FITC Delivery into Plant Cells Using Magnetic Single-Walled Carbon Nanotubes

Yuzhi Hao1, 2, 3, 4, Xiaoyan Yang2, Yongzhong Shi5, James Xing6, Janet Marowitch5, Jianmin Chen7, and Jie Chen1, 2, 3, *

1 Department of Biomedical Engineering, University of Alberta, Edmonton, Alberta, Canada
2 Department of Electrical and Computer Engineering, University of Alberta, Edmonton, Alberta, Canada
3 National Institute for Nanotechnology, Edmonton, Alberta, Canada
4 School of Chemical Engineering, Hebei University of Technology, Tianjin, China
5 Alberta Innovates - Technology Futures, 250 Karl Clark Road, Edmonton, Alberta, Canada
6 Department of Pathology and Public Health, University of Alberta, Edmonton, Alberta, Canada
7 College of Biosciences and Biotechnology, Yangzhou University, Jiangsu, China

In this paper, fluorescein isothiocyanate (FITC) was covalently bonded with magnetic single-walled carbon nanotubes (mSWCNTs) that were purified using our previous method. To demonstrate our design, mSWCNT-FITC was delivered into plant cells (canola and carrot cells) driven by external magnetic forces. From FACS results, the FITC delivery efficiency was about 100% for both two canola and carrot protoplasts, which were further confirmed by the confocal and sectional TEM images. Some mSWCNTs were found trapped both inside the endosomes of canola protoplast and outside endosome near the nuclear membrane of carrot protoplast according to the sectional TEM images. All results showed that mSWCNT is a good delivery carrier for biomolecules.

Keywords: Magnetic Single-Walled Carbon Nanotubes (SWCNTs), Protoplast of Canola and Carrot, FITC Delivery.

1. INTRODUCTION

Several research articles on delivering drugs and genes using carbon nanotubes (CNTs) have been published.1–4 However, the ability of CNTs diffused or endocytosed by cells is not strong, and thus magnetic forces are used to help drive nanomaterials into some difficult-to-deliver cell lines.1, 5–7 However, most investigations were focused on exploring the interactions between nanomaterials and mammalian cells. The research about whether nanomaterials can be used as biomolecular transporters for plant cells, however, is very few. In particular, no research has been reported using magnetic carbon nanotubes for plant cell delivery because delivering biomolecules into plant cells is much more difficult than mammalian cells due to the plant cell walls. Most previous researches have been focused on using liposome as carriers and delivering biomolecules into protoplasts, in which cell walls and certain cell surface proteins were removed enzymatically.8–9 A few researches using nanomaterial delivery into plant cells were reported in recent years.10–14 Etxeberria et al. exploited the uptake of fluorescent quantum dots directly into the cytoplasm of walled plant cells after the cells were starved for 24 h.10 Torney et al. utilized the mesoporous silica nanoparticles to deliver DNA and chemicals into plant cells.11 Liu et al. reported that single-walled carbon nanotubes (SWCNTs) can penetrate cell wall and deliver FITC into walled plant cell,12 and the authors gave the confocal images to support the conclusion. Khodakovskaya et al. studied the relationship between seed germination, plant growth and carbon nanotubes.13 Serag et al. exploited the subcellular localization of multi-walled carbon nanotubes in plant cells, and concluded that multi-walled carbon nanotubes can penetrate cell membrane of protoplast.14

In this paper, we exploited the use of magnetic single-walled carbon nanotubes (mSWCNTs) as carriers to deliver fluorescein isothiocyanate (FITC) with the assist of external magnetic forces into plant cells. To achieve our goal, we covalently bonded FITC with mSWCNT. Two plant cells were chosen – canola (Brassica napus L. var. Jet Neuf) and carrot (Dacus carota L. var. Konsevnaaja), and their protoplasts were isolated. We used the static-standing method (directly putting cells on the top of magnet) to drive mSWCNT-FITC into plant cells. The FITC delivery...
efficiency is about 100% for both canola and carrot protoplasts according to Flow Cytometry (FACS) results, which were further confirmed by both the confocal and sectional TEM images. mSWCNTs were found both inside endosomes of canola protoplast and outside endosome near nuclear membrane of carrot protoplast.

