Supporting Information for:

Comparison of cRGDfK Peptide Probes with Appended Shielded Heptamethine Cyanine Dye (s775z) for Near Infrared Fluorescence Imaging of Cancer

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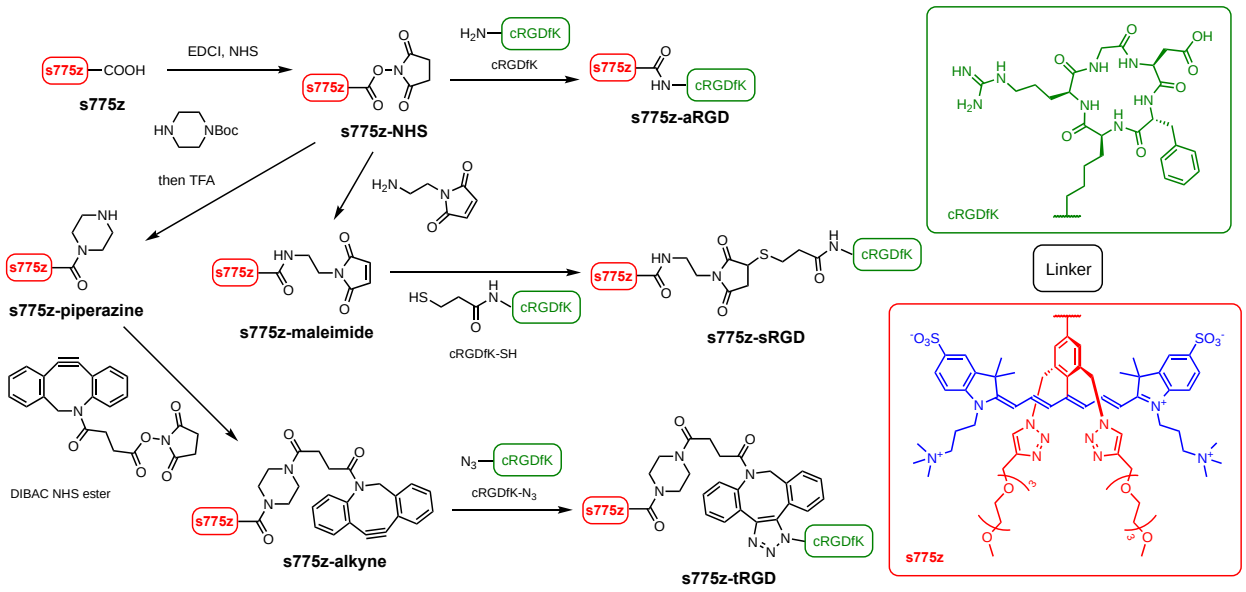
General

Reagents and solvents were purchased from Sigma-Aldrich, VWR, Oakwood, Thermo Fisher and TCI and used without further purification unless stated otherwise. Column chromatography was performed using Biotage Sfär columns. ^{1}H NMR spectra were recorded on a Bruker 500 NMR spectrometer. Chemical shifts are presented in ppm and referenced by residual solvent peak. High-resolution mass spectrometry (HRMS) was performed using a time-of-flight (TOF) analyzer with electrospray ionization (ESI). Absorption spectra were recorded on an Evolution 201 UV/vis spectrometer with Thermo Insight software. Fluorescence spectra were collected on a Horiba Fluoromax-4 fluorometer with FluoroEssence software. Analyte solutions were prepared in phosphate buffered saline (Thermo Fisher). All absorption and fluorescence spectra were collected using quartz cuvettes (1 mL, 1 cm path length; for emission spectra, slit width = 3 nm).

Synthesis and Compound Characterization



Scheme S1. (Copy of manuscript Scheme 2) Synthesis and characterization of **s775z** and **s775z-aRGD** have been reported previously by our group.[1] DIBAC NHS ester[2], cRGDfK-SH[3] and cRGDfK-N_3[4] were synthesized according to literature procedures.

Zwitterionic pentamethine cyanine dye **650z** was synthesized according to a literature procedure.[5]

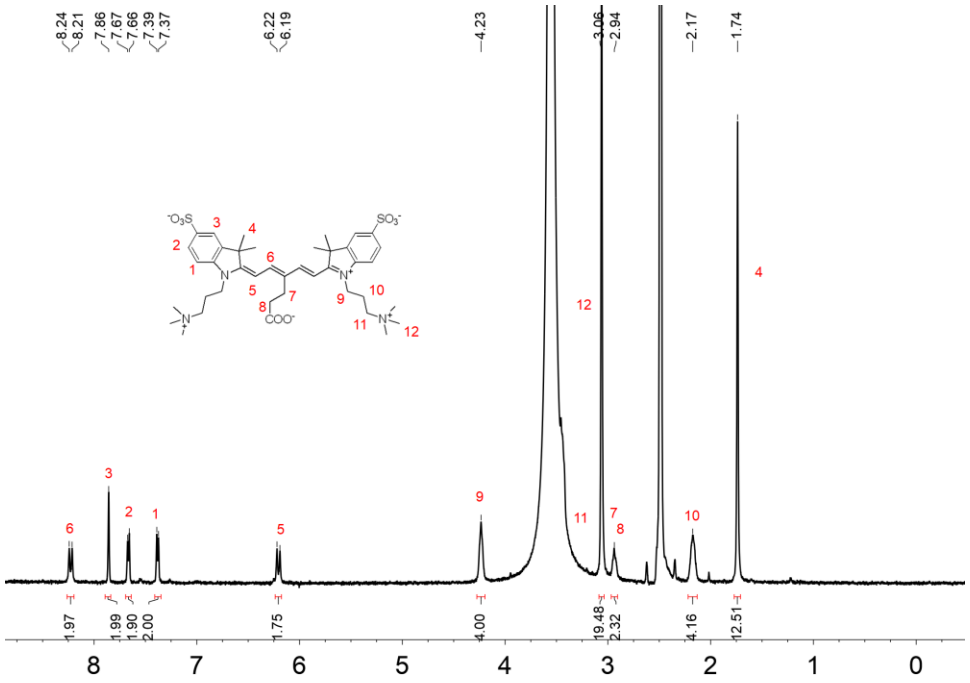
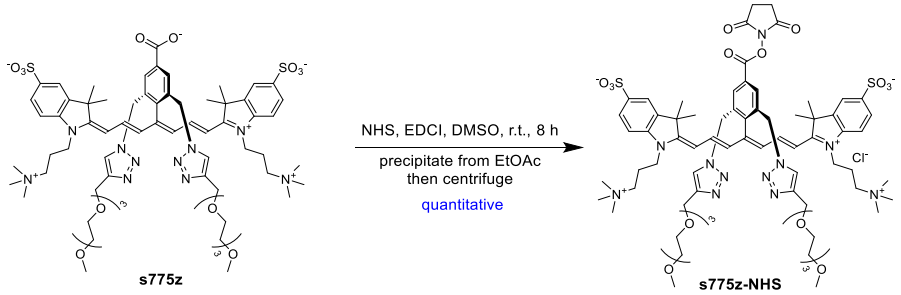


Figure S1. ^{1}H NMR spectrum (500 MHz, DMSO-d_6, 25 ^{o}C) of **650z**



Scheme S2. Synthesis of **s775z-NHS**

A mixture of the **s775z**, N-hydroxysuccinimide (3 eq) and N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (3 eq) in DMSO (0.1 mL for 1 mg of **s775z**) was stirred at room temperature for 8 h in the dark. The reaction mixture was transferred into a centrifuge tube. Ethyl acetate (5 mL for 1 mL of DMSO) was added to the tube and mixture was centrifuged at 3600 rpm for 5 min. The supernatant was discarded and the green solid was washed with ethyl acetate (2x) and diethyl ether (1x) then dried in vacuo to afford **s775z-NHS** with quantitative yield.

^{1}H NMR (500 MHz, D_2O, 25 ^{o}C) δ (ppm): 8.67 (s, 2H), 7.99 (s, 2H), 7.72 (d, *J* = 8.4 Hz, 2H), 7.66 (s, 2H), 7.25 (d, *J* = 8.4 Hz, 2H), 6.68 (d, *J* = 13.2 Hz, 2H), 6.57 (dd, *J* = 13.2, 13.2 Hz, 2H), 6.22 (d, *J* = 13.5 Hz, 2H), 5.52 (s, 4H), 4.33 (s, 4H), 4.08 (br s, 4H), 3.45-3.30 (m, 28H), 3.19 (s, 6H), 3.05 (s, 18H), 2.98 (s, 4H), 2.19 (br s, 4H), 1.02 (s, 12H).

HRMS (ESI-TOF) m/z: $[M + H]^{2+}$ calcd for $C_{72}H_{101}N_{11}O_{18}S_2^{2+}$ 735.8378, found 735.8379.

