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Controllable Delivery of Small-Molecule Compounds to Targeted Cells Utilizing Carbon Nanotubes

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Supporting Information

ABSTRACT: Carbon nanotubes (CNTs) have emerged as a new alternative and efficient tool for transporting molecules with biotechnological and biomedical applications, because of their remarkable physicochemical properties. Encapsulation of functional molecules into the hollow chambers of CNTs can not only stabilize encapsulated molecules but also generate new nanodevices. In this work, we have demonstrated that CNTs can function as controllable carriers to transport small-molecule compounds (SMCs) loaded inside their hollow tunnels onto targeted cells. Using indole as model compound, CNTs can protect indole molecules during transportation. Labeling indole-loaded CNTs (indole@CNTs) with EphB4-binding peptides generates cell-homing indole@CNTs (CIDs). CIDs can selectively target EphB4-expressing cells and release indole onto cell surfaces by near-infrared (NIR) irradiation. Released indole molecules exhibit significant cell-killing effects without causing local overheating. This establishes CNTs as excellent near-infrared controllable delivery vehicles for SMCs as selective cell-killing agents.

Progress in understanding the biological interactions of carbon nanotubes (CNTs) has led to demonstration of their potential application in biotechnology and biomedicine.[1−3] The ultrahigh surface area of carbon nanotubes allows for the loading and the shuttling of various biomolecules into cells,[4−6] and lately their hollow tunnels have been utilized to encapsulate chains of atoms, organic molecules, inorganic crystals, and DNA.[7−10] Due to the unique structure and high crystallinity, both single-walled and double-walled carbon nanotubes (SWNTs and DWNTs) have become the focus of much research interest in these areas. Recent studies have suggested that DWNTs have advantages over SWNTs or MWNTs (multiwalled carbon nanotubes) in various applications because their mechanical properties, thermal conductivity, and structural stability are likely to be superior owing to their coaxial structure.[11,12]These CNTs can transform noninvasive near-infrared (NIR) light into heat,[13,14] opening up new venues not only for direct thermal ablation of cancer cells as demonstrated with SWNTs,[15,16] but also for controllable molecule transportation. Very recently, a molecular dynamics study supported the idea that optical heating of CNTs can assist in releasing encapsulated drugs.[17] Here we show that CNTs can function as molecule transporters for controllably delivering small molecules (SMCs) through NIR excitation (Figure 1).

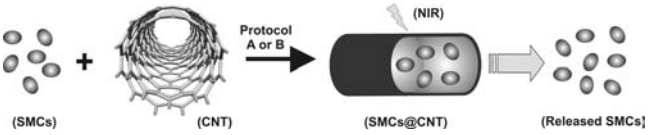

Figure 1. Encapsulation of SMCs into CNTs generates NIR-controllable molecule transporters as potential drug delivery systems.

We selected indole as a model SMC as it can disrupt cell membranes and inhibit cell growth at enriched concentrations.[18] Indole derivative-based libraries have been widely used for screening cancer drugs.[19] The indole moiety of tryptophan exhibits high affinity for CNTs.[20,21] Therefore, CNTs encapsulated with indole (i.e., indole@CNTs) can be a feasible system for evaluating the function of CNTs as potent molecule transporters to deliver SMCs to targeted cells. In this study, as-purified CNTs were prepared by density gradient centrifugation (Supporting Information [SI]), and characterized with Raman microscopy, atomic force microscopy (AFM), and high-resolution transmission electron microscopy (HRTEM). It was found that the as-purified CNTs contained a mixture of SWNTs and DWNTs, but the majority were DWNTs (Figures S1 and S2 [SI]). The indole@CNTs were prepared utilizing two protocols which were modified from the molten-phase method[7] and the nanoextraction technique using ethanol[22] or supercritical CO_2.[23] Briefly, CNTs were first functionalized by acid treatment,[24] and functionalized CNTs (f-CNTs) were mixed with excess indole molecules. The mixtures were subjected to heating and/or high-pressure treatments with one of the two following protocols. Protocol A: Mixtures were rinsed in ethanol in a tightly closed vial at 70 °C for 12 h; and Protocol B: Mixtures were rinsed in supercritical CO_2 (1500 psi) at 70 °C for 12 h (see [SI] for details). Encapsulation of indole molecules in indole@CNTs was confirmed using a complex approach of Raman and FT-IR attenuated total reflectance (ATR) spectroscopies, which have already been successfully applied to identify C60 within CNTs.[25] Figure 2a compares the Raman spectra of indole@CNTs with those of indole and CNTs. As a result, the encapsulated indole molecules in CNTs were detectable. To ensure that these indole molecules were not ones absorbed on the outside surfaces of CNTs, FT-IR ATR spectroscopy was utilized to detect any indole absorbed on CNTs. As indicated in Figure 2b, indole molecules absorbed on the outside surface of CNTs could be washed away. Furthermore, indole fluorescence quenching experiments confirmed indole encapsulation within the

