Supporting Information for

The PEG-Fluorochrome Shielding Approach for Targeted Probe Design

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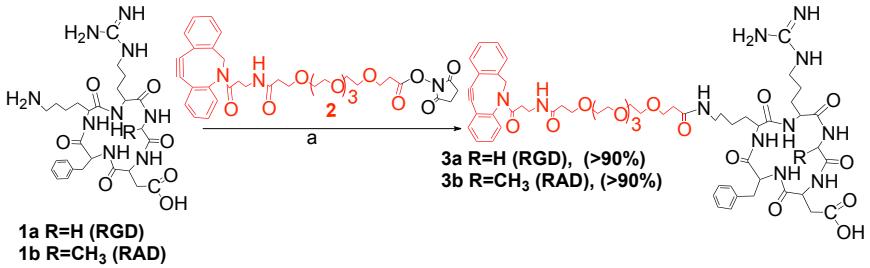
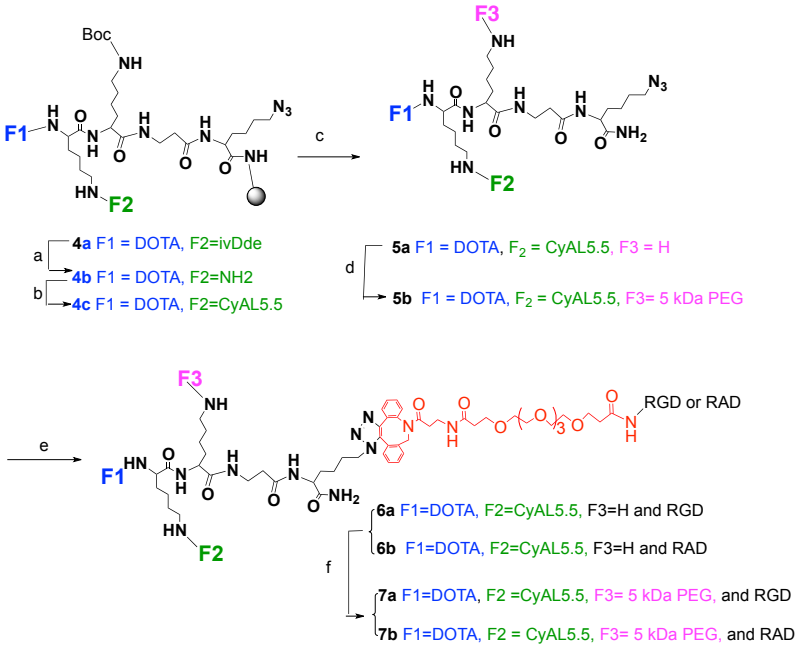
Materials and Methods

Protected L-amino acids, PyBOP and Rink Amide MBHA resin were from Novabiochem (EMD Biosciences). Other special chemicals were from other sources: DOTA(CO_2Bu^t)$_3$ (Macrocyclics), mPEG-NHS ester (5 kDa; Creative PEGworks), Fmoc-Lys(N_3)–OH (AnaSpec), and DBCO-PEG4-NHS (Click Chemistry Tools). The fluorescent dye CyAL5.5 was synthesized as described [1]. All the other solvents and chemicals were from Sigma-Aldrich. Molecular weights were obtained by MS-ESI Micromass (Waters) and MALDI-TOF analyses at the Tufts University Core Facility. RP-HPLC (Varian ProStar detector and delivery modules) employed an eluant A (0.1% TFA /water) and eluant B (0.1% TFA in 9.9% water in acetonitrile). RGD and RAD peptides were cRGDfK and cRADfK from Peptides International. Synthetic schemes and nomenclature are given in Figure 1.



Scheme 1. Synthesis of linker RGD (or RAD) targeting vehicles. a: DMF/DiPEA.

For (**3a**): A stock solution of DBCO-PEG4-NHS ester (**2**) (containing 7.5mg, 10.8μmol) in anhydrous DMSO was added to the solution of the RGD peptide, cRGDfK (**3a**) (5.6 mg, 9.28 μmol from Peptides International) in anhydrous DMSO (0.4ml). After DiPEA (9μl) was added, the mixture was incubated at room temperature overnight. After diluted with buffer A, the mixture was purified by HPLC with gradient of 20%B-100%B in 15 min, then back to 20% B in 5 min and isocratic for 5min; flow: 12ml/min; λmax: 226nm; column: Higgins Analytical Inc. Clipeus C18, 10μm, 250x20mm, P/N: CS-2520-C181, S/N: 186532. A white powder (**5a**) was obtained. Yield: >90% $C_{59}H_{79}N_{11}O_{15,}$ MW: 1182.32, MS: Cal. 1181.58, Observed: 1182.11. For (**3b**), the procedure was followed **3a** by using the RAD peptide cRADfK with similar results. $C_{60}H_{81}N_{11}O_{15,}$ MW: 1196.35, MS: Cal. 1195.59, Observed: 1196.30.



