Supplementary Information

# Cationic Supramolecular Nanoparticles for Co-delivery of Gene and Anticancer Drug

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The Supplementary Information includes:

1. Supporting Information, Fig. S1-S15.
2. Experimental Details.
3. Table S1 and S2.

## 1. Supporting Information

In Fig. S1, the molar ratio of PEI and CyD was calculated by stoichiometry of integral values from the $^1$H NMR spectrum of PEI-CyD in $D_2O$. The characteristic peaks of doxorubicin and adamantyl group were identified in the spectrum of PEI-CyD/Ada-Dox with chemical shift variation.

In Fig. S2, the wavelength of UV-Vis absorbance peak of PEI-CyD/Ada-Dox at 508.9 nm, distinguished from doxorubicin at 480.1 nm with a red shift of c.a. 29 nm, demonstrated the covalent conjugation between doxorubicin and adamantyl group. The drug loaded amounts were determined from UV-Vis absorbance by calibration curve measurement. Three PEI-CyD/Ada-Dox conjugates were synthesized by controlling the feed ratio of the starting materials. The loaded doxorubicin amount was estimated to be 2.0, 5.1 and 9.8 % (w/w) when the feed ratio of PEI-CyD and Ada-Dox were 5:1, 15:1 and 45:1, respectively. The host-guest structure between cyclodextrin and adamantyl group took advantage of numerous binding sites of cyclodextrin in PEI-CyD polymer and enlarged the drug loading capacity of cationic polymer.

In Fig. S3, no peaks of doxorubicin could be identified in XRD spectrum of PEI-CyD/Ada-Dox, indicating crystalline doxorubicin transformed to amorphous PEI-CyD/Ada-Dox in the synthesis.

In Fig. S4, the thermal stability of PEI-CyD/Ada-Dox was evaluated using TGA and compared with free doxorubicin and the PEI-CyD/Ada conjugates. The decomposition temperature ($T_d$) for PEI-CyD/Ada-Dox decreased by 3 °C, as compared with its respective PEI-CyD/Ada, indicating PEI-CyD/Ada being the majority in the conjugates with doxorubicin being the minority.

2D-NOESY NMR measurements were conducted to further confirm the host-guest complexation between CyD-PEI and Ada-Dox (Fig. S5). It was found that all three signals of methylene and methine protons of adamantyl moiety were well correlating with inner protons C(3)H of β-CyD core, indicating the formation of supramolecular inclusion complexes.

Luciferase expression in B16F10 and COS7 cells were used to assess the *in vitro* transfection efficiency (Fig. S14). PEI-CyD/Ada-Dox can mediate highly efficient gene transfection, which is slightly lower than those mediated by PEI-CyDPEI (25 kDa). The decreased transfection activity of PEI-CyD/Ada-Dox polyplexes was probable due to of the decreased amino density after the introduction of guest Ada-Dox to PEI-CyD polymer.

