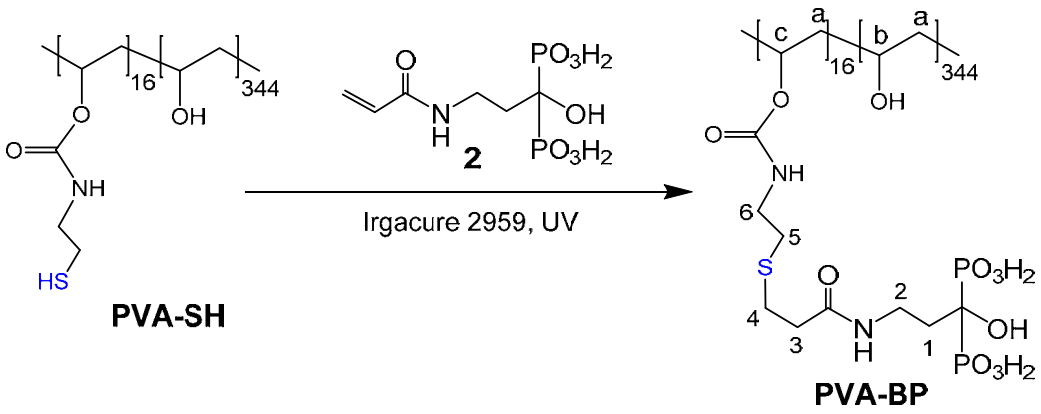
Supporting Information

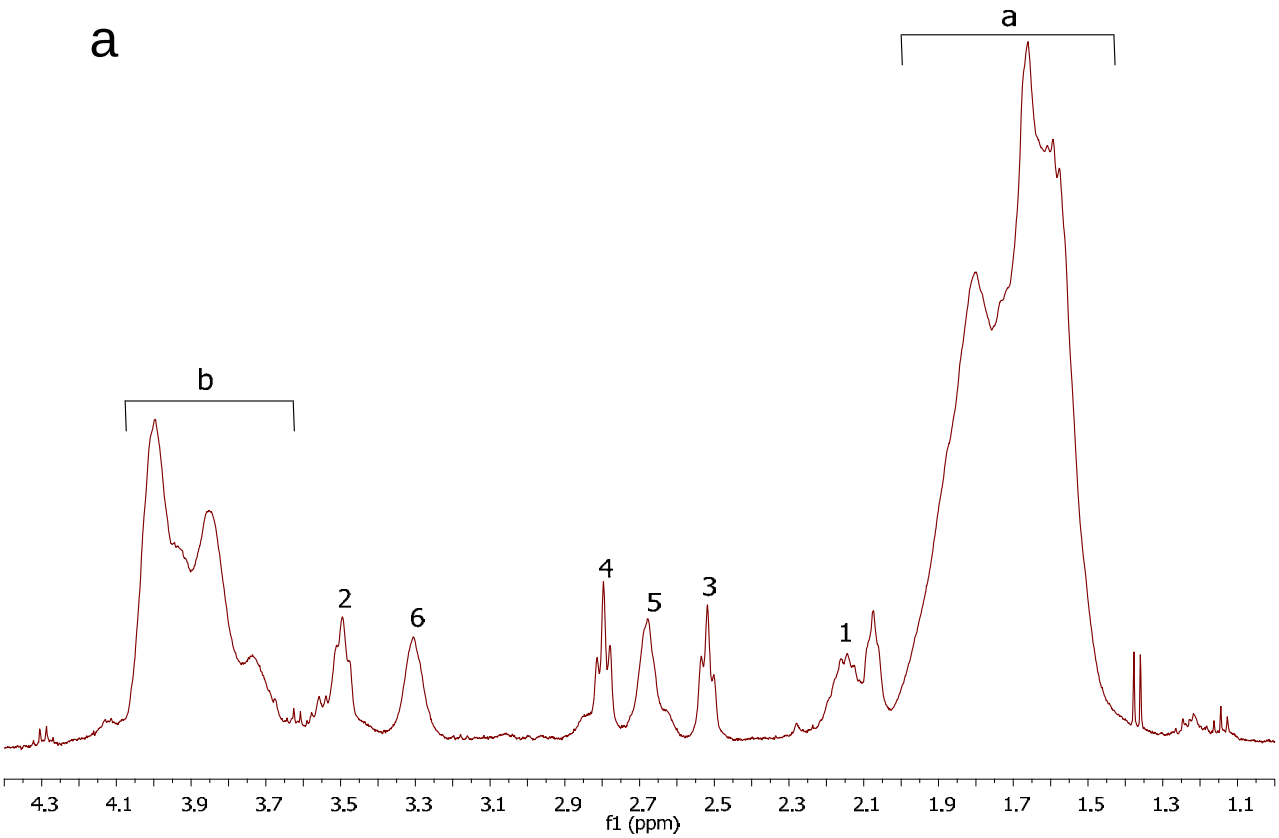
Bisphosphonate-functionalized Hyaluronic Acid Show Selective Affinity For Osteoclasts As A Potential Treatment For Osteoporosis

