Supporting Information for:

A Carbon Nanotube Optical Sensor Reports Nuclear Entry *via* a Non-Canonical Pathway

Januka Budhathoki-Uprety[1], Rachel E. Langenbacher[1,2], Prakrit V. Jena[1], Daniel Roxbury[1,3], and Daniel A. Heller[1,2*]

[1]Memorial Sloan Kettering Cancer Center, New York, New York 10065, United States

[2]Weill Cornell Medical College, New York, New York 10065, United States

[3]Department of Chemical Engineering, University of Rhode Island, Kingston, Rhode Island 02881, United States

*Correspondence to: hellerd@mskcc.org

Polycarbodiimide synthesis and characterization

Reagents were purchased from Sigma-Aldrich, Milwaukee, WI, Acros Organics, and Fisher Scientific, Fair Lawn, NJ, and used as received. Neutral silica gel (Ultrapure 60–200 μm, 60 Å, Acros Organics) was used for column chromatography purification of monomers. Anhydrous and inhibitor-free tetrahydrofuran (THF) was used for click chemistry. NMR data were acquired using a Bruker Advance III Ultrashield Plus 500 MHz spectrometer at room temperature. The chemical shift values were reported relative to TMS (δ = 0.00 ppm) as an internal standard. Fourier transform infrared (FTIR) spectra were acquired using a Bruker Optics Tensor 27 FTIR spectrometer using ATR cell (Pike technologies) or NaCl disc. Wavenumbers in cm^{-1} were reported for characteristic peaks. High resolution mass spectra (HRMS) were obtained using a Waters LCT-Premier XE mass spectrometer by electrospray ionization. Size exclusion chromatography (SEC) was conducted using a Viscotek GPCmax system (Malvern Instruments) equipped with ViscoGEL columns (I-MBMMW-3078 and I-MBLMW-3078 in series) paired to a Viscotek TDA 305 triple detector array at 30 °C using THF as an eluent to determine relative molecular weights of the polymers. Polystyrene standards were used for the calibration of the instrument. Polymer samples were dissolved in THF, and the solutions were filtered through 0.45 μm PTFE filters prior to injection. The flow rate was 1.0 mL/min, and injector volume was 100 μL. OmniSEC software was used to calculate the molecular weight.

Urea derivative (**1),** monomer (**2**) and corresponding alkyne polycarbodiimide polymer were synthesized according to literature procedure[1], and the characterization data obtained were in agreement with the reported values. **Alkyne polycarbodiimide polymer**. FTIR (thin film, cm^{-1}): characteristic absorption from terminal alkyne group and polymer backbone; 3304 (terminal alkyne C–H), 2123 (alkyne triple

bond, C≡C), 1631 (polymer backbone, C=N). Mn = 36, 608, PDI = 1.35. ^{1}H NMR (500 MHz, $CDCl_3$, δ ppm): reference TMS = 0 ppm, δ = 7.28–6.84 (br), 5.35–5.29 (br), 4.37–4.20 (br), 3.14 (br), 2.07–0.75 (br). 11-Azido-3,6,9-trioxaundecan-1-amine was used as received and also derivatized to its corresponding guanidine derivative for 'click' reactions. ***Synthesis of the guanidinium azide (1-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethyl)guanidine*,3**, was adopted from literature procedure[2] and outlined in figure S1. Briefly, 11-Azido-3,6,9-trioxaundecan-1-amine (25 mg) was added slowly to the stirring solution of 2-Ethyl-2-thiopseudourea hydrobromide in the presence of *N*,*N*-Diisopropylethylamine (1:3:5) in dichloromethane (8 mL), and the mixture was refluxed for 8 h. The reaction proceeded with evolution of pungent ethanethiol by product. Conversion of amine to guanidine was monitored by mass spectrometry and the reaction was stopped at >95% conversion as confirmed using mass spectrometry, **LRMS** (ESI) $[M+H]^+$ m/z calcd for $C_9H_{21}N_6O_3$, 261.17; found 261.1. The reaction mixture was washed with saturated sodium chloride (3x), dried over anhydrous Na_2SO_4, filtered and concentrated using rotary evaporator. **FTIR** (thin film, NaCl, 4000 cm^{-1} – 800 cm^{-1}): 3347.4, 2923.5, 2854.9, 2103.2, 1669.4, 1541.2, 1458.1, 1347.1, 1285.5, 1121.6, 933.3, 810.6. The azides were coupled to the alkyne polymer by Cu(I) catalyzed azide-alkyne cycloaddition 'click' chemistry following a literature procedure[3]. Manipulations were performed under inert atmosphere. Briefly, azide solution (1:1.5 mol equivalents per alkyne unit in the polymer) was added to stirring alkyne polymer (20 mg) dissolved in tetrahydrofuran (5 mL, anhydrous, inhibitor free) at room temperature. After five minutes, CuI (5.0 equiv) and triethyl amine (6.0 equiv) were added, and the reaction mixture was stirred overnight (12 h). Completion of the coupling reaction was confirmed by FTIR analysis of the resulting polymers. The products were washed with THF (10 mL, 3X) to remove unreacted small molecules, dried and characterized by FITR spectroscopy. Limited solubility of the final polymers posed difficulty in GPC analysis and NMR spectroscopies.

