Supporting Information

Lipid-insertion enables targeting functionalization of erythrocyte membrane-cloaked nanoparticles

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1. Experimental Section

Synthesis of ligand-linker-lipid conjugates. 1,2-distearoyl-*sn*-glycero-3-phosphoethanol amine conjugated to polyethylene glycol 2000 with fluorescein isothiocyanate (FITC-PEG-lipid) was purchased from Nanocs (New York, NY). 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[folate(polyethylene glycol)-2000] (folate-PEG-lipid) was purchased from Avanti Polar Lipids (Alabaster, AL). AS1411 aptamer, with the sequence of GGT GGT GGT GGT TGT GGT GGT GGT GG, was custom synthesized by Integrated DNA Technologies (San Diego, CA) with a 3' thiol modifier. 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-

[maleimide(polyethylene glycol)-2000] (maleimide-PEG-lipid) was purchased from Avanti Polar Lipids. For the synthesis of AS1411-PEG-lipid, the aptamer was suspended at 1 mg/mL in a buffer consisting of 10 mM Tris pH 8.0 (Cellgro) and 0.1 mM ethylenediaminetetraacetic acid (EDTA) (USB Corporation) with a 100× molar excess of tris[2-carboxyethyl] phosphine (TCEP) (Millipore) to reduce the disulfide bond of the thiol modifier. The solution was allowed to incubate for 4 hr at room temperature before washing the aptamer in the same reducing solution 3 times using a 10 kDa molecular weight cutoff Amicon Ultra-4 centrifuge filter (Millipore). The reduced aptamer was then incubated overnight with an equimolar amount of maleimide-PEG-lipid before washing again 3 times in water.

Preparation of ligand-inserted RBC ghosts. Whole blood was obtained from 10 week-old male ICR mice (Charles River Laboratories) via cardiac puncture using syringes preconditioned with heparin (Sigma Aldrich) and EDTA. All animal experiments were reviewed, approved and performed under the regulatory supervision of The University of California, San Diego's institutional biosafety program and the Institutional Animal Care and Use Committee (IACUC). RBCs were extracted from the collected blood by centrifuging at 800 × g for 5 min at 4°C and washed 3 times in cold phosphate buffered solution (PBS) (Invitrogen). The buffy coat was removed in the process. The washed RBCs were then lysed in 0.25× PBS, and the ghosts were separated by collecting the pink pellet after centrifuging at 800 × g for 5 min at 4°C. The pellet was then resuspended in water and incubated with ligand-PEG-lipid (ligand = FITC, folate, or AS1411) for 30 min to form ligand-inserted RBC ghosts. All samples were then washed by pelleting at 800 × g for 5 min at 4°C before further use.

Characterization of ligand-inserted RBC ghosts. To quantify ligand incorporation onto RBC ghosts, different amounts of FITC-PEG-lipid were incubated with RBC ghosts derived from 1 mL of mouse blood for 30 min. The total fluorescence of the solution was first quantified to obtain a value for the initial input. The ghosts were then washed 3 times and resuspended to the original volume. Another fluorescence reading was taken to determine the amount of ligand retained on the RBC ghosts relative to the initial input. For flow cytometry measurements, 40 μg of FITC-PEG-lipid was added into RBC ghosts derived from 1 mL of blood while plain RBC ghosts were used as a negative control. The samples were run on a FACSCanto II flow cytometer from Becton, Dickinson, and Company (Franklin Lakes, NJ) and the resulting data was analyzed using FlowJo software from Tree Star (Ashland, OR). Fluorescence imaging studies were conducted by dropping the FITC-modified RBC ghosts on a poly-L-lysine coated slide from Polysciences (Warrington, PA) and imaged using a 60X oil immersion objective on a DeltaVision Deconvolution Scanning Fluorescence Microscope from Applied Precision (Issaquah, WA).

