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Functionalization of Carbon Nanotubes via Cleavable Disulfide Bonds for Efficient Intracellular Delivery of siRNA and Potent Gene Silencing

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Much progress has been made in developing functionalization schemes for carbon nanotubes to impart solubility and chemical and biological functions to these novel 1D materials.[1−3] Relatively unexplored is the incorporation of cleavable bonds into the functionalization to enable controlled molecular releasing from nanotube surfaces, thus creating 'smart' nanomaterials with useful functionality in chemical and biological settings. For instance, intracellular delivery of biological molecules by carbon nanotube transporters[1−4] should be significantly facilitated if efficient strategies are devised to release biological cargos from nanotube sidewalls via biologically triggered bond cleavage of nanotube bioconjugates. Here, we present a novel functionalization scheme for single-walled carbon nanotubes (SWNTs) to afford nanotube−biomolecule conjugates with several interesting properties. First, the SWNT conjugates form highly stable suspensions in aqueous solutions including physiological buffers. Second, a cleavable disulfide linkage exists between the attached molecules and SWNT sidewall. Third, the cleavable functionalization scheme is general for a wide range of biological cargos including nucleic acids (DNA, RNA) and proteins.

A frontier in the field of gene and protein therapy is RNA interference (RNAi) for gene silencing by short interfering RNA (siRNA) delivered to mammalian cells.[5] Efficient intracellular transport and delivery of siRNA are critical to RNAi potency. As an application of our cleavable functionalization of SWNTs, we show transport, release, and delivery of siRNA in mammalian cells by SWNT carriers and achieve highly efficient lamin A/C gene silencing compared to existing transfection agents.

Our functionalization approach involves first making stable aqueous suspensions of short SWNTs by noncovalent adsorption of phospholipid molecules with poly(ethylene glycol) (PL-PEG, MW of PEG = 2000) chains and terminal amine or maleimide groups (PL-PEG-NH_2 or PL-PEG-maleimide, Figure 1a). The PL-PEG binds strongly to SWNTs via van der Waals and hydrophobic interactions between two PL alkyl chains and the SWNT sidewall, with the PEG chain extending into the aqueous phase to impart solubility in water (SI). The amine or maleimide terminal on the PL-PEG immobilized on SWNT can then be used to conjugate with a wide range of biological molecules. For incorporation of a disulfide bond, we employed a heterobifunctional cross-linker sulfosuccinimidyl 6-(3′-[2-pyridyldithio]propionamido)hexanoate (sulfo-LC-SPDP) for any thiol-containing biomolecule (X) to afford SWNT-PL-PEG-SS−X (**1**−X, Figure 1a). Specifically, we formed **1**−DNA (15-mer DNA with fluorescence label Cy3) and **1**−siRNA. For control experiments, we also prepared SWNT-PL-PEG−X (**2**−X, X = DNA, siRNA, Figure 1a) conjugates with no disulfide linkage by conjugating X to SWNT-PL-PEG-maleimide.

UV−vis−NIR (Figure 1b) and atomic force microscopy (AFM) were used to characterize spectroscopic and structural (length ∼50−300 nm) properties of the nanotubes (SI). To verify the disulfide linkage in **1**−X, we added dithiothreitol (DTT) to a solution of

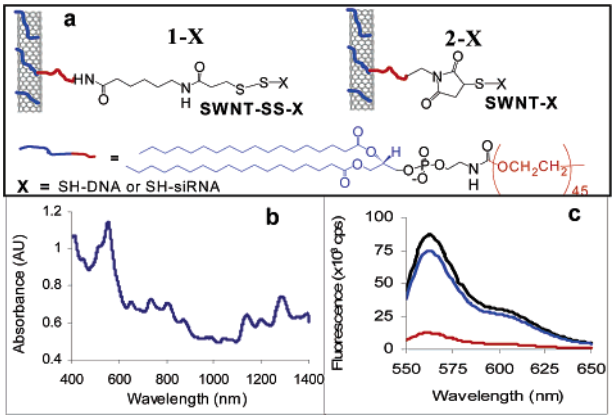

Figure 1. (a) Two schemes of SWNT functionalization by thiolated biological molecule X with (**1**−X) and without (**2**−X) disulfide bond respectively. Both DNA and RNA cargos contain a thiol functional group and a six-carbon long spacer at the 5′ end of the DNA or RNA. (b) UV−vis−NIR of a **1**−DNA solution (peak at ∼550 nm due to Cy3 label on DNA) and (c) fluorescence spectra (for Cy3 label) of a **1**−DNA and **2**−DNA, respectively, before (black) and after DTT treatment and filtration (blue for **2**−DNA and red for **1**−DNA).