2. MATERIALS AND METHODS

Cell culture: MD cell suspensions of canola (B. napus L. var. Jet Neuf) were kindly provided by Dr. Randall J. Weselake. Cells were 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). At 2-week intervals, one third of the mass of cells grown in 125 ml flasks was transferred to 50 ml of fresh NLN medium. Seeds of carrot (D. carota L. var. Konsernaja 63) were obtained from Plant Gene Resources of Canada (Saskatoon, Saskatchewan). Cells derived from leaves of \textit{in vitro} plants were cultured in MS media, 3% sucrose, 0.2 mg/L BA, 1.0 mg l-1 NAA (pH = 6). Two to Three days after subculture, cells were used for protoplast isolation.

Protoplast isolation: Plant cells were preplasmolyzed by incubation in CPW13M solution for 1 h at room temperature. The solution was then replaced with a digestion solution, consisting of 1/2 MS salts, 0.06% 2-(N-Morpholino) ethanesulfonic acid (MES), 13% manitol, 0.1% Macerozyme R-10 (Yakult Honsha Co., Japan) and 0.5% Cellulase Onozuka R-10 (Yakult Honsha Co., Japan), pH 5.8. The incubation was carried out overnight (16 h) at 25 °C in the dark. The digestion mixture was filtered through a sterile nylon cell strainer (40 μm, BD Falcon, USA) to remove the debris, and then centrifuged (1000 rpm) for 10 min. The pellet was resuspended in CPW25S and 2 ml of CPW13M was added to the top. The protoplasts were then collected with sterilized Pasteur pipettes following centrifugation (1000 rpm) for 10 min. The pellet was resuspended in CPW25S and 2 ml of CPW13M was added to the top. The protoplasts were then collected with sterilized Pasteur pipettes following centrifugation (1000 rpm) for 10 min, washed twice, and finally resuspended in 1/2 NLN medium supplemented with 13% manitol. The protoplast solution was used for the mSWCNT-FITC delivery experiment.

Synthesis of mSWCNT-FITC: 2 mg of purified mSWCNT (please refer to our previous article (15) for the purified method) was dissolved into a 120 ml flask containing 5 ml of concentrated H2SO4/HNO3 (V:V = 3:1). The solution was sonicated for 10 minutes, then washed completely. The mSWCNTs were resuspended into a 120 ml flask containing 200 ml of MilliQ water. 5 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiamide hydrochloride (EDC) and 1 ml of ethyl diamine were added into the flask. The mixture was stirred for 30 minutes in dark. The solution was dialysed until no free ethyl diamine and EDC in solution. 100 mg of FITC was dissolved into 10 ml of DMF, and added into the dialysed solution. The mixture was stirred for 5 minutes and kept in room temperature overnight. The mixture solution was dialysed until no free FITC molecules in solution.

Magnetic-field-driven cellular uptake experiment: Protoplasts with a density of 5 × 10^5 cells/plate were placed in 35 mm culture dishes, the dishes were sealed with parafilm. The magnetic-field-driven delivery method was carried out by placing the culture dishes containing 1 ml of medium with 0.25 μg/ml mSWCNT-FITC or mSWCNT on the top of an Nd–Fe–B permanent magnet for 12 h, then the protoplasts were collected, fixed in 2% paraformaldehyde and completely washed twice with PBS and 70% ethanol, respectively.

Cell viability: Protoplasts were seeded in 35 mm Petri dishes in culture medium. 30 μl of mSWCNTs was added into each dish. The Petri dishes were put on top of the Nd–Fe–B magnet at room temperature overnight. A drop of cell solution was deposited on a microscope glass slide and stained with FDA. Images were taken with both bright and green channels under a fluorescent microscope (Leica CW 225 A with Nikon digital camera DXM1200). The protoplast numbers were counted under bright channel and fluorescent channel. Then cell viability or NPs cytotoxicity was calculated. The total cell number is 100 in bright channel.

Flow Cytometry Measurement: Protoplasts exposed to mSWCNT-FITC at different concentration were collected and centrifuged at 1000 rpm for 10 min. The collected cells were extensively washed using PBS, then fixed in 2% paraformaldehyde. The fixed cells were washed with 70% ethanol twice again, and then resuspended in 400 μl PBS. The mSWCNT-FITC delivery efficiency was evaluated with Flow Cytometry (FACSan, Becton-Dickinson, San Jose, CA, USA) at an excitation wavelength of 488 nm.

