Supplementary material Site-Specific Dual-Labeling of a VHH with a Chelator and a Photosensitizer for Nuclear Imaging and Targeted Photodynamic Therapy of EGFR-Positive Tumors

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Abbreviations

BCN: Bicyclo[6.1.0]nonyne
CV: Column volume
DIPEA: *N,N*-Diisopropylethylamine
DMF: *N,N*-Dimethylformamide
DOL: Degree of labeling
DTPA: Diethylenetriaminepentaacetic acid
ESI: Electrospray ionization
FA: Formic acid
HATU: 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate
HPLC: High performance liquid chromatography
HRMS: High resolution mass spectrometry
LRMS: Low resolution mass spectrometry
MALDI-TOF: Matrix-assisted laser desorption ionization - Time of flight
MES: 2-(*N*-morpholino)ethanesulfonic acid
NHS: *N*-hydroxysuccinimide
PB: Phosphate buffer
PBS: Phosphate buffered saline
PEG: Polyethylene glycol
RT: Room temperature
TCEP: Tris(2-carboxyethyl)phosphine
TEAB: Triethylammonium bicarbonate
TFA: Trifluoroacetic acid
THF: Tetrahydrofuran
TSTU: *N,N,N′,N′*-tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate
UV: Ultraviolet

Materials and general procedures

Chemicals. All chemicals were purchased from Sigma-Aldrich, Acros Organics, Alfa Aesar and TCI Europe and used without further purification. IRDye700DX-NHS was purchased from LICOR (USA). DTPA was kindly provided by CheMatech (France). Moisture sensitive reactions were performed under nitrogen or argon atmosphere. HPLC-gradient grade MeCN was obtained from Biosolve or Carlo Erba. LC-HRMS grade MeCN was obtained from Fisher Scientific. All aqueous buffers used in this work and aqueous mobile-phases for HPLC were prepared using water purified to 18.2 MΩ cm with a PURELAB Ultra system from ELGA.

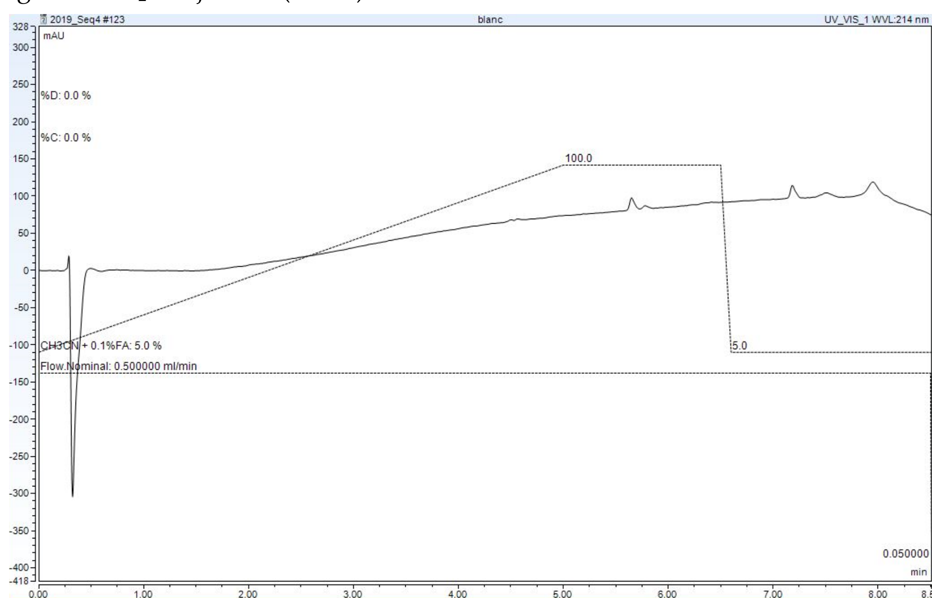
Ion chromatography. The number of TFA counterions was quantified by ionic chromatography. Analyses were performed using an ICS 5000 system from Thermo Scientific equipped with a conductivity detector CD (Thermo Scientific)

and a conductivity suppressor ASRS-ultra II 4 mm (Thermo Scientific). TFA was separated using an Ion Pac AS11-HC analytical column (4 µm, 4 × 250 mm, Thermo Scientific) equipped with an Ion Pac AS11-HC guard column (4 µm, 4 × 250 mm, Thermo Scientific). Chromatographic analyses were recorded with Chromeleon software, 7.2. The elution of TFA was conducted with a 15 min isocratic elution with 90% eluent A (NaOH 30 mM) and 10% eluent B (H_2O) at a flow rate of 1.5 mL/min and a column temperature of 30 °C.

Semi-preparative HPLC. Purifications were performed on an UltiMate 3000 system Dionex (Thermo Scientific) equipped with an UV-visible detector, on one of the following columns: BetaBasic-18 column (Thermo Scientific; 5 µm, 150 Å, 150 × 30 mm) at 20 mL/min or Hypersil gold C18 column (Thermo Scientific, 100 Å, 250 × 10 mm) at 3.5 mL/min, with HPLC grade eluents. The fractions of interest were analyzed by HPLC-MS, pooled, concentrated under reduced pressure to remove organic solvents and freeze-dried.

HPLC-MS. The compounds were analyzed by HPLC-MS, performed on an UltiMate 3000 system Dionex (Thermo Scientific) equipped with a DAD detector and coupled to a low-resolution mass spectrometry detector MSQ Plus (Thermo Scientific) equipped with an ESI source. Separation was achieved using an RP Kinetex™ column (Phenomenex) (2.6 µm, 100 Å, 50 × 2.1 mm) with ultrapure water and HPLC-grade MeCN (A: H_2O 0.1% FA and B: MeCN 0.1% FA). Analyses were performed with the following gradient program: 5% to 100% of B in 5 min, 100% B for 1.5 min, 100% to 5% B in 0.1 min and 5% B for 1.9 min, at a flow rate of 0.5 mL/min. The purity of compounds was determined from the integration of RP-HPLC-MS chromatograms at 214 nm.

HPLC-MS chromatogram of H_2O injection (blank) at 214 nm:



HRMS. Spectra were recorded on a mass spectrometer LTQ Orbitrap XL (Thermo Scientific) using an ESI source. Spectra were recorded in positive mode.

RP-HPLC-HRMS. The protein 7D12 and related conjugates were characterized by RP-HPLC-HRMS performed on a HPLC UltiMate 3000 system (Thermo Scientific) equipped with a UV-visible detector and coupled to a high-resolution mass spectrometer LTQ Orbitrap XL (Thermo Scientific) equipped with an ESI source. The separation was achieved on a Thermo Scientific™ MAbPac™ column (4 µm, 50 × 21 mm) with the following eluents: A: H_2O, 0.1% FA + 0.02% TFA and B: MeCN/H_2O (90 : 10, v/v), 0.1% FA + 0.02% TFA, and a linear gradient program: 5% to 100% of B in 15 min, 100% B for 3 min, 100% to 5% B in 0.1 min and 5% B for 6 min, at a flow rate of 0.3 mL/min, at 80 °C. Multicharged mass spectra were deconvoluted with Protein Deconvolution 2.0 software (Thermo Scientific).

MALDI-TOF. Analyses were recorded on a Bruker Daltonics Ultraflex II spectrometer equipped with a Smartbeam (Nd-YAG 355 nm) and used in the MALDI-TOF linear positive mode (10-25 kDa mass range). Samples were desalted

using C4 ZipTips, mixed with a freshly prepared 3,5-dimethoxy-4-hydrowycinnamic acid matrix solution and spotted on an MSP 365 MALDI plate.

^{1}H- and ^{13}C-NMR. Spectra were recorded on Bruker Avance Neo 500 MHz spectrometers under routine conditions at 298 K and were referenced to TMS or residual solvent signals. The following abbreviations and their combinations are used: s, singlet; d, doublet; t, triplet; q, quadruplet; m, multiplet. **Protein concentrations**. Concentrations of protein and protein conjugates were determined by measuring the absorbance on a Varian Cary 50 (double beam) spectrophotometer by using a TrayCell (Hellma, pathlength: 0.2 (factor 50) mm or 1.0 (factor 10) mm, chamber volume 0.7-4 µL), at 25 °C.**UV-visible spectra**. Spectra were obtained on an Agilent Cary 50 UV-Vis-Spectrophotometer by using a rectangular quartz cell (Hellma, 100-QS, 45 × 12.5 × 12.5 mm, pathlength: 10 mm, chamber volume: 3.5 mL), at 23 °C. Fluorescence spectroscopic studies were recorded on a JASCO FP8500 spectrofluorometer in a 10 mm path-length semi-micro quartz cuvette (Starna), at 25 °C.

