Supporting Information for

Design and Assembly of Supramolecular Dual-modality Nanoprobes

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S1. Materials

All 9-fluorenylmethoxycarbonyl (Fmoc) protected amino acids, 2-(1H-benzotriazole-1-yl)- 1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and Fmoc-Glu (OtBu)-Wang resin were purchased from Advanced Automated Peptide Protein Technologies (AAPPTEC, Louisville, KY). Ninhydrin test reagents and 5-carboxyfluorescein (5-FAM) were purchased from AnaSpec, Inc. (Fremont, CA); Tri-tert-butyl-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA tris(*t*Bu ester)) was purchased

from TCI America (Portland, OR); N-Fmoc-6-aminohexanoic acid was purchased from Bachem Co. (Torrance, CA); $GdCl_3.6H_2O$ was purchased from Alfa Aesar (Ward Hill, MA) and palmitic acid was purchased from Sigma-Aldrich (St. Louis, MO). All the other general chemicals were purchased from VWR.

S2. Synthesis and characterization of molecules **1** and **2**

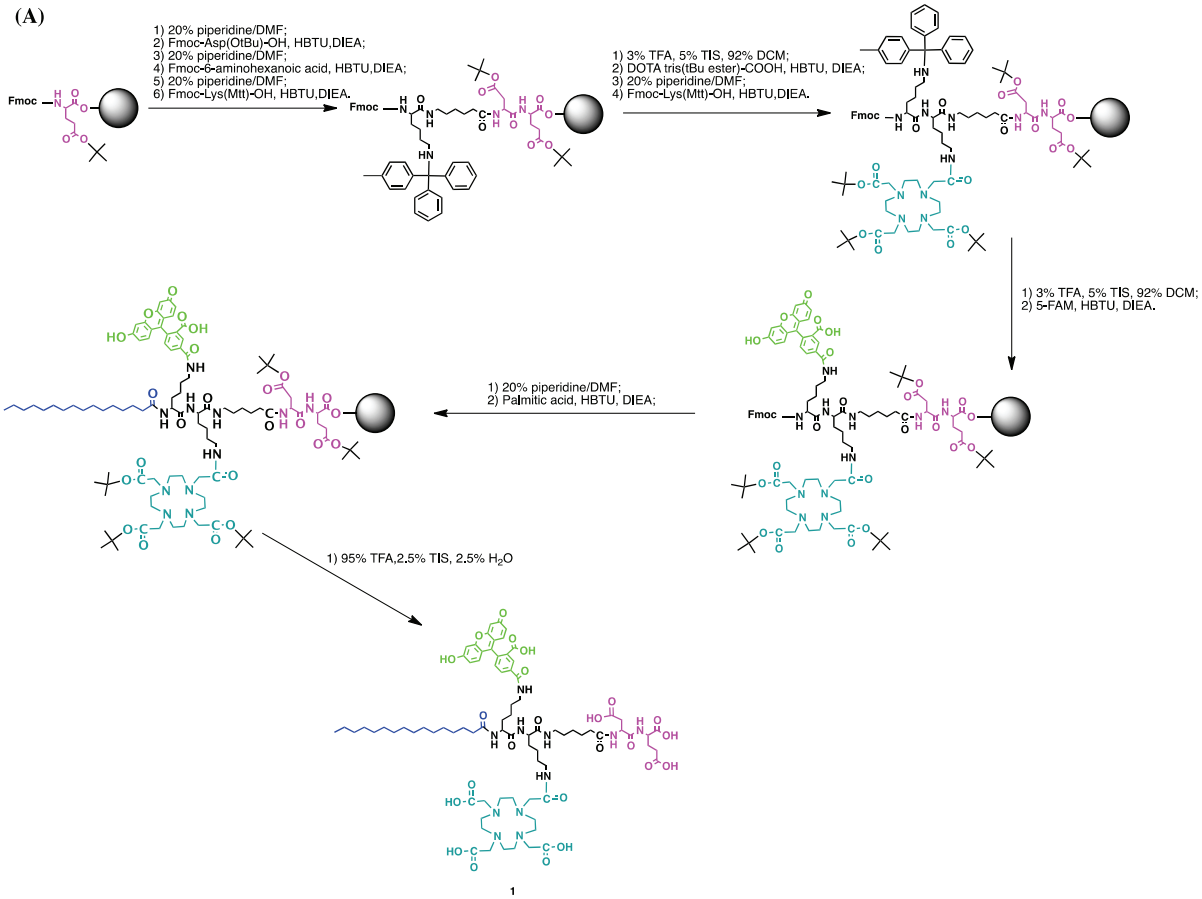
Standard Fmoc solid phase peptide synthesis strategies were used to synthesize the two peptide-based molecules: 2-(15-(4-(3-carboxy-4-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)benzamido)butyl)-2-(carboxymethyl)-4,11,14,17-tetraoxo-12-(4-(2-(4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecan-1-yl)acetamido)butyl)-3,10,13,16-tetraazadotriacontanamido) pentanedioic acid (**1**), and 2-(15-(4-(3-carboxy-4-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)benzamido)butyl)-2-(carboxymethyl)-4,11,14,17,24-pentaoxo-18-palmitamido-12-(4-(2-(4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecan-1-yl)acetamido)butyl)-3,10,13,16,23-pentaazanonatriacontanamido) pentanedioic acid (**2**). Reagents and conditions used for each step are shown in Figure S1 (A) and (B). Commercially available Fmoc-Glutamic acid (O-*t*Bu)-Wang resin was swelled in DCM for 15 min before the deprotection of Fmoc group on the glutamic acid residue using a 20% piperidine DMF solution for 5 min and repeated once for 10 min. After washed with DMF and DCM thoroughly, 4-fold excess of Fmoc-protected amino acid, 4-fold excess of HBTU and 10-fold excess of DIEA were dissolved in DMF and added into the reaction vessel for standard amine-carboxylic acid coupling. The coupling time of 1 hour was used for all the amino acids and longer coupling time for 5-FAM and DOTA conjugation was used to ensure the completion of coupling. After each coupling step, the excess of reactant was washed off thoroughly from resin using DMF and DCM, and the Fmoc was deprotected before conjugation of the next residue. After each step of conjugation and deprotection of amine protecting groups, ninhydrin test was carried out to monitor the complete of conjugation and degree of deprotection. With the existence of unprotected amine groups, ninhydrin reagents turn into blue color quickly; on the other hand, negative results of ninhydrin test indicate the completion of conjugation on amine group.

