International Journal of Pharmaceutics 425 (2012) 1–8

Contents lists available at SciVerse ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

HP55-coated capsule containing PLGA/RS nanoparticles for oral delivery of insulin

Zhi Min Wu[a,1], Liying Zhou[b,1], Xin Dong Guo[a], Wei Jiang[a], Li Ling[a], Yu Qian[a], Kathy Qian Luo[c], Li Juan Zhang[a,*]

[a] School of Chemistry and Chemical Engineering, South China University of Technology, Guangzhou 510640, PR China
[b] Department of Chemical and Biomolecular Engineering, The Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong
[c] Division of Bioengineering, School of Chemical and Biomedical Engineering, Nanyang Technological University, 70 Nanyang Drive, Singapore 637457, Singapore

ARTICLE INFO

Article history:
Received 3 October 2011
Received in revised form
20 December 2011
Accepted 29 December 2011
Available online 12 January 2012

Keywords:
Insulin
Nanoparticles
Enteric capsule
Oral delivery
Hypoglycemic effect

ABSTRACT

In this work, we designed and developed a two-stage delivery system composed of enteric capsule and cationic nanoparticles for oral delivery of insulin. The enteric capsule was coated with pH-sensitive hydroxypropyl methylcellulose phthalate (HP55), which could selectively release insulin from nanoparticles in the intestinal tract, instead of stomach. The biodegradable poly(lactic-*co*-glycolic acid) (PLGA) was selected as the matrix for loading insulin. Eurdragit® RS (RS) was also introduced to the nanoparticles for enhancing the penetration of insulin across the mucosal surface in the intestine. The nanoparticles were prepared with the multiple emulsions solvent evaporation method via ultrasonic emulsification. The optimized nanoparticles have a mean size of 285 nm, a positive zeta potential of 42 mV. The encapsulation efficiency was up to 73.9%. In vitro results revealed that the initial burst release of insulin from nanoparticles was markedly reduced at pH 1.2, which mimics the stomach environment. In vivo effects of the capsule containing insulin PLGA/RS nanoparticles were also investigated in diabetic rat models. The oral delivered capsules induced a prolonged reduction in blood glucose levels. The pharmacological availability was found to be approximately 9.2%. All the results indicated that the integration of HP55-coated capsule with cationic nanoparticles may be a promising platform for oral delivery of insulin with high bioavailability.

1. Introduction

Multiple daily injection of insulin remains to be the traditional approach for the treatment of insulin-dependent diabetic patients (Pillai and Panchagnula, 2001). However, suboptimal control of blood glucose level and poor patient compliance are associated disadvantages with this treatment (Lin et al., 2004). Oral insulin delivery is the convenient way to diabetic patients as it is the most physiological and comfortable means (Agarwal and Khan, 2001; Wong, 2010). However, it is a tough task for orally delivering bioactive macromolecules, due to the highly organized array of barriers existed in the gastrointestinal (GI) tract, such as rapid enzymatic degradation and the poor intestinal absorption (Stoll et al., 2000b). Only small amounts of protein could reach the intestinal wall to be systemically absorbed (Paulson et al., 2001; Stoll et al., 2000a). For many years, various approaches have been developed to enhance oral delivery of insulin (Iyer et al., 2010; Khafagy et al., 2007). Although the possible safety of nanoparticles has not yet been proved, the polymeric nanoparticles are of especial interest due to the pharmaceutical advantages such as enabling modulation of physicochemical characteristics (Card and Magnuson, 2011; Galindo-Rodriguez et al., 2005). Moreover, their submicron size and large specific surface area favor their absorption compared to larger particles (Kesisoglou et al., 2007; Wu et al., 2004).

In recent years, a number of potential oral insulin-loaded polymeric nanoparticles have been developed to overcome the barriers in the GI tract by testing their biological activity in experimental animals (Damge et al., 2008; Des Rieux et al., 2006; Woitiski et al., 2008). The first obstacle for oral delivery of insulin exists in the stomach which forms the boundary between the intestine and the external environment. Special pH-sensitivity of nanoparticles, which prevents insulin from contacting the highly acidic medium in the stomach, provides the protection to confront this early barrier (Cui et al., 2007; Jelvehgari et al., 2009; Lin et al., 2008; Reis et al., 2007; Sajeesh and Sharma, 2006; Sonaje et al., 2009). In order to overcome the secondary barrier of poor intestine absorption, the mucoadhesive nanoparticles can prolong the intestinal residence time and increase the permeability of insulin to the systemic circulation (Deutel et al., 2008; Jintapattanakit

* Corresponding author. Tel.: +86 20 87112046; fax: +86 20 87112046.
E-mail address: celjzh@scut.edu.cn (L.J. Zhang).
[1] These authors have contributed equally to this work.

0378-5173/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2011.12.055

et al., 2009, 2007; Pan et al., 2002; Sonaje et al., 2009; Woitiski et al., 2010; Yin et al., 2009). The positive charge of mucoadhesive nanoparticles was suggested as a major factor for promoting insulin absorption through the electrostatic force with epithelium (Bernkop-Schnurch, 2000). The nanoparticles with more positive charge appeared to be more effective on opening tight junction, leading to an increase in paracellular permeability of drug (Lin et al., 2007). The double-functional nanoparticles with the pH-sensitivity and mucoadhesivity can be designed to against the barriers (Sonaje et al., 2010b). However, the mucoadhesivity and pH-sensitivity may be not synergistic, since the positive charge of mucoadhesive polymer may reduce the stability of the nanoparticles in stomach, while the pH-sensitive polymer would weaken the positive charge of the nanoparticles in intestine (Sarmento et al., 2007a,b; Sonaje et al., 2009). It was recently suggested that filling the double-functional nanoparticles into the enteric-coated capsule could promote the synergistic efficacy (Nguyen et al., 2011; Sonaje et al., 2010a).

