

siRNA Delivery into Human T Cells and Primary Cells with Carbon-Nanotube Transporters**

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Molecular transporters and delivery agents have been pursued actively in the past.^[1] RNA interference (RNAi) has emerged recently as a powerful method for biological research and the potential treatment of human diseases, including AIDS and cancer.^[2,3] It has been shown that the delivery of short interfering RNA (siRNA) to human T cells to silence the expression of the HIV-specific cell-surface receptors CD4 and/or coreceptors CXCR4/CCR5 can block HIV-virus entry and reduce infection.^[4–6] Much remains to be done to enable the efficient delivery of molecules both in vitro and in vivo. Existing delivery methods include viral and nonviral approaches.^[2,7] In vitro, certain T cells and primary cells are still difficult to transfect by nonviral agents, such as liposomes, as in the case of siRNA delivery to T cells.^[4] Electroporation is an efficient in vitro transfection method, but cell damaging may occur.^[8] A simple nonviral method for molecular delivery to various types of cells should improve understanding of cellular behavior and functions, and lead to potential in vivo applications.

Recently, carbon nanotubes have been shown to traverse cellular membranes by endocytosis and shuttle biological molecules, including DNA, siRNA, and proteins, into immortalized cancer cells.^[9–15] Little cytotoxicity is observed for nanotubes with appropriate functionalization and high aqueous solubility. The ability of nanotubes to transport molecules into biologically and medically more relevant T cells and primary cells remains untested. It is important to investigate whether nanotube transporters exhibit advanced capabilities over conventional nonviral delivery agents.

Herein, we explore single-walled carbon nanotubes (SWNTs)^[16] as nonviral molecular transporters for the delivery of siRNA into human T cells and primary cells. We show that nanotubes are capable of siRNA delivery to afford efficient RNAi of CXCR4 and CD4 receptors on human T cells and peripheral blood mononuclear cells (PBMCs). The delivery ability and RNAi efficiency of nanotubes far exceed those of several existing nonviral transfection agents, including four formulations of liposomes. A dependence of the delivery ability of nanotubes on functionalization and the degree of hydrophilicity was also observed upon probing the interaction of SWNTs with single cells by micro-Raman spectroscopic imaging. This result showed that hydrophobic interactions are an underlying factor in nanotube-mediated molecular delivery.

SWNTs (average length ≈ 200 nm, diameter ≈ 1 – 3 nm; Figure 1 a,b) were functionalized and made water soluble by the strong adsorption of phospholipids (PLs) grafted onto amine-terminated polyethylene glycol (PEG; PL-PEG₂₀₀₀-NH₂, Figure 1 a),^[13,14] to give a nanotube suspension stable as a solution in PBS buffer or in a serum-containing cell medium, without aggregation (Figure 1 c). The two alkyl chains on a PL molecule strongly bind to SWNTs without detachment in PBS even upon heating to 70 °C for weeks. Thiol-modified siRNA cargo molecules were linked to the amine groups on the sidewalls of SWNTs through cleavable

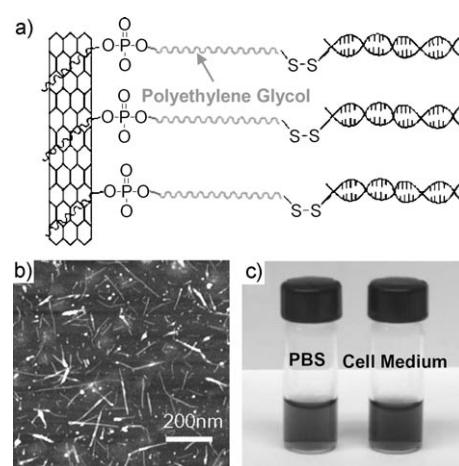


Figure 1. Carbon nanotubes for siRNA delivery into human T cells: a) Functionalization of SWNTs with PL-PEG₂₀₀₀-NH₂ (PL = phospholipid) for the conjugation of thiol-siRNA through disulfide linkages; b) atomic force microscopy (AFM) image of SWNTs functionalized with PL-PEG₂₀₀₀; c) photograph showing a suspension of PL-PEG₂₀₀₀-functionalized SWNTs in phosphate buffer saline (PBS; left) and RPMI-1640 serum-containing cell medium (right) without any aggregation of nanotubes.

