Electronic Supplementary Material (ESI) for Nanoscale
This journal is © The Royal Society of Chemistry 2013

Supporting Information

Biodegradable Cationic Polymer Nanocapsules for Overcoming Multidrug Resistance and Enabling Drug-Gene Co-Delivery to Cancer Cells

Chih-Kuang Chen,[‡a] Wing-Cheung Law,[‡b] Ravikumar Aalinkeel,[c] Yun Yu,[a] Bindukumar Nair,[c] Jincheng Wu,[a] Supriya Mahajan,[c] Jessica L.Reynolds,[c] Yukun Li,[a] Cheng Kee Lai,[a] Emmanuel S. Tzanakakis,[a] Stanley A. Schwartz,*[c] Paras N. Prasad,*[b,d] and Chong Cheng*[a]

[a] Department of Chemical and Biological Engineering, University at Buffalo, The State University of New York, Buffalo, NY 14260, USA. E-mail: ccheng8@buffalo.edu

[b] Institute for Lasers, Photonics and Biophotonics, and Department of Chemistry, University at Buffalo, The State University of New York, Buffalo, NY 14260, USA. E-mail: pnprasad@buffalo.edu

[c] Department of Medicine, Division of Allergy, Immunology, and Rheumatology, University at Buffalo, The State University of New York, Buffalo General Hospital, Buffalo, NY 14203, USA. Email: sasimmun@buffalo.edu

[d] Department of Chemistry, Korea University, Seoul, 136-701, Korea.

Experimental Section

Characterization and Processing Methods. All ^{1}H NMR spectra were recorded at 500 MHz on solutions in $CDCl_3$ on a Varian INOVA-500 spectrometer maintained at 25 ºC, with tetramethylsilane (TMS) as an external reference standard. FT-IR spectra were obtained on a Bruker Tensor 27 system using attenuated total reflectance (ATR) sampling accessories. Gel permeation chromatography (GPC) data were obtained using a Viscotek GPC system equipped with a VE-3580 refractive index (RI) detector, a VE 1122 pump, and two mixed-bed organic columns (PAS-103M and PAS-105M). DMF (HPLC) containing 0.01 M LiBr was used as the mobile phase with a flow rate of 0.5 mL/min at 57 ºC. The GPC instrument was calibrated using narrowly-dispersed linear polystyrene standards purchased from Varian.

The average hydrodynamic diameters (D_h) and zeta-potential of miniemulsion nanodroplets and CPLA NCs (with or without therapeutic loadings) were measured by dynamic light scattering (DLS) on a nano-ZS90 (Malvern, Inc.) in water at 25°C. All experiments were conducted by using a 4 mW 633 nm HeNe laser as the light source at a fixed measuring angle of 90° to the incident laser beam. The correlation decay functions were analyzed by cumulant method coupled with Mie theory to obtain volume and number distribution.

Transmission electron microscopy (TEM) images were obtained using a JEOL 2010 microscope. Each TEM sample was prepared by dropwise addition of 10 μL of aqueous solution of NCs (1.3 mg/mL) on a carbon-coated copper grid, followed by negative staining using 10 μL of 0.5% uranyl acetate solution.

Ultrasonication was conducted by using a 150 Series Digital Sonic Dismembrator (Fisher Scientific) equipped with a SLP Microtip (0.08 inch in diameter, Brason Ultrasonics). Continuous mode was used in the miniemulsification process. UV irradiation was conducted by using a UVGL-58 handheld UV lamp (6 Watt, 0.12 Amps; λ_{max} = 365 nm). The concentration of Dox was determined using a Shimadzu 3101PC UV–Vis-NIR scanning spectrophotometer, based on the characteristic UV-Vis adsorption of Dox at 490 nm.