Guanidine polycarbodiimide polymer **FTIR** (thin film, 4000 cm^{-1} – 800 cm^{-1}): 3415.9 (vs, br), 3007.3 (m), 2919.0 (m), 1651.8 (s, br, guanidine and polymer backbone overlap, C=N), 1437.5 (m), 1407.5 (m), 1317.3 (w), 1020.1 (s), 953.2 (m), 902.4 (w).

Amine polycarbodiimide polymer **FTIR** (thin film, 4000 cm^{-1} – 800 cm^{-1}): 3424.0 (vs), 3002.3 (m), 2916.5 1660. 3 (s, polymer backbone, C=N), 1437.5 (m), 1407.7 (m), 1315.2 (w), 1024.3 (s), 954.0 (m).

Characterization of polymer-nanotube complexes

The suspensions were characterized by visible-near-infrared (VIS−NIR) absorbance and NIR fluorescence spectroscopies. The VIS−NIR absorption spectra were measured with a JASCO V-670 spectrophotometer. Near infrared photoluminescence excitation/emission (PL) measurements were performed on a home-built instrument consisting of an IsoPlane SCT 320 spectrograph and PioNIR InGaAs detector (Princeton Instruments) connected to an Olympus IX71 inverted microscope. A 20×

objective was used. Samples were excited using a SuperK Extreme supercontinuum laser connected to a Varia variable bandpass filter (NKT Photonics). The excitation wavelength was varied from 491 to 824 nm, and the emission was recorded from 915 to 1354 nm. Data were collected using a custom Labview (National Instruments) automation program. These data were then analyzed and plotted using Matlab (The MathWorks) code.

Zeta potential (surface charge) measurements were conducted by suspending polymer-nanotube complexes (~ 3 mg/L) in ultrapure water (18.2 mΩ) in a 1 mL folded capillary cell (Malvern). Measurements were conducted at room temperature using a Zetasizer Nano-ZS instrument (Malvern).

Atomic force microscopy measurements were conducted by depositing polymer-nanotube complexes (1 mg/L) onto a freshly cleaved mica surface (Pelco Mica Disc, V1, Ted Pella) and allowed to stand for 1 min before washing the surface with deionized water two times to remove unbound carbon nanotubes. The mica surface was dried at room temperature with ultrapure nitrogen prior to imaging. AFM images were collected using Asylum MFD-3D-BIO in AC mode using AC240TS tips (Asylum Research). The typical scan size was 2 μm and scan rate was 0.25 Hz–0.5 Hz. The images were processed with Igor software.

Cell surface proteoglycan deactivation

Cells were plated onto 35 mm glass bottom dishes (MatTek) as described and incubated in the presence of 75 mM sodium chlorate for 48 h to deactivate proteoglycans (Kalia et al. J Virology, 2009). Cells were treated with guanidinium-nanotube complexes (0.2 mg/L) for 30 minutes in the presence of sodium chlorate. Cells were washed with PBS and placed in fresh media prior to imaging.

Western blot analysis

HeLa and SiHa cells were trypsinized and washed in ice-cold PBS then resuspended in a microcentrifuge tube with ice-cold lysis buffer (RIPA lysis and extraction buffer, Thermo Fisher). These samples were incubated at 4 $^{\circ}$C for 30 minutes with vortexing. Solutions were then centrifuged to separate cell debris from cell lysate. Protein content of the supernatant was quantified using the Bradford assay. Samples were mixed with 2x Laemmli sample buffer and subsequently boiled at 100 $^{\circ}$C for five minutes. Equal amounts of protein were loaded into the wells of a 10% SDS-page gel (Bio Rad). A molecular weight marker was used in a separate well (Bio Rad, Precision Plus Protein Dual Color Standards). The gel was run for 1 h at 100 V. Transfer to a nitrocellulose membrane (Thermo Fisher) was carried out overnight at 20 V. The membrane was blocked for 1 hr at room temperature using blocking buffer (Pierce clear milk blocking buffer, Thermo Scientific), and then incubated with the primary antibody, Abcam Ab9485 Rb

pAb to GAPDH, or Abcam Ab2811 Ms mAb to NTF97/importin β overnight at 4 °C in blocking buffer. The membrane was then washed three times in TBST buffer for five minutes, before incubation with the secondary antibody, Abcam Ab9720 goat pAb to Rb igG(HRP) or Abcam Ab131368 Rat mAb to Ms IgG (HRP), in blocking buffer at room temperature for one hour. The membrane was then washed three times in TBST buffer for five minutes. Signal development was carried out using incubation in Immobilon western chemiluminescent HRP substrate (Millipore) for five minutes. Excess reagent was removed and the membrane was covered in a transparent plastic sleeve before chemiluminescence was read using a ChemiDoc XRS system. GAPDH was used as a loading control.