Synthesis and analytical characterization of the bimodal probe DTPA-NH-Tz-S-IRDye700DX

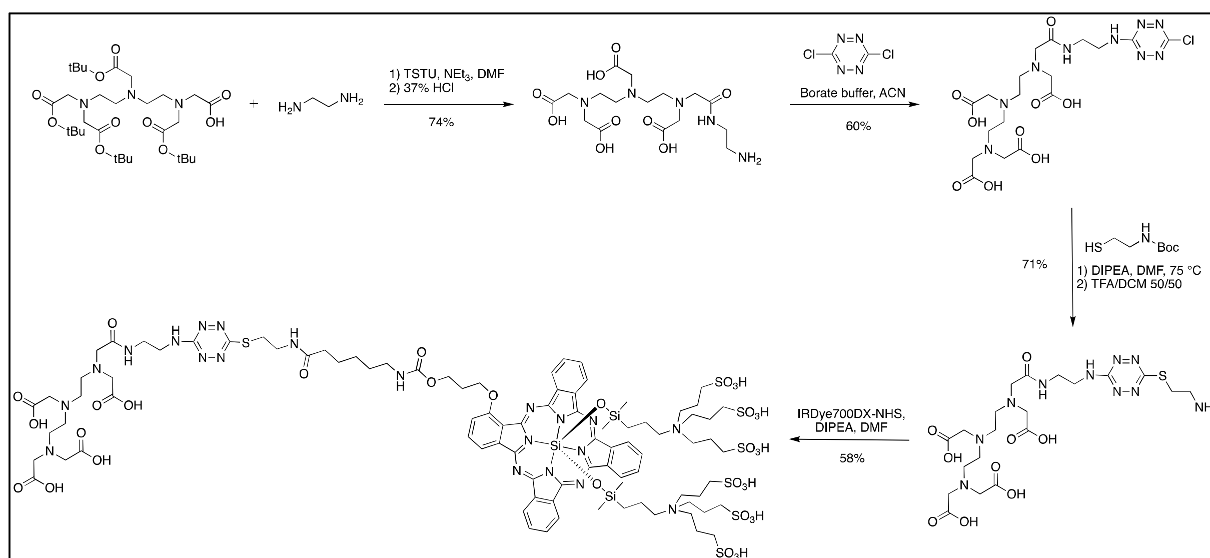
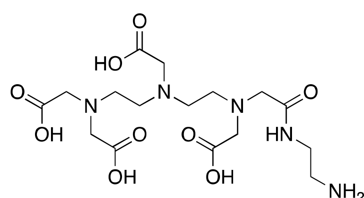


Figure S1. Synthesis of the bimodal probe DTPA-NH-Tz-S-IRDye700DX.

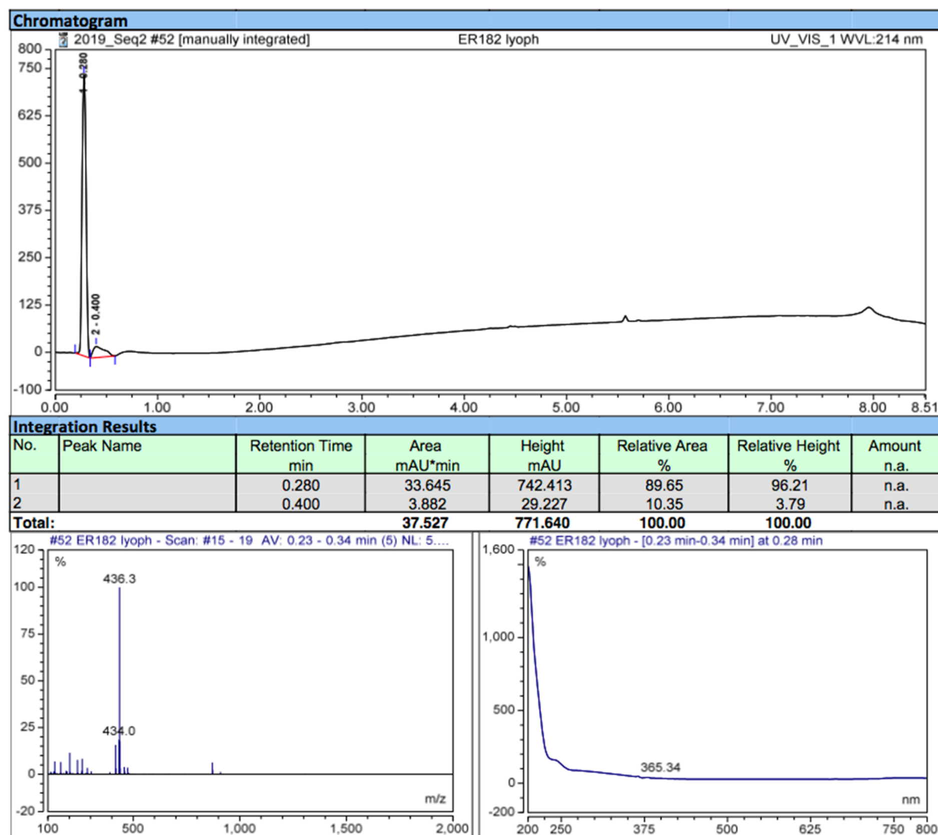


DTPA-NH₂. TSTU (121 mg, 0.402 mmol, 1.2 equiv.) was added to a solution of DTPA(tBu)$_4$ ester (207 mg, 0.335 mmol, 1 equiv.) in DMF (2 mL) with NEt_3 (140 µL, 1.005 mmol, 3 equiv.). The solution was stirred at room temperature for 1 h. Ethylenediamine (224 µL, 3.35 mmol, 10 equiv.) was then added to the activated ester and the reaction mixture was stirred for 2 h at room temperature to give a white suspension. This mixture was concentrated under vacuum and purified on semi-preparative HPLC on a BetaBasic-18 column (A: H_2O 0.1% TFA, B: MeCN 0.1% TFA; with the following gradient program: 5% of B for 10 min, 5% to 70% of B in 50 min, at a flow rate of 20 mL/min) to obtain a white solid. HCl 37% (6 mL) was added to the solid and the mixture was concentrated under vacuum. After lyophilization, the expected compound (brown solid) was obtained as a hydrochloride (3 HCl) salt (134.4 mg, 74%). The compound was analyzed with RP-HPLC-MS (Figure S2), ^{1}H- and ^{13}C-NMR and HRMS (Figure S3).

RP-HPLC-MS: t_r = injection peak, *m/z* calculated for $C_{16}H_{29}N_5O_9$ [M+H]$^+$ 436.2, found 436.3.

^{1}H-NMR (500 MHz, D_2O): δ = 3.1-3.25 (m, 6H), 3.45-3.6 (m, 6H), 3.68 (s, 2H), 4.14 (s, 4H), 4.19 (s, 4H) ppm; ^{13}C-NMR (126 MHz, D_2O): δ = 37.0 (CH_2), 38.8 (CH_2), 49.6 (CH_2), 49.9 (CH_2), 52.9 (CH_2), 53.9 (CH_2), 55.4 (CH_2), 56.2 (CH_2), 167.5 (C), 169.2 (C), 169.4 (C), 173.3 (C) ppm.

HRMS: *m/z* calculated for $C_{16}H_{29}N_5O_9$ [M+H]$^+$ 436.20447, found 436.20408.



No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		0.280	33.645	742.413	89.65	96.21	n.a.
2		0.400	3.882	29.227	10.35	3.79	n.a.
Total:			37.527	771.640	100.00	100.00	

Figure S2. RP-HPLC-MS analysis of DTPA-NH_2. (Top) RP-HPLC chromatogram. (Bottom) ESI (+)-LRMS and UV-Vis absorbance spectrum.

