Published in final edited form as:

J Control Release. 2019 January 28; 294: 259–267. doi:10.1016/j.jconrel.2018.12.028.

Long-term oral administration of Exendin-4 to control type 2 diabetes in a rat model

Kenichi Suzuki^{a,b}, Kyoung Sub Kim^a, and You Han Bae^{a,*}

^aDepartment of Pharmaceutics and Pharmaceutical Chemistry, University of Utah, Salt Lake City, UT 84112, USA

^bFuji Research Laboratories, Pharmaceutical Division, Kowa Co. Ltd., 332–1 Ohnoshinden, Fuji, Shizuoka, Japan

Abstract

Exendin-4 is a glucagon-like peptide-1 (GLP-1) receptor agonist and potent insulinotropic agent for type 2 diabetes patients; however, its therapeutic utility is limited due to the frequent injections required. Long-acting agonists reduce the number of injections, but they can compromise potency. In this study, chondroitin sulfate-g-glycocholic acid-coated and Exendin-4 (Ex-4)-loaded liposomes (EL-CSG) were prepared for oral administration of Ex-4. The Ex-4 loading efficiency was 77% and the loading content in the nanoparticles was 1 wt-%. In rat models, a single oral dose (200 µg/kg) of EL-CSG showed a relative oral bioavailability of 19.5%, compared with subcutaneous administration (20 µg/kg), and sustained pharmacokinetics for up to 72 h. The overall long-term pharmacodynamic effects, assessed by hemoglobin A1c (HbA1c), body weight, and blood lipid concentrations, of daily oral EL-CSG (300 µg/kg) for four weeks were equivalent to or better than daily subcutaneous injections of free Ex-4 solution (20 µg/kg).

Keywords

Exendin-4; oral drug delivery; bile acid; liposome; liposome intestinal absorption; Type 2 diabetes

1. Introduction

Type 2 diabetes mellitus (T2DM) with impaired insulin secretion is in part caused by decreased secretion of glucagon-like peptide-1 (GLP-1) [1]. Exendin-4 (Ex-4) is a GLP-1 receptor agonist comprised of 39 amino acids that has been used in the treatment of T2DM.

*Corresponding author. you.bae@utah.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Declaration of Interest

The authors declare no competing financial interest.

Additional information

Supplementary information is available in the online version of the paper.

Correspondence and requests for materials should be addressed to Y.H.B (you.bae@utah.edu).

Ex-4 has a significantly longer half-life *in vivo* compared to that of endogenous GLP-1 [2,3], while possessing GLP-1-like glucoregulatory activities, such as glucose-dependent enhanced insulin secretion, suppressed glucagon secretion, reduced gastric mobility, and decreased food intake [4,5]. Exenatide, a synthetic version of Ex-4, has been approved as an adjunctive therapy for patients with T2DM who fail to achieve glycemic control with oral antidiabetic agents [4,6]. Ex-4 has been widely used for T2DM and has great potential as a future therapeutic for neurodegenerative diseases, such as Alzheimer's and Parkinson's disease, which are linked to insulin resistance in the brain [7,8]. However, patients are administered frequent subcutaneous (SC) injections, resulting in high costs and poor patient compliance in addition to side effects, such as injection site infection [6,9]. Despite injectable long-acting GLP-1 receptor agonists, peroral administration of such active pharmaceutical ingredients may represent a more convenient and comfortable alternative for diabetic patients [10].

Various attempts have been made to develop oral delivery systems for Ex-4 [11–13]. However, the oral bioavailability, particularly in humans, of protein/peptide drugs such as Ex-4 is limited due to enzymatic inactivation and absorption barriers present in the gastrointestinal tract (GIT) [10]. These typically include physical barriers of viscous mucous layers and tight junctions of aligned enterocytes in the GIT, chemical barriers of low stomach pH, and biological barriers such as enzymatic degradation. Overcoming these problems is essential for enhancing absorption of orally administered peptide-based drugs [10,14]. Consequently, the development of a delivery vehicle for oral administration of protein/peptide drugs presents an interesting challenge. Recent reports have indicated promising oral absorption results of Fc-modified poly(lactic-co-glycolic acid) nanoparticles loaded with Ex-4 for targeting Fc receptors [15]. However, the binding capacity in adults of Fc receptors in the GIT is low and differences in the intestinal environment between rodents and humans are likely to confound extrapolation of animal model data to the clinic. Since it seems that problems still remain in clinical applications, we focused on the enterohepatic circulation of bile acid and started developing an oral drug delivery carrier modified with bile acid.

Enterohepatic circulation of bile acids is composed of two major processes: secretion from the liver and absorption in the intestines. Bile acids are secreted into the duodenum, where they emulsify water-insoluble nutrients to facilitate absorption [16]. In the distal small intestine, bile acids are absorbed by both passive and active mechanisms. Passive absorption occurs in the proximal regions of the small intestine and colon, while active absorption is restricted to the ileum [17]. In humans and other vertebrates examined to date, the ileal epithelium has developed efficient transport mechanisms for the active reclamation of bile acids [18]. This enterohepatic circulation is an efficient process; only 5–10% of the intestinal bile acids are eliminated in the feces [19] and as much as 30 g/day and 20 mg/day of intestinal bile acids are recycled in humans [20] and mice [21], respectively. This capacity is several orders greater in magnitude than the reabsorption capacity of gastric leptin (its secreted amount is unknown, but very limited [22]) or vitamin B₁₂ (cyanocobalamin, 2–4 µg/day in humans [23]) mediated by oral absorption. A recent study revealed that low-molecular-weight heparin modified with deoxycholic acid enters distal ileocytes using the apical sodium-dependent bile acid transporter (ASBT) [24]. The observed entry mechanism

of this modified heparin was clearly distinguished from the pumping mechanism for small-molecule bile acids, of which details were recently elucidated. While the specific entry and transport mechanisms are largely unknown and widely open for investigation, it was reported that nanoparticles, self-assembled from heparin-taurocholic acid-docetaxel conjugates, were orally absorbed to elicit an antitumor effect [25]. It was also shown that intact nanoparticles were orally absorbed, using anionic probe polystyrene nanoparticles with glycocholic acid (GCA)-decorated surfaces [26].

In addition, micro- to nano-sized drug delivery carriers are available, each having distinct pharmaceutical characteristics. For micro-sized formulations, high encapsulation rates are expected due to their large physical dimensions, and their release behavior can be characterized as slow kinetics [27]. For nano-sized formulations, the encapsulation rate is generally low, and their release behavior is comparatively fast. Various nano-sized pharmaceutical formulations have been developed, and preparations to improve absorbability have been studied extensively [28]. When considering GIT transit time in humans, especially through the small intestine, a sustained release of ~24 h deemed sufficient for a formulation to act as a drug carrier. One absorption mechanism is the transcellular pathway that involves either passive transport or endocytosis of nanoparticles through epithelial cells. The cut-off size for nanoparticles to be taken up by epithelial cells has been reported as 300 nm [29]; maintaining nanoparticles below this cut-off size is expected to improve intestinal absorption.

