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Biocompatible Organic Charge Transfer Complex Nanoparticles Based on a Semi-Crystalline Cellulose Template

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Supplementary Information

Equipment

a) Nuclear magnetic resonance (NMR) spectroscopy. ^{1}H and ^{13}C NMR spectra were performed on a Varian 400 MHz spectrometer in DMSO-d6.

b) Molecular weight analysis. The molecular weight was measured by Gel Permeation Chromatography (GPC) (Viscotek) equipped with RI detection and ViscoGEL I-series columns (Viscoteck I-MBLMW-3078) using DMF as the eluent at 0.75 mL/min and 45 °C. The instrument was calibrated with a series of 10 narrow polydispersity polystyrene standards (500 to 200,000 g/mol).

c) NP size analysis. Particle sizes were measured by Dynamic Light Scattering (DLS) using a Malvern Zetasizer Nano ZS (He-Ne laser, λ = 632 nm).

d) UV–Vis measurements were performed on a Shimadzu UV-1800 spectrophotometer.

e) The fluorescence emission spectra were obtained on a Hitachi F-7500 fluorometer.

f) Thermogravimetric analyses (TGA) were performed on a Mettler Toledo TGA/DSC-1 system under nitrogen at a heating rate of 10 °C/min.

g) Infrared spectroscopy was performed on an AVATAR 360 FT-IR. Samples were gently ground with KBr, and pellets were pressed manually.

h) Confocal laser scanning microscopy was performed on a Zeiss LSM-700 microscope, and images were processed using ImageJ (NIH).

i) Luminescence measurements for toxicity assays were performed on a Tecan Infinite M200 pro plate reader.

Materials

Cellulose (Sigmacell cellulose, type 101, highly purified, fibers), 1-pyrenebutyric acid (97%), TCNQ (98%), TCNB (97%), and TCNE (98%) were obtained from Sigma-Aldrich and used as received. 2,3-Di-O-methyl cellulose and 1-pyrenebutanoyl chloride were prepared according to ref.1S and 2S, respectively. Other reagents were used as received without further purification.

Methods

Synthesis of Pyrene-conjugated 2,3-di-O-methyl cellulose (Py-DOMC): 2,3-di-O-methyl cellulose (DOMC) (0.70 g, 3.43 mmol, M_n = 27,190, M_w/M_n = 3.12) was dissolved in the mixture of anhydrous DMAc (50 mL) and pyridine (0.6 mL, 6.94 mmol). The reaction was cooled in an ice bath and a solution of 1-pyrenebutanoyl chloride (2.0 g, 6.51 mmol) in DMAc (20 mL) was slowly added drop-wise. The reaction mixture

warmed to room temperature and stirred overnight. The product was precipitated in ethanol and filtered, and the residue was washed with ethanol. The product was dissolved DMAc and re-precipitated into ethanol. Yield = 0.98 g (2.06 mmol, 60%, M_n = 39,440, M_w/M_n = 3.30). IR (KBr): δ = 3342.1, 2932.7, 1737.8 (>C=O), 1456.7, 1371.0, 1316.2, 1059.7, and 843.9 cm^{-1}

Preparation of CT-cellulose-acceptor complexes: A toluene/DMAc (9/1 = v/v, 0.2 or 0.02 mol/L) mixture of Py-DOMC (47.5 mg, 0.1 mmol) and acceptors (20.4 mg, 0.1 mmol) in 10 mL glass vials was closed and heated at 100°C for 2 days. The resulting precipitate was collected by centrifugation, washed with ethanol and acetone, and dried at 50 °C under vacuum, to produce acceptor-CT-cellulose as a colored solid in 49-51% (0.02 g/L) and 64-70% (0.2 g/L).

IR (TCNQ-CT-cellulose (0.2 M)) (KBr): δ = 3577.4, 3468.8, 3277.1, 2944.1, 2217.0 (-CN), 2181.2 (-CN), 1738.7 (>C=O), 1639.0, 1543.8, 1058.1, and 848.0 cm^{-1}

IR (TCNQ-CT-cellulose (0.02 M)) (KBr): δ = 3438.6, 2925.9, 2218.4 (-CN), 1737.9 (>C=O), 1636.0, 1457.2, 1370.0, 1115.7, 1064.5, and 846.3 cm^{-1}

IR (TCNE-CT-cellulose (0.2 M)) (KBr): δ = 3039.8, 2932.0, 2215.6 (-CN), 1737.7 (>C=O), 1637.9, and 845.2 cm^{-1}

