Supporting Information for

ORIGINAL ARTICLE

Legumain-mediated self-assembly of ^{131}I-labelled agent for targeted radiotherapy of tumor

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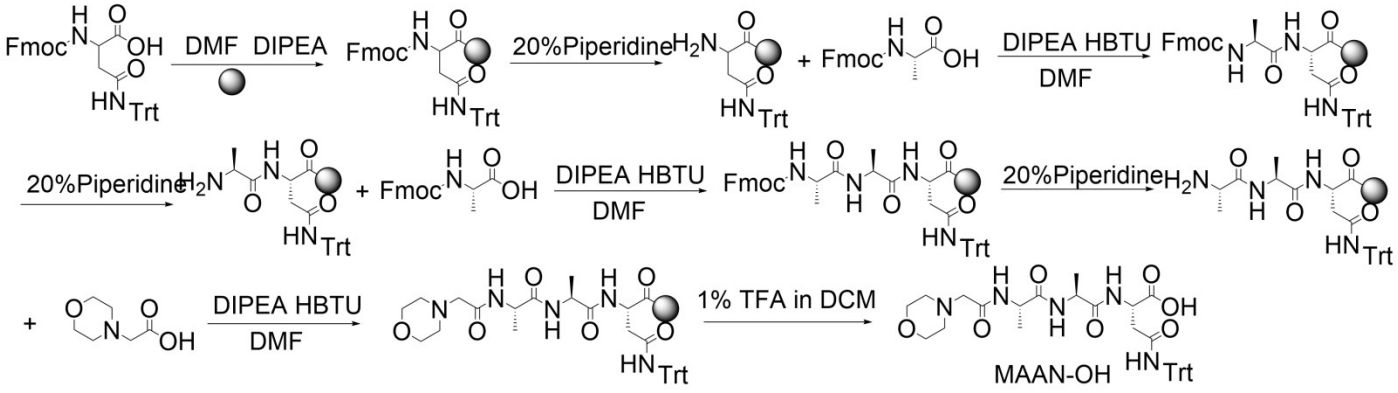
1. Synthesis of non-radioactive agent [^{127}I]MAAN.

The synthesis of compound 1 (230 mg, 0.22 mmol) was similar to compound JG03 reported in our previous work[1]. Compound 1 was dissolved in dichloromethane (DCM) and trifluoroacetic acid (TFA) (v/v, 1:1) containing 2-3% triisopropyl silane (TIPS) to remove the Trt and Boc protecting group. After stirring at 25℃ for 0.5 h, the solution was evaporated under reduced pressure, and the crude product was precipitated with diethyl ether. Then compound 2 was obtained after centrifugation (4000r/min) for 5 min and quickly dissolved in CH_3OH containing 2-3% TIPS. Meanwhile SEt (1.2 eq) was added to the solution to react at 25 °C for 1 h under nitrogen atmosphere, and compound 3 was acquired by precipitating with diethyl ether. Compound 3 (149 mg, 0.20 mmol), MAAN-OH (142 mg, 0.22 mmol) and HBTU (88 mg, 0.23 mmol) were dissolved in dry tetrahydrofuran (THF). Subsequently, DIPEA (82 μL, 0.50 mmol) was added to the reaction mixture to adjust the pH to 8-9 and reacted at 25 ℃ under nitrogen atmosphere for 3 h. MAAN-OH was prepared by solid phase peptide synthesis (SPPS). Afterwards, the crude product of compound 4 was obtained by precipitating with diethyl ether. And then compound 4 (233 mg, 0.17 mmol) was dissolved by 5% piperidine (2 mL), and then reacted for about 15 min under nitrogen atmosphere and ice bath to remove the Fmoc protecting group. Then 1M HCl (2 mL) was added drop by drop to adjust pH to about 4-5. Afterwards, the crude product was purified with preparative HPLC to yield the desired compound 5. Solid compound 5 (57 mg, 0.05 mmol) was prepared by removing water using the freeze dryer. After lyophilization, compound 5, PHPAA (9 mg, 0.055 mmol), and HBTU (22 mg, 0.058 mmol) were added to the reaction flask in turn and then were dissolved by dry THF. After adjusting the pH with DIPEA (21 μL, 0.13 mmol) to 8-9, the reaction solution was stirred at 25 ℃ for 1 h under nitrogen atmosphere to produce the compound 6

(52 mg, 0.04 mmol). Subsequently, the Trt protecting group of compound 6 was cleaved with 50% TFA in DCM at 25 °C for 0.5 h. The mixture was evaporated under reduced pressure and precipitated from the solution with cold diethyl ether. The crude product MAAN was separated by centrifugation. The pure compound MAAN (31 mg, 0.03 mmol) was obtained after preparative HPLC purification. Finally, MAAN (0.1 mg, 1×10^{-4} mmol) reacted with KI solution (0.036 mg, 2.2×10^{-4}) and iodogen (0.08 mg, 1.9×10^{-4}) attached to the centrifugal tube at 25 °C for 3 min and the nonradioactive agent [^{127}I]MAAN was obtained (yield: 34 %).

^{1}H NMR of MAAN (400 MHz, DMSO-d_6): δ (ppm) 10.51 (s, 1H), 8.79 (d, J = 7.1 Hz, 1H), 8.74 (d, J = 2.0 Hz, 1H), 8.31 (t, J = 3.0 Hz, 1H), 8.29 (s, 1H), 8.20 (d, J = 8.9 Hz, 2H), 8.13 (d, J = 7.6 Hz, 1H), 8.09 (d, J = 7.5 Hz, 1H), 7.93 (t, J = 5.6 Hz, 1H), 7.80 (dd, J = 9.1, 2.2 Hz, 1H), 7.49 – 7.43 (m, 1H), 7.03 (d, J = 2.3 Hz, 1H), 7.02 – 6.98 (m, 2H), 6.70 – 6.61 (m, 2H), 4.59 – 4.21 (m, 6H), 4.03 – 3.87 (m, 4H), 3.77 (d, J = 5.7 Hz, 4H), 3.23 (s, 2H), 3.15 (dd, J = 13.5, 4.2 Hz, 2H), 3.08 – 2.83 (m, 4H), 2.68 (q, J = 7.3 Hz, 2H), 2.62 – 2.52 (m, 2H), 1.84 – 1.60 (m, 2H), 1.48 – 1.16 (m, 14H).

^{13}C NMR of MAAN (101 MHz, DMSO-d_6): δ (ppm) 172.54, 172.03, 171.78, 171.75, 170.98, 169.13, 163.99, 156.27, 148.18, 139.76, 137.12, 135.56, 130.28, 127.04, 125.26, 121.37, 115.40, 114.02, 111.94, 63.50, 56.97, 54.08, 52.85, 52.30, 50.22, 48.82, 42.67, 42.03, 40.76, 38.91, 37.49, 32.05, 31.90, 29.28, 23.33, 18.55, 18.37, 14.71.



Scheme S1. Synthesis route of peptide MAAN-OH.

Scheme S2. Synthesis route of non-radioactive compound [^{127}I]MAAN.

