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Research paper

# Design and evaluation of solid lipid nanoparticles modified with peptide ligand for oral delivery of protein drugs

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ABSTRACT

Designing feasible and effective peptide ligand modified solid lipid nanoparticles (SLNs) to improve oral bioavailability of protein drugs and evaluating the influence of mucus remains important. In the present work, two kinds of peptide ligand modified SLNs loaded with salmon calcitonin (sCT), namely, sCT CSK-SLNs and sCT IRQ-SLNs, were prepared by coupling the peptide ligand CSKSSDYQC (CSK) which was reported to show affinity with goblet cells, or IRQRRRR (IRQ), a cell penetrating peptide, to polyoxyethylene (40) stearate (SA-$PEG_{2000}$). Compared with unmodified SLNs, CSK or IRQ modified SLNs with better drug protection ability could facilitate the internalization of drug on Caco-2/HT29-MTX co-cultured cells and permeation in excised rat duodenum mucosa. The internalization mechanism of two kinds of peptide ligand modified SLNs was mainly active transport via both clathrin- and caveolae-dependent endocytosis. Although mucus was an impediment to the transport of SLNs, the peptide ligand modified SLNs still showed improved drug absorption. The absolute bioavailability of sCT CSK-SLNs (12.41 ± 3.65%) and sCT IRQ-SLNs (10.05 ± 5.10%) raised to 2.45-fold and 1.98-fold compared with unmodified SLNs (5.07 ± 0.54%), implying the feasibility and effectiveness of CSK and IRQ peptide modification for the enhancement of the oral bioavailability of protein drugs. In summary, the nanoparticles modified with CSK or IRQ peptide ligand could be the potential carriers for the transport of protein drugs across intestinal barriers.



## 1. Introduction

Although oral delivery of protein drugs has attracted much attention during the recent years, increasing their bioavailability still remains challenging. Apart from unfavorable physicochemical properties of drugs, gastrointestinal enzymatic barrier and absorption barrier which consists of intestinal epithelial cells as well as mucus layer are needed to be overcome [1].

Solid lipid nanoparticles (SLNs), introduced as an oral drug–carrier system in the middle of 1990s, attracted a lot of interests owing to their stabilization and absorption-promoting effects as well as availability of large-scale production [2]. Although nanoparticles modified with peptide ligand at their surface could present a more efficient way to enhance oral absorption of protein drugs [3], reports about peptide ligand modified SLNs were limited. Furthermore, it seemed that mucus plays an obstacle role for SLNs. On the one hand, the electrostatic repulsion between the both negatively charged mucus and SLNs hampered the access of SLNs to mucus. Moreover, even if some SLNs entered into the mucus, they

*Abbreviations:* SLNs, solid lipid nanoparticles; sCT, salmon calcitonin; CSK, CSKSSDYQC; IRQ, IRQRRRR; SA, stearic acid; PEG, polyethylene glycol; SA-$PEG_{2000}$, polyoxyethylene (40) stearate; sCT SLNs, sCT-loaded solid lipid nanoparticles; sCT CSK-SLNs, sCT-loaded solid lipid nanoparticles prepared with SA-$PEG_{2000}$-CSK; sCT IRQ-SLNs, sCT-loaded solid lipid nanoparticles prepared with SA-$PEG_{2000}$-IRQ; FITC, fluorescein isothiocyanate; FITC-sCT, sCT-labeled with FITC; EE, entrapment efficiency; DL, drug loading; PDI, polydispersity index; TEM, transmission electron microscopy; DCC, N,N-dicyclohexyl carbodiimide; DMAP, 4-dimethylaminopyridine; HCl, hydrochloric acid; $CDCl_3$, deuterated chloroform; $^1H$ NMR, $^1H$ nuclear magnetic resonance; HPLC, high-performance liquid chromatography; MS, mass spectrometry; CVM, human cervicovaginal mucus; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; HBSS, Hank's Balanced Salt Solution; PBS, Phosphate-Buffered Saline; K–R solution, Kreb's–Ringer solution; Papp, apparent permeability coefficient; i.d., intraduodenal administration; i.v., intravenous administration; *F*, absolute bioavailability; D, total calcium decreases; RPA, relative pharmacological activity.

