Supporting Information for

A Europium-Lipoprotein nanocomposite for high-sensitive MR-fluorescence multimodal imaging

Qi Wang,[a] Shizhen Chen,[a] Qing Luo,[a] Maili Liu[a] and Xin Zhou*[a]

[a]Key Laboratory of Magnetic Resonance in Biological Systems, State Key Laboratory for Magnetic Resonance and Atomic and Molecular Physics, National Center for Magnetic Resonance in Wuhan, Wuhan Institute of Physics and Mathematics, Chinese Academy of Sciences, Wuhan 430071, China. E-mail: xinzhou@wipm.ac.cn

Materials

1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine (DMPE) and 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-(lissminerhodamine B sulfonyl) (Rhod-DMPE) were purchased from Avanti Polar Lipids (Alabaster, AL). Tri-tert-butyl 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetate (tri-tBu-DOTA) was purchased from Tokyo Chemical Industries (Tokyo, Japan). Human ApoA-I apolipoprotein (ApoA-I) was purchased from Merck Millipore. N,N,N',N'-Tetramethyl-O-(N-succinimidyl)uroniumhexafluorophosphate (HSTU), N,N-Diisopropylethylamine (DIPEA), trifluoroacetic acid (TFA), sodium cholate hydrate and Europium (III) triflate were purchased from Aladdin Reagent (Shanghai, China). Glycine ethyl ester hydrochloride was from Sigma-Aldrich. CH_2Cl_2, N,N-Dimethylformamide (DMF) were dried before use.

Synthesis of Eu-DOTA-3AmCE-DMPE

The procedure of synthesis of Eu-DOTA-3AmCE-DMPE was shown as Fig. S1. Tri-tBu-DOTA (0.173 mmol, 99 mg), HSTU (0.173mmol, 62 mg) and DIPEA (0.786mmol, 102 mg) were dissolved in dry DMF (3 mL), and then stirred for 1 h under nitrogen atmosphere. To the yellowish solution obtained, a solution of DMPE (0.157mmol, 100mg) in CH_2Cl_2 (5 mL) was added dropwise under nitrogen atmosphere. The temperature was raised to 45 ºC until the solution became clear, and kept the temperature at 45 ºC for another 2 h. After cooling, the solution was stirred at room temperature overnight. The reaction was monitored by Thin-Layer Chromatography (TLC) colored by ninhydrin. The solution was concentrated under reduced pressure, co-evaporated with toluene to remove the DMF. After precipitated in 1:1 v/v water/MeOH and isolated by centrifugation, the obtained yellowish pellet was dissolved in CH_2Cl_2 and rotary evaporated. The resulting solid was redissolved in 10 mL diethyl ether (some heating was needed), and washed with demi water (10 mL). Then the organic phase was rotary

evaporated, resulting in as a yellowish solid **1**.

TFA (2 mL) was added to the **1** solution in CH_2Cl_2 (10 mL) under nitrogen atmosphere and vigorously stirred for 5 h at room temperature. The solution was rotary evaporated without further treatment for next step reaction. **2** was obtained.

