Supporting Information

Harnessing Frémy's Salt for Tyrosine-Directed Bioconjugations

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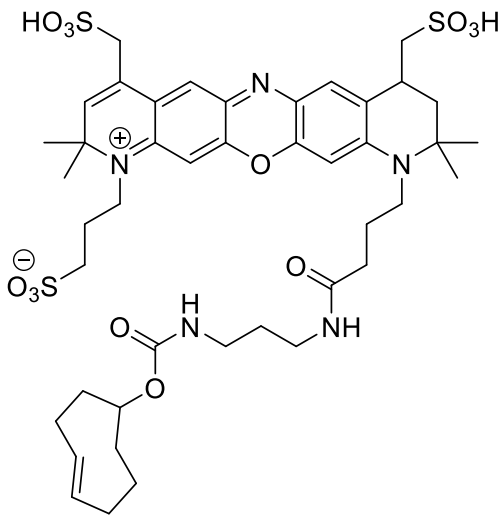
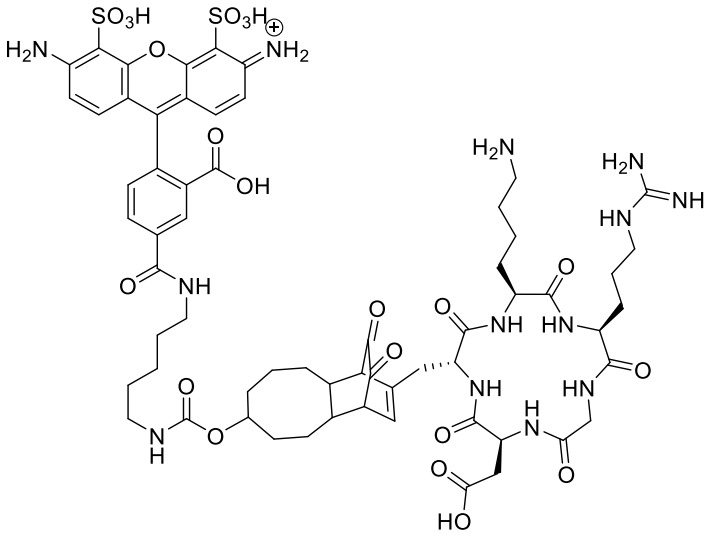
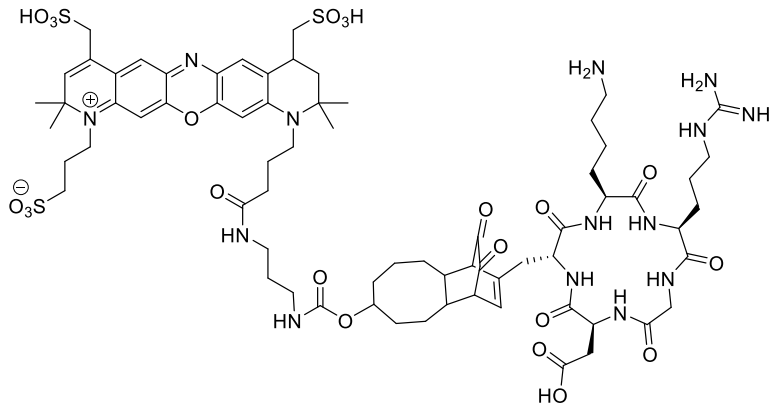
Supplemental Methods and Materials



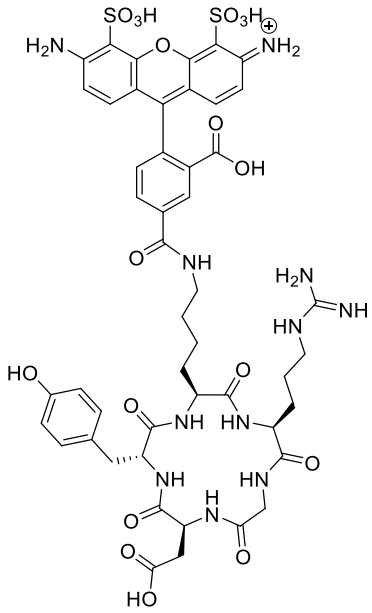
Synthesis of TCO-MB680R*:* MB680R-NHS (5.0 mg, 6.0 μmol) and TCO-NH_2 HCl (3.2 mg, 12.0 μmol) were dissolved in 0.6 mL of DMF in a 1.5 mL vial. 4.2 μL of DIPEA (3.1 mg; 24.0 μmol) was added, and the solution was covered with foil to protect it from light. After stirring at room temperature for 1 hour, the reaction was dry-loaded onto C_{18}-functionalized silica powder in a Biotage V10 and purified with a Biotage (Uppsala, Sweden) Isolera One flash chromatography system with a Biotage Sfar C18 Duo column and a 6 mL/min 5-95% MeCN/water gradient over 15 minutes. The blue eluent was collected and lyophilized overnight to afford a blue powder (3.6 mg, 64% yield). ESI-MS m/z: $[M+H^+]^+$ calculated 949.3; observed 950.7 (*Figure S1*).



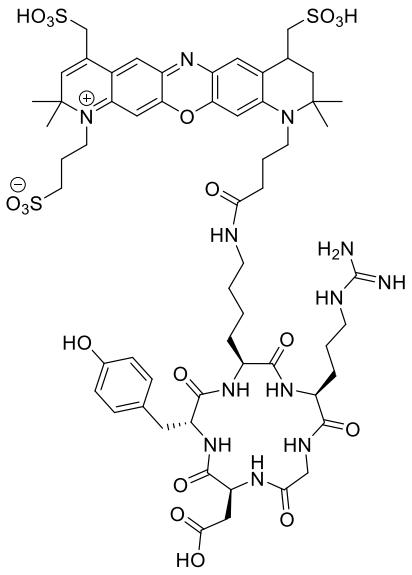
Synthesis of AF488-^{Y}c(RGDyK)*:* AF488-TCO (2 mg; 2.6 μmol) and c(RGDyK) (2.4 mg; 3.9 μmol) were dissolved in a 50% MeCN/10 mM tris (pH 8) solution, combined in a 1.5 mL vial to a final volume of 0.7 mL and placed on ice. Frémy's salt (2.8 mg; 10.4 μmol) was added, and the reaction was shaken on ice for 1 hour. Dissolved salts were removed by SPE with a C_{18} SepPak, and the solution was purified by RP-HPLC with a Phenomenex Jupiter 5 μm C18 300 Å 250 × 10 mm column and a 5 mL/min 5-95% MeCN/water (+ 0.1% TFA) gradient over 30 min. The purified product was lyophilized overnight to afford a bright red powder (1.8 mg, 63% yield). ESI-MS m/z: $[M+2H^{+}]^{2+}$ calculated 703.5; observed 703.0 (*Figure S2*)



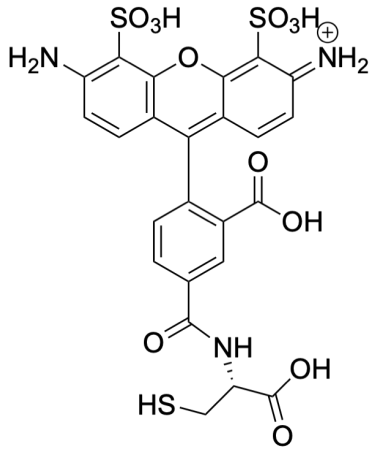
Synthesis of MB680R-^{Y}c(RGDyK)*:* MB680R-TCO (2 mg, 2.1 μmol) and c(RGDyK) (1.9 mg, 3.1 μmol) were dissolved in a 50% MeCN/10 mM tris (pH 8) solution, combined in a 1.5 mL vial to a final volume of 0.7 mL and placed on ice. Frémy's salt (2.3 mg; 8.6 μmol) was added, and the reaction was shaken on ice for 1 hour. Dissolved salts were removed by SPE with a C18 SepPak, and the solution was purified by RP-HPLC with a Phenomenex Jupiter 5 μm C_{18} 300 Å 250 × 10 mm column and a 5 mL/min 5-95% MeCN/water (+ 0.1% TFA) gradient over 30 min. The purified product was lyophilized overnight to afford a bright red powder (2.1 mg, 66% yield). ESI-MS m/z: $[M+2H^{+}]^{2+}$ calculated 792.9; observed 792.5 (*Figure S3*).



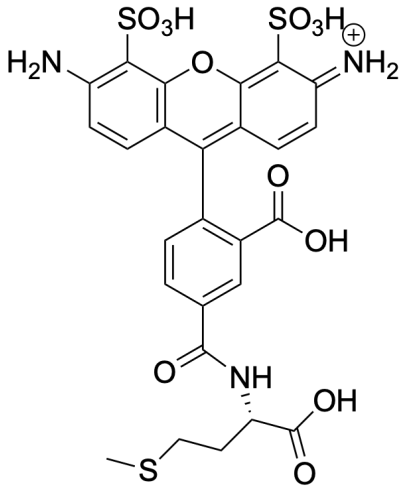
Synthesis of AF488-^{K}c(RGDyK)*:* AF488-NHS (2.5 mg, 4.0 μmol) and c(RGDyK) (3.7 mg, 6.0 μmol) were dissolved in DMF and combined in a 1.5 mL vial to a final volume of 0.3 mL. 2.8 μL of DIPEA (2.1 mg; 16 μmol) was added and the solution was covered with foil to protect it from light. After stirring at room temperature for 1 hour, the reaction was diluted with water, lyophilized overnight, redissolved in 50% MeCN/water, filtered, and purified by RP-HPLC with a Phenomenex Jupiter 5 μm C_{18} 300 Å 250 × 10 mm column and a 5 mL/min 5-95% MeCN/water (+ 0.1% TFA) gradient over 30 min. The purified product was lyophilized overnight to afford a bright red powder (3.8 mg, 85% yield). ESI-MS m/z: $[M+H^+]^+$ calculated 1137.3; observed 1136.8 (*Figure S4*).