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were supposed to be easily trapped by mucus due to their hydrophobic properties. Regretfully, no reports have been concerned with the effects of mucus on SLNs. Therefore, it is important to design feasible and effective peptide ligand modified SLNs to improve the oral bioavailability of protein drugs and evaluating the influence of mucus.

Recently, two categories of peptide ligands were applied: epithelium targeting peptide and permeation enhancing peptide. A CSKSSDYQC (CSK) peptide was found to be an effective ligand exhibiting high affinity with goblet cells on the epithelium [4]. Previous studies of our group demonstrated that the trimethyl chitosan nanoparticles modified with CSK peptide possessed goblet cell targeting property and produced a higher bioavailability compared to unmodified ones *in vivo* after oral administration [5]. In addition, cell-penetrating peptides (CPP), a series of short peptides, were found supremely effective in enhancing the penetration through biological membranes for different cargos. Insulin-loaded SLNs modified with R8 peptide were reported to significantly improve the insulin transportation efficiency on Caco-2 cells and the *in vivo* hypoglycemic effect in diabetic rats [3], demonstrating that the CPP played an important role on the effective delivery of protein drugs. Recently, liposome modified with IRQRRRR (IRQ) peptide, a CPP, exhibited greatly improved cellular internalization via different mechanisms [6]. However, no further reports were found about their modification on SLNs for oral protein delivery.

In the present work, therefore, based on the above mentioned research, two kinds of peptide ligand modified salmon calcitonin (sCT)-loaded SLNs, namely, sCT CSK-SLNs and sCT IRQ-SLNs, were prepared by conjugating the peptide ligands CSKSSDYQC (CSK) or IRQRRRR (IRQ) to polyoxyethylene (40) stearate (SA-$PEG_{2000}$), with the unmodified SLNs (sCT SLNs) as the control. The drug protection abilities of SLNs in phosphate buffer (pH 6.8) with pancreatin were investigated. The cellular uptake especially the internalization mechanism as well as the influence of mucus, was studied using Caco-2/HT29-MTX co-cultured cells. Moreover, the excised rat intestinal mucosa or human cervicovaginal mucus (CVM) has also been used to investigate the permeation or diffusion properties of SLNs through mucus via Ussing chamber. Finally, the *in vivo* pharmacokinetic and pharmacodynamic studies in rats were performed after intraduodenal administration of SLNs.

## 2. Materials and methods

### 2.1. Materials

Salmon calcitonin (sCT), CSKSSDYQC (CSK) and IRQRRRR (IRQ) were chemically synthesized by Chengdu Kaijie Biopharm Co., Ltd. (Sichuan, China). Stearic acid was obtained from Huzhou Zhanwang Pharmaceutical Co., Ltd. (No. 20090312, Zhejiang, China). Tripalmitin was gained from TCI (Tokyo, Japan). Soybean phosphatidyl choline was purchased from Taiwei Pharmaceutical Co., Ltd. (No. 20110704, Shanghai, China). Sodium cholate was supplied by Aoboxing Biotechnologies Co., Ltd. (No. 20120106, Beijing, China). Poloxamer 188 was obtained from Nanjing Weier Chemical Co., Ltd. (No. 20081101, Jiangsu, China). Polyoxyethylene (40) stearate, fluorescein isothiocyanate (FITC), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and sodium pentobarbital were purchased from Sigma–Aldrich (St. Louis, MO, USA). Pancreatin (No. 20100108) and heparin sodium (No. 20110508) were gained from Huishi Biochemical Reagent Co., Ltd. (Shanghai, China). N-acetyl-L-cysteine was obtained from Aladdin Chemistry Co., Ltd. (Shanghai, China). All the other reagents and solvents were of analytical or chromatographic grade. The colorimetric $Ca^{2+}$ assay kit and salmon calcitonin-radioimmunoassay (RIA) kit were supplied by BHKG Clinical Reagents Co., Ltd. (No. 20120502, Beijing, China) and Phoenix Pharmaceuticals, INC. (California, USA), respectively.

