Electronic Supplementary Information

Rituximab Conjugated Iron Oxide Nanoparticles for Targeted Imaging and Enhanced Treatment against CD20-Positive Lymphoma

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S1. Experiment Methods

S1.1. Characterization of nanoparticles and nanoprobes

The size and morphology of the Fe₃O₄-PEG nanoparticles were examined by transmission electron microscopy (TEM, JEOL, Tokyo, Japan) at a working voltage of 200 kV. The Fe concentration in the aqueous solution of Fe₃O₄ nanoparticles was measured via the phenanthroline method on a UV-visible spectrophotometer (UV-3600, Japan). The hydrodynamic diameter and zeta potential of the nanoparticles and nanoprobes were determined using a particle size analyzer (Malvern Instruments, Malvern Zetasizer, UK) at 25°C. The saturation magnetization was determined using a vibrating sample magnetometer (VSM, Lakeshore VSM 7407, USA). Thermogravimetric analysis (TGA, Pyris 1 DSC, USA) was used to study the surface of the PEG-modified Fe₃O₄ nanoparticles. Clinical MRI (Avanto-1.5T, Siemens, Germany) was employed to measure the r2 value of the nanoparticles and nanoprobes.

S1.2. Calculate the number of rituximab (RTX) in Fe₃O₄-PEG-nAb nanoprobes

The number of RTX molecules on the surface of the Fe_3O_4 -PEG-nAb nanoprobes could be controlled by varying the amount used in the reaction. The total amount of RTX used in the reaction was denoted as A. The antibody level in percolate filter after gel chromatography was quantitatively detected by BCA assay and denoted as B. Hence, the total amount of RTX molecules that had been conjugated onto the surface of the nanoprobes could be calculated as C=A-B.

The number of RTX on the surface of the Fe_3O_4 -PEG-nAb nanoprobes was measured and calculated as follows:

$$m_{1} = \frac{4}{3}\pi \left(\frac{D}{2}\right)^{-3}\rho_{Fe_{3}O_{4}}$$

$$m_{Fe_{3}O_{4}} = \frac{m \times M_{Fe_{3}O_{4}}}{3 \times M_{Fe}}$$

$$N_{Fe_{3}O_{4}} = \frac{m_{Fe_{3}O_{4}}}{m_{1}} = \frac{m \times M_{Fe_{3}O_{4}}}{3 \times M_{Fe} \times \frac{4}{3}\pi \left(\frac{D}{2}\right)^{-3}\rho_{Fe_{3}O_{4}}} = \frac{m \times 2M_{Fe_{3}O_{4}}}{\pi D^{3} \times \rho_{Fe_{3}O_{4}} \times M_{Fe}}$$

$$N_{RTX} = \frac{m_{RTX}}{150KDa} \times N_A$$

$$N = \frac{N_{RTX}}{N_{Fe_3o_4}} = \frac{m_{RTX} \times N_A \times \pi D^3 \times \rho_{Fe_3o_4} \times M_{Fe}}{150KDa \times m \times 2M_{Fe_3o_4}} = \frac{N_A \times \pi D^3 \times \rho_{Fe_3o_4} \times M_{Fe}}{150KDa \times 2M_{Fe_3o_4}} \times \frac{m_{RTX}}{m}$$

Where m_1 represents the weight of one Fe₃O₄ nanoparticle, D is the diameter of the Fe₃O₄ nanoparticles. ${}^{\rho_{Fe_3}o_4}$ is the density of the Fe₃O₄. ${}^{m_{Fe_3}o_4}$ and m represents the total weight of tested Fe₃O₄ nanoparticles and Fe in the solution. ${}^{M_{Fe_3}o_4}$ and ${}^{M_{Fe}}$ are the molar mass of Fe₃O₄ and Fe. ${}^{N_{Fe_3}o_4}$ and ${}^{N_{RTX}}$ are the number of Fe₃O₄ nanoparticles and RTX antibodies in the solution. ${}^{m_{RTX}}$ represents the content of RTX antibodies in the tested nanoprobes solution. Then, the number of PEG molecules per nanoparticle, denoted as N, was calculated as 2, 5 or 8. Hence, 2, 5 or 8 RTX antibodies were present on the surface of one Fe₃O₄-PEG particle.