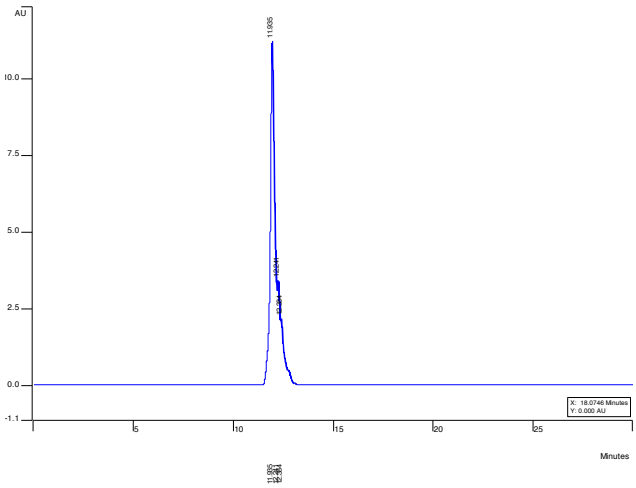
Scheme 2. The synthesis of trifunctional probes. a: NH2NH2, DMF; b: CyAL5.5 Acid, PyBOP, DMF, DiPEA; c: TFA; d: 5 kDa PEG-NHS, DMSO; e: **5a**, RGD-PEG4-DBCO (**3a**) or RAD-PEG4-DBCO (**3b**), DMSO; f: 5 kDa PEG-NHS.

Synthesis of 5a: The DOTA(CO_2-Bu^t)$_3$-Lys(ivDde)-Lys(Boc)-β-Ala-Lys(N_3) peptide (**4a**) was manually synthesized on Rink Amide MBHA resin (0.15 mmol) with an Fmoc/*t*-Bu strategy using a polypropylene 5 mL disposable syringe fitted with a sintered frit. Coupling reactions employed 2 equiv. (relative to resin) of *N*-α-Fmoc-protected amino acid activated in situ with 2 equiv. of PyBOP and 4 equiv. of DiPEA in DMF (10 mL/g resin) for 1-2 hrs. Coupling efficiency was assessed with picrylsulfonic acid. *N*-α-Fmoc groups were removed with a piperidine/DMF solution (1:4) for 4x10 min (10 mL/g resin). The coupling of DOTA was overnight with same equivalent of other reagents. After intermediate (**4b**) was obtained by *N*-ε-ivDde group removal with 2% hydrazine in DMF for 5 min (10 mL/g resin), the attachment of CyAL5.5 (for intermediate **4c**) was carried out on the solid phase for overnight by using CyAL5.5 acid (2 equiv.) under the in situ activation of PyBOP (2 equiv.) and DiPEA (8 equiv.). Intermediate DOTA-Lys(CyAL5.5)-Lys(NH_2)-β-Ala-Lys(N_3) (**5a**) was released from the solid support with TFA/H_2O/TIS/EDT 88:2:5:5 (twice, 4 h, 20 mL/g resin). After the solvent was evaporated, the residue was precipitated and triturated with cold ether. A blue solid could be obtained by centrifuge. The solid was purified further by preparative HPLC with a gradient of 20% - 80%B in 15min, back to 20%B in 3min, and isocratic for 3 min; λmax: 670 nm; flow: 21ml/min; column: Higgins Analytical Inc., Clipeus C18 10μm, 250x20 mm. A blue powder of compound (**5a**) was obtained after lyophilization with a yield of 40%. $C_{81}H_{117}N_{16}O_{18}S_2^+$; MW: 1667.02; MS: cal. 1665.82; found (m/z): 1666.2 and 833.8.

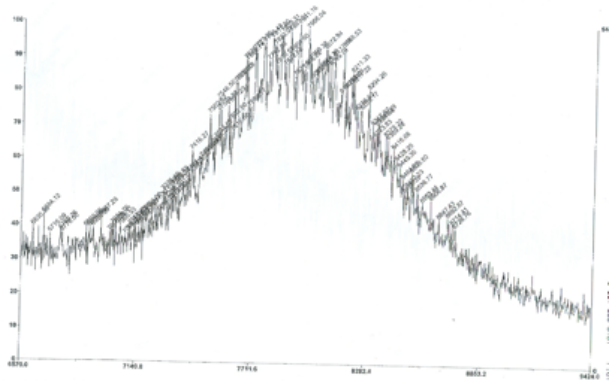
Synthesis of 5b: To a solution of DOTA-Lys(CyAL5.5)-Lys(NH_2)-**ß**-Ala-Lys(N_3)-NH_2 (**5a**) (1.0 mg, 0.6 μmol) in anhydrous DMSO (0.4 ml), was added the solution of 5 kDa PEG-NHS (13.8 mg, 2.76 μmol) in anhydrous DMSO (0.5ml). After DiPEA (10 μL) was added, the reaction mixture was incubated at room temperature for 3 days. The mixture was diluted by acetonitrile and water (0.1% TFA, 1:1 v/v) and purified by HPLC with a gradient of 20%-100%B in 20 min, then back to 20% B in 5 min and isocratic for 5 min; flow: 5ml/min; λmax: 670 nm; Varian Pursuit XRs 5 C18, 250x10 mm column, P/N: A6000250X100, S/N: 1007962. A blue powder (**5b**) was obtained after lyophilization. Yield: >90%. Mass was in a wide range from 6200 to 7100 due to PEG.