Materials. 1,4-Butanediol bis(3-mercaptopropionate) (98%), trimethylolpropane tris(3-mercaptopropionate) (95%), chloroform (HPLC) and bis-(2-hydroxyethyl)-imino-tris-(hydroxy methyl)-methane (bis-tris; 99%) were purchased from Fisher. 2,2'-Dimethoxy-2-phenylacetophenone (DMPA; 98%) was purchased from Acros Organics. 2-(Diethylamino)ethanethiol hydrochloride (DEAET, 98+%) was purchased from Amfinecom Inc. Near infrared dye IRDye® 800CW maleimide (NIR dye; 99%) was purchased from LI-COR. Doxorubicin hydrochloride (Dox•HCl; 98-102%) was purchased from Sigma, and converted into water-insoluble Dox following a literature method.[1]

Synthesis of CPLA. Following the synthetic method we reported previously,[2] the CPLA sample was prepared from a functional polylactide (PLA) with 50 mol% of allyl-containing repeat units (DP_n^{NMR} = 80, M_n^{NMR} = 12.7 kDa, PDI^{GPC} = 1.16). The synthesis of the allyl-functionalized PLA was described in a previous publication from us.[2] In a 50 mL flask, the allyl-functionalized PLA (200 mg; with 0.63 mmol of ally groups), DEAET (53.4 mg; 0.32 mmol) and photoinitiator DMPA (32.4 mg; 0.13 mmol) were dissolved in $CDCl_3$ (5 mL). After degassing by three cycles of freeze-pump-thaw, the reaction mixture was irradiated by UV light

(λ_{max} = 365 nm) for 30 min. The resulting solution was dialyzed against acetone for 5 days using molecular porous membrane tubing with approximate molecular weight cut off (MWCO) at 3500 Da, and then dried in vacuo to give CPLA with 90% yield. FT-IR: 2927, 1748, 1640, 1451, 1355, 1182, 1129, 1086, 1046, 927, 870, 756, 702 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$, ppm): δ 1.21-1.26 (br m, $(CH_3CH_2)_2NH^+Cl^-$ from amine-functionalized units), 1.55-1.59 (br m, CH_3 of units from LA, allyl-functionalized units and CH_3 from amine-functionalized units), 1.76-1.77 (br m, $CH_2CH_2CH_2SCH_2$ from amine-functionalized units), 2.00-2.08 (br m, $CH_2CH_2CH_2SCH_2$ from amine-functionalized units), 2.49-3.20 (br m, $CH_2CH{=}CH_2$ of units from allyl-functionalized units and $CH_2CH_2CH_2SCH_2$, $SCH_2CH_2NH^+Cl^-(CH_2CH_3)_2$ from amine-functionalized units), 5.14-5.20 (br m, $CHCH_3$ of units from LA, $CHCH_3$, $CHCH_2CH{=}CH_2$, and $CH_2CH{=}CH_2$ of units from allyl-functionalized units**,** $CHCH_3$ and $CHCH_2CH_2CH_2SCH_2CH_2NH^+Cl^-(CH_2CH_3)_2$ from amine-functionalized units), 5.78 (br m, $CH_2CH{=}CH_2$ of units from allyl-functionalized units), 7.30-7.37 (m, Ar-H from α-terminal). As determined by ^{1}H NMR analysis based on a comparison of the resonance intensities of allyl C*H* protons ($CH{=}CH_2$) from the allyl-functionalized units (at 5.78 ppm) and the methyl protons of the tertiary amine-based units (at 1.21-1.26 ppm), the CPLA sample had 30 mol% of ally-functionalized units and 20 mol% of tertiary amine-based units. M_n^{NMR} = 15.4 kDa, M_n^{GPC} = 12.0 kDa (relative to linear polystyrene), PDI^{GPC} = 1.17 (relative to linear polystryrene).

