Electronic Supplementary Information for

Synthesis and Toxicity Testing of Cysteine-Functionalized Single-Walled Carbon Nanotubes with *Caenorhabditis elegans*

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Fluorescence Microscopy Experiments

A 0.08 mg/mL solution of oxidized SWNTs in DI H_2O was prepared, 5 mL of which was combined with 50 uL of 1 mg/mL fluorescein isothiocyanate (FITC) solution and stirred for one hour. A change in maximum UV-vis absorption wavelength was used to confirm the attachment. Approximately 100 μL of this 250 μg/mL stock solution described above was spread on a nematode growth medium agar plate containing an *E. coli* OP50 food source. Ten adult worms (All worms used in this research were N2 wild-type strain.) were individually picked and transferred from base population plates to the experimental plate and allowed to incubate for 30 min. One drop of phosphate buffered saline (pH 7.4) was placed on a microscope slide, onto which the worms were then transferred for imaging. Throughout this process, strict protocols were followed to prevent light from illuminating the sample and photobleaching the FITC. Fluorescence microscopy images were obtained using a Nikon Eclipse EZ-C1 Plus confocal microscope system. Panel a of Figure ESI1 shows a control image of a *C. elegans* adult that has been exposed to just FITC. The image shows bright spots from the worm's autofluorescence and

a diffuse glow from the FITC. Likewise, panel b shows a control image of a *C. elegans* adult exposed to Rhodamine B. This image also shows spots from the worm's autofluorescence and bright fluorescence from the Rhodamine B.

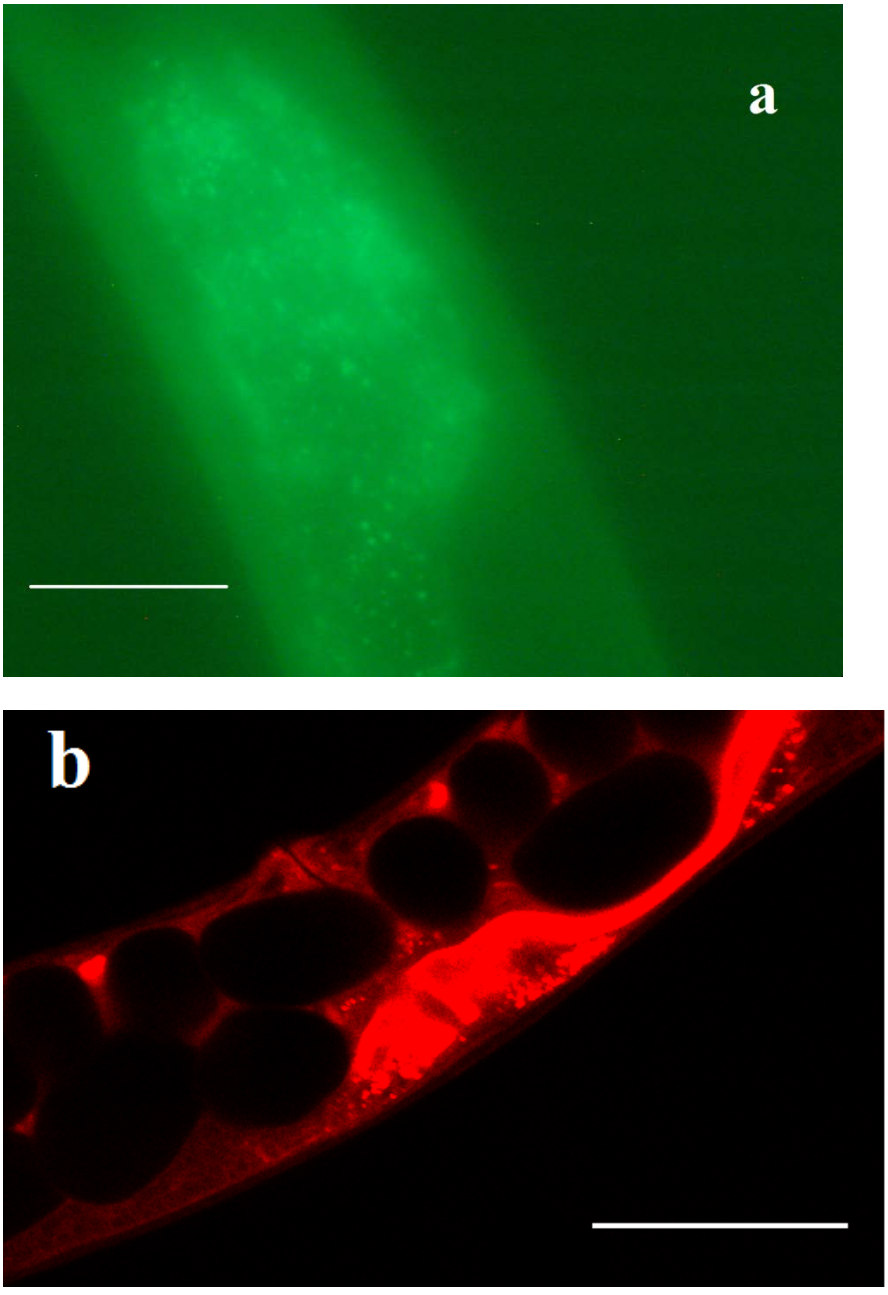


Figure ESI1. Fluorescence microscope images (60x) of *C. elegans* exposed to (a) fluorescein isothiocyanate, and (b) Rhodamine B. Each scale bar is 0.05 mm.