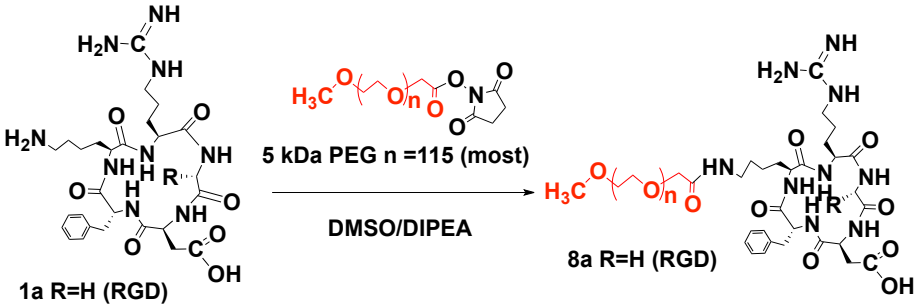
Synthesis of unPEGylated RGD and RAD probes, 6a, 6b: For **6a**, a mixture of the solution of DOTA-Lys(CyAL5.5)-Lys(NH_2)-β-Ala-Lys(N_3)-NH_2 (**5a**) (3.2 mg, 1.92 μmol) in DMSO (0.4 ml) with the solution of DBCO-PEG4-RGD (**3a**) (2.5 mg, 2.11 μmol) in DMSO (0.4ml) was incubated for 2 h at room temperature. The product was purified by HPLC with a gradient of 20%-100%B in 20 min, then back to 20% B in 5 min and isocratic for 5min; flow: 5 ml/min; λmax: 670 nm; column: Varian Pursuit XRs 5 C18, 250x10 mm, P/N: A6000250X100, S/N: 1007962. A blue powder (**6a**) was obtained. Yield: ~99%. $C_{140}H_{196}N_{27}O_{33}S_2^+$, MW: 2849.34, MS: cal. 2847.39, found: 2848.29. For (**6b**), the procedure was followed **6a** by using **3b** with similar results. $C_{141}H_{198}N_{27}O_{33}S_2^+$, MW: 2863.37, MS: cal. 2861.41, found: 2862.22.

Synthesis of multifunctional PEGylated RGD and RAD probes, 7a and 7b: To a solution of DOTA-Lys(CyAL5.5)-Lys(NH_2)-**ß**-Ala-Lys(N_3-DBCO-PEG4-RGD)-NH_2 (**6a**) (1.54 mg, 0.54 μmol) in DMSO (0.9 ml), was added the solution of 5 kDa PEG-NHS (18 mg, 3.6 μmol). After DiPEA (10 μL) was added, the reaction mixture was incubated at room temperature for 3 days. The mixture was diluted by acetonitrile and water (0.1% TFA, 1:1 v/v) and purified by HPLC purification with a gradient of 20%-100%B in 20 min, then back to 20% B in 5 min and isocratic for 5min; flow: 5 ml/min. λmax: 670 nm; column: Varian Pursuit XRs 5 C18, 250x10mm, P/N: A6000250X100, S/N: 1007962. A blue powder (**7a**) was obtained. Yield: >90%. Mass was observed in a wide range from 7300 to 8300 due to PEG. For (**7b**), the procedure was followed **7a** by using **6b** with similar results. Masses ranged from 7300 to 8300 Da due to PEG polydispersity. Compounds 7a and 7b were more than 90% pure by RP-HPLC (below) and FPLC (Figure 2b). The RP-HPLC purification of 7a is shown below.



MALDI-TOF Mass spec of 7a is shown below,

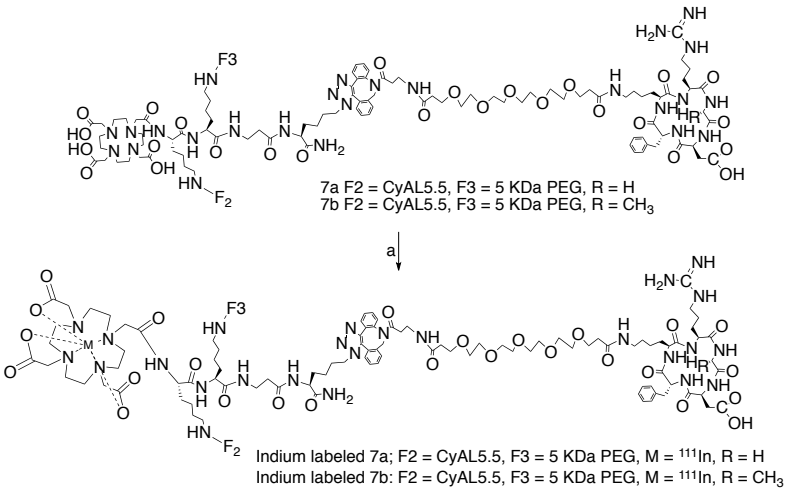




Scheme 3. Reaction of RGD with 5 kDa PEG-NHS. See Figure S4a.

