

A Carbon Nanotube Optical Sensor Reports Nuclear Entry *via* a Noncanonical Pathway

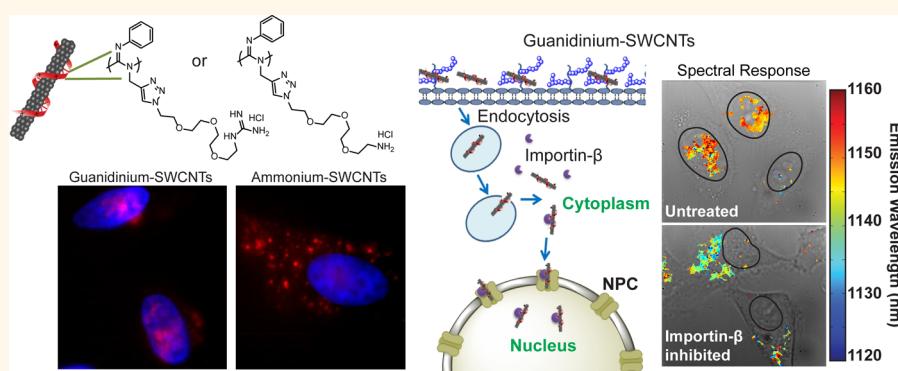
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S Supporting Information



ABSTRACT: Single-walled carbon nanotubes are of interest in biomedicine for imaging and molecular sensing applications and as shuttles for various cargos such as chemotherapeutic drugs, peptides, proteins, and oligonucleotides. Carbon nanotube surface chemistry can be modulated for subcellular targeting while preserving photoluminescence for label-free visualization in complex biological environments, making them attractive materials for such studies. The cell nucleus is a potential target for many pathologies including cancer and infectious diseases. Understanding mechanisms of nanomaterial delivery to the nucleus may facilitate diagnostics, drug development, and gene-editing tools. Currently, there are no systematic studies to understand how these nanomaterials gain access to the nucleus. Herein, we developed a carbon nanotube based hybrid material that elucidate a distinct mechanism of nuclear translocation of a nanomaterial in cultured cells. We developed a nuclear-targeted probe *via* cloaking photoluminescent single-walled carbon nanotubes in a guanidinium-functionalized helical polycarbodiimide. We found that the nuclear entry of the nanotubes was mediated by the import receptor importin β without the aid of importin α and not by the more common importin α/β pathway. Additionally, the nanotube photoluminescence exhibited distinct red-shifting upon entry to the nucleus, potentially functioning as a reporter of the importin β -mediated nuclear transport process. This work delineates a noncanonical mechanism for nanomaterial delivery to the nucleus and provides a reporter for the study of nucleus-related pathologies.

KEYWORDS: helical polymer, hyperspectral imaging, cervical cancer, near-infrared sensor, biosensor, polycation, nuclear pore complex

The cell nucleus, nuclear transport receptors, and the nuclear pore complex (NPC) are potential therapeutic targets for diverse pathologies including cancer and infectious diseases.¹ The targeted delivery of exogenous macromolecular materials such as transcription factors and nucleic acids for gene therapy to the nucleus is currently inefficient² and challenging to engineer in part because macromolecule entry into the nucleus is tightly regulated by the NPCs,³ which are typically 20–50 nm in diameter⁴ and allow transit of import cargoes presenting active nuclear localization signals (NLSs), commonly arginine, and lysine-

rich short peptide sequences derived from nuclear and viral proteins.⁵

Several strategies have been applied, with mixed success, to localize macromolecules within the cell nucleus. One such strategy involves conjugating canonical NLS peptides to nanomaterials.^{6–8} Guanidine, the functional side chain in arginine, is a small moiety that can be integrated into molecular probes with minimal structural perturbations and has been

