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Supporting Information

Erythrocyte Membrane Concealed Paramagnetic Polymeric Nanoparticle for Contrast-Enhanced Magnetic Resonance Imaging

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Synthesis of DSPE-DOTA-Gd

The synthesis of Gadolinium ion chelated DSPE-DOTA was performed using a two-step preparation method as previously described with some modifications.[1] In brief, 75 mg of DSPE was first dissolved in 10 mL chloroform containing 2% (v/v) triethylamine (TEA). The mixture was stirred for 30 mins at room temperature to activate the amine end group of DSPE. After that, 86 mg DOTA-NHS ester was added and stirred vigorously until all reagents were completely dissolved. The reaction was allowed to take place for another 3 h at 40ºC. At the end of incubation, the obtained clear reaction mixture was concentrated and dried under vacuum to recover white powder. To purify the conjugating DSPE-DOTA lipid, 6 mL of deionized water was used to disperse reaction crude, the suspension underwent 5 cycles of freezing and thawing in liquid nitrogen and 60ºC water bath, respectively, and then centrifuged at 4500 g for 10 mins. The resulted DSPE-DOTA suspension was filtered through a 0.2 μm membrane to remove insoluble by-product and lyophilized to get dry powder. The formation of DSPE-DOTA was confirmed by FT-IR and ^{1}H-NMR.

The gadolinium ion was thermodynamically chelated to DSPE-DOTA conjugated lipid under an acidic condition at 50ºC. Specifically, 50 mg DSPE-DOTA (0.05 mmole) was hydrated in 10 mL of acetate buffer (pH = 5.5) and further treated with 0.1 mmole of $Gd(OAc)_3$ 50ºC for 12 h. After incubation, the Gd-DOTA-DSPE was purified by centrifugation at 4500g for 10 min. The obtained white product was washed 3 times with acetate buffer (pH 5.5) and 2 times with water to remove all non-chelated Gd^{3+} ion. The samples were lyophilized to obtain a dry powder. The percentage yield of the gadolinium concentration in the reaction mixture was analyzed by using Inductively Coupled Plasma Mass spectrometry (ICP-MS, Perkin Elmer, NEXion 350X). The resulting yield was 98%.

Red blood cell membrane extraction

Red blood cell membrane (RBC-M) was extracted from whole blood using hypotonic treatment.[2] In brief, whole blood was centrifuged to remove any buffy coat and plasma and mixed with 10 volume of Mili-Q water to disrupt RBC. The aliquot was sonicated briefly using bath sonication for 5 mins to hemolysis RBCs. The blood suspension was then centrifuged at 5,000 rpm for 5 min to separate RBC-M from the hemoglobin by carefully removing the suspension. The RBC-M pellet was washed several times to remove hemoglobin completely. The purified RBC-M was stored at -20ºC for further use. The amount of protein in extracted RBC-M was quantified by bicinchoninic acid (BCA) assay.

Fluorescence resonance energy transfer

To track the insertion of RBC-M onto the surface of Gd-PLGA, fluorescence resonance energy transfer study (FRET) was conducted. For the experiment, a FRET pair of chromophores, a fluorescent donor (L-α-phosphatidylethanolamineN-(4-nitrobenzo-2-oxa-1,3-diazole) (Ammonium Salt) (Egg-transphosphatidylated, Chicken), PE-NBD and a fluorescent acceptor (L-α-Phosphatidylethanolamine-N-(lissamine rhodamine B sulfonyl) (Ammonium Salt) (Egg-transphosphatidylated, Chicken) PE-RhB, was incorporated separately into RBC-M vesicle and Gd-PLGA (or PLGA) with a 3:7 mole ratio, respectively. In particular, the NBD labeled RBC-M vesicles were prepared by sonicating 250 μg RBC-M with 30 μg PE-NBD for 10 mins. Similarly, 99.4 μg PE-RhB was used to label 1 mg PLGA or Gd-PLGA by dispersing in 1 mL water/Gd-lipid suspension prior to polymeric core fabrication.

Typical fusion study was performed by mixing 100 μL of NBD-RBC with 100 μL RhB-PLGA/RhB-Gd-PLGA. The mixture was incubated for a varying period (5, 30, and 60 mins) at

37ºC with gentle shaking or bath sonicated for 10 mins. Thereafter, 100 μL of the suspension was used to record the fluorescence emission spectrum at λ_{ex} = 470 nm and λ_{em} = 500-700 nm.

