

Electronic Supplementary Information for :

Facile Fabrication of Positively-Charged Helical Poly(phenyl isocyanide)s Modified Multi-Stimuli-Responsive Nanoassembly Capable of High Efficiency Cell-Penetrating, Ratiometric Fluorescence Imaging, and Rapid Intracellular Drug Release

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Instruments

The ^1H nuclear magnetic resonance (NMR) spectra were recorded using a Bruker 600 MHz spectrometer operated in the Fourier Transform mode. Chemical shifts are reported in delta (δ) units and expressed in parts per million (ppm) downfield from tetramethylsilane using the residual proton solvent as an internal standard. Molecular weights and molecular weight distributions were determined using a size exclusion chromatograms (SEC) equipped with a Waters 1515 pump and a Waters 2414 differential refractive index detector (set at 40 °C). A series of three linear Styragel columns (HR0.5, HR2, and HR4; 3.6×300 mm) was used at a temperature of 40 °C. The eluent used was THF at a flow rate of 0.3 mL/min. FT-IR spectra were recorded on Perkin-Elmer Spectrum BX FT-IR system using KBr pellets at 25 °C. Circular dichroism (CD) and UV-vis spectra were performed on JASCO J1500 and UNIC 4802 UV/vis double beam spectrophotometers, respectively. Quartz cells with 10.0 or 1.0 mm lengths were used in CD and UV-vis measurements. Fluorescence spectra were recorded using a RF-5301/PC (Shimadzu) spectrofluorometer. The temperature of the water-jacketed cell holder was controlled by a programmable circulation bath. The slit widths were set at 5.0 nm for both excitation and emission. Transmission electron microscopy (TEM) observations were conducted on a JEM-2100F electron microscope operating at an acceleration voltage of 200 kV. The samples for TEM observation were prepared by casting the corresponding solutions of polymers onto copper mesh grids and drying in air at room temperature. Dynamic light scattering (DLS) measurements were carried on a Nano-ZS90 Zetasizer of Malvern (UK) instrument, all data were averaged over three time measurements.

Materials

All solvents were obtained from Sinopharm. Co. Ltd. and were purified by the standard procedures before use. Ethylene glycol, α -bromoisobutyryl bromide, sodium azide and Nile red (NR) were purchased from Aladdin and Sigma-Aldrich and used as received without further purification. Water was deionized with a Milli-Q SP reagent water system (Millipore) to a specific resistivity of 18.0 M Ω cm. Tetraphenylethene

(TPE)-functionalized phenyl isocyanide monomers (TPEPI),¹ L-hydrophilic phenyl isocyanide monomer (HPPI),² H₂O₂-responsive phenyl isocyanide monomer (HBPI),³ pH-responsive monomer (TTAMA),⁴ light-responsive monomer (NBOC),⁵ alkynyl modified guanidinium compounds,⁶ Pd(II) containing RAFT chain transfer agent (BSTP-Pd(II)),⁷ and phenylethynyl Pd(II) complex⁸ were directly used as the same batch in our previously reported literatures.

Sample Preparation

Synthesis of 2-Hydroxyethyl 2-Bromo-2-Methylpropanoate (1). The synthesis procedure was shown in Figure S1a. Into a 250 mL dried round-bottom flask, anhydrous ethylene glycol (4.5 mL, 81 mmol), triethylamine (11.2 mL, 81 mmol), and dichloromethane (CH₂Cl₂; 40 mL) were added. The flask was put into an ice bath and stirred with magnetic stir bar. After that, 2-bromoisobutyryl bromide (9.0 mL, 73 mmol) in 10 mL of CH₂Cl₂ was added dropwise over 1 h. Then, the reaction mixture was stirred at room temperature for another 5 h. After removing the insoluble salts by suction filtration, the filtrate was concentrated and further purified by silica gel column chromatography using petroleum ether/ethyl acetate (v/v = 6/1) as the eluent. After removing all the solvents, the product **1** was obtained as a liquid (12.5 g, yield: 81 %). ¹H NMR (600 MHz, CDCl₃, 25 °C; Figure S1b): δ 4.29 (t, 2H, HOCH₂CH₂COO-), 3.82 (t, 2H, HOCH₂CH₂COO-), 2.47 (s, 1H, -OH), 1.92 (s, 6H, -C(CH₃)₂Br).

Synthesis of 2-Hydroxyethyl 2-Azido-2-Methylpropanoate (2). The synthesis procedure was shown in Figure S1a. Into a 100 mL dried round-bottom flask, compound **1** (4.0 g, 19 mmol) was added to a solution of sodium azide (1.48 g, 22.7 mmol) in 40 mL of DMF, and the reaction mixture was stirred for 12 h at room temperature. Then, 50 mL of CH₂Cl₂ was added and the solution was washed with water (3 × 50 mL) to remove the excess NaN₃ and DMF. The organic layers were dried over anhydrous MgSO₄, and CH₂Cl₂ was removed using a rotary evaporator. The product **2** was obtained as a liquid (3.02 g, yield: 92%). FT-IR (KBr, cm⁻¹, 25 °C; Figure S1d): 2110 (-N₃).

Synthesis of Azido-Functionalized Phenyl Isocyanide Monomers (PI(N₃)). The synthesis procedure was shown in Figure S1a. In a typical run, into a 50 mL round bottom flask, pentafluorophenyl (PFP) ester-functionalized phenyl isocyanide monomer (1.8 g, 5.8 mmol), compound **2** (1.20 g, 6.9 mmol), 4-dimethylaminopyridine (0.7 g, 5.8 mmol), and anhydrous THF (15 mL) were charged. The resulting mixture was stirred at 30 °C for 12 h. The solvent was removed by evaporation under reduced pressure. Then the residue was dissolved in CH₂Cl₂ (30 mL) and washed successively with brine (20 mL × 2). The solution was further dried over anhydrous MgSO₄. After filtration, the solvent was removed by rotary evaporation under reduced pressure. The crude product was purified by column chromatography using CH₂Cl₂ as eluent to afford PI(N₃) as a brown oil liquid (1.31 g, 75% yield). ¹H NMR (600 MHz, CDCl₃, 25 °C; Figure S1c): δ 8.07 and 7.45 (m, 4H, Ar-H), 4.58 (t, 2H, HOCH₂CH₂COO-), 4.53 (t, 2H, HOCH₂CH₂COO-), 1.92 (s, 6H, -C(CH₃)₂N₃).

