The Synthesis of a Multiblock Osteotropic Polyrotaxane by Copper(I)-Catalyzed Huisgen 1,3-Dipolar Cycloaddition[a]

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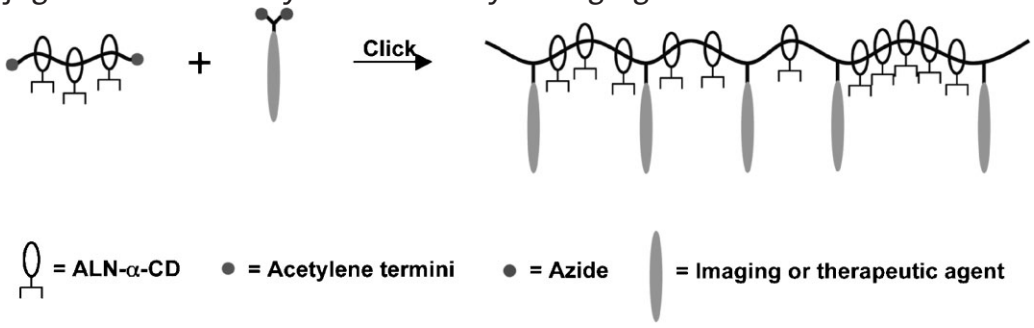
The design and synthesis of a novel bone-targeting polyrotaxane delivery system that utilizes alendronate (ALN) as targeting moiety is presented in this manuscript. For the introduction of ALN, it is first conjugated to α-cyclodextrin (α-CD) and subsequently threaded onto a short poly(ethylene glycol) (PEG) chain, forming a pseudopolyrotaxane. Using click chemistry, this assembly is copolymerized with bulky monomers that bear imaging and/or therapeutic agent(s) to prevent ALN-functionalized α-CD from dethreading. Overall bone affinity of this novel polymer conjugate can be easily controlled by changing the number of ALN-α-CD incorporated. The osteotropicity of the delivery system was also confirmed in vivo.



Introduction

As the human life span continues to increase, so has the prevalence of musculoskeletal diseases. There are currently over 24 million Americans that suffer from osteoporosis, afflicting approximately one in four women over the age of 50.[1] Over half of all cancer patients, excluding those with skin cancer, will develop bone metastasis at some point over the course of their disease, resulting in 350 000 deaths annually in the US.[2,3] Arthritis, an umbrella term for over 100 different types of musculoskeletal diseases that afflicts joints, affects over 70 million adult Americans.[4] The total arthritis-related expenditures in the US for the year 2005 were $353 billion.[5] All of these statistics are anticipated to increase significantly in the near future as the human population continues to grow older.

Many different therapies have been developed to treat these various bone diseases. However, they typically involve the administration of therapeutic agents that are not site-specific for bone, resulting in adverse side effects. For example, treating osteoporosis with estrogen replacement therapy can lead to intrauterine hemorrhage and occasionally both endometrial and breast cancer.[6–7] Docetaxel (Taxotere) chemotherapy for the treatment of cancer that has metastasized to bone can result in a number of adverse effects, including neutropenia, alopecia, arthalgia, myelosuppression, etc.[8–11] A method to improve

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the therapeutic indexes of these therapeutic agents is to first load them into an osteotropic (bone-targeting) drug delivery system to allow targeted delivery to the skeleton.

Over the past two and a half decades, many different osteotropic delivery systems have been developed that use a wide range of bone-targeting moieties, including tetracycline, polymalonic acid, alendronate (Fosamax, ALN), aspartic acid octapeptide (Asp_8), etc.[12–16] ALN, in particular, belongs to a group of compounds known as bisphosphonates that are widely known for their ability to bind to hydroxyapatite (HA),[16–18] the major mineral component of bone.[19] As a targeting moiety, ALN offers a number of advantages: (i) it is stable both during chemical modifications and in vivo, (ii) it has a long terminal half-life of ≈10 years, (iii) binding affinity for HA is higher than the other bone-targeting moieties, (iv) high hydrophilicity limits cellular membrane crossing to undetectable levels, making ALN highly selective for bone and limiting toxicity, and (v) it contains a single primary amino functional group that serves as an ideal chemical handle for conjugation reactions.[20] A major limitation of ALN, however, is it only freely dissolves in aqueous solvent, greatly restricting the number of conjugation synthetic pathways that can be followed. This makes their incorporation into delivery systems challenging and perhaps explains the limited research published on ALN-containing macromolecular polymeric delivery systems. As one of the pioneers of the field, Kopeček and coworkers synthesized the first *N*-(2-hydroxypropyl)-methacrylamide (HPMA) copolymer with ALN targeting moieties,[17,21] while Satchi-Fainaro and coworkers went a step further and recently reported a similar HPMA copolymer that was loaded with paclitaxel.[22] Choi and Kim developed poly(D,L-lactide-*co*-glycolide) (PLGA) nanoparticles[23] and our laboratory synthesized a linear multifunctional poly(ethylene glycol) (PEG) copolymer,[24] both with ALN as the bone-targeting moiety. In most of these studies, however, there were batch-to-batch variations in how much ALN was incorporated into the systems.

To address these challenges, we designed and synthesized a novel osteotropic α-cyclodextrin (α-CD)/PEG polyrotaxane delivery system utilizing click chemistry.[25–27] Pseudopolyrotaxanes are the supermolecular assembly of many cyclic molecules threaded onto a polymer backbone. When sterically bulky structures are used to cap the polymer termini, pseudopolyrotaxanes become polyrotaxanes, which are not subjected to entropy-driven dethreading upon dilution.[28] The most investigated polyrotaxane/pseudopolyrotaxane is the α-CD/PEG system.[28–31] Since its discovery in 1990,[29] this molecular assembly has been extensively scrutinized and has led to interesting developments of biodegradable polyrotaxanes for controlled drug delivery.[32–35] In these studies, drugs are typically conjugated to the α-CD rings post polyrotaxane formation and environment (e.g., low pH, enzymes) triggered decapping is employed as the drug releasing mechanism.

In the novel osteotropic α-CD/PEG polyrotaxane design presented here, ALN is first conjugated to α-CD via the copper(I)-catalyzed Huisgen 1,3-dipolar cycloaddition (a click chemistry reaction) and subsequently threaded onto a PEG backbone, forming a pseudopolyrotaxane. Using click chemistry again, this assembly is copolymerized with sterically bulky monomers to prevent alendronate-functionalized α-cyclodextrin (ALN-α-CD) from dethreading, thereby forming a polyrotaxane. Imaging and/or therapeutic agents can be introduced into the polyrotaxane by covalent conjugation to the monomers prior to copolymerization. The most unique feature of this delivery system is the ability to easily control overall bone-binding affinity by freely adjusting the number of ALN-α-CD incorporated.

Experimental Part

Materials

α-Cyclodextrin (α-CD) was purchased from TCI America (Portland, OR, USA) and dried in a vacuum oven overnight. Sodium alendronate trihydrate was purchased from Ultratech India Ltd. (New Mumbai, India). PEG monomethyl ether ($\overline{M}_w = 1\,900$ Da) was purchased from Alfa Aesar (Ward Mill, MA, USA). HA (DNA-grade) was purchased from BioRad (Hercules, CA, USA). PD-10 columns (Sephadex G-25 resin) were purchased from GE HealthCare (Piscataway, NJ, USA). Dialysis membrane [molecular weight cutoff (MWCO) = 12–14 kDa] was purchased from Spectrum Laboratories (Rancho Dominguez, CA, USA). Thin layer chromatography or TLC plates (silica gel 60 F_{254}) were purchased from VWR (Darmstadt, Germany). All other compounds and solvents were purchased from either Sigma–Aldrich (St. Louis, MO, USA) or Acros Organics (Morris Plains, NJ, USA). Unless otherwise stated, all compounds were reagent grade and used without further purification.

Methods

^{1}H and ^{13}C NMR spectra were recorded with a Varian Inova Unity 500 MHz NMR spectrometer at 20 °C (Varian, Inc., Palo Alto, CA, USA). The ALN content of the polyrotaxanes and amine concentration were estimated by ^{1}H NMR integration values. The weight average molecular weight ($\overline{M}_w$), number average molecular weight ($\overline{M}_n$), and polydispersity index (PDI) of the copolymers were determined by size-exclusion chromatography (SEC) using an ÄKTA FPLC system (GE Healthcare) equipped with UV and refractive index (Knauer, Berlin, Germany) detectors, based on a PEG calibration curve. SEC measurements were performed on a Superdex 200 (10/300 GL) column with phosphate-buffered saline (PBS; pH 7.3) as the eluent. HPLC spectra were recorded with an Agilent 1100 HPLC system (Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a diode-array based UV detector. A reverse phase C_{18} column (Agilent, 4.6 × 250 mm, 5 μm) was used with a

flow rate of 1 mL · min^{-1}. UV-vis spectra were recorded with a UV-1601PC UV-vis spectrophotometer (Shimadzu, Kyoto, Japan). Differential scanning calorimetry (DSC) analyses of the samples were performed with a Shimadzu DSC-50 (Shimadzu, Kyoto, Japan) under nitrogen atmosphere with a scanning speed of 10 °C · min^{-1}. Rhodamine B concentrations in the rhodamine B-labeled polymers were determined by UV-vis spectrophotometer analysis at wavelength 558 nm in methanol/PBS (pH 7.3, 1:2) solution based on a rhodamine B-labeled monomer (**11**) standard curve. Fluorescein isothiocyanate (FITC) concentrations in the FITC-labeled polymers were determined by UV-vis spectrophotometer analysis at wavelength 498 nm in PBS (pH 7.8) solution. The extinction coefficient of conjugated FITC at 498 nm and pH 7.8 is estimated to be 69 000 L · mol^{-1} · cm^{-1}.[36] Triplicate measurements were performed and standard deviations were calculated. A ninhydrin assay was used to qualitatively determine the presence of amine functional groups.[37–38]

