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# Supplementary Materials for

## Erythrocyte leveraged chemotherapy (ELeCt): Nanoparticle assembly on erythrocyte surface to combat lung metastasis

Zongmin Zhao, Anvay Ukidve, Yongsheng Gao, Jayoung Kim, Samir Mitragotri*

*Corresponding author. Email: mitragotri@seas.harvard.edu

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## Supplementary Materials and Methods

### Materials

Poly(lactic-co-glycolic acid) (PLGA) (65:35) Resomer® 653, methotrexate, camptothecin, 5- fluorouracil, sodium heparin, fluroshield®, DMEM, fetal bovine serum (FBS), Penicillin Streptomycin (Pen Strep) were obtained from Sigma Aldrich (MO, USA). Doxorubicin hydrochloride, docetaxel, paclitaxel and gemcitabine hydrochloride were obtained from L.C. Laboratories (MA, USA). Nunc™ Lab-Tek™ II Chamber Slide™ System, cell staining buffer, puromycin, phosphate buffered saline (1X), Axygen™ 1.5mL Self-Standing Screw Cap tubes were obtained from Thermo Fischer Scientific (MA, USA). B16-F10 melanoma cell line (B16F10-Luc) expressing luciferase were obtained from Imanis Life Sciences (MN, USA). Human whole blood and serum was obtained from BioIVT (NY, USA). Xenolight-D-luciferin potassium salt was obtained from Perkin Elmer (MA, USA). Lithium heparin coated microtainer tubes were obtained from BD medical technology (NJ, USA). CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) kit was obtained from Promega (CA, USA). Tissue Tek OCT compound was obtained from Sakura Finetek (CA, USA). 0.9 % saline solution was obtained from Teknova (CA, USA). Paraformaldehyde was obtained from Electron Microscopy sciences (PA, USA). All other chemicals were reagent grade and obtained from Sigma Aldrich (MO, USA).

### Cell culture

B16F10-Luc cells were cultured in a humidified incubator maintained at 37° C and 5 % $CO_2$. They were cultured in DMEM media supplemented with 10 % FBS, 1% Pen Strep and 1 µg/mL Puromycin. Cells were passaged 3-4 times before their use.

### *In vitro* drug release study

DOX containing nanoparticles were resuspended in 1 mL complete medium (DMEM + 10% FBS) and incubated at 37° C on a tube revolver. At regular time points, the particles were centrifuged at 12000 g for 15 mins and the supernatant was collected for analysis. The particles were further resuspended in

1mL of fresh release media and incubated at 37° C until the next time point. Samples were taken at 1, 2, 4, 6, 12 and 24 h after starting the incubation. The cumulative release was quantified using DOX as fluorophore (Ex/Em 470/590 nm) on a plate reader (Tecan Safire 2®, NY, USA).

**Particle internalization and cytotoxicity studies**

Particle internalization was confirmed using flow cytometry and confocal microscopy. For flow cytometry analysis, 2 x $10^6$ B16F10-Luc cells were plated in a 12-well plate and allowed to adhere overnight. Plates were then aspirated, and 1 mL of fresh media was added to each well. 30 µg of nanoparticles were added to each well and allowed to incubate for 20 mins, 2 h or 6 h at 37° C in an incubator. After the stipulated time points, media in the wells was completely aspirated and washed 3 times with PBS and the cells were detached from plate using 0.25 Trypsin/EDTA solution. After being washed with PBS, these cells were analyzed by flow cytometry (BD LSR Analyser II, CA, USA) using DOX fluorophore.

For confocal microscopy, 2 x $10^5$ B16F10-Luc cells were plated in individual chambers of Nunc™ Lab-Tek™ II Chamber Slide™ System (Thermo Fischer Scientific) and allowed to adhere overnight. Plates were then aspirated, and 1 mL of fresh media was added to each well. 30 µg of nanoparticles were added to each well and allowed to incubate for 20 mins, 2 h or 6 h at 37° C in an incubator. After the stipulated time points, media in the wells was completely aspirated and cells were washed 3 times with PBS before fixing with 4% paraformaldehyde. The fixed cells were mounted using Fluroshield ® to stain for DAPI (Ex/EM 340/488 nm) and were analyzed using confocal microscopy (Upright Zeiss LSM 710 NLO ready, Germany).

