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### Supporting Information

### Post-targeting strategy for ready-to-use targeted nanodelivery post cargo loading

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Fig. S1 Post-targeting preparation of typical  $N_POC$  nanoplatform post loading drug/gene cargoes and Structures of OEI-EHDO, Chol-PBA and PBA-PEG-FA.



Fig. S2 <sup>1</sup>H NMR spectra of mPEG-PBA (A) and PBA-PEG-FA (B) in  $D_2O$ .



**Fig. S3** Particle size (A) and zeta potential (B) of various samples measured by DLS. Values were expressed as means  $\pm$  S.D. \*\*p < 0.01 were determined by a Student's t-test when the group treated with N<sub>P</sub>OC(DG)-PF were compared with those treated with N<sub>P</sub>OC(DG).



Fig. S4 TEM images of  $N_POC$  (A),  $N_POC(DG)$  (B),  $N_POC(DG)$ -PF (C).



**Fig. S5** DLS profiles of NpOC(DG)-PF solutions over time in the medium containing 10% serum and a normal blood sugar concentration of 1.0 mg/mL at pH = 7.4.



Fig. S6 CLSM images of HeLa cells recorded at 2 h and 8 h post the incubation with  $N_POC(DG)$  and  $N_POC^{\#}(DG)$ . Nuclei were stained blue with Hoechst 33342. pGL-3 was stained green with YOYO-1. Red fluorescence referred to DOX. Scale bars: 20  $\mu$ m.



Fig. S7 Cytotoxicity profiles of  $N_POC$  and  $N_POC^{\#}$  nanoassemblies in HeLa cells after 48-h co-culture. Values were expressed as means  $\pm$  S.D.



**Fig. S8** Luciferase expression in HeLa cells mediated by vector/pGL-3 complexes at various w/w ratios. Data were shown as the mean  $\pm$  S.D. (n=3)



**Fig. S9** Flow cytometric profiles of KB cells and COS7 cells upon 4 h coincubation with  $N_POC(DG)$  (A) and  $N_POC(DG)$ -PF (B) in the absence/presence of free FA inhibitor. DOX concentration was fixed at 2.5 µg/mL. Purple line: KB cells in the absence of FA, green line: KB cells in the presence of FA, blue line: COS7 cells in the absence of FA, orange line: COS7 cells in the presence of FA.

#### **Experimental Section**

#### Materials

Branched oligoethylenimine (OEI, Mw~1800 Da) was purchased from Sigma Aldrich (St. Louis, MO, USA). 5-ethyl-5-(hydroxymethyl)-1,3-dioxan-2-oxo (EHDO) was prepared according to an amended method in the literature.<sup>[1]</sup> 5-ethyl-5-(hydroxymethyl)-1,3-dioxan-2-oxo grafted oligoethylenimine (OEI-EHDO) and cholest-5-en-3-ol(3β)-(3-boronophenyl) carbamate (Chol-PBA) was prepared according to the previous work.<sup>[2]</sup> 3-aminophenylboronic acid (PBA, 98%), 2-(Bromomethyl) phenylboronic acid and cholesteryl chloroformate (Chol, 98%) were obtained from Aldrich Chemical Co. Ltd. Folate (FA), dual amine-terminated polyethyleneglycol (NH<sub>2</sub>-PEG-NH<sub>2</sub>, Mw~ 1900 Da) and amine-functionalized PEG (mPEG-NH<sub>2</sub>, Mw~ 1900 Da) were purchased from Aladdin. Doxorubicin hydrochloride (DOX HCI) was purchased from Zhejiang Hisun Pharmaceutical Co. (China). All other chemicals were purchased from Shanghai Chemical Reagent Ltd. and used without any treatments. QIAfilter<sup>™</sup> plasmid Purification Giga Kit (5) was purchased from Qiagen (Hilden, Germany). GelRed<sup>™</sup> was purchased from Biotium (CA, USA). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), trypsin. YOYO-1 iodide. Hoechst 33342. 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide (MTT), penicillin-streptomycin and phosphate buffered saline (PBS) were purchased from Invitrogen Corp (Carlsbad, CA, USA).

