Peptide-based Carbon Nanotubes for Mitochondrial Targeting

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Electronic Supplementary Information

Instrumentation

HPLC analyses were performed on a Varian ProStar 240 instrument or on a Beckman Coulter System Gold. Eluents: A: H_2O + 0.1 % TFA; B: CH_3CN + 0.08 % TFA. UV-Vis spectra were recorded using a Varian Cary 5000 spectrophotometer, using 1 cm path quartz or optical glass cuvettes. Fluorescent spectra were recorded on a Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies) using 1 cm path quartz cuvettes. TEM analysis was performed on a Hitachi 600 microscope with an accelerating voltage of 75 kV. Pictures were taken using a CCD high resolution digital Hamamatsu camera (RIO Microscopy Facility Platform of Esplanade Campus, Strasbourg, France). TEM analysis was performed also with a Philips EM 208 microscope with an accelerating voltage of 100 kV. Images were acquired using an Olympus Morada CCD camera. The samples were dispersed in DMF with a concentration of 0.1 mg/ml and the suspension was sonicated for 30 minutes. 20 µl of this suspension were then drop-casted on a carbon-coated copper grid and dried under vacuum overnight. Thermogravimetric Analysis were performed using TGA Q500 TA instrument with the following procedure: isotherm at 100 °C for 20 minutes (to remove residual solvents), ramp of 10 °C min^{-1} from 100 to 1000 °C under N_2 using a flow rate of 60 ml min^{-1}. ESI Mass spectra were recorded on a Thermo Fisher Finnigan LCQ Advantage Max Instrument.

Characterization of MTS peptide

MTS peptide was purified by semi-preparative HPLC chromatography (gradient: 5-65% B in 20 minutes), and characterized by HPLC chromatography (t_R 16.95) and mass spectrometry (ESI-MS (calcd: 3475.6): 1739.5 $[M+H]^{2+}$, 696.2 $[M+H]^{5+}$, 577.6 $[M+H]^{6+}$).