Synthesis of ligand-functionalized RBC-NPs. The synthesis of ligand-functionalized RBC-NPs was adapted from a previously published protocol[1]. Briefly, RBC ghosts derived from 1 mL of blood that were functionalized with 40 μg of FITC-PEG-lipid were extruded serially through a 400 nm and then 100 nm polycarbonate membranes using a mini extruder (Avanti Polar Lipids). Polymeric cores of about 70 nm in diameter were prepared via the nanoprecipitation of 0.67dL/g carboxyl-terminated poly($_{D,L}$-lactic-*co*-glycolic acid) (PLGA) (LACTEL Absorbable Polymers). The ligand-inserted membrane vesicles and the polymeric cores were then extruded together at a ratio of 1 mL of blood worth of vesicles to 1 mg of cores

through a 100 nm polycarbonate membrane to form the final RBC-NPs. To measure average particle size and zeta potential, functionalized and non-functionalized RBC-NPs were suspended at 1 mg/mL in clear disposable capillary cells (DTS1061) from Malvern and measured by DLS using a Zetasizer (ZEN3600) from Malvern (United Kingdom). All measurements were taken at a backscattering angle of 173° and were performed in triplicate. The morphology of the particles were imaged by scanning electron microscopy (SEM) using an XL30 ESEM from FEI/Phillips (Hillsboro, OR) at a beam intensity of 20 kV. Samples were prepared at 1 μg/mL and 5 μL drops were dried onto silicon wafers. The samples were then coated with iridium at 85 mA for 7 sec on a K575X Sputter Coater from Emitech (Fall River, MA) followed by SEM imaging.

Colocalization studies of ligand-functionalized RBC-NPs. KB cells (ATCC: #CCL-17) were maintained in folate free RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (Hyclone) and penicillin-streptomycin (Gibco). Before the experiment, cultures were detached with 0.25% trypsin-EDTA (Gibco) and plated onto 8-chamber Labtek II slides (Nunclon) at 80% confluency. After allowing the cells to adhere overnight, they were incubated with RBC-NPs, of which the polymeric core was loaded with 0.05 wt% 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindo dicarbocyanine (DiD) (Invitrogen) and the RBC membrane shell contained FITC (40 μg FITC-PEG-lipid per 1 mL of blood worth of RBC ghosts). The cells were incubated with the RBC-NPs at a concentration of 0.25 mg/mL for 2 hr. The cells were then washed with media and allowed to incubate for another 4 hr before fixing with 10% formalin (Millipore) and mounted with DAPI-containing Vectashield® (Invitrogen). Imaging was done using a 60X oil immersion objective on an Applied Precision DeltaVision Deconvolution Scanning Fluorescence Microscope.

Cellular uptake studies of ligand-functionalized RBC-NPs. All flow cytometry studies were conducted on a FACSCanto II flow cytometer (Becton Dickinson) and the resulting data was analyzed using FlowJo software (Tree Star). 10,000 events were collected per sample and gated using control cells with no RBC-NP incubation. Histograms were plotted with fluorescence intensity on the x-axis using a biexponential scale. All imaging studies were conducted using a 20X objective on a DeltaVision Deconvolution Scanning Fluorescence Microscope from Applied Precision.

Both KB Cells (ATCC: #CCL-17) and A549 cells (ATCC: #CCL-185) were maintained as described above in supplemented folate-free RPMI (Gibco). Folate-functionalized particles with 0.05 wt% DiD loaded into the polymeric cores were prepared using folate-PEG-lipid at a ratio of 20 μg folate-PEG-lipid per 1 mL of blood worth of RBC ghosts. Cells were plated at a density of 10^5 cells per well on 12-well plates (Becton Dickinson) for flow cytometry or at 80% confluency on 8-chamber Labtek II slides (Nunclon) for fluorescence imaging and were allowed to adhere overnight before use. Folate-functionalized and non-targeted particles were incubated at a concentration of 250 μg per 1 mL of media. For samples with free folate, the concentration was adjusted to 1 mM using a 100 mM stock solution of folate in PBS (Gibco) 10 min before incubation with nanoparticles. All samples were incubated for 30 min, washed 3 times with media, and incubated in fresh media for another 30 minutes. For flow cytometry, cells were detached with trypsin EDTA (Gibco), washed with PBS (Gibco), and taken for analysis. For fluorescence imaging, the chambers were gently washed with PBS, fixed with 10% formalin (Millipore), and mounted with DAPI-containing Vectashield® (Invitrogen).