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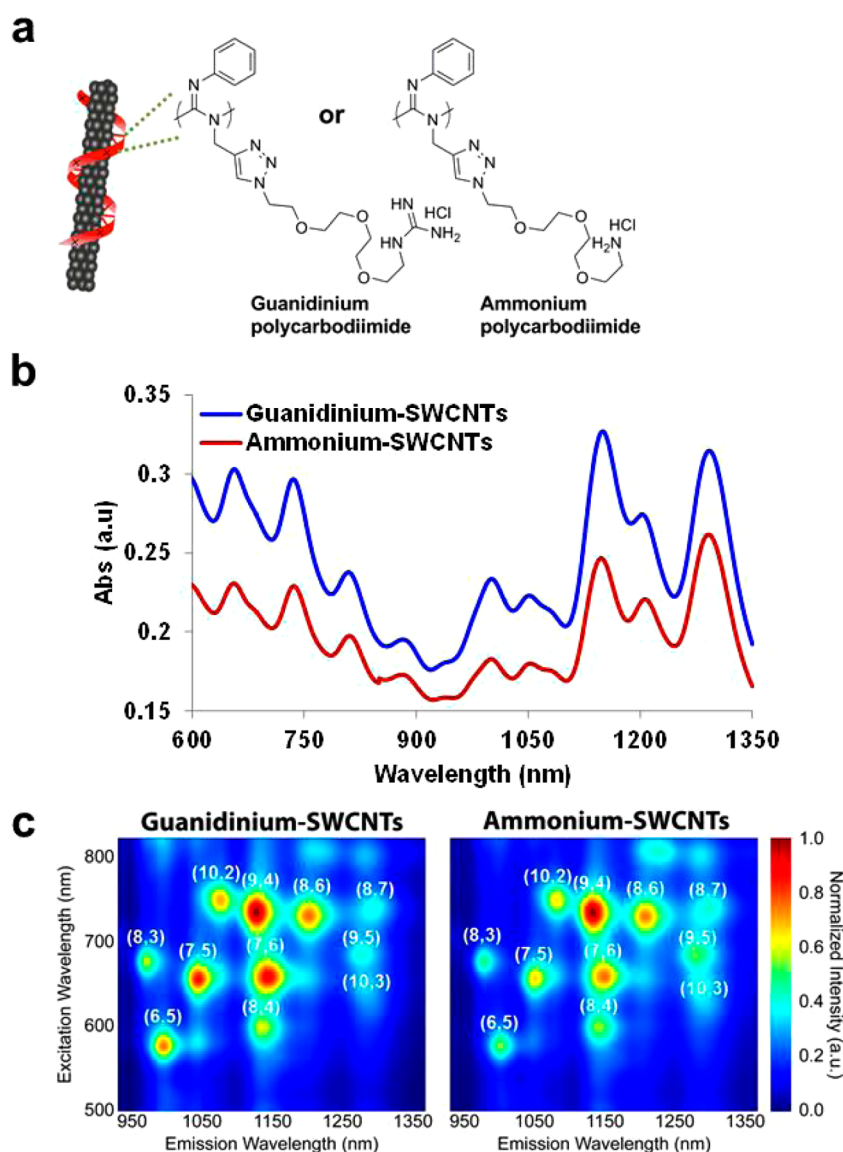


Figure 1. Cationic helical polycarbodiimide-cloaked single-walled carbon nanotubes. (a) Chemical structures of two polycarbodiimide polymers used for noncovalent encapsulation of the carbon nanotubes. (b) Vis–NIR absorbance spectra of aqueous suspensions of guanidinium– and ammonium–nanotube complexes. (c) Normalized photoluminescence excitation–emission (PL) plots of guanidinium– and ammonium–nanotube complexes.

widely incorporated in synthetic drugs and drug carriers, including polymers and nanoparticles, to improve cell uptake.⁹ Guanidine-functionalized oligomers and small polymers have been found to translocate to the cell nucleus in cultured cells,¹⁰ making guanidine a potential class of noncanonical nuclear localization signals to facilitate nuclear delivery. Noncanonical NLSs may enhance stability, obviating problems with the *in vivo* use of peptide-based targeting moieties, which are susceptible to enzymatic degradation and immunogenic responses. Currently, the mechanisms by which nonpeptide based synthetic nanomaterials are trafficked to the cell nucleus, including guanidine-functionalized materials, are unknown.

Nucleo-cytoplasmic transport of macromolecules often involves karyopherin β (importin β), a major nuclear import receptor that acts as a chaperone for highly charged nuclear proteins.¹¹ Structural analyses show that importin β has a large degree of flexibility that it uses to import cargoes directly or, more commonly, through adaptor proteins such as importin

α .¹² Most nucleoproteins incorporate a canonical NLS peptide sequence that binds to adaptor proteins to form a complex.¹³ The most common (canonical) mechanism¹⁴ involves a heterodimer, importin α/β , which binds to NLS-bearing cargoes resulting in translocation of the importin/cargo complex through the NPC.¹⁵ Although some proteins and viruses have been found to translocate from the cytoplasm to the nucleus facilitated by importin β without the aid of importin α ,^{16,17} this noncanonical mechanism of nucleocytoplasmic transport is relatively underexplored. Importin β is overexpressed in cervical, ovarian, and esophageal cancers,¹⁸ among others, and its function is also involved in the internalization of viruses,¹⁷ making it a potential therapeutic target in cancers and viral infections.