Caco-2 cells were obtained from Institute of Biochemistry and Cell Biology (Shanghai, China). HT29-MTX cell line was a kind gift from Dr. Thécla Lesuffleur (INSERM, Paris, France). Male Sprague–Dawley rats weighing 250 ± 20 g were supplied by the Experimental Animal Center of West China Medical Center of Sichuan University (protocol number for animal study: CSDGZ-10). The rats were housed at a room temperature of 22 ± 2 °C and a relative humidity of 50 ± 10%.

### 2.2. Synthesis of SA-$PEG_{2000}$-CSK and SA-$PEG_{2000}$-IRQ [7]

The synthetic routes of SA-$PEG_{2000}$-CSK and SA-$PEG_{2000}$-IRQ are shown in Fig. 1. For the synthesis of $PEG_{2000}$-stearate (SA-$PEG_{2000}$), stearic acid (SA, 1 mmol) was dissolved in tetrahydrofuran (THF) followed by the addition of N,N-dicyclohexyl carbodiimide (DCC, 2.4 mmol) and 4-dimethylaminopyridine (DMAP, 0.1 mmol). After removing the precipitated dicylcohexyl urea (DCU), the resulted filtrate was slowly dropped into THF with polyethylene glycol 2000 ($PEG_{2000}$, 2 mmol) at 40 °C. The reaction mixture was incubated at 40 °C for 24 h with stirring. Then the organic solvents were removed using a rotary evaporator. The obtained residue dissolved in dichloromethane (DCM) was extracted with water to remove partial excess $PEG_{2000}$. The organic layer was dried using anhydrous sodium sulfate. After the removal of sodium sulfate, the resultant mixture was purified on a silica-gel chromatography column to obtain $PEG_{2000}$-stearate (compound 1).

For the synthesis of $PEG_{2000}$-stearate carboxyl derivative, the prepared $PEG_{2000}$-stearate (1 mmol) and succinic anhydride (1.5 mmol) were dissolved in anhydrous pyridine and incubated for 48 h at 80 °C to ensure the completion of reaction. Subsequently, the organic solvents were removed under vacuum and then the reaction mixture was purified on a silica-gel chromatography column to get the $PEG_{2000}$-stearate carboxyl derivative (compound 2). $^1H$ nuclear magnetic resonance ($^1H$ NMR) spectrums of compound 1 and compound 2 were measured in deuterated chloroform ($CDCl_3$) at 400 MHz.

The CSK peptide was synthesized on Wang resin in an automated peptide synthesizer using a standard Fmoc solid-phase synthesis protocol. The 6-aminocaproic acid (EACA) as a spacer was attached to the N-terminal of CSK peptide to obtain intermediate product, EACA-CSK peptide (EACA-ligand). Then the SA-$PEG_{2000}$-CSK was synthesized by the introduction of a covalent amide bond between the carboxyl group of $PEG_{2000}$-stearate carboxyl derivative and the terminal amino group of EACA-CSK peptide. This reaction was carried out on the solid phase using N,N′-diisopropylcarbodiimide (DIC) as catalyst. The purification was accomplished using the reversed-phase preparative high-performance liquid chromatography (HPLC) to yield SA-$PEG_{2000}$-CSK. Synthesis of SA-$PEG_{2000}$-IRQ was carried out following the procedure used for the synthesis of SA-$PEG_{2000}$-CSK. The structures of SA-$PEG_{2000}$-CSK and SA-$PEG_{2000}$-IRQ were characterized by mass spectrometry (MS).

### 2.3. Preparation and characterization of SLNs

#### 2.3.1. Preparation of SLNs

According to the procedure previously developed by our group, sCT SLNs were prepared by double emulsion technique [8]. Briefly, sCT (2.5 mg) was dissolved in 0.5 ml of hydrochloric acid (HCl) solution (0.01 M), and then sodium cholate (25 mg) was added and vortex-mixed. Subsequently, the aqueous solution was mixed with 1 ml of dichloromethane, in which soybean phosphatidyl choline (20 mg), stearic acid (1 mg) and tripalmitin (10 mg) were dissolved. The mixture was dispersed by ultrasonication (JY92-II