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Intracellular Delivery of Quantum Dots Tagged Antisense Oligodeoxynucleotides by Functionalized Multiwalled Carbon Nanotubes

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ABSTRACT

With the goal of identifying an improved delivery scheme for intracellular tracking and anticancer therapy, we explored a novel double functionalization of a carbon nanotube delivery system containing antisense oligodeoxynucleotides (ASODNs) as a therapeutic gene and CdTe quantum dots as fluorescent labeling probes via electrostatically layer-by-layer assembling. This is the first time that we used mercaptoacetic acid-capped CdTe quantum dots as fluorescent labeling probes for clearly tracking the intracellular transport and evaluating delivery efficiency of ASODNs by functionalized multiwalled carbon nanotubes (MWNTs).

Telomerase activity has been found in ∼85−90% of all human tumors but not in adjacent normal cells.[1−3] This makes telomerase a target not only for cancer diagnosis but also for the development of novel gene therapeutic agents. Among nucleic acid-based methods for controlling gene expression, antisense (AS) therapies are potentially powerful candidates for clinical treatments of various ailments, including cancer.[4,5] In conventional AS approaches, antisense oligodeoxynucleotides (ASODNs) designed to hybridize with target mRNA sequences down-regulate the expression of the corresponding translated proteins. Although ASODNs have emerged as an exciting and promising strategy in the field of cancer therapy in recent years,[6] their development in viable therapeutic systems has faced challenges due to their susceptibility to enzyme degradation and poor intracellular uptake. Therefore, suitable drug delivery systems are needed to develop for effectively improving cellular uptake and stability of ASODNs.[7]

Carbon nanotubes (CNTs), with unique structural, electronic, and mechanical properties, have been rapidly exploring in the biological and medical applications, especially the use of functionalization CNTs as carriers of bioactive molecules and drugs.[8−24] Furthermore, semiconductor quantum dots (QDs) with unique optical properties have recently received tremendous attention for biological labeling and long-term tracking of biological processes.[25−28] The aim of the work described herein is to explore a novel double functionalization of multiwalled carbon nanotubes (MWNTs) delivery scheme via electrostatically layer-by-layer assembling. This strategy enabled to simultaneously link anticancer agents, ASODNs, as the therapeutic active molecules as well as fluorescent probes, semiconductor quantum dots (CdTe), to the MWNTs for tracking the uptake of material (Scheme 1). To the best of our knowledge, it is the first time that we used mercaptoacetic acid-capped CdTe quantum dots as fluorescent labeling probes for clearly tracking the intracellular transport and evaluating delivery efficiency of ASODNs by functionalized MWNTs.

The combined treatment of strong acids and sonication is known to reduce the CNTs length and generate anionic groups (mainly carboxylate) along the sidewalls and ends of the nanotubes.[29−31] In this work, we prepared a series of oxidized MWNTs by applying different duration of oxidation. The MWNTs-length distribution was assessed by transmission electron microscopy (TEM) (Figure 1). For efficient loading and intracellular delivery of ASODNs, we selected MWNTs acid-treated for 16 h, i.e., about 50−150 nm of the nanotube length, as the suitable drug carriers.

As solubility under physiological conditions is a key prerequisite to make CNTs biocompatible, four types of cationic polyelectrolytes, polyethylenimine (PEI), poly(diallyldimethylammonium) chloride (PDDA), poly(amidoamine) (PAMAM) dendrimers, and Chitosan were used to modify oxidized MWNTs to obtain functionalized MWNTs (f-MWNTs), respectively. The properties of oxidized MWNTs

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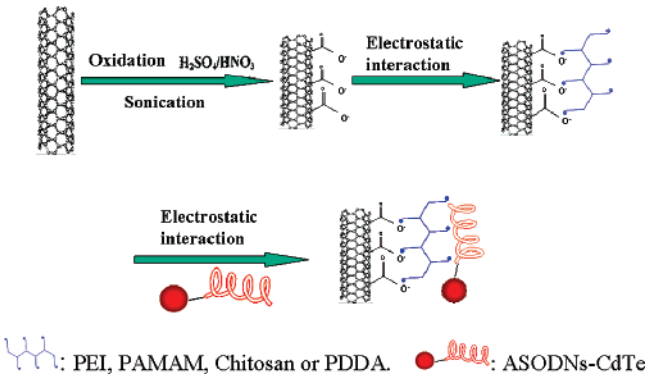
Scheme 1. Approach and Mechanism for Preparing MWNT−PEI−ASODNs−CdTe



