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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

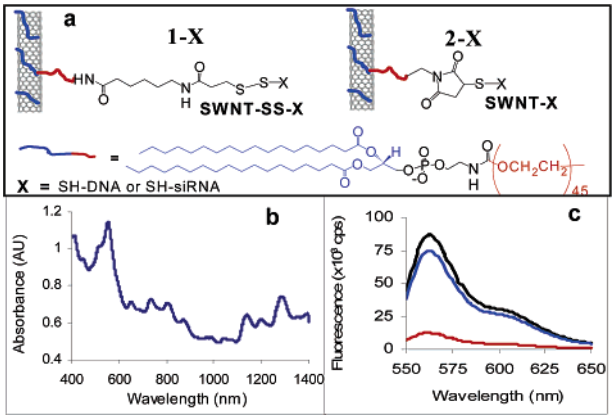

Figure 1. (a) Two schemes of SWNT functionalization by thiolated biological molecule X with (**1**−X) and without (**2**−X) disulfide bond respectively. Both DNA and RNA cargos contain a thiol functional group and a six-carbon long spacer at the 5′ end of the DNA or RNA. (b) UV−vis−NIR of a **1**−DNA 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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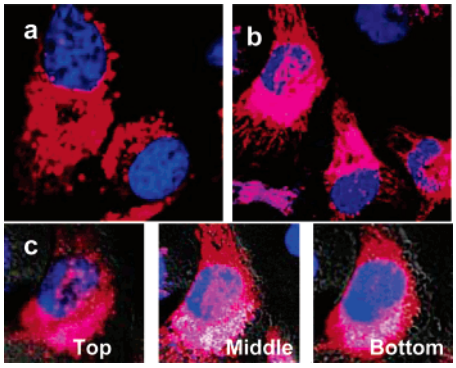

Figure 2. Confocal fluorescence images of HeLa cells (nucleus stained blue by Draq 5) after incubation in (a) **2**−DNA and (b) **1**−DNA. Red color surrounding the nucleus corresponds to Cy3-labeled DNA molecules transported inside cells by SWNT carriers. (c) Three confocal images recorded with different 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

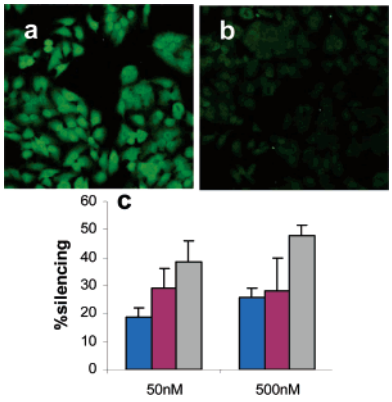

Figure 3. Confocal microscopy RNAi assay for (a) untreated control HeLa cells and (b) cells incubated with **1**−siRNA showing much weaker fluorescence than (a) due to silencing of the expression of lamin protein by RNAi. (c) Silencing efficiency of lipofectamin−siRNA (blue), **2**−siRNA (purple) and **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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