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Supporting Information

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Preparation, Purification, Characterization and Cytotoxicity of Water-soluble, Transition-metal Free Carbon Nanotube Aggregates

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Materials and Methods

Synthesis of a-NHA 2. A mixture of NHA (200 mg) and sodium amide (200 mg) in liquid ammonia (320 mL) was refluxed (-33 °C) for 3 h in a flask equipped with a dry-ice condenser. A black solid was obtained after removal of ammonia. The crude material was washed with a saturated aqueous solution of NH_4Cl (100 mL), and a black solid of a-NHA **2** (205 mg) was obtained after filtration and drying at 25 °C, 0.2 mmHg for 12 h (Scheme 1). We can scale up the reaction without particular precautions to 1.00-g scale to obtain 1.03 g of **2**. TEM (JEM2100F, JEOL) was used to analyze the structure of a-NHA **2** (Figure 1f & Figure S1).

Preparation and Characterization of Aqueous Solution of a-NHA 2. Amino NHA **2** (1.00 mg) gave a grey clear solution in water (5.00 mL) upon sonication (38 kHz; US-3, As One Co.) for 2 min. No visible particles were present in the solution, and no materials remained on the membrane (pore size 5 μm, Advantec) upon filtration. The concentration of a-NHA **2** can be increased further beyond 1.0 mg/mL, but the solution becomes too dark to examine whether the solution is homogenous or not. The size of the particles in solution was analyzed by DLS (Zetasizer Nano ZS, Malvern) (Figure 1b). The solution was also deposited on mica and was analyzed by AFM (JSPM-4200, JEOL) after deposition on mica (Figure 1b, d&e).

Fractionation of a-NHA 2 by Column Chromatography. An aqueous solution of a-NHA **2** (0.2

mg/mL, 0.50 mL) was loaded on a column of Sephacryl 500HR (bed volume: 17 mL, column diameter: 1 cm, column height: 22 cm) and eluted with water at a flow rate of 0.5 mL/min. The chromatogram (Fig. 2) was obtained using a UV detector (detection at 260 nm, UV-2075, JASCO), and the eluent was collected in fractions of 0.25 mL. The size of particles in each fraction was analyzed by DLS. UV absorbance of the fractions at 260 nm showed that 92% of loaded particles were elueted from the column. The amount of 99% of the material was remained in the column, when we used Sephacryl gels of smaller or larger pore sizes (Sephacryl 100HR and 1000SF).

Detection and Quantification of Amino Group of a-NHA 2 by Sanger's Method. The amino NHA **2** (5.00 mg) was mixed with 1-fluoro-2,4-dinitrobenzene (1.00 mg) in an aqueous solution of $NaHCO_3$ (0.10 M, 100 μL) and ethanol (50 μL) at 60 °C for 20 min. A black solid was obtained after filtration of the reaction mixture. The black solid was washed with water (1.0 mL) and ethanol (1.0 mL) and dried in vacuo to obtain DNP-a-NHA **3** (5.28 mg) (Scheme 1). Absorption spectra of the aqueous solution of DNP-a-NHA **3** (1.00 mg) in water showed a peak at 355 nm due to the dinitrophenylamino group (Figure S2b). Note that none of the starting materials shows this absorbance at 355 nm. The experiments indicated that amino groups are introduced to NHA by the reaction with sodium amide. The absorbance of dinitrophenylamino group at 355 nm was measured 0.076 for 0.02 mg/mL of **3** in water. Molar absorption coefficient (ε) is reported[1] in a range of 16,000-18,000 $M^{-1}•cm^{-1}$, which showed that the dinitrophenyl group is present at 0.22 μmol/mg. When we increased the amount of 1-fluoro-2,4-dinitrobenzene (up to 500 mg) or the time of reaction (up to 48 h), the amount of DNP in **4** was not affected (0.22-0.27 μmol/mg). The results indicated that all the available amino groups of NHA **2** were dinitrophenylated under these conditions.