Synthesis of 3-(acrylamido-1-hydroxypropane-1,1-diyl) bis(phosphonic acid) (pamidronate acrylamide). Pamidronate hydrochloride (543 mg, 2 mmol) was dissolved in 20 mL of 2 wt. % NaOH. The solution was cooled to 0°C and acryloyl chloride (0.64 mL, 8 mmol) was added into 4 portions (162 µL for each portion) over 45 min while the reaction mixture was kept at 0°C by an external ice-bath cooling. After the third addition of acryloyl chloride, the pH was adjusted with 2 wt. % NaOH from 2 to 10. After the addition, the mixture was stirred for 1.5 h while the temperature was raised to room temperature. The resulting mixture was extracted with ethyl acetate (2x). The aqueous phase was evaporated and the residue was triturated with methanol. Crystals which were insoluble in methanol were collected by filtration and dried in vacuo to give 468 mg (81% yield) of acrylated pamidronate as a white solid. ^{1}H-NMR (D_2O): 6.21 and 6.12 (2H, dd and d, CH_2=, *J* = 16.1 Hz, *J* = 9.9 Hz), 5.69 (1H, d, –CH=, *J* = 10.3 Hz), 3.53 (2H, t, –NHC$\underline{H_2}$–, *J* = 7.7 Hz), 2.20 – 2.09 (2H, m, –C$\underline{H_2}$C(OH)(PO_3H_2)$_2$). ^{31}P-NMR: 18.6.

Syntheses of PVA-SH and PVA-BP. Thiolated PVA (PVA-SH) was synthesized according to our previously published procedure.[1] It was then used as a starting material in the synthesis of bisphosphonate derivative of PVA (PVA-BP). Conversion of PVA-SH to PVA-BP is shown in Scheme S1 and was accomplished by photo-initiated thiol-ene addition reaction using pamidronate acrylamide **2**. ^{1}H-NMR and ^{31}P-NMR spectra of PVA-BP in D_2O are shown in Figure S1.



Scheme S1. Synthesis of PVA-BP from PVA-SH.


a
a
b
2
6
4
5
3
1
4.3
4.1
3.9
3.7
3.5
3.3
3.1
2.9
2.7
2.5
2.3
2.1
1.9
1.7
1.5
1.3
1.1
f1 (ppm)