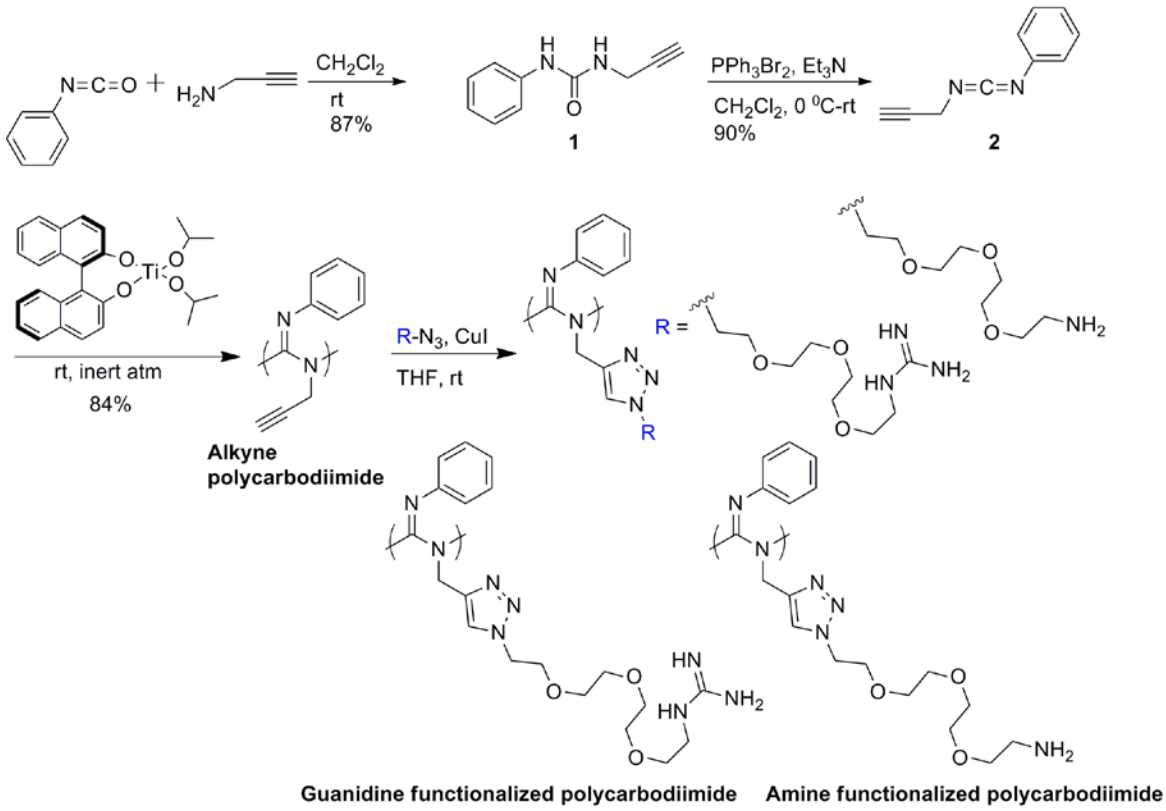
Cell viability assay

Cells were plated in a 35 mm Petri dish. Cells at 70-80% confluence were incubated with 0.1 mg/L to 2 mg/L guanidinium polymer-nanotube complexes for 24 h at 37 °C. Cells incubated under the same conditions but without nanotubes were used as the control. A Tali Image-Based Cytometer was used to measure cell viability tests performed using the Tali Viability Kit - Dead Cell Red (Invitrogen) following the manufacturer's protocol. The results were compared to control cells in the absence of nanotubes. Each sample was tested in triplicate.

Salmon sperm DNA experiments

The guanidinium-nanotube complexes (20 µL, 4.5 mg/L) were incubated with salmon sperm DNA (40 µL, 1 mg/mL) at room temperature for 1 h. An aliquot of the resulting sample was imaged on a 35 mm glass bottom dish using hyperspectral microscopy (Photon etc) measurements. A second aliquot was centrifuged at 30,000 rcf for 5 min. The pellet was transferred to a 35 mm glass bottom dish for hyperspectral microscopy.

Figures



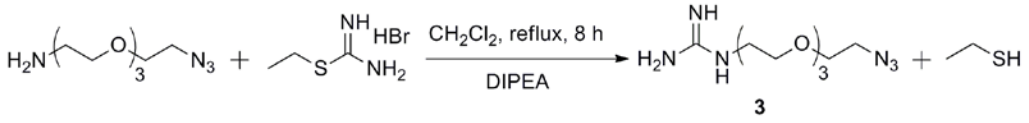


Figure S1. Synthesis of polycarbodiimide polymers.

