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Supporting Information

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Targeted Charge Reversal Nanoparticles for Nuclear Drug Delivery

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Experimental Procedures

Materials N-hydroxysuccinimide (NHS) (98%) was purchased from Alfa Aesar. Polyethylenimine (PEI, branched, Mw 1.8 KDa, 25% NH_2, 50% NH and 25% N) was purchased from Polysciences. ε-Caprolactone (ε-CL) (Aldrich) was dried over calcium hydride. Dimethyl sulfoxide (DMSO) and octanoic acid (Aldrich) were dried over 4 Å molecular sieve. All other chemicals otherwise stated were from Aldrich and used without further purification.

Polymer Synthesis (Scheme S1)

Synthesis of poly(ε-caprolactone) (PCL) (a): ε-Caprolactone (ε-CL) (12.5 mL, 113mmol) and octanoic acid (1.75 mL, 11 mmol) were charged into a flask. The flask was sealed with a rubber septum and degassed. It was heated at 225 °C for 3.5 h with magnetic stirring. The solid was cooled to about 60 °C and dissolved in THF. The solution was poured into 10-fold cold methanol to remove the unreacted monomer. The solid was isolated and purified by reprecipitation. It then was dried under high vacuum at 60 °C. PCL with a terminal carboxylic acid (PCL-COOH) (11.8 g, yield 47%) was obtained. ^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm): 4.08 (t), 2.32 (t), 1.71-1.57 (m), 1.42-1.34 (m), 0.88 (t). Its molecular weight was 3.8 KDa determined by NMR, and 3.2KDa determined by gel permeation chromatography with polydispersity index of 1.15.

Synthesis of PCL-COO-NHS (b): PCL-COOH (2.45 g, 0.64 mmol), N-hydroxysuccinimide (NHS, 0.38 g, 3.3 mmol) and 1,3-dicyclohexylcarbodiimide (DCC, 0.67 g, 3.3 mmol) were charged into a 50 mL

flask and dissolved in 20 mL of dichloromethane. The reaction solution was stirred at room temperature for 48 h, and then filtered. The filtrate was poured into a large excess of dry ether. The solid was isolated and reprecipitated twice. The product was dried under high vacuum at room temperature for 8 h. PCL with a terminal NHS ester (PCL-COO-NHS) was obtained (1.74 g, 71%). ^{1}H-NMR (400 MHz, CDCl3): δ (ppm): 4.08 (t), 2.85 (m), 2.32 (t), 1.71-1.57 (m), 1.45-1.32 (m), 0.88 (t).

