Nanotechnology

DOI: 10.1002/ange.200503389

Carbon Nanotubes as Intracellular Transporters for Proteins and DNA: An Investigation of the Uptake Mechanism and Pathway**

*Nadine Wong Shi Kam, Zhuang Liu, and Hongjie Dai**

New materials for the intracellular transport of biological cargos such as DNA, proteins, and drug molecules have been actively sought to effectively breach the cell-membrane barriers for delivery and enabling functionality of extracellular agents. Single-walled carbon nanotubes (SWNT) have been recently shown to shuttle various molecular cargos inside living cells including proteins, short peptides, and nucleic acids.[1–8] The internalized nanotubes were found to be biocompatible and nontoxic at the cellular level. Furthermore, a very recent study has established targeted internalization of SWNTs into cancer cells that express specific cell-surface receptors and subsequent use as high near-infrared (NIR) absorbing agents for cancer-cell destruction without harming normal cells.[7] Release of oligodeoxynucleotides from SWNT transporters in vitro has also been demonstrated by NIR excitation of nanotubes that are located inside living cells. The utilization of the intrinsic physical properties of SWNTs allows the realization of a new class of biotransporters and opens up new possibilities in drug delivery and NIR radiation therapy.

At the present time, several fundamental issues remain to be addressed for the use of carbon nanotubes as potential biological transporters. One such issue is the entry mechanism that regulates the cellular internalization of SWNTs and their cargos. We[1,2] have suggested that SWNTs traverse the cellular membrane through endocytosis, whereas Pantarotto et al.[5] have suggested an energy independent nonendocytotic mechanism that involves insertion and diffusion of nanotubes through the lipid bilayer of the cell membrane. Detailed work to establish the cellular uptake mechanism and pathway for SWNTs is currently lacking.

Herein, we present the first systematic investigation of the cellular uptake mechanism and pathway for carbon nanotubes. We first show that intracellular transportation of proteins and DNA by SWNTs is indeed general, thus further confirming the transporter ability of these materials. We then present evidence that shows clathrin-dependent endocytosis as the pathway for the uptake of various SWNT conjugates with proteins and DNA. We also discuss the differences between the nanotube materials and the experimental procedures used in our work and by Pantarotto et al. who suggested an energy-independent nonendocytotic uptake of nanotubes. In our current work, we aim to clearly establish the intracellular uptake mechanism of SWNTs in the form of individual and small bundles with lengths of <1 micron and to avoid any confusion and controversy over the cellular-uptake mechanism for these materials.

To investigate the generality of carbon nanotubes for the transportation of proteins and DNA inside mammalian cells, we used, in our current work, two different mammalian cells lines, adherent HeLa cells and non-adherent HL60 cells. Control experiments were carried out through the incubation of cells in protein and DNA solutions in the absence of nanotubes. In parallel experiments, HeLa and HL60 cells were incubated with various noncovalent protein–SWNT and DNA–SWNT conjugates (Figure 1a, b) and analyzed by both

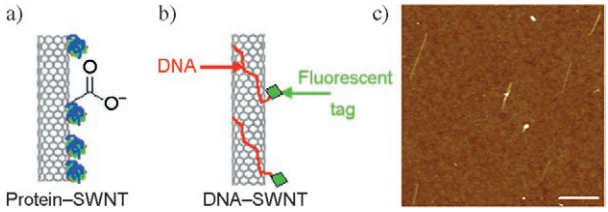

Figure 1. Schematic representation of a) protein–SWNT and b) DNA–SWNT. c) AFM image of DNA–SWNT deposited on a SiO_2 substrate (scale bar = 200 nm).

flow cell cytometry and confocal fluorescence microscopy. Unless otherwise stated, the SWNTs that were used were short ($\approx$50–200 nm, afforded by sonication), individual, and small bundles of nanotubes as characterized by atomic force microscopy (AFM; Figure 1c). Acid oxidized SWNTs were used for noncovalent conjugation with proteins and non-oxidized, as-grown SWNTs were used to complex with DNA molecules (see Experimental Section).

