Noncovalent Functionalization of Carbon Nanotubes by Fluorescein-Polyethylene Glycol: Supramolecular Conjugates with pH Dependent Absorbance and Fluorescence

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Supplementary Information with Supplementary Figures

Functionalization of SWNTs by Fluorescein-PEG-COOH.

1 mM of fluorescein-PEG-NHS (PEG MW 5000 Da, Nektar Therapeutics) was sonicated with 0.25 mg/mL of HiPco SWNTs (Carbon Nanotechnologies Inc) in water for ~0.5-1h, and the resulting dark suspension was centrifuged at 25,000 g for 6 hrs. The pellet formed at the bottom of the centrifuge tube containing aggregates such as bundles and impurities was discarded. The supernatant was collected and filtered through a centrifugal filter device (100k Da MWCO, Millipore Amicon) to remove excess pegylated fluorescein, washed with water several times, and re-suspended in water. The solution was subsequently dialyzed (1 M Da MWCO, SpectrumLabs Spectra/Por Biotech CE membrane) over a few days to ensure complete excess removal. Note that the NHS group of the fluorescein-PEG-NHS hydrolyzes over the preparation period and becomes a carboxyl group in the final product.

Characterization of Fluor-PEG/SWNT.

Atomic Force Microscopy (AFM). Fluor-PEG/SWNT was deposited on SiO_2 by soaking a piece of SiO_2 in the Fluor-PEG/SWNT solution in 10 mM HCl for ~15 min. The acid neutralizes the charge on Fluor-PEG and helps the Fluor-PEG/SWNT conjugate to adsorb on SiO_2. The SiO_2 substrate was rinsed briefly with water, blow dried with air, and used for AFM imaging. Lengths of 100 SWNTs were measured, and the average length was estimated to be ~158 nm.

UV-vis-NIR Spectroscopy. UV-vis-NIR measurement was carried out using Cary 6000i spectrophotometer. The nanotube concentration in Fluor-PEG/SWNT solution was estimated by the absorbance value at 808 nm and using the extinction coefficient 7.9×10^6 $M^{-1}cm^{-1}$ (assumes ~150 nm 170k Da SWNTs).[1] The path length used for the measurements was 1 cm. The absorption spectrum of the nanotube solution shows distinct peaks in the UV-vis-NIR region, characteristic of highly dispersed individualized SWNTs.

Stability. The aqueous stability of Fluor-PEG/SWNT at high temperature and in high salt and protein environment was assessed by the following: 25 nM of Fluor-PEG/SWNT in water was heated to 70 ºC for 2 days. A small volume of a concentrated Fluor-PEG/SWNT solution was added to cell culture medium (Dulbecco's Modified Eagle's Medium, Invitrogen) with 10% fetal bovine serum (FBS) such that the final concentration of Fluor-PEG/SWNT was 25 nM. Fluor-PEG/SWNT remained soluble in both cases and no aggregation was observed, even after a centrifugation step at 2000 g for 10 min.

[1] Kam, N. W. S.; O'Connell, M.; Wisdom, J. A.; Dai, H. J. *PNAS* **2005**, *102*, 11600.

Estimation of Number of Fluor-PEG per SWNT. To determine the amount of Fluor-PEG adsorbed on SWNT, a high concentration of another surfactant known to be efficient at adsorbing and solubilizing SWNT in water was added to Fluor-PEG/SWNT solution to displace the Fluor-PEG adsorbed on SWNT. 1 mg/mL (~360 μM) of 1,2-Distearoyl-*sn*-Glycero-3-Phosphoethanolamine-N-[Amino(Polyethylene Glycol)2000] (PL-PEG-NH_2, Avanti Polar Lipids)[2] was added to Fluor-PEG/SWNT solution with the following 5 concentrations of nanotubes: 2.0, 3.2, 4.8, 6.6, and 7.8 nM. (Note: Fluor-PEG/SWNT suspension used was after removal of excess Fluor-PEG by dialysis to prevent excess Fluor-PEG from contributing to the fluorescence signal.) It was allowed to react overnight followed by 10 min sonication. Emission spectrum of the resulting solutions containing freed Fluor-PEG and PL-PEG-NH_2/SWNT in 1 mg/mL of PL-PEG-NH_2 at the 5 different SWNT concentrations was measured with a spectrofluorometer ($\lambda_{excitation}$ = 495 nm, $\lambda_{emission}$ scanned from 500-600 nm, Spex Fluorolog 3). To determine the concentration of this freed Fluor-PEG, calibration curve of Fluor-PEG fluorescence intensity was obtained by adding known concentrations of Fluor-PEG to a background of 5 nM of PL-PEG-NH_2/SWNT in 1 mg/mL of PL-PEG-NH_2. The concentrations of freed Fluor-PEG, which is the concentration of Fluor-PEG adsorbed on SWNTs, are plotted against the corresponding Fluor-PEG/SWNT concentration in S fig. 1. The number of Fluor-PEG attached to each SWNT was estimated and is also plotted. Fluor-PEG concentration increased linearly with SWNT concentration in the range of 1-5 nM, and correspondingly, the number of Fluor-PEG per SWNT remained constant in this range at an average of ~88 Fluor-PEG per SWNT. This value can be more accurately described