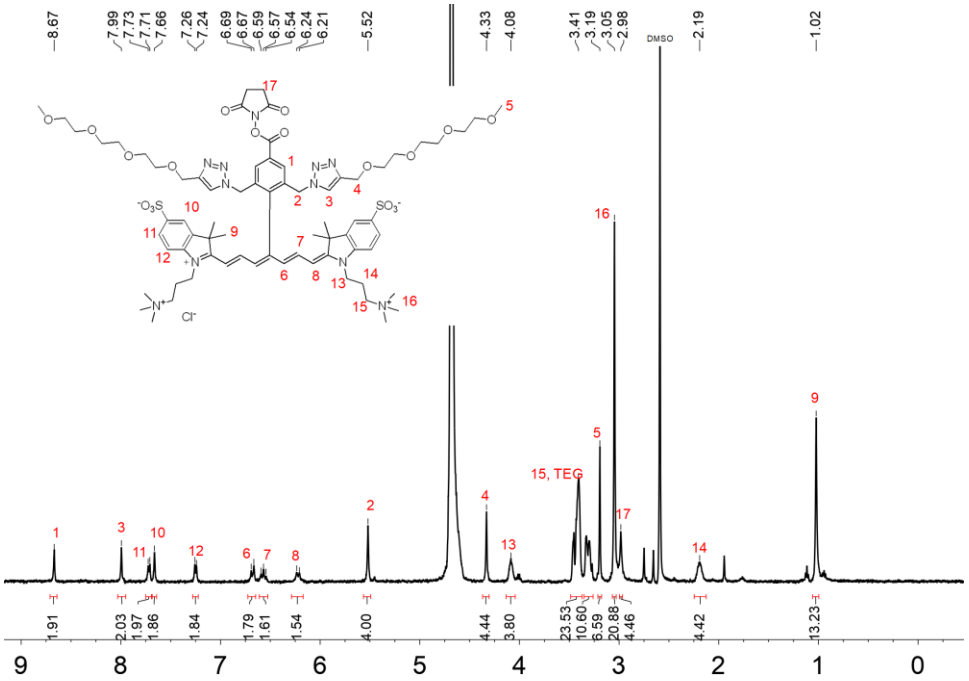
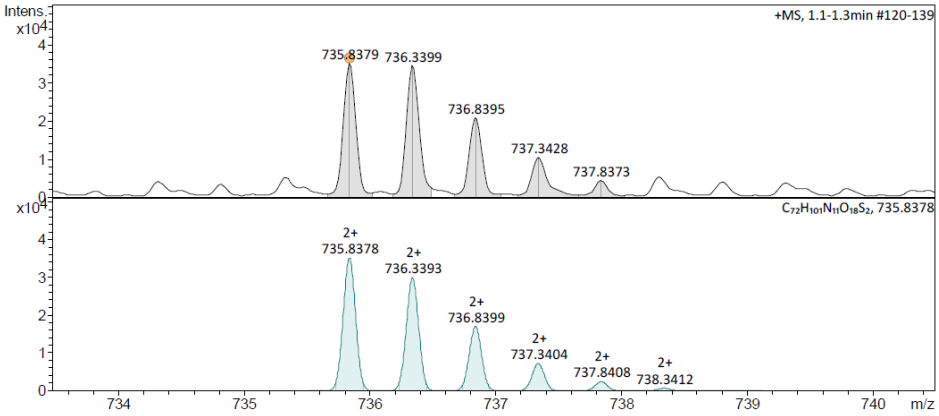
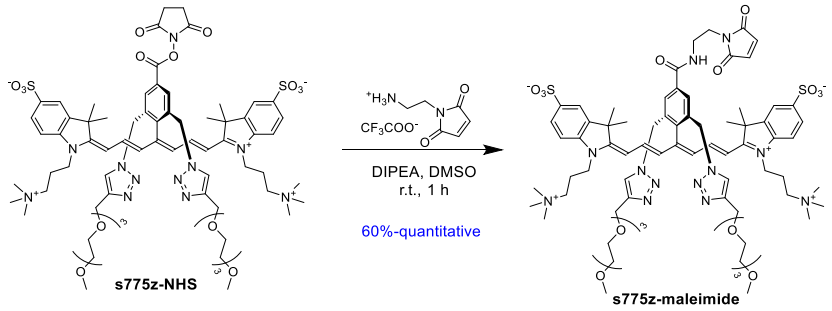


Figure S2. ^{1}H NMR spectrum (500 MHz, D_2O, 25 ^{o}C) of **s775z-NHS**



Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# mSigma	Score	rdb	e¯ Conf	N-Rule
735.8379	1	C72H101N11O18S2	735.8378	-0.1	83.0	1	100.00	28.0	even	ok

Figure S3. HRMS spectrum of **s775z-NHS**



Scheme S3. Synthesis of **s775z-maleimide**

A mixture of the **s775z-NHS**, *N*-(2-aminoethyl) maleimide trifluoroacetate salt (3 eq) and DIPEA (6 eq) in DMSO (0.1 mL for 1 mg of **s775z-NHS**) was stirred at room temperature for 1 h in the dark.

For small scale reactions (<10 mg), the reaction was transferred into a centrifuge tube. Ethyl acetate (5 mL for 1 mL of DMSO) was added to the tube and mixture was centrifuged at 3600 rpm for 5 min. The supernatant was discarded and the green solid was washed with ethyl acetate (2x) and diethyl ether (1x) then dried in vacuo to afford **s775z-maleimide** with quantitative yield.

For larger scale reactions (>50 mg), there is significantly increased amount of by product. The reaction mixture was directly purified by reverse phase column chromatography (C18, 20% - 35% MeOH in H_2O with 0.5% TFA) to afford **s775z-maleimide** (green solid, ~60% yield).

^{1}H NMR (500 MHz, D_2O, 25 ^{o}C) δ (ppm): 8.10 (s, 2H), 7.95 (s, 2H), 7.70 (d, *J* = 8.5 Hz, 2H), 7.64 (s, 2H), 7.23 (d, *J* = 8.5 Hz, 2H), 6.77 (s, 2H), 6.70 (d, *J* = 13.1 Hz, 2H), 6.59 (dd, *J* = 13.1, 13.1 Hz, 2H), 6.21 (d, *J* = 13.1 Hz, 2H), 5.45 (s, 4H), 4.33 (s, 4H), 4.07 (br s, 4H), 3.73 (br s, 2H), 3.60 (br s, 2H), 3.44-3.28 (m, 28H), 3.18 (s, 6H), 3.03 (s, 18H), 2.18 (br s, 4H), 0.97 (s, 12H).

HRMS (ESI-TOF) m/z: $[M]^+$ calcd for $C_{74}H_{103}N_{12}O_{17}S_2^+$ 1495.7000, found 1495.7120.

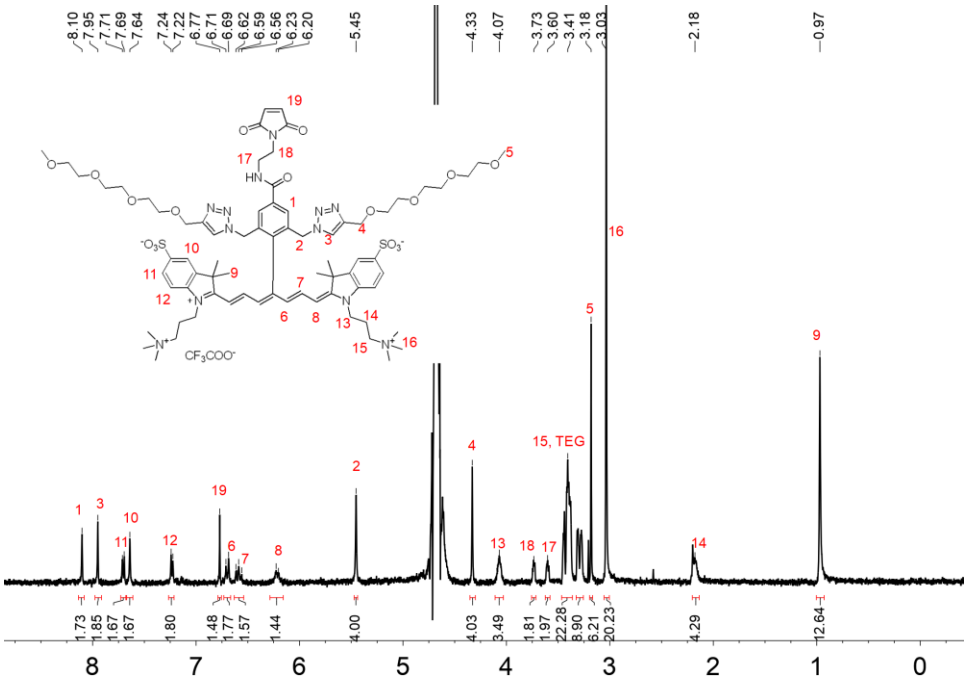
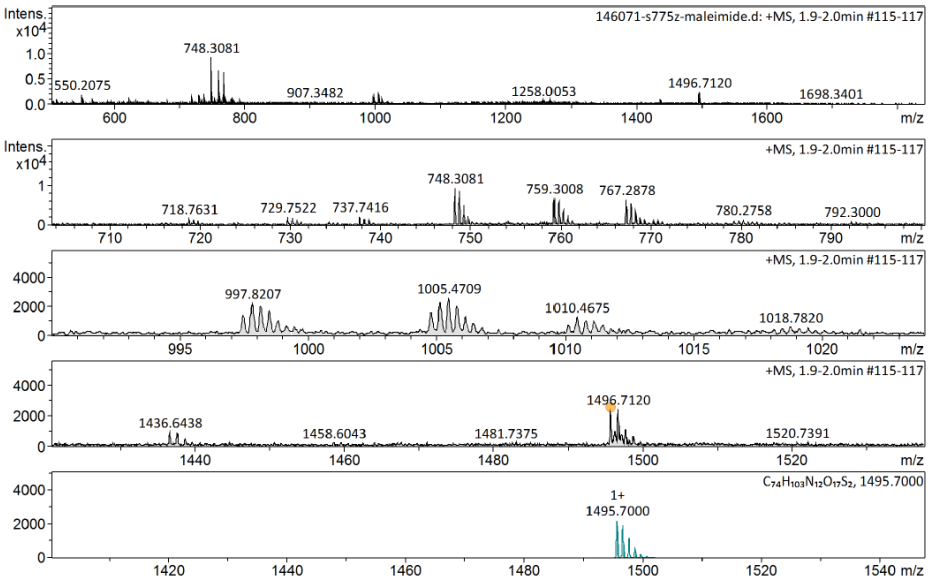
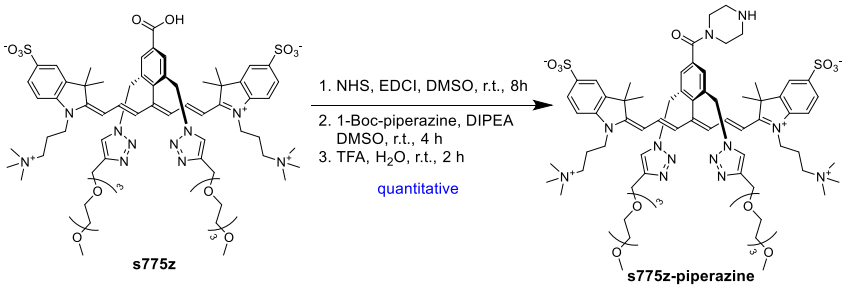


