# Supporting Information

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### SI Materials and Methods

**Synthesis and Characterization of the SWCNT-[(DOTA)(AF488)(AF680)] Construct.** ***Sep-Pak purification of SWCNT-$NH_2$.*** After the Boc-amine was deprotected, the SWCNT-$NH_2$ product was purified from carbonaceous impurities by reverse-phase C18 chromatography. In brief, SWCNT-$NH_2$ was dispersed in 0.1 M triethylamine acetate (TEAA; Fisher) and adjusted to pH 7. Acetonitrile was added to a final vol/vol of 20%. A Sep-Pak Plus C18 cartridge (Waters) was equilibrated with 20% acetonitrile/0.1 M TEAA. The SWCNT-$NH_2$ was loaded onto the cartridge and washed extensively with 20% acetonitrile and 0.1 M TEAA at 1 mL/min. The purified SWCNT-$NH_2$ was eluted from the cartridge in 50% acetonitrile/water, and the solvent was evaporated to yield the purified SWCNT-$NH_2$ solid. This solid was analyzed by reverse-phase HPLC as described below using method II. The crude SWCNT-$NH_2$ yielded three chromatographic peaks (1). The Sep-Pak–purified SWCNT-$NH_2$ yielded a single HPLC peak, and TEM and Raman analyses of the Sep-Pak–purified SWCNT-$NH_2$ confirmed the CNT identity of the product (Fig. 1 *B* and *C* and Fig. S1).

***Functionalization of purified SWCNT-$NH_2$.*** Purified SWCNT-$NH_2$ was dissolved in a reaction buffer of 0.1 M phosphate, 0.15 M NaCl (pH 7.5), and 20% acetonitrile (vol/vol) at a concentration of 3.4 g/L. To this reaction mixture was added an amine-reactive Alexa Fluor 488 tetrafluorophenyl ester (AF488-TFP; Invitrogen). The AF488 was reacted at a ratio of 1 dye per 2 amines at ambient temperature for 2 h, with the pH adjusted to 8.5 with 10 N NaOH to hydrolyze any remaining unreacted dye. This mixture was purified using a C18 cartridge. The AF488 stoichiometric substitution was quantified by UV/Vis spectrophotometry using a SpectraMax M2 Microplate Reader (Molecular Devices). The SWCNT-[($NH_2$)(AF488)] product was further reacted with AF680-SE (Invitrogen). The SWCNT-[($NH_2$)(AF488)] in 50% acetonitrile/water was diluted into a buffer of 0.1 M phosphate, 0.15 M NaCl (pH 7.5), and 20% acetonitrile/water. Again, a ratio of 1 dye per 2 amines was used under similar reaction conditions. Unreacted dye was removed by dialysis with a 10,000-MW cassette (Slide-a-lyzer; Thermo Scientific), followed by purification with a centrifugal filter device of 30,000 MW (Amicon Ultra15; Millipore). This product (SWCNT-[($NH_2$)(AF488)(AF680)]) was analyzed by UV/VIS spectrophotometry to quantify the AF680 stoichiometry.

The dual-fluorescently labeled SWCNT-[($NH_2$)(AF488)(AF680)] was concentrated in distilled water (to 0.15 mL total volume) and added to 0.150 mL of 0.2 M sodium bicarbonate (pH 9), which had been previously treated with Chelex (Bio-Rad) resin to render the buffer metal-free. This solid was reacted with the amine reactive bifunctional chelate 2-(4-isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA-SCN; Macrocyclics) at a ratio of 6 mmol of DOTA/g SWCNT to modify all remaining amines with the DOTA chelating group. The reaction was allowed to proceed for 2 h at room temperature, after which the mixture was purified on a 10 DG size-exclusion chromatography column (Bio-Rad) to remove any free chelate from the product, as described previously (2). The final construct, SWCNT-[(DOTA)(AF488)(AF680)], was lyophilized to yield the solid green-brown product used for subsequent radiolabeling and PK studies. This product was analyzed by HPLC using method II (Fig. 1).

