Plant nanobionics approach to augment photosynthesis and biochemical sensing

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Supplementary Figure S1 | Control nIR images of *A. thaliana* leaves with infiltration medium. a, CRi Maestro nIR images of leaf lamina, and cross sections of b, leaf veins (20x) c, parenchyma cells with chloroplasts (20x) d, and chloroplasts *in vivo* (63x). Laser excitation 785 nm.
**Supplementary Figure S2 | PAA-NC and SWNT-NC characterization.**

- **a,** X-ray Photoelectron Spectra for PAA-NC showing cerium alternation between Ce$^{3+}$ and Ce$^{4+}$ oxidation states.
- **b,** Oxygen binding energy corresponding to metal oxide.
- **c,** FTIR indicates amide bond between 5’amino ss(AT)$_{15}$-SWNT and PAA-NC.
- **d,** AFM, **e,** height profile of SWNT-NC complex.
Supplementary Figure S3 | SWNT-NC near Infrared spectrum and Images. 

a, SWNT nIR fluorescence was not modified after conjugation with PAA-NC. 
b, nIR image of single particle SWNT-NC in buffer solution. Laser excitation 658 nm.
Supplementary Figure S4a | TEM images of SWNT-NC inside extracted chloroplasts. Nanoparticles localized both in the chloroplasts thylakoid membranes (red arrows) and the stroma (yellow arrows) in which the light reactions and carbon reactions of photosynthesis take place, respectively.
Supplementary Figure 4b | TEM images of extracted chloroplasts without nanoparticles.
Supplementary Figure S5 | Chloroplast intactness after interfacing with SWNTs and SWNT-NC. a, Chloroplasts only, and b, Chloroplasts + SWNT-NC with example intact (green arrow) and ruptured (purple arrow) chloroplasts. c, Percentage of intact chloroplasts for three samples. Phase contrast microscopy was used to determine the intactness of spinach chloroplasts (Lilley et al. 1975). The chloroplast envelope is distinguished by the presence of a bright halo, which is observed as either intact or broken. The intactness of chloroplast membranes was indistinguishable between treatments (n = 354). Error bars are standard deviations.
Supplementary Figure S6 | Initial rates of DCPIP reduction of chloroplasts with PAA-NC, SWNT-NC, SWNTs and metallic SWNTs (m-SWNTs). a, Increased photosynthetic activity of chloroplasts with nanoceria and SWNT-NC in the first 30 minutes of reduction of the electron acceptor dye DCPIP. b, Both SWNTs and m-SWNTs increased the photosynthetic activity of chloroplasts in the first 30 minutes but m-SWNTs were less effective than SWNTs. c and d, are insets of figures a and b for the initial five minutes. e, Comparison of DCPIP (µM) change between chloroplasts with nanoparticles minus chloroplasts without nanoparticles (Chloroplasts) vs. DCPIP reduced by nanoparticles in buffer after 30 minutes and 6 hrs. While reduction of DCPIP by PAA-NC in buffer contributed significantly to the observed DCPIP reduction by chloroplasts in the first 30 minutes, DCPIP reduction in buffer by SWNT-NC cannot account for the effect of these nanoparticles in chloroplast photosynthetic activity both in the initial 30 min and after 6 hrs. Similarly, both SWNTs and metallic SWNTs (m-SWNT) showed a low dye reduction in buffer relative to their effect on chloroplast DCPIP change. Data represent averages (n = 3). Error bars are standard deviations.
Supplementary Figure S7 | Electron transport rate light curves for SWNT chloroplasts and SWNT leaves. Extracted chloroplasts of *S. oleraceae* assembled with SWNTs at (a) 2.5 mg/L and (b) 5 mg/L showed no average enhancement in the light reactions of photosynthesis for all light levels (n = 3). (c) Higher electron transport rates in leaves of *A. thaliana* infiltrated with SWNTs at 2.5 mg/L concentration were observed for different light levels (n = 5).
Supplementary Figure S8 | SWNT and metallic SWNT (m-SWNTs) absorbance spectrum (5 mg / L). The m-SWNTs have two characteristic peaks at 532 and 561 nm. In contrast, a mix of HiPCo SWNTs (Unidym) has several absorbance peaks in both the visible and the nIR.