1−DNA (Cy3-labeled DNA). DTT is commonly used to cleave and reduce disulfide linkage into free sulfhydryls. We treated **1**−DNA with DTT for cleaving and detaching Cy3−DNA from nanotubes, and filtered the solution through a 100 nm membrane filter to remove any free Cy3−DNA in the solution. The resulting SWNT solution exhibited a drastic decrease in Cy3-fluorescence (Figure 1c). No such reduction was observed for a similarly filtered **1**−DNA solution without DTT exposure or for **2**−DNA (no SS linkage) after similar DTT treatment and filtration. These clearly confirmed the presence of a cleavable disulfide linkage in the **1**−DNA conjugates, representing the first time such functionalization was demonstrated for SWNTs.

Next, we explored transporting biological molecules inside mammalian cells via SWNT transporters and exploited enzymatic cleavage of disulfide bonds in lysosomal compartments for releasing and delivering molecules to the cytosol to perform biological functions. We have shown that SWNTs transport various biological molecules inside living cells with no ill-effect on cell viability and proliferation[2] and have recently found that the cellular uptake is mediated by endocytosis[1−3] known to involve containment of SWNT conjugates in endosomal or lysosomal compartments upon cellular internalization. It is known that active releasing of endocytosed species from endosomes/lysosomes can allow molecule cargos to reach their intended destinations and prevent degradation inside the lysosomes.[6]

On the basis of previous work[7] we expect that upon endocytotic entry, disulfide-containing **1**−X conjugates can be cleaved by thiol-reducing enzymes aided by the acidic pH in the lysosomes. The released molecules from SWNTs could then be freed from

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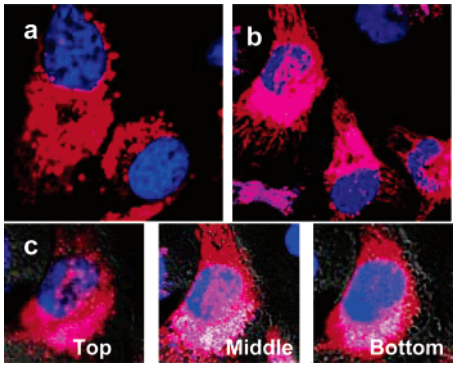

Figure 2. Confocal fluorescence images of HeLa cells (nucleus stained blue by Draq 5) after incubation in (a) **2**−DNA and (b) **1**−DNA. Red color surrounding the nucleus corresponds to Cy3-labeled DNA molecules transported inside cells by SWNT carriers. (c) Three confocal images recorded with different focal planes (top, middle, and bottom of cells) along the viewing direction for a cell shown in (b).

lysosomal lipid vesicles to reach the cell cytosol. Indeed, for HeLa cells incubated in a solution of **1**−DNA for 24 h in the presence of a blue nuclear dye (Draq 5), confocal microscopy imaging revealed colocalization of fluorescence of Cy3−DNA (red) in the cell nucleus, giving rise to pink fluorescent spots within the nucleus (Figure 2b). The colocalization was confirmed by images recorded at different focal planes along the viewing direction, as the pink fluorescence originated from the "middle" *z*-slice passing through the cell interior (Figure 2c). This result suggested the intended disulfide cleaving/releasing of molecular cargos into the cytosol and subsequent nuclear translocation of DNA. It is known that short oligonucleotide delivered to the cell cytosol can readily internalize into the cell nucleus.[8] In contrast, for **2**−DNA conjugates, we observed cellular internalization but no nuclear translocation (Figure 2a). This suggested that without the release of DNA from SWNT transporters, the **2**−DNA SWNT conjugates accumulated in the perinuclear region and were unable to penetrate through the nuclear membrane.