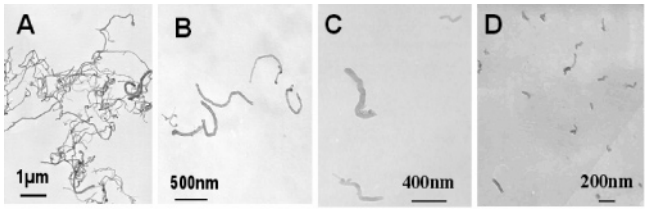


Figure 1. TEM image of (A) pristine MWNTs; MWNTs treated with sulfuric acid/nitric acid for (B) 4 h, (C) 8 h, (D) 16 h.

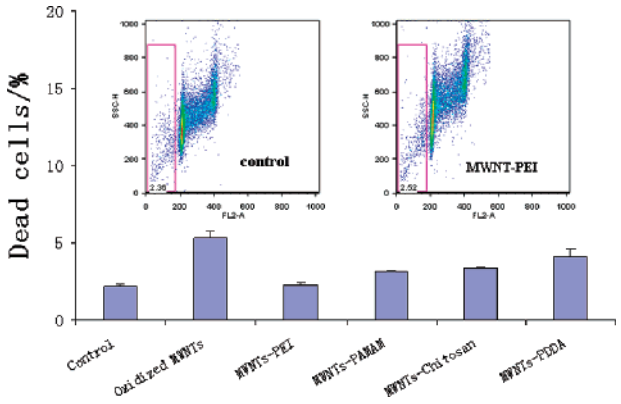


Figure 2. Percentage of dead HeLa cells after treatment with either oxidized MWNTs or f-MWNTs for 1 h. HeLa cells ($2-3 \times 10^5$ cells/well) were either left untreated or incubated with 1.25 mg/L of oxidized MWNTs or f-MWNTs. After incubation and washings, the cells were stained with propidium iodide (PI) and analyzed by flow cytometry (Inset: each blue point corresponds to a single cell. The right quadrant corresponds to living cells, the left quadrant corresponds to apoptotic cells).

and f-MWNTs were characterized by FTIR spectra (see Figures S1−S2 in the Supporting Information). These results clearly show that highly stable aqueous suspensions of f-MWNTs were obtained via electrostatic interaction between the $-COO^-$ of oxidized MWNTs and the positively charged cationic polyelectrolytes backbone.

To assess the biological properties of oxidized MWNTs and f-MWNTs, we initially studied the cytotoxicity effects of oxidized MWNTs and f-MWNTs on Human cervical cancer HeLa cells. As shown in Figure 2, the cationic polymers modified MWNTs clearly reduced the toxic effects compared with the oxidized MWNTs. Especially, nearly all the cells were observed to survive upon treatment with PEI

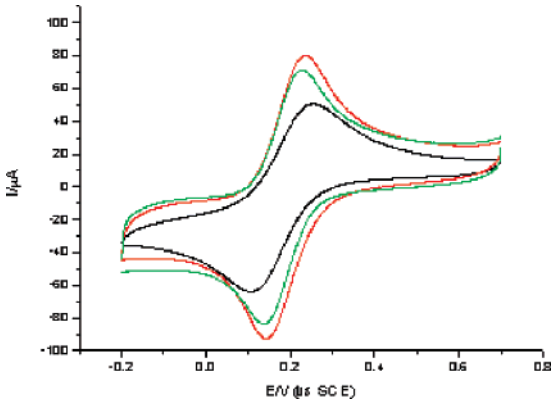


Figure 3. Cyclic voltammograms of the MWNTs/GC-modified electrode (light black), the PEI−MWNTs/GC-modified electrode (light green), and the CdTe−ASODNs−PEI−MWNTs/GC-modified electrode (light red) in 5 mM $K_3Fe(CN)_6$/0.1 M KCl solution at a scan rate of 80 mV/s, respectively.