***Radiolabeling of the construct with $^{86}Y$.*** Yttrium-86 was produced and chemically separated as described previously (3). For this, 300 MBq (8.1 mCi) of "no carrier added" $^{86}Y$ in 0.05 M HCl was provided for labeling. Activity was assayed in a Squibb CRC-15R radioisotope calibrator set at 711, with the displayed activity value divided by 2.

The CNT-[([$^{86}Y$]DOTA)(AF488)(AF680)] construct was prepared by adding 300 MBq (8.1 mCi) of acidic $^{86}Y$ chloride to 0.400 mg of a 1 g/L solution of CNT-[(DOTA)(AF488)(AF680)] in MFW and 0.050 mL of 3 M ammonium acetate (Aldrich) and 0.015 mL of 150 g/L *l*-ascorbic acid (Aldrich) to yield a clear, dark green-brown solution of pH 5.0. The reaction was heated at 61 °C for 45 min, quenched with 0.040 mL of 50 mM diethylenetriaminepentaacetic acid (DTPA; Aldrich), and then purified by size-exclusion chromatography using a P6 resin (Bio-Rad) as the stationary phase and 1% human serum albumin (Swiss Red Cross) in 0.9% NaCl (Abbott Laboratories) as the mobile phase (3). An aliquot of the final product, CNT-[([$^{86}Y$]DOTA)(AF488)(AF680)], was used to determine the radiochemical purity by instant TLC using silica gel (ITLC-SG), as described previously. Further spectroscopic, radiometric, and chromatographic characterization of the construct was performed by reverse-phase HPLC using method I (3, 4).

***Radiolabeling of the construct with $^{111}In$.*** The $^{111}In$ was obtained from MDS Nordion for use in other tracer experiments. The same construct, CNT-[(DOTA)(AF488)(AF680)], was labeled using materials and methods similar to those described above for the $^{86}Y$ radiochemical labeling process. These two radionuclides have been demonstrated to have similar labeling kinetics, product purities, and yields in labeling reactions with CNT-DOTA constructs (2–4).

**Reverse-Phase HPLC Analyses.** All HPLC analyses was performed on a Beckman Coulter System Gold HPLC system equipped with an inline UV/Vis spectrum detector and tunable multi-wavelength fluorescence detector (Jasco FP-2020). Radioactivity was monitored through the use of an inline γ-RAM Model 3 radioactivity detector. The raw data obtained from each chromatograph was processed and plotted using GraphPad Prism (GraphPad Software). The stationary phase was a Gemini C18 column (5 μ, 250 × 4.6 mm) (Phenomenex). Method I used a 0–100% mobile phase gradient (30 min) of 0.1 M TEAA (pH 6.5) (Aldrich) and acetonitrile (Aldrich) at a flow rate of 1.0 mL/min. Method II used a 20–100% mobile phase gradient (40 min) of 0.1 M TEAA (pH 6.5) and acetonitrile at a flow rate of 1.0 mL/min.

The covalently appended dye and DOTA moieties either react with the amines on the SWCNT or undergo hydrolysis during the course of the modification reaction. Unattached ("free") DOTA will elute at ≈8–9 min (3); unattached AF488 will elute at ≈3–5 min, and unattached AF680 will at ≈12–14 min (Fig. S3*A*). Note that the free dyes have a much sharper chromatographic distribution, and that their fluorescence is not quenched; for example, the free AF488 was removed from the reaction mixture by C18 chromatography, and UV-Vis analysis demonstrated the product to be >99% pure. The fluorescence trace shown in Fig. 1*E*, however, reveals that that 1% impurity had ∼10% the signal intensity as the attached AF488 in the SWCNT-[(DOTA)(AF488)(AF680)] construct. We estimate that "bound" dye is quenched ≈1–2 orders of magnitude relative to the free dye.

We performed another control experiment to examine the HPLC elution profile of unattached dyes in the urine of mice after i.v. injection. In brief, hydrolyzed AF488 dye (i.e., not bound to CNTs or reactive with any amine moieties present in vivo) was injected i.v. into a mouse, after which urine was collected and analyzed by HPLC. The results revealed that the hydrolyzed AF488 dye in urine eluted at 3 min, much earlier than when the

SWCNT constructs eluted (19–20 min). The same experiment was performed with hydrolyzed AF680 dye as described above. The HPLC results revealed that the hydrolyzed AF680 dye in urine eluted at 12 min, much earlier than when the SWCNT constructs eluted (19–20 min). The hydrolyzed dye (i.e., with the amine-reactive moiety on the dye consumed by hydrolysis and rendered not amine-reactive) was examined to ensure that the dye could not react with a serum protein and confound the analysis. The unbound $^{86}$Y-DOTA moiety is also quite hydrophilic and elutes at ≈5 min, well in advance of the construct elution time. Fig. S3*B* shows the chromatographic elution profiles of the AF dyes in the urine.

