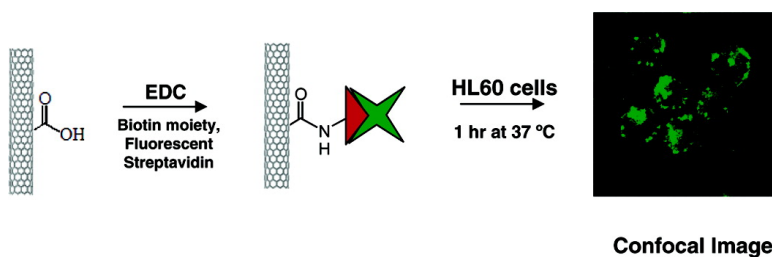## Communication

Nanotube Molecular Transporters: Internalization of Carbon Nanotube–Protein Conjugates into Mammalian Cells

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Nanotube Molecular Transporters: Internalization of Carbon Nanotube−Protein Conjugates into Mammalian Cells

Nadine Wong Shi Kam, Theodore C. Jessop, Paul A. Wender,* and Hongjie Dai*

Department of Chemistry Stanford University, Stanford, California 94305

Received March 10, 2004; E-mail: hdai@stanford.edu; wenderp@stanford.edu

The design of new strategies for the delivery of drugs and molecular probes into cells is necessitated by the poor cellular penetration of many small molecules and an increasing number of macromolecules including proteins and nucleic acids.[1] Strategies in which a poorly permeating drug or probe molecule is covalently attached to a transporter to produce a cell-penetrating conjugate offer a solution to this problem. Several classes of transporters have been investigated including lipids, PEGs, and more recently peptides.[2−5] The ability of new materials such as nanotubes[6,7] to serve as biocompatible transporters has received relatively little attention. Thus far, the main activities at this interface of materials and life sciences include functionalization and immobilization of biomolecules on nanotubes for characterization, manipulation, separation, and for device applications such as biosensors.[8−14] Few reports exist thus far on how carbon nanotubes interact with and affect living systems. Mattson et al. have investigated the growth pattern of neurons on as-grown and functionalized multiwalled nanotubes.[15] Recently, Pantarotto et al. reported the internalization of fluorescently labeled nanotubes into cells with no apparent toxicity effects observed, although without identifying the uptake mechanism.[16] Here, we present our findings on the uptake of single-walled nanotubes (SWNT) and SWNT−streptavidin (a protein with clinical applications in anticancer therapies[17]) conjugates into human promyelocytic leukemia (HL60) cells and human T cells (Jurkat) via the endocytosis pathway.

Stable aqueous suspensions of purified, shortened, and functionalized nanotubes were obtained by oxidation and sonication[9,10] of laser-ablated SWNT. Specifically, SWNTs were refluxed in 2.5 M HNO₃ for two 36-h periods separated by cup-horn sonication for 30 min [see Supporting Information (SI)]. The resulting mixture was then filtered through a 100-nm pore size polycarbonate filter, rinsed, and resuspended in pure water with sonication. Centrifugation (7000 rpm, 5 min) removed larger unreacted impurities from the solution to afford a stable suspension of 1.

Analysis of 1 by atomic force microscopy revealed mostly short (∼100 nm to 1 μm) SWNTs with diameters in the range of 1−5 nm corresponding to mostly isolated individual SWNTs and small bundles (see SI). No significant amounts of particles were observed on the substrate, suggesting good purity of the SWNTs in solution. Zeta potential measurement revealed a surface potential of ∼ −75 mV at pH 7 on 1 (see SI), confirming the existence of numerous negatively charged acidic groups at the sidewalls of the nanotubes. In pure water, 1 was stable for extended periods of time and did not agglomerate. In physiological buffer solutions containing ∼0.2 M salt, the suspension was less stable and started to aggregate after 2−3 h.

Besides providing a highly stable aqueous suspension of purified, shortened nanotubes, the oxidation/sonication procedure introduced surface carboxylates on the nanotubes for chemical derivatization. Reaction of 1 with EDC and 5-(5-aminopentyl)thioureidyl fluorescein afforded fluorescein-functionalized SWNTs, 2 (Figure 1).
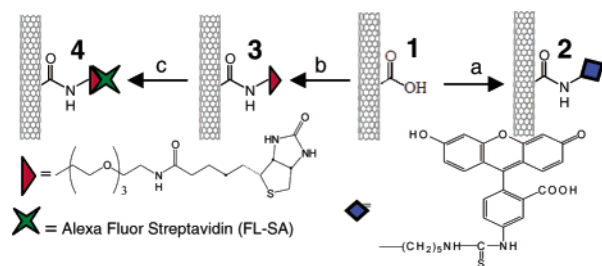

Figure 1. Synthesis and schematic of various SWNT conjugates. (a) EDC, 5-(5-aminopentyl)thioureidyl fluorescein, phosphate buffer; (b) EDC, biotin-LC-PEO-amine, phosphate buffer; (c) fluoresceinated streptavidin.
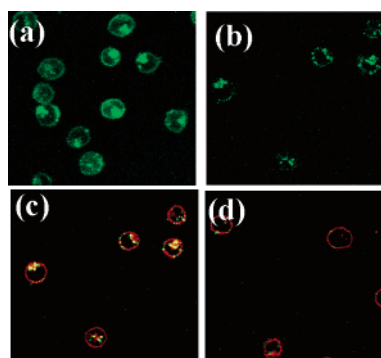

Figure 2. Confocal images of cells after incubation in solutions of SWNT conjugates: (a) after incubation in 2, (b) after incubation in a mixture of 4 (green due to SA) and the red endocytosis marker FM 4-64 at 37 °C (image shows fluorescence in the green region only), (c) same as b with additional red fluorescence shown due to FM 4-64 stained endosomes, (d) same as b after incubation at 4 °C.