5KDa PEG-NHS ester (30 mg, 0.006 mmol) in anhydrous DMSO (1 ml) was added to the powder of cRGDfK (**1a**, 2.4 mg, 0.004 mmol). After DiPEA (4 µl, 0.024 mmol, 6 eq.) was added, the mixture was incubated at room temperature for 3 days. After diluted with water (0.1% TFA), the mixture was purified by C18 reverse phase HPLC with a gradient of 5%-80% buffer B in 20 min with a flow of 3 ml/min at λmax: 226 & 254 nm. After lyophilization, a white powder was obtained and identified by a mass spectrum. MS: 5700.

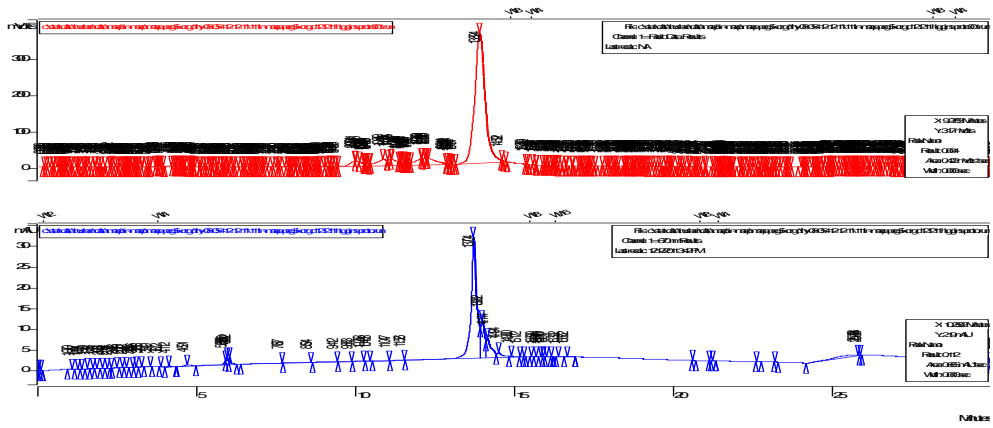
Synthesis of ^{111}indium labeled probes:



Scheme 4. ^{111}In labeling of 7a or 7b. a: $^{111}InCl_3$, 1M HEPES, pH 5, 70 °C.

To the solution of 7a or 7b (20 nmoles), reconstituted with 1M HEPES buffer (pH 5.0) (0.7 ml) in a 5 ml conic react vial, was added the solution of $^{111}InCl_3$ in 0.05N HCl (150 µl, 333 MBq, 9 mCi). After 45 minutes at 70 °C, the mixture was cooled in a water bath for 2 min. A stock solution of EDTA (70 mM, 50 µl) was added and the solution was allowed to stay at RT for 15 min. After the mixture was diluted with ammonium acetate buffer (0.5 ml, 1 M, pH 6), and loaded onto a C18 cartridge preconditioned by ethanol (0.5 ml, 0.1% acetic acid) and water (1 ml, 0.1% acetic acid) sequentially. The labeled tracers were purified by washing the cartridge with water (1 ml, 0.1% acetic acid) and collected by eluting with acetonitrile (200 µl, 0.1% TFA). The acetonitrile and TFA was removed by co-evaporation with ethanol (3 x 200 µl) together with Ar flow and reconstituted with 0.9% saline for injection. Radioactive products were identified by their co-chromatography with the corresponding nonradioactive indium labeled compounds. Radiochemical yield (RCY) was 50-70%.

HPLC radiogram (red) and UV chromatogram (blue) of ^{111}In labeled 7a is shown below. The radioactive detector was set as about 20 seconds behind UV detector.



Adsorption Spectra

The concentrations of compounds 5a, 5b, 6a, 6b, 7a, and 7b were adjusted about 4 µM. Absorption spectra were recorded on an Evolution 300 UV-Vis spectrometer from Thermo Scientific.

Quantum yield

Quantum yields were determined as described in [1] and [2] using Cy5.5 as a reference fluorochrome and quantum yield of 0.28 (GE Healthcare Lifesciences). Excitation wavelength used was 630 nm and emission spectra were recorded from 650 nm to 800 nm in PBS and maximum emission used. Concentrations were below 0.7 µM (absorbance less than 0.1). Measurements were made in triplicate and are expressed as mean ± SD. Absorption and emission spectra are shown in Figure S2.

