Original Research Paper

Mimicking natural cholesterol assimilation to elevate the oral delivery of liraglutide for type II diabetes therapy

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Abstract

Glucagon-like peptide-1 receptor agonists (GLP-1 RA) are a series of polypeptides broadly applied in the long-term treatment of type II diabetes. However, administration of GLP-RA is mainly through repetitive subcutaneous injection, which may seriously decrease the compliance and safety. Herein, a bio-inspired oral delivery system was designed to enhance the oral absorption of liraglutide (Lira), a kind of GLP-1 RA, by mimicking the natural cholesterol assimilation. 25-hydroxycholesterol (25HC), a cholesterol derivative, was modified on the surface of Lira-loaded FLGA nanoparticles (Lira 25HC NPs) and functioned as a “top-down” actuator to facilitate unidirectional transcytosis across the intestinal epithelium. After oral delivery, Lira 25HC NPs displayed improved therapeutic effect as compared with oral free Lira on type II diabetes db/db mice, as evidenced by multiple relieved diabetic symptoms including the enhanced glucose tolerance, repressed weight growth, improved liver glucose metabolism, decreased fasting blood glucose, HbA1c, serum lipid, and increased β cells activity. Surprisingly, the fasting blood glucose, liver glucose metabolism, and HbA1c of oral Lira-loaded 25HC NPs were comparable to subcutaneous injection of free Lira. Further mechanisms revealed that 25HC ligand could mediate the nanoparticles to mimic natural cholesterol absorption by exerting high affinity towards apical Niemann-Pick C1 Like 1 (NPC1L1) and then basolateral ATP binding cassette transporter A1 (ABCA1) overexpressed on the opposite side of intestinal epithelium. This cholesterol assimilation-mimicking strategy achieve the unidirectional transport across the intestinal epithelium, thus improving the oral absorption of liraglutide. In general, this study established a cholesterol simulated platform and provide promising insight for the oral delivery of GLP-1 RA.

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1. Introduction

Diabetes, is a chronic disease characterized by hyperglycemia with high prevalence and numerous complications, and represents as one of the severest challenges of global public health problems [1]. Glucagon-like peptide-1 (GLP-1) is an insulin-releasing hormone mainly secreted by intestinal L cells. Its analogue, named GLP-1 receptor agonists (GLP-1 RA), possesses glucose-dependent hypoglycemic ability and is clinically used for diabetes that cannot be controlled by oral hypoglycemic drugs (metformin, etc.) [2]. Furthermore, GLP-1 RA can also stimulate the proliferation of β cells, reduce weight, and protect the cardiovascular, kidney and nervous systems for diabetics [2–4].

At present, the vast majority of GLP-1 RA are administered via subcutaneous injection, but the repetitive injection leads to poor patient compliance and various side effects. Oral administration can significantly improve patient compliance. In addition, the oral absorption of GLP-1 RA through the intestinal tract coincides with endogenous secretion behavior of GLP-1, which can avoid sharp drug explosion in systemic circulation [5,6]. However, the degradation in gastrointestinal tract and low efficiency of trans-epithelium transport substantially reduce the oral absorption of GLP-1 RA [7]. Although FDA approved the first oral GLP-1 RA semaglutide for the treatment of type II diabetes in 2019 [8], the concurrent use of absorption enhancer SNAC might unspecifically open the tight junctions, resulting in safety issues [9,10]. Moreover, the mechanism of SNAC as absorption enhancer is closely related to the molecular structure of semaglutide, which may not be broadly applicable to other GLP-1 RA [8,11]. Therefore, it is of great importance to develop a general strategy that makes oral delivery of GLP-1 RA feasible.

Ligand modified nanoparticles have been widely applied to improve the epithelial absorption after oral administration. However, due to the polar distribution of membrane transport protein in intestine epithelium, the ligand modified nanoparticles may achieve receptor-mediated active endocytosis but encounter the difficulty in basolateral exocytosis [12]. Based on this, we sought to develop strategies to facilitate the unidirectional trans-epithelial transport of nanoparticles. To this end, efficient absorption of natural nutrients in intestine gave us inspiration. Cholesterol is an important nutrient, and the human body needs at most 200 mg/d to complement the consumed cholesterol [13,14]. There is a natural cholesterol absorption pathway in the intestine: the ingested cholesterol first binds to Niemann-Pick C1 Like 1 (NPC1L1) on the apical side of intestinal epithelial to enter the cell, and then transport to the basolateral side of cells by ATP binding cassette transporter A1 (ABCA1), forming a “top-down” unidirectional cholesterol absorption pathway [15–18]. 25-hydroxycholesterol (25HC) is a cholesterol derivative that could be absorbed through the NPC1L1–ABCA1 pathway [19,20]. Inspired by this, we construct a 25HC-modified oral drug delivery system, utilizing one ligand to simultaneously enhance the apical endocytosis and basolateral exocytosis.