Having established in vitro disulfide cleavage and release of molecules from SWNTs, we extended our work to transporting and delivery of siRNA via SWNTs. We employed a siRNA known to silence the gene encoding lamin A/C protein present inside the nuclear lamina of cells.[5] Similar to the DNA case, we prepared both **1**−siRNA and **2**-siRNA. HeLa cells were incubated with **1**−siRNA ([SWNT] ≈ 10 nM or 1.5−2 mg/L and [siRNA] = 50−500 nM, [cell] = 40,000/well) for up to 24 h (in the presence of 5% fetal bovine serum), fixed 48−72 h later and stained with anti-lamin and a fluorescently labeled secondary antibody. For comparison, we also employed a commercial transfecting agent lipofectamine for siRNA delivery ([lipofectamine] ≈ 1 mg/L). Confocal imaging revealed significant reduction in lamin A/C protein expression by **1**−siRNA (weak fluorescence in Figure 3b) relative to untreated control cells (Figure 3a). Further, flow cytometry data showed that for a given siRNA concentration, the potency of RNAi or percentage of silencing followed **1**−siRNA > **2**−siRNA > lipofectamine−siRNA (Figure 3c). We attribute the higher silencing efficiency of **1**−siRNA than that of **2**−siRNA to active releasing of siRNA from SWNTs by enzymatic disulfide cleavage, which maximizes the endosome/lysosome escape of siRNA. Also, the functionality of siRNA may be less perturbed when in a free, released form than when attached to SWNT

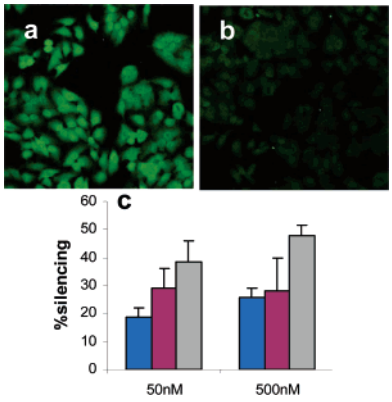

Figure 3. Confocal microscopy RNAi assay for (a) untreated control HeLa cells and (b) cells incubated with **1**−siRNA showing much weaker fluorescence than (a) due to silencing of the expression of lamin protein by RNAi. (c) Silencing efficiency of lipofectamin−siRNA (blue), **2**−siRNA (purple) and **1**−siRNA (gray) for 50 and 500 nM siRNA concentrations. The cells were fixed and stained with anti-lamin and a fluorescently labeled secondary antibody prior to analysis. The confocal images were captured at similar experimental settings for (a) and (b).

sidewalls. Our **1**−siRNA exhibits a 2-fold advantage over transfection by lipofectamine (for the same 500 nM siRNA concentration), a widely employed transfection agent. We attribute this to the high surface area of SWNTs for efficient siRNA cargo loading, high intracellular transporting ability of SWNTs, and high degree of endosome/lysosome escape owing to the disulfide approach. Note that we have also obtained excellent siRNA delivery and silencing results for the luciferase gene with SWNT transporters (Supporting Information(SI)).

In summary, we have attached various biological molecules to phospholipids functionalized SWNTs via cleavable disulfide linkage. With this novel functionalization, we have demonstrated transporting, releasing, and nuclear translocation of DNA oligonucleotides in mammalian cells with SWNT transporters. We have further shown highly efficient delivery of siRNA by SWNTs and more potent RNAi functionality than a widely used transfection agent, lipofectamine. Carbon nanotube molecular transporters are promising for various applications including gene and protein therapy.

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Supporting Information Available: Experimental procedures and other results including AFM, loading of molecules on SWNTs, stability of SWNT solutions, and luciferase silencing. This material is available free of charge via the Internet at http://pubs.acs.org.