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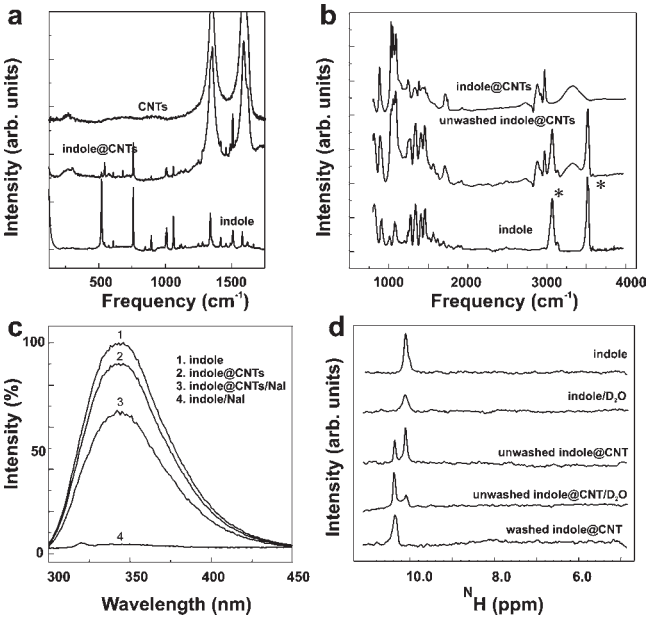

Figure 2. Confirmation of indole encapsulation inside CNTs prepared using Protocol B. (a). Raman spectra of CNTs (top), indole@CNTs (middle), and indole (bottom) were recorded with an excitation at 532 nm. (b). ATR spectra of indole@CNTs were compared with those of unwashed indole@CNTs and indole. Unique indole peaks (*) disappeared in the spectrum of the indole@CNTs after thorough washing. (c). Fluorescence spectra were compared for indole (0.3 μM)/CNT mixtures and indole@CNTs containing 0.3 μM indole, respectively, in the absence or in the presence of 1 M NaI. (d). One-dimensional ^{15}N-HSQC spectra were recorded for ^{15}N-indole, unwashed ^{15}N-indole@CNTs, and ^{15}N-indole@CNTs.

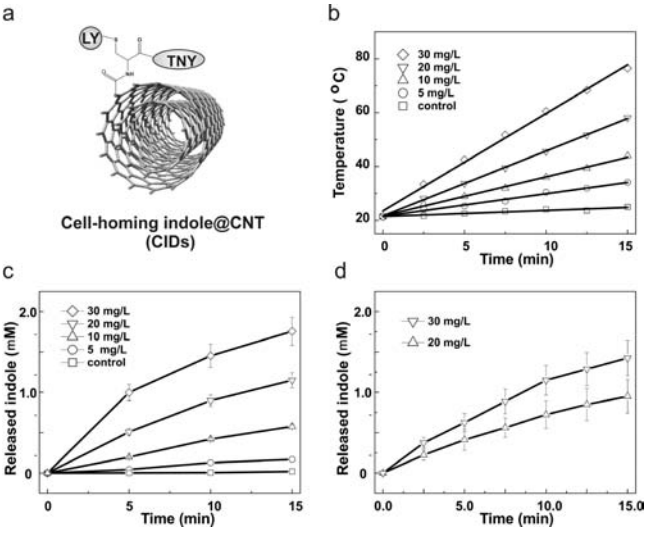

Figure 3. Release of indole molecules from CIDs through NIR irradiation in vitro. (a) The construct of the cell-homing indole@CNTs (CIDs). Each CID was composed of CNTs, an EphB4-binding peptide (TNY), and Lucifer yellow (LY). Indole molecules are not shown for clarity. (b) CIDs burst solution temperature through continuous irradiation at different CID concentrations. The values of R^2 for these linear fitting are > 0.996. (c) Concentration of released indole molecules in solution increased with continuous NIR irradiation. (d) Concentration of released indole in solution following pulsed NIR irradiation at 37 °C.