Therefore, based on simulating the “top-down” absorption pathway of natural cholesterol, 25HC-modified nanoparticles (25HC NPs) were constructed to deliver the GLP-1 RA Lira as model drug. First, type II diabetic db/db mice were used for long-term pharmacodynamics evaluation. The intraperitoneal injection glucose tolerance test (IPGTT), weight growth, liver glucose metabolism, fasting blood glucose, HbA1c, serum lipid, and β cells activity were evaluated. Next, the mechanisms on the intestinal absorption of 25HC NPs were explored, including apical endocytosis and basolateral exocytosis. As a result, oral delivery of Lira-loaded 25HC NPs could effectively relieve the diabetic symptom of db/db mice. This study established an absorption enhancer-free delivery system, complying the absorption of natural nutrient and broadening the idea of oral GLP-1 RA development.

2. Materials and methods

2.1. Materials

PLGA (50/50, viscosity: 0.26–0.54 dL/g) with methoxy ester end was purchased from Lactel absorbable polymers (California, USA). 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)] (DSPE–PEG) was purchased from Avanti Polar Lipids (Alabama, USA). DSPE–PEG with an amino end group (DSPE–PEG–NH₂) was purchased from Ponsure Biotechnology (Shanghai, China). 25HC was purchased from Kangshan biotechnology (Zhengzhou, China). C6 was purchased from Sigma-Aldrich (Missouri, USA). Lira was a kind gift from HEC Pharmaceutical (Guangdong, China). Mouse anti-insulin antibody was purchased from Cell Signaling Technology (Massachusetts, USA) and rabbit anti-glucagon antibody was purchased from Santa Cruz Biotechnology (Texas, USA). Goat anti-mouse IgG Alexa Fluor488 and donkey anti-rabbit IgG Alexa Fluor647 were purchased from Thermo Fisher Scientific (Massachusetts, USA). Rabbit anti-ABCA1 antibody was purchased from Biosynthesis Biotechnology (Beijing, China). All other reagents and chemicals were purchased from commercial products of analytical or reagent grade.

2.2. Synthesis of DSPE–PEG–25HC

25HC–COOH was adopted as an intermediate to synthesize DSPE–PEG–25HC. Briefly, 25HC (80 mg), succinic anhydride (40 mg) and dimethyl aminopropidine (DMAP, 20 mg) were dissolved in dichloromethane (1 mL). Then the reaction liquid was stirred at room temperature for 24 h. Subsequently, dichloromethane was removed by rotary evaporation at 40 °C and 5 mL saturated sodium chloride solution was added to hydrolyze unreacted succinic anhydride at 40 °C for 2 h. Finally, the hydrolyzed liquid was mixed with dichloromethane to extract 25HC–COOH and then dried 25HC–COOH was obtained via rotary evaporation.

25HC–COOH and DSPE–PEG–NH₂ were linked by amido linkage. Briefly, 25HC–COOH (20 mg), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI, 11.5 mg) and N-hydroxy succinimide (NHS, 6.9 mg) were dissolved in DMSO. After activating -COOH for 0.5 h at 37 °C, DSPE-PEG-NH₂ (56 mg) was added and the pH value was
adjusted to 8–9 by triethanolamine. After being stirred at 37°C for 24 h, the reaction liquid was dialyzed by DMSO for the first 12 h and purified water for later 24 h at 25°C. In the end, dialysis solution was lyophilized for 48 h. \(^1\)H NMR was used to confirm the structure of 25HC-COOH and DSPE-PEG-25HC. The linking efficiency of 25HC-COOH with DSPE-PEG-NH\(_2\) was determined by quantifying 25HC hydrolyzed from DSPE-PEG-25HC after incubation with alkali. Briefly, DSPE-PEG-25HC was dissolved in the mixture of DMSO and 6 M NaOH (1:9, v/v) at 2 mg/ml. After incubation at 37°C for 24 h, 1 equal volume of 6 M HCl was added to neutralize NaOH and the mixture was mixed with dichloromethane to extract free 25HC. Finally, dichloromethane was removed by nitrogen blow at 40°C. The dry powder was dissolved by methanol and measured by reverse-phase high-performance liquid chromatography (HPLC) (Agilent 1260 series; Diamonsil C\(_18\) column: 150 mm × 4.6 mm, 5 μm; Detection wavelength 208 nm; Mobile phase: Methanol: \(\text{H}_2\text{O}=9:1;\) Flow rate:1 ml/min).

### 2.3 Preparation and characterization of 25HC modified NPs

The PLGA nanoparticles were prepared by nano-precipitation with the microfluidics chip. Briefly, PLGA, DSPE-PEG, DSPE-PEG-25HC and phospholipid (PL) were dissolved in DMSO individually as stock solution (16 mg/ml). The mixture of PLGA, DSPE-PEG, and PL stock solution (4:3:1, v/v) was diluted with purified water in the volume ratio of 1:10 to 1:15 in cooperation with the microfluidic chip. The flow rate of the organic phase and aqueous phase was 0.16 and 1 ml/min, respectively. For drug-loaded nanoparticles, C6 (0.2%, w/w) or Lira (16 mg/ml) was added. 25HC NPs were prepared by replacing different percentage of DSPE-PEG with DSPE-PEG-25HC. DSPE-PEG-25HC accounted for 0, 50% and 100% of the total amount of DSPE-PEG were recorded as 0, 50% and 100% 25HC NPs, respectively. Then the organic solvent and unloaded ingredient were removed by ultrafiltration (MWCO 100 kDa).