Probe volume determinations

Size (volume) was determined by FPLC using an ÄKTA Purifier 10 and Superdex^{TM} 75 10/300GL column (GE Healthcare Lifesciences) with a running buffer of 0.05 M sodium phosphate, 0.15 M NaCl (0.1% Tween, pH 7.2) and flow rate of 0.5 ml/min. The protein standards (Gel Filtration Calibration Kit LMW, code no. 28-4038-41, GE Healthcare) (0.3 mg/ml, mixture of Aprotinin, Ribonuclease A, Ovalbumin, and Conalbumin) and Blue Dextran 2000 were used. To obtain volumes, Mr (apparent molecular weight based on size exclusion retention) was ploted versus K_{av}. $K_{av} = (V_e-V_o)/(V_t-V_o)$, V_t = total volume, V_e = elution volume, V_o = void volume.

Fluorescence lifetime

Fluorescence lifetime images were acquired using a time domain fluorescence system described [3]. Briefly, the system consists of a gated intensified CCD camera (Lavision, Gmbh), which allows noncontact detection with 250 ps time resolution. A home-built supercontinuum (SC) laser source (NL-PM-750, Thorlabs) in conjunction with a Ti:Sapph laser (MaiTai, Spectra Physics) was used to excite the samples. For an input of 750 nm TiSapph (~2 W), the SC provides a broad output spectrum of picosecond pulses in the range of 450 nm–800 nm. For excitation of the samples reported in this work, the output of the SC was filtered using a 650 nm band pass filter (40-nm width) and coupled to a 200 µm fiber. The other end of the fiber was coupled to a collimation package to illuminate the sample in the reflectance mode. Fluorescence was collected using a 700 nm long pass filter. Images were acquired as a function for a range of time delays with respect to the incident pulse. The camera integration time was set to 1 s and the gain of the intensifier was set to 660 volts. The time resolved data were acquired over the

entire area of illumination of the tube and lifetimes were analyzed on a pixel by pixel basis in MATLAB using a simple non-linear least squares fit algorithm based on the Nelder Mead simplex approach, which recovers both the lifetimes and decay amplitudes. For the samples reported here, a single exponential fit was found to be sufficient.

Cell binding assay

The concentrations of compounds 5a, 5b, 6a, 6b, 7a, and 7b were determined spectrophotometrically at 694 nm using an extinction coefficient of 130,000 cm^{-1} M^{-1} for CyAL5.5 [1]. BT-20, a human breast carcinoma cell line, was from the American Tissue Culture Collection and maintained according to their instructions.

BT-20 cells were seeded into 12-well plates at $5X10^5$ cells/well in culture medium (EMEM with 10% FBS) on day before. The following day, medium was removed, each well was rinsed with DPBS (+Ca, +Mg) twice, and then 100 µl of 1% FBS / DPBS (+Ca, +Mg) was added to each well. 100 µl of compounds 5a, 5b, 6a, 6b, 7a, and 7b at concentrations of 2 µM in DPBS were added, which means that, the cells were treated with compounds at concentrations 1 µM in 0.5% FBS / DPBS (+Ca, +Mg). The plates were kept in incubator at 37 ^oC with 5% CO_2 for 30 min. Thereafter, the plates were washed twice with DPBS (+Ca, +Mg), treated with 0.05%Trypsin-EDTA (GIBCO), then inhibited with 1% FBS / DPBS (+Ca, +Mg) and centrifuged, washed with DPBS once more, and redispersed in DPBS (+Ca, +Mg), ready for flow cytometry with the BD 3 laser LSR2. All data was obtained in triplicate, with data expressed as the mean ± a standard error.

To assess integrin binding interactions by displacement, the "RGD" peptide was cRGDfK (Peptides International, PCI-3661-PI)) while "RAD" was cRADfK (PCI-3883-PI). Peptides or 8a (350 µM) in 1% FBS / DPBS (+Ca, +Mg) (100 µL/well) was added to cells and incubated for 1h. Then 100 µL of compound 7a at (2 µM) was added, with incubation for another 30 min. Cells were detached and assayed for fluorescence by FACS as above. All was obtained in triplicate, with data expressed as the mean ± a standard error.

BT-20 tumor model

All animal experiments were approved by the Institutional Review Committee of Massachusetts General Hospital. Female nude mice (25-30g; 6-8 weeks old; nu/nu; Cox 7, MGH, Boston, MA) were anesthetized with isoflurane/O_2. Tumor cell implantation was performed both sides around the shoulder. 200 µl of cell suspension containing 10^6 cells in Matrigel (BD) was injected subcutaneously. Tumors were allowed to grow for 7 to 10 days.