functionalized MWNTs, showing a minimum level of cell death similar to the untreated cells. These results indicate that f-MWNTs interacted with cationic polymers, and even the nanotubes themselves exhibit little toxicity to HeLa cells. It is well documented that polyethylenimine (PEI) is a versatile vector that could greatly enhance the efficiency of gene transfer in vitro and in vivo.[32,33] Therefore, we selected MWNTs−PEI as a therapeutic gene (i.e., ASODNs) delivery vector.

Sequently ASODNs were allowed to interact with positively charged amine groups on PEI−MWNTs. Such interaction leads to varying degrees of ASODNs condensation depending on the charge density, thus it is pivotal to quantify the density of amine groups from PEI on MWNTs surface. The density of amine groups was determined by a colorimetric titration method involving their reaction with a UV-sensitive reagent (4-nitrobenzaldehyde).[34] The reaction between amine and aldehyde groups under anhydrous conditions generates an imine group, which may be hydrolyzed back to the precursors in a known volume of water to produce 4-nitrobenzaldehyde, whose absorbance can be measured by UV spectroscopy at 267 nm (see Scheme S3 in the Supporting Information). The absorbance measurements revealed that the density of amine groups on MWNTs was about 66.33 μmol/g. Combining this with the data of the surface area of MWNTs (Supporting Information), the loading of amine groups on MWNTs was 0.715 μmol/ m^2.

To obtain a double functionalization of therapeutic gene delivery scheme, ASODNs (20-mer, modified with amino group on the 5′ end) were first labeled with mercapoacetic acid-capped CdTe on the 5′ end by amide reaction. The negatively charged CdTe fluorescent-tagged ASODNs were then anchored to the surface of the positively charged PEI−MWNTs through the electrostatic interaction.

As cyclic voltammetry (CV) in electrochemistry is often used as an effective method for monitoring the layer-by-layer assembling process,[35−37] we explored the application of CV in qualitatively investigating the availability of the electrostatic interaction among PEI, ASODNs−CdTe, and oxidized MWNTs. In Figure 3, a comparison between the

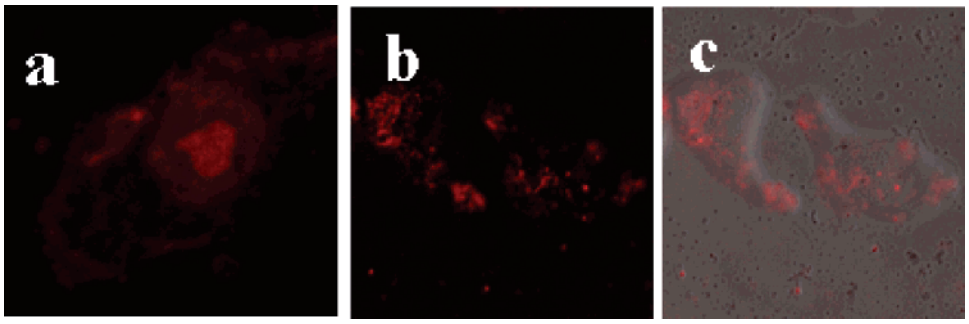


Figure 4. Confocal fluorescence images of HeLa cells after incubation in solutions of (a) MWNTs−PEI−ASODNs−CdTe, (b) MWNTs−PEI−CdTe for 3h at 37 °C, (C) merged image of (b).

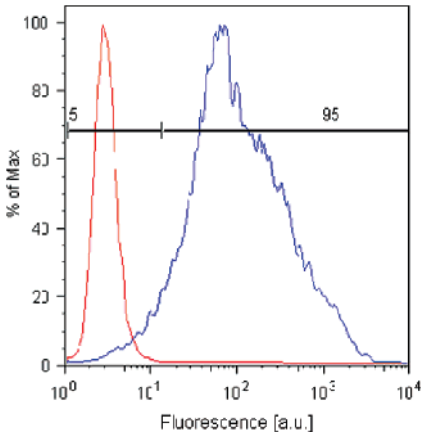


Figure 5. Flow cytometry of cells incubated with MWNTs−PEI−ASODNs−CdTe (blue curve) as compared to unlabeled cells (red curve) demonstrates the ability to quantify the delivery efficiency of MWNTs−PEI for ASODNs delivery in HeLa cells. After washings, the cells were analyzed by flow cytometry. The fluorescence was detected from the CdTe tagged on ASODNs.