Red Emission of *C. elegans* Autofluorescence

In conducting the studies of fluorescence of Rhodamine B with SWNTs, we observed bright red spots, similar to the pattern of green autofluorescence in *C. elegans*. To determine whether this was from Rhodamine B or red emission from the autofluorescence, we collected confocal fluorescent microscope images of worms that were not exposed to any fluorescent dye. Panels a and b of Figure ESI2 show the fluorescence from the same region of the same worm, each excited with 488 nm light, which is appropriate for exciting autofluorescence. These images demonstrate that the autofluorescence spots emit red fluorescence when excited with 488-nm light. Figure ESI2c shows red fluorescence from the same section of the same worm, excited with 544 nm light, which is appropriate for exciting red fluorescence. The fact that panels a, b, and c show the same pattern of spots indicate that all of the fluorescence is coming from the autofluorescence spots. We therefore conclude that the autofluorescence spots fluoresce red light when excited with light appropriate for exciting green and red fluorescence. This means that the bright red spots in Figure 1f arise from the autofluorescent spots.

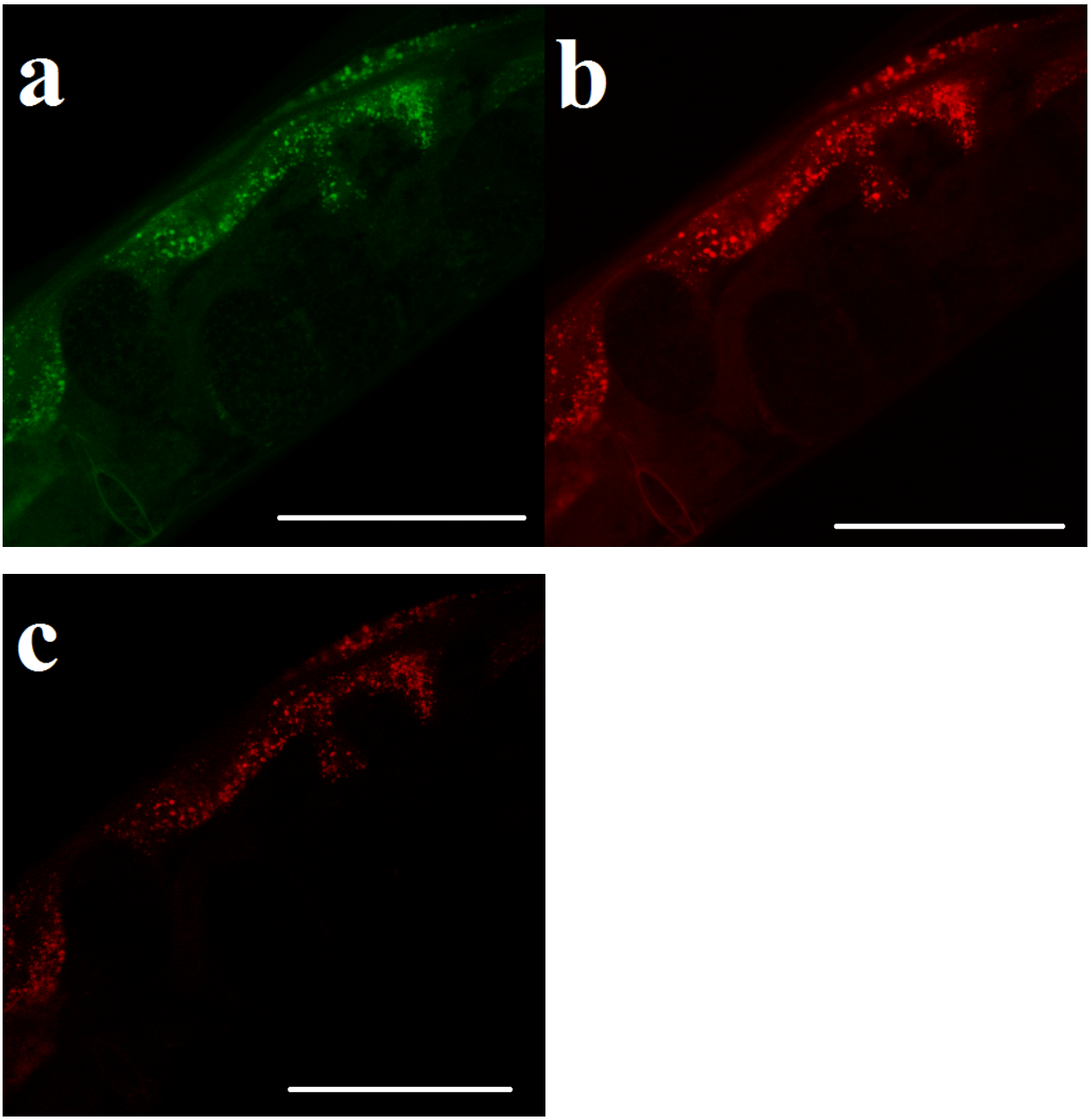
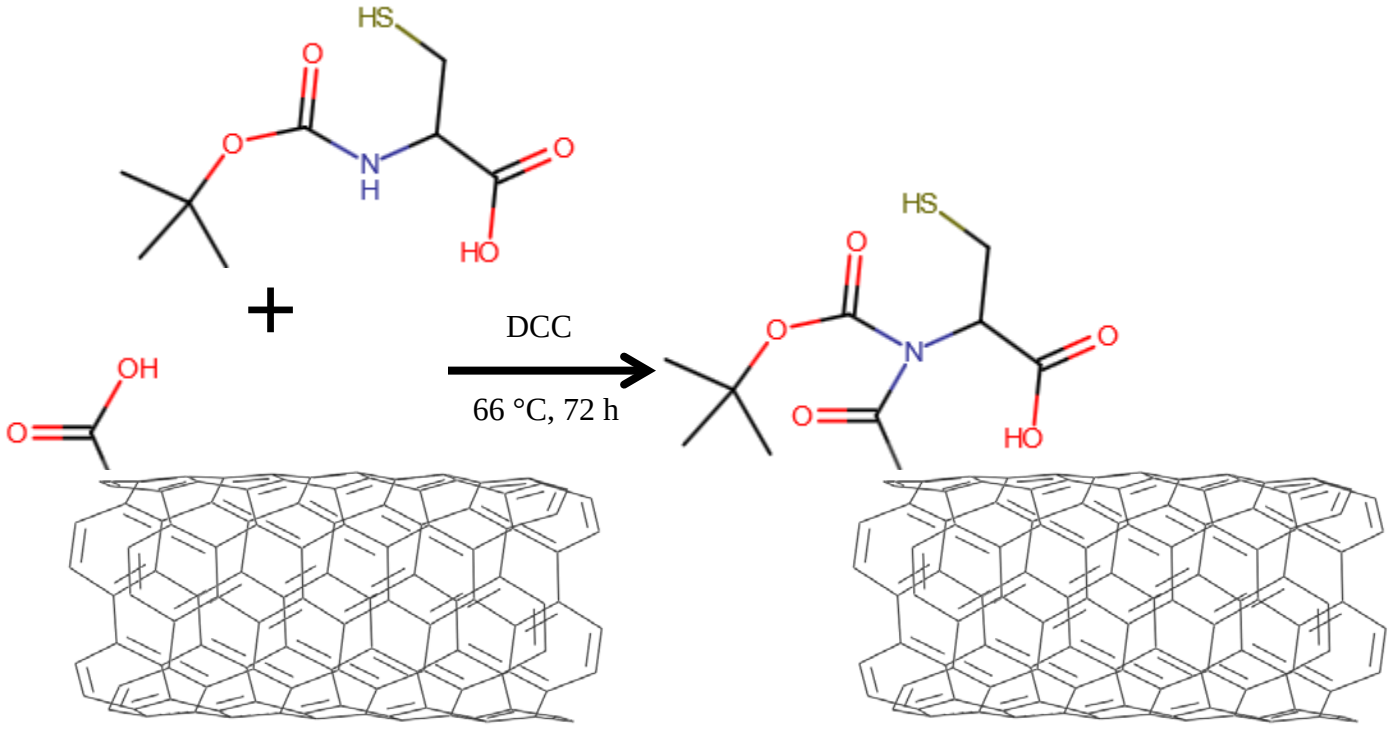