With these nanotubes of different functionalization, molecular cargos, and fluorescent tagging, we consistently observed, by confocal fluorescence microscopy imaging, cellular uptake of the protein–SWNT and DNA–SWNT conjugates by both HeLa and HL60 cells (representative image of uptake shown in Figure 2a) after incubation at 37 °C. The successful uptake of both SWNT conjugates was further confirmed by flow cell cytometry measurements (Figure 2d, see fluorescence signal under 'protein–SWNT' and 'DNA–SWNT' labels, respectively) from a population of $\approx$10000 cells. The noncovalent conjugations between proteins and DNA and SWNTs are sufficiently strong for entry as carrier–cargo complexes into living cells. This was shown by the little intracellular uptake of fluorescently tagged proteins and DNA molecules without nanotube transporters (Figure 2d low fluorescence levels under 'protein' and 'DNA' respectively). The existence of SWNTs inside cells was also evidenced by a recent finding that SWNTs inside living cells can be excited optically by NIR laser light. Continuous NIR radiation of SWNTs inside cells was also shown to lead to cell death owing to the high NIR absorbance of SWNTs and the

[*] N. W. S. Kam, Z. Liu, Prof. H. Dai
Department of Chemistry
Stanford University
Stanford, CA 94305 (USA)
Fax: (+1) 650-725-0259
E-mail: hdai@stanford.edu

[**] This work was supported by a Stanford Interdisciplinary Translational Research Grant.

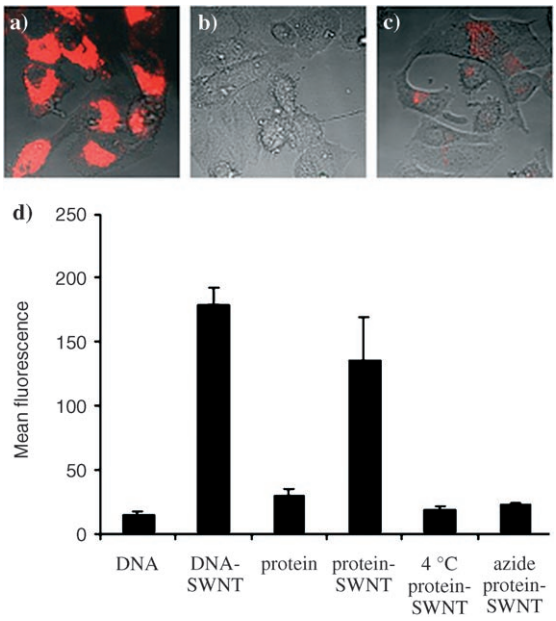

Figure 2. Confocal microscopy images of HeLa cells after incubation in fluorescently labeled DNA–SWNT at a) 37 °C, b) 4 °C, and c) after pretreatment with NaN_3. d) Flow cell cytometry data for HL60 cells that were incubated in fluorescently labeled pure DNA or protein solutions without nanotubes (labeled 'DNA' and 'protein', respectively), DNA–SWNT and SA–SWNT at 37 °C, and for protein–SWNT at 37 °C in cells incubated at 4 °C or pretreated with sodium azide.

resultant heating in vitro.[7] In the case of DNA–SWNT conjugates, DNA cargos can be detached from the SWNT carriers, after cellular internalization, by short NIR pulsing to transiently excite the nanotubes. The detached DNA molecules can subsequently undergo nuclear translocation and enter the cell nucleus.[7]

