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Relationship between drug effects and particle size of insulin-loaded bioadhesive microspheres

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KEY WORDS particle size; drug effects; insulin; microspheres; oral administration

ABSTRACT

AIM: To formulate and characterize insulin-loaded adhesive microspheres (MP) and evaluate drug effects of MP with various sizes, 120, 350, and 1000 nm in diameter, in the alloxan-induced diabetic rats. **METHODS:** Insulin-loaded MP were formulated by an ionotropic gelation procedure. Particle size distributions were determined by photon correlation spectroscopy and optical microscopy. The factors that influenced the particle sizes and loading capacity were investigated, and the release properties were assessed *in vitro*. The hypoglycemic effect was investigated by monitoring the plasma glucose level of the alloxan-induced diabetic rats after oral administration. **RESULTS:** All the MPs with three sizes formulated were in the desired size range, and the loading capacity was 15.3 %±1.7 % (120 nm), 32.4 %±2.4 % (350 nm), and 53.3 %±2.7 % (1000 nm) respectively. The particle size also had an influence on the release property of the MPs. Half an hour later, 25 %±4 % (120 nm), 18.3 %±2.4 % (350 nm), and 8.6 %±1.3 % (1000 nm) of insulin were released. MP with different sizes had various degree of hypoglycemic effects after 10 h ($P<0.05$ *vs* control insulin solution). The plasma glucose level of 350 nm size particles remarkably decreased 15 h later ($P<0.05$ *vs* 120 nm) or 35 h later ($P<0.01$ *vs* others). The relative pharmacological availability was 10.2 %±0.5 % (120 nm), 14.9 %±1.3 % (350 nm), and 7.3 %±0.8 % (1000 nm) respectively. Particles of 350 nm showed a comparatively higher availability ($P<0.05$). **CONCLUSION:** Adhesive CS-MP were helpful in increasing the relative pharmacological bioavailability of insulin, and a distinct advantage of proper particle size helped to increase the drug effects.

INTRODUCTION

Adhesive particulate carrier systems are of interest as a potential means for oral or nasal administration to enhance drug absorption[1,2], improve bioavailability[3], and as a carrier for macromolecular such as DNA, vaccine in oral immunization[4]. The adhesive property results in increased gastrointestinal transit time, and a distinct increase in efficiency [5]. Particulate matters administered orally may gain entry into the follicle associated epithelium overlying the dome region of the Peyer's patch, via M cells[6]. Oral uptake of methotrexate administered in non-ionic surfactant vesicles of about 120 nm in diameter and elevated liver levels of methotrexate suggested the uptake of intact vesicles and their subsequent sequestration by the reticuloendothelial system (RES)[7]. Micro- and nanoparticles loading antigen, in addition to protecting antigen from the gastric pH and proteolytic enzymes, also act as an adjuvant because they produce the elevated immune

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response compared to soluble antigen[8]. It has proved that cyclosporine A binding to polylactic acid nanosphere produced 20-fold increase in the uptake of it by macrophages *in vitro*[9].

Various types of polymer materials were used as carriers for investigating the factors which influence the gastrointestinal uptake of MP. The hydrophilic polysaccharide CS [(1 → 4)-2-amino-2-deoxyl-β-*D*-glucan] (CS) could be derived by partial deacetylation of chitin from crustacean shells. It has been reported that CS had the special feature of adhering to mucous surface and transiently opening or widening the tight injunction between epithelial cells recently[10]. Chitosan microparticles (CS-MP), cross-linked with the polyanion sodium tripolyphosphate (TPP) and incorporated polyethyleneoxide-polypropyleneoxide copolymers, were suggested to be used as vehicles of proteins and vaccine for oral administration[11].

The main goal of this work was to investigate the potential of CS-MP for oral delivery of insulin and the size-dependent drug effects *in vivo*. Therefore, we expected to have information not only about the potential of CS-MP for increasing insulin intestinal absorption, but also about the suitability of the MP formulation process for preserving the insulin activity and the hypoglycemic effect of the MP with various sizes.

MATERIALS AND METHODS

Experimental animals Alloxan-induced diabetic male Wistar rats (250 g±30 g) were provided by Pharmacological Laboratory of Shenyang Pharmaceutical University, China. The plasma glucose levels of them were in the range of 13.9-16.7 mmol/L. The rats were fasted with water *ad libitum* for 12 h before the experiments and divided into 5 groups: control insulin solution, insulin-loaded CS-MP suspension (120, 350, and 1000 nm) at a dosage of 10 U/kg, and insulin solution (sc, 1 U/kg). All the rats were allowed to drink freely during the experiment.