Whole animal surface fluorescence imaging

In vivo evaluation of compounds 6b and 7b with normal nude mice (female, 25-30g; 6-8 weeks old; nu/nu; Cox 7, MGH, Boston, MA) was done by intramuscular injection. 10 µl of compound 6b or 7b (100 µM) was injected intramuscularly at both back legs of each mouse. *In vivo* evaluation of compound 7a with BT-20 tumor-bearing animal was done by intravenous injection. Dosage was 200 µl of 7a at 10 µM. The fluorescent intensity was monitored via KODAK imaging system FX (Carestream Molecular Imaging, Rochester, NY). Two steps took multispectra: fluorescence with multi-excitation wavelengths (600, 610, 620, 630, and 650 nm) with the emission set at 700 nm; the second step was an x-ray. Multispectra were unmixed with CyAl5.5 spectrum, nude skin spectrum, and food chlorophyll spectrum. The images shown in Figure 3c are unmixed CyAl5.5 images.

Biodistribution of ^{111}Indium labeled 7a or 7b by dissection and gamma counter

150 µl of ^{111}Indium (300 µCi) labeled compounds 7a or 7b was i.v. injected into BT-20 tumor-bearing animals (80 nmoles/kg). 24h later, animals were sacrificed and organs (tumor, blood, liver, spleen, stomach, kidneys, small

intestine, lung, heart, tail, fat, muscle,) were collected. The radioactivities of those organs were measured by a gamma counter (Perkin Elmer, Wizard^2 2480).

SPECT/CT imaging

150 µl of ^{111}Indium (300 µCi) labeled compounds 7a (80 nmoles/kg) was i.v. injected to BT-20 tumor-bearing animals. 24h later, SPECT/CT images were taken with a triple modality microPET-SPECT-CT imaging device (Triumph, GE Healthcare) in Martinos Center for Biomedical Imaging.

Supplementary Data

Structures: The structures of compounds 5a to 7b are given in **Figure S1.** The most prominent species in the polydisperse 5 kDa PEG is n = 115, and is shown for 5b, 7a and 7b.