[2] Kam, N. W. S.; Liu, Z.; Dai, H. J. *J. Am. Chem. Soc.* **2005**, *127*, 12492.

as 11 μM of Fluor-PEG per absorption unit at 808 nm (or 0.5 μmols Fluor-PEG per 1 mg of SWNT) since this does not make any assumptions on the molar extinction coefficient.

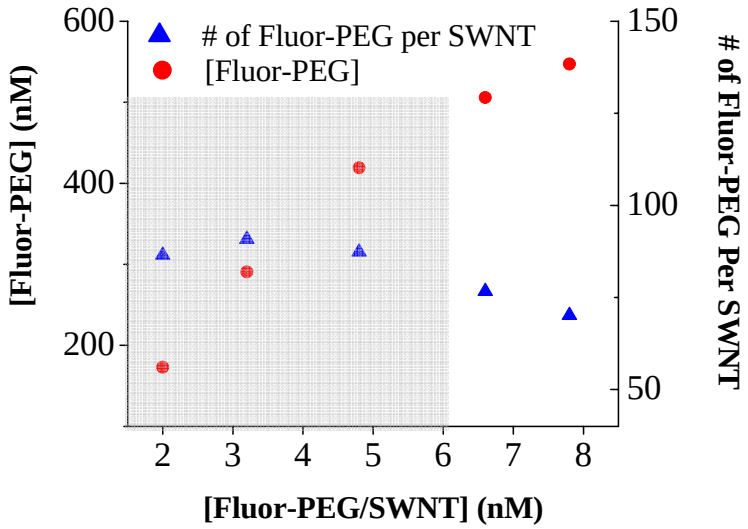


Figure S1. Plot of concentration of free fluor-PEG detached from the SWNT as a function of concentration of SWNT (red dots) and the number of fluor attached on each SWNT (blue triangle). The gray box indicates the linear region that was used to obtain the average number of fluor per SWNT, ~90.

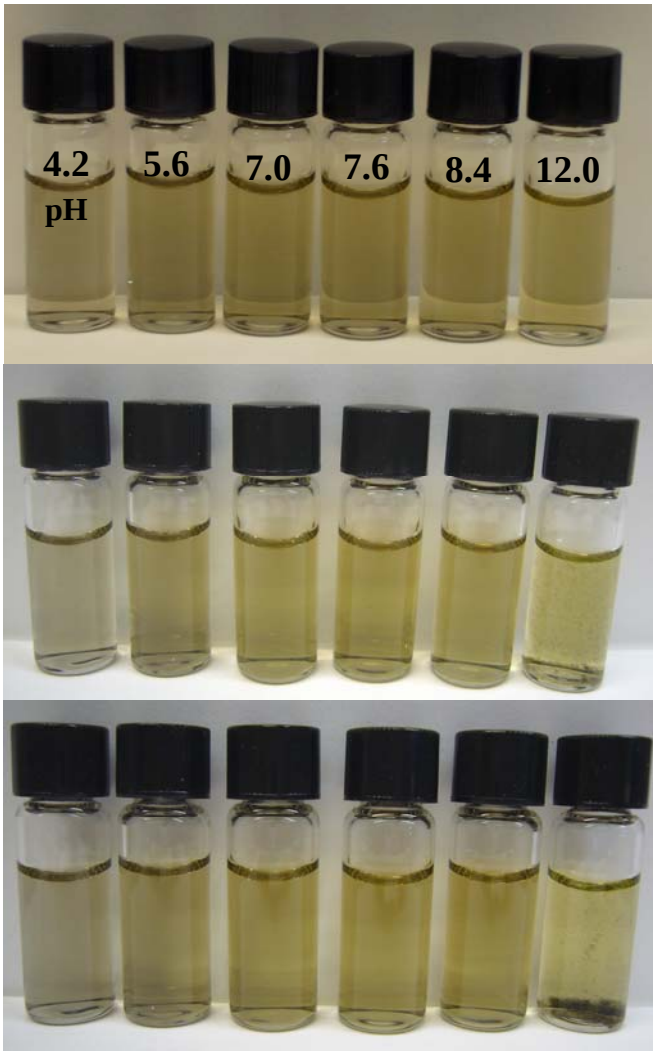
Estimation of SWNT coverage by Fluor-PEG. Using 170k Da as the molar mass of a ~150nm long SWNT, the number of carbons per SWNT was calculated to be 170kg/mole ÷12g/mole = 1.4×10^4. If we assume that all the atoms in the ring system of fluorescein are interacting with the SWNT via π-stacking, there are 20 atoms interacting with the SWNT aromatic rings. Since there are ~88 fluor attached to each tube, 20 atoms/fluor × 88 fluor/SWNT = 1760 carbon atoms of a SWNT are being covered by the adsorbed fluor. This corresponds to ~12% coverage ($1760 \div 1.4 \times 10^4 = 0.12$).