Figure S4. ^{1}H NMR spectrum (500 MHz, D_2O, 25 ^{o}C) of **s775z-maleimide**



Meas. m/z	#	Ion Formula	m/z	err [ppm]	Mean err [ppm]	rdb	N-Rule	e¯ Conf
1495.700388	1	C74H103N12O17S2	1495.700008	-0.3	583.1	29.5	ok	even

Figure S5. HRMS spectrum of **s775z-maleimide**



Scheme S4. Synthesis of **s775z-piperazine**

A mixture of the **s775z-NHS**, 1-Boc-piperazine (3 eq) and DIPEA (5 eq) in DMSO (0.1 mL for 1 mg of **s775z-NHS**) was stirred at room temperature for 4 h in the dark. The reaction mixture was transferred into a centrifuge tube. Ethyl acetate (5 mL for 1 mL of DMSO) was added to the tube and mixture was centrifuged at 3600 rpm for 5 min. The supernatant was discarded and the green solid was washed with ethyl acetate (2x) and diethyl ether (1x) then dried in vacuo to afford the Boc protected intermediate. The Boc protected intermediate was dissolved in TFA/H_2O mixture (95/5, 10 mg/mL) and stirred at room temperature for 2 h in the dark. TFA and H_2O was removed under reduced pressure, the residue was washed with acetone and dried to afford **s775z-piperazine** (green solid, trifluoroacetate salt, quantitative yield).

^{1}H NMR (500 MHz, D_2O, 25 ^{o}C) δ (ppm): δ 7.97 (s, 2H), 7.91 (s, 2H), 7.71 (d, *J* = 8.5 Hz, 2H), 7.63 (s, 2H), 7.24 (d, *J* = 8.5 Hz, 2H), 6.70 (d, *J* = 13.2 Hz, 2H), 6.61 (dd, *J* = 13.2, 13.2 Hz, 2H), 6.23 (d, *J* = 13.2 Hz, 2H), 5.45 (s, 4H), 4.33 (s, 4H), 4.07 (br s, 4H), 4.00 (s, 2H), 3.69 (s, 2H), 3.48 – 3.24 (m, 28H), 3.18 (s, 6H), 3.03 (s, 18H), 2.18 (br s, 4H), 0.99 (s, 12H).

HRMS (ESI-TOF) m/z: $[M+H]^{2+}$ calcd for $C_{72}H_{106}N_{12}O_{15}S_2^{2+}$ 721.3666, found 721.3663.

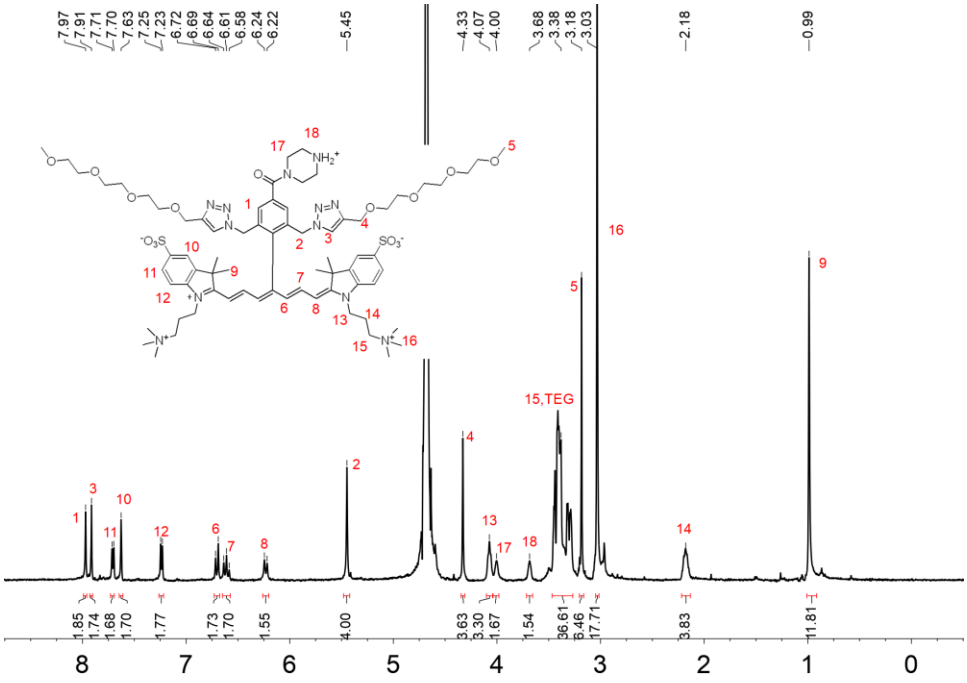


Figure S6. ^{1}H NMR spectrum (500 MHz, D_2O, 25 ^{o}C) of **s775z-piperazine**

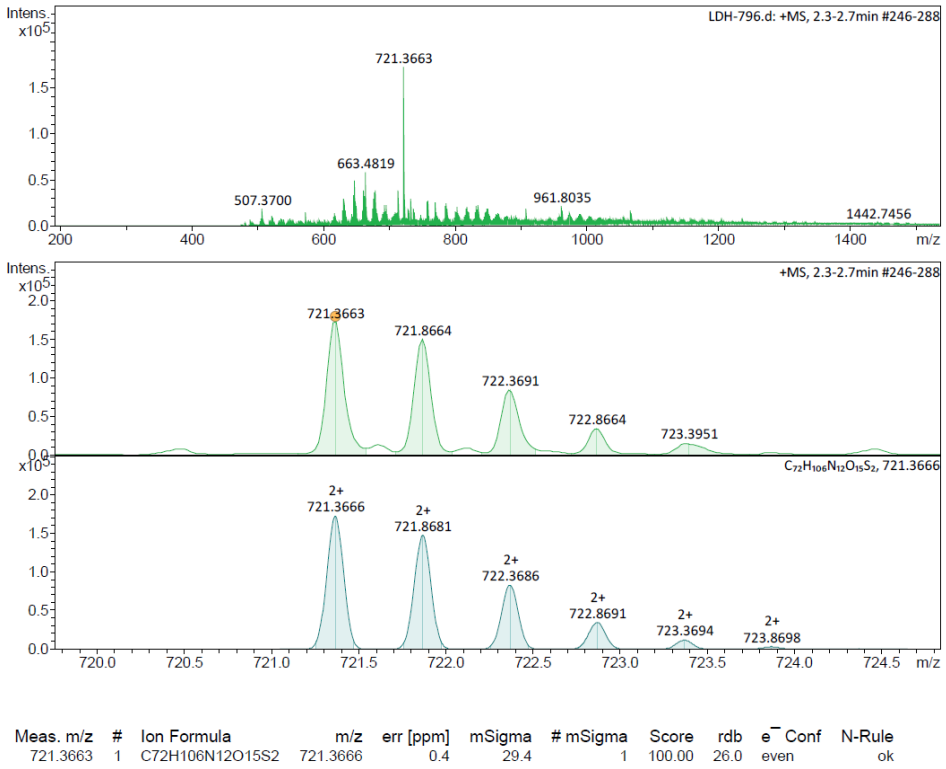
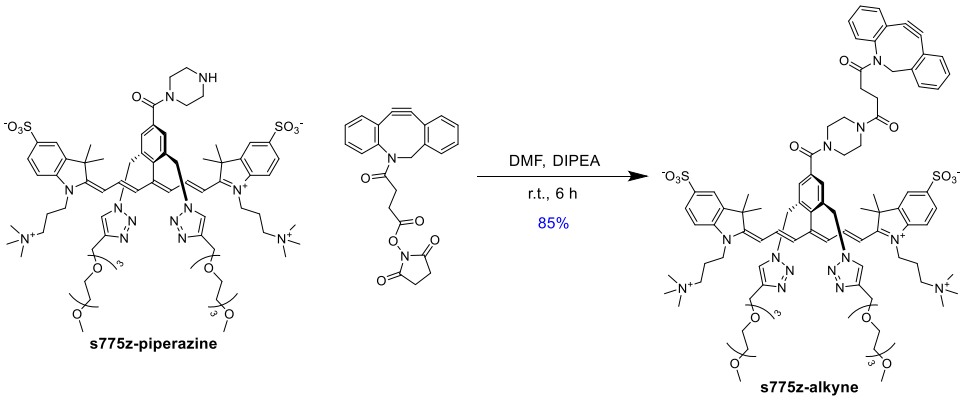


