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Efficiency of high molecular weight backbone degradable HPMA copolymer–Prostaglandin E_1 conjugate in promotion of bone formation in ovariectomized rats

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ABSTRACT

Multiblock, high molecular weight, linear, backbone degradable HPMA copolymer-prostaglandin E_1 (PGE_1) 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 postpolymerization aminolysis and thiol-ene chain extension. The multiblock conjugate contains Asp_8 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.

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 *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 (Asp_8) [10–12] or alendronate [11,13–17] as targeting moieties. Prostaglandin E_1 (PGE_1), 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, Asp_8-targeted HPMA copolymer-PGE_1 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

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retention time of the conjugate in the circulation with concomitant decrease in pharmaceutical efficiency [24]. Yet, higher molecular weight drug carriers with a non-degradable 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–PGE_1 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 Asp_8-targeted HPMA copolymer-PGE_1 conjugates. Low molecular weight Asp_8-targeted HPMA copolymer-PGE_1 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 E_1 ester (MA-GlyGlyProNle-4AB-PGE_1) [19], 3-(*N*-methacryloylglycylglycyl) thiazolidine-2-thione (MA-GlyGly-TT) [33], *N*-methacryloyltyrosinamide (MA-Tyr-NH_2) [34], and peptide2CTA(N^{α},N^{ε}-bis(4-cyano-4-(phenylcarbonothioylthio)pentanoylglycylphenyl-alanylleucylglycyl)-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). ^{1}H NMR spectra were recorded on a Mercury400 spectrometer using DMSO-d_6 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 H_2O) 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 Optilab rEX 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 XK 50/100 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.

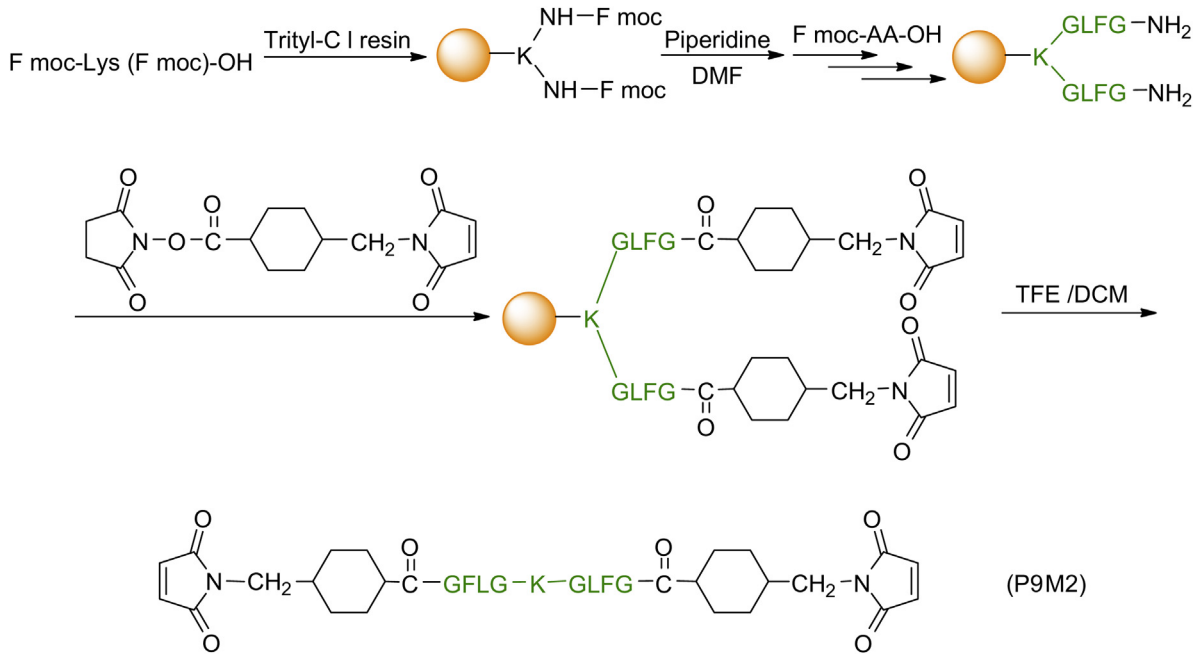
2.3. Synthesis of low molecular weight reactants

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 $[M + 2H]^{2+}$.

2.3.2. Synthesis of aminohexanoylglycylprolylnorleucyl(ε-carboxyfluorescein)-lysylocta-D-aspartic acid (NH_2HexGlyProNleLys(CF)-Asp_8, FAsp_8)

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/H_2O (95/2.5/2.5) for 2 h. Yield 220 mg (81%). ESI-MS: m/z = 1806.6 $[M + H]^+$.



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