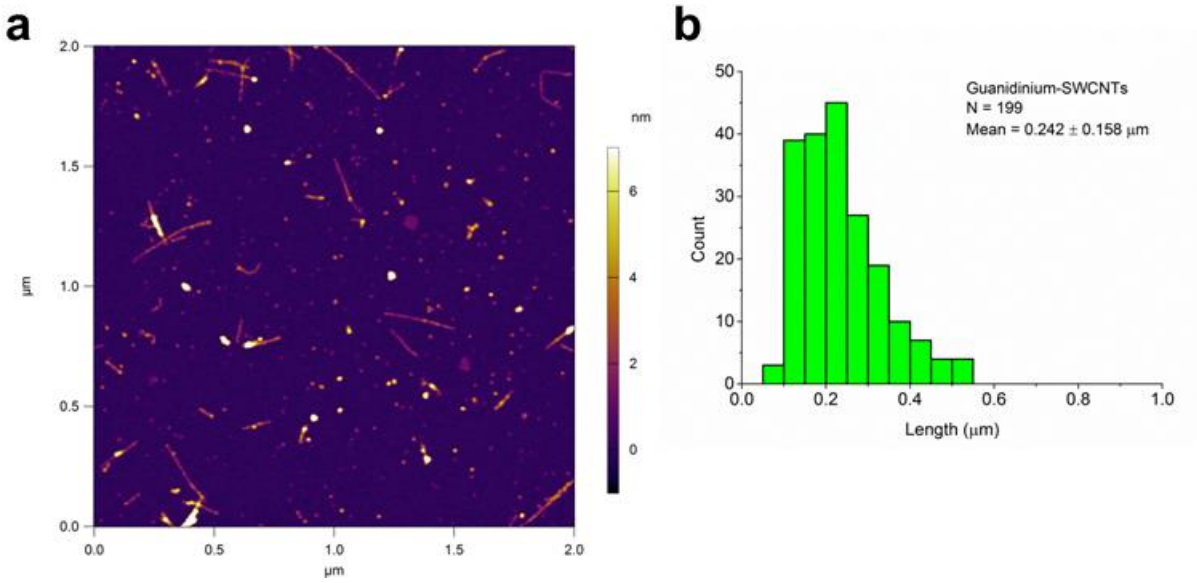


Figure S2. Atomic force microscopy (AFM) of guanidinium-carbon nanotube complexes. **a**, Height profile AFM image of guanidinium-SWCNT complexes. **b**, Length distribution of the sample as quantified from AFM images.

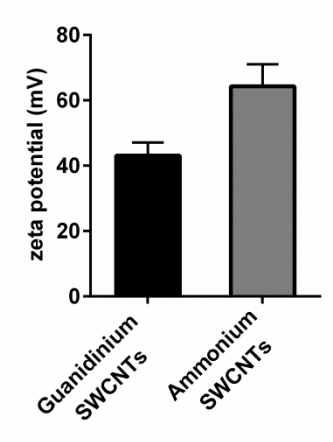


Figure S3. Zeta potential measurements of polycarbodiimide-carbon nanotube complexes.