Figure S7. HRMS spectrum of **s775z-piperazine**



Scheme S5. Synthesis of **s775z-alkyne**

A mixture of the **s775z-piperazine**, DIBAC NHS ester (2 eq) and DIPEA (5 eq) in DMF (0.2 mL for 1 mg of **s775z-piperazine**) was stirred vigorously at room temperature for 6 h in the dark. The reaction mixture was transferred into a centrifuge tube. Diethyl ether (5 mL for 1 mL of DMF) was added to the tube and mixture was centrifuged at 3600 rpm for 5 min. The supernatant was discarded and the green solid was washed with Diethyl ether serval times and dried to afford **s775z-alkyne** (green solid, 85% yield).

HRMS (ESI-TOF) m/z: $[M+Na]^{2+}$ calcd for $C_{91}H_{118}N_{13}O_{17}S_2Na^{2+}$ 857.9048, found 857.9050.

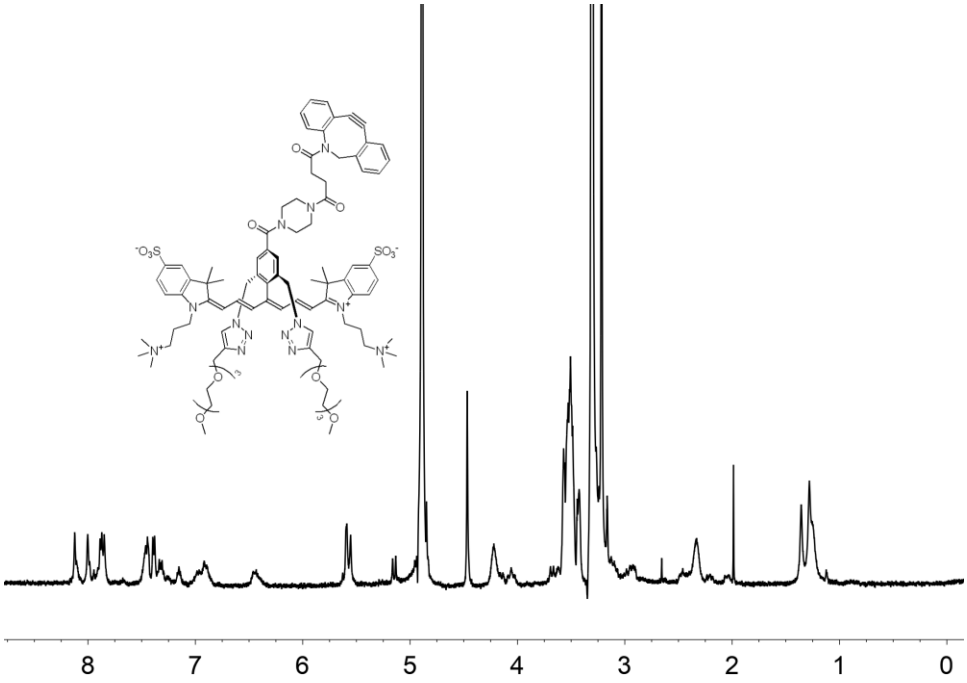
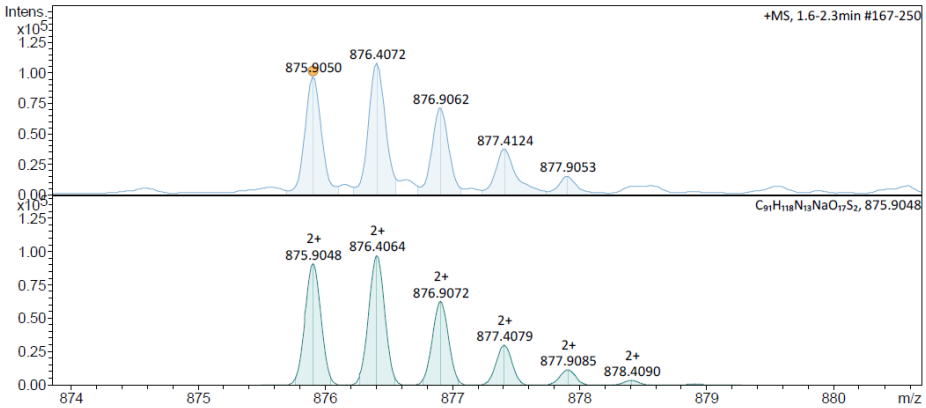


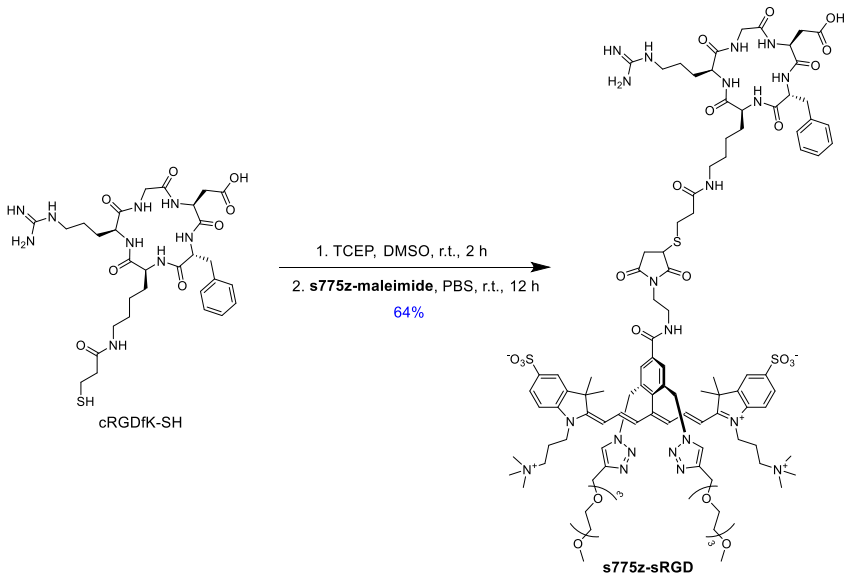
Figure S8. ^{1}H NMR spectrum (500 MHz, methanol-d_4, 25 ^{o}C) of **s775z-alkyne**



Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# mSigma	Score	rdb	e¯ Conf	N-Rule
875.9050	1	C91H118N13NaO17S2	875.9048	-0.2	30.3	1	100.00	39.0	even	ok

Figure S9. HRMS spectrum of **s775z-alkyne**

Synthesis of **s775z** probes



Scheme S6. Synthesis of **s775z-sRGD**

Thiol cRGDfK-SH is dissolved in DMSO. Then TCEP (freshly prepared, 1 M in H_2O, 0.5 eq) was added. The solution was stirred at room temperature for 2 h under nitrogen atmosphere. A solution of **s775z-maleimide** (2 eq) in PBS buffer (pH 7.4, 10 mg/mL) was added. The mixture was stirred at room temperature for 12 h under nitrogen atmosphere in the dark. The product was purified by reverse-phase column chromatography (C18) to give **s775z-sRGD**.

Important notes: free thiol forms disulfide in air, which is usually reduced by TCEP before thiol-maleimide coupling. However, excess phosphine can quench the heptamethine fluorophore.[6] Therefore, only 0.5 equiv. of TCEP was used and the coupling was performed in PBS buffer to quench excess TCEP (TCEP is unstable in phosphate buffer). All solvents were bubbled with nitrogen before usage.

Green solid, 64% yield, reverse phase column chromatography: C18, 20-50% MeOH contains 0.5% TFA in H_2O.

HRMS (ESI-TOF) m/z: $[M]^+$ calcd for $C_{104}H_{149}N_{21}O_{25}S_3^+$ 2188.0190, found 2188.0193.