Since the first barrier of oral insulin in GI tract is a rapid enzymatic degradation, then followed by the second barrier of the poor intestinal absorption, bypassing two barriers may be also sequential to reach the therapeutic objective (Ferrari, 2005). The multistage delivery system can efficiently overcome various barriers (Ferrari and Tasciotti, 2008; Serda et al., 2010; Tasciotti et al., 2008; Wong et al., 2011). In this study, a two-stage delivery system was designed (Fig. 1). The delivery system with pH-sensitivity (stage-1) and the mucoadhesive property (stage-2) could have an excellent synergistic effect. The hard gelatin capsules coated with pH sensitive enteric polymer, hydroxypropyl methylcellulose phthalate (HP55, pK_a 5.5) was designed to overcome the first barrier. By dissolving HP55 in the solvent mixture of acetone and methylene chloride, the HP55 coating is easier to be dried at room temperature with preparation convenience. Whereas the insulin-loaded cationic nanoparticles composed of the poly(lactic-*co*-glycolic acid) (PLGA) and Eurdragit® RS (RS) (a nondegradable but biocompatible mucoadhesive polymer) was designed to overcome the second barrier. The capsule coated with HP55 in stage-1 was not soluble in stomach (pH = 1.2–2.2), but could dissolve rapidly in the upper region of the small intestine (pH > 5.5) to release the cationic nanoparticles in stage-2. The cationic nanoparticles aim to open the tight junction and enhance the absorption of released insulin.

The insulin loaded PLGA/RS nanoparticles were prepared by the multiple emulsions solvent evaporation method via ultrasonic emulsification. Effects of preparation parameters on the encapsulation efficiency (EE) and size of nanoparticles were investigated. The physicochemical characterization of nanoparticles, such as morphology, diameter, zeta potential, insulin loading efficiency, and the stability of nanoparticles in the GI medium were examined. Then the stage-2 cationic nanoparticles were filled into the stage-1 enteric-coated capsules forming the two-stage delivery system. The capsules were evaluated for in vitro release of insulin, and in vivo biological efficacy after oral administration in diabetic rats.

2. Materials and methods

2.1. Materials

Pure crystalline porcine insulin (27 IU/mg) was purchased from Xuzhou Wanbang Bio-Chemical Co., Ltd. (No. 0312A02, Jiangsu, China). Poly(lactide-*co*-glycolide) (PLGA 50/50, M_w is about 20,000) was acquired from Shandong Medical Instrument Institute. Hydroxypropyl methylcellulose phthalate (HP55, M_w is about 45,000) and polyvinyl alcohol (PVA, 87% hydrolysed, M_w is about 31,000–50,000) were purchased from Acros Organics (New Jersey, USA). Eudragit® RS100 (RS) was obtained from Shanghai Chineway Pharmaceutical Tech. Co., Ltd. (Shanghai, China). The hard-shell gelatin capsule (size 9, length 8.6 mm, diameter 2.65 mm and volume 0.025 mL) was purchased from Torpac Inc. (NJ, USA). All other reagents and solvents were analytical grade. Distilled and deionized water (Milli-Q water systems, Bedford, USA) was used for preparation of all sample solutions.

2.2. Animals

Male Sprague–Dawley rats weighing ~250 g were provided by the Animal & Plant Care Facility of the Hong Kong University of Science and Technology (HKUST). The rats received standard laboratory chow diet and tap water, available ad libitum. All experiments were carried out in accordance with the guidelines for the Care and Use of Experiment Animals in HKUST.

2.3. Preparation of insulin-loaded PLGA/RS nanoparticles

The preparation of insulin-loaded PLGA/RS nanoparticles was carried out by the multiple emulsions solvent evaporation technique. Briefly, the inner aqueous phase of insulin solution was added into oil phase of polymer solution (PLGA/RS, 50/50, w/w) using methylene chloride as solvent, and then the resulting mixture was emulsified by sonification to form the primary emulsion. The primary emulsion was thereafter poured into the external aqueous phase of PVA solution (1%) and sonicated at power of 60 W for 1 min, involving the formation of the multiple emulsions. After evaporation of methylene chloride under reduced pressure, the nanoparticles were collected by centrifugation at 20,000 rpm/min for 10 min and then washed for three times with distilled water. The insulin loaded PLGA/RS nanoparticles were vacuum freeze-dried for 24 h after prefreezing the resultant dispersion (concentration of cryoprotectant was 1.5%) at −20 °C overnight.

2.4. Characterization of the nanoparticles

The size and zeta potential of nanoparticles were determined by photon correlation spectroscopy (PCS) at 25 °C with a detection angle of 90° by using a Malvern Zetasizer II (Malvern Instruments, UK). The measurement was executed in an aqueous dilute nanoparticles suspension. The mean particle sizes and zeta potential values of nanoparticles at pH 1.2, pH 2.0, pH 2.5 (HCl solution), and pH 6.0, pH 6.6, pH 7.0, pH 7.2 (phosphate buffered saline, PBS), simulating the pH environments in the GI tract, were investigated (Lin et al., 2008).

The morphologies of nanoparticles were determined by scanning electron microscopy (SEM, LEO 1530 VP, Germany) and transmission electron microscopy (TEM, Hitachi JEM-100 CXII, Japan). For SEM measurement, the powder of nanoparticles was fixed thinly on an aluminum stub and coated with gold, and performed at an accelerating voltage of 10 kV and a magnification of 20,000× in the transmission electron mode. For TEM, an aqueous droplet of nanoparticles suspension was immobilized on copper grids and negatively stained with phosphotungstate solution (2%, w/v), then dried at room temperature.