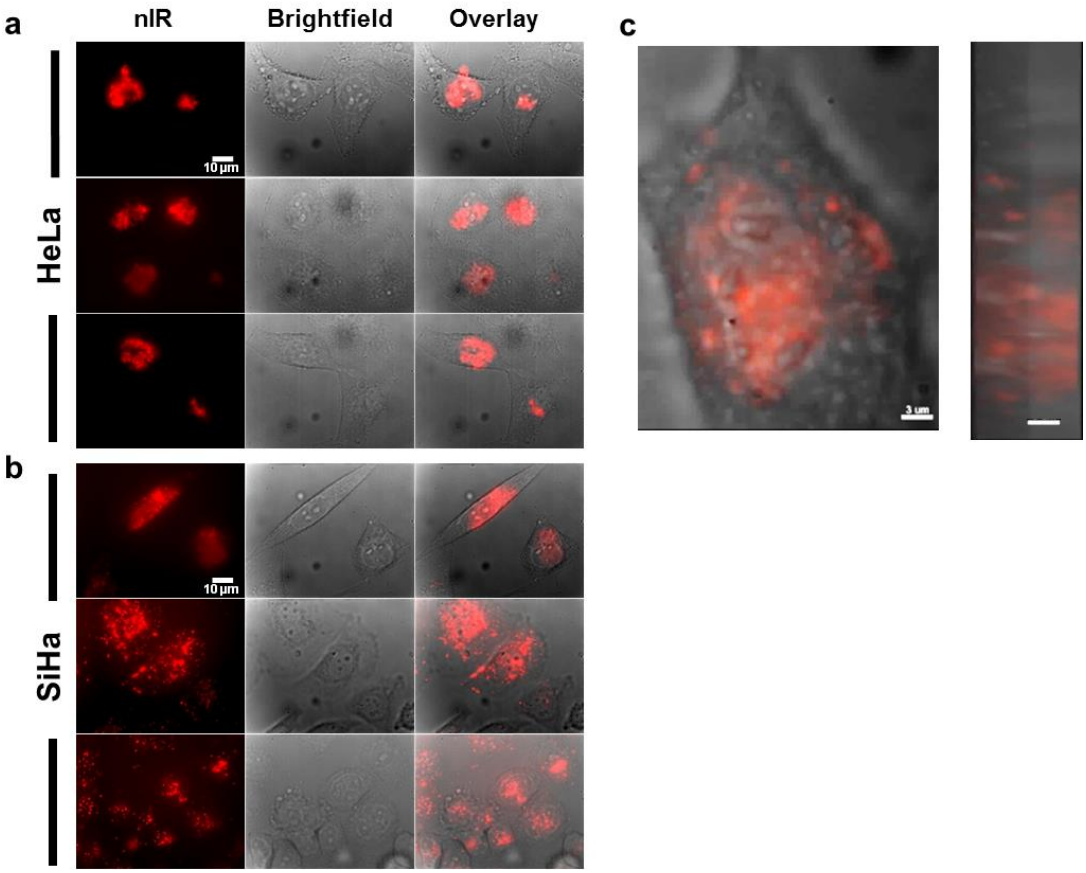


Figure S4. Near-infrared emission of guanidinium-nanotube complexes in live cells. **a**, HeLa cells. **b**, SiHa cells. All images in panels a and b are at the same scale. **c**, Snapshots from Movie S3 showing emission from carbon nanotubes within the nuclear volume of a HeLa cell. Scale bar is 3 µm. The 3D representation was prepared *via* deconvolution of successive near infra-red images acquired with a 0.2 s exposure using a piezo-controlled stage with a z-plane spacing of 50 nm through the entire cell volume using an automated xyz-stage (MS-2000, Applied Scientific Instrumentation). The images were analyzed using Bitplane Imaris 8.0.2 software.

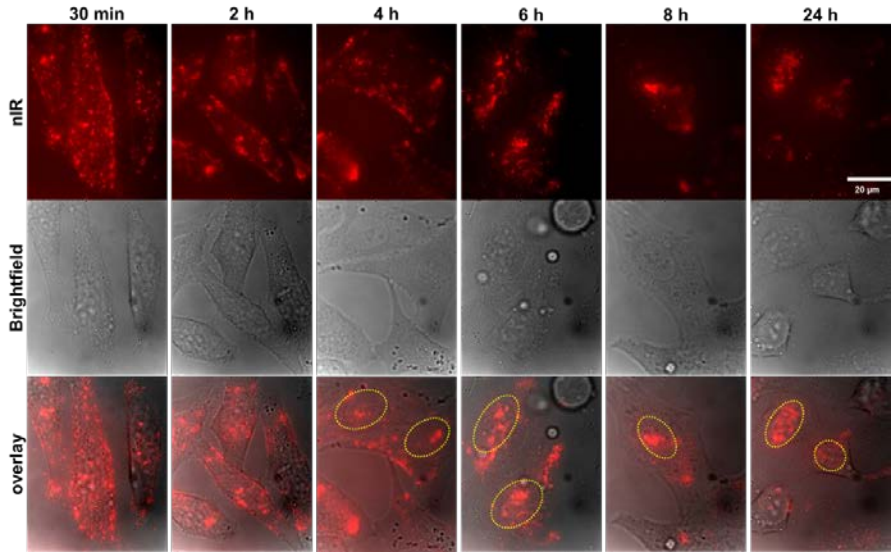


Figure S5. Time-course images of guanidinium-nanotube complex near infrared emission in HeLa cells. Cell nuclei are outlined with yellow dotted lines for visualization.

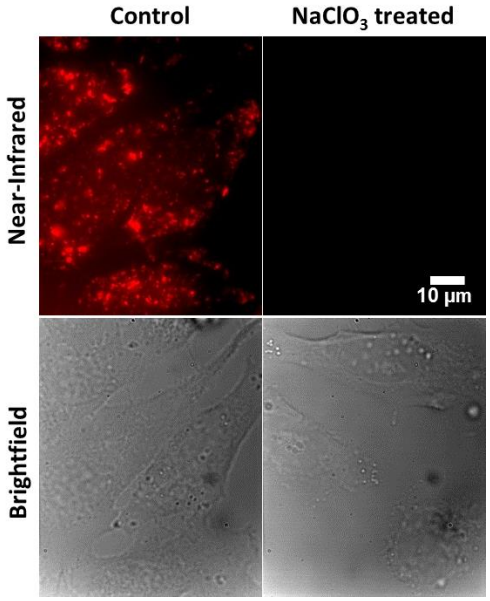


Figure S6. Near-infrared emission from guanidinium-nanotube complexes in HeLa cells treated with $NaClO_3$ before introducing guanidinium-nanotube complexes.

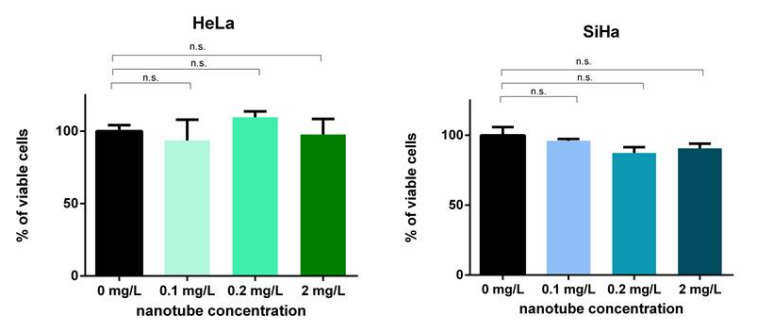


Figure S7. Cell viability assays with guanidinium-nanotube complexes.