Synthesis of Mono-(6-azido-6-deoxy)-α-cyclodextrin (2)

Mono-6-(ρ-tolylsulfonyl)-α-cyclodextrin (synthesized from α-CD according to ref.[39] and purified according to ref.[40], 1.03 g, 0.914 mmol) and sodium azide (0.122 g, 1.86 mmol) were added to water (12 mL) and the suspension was allowed to stir at 90 °C overnight. TLC (7:7:4 by volume, 2-propanol/ethyl acetate/water) showed that limiting reagent had been consumed. The solution was precipitated in cold acetone (120 mL) and the resulting white precipitate was collected and washed with acetone. Precipitation was carried out three additional times. Yield = 0.660 g (72.4%). ^{1}H NMR (D_2O): δ(ppm) = 5.01–4.98 (m, 6H), 3.93–3.48 (m, 36H). ^{13}C NMR (D_2O): δ = 102.1, 82.4, 81.6, 74.0, 72.4, 72.2, 71.1, 60.6, 51.5. HPLC (85:15 v/v, H_2O/CH_3CN, 205 nm): λ_{max} = 2.72 min.

Synthesis of Acetylene-Functionalized Alendronate (5)

Sodium alendronate trihydrate (**4**, 3.15 g, 9.69 mmol) was dissolved in water (60 mL) and NaOH solution (1 M) was added dropwise until pH 8.5 was reached. 2,5-Dioxopyrrolidin-1-yl pent-4-ynoate [synthesized from 4-pentynoic acid (compound **3**) according to ref.[41], 2.40 g, 12.3 mmol] was dissolved in acetonitrile (60 mL) and added to the solution in four portions, 2 h apart, stirring at 22 °C. Before each portion was added, the pH was readjusted to 8.5. After the last portion was added, the reaction was continued for 2 h at 22 °C. The solution was then concentrated and precipitated in ethanol (10× excess) three times. The solid product was collected and dried. Yield = 3.25 g (89.9%). ^{1}H NMR (D_2O): δ(ppm) = 3.23–3.21 (m, 2H), 2.90 (t, J = 7.8 Hz, 1H), 2.48–2.45 (m, 4H), 1.93–1.88 (m, 2H), 1.81–1.74 (m, 2H). ^{13}C NMR (D_2O): δ = 175.4, 84.3, 74.7, 71.1, 41.1, 35.3, 32.2, 24.3, 15.4.

Synthesis of Alendronate-Functionalized α-Cyclodextrin (ALN-α-CD, 6)

Compound **2** (0.791 g, 0.792 mmol) and **5** (0.293 g, 0.785 mmol) were dissolved in water/methanol (1:1 v/v, 28 mL). Tris(benzyltriazolylmethyl)amine (TBTA, synthesized according to ref.[39], 39.0 mg, 73.5 μmol) was dissolved in a minimal amount of methanol, tetrakis(acetonitrile)copper(I) hexafluorophosphate (27.2 mg, 73.0 μmol) was dissolved in a minimal amount of acetonitrile, and then both solutions were combined under argon atmosphere. This combined solution was added to the previous solution under argon atmosphere and allowed to stir at 22 °C overnight. Organic solvents were then removed and the resulting aqueous solution was filtered. The filtrate was concentrated and precipitated in ethanol (10× excess). The blue solid was collected and dried and indicated that some copper impurity still remained. TLC (1:1 v/v, methanol/water) showed that excess **2** had been removed. Yield = 1.00 g (90.5%). ^{1}H NMR (D_2O): δ(ppm) = 7.76 (s, 1H), 5.10–5.09 (m, 1H) 5.01–4.90 (m, 5H), 4.55–4.50 (m, 1H), 4.13–4.09 (m, 1H), 3.98–3.42 (m, 32H), 3.11–3.06 (m, 3H), 2.95 (t, J = 7.6 Hz, 2H), 2.73–2.71 (m, 1H), 2.55 (t, J = 8.3 Hz, 2H), 1.84 (br, 2H), 1.71 (br, 2H). ^{13}C NMR (D_2O): δ = 175.1, 146.6, 125.2, 102.0, 83.1, 81.3, 80.8 73.8, 73.3, 72.4, 72.1, 71.1, 60.5, 59.2, 51.3, 40.4, 35.4, 31.5, 23.8, 21.3. HPLC (85:15 v/v, H_2O/CH_3CN, 205 nm): λ_{max} = 1.36 min.

Synthesis of ALN-α-CD/PEG 2000 Pseudopolyrotaxane (8)

To a solution of acetylene-terminated PEG 2000 (compound **7**, synthesized according to ref.[24], 92.8 mg, 42.9 μmol) in water (2.5 mL) was added **6** (0.253 g, 0.180 mmol). The solution was sonicated for 30 min and the solvent was then removed in vacuum at 22 °C. Crude yield = 0.36 g. DSC was employed for qualitative characterization (Supporting Information Figure S1). ^{1}H NMR (D_2O): δ(ppm) = 7.76 (s, 1H), 5.10–4.92 (m, 6H), 4.56–4.52 (m, 1H), 4.20 (br, 1H), 4.11–4.09 (m, 1H), 3.94–3.44 (m, 76H), 3.10 (br, 3H), 2.94 (br, 2H), 2.75 (br, 2H), 2.55 (br, 2H), 1.74 (m, 4H). ALN-α-CD:PEG 2000 = 4:1.

Synthesis of Rhodamine B-Labeled Polyrotaxane (12)

Compound **6** (226 mg, 158 μmol) and **7** (62.8 mg, 29.0 μmol) were dissolved in water (1.5 mL) and stirred at 22 °C for 24 h. Acetylene-terminated monomethyl ether PEG 1 900 (compound **9**, synthesized according to ref.[24], 12.8 mg, 6.46 μmol), 2,2-bis(azidomethyl)-propane-1,3-diol (compound **10**, synthesized according to ref.[24], 4.84 mg, 26.0 μmol), rhodamine B-labeled monomer (compound **11**, synthesized according to ref.[24], 4.01 mg, 6.19 μmol), tris(hydroxypropyltriazolylmethyl)amine (THPTA, synthesized according to ref.[24], 6.50 mg, 15.0 μmol), $CuSO_4 \cdot 5H_2O$ (165 μg, 0.661 μmol), and water (500 μL) were added to the solution. Sodium L-ascorbate (1.65 mg, 8.25 μmol) was dissolved in water (500 μL) and added under argon. The solution was allowed to stir at 22 °C in the absence of light under argon for 3 d. Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA, 63.0 mg, 169 μmol) was then added and stirred for 1 h. The solution was subjected to dialysis (MWCO = 12–14 kDa) in the absence of light and passed through a Sephadex G-25 column for purification. Yield = 0.124 g. The polyrotaxane stained HA pink in an in vitro binding study (Supporting Information Figure S2), qualitatively characterizing osteotropicity. Apparent $\overline{M}_w$ = 22.2 kDa, PDI = 3.4. ^{1}H NMR (D_2O): δ(ppm) = 7.91 (s, PEG triazole, =CH–), 7.75 (s, ALN-α-CD triazole, =CH–), 5.09 (m, α-CD, –CH–), 5.00–4.89 (m, α-CD, –CH–), 4.54–

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4.49 (m, α-CD, −CH−), 4.42 (s, PEG triazole−CH_2−carbamate, −CH_2−), 4.33 (m, diazide monomer, −CH_2−), 4.15–4.08 (m, α-CD and PEG, −CH− and −CH_2−), 3.96–3.42 (m, α-CD and PEG, −CH− and −CH_2−), 3.30 (s, mPEG, −CH_3), 3.27 (s, diazide monomer, −CH_2−), 3.11–3.05 (m, ALN-α-CD, −CH− and −CH_2−), 2.94 (t, $J = 7.3$ Hz, ALN linker to α-CD, −CH_2−), 2.72–2.70 (m, α-CD, −CH−), 2.54 (t, $J = 7.3$ Hz, ALN linker to α-CD, −CH_2−), 1.86–1.82 (m, ALN, −CH_2−), 1.73–1.69 (m, ALN, −CH_2−). ALN-α-CD:PEG 2000 = 2.5:1. [Rhodamine B] = $9.32 \pm 0.05 \times 10^{-7}$ mol · g^{-1}.