The drug encapsulation efficiency (EE) and loading capacity (LC) were calculated by formulas:

$$\text{EE}\ (\%) = \frac{M_{\text{total}} - V_{\text{supernatant}} \times C_{\text{supernatant}}}{M_{\text{total}}} \times 100\% \quad (1)$$

$$\text{LC}\,(\%) = \frac{M_{\text{total}} - V_{\text{supernatant}} \times C_{\text{supernatant}}}{W_{\text{nanoparticle}}} \times 100\% \quad (2)$$

where M_{total} is initial amount of insulin (mg), $V_{\text{supernatant}}$ is the volume of supernatant (mL), $C_{\text{supernatant}}$ is the concentration of insulin

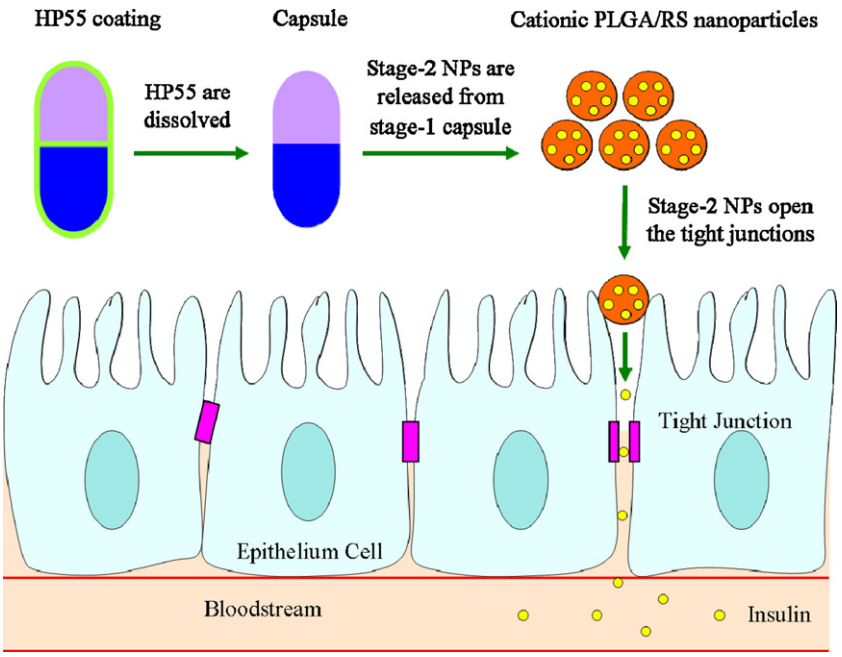

Fig. 1. Schematic illustration of the presumed mechanism of the paracellular transport of insulin using the enteric capsule filled with cationic nanoparticles.

in supernatant (mg/mL), and $W_{nanoparticle}$ is the weight of nanoparticles (mg).

The concentration of insulin was determined by reverse phase HPLC (Agilent 1200, ZORBAX 300 SB-C18 column, 150 mm × 4.6 mm, 5 μm, USA) (Xu et al., 2006). The mobile phase is consisted of a premixed isocratic mixture of 0.2 M sodium sulfate anhydrous solution adjusted to pH 2.3 with phosphoric acid and acetonitrile (73:27, v/v). Samples and standards (20 μL) were injected and eluted at a flow rate of 0.8 mL/min at 30 °C. The absorbance of insulin was determined using the UV trace at 214 nm.

2.5. Preparation of HP55-coated capsule containing insulin loaded PLGA/RS nanoparticles

Hard gelatin capsules were filled with insulin loaded PLGA/RS nanoparticles at a dose of 50 IU/kg. The capsules were immersed in HP55 solution (40 mg/mL) with a mixture solvent of methylene chloride and acetone (4:1, v/v), and followed by air drying at room temperature. The procedure was repeated three times. The prepared enteric-coated capsules were kept in a desiccator until being used.

2.6. In vitro insulin release study

The insulin loaded nanoparticles (5 mg) with or without HP55-coated capsules were dispersed in 5 mL simulated gastric fluid (SGF, pH 1.2) and simulated intestinal fluid (SIF, pH 7.4), respectively, and shaken at 100 rpm at 37 °C using a constant-temperature shaker (SHA-B, Guohua Co., Ltd., China). At specified time intervals (0, 30, 60, 120 and 240 min), the supernatant was collected by centrifugation. The concentrations of insulin in the supernatant were determined by reverse phase HPLC method, and the total amount of insulin released from the nanoparticles was calculated. Each experiment was carried out in triplicate.

2.7. In vivo study for detecting the hypoglycemic effects

Male Sprague–Dawley rats weight about 250 g were fasted overnight and induced diabetics by single intraperitoneal injection of 65 mg/kg streptozotocin (STZ) in citrate buffer at pH 4.5. After 2 weeks of STZ treatment, rats with symptoms of frequent urination, loss of weight, and the fasted blood glucose levels higher than 250 mg/dL were selected as diabetic rats and randomly divided into several groups. The diabetic rats were fasted overnight with free access to water before testing. To minimize the diurnal blood glucose fluctuations, experiments were started from the morning. The diabetic rats were then used to evaluate the hypoglycemic effects of HP55-coated capsules containing the insulin loaded PLGA/RS nanoparticles via oral feeding at 50 IU/kg. The positive control was rats injected with 5 IU/kg insulin solution by subcutaneous (S.C.) injection. The negative control was diabetic rats receiving oral feeding of 50 IU/kg insulin-free nanoparticles. The dosed rats were fasted for 12 h with free access of water. Twelve hours after application the rats were fed. Blood samples were collected from the tail vein of the rat prior to drug administration and at different time intervals after dosing. The blood glucose levels were then measured using a Roche Accu-check glucometer device (Roche Diagnostics, Mannheim, Germany).

Several pharmacodynamic parameters were calculated, which include (1) the hypoglycemic effect, quantified by the area above the curve ($AAC_{0-24\,h}$) by linear trapezoidal method, (2) relative pharmacological availability (PA), (3) the time of minimum glycemia (T_{max}) and (4) the minimum glucose concentration in the blood (C_{min}). The PA of the orally administered insulin was calculated according to the following Eq. (3):

$$PA\,(\%) = \frac{[AAC_{oral}] \times dose_{S.C.}}{[AAC_{S.C.}] \times dose_{oral}} \times 100\% \quad (3)$$

2.8. Statistical analysis

Data were presented as mean ± standard deviation (SD). Comparison between two groups was analyzed by the one-tailed Student's *t*-test. A statistical difference was considered when significance *p* value was less than 0.05.

Table 1
Preparation parameters of multiple emulsions by ultrasonic emulsification method.