Glycine ethyl ester hydrochloride (0.573 mmol, 80 mg) was dissolved in DMF (4 mL). Then equivalent Et_3N was added and solid was precipitate out. After filtered, the filtrate was added slowly to **2** (0.157 mmol, 160mg), HSTU (0.550 mmol, 198 mg) and DIPEA (3.144 mmol, 407 mg) solution in CH_2Cl_2 (10 mL), which had been stirred under nitrogen atmosphere for 2 h. The mixture was stirred at room temperature under nitrogen atmosphere for another 12 h. The solution was concentrated under reduced pressure, co-evaporated with toluene. The product was redissolved in CH_2Cl_2 and loaded onto a silica column (CH_2Cl_2/MeOH 4/1 to 1/8, v/v). Yellowish solid **3** was obtained[1]. ^{1}H NMR (500 MHz, $CDCl_3$+drops of CD_3OD): δ=4.1-3.8 (br m, 19H), 3.6-2.6 (br m, 26H, NCH_2CH_2N {16H} + NCH_2CO {8H} + $POCH_2CH_2NHCO$ {2H}), 2.3-2.2 (t, 4H, $CH_3(CH_2)_{11}CH_2CO$), 1.5 (m, 4H), 1.3-1.1 (m, 49H, $CH_3(CH_2)_{11}CH_2CO$ {40H}+CH_3CH_2O {9H}), 0.8 (t, 6H, CH_3CH_2). Hydrogens on –NH and –P-OH were not found. ESI-MS (positive mode): m/z $[C_{61}H_{113}N_8O_{18}P+H]^+$Calcd. 1277.79 Da; Obsd. 1277.779 Da.

To a solution of **3** (0.016 mmol, 20 mg) in 5 mL CH_2Cl_2/MeOH (1:1, v/v) was added Eu (III) triflate (0.012 mmol, 9.6 mg) solution in 5 mL MeOH/H_2O (4:1, v/v) and the pH was adjust to 7 using pyridine. Subsequently, the solution was stirred at room temperature overnight. To check if there was excess Eu (III) ion left, the xylenol orange indicator test was used. After confirmation of no Eu (III) ion existing, the solution was rotary evaporated and dried *in vacuo*. The product **4** was used without further treatment[2].