Fluorescence and Absorbance Properties of SWNT bound Fluorescein.

Emission and absorption spectrum of Fluor-PEG/SWNT suspension in phosphate buffered saline (PBS) at pH 7.4 (Invitrogen) was measured at the following 5 concentrations ($\lambda_{excitation}$ = 495 nm): 2.3, 4.2, 6.0, 7.4, and 9.5 nM. Using ~88 Fluor-PEG per SWNT, the corresponding Fluor-PEG concentration attached to the SWNT at the 5 concentrations was calculated to be 202, 370, 528, 651, and 836 nM, and free Fluor-PEG solutions at these concentrations were prepared in PBS. The emission spectra of these free Fluor-PEG solutions in PBS were measured for comparison. The absorption and emission curves for 6.0 nM Fluor-PEG/SWNT and 528 nM Fluor-PEG are shown in Fig. 2a and b respectively. The plot of the fluorescence intensity at 517 nm of Fluor-PEG/SWNT and Fluor-PEG with respect to Fluor-PEG concentration is shown in Fig.2c. The fluorescence intensity of Fluor-PEG/SWNT at 517 nm is ~33% that of free Fluor-PEG at all 5 concentrations of Fluor-PEG.

pH Dependence of SWNT bound Fluorescein Emission and Absorption. 4.6 nM Fluor-PEG/SWNT solutions at various pHs were prepared in 10 mM phosphate buffer with 150 mM NaCl at pHs: 3.0, 4.2, 5.6, 7.0, 7.6, 8.4, and 12. The absorption and emission ($\lambda_{excitation}$ = 495 nm) spectra of each solution were measured (Fig. 3a and b). For comparison, 405 nM (88 × 4.6 = 405 nM) Fluor-PEG solutions at the same pHs were also prepared, and the emission spectra of these solutions were measured. The emission peak intensity (at 517 nm) of Fluor-PEG/SWNT and free fluor as a function of pH is plotted in fig. 3c.

pH Dependence of Aqueous Stability of Fluor-PEG/SWNT. Fluor-PEG/SWNT solutions at different pHs were prepared at the same concentration and observed over several days for precipitates.



Day 1
No precipitation is observed.

Day 4
After >48hs, we can start to see black precipitates from the pH 12 solution.

Day 5
SWNT precipitates have settled at the bottom from the pH 12 solution, but more are still precipitating out. No precipitates were observed for the lower pHs.

Figure S2. Photographs of Fluor-PEG/SWNT solutions (with same nanotube concentration) at various pHs recorded after standing for various times indicated. Precipitation of nanotubes was seen in the pH 12 solution but not in the others.

Fluor-PEG/SWNTs are less stable at high pHs, suggesting weaker interaction between SWNT and Fluor-PEG. Fluor-PEG desorbs slowly from the SWNT surface over time.

pH Dependence of SWNT Photoluminescence of SWNTs solubilized by Fluor-PEG. We have measured the pH dependence of SWNT photoluminescence in the near IR using an InGaAs detector at 785nm laser excitation. We observed no significant pH dependence in the pH range studied as shown in Fig.S3, which differed from the results in ref. 9 and 10. This difference was currently not understood, but could be related to the different coatings on the nanotubes (neutral PEGylation in our case and surfactants with charged groups in other cases). That is, the response of SWNT photoluminescence emission to pH could depend on the type of functionalization. Future work is needed to reconcile the different observations.

