Supporting Information for:

Chemically Functionalized Water-Soluble Single-Walled Carbon Nanotubes Modulate Morpho-Functional Characteristics of Astrocytes

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Materials and Methods

Purified astrocytic culture. Astrocytic cell cultures were prepared using a previously described procedure [1]. Briefly, visual cortices of 0- to 2- day old C57BL/6 mice pups were isolated, treated with papain (20 IU/mL; Sigma) in the presence of L-cysteine (0.2 mg/mL) for 1 hour at 37°C, neutralized with trypsin inhibitor (type II-O, 10 mg/mL; Sigma) for 5 minutes at room temperature (22-25°C), and triturated in culture media. The resulting cell suspension was plated into 25 cm^2 tissue culture flasks. One hour post plating, the cell suspension was replaced with fresh culture media containing α-minimum essential medium (without phenol red; Invitrogen) supplemented with fetal bovine serum (10% v/v; Hyclone), sodium bicarbonate (14 mM), sodium pyruvate (1 mM), D-glucose (20 mM), L-glutamine (2 mM), penicillin (100 IU/mL) and streptomycin (100 µg/mL) (pH 7.35). Cells were maintained at 37°C in a 95% air/ 5% CO_2 incubator for 1-2 weeks to obtain cell growth and proliferation to ~60% confluency after which the cell cultures were purified for astrocytes using a previously described procedure [2]. Briefly, flasks were shaken twice on an orbital shaker, first for 1.5 hours followed by two exchanges with culture media and again for 18 hours, at 37°C and 260 rpm. Purified astrocytes were detached by adding trypsin (10000 N_α-benzoyl-L-arginine ethyl ester hydrochloride units/mL; Invitrogen) for 2 minutes and pelleted by centrifugation at 100 x g for 10 minutes. The pellet was resuspended in culture media. Cells were then plated onto round (12 mm in diameter) glass coverslips pre-coated with polyethyleneimine [(PEI), 1 mg/ml; for detailed coating procedure see [3]] and maintained in the incubator for 3 days until used in the experiments. At 1 hour post plating, the astrocytes on coverslips received treatments of ws-SWCNTs or db-cAMP added to culture medium

and returned to the incubator. In control dishes, we applied the ws-SWCNT/db-cAMP vehicle, 2.5 µL of water. Unless specifically indicated otherwise, in all procedures, we used water purified by the Milli-Q® Synthesis system (Millipore Corp.; http://www.millipore.com/pressroom/cp3/5khpn7). This ultra-pure water has 18.2 MΩ*cm resistivity, less than 5 parts per billion (ppb) of organics content and pyrogen content less than 0.001 EU/mL. Our culturing method yields purified astrocytes (>99%), as we described elsewhere [4, 5]. In order to reduce the influence of astrocyte-astrocyte communication on the measurements [6], all experiments were done on solitary astrocytes devoid of contact with other astrocytes present on a coverslip.

Calcein loading and imaging. Live mouse cortical astrocytes were loaded with calcein and studied at room temperature using light microscopy [1, 6]. Briefly, coverslips with astrocytes were incubated for 20 minutes with calcein acetoxymethyl (AM) ester (1 µg/mL; Invitrogen) along with pluronic acid (0.025% w/v; Invitrogen), in external solution containing (in mM): NaCl (140), KCl (5), $CaCl_2$ (2), $MgCl_2$ (2), D-glucose (5), and Hepes (10) (pH 7.4); dye loaded in cells was allowed to de-esterify for 20 minutes at room temperature in external solution. These coverslips were then rinsed in the external solution and mounted onto an imaging chamber filled with external solution and examined using a microscope (Nikon TE300) equipped with differential interference contrast (DIC) and epifluorescence illumination (xenon arc lamp; 100W). Cells were visualized under a standard fluorescein isothiocyanate (FITC) filter set and a 60x Plan Apo objective (numerical aperture, 1.4). To acquire the images, we used a CoolSNAP®-HQ^2 cooled, charge coupled device (CCD) camera driven by MetaMorph® imaging software ver. 7.0. The width of one pixel in all the images corresponds to 105 nm,

imposed by the calculated Rayleigh's transverse resolution of 233 nm (at 535 nm emission) and by the Nyquist-Shannon sampling theorem. Neutral density filters and an electronic shutter that was controlled by the software were inserted in the excitation pathway to reduce photo-bleaching. The calcein-loaded cells were analyzed using a self-designed algorithm for automatic tracing (see Morphometic Analysis below).