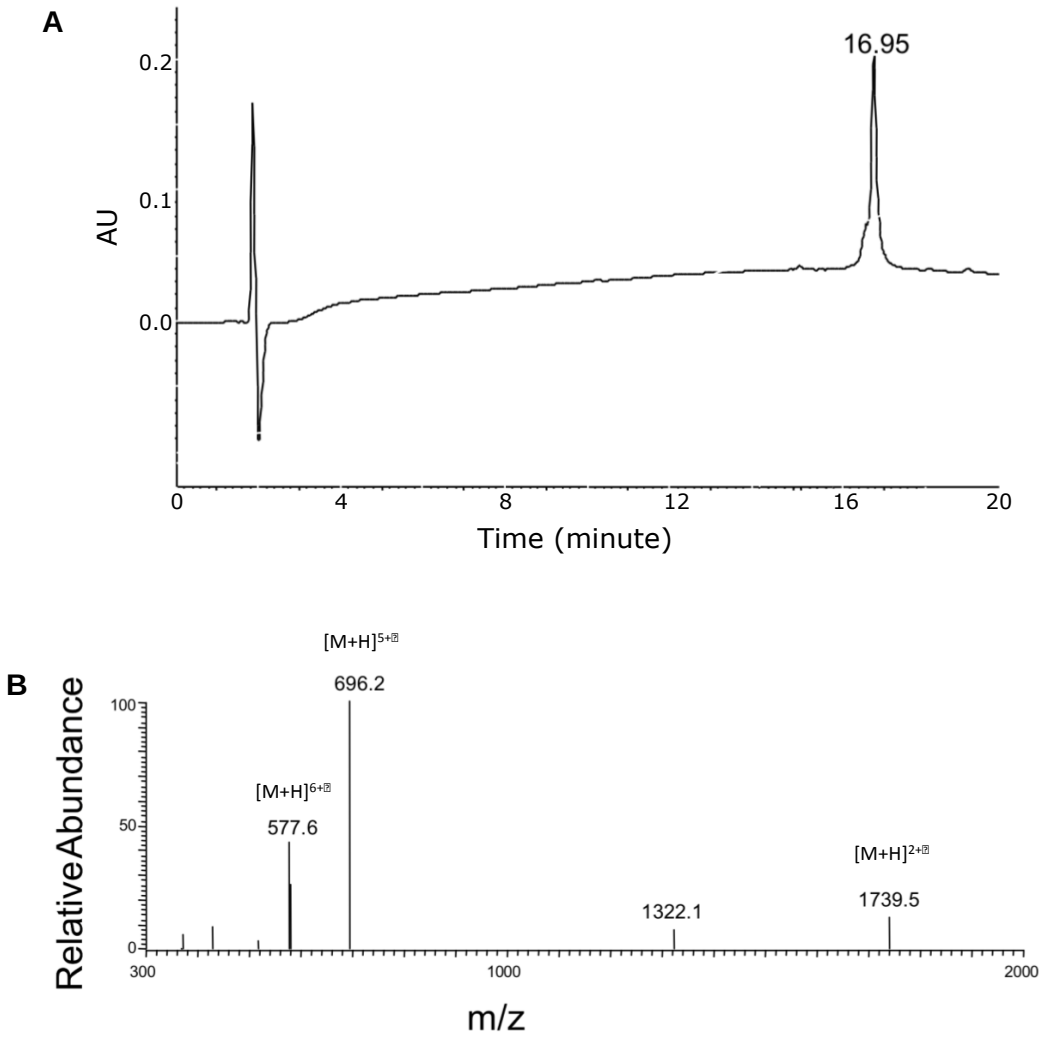

Figure S1. HPLC chromatogram (A) and mass spectrum (B) (m/z: calcd 3475.6) of MTS peptide.

Functionalization of MWCNTs

Functionalization of MWCNTs 1. 500 mg of MWCNTs from "Nanostructured and Amorphous Materials" were sonicated in a water bath (20 W, 40 kHz) for 24 hours in a sulfuric acid/nitric acid solution (3:1 v/v, 98% and 65%, respectively) at room temperature. Deionized H_2O was then carefully added and the oxidized MWCNTs were filtered (Omnipore® PTFE membrane filtration, 0.45 μm), re-suspended in water and filtered again until the pH became neutral.

MWCNTs were then dialyzed against water (Spectra/Por® dialysis membrane MWCO 12-14,000 Da) for 3 days and then lyophilized, affording MWCNTs **1**. MWCNTs **1** were characterized by TEM to determine the average length of oxidized MWCNTs: 0.26 µm (n=150). The degree of functionalization was determined by TGA at 550 °C: weight loss 7.88%, corresponding to a functionalization of 1.75 mmol/g.

Functionalization of MWCNTs 2. MWCNTs **1** (100.0 mg) were suspended in DMF (100 ml) and sonicated in a water bath for 30 minutes. Mono Boc protected TEG amino acid (100 mg per 5, 1.63 mmol) and paraformaldehyde (150 mg per 5) were added by portions over 5 days (one addition per day). The reaction mixture was stirred at 115 °C for five days. After cooling the suspension to room temperature, CNTs were filtered over a PTFE membrane (0.45 µm). The solid recovered on the filter was dispersed in DMF, sonicated in a water bath until thorough dispersion of the CNTs and filtered over a PTFE membrane (0.45 µm). This sequence was repeated 3 times with DMF, twice with MeOH and once with Et_2O and then CNTs were dried under vacuum. MWCNTs were dialyzed against water and then lyophilized. The obtained material (100 mg) was suspended in a 4M HCl solution in dioxane (100 ml) and sonicated in a water bath for 30 minutes. The reaction mixture was stirred overnight at room temperature. CNTs were filtered over a PTFE membrane (0.45 µm) and washed as before, affording MWCNTs **2**. The amount of free amines present on the material was determined by Kaiser Test: 70 µmol/g.

Functionalization of MWCNTs 3. MWCNTs **2** (50 mg) was suspended in DMF (20 ml) and sonicated in a water bath for 30 minutes. DIEA (2 ml, 11.5 mmol) and *N*-succinimidyl 3-maleimidopropionate (250 mg, 0.9 mmol) were added to the suspension and the reaction mixture was stirred at room temperature for 48 hours. CNTs were filtered over a PTFE membrane (0.45

μm) and washed as before, affording MWCNTs **3**. The amount of free amines was determined by Kaiser Test: 10 μmol/g. Hence, the amount of functionalization was 60 μmol/g. Yield: 86%.

Characterization by fluorescence spectroscopy of MWCNTs 4

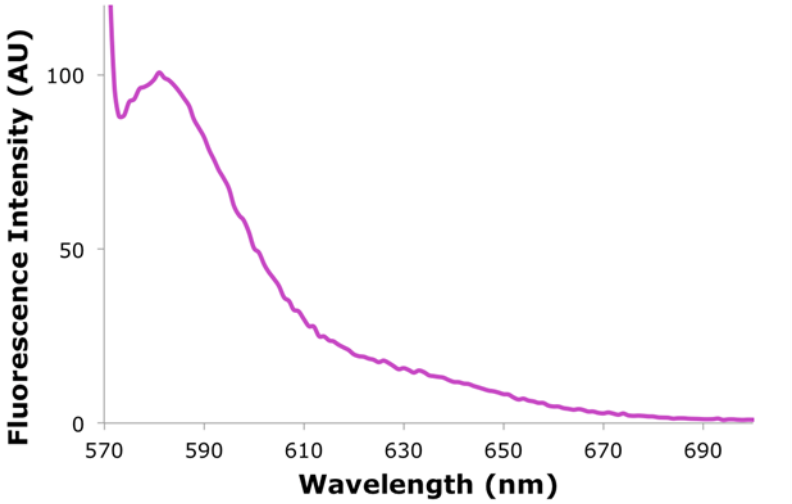

Figure S2. Fluorescence spectrum of MWCNTs **4**.

