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Cationic and thermosensitive protamine conjugated gels for enhancing sustained human growth hormone delivery

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ABSTRACT

Thermosensitive and cationic poly(organophosphazenes) were designed and synthesized for the sustained delivery of human growth hormone (hGH) charged negatively at the physiological conditions to enhance greatly patient convenience and to improve efficacy and stability. Protamine for a complex formation with hGH was chosen and conjugated to carboxylic acid-terminated poly(organophosphazenes) by a covalent amide linkage. The aqueous solution of the cationic polymer conjugates formed a gel at 37 °C regardless of hGH presence. When the conjugate solution was mixed with hGH solution, a complex was formed and free hGH could be released from the complex. In the *in vitro* and *in vivo* release studies of hGH/polymer-protamine conjugate, the initial burst release was suppressed and the release period was prolonged as the protamine amount was increased. In the PK and PD studies with cynomolgus monkeys, a single administration of hGH/cationic polymer conjugate induced the elevated plasma level of hGH until 5 days and also elevated plasma level of IGF-1 as a function of free hGH until 13 days. These results suggest that the injectable, thermosensitive, and cationic poly(organophosphazene)-protamine conjugate may hold a great potential as an effective carrier for sustained release of hGH with improved patient convenience, stability and efficacy.

1. Introduction

Human growth hormone (hGH) is a single chain polypeptide hormone consisted of 191 amino acids that stimulate growth and cell reproduction. It is used clinically to treat short stature caused by growth hormone deficiency and also used to other diseases which are not related with hGH deficiency such as Turner syndrome and growth failure due to chronic renal failure [1]. It is one of the protein therapeutics which is in great request [2]. Likewise other protein drugs, however, it has to be administered subcutaneously by daily injection due to its short plasma half-life, and results in poor patient compliance. Furthermore, each protein drug has its own unique characteristic properties for its functionality, stability and efficacy. Therefore, protein drugs need their own formulation and delivery manners.

Sustained protein release technology can improve greatly not only patient convenience, but also stability and efficacy of target proteins. Although hGH is secreted in a pulsatile manner, continuous infusion of hGH via a pump has resulted in the elevated insulin-like growth factor-1 (IGF-1) levels, which is comparable with daily injection, indicating that the pulsatile hGH release may not be required for clinical efficacy [3,4]. For sustained hGH delivery various strategies including fusion of stabilizing peptide [5], crystal formulation [6], encapsulation to microsphere and microparticle [1,7,8], and loading to hydrogel [9,10] have been proposed.

The most investigated method is an encapsulation of hGH to poly(lactic acid-co-glycolic acid) (PLGA) microsphere. PLGA is a biodegradable polymer and approved in market for sustained release formulation of peptides such as luteinizing hormone-releasing hormone (LHRH) analogues [11,12]. However, developing sustained release formulation of proteins including hGH has been hampered due to their complex structure which is related to protein activity. In this system, protein aggregation can be occurred by a formulation and an adsorption onto the microsphere surface as well as an initial burst release by the rapid dissolution of non-aggregated water soluble protein [13]. Furthermore, it has been reported that this system can cause protein denaturation and inflammation by hydrophobic interactions and/or acidic degradation products [8,14,15].

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Hydrogel is a three dimensional hydrophilic polymer network which can absorb large amount of water or biological fluids [16]. Because of its compatibility with protein and living tissue which are attributed to high water contents and soft consistency, it can be an alternative strategy instead of PLGA microsphere for sustained hGH delivery. Especially, thermosensitive and injectable hydrogel has been attended since it can provide patient convenience by an easy administration to the body through a simple injection without surgery [17]. In addition, its thermosensitive gelation without toxic chemical cross linkers makes it more biocompatible. Various therapeutic drugs with the thermogelation property can be formulated by a simple mixing without loss of drug [18] to improve the loading capacity. However, the release of protein drug from the hydrogel is generally diffusion-controlled, and the sustained release over a long time period may not be expected when the protein is physically entrapped in the gel due to the hydrophilicity and the small hydrodynamic sizes of the protein drugs [19].