Figure ESI2. Confocal fluorescent images of the same section of the same *C. elegans* adult at 60x magnification showing (a) green autofluorescence using 488 nm excitation appropriate for green emission, (b) red fluorescence using 488 nm excitation appropriate for green emission, and (c) red fluorescence using 544 nm excitation appropriate for red emission. The scale bars are 0.05 mm.

L-Cysteine Functionalization of SWNTs

Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich and used without further purification. The reaction scheme is shown in Scheme ESI1. Cys-SWNT's were created by sonicating a suspension 15 mg oxidized SWNTs (NanoLab Inc., Waltham MA, >95% carbonaceous purity) 60 mg boc-L-cysteine, 30 mL tetrahyrdofuran (THF), and 60 mg n,n-dicyclocarbodiimide (DCC) in an Elmasonic S 30 H sonicator (Singen, Germany), then stirring



Scheme ESI1. Covalent attachment of boc-L-cysteine to oxidized SWNTs

under reflux at 66°C for 72 hours. The butoxycarbonyl (boc) protection group on the cysteine prevents the cysteine from polymerizing during the reaction. Upon completion of the reflux, 30 mL THF was added as the solution was stirred and cooled to room temperature. The product was filtered using a 0.1-μm PTFE filter membrane and washed repeatedly with THF and water to

remove excess cysteine. To assess purity of the final product, light microscopy was used to inspect the sample for excess cysteine crystals. The product being sufficiently pure, FTIR spectroscopic analysis of the Cys-SWNTs was used to confirm successful attachment. Infrared analysis was conducted using a Nicolet 6700 Fourier Transform Infrared Spectrometer with an attenuated total reflection (ATR) attachment and a liquid-nitrogen-cooled HgCdTe detector (Thermo Electron Corporation, Madison, WI).

The FTIR spectra of oxidized SWNTs and Cys-SWNTs are shown in Figure ESI3. The spectrum of oxidized SWNTs (black trace) shows a broad –OH peak at 3350 cm^{-1} from carboxylic acid and hydroxyl groups and a small carbonyl (C=O) peak at 1700 cm^{-1}. The similarity in the spectra of Cys-SWNTs (red trace) and of boc-L-cysteine (green trace) indicates that the cysteine is present on the SWNTs.

