Experimental

Materials. Exenatide with a sequence of HEGTFTS-DLSDKQMEEEAVRLFIEWLKNNGGPPSSGAPPSS was purchased from Anygen Co. (Gwangju, Korea). LCA, GC, βCD, 1-ethyl-3(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl), N-hydroxysuccinimide (NHS), triethyl amine (TEA), trifluoro acetic acid (TFA), and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (Saint Louis, MO, USA). An exenatide enzyme immunoassay (EIA) kit was purchased from Roche Diagnostics (Seoul, Korea). All other chemicals were of analytical grade and used without further purification.

Synthesis of GC-βCD. GC-βCD conjugate was synthesized by the chemical conjugation of carboxymethyl-βCD (CM-βCD) to a GC backbone by a condensation reaction, as previously described. In brief, CM-βCD (122.8 mg, 0.1 mmol) was dissolved and stirred for 30 min in 25 mL of distilled water containing EDC (29.6 mg, 0.15 mmol) and NHS (18.3 mg, 0.15 mmol). GC (100 mg, 0.4 µmol) in 10 mL of distilled water was slowly added to the solution, and the resulting solution was stirred at room temperature for 1 day. The product was purified by dialysis (MWCO=12,000-14,000 Da) against distilled water for 1 day, followed by lyophilization. The samples were stored at 4°C until further use. The chemical structure of the conjugate was confirmed by 1H NMR spectrometer (JEOL, JNM-AL300) operating at 300 MHz, for which the sample was dissolved in D2O. The degree of substitution, defined as the number of βCD per 100 sugar residues of GC, was found to be 25.7, which was calculated from 1H NMR spectra based on the peak integration ratio of the N-acetyl group in GC (δ=1.9 ppm) to the anomeric proton of βCD (δ=4.9 ppm).

Preparation of LCA-Exenatide. The conjugation of LCA into the exenatide was carried out using a previously reported procedure. In brief, 100 µL of NHS-activated LCA (LCA-NHS) (1.6 mg/mL in DMSO containing 0.3% TEA) was mixed with 100 µL of exenatide (5 mg/mL in DMSO containing 0.3% TEA). The molar ratio of exenatide to LCA-NHS was fixed at 1.5. The resulting solution was gently stirred at room temperature for 1 h, and the reaction was stopped by adding 200 µL of 1% TFA. The LCA-conjugated exenatide on the residue of Lys was obtained by using reverse-phase high-performance liquid chromatography (RP-HPLC) through a Capcell-pak RP-18 column (250×10 mm, 5 µm, Shiseido, Japan) using a gradient elution. UV detection was performed at 215 nm. The column was eluted at a flow rate of 4 mL/min.

Preparation of LCA-Exenatide/GC-βCD Complexes. In order to prepare LCA-exenatide/GC-βCD complexes, LCA-exenatide solution (0.5 mg/mL in distilled water) was added slowly to the GC-βCD solution (3.3 mg/mL in DMSO). The resulting solutions were mixed gently for 10 min, followed by brief sonication using a probe-type sonifier (VCX-750, Sonics & materials, CT, USA) at 90 W for 30 s operated in pulsed mode with on-off cycles for 5 and 1 s, respectively. Then, unbound LCA-exenatide was removed by dialysis against distilled water for 1 day (MWCO=12,000-14,000 Da) at 4°C and lyophilized. The lyophilized samples were stored in the deep-freezer at -50°C until further use. The amount of LCA-exenatide in the complex was estimated using a RP-HPLC.

In vitro Enzymatic Stability Test. To determine the enzymatic stability of the samples in vitro, exenatide, LCA-exenatide, and LCA-exenatide/GC-βCD were separately dissolved in PBS (pH 7.4). Thereafter, trypsin solution (2 nM) was mixed with the sample solution, followed by incubation at 37°C. Samples (40 µL) were withdrawn at specific time intervals, and the enzymatic reaction was stopped by the addition of 2% TFA solution. The concentrations of exenatide and LCA-exenatide were determined using a RP-HPLC, for which gradient elution was carried out by using the eluents of 0.1% TFA in distilled water and 0.1% TFA in acetonitrile.

In vivo Antidiabetic Effects. Type 2 diabetic C57BL/6 db/db mice (male, 6–8 weeks old) were supplied by the Korea Research Institute of Bioscience and Biotechnology (Daejon, Korea). All experiments with live animals were performed in compliance with the relevant laws and institutional guidelines of Sungkyunkwan University.

The mice were used for the acute anti-diabetic activity tests, after being acclimatized for one week in an animal facility. Under non-fasting conditions with free access to water and food, mice were administered a single subcutaneous injection of exenatide, LCA-exenatide, or LCA-exenatide/GC-βCD (100 nmol exenatide/kg, 200 µL). Blood samples were collected from tail veins with predetermined intervals, and the blood glucose levels were determined using a One-Touch blood glucose meter.

References