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disulfide bonds (Figure 1 a). We chose to conjugate molecules onto SWNTs through disulfide linkages to facilitate cargo release upon cellular uptake.^[13]

We investigated SWNT delivery of siRNA against CXCR4 (Figure 2), a cell-surface coreceptor required for HIV entry into human T cells and infection.^[4–6] We observed

to the T cells (Figure 2 f, labeled as CXCR4b). In control experiments, cells incubated in solutions of SWNTs conjugated with scrambled siRNA-targeting luciferase and SWNTs alone exhibited no CXCR4-knockdown effect (Figure 2 f). These results demonstrated specific knockdown of cell-surface CXCR4 by SWNT-mediated siRNA_{CXCR4} delivery into T cells (see the Supporting Information for an siRNA-dose-dependent RNAi effect mediated by SWNT delivery).

No silencing effect was observed with T cells incubated with lipofectamine2000–siRNA complexes for 1 day or 3 days (Figure 2 a,b,d (confocal data)), which is consistent with previous findings for T cells with liposomes. Besides lipofectamine2000 (referred to as “Lipo1” in Figure 2 f), we also investigated several recently formulated liposomes and found that none of these agents had an RNAi effect on T cells (Figure 2 f). However, we did find that both liposomes and SWNTs were capable of siRNA delivery to immortalized cancer cells, with a CXCR4- and CD4-silencing effect on HeLa-derived MAGI cells (see the Supporting Information).

As both CD4 receptors and CXCR4 coreceptors are required for HIV viral entry into human T cells, we also investigated the delivery of CD4 siRNA into T cells by SWNTs and observed approximately 60 % knockdown of CD4 expression (Figure 3 a,b). The down regulation of CD4 receptors on T cells by treatment with SWNT–siRNA_{CD4} had no nonspecific effect on the CXCR4 receptors, and vice versa (Figure 3 b). We also observed simultaneous knockdown of CD4 and CXCR4 receptors after incubating T cells in a solution containing a 1:1 mixture of SWNT–siRNA_{CD4} and SWNT–siRNA_{CXCR4} conjugates (Figure 3 b). This experiment demonstrates the cosilencing of two types of receptors on T cells by nanotube siRNA delivery.

Next, we investigated the possibility of carbon-nanotube delivery of siRNA into human primary cells, that is, peripheral blood mononuclear cells (PBMCs) donated by a patient. The incubation of PBMCs in a solution of SWNT–siRNA_{CXCR4} conjugates led to approximately 60 % knockdown of CXCR4 receptors, thus indicating the effectiveness of siRNA delivery to human primary cells by nanotube transporters (Figure 4 a,b). No RNAi effect was observed with PBMCs after incubation in a solution of lipofectamine–siRNA complex (Figure 4 a,b).

The biocompatibility of foreign matter in living systems is an important point of concern. Recent data show that the degree of cytotoxicity of nanotubes is dependent on chemical functionalization.^[17–19] Nanotubes functionalized to a high degree with groups that promote solubility and stability in water are nontoxic to cells,^[9–15,17–19] even at high concentrations. To prove the nontoxicity of our nanotube delivery system, we carried out systematic investigations on T cells and immortalized cells (MAGI cells) after incubation in solutions of SWNTs, and compared them with lipofectamine-treated cells. A standard

