Supporting Information

Bidentate iminodiacetate modified dendrimer for bone imaging

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Figure S1. Synthetic scheme of IMPLD **9**: a) HATU, DIPEA, dry DMF, 5 h; b) TFA/DCM 1/1, 5 h; c) K_2CO_3, DMF/H_2O 1/1, 60°C, 30 min, 42 % over 3 steps; d) HATU, DIPEA, dry DMF, overnight; e) hydrazine 2 % in DMF, 30 min; f) FITC, DIPEA, dry DMSO, overnight; g) TFA/TIS/H_2O 95/2.5/2.5.

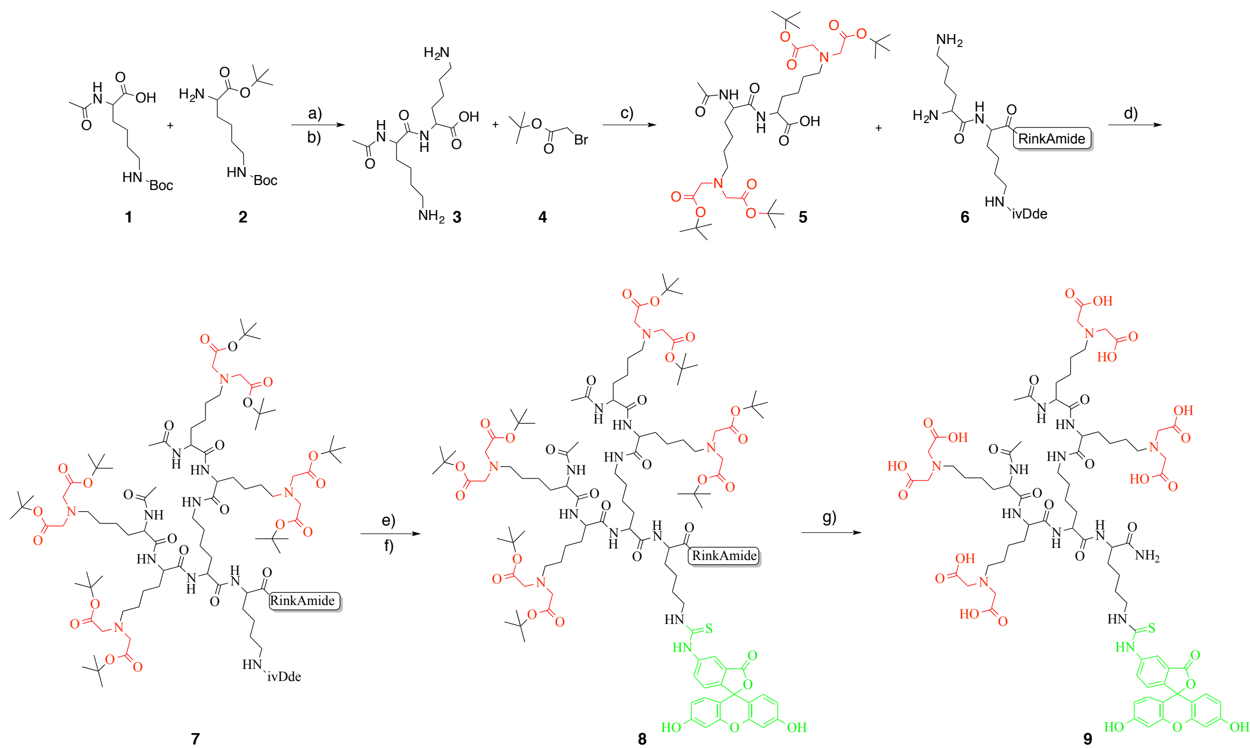


Figure S2: Chemical structure of Cy5.5-IMPLD.