To the Glutamic acid (O-*t*Bu)-wang resin, Fmoc-Aspartic acid (O-*t*Bu)-OH, linker N-Fmoc-6-aminohexanoic acid and Fmoc-Lysine (Mtt)-OH were conjugated subsequently. Before deprotection of Fmoc on lysine residue, the side chain protection group 4-methyltrityl (Mtt) was deprotected for DOTA conjugation. The deprotection of Mtt group was done with a mixed solution of TFA/TIS/DCM (3/5/92) until no yellow color was observed. DOTA tris(*t*Bu ester)-COOH was coupled on the lysine side chain through standard amine-carboxylic acid coupling. Another Fmoc-Lysine (Mtt)-OH was conjugated on the peptide backbones and 5-FAM was conjugated on the side chain of lysine residue using the same strategy. The reaction vessels were covered with aluminum foil to prevent photobleaching of 5-FAM fluorophore. Under the same coupling conditions, palmitic acid was conjugated on the N-terminal of peptide to obtain molecule **1**. For molecule **2**, after 5-FAM conjugation, Fmoc-Lys(Fmoc)-OH was conjugated on peptide and palmitic acid was conjugated to both the N-terminal and side chain of the final lysine residue after Fmoc deprotection. After synthesis of the complete molecules, peptides were cleaved off from resins with a TFA/TIS/H_2O (95/2.5/2.5) cleavage cocktail for 3 hours to generate molecules **1** and **2**, which also resulted in simultaneous

deprotection of O-*t*Bu groups on peptides and DOTA molecules. The solution was then concentrated using rotavapor and then peptides were precipitated in cold ether. The precipitate was filtered and dried for purification.

Abbreviations:

DCM: dichloromethane
DIEA: diisopropylethylamine
DMF: *N*,*N*-dimethylformamide
DOTA: Tri-tert-butyl-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid
Fmoc: Fluorenylmethyloxycarbonyl
HBTU :*O*-benzotriazole-*N*,*N*,*N*',*N*'-tetramethyluroniumhexafluorophosphate
Mtt: 4-methyltrityl
OtBu: *O*-tert-butyl
TFA: trifluoroacetic acid
TIS: triisopropylsilane