Synthesis of Light-Responsive PNBOC-PTPEPI-PHPPI (S1). The synthesis procedure was shown in Scheme S1. A solution of NBOC (92.49 mg, 0.3 mmol; [M]₀/[I]₀ = 50) in 1,4-dioxane (1.0 mL) was added to a degassed 1,4-dioxane solution (1.0 mL) of BSTP-Pd(II) (4.12 mg, 0.006 mmol) and azoisobutyronitrile (AIBN; 0.5 mg, 0.003 mmol). The reaction flask was then immersed into an oil bath at 80 °C and stirred for 36 h in dark. After cooled to room temperature, the polymerization solution was precipitated into an excess of cold methanol. The generated polymer was isolated via centrifugation. After dried under vacuum, PNBOC-Pd(II) was obtained. The SEC trace was shown in Figure S2a and ¹H NMR spectrum was shown in Figure S3.

Then, the one-pot sequential copolymerization of phenyl isocyanide monomers with PNBOC-Pd(II) complex as a single catalyst was performed, the synthesis procedure was shown in Scheme S1. In a typical run, a 20 mL oven-dried flask was charged with PNBOC-Pd(II) catalyst (54.54 mg, 0.004 mmol), TPEPI (28.65 mg, 0.06 mmol; [M]₀/[I]₀ = 15), and anhydrous methylbenzene (5.0 mL). The mixture was allowed to stir at 55 °C in dark, and the copolymerization progress was monitored by size exclusion chromatograms (SEC) until the molecular weight of PNBOC-PTPEPI

ceased to increase ($M_n = 15.5$ kDa, $M_w/M_n = 1.19$; Figure S2a). Subsequently, under nitrogen atmosphere, HPPI (89.80 mg, 0.22 mmol; $[M]_0/[I]_0 = 55$) was added via a double-tipped needle, and the copolymerization was stirred at 55 °C for another 24 h in dark. After the reaction was complete, the copolymerization solution was precipitated into a large amount of cold diethyl ether, collected by centrifugation, and dried in vacuum at ambient temperature overnight to afford the final light-responsive block copolymer PNBOC-PTPEPI-PHPPI (S1). The molecular weight and molecular weight distribution of P1 were determined by SEC, revealing a M_n of 38.5 kDa and an M_w/M_n of 1.20. The chemical structure was also confirmed by ^1H NMR (Figure S2b) and the actual DP were determined to be PNBOC₄₂-PTPEPI₁₀-PHPPI₅₁ (S1) based on ^1H NMR and SEC results (Figure S2a).

Synthesis of pH-Responsive PTTAMA-PTPEPI-PHPPI (S2). Following the same procedure, the other pH-responsive block copolymers were also synthesized with the initial feed amount of TTAMA (136.05 mg, 0.3 mmol; $[M]_0/[I]_0 = 50$), BSTP-Pd(II) (4.12 mg, 0.006 mmol), AIBN (0.5 mg, 0.003 mmol), and 1,4-dioxane (2.0 mL). The synthesis procedure was shown in Scheme S1. The generated PTTAMA-Pd(II) was isolated and the SEC trace was shown in Figure S4a. Then, PTTAMA-Pd(II) catalyst (75.30 mg, 0.004 mmol), anhydrous methylbenzene (5.0 mL), TPEPI (28.65 mg, 0.06 mmol; $[M]_0/[I]_0 = 15$), HPPI (89.80 mg, 0.22 mmol; $[M]_0/[I]_0 = 55$) and were sequentially added. The molecular weight and molecular weight distribution of S2 were determined by SEC (Figure S4a), revealing a M_n of 42.0 kDa and an M_w/M_n of 1.16. The chemical structure was also confirmed by ^1H NMR (Figure S4b), and the actual DP were determined to be PTTAMA₄₀-PTPEPI₉- PHPPI₄₈ (S2) based on ^1H NMR and SEC results.

Synthesis of H₂O₂-Responsive PHBPI-PTPEPI-P[HPPI-(N₃)] (S3). The one-pot sequential copolymerization of HBPI, TPEPI, HPPI, and PI(-N₃) monomers with phenylethynyl Pd(II) complex as a single catalyst was performed in a similar way to the previously reported reference.⁸ Briefly, a 20 mL oven-dried flask was charged with phenylethynyl Pd(II) (2.0 mg, 0.004 mmol), HBPI (72.64 mg, 0.2 mmol; $[M]_0/[I]_0 = 50$), and anhydrous methylbenzene (5.0 mL). The mixture was allowed to

stir at 80 °C, after the molecular weight of PHBPI-Pd(II) ceased to increase, TPEPI (30.56 mg, 0.064 mmol; $[M]_0/[I]_0 = 16$), and a mixture of HPPI (97.97 mg, 0.24 mmol; $[M]_0/[I]_0 = 60$) and PI(-N₃) (9.67 mg, 0.032 mmol; $[M]_0/[I]_0 = 8$) were consecutively added into the mixture via a double-tipped needle. The synthesis procedure was shown in Scheme S2. The copolymerization progress was also monitored by SEC (Figure S5a) until the molecular weight ceased to increase, revealing a M_n of 42.3 kDa and an M_w/M_n of 1.17. The actual DP was determined to be PHBPI₄₃-PTPEPI₁₀-P[HPPI₅₀-(N₃)₅] (S3).

Synthesis of Positively Charged H₂O₂-Responsive PHBPI-PTPEPI-P[HPPI-(Gu)] (S4). This polymer was synthesized through a classical “click chemistry” between S3 and alkynyl modified guanidinium compounds. Typically, S3 (0.4 g, 0.01 mmol; [-N₃] = 0.05 mmol), alkynyl modified guanidinium (0.1 mmol), DBU (0.1 mmol), and 20 mL of DMF were charged in a 50 mL of reaction flask. Under the atmosphere of N₂, CuI (0.1 mmol) was added and the reaction mixture was allowed to stir at 40 °C for 48 h. Then, the mixture was precipitated with large amounts of anhydrous diethyl ether to give the final PHBPI-PTPEPI-P[HPPI-(Gu)] copolymers. The chemical structure was also confirmed by SEC (Figure S5a) and ¹H NMR (Figure S5b) and the actual DP were determined to be PHBPI₄₃-PTPEPI₁₀-P[HPPI₅₀-(Gu)₅].