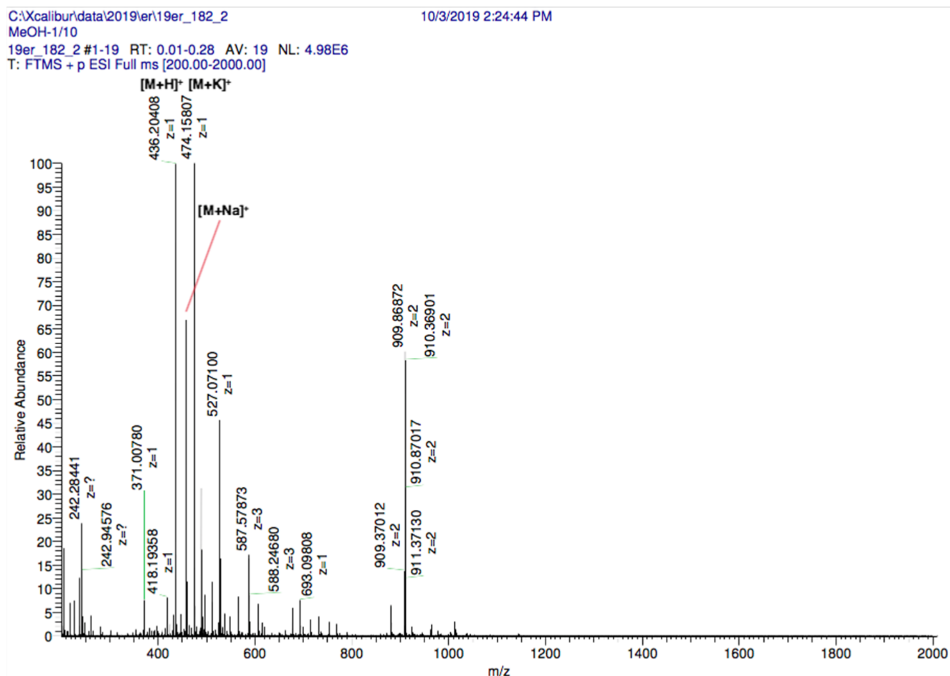
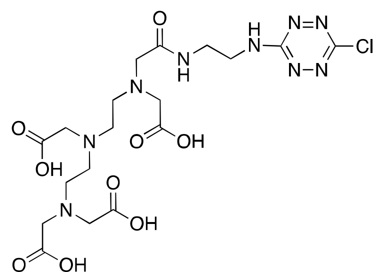


Figure S3. HRMS analysis of DTPA-NH_2.



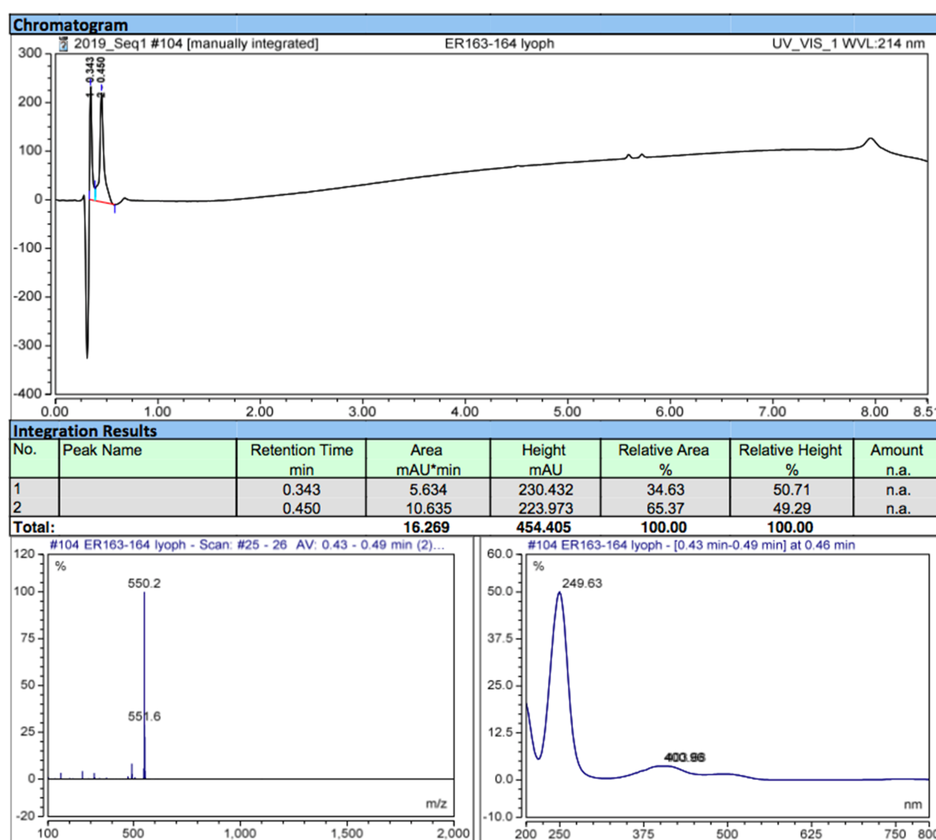
DTPA-NH-Tz-Cl. A solution of 3,6-dichloro-1,2,4,5-tetrazine (44.9 mg, 0.298 mmol, 1.2 equiv.) in acetonitrile (1 mL) was added to a solution of DTPA-NH_2 (134.4 mg, 0.248 mmol, 1 equiv.) in 0.1 M borate buffer pH 8.0 (4 mL). The resulting orange solution was stirred at room temperature for 1 h. The mixture was concentrated under vacuum and purified by semi-preparative HPLC on a BetaBasic-18 column (A: H_2O 0.1% TFA, B: MeCN 0.1% TFA; with the following gradient program: 2% of B for 10 min, 2% to 40% of B in 50 min, at a flow rate of 20 mL/min) to give an orange powder (98 mg, 60%). The compound was analyzed with RP-HPLC-MS (Figure S4), ^{1}H- and ^{13}C-NMR and HRMS (Figure S5).

Ionic chromatography: 1 TFA salt.

RP-HPLC-MS: tr = 0.45 min, m/z calculated for $C_{18}H_{28}ClN_9O_9$ $[M+H]^+$ 550.2, found 550.2.

^{1}H-NMR (500 MHz, D_2O): δ = 3.26 (t, J=5.8 Hz, 2H), 3.30 (t, J=6.0 Hz, 2H), 3.43 (t, J=5.8 Hz, 2H), 3.53 (t, J=6.1 Hz, 2H), 3.59 (t, J=5.4 Hz, 2H), 3.73 (t, J=5.5 Hz, 2H), 3.77 (s, 2H), 3.92 (s, 2H), 3.97 (s, 2H), 4.05 (s, 4H) ppm; ^{13}C-NMR (126 MHz, D_2O): δ = 38.4 (CH_2), 40.1 (CH_2), 50.3 (CH_2), 50.6 (CH_2), 52.2 (CH_2), 54.0 (CH_2), 55.8 (CH_2), 56.0 (CH_2), 56.4 (CH_2), 158.7 (C), 161.6 (C), 168.1 (C), 170.5 (C), 170.8 (C), 172.6 (C) ppm.

HRMS: m/z calculated for $C_{18}H_{28}ClN_9O_9$ $[M+H]^+$ 550.17779, found 550.17602.



No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		0.343	5.634	230.432	34.63	50.71	n.a.
2		0.450	10.635	223.973	65.37	49.29	n.a.
Total:			16.269	454.405	100.00	100.00	

Figure S4. RP-HPLC-MS analysis of DTPA-NH-Tz-Cl (Top) RP-HPLC chromatogram. (Bottom) ESI (+)-LRMS and UV-Vis absorbance spectrum.