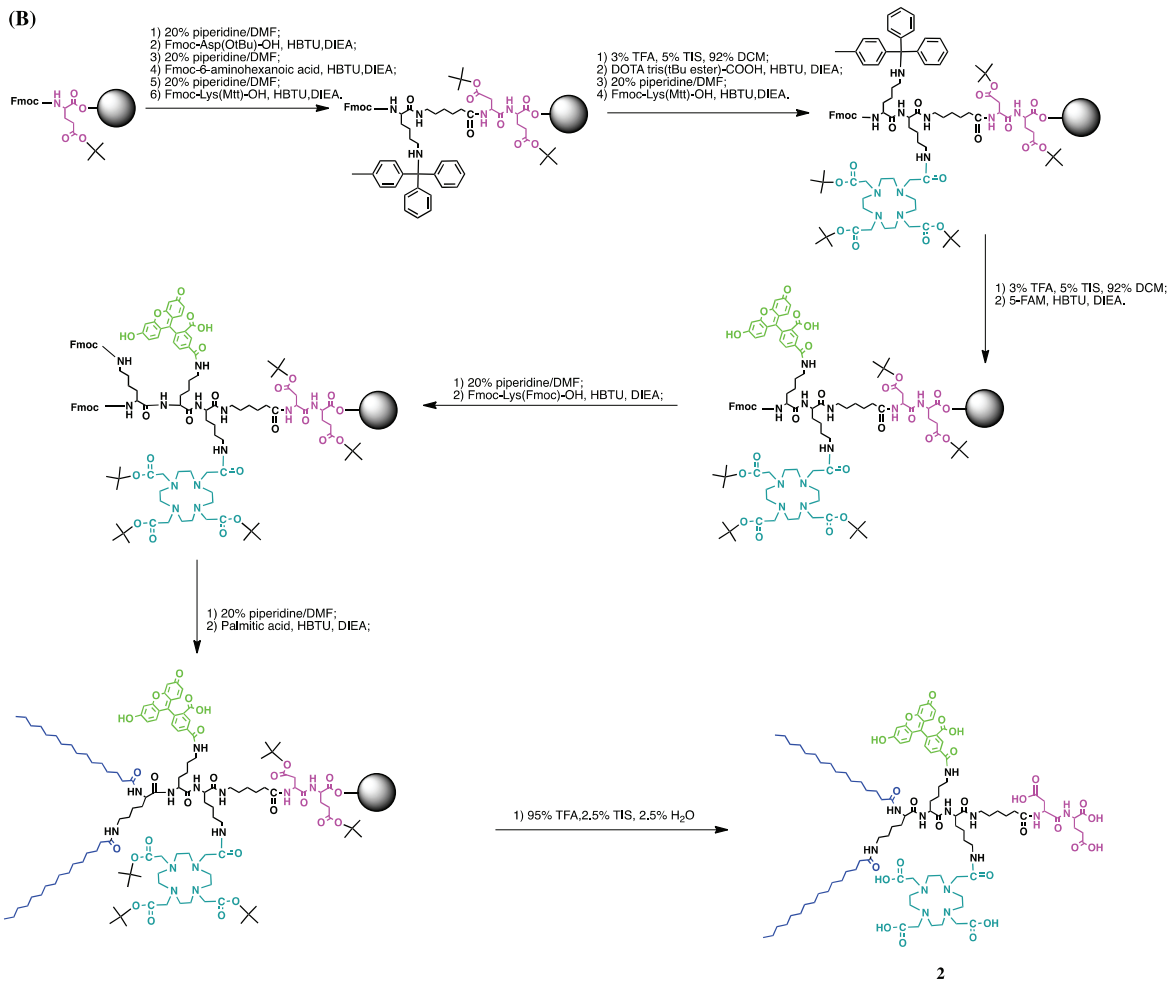


Figure S1. Synthesis of molecules **1** (A) and **2** (B) *via* standard Fmoc-based solid phase peptide synthesis strategies.

All the molecules were purified with reverse-phase high-performance liquid chromatography (HPLC) on a Varian ProStar Model 325 preparative HPLC (Agilent Technologies, Santa Clara, CA) equipped with a fraction collector and UV-vis detector using a Varian Polymeric Column (PLRP□S, 100 Å, 10 µm, 150 × 25 mm) at 25°C. A water/acetonitrile gradient containing 0.1 % *v*/*v* NH_4OH as solvent system and a flow rate of 25 mL/min were used. The absorbance at 220 nm and 490 nm were monitored based on peptide backbone absorbance and 5-FAM peak absorbance. Crude products were dissolved in water containing 0.1% *v*/*v* NH_4OH and a 10 mL solution was injected each time. Collected fractions were analyzed by electrospray mass spectrometry (ESI□MS) (LDQ Deca ion□trap mass spectrometer, Thermo Finnigan, San Jose, CA) or MALDI (Bruker Autoflex III MALDI-TOF instrument (Bruker Daltonics, Billerica, MA)) and the final products were lyophilized using a FreeZone □105°C 4.5 L freeze dryer (Labconco, Kansas City, MO) and stored in -30°C freezer. Analytical HPLC was used to characterize the purity of molecules on the same HPLC instrument with a Varian Column (PLRP-S 100 Å, 10 µm, 150 × 4.6 mm) with a flow rate of 1 mL/min. The elution gradient started from 95% A and 5% B for 5 min and ramp to 40% A and 60% B in 20 min and then ramp

to 100% B in 5 min for molecule **1**. A gradient ramping from 5% to 100% B in 20 min was used for molecule **2**. Analytical HPLC chromatograms of molecules **1** and **2** are shown in Figure S2. For molecule **1**, ESI-MS m/z ($[M+H]^+$): calculated for $C_{80}H_{116}N_{11}O_{24}$, 1615.8, found 1615.2; for molecule **2**, ESI-MS m/z ($[M+H]^+$): calculated for $C_{102}H_{157}N_{13}O_{26}$, 1981.4, found 1981.6.