Nanoparticles were re-dispersed with PBS or other solution. The particle size and zeta potential of nanoparticles were determined by Zetasizer Nano ZS90 (Malvern Instruments, UK). The morphology of nanoparticles was observed by Transmission electron microscopy (TEM). To determine the dynamic stability of nanoparticles solution, the change of particle size was evaluated within 24 h at 37°C. The entrapment efficiency (EE) and drug loading efficiency (DL) of Lira-loaded nanoparticles were determined after ultrafiltration (3000 g, 30 min, MWCO 100 kDa). The Lira amount in the filter liquor and filter residue was quantified by HPLC(Agilent 1260 series. Diamonsil C18 column, 150 mm × 4.6 mm, 5 μm). Detection wavelength, 214 nm. The mobile phase, \(\text{H}_2\text{O} (0.2\% \text{TFA})\): Acetonitrile = 49:51. Flow rate, 1 ml/min) and the mass of nanoparticles was measured after lyophilization. All assays were performed in triplicate. The EE and DL were calculated using the following formulas:

\[
\text{EE} (%) = \frac{\text{Lira loaded in nanoparticles}}{\text{Lira loaded in nanoparticles} + \text{free Lira}} \times 100%
\]

\[
\text{DL} (%) = \frac{\text{Lira loaded in nanoparticles}}{\text{weight of nanoparticles}} \times 100%
\]

### 2.4 Stability, release, and anti-enzymolysis of Lira-loaded NPs

To determine the dynamic stability of Lira-loaded NPs, the change of particle size was evaluated over time incubated with simulated gastric fluid (SGF) or simulated intestinal fluid (SIF) at 37°C. To investigate the Lira release, Lira-loaded NPs were placed into a dialysis tube (MWCO 100 kDa, Spectrum Laboratories, Massachusetts, USA). The tubes were floated on 21.6 SGF (without pepsin) for the first 2h and on 21.6 SIF (without trypsin) for the later 6h. 100 μl nanoparticles was taken out at 0.5, 1, 2, 3, 4, 6 and 8 h for quantitative measurement of Lira by HPLC. The protection of nanoparticles for Lira against trypsin was studied. Nanoparticles loading Lira were incubated with SGF (trypsin, 20 μg/ml) for 4h. 100 μl sample was trapped out at determined time and mixed with 2% trifluoroacetic acid to terminate enzymolysis. The remaining Lira was measured by HPLC.

### 2.5 Animals and cells

Animals received care in accordance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Sichuan University. C57BL/6 mice were purchased from Chengdu Dashuo Bio-tech (Sichuan, China). Db/db mice were purchased from Gempfarmatech (Jiangsu, China). Caco-2 cells were cultured in Dulbecco’s modified eagle medium (DMEM) with high glucose, 15% (v/v) fetal bovine serum, 1.5% (v/v) nonessential amino acid, 1.5% (v/v) L-glutamine, 1.5% penicillin and streptomycin (100 IU/ml). Cells were incubated at 37°C and 95% relative humidity with 5% CO\(_2\) (v/v).

### 2.6 In vivo absorption of NPs in healthy mice

C57BL/6 mice were used to evaluate the oral absorption of C6-labeled 25HC NPs. After 12 h fasting, different formulations (C6: 1 mg/kg) were administered to the mice via oral gavage. The peptide INF-7 was orally administrated to exclude the influence of the lysosome trap. At determined time, 20 μl blood were collected from the orbit. The fluorescence intensity of C6 was measured and the concentration was quantified by standard curve.

On the other hand, the intraperitoneal injection glucose tolerance test (IPGTT) after administration of Lira was studied. After 12 h fasting, C57BL/6 mice were ingested with Lira-loaded NPs (5 mg/kg), whereas oral free Lira (5 mg/kg), s.c. free Lira (0.2 mg/kg), and saline as control. Then, 2 g/kg glucose was intraperitoneal injected. At determined time, the blood glucose was tested from tail vein by the glucometer.

### 2.7 Treatment of type II diabetes with Lira-loaded NPs

#### 2.7.1 IPGTT of single dose Lira in db/db mice

Furtherly, the intraperitoneal injection glucose tolerance test (IPGTT) after administration of Lira was studied in db/db mice.
Excepting the different glucose dosage of 1 g/kg, the other protocol was the same as to IPGTT experiment in 2.6.

2.7.2. Long-term treatment of Lira in db/db mice
For long-term therapeutic evaluation, the db/db mice were randomly divided into five groups: oral Lira-loaded 25HC NPs (5 mg/kg); s.c. free Lira (0.2 mg/kg); oral Lira-loaded PEG NPs (5 mg/kg); oral free Lira (5 mg/kg); oral saline. The mice were given a dose once a day after 4 h fasted, and the fasting weight and fasting blood glucose were recorded every 4 d after 28 d of therapeutic, the HbA1C was measured by NycoCard® READER II (Kjelsasveien, Norway). Then, the IPGTT of db/db mice was studied to evaluate the anti-hyperglycemia potential after long-term administration.