Indirect immunocytochemistry (ICC). Labeling of astrocytes using ICC for GFAP was done as previously described [4, 6]. Briefly, astrocytes on coverslips were fixed with freshly prepared Dent's fixative (80% methanol and 20% dimethyl sulfoxide) for 30 minutes and then permeabilized with 0.25% (v/v) Triton X-100 for 10 minutes. To prevent non-specific binding, the cells were incubated with 10% (v/v) goat serum in phosphate buffered saline (PBS) for 30 minutes followed by an overnight (> 12h) incubation of the cells at 4^{o}C with primary antibody against GFAP (mouse monoclonal, 1:500 dilution; ICN Cat. No. 69110; MP Biomedicals; Solon, OH). Cells were then washed three times with PBS and incubated for 1h with tetramethylrhodamine isothiocyanate (TRITC)-conjugated secondary antibody (1:200; Millipore) at room temperature. After triple wash with PBS and double wash with water, the coverslips were mounted onto glass microscopic slides in ProLong® Gold antifade reagent (Invitrogen) to prevent photo bleaching. The PBS solution was composed of (in mM): NaCl (137), KCl (2.7), Na_2HPO_4 (10) and KH_2PO_4 (1.8) (pH 7.2). GFAP labeled astrocytes were imaged using DIC and fluorescence microscopy. Immunoreactivity (ir) was visualized using a standard TRITC filter set under the above mentioned microscope and associated equipment. To test for the non-specific binding of the secondary antibody, parallel controls were run, in which the primary antibody was omitted. These

controls were used to calculate the threshold value for GFAP labeled astrocytes, where the background subtracted mean intensity of the control cells (autofluorescence) + 6 standard deviation was used as the threshold value; the background fluorescence was obtained from regions of coverslips containing no cells. All raw fluorescence images had pixel intensities within the camera's dynamic range (0-16383). Total area of the GFAP labeled cells was calculated using the DIC images, where the outline of the cells was manually traced (see Morphometric Analysis below). Here, only astrocytes that could be imaged within a single frame were analyzed in order to avoid errors in quantification of GFAP fluorescence intensity that would otherwise occur during the stitching/auto-leveling step of the automatic morphometric analysis described next.

***Morphometric Analysis*.** To quantify the morphology of mouse cortical astrocytes, we used images of calcein-loaded solitary astrocytes, which were run through a self-designed algorithm (Fig. S1, which steps are parenthetically referred to below) to get the area and perimeter values of the cells. These values were further used to calculate the form factor (FF) of the cell. When the cell size exceeded an individual image frame, two or more images were taken and processed by image stitching and auto-leveling using Adobe Photoshop CS (Adobe Systems Inc., San Jose, CA) (Step 1). The stitched images were passed through a 3x3 kernel (a low-pass) filter, to reduce the effect of noise along the perimeter (Step 2). In order to detect the outline of the cells, the stitched images were processed using the ImageJ software 1.43 (NIH, Washington, DC) to apply a Laplacian of Gaussian (LoG) 3D filter to the image (Step 3), which calculates the second derivative of the image based on the intensity of calcein and facilitates edge detection [7]. These images were then analyzed using Metamorph 6.1 (Molecular

Devices, Chicago, IL) and the included integrated morphometry analysis tool. Using the LoG 3D image, a circle containing octants was centered over the astrocyte (Step 4). Regions of interest (10 x 10 pixels) were created at the intersections between the octant radii and the cell edge/perimeter. These regions were then transposed to the stitched image (Step 5) and the average intensities of the regions were obtained using the region statistics tool in Metamorph. These values were further averaged and the resulting overall mean intensity was used as the intensity threshold value. Stitched images were then thresholded based on the mean intensity value calculated (Step 6), and the area and perimeter (Step 7) were calculated using the integrated morphometry. Furthermore, the form factor (FF) was calculated for each cell using the equation [8]:

$$\text{Form Factor (FF)} = 4\pi\,[\text{Area } (\mu m^2)] \,/\, [\text{Perimeter } (\mu m)]^2 \quad (1).$$

To verify if the area of the astrocytes calculated using the algorithm is accurate, we used calcein-loaded control astrocytes (n=12) and their corresponding DIC images, which were used to manually trace the outline of the cells using Metamorph (Fig. S2). Manual tracing was done based on the edge or perimeter of the cell that appears in a DIC image. Area obtained through this manual tracing was compared to the corresponding algorithm derived area of the calcein-loaded control astrocytes. The median percentage error of the algorithm derived area to the manually traced area was −2.2% (interquartile range: −3.5% to 1.2%). We find no statistical difference between these two correlated groups (Wilcoxon signed-rank test, p=0.116) indicating that automatic and manual analysis cross-check.