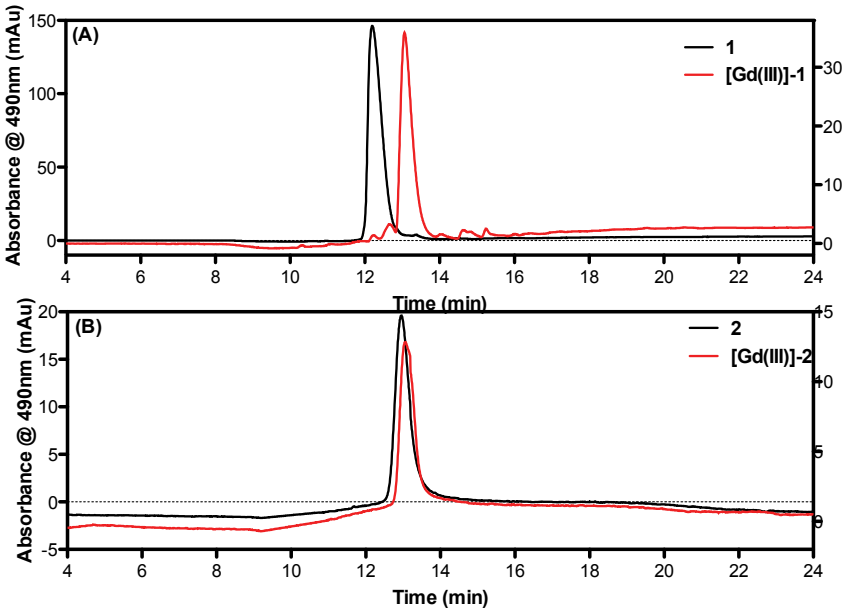


Figure S2. Analytical HPLC chromatograms of molecules **1** and **[Gd(III)]-1** (A) and **2** and **[Gd(III)]-2** (B) UV-vis absorbance at 490 nm was monitored, which is the absorbance maximum of 5-FAM fluorophore.

S3. Synthesis and characterization of complexes **[Gd(III)]-1** and **[Gd(III)]-2**

To synthesize gadolinium complexes of molecules **1** and **2**, dilute solutions of **1** and **2** (50 μM and 10 μM, respectively) in H_2O were used to ensure molecules exist in solution as single molecules, so that the chelation of DOTA of gadolinium are not hindered by self-assembled structures. A 4-fold excess of 100 μM $GdCl_3.6H_2O$ was added into each solution. The solutions were heated up to 80 °C and the pH were adjusted to 10 to 11 with 1 M NaOH. After heated up for 2 hours, solutions were cooled down and preparative HPLC was used to purify gadolinium complexes of molecules **1** and **2** and free $GdCl_3$ were removed during HPLC purification. After complexation, the negative charges on DOTA molecules were neutralized by gadolinium cations, so that the interactions between complexes and the HPLC columns are therefore affected, which resulted in the elution peak shift on HPLC chromatography, as shown in Figure S2. For **[Gd(III)]-1**, MALDI-TOF m/z ($[M+H]^+$): calculated for $C_{80}H_{112}GdN_{11}O_{24}^{3+}$, 1767.1, found 1767.7; for **[Gd(III)]-2**, ESI-MS m/z ($[M+2H]^{2+}$): mass calculated for $C_{102}H_{154}GdN_{13}O_{26}^{3+}$, 2137.6, found 1068.6 for $[M+2H]^{2+}$, mass after deconvolution is 2137.2 .

S4. Morphology of self-assembled nanostructures

TEM were used to characterize the self-assembly morphology of these nanoprobes. A 10μL sample solution in PBS buffer at pH 7.4 was loaded on a Cu grid with carbon film. The excess of solution was blotted with filter paper to leave a thin layer of sample solution on grid. Staining solution 2 wt% uranyal acetate was added to the sample and the excess of solution was blotted with filter paper after staining for 30 seconds. Samples were dried at room temperature for at least 3 hours before TEM images were taken. TEM images were taken using a Tecnai 12 TWIN TEM instrument equipped with a FEI Eagle bottom mount camera (FEI, Hillsboro, OR) and SIS Megaview III wide-angle CCD camera. Diameters of micelles were averaged from at least 30 samples from 3 images for each sample.