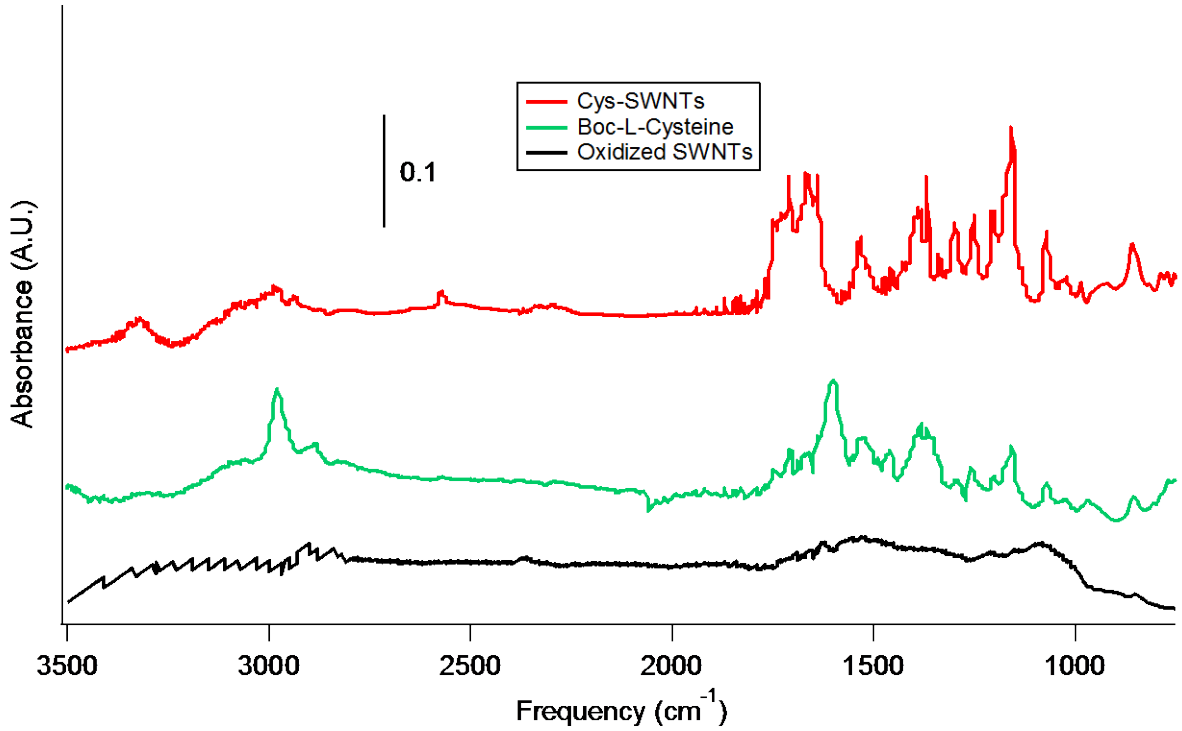
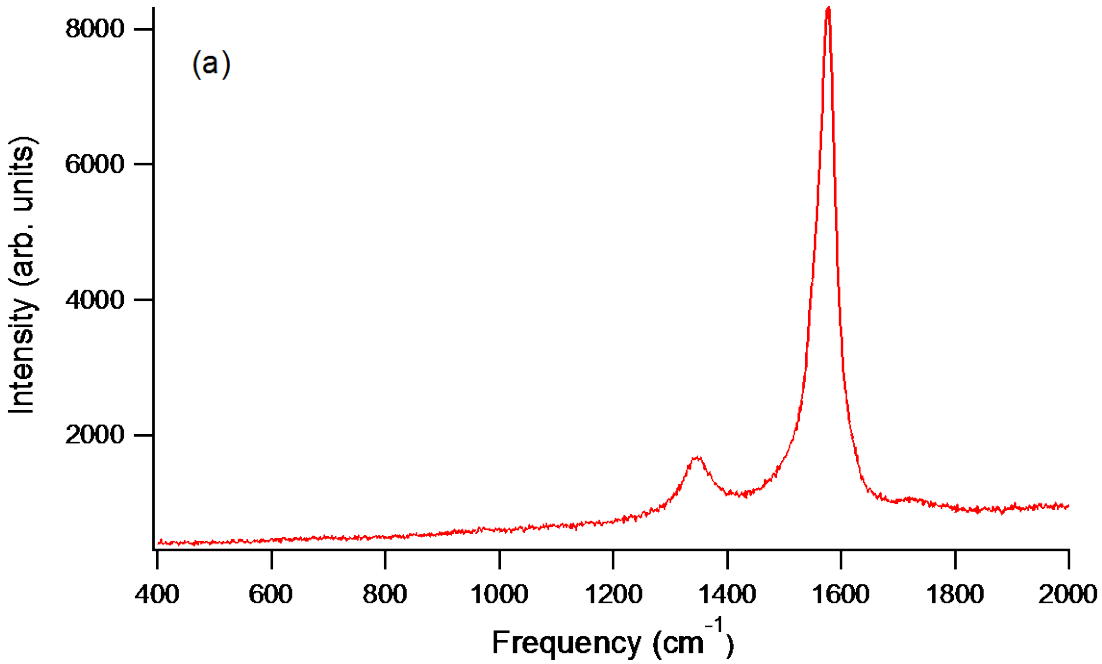


Figure ESI3. FTIR spectra of oxidized SWNTs (black) boc-L-cysteine (green), and Cys-SWNTs (red)

Raman spectra of oxidized SWNTs and Cys-SWNTs are shown in Figures ESI4a and ESI4b, respectively. Each figure shows a G band at 1580 cm^{-1}, indicative of sp^2-hybridized carbon, and a D band at 1348 cm^{-1}, sp^3-hybridized carbon from functionalization on the nanotube sidewall. In Figure ESI4b, the D band has greater relative intensity, indicating significant functionalization of the Cys-SWNTs.