The db/db mice were sacrificed and the blood was collected to measure triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDLC) and low-density lipoprotein cholesterol (LDLC). Heart, liver, spleen, lung, and kidney were collected for histological analysis. The hepatic glycogen was measured by Glycogen Assay Kit (Solarbio Science & Technology, Beijing, China). In addition, pancreas was collected, immuno-labeled and embedded by paraaffin. Immunofluorescence staining of insulin and glucagon in islets were performed using mouse anti-insulin primary antibody and rabbit anti-glucagon primary antibody, and goat anti-mouse IgG Alexa Fluor488 and donkey anti-rabbit IgG Alexa Fluor647 served as the secondary antibodies, respectively. Finally, images were collected by confocal laser scanning microscope (CLSM, Zeiss LSM 800) at equal exposure conditions analyzed by using the Image-Pro-Plus.

2.8. Apical endocytosis of NPs

To evaluate cellular uptake of nanoparticles, Caco-2 cells were seeded on 96-well plates at the density of 1 × 10⁵/well and were cultured 4–5 d before use. Cell number was first normalized by relative viability using Alamar blue assay. Then, cells were incubated with C6-labeled nanoparticles for 2 h. After being washed by PBS three times, the intracellular fluorescence intensity (485/520 nm) was measured. The uptake efficiency was presented by the ratio of nanoparticles to resazurin and normalized by PEG NPs.

Additionally, the cellular uptake was qualitatively analyzed via fluorescence microscopy. Briefly, cells were seeded on a glass coverslip at 100 cells/well and were cultured 4–5 d before use. Cells were incubated with C6-labeled nanoparticles for 2 h. After incubation, the cells were washed with PBS, immobilized by 4% paraformaldehyde. At last, DAPI (5 μg/ml) was applied to stain the nucleus before observation by CLSM.

To identify the endocytosis pathways of different nanoparticles, Caco-2 cells were incubated with specific internalization inhibitors. Cell viability was first determined by Alamar blue assay. Thereafter, cells were treated by inhibitors for the first 0.5 h and were exposed to C6-labeled nanoparticles containing inhibitors for another 2 h. The inhibitors included NaN₃ (1 mM), MβCD (10 mM), Chlorpromazine (CPZ, 30 μM), Amiloride (12 μg/ml), Simvastatin (10 μg/ml), Hypertonic sucrose (0.4 M) and Ezetimibe (10, 20, 50 μM). Finally, the nanoparticles were washed out and cells were dissolved by DMSO. The intracellular fluorescence intensity was measured and the groups without inhibitors were set as control.

2.9. Basolateral exocytosis of NPs

The expression of ABCA1 transporter was visualized by CLSM. Briefly, Caco-2 cells were seeded on Transwell® at the density of 3–5 × 10⁴ cells/well and cultured for 14–21 d until the TEER value reached the 400 Ω·cm². Nanoparticles were incubated with the Caco-2 cell monolayer for 4 h. Then, the polycarbonate membrane seeding Caco-2 cell monolayer was cut out and immersed in 4% paraformaldehyde immobilization. Then, the membrane was incubated with 0.2% Triton X100 for 5 min and with 5% BSA for 5 min at 37 °C. The immune-fluorescent staining of ABCA1 was performed by incubating with rabbit anti-ABCA1 primary antibody for 24 h at 4 °C and goat anti-rabbit IgG Alexa Fluor647 for 1 h at 37 °C. The following steps are the same as 2.7.1. In order to evaluate the effect of 25HC on ABCA1 expression, a group of cell monolayer was incubated with 25HC.

2.10. Transcytosis of NPs

For the transcytosis tests, Caco-2 cells were seeded on Transwell® at the density of 3–5 × 10⁴ cells/well and cultured for 14–21 d until the TEER value reached the 400 Ω·cm². Prior to the study, Caco-2 monolayers were washed by HBSS and the chambers were equilibrated for 0.5 h at 37 °C. 200 μl C6-labeled nanoparticles were added to the apical chamber and 800 μl blank HBSS was added to the basolateral chamber. The peptide INF-7 was used to exclude the influence of the lysosome trap. At the determined time, 80 μl HBSS was sampled from the basolateral chamber followed by restituted of blank HBSS. The samples were dissolved in DMSO before measured by Varioskan Flash multimode reader. The apparent permeability coefficient (Papp) was calculated using the following formula

\[ Papp = \frac{dQ}{dt} \times \frac{1}{A \times C_0} \]

Where, the dQ/dt is the flux of C6-labeled nanoparticles from chamber A to chamber B, C₀ is the initial fluorescence intensity of nanoparticles, and A is the membrane area (cm²).