Parameters	Values			
Ultrasonic power (W) in preparation of primary emulsion	40	60	80	95
Ultrasonic time (s) in preparation of primary emulsion	30	60	120	240
Polymer concentration in oil phase (mg/mL)	10	20	30	40
Oil phase volume (mL)	2	5	10	15
External aqueous phase volume (mL)	10	20	30	40

3. Results and discussion

3.1. Effect of preparation parameters on the size and encapsulation efficiency (EE) of nanoparticles

The insulin loaded PLGA/RS nanoparticles was prepared by multiple emulsions solvent evaporation method, which comprises the formation of primary emulsion and multiple emulsions using ultrasonic emulsification. However, producing nanoparticles with desired properties (especially high encapsulation efficiency, small size and narrow size distribution) is still a challenge. Many factors could affect the properties of nanoparticles, such as ultrasonic power, ultrasonic time, polymer concentration and the oil phase volume. The effects of main factors on the size and EE of nanoparticles were discussed below (Table 1). The ultrasonic power and time during formation of multiple emulsions were 60 W and 60 s, respectively. The volume and insulin concentration of inner aqueous phase were 1 mL and 10 mg/mL, respectively.

Influences of ultrasonic power and ultrasonic time during preparation of primary emulsion were firstly studied. Generally, there is an optimum power existed (Canselier et al., 2002). Beyond this optimum, coalescence may become predominant due to the cavitation phenomena. The strong ultrasonic power between 40 W and 80 W (Fig. 2a) and long ultrasonic time (Fig. 2b), which favor the formation of smaller drops of primary emulsions, can cause the decrease in size of nanoparticles (Abismail et al., 1999). The ultimate size of an emulsion is determined by the balance between droplet break-up and re-coalescence (Djenouhat et al., 2008; Walstra, 1993). However, at ultrasonic power 95 W, extremely large nanoparticles were obtained, perhaps because the coalescence of the inner aqueous droplets became dominating. On the other hand, we found that the encapsulation efficiency was low with high ultrasonic power and long ultrasonic time, which may be due to the increased destruction of drops of inner aqueous phase, thus resulted in leaking out of insulin from inner aqueous phase. For overall consideration, the ultrasonic power of 40 W and time of 60 s were selected to prepare the primary emulsion in this work.

The polymer concentration, oil phase volume and external aqueous volume may also play critical roles in influencing the size and EE of nanoparticles. Fig. 3a shows that the concentration of polymer PLGA/RS (50/50, w/w), when it was above 20 mg/mL, did not significantly influence the EE, whereas when it was at 10 mg/mL, the EE decreased significantly. There may be existed a threshold concentration of polymer solution, which inhibited the diffusion of insulin to the external aqueous phase. We observed that size of the nanoparticles slightly decreased with increase of oil phase concentration. The increase in concentration of polymer solution may produce a higher solution viscosity of oil phase, thus tend to reduce the coalescence of the water droplet, resulting in smaller w/o emulsion droplet (Behrend et al., 2000; Gaikwad and Pandit, 2008). Thus, the polymer concentration at 20 mg/mL was applied in this study.

The effects of oil phase volume on the size and EE of nanoparticles were presented in Fig. 3b. It showed that the nanoparticles have the smaller size when the oil phase volume ranged from 5 to 10 mL. This effect is presumably attributed to the balance of

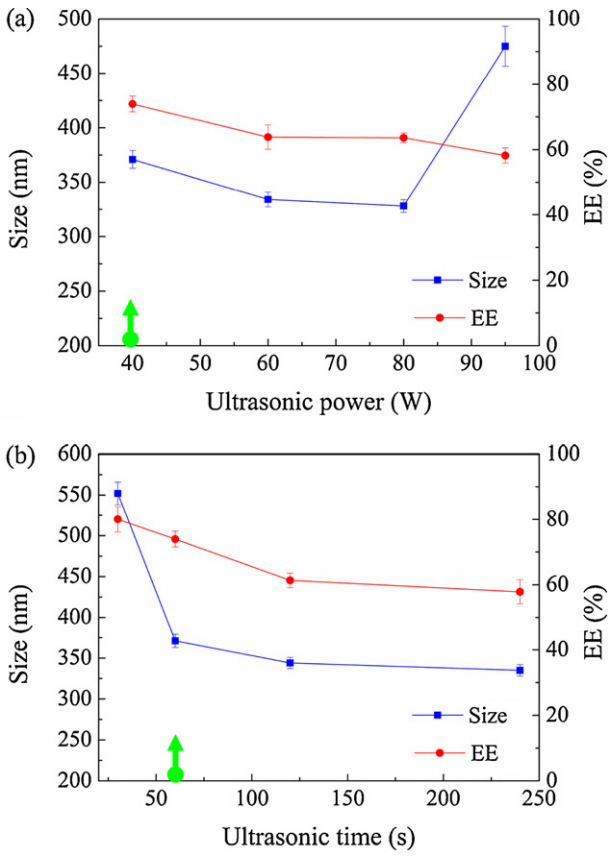

Fig. 2. The effects of (a) ultrasonic power and (b) ultrasonic time on the size and EE ($n = 3$).

stability in the multiple emulsions. As water has a high surface energy (72.8 mJ/m^2), the droplets of inner aqueous phase can coalesce into one another if the oil phase volume is too low, which decrease the stability of w/o emulsion. Thus, the size of nanoparticles decreases rapidly with the increase of oil phase volume from 2 mL to 5 mL. However, the size of nanoparticles increased slightly when the oil phase volume increased from 10 to 15 mL, showing 400 nm at 15 mL. This may be due to a reduction in ultrasonic power density with large volumes of oil phase. The reduction of ultrasonic density probably decreased the stability of multiple emulsion droplets during the preparation process, resulting in the formation of larger nanoparticles (Canselier et al., 2002). On the other hand, the EE increased with the oil phase volume from 2 to 5 mL, which may be also due to the increased stability of w/o emulsion mentioned above. Although increasing oil phase volume produce more stable w/o emulsion, the stability of the multiple emulsions become poor by the decreased ultrasonic density. As a consequence, further increase in the oil phase volume (from 5 to 15 mL) results in a reduction in the EE. Consequently, the oil phase volume at 5 mL was optimized for achieving acceptable size and EE.