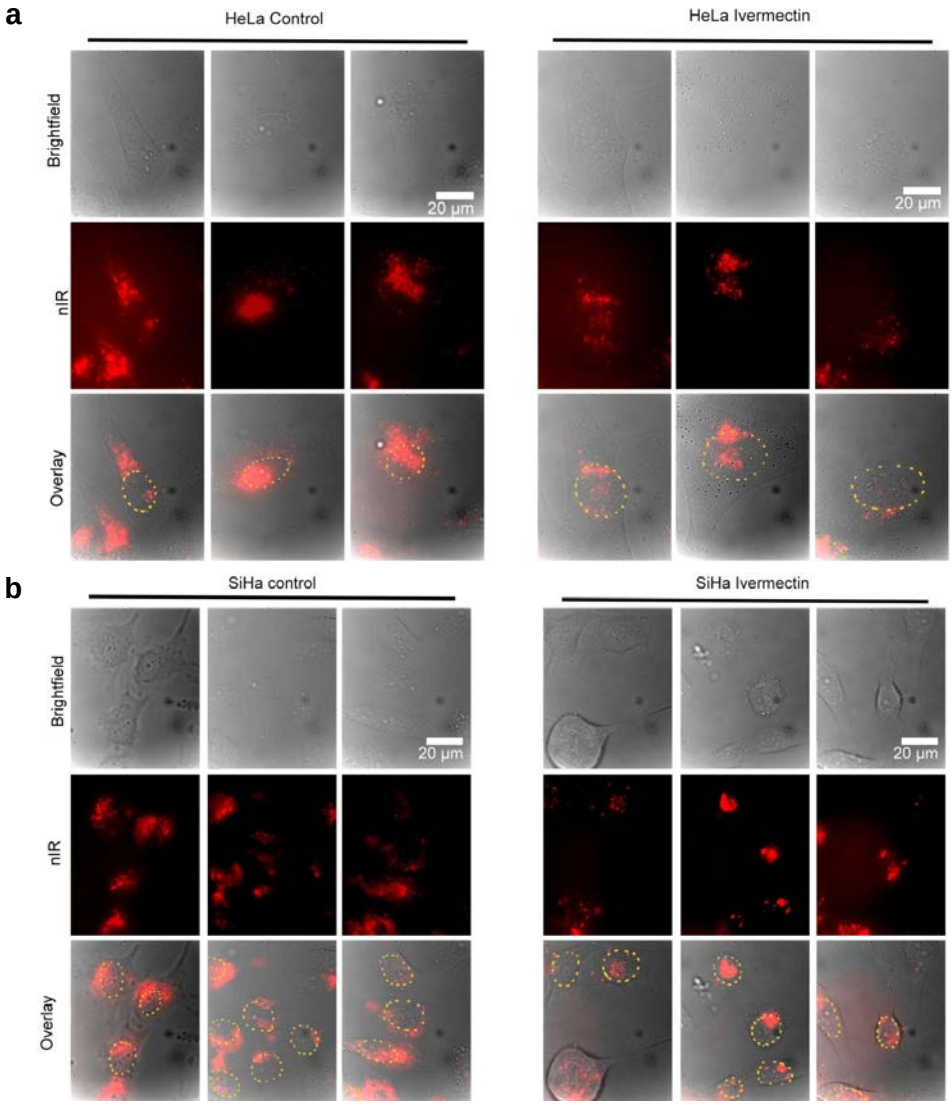


Figure S8. Near-infrared emission of guanidinium-nanotube complexes introduced after adding ivermectin to the cell media. **a**, HeLa cells. **b**, SiHa cells. Cell nuclei are outlined in yellow for visualization.