The cytotoxicity of loaded PLGA particles, unloaded PLGA particles and free DOX was assessed using CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) according to manufacturer's instructions. Briefly, 2000 B16-F10-Luc cells were seeded in a 96-well plate and allowed to adhere overnight. The media was then aspirated and replaced with media containing various formulations at

different concentrations and allowed to incubate for 24 h at 37° C. 20 µl of CellTiter 96® AQueous One Solution reagent was added to the wells and allowed to incubate in a humidified incubator at 37° C for 4 h. The absorbance was read at 490 nm using a plate reader (Epoch II, Biotek systems, VT, USA). Dose response curves were fit to each formulation with the Variable slope model (Four parameter-dose response curve) using Graphpad Prism 6 and $IC_{50}$ values were calculated using the same software.

**Preparation of different chemotherapeutic agent-loaded biodegradable PLGA nanoparticles**

All chemotherapeutic agent-loaded biodegradable PLGA nanoparticles were prepared using a nanoprecipitation method with minor modifications. To prepare nanoparticles loaded with methotrexate, 5-fluorouracil, 5-fluorouracil + methotrexate, and camptothecin, 2 mg of drug (1 mg of each for 5-fluorouracil + methotrexate) was dissolved in 200 µL of DMSO. The drug solution was then mixed with 20 mg of PLGA dissolving in 1 mL acetone. The following steps are same as in preparing DOX-loaded PLGA nanoparticles. To prepare nanoparticles loaded with docetaxel and paclitaxel, 20 mg of PLGA and 2 mg of drug was dissolved in 1 mL acetone to form the organic phase. The following steps are as in preparing DOX-loaded PLGA nanoparticles. To prepare nanoparticles loaded with gemcitabine, 2 mg of gemcitabine hydrochloride was dissolved in 0.5 mL methanol and 5 µL Triethylamine (TEA), and this drug solution was added to 20 mg of PLGA dissolved in 1 mL acetone. The following steps are same as in preparing DOX-loaded PLGA nanoparticles. All particles were collected at 12000 *g* for 15 mins and washed three times using deionized water. Nanoparticle size and zeta potential were measured using dynamic light scattering (Malvern Zen3600).

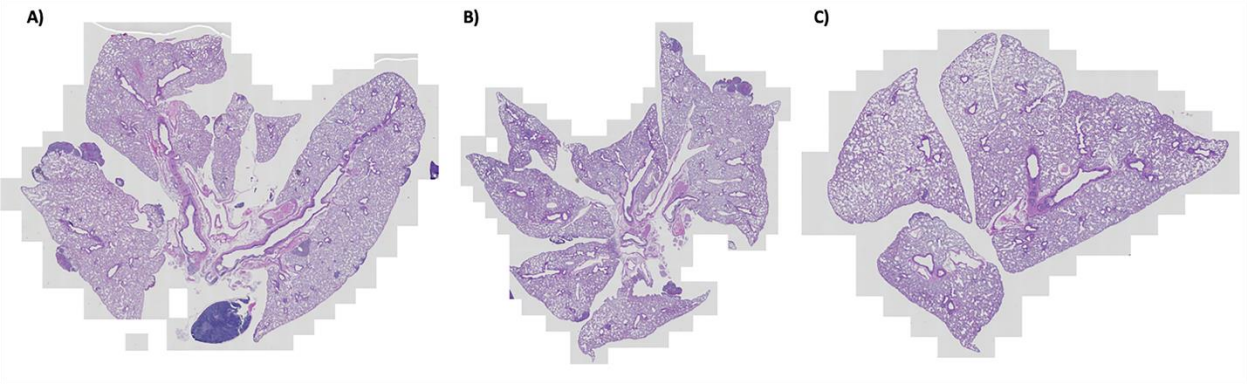


**Fig. S1. Representative H&E staining images of lungs of mice.** Mice treated with (**A**) control (Saline), (**B**) DOX-loaded NPs, and (**C**) drug nanoparticles assembled on erythrocytes (RBC-NPs) were scarified 16 days after tumor inoculation in the late-stage lung metastasis model. Lungs were processed by H&E staining.

