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Efficiency of High Molecular Weight Backbone Degradable HPMA Copolymer – Prostaglandin E₁ Conjugate in Promotion of Bone Formation in Ovariectomized Rats

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Abstract

Multiblock, high molecular weight, linear, backbone degradable HPMA copolymer-prostaglandin E₁ (PGE₁) conjugate has been synthesized by RAFT polymerization mediated by a new bifunctional chain transfer agent (CTA), which contains an enzymatically degradable oligopeptide sequence flanked by two dithiobenzoate groups, followed by post-polymerization aminolysis and thiol-ene chain extension. The multiblock conjugate contains Asp₈ as the bone-targeting moiety and enzymatically degradable bonds in the polymer backbone; in vivo degradation produces cleavage products that are below the renal threshold. Using an ovariectomized (OVX) rat model, the accumulation in bone and efficacy to promote bone formation was evaluated; low molecular weight conjugates served as control. The results indicated a higher accumulation in bone, greater enhancement of bone density, and higher plasma osteocalcin levels for the backbone degradable conjugate.

Keywords

Biodegradation; multiblock copolymer; backbone degradable HPMA copolymer; bone targeting; anabolic agent; osteoporosis; ovariectomized rat model

1. Introduction

Water-soluble polymers have been widely used as drug carriers for several decades [1]. Well-known advantages of binding drugs to polymer carriers have been identified [2]: a) uptake by fluid-phase pinocytosis (non-targeted polymer-bound drug) or receptor-mediated endocytosis (targeted polymer-bound drug), b) increased *passive* accumulation of the drug at the tumor site by the enhanced permeability and retention (EPR) effect [3], c) increased

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active accumulation of the drug at the tumor site by targeting, d) decreased non-specific toxicity of the conjugated drugs, e) potential to overcome multidrug resistance, f) decreased immunogenicity of the targeting moiety, g) immunoprotecting and immunomobilizing activities, and h) modulation of cell signaling and apoptotic pathways.

Copolymers of *N*-(2-hydroxypropyl)methacrylamide (HPMA) have been extensively studied as drug carriers and the design rationale described [4-6]. In addition to anticancer drug conjugates, HPMA copolymer-based therapeutics have been developed for the treatment of musculoskeletal diseases [7-9]. Selective targeting to bone was achieved by attaching D-aspartic acid octapeptide (Asp₈) [10-12] or alendronate [11,13-17] as targeting moieties. Prostaglandin E₁ (PGE₁), a potent and well-established anabolic agent in bone [18], has been used as the anabolic agent for the promotion of bone formation. It was attached to the HPMA copolymer via a cathepsin K sensitive oligopeptide sequence, Gly-Gly-Pro-Nle, and a self-eliminating 4-aminobenzyl alcohol structure [19,20]. Such conjugates have a potential as therapeutics for the treatment of osteoporosis [21]. Indeed, Asp₈-targeted HPMA copolymer-PGE₁ conjugate given as a single injection resulted in greater indices of bone formation (than controls) in aged, ovariectomized (OVX) rats [12].

It is well established that high-molecular weight (long-circulating) polymer conjugates accumulate efficiently in solid tumor tissue due to the EPR effect. The higher the molecular weight of the conjugate, the higher the accumulation in tumor tissue with concomitant increase in therapeutic efficacy [3,22,23]. However, the renal threshold limits the molecular weight of the first generation of polymeric carriers to below ~50 kDa; this lowers the retention time of the conjugate in the circulation with concomitant decrease in pharmaceutical efficiency [24]. Yet, higher molecular weight drug carriers with a nondegradable backbone deposit and accumulate in various organs, impairing biocompatibility. To solve this dilemma, we designed second-generation HPMA copolymer-based nanomedicines based on high molecular weight HPMA copolymer - drug carriers containing enzymatically degradable bonds in the main chain (polymer backbone). These multiblock copolymers are synthesized by RAFT polymerization followed by click (alkyne-azide [25,26] and/or thiol-ene [27]) reactions. Recently, the advantage of backbone degradable HPMA copolymer-doxorubicin [28], paclitaxel [29], and gemcitabine [30] conjugates has been demonstrated on ovarian carcinoma animal models.

The aim of this study is to evaluate the potential of second generation of linear, multiblock, high molecular weight backbone degradable HPMA copolymer – PGE₁ conjugates in the treatment of musculoskeletal diseases. In particular, we employed the OVX rat model and evaluated the biodistribution and efficacy in promoting bone formation of long circulating backbone degradable Asp₈-targeted HPMA copolymer-PGE₁ conjugates. Low molecular weight Asp₈-targeted HPMA copolymer-PGE₁ conjugates and non-targeted HPMA copolymer served as controls.

2. Materials and methods

2.1 Materials

N-α-Fmoc protected amino acids, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and 2-Cl-trityl chloride resin (100-200 mesh, 1.27 mmol/g) were purchased from EMD Biosciences (San Diego, CA). Papain (EC 3.4.22.2, from papaya latex) and 2,2'-azobisisobutyronitrile (AIBN) were purchased from Sigma-Aldrich (St. Louis, MO). 1-hydroxybenzotriazole (HOBt) was purchased from AnaSpec (Fremont, CA). *N,N'*-diisopropylcarbodiimide (DIC), 2,2,2-trifluoroethanol (TFE), *N,N'*-diisopropylethylamine (DIPEA; 99%) was from Alfa Aesar (Ward Hill, MA), 4-(*N*-maleimidomethyl)cyclohexanecarboxylic acid *N*-hydroxysuccinimide ester (SMCC) was

from Soltec Ventures (Beverly, MA). All other reagents and solvents were from Sigma-Aldrich (St. Louis, MO). HPMA [31], 4-cyanopentanoic acid dithiobenzoate [32], *N*-methacryloylglycylglycyl-L-prolyl-L-norleucyl(4-aminobenzyl alcohol) prostaglandin E₁ ester (MA-GlyGlyProNle-4AB-PGE₁) [19], 3-(*N*-methacryloylglycylglycyl)thiazolidine-2-thione (MA-GlyGly-TT) [33], *N*-methacryloyltyrosinamide (MA-Tyr-NH₂) [34], and peptide2CTA (*N*^α,*N*^ε-bis(4-cyano-4-(phenylcarbonothioylthio)pentanoylglycylphenylalanylleucylglycyl)-lysine) [27] were prepared according to described procedures.