The effects of external aqueous phase volume on the size and EE of nanoparticles were shown in Fig. 3c. The increase of the external aqueous phase volume resulted in an increase in both size and EE. It has been suggested that the ultrasonic density decreased with the increase in the volume of external aqueous phase, which resulted in formation of larger emulsion droplet (Gaikwad and Pandit, 2008; Leong et al., 2009). At a stable dispersion of primary emulsion, it was associated with an increase in particle volume by increasing the size of multiple emulsions, which enabled more insulin to be incorporated into the nanoparticles. In this study, the best value of the external aqueous phase volume was found to be 20 mL.

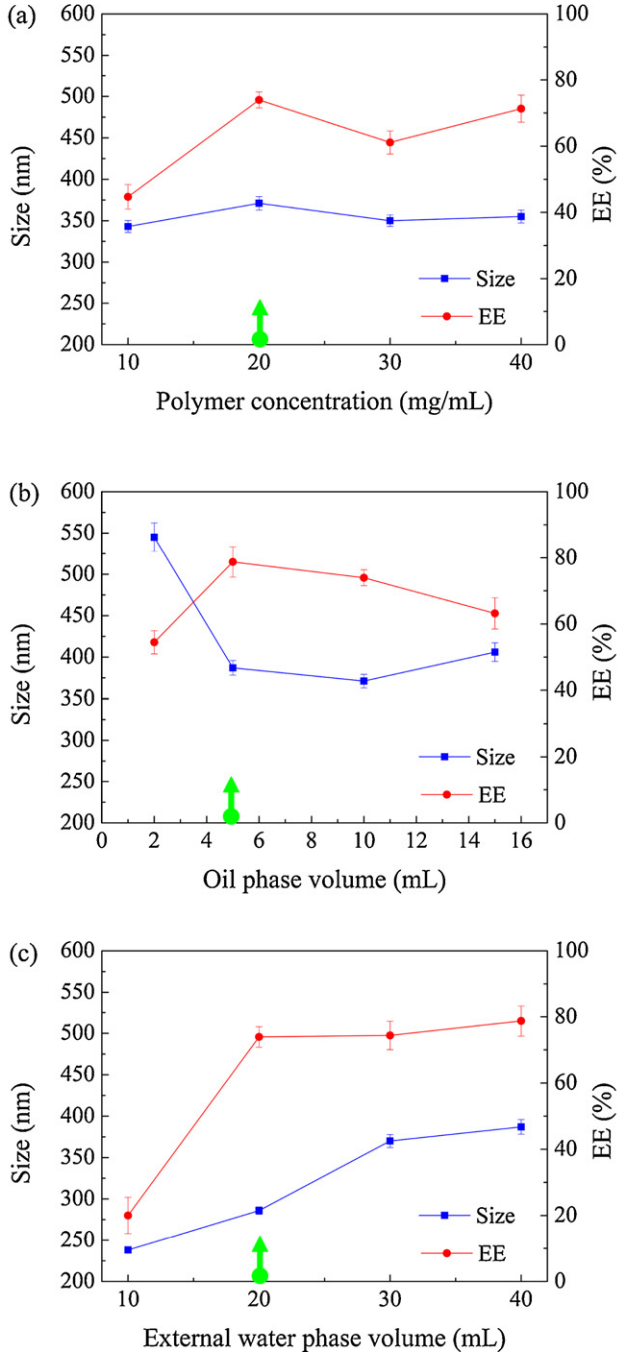

Fig. 3. The effects of (a) polymer concentration, (b) oil phase volume and (c) external water phase volume on the size and EE ($n = 3$).

As a result of the process described above, the optimized PLGA/RS nanoparticles had a mean particle size of 285.6 ± 4.5 nm with a positive zeta potential 42.9 ± 1.4 mV, showing a spherical shape and uniform size (Fig. 4). The EE and loading capacity (LC) of the nanoparticles were 73.9% and 6.7%, respectively.

3.2. *Effect of freeze-drying on the PLGA/RS nanoparticles*

Freeze-drying techniques have been widely employed to yield solid products and aid in conserving the structural integrity of microspheres/nanoparticles and the bioactivity of protein drugs upon storage (Abdelwahed et al., 2006; Ma et al., 1994). Nevertheless, this process generates stresses of freezing and desiccation,

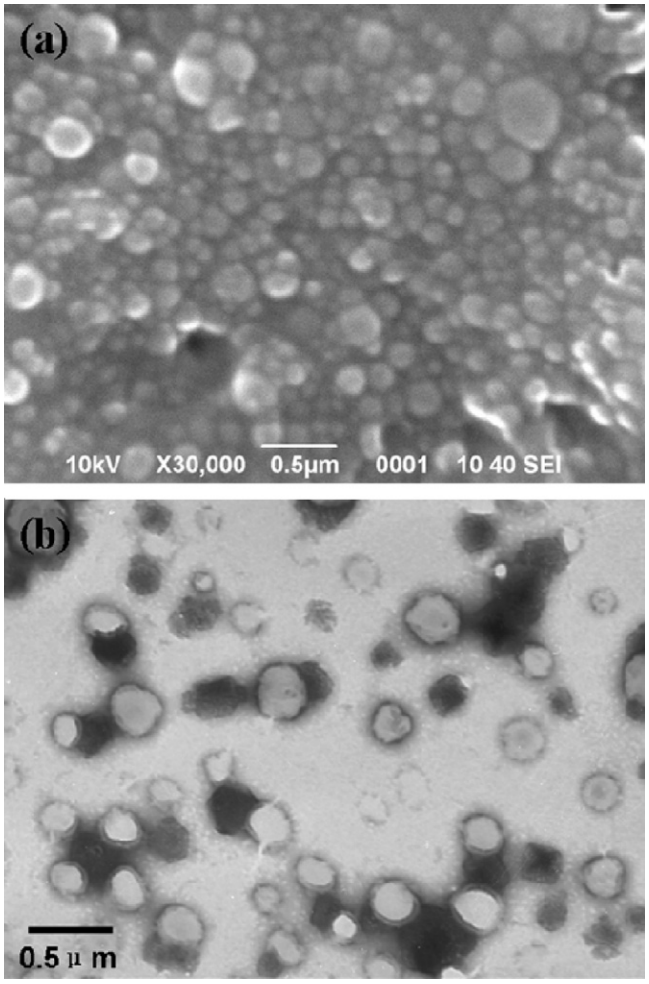

Fig. 4. (a) SEM and (b) TEM images of insulin loaded PLGA/RS nanoparticles.

which could affect the physical instability, such as nanoparticles aggregation and fusion (Abdelwahed et al., 2006). Cryoprotectants are usually added into the formulation to preserve the structural integrity of the nanoparticles. In this study, the effects of trehalose as the cryoprotectant at the different concentrations were evaluated during the freeze-drying process.