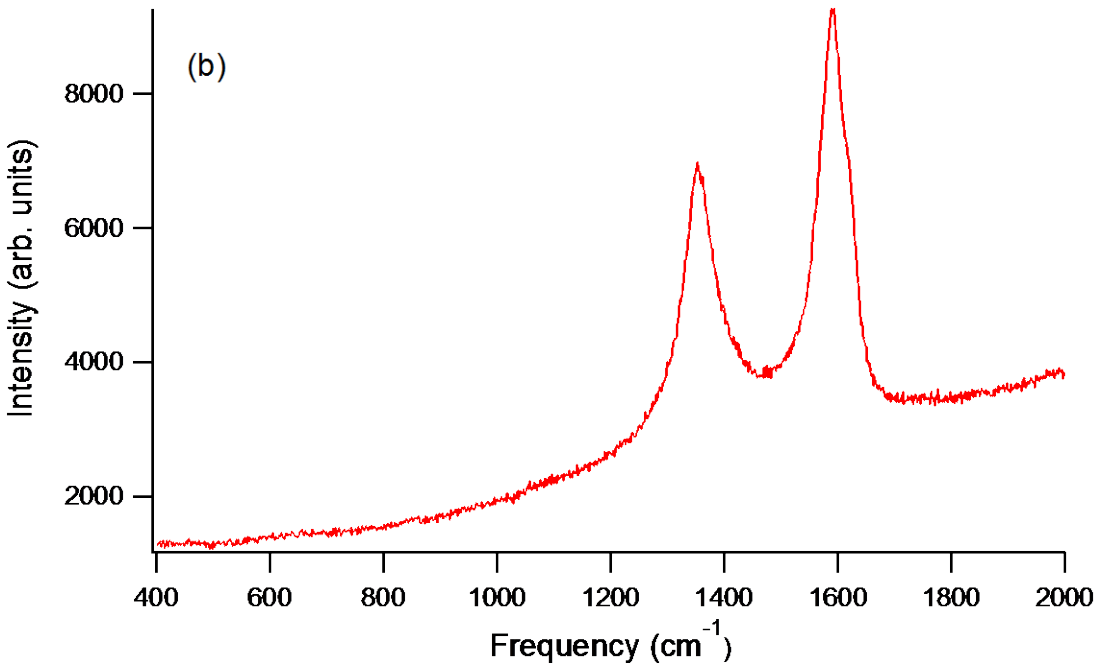


Figure ESI4. Raman spectra of (a) oxidized SWNTs and (b) Cys-SWNTs

The solubility of Cys-SWNTs in water was determined as follows. Approximately 40 mg of Cys-SWNTs were dried in an oven and weighed. These SWNTs were added to 10 mL of deionized water, sonicated, and left undisturbed for 1 h. The solution was centrifuged at 3600 rpm for 15 min, shaken, and then centrifuged again at 3600 rpm for 1 h. The supernatant was decanted, and the undissolved Cys-SWNTs were dried in an oven and weighed. Small samples of the supernatant were centrifuged at 8000 rpm for 1 h, resulting in no further settling of aggregates, confirming that the cys-SWNT were stably suspended. Using this method, the solubility of Cys-SWNTs in deionized water was determined to be about 1.6 mg/mL.

UV Sterilization

Approximately three milligrams of nanotubes were suspended in deionized (DI) H_2O, sonicated, and exposed to a pen lamp emitting UV radiation at 255 nm for 15 minutes. These

nanotubes were then introduced into 10 mL of autoclaved YPD growth medium and incubated at 37^{o}C for 24 hours and examined for any bacterial growth. Absence of bacterial growth affirmed the effectiveness of this sterilization process.

Toxicity Survival Assay

Ten-milliliter suspensions of Cys-SWNTs in 1X M9 buffer (KH_2PO_4, Na_2HPO_4, NaCl, $MgSO_4$, in DI H_2O) were created at 50, 100, and 250 μg/mL concentrations. Approximately twelve to twenty individual worms in the L4 stage of the *C. elegans* life cycle were picked and suspended in each solution, which was then covered (but not capped, to allow access to oxygen) and shaken for three hours. The suspensions were poured onto plates so worms could be located under microscope visualization and transferred to fresh NGM plates containing OP50 food source. These worms were then monitored over three days to record any deaths. This trial was repeated with oxidized SWNTs. For both experiments, control worms were subjected to three hours of shaking in 1X M9 buffer and then plated and observed over three days.

To observe the effect of long-term nanotube exposure on worm mortality, 250 μg/mL solutions of cysteine, oxidized SWNTs, and Cys-SWNTs were created in 1X M9 buffer. Plates were spread with 200 μL of each solution, and ten to twenty adult *C. elegans* were transferred onto the plates for 4 hours and then removed. The progeny of these worms was then counted each day and transferred to new plates every day, with fresh applications of 200 μL of the experimental solutions. This procedure was continued until all worms had expired, as determined by response to touch stimulus.

Lifespan Analysis

To observe the effects of long-term nanotube exposure on worm mortality, 250 μg/mL solutions of oxidized SWNTs, and Cys-SWNTs were created in 1X M9 buffer. Plates were spread with 200 μL of each solution, including a control plate with 1X M9 buffer, and 20 adult *C. elegans* were transferred onto each plate. The worms were then counted daily and transferred to new plates every 3–4 days, with fresh applications of 200 μL of the experimental solutions. This procedure was continued until all worms had expired, as determined by response to touch stimulus. Four plates were made for each solution, yielding a total sample size of 80 worms tested in each solution.

