

## **Design of lipid modified polymeric nanoparticles for improvement of oral absorption of insulin**

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### **Abstract**

In this study, lipid modified polymeric nanoparticles for oral delivery of insulin was developed. Firstly, chitosan nanoparticles (CS NPs) was prepared by cross-linking of chitosan with triphosphate TPP as the core of the core-shell structured nanocarriers. Then lipid coating chitosan nanoparticles (LCS) was prepared by co-incubation of the CS NPs with EPC liposomes. The morphologies of these nanocarriers were observed using transmission electron microscope (TEM). These nanocarriers were also characterized in terms of the stability of insulin in the nanocarriers, mucus penetrating properties, and cellular association efficiencies. Under TEM a core-shell structure could be observed in LCS indicating CS NPs formed the core which was coated with lipids. LCS had an average diameter of 202 nm with a zeta potential of -7.1 mV. *In vitro* degradation study showed that with the phospholipids layer, LCS could protect the chitosan associated insulin from degradation by trypsin and  $\alpha$ -chymotrypsin. Cellular association study performed on Caco-2 revealed that the cell association of LCS was slightly higher than CS NPs. However compared to CS NPs, LCS did not enhance mucus penetration properties in *in vitro* mucus penetration study. In summary, the core-shell structured nanocarriers present promising for effective oral insulin delivery by combining the effects of enhancing the stability of insulin in GI tract and enhanced cell association study. However, the functionality of the nanocarriers needs to be further improved by enhancing mucus penetration property.

**Key words:** insulin; chitosan nanoparticles; liposomes ; mucus penetration

## 1. Introduction

Oral administration of protein and peptide presenting a series of attractive advantages, include avoidance of additional risks, pain and discomfort associated with injections. However, orally administered peptides and proteins is encountered with many difficulties, as it has to confront various barriers in the gastrointestinal (GI) tract(1). Ideal nanocarriers for oral protein delivery should be stable in GI tract, able to diffusion through the mucus and uptake by endothelial cell(2). However few nanoparticles have all these characteristics.

In recent years, a kind of core-shell liponanoparticles (nanoparticles coated by lipid bilayer) have shown great potential or drug delivery (3-7).The structures include the biomimetic property of lipid bilayers, and are imparted a mechanical stability attributed to the solid core. Moreover, the resulting capsules permit transporting and stabilizing the fragile biomolecules, which are packaged in the core, against the harsh surrounding environment. However, to date, most of the explored core-shell liponanoparticles were of hydrophobic core such as PLGA, which has low protein encapsulation efficiency. Less attention has been paid to enhance mucus diffusion efficiency of core-shell liponanoparticles, which is a long standing challenge in mucosal membrane delivery of bioactives (8).

In view of these, a core-shell nanoparticle was designed by encapsulating chitosan-TPP nanocomplex into liposomes. Chitosan here is utilized to fabricate the hydrophilic core, which has more potential to load proteins such as insulin than PLA nanoparticle (9). The chitosan nanoparticles were loaded into liposomes by incubation with preformed liposomes. Our hypothesis is that the core-shell nanoparticles could render protein drugs like insulin with enhanced stability in GI tract, improved mucus penetrating properties and cellular uptake efficiency.

## 2. Materials and Methods

### 2.1 Materials

Egg phosphatidylcholine (EPC) was purchased from by Q.P. Corp (Tokyo, Japan). Chitosan (CS) in the form of hydrochloride salt (Protasan 213 CL) was purchased from FMC Biopolymer AS (Norway). FITC-insulin, FITC- dextran 4000 and Hank's balanced salt solution (HBSS) were all purchased from Sigma-Aldrich (Saint Louis, Missouri, USA). All other chemicals were of analytical reagent grade.

### 2.2 Preparation of LCS nanocarriers

Core-shell nanoparticles were obtained by coincubating chitosan nanoparticles with preformed liposomes under room temperature for 1 h hour. The chitosan nanoparticles was prepared by ionic gelation of chitosan with tripolyphosphate anions(TPP) as described by Seijo et al(10). The preformed liposomes were prepared by the thin film hydration technique followed by extrusion.

### 2.3 Characterization of LCS nanocarriers

The particle size and size and  $\zeta$ -potential of LCS were determined at room temperature in distilled water with a Malvern Zetasizer NanoZS (Malvern Instruments, London, UK).The morphology of LCS and, CS NPs were observed using transmission electron microscope (TEM, Philips CM200) at an accelerating voltage of 160 kV. Prior to observation under TEM, the samples were deposited on carbon coated copper grids and stained with phosphotungstic acid (1 % w/v)(11).