**Instant TLC Analysis.** A small aliquot of the final product, CNT-[([$^{86}$Y]DOTA)(AF488)(AF680)], was used to determine the radiochemical purity by ITLC-SG using silica gel–impregnated paper (Gelman Science) (3, 4). The paper strips were developed using two different mobile phases: mobile phase I, with 10 mM EDTA, and mobile phase II, with 9% NaCl/10 mM NaOH. The $R_f$ of the radiolabeled CNT construct was 0, and any unchelated $^{86}$Y species or unattached metal chelates were characterized by an $R_f$ of 1.0 in mobile phase I. In mobile phase II, the radiolabeled CNT construct and any free metal species were characterized by an $R_f$ of 0, and the $R_f$ of any unattached metal chelate species was 1.0. The strips were spotted, developed, dried, and counted intact using an Ambis Model 4000 gas ionization detection system.

**DLS and ζ-Potential Results.** DLS yielded an average diameter value of 105 ± 2.9 nm for the construct, with lengths ranging from 90 to 900 nm. The average SWCNT length was calculated as ∼300 nm (assuming that $d \sim 1$ nm). DLS measurements of rod-shaped CNTs rely on spherical geometric assumptions to yield a value for size. The DLS data provide an average hydrodynamic radius ($R_h$) using this spherical assumption. We assume a rod shape to relate $R_h$ to the radius of gyration ($R_g$). The $R_g$ for rods depends on the aspect ratio (length/diameter ratios). The TEM images (Fig. S1) revealed a small amount of dried SWCNTs with sizes consistent with the DLS predictions. Measurement of the ζ-potential yielded a value of −8.9 ± 3.3 mV, indicating that the construct had an overall negative charge.

**TEM Analysis.** The Sep-Pak–purified SWCNT-$NH_2$ was analyzed by TEM as described previously (2–4). In brief, images were obtained using a Philips EM-201 transmission electron microscope with 1-nm resolution (high voltage = 80 kV). Samples were adsorbed onto plasma-treated Formvar-coated copper grids. Fig. S1 *A–C* shows three representative images at 100 nm, 500 nm, and 2,000 nm scales. The TEM images show bundles of CNTs of varying thicknesses (≈20 nm) and lengths. TEM image analysis was performed using ImageJ software (http://rsb.info.nih.gov/ij/), and GraphPad Prism software (Graphpad Software) was used for statistical analysis and data plotting (3). These data show a mean CNT length distribution of 195 ± 69 nm ($n$ = 644). Fig. S1*D* is a histogram of these data, showing a range of CNT lengths spanning ≈500 nm.

**UV-Vis CNT Spectral Signature in the Urine of Mice Receiving SWCNT-[(DOTA)(AF568)]: Direct Evidence for Intact CNT Filtration and Elimination.** Additional HPLC analyses of urine samples from mice that received 6- to 40-fold larger doses of SWCNT construct showed the characteristic UV-Vis spectrum of the SWCNTs in the chromatographic peak attributed to the construct (Fig. S2). The particular construct that was injected i.v. to yield this figure, SWCNT-[(DOTA)(AF568)], was prepared from the same batch of purified SWCNT-$NH_2$ used to prepare the construct for these studies. AF568 dye (Invitrogen) was substituted, and the final construct was purified as described above.

We purposely injected much larger mass doses compared with those for the imaging studies to look for the CNT absorbance spectrum in the urine. The SWCNT-[(DOTA)(AF568)] construct was injected i.v., and after 1 h, the urine was harvested as described above. A sample of the injected material and the urine were analyzed chromatographically, and both exhibited a peak at about 19–20 min that showed the CNT and AF568 spectral signatures before injection and in the urine. The urine from this experiment was dark-brown, compared with the yellow urine of an uninjected control mouse, and the chromatographic peak clearly exhibited the CNT spectral signature. (Note that the 6- to 40-fold increase describes 0.06–0.24 mg of construct injected per mouse, whereas the "tracer"-based data presented in the main text represents ≈0.01 mg injected per mouse.)