2.2 Methods

UV-vis spectra were measured on a Varian Cary 400 Bio UV-visible spectrophotometer. Mass spectra were measured on an FTMS mass spectrometer (LTQ-FT, ThermoElectron, Waltham, MA). ¹H-NMR spectra were recorded on a Mercury400 spectrometer using DMSO-d₆ as the solvent. HPLC profiles were measured on RP-HPLC (Agilent Technologies 1100 series, Zorbax C8 column 4.6×150 mm) with gradient elution from 2 to 90% of Buffer B within 30 min at flow rate of 1.0 mL/min (Buffer A: deionized water (DI H₂O) with 0.1% TFA, Buffer B: acetonitrile with 0.1% TFA). The molecular weight and polydispersity index (PDI) of polymers were measured on an ÄKTA FPLC (fast protein liquid chromatography) system (GE Healthcare, formerly Amersham) equipped with miniDAWN TREOS and OptilabEX detectors (Wyatt Technology, Santa Barbara, CA) using a Superose 6 or 12 HR10/30 column with acetate buffer/acetonitrile (70/30, pH 6.5) as the mobile phase and flow rate 0.4 mL/min. The multisegment copolymers were fractionated on the same FPLC system using XK50/100 preparative column. Acetate buffer/acetonitrile (70/30, pH 6.5) was used as the mobile phase. The flow rate was 2.5 mL/min.; fractions were collected every 20 min. The salt in the fractions was removed by dialysis. Narrow polydispersity polymer fractions were obtained after freeze-drying.

2.3 Synthesis of low molecular weight reactants

2.3.1 Synthesis of enzyme-sensitive maleimide linker, *N*^α,*N*^ε-bis((4-maleimidomethyl)cyclohexanecarbonyl-glycylphenylalanylleucylglycyl)-lysine (P9M2)—The maleimido linker containing an enzyme-sensitive peptide sequence was synthesized by solid phase peptide synthesis (SPPS) methodology and manual Fmoc/tBu strategy on 2-chlorotrityl chloride resin (Scheme 1). HBTU was used as the coupling agent and 20% piperidine in *N,N*-dimethylformamide (DMF) as the deprotection agent for Fmoc protected amino acids (Fmoc-AA-OH). Briefly, Fmoc protected amino acids, Fmoc-Lys(Fmoc)-OH, Fmoc-Gly-OH, Fmoc-Leu-OH, Fmoc-Phe-OH, and Fmoc-Gly-OH were coupled sequentially to the 2-Cl-trityl chloride resin beads (60 mg, 0.02 mmol loading). After deprotection, SMCC (3 times excess) was coupled to the terminal glycyl residue in DMF. The peptide was isolated following cleavage from resin by 30 % TFE in DCM for 2 h. Yield 20 mg (75%). ESI-MS: *m/z* = 1333.5 [*M* + *H*]⁺, 1355.6 [*M* + *Na*]⁺, 667.4 [*M* + 2*H*]²⁺.

2.3.2 Synthesis of aminohexanoylglycylprolylnorleucyl(ε-carboxyfluorescein)-lysyllocta-D-aspartic acid (NH₂HexGlyProNleLys(CF)-Asp₈, FAsp₈)—See Scheme 2 for structure. The fluorescein-aspartic acid octapeptide was synthesized by SPPS using HBTU as the coupling agent and 20% piperidine in DMF (for Fmoc protecting group) and 3% hydrazine in DMF (for Dde protecting group) as the deprotection agents. Briefly, Fmoc-D-Asp(tBu)-OH × 8, Dde-Lys(Fmoc)-OH, carboxyfluorescein, Fmoc-Nle-OH, Fmoc-Pro-OH, Fmoc-Gly-OH and Fmoc-Ahx-OH were coupled sequentially to the beads (0.3 g, 0.15 mmol loading). After deprotection, the peptide was cleaved from resin by TFA/TIS/H₂O (95/2.5/2.5) for 2 h. Yield 220 mg (81%). ESI-MS: *m/z* = 1806.6 [*M* + *H*]⁺.

2.4 Polymers for biodistribution study

2.4.1 Synthesis of low molecular weight Asp₈-targeted HPMA copolymer (P-Asp₈) and multiblock, backbone degradable HPMA copolymer (mP-Asp₈)—The copolymers were synthesized in several steps (Scheme 2). First, P-TT - an HPMA copolymer containing reactive thiazolidine-2-thione groups at side chain termini was synthesized. Reaction of P-TT with NH₂HexGlyProNleLys(CF)-Asp₈ produced P-Asp₈. Following chain extension the polymer conjugate was fractionated to produce a low molecular weight and a high molecular weight fraction.

2.4.1.1 P-TT: HPMA (300 mg, 2.09 mmol), MA-GG-TT (75 mg, 0.25 mmol, 6 mol%), MA-Tyr-NH₂ (11.2 mg, 0.05 mmol, 2 mol%), peptide2CTA (13.6 mg) and 2,2'-azobisisobutyronitrile (AIBN) (1 mg) were dissolved in 2 mL of methanol (0.3% of acetic acid), bubbled with N₂ for 30 min and polymerized at 50 °C for 36 h. The polymer was purified by dissolution-precipitation method in methanol-acetone and dried under reduced pressure at room temperature. Yield 230 mg.

2.4.1.2 Binding of Asp₈: The HPMA-TT copolymer (230 mg) was dissolved in 1 mL of DMF, and added to DMF solution of H₂N-R-Asp₈ (NH₂HexGlyProNleLys(CF)-Asp₈) (120 mg) in 1 mL of DMF. Then 120 µL of *N,N*-diisopropylethylamine (DIPEA) was added. The reaction mixture was stirred at room temperature overnight. After reaction, 100 µL of butylamine was added to the reaction system and stirred for additional 1 h. the conjugate was precipitated in ether, washed with ether then dried under reduced pressure, yield 280 mg.

2.4.1.3 Chain extension: Telechelic SH P-Asp₈ (390 mg) was dissolved in DMF (~2 mL); 0.5 mL of divinyl sulfone and a drop of DIPEA were added. The reaction mixture was stirred overnight, then purified by dissolution-precipitation method in methanol-acetone and dried under reduced pressure. Vinylsulfone end-modified telechelic P-Asp₈ (370 mg) and telechelic SH P-Asp₈ (370 mg) were dissolved in 4 mL of DMF. The reaction mixture was stirred at room temperature for 3 days. The multiblock conjugate was isolated by precipitation in acetone, washed with acetone and dried under reduced pressure. Yield 720 mg.

2.4.1.4 Fractionation: The multiblock copolymer was dissolved in water, filtered through a 0.22 µm filter, and fractionated on Superose 6 HR16/60 preparative column. Sodium acetate buffer (0.1 M, pH 6.5) with 30% of acetonitrile was used as the mobile phase. Fractions were collected, concentrated using an Amicon ultrafiltration cell with a 30 kDa MWCO membrane, and dialyzed (MWCO 6-8 kDa) against DI water at 4 °C for 20 h, then freeze-dried. The molecular weights and polydispersity of the fractions were measured using FPLC. The content of D-aspartic acid octapeptide was determined by amino acid analysis as well as UV-vis spectrophotometry using the fluorescein groups attached to D-aspartic acid octapeptide. High molecular weight (polymer #1, Mw 315 kD, PDI 1.26) and low molecular weight (polymer #2, Mw 40 kD, PDI 1.08) fractions were selected for biodistribution study (Scheme 2).

