

Original Research Article

Development and Optimization of Insulin-Chitosan Nanoparticles

Liang Zhao^{1*}, Chang Su^{2*}, Bingya Zhu¹ and Yunhong Jia¹

¹College of Pharmacy, ²College of Veterinary Medicine, Liaoning Medical University, Jinzhou 121000, Liaoning Province, China.

*For correspondence: Email: lypharmacy@163.com; Tel: +86-15804168627; Fax: +86-04164673320

Received: 21 September 2012

Revised accepted: 25 November 2013

Abstract

Purpose: To optimize the preparation of insulin-chitosan nanoparticles (ICNS) using response surface methodology (RSM).

Methods: ICNS were formulated through ionic cross linking method. The effects of the ratio between insulin and chitosan, pH of the medium and rotation speed on insulin encapsulation efficiency (EE) were investigated. Box-Behnken experimental design coupled with response surface method was employed to optimize formulation. Properties such as particle shape, size, zeta potential and release behavior were analyzed.

Results: The best formulation was produced under the following conditions: the ratio between insulin and chitosan was 0.08, pH 3.0, and rotation speed 187.4 rpm. Verifying experiments were established under the optimal conditions and EE was 93.1 %. Nanoparticles showed fine degree of sphericity and homogenous distribution of particle size. The particle size of nanoparticles was between 91.3 ± 7.9 and 220.2 ± 9.5 nm and the average zeta potential was 14.4 ± 2.9 mv. More than 16.8 % of total drug was released rapidly in the first 1 h. Thereafter, the insulin trapped in ICNS was released into the medium slowly and > 93.0 % was released completely within 24 h. Ritger-Peppas model was the best-fit drug release from all the formulations. The diffusion exponent (n) indicates that drug release pattern was non-Fickian diffusion.

Conclusion: Response surface method was a useful tool to predict the optimal formulation. ICNS showed excellent characteristics of homogenous particle size distribution, good spherical property, positive zeta potential and longer drug delivery. It could be a promising carrier for the oral administration of insulin.

Keywords: Nanoparticles, Response surface methodology, Insulin release, Encapsulation efficiency, Zeta potential, Ritger-Peppas model, Box-Behnken design.

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Research on insulin in the management of diabetes mellitus and its preparations is still attracting increasing attention. Researchers tend to design a variety of insulin formulations based on drug delivery theory as a foundation and after a thorough research on physico-chemical properties *in vitro* and *in vivo* pharmacodynamics. Insulin formulations for oral delivery

are emulsions [1], liposomes [2], microspheres [3] and nanoparticles [4]. Nanoparticles, as a drug delivery system, have become an excellent oral insulin delivery carrier [5]. Research shows that nanoparticles produced intestinal uptake in M cells and transport across membrane in the intestinal villi positions. When the small intestinal epithelial cells and Cells which had similar functions with M cells were co-cultured *in vitro*, transport velocity of polystyrene particles with 200 and 500 nm were increased 200-fold [6].

Chitosan (CS) as the only alkaline polysaccharide in nature has a good biocompatibility, biodegradability, adhesion and non-toxicity and has been widely used in medical and pharmaceutical field [7-9]. It has been demonstrated that the positively charged chitosan nanoparticles had strong electrostatic interaction with the negatively charged mucin glycosylation, leading to strong mucosal adhesion. This interaction is responsible for prompting the contact between nanoparticles and epithelial cells and increasing uptake of particles in aggregate lymphoid nodule [10-11]. The objective of this study was to prepare insulin-chitosan nanoparticles (ICNS) using ionic cross-linking method. Response surface method was applied to optimize the formulation and analyze the influence of variables on insulin encapsulation efficiency. Some properties of particles such as shape, size, zeta potential and release behavior were analyzed.