Materials and reagents The hydrophilic CS (viscosity 45 mPa•s, degree of deacetylation 88.9 %) was generously supplied by Dr ZHAO Rui-Lan (Department of Pharmacy, Shenyang Pharmaceutical University). Porcine insulin (27.6 U/mg) was purchased from Xuzhou Biochemical Plant (China). Tianjing Tianhe Reagents Company (China) produced polyanion tripolyphosphate sodium. Poloxamer188 was purchased from BASF Corporation (USA). All the other reagents were of the chemical grade.

Investigation of the conditions for the formulation of CS-MP CS-MP were prepared according to the procedure firstly reported by Fernandez-Urrusuno *et al* with little modification based on the ionotropic gelation of CS with TPP anions[12]. Preliminary experiments were done to determine the formation zone of the nanoparticles. CS was dissolved in the pH 4 acetic acid aqueous solution at various concentrations of 1.0 , 2.0, 3.0, 4.0, and 5.0 g/L, and TPP was dissolved in purified water at concentrations of 0.36, 1.0, 1.5, 3.0, and 5.0 g/L. Finally, 2 mL of TPP solution was added to 4 mL of the CS solution through a syringe needle under magnetic stirring at room temperature. Then the samples were visually analyzed and three different systems were identified: solution, opalescent suspension, and aggregates. The opalescent suspension was a suspension of nanoparticles we hoped to acquire.

Preparation of MP with various size Insulin-loaded CS-MP were formed spontaneously upon incorporation of TPP aqueous solution containing insulin to pH 4 CS acidic solution according to the methods mentioned above. The CS-MP containing poloxamer188 were formed by dissolving various amounts of poloxamer188 to CS-MP suspension under magnetic stirring ambient. The concentration of poloxamer188 was in the range of 0.5 % to 10 % (w/v).

Particle size distribution Particles smaller than 500 nm diameter were characterized for the size distribution by photon correlation spectroscopy using a Zetasizer III (Malvern Instruments, UK). Optical microscopy (Olympus CHT-001, Olympus Corporation, Lake Success, NY) was used to measure the particles larger than 500 nm. Each batch was analyzed in triplicate.

Insulin loading of these MP The association efficiency of insulin was determined upon separation of MP from the aqueous medium containing nonassociated insulin by centrifugation at 10 000×*g* for 30 min. The amount of free insulin in the supernatant was measured by using HPLC. Twenty microliters were injected into a chromatograph (Shimadzu LC-10A, Kyoto, Japan) equipped with a UV detector (Skimadzu SPD-10A) and reversed phase column (Kromasil C-18, 5 μ, 4.6 mm×200 mm, Zirchrom, Tianhe-Chromatography Industry, China). The mobile phase was a mixture of acetonitrile: NaH_2PO_4 0.1 mol/L: Na_2SO_4 0.05 mol/L=30:35:35 (adjusted to pH 2.5 by phosphate acid). The flow rate was 1.0 mL/min, the wavelength was set

at 214 nm and the column was operated at 35 ºC. Insulin association efficiency (AE) and loading capacity (LC) of the MP were calculated according to the equation (1) and (2) below established by Fernandez-Urrnsuno *et al*[12].

$$AE = \frac{\text{Total amount insulin}-\text{Free amount insulin}}{\text{Total amount insulin}} \quad (1)$$

$$AE = \frac{\text{Total amount insulin}-\text{Free amount insulin}}{\text{Microparticles weight}} \quad (2)$$

Release of insulin from MP *in vitro* The release of insulin from CS-MP with various sizes *in vitro* was carried out by using a shaker (ZS501-A, Liaoning Boda Scientific Apparatus Limited Company) under physiologic conditions at 37 ºC with a shake frequency of 50 times/min. A sample of MP was suspended in 5 mL phosphate buffer solution (PBS, pH 7.4). The amount of MP in the release media was adjusted to guarantee the sink conditions for insulin. At appropriate time intervals, individual samples were centrifuged at 10 000×*g* for 30 min. The supernatant was separated and assayed by HPLC for determining the amount of insulin released from CS-MP. The sedimented MPs were redispersed in the same volume of release medium. The following procedure was carried out according to the method mentioned above.