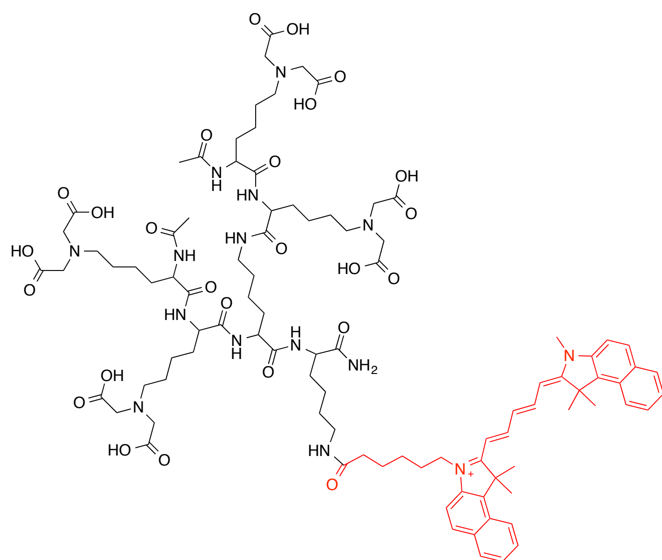


Figure S3. *In vivo* fluorescence images, dorsal view, of the representative animal with 20 nmol of IMPLD at the indicated time points. The probe initially accumulated in the kidney (images at 6 hr and 1 day). The spine signal was seen 3 hours after the injection, and lasted more than 4 weeks.

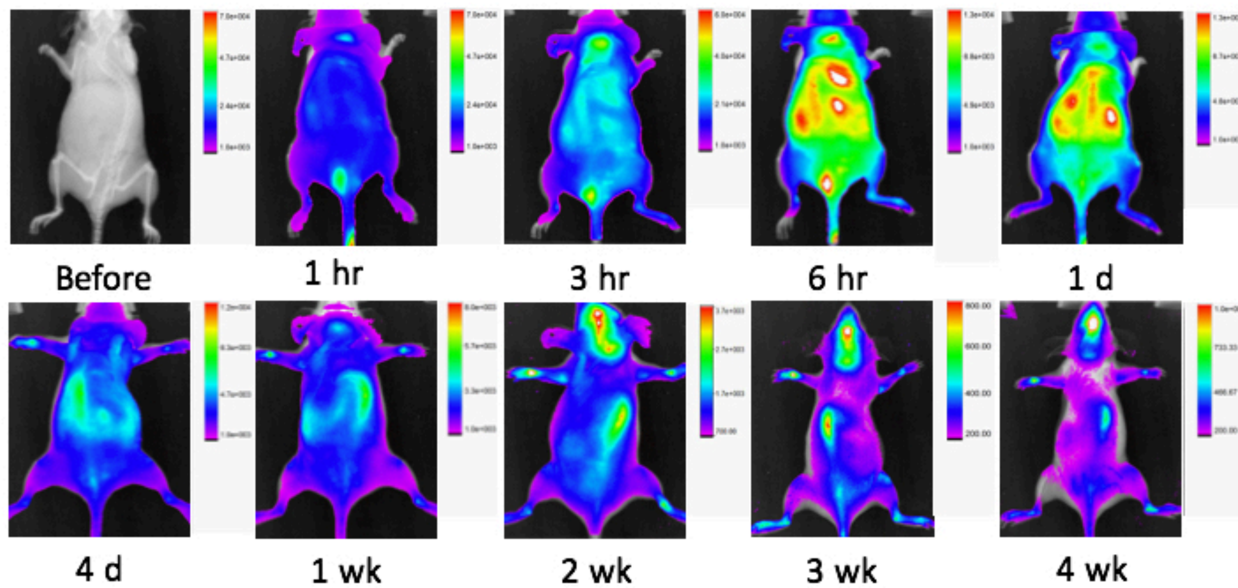


Figure S4. *Ex vivo* organ fluorescent images revealed the clearance of IMPLD from the kidneys and liver over time.