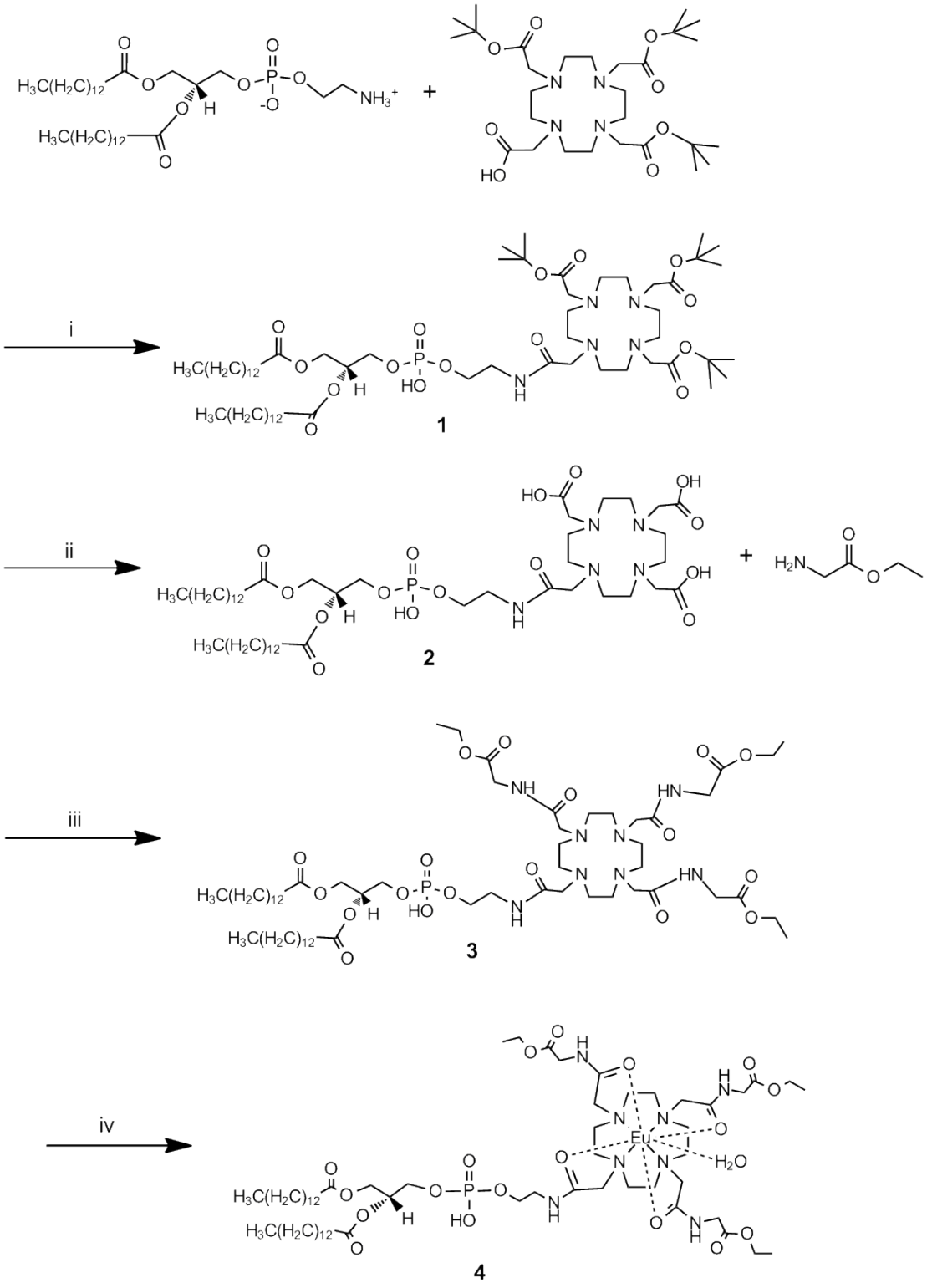

Fig. S1 The synthesis procedure of Eu-labeled phospholipid **4**, Eu-DOTA-3AmCE-DMPE. (i) HSTU, DIPEA, r.t., overnight. (ii) TFA, r.t., 5 h. (iii) HSTU, DIPEA, r.t., overnight. (iv) $(CF_3SO_3)_3Eu$, r.t.

Preparation of Eu-labeled rHDL nanocompisites

Eu-DOTA-3AmCE-DMPE, DPPC and Rhod-DMPE were dissolved in CH_2Cl_2/MeOH (4:1,

v/v). Then the solution was dried into a lipid film in vacuum. The lipid mixture was dispersed in PBS (0.1 M, pH 7.4) containing sodium cholate and the temperature was increased to 55 ºC, making the lipid fully hydrate. After cooling to the room temperature, ApoA-I (0.03 μmol, 1 mg) in PBS was added to the solution. The molar ratio was 1:300:50:5:450 for ApoA-I:Eu-DOTA-3AmCE-DMPE:DPPC:Rhod-DMPE:sodium cholate. After incubation for 12 h at 37 ºC, the sample was dialyzed against PBS (0.1 M, pH 7.4) for 24 h with three buffer changes to remove the sodium cholate. Filtered through a 0.22 μm syringe filter, Eu-Rhod-rHDL was prepared[3, 4].

The protocol above was also used to prepare the Rhod-rHDL without labeled Eu (III), in which the Eu-DOTA-3AmCE-DMPE was changed to equal molar DMPE. The molar ratio of ApoA-I:DMPE:DPPC:Rhod-DMPE:sodium cholate was 1:300:5:450.

Characterization of rHDLs nanocompisites

The concentration of Europium (III) was determined by Inductive Coupled Plasma Atomic Emission Spectrometry (ICP-AES) (IRIS Intrepid II XSP, Thermo Electron, USA). The sample was preprocessed with hot concentrated nitric acid for 1 h. The amount of Eu(III) per rHDL particle was estimated by the ratio of concentration of Eu(III) and the concentration of rHDL particle, which was from the concentration of ApoA-I dividing the ApoA-I amount per rHDL. In this case, there are three ApoA-I molecules per rHDL[5].

The standard curve for the determination of Eu(III) is as Fig. S2. The concentration of Eu(III) in the Eu-Rhod-rHDL solution, diluted for 1000 folds before testing, was read at 1.82 mg L^{-1}, which is 12 mM of Eu(III) for the original solution.