S5. Live-cell fluorescence imaging and cytotoxicity studies of dual-modality probes

Human cervical cancer cell line KB-3-1 was used to study the fluorescence measurement of probe **1** and **2**. KB-3-1 cells were cultured in DMEM media (Invitrogen, Carlsbad, CA) with 10% FBS and 1% Pen-Strep (Invitrogen, Carlsbad, CA). Cells were maintained at 5% carbon dioxide (CO2), at 37.0 °C in a humidified incubator. KB-3-1 cells were seeded on a 24-well plate with a density of 1×10^5 cells per well and incubated overnight allowing attachment to the plate. To the plate, probe **1** and **2** solution in fresh culture media with a series of concentrations were added and incubated for 2 hrs for concentration dependence studies. Probe **1** of 200 μM and probe **2** of 50 μM were added and incubated for 0.5, 1, 2 and 4 hours for time dependence studies. After incubation, sample solutions were removed carefully and the wells were washed with cell culture media twice and live cell fluorescence imaging were recorded with a JENCO BC-366 optical microscope (San Diego, CA) equipped with a JENCO 5M CMOS camera for color imaging. The PS-2 mercury lamp and blue filter with 420-485nm excitation wavelengths and 520 ± 10% nm emission wavelengths were used for 5-FAM fluorescence measurement. Representative fluorescent images of probe **1** and **2** of 200 μM and 50 μM after incubation for 2hrs are shown in Figure 2. Then cells were then trypsinized, collected and resuspended in cold PBS for flow cytometry studies. Cells were analyzed with FL1 channel (Green, 530/30 nm) using a FACSCalibur flow cytometer (Becton Dickinson, NJ). Live cells were gated using forward scatter (FSC) and side scatter (SSC) parameters, and 10,000 gated events were collected for analysis. The fluorescence histograms are shown in Figure 2 and geometric mean intensity are plotted in Figure S3 and S4.

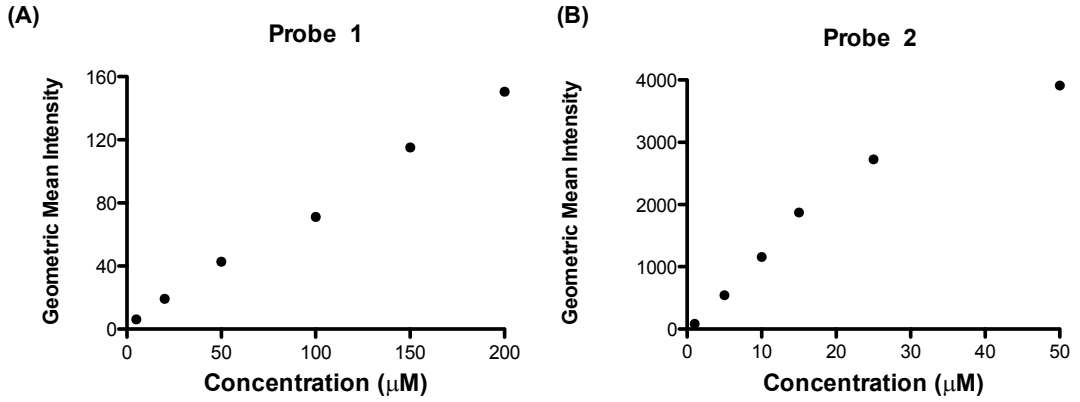


Figure S3. Geometric mean intensity of KB-3-1 cells incubated with Probe **1** (A) and **2** (B) at a series of concentrations for 2 hrs.

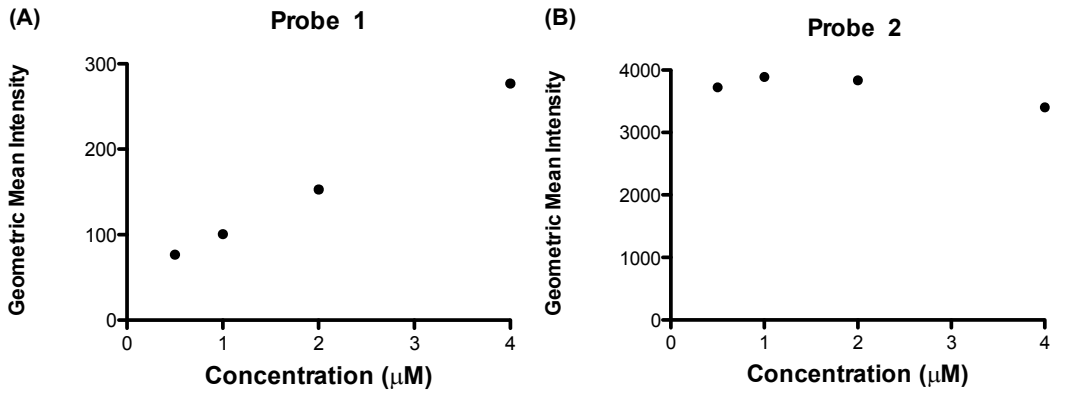


Figure S4. Geometric mean intensity of KB-3-1 cells incubated with Probe **1** of 200 μM (A) and **2** of 50 μM (B) for 0.5, 1, 2 and 4 hrs.