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Supplementary Information with Supplementary Figures

Functionalization of SWNTs by phospholipids with PEG chains (PL-PEG). Hipco SWNTs were sonicated extensively (1-1.5h) in a solution of 1,2-Distearoyl-*sn*-Glycero-3-Phosphoethanolamine-N-[Amino(Polyethylene Glycol)2000] (Avanti Polar Lipids). The phospholipid molecule contained a polyethylene glycol chain (45 units) terminated with either an amine group (PL-PEG-NH_2) or a maleimide group (PL-PEG-maleimide). The typical PL-PEG concentration used was in the range of 0.1-1 mg/mL. The suspension was then filtered through a membrane filter (Whatman, pore size 100 nm) to remove excess phospholipids, rinsed thoroughly with H_2O and re-suspended in either H_2O or buffer. The suspension was centrifuged at 24,000 g for 6 h to remove impurities and large nanotubes bundles that aggregated as the sediment. The supernatant was collected and re-centrifuged under similar conditions, and the sediment was again discarded. The nanotubes present in the supernatant (after the two rounds of centrifugation) were short with tube lengths mainly in the range of 50-300 nm as revealed by AFM imaging (S_Figure 1). Both AFM imaging and UV-vis-NIR (Fig. 1b in main text) spectroscopic characterization revealed high purity of SWNTs in the final solution of SWNTs.

Preparation of 1-X (SWNT-PL-PEG-SS-X). 2.5 mg of sulfosuccinimidyl 6-(3'-[2-pyridyldithio]-propionamido)hexanoate (sulfo-LC-SPDP, Pierce) was added to a solution of SWNTs functionalized with PL-PEG-NH_2 in 50mM phosphate buffer (supplemented with 0.15M NaCl, pH=7.4). The mixture was allowed to react at room temperature for 1 h, after which the conjugate was centrifuged through a centrifugal filter with molecular weight cutoff of 100kD (Millipore) for 5 min twice to remove excess sulfo-LC-SPDP cross-linkers. The nanotube conjugates were then re-suspended in H_2O for conjugation with thiolated biological molecules including SH-DNA or SH-siRNA. The DNA sequence used in the current work was fluorescently labeled Cy3-SH-$(CH_2)_6$-CATTCCGAGTGTCCA, and the siRNA was a double stranded with sense sequence 5'-SH-$(CH_2)_6$CUGGACUUCCAGAAGAACAdTdT-3' and antisense sequence 5'-dTdT-GACCUGAAGGUCUUCUUGU-3' (no fluorescent labeling of the siRNA). The conjugation was allowed to last overnight and the resulting **1**-DNA or **1**-SiRNA solution was then ready for further experiments.

Preparation of 2-X (SWNT-PL-PEG-X). SWNTs dispersed in PL-PEG-maleimide in a phosphate buffer of pH ~ 8 were mixed with either SH-DNA or SH-siRNA and allowed to react for ~3-4 h at room temperature. The resulting **2**-DNA or **2**-SiRNA conjugates (containing no disulfide bonds) were used to compare with the **1**-counterpart with disulfide linkage.

Cellular Incubation for DNA and SiRNA transfection. HeLa cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (all reagents from Invitrogen). Cells were plated into chambered coverslides or 24 well plates 24 h prior to incubation with **1**- or **2**-SWNT conjugates at a concentration of ~8 x 10^4 cells per well.

1-DNA and **2**-DNA incubation: The SWNT conjugate was added to the chambered cover-slides containing HeLa cells in the DMEM medium supplemented with FBS and pen-strep. The concentration of SWNT and DNA was 1.5-2 mg/L and ~200 nM respectively and the concentration of serum in the incubation is determined to be ~ 5%. The cells were incubated for ~ 3h at 37 °C and 5% CO_2. After incubation, the cells were thoroughly washed and observed by confocal microscopy either immediately or up to~ 24h later.