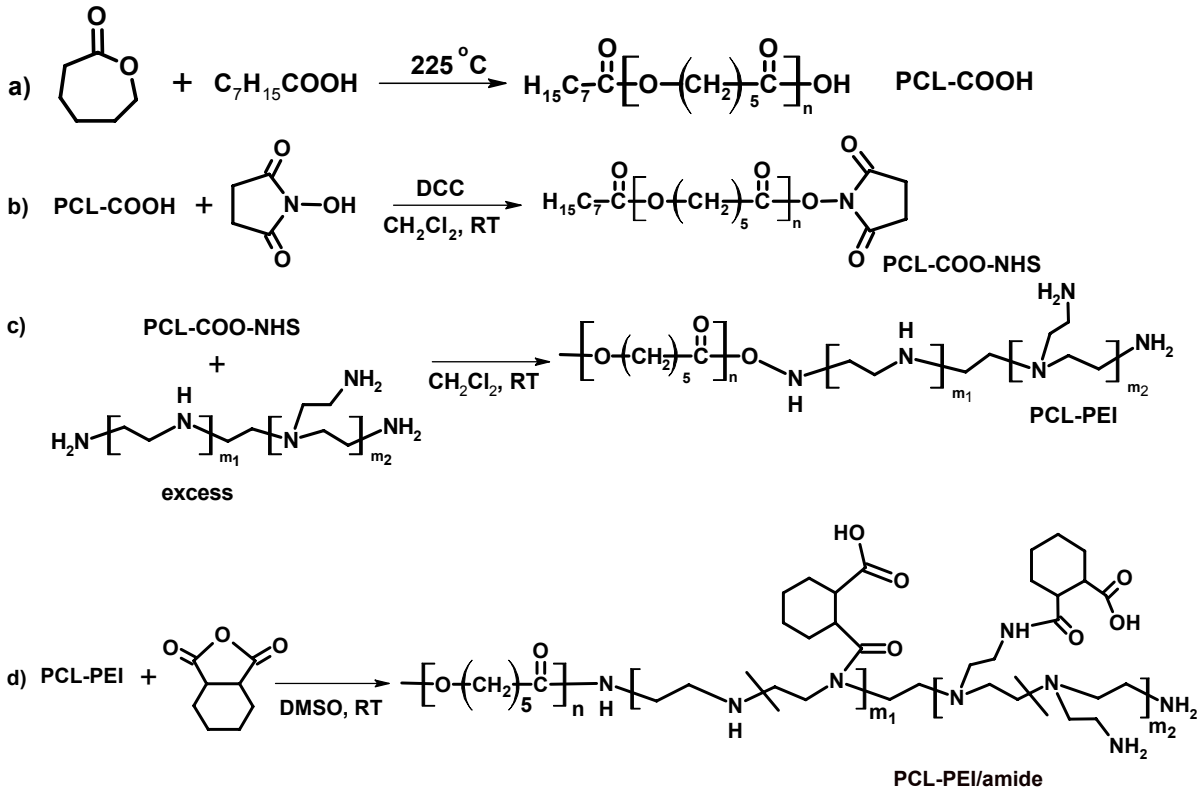
Synthesis of PCL-PEI (c): PCL-COO-NHS (1.74 g, 0.45 mmol) and PEI (Mn of 1.8KDa, 8.3 g, 4.6 mmol) were separately dissolved in 20 mL of dichloromethane. The PCL-COO-NHS solution was dropwise added into the PEI solution with stirring. The reaction was continued with the protection of nitrogen at room temperature for 48 h. The solution was then washed twice with 100 mL of water. The organic phase was precipitated in diethyl ether and dried under high vacuum at 40 °C for 8 h. PCL-block-PEI (PCL-PEI) was obtained (0.92 g, 53% yield). ^{1}H NMR (400 MHz, CDCl3): δ (ppm): 4.08 (t), 2.81-2.60 (m), 2.32 (t), 1.69-1.61 (m), 1.43-1.30 (m), 0.88 (t). The chain ratio PCL/PEI calculated from the NMR spectrum was 1.07. GPC showed that there was no unreacted PCL in the block copolymer.