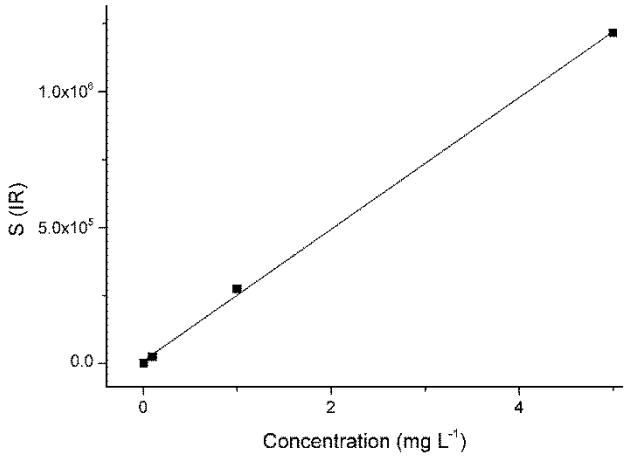

Fig. S2 The ICP-AES standard curve for Eu(III) concentration determination.

The size distribution and the morphology of the rHDL were investigated by transmission electron microscopy (TEM) and dynamic light scattering (DLS). The particle size and morphology of the particle was observed by transmission electron microscopy on a FEI Tecnai G^2 20 TWIN (FEI Co., USA) transmission electron microscope. The mean particle size and size distribution were determined by DLS at 25 ºC in PBS at pH 7.4 on a Zetasizer Nano ZS instrument (Malvern Instruments, Malvern, UK). Zeta potential of rHDL was also measured in PBS at pH 7.4 on the

same apparatus. (Fig. S3a)

Comment [WQ]: Revised.

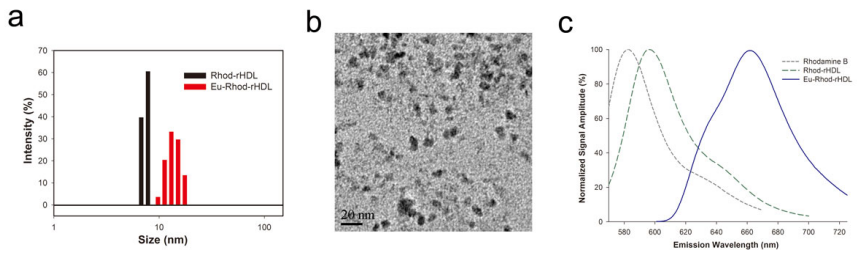

Fig. S3 The characterization of the rHDLs. (a) The size of Eu-Rhod-rHDL and Rhod-rHDL were investigated by the DLS. The mean size of Rhod-rHDL and Eu-Rhod-rHDL were 8.8 and 14.3 nm, respectively. (b) TEM photographs of Eu-rHDL, which confirmed the morphology and size. size distribution of Eu-Rhod-rHDL. (c) Emission fluorscence spectra showed the conjugation of dye to rHDL caused a 12 nm red shift, while the introducing of Eu (III) labeled phospholipid caused a rather large red shift of 70 nm.

The fluorescence property of samples was investigated with a HORIBA FM-4 spectrofluorometer. All samples were dissolved in PBS at pH 7.4 and with excitation wavelength was 552 nm, the fluorescence spectra were recorded. All spectra were normalized with signal amplitude. (Fig. S3b)

***In vitro* CEST MR imaging**

Solution of Eu-Rhod-rHDL in concentration of 77.4 μM (on a per rHDL particle basis) were prepared in PBS at pH 7.4 and 10% D_2O for nuclear magnetic resonance spectroscopy. The CEST Z-spectra were acquired on a Bruker AVANCE III 500 MHz NMR spectrometer, with a selective saturation pulse at 25 μT for 5 s at MR frequencies ranging from +80 to -80 ppm with 1 ppm increment (where the water resonance is referenced to 0 ppm). The Z-spectra of Rhod-rHDL and PBS were also acquired with the above procedure as control experiments. To describe the CEST effect of the contrast agent, CEST % was defined as the Equation 1[6], where M_s is the signal intensity of selective saturation at the exchanging site of interest, while M_0 is of the opposite side of the water resonance from the site of interest.