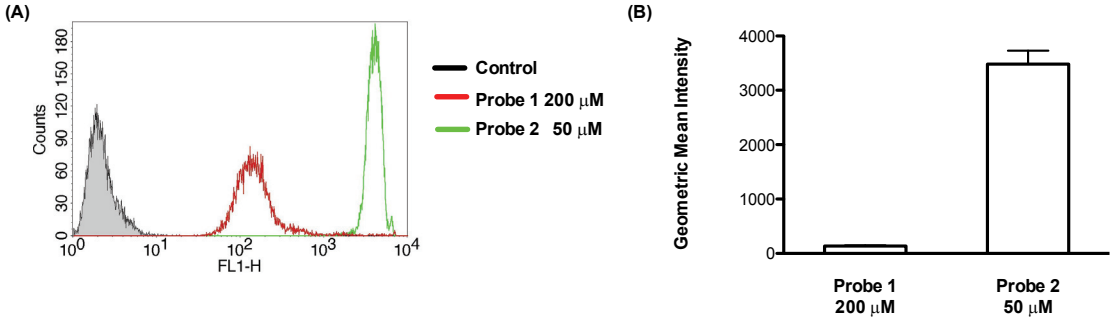


Figure S5. Representative flow cytometry histograms (A) and geometric mean intensity (B) of KB-3-1 cells incubated with probe **1** of 200 μM and **2** of 50 μM for 2 hrs.

Confocal fluorescence imaging was also used to study cellular uptake in more details. KB-3-1 cells were seeded onto a 8-well glass bottom plate (Labtek, Scott's Valley, CA) pretreated with type I rat tail collagen (Invitrogen) at 4×10^4 cells/well, and allowed to attach overnight. The cells were incubated with probe **1** of 100 μM and probe **2** μM of 50 for 2 hours. Thirty minutes before the washing step, 100 nM Lysotracker® Red (Invitrogen) and 10 μg/mL Hoechst 33342 (Invitrogen) were added. The cells were then washed with phenol red free DMEM (Corning, Tewksbury, MA) supplemented with 10% FBS and imaged using a Zeiss 510 confocal laser scanning fluorescent microscope (Frankfurt, Germany). The representative confocal images of cells at DIC, 5-FAM, Lysotraker® Red, Hoechst 33342 fluorescence, and merged channels are shown in Figure S6.

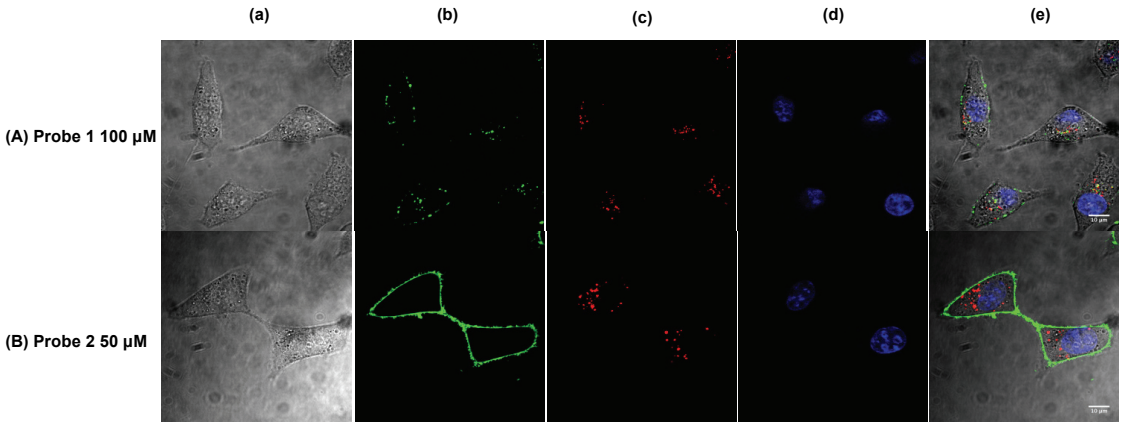


Figure S6. KB-3-1 cells were incubated with probe **1** of 100 μM (A) and probe **2** of 50 μM (B) for 2 hours. Representative confocal images of cells are shown in panel (a)-(e). Panels (a) are DIC images, (b) are 5-FAM (green), (c) are Lysotraker® Red (red), (d) are Hoechst 33342 (blue) and (e) are the merged channels.

Cytotoxicity studies of both molecules **1** and **2** were carried out using sulforhodamine B (SRB) staining assay. After being trypsinized from cell culture petri dish, cells were seeded on to a 96-well plate with a 16,000 cells per well density overnight at 37°C. Samples of various concentrations in culture media were added to the plate after the old culture media were removed. Cells were incubated with samples for 20 hrs at 37°C. After culture media were completely removed from each well, cells were fixed with 100μL 10% (Trichloroacetic acid) TCA solution at 4 °C for 1 hour. TCA solution was removed and plate was rinsed with water 3 times to wash off the residue chemicals and dried with air completely. To each well, 30 μL of 0.4 % SRB solutions were added to stain the live cells for 30 min. The staining solution was then removed and plate was rinsed with 1 % acetic acid till the wash solution is colorless. The plate was dried with air and 100 μL 10 mM Tris buffer was added to each well to solubilize SRB for 5 min. The solution was mixed well and the absorbance at 565 nm was recorded using an absorbance plate reader. The cell viability of molecules **1** and **2** of a series of concentration were plotted after normalized based on the blank control on the same plate, as shown in Figure S7. The results indicate that almost no effects on cell viability of all samples were observed at these concentrations after 20 hrs incubation.