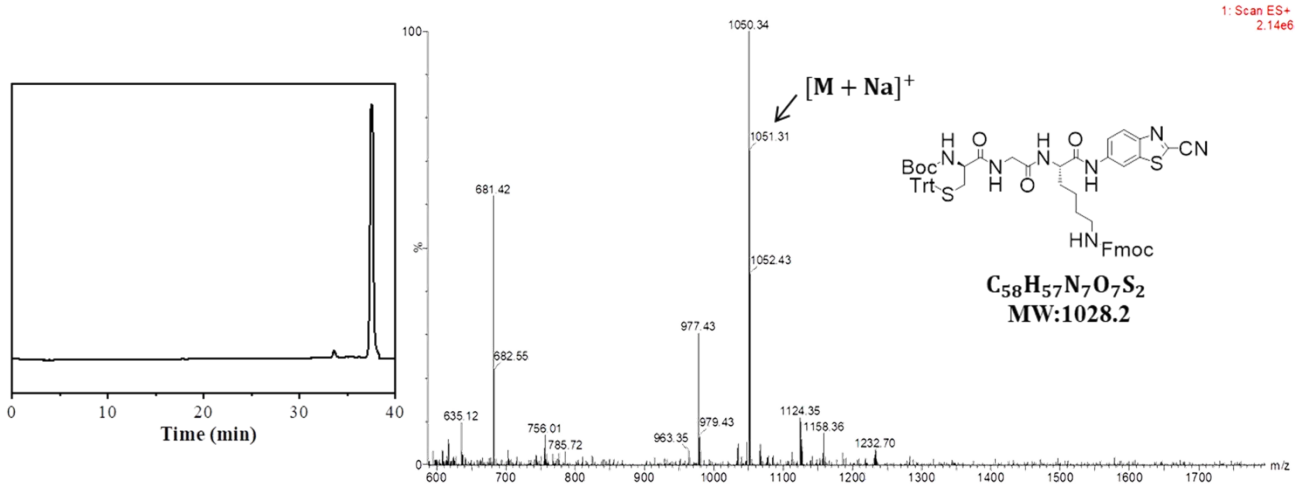


Figure S1. HPLC and ESI-MS analysis of compound 1.

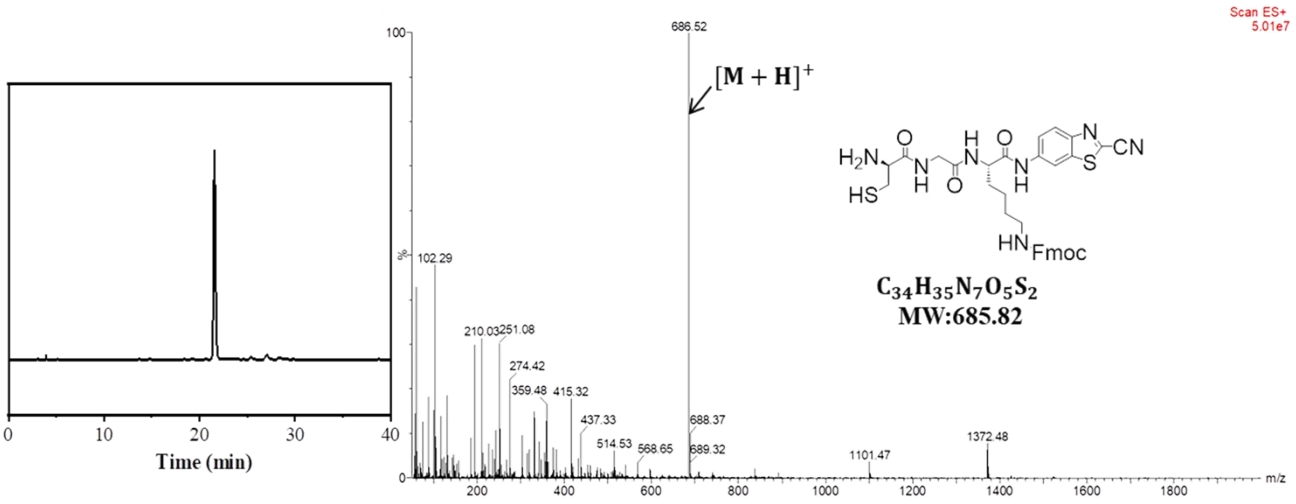


Figure S2. HPLC and ESI-MS analysis of compound 2.

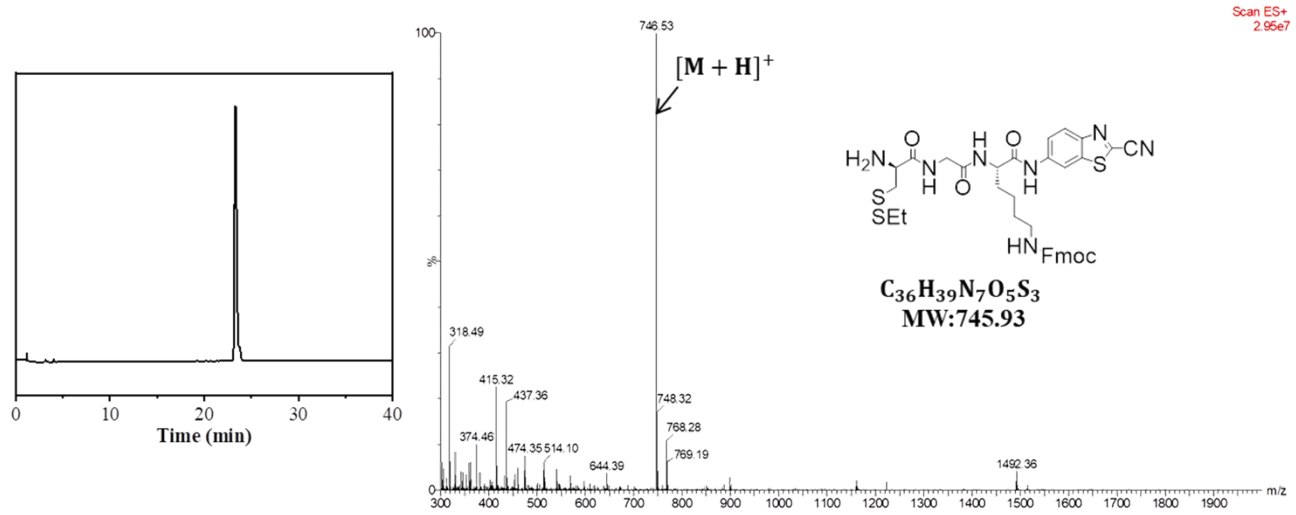


Figure S3. HPLC and ESI-MS analysis of compound 3.

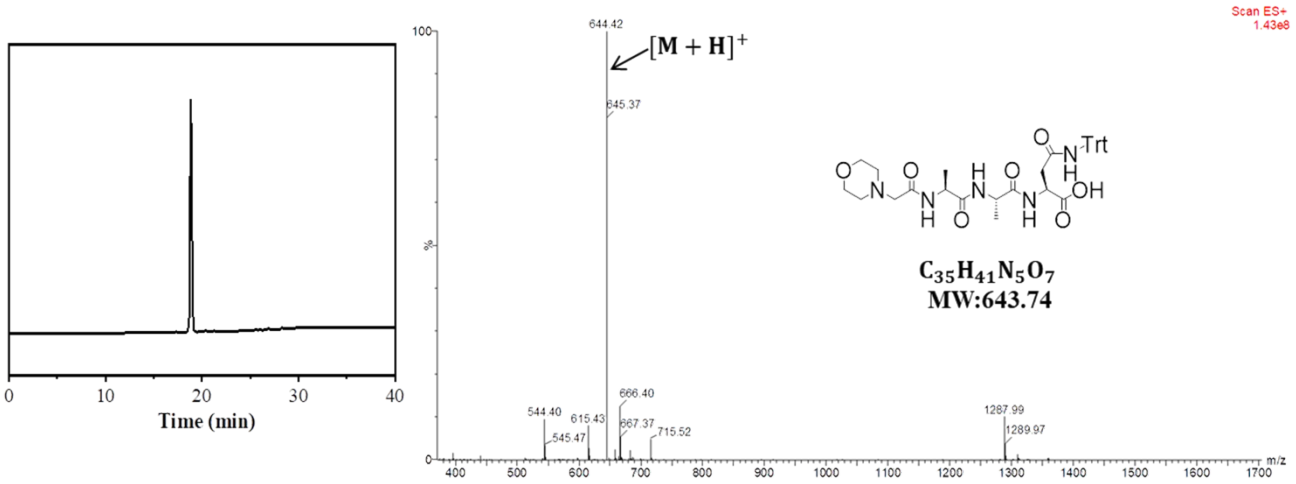


Figure S4. HPLC and ESI-MS analysis of MAAN-OH.