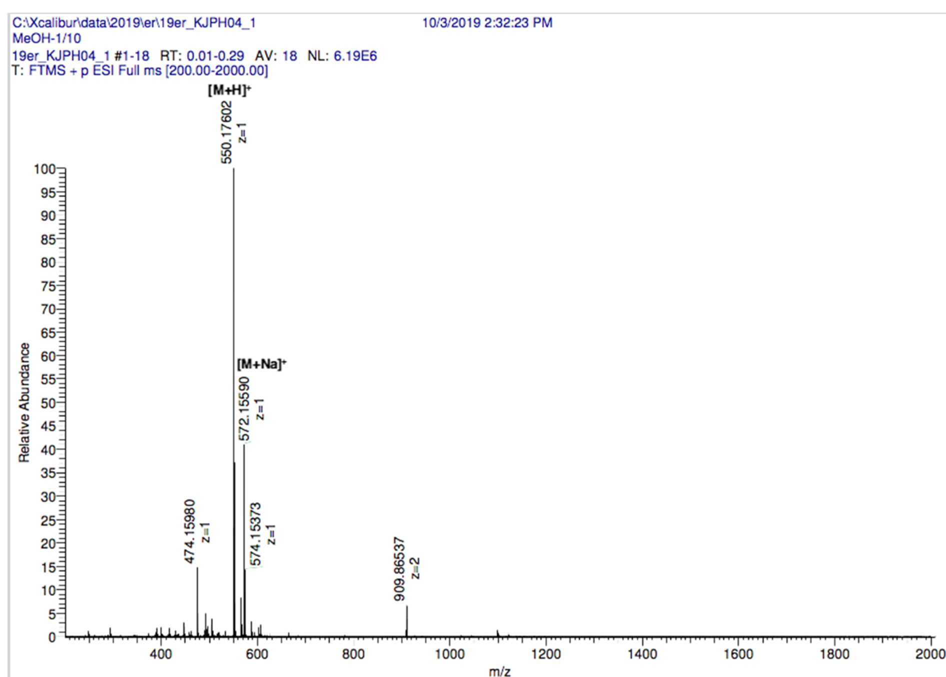
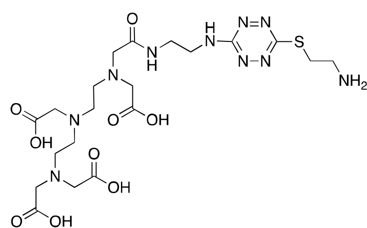


Figure S5. HRMS analysis of DTPA-NH-Tz-Cl.



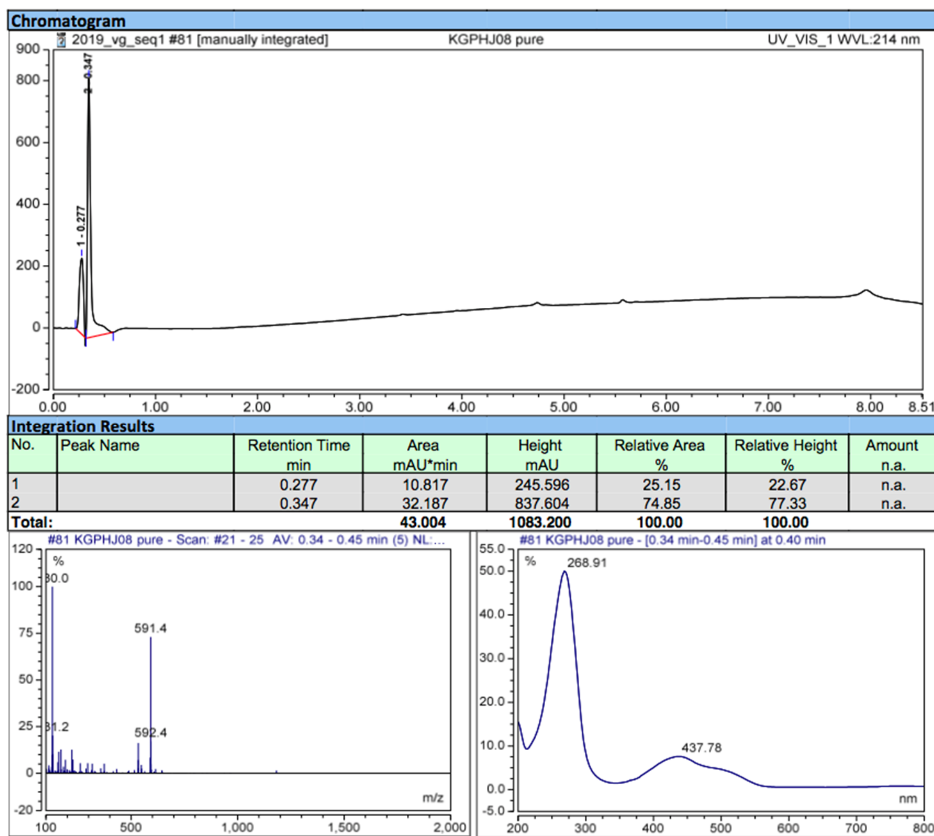
DTPA-NH-Tz-NH_2. Tert-butyl (2-mercaptoethyl)carbamate (14.9 mg, 84.1 µmol, 1.2 equiv.) in DMF (2 mL) was added to a solution of DTPA-NH-Tz-Cl (50 mg, 75.3 µmol, 1 equiv.) in DMF (2 mL) and DIPEA (104.5 µL, 602 µmol, 8 equiv.). The resulting orange solution was stirred at 75 °C. After 2 h, the solvent was removed under vacuum. CH_2Cl_2 (2 mL) and TFA (2 mL) were added and the resulting solution was stirred at room temperature for 2 h. After concentration under vacuum the mixture was purified by semi-preparative HPLC on a BetaBasic-18 column (A: H_2O 0.1% TFA, B: MeCN 0.1% TFA; with the following gradient program: 2% of B for 10 min, 2% to 40% of B in 50 min, at a flow rate of 20 mL/min) to afford an orange powder (43.8 mg, 71%). The compound was analyzed with RP-HPLC-MS (Figure S6), ^{1}H- and ^{13}C-NMR and HRMS (Figure S7).

Ionic chromatography: 2 TFA salt.

RP-HPLC-MS: t_r = 0.35 min, *m/z* calculated for $C_{20}H_{34}N_{10}O_9S$ $[M+H]^+$ 591.2, found 591.4.

^{1}H-NMR (500 MHz, D_2O): δ = 3.27 (m, 4H), 3.43 (m, 4H), 3.55 (m, 6H), 3.70 (m, 2H), 3.76 (s, 2H), 3.91 (s, 2H), 3.98 (s, 2H), 4.05 (s, 4H) ppm; ^{13}C-NMR (126 MHz, D_2O): δ = 27.8 (CH_2), 38.3 (CH_2), 38.8 (CH_2), 39.9 (CH_2), 50.3 (CH_2), 50.6 (CH_2), 52.2 (CH_2), 54.0 (CH_2), 55.8 (CH_2), 56.0 (CH_2), 56.4 (CH_2), 161.0 (C), 163.9 (C), 168.1 (C), 170.4 (C), 170.7 (C), 172.6 (C), ppm.

HRMS: *m/z* calculated for $C_{20}H_{34}N_{10}O_9S$ $[M+H]^+$ 591.23103, found 591.22921.



No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		0.277	10.817	245.596	25.15	22.67	n.a.
2		0.347	32.187	837.604	74.85	77.33	n.a.
Total:			43.004	1083.200	100.00	100.00	

Figure S6. RP-HPLC-MS analysis of DTPA-NH-Tz-NH2. (Top) RP-HPLC chromatogram. (Bottom) ESI (+)-LRMS and UV-Vis absorbance spectrum.

