at 252×g (1500 RPM) for 4 min afterwards.

### 2.4. FACS analysis and cell viability detection

The protoplasts were resuspended in 300 μl CPW13M solution after the delivery and washing in order to evaluate the delivery efficiency using a FACS Calibur (Becton–Dickinson). For a cell viability assay, the protoplasts were stained with 3 μl FDA, and 10 μl protoplast solution was then transferred onto a hemocytometer. Images were taken under both the bright and fluorescent fields, and finally merged together by Photoshop CS4. Only the fluorescent cells will be considered as living. The number of fluorescent cells would be counted based on the merged image.

### 2.5. Confocal microscopy and SEM analysis

The canola protoplasts were firstly attached onto the cover slip by polylysine. The protoplasts were then dehydrated by 4%
paraformaldehyde for 1.5 h. The cover slip was then turned over and transferred to a slide using Vectashield mounting medium containing DAPI. Images were taken under a Leica SP5 II confocal microscope. For SEM images, 40 × 10⁴ canola protoplasts were centrifuged with 45 μl microbubble solution at 1006 × g (3000 RPM) as mentioned before. After that the protoplasts were fixed in pure methanol for 5–10 min, and then underwent critical point drying as mentioned in [21]. SEM images were taken by a JSM 6301 FX emission scanning electron microscope (JEOL) operated at 5 kV.

3. Results and discussion

3.1. Microbubble size and morphology

Size distribution of the microbubbles was analyzed by a Multi- sizer™ 3 and the results are shown Fig. 2a. The horizontal axis denotes the particle size, while the vertical axis conveys the particle number at a particular size. Since the measurement was performed under a 20 μm aperture, which had a proper measurement range from 0.4 to 12 μm. The microbubble size distribution curve started around 500 nm. The curve clearly shows that the microbubble size distribution peak was around 1 μm. DSPC and Tween80 are the main components of the microbubble monolayer, while air is trapped inside [22–23] (Fig. 2b). Glycerol is added in order to further stabilize the microbubble structure by slowing down the air diffusing rate to the surrounding environment.

3.2. FITC delivery into canola protoplasts

The effect of centrifuge speed and the amount of microbubble solution on the delivery efficiency were separately identified and quantified by flow cytometry. Fig. 3a show the result of delivery efficiency at different centrifuge speeds: different centrifuge speeds have no significant impact on delivery efficiency. The delivery efficiency could reach 90% even when the centrifuge speed is only 1 × g (100 RPM). Similarly, the delivery efficiencies were independent of the amount of microbubble solution, as shown in Fig. 3b. The efficiencies are around 85–90%. The FACS histograms of cell fluorescence are shown in Fig. 3c and d. The horizontal axis is the fluorescence strength, while the vertical axis stands for the cell number at certain fluorescence strength. On Fig. 3c protoplasts centrifuged alone are shown as a single population with low fluorescent background on the left. The peak of the fluorescence distribution of protoplasts mixed with FITC for 6 min (blue curve) shifted to the right. The fluorescence distribution of protoplasts centrifuged with microbubbles at 1 × g (100 RPM) (green curve) shows two peaks and shifted to the far right. This phenomenon shows that after the microbubble assisted centrifugation process, the protoplasts have a stronger fluorescence signal, which also means the microbubbles assisted centrifugation process did help improve the delivery efficiency. To clearly present the delivery difference between samples centrifuged with different amounts of microbubble solution, the FACS histograms were compared between samples centrifuged with 5 and 65 μl of microbubble solution as shown in Fig. 3d. The fluorescence distribution for canola protoplasts centrifuged with 65 μl of microbubble solution was much sharper than that with 5 μl of microbubble solution. In other words, more canola protoplasts have taken a similar amount of FITC in the sample centrifuged with 65 μl of microbubble solution. The reasons might be that the extra amount of microbubbles opened extra pores on the cell membrane of one single protoplast, or a greater population of overall protoplasts increases diffusing FITC molecules into the cells. Fig. 3e and f show the confocal microscopy images of canola protoplasts centrifuged alone and canola protoplasts with FITC delivered by microbubbles assisted centrifugation. The blue color stands for the cell nucleus, while the green fluorescent color was emitted by FITC

![Confocal microscopy images](image-url)
under the proper laser stimulation. Fig. 3f clearly shows that most of the canola protoplasts were green confirming that FITCs have been delivered into the protoplasts.