Single-walled carbon nanotubes (SWCNTs) are multifaceted nanomaterials with mechanical, photophysical, and thermal properties which are of interest for applications such as nanocarriers, bioimaging tools, and molecular sensors.^{19,20}

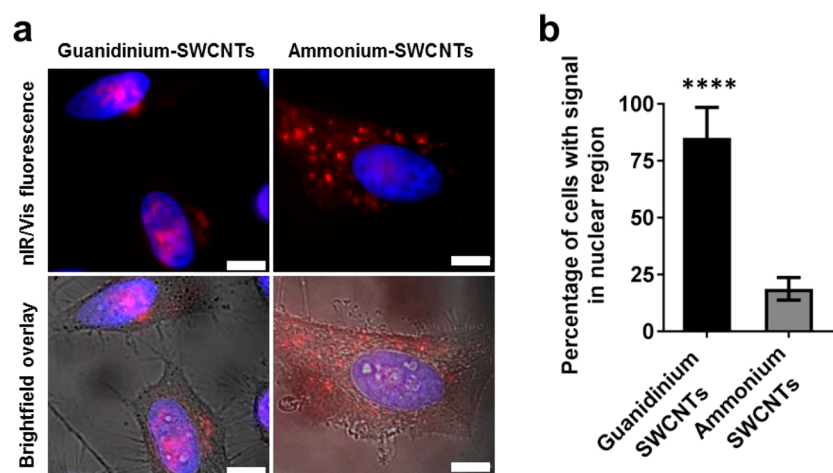


Figure 2. Subcellular localization of polycarbodiimide-single-walled carbon nanotube complexes. (a) Fluorescence and brightfield overlay images of polymer–nanotube complexes in HeLa cells/nuclei. Red = carbon nanotube near-infrared emission, blue = Hoechst 33258 nuclear stain. (b) Quantification of nuclear localization of guanidinium–polymer complexes and ammonium–polymer complexes. Graph represents mean $\pm$ standard deviation from three replicate experiments. Scale bars = 10 μ m. **** $P < 0.0001$ ($P < 0.05$ significant).

SWCNTs exhibit photostable, intrinsic photoluminescence in the near-infrared (NIR) spectral window²¹ where there is minimal absorption, autofluorescence, and scattering of light in biological specimens.²² Therefore, SWCNTs are promising for the study of cellular transport processes. Importantly, nanotube photoluminescence also undergoes spectral changes mediated by redox and pH,^{23,24} solvent dielectric,^{25,26} and electrostatic charge,²⁷ potentiating nanotube-based optical sensors²⁸ in complex biological environments. A large surface area provides opportunities for multivalent functionalization for subcellular targeting and molecular loading for targeted delivery.²⁹ Nanotubes have been used to deliver various molecular cargos including chemotherapeutic drugs, oligonucleotides, peptides, and proteins inside live cells.¹⁹ Recent studies have demonstrated that surface functionalization can modulate nanotube physicochemical behavior and biological interactions, including cytotoxicity.²² Carbon nanotubes have been targeted *via* nuclear protein fragments containing NLS peptide sequences³⁰ and, in their absence, observed to localize to the nucleus in certain cases.³¹ As with other nanomaterials, the mechanisms underlying the nuclear transport of carbon nanotubes remain elusive.³²

In our previous work, we developed polycarbodiimide polymer derivatives with functional moieties³³ that can effectively cloak and suspend SWCNTs in aqueous media, resulting in photoluminescent complexes that are stable under ambient conditions for several months.³⁴ Here, we developed functional derivatives of this class of materials and present a polycarbodiimide–nanotube complex that functions as a nanoscale probe to efficiently target the cell nucleus, discern its mechanism of nuclear transit, and report the mechanism *via* an optical response. Although carbon nanotubes have been made to localize in the nucleus, their mechanisms of nuclear translocation and optical response upon entering the nucleus were unexplored. We found that our probe, a photoluminescent single-walled carbon nanotube encapsulated in a multimeric guanidine-functionalized helical polycarbodiimide, translocated to the cell nucleus through the nuclear pore complex (NPC) *via* a distinct mechanism mediated by the importin β transport receptor without the aid of importin α . Upon entering the nucleus, the probe exhibited a distinct red-shift in its emission

wavelength, thus functioning as a sensor for nuclear localization *via* importin β -mediated translocation through the NPC, potentiating an optical reporter for this cellular process.