**Dynamic PET Imaging.** An energy window of 350–700 keV and a coincidence timing window of 6 ns were used. The resulting list-mode data were sorted into fifteen 12-s (0–3 min), fourteen 30-s (3–10 min), forty 60-s (10–50 min), one 180-s (50–53 min), and one 420-s (53–60 min) time bins and into 2D histograms by Fourier rebinning, and transverse images were reconstructed in a 128 × 128 × 96 matrix by filtered back-projection. The image data were corrected for nonuniformity of the scanner response, dead time count losses, and physical decay to the time of injection. No correction was applied for attenuation, scatter, or partial volume averaging. The measured reconstructed spatial resolution of the Focus 120 micro-PET scanner was 1.6 mm FWHM at the center of the field of view. The counting rates in the reconstructed images were converted to activity concentrations (%ID/g) using an empirically determined system calibration factor (MBq/mL/cps/voxel) derived from the imaging of a mouse-sized phantom containing $^{18}$F.

**Organic Cationic Transport, Organic Anionic Transport, and Megalin Receptor Competition Studies.** A 5 g/L solution (0.105 g in 20.9 mL) of the OCT inhibitor cimetidine (Sigma-Aldrich) was prepared in normal saline, sterile-filtered, and stored in a septa-sealed vial. This solution had a pH of 7. A 1 g/L solution (0.036 g in 36.5 mL) of the OAT inhibitor probenecid (Sigma-Aldrich) was prepared in normal saline. The pH of this solution was adjusted to 6 with the addition of 0.14 mL of 1 M NaOH (Sigma-Aldrich). The solution was then sterile-filtered and stored in a septa-sealed vial. A 50 g/L solution of the megalin receptor inhibitor gentamicin (Sigma-Aldrich) was diluted 1:100 in normal saline to prepare a 0.5 g/L solution.

**IHC and IF Staining and Microscopy.** IHC staining with an anti-AF488 antibody was performed at MSKCC's Molecular Cytology Core Facility using a Discovery XT processor (Ventana Medical Systems). The tissue sections were blocked for 30 min in 10% normal goat serum and 2% BSA in PBS. The primary antibody incubation (rabbit polyclonal anti-AF488 antibody; Molecular Probes) was used in a 0.005 g/L concentration. Incubation with the primary antibody was done for 4 h, followed by a 1 h incubation with biotinylated goat anti-rabbit IgG (Vector Laboratories) diluted 1:200. The detection was performed with Secondary Antibody Blocker, Blocker D, Streptavidin-HRP D, and the DAB Detection Kit (Ventana Medical Systems) in accordance with the manufacturer's instructions. The IF detection of anti-AF488 was performed at MSKCC's Molecular Cytology Core Facility using a Discovery XT processor. The tissue sections were processed as described above for IHC, except that incubation was followed by biotinylated goat anti-rabbit IgG and detection was performed with Streptavidin-HRP D, followed by incubation with Tyramide AF488 (Invitrogen). Controls included normal tissue (no construct injection), AF488 dye not conjugated to SWCNTs (at 1 and 60 min after injection), and isotype control staining IgG (with nonspecific IgG as the primary antibody).

Wide-field microscopy was performed with an Axioplan2 imaging microscope equipped with AxioCam MRm Camera (Zeiss), using filter cubes for DAPI, AF488, and TRITC. Confocal microscopy was performed using an inverted Leica TCS AOBS SP2 microscope. Z-series stacks were recorded at 1 μm increments.

**Component and Composite IF Images of the Kidney Nephron Sections for SWCNT-[(DOTA)(AF488)(AF680)]-Treated and Control Animals.** Imaging studies focusing on the local nephron distribution of the construct used IHC and IF microscopy. The nephron distribution of the CNT construct was evaluated as a function of time from 1 min to 7 d by harvesting kidneys and preparing tissue sections (Fig. S4).