#### Instruments

<sup>1</sup>H nuclear magnetic resonance (<sup>1</sup>H NMR) spectra were recorded on a Varian Unity 300 M Hz spectrometer using  $D_2O$  as the solvent. The hydrodynamic size and zeta potential were determined by dynamic light scattering (DLS) on a Malvern Zetasizer Nano-ZS ZEN3600 instrument. Transmission electron microscopy (TEM) was carried out using a JEOL JEM-100CXII instrument operating at an acceleration voltage of 80 KV. Confocal microscopy was performed on a confocal laser scanning microscope (CLSM) (Nikon C1-si TE2000, Japan) and recorded by EZ-C1 software. Flow cytometric assay was carried out by flow cytometry (BD FACSAria<sup>™</sup> III, USA). Image acquisition was performed on a Maestro in vivo imaging system (Cambridge Research & Instrumentation, Inc., USA) with the excitation wavelength 480 nm and emission wavelength 600 nm. Results were analyzed using Living Image 3.1 software (Caliper Life Sciences).

# Synthesis of phenylboronic acid functionalized PEG (mPEG-PBA) and dual functionalized PEG (PBA-PEG-FA)

Phenylboronic acid functionalized mPEG-PBA was synthesized according our previous method. In brief, mPEG-NH<sub>2</sub> (820 mg) and 2-(bromomethyl) phenylboronic acid (800 mg) were dissolved in 30 mL of methanol, and stirred at 70 °C for 24 h. Then, the reaction solution was concentrated by rotary evaporation followed by precipitation in a great amount of diethyl ether thrice. The product was dried under high vacuum to obtain the light yellow powder in ~62% yield.[3] For preparation of phenylboronic acid and folate dual-functionalized PEG, 2-(bromomethyl) phenylboronic acid (108 mg) was dissolved in 5 mL of dry methanol, and then added dropwise into 25 mL dry methanol containing 950 mg NH<sub>2</sub>-PEG-NH<sub>2</sub>, and stirred at 70 °C for 24 h. After that, folate (220 mg), DIC (127 mg) and HOBt (135 mg) dissolved in 5 mL of dry DMSO was added dropwise into the abovementioned reaction mixture, and further stirred for another 48 h at room temperature. The reaction solution was precipitated with a large amount of diethyl ether, collected by centrifugation, and dried at 40 °C under vacuum overnight. The chemical structure of mPEG-PBA and PBA-PEG-FA was verified by <sup>1</sup>H NMR spectrometry.

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#### Preparation of OEI-EHDO/Chol-PBA nanoassembly (N<sub>P</sub>OC)

OEI-EHDO/Chol-PBA nanoassembly was prepared by the solvent evaporation method. According to our previous study, the molar ratio of OEI-EHDO and Chol-PBA was optimized at 1:1. In brief, Chol-PBA (30 mg) dissolved in 150  $\mu$ L of THF was added to 30 mL of deionized water containing OEI-EHDO (90 mg) under stirring for 24 h. The organic solvent was completely removed by rotary evaporation. After centrifugation treatment, the obtained solution was lyophilized to provide the N<sub>P</sub>OC nanoassembly.

#### Preparation of drug loaded N<sub>P</sub>OC (N<sub>P</sub>OC(D)) and in vitro drug release study

DOX loading into the N<sub>P</sub>OC nanoassembly was performed as follows: 5.0 mg of N<sub>P</sub>OC were dispersed in 10 mL of distilled water. 1.0 mg of DOX dissolved in 2.0 mL of DMSO was added to the above solution, which was allowed for stirring for 6 h. The obtained solution was then placed into a dialysis tube with molecular weight cut-off 3500 Da and dialyzed against 2 L of deionized water for 24 h. The deionized water was replaced every 4 h to remove DMSO and unloaded DOX. After that, the solution in dialysis tube was collected. A part of drug-loaded nanoassembly solution was lyophilized and then dissolved in 3 mL of DMSO for UV-Vis measurement. The drug loading content (DLC) was determined on the basis of the UV absorbance intensity at 480 nm for DOX, according to the standard calibration curve experimentally obtained. The data were shown as the mean value from triplicate independent experiments. The DLC is defined as follows:

DLC (wt. %) = (mass of loaded drug)/(mass of drug-loaded micelles) ×100%

3 mL of DOX-loaded N<sub>P</sub>OC nanoassembly solution obtained as above was dialyzed against 8 mL of pH~7.4 PBS buffer and pH~5.0 acetate buffer at 37 °C,

respectively. At the pre-set time intervals, the solution was withdrawn and replaced with same volume of fresh buffer solution. The amount of DOX released from  $N_POC$  nanoassembly was determined according to the fluorescence absorbance intensity at 560 nm, using an experimentally obtained standard calibration curve.

#### Preparation of drug and gene co-loaded nanocomplexes (N<sub>P</sub>OC(DG))

In brief, N<sub>P</sub>OC(DG) nanocomplexes at different w/w ratios (weight ratio of OEI-EHDO versus DNA) were prepared by adding known volume N<sub>P</sub>OC(D) solution (DOX: 0.5 mg/mL) to 5  $\mu$ L of DNA solution (200 ng/ $\mu$ L in TE water), followed by incubation at 37 °C for 30 min.

