Supporting Information

Bone-targeting poly(ethylene sodium phosphate)

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1. Experimental Procedures

1.1. Materials

2-Chloro-2-oxo-1,3,2-dioxaphospholane was kindly donated by the Nof Corporation, Tokyo, Japan and was used without further purification. Poly(phosphate) (polyP; Na^+ salt, m=60-70) was purchased from Bioenex Inc., Hiroshima, Japan and was used without further purification. 2-Methoxy-2-oxo-1,3,2-dioxaphospholane (MP)[1] and 2-(but-3-yn-1-yloxy)-2-oxo-1,3,2-dioxaphospholane (BYP)[2] were synthesized as previously reported, purified by vacuum distillation, and stored under argon at 4°C until use. Cy5-Az was purchased from Sigma-Aldrich Co., St. Louis, MO, USA. The other chemicals for polymer synthesis were obtained as extra-pure grades and used without further purification. Water was purified using a Millipore Milli-Q system, which involves UV irradiation, ion exchange, and filtration (18.2 MΩ cm).

1.2. Polymer synthesis

Poly(ethylene sodium phosphate) (PEP·Na) was synthesized using a previously reported method.[1] Briefly, MP (20 mmol) was placed into a thoroughly dried 30 mL round-bottom flask equipped with a three-way stopcock. After the mixture was dried under reduced pressure for 2 h, methanol as an initiator and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) as a catalyst were added under an argon atmosphere while cooling with ice. The reaction was allowed to proceed for 3 h. Poly(2-methoxy-2-oxo-1,3,2-dioxaphospholane) (PMP) was purified by re-precipitation from diethyl ether. The number-average molecular weight (M_n) and weight-average molecular weight (M_w) were determined by gel permeation chromatography (GPC) through a Polymer Laboratories MIXED-C column using a calibration curve based on linear polystyrene standards. For this measurement, chloroform was used as the GPC solvent. PMP (2.5 g) was dissolved in 50 mL of deionized water (dH_2O) and stirred with 2 equivalents of trimethyl amine relative to the MP unit. After 24 h, 20 g of cation-exchange resin (Amberlite® IR-120, Merck KGaA, Darmstadt, Germany) was added to the polymer solution followed by stirring for 1 h. This solution was then filtered to remove the cation-exchange resin. The filtrate was dialyzed overnight through a dialysis membrane (MWCO 1000) against distilled water. To form a polymer sodium salt, the pH of the dialyzed poly(ethylene phosphate) (PEP) solution was adjusted to 7.0 by adding 0.1 N and 0.01 N NaOH. The solution was dialyzed against distilled water for one day. Freeze-drying of the aqueous solution yielded PEP·Na (Figure 1), which is a colorless, highly water-soluble polyelectrolyte. The structures of PMP and PEP·Na were characterized by ^1H NMR (ESC400, JEOL Ltd., Tokyo, Japan).

In order to obtain fluorescent PEP·Na (Cy5-PEP·Na), MP and BYP were copolymerized. MP (18 mmol) and BYP (2 mmol) were placed into a thoroughly dried 30 mL round-bottom flask equipped with a three-way stopcock. The following synthetic procedures are similar to those described above for PMP. The demethylation of P(MP/BYP) was also performed by the reaction with trimethyl amine and the cation-exchange resin. P(EP·Na/BYP) was then obtained after neutralization with aqueous NaOH. Immobilization of Cy5-Az with P(EP·Na/BYP) was achieved by a copper(I)-catalyzed azide–alkyne cycloaddition, which is well known as a "click reaction".[3] P(EP·Na/BYP) (550 mg) was dissolved in a 4:1 mixture (2.34 mL) of water and tert-butyl alcohol. Cy5-Az (10 mg/mL in DMSO; 220 μL), sodium ascorbate (2.6 M; 58.6 μL), and copper(II) sulfate (1.6 M; 9.5 μL) were added to the solution. The reaction proceeded for 24 h at room temperature. Cy5-PEP·Na was purified via dialysis (MWCO 3,500 g/mol). The density of Cy5-Az of Cy5-PEP·Na was determined using a fluorescence spectrophotometer (F-2500, Hitachi, Tokyo, Japan). The apparent molecular weights of PEP·Na and Cy5-PEP·Na were determined by GPC using a Jasco GPC system equipped with a refractive index detector and size-exclusion columns, Shodex, SB-803 HQ, and SB-806 M, with a poly(ethylene glycol) (Tosoh standard sample) standard in acetate buffer containing 0.1 M CH_3COONa, 0.3 M NaCl, and 1.0 mM EDTA·2Na.