Red blood cell membrane protein characterization

The retention of characteristic proteins of the red blood cell membrane and its translocation into the paramagnetic polymeric core was confirmed by SDS-PAGE analysis, dot blots and western blot. For SDS-PAGE analysis, whole cell lysate, RBC-M, RBC-Gd-PLGA samples were prepared at a protein concentration of 300 μg/mL. RBC-Gd-PLGA were collected by centrifugation at 13,000 rpm for 30 mins and redispersed in gel loading dye. All samples were heated at 90 °C for 5 min, and 20 μL of samples were loaded into wells of 4–20% Mini-PROTEAN® TGX Protein Gels and stained using Coomassie Brilliant Blue. For Western blot analysis, proteins were transferred to PVDF membrane by the wet-blot method. The transfer was carried out for 1 hr at 2500 mA, 500 W, and 100 V. After that, the membranes were blocked in 5% milk in TBST for 30 mins at room temperature under rocking condition. The membrane was subsequently treated with primary antibodies for Glycophorin A (Santa Cruz), β-actin (Santa Cruz), and CD47 (Santa Cruz) along with HRP-conjugated anti-mouse IgG secondary antibody (Cell Signaling). The blotted films were further developed using SignalFire™ ECL Reagent (Cell signaling) and imaged for chemiluminescence signal under Bio-imager (Kodak).

Gadolinium loading and release study

The cumulative gadolinium release from the RBC-Gd-PLGA was assessed at 37°C under physiological and endogenous ionic condition. In brief, RBC-Gd-PLGA (1 mg/mL, 2 mL) was placed in a dialysis bag membrane (Mw. Cutoff=500Da) and dialyzed against 250 mL of PBS (pH=7.4) under shaking condition (100rpm). At predetermined time intervals, 500 μL of the sample was collected and replaced by 500 μL fresh media. The amount of released Gd^{3+} was

quantified by ICP-MS. As a control experiment, the same amount of clinically used contrast agent (Magnevist®) was placed in a dialysis bag and processed under the same experimental condition.

In vitro immunogenicity assay

The immunoregulatory potential of the prepared nanoparticles was tested standard cytokine analysis assay. In brief, 4×10^5 human monocytic cell line (THP-1) were seeded in a 24-well plate and treated with bare PLGA NPs (10μg/mL), RBC-M (10μg/mL), Gd-lipid (10μg/mL), and RBC-Gd-PLGA (10μg/mL) at 37°C for 24h. After 24h incubation, cell culture supernatants were collected, centrifuged to remove cell debris and stored at -20°C for further analysis. For cytokine assay, samples were thawed and analyzed for pro-inflammatory cytokines, IL-1β (LOD:1.7 pg/ml), IL-6 (LOD:1.5 pg/mL) and TNF-α (LOD:1 pg/mL) using Magnetic Human Cytokine Multiplex Assays Kit (R&D Systems, Inc. Minneapolis, MN) in Luminex MagPix® instrument (Millipore Inc.,) as manufacturer recommendations. For positive control, cells were treated with 5μg/mL of lipopolysaccharide for 24h.

In vitro macrophage uptake study

J774 murine macrophage cells were plated at a density of 20,000 cells per well on an 8-well chamber slide (BD Biosciences). After 24hr, cells were treated with RhB labeled PLGA, RBC-Gd-PLGA, and PEGylated NPs with a final NP concentration of 50 μg/mL and incubated over varying periods (0.5, 1.5, 3, and 6 h). After incubation, the treated cells were washed twice with 1× PBS (pH 7.4), fixed with 4% paraformaldehyde for 15 min at room temperature, stained with DAPI for an additional 10 min, mounted with Fluoromount aqueous medium (Sigma-Aldrich), and imaged using CLSM (Carl Zeiss, LSM-700). The cellular uptake was assessed by

measuring RhB fluorescence intensity using Image-J software (National Institute of Health). The data were analyzed using one-way ANOVA with Turkey's multiple comparison post-test.