EXPERIMENTAL

Materials

Chitosan (CS, 400kDa) was obtained from Haixin Biological Product Co., Ltd, China, while insulin (27.5IU.mg⁻¹) was purchased from Jiangsu Wangbang Bio-Technology Co., Ltd, China. Trimeric sodium phosphate (TPP) and acetic acid were obtained from Sigma Chemicals, St Louis, USA. All other chemicals purchased were

of analytical grade and were used without further purification.

Experimental design

The ratio between insulin and chitosan (A), pH of medium (B) and rotation speed (C) were the 3 main effective factors and drug encapsulation efficiency as response surface experimental factors were selected to carry out the experiments. Range of the ratio between insulin and chitosan (g/g) was 0.05~ 0.15, pH of medium was 3.0~6.0, rotation speed was 100~300 rpm. Box-Behnken central composite design principle was adopted to optimize experimental design and the data analyzed using Design-Expert®, version 8.0.6.1 (Stat-Ease Inc, USA) to generate a Box-Behnken matrix design as shown in Table 1. Out of a total test point of 17 obtained, 12 were factorial points while 5 were zero points to evaluate error.

Preparation of ICNS

CS (50 mg) was dissolved in 1% (v/v) acetic acid solution, stirring with a magnetic stirrer and swelling over night. Saturated NaOH solution was used to adjust the pH of the medium to the exact pH used. NaOH (0.01 mol/L) solution containing insulin was added with a dropper at 2 ml/min into the chitosan solution and stirring continued for 2 h. TPP reserve liquid (0.5 mg/mL) was prepared and filtered through 0.45 µm filter

Table 1: Box-Behnken matrix design scheme and response values

Fomulation code	A(g/g)	B	C/rpm	Y EE(%)
F1	0.10	4.50	200.00	88.0±3.4
F2	0.10	4.50	200.00	87.6±5.6
F3	0.10	4.50	200.00	86.9±4.1
F4	0.10	6.00	300.00	86.5±3.9
F5	0.10	4.50	200.00	86.8±6.1
F6	0.15	4.50	300.00	79.8±5.2
F7	0.15	4.50	100.00	64.7±3.5
F8	0.05	3.00	200.00	91.5±4.9
F9	0.05	4.50	300.00	82.5±4.7
F10	0.05	6.00	200.00	81.9±3.9
F11	0.15	3.00	200.00	81.2±5.1
F12	0.15	6.00	200.00	69.9±3.6
F13	0.10	3.00	100.00	87.8±4.2
F14	0.10	6.00	100.00	73.4±3.1
F15	0.10	3.00	300.00	85.6±5.9
F16	0.05	4.50	100.00	78.7±4.4
F17	0.10	4.50	200.00	88.3±5.5

(Jiuding Inc, China). ICNS were prepared by dropping 3 ml of TPP reserve liquid quickly into the system at 40 °C and continuously stirred at certain speed ranging from 100 to 300 rpm for 1 h until the mixture turned creamy. ICNS collected was washed 3 times with deionized water and centrifuged at 16000 rpm for 20 min. The particles were freeze-dried using vacuum freeze drier (FD-1, Boyikang, China) to obtain the powders.

Characterization of ICNS

Scanning electron microscopy (S-4800, Hitachi, Japan) was used to observe the morphology of ICNS. Particle size and zeta potential were determined using a Zetasizer (Nano ZS90, Malvern, UK).

Determination of drug encapsulation efficiency

Dried ICNS (5 mg) was dispersed in 10 ml of 0.01 mol/L HCl solution. Ultrasonic extraction was performed for 15 min at 4 °C. The product obtained was centrifuged at 16000 rpm for 30 min and the supernatant was collected and filtered through a 0.22 mm millipore filter. The absorbance of the filtrate was taken at 276 nm using a UV/Visible spectrophotometer (model 1601, Shimadzu, Japan) to determine the amount of insulin trapped in the ICNS. The encapsulation efficiency (EE, %) was calculated using Eq 1.

$$EE (\%) = \{(S_i - S_f)/S_i\}100 \dots\dots\dots (1)$$

where S_i is the initially added insulin and S_f is the free insulin in the supernatant,