This manual tracing of DIC images was also used in all the GFAP experiments, where the DIC images of immunolabeled astrocytes were used to calculate the total

area of the astrocyte as GFAP covers only a portion of the total cell by area i.e. the median GFAP-ir occupancy of control astrocytes is ~68.9% (interquartile range: 61.5% to 76.2%) (Fig. 1and Fig. S3). Since the astrocytes are fixed and permeabilized for ICC, loading with calcein is not an option as calcein leaks out from cells with compromised plasma membrane [6].

Synthesis and characterization of graft co-polymers. In this study, we prepared ws-SWCNTs using previously described procedures [9, 10]. Briefly, we modified commercially available, purified P3-SWNT (Carbon Solutions, Inc., Riverside, CA) with carboxylic acid groups at their ends (SWCNT-COOH) to make an acyl chloride intermediate (SWCNT-COCl). P3-SWNT material (1 g) was dispersed in 1 L of dry DMF by ultrasonication for 2 h and high-shear mixing for 1 h to give a homogeneous suspension. Oxalyl chloride (20 mL) was added drop-wise to the SWCNT-COOH solution at 0°C under argon. The reaction mixture was stirred at 0°C for 2 h and again at room temperature for 2 h, followed by overnight heating at 70°C to remove the excess oxalyl chloride (boiling point 63°C). The functionalization of the resulting SWCNT-COCl intermediate was performed by the addition of either poly-*m*-aminobenzene sulphonic acid (PABS, 5 g) [11] or poly-ethylene glycol (PEG, 5 g) to form the corresponding graft copolymers [9, 10, 12] by reaction of the components at 120^{o}C for 5 days. After that, the mixture was filtered through a membrane (pore size 0.22 μm), repeatedly rinsed with ethanol (95%) and then with distilled water to remove any excess of PABS or PEG. The resulting final product, SWCNT-PABS or SWCNT-PEG (Fig. S4), was collected on a membrane, dried under vacuum overnight, and then reconstituted in distilled water in 2.0-5.0 mg/mL stock solutions. Further dilution of ws-SWCNTs, prior to their application

to cultured cells, was done using purified water. Grafts copolymers were characterized using atomic force microscopy (AFM), thermogravimetric analysis (TGA) and infrared (IR) spectroscopy[10]; data are summarized in Fig. S5-S8. SWCNT-PABS has a composition of 33 weight percent (wt%) SWCNTs and 67 wt% PABS, while SWCNT-PEG contains 76 wt% SWCNTs and 24 wt% PEG. Similar SWCNT-PABS and SWCNT-PEG copolymers are commercially available as P8-SWCNT and P7-SWCNT, respectively (Carbon Solutions Inc., www.carbonsolution.com).

