Introducing Carbon Nanotubes into Living Walled Plant Cells through Cellulase-Induced Nanoholes

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Two movies: Movie 1 and Movie 2.

MATERIALS AND METHODS

Carbon nanotubes and cellulase immobilization

CSCNT was a gift from GSI Creos Corporation, Japan (Lot No. 24PS, medium). The as-received tubes have lengths between (1 μm-100 μm) and the mean diameter is 60~100 nm. After functionalization, tubes have lengths ~500 nm. All chemicals used for nanotube functionalization and cellulase immobilization were purchased from Sigma (St. Louis, MO, USA) except sulphuric and nitric acids, which were purchased from Wakao, Inc., Japan. CSCNT were shortened and oxidized by sonication in mix of concentrated sulphuric and nitric acids (3:1) for 12 h at room temperature followed by reflux at 120°C overnight. The resulting dispersion was washed repeatedly with MilliQ water and filtered until the pH was ~7. The resulting functionalized CSCNT were then dried under vacuum overnight. This procedure removes metallic and carbonaceous impurities and generates carboxylate groups on tips and exposed surfaces of CSCNT. For cellulase immobilization, 2 mg of functionalized CSCNT were dispersed in 2 mL pH 6 MES buffer and sonicated for 10 minutes to obtain a homogenous dispersion, which indicated that CSCNT was well functionalized with hydrophilic carboxylate groups. This dispersion was then mixed with 1 mL of 400 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 100 mM N-hydroxysulfosuccinimide (NHSS) in pH 6 MES buffer and vortexed at room temperature for 15 min. The resulting mixture was spun at 15,000 rpm for 5 minutes, and the supernatant was discarded. The buffer wash was repeated to remove excessive EDC and NHSS. Cellulase at 10 mg/mL in pH 7 phosphate buffer was added to the mixture and stirred in a small vial overnight, at room temperature. The reaction mixture was then spun at 15,000 rpm at 4°C for 10 minutes, and the resulting supernatant was discarded. This step served to remove any free cellulase and was

repeated 4 additional times. One ml of 10% OG/Murashig and Skoog (MS) medium was added to the CSCNT-cellulase precipitate collected, vortexed to form a homogenous dispersion, and stored at 4°C. Prior to use, CSCNT-cellulase stock was diluted by 0.1% OG in MS. The CSCNT-cellulase was characterized by tapping mode AFM and enzyme activity assays. CSCNT-cellulase dispersions were stable for a week at 4°C as shown by reproducible hydrolytic activity against cellulose microfibrils. Accurate CSCNT-cellulase size determination is a topic of current research because some of the dispersed particles organize themselves into groups.

Qdot labeling

Core Qdot (CdSe) (diameter; around 5 nm), passivated in Tri-octyl phosphine oxide (TOPO), with photoluminescence emission maximum at 567 nm were sonicated in PBS buffer for 1 h, then filtered with a 0.2 µm syringe filter. Prior to OG addition, 0.2 µM of CdSe dispersion were allowed to interact with 0.5 mg CSCNT-cellulase for 2 h at room temperature followed by centrifugation at 15,000 rpm for 5 min and the resulting supernatant was discarded. Nanotube residue was washed 4 times with MilliQ water-prepared MS medium to remove excess CdSe. Microscopic examination of CSCNT/CdSe nanohybrids revealed strong adsorption of CdSe onto CSCNT-cellulase. Core Qdot were used for labeling instead of Qdot-streptavidin conjugate to circumvent the sonication process necessary for labeling nanotubes with Qdot-streptavidin, which might affect enzyme activity.

Plant material and uptake experiments

Arabidopsis thaliana ecotype Columbia Col-0 seeds were obtained from Riken Bioresource Center, Tsukuba, Japan. All chemicals used for tissue culture were

purchased from Sigma (St. Louis, MO, USA). *A. thaliana* cell suspension culture was initiated in MS medium supplemented with 1µM of 2,4D and kinetin. Twenty five mL of cell suspension was filtered through cell strainer to remove large cell clumps and obtain homogenous suspension. One mL of cell suspension was mixed with 10 µg of CSCNT-cellulase for 3 hours at 25°C in the presence of 0.1% OG.

For toxicity testing, cultured cells were incubated in MS medium, 25°C, dark, shaking 60 rpm, with the CSCNT system at two different concentrations (10 and 1000 µg/ml). The later represents 100 times higher concentration used in uptake experimenst. As a control, cells were incubated in MS medium without CSCNT. Cell viability was evaluated using a plant cell viability assay kit (Sigma, St. Louis, MO, USA) following the accompanying protocol.