2.4.1.5 P (control): HPMA (280 mg, 1.96 mmol), MA-Tyr-NH₂ (10 mg, 0.04 mmol, 2 mol %), MA-FITC (10 mg, 0.02 mmol, 1 mol%), 4-cyanopentanoic acid dithiobenzoate (1.4 mg) and VA-044 (0.5 mg) were dissolved in methanol/H₂O (2/1, 2.4 mL). Polymerization mixture was bubbled with N₂ for 30 min, sealed and polymerized at 40 °C for 24 h, then the polymer was purified by dissolution-precipitation method in methanol-acetone and dried under reduced pressure at room temperature. Yield 198 mg (66%). Polymer #3, Mw 31.5 kDa.

2.5 Polymers containing PGE₁ for treatment

2.5.1 mP-Asp₈-PGE₁—The conjugate was synthesized in several steps. First, a PGE₁ containing copolymer precursor, P-TT-PGE₁, containing reactive TT groups was synthesized. Then NH₂HexGlyProNleLys(CF)-Asp₈ was attached, followed by chain extension with maleimide linker P9M2 and the polymer was fractionated to yield a high molecular weight conjugate with narrow molecular weight distribution. The procedure is briefly described below:

2.5.1.1 P-TT-PGE₁: HPMA (164 mg, 1.144 mmol), MA-GlyGlyProNle-4AB-PGE₁ (55 mg, 0.065 mmol, 5 mol%) and MA-GlyGly-TT (24 mg, 0.078 mmol, 6 mol%) were placed into a 5 mL ampoule with a stirring bar, and dissolved in DMF/methanol (1/1, with 1/200 of acetic acid, 1.2 mL). Then, peptide2CTA (2.1 mg, 1.5 μmol) and 2,2'-azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride (VA-044) (0.3 mg, 1.0 μmol) were added. The solution was bubbled with N₂ for 30 min, flame sealed and polymerized at 40 °C for 24 h. After polymerization, the copolymer was purified by precipitation into acetone, filtered, washed with acetone 3 times, and dried under reduced pressure at room temperature. Yield 136 mg (56%); The PGE₁ content was determined by UV spectroscopy using molar extinction coefficient 21,000 M⁻¹cm⁻¹ (λ_{max} 247 nm, methanol; background from polymer was subtracted – absorbancy of control polymer with aminolyzed TT groups) [19].

2.5.1.2 Binding of Asp₈: P-TT-PGE₁ (543 mg) and (NH₂HexGlyProNleLys(CF)-Asp₈ (134 mg) were dissolved in DMF (10 mL), and DIPEA (170 μL) was added under stirring. The reaction mixture was stirred for 6 h at room temperature. After reaction, hexylamine (100 μL) was added to the reaction mixture and stirring continued for 5 min. Then the product was precipitated into acetone, filtered, washed with acetone 3 times, and dried under reduced pressure. Yield 670 mg.

2.5.1.3 Chain extension: The product was re-dissolved in DMF, maleimide linker P9M2 (10 mg, 3% excess) was added to the reaction mixture and the reaction continued for 36 h. Following chain extension, the product was precipitated in acetone, washed with acetone 3 times and dried under reduced pressure at room temperature.

2.5.1.4 Fractionation: The copolymer was dissolved in water, filtered through a 0.22 μm filter, and the sample was fractionated on XK50/100 column. The isolation procedure was as described above. High molecular weight fraction was selected for the treatment study (polymer #4, Mw 329.0, PDI 1.39; Scheme 3).

2.5.2 P-Asp₈-PGE₁—First, P-TT-PGE₁ was synthesized by RAFT polymerization using HPMA (164 mg, 1.144 mmol), MA-GlyGlyProNle-4AB-PGE₁ (55 mg, 0.065 mmol), and MA-GlyGly-TT (24 mg, 0.078 mmol), 4-cyanopentanoic acid dithiobenzoate (2.1 mg, 7.5 μmol), and VA-044 (1.6 mg, 5.0 μmol) in DMF/methanol (1/1, with 1/200 of acetic acid, 1.2 mL). The polymerization solution was bubbled with N₂ for 30 min, flame sealed and polymerized at 40 °C for 24 h. After polymerization, the copolymer was purified by precipitation into acetone, filtered, washed with acetone 3 times, and dried under reduced pressure at room temperature. Yield 197 mg (81%), Mw 34.4 kDa, PDI 1.10. The CTA end groups were removed by reaction with 10 × of VA-044 in water at 40 °C for 2 h. P-TT-PGE₁ (197 mg) was reacted with NH₂HexGlyProNleLys(CF)-Asp₈ (53 mg) to introduce Asp₈ moieties. After removal of unreacted TT groups by aminolysis with hexylamine as described above, the polymer was purified by dissolution-precipitation in methanol-acetone 3 times, washed with acetone and dried under reduced pressure. Yield 230 mg (polymer #5, Mw 51.2, PDI 1.19, Scheme 4).

2.6 Enzyme-catalyzed degradation of multisegment polyHPMA

The degradation of multiblock polyHPMA obtained from thiol-ene reactions was performed in McIlvaine's buffer (50 mM citrate/0.1 M phosphate) at pH 6.0, 37 °C, using papain as the model enzyme. Papain (1 mg) was dissolved in 0.9 mL buffer containing 5 mM GSH and the mixture was pre-incubated for 5 min at 37 °C. Multiblock HPMA copolymer-PGE₁ conjugate mP-Asp₈-PGE₁ (329.0 kDa fraction, 3 mg) in 0.1 mL buffer was added and incubated at 37 °C overnight. The sample was analyzed by SEC (size exclusion chromatography) on a Superose 6 HR/10/30 column using the ÄKTA FPLC system equipped with miniDAWN TREOS and OptilabEX detectors.

2.7 Biodistribution study

The copolymers were radioiodinated with ¹²⁵I by a standard chloramine-T assay [30] and purified twice on a Sephadex PD-10 column. The specific activity of the hot samples was about 50 Ci/mg. The hot sample was diluted 200 times with cold sample (same conjugate without ¹²⁵I labeling). Animal experiments were performed according to the University of Utah IACUC approved protocols. ¹²⁵I-Labeled HPMA copolymer – D-Asp₈ conjugates, mP-Asp₈ and P-Asp₈, (2 mg/kg, 0.5 µCi/rat) in 200 µL 0.9% NaCl were administered intravenously (i.v.) to Sprague-Dawley rats (Charles River, 2.5 month old, ~165 g, 3 rats/group) via tail vein injection. Low molecular weight HPMA copolymer without targeting moiety (P) was used as control. Animals were sacrificed at designated time points, blood samples were withdrawn via the caudal vena cava. Major organs such as heart, lungs, kidneys, liver, spleen, and long bones (tibia and femur, ×2) were isolated, processed, and counted with a γ counter (Packard, Minaxi γ, Autogamma 5000 series, calibrated with ¹³⁷Cs, nominally 0.25 µCi).