Supplementary Figure S9 | XTT cumulative reduction in the presence of chloroplasts incubated with scavenger superoxide dismutase (SOD). To allow the transport of the membrane impermeable SOD to the sites of superoxide generation, chloroplasts were ruptured in water then suspended in buffer during incubation with SOD for an hour. The significantly lower levels of XTT formazan production in the presence of the superoxide scavenger SOD confirms previous studies in plant cells (Able et al. 1998 42) demonstrating that XTT is responsive to changes in superoxide concentration.
Supplementary Figure S10 | Response of ss(AT)$_{15}$-SWNTs inside chloroplasts and leaves to water.

a, Deionized water slightly enhanced the fluorescence intensity of all SWNT chiralities inside extracted chloroplasts while b, SWNTs inside leaves exhibited a slight reduction in nIR fluorescence. c, 20x view of ss(AT)$_{15}$-SWNT inside a leaf with incisions before (left) and after (right) addition of 20 μL deionized water with three ss(AT)$_{15}$-SWNT regions of interest highlighted; d, Peak intensity-time traces of three ss(AT)$_{15}$-SWNT regions showing stable SWNT intensity after addition of 20 μL deionized amidst a slight reduction in overall nIR fluorescence from the leaf. Laser excitation 785 nm.
Supplementary Figure S11 | Response of chloroplast ROS, superoxide scavenging, and photosynthetic activity to cerium ion concentration. 

- **a**, Ce$^{3+}$ had no effect on ROS scavenging quantified by the conversion of H$_2$DCFDA to DCF; 
- **b**, chloroplast superoxide concentration, and 
- **c**, reduction of the electron acceptor DCPIP (0.01 mg Chlorophyll / mL). Error bars are standard deviations (n = 3).
Supplementary Materials and Methods

**Chloroplast isolation and chlorophyll concentration.** Chloroplasts were isolated from commercially available baby spinach leaves (*Spinacia oleracea* L.) as described by Sharkey *et al.*¹ with modifications. Chloroplasts were isolated in sucrose buffer (pH 7.3, 28 mM Na₂HPO₄, 22 mM KH₂PO₄, 2.5 mM MgCl₂, 400 mM sucrose, and 10 mM KCl) by two cycles of centrifugation at 4000 RCF for 10 min, then separated in a Percoll gradient consisting of 1 mL layers of 80%, 60%, 40%, and 20% Percoll in buffer. After 20 min of centrifugation at 4000 RCF, chloroplasts were selected from the 40% to 60% bands and washed with buffer. Chlorophyll concentration was determined according to Arnon *et al.*². 100 µL of the chloroplast solution was added to 1 mL 80:20 acetone:water to suspend chloroplasts’ chlorophyll molecules, vortexed for 1 min, and centrifuged at 3000 g (Spectrafuge 24D by Labnet International) for 2 min. Absorption of supernatant was recorded at 652 nm (Shimadzu UV-3101PC) and chlorophyll content calculated based on extinction coefficient of 36 mL mg⁻¹.