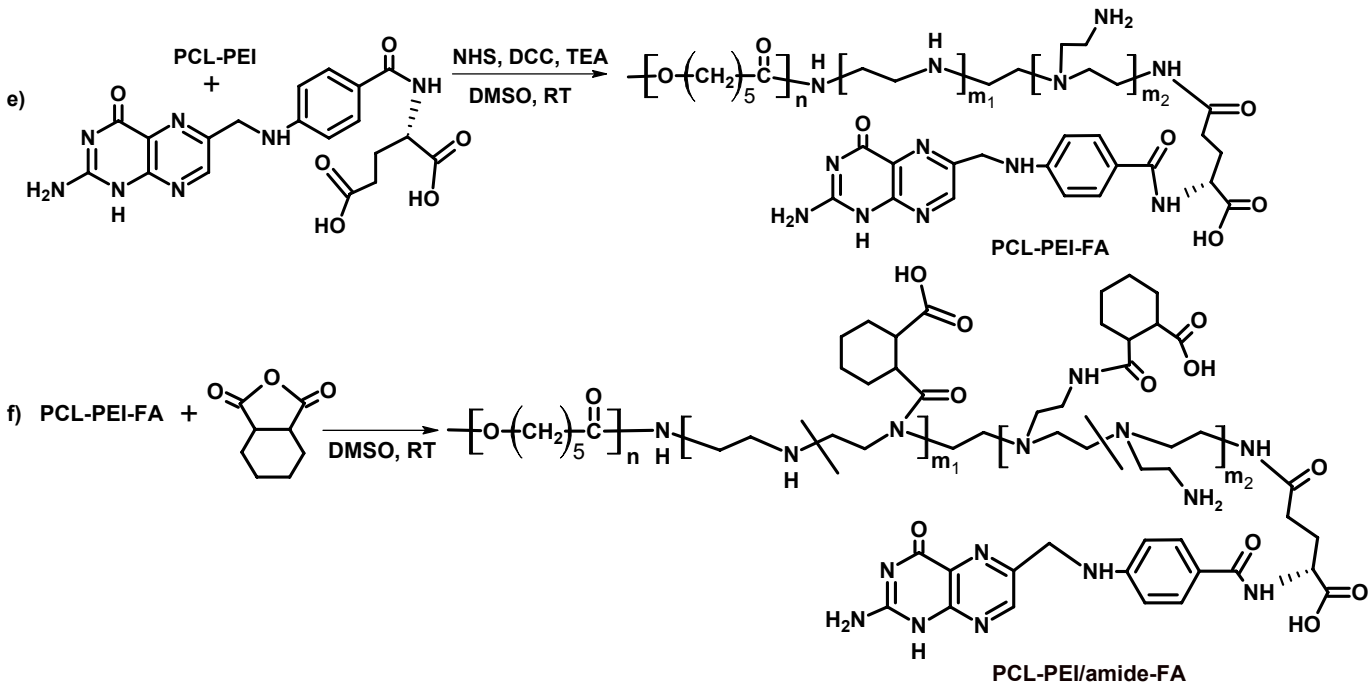
Synthesis of PCL-PEI/amide (d): PCL-PEI (0.1 g, 0.018 mmol, equivalent to 0.567 mmol of NH_2 and NH) was dissolved in 5 mL of DMSO in a 25 mL flask with a magnetic stirring bar. 1,2-*cis*-Cyclohexanedicarboxylic anhydride (16 mg, 0.10 mmol) was added. The reaction was kept at room temperature with the protection of nitrogen for 48 h. The mixture was precipitated in diethyl ether. The solid was isolated and purified by reprecipitation twice. It was dried under high vacuum at 40 °C for 8 h. PCL-PEI/amide was obtained (0.06 g, 60 % yield). ^{1} H NMR (400 MHz, d_6-DMSO): δ (ppm): 4.00 (t), 2.81-2.50 (m, overlapped with the solvent), 2.26 (t), 1.64-1.49 (m), 1.47-1.21 (m), 0.88 (t). The calculation from the ^{1}H-NMR spectrum showed that 23% of the amine groups (NH_2+NH) of the PEI block were converted to their amides.

Synthesis of PCL-PEI-FA (e): PCL-PEI (0.5 g, equivalent to 2.84 mmol of NH2 and NH), DCC (344.6 mg, 1.7 mmol), NHS (204 mg, 1.7 mmol), folic acid (39.3 mg, 0.09 mmol) were charged into a 25 mL flask and dissolved in 10 mL of DMSO. Triethylamine (TEA, 1.7 mL) was added to the solution. The reaction was kept at room temperature with the protection of nitrogen for 48 h. The mixture was

purified by repeated precipitated in diethyl ether. The raw product was further purified by dialysis in DI water (Spectra Por -7, MWCO 3,500) to remove the unreacted folic acid. The resulting product was dried under high vacuum at 40 °C for 8 h to give final product 0.35 g (yield 70%). ^{1}H-NMR (400 MHz, d_6-DMSO): δ (ppm): 8.52 (s), 8.00 (s), 7.56 (s), 6.62 (s), 4.42 (s), 3.98 (t), 2.81-2.50 (br, overlapped with the solvent), 2.26 (t), 1.64-1.49 (m), 1.47-1.21 (br), 0.88 (t). The ^{1}H-NMR spectrum showed that on average each PCL-PEI chain had 0.79 folic acid molecule.

Synthesis of PCL-PEI/amide-FA (f): PCL-PEI-FA (0.35 g, equivalent to 1.89 mmol of NH_2 and NH) was dissolved in 10 mL of DMSO in a 25 mL flask with a magnetic stirring bar. 1,2-*cis*-Cyclohexanedicarboxylic anhydride (52.5 mg, 0.34 mmol) was added. The reaction was kept at room temperature with the protection of nitrogen for 48 h. The mixture was precipitated in diethyl ether. The solid was isolated and purified by repeated precipitation. It was dried under high vacuum at 40 °C for 8 h. PCL-PEI/amide-FA was obtained (0.25 g, 71% yield). ^{1} H NMR (400 MHz, DMSO): δ (ppm): 8.65 (s), 8.14 (s), 7.65 (s), 6.64 (d), 4.49 (s), 4.02 (t), 2.80-2.45 (br, overlapped with the solvent), 2.27 (t), 1.74 (s), 1.64-1.49 (m), 1.47-1.21 (br), 0.88 (t). The ^{1}H-NMR spectrum showed that about 17.6% of the PEI amine groups were amidized.