1-SiRNA **and 2**-SiRNA incubation: Cells were plated into 24 well plates or 4 well chamber microscope slides and incubated with **1**- or **2**-SiRNA ([SWNT] ~ 1.5-2 mg/L and [siRNA]~ 50 or 500nM) for ~ 24 h at 37 °C in a 5% CO_2 environment. The serum concentration in the incubation medium is estimated to be ~2-5%. After incubation, the cells were washed and supplemented with fresh medium and allowed to incubate for an additional 24-72 h for RNAi to take effect. For confocal microscopy analysis, cells were fixed on chambered slides, and for flow cytometry analysis, cells were first trypsinized and subsequently fixed. Following cell fixation, cells were incubated with primary antibody to lamin (Abcam) and then with a fluorescently labeled secondary antibody (Aldrich). Confocal microscopy imaging and flow cytometry probed the fluorescence signals of the secondary antibody.

Lipofectamine-SiRNA incubation: Lipofectamine was purchased from Invitrogen and used according to the manufacturer's directions. Briefly lipofectamine was incubated with siRNA at room temperature for 15 min. The lipofectamine-siRNA mixture was then added to HeLa cells under the same conditions as above, with [lipofectamine] = 1mg/L and [siRNA] = 50 or 500 nM in the incubation. The cells were allowed to incubate as described above and observed either by confocal microscopy or flow cytometry.

Confocal microscopy. Adherent HeLa cells were imaged directly on the chambered cover-glass or glass slide using a Zeiss LSM 510 confocal microscope. For assessment of nuclear translocation of DNA, HeLa cells were co-stained with a blue nuclear dye, Draq 5 (Axxora) for ~ 5min prior to confocal analysis.

Cell Cytometry. After incubation in **1**-DNA or **2**-DNA, cells were washed and re-suspended in ice-cold PBS supplemented with 2% propidium iodide (PI). The mean fluorescence was measured from 10,000 cells by flow cytometry (Becton Dickinson FACScan). Cells that were co-stained with propidium iodide (corresponding to naturally occurring dead cells) were excluded from the data analysis.

For RNAi assay, cells transfected by SiRNA via SWNT carriers or Lipofecamine were first trypsinized and subsequently fixed. Following cell fixation, cells were incubated with primary antibody to lamin (Abcam) and then with a fluorescently labeled secondary antibody (Aldrich). Flow cytometry was then used to measure the mean fluorescence of 5,000 -10000- cells. The fluorescence detected was from the FITC tag on the secondary

antibody. The fluorescence measurement from fixed, untreated cells was also recorded. The percent silencing reported in Figure 3 was derived from the difference between the fluorescence intensities of the samples that were treated with siRNA and that of the control cells.

SWNT-DNA conjugates characterization.

UV-vis-NIR spectroscopy .UV-vis-NIR measurement was carried out using a Cary 6000i spectrophotometer. The distinct peaks in UV-vis-NIR spectrum for **1**-DNA shown in Fig. 1c are characteristic of highly dispersed single-walled nanotubes in the suspension. The presence of the peak around 550 nm is due to the Cy3 label on the DNA strand. Following the combined sonication, filtration and centrifugation steps, the nanotubes in the suspension were mostly individualized or small bundles of SWNTs without large aggregates or impurities in the solution.

Atomic Force Microscopy (AFM). AFM analysis of the conjugates were carried out by dropping ~20 μL of SWNT suspension onto a silicon oxide substrate. The droplet was allowed to stand for ~20 min at room temperature after which the substrate was rinsed with H_2O, dried under N_2 flow and used for AFM imaging.

Determination of number of DNA strands per nanotube. UV-vis-NIR and fluorescence spectroscopy were used to estimate the number of DNA strands conjugated to a SWNT. **1**-DNA conjugates with various loadings of DNA onto the tubes were made by conjugating a stock solution of SWNT-PEG-PL with various concentrations of cy3 labeled SH-DNA. The concentration of SWNTs was 10 nM (as determined by UV-vis-NIR absorbance spectrum of SWNTs at 808 nm with an extinction coefficient of 7.9 x