In the present study, we provide new evidence for enhanced oral delivery of Ex-4 using liposome-based nanocarriers and surface GCA. GCA was selected because it is the richest component in human bile salts [30] with a relatively low logP value [31], which allows more exposure to the water phase, and requires a relatively straightforward conjugation chemistry that simplifies analysis. Ex-4-loaded cationic liposomes (EL) comprised of 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) were fabricated by conventional methods. The resulting liposomes were further coated with chondroitin sulfate to yield EL-CS or GCA-conjugated chondroitin sulfate to yield EL-CSG. Using a T2DM rat model, the biological efficacy of orally administered EL-CSG was compared with that of EL-CS, diabetes and non-diabetes controls, and SC-injected free solution by using oral glucose tolerance tests (OGTTs) and analyzing the pharmacodynamic effects after 4 weeks of treatment.

2. Materials and Methods

2.1. Materials

GCA, dimethylformamide, ethylenediamine (EDA), dicyclohexylcarbodiimide, N-hydroxysuccinimide, ethyl acetate, acetonitrile, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, diethyl ether, streptozocin, and 2-ethanesulfonic acid (MES) buffer were purchased from Sigma-Aldrich (St. Louis, MO, USA). Potassium sodium-encapsulated Preyssler-type phosphotungstate was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Exenatide (BYETTA®) was purchased from AstraZeneca (Cambridge, United Kingdom). DOPC was purchased from Echelon Bioscience (Salt Lake City, UT, USA) and DOTAP was purchased from CordenPharma

(Boulder, CO, USA). CS (molecular weight approximately 25 kDa, injectable grade) was obtained from Yantai Dongcheng Biochemicals Co., Ltd. (Shandong, China). Fasted State Simulated Gastric Fluid (FaSSGF, FFF01) and Fasted State Simulated Intestinal Fluid (FaSSIF-V2, V2FAS01) were purchased from Biorelevant.com Ltd. (Surrey, United Kingdom). Dialysis membrane (Biotech Cellulose Ester Membrane, molecular weight cut-off 50 kDa) was purchased from Repligen (Waltham, MA, USA). High fat diet (TD 96132) was purchased from Envigo (Cambridgeshire, United Kingdom). The Diazyme Direct HbA1c Assay kit (DZ168A-K) was purchased from Diazyme Laboratories (San Diego, CA, USA). The Cholesterol Assay Kit (ab65390) was purchased from Abcam (Cambridge, MA, USA).

2.2. Synthesis of glycocholic acid -conjugated chondroitin sulfate (CSG)

The procedure for the synthesis of CSG is presented in Fig. S1. To synthesize GCA-EDA, GCA (644 μmol), dicyclohexylcarbodiimide (838 μmol), N-hydroxysuccinimide (838 μmol), and EDA (32.2 mmol) were dissolved in 3 mL of dimethylformamide. The reaction mixture was stirred for 24 h at room temperature. The reacted solution was filtered and precipitated in ethyl acetate. The collected pellet was dried *in vacuo* for 24 h. The dried powder was dissolved in 10 mL of distilled water, filtered, and lyophilized [32]. To synthesize CSG, the GCA-EDA (425 μmol), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (550 μmol), N-hydroxysuccinimide (550 μmol), and CS (8 μmol) were dissolved in 3 mL of 0.1 M MES buffer, pH 6.0. The reaction mixture was stirred for 24 h at room temperature. The reacted solution was filtered and precipitated three times in 60% cold ethanol. The collected pellet was dried *in vacuo* for 24 h. The synthesized CSG was characterized using a Mercury 400 ^1H NMR spectrophotometer (Fig. S1).

2.3. Preparation of EL

EL were prepared as outlined in a previous report [33]. In brief, DOPC (38 mg) and DOTAP (39 mg) were dissolved in 15 mL of diethyl ether and 6 mL Ex-4 solution (0.5 mg/mL) was added. To produce an emulsified mixture, the solution was placed in a VWR Scientific Aquasonic 75T Ultrasonic cleaner for 5 min. After sonicating the flask, the solvent was removed by rotary evaporation at 60 °C. The obtained EL was then purified via dialysis (Spectra/Por[®] molecular weight cut-off 50 kDa membrane) against distilled water for 24 h to remove unloaded Ex-4. The purified liposome solution was passed through a 200-nm filter (Whatman Nuclepore, Maidstone, UK) at 60 °C using a membrane extruder LiposomeFast[®] LF-50 (Avestin, Inc., ON, Canada).

The Ex-4 loading efficiency was estimated using reversed-phase high-performance liquid chromatography (HPLC) [13,33]. Specifically, the DIONEX UltiMate 3000 serial HPLC system consisted of a quaternary pump, degasser, autosampler, column oven, UV detector, and HP ChemStation Software (all from Thermo Scientific, USA). The chromatographic conditions were optimized with a Kromasil[®] C4 column (4.6 mm $\times$ 250 mm, i.d., 5 μm particles, AkzoNobel, USA). The column temperature was maintained at 40 °C. Mobile phase A was potassium dihydrogen phosphate (KH_2PO_4 , 20 mmol/L) adjusted to pH 2.5 with phosphoric acid, and mobile phase B was acetonitrile. The mobile phase was filtered through a 0.45- μm membrane filter and degassed with an ultrasonic water bath prior to use.

The following gradient conditions were used for Ex-4 determination: 30–45% mobile phase A over 30 min and then returned to 30% mobile phase A over 10 min. The flow rate was 1.0 mL/min and the injection volume was 50 μ L. The UV signal was detected at 214 nm because the absorption of the amide group was more sensitive than that of Phe, Tyr, and Trp at 280 nm.

The Ex-4 loading efficiency and loading capacity in the nanoparticles were calculated using the equations (1) and (2) listed below [34,35].

$$\begin{aligned} \text{Loading efficiency (\%)} & \quad (1) \\ &= \frac{(\text{Total amount of Ex - 4 added}) \text{ minus } (\text{Free Ex - 4})}{(\text{Total amount of Ex - 4 added})} \times 100\% \end{aligned}$$

$$\begin{aligned} \text{Loading capacity (\%)} & \quad (2) \\ &= \frac{(\text{Total amount of Ex - 4 added}) \text{ minus } (\text{Free Ex - 4})}{(\text{weight of nanoparticles})} \times 100\% \end{aligned}$$

The EL-CSG and EL-CS were prepared by electrostatic interaction by mixing cationic liposomes with anionic CS or CSG complexes. In brief, 1 mL of EL was mixed with 1.0 mL of CS or CSG (10 mg/mL) and then stirred for 2 min.

2.4. Size and zeta-potential measurements

The size and zeta-potential of each liposomal formulation were measured using dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments, USA). Prior to measurement, the liposome solution was stabilized at room temperature for 2 h. The average of three measurements was used.

