Supporting Information

‘Marker-of-self’ functionalization of nanoscale particles through a top-down cellular membrane coating approach

Che-Ming J. Hu,[ab] *Ronnie H. Fang,*[ab] *Brian T. Luk,*[bc] *Kevin N.H. Chen,*[a] *Cody Carpenter,*[a] *Weiwei Gao,*[ab] *Kang Zhang*[ade] *and Liangfang Zhang*[ab]*

[a]Department of NanoEngineering, [b]Moores Cancer Center, [c]Department of Bioengineering, [d]Department of Ophthalmology and Shiley Eye Center, University of California, San Diego, La Jolla, CA 92093, USA

[e]State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangzhou 510275, China.

*Corresponding author: zhang@ucsd.edu, Tel: 858-246-0999, Fax: 858-534-9553.

TABLE OF CONTENTS

1. Experimental Section

Preparation of RBC Membrane-Coated Nanoparticles (RBC-NPs). RBCs were collected from 10 week-old male ICR mice (Charles River Laboratories) by centrifuging the whole blood at 2000 x g for 5 min, following which the supernatant and buffy coat were removed. Collected RBCs were then subject to hypotonic treatment to remove interior contents. The resulting RBC ghosts were extruded through 100 nm polycarbonate porous membranes using an extruder (Avanti Polar Lipids) to prepare RBC membrane-derived vesicles with a diameter of approximately 120 nm. Poly(lactic-co-glycolic acid) (PLGA) polymeric cores were prepared using 0.67 dL/g carboxy-terminated 50:50 PLGA polymer (LACTEL Absorbable Polymers) through a solvent displacement process, during which 1 mg of PLGA was dissolved in 200 μL of acetone and added drop-wise to 3 mL of water. Following solvent evaporation for 2 hr, the particles were washed using 10 kDa molecular weight cutoff (MWCO) Amicon Ultra-4 Centrifugal Filters (Millipore). The RBC-NPs were then prepared by fusing the RBC membrane-derived vesicles onto the PLGA particles by extruding the particles with the RBC membrane-derived vesicles through 100 nm polycarbonate porous membranes. The size and the zeta potential of the resulting RBC-NPs were obtained from three dynamic light scattering (DLS) measurements using a Malvern ZEN 3600 Zetasizer, which showed an average hydrodynamic diameter of 70 and 85 nm before and after the extrusion process, respectively (Figure S1). The particle morphology was characterized using scanning electron microscopy (SEM). Samples for SEM imaging were prepared by dropping 5 μL of the RBC-NP solution onto a polished silicon wafer. After drying the droplet at room temperature overnight, the sample was coated with chromium and then imaged by SEM.

Identification of Membrane Proteins and CD47 on the RBC-NPs: RBC-NPs were isolated from free RBC membrane materials by ultracentrifugation at 14000 x g for 30 min. The resulting RBC-NPs were lyophilized, prepared in lithium dodecyl sulfate (LDS) sample loading buffer (Invitrogen), and separated on a 4-12% Bis-Tris 10-well minigel in MOPS running buffer using a Novex® Xcell SureLock Electrophoresis System (Invitrogen). For membrane protein identification, the gel was stained using SimplyBlue^{TM} SafeStain solution (Life Technologies)

following the manufacturer's instructions and imaged using a gel imager. For CD47 identification, the resulting gel was transferred to a nitrocellulose membrane. The membrane was then stained with a primary rat anti-mouse CD47 antibody (BD Biosciences) and a secondary goat anti-rat IgG HRP conjugate (Millipore). The membrane was then subject to ECL western blotting substrate (Pierce) and developed with the Mini-Medical/90 Developer (ImageWorks).

Quantification of CD47 on the RBC-NPs: The amount of CD47 retained on the RBC-NPs was quantified by comparing the CD47 protein intensity to protein standards prepared on the same western blotting membrane. The protein standards were prepared from predetermined volumes of blood, which yielded a positive linear correlation between the CD47 band intensity and the blood volume following western blotting (Figure S2). The band intensities were quantified through an image analysis with Adobe Photoshop software. A CD47 standard curve was then established by converting the blood volumes to their corresponding CD47 quantity, using an estimated concentration of mouse RBCs in the blood (10^{10}/mL)[1] and an average number of CD47 per mouse RBC (16,500 copies/cell)[2] (Figure S2).