$10^6\ M^{-1}cm^{-1}$, see ref. 3). The resulting conjugates were filtered by using a centrifugal filter with MWCO 30kD to remove excess unbound cy3-DNA. The filtration step was repeated twice and the filtrates for each concentration were pooled. A standard titration curve for the absorbance (λ= 550 nm) cy3 labeled SH-DNA was determined and used to calculate the concentration of excess DNA in the filtrates from the various **1**-DNA conjugates. The data is then used to estimate the loading of DNA molecules on a SWNT. Alternatively, experiments were carried out to use the fluorescence intensity of the cy3 label ($\lambda_{excitation}$ = 545 nm and $\lambda_{emmission}$=565 nm) as a measure of DNA molecules that were in excess in the solution after conjugation reactions to SWNTs. Both the absorbance and fluorescence measurements revealed that the loading of DNA for the **1**-DNA conjugate was about ~20 DNA molecules per SWNT (average length 200nm) after conjugation reaction of 2μM DNA with ~ 10nM of SWNT used for the experiments in Fig.2b-c. Note that negligible amount of excess DNA was found in the filtrate when the original concentration of DNA used in the conjugation mixture was below 100 nM with the concentration of SWNTs being ~ 10 nM.

Stability of SWNTs functionalized by phospholipids (SWNT-PL). The typical SWNT-PL suspensions were originally made with SWNT concentration of 250 mg/L and the final concentration of short SWNT in the supernatant following the centrifugation steps was estimated to be ~25mg/L. The aqueous stability of our SWNT-PL conjugates was assessed at high temperatures and in high salt buffer solutions. In comparison with SWNT functionalized with other surfactants (tween-20, sodium dodecyl sulfate (SDS), gum arabic), SWNT-PL were the only ones that remained stable both under high

temperature (80°C) and high salt environment (0.2 M phosphate buffer, pH 7.0) (see S_Figure 2). The suspension of SWNT-PL conjugated with either DNA or siRNA was stable for weeks and even up to months when stored at 4 °C (S_Figure 2).

Ex-vitro disulfide cleavage of 1-DNA by DTT and monitoring of the cleavage by fluorescence measurement. A solution of **1**-DNA (SWNT-PL-PEG-SS-cy3DNA) (in 10 mM phosphate buffer, pH=7.0) was exposed to dithiothreol (DTT, 10 mM, Aldrich) and allowed to react for 2 h at room temperature. Following the reaction, the mixture was filtered twice through a centrifugal membrane filter (molecular weight cutoff 50KD) and re-suspended in H_2O. Control solutions of **1**-DNA and **2**-DNA not exposed to DTT as well as or **2**-DNA treated with DTT (as described for **1**-DNA) were also filtered twice and re-suspended in H_2O. Fluorescence measurements for cy3 were carried out using a Fluorolog 3 fluorimeter for all solutions to obtain Fig. 1c (excitation = 545 nm and emission = 565 nm). The fluorescence levels were normalized for the **1**-DNA and **2**-DNA samples. Note that the fluorescence signal for **2**-DNA was not significantly changed after DTT treatment since no cleavable disulfide bond exists for DNA-Cy3 conjugated to SWNTs via the maleimide covalent chemistry.

Silencing of Firefly Luciferase expression by SWNT delivered SiRNA.

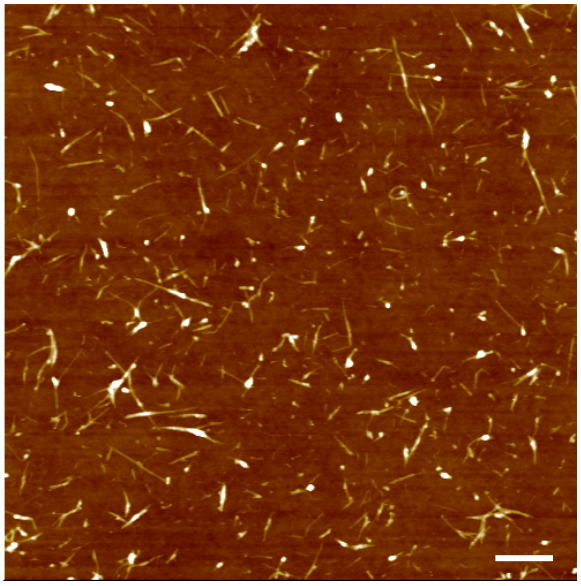
We have also obtained excellent siRNA delivery and silencing result with siRNA against the firefly luciferase gene by using SWNT transporters. This combined with the Lamin A/C silencing result proves the generality of SWNT transporters for siRNA delivery (all via the disulfide attachment) and potent RNAi functionality.

