Supporting Information

^{68}Ga-labeled maleimide for blood pool and lymph PET imaging through covalent bonding to serum albumin *in vivo*

Lixia Feng[1], Jianyang Fang[1], Xinying Zeng[1], Huanhuan Liu[1], Jingru Zhang[1], Lumei Huang[1], Zhide Guo[1], Rongqiang Zhuang[1]*, Xianzhong Zhang[1]**

[1]State Key Laboratory of Molecular Vaccinology and Molecular Diagnostics & Center for Molecular Imaging and Translational Medicine, School of Public Health, Xiamen University, 4221-116 Xiang'An South Road, Xiamen 361102, China

***Correspondence to:**

Xianzhong Zhang, E-mail: zhangxzh@xmu.edu.cn;

Rongqiang Zhuang, E-mail: zhrq@xmu.edu.cn;

Zhide Guo, E-mail: gzd666888@xmu.edu.cn

1. Methods of HPLC

As the UV retention time of the precursor was very close to that of the labeled product, High-Performance Liquid Chromatography (HPLC) conditions and the retention time of the precursor were explored to distinguish the radioactive product from others.

Method 1: mobile phases were acetonitrile with 0.1% trifluoroacetic acid (A) and water with 0.1% trifluoroacetic acid (B), 5% A, 95% B for 0-10 min, 10% A, 90% B for 10-20 min, flow rate:1 mL/min, UV monitoring wavelength = 220 nm. Hypersil GOLD C18 selectivity HPLC column (25005-254630, 250×4.6 mm, 5 μm, Thermo Scientific, USA) was used.

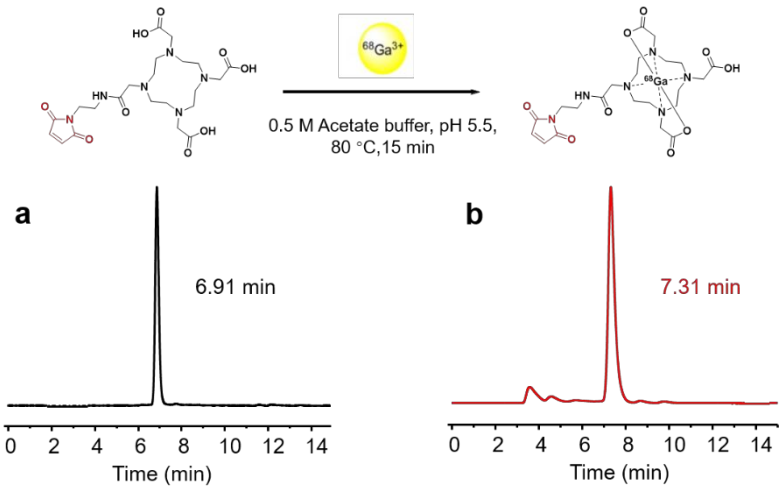

Figure S1. (a) UV-HPLC analysis of precursor DM and (b) radio-HPLC analysis of [^{68}Ga]Ga-DM

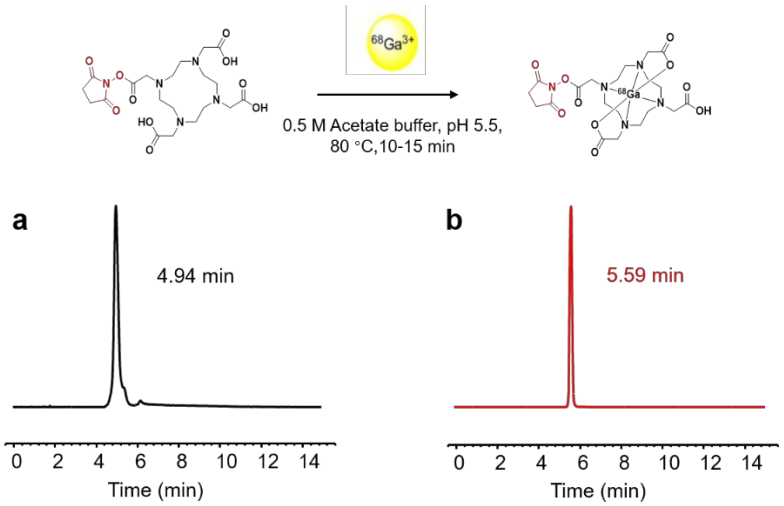

Figure S2. (a) UV-HPLC analysis of precursor DOTA-NHS and (b) radio-HPLC analysis of [^{68}Ga]Ga-DOTA-NHS

About 37 MBq/50 μL of purified tracer [^{68}Ga]Ga-DM was added to the albumin solution (HSA & BSA), co-incubated at 37 °C for 30 min, and transferred to an ultrafiltration centrifuge tube of 0.5 mL/30 kDa for separation and purification, centrifuged at 14,000 rpm, 4 °C for 15 min to obtain an albumin-bound tracer.