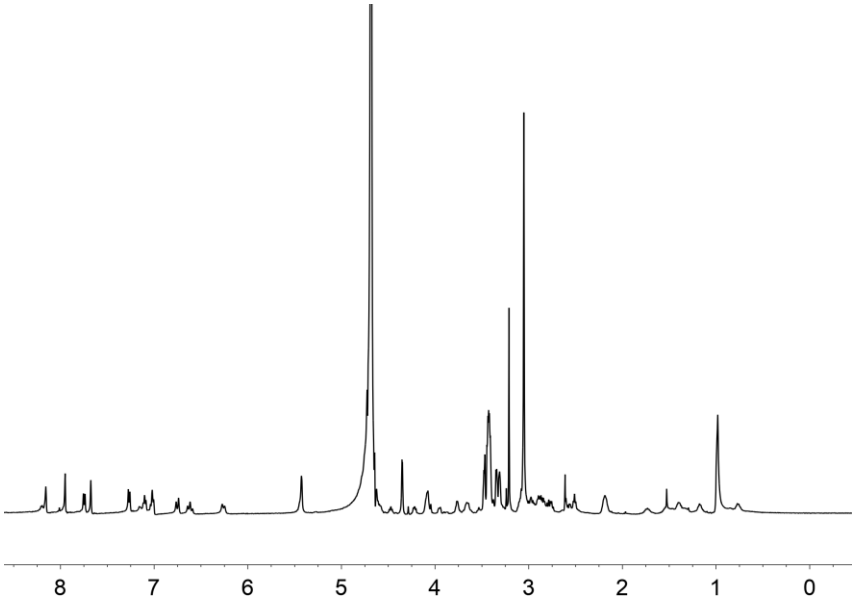


Figure S10. ^1H NMR spectrum (500 MHz, D_2O, 25 ^oC) of **s775z-sRGD**

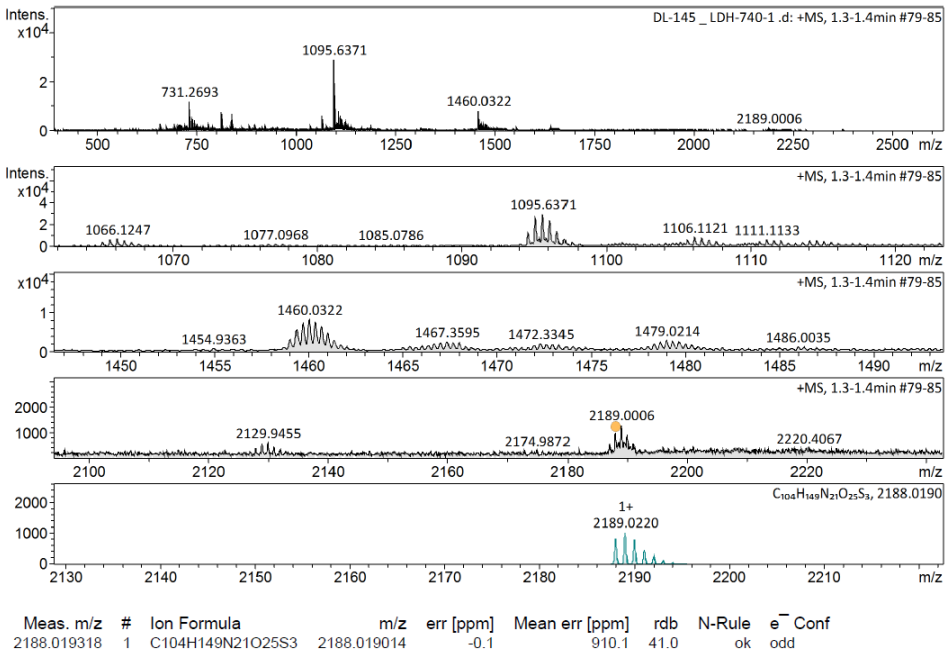


Figure S11. HRMS spectrum of **s775z-sRGD**

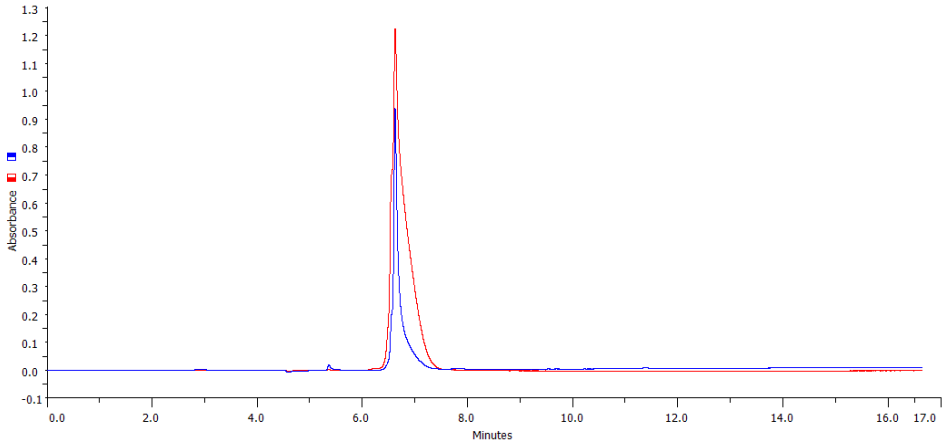
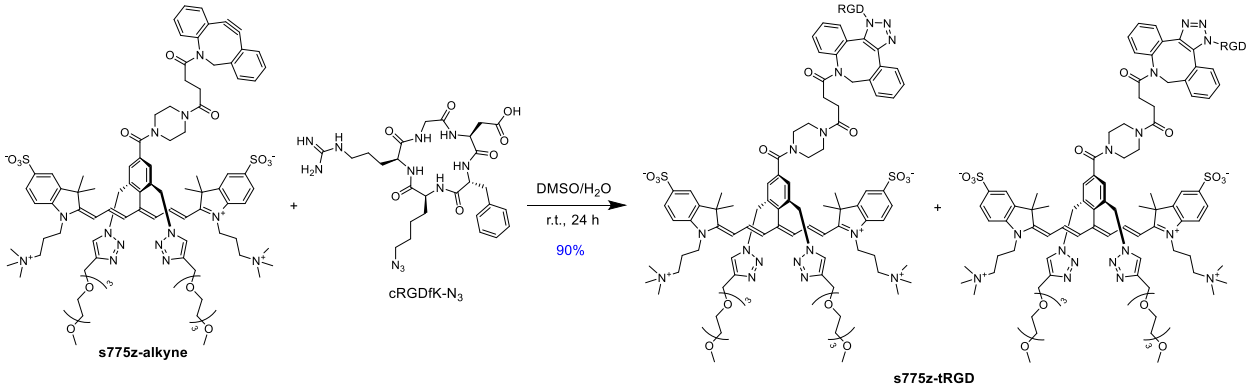


Figure S12. HPLC spectrum of **s775z-sRGD** showing high purity. Solvent: 0-95% acetonitrile in water with 0.1% TFA; flow rate: 1 mL/min; detector: 750 nm (red) and 260 nm (blue). Purity: 98%.



Scheme S7. Synthesis of **s775z-tRGD**

A solution of azide cRGDfK-N_3 (MeOH, DMSO, or H_2O, depends on the solubility of azide) was added to a solution of **s775z-alkyne** in water or DMSO (make sure there is no insoluble material in the mixture). The mixture was stirred at room temperature for 24 h in the dark. After that the mixture was directly purified by reverse phase column chromatography to give **s775z-tRGD**.

Green solid, 90% yield, reverse phase column chromatography: C18, 40-60% MeOH contains 0.5% TFA in H_2O.

HRMS (ESI-TOF) m/z: $[M + H]^{2+}$ calcd for $C_{118}H_{159}N_{24}O_{24}S_2^{2+}$ 1180.0695, found 1180.0689.