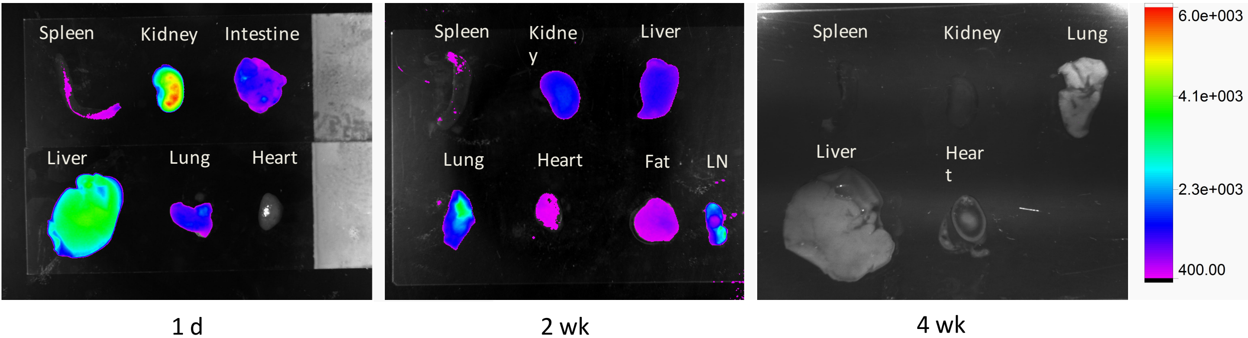
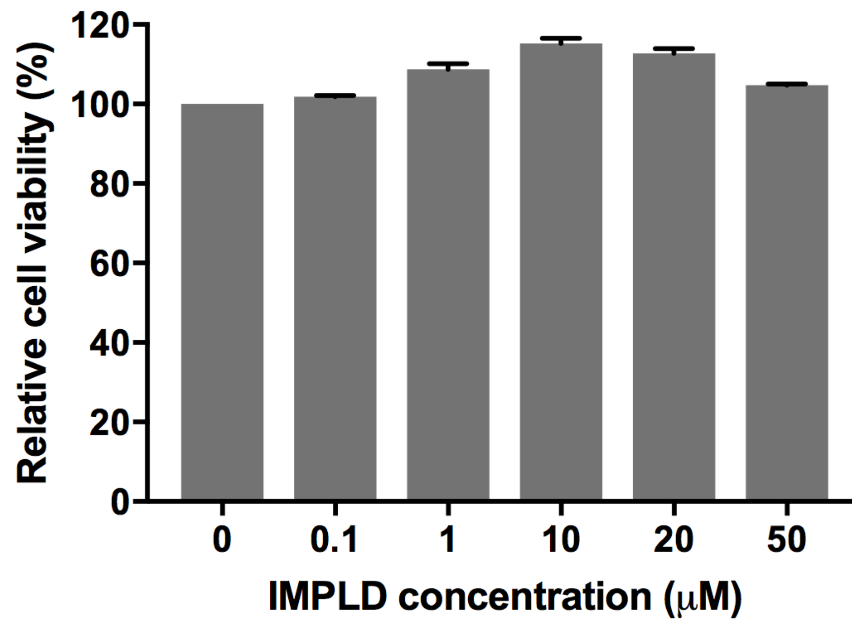


Figure S5. Cytotoxicity of IMPLD in NIH-3T3 fibroblast cells. The cells were treated with IMPLD (0, 0.1, 1, 10, 20 and 50 μM) for three days prior to the MTS proliferation assay. The result showed no cytotoxicity up to 50 μM of IMPLD.



Material and methods

Materials for chemical synthesis

Acetic acid, acetic anhydride, acetonitrile (MeCN), diisopropylethylamine (DIPEA), dimethylsulfoxide (DMSO), fluorescein isothiocyanate isomer I (FITC), hydrazine, piperidine, potassium carbonate (K_2CO_3), *tert*-butyl bromoacetate, trifluoroacetic acid (TFA), triisopropylsilane (TIS) and sodium sulfate anhydrous ($NaSO_4$) were purchased from Sigma Aldrich (St. Louis, MO, USA); dichloromethane (DCM), ethylacetate (EtOAc), hexane, methanol (MeOH), methyl *tert*-butyl ether (MTBE), *N,N'*-dimethylformamide (DMF) and sodium chloride were supplied by VWR (Radnor, PA, USA); *N*α-Acetyl-*N*ε-Boc-L-lysine (Ac-Lys(Boc)-OH), O-(7-Aza-1H-benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HATU) and (S)-2-(Fmoc-amino)-4-aminobutyric acid (Fmoc-L-Dab-OH) from Alfa Aesar (Ward Hill, MA, USA); (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), Fmoc-L-Lys(Fmoc)-OH, Fmoc-L-Lys(ivDde)-OH and *N*-methylmorpholine (NMM) were supplied by Protein Technologies (Tuscon, AZ, USA); Fmoc-L-Lysine-OH and Nε-Boc-L-lysine *tert*-butyl ester hydrochloride (H-Lys(Boc)-O*t*Bu) from Chem Impex International (Wood Dale, IL, USA), Fmoc-L-Orn-OH·HCl from Bachem (Torrance, CA, USA) and Cyanine5.5 NHS ester from Lumiprobe (Hallandale Beach, FL, USA).