Investigation of drug release behavior

The *in vitro* release profile of insulin from nanoparticles was investigated by determining the residual amount of insulin present in the nanoparticles. Several amount (2 mg) of the same freeze dried ICNS were suspended in tubes with caps filled with 5ml phosphate buffer (pH 7.4) at 37 °C and shaken horizontally at 50 min⁻¹ by using an orbital shaker bath (SHA-C, Kaihang, China). At preselected time intervals, three of tubes were withdrawn and centrifuged at 16000 rpm for 30 min and the precipitated ICNS was collected and washed 3 times with deionized water. The amount of residual insulin in nanoparticles was analyzed at 276 nm by using the same method as described in the section of determination of drug encapsulation efficiency. The cumulative amount of insulin (AR) released

from ICNS at different time was calculated using Eq 2.

$$AR (\%) = \{(S_t - S_r)/S_t\}100 \dots\dots\dots (1)$$

where S_t is the initially trapped insulin and S_r is the residual insulin in ICNS at various times.

Statistical analysis

One-way ANOVA was applied to determine significant difference between various values. The level of significance was set at $p < 0.05$.

RESULTS

Response surface methodology (RSM)

The encapsulation efficiency of ICNS (Y) ranged from 64.7±3.5 to 91.5±4.9 %. RSM results for response Y (% encapsulation efficiency) are given in Table 1. The resultant equation for response Y is shown in Eq 3.

$$Y = +87.52 - 4.87A - 4.30B + 3.72C - 43AB + 2.83AC + 3.82BC - 6.65A^2 + 0.25B^2 - 4.45C^2 \dots\dots\dots (3)$$

The result of regression variance analysis and significance test on the model was investigated. F value is 31.24 and the p value < 0.0001 , which demonstrates that the model is highly significant. Model determination coefficient value was 0.9445, which showed that there was linear relationship between the equation dependent variables and all independent variables. The related influence of three factors classified in response value from high to low was the ratio between insulin and chitosan (A), pH of medium (B) and rotation speed (C). Factors that affected the response value remarkably were A, B, C, AC, BC, A², C² and no simple linear relationship was established between the response values and factors. Three factors had a strong interaction on the response value.

The regression optimization response surface plot is shown in Figure 1. The ratio between insulin and chitosan (A) can affect EE. As shown in Figure 1-I, EE value was increased with the increase of the ratio. When the ratio between insulin and chitosan (A) was 0.08 g/g, maximum EE value was 92.82 %. After that, EE value decreased with increase in the ratio. With the decrease of pH of medium (B), EE value was increased gradually.