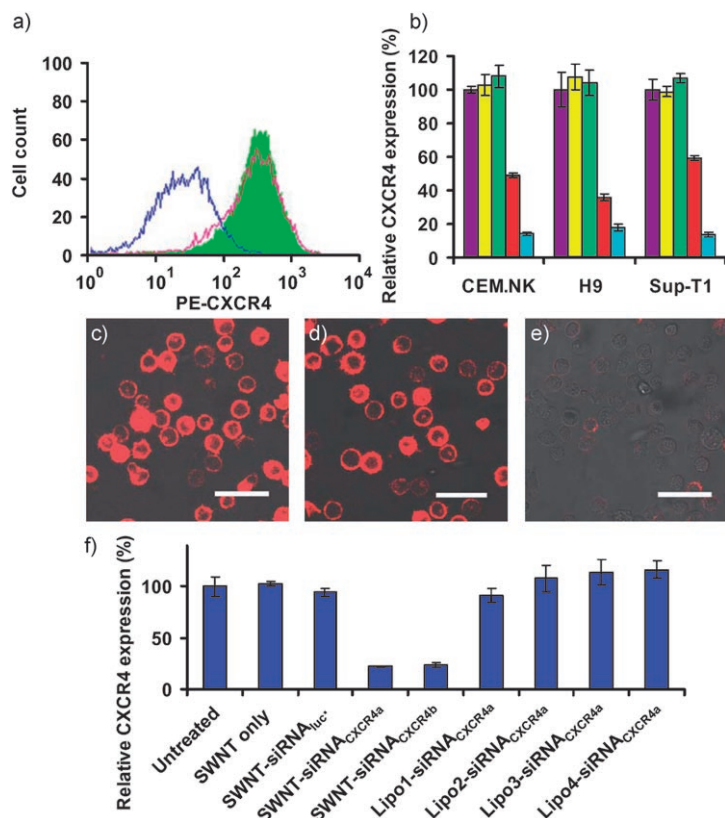


Figure 2. Delivery of siRNA against CXCR4 cell-surface receptors to human T cells by carbon nanotubes: a) Flow cytometry data of CEM cells stained with fluorescent PE-anti-CXCR4; green area: untreated cells; pink curve: cells incubated with lipofectamine2000–siRNA_{CXCR4} for 3 days and then stained; blue curve: cells incubated with SWNT–siRNA_{CXCR4} for 3 days and then stained; b) relative CXCR4 expression level on untreated cells (purple), on cells treated for 1 day (yellow) and 3 days (green) with lipofectamine2000–siRNA_{CXCR4}, and on cells treated for 1 day (red) and 3 days (blue) with SWNT–siRNA_{CXCR4}; the expression levels were calculated on the basis of the mean fluorescence intensity of PE-anti-CXCR4-stained cells from cytometry data; c)–e) confocal images of untreated cells, cells treated with lipofectamine2000–siRNA_{CXCR4}, and cells treated with SWNT–siRNA_{CXCR4}, respectively, after staining with PE-anti-CXCR4 (all images were recorded under identical imaging settings); scale bars: 40 μ m; f) CXCR4 expression levels on CEM cells after various treatments, as indicated, including treatment with four types of liposomes (Lipo1–4) and luciferase (Luc), siRNA control; the expression levels were determined by FACS analysis (fluorescence-activated cell sorting).

50–60 % knockdown of CXCR4 receptors on H9, Sup-T1, and CEM cells incubated in a solution of SWNT–siRNA_{CXCR4} ([siRNA] $\approx$ 50 nM) for 24 h (Figure 2 b, red bars), and approximately 90 % silencing efficiency was observed upon incubation for 3 days (Figure 2 a,b (cytometry data) and Figure 2 e (confocal image)). A second sequence of CXCR4 siRNA also had an efficient silencing effect when transported by SWNTs