General analytical methods for chemical synthesis

NMR spectra were obtained on a Bruker 2014 AvanceHD III 500MHz spectrometer at 500 MHz for ^1H and 126 MHz for ^{13}C spectra. The middle solvent peak for ^1H NMR spectra was referenced to 7.26 in deuterated chloroform ($CDCl_3$) and 2.50 in deuterated dimethylsulfoxyde (DMSO-d_6). The middle solvent peak for ^{13}C NMR spectra was referenced to 77.16 in $CDCl_3$ and 39.52 in DMSO-d_6. The coupling constants (J) are in Hz and the chemical shifts (δ) are given in parts per million.

High-performance liquid chromatography (HPLC) was performed on an Agilent Technologies preparative HPLC system. A Grace Vydac 218TP C18 5 μm column was used for analytical HPLC (flow rate 1.0 mL/min), while a Phenomenex Luna C18(2) 10 μm column was used for preparative HPLC (flow rate 10.0 mL/min). The mobile phases used were 0.1 % v/v trifluoroacetic acid in water (phase A) and 0.1 % v/v trifluoroacetic acid in acetonitrile/water 9/1 (phase B).

Liquid chromatography-mass spectroscopy (LC-MS) analyses were obtained on a Waters Acquity UPLC-H class system operating under electrospray ionization conditions (ESI). The mobile phases used were 0.05 % v/v trifluoroacetic acid in water (phase A) and 0.05 v/v % trifluoroacetic acid in acetonitrile (phase B). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analyses were obtained from the Tufts Medical School, Core facilities, Boston, MA.

Evaporation *in vacuo* refers to the removal of solvent on a Heidolph rotary evaporator with an integrated vacuum pump. Thin-layer chromatography (TLC) was executed on aluminum backed 60 F254 silica gel. When necessary, dry solvents were obtained from a Pure Process Technology solvent purification system.

Synthetic procedure

Synthesis of N^2-(N^2-acetyl-N^6,N^6-bis(2-(*tert*-butoxy)-2-oxoethyl)lysyl)-N^6,N^6-bis(2-(*tert*-butoxy)-2-oxoethyl)lysine (Ac-Lys($CH_2COOtBu$)$_2$-Lys($CH_2COOtBu$)$_2$-OH, **5**):