Preparation of Nile Red (NR) and Anti-Cancer Drug (CPT) Loaded Polymeric Micelles. Hydrophobic NR and CPT molecules could be loaded into the hydrophobic cores during the solvent selective self-assembly. Taking PHBPI-PTPEPI- P[HPPI-(Gu)] as an example. Typically, the DMF solutions of polymer (10.0 g/L) and NR (1.0 g/L) were prepared in advance and mixed together. Under vigorous stirring, DI water was added via a syringe pump at a flow rate of 0.05 mL/min. After the addition was completed, the dispersion was left stirring for another 3 h. DMF was then removed by dialysis (MWCO 3.5 kDa) against pure water for 24 h. Fresh water was replaced approximately every 6 h. The obtained dispersion with a characteristic of colloidal aggregates did not exhibit any macroscopic phase separation upon standing at room temperature for more than a week, suggesting the formation of stable aggregates. To determine the contents of NR, an aliquot of NR@PHBPI-PTPEPI- P[HPPI-(Gu)]

dispersion was dissolved in DMSO. The NR encapsulation efficiency and loading content were calculated to be ~ 75.2 wt/wt % and ~ 3.36 wt/wt % based on the absorbance of NR against its standard calibration curves. Encapsulation efficiency (%) = (weight of molecules in the micelles / weight of the feeding molecules) $\times 100\%$; Loading content (%) = (weight of molecules) / (weight of molecules + weight of polymer) $\times 100\%$. Following the same procedure, the other samples NR@PTTAMA-PTPEPI-PHPPI and NR@PNBOC-PTPEPI-PHPPI were also prepared.

Preparation of NR and Anti-Cancer Drug (CPT) Loaded Complex Nanoassembly. The CPT/NR@nanoassembly fabricated by the three different kinds of stimuli-responsive copolymers were as follows: the DMF solutions of PTTAMA-PTPEPI-PHPPI (10 g/L), PNBOC-PTPEPI-PHPPI (10 g/L), PHBPI-PTPEPI-P[HPPI-(Gu)] (10 g/L), NR (1.5 g/L), and CPT (1.5 g/L) were prepared in advance and mixed together. Under vigorous stirring, DI water was added via a syringe pump at a flow rate of 0.05 mL/min. After the addition was completed, the dispersion was left stirring for another 5 h. DMF was then removed by dialysis (MWCO 3.5 kDa) against pure water for 24 h. Fresh water was replaced approximately every 6 h. The obtained dispersion of CPT/NR@nanoassembly with a characteristic of colloidal aggregates did not exhibit any macroscopic phase separation upon standing at room temperature for more than a week, suggesting the formation of stable aggregates. The encapsulation efficiency (%) and loading content (%) of NR and CPT were also calculated by using the above mentioned method.

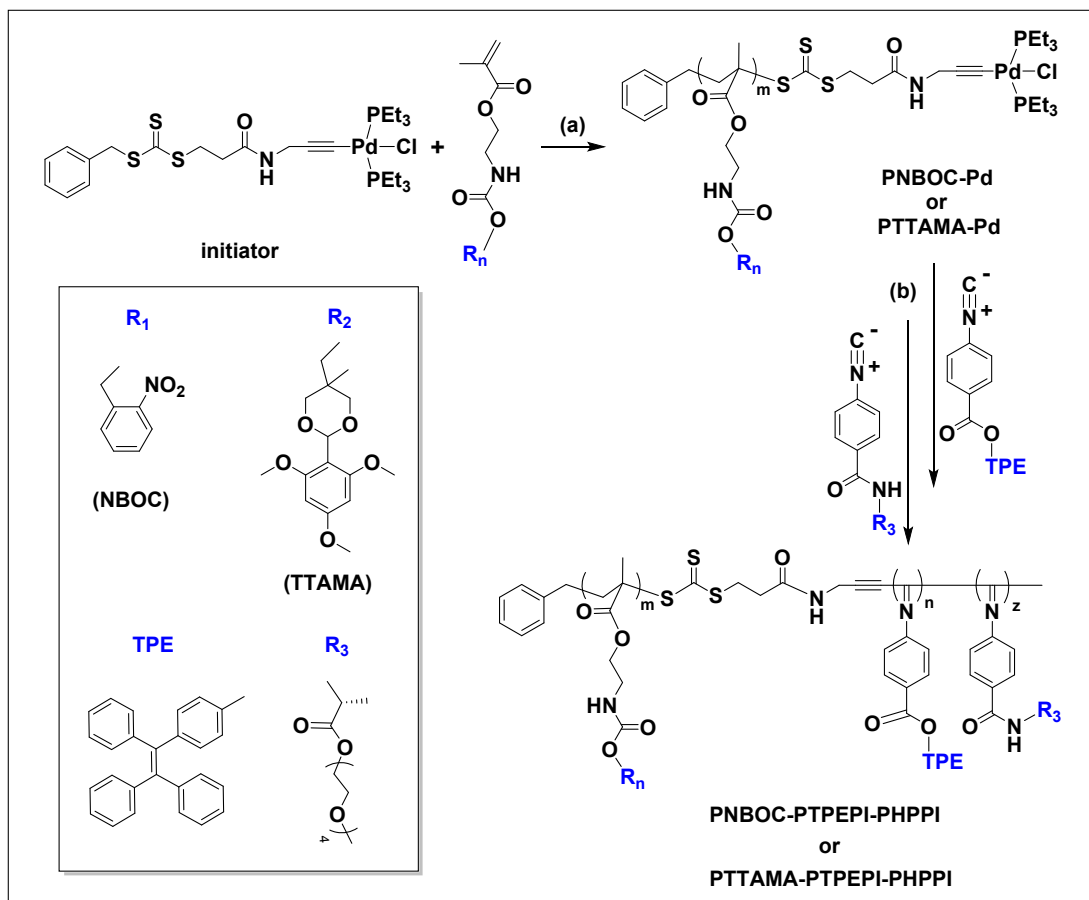
In Vitro Cargo Release Profile. The cargo release from CPT/NR@nanoassembly was measured by the dialysis method. Briefly, the nanoassembly dispersion (1.0 g/L; 10.0 mL) was placed in a dialysis tube (MWCO 3.5 kDa) and then immersed into 500 mL of water with Tween 20 (1% total volume) under gentle stirring at 37 °C. Then, the system was treated by H₂O₂ (50 mM), pH (5.5), and 365 nm UV light in turn or simultaneously according to the need. At different time intervals, 20 mL external water solution was removed and replaced with equal volume of fresh water. The separated solution was lyophilized and then dissolved in DMSO, the cargo concentration was quantified by measuring the absorbance against a standard

calibration curve.