Next, we carried out a systematic investigation of the cellular internalization mechanism and pathway for SWNT conjugates. Endocytosis is known as a general entry mechanism for various extracellular materials and is an energy-dependent uptake[9–11] that is hindered when incubations are carried out at low temperature (4 °C instead of 37 °C) or in ATP (adenosine triphosphate) depleted environments.[10–12] Cellular incubations with our SWNT conjugates were carried out at 4 °C and with cells pretreated with NaN_3 in parallel with our regular incubation conditions. Treatment with NaN_3 is known to disturb the production of ATP in cells, thus hindering the endocytotic pathway. Indeed, the fluorescence levels observed with confocal microscopy from cells (after incubation in SWNT conjugates at 4 °C (Figure 2b) and ATP depletion by NaN_3 (Figure 2c)) were low. This therefore indicates endocytosis as the internalization mechanism for the uptake of SWNT conjugates at 37 °C. This was further confirmed by flow cytometry measurements, which indicated a significant reduction in cellular uptake at 4 °C and under the azide ATP depletion conditions (Figure 2d).

The endocytosis pathway encompasses several subcategories, including phagocytosis, pinocytosis, and clathrin-dependent receptor-mediated and clathrin-independent endocytosis.[10] Internalization through clathrin-dependent endocytosis occurs when the clathrin coat on the plasma membrane forms invaginations in the membrane leading to the budding of clathrin-coated vesicles.[10] The extracellular species located on the cell membrane are trapped within the vesicles and brought inside the cells. On the other hand, clathrin-independent endocytosis can occur through the caveolae or lipid-raft pathway. Caveolae are flask-shaped membrane invaginations on cell surfaces that are rich in cholesterol and sphingomyelin.[13]

Receptor-mediated endocytosis through clathrin-coated pits is the most common pathway of endocytosis. To assess the role of clathrin in the internalization of SWNTs, we carried out incubations under conditions that are known to disrupt the formation of clathrin-coated vesicles on the cell membrane. Such a treatment consisted of pretreating the cells with either sucrose (hypertonic treatment) or a K^+-depleted medium prior to exposure to the SWNT conjugates.[14–17] These pretreatments drastically reduced the level of cellular uptake of SWNTs as deduced from the cell cytometry data (Figure 3a), therefore suggesting the clathrin pathway for endocytotic cellular uptake of SWNTs.

As transferrin is well established to be internalized through the clathrin-mediated endocytosis pathway, we carried out control experiments in which the cells were

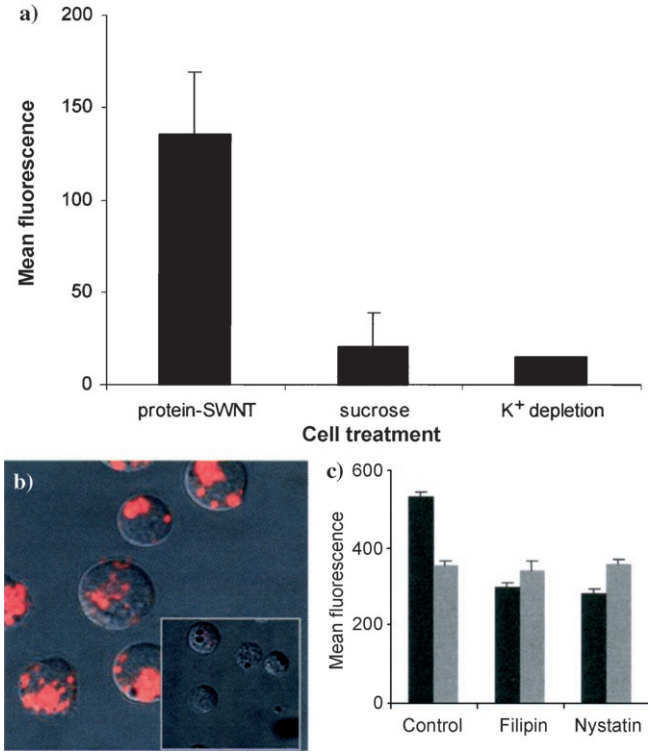

Figure 3. a) Flow cell cytometry data obtained after incubation in protein–SWNT solutions for untreated cells, cells pretreated with 0.45 M sucrose, and K^+-depleted medium, respectively. b) A confocal image that shows cellular uptake of a rhodamine-labeled transferrin protein in HL-60 cells at 37 °C. The inset shows the lack of uptake of the transferrin protein after pretreatment of cells in sucrose. c) Flow cytometry data of cells after incubation in cholera-toxin B (black bars) and BSA–SWNT (gray bars) for HeLa cells without any pretreatment (control) and cells pretreated with filipin and nystatin, respectively.