Synthesis of CPLA NCs. In a 10-mL small vial, a chloroform solution (0.113 mL) containing 1,4-butanediol bis(3-mercaptopropionate) (1.04 mg; with 7.8×10^{-3} mmol of thiol group) and DMPA (0.2 mg; 7.8×10^{-4} mmol) was added to 3.5 mL of aqueous solution of CPLA

(5.0 mg; with 7.8×10^{-3} mmol of allyl group). Transparent oil-in-water (O/W) miniemulsion was obtained after 20 min ultrasonication of the mixture (using 40 and 68 μm microtip amplitudes for the trials of NCs **1** and **2**, respectively). Then thiol-ene cross-linking reaction was induced by UV irradiation (λ_{max} = 365 nm) of the transparent miniemulsion for 30 min at room temperature to give CPLA NCs. The aqueous solution of NCs was obtained after the removal of chloroform by simple evaporation using rotavapor at room temperature. FT-IR: 2923, 2854, 1732, 1454, 1354, 1183, 1130, 1087, 1046, 869, 757, 705 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$, ppm): δ 1.21-1.26 (br m, $(CH_3CH_2)_2NH^+Cl^-$ from amine-functionalized units), 1.55-1.72 (br m, CH_3 of units from LA, CH_3 from amine-functionalized units and $COOCH_2CH_2CH_2CH_2OCO$ from cross-linkage), 1.76-1.77 (br m, $CH_2CH_2CH_2SCH_2$ from amine-functionalized units), 2.00-2.08 (br m, $CH_2CH_2CH_2SCH_2$ from amine-functionalized units), 2.41-3.85 (br m, $CH_2CH_2CH_2SCH_2CH_2NH^+Cl^-(CH_2CH_3)_2$ from amine-functionalized units, 2 × $CH_2SCH_2CH_2COO$ from cross-linkage), 4.06-4.25 (br m, $COOCH_2CH_2CH_2CH_2OCO$ from cross-linkage), 5.14-5.20 (br m, CH from all units), 7.30-7.37 (m, Ar-H from from α-terminal of CPLA).

DLS results of CPLA NCs **1**: miniemulsion nanodroplets: $D_{h,v}$ = 48 ± 20 nm; NCs before chloroform removal: $D_{h,v}$ = 50 ± 19 nm; NCs after chloroform removal: $D_{h,v}$ = 35 ± 18 nm, ζ-potential = 54 ± 3 mV.

DLS results of CPLA NCs **2**: miniemulsion nanodroplets: $D_{h,v}$ = 24 ± 12 nm; NCs before chloroform removal: $D_{h,v}$ = 24 ± 10 nm; NCs after chloroform removal: $D_{h,v}$ = 22 ±10 nm, ζ-potential = 45 ± 2 mV.

Synthesis of Near Infrared (NIR) Dye-labeled CPLA NCs (NIR-CPLA NCs). In a 10-mL small vial, a chloroform solution (0.113 mL) containing 1,4-butanediol bis(3-mercaptopropionate) (0.93 mg; with 7.0×10^{-3} mmol of thiol group), trimethylolpropane tris(3-mercaptopropionate) (0.16 mg; with 1.2×10^{-3} mmol of thiol group) and DMPA (0.2 mg; 7.8×10^{-4} mmol) was added to 3.5 mL of aqueous solution of CPLA (5.0 mg; with 7.8×10^{-3} mmol of allyl group). Transparent oil-in-water (O/W) miniemulsion was obtained after ultrasonication of the mixture with microtip amplitude of 68 μm for 20 min. Then thiol-ene cross-linking reaction was induced by UV irradiation (λ_{max} = 365 nm) of the transparent miniemulsion for 30 min at room temperature to give thiol-functionalized CPLA NCs. After the removal of chloroform to get the aqueous solution of thiol-functionalized CPLA NCs, a maleimide-functionalized NIR dye solution (100 μL, 0.25 μg/μL; 0.021×10^{-3} mmol) was added dropwise. Then the solution was stirred gently at room temperature for 2 h, for the occurrence of highly efficient thiol-maleimide conjugation of NIR dye with the CPLA NCs. Subsequently, the reaction solution was dialyzed against ice-cold water for one day using molecular porous membrane tubing with approximate molecular weight cut off (MWCO) at 8000 Da, to give the aqueous solution of NIR-CPLA NCs.