The size and zeta potential of the tested nanoparticles after freeze-drying were investigated and compared as shown in Table 2. We prepared our initial nanoparticles with external aqueous volume 40 mL. Before freeze-drying, the mean particle size of nanoparticles was 374.6 ± 3.8 nm, and the zeta potential was 37.9 ± 2.2 mV. Nanoparticles without the addition of trehalose aggregated after freeze-drying. When the trehalose concentration increased from 0.25% to 1%, the size and polydispersity

Table 2
Influences of the freeze-drying process with or without trehalose on the size and zeta potential of insulin loaded PLGA/RS nanoparticles ($n = 3$).

Trehalose (%)	Mean particle size (nm)	PDI	Zeta potential (mV)
Before freeze-drying	374.6 ± 3.8	0.39 ± 0.03	37.9 ± 2.2
0%	N/A	N/A	11.46 ± 3.9
0.25%	1426 ± 277.8	0.75 ± 0.27	15.85 ± 2.3
0.5%	664.6 ± 23.2	0.54 ± 0.12	30.29 ± 1.8
1.0%	483.0 ± 14.1	0.42 ± 0.06	31.43 ± 1.2
1.5%	370.6 ± 4.1	0.40 ± 0.01	30.39 ± 1.5
2.0%	374.7 ± 6.7	0.44 ± 0.07	31.74 ± 1.3

N/A: precipitation of nanoparticles was observed.

Table 3
Mean particle sizes and zeta potential values of nanoparticles after freeze-drying at distinct pH environments ($n = 3$).

pH values	Mean particle size (nm)	PDI	Zeta potential (mV)
1.2	244.3 ± 2.3	0.238 ± 0.019	18.3 ± 3.7
2.0	246.3 ± 1.2	0.285 ± 0.035	25.4 ± 4.2
2.5	250.7 ± 2.5	0.245 ± 0.020	33.9 ± 2.1
6.0	350.2 ± 21.2	0.359 ± 0.065	32.6 ± 0.5
6.6	393.7 ± 12.2	0.409 ± 0.058	23.7 ± 0.5
7.0	437.7 ± 19.0	0.491 ± 0.019	8.6 ± 0.4
7.2	543.2 ± 22.1	0.528 ± 0.018	7.9 ± 0.1

The freeze-dried nanoparticles were prepared using 1.5% trehalose as cryoprotectant.

(PDI) of nanoparticles were reduced significantly due to the decrease in freezing stress. And no obvious changes in size and PDI were observed when the trehalose concentration was above 1.5%. However, it was found that the addition of trehalose to PLGA/RS nanoparticles suspension decreased the positive charge of nanoparticles surface from +37.9 mV to approximately +30 mV. This may be due to the hydrogen bonding between hydroxyl groups of trehalose and the positive groups in nanoparticles surface. At the trehalose concentration below 0.25%, the lack of an amorphous trehalose matrix induced the large aggregation and low zeta potential. It was proved that trehalose was more effective for stabilizing PLGA/RS nanoparticles during freeze-drying at concentration of 1.5%.

3.3. Zeta potential values of PLGA/RS nanoparticles at different pH conditions

The zeta potential value is an important particle character as it can influence both particle stability and mucoadhesion in GI conditions (Hariharan et al., 2006; Vandervoort and Ludwig, 2002). The mucus layer itself is an anionic polyelectrolyte at neutral pH value (Baeyens and Gurny, 1997). Consequently, the positive charge groups on the nanoparticles attract with the mucus by the electrical force. As shown in Table 3, the zeta potential values and sizes of the PLGA/RS nanoparticles were investigated in the pH range 1.2–7.2, representing the GI conditions. As a result of the incorporation of quaternary ammonium functional groups, Eudragit® RS possesses a defined swelling capacity and permeability with respect to water, which is independent of pH (Vachon and Nairn, 1998). Insulin [isoelectric point (pI) = 5.4] changes into a positively charged molecule at pH 1.2. Theoretically, the insulin loaded PLGA/RS nanoparticles would have a high zeta potential due to the increased positive charge of insulin with increase in acidity. Nevertheless, the zeta potentials of PLGA/RS nanoparticles became less positive from pH 2.5 to 1.2. This may be because of the presence of too much free PVA on the highly positive charged nanoparticles and a shielding effect of PVA layer in strong acidic condition. The anionic character of PVA chains containing ionized acetate groups and remaining in suspensions of freeze-drying nanoparticles, reduces the zeta potential of nanoparticles (Wi niewska, 2011). On the other hand, the steric stability of PVA layer on the surface of nanoparticles can prevent the self-aggregation of the nanoparticles, thus the size of nanoparticles in acidic conditions showed no obvious change. In the case of the simulated intestinal medium of pH > 6.0, the zeta potential of PLGA/RS nanoparticles reduced due to the neutralization of the negatively charged insulin as well as increased counter ions (phosphate), leading to the part aggregation of the nanoparticles.