MB680R-^{K}c(RGDyK)*:* MB680R-NHS (2.5 mg; 3.0 μmol) and c(RGDyK) (2.8 mg; 4.5 μmol) were dissolved in DMF and combined in a 1.5 mL vial to a final volume of 0.3 mL. 2.1 μL of DIPEA (1.6 mg; 12 μmol) was added and the solution was covered with foil to protect from light. After stirring at room temperature for 1 hour, the reaction was diluted with water, lyophilized overnight, redissolved in 50% MeCN/water, filtered, and purified by RP-HPLC with a Phenomenex Jupiter 5 μm C_{18} 300 Å 250 × 10 mm column and a 5 mL/min 5-95% MeCN/water (+ 0.1% TFA) gradient over 30 min. The purified product was lyophilized overnight to afford a blue powder (3.6 mg, 90% yield). ESI-MS m/z: $[M+H^+]^+$ calculated 1343.5; observed 1343.8 (*Figure S5*).



Synthesis of AF488-L-cysteine*:* AF488-NHS (0.5 mg, 0.8 μmol) and L-cysteine (0.2 mg, 1.6 μmol) were dissolved in water and combined in a 1.5 mL vial to a final volume of 80 μL. 0.6 μL of DIPEA (0.4 mg; 3.2 μmol) was added and the solution was covered with foil to protect it from light. After stirring at room temperature for 1 hour, the reaction was purified by RP-HPLC with a Phenomenex Jupiter 5 μm C_{18} 300 Å 250 × 10 mm column and a 5 mL/min 5-95% MeCN/water (+ 0.1% TFA) gradient over 30 min. The purified product was lyophilized overnight to afford a bright red powder (0.3 mg, 59% yield). ESI-MS m/z: $[M+H^+]^+$ calculated 638.0; observed 638.2 (*Figure S16*).



Synthesis of AF488-L-methionine*:* AF488-NHS (0.5 mg, 0.8 μmol) and L-methionine (0.2 mg, 1.6 μmol) were dissolved in a 1:1 mixture of water and DMF and combined in a 1.5 mL vial to a final volume of 80 μL. 0.6 μL of DIPEA (0.4 mg; 3.2 μmol) was added and the solution was covered with foil to protect it from light. After stirring at room temperature for 1 hour, the reaction was purified by RP-HPLC with a Phenomenex Jupiter 5 μm C_{18} 300 Å 250 × 10 mm column and a 5 mL/min 5-95% MeCN/water (+ 0.1% TFA) gradient over 30 min. The purified product was lyophilized overnight to afford a bright red powder (0.3 mg, 40% yield). ESI-MS m/z: $[M+H^+]^+$ calculated 666.0; observed 666.3 (*Figure S17*).

Evaluation of Amino Acid Reactivity**:** L-phenylalanine, L-tryptophan, AF488-L-cysteine, or AF488-L-methionine (0.15 mg, 0.2-0.7 μmol) were dissolved in a 50% MeCN/10 mM tris (pH 8) solution and combined in a 1.5 mL vial to a final concentration of 1 mM (220-750 μL) and placed on ice. Frémy's salt (0.2-0.8 mg; 0.9-3.0 μmol) was added, and the reaction was shaken on ice for 1 hour. Crude reaction mixtures were concentrated with a Biotage V-10 if required and analyzed by analytical RP-HPLC using a Phenomenex Jupiter 5 μm C_{18} 300 Å 250 × 4.6 mm column and a 1 mL/min 5-95% MeCN/water (+ 0.1% TFA) gradient over 15 min. Chromatograms of the crude reaction mixtures were compared to chromatograms of the pure standards (*Figures S14, S15, S18, S19).*

UV-Vis Measurements of O-Quinone Formation: c(RGDyK) (1.0 mg; 1.6 μmol) and Frémy's salt (2.1 mg; 8 μmol) were dissolved in ice-cold 10 mM tris (pH 8) and combined into a total volume of 0.5 mL. The samples were shaken on ice for the duration of the experiment. UV-Vis spectra were measured with a Thermo Scientific NanoDrop One immediately after combining the reagents and every 5 minutes up to 1 hour.

Stability Assays: 200 μM solutions of AF488-^{Y}c(RGDyK), MB680R-^{Y}c(RGDyK), AF488-^{K}c(RGDyK), and MB680R-^{K}c(RGDyK) in PBS were prepared in 1.5 mL tubes and placed on a thermomixer (37 °C, 500 RPM) for 7 days. Samples were analyzed by analytical RP-HPLC at 0, 1, 3, 5, and 7 days using a Phenomenex Jupiter 5 μm C_{18} 300 Å 250 × 4.6 mm column and a 1 mL/min 5-95% MeCN/water (+ 0.1% TFA) gradient over 30 min. Purity was assessed by integrating peaks at 488 or 680 nm. (*Figure S6*).

Cell Culture: U-87 MG cells were purchased from ATCC and cultured in Eagle's Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum and penicillin-streptomycin in an incubator at 37 °C and 5% CO_2.

Confocal Microscopy: 5×10^4 U-87 MG cells were plated in triplicate onto 4-well cell culture microscope slides with 0.2 mL of media and allowed to adhere in an incubator overnight (see *Cell Culture*). The wells were washed with PBS, filled with another 0.2 mL of PBS supplemented with 10% fetal bovine serum to block nonspecific binding, and incubated for 1 hour in the presence of 35 μM DAPI (4′,6-diamidino-2-phenylindole) and either 10 μM AF488-^{Y}c(RGDyK) or AF488-^{K}c(RGDyK). The wells were washed with PBS, fixed with a 10% formalin solution, and mounted with Fluoroshield (Sigma Aldrich) mounting medium. The slides were imaged with a Nikon A1 confocal microscope (Melville, NY) using a 60× oil objective and an 8 ms exposure time. AF488 signal was acquired with a 488 nm laser at 1.0% power with a PMT HV of 15 and a 525/50 nm filter. DAPI signal was acquired with a 405 nm laser at 2.5% power with a PMT HV of 95 and a 450/50 nm filter. All images were acquired using the same system settings.

Fluorescence Quantification: 5 μM stock solutions of each fluorophore-bearing conjugate were prepared in water. 100 μL of each respective conjugate stock solution were added to wells (n = 7) of a clear-bottom 96-well plate and their relative fluorescence was measured with a SpectraMax i3 plate reader. AF488-conjugates were analyzed with an excitation wavelength of 494 nm and an emission wavelength of 517 nm. MB680R-conjugates were analyzed with an excitation wavelength of 685 nm and an emission wavelength of 709 nm. An excitation bandwidth of 9 nm and an emission bandwidth of 15 nm was used for all measurements.

Subcutaneous Xenografts: All animal work was performed under the guidance and approval of the Institutional Animal Care and Use Committee (IACUC) of Weil Cornell Medical College. U-87 MG cells were harvested, resuspended in media, and mixed with ice-cold Matrigel (50% v/v) for xenografting. Eight athymic mice (female, 5-7 weeks old) were anesthetized with a 2% isoflurane/oxygen gas mixture, and 5 ×

10^6 U-87 MG cells in 100 μL of the media/Matrigel mixture were subcutaneously injected above their right shoulders. Tumors were grown for two weeks prior to *in vivo* experimentation.

NIRF Imaging*:* All mice were fed AIN-93G food (Research Diets; New Brunswick, NJ) for two weeks prior to imaging in order to reduce autofluorescence caused by their diet. U-87 MG tumor-bearing mice were injected intravenously with either MB680R-^{Y}c(RGDyK) or MB680R-^{K}c(RGDyK) (13 nmol, 100 μL). The mice were anesthetized with a 2% isoflurane/oxygen gas mixture and imaged with an IVIS Spectrum using default settings and an excitation/emission of 675/720 nm. Images were collected 1, 4, and 24 hours after injection. After the final imaging timepoint (*i.e.* 24 hours), all mice were euthanized via CO_2 asphyxiation and dissected. Organs of interest (tumor, heart, liver, spleen, and kidneys) from each mouse were placed in the scanner and imaged using the same settings. Images were processed with Living Image software. Regions of interest (ROIs) were automatically drawn by the software, and the average signal from each organ was normalized to the area of the respective ROI.