S1.3. In vitro MRI

An *in vitro* study of the ability of the nanoprobe to target and enter Raji cells was performed by MRI. More specifically, CD20-positive Raji cells and CD20-negative K562 cells (1×10⁶ mL⁻¹) in the logarithmic phase were seeded in 6-well plates and cultured in RPMI-1640 containing 10% fetal bovine serum (FBS). The cells were incubated for 24 h at 37°C with different concentrations of nanoparticles and nanoprobes suspended in fresh media (0, 20, 40, 60, 80, 100 μ g[Fe]/mL). Following 3 washes with PBS, 5×10^5 cells were prepared for each MRI sample. The cells were suspended in 4 mL of 1% Sepharose bio-gel prepared beforehand and transferred into 5-mL sterilized glass bottles for imaging. Following rapid vibration on a vibrator apparatus, the cells were evenly distributed in agarose solution. All samples were cooled at room temperature to form a gel. MR images were obtained using a clinical 1.5-T MR scanner (Avanto-1.5T, Siemens, Germany) with a head and neck coil. Spin-echo imaging sequences were used to obtain T2-weighted images. The parameters were as follows: repetition time (TR) =2500 ms; echo time (TE)=22.0, 44.0, 66.0, 88.0, 110.0, 132.0, 154.0, 176.0, 198.0, 220.0, 242.0, 264.0, 286.0, 308.0, 330.0, and 352.0 (n=16); field of view (FOV)=230 mm×230 mm; matrix=256×256; number of excitations (NEX)=1; and slice thickness=5 mm. Under the viewing model, the obtained maps were evaluated by dynamic

analysis. The T2 values of the area of interest were measured within a region of interest (ROI) of 0.25 cm^2 and plotted.

S1.4. Hemolysis Evaluation

Blood (5 mL) from healthy rabbits was collected in 10-mL heparin-coated tubes. The blood was purified by centrifugation at 1500 rpm for 5 min, and the red blood cells (RBCs) were washed three times with sterile physiological saline solution. When the supernatant was not red colored, the precipitated RBCs were resuspended in sterile physiological saline solution. Then, Fe₃O₄-PEG (20, 40, 60 μ g/mL) and Fe₃O₄-PEG-nAb (20, 40, 60 μ g/mL) were mixed with the RBC suspension. A negative control was established by mixing 2.5 mL of RBC suspension and 2.5 mL of physiological saline solution. A positive control was prepared by mixing 2.5 mL of RBC suspension with 2.5 mL of pure water. The hemolysis and clots were examined every 15 min over the first 1 h and hourly thereafter. After incubation for 3 h, images were captured to represent the hemolytic reaction of all the samples. Then, the supernatant at 540 nm was measured on a UV-visible spectrophotometer (UV-3600, Shimadzu, Japan). The hemolysis rate (HR) was calculated using the following equation: HR=(OD_{MNPs}-OD_{C1}) ×100%/(OD_{C2}-OD_{C1}).

In this equation, OD_{MNPs} is the absorbance of the supernatant in the nanoparticle and nanoprobe groups, OD_{C1} is the absorbance of the negative control, and OD_{C2} is the absorbance of the positive control.

S1.5. Cell viability assay by CCK-8 and AO/EB staining

Cells were seeded at 1.0×10^4 cells per well in 50 µL of medium in 96-well plates. Then, every kind of RTX (34.5 µg/mL), Fe₃O₄-PEG (50 µg[Fe]/mL) nanoparticles and Fe₃O₄-PEG-8Ab (50 µg[Fe]/mL) nanoprobes was added to the wells at each concentration in triplicate such that the final volume in each well was 100 µL. The mixtures were incubated for 72h. Cell viability was evaluated by CCK-8 assay according to the manufacturer's instructions. The absorbance at 450 nm was determined using a fully automatic microplate reader (ELx808, BioTek, USA).