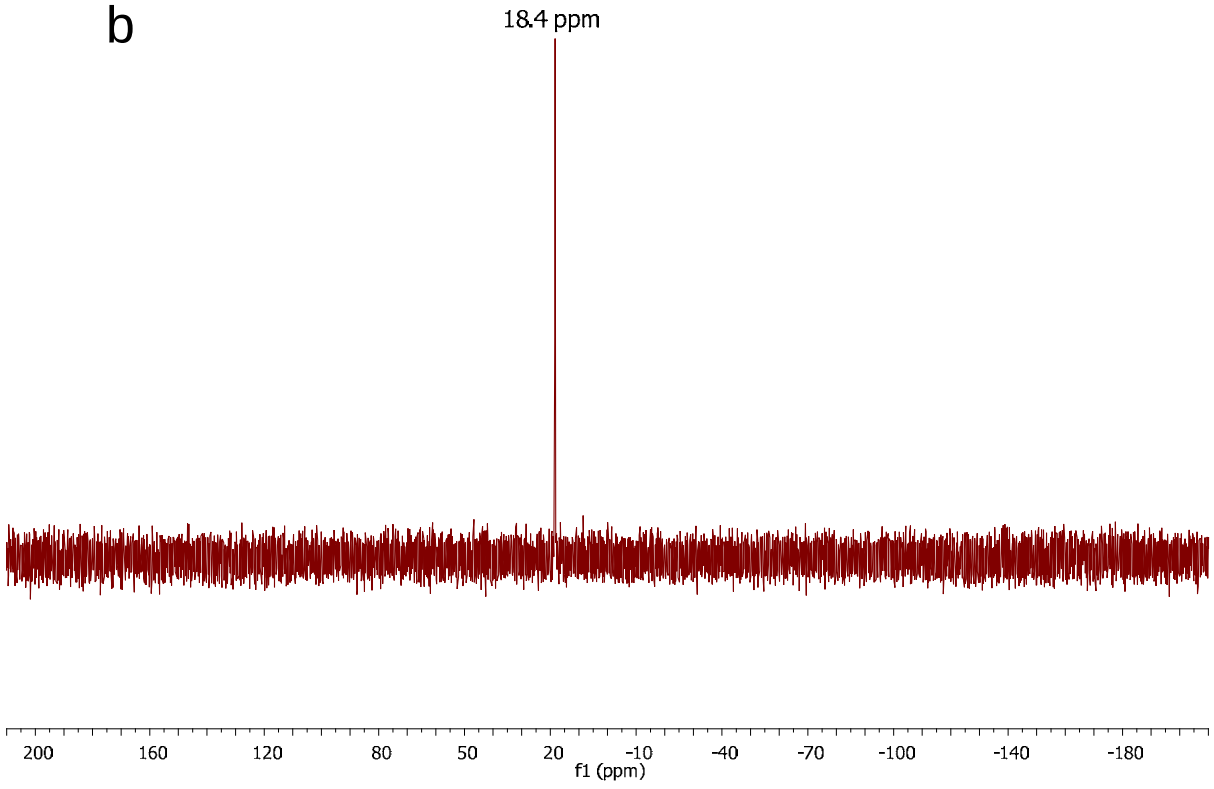
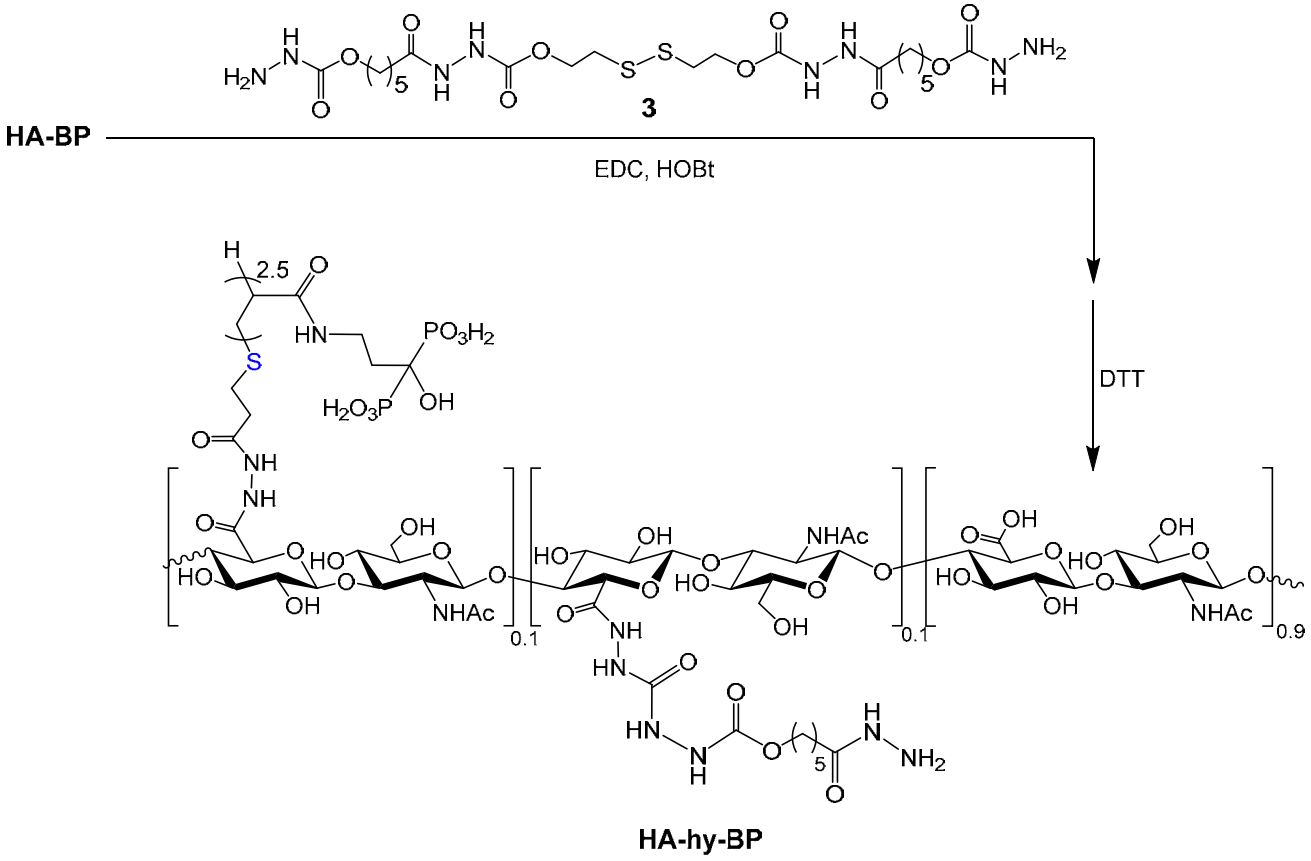