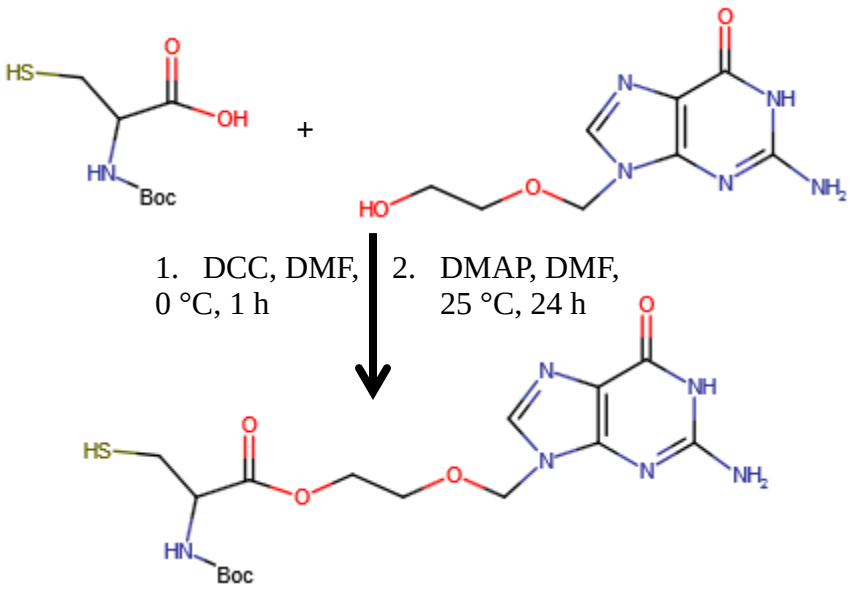
Brood Size Assay

To observe reproductive effects of nanotube exposure, plates were spread with 200 μL of cysteine, oxidized NT, and cys-NT experimental solution. Adult N2 worms were picked onto the plates and allowed to lay eggs overnight. The adults were then removed and progeny was counted daily, with a transfer to a new plate and reapplication of the experimental solutions after each count.

Cysteine-Acyclovir Synthesis

The acyclovir-cysteine conjugate was synthesized by refluxing 40 mg boc-L-cysteine, 25 mg DCC, and 250 uL dimethylformamide (DMF) under N_2 atmosphere for 1 hour. A solution of 25 mg acyclovir, 1.25 mg dimethylaminopyridine (DMAP), and 1.5 mL DMF was added dropwise, and the solution was stirred for 12 hours as it warmed to room temperature. The reaction scheme is shown in Scheme ESI2. The product was filtered using 0.2 μm PES filter paper and washed

with 10 mL DMF. The FTIR spectrum, shown in Figure ESI5, was used to confirm the identity of the final product.



Scheme ESI2. Reaction scheme for the covalent attachment of boc-L-cysteine to acyclovir

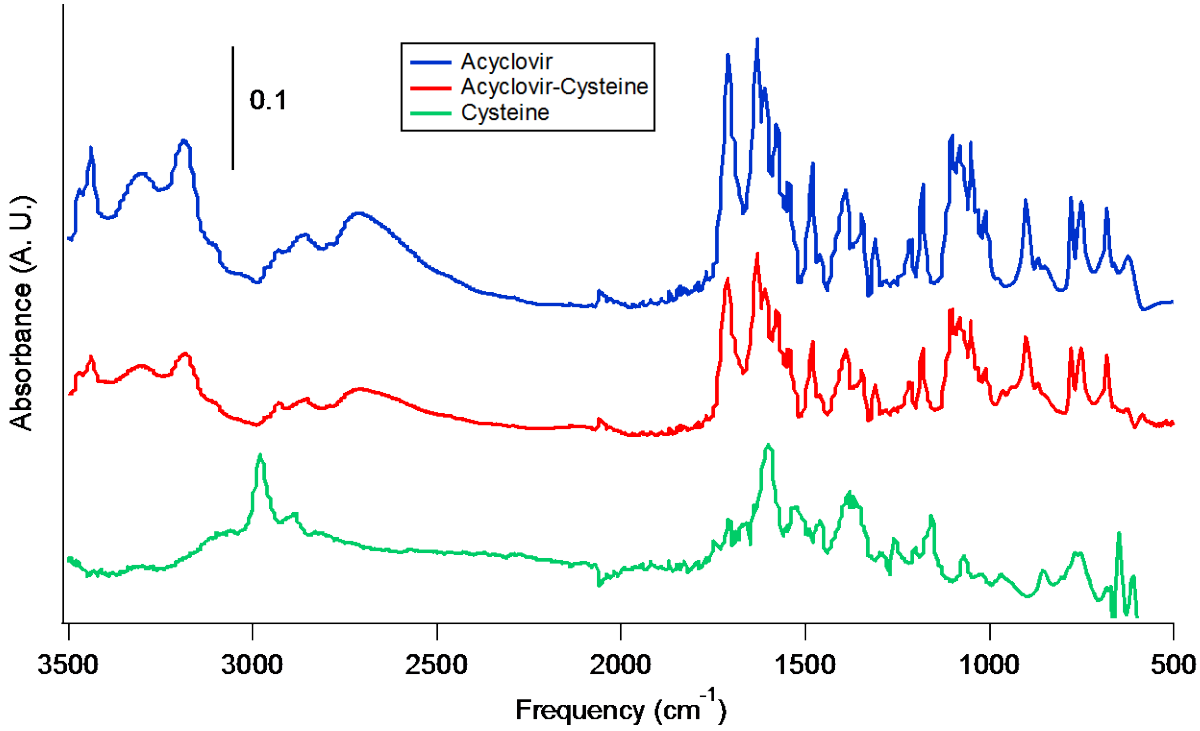
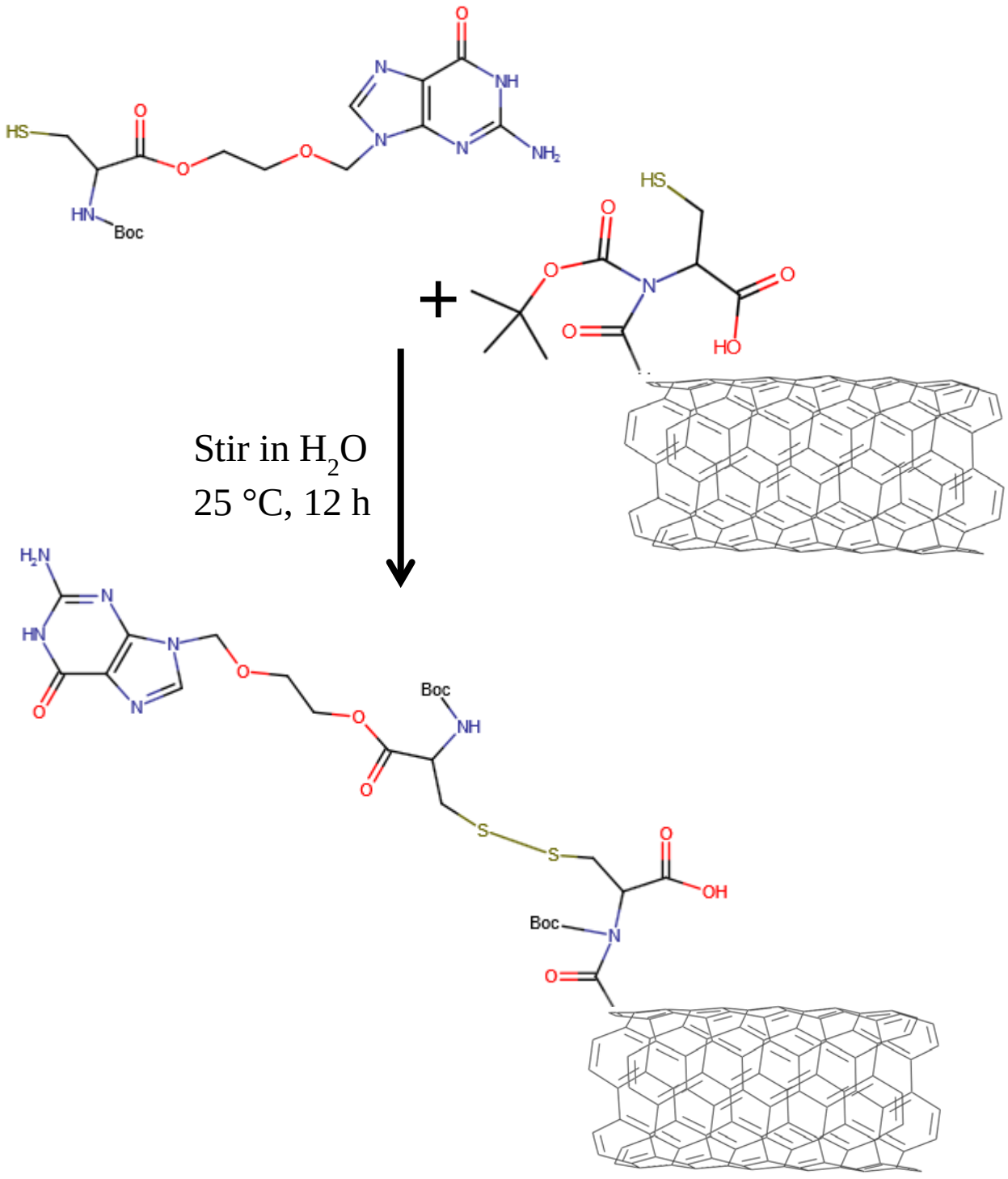