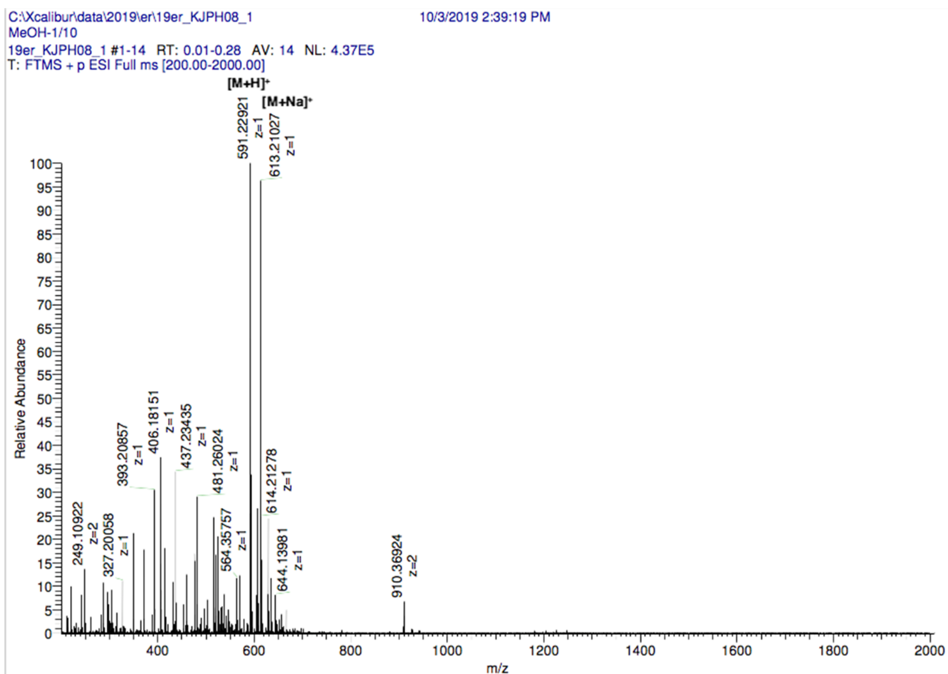
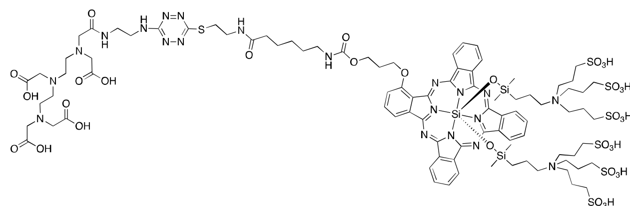
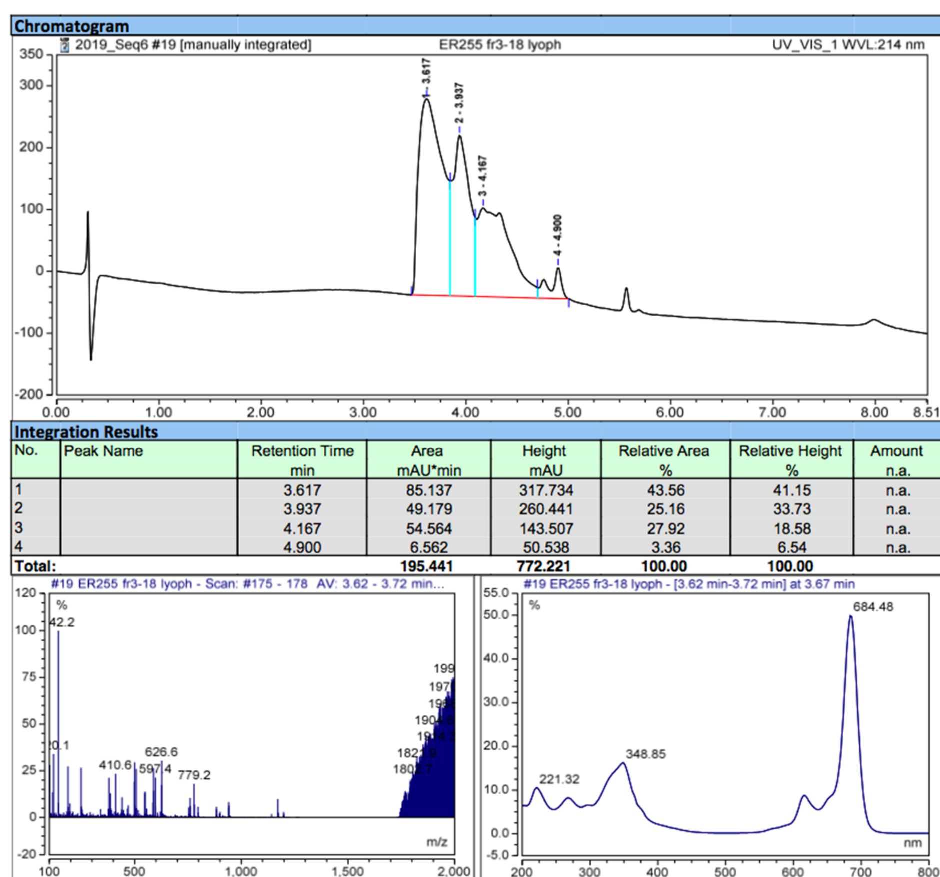


Figure S7. HRMS analysis of DTPA-NH-Tz-NH2.



DTPA-NH-Tz-S-IRDye700DX. IRDye700DX (6.3 mg, 3.22 µmol, 1 equiv.) in DMF (1 mL) with DIPEA (8 µL, 45.9 µmol, 14 equiv.) was added to a suspension of DTPA-NH-Tz-NH$_2$ (3.43 mg, 4.19 µmol, 1.3 equiv.) in DMF (1 mL) with DIPEA (8 µL, 45.9 µmol, 14 equiv.). The green solution was stirred at RT overnight. After concentration under vacuum, the mixture was purified by semi-preparative HPLC on an Hypersil column (A: TEAB 50 mM, B: MeCN with the following gradient program: 20% of B for 5 min, 20% to 60% of B in 60 min, at a flow rate of 3.5 mL/min) to give a blue/green powder (4.4 mg, 58%). The compound was analyzed with RP-HPLC-MS (Figure S8).

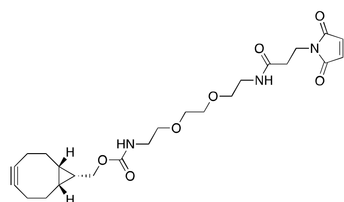
RP-HPLC-MS: t_r = 3.6 min., *m/z* calculated for $C_{90}H_{129}N_{21}O_{33}S_7Si_3$ $[M+2H]^{2+}$ 1170.8, found 1171.4, *m/z* calculated for $[M+3H]^{3+}$ 780.9, found 779.2.



No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		3.617	85.137	317.734	43.56	41.15	n.a.
2		3.937	49.179	260.441	25.16	33.73	n.a.
3		4.167	54.564	143.507	27.92	18.58	n.a.
4		4.900	6.562	50.538	3.36	6.54	n.a.
Total:			195.441	772.221	100.00	100.00	

Figure S8. RP-HPLC-MS analysis of compound DTPA-NH-Tz-S-IRDye700DX (Top) RP-HPLC chromatogram. (Bottom) ESI (+)-LRMS and UV-Vis absorbance spectrum.

Site-specific labeling of 7D12 with the bimodal probe



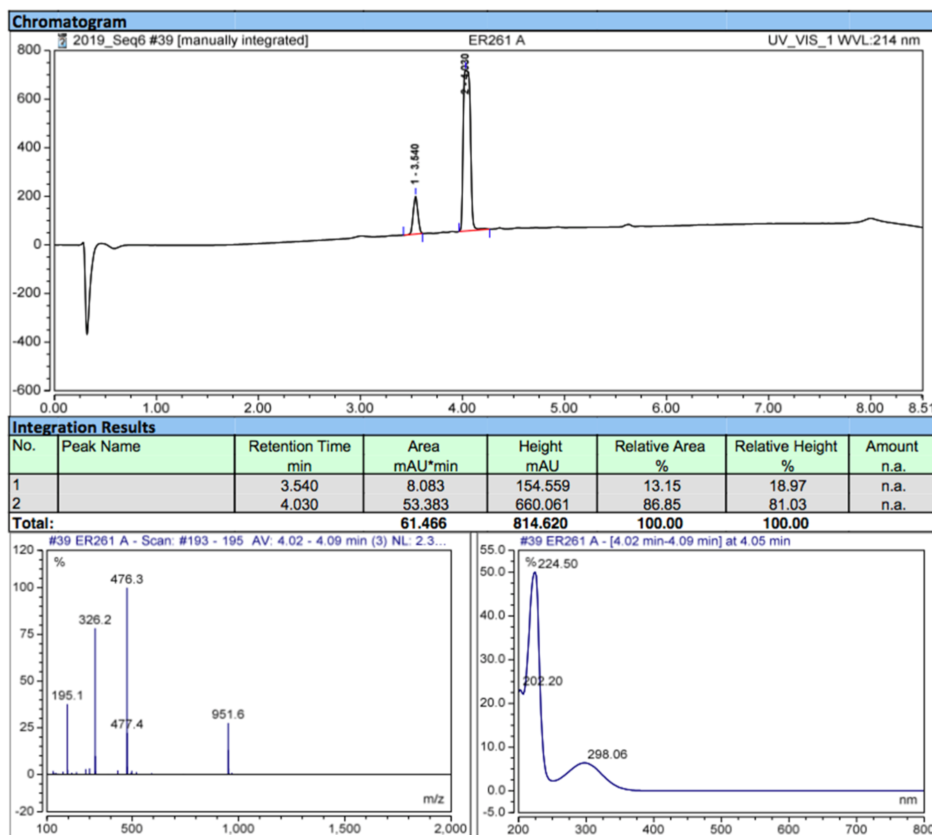
BCN-PEG$_2$-maleimide. A solution of maleimide-NHS (13.5 mg, 50.1 µmol, 1.1 equiv.) in DMF (0.5 mL) was added to a solution of BCN-PEG$_2$-NH$_2$ (15 mg, 46 µmol, 1 equiv.) in DMF (0.5 mL) and DIPEA (16.1 µL, 92 µmol, 2 equiv.). The resulting yellowish solution was stirred at RT. After 1 h, the reaction mixture was concentrated under vacuum and purified by semi-preparative HPLC on a BetaBasic-18 column (A: H_2O 0.1% FA, B: MeCN 0.1% FA; with the following gradient program: 15% of B for 5 min, 15% to 65% of B in 50 min, at a flow rate of 20 mL/min) to give a colorless oil (9.1 mg, 41%, purity: 87%). The compound was analyzed with RP-HPLC-MS (Figure S9), ^1H- and ^{13}C-NMR and HRMS (Figure S10).