2.5. *In vitro* Ex-4 release study

In vitro Ex-4 release was measured by a dialysis method [36]. In brief, the EL, EL-CS, and EL-CSG were dispersed in distilled water. The suspension was placed into a dialysis membrane bag (Spectra/Por® molecular weight cut-off 50 kDa). The bag was sealed and subsequently immersed in a vial containing fresh release medium, such as Simulated Intestinal Fluid, pH 6.81; USP 26 [37]. The release of Ex-4 from the liposomes was performed with mechanical mixing (100 revolutions/min) at 37 °C. Two hundred microliters of the outer solution was sampled and replaced with fresh buffer at predetermined time intervals (0–48 h). The Ex-4 concentration in each sample (n=3) was measured by HPLC, as indicated in section 2.3.

2.6. Particle degradation in solutions, including simulated GIT fluids

To prepare freeze-dried EL-CSG (from 1 mL), 0.8 mL trehalose solution (50 mg/mL) was used as a cryoprotectant during the freeze-drying process [38,39]. To evaluate the particle

stability of EL-CSG, 1.0 mL of test media (distilled water, phosphate-buffered saline (PBS, pH 7.4), FaSSGF, or FaSSIF-V2) was mixed with the freeze-dried powder. After reconstitution, the nanoparticles were characterized using the Zetasizer Nano ZS.

2.7. *Ex vivo* nanoparticle transport efficacy in the small intestine

All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Utah. To perform the everted gut sac (EGS) assay, the small intestines were isolated from 200–225 g male Sprague-Dawley rats (Charles River Laboratories International, Inc.). The distal ileum was isolated at a length of 5 cm and each segment was inverted lumen-side out. After one end of the segment was tied, the inner space was filled with oxygenated culture medium (800 μ L) and the other end was then tied to make a 4-cm-long EGS. The EGS was placed in Krebs-Ringer solution (J67591, Alfa Aesar, USA) containing 0.05 mg/mL free Ex-4 (BYETTA[®] appropriately diluted with PBS), EL, EL-CS, or EL-CSG at 37 °C; the amount of Ex-4 transported into each sac was measured after 90 min [40]. The amount of Ex-4 transported per unit tissue area (calculated by tissue dimension and not surface area expanded by villi and microvilli) was analyzed by an Ex-4 ELISA kit (EK-070–94, Phoenix Pharmaceuticals Inc., Mountain View, CA, USA). Cellular integrity and tissue viability over 90 min were confirmed by monitoring lactate dehydrogenase (LDH) concentration per unit area of tissue [41].

2.8. *In vivo* blood Ex-4 level and bioavailability

To evaluate the blood insulin level and bioavailability of EL-CS and EL-CSG, 200–225 g male Sprague-Dawley rats (Charles River Laboratories International, Inc.) were randomly divided into three groups (n=6 per group). The rats were water fasted overnight prior to nanoparticle administration and the fasting continued for 12 h thereafter. The following formulations were administered to rats individually: oral EL-CS (200 μ g/kg), oral EL-CSG (200 μ g/kg), and SC injection of free Ex-4 solution (20 μ g/kg). Blood samples (200 μ L) were collected from the jugular vein, centrifuged (1,800 $\times$ g, 4 °C, 10 min), and subsequently quantified for plasma Ex-4 concentration using the Ex-4 ELISA kit. Before *in vivo* experiments, we examined whether the applied ELISA kit shows selectivity for exendin-4 and confirmed that there is no cross-reactivity between Ex-4 and GLP-1. The relative bioavailability (BA_R) was calculated using equation (3) [42], where AUC is the area under the plasma concentration vs. time curve.

$$BA_R = \frac{AUC_{(Oral)} \times Dose_{(SC)}}{AUC_{(SC)} \times Dose_{(oral)}} \times 100 \quad (3)$$

2.9. *In vivo* blood glucose level

To establish the T2DM model, 150–170 g male Sprague-Dawley rats were divided into five groups (n=6 per group). The rats were fed a high fat diet consisting of 18 %kcal protein, 40 %kcal carbohydrate, and 40 %kcal fat (TD 96132, Envigo) for 2 weeks. After 2 weeks, the rats were subjected to an overnight fast and injected SC with streptozocin (35 mg/kg body weight in 0.1 M citrate buffer, pH 4.5). The rats had free access to food and water after

the streptozocin injection. After a 12-h fast, the rats with fasting glucose levels >200 mg/dL were considered diabetic and were used for further study [43]. The biological efficacy of orally administered EL-CSG was compared with that of EL-CS, and SC-injected free Ex-4 solution, and with diabetes control (DC) and non-diabetic control (NC, normal control) rats, using an OGTT as previously reported [44,45]. Specifically, the rats were fasted overnight and then administered enteric-coated capsules containing EL-CS (300 µg/kg) or EL-CSG (300 µg/kg), or they were injected SC with free Ex-4 solution (20 µg/kg). To initiate the OGTT, an oral dose of glucose (1 g/kg) was administered to the animals 2 h after receiving the Ex-4 treatment in order to imitate the postprandial state, which is characterized by a rapid and significant increase in blood glucose after a meal [46]. Blood samples were collected from the tail veins of the test rats, and their blood glucose levels were evaluated using a glucose meter (Contour Next EZ, Bayer).

2.10. *In vivo* pharmacodynamic study

To evaluate therapeutic effects, the T2DM rats were randomly divided into four groups (n=6 per group). Over a period of 4 weeks, free Ex-4 solution (20 µg/kg, daily) was administered by SC injection, while EL-CS (300 µg/kg, daily) and EL-CSG (300 µg/kg, daily) were administered by gavage at 6 PM, one hour prior to the first meal of the day. After 4 weeks administration, blood samples (200 µL) were collected from the jugular vein, centrifuged (1,800 ×g, 4 °C, 10 min), and the content of Ex-4 (EK-070-94, Phoenix Pharmaceuticals Inc.), hemoglobin A1c (HbA1c, Diazyme Lab., San Diego, CA, USA), total cholesterol (T-Chol, ab65390, Abcam), high-density lipoprotein (HDL, ab65390, Abcam), and low-density lipoprotein (LDL, ab65390, Abcam) in the plasma was quantified using each assay kit.

2.11. Statistical analysis

Each result from the *in vitro* studies is expressed as the mean ± standard deviation (SD). Each result from the *ex vivo* and *in vivo* studies is expressed as the mean ± standard error of the mean (SEM). Differences between the values were assessed using Student's t-tests.