**Construct Uptake and Component and Composite IF Images of HK-2 Cells in Vitro.** HK-2 cells derived from normal kidney were obtained from American Type Culture Collection. HK-2 cells were cultured in Keratinocyte Serum-Free Medium (Invitrogen) containing 0.05 g/L bovine pituitary extract and 5 ng/mL EGF at 37 °C and 5% $CO_2$. The HK-2 cells were seeded on 24 mm-diameter polyester filters ($1 \times 10^5$ cells/well) with a pore size of 0.4 μm (Transwell Clears; Corning-Costar). The cells were cultured for 7 d to allow the cells to attach and polarize.

Construct uptake was quantified by incubating seeded, polarized cells with a radiolabeled construct. In brief, SWCNT-[([$^{111}$In]DOTA)(AF488)(AF680)] was added to the apical or basolateral compartments of the Transwell plates (see Fig. S6 for a diagram of the experimental apparatus and design) at a final concentration of 0.06 g/L, and the cells were incubated at 37 °C for 1, 3, 6, 12, or 24 h (in duplicate). The experiment was terminated by washing the cell monolayer three times with ice-cold PBS (8 g/L NaCl, 0.2 g/L KCl, 1.56 g/L $Na_2HPO_4$ and 0.2 g/L $KH_2PO_4$) in water at pH 7.2. The cells were then trypsinized and transferred to tubes for radioactivity γ-counting with a Packard Cobra Gamma counter using a 15- to 550-keV window. The uptake of the added construct radioactivity (via the upper or lower chamber) by the cells was expressed as the ratio of cell-associated activity per compartment (Fig. 4*I*). The Transwell plates were used to investigate the route of HK-2 cellular uptake of SWCNTs. As shown in Fig. 4*I*, the uptake of SWCNTs was greater from the apical side than from the basolateral side ($P < 0.05$), suggesting that SWCNTs are taken up by polarized cells by the brush border on the luminal side.

We also performed fluorescence microscopy on seeded and polarized HK-2 cells using the nonradiolabeled construct SWCNT-[(DOTA)(AF488)(AF680)]. In brief, the cells were seeded ($0.6 \times 10^5$/well) in a four-well Lab-Tek chamber slide system (Nunc) and cultured for 6 d, to allow the complete spreading of the cells. The cells were then incubated with the SWCNT-[(DOTA)(AF488)(AF680)] for 0.5, 1, 6, 12, and 24 h. The cells were washed three times in ice-cold PBS and fixed in 4% paraformaldehyde. The primary antibody incubation (rabbit polyclonal anti-AF488 antibody; Molecular Probes) was used at a 0.005 g/L concentration. The incubation with the primary antibody was done for 4 h, followed by a 1 h incubation with goat anti-rabbit IgG-AF488 (Molecular Probes) diluted 1:500. Controls included no construct and normal rabbit IgG (instead of the primary antibody) (Fig. 4 *C–H*). All of the DAPI, AF488, DIC, and composite images for the time course and all controls are presented in Fig. S5.


1. Zhao B, et al. (2001) Chromatographic purification and properties of soluble single-walled carbon nanotubes. *J Am Chem Soc* 123:11673–11677.
2. Villa CH, et al. (2008) Synthesis and biodistribution of oligonucleotide-functionalized, tumor-targetable carbon nanotubes. *Nano Lett* 8:4221–4228.
3. McDevitt MR, et al. (2007) PET imaging of soluble yttrium-86–labeled carbon nanotubes in mice. *PLoS ONE* 2:e907.
4. McDevitt MR, et al. (2007) Tumor targeting with antibody-functionalized, radiolabeled carbon nanotubes. *J Nucl Med* 48:1180–1189.