Hence, we could infer that the mucoadhesivity of PLGA/RS nanoparticles in luminal surface of duodenal region (pH 6.0–6.6) should be greater than those of jejunum (pH 7.0) and ileum region (pH 7.4), due to the higher positive zeta potential at pH 6.0–6.6

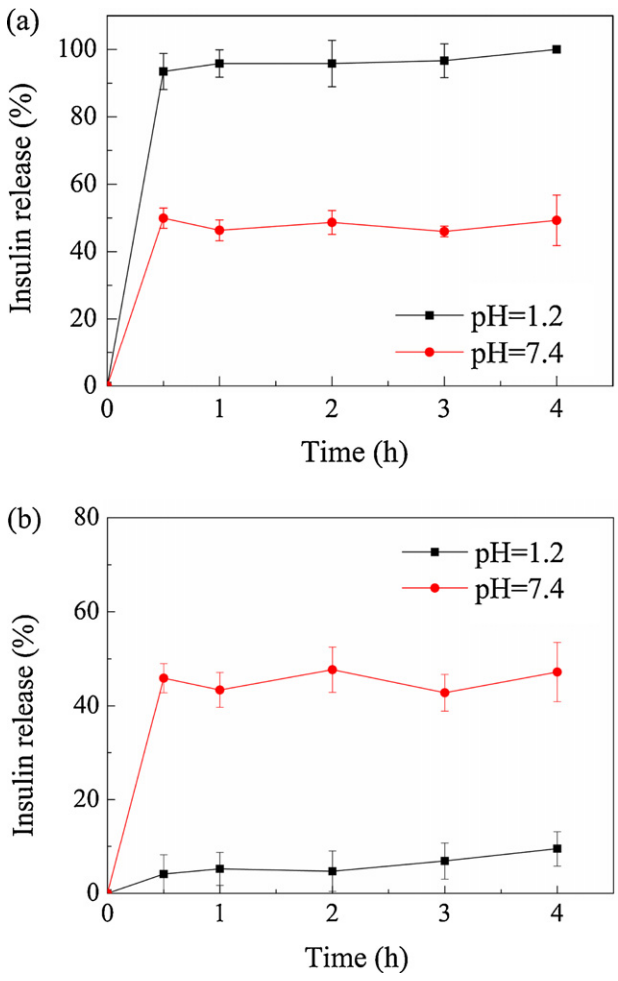

Fig. 5. In vitro release of insulin from (a) PLGA/RS nanoparticles without capsule and (b) PLGA/RS nanoparticles within HP55-coated capsule ($n = 3$).

medium compared to that of at pH 7.0–7.2. The enteric capsules coated with HP55 ($pK_a = 5.5$) can be employed for targeted delivery of nanoparticles to the upper of small intestine, and allow the adsorption of insulin for a long period of time.

3.4. In vitro release study

The in vitro insulin release profiles of PLGA/RS nanoparticles with and without HP55-coated capsule were evaluated at acidic and alkaline pH environment. As shown in Fig. 5a, the nanoparticles without HP55-coated capsule displayed an unfavorable pH-sensitive release profiles. Above 90% of insulin released at pH 1.2 and around 50% released at pH 7.4 medium within the first 0.5 h. The release of insulin from PLGA/RS nanoparticles may be modulated by a swelling-diffusion process. At low pH, RS molecules repel mutually, resulting in a swelling and permeable polymer matrix. As well, at pH 1.2, the positively charged insulin molecules interact repulsively with the positively charged RS, promoting release of insulin. In contrast, insulin with negative charge in the intestinal medium attracts with RS polymer by the electrostatic attraction. Thus, incomplete release of insulin from PLGA/RS nanoparticles was observed at pH 7.4 PBS condition.

When the stage-2 PLGA/RS nanoparticles were filled into the stage-1 HP55-coated capsule as the two-stage delivery system, the initial release of insulin dramatically reduced at pH 1.2, as shown in Fig. 5b. Indeed, the tested capsules remained intact in acid environment during the experimental process. It suggested that HP55

Table 4
Pharmacodynamic parameters after oral administration of enteric-coated capsule filled with insulin loaded PLGA/RS nanoparticles at 50 IU/kg doses, and S.C. injection of 5.0 IU/kg insulin solution ($n = 3$).

Sample	$AAC_{0-24\,h}$ (% h)	Hypoglycemic effect (%)	PA (%)	T_{max} (h)	C_{min} (%)
Enteric capsule (oral)	931 ± 237	32.9 ± 8.4	9.2 ± 2.4	10	58.9 ± 20
Insulin solution (S.C.)	1008 ± 315	35.2 ± 11.2	100	2	11.7 ± 1.1

T_{max}: time at minimum relative basal glucose concentration in the blood.
C_{min}: minimum relative basal glucose concentration in the blood.

played an important role for selectively releasing insulin at pH 7.4, instead of pH 1.2. We also found that at pH 7.4, the insulin release profiles from PLGA/RS nanoparticles were similar with or without capsule, indicating that the stage-2 capsule had no effect on the release of insulin from particles in intestinal condition. Thus there is a synergistic efficacy between the first stage capsule and the second stage nanoparticles. The HP55-coated capsule firstly dissolved, and then the nanoparticles were released. The HP55-coated capsule was functional as the carrier to protect the delivery process of the nanoparticles, while the nanoparticles with cationic property may have the ability to open tight junction and enhance the permeation of the released insulin.

3.5. Oral administration of the capsule filled with PLGA/RS nanoparticles in diabetic rats

Fig. 6 shows the blood glucose level-time profiles after oral administration of HP55 coated capsule containing insulin loaded PLGA/RS nanoparticles (50 IU/kg), blank PLGA/RS nanoparticles (50 IU/kg, negative control) and S.C. injection of free insulin solution (5 IU/kg, positive control) in diabetic rats. As expected, no hypoglycemic effect was observed in the negative control group. However, oral administration of the capsule filled with insulin loaded PLGA/RS nanoparticles and S.C. injection of free insulin solution showed a significant hypoglycemic effect in the diabetic rats. The blood glucose levels of diabetic animals decreased sharply (90% in 2 h) after S.C. injection of the insulin solution, and returned gradually to the basal levels at 10 h. Whereas, the blood glucose levels reduced slowly after oral administration of the enteric-coated capsules. The slight increase of the blood glucose levels during the first 4 h was possibly due to physical stress to the diabetic rats during handling and blood sampling (Sajeesh et al., 2010). The hypoglycemic effect and the pharmacological availability (PA) of the enteric-coated capsule, as noted in Table 4, were 32.9% and 9.2%, respectively, which were determined by the dose-corrected $AAC_{0-24\,h}$ over 24 h relative to that of S.C. injection.