**SWNT and nanoceria synthesis.** Raw HiPCo SWNTs (Unidym) were washed using organic phase separation as in Tvrdy *et al.*³. The SWNTs were wrapped with a 30-base (dAdT) sequence of ssDNA (AT₁₅) (Integrated DNA Technologies) as in Zhang *et al.*⁴, in chitosan as in Reuel *et al.*⁵, in PVA as in Zhang *et al.*⁴, and in phospholipid–polyethylene glycol coated SWNTs as described by Welsher *et al.*⁶. Metallic SWNT were separated from HiPCo SWNTs (Unidym) by adsorption onto a magnetite-polymer construct. In brief, a mixture of SWNTs (1 mg / mL) in 1 wt % SDS was bath sonicated for 10 min followed by tip sonication at 20 W for 2 hrs. Then 3.5 mLs of equilibrated magnetic sephacryl was added to 10 mLs aliquots of SDS-SWNT and the
mixture stirred vigorously for 18 hrs. The solution was centrifuged at 4000 rpm for 10 min to settle out the sephacryl and the supernatant collected. The SDS-SWNT solution was characterized via photoabsorption spectroscopy to ensure isolation of m-SWNTs (Fig. S8). Finally, the m-SWNT SDS coating was exchanged with 30-base (dAdT) sequence of ssDNA (AT\textsubscript{15}) (Integrated DNA Technologies). Synthesis of poly (acrylic acid)-coated nanoceria proceeded as in Asati \textit{et al.}\textsuperscript{7} with modifications. Cerium (III) nitrate (2.5 mL, 1.09 g, 1 M, Sigma Aldrich, 99%) and an aqueous solution of 1,800 M.W. poly (acrylic acid) (2.5 mL, 2.25 g, 0.5 M, Sigma Aldrich) were added dropwise to 12.5 mL of HEPBS buffer (1.3 g, 0.4 M, Sigma Aldrich). The resulting mixture was adjusted to pH 8.5 with NaOH (8 M) and placed under continuous stirring for 24 hr at room temperature. The preparation was then centrifuged at 4000 RCF for 60 min to settle any debris and large agglomerates. The supernatant solution was then concentrated and purified by centrifugation at 4000 RCF for 10 min using a 10K Amicon cell (Millipore Inc.).

**Nanoparticle characterization.** SWNT concentration was calculated from absorbance measurements at 632 nm in a UV-VIS-nIR scanning spectrometer (Shimadzu UV-3101PC) using an extinction coefficient of 0.036 (mg/L)\textsuperscript{-1} cm\textsuperscript{-1}. The PAA-NC concentration was determined by recording the absorbance at 240 nm and a molar absorption coefficient of 20 cm\textsuperscript{-1} mM\textsuperscript{-1} as in Safi \textit{et al.}\textsuperscript{8} Carbon nitrile groups in the amide bonds of SWNT-NC complexes were detected by Fourier transform infrared spectroscopy (FTIR) Nicolet 4700 (Thermo). Atomic Force Microscopy (AFM) images of SWNT-NC were taken in an Asylum Research AFM with a silicon tip in air. Samples were mounted on clean silicon dioxide plates coated with (3-aminopropyl) triethoxysilane APTES 1% (v/v). X-ray photoelectron spectroscopy (XPS) was
performed on a Kratos AXIS Ultra X-ray Photoelectron Spectrometer with x-ray irradiation at 150 W. The TEM samples were mounted on Lacey-CA 300 mesh Cu grids (Ted Pella) and imaged in a 2000FX TEM microscope (JOEL) operating at 200kV. The SWNT zeta potentials were quantified in a ZetaPALS Zeta potential analyzer (Brookhaven Instruments).

**SWNT chloroplast uptake videos.** Videos were recorded in an AxioVision inverted microscope (Zeiss, Axiovert 200) coupled to a 2D InGaAs CCD array (OMA-V 2D, Princeton Instruments). Chloroplast autofluorescence was masked using 785 nm Invictus photodiode laser excitation (Kaiser) and 1100 nm long pass emission filter (Chroma). SWNTs (5 mg L⁻¹) were added to chloroplasts (0.05 mg Chl mL⁻¹) mounted on a glass slide and fluorescence recorded at 0.5 s (ss(AT)₁₅, lipid) or 1s (Chitosan and PVA) per frame. Chloroplasts nIR imaging was performed under 658 nm Cl-200 laser excitation (CrystaLaser) and 860 nm long pass emission filter (Chroma).

