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Decanoic acid-modified glycol chitosan hydrogels containing tightly adsorbed palmityl-acylated exendin-4 as a long-acting sustained-release anti-diabetic system

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ABSTRACT

Decanoic acid-modified glycol chitosan (DA-GC) hydrogels containing tightly adsorbed palmitic acid-modified exendin-4 (Ex4-C16) were prepared, and their pharmaceutical abilities as a long-acting sustained-release exendin-4 system for the treatment of diabetes were evaluated. Glycol chitosan (GC) was conjugated with N-hydroxysuccinimide-activated decanoic acid (DA) in anhydrous 0.4% dimethylaminopyridine/dimethylsulfoxide at different feed ratios. DA-GC hydrogels formed by physical self-assembly during dialysis vs. deionized water, and their inner network structures, swelling or gel-forming abilities and release properties were examined. The hypoglycemia caused by Ex4-C16-loaded DA-GC hydrogels was evaluated by subcutaneous administration in type 2 diabetic *db*/*db* mice. The results obtained showed that GC prepared at a DA:GC feed ratio of 1:100 had optimal properties with respect to hydrogel swelling, stiffness and Ex4-C16 incorporation, whereas DA-GC hydrogels prepared at a feed ratio of greater than 1:100 formed gels that were too stiff. The in vitro and in vivo release of Ex4-C16 from DA-GC hydrogels was dramatically delayed compared with native Ex4 probably due to strong hydrophobic interactions. In particular, Ex4-C16 in DA-GC hydrogels was found to be present around the injection site up to 10 days after subcutaneous administration, whereas Ex4 in DA-GC hydrogels was cleared from injection sites in ~2 days in ICR mice. Finally, the hypoglycemia induced by Ex4-C16 DA-GC hydrogels was maintained for >7 days. Our findings demonstrate that Ex4-C16 DA-GC hydrogels offer a potential delivery system for the long-term treatment of type 2 diabetes.

1. Introduction

Hydrogels have a 3-D network structure of cross-linked hydrophilic polymer, and have attracted much attention because of their potential uses in a variety of therapeutic and biomedical applications [1–3]. Hydrogels can be prepared using many different methods for specific purposes and are versatile enough to accept a wide range of drugs, such as, synthetics [4], proteins [3], vaccine components [5], oligonucleotides or genes [6], and even living cells [1]. In particular, the tightly cross-linked polymer backbones of hydrogels offer the sustained, long-term delivery of many therapeutic agents.

Based on the mesh size theory of hydrogels, the release of peptide or protein drugs is retarded because their molecular sizes are much greater than those of chemical drugs [2,7]. However, hydrogels' lattices contain a large amount of water due to their hydrophilic backbones, thus hydrophilic proteins are likely to be rapidly lost by diffusion when the stiffness and rigidity of hydrogels is weak. Consequently, fully swollen hydrogels with moderate stiffness show much more rapid protein release patterns than expected, whereas compact, stiff hydrogels are likely to release proteins slowly, and in some cases retain proteins for a long time [3,8–10]. Accordingly, to provide long-term therapy, effective approaches to induce sustained/controlled release of peptides or proteins from hydrogels are required to prevent rapid initial burst-out release on the one hand and to continue having a constant therapeutic effect on the other.

Of the many natural polymers, chitosan displays numerous advantages as a hydrogel carrier material because it is biocompatible/biodegradable in vivo and has no significant toxicity [2,11,12]. Moreover, chitosan contains a number of primary aliphatic amines that can be modified by a variety of functional chemical moieties

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and utilized to form stable hydrogels. Chitosan hydrogels have been prepared using several cross-linking agents, such as glutaraldehyde, genipin and isocyanate derivatives [2], and, furthermore, hydrophobically modified chitosan self-assembles to form hydrogels with particle sizes ranging from nanometers to centimeters [10]. In particular, glycol chitosan (GC) has the advantage of high water solubility as a pharmaceutical excipient due to the presence of ethylene oxide [12].

Hydrogels have been extensively used as sustained-release systems for the treatment of diabetes. The block copolymer-based in situ gels with thermoreversible or pH-sensitive ability were prepared for sustained insulin release without invasive surgery [13,14]. However, despite many advantages for diabetes, hydrogels have not been approved as a pharmaceutical product for human use due to burst-out release and short therapeutic duration. In an attempt to achieve prolonged hypoglycemia (>1 week) in diabetic patients after a single subcutaneous administration, we sought to develop a sustained-release glycol chitosan hydrogel system conjoined with long-acting palmityl acylated exendin-4 (Ex4-C16). For this purpose, we fabricated self-associated hydrogels composed of decanoic acid-modified glycol chitosan (DA-GC) containing hydrophobically adsorbed Ex4-C16. The physical assembly of DA-GC and the affinity of Ex4-C16 for the hydrogels formed were optimized by taking advantage of the hydrophobic interaction between fatty acids in the chitosan and the Ex4. In addition, the in vivo distributions and hypoglycemic efficacies of prepared hydrogels were evaluated in a *db*/*db* diabetic mouse model after subcutaneous (s.c.) administration.

2. Materials and methods

2.1. Materials

Ex4 was purchased from the American Peptide Company (Sunnyvale, CA). GC (Mw > 82 kDa, 60% deacetylated) and palmitic acid N-hydroxysuccinimide (Pal-NHS) were purchased from Sigma Aldrich (St. Louis, MO). Decanoic acid was purchased from the Tokyo Chemical Industry (Tokyo), and Cy5.5 NHS ester from GE Healthcare (Piscataway, NJ). All other reagents were obtained from Sigma–Aldrich, unless otherwise specified.