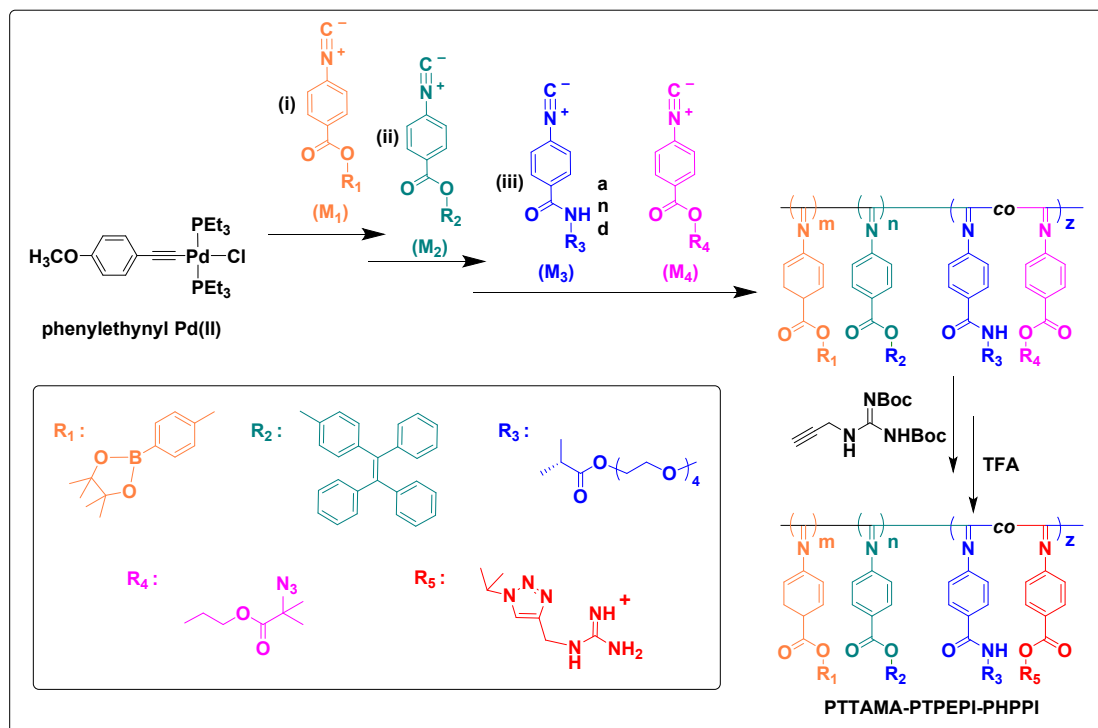
Cell Culture and in Vitro Cytotoxicity Assessment. HeLa cells (5×10^3 cells/well) in Dulbecco's modified Eagle's medium (DMEM) complete medium were plated into a 96-well plate and incubated overnight. HeLa cells were then exposed to different micelles with different concentrations at 37 °C for up to 10 h in DMEM complete medium. Then, cells were rinsed with PBS buffer and DMEM complete medium. Cytotoxicity was assessed by adding 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) for another 4h. Cells incubated with PBS only were served as positive control.

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Scheme S1. Synthetic routes employed for the preparation of pH-responsive PTTAMA-PTPEPI-PHPPI and light-responsive PNBOC-PTPEPI-PHPPI block copolymers through the combination of successive reversible addition-fragmentation chain transfer (RAFT) polymerization and Pd(II)-catalyzed living polymerization.



Scheme S2. Synthetic routes employed for the preparation of H_2O_2 -responsive PHBPI-PTPEPI-P[HPPI-(Gu)] block copolymers through the successive Pd(II)-catalyzed living polymerization.

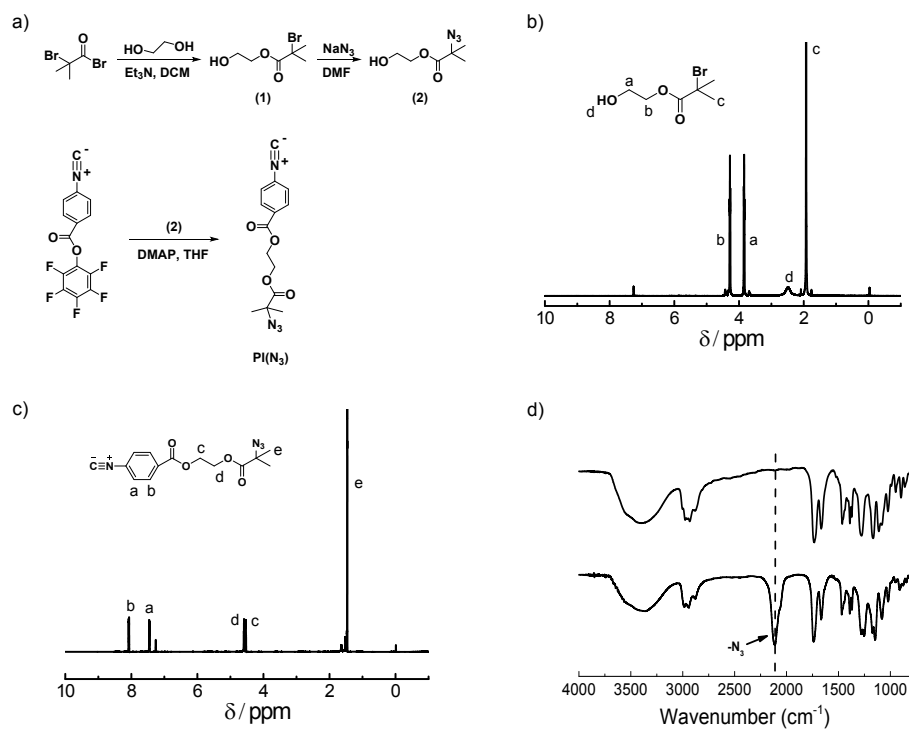


Figure S1. (a) Synthetic routes employed for the preparation of azide functionalized phenyl isocyanide monomers (PI(-N₃)). ¹H NMR spectra obtained for the intermediate compound 1 (b) and the final PI(-N₃) (c). (d) FT-IR spectra obtained for compounds 1 (up) and 2 (down) at 25 °C using KBr pellets.