Synthesis of Rhodamine B-Labeled PEG Copolymer

7 (100. mg, 46.4 μmol), **9** (24.0 mg, 12.1 μmol), **10** (8.12 mg, 43.6 μmol), **11** (6.09 mg, 9.41 μmol), $CuSO_4 \cdot 5H_2O$ (131 μg, 0.523 μmol), and THPTA (2.45 mg, 5.64 μmol) were dissolved in water (2 mL). Sodium L-ascorbate (1.08 mg, 5.55 μmol) was dissolved in water (0.5 mL) and added to the solution dropwise under argon. The reaction was allowed to stir at 22 °C under argon for 2 d in the absence of light. EDTA (1.96 mg, 5.16 μmol) was then added and stirred for 1 h. The solution was subjected to dialysis (MWCO = 12 to 14 kDa) in the absence of light and passed through a PD-10 column for purification. Yield = 97.1 mg. The polymer failed to stain HA in an in vitro binding study, suggesting lack of osteotropicity (Supporting Information Figure S2). Apparent $\overline{M}_w$ = 32.1 kDa, PDI = 2.6. ^{1}H NMR (D_2O): δ(ppm) = 7.97 (s, triazole, =CH−), 4.48 (s, triazole−CH_2−carbamate, −CH_2−), 4.39 (s, diazide monomer, −CH_2−), 4.21 (br, PEG, −CH_2−), 3.76–3.47 (m, PEG, −CH_2−), 3.36 (s, mPEG, −CH_3), 3.34 (s, diazide monomer, −CH_2−). [Rhodamine B] = $2.32 \pm 0.04 \times 10^{-6}$ mol · g^{-1}.

Synthesis of Methyl 2-(3-azido-2-[azidomethyl]-2-[hydroxymethyl]propoxy)acetate

Compound **10** (4.10 g, 22.0 mmol) was dissolved in anhydrous tetrahydrofuran (THF, 80 mL) and sodium hydride (60 wt.-% in mineral oil, 0.97 g, 24 mmol) was slowly added at 0 °C. The solution was allowed to stir for 3 h at 22 °C. Methyl 2-bromoacetate (6.5 mL, 68 mmol) was dissolved in anhydrous THF (6 mL) and then added to the solution. The reaction was stirred overnight at 22 °C. Water (20 mL) was added to quench the reaction and THF was removed. The solution was extracted with ethyl acetate three times. The organic fractions were collected, dried with anhydrous $MgSO_4$, filtered, and the volatiles were removed. The resulting liquid was subjected to flash chromatography (1:3 v/v, ethyl acetate/hexane) for purification. Yield = 0.563 g (9.91%). ^{1}H NMR ($CDCl_3$): δ(ppm) = 4.87 (t, $J = 4.9$ Hz, 1H), 4.13 (s, 2H), 3.66 (s, 3H), 3.36 (s, 2H), 3.35 (s, 2H), 3.34 (s, 2H), 3.30 (d, $J = 4.9$ Hz, 2H). ^{13}C NMR ($CDCl_3$): δ = 171.8, 70.4, 67.4, 62.3, 52.2, 51.1, 45.2.

Synthesis of *N*-(2-Aminoethyl)methyl 2-(3-azido-2-[azidomethyl]-2-[hydroxymethyl]propoxy)acetamide (13)

Methyl 2-(3-azido-2-[azidomethyl]-2-[hydroxymethyl]propoxy)-acetate (0.550 g, 2.13 mmol) was dissolved in ethylene diamine (10 mL, 150 mmol) under argon and allowed to stir at 55 °C overnight. TLC (1:2 v/v, ethyl acetate/hexane) verified that limiting reagent had been fully consumed. Solvent was then removed. Yield = 0.610 g (100%). ^{1}H NMR (DMSO-d_6): δ(ppm) = 7.69 (br, 1H), 3.84 (s, 2H), 3.39 (s, 4H), 3.33 (s, 2H), 3.31 (s, 2H), 3.12–3.08 (m, 2H), 2.58 (t, $J = 6.6$ Hz, 2H). ^{13}C NMR (DMSO-d_6): δ = 168.8, 70.2, 69.8, 59.8, 51.1, 45.1, 41.7, 41.3.

Synthesis of Fluorescein Isothiocyanate-Labeled Monomer (14)

Compound **13** (92.2 mg, 0.322 mmol) and triethyl amine (100. μL, 0.294 mmol) were dissolved in anhydrous *N*,*N*-dimethylformamide (DMF, 1.5 mL). Fluorescein isothiocyanate (FITC), isomer I (90% pure, 127 mg, 0.294 mmol) was added and the reaction was stirred at 22 °C for 4 h in the absence of light. TLC (1:5 v/v, methanol/ethyl acetate) verified that the limiting agent had been consumed. The solution was concentrated and triturated with ethyl acetate (10× excess) twice. The solid was collected and dried overnight. Yield = 136 mg (68.8%). ^{1}H NMR (DMSO-d_6): δ(ppm) = 8.31–8.26 (m, 2H), 7.93 (br, 1H), 7.90 (t, $J = 5.6$ Hz, 1H), 7.76 (br, 1H), 7.18 (d, $J = 8.3$ Hz, 1H), 6.67–6.55 (m, 6H), 3.88 (s, 2H), 3.42 (s, 4H), 3.36 (s, 2H), 3.33 (s, 2H), 3.31–3.27 (m, 2H), 2.78 (t, $J = 6.3$ Hz, 2H). ^{13}C NMR (DMSO-d_6): δ = 180.9, 169.5, 168.7, 161.3, 152.4, 145.7, 141.1, 129.2, 128.0, 124.6, 117.3, 113.6, 113.5, 110.0, 102.3, 70.2, 69.8, 59.8, 51.1, 45.1, 43.4, 37.9.

Representative Procedure for the Synthesis of Fluorescein Isothiocyanate-Labeled Polyrotaxanes Using Copolymerization

Compound **6** (88.8 mg, 61.7 μmol) and **7** (26.7 mg, 12.3 μmol) were dissolved in water (600 μL) and stirred at 22 °C for 2 h. Compound **9** (5.35 mg, 2.70 μmol), **13** (3.13 mg, 10.9 μmol), **14** (1.84 mg, 2.73 μmol), THPTA (2.04 mg, 4.69 μmol), $CuSO_4 \cdot 5H_2O$ (53 μg, 0.21 μmol), and water (300 μL) were added to the solution. Sodium L-ascorbate (545 μg, 2.72 μmol) was dissolved in water (100 μL) and added under argon. The solution was allowed to stir at 22 °C in the absence of light under argon for 3 d. EDTA (23.9 mg, 64.0 μmol) was then added and stirred for 1 h. The solution was subjected to dialysis (MWCO = 12 to 14 kDa) in the absence of light and passed through a PD-10 column for purification. Yield = 52.1 mg. Apparent $\overline{M}_w$ = 24.0 kDa, PDI = 3.1. ^{1}H NMR (D_2O): δ(ppm) = 7.96 (s, PEG triazole, =CH−), 7.79 (s, ALN-α-CD triazole, =CH−), 5.13 (m, α-CD, −CH−), 5.02–4.94 (m, α-CD, −CH−), 4.59–4.56 (m, α-CD, −CH−), 4.38 (s, amine monomer, −CH_2−), 4.19–4.12 (m, α-CD and PEG, −CH− and −CH_2−), 4.01–3.48 (m, α-CD and PEG, −CH− and −CH_2−), 3.35 (br, amine monomer, −CH_2−), 3.22–3.11 (m, ALN-α-CD and amine monomer, −CH− and −CH_2−), 2.98 (t, $J = 6.8$ Hz, ALN linker to α-CD, −CH_2−), 2.78–2.76 (m, α-CD, −CH−), 2.59 (t, $J = 7.6$ Hz, ALN linker to α-CD, −CH_2−), 1.92–1.89 (m, ALN, −CH_2−), 1.76 (br, ALN, −CH_2−). ALN-α-CD:PEG 2000 = 2.2:1. FITC concentration = $1.02 \pm 0.01 \times 10^{-5}$ mol · g^{-1}.

Polyrotaxanes of different ALN-α-CD:PEG 2000 ratios were obtained by incorporating different amounts of **6**. The apparent $\overline{M}_w$ and PDI, ALN-α-CD:PEG 2000 ratios, and FITC concentrations of these polyrotaxanes can be found in Supporting Information Table S1.

Synthesis of FITC-Labeled PEG Copolymer Using Copolymerization

Compound **7** (26.5 mg, 12.3 μmol), **9** (5.43 mg, 2.74 μmol), **13** (3.13 mg, 10.9 μmol), **14** (1.88 mg, 2.80 μmol), THPTA (1.15 mg, 2.64 μmol), and $CuSO_4 \cdot 5H_2O$ (106 μg, 0.424 μmol) were dissolved in water (900 μL). Sodium L-ascorbate (1.09 mg, 5.45 μmol) was dissolved in water (100 μL) and added to the solution under argon. The solution was allowed to stir at 22 °C under argon in the absence of light for 3 d. EDTA (0.784 μg, 2.10 μmol) was then added and stirred for 1 h. The solution was subjected to dialysis (MWCO = 12 to 14 kDa) in the absence of light and passed through a PD-10 column for purification. Yield = 19.1 mg. Apparent $\overline{M}_w$ = 16.7 kDa, PDI = 2.8. ^{1}H NMR (D_2O): δ(ppm) = 7.92 (s, PEG triazole, =CH−), 4.43 (s, PEG triazole−CH_2−carbamate, −CH_2−), 4.33 (s, amine monomer, −CH_2−), 4.16–4.12 (br, PEG, −CH_2−), 3.98 (s, amine monomer, −CH_2−), 3.78–3.45 (m, PEG and amine monomer, −CH_2−), 3.33 (s, mPEG, −CH_3), 3.31 (s, amine monomer, −CH_2−), 3.30 (s, amine monomer, −CH_2−), 3.12 (t, J = 5.9 Hz, amine monomer, −CH_2−). FITC concentration = $2.54 \pm 0.03 \times 10^{-5}$ mol · g^{-1}.