2.8 Efficacy study in ovariectomized rats

Ovariectomized (OVX) Sprague-Dawley rats (Charles River; 7 month old; ~350 g) were ovariectomized 4 months before the study. The HPMA copolymer-PGE₁ conjugates, mP-Asp₈-PGE₁ and P-Asp₈-PGE₁, were administered i.v. on days 1, 8 and 15 (0.5 mg PGE₁ equivalent per dose per rat). Control group was administered saline. The OVX rats were euthanized on day 33. Femurs, tibias, 5th lumbar vertebrae, and mandibles were harvested.

Bone density (BMD) was measured on a pDEXA Sabre X-ray Bone Densitometer (Norland Medical Systems) and analyzed with the Sabre software (Version 3.9.2). Serum osteocalcin levels were measured using Rat Osteocalcin EIA Kit (Cat # BT-490 (Biomedical Technologies)).

2.9 Bone growth imaging

To image the bone growth the rats (~0 g, 5 rats/group) were given Alizarin Red S (100 mg/kg) (i.p.) to label bone formation surfaces [35] 4 days before treatment (day -4). Tetracycline.HCl (25 mg/kg), a bone formation marker, was administered i.p. on days 17 and 24 to label bone formation surfaces. Following euthanization on day 33, the bones were fixed with formalin and dehydrated in ethanol (70% to 100% in 1 week), and embedded undecalcified in poly(methyl methacrylate). Sections of the bone were cut with a bone saw, mounted on plastic slides, ground to about 30 µm in thickness and viewed under the confocal fluorescence microscope for the presence of the alizarin red S, tetracycline which marked bone formation surfaces and the FITC indicating the uptake of the conjugates.

Spectral Separation: Single optical sections were imaged using a Nikon AR1si confocal microscope and a 20x PLANAPO objective NA 0.75. FITC, Alizarin Red and tetracycline were discriminated using the spectral unmixing feature of the Nikon A1Rsi confocal. Images were obtained using the 32 PMT array detector and 405, 488 and 561 nm lines

simultaneously. Tetracycline, Alizarin Red and bone autofluorescence spectra were measured on control samples not containing FITC and these signals were unmixed from the experimental samples using the software preset FITC spectra. The signals from Alizarin Red, FITC and tetracycline were pseudocolored red, green and yellow respectively for visualization in the figure using Photoshop.

2.10 Statistical Analysis

The one-way ANOVA with post-hoc testing was used to determine the statistical significance of the differences in the organ deposition and bone growth among the biodegradable high molecular weight, traditional and control conjugates. $p < 0.05$ was considered statistically significant.

3. Results

3.1 Synthesis of polymer conjugates

The aim of the study was to compare the biological properties of the (second generation) multiblock, backbone degradable HPMA copolymer conjugates with the traditional conjugates (low molecular weight, non-degradable). Two types of conjugates have been used (Table 1, Schemes 2 - 4). For the biodistribution study we used multiblock (mP-Asp₈) and low molecular weight (P-Asp₈) HPMA copolymer-Asp₈ conjugates. Low molecular HPMA copolymer (P) served as control. For the evaluation of the potential to promote bone formation in an OVX rat model we used Asp₈-targeted HPMA copolymer – PGE₁ conjugates: multiblock, degradable conjugate, mP-Asp₈-PGE₁, and traditional, low molecular weight, nondegradable conjugate, P-Asp₈-PGE₁.

3.1.1 Conjugates for biodistribution study—The bone targeting biodegradable HPMA copolymer was synthesized first by RAFT polymerization of HPMA and MA-GlyGly-TT using peptide2CTA as the chain transfer agent to introduce an enzyme sensitive linker into the copolymer main chain and AIBN as the initiator. We tried to introduce D-aspartic acid octapeptide (Asp₈) by copolymerization of Asp₈ monomer with HPMA. Probably due to the large size of the Asp₈ monomer, when compared with HPMA, the copolymerization of HPMA with Asp₈ monomer was not efficient and resulted in low Asp₈ content. Consequently, instead of using Asp₈ containing monomer, we used monomer MA-GlyGly-TT that contained reactive groups and prepared a polymer precursor. Bone-targeting Asp₈ moieties were attached to the precursor via nucleophilic substitution of reactive TT groups with NH₂HexGlyProNleLys(CF)-Asp₈. The unreacted TT groups were aminolyzed with n-butylamine; simultaneously, the terminal chain transfer (dithiobenzoate) groups of the copolymer were also aminolyzed to form a telechelic copolymer with -SH groups at both chain ends. The -SH terminated telechelic conjugate was extended by reaction with divinyl sulfone and fractionated by SEC (Scheme 2). The conjugates are characterized in Table 1 and their FPLC profiles are shown in Figure 1.

3.1.2 PGE₁ conjugates for the treatment study—We synthesized a biodegradable maleimide linker and used it as a chain extender for the synthesis of multiblock degradable polymers. Previously, we used a dimaleimido compound where two maleimido groups were bridged by a short PEG spacer [27]. Here we replaced the PEG spacer with an enzyme sensitive GFLG spacer, to insert more degradable sites into the polymer main chain. The maleimide linker was synthesized by SPPS methodology by sequentially coupling Lys(Fmoc)-OH, Fmoc-Gly-OH, Fmoc-Leu-OH, Fmoc-Phe-OH and Fmoc-Gly-OH. The maleimido groups were introduced by reaction of SMCC with end amino groups of glycine. The linker was cleaved from beads under mild condition as shown in Scheme 1.

The bone targeted biodegradable HPMA copolymer – PGE₁ conjugate was synthesized by RAFT polymerization of HPMA, MA-GlyGly-TT and MA-GlyGlyProNle-4AB-PGE₁ using the enzyme sensitive peptide2CTA as the chain transfer agent. Low temperature free-radical initiator VA-044 was used as the initiator, so that the polymerization was processed at low temperature, which is favorable for the stability of PGE₁ and reactive TT groups. D-Aspartic acid octapeptide (Asp₈) groups were also introduced by reaction of the reactive TT containing copolymer precursor with NH₂HexGlyProNleLys(CF)-Asp₈ as described above. The unreacted TT groups were aminolyzed with n-hexylamine in 5 min; simultaneously the terminal dithiobenzoate groups of the copolymer were also aminolyzed into -SH groups to form a homotelechelic copolymer with thiol groups at both chain ends. During the short time aminolysis reaction PGE₁ remained stable. The -SH terminated telechelic conjugate was extended by a reaction with the enzyme sensitive maleimide linker and the product was fractionated by SEC (Scheme 3). The traditional bone targeting HPMA copolymer – PGE₁ conjugate was synthesized by RAFT polymerization of HPMA, MA-GlyGly-TT and MA-GlyGlyProNle-4AB-PGE₁ using 4-cyanopentanoic acid dithiobenzoate as the chain transfer agent. D-Aspartic acid octapeptide (Asp₈) groups were also introduced by reaction of the reactive TT containing copolymer precursor with NH₂HexGlyProNleLys(CF)-Asp₈ after the CTA end groups were replaced by reaction with 10 times excess of VA-044 (Scheme 4). The conjugates for the treatment study are characterized in Table 1 and their FPLC profiles are shown in Figure 1.