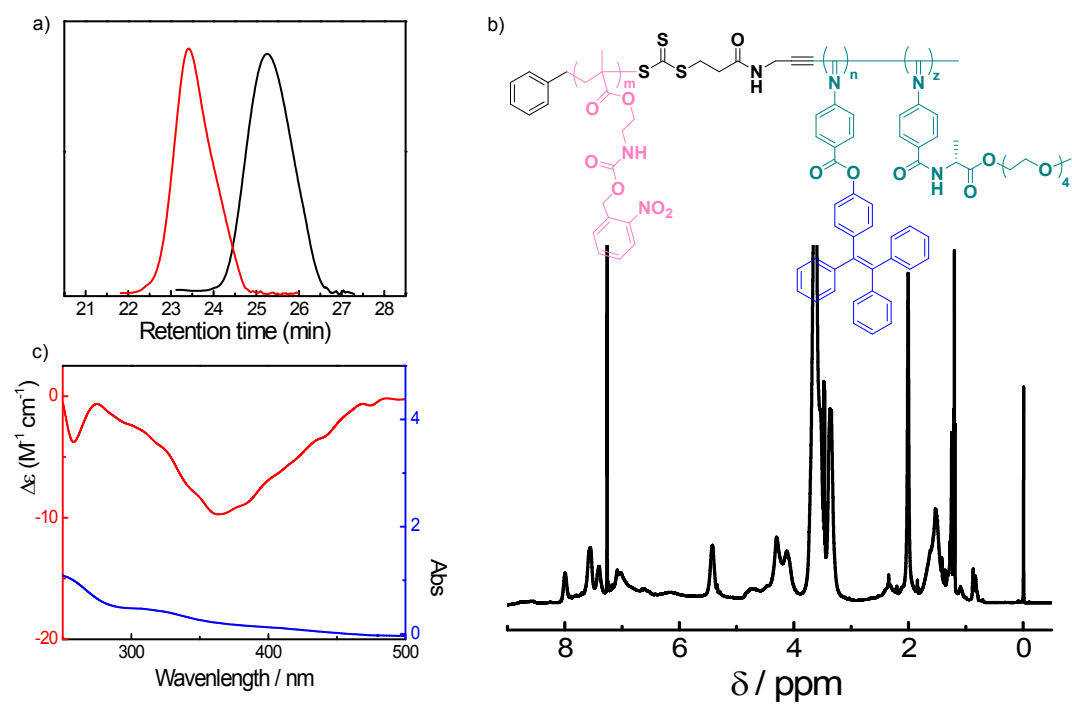


Figure S2. (a) SEC (red line), (b) ¹H NMR, and (c) CD spectra obtained for the light-responsive PNBOC-PTPEPI-PHPPI (S1 in Table 1) block copolymers. The black line in (a) showed the SEC trace for PNBOC-Pd(II) intermediate.

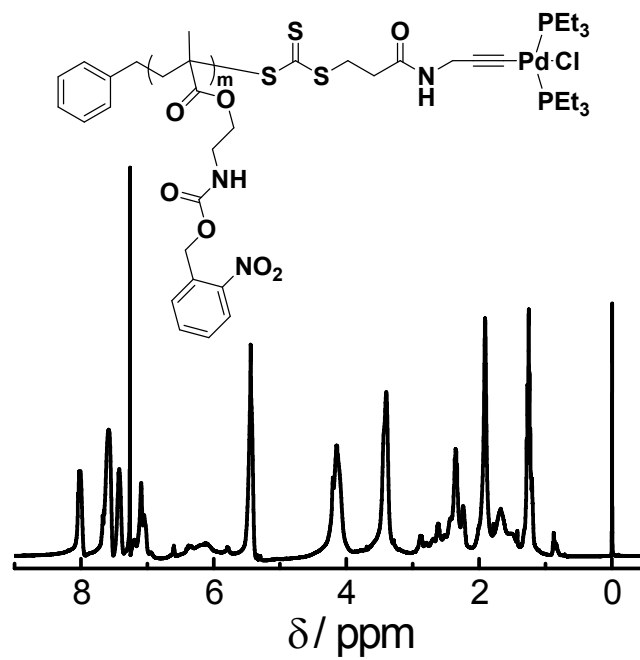


Figure S3. ¹H NMR spectrum obtained for the light-responsive PNBOC-Pd(II) intermediate.

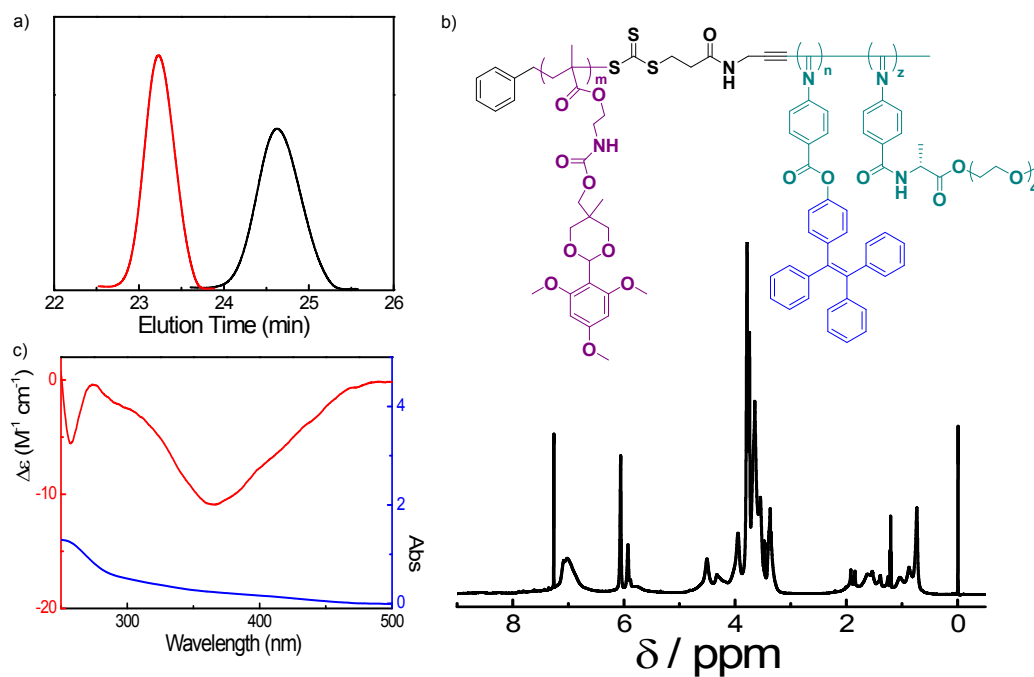


Figure S4. (a) SEC (red line), (b) ¹H NMR, and (c) CD spectra obtained for the pH-responsive PTTAMA-PTPEPI-PHPPI (S2 in Table 1) block copolymers. The black line in (a) showed the SEC trace for PTTAMA intermediate.