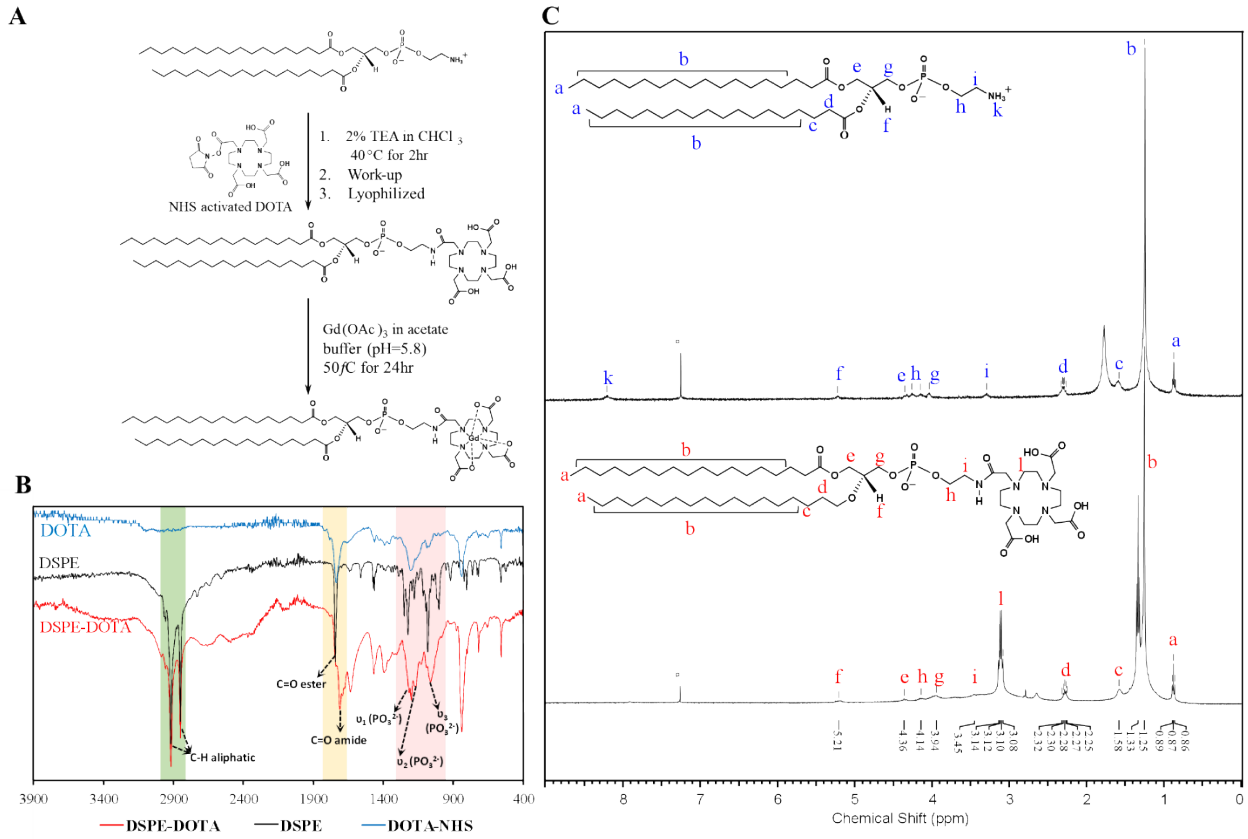

Figure S1. Synthesis and characterization of DSPE-DOTA-Gd (Gd-Lipid). (A) Reaction scheme. (B) FT-IR spectrum showing functional peaks of starting materials (DOTA, DSPE lipid) and product (DSPE-DOTA). (**C**) ^{1}H-NMR spectra of DSPE and DSPE-DOTA and its corresponding proton signals.