Synthesis of Fluorescently Labeled a-NHA (4). To an aqueous solution of **2** (0.40 mg/mL, 20 mL) was added a solution of

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[1] V. Rehak, V. Kaderabek, *Coll. Czech. Chem. Commun.* **1979**, *44*, 1613-1618.

2-(2,7-difluoro-6-hydroxy-3-oxo-3H-xanthen-9-yl)-N-[5-(2,5-dioxo-pyrrolidin-1-yloxycarbonyl)-pentyl]-terephthalamic acid (2.8 mg; Oregon Green 488-X, Invitrogen) in methanol (20 mL), and the mixture was stirred for 16 h at ambient temperature. A black solid was obtained after filtration of the reaction mixture. The black solid was further washed with water (20 mL) and methanol (20 mL) and dried in vacuo to give OG-a-NHA **4** (8.23 mg) (Scheme 1). When we treated **2** with methyl ester of OG under the same reaction conditions, the resulted NHA did not show absorption and excitation of the fluorescent dye. The result indicated that the fluorescent probe is not adsorbed on NHA but is covalently attached.

Quantification and Visualization of Fluorescently Labeled a-NHA 4 in Cells by Flow Cytometry and Confocal Microscope. Cellular uptake of NHA was proved by FCM and confocal microscopic analysis using the fluorescently labeled NHA **4**. For the FCM analysis, approximately 1.5×10^5 cells were cultured on a 24-well plate and incubated 24 h prior to treatment with **4**. Cells (3T3, mouse embryonic fibroblast cells) were then incubated for 6 h with **4** at the concentration of 0.01 and 0.05 mg/mL, respectively, dissociated with 0.05% trypsin and 0.02% EDTA (100 μL), washed twice with phosphate buffered saline (PBS; 1 mL) and resuspended in PBS containing 2% FBS (500 μL). The sample was then analyzed by FACScan instrument (Becton Dickinson). Fluorescence was detected at excitation and emission settings of 488 nm and 530 nm (FL-1 channel). FCM data represent the mean fluorescence obtained with a population of 10,000 live cells. Figure 3a illustrates a concentration-dependent increase of the cellular uptake of OG-a-NHA **4**. We also observed time-dependent uptake of OG-a-NHA **4** (Figure 3b), when 3T3 and HeLa (human cervix) cells were incubated with **4** at the concentration of 0.05 mg/mL, respectively. For the confocal microscopic analysis (Figure 3d), 3T3 cells were incubated with NHA **4** (0.05 mg/mL) for 20 h, washed with Hanks' balanced salt solutions (HBSS), stained with FM4-64 (Molecular Probes; excitation at 543 nm and emission at 590 nm) and analyzed by ZEISS LSM 510 confocal microscope (excitation at 488 nm and emission at 518 nm). The cells were stained 5-6 hours prior to the observation.

Cytotoxicity of a-NHA 2. Cytotoxicity of a-NHA **2** was evaluated using 3T3 cells and HeLa cells by Bradford assay. Approximately 2×10^4 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin-streptomycin, Gibco) on a 96-well plate in a humidified atmosphere at 37 °C with 5% CO_2 for 24 h. The cells were then incubated with **2** at various concentrations for 48 h. The cells were washed with PBS to remove **2** and dead cells, and the remained live cells were lysed with cell lytic-M (Sigma). The lysate was centrifuged at 12,000 rpm for 10 min to separate proteins further from **2**. To the lysate was added protease inhibitors (phenylmethylsulfonyl fluoride, aprotinin, and leupeptin), and the amount of total protein was determined by Bradford assay using Coomassie Brilliant Blue G-250. The experiments were carried out in triplicate, and the standard deviation from the mean value is shown as an error bar (Figure 4). When we added **2** in a control lysate that was obtained from intact cells, the viability was not affected (100% viability). The result showed that the removal of **2** by centrifuge afforded reliable values of viability. As reference experiments, Bradford assay was carried out with quarts particles (Min-U-Sil 5, US Silica) and TiO_2 nanoparticles (25-70 nm, primarily anatase, Aldrich), respectively. We found that a viability assay using an in-situ probe had a problem in quantification. For instance, when the viability assay was carried out using an in-situ redox indicator, resazurin (Alamar Blue®[2,3,4]), the cell viability value was found to be much lower than that of Bradford assay (Figure S4). When we washed the cells with PBS and removed a-NHA **2** before the resazurin treatment, the viability value became comparable to the Bradford data (Figure S4). To the contrary, when the viability assay was carried out using another in-situ probe, propidium iodide (PI), in FCM experiment, the viability value was higher than that of Bradford assay. Thus, the viability value of HeLa cells was 99% by PI assay, when the viability value was 74% by Bradford assay (dose of **2**: 1 mg/mL, incubation time: 48 h).

