Supporting Information

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SI Text

Particle Characterization. Particle dimensions were determined by analysis of images from a microscope mounted camera (Zeiss AxioCam MRm) using a 100× objective (Zeiss Axio Imager D.1M). Over 50 fully hydrated particles in pH 7.4 PBS buffer were measured for each case. Mouse RBCs used for zeta potential measurements were pelleted and washed three times with cold PBS prior to cross-linking with 1% glutaraldehyde for 5 min. The mouse RBCs were washed with PBS and diluted 1,000-fold with cold deionized water for zeta potential measurements. Zeta potentials for particles and RBCs were measured on a nano ZS zetasizer (Malvern Instruments) in water (Table S2).

Assessment of Biocompatibility and Interaction with Endothelial Cells. Human umbilical vein endothelial cell (HUVEC) or HeLa cells were seeded in 200 μL of media (HuMEC containing HuMEC supplement and bovine pituitary extract or MEM containing Earle's salts and both supplemented with 10% fetal bovine serum) at a density of 5,000 cells per cm^2 into a 96-well microtiter plate. Cells were allowed to adhere for 24 h and subsequently incubated with PRINT particles at concentrations ranging from 200 μg/mL to 1.56 μg/mL for 72 h at 37 °C in a humidified 5% CO_2 atmosphere. After the incubation period, all medium/particles were aspirated off cells. The ATP-luciferase assay requires 100 μL fresh medium, which was added back to cells, followed by the addition of 100 μL CellTiter-Glo® Luminescent Cell Viability Assay reagent (Promega). Plates were placed on a microplate shaker for 2 min, then incubated at room temperature for 10 min to stabilize luminescent signal. The luminescent signal was recorded on a SpectraMax M5 plate reader (Molecular Dynamics). The MTS [(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)] viability assay utilizes 20 μL of CellTiter 96® AQueous One Solution Reagent (Promega) into each well of the 96-well assay plate containing the samples in 100 μL of culture medium. Plates were incubated at 37 °C for 1–4 h in a humidified, 5% CO_2 atmosphere. The absorbance at 490 nm was recorded using a SpectraMax M5 plate reader (Molecular Dynamics). The viability of the cells exposed to PRINT particles was expressed as a percentage of the viability of cells grown in the absence of particles.

The HUVEC cell line was used to investigate the uptake of red blood cell mimics (RBCMs) with 1, 2, 5, or 10% cross-linker and which contained 1% fluorescein-o-acrylate as the covalently bound fluorescent dye. Cells were plated into 24-well tissue culture flat bottom plates (Corning/Costar 3526) and allowed to incubate overnight at 37 °C, 5% CO_2. The following day, particles were diluted in complete medium to obtain a final assay concentration of 15 μg/mL. Particle solution (300 μL) was then incubated with cells over a time course comprised of 15 m, 1 h, 2 h, 4 h, and 24 h (37 °C, 5% CO_2). After cell/particle incubation, the cells were washed with 1× Dulbecco's Phosphate Buffers Saline (DPBS) and detached by trypsinization (300 μL/well). Cells were resuspended in a 1:1 solution of 0.4% trypan blue (TB) solution in 1× DPBS containing 10% FBS (500 μL/well; total sample volume 800 μL) and transferred to a 5-mL Falcon (352063) polypropylene round-bottom tube. This assay is based on the observation that, for noninternalized particles, the vital dye TB acts to quench the fluorescence emission of the fluorescein bound to the particles, shifting the emission to red, whereas an internalized particle will fluoresce green (1). Cells were analyzed by flow cytometry (CyAn ADP, Dako) for green and red fluorescence. There were 10,000 cells measured in each sample.