Raji and K562 cells viability after treatment with RTX, Fe₃O₄-PEG nanopartcles, and Fe₃O₄-

PEG-8Ab nanoprobes were also tested with acridine orange (AO) and ethidium bromide (EB) staining. According to the standard process, the treated cells were centrifuged at 2000 rpm for 5 min to remove the nanoparticles and nanoprobes, and the resuspended cells were incubated with a mixture of AO and EB for 30 min at 37°C without light, and examined under inverted fluorescence microscope (Axioskop 200, Carl Zeiss, Germany).

S1.6. Western blot analyses

Following treatment with RTX, Fe₃O₄-PEG, and Fe₃O₄-PEG-8Ab for 72 h, the cell lysates of all samples were collected and analyzed by western blotting to detect the expression levels of associated proteins. The protein concentration of the cell lysate was measured by protein assay kit. The primary antibodies Bcl-2, BAX and Caspase-3 are used in this experiment. The secondary antibody was horseradish peroxidase conjugated anti-rabbit IgG. According to standard procedures, samples in each group were separated in SDS-PAGE gels and transferred electrophoretically to polyvinylidenedifluoride (PVDF) membranes. The membrane was incubated overnight with the primary antibody (1:1000) at 4°C after blocking with tris-buffered saline (TBS) containing 0.1% tween-20 and 5% non-fat milk at room temperature for 2 h. After washing and subsequent blocking, the membrane was incubated with peroxidase conjugated secondary antibody (1:500) for 2 h at room temperature, and subsequently stained with Diaminobenzidine (DAB). Anti- β -actin was used as a control to ensure equal loading. Detection was performed using an enhanced chemiluminescence kit and an ECL system.

S1.7. Assay of CDC efficacy with heat-inactivation of human serum

The serum of specific-pathogen-free (SPF) NOD-SCID mice and Kunming mice was separated from heart blood by centrifugation. Normal human serum was obtained from the healthy volunteer, whose blood was collected in Southeast University-Affiliated Zhongda Hospital. The serum C3 and C4 levels were tested using a BNII system (Siemens) at the inspection center of Southeast University-Affiliated Zhongda Hospital. Changes in the CDC efficacy induced by the heat-inactivation of human serum (60°C, 30 min) were tested as follows. Heat-inactivated control human serum and human serum were mixed with Raji cells in RPMI-1640 culture medium containing Fe₃O₄-PEG-8Ab (5

5

µg[Fe]/mL). Following 6 h of co-culture at 37°C, the cells were washed with ice-cold PBS and stained with annexin V-FITC/PI to measure cell viability by flow cytometry.

S1.8. Synthesis of Fe₃O₄-PEG-nAb-Cy7 nanoprobes

Multivalent Fe₃O₄-PEG-nAb nanoprobes were conjugated with Cy7-NHS by a reaction between the amino terminus of the antibodies and the carboxyl terminus of NHS to form Fe₃O₄-PEG-nAb-Cy7. The Fe₃O₄-PEG-nAb-Cy7 was purified by gel chromatography (GE Sephacryl s-300), and the filtrate was tested using a UV-visible spectrophotometer to determine the quantity of Cy7 in the nanoprobe. Nanoprobes in the blood samples were quantified based on a standard curve derived from the iron content and the Cy7 fluorescence intensity. Typically, the absolute iron concentration was determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES). Ten normative samples in the sequence shown in **Table S3** were added to a clean, opaque 96-well plate. The fluorescence intensity of each well was measured by a microplate reader. Then, a standard curve was created according to the iron concentration as the abscissa and the fluorescence intensity as the coordinate (**Fig. S11**).

S1.9. Establish the model of mouse subcutaneous lymphoma tumor

To produce xenografts, Raji cells were collected in a pellet by centrifugation, resuspended in PBS, and mixed with BD MatrigelTM Matrix (San Jose, CA, USA) kept on ice at a 1:1 (v/v) ratio. Finally, NOD-SCID mice were inoculated subcutaneously with 200 μ L of the cell mixture to develop xenografts. Animal weight and tumor size were monitored every two days. At approximately 2 weeks post-inoculation, the tumor volumes reached approximately 100 mm³.