MCF-7 Cells (ATCC: #HTB-22) were maintained in DMEM (Gibco) supplemented with 10% fetal bovine serum (Hyclone) and penicillin-streptomycin (Gibco). AS1411-functionalized

particles with 0.05 wt% DiD loaded into the polymeric cores were prepared using AS1411-PEG-lipid at a ratio of 100 μg AS1411-PEG-lipid per 1 mL of blood worth of RBC ghosts. Cells were plated at a density of 10^5 cells per well on 12-well plates (Becton Dickinson) for flow cytometry or at 80% confluency on 8-chamber Labtek II slides (Nunclon) for fluorescence imaging and were allowed to adhere overnight before use. AS1411-functionalized and non-targeted particles were incubated at a concentration of 250 μg per 1 mL of media. For the sample with free AS1411, 100 μg AS1411 in 10 μL PBS (Gibco) was added 10 min before incubation with nanoparticles. All samples were incubated for 30 min and then washed 3 times with media followed by incubating for another 30 minutes. For flow cytometry, cells were detached with trypsin EDTA (Gibco), washed with PBS (Gibco), and taken for analysis. For fluorescence imaging, the chambers were gently washed with PBS, fixed with 10% formalin (Millipore), and mounted with DAPI-containing Vectashield® (Invitrogen).

Cytotoxicity study of RBC-NPs. Human umbilical vein endothelial cells (HUVECs) (Cell Applications) were maintained in endothelial cell growth medium (Cell Applications). Before the experiment, the cells were detached and plated at a density of 5 x 10^3 cells per well in a 96-well plate and allowed to attach overnight. Both unmodified and folate-functionalized RBC-NPs were prepared as previously described and serially diluted 3X starting from a concentration of 2 mg/mL. The samples were added to an equal volume of media in each well and allowed to incubate for 16 hr. Each sample was carried out in triplicate. The wells were then washed with media and allowed to incubate for another 48 hr. To conduct the cytotoxicity assay, all solution was removed from the wells and 100 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Invitrogen) in PBS was added to each well at a concentration of 0.5 mg/mL.

After 3 hr incubation at 37°C, the solution was removed and the crystals were solubilized with 100 µL of dimethyl sulfoxide (DMSO) (Sigma Aldrich) and the plate was allowed to incubate for another 20 min. The data was analyzed after reading the absorbance at 540 nm.