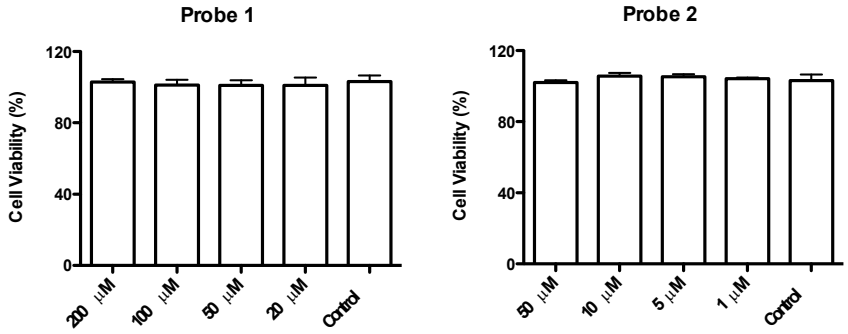


Figure S7. Normalized cell viability of molecules **1** and **2** of up to 50 μM and 200 μM after 20 hours incubation with KB-3-1 cells.

S6. Determination of ^{1}H longitudinal relaxivity r_1 of **[Gd(III)]-1** and **[Gd(III)]-2**.

Proton T_1 relaxation time with existence of nanoprobes **[Gd(III)]-1** and **[Gd(III)]-2** with a series of concentrations were measured in order to study the effects of micelle formation on contrast agents relaxivity. Water proton longitudinal relaxivity r_1 of **[Gd(III)]-1** and **[Gd(III)]-2** were determined on an Aspect M2 1T MRI scanner (Shoham, Israel) with a 35 mm diameter coil at room temperature. An inversion recovery-spin echo (IR-SE) sequence was used with different recovery times for **[Gd(III)]-1** and **[Gd(III)]-2**. Inversion time 15, 30, 60, 100, 200, 400, 600, 800, 1200, 1500, 2500, 3500 and 4500 ms were used for **[Gd(III)]-1**; while 50, 400, 600, 800, 1200, 1500, 2500, 3500, 4500 and 6000 ms were used for **[Gd(III)]-2**, respectively. Repetition time of 10 seconds was used for all measurements. Concentrations of 20 to 300 μM for **[Gd(III)]-1** and 0 to 50 μM for **[Gd(III)]-2** were chosen in order to cover the CMC concentration range. All sample solutions were in 1 × PBS buffer at pH 7.4. A nonlinear fitting algorithm was used to fit the observed MR amplitudes to the exponential recovery function to yield the T_1 relaxation time for each concentration.
Enhancement of relaxation rate R, which is the reciprocal of relaxation time T_1, can be calculated and plotted against probe concentrations (mM^{-1}). Relaxivity of monomer (r_1^m) and self-assembled micelle (r_1^s) are determined from the slopes of linear fits of plots as previously reported. [1]

When concentration is below CMC, the enhancement of relaxation rate was determined by the monomer concentration of probes, therefore, the slope is determined as relaxivity r_1^m (equation (1)).

$$R_1^{obs} - R_1^{PBS} = r_1^m c_{Gd} \quad (1)$$

After concentration reaches CMC, there are two contributions for enhancement of relaxation rate from both monomer and micelles. Therefore, the relaxivity of micelles can be determined from the slopes at the second part described as equation 2 and CMC was calculated accordingly based on equation 3.

$$R_1^{obs} - R_1^{PBS} = r_1^m \times CMC + r_1^s(C_{Gd} - CMC) \quad (2)$$

$$R_1^{obs} - R_1^{PBS} = (r_1^m - r_1^s) \times CMC + r_1^s C_{Gd} \quad (3)$$

S7. CMC measurement of dual-modality nanoprobes with fluorescence measurement

Normalized absorbance spectra of 5-FAM and molecules **1** and **2**, obtained using a Varian Cary 300 Bio UV-visible spectrophotometer (Agilent Technologies, Santa Clara, CA), are shown in Figure S8 (A). Compared with the peak absorbance of 5-FAM at 492 nm, the absorbance peaks of molecules **1** and **2** shifted to 498 nm. However, the maximum excitation wavelengths for both molecules **1** and **2** remain at 492 nm as of 5-FAM. The self-assembly of molecules **1** and **2** was also studied through the measurement of their fluorescence signals. Samples were dissolved in PBS buffer at pH 7.4 and a series of concentrated samples were obtained through series dilution from higher concentrated stock solutions. The fluorescence spectra were measured with a Fluorolog Spectrofluorometer (Horiba Jobin Yvon, Inc., Edison, NJ) using a quartz fluorometer cuvette with a 10 mm path length (Starna Cells, Inc., Atascadero, CA). Excitation wavelength at 492 nm and emission spectra were taken between 500 to 540 nm with a slit width of 1.5 nm. The emission wavelength step was set to be 1 nm with an integration time of 0.1 second. The emission spectra of molecule **1** from 4 to 500 μM concentration and of molecule **2** from 0.5 to 64 μM in PBS buffer at pH 7.4 are shown in Figure S8 (C) and (D), respectively. For both molecules **1** and **2**, the maximum fluorescence emission shifts to higher wavelength with increasing concentration. The plots of the emission peak position versus concentration for both molecules **1** and **2** are shown in Figure S8 (E).