incubated in solutions of fluorescently (rhodamine) labeled transferrin under similar experimental conditions as those used for the nanotubes. Indeed, we observed that transferrin uptake was blocked (similar to our SWNTs) after the cells were incubatated in transferrin solutions at low temperature and after pretreatment with a sucrose solution or K^+ depleted buffer. As an example, Figure 3b shows a confocal image of cells with normal transferrin uptake and a lack of uptake after sucrose pretreatment (inset).

To further elucidate SWNT cellular uptake, we investigated the possibility of cellular entry by SWNT conjugates through the caveolae or lipid-rafts pathway. As caveolae-dependent cell entry relies on the presence of cholesterol domains, we pretreated cells with the drugs filipin and nystatin, which are known to disrupt the cholesterol distribution within the cell membrane. Figure 3c shows the flow cytometry data that were obtained after incubations in protein–SWNT conjugates for cells with and without filipin and nystatin pretreatment, respectively. In stark contrast to the clathrin-blocking experiments, we observed that pretreatment of cells with these drugs had no blocking effect on the cellular uptake of SWNTs (Figure 3c gray bars), which suggests little or no involvement of the caveolae-dependent cell-entry pathway for nanotubes. As a control experiment, we carried out cellular-uptake experiments of fluorescently labeled cholera-toxin B (CTX-B), which is a protein with multivalent ligand whose receptor GMl resides in the caveolae and is known to be internalized through a caveolae-mediated endocytosis pathway. Indeed, we observed that the uptake of CTX-B by our cell lines was substantially blocked (by $\approx 50\,\%$) by pretreatment with filipin and nystatin (Figure 3c black bars) under the same experimental conditions as those used for our nanotubes. Taken together, our experimental data suggest that cellular internalization of SWNT conjugates with proteins and DNA is through the clathrin-dependent endocytosis pathway.

Our results above clearly differ from the suggestion by Pantorotto et al.[5] that nanotube uptake by living cells through an energy-independent nonendocytotic pathway involves insertion and diffusion of nanotubes across cell membranes. We note, however, that there are several differences in both the nanotube material and experimental procedures that were used between the two studies. Pantorotto et al. employed materials that contained positively charged peptide-functionalized bundles and aggregates of SWNTs (which were microns in length and submicron in bundle size) as characterized by microscopy (see supplementary information of Reference [5]). The coexistence of nanotubes and cells was observed after incubation at 4 °C followed by rinsing and fixing of the cells. This led to the suggested nonblocking of nanotube uptake at 4 °C and thus nonendocytotic uptake.[5]

In an effort to investigate the effect of size on SWNT uptake, we obtained DNA–SWNT conjugates that contained longer (200 nm–2 μm, mean ≈ 750 nm) and larger (up to 15 nm measured by AFM, Figure 4a) SWNT bundles through a reduction in sonication time for the raw hipco material and by using only mild centrifugation to remove the large aggregates. Confocal imaging revealed cellular uptake of

