Hypoglycemic efficacy of chitosan-coated insulin liposomes after oral administration in mice[1]

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KEY WORDS chitin; chitosan; insulin; liposomes; blood glucose; oral administration

ABSTRACT

AIM: To evaluate the hypoglycemic efficacy of insulin liposomes coated by chitosan with different molecular weights and concentrations after oral administration in mice. **METHODS:** Insulin-liposomes were prepared by reversed-phase evaporation. Chitosan coating was carried out by incubation of the liposomal suspensions with the chitosan solution. The hypoglycemic efficacies of chitosan-coated insulin liposomes were investigated by monitoring the blood glucose level using the glucose oxidase method after oral administration to healthy mice. **RESULTS:** In all the insulin liposomes, the insulin liposomes coated by 0.2 % chitosan (M_r 1000 kDa) showed a better hypoglycemic efficacy as compared with the other liposomes coated by chitosan. The minimum blood glucose level was 15.1 %±6.0 % of the initial (n=6). The hypoglycemic efficacy lasted for 4 h after oral administration to mice. **CONCLUSION:** Chitosan-coated liposomes could reduce tryptic digestion on insulin, and enhance enteral absorption of insulin. The molecular weights and concentrations of chitosan had significant effects on hypoglycemic efficacy of chitosan-coated insulin liposomes after oral administration to healthy mice.

INTRODUCTION

In the past two decades the potential usefulness of liposomes as drug carriers for improving enteral absorption of poorly absorbed drugs including peptide drugs such as insulin has attracted considerable interest. These phospholipid vesicles are capable of encapsulating both hydrophobic and hydrophilic drugs; they are biodegradable and are not toxic *in vivo*. The drugs encapsulated in liposomes are sufficiently protected from enzymatic attack and immune recognition[1]. Liposomes can improve enteral absorption of poorly absorbed drugs including peptide drugs. However, the results of these studies indicate the influences of the liposome formulations on drug absorption are not predictable or reproducible[2].

Chitosan is natural cationic polysaccharide derived from deacetylation of chitin, which is, after cellulose, the most abundant polymer found in nature. It is a hydrophilic, biocompatible, and biodegradable polymer with low toxicity[3,4]. Because of its bioadhesive properties, chitosan has also received substantial attention in novel bioadhesive drug delivery systems with aim to improve the bioavailability of drugs by prolonging the residence time at the site of absorption[5,6]. Chitosan can induce a redistribution of cytoskeletal F-actin and the tight junction protein ZO-1 via interaction between its positive charges and mucosal negative charges, which results in increased paracellular permeability of hydrophilic macromolecules[7]. Chitosan was used as a

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stabilizing constituent of liposomes[2,8]. The mucoadhesive property of chitosan-coated liposomes helps in delaying intestinal transit time so as to increase absorption of insulin[9]. Several studies have highlighted the potential use of chitosan[9,10].

The purpose of this study was to evaluate the hypoglycemic efficacy of insulin liposomes coated by chitosan with different molecular weights and at different concentrations after oral administration in healthy mice.

MATERIALS AND METHODS

Reagents and chemicals Porcine insulin (27.6 kU/g) was purchased from Xuzhou Biochemical Pharmaceutical Factory (China). E200 Soy lecithin was a gift from Lucas Meyer GmbH & Co (Hamburg, Germany). Cholesterol (Sigma C-8667, purity ≥99 % Sigma grade), sodium cholate (Sigma C-1254, purity ≥99 %), pepsin (Sigma P-7125, 600-1800 kU/g protein) and trypsin (Difco 0152-17, 2-4 kU/g) were obtained from Xinjingke Biotechnology Co, Ltd (Beijing, China). The glucose oxidase kit was obtained from Shanghai Rongsheng Biotech Co, Ltd (China). Low, medium, and high weight chitosan (Ch, M_r 65, 140, 680, and 1000 kDa) with a deacetylation grade of about 90 % were provided by Nantong Shuangling Biomaterial Co (China). Purification of chitosan: 5 g of chitosan (food grade) was dissolved in 400 mL of 1 % (v/v) acetic acid aqueous solution, then filtered and reneutralized with NH_4OH. The flocculated polymer is recovered by filtration, washed, and lyophilized[11]. All other reagents were of analytical grade and commercially available. Water was demineralized and twice distilled. Phosphate buffered saline (PBS) consisted of NaCl 137 mmol/L, KCl 2.6 mmol/L, $Na_2HPO_4 \cdot 12H_2O$ 6.4 mmol/L, NaH_2PO_4 1.4 mmol/L, pH 7.4.