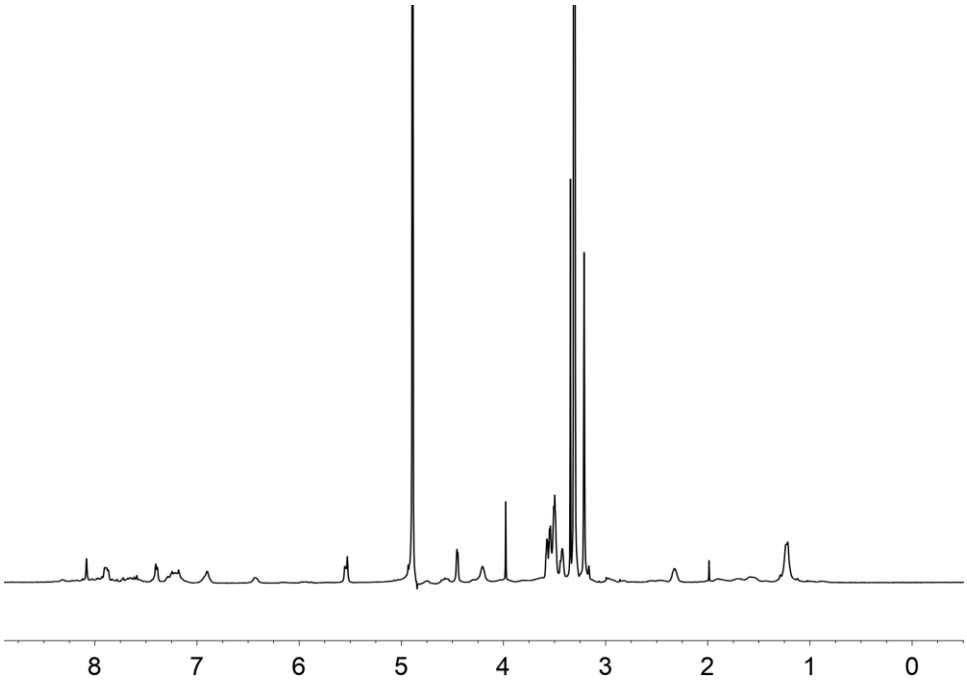


Figure S13. ^1H NMR spectrum (500 MHz, D_2O, 25 ^oC) of **s775z-tRGD**

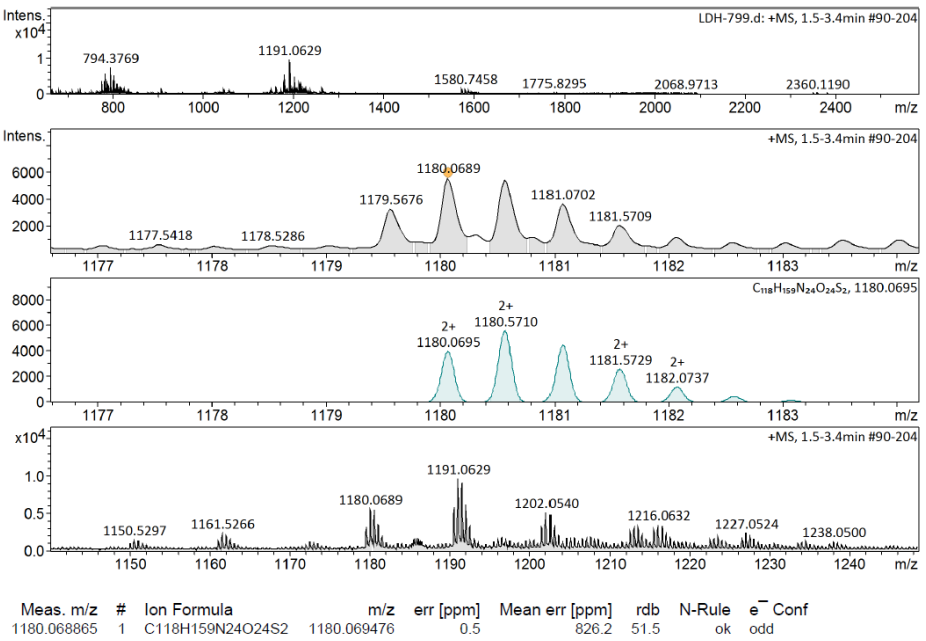


Figure S14. HRMS spectrum of **s775z-tRGD**

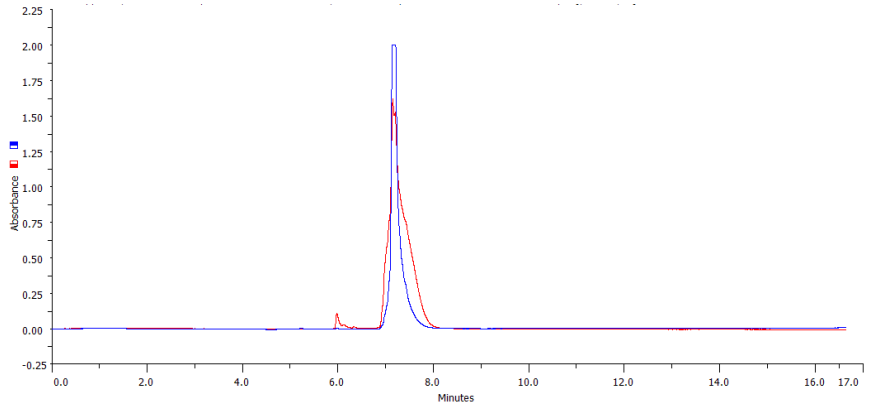


Figure S15. HPLC spectrum of **s775z-tRGD** showing high purity. Solvent: 0-95% acetonitrile in water with 0.1% TFA; flow rate: 1 mL/min; detector: 750 nm (red) and 260 nm (blue). Purity: 95%.

Photophysical Properties

Analyte solutions were prepared in phosphate buffered saline (Thermo Fisher. All absorption and fluorescence spectra were collected using quartz cuvettes (1 mL, 1 cm path length; for emission and excitation spectra, slit width = 3 nm).

Quantum yield measurements used **s775z** (Φ_F = 9.0% in pH 7.4 PBS) as a reference standard.[1] The concentrations of **s775z** and other cyanine dyes were adjusted to the absorption value of 0.08 at 730 nm. The fluorescence spectrum of each solution was obtained with excitation at 730 nm, and the integrated area was used in the quantum yield calculation by the following equation:

$$\Phi_{sample} = \Phi_{ref} \times \frac{\eta^2_{sample}\ I_{sample}\ A_{sample}}{\eta^2_{ref}\ I_{ref}\ A_{ref}}$$

where η is the refractive index of the solvent, I is the integrated fluorescence intensity, and A is the absorbance at a chosen wavelength. The estimated error for this method is ±10%.

Table S1. Chemical properties of fluorescent probes.

Dye[a]	MW (g/mol)	pK_a, H_2O[b]	LogP[c]	LogD, pH 7.4[c]
s775z	1373.69	9.27	- 2.41	- 4.72
s775z-aRGD	1959.36	9.44	- 7.03	- 10.84
s775z-sRGD	2187.62	9.42	- 8.23	- 12.04
s775z-tRGD	2358.81	9.73	- 5.21	- 9.02
650z	785.03	8.02	- 3.02	- 5.65

[a]Carboxylic acid residues were deprotonated when preforming calculation. [b]Calculated in BondEnergy software.[7,8] [c]Calculated in ChemAxon Chemicalize software.[9]

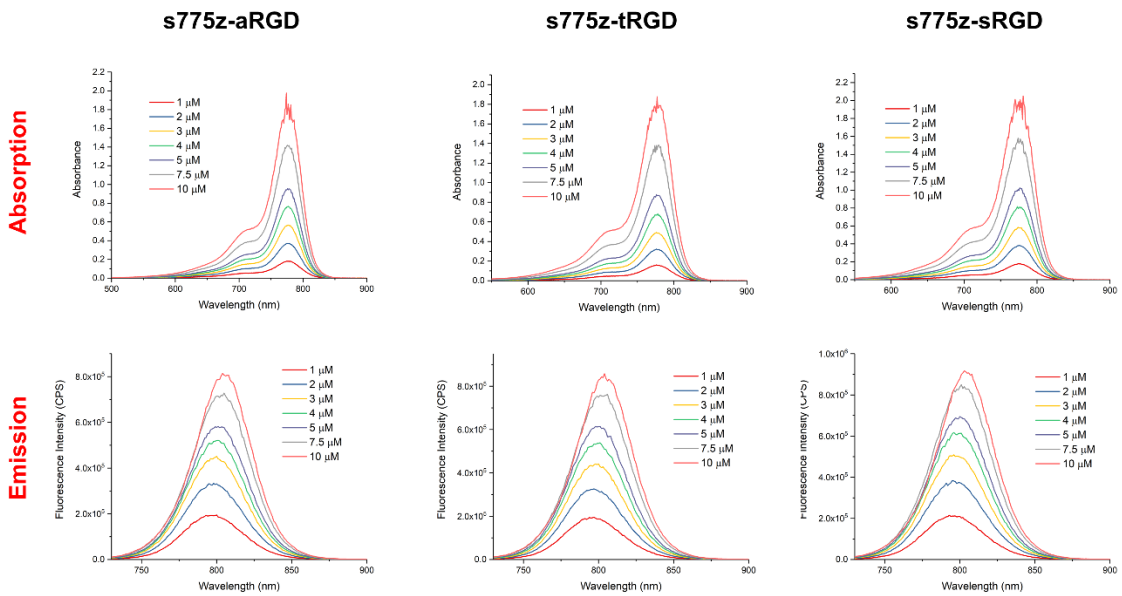


Figure S16. Spectral properties and absorption-emission spectra of **s775z-aRGD, s775z-sRGD,** and **s775z-tRGD** at different concentrations (pH 7.4 PBS buffer at room temperature). λ_{ex} = 720 nm, slit width = 3 nm.