RESULTS AND DISCUSSION

We synthesized helical polycarbodiimide polymers³⁴ to non-covalently suspend single-walled carbon nanotubes in aqueous media. The polymers contained phenyl groups to promote adhesion to the graphitic carbon sidewall of the nanotube *via* π – π and hydrophobic interactions and either (1) guanidine or (2) amine side chains to mimic arginine and lysine, respectively (Figure 1a and Supporting Figure S1). Such amino acids are common in cell-penetrating and NLS peptide sequences.⁵ The polymer–SWCNT complexes were water-soluble and stable under ambient conditions without visual aggregation over several months. Atomic force microscopy (AFM) images suggested that the polymers “cloak” the nanotubes (Supporting Figure S2) similarly to ssDNA.³⁵ The polycarbodiimide–nanotube complexes exhibited spectral features (Figure 1b, 1c) characteristic of SWCNTs produced by the HiPco method²¹ and positive surface potentials consistent with the charge of the polymers (Supporting Figure S3).

Human cervical cancer cell lines, HeLa and SiHa, were interrogated with the polycarbodiimide–nanotube complexes. Under standard cell culture conditions, the guanidinium–nanotube complexes exhibited substantial uptake and preferential accumulation in the cell nucleus (Figure 2a and Supporting Figure S4). Approximately 92% of SiHa cell nuclei and 84% of HeLa cell nuclei were positive for NIR emission from the nanotubes. Under the same conditions, the ammonium–nanotube complexes also exhibited high cell uptake but minimal nuclear localization (Figure 2b). Ammonium functional moieties are known to be less efficient in facilitating endosomal escape of nanomaterials compared to guanidine functional groups, potentially contributing to the reduced nuclear localization of ammonium–SWCNTs.³⁶ The nuclear translocation of the guanidinium polycarbodiimide-functionalized nanotubes over the ammonium functionalization apparently mimicked behavior seen with peptides.³⁷

Cells treated with the guanidinium–nanotube complexes were monitored *via* near-infrared microscopy to evaluate the