**Raman spectroscopy 3D mapping.** Maps were acquired in a confocal Raman spectrometer HR-800 (Horiba BY) using a 632 nm laser source. Chloroplasts (0.05 mg Chl mL⁻¹) were mixed for 15 minutes with SWNTs (5 mg L⁻¹) at 1:1 v/v. Samples were mounted on silicon dioxide plates, focused on a 100x objective and Silicon Raman peak used as reference position in Z axis. The confocal hole was reduced to 50 μm for a Z-axis resolution of ±1 μm. Raman spectra were taken after chloroplast exposure to 30 s under laser beam illumination when background levels were negligible. Raman 3D maps were plotted in Matlab R2011b.
**Laurdan labeled liposomes and SWNT interaction.** Laurdan (15 µM) was suspended with the most common chloroplast lipids, DGDG (0.7 mM) and MDGD (0.3 mM) (Avanti lipids) in chloroform-methanol (1:1). The solution was evaporated in vacuum chamber for 3 hr followed by nitrogen gas drying. The lipid layer was pre-hydrated in 3 mL of sterile PBS buffer 1X warmed above the lipids gel-fluid transition temperature (70 °C). The preparation was immediately submerged in a water bath above 80 °C while spun in a rotary for 60 min. The laurdan-labeled liposome change in fluorescence in the presence of ss(AT)_{15}-SWNTs was measured in the 410-650 nm range on a Varioskan flash plate reader 3001 (Thermo) under wavelength excitation of 390 nm. Laurdan generalized polarization (Gp) was calculated as in Szilágyi et al.\(^9\)

**Leaf infiltration with SWNTs.** Leaves of *Arabidopsis thaliana* were infiltrated with 100 µL of ss(AT)_{15}-SWNTs as in Huang et al.\(^10\). The SWNT stock suspension was dissolved in 10 mM MgCl\(_2\) and 10 mM MES. A 1 ml needleless syringe was used to push the SWNT solution through several areas on the abaxial side of the leaf lamina. For *in vivo* near infrared imaging, plants were uprooted from soil and roots wrapped with a water-moistened cheese cloth to maintain plants hydrated during imaging. Leaves were immobilized on a no. 1 thickness coverslip using double-sided sticky tape.

**Cri Maestro imaging.** Whole leaf imaging was performed on a CRi Maestro (PerkinElmer) containing a liquid crystal tuning element that allows transmitted light to be electronically tuned with a maximum wavelength range of 650 - 1050 nm and a 40 nm bandpass under a laser
excitation source of 785 nm. By analyzing the spectral emission wavelengths of the SWNT signal and leaf fluorescence, the entire signal’s fingerprint is separated into these components and the signal of interest determined. The emission window for leaves infiltrated with ss(AT)$_{15}$-SWNTs (30 mg L$^{-1}$) was set from 950 to 1050 nm with a 5 nm step size and 20 second reading at each step.

**nIR imaging of SWNTs in leaf cross sections.** Leaf cross sections were imaged in the nIR by an Axiovision Zeiss inverted microscope with an InGAs array nIR detector. Leaf brightfield images were taken with a Zeiss brightfield camera (Zeiss, Axiovert 200). The SWNT nIR fluorescence images were collected at 0.5 s exposure with an emission filter of 1100 nm and laser excitation of 785 nm off resonance of photosynthetic pigments. Leaves were infiltrated with 100 ul of SWeNT ®SG76 (SouthWest Nanotechnologies Inc.) ss(AT)$_{15}$-SWNTs (30 mg L$^{-1}$). Manual cross sections were imaged to detect SWNTs in the proximity of leaf veins. Imaging of leaf parenchyma cells and chloroplast was performed in cross sections fixed in 2.5% gluteraldehyde, 3% paraformaldehyde with 5% sucrose in 0.1M sodium cacodylate buffer (pH 7.4), and fixed in 1% OsO$_4$ in veronal-acetate buffer. The cell pellet was stained in block overnight with 0.5% uranyl acetate in veronal-acetate buffer (pH 6.0), then dehydrated and embedded in Embed-812 resin. Sections were cut on a Reichert Ultracut E microtome with a Diatome diamond knife at a thickness setting of 50 nm. Chloroplast fluorescence was imaged with a 658 nm 200 mW diode pumped solid state laser (CrystaLaser, RCL-100-660) and a band pass filter 700-750 nm (Chroma). Leaf images were colocalized to SWNT sources of nIR fluorescence using a custom MATLAB mapping file using leaf autofluorescence as a broadband emission source. A similar mapping file was created to map regions of interest from the SWNT
sources of fluorescence and the brightfield images. SWNT images and either leaf fluorescence or leaf brightfield images were overlaid using coordinates from our mapping files.