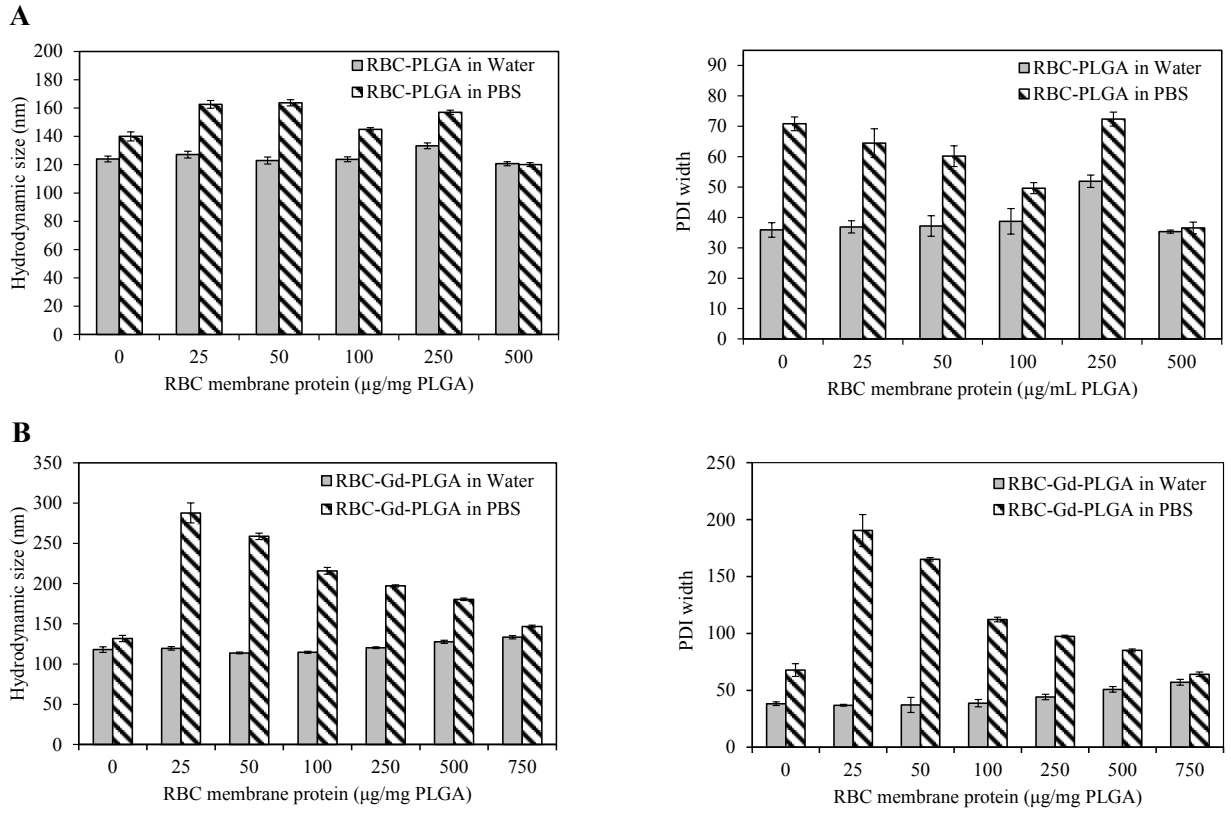

Figure S2. Optimization of RBC coating condition with various RBC membrane protein concentration by tracking the stability in hydrodynamic size and polydispersity index of nanoparticle in physiological pH=7.4 for (A) RBC coated PLGA NPs and (B) RBC coated Gd labeled PLGA NPs. (n=3)