DLS data of NIR-CPLA NCs: miniemulsion nanodroplets: $D_{h,v}$ = 19 ± 8 nm; thiol-functionalized CPLA NCs before chloroform removal: $D_{h,v}$ = 18 ± 11 nm; NIR-CPLA NCs after dialysis: $D_{h,v}$ = 18 ± 8 nm, ζ-potential = 25 ± 1 mV.

Preparation of Dox-Encapsulated CPLA NCs (Dox-CPLA NCs). Dox-CPLA NCs was prepared following the same procedure for the synthesis of CPLA NCs **2**, except that Dox (0.78 mg) was present in the chloroform solution prior to the miniemulsion process and chloroform (5 × 2 mL) was used for extraction to remove the free Dox in water phase after UV irradiation. After the removal of chloroform, 11.6 wt% of encapsulated Dox loading (relative to CPLA NCs) was determined based on UV absorbance of Dox at 490 nm, corresponding to 74% of encapsulation efficiency.

DLS results of Dox-CPLA NCs: miniemulsion nanodroplets: $D_{h,v}$ = 21 ± 7 nm; Dox-CPLA NCs before chloroform removal and extraction: $D_{h,v}$ = 20 ± 9 nm; Dox-CPLA NCs after extraction and chloroform removal: $D_{h,v}$ = 20 ± 8 nm, ζ-potential = 42 ± 4 mV.

The Preparation of IL-8 siRNA-loaded CPLA NCs (IL-8-siRNA-CPLA NCs). IL-8 siRNA (5' GGAUUUUCCUAGAUAUUGC dtdt) was obtained from Thermo Scientific Dharmacon. Various amounts of CPLA stock solution (1.4 mg/mL) were mixed with 10 μL of IL-8 siRNA (10 μM) (weight ratio of CPLA NC/IL-8-siRNA= 30, 65, 100 and 150) with gentle vortex. The mixtures were left undisturbed for 30 min for the formation of IL-8-siRNA-CPLA NCs.

In Vitro Degradation Study of CPLA NCs. A 10 mL solution of CPLA NCs **1** in bis-tris-HCl (pH = 7.4) buffer was prepared. The solution was kept at 37 ºC using a thermostat oil bath with gentle stirring. The variations of relative intensity of scattered light (I/I_0) of the buffer solution of CPLA-NCs were traced at different time intervals by DLS. The initial intensity of scattered light of the solution was measured and defined as I_0.

Cell Viability Study. The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy -phenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt, Promega) assay was performed to evaluate the cytotoxicity of free Dox, CPLA NCs, Dox-CPLA NCs and Dox/IL-8-siRNA CPLA NCs. PC3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. In each assay, initial number (~2500 or ~5000) of PC3 cells were dispensed into each well of a 96-well flat bottomed microtiter plate and cultured overnight for 48 or 72 h incubation study, respectively. For MCF7/ADR and MCF7 cells, same preparation procedures were used as PC3 cells. Various concentrations of CPLA NCs (3, 6, 12, 30, 60, 120, 240 and 360 μg/mL), free Dox ([Dox] = 0.2, 0.4, 1, 2, 4, 10, 20, 40 and 60 μM), Dox-CPLA NCs ([Dox] = 0.2, 0.4, 1, 2, 4, 10, 20, 40 and 60 μM), and Dox/IL-8-siRNA CPLA NCs ([Dox] = 0.2, 0.4, 1, 2, 4, 10 and 20 μM; [siRNA] = 0.1 μM) were exposed to PC3 cells for 48 or 72 h and the cell viabilities were compared with the untreated control. After the incubations of 48 or 72 h, sample solutions were removed and 10 μL of MTS reagent with 100 μl of fresh cultural medium was added to each well and thoroughly mixed. The absorbance of formazan (produced by the cleavage of MTS by dehydrogenases in living cells) is directly proportional to the number of live cells. One hour later, the absorbance of each well at 490 nm was measured using a multi-well plate reader. Assays were performed in quadruplicate and the results were presented in the form of average ± standard deviation (the viability of untreated cells was assigned as 100%). As before, cell viability results of MCF7/ADR or MCF7 cells treated by

free Dox, Dox-CPLA NCs and CPLA NCs with studied concentrations were obtained using the same analysis procedures as PC3 cells.