Supplemental Figures

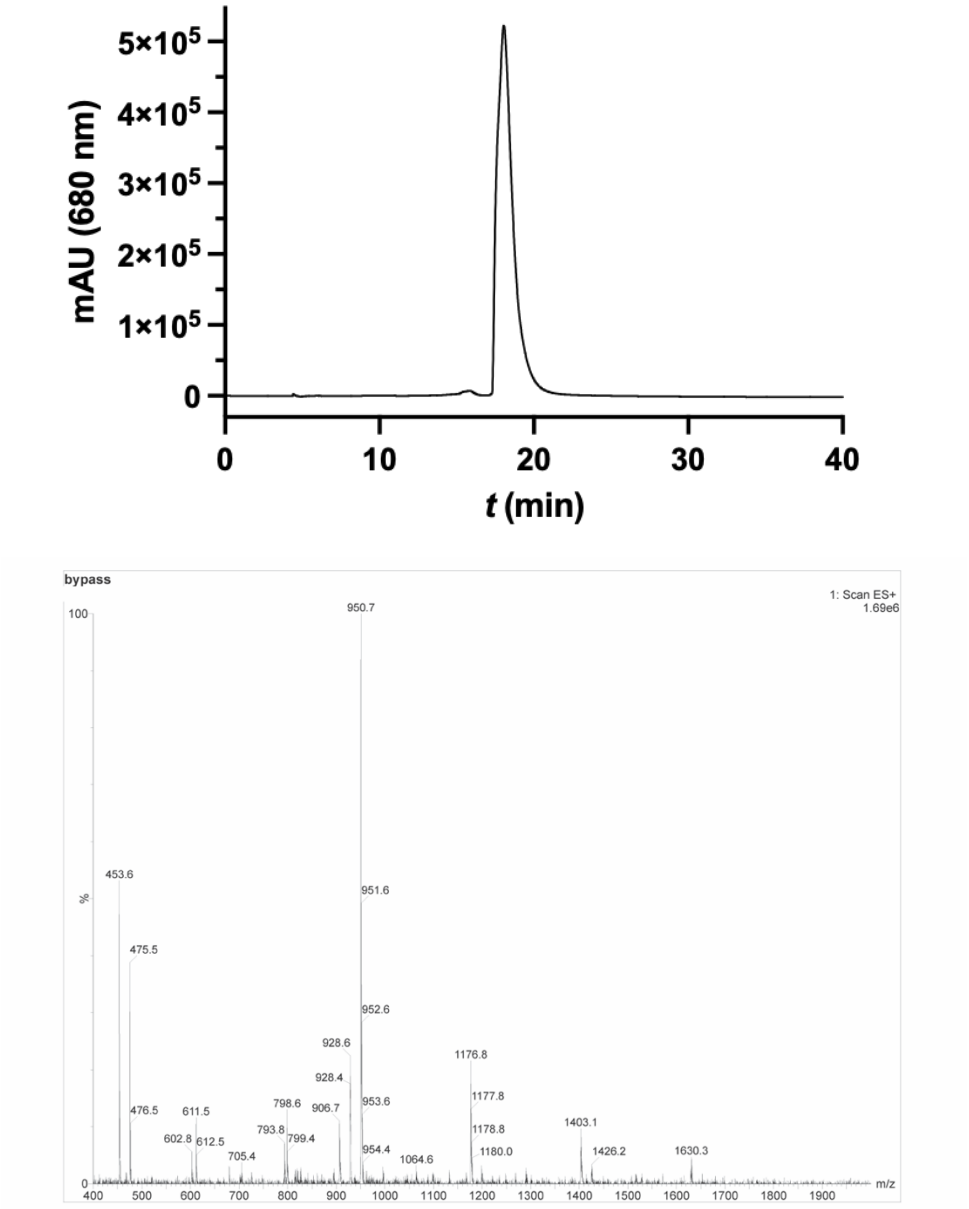
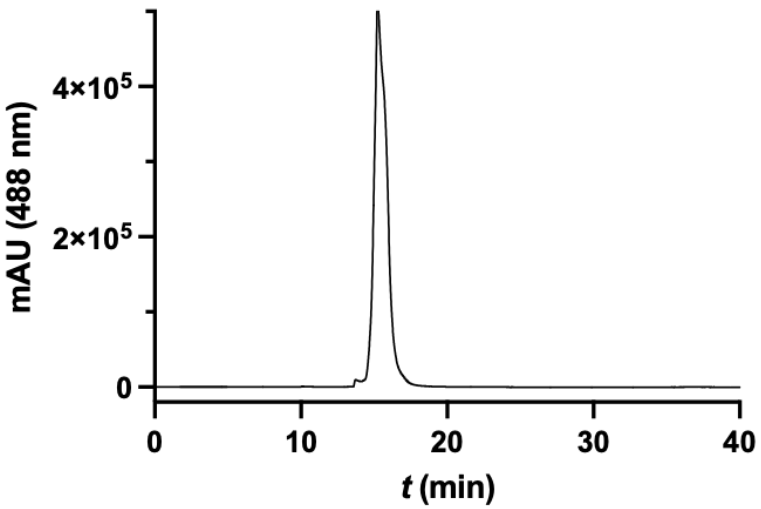


Figure S1. HPLC chromatogram (top) and ESI-MS (bottom) of purified TCO-MB680R.



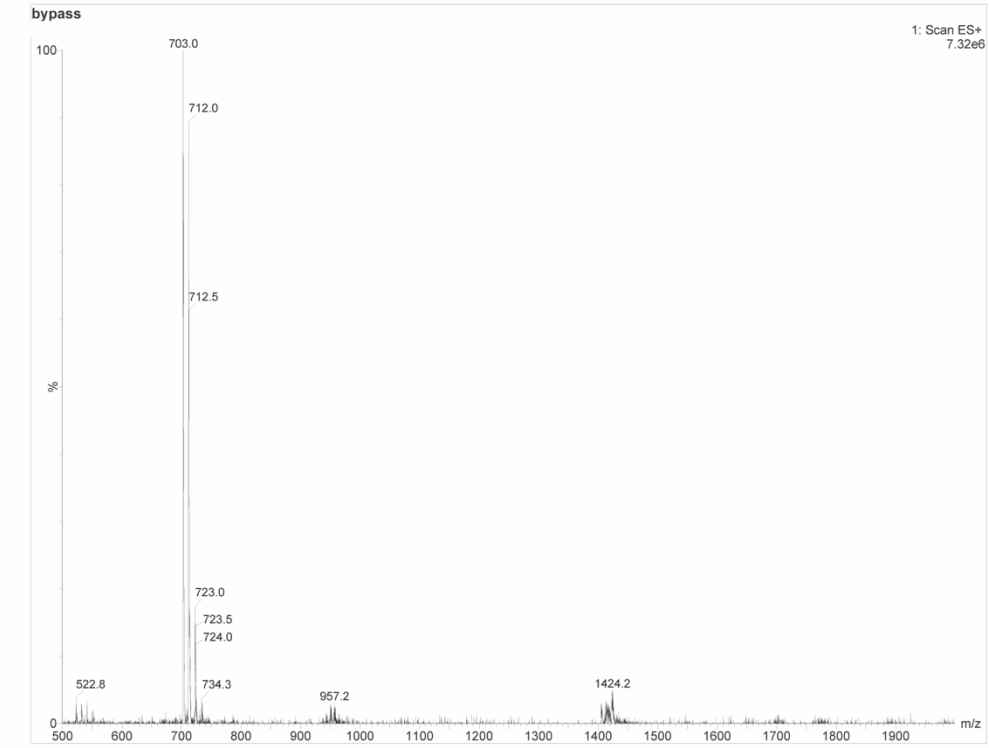


Figure S2. HPLC chromatogram (top) and ESI-MS (bottom) of purified AF488-^{Y}c(RGDyK).