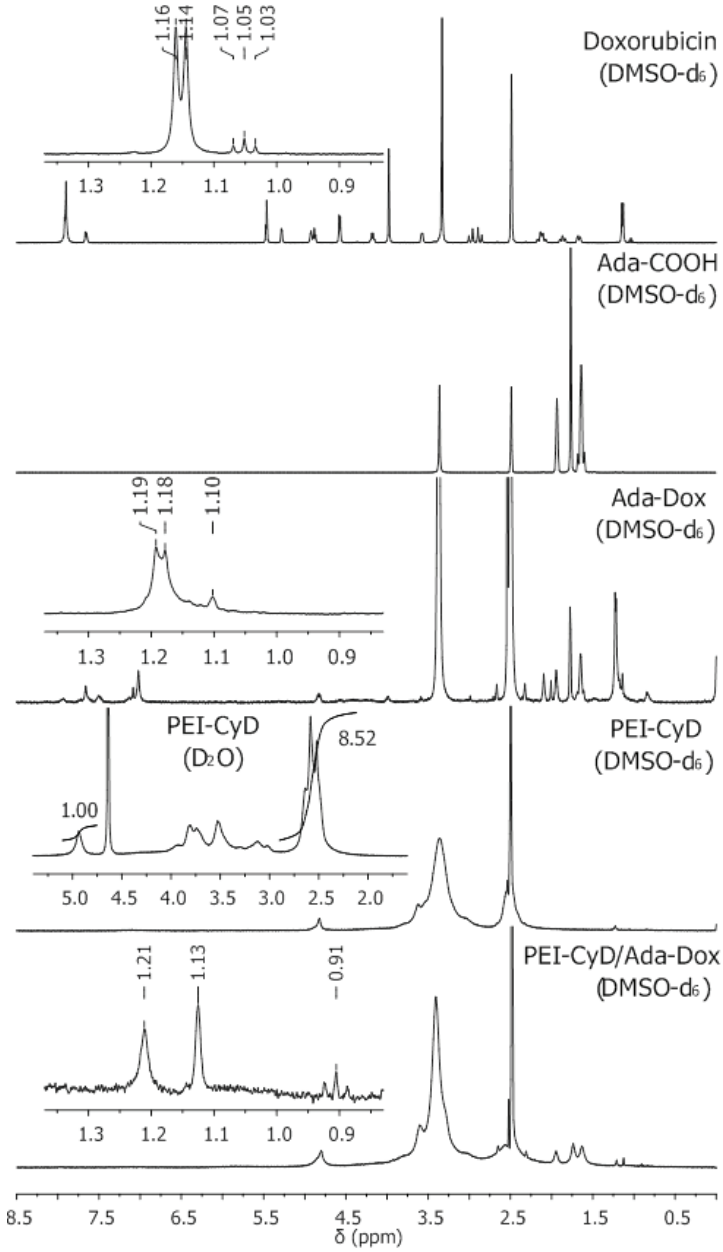


Fig. S1. $^{1}H$ NMR spectra of Doxorubicin, Ada-COOH, Ada-Dox, PEI-CyD, PEI-CyD/Ada-Dox in DMSO-$d_6$ and PEI-CyD in $D_2O$.

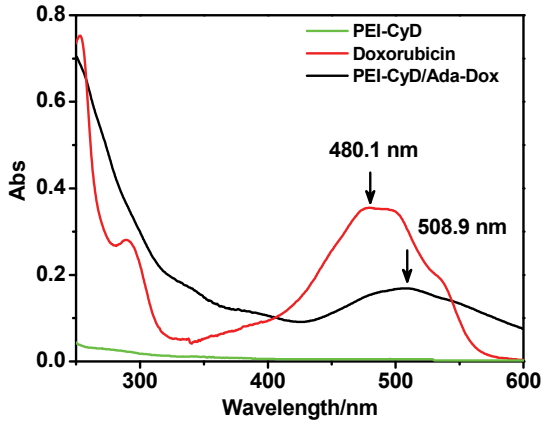


Fig. S2. Ultraviolet-Visible spectra of PEI-CyD, Doxorubicin and PEI-CyD/Ada-Dox. The peak wavelengths of doxorubicin and PEI-CyD/Ada-Dox were noted in the spectra.

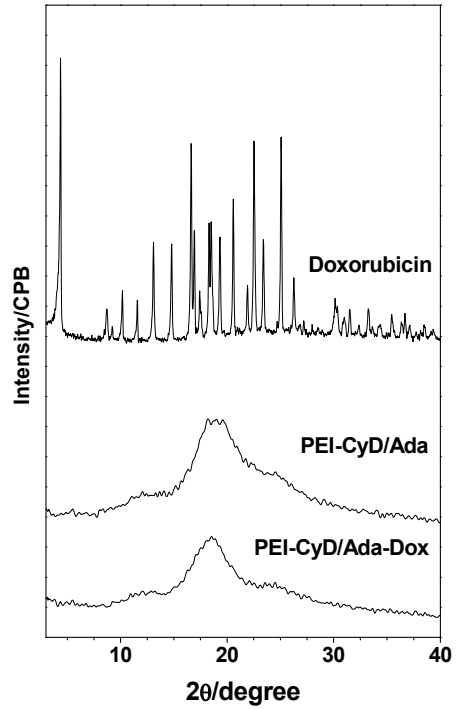


Fig. S3. XRD analysis spectra of Doxorubicin, PEI-CyD/Ada and PEI-CyD/Ada-Dox.

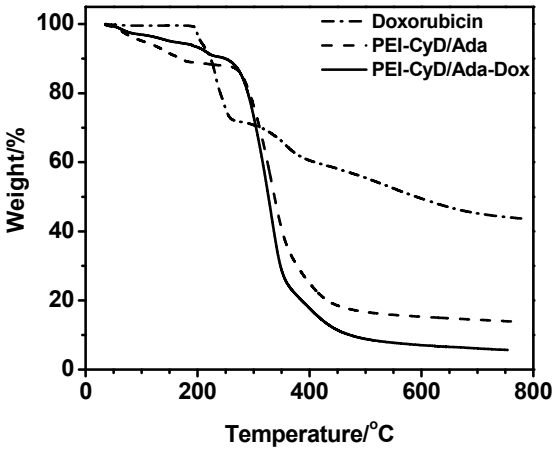


Fig. S4. Thermogravimetric analysis of doxorubicin, PEI-CyD/Ada and PEI-CyD/Ada-Dox determining their decomposition temperatures ($T_d$) of 194.6, 282.3 and 278.7 °C, respectively.