**PAA-NC confocal imaging.** Images were taken in a Zeiss LSM 710 NLO microscope. Nanoceria were labeled via carbodiimide reaction with Alexa fluor 405 Cadaverine (Invitrogen). The preparation was filtered in 3 cycles of 5 min centrifugation through a 10K Amicon membrane (Milipore) at 14 g. Labeled PAA-NC were mixed with chloroplasts (0.03 mg Chl mL\(^{-1}\)) and incubated for 2 hours.

**Transmission electron microscope imaging.** A FEI Technai Spirit TEM microscope at 80KV was used to image chloroplast uptake of SWNT-NC complex. Chloroplasts (0.03 mg Chl mL\(^{-1}\)) were incubated for 2 hours with SWNT-NC (5 mg SWNT L\(^{-1}\)), and fixed (2.5% gluteraldehyde, 3% paraformaldehyde with 5% sucrose in 0.1M sodium cacodylate buffer pH 7.4). Then chloroplasts were post fixed in 1% osmium in veronal-acetate buffer, dehydrated and embedded in Spurr’s resin, and sectioned at 50 nm thickness with a Leica Ultracut UCT microtome. Cerium analysis by inductively coupled mass spectroscopy (ICP-MS) was performed on chloroplasts after purification from free SWNT-NC by three cycles of 5 min centrifugation at 12000 g using 5 \(\mu\)m pore size Ultrafree-MC centrifugal filters (Millipore). Samples were characterized by ICP-MS at Elemental Analysis Inc. (Lexington, KY) under project number 6197-12.
Nanoceria scavenging of reactive oxygen species. In DCF experiments, chloroplasts were illuminated at approximately 3% of photosynthesis saturation light levels to reduce H$_2$DCFDA light induced autofluorescence (Fig. S11a), while in XTT experiments, illumination with a LED flood lamp FL-70W (LED wholesalers) was 30% of photosynthesis light saturation (200 µmols m$^{-2}$ s$^{-1}$ photosynthetic active radiation, PAR, or 40 W m$^{-2}$). DCF fluorescence at 520 nm and XTT absorbance at 470 nm was recorded every 30 min for six hours with a Varioskan well plate reader (Thermo). Superoxide concentration based on XTT measurements was calculated from background corrected absorbance values using an extinction coefficient of 21.6 mM$^{-1}$cm$^{-1}$ 11. Controls with cerium nitrate addition at the same concentrations of PAA-NC show no association of ROS and superoxide scavenging with cerium concentration (Supplementary Fig. S11 a-b).

Nitric oxide solution. Saturated NO solution was prepared using a method similar to that reported previously 4. In brief, 5 mL of H$_2$O was introduced into a 10 mL round-bottom flask and sealed with a septum with an inlet and an outlet needle. Argon gas (Airgas) was bubbled into the H$_2$O for 1 hr to remove dissolved oxygen, NO gas (99.99%, Electronicfluorocarbons) was then bubbled for 1 hr at an outlet pressure of 2 psi. The final NO concentration of approximately 50 mM was determined using the horseradish peroxidase assay 12,13.

References