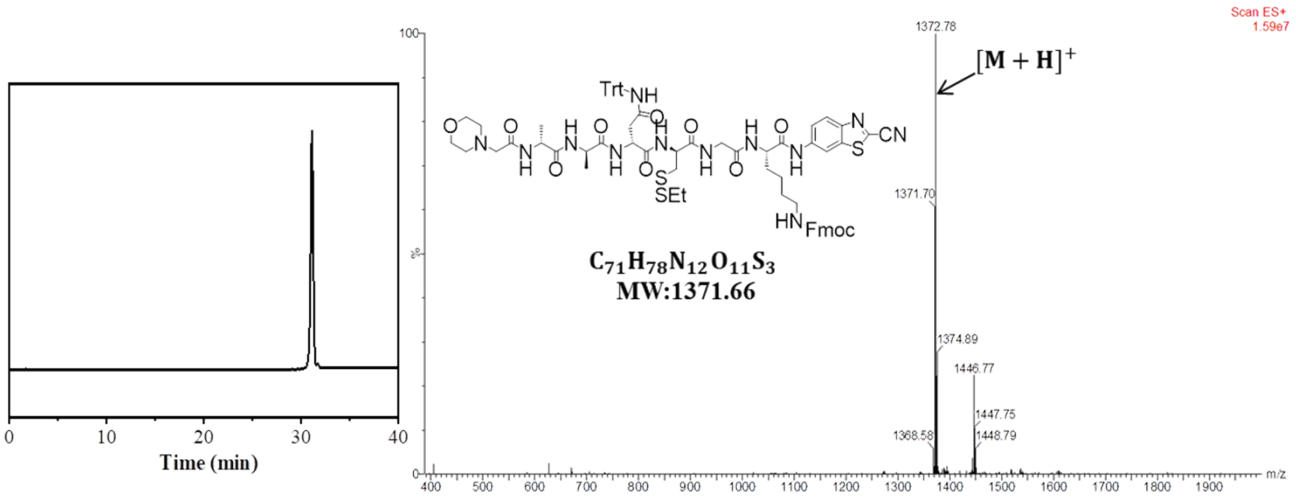


Figure S5. HPLC and ESI-MS analysis of compound 4.

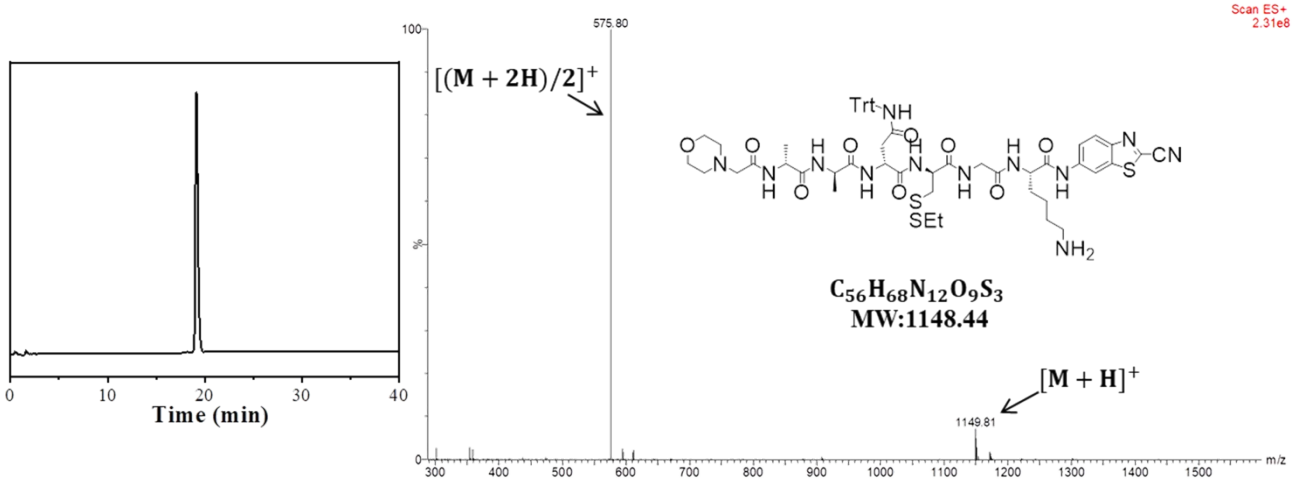


Figure S6. HPLC and ESI-MS analysis of compound 5.

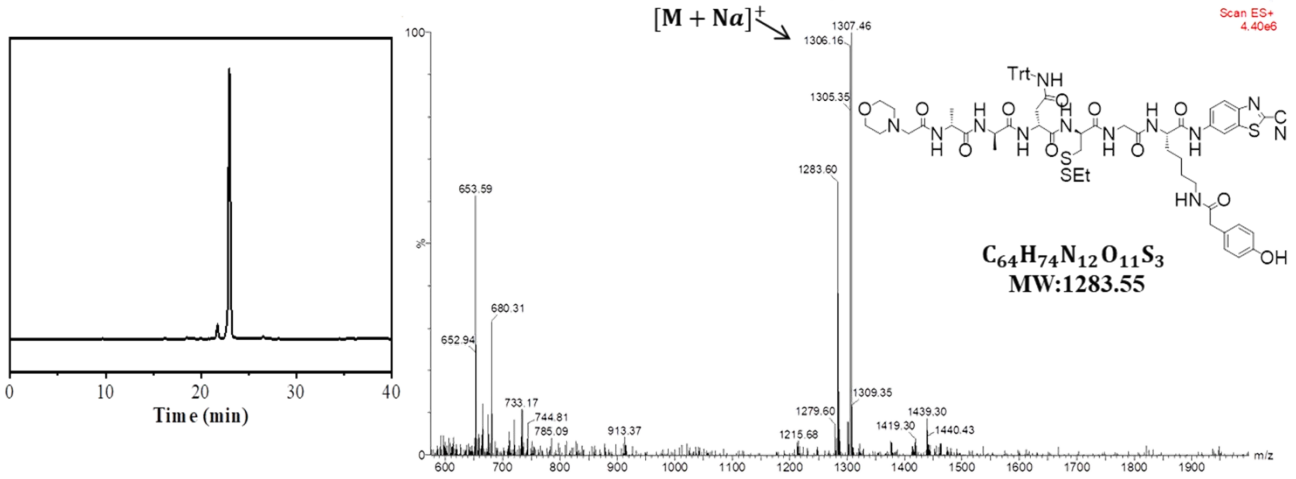


Figure S7. HPLC and ESI-MS analysis of compound 6.

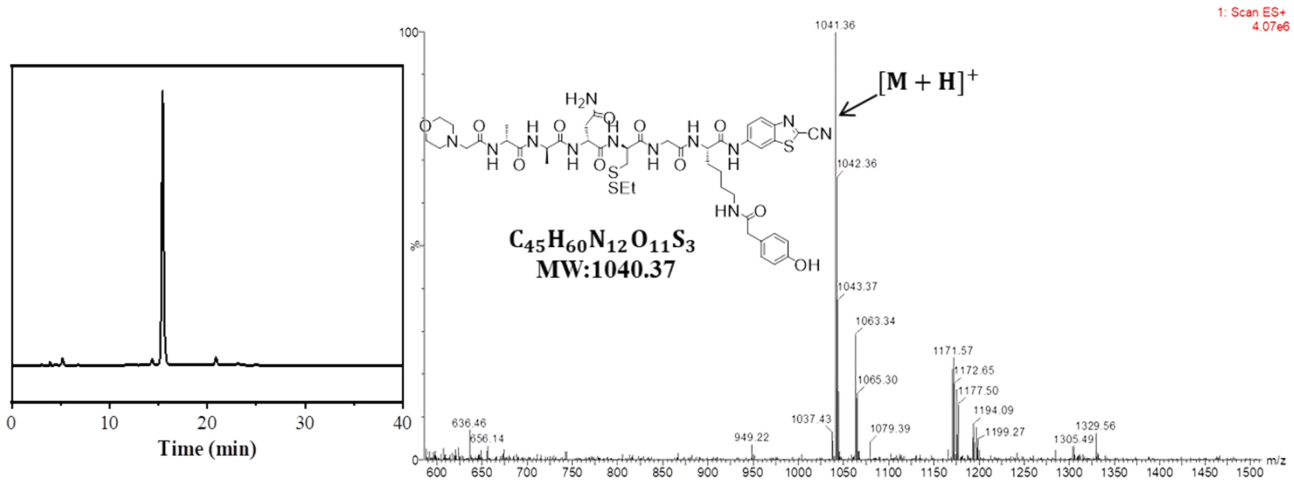


Figure S8. HPLC and ESI-MS analysis of MAAN.