Method 2: mobile phase was 1×PBS solution with a pH of 7.4 (C), 100% C for 20 min, flow rate:1 mL/min, and UV monitoring wavelength = 278 nm. Acclaim SEC-300 size exclusion chromatography HPLC column (0797233, 300×4.6 mm, 5 μm, Thermo Scientific, USA) was used.

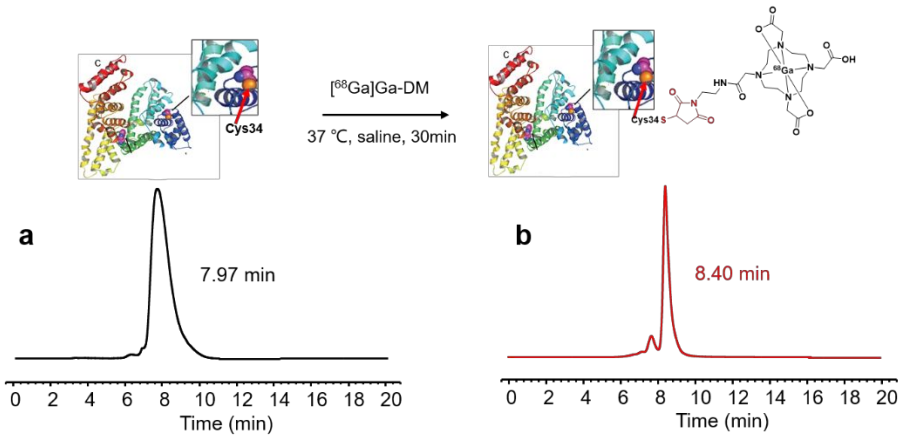

Figure S3. (a) UV-HPLC analysis of precursor HSA and (b) radio-HPLC analysis of [^{68}Ga]Ga-DM-HSA

2. Methods of iTLC

The R_f value of the albumin-bound tracer was 0–0.1, which was lower than the unbound [^{68}Ga]Ga-DM (R_f value was 0.7–0.9) and the free ^{68}Ga ion (R_f value was 0.9–1.0), so, the iTLC method was established to judge the stability of the tracer binding with albumin in saline and serum.

Method 3: the samples were loaded on an iTLC-SG strip (5×70mm) and 0.1 M sodium citrate solution (pH = 5.0) as the developing agent. The iTLC-SG strip was detected by a MiniScanTLC scanner.

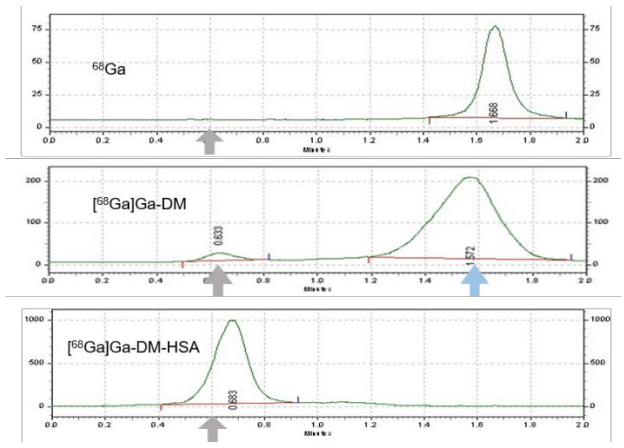

Figure S4. iTLC analysis of ^{68}Ga, [^{68}Ga]Ga-DM, and [^{68}Ga]Ga-DM-HSA

1

3. Methods of purification

Method 4: Tracer was co-incubated with HSA for several minutes. Samples were transferred to an ultrafiltration centrifuge tube of 0.5 mL/30 kDa for separation and purification, centrifuged at 14,000 rpm, 4 °C for 15 min to obtain the concentrated solution, and filtered solution represented the albumin-bound tracers, and unbound tracers, respectively. The radioactivity counts of the two parts were detected by the γ - counter and the radioactivity bound fraction was calculated as follows:

$$\text{Bound fraction} = \frac{\text{CPM}_{(\text{concentrated})}}{\text{CPM}_{(\text{concentrated})} + \text{CPM}_{(\text{filtered})}}$$