The biodegradability was tested by incubation of the multiblock conjugate mP-Asp₈-PGE₁ (329.0 kDa) with papain. The concentration of the conjugate was 3 mg/mL, papain 1 mg/mL. The cleavage was performed at 37 °C overnight. The cleavage product had a molecular weight of 40.2 kDa (Figure 1).

3.2 Biodistribution

The ¹²⁵I-labeled HPMA copolymer-D-Asp₈ conjugates, mP-Asp₈ (Mw 315.2 kDa, PDI 1.26) and P-Asp₈ (Mw 40.8 kDa, PDI 1.08) and non-targeted control polyHPMA, P (Mw 31.5 kDa, PDI 1.09) were administered intravenously to Sprague-Dawley rats (2.5 months old, 3 rats/group) and their distribution to different organs was measured by gamma counter following the isolation of the organs. The biodistribution of the bone targeted HPMA copolymer conjugates and of the control conjugate is shown in Figures 2 and 3. Bone targeting ability of the conjugates showed an order of: high molecular weight biodegradable conjugate > low molecular weight traditional conjugate > non-targeted conjugate (Figure 2). The low molecular weight P-Asp₈ conjugate showed a higher accumulation in kidney, whereas the high molecular weight mP-Asp₈ conjugate had a higher accumulation in liver and spleen (Figure 3).

The higher accumulation of the high molecular weight biodegradable conjugate in bone was consistent with the longer circulation time; this permitted extravasation of a larger amount of the conjugate through the discontinuous blood vessels of bone marrow.

3.3 Efficacy study in ovariectomized rats

Three groups of ovariectomized rats (n=5) were used with the aim to assess the potential of second generation of HPMA copolymer - PGE₁ conjugates in promoting bone growth: a) multiblock backbone degradable conjugate, mP-Asp₈-PGE₁; b) traditional (first generation) low molecular weight conjugate, P-Asp₈-PGE₁; c) untreated controls (saline). After three doses of 0.5 mg PGE₁ equivalent (days 1, 8, and 15) the rats were sacrificed on day 33 and the results analyzed.

3.3.1 Bone mineral density (BMD) analysis—The bone density was measured using pDEXA Sabre X-ray Bone Densitometer (Norland Medical Systems) combined with the Sabre software (Version 3.9.2). Resolution was 0.5×0.5 mm. The bone density of right femur, right tibia and lumbar vertebrae (LVB) before treatment (day minus 2) and after treatment (day 33) were measured to compare the increase of the bone density. The results are shown in Figure 4. Both low molecular weight conjugate and high molecular weight conjugate treatment groups produced higher increases in BMD than the control group. The increase in BMD following administration of the high molecular weight multiblock conjugate mP-Asp₈-PGE₁ was greater than that of the low molecular weight conjugate; the difference was statistically significant. The results indicate that an increase of the molecular weight enhances the treatment efficacy of the bone targeted HPMA copolymer – PGE₁ conjugate.

3.3.2 Serum osteocalcin concentration levels—Osteocalcin, a specific product of osteoblasts, is a noncollagenous protein with a molecular weight of 5.8 kDa. Serum osteocalcin can be used as a marker of osteoblast activity and the bone formation process. The serum osteocalcin concentration was measured on days 1, 15 and 33. The results are shown in Figure 5. On day 1 before the administration of the PGE₁ conjugates, all 3 groups of rats gave a similar serum osteocalcin level. On day 15, there was an increase in serum osteocalcin level for both PGE₁ conjugates treatment groups when compared to day 1. On day 33, 18 days after the last treatment, the serum osteocalcin level for control and low molecular weight PGE₁ conjugate (P-Asp₈-PGE₁) treatment groups gave a similar serum osteocalcin level as on day 1, but the high molecular weight PGE₁ conjugate (mP-Asp₈-PGE₁) treatment group gave a high serum osteocalcin level. The reason is probably that 18 days after the treatment, the low molecular weight PGE₁ conjugate (P-Asp₈-PGE₁) was partially cleared from the organism, but the high molecular weight PGE₁ conjugate, mP-Asp₈-PGE₁, having a longer residence time in the body and a higher bone accumulation, created a larger anabolic effect in bone formation.

3.3.3 Detection of bone formation using confocal fluorescence microscopy—Selected bone samples were fixed with formalin, dehydrated in ethanol and embedded undecalcified in poly(methyl methacrylate). Sections of bone were cut with a bone saw, ground to about 30 μ m in thickness and viewed under confocal fluorescence microscope. The separation of the Alizarin Red, FITC, and tetracycline signal was performed as described in Methods.

Figure 6 shows the bone growth of a typical sample, rat femur retrieved from a rat exposed to the (FITC-labeled) multiblock HPMA copolymer-Asp₈-PGE₁ conjugate (mP-Asp₈-PGE₁). Spectral separation (see 2.8) permitted to individually evaluate the images of the bone before experiment (Alizarin Red, Fig. 6A), accumulation of FITC labeled P-Asp₈-PGE₁ (Fig. 6B), combined image of FITC labeled P-Asp₈-PGE₁ (arrow) and tetracycline (Fig. 6C) and the image of tetracycline (arrow; Fig. 6D). The images seem to indicate that growth of bone occurred after each administration of P-Asp₈-PGE₁.

4. Discussion

HPMA copolymers have been studied extensively as carriers of biologically active compounds [1-6]. In addition to anticancer drug conjugates, HPMA copolymer-based macromolecular therapeutics for the treatment of musculoskeletal [7,8], infectious, and inflammatory diseases [9] have been evaluated.

Bone is one of the tissues with discontinuous blood vessels in bone marrow, which may result in extravasation of macromolecules (active EPR effect [3]). As we have demonstrated

previously with model (non-degradable) conjugates, higher molecular weight HPMA copolymer-D-Asp₈ conjugates showed enhanced accumulation to bone of mice due to prolonged half-life in circulation [10]. Here we tried to validate the hypothesis that higher accumulation of backbone degradable HPMA copolymer - PGE₁ conjugates will result in enhanced indices of bone formation. This hypothesis is supported by our previous data on the preferential accumulation of D-Asp₈ targeted conjugates to bone resorption surfaces [11], and on the enhanced antitumor activity of higher molecular weight linear backbone degradable multiblock HPMA copolymer conjugates with anticancer drugs doxorubicin [28], paclitaxel [29], and gemcitabine [30].