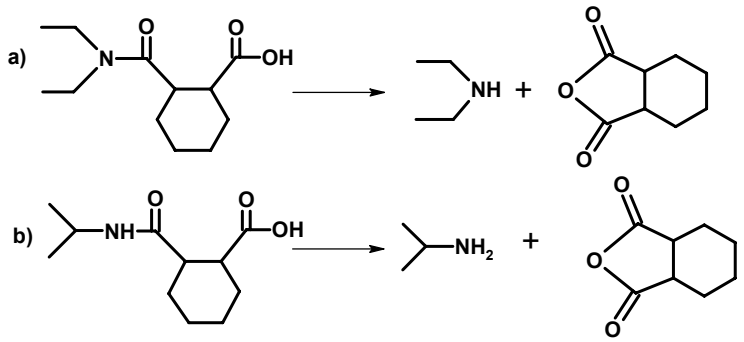
Scheme S1: Synthesis of folic acid functionalized poly(ε-caproactone)-block-polyethyleneimine (PCL-PEI-FA) and the subsequent amide product (PCL-PEI/amide-FA).

Model compound synthesis and hydrolytic kinetics measurement (Scheme 2):

Briefly, N, N-diethylamine (415 μL, 4 mmol) and 1,2- *cis*-cyclohexanedicarboxylic anhydride (617 mg, 4 mmol) were dissolved in 10 mL dichloride methane. The reaction was kept at room temperature for 2 h with stirring. The solvent was then removed by rotary evaporation to obtain the raw product. The raw product was purified by recrystallizing from benzene to get 2-[(diethylamino)carbonyl]cyclohexanecarboxylic acid. ^{1}H NMR (400 MHz, CDCl3): δ (ppm): 3.60-3.22 (m), 2.77 (m), 2.51-2.45 (m), 1.87-1.66 (m), 1.58-1.46 (m), 1.49-1.39 (m), 1.28 (q), 1.17 (t). Similarly, 2-[(isopropylamino)carbonyl]cyclohexanecarboxylic acid was synthesized. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm): 3.83 (q), 2.73 (q), 2.64 (q), 1.87 (m), 1.79 (m), 1.64 (m), 1.45 (m), 1.33 (m), 1.01 (d).

The hydrolysis of the model compounds was monitored by ^{1}H-NMR. Briefly, 2-[(isopropylamino)carbonyl]cyclohexanecarboxylic acid (10 mg) was dissolved in 2 mL D_2O. Sodium carbonate was used to adjust the solution pH to 5.0, 6.0 or 7.4 at 37 °C. At predesigned time intervals, ^{1}H-NMR spectra of the solution were measured on a Bruker Avance DRX-400 spectrometer. The hydrolysis

was monitored by measuring the integrations of the peaks at 3.77-3.9 ppm (CH in the amide of propan-2-amine) and the 3.3-3.5 ppm (CH in the propan-2-amine).



Scheme S2: Hydrolysis of model compounds: 2-[(diethylamino)carbonyl]cyclohexanecarboxylic acid and 2-[(isopropylamino)carbonyl]cyclohexanecarboxylic acid.