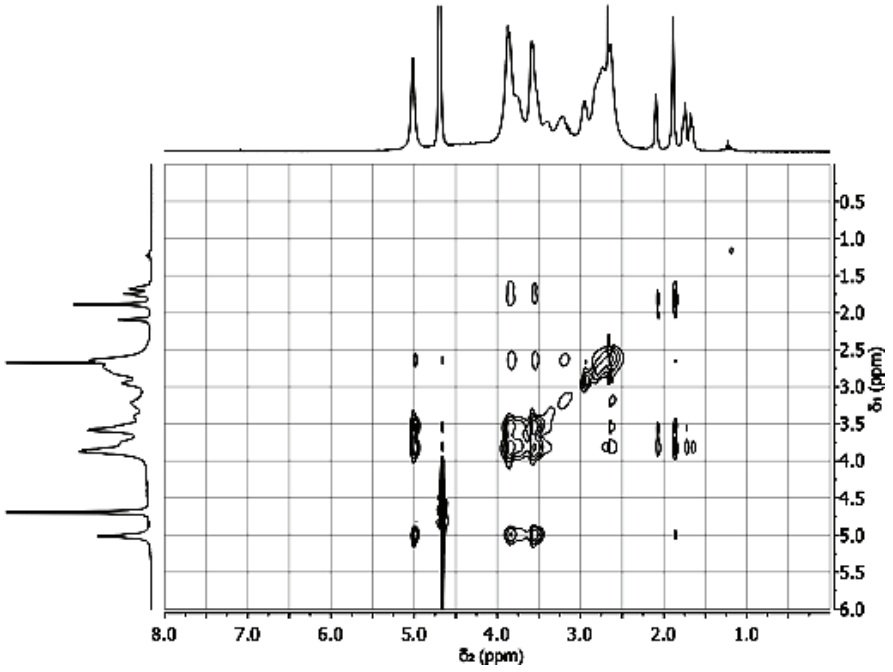


Fig. S5. 2D-NOESY NMR spectra of a 2:1 (guest:host) mixture of the CyD-PEI and AD-Dox in $D_2O$ at 25 °C

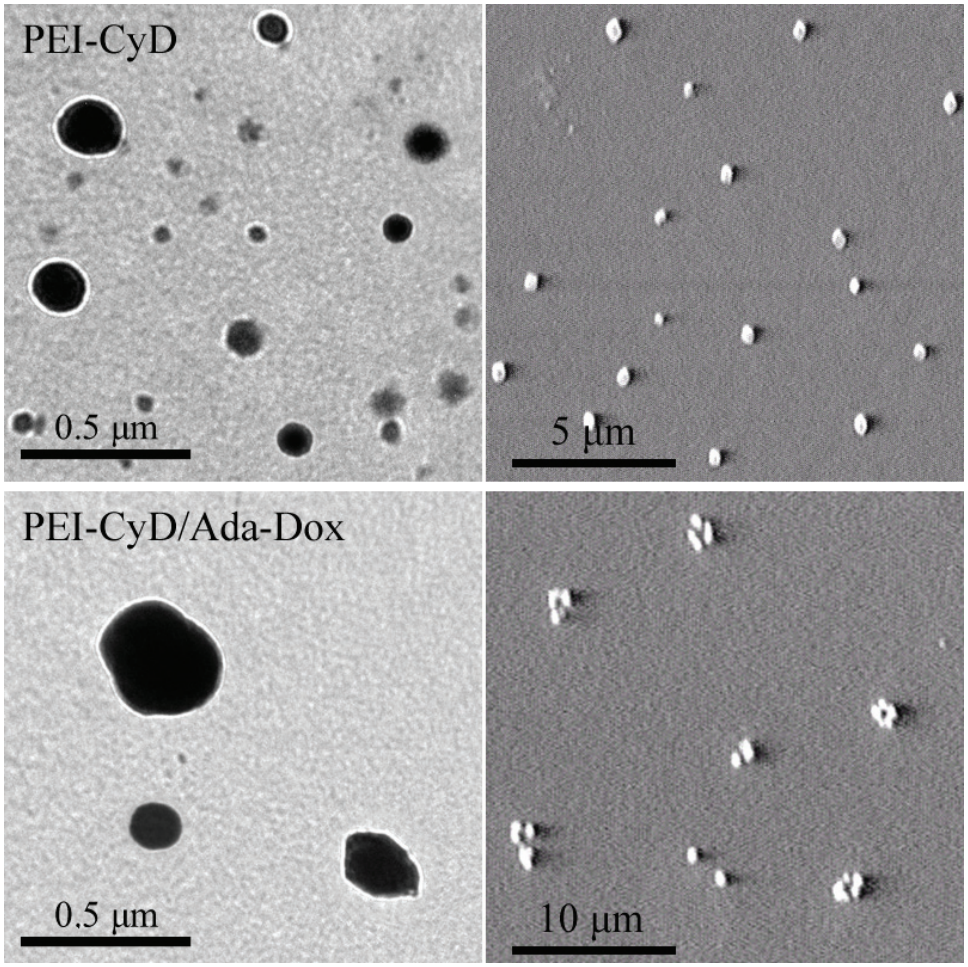


Fig. S6. TEM and SEM images of complexes formed by DNA with PEI-CyD polymer or supramolecular PEI-CyD/Ada-Dox conjugates (5.1% drug loaded).