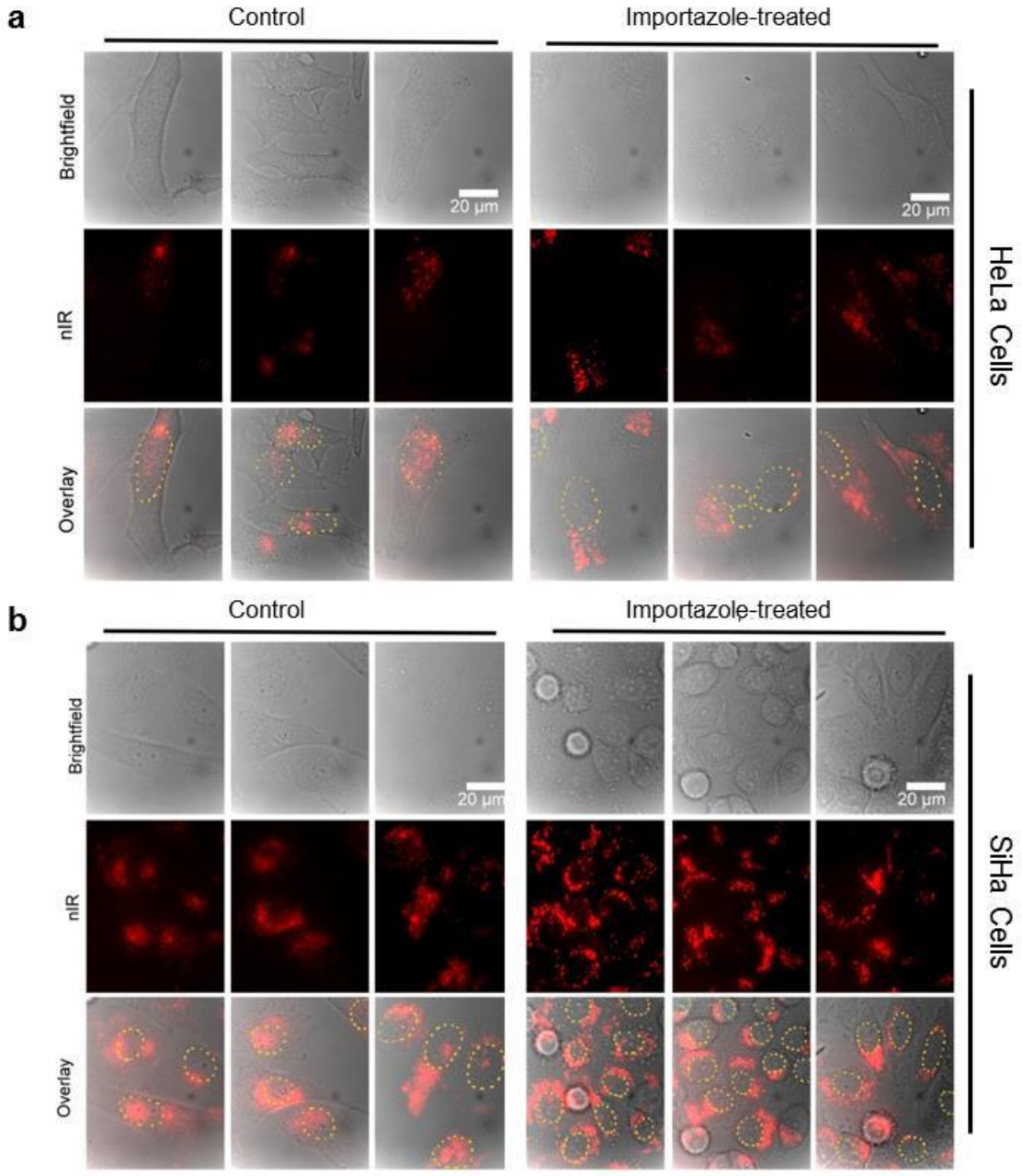


Figure S9. Near-infrared emission of guanidinium-nanotube complexes introduced after adding importazole to the cell culture media. **a**, HeLa cells. **b**, SiHa cells. Cell nuclei are outlined in yellow for visualization.

