Supporting Information

^{129}Xe hyper-CEST/^{19}F MRI multimodal imaging system for sensitive and selective tumor cells detection

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1. Materials

Pluronic F-68 (average MW = 8.4 kD) was purchased from Energy Chemical. Phospholipid Lipoid S75 was purchased from Lipoid AG (Ludwigshafen, Germany). Perfluorooctyl bromide was purchased from Shanghai Qinba Chemical Co., Ltd. Peptide cyclo-(Arg-Gly-Asp-D-Tyr-Cys) (c-(RGDyc)) was purchased from GL Biochem (Shanghai, China). Cholesterol PEGs Maleimide was purchased from Shanghai Peng Sheng Biological Technology Co., Ltd. Dialysis bag (Mr 1000Da) was purchased from Biosharp (Hefei, China). Human lung adenocarcinoma cell line A549 was obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). RAW 264.7 macrophage was obtained from BOSTER Biological Technology Co., Ltd (WuHan, China). Normal human lung fibroblast cell line WI-38 was obtained from China Center for Type Culture Collection (CCTCC).

2. Synthesis of Cls-PEG-RGDyc

5 mg of c-(RGDyc) and 21 mg cholesterol-PEG2000-maleimide (1 eq) was dissolved in phosphate buffer (5 mM, pH = 7.4) under a nitrogen atmosphere and the resulting mixture was shaken at 25 °C for 20 h at 300 rpm. The crude product was purified by dialysis (MW cut-off = 1000 Da) in buffered water at pH = 7.4. The resulting solution was freeze-dried to give the product Cls-PEG-RGDyc (Figure S1), which was verified by mass spectroscopy (Figure S2).

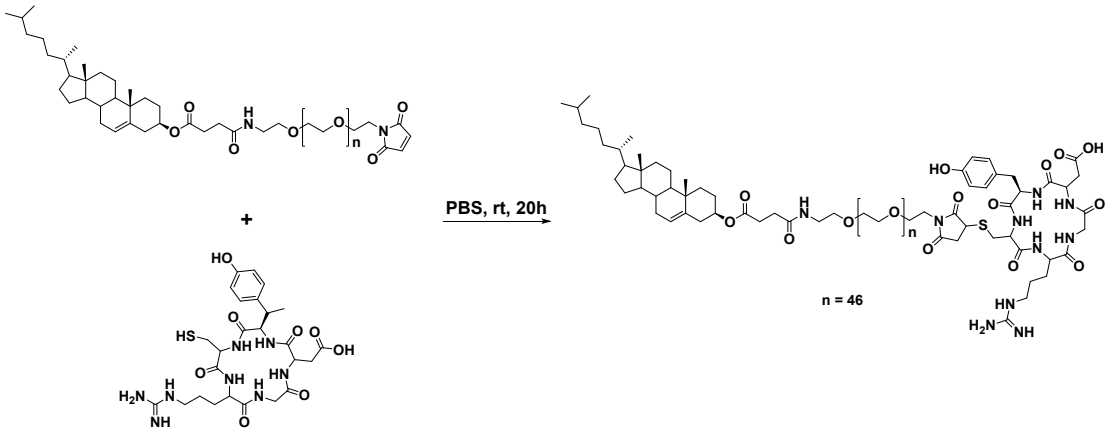

Figure S1. Synthesis of Cls-PEG-RGDyc.

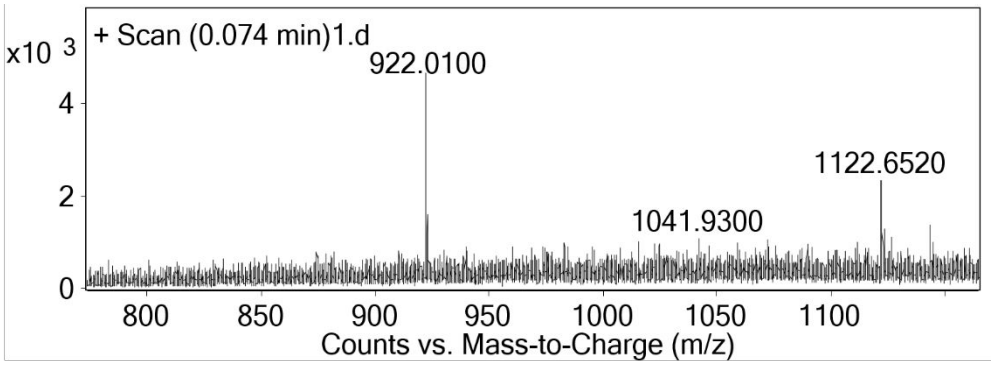

Figure S2. HRMS (ESI) of Cls-PEG-RGDyc (calcd for $C_{155}H_{278}N_{10}O_{60}S$ $[M+3(CH_3OH)]^{3+}$:1122.6500, found 1122.6520).