indole@CNTs when excess NaI was used as quenching agent (Figure 2c). The fluorescence of the indole molecules absorbed on the surface of CNTs were completely quenched by 1 M NaI. The indole encapsulated in indole@CNTs was protected from interaction with NaI. Indole encapsulation in indole@CNTs was also evidenced using hydrogen−deuterium (H−D) exchange experiments monitored by NMR spectroscopy, as CNTs protected the encapsulated N−H proton from H−D exchange with deuterated solvent. To enhance the NMR signal of the N−H proton, ^{15}N-indole@CNTs were prepared, and their N−H signals in one-dimensional (1D) ^{15}N-HSQC (heteronulear single quantum coherence) spectra (Figure 2d) were significantly enhanced, compared with the 1D ^{1}H spectra (Figure S3 [SI]). It is notable that the N−H proton resonance of encapsulated ^{15}N-indole molecules was downfield-shifted by 0.27 ppm (Figure 2d), possibly due to the molecular interaction between indole and CNT. The amount of encapsulated indole in indole@CNTs was spectroscopically quantitated and both aforementioned protocols gave efficient encapsulation (Figures S4 and S5 [SI]). As a result, the indole@CNTs prepared by Protocols A andj B contained 3.5 ± 0.8 and 7.6 ± 0.6 mg indole/mg CNTs, respectively. Thus, in further experiments, only indole@CNTs prepared utilizing Protocol B were used. In this work, we used L-ascorbic acid (L-AA) as a control, as L-AA is hydrophilic. L-AA was encapsulated into CNTs using Protocol B. The amount of encapsulated L-AA in L-AA@CNTs was determined to have 8.9 ± 0.7 mg/mg CNTs (Figure S5 [SI]). Though it is not fully clear what factors determine encapsulation efficiency, it is likely that molecule solubility in sCO_2 plays a contributing role in encapsulation (see SI for more discussion).

In order to facilitate indole@CNTs delivery to target-specific cells, indole@CNTs were labeled with a cell-homing peptide, the amino acid sequence of which was Thr-Asn-Tyr-Leu-Phe-Ser-Pro-Asn-Gly-Pro-Ile-Ala-Arg-Ala-Trp (TNY). The TNY peptide, previously selected using phage display techniques, exhibited high affinity to the EphB4 receptor ($K_d \approx 15$ nM).[26] The receptor has been identified as a common tumor suppressor in breast, colorectal, and prostate cancer.[31] Therefore, the TNY−EphB4 interaction should enable TNY-labeled indole@CNTs to target cells expressing the EphB4 receptor. For this purpose, the TNY peptides were conjugated with indole@CNTs with EDC and sulfo-NHS (SI). In order to visualize indole@CNTs by fluorescence microscopy, the TNY-labeled indole@CNTs were also labeled with a fluorescence probe, Lucifer yellow (LY), through introduction of an additional amino acid residue of Cys at the N-terminal of the TNY peptide (SI). The amount of TNY peptide on each CNT was determined to be in a ratio of 1:8 (CNT:TNY). Indole@CNTs attached with LY-labeled TNY were designated CIDs, and their construction is described in Figure 3a.

To evaluate how efficient CIDs can unload encapsulated indole molecules by NIR irradiation, four different concentrations of CID suspensions (i.e., 5 mg/L, 10 mg/L, 20 mg/L, and 30 mg/L) in 1 mL of cell culture medium were placed in a 3 mL quartz cuvette. Note that these concentrations referred to CNT mass. Each sample was irradiated with an 808 nm NIR laser at 1.2 W/cm^2 for 15 min (Figure S6 (SI). Upon NIR irradiation, CIDs in aqueous suspensions were very efficient at converting NIR energy into heat. At a given concentration of CIDs, solution heating was observed to be linear over time (Figure 3b). Control experiments indicated that the heating rates of buffer