Equipment ZFQ-85A rotary evaporator and GH-82 bath type shaker was from Shanghai Medical Special Appliance Factory. J-250 hydrologic cycle vacuum pump was from Gongyi Yingxia Appliance Factory, Henan. JY-92 II probe ultrasonifier was from Ningbo Xingzhi Scientific Instruments Institute. AE 200 electronic Analytical Balance and 320 pH meter was from Mettler Toledo. Alpha 1-2 lyophilizer was from Martin Christ, Germany. UV-9200 spectrophotometer was from Beijing Reili Analytical Instruments Co. H600A-2 transmission electron microscopy was from Hitachi Ltd, Japan, Zetasizer 3000SH was from Malvern Instruments Ltd, UK. L8-60M ultracentrifuge was from BECKMAN, USA. LC-10AT liquid chromatograph pump and SPD-10A UV-Vis Detector was from Shimadzu Ltd, Japan.

Animals Male Kunmin mice (20±2 g) were supplied by Experimental Animal Center of China Pharmaceutical University.

Preparation of chitosan-coated liposomes Liposomes were prepared by reversed-phase evaporation. A total phospholipid mixture of 225 mg (soy lecithin/cholesterol in the weight ratios of 4:1) was dissolved in 10 mL of ether in flask. Four milligram of insulin dissolved in 3 mL of PBS (pH 7.4) was added to the flask, then dispersed with a shaker at room temperature, and emulsified with a bath-type ultrasonifier. The ether was evaporated using rotary evaporator in 20 ºC water bath under reduced pressure. The resulting liposomal dispersion was vortex-mixed for 15 min to form the liposomal suspensions, then homogenized with probe-type ultrasonifier for 1 min. Chitosan-coated liposomes were prepared by adding 3 mL of chitosan solution to 3 mL of the homogenized liposomal suspensions, incubated at 10 ºC for at least 1 h.

Morphological examinations of liposomes Negative stain electron micrographs were prepared by the following technique: liposomes (1 mL) were diluted to 5 mL with PBS, then were applied onto carbon-coated grids (300 mesh) and drawn off by filter paper. A drop of 2 % (w/v) sodium phosphotungstate aqueous solution was applied to the grid, drawn off with a piece of filter paper and allowed to dry for 1 h. Morphological examinations of liposomes were performed by transmission electron microscopy (TEM).

Particle size and ζ potential measurements Liposomal suspension (100 μL) was diluted to 10 mL with distilled water, then subjected to photon correlation spectroscopy and laser Doppler anemometry (Zetasizer 3000SH, UK) .

Determination of encapsulation efficiency (EE) Liposomal suspension (4 mL) was ultracentrifuged (100 000×g, 10 ºC, 5 h). After centrifugation, the supernatant or the liposomal suspensions (0.2 mL) were diluted to 10 mL with PBS, then 0.5 mL of 10 g/L sodium cholate, 2.3 mL of PBS and 1 mL of chloroform were added to 2.5 mL of the diluted solution. The mixture was vortex-mixed for 1 min, and then centrifuged at 350×g for 15 min. HPLC analysis was performed immediately. Encapsulation efficiency was calculated as below:

$$EE=(W_T-W_F)/W_T\times 100\ \%,$$

Where EE is encapsulation efficiency, W_T is the total amount of insulin in liposomal suspensions; W_F is the free amount of insulin that was found in the supernatant.

HPLC analysis The HPLC system consisted of a Shimadzu LC-10AT liquid chromatograph, a Shimadzu SPD-10A UV-Vis monitor, and a Shimadzu CR 6A chromatopac. The column is 250 mm×4.6 mm, Lichrospher ODS-C18, 5 μm. The mobile phase was a mixture of acetonitrile and sulfate buffer solutions [Na_2SO_4 0.025 mmol/L+NaH_2PO_4 0.05 mmol/L , pH value was adjusted to 3.0 with H_3PO_4] (28:72). The flow rate was 1.0 mL/min. The temperature of column was 40 ºC. Detection wavelength was at 214 nm.