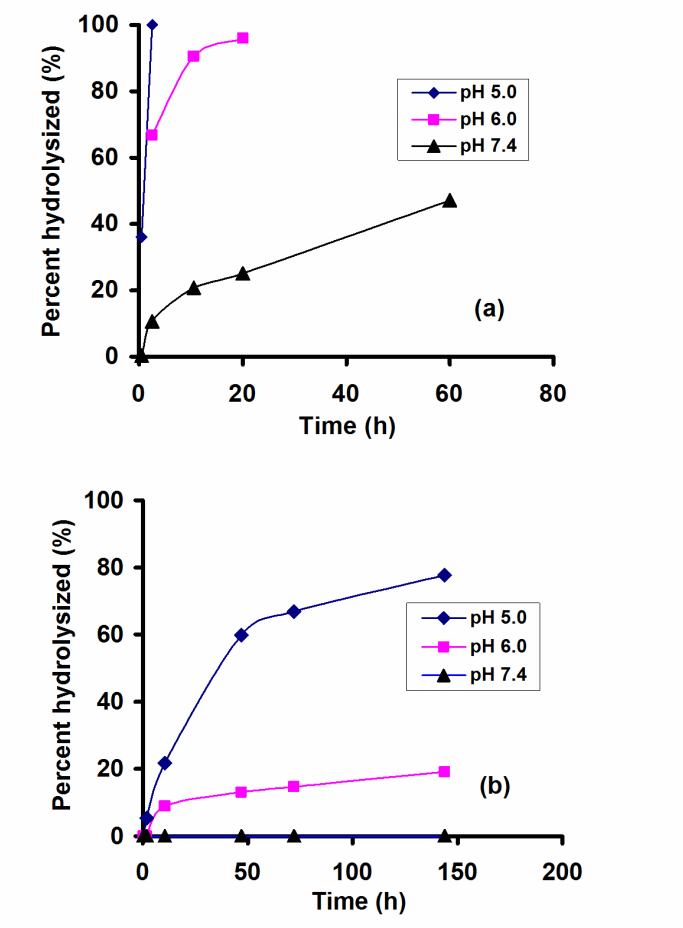


Figure S1. The hydrolysis kinetics of the model compounds 2-[(diethylamino) carbonyl] cyclohexanecarboxylic acid (a), 2-[(isopropylamino)carbonyl]cyclohexanecarboxylic acid (b) at 37 °C.

Transmission electron microscopy (TEM) of the nanoparticles. The nanoparticles solution (8 μL) was applied onto a 150-mesh carbon-coated copper grid for 10 s. The excess solution was wicked off with filter paper. This coating procedure was repeated 5 times. Images were recorded using a transmission electron microscope (HITACHI H-7000 TEM) operated at a voltage of 75 kV with an original magnification of 30,000 × or high resolution 100,000 ×.

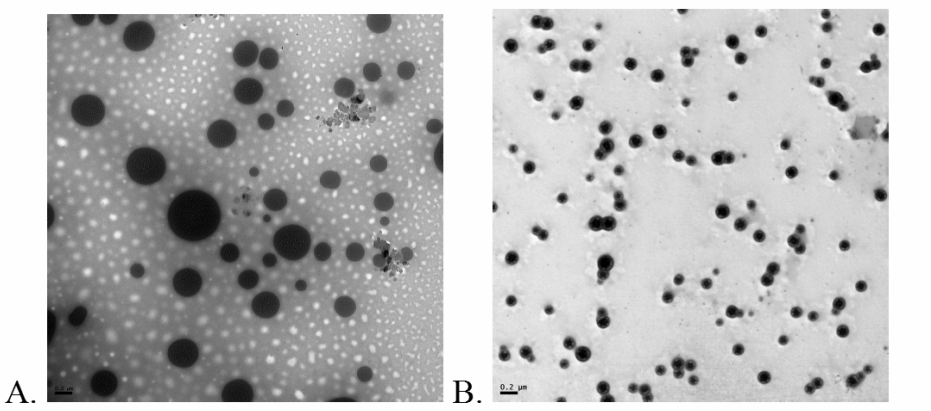


Figure S2. TEM images of PCL-PEI/amide-FA-formed nanoparticles (A) and the nanoparticles loaded with 14.6 wt% doxorubicin (TCRNs/DOX, B). Scale bars are 200 nm.

The amide hydrolysis kinetics of TCRNs: The hydrolysis of the amides in the TCRNs was monitored by ^{1}H-NMR. The TCRNs in DI water were prepared as described above. The nanoparticle solution was adjusted to pH of 5.0, 6.0 or 7.4, respectively, at a concentration of 1 mg/mL. DMF (1μl) was added to the solution as the internal standard. These solutions were immersed in a 37 °C water bath. At predesigned time intervals, the TCRN solution (0.5 ml) was sampled and filtrated using Centricon centrifugal filter devices (YM-3, 3,000 MWCO, Millipore Corp., Bedford, MA). The percentage of hydrolyzed amides was calculated from the integrations of the reference peak at 3.0-2.7 ppm (DMF signal) and the peak at 1.7-1.0 ppm of free 1,2-*cis*-cyclohexanedicarboxylic acid hydrolyzed from the amides.