Furtherly, an in-situ model was used to evaluate the transcytosis of nanoparticles. SD rats were fasted but free to water for 24 h before the experiment. Rats were anesthetized and the abdominal cavity was opened. At the jejunum, a loop was ligated and 500 μl C6-labeled PEG NPs or 25HC NPs were injected into the loop. The loop was placed back into the abdominal cavity. After 2 h, the rats were sacrificed and the loop was removed, washed, fixed in 4% paraformaldehyde, and dehydrated with 30% sucrose overnight. Then, the loop was embedded by the optimal cutting temperature compound and cut into 10 μm slices. The ABCA1 was stained by rabbit anti-ABCA1 primary antibody for 1 h at 37 °C and goat anti-rabbit IgG Alexa Fluor647 for 1 h at 37 °C. The following steps are the same as the above-mentioned.
3. Results and discussions

3.1. Synthesis of 25HC modified DSPE-PEG

DSPE-PEG is a phospholipid-polyethylene glycol polymer widely used in drug delivery. One of its major features is its ability to modify a variety of terminal functional groups, such as histidine [21], butyric acid [22] and R8 [23]. Besides, PEG shielding permits the nanoparticles to penetrate the mucus [22,23]. 25HC is a hydroxyl derivative of cholesterol with strong hydrophobicity. The hydroxyl at C25 has low reactivity due to steric hindrance. In the human body, esterification of the C3 hydroxyl group of cholesterol is the most important metabolic reaction [24]. Inspired by this, succinic anhydride was used to react with 25HC [25] so that the C3 hydroxyl group could alcoholize succinic anhydride, thereby exposing the free carboxyl group to obtain carboxylated 25HC (25HC-COOH). Then DSPE-PEG-NH2 and 25HC-COOH were conjugated together by amide reaction.

The structure of the product was verified by proton 1H NMR spectroscopy. The hydrogen peak (C3) of 25HC-COOH originally located at 3.5 ppm moved to 4.6 ppm as compared with the 25HC spectrum, which was affected by adjacent succinic acid groups. At the same time, a typical peak of methylene succinate appeared at 2.5–2.7 ppm, confirming the successful linkage of succinic acid (Fig. S1A). Based on spectroscopy of DSPE-PEG-NH2, the characteristic peak of 25HC appeared at 0.5–1.5 ppm, indicating that DSPE-PEG-25HC was successfully synthesized (Fig. S1B-C). To quantitatively determine the conjugation efficiency between DSPE-PEG and 25HC, the linked 25HC was hydrolyzed and then measured (Fig. S1D). The HPLC results showed that free 25HC and linked 25HC could be effectively distinguished. And according to the standard curve (y = 5491.8x–1.0396, R2=0.9999), the conjugation efficiency was 92.3±12.3%.

3.2. Preparation and characterization of 25HC NPs

As illustrated in Fig. 1A, phospholipids and PLGA constituted the core of the nanoparticle, in which DSPE-PEG-25HC was inserted on the surface, and Lira was loaded inside. Initially, the mixed DMSO was dropped into water. The materials dissolved in the organic phase assembled into nanoparticles by nanoprecipitation as previous reported [22]. However, the PDI of 25HC NPs was higher than 0.4, which may be attributed to the hydrophobicity of 25HC. Consequently, a microfluidic chip was adopted to optimize the preparation. The organic phase and aqueous phase were injected into the microfluidic chip with individual flow rate (Fig. 1B). Because of the laminar flow of two phases in the chip, the materials could be sufficiently dispersed [26]. After adjusting the ratio and injection rate of two phases, the final obtained nanoparticles showed the size of 139.8±2.4 nm and PDI of 0.227±0.026 (Fig. 1C). Interestingly, the size of 25HC NPs was smaller than PEI NPs (167.7±2.4 nm). This might be due to the hydrophobic 25HC that compressed the PLGA core [27]. Besides, the neutral zeta potential of nanoparticles was beneficial to avoid mucus trapping [23]. TEM image presented in Fig. 1D showed the spherical shape of 25HC NPs with a hydration shell of PEG.

To verify the stability of the nanoparticles during cell experiments and in vivo experiments, the nanoparticles were dispersed in PBS (pH 7.4), simulated gastric fluid (SGF), or simulated intestinal fluid (SIF), and incubated at 37 °C. The particle size was measured at pre-determined time intervals. The results showed that the particle size did not change significantly during 24 h when dispersed in PBS, SGF, or SIF, indicating the good stability (Fig. S2A and S2B). The fluorescent agent coumarin 6 (C6)-labeled nanoparticles were prepared to calculate the relative amount of nanoparticles. The loaded C6 exhibited low diffusion in medium (Fig. S2E), which could be applied in quantitative analysis of cell experiments.