Note: this compound proved to be unstable, giving rise to unidentified products over time, even when stored at -80 °C. It was aliquoted as a 20 mM solution in DMSO and kept at -20 °C.

RP-HPLC-MS: t_r = 4 min, *m/z* calculated for $C_{24}H_{33}N_3O_7$ $[M+H]^+$ 476.2, found 476.3.

^{1}H-NMR (500 MHz, $CDCl_3$): δ = 0.8-1.0 (m, 3H), 1.25 (m, 4H), 2.1-2.3 (m, 4H), 2.53 (t, J=7.2 Hz, 2H), 3.3-3.7 (m, 10H), 3.84 (t, J=7.2 Hz, 2H), 4.1 (m, 2H), 5.22 (br, 1H), 6.12 (br, 1H), 6.70 (d, 2H) ppm.

HRMS: *m/z* calculated for $C_{24}H_{33}N_3O_7$ $[M+H]^+$ 476.23185, found 498.22166.



No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		3.540	8.083	154.559	13.15	18.97	n.a.
2		4.030	53.383	660.061	86.85	81.03	n.a.
Total:			61.466	814.620	100.00	100.00	

Figure S9. RP-HPLC-MS analysis of BCN-PEG2-maleimide. (Top) RP-HPLC chromatogram. Note: the impurity at tr = 3.54 min corresponds to the hydrolyzed or hydrated product (mass m/z= 494). (Bottom) ESI (+)-LRMS and UV-Vis absorbance spectrum.

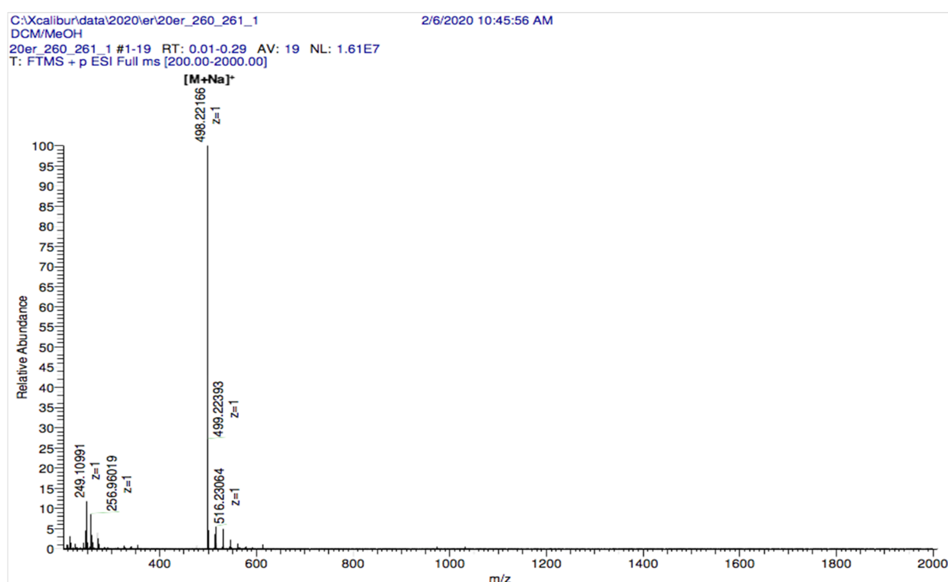


Figure S10. HRMS analysis of BCN-PEG2-maleimide.

7D12-BCN.

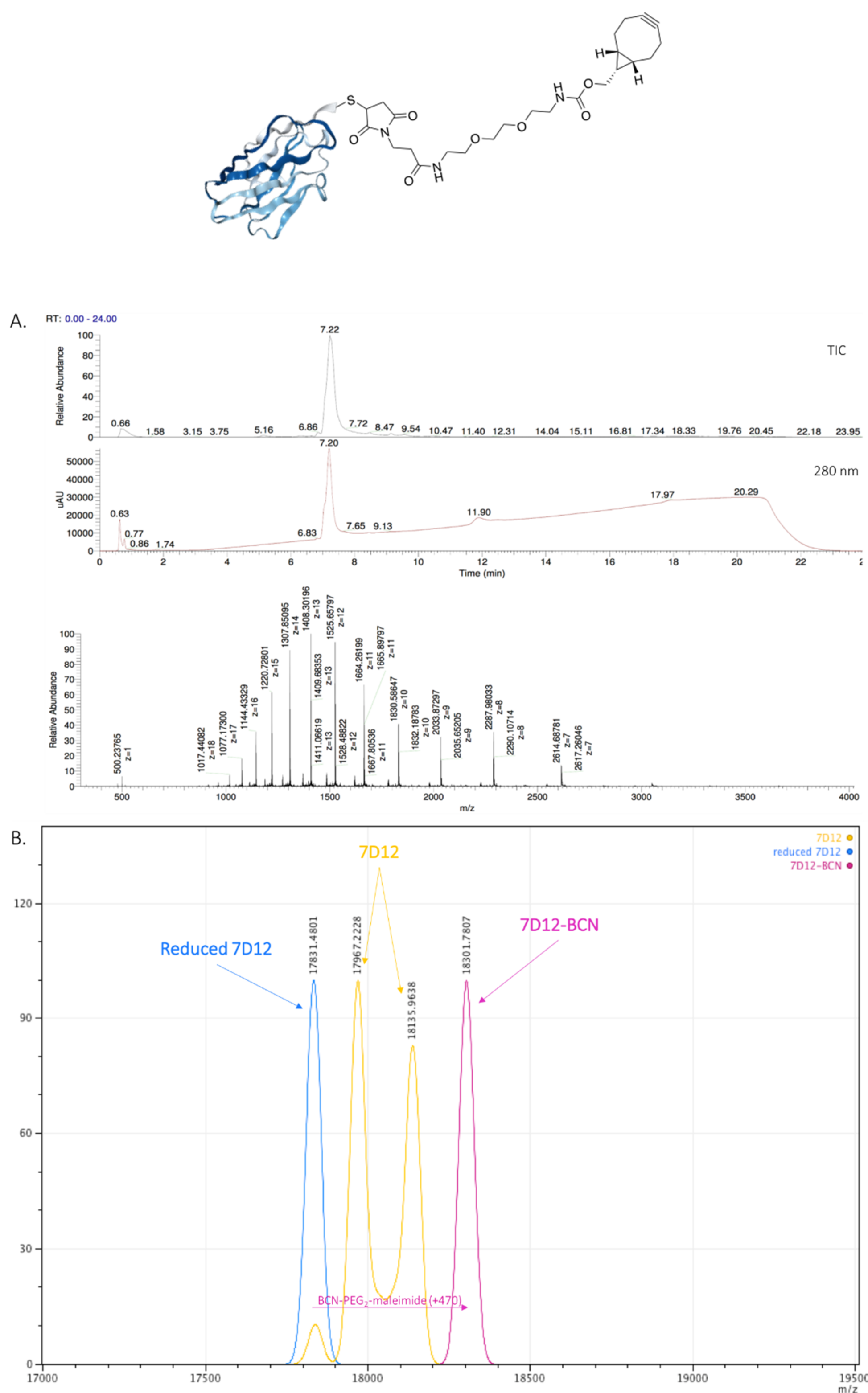


Figure S11. RP-HPLC-MS (Orbirap) analysis of compound 7D12-BCN. A. (Top) RP-HPLC chromatograms (TIC and 280 nm). (Bottom) Multicharged ESI-MS spectrum. B. Deconvoluted MS spectrum of native 7D12, reduced 7D12 and 7D12-BCN.