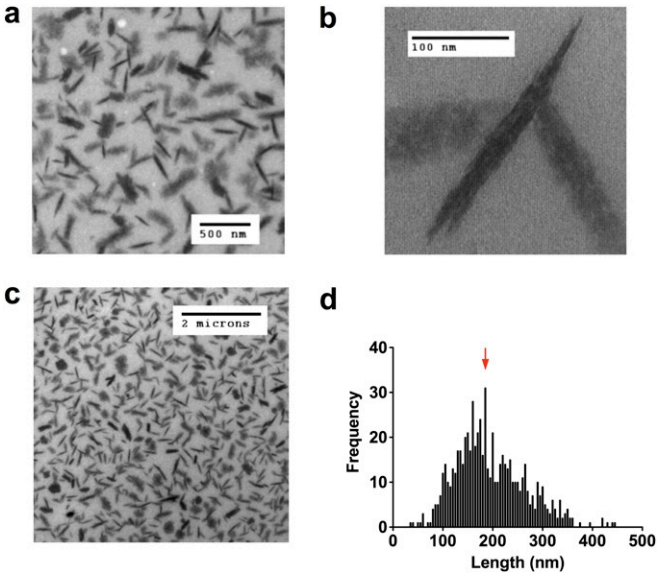


**Fig. S1.** (*A–C*) Representative TEM images of the Sep-Pak–purified SWCNT-$NH_2$ at scales of 500 nm (*A*), 100 nm (*B*), and 2,000 nm (*C*). (*D*) A histogram of these data showing a range of CNT lengths spanning ≈500 nm. The mean CNT length (red arrow) was 195 ± 69 nm ($n$ = 644).