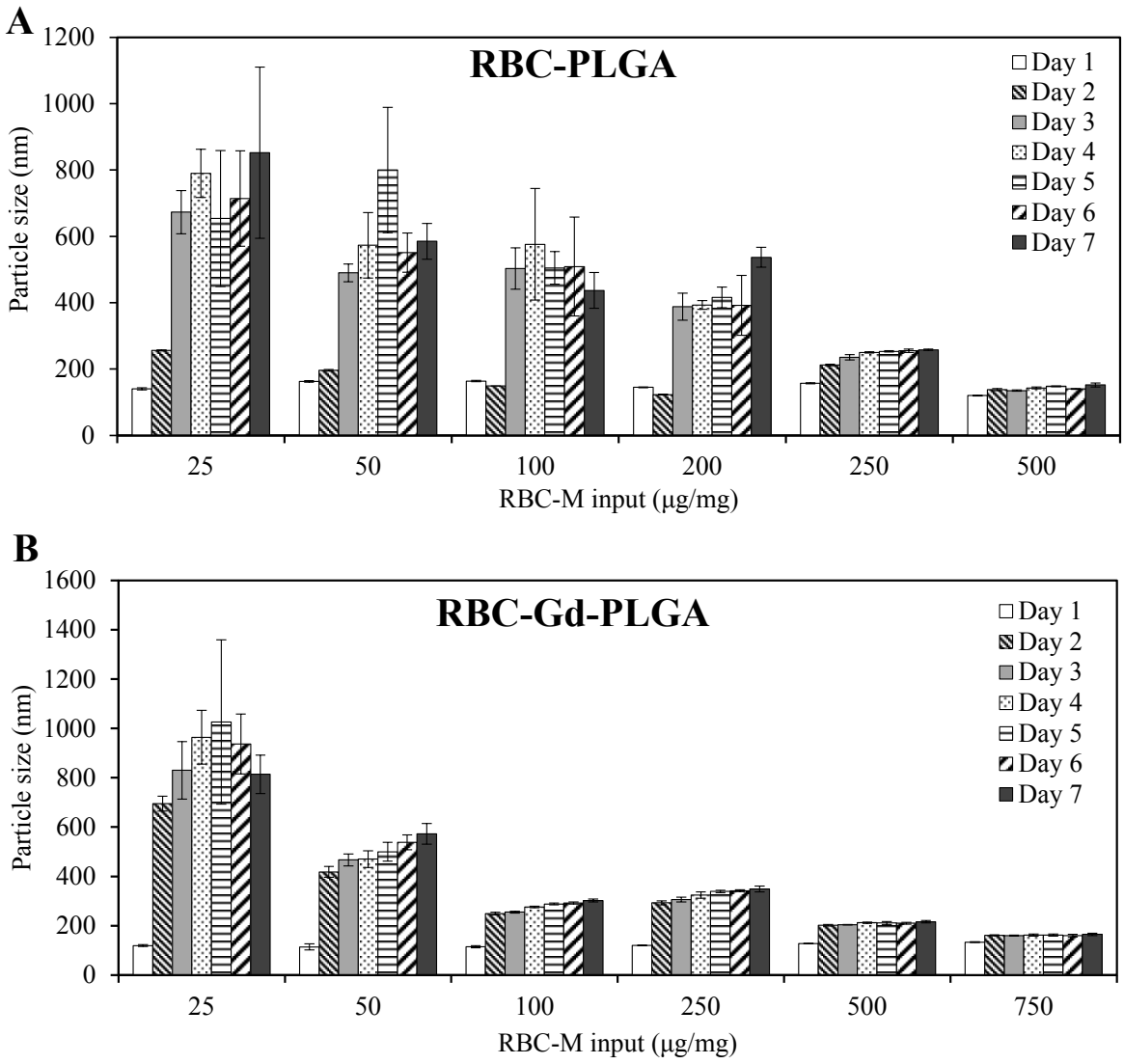

Figure S3. Stability of RBC-PLGA and RBC-Gd-PLGA under physiological condition (pH 7.4) of (A) RBC coated PLGA and (B) RBC coated Gd labeled PLGA (n=3)

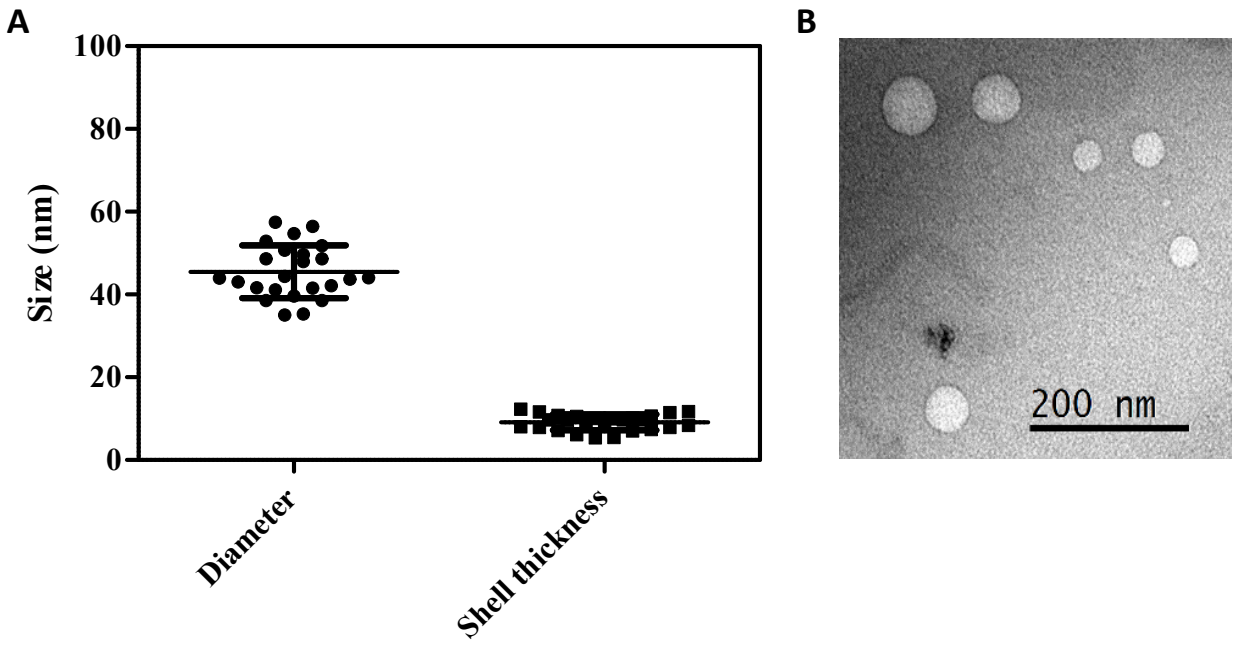

Figure S4. (A) TEM analysis for core diameter and shell thickness of RBC-Gd-PLGA (n=30). (B) TEM micrograph of Gd-PLGA nanoparticle.