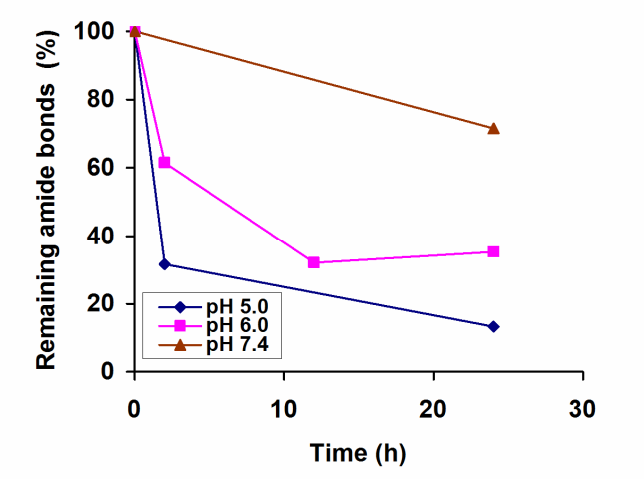


Figure S3. The overall hydrolytic kinetics of the amides in the PCL-PEI/amide-FA at different acidities at 37 ^{o}C.

TCRN size and zeta potential measurements. The sizes (diameter) of CRNs, TCRNs, and TCRNs/DOX nanoparticles were determined using a Nano-ZS zetasizer (Malvern Instrument Ltd., UK) with a laser light wavelength of 632.8 nm and a scattering angle at 173°. The nanoparticles were prepared as described above. The zetasizer was routinely calibrated with a 60 nm nanosphere™ standard (Duke Scientific Corp. CA). Each measurement was performed in triplicate, and the results were processed with DTS software version 3.32.

The zeta-potentials of the nanoparticles were determined by phase analysis light scattering technology using the zetasizer (Malvern Instrument), which was routinely calibrated with a -50 mV zeta potential standard (Malvern Instruments). The nanoparticles were dispersed in 20 mL of buffer at pH 5.0, 6.0, or 7.4 at 0.1 mg/mL with stirring. The measurements were performed in disposable zeta capillary cells at 37 °C. The attenuator was set at 9 and the F (Ka) value was set at 1.5. Each measurement was performed for 30 runs, and the results were processed with DTS software version 3.32.

Hemolytic activity of nanoparticles on red blood cell (RBCs). All experiments were conducted with the approval of the University of Wyoming Animal Care and Use Committee and followed the procedures used in our lab. Mouse blood was collected in heparin-containing eppendorf, and then centrifuged at 1,000 ×g for 5 min to separate the RBCs from the plasma. The RBCs were dispersed in Alsever's Buffer. The RBC

suspension (600 μL) was washed 5-6 times by centrifugation (1,000 ×g, 3 min) until the cell suspension became clear, and then diluted in 4000 μl GVB buffer to obtain RBC stock solution. The nanoparticle solution (100 μL at 0, 2, 4, 8, 40, or 80 μg/mL in PBS), GVB buffer (200 μL), and the RBC stock solution (100 μL) were added to tubes respectively. The tubes were incubated at 37 °C for 1 h. Then, 2 mL of 0.15 M NaCl was added to each tube. The tubes were centrifuged (1,000 ×g, 3 min) to separate the intact RBCs. The supernatant solutions were collected. Absorbance of hemoglobin in the supernatant was measured at 412 nm using a UV-VIS spectrophotometer. Standard 100%, 50%, 0% hemolysis solutions were made from following solutions: 50μL cell solution/450μL H_2O/500μL 0.3M NaCl /50 μL PBS/100μL GVB buffer, 25μL cell solution/475 μL H_2O/500 μL 0.3M NaCl /50μL PBS /100μL GVB buffer, and 500μL H_2O/500 μL 0.3 M NaCl/50 μL PBS/100 μL GVB buffer, respectively. The cell hemolysis percentage was calculated by Hemolysis (%) = $(Abs-Abs_0)/(Abs_{100}-Abs_0) \times 100$, Where Abs, Abs_{100}, and Abs_0 are the absorbances of the sample, the 100% hemolysis solution, and the 0% hemolysis solution respectively. All hemolytic experiments were carried out in triplicates.