1.3. Surface plasmon resonance analyses

Binding studies using surface plasmon resonance (SPR) were performed with a Biacore X100 instrument (Biacore). Thrombin-immobilized CM5 sensor chips were prepared using an amine coupling kit following the manufacturer's instructions. 1 μM PolyP or PEP·Na in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (10 mM HEPES containing 150 mM NaCl and 0.005% surfactant P20, pH 7.4) was injected onto the constructed thrombin-immobilized sensor chip at a flow rate of 10 μL/min for 1 min at 25°C. Thrombin-immobilized sensor chips were regenerated after measurements by washing with 1 M NaCl before sample injection.

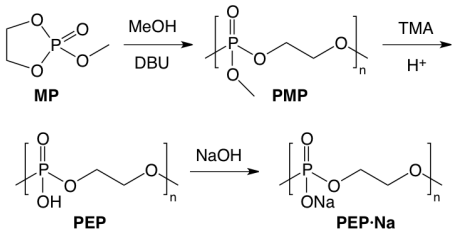
1.4. Cytotoxicity test

The viability of MC-3T3E1 cells in contact with polymers was investigated by WST-8 assay. Briefly, a suspension of MC3T3-E1 cells (100 μL; 5×10^4 cell/mL) in α-MEM was introduced into a 96-well tissue culture plate (Thermo Scientific, Rochester, U.S.A.) and incubated in a culture medium at 37°C with 5% CO_2. After 24 h of cultivation, the medium was replaced with one containing polymers and the cells were further cultivated for 24 h. The viability of MC3T3-E1 cells in each well was evaluated using a WST-8 Cell Counting Kit (Dojindo, Kumamoto, Japan).

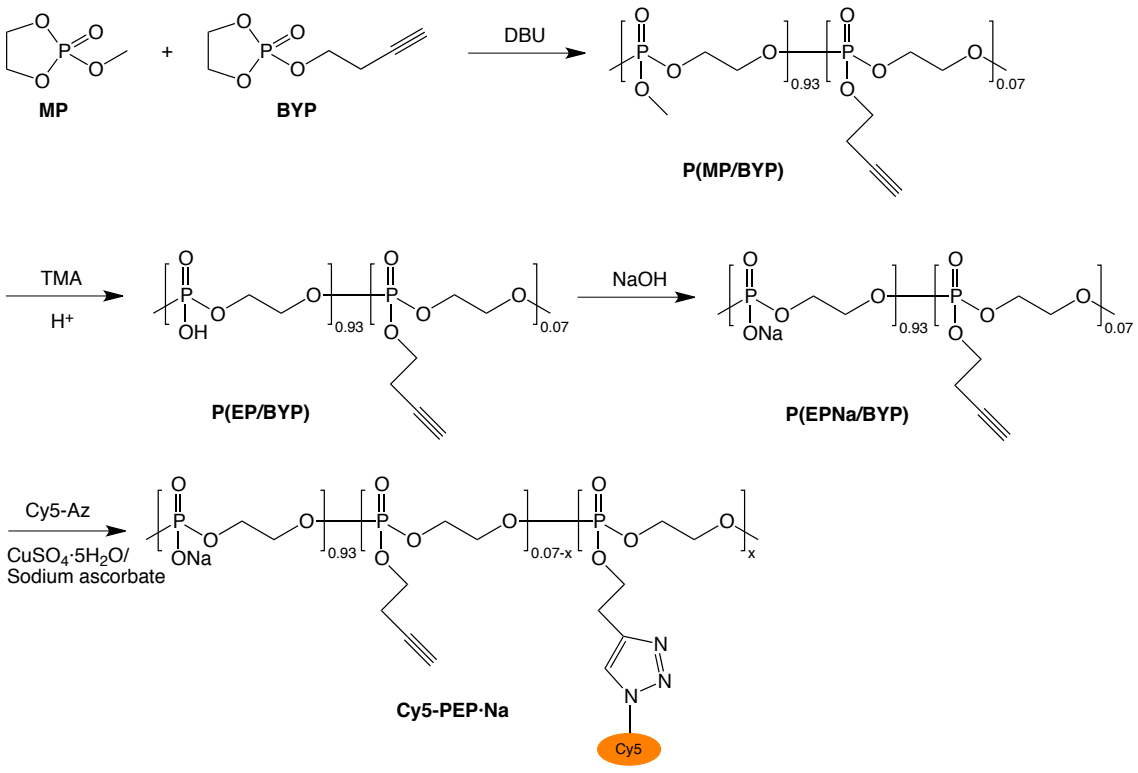
1.5. Biodistribution assay (*In vivo* imaging)

