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Biotinylated liposomes as potential carriers for the oral delivery of insulin

Xingwang Zhang, PhD, Jianping Qi, PhD, Yi Lu, PhD, Wei He, PhD, Xiaoyang Li, MSc, Wei Wu, PhD*

School of Pharmacy, Fudan University, Shanghai, PR China

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Abstract

This study aimed to explore biotinylated liposomes (BLPs) as novel carriers to enhance the oral delivery of insulin. Biotinylation was achieved by incorporating biotin-conjugated phospholipids into the liposome membranes. A significant hypoglycemic effect and enhanced absorption were observed after treating diabetic rats with the BLPs with a relative bioavailability of 12.09% and 8.23%, based on the measurement of the pharmacologic effect and the blood insulin level, respectively; this achieved bioavailability was approximately double that of conventional liposomes. The significance of the biotinylation was confirmed by the facilitated absorption of the BLPs through receptor-mediated endocytosis, as well as by the improved physical stability of the liposomes. Increased cellular uptake and quick gastrointestinal transport further verified the ability of the BLPs to enhance absorption. These results provide a proof of concept that BLPs can be used as potential carriers for the oral delivery of insulin.

From the Clinical Editor: Diabetes remains a major source of mortality in the Western world, and advances in its management are expected to have substantial socioeconomic impact. In this paper, biotinylated liposomes were utilized as carriers of insulin for local delivery, demonstrating the feasibility of this approach in a rat model.

Key words: Insulin; Biotin; Liposomes; Oral delivery; Receptor-mediated endocytosis

Insulin (INS) is one of the most important therapeutic drugs for the treatment of both INS-dependent (type I) and INS-independent (type II) diabetes mellitus and is commonly administered subcutaneously, which can lead to pain, allergic reactions, hyperinsulinemia, lipodystrophy around the injection site and even failed glycemic control due to noncompliance.[1] In recent years, much attention has focused on peroral administration of INS. This mode of delivery has several advantages, such as good patient compliance, low cost, favorable glucose homeostasis and entrance into the circulation via the portal circulation, a mechanism that is very similar to endogenous INS.[2] For this end, a variety of oral INS delivery systems have been explored, including hydrogels,[3] microemulsions,[4] nanospheres,[5] nanocubicles,[6] polymeric nanoparticles[7,8] and liposomes.[9-11]

Liposomes have shown promising potential in oral INS delivery due to their facilitated absorption and their ability to protect the payload from the harsh gastrointestinal (GI) environment. However, conventional phospholipid/cholesterol liposomes are sensitive to damage caused by gastric acid or GI enzymes, resulting in reduced oral bioavailability. To elongate the GI survival of liposomes, the vesicles have been modified in several ways; these include the incorporation of bile salts,[12] coating the liposomes with polymers[13,14] and the design of multilayered or multi-vesicular carriers.[15,16] Further efforts have focused on how to increase the intestinal adhesion of the liposomes, as well as how to open the tight junctions and enhance lymphatic absorption. In spite of these efforts, the oral bioavailability of INS-loaded liposomes has not reached our expectation. It seems that the field has reached a bottleneck, and alternative strategies should be explored to further enhance the bioavailability of orally administered INS. Until now, the ligand-mediated active uptake pathway has not been well studied for this purpose. It has been established that receptor-mediated

Abbreviations: BLPs, biotinylated liposomes; CD, circular dichroism; CLPs, conventional liposomes; CLSM, confocal laser scanning microscope; DSPE, 1,2-distearoyl-sn-glycero-3-phosphatidyl ethanolamine; EE, entrapment efficiency; FITC, fluorescent isothiocyanate; GI, gastrointestinal; MFI, mean fluorescence intensity; PA, pharmacological bioavailability; P_{app}, apparent permeability; *s.c*, subcutaneous; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; SPC, soybean phosphatidylcholine; TEM, transmission electron microscopy; T_t, phase transition temperature.

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*Corresponding author: School of Pharmacy, Fudan University, Shanghai 201203, PR China.

E-mail address: wuwei@shmu.edu.cn (W. Wu).

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endocytosis is an important absorption mechanism for certain substances, such as proteins and hormones. There are a variety of receptors expressed in the membranes of enterocytes, such as vitamins, transferrins, amino acids and sugar receptors.[17] Targeting these receptors is an important strategy to improve the oral bioavailability of poorly permeable therapeutic agents.[18-20] Indeed, some vitamin ligands have been studied in the oral delivery of water-insoluble drugs and biomacromolecules.[21-24]

Biotin (vitamin B7) is a vitamin that cannot be synthesized endogenously. The GI uptake of this vitamin is through Na^+-dependent and carrier-mediated endocytosis; biotin receptors are non-specific and are distributed throughout the small intestine.[25] Biotin-conjugation to glucagon-like peptide-1 significantly enhanced its cellular permeability and hypoglycemic efficacy by oral delivery.[26] Although biotinylated vehicles have been studied for the non-parenteral delivery of active ingredients,[27-30] the potential of biotin as a decorating ligand of nanocarriers for oral drug delivery has not been investigated.