3. Preparation and characterization of NEs and NEs-RGD

To a solution of 4% (w/w) Lipoid S75 and 0.5% (w/w) Pluronic F-68 in phosphate buffer (5 mM, pH = 7.4) was added 20% (w/w) PFOB. The crude emulsion was sonicated in a water bath for 10 min and shaken with a test tube Shaker for 15 min, followed by filtration through a 0.2 μm syringe filter 3 times. The emulsion were diluted 20-fold with deionized water for storage prior to use. NEs were incubated with Cls-PEG-RGDyc (phospholipid : Cls-PEG-RGDyc = 20: 1) on a rotary shaker at 25°C for 1 h to provide RGD modified NEs which was then shaken with fluorescent dye DiI (5

mg/mL) for 1 hour to provide NEs-RGD. NEs-RGD give a characteristic UV absorbance of c-(RGDyc) around 275 nm (Figure S3).

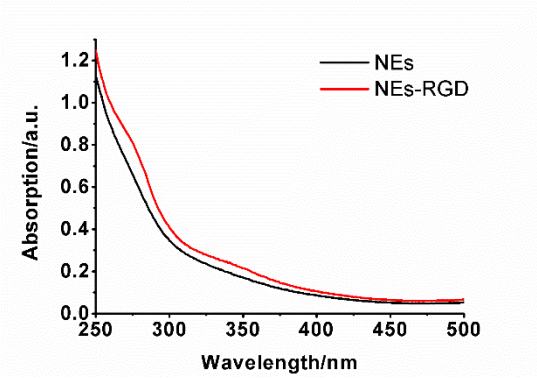

Figure S3. UV absorption of NEs-RGD and NEs.

The size distribution and ζ-potential of NEs and NEs-RGD were measured by DLS (Nano ZS 90, Malvern, UK) and TEM (Tecnai G20, FEI, USA, negative staining with phosphotungstic acid at 1%, w/v).

Table S1. Characterization of NEs and NEs-RGD.

	NEs	NEs	NEs	NEs-RGD
Diameter	296±4	260±2	195 ±1	210±1
PDI	0.25	0.19	0.19	0.17
Z (mV)	-32.1±0.6	-31.1±0.4	-12.9±0.9	-15.6±0.6

All data is presented as the mean ± SD (n = 3).

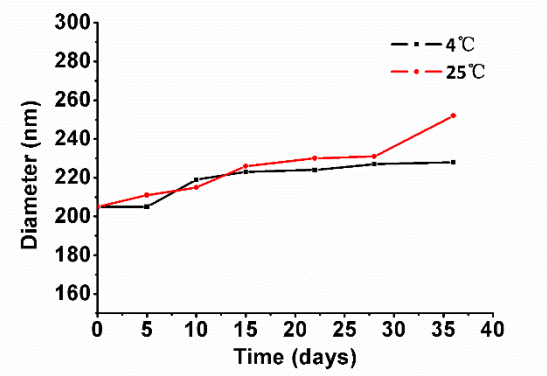

Figure S4. Stability of NEs-RGD at 4 °C and 25 °C by DLS.

4. Cellular uptake study and cytotoxicity assay

A549 cells, WI-38 cells and RAW 264.7 cells were cultured in F-12K (Boster, China), MEM, and DMEM-High glucose medium, respectively, with 10% fetal bovine serum and 100 units/mL penicillin and 0.1 mg/mL streptomycin under a humidified air with 5% CO_2 at 37 °C.

Cells were seeded in a 6-well chamber slide at a density of 2×10^5 /mL and incubated for 12 h which was then treated with NEs at 37 °C for 1 h. The blocked A549 cells was protreated with RGD molecule at a concentration of 0.05 mg/mL for 0.5 h. After washed with PBS for 3 times, cells were fixed with 4% paraformaldehyde for 10 min at room temperature. Fixed cells were stained with DAPI for 5 min and washed with PBS 4 times. Finally, cells were mounted on slides in fluoromount with coverslips imaged under Confocal Laser Scanning Microscope (A1R/A1, Nikon, Japan) and Confocal Laser Scanning Microscope (TCS SP8, Leica, Germany).