Cellular localization of TCRNs by confocal scanning laser microscopy.

a) TCRNs/DOX: SKOV-3 ovarian cancer cells were obtained from American Type Culture Collection (ATCC, Rockville, MD). The cells were plated into glass-bottom petri dishes (MatTek, Ashland, MA, no. P35G-1.0-14-C) at 80,000 cells per plate in 2 mL of RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum, 10 μg/ml insulin, and antibiotic/antimycotic solution. They were incubated for 24 h at 37 °C and 5% CO_2 before the treatments. Treatments were prepared in the RPMI medium containing 10 mM HEPES (pH 7.4). The TCRNs/DOX solution was added to the medium at the DOX-equivalent dose of 1μg/mL. Control experiments were carried out at the same time. After 1 h, lysotracker (Molecular Probes, Carlsbad, CA) was added to the wells at a concentration of 150 nM. The images were taken 1 h later using a confocal scanning laser microscope (Leica TCS SP2 microscope). Lysotracker was observed by using a 488-nm laser, and the emission wavelength was read from 510 to 540 nm and expressed as green. TCRNs/DOX were observed by using a 488-nm laser, and the emission wavelength

was read from 560 to 610 nm and expressed as red. Images were produced by using the lasers sequentially with a 63 × objective lens. Cells were kept at 37 °C and 5% CO_2 except when being observed on the microscope.

b) TCRNs/PKH26 and the nuclear localization: TCRNs/PKH26 nanoparticles were prepared similarly to the preparation of TCRNs/DOX except that PKH26 were used. Treatments were prepared in RPMI medium containing 10 mM HEPES at pH 7.4. The TCRNs/PKH26 solution was diluted in the HEPES-containing RPMI medium to make the same polymer concentration as the TCRNs/DOX treatment in *a*, and the cell culture with TCRNs/PKH26 was the same as that with TCRNs/DOX in *a*. After 12 or 24 h, DRAQ5 ™ (AXXORA LLC, San Diego, CA) was added to the wells at a concentration of 5 μM. The images were taken using confocal microscope. The nuclear staining was observed by using a 633-nm laser, and the emission wavelength was read from 660 to 810 nm and expressed as blue. TCRNs/PKH26 were observed using a 543-nm laser, and the emission wavelength was read from 560 to 610 nm and expressed as red. Images were processed with NIH ImageJ.

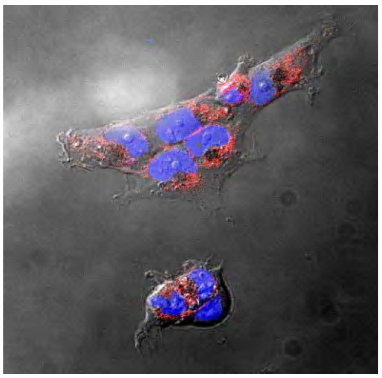


Figure S4. Nuclear localization of TCRNs/DOX observed by confocal laser scanning microscopy after cultured with SKOV-3 cells for 8 h at 37 ℃. The nuclei were stained with DRAQ5 (blue). DOX was assigned to red. Original magnification is 63 ×.