S1.10. Scanning parameters of in vivo MRI

The TurboRARE T2 scanning sequence was used with the following parameters: TR=2500.0 ms; TE=33.0 ms; flip angle=180.0°; average=1; FOV= 4 cm × 4 cm; matrix=256×256; slice thickness=1.0 mm. The fast low-angle shot (FLASH) multislice T2* scanning sequence was used with the following parameters: TR=333.1 ms; TE=5.0 md; flip angle = 30.0° ; average=1; FOV= 4 cm× 4 cm; matrix= 512×512 ; slice thickness=1.0 mm.

S1.11. Histopathological staining

The main organs (heart, liver, spleen, lungs and kidneys) and tumor tissue of the mice were harvested for histopathological analysis. Hematoxylin and eosin (H&E) staining was used to evaluate the pathological features. Prussian blue staining was used to observe the iron content in the tissues. Typically, the tissues were fixed in 10% formalin (less than 24 h) before paraffin processing. Then, they were sectioned at a thickness of 5 μ m, stained with H&E or Prussian blue and examined by optical microscopy.

S2. Results and Discussion

S2.1. PEG calculation on the surface of Fe₃O₄-PEG nanoparticles

The thickness of the PEG layer on the surface of Fe_3O_4 -PEG nanoparticles was measured by TGA and calculated as follows:

$$\begin{split} M_{Fe_{3}0_{4}} &= \frac{4}{3}\pi \left(\frac{D}{2}\right)^{-3}\rho_{Fe_{3}0_{4}} \\ S_{Fe_{3}0_{4}} &= 4\pi \left(\frac{D}{2}\right)^{-2} \\ N_{PEG} &= \frac{m_{Fe_{3}0_{4}} - PEG \times x}{M_{PEG}} \times N_{A} \\ N_{Fe_{3}o_{4}} &= \frac{m_{Fe_{3}0_{4}} - PEG \times (1-x)}{M_{Fe_{3}o_{4}}} \\ R_{PEG} &= \frac{N_{PEG}}{N_{Fe_{3}0_{4}} \times S_{Fe_{3}0_{4}}} = \frac{m_{Fe_{3}0_{4}} - PEG \times x \times \frac{4}{3}\pi \left(\frac{D}{2}\right)^{-3}\rho_{Fe_{3}0_{4}} \times N_{A} \\ R_{PEG} &= \frac{N_{PEG}}{N_{Fe_{3}0_{4}} \times S_{Fe_{3}0_{4}}} = \frac{m_{Fe_{3}0_{4}} - PEG \times x \times \frac{4}{3}\pi \left(\frac{D}{2}\right)^{-3}\rho_{Fe_{3}0_{4}} \times N_{A} \\ &= 1.2 \end{split}$$

 $N = R_{PEG} \times S_{Fe_3O_4} = 487$ where ${}^{M_{Fe_3O_4}}$ and ${}^{S_{Fe_3O_4}}$ represent the weight and surface area of one Fe₃O₄ nanoparticle, D is the TEM diameter of the Fe₃O₄ nanoparticles, ${}^{\rho_{Fe_3O_4}}$ is the density of the Fe₃O₄, ${}^{N_{Fe_3O_4}}$ and ${}^{N_{PEG}}$ represent the total number of Fe₃O₄-PEG nanoparticles and PEG molecules, respectively, ${}^{m_{Fe_3O_4-PEG}}$ is the total weight of the tested Fe₃O₄-PEG nanoparticles, x is the ratio of weight loss, and ${}^{M_{PEG}}$ is the molecular weight of modified PEG. Then, the number of PEG molecules per square nanometer, denoted by ${}^{R_{PEG}}$, was calculated as 1.2 per nm². Hence, 487 PEG molecules were present on the surface of one Fe₃O₄-PEG nanoparticle.