IR (TCNE-CT-cellulose (0.02 M)) (KBr): δ = 3343.8, 2935.5, 1738.0 (>C=O), 1457.3, 1160.8, 1112.5, 1060.7, and 845.2 cm^{-1}

IR (TCNB-CT-cellulose (0.2 M)) (KBr): δ = 3342.0, 2931.3, 1737.7 (>C=O), 1371.1, 1059.6, and 845.3 cm^{-1}

IR (TCNB-CT-cellulose (0.02 M)) (KBr): δ = 3341.2, 2908.9, 1737.8, 1060.2, and 844.7 and 845.2 cm^{-1}

Nanoprecipitation: Stock solutions of Py-DOMC and TCNQ-CT cellulose were prepared at a concentration of 0.2 g/L or 1.0 g/L in DMSO either with or without 5 wt% Tween20 relative to the cellulose mass. The DMSO solutions were added drop wise to a rapidly stirring deionized water to a final concentration of 10% DMSO solution and a final cellulose concentration of 0.02 g/L or 0.1 g/L. Absorbance and fluorescence measurements were made using nanoparticles dispersed in water while dialysis against 1X PBS was performed in mini dialysis cups (Pierce, MWCO 3,500 Da) or Pur-A-Lyzer Midi dialysis apparatuses (Sigma-Aldrich, MWCO 3,500 Da) for 1 h prior to use in biological studies.

Cell viability assays (CellTiter-Glo Assay): For the biological studies, unless otherwise indicated, all cells were cultured in HyClone Dulbecco's High Glucose Modified Eagles Medium (with L-glutamine, without Phenol red and sodium pyruvate, supplemented with 5% FBS) at 37 ˚C, 5% CO_2, in a humidified atmosphere. 48 hours prior to the assay, HeLa cells were seeded in 96-well white opaque tissue culture plate at a density of 2,500 cells per well, in 100 μL growth medium. Following this initial adhesion period, the medium was replaced with 200 μL fresh growth medium. Nanoparticles were prepared by nanoprecipitation in water as detailed above at a concentration of 0.1 g/L and dialyzed against 1X PBS for 4 h. After dialysis, the nanoparticles were serially diluted two-fold to 0.05 g/L, 0.025 g/L, 0.0125 g/L, 0.00625 g/L, 0.003125 g/L, and 0.00156 respectively and added to the cells (80 μL). The nanoparticles were incubated in growth conditions for 36 h. After this period the CellTiter-Glo Assay (Promega) was performed by removing the medium and replacing with 50 μL of a 1:1 volumetric ratio dilution of CellTiter-Glo reagent and serum free DMEM. The cells were incubated on an orbital mixer for 10 minutes prior to reading luminescence and normalized against untreated cells (N = 4 or 5, +/- standard deviation).

Cellular uptake studies: HeLa cells were seeded at a density of 30,000 cells/well in 300 μL growth medium in 8-well chamber slides (LabTek) and incubated overnight for 24 h prior to incubation. After this adhesion period, the medium was exchanged with 300 μL fresh growth medium. Nanoparticles containing 5 wt% Tween20 were prepared by nanoprecipitation and dialyzed against 1X PBS for 1 h. The nanoparticles (100 μL) were added by pipette to the cells and incubated for 2 h prior to imaging. Confocal laser scanning microscopy was performed on a Zeiss LSM 700 with 20X dry and 63X oil immersion objectives. Pyrene fluorescence was visualized by excitation with the 405 nm laser, while CT complex fluorescence was visualized by excitation with the 639 nm laser. Images were processed with ImageJ (NIH) using a median filter (radius = 1 pixel).

***In vivo* studies:** All experiments were approved by the Institutional Animal Care & Use Committee (IACUC) of The University of Texas Southwestern Medical Center and were consistent with local, state and federal regulations as applicable. Athymic Nude-Foxn1^{nu} mouse (Harlan Laboratories) were injected with RPMI-7951 melanoma cells subcutaneously on both flanks. After the tumors reached adequate size, intratumoral injections (150 μL) of Tween20 coated nanoparticles (0.1 g/L) were performed. Tumors on separate flanks of the same animal were injected either with Py-DOMC NPs or TCNQ-CT NPs, respectively with a second injection (150 μL) at the two-hour time point. After an additional two hours incubation time, the animal was sacrificed, the tumors resected, embedded in Tissue-Tek (Sakura) and snap-frozen at -80 ºC. The tumor tissue was sectioned on a Leica CM3050 S cryostat (8 micron sections) and mounted on slides. Confocal laser scanning microscopy was performed on a Zeiss LSM 700 with a 63X oil immersion objective. Pyrene fluorescence was visualized by excitation with the 405 nm laser, while CT complex fluorescence was visualized by excitation with the 639 nm laser. Images were processed with ImageJ (NIH) using a median filter (radius = 1 pixel).