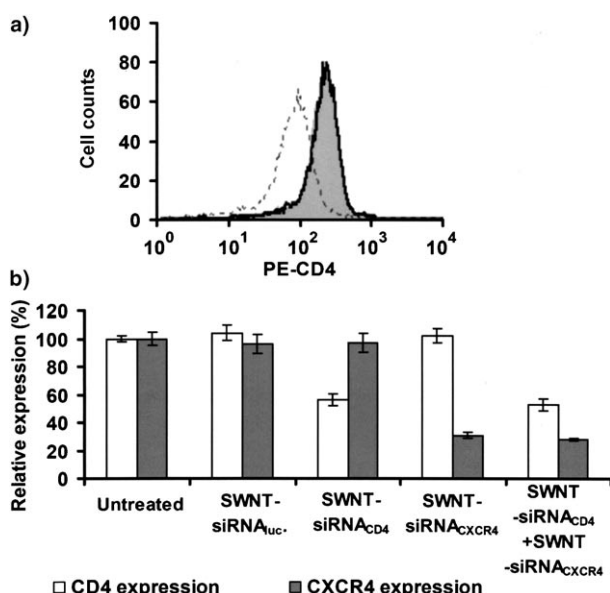


Figure 3. SWNT-mediated knockdown of CD4 and CXCR4 receptors on T cells by RNAi: a) Flow cytometry data obtained with CEM cells stained with the PE-labeled anti-CD4 antibody; gray area: control cells without any treatment; solid curve: cells after incubation with lipofectamine-siRNA_{CD4} for 3 days followed by staining; dashed curve: cells after incubation with SWNT-siRNA_{CD4} for 3 days followed by staining; b) CD4 and CXCR4 expression levels on CEM cells treated under the various conditions indicated; the data were obtained by flow cytometry measurements of CEM cells costained with PE-labeled anti-CD4 and anti-CXCR4 antibodies.

proliferation assay (Figure 5a–c) and cytotoxicity assays (Figure 5d) found no obvious toxic effects of our PL-PEG-functionalized SWNTs on either T cells or MAGI cells. Three T cell lines treated with SWNTs proliferated at the same rate as control untreated cells. lipofectamine2000 was found to be slightly toxic to MAGI cells at high doses (Figure 5a,d, “MAGI-CCR5” panel); this high-dose toxicity is a known effect described in the literature.^[26] No toxicity effect was observed with T cells after incubation in solutions of lipofectamine2000 (Figure 5b,d, “H9” panel), which is consistent with the lack of liposome internalization into the T cells.

Our results suggest that nanotubes are generic molecular transporters for various types of biologically important cells, from cancer cells to T cells and primary cells. To glean insight into the mechanism by which nanotubes interact with cells, we varied the chemical functionalization of the nanotubes and investigated the uptake behavior. We employed the micro-Raman technique to probe directly SWNTs in living cells with an approximately 2- μ m spatial resolution. SWNTs are quasi-one-dimensional systems

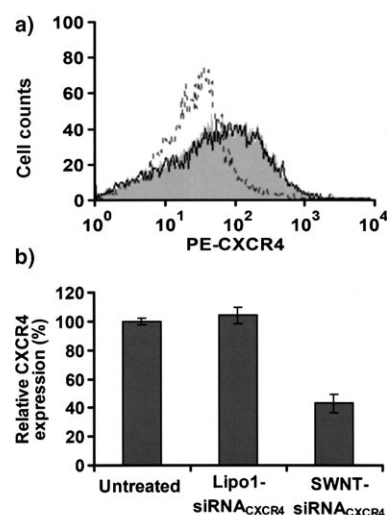


Figure 4. Delivery of CXCR4 siRNA into human primary cells by SWNTs: a) Flow cytometry data obtained with PBMC cells stained with PE-labeled anti-CXCR4; gray area: untreated control cells; solid curve: cells incubated with lipofectamine-siRNA_{CXCR4} for 3 days; dashed curve: cells incubated with SWNT-siRNA_{CXCR4} for 3 days; b) expression level of CXCR4 receptors on PBMC cells after treatment with SWNT-siRNA or lipofectamine-siRNA relative to that of CXCR4 receptors on untreated control cells.

which exhibit a strong resonance Raman shift at $\tilde{\nu} \approx 1580 \text{ cm}^{-1}$ (G band, characteristic of graphitic carbon; Figure 6a).^[20] As expected, we observed strong Raman