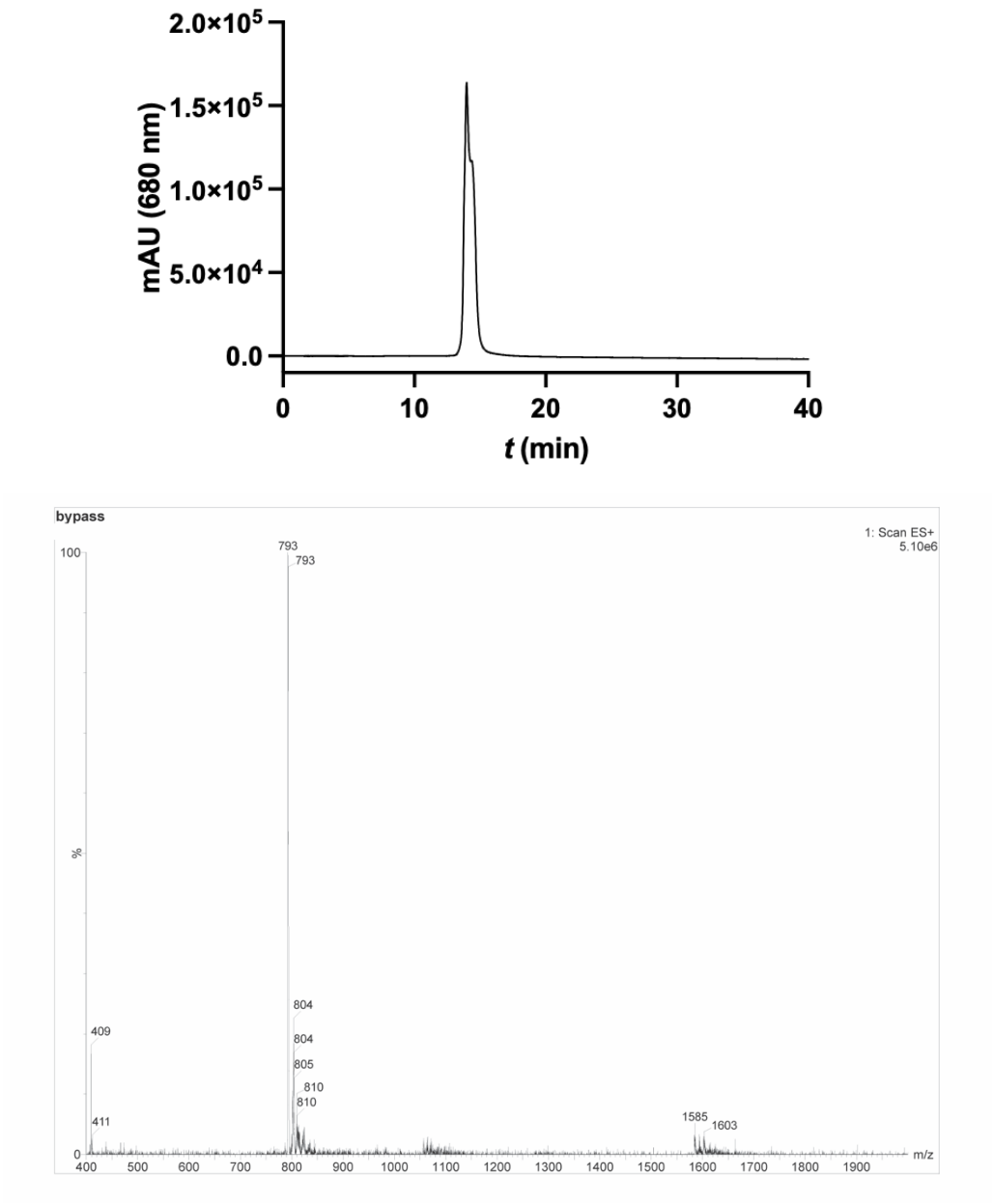


Figure S3. HPLC chromatogram (top) and ESI-MS (bottom) of purified MB680R-^{Y}c(RGDyK).

Elemental Composition Report **Page 1**

Single Mass Analysis
Tolerance = 5.0 PPM / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
995 formula(e) evaluated with 1 results within limits (up to 50 closest results for each mass)
Elements Used:
C: 0-62 H: 0-79 N: 0-14 O: 0-22 S: 0-2

RW_Dec_12-HRMS_2025
XEVO-G3QTOF#YGA0553

NMR Analytical Core Facility
Waters Acquity Xevo G3
C62H78N13O21S2

03-Dec-2025
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Wei Siang Kao 1:43
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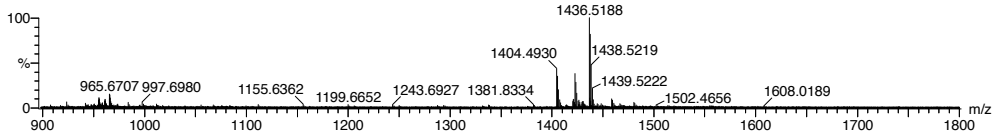
2.07e+007

AF_Y_02 35 (0.159) Cm (35:54)



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Maximum:		5.0	5.0	50.0				
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
1404.4930	1404.4877	5.3	3.8	30.5	583.8	n/a	n/a	C62 H78 N13 O21 S2

Figure S4. HRMS for AF488-^{Y}c(RGDyK).

Elemental Composition Report **Page 1**

Single Mass Analysis
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Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
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Elements Used:
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RW_Dec_12-HRMS_2025
XEVO-G3QTOF#YGA0553

NMR Analytical Core Facility
Waters Acquity Xevo G3
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03-Dec-2025
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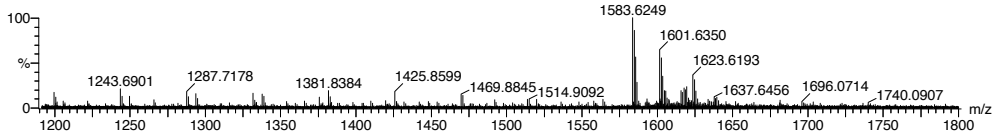
1: TOF MS ES+
1.25e+005

MB_Y 47 (0.205)



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Maximum:		5.0	5.0	50.0				
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1583.6249	1583.6220	2.9	1.8	28.5	342.1	n/a	n/a	C70 H99 N14 O22 S3

Figure S5. HRMS for MB680R-^{Y}c(RGDyK).

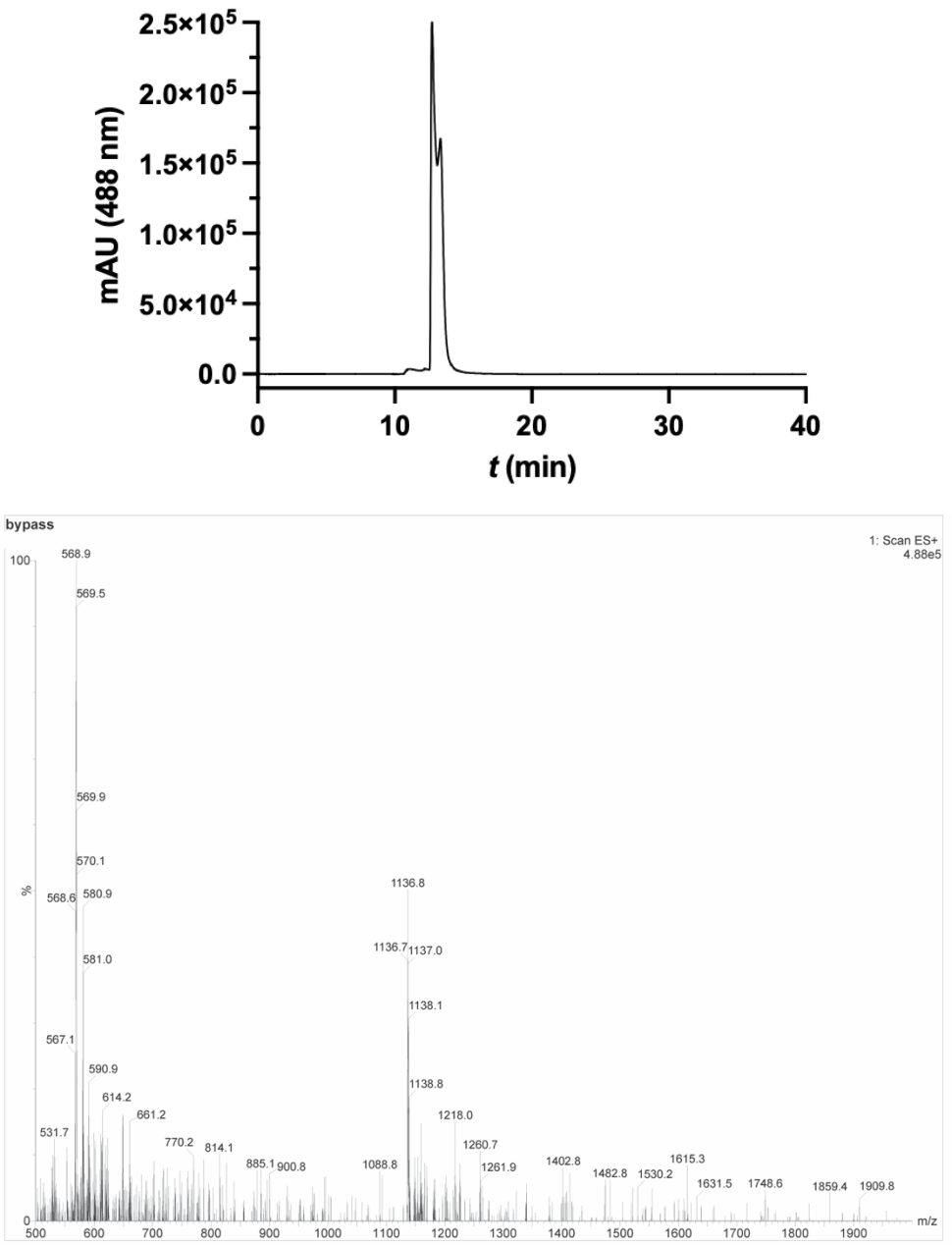

Figure S6. HPLC chromatogram (top) and ESI-MS (bottom) of purified AF488-^{K}c(RGDyK).