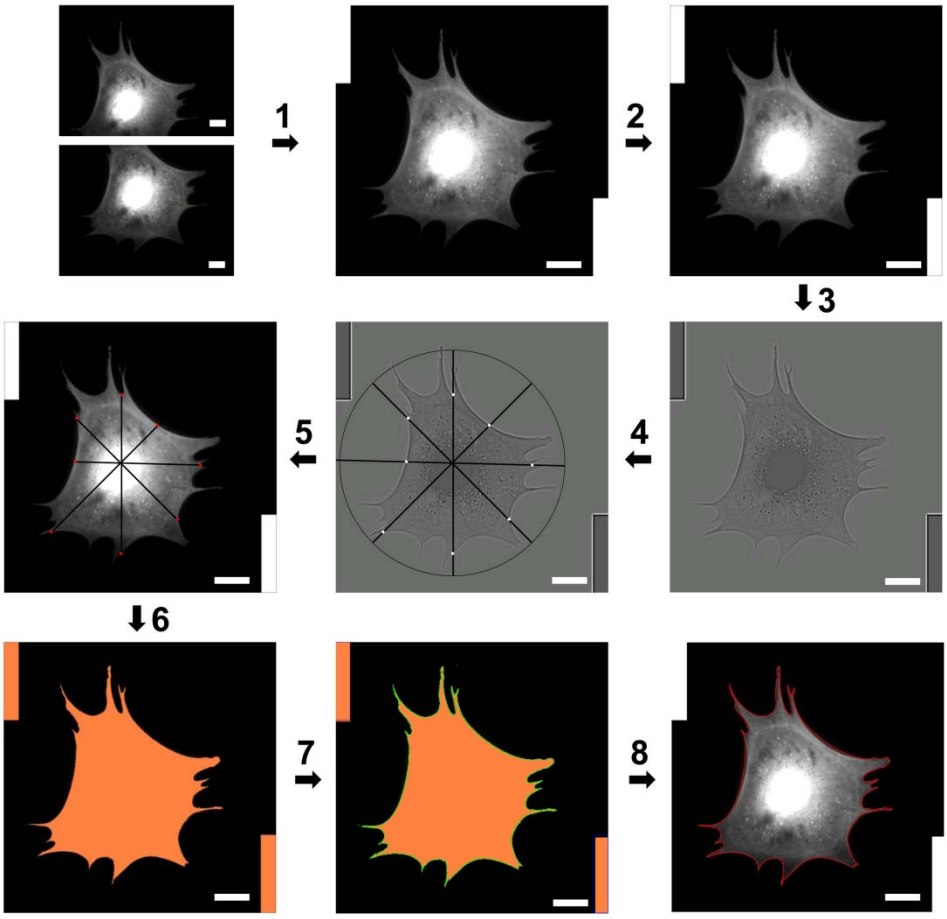


Figure S1. Step by step description of the algorithm used in the automatic morphometric analysis. Two raw fluorescence images (top left) represent a calcein-loaded solitary astrocyte treated with 5 μg/mL of SWCNT-PEG. Steps involved in the quantification of morphology are as follows:

1. Stitching and auto-leveling the images to visualize the whole cell;
2. Passing the stitched image through a 3x3 kernel filter to remove the noise along the perimeter;
3. Applying a Laplacian of Gaussian (LoG) 3D filter to facilitate edge detection;
4. Creating 10 x 10 pixel regions at intersects between the radii of the centered octants and the cell edge;
5. Transposing the 10 x 10 pixel regions to the stitched image;
6. Calculating the average intensities of the regions and using the average of averages to threshold the image (positive pixels indicted in orange); and
7. Creating region around astrocyte on the thresholded image (orange represents the cell area, while green represents the cell perimeter).

Step 8 is not performed in analysis, but presented here to visualize the region of interest (outlined by a red form) from the step 7 transposed onto a stitched image. Scale bars, 20 μm.