Then the model drug Lira was encapsulated into nanoparticles. The EE and DL were about 80% and 20%, respectively. After loading Lira, the size of 25HC NPs became larger, which was similar with PEG NPs (Fig. 1G). This could be attributed to the fact that when loaded into PEG NPs, Lira occupied the gap in the core without changing size. But as for 25HC NPs, Lira squeezed into the compressed core and led to swelling of size. When incubated within SGF and SIF, the size of Lira-loaded PEG NPs remained unchanged, whereas that of Lira-loaded 25HC NPs slightly increased in SIF. This was possibly because that the released Lira adhered to 25HC on the surface by hydrophobic interaction, leading to larger hydrated particle size. Furthermore, we measured the Lira released to simulated fluid, and the results showed no difference between two NPs with sustained drug release (Fig. 1E). Besides, the nanoparticles could effectively protect Lira against trypsin (Fig. 1F). It was interesting that the resistance to trypsin was stronger after modifying the 25HC on the surface NPs, which may be due to that the released Lira adhered to 25HC could decelerate the enzymolysis of Lira. The released Lira adhered to 25HC could decelerate the enzymolysis of Lira. Compared with insulin in our previous studies, Lira had higher loading ability and slower drug release [28,29]. This might be attributed to the hydrophobic palmilig acid in Lira [30], possessing high affinity to PLGA.

3.3. 25HC NPs show improved oral absorption in healthy mice

To evaluate the efficiency of this oral delivery system, healthy C57BL/6 mice were administrated with C6-labeled NPs, and the profiles of serum C6 concentration with time were shown in Fig. 2A. The results showed that the C6 25HC NPs had enhanced concentration as the Cmax was 1.91-fold higher than that of C6 PEG NPs. The area under curve (AUC) calculated in Fig. 2C was in accordance with Cmax, showing that C6 25HC NPs had the significantly higher AUC with 1.59-fold increase as compared with C6 PEG NPs. For the purpose of oral delivery of Lira, the intraperitoneal injection glucose tolerance test (IPGTT) was firstly investigated in C57BL/6 mice. As shown in Fig. 2B, due to directly perfusing into blood, s.c. free Lira could maintain the blood glucose at the initial level. Of note, oral Lira 25HC NPs also exhibited the capacity to control blood glucose, which appeared to be more efficient than oral free Lira and oral Lira PEG NPs. The AUC of blood glucose-time profile showed s.c. free Lira and oral Lira 25HC NPs groups were significantly lower than oral free Lira and oral Lira PEG NPs (Fig. 2D) (P<0.001). Lira can stimulate glucose-dependent
insulin secretion by binding to G protein-coupled receptors expressed on the surface of pancreatic β cells [31]. Therefore, the change in blood glucose after glucose administration was capable of indirectly reflecting the content of Lira in the body [32], thereby revealing the absorption of oral Lira. Referring to the IPGTT, oral delivered 25HC NPs could effectively enhance the oral absorption of Lira in healthy mice.

3.4. Lira-loaded 25HC NPs could relieve the diabetic symptom of db/db mice

In the following study, the type II diabetic db/db mice were adopted as the model to evaluate our delivery system. According to the above results, mice were divided into five groups, oral free saline as the negative control, s.c. free Lira as the positive control, oral free Lira, oral Lira PEG NPs, and oral Lira 25HC NPs as the experimental group. Firstly, the IPGTT of db/db mice with single-dose Lira was conducted. As shown in Fig. 3A, the s.c. free Lira had the best hypoglycemic effect at 120 min after glucose injection with a blood glucose of 5.2 ± 1.1 mmol/l, which was extremely close to normal level. Although the blood glucose of oral Lira 25HC NPs group could not decrease to normal level, it was significantly lower as compared with oral saline and oral free Lira both at 60 and 90 min (P<0.05), while no significant difference among oral Lira PEG NPs, oral saline, and oral free Lira. Furthermore, due
Fig. 2 - Pharmacokinetic and pharmacodynamic evaluation in healthy mice. (A) Blood concentration of C6 in C57BL/6 mice after oral administration of different formulations (n = 5). *P < 0.05 compared to the C6 PEG NPs. (B) Blood glucose of C57BL/6 mice in the IPGTT (n = 5). *P < 0.05 compared to the Oral free Lira. (C) AUC of C6 Blood concentration (n = 5). **P < 0.01 compared to the C6 PEG NPs. (D) AUC of blood glucose in the IPGTT (n = 5). ***P < 0.001 compared to the Oral free Lira. *P < 0.05. Error bars represent SD.

Fig. 3 - Effects of the formulations on diabetes mellitus. (A) Oral glucose tolerance test (OGTT). *P < 0.05 compared to the Oral free Lira. #P < 0.05 compared to the Oral Lira 25HC NPs. (B) Fasting blood glucose (BG) levels in the different groups. *P < 0.05 compared to the Oral free Lira. (C) Blood glucose level in the IPGTT (n = 5). ***P < 0.001 compared to the Oral free Lira. (D) AUC of blood glucose in the IPGTT (n = 5). ***P < 0.001 compared to the Oral free Lira. *P < 0.05. Error bars represent SD.

Fig. 4 - Effects of the formulations on body weight and body fat. (A) Body weight changes in the different groups. *P < 0.05 compared to the S.C. free Lira. (B) Body fat changes in the different groups. *P < 0.05 compared to the S.C. free Lira.