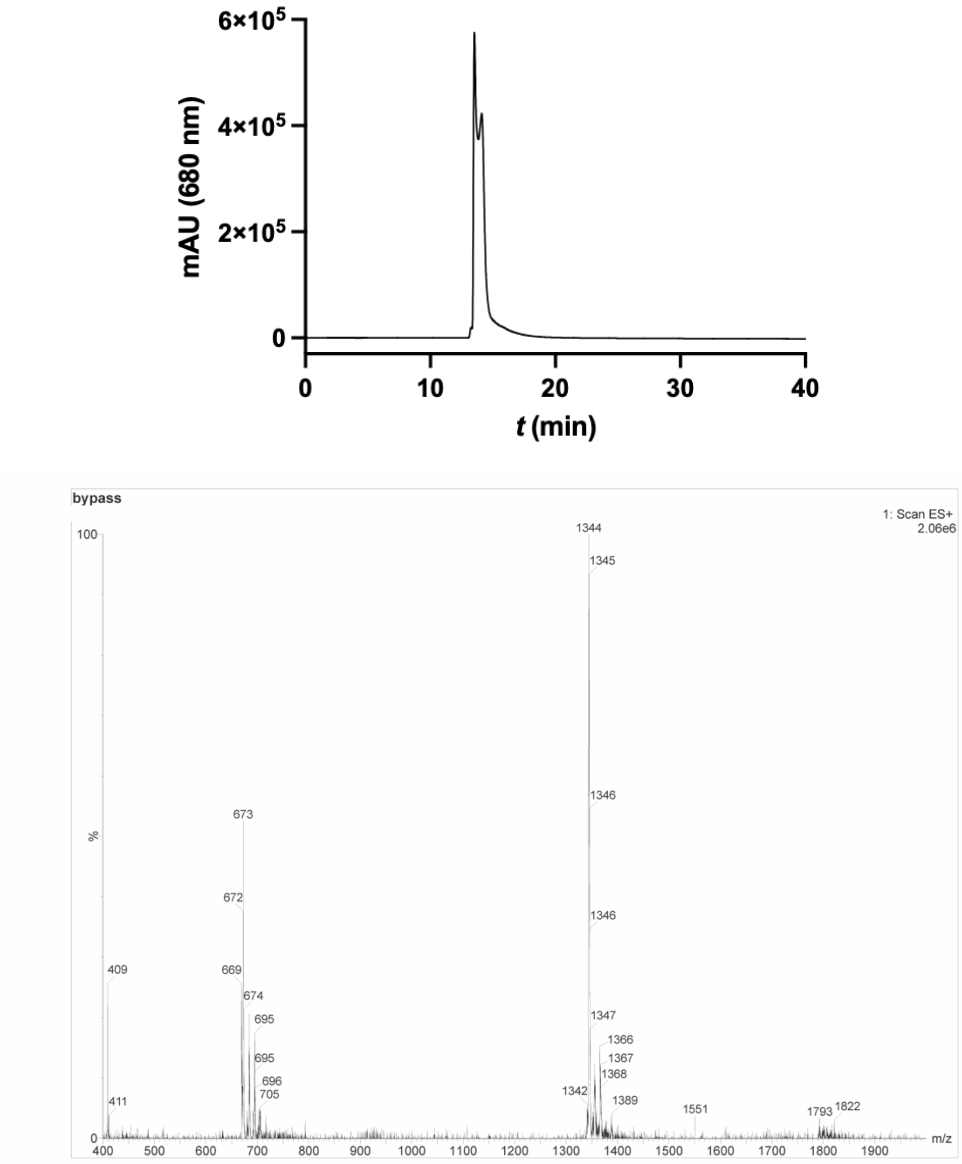

Figure S7. HPLC chromatogram (top) and ESI-MS (bottom) of purified MB680R-^{K}c(RGDyK).

Elemental Composition Report **Page 1**

Single Mass Analysis
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Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
873 formula(e) evaluated with 1 results within limits (up to 50 closest results for each mass)
Elements Used:
C: 0-48 H: 0-55 N: 0-11 O: 0-18 S: 0-3

RW_Dec_12-HRMS_2025
XEVO-G3QTOF#YGA0553

NMR Analytical Core Facility
Waters Acquity Xevo G3
C48H54N11O18S2

03-Dec-2025
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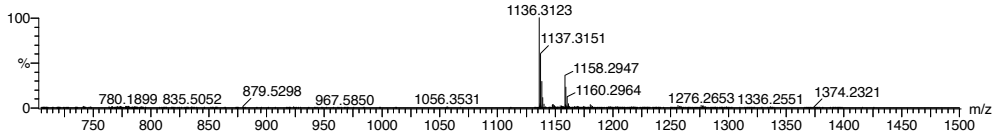
2.04e+007

AF_K 50 (0.216) Cm (50:69)



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Maximum:		5.0	5.0	50.0				
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
1136.3123	1136.3090	3.3	2.9	27.5	661.5	n/a	n/a	C48 H54 N11 O18 S2

Figure S8. HRMS for AF488-^{K}c(RGDyK).

Elemental Composition Report **Page 1**

Single Mass Analysis
Tolerance = 5.0 PPM / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
1026 formula(e) evaluated with 1 results within limits (up to 50 closest results for each mass)
Elements Used:
C: 0-58 H: 0-79 N: 0-12 O: 0-19 S: 0-3

RW_Dec_12-HRMS_2025
XEVO-G3QTOF#YGA0553

NMR Analytical Core Facility
Waters Acquity Xevo G3
C58H78N12O19S3

03-Dec-2025
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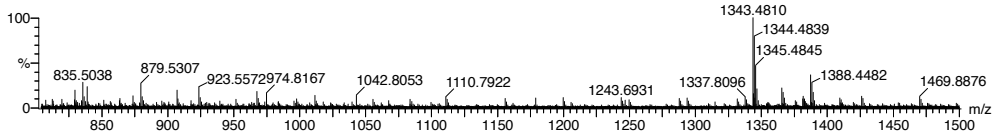
9.50e+006

MB_K 27 (0.129) Cm (25:40)



Minimum:				-1.5				
Maximum:		5.0	5.0	50.0				
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
1343.4810	1343.4747	6.3	4.7	25.5	598.0	n/a	n/a	C58 H79 N12 O19 S3

Figure S9. HRMS for MB680R-^{K}c(RGDyK).

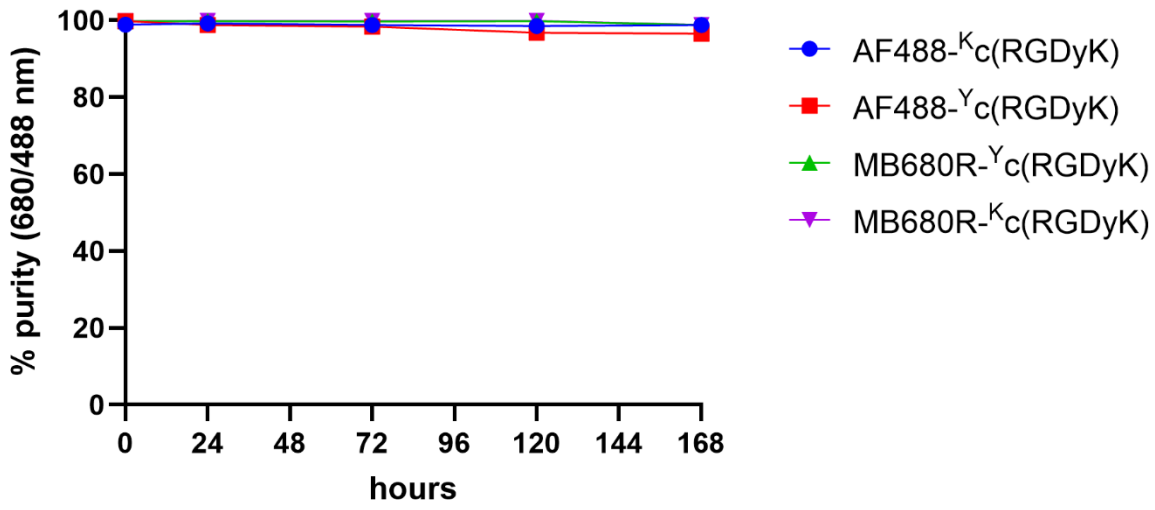


Figure S10. Stability of fluorophore-bearing c(RGDyK) conjugates in PBS at 37 °C over 7 days.