3. Results and Discussion

3.1. Synthesis and characterization of liposome coating materials

In order to stabilize cationic liposomes in the GIT environment by anionic polymer coating and provide a surface to enhance intestinal absorption, we synthesized CSG. The procedure for the synthesis of CSG is presented in Fig. S1. The carboxyl group of GCA was modified to yield a primary amine by reaction with EDA. The modified GCA was coupled to the carboxylic groups of CS through a conventional carbodiimide coupling reaction. Successful synthesis of CSG was confirmed by the appearance of the -CH₃ peaks of GCA at 0.5–1 ppm using ¹H-NMR. The degree of GCA conjugation on CS was estimated by comparing the intensity of the -CH₃ peak of CS at 1.8 ppm to that of GCA at 0.5–1 ppm. The degree of GCA conjugation was determined to be ~18 units of GCA per 100 units of CS. Varying amount of CSG was also added to Caco-2 cell culture medium to optimize its concentration; in this cell culture model, the non-cytotoxic concentration was estimated as 1 mg/mL (Fig. S2).

3.2. Preparation and characterization of EL-CSG

Three liposomal formulations, shown in Table 1, were prepared according to the method outlined in a previous report [47]. The conventional liposomes were prepared with a Reverse-Phase Evaporation technique, using DOPC and DOTAP. This method has been reported to be capable of encapsulating relatively water soluble drugs in the inner aqueous phase of liposomes at high concentrations [48]. The size, size distribution, and zeta potential of the Ex-4 loaded liposomes are summarized in Table 1. The average size of the EL-CS (205 nm) and EL-CSG (230 nm) was larger than that of the corresponding liposomes without CS or CSG coating (154 nm). The size changes were more than anticipated, which is probably due to alterations in electrostatic repulsion forces and surface properties by GCA modification. The zeta potential of the EL-CS and EL-CSG changed from cationic (+57 mV) to anionic as the Ex-4-loaded cationic liposomes were coated with the anionic CS or CSG (−33 mV and −31 mV, respectively). The Ex-4 loading efficiencies of EL, EL-CS, and EL-CSG were 77.2, 72.8, and 74.2%, respectively, and the content of EL-CSG in the nanoparticles was 1.2 wt-%, which is high enough to meet the therapeutic window of extremely potent Ex-4 (minimum therapeutic concentration (MTC) of Ex-4 is 50–350 pg/mL in humans [49], 5–10 µg/SC injection) after oral administration. Due to a large water phase volume used in this experiment and electrostatic interactions between cationic lipid and anionic Ex-4, it is assumed that Ex-4 is located on the inner and outer surfaces of the resulting liposomes. The low loading content of Ex-4 in the liposomes still resulted in a strong cationic surface for polymer coating. The formulations were immediately used for oral uptake studies or freeze-dried in the presence of trehalose for storage. When the lyophilized powder was reconstituted, the particle properties were equivalent to those of the fresh formulations.

The size and shape of blank liposomes, EL, EL-CS, and EL-CSG in distilled water was measured by transmission electron microscopy after negative staining, such as potassium sodium-encapsulated Preyssler-type phosphotungstate (Fig. 1c–f). Negative staining usually retains the concavo-convex structure of the surface, which often collapses during drying, and allows observation of the liposome multilayer structure [50]. Compared with blank liposomes, these nanostructures were also observed in a previous report [51] where cationic liposomes were prepared with a single-stranded oligodeoxynucleotide. The addition of oligodeoxynucleotide to the cationic liposomes induced both liposome aggregation and the formation of a novel condensed lamellar phase. This phase was proposed to be stabilized by anionic single-stranded oligodeoxynucleotide molecules intercalated between cationic bilayers. The layered structure observed in EL suggests that the interaction between the cationic surface of the liposomes and Ex-4 (anionic single-chain peptide) in the multilayered structure allows high encapsulation efficiency of Ex-4 into the liposomes.

3.3. Ex-4 release profile and protection from intestine-simulated fluid

Ex-4 release profiles under GIT-simulated pH conditions were measured. EL, EL-CS, and EL-CSG showed 88, 62, and 51% cumulative release over 24 h, respectively (Fig. 1b), indicating that the coating layers delayed the release rates over 72 h. At the end point (~72 h), the release efficiencies of EL, EL-CS, and EL-CSG were 90.5, 66.7, and 55.7 %, respectively. These results suggest that these liposomes are generally stable and will lose a

limited amount of Ex-4 during transit time in the GIT. To demonstrate further the particle stability under GIT conditions, the particle properties were evaluated in simulated fluid (Table 2). PBS was used as a negative control. EL-CSG was rapidly reconstituted within a few seconds under the four media conditions. In addition, the initial particle properties after reconstitution with all media were maintained for at least 1 day at 25 °C (data not shown). However, liposome aggregation in the simulated gastric fluid under fasting conditions (i.e., FaSSGF) was slightly higher than that in simulated intestinal fluid under fasting conditions (i.e., FaSSIF-V2). Ex-4 is comparatively stable within a pH range of 1.2–8 [52] and CSG-coated liposomes are stable in the GIT after oral administration; these features would play an important role in increased Ex-4 absorption because they increase the opportunity for the Ex-4-loaded nanoparticles to reach the distal small intestine.

3.4. *Ex vivo* nanoparticle transport efficacy using the EGS model

We used the EGS model due to the unavailability of an acceptable monolayer cell culture model that mimics ileocyte absorption at a tissue level. The Ex-4 transport of EL, EL-CS, and EL-CSG was compared with that of free solution (Fig. 2a). The control cationic EL was 2.1 times less permeable than was anionic EL-CSG at the same time point, which was considered non-specific transport. All results support the supposition that the transport is rather specific at the distal ileum, despite a certain degree of non-specific transport that is observed in the EGS model. The cellular integrity and viability of the tissue over 90 min was confirmed by monitoring LDH concentration normalized per unit area of the tissue (Fig. 2b). All concentrations of LDH released were below 40 U/L/cm², which indicates that the tissue remained viable during the test period [41].

3.5. *In vivo* pharmacokinetic study and absorption pathway

To demonstrate the Ex-4 absorption efficacy of EL-CSG in the GIT, we measured time-dependent plasma Ex-4 levels and their related pharmacokinetic parameters (Table 3). The plasma Ex-4 levels of the SC-injected free Ex-4 solution increased and decreased rapidly within 12 h post-administration, while those of EL-CSG gradually increased for 12 h, with an early peak at 3 h, and then slowly decreased over the next 60 h (Fig. 3). We hypothesized that EL-CSG is absorbed into the intestine with bile acids via ASBT, and then transferred to the bloodstream via the enterohepatic circulation, similar to the mechanism recognized with other drugs, such as meloxicam [53]. The temporary increase in plasma concentration at 3 h seems triggered by reabsorption or recycling event. It is difficult to reach a solid conclusion regarding the contribution of enterohepatic circulation because these data were derived from only a peripheral pharmacokinetic study.