Additional Supporting Information Figures

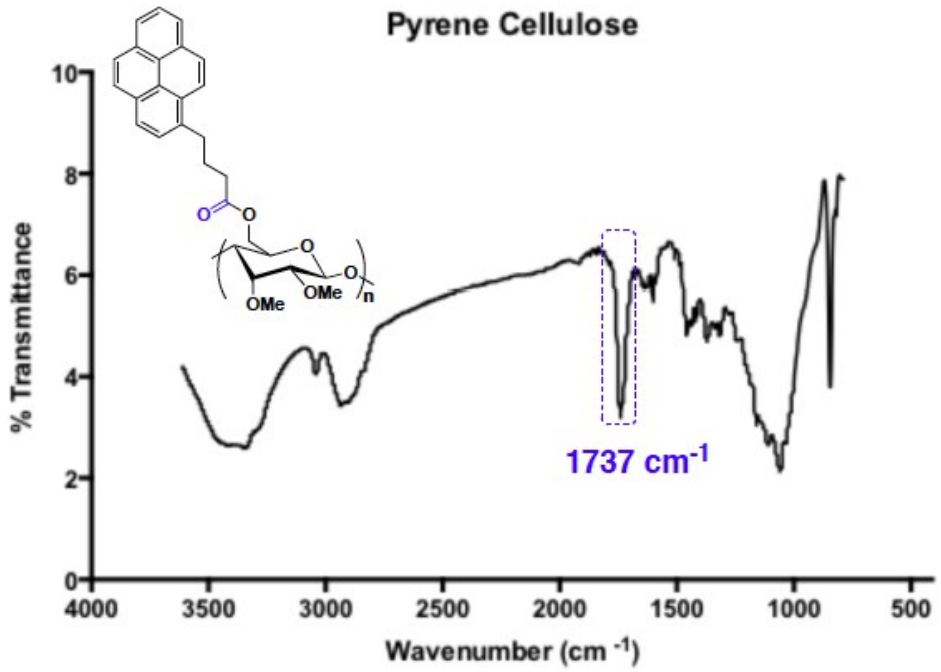

Figure S1. FT-IR spectrum of Py-DOMC from a KBr pellet.

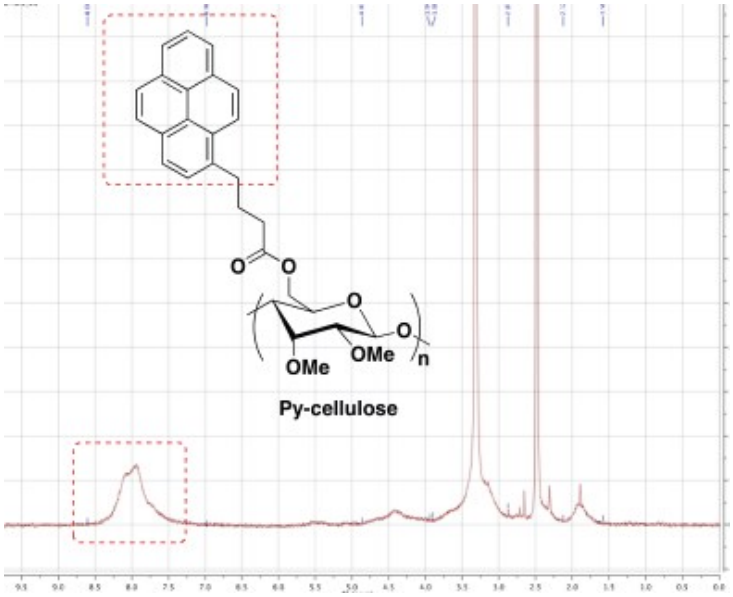

Figure S2. ^{1}H NMR spectrum of Py-DOMC in DMSO-D6.