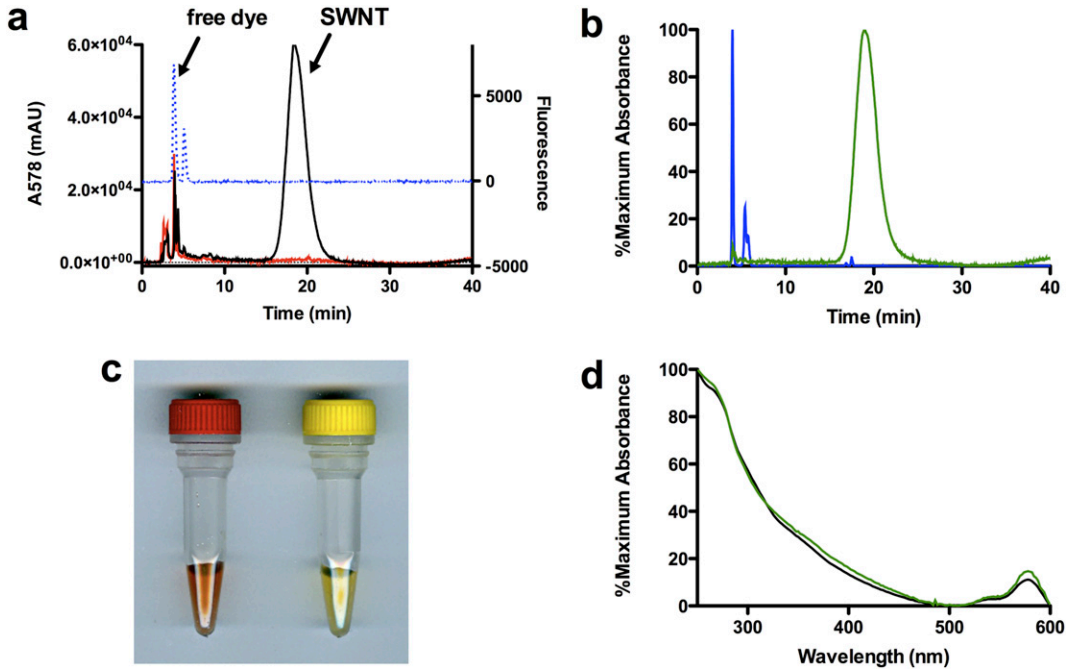


**Fig. S2.** Direct chromatographic and spectroscopic evidence of the CNTs (with attached AF dye) in the urine of mice was obtained through i.v. injection of larger doses of construct. (*A*) Reverse-phase HPLC chromatographs of urine extracted from mice receiving SWCNT-[(DOTA)(AF568)] (black trace) or saline control (red trace) demonstrating SWCNT elution at 17–22 min. The fluorescence trace of only hydrolyzed AF568 dye (blue trace) in the urine was included as a control. (*B*) Reverse-phase HPLC chromatographs of AF568 dye alone (blue trace) and SWCNT-[(DOTA)(AF568)] alone (green trace) demonstrating the large shift in retention time between free unattached dye and SWCNT-incorporated dye. (*C*) Photograph of vials containing urine from mice injected with SWCNT-[(DOTA)(AF568)] (*Left*, red cap) and saline control (*Right*, yellow cap). The dark-brown color of the functionalized SWCNT is visible compared with the yellow color of the saline-injected control. (*D*) UV-Vis spectra of the major HPLC peak (17- to 22-min elution peak) of urine collected from SWCNT-[(DOTA)(AF568)]-injected mice (black trace) and the injection formulation (green trace) demonstrating identical spectral features and attachment of the dye to the SWCNTs.

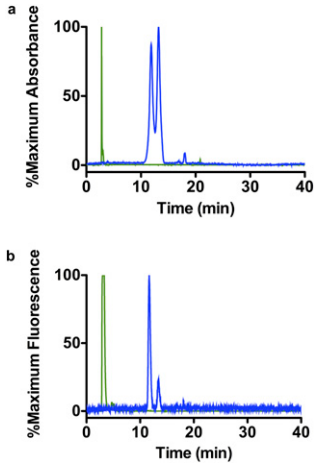


**Fig. S3.** HPLC chromatographs showing the absorbance of unattached AF488 (green trace), which elutes at ≈3–5 min, and unattached AF680 (blue trace), which elutes at ≈12–14 min (*A*), and the fluorescence of urine samples of mice injected with hydrolyzed AF488 dye (green trace), which eluted at 3 min, and hydrolyzed AF680 dye (blue trace), which eluted at 12 min (*B*).

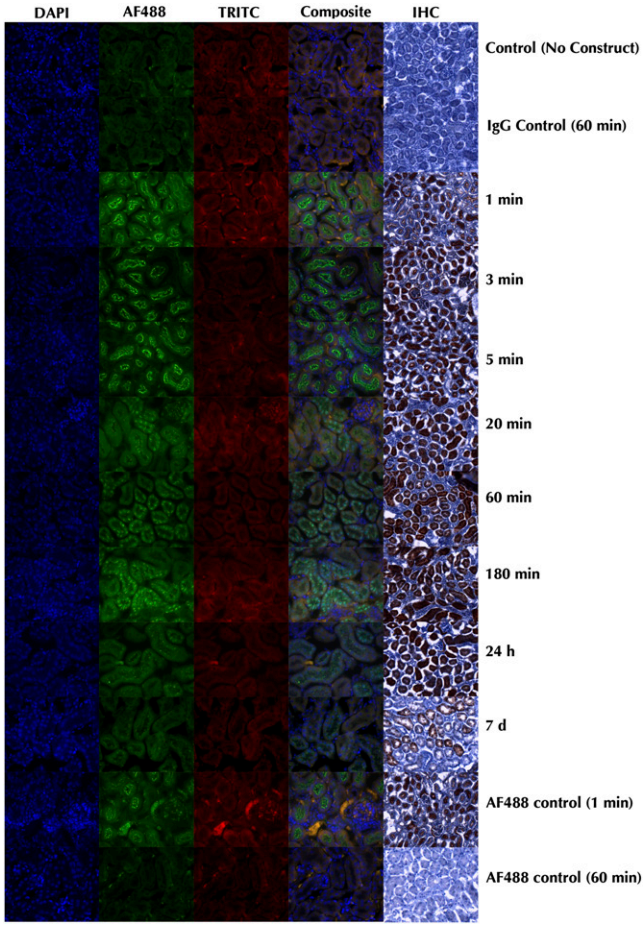


**Fig. S4.** Component (DAPI, AF488, and TRITC) and composite IF images of the kidney nephron sections for SWCNT-[(DOTA)(AF488)(AF680)]-treated and control animals and corresponding IHC images. The nephron distribution of the CNT construct was evaluated as a function of time from 1 min to 7 d by harvesting kidneys and preparing tissue sections. Controls included untreated mice (no construct) and normal rabbit IgG-injected (instead of the primary antibody) and AF488 (dye only)-injected mice at 1 and 60 min.