2.2. Experimental animals

Type 2 diabetic C57BL/6 *db*/*db* mice (male, ~5 weeks old) were purchased from the Korean Research Institute of Bioscience and Biotechnology (Daejon, Korea). ICR mice (male, ~6 weeks old) were purchased from the Hanlim Experimental Animal Laboratory (Seoul). Animals were cared for in accordance with the National Institute of Health (NIH) guidelines for the care and use of laboratory animals (NIH Publication 80–23, revised in 1996). Animals were housed in groups of 6–8 under a 12 h light/dark cycle (lights on at 6 a.m.), allowed food and water ad libitum, and acclimatized for 2 weeks. This study was approved by the Ethical Committee on Animal Experimentation at Sunkyunkwan University.

2.3. Preparation of Ex4-C16

Ex4-C16 was prepared using a modification of a previously described procedure [15–19]. Briefly, 10 mg of Ex4 was mixed with 1.3 mg of Pal-NHS (molar ratio 1:1.5) in 3 ml of 0.3% triethylamine/anhydrous dimethylsulfoxide (DMSO) and allowed to react at room temperature for 90 min. The reaction mixture was then subjected to reverse-phase high-performance liquid chromatography (RP-HPLC) on a LiChrospher 100 RP-18 column (250 × 4.0 mm, 5 μm, Merck, Germany) at ambient temperature. Gradient elution was carried out at a flow-rate of 1.0 ml min^{-1} using solvent A (0.1% trifluoroacetic acid (TFA) in deionized water (DW)) and solvent B (0.1% TFA in acetonitrile (ACN)). The following gradient profile was used: 30–50% B for 10 min followed by 50–90% B for 20 min. Eluates were monitored at 215 nm, and the fraction corresponding to Ex4-C16 was collected, dried under nitrogen and stored in 10 mM phosphate-buffered saline (PBS, pH 7.4) at 4 °C until needed.

2.4. Synthesis of decanoic (C10) or palmitic (C16) acid-modified glycol chitosan

Fatty acid-modified glycol chitosan (FA-GC) was prepared using a modification of a previously described procedure [10,20]. Briefly, 1 g of glycol chitosan was dissolved in 50 ml of DW and dialyzed using a semi-permeable dialysis membrane tube (MWCO = 10 kDa, Spectrum®, Rancho Dominquez, CA) for 24 h in order to remove any salts. The resulting solution was then lyophilized to obtain powdered GC, 60 mg of which was then dissolved in 6 ml of DMSO containing 0.4% dimethylaminopyridine and mixed with different amounts of the NHS ester of decanoic acid or palmitic acid (25, 50, 100 and 200 eq. vs. GC) at 37 °C for 12 h. Acetone (30 ml) was then added to the reaction mixture to precipitate the FA-GC chitosan derivatives. Precipitates were washed with excess ethyl acetate and acetone and then rewashed with DW. After discarding supernatants, fresh precipitates were freeze-dried and stored at −70 °C until needed. The synthesized FA-GCs were analyzed by ^{1}H NMR (Varian Unity-Inova 500, St. Louis, MO).

2.5. Field emission scanning electron microscopy (FE-SEM) characterization of FA-GCs

Dry powdered FA-GC samples were attached to specimen stubs using double-sided tape and sputter-coated with gold–palladium in an argon atmosphere using a Hummer I sputter coater (Anatech Ltd., St. Alexandria, VA). The surface morphologies of FA-GCs were observed by FE-SEM (LEO SUPRA 55 GENESIS 2000, Carl Zeiss, Germany) at an accelerating voltage of 15,000 V.

2.6. Swelling of FA-GC hydrogels

Thirty milligrams of decanoic acid- or palmitic acid-modified GCs (DA-GC or PA-GC, respectively) prepared using different GC to FA feed ratios were immersed in 10 ml of 10 mM PBS (pH 7.4) and kept at 37 °C. At predetermined times, the hydrogels were withdrawn and weighed after being blotted by a filter paper to remove surface water.

2.7. Incorporation and release of Ex4 and Ex4-C16 in DA-GC hydrogels

Aliquots (3 mg) of lyophilized FA-GC powder were immersed in 100 μl of a 1:1 mixture of 0.1% TFA/DW and 0.1% TFA/ACN containing either Ex4 or Ex4-C16 (10, 20, 40 or 80 μg each). Next, 0.9 ml of 50 mM PBS (pH 7.4) buffer was added dropwise to the DA-GC hydrogels formed to induce hydrophobic interactions between the fatty acid moieties in both the Ex4 and the GC. Three hours later, ACN was thoroughly removed by nitrogen purging for 15 min. For the animal study, DA-GC containing either Ex4 or Ex4 C16 was sterilized overnight (>12 h) by UV light emission to prevent bacterial contamination. Five aliquots each (3 mg, 1 ml) of DA-GC or PA-GC containing either Ex4 or Ex4-C16 (50 μg each) in 50 mM PBS (pH 7.4) were gently agitated at 37 °C. At 0, 0.5, 1, 2, 3, 7, 14 and 21 days, each hydrogel sample was centrifuged at 2250*g* for 5 min and the supernatants were discarded. The amounts of drug remaining in the hydrogels were determined by RP-HPLC analysis (as described above) to determine the incorporation efficiencies (%)