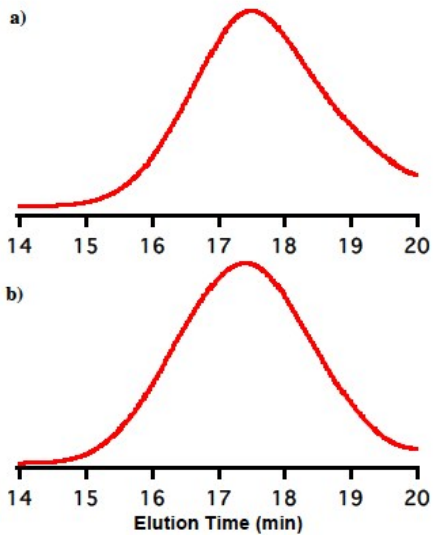

Figure S3. GPC curves of DOMC (**a**) and Py-DOMC (**b**) show monomodal peaks.

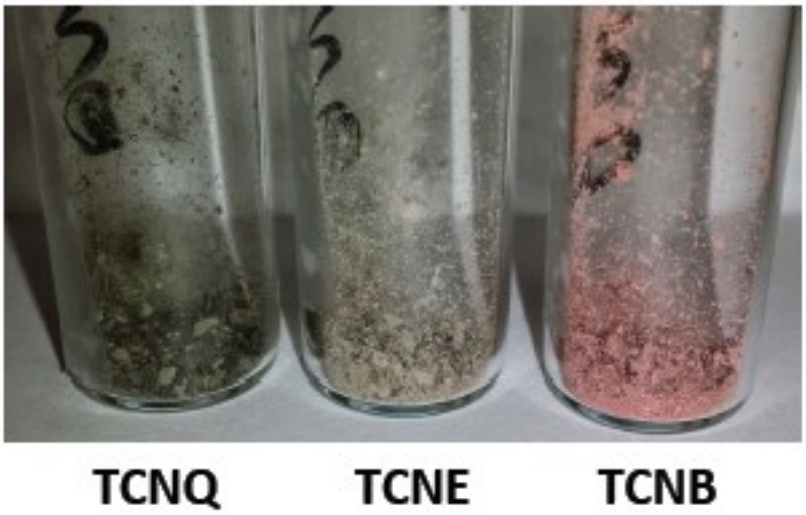

Figure S4. Photographs of linear TCNQ-, TCNE-, and TCNB-CT cellulose solids.

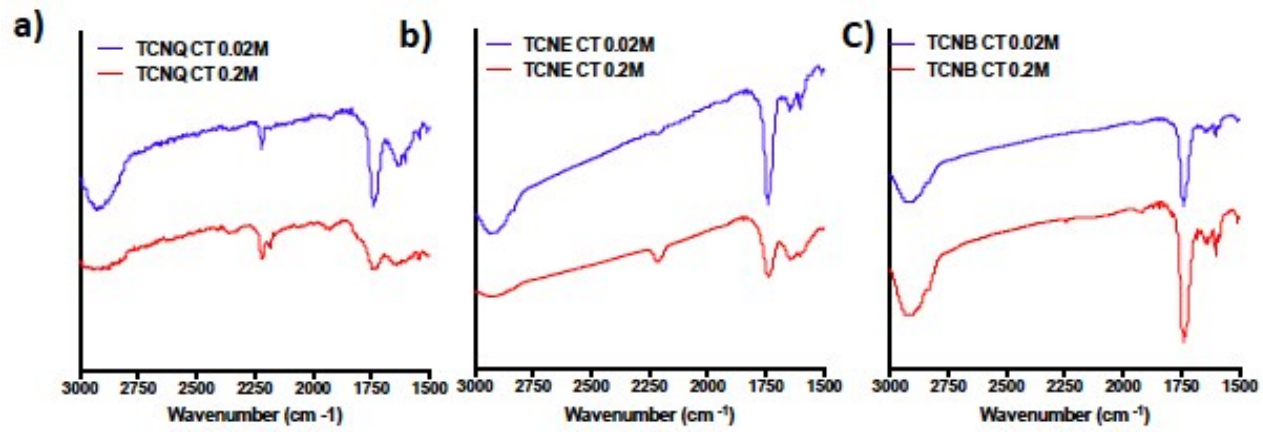

Figure S5. FT-IR spectra from KBr pellets of (**a**) TCNQ-CT-cellulose (blue; 0.02 M, red; 0.2 M), (**b**) TCNE-CT-cellulose (blue; 0.02 M, red; 0.2 M), and (**c**) TCNB-CT-cellulose (blue; 0.02 M, red; 0.2 M).