DTPA-IRDye700DX-7D12

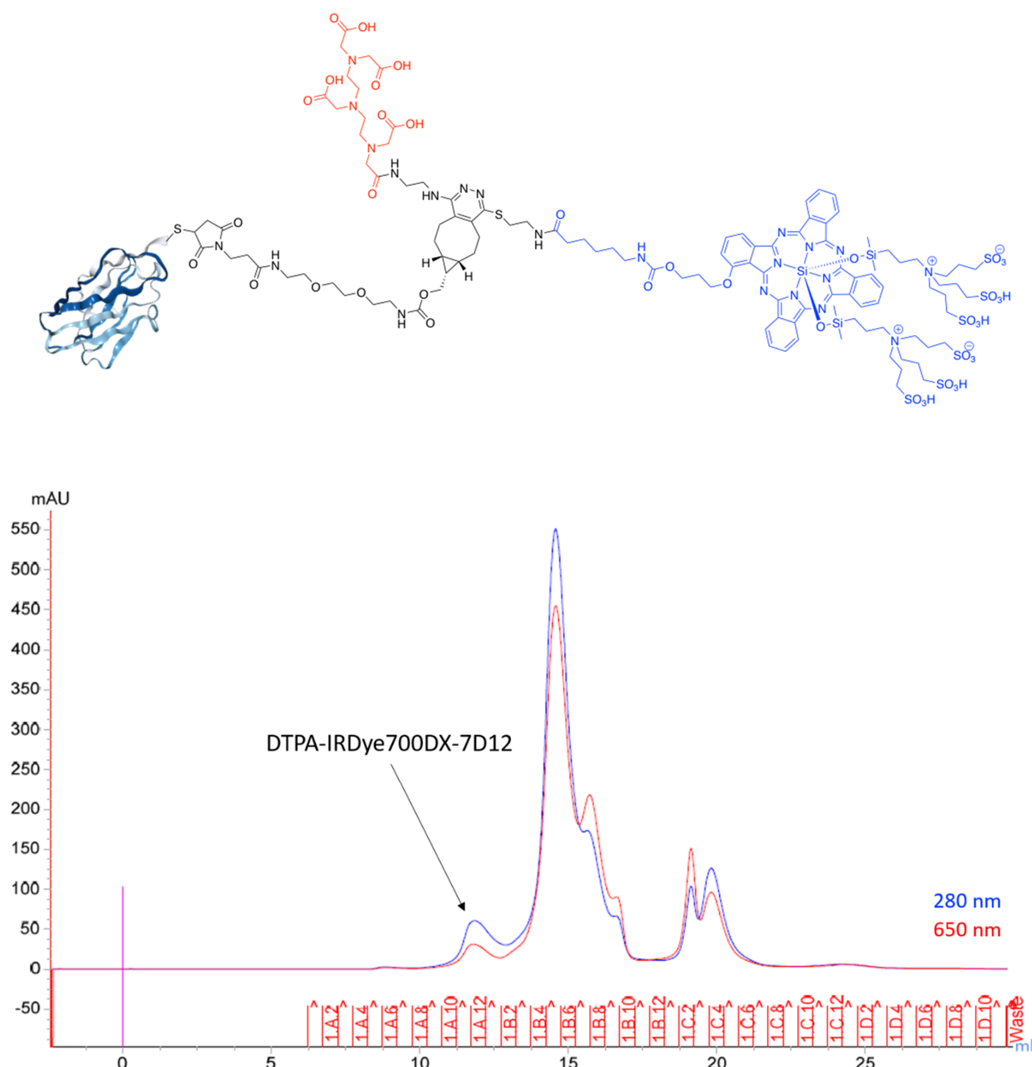


Figure S12. FPLC purification chromatogram of the compound DTPA-IRDye700DX-7D12.

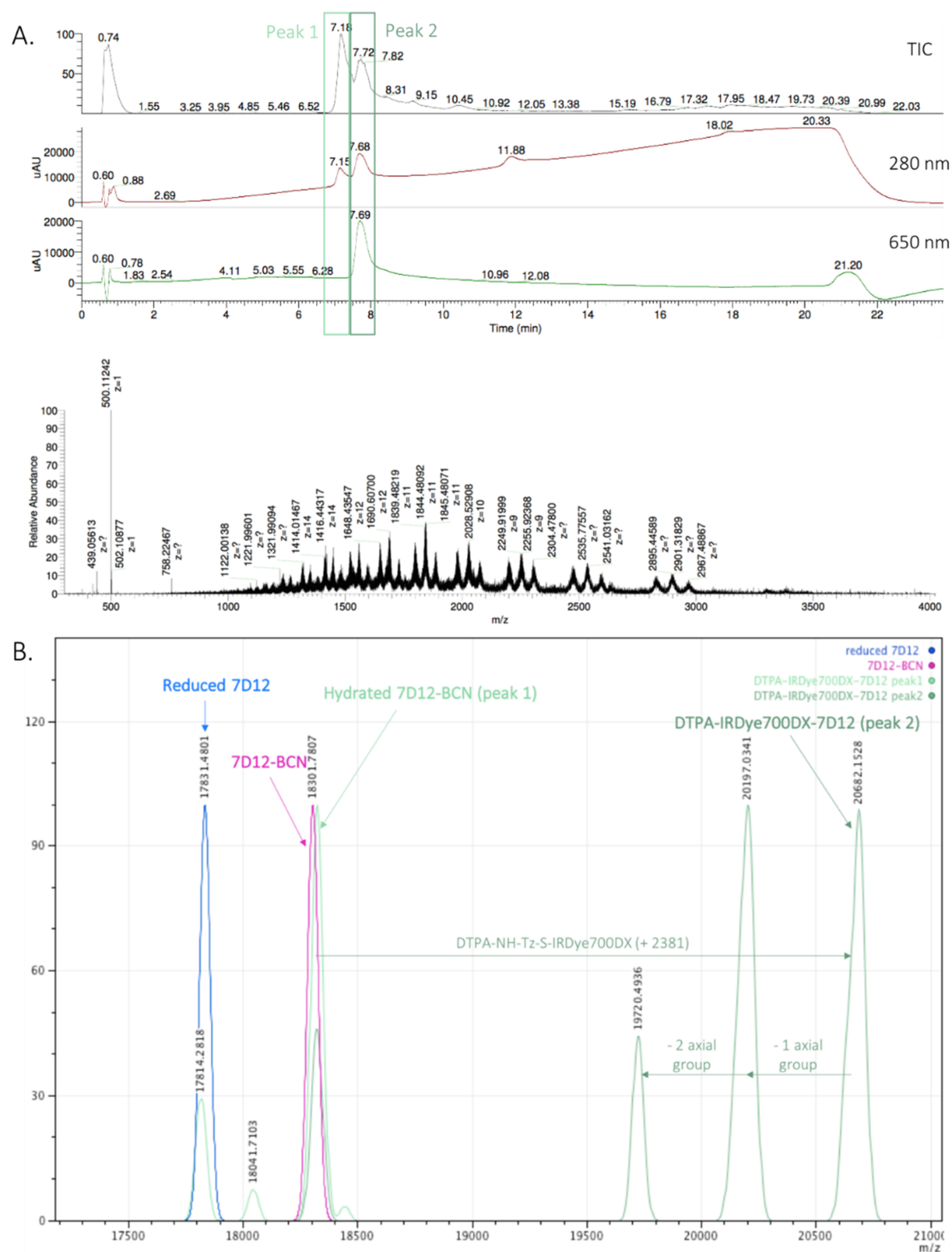


Figure S13. RP-HPLC-MS (Orbirap) analysis of compound DTPA-IRDye700DX-7D12. A. (Top) RP-HPLC chromatograms (TIC, 280 nm and 650 nm). (Bottom) Multicharged ESI-MS spectrum. B. Deconvoluted MS spectrum of reduced 7D12, 7D12-BCN and DTPA-IRDye700DX-7D12.