Mouse Experiments. ***Long time points for 1% cross-linked RBCMs.*** To verify the long circulating behavior of the 1% cross-linked RBCMs, we administered 33 mg particles/kg mouse weight with a 3.5 mg/mL solution of RBCMs in PBS with 0.1% poly(vinyl alcohol) via tail–vein injection to female BALB/c mice of 20- to 22-g body weight. We examined three mice per time point, including 2, 8, 24, 72, and 120 h postinjection. At each time point examined, we sacrificed the mice, harvested lung, liver, kidney, and spleen tissues, collected blood via cardiac puncture, and measured the fluorescent signal from the particles using an IVIS Kinetic fluorescent imager (Caliper Life Sciences) with excitation at 675 nm and emission measured at 720 nm. Blood was pipetted in 100-μL aliquots to black 96-well plates for analysis on the IVIS Kinetic imager, with blood measured in triplicate for each mouse. To obtain values for particle concentration in blood, we performed serial dilutions in triplicate of particles in freshly harvested mouse blood (from undosed mice) and plotted a standard curve (Fig. S3). To track biodistribution of 1% RBCMs in lung, liver, kidney, and spleen tissues over time, tissues were imaged as above and plotted as fluorescence efficiency per gram of tissue weight (Fig. S4).

Procedure for Staining Lung Tissue. Tissues were frozen on dry ice shortly after harvesting, and 10-μm frozen sections were prepared for staining. Microscope slides with frozen tissue sections were allowed to thaw at room temperature for 20–30 min, at which point they were washed briefly with Tris buffered saline (TBS, Fisher Scientific). The sections were fixed with 2.5% paraformaldehyde in TBS for 5 min, followed by two 5-min washes with TBS. The excess paraformaldehyde was quenched with 10 mM ethanolamine in PBS for 5 min, followed by washing twice with TBS for 2 min. The sections were permeablized with 0.1% Triton X-100 (Alpha Aesar) in TBS for 5 min and then washed with TBS twice for 5 min. The sections were incubated overnight at 4 °C with 33 nM $\mathrm{AlexaFluor}_{488}$ conjugated phalloidin (Invitrogen) and 1 μM To-Pro-3 iodide (Invitrogen), both solutions having been prepared using 0.025% Tween 20 (Fisher Scientific) in TBS. Following incubation, the sections were washed 4 times for 5 min prior to mounting with ProLong Gold antifade reagent (Invitrogen).

All samples were imaged with an Olympus FV 500 confocal laser scanning microscope with filters optimized for the different fluorescent probes, including the rhodamine B dye in the RBCMs. Images were taken with a 40× objective lens.

Compartmental Analysis. Several pharmacokinetic models were investigated to determine the best model to describe the plasma concentration over time profiles collected with intravital microscopy, as well as the longer time-point data collected via blood draws. PK analysis was performed separately on each mouse using WinNonlin (version 5.2.1; Pharsight Corp.) software. Both one- and two-compartment models with elimination from the central compartment were investigated using models from the WinNonlin model library. To determine if the RBCMs followed a dual elimination mechanism, a two-compartment model with elimination from central and peripheral compartments built by our group was also investigated. The data were weighted according to the equation $1/C_i$, where C_i denotes the predicted concentration or normalized fluorescent signal. All iterations were conducted using the Gauss–Newton algorithm (2). The evaluation of the goodness of fit was based on the Akaike information criterion, the variability of the parameter estimates, and the ab-

sence of a significant correlation between independent model parameters (<0.95). Secondary PK parameters [$t_{1/2(\alpha)}$, $t_{1/2(\beta)}$, CL_s, CL_d, V_p, and area under the curve (AUC)] were calculated from the estimated pharmacokinetic parameters for the long time-point scans (Fig. S5). The two-compartment model from the WinNonlin library was found to best describe the data in all cases.

For determination of alpha and beta half-lives from the intravital microscopy scans, we fit the data to the best-fit model from the WinNonlin analysis. The two-compartment model assumes that particles are transported between the central and peripheral compartments with first-order kinetics and that particles are eliminated from the central compartment with first-order kinetics. The data were fit to Eq. **S1**, where Cp is the concentration of the particle in the plasma, and α and β are rate constants for the distribution and elimination processes, respectively. The fit was

$$Cp = Ae^{-\alpha t} + Be^{-\beta t} \qquad \textbf{[S1]}$$

calculated by nonlinear regression, maximizing the R^2 value for the fit and producing best-fit values for the coefficients and rate constants (3). Half-lives for the distribution and elimination process were calculated by Eqs. **S2** and **S3**, respectively.

$$\alpha_{t1/2} = \frac{\ln 0.5}{\alpha}, \qquad \textbf{[S2]}$$

$$\beta_{t1/2} = \frac{\ln 0.5}{\beta}. \qquad \textbf{[S3]}$$

Although exact values for particle concentrations in the plasma could not be calculated for this study, the well-defined shape of the elimination curves (350 points over 2 h) allowed for determination of the half-life values, which are not dependant on absolute concentration.