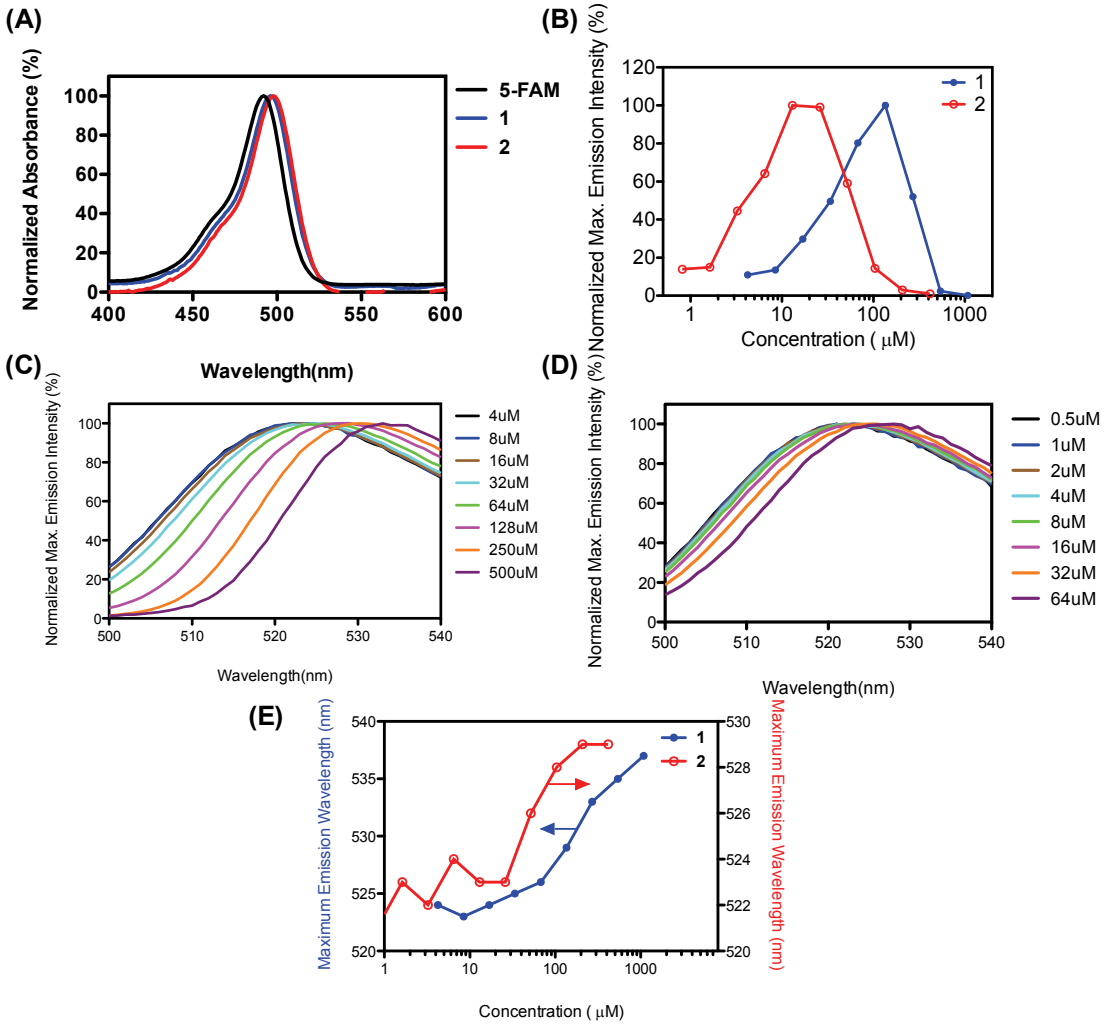


Figure S8. (A) Absorbance spectra of 5-FAM and molecules **1** and **2**. (B) Emission spectra of molecule **1** from 4 to 500 μM in PBS buffer at pH 7.4. (C) Emission spectra of molecule **2** from 0.5 to 64 μM in PBS buffer at pH 7.4. (D) Maximum emission wavelengths of molecules **1** and **2** at different concentrations.

Reference:

1. G. M. Nicolle, E. Toth, K.-P. Eisenwiener, H. R. Macke and A. E. Merbach, *J Biol Inorg Chem*, 2002, **7**, 757-769.