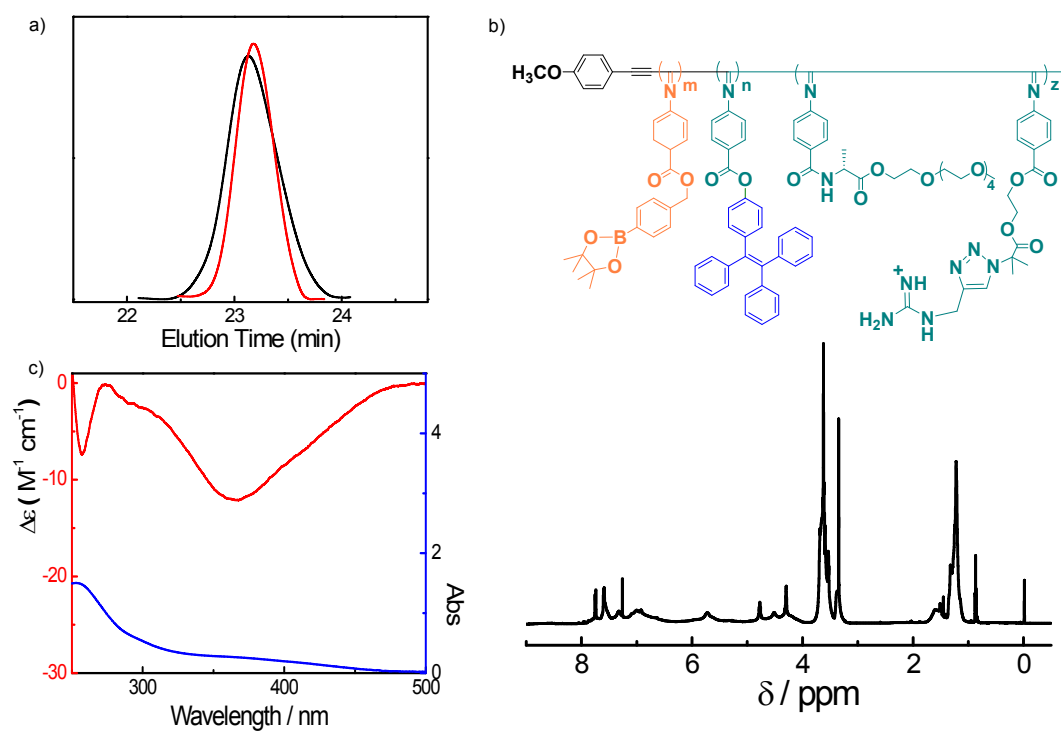


Figure S5. (a) SEC (black line), (b) ^1H NMR, and (c) CD spectra obtained for the H_2O_2 -responsive PHBPI-PTPEPI-P[HPPI-(Gu)] (S4 in Table 1) block copolymers. The red line in (a) showed the SEC trace for PHBPI-PTPEPI-P[HPPI-(N_3)] (S3 in Table 1) block copolymers.

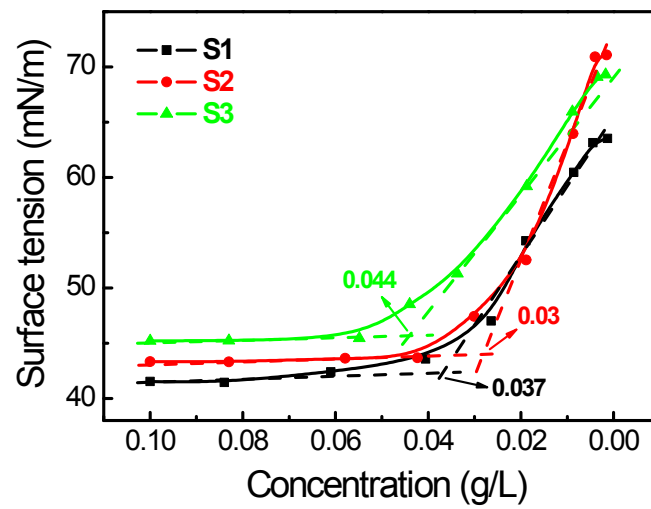


Figure S6. Surface intension of micelles prepared by S1 (black line), S2 (red line), and S4 (green line) in water as a function of concentration at 25 °C.

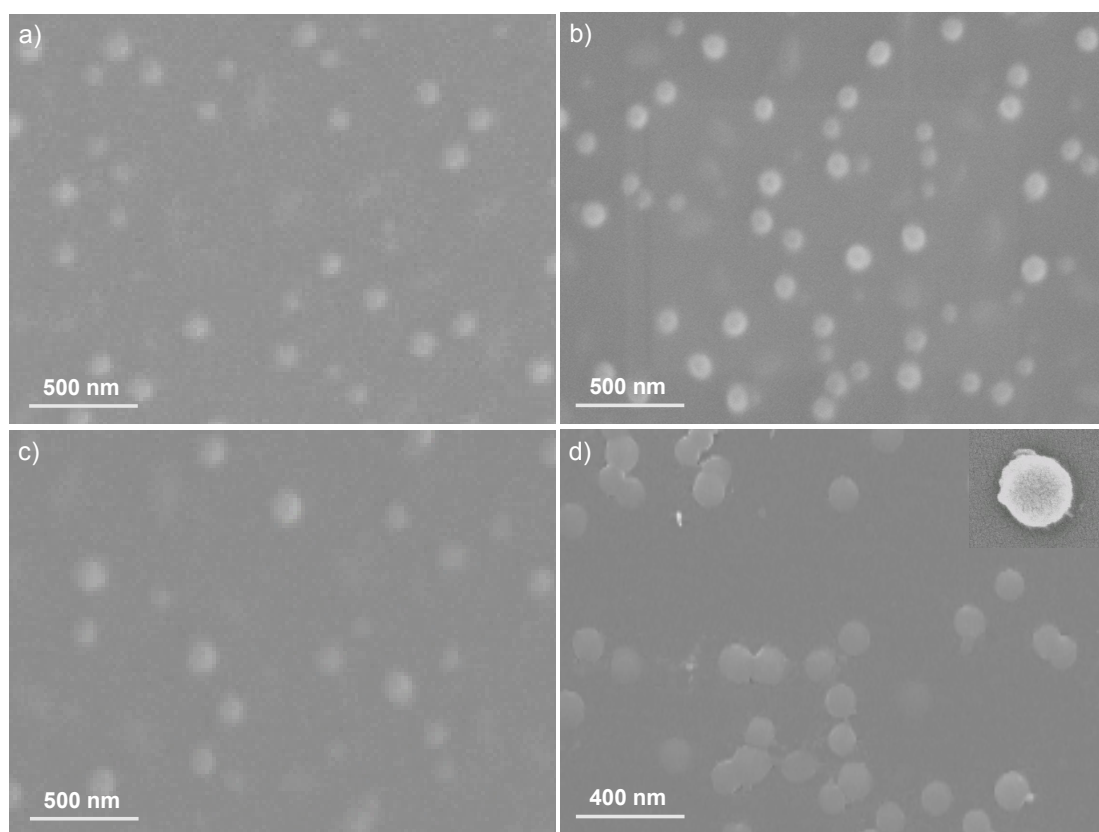


Figure S7. SEM images obtained for the spherical morphology of (a) PHBPI-PTPEPI-P[HPPI-(Gu)], (b) PTTAMA-PTPEPI-PHPPI, (c) PNBOC-PTPEPI-PHPPI block copolymers and (d) CPT/NR@nanoassembly complex dried from water.