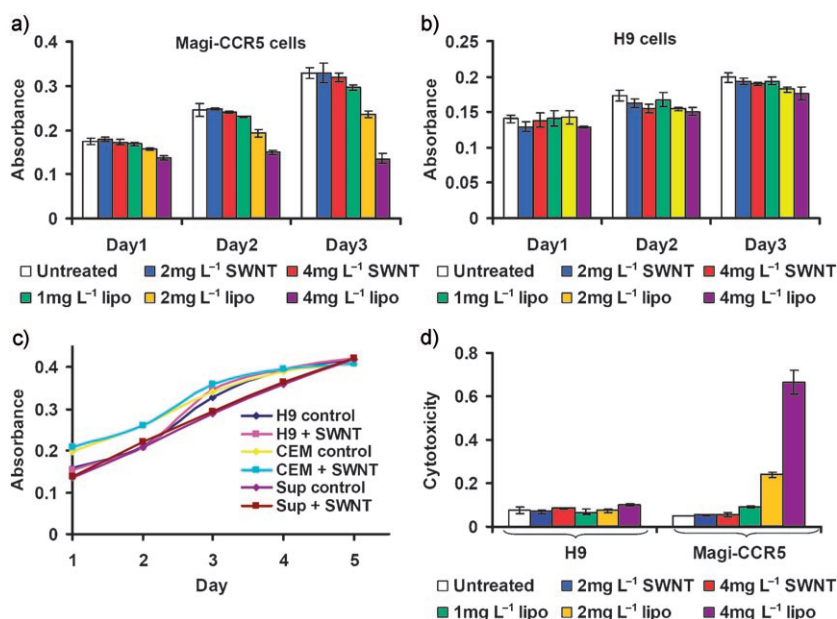


Figure 5. Proliferation and cytotoxicity assays: a), b) proliferation of Magi-CCR5 and H9 cells, respectively, after overnight incubation with SWNTs or lipofectamine2000 at the concentrations indicated; the absorbance is linearly dependent on the number of viable cells; c) proliferation assay for three T cell lines after incubation for 3 days with SWNTs (4 mg L⁻¹) functionalized with PL-PEG₂₀₀₀; no obvious difference was observed between untreated cells and cells treated with SWNTs; d) cytotoxicity assay for H9 cells and MAGI cells carried out after 3 days of incubation with the conjugates indicated; cytotoxicity values correspond to the percentage of dead cells.

signals at $\tilde{\nu} \approx 1580 \text{ cm}^{-1}$ in T cells after incubation with SWNTs functionalized with PL-PEG₂₀₀₀-NH₂ (by “Raman mapping” of single cells; Figure 6b, left panel). This spectroscopic evidence for the existence of SWNTs inside T cells is