2. Supporting Figures

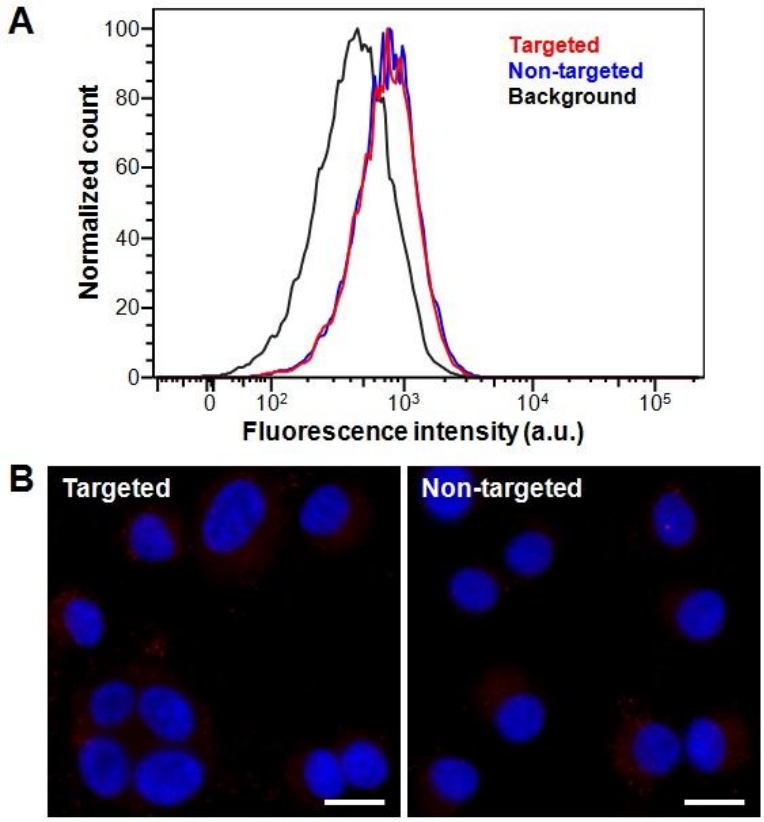

Fig. S1 Folate-functionalized RBC-NPs do not preferentially target a folate receptor-negative cell line. (A) Flow cytometry histograms of A549 cells alone (black) and the cells incubated with folate-functionalized RBC-NPs (red) and non-targeted RBC-NPs (blue). (B) Fluorescence microscopy of A549 cells incubated with folate-functionalized RBC-NPs and non-targeted RBC-NPs. A fluorescent probe DiD was loaded inside the RBC-NPs for visualization (red) and cellular nuclei were stained with DAPI (blue). Scale bars = 25 µm.

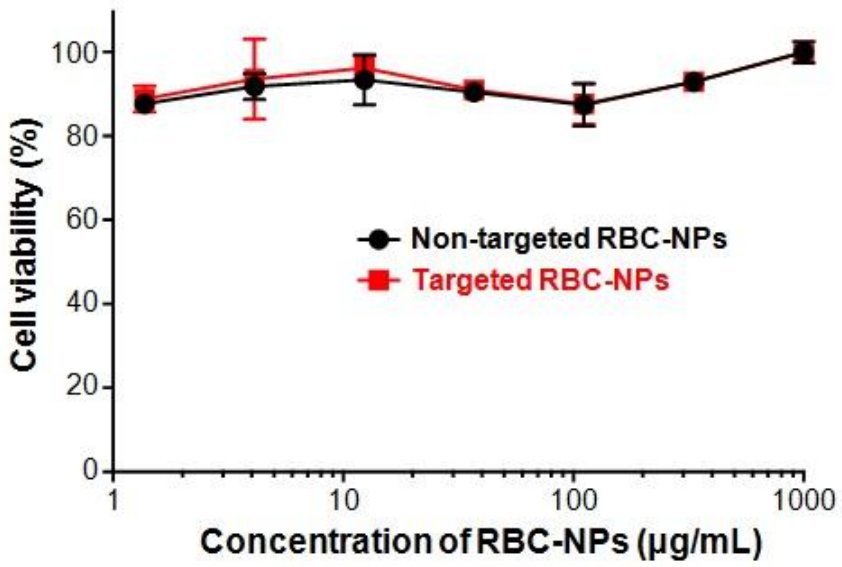

Fig. S2 RBC-NPs do not exhibit cytotoxicity when incubated with normal cells. HUVECs were incubated with RBC-NPs, both unmodified and folate-functionalized, for 16 hr followed by an additional incubation of 48 hr in media. Cell viability was determined using an MTT assay (n=3).

3. References:

(1) C. M. Hu, L. Zhang, S. Aryal, C. Cheung, R. H. Fang, L. Zhang, *Proc. Natl. Acad. Sci. USA*, 2011, **108**, 10980-10985.