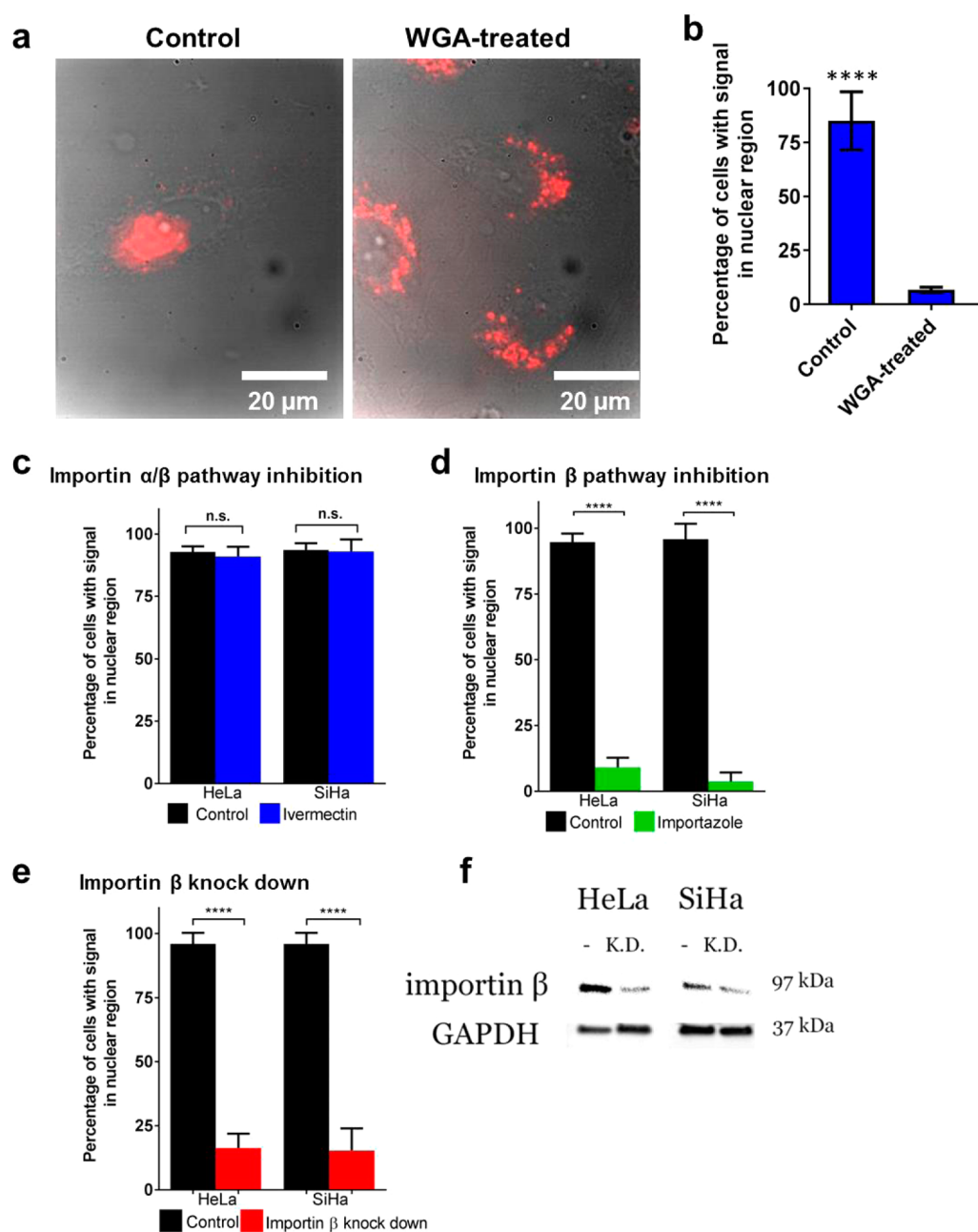


Figure 3. Mechanistic studies of nanoprobe nuclear translocation. (a) Near-infrared emission/brightfield overlay images of HeLa cells upon treatment with wheat germ agglutinin (WGA) and guanidinium–nanotube complexes. (b) Graph quantifying nuclear localization from the experiment. (c) Nuclear localization of nanotubes in cells treated with ivermectin, a pharmacological inhibitor of importin α/β -mediated nuclear import, quantified from NIR fluorescence images. (d) Nanotube localization in cells treated with importazole, an inhibitor of importin β -mediated nuclear import. (e) Nanotube localization in cells after knockdown of importin β via shRNA transfection. (f) Western blot analysis of the shRNA-treated cells. Graphs represent mean $\pm$ standard deviation from three replicate experiments. **** $P < 0.0001$ ($P < 0.05$ significant).

timing of nuclear localization. After incubation, the guanidinium–nanotubes associated with the cell membranes, appearing as puncta (Supporting Figure S5). After approximately 4 h, the nanotubes began to localize in the cell nucleus and accumulation continued over 24 h. The guanidinium–nanotube complexes became motionless upon nuclear localization, whereas the ammonium–nanotube complexes were mobile (movies S1–S3).

As proteoglycans are the primary receptors on cell surfaces for many cationic macromolecular cargos, including polyarginine derivatives,³⁸ we investigated whether guanidinium–

nanotube complexes would associate with proteoglycans on the cell surface to facilitate internalization. We deactivated proteoglycans by chemical treatment with sodium chlorate³⁹ prior to addition of guanidinium–nanotube complexes to the cells. The treatment distinctly reduced the uptake of the complexes (Supporting Figure S6), suggesting that proteoglycans mediated their uptake.

We assessed whether nanotubes gain entry to the nucleus via NPCs by inhibiting this pathway with wheat germ agglutinin (WGA). Inside cells, WGA binds to carbohydrates on nuclear pore complex proteins and cross-links nucleoporins, limiting

entry of macromolecules *via* the NPCs.⁴⁰ We pretreated the cells with WGA to block NPCs before administering guanidinium–nanotube complexes to cells incubated in WGA-free cell culture medium. Under these conditions, the nuclear translocation of nanotubes was attenuated by up to 92% compared to control cells that did not receive WGA treatments (Figure 3a, 3b), suggesting that the nanotubes entered the nucleus *via* NPCs.

Inside cells, the nanotubes presumably escape the endolysosomal pathway, most likely by membrane disruption in endosomes/lysosomes due to guanidine-rich sequences.³⁶ As certain materials that cause such disruption often lead to cytotoxicity,⁴¹ cell viability was evaluated upon treatment with the guanidinium–nanotube complexes. The assays found that the complexes conferred no discernible cytotoxicities (Supporting Figure S7).

To determine whether nanotube translocation followed the canonical importin α/β pathway, we treated cells with ivermectin, a pharmacological inhibitor of importin α/β -mediated nuclear transport.⁴² Nuclear entry was not significantly blocked in ivermectin-treated cells compared to control cells (Figure 3c and Supporting Figure S8). Ruling out the importin α/β pathway, we then explored whether importin β alone resulted in nuclear translocation of the nanotubes. Treatment of the cells with importazole, an inhibitor of importin β -mediated nuclear translocation,⁴³ caused a significant reduction in nuclear localization (Figure 3d and Supporting Figure S9). We further confirmed this mechanism by knocking down importin β expression. Cells were transfected with shRNA against importin β before administering the nanotube complexes. The knockdown resulted in attenuated nuclear localization of the nanotubes by 83–84% compared to cells that were transfected with an empty vector, according to imaging studies and consistent with the pharmacological inhibition experiment (Figure 3e and Supporting Figure S10). Importin β knockdown was confirmed by Western blot analysis (Figure 3f). These results imply that guanidinium–nanotube nuclear import is mediated by importin β without the aid of importin α .