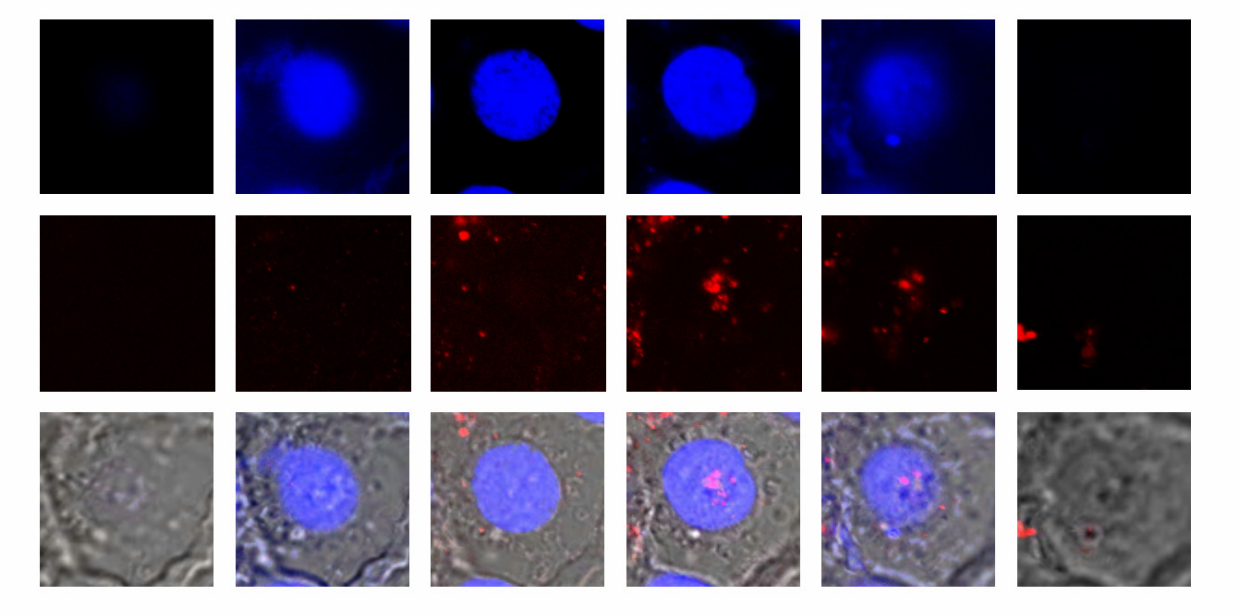


Figure S5. Confocal z-serial section images collected from the top towards the bottom of the cell at a step of 3 μm (from left to right). TCRNs/PKH26 were observed by confocal microscopy after cultured with SKOV-3 cells for 24 h at 37 ℃. The upper panel was collected from the DRAQ5 channel; the middle panel was collected from the PKH26 channel; the lower panel was the overlay of the DRAQ5, PKH26 and transmittance channels. The nuclei were stained with DRAQ5 (blue). TCRNs/PKH26 were assigned to red. Original magnification was 63 ×.

Cellular uptake measured by flow cytometry: SKOV-3 cells were seeded in 6-well plates at a density of 1×10^6 cells per well in 2.5 mL RPMI-1640 medium and incubated in a humidified 5% CO_2 atmosphere for 48 h. The original medium was replaced by fresh medium that were supplemented with free DOX, blank TCRNs, TCRNs/DOX, or TCRNs/DOX pretreated at pH 6 for 2 h, or DOX-loaded control nanoparticles CRNs (CRNs/DOX) at the same dose. The cells were incubated for 1 h at 37 °C, and then washed three times with cold PBS, and harvested by trypsin treatment. The harvested cells were suspended in 1 mL of PBS containing 3% FBS (0.5 mL). The cell suspensions were centrifuged at 1000 rpm for 4 min at 4 °C. The supernatants were discarded and the cell pellets were washed with 1 mL of PBS to remove the background fluorescence in the medium. After two cycles of washing and centrifugation, cells were resuspended and diluted to a final volume of 1 mL in PBS. Cells having DOX

fluorescence were enumerated by fluorescence-activated cell sorting (FACS) (NPE Quanta™ system). Cells were excited with an argon laser (488 nm) and the signals were collected in the FL2 channel. Forward and side-scatter were "gated" to exclude dirt and clumped cells; gating was used identically on all analyses. Data were analyzed with WinMDI (version 2.8) software to obtain the DOX-positive cell percentage and relative fluorescent unit (RFU)/cell.

In vitro cytotoxicity MTT assay: The cytotoxicity assay was carried out using the (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) (MTT) cell proliferation kit (ATCC, Manassas, VA) according to the modified manufacturer's protocol. SKOV-3 cells were seeded in 96-well plates at an initial density of 15,000 cells/well in 200 μL of RPMI medium. The cells were allowed to grow for 24 h. The original medium in each well was replaced with 100 μL of fresh medium. The free DOX or TCRNs/DOX solutions was added to the medium at concentrations ranging from 0.1 μg/mL to 10 μg/mL. Each dosage was replicated in 3 wells. Treated cells were incubated at 37 °C under a humidified air with 5% CO_2 for 4 h. The medium in each well was then replaced with fresh culture medium and the cells were allowed to incubated for another 20 h. MTT reagent (10 μL) was added to each well and the cells were incubated for 2 h at 37 °C or until purple crystals were visible. Detergent reagent (100 μL) was added to each well and then the plates were placed in a 37 °C incubator for 2 h, or until all the crystals dissolved. The absorbance at 570 nm of the solution in each well was recorded using a microplate UVspectrometer (SpectraMax 384 Plus). Cell viability was calculated relative to the control.