# Preparation of post-targeting nanocomplexes (N<sub>P</sub>OC(DG)-PF) via coupling with PBA-PEG-FA (PF)

In brief, N<sub>P</sub>OC(DG) complexes were prepared as mentioned above (w/w ratio~25), and then 20  $\mu$ L of PBA-PEG-FA (mPEG-PBA) (1.0 mg/mL) was added into the complex solution. The N<sub>P</sub>OC(DG)-PF was obtained after stirring for 1 h at room temperature. The pre-targeting nanocomplexes were prepared by coupling with PBA-PEG-FA prior to DNA complexation.

# Synthesis of cholesterol conjugated OEI-EHDO (OEI-EHDO-Chol) and preparation of OEI-EHDO-Chol ( $N_POC^*$ ) nanoassembly

OEI-EHDO (196 mg, 0.1 mM) and triethylamine (20.2 mg, 0.2 mM) were dissolved in 10 mL of dry DMSO. Chol (67.4 mg, 0.15 mM) dissolved in 5 mL of dry  $CH_2CI_2$  was added to the above solution dropwise, and the reaction was allowed to proceed for 12 h. Thereafter, the reaction mixture was dialyzed against DMSO and distilled water to obtain the N<sub>P</sub>OC<sup>#</sup> nanomicelles. The hydrodynamic size of N<sub>P</sub>OC<sup>#</sup> nanomicelles

was ~180 nm determined by DLS (data not shown), and the drug loading content was calculated to be 8.1%.

#### Measurement of hydrodynamic size and zeta potential

The hydrodynamic size ( $D_h$ ) and zeta potential of various samples were determined by the dynamic light scattering (DLS) technique using Zeta sizer Nano-ZS ZEN3600 (Malvern instruments). The prepared solution was all passed through a 0.45 µm pore size filter to avoid interference of the dust prior to the experiment.

#### Transmission electron microscopy (TEM) observation

In brief, a drop of sample solution was placed on a copper grid with Formvar film and stained by phosphotungstic acid solution (0.2% w/v) prior to photography by TEM (JEOL JEM-100CXII microscope).

#### Agarose gel retardation assay

 $N_POC(DG)$ , post-targeting  $N_POC(DG)$  and pre-targeting  $N_POC(DG)$  at different w/w ratios were obtained by mixing 0.1 µg of pGL-3 with a certain volume of various sample solutions, followed by diluting with PBS to a total volume of 8 µL before incubation at 37 °C for 30 min. Then, the agarose gel retardation assays were conducted according to the reference.<sup>[2]</sup> As for the post-targeting group, PBA-PEG-FA was added to further incubate for 1 h after DNA complexation.

#### Cell culture and amplification of plasmid DNA

African green monkey SV40-transformed kidney fibroblast (COS7) cells and human cervix carcinoma (HeLa) cells were incubated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% antibiotics

(penicillin-streptomycin, 10,000 U·mL<sup>-1</sup>) at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>. Human mouth epidermal carcinoma (KB) cells were incubated in folic acid-depleted RPMI-1640 medium containing 10% FBS and 1% antibiotics (penicillin-streptomycin, 10,000 U mL<sup>-1</sup>) in a humidified atmosphere containing 5% CO<sub>2</sub>. The p53 and pGL-3 plasmid used in this study was transformed in E. coli JM109. Green fluorescent protein-tagged p53 and luciferase reporter gene pGL-3 were amplified in the terrific broth media at 37 °C overnight followed by purification using an EndoFree QiAfilterTM Plasmid Giga Kit (5). Then, the purified plasmids were diluted with Tris-EDTA (TE) buffer solution to a final concentration of 200 ng  $\mu$ L<sup>-1</sup> and then stored at -20 °C for later use.