In order to understand these contributions, we will need to cannulate the portal vein and compare the pharmacokinetic profiles between the portal and peripheral circulation after feeding with EL-CSG; this will be the focus of future studies. However, considering the current data, these observations are probably related to the transit time profiles of individual nanoparticles in the GIT. A certain fraction of the nanoparticles may migrate along the GIT with water, while the remainder may interact with the mucus layer, slowing its movement. The sustained pharmacokinetic profiles produce beneficial effects, mimicking a long-acting drug, which could be more important than oral bioavailability. For instance, to meet the

minimum therapeutic concentration of Ex-4, it is injected twice a day in clinical settings due to rapid wash-out 12 h after SC injection (Fig. 3). Although a 10 times higher oral dose was used in this study (20 µg/kg SC vs. 200 µg/kg Oral), the blood concentration of the drug was maintained for 3 days after this single oral dose. The Ex-4 therapeutic range, expressed as the MTC, is well-known to be 50–350 pg/mL in human clinical use. These results strongly suggest that this liposome has sufficient potency to be used as an oral antidiabetic therapy.

For comparing oral bioavailability, EL-CS was used as a negative control group. The rats orally administered EL-CSG exhibited a 4.7-fold higher AUC than the group orally administered EL-CS. EL-CSG exhibited an enhanced oral bioavailability (oBA; 19.5%) despite a series of barriers encountered with the intestinal absorption of Ex-4. These results demonstrate that the EL-CSG formulation supports ASBT-mediated, sustained absorption and release of a significant fraction of Ex-4 in the plasma. The Ex-4 formulation is likely influenced also by the stomach conditions. The oBA can be affected by various parameters, such as physiological factors (GIT movement and pH), drug characteristics (vulnerability to pH and digestive enzymes, and stability during formulation), and formulation characteristics (release rate and protection capacity)[54,55]. The oBA was remarkably high, considering that the formulation is exposed first to gastric conditions without enteric protection and Ex-4 is then lost by release until it reaches the absorption site.

3.6. *In vivo* pharmacodynamics with T2DM rats

To ascertain the biological efficacy of EL-CSG after oral administration, OGTT was conducted on diabetic rats treated with EL-CSG, free Ex-4 solution, EL-CS, or PBS (DC), as well as on normal rats (NC). When T2DM rats treated with PBS, EL-CS, or free Ex-4 solution were given an oral solution of glucose (1 g/kg body weight), their blood glucose levels increased immediately, reaching a maximum of 238, 213, and 194%, respectively, after 30 min. Moreover, the blood glucose levels of EL-CSG-treated diabetic rats increased less (maximum 171%, $P < 0.01$) than the other groups, indicating that oral EL-CSG improved their glycemic response (Fig. 4a and 4b). These results show that the experimental T2DM rats exhibited a hyperglycemic state in response to oral glucose and that these rats were valid as a pathological model for pharmacodynamic studies of T2DM. EL-CSG also can be used as an appropriate oral T2DM treatment. Extrapolation of pharmacological effects in rat models to those in human patients is still debatable. Regardless, the pharmacological effect of EL-CSG was positive in the rat diabetes model of high fat diet combined with streptozocin [56], which is an accepted model of T2DM in humans.

To evaluate therapeutic effects and safety, high doses of free Ex-4 solution, EL-CS, and EL-GCS were administered daily to T2DM rats for 4 weeks. After the 4-week administration, body weights, as well as HbA1c, T-Chol, LDL cholesterol, and HDL cholesterol levels in blood plasma were measured as T2DM-treatment indicators. There are many metabolic disorders in T2DM that are based on decreased insulin action, such as chronic hyperglycemia, abnormal serum lipid metabolism, and hypertension. As a diabetic treatment, there are three well-known pharmacological effects of Ex-4, namely (1) promoting insulin secretion from pancreatic β cells depending on blood glucose levels, (2) delaying gastric emptying and suppressing appetite and food intake, and (3) protecting the

function of β cells. These are important parameters for controlling the diabetic condition. With respect to blood glucose management, it is important to reduce HbA1c levels while minimizing blood glucose fluctuations without causing hypoglycemia, in order to suppress vascular complications.

The change in body weight from 0 to 4 weeks, as well as the HbA1c, T-Chol, and LDL cholesterol levels significantly decreased (Fig. 5a–d), while the HDL cholesterol levels increased (Fig. 5e) in the EL-CSG-treated T2DM rats compared to the corresponding values in the DC and NC rats. Ileum tissue morphology after 4 weeks of EL-CS and EL-CSG oral administration was also assessed by hematoxylin and eosin staining and no morphological abnormalities were observed (Fig. S2). The side effects of Ex-4 in human clinical use are well-known to be milder than those of insulin, the latter including severe hypoglycemia. For example, the main side effects of Ex-4 are gastrointestinal in nature, including acidic or sour stomach, belching, diarrhea, heartburn, indigestion, nausea, and vomiting. During the 4-week administration period, the rats appeared healthy with respect to general appearance, behavior, and excretion, thus no side effects were noted in this study.

In addition to its action on pancreatic β cells to promote insulin secretion, Ex-4 has a variety of other effects, such as suppressing glucagon secretion from pancreatic α cells, suppressing appetite through the central nervous system, and delaying gastric emptying. Intestinal absorption of Ex-4 after oral administration of EL-CSG is believed to reduce HbA1c (-3.1%) and lead to weight loss (-13.6%) via these mechanisms. With regard to cholesterol levels, T-Chol decreased by 22.8%, HDL increased by 66.3%, and LDL decreased by 38.1% in the EL-CSG administration group, showing the same trends as in the group administered Ex-4 free solution by SC injection. It is known that GLP-1 decreases chylomicron absorption from the small intestine, inhibits fat synthesis through activation of 5' adenosine monophosphate-activated protein kinase in the liver, accumulates fat in the liver, and decreases triglycerides in the blood [57]. It has also been reported to suppress cholesterol absorption in the small intestine and enhance expression of LDL receptors in the liver [58].

These results suggest that intestinal absorption of Ex-4 via ASBT is unlikely to significantly affect cholesterol homeostasis. Regarding diabetes treatment, it is important to comprehensively manage blood sugar, blood lipids, and blood pressure. The Ex-4 formulation has a multifaceted action in addition to its hypoglycemic effect, and is expected to inhibit diabetic complications. Therefore, this Ex-4 formulation has potential as a future antidiabetic drug. In addition, the present pharmaceutical technology, which can extend drug absorption after oral administration, is a promising preparation for the oral delivery of many drugs, including those targeting diabetes.

4. Conclusions

To evaluate the translational potential of oral nanoparticle absorption utilizing bile acid transporters, a new cationic liposomal preparation loaded with Exendin-4 and coated with anionic chondroitin sulfate-*g*-glycocholic acid was developed. The observations of oral bioavailability, sustained pharmacokinetics, good glucose response rates, and long-term

pharmacodynamic effects provide support that the formulation and delivery route are safe and effective for treating type 2 diabetes in rats.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was partially supported by the National Institutes of Health (NIH DK114015-01).