Peptic and tryptic digestion of insulin Liposomal suspensions were incubated with equivolumetric peptic solution 0.05 g/L in Tris-HCl-buffered saline 10 mmol/L (pH 2.0), or equivolumetric tryptic solution 3.6 g/L in Tris-HCl-buffered saline 10 mmol/L (pH 7.4) at 37 ºC while shaking. Aliquot volumes of 200 μL were taken at predetermined time points and the reaction was terminated by adding 200 μL of NaOH 0.05 mol/L or HCl 0.1 mol/L. The samples were stored at -10 ºC until HPLC analysis.

Animal experiments The mice fasted with water *ad libitum* for 8 h before experiments were divided into 10 groups. The liposomal suspensions were administered intragastrically at a dosage of 250 U/kg. As a reference, an equivalent amount of insulin solution was administered. About 60 μL blood sample was obtained from the eye ground vein cluster at an appropriate interval after oral administration. Serum was separated from plasma by centrifugation at 2500 r/min for 10 min. The blood glucose levels were measured by using the glucose oxidase method.

Statistical analysis All the data with the exception of particle size and ζ potential were expressed as mean±SD. Statistical analysis was performed using *t*-test.

The mean blood glucose level before oral administration was taken as a 100 percent glucose level. The percentage of glucose reduction at each time after dosing was calculated and plotted against time.

RESULTS

Morphology, particle size, and ζ potential of liposomes The liposomal suspensions were of ivory-white. All insulin-liposomes were of spherical or ellipsoidal shape. The fingerprint characteristic of uncoated liposomes was very clear. However, because of hydrophilicity of chitosan, the stained areas of chitosan-coated liposomes corresponded to chitosan/dye complex was represented by a dark mesh around the membrane (Fig 1). Particle size and ζ potential of liposomes were shown in Tab 1 and Tab 2.

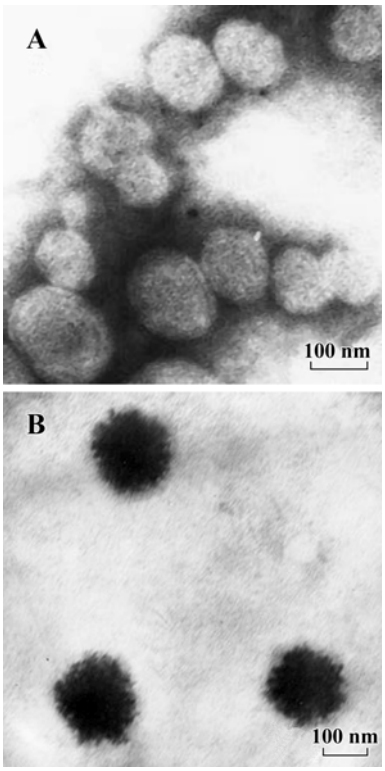

Fig 1. Transmission electron photomicrograms of insulin liposomes. (A) Uncoated liposomes; (B) Chitosan-coated liposomes.

Tab 1. Effects of chitosan with different molecular weights on size and ζ potential of insulin-liposomes.

M_r	Zeta potential/mV	Width/mV	Size/nm	Polyindex
Uncoated	-2.9	0.2	168.6	0.143
65 kDa	4.9	0.4	206.2	0.246
140 kDa	5.0	0.3	203.0	0.134
680 kDa	5.5	0.5	199.7	0.227
1000 kDa	6.7	0.3	203.4	0.132

EE of insulin in liposomes EE was influenced by chitosan with different molecular weight and at different concentrations (Tab 3, 4). As compared with

Tab 2. Effects of chitosan at different concentrations on size and ζ potential of insulin-liposomes.

Concentration	Zeta potential/ mV	Width/ mV	Size/nm	Polyindex
0.1 % Ch1000 kDa	6.2	0.4	188.1	0.211
0.2 % Ch1000 kDa	6.7	0.3	202.4	0.132
0.3 % Ch1000 kDa	7.3	0.5	222.1	0.228
0.4 % Ch1000 kDa	10.5	0.2	231.2	0.167
0.5 % Ch1000 kDa	17.0	0.4	246.3	0.142

Tab 3. Entrapment efficiencies of insulin-liposomes coated by chitosan with different molecular weights. *n*=3. Mean±SD.

M_r	Entrapment efficiency/%
Uncoated	81.6±2.4
65 kDa	69.7±5.1
140 kDa	71.2±3.6
680 kDa	75.8±4.3
1000 kDa	75.9±4.9

other chitosan-coated liposomes, EE of the liposomes coated by 0.2 % chitosan with M_r 1000 kDa was higher (75.9 %±4.9 %, *n*=3), but lower than that of uncoated insulin liposomes (81.6 %±2.4 %, *n*=3).