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[2] S. A. Ahmed, R. M. Gogal, Jr., J. E. Walsh, *J. Immunol. Methods* **1994**, *170*, 211-224.
[3] M. R. Slaughter, P. J. Bugelski, P. J. O'Brien, *Toxicol. in vitro* **1999**, *13*, 567-569.
[4] C. M. Holst, S. M. Oredsson, *Toxicol. in vitro* **2005**, *19*, 379-387.

This and other observations indicated that probe dyes were absorbed on a-NHA and that the in-situ fluorescent measurement gave erroneously low (AlamarBlue assay) or high (PI assay) values.

Supporting Data

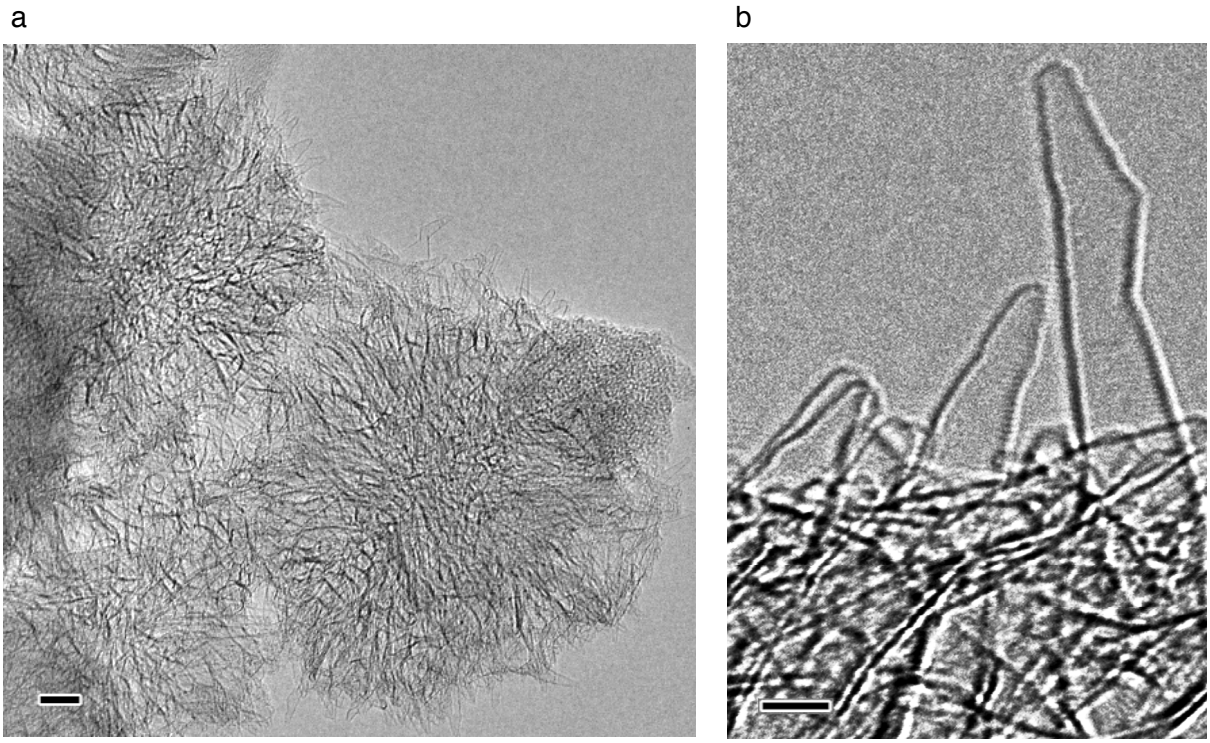

