Peptidic PEGylated solid lipid nanoparticles for oral delivery of salmon calcitonin (sCT) and the influence of mucus

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ABSTRACT SUMMARY
Two kinds of peptidic PEGylated salmon calcitonin (sCT)-loaded solid lipid nanoparticles (SLNs), namely, sCT CSK-SLNs and sCT IRQ-SLNs, were prepared by conjugating the peptidic ligands CSKSSDYQC (CSK) or IRQRRRR (IRQ) to polyoxyethylene (40) stearate (SA-PEG2000) with unmodified SLNs (sCT SLNs) as control. The drug protection abilities of all SLNs were improved in simulated intestinal fluid with pancreatinum. Moreover, both of modified SLNs showed higher uptake than unmodified ones on Caco-2-HT29-MTX co-cultured cells. The presence of mucus reduced the uptake of all SLNs, especially unmodified SLNs. Pharmacokinetic study demonstrated that the absolute bioavailabilities of sCT for sCT CSK-SLNs and sCT IRQ-SLNs were significantly enhanced compared with sCT SLNs.

INTRODUCTION
Although oral delivery of protein drugs has attracted much attention during recent years, increasing of their bioavailability still remained challenging. Apart from unfavorable physicochemical properties of drugs, the absorption barrier of intestinal mucosa, consisting of intestinal epithelial cells as well as mucus layer are needed to be overcome.

Solid lipid nanoparticles (SLNs), introduced as an oral drug-carrier system in the middle of 1990s, attracted a lot of interests owing to their availability of large-scale production and low cytotoxicity. Although modified nanoparticles with a peptide ligand at their surface could present a more efficient way to enhance oral absorption of protein drugs, limited reports were found about peptidic SLNs. Furthermore, it seemed that mucus plays an obstacle role for SLNs. On one hand, the electrostatic repulsion between the negatively charged mucus and SLNs hampered the access of SLNs to mucus. Moreover, even if some SLNs enter into the mucus, they were supposed to be easily trapped by mucus due to their hydrophobic properties. Regrettfully, no reports have been concerned with the effects of mucus on SLNs. Therefore, designing feasible and effective peptidic SLNs to improve the bioavailability of oral protein drugs and evaluating the influence of mucus remains important.

In the present work, two kinds of peptidic PEGylated salmon calcitonin (sCT)-loaded SLNs, namely, sCT CSK-SLNs and sCT IRQ-SLNs, were prepared by coupling the peptidic ligands CSKSSDYQC (CSK) which was reported to show affinity with goblet cells, or IRQRRRR (IRQ), a cell penetrating peptide, to polyoxyethylene (40) stearate (SA-PEG2000). The stability and drug protection abilities of SLNs in simulated intestinal fluid were investigated. The cellular uptake especially the internalization mechanism as well as the influence of mucus, were studied using Caco-2-HT29-MTX co-cultured cell monolayer. Finally, the pharmacokinetic bioavailabilities after intra-duodenum administration of SLNs were evaluated in vivo.

EXPERIMENTAL METHODS
The sCT-loaded SLNs (sCT SLNs) were prepared with SA-PEG2000 by double emulsion technique. sCT CSK-SLNs and sCT IRQ-SLNs were prepared by conjugation of CSK or IRQ peptide with SA-PEG2000. Fluorescent SLNs incorporated with fluorescein isothiocyanate labeled sCT (FITC-sCT) were prepared accordingly.

The stability and drug protective properties of SLNs were investigated in the simulated intestinal fluid (pH 6.8) in the absence or presence of pancreatinum.

Caco-2/HT29-MTX co-cultured cells with a ratio of 1:1 were used for the cellular uptake and internalization mechanism studies. The amounts of internalized FITC-sCT were measured at 0.5, 1, 1.5 and 2h, respectively. Besides, in order to further study the endocytosis mechanism and the effect of mucus, the co-cultured cells were pre-incubated with different endocytosis inhibitors or N-acetyl-L-cysteine. The latter was used to remove the mucus layer.

Pharmacokinetic experiment was performed on normal male Sprague-Dawley rats following intra-duodenum (i.d) administration with the formulations including free sCT solution, sCT SLNs, sCT CSK-SLNs and sCT IRQ-SLNs (equivalent to 250 IU/kg of sCT). The concentration of sCT in the plasma was determined using salmon calcitonin-radioimmunoassay (RIA) kit at certain time intervals (3, 10, 20, 40, 90, 120, 240, 480 and 720 min), respectively. In addition, the plasma profile of sCT following intravenous (i.v) administration of the sCT solution (10 IU/kg) via the tail vein of rats was used to assess the absolute bioavailability of sCT.