Response surface analysis

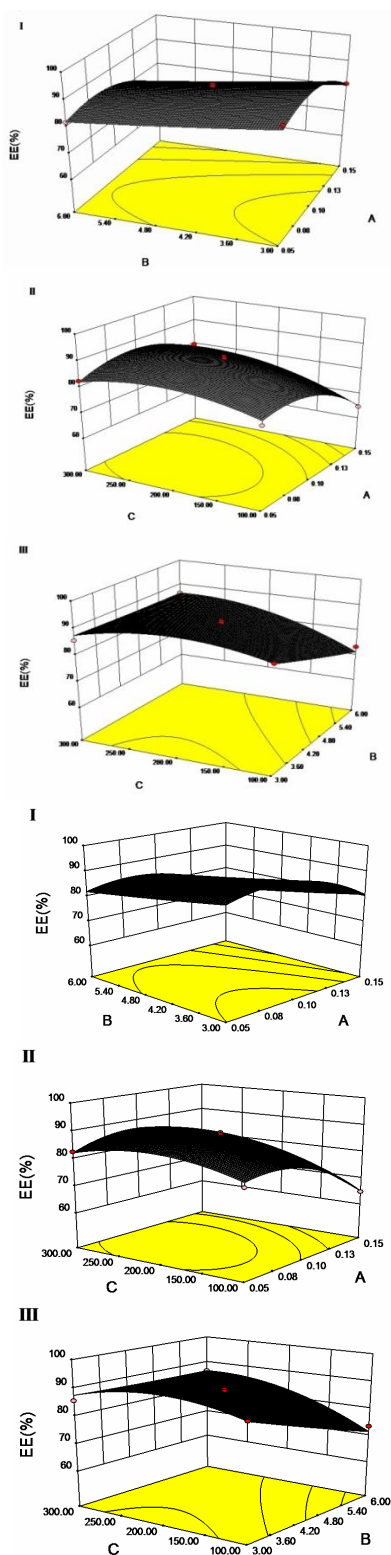


Figure 1: Effect of (I) insulin:chitosan ratio (A) and pH of medium (B); (II) insulin:chitosan ratio (A) and rotation speed (C); (III) pH of medium (B) and rotation speed (C) on encapsulation efficiency (EE)

The regression optimization response surface plot is shown in Figure 1. The ratio between insulin and chitosan (A) can affect EE. As shown in Figure 1-I, EE value was increased with the

increase of the ratio. When the ratio between insulin and chitosan (A) was 0.08 g/g, maximum EE value was 92.82 %. After that, EE value decreased with increase in the ratio. With the decrease of pH of medium (B), EE value was increased gradually. Flatness of surface suggested that interaction between factors A and B was weak. As shown in Figure 1 (II), as ratio and rotation speed (C) increased, EE value showed a curved surface. When the ratio was 0.09 g/g and rotation speed was 232.46 rpm, maximum EE value obtained was 88.85 %. The curves displayed a steep slope, indicating that factors A and C had significant interaction ($p < 0.05$). The interaction graph between pH of medium (B) and rotation speed (C) is shown in Figure 1 (III). Maximum EE (92.07 %) was obtained when pH and rotation speed were 3 and 198.93 rpm, respectively. The sharp slope of the surface implies that the interaction between B and C was significant ($p < 0.05$). The optimal condition for formulation of ICNS was as follows: insulin/chitosan ratio 0.08, pH 3.0, and rotation speed 187.4 rpm. The highest encapsulation efficiency of 92.88% was obtained. Verifying experiments were established under the optimal conditions and EE was 93.10 % with low percentage bias (3.78%).

Morphology of ICNS

The size range of ICNS was between 91.28 ± 7.9 and 220.2 ± 9.5 nm and the average zeta potential was 14.4 ± 2.9 mv. It can be seen from Figure 2 that ICNS showed good sphericity with good monodispersity and homogenous particle size distribution.

Figure 3 shows that three ICNS formulations (F1, F8 and F17) prepared using the matrix design and the optimal formulation (F_{of}) exhibited good drug release behavior. More than 16.8% of total drug was released rapidly in the first 1 h. Thereafter, insulin trapped in ICNS was released into the medium slowly and > 93 % was released completely within 24 h. The *in vitro* release data were fitted into release kinetics models with the aid of using Origin Version 8.0 to determine the best-fit release model .

The release kinetic data analysis and correlation coefficient (R^2) are shown in Table 2. The relationship between cumulative release rate and time was tested using Zero-order, First-order, Higuchi and Ritger-Peppas models. For all the formulations, *in vitro* drug release was best fitted to Ritger-Peppas model which showed the highest correlation coefficient (R^2). The diffusional exponent (n) in Ritger-Peppas model was greater than 0.45, which suggests that drug

release from ICNS was non-Fickian diffusion and therefore was influenced by drug diffusion and matrix erosion [12].