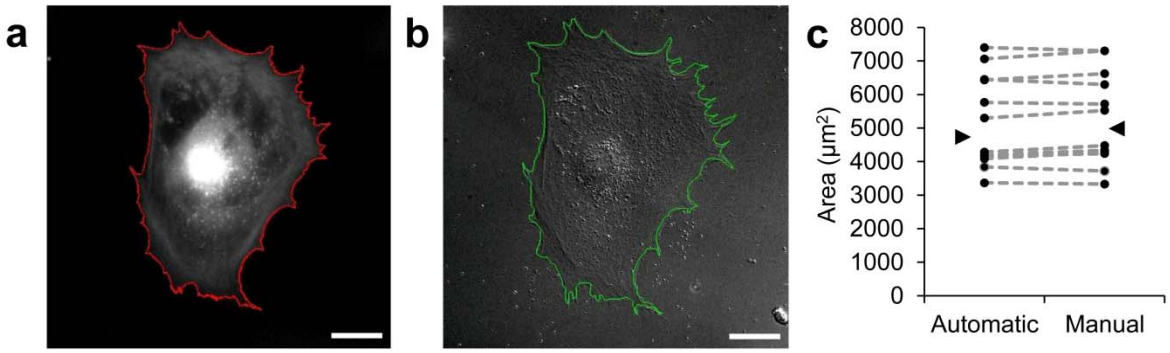


Figure S2. Comparison between the area values obtained using automatic and manual tracing. a) Fluorescence image of a solitary control astrocyte loaded with calcein and analyzed using the self-designed algorithm. Automatic tracing of the cell perimeter is shown in red; it outlines an area of 5491 μm^2. b) Corresponding DIC image of the calcein-loaded astrocyte in a). Manual tracing of the cell perimeter is shown in green; it outlines an area of 5638 μm^2. Scale bars, 20 μm. c) Summary graph comparing the areas of 12 control (sham-treated) astrocytes obtained using the automatic and manual tracing approaches. The dotted line connects the matching measurements (black dots) from a single cell, while the triangles represent the median values from all cells. The median percentage error of the automatic/algorithm obtained area to the manually traced area is −2.2%.

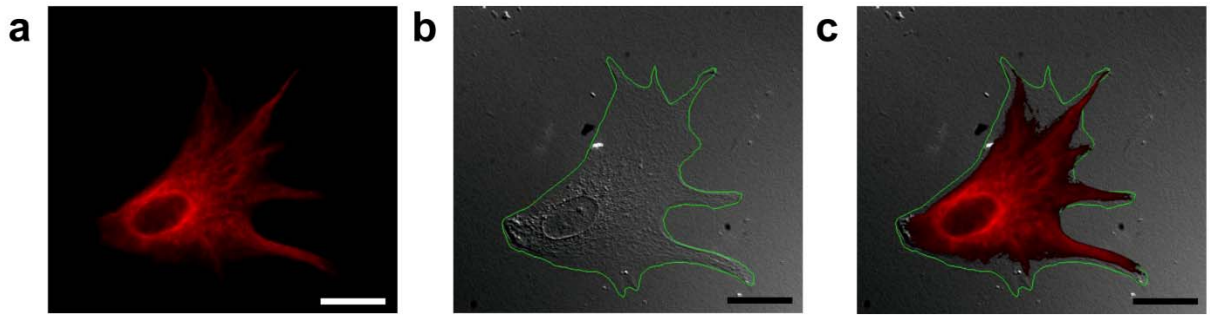
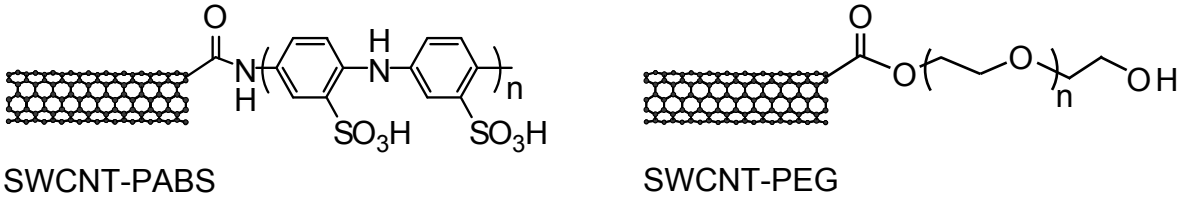


Figure S3. Glial fibrillary acidic protein immunoreactivity (GFAP-ir) occupies a portion of the total cell area. a) Fluorescence image of a solitary control astrocyte immunolabeled for GFAP (red). b) Corresponding DIC image of the astrocyte in a) with its perimeter manually traced (green) and outlining the actual area of the cell. c) Overlay of the GFAP immunolabeled astrocyte image over its corresponding DIC image. GFAP-ir occupies ~69% of the total astrocyte area. Scale bars, 20 μm.



SWCNT-PABS SWCNT-PEG

Figure S4. Schematic illustration of the structures of water-soluble SWCNTs: SWCNT-PABS and SWCNT-PEG.

