Translocation of bioactive peptides across cell membranes by carbon nanotubes

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Supplementary Information

Experimental Section

General. All reagents and solvents were obtained from commercial suppliers and used without further purification. CNTs were purchased from Carbon Nanotechnologies, Inc. (USA). PEGA-NH_2 resin was purchased from Novabiochem (Läufelfingen, Switzerland), MEM was purchased from GIBCO BRL (Pasley, Scotland) and RPMI 1640 from Bio Whittaker Europe (Verviers, Belgium). Cells were purchased from ATCC (Manassas, USA).

The electronic transmission microscope analysis were performed on a TEM Hitachi 600 HS. The epifluorescence observations were conducted on a Zeiss Axioskop II and the confocal analysis on a Zeiss Axiovert 100M. The image editing was performed using Adobe Photoshop® version 7.0. For the flow cytometry analysis a FACSCalibur® (BectonDickinson, USA) was used.

Abbreviations. Symbols and abbreviations for amino acids and peptides are in accord with the recommendations of the IUPAC-IUB Commission on Nomenclature (*J. Biol. Chem.* 1972, *247*, 977). Other abbreviations used are: *t*Bu, *tert*-butyl; CNT, carbon nanotubes; DAPI, 4',6-diamidino-2-phenylindole; DIEA, diisopropylethylamine; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; Fmoc, fluorenylmethyloxycarbonyl; MALDI-Tof, Matrix Assisted Laser Desorption Ionization Time-of-flight; MEM, minimum essential medium; MWNT, multi-walled nanotubes; SWNT, single-walled nanotubes; TEM, transmission electronic microscopy.

Synthesis of 1. To a solution of the amino functionalized SWNT prepared as described in reference 1 (4 mg, 1.8 μmol, based on the loading calculated with the quantitative Kaiser test)[2] in 200 μl of DMF, DIEA (2 μL, 11.5 μmol) and a solution of fluorescein isothiocyanate FITC (2.5 mg, 6.4 μmol) in 200 μL of DMF were added. The reaction mixture was stirred for 4 h at room temperature. The solvent was evaporated and the product was precipitated several time from methanol/diethyl ether and finally dried under vacuum. The removal of FITC was monitored by TLC (eluant

DCM/MeOH 95:5). The yield of the reaction was quantitative. Figure S1 shows the TEM analysis of **1**.

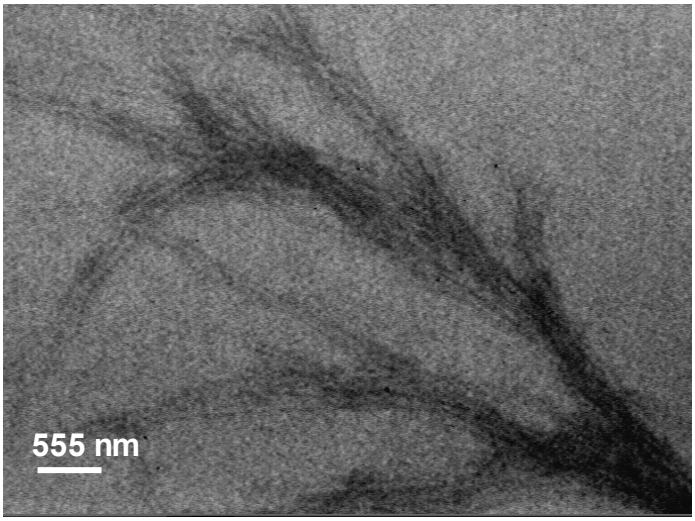

Figure S1. TEM images of **1** suspended in diethyl ether, deposited on the grid (Formvar support film on copper 200 mesh-grid) and, after evaporation of the solvent, analyzed on a TEM Hitachi 600 HS at 110 kV.

Synthesis of 2. To a solution of SWNT functionalized with the succinimidyl group as described in Ref. 3 (5.0 mg 2.2 μmol, based on the loading calculated with the quantitative Kaiser test)[2] in 1.5 ml of water, the peptide H-Lys(FITC)-QRMHLRQYELLC-OH (6.0 mg, 2.8 μmol) was added. The reaction was stirred for 6 h at room temperature and 40 mg of PEGA-NH_2 resin previously derivatized with N-succinimidyl 3-maleimidopropionate were added overnight to eliminate the excess of peptide. The complete removal of the excess of peptide was followed by TLC (DCM/MeOH 7:3) and confirmed by mass MALDI-Tof analysis. The scavenger resin was then filtered and the conjugate peptide-SWNT **2** was lyophilized. The yield was quantitative, obtaining 6 mg of the peptide CNT **2**. Figure S2 shows the TEM analysis of **2**.