In this study, we have designed and synthesized a thermosensitive and cationic poly(organophosphazene) hydrogel for sustained delivery of hGH. Poly(organophosphazene) hydrogel bearing of hydrophobic amino acid and poly(ethylene glycol) exhibits a sol–gel transition behavior by temperature [20,21]. It is known that the degraded products are non-toxic and biocompatible [22,23]. The gelation property can be controlled by the substituents introduced to the polymer backbone. To hold negatively charged hGH by an electrostatic interaction within the hydrogel, we conjugated protamine as a cationic moiety. Protamine is a FDA approved small basic protein and used as heparin antagonist and long-acting formulation of insulin (NPH insulin) [24]. The protamine was introduced to carboxylic acid terminated poly(organophosphazene) bearing hydrophobic L-isoleucine ethyl ester (IleOEt), hydrophilic α-amino-ω-methoxy-poly-(ethylene glycol) with molecular weight of 550 Da (AMPEG550), and carboxylic acid terminated glycylglycine. After the synthesis, the physicochemical properties, ionic complexation with hGH, and release profile *in vitro* and *in vivo* were examined and the *in vivo* activity of released hGH was also monitored by measuring the insulin-like growth factor-1 (IGF-1) level.

2. Materials and methods

2.1. Materials

Hexachlorocyclotriphosphazene was acquired from Aldrich and purified by sublimation at 55 °C under vacuum (about 0.1 mmHg). α-Amino-ω-methoxy-PEG (AMPEG) was prepared by a published method [25]. The ethyl esters of isoleucine (IleOEt) were prepared according to the literature [26]. Glycylglycine allyl ester (GlyGlyOAll) was synthesized as described previously [27]. Ethyl-2-(*O*-glycyl)lactate (GlyLacOEt) was prepared as described by Crommen et al. [28]. Tetrahydrofuran (THF) was dried by reflux over sodium metal and distilled, and triethylamine (TEA) was distilled over BaO under dry nitrogen. Protamine sulfate was obtained from Sigma–Aldrich (St. Louis, MO, USA). Recombinant human growth hormone (hGH) was provided from Chong Ken Dang Pharmaceutical Corp. (Seoul, Korea). Zinc acetate was purchased from Sigma–Aldrich (St. Louis, MO, USA). All other reagents were purchased from commercial suppliers and used as received. All animal experiments were approved by Animal Care Ethnic Committee (ACEC) of Korea Institute of Science and Technology (KIST).

2.2. Synthesis of protamine conjugated poly(organophosphazenes)

All reactions were carried over an atmosphere of dry nitrogen by using standard Schlenk-line techniques. Protamine conjugated poly(organophosphazene)s were synthesized by according to the procedure of Scheme 1, which was a modified version of a protocol described in a previous report [27]. Briefly, poly(dichlorophosphazene) (I) dissolved in THF reacted stepwise with IleOEt, GlyGlyOAll, and AMPEG550 to produce polymer (II). The carboxylic acid-terminated polymer (III) was prepared by the allyl ester cleavage reaction of polymer (II) using 0.2 equivalent of tetrakis(triphenylphosphine)palladium(0) and 20 equivalent of morpholine. The protamine conjugated poly(organophosphazene) (IV) was then obtained an 80–90% yield by reacting with activated carboxylic acid-terminated polymer (III) by isobutyl chloroformate (IBCF) and TEA to protamine solution at 0 °C-RT.