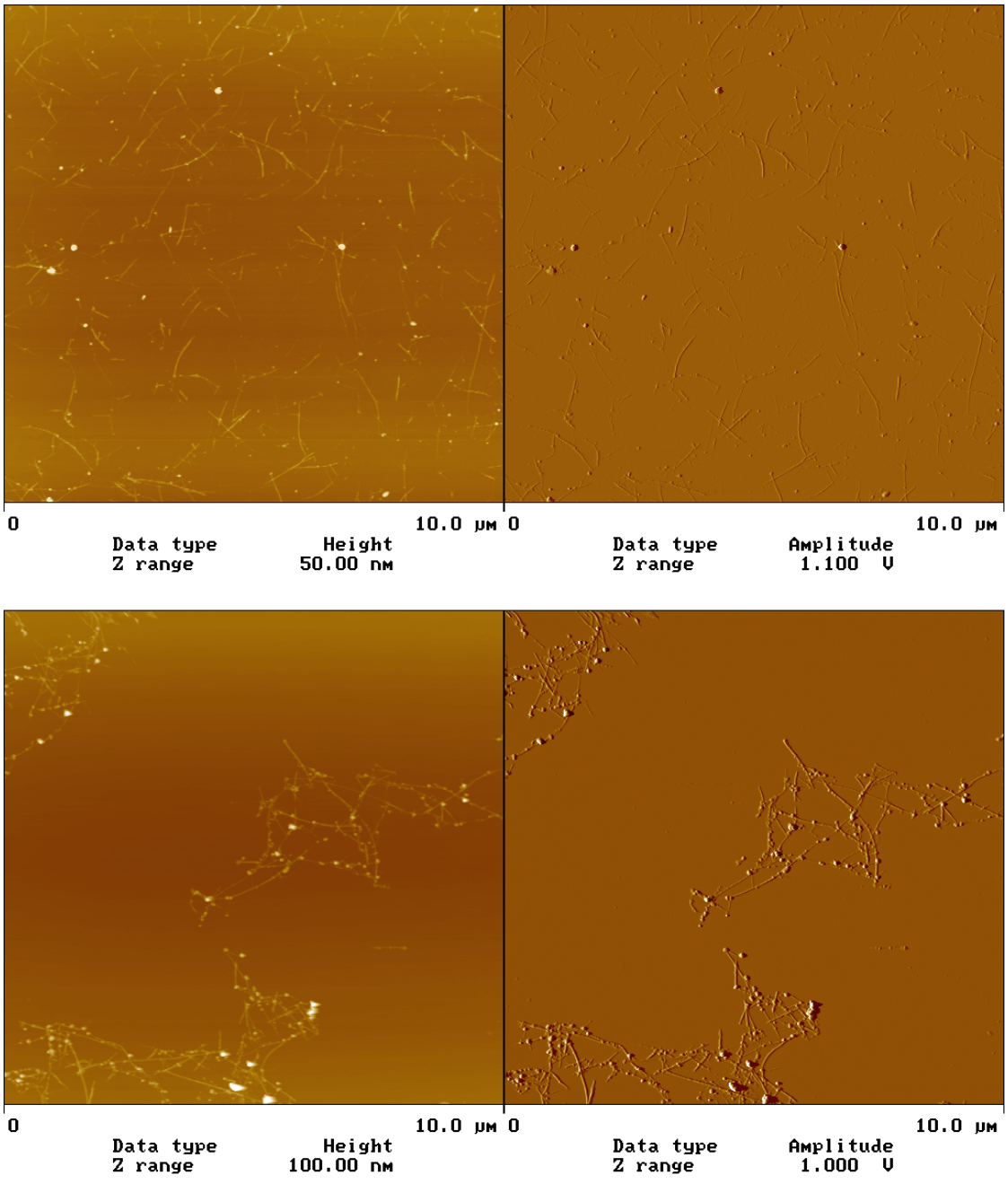


Figure S5. AFM images of the functionalized SWCNT materials. (top) SWCNT-PABS and (bottom) SWCNT-PEG. The samples were prepared from a diluted aqueous dispersion dropped on a mica substrate and air dried. The images were recorded using AFM (Digital Instruments Nanoscope IIIA) in tapping mode. We used the SWCNT height as a measure of their diameters. Both materials had similar diameters (range 1–6 nm) and lengths (range 0.1–1.9 µm). These diameter measurements are consistent with the presence of both individual as well as bundled SWCNTs in our preparations.