### 2.4 In vitro degradation study

In vitro protein degradation was studied by incubating different insulin-loaded nanocarriers with trypsin and  $\alpha$ -chymotrypsin. Aliquots were withdrawn at various time intervals and non-degraded insulin was determined by a reversed phase HPLC.

### 2.5 Cell associate study

An inverted confocal microscope (CLSM) was used to visualize the cellular association of FITC-insulin loaded nanocarriers in a Caco-2 cell model.

### 2.6 In vitro mucus penetration study

In vitro mucus penetration was studied by a method described by Y. Barenholz (12). In order to avoid the enzyme degradation, FITC-dextran 4000(FD4) was used to label the nanocarriers. The amount of penetrated FD4 in the in vitro intestine issue was determined using a microplate-reader.

## 3. Results and discussion

### 3.1 Preparation and characterization of the nanocarriers.

As shown in Tab. 1, un-encapsulated chitosan nanoparticles had an average hydrodynamic diameter of  $210.5 \pm 45.3$  nm with a polydispersity of  $0.311 \pm 0.075$  and a strong positive zeta potential of approximately +36 mV. The incorporation of the chitosan nanoparticles into the liposomes did not have a distinct effect on particle size, but led to a significant decrease ( $P < 0.05$ ) in the zeta potential, which were reduced to -7.1 mV. The zeta potential conversion in some way indicated the formation of chitosan encapsulated lipoparticles. As shown in Fig. 1, core shell structure was observed for LCS, which further confirmed that chitosan nanoparticle has been encapsulated into liposomes. A schematic presentation of the core-shell structure can be seen in Fig.2.

Table.1 Physicochemical properties of insulin-loaded CS NPs and LCS (mean $\pm$ SD, n=3)

Formulations	MeanDiam (nm)	PI	$\zeta$ potential (mV)
CS NPs	210.5 $\pm$ 45.3	0.311 $\pm$ 0.075	+36.6 $\pm$ 4.5
LCS	202.8 $\pm$ 22.9	0.175 $\pm$ 0.069	-7.1 $\pm$ 3.2

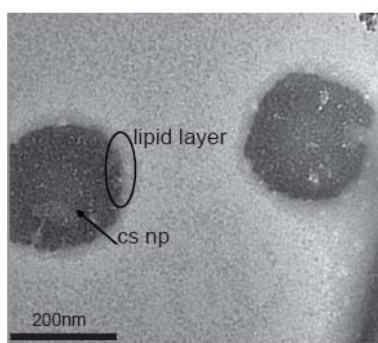


Figure. 1 TEM images of LCS

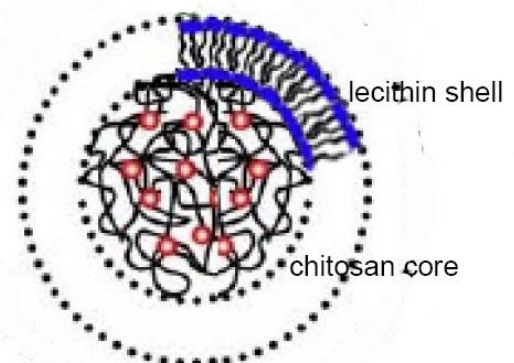


Figure 2 The schematic presentation of LCS

### 3.2 Protective ability of LCS against gut enzymes in vitro

LCS protected 40% and 70% of encapsulated insulin from degradation of trypsin and  $\alpha$ -chymotrypsin (Fig. 3) whereas plain insulin solutions and CS NPs were almost degraded under these same conditions, which indicated that this core-shell structure successfully improved the stability of protein encapsulated in the core of the nanocarrier.