2.2.1. $[NP(IleOEt)_{1.18}(GlyGlyOH)_{0.23}(AMPEG550)_{0.59}]_n$ (polymer **1**)

Poly(dichlorophosphazene) (2.00 g, 17.26 mmol), IleOEt (3.99 g, 20.36 mmol), GlyGlyOAll (1.48 g, 5.18 mmol), AMPEG 550 (9.87 g, 17.95 mmol), $(Ph_3P)_4Pd(0)$ (0.74 g, 0.64 mmol), and morpholine (5.61 g, 64.39 mmol) were used. Yield: 68%. ^{1}H NMR ($CDCl_3$), δ (ppm): 0.8–1.0 (s, 6H), 1.1–1.3 (b, 3H), 1.3–1.6 (b, 2H), 1.6–1.9 (b, 1H), 3.2 (s, 2H), 3.3 (s, 3H), 3.4–3.8 (b, 44H), 3.9 (s, 2H), 4.0–4.1 (b, 1H), 4.1–4.3 (b, 2H).

2.2.2. $[NP(IleOEt)_{1.16}(GlyGlyOH)_{0.17}(AMPEG550)_{0.67}]_n$ (polymer **2**)

Poly(dichlorophosphazene) (4.00 g, 34.52 mmol), IleOEt (8.04 g, 41.07 mmol), GlyGlyOAll (2.96 g, 10.35 mmol), AMPEG 550 (19.36 g, 35.21 mmol), $(Ph_3P)_4Pd(0)$ (1.44 g, 1.25 mmol), and morpholine (10.85 g, 124.58 mmol) were used. Yield: 69%. ^{1}H NMR ($CDCl_3$), δ (ppm): 0.8–1.0 (s, 6H), 1.1–1.3 (b, 3H), 1.3–1.6 (b, 2H), 1.6–1.9 (b, 1H), 3.2 (s, 2H), 3.3 (s, 3H), 3.4–3.8 (b, 44H), 3.9 (s, 2H), 4.0–4.1 (b, 1H), 4.1–4.3 (b, 2H).

2.2.3. $NP(IleOEt)_{1.18}(GlyGlyOH)_{0.17}(GlyGlyPro)_{0.06}(AMPEG550)_{0.59}]_n$ (conjugate **1**)

Polymer **1** (2.50 g, 4.26 mmol) was dissolved in 50 ml of distilled THF. After cooling at 0 °C, TEA (0.27 ml, 1.96 mmol) and IBCF (0.13 ml, 0.98 mmol) were added and stirring for 40 min in order to activate carboxyl groups of polymer. The mixture was transferred to protamine (10.00 g, 1.96 mmol) dissolved in 0.01 M HEPES, pH 7.5 contained TEA (0.55 ml, 2.75 mmol). The reaction mixture was stirred at 4 °C for 18 h and then at room temperature for 6 h. After reaction, the solution was concentrated and purification was performed by precipitation with 1 M KF. The precipitate was dialyzed in distilled water at 4 °C for 3 days and the dialyzed solution was freeze-dried to obtain final product. Yield: 85%. ^{1}H NMR (D_2O), δ (ppm): 0.6–1.0 (br, 12H), 1.0–1.3 (br, 3H), 1.4–2.0 (br, 93H), 2.1–2.3 (s, 3H), 2.9–3.1 (s, 42H), 3.1–3.3 (s, 3H), 3.3–4.1 (br, 60H), 4.1–4.2 (br, 21H), 4.2–4.6 (br, 9H).

2.2.4. $[NP(IleOEt)_{1.16}(GlyGlyOH)_{0.14}(GlyGlyPro)_{0.03}(AMPEG550)_{0.67}]_n$ (conjugate **2**)

Polymer **2** (2.50 g, 4.04 mmol), protamine (7.00 g, 1.37 mmol), IBCF (0.09 ml, 0.69 mmol), and TEA (0.57 ml, 4.12 mmol) were used. Yield: 89%. ^{1}H NMR (D_2O), δ (ppm): 0.6–1.0 (br, 12H), 1.0–1.3 (br, 3H), 1.4–2.0 (br, 93H), 2.1–2.3 (s, 3H), 2.9–3.1 (s, 42H), 3.1–3.3 (s, 3H), 3.3–4.1 (br, 60H), 4.1–4.2 (br, 21H), 4.2–4.6 (br, 9H).