To visualize the interaction of nanotubes with cells, fluorescently labeled nanotubes 2 (0.05 mg/mL SWNT) were incubated with HL60 cells for 1 h at 37 °C. The cells were washed twice, collected by centrifugation, and resuspended in growth medium. Confocal microscopy revealed appreciable fluorescence on the surface and, more importantly, in the cell interior (Figure 2a).

Having discovered the ability of 2 to enter cells, we sought to utilize the nanotubes to carry proteins into cells. Toward this end, 1 was treated with EDC and biotin-LC-PEO-amine followed by dialysis to afford biotin-functionalized SWNTs 3, which was then incubated with fluoresceinated streptavidin (SA) to afford SWNT−biotin−SA conjugate 4 (Figure 1). To evaluate the ability of nanotubes to enable the cellular uptake of the attached protein, HL60 cells were incubated with 4 as described above. Visualization of the SA revealed intense fluorescence inside the cells (Figure 2b). Importantly, the internalization of SWNT−biotin−SA conjugate 4 illustrates that nanotubes can carry large cargos, in this case SA (MW ≈ 60 kD), and transport them into cells. The uptake of SA was further confirmed by flow cytometry (Figure 3). The fluorescence of cells incubated with SA alone was only slightly
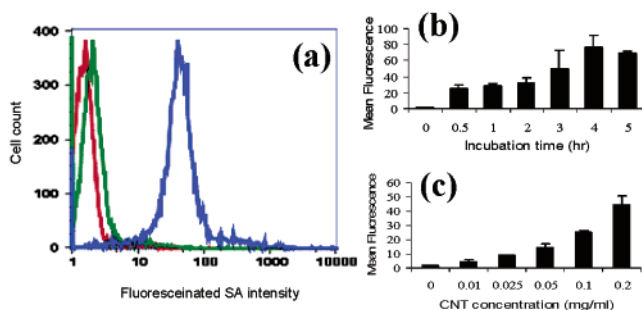
Figure 3. Flow cytometry data. (a) Fluorescence histogram for untreated cells (red curve), cells after 1 h incubation in a solution containing Alexa Fluor-labeled SA only (green curve) and after 1 h incubation in a solution of **4** (blue curve). (b) Mean green fluorescence of cells vs time of incubation in **4** ([SWNT] = 0.05 mg/mL). (c) Mean green fluorescence of cells vs concentration of **4** for 1 h incubation.

greater than the background fluorescence of untreated cells (Figure 3a, green and red curves respectively). We systematically varied the time of cell incubation of **4** ([SWNT] $\approx$ 0.05 mg/mL in the solution) and found uptake increased with longer incubations, up to ~4 h (Figure 3b). Upon increasing the concentration of **4** in the incubation solution, we observed a monotonic increase in the cellular fluorescence (Figure 3c).

To examine the potential toxicity of SWNT, HL60 cells were incubated with **1**, **2**, **3**, and **4** (1 h, 0.05 mg/mL tubes), isolated by centrifugation and observed after 24 and 48 h. In the case of **1**, **2**, and **3**, no appreciable cell death was observed (see SI). These results indicate that the functionalized SWNT themselves exhibit little toxicity to HL60 cells.

The SWNT−biotin−SA conjugate **4**, however, was found to cause extensive cell death when examined 48 h after the 1-h incubation with HL60. The degree of cell death was substantial as evidenced by the large amounts of cell debris observed (see SI). We found that the onset of appreciable cell death occurred typically ~12 h after the incubation with **4**. To further confirm that the toxicity was due to the delivery of SA into cells, the amount of SA on the SWNT was reduced by decreasing the [SA] used to load the SWNT from 2.5 to 0.0625 μM (Figure 1c). The observed toxicity was nearly nonexistent at [SA] < 1.25 μM. The dependence of cell viability on the amount of SA uptake was similar to a previous observation.[17] Consistent with the inability of SA to transverse the cell membrane alone, no toxicity was observed after cells were incubated even in highly concentrated solutions of SA. Significantly, these studies show that the SWNTs not only internalize the protein but the internalized conjugate also elicits a functional dose-dependent response.