Immunostaining and Negative Staining for Transmission Electron Microscopy (TEM) Imaging: A drop of the RBC-NP or bare PLGA nanoparticle (bare NP) solution (1 mg/mL) was deposited onto a glow-discharged carbon-coated grid. For immunostaining, the sample droplet was washed with 3 drops of 0.5 mg/mL rat anti-mouse CD47 antibody solution. For staining CD47's intracellular domains, a rabbit anti-CD47 antibody (GeneTex) that specifically targets an intracellular sequence of CD47 was used. Following 30 sec of incubation, the sample was rinsed with 3 drops of either a goat anti-rat IgG gold conjugate (5 nm) solution (Canemco, Inc.) or a anti-rabbit IgG gold conjugate (5 nm) solution (Sigma-Aldrich) and then washed with 10 drops of distilled water. For negative staining, the particle sample droplet was washed with 10 drops of distilled water and stained with 1% uranyl acetate. These samples were then imaged using an FEI Sphera Microscope at 200kV.

Macrophage Uptake Study: PLGA nanoparticles encapsulating 0.05% (w/w) 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt (DiD) dye (Life Technologies) were prepared for fluorescence quantification using flow cytometry. CD47-

blocked RBC-NPs were prepared by incubating 1 mg of the DiD-loaded RBC-NPs with 400 μg of rat anti-mouse CD47 antibodies (BD Biosciences) for 1 hr. For the macrophage uptake study, J774 murine macrophage cells were cultured in DMEM media (Invitrogen) supplemented with 10% FBS (Sigma-Aldrich) and plated at a density of 10^5 cells/well on 12-well plates (BD Biosciences). On the day of the experiment, the cells were washed and cultured in fresh culture media. Bare PLGA nanoparticles (bare NPs), RBC-NPs, and CD47-blocked RBC-NPs were incubated at a concentration of 25 μg/mL with the macrophage cells at 37°C for 10 min. Non-internalized nanoparticles were washed away with PBS. The macrophage cells were then scraped off the plates and analyzed using flow cytometry. All flow cytometry studies were conducted on a FACSCanto II flow cytometer (BD Biosciences) and the resulting data was analyzed using FlowJo software from Tree Star. Thirty thousand events were collected per sample and gated using control cells that were not incubated with any nanoparticles. Histograms were plotted with fluorescence intensity as the x-axis using a biexponential scale. The mean fluorescence was plotted in a bar chart with error bars representing the standard error. Statistical analysis was performed based on a two-tailed, unpaired t-test.

2. Theoretical Calculations of RBC-NP Surface Area and CD47 Density

Density of PLGA polymer: $\rho = 1.25$ g/mL [3]

Radius of the PLGA core: r = 35 nm

Radius of the RBC-NP: $r_{np} = 42.5$ nm

Mass per PLGA NP core: $M_{core} = \rho \times \frac{4}{3}\pi r^3 = 2.24\times10^{-16}$ g per particle

Number of nanoparticles made of 1 mg of PLGA polymer: N_{np} =1 mg / M_{core} = **4.45×10^{12}**

Surface area of each RBC-NP: $S_{np} = 4\,\pi\, r_{np}^2 = 0.023$ μm^2 per particle

Total surface area of RBC-NPs made of 1 mg of PLGA polymer:

$$S_{total} = N_{np} \times S_{np} \approx 1\times\mathbf{10^{11}}\ \boldsymbol{\mu m^2}$$

CD47 density on the RBC-NPs (at saturation)

From data showing in Figure 2D, 2×10^{13} copies of CD47 per mg of PLGA nanoparticles:

CD47 number per nanoparticle: 2×10^{13} copies / $N_{np} \approx$ **5 copies/nanoparticle**

CD47 number per particle area: 2×10^{13} copies / $S_{total} \approx$ **200 copies/μm^2**

Theoretical amount of blood for complete coating of 1 mg of 70 nm PLGA nanoparticles