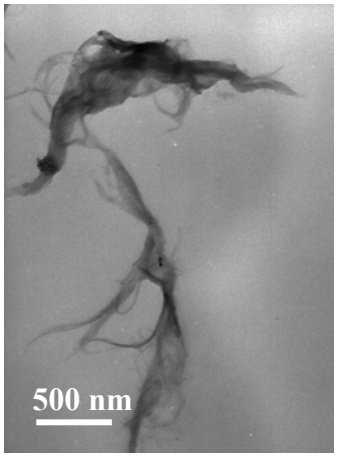

Figure S2. TEM images of **2** suspended in diethyl ether, deposited on the grid (Formvar support film on copper 200 mesh-grid) and, after evaporation of the solvent, analyzed on a TEM Hitachi 600 HS at 110 kV.

Peptide synthesis. The peptide QRMHLRQYELLC was prepared by standard Fmoc/tBu solid phase chemistry on a automatic peptide synthesizer.[4,5] Then, Boc-Lys(Fmoc)-OH was coupled using the standard conditions, the Fmoc was cleaved and the resin was washed. A solution of FITC (30 mg, 0,13 mmol) in 1 ml of DMF was added and stirred for 2 h. The peptide was cleaved from the resin, purified by RP-HPLC and analyzed by MALDI-Tof mass spectrometry. RP-HPLC characterization was carried out on a Macherey-Nagel C_{18} column using a linear gradient of A: 0.1% TFA in water and B: 0.08% TFA in acetonitrile, 5-65% B in 20 min at 1.2 mL/min flow rate. The elution time was 13.9 min. MALDI-Tof: (*m/z*) 2106.38 [$M+H^+$].

Cells and Cell Cultures. Human fibroblasts 3T6 and murine 3T3 were cultured as exponentially growing confluent monolayers on 75 cm^2 flask in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum. Human keratinocytes were cultured as exponentially growing confluent monolayers on 25 cm^2 flask in MEM (minimum essential medium) supplemented with 10% (v/v) fetal calf serum.

Fluorescence microscopy. Exponentially growing cells were dissociated by scraping. 1.5×10^5 cells were cultured on 15 mm glass coverslips until confluence. The culture medium was discarded and the cells were incubated at 37 °C with CNTs **1** and **2** dissolved in physiological buffer solution (PBS) at different concentrations (1 μmol, 5 μmol and 10 μmol). Subsequently, cells were rinsed once with PBS. The fixation step was performed using 3.7 % *para*formaldehyde in PBS for 10 min and washed three times with PBS. In order to stain the nuclei, cells were incubated for 5 min with DAPI and washed with distilled water. The coverslip was then mounted onto microscopy glass with Moviol and ProLong™ antifade agent. The distribution of fluorescence was analyzed on a Zeiss Axioskop II fluorescence microscope and on Zeiss Axiovert 100M confocal microscope. Two different wavelengths, 494 nm and 358 nm, were used to excite FITC and DAPI, respectively. The images were taken with a CCD camera HRDO76-NIK (0,76X).

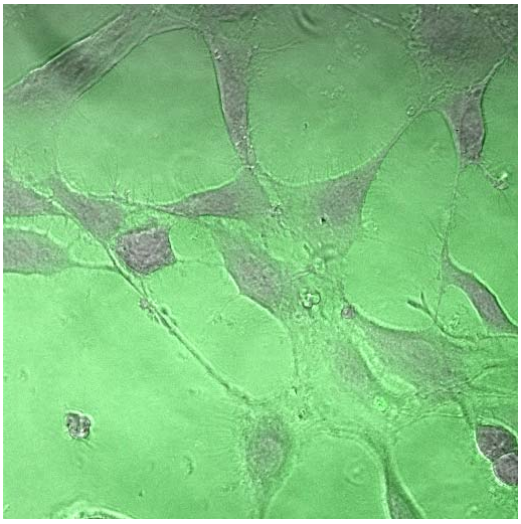

Figure S3. Human fibroblasts 3T6 incubated with 1 μM concentration of fluorescein for 20 min at 37 °C and directly analyzed by Zeiss Axiovert 100M confocal microscope. The image shows that fluorescein is not uptaken by the cells.

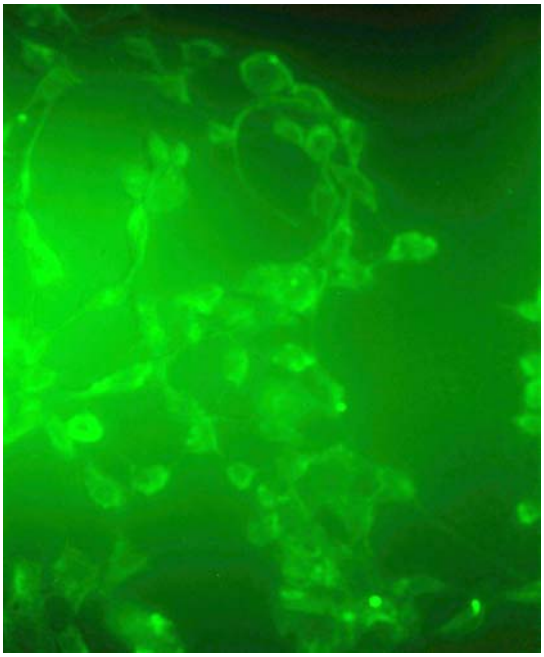

Figure S4. Human fibroblasts 3T6 incubated with 5 μM concentration of CNT **1** for 5 min at 37 °C, washed and analyzed by epifluorescence microscope Zeiss Axioskop II. The image shows the localisation of the fluorescence mainly within the cytoplasm.