Fig. 5 - Histological analysis of the liver in db/db mice. (A) H&E staining of the liver in db/db mice. *P < 0.05 compared to the S.C. free Lira. (B) Histological analysis of the liver in db/db mice. *P < 0.05 compared to the S.C. free Lira.
Fig. 3 – Pharmacodynamic evaluation for long-term treatment in type II diabetic db/db mice. (A) Blood glucose profile of db/db mice in the IPGTT with single dose before the long-term treatment (n = 4–6). *P < 0.05 compared to the Oral saline and Oral free Lira. (B) Schedule diagram of long-term treatment in db/db mice, the mice were given dosage once a day after 4 h fasted, and every four days the fasting weight and fasting blood-glucose were recorded. (C) Fasting body weight of db/db mice with long-term treatment (n = 4–6). (D) Fasting blood-glucose of db/db mice with long-term treatment (n = 4–6). *P < 0.05 Oral Lira 25HC NPs compared to the Oral saline and Oral free Lira. *P < 0.05 S.C. free Lira compared to the Oral saline and Oral free Lira. (E) HbA1c of db/db mice before and after long-term treatment (n = 4–6). *P < 0.05 compared to day 0. (F) Blood glucose profile of db/db mice in the IPGTT after the long-term treatment (n = 4–6). *P < 0.05 compared to the Oral saline and Oral free Lira. Error bars represent SD.

TG was reduced remarkably after treatment with s.c. free Lira and oral Lira 25HC NPs (P < 0.05), while TC, LDL-C and HDL-C after treatment with oral Lira 25HC NPs were not significantly decreased. Interestingly, the HDL-C was reduced rather than enhanced in other reports [38,39]. This might be because the value of HDL-C of db/db mice had already exceeded the normal level [40], thus controlling it to the normal range satisfy the purpose of therapy. Certainly, the variation trend of HDL-C was still controversial.

Furthermore, immunofluorescence staining of insulin and glucagon was used to reflect the amount and function of β cells and α cells (Fig. 4G). Treatment with s.c. free Lira or oral Lira 25HC NPs could increase insulin secretion and reduce glucose secretion, and β cells were driven to the center of islets. Such a β-cell centric architecture was typically observed in the islets of normal mice. The relative areas of β cells and α cells were calculated and shown in Fig. 4H. Type II diabetes is invariably associated with a decline in functional β cells mass. Agonists of the GLP-1 receptor, such as Lira, can improve glycemic control via both their acute insulinotropic function and, under certain circumstances, also by chronic action to preserve β cells by stimulating β cells proliferation and inhibiting apoptosis [5,41]. On the other hand, in vivo toxicity of the oral Lira 25HC NPs to multiple organs was assessed. H&E
staining revealed no obvious pathological changes in major organs (Fig. S3), showing the eligible biocompatibility of our delivery system for long-term administration.

These studies demonstrated long-term administration of oral Lira 25HC NPs could elevate glucose metabolism and relieve the diabetic symptom of db/db mice, with a similar degree achieved by s.c. free Lira, including decreasing glucose tolerance, weight growth, fasting blood glucose, and HbA1c, and improving liver glucose metabolism, serum lipid, and β cells activity.

3.5. 25HC NPs have efficient cellular uptake with mediation of NPC1L1

In the following studies, the Caco-2 cells were used to explore the mechanism of the therapeutic effect. Firstly, nanoparticles
had no significant cytotoxicity at tested concentration (Fig. S4). And to some extent, 25HC NPs could enhance the cell viability, probably due to the trophic function of 25HC.

As shown in Fig. 5A, 25HC NPs had higher uptake. The uptake of PEG NPs was standardized to 1, showing that the uptake of 50% and 100% 25HC NPs was about 2.1 and 3.5 times higher than PEG NPs respectively ($P < 0.001$). In addition, the CLSM revealed the effective uptake after 25HC modification (Fig. 5B). Results showed that the cellular uptake efficiency increased with the enhancement of 25HC modification percentage, suggesting the crucial role of 25HC for cellular uptake.

In order to explore the mechanism underlying the increased uptake of 25HC NPs, firstly NPC1L1 specific inhibitor ezetimibe was mixed with nanoparticles and co-incubated with Caco-2 cells [42]. As shown in Fig. 5C, ezetimibe had an inhibitory effect on the uptake of 25HC NPs at low, medium, and high concentrations ($P < 0.05$). However, ezetimibe did not affect the uptake of PEG NPs, indicating 25HC NPs was actively transported into the cells through the specific binding to NPC1L1. Then, various uptake inhibitors were applied to investigate the uptake pathway of nanoparticles (Table S1). The results revealed that PEG NPs were endocytosed into cells mainly through clathrin-mediated and lipid raft/caveolin-mediated pathway. In addition to the above two pathways, 25HC NPs entered through the additional caveolin pathway that was inhibited by simvastatin (Fig. 5D). It is unexpected the uptake of 25HC NPs after treatment of CPZ was higher than control group. CPZ is an amphipathic molecule, it can easily incorporate into the lipid bilayers and can increase lipid fluidity within the plasma membrane [43]. So, we guess the lipid fluidity increase might stimulate the uptake of 25HC NPs because cholesterol constitutes the membrane lipid. The modification of 25HC increased the endocytosis pathways involved, which might also facilitate the cellular uptake of nanoparticles.