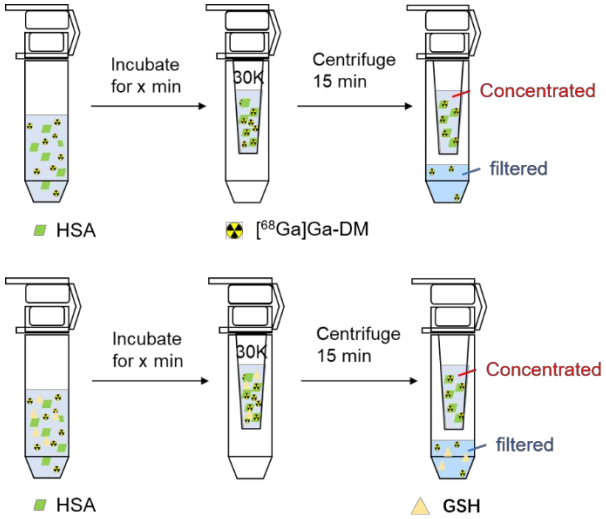

Figure S5. Diagram of purification

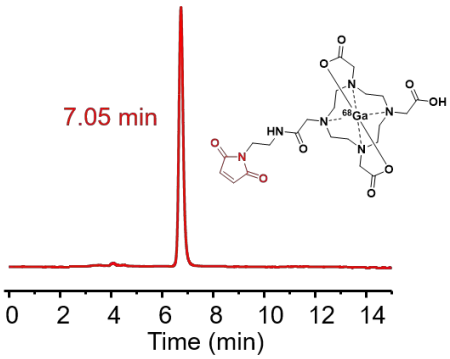

Figure S6. Radio-HPLC analysis of [^{68}Ga]Ga-DM injection

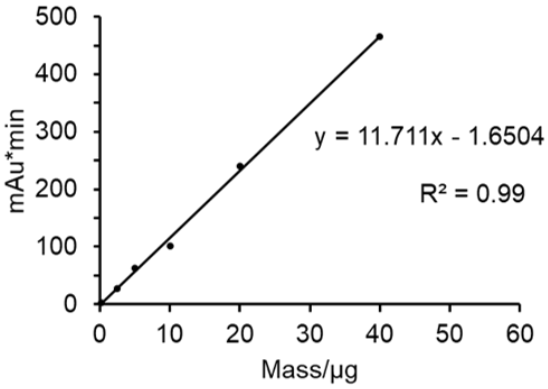

Figure S7. Ultraviolet standard curve for DM with a detection wavelength of 220 nm