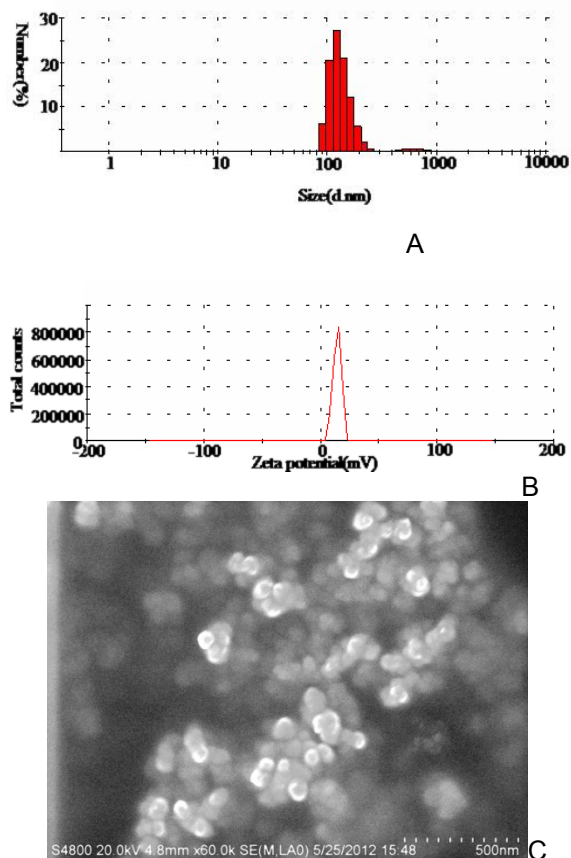


Figure 2: Morphology of ICNS (**Note:** A = particle distribution of ICNS, B = zeta potential distribution of ICNS, and C = SEM of ICNS)

In vitro drug release

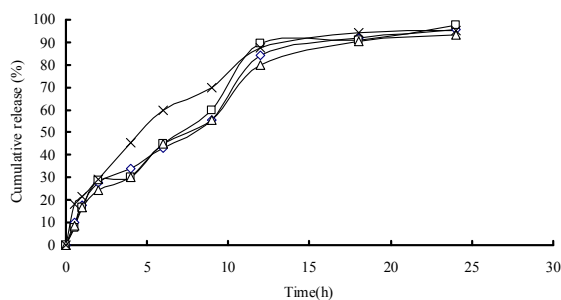


Figure 3: Drug release of ICNS (**Note:** F1 = Δ, F8 = ×, F_of = ◇, F17 = □) (n = 3)

Table 2: Drug-release kinetic data for ICNS

Code	Zero order R2	First order R2	Higuchi R2	Ritger-Peppas R2	n
F1	0.9091	0.9788	0.9732	0.9794	0.6269
F2	0.838	0.9716	0.9700	0.9806	0.4845
F_of	0.9024	0.9718	0.9679	0.9819	0.5831
F17	0.8871	0.9557	0.9574	0.9670	0.6350

DISCUSSION

Response surface method was a useful tool to predict formulation and was adopted to establish the function of relation between regression factors and experimental results, finding explicit functions, regression equation on factors and response value through the whole region. The highest response value and the optimal formulation were obtained with good prediction [13,14]. Using RSM, it is possible to optimize the formulation with optimum drug encapsulation efficiency, good sphericity and homogenous particle size distribution [15]. ICNS showed the rapid drug release during the initial period and more than 16.8% of total drug were released from ICNS within the first 1 h. This is perhaps due to the fact that free insulin was attached to the surface of ICNS or migrated toward the surface with evaporation of water during the freeze-drying process, leading to rapid dumping from ICNS at the initial stage. Drug release decreased after rapid release was completed. As the formation of a porous structure through the whole ICNS by continuous dissolution and erosion of chitosan continued, drug resistance to dissolution and diffusion reduced, and thus drug release rate increased rapidly.

CONCLUSION

ICNS prepared showed excellent properties on morphology and also displayed a prolonged drug release within 24 h. It could be a promising carrier for the oral administration of insulin.

ACKNOWLEDGMENT

Both Liang Zhao and Chang Su contributed equally to the authorship of this manuscript. This work is supported Natural Science Foundation of Liaoning Province (No. 2013010135-401) and Grant of Liaoning Medical University (No. XZJJ20130104-05). The authors thank Dr Rongjian Su (Central Laboratory, Liaoning Medical University) for his help. The authors also wished to acknowledge the support of Prof Wei Cheng and other staff of Pharmacy Department, Liaoning Medical University.