Based on our findings, we propose a model for nuclear translocation of the guanidinium–nanotube complexes (Figure 4). The complexes enter the cells *via* endocytosis facilitated by proteoglycans. The complexes then escape the endolysosomal pathway to enter the cytosol, where the nanotubes interact with importin β . The importin β then facilitates guanidinium–nanotube translocation *via* the nuclear pore complex, without the aid of importin α .

We assessed the optical response of the guanidinium–nanotube complexes in the nuclear environment. Using near-infrared hyperspectral microscopy,⁴⁴ we collected spectral information from the nanotube complexes in the live cells and spatially resolved the spectral data. Upon measuring the nanotube emission, focusing on the (9,4) species, we found that the emission was red-shifted approximately 11 nm in the nucleus, compared with nanotubes in aqueous solution (Supporting Figure S11). Upon interrogation with WGA and importazole to inhibit nuclear localization, the emission wavelength did not red shift and remained similar to the values in solution (Figure 5a,b). The reagents themselves, WGA and importazole, did not cause significant changes in nanotube emission (Supporting Figure S12).

We hypothesized that the highly charged character of chromatin may play a role in modulating the emission of the

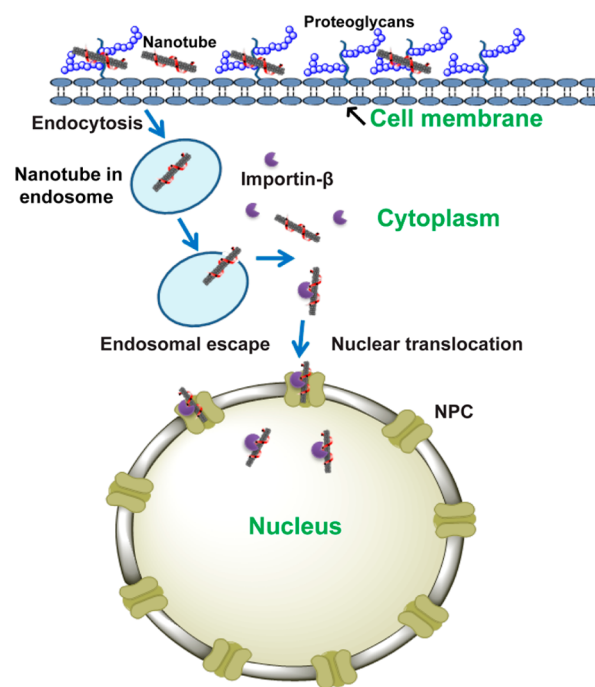


Figure 4. Model for proposed mechanism of nanoprobe translocation to the nucleus. The nanotube complex enters the cell *via* endocytosis upon binding to proteoglycans on the cell membrane. The nanotube then escapes the endosome and binds to importin β . The importin– β –nanotube complex then transits the nuclear membrane *via* the NPC.

carbon nanotubes upon entry to the nucleus. Previous work found that carbon nanotubes, once in the nucleus, can directly interact with DNA.³⁰ We also recently discovered that increased anionic charge due to polyelectrolytic biomacromolecules in the local environment of carbon nanotubes can result in distinct red-shifting of nanotube emission.²⁷ To test whether DNA, found predominantly in the nucleus, could potentially cause a similar modulation of the optical bandgap, we introduced the guanidinium–nanotube complexes to salmon sperm DNA in solution. The DNA resulted in red-shifting of the nanotube emission by approximately 6 nm (Supporting Figure S12). To attempt to recapitulate the magnitude of the shift in nuclei, the mixture of salmon DNA and nanotube complexes was centrifuged, yielding a pellet. The emission wavelength from the pellet was 12 nm red-shifted as compared to the emission from nanotube complexes in the absence of DNA. The resulting emission wavelength of 1148 nm under these conditions was comparable to that in cell nuclei. This experiment suggests that the interaction of the guanidinium–nanotube complexes with DNA in the nucleus may mediate the spectral response. In the case where the aggregates were pelleted *in vitro*, it is currently unclear whether the increased nanotube contact with DNA or the aggregation itself caused an exacerbation of the red-shifting, and thus, further studies on the exact nature of this shift, and the nanotube–biomolecule interactions that cause it in the nucleus, are warranted.