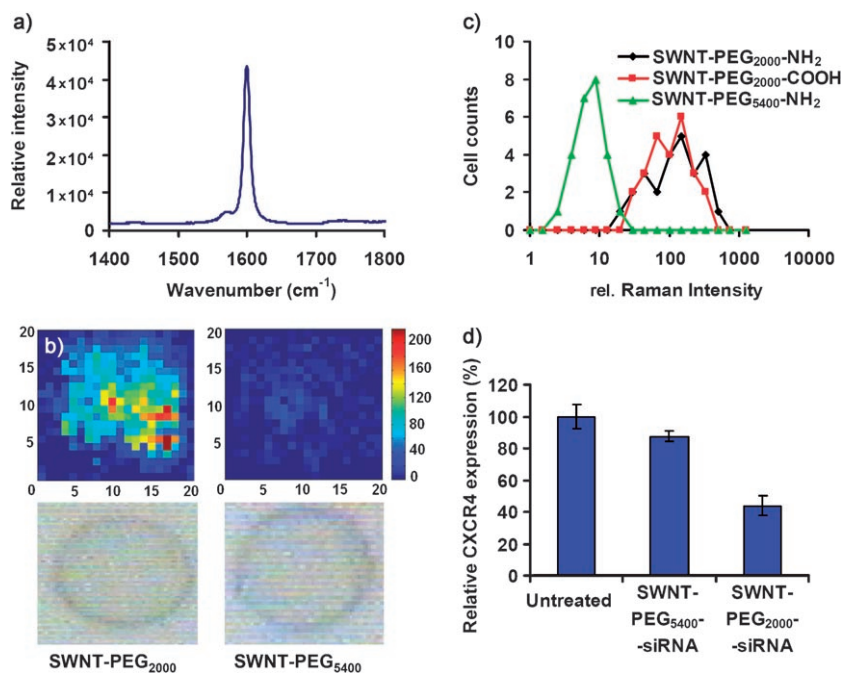


Figure 6. Functionalization dependence of SWNT uptake and molecular-delivery ability in T cells probed by micro-Raman spectroscopy: a) Raman spectrum of SWNTs made water soluble by PL-PEG₂₀₀₀; b) Raman intensity maps (top panel; colors correspond to the Raman intensities of the SWNT G bands according to the vertical bar on the right) and light-microscope images (bottom panel) of single CEM cells after incubation for 1 day with SWNTs functionalized with PL-PEG₂₀₀₀ (left) and PL-PEG₅₄₀₀ chains (right); c) Raman “cell-sorting” data; that is, the number of CEM cells (y axis) that exhibit various G-band Raman intensities (x axis) after incubation with conjugates of SWNT-(PL-PEG₂₀₀₀)-NH₂ (black), SWNT-(PL-PEG₂₀₀₀)-COOH (red), and SWNT-(PL-PEG₅₄₀₀)-NH₂ (green); the data in (b) and (c) show that much fewer SWNTs with longer PEG chains are internalized into T cells; d) expression level of CXCR4 surface receptors on untreated control CEM cells and CEM cells after incubation for 1 day with conjugates of SWNT-(PL-PEG₅₄₀₀)-siRNA or SWNT-(PL-PEG₂₀₀₀)-siRNA.

consistent with the efficient delivery of siRNA by PL-PEG₂₀₀₀-functionalized SWNTs. A similar Raman signal was observed for SWNTs inside cells when the terminal functional group on the nanotubes was changed from NH₂ to COOH (Figure 6c). In contrast, much lower Raman intensities were observed for SWNTs in T cells after incubation of the T cells with SWNTs functionalized with PL-PEG₅₄₀₀-NH₂, that is, with longer PEG chains (Figure 6b, right panel, and Figure 6c). Furthermore, we found that T cells treated with SWNT-(PL-PEG₅₄₀₀)-siRNA exhibited a significantly lower degree of RNAi than those treated SWNT-(PL-PEG₂₀₀₀)-siRNA (Figure 6d). These results reveal the reduced cellular uptake of SWNTs when functionalized with more hydrophilic and longer PEG chains.

Carbon nanotubes in as-made forms are highly hydrophobic. We suggest that our SWNTs functionalized with PEG₂₀₀₀ retain certain hydrophobicity (because of incomplete coverage of PL-PEG on the tubes), which promotes their binding and association with cells through hydrophobic

interactions with hydrophobic domains on cell membranes. The relatively long length (ca. 200 nm) of SWNTs may facilitate interactions and binding with cells, which is an important first step for cellular entry by endocytosis.^[21] When longer PEG chains are attached to render higher hydrophilicity and “inertness” (i.e., resistance to nonspecific binding to biological species),^[22,23] nanotubes exhibit reduced cellular association (Figure 6b,c), accompanied by reduced cellular uptake and RNAi (Figure 6d), as seen in our experiments. Thus, balanced chemical functionalization schemes that impart sufficient aqueous solubility to nanotubes but do not impair their ability to bind with cell surfaces are important for the development of nontoxic and efficient nanotube transporters.

In the case of immortalized cancer cells, cationic liposomes, peptides, and polymers can bind to cell-surface constituents with high surface negative charges through electrostatic forces to initiate cellular uptake and molecular delivery.^[24] Liposomes are incapable of delivery into T cells, which suggests that the electrostatic driving force for cellular binding and uptake may not be generic to all cell types. Hydrophobic interactions between nanomaterials and cell surfaces could be exploited as a more generic driving force for cellular binding and internalization. Other hydrophobic nanomaterials with suitable functionalization may also be utilized like SWNTs for molecular delivery into cells that are difficult to transfect by existing nonviral agents.

We have found that SWNTs can be used as molecular transporters for human T cells and primary cells, with superior silencing effects over conventional liposome-based nonviral agents. The cellular uptake of nanotubes has been shown to be functionalization dependent and dependent on the polyethylene glycol (PEG) chain length. Micro-Raman spectroscopic experiments on single cells and SWNT-based Raman “cell sorting” showed hydrophobic interactions to be one of the driving forces for nanotube transporters. The development of generic transporter vehicles for a wide range of cell types should facilitate the manipulation of genes and the investigation of cell functions in cell culture, with potential extensions to in vivo applications.

Experimental Section

Cell culture: T cell lines and MAGI cell lines were obtained from the NIH AIDS Reagents Program and cultured in the recommended medium. Human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density-gradient centrifugation from the blood of a patient and cultured in RPMI-1640 containing FBS (10%)

and Interleukin-2 (10 U mL^{-1}). All chemicals were obtained from Invitrogen and Aldrich.