REFERENCES

- Cournarie F, Savelli MP, Rosilio V, Bretez F, Vauthier C, Grossiord JL, Seiller M. Insulin loaded W/O/W multiple emulsions: comparison of the performances of systems prepared with medium-chain-triglycerides and fish oil. *Eur J Pharm Biopharm.* 2004; 58: 477-482.
- Niu M, Lu Y, Hovgaard L, Guan P, Tan Y, Lian R, Qi J, Wu W. Hypoglycemic activity and oral bioavailability of insulin-loaded liposomes containing bile salts in rats: the effect of cholate type, particle size and administered dose. *Eur J*

- Pharm Biopharm.* 2012; 81(2): 265-272.
3. He P, Tang Z, Lin L, Deng M, Pang X, Zhuang X, Chen X. Novel biodegradable and pH-sensitive poly(ester amide) microspheres for oral insulin delivery. *Macromol Biosci.* 2012; 12(4): 547-556.
 4. Zhang ZH, Zhang YL, Zhou JP, Lv HX. Solid lipid nanoparticles modified with stearic acid-octaarginine for oral administration of insulin. *Int J Nanomedicine.* 2012; 7: 3333-3339
 5. Mesiha MS, Sidhom MB, Fasipe B. Oral and subcutaneous absorption of insulin poly(isobutylcyanoacrylate)nanoparticles. *Int J Pharm.* 2005; 288: 289-293.
 6. Lai SK, Wang YY, Hanes J. Mucus-penetrating nanoparticles for drug and gene delivery to mucosal tissues. *Adv Drug Deliv Rev.* 2009; 61(2): 158-171.
 7. Di Martino A, Sittinger M, Risbud MV. Chitosan: a versatile biopolymer for orthopaedic tissue-engineering. *Biomaterials.* 2005; 26(30): 5983-5990.
 8. Sinha VR, Singla AK, Wadhawan S, Kaushik R, Kumria R, Bansal K, Dhawan S. Chitosan microspheres as a potential carrier for drugs. *Int J Pharm.* 2004; 274(1-2): 1-33.
 9. Grenha A, Grainger CI, Dailey LA, Seijo B, Martin GP, Remuñán-López C, Forbes B. Chitosan nanoparticles are compatible with respiratory epithelial cells in vitro. *Eur J Pharm Sci.* 2007; 31(2): 73-84.
 10. Valerie D, Amin KM, Merw in JR. Effect of chitosan on epithelial permeability and structure. *Int J Pharm.* 1999; 182: 21- 32.
 11. Park JH, Saravanakumar G, Kim K, Kwon IC. Targeted delivery of low molecular drugs using chitosan and its derivatives. *Adv Drug Deliv Rev.* 2010; 62(1): 28-41.
 12. Ritger RL, Peppas NA. A simple equation for description of solute release. I. Fickian and non-Fickian release from non-swellable devices in the form of slabs, spheres, cylinders or discs. *J Controlled Release.* 1987; 5(1): 23-36.
 13. Sharma S, Malik A, Satya S. Application of response surface methodology (RSM) for optimization of nutrient supplementation for Cr (VI) removal by *Aspergillus lentulus* AML05. *J Hazard Mater.* 2009; 164(2-3): 1198-1204.
 14. Bezerra MA, Santelli RE, Oliveira EP, Villar LS, Escaleira LA. Response surface methodology (RSM) as a tool for optimization in analytical chemistry. *Talanta.* 2008; 76(5): 965-977.
 15. Shahzad MK, Ubaid M, Murtaza G. Formulation and Optimization of Celecoxib-Loaded Microspheres Using Response Surface Methodology. *Trop J Pharm Res.* 2012; 11(5): 695-702.