CONCLUSIONS

In this work, we developed a nanoscale probe that targeted the cell nucleus *via* a distinct mechanism for carbon nanomaterial transport and reported that mechanism *via* an optical response. Using chemical inhibition, shRNA knockdown, and biochem-

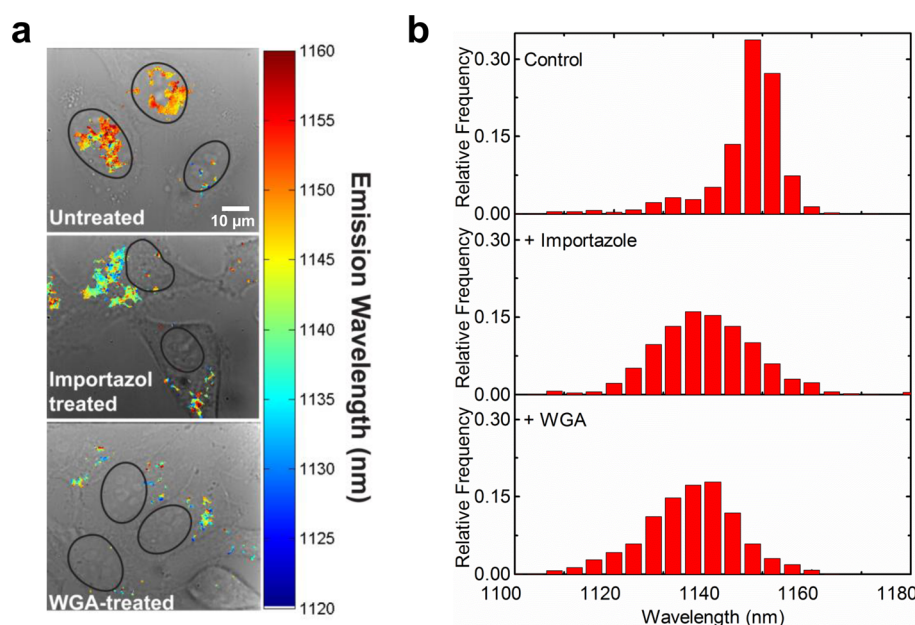


Figure 5. Nanotube photoluminescence response on nuclear localization and imaging *via* hyperspectral microscopy. (a) Hyperspectral maps of guanidinium-nanotube complex emission in live HeLa cells, overlaid on the brightfield images. Nuclear outlines are shown as black lines. (b) Histograms of peak emission wavelength from the hyperspectral data, obtained from three replicate experiments.

ical assays, we found that the nuclear translocation of the nanoprobe was mediated by the import receptor importin β and not by the more common importin α/β pathway. Upon entry to the nucleus, the probe emission wavelength was found to red-shift significantly. The photoluminescence shift was spatially mapped using NIR hyperspectral microscopy, resulting in quantitative images of nanotube optical bandgap modulation. The optical modulation in the nuclear environment constitutes a reporting event of the translocation mechanism and provides the optical reporter for this phenomenon. The work illustrates mechanistic evidence for the nuclear translocation of a synthetic nanomaterial through NPCs mediated specifically by importin β . The structural flexibility of importin β may be exploited for other nanomaterial delivery to the nucleus, especially in diseases where this import receptor is overexpressed.¹⁸ A probe for this process may have applications in drug delivery, in the screening/development of inhibitors of importin β and nucleo-cytoplasmic transport processes, and as a tool for investigations in cell and disease biology.

EXPERIMENTAL SECTION

Preparation of Polymer–Nanotube Complexes. Polymer–nanotube complexes were prepared by introducing HiPco nanotubes (1 mg) to aqueous acidic solutions of either ammonium or guanidinium polycarbodiimide polymers (1 mL, 5 mg/mL water). The mixtures were probe sonicated for 20 min (750 W, 20 kHz, 40% Amplitude, SONICS VibraCell) at low temperature using a CoolRack M30 PF (BioCision) stored at -20°C prior to use. Solutions were centrifuged (SORVALL Discovery 90SE, HITACHI) at 280000g for 30 min at room temperature. After centrifugation, ca. 80% of the supernatant was collected and filtered through a 100 kDa molecular weight cutoff centrifugal filter (Millipore Amicon) to remove excess polymer and acid. The washing step was repeated three more times before the nanotubes were resuspended in water. Removal of acid from the suspension was confirmed with litmus paper. Ultrapure water (18.2 m Ω) was used for all aqueous solutions. The suspensions were characterized by visible–near-infrared (vis–NIR) absorbance, NIR fluorescence spectroscopies, zeta potential (surface charge) measurements, and atomic force microscopy.