2.3. Synthesis of poly(organophosphazenes) with depsipeptide

Poly(organophosphazenes) with depsipeptide were synthesized similarly by the procedure stated in the previous report [29].

2.3.1. $[NP(IleOEt)_{1.14}(GlyLacOEt)_{0.03}(AMPEG550)_{0.83}]_n$ (polymer **3**)

Poly(dichlorophosphazene) (2.00 g, 17.26 mmol), IleOEt (3.95 g, 20.19 mmol) and GlyLacOEt (0.23 g, 1.04 mmol), and AMPEG 550 (14.62 g, 26.58 mmol) were used. Yield: 78%. ^{1}H NMR ($CDCl_3$), δ (ppm): 0.8–1.0 (s, 6H), 1.1–1.3 (br, 6H), 1.3–1.6 (br, 5H), 1.6–1.9 (br, 1H), 2.8–3.1 (br, 2H), 3.4 (s, 3H), 3.5–3.9 (br, 42H), 3.9–4.1 (br, 4H), 4.1–4.3 (br, 5H), 5.0–5.1 (br, 1H).

2.3.2. $[NP(IleOEt)_{1.14}(GlyLacOEt)_{0.02}(AMPEG550)_{0.83}]_n$ (polymer **4**)

Poly(dichlorophosphazene) (8.00 g, 69.03 mmol), IleOEt (15.40 g, 78.70 mmol) and GlyLacOEt (0.92 g, 4.14 mmol), and AMPEG 550 (63.79 g, 115.98 mmol) were used. Yield: 62%. ^{1}H NMR ($CDCl_3$), δ (ppm): 0.8–1.0 (s, 6H), 1.1–1.3 (br, 6H), 1.3–1.6 (br, 5H), 1.6–1.9 (br, 1H), 2.8–3.1 (br, 2H), 3.4 (s, 3H), 3.5–3.9 (br, 42H), 3.9–4.1 (br, 4H), 4.1–4.3 (br, 5H), 5.0–5.1 (br, 1H).

2.4. Characterization of poly(organophosphazenes)

The structures of prepared polymers were estimated by measuring ^{1}H NMR (Varian Gemini-300 spectrometer operating at 300 MHz in the Fourier transform mode with $CDCl_3$ and D_2O) and FT-IR (Spectrum GX FT-IR, Perkin–Elmer). The substituted amount of protamine was determined by micro BCA assay (Pierce, Rockford, IL, USA). The viscosity measurements on the aqueous polymer solutions were performed on a Brookfield RVDV-III+ viscometer between 5 and 70 °C under a fixed shear rate of 0.1^{-1}. The measurements were carried out with a set spindle speed of 0.2 rpm and with a heating rate of 0.33 °C/min. Molecular weights (MWs) of polymers (polymer **1**–**4**) were measured by gel permeation chromatography (GPC) system (Waters 1515) with a refractive index detector (Waters 2410) and two styragel columns (Waters styragel HR 4E and HR 5E) connected in line at a flow rate of 1 ml/min at 35 °C. THF containing 0.1 wt% of tetrabutylammonium bromide was used as a mobile phase. Polystyrenes (MW: 1270; 3760; 12,900; 28,400; 64,200; 183,000; 658,000; 1,050,000; 2,510,000; 3,790,000) were used as standards to calibrate the column. In case of conjugates **1** and **2**, the MWs were measured in water system due to the solubility of polymers. Except column, mobile phase, and standards, same conditions were used. For measurement, two ultrahydrogel columns (Waters linear) were used and water containing 0.1% trifluoroacetic acid and 40% acetonitrile was used as a mobile phase. Polyethylenimines (MW: 1800; 10,000; 25,000; 70,000) were used as standards.

2.5. Preparation of zinc-complexes hGH

To stabilize hGH during release periods, zinc complex hGH (Zn-hGH) was employed. This complex was formed by adding six molar equivalents of zinc acetate