The biodistribution study revealed enhanced accumulation in kidneys for P-Asp₈ than HPMA copolymer (P; control). This may be the result of the high D-Asp₈ concentration in the polymer (P-Asp₈) and its low molecular weight; this results in the avoidance of recognition by the MPS (mononuclear phagocyte system) and preferential accumulation in the kidney. Lam et al. have shown that accumulation in the liver of PEG-oligocholeic acid micellar nanoparticles derivatized with different number of aspartic acids was dependent on charge (aspartic acid content). Highly negatively charged nanoparticles accumulated in the liver to a much higher extent than those with a slightly negative charge apparently due to phagocytosis by Kupffer cells [36]. In future work the amount of D-Asp₈ in our conjugates can be decreased without impairing bone recognition.

High molecular weight multiblock mP-Asp₈ showed enhanced accumulation in spleen and liver. The amount of radioactivity in kidney and spleen decreased from 12 h to 72 h, indicating the elimination of the conjugate from the organism. Accumulation of macromolecules in the liver depends on molecular weight, charge, and architecture. For example, PAMAM dendrimers (G5.0-OH and G6.0-OH) accumulated in the liver of mice to a larger extent than HPMA copolymers of similar molecular weight [37]. Increased molecular weight of polymer carriers may contribute to augmented capture by macrophages of the reticuloendothelial system [38]. In addition to size, the surface charge [39] and opsonization will have an impact on the fate of macromolecules [40,41]. Consequently, decreasing the Asp₈ content in the conjugates evaluated should be beneficial. The Asp₈ in the conjugates was about 30% wt.; for bone targeting 5-10% wt. of Asp₈ should be sufficient. However, the high accumulation in liver and spleen will reduce the accumulation of the conjugates in other organs. On the other hand, PGE₁ will be metabolized in the liver resulting in further reduction of adverse effects.

The bone mineral density and the osteocalcin concentration levels provide information on bone formation activity. The bone mineral density can be directly used to compare the same bone before and after the treatment. The use of multiblock, biodegradable high molecular weight HPMA copolymer carrier resulted in enhancement of mP-Asp₈-PGE₁ bone accumulation (Figure 2) and higher bone mineral density increase (Figure 4). The higher accumulation of the high molecular weight conjugate is in agreement with results on the accumulation of non-degradable HPMA copolymer carriers of 96 kDa molecular weight [10]. At the end of the experiment (day 33) the osteocalcin concentration level was significantly higher in rats treated with multiblock mP-Asp₈-PGE₁ (Figure 5). This indicates higher osteoblast activity and bone formation process in agreement with results on bone mineral density determination. Thus it appears that we validated the hypothesis; the new design of backbone degradable multiblock, high molecular weight Asp₈-targeted HPMA copolymer PGE₁ conjugates results in the enhancement of bone accumulation with concomitant increase in efficacy.

PGE₁ is an effective stimulator of bone formation that is rapidly metabolized following systemic administration [18]. Attachment to a polymeric carrier enhances PGE₁ stability. In

addition, unlike the free PGE₁, PGE₁ bound to the polymer carrier would not trigger the activation of the PGE receptors, because the C-1 carboxyl group (needed as an attachment point) has been shown to play an important role in PGE₁ binding to receptors [42]. Consequently, PGE₁ has to be released from the polymer carrier to become biologically active. We attached PGE₁ to HPMA copolymer via a cathepsin K sensitive sequence (Gly-Gly-Pro-Nle) and a 1,6 self-eliminating 4-aminobenzyl alcohol unit. Previously, it was shown that cathepsin K releases unmodified PGE₁ from such a conjugate [19]. The bone formation activity of first generation (low molecular weight) HPMA copolymer-PGE₁ conjugates in an OVX rat model was demonstrated previously [12]. Here we have demonstrated that the new carrier design, backbone degradable HPMA copolymer is a novel platform for the synthesis of more efficient polymer – anticancer drug [28-30] and polymer – anabolic agent conjugates.

The design of conjugates may be further optimized and conjugates with a lower content of Asp₈ and molecular weights of 100 – 200 kDa evaluated. In accordance with our previous results in bone targeting [14] and in the treatment of experimental ovarian cancer [28-30] such conjugates may possess high efficacy and permit manipulation of organ distribution. A detailed evaluation of the relationship between the molecular weight of biodegradable HPMA copolymer conjugates and their efficacy in the treatment of musculoskeletal diseases is warranted.

5. Conclusions

High molecular weight, multiblock, backbone degradable Asp₈-bone targeted HPMA copolymer – PGE₁ conjugate was synthesized by combination of RAFT polymerization mediated by a new bifunctional CTA that contains an enzymatically degradable oligopeptide sequence and thiol-ene extension reaction with a degradable maleimide linker. These polymer carriers are enzymatically degradable and the degradation products have molecular weight distributions below the renal threshold. When compared to traditional (low molecular weight) HPMA copolymer conjugate, the multiblock PGE₁ conjugate showed higher accumulation in bone of OVX rats and a higher efficacy in promoting bone formation in an osteoporosis rat model.

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References

- [1]. Kopeček J. Soluble biomedical polymers. *Polim Med.* 1977; 7:191–221. [PubMed: 593972]
- [2]. Kopeček J, Kopecková P. HPMA copolymers: Origins, early developments, present, and future. *Adv Drug Deliv Rev.* 2010; 62:122–149. [PubMed: 19919846]
- [3]. Maeda H. Tumor-selective delivery of macromolecular drugs via the EPR effect: background and future prospects. *Bioconjug Chem.* 2010; 21:797–802. [PubMed: 20397686]
- [4]. Kopeček J, Kopecková P. Design of polymer-drug conjugates. In: Kratz, F.; Senter, P.; Steinhagen, H., editors. *Drug delivery in oncology*. Vol. 2. Wiley-VCH; Weinheim: 2012. p. 485-512.
- [5]. Kopeček J. Polymer – drug conjugates: Origins, progress to date and future directions. *Adv Drug Deliv Rev.* 2013; 65:49–59. [PubMed: 23123294]
- [6]. Zhou Y, Kopeček J. Biological rationale for the design of polymeric anti-cancer nanomedicines. *J Drug Target.* 2013; 21:1–26. [PubMed: 23009337]