Cell Culture and Nuclear Import Assay. HeLa cells were purchased from the American Type Culture Collection (ATCC). SiHa cells were obtained from the Antibody and Bioresource Core Facility at Memorial Sloan Kettering Cancer Center. STR analysis was performed for cell authentication. Cells were cultured in DMEM supplemented with 10% (v/v) heat-inactivated FBS, 1% penicillin streptomycin, 1% glutamine, and 2.5% HEPES at 37°C in humidified air containing 5% CO_2 . All reagents were purchased from Gibco (Life Technologies). Cells were trypsinized, washed, and plated onto a 35 mm glass bottom Petri dish (MatTek). Cells were used after 24 h at 70–80% confluence. Polymer–nanotube complexes were diluted to 3 mg/L in water to avoid potential agglomeration of cationic carbon nanotubes at high concentration in complete cell culture media. The resulting solutions were then added to a cell culture medium containing serum to a final concentration of 0.2–2 mg/L of nanotubes. The cell culture medium was removed from the cells in the dishes and replaced with media containing polymer–nanotube complexes. The cells were incubated with the polymer–nanotube/media solutions for the time periods specified in the text, washed with PBS three times, and incubated in fresh cell culture media for up to 16 h prior to imaging.

Live Cell NIR Imaging and Hyperspectral Microscopy. The near-infrared photoluminescence imaging and spectroscopy of the cells were performed using a hyperspectral microscope under 730 nm excitation and 0.2 s exposure, as described.⁴⁴ Broadband images and 3D stacks/overlays were collected using the same microscope without the hyperspectral functionalities. DAPI fluorescence images were collected using an X-cite 120Q lamp (Lumen Dynamics) with DAPI filter set and CCD camera (QIClick, QImaging), while near-infrared images were collected using a 2D InGaAs array. Images and movies were processed using ImageJ software (NIH) and Bitplane Imaris 8.0.2 software.

Nuclear Pore Complex Blocking Experiment. Cells were plated onto 35 mm glass bottom dishes as described and incubated overnight. Cells were incubated with fresh cell culture media containing wheat germ agglutinin (WGA) (50 $\mu\text{g}/\text{mL}$) at 37°C for 30 min. Cells were washed three times with PBS and placed into fresh media. The cells were then treated with guanidinium nanotube complexes (0.2 mg/L) for 3 h. The cells were washed 3 $\times$ with PBS, fresh medium was added, and the mixture was incubated for 16 h before imaging. Cells not treated with WGA were used as the control.

Pharmacologic Inhibition of Importin α/β and Importin β Mediated Nuclear Translocation. Cells were preincubated for 30 min with either importazole (50 μ M), ivermectin (25 μ M), or vehicle (DMSO) before introduction of guanidinium–nanotube complexes (0.2 mg/L) for 3 h. The cells were washed with PBS, and fresh media with importazole, ivermectin, or vehicle was added. The cells were then incubated for approximately 16 h before imaging.

Importin β Knockdown by shRNA. Cells were seeded onto 35 mm Petri dishes and grown to 90% confluence in complete media. Cells were transfected following a protocol described in ref 45 and using a mixture of 9 μ L of FuGene HD (Promega) and 3 μ L of either a plasmid for importin β knockdown (Mission shRNA plasmid DNA (human) against karyopherin (importin β 1) or an empty plasmid (Mission pLKO 1-puro Vector control plasmid DNA). Cells were incubated in transfection media for 1 h then washed with PBS and placed onto fresh media. Twenty-four hours after transfection, the cells were incubated with guanidinium–nanotube complexes (0.2 mg/L) for 30 min, and the cells were then incubated for approximately 16 h before imaging.

Statistical Analysis. Each experiment was performed in triplicate. Total numbers of cells examined for nuclear localization of guanidinium–nanotube complexes are as follows for HeLa cells: importazole-treated cells, $n = 95$; vehicle control, $n = 83$; ivermectin-treated cells, $n = 84$; vehicle control, $n = 91$; shRNA knockdown, $n = 117$; empty vector control, $n = 96$. For SiHa cells: importazole treated cells, $n = 113$; vehicle control, $n = 131$; ivermectin-treated cells, $n = 93$; vehicle control, $n = 77$; shRNA knockdown, $n = 100$; empty vector control, $n = 111$. In each case, the percentage of cells with NIR emission in the nuclear localization was calculated separately for three replicates. The significance was calculated using a two-tailed, unpaired t test.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsnano.7b00176.

Additional experimental procedures, supporting figures, and characterization data (PDF)

Guanidinium–nanotube complexes in HeLa cells (AVI)

Ammonium–nanotube complexes in HeLa cells (AVI)

Guanidinium–nanotube complexes in HeLa cells (AVI)

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Notes

The authors declare no competing financial interest.

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