Spectral properties of compounds

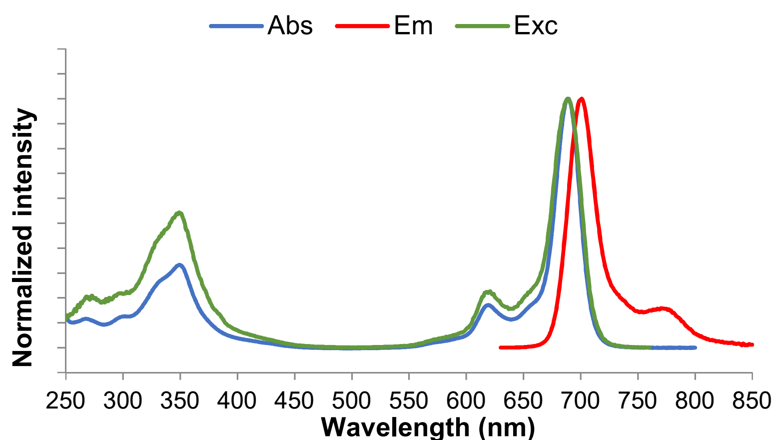


Figure S14. Normalized photophysical spectra (absorbance, excitation and emission) of compound IRDye700DX. $\lambda_{Ex, max}$ = 689 nm, $\lambda_{Em, max}$ = 701 nm.

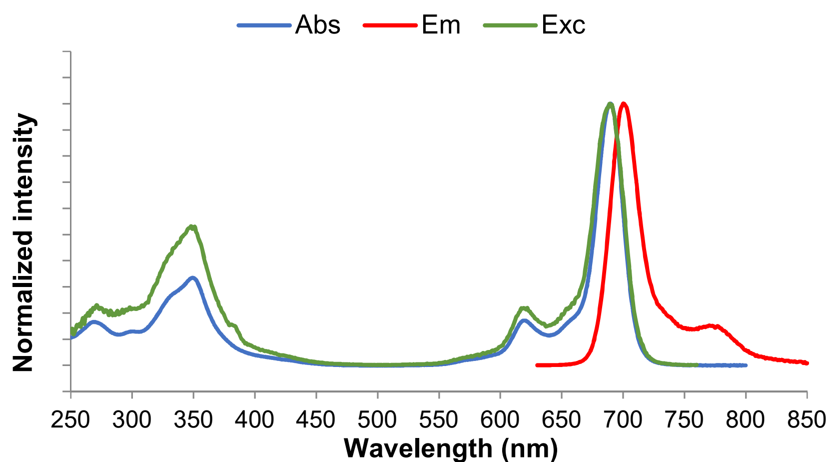


Figure S15. Normalized photophysical spectra (absorbance, excitation and emission) of bimodal probe DTPA-NH-Tz-S-IRDye700DX. $\lambda_{Ex, max}$ = 689 nm, $\lambda_{Em, max}$ = 700 nm

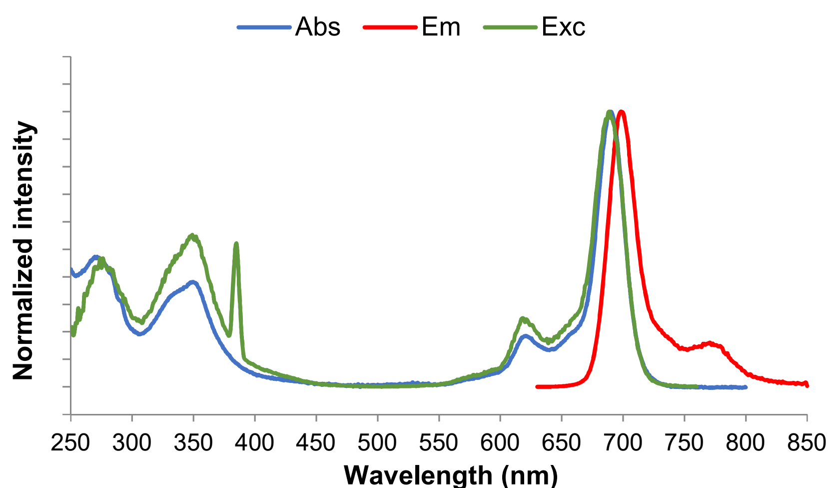


Figure S16. Normalized photophysical spectra (absorbance, excitation and emission) of bimodal conjugate DTPA-IRDYe700DX-7D12. $\lambda_{Ex, max}$ = 689 nm, $\lambda_{Em, max}$ = 698 nm

Stability test by RP-HPLC in radiolabeling-like conditions

8.3 µL of 0.05 M HCl and 16.6 µL of 0.5 M MES were added to a solution of DTPA-IRDye700DX-7D12 in PBS (1.04 mg/mL, 20 µg). The resulting solution was stirred in a thermomixer (900 rpm, 25 °C). After 0 min, 1 h, 2 h, 4 h a sample (15 µL) of solution was collected and analyzed by RP-HPLC-MS (Orbitrap) at a wavelength of 280 nm and 650 nm (Figure S17). The separation was achieved on a Thermo Scientific™ MAbPac™ column (4 µm, 50 × 21 mm) with the following eluents: A: H_2O, 0.1% FA + 0.02% TFA and B: MeCN/H_2O (90 : 10, v/v), 0.1% FA + 0.02% TFA, and a linear gradient program: 5% to 100% of B in 15 min, 100% B for 3 min, 100% to 5% B in 0.1 min and 5% B for 6 min, at a flow rate of 0.3 mL/min, at 80 °C.

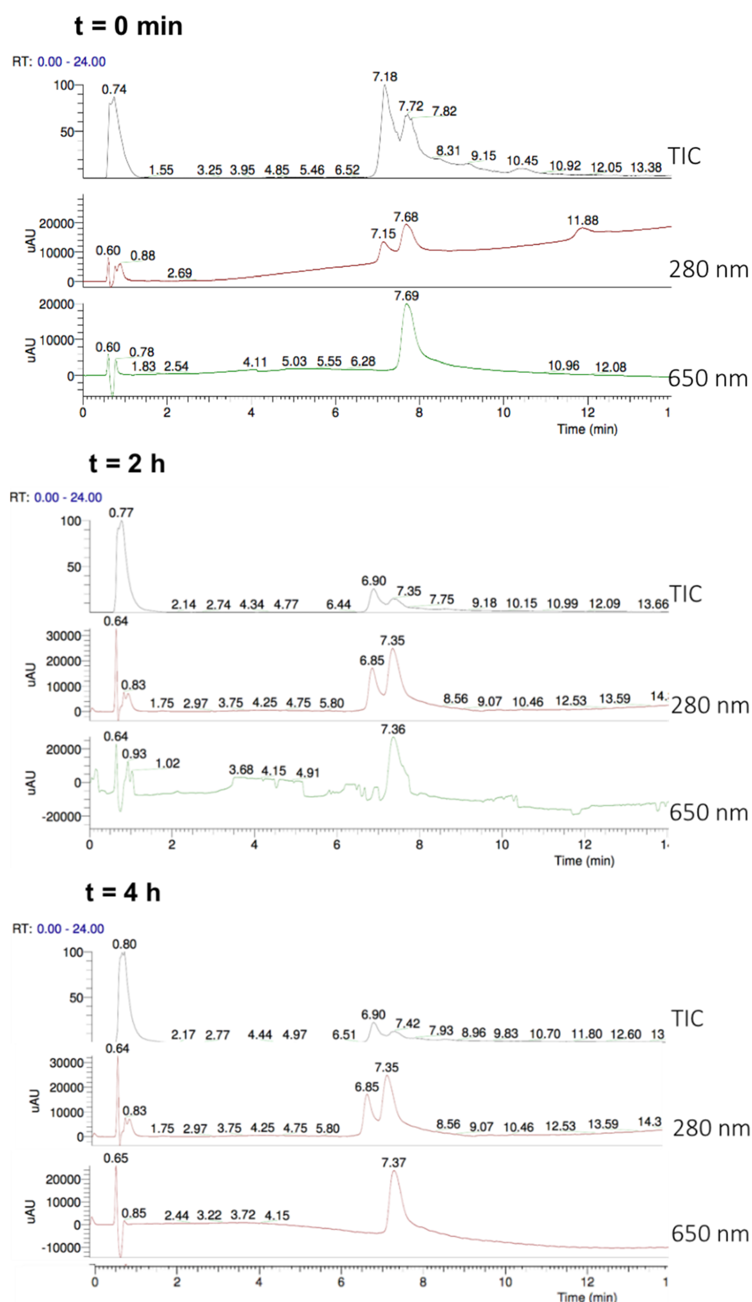


Figure S17. RP-HPLC-MS chromatograms (TIC, 280 nm and 650 nm) of compound DTPA-IRDye700DX-7D12 after 0 min, 2 h and 4 h of incubation in radiolabeling-like conditions.