Bovine Serum Albumin Binding Studies

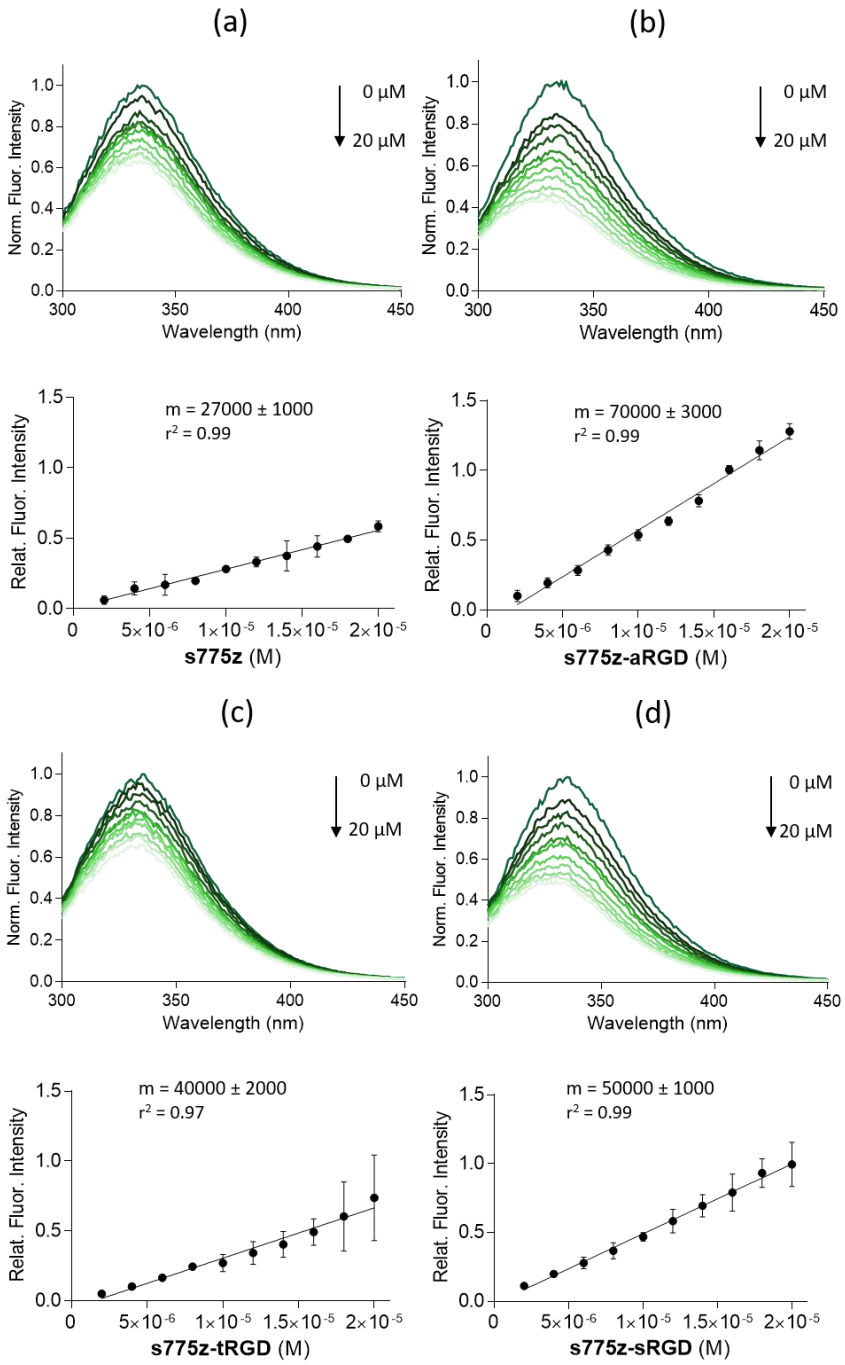


Figure S17. Dye/BSA association studies of: (a) **s775z**, (b) **s775z-aRGD**, (c) **s775z-tRGD** and (d) **s775z-sRGD** in water at 37 °C. Aliquots (4 µL) of probe stock solution (1 mM) was added to 2 mL of 2 µM BSA, thus varying the probe concentration from 0-20 µM. BSA tryptophan fluorescence intensity (excitation: 280 nm; slit width: 2 nm) was plotted as a function of the dye concentration to obtain association constants K_a where m = K_a ± SD (M^{-1}).

***In vitro* Cell Studies**

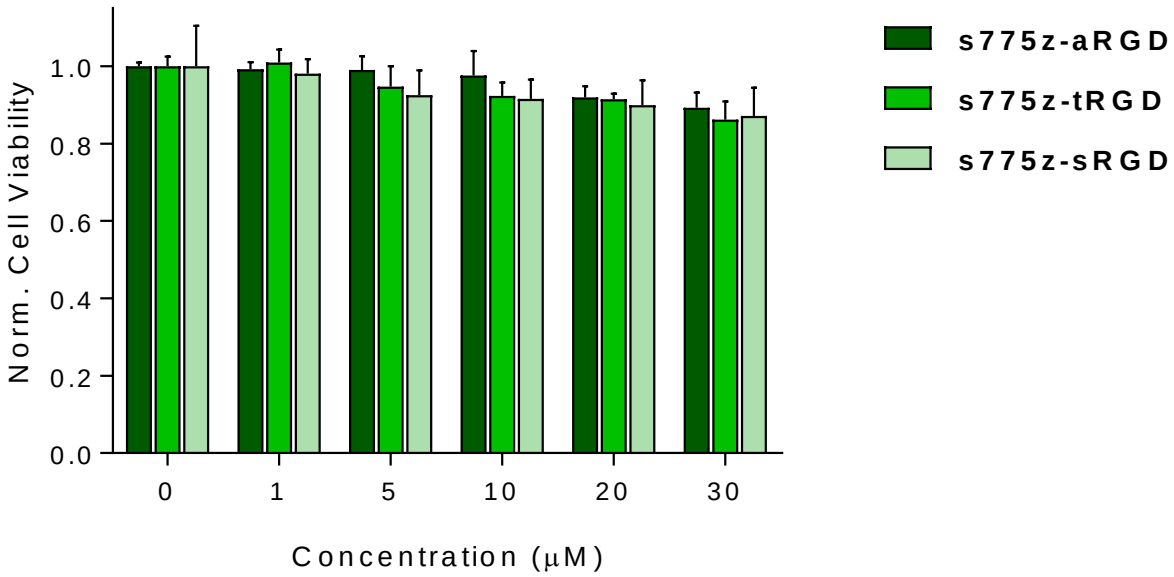


Figure S18. MTT cell viability assays of **s775z-aRGD, s775z-sRGD,** and **s775z-tRGD**. Separate wells containing A549 cells were treated with one of the probes for 24 hr at 37 ^{o}C and 5% CO_2. Readings are normalized to untreated cells and reflect triplicate experiments.

***In vivo* Imaging Studies**

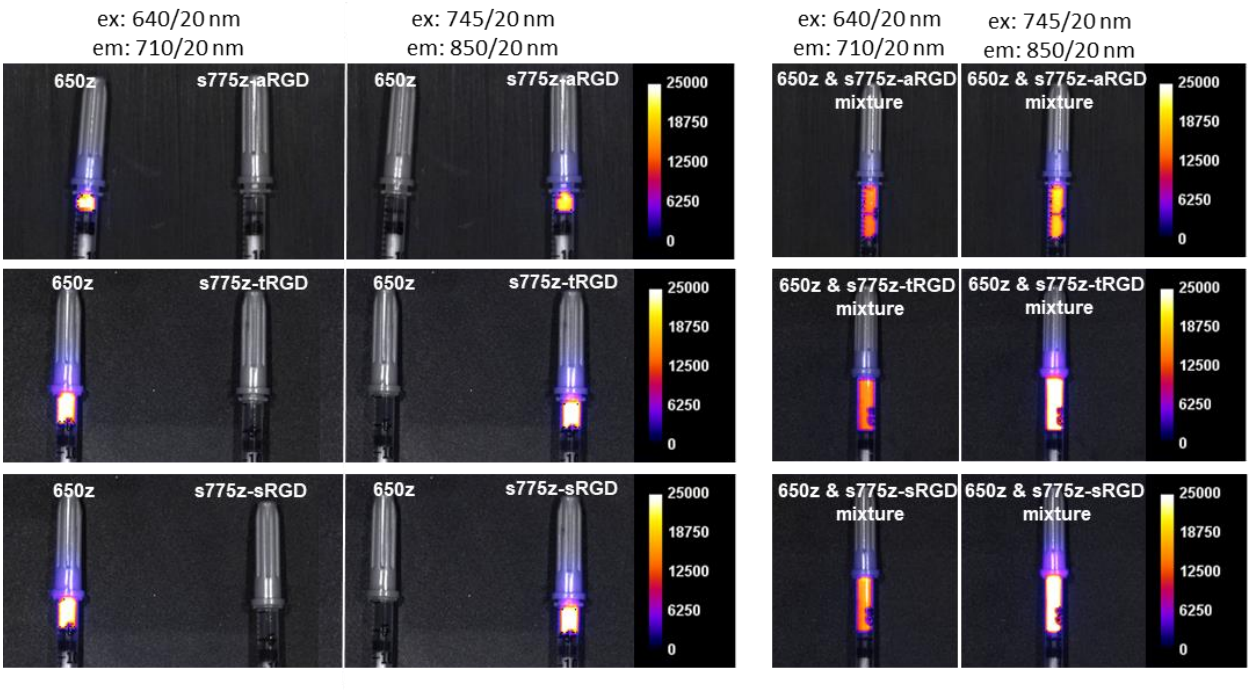


Figure S19. Fluorescent and brightfield images of syringes containing: (*left panel*) dilute aqueous solution of **650z** dye or one of the s775z probes; (*right panel*) binary 1:1 mixture of **650z** dye and one of the s775z probes. Images acquired using *in vivo* imaging station with two different filter settings [(ex: 640/20 nm, em: 710/20 nm, exposure: 3 s, percent power: 50%, F-stop: 2, FOV:20, binning: low) or (ex: 745/20 nm, em: 850/20 nm, exposure: 3 s, percent power: 50%, F-stop: 2, FOV:20, binning: low)].