The animal protocol was approved by the Animal Experimentation Committee of Osaka Medical College (Permit Number: 29079). Retired female ICR mice (Oriental Yeast Co., Ltd., Tokyo, Japan) were obtained at about three weeks of age and bred with alfalfa-free diet (iVid#2, Oriental Yeast Co., Ltd., Tokyo, Japan) for an additional week before *in vivo* experiments. One hundred microliters of solutions containing 100 mg/mL fluorescent PEP·Na (Cy5-PEP·Na) or 100 μg/mL free Cy5-Az were administrated to mice (n = 4 for each condition) by tail vein injection under anesthesia with O_2/sevoflurane (3–5% sevoflurane). *In vivo* fluorescence signals of major bones, such as spine, limb, and tail, were recorded by using a Caliper IVIS Lumina II in vivo imaging system (Caliper Life Science, USA) at various times. For the in vivo imaging, mice were briefly anesthetized with O_2/isoflurane (1–3% isoflurane) to keep them calm. For quantitative analyses of the optical imaging data, regions of interest (ROI) were drawn in the spine region of each animal. Half of the mice (n = 2 for each condition) was anesthetized and sacrificed at 75 h by an intraperitoneal overdose with pentobarbital to perform *ex vivo* observations. The other half was used for the investigation of long-term biodistribution.

2. Additional Figures



Scheme S1. Synthetic route PEP·Na.



Scheme S2. Synthetic route of Cy5-PEP·Na.