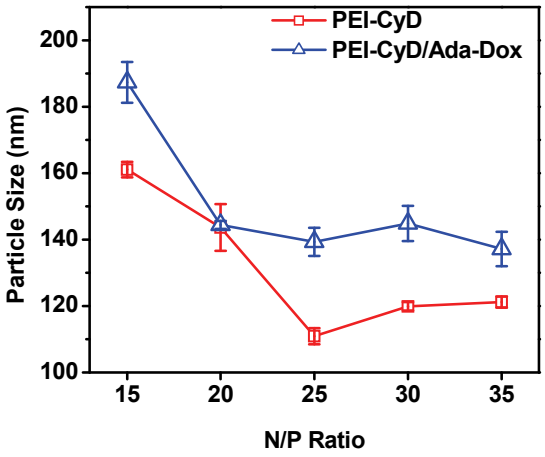


Fig. S7. Particle size measurements of at various N/P ratios of 15, 20, 25, 30 and 35.

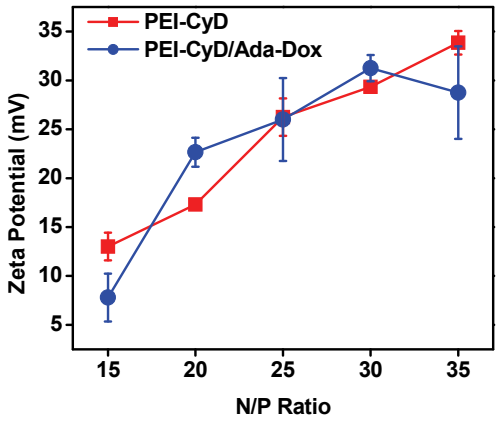


Fig. S8. Zeta potential measurements of at various N/P ratios of 15, 20, 25, 30 and 35.

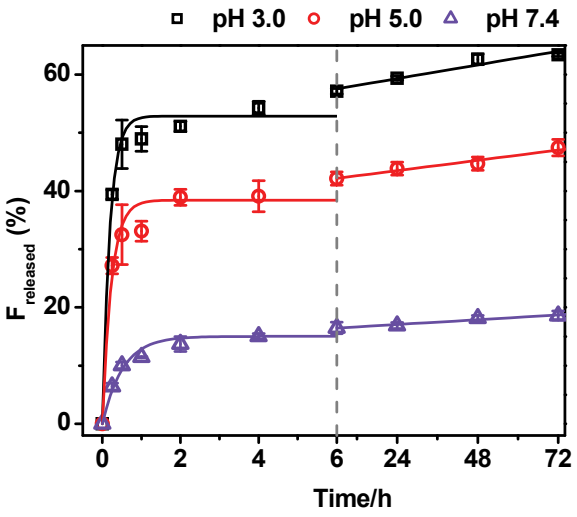


Fig. S9. pH-dependent release of doxorubicin from PEI-CyD/Ada-Dox nanoparticles at pH 3.0, 5.0 and 7.4.

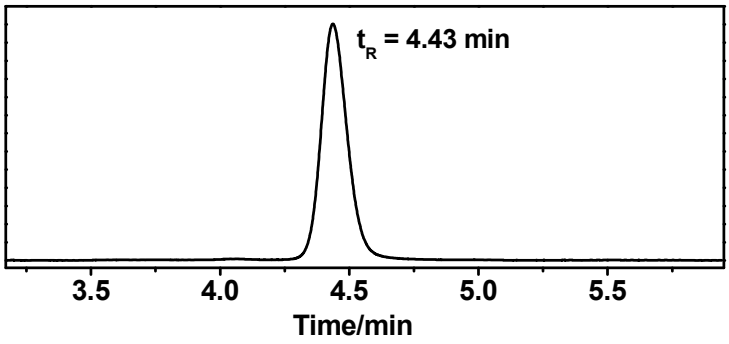


Fig. S10. HPLC diagram of dialyzed PEI-CyD/Ada-Dox solution where $t_R$ was shown to be 4.43 min.