Ac-Lys(Boc)-OH (**1,** 0.80 g, 2.77 mmol) and HATU (1.26 g, 3.33 mmol) were dissolved in anhydrous DMF (30 mL). H-Lys(Boc)-O*t*Bu (**2**, 0.94 g, 2.77 mmol) was added together with DIPEA (1.68 mL, 9.70 mmol) to the solution. The reaction was stirred for 5 h. the solvent was then removed *in vacuo*. Crude oil was dissolved in DCM, water was added and the aqueous layer was extracted with DCM (3 x 20 mL). The organic layers were combined, washed with brine, dried over $NaSO_4$ and concentrated *in vacuo.* The crude product was dissolved in TFA/DCM 1/1 (10 mL) and stirred for 5 hours. The solvent was removed under gentle nitrogen flow and the product (**3**) was redissolved in water and DCM. The aqueous layer was extracted with DCM (3 x 20 mL), the organic layers were combined, washed with brine, dried over $NaSO_4$ and concentrated *in vacuo.* Half of the crude product (**3**) was redissolved in DMF/H_2O (20 mL) and K_2CO_3 (1.30 g, 9.66 mmol) was added together with *tert*-butyl bromoacetate (**4**, 917 μL, 6.21 mmol). The temperature was brought to 60 °C and the reaction was stirred for 30 minutes. The temperature was brought down to room temperature and acetic acid 5 % (20 mL) was mixed into the solution. DCM was added and the aqueous layer was extracted with DCM (3 x 20 mL). The organic layers were combined, washed with brine, dried over $NaSO_4$, and concentrated *in vacuo.* The crude product was purified via silica gel chromatography (hexane/EtOAc/MeOH 50/50/0 to 0/80/20) to achieve **5** as an off white solid (0.45 g, 0.58 mmol, 42 % yield over three steps). TLC r.f. in EtOAc/MeOH 8/2 = 0.17. NMR, δ (500 MHz, DMSO), 7.93 (2 H, d, J = 8.1 Hz, 2 NH), 4.23 (1 H, m, CH Lys), 4.07 (1 H, m, CH Lys), 3.31 (8 H, s, 4 CH_2COO*t*Bu), 2.55 (4 H, t, J = 6.9 Hz, 2 Lys CH_2N), 1.82 (3 H, s, Ac CH_3), 1.66-1.39 (4 H, m, 2 Lys $CHCH_2$), 1.39-1.33 (4 H, m, 2 Lys CH_2CH_2N), 1.26-1.23 (4 H, m, 2 Lys $CHCH_2CH_2$), 1.39 (36 H, s, 4 *t*Bu). ^{13}C-NMR δ (126 MHz, DMSO) 171.68 (COOH), 170.04 (4 COO*t*Bu), 168.94 (2 CONH), 79.99 (4 $C(CH_3)_3$), 55.36 and 55.27 (4 CH_2COO*t*Bu), 53.26 and 53.19 (CH_2N), 52.19 and 51.94 (CH), 32.00 and 30.96 ($CHCH_2$), 27.69 (4 $C(CH_3)_3$), 27.02 and 26.94 (CH_2CH_2N), 22.92 ($CHCH_2CH_2$), 22.39 (Ac CH_3). ESI-MS m/z, $M+H^+$ = 773.8, calculated for $C_{36}H_{26}N_2O_7S$ = 772.5.

Synthesis of 8,24-diacetamido-14-((1-amino-6-(3-(3',6'-dihydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthen]-5-yl)thioureido)-1-oxohexan-2-yl)carbamoyl)-11,21-bis(4-(bis(carboxymethyl)amino)butyl)-3,29-bis(carboxymethyl)-9,12,20,23-tetraoxo-3,10,13,19,22,29-hexaazahentriacontanedioic acid (IMPLD, **9**)

Solid phase peptide synthesis (SPPS) was performed on an automatic synthesizer (PS3, Protein Technologies, Tuscon, AZ, USA) using standard Fmoc chemistry. Rink amide-MBHA LL resin (Novabiochem, Billerica, MA, USA) with a loading capacity of 0.38 mmol/g was employed to afford peptide amides. Fmoc-Lys(ivDde)-OH and Fmoc-Lys(Fmoc)-OH (4 equiv) were coupled in this order on the resin (0.1 mmol) using HBTU (4 equiv) and the base NMM. Fmoc deprotection was achieved by exposure to 20 % piperidine in DMF to achieve **6. 6** was added together with DIPEA (1 mL) to a previously dissolved solution of **5** (6 equiv) and HATU (6 equiv) in anhydrous DMF (10 mL). The reaction was shaken, over a period of two nights, to achieve **7**. **7** was dissolved in hydrazine 2% in DMF (10 mL) and shaken for 30 min to cleave the ivDde protecting group. FITC (6 equiv) was subsequently dissolved in DMSO anhydrous (4 mL) and added, together with 1 mL of DIPEA, to the resin-bound peptide. The mixture was agitated in darkness overnight to achieve **8**. **8** was cleaved from the resin for 4 hours with 95 % TFA, 2.5 % TIS, 2.5 % H_2O (5 mL) and precipitated from MTBE. The precipitate was centrifuged, washed further with MTBE, and dried in a vacuum dessicator. The crude fully deprotected FITC labeled peptide was purified by prep HPLC (220 nm and 460 nm) from 0 to 100 % of phase B over 45 min to achieve **9** (21.2 min retention time) as a yellow powder. Retention time analytical HPLC from 0 to 100 % of phase B over 30 min: 11.6 min. NMR, δ (500 MHz, DMSO), 10.27 (2 H, s, OH FITC), 8.45 (1 H, br. s, Lys-NH-FITC), 8.30 (1 H, br. s, CH FITC), 8.04-7.01 (10 H, 8 NHCO and 1 $CONH_2$), 7.75 (1 H, s, CH FITC), 7.17 (1