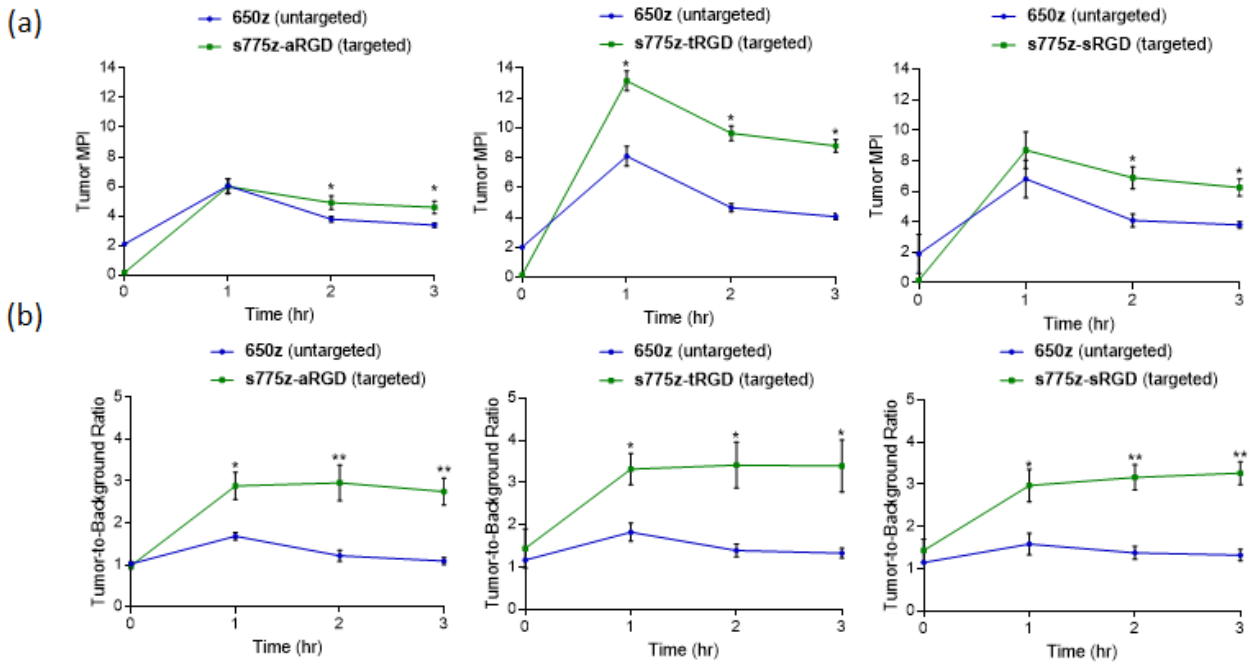


Figure S20. (a) Tumor Mean Pixel Intensity (MPI), and (b) Tumor-to-Background Ratio for living mice bearing subcutaneous tumor (human A549 lung adenocarcinoma), after intravenous co-injection of **650z** dye and one of the s775z probes (**s775z-aRGD**, **s775z-tRGD** or **s775z-sRGD**) (20nmol/mouse). *indicates $p \leq 0.05$, ** $p < 0.01$

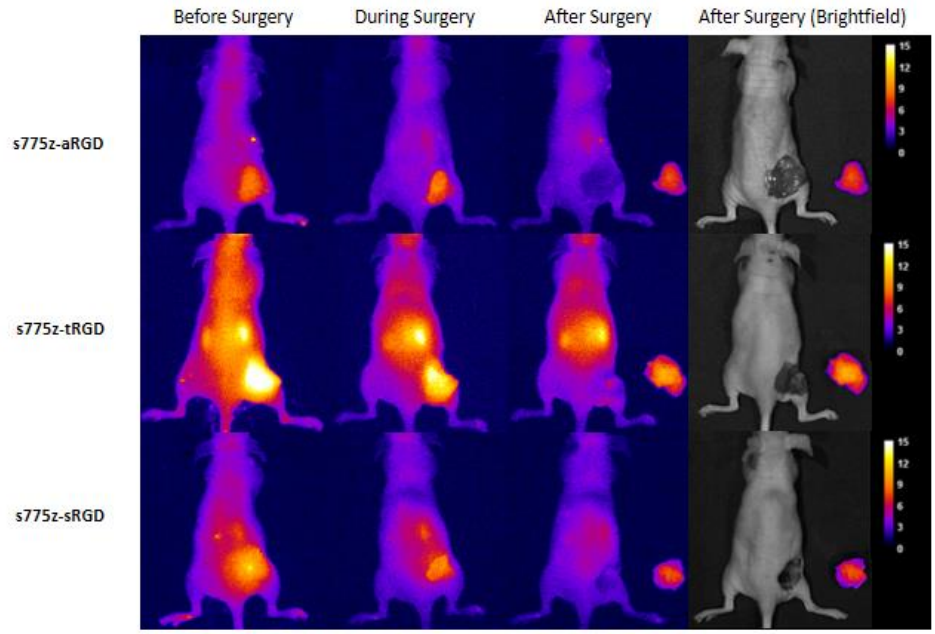


Figure S21. Mock surgery process using a tumor-bearing mouse at 3 h post injection of **s775z-aRGD**, **s775z-tRGD** or **s775z-sRGD**. Representative images were taken *Before Surgery* (living mouse), *During Surgery* (sacrificed mouse with surrounding skin removed) and *After Surgery* (excised tumor next to the sacrificed mouse body).

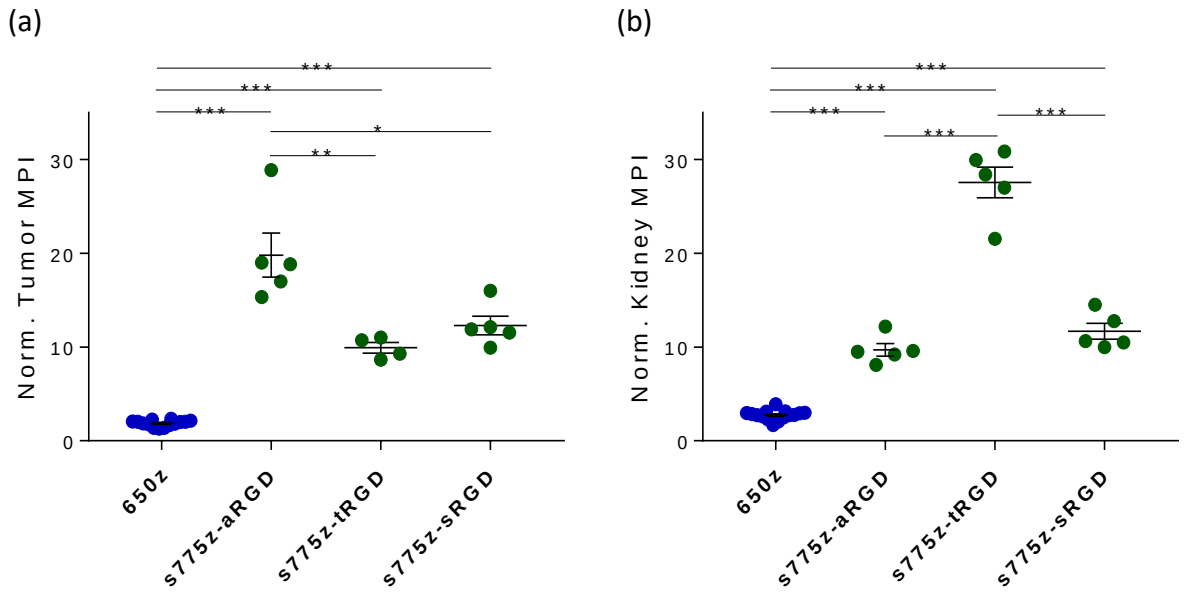


Figure S22. Expansion of the biodistribution data in article Figure 3. The normalized mean pixel intensity (MPI) for untargeted **650z** and each of the three targeted s775z probes in the excised: (a) tumor tissue, or (b) kidney tissue. The MPI values for each probe are normalized to the thigh muscle MPI from the same animal (N = 5 for each probe). Fluorescence filter settings for the untargeted **650z** [(ex: 640/20 nm, em: 710/20 nm, exposure time: 3 sec, percent power: 50%, F-stop: 2, FOV: 20, binning – low) and targeted s775z probes (ex: 745/20 nm, em: 850/20 nm, exposure time: 3 sec, percent power: 50%, F-stop: 2, FOV: 20, binning – low). The average for each cohort (N = 5) is indicated by the black line with error bars representing ± SEM. *indicates $p < 0.05$, **$p < 0.01$, *** $p < 0.001$

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