Synthesis of Amine-Functionalized Polyrotaxane (15)

Compound **6** (149 mg, 104 μmol) and **7** (44.1 mg, 20.4 μmol) were dissolved in water (500 μL) and stirred at 22 °C for 2 h. **9** (8.91 mg, 4.50 μmol), **13** (6.49 mg, 22.7 μmol), THPTA (3.42 mg, 7.87 μmol), $CuSO_4 \cdot 5H_2O$ (885 μg, 0.354 μmol), and water (1.5 mL) were added to the solution. Sodium L-ascorbate (924 μg, 4.66 μmol) was dissolved in water (500 μL) and added under argon. The solution was allowed to stir at 22 °C under argon for 2 d. EDTA (42.6 mg, 114 μmol) was then added and stirred for 1 h. The solution was subjected to dialysis (MWCO = 12 to 14 kDa) and passed through a PD-10 column for purification. A ninhydrin assay verified the presence of amine. Yield = 80.6 mg. Apparent $\overline{M}_w$ = 36.8 kDa, PDI = 3.2. ^{1}H NMR (D_2O): δ(ppm) = 7.92 (s, PEG triazole, =CH−), 7.76 (s, ALN-α-CD triazole, =CH−), 5.09 (m, α-CD, −CH−), 5.00–4.90 (m, α-CD, −CH−), 4.54–4.50 (m, α-CD, −CH−), 4.33 (s, amine monomer, −CH_2−), 4.15–4.04 (m, α-CD and PEG, −CH− and −CH_2−), 3.97–3.42 (m, α-CD, PEG, and amine monomer, −CH− and –CH_2–), 3.31–3.29 (m, amine monomer and mPEG, −CH_2− and –CH_3), 3.16–3.07 (m, ALN-α-CD and amine monomer, −CH− and −CH_2−), 2.95 (t, J = 7.3 Hz, ALN linker to α-CD, −CH_2−), 2.74–2.71 (m, α-CD, −CH−), 2.55 (t, J = 7.3 Hz, ALN linker to α-CD, −CH_2−), 1.87–1.82 (m, ALN, −CH_2−), 1.72 (br, ALN, −CH_2−). ALN-α-CD:PEG 2000 = 2.9:1.

Synthesis of FITC-Labeled Polyrotaxane Using Polymer Analogous Reaction (16)

Compound **15** (55.1 mg) was dissolved in carbonate-buffered solution (2 mL, pH 8.5), FITC (41.5 mg, 95.9 μmol) was dissolved in DMF (1 mL), and the two solutions were combined. The combined solution was stirred overnight at 22 °C in the absence of light and subsequently triturated in water (20 mL). The suspension was filtered and the filtrate was subjected to dialysis (MWCO = 12 to 14 kDa) in the absence of light and passed through a Sephadex G-25 column for purification. Yield = 51.3 mg. The polyrotaxane stained HA yellow in an in vitro binding study, suggesting osteotropicity (Supporting Information Figure S3). Apparent $\overline{M}_w$ = 38.1 kDa, PDI = 3.5. ^{1}H NMR (D_2O): δ(ppm) = 7.92–7.81 (m, PEG triazole and FITC, =CH−), 7.75 (s, ALN-α-CD triazole, =CH−), 7.60–7.58 (br, FITC, =CH−), 7.42 (br, FITC, =CH−), 7.15–6.90 (m, FITC, =CH−), 6.53–6.38 (m, FITC, =CH−), 5.08–4.90 (m, α-CD, −CH−), 4.50–4.40 (m, α-CD, −CH−), 4.24 (s, amine monomer, −CH_2−), 4.14–4.06 (m, α-CD and PEG, −CH− and −CH_2−), 3.93–3.44 (m, α-CD, PEG, and amine monomer, −CH− and −CH_2−), 3.30–3.27 (m, amine monomer and mPEG, −CH_2− and −CH_3), 3.18–3.06 (m, ALN-α-CD and amine monomer, −CH− and −CH_2−), 2.94 (br.t, ALN linker to α-CD, −CH_2−), 2.74–2.71 (m, α-CD, −CH−), 2.55 (t, J = 7.6 Hz, ALN linker to α-CD, −CH_2−). FITC concentration = $2.54 \pm 0.06 \times 10^{-5}$ mol · g^{-1}.

Synthesis of Amine-Functionalized PEG Copolymer

Compound **7** (91.0 mg, 42.1 μmol), **9** (18.4 mg, 9.29 μmol), **13** (13.8 mg, 48.3 μmol), THPTA (21.1 mg, 48.6 μmol), and $CuSO_4 \cdot 5H_2O$ (1.21 mg, 4.85 μmol) were dissolved in water (4.5 mL). Sodium L-ascorbate (9.57 mg, 48.3 μmol) was dissolved in water (600 μL) and added to the solution under argon. The solution was stirred at 22 °C under argon for 3 d. EDTA (18.3 mg, 49.1 μmol) was then added and stirred for 1 h. The solution was subjected to dialysis (MWCO = 12 to 14 kDa) and subsequently passed through a PD-10 column for purification. A ninhydrin assay verified the presence of amine. Yield = 90.3 mg. Apparent $\overline{M}_w$ = 31.1 kDa, PDI = 3.4. ^{1}H NMR (D_2O): δ(ppm) = 7.91 (s, PEG triazole, =CH−), 4.56–4.48 (m, PEG triazole-CH_2-carbamate, −CH_2−), 4.33 (s, amine monomer, −CH_2−), 4.14–4.11 (br, PEG, −CH_2−), 3.92 (s, amine monomer, −CH_2−), 3.76–3.47 (m, PEG and amine monomer), 3.30–3.27 (m, amine monomer and mPEG, −CH_2− and −CH_3), 3.14 (t, J = 5.9 Hz, amine monomer, −CH_2−).

Synthesis of FITC-Labeled PEG Copolymer Using Polymer Analogous Reaction

Amine-functionalized PEG copolymer (see previous step, 28.7 mg) was dissolved in carbonate-buffered solution (1.5 mL, pH 8.5), FITC (24.8 mg, 57.3 μmol) was dissolved in DMF (750 μL), and the two solutions were combined. The combined solution was stirred overnight at 22 °C in the absence of light and subsequently triturated in water (20 mL). The suspension was filtered and the filtrate was subjected to dialysis (MWCO = 12 to 14 kDa) in the absence of light and passed through a Sephadex G-25 column for purification. Yield = 24.1 mg. The resulting yellow polymer did not stain HA in an in vitro binding study, qualitatively characterizing lack of osteotropicity (Supporting Information Figure S3). Apparent $\overline{M}_w$ = 25.5 kDa, PDI = 2.9. ^{1}H NMR (D_2O): δ(ppm) = 7.98–7.80 (m, PEG triazole and FITC, =CH−), 7.58 (br, FITC, =CH−), 7.00–6.48 (m, FITC, =CH−), 4.52–3.12 (m, PEG and amine monomer, −CH−). FITC concentration = $1.61 \pm 0.07 \times 10^{-4}$ mol · g^{-1}.

Hydroxyapatite in vitro Binding Study

Polyrotaxane, or control compounds, were dissolved in PBS (pH 7.4) with a concentration of 1.00 mg · mL^{-1} and the UV-vis absorbance (λ = 495 nm) was measured (absorbance not measured for the

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rhodamine B-labeled polyrotaxane and related controls). The solution (1.00 mL) was incubated with HA powder (25 mg) for 5 min, unless otherwise stated, at 22 °C. The suspension was centrifuged and the UV-vis absorbance ($\lambda = 495$ nm) of the supernatant was measured and compared to the initial measurement. Background correction was applied. Data was measured in triplicate and the standard deviations were calculated.