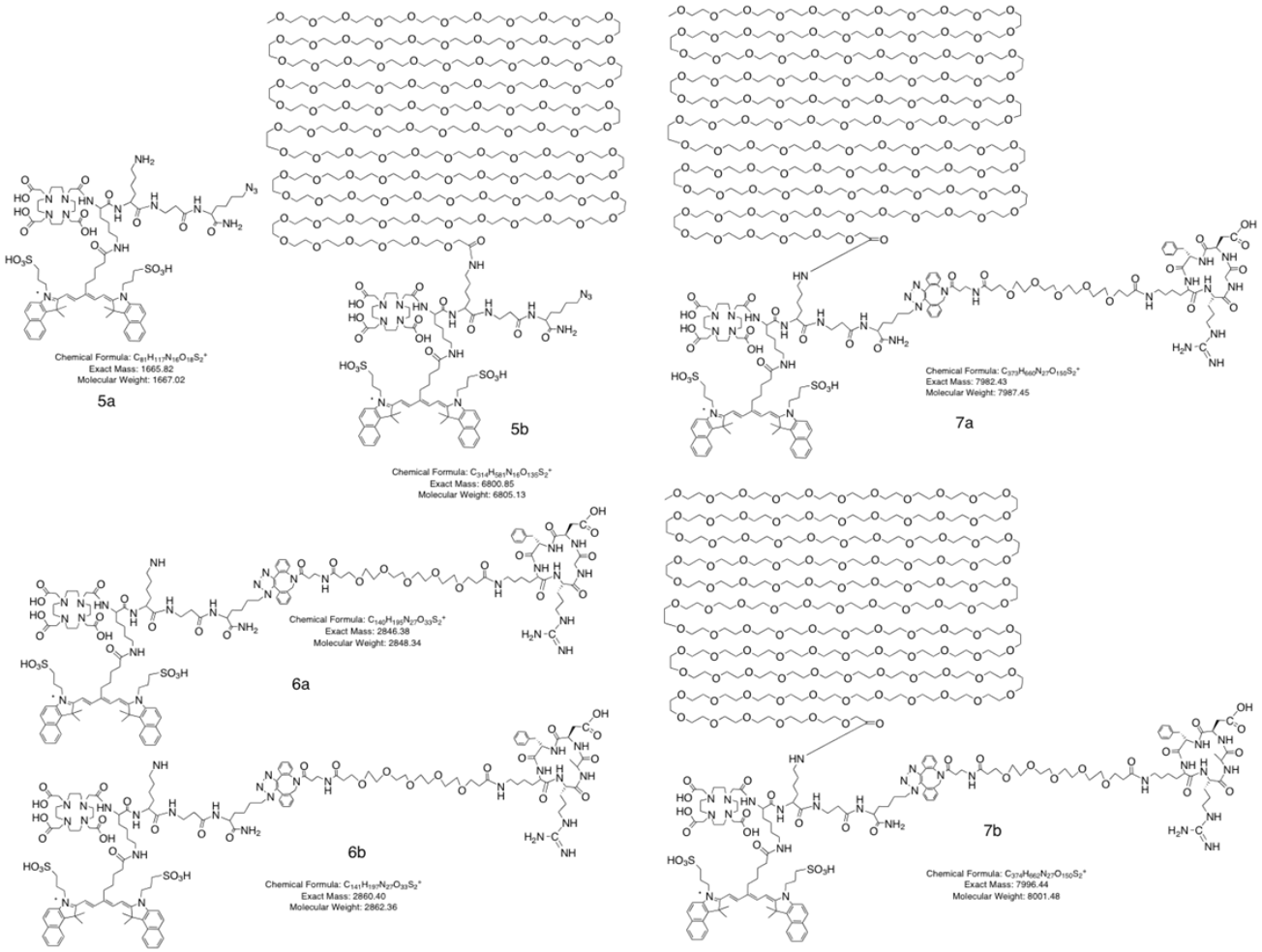


Figure S1. Structures of 5a through 7b are shown.

The absorption and emission spectra of multifunctional agents and multifunctional probes are shown in **Figure S2.**

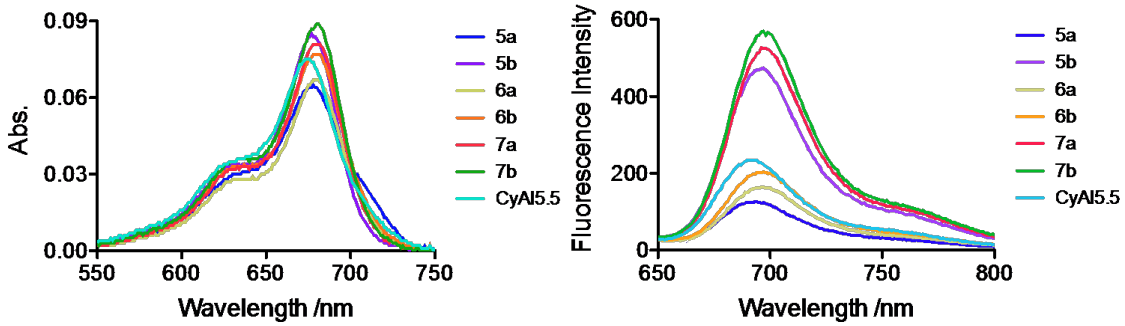


Figure S2. Absorption and emission spectra of PEG fluorochrome shielded reagents and probes are shown.

Figure S3

Fluorescence lifetime determination: Exemplary time versus fluorescence versus time curves generating the lifetimes in **Figure 2c** are shown in **Figure S3.**

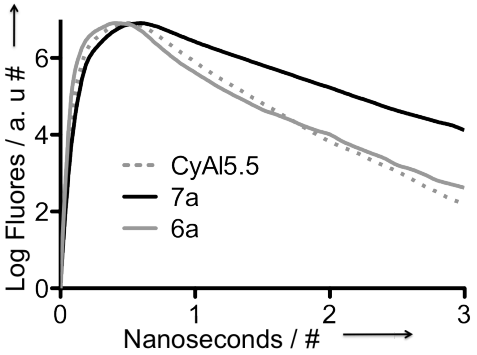


Figure S3. Fluorescence lifetime curves for selected compounds used to determine values shown in Figure 2c. PEGylated 7a has a longer lifetime than unPEGylated 6a.

Need for a short PEG linker: The short PEG linker (Figure 1, red) used in the design of the PEG fluorochrome shielded, integrin-binding probe (7a) was essential to obtain selective fluorochrome shielding and integrin interctions. To demonstrate this point, the 5 kDa PEG used for fluorochrome shielding was reacted with the RGD peptide as shown in **Figure S4a,** to yield 8a. The binding of 8a to integrins was determined from its ability to displace 7a from BT-20 cells, as shown in (4b) with peak fluorescence tabulated in (4c). 8a was significantly less able to displace 7a than the RGD peptide ($p = 0.0002$), indicating that the direct attachment of the 5 kDa PEG to the RGD peptide partially blocked RGD/integrin interactions and lead to the conclusion that the linker used in the design of 7a was essential.