The results described above with HL60 cells appear to be general with other cells as well, including Jurkat, Chinese hamster ovary (CHO), and 3T3 fibroblast cell lines. It has been shown that hydrophobic forces are responsible for nonspecific binding between nanotubes and proteins.[14] Although the nanotubes used in the current work contain negatively charged carboxylates along the sidewalls, such groups are likely only present on defect sites along the tubes. The unoxidized areas of the nanotubes may still afford regions of appreciable hydrophobicity. We propose that the nanotubes nonspecifically associate with hydrophobic regions of the cell surface and internalize by endocytosis.[18,19] We detected no green fluorescence from the interior of cells after incubation in **4** at 4 °C (Figure

2d), consistent with the blockage of endocytosis at 4 °C.[18,19] Further, we used a red FM 4-64 marker to stain[19,20] endosomes formed around nanotubes during endocytosis and observed yellow color (Figure 2c) inside cells due to overlapping of green fluorescence (SWNT conjugates) (Figure 2b) and red-stained endosomes (also see SI). This provides a direct evidence for endocytosis of nanotubes conjugates. The nanotubes appear to accumulate in the cytoplasm in the cells after internalization.

In conclusion, we have prepared modified nanotubes and have shown that these can be derivatized to enable attachment of small molecules and proteins. The functionalized SWNT enter nonadherent as well as adherent cell lines (CHO and 3T3) and by themselves are not toxic. While the fluoresceinated protein SA by itself cannot enter cells, it readily enters cells when complexed to a SWNT−biotin transporter, exhibiting dose-dependent cytotoxicity. The uptake pathway is consistent with endocytosis. SWNT could be exploited as molecular transporters for various cargos. The biocompatibility, unique physical, electrical, optical, and mechanical properties of SWNT provide the basis for new classes of materials for drug, protein, and gene delivery applications.

Supporting Information Available: Experimental procedures and additional results and discussions. This material is available free of charge via the Internet at http:://pubs.acs.org.

References

(1) Smith, D. A.; van de Waterbeemd, H. *Curr. Opion. Chem. Biol.* **1999**, *3*, 373−378.
(2) Bendas, G. *Biodrugs* **2001**, *15*, 215−224.
(3) *Adv. Drug Delivery Rev.* **2002**, *54*.
(4) Wright, L. R.; J. B. R.; Wender, P. A. *Curr. Protein Peptide Sci.* **2003**, *4*, 105−124.
(5) Langel, Ü., Ed.; *Cell-Penetrating Peptides: Processes and Applications*; CRC Press: Boca Raton, Fla, 2002.
(6) Dresselhaus, M. S., Dresselhaus, G., Avouris, P., Eds. *Carbon Nanotubes*; Springer: Berlin, 2001; Vol. 80.
(7) Dai, H. *Surf. Sci.* **2002**, *500*, 218−241.
(8) *Acc. Chem. Res.* **2002**, *35*. Special issue on carbon nanotubes.
(9) Chen, J.; Hammon, M. A.; Hu, H.; Chen, Y. S.; Rao, A. M.; Eklund, P. C.; Haddon, R. C. *Science* **1998**, *282*, 95−98.
(10) Liu, J.; Rinzler, A. G.; Dai, H.; Hafner, J. H.; Bradley, R. K.; Boul, P. J.; Lu, A.; Iverson, T.; Shelimov, K.; Huffman, C. B.; Rodriguez-Macias, F.; Shon, Y.-S.; Lee, T. R.; Colbert, D. T.; Smalley, R. E. *Science* **1998**, *280*, 1253−1256.
(11) O'Connell, M. J.; Boul, P.; Ericson, L. M.; Huffman, C.; Wang, Y.; Haroz, E.; Kuper, C.; Tour, J.; Ausman, K.; Smalley, R. E. *Chem. Phys. Lett.* **2001**, *342*, 265−271.
(12) Chen, R.; Zhang, Y.; Wang, D.; Dai, H. *J. Am. Chem. Soc.* **2001**, *123*, 3838−3839.
(13) Erlanger, B. F.; Chen, B.; Zhu, M.; Brus, L. E. *Nano Lett.* **2001**, *1*, 465−467.
(14) Chen, R. J.; Bangsaruntip, S.; Drouvalakis, K. A.; Kam, N. W. S.; Shim, M.; Li, Y. M.; Kim, W.; Utz, P. J.; Dai, H. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 4984−4989.
(15) Mattson, M. P.; Haddon, R. C.; Rao, A. M. *J. Mol. Neurosci.* **2000**, *14*, 175−182.
(16) Pantarotto, D.; Briand, J.; Prato, M.; Bianco, A. *Chem. Commun.* **2004**, *1*, 16−17.
(17) Hussey, S. L.; Peterson, B. R. *J. Am. Chem. Soc.* **2002**, *124*, 6265−6273.
(18) Silverstein, S. C.; Steinman, R. M.; Cohn, Z. A. *Annu. Rev. Biochem.* **1977**, *46*, 669−722.
(19) Vida, T. A.; Emr, S. D. *J. Cell Biol.* **1995**, *128*, 779−792.
(20) Richard, J. P.; Melikov, K.; Vives, E.; Ramos, C.; Verbeure, B.; Gait, M. J.; Chenomordik, L. V.; Lebleu, B. *J. Biol. Chem.* **2003**, *278*, 585−590.

JA0486059