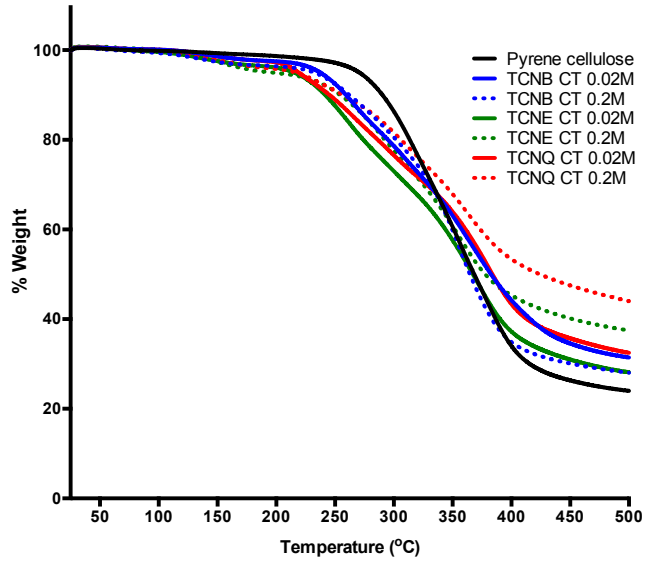

Figure S7. TGA measurements of TCNQ-CT-cellulose (red solid, 0.02 mol/L; red dotted, 0.2 mol/L), TCNE-CT-cellulose (green solid, 0.02 mol/L; green dotted, 0.2 mol/L), and TCNB-CT-cellulose (blue solid, 0.02 mol/L; blue dotted, 0.2 mol/L).

Table S1. TGA data for DOMC, Py-DOMC, and CT complexes.

Sample	$T_{5\%wt\ loss}$ (°C)	$T_{10\%wt\ loss}$ (°C)	$\%wt_{final}$ (500 °C)
Py-DOMC	270.67	290.17	24.00
TCNB	281.83	298.33	4.09
TCNB CT 0.02M	237.33	259.33	31.45
TCNB CT 0.2M	232.67	262.17	28.05
TCNE	151.17	161.50	1.36
TCNE CT 0.02M	215.67	241.67	28.15
TCNE CT 0.2M	196.83	254.50	37.49
TCNQ	284.83	297.50	11.49
TCNQ CT 0.02M	219.00	245.33	32.49
TCNQ CT 0.2M	217.00	256.67	44.03

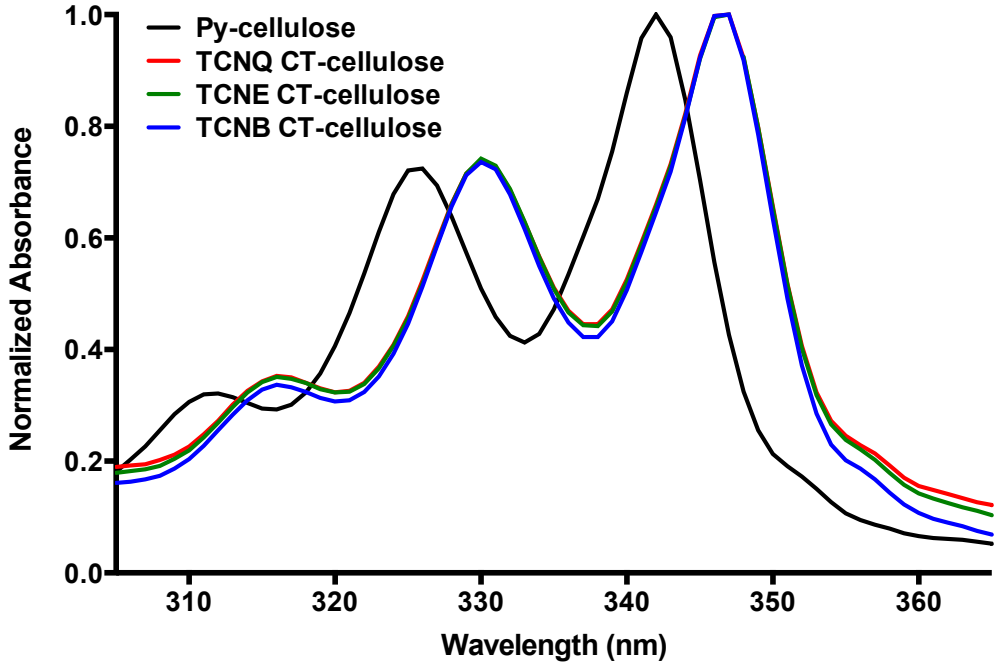

Figure S8. Absorbance spectra of CT complexes dissolved in DMSO.