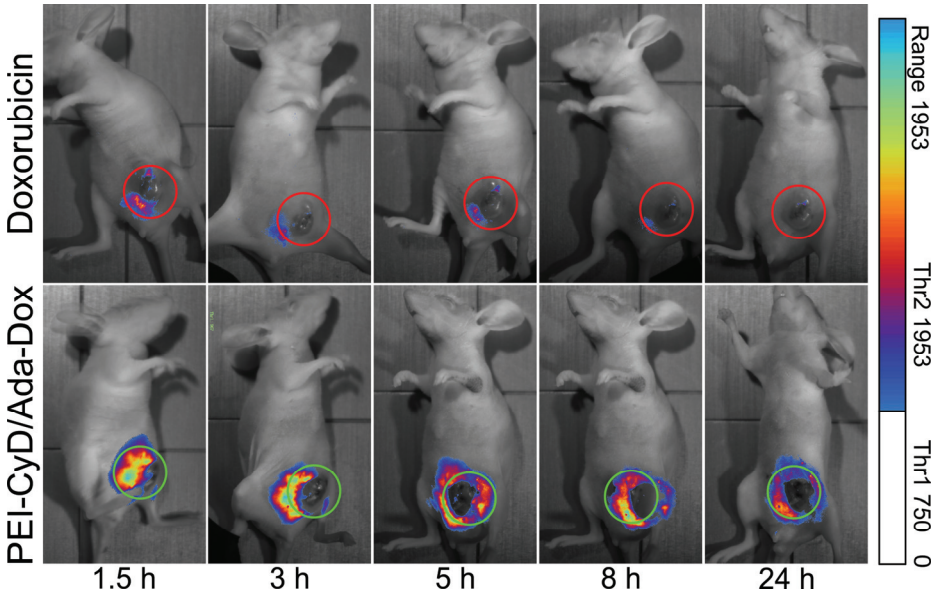


Fig. S11. In vivo retention of doxorubicin (upper panel) and PEI-CyD/Ada-Dox (lower panel) in nude mice for 1.5, 3, 5, 8, 24 h after a single dose administration.

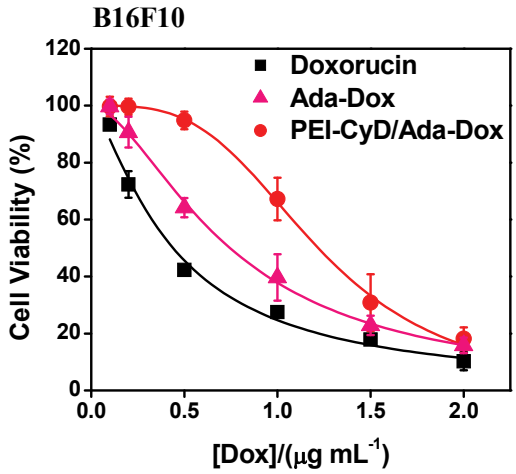


Fig. S12. Cytotoxicity of doxorubicin (n = 4; $IC_{50}$ = 0.44 μg·mL$^{-1}$), Ada-Dox (n = 4; $IC_{50}$ = 0.75 μg·mL$^{-1}$) and PEI-CyD/Ada-Dox (n = 4; $IC_{50}$ = 1.2 μg·mL$^{-1}$) in B16F10 cells after 72 h of incubation measured by MTT assays.

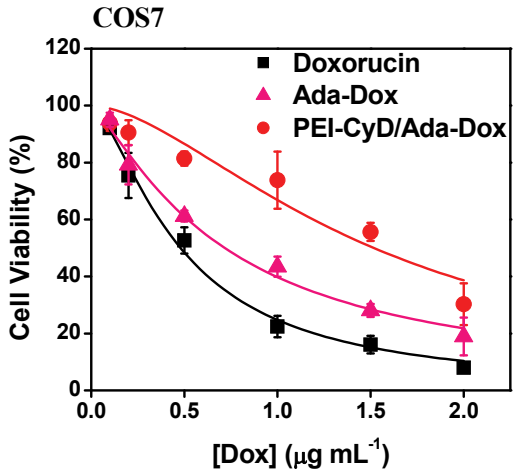


Fig. S13. Cytotoxicity of doxorubicin, Ada-Dox and PEI-CyD/Ada-Dox in COS7 cells after 72 h of incubation measured by MTT assays.