Validation of Osteotropicity of FITC-Labeled ALN-α-CD-Containing Polyrotaxane (16) in Healthy Mice

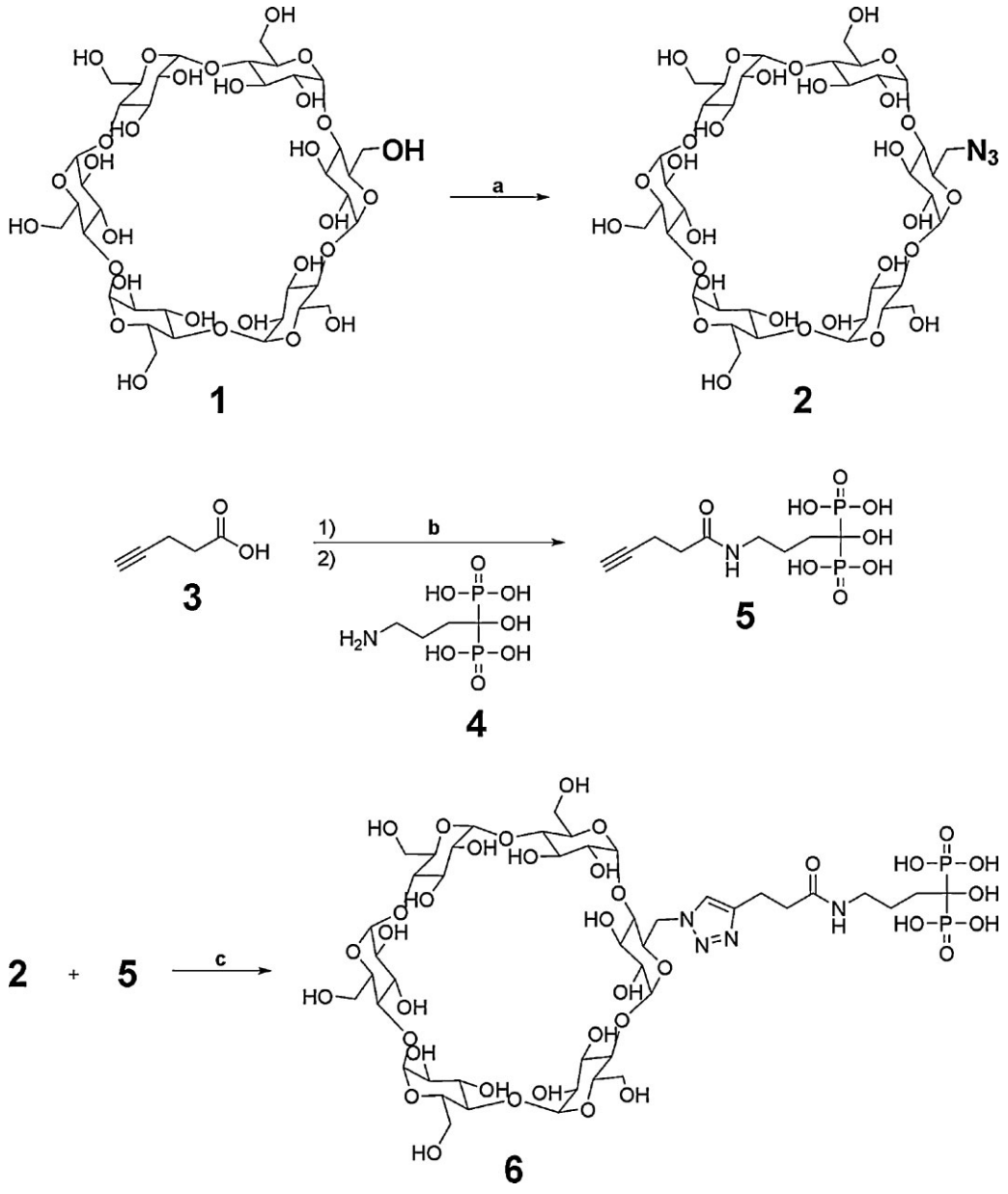
FITC-labeled polyrotaxane (**16**, 12.7 mg) was dissolved in saline solution (700 μL), filtered (0.2 μm filter), and administered via tail vein injection (200 μL) into healthy Swiss Webster mice (3/group). To serve as a control, FITC-labeled PEG copolymer (12.5 mg) was dissolved in saline solution (700 μL), filtered (0.2 μm filter), and administered via tail vein injection (700 μL) into healthy Swiss Webster mice (3/group). As a second control, saline solution (700 μL) was filtered (0.2 μm filter), and administered via tail vein injection (200 μL) into healthy Swiss Webster mice (3/group). After 24 h, the mice were sacrificed and their femurs were harvested. The distal femur was removed and placed in Villanueva osteochrome (Polysciences, Warrington, PA, USA) for 72 h. The specimen was then dehydrated in a graded series of alcohol and acetone, and then embedded un-decalcified in polymethylmethacrylate. 14 μm sections were obtained in the longitudinal plane using a vertical bed microtome (Leica RM2255, Leica Microsystems, Wetzlar, Germany) and affixed to slides coated with a 1% gelatin solution. Fluorescence imaging was performed using a semi-automatic image analysis system (Bioquant Image Analysis Corporation, Nashville, TN, USA) linked to a microscope (BX51, Olympus Corporation, Center Valley, PA, USA) equipped with fluorescent light. All images were taken under the same conditions.

Results and Discussion

In an effort to develop an osteotropic delivery system in which ALN targeting moiety content can be freely adjusted, a polyrotaxane approach was taken using the α-CD/PEG assembly. The initial synthetic step of this system is the conjugation of ALN to α-CD. To account for ALN's exclusive solubility in water, the copper(I)-catalyzed Huisgen 1,3-dipolar cycloaddition of azides and acetylenes was selected as the linking reaction. This click chemistry reaction can be performed in water, requires mild reaction conditions, and is high-yielding, making purification simpler.[27] A primary hydroxyl functional group of α-CD (**1**) was first activated by tosylation and allowed to undergo a S_N2 substitution with sodium azide to form mono-(6-azido-6-deoxy)-α-cyclodextrin (**2**). It was extensively purified by repeat precipitation in acetone, as any free azide present may potentially interfere with the click reaction. Presence of a single peak at $\lambda_{max} = 2.72$ min in the HPLC spectrum (85:15 v/v, H_2O/CH_3CN, 205 nm) verified that **2** was pure. An acetylene functional group was then introduced onto ALN (**4**) by reaction of ALN with 4-pentynoic acid through EDC/NHS coupling in aqueous mixed solvent. The resulting molecules were "clicked" together under inert atmosphere using a Cu(I) catalyst and a Cu(I) stabilizing agent, TBTA,[26,42] forming ALN-α-CD (**6**, Scheme 1). A Cu(I) stabilizing agent is required for this reaction to protect Cu(I) active catalyst from degradation pathways (e.g., oxidation). Emergence of a singlet peak at 7.76 ppm in the ^{1}H NMR spectrum verified the presence of 1,2,3-triazole in the product. The low overall yield of this step, calculated to be 13.0% with respect to α-CD, can be attributed to difficulty in tosylating a single hydroxyl group in α-CD. More efficient synthetic routes are currently being explored.

As the second step, PEG diol ($\overline{M}_w = 2000$ Da) was capped with acetylene functional groups (**7**), to serve as chemical handles for which sterically bulky monomers can be later conjugated to prevent dethreading. Complete conversion of the two hydroxyl termini into acetylene is of critical importance, as mono-acetylene PEG would act as a chain terminator and result in low-molecular-weight polyrotaxane in the final copolymerization step. To ensure 100% conversion, the hydroxyl termini were first activated with highly reactive phosgene and subsequently treated with propargylamine. After purification by precipitation and LH-20 column separation, NMR and SEC analysis verified **7** to be highly pure, but with a small amount of acetylene-terminated PEG dimer present from phosgene cross-linking. ALN-α-CD was then threaded onto **7**, as shown in Scheme 2, forming a pseudopolyrotaxane (**8**). This was achieved by adding ALN-α-CD to a concentrated aqueous solution of **7** and sonicating for 30 min at room temperature. Water solvent was slowly removed at room temperature in vacuum to afford the product. Using too dilute of solution and/or high temperatures would not result in pseudopolyrotaxane formation, as entropy becomes the major driving force in such conditions.[28] DSC analysis qualitatively confirmed the pseudopolyrotaxane formation. A large endothermic peak appeared at 50 °C in the DSC spectrum of **7**, corresponding to its melting point, but was nearly absent in the spectrum of **8** (Supporting Information Figure S1). A small amount of **7** still remained, however, which is typical of α-CD/PEG pseudopolyrotaxanes/polyrotaxanes.[28]

In the final step, the copper(I)-catalyzed Huisgen 1,3-dipolar cycloaddition is used again to copolymerize the pseudopolyrotaxane with a diazide monomer, 2,2-bis(azidomethyl)propane-1,3-diol (**10**, Scheme 3) or its functional derivatives, to form the polyrotaxane delivery system. This monomer is known to undergo click chemistry rapidly due to a self-catalyzing effect[43] and will form polymers of high molecular weights. In order to prevent gel formation, a chain terminator, mono-acetylene-terminated PEG (**9**), must also be included.[24] **9** was synthesized following the same protocol for **7**, but using monomethyl ether PEG



Scheme 1. Synthesis of ALN-α-CD. (a) TsCl, pyridine, rt; NaN_3, H_2O, 90 °C; (b) EDC, NHS, CH_2Cl_2, rt; (c) $Cu(CH_3CN)_4PF_6$, TBTA, 1:1 CH_3OH:H_2O, rt.

($\overline{M}_w = 1900$ Da) as starting material. $CuSO_4 \cdot 5H_2O$ and sodium ascorbate (10× molar excess) are used under inert atmosphere for the in situ generation of active Cu(I) catalyst.[43] A water-soluble Cu(I) stabilizing agent, THPTA (10× molar excess), is also included to prevent oxidation and disproportionation of the catalyst and to have better control over the PDI of the copolymers.[24,42,44] The reaction is performed at room temperature in aqueous solvent and is complete within 2 d. Therapeutic agents or imaging agents can be incorporated into the polyrotaxane by conjugation to **10** prior to copolymerization (e.g., **11** in Scheme 3). The amount incorporated can be adjusted by changing the reactants ratio (not shown). Similarly, the overall bone-binding affinity of the polyrotaxanes can be altered by changing the ALN-α-CD (**6**) to acetylene-terminated PEG 2000 (**7**) feed-in ratio. For general purification, the reaction solution is incubated with EDTA for 1 h to chelate copper catalyst, followed by dialysis (MWCO = 12 to 14 kDa) and gel filtration chromatography over Sephadex G-25 resin.