Figure S1. TEM image of a-NHA **2**. a) A low magnification TEM image showing the aggregate structure of nanotubes. Scale bar shows 10 nm. b) A high magnification TEM image showing that no damage was caused by the $NaNH_2$ treatment. Scale bar shows 2 nm. This is the same image as the one in Figure 1f in the main text.

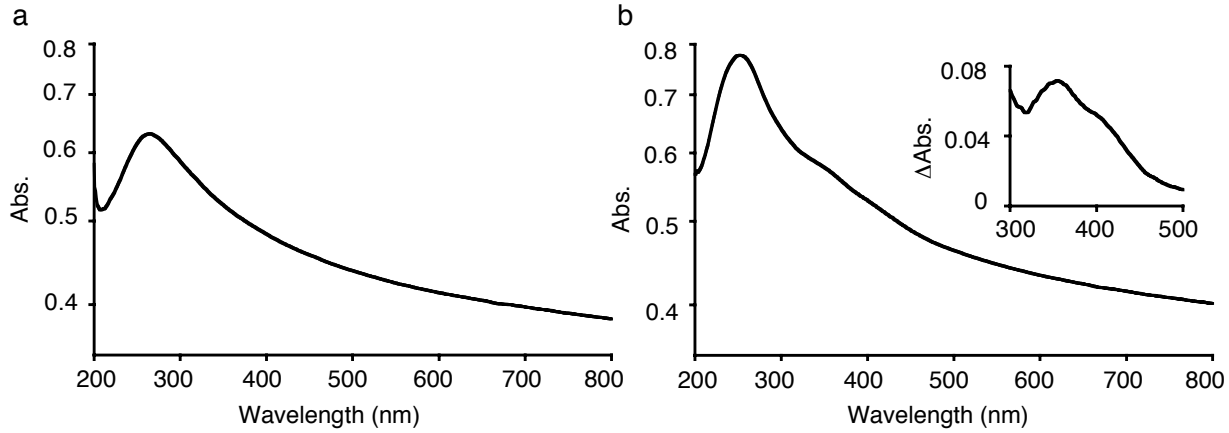

Figure S2. a) UV-vis spectrum of amino NHA **2** in water at the concentration of 0.02 mg/mL. The absorption at 260 nm corresponds to the plasmon absorption of tubular graphite. b) UV-vis spectrum of DNP-a-NHA **3** at the concentration of 0.02 mg/mL in water. Inset shows the differential spectrum of **3** and **2**. The absorption at 350 nm is due to the dinitrophenylamino group.