S2.2. Calculation of RTX number in multivalent Fe₃O₄-PEG-nAb nanoprobes

The number of RTX molecules in multivalent Fe₃O₄-PEG-nAb nanoprobes was measured and calculated. The prepared Fe₃O₄-PEG-nAb were purified by s-300 gel permeation chromatography. Qualitative analysis of RTX in percolate was performed using a UV-visible spectrophotometer. The percolate containing Fe₃O₄-PEG-nAb nanoprobes was defined as the zeroth milliliter of percolate. Then the first milliliter, second milliliter, until the twelfth milliliter of percolate was obtained in turn. The line at 1 mL (4, 5, 6, and 7 mL) in the figures represents the first milliliter (fourth, fifth, sixth, and seventh milliliter) of the percolate of the nanoprobes sample after filtration, as shown in Fig. S2. It is obvious that no absorption peak corresponding to the antibodies appeared until the fifth milliliter, illustrating that the nanoprobes completely separated from the RTX in the s-300 gel. The uncoupled RTX antibodies were mainly isolated in the fifth, sixth and seventh milliliters. By the eighth milliliter of the percolate, all the antibodies had been filtered out, and no protein absorption peak was detected on the absorption spectrum. Quantitative analysis of RTX in percolate was performed using a BCA assay kit. The input dosage of the antibodies minus the quantity of antibodies in the percolate yielded the content of antibodies in the nanoprobes, as shown in Table S1. The average number of conjugated antibodies per nanoparticle was calculated. The results indicated that multivalent Fe₃O₄-PEG-nAb nanoprobes (Fe₃O₄-PEG-2Ab, Fe₃O₄-PEG-5Ab, and Fe₃O₄-PEG-8Ab) were successfully fabricated.

serial number	Input of Rituximab(ug)	the A	b content i	n percolate	e (µg)	Ab content in nanoprobe	Fe content	number of Ab in	
		5 mL	6mL	7mL	total	(μg)	(µg)	. I	
1	300	40.7	51.1	0	91.8	208.2	1000	2.49	
2	500	27.5	31.4	17.8	76.7	423.3	1000	5.07	
3	400	27.5	60.25	0	87.75	312.25	450	8.3	

Table. S1. The input dosage of RTX antibodies, the RTX antibodies content in percolate and the calculate of number of RTX in Fe₃O₄-PEG-nAb nanoprobes

Table. S2. The complement detection in blood serum samples					
comple	content of complement (g/L)				
sample	C3	C4			
human blood serum	1.31	0.289			
NOD-SCID mouse blood serum	0.318	<0.0615			
mouse blood serum	0.27	<0.0615			
reference	0.9-1.8	0.1-0.4			

Table. S3. The addition of multivalent nanoprobes in each well to build a standard curve

sample	1	2	3	4	5	6	7	8
mice blood control (µL)	30	30	30	30	30	30	30	30
nanoprobe solution (µL)	0	1	4	6	8	12	14	18
distilled water (µL)	20	19	16	14	12	8	6	2

Table. S4. The med	lian survival time o	f NOD-SCID	mice in each	treatment group (n=8)
Survival	control	RTX	Fe ₃ O ₄ -PEG	Fe ₃ O ₄ -PEG-8Ab

Data summary	control	MIM	103041110	10,041100 0110
Median survival (day)	25	29	26	37



Fig. S1. The Fe₃O₄-PEG nanoparticle diameter distribution range was obtained by average diameter analysis software. The mean sizes of Fe₃O₄-OA (A), Fe₃O₄-PEG nanoparticles (B) and Fe₃O₄-PEG-2Ab nanoprobe (C) as determined by TEM. (Fe₃O₄-OA, 11.3 \pm 2.9 nm, Fe₃O₄-PEG 11.5 \pm 2.7 nm and Fe₃O₄-PEG-2Ab, 11.2 \pm 2.7 nm)



Fig. S2. The qualitative detection of percolate of the nanoprobe samples after filtration was measured by UV-visible. The percolate of Fe₃O₄-PEG-2Ab (A), Fe₃O₄-PEG-5Ab (B) and Fe₃O₄-PEG-8Ab (C). The Zeta potentials of Fe₃O₄-PEG nanoparticles (D) and Fe₃O₄-PEG-nAb (n=2, 5 or 8) multivalent nanoprobes (E).