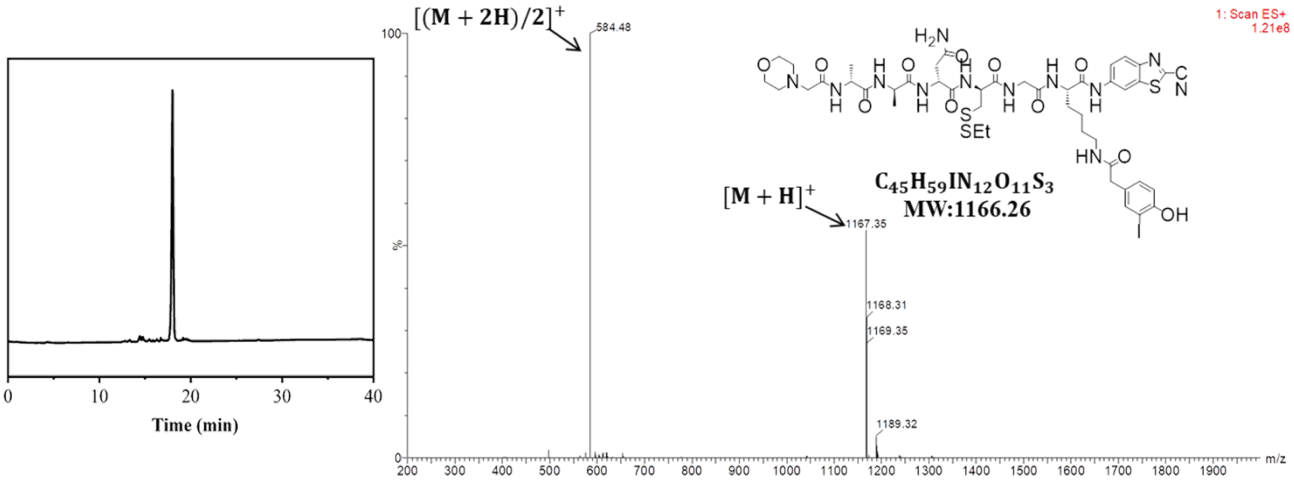


Figure S9. HPLC and ESI-MS analysis of [^{127}I] MAAN.

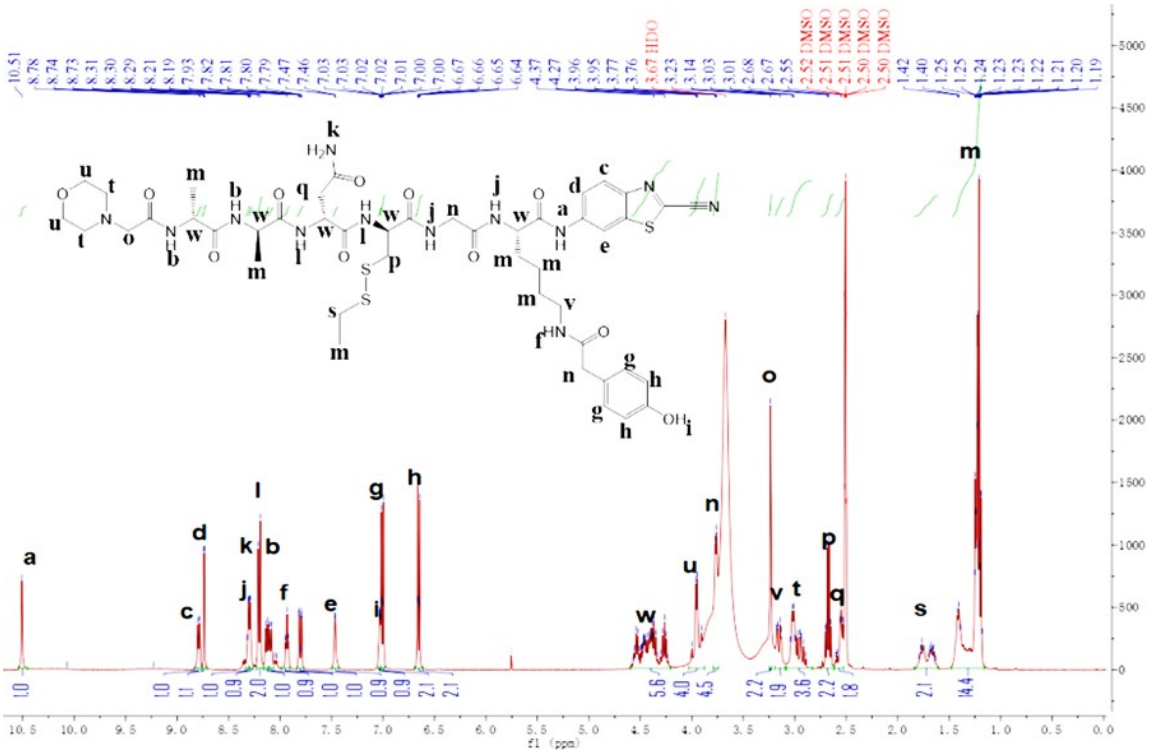


Figure S10. ^{1}H-NMR of precursor MAAN.

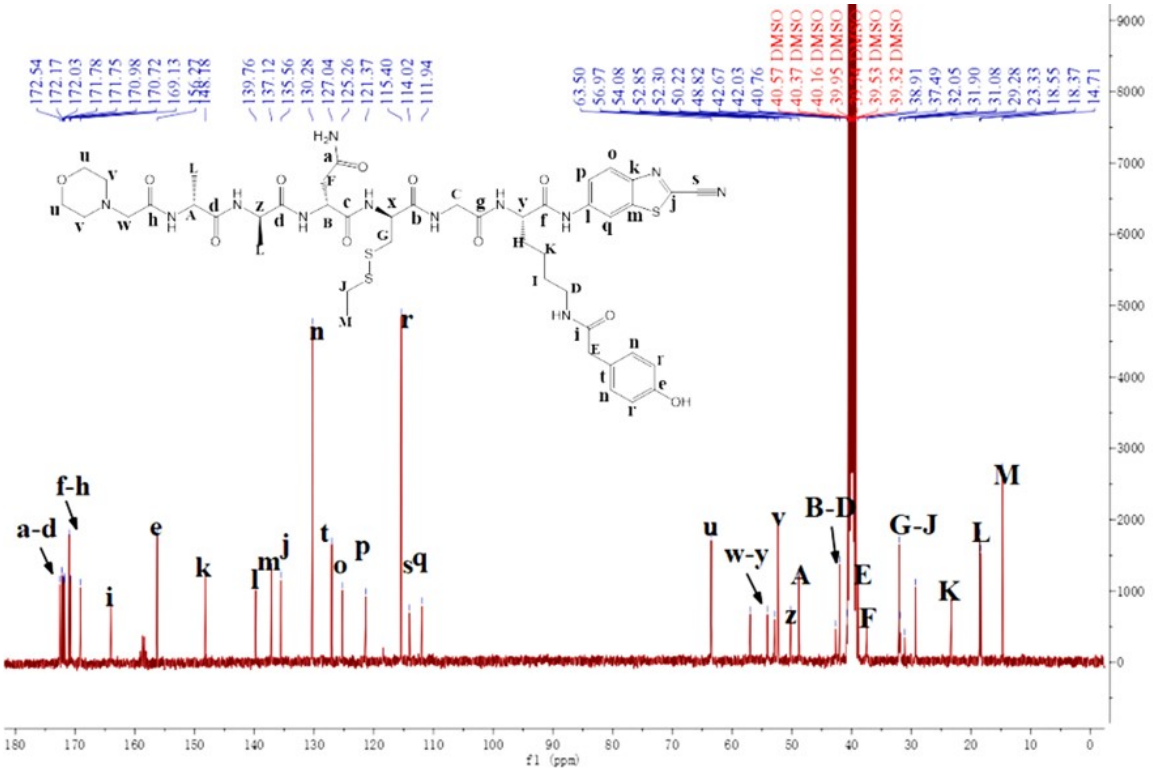


Figure S11. ^{13}C-NMR of precursor MAAN.