Blood volume = S_{total} / average surface area of mouse RBC (75 μm^2)[4]/ concentration of RBCs in mouse blood (10^{10}/mL)[1]

≈ **125 µL**

3. Supporting Figures

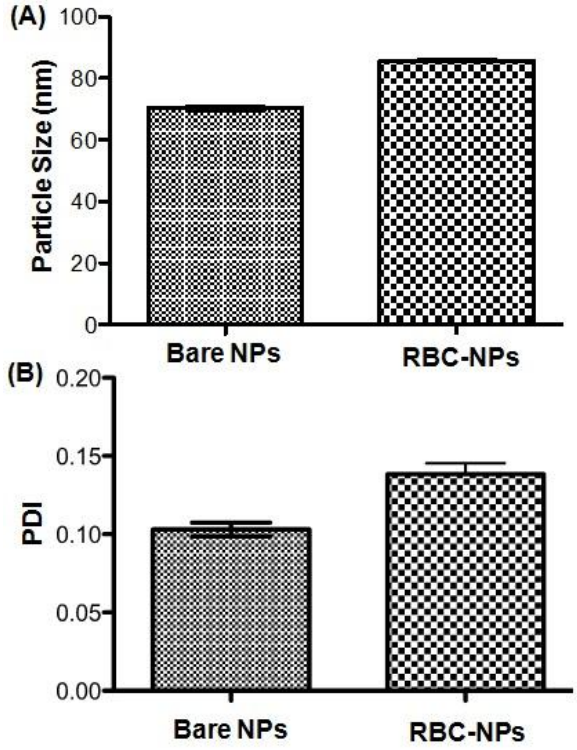

Fig. S1 (A) Particle size (diameter, nm) and (B) polydispersity index (PDI) of the PLGA nanoparticles before and after the RBC membrane coating determined by dynamic light scattering (DLS) measurements.

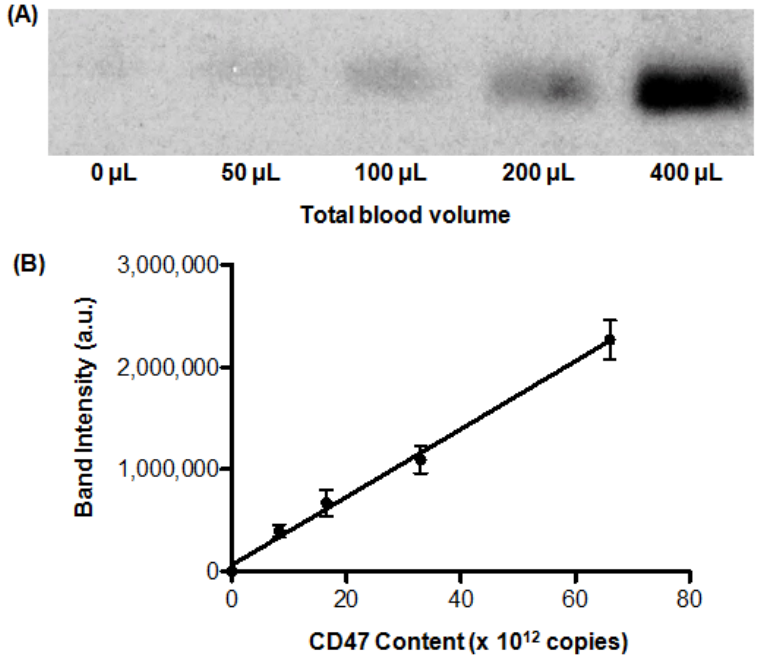

Fig. S2 (A) Western blotting analysis of CD47 contents in various volumes of mouse blood. (B) A standard curve of the CD47 band intensities on the western blot membranes versus the estimated CD47 contents from the corresponding blood volumes (n = 3).