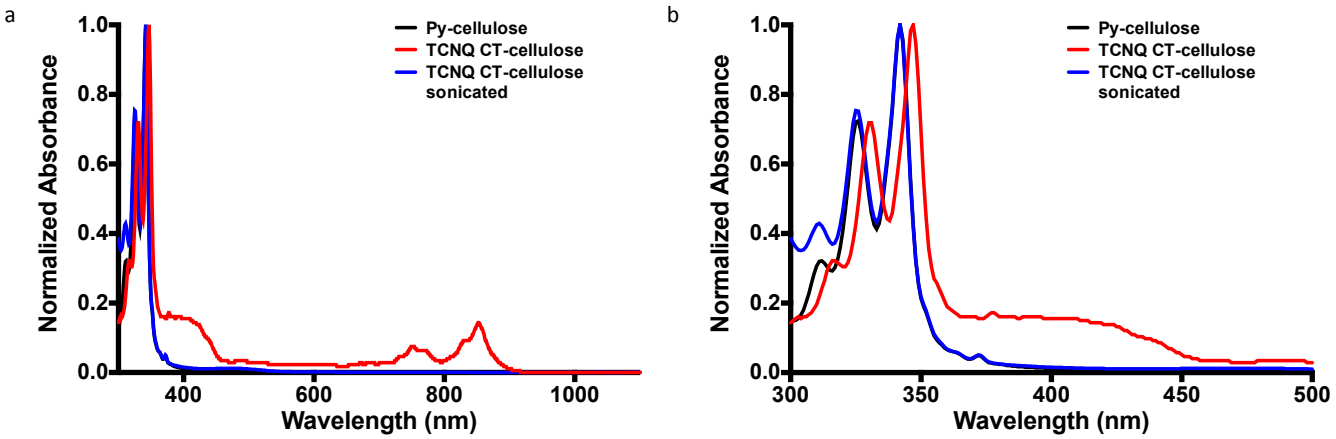

Figure S9. The effect of sonication on DMSO solutions for TCNQ-CT cellulose.