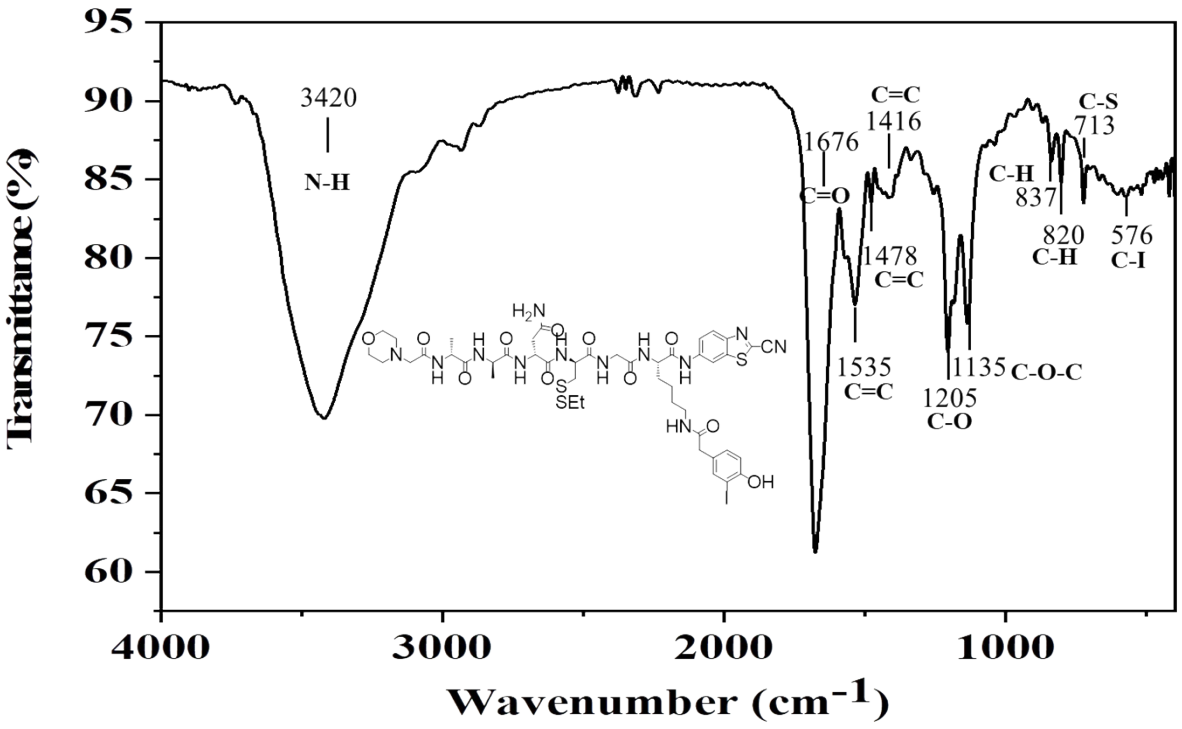


Figure S12. Fourier Transform Infrared Spectra analysis of [^{127}I]MAAN.

2. Radiosynthesis of [^{131}I]MAAN.

2.1 Optimization of Radiolabeling Conditions.

The radioactive agent [^{131}I]MAAN was synthesized using the traditionally iodogen labelling method. Iodogen is an oxidant reagent, which is soluble in dichloromethane but insoluble in water. As a result, we

dissolved iodogen in DCM and blow-dried it with nitrogen atmosphere to make it adhere to the inner wall of the tube for subsequent marking experiments. First, to identify the optimal dosage of iodogen and reaction time, MAAN (30 μg) was reacted with different dosage of iodogen (20, 15, 10 and 7.5 μg) and [^{131}I]NaI (37 MBq) in 40 μL reaction solution volume at 20 ℃ sequentially. A small amount of reaction liquid was taken out for radio-HPLC analysis to determine the conversion rate of [^{131}I]MAAN at various time point (1, 2, 3, 5 and 7 min). Subsequently, the influence of temperature and reaction time on the conversion rate was also studied with the optimized quantity of iodogen.

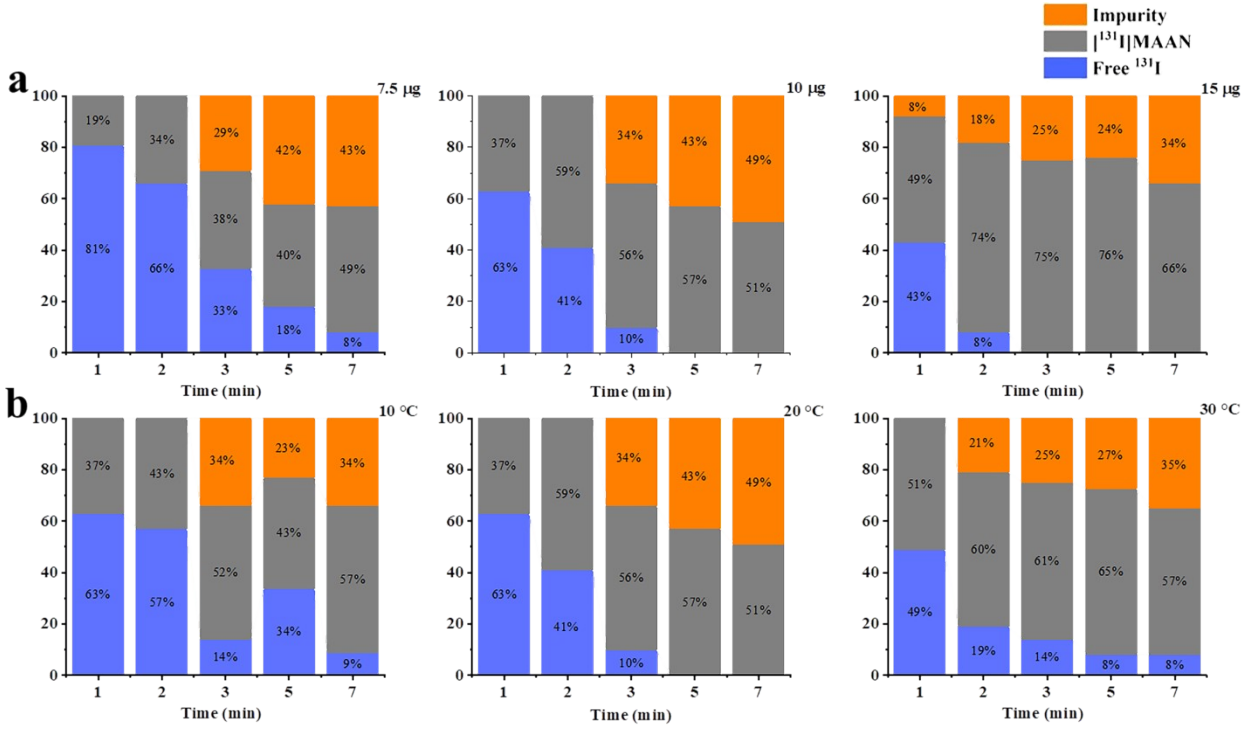


Figure S13. Optimization of radiolabeling conditions. (a) Effect of iodogen dosage and reaction time on the radiolabeling yield. (b) Effect of reaction temperature and time on the radiolabeling yield.