#### Confocal laser scanning microscopy (CLSM)

HeLa, KB and COS7 cells were seeded in single dish with the density of  $2.0 \times 10^5$  cells/dish and incubated at 37 °C for 24 h. Plasmid pGL-3 (1 µg) was firstly labeled with YOYO-1 (3.0 µL,  $1 \times 10^{-2}$  M) by 15 min of coincubation at 37 °C. To observe the intracellular trafficking of loaded DNA and DOX, HeLa cells were then treated with N<sub>P</sub>OC(DG) and N<sub>P</sub>OC#(DG) at a DOX dosage of 2.5 µg/mL and w/w ratio of 25, and co-incubation were proceeded for 2 h or 8 h. Afterward, the media were discarded and the cells were washed with PBS thrice. The nuclei were stained with Hoechst 33342 for 15 min and washed several times with PBS, and photographed by CLSM (Nikon C1-si TE2000, Japan). To study the targeting capability of N<sub>P</sub>OC(DG)-PF prepared by our post-targeting strategy, KB cells were incubated with the post- and pre-targeting with Hoechst 33342, the cells were visualized by CLSM. To further investigate the targeting ability, free FA with a concentration of 2.5 µM (~1.0 µg/mL) was firstly added to block the folic acid receptor on KB cell. Then, the FA-treated cells

were incubated with the N<sub>P</sub>OC(DG)-PF prepared via post-targeting methodology for 1 h at 37 °C. In comparison, the case without addition of free FA was conducted under the same conditions. COS7 cells were used as FA receptor-negative control cells. The PF-absent N<sub>P</sub>OC(DG) was selected as control counterpart upon the same treatment in both cells. The nuclei were then stained prior to CLSM observation.

#### Flow cytometry

The cellular uptake of N<sub>P</sub>OC(DG)-PF and N<sub>P</sub>OC(DG) in the presence/absence of free FA was quantitatively evaluated by flow cytometry. KB and COS7 cells were seeded in 6-well plates ( $6 \times 10^4$  cells/well) and cultured for 24 h. Then, cells in some well were pre-incubated with 2.5 µM of free FA (~1.0 µg/mL) for 2 h to block the FA receptor. Afterward, the media were replaced with fresh cell culture medium containing N<sub>P</sub>OC(DG)-PF and N<sub>P</sub>OC(DG) with DOX concentration of 2.5 µg/mL and w/w ratio of 25. After further incubation for 1 h, the media were discarded and the cells were washed with PBS, digested by trypsin and collected by centrifugation treatment (1000 rpm, 5 min). The bottom cells were resuspended with PBS, filtrated and examined by flow cytometry (BD FACSAria<sup>TM</sup> III, USA). The non-treated cells as negative control were calibrated to identify viable cells.

#### In vitro gene transfection

Transfection of pGL-3 plasmids mediated by N<sub>P</sub>OC(G) and N<sub>P</sub>OC(DG) in HeLa cells was studied and PEI25K was used as positive control. Cells were seeded in 24-well plate (6×10<sup>4</sup> cells/well) and incubated for 24 h. The prepared N<sub>P</sub>OC(G) and N<sub>P</sub>OC(DG) complexes at various w/w ratios were diluted to 1 mL with 10% FBS-containing DMEM and added to the cell wells. After 4 h incubation, the cells was fed with fresh cell culture and further incubated for 44 h at 37 °C. The luciferase assay

was then performed.<sup>[2]</sup> Data were shown as mean  $\pm$  standard deviation (SD) based on three independent measurements.

#### In vitro cytotoxicity assay

The in vitro cytotoxicity of various samples was examined by MTT assay. p53 was used as a therapeutic gene to demonstrate the combination effect together with DOX. HeLa, KB and COS7 cells were seeded in 96-well plate (6×10<sup>3</sup> cells/well) and incubated at 37 °C for 24 h. To study the cytotoxicity of single or dual loaded samples, HeLa cells were treated with various samples at a series of concentrations for 48 h while DNA-present samples (w/w ratio ranging from 5 to 30) for 4 h and further 44 h with fresh cell culture. After that, 20 µL of MTT solution (5 mg mL<sup>-1</sup>) was added and further cultured for 4 h. Then, the medium in each well was replaced with 150 µL of DMSO. The optical density (OD) was measured at 570 nm with a microplate reader (BIO-RAD 550, USA). The relative cell viability was calculated by the following equation: (OD570<sub>sample</sub>-OD570<sub>background</sub>)/(OD570<sub>control</sub>-OD570<sub>background</sub>)×100%, where OD570<sub>sample</sub> was obtained in the presence of samples, OD570<sub>control</sub> was obtained in the absence of samples and OD570<sub>background</sub> was obtained in the absence of samples and cells. Data were shown as the mean ± standard deviation (SD) based on triplicate independent experiments. To verify the targeting capability of N<sub>P</sub>OC(DG)-PF, free FA (2.5 µM) was pre-incubated with KB cell to block the folic acid receptor. Then, the FA-treated cells were treated with the post-targeting N<sub>P</sub>OC(DG)-PF for 4 h and further incubated with fresh cell culture for 44 h. The cell viability was then calculated as described above. The case without free FA addition was compared under the same conditions. COS7 cells were used as FA receptor-negative control cells. The N<sub>P</sub>OC(DG) without targeting groups (PF) was selected as control counterpart upon the same treatment in both cells.