Hypoglycemic effect of MP *in vivo* Blood samples were collected from the eye orbital vein before oral administration to establish baseline glucose levels, and at different time the blood samples were collected in the same way. Hypoglycemia was determined in the plasma sample by glucose-oxidase method (Glucose GOD-PAD kit, Beijing Ruikang Biochemical Reagent Industry, Beijing, China).

Statistical analysis *in vivo* The mean plasma glucose levels before oral administration were taken as the baseline levels. The percentage of glucose reduction at each time after dosing was calculated and plotted against time.

Relative pharmacological availability was calculated by utilizing equation (3):

$$f = \frac{AAC_{po} \times (\text{weight/dose})_{po}}{AAC_{sc} \times (\text{weight/dose})_{sc}} \quad (3)$$

f was the pharmacological efficacy of the dose *vs* sc, AAC_{po} and AAC_{sc} were the areas above the curve of insulin-loaded CS-MP (po) and insulin solution (sc). The weight/dose was determined experimentally. The average standard deviations of plasma glucose levels measured (n=8) were graphed *vs* time and the trapezoid rule was used to calculate the AAC.

All the data were expressed as mean±SD. Statistical analysis was performed using *t* test.

RESULTS

Formulation MP with various size All the MP with three sizes formulated were in the desired size range (Fig 1). The particle size was greatly influenced by the concentrations of CS and TPP solution and could also be modulated by varying the weight ratio of CS to poloxamer188 (from 200 nm up to 1000 nm). Insulin association efficiency was in the range of 50.4 % to 91.5 % with different loading capacity aquired (Tab 1 and Tab 2).

Release property *in vitro* Various sizes of particles demonstrated a burst release of less than 30 % at

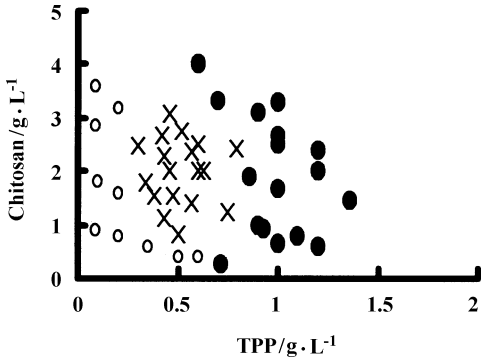

Fig 1. Investigation on the conditions for the formation of CS-MP. ○ represented the zone of a solution; × and ● were the zones with the particle size smaller than 500 nm, larger than 500 nm, respectively. *n*=5. Mean±SD.

Tab 1. Formulation and association insulin to various sizes of CS-MP. The concentration of poloxamer188 was 1 % (w/v) in all the formulations. Mean±SD.

CS solution /g·L^{-1}	TPP solution /g·L^{-1}	Desired diameter /nm	Actual diameter /nm (n=10)	Association efficiency /% (n=6)	Loading capacity /% (n=6)
1.34	0.32	120	123±40	50.4±2.2	15.3±1.7
2.00	0.45	350	345±60	71±3	32.4±2.4
3.21	1.62	1000	1061±200	92±4	53.3±2.7

Tab 2. Influence of poloxamer188 on the characteristics of insulin-loaded CS-MP subsequently incorporated after the formation of MP. The concentration of CS and TPP are 1.45 g/L and 0.50 g/L, respectively in all the formulations. *n*=6. Mean±SD.

$C_{poloxamer188}$ (w:v)	Mean particle size/nm	Association efficiency/%	Loading capacity/%
0	265±34	88.6±2.4	42.3±1.9
0.5 %	274±22	87±3	41.4±2.4
1.0 %	289±31	87.3±2.0	41.6±1.6
10.0 %	387±36	85.4±1.3	40.7±1.3

the first half an hour. During this time interval, 25 %± 4 % (120 nm), 18.3 %±2.4 % (350 nm), and 8.6 %± 1.3 % (1000 nm) of insulin were released; 1 h later, about 53.4 % of insulin were released from MPs with 120 nm, however, insulin released from other particles were not more than 40 %. The cumulate amount of 60 %-76 % insulin in all sizes of MP was released 7 h later. In particles with 1000 nm, the releasing rate was kept an ascending tendency until the end of the experiment (Fig 2). Besides, for the MP with the same size, the release rate was accelerated when hydrophilic poloxamer188 was contained in the CS-MP. The influence of poloxamer188 concentration on the release property of CS-MP is in research.