Figure ESI5. FTIR spectra of acyclovir (blue), the cysteine-acyclovir conjugate (red), and boc-L-cysteine (green)

Disulfide Bond Formation and Decoupling

The reaction process for attaching the acyclovir-cysteine conjugate to Cys-SWNTs is shown in Scheme ESI3. In 4 mL of deionized water (deionized through a Millipore Academic Gradient System with a resistivity > 18.2 MΩ·cm), 10 mg of Cys-SWNTs and 4 mg of cysteine-acyclovir were stirred constantly for 12 h. The solution was then filtered using a 0.6 µm isopore membrane (Millipore) before being dried under vacuum. The attached acyclovir-functionalized SWNT (acyc-SWNTs) samples were analyzed by FTIR spectroscopy. Figure ESI6 shows the spectra of the acyclovir-cysteine conjugate and the acyc-SWNTs. The spectra have multiple common peaks, including the C–O stretch at 1100 cm^{-1}, the C=C ring stretch at 1630 cm^{-1}, C–H stretches

at 2850 and 2920 cm^{-1}, the O–H stretch at 3200 cm^{-1}, and the N–H stretch at 3320 cm^{-1}. Notably, the S–H stretch at 750 cm^{-1} that is present in the spectrum of the acyclovir-cysteine conjugate is absent in that of the acyc-SWNTs, providing evidence for attachment via a disulfide bond. For cleavage of the disulfide bond, the acyc-SWNTs were added to a 10 mM solution of β-mercaptoethanol. The reduced acyc-SWNTs solution was filtered using a 0.6 µm membrane then dried under a vacuum. The reduced acyc-SWNTs sample was analyzed using FTIR, as shown in Figure ESI7. The change in the spectrum, particularly the appearance of the S–H stretch at750 cm^{-1}, indicates the loss of acyclovir from the nanotubes.