Peptic and tryptic digestion of insulin Uncoated liposomes did not protect insulin from peptic digestion compared with insulin in PBS. After 30 min peptic digestion, the residual percents of insulin in PBS, and in uncoated liposomes were 6.4 %±5.4 % and 3.9 %±4.7 %, respectively. On the other hand, insulin encapsulated in chitosan-coated liposomes was protected at some extent, and the percentage of residual insulin was from 15.6 %±1.9 % to 46.3 %±3.9 % after 30 min (Tab 5, 6).

Tab 4. Entrapment efficiencies of insulin-liposomes coated by chitosan at different concentrations (M_r 1000 kDa). *n*=3. Mean±SD.

Concentration	Entrapment efficiency/%
0.1 % Ch1000 kDa	58.5±3.4
0.2 % Ch1000 kDa	75.9±4.9
0.3 % Ch1000 kDa	74.6±4.7
0.4 % Ch1000 kDa	59.3±3.1
0.5 % Ch1000 kDa	56.2±2.6

Insulin in uncoated liposomes was not protected from tryptic digestion compared with insulin in PBS (27.8 %±3.2 % and 22.6 %±3.0 % 5 h later, respectively). In addition, insulin in the liposomes coated by chitosan at different concentrations and with different molecular weights strongly protected residual insulin from tryptic digestion (from 63.2 %±1.5 % to 75.4 % ±5.0 % 5 h later, Tab 7, 8).

Hypoglycemic effect of chitosan-coated insulin liposomes *in vivo* Oral administrations of insulin solution and uncoated insulin liposomes had no hypoglycemic efficacies; while in contrast all chitosan-coated insulin liposomes were found effective orally. The insulin liposome coated by 0.2 % of Ch1000k resulted in a significant decrease in the blood glucose level from 0.25 h to 4 h. This hypoglycemic state was maintained up to at least 4 h (P<0.01 *vs* insulin or uncoated), and

Tab 5. Digestion of insulin and insulin-liposomes coated by chitosan with different molecular weights in pepsin solution. *n*=3. Mean±SD.

Time/min	Ins/%	Uncoated/%	Ch1000 kDa/%	Ch680 kDa/%	Ch140 kDa/%	Ch65 kDa/%
0	100.0	100.0	100.0	100.0	100.0	100.0
0.5	77.6±4.6	98.6±2.4	96.0±3.1	86.1±3.6	85.5±4.3	96.4±3.0
1	70.5±2.9	85.9±3.2	89.2±4.2	76.0±4.3	82.4±3.7	81.4±2.7
2	58.9±4.1	75.3±5.5	87.1±1.9	72.1±2.5	72.9±4.6	78.6±4.4
5	45.1±3.7	58.9±3.8	77.0±5.0	50.8±4.7	57.5±2.9	70.7±2.3
10	32.3±4.4	22.7±2.6	73.1±4.8	48.5±3.9	55.4±3.6	68.3±5.3
15	19.8±2.2	6.9±3.4	61.8±3.0	30.7±2.3	40.6±1.6	36.3±3.2
30	6.4±5.4	3.9±4.7	24.7±2.1	20.1±5.1	17.5±3.3	15.6±1.9

Tab 6. Digestion of insulin and insulin-liposomes (Ch1000 kDa) coated by chitosan at different concentrations in pepsin solution. *n*=3. Mean±SD.

Time/min	Digestion rate/%				
	0.1 %	0.2 %	0.3 %	0.4 %	0.5 %
0	100.0	100.0	100.0	100.0	100.0
0.5	93.2±2.9	96.0±3.1	95.5±3.4	99.4±4.0	91.8±2.3
1	92.7±2.8	89.2±4.2	83.8±2.3	93.3±3.2	91.1±3.1
2	85.3±4.1	87.1±1.9	83.6±3.5	92.1±2.1	87.6±3.3
5	76.0±3.6	77.0±5.0	77.9±2.7	86.1±4.2	82.6±4.0
10	45.5±2.5	73.1±4.8	66.1±5.3	73.7±2.8	75.1±2.6
15	44.2±3.7	61.8±3.0	63.6±4.7	68.9±4.5	74.2±5.0
30	20.8±4.3	24.7±2.1	35.7±5.1	43.6±2..7	46.3±3.9

Tab 7. Digestion of insulin and insulin-liposomes coated by chitosan with different molecular weights in trypsin solution. *n*=3. Mean±SD.