CVs shows that the difference in potential between the anodic and cathodic peaks for ferricyanide (ΔE_p) is 127 mV for the oxidized MWNTs/GC-modified electrode, 90 mV for the PEI−MWNTs/GC-modified electrode and 93 mV for the CdTe−ASODNs−PEI−MWNTs/GC-modified electrode. Furthermore, the electrochemical response current at the CdTe−ASODNs−PEI−MWNTs/GC-modified electrode is much larger when compared to the oxidized MWNTs-modified electrode and PEI−MWNTs/GC-modified electrode. These results suggest that the PEI and the ASODNs−CdTe were successively adsorbed to the MWNTs by electrostatic interaction. It can be explained that the positively charged PEI polymer coating on the surface of the MWNTs facilitate the electron transfer of $Fe(CN)^{3-/4-}$ redox couple, and further adsorption of negatively charged CdTe−ASODNs relatively restricted the electron transfer of this redox couple, whereas their layer-by-layer coatings on the surface of MWNTs mainly increase the charging current.

A combination of confocal microscopy and flow cytometry enabled the qualitative and quantitative assay of MWNTs−ASODNs−CdTe delivery into the HeLa cells. HeLa cells incubated with either MWNTs−PEI−ASODN−CdTe or MWNTs−PEI−CdTe for 3 h were analyzed by using confocal microscopy. Figure 4 clearly shows that both MWNTs−PEI−ASODNs−CdTe and MWNTs−PEI−CdTe have entered into the cell cytoplasm from the observation of the CdTe fluorescent signal (red) within cells. Interestingly, the confocal microscopy imaging reveals that the localization of intense fluorescence of CdTe-labeled ASODNs−PEI−MWNTs is mainly in the cell nucleus, whereas the CdTe-labeled MWNTs−PEI is mainly localized at the cell cytoplasm. This result suggests that after transported inside the cell cytosol by the MWNTs−PEI carriers, ASODNs was almost entirely delivered into cell nuclei for inhibiting the target mRNA of telomerase. It could be ascribed to its special characteristic of strong nucleus localization of ASODNs as reported.[38,39] Therefore, we used flow cytometry to further evaluate the delivery efficiency of PEI−MWNTs for ASODNs delivery by CdTe quantum dots as fluorescence labeling. Figure 5 shows the delivery efficiency of MWNTs−PEI−

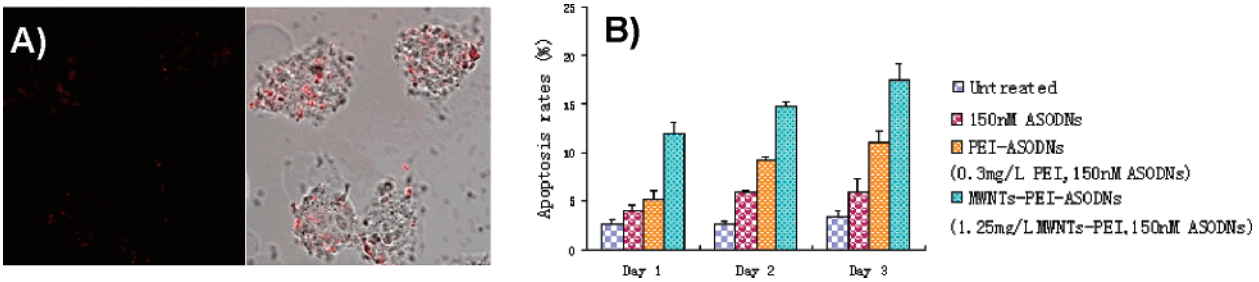


Figure 6. (A) Confocal fluorescence image of HeLa cells taken 24 h after 1 h long incubation with MWNTs−PEI−ASODNs−CdTe at 37 °C (left), and merged image (right). (B) Flow cytometric analysis. After culturing the cells in the presence of naked ASODNs, PEI−ASODNs, and MWNTs−PEI−ASODNs, cells were harvested, washed in PBS, fixed in 1% paraformaldehyde, permeabilized with 96% ethanol, RNase treated, and then stained with propidium iodide (50 μg/mL). The cells were allowed to analyze at 24 h interval for a period of 72 h.