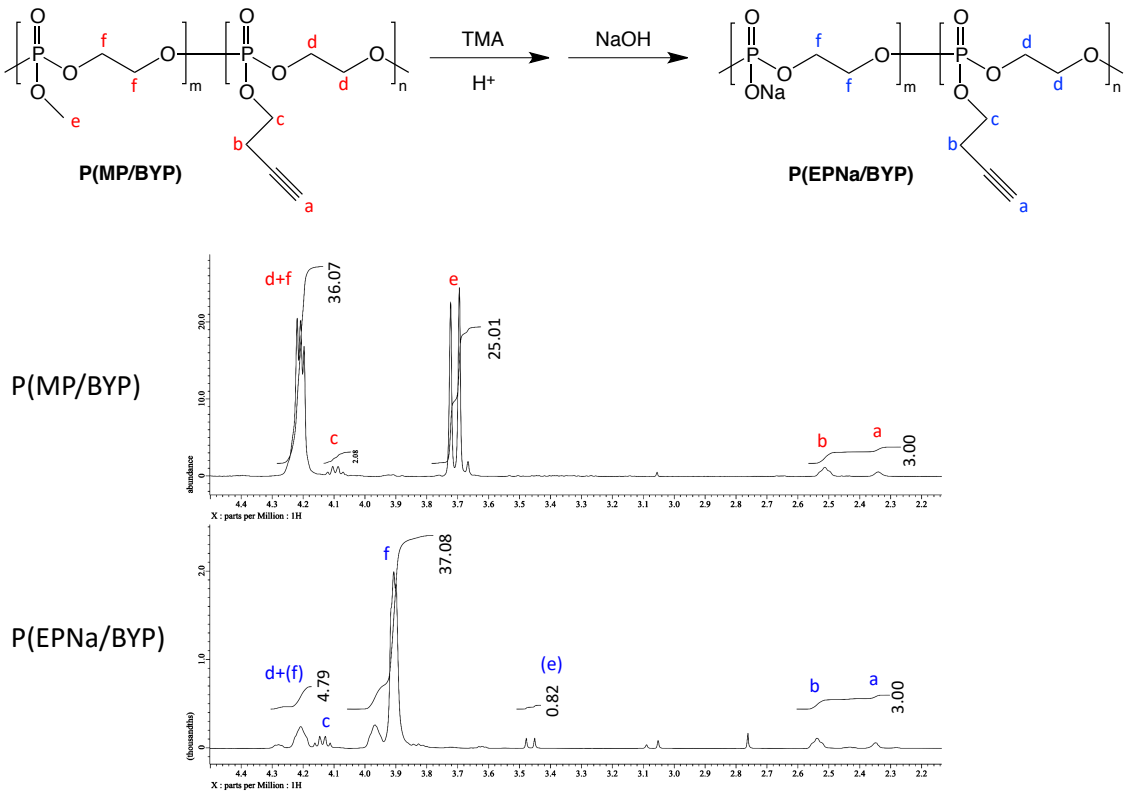


Figure S1. ^{1}H NMR spectra of P(MP/BYP) and P(EPNa/BYP).

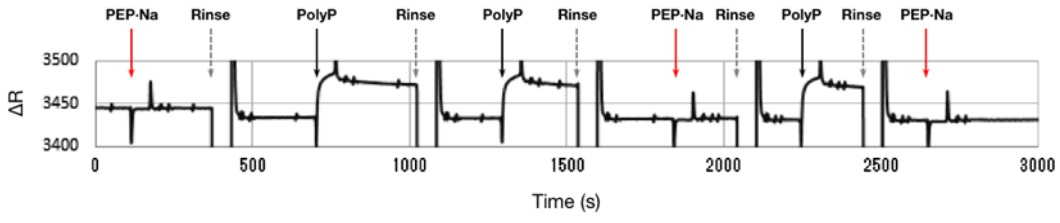


Figure S2. Study of repetitive polymer binding with thrombin.

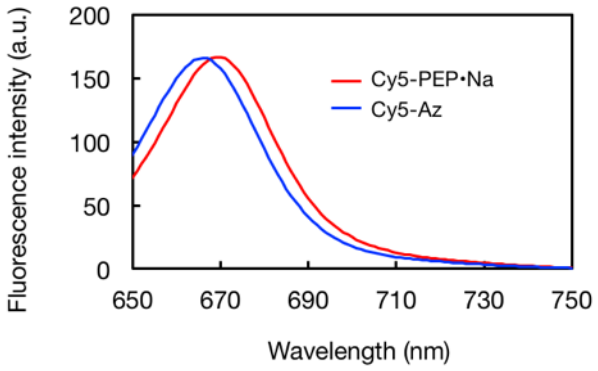


Figure S3. Fluorescence spectra of Cy5-PEP·Na (100 mg/mL ≈ 10 mM) and Cy5-Az (100 μg/mL ≈ 0.1 mM).