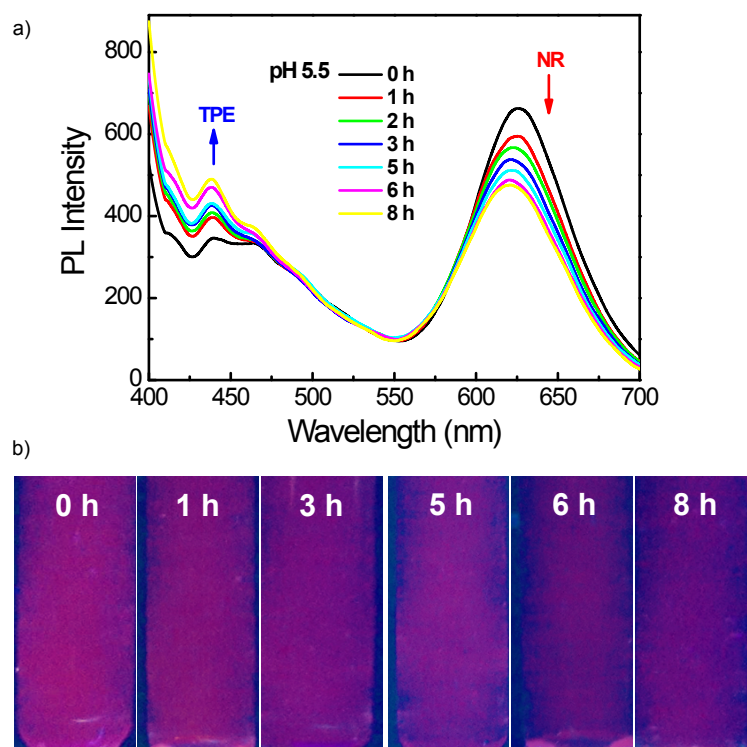


Figure S8. (a) Fluorescence emission spectra obtained for the aqueous dispersion of NR@nanoassembly at different times in the exposure of HCl (pH 5.5). (b) Typical fluorescent photographs taken under 365 nm UV irradiation from a hand-held UV lamp.

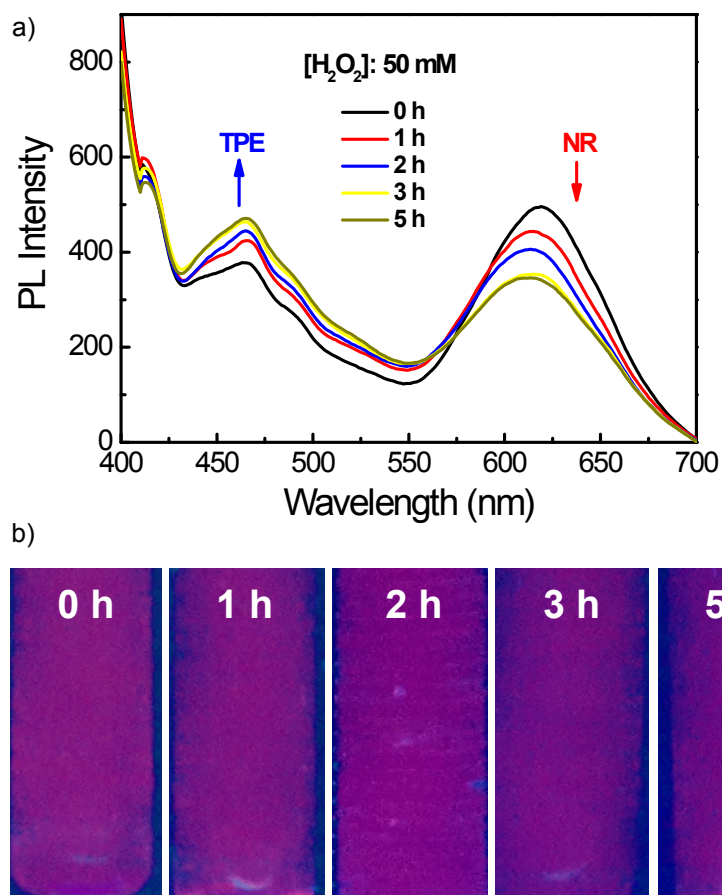


Figure S9. (a) Fluorescence emission spectra obtained for the aqueous dispersion of NR@nanoassembly at different times in the exposure of H₂O₂ (50 mM). (b) Typical fluorescent photographs taken under 365 nm UV irradiation from a hand-held UV lamp.

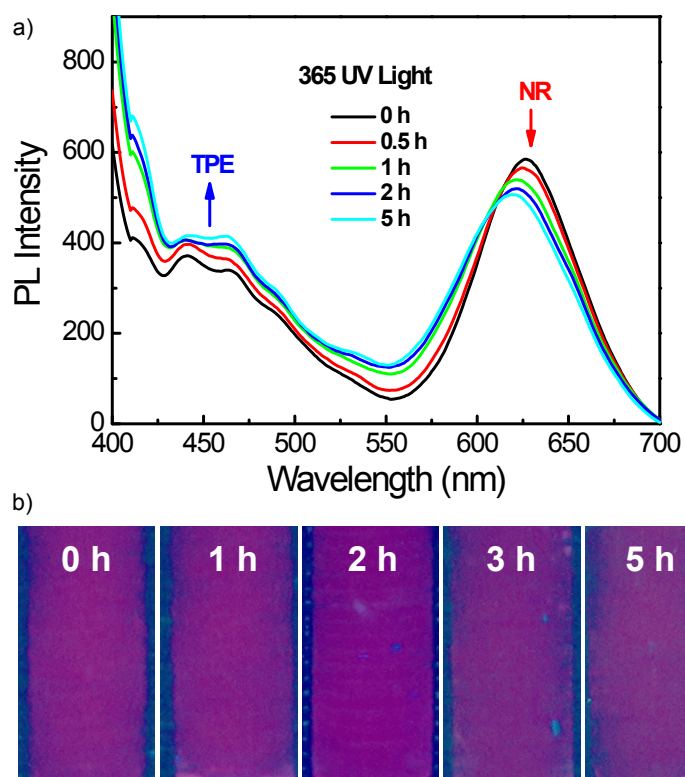


Figure S10. (a) Fluorescence emission spectra obtained for the aqueous dispersion of NR@nanoassembly at different times in the exposure of 365 nm UV light. (b) Typical fluorescent photographs taken under UV irradiation from a hand-held UV lamp.