ASODNs−CdTe is 95%, indicating that the amine-terminal PEI−MWNTs had a vast increase in delivery efficiency for ASODNs. Furthermore, in order to further evaluate the gene-transfer efficiency of MWNTs−PEI, we also carried out delivering the enhanced green fluorescence protein (EGFP) gene, pEGFPN1, with the MWNTs−PEI transporters into cells (see Figure S5 in the Supporting Information). As comparison, cells were also incubated with either PEI−pEGFPN1 or naked pEGFPN1. The data demonstrate that MWNTs−PEI−pEGFPN1 exhibit much higher EGFP-transfection efficiency than PEI−pEGFPN1 and naked pEGFPN1, suggesting that MWNTs−PEI could efficiently deliver the EGFP gene into cells and the protein was successfully expressed in transfected cells. Therefore, the above results show that PEI−MWNTs could be utilized as efficient gene delivery vectors, which were attributed to the stable f-MWNTs conjugates preventing DNA from enzyme degradation and the enhanced proton-sponge effects of PEI coating on the surface of MWNTs.[32]

Next, we monitored cellular apoptosis induced by the ASODNs transfected with PEI−MWNTs. Confocal microscopy imaging (Figure 6A) reveals extensive cell death when examined 24 h after the hour-long incubation with PEI−MWNTs−ASODNs−CdTe. Furthermore, we carried out flow cytometry to quantify the cell apoptosis efficiency after incubating HeLa cells with MWNTs−PEI−ASODNs, PEI−ASODNs, and naked ASODNs, respectively, for different periods of time (24−72 h). As shown in Figure 6B, the cells transfected with MWNTs−PEI−ASODNs, PEI−ASODNs, or ASODNs showed increased cell apoptosis with time elongation. Especially, HeLa cells treated with MWNTs−PEI−ASODNs show much higher cellular apoptosis than those treated with PEI−ASODNs or naked ASODNs, indicating the enhanced cellular delivery of ASODNs by functionalized nanotube transporters can much more effectively inhibit telomerase activity and subsequently induce the cell apoptosis, which can be attributed to the high surface area and high intracellular delivery efficiency of PEI−MWNTs for efficient ASODNs loading and transporting.

Finally, we also investigated the cellular uptake mechanism for MWNTs−PEI−ASODNs by using CdTe quantum dots as fluorescent tagging. By confocal microscopy imaging (see Figure S6 in the Supporting Information), we observed that MWNTs−PEI−ASODNs−CdTe entered the HeLa cells after incubation for 1 h at 37 °C. The strong red fluorescence corresponds to CdTe labels on MWNTs−PEI−ASODNs inside HeLa cells. However, very weak red fluorescence could be detected after incubation for 1h at 4 °C, showing very little uptake of MWNTs−PEI−ASODNs inside cells. This therefore indicates the endocytosis mechanism[8,24] for the cellular uptake of MWNTs−PEI−ASODNs at 37 °C.

In summary, we have prepared a novel double functionalization of carbon nanotube delivery scheme containing therapeutic gene and QDs labeling, CdTe tagged ASODNs-f-MWNTs, via electrostatically layer-by-layer assembling. With this novel functionalization, we have demonstrated efficient intracellular transporting, strong cell nucleus localization and high delivery efficiency of ASODNs by the PEI−MWNTs carriers. Furthermore, the ASODNs bound to PEI−MWNTs show their effective anticancer activity. Therefore, these functionalized MWNTs could hold great promising for biological delivery applications and gene therapy.

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Supporting Information Available: Materials and methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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