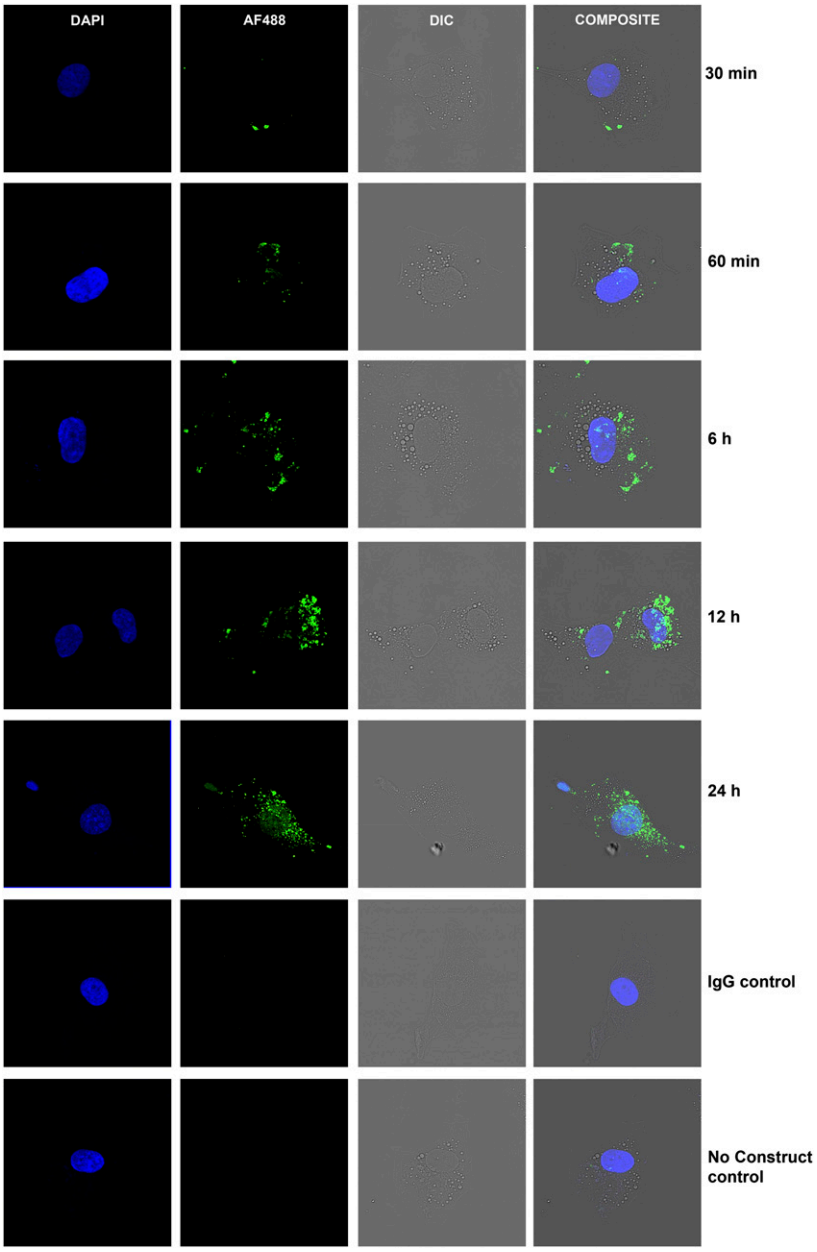


**Fig. S5.** Component (DAPI, AF488, and DIC) and composite time-course IF images of the HK-2 cells using the SWCNT-[(DOTA)(AF488)(AF680)] construct. Controls included no construct and normal rabbit IgG (instead of the primary antibody).

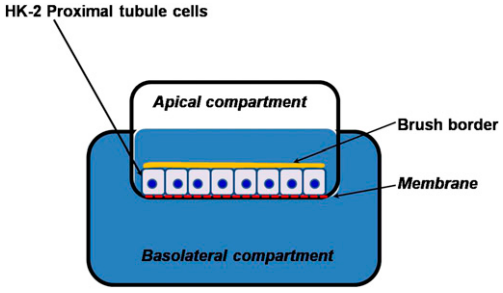


**Fig. S6.** An annotated illustration of the experimental apparatus and design of the Transwell plates used for the in vitro experiments with HK-2 cells.