Cells and cell culture

Mouse macrophages (RAW 264.7) or human HeLa cells (cervix epithelioid carcinoma) were obtained from American Type Culture Collection (ATCC). Both cellular types were cultured under controlled atmosphere (37 °C, 5% CO_2) in RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum (FBS) and 100 U/ml gentamycin. In addition, for the murine cell line, the culture medium contained β-mercaptoethanol (50 μM) and HEPES (20 mM). When confluence reached 70-80%, RAW 264.7 macrophages and HeLa cells were detached with a phosphate buffer saline (PBS)-EDTA (2 mM) solution or trypsinized, respectively, and subcultured in T75 cm^2 flasks. Before *in vitro* experiments, either RAW 264.7 or HeLa cells were removed from culture flasks and reseeded in 24 well plates ($5 \cdot 10^5$ cells/well, 400 μl/well)

and allowed to adhere overnight prior experimentation. For microscopy procedures, cells were plated in 24 well plates as described using glass coverslips in addition.

Cellular uptake studies performed by epifluorescence microscopy

For epifluoresce microscopy studies, RAW 264.7 cells were exposed to MWCNTs **4** (100 µg/ml) during 24 hours (37 °C, 5% CO_2). To avoid any possible degradation of peptide-nanotube conjugate, we prepared the dispersion of the CNTs in cell medium immediately before incubation into cells. After incubation, cells were washed twice with TBS and saturation was performed in TBS supplemented with 1% BSA (20-30 minutes at 4 °C) in order to avoid non specific binding of the antibodies used for staining. For cellular membrane staining, cells were first incubated with an anti-mouse CD11b-biotinylated primary antibody (BD Pharmigen; diluted 1:100) for 1 hour at 4 °C and then with Streptavidin-Alexa488 (Molecular Probes; diluted 1:500) for 30 minutes at 4 °C. RAW 264.7 cells were then fixed with 4% paraformaldehyde for 1 hour at 4 °C and nuclei were counterstained with DAPI (Sigma; 0.1 µg/ml) for 10 minutes at room temperature. Finally cells were mounted on glass slides with Vectashield (Vector Laboratories) and images were acquired using epifluorescence microscope Zeiss Axioskop 2 microscope with a 40x objective (Carl Zeiss Inc.).

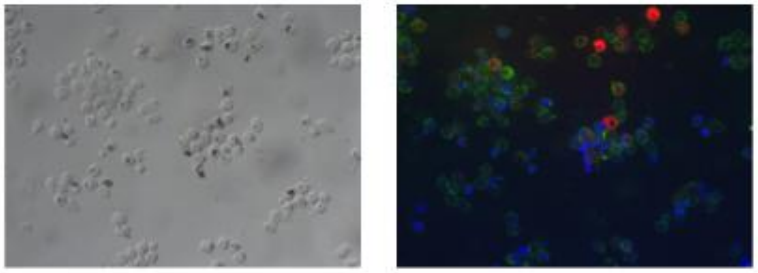

Figure S3. DIC (left) and overlay (b) of fluorescence microscope images of macrophages incubated with MTS-MWCNTs **4**. Cell membrane was stained in green and nucleus in blue. MTS-MWCNTs (red) showed the ability to enter inside macrophages and localize in the perinuclear zone.

Cellular uptake studies performed by confocal microscopy

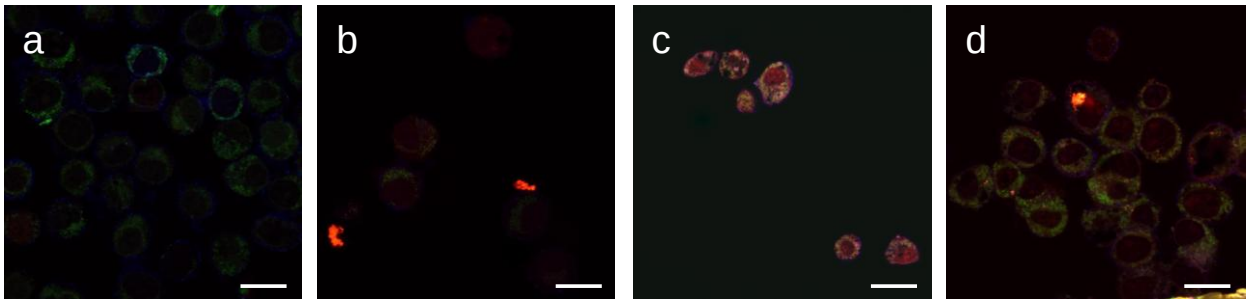

Figure S4. CLSM images of HeLa cells incubated for 24 hours with MTS-MWCNTs **4** at 10 (a), 25 (b), 50 (c) and 100 (d) µg/ml. MWCNT localization is indicated by red fluorescence, while cell membrane is evidenced by blue fluorescence and mitochondria by green fluorescence. Co-localization of nanotubes and mitochondria is highlighted in yellow. Scale bars correspond to 15 µm.