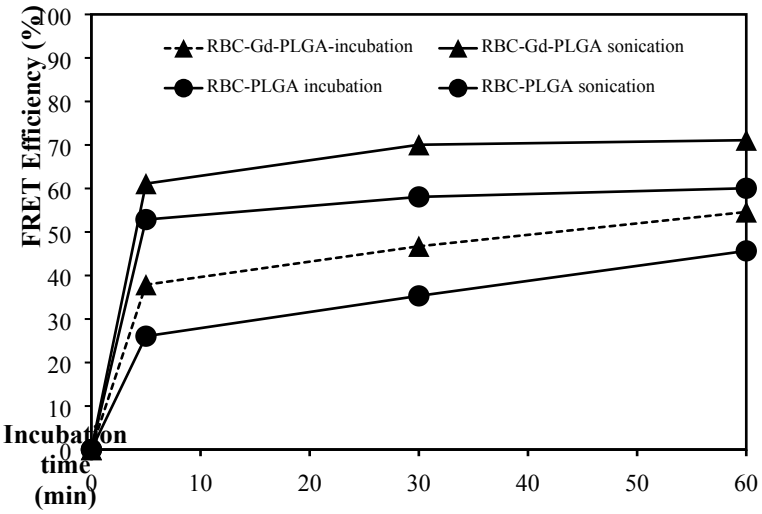

Figure S5. Time dependent fluorescent resonance energy transfer efficiency of NBD-RBC-M and RhB-PLGA/RhB-Gd-PLGA.

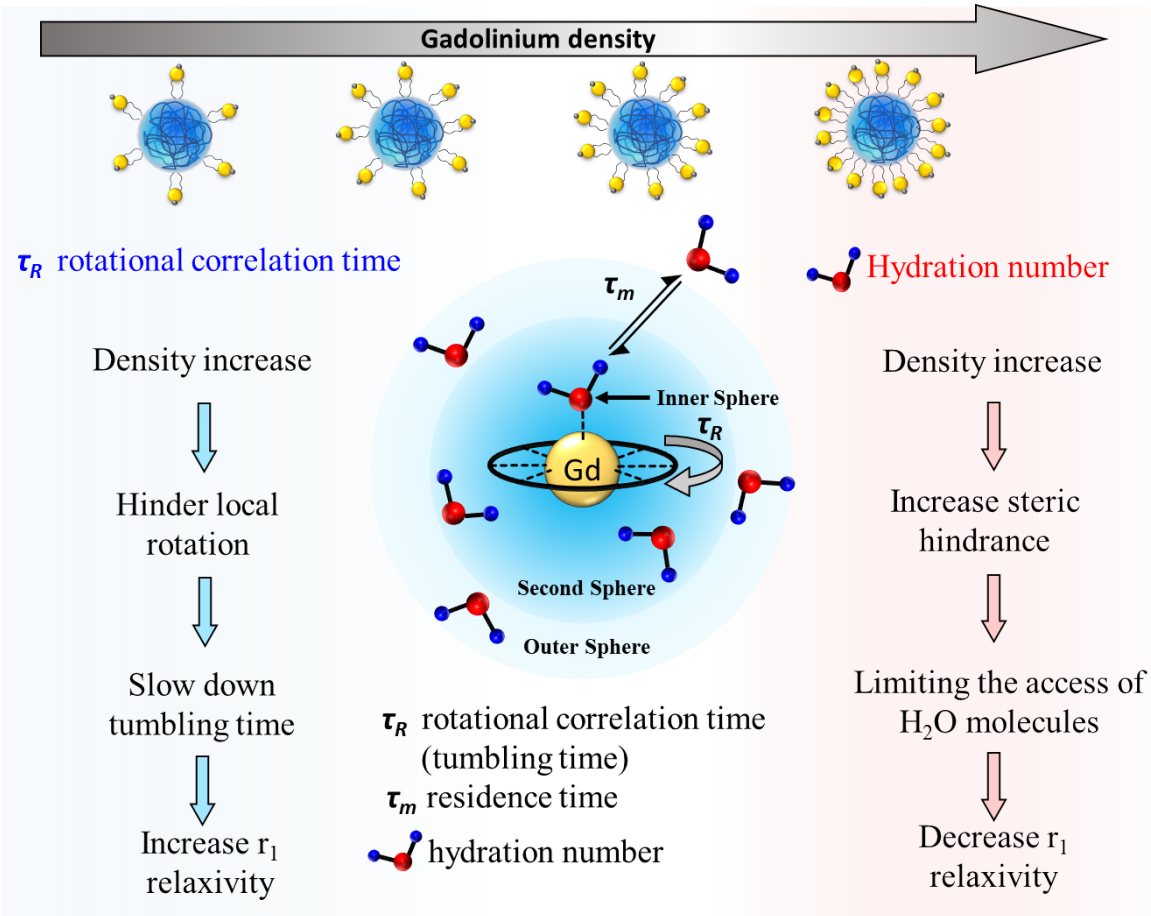

Figure S6. Influence of Gd-Lipid packing density on the relaxation mechanism of water proton surrounding the paramagnetic core. The T_1-weighted MRI phantoms were taken using Fast Spin Echo Sequence at 3 T with TR = 1000 ms, TE = 10 ms, FA = 90, FOV 256 × 256, and slice thickness = 2 mm.