Microscopy and image analysis

AFM imaging was carried out using the nanoscope llla MultiMode system (Digital Instruments, Santa Barbara, CA, USA). CSCNT-cellulase was dispersed at 1 ng/µL in either MilliQ water or in 1% OG solution. The dispersion (3 µL) was spread over a recently cleaved AP-mica surface. After 20 minutes, AFM observations were started. The nanoscope was operated in the tapping mode, using standard Si_3N_4 cantilevers (100 µm long, 5-40 nm tip curvature radius; 5-50 kHz resonance frequency, 0.32 N/m force constant; Digital Instruments specifications). Scanning parameters consisted of a 8.9-9.0 kHz driving frequency and a 2 Hz scanning rate.

Epifluorescence microscopy was carried using an inverted microscope (Axiovert 135TV, Carl Zeiss, Tokyo, Japan) illuminated by a 100W mercury arc lamp and a 10x/0.3 NA objective lens (Carl Zeiss). Photos and movies were captured by EB-

CCD camera (C7190-21, Hamamatsu Photonics, Hamamatsu, Japan). Movies were captured at 15 frames per second (fps).

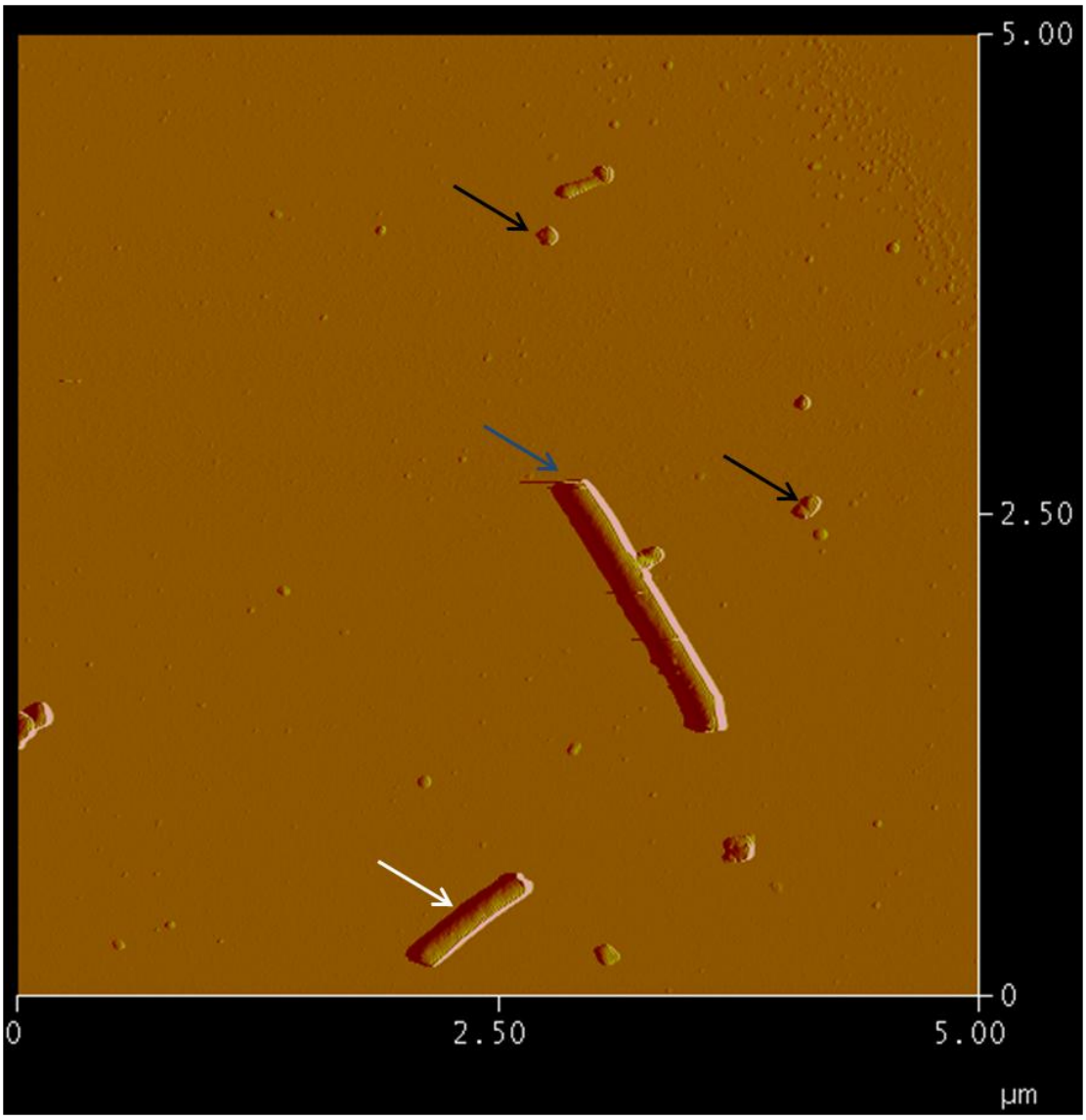
Confocal imaging were taken using 40x objectives with a laser scanning confocal microscope (Olympus, FV1000) equipped with multi-line Ar laser (458 nm, 488 nm, 515 nm), HeNe(G) laser (543 nm, 1 mW), HeNe (R) laser (633-nm, 10 mW), LD laser (405 nm, 25 mW) and AOTF laser combiner plus a set of ion deposition and barrier filters. Quantum dots luminescence was detected in Qdot 565 channel using a 543 nm laser. Cell autofluorescence was detected in Fura-red channel with a high autofluorescence signal using a 515 nm laser. Images were acquired and analyzed using Fluoview software.