Each elimination curve was analyzed individually for the above values, with the averages representing three to four mice per particle modulus.

1. Fattorossi A, Nisini R, Pizzolo JG, D'Amelio R (1989) New, simple flow cytometry technique to discriminate between internalized and membrane-bound particles in phagocytosis. *Cytometry* 10:320–325, 10.1002/cyto.990100311.
2. Pharsight (2008) *WinNonlin User's Guide*.
3. Brown AM (2001) A step-by-step guide to non-linear regression analysis of experimental data using a Microsoft Excel spreadsheet. *Comput Methods Programs Biomed* 65:191–200.

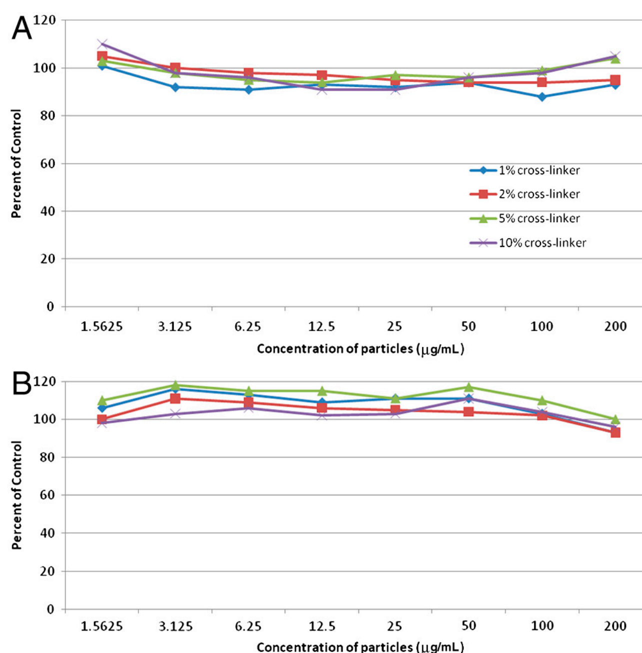


Fig. S1. Dose-dependent cell viability of HUVEC (endothelial) cells when dosed with RBCMs with varied crosslink density (1–10%) after 72 h of incubation with the particles as determined by MTS (*A*) and ATP/luciferase (*B*) assays.

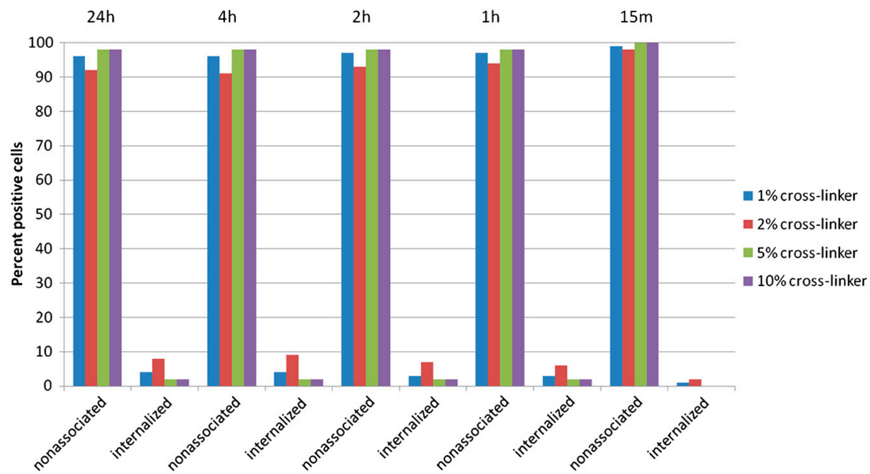


Fig. S2. Uptake of 1, 2, 5, and 10% cross-linked RBCMs in HUVEC cells after dosing times from 15 min to 24 h. Results are expressed in terms of percentage of cells that had taken up RBCMs (internalized) and that were unassociated with RBCMs (unassociated).