H, d, J = 8.3, CH FITC), 6.67 (2 H, s, 2 CH FITC), 6.59-6.53 (4 H, m, 4 CH FITC), 4.24-4.11 (6 H, m, 6 CH), 3.93 (16 H, s, 8 CH_2COOH), 3.47 (2 H, br. s., CH_2NH-FITC), 3.10-2.93 (12 H, m, 5 CH_2N), 1.84 (6 H, s, 2 CH_3), 1.71-1.41 (24 H, m, 6 CH_2CH_2N and 6 $CHCH_2$), 1.34-1.12 (12 H, m, 6 $CHCH_2CH_2$). ^{13}C-NMR δ (126 MHz, DMSO) 180.38 (CS FITC), 173.42-141.49 (8 COOH, 6 CONH, 2 $COCH_3$, COO, 2 C-OH), 128.88-102.10 (CH FITC), 55.06-52.10 (CH_2N, CH, CH_2COOH), 43.55 (CH_2NH-FITC), 32.04-22.33 (CH_3, $CHCH_2$, $CHCH_2CH_2$, CH_2CH_2N). MALDI-TOF, $M+H^+$ = 1723.7, calculated for $C_{79}H_{92}N_{18}O_{16}S$ = 1722.7.

Synthesis of Cy5.5-IMPLD

Cy5.5-IMPLD was synthesized following the convergent synthetic method. Instead of FITC, Cy5.5-NHS (2 equiv) was grafted to the resin-bound peptide in DMSO (4 mL) and DIPEA (1mL) for 3 hours. The crude fully deprotected Cy5.5-labeled peptide was purified by prep HPLC (220 nm and 670 nm) from 20 to 100 % of phase B over 45 minutes to achieve the product (22.9 min retention time) as a blue powder. Retention time analytical HPLC from 20 to 100 % of phase B over 30 minutes: 16.7 min. MALDI-TOF: $M+H^+$ = 1900.1, calculated for $C_{79}H_{92}N_{18}O_{16}S$ = 1899.0.

General analytical methods for in vitro and in vivo tests

The IMPLD treated calcium salts were quantitated by measuring the ultraviolet absorbance of the supernatant solutions with an Agilent Technologies Cary 60 UV-Vis. The fluorescence on the surface of the treated salts was observed with a Bruker in-vivo F PRO fluorescence microscope (ex: 410 nm, em: 535/72 nm). All animal studies were performed in compliance with the approved animal protocols and guidelines of Institutional Animal Care and Use Committee of Weill Cornell Medicine. IMPLD treated mice were analyzed with a Bruker In-Vivo Extreme fluorescent microscope (ex: 650 nm, em: 700 nm) for the in-vivo imaging, under anesthesia with 2% isoflurane, and with Bruker in-vivo F PRO (ex: 650 nm, em: 700 nm) for the ex-vivo imaging.

Materials for in vitro tests

Hydroxyapatite (HA) and calcium pyrophosphate (Py) were purchased from Sigma Aldrich (S. Louis, MO, USA), calcium phosphate (Ph) was purchased from Thermo Fisher Scientific (Waltham, MA, USA), calcium oxalate (CaOx) from Alpha Aesar (Ward Hill, MA, USA), calcium carbonate ($CaCO_3$) from Avantor Performance Materials (Center Valley, PA, USA), TRIS buffer from Amresco, Inc. (Solo, OH, USA), PBS pH 7.4 from Corning (Tewksbury, MA, USA).