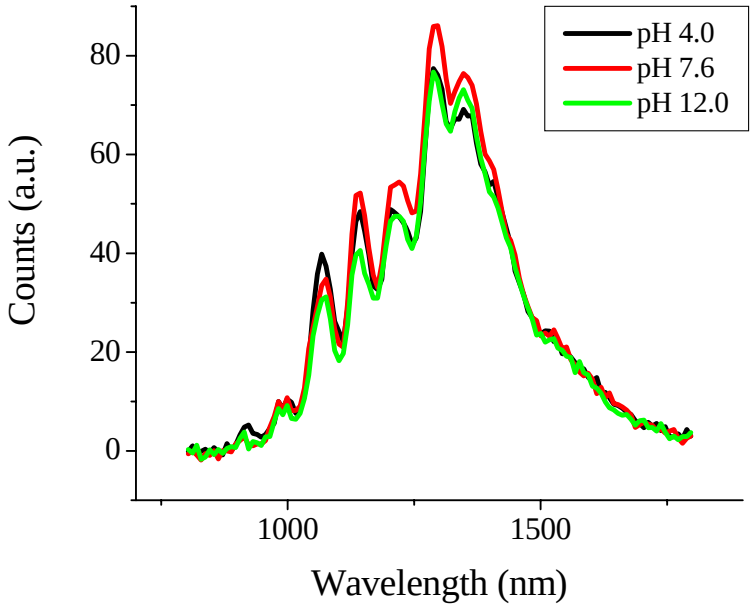


Figure S3. NIR photoluminescence spectra of Fluor-PEG/SWNT solutions (with same nanotube concentration) in phosphate buffers at the various pHs.

Internalization of Fluor-PEG/SWNT by mammalian cells.

Incubation of Fluor-PEG/SWNT with BT474 Cells. A breast cancer cell line, BT474, was grown in cell culture medium (Dulbecco's Modified Eagle's Medium, Invitrogen) supplemented with 10% FBS. Prior to incubation, the cells were collected by centrifugation and resuspended in the culture medium at a cell density of ~1×10^6 cells/mL. 200 µL of the cell suspension was mixed with 50 µL of a 500 nM Fluor-PEG/SWNT solution. The cells were incubated in the Fluor-PEG/SWNT solution for 24 h at 37^{o}C, in 5% CO_2 atmosphere.

Confocal Microscopy Imaging of Cells. After the incubation step above, the BT474 cells were washed twice with fresh cell medium to remove uninternalized Fluor-PEG/SWNT, then immediately imaged in chambered cover slides by Zeiss LSM 510 confocal fluorescence microscope.

Flow Cytometry. Also after incubation, the cells were washed to remove excess Fluor-PEG/SWNT, detached by trypsin-EDTA (Gibco), collected by centrifugation and re-suspended in cell culture medium. The cells were analyzed by a Becton-Dickinson FACScan instrument. 2% propidium iodide (PI, Fluka chemicals) was added to the cell suspension prior to the measurement. PI is a membrane impermeable dye and does not stain live cells. It can enter dead cells and intercalate into DNA, thereby selectively staining the dead cells. We carried out dual detection of red (PI) and green fluorescence (fluorescein) with the cells. The data presented here represent the mean green (fluorescein) fluorescence obtained with a population of 6, 000 live cells.

Micro-Raman Mapping of Incubated Cells. After incubation in 48-well culture plates (250 µl cell medium in each well) for 24 h, the cells were washed twice to remove

uninternalized Fluor-PEG/SWNT, detached by trypsin-EDTA (Gibco), collected by centrifugation and re-suspended in 50 μl cell culture medium. 10 μl of the cell suspension was dropped on a thin plastic film and covered by another thin plastic film. The scanning area was selected by focusing on the cells using the microscope, and the micro-raman laser was then focused at this plane ($\lambda_{excitation}$ = 785 nm). A line scanning mode was applied to get a raman spectrum at every point in the selected area (1μm × 1μm for each point). The image was obtained by plotting the integrated area in the range 1580-1610cm^{-1} of the raman spectra obtained in the cell area.

A raman image of the cells incubated with Fluor-PEG/SWNT is shown in figure 4c. While the presence of the G-band signal from the cells do not directly show that SWNTs are inside of the cells, this shows that SWNTs are present at the cells and suggests that Fluor-PEG/SWNTs are inside because SWNTs and Fluor-PEG are attached, and the fluorescein fluorescence was shown to be coming from the inside. Control cells without Fluor-PEG/SWNT have no G-band signal showing that cells do not have contributing peaks at 1600 cm^{-1} (data not shown).