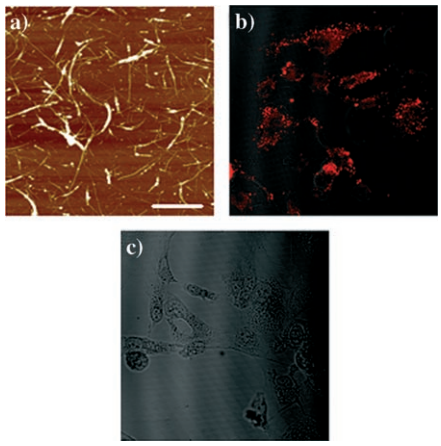

Figure 4. a) Atomic force microscopy image of relatively long bundles of nanotubes (scale bar = 500 nm). b) and c) Confocal image of HeLa cells after incubation in solutions of long nanotubes at b) 37 °C and c) 4 °C, respectively.

these ≈ 750-nm SWNT conjugates upon incubation with HeLa cells at 37 °C (Figure 4b) and the lack of uptake at 4 °C (Figure 4c). These results were similar to those obtained with our regular, short (≈ 200 nm) SWNTs. Nevertheless, a quantitative comparison of uptake for the two materials is difficult owing to inhomogeneous SWNT length distributions between the two samples and the tendency of lower aqueous stability for the longer SWNTs. Our attempt to obtain even larger SWNT aggregates for cellular-uptake experiments, as in Reference [5], failed owing to the poor stability of large SWNT aggregates.

It remains unclear what the precise uptake pathway is for micron-scale SWNT aggregates, as was observed by Pantorotto et al., both at 37 °C and 4 °C. The proposed insertion, diffusion, and penetration mechanism should not operate for such large micron-sized structures. It has been shown previously that materials >1 μm in size generally have difficulty undergoing endocytotic cellular uptake, even at 37 °C.[18] One possibility is that large positively charged peptide-functionalized SWNT aggregates exhibit a high tendency to associate with negative cell-surface species, which gives rise to a false uptake result. This has been shown to be the cause of artifactual cellular uptake of HIV-tat peptides at 4 °C.[19] Regardless of the cause, we believe that large SWNT aggregates are clearly not desired for biological transporter applications owing to the lower aqueous stability, higher tendency for nonspecific association with cell surfaces, and lower ability to traverse various biological membrane barriers.

In summary, we have shown that short SWNTs with various functionalizations are capable of the transportation of proteins and oligonucleotides into living cells and that the cellular-uptake mechanism is energy-dependent endocytosis. The detailed endocytosis pathway for short, well-dispersed SWNT conjugates is mainly through clathrin-coated pits rather than caveolae or lipid rafts. Establishment of the entry mechanism is of fundamental importance and will facilitate future developments of carbon-nanotube transporters for biological-delivery applications.[20]

Experimental Section

Materials: Bovine serum albumin (BSA) and streptavidin (SA) labeled with an alexa fluor 488 dye were obtained from Molecular Probes Inc. DNA oligonucleotides were obtained from the PAN Facility at Stanford University. All tissue culture materials were obtained from Invitrogen and all chemicals used for the endocytosis-mechanism experiments were obtained from Sigma-Aldrich.

SWNT conjugate preparation.

Protein–SWNT: Single-walled carbon nanotubes (SWNT) made through the Hipco process were treated by sonication and acid reflux as described previously to afford short, ($\approx$200 nm) water-soluble nanotubes that contain acidic surface groups.[1,2] Fluorescently labeled BSA or SA in a concentration range of 100 nM–1 μM was then mixed with the oxidized nanotubes for 1–2 h. The proteins were found to bind nonspecifically to the nanotubes to form protein–SWNT conjugates.[2]

DNA–SWNT: SWNTs made through the Hipco process was sonicated in a solution of a fluorescently labeled 15-mer oligonucleotide (CATTCCGAGTGTCCA-X in which X = Cy3 or fluorescein isothiocyanate (FITC)) for $\approx$30 min to 1 h (DNA concentration $\approx$2–10 μM). The resultant suspension was centrifuged at 24000 g for $\approx$6 h to remove large impurities in the sample. The sonication/centrifugation process was repeated twice. The final decanted solution contained mostly SWNTs that are 50–200 nm in length (the apparent height or diameter of the tubes is in the range of $\approx$1–5 nm as determined by AFM topography measurements that correspond to individual and small bundles of SWNTs) wrapped by DNA oligonucleotides.[21]

Long SWNT conjugate preparation: DNA–SWNT conjugates were prepared as described above, with the sonication time reduced to $\approx$10–20 min. The resultant suspension was centrifuged at 10000 g for $\approx$15 min to remove large impurities in the sample. The final supernatant was analyzed by AFM and found to contain bundles of nanotubes that ranged from 200 nm–2 μm in length and had an apparent height or diameter between 3 and 15 nm.