Scheme ESI3. Attachment of acyclovir to Cys-SWNTs via a disulfide bond

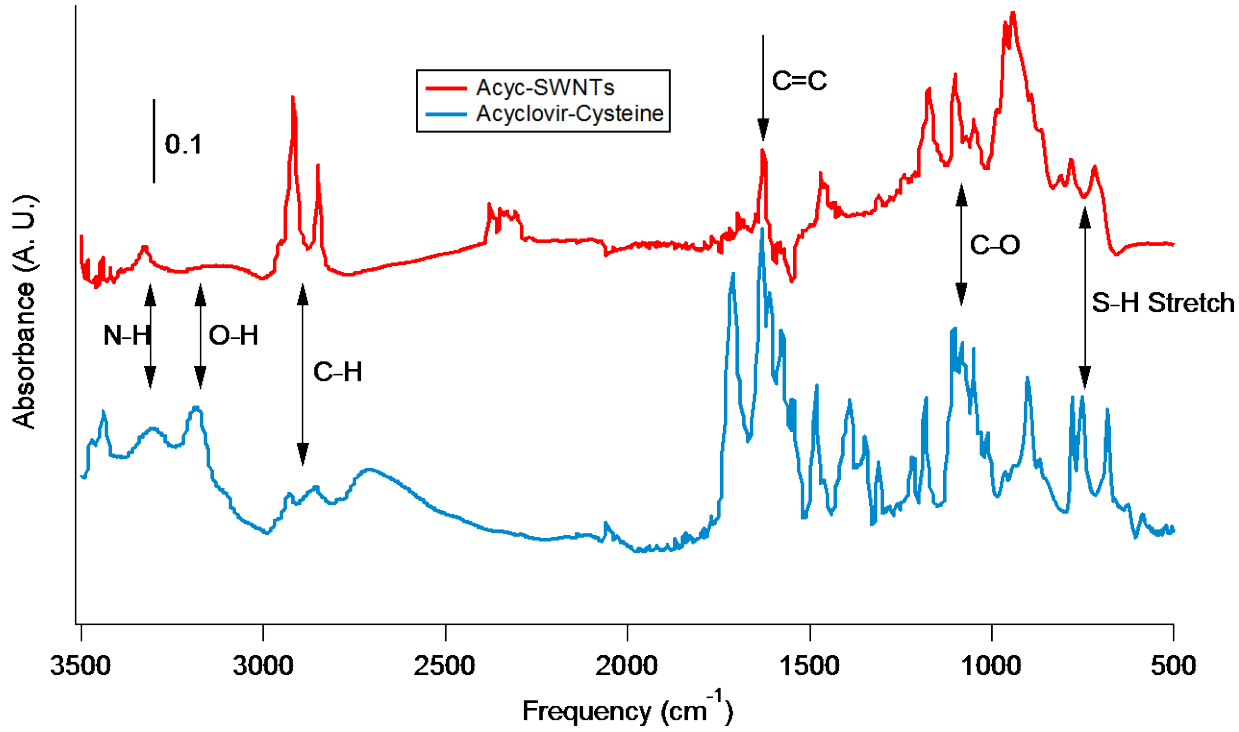


Figure ESI6. FTIR spectra of acyclovir-cysteine (blue) and acyclovir-SWNTs (red), illustrating some of the important vibrations common to both substances

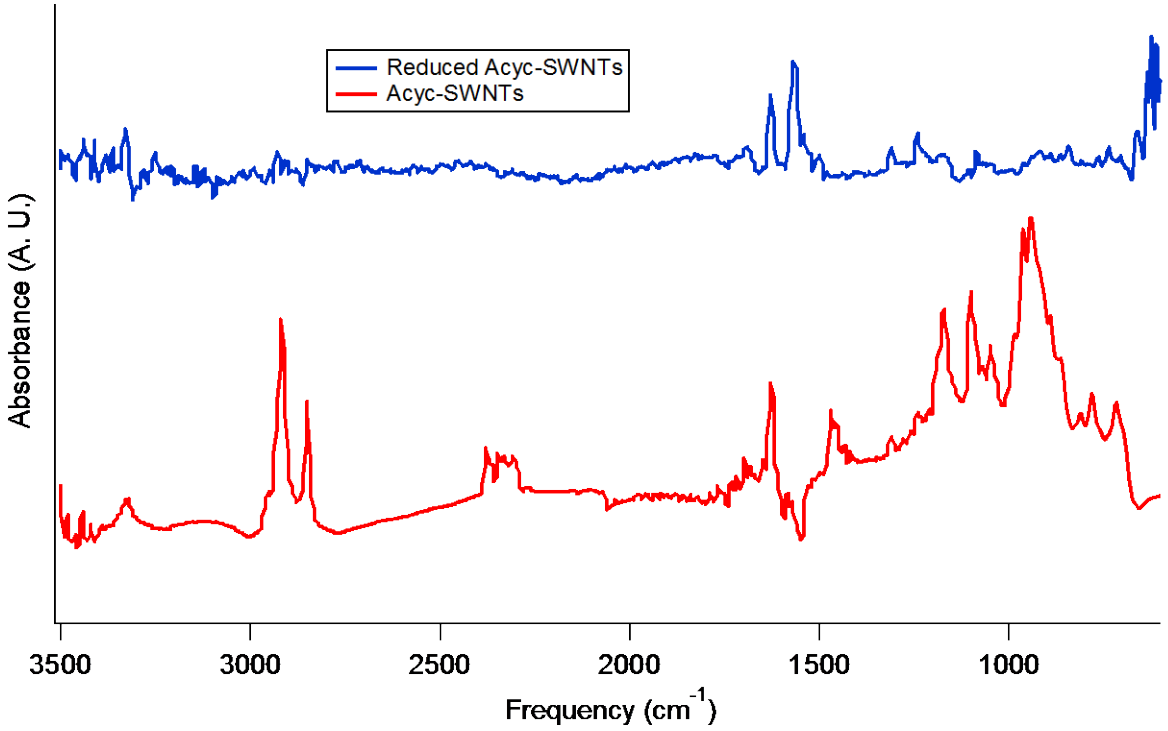


Figure ESI7. FTIR spectra of acyc-SWNTs (red) and acyc-SWNTs that have been reduced (blue) by exposure to β-mercaptoethanol