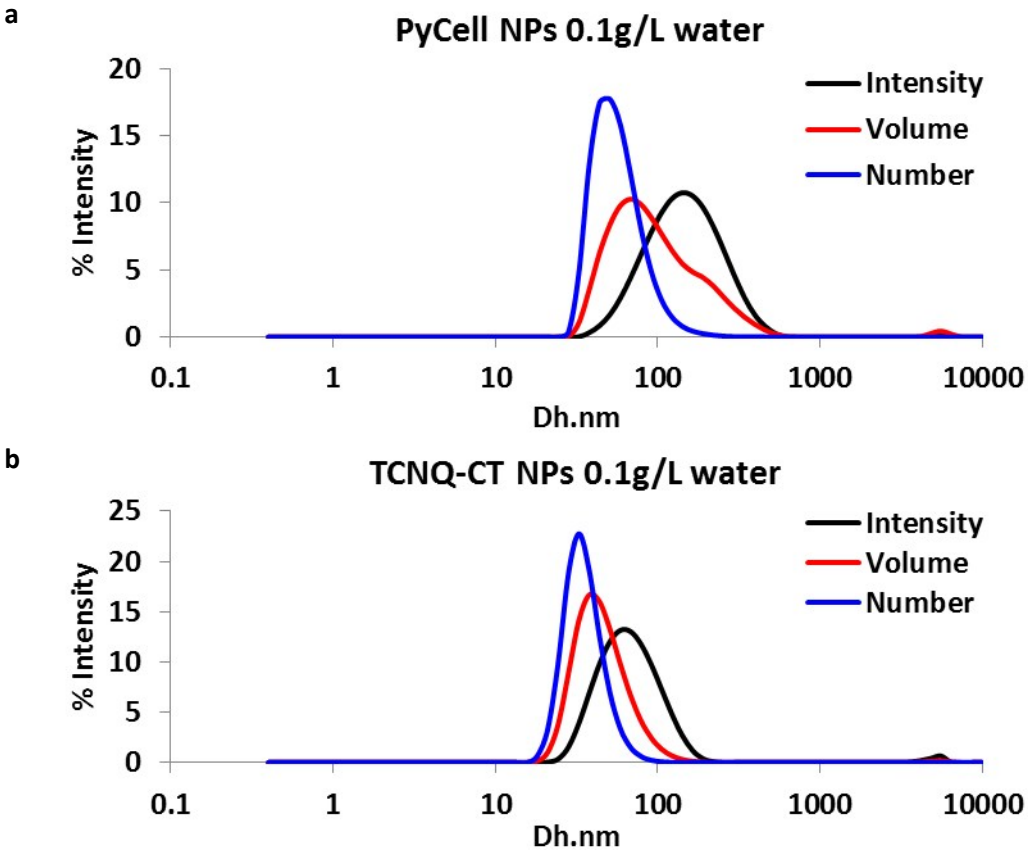

Figure S10. Size distribution plots of nanoparticles of (**a**) Py-DOMC + 5 wt. % Tween20 and (b) linear TCNQ-CT + 5 wt. % Tween20 at a concentration of 0.1 g/L after dialysis to 1X PBS pH 7.4.

Table S2. Detailed size distribution results.

Sample	Number Mean	Intensity Mean	Volume Mean	PDI
Py-DOMC NPs (0.1 g/L water)	60 ± 12	184 ± 22	163 ± 86	0.24
TCNQ-CT NPs (0.1 g/L water)	36 ± 4	135 ± 108	70 ± 40	0.20
Py-DOMC + 5% Tween20 NPs (0.1 g/L PBS)	18 ± 12	190 ± 97	37 ± 33	0.40
TCNQ-CT + 5% Tween20 NPs (0.1 g/L PBS)	16 ± 6	49 ± 1	25 ± 5	0.19

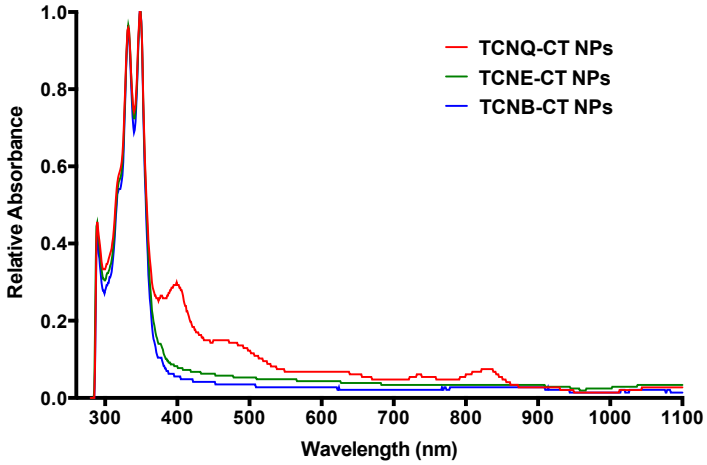

Figure S11. Absorbance measurements of nanoparticles formed from all acceptor-CT cellulose showed that only TCNQ had peaks in the expected region for CT complexes.