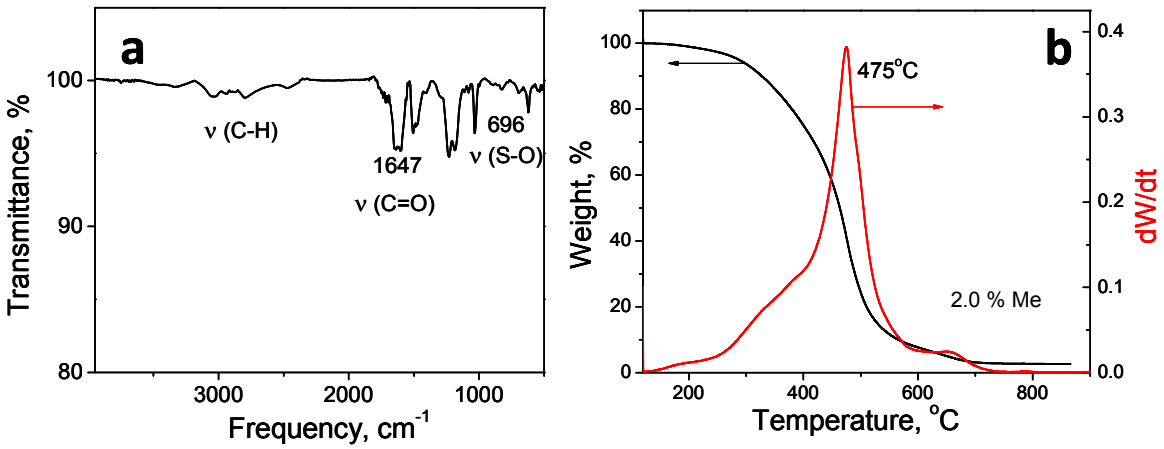


Figure S6. Characterization data for SWCNT-PABS. a) Mid-IR spectrum showing the carbonyl stretch ν (C=O) in amide groups formed between PABS and SWCNT-COOH. b) TGA weight change (black line) and its derivative (red line). The measurement was performed in air at a heating rate of 5°C/min. The metal content (Me) was estimated from the TGA residue.

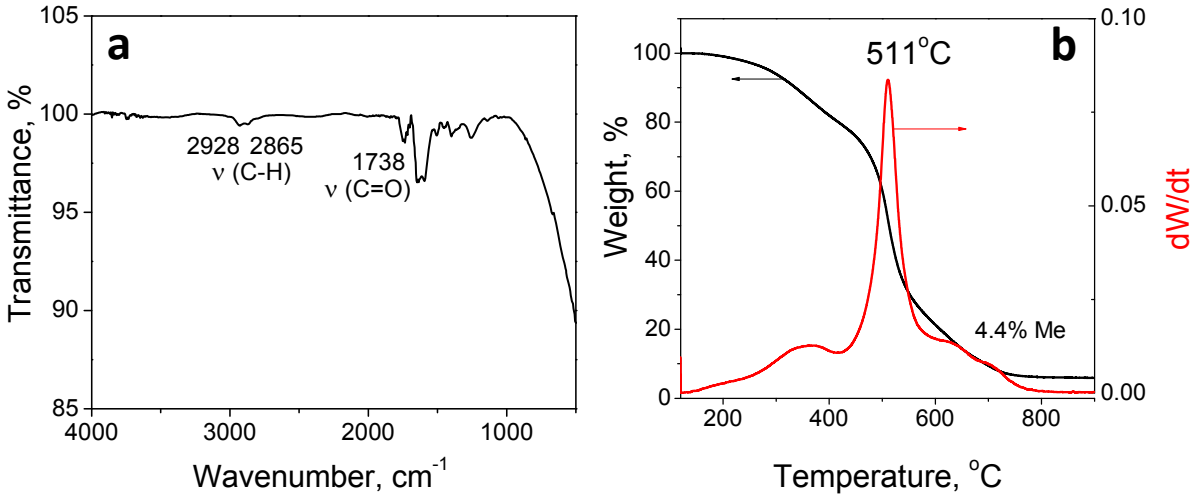


Figure S7. Characterization data for SWCNT-PEG. a) Mid-IR spectrum showing the carbonyl stretch ν (C=O) in ester groups formed between PEG and SWCNT-COOH. b) TGA weight change (black line) and its derivative (red line); the metal content (Me) was estimated from the TGA residue. The measurement was performed in air at a heating rate of 5°C/min.