4. Calculation of the covalent binding reaction rate

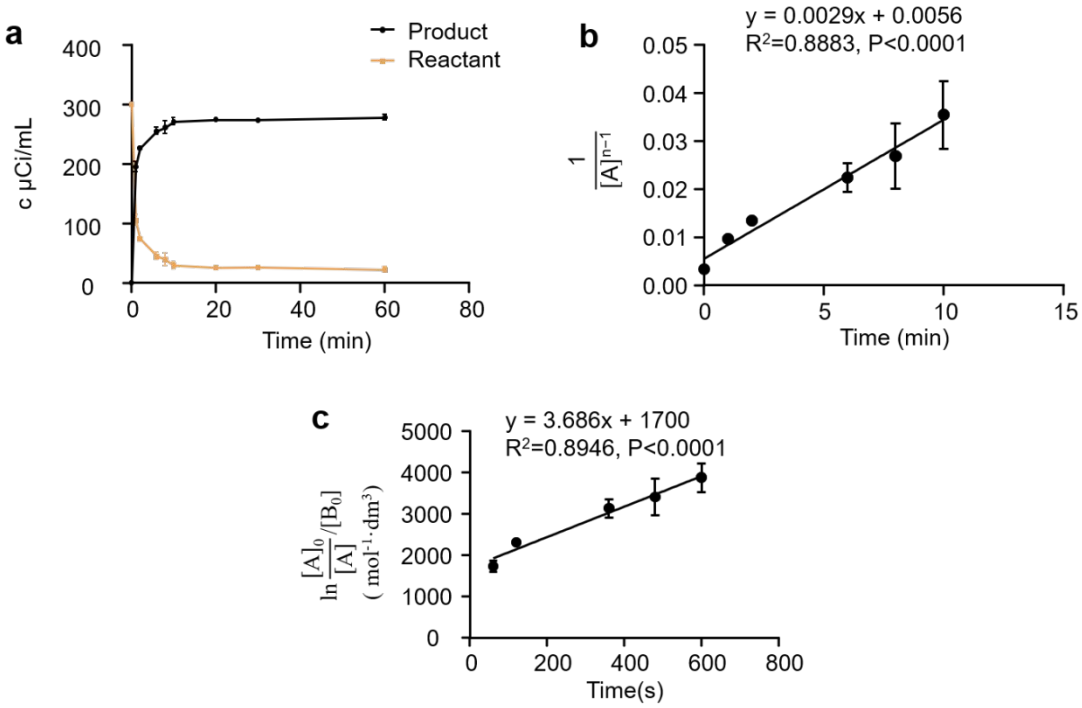

Figure S8. (a) Curves of reactant and product concentrations over time; (b) $\frac{1}{[A]^{n-1}}$-time diagram when the reaction order is 2; (c) $\ln\frac{[A]^0}{[A]}/[B^0]$-time chart

Consider the second-order reaction:

$$a[^{68}\mathrm{Ga}]\mathrm{Ga\text{-}DM} + b\mathrm{HSA} \rightarrow [^{68}\mathrm{Ga}]\mathrm{Ga\text{-}DM\text{-}HSA} \quad (\frac{[A]^0}{[B]^0} \neq \frac{a}{b})$$

Concentration [A] [B] → [P]

Initial concentration $[A]_0$ $[B]_0$ → $[P]_0$

The dose of reactant B is much higher than that of reactant A, so, $[B] \approx [B]^0$, the rate equation corresponds to:

$$k=\frac{1}{t}\ln\frac{[A]^0}{[A]}/[B]^0 \ \mathrm{mol^{-1}\cdot dm^3\cdot s^{-1}}$$

Serve $\ln\frac{[A]^0}{[A]}/[B]^0$ as y and t as x to obtain the linear ship as shown in Figure S8c, therefore, the covalent binding reaction rate K is about 3.69 $\mathrm{mol^{-1}\cdot dm^3\cdot s^{-1}}$.

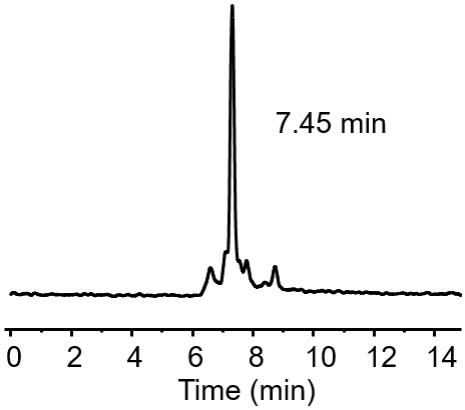

Figure S9. Radio-HPLC analysis of urine at 5 min after administration

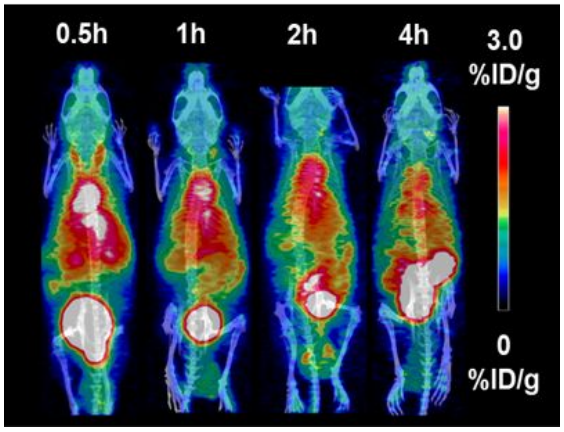

Figure S10. Representative PET-CT images of [^{68}Ga]Ga-DM-BSA

Table S1. Uptake ratio of blood/organs at selected time points

Time (min)	Blood /organ (Mean ± SEM, n=4)			
	Heart	Liver	Lung	Kidney
2	2.72 ± 0.15	3.77 ± 0.10	1.16 ± 0.09	0.50 ± 0.10
5	3.72 ± 0.23	4.67 ± 0.28	1.72 ± 0.07	0.97 ± 0.27
30	4.28 ± 0.13	3.84 ± 0.67	1.78 ± 0.12	1.54 ± 0.32
60	2.64 ± 0.68	3.22 ± 0.78	1.51 ± 0.42	1.63 ± 0.44
120	3.16 ± 0.38	3.70 ± 0.32	1.65 ± 0.17	1.97 ± 0.12
180	3.35 ± 0.21	3.19 ± 0.23	1.88 ± 0.17	1.71 ± 0.03 *