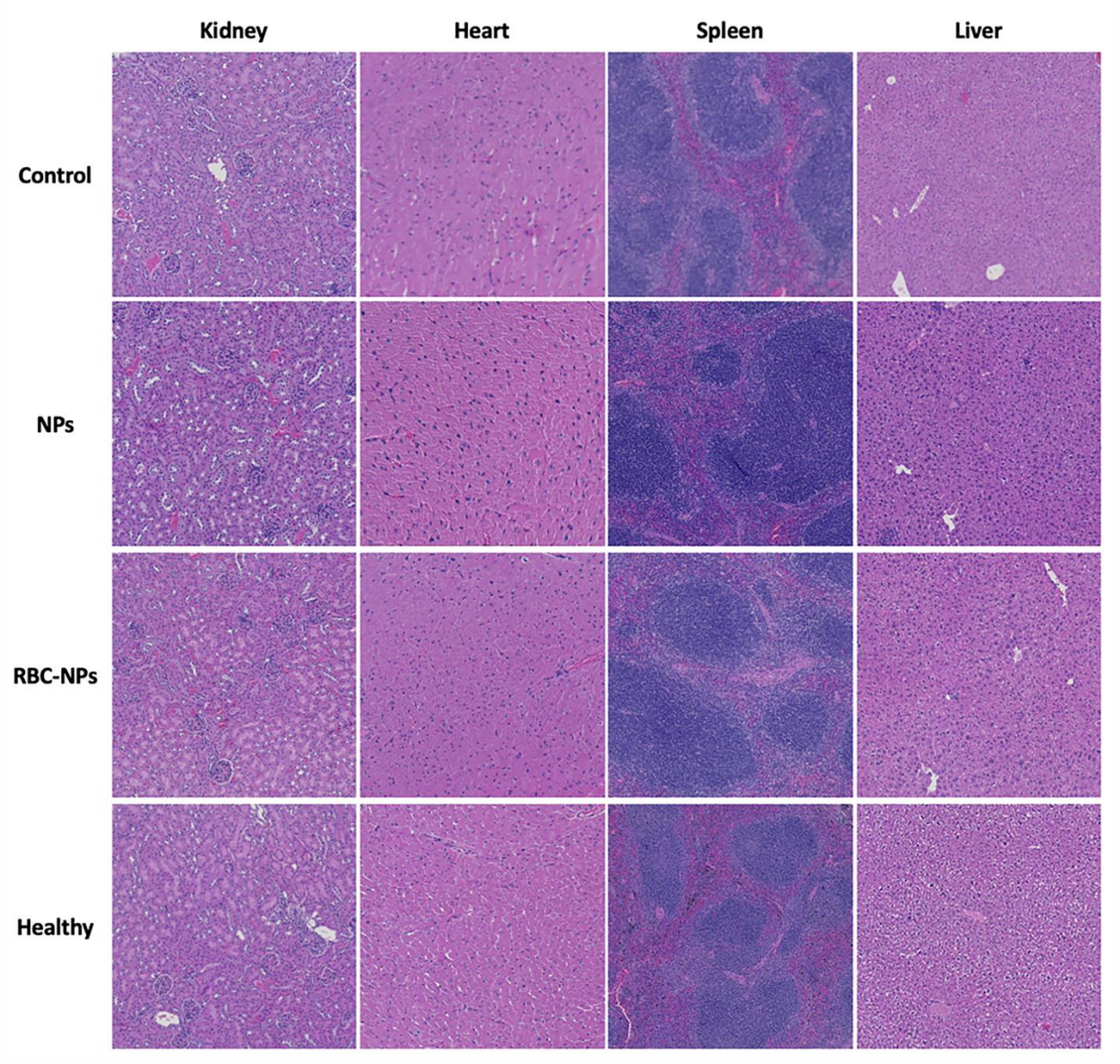


**Fig. S2. Representative H&E staining images of organs of mice treated with different drug formulations.** 16 days after tumor inoculation in the late-stage lung metastasis model, mice were scarified and organs were processed by H&E staining.