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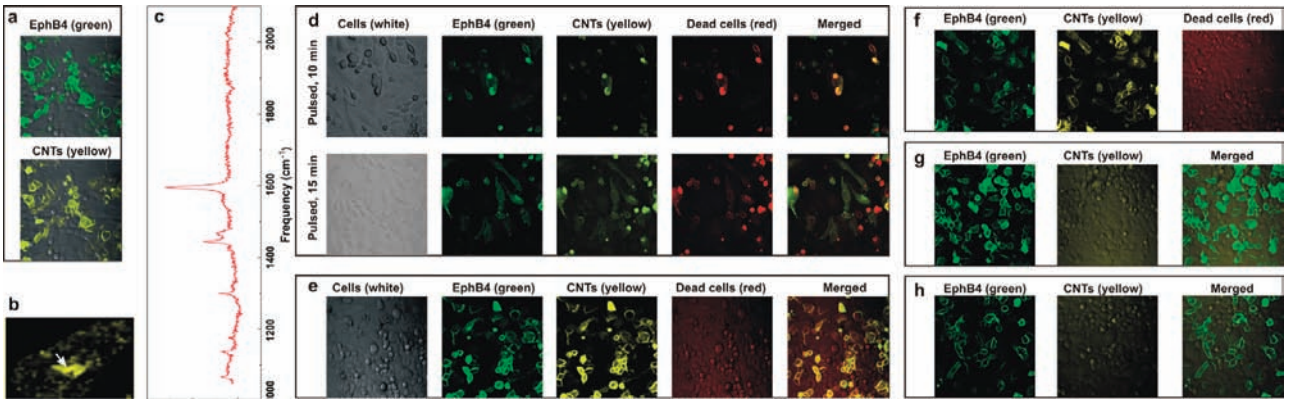

Figure 4. Microscopic evaluation of the effects of CIDs and controls on EphB4-expressing HeLa cells. The cells were treated with or without NIR irradiation for 15 min (except where mentioned). Biomarkers (i.e., EphB4), CNTs, and dead cells are visualized in green, yellow, and red, respectively. (a) Cells expressed GFP-tagged EphB4 on their surfaces (top), and CIDs bound to the expressed EphB4 (bottom). (b,c) CNTs bound on a cell surface were characterized with Raman spectrometry. Raman spectrum in panel c was measured on the spot labeled by the arrow in panel b. (d) CIDs released indole on EphB4-expressing cells upon NIR irradiation. The cells were treated with pulsed NIR irradiation for 10 min (top panels) and 15 min (bottom panels), respectively, in the presence of 20 mg/mL CIDs. (e) EphB4-expressing cells were irradiated for 15 min in the presence of 20 mg/mL CADs. (f) CIDs lacking indole in CNTs (i.e., CDs) bound but were not harmful to EphB4-expressing HeLa cells with NIR irradiation. (g,h) EphB4-expressing cells were treated with indole@CNTs or nCID, respectively.

alone and indole solution alone were 0.02 and 0.05 °C/min, respectively. The fractional heating provided by CNTs was calculated to be 3.41 °C/min at 20 mg/L of CIDs. The high concentration of CIDs in solution could significantly raise solution temperature. For instance, NIR irradiation on the solution containing 30 mg/L of CIDs caused rapid temperature increases in bulk solution to over 50 °C within 8 min (Figure 3b). As shown in Figure 3c, such NIR irradiation could efficiently release indole molecules from the tunnels of CNTs. For a given irradiation time, the higher the concentration of CIDs the solution contained, the higher temperature the irradiated CNTs generated, and the greater the amount of released indole that was observed. Therefore, a desired indole concentration in solution could be achieved by appropriate control of the CID concentration and the NIR irradiation duration. Considering that heating is caused only by NIR absorption on CNTs, it is likely that molecule diffusion is the predominant process for releasing the encapsulated molecules. A recent theoretical study by Chaban et al. on the effect of heating on the release of encapsulated molecules from CNTs support our experimental data, indicating that optical heating of CNTs can assist in releasing encapsulated molecules.[27]