Unlike other imaging modality, the contrast agent used in MRI does not directly emit a signal for detection but rather influence the microenvironment surrounding it to shorten the relaxation time of proton spin. The magnitude of this effect strongly depends on chemical properties and structural complexity when the contrast agent is incorporated into a nanoparticulate

system.[3–5] In the case of GBCA, the presence of paramagnetic ion will mainly increase the longitudinal relaxation rate ($1/T_1$) of water. According to Solomon-Bloembergen-Morgan (SBM) and outer sphere quantum mechanical theory, the enhancement of relaxivity is governed by rotational correlation time, the distance between metal-water hydrogen, number of coordinated water molecules, the exchange rate of a water molecule from inner sphere to outer sphere, and water residence time.[4,5] Therefore, by infusing Gd-Lipid onto the surface of cell membrane coated PNP, we could simultaneously slow down rotational correlation time, shorten the distance between the paramagnetic ion and water hydrogen, prolong water residence time, and enrich the number of water molecules nearby metal ion. The combination of these effects together with an optimal packing density of Gd^{3+} generated by our nanoconstruct leads to the significant increase in observed r_1 relaxivity.

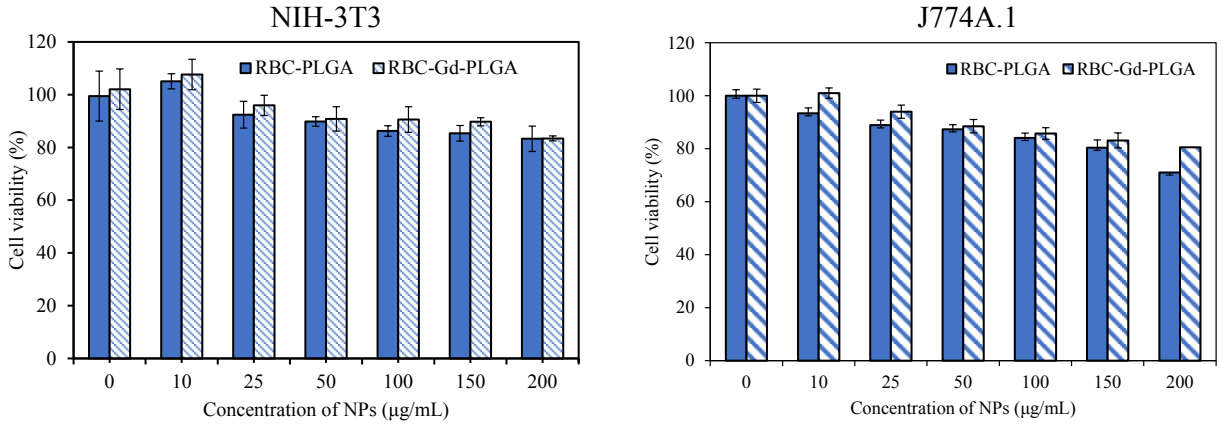

Figure S7. Biocompatibility of RBC-PLGA and RBC-Gd-PLGA in mouse normal fibroblast (NIH/3T3) and mouse macrophage (J774A.1) in terms of % cell viability quantified via MTT assay. Cells were treated with varying concentrations of particles for 24h.