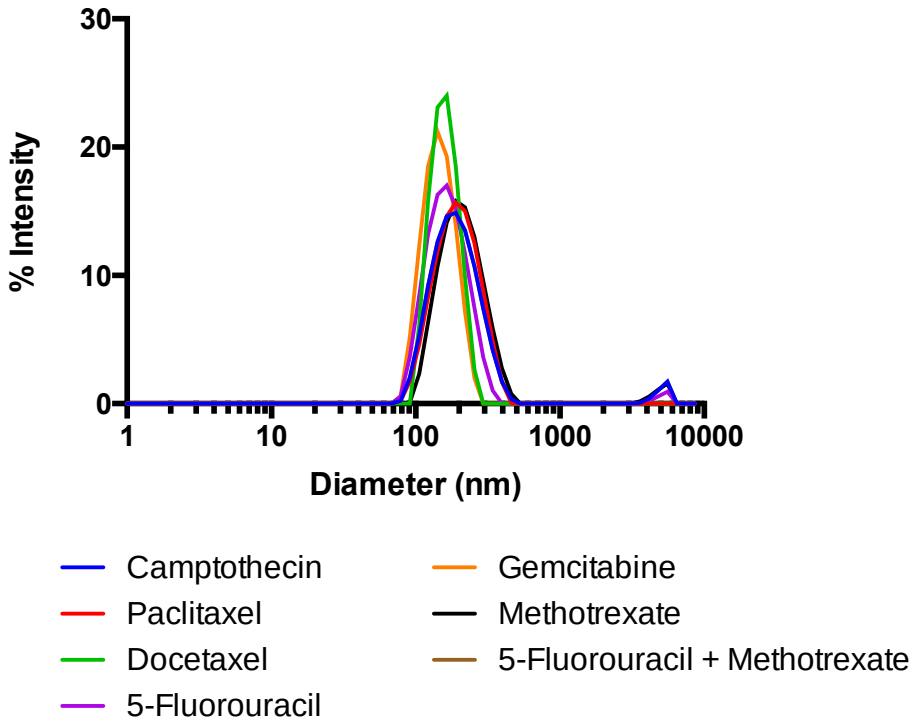


**Fig. S3. Size distribution of different chemotherapeutic agent–loaded biodegradable PLGA NPs.**

**Table S1. Physicochemical properties of different chemotherapeutic agent–loaded biodegradable PLGA NPs.**

| | Average diameter (nm) | Zeta-potential (mV) | PDI |
|---|---|---|---|
| **Camptothecin** | 231.3 ± 6.5 | -27.9 ± 1.3 | 0.250 ± 0.038 |
| **Paclitaxel** | 222.0 ± 9.7 | -30.3 ± 1.4 | 0.342 ± 0.043 |
| **Docetaxel** | 182.2 ± 8.1 | -26.5 ± 1.2 | 0.256 ± 0.092 |
| **5-Fluorouracil** | 167.7 ± 4.7 | -29.4 ± 0.5 | 0.196 ± 0.020 |
| **Gemcitabine** | 139.5 ± 1.8 | -28.0 ± 0.8 | 0.068 ± 0.044 |
| **Methotrexate** | 211.1 ± 2.5 | -21.8 ± 0.5 | 0.236 ± 0.016 |
| **5-Fluorouracil + Methotrexate** | 197.7 ± 6.2 | -25.7 ± 0.3 | 0.278 ± 0.021 |