Expression of Luciferase plasmids in HeLa cells. This first step of our experiment was used to deliver plasmid DNA into cells. Plasmid DNAs (pDNA) encoding firefly luciferase (pGL3-Control, 5256 bpa) and renilla luciferase (pRL-TK, 4045 bpa) were obtained from Promega and used for the expression of luciferase inside cells. Luciferase expression was achieved by using Lipofectamine 2000 as the transfection agent for the plasmids according to the manufacturer's recommended procedure. Briefly, HeLa cells were plated in a 48-well plate (2×10^4 cells/well) for 24h until they reached 50% confluency, after which the medium was replaced with DMEM without serum. The cells were co-transfected with two luciferase plasmids (firefly luciferase, pGL3-control and renilla luciferase, pRL-TK) in the presence of lipofectamine by adding 0.1µg of pGL3 and 0.1µg of pRL-TK to each well. The final volume of each well was adjusted to 250µl. After 4-5 hours of transfection, the cell medium was adjusted such that it contained 10% FBS.

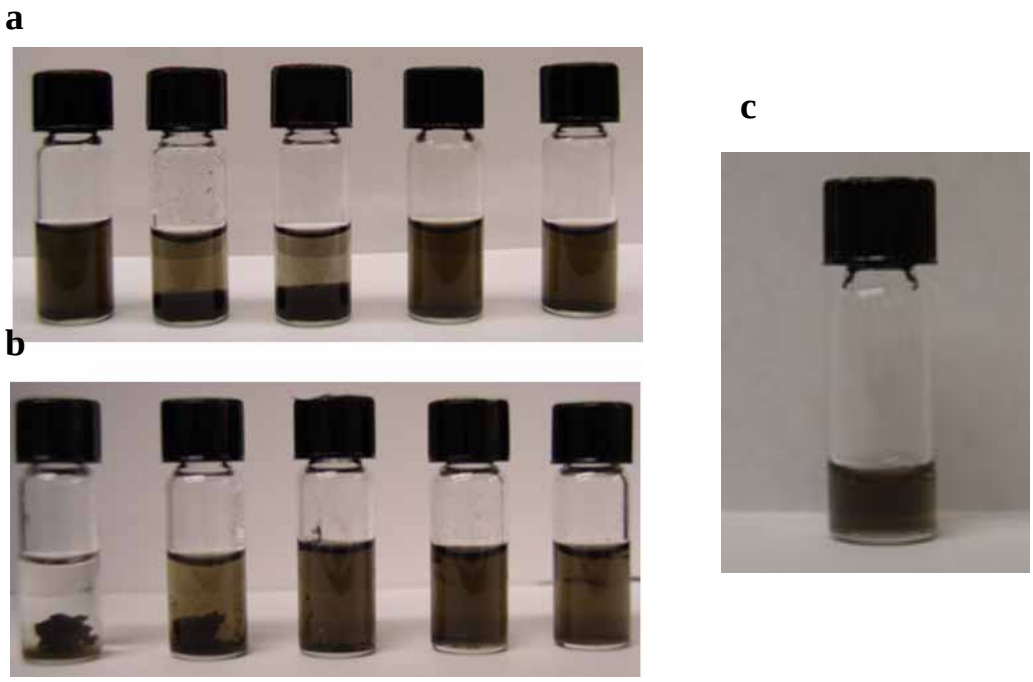
Luciferase silencing. Our RNAi study was specifically aimed at targeting the firefly luciferase gene (pGL3) by the use of a double-stranded RNA with sense sequence (5'-SH-$(CH_2)_6$ CUUACGCUGAGUACUUCGAdTdT-3') and antisense sequence (sequence 5'-UCGAAGUACUCAGCGUAAGdTdT-3'). Lipofectamine-siRNA and SWNT-S-S-siRNA conjugates were prepared as described for the lamin study. The cells that were pre-transfected by the Luciferase plasmids as described above were exposed to control siRNA (free siRNA), lipofectamine-siRNA and SWNT-PL-PEG-SS-siRNA respectively for 12 h. The cells were then washed and allowed to incubate for an additional 36 h in fresh medium. The Luciferase expression level was then investigated by using a dual Luciferase assay kit that contains substrates for both the firefly and renilla