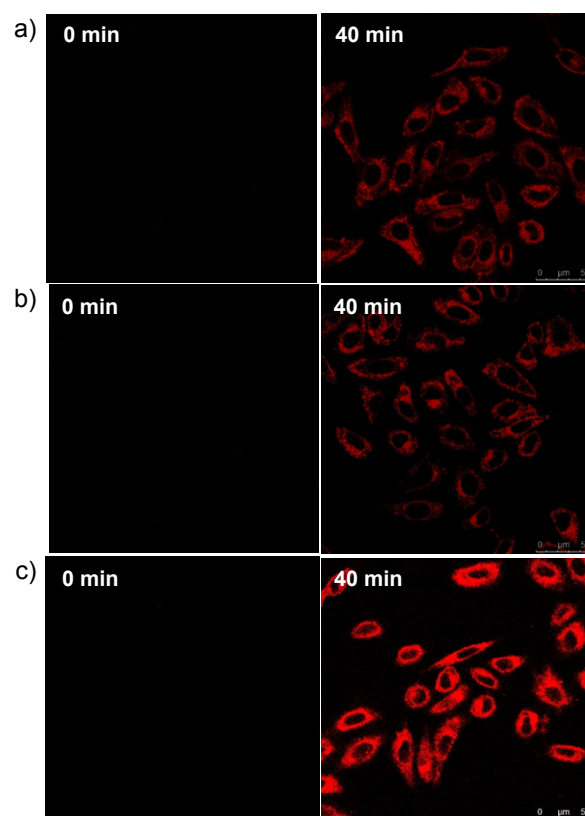


Figure S11. Incubation duration-dependent CLSM images of live HeLa cells when culturing at 37 °C with (a) NR@S1, (b) NR@S2, and (c) NR@S4. The red channel was excited at 550 nm and collected between 580 and 700 nm.

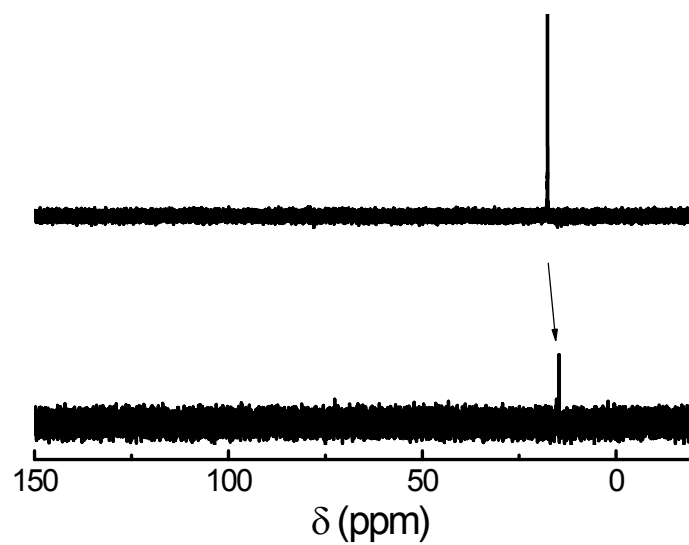


Figure S12. ^{31}P NMR spectra obtained for BSTP-Pd(II) (top) and PNBOC₄₂-PTPEPI₁₀-PHPPI₅₁ (down) in CDCl₃ at room temperature.

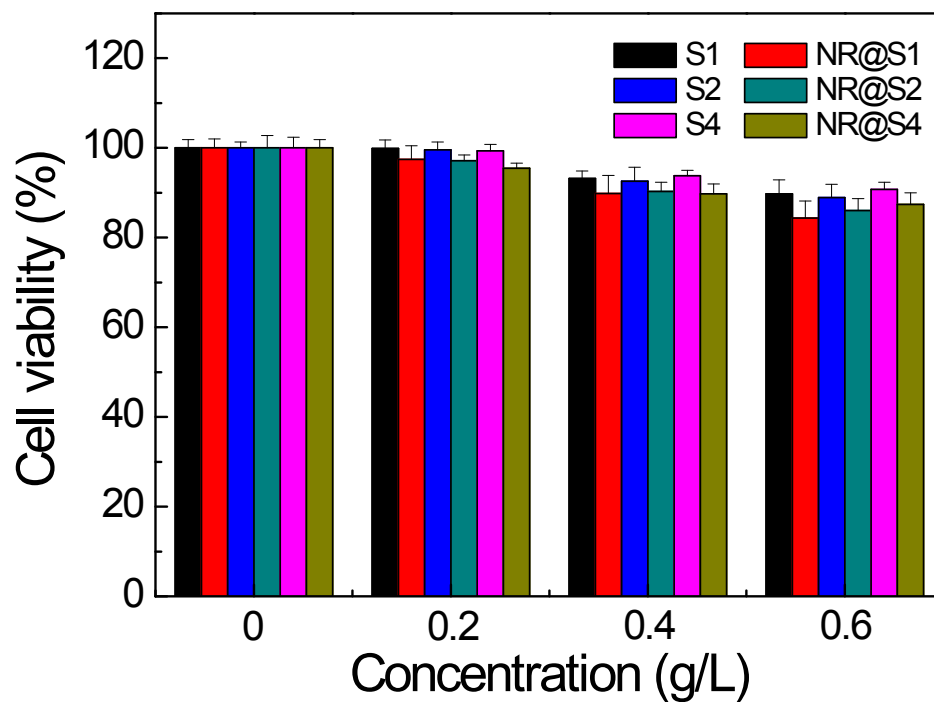


Figure S13. Viability of HeLa cells after being incubated with different NR loaded nanoassemblies with various concentrations (0-0.6 g/L) for 12 h. The error bars are based on the standard deviations of four parallel samples.

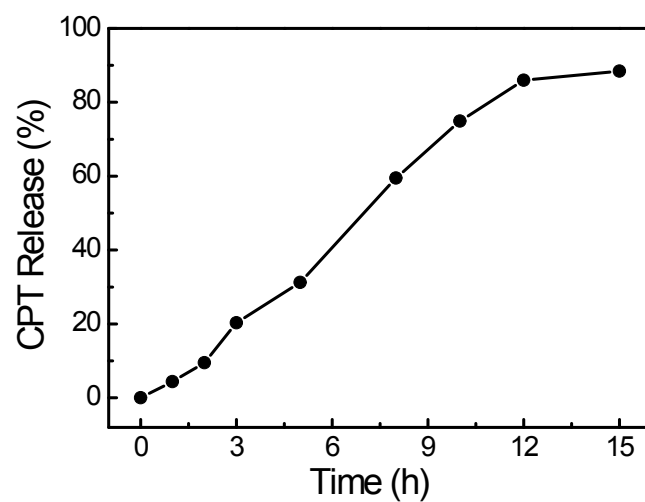


Figure S14. In-vitro CPT release profile of the aqueous dispersion of CPT/NR@nanoassembly at different times in the simultaneous exposure of H₂O₂ (50 mM), HCl (pH 5.5), and UV light (365 nm).