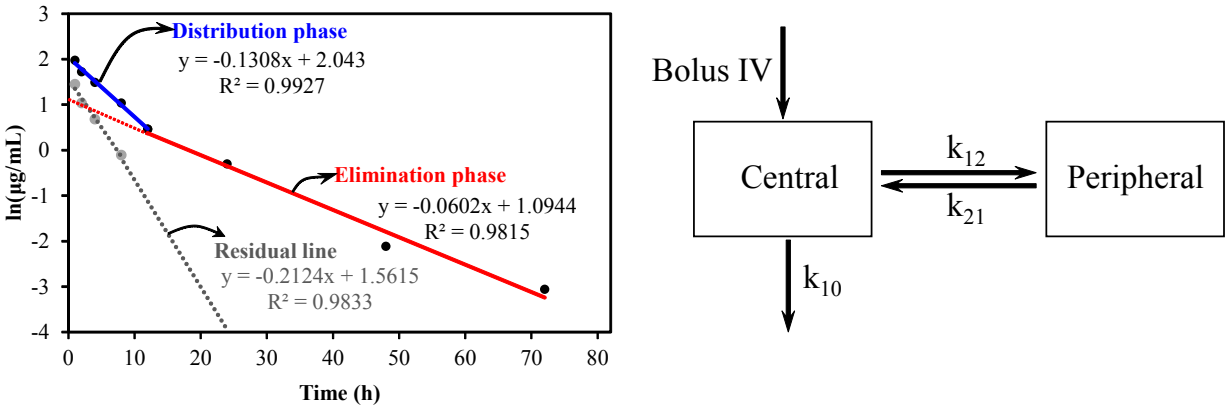

Figure S8. Plasma RBC-Gd-PLGA concentrations in natural logarithmic scale with linear components of a two-compartment model after an intravenous bolus dose.

The natural logarithmic scale with linear components for RBC-Gd-PLGA plasma concentration depicted by 2 main phases, a short distribution phase with $t_{1/2\alpha}$=3.12 h and a long elimination $t_{1/2\beta}$=11.6 h (**Figure 5A inset and Table S3**). The volume distribution of RBC-Gd-PLGA at steady state (V_{dss}) was found to be 2.78 mL which is similar to the volume of blood in a mouse (2-3 mL). The similarity between V_{dss} and plasma volume suggest that the RBC-Gd-PLGA mainly stays in the circulation system and poorly distributed in the tissue compartment.

Table S1. In vitro macrophage uptake study of PEGylated, PLGA and RBC-Gd-PLGA NPs after 0.5, 1.5, 3, and 6hr incubations analyzed using one-way ANOVA with Turkey's multiple comparison post-test.

Incubation time	Tukey's Multiple Comparison Test	Mean Diff.	q	Significant (P < 0.05)	95% CI of diff
0.5 hr	PEGylated NPs vs PLGA	-3916	15.07	Yes	-4789 to -3044
	PEGylated NPs vs RBC-Gd-PLGA	1832	6.632	Yes	905.3 to 2759
	PLGA vs RBC-Gd-PLGA	5749	21.61	Yes	4856 to 6641
1.5 hr	PEGylated NPs vs PLGA	-3732	13.50	Yes	-4660 to -2805
	PEGylated NPs vs RBC-Gd-PLGA	2093	7.841	Yes	1198 to 2989
	PLGA vs RBC-Gd-PLGA	5826	19.09	Yes	4802 to 6849
3 hr	PEGylated NPs vs PLGA	446.1	1.250	No	-751.5 to 1644
	PEGylated NPs vs RBC-Gd-PLGA	3014	8.475	Yes	1821 to 4208
	PLGA vs RBC-Gd-PLGA	2568	7.755	Yes	1457 to 3679
6 hr	PEGylated NPs vs PLGA	3721	7.707	Yes	2101 to 5340
	PEGylated NPs vs RBC-Gd-PLGA	2959	7.089	Yes	1559 to 4360
	PLGA vs RBC-Gd-PLGA	-761.2	1.538	No	-2421 to 899.0

Table S2. T_1 (ms) relaxation time of RBC-Gd-PLGA NPs and Magnevist®.

Gd (μg/mL)	Gd (mM)	Magnevist®	Amount of Gd-Lipid input per polymer (μg/mg)			
			50	100	200	400
11	0.07	1643.492	1185.409	884.513	1111.593	1257.481
5.5	0.035	2447.137	2147.917	1820.082	2086.443	2203.982
2.7	0.017	3319.314	3101.053	2633.398	3040.981	3184.265
water	0	4794.243	4794.243	4794.243	4794.243	4794.243

Table S3. Pharmacokinetic parameters of RBC-Gd-PLGA calculated based on two compartment model using method of residuals.

Pharmacokinetic parameters	Two-compartment model
Elimination phase	
β (h^{-1})	0.06
B_0 (μg/mL)	3.12
$t_{1/2\beta}$ (h)	11.6
Distribution phase	
α (h^{-1})	0.21
A_0 (μg/mL)	4.88
$t_{1/2\alpha}$ (h)	3.27
Concentration at time zero	
C_0 (μg/mL)	8.00
Rate constant	
k_{21} (h^{-1})	0.12
k_{10} (h^{-1})	0.11
k_{10} (h^{-1})	0.05
Volume of distribution	
V_c (mL)	2.00
V_p (mL)	0.77
V_{dss} (mL)	2.78
Area under the curve	
AUC (μg.h/mL)	75.02
Clearance total	
Cl_{total} (mL/h)	0.21

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