Time/h	Digestion rate/%					
	Ins	Uncoated	Ch 1000 kDa	Ch680 kDa	Ch140 kDa	Ch65 kDa
0	100.0	100.0	100.0	100.0	100.0	100.0
0.25	97.3±4..9	99.4±3.7	98.9±3.5	88.1±1.8	81.5±5.8	96.7±3.3
0.5	96.6±4.4	95.8±3.3	93.5±4.0	79.9±2.9	75.6±2.3	94.7±2.8
1	85.8±1.8	84.3±4.2	88.1±2.6	78.9±3.7	75.1±3.2	89.9±4.7
1.5	74.8±3.4	62.8±2.7	87.8±1.9	76.3±2.4	73.0±3.1	86.4±3.6
2	64.5±5.1	57.5±4.6	87.1±3.0	74.5±1.6	73.0±4.4	82.9±3.0
3	48.9±4.0	50.4±3.1	86.7±2.4	72.1±3.3	72.6±2.3	82.9±5.9
4	31.4±2.6	29.6±4.1	85.2±5.0	70.2±4.5	68.9±3.9	74.1±1.8
5	27.8±3.2	22.6±3.0	73.4±3.9	68.5±2.8	67.0±2.1	63.6±4.7

Tab 8. Digestion of insulin and insulin-liposomes (Ch1000 kDa) coated by chitosan at different concentrations in trypsin solution. *n*=3. Mean±SD.

Time/h	Digestion rate/%				
	0.1 %	0.2 %	0.3 %	0.4 %	0.5 %
0	100.0	100.0	100.0	100.0	100.0
0.25	93.1±3.6	98.9±3.5	99.7±4.3	95.5±4.7	90.9±2.8
0.5	88.7±2.3	93.5±4.0	96.7±2.5	95.2±3.4	86.3±3.6
1	84.7±4.5	88.1±2.6	93.3±3.1	92.7±1.8	85.8±4.4
1.5	83.4±2.1	87.8±1.9	92.8±4.8	89.5±2.4	81.2±2.0
2	78.2±3.7	87.1±3.0	89.9±2.7	87.4±5.2	79.9±3.2
3	75.8±4.2	86.7±2.4	81.8±3.0	86.3±3.5	75.7±4.8
4	72.4±3.0	85.2±5.0	79.7±2.9	80.1±5.3	73.7±3.1
5	63.2±1.5	73.4±3.9	75.4±5.0	72.3±3.3	70.6±2.5

the minimum blood glucose level was 15.1 %±6.0 % of the initials at 15 min after administration (Fig 2, 3).

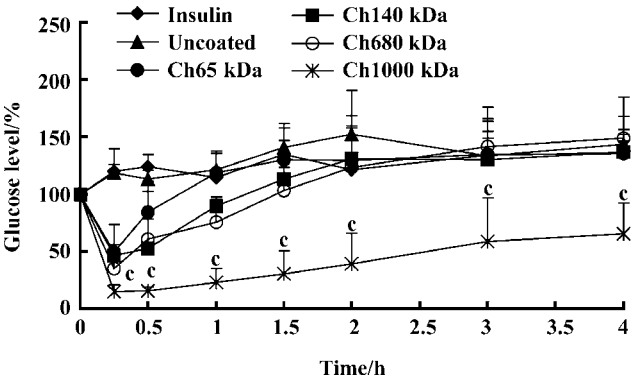

Fig 2. Hypoglycemic effect of insulin-liposomes coated by chitosan with different molecular weights in healthy mice after oral administration. *n*=5. Mean±SD. $^{c}P<0.01$ *vs* insulin.

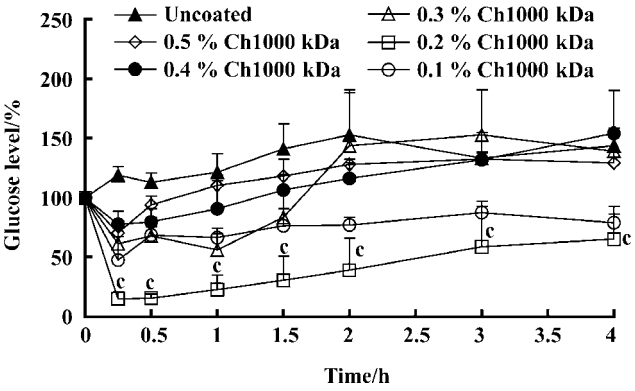

Fig 3. Hypoglycemic effect of insulin-liposomes coated by chitosan with different concentration in healthy mice after oral administration. *n*= 6. Mean±SD. $^{c}P<0.01$ *vs* uncoated.