- [7]. Wang D, Miller SC, Kopecká P, Kopeček J. Bone-targeting macromolecular therapeutics. *Adv Drug Deliv Rev.* 2005; 57:1049–1076. [PubMed: 15876403]
- [8]. Low SA, Kopeček J. Targeting polymer therapeutics to bone. *Adv Drug Deliv Rev.* 2012; 64:1189–1204. [PubMed: 22316530]
- [9]. Yuan F, Quan LD, Cui L, Goldring SR, Wang D. Development of macromolecular prodrug for rheumatoid arthritis. *Adv Drug Deliv Rev.* 2012; 64:1205–1219. [PubMed: 22433784]
- [10]. Wang D, Sima M, Mosley RL, Davda JP, Tietze N, Miller SC, et al. Pharmacokinetic and biodistribution studies of a bone-targeting drug delivery system based on *N*-(2-hydroxypropyl)methacrylamide copolymers. *Mol Pharm.* 2006; 3:717–725. [PubMed: 17140259]
- [11]. Wang D, Miller SC, Shlyakhtenko LS, Portillo AM, Liu XM, Papangkorn K, et al. Osteotropic peptide that differentiates functional domains of the skeleton. *Bioconj Chem.* 2007; 18:1375–1378. [PubMed: 17705416]
- [12]. Miller SC, Pan H, Wang D, Bowman BM, Kopecká P, Kopeček J. Feasibility of using a bone-targeted, macromolecular delivery system coupled with prostaglandin E₁ to promote bone formation in aged, estrogen-deficient rats. *Pharm Res.* 2008; 25:2889–2895. [PubMed: 18758923]
- [13]. Hrubý M, Etrych T, Kučka J, Forstrová M, Ulbrich K. Hydroxybisphosphonate-containing polymeric drug-delivery systems designed for targeting into bone tissue. *J Appl Polym Sci.* 2006; 101:3192–3201.
- [14]. Pan H, Sima M, Kopecká P, Wu K, Gao S, Liu J, et al. Biodistribution and pharmacokinetic studies of bone-targeting *N*-(2-hydroxypropyl)methacrylamide copolymer-alendronate conjugates. *Mol Pharm.* 2008; 5:548–558. [PubMed: 18505266]
- [15]. Segal E, Pan H, Benayoun L, Kopecká P, Shaked Y, Kopeček J, et al. Enhanced anti-tumor activity and safety profile of targeted nano-scaled HPMA copolymer-alendronate-TNP-470 conjugate in the treatment of bone malignancies. *Biomaterials.* 2011; 32:4450–4463. [PubMed: 21429572]
- [16]. Miller K, Eldar-Boock A, Polyak D, Segal E, Benayoun L, et al. Antiangiogenic activity of HPMA copolymer – paclitaxel – alendronate conjugate on breast cancer bone metastasis mouse model. *Mol Pharm.* 2011; 8:1052–1062. [PubMed: 21545170]
- [17]. Miller K, Clementi C, Eldar-Boock A, Benayoun L, Barshack I, Shaked Y, et al. Poly(ethylene glycol)-paclitaxel-alendronate self-assembled micelles for the targeted treatment of breast cancer bone metastases. *Biomaterials.* 2013; 34:3795–3806. [PubMed: 23434349]
- [18]. Vrotsos Y, Miller SC, Marks SC Jr. Prostaglandin E – a powerful anabolic agent for generalized or site-specific bone formation. *Crit Rev Eukaryot Gene Expr.* 2003; 13:255–263. [PubMed: 14696972]
- [19]. Pan H, Kopecká P, Wang D, Yang J, Miller S, Kopeček J. Water-soluble HPMA copolymer – prostaglandin conjugates containing a cathepsin K sensitive spacer. *J Drug Target.* 2006; 14:425–435. [PubMed: 17092842]
- [20]. Pan H, Liu J, Dong Y, Sima M, Kopecká P, Brandi ML, et al. Release of prostaglandin E₁ from *N*-(2-hydroxypropyl)methacrylamide copolymer conjugates by bone cells. *Macromol Biosci.* 2008; 8:599–605. [PubMed: 18401866]
- [21]. Raisz LG. Pathogenesis of osteoporosis: concepts, conflicts, and prospects. *J Clin Inv.* 2005; 115:3318–3325.
- [22]. Seymour WL, Duncan R, Strohalm J, Kopeček J. Effect of molecular weight of *N*-(2-hydroxypropyl)methacrylamide copolymers on body distribution and rate of excretion after subcutaneous, intraperitoneal and intravenous administration to rats. *J Biomed Mater Res.* 1987; 21:1341–1358. [PubMed: 3680316]
- [23]. Shiah J, Dvořák M, Kopecká P, Sun Y, Peterson CM, Kopeček J. Biodistribution and antitumor efficacy of long-circulating *N*-(2-hydroxypropyl)methacrylamide copolymer-doxorubicin conjugates in nude mice. *Eur J Cancer.* 2001; 37:131–139. [PubMed: 11165140]
- [24]. Lammers T, Kühnlein R, Kissel M, Subr V, Etrych T, Pola R, et al. Effect of physicochemical modification on the biodistribution and tumor accumulation of HPMA copolymers. *J Control Release.* 2005; 110:103–118. [PubMed: 16274831]