Confocal Imaging. The day prior to the experiments, studied cells were seeded on a square glass coverslip in 35 mm cell culture dishes at 40-50% confluence. For Dox delivery experiments, culture dishes were treated with 125 μL solution of Dox-CPLA NCs (1.4 mg/mL) with final concentration of Dox 40 μM. The samples were then cultured at 37 ºC in a humidified atmosphere containing 5% CO_2 for 4, 8, 24 and 48 h of MCF7/ADR cells and 1, 8 and 24 h of PC3 cells. For siRNA delivery experiments, IL-8-siRNA CPLA NCs were prepared by mixing 10 μL solution of IL-8 $siRNA^{FAM}$ (10 μM) with 28.6μl solution of CPLA-NCs (1.4 mg/mL) for 20 min. The samples treated with IL-8-$siRNA^{FAM}$-CPLA NCs with final concentration of IL-8-$siRNA^{FAM}$ of 0.1 μM and weight ratio of CPLA NC/$siRNA^{FAM}$ of 30 were cultured at 37 ºC in a humidified atmosphere containing 5% CO_2 for 1, 8 and 24 h. To demonstrate the co-delivery capability, Dox/IL-8-$siRNA^{FAM}$-CPLA NCs were prepared by mixing 44 μL solution of IL-8 $siRNA^{FAM}$ (10 μM) with 125 μL solution of Dox-CPLA NCs (1.4 mg/mL) for 20 min. The treated dishes were cultured at 37 ºC in a humidified atmosphere containing 5% CO_2 for 4 h. All dishes were rinsed with DMEM for 3 times to wash out any free particles before imaging.

Cell imaging was carried out using a Leica TCS-SP2/AOBS confocal microscope, equipped with excitation laser lines of 405, 442, 458, 476, 488, 496, 543, 633 nm and capable of spectral detection in the range of 400-720 nm. All of the confocal images were taken under the same conditions (the parameters of photodetector gain, pinhole size and exposure time were kept constant).

Flow Cytometry Study. The day before the experiments, MCF7/ADR cells were seeded on a square glass coverslip in 35 mm cell culture dishes at 40-50% confluence. MCF7/ADR cells were treated with free Dox and Dox-CPLA NCs with final concentration of Dox 0.4 μM. After 4 h treatment, cells were dissociated with TrypLE (Invitrogen) and collected by centrifugation at 200×g for 5 mins and were washed twice with PBS (Mediatech) before flow cytometric analysis. Data acquisition was carried out in a FACS Calibur flow cytometry with the CellQuest software (Becton Dickinson). Data analysis was further performed with the Flow Express V4.0 suite (De Novo Software, Los Angeles, CA).

Given that Dox has an intrinsic fluorescence emission of ~600 nm that can also be detected by flow cytometryintracellular amount of Dox were further measured using FL-2 channel. As shown in Fig. S5, Dox-CPLA NCs resulted in a 92.43% ± 2.65% Dox positive cells while free Dox had a 41.29% ± 2.15% Dox positive cells after 4 h incubation. Thus, Dox-CPLA NCs exhibited a ~50% increase in Dox positive cells as compared with free Dox. The untreated control was used to determine the positive threshold of Dox positive cells.

Gel Retardation Assay. The siRNA binding affinity of CPLA NC was studied by agarose gel electrophoresis. IL-8-siRNA-CPLA NC nanoplexes were prepared with different weight ratios (CPLA NC/IL-8-siRNA = 30, 65, 100 and 150). Agarose gel (1%) was prepared and mixed with ethidium bromide. Nanoplexes and unconjugated IL-8-siRNA were loaded into the wells in tris-acetate-EDTA (TAE) solution. Then a voltage of 70 V was applied for 40 min. The retardation of the nanoplexes can be visualized by the fluorescence from ethidium bromide under UV light.