Cellular incubation in nanotube conjugates: Two cell lines, human promyelomonocytic HL60 cells and cervical cancer HeLa cells were used in the current work. HL60 were cultured in RPMI (Roswell Park Memorial Institute) 1640 medium supplemented with 10% fetal bovine serum (FBS), whereas HeLa cells were cultured in DMEM medium supplemented with 10% FBS and 1% penicillin–streptomycin. The cells were suspended in phosphate-buffered saline (PBS) solution, mixed with a SWNT conjugate, rhodamine-labeled transferring, or FITC-labeled cholera-toxin B, and incubated for 1–3 h at 37°C (except for the low-temperature 4°C incubations). The concentration of SWNTs in the incubation solution was typically $\approx$2.5 mg L^{-1} as measured by the UV/Vis–NIR absorption spectrum, and the cell density was $\approx 2–3 \times 10^5$ cells/well. Adherent HeLa cells were either trypsinized to detach from the petri dishes to form a suspension, or were seeded overnight in chambered cover slides.

Endocytosis mechanism investigation.

Low temperature incubation at 4°C: Cellular incubations were carried out as described above with the solution kept at 4°C, instead of the regular 37°C condition.

Incubation of cells in SWNT conjugates under ATP depletion: For the ATP-depletion studies, the cells were preincubated in PBS buffer solution and supplemented with 10 mM NaN_3 and 50 mM 2-deoxy-D-glucose for 30 min at 37°C followed by incubation in a solution of either DNA–SWNT or protein–SWNT conjugates.

Hypertonic incubation: For the hypertonic treatment, the cells were preincubated for 30 min in PBS buffer solution and supplemented with 0.45 M sucrose before exposure to the SWNT conjugate 37°C.

Potassium-depletion incubation. The K^+ depletion was achieved by preincubation of the cells in a potassium-free buffer that consists of HEPES *N*-(2-hydroxyethyl)piperazine-*N*-[2-ethanesulfonic acid], NaCl, $MgCl_2$, and D-glucose for 30 min at 37°C prior to incubation in a nanotube solution.

Filipin and nystatin treatment. For these incubations, the cells are pretreated in PBS buffer solution and supplemented with filipin (5 μg mL^{-1}) and nystatin (10 μg mL^{-1}) for 30 min followed by exposure to a SWNT conjugate at 37°C.

Confocal microscopy. After incubation, the non-adherent cells were washed by centrifugation twice and resuspended in ice-cold PBS buffer solution. For adherent cells, the cell medium was removed from the chambered cover slides, washed, and replaced with ice-cold PBS buffer solution. For analysis, about 20 μL of non-adherent cell suspension was dropped onto a cover glass slide and then imaged immediately with a Zeiss LSM510 confocal microscope. Adherent HeLa cells were imaged directly on the chambered cover glass.

Flow Cell Cytometry. For cell cytometry, adherent cells were detached from the surface by treatment with trypsin–EDTA and received similar subsequent treatment as the non-adherent cells. After incubation, the cells were washed, resuspended in ice-cold PBS buffer solution, and supplemented with 2% propidium iodide (PI). The mean fluorescence was measured from $\approx$10000 cells by using flow cytometry (Becton Dickinson FACScan). Cells that were stained with propidium iodide (corresponding to naturally occurring dead cells) were excluded from the data analysis.