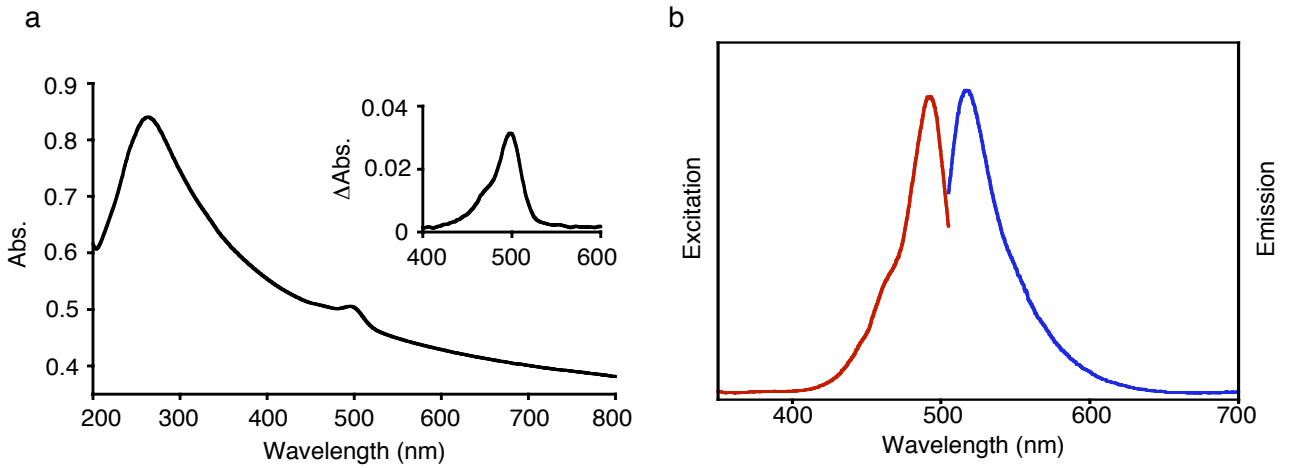

Figure S3. Spectra of OG-a-NHA **4**. a) UV-vis spectrum of **4** in water at the concentration of 0.02 mg/mL in water. Inset shows the differential spectrum of **4** and **2**. The absorbance of OG at 500 nm was measured 0.038. Taking into account the molar absorption coefficient of the chromophore (84,000 M^{-1}•cm^{-1})[5], we determined the amount of OG as 0.017 μmol/mg. b) Excitation (red) and emission spectra (blue) of **4** in water.

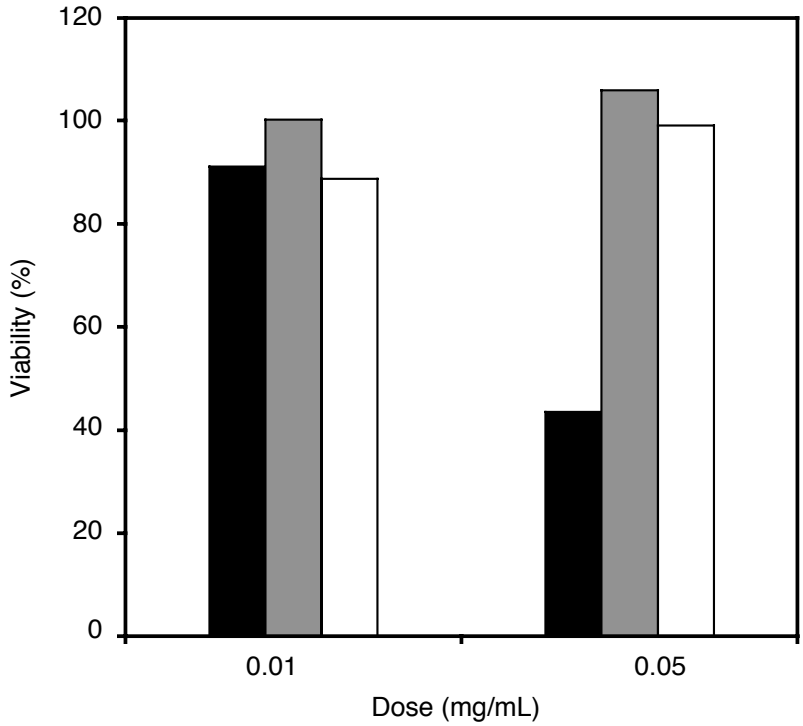

Figure S4. Method dependence of the measured viability of HeLa cells after incubation with **2** for 48 h. Black and gray bars show the viability based on Alamar Blue® assay (black, in-situ staining; gray, staining after removal of a-NHA **2**), and white bars show the viability based on Bradford assay that gives correct assessment of the viability. The very short black bar at the 0.05 mg/mL dose indicates that the Alamar Blue in-situ assay gave erroneous results.

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[5] R. P. Haugland, *The Handbook, A Guide to Fluorescent Probes and Labeling Technologies*, 10th ed., pp. 7-92, Molecular Probes, Eugene, 2005.