b
18.4 ppm
200
160
120
80
50
20
-10
-40
-70
-100
-140
-180
f1 (ppm)

Figure S1. ^{1}H-NMR (a) and (b) ^{31}P-NMR spectra of PVA-BP derivative in D_2O. Designation of PVA-BP protons is given in Figure S1.

Synthesis of hydrazide and bisphosphonate dually modified HA (HA-hy-BP). L-HA-BP and H-HA-BP were further derivatized with hydrazide groups according to Scheme S2. Hydrazide-modified linker **3** was synthesized according to the literature procedure.[2] 70 mg of HA-BP (either low or high molecular weight) was dissolved in 4 mL of de-ionized water. Linker **3** (15 mg, 0.0175 mmol) was first dissolved in 0.2 mL of methanol and the obtained methanolic solution was added to the HA solution. *N*-hydroxybenzotriazole (HOBt) (26.8 mg, 0.175 mmol) was separately dissolved in 0.5 mL of *N*-methyl-2-pyrrolidone and added to the solution of HA. The pH of the resultant solution was adjusted to 4.5 after which the coupling reaction was initiated by addition of solid EDC (10 mg, 0.52 mmol) to the reaction mixture. The mixture was stirred overnight. The reaction solution was basified to 8.5 with 1M NaOH and DTT (25 mg, 0.162 mmol) was added to the solution to provide cleavage of disulfide bond of the coupled linker. The mixture was stirred overnight, after which the solution was acidified to 3.5 with 1M HCl and transferred to a dialysis tube (M_w cutoff = 3500). After exhaustive dialysis against dilute HCl (pH 3.5) containing 0.1 M NaCl, followed by dialysis against dilute HCl, pH 3.5 two times, the solution was lyophilized to give L-HA-hy-BP and H-HA-hy-BP derivatives with 81% and 91% yields respectively. The incorporation of hydrazide groups was verified by ^{1}H-NMR (Figure S2). Specifically, the peaks corresponding to the native HA protons, such as acetamide protons at 1.9 ppm, 2', 3', 4', 5', and 6'-protons of HA disaccharide unit at 3.2 – 4.0 ppm, as well as anomeric 1'-protons at 4.4 ppm, were compared with newly appeared peaks at 4.90, 2.23, 1.57 and 1.30 ppm corresponding to *α*, *β*, *γ*, *δ*, and *ε* methylene protons of –OCH_2CH_2CH_2CH_2CH_2CONHNH_2 side chain (Figure S2). This indicated that 10% of the HA disaccharide units were modified with hydrazide-terminated side chains.