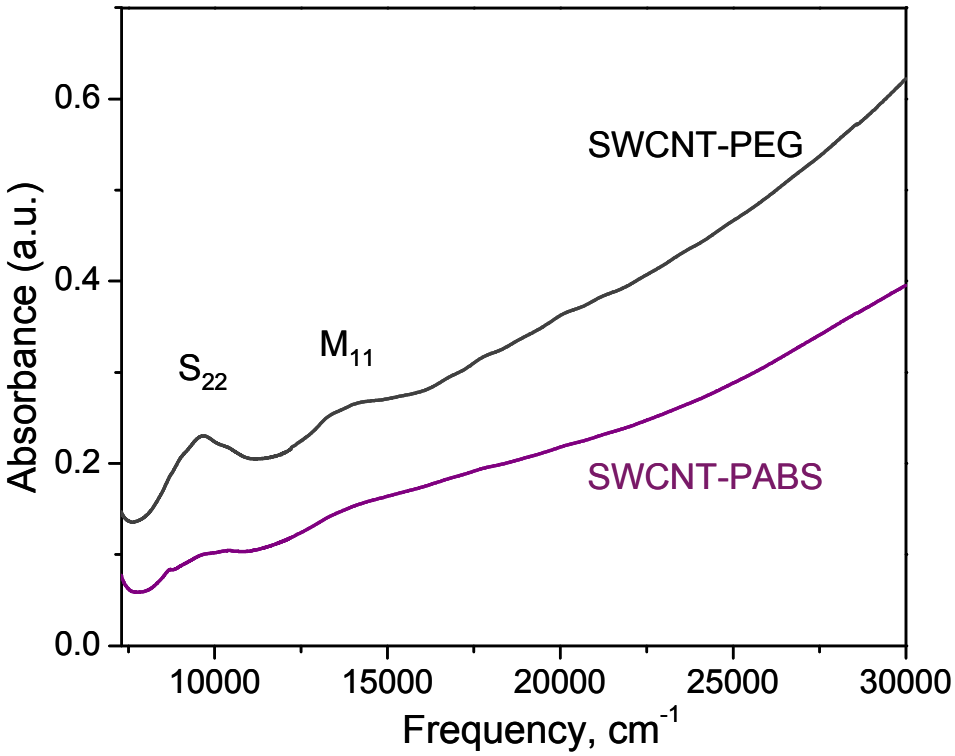


Figure S8. Near-IR spectra of aqueous dispersions of SWCNT-PABS and SWCNT-PEG. S_{22} and M_{11} indicate the second semiconducting interband transition and first metallic transition in SWCNTs, respectively. Absorbance is given in units (a.u.).

References:

1. Reyes, R. C.; Perry, G.; Lesort, M.; Parpura, V. *Cell Calcium* **2011,** 49, (1), 23-34.
2. McCarthy, K. D.; de Vellis, J. *J Cell Biol* **1980,** 85, (3), 890-902.
3. Lee, W.; Parpura, V. *Methods Mol Biol* **2012,** 846, 261-76.
4. Montana, V.; Ni, Y.; Sunjara, V.; Hua, X.; Parpura, V. *J Neurosci* **2004,** 24, (11), 2633-42.
5. Malarkey, E. B.; Reyes, R. C.; Zhao, B.; Haddon, R. C.; Parpura, V. *Nano Lett* **2008,** 8, (10), 3538-42.
6. Hua, X.; Malarkey, E. B.; Sunjara, V.; Rosenwald, S. E.; Li, W. H.; Parpura, V. *J Neurosci Res* **2004,** 76, (1), 86-97.
7. Sage, D.; Neumann, F. R.; Hediger, F.; Gasser, S. M.; Unser, M. *IEEE Trans Image Process* **2005,** 14, (9), 1372-83.
8. Wilms, H.; Hartmann, D.; Sievers, J. *Cell Tissue Res* **1997,** 287, (3), 447-58.
9. Zhao, B.; Hu, H.; Yu, A.; Perea, D.; Haddon, R. C. *J Am Chem Soc* **2005,** 127, (22), 8197-203.
10. Ni, Y.; Hu, H.; Malarkey, E. B.; Zhao, B.; Montana, V.; Haddon, R. C.; Parpura, V. *J Nanosci Nanotechnol* **2005,** 5, (10), 1707-12.
11. Roy, B. C.; Dutta Gupta, M.; Bhowmik, L.; Ray, J. K. *Synth Met* **1999,** 100, 233-236.
12. Zhao, B.; Hu, H.; Haddon, R. C. *Adv Func Mater* **2004,** 14, 71-76.