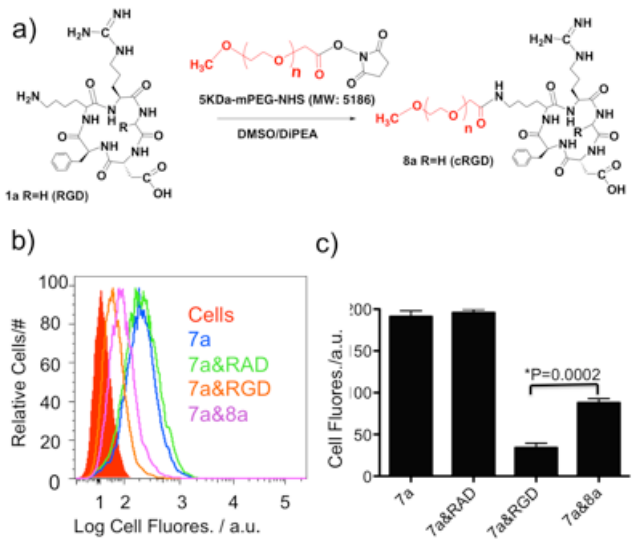


Figure S4. Effect of direct PEG attachment to an RGD peptide on integrin binding. (a) Synthesis of a PEGylated RGD where a 5 kDa PEG was attached directly to an RGD peptide (8a). (b) Activity of RGD, RAD and 8a displacing 7a binding to BT-20 cells. (c) Displacement of 7a by RGD, RAD, or the PEGylated 8a. 8a had decreased displacement. A summary of statistical tests performed is provided in Table S1.

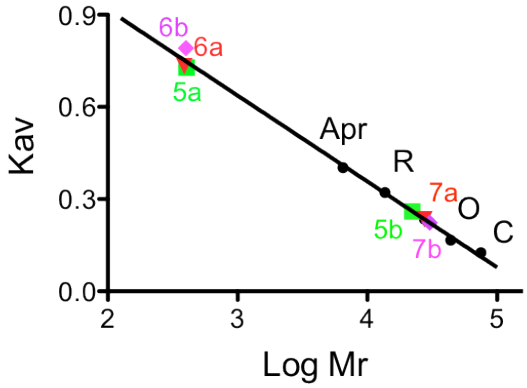


Figure S5. Volumes (Mv, molecular volume by retention) of unPEGylated and PEGylated probes were determined using conalbumin (C), ovalbumin (O), ribonuclease (R), and aprotinin (A) as standards. Values are summarized in Table 1.

Statistical tests of significance

Table S1. Summary of statistical tests

Figure	Parameter	Comparison	P value
2b	Quantum Yield	5a vs 5b	<0.0001
2b	Quantum Yield	6a vs 7a	<0.0001
2b	Quantum Yield	6b vs 7b	<0.0001

2c	Fluores. lifetime	5a vs 5b	<0.0001
2c	Fluores. lifetime	6a vs 7a	<0.0001
2c	Fluores. lifetime	6b vs 7b	<0.0001
4b	Cell Fluores. (binding)	5b vs 7b	0.67
4b Inset	Cell Fluores. (binding)	6a-6b vs 7a-7b	0.74
Suppl 4c	Cell Fluores. (7a displacement)	7a, RGD vs. 7a, 8a	0.0002
Suppl 4c	Cell Fluores. (7a displacement)	7a, RAD vs 7a	0.37

Biodistributon of PEG-fluorochrome shielded probes: The biodistributions of 7a and 7b are provided in Table S2. The %ID/gm data is from Figure 5. A measure of the amount of RGD/integrin binding in a tissue is the difference between 7a and 7b. High RGD/integrin binding is seen in the tumor, stomach, small intestine and lung.

Table S2. Biodistributions of the PEGylated 7a RGD probe and PEGylated 7b RAD probe in BT-20 tumor bearing mice. Bold = significant = p<0.01 for 7a versus 7b. Values are means ± 1 SD for n= 4.

Organ	%ID/gm			
	7a	7b	p value (%ID/gm)	7a-7b (%ID/gm)
blood	0.16±0.09	0.12±0.044	0.4795	
liver	12.8±2.6	7.94±2.1	0.0274	High NSB
kidney	13.18±3.21	12.3±4.5	0.7680	High NSB
spleen	5.08±0.67	0.74±0.18	<0.0001	
stomach	4.09±0.87	1.09±0.46	**0.0009**	**3.00±1.33**
small intest.	6.10±0.98	0.69±0.24	**<0.0001**	**5.41±1.22**
muscle	0.68±0.20	0.49±0.20	0.2297	
fat	0.91±0.39	0.50±0.13	0.1002	
tail	3.21±1.34	2.40±1.40	0.4376	
lung	2.71±0.69	0.68±0.15	**0.0012**	**2.03±0.84**
heart	1.61±0.37	0.82±0.17	**0.0082**	**0.79±0.54**

tumor	4.14±0.66	1.06±0.24	**0.0001**	**3.08±0.90**

References:

(1) Shao, F.; Yuan, H.; Josephson, L.; Weissleder, R.; Hilderbrand, S. A. *Dyes Pigm* **2011**, *90*, 119.

(2) Demas, J. N.; Crosby, G. A. *J. Phys. Chem.* **1971**, *75*, 991.

(3) Kumar, A. T.; Raymond, S. B.; Dunn, A. K.; Bacskai, B. J.; Boas, D. A. *IEEE Trans Med Imaging* **2008**, *27*, 1152.