Scheme S2. Synthesis of LMW and HMW HA-hy-BP derivatives.

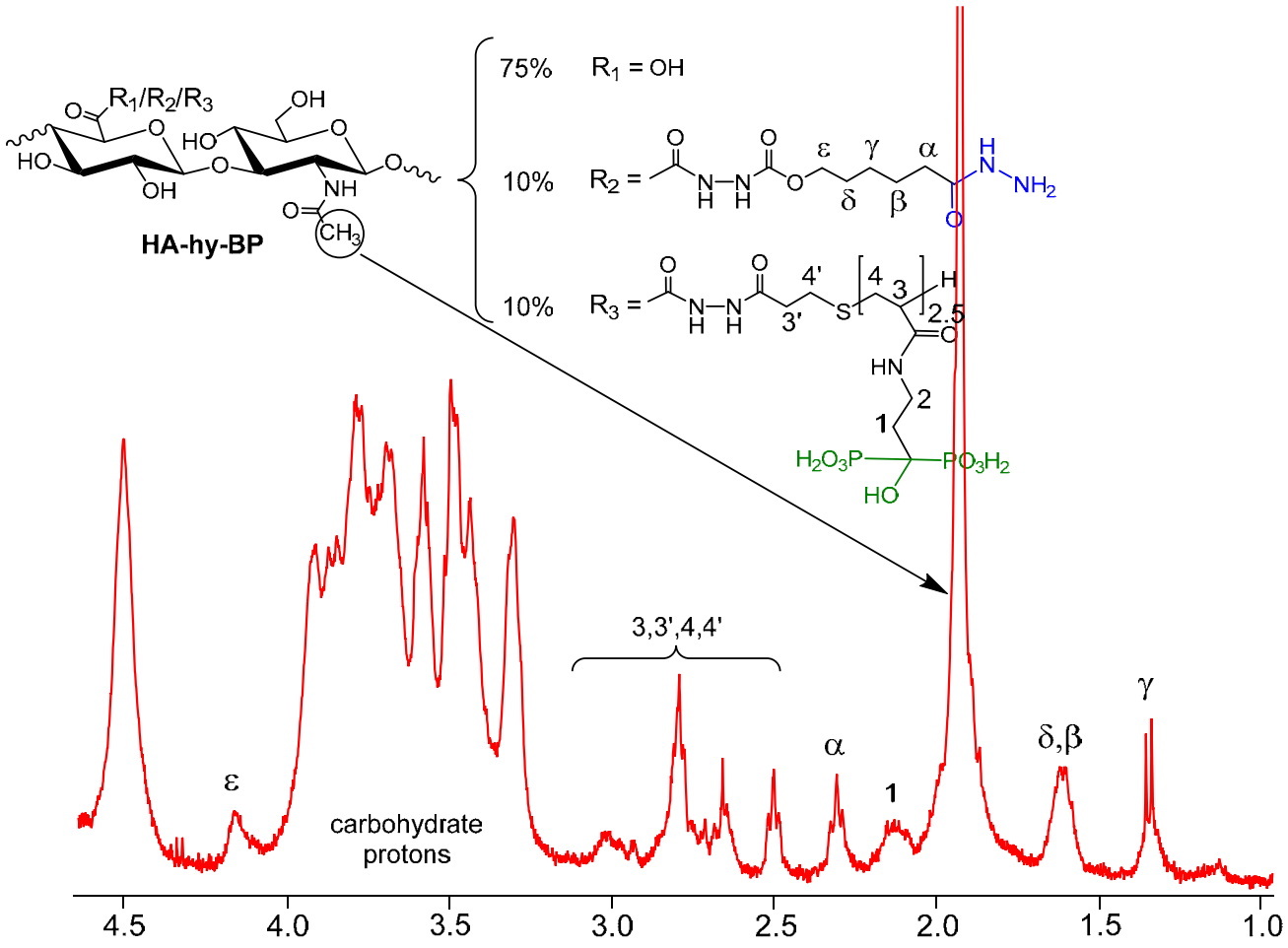


Figure S2. ^{1}H-NMR spectrum of L-HA-hy-BP derivative in D_2O.