Transfection and RNA Extraction. Gene expression was monitored at 48 h post treatment. Twenty-four hours before siRNA transfection, ~20,000 PC3 cells were seeded onto 6-well plates in OPTI-MEM containing 4% FBS with no antibiotics to give 30 to 50% confluence at the time of transfections. The siRNA was reconstituted in DNase-RNase free water to a final concentration of 10 μM and mixed with different amount of CPLA-NCs as described above. The final concentration of siRNA for in vitro transfection of cancer cell was 0.1 μM. After 48 h, cytoplasmic RNA was extracted by an acid guanidinium-thiocyanate-phenol-chloroform method as described using TRIzol reagent (Invitrogen-Life Technologies). The amount of RNA was quantitated using a Nano-Drop ND-1000 spectrophotometer (Nano-Drop). RNA was reverse transcribed to cDNA using the reverse transcriptase kit from Promega, this was followed by real-time quantitative PCR which was used to determine the relative abundance of the mRNA species. Relative IL-8 gene expression was calculated using the comparative threshold cycle number (CT) method.

Biodistribution of NIR-CPLA NCs. All animal experiments and maintenance were approved by the University at Buffalo Institutional Animal Care and Use Committee (IACUC). Solutions of NIR–CPLA NCs (5 mg/mL) were intravenously injected to 6 groups of nude mice (n = 3 per group; concentrations of NIR-CPLA NCs in mice: 25 mg/kg). Maestro in vivo optical imaging system (CRI, Inc.,Woburn, MA) was used for small animal imaging. The major organs (liver, spleen, kidney, heart and lung) of mice were harvested at different time points post injection (1, 4, 8, 16, 30 h and 1.5 wk) and illuminated by the built-in NIR light source and the built-in liquid crystal tunable filter. To acquire the fluorescence images, the system was operated

This journal is © The Royal Society of Chemistry 2013

in the cube acquisition mode and the "Red" filter set (a 615–665 nm band pass filter for excitation and a 700 nm long-pass filter for emission) was applied. Image cube files, each containing a series of picture information from 700 to 950 nm (with 10 nm steps), were collected. Based on the cube files, fluorescent images and the corresponding spectra were obtained by the software provided. The fluorescent images were analyzed using the built-in software under the same condition and parameters.

References:

1. Benoit, D. S. W.; Henry, S. M.; Shubin, A. D.; Hoffman, A. S.; Stayton, P. S. *Mol. Pharmaceutics* **2010**, *7*, 442–455.
2. Chen, C.-K.; Law, W.-C.; Aalinkeel, R.; Nair, B.; Kopwitthaya, A.; Mahajan, S. D.; Reynolds, J. L.; Zou, J.; Schwartz, S. A.; Prasad, P. N.; Cheng, C. *Adv. Healthcare Mater.* **2012**, *1*, 751-761.

Supporting Figures

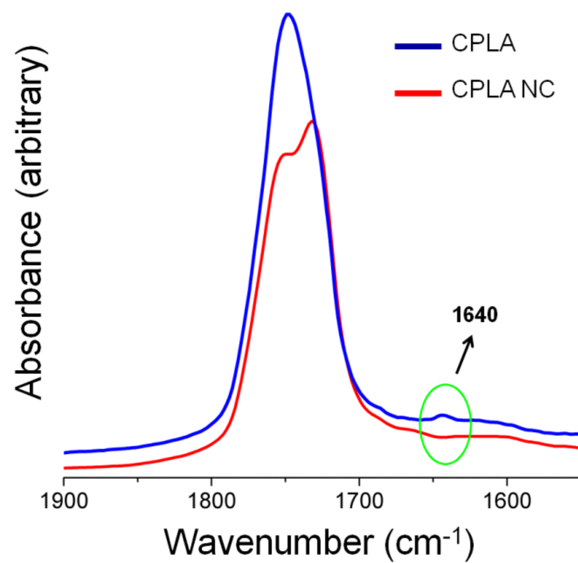

Fig. S1 Partial FT-IR spectra of CPLA and CPLA NCs **1**.