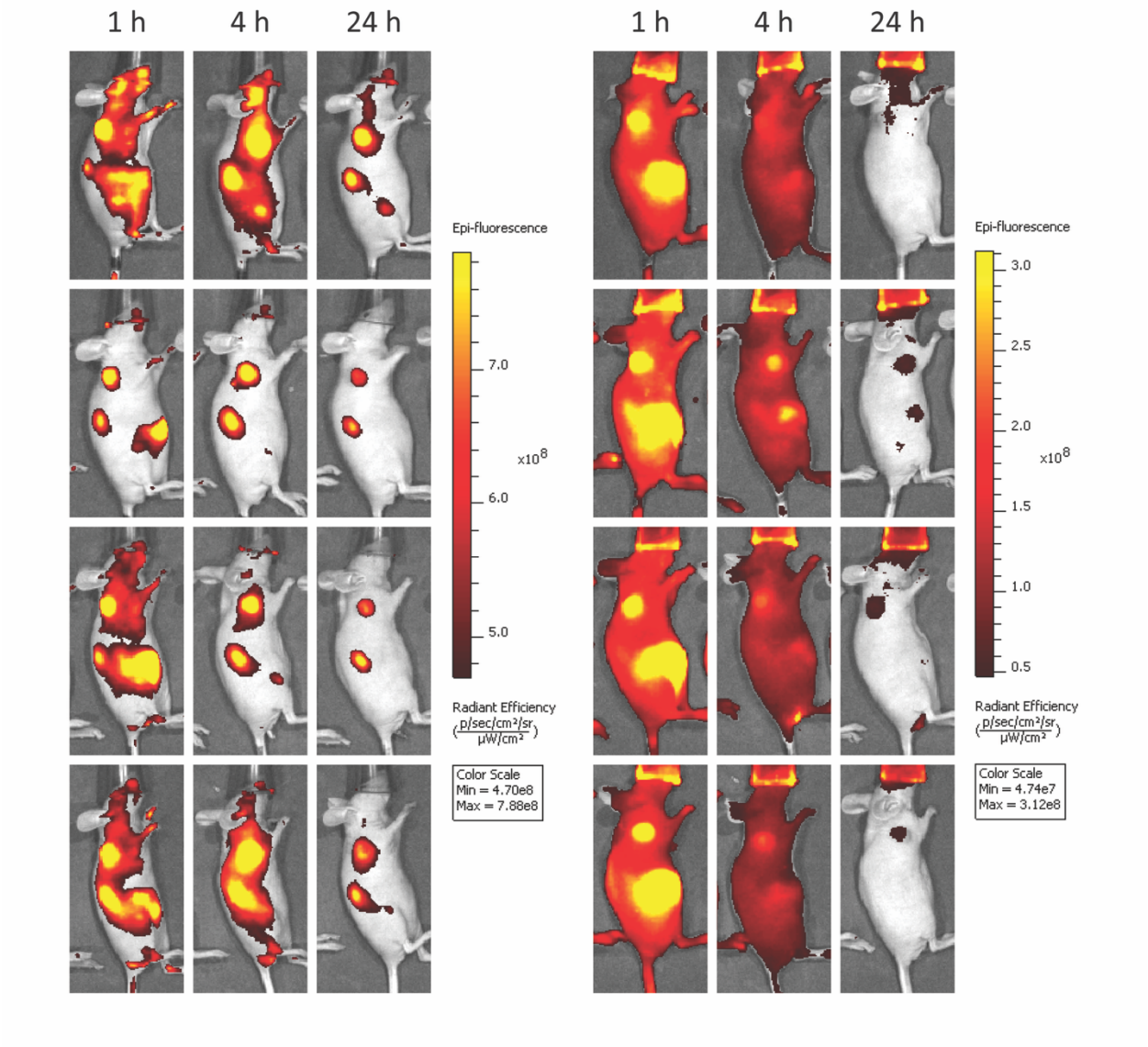


Figure S11 NIRF images of mice 1, 4, and 24 h after intravenous injection of MB680R-^{Y}c(RGDyK) (left) or MB680R-^{K}c(RGDyK) (right).

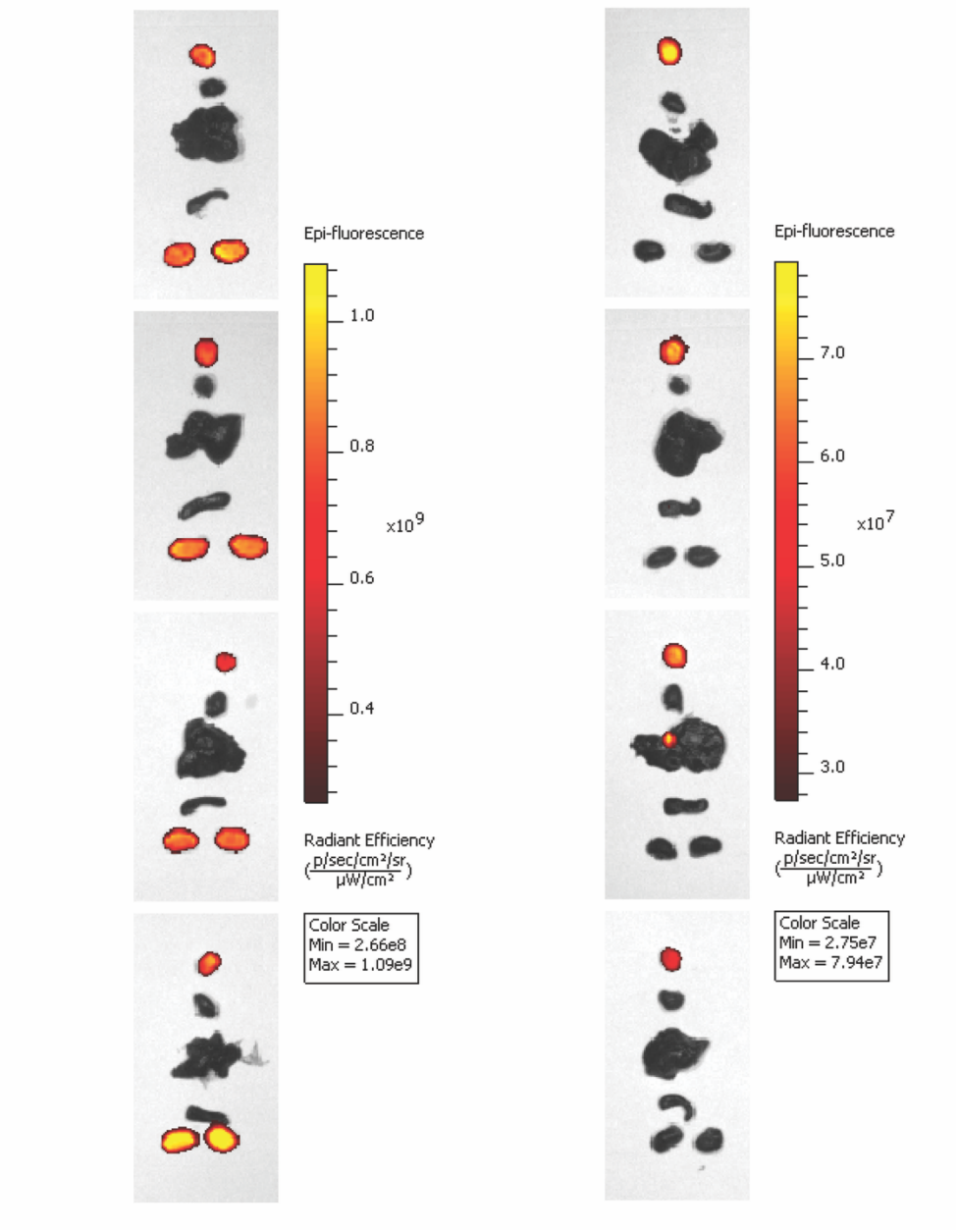


Figure S12. *Ex vivo* NIRF imaging of select organs 24 h after intravenous injection of MB680R-^{Y}c(RGDyK) (left) or MB680R-^{K}c(RGDyK) (right). Organs, from top to bottom, are tumor, heart, liver, spleen, kidneys.

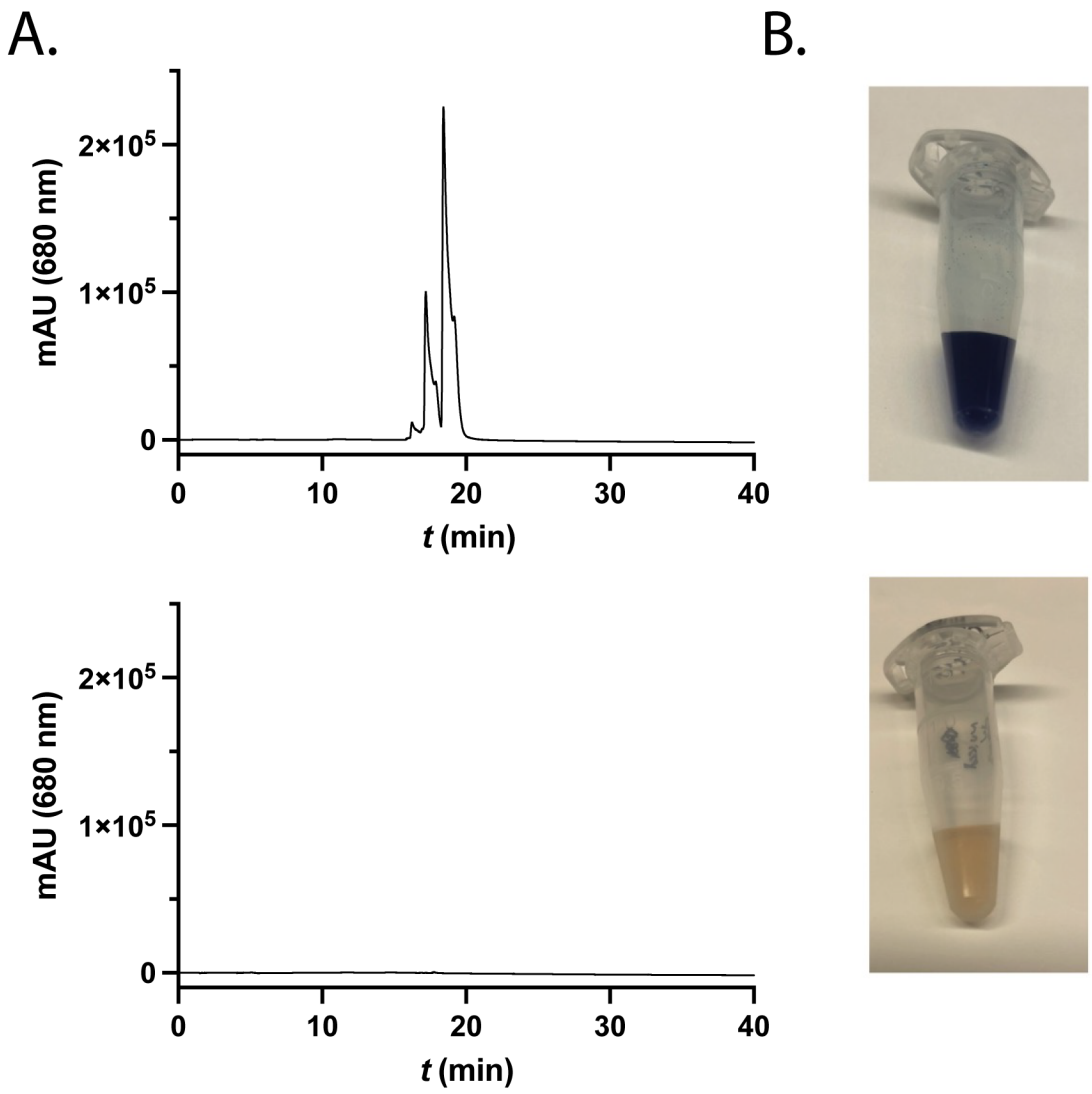


Figure S13. HPLC chromatograms (A) and images (B) showing Cy5.5-NHS before (top) and after (bottom) the addition of excess Frémy's salt.