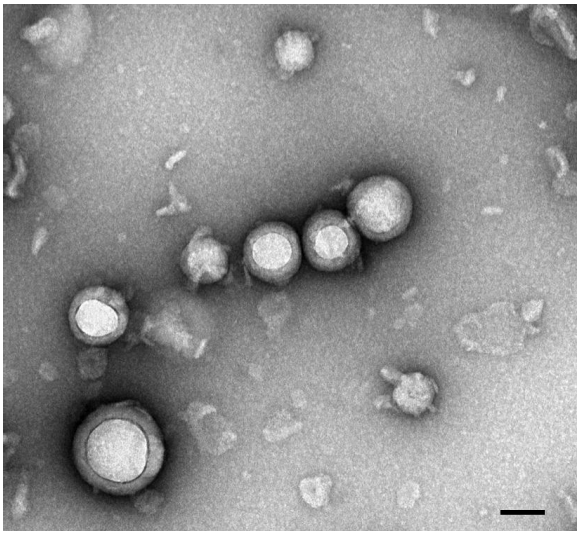

Fig. S3 TEM visualizations of the RBC-NPs prepared with excess RBC membrane to polymer ratio (250 μL of blood per mg of polymer). Unilamellar membrane coating was observed on the RBC-NPs and excess RBC membranes remained in vesicular forms (scale bar = 50 nm).

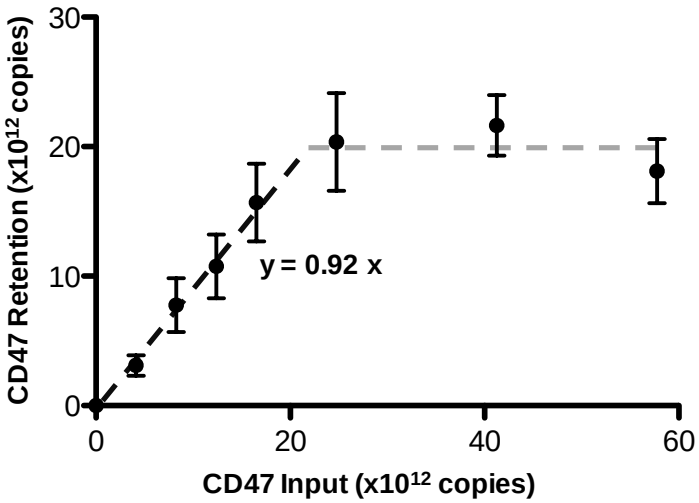

Fig. S4 CD47 retention on the RBC-NPs was plotted against the CD47 input estimated from the corresponding RBC membrane materials. Approximately 92% of the input CD47 proteins were translocated from the RBC membranes to the nanoparticle surfaces (n = 5).