References

- [1]. Todd JF, Bloom SR, Incretins and other peptides in the treatment of diabetes, *Diabet. Med* 24 (2007) 223–232. [PubMed: 17263764]
- [2]. Drucker DJ, Biologic actions and therapeutic potential of the proglucagon-derived peptides., *Nat. Clin. Pract. Endocrinol. Metab* 1 (2005) 22–31. [PubMed: 16929363]
- [3]. Holst JJ, Gromada J, Role of incretin hormones in the regulation of insulin secretion in diabetic and nondiabetic humans., *Am. J. Physiol. Endocrinol. Metab* 287 (2004) E199–206. [PubMed: 15271645]
- [4]. Davidson MB, Bate G, Kirkpatrick P, Exenatide, *Nat. Rev. Drug Discov* 4 (2005) 713–714. [PubMed: 16178120]
- [5]. Nielsen LL, Young AA, Parkes DG, Pharmacology of exenatide (synthetic exendin-4): A potential therapeutic for improved glycemic control of type 2 diabetes, *Regul. Pept* 117 (2004) 77–88. [PubMed: 14700743]
- [6]. Gedulin BR, Smith PA, Jodka CM, Chen K, Bhavsar S, Nielsen LL, Parkes DG, Young AA, Pharmacokinetics and pharmacodynamics of exenatide following alternate routes of administration, *Int. J. Pharm* 356 (2008) 231–238. [PubMed: 18291606]
- [7]. Talbot K, Brain insulin resistance in Alzheimer's disease and its potential treatment with GLP-1 analogs, *Neurodegener. Dis. Manag* 4 (2014) 31–40. [PubMed: 24640977]
- [8]. Aviles-Olmos I, Dickson J, Kefalopoulou Z, Djamshidian A, Ell P, Soderlund P, Whitton P, Wyse R, Isaacs T, Lees A, Limousin P, Foltynie T, Exenatide and the treatment of patients with Parkinson's disease, *J. Clin. Invest* 123 (2013) 2370–2736.
- [9]. Simonsen L, Holst JJ, Deacon CF, Exendin-4, but not glucagon-like peptide-1, is cleared exclusively by glomerular filtration in anaesthetised pigs, *Diabetologia* 49 (2006) 706–712. [PubMed: 16447056]
- [10]. Hamman JH, Enslin GM, Kotzé AF, Oral delivery of peptide drugs: Barriers and developments, *BioDrugs* 19 (2005) 165–177. [PubMed: 15984901]
- [11]. Su YC, Jin CH, Han JS, Yu SY, Lee S, Kang CL, Preparation, characterization, and application of biotinylated and biotin-PEGylated glucagon-like peptide-1 analogues for enhanced oral delivery, *Bioconjug. Chem* 19 (2008) 334–341. [PubMed: 18078308]
- [12]. Jin CH, Chae SY, Son S, Kim TH, Um KA, Youn YS, Lee S, Lee KC, A new orally available glucagon-like peptide-1 receptor agonist, biotinylated exendin-4, displays improved hypoglycemic effects in db/db mice, *J. Control. Release* 133 (2009) 172–177. [PubMed: 18977255]
- [13]. Nguyen HN, Wey SP, Juang JH, Sonaje K, Ho YC, Chuang EY, Hsu CW, Yen TC, Lin KJ, Sung HW, The glucose-lowering potential of exendin-4 orally delivered via a pH-sensitive nanoparticle vehicle and effects on subsequent insulin secretion in vivo, *Biomaterials* 32 (2011) 2673–2682. [PubMed: 21256586]
- [14]. Goldberg M, Gomez-Orellana I, Challenges for the oral delivery of macromolecules, *Nat. Rev. Drug Discov* 2 (2003) 289–295. [PubMed: 12669028]
- [15]. Shi Y, Sun X, Zhang L, Sun K, Li K, Li Y, Zhang Q, Fc-modified exenatide-loaded nanoparticles for oral delivery to improve hypoglycemic effects in mice, *Sci. Rep* 8 (2018).

- [16]. Malagelada JR, DiMagno EP, Summerskill WHJ, Go VLW, Regulation of pancreatic and gallbladder functions by intraluminal fatty acids and bile acids in man, *J. Clin. Invest* 58 (1976) 493–499. [PubMed: 956380]
- [17]. Schiff ER, Small NC, Dietschy JM, Characterization of the kinetics of the passive and active transport mechanisms for bile acid absorption in the small intestine and colon of the rat., *J. Clin. Invest* 51 (1972) 1351–1362. [PubMed: 5024036]
- [18]. Inagaki T, Moschetta A, Lee Y-K, Peng L, Zhao G, Downes M, Yu RT, Shelton JM, Richardson JA, Repa JJ, Mangelsdorf DJ, Kliewer SA, Regulation of antibacterial defense in the small intestine by the nuclear bile acid receptor, *Proc. Natl. Acad. Sci* 103 (2006) 3920–3925. [PubMed: 16473946]
- [19]. Alrefai WA, Gill RK, Bile acid transporters: Structure, function, regulation and pathophysiological implications, *Pharm. Res* 24 (2007) 1803–1823. [PubMed: 17404808]
- [20]. Hofmann AF, The enterohepatic circulation of bile acids in man, *Clin Gastroenterol* 6 (1977) 3–24. [PubMed: 330051]
- [21]. Hofmann AF, Hofmann F, Hofmann Alan, A.F., The enterohepatic circulation of bile acids in mammals: form and functions, *Front. Biosci Volume* (2009) 2584.
- [22]. Cammisotto PG, Renaud C, Gingras D, Delvin E, Levy E, Bendayan M, Endocrine and exocrine secretion of leptin by the gastric mucosa, *J. Histochem. Cytochem* 53 (2005) 851–860. [PubMed: 15995144]
- [23]. Carmel R, How I treat cobalamin (vitamin B12) deficiency, *Blood* 112 (2008) 2214–2221. [PubMed: 18606874]
- [24]. Al-Hilal TA, Chung SW, Alam F, Park J, Lee KE, Jeon H, Kim K, Kwon IC, Kim IS, Kim SY, Byun Y, Functional transformations of bile acid transporters induced by high-affinity macromolecules, *Sci. Rep* 4 (2014).
- [25]. Khatun Z, Nurunnabi M, Reeck GR, Cho KJ, Lee YK, Oral delivery of taurocholic acid linked heparin-docetaxel conjugates for cancer therapy, *J. Control. Release* 170 (2013) 74–82. [PubMed: 23665255]
- [26]. Kim KS, Suzuki K, Cho H, Youn YS, Bae YH, Oral Nanoparticles Exhibit Specific High-Efficiency Intestinal Uptake and Lymphatic Transport, *ACS Nano* 12 (2018) 8893–8900. [PubMed: 30088412]
- [27]. Zhu C, Huang Y, Zhang X, Mei L, Pan X, Li G, Wu C, Comparative studies on exenatide-loaded poly (D,L-lactic-co-glycolic acid) microparticles prepared by a novel ultra-fine particle processing system and spray drying, *Colloids Surfaces B Biointerfaces* 132 (2015) 103–110. [PubMed: 26037698]
- [28]. Griffin BT, Guo J, Presas E, Donovan MD, Alonso MJ, O'Driscoll CM, Pharmacokinetic, pharmacodynamic and biodistribution following oral administration of nanocarriers containing peptide and protein drugs, *Adv. Drug Deliv. Rev* 106 (2016) 367–380. [PubMed: 27320644]
- [29]. He C, Yin L, Tang C, Yin C, Size-dependent absorption mechanism of polymeric nanoparticles for oral delivery of protein drugs, *Biomaterials* 33 (2012) 8569–8578. [PubMed: 22906606]
- [30]. Rees DO, Crick PJ, Jenkins GJ, Wang Y, Griffiths WJ, Brown TH, Al-Sarireh B, Comparison of the composition of bile acids in bile of patients with adenocarcinoma of the pancreas and benign disease, *J. Steroid Biochem. Mol. Biol* 174 (2017) 290–295. [PubMed: 29031685]
- [31]. Roda a, Minutello a, Angellotti M. a, Fini a, Bile acid structure-activity relationship: evaluation of bile acid lipophilicity using 1-octanol/water partition coefficient and reverse phase HPLC., *J. Lipid Res* 31 (1990) 1433–1443. [PubMed: 2280184]
- [32]. Suzuki AZ, Watanabe T, Kawamoto M, Nishiyama K, Yamashita H, Ishii M, Iwamura M, Furuta T, Coumarin-4-ylmethoxycarbonyls as Phototriggers for Alcohols and Phenols, *Org. Lett* 5 (2003) 4867–4870. [PubMed: 14653694]
- [33]. Sarmiento B, Ribeiro A, Veiga F, Ferreira D, Development and validation of a rapid reversed-phase HPLC method for the determination of insulin from nanoparticulate systems, *Biomed. Chromatogr* 20 (2006) 898–903. [PubMed: 16389645]
- [34]. Lin YH, Chen CTCH, Liang HF, Kulkarni AR, Lee PW, Chen CTCH, Sung HW, Novel nanoparticles for oral insulin delivery via the paracellular pathway, *Nanotechnology* 18 (2007).