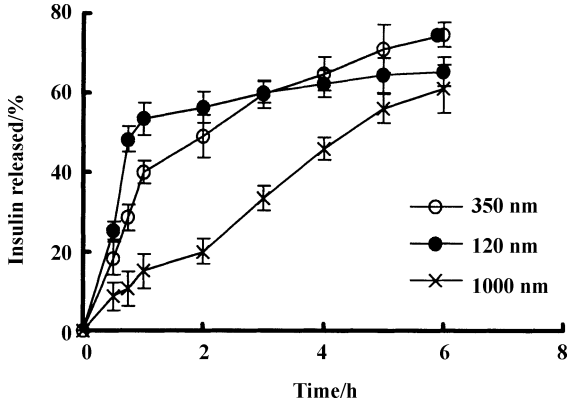

Fig 2. Insulin released from various sizes of CS-MPs *in vitro* in pH 7.4 phosphate buffer solution. *n*=4. Mean±SD.

Drug effects *in vivo* Insulin-loaded CS-MP with three sizes of were found effective orally. CS-MP of 350 nm oral administered to the alloxan-diabetic rats with insulin dosage of 10 U/kg resulted in a significant decrease of the plasma glucose 10 h later and this hypoglycemic state lasted for 6-10 h (P<0.01 *vs* control). The plasma glucose level of particles with 350 nm size remarkably decreased 15 h later (P<0.05 *vs* 120 nm), 35 h later (P<0.01 *vs* others, Fig 3). The pharmacological availability of various sizes (120, 350, and 1000 nm) relative to insulin solution (sc) were 10.2 %, 14.9 %, and 7.3 % respectively. The results of the *t* test showed that the availability of 350 nm was distinctively higher than that of 120 nm or 1000 nm particles respectively (P<0.05).

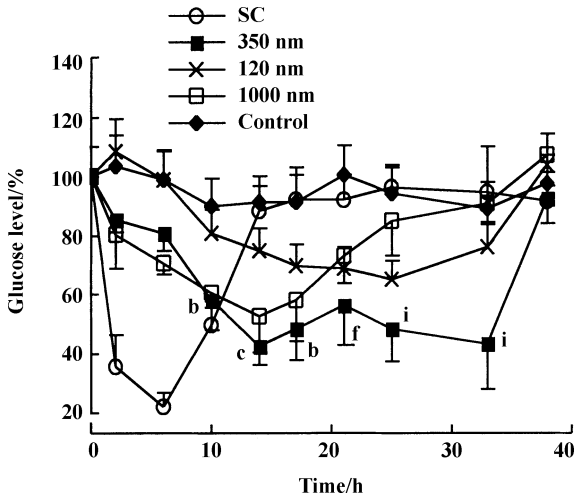

Fig 3. Hypoglycemic effect of various sizes of CS-MPs in alloxan-induced diabetic rats relative to insulin pharmacological saline solution (sc). ^{b}P<0.05, ^{c}P<0.01 *vs* 120 nm. ^{f}P<0.01 *vs* control. ^{i}P<0.01 *vs* 1000 nm. *n*=8. Mean±SD.

DISCUSSION

The use of colloidal carriers made of CS has arisen as promising alternative vehicles for improving the transport of macromoleculars such as peptides and proteins. According to many reports, the M-cells, specialized epithelial cell overlying the lymphoid tissue of the intestinal epithelium, appeared to have a higher uptake of smaller particles[6]. A size-dependent particles deposition in the gastrointestinal tracts of rats was reported in earlier research work and has been combined with adhesion depending on the particle's surface properties[13]. It has been reported that there is a relationship between release property of microspheres *in vitro* and drug effects *in vivo*[14], nevertheless, drug effects of different sizes of insulin-loaded CS-MP has not been

reported until now.

Studies on the conditions for preparing CS-MP indicated that the ionic interaction between negative polyanion TPP and the positively charged amino groups of CS (pK_a 6.5) in pH 4 acetic acid was responsible for the formation of CS-MP. From above, we may conclude that the electrostatic interaction between the acidic insulin groups and the amino CS groups played a role in association insulin to CS-MP. These results were coincident with the conclusion early drawn by Calvo *et al*[11]. It has been proved that the incorporation of poloxamer188 also had an influence on the particle size. However, the negative oxygen atom of poloxamer188 may inhibit the association of insulin by competing in their interaction with amino groups of CS (results not shown in this paper). Therefore, poloxamer188 was incorporated after insulin was entrapped in the CS-MP.