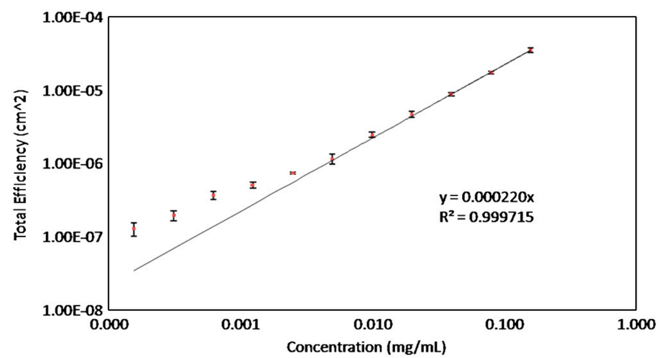


Fig. S3. A standard curve that correlates fluorescent efficiency (signal intensity) and concentration of 1% cross-linked RBCMs in whole blood. Deviation from linearity defines the lower limit of quantification for fluorescent signal. Data collected for the blood draw experiments fell within the linear range of this plot. Scale bars represent one standard deviation, with $n = 3$.

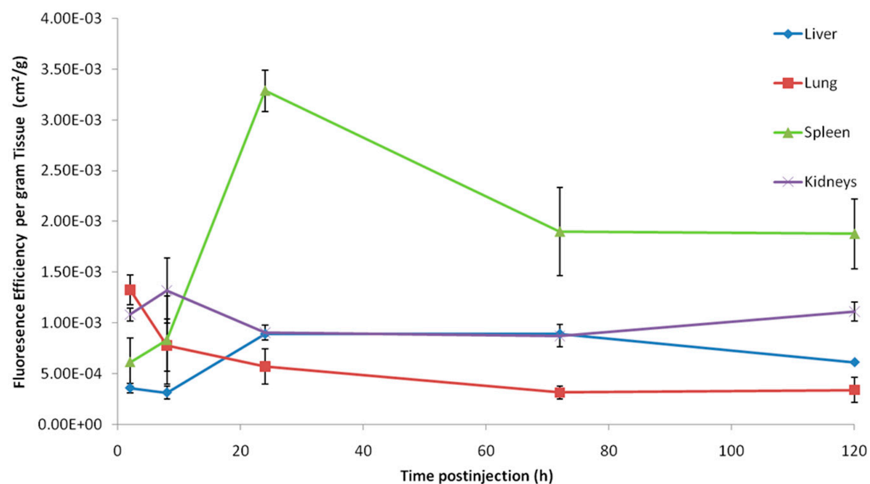


Fig. S4. A plot showing the biodistribution of 1% cross-linked RBCMs in mice over a 2- to 120-h time course in the liver, lung, spleen, and kidneys. Data are presented as fluorescent efficiency per gram of tissue weight. Error bars represent one standard deviation, with $n = 3$ for each case.

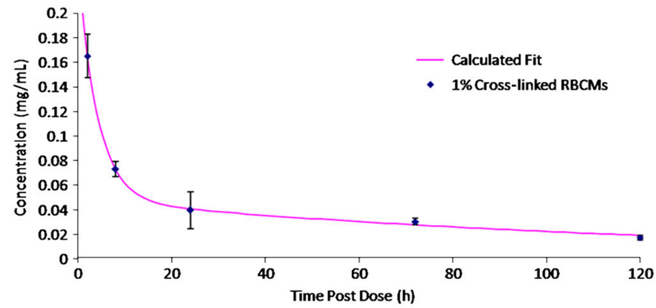
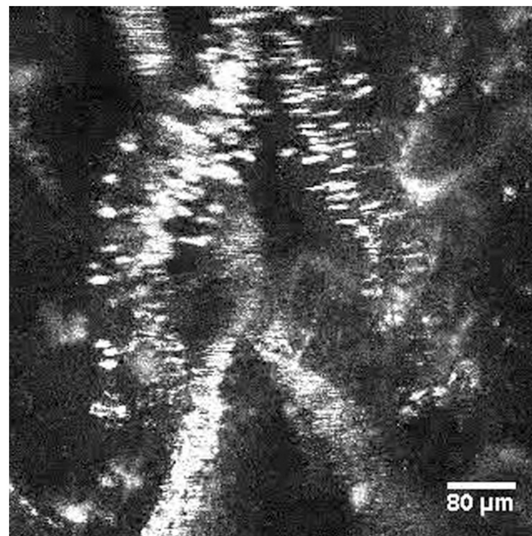