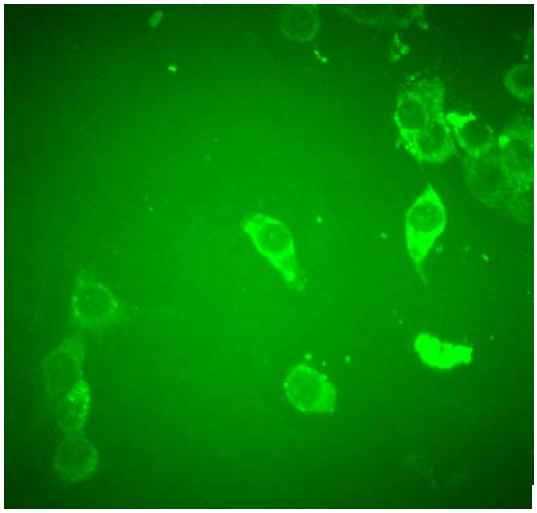

Figure S5. Human keratinocytes incubated with 1 μM concentration of CNT **1** for 20 min at 4 °C, washed and analyzed by epifluorescence microscope Zeiss Axioskop II. The image shows the localisation of the fluorescence mainly within cell cytoplasm.

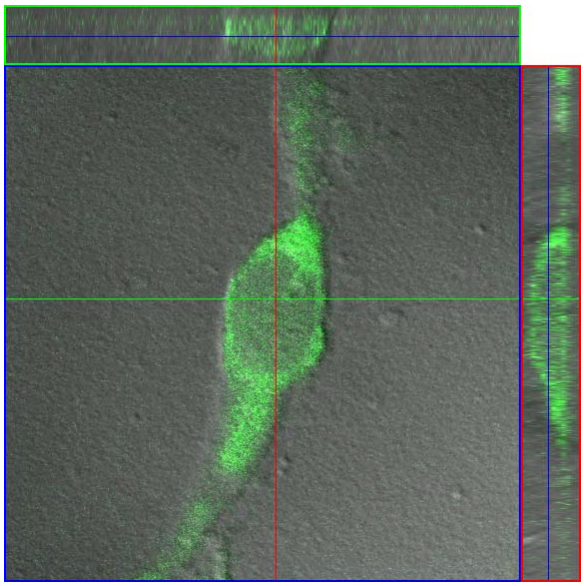

Figure S6. Human fibroblasts 3T6 incubated with 1 μM concentration of CNT **1** for 30 min at 37 °C, washed and analyzed by Zeiss Axiovert 100M confocal microscope sectioning the cells each 0.55 μm layer. The elaboration was made using Zeiss LSM Image Browser version 3,1,0,99. The image shows the fluorescence location within the cell on the X and Y section.

Flow Cytometry. To analyse the cell viability by FACS, exponentially growing fibroblast cells were dissociated with trypsin for 5 min, centrifuged at 1000 tr/min and washed three times with annexin V buffer solution. 100 μL of the same buffer and 0.5 μL of annexin V-APC were added to

the cells and incubated for 15 min in the dark. Then, 5 µL of propidium iodide staining solution (50 µg/ml) were added. The analysis was performed using a cytofluorimetry machine FACSCalibur® operating at two different excitation wavelengths (543 nm and 647 nm). CellQuest® software was used for the data analysis. A minimum of 20,000 events per sample were analysed.

References

1. Georgakilas, V.; Tagmatarchis, N.; Pantarotto, D.; Bianco, A.; Briand, J.-P.; Prato, M. *Chem. Commun.* **2002**, 3050-3051.
2. Sarin, V. K.; Kent, S. B. H.; Tam, J. P.; Merrifield, R. B. *Anal. Biochem.* **1981**, *117*, 147-157.
3. Pantarotto, D.; Partidos, C. D.; Graff, R.; Hoebeke, J.; Briand, J.-P.; Prato, M.; Bianco, A. *J. Am. Chem. Soc.* **2003**, *125*, 6160-6164.
4. Goodman, M.; Felix, A.; Moroder, L.; Toniolo, C. *Methods of Organic Chemistry* (Houben-Weyl), Vol. E22a, Thieme, Stuttgart, **2002**.
5. Neimark, J.; Briand, J.-P. *Pept. Res.* **1993**, *6*, 219-228.