Atomic force microscope (AFM) image: A small amount of sample solution was directly transferred drop-wise onto the silicon wafer. The sample was covered and kept at room temperature until the solution was dry. AFM images were taken using a Veeco Multimode V SPM operating in tapping mode.

Confocal microscopy imaging of plant cells: Protoplasts were seeded at a density of 1 × 10^5 cells/cm² on cover slips previously coated with poly-L-lysine (10 μg/ml) for 45 min. The protoplasts were exposed to 0.25 μg/ml mSWCNT-FITC and mSWCNT (the control) on an Nd–Fe–B permanent magnet. After 12 hours of incubation on an Nd–Fe–B permanent magnet, the cells were fixed in 2% paraformaldehyde and washed twice with PBS buffer and twice with 70% ethanol, respectively. The sample was examined under a confocal laser scanning microscope (Quorum Wave FX-Sinning Disk) equipped with imaging software – Hamamatsu EMCCD (C9100-13).

TEM image: TEM images were taken using a Philips FEI Morgagni 268 instrument, operated at 80 kV. The sample solution was deposited on the copper support, which
was coating with carbon. Protoplasts were fixed in 2% glutaraldehyde in 4% PEA/cacodylate buffer, pH 7.2, for 2 hours at room temperature. (a) The fixative solution was drained off and replaced with 0.1 M PBS buffer. Two further changes were done 10 minutes apart. (b) The buffer was drained off and the sample was post-fixed with 1% osmium tetroxide (OsO₄ in 0.12 M Cacodylate buffer, pH 7.2) for one hour. (c) The sample was washed using 0.1 M phosphate buffer 3 times for a total of one half hour. (d) The sample was dehydrated through a graded ethanol series as follows: 50%, 70%, 90%, 100% × 3 changes; one change every 15 minutes. (e) The ethanol was drained off from specimen and new ethanol: Spurr mix was added for 3 hours. The ethanol: Spurr mix was replaced with pure Spurr resin. The Petri dish was sealed overnight. (f) The Spurr resin was replaced again and the sample was dried at 70–80°C in an oven for 18 hours. (g) The sample was cooled and then removed from molds. (h) The sample was ultracut by Reichert-Jung Ultramicrotome and stained with uranyl acetate and lead citrate.

3. RESULTS AND DISCUSSION

3.1. Synthesis of mSWCNT-FITC

Nickel nanoparticles were catalyst for SWCNT growth, therefore, most nickel nanoparticles should remain in SWCNT after synthesis. Some nickel nanoparticles remained on the surface or were trapped inside SWCNT after using our previous purified method¹⁵ (black dots in Figure 1(C)), indicating these nanotubes were magnetic SWCNTs (mSWCNTs). After purification, these mSWCNTs still existed in bundle, and the diameters are about 20–40 nm (Figs. 1(A and B)), which suggested at least 10 SWCNTs were bundled together because the diameter of a single SWCNT is about 2–3 nm. Figure 1(D) is the synthetic process of making mSWCNT-FITC. Firstly, the mSWCNT was oxidized in concentrated H₂SO₄/HNO₃, to produce —COOH functional groups. Secondly, these —COOH functional groups reacted with ethyl diamine. By controlling the ratio of ethyl diamine to SWCNT, a free amino functional group can be remained after cross-linking
Fig. 2. FITC delivery efficiency (FACS results) of mSWCNT-FITC before and after 70% ethanol washing. (A) 70% ethanol and PBS washing; (B) PBS washing only.

Fig. 3. Microscope image of canola protoplast and Canola and carrot protoplast viability treated with mSWCNT-FITC. (A) Microscope image of canola protoplast (20 x 100). (B) Canola and carrot protoplast viability treated with mSWCNT-FITC.
reaction. Finally, these free amino functional groups reacted with FITC molecules to form covalent bond, and the covalent bond can ensure that, during the mSWCNT delivery process, FITC molecules and mSWCNTs are not separated. In order to ensure the FITC molecules in cells were from SWCNT-FITC, not from free FITC molecules, the reaction solution was dialyzed until no free FITC molecule exists in solution.