3.3. FITC-Dextran delivery into canola protoplasts

Fig. 4b and c show the confocal microscopy images of samples centrifuged with the mixture of microbubbles and FITC-Dextran. Both of the images show an intense green fluorescent signal released by FITC-Dextran closely surrounding the cell nucleus (blue colored). On the top right corner of Fig. 4b, an image shows that the FITC-Dextran could even enter walled canola cells after treatment using microbubble assisted delivery. A small population of individual walled canola cells exist due to the fact that cell walls were not fully removed by the enzyme solution. Unlike canola protoplast cells, the walled cells usually do not have a round morphology. The image on the top right of Fig. 4b shows that the bursting of microbubbles could even create pores on the cell wall when centrifuged with microbubbles. The FACS histogram is shown in Fig. 4d. Similar to the results obtained under FITC delivery experiment, when centrifuged with microbubbles, the fluorescence distribution clearly shifted to the right compared to the canola protoplasts centrifuged alone or canola protoplasts only mixed with FITC-Dextran (70 or 250 kDa).

3.4. Viability of canola protoplasts treated with microbubble assisted centrifuge process

The canola protoplast viability was checked using the FDA staining method. Only the fluorescent protoplasts are considered to be viable, and the number of fluorescent cells was counted using the FACS histogram. Fig. 5 shows the viable cell count with different amounts of microbubble solution. The viable cell count clearly shifted to the right compared to the canola protoplasts centrifuged alone or canola protoplasts only mixed with FITC-Dextran (70 or 250 kDa).

Fig. 6 shows the SEM images of canola cells. (a) A canola protoplast with centrifugation alone; (b) canola protoplasts treated by microbubbles assisted centrifugation; the pores formed are pointed out by the red arrows; (c) canola cells with cell wall centrifuged alone; (d) canola cells with cell wall centrifuged with microbubbles. The red dashed circle marks the area with small pores.
a hemocytometer. Although different amounts of microbubble solution were used, the number of live cells had no significant change compared to cell control as shown in Fig. 5a. The percentages of the number of viable protoplasts in various amount of microbubble solution compared to the protoplasts centrifuged alone are also shown in Fig. 5a. Except at the point where 25 μl of microbubble solution was used, the values of the percentages go down as the amount of microbubble solution increases. The cell viability of the sample treated with 447 g (2000 RPM) was also measured using the same method shown in Fig. 5b. The number of viable canola protoplasts when centrifuged with microbubbles is very similar to the number of viable canola protoplasts when centrifuged alone. All of this data indicates that the damage caused by the microbubble assisted centrifuge process to the protoplasts is very limited. It was hypothesized that the bursting of microbubbles induces pores on the cell membrane, but protoplasts might recover from this temporary pore opening and most of the protoplasts survive after the treatment. This is an obvious advantage to the other physical-based methods (bombardment, sonoporation and electroporation), because the high delivery efficiency of the other methods usually leads to low cell viability.

3.5. SEM images of canola protoplasts treated with the microbubble assisted centrifuge process

Fig. 6a shows the canola protoplasts morphology when centrifuged alone. The cell surface is intact and smooth. Canola protoplasts’ surfaces change significantly when centrifuged with microbubbles (Fig. 6b). There are many small pores on the cell surface as pointed out by red arrows. The cell surface also becomes rougher. For canola cells with cell walls, the cells accumulate together and attach to each other (Fig. 6c and d). When centrifuged alone, the canola cells show an integrated cell wall (Fig. 6c). Contrary to the pores scattered on the canola protoplasts’ membranes, pores on cell walls are formed only in certain regions and are much smaller when centrifuged with microbubbles. The regions marked by the red dashed line are magnified and shown in the left part of Fig. 6d. The pores formed on the protoplasts’ membranes and cell walls might due to the bursting of microbubbles during the centrifuge process. Once the intracellular pathway had been created, the biomolecules such as FITC or FITC-Dextran could then be more easily diffused into the cells. The protoplasts’ membranes are mainly composed of a lipid double layer, while the cell walls are made of cellulose. As a result, the cell wall is much more rigid than the cell membrane. The pores created by the impact force are therefore much smaller on the cell wall than on the cell membrane.

4. Conclusion

Not much research has been reported so far on biomolecule delivery into plant cells. In this study, we have successfully delivered biomolecules of up to 250 kDa into canola protoplasts using a microbubble assisted delivery method. The delivery efficiency was around 90%. Although the delivery efficiency is similar at a variety of centrifuge speeds and amounts of microbubble solution, the FACS histogram showed that a higher amount of microbubble solution could lead to stronger fluorescence. The advantage of this method is the cost is extremely low. In addition, the number of viable canola protoplast after microbubble assisted centrifugation is as high as canola protoplasts which are centrifuged alone with around 90% delivery efficiency. From confocal microscopy images, we observed that FITC-Dextran could be delivered even into canola cells with intact cell walls. This new delivery method holds a great potential. Studies of this method’s applications in plant cell biology and transformation technology are the subjects for our future investigations.

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