Received: September 25, 2005
Revised: October 10, 2005
Published online: December 13, 2005

Keywords: cellular uptake · DNA · drug delivery · endocytosis · nanotubes

[1] N. W. S. Kam, H. Dai, *J. Am. Chem. Soc.* **2005**, *127*, 6021.
[2] N. W. S. Kam, T. C. Jessop, P. A. Wender, H. Dai, *J. Am. Chem. Soc.* **2004**, *126*, 6850.
[3] Q. Lu, J. M. Moore, G. Huang, A. S. Mount, A. M. Rao, L. L. Larcom, P. C. Ke, *Nano Lett.* **2004**, *4*, 2473.
[4] A. Bianco, J. Hoebeke, S. Godefroy, O. Chaloin, D. Pantarotto, J.-P. Briand, S. Muller, M. Prato, C. D. Partidos, *J. Am. Chem. Soc.* **2005**, *127*, 58.
[5] D. Pantarotto, J. Briand, M. Prato, A. Bianco, *Chem. Commun.* **2004**, *1*, 16.
[6] D. Pantarotto, R. Singh, D. McCarthy, M. Erhardt, J. P. Briand, M. Prato, K. Kostarelos, A. Bianco, *Angew. Chem.* **2004**, *116*, 5354; *Angew. Chem. Int. Ed.* **2004**, *43*, 5242.
[7] N. W. S. Kam, M. O'Connell, J. A. Wisdom, H. Dai, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 11600.
[8] N. W. S. Kam, Z. Liu, H. Dai, *J. Am. Chem. Soc.* **2005**, *127*, 12492.
[9] M. Marsh, H. T. McMahon, *Science* **1999**, *285*, 215.
[10] S. Murkherjee, R. N. Ghosh, F. R. Maxfield, *Phys. Rev.* **1997**, *77*, 759.
[11] S. C. Silverstein, R. M. Steinman, Z. A. Cohn, *Annu. Rev. Biochem.* **1977**, *46*, 669.
[12] S. L. Schmid, L. L. Carter, *J. Cell Biol.* **1990**, *111*, 2307.
[13] R. D. Singh, V. Puri, J. T. Valiyaveettil, D. L. Marks, R. Bittman, R. E. Pagano, *Mol. Biol. Cell* **2003**, *14*, 3254.
[14] J. Heuser, R. G. W. Anderson, *J. Cell Biol.* **1989**, *108*, 389.
[15] J. M. Larkin, M. S. Brown, J. L. Goldstein, R. G. W. Anderson, *Cell* **1983**, *33*, 273.
[16] I. H. Madshus, K. Sandvig, S. Olsnes, B. van Deurs, *J. Cell. Physiol.* **1987**, *131*, 14.
[17] M. G. Qaddoumi, H. J. Gukasyan, J. Davda, V. Labhasetwar, K. J. Kim, V. H. Lee, *Mol. Vision* **2003**, *9*, 559.
[18] J. Rejman, V. Oberle, I. S. Zuhorn, D. Hoesktra, *Biochem. J.* **2004**, *377*, 159.

[19] T. B. Potocky, A. K. Menon, S. H. Gellman, *The J. Biol. Chem.* **2003**, *278*, 50188.
[20] During the writing of this paper, a publication appeared on the uptake of multiple-walled carbon nanotubes and DNA cargos that revealed inhibition of uptake at 4°C and suggested the endocytosis pathway.Y. Liu, D.-C. Wu, W.-D. Zhang, X. Jiang, C.-B. He, T. S. Chung, S. H. Goh, K. W. Leong, *Angew. Chem.* **2005**, *44*, 4860; *Angew. Chem. Int. Ed.* **2005**, *44*, 4782.
[21] M. Zheng, A. Jagota, E. D. Semke, B. A. Diner, R. S. Mclean, S. R. Lustig, R. E. Richardson, N. G. Tassi, *Nat. Mater.* **2003**, *2*, 338.