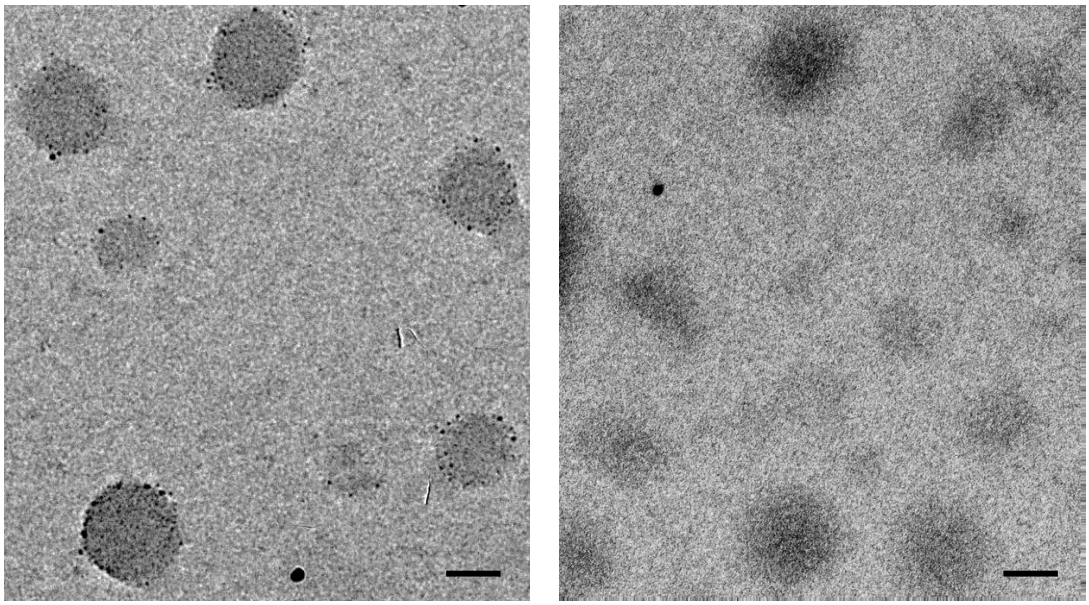

Fig. S5 Goat anti-rat IgG gold conjugates (~ 5 nm) were applied to RBC-NPs either with the rat anti-mouse CD47 primary stain (left panel) or without the primary stain (right panel). TEM visualizations reveal that attachment of the gold conjugates is specific to the primary immunostain of rat anti-mouse CD47 (scale bar = 50 nm).

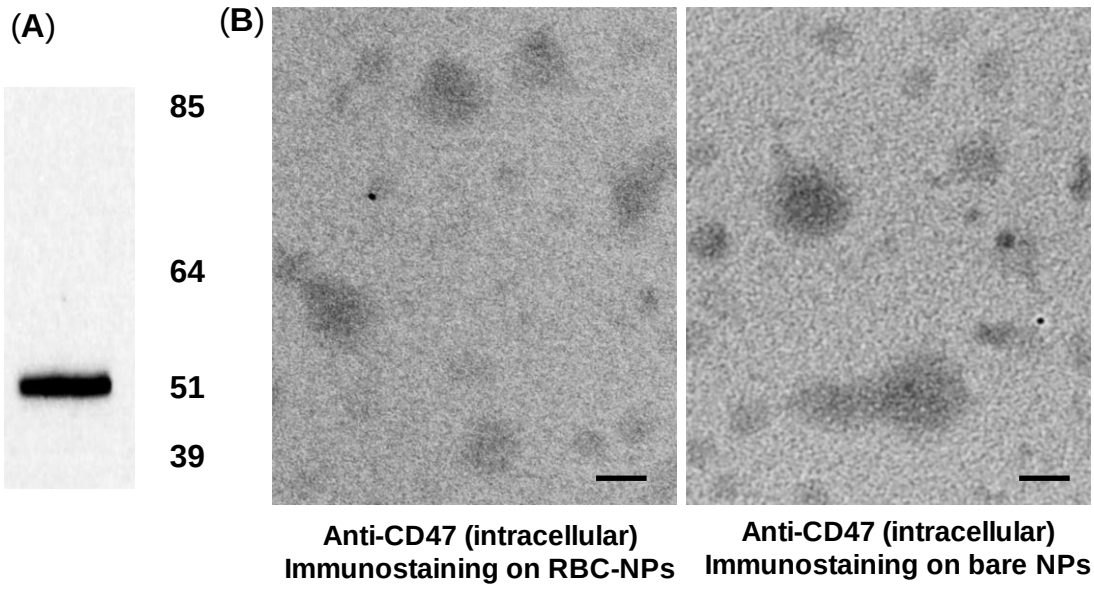

Fig. S6 (A) An anti-CD47 antibody (CD47's intracellular sequence specific) binds to RBC-NPs' CD47 protein resolved by SDS-PAGE. (B) Goat anti-rabbit IgG gold conjugates (~5 nm) were applied to RBC-NPs (left panel) and bare NPs (right panel) with the rabbit anti-CD47 (intracellular sequence specific) primary stain. No immunogold staining on either RBC-NPs or bare NPs was observed under TEM (scale bar = 50 nm).

4. References

1 E. S. Russell, E. F. Neufeld and C. T. Higgins, *Proc. Soc. Exp. Biol. Med.*, 1951, **78**, 761-766.

2 S. Subramanian, R. Tsai, S. Sen, K. N. Dahl and D. E. Discher, *Blood Cells Mol. Dis.*, 2006, **36**, 364-372.

3 R. Qi, R. Guo, M. Shen, X. Cao, L. Zhang, J. Xu, J. Yu and X. Shi, *J. Mater. Chem.*, 2010, **20**, 10622-10629.

4 R. E. Waugh and I. H. Sarelius, *Am. J. Physiol.*, 1996, **271**, C1847-1852.