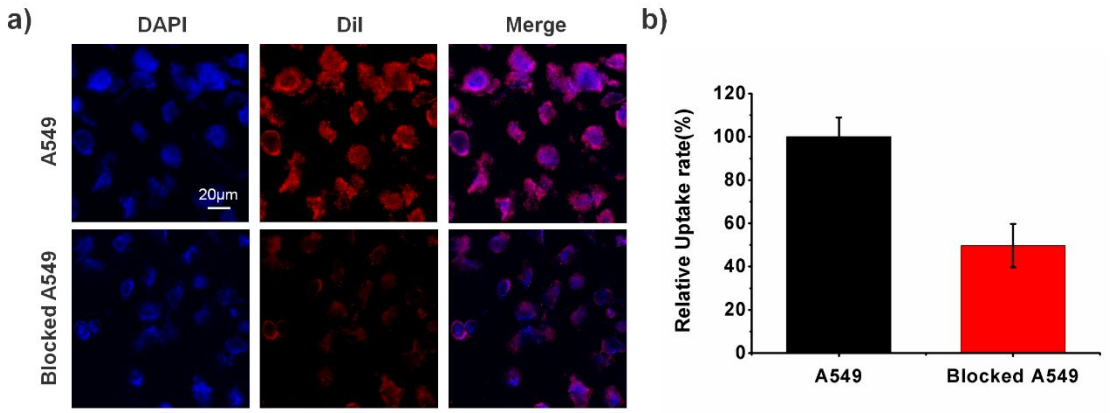

Figure S5. (a) Confocal images of NEs-RGD treated A549 cells and blocked A549 cells. (b) The relative fluorescence intensity of NEs-RGD treated A549 cells and blocked A549 cells.

Methylthiazolytetrazolium (MTT) assay kit was employed to evaluate cell toxicity of NEs and NEs-RGD. Briefly, A549 cells were seeded into 96-well plates and incubated overnight. NEs samples were diluted with the serum-free medium to different concentration (C_{PFOB}=0.5, 1, 2, 5, 10, 20 mM, respectively) and incubate with cells at 37 °C for 6 h. Then remove the sample and washed 2 times with PBS, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to the culture medium at a final concentration of 0.5 mg/mL, and incubated at 37 °C for another 4 h. After removal of the medium, 200 μL DMSO was added per well and incubated at 37 °C for 30 min to dissolve the formed formazan, which was only produced by living cells react with MTT. Cell viability was measured using ELISA plate reader (Spectra MAX 190, Molecular Devices, USA). Data are presented as mean ± SD, n = 3.

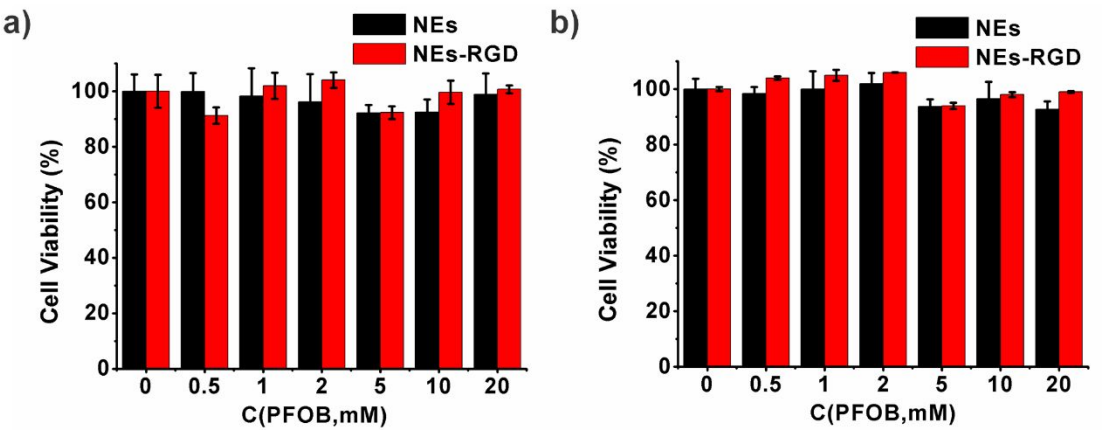

Figure S6. Cytotoxicity assay of NEs and NEs-RGD on A549 cells (a) and MCF-7 cells (b).