Initially, an ALN-α-CD containing polyrotaxane was synthesized in which 20% of the diazide monomer was labeled with rhodamine B (**11**), a bright pink dye (Scheme 3). An ALN-α-CD:acetylene-terminated PEG 2000 feed-in ratio of 5:1 was used. **11** was synthesized through EDC/NHS

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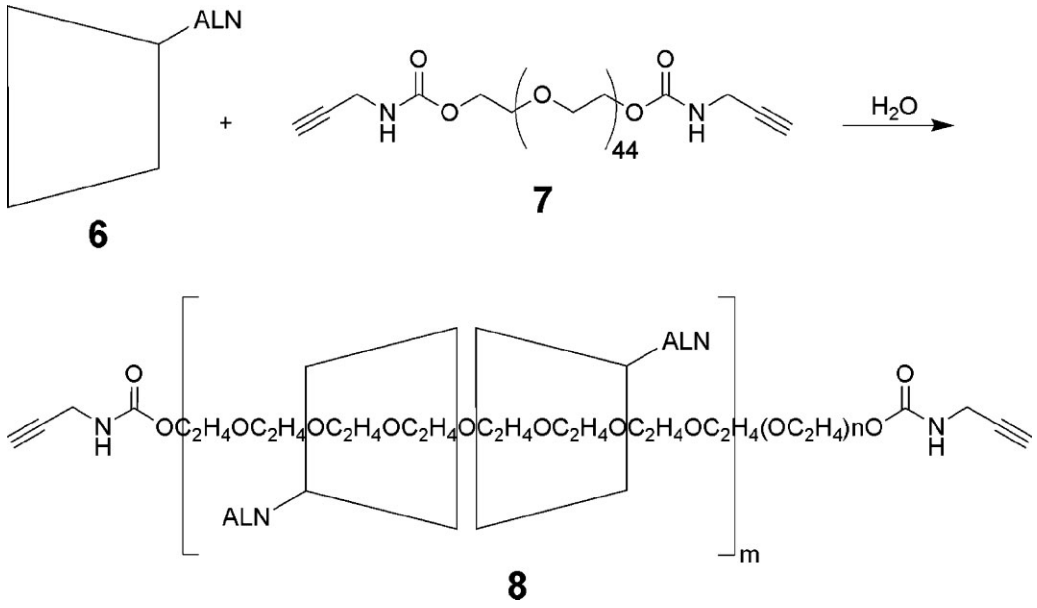
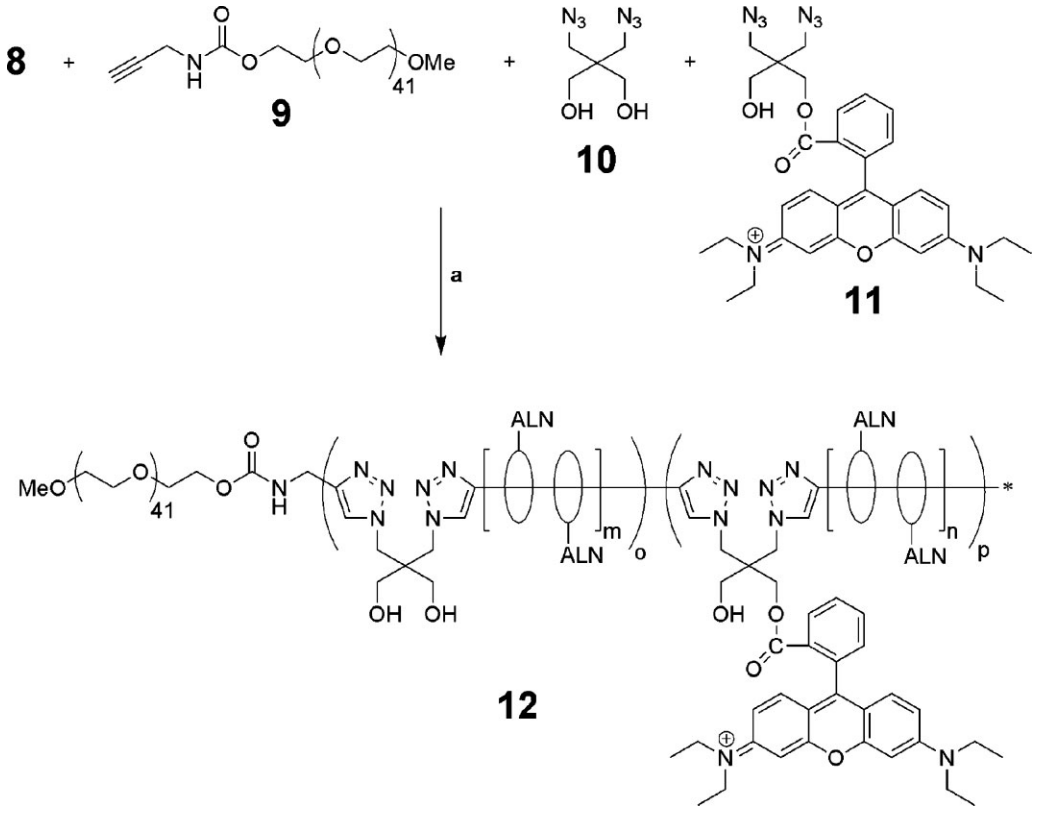


Scheme 2. Formation of the osteotropic pseudopolyrotaxane, in where ALN-α-CD (**6**) has been simplified. α-CD is commonly depicted as a truncated cone structure to better portray its 3-dimensional configuration.



Scheme 3. Synthesis of a rhodamine B-labeled osteotropic polyrotaxane (**12**). (a) $CuSO_4 \cdot 5H_2O$, sodium ascorbate, THPTA, H_2O, rt. PEG and α-CD are represented as extended lines and ovals, respectively, in the product. A rhodamine B-labeled monomer was incorporated into the polyrotaxane to prevent dethreading of ALN-α-CD, simultaneously serve as a drug surrogate, and for the convenience of an in vitro HA binding study.

coupling of one of the hydroxyl functional groups of **10** with the carboxylic acid functional group of rhodamine B, forming an ester linkage. The steric bulk of rhodamine B, along with a molar excess of **10** used in the reaction, effectively prevented the second hydroxyl functional group of the monomer from reacting. Flash chromatography was employed for purification to ensure complete removal of unlabeled monomer. After copolymerization and purification, the final polyrotaxane (**12**) displayed a pink color, suggesting the successful incorporation of **11**. Using SEC, the $\overline{M}_w$ and PDI of **12** were estimated to be 22.2 kDa and 3.4, respectively, based on a PEG calibration curve (Figure 1). By comparison of integral values of corresponding peaks in the ^{1}H NMR spectrum, the average ALN-α-CD:PEG 2000 ratio of **12** was estimated to be 2.5:1. This ratio is greatly reduced from the feed-in ratio, indicating that a significant portion of the ALN-α-CD had been lost. The most probable explanations are a portion of ALN-α-CD was not threaded onto the PEG backbone prior to copolymerization and/or the concentration of **11** in the polyrotaxane was not high enough to prevent dethreading (rhodamine B concentration $= 6.21 \pm 0.05 \times 10^{-7}$ mol · g^{-1}). In the former explanation, the conjugation of the sterically bulky ALN side chain to α-CD may disrupt the hydrogen bonding network of adjacent α-CDs threaded on the pseudopolyrotaxane. It is known that the driving force for successful pseudopolyrotaxane formation is hydrogen bonding between threaded α-CDs.[28] Additionally, ALN-α-CD may form inclusion complexes with other molecules that were introduced into the system (e.g., monomers **10** and **11**, THPTA, etc.), thereby forming a competition with **7** for pseudopolyrotaxane formation. A similar competition between pseudopolyrotaxane formation and inclusion complexation with HPMA monomer was observed when Feng and coworkers developed β-cyclodextrin/Pluronic F127 polyrotaxanes.[47–49] In the latter explanation, the low concentration of rhodamine B may be attributed to the low incorporation of **11** during the copolymerization and/or cleavage of rhodamine B from the polymer via hydrolysis due to the long-term exposure of the ester linkage to water during adjacent purification. **12** was then evaluated for osteotropicity in vitro, where rhodamine B served as a traceable drug surrogate. After dissolution in PBS (pH 7.4), **12** was incubated with HA. HA, typically a white solid, was immediately stained pink (<10 s) and the color could not be dissipated by successive washings with PBS and acetone, indicating that **12** can bind tightly and quickly to HA in vitro (Figure 2). All of the controls tested, including **11** and rhodamine B-labeled PEG copolymer lacking ALN-α-CD, were unable to stain the mineral (Supporting Information Figure S2).