- [35]. Chen MC, Wong HS, Lin KJ, Chen HL, Wey SP, Sonaje K, Lin YH, Chu CY, Sung HW, The characteristics, biodistribution and bioavailability of a chitosan-based nanoparticulate system for the oral delivery of heparin, *Biomaterials* 30 (2009) 6629–6637. [PubMed: 19767097]
- [36]. Pandya NT, Jani P, Vanza J, Tandel H, Solid lipid nanoparticles as an efficient drug delivery system of olmesartan medoxomil for the treatment of hypertension, *Colloids Surfaces B Biointerfaces* 165 (2018) 37–44. [PubMed: 29453084]
- [37]. Plasencia AMA, Mowry J, Smith J, Quigley K, In vitro release of fentanyl from transdermal patches in gastric and intestinal fluid, *Clin. Toxicol* 52 (2014) 945–947.
- [38]. Abdelwahed W, Degobert G, Stainmesse S, Fessi H, Freeze-drying of nanoparticles: Formulation, process and storage considerations, *Adv. Drug Deliv. Rev* 58 (2006) 1688–1713. [PubMed: 17118485]
- [39]. Chang L, Shepherd D, Sun J, Ouellette D, Grant KL, Tang X, Pikal MJ, Mechanism of protein stabilization by sugars during freeze-drying and storage: Native structure preservation, specific interaction, and/or immobilization in a glassy matrix?, *J. Pharm. Sci* 94 (2005) 1427–1444. [PubMed: 15920775]
- [40]. Li M, Si L, Pan H, Rabba AK, Yan F, Qiu J, Li G, Excipients enhance intestinal absorption of ganciclovir by P-gp inhibition: Assessed in vitro by everted gut sac and in situ by improved intestinal perfusion, *Int. J. Pharm* 403 (2011) 37–45. [PubMed: 20969937]
- [41]. Surampalli G, Nanjwade BK, Patil PA, Safety evaluation of naringenin upon experimental exposure on rat gastrointestinal epithelium for novel optimal drug delivery, *Drug Deliv* 23 (2016) 512–524. [PubMed: 24937377]
- [42]. Teply BA, Tong R, Jeong SY, Luther G, Sherifi I, Yim CH, Khademhosseini A, Farokhzad OC, Langer RS, Cheng J, The use of charge-coupled polymeric microparticles and micromagnets for modulating the bioavailability of orally delivered macromolecules, *Biomaterials* 29 (2008) 1216–1223. [PubMed: 18082254]
- [43]. Khan HBH, Vinayagam KS, Moorthy BT, Palanivelu S, Panchanatham S, Anti-inflammatory and anti-hyperlipidemic effect of semecarpus anacardium in a high fat diet: STZ-induced type 2 diabetic rat model, *Inflammopharmacology* 21 (2013) 37–46. [PubMed: 22274733]
- [44]. Stern MP, Williams K, Haffner SM, Identification of persons at high risk for type 2 diabetes mellitus: Do we need the oral glucose tolerance test?, *Ann. Intern. Med* 136 (2002) 575–581. [PubMed: 11955025]
- [45]. Chuang EY, Nguyen GTH, Su FY, Lin KJ, Chen CT, Mi FL, Yen TC, Juang JH, Sung HW, Combination therapy via oral co-administration of insulin- and exendin-4-loaded nanoparticles to treat type 2 diabetic rats undergoing OGTT, *Biomaterials* 34 (2013) 7994–8001. [PubMed: 23891516]
- [46]. Avignon A, Radauceanu A, Monnier L, Nonfasting plasma glucose is a better marker of diabetic control than fasting plasma glucose in type 2 diabetes, *Diabetes Care* 20 (1997) 1822–1826. [PubMed: 9405900]
- [47]. Cortesi R, Esposito E, Gambarin S, Telloli P, Menegatti E, Nastruzzi C, Preparation of liposomes by reverse-phase evaporation using alternative organic solvents, *J. Microencapsul* 16 (1999) 251–256. [PubMed: 10080118]
- [48]. Szoka F, Papahadjopoulos D, Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation., *Proc. Natl. Acad. Sci* 75 (1978) 4194–4198. [PubMed: 279908]
- [49]. Taylor K, Kim D, Nielsen LL, Aisporna M, Baron AD, Fineman MS, Day-long subcutaneous infusion of exenatide lowers glycemia in patients with type 2 diabetes, *Horm. Metab. Res* 37 (2005) 627–632. [PubMed: 16278786]
- [50]. Almeida JD, Edwards DC, Brand CM, Heath TD, Formation of virosomes from influenza subunits and liposomes, *Lancet* 306 (1975) 899–901.
- [51]. Weisman S, Hirsch-Lerner D, Barenholz Y, Talmon Y, Nanostructure of cationic lipidoligonucleotide complexes, *Biophys. J* 87 (2004) 609–614. [PubMed: 15240493]
- [52]. Sun Y, Wang M, Sun B, Li F, Liu S, Zhang Y, Zhou Y, Chen Y, Kong W, An investigation into the gastrointestinal stability of exenatide in the presence of pure enzymes, everted intestinal rings and intestinal homogenates, *Biol. Pharm. Bull* 39 (2016) 42–48. [PubMed: 26725426]