Enzyme activity assay

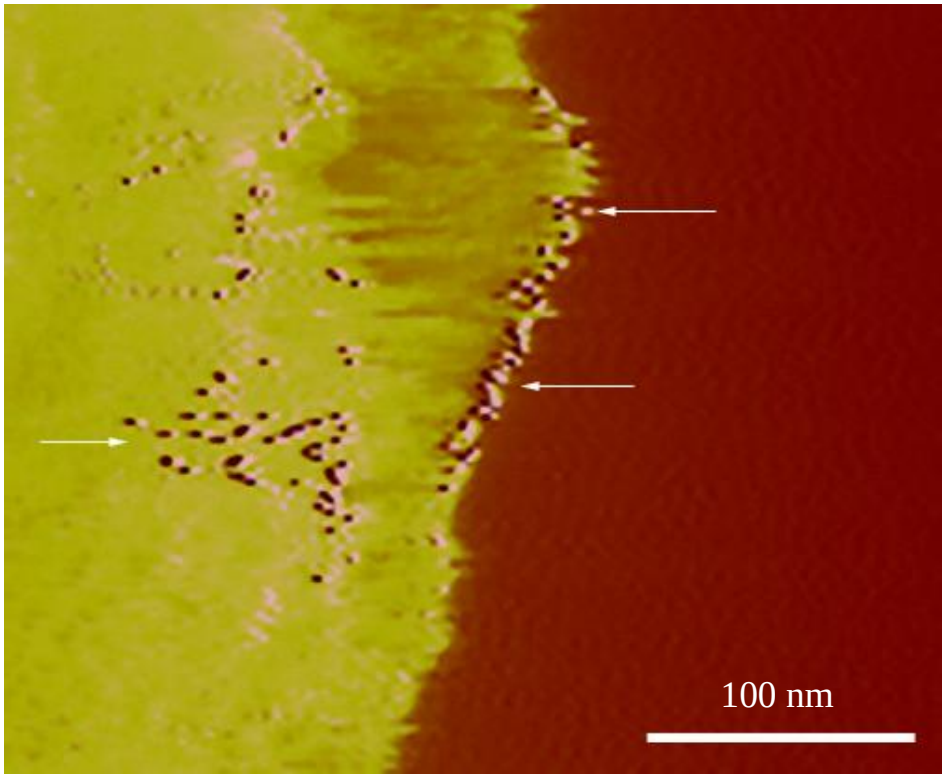
All chemicals used for enzyme activity assays were purchased from Sigma (St. Louis, MO, USA) unless otherwise stated. Activity of cellulase immobilized on CSCNT was assessed against cellulose microfibrils (medium lot). Typically, 1 mg of CSCNT-cellulase was added to 1 mg of cellulose suspended in 1 mL MilliQ water and incubated for 3 hours at 37°C. Glucose and cellobiose released after cellulose hydrolysis were labeled with 2-aminoacridone (AMAC; Invitrogen, Molecular Probes). The AMAC solution (0.1 M) was prepared using 5 µL of a mixture of DMSO and acetic acid (17:3 v/v). A 10 µL aliquot of the hydrolysate solution was transferred to a 500 µL screw capped microcentrifuge tube, and a 5 µL aliquot of the AMAC solution was added, followed by a 10 µL addition of 1 M $NaBH_3CN$. This mixture was kept in a water bath at 55°C for 2 h and diluted to suitable concentrations with MilliQ water prior to analysis. Electrophoresis experiments were carried out on a Hitachi SV1100 microchip electrophoresis instrument with a light-emitting diode confocal fluorescence detector and an external power supply capable of providing