- [25]. Yang J, Luo K, Pan H, Kopecká P, Kopeček J. Synthesis of biodegradable multiblock copolymers by click coupling of RAFT-generated heterotelechelic polyHPMA conjugates. *React Funct Polym.* 2011; 71:294–302. [PubMed: 21499527]
- [26]. Luo K, Yang J, Kopecká P, Kopeček J. Biodegradable multiblock *N*-(2-hydroxypropyl)methacrylamide copolymers via reversible addition-fragmentation chain transfer polymerization and click chemistry. *Macromolecules.* 2011; 44:2481–2488. [PubMed: 21552355]
- [27]. Pan H, Yang J, Kopecká P, Kopeček J. Backbone degradable multiblock *N*-(2-hydroxypropyl)methacrylamide copolymer conjugates via reversible addition fragmentation chain transfer polymerization and thiol-ene coupling reaction. *Biomacromolecules.* 2011; 12:247–252. [PubMed: 21158387]
- [28]. Pan H, Sima M, Yang J, Kopeček J. Synthesis of long-circulating backbone degradable HPMA copolymer-doxorubicin conjugates and evaluation of molecular weight dependent antitumor efficacy. *Macromol Biosci.* 2013; 13:155–160. [PubMed: 23339052]
- [29]. Zhang R, Luo K, Yang J, Sima M, Sun Y, Janát-Amsbury MM, et al. Synthesis and evaluation of a backbone biodegradable multiblock HPMA copolymer nanocarrier for the systemic delivery of paclitaxel. *J Control Release.* 2013; 166:66–74. [PubMed: 23262201]
- [30]. Yang, J.; Kopeček, J. Recent development and preclinical evaluation of HPMA copolymer-drug conjugates as anticancer therapeutics; Proceedings, 40th Annual Meeting & Exposition of the Controlled Release Society; Honolulu, Hawaii, USA. July 21–24, 2013;
- [31]. Kopeček J, Bazilová H. Poly[*N*-(2-Hydroxypropyl)methacrylamide]. 1. Radical Polymerization and Copolymerization. *Eur Polym J.* 1973; 9:7–14.
- [32]. Mitsukami Y, Donovan MS, Lowe AB, McCormick CL. Water-soluble polymers. 81. Direct synthesis of hydrophilic styrenic-based homopolymers and block copolymers in aqueous solution via RAFT. *Macromolecules.* 2001; 34:2248–2256.
- [33]. Subr V, Ulbrich K. Synthesis and properties of new *N*-(2-hydroxypropyl)methacrylamide copolymers containing thiazolidine-2-thione reactive groups. *React Funct Polym.* 2006; 66:1525–1538.
- [34]. Lee JH, Kopecká P, Kopeček J, Andrade JD. Surface properties of copolymers of alkyl methacrylates with methoxy (polyethylene oxide) methacrylates. *Biomaterials.* 1990; 11:455–464. [PubMed: 2242394]
- [35]. Vilman H. The in vivo staining of bone with alizarin red S. *J Anat.* 1969; 105:533–545. [PubMed: 4187091]
- [36]. Xiao K, Li Y, Luo J, Lee JS, Xiao W, Gonik AM, et al. The effect of surface charge on in vivo biodistribution of PEG-oligocholeic acid based micellar nanoparticles. *Biomaterials.* 2011; 32:3435–3446. [PubMed: 21295849]
- [37]. Sadekar S, Ray A, Janát-Amsbury M, Peterson C, Ghandehari H. Comparative biodistribution of PAMAM dendrimers and HPMA copolymers in ovarian-tumor-bearing mice. *Biomacromolecules.* 2011; 12:88–96. [PubMed: 21128624]
- [38]. Duncan R, Pratten MK, Cable HC, Ringsdorf H, Lloyd JB. Effect of molecular size of 125I-labelled poly(vinylpyrrolidone) on its pinocytosis by rat visceral yolk sac and rat peritoneal macrophages. *Biochim J.* 1980; 196:49–55.
- [39]. Liu J, Bauer H, Callahan J, Kopecká P, Pan H. Endocytic uptake of a large array of HPMA copolymer: Elucidation into the dependence on the physiological characteristics. *J Control Release.* 2010; 143:71–79. [PubMed: 20043962]
- [40]. Kamei S, Kopeček J. Prolonged blood circulation in rats of nanospheres surface-modified with semitelechelic poly[*N*-(2-hydroxypropyl)methacrylamide]. *Pharm Res.* 1995; 12:663–668. [PubMed: 7479550]
- [41]. Bertrand N, Leroux JC. The journey of a drug-carrier in the body: An anatomo-physiological perspective. *J Control Release.* 2012; 161:152–163. [PubMed: 22001607]
- [42]. Ungrin MD, Carriere MC, Denis D, Lamontagne S, Sawyer N, Stocco R, et al. Key structural features of prostaglandin E(2) and prostanoid analogs involved in binding and activation of the human EP(1) prostanoid receptor. *Mol Pharmacol.* 2001; 59:1446–1456. [PubMed: 11353805]

Figure 1.

FPLC profiles of bone targeted HPMA copolymer-PGE₁ conjugates. mP-Asp₈, high molecular weight conjugate for biodistribution study; P-Asp₈, low molecular weight conjugate for biodistribution study; P, control conjugate for biodistribution study; mP-Asp₈-PGE₁, high molecular weight conjugate for treatment; P-Asp₈-PGE₁, low molecular weight conjugate for treatment; Degraded, degradation product after exposure of mP-Asp₈-PGE₁ to papain.

Figure 2.

Accumulation of bone targeted HPMA copolymer conjugates in bone of Sprague-Dawley rats at different time points. Left columns – P (control); middle columns – P-Asp₈; right columns – mP-Asp₈. * $P < 0.05$ for mP-Asp₈ group compared to P-Asp₈ and control P groups. ** $P < 0.05$ for P-Asp₈ group compared to control P group. $n = 3$ per group. Data are means $\pm$ s.d.

Figure 3. Biodistribution of bone targeted HPMA copolymer conjugates in various organs of Sprague-Dawley rats at different time points. Left columns – P (control); middle columns – P-Asp8; right columns – mP-Asp8. Data are means μ s.d.

Figure 4.

Percentage of bone mineral density increase in OVX Sprague-Dawley rats following administration of Asp₈-targeted HPMA copolymer-PGE₁ conjugates. The BMD was measured on day -2 and day 33. Left columns – untreated controls (saline); middle columns – P-Asp₈-PGE₁; right columns - mP-Asp₈-PGE₁. * $P < 0.05$ for mP-Asp₈-PGE₁ group compared to control. ** $P < 0.05$ for mP-Asp₈-PGE₁ group compared to P-Asp₈-PGE₁ and control. $n = 5$ per group. Data are means $\pm$ s.d.

Figure 5.

Serum osteocalcin concentration levels in Sprague-Dawley OVX rats on day 1 before administration of the first dose of Asp₈-targeted HPMA copolymer-PGE₁ conjugates, on day 15 before administration of the third dose, and on day 33. Left columns – untreated controls (saline); middle columns - P-Asp₈-PGE₁; right columns - mP-Asp₈-PGE₁. * $P < 0.05$ for mP-Asp₈-PGE₁ group compared to P-Asp₈-PGE₁ and control groups. $n = 5$ per group. Data are means $\pm$ s.d.

Figure 6.

Detection of bone growth in retrieved bones of OVX Sprague-Dawley rats by confocal fluorescence microscopy. The rats received Alizarin Red on day -4; mP-Asp₈-PGE₁ was administered 3-times, on days 1, 8, and 15, and the bone growth marker, tetracycline on days 17 and 24. Images: (A) Complete image with Alizarin Red, green mP-Asp₈-PGE₁ and yellow tetracycline label. The red line is the surface of femur marked with Alizarin Red 4 days before the experiment. Two yellow images appear to be tetracycline bone growth markers administered on days 17 and 24. Inset shows also the green fluorescence of FITC associated with the polymer conjugate. (B) This image shows the FITC fluorescence of mP-Asp₈-PGE₁ and demonstrates the incorporation of the polymer into the bone after each of the administrations on days 1, 8, and 15. (C) Comparison of green FITC fluorescence of mP-Asp₈-PGE₁ (arrow) and yellow tetracycline fluorescence (double arrow). (D) Two lines of tetracycline fluorescence (arrow). The line marked with double arrow is the bleed-over of Alizarin Red.

Scheme 1.
 Synthesis of enzyme-sensitive maleimide linker P9M2.

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Scheme 3.

Synthesis of bone targeted biodegradable HPMA copolymer – PGE₁ conjugate.

Scheme 4.

Synthesis of traditional bone targeted HPMA copolymer – PGE₁ conjugate.

Table 1

Characterization of bone-targeted HPMA copolymer conjugates

Polymer #	Structure	Mw (kDa)	PDI	Asp ₈ (wt%)	PGE ₁ (wt%)
1	mP-Asp ₈	315.2	1.26	36.6	-
2	P-Asp ₈	40.8	1.08	29.1	-
3	P	31.5	1.09	-	-
4	mP-Asp ₈ -PGE ₁	329.0	1.39	27.2	5.05
5	P-Asp ₈ -PGE ₁	51.2	1.19	27.2	4.96