Fluorescent probes preparation

Stock solutions were prepared dissolving a small portion of IMPLD in 50 μL of de-ionized water. The concentration of the fluorescently labeled probe was determined, after dilution in PBS, by UV absorbance, according to the predetermined extinction coefficient of FITC (ε_{PBS} = 67,000 $M^{-1}cm^{-1}$ at 493 nm), or after dilution in MeOH in the case of Cy5.5-IMPLD (ε_{MeOH} = 209,000 $M^{-1}cm^{-1}$ at 673 nm). Final working solutions (10 μM) were obtained diluting the appropriate volume of stock solution in TRIS buffer pH 7.4.

Calcium salts binding assay

2.5 mg of HA, CaOx, $CaCO_3$, Ph and Py were incubated with 100 μl of IMPLD working solution in TRIS buffer pH 7.4 at room temperature for 3 h with constant agitation. The samples were centrifuged and the supernatants were transferred on a 96 well pate for UV measurement. The amount of the probe bound to the calcium salts was determined indirectly by measuring the unbound probe in solution at 494 nm, and comparing these values with a control solution of the untreated probe. The same experiment was executed for IMPLD using 5 and 10 mg of salts and 500 μl of solution obtaining the same results (data not shown).

Cell culture and IMPLD cytotoxicity assay

The murine fibroblast cells (NIH/3T3, ATCC, Manassas, VA) were cultured in DMEM (Corning, Manassas, VA) containing 100U/mL penicillin, 100mg/ml streptomycin and 10% fetal bovine serum (FBS, v/v; Gibco). The cell ($1x10^4$) were seeded in a 96 well plate for 18 hours, and then treated with IMPLD (0, 0.1, 1, 10, 20 and 50 μM) for 3 days. Then MTS cell proliferation assay was performed with Tecan infinite M1000 Pro microplate reader (Tecan Trading AG, Switzerland) after incubation with CellTiter 96®Aqueous one solution cell proliferation assay (Promega) for 1.5 hour.

In vivo and ex vivo imaging of IMPLD bone binding

20 nmol of IMPLD were injected through the tail vein to 6-7 weeks old female nude mice (n=4, Charles River, Wilmington, MA). Time-dependent optical fluorescence images were obtained at 0, 1, 3, 6 hours, 1 day, 4 days, 1 wk, 2 wks, and 4 wks. *Ex vivo* imaging was performed as time point analysis at 1 day, 1 wk, 2 wks, and 4 wks after the skin and muscles were removed.

In vivo fluorescence images from knee and thigh muscle were analyzed 5 times at each time points with a Bruker molecular imaging software v. 7. 1. 1 using the ROI analysis function. Ratio of knee-to-background fluorescence intensity for IMPLD was calculated with the following formula:

K/B= Mean intensity of Knee/ Mean intensity of background (Thigh muscle)

Statistical analysis

Statistical analyses were performed using one-way and two-way Anova with Prism 6 (GraphPad Software, Inc.). Values of P less than 0.05 were considered statistically significant.

NMR spectra

N^2-(N^2-acetyl-N^6,N^6-bis(2-(*tert*-butoxy)-2-oxoethyl)lysyl)-N^6,N^6-bis(2-(*tert*-butoxy)-2-oxoethyl)lysine (Ac-Lys($CH_2COOtBu$)$_2$-Lys($CH_2COOtBu$)$_2$-OH, **5**)

^{1}H NMR

^{13}C NMR

8,24-diacetamido-14-((1-amino-6-(3-(3',6'-dihydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthen]-5-yl)thioureido)-1-oxohexan-2-yl)carbamoyl)-11,21-bis(4-(bis(carboxymethyl)amino)butyl)-3,29-bis(carboxymethyl)-9,12,20,23-tetraoxo-3,10,13,19,22,29-hexaazahentriacontanedioic acid (IMPLD, **9**)

^{1}H NMR