Nevertheless, the continuous NIR irradiation could overheat the cell media and damage living cells, although it could unload indole molecules efficiently. CIDs would be more useful if NIR irradiation could be utilized to control the unloading of the encapsulated molecules from CIDs without overheating the solution. Previously, Kam et al. demonstrated that the use of pulsed NIR irradiation could release noncovalently attached DNA from the surfaces of CNTs without causing cell death.[14] In this study, we aimed to examine whether pulsed NIR irradiation could also drive internalized indole molecules to egress from CIDs without overheating the surrounding solution. Such experiments were undertaken at 37 °C. Two concentrations of CIDs (i.e., 20 and 30 mg/L) were examined with a 10-s pulsed NIR irradiation for 15 min, and the amount of released indole was quantitated thereafter. As a result, the use of pulsed NIR irradiation could achieve more than 80% of the yield observed by use of continuous NIR irradiation. The 10-s pulsed NIR irradiation on 20 mg/L of CID solution released as much as 0.95 ± 0.087 mM indole into the solution within 15 min (Figure 3d). Increasing the CID concentration to 30 mg/L released more indole molecules into solution (1.43 ± 0.076 mM). At both CID concentrations, after pulsed irradiation for 45 min, 90% of encapsulated indole molecues could be released, while it took at least 15 min to achieve the same yield with continuous irradiation. However, at the higher CID concentration as well as for longer irradiation times, the solution temperature was significantly altered and increased over 41 °C by the end of the NIR irradiation. On the other hand, we did not observe release of indole from CNTs by incubation of CIDs at either 25 or 37 °C for 30 min (data not shown), suggesting that indole/CNT formed stable composites. Therefore, the CID concentration of 20 mg/L and the 10-s pulsed NIR irradiation time of 10 min were used to examine the selectivity and toxicity of CIDs on cell culture in order to maintain the temperature of the solution at 37 °C.

To evaluate the selectivity of CIDs for cell-targeting and killing, EphB4 tagged with green fluorescence protein (EphB4−GFP)[28] was expressed on HeLa cells as a biomarker (Figure 4a. top). Through TNY−EphB4 interactions, CIDs that bound to the surface of HeLa/EphB4 cells were monitored by LY fluorescence (Figure 4a, bottom). CNTs bound on cell surfaces were further confirmed with Raman spectrometry, as indicated in Figure 4b,c. When HeLa cells were transfected with limited EphB4−GFP plasmids, Eph−GFP proteins were discriminately expressed on different cells (Figure 4d). These cells were then incubated with CIDs for 30 min, and washed with culture medium to remove unbound CIDs. The cells were irradiated by a 10-s pulsed NIR laser for 10 or 15 min. After 12 h, the EphB4-expressing cells significantly changed their morphology, while those cells with less or no EphB4−GFP biomarkers on their surfaces were insignificantly affected (Figure 4d). These results indicated that CIDs targeted EphB4-expressing cells and released indole molecules to targeted cells to cause cell death. When we replaced the indole inside CIDs with L-ascorbic acid as a control (designated CADs), and such constructs bound to EphB4-expressing cells but were not harmful to the cells when irradiated with NIR (Figure 4e). In other control experiments, CIDs lacking indole (designated CDs) also exhibited high-

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selectivity to EphB4-expressing HeLa cells similar to that of CIDs (Figure 4f), but these cells remained healthy even after NIR irradiation for 15 min. Control experiments were also done with indole@CNTs as well as a variation of CIDs which had their cell-homing peptides replaced with a non-EphB4-binding peptides (nCIDs, Table S2 [SI]). Neither indole@CNTs (Figure 4g) nor nCIDs (Figure 4h) exhibited cell-killing effects on EphB4-expressing cells, as they were unable to bind to cells.

Cell death caused by indole molecules released from the indole@CNTs system were further evaluated by measurement of the ADP:ATP ratio in cell cultures, as a high ADP:ATP ratio indicates the occurrence of cell apoptosis related to cell death. Cell death was detected only for cells targeted by fully functional CIDs (see Figure S7 [SI]).

In summary, the possibility of packing small-molecule compounds (indole molecules in this study) into CNTs has been demonstrated. Analyses of the Raman, FT-IR ATR spectroscopy and fluorescence quenching assays as well as ^{15}N HSQC NMR spectroscopy results have confirmed that indole molecules form a condensed phase within CNTs. Additionally, indole molecules inside CNTs have been shown to be driven from the CNTs by use of NIR irradiation. The current work has also demonstrated that these indole-packed CNs (indole@CNTs) can serve as controllable vehicles to deliver indole molecules to targeted cells in combination with a cell-homing agent and NIR irradiation. Locally released indole molecules exhibited significant cell-killing effects. Combined with previous investigations[15,16] which showed NIR pulses can induce local heating of CNTs for cell-killing, our results suggest that drug-loaded CNTs are potent cell-killing agents with selectivity and controllability. Nevertheless, encapsulation of other small-molecule compounds into CNTs will generate new functional nanomaterials with novel properties.

■ ASSOCIATED CONTENT

Supporting Information. All experimental procedure, additional data and discussion. This material is available free of charge via the Internet at http://pubs.acs.org.

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