To determine if concentrations of ALN-α-CD in the polyrotaxane could be freely adjusted, four polyrotaxanes were synthesized using different feed-in ratios of ALN-α-CD to acetylene-terminated PEG 2000: 1:1, 2:1, 5:1, and 10:1. For this a different set of monomers were used to better prevent dethreading. In favor of finding a monomer more stable in aqueous conditions than the ester-linked **11**, a FITC-labeled monomer was synthesized (Scheme 4). Like rhodamine B, FITC is sterically bulky and emits fluorescence upon excitation. It can be easily conjugated to primary amines through an thiourea linkage. Unlike ester functional groups, thiourea linkages are not as susceptible to hydrolysis and are more stable in vivo.[45–46] One of the hydroxyl functional groups of **10** was first deprotonated with sodium hydride to form a stronger nucleophile and then mixed with methyl 2-bromoacetate to introduce a

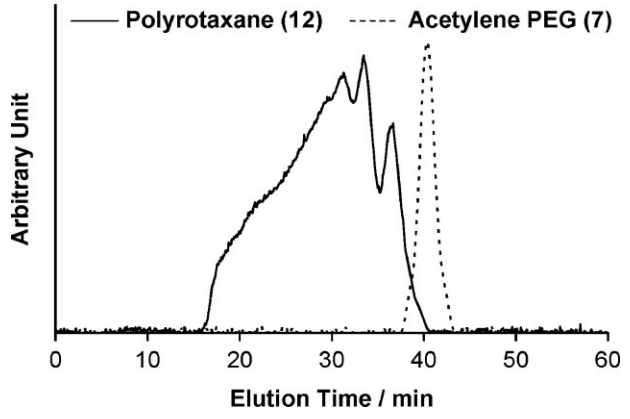


Figure 1. SEC analysis of rhodamine B-labeled polyrotaxane (**12**, $\overline{M}_w = 22.2$ kDa, PDI = 3.4) and acetylene-terminated PEG (**7**, $\overline{M}_w = 2.2$ kDa, PDI = 1.1) for comparison. $\overline{M}_w$ and PDI values were estimated based on a PEG calibration curve.

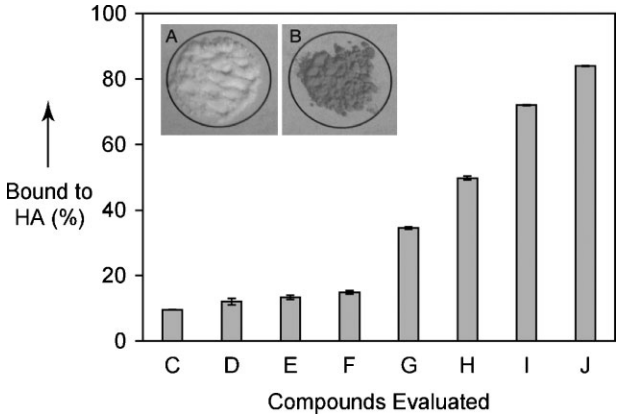


Figure 2. HA binding study. Inset: Untreated HA (A) is shown as a white powder. After mixing **12** with HA and extensive washing, the HA powder remained bright pink (B). Chart: The binding of four FITC labeled-polyrotaxanes containing different concentrations of ALN-α-CD to HA, along with four controls. The controls are **14** (C), a 1:1 physical mixture of **14**/**6** (D), a FITC-labeled PEG copolymer void of **6** (E), and a 1:1 wt.-% physical mixture of E/**6** (F). The average ALN-α-CD:PEG 2000 ratios of the polyrotaxanes evaluated are 0.7:1 (G), 1.2:1 (H), 2.2:1 (I), and 5.4:1 (J).

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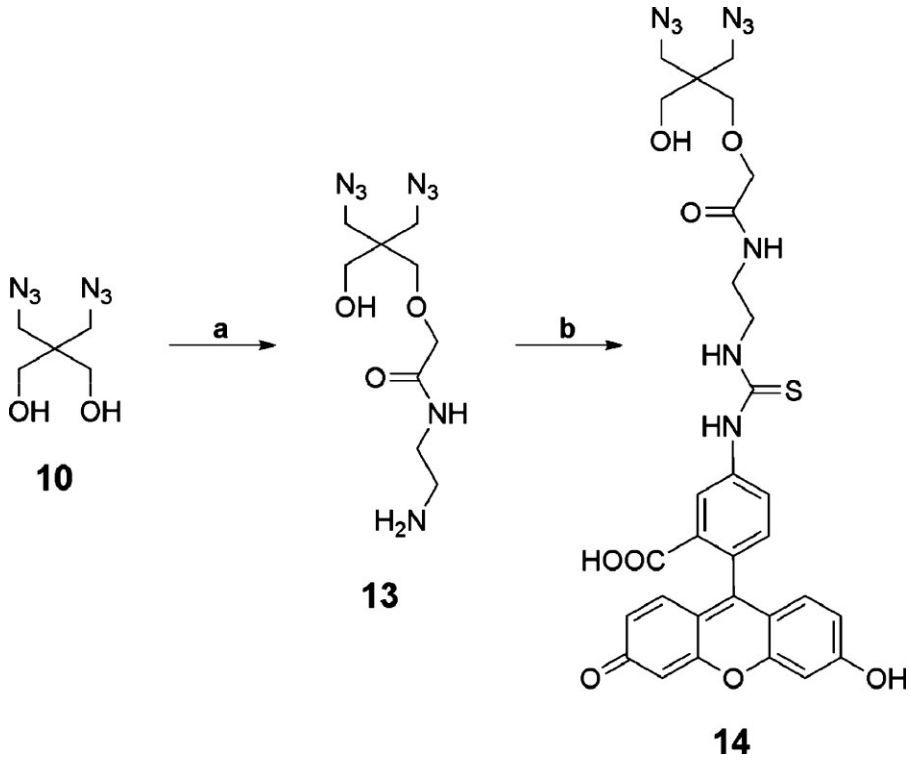


Scheme 4. Synthesis of the FITC-labeled monomer. (a) NaH, methyl 2-bromoacetate, THF, rt; ethylene diamine, 55 °C; (b) FITC, TEA, DMF, rt.

methyl ester functional group. After workup and purification by flash chromatography, this monomer was subsequently treated with ethylene diamine to become amine-functionalized (**13**). A high volume of ethylene diamine was used to prevent potential cross-linking, which was verified by comparison of integral values in the ^{1}H NMR spectrum. **13** was then conjugated to FITC, isomer 1 (**14**). **13** and **14**, ratio of 80/20%, were utilized to produce the polyrotaxanes. The copolymerizations were performed in the absence of light to prevent quenching. After purification, the average ALN-α-CD:PEG 2000 ratios of the FITC-labeled polyrotaxanes were estimated to be 0.7:1, 1.2:1, 2.2:1, and 5.4:1 by ^{1}H NMR. In each case, approximately half of the ALN-α-CD original content was lost, which is similar to the amount lost by the rhodamine B-labeled polyrotaxane. This may again be attributed to low incorporation of dye-labeled monomer (the FITC concentrations ranged from 4.9 ± 0.1 to $19.4 \pm 0.5 \times 10^{-6}$ mol · g^{-1}) and/or a portion of ALN-α-CD was not threaded onto the PEG backbone prior to copolymerization.

The biomineral-binding potential of these four FITC-labeled polyrotaxanes were evaluated in an in vitro HA binding assay, using FITC as a traceable drug surrogate. As shown in Figure 2, the higher the ALN-α-CD concentration in the polyrotaxane the greater its binding affinity for HA, with percent binding values ranging from 34.5 to 84.0%. Such markedly different osteotropicities can be easily achieved, potentially allowing personalized treatment arrangements according to the type and severity of the bone diseases of the subjects. High osteotropicity, such as 84.0%, also requires less polyrotaxane needed to be administered to achieve desired therapeutic effects. To serve as controls, **14**, a 1:1 physical mixture of **14**/**6** (α-CD is known to form inclusion complexes with a variety of compounds), a FITC-labeled PEG copolymer containing no ALN-α-CD, and a 1:1 wt.-% physical mixture of FITC-labeled