3.2. FITC Delivery into Plant Cells

After the canola or carrot protoplast were prepared and purified, these protoplasts were planted in Petri dish, the cell number is $5 \times 10^5$/plate. Then the protoplasts were mixed with purified mSWCNT-FITC solution, and put on the magnet for 12 hours. The protoplasts were harvested and fixed using cell fixing solution. Before taking FACS determination, the fixed protoplasts were washed by using PBS buffer or 70% ethanol. According to Figure 2, FITC delivery efficiency from FACS results is about 100% for both canola and carrot protoplasts, in which mSWCNT concentration is in the range of 0.06–0.25 μg/ml. Regardless they are canola or carrot protoplast, the higher concentration of mSWCNT resulted in the stronger fluorescence signal. This result shows that higher mSWCNT concentration corresponds to more mSWCNT-FITC entering cells. In order to ensure the mSWCNT-FITC attached on the surface of cells was completely removed, the protoplasts were washed twice using 70% ethanol after twice PBS washing. In Figure 2(B), the protoplasts were washed twice only using PBS. Compared with the FACS results in Figure 2(B), a little left shift for canola protoplast and a bigger left shift for carrot protoplast after ethanol washing were observed (Fig. 2(A)), which indicates most mSWCNT-FITC outside cells were washed away because SWCNT is more soluble in ethanol than in water. The distributive curves of cell counts from the controls in FACS were different from that of normal mammalian cell lines. For instance, the fluorescent strength of normal mammalian cell lines was at the order of $10^0 – 10^1$, but those of two protoplasts were at the order of $10^0 – 10^2$, which indicated the protoplasts may exist in different size after purification, which was confirmed by microscope images in Figure 3(A). According to the image in Figure 3(A), the size of canola protoplast after purification is different, the bigger one is about 4 times of smaller one. Though there are two peaks for control, we still can see all fluorescent signals shifted toward stronger fluorescent strength position after mSWCNT-FITC delivery. It showed that mSWCNT-FITC did enter into almost all protoplasts.

![Canola protoplast](image1.png) ![Canola protoplast/mSWCNT-FITC](image2.png) ![Carrot protoplast](image3.png) ![Carrot protoplast/mSWCNT-FITC](image4.png)

**Fig. 4.** Confocal images of canola and carrot protoplasts/mSWCNT-FITC. (Because the size of carrot cell is much smaller than that of canola cell, the green fluorescent signal in carrot cell is weaker than the canola cell).
Figure 3(B) showed that mSWCNT was not cytotoxicity for these two protoplasts because the cell viability after treated with mSWCNT-FITC remained similar as the control by using FDA staining method. We took several images, and counted 100 protoplasts in bright channel.

In order to confirm our observations, we did confocal and sectional TEM images of these two protoplasts. Compared to the control cell, green fluorescent signals appeared in most cells after mSWCNT-FITC delivery. The signal strength was different for different cells, which reflected how many FITCs entered the cells (Fig. 4). Even if the size of carrot protoplast is smaller than that of canola protoplast, the mSWCNT-FITC can also enter them. There were some green fluorescent signals appeared near the nucleus, which meant the FITC was near the nucleus. Figure 5 is the sectional TEM images of these two protoplasts. For canola protoplast, the mSWCNTs were found in endosomes (Fig. 5-canola A–D). However, for carrot protoplast, an mSWCNT was found outside the cell and an mSWCNT was found near nuclear membrane. All these results showed that mSWCNT not only entered cells but also distributed in different organelle inside plant cells. Due to the contrast between SWCNT and cell itself is not bigger, it is difficult to find SWCNT in relative dark position in cells.

4. CONCLUSION

To ensure the delivery of FITC, it was covalently bonded with mSWCNT. FACS results showed that mSWCNT-FITC can enter canola and carrot protoplasts driven by external magnetic force. The FITC delivery efficiency was
about 100% according to FACS results. The confocal and sectional TEM images further confirmed that mSWCNT-FITCs were inside these plant cells. mSWCNTs were also found both in the endosomes of canola protoplast and outside endosome near nuclear membrane of carrot protoplast.

**Acknowledgments:** The authors are grateful to Mr. Curtis Kuzyk, Dr. Minghui Du of Alberta Innovates - Technology Futures, and Dr. Weikai Yan of Agriculture and Agri-Food Canada (AAFC) for discussions on experiment designs. The research was funded by the Alberta Innovates - Bio Solutions and NSERC/CIHR Collaborative Health Research Projects Program.

**References and Notes**


Received: 11 April xxxx. Accepted: 24 April xxxx.