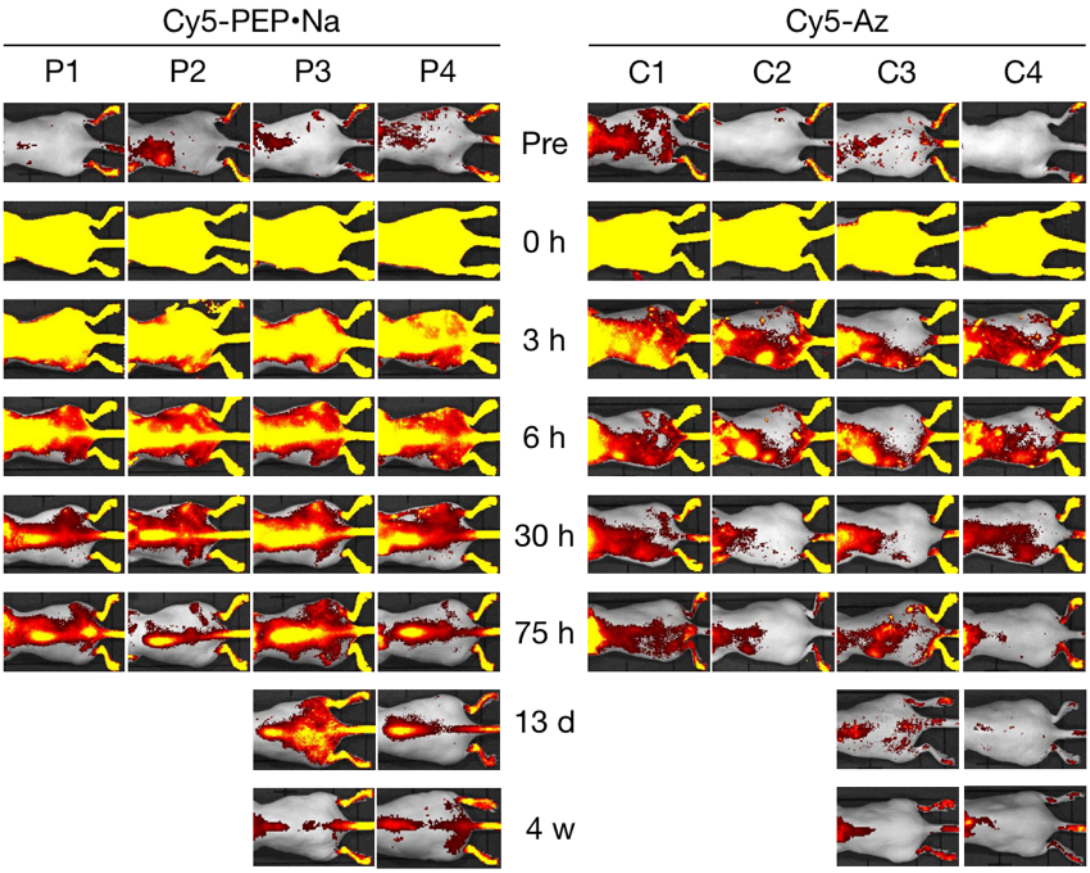


Figure S4. *In vivo* study of accumulation of Cy5-PEP·Na and Cy5-Az on bone.

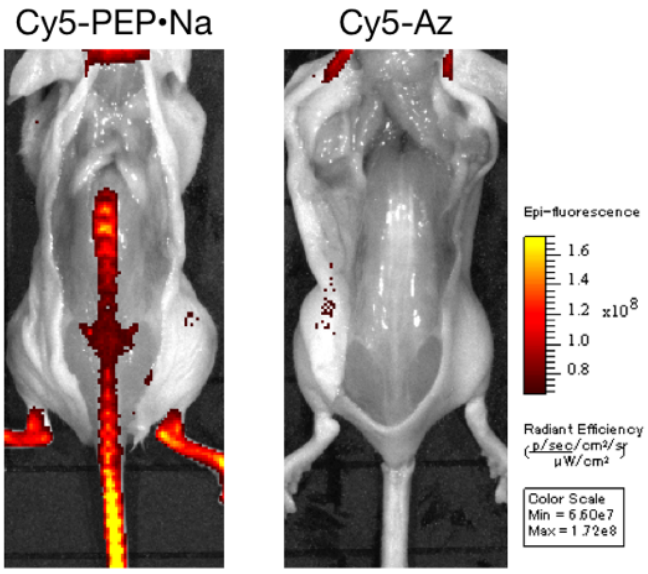


Figure S5. *Ex vivo* images of accumulation of Cy5-PEP·Na and Cy5-Az on bone 75 h after single intravascular injection.

3. References

1. S. Kootala, M. Tokunaga, J. Hilborn and Y. Iwasaki, *Macromol. Biosci.*, **2015**, 15, 1634.
2. S. Zhang, A. Li, J. Zou, L. Y. Lin and K. L. Wooley, *ACS Macro. Lett.*, **2012**, 1, 328.
3. V. V. Rostovtsev, L. G. Green, V. V. Fokin and K. B. Sharpless, *Angew. Chem. Int. Ed. Engl.*, **2002**, 41, 2596.