5. ^{19}F-NMR Measurements

To determine the exact concentration of PFOB in nanoemulsion, an aqueous suspension of nanoemulsion containing 10% D_2O and trifluoroethanol (10 mM) was introduced in the sample tube. Trifluoroethanol (at -76 ppm) was used as an internal standard to calibrate the concentration of PFOB in nanoemulsion (Figure S7).

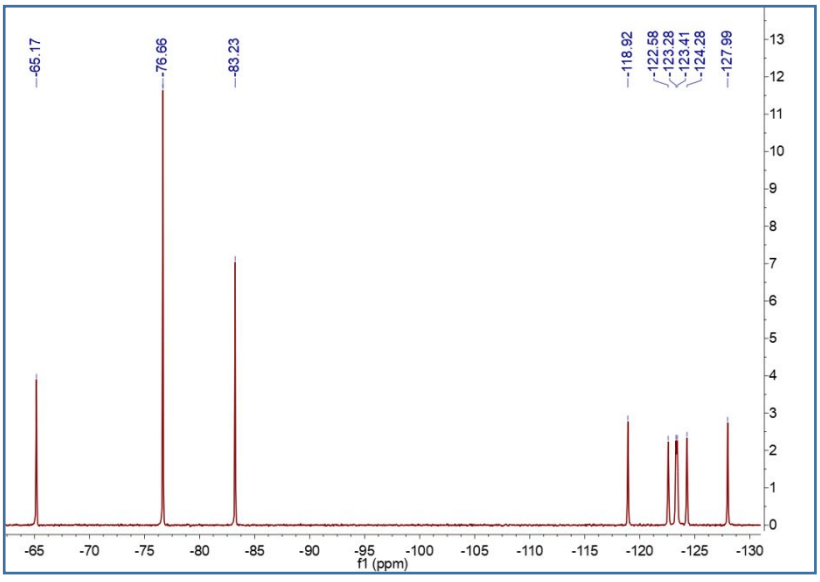

Figure S7. ^{19}F NMR of nanoemulsion (trifluoroethanol peak at -76 ppm).

Absolute encapsulation efficiency of PFOB was calculated as follows:

$$\eta PFOB = n/n_0.$$

(n_0 is the theoretically content of PFOB)

All the ^{19}F NMR experiments were performed on a Bruker Ascend WB 500 MHz spectrometer. The longitudinal relaxation time T_1 was measured through the inversion recovery method and the transverse relaxation time T_2 was measured through the spin-echo method (Table S2).

Table S2. T_1, T_2 of PFOB and PFOB nanoemulsion.

Sample	T_1	T_2
PFOB (-CF_3 at -83 ppm)	1.112 s	885.556 ms
PFOB (-CF_2 at -65 ppm)	915.729 ms	672.612 ms
Emulsion (-CF_3 at -83 ppm)	1.389 s	1.09 s
Emulsion (-CF_2 at -65ppm)	1.105 s	702.368 ms

6. *In vitro* and *in vivo* ^{19}F-MRI

A549 cells and WI-38 cells were treated with NEs or NEs-RGD (NEs concentration = 26 pM) in serum-free culture medium. After 1 h co-incubation at 37 °C, cells were washed 3 times with PBS, harvested and suspended in 2 mL PBS for ^{19}F MRI. ^{19}F MRI images were acquired through RARE method. (TR = 1500 ms, TE = 3 ms; FOV = 4.9×4.9 cm, 35 mm slice thickness; 102 min of data acquisition; RARE factor =4; matrix size = 32×32; 512 averages).

BALB/C male nude mice (6 weeks, 18-21 g) were purchased from Human SJA Laboratory Animal Co., Ltd. For the xenograft tumor mice, A549 cells (1×10^6/100 μL) were subcutaneously injected into the left hind of the mice. The mice had free access to water and food until tumor size reached about 100 mm^3. All experimental protocols in this study were approved by Animal Care and Use Committees at the Wuhan Institute of Physics and Mathematics, the Chinese Academy of Sciences.

The tumor-bearing mice were anesthetized with chloral hydrate (0.01 mL/g). After 200 μL nanoemulsion (PFOB concentration = 18.2 mM) was injected into the tumor, ^{19}F MRI was performed on 400 M Bruker BioSpec MRI system 3 h later. After a ^{1}H MRI scan using a RARE sequence (TR = 5000 ms, TE = 11 ms, 22 ms, 33 ms, 44 ms, 55 ms, 66 ms, 77 ms, 88 ms; FOV = 4×4 cm, 1 mm slice thickness; 160 s of data acquisition; RARE factor =8; matrix size = 256×256), ^{19}F MRI was performed through a RARE sequence (TR = 2000 ms, TE = 3 ms, FOV = 4.94×4.94 cm, 30 mm slice thickness, 17 min of data acquisition, matrix size = 32×32, 64 averages). The ^{19}F MR image was segmented using 0.2*maximum value as threshold and interpolated into 128*128 matrix.