voltages ranging from 0 to 5000 V (Hitachi Electronics Engineering, Tokyo, Japan). Data acquisition and analysis were performed using the software supplied with the system. A blue light-emitting diode, with a median excitation wavelength of 470 nm, was used as the excitation source. Fluorescence was collected with a condensing lens, spectrally filtered by a beam splitter (transmission > 530 nm) and an emission filter (transmission > 580 nm), and then detected by an avalanche photodiode (Hamamatsu Photonics, Hamamatsu, Japan). The electrophoresis PMMA chip specifications were provided in previous studies (Zhang, L. *et al.* Electrophoresis 2002, 23, 2341-2346). Running buffer with cellulose polymer additives was prepared by adding methylcellulose ((MC; viscosity of 2% aqueous solution at 20ºC, 4000 cP) to 100 mM boric acid and stirring slowly until the solution appeared homogeneous and transparent. The pH was adjusted to 8.5 with 1 N KOH and 1 N HCl. The solution was filtered through a 0.2 µm syringe filter (Millipore, Bedford, MA, USA). MilliQ water was used for the preparation of buffer and all other solutions. The activity of CSCNT-cellulase was compared with that of crude cellulase by adding 1mg of both materials to 1mg/mL of suspended cellulose microfibrils. The activity was reported by comparing the area under the glucose and cellobiose peaks in both cases. We recorded ~35% loss in activity after labeling the nanotubes with CdSe which we attribute to the presence of relatively high conc. of TOPO surfactant. However, we believe that this is a minor issue because; in a real biomolecular delivery experiment the fluorescent label would be excluded.

SUPPLEMENTARY FIGURES

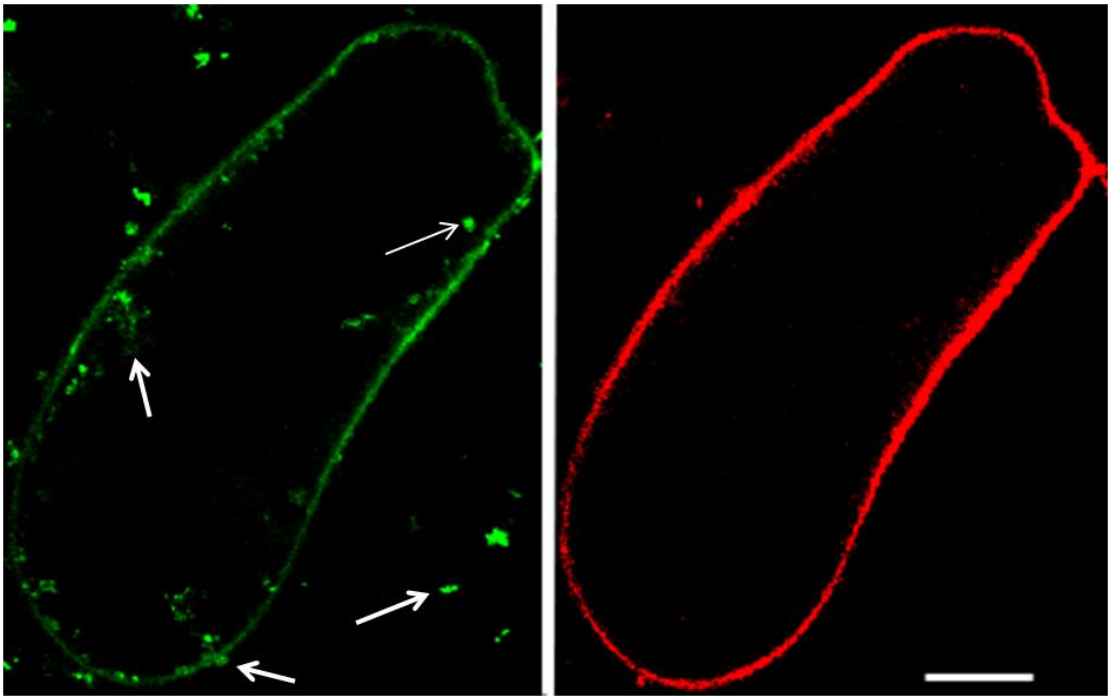


Supplementary Figure S1. Amplitude AFM characterization of cellulase immobilization on the surface of carbon nanotubes. A white arrow indicates cup-stacked carbon nanotubes without cellulase. Black arrows indicate cellulase molecules. A blue arrow indicates cellulase-immobilized cup-stacked carbon nanotubes.