### 3.6. 25HC NPs are basolaterally exocytosed by ABCA1

Another difficulty of transcytosis is the basolateral exocytosis. As for the absorption of natural cholesterol, ABCA1 expressed in enterocytes facilitate the basolateral exocytosis, which
Fig. 6 – Transcellular studies of 25HC NPs in vitro. (A) Representative images of CLSM of NPs and ABCA1. (B) The co-localization of NPs with ABCA1. (C) Orthogonal CLSM images of the Caco-2 cell monolayer after transcytosis of NPs. X-Z and Y-Z presents the orthogonal distribution. Blue: nucleus. Green: C6-labeled NPs. Red: ABCA1. Scale bar = 20 µm. (D) Papp of different formulations on Caco-2 monolayer (n = 6). *** P < 0.001 compared to PEG NPs. Error bars represent SD. (E) The in situ absorption of NPs. Blue: nucleus. Green: C6-labeled NPs. Red: ABCA1. Scale bar = 50 µm.

might also be beneficial to the absorption of cholesterol-mimicking 25HC NPs [44,45]. To verify the hypothesis, we first characterized the distribution of ABCA1 in Caco-2 cells. The results of CLSM showed that cells expressed a large number of ABCA1, and the expression of ABCA1 on basolateral side was higher than the apical side, showing the characteristics of polar distribution (Fig. S5A). Next, the free 25HC was incubated with the Caco-2, and the fluorescence of ABCA1 was found to increase (Fig. S5B), meaning that 25HC significantly promoted the expression of ABCA1. ABCA1 plays a role in the cholesterol transport across the basolateral side when stimulated by Liver nuclear X receptors/ retinoic X receptors (LXR/RXR) ligands [45]. LXR act as oxysterol sensors of intracellular cholesterol homeostasis. LXR and LXRβ form obligate heterodimers with retinoid receptors, RXRs, and are bound and activated by a class of naturally occurring oxysterols, thus activating the expression of ABCA1 [46].

Supported by the above results, 25HC NPs were expected to stimulate the expression of ABCA1. Excitingly, a brighter fluorescence of ABCA1 was exhibited after incubated with 25HC NPs when compared with PEG NPs (Fig. 6A), reflecting that 25HC maintained the ability to enhance ABCA1 expression after covalent linkage to nanoparticles. Besides, 25HC NPs had stronger co-localization with ABCA1. The Pearson’s correlation coefficient of 25HC NPs with ABCA1 was 0.197, higher than that of PEG NPs (Fig. 6B). Furthermore, as pointed out by arrows in Fig. 6C, 25HC NPs were more likely to distribute on the basolateral side while PEG NPs were still trapped in the cells mostly. In summary, 25HC NPs could not only bind to ABCA1 but also stimulate ABCA1 expression, so
more 25HC NPs localized at the basolateral membrane for subsequent exocytosis.

3.7. 25HC NPs facilitate transepithelial transport

The above experiment verified the unidirectional transport of nanoparticles from apical endocytosis to basolateral exocytosis. Next, the Transwell® model was constructed to simulate the monolayer structure of the intestinal epithelium and investigate the transmembrane efficiency of nanoparticles. Without influencing the TEER of cell monolayer (Fig. 56), 25HC modification could effectively enhance the apparent permeability coefficient (Papp) of 25HC NPs from 2.11 × 10−6 cm/s to 3.99 × 10−6 cm/s (Fig. 6D). In general, through the active uptake and the exocytosis regulation of 25HC, the transepithelial efficiency of nanoparticles could be effectively improved.

Subsequently, in situ model was used to investigate the absorption of nanoparticles. As shown in Fig. 6E, more ABCA1 were expressed on the basolateral side of the intestinal epithelium. The green fluorescence of nanoparticles displayed that PEG NPs were only adsorbed on the margin of villi, while 25HC NPs entered the basolateral region. And the red fluorescence of ABCA1 overlapped with the green fluorescence of 25HC NPs could be observed. This qualitative result correlated well with the process of in vitro cell models, further illustrated the mechanism of our delivery system. In general, based on enhanced apical endocytosis and promoted basolateral exocytosis, 25HC modification improved the transmembrane efficiency of oral nanoparticles.

4. Conclusion

In summary, to improve the oral absorption of Lira, we developed a nano-delivery system by simulating the absorption of cholesterol. The in vivo studies in db/db mice showed that this oral Lira delivery system could improve glucose metabolism and relieve the diabetic symptom, achieving good therapeutic efficacy. The in vitro cell studies revealed that 25HC NPs had efficient cellular uptake mediated by NPC1L1 on the apical side of the cells. 25HC NPs enhanced ABCA1-mediated basolateral exocytosis, and finally showed increased trans-epithelial transport. Overall, our study provides a more universal approach to orally deliver GLP-1 RA rather than adopting specific coordination agents, broadening the thinking for developing oral GLP-1 RA.

Conflicts of interest

The authors have declared no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ajps.2022.08.002.

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