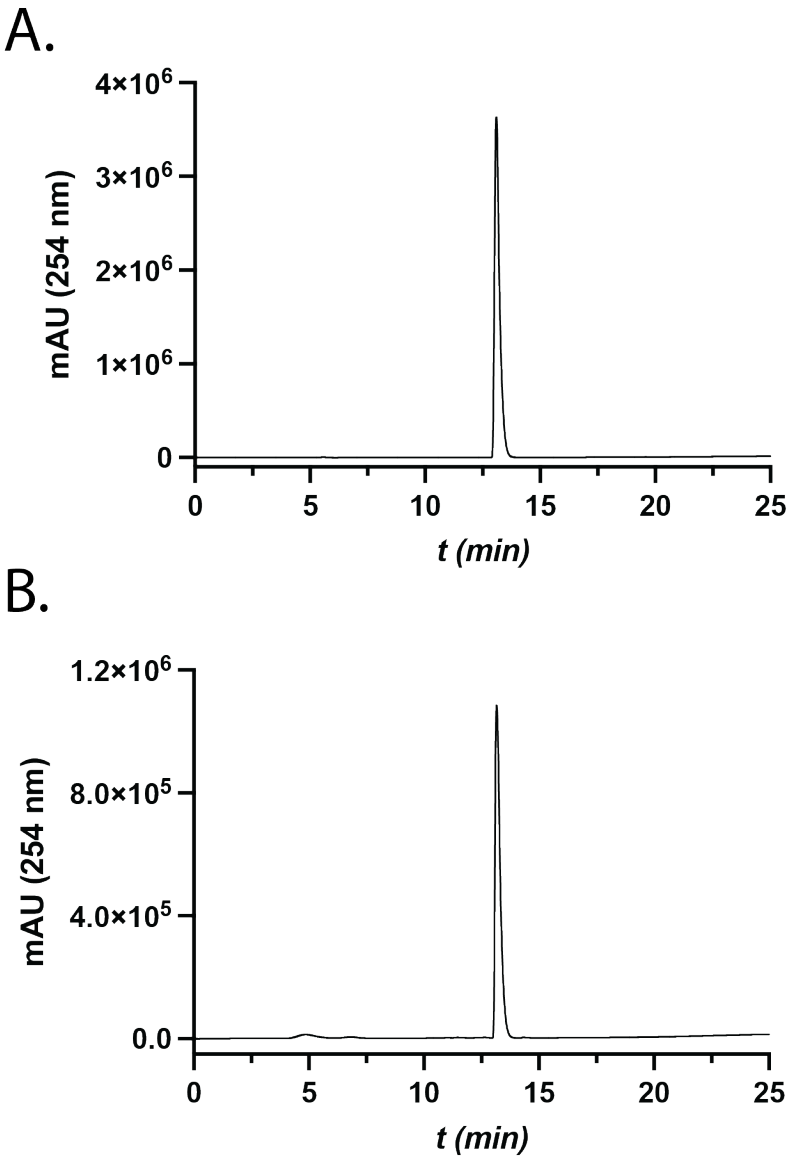


Figure S14. HPLC chromatogram (254 nm) of phenylalanine collected before (A) and after (B) the addition of Frémy's salt.

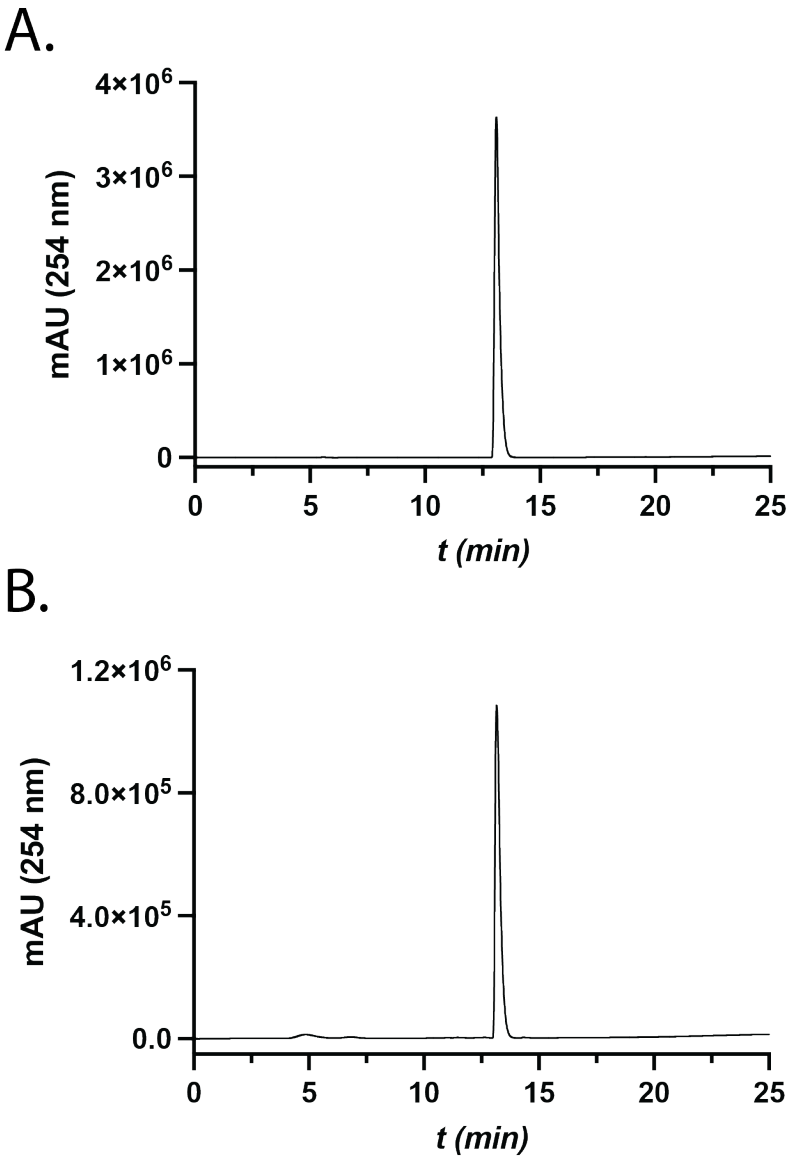


Figure S15. HPLC chromatogram (254 nm) of tryptophan collected before (A) and after (B) the addition of Frémy's salt.