7. ^{129}Xe Hyper-CEST NMR and MRI

^{129}Xe NMR and MRI measurements were performed on a 400 MHz (9.4 T) Bruker AV400 wide-bore spectrometer (Bruker Biospin, Ettlingen, Germany), equipped with microimaging gradient coils and RF pulse frequency for ^{129}Xe was 110.7 MHz. Use a home-built continuous-flow apparatus to produce hyperpolarized ^{129}Xe gas by spin-exchange optical pumping method. The gas mixture consisting of 10 % N_2, 88 % He, and 2 % Xe (86 % enriched ^{129}Xe or natural abundance ^{129}Xe) and directly bubbled into a 10 mm NMR tube for 20 s, ^{129}Xe NMR spectra was obtained using a 10 mm double resonant probe (^{129}Xe and ^{1}H, PA BBO 400 W1/S2 BB-H-D-10Z) with rectangle pulse

of flip angle (90°). Approximately 20% of ^{129}Xe spin polarization was achieved. The sample temperature was set at 300 K controlled by VT unit on NMR spectrometer. For the hyper-CEST NMR experiment, nanoemulsion sample or cells sample was put into NMR tube and bubbled for 20 s following a delay of 3 s to ensure the bubbles to collapse before signal acquisition. Using a RF-pulse 5 s, 6.5 μT cw saturation for varying offset frequencies, the chemical shift of Xe range from 50 to 100 ppm、110 to 255ppm in 5 ppm steps and 100 to 110ppm in 2ppm.

For the ^{129}Xe hyper-CEST MRI, A549 cells and WI-38 cells were treated with NEs and NEs-RGD (NEs concentration = 26 pM) for 1 hour at 37 °C, followed by washed with PBS for 3 times and resuspended (5×10^6 /mL) in 2 mL PBS for the Hyper-CEST experiment (saturation: 5 s, 13 μT). MR images were acquired using RARE sequence (FOV = 2x2 cm; matrix size = 32x32; slice thickness = 30 mm; echo time = 6 ms, repetition time = 99.3 ms, RARE factor =16). The ^{129}Xe MR image was segmented using 0.2*maximum value as threshold and interpolated into 64*64 matrix.

8. H&E stains of NEs-RGD treated mice organs

To further investigate the toxicity of NEs-RGD on the organs of mice, the histopathological study was performed on the main organs (heart, liver, spleen, lung and kidney) both of the treatment and control groups, after injection 1 day (Figure S8).

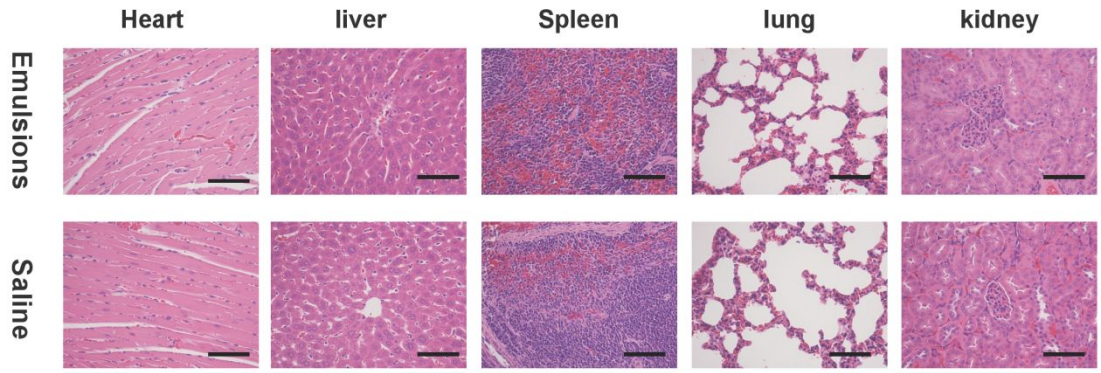

Figure S8. Haematoxylin and eosin (H&E) stains of heart, liver, spleen, lung and kidney from NEs-RGD and saline treated nude mice. Scale bar, 100 μm.