SWNT functionalization and SWNT-siRNA conjugates: Raw Hipco SWNTs were sonicated with PL-PEG₂₀₀₀-NH₂ for 1 h, then centrifuged at 24000 g for 6 h to remove aggregates. Short functionalized SWNTs were obtained from the supernatant.^[13] PL-PEG₅₄₀₀-NH₂ functionalization was used in the Raman imaging experiment. Excess phospholipids in nanotube suspensions were removed by thorough filtration through a 100-kD filter (Millipore). Thiol-modified siRNA (Dharmacon) was attached to SWNT-(PL-PEG₂₀₀₀-NH₂) through disulfide bonds by using a sulfo-LC-SPDP (2 mM; sulfosuccinimidyl 6-(3'-(2-pyridyldithio)propionamido)hexanoate, Pierce) linker in the presence of EDC (10 mM; 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) and sulfo-NHS (10 mM; N-hydroxysulfosuccinimide, Pierce).^[13] Excess linkers and reagents were removed by filtration prior to siRNA attachment. 5'-Thiol-modified siRNAs with the following sequence were used: CXCR4 (two sequences used) sequence a: 5'-thiol-GCG GCA GCA GGU AGC AAA GdTdT-3' (sense) and sequence b: 5'-thiol-AUG GAG GGG AUC AGU AUA UdTdT; CD4: 5'-thiol-GAU CAA GAG ACU CCU CAG UdGdA-3' (sense); luciferase (scramble, used as a control): 5'-thiol-CUU ACG CUG AGU ACU UCG AdTdT-3'. Prior to conjugation, the thiolated siRNAs were treated with dithiothreitol (1 mM) for 2 h and then desalted with a Sephadex G25 MicroSpin column (Amersham).

Cellular incubation: T cells or PBMC cells were placed in 48-well plates with approximately 100000 cells per well. SWNT-siRNA conjugates were added to a SWNT concentration of 2–3 mg L⁻¹ (determined by UV/Vis-NIR absorbance^[14]) with an associated siRNA concentration of $\approx 50 \text{ nM}$ unless otherwise stated. The cells were incubated for various lengths of time and washed with PBS before analysis. Four types of liposomes, lipofectamine2000 (Invitrogen), lipofectamineRNAiMAX (Invitrogen), siPORT (Ambion), and HiPerFect (Qiagen) were used according to the instructions of the manufacturer for siRNA-transfection tests. RNAi assays by confocal and flow cytometry were carried out on the third day after initiation of the incubation.

Confocal fluorescence microscopy and flow cytometry: A Zeiss LSM 510 microscope was used for confocal fluorescence imaging. Anti-CXCR4 and anti-CD4 monoclonal antibodies labeled with phycoerythrin (PE) were used to stain the CXCR4 and CD4 receptors, respectively, on T cells prior to analysis by confocal and flow cytometry (Becton Dickinson). All cytometry data was obtained in triplicate.

Toxicity and proliferation assays: Cell proliferation and cytotoxicity assays were performed by using a CellTiter-96 one-solution kit (Promega Inc.) and a CytoTox-96 kit (Promega Inc.), respectively.

Micro-Raman spectroscopy and imaging: SWNTs exhibit strong resonance Raman bands, such as the G band at $\tilde{\nu} = 1580 \text{ cm}^{-1}$.^[16] The characteristic intensity of the Raman band can provide a measure of the relative number of nanotubes^[25] in cells as a function of chemical functionalization. Cells were incubated for 1 day with SWNTs (ca. 3 mg L^{-1}) with PL-PEG₂₀₀₀-X (X = NH₂, COOH) or PL-PEG₅₄₀₀-NH₂, washed, and resuspended in PBS. A drop of the cell suspension in PBS was sealed between two thin plastic coverslips and observed under a Renishaw Raman microscope. Raman intensity mapping over individual cells (laser beam focused down to a spot size of approximately $2 \mu\text{m}$) was performed at 1580 cm^{-1} . To obtain statistical results, the Raman intensity of the G band was averaged for 20–30 cells after incubation with differently functionalized SWNTs.

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