DISCUSSION

The fingerprint characteristic of uncoated liposomes was very clear. However, because of hydrophilicity of chitosan, the stained areas of chitosan-coated liposomes corresponded to chitosan/dye complex was represented by a dark mesh around the membrane. This result confirmed that chitosan was incumbent on the surface of the liposomes.

The formation of a chitosan layer on the surface of the liposomes was confirmed by comparing the particle size and the zeta potential of liposomes before and after chitosan coating too.

When the coating amount of chitosan was increased by increasing the concentration of chitosan, the particle size of chitosan-coated liposomes increased. The increase was probably due to a combination of adsorption coagulation and bridging between chitosan and liposomes[12]. But chitosan molecular weight did not significantly affect on the particle sizes. The increased ζ potentials of chitosan-coated liposomes were found with increase in both chitosan molecular weights and concentrations. The changes of ζ potentials were attributed to the more cationic polymers fixed on the surface of the liposomes.

EE of insulin in chitosan-coated liposomes was lower than that in uncoated liposomes and slightly increased with increase of the chitosan molecular weights. It was likely that chitosan with lower molecular weight increased the leakage of chitosan-coated liposome. It seemed that the EE was less concentration-dependent. the leakage increased no matter the chitosan concentration was increased or decreased[12].

Insulin encapsulated in chitosan-coated liposomes was protected from pepsin at some extent; this protective action was enhanced by increasing chitosan molecular weight and its concentration, which might result in the increased mucosity. Pepsin was positively charged in pH 2.0 solution. Soy lecithin was negatively charged and pepsin could easily bind to the surface of uncoated liposomes as result of electrostatic effect. The interaction between pepsin and uncoated liposomes enhanced the peptic digestion of insulin encapsulated in uncoated liposomes. But pepsin was not likely to bind to the surface of chitosan-coated liposomes because chitosan was positively charged. Therefore, chitosan-coated liposomes were in favor of protection of insulin from pepsin at some extent

Insulin in the liposomes coated by chitosan with different concentrations and molecular weights was strongly protected from trypsin. This protective action was enhanced by increasing molecular weight of chitosan and had less correlation with concentration of chitosan. In pH 7.4 solution, chitosan molecule existed in more coiled configuration[13,14]. It was fixed on the surface of liposome to form a protective layer. Trypsin is positively charged, and did not easily bind to the surface of chitosan-coated liposomes.

Increase in chitosan molecular weight caused increase in the hypoglycemic efficacy of chitosan-coated insulin liposomes. The hypoglycemic efficacy of the liposomes coated by Ch1000 kDa was markedly superior to that of the liposomes coated by other chitosan. However, some differences were observed in insulin

liposomes coated by chitosan at different concentrations. Either increasing or decreasing chitosan concentration, the hypoglycemic efficacies of chitosan-coated insulin liposomes were decreased. It is suspected that the leakage also increased as chitosan concentration was increased[15], and insulin was released fast from coated liposome when chitosan concentration decreased because the chitosan only formed a thinner membrane in lower concentration. It suggested that chitosan at both higher or lower concentration was not beneficial to protect insulin from enzymatic digestion. Thus, the best chitosan concentration in the coating was 0.2 %.

The zeta potential and the mucoadhesive property of the chitosan-coated liposomes were increased with increase of the concentration of chitosan. In addition, the adhesive ability to the mucin layer was also increased with increase of the molecular weight and concentration of chitosan. The adhesive ability was an important factor in prolonging retention in the gastro-intestinal tract and promoting penetration into the mucus layer. But increase of the values of the zeta potential of the positively charged liposome coated by chitosan caused leakage of insulin as a result of electrostatic interaction between chitosan on the surface of liposomes and insulin entrapped. Thus, the hypoglycemic efficacy of chitosan-coated insulin liposomes was increased with increase of the molecular weight of chitosan, increase or decrease of the concentration of chitosan. The liposomes coated by 0.2 % Ch1000 kDa had the best effect.

In summary, chitosan-coated liposomes could reduce tryptic digestion on insulin, and enhance enteral absorption of insulin. The molecular weights and concentrations of chitosan had significant effects on hypoglycemic efficacy of chitosan-coated insulin liposomes after oral administration to healthy mice.

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