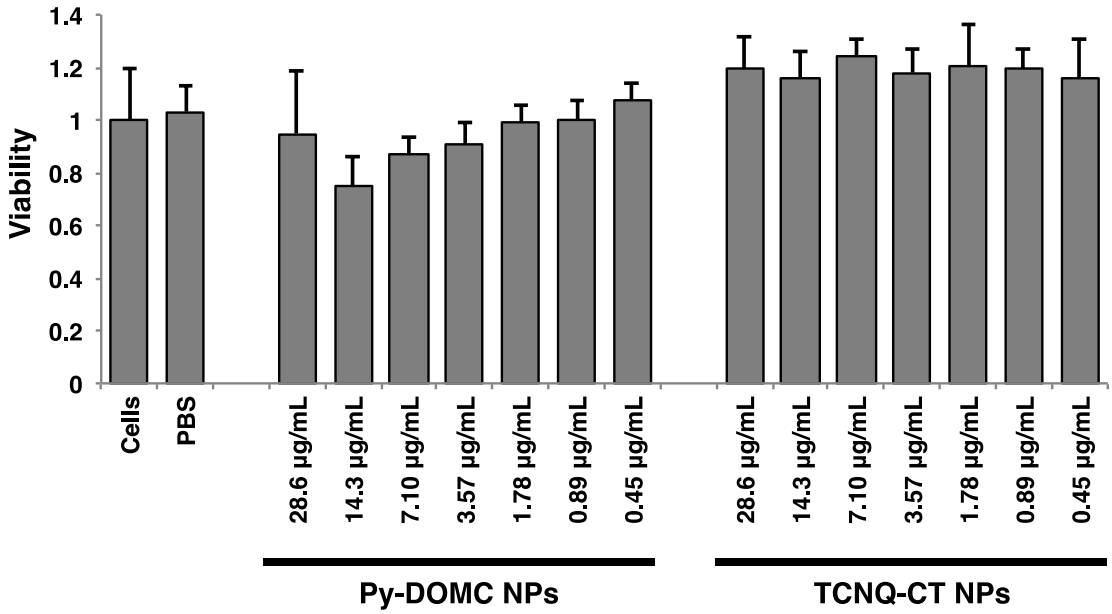

Figure S12. CellTiter-Glo assay to determine cytotoxicity of the TCNQ-CT NPs and Py-DOMC NPs. Formulations were prepared by nanoprecipitation in water at 0.1 g/L final concentration and dialyzed against 1X PBS for 4 h. The nanoparticles (80 µL) were added to HeLa cells in a 96-well plate in 200 µL growth medium and incubated for 36 h prior to reading the assay. Values are normalized to untreated cells and plotted +/- standard deviation (N=4 or 5).

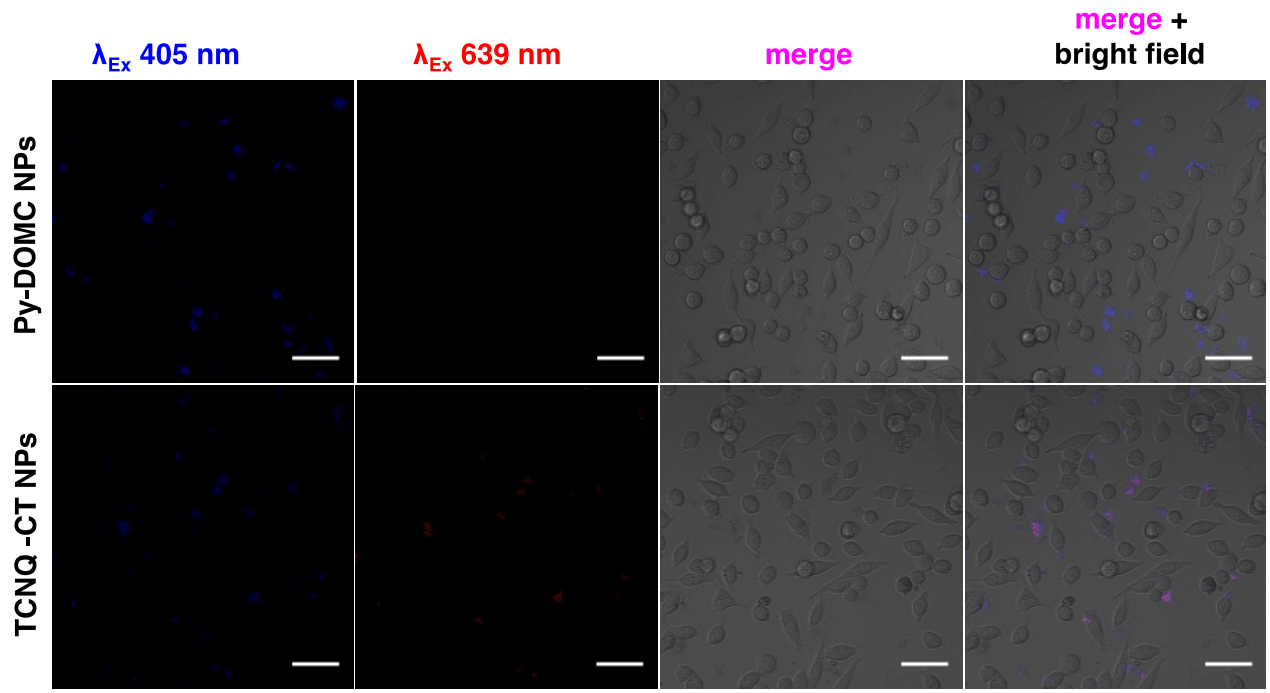

Figure S13. Imaging of the aggregates of Py-DOMC and TCNQ-CT in the presence of HeLa cells (20X objective). When Py-DOMC and TCNQ-CT were not in nanoparticle form, aggregation occurred in biological media. Nevertheless, strong aggregation-induced emission (AIE) was measured in serum-containing media and in the presence of cells. **Figure 3** in the main text shows cellular uptake of Py-DOMC and TCNQ-CT nanoparticles. Scale bar = 50 µm.