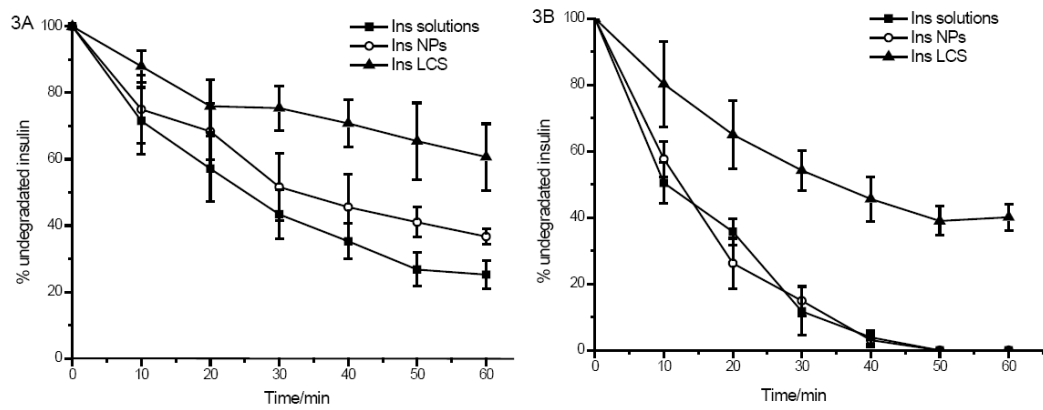


Figure 3A. Degradation profiles of insulin by trypsin as a function of time  
 3B. Degradation profiles of insulin by  $\alpha$ -chymotrypsin as a function of time

### 3.3 Cell association study in Caco-2 cells

As shown in Fig. 4, the Caco-2 cell association efficiency of FITC-insulin was significantly enhanced by LCS and CS NPs compared to insulin solution group. Slightly stronger green fluorescence was observed in LCS compared with CS NPs, which indicated that LCS had higher association efficiency with Caco-2 cells than CS NPs.

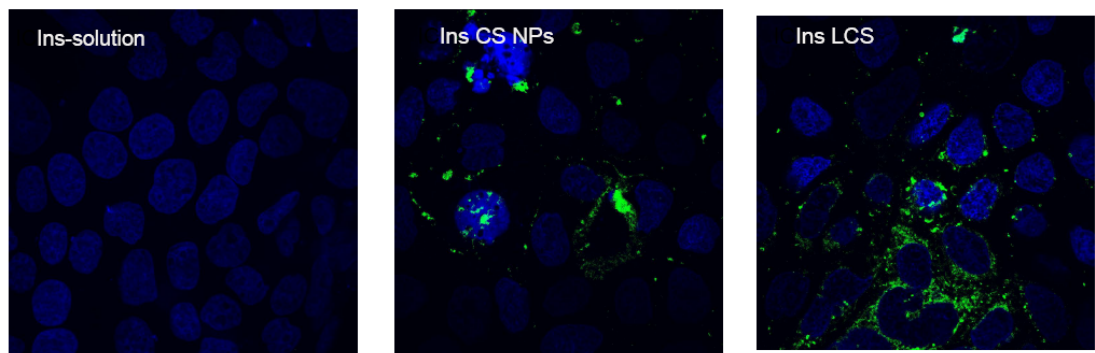


Figure 4. Confocal laser scanning microscopy (CLSM) cell uptake of FITC-Insulin loaded in different nanocarriers

### 3.4 In vitro mucus penetrating study

As shown in Fig. 5, the cumulatively penetrated amounts of FD4 in CS NPs and LCS in 2 h were less than 35% and there was not significant difference between CS NPs and LCS group, which indicated that LCS had limited mucus penetration properties. In order to efficiently improve oral absorption of proteins or peptides, the problem should be overcome by some strategies.

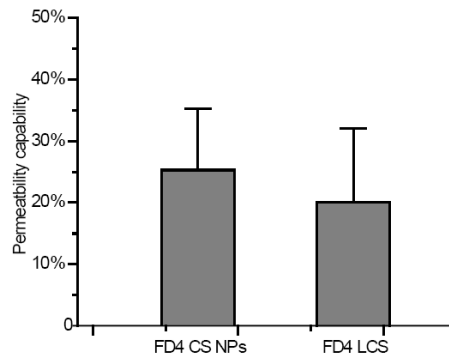


Figure 5. In vitro intestinal permeability capacity of different carriers

#### 4. Conclusion

A core shell structured nanocarriers for oral protein delivery were prepared by incorporating chitosan nanoparticles with EPC liposomes. *In vitro* enzyme degradation studies showed that the lipid modified polymeric nanoparticles could significantly protect the encapsulated proteins from enzyme degradation and had slightly higher cell association efficiency. However, *in vitro* study revealed that LCS had limited mucus penetration. Therefore, further improvement on the functionality of the carriers needs to be done. In the following study, pluronic F127 modified EPC liposome will be used to encapsulate chitosan nanoparticles instead of unmodified EPC liposome as we discovered that pluronic F127 modified EPC liposomes had higher mucus penetration compared with unmodified liposomes(13).

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