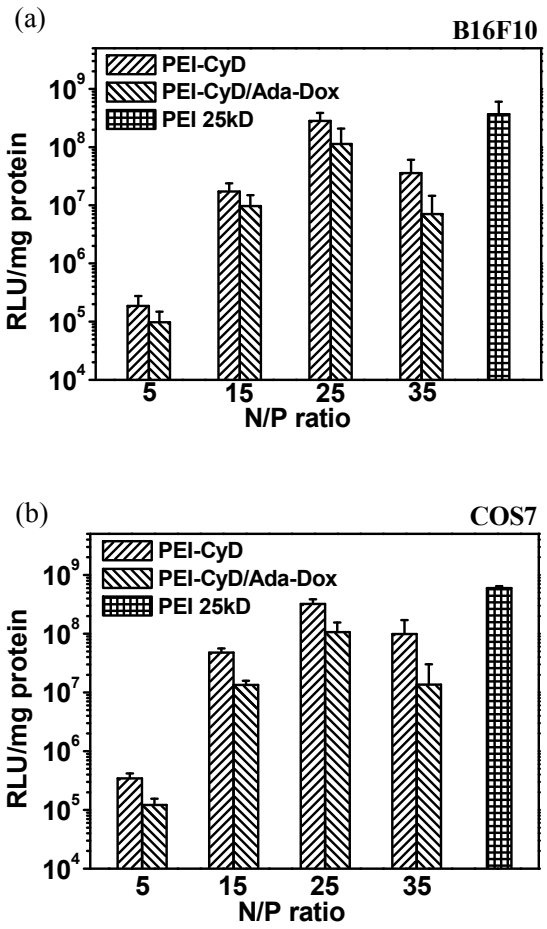


Fig. S14. In vitro transfection efficiency of DNA-condensing PEI-CyD and PEI-CyD/Ada-Dox complexes in (a) B16F10 and (b) COS7 cells using pGL3-luc plasmid after 48 h of incubation. PEI 25kD was used as a control group at weight ratio of 1.2.

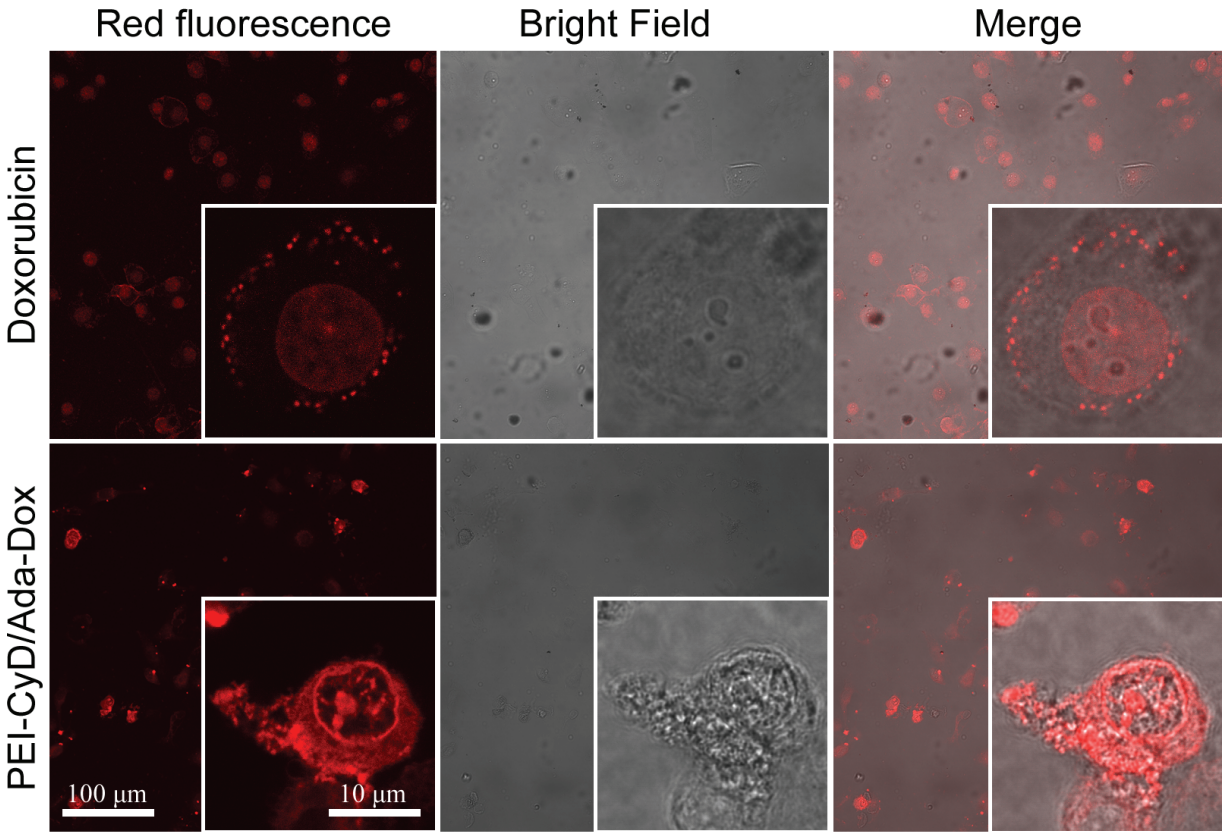


Fig. S15. Confocal laser scanning microscopy images of B16F10 cells treated with doxorubicin or PEI-CyD/Ada-Dox for 4 h.