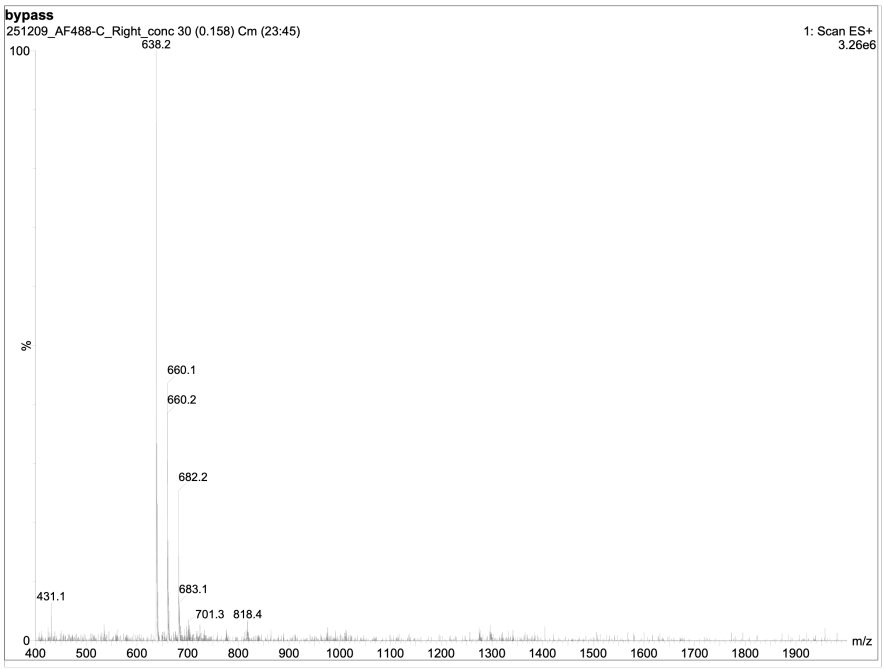


Figure S16. ESI-MS of AF488-cysteine

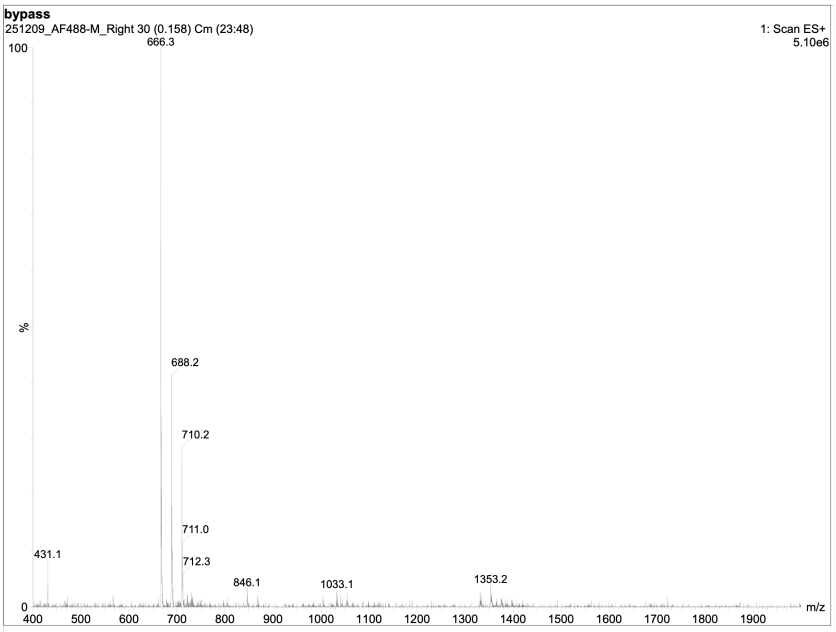


Figure S17. ESI-MS of AF488-methionine

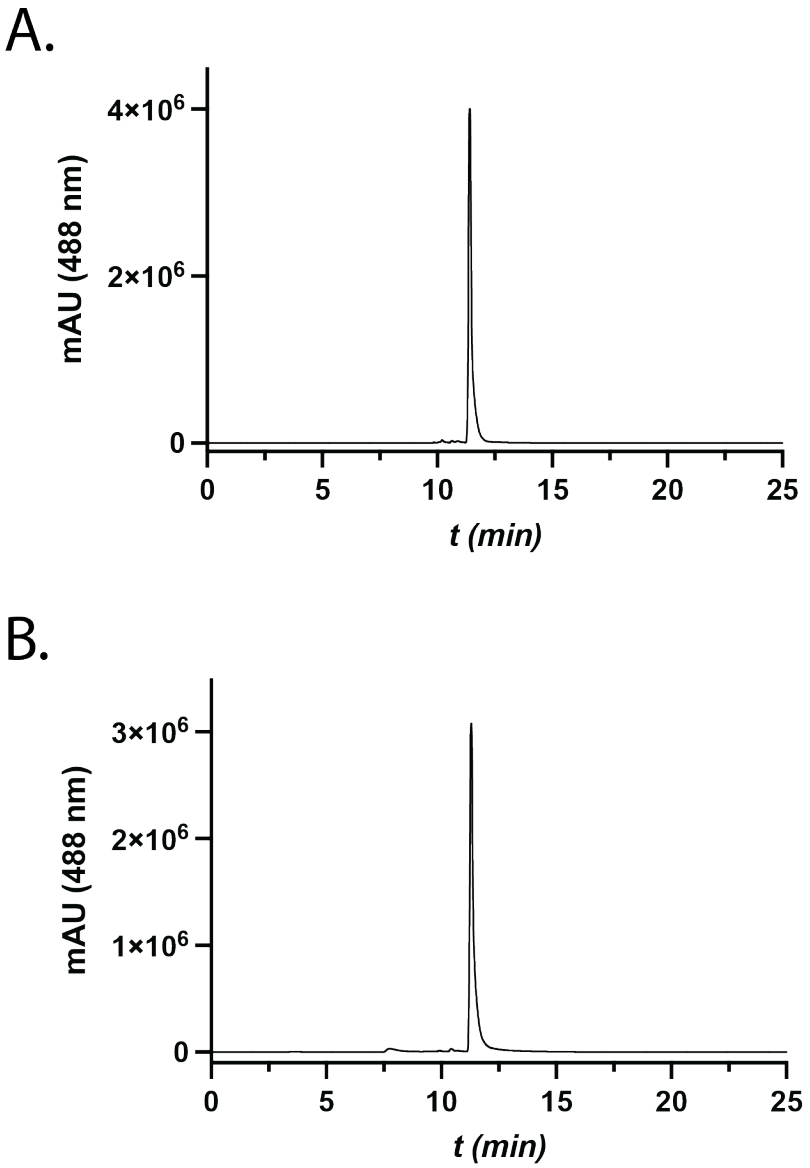


Figure S18. HPLC chromatogram (480 nm) of AF488-cysteine collected before (A) and after (B) the addition of Frémy's salt.

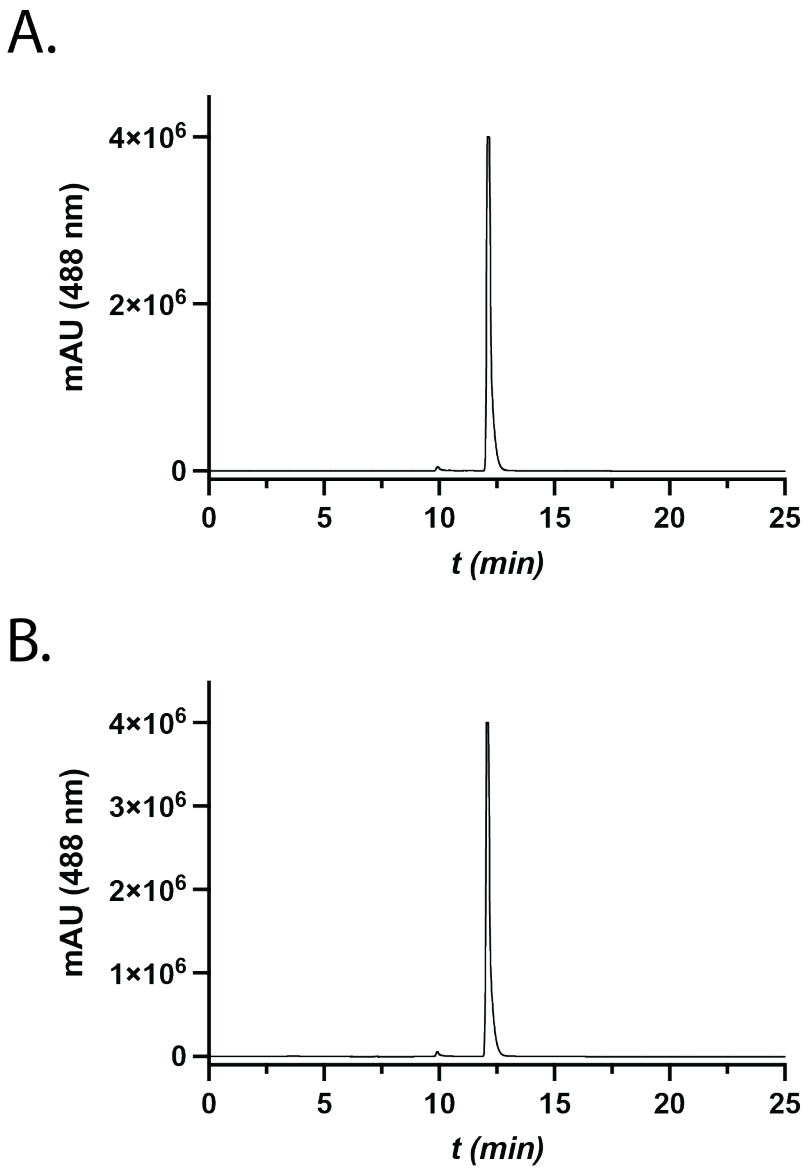


Figure S19. HPLC chromatogram (480 nm) of AF488-methionine collected before (A) and after (B) the addition of Frémy's salt.

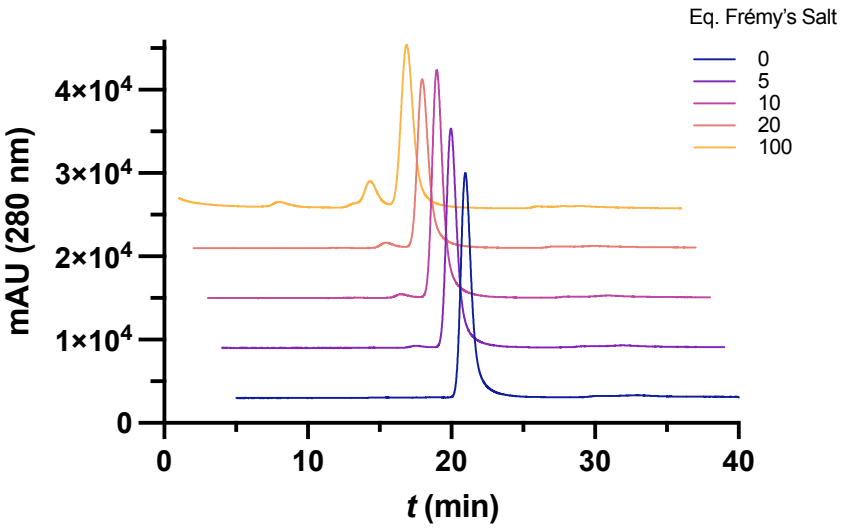


Figure S20. Size-exclusion HPLC chromatograms of the monoclonal antibody 5B1 after shaking in solution with various molar equivalents of Fremy's salt for 1 h.