In this proof-of-concept study, we explored the potential of biotinylated liposomes (BLPs) as novel nanocarriers for the oral delivery of INS. Biotinylation was achieved through the incorporation of biotin-conjugated 1, 2-distearoyl-sn-glycero-3-phosphatidyl ethanolamine (DSPE) into the liposome membranes. The hypoglycemic effect and the blood INS level were monitored to calculate the bioavailability after oral administration of the BLPs. Mechanistic studies were performed to investigate the biotin receptor expression, cellular uptake, and the endocytosis pathways, as well as how the BLPs were transported through the GI tract. The effect that the biotin decoration had on the physical stability of the liposomes and the implications this had on the liposome's survival in the GI tract were also studied.

Methods (see supporting information for details)

INS-loaded BLPs were prepared using a reversed-phase evaporation method. The particle size of the liposomes was measured using dynamic light scattering. The morphology of the liposomes was observed by transmission electron microscopy (TEM). The entrapment efficiency (EE) of INS in the liposomes was determined after separating the free INS from the liposomes through gel permeation chromatography. Conformational stability was evaluated using circular dichroism (CD) spectroscopy, and bioactivity was evaluated after subcutaneous (*s.c.*) injection of INS released from the liposome formulations. Leakage of INS from the liposomes was also evaluated by monitoring the change in EE over time.

The physical stability of the liposomes was characterized by measuring the phase transition temperature (T_t) and by monitoring the INS levels after incubation in simulated physiological fluids. The T_t of the liposomes was determined by measuring the fluorescence intensity of the calcein released from the liposomes as a function of temperature by fluorospectrophotometry. The differentiated fluorescence intensity to that of 25 °C, which denotes the release of calcein from liposomes, was plotted as a function of temperature, and T_t was estimated from the sharp change in the fluorescence intensity.[31]

The hypoglycemic effect was evaluated in gene-knocked out diabetic (SLAC/GK) rats by monitoring the blood glucose level, and the oral bioavailability was calculated using *s.c.* INS administration as a reference. All animal experiments, including those described in other parts of this article, were conducted in accordance with the approval of Experimental Animal Ethical Committee of Fudan University.

The expression of the biotin receptor in enterocytes was investigated using the Caco-2 cell model, which is based on the principle of specific receptor-ligand interaction. Biotin labeled with 5-aminofluorescein was used to specifically bind the biotin receptors, and this event was photographed using a confocal laser scanning microscope (CLSM). Caco-2 cell monolayers and Caco-2/Raji co-cultured monolayers with M cell characteristics were used to assess the transport of the INS preparations. To investigate the influence of the liposomes on cell tight junctions, changes in the peripheral filaments of cells were observed by CLSM after incubation with the INS solution, INS-loaded conventional liposomes (CLPs) or BLPs. The cellular uptake of fluorescent isothiocyanate-labeled insulin (FITC-INS)-loaded liposomes under different conditions was visualized and quantified by CLSM and flow cytometry, respectively.

To study the pathways involved in the endocytosis of the liposomes, cells were pre-treated with various inhibitors (Table S1), and the uptake of the FITC-INS loaded liposomes was studied using flow cytometry to identify the different pathways.

The gastrointestinal transport of the liposomes was visualized after oral administration of liposomes loaded with rhodamine B. Transmembrane permeability was evaluated by fluorescent imaging of intestinal tissue slices after oral administration of the FITC-INS loaded liposomes.

Results

Synthesis and characterization of biotin-DSPE

The synthetic scheme of biotin-DSPE and the ^{1}H NMR spectra of the purified products are shown in Figure S1. The ^{1}H NMR spectrum of biotin-DSPE clearly showed signals for DSPE (δ 0.92, 1.25, and 2.31) and biotin (δ 1.31, 1.49, 2.18, 2.48, 6.35 and 6.42). Meanwhile, the disappearance of the signal at δ 12.0, which corresponds to the carboxyl proton of biotin, and the appearance of a proton signal at δ 8.18, which is a consequence of forming an amide group, indicated the successful conjugation of biotin to DSPE.

Formulation, preparation and characterization of BLPs

A typical BLP formulation was made up of 385.4 mg soybean phosphatidylcholine (SPC), 114.6 mg biotin-DSPE, 81.6 mg cholesterol and 1.0 mL INS solution as an aqueous phase. By controlling the preparative parameters, BLPs prepared under optimal conditions had a mean particle size of ~150 nm with a narrow distribution (Figure 1, *A*). High INS payloads were obtained for both BLPs and CLPs, with EEs that ranged from 35% to 42%. The morphology of the BLPs was nearly spherical, as observed by TEM (Figure 1, *B*).