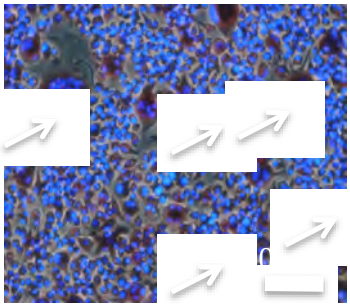


Fig S3. Effect of PVA-BP on osteoclast-induced TRAP activity. Raw264.7 cells were induced with (20ng/mL) RANK ligand for 5 days. Subsequently they were treated with PVA-BP for 24 hours. TRAP activity was measured as described in ''Materials and Methods''. The images show the formation of giant multi-nucleated cells indicating the formation of osteoclast-like cells.

Sample	Name	Conc. of BP (μM)	Conc. of HA (μM)	Group
Native high-molecular-weight hyaluronic acid	H-HA	0	100	-
Native low-molecular-weight hyaluronic acid	L-HA	0	100	-
High-molecular-weight hyaluronic acid without BP	H-HA	0	100	A
Low-molecular-weight hyaluronic acid without BP	L-HA	0	100	B
Control	C	0	0	C
High-molecular-weight hyaluronic acid with BP	H-HA-BP	100	-	D
Low-molecular-weight hyaluronic acid with BP	L-HA-BP	100	-	E
Free BP	Free BP	100	0	F

Table 1. Concentration of HA and BP in each compound in μM.

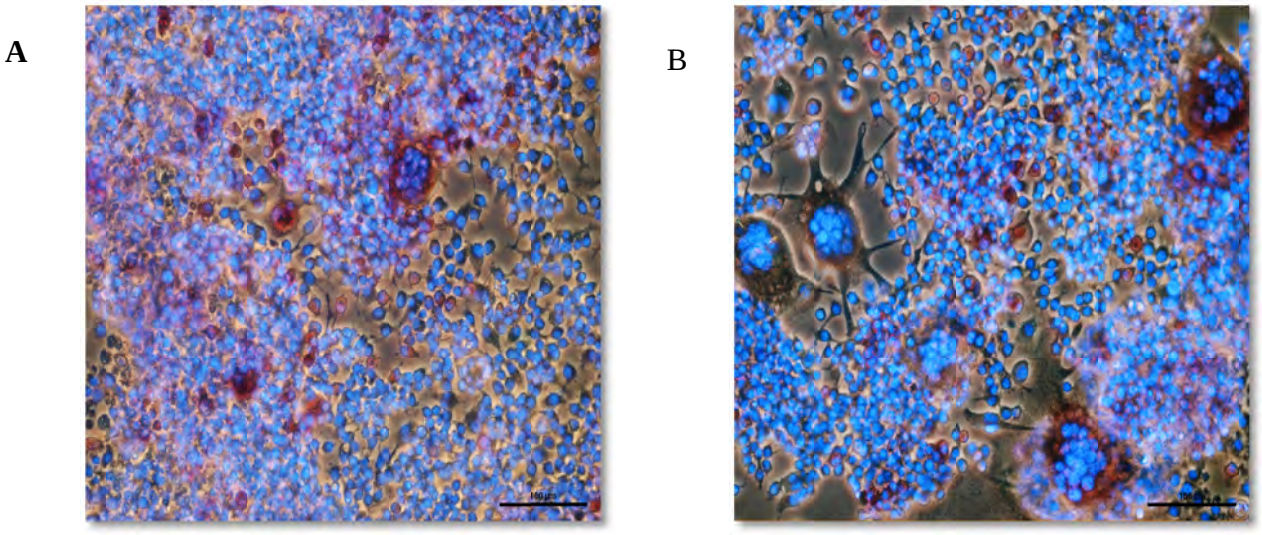


Fig S4. Effect of fluorescent analogues of L-HA-hy-BP (A) and L-HA-BP (B) on osteoclast-induced TRAP activity. Raw264.7 cells were induced with (20ng/mL) RANK ligand for 5 days. Subsequently they were treated with the fluorescent analogues of H-HA-hy-BP (A) and H-HA-BP (B) for 24 hours. TRAP activity was measured as described in ''Materials and Methods''. The images show the formation of giant multi-nucleated cells indicating the formation of osteoclast-like cells. Quantification through image analysis showed no significant differences in osteoclast numbers.