Fig. S5. A plot of particle concentration in whole blood over time for 1% cross-linked RBCMs where $n = 4$ for each data point. These data were fit to a two-compartment pharmacokinetic model with elimination from the central compartment (red) by nonlinear regression analysis. The fit was used to calculate pharmacokinetic parameters for these particles. Scale bars represent one standard deviation, with $n = 3$.

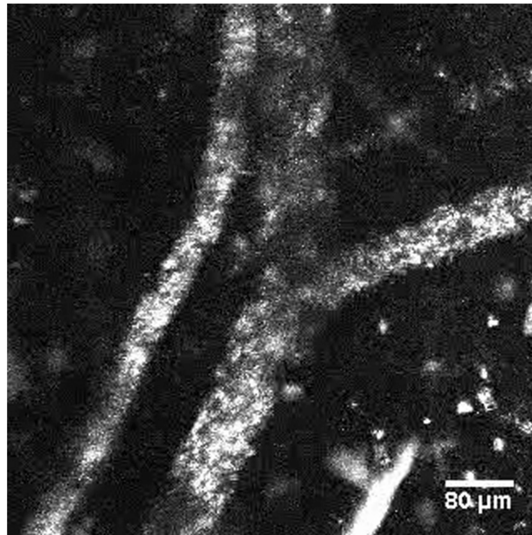
Movie S1. RBCM particles (1% cross-linked) that are 6 μm in diameter deforming in flow to traverse a 3-μm wide restriction in a 3-μm tall poly(dimethyl siloxane) microfluidic channel. Observed restrictions are in the middle of the device, implying that the RBCMs in this movie have traversed approximately 50 such pores immediately prior to imaging without noticeable loss of structure or elasticity. The flow rate was 0.06 μL/ min.

Movie S1 (AVI)



Movie S2. Intravital microscopy for 10% cross-linked RBCMs. The movie represents 2 h of scan time on a typical subject compressed to play in 10 s. Imaging scans used a 633-nm laser and proceeded for 2 h, with an image taken every 2 s at a scan rate of 0.573 s/frame.

Movie S2 (MOV)



Movie S3. Intravital microscopy for 1% cross-linked RBCMs. The movie represents 2 h of scan time on a typical subject compressed to play in 10 s. Imaging scans used a 633-nm laser and proceeded for 2 h, with an image taken every 2 s at a scan rate of 0.573 s/frame.
Movie S3 (MOV)

Table S1. The composition of hydrogels used for RBCMs

Monomer	%
PEG 4000 diacrylate	1–10
2-Carboxyethyl acrylate	10
1-Hydroxycyclohexyl phenyl ketone	1
Methacryloxyethyl thiocarbonyl rhodamine B (PolyFluor 570)	0.1
2-Hydroxyethylacrylate	Remainder
Fluorescein-o-acrylate	0–1
Total	100

Table S2. Characterization of RBCM particles and mouse RBCs

	1% cross-linker	2% cross-linker	5% cross-linker	10% cross-linker	Mouse red blood cell
Particle diameter (µm)*	5.96 ± 0.27	5.68 ± 0.32	5.66 ± 0.34	5.18 ± 0.30	6.0 (ref. 1)
Zeta Potential (mV)†	−24.2 ± 5.4	−23.6 ± 3.4	−19.9 ± 6.6	−17.2 ± 3.4	−46.6 ± 4.84

*Error represents one standard deviation from the mean with $n = 50$ for particle diameters.
†Error represents standard deviations with $n = 16$.

1. Snyder GK, Weathers WW (1977) Hematology, viscosity, and respiratory functions of whole blood of the lesser mouse deer, Tragulus javanicus. *J Appl Physiol* 42:673–678.