## 2. Experimental Details

*Preparation of PEI-CyD Copolymer:* CyD (2.17 g, 1.91 mmol) and CDI (2.43 g, 15.0 mmol) were dissolved in DMSO (20 mL), and then mixed with 200 μL of Et3N. The mixture was stirred at room temperature for 3 h under nitrogen. PEI 600 (8.83g, 14.7 mmol) was dissolved in DMSO (15 mL). After addition of Et3N (200 μL), PEI solution was added dropwise to CyD-CDI over 2.5 h with stirring, followed by overnight reaction. The crude product was dialyzed in water for 2 days and freeze-dried for another 2 days.

*Preparation of PEI-CyD/Ada-Dox*: PEI-CyD/Ada-Dox conjugates were synthesized by host-guest interaction. Ada-COOH (35.2 mg, 0.19 mmol) and CDI (45.5 mg, 0.28 mmol, 1.50 equiv) were dissolved in DMSO (2 mL), mixed with $Et_3N$ (200 μL), and then stirred at room temperature for 3 h under nitrogen. Doxorubicin hydrochloride (93.3 mg, 0.17 mmol) dissolved in DMSO (1 mL) was slowly added to Ada-CDI and the mixture was further stirred overnight under nitrogen. Upon addition of distilled water to remove excess CDI, different amounts of PEI-CyD (0.60 g, 1.80 g or 5.42 g) dissolved in $H_2O$ (10-30 mL) were added dropwise to Ada-Dox. The resultant mixture was stirred for 8 h. Then the crude product was dialyzed in water for 1 day and freeze-dried. Ada-Dox was obtained by filtering the overnight-reacted mixture through silica gel.

*Preparation of SNPs*: SNPs were prepared in electrostatic interaction between negatively charged plasmid DNA and the positively charged conjugates. PEI-CyD/Ada-Dox (10.0 mg) and plasmid DNA (1.0 mg) were mixed in distilled water (20 μL) at N/P ratio of 25 and incubated at room temperature for 30 min.

*Determination of Particle Size and Zeta Potential:* DLS measurements of the SNPs were carried out in triplicate at 25 °C on a Zetasizer Nano ZS (Malvern Instruments, Southborough, MA) with a laser light wavelength of 633 nm at a 173° scattering angle.

*Electron Microscopy Visualization*: TEM and SEM were used to obtain the optical properties. SNPs were observed using a Tecnai 10 TEM (Philips Electron Optics, Eindhoven, NL) and photographed with a Gatan Erlangshen 500W digital camera (Pleasanton, CA). For SEM viasulization, the gold-coated complexes were observed using a Cambridge Stereoscan 260 SEM (Cambridge, UK) at 20kV.

*pH Dependent Drug Release*: HPLC was performed using an HP 1100 HPLC (Agilent Technologies, Santa Clara, CA) and a TSK-gel ODS-100V column (Tosoh Bioscience, Stuttgart, Germany) with

isocratic flow of 1.0 mL·min$^{-1}$ of water: acetonitrile: formic acid [70:30:0.05]. The relationship between the integrated area of absorbance at 495 nm and standard Dox concentration was successfully fit by linear regression ($r^2$ = 0.999). PEI-CyD/Ada-Dox (2 mg) in phosphate buffer solutions (4 mL, pH = 3.0, 5.0 or 7.4) were incubated at 37 °C in dialysis bags in solutions with relative pH for 0.0, 0.25, 0.5, 1, 2, 4, 6, 24, 48 or 72 h. Samples were collected from solutions outside the dialysis bags and adjusted to pH 7.4 with regulated volume. 25 μL of each sample was loaded onto the column and peaked at 4.50±0.08 min. The quantities of doxorubicin at different time point were converted to release percentage ($F_{\%,\ released}$). For samples in 6 h, data points were fit by nonlinear regression to a first-order release model:

$$F_{\%,released} = a\left[1 - e^{\frac{-(\ln 2)t}{t_{1/2}}}\right]$$

Where $t$ is the time (h) after incubation, $t_{1/2}$ is the half-life (h) of release, and $a$ (%) is the maximum extent of drug release. For samples of 6, 24, 48 and 72 h, data points were fit by linear regression.

*MTT Assay*: B16F10 cells were seeded into 96-well microplates at the density of 8 × 10$^3$ cells in complete medium (200 μL) per well. After incubation for 20 h, the cultural medium was replaced by 10% FBS-medium with different concentrations of selected chemicals. After further incubation for 72 h, 100 μL of 10% sterilized MTT (5 mg·mL$^{-1}$) stock solution in serum-free medium was added to each well. After 4 h, unreacted dye was removed by aspiration, and the formazan crystals were dissolved in DMSO (100 μL) per well and measured in a microplate reader at the length of 570 nm. The relative cell viability (%) related to control cells cultured in medium without chemicals was calculated by the formula below:

$$V\% = \frac{[A]_{experimental} - [A]_{blank}}{[A]_{control} - [A]_{blank}} \times 100\%$$