It has been reported that CS in a proper particulate system enhanced the intestinal absorption to a greater extent than the CS solution[1]. The results of drug effects may provide some evidence to prove the ability of CS-MP to enhance the intestinal absorption of insulin. The positive behavior of CS-MP could also demonstrate the conclusions, which was drawn by other authors, that the interaction of CS with the cell membrane resulted in a structural reorganization of tight junction which was followed by an enhanced transport through the paracellular pathway[15].

MP with 350 nm had a greater effect on the glucose level. This result seemed inconsistent with some earlier researches[13,16]. Now we have to concern some other factors such as the viscosity of the suspension, the nature of the CS, and the release property of various sizes of particles. The increased viscosity resulted from higher concentration of CS would prolong the transit time of the MP in the gastrointestinal tract, and at the same time, help to protect insulin from degradation by reducing the movement velocity of molecules. Besides, larger particles released relatively less amount of insulin at the initial burst stage and at this time this part of insulin would have higher possibility to be destroyed in the harsh condition of the gut. However, particles of 1000 nm diameter taken up to a much lesser extent was due to an extremely greater difficulty to gain entry in the tissue through intracellular spaces, therefore, were mainly localized in the epithelial lining of the Peyer's patch and the nonpatch microvilli although they retained a relatively longer period in the gastrointestinal tract. Besides, it was found that CS-MP smaller than 120 nm in pH 1 hydrochloride acid solution appeared transparent several minutes later. While for particles larger than 120 nm, this phenomenon did not occur. Smaller particles may have lower capability of resisting acidic condition in the gastric juice, which may explain why the CS-MP with a diameter of 120 nm has lower drug effects *in vivo*.

In general, an optimal particle size for the design of a particulate carrier system must be chosen based on two major influencing factors. It should be kept in mind that increased the particles allowed the transport of higher drug amounts with less polymer. On the contrary, higher drug effects may exist in smaller particles within limited range. The results of this study showed that a distinct advantage of proper particle size might increase the drug effects *in vivo*. Polymer MP with proper sizes may be particularly well suited for the use with desired project.

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口服胰岛素生物粘附性微球的药效与粒度的关系

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关键词　粒径；药效；胰岛素；微球体；口服给药

目的：以脱乙酰壳聚糖为载体，制备了粒径分别为120，350和1000 nm的胰岛素生物粘附性微球并考察各类微球对四氧嘧啶糖尿病大鼠的降血糖作用. **方法**：采用离子趋向凝胶化方法制备了粒度不同的各类微球. 光子相关光谱法及光学显微镜法测定了各类微球粒径及粒度分布. 考察了处方各因素对微球粒径大小和载药量的影响及各类微球的体外释药特性. 以葡萄糖氧化酶法测定血糖浓度来评价各类微球口服给药后对糖尿病大鼠的降血糖作用. **结果**：制备微球粒径均在所需范围，载药量分别为15.3 %±1.7 %（120 nm），32.4 %±2.4 %（350 nm），53.3 %±2.7 %（1000 nm）. 粒径对微球的体外释放特性有一定的影响，0.5 h后各类微球释放25 %±4 %（120 nm），18.3 %±2.4 %（350 nm），8.6 %±1.3 %（1000 nm）. 口服给药10 h后，各类微球均表现出明显的降血糖作用（$P<0.05$）. 15 h，粒径为350 nm微球所引起的降血糖作用明显高于120 nm；350 nm的微球与其它微球比，35 h后，仍具有显著的降血糖作用（$P<0.01$）. 与皮下注射相对照，口服各类微球的相对生物利用度分别是10.2 %±0.5 %（120 nm），14.9 %±1.3 %（350 nm），7.3 %±0.8 %（1000 nm）. t检验结果表明，口服350 nm微球的生物利用度明显高于其它粒度的微球（$P<0.05$）. **结论**：生物粘附性壳聚糖微球能够提高胰岛素的相对生物利用度，粒径对药物作用有影响且制备合适粒度的微球将有助于进一步提高药效. 载体聚合物对药物的胃肠道吸收发挥重要作用.

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