RESULTS AND DISCUSSION
The obtained sCT IRQ-SLNs showed an average diameter of 409.9 ± 4.5 nm, larger than those of sCT SLNs (240.5 ± 15.0 nm) and sCT CSK-SLNs (244.4 ± 8.5 nm). The zeta potentials of sCT SLNs, sCT CSK-SLNs and sCT IRQ-SLNs were -27.5 ± 0.3 mV, -29.1 ± 0.7 mV and -19.8 ± 0.5 mV, respectively. The entrapment efficiency of all SLNs exhibited around 50 %.

The stability study indicated that the particle sizes of
sCT SLNs and sCT CSK-SLNs increased slightly until 12h in the simulated intestinal fluid in the absence of mucatinium, while those of sCT IRQ-SLNs increased significantly from 4h. Besides, the absolute value of zeta potential of sCT SLNs and sCT CSK-SLNs remained unaltered within 12h, while the sCT IRQ-SLNs showed a decrement of 22.3 % at 12h (p<0.01).

In drug protective properties study, free sCT was almost totally degraded at the first 15min with the residual sCT less than 5 %. Due to the protection of PEGylated SLNs, the gradual degradation of sCT encapsulated in SLNs was observed for a period of 12h. Fig. 1 demonstrated that all SLNs could significantly improve the internalization of drugs. The internalized sCT from FITC-sCT SLNs at 1 h was 2.12-fold higher than that of free sCT (p<0.01). Meanwhile, the uptake amount of FITC-sCT CSK-SLNs and FITC-sCT IRQ-SLNs were 2.29-fold (p<0.01) and 1.37-fold (p<0.01) higher than that of unmodified SLNs. It should be noticed that the CSK modification presented higher internalization than IRQ peptide.

The internalization mechanism study (Fig.2A) showed that the uptake of FITC-sCT from all SLNs was significantly reduced at 4°C with the addition of sodium azide (NaN₃), both of which could block the active transport. The uptake of FITC-sCT from CSK-SLNs and IRQ-SLNs could be significantly inhibited by chlorpromazine (Chl) and filipin (Fil), indicated that the uptake mechanism of peptide SLNs was mainly active transport via both clathrin- and caveolea-dependent endocytosis.

Fig.2B showed the uptake of FITC-sCT SLNs, FITC-sCT CSK-SLNs and FITC-sCT IRQ-SLNs were 1.26-fold (p<0.01), 1.11-fold (p<0.01) and 1.20-fold (p<0.01) higher with the absence of mucus than those with the presence of mucus, which was different from the observations in previous studies of chitosan nanoparticles, maybe because of the surface charge and hydrophilic properties of the latter. The internalization enhancing ratio of CSK or IRQ peptide modification (the FITC-sCT uptake of modified SLNs versus that of unmodified SLNs) were 1.88 or 1.66 (absence of mucus) and 1.65 or 1.59 (presence of mucus), respectively, implying the improved uptake of peptide SLNs by Caco-2/HT29-MTX co-cultured cell monolayer even in the presence of mucus.

As illustrated in Fig. 3, the plasma concentrations of sCT in free sCT solution and sCT SLNs gradually decreased from 40 min to 120 min while those of peptide SLNs still remained high during 120-720 min and 90-120 min, respectively. The AUC of sCT SLNs, sCT CSK-SLNs and sCT IRQ-SLNs was 2.53-fold, 6.20-fold and 5.02-fold higher than that of sCT solution. The absolute bioavailability of sCT CSK-SLNs (12.41 ± 3.65 %, p<0.01) and sCT IRQ-SLNs (10.05 ± 5.10 %, p<0.05) was 2.45-fold and 1.98-fold higher than that of sCT SLNs (5.07 ± 0.54 %), implying the feasibility and effectiveness of CSK and IRQ peptide ligands used to enhance the bioavailability of protein drugs for oral administration.

**CONCLUSION**

The peptide PEGylated SLNs could improve the drug protection ability of SLNs and facilitate the uptake on Caco-2/HT29-MTX co-cultured cell monolayer. Although mucus was an impediment to internalization of SLNs, the peptide SLNs still showed improved drug absorption. The absolute bioavailability of sCT CSK-SLNs and sCT IRQ-SLNs was significantly enhanced compared to sCT SLNs. Therefore, this oral delivery system might be a promising strategy for the oral absorption of peptide and protein drugs.

**REFERENCES**


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