$$CEST\% = \frac{M_0 - M_s}{M_0} \times 100 \qquad \text{(Equation 1)}$$

The CEST images were conducted at 9.4 T by a Bruker AVANCE III 400 MHz wide bore microimaging system, using a Fast Low-Angle Shot (FLASH) pulse sequence (TR=5012 ms, TE= 6 ms, matrix 128×128, field of view 20×20 mm^2, slice thickness 4 mm, one average) preceded by a 8 μT selective saturation pulse at 50 ppm centered on the bulk water frequency for 5 s. Image with saturation at -50 ppm was also acquired as a reference. The percent of CEST was calculated by comparing the images acquired with selective saturation at +50 ppm with images acquired with selective saturation at -50 ppm. Because we used the FLASH pulse sequence, the contrast of the original MR image was lost at a certain degree. However, as the CEST MR image was obtained from the ratio of two different images, the contrast losing in the CEST images was small.

Cell culture

Murine macrophage RAW 264.7 cells and normal human WI-38 cells were purchased from the Type Culture Collection of Chinese Academy of Sciences, Shanghai. RAW 264.7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) Fetal Bovine Serum (FBS), 100 μgmL^{-1} penicillin, and 10 μgmL^{-1} streptomycin in cell culture flask at 37 °C in 5% CO_2. The WI-38 cells were cultured in Minimum Essential Medium (MEM) in the same condition. The medium was changed every 2–3 days. Cells were passaged when reached 80%–90% confluence, trypsinized by 0.25% trypsin and 0.02% EDTA solution, and the cell concentration was determined using a hemacytometer.

***In vitro* cellular fluorescence imaging**

To test the specificity of the rHDL particles, RAW 264.7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) on cover glasses in 6-well plate for 12 h. Then, the medium was replaced by the DMEM containing rHDL (20 μg mL^{-1}) and incubated at 37 °C in 5% CO_2 for 4 h. Afterwards the medium was removed and the cover glasses were washed with PBS for three times. 4% paraformaldehyde solution was added to the wells and removed after 10 min for cell fixation; the nuclei were stained with DAPI. The cover glasses were washed by double distilled water for three times before mounted to slides. The WI-38 cells were treated in the same protocol except the medium was replaced by the Minimum Essential Medium (MEM). The fluorescence images were taken under a Nikon A1 Laser Scanning Confocal Microscope (Nikon, Japan).

References

1 S. Hak, H. M. Sanders, P. Agrawal, S. Langereis, H. Grull, H. M. Keizer, F. Arena, E. Terreno, G. J. Strijkers and K. Nicolay, *Eur. J. Pharm. Biopharm.*, 2009, **72**, 397-404.

2 S. Viswanathan, S. J. Ratnakar, K. N. Green, Z. Kovacs, L. M. De Leon-Rodriguez and A. D. Sherry, *Angew. Chem. Int. Ed.*, 2009, **48**, 9330-9333.

3 A. Jonas, *Methods Enzymol.*, 1986, **128**, 553-582.

4 P. C. Rensen, R. L. de Vrueh, J. Kuiper, M. K. Bijsterbosch, E. A. Biessen and T. J. van Berkel, *Adv. Drug Deliver. Rev.*, 2001, **47**, 251-276.

5 A. Jonas, K. E. Kezdy and J. H. Wald, *J. Biol. Chem.*, 1980, **255**, 877-881.

6 K. M. Ward, A. H. Aletras and R. S. Balaban, *J. Magn. Reson.*, 2000, **143**, 79-87.