- [53]. Busch U, Schmid J, Heinzel G, Schmaus H, Baierl J, Huber C, Roth W, Pharmacokinetics of meloxicam in animals and the relevance to humans, *Drug Metab. Dispos* 26 (1998) 576–584. [PubMed: 9616195]
- [54]. Martinez MN, Amidon GL, A mechanistic approach to understanding the factors affecting drug absorption: A review of fundamentals, *J. Clin. Pharmacol* 42 (2002) 620–643. [PubMed: 12043951]
- [55]. Wilkinson GR, The effects of diet, aging and disease-states on presystemic elimination and oral drug bioavailability in humans, *Adv. Drug Deliv. Rev* 27 (1997) 129–159. [PubMed: 10837555]
- [56]. Skovsø S, Modeling type 2 diabetes in rats using high fat diet and streptozotocin, *J. Diabetes Investig* 5 (2014) 349–358.
- [57]. Ben-Shlomo S, Zvibel I, Shnell M, Shloma A, Chepurko E, Halpern Z, Barzilai N, Oren R, Fishman S, Glucagon-like peptide-1 reduces hepatic lipogenesis via activation of AMP-activated protein kinase, *J. Hepatol* 54 (2011) 1214–1223. [PubMed: 21145820]
- [58]. Li L, Miao Z, Liu R, Yang M, Liu H, Yang G, Liraglutide prevents hypoadiponectinemia-induced insulin resistance and alterations of gene expression involved in glucose and lipid metabolism., *Mol. Med* 17 (2011) 1168–1178. [PubMed: 21785811]

Fig. 1.

a) Schematic representation of EL-CSG. b) Drug release profile of EL, EL-CS, and EL-CSG in Simulated Intestinal Fluid (pH 6.8) (mean $\pm$ SD, n=3). Transmission electron micrographs of the freeze-dried nanoparticles c) Blank liposome, d) EL, e) EL-CS, and f) EL-CSG in the presence of trehalose after being resuspended in distilled water.

Fig. 2.

a) Nanoparticle transport into EGS tissue in Krebs-Ringer solution containing EL, EL-CS, EL-CSG, or free Ex-4 solution (0.05 mg/mL, 37 °C, 90 min incubation). b) LDH release from 50 µg/mL EL-CS-, EL-CSG-, or free solution-treated EGS tissue of the distal ileum after a 90-min incubation (mean $\pm$ SD, n=4). **p < 0.01.

Fig. 3. Plasma Ex-4 level versus time profiles of Sprague-Dawley rats. Free Ex-4 solution (20 $\mu\text{g/kg}$) was administered by SC injection. EL-CS and EL-CSG (200 $\mu\text{g/kg}$) were administered by oral gavage. (mean $\pm$ SEM, $n=6$)

Fig. 4.

a) Blood glucose level versus time profiles of T2DM rats. Glucose (1 g/kg) was administered orally at 0 h. Two hours prior to glucose administration, 20 μ g/kg of free Ex-4 solution was injected SC, while 300 μ g/kg of EL-CS or EL-CSG was administered orally. b) Comparison of blood glucose concentrations 1 h post-glucose administration. (mean $\pm$ SEM, n=6) [#]p < 0.1, ^{*}p < 0.05, ^{**}p < 0.01 compared to DC group.

Fig. 5.

Comparison of a) HbA1c, b) body weight change, c) T-Chol, d) LDL, and e) HDL levels after 4 weeks of treatment with Free Ex-4 solution (20 $\mu\text{g/kg}$, daily SC injection), EL-CS, and EL-CSG (300 $\mu\text{g/kg}$, daily, oral) into T2DM rats (mean $\pm$ SEM, n=6). DC is the group of PBS-treated diabetic rats. NC is the group of PBS-treated normal rats. ** $p < 0.01$, * $p < 0.05$.

Table 1.

Characteristics of CS and CSG-coated Ex-4-loaded liposomes

Formulation	Ex-4 (mg)	Liposome composition		Coating materials		Ex-4 loading efficiency (%) ^a	Ex-4 content (%) ^a	Size (nm) ^a	Zeta potential (mV) ^a
		DOPC (mg)	DOTAP (mg)	CS (mg)	CSG (mg)				
Blank liposome	-	38	39	-	-	-	-	136 ± 1.8	+61 ± 0.4
EL	3	38	39	-	-	77.2 ± 3.4	2.9 ± 0.1	154 ± 0.2	+57 ± 0.6
EL-CS	3	38	39	100	-	72.8 ± 2.2	1.2 ± 0.1	205 ± 2.6	- 33 ± 0.2
EL-CSG	3	38	39	-	100	74.2 ± 2.5	1.2 ± 0.1	229 ± 4.0	- 31 ± 0.2

^aEach value represents the mean ± SD, n=3

Table 2.

Stability of EL-CSG in simulated gastrointestinal fluids

Formulation		Reconstitution media	Size (nm)	PDI ^a
EL-CSG	Freeze drying	Initial	181 ± 1.4	0.12 ± 0.04
		Distilled water	175 ± 2.0	0.13 ± 0.03
		PBS	195 ± 1.3	0.19 ± 0.03
		FaSSGF	201 ± 3.9	0.23 ± 0.05
		FaSSIF-V2	171 ± 1.4	0.13 ± 0.03

^aPDI: polydispersity index

Each value represents the mean ± SD, n=3

Table 3.

Pharmacokinetic analysis of free Ex-4 solution (SC administration), EL-CS, and EL-CSG (oral administration) in normal rats

Formulation	Admin route	Dose volume (μL)	Dose (μg/kg)	T _{max} (h) ^a	C _{max} (ng/mL)	AUC _(0-72h) (ng·h/mL)	BA _R (%)
Free Ex-4 solution	SC	20	20	-	-	111 ± 5	100
EL-CS	Oral	500	200	12	1.3 ± 0.2	46 ± 8	4.1 ± 0.7
EL-CSG	Oral	500	200	3	7.8 ± 1.1	216 ± 23	19.5 ± 2.0

^aC_{max}: maximum plasma concentration; T_{max}: time at which C_{max} occurs; T_{1/2}: terminal half-life; AUC_(0-72h): area under the plasma concentration vs. time curve from 0 to 72 h; BA_R: relative bioavailability.

Where indicated, each value represents the mean ± SEM, n=6