#### Live/Dead cell staining assay

p53 was used as a therapeutic gene to demonstrate the combination effect together with DOX. HeLa cells were seeded in 6-well plates ( $5 \times 10^5$  cells/well) and incubated for 24 h. Then, the cells were treated with various samples including PBS, N<sub>P</sub>OC(G), N<sub>P</sub>OC(D), N<sub>P</sub>OC<sup>#</sup>(DG), DOX and N<sub>P</sub>OC(DG) at a DOX dosage of 2.5 µg/mL and w/w ratio of 25. After 4-h incubation, the cells were stained with Calcein-AM ( $4 \times 10^{-6}$  M) and PI ( $4 \times 10^{-6}$  M) in PBS buffer solution and further incubated for 15 min at 37 °C. Then, the cells were washed with PBS thrice prior to exposure to CLSM (Nikon C1-si, Japan). Calcein-AM, and PI were excited at 488 and 543 nm respectively, and the corresponding emission spectra were collected under wavelength coverage at 510-540 nm (green) and 570-620 nm (red), respectively.

#### In vivo anti-tumor effect

The animal experiments were conducted according to the guidelines for laboratory animals established by the Wuhan University Center for Animal Center Experiment/A3-Lab. Mice were subcutaneously injected with 100  $\mu$ L of hepatoma H22 cells (1×10<sup>6</sup> cells/mouse) on the right hind limb. When tumor volume reached ~100 mm<sup>3</sup>, the mice were divided into six groups randomly (6 mice per group). The mice were subcutaneously injected with different formulations including PBS, N<sub>P</sub>OC(G), N<sub>P</sub>OC(D), N<sub>P</sub>OC<sup>#</sup>(DG), DOX and N<sub>P</sub>OC(DG) at a DOX dosage of 2.5 mg/kg and plasmid p53 of 0.75 mg/kg every day. The mice body weight and tumor volume were measured every day and tumor volume were calculated as V = W<sup>2</sup>L/2, where W and L are the shortest and longest diameters, respectively. Relative tumor volume was calculated as the recorded tumor volume versus the initiated tumor volume before treatment. Relative body weight was defined as the recorded body

weight versus the initiated body weight before treatment. After 12 days, mice were sacrificed, and the tumors were exfoliated, weighed and photographically recorded. Hematoxylin and eosin (H&E) staining were performed for histological examinations. The tumors were fixed using 4% formalin, embedded in paraffin, and sectioned. Sections with 5 µm thickness were placed on polylysine-coated slides and stained with hematoxylin and eosin. The stained slides were photographed under microscope (BX60, Olympus, Japan) at 200× magnifications. To examine the p53 expression presenting green fluorescence in tumor tissues, cryosections of tumor tissues were conducted. Cryosections with 5 µm thickness were fixed with cold acetone, stained with 4',6-diamidino-2-phenylindole (DAPI) for 10 min, and washed with PBS thrice. The stained cryosections were finally photographed by CLSM (Nikon C1-si TE2000, Japan).

#### In vivo fluorescence imaging and tissue distributions of N<sub>P</sub>OC(DG)-PF

For comparison, FA-absent PEG-PBA was coupled to N<sub>P</sub>OC(DG) in the same fashion to provide N<sub>P</sub>OC(DG)-P control. Mice bearing H22 tumor on the right hind limbs were developed by subcutaneous injection of  $1 \times 10^6$  cells per mouse. Then, mice were intravenously injected with 100 µL of sample solutions including N<sub>P</sub>OC(DG)-PF and N<sub>P</sub>OC(DG)-P at a DOX dosage of 2.5 mg/kg and plasmid p53 of 0.75 mg/kg. After 24 h post injection, the mice were anesthetized and imaged via Maestro *in vivo* Imaging System (Cambridge Research &Instrumentation, Inc., USA). A single-filter set with the excitation wavelength of 480 nm and emission wavelength of 600 nm was exploited to detect DOX-related fluorescence, due to the absence of autofluorescence produced by the skin and blood vessels.<sup>[4]</sup> For *ex vivo* fluorescence imaging, tumors and major organs (heart, liver, spleen, lung and kidney) were

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dissected, washed with cold saline and then subjected to Maestro *in vivo* Imaging System to obtain the fluorescence images.

#### **Statistical analysis**

Statistical analysis was performed by Student's t test. The data were expressed as mean  $\pm$  standard deviation unless otherwise noted. A value of p < 0.05 was considered statistically significant.

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