2.2 Measurement of molar activity.

At first, five different standards (20 μL) of reference substance with a series of identified concentration were used to establish a standard curve by using HPLC-UV detector. Then, the purified probe (20 μL) was injected to analytical HPLC with UV detection. The amount of carrier MAAN in above solution was determined by the standard curve and subsequently the amount of [^{131}I]MAAN can be counted. Finally, the molar activity of [^{131}I]MAAN is obtained by dividing the radioactivity (MBq) of the [^{131}I]MAAN by the amount of its substance (nmol).

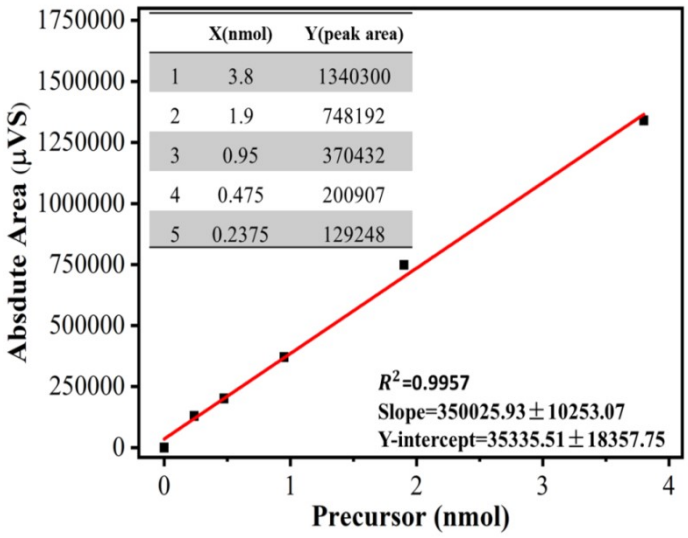


Figure S14. Standard curve for the absorbance of precursor MAAN at different concentration.

3. Legumain-controlled self-assembly and enzyme kinetics study.

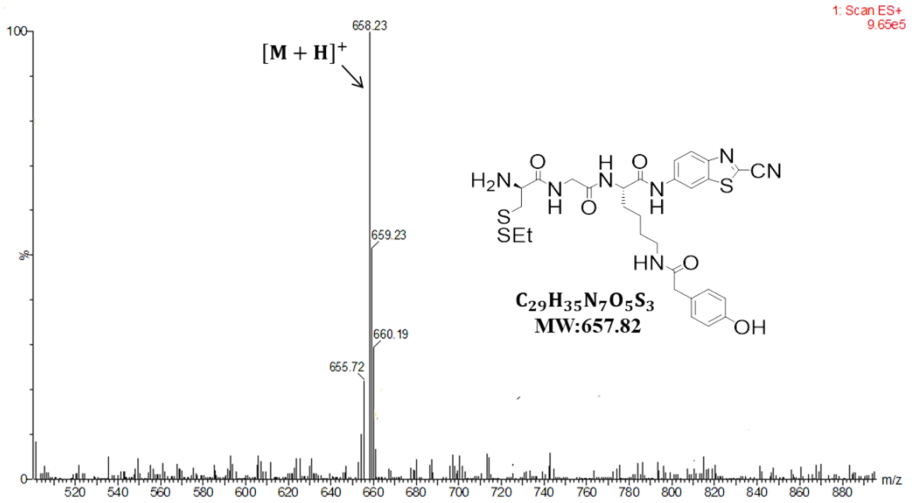


Figure S15. ESI-MS of compound MAAN-Cleaved.

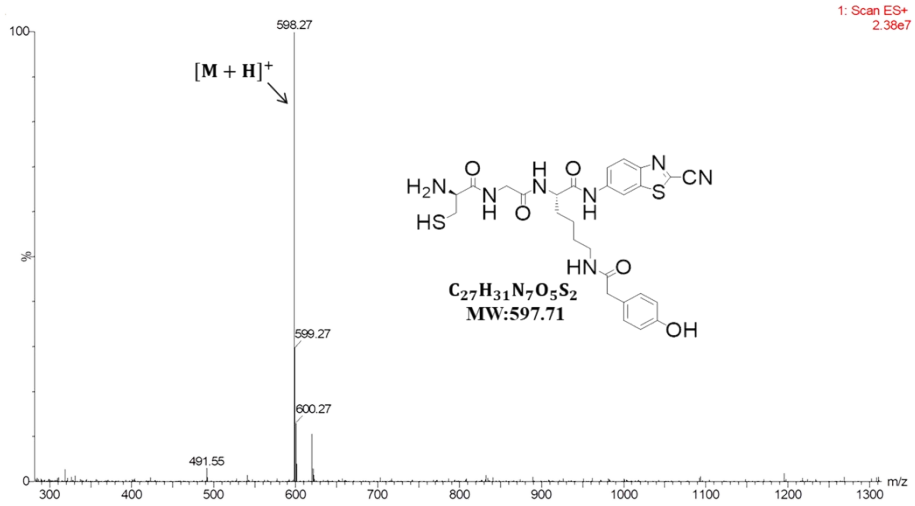


Figure S16. ESI-MS of compound MAAN-Core.

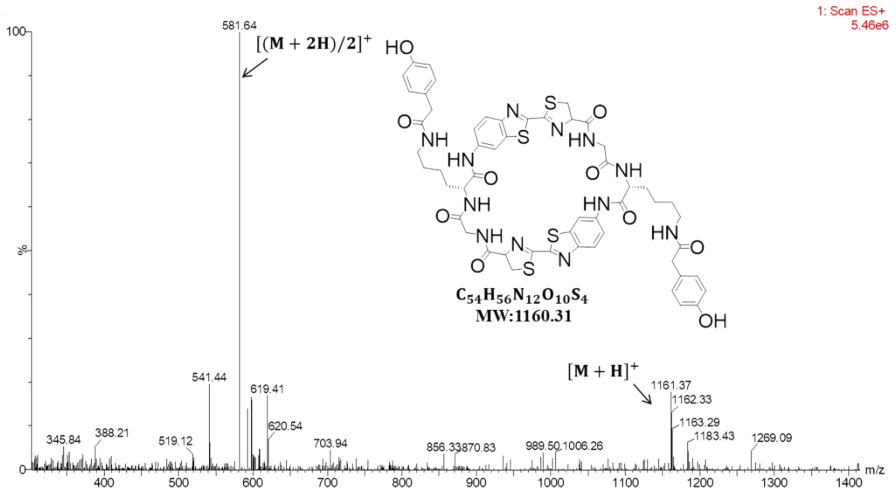


Figure S17. ESI-MS of compound MAAN-Dimer.

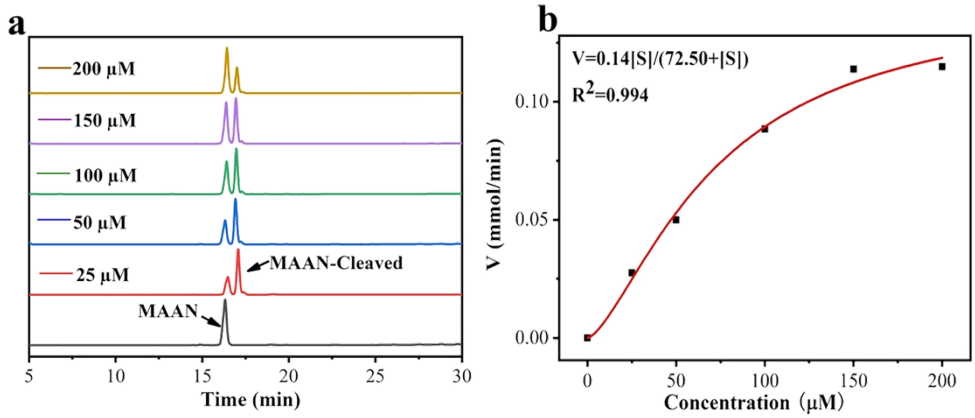


Figure S18. Enzyme kinetics of MAAN towards legumain. (a) HPLC trace of MAAN at different concentration (25-200 μM) incubated with legumain (1 ng/μL) at 37 °C for 1 h. (b) Michaelis -Menton plot of MAAN towards legumain.