Fig. S3. Flow cytometric analysis of Raji cells (A) and K562 cells (B) treated with RTX, Fe_3O_4 -PEG nanoparticles, and Fe_3O_4 -PEG-nAb multivalent nanoprobes for 24h. Compare the differences of the cell viability among these groups and analyzed them by independent sample T-test (C). (p>0.05 (ns), no statistical significance; p<0.05 (*), statistical significance; p<0.001(**), highly statistical significance)



Fig. S4. Flow cytometric analysis of Raji cells (A) and K562 cells (B) treated with RTX, Fe_3O_4 -PEG nanoparticles, and Fe_3O_4 -PEG-nAb multivalent nanoprobes for 48h. Compare the differences of of the cell viability among these groups and analyzed them by independent sample T-test (C). (p>0.05 (ns), no statistical significance; p<0.05 (*), statistical significance; p<0.001(**), highly statistical significance)



Fig. S5. The cell viability of Raji cells (A) and K562 Cells (B) was detected using CCK-8 after incubating with RTX and Fe_3O_4 -PEG nanoparticles for 24h, 48h and 72h.



Fig. S6. The cell viability of Raji cells (A) and K562 Cells (B) was detected using CCK-8 after incubating with Fe_3O_4 -PEG-nAb multivalent nanoprobes for 24h 48h and 72h.



Fig. S7. The Raji and K562 cell viability in BB buffer (0.01M) after incubating for 24h, 48h and 72h.



Fig. S8. The apoptosis detection of Raji cells after incubating with Fe₃O₄-PEG-8Ab (5 μ g[Fe]/mL, Ab 3.45 μ g/mL) multivalence nanoprobes and Fetal calf serum (5%) (control group, A), human serum (5%) (test group, B) and heat-inactivated human serum (5%) (test group, C) for 6 h.



Fig. S9. The histogram of enhanced Raji cell apoptosis tested by CCK-8 after incubating with Fe_3O_4 -PEG (5 µg[Fe]/mL), RTX (3.5 µg/mL) and Fe_3O_4 -PEG-8Ab (5µg[Fe]/mL, Ab 3.45µg/mL) in the presence of different serums (2%) (Fetal calf serum, Mouse serum, NOD-SCID serum and human serum) for 24h (A).

Scatter diagram of enhanced Raji cell CDC as detected by FACS after treatment with RTX (3.5 μ g/mL), Fe₃O₄-PEG (5 μ g[Fe]/mL) and Fe₃O₄-PEG-8Ab (5 μ g[Fe]/mL, Ab 3.45 μ g/mL) in the presence of different sera (5%; human serum, NOD-SCID serum and mouse serum) for 24h (B-K).



Fig. S10. The Fluorescence emission spectrum of Fe_3O_4 -PEG-5Ab-Cy7 multivalent nanoprobes



Fig. S11. Standard curve of the variation of fluorescence intensity of Fe_3O_4 -PEG-5Ab-Cy7 nanoprobes with the Fe concentration



Fig. S12. T2 and T2*-weighted MR images of NOD-SCID mice bearing subcutaneous xenotransplanted tumor model of Raji cells pre and 4, 6, 12, 24, 48, 72h after the injection of multivalent nanoprobes Fe_3O_4 -PEG (A). T2 and T2*-weighted MR images of NOD-SCID mice bearing subcutaneous xenotransplanted tumor model of Raji cells pre and 1, 4, 6, 12, 24, 48, 72h after the injection of Fe₃O₄-PEG-8Ab multivalent nanoprobes (C). Quantification of T2 and T2* signal changes of tumor signal noise radio (SNR) at the corresponding time points after injection of Fe₃O₄-PEG (B) and Fe₃O₄-PEG-8Ab (D).



Fig. S13. The body weight changes of NOD-SCID mice in each group during the process of treatment.



Fig. S14. The H&E of heart, liver, spleen, lung, kidney and tumor tissues, which were harvested from NOD-SCID mice treated without (control) and with RTX, Fe_3O_4 -PEG nanoparticles, Fe_3O_4 -PEG-8Ab multivalent nanoprobes at the end of the experiment (Scale Bar 100 μ m).



Fig. S15. The Prussian blue stained images of heart, liver, spleen, lung, kidney and tumor tissues, which were harvested from NOD-SCID mice treated without (control) and with RTX, Fe_3O_4 -PEG nanoparticles, Fe_3O_4 -PEG-8Ab multivalent nanoprobes at the end of the experiment (Scale Bar 100 μ m).