luciferase (Promega) using a LB96V microplate luminometer (Berthold Tech.). The expression of the firefly luciferase was recorded for the cells transfected with the various siRNA conjugates and was normalized against the Renilla expression. Shown in S_Figure 3 is the expression of firefly Luciferase relative to renilla Luciferase in HeLa cells treated with siRNA alone, lipofectamine-siRNA and SWNT-PL-PEG-SS-siRNA respectively. Consistent with our data for LaminA/C RNAi, the SWNT-SS-siRNA affords excellent silencing of the firefly Luciferase in HeLa cells.

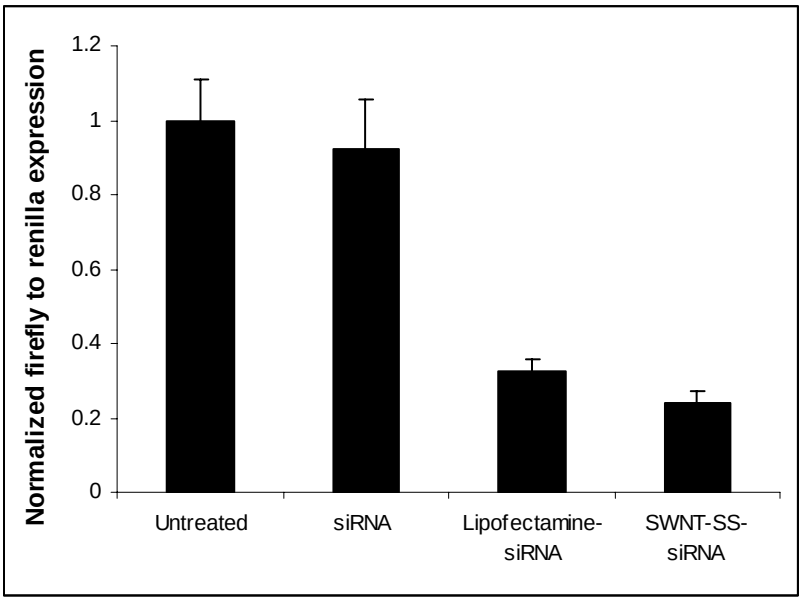
Supplementary Figures



S_Figure 1. Atomic force microscopy (AFM) image of **1**-DNA, revealing that most nanotubes were individualized or in small bundles with lengths ranging from 50-300 nm. Scale bar = 300 nm



S_Figure 2. Photographs of different SWNT aqueous suspensions. SWNT functionalized with (i) tween-20 (ii) Gum Arabic (iii) Sodium dodecyl sulfate (SDS) and (iv) PL-PEG terminated with a methyl group and (v) PL-PEG with an amine group after (a) heating to 80 °C and (b) adding 0.2 M phosphate buffer, pH 7.0 respectively. The PL-PEG functionalized SWNTs in (iv) and (v) are stable after both treatments. (c) A solution of SWNT-PL-PEG-SS-siRNA (**1**-siRNA) showing sustained stability after standing for 2 months at 4°C.



S_Figure 3. Firefly Luciferase expression assay after SiRNA treatment of HeLa cells (~20,000 cells). The graph shows normalized expression level of firefly Luciferase relative to renilla luciferase after RNAi (specific to Firefly Luciferase) induction by siRNA alone, lipofectamine-siRNA and SWNT-PL-PEG-SS-siRNA respectively. The concentration of siRNA used for these studies was 500 nM. The Luciferase expression levels were obtained by measurement of the luminescence using a dual Luciferase assay kit.