4. Confocal fluorescence imaging.

First, HCT116 or SKOV3 cells were incubated with 40 μM MAAN for 3 h. Then, the medium was removed and cells were further incubated with 1 mL fresh DMEM containing 1 μL Lyso tracker to confocal dish for 10 min. After washing with PBS for three times, 1 mL PBS was added to confocal dish to perform confocal fluorescence imaging.

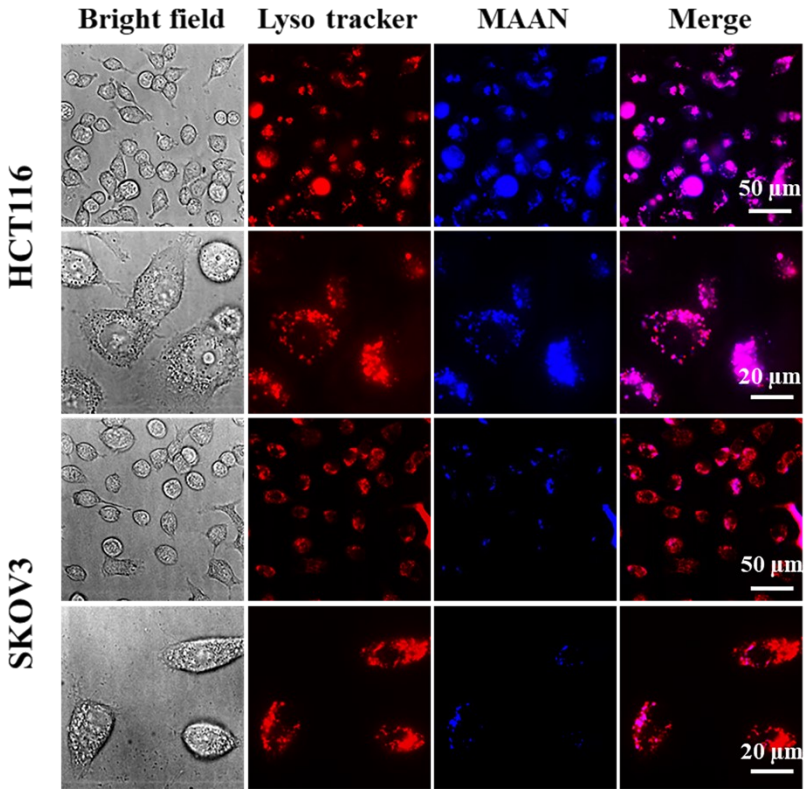


Figure S19. Confocal imaging of HCT116 and SKOV3 cells incubated with MAAN.

5. *In vitro* Cerenkov imaging of [^{131}I]MAAN.

Different concentration of [^{131}I]MAAN (0, 0.4, 0.9, 1.8, 3.7, and 7.4 MBq) was dispersed in 200 μL PBS and then added into a 96-well plate. Cerenkov imaging was conducted with an IVIS Spectrum (Perkin Elmer, Wellesley, MA, USA). Similarly, Cerenkov imaging of [^{131}I]NaI was also carried out as the control group.

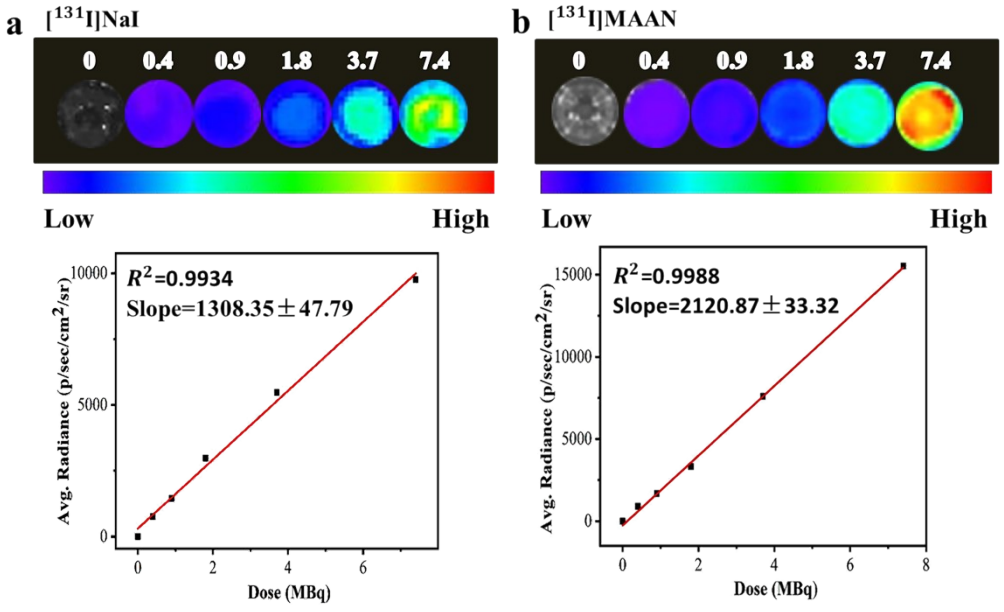


Figure S20. Cerenkov images and average radiance of (a) [^{131}I]NaI and (b) [^{131}I]MAAN at various dose (0, 0.4, 0.9, 1.8, 3.7 and 7.4 MBq).

6. *In vivo* Cerenkov imaging of [^{131}I]MAAN and [^{131}I]NaI.

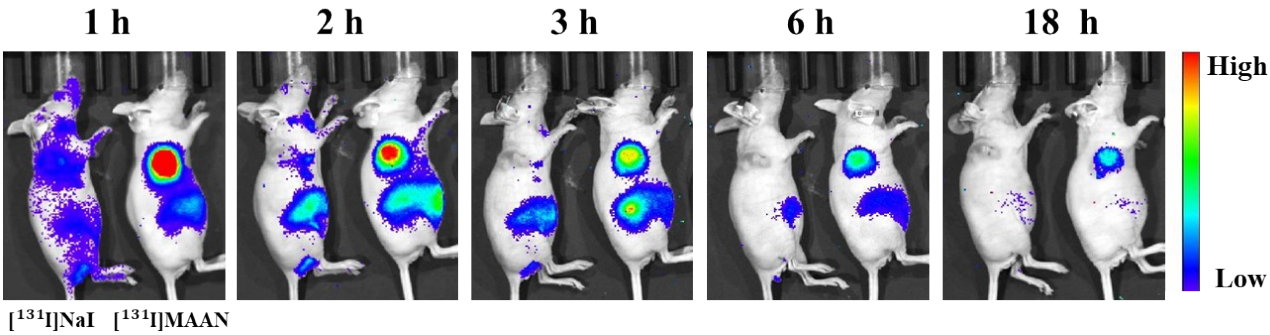


Figure S21. Cerenkov images of HCT116-bearing mice after intratumor injection of [^{131}I]NaI (left) and [^{131}I]MAAN (right) from 1 h to 18 h.