where V% is the cell viability (%), $[A]_{experimental}$ is the absorbance of the wells culturing cells treated with chemicals, $[A]_{blank}$ is the absorbance of media blanks, $[A]_{control}$ is the absorbance of the wells culturing cells treated with chemical-free 10%FBS-medium. The 50% inhibitory concentration ($IC_{50}$) was determined by fitting data to the following equation:

$$V\% = \frac{100}{1 + \left(\frac{[Dox]}{IC_{50}}\right)^p}$$

Where V% is the viability (%), [Dox] is well concentration (μg·mL$^{-1}$) of doxorubicin or doxorubicin equivalent of PEI-CyD/Ada-Dox conjugate, and p defines the slope of the sigmoid curve.

*In vitro Transfection*: B16F10 cells or COS7 cells were seeded in 24-well microplates at a density of 3 × 10$^4$ cells in 500 μL of complete medium per well within 24 h. The transfection of EGFP pDNAs was

performed with SNPs in serum-free medium. After 4 h, the transfection medium was replaced with complete medium containing 10% FBS and incubated for additional 44 h. After a total transfection time of48 h, cells were washed with preheated PBS twice, lysed with 100 μl of cell lysis buffer (Promega, USA) for 30 min. The luciferase activity in cell extracts was measured using a luciferase assay kit (Promega, USA) on a single-well luminometer (Berthold lumat LB9507, Germany) for 10 s. The relative light units (RLU) were normalized against protein concentration in the cell extracts, which was measured using bicinchoninic acid assay kit (Biorad, CA, USA). Absorption was measured on a microplate reader (Spectra Plus, TECAN) at 570 nm and compared to a standard curve calibrated with BSA samples of known concentration. Results are expressed as relative light units per milligram of cell protein lysate (RLU/mg protein).

*Confocal Laser Scanning Microscopy (CLSM) Observation*: Confocal images were acquired using a Zeiss LSM 510 laser scanning microscope (Caril Zeiss, Jena, Germany) using a oil-immersion 100× objective. For cellular uptake observation, the cells were treated with doxorubicin or SNPs for 4 h. For co-delivery observation, the cells were treated with SNPs for 4 h and further incubated in complete medium containing 10% FBS for 48 h. The nuclei were stained with DAPI (Sigma). Excitation wavelengths were 488 nm, 395 nm and 350 nm for doxorubicin, EGFP and DAPI, respectively.

*Flow Cytometry*: The cellular uptake of doxorubicin and SNPs was carried out in FL2-H channel with a BD FACSCalibur flow cytometer (BD Biosciences, San Diego, CA).

*In vivo Retention and Transfection Assay*: BALB/c mice (6-8 weeks old) bearing ventral B16F10 tumors were treated at 8 days after implantation. Retention assay was performed with both free drug and EGFP pDNA conjugated SNPs (N/P ratio = 25:1) at the equivalent doxorubicin dose of 1.0 $mg \cdot kg^{-1}$ BW. Florescence images were acquired at 1.5, 3, 5, 8 or 24 h post-injection using a NightOWL II LB 983 NC320 in vivo optical imaging system (Berthold, Oak Ridge, TN) at excitation wavelength of 488 nm. Tumors were taken out after 48 h post-transfection and scanned at excitation wavelength of 395 nm. Animal experiments were performed in accordance with the Guidelines of the Zhejiang University Animal Experimentation Committee.

## 3. Table S1 and S2

**Table S1** Pharmacokinetics of free drug release from PEI-CyD/Ada-Dox

| pH | $F_{\%,released} = a\left[1 - e^{\frac{-(\ln 2)t}{t_{1/2}}}\right]$ | | $F_{\%,released} = k_0 t + b$ | |
|---|---|---|---|---|
| | *a* | $t_{1/2}$ (h) | $k_0$ ($10^{-2}\cdot h^{-1}$) | *b* |
| 7.4 | 15.0±0.7 | 0.36±0.06 | 3.45±0.49 | 16.2±0.2 |
| 5.0 | 38.4±1.5 | 0.16±0.03 | 7.34±2.52 | 41.7±1.0 |
| 3.0 | 52.9±1.4 | 0.13±0.02 | 9.79±0.02 | 56.9±0.8 |

**Table S2** $IC_{50}$ values in cytotoxicity assays

| Compounds | $IC_{50}$ in B16F10 cells ($\mu g\cdot mL^{-1}$) | $IC_{50}$ in COS7 cells ($\mu g\cdot mL^{-1}$) |
|---|---|---|
| Doxorubicin | 1.22±0.02 | 1.52±0.17 |
| Ada-Dox | 0.75±0.02 | 0.72±0.04 |
| PEI-CyD/Ada-Dox | 0.44±0.03 | 0.48±0.03 |