* n=3

5. Gel electrophoresis and autoradiography analysis of [^{68}Ga]Ga-DM cultured plasma

Plasma protein concentration was measured at 79 mg/mL via bicinchoninic acid assay (BCA)[1], 4.9 MBq of [^{68}Ga]Ga-DM was incubated with mouse whole blood with anticoagulant for 30 min, then, centrifuged at 14000 rpm for 10 min to obtain a clear plasma sample. The sample was diluted 20 times, then, 10 μL of it was taken for protein electrophoresis, and commercially obtained BSA and mouse serum protein were used as controls. After electrophoresis, the radioactive signal was localized by autoradiography of the gel. Gel electrophoresis was carried out at 80 V for 30 min, and 120 V for 1.5 h. Exposure duration was 30 min for autoradiography.

The results of gel electrophoresis were shown in **Figure S11a**, each protein can be well separated. Albumin in plasma samples can be localized by the electrophoretic band of BSA (1, 6), where the molecular weight was between 75 kDa and 63 kDa. Compared to the electrophoretic band of mouse serum protein (3, 4), [^{68}Ga]Ga-DM co-incubation with blood did not change the properties of plasma proteins. The results of gel autoradiography were shown in **Figure S11b.** There were only two radioactive signals, one with molecular weights between 75 kDa and 63 kDa (I, III), and another with molecular weights less than 17 kDa (II, IV). The quantitative analysis data were shown in **Table S2.** Fusion of coomassie brilliant blue staining and autoradiography (**Figure S11c**) showed that 75% of the agent was bound to albumin.

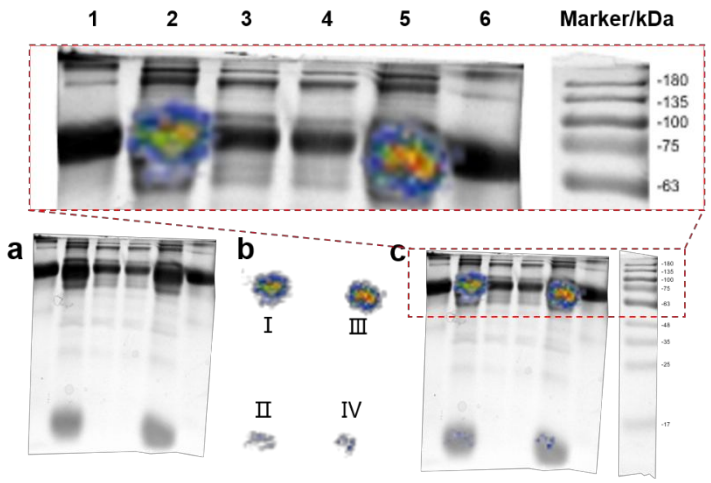

Figure S11. (a) Coomassie brilliant blue staining after gel electrophoresis; (b) Autoradiography after gel electrophoresis; (c) Merge of coomassie brilliant blue staining and autoradiography. Lanes 1, 6 represent commercially obtained BSA; Lanes 2, 5 represent diluted plasma samples; Lanes 3,4 represent mouse serum protein.

Table S2. Quantitative analysis data of gel autoradiography

No.	DLU /mm2	Percentage (%)
□	**61622.83 ± 2755.40**	**75.16 ± 1.08**
□	**20356.20 ± 861.71**	**24.84 ± 1.08**
□	**76008.97 ± 3949.14**	**75.73 ± 0.36**
□	**24347.23 ± 841.43**	**24.27 ± 0.36**

References

(1) Arslan A, Duman H, Kaplan M, Uzkuç H, Bayraktar A, Ertürk M, Alkan M, Frese SA, Duar RM, Henrick BM, Karav S. Determining Total Protein and Bioactive Protein Concentrations in Bovine Colostrum. J Vis Exp. **2021** Dec 10;(178). doi: 10.3791/63001.