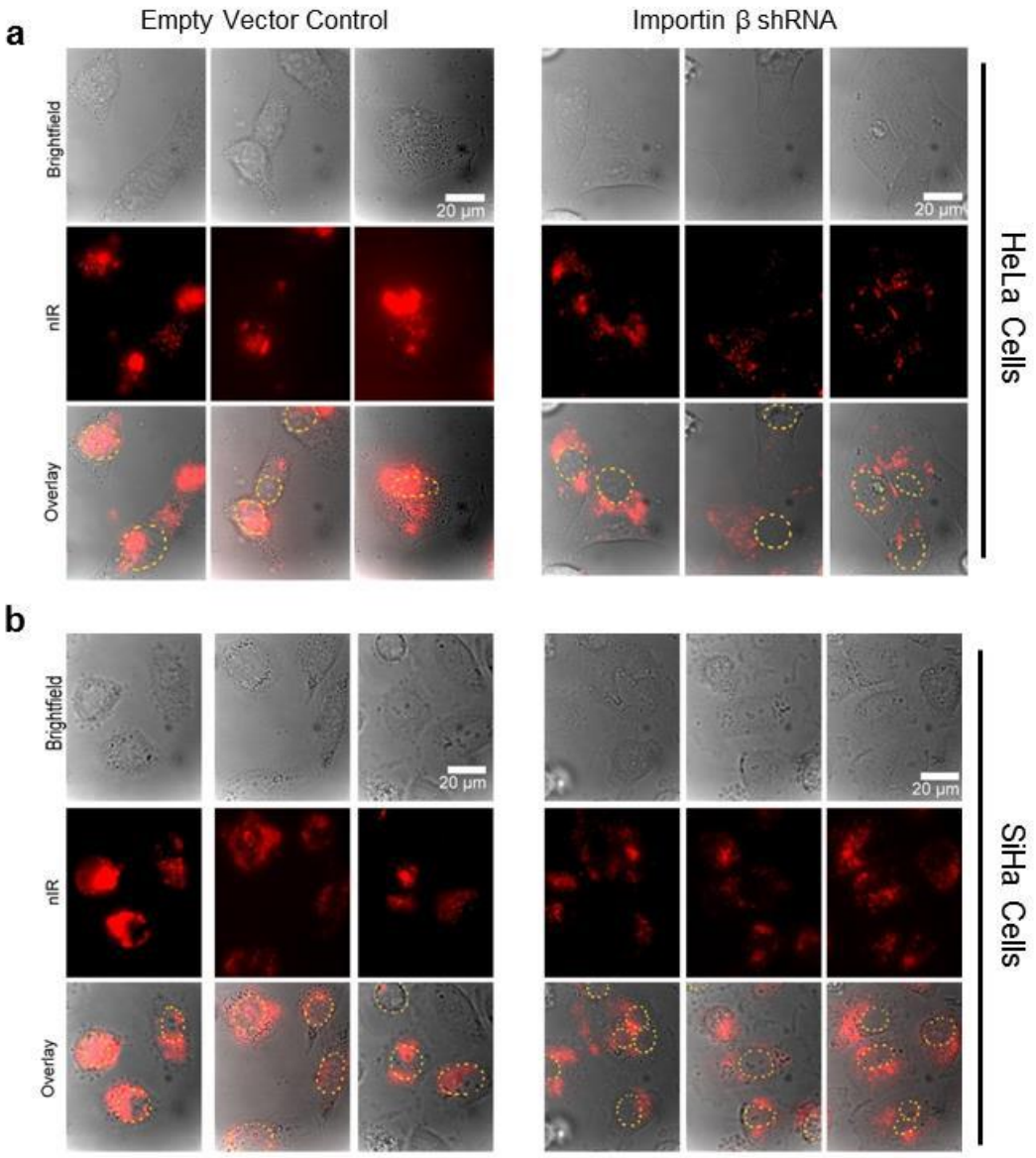


Figure S10. Near-infrared emission of guanidinium-nanotube complexes introduced after knockdown of importin β using shRNA. **a**, HeLa cells. **b**, SiHa cells. Cell nuclei are outlined in yellow for visualization.

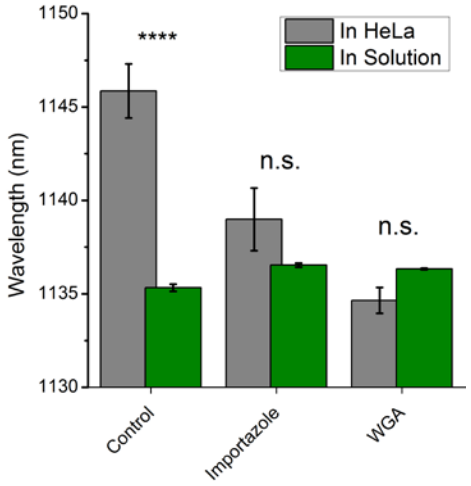


Figure S11. Comparisons of carbon nanotube emission shifts in cells (HeLa) and under cell-free conditions (in aqueous solution) with inhibitors of nuclear translocation. Graphs represent mean ± standard deviation from 3 replicate experiments. ****P<0.0001 (P<0.05 significant).

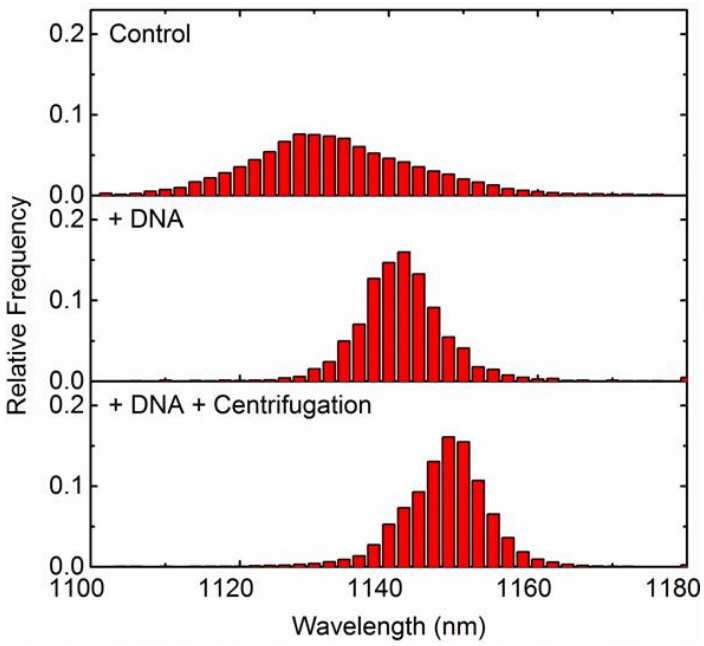


Figure S12. Histogram of center emission wavelength of guanidinium-nanotube complexes upon interaction with salmon sperm DNA in solution and of pellet after centrifugation, measured by near-infrared hyperspectral microscopy.

References

1. Budhathoki-Uprety, J.; Jena, P. V.; Roxbury, D.; Heller, D. A., Helical Polycarbodiimide Cloaking of Carbon Nanotubes Enables Inter-Nanotube Exciton Energy Transfer Modulation. *J Am Chem Soc* **2014**, *136*, 15545-15550.

2. Lim, H. D.; Smits, R. A.; Bakker, R. A.; van Dam, C. M. E.; de Esch, I. J. P.; Leurs, R., Discovery of S-(2-guanidylethyl)-isothiourea (VUF 8430) as a Potent Nonimidazole Histamine H-4 Receptor Agonist. *J Med Chem* **2006**, *49*, 6650-6651.
3. Budhathoki-Uprety, J.; Novak, B. M., Synthesis of Alkyne-Functionalized Helical Polycarbodiimides and their Ligation to Small Molecules using 'Click' and Sonogashira Reactions. *Macromolecules* **2011**, *44*, 5947-5954.