7. Toxicity of TRT in normal organs.

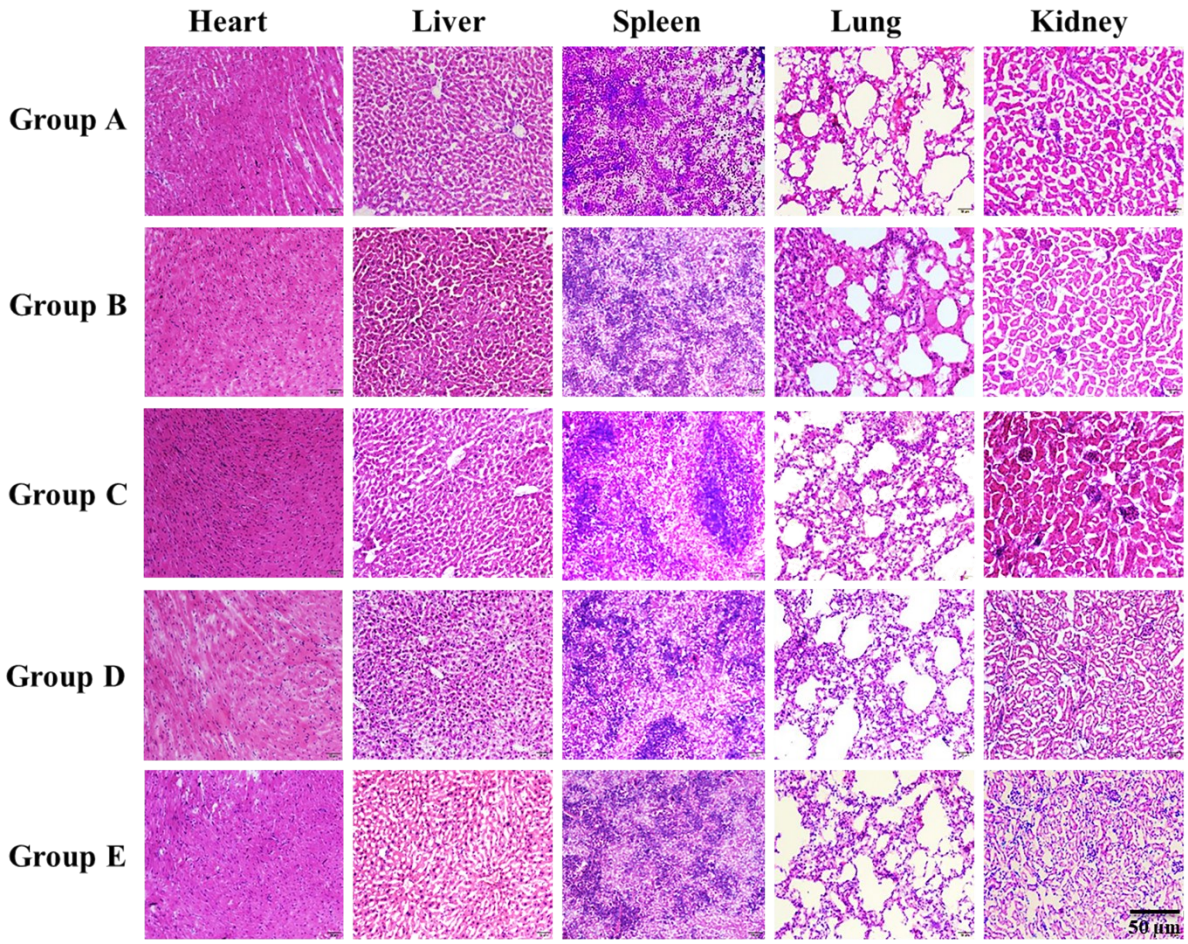


Figure S22. H&E staining of main normal organs (heart, liver, spleen, lung and kidney) after treatment in the group A-E. Scale bar: 50 μm.

8. Western blot image

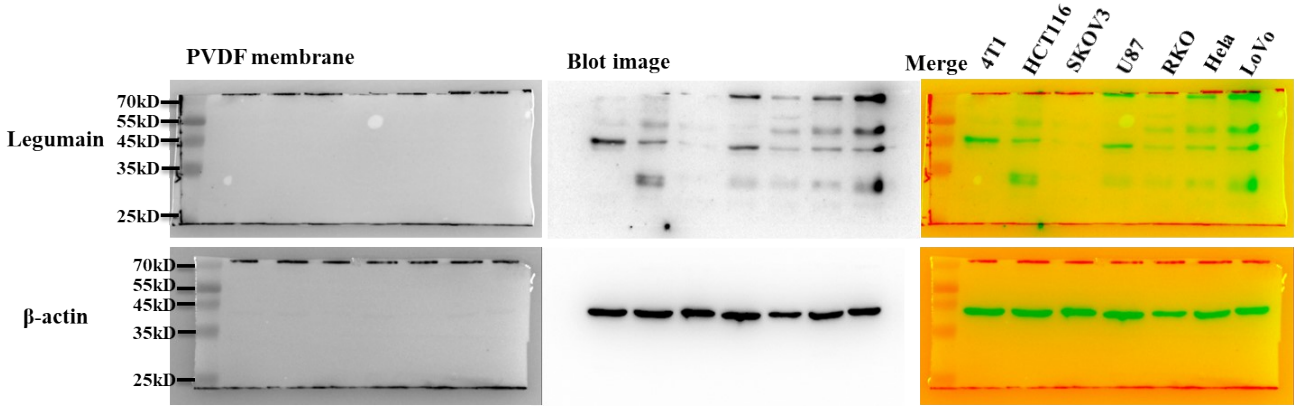


Figure S23. The uncropped and unprocessed western blot image.

9. Red blood cell hemolysis assay

The *in vitro* hemolysis assay was performed by the co-incubation of [^{127}I]MAAN with red blood cells to further evaluate the biocompatibility of the polymeric micelles. Briefly, red blood cells were acquired by the centrifugation (5000 r for 5 min) of fresh rat blood, followed by adding PBS to obtain a 2% red blood cell suspension. Then, the resulting red blood cell suspension (200 μL) was incubated with [^{127}I]MAAN solution (800 μL) at different concentrations (20, 40, 60, 80, 100 μg mL $^{-1}$) at 37 °C for 4 h. The negative control and positive control were designed by the co-incubation of saline (200 μL) or H_2O (200 μL) with the red blood cell suspension (800 μL). Finally, the incubation fluid was centrifuged (5000 r for 3 min), and the supernatant (200 μL) was removed for the measurement of the optical absorbance at 570 nm using a microplate reader. The hemolysis ratio was measured according to the formula: hemolysis ratio (%) = (absorbance of sample – absorbance of negative control)/(absorbance of positive control − absorbance of negative control) × 100%.

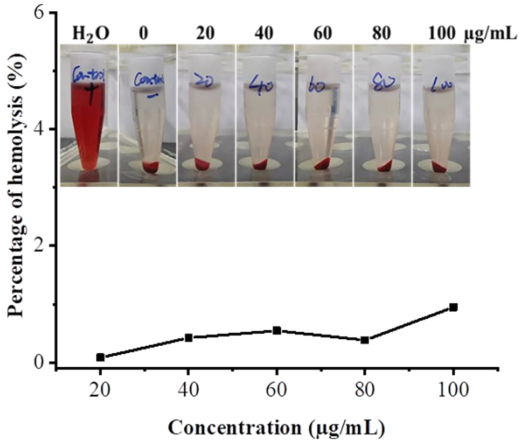


Figure S24. The hemolysis assay of [^{127}I]MAAN at various concentration solution varying from 20 to 100 μg mL $^{-1}$. Red blood cells incubated with saline and H_2O were used as the negative (−) and positive (+) controls.

Table S1. HPLC conditions for the analysis of each compound.

Time (minute)	Flow (mL/min)	H_2O% (0.1%TFA)	CH_3CN% (0.1%TFA)
0	1.0	80	20
3	1.0	80	20
35	1.0	10	90
40	1.0	80	20

Table S2. HPLC conditions for the purification of compound **5**.

Time (minute)	Flow (mL/min)	H_2O% (0.1%TFA)	CH_3CN% (0.1%TFA)
0	3.0	80	20
3	3.0	80	20
15	3.0	55	45
20	3.0	50	50
25	3.0	47	53
30	3.0	5	95
35	3.0	80	20

Table S3. HPLC conditions for the purification of **MAAN**.

Time (minute)	Flow (mL/min)	H_2O% (0.1%TFA)	CH_3CN% (0.1%TFA)
0	3.0	80	20
3	3.0	80	20
13	3.0	50	50
18	3.0	47	53
26	3.0	40	60
31	3.0	5	95
35	3.0	80	20

References

1. L. Qiu, K. Li, W. Dong, Y. Seimbille, Q. Liu, F. Gao and J. Lin, *ACS Nano*, 2021, **15**, 18250−18259.