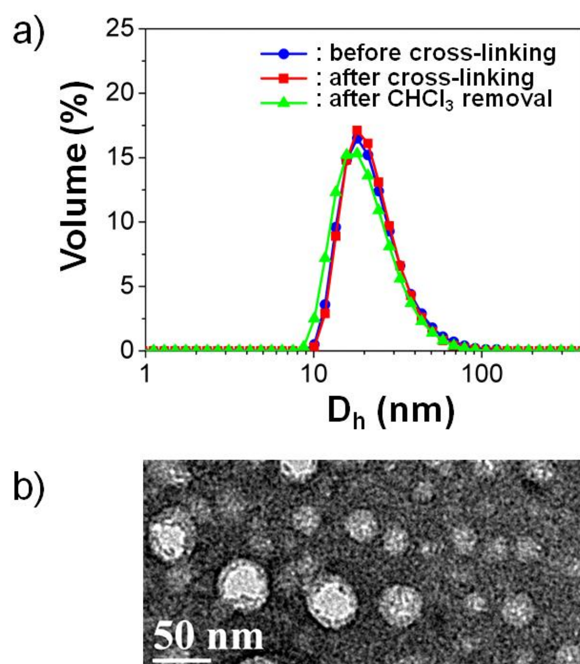

Fig. S2 (a) DLS results for the synthesis of CPLA NCs **2**. (b) TEM image of CPLA NCs **2**.

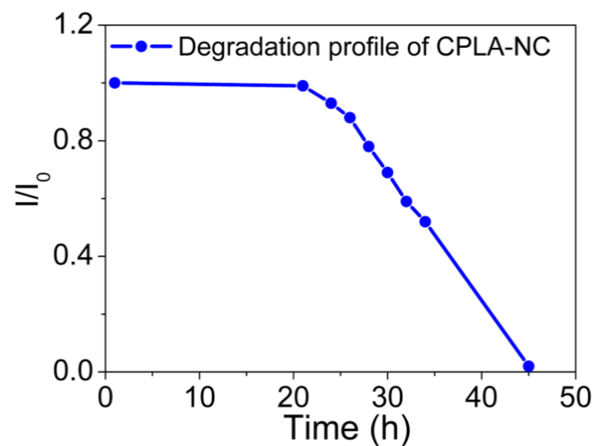

Fig. S3 Hydrolytic degradation of CPLA NCs **1** in a bis-tris HCl buffer solution (pH = 7.4) monitored by DLS.

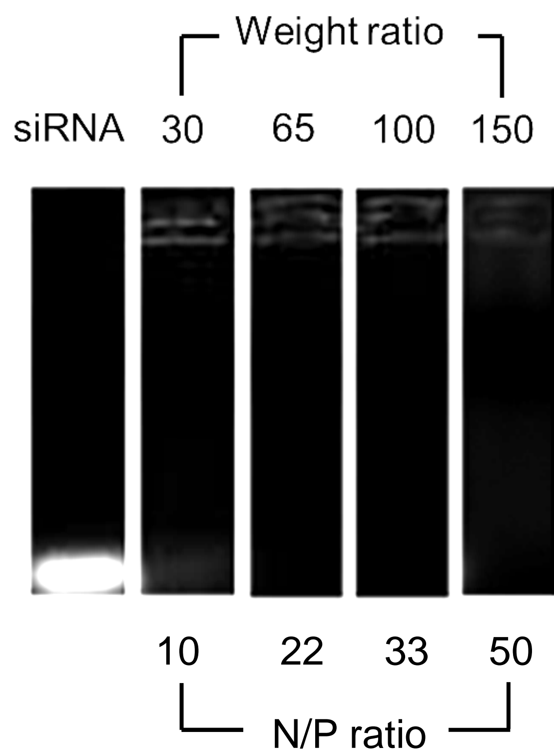

Fig. S4 Electrophoretic mobility of IL-8-siRNA CPLA NCs at different weight ratios of CPLA NCs to siRNA.