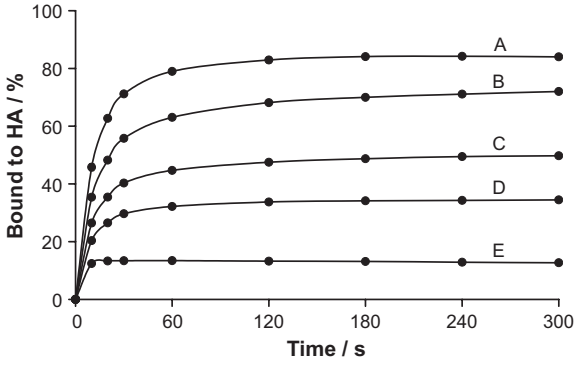
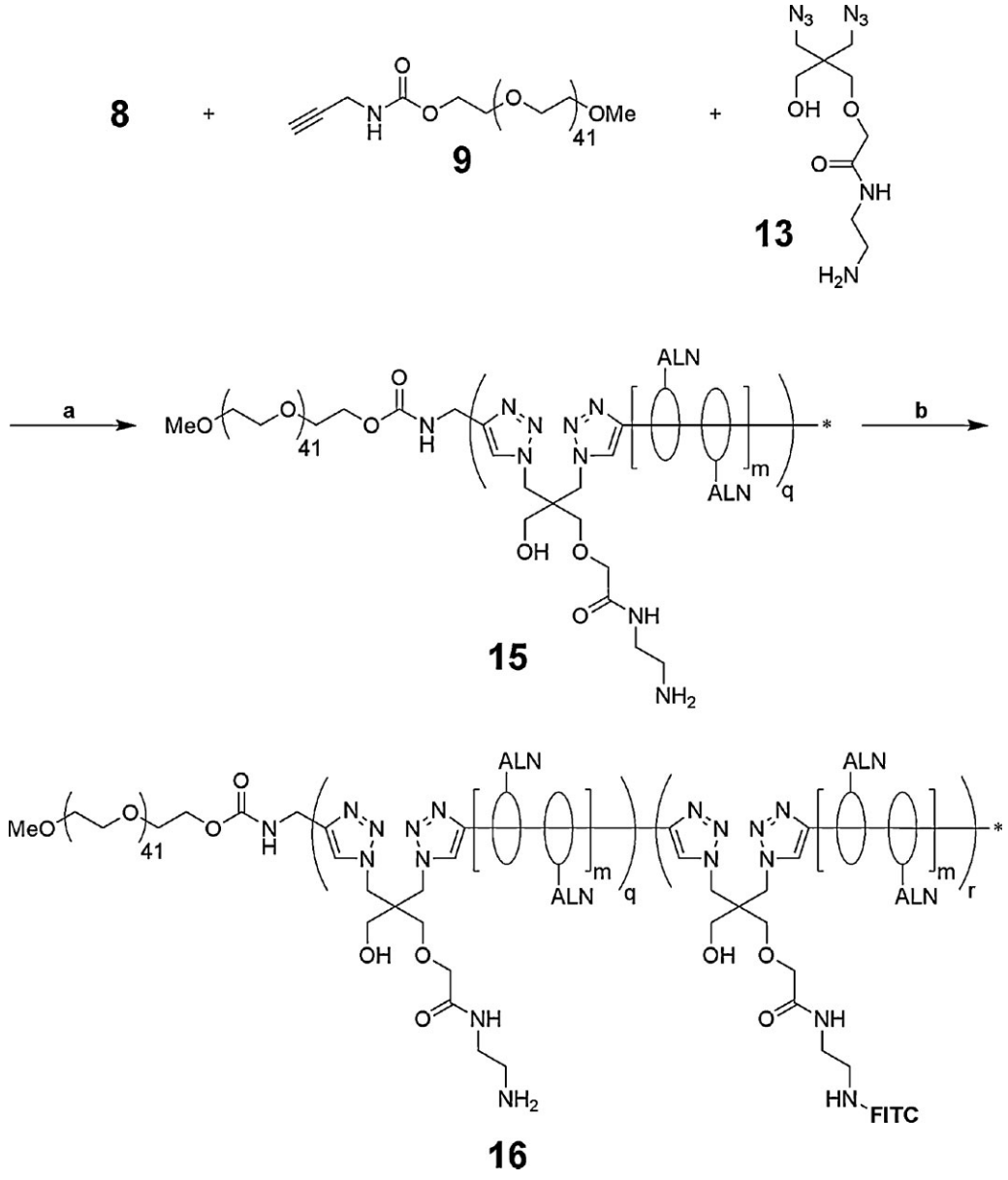


Figure 3. The initial binding kinetics of four polyrotaxanes with different concentrations of ALN-α-CD to HA. The average ALN-α-CD:PEG 2000 ratios are 5.4:1 (A), 2.2:1 (B), 1.2:1 (C), and 0.7:1 (D). A 1:1 wt.-% physical mixture of **6** and FITC-labeled PEG copolymer void of **6** was used as a control (E).

PEG copolymer/**6** were subjected to the HA binding study. All controls displayed a rather low affinity to HA (9.5 to 14.9%), which may be attributed to non-specific binding of FITC.

The binding kinetics of the four FITC-labeled polyrotaxanes toward HA was evaluated. In each case, binding occurred quickly. A binding plateau was reached within two to three minutes, with 50% of the total binding occurring in less than 10 s (Figure 3). These kinetics are typical for bisphosphonates, which bind tightly and quickly to bone.[11] Each of the polyrotaxane binding curves was nearly identical in curvature, indicating that the binding kinetics are independent of the concentration of ALN-α-CD. As a control, a 1:1 wt.-% physical mixture of FITC-labeled PEG copolymer/ALN-α-CD was tested. In this trial, a non-specific binding plateau was also reached within 10 s.

The osteotropicity of the polyrotaxane was also confirmed in vivo. To address the problem of the low incorporation of the FITC-labeled monomer into the aforementioned polyrotaxanes, a FITC-labeled polyrotaxane was synthesized following a polymer analogous approach (**16**, Scheme 5). An amine-functionalized poly-



Scheme 5. Synthesis of the FITC-labeled polyrotaxane (**16**) for in vivo evaluation. (a) $CuSO_4 \cdot 5H_2O$, THPTA, sodium ascorbate, H_2O, rt; (b) 1:2 DMF:CBS (pH 8.5), FITC, rt. PEG and α-CD are represented as extended lines and ovals, respectively, in the polymers. The FITC-labeled PEG copolymer for in vivo evaluation was also synthesized following this synthetic scheme, except compound **8** was replaced with **7**.

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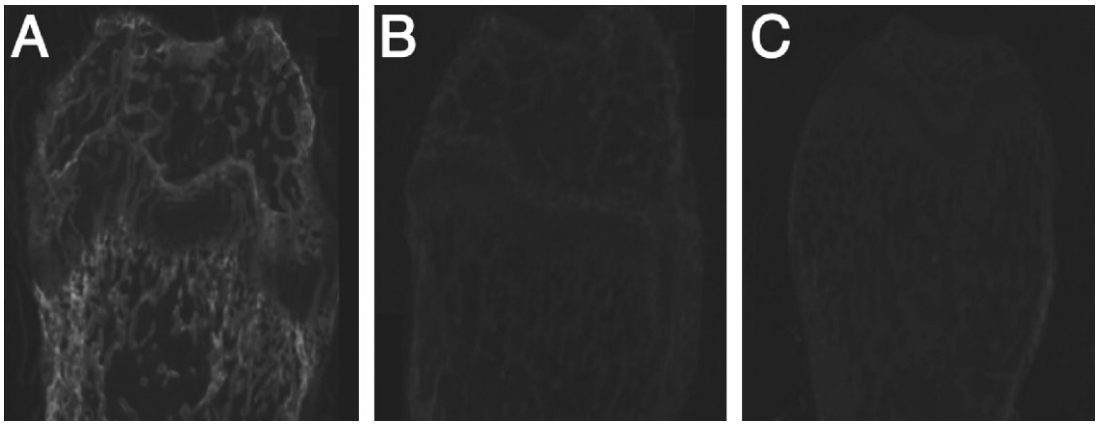

Figure 4. Distal femur of mice evaluated for fluorescence post injection of (A) FITC-labeled polyrotaxane, (B) FITC-labeled PEG copolymer void of ALN-α-CD, and (C) saline solution. Only the distal femur from the polyrotaxane injection displayed an appreciable amount of fluorescence, suggesting a strong binding and retention of the polyrotaxane to the bone.

rotaxane was first synthesized (**15**) using only monomer **13**. The ALN-α-CD:PEG 2000 ratio was estimated to be 2.9:1 by ^{1}H NMR. **15** was then labeled with FITC by dissolving in DMF/carbonate-buffered solution (pH 8.5) and adding a molar excess of FITC, isomer I. The reaction was stirred overnight at room temperature in the absence of light. After purification, the FITC concentration of **16** was determined to be $2.54 \pm 0.06 \times 10^{-5}$ mol · g^{-1}, which is significantly higher compared to the other FITC-labeled polyrotaxanes. When subjected to a HA binding study, the HA was immediately stained yellow, confirming in vitro osteotropicity (Supporting Information Figure S3). **16**, along with two controls, FITC-labeled PEG copolymer void of ALN-α-CD (synthesized by polymer analogous approach, see Scheme 5 caption) and saline solution, were then administered to Swiss Webster mice via tail vein injections. After 24 h, the mice were sacrificed and their femurs were isolated and processed for assessment of fluorochrome labeling. As shown in Figure 4, the bone at the distal femur metaphysis displayed high fluorescent signal in mice injected with FITC-labeled polyrotaxane while mice injected with FITC-labeled PEG copolymer or saline showed no fluorescence.

All copolymers synthesized were subjected to FPLC analysis. The $\overline{M}_w$ and PDI of each were estimated based on a PEG calibration curve and ranged from 16.7 to 38.1 kDa and 2.6 to 3.5, respectively. Such variability may be partially attributed to the polycondensation reaction nature of the click copolymerization and the use of different monomers in the copolymerization. To reduce the variability, experimental conditions may be further optimized, but it is likely that PDI will remain above 2.0. A reliable method to further control PDI is through column fractionation using SEC. Biodegradability can be easily introduced into this delivery system by conjugating a biodegradable linker (e.g., polyester) to PEG 2000 termini prior to functionalization with acetylene.

Conclusion

In summary, a novel multiblock osteotropic α-CD/PEG polyrotaxane was developed in which the concentration of bone-targeting moiety (ALN) can be freely adjusted, thereby affecting the overall binding affinity for bone. The system was shown to bind to bone in vivo extensively within 24 h post injection. Therapeutic agents and imaging agents can be conveniently introduced via the functionalized diazide monomers when the multiblock polyrotaxane is constructed. This uniquely structured conjugate may become a powerful tool for the diagnosis and treatment of various bone diseases. The design principle validated in this case may also be beneficial in construction of other polymeric drug delivery systems.

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