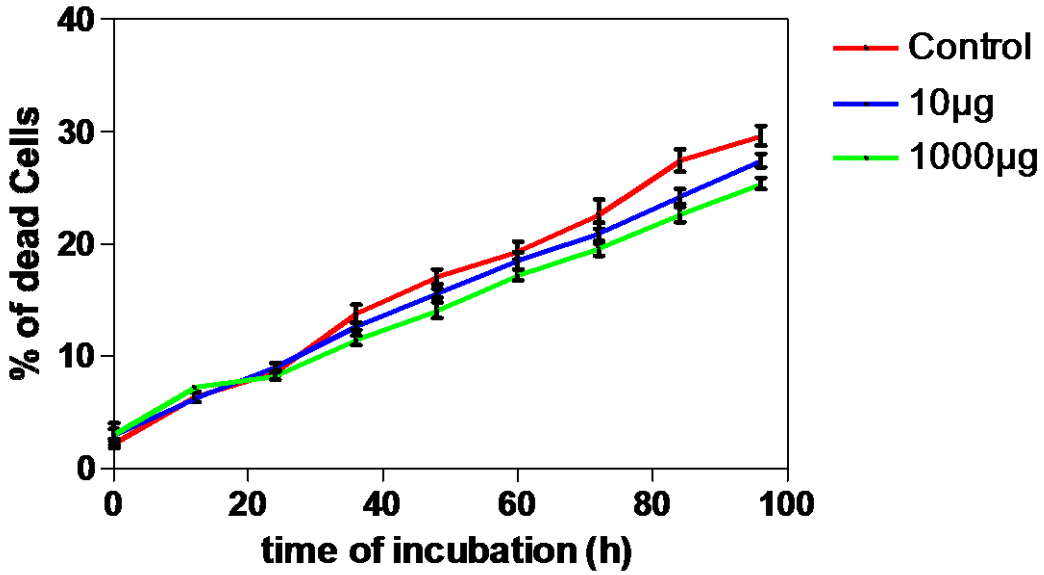


Supplementary Figure S2. Amplitude AFM characterization of core Qdots (arrows) adsorbed onto CSCNT ropes. Mica surface was free from Qdots. CNT and Qdot concentrations were 200 mg/ml and 0.04 µM respectively.

It was difficult to monitor Qdot adsorbed onto a single CSCNT due to large difference in objects' sizes. Therefore, the surface of an aggregated mass of CSCNT away from mica surface was examined. The walls of individual nanotubes within nanotubes ropes are not resolved due to the thickness of the cantilever (200 nm). Since, cellulase molecules hold nanotubes together; the walls are resolved in Figure 1. The cellulase molecules create molecular spacing between nanotubes (C.F. adsorbed nanotubes in nanotubes ropes), which helps the resolution of nanotubes' walls.



Supplementary Figure S3. Single focal plane imaging of CSCNT-cellulase uptake by *Arabidopsis Thaliana* cells. $CSCNT_{CdSe}$-cellulase was observed outside as well as inside the cell (White arrows). Qdot fluorescence is false-colored in green. Cell autofluorescence is in red. (scale bar: 20 µm).



Supplementary Figure S4. Evaluation of CSCNT toxicity on *Arabidopsis* mesophyll cells. Ten µg and 1000 µg of the material were added to 20 mL of 4 days *Arabidopsis* suspension culture.

Movies-related texts

- **Supplementary movie 1. Real-time tracking of CSCNT-cellulase inside *Arabidopsis thaliana* cells (arrow).**
- **Supplementary movie 2. Real-time tracking of $CSCNT_{CdSe}$-cellulase inside *Arabidopsis thaliana* cells.**

CSCNT-cellulase can be observed leaking into the cell through local sites at cell periphery where local cellulolysis assists internalization. Several CSCNT-cellulase can be well seen inside the cells during the first 10 seconds. The arrow in the movie indicates $CSCNT_{CdSe}$-cellulase. Arrows in the figure indicate cell vesicles.