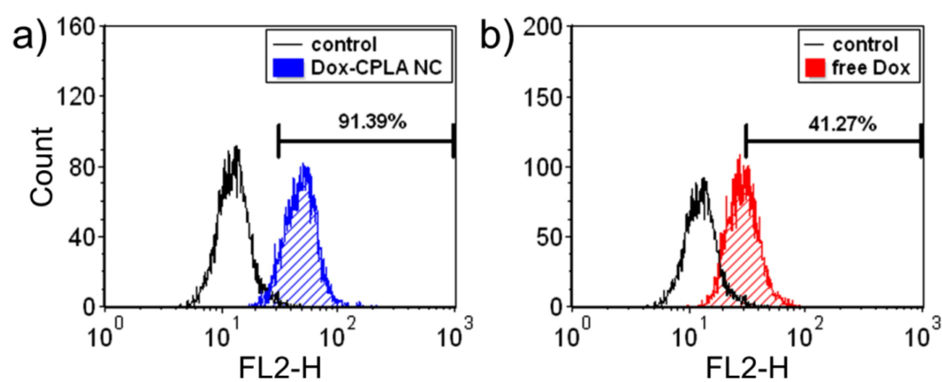

Fig. S5 Flow cytometric histograms of MDR7/ADR cells after 4 h of incubation with a) Dox-CPLA NCs and b) free Dox ($[Dox]_0$ = 0.4 μM). Untreated MDR7/ADR cells were used as control.

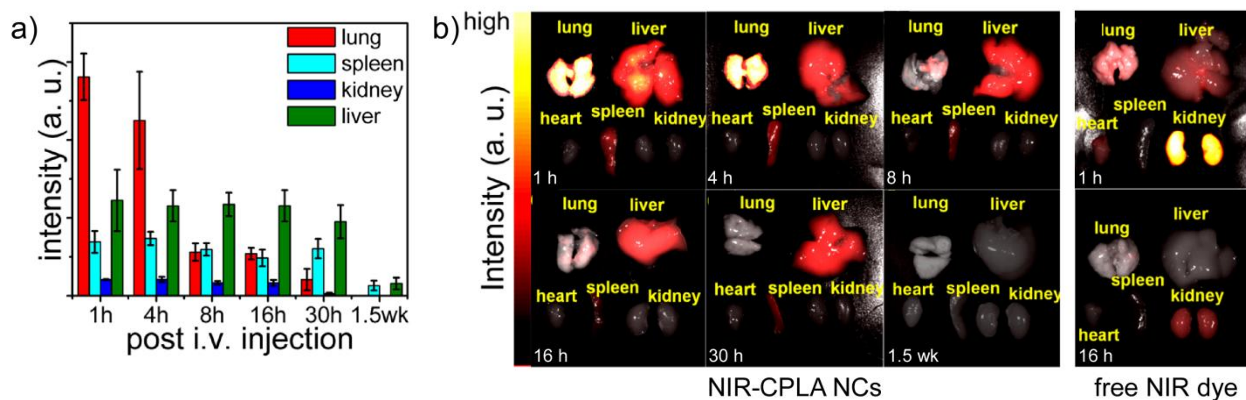

Fig. S6 a) Biodistribution of NIR-CPLA NCs in nude mice according to the fluorescence intensities of individual organs. b) Major organs of nude mice treated with NIR-CPLA NCs harvested at 1, 4, 8, 16, 30 h and 1.5 wk post injections (left six images), and treated with free NIR dye harvested at 1 and 16 h post injections (right two images). The organ images show the overlapping pictures of NIR fluorescence intensity and gray scale pictures, and the color bar represents the amplitude of NIR fluorescence intensity.

Note: In contrast to free NIR dye, the NIR-CPLA NCs escaped fast renal clearance due to their nanoscopic dimensions; they were accumulated in lung in the first few hours, and then remained substantially in liver and spleen. In this context, these NCs may have chance to deliver cargoes to organs of interest. However, fluorescence signals could hardly be detected after 1.5 wk, indicating NCs were eventually degraded and the residues were effectively eliminated; no ill effects or abnormal behaviors of the mice were observed during the period.