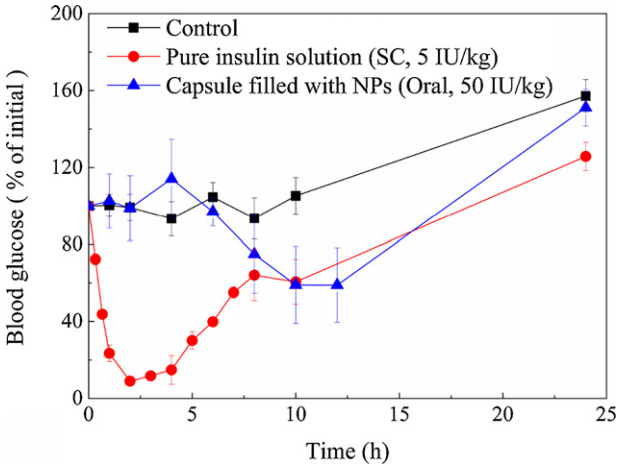

Fig. 6. Plasma glucose levels after oral administration of the HP55-coated capsule containing insulin loaded PLGA/RS nanoparticles (50 IU/kg), blank nanoparticles, and S.C. injection of insulin solution (5.0 IU/kg) in diabetic rats ($n = 3$). Significant difference from negative blank nanoparticles control ($p < 0.05$).

The observed hypoglycemic efficacy of the capsule filled with insulin loaded PLGA/RS nanoparticles in the diabetic rat model can be illuminated by the synergistic effect of the two-stage delivery system. The stage-1 HP55-coated capsule could serve as a platform for encapsulation of insulin-loaded nanoparticles and reduce its proteolytic break-down in hash environment of stomach. The mucoadhesive property of the stage-2 nanoparticles was achieved by attachment of cationic ammonio-methyl groups of RS to the mucous (Damge et al., 2007). The RS-based nanoparticles may induce the opening of epithelial tight junctions between enterocytes, and allow the transport of macromolecular drugs (Damge et al., 2010; Thanou et al., 2001). Once the stage-1 capsule reached the upper intestine, the encapsulated stage-2 nanoparticles would be released very rapidly and reached a high particle gradient concentration. The colloidal stability of PLGA/RS nanoparticles in basic medium may increase the chance of the nanoparticles to distribute and adhere in large region of intestine. The released insulin from the PLGA/RS nanoparticles could be transported via the intercellular pathway. These effects may act synergistically to enhance and prolong the efficacy of insulin delivered by using HP55-coated capsule containing PLGA/RS nanoparticles as two-stage delivery system.

4. Conclusions

HP55-coated capsule containing insulin loaded PLGA/RS nanoparticles was developed as a two-stage delivery system for the oral delivery of insulin. The optimized stage-2 PLGA/RS nanoparticles had a mean particles size of 285.6 ± 4.5 nm with a positive zeta potential 42.9 ± 1.4 mV. The encapsulation efficiency and loading content of the nanoparticles were 73.9% and 6.7%, respectively. The stage-1 HP55-coated capsule prevented the release of insulin from the PLGA/RS nanoparticles in acidic environment, thereby against rapid enzymatic degradation. PLGA/RS nanoparticles, as the second stage, adhered to the intestine mucosa and released insulin to improve the absorption of insulin across the intestinal epithelial cells. The two-stage delivery system showed a prolonged hypoglycemic effect in a diabetic rat model after oral administration. The pharmacological availability was approximately 9.2%. These results suggested that the two-stage delivery system developed in this study might be employed as a potential approach to improve the efficacy for oral delivery of protein/peptide drugs.

Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (No. 21176090), Hong Kong ITF (No. ITP/006/08NP), the Team Project of Natural Science Foundation of Guangdong Province, China (No. S2011030001366), Guangzhou Municipal Bureau of Science and Technology (No. 2009J1-C511-2), and the Fundamental Research Funds for the Central Universities (No. 2011ZM0041).

References

Abdelwahed, W., Degobert, G., Stainmesse, S., Fessi, H., 2006. Freeze-drying of nanoparticles: formulation, process and storage considerations. Adv. Drug Del. Rev. 58, 1688–1713.

Abismail, B., Canselier, J.P., Wilhelm, A.M., Delmas, H., Gourdon, C., 1999. Emulsification by ultrasound: drop size distribution and stability. Ultrason. Sonochem. 6, 75–83.

Agarwal, V., Khan, M.A., 2001. Current status of the oral delivery of insulin. Pharm. Technol. 10, 76–90.

Baeyens, V., Gurny, R., 1997. Chemical and physical parameters of tears relevant for the design of ocular drug delivery formulations. Pharm. Acta Helv. 72,191–202.

Behrend, O., Ax, K., Schubert, H., 2000. Influence of continuous phase viscosity on emulsification by ultrasound. Ultrason. Sonochem. 7, 77–85.

Bernkop-Schnurch, A., 2000. Chitosan and its derivatives: potential excipients for peroral peptide delivery systems. Int. J. Pharm. 194, 1–13.

Canselier, J.R., Delmas, H., Wilhelm, A.M., Abismail, B., 2002. Ultrasound emulsification—an overview. J. Disper. Sci. Technol. 23, 333–349.

Card, J.W., Magnuson, B.A., 2011. A review of the efficacy and safety of nanoparticle-based oral insulin delivery systems. Am. J. Physiol-Gastr. L., doi:10.1152/ajpgi.00107.2011.

Cui, F.D., Tao, A.J., Cun, D.M., Zhang, L.Q., Shi, K., 2007. Preparation of insulin loaded PLGA-Hp55 nanoparticles for oral delivery. J. Pharm. Sci. 96, 421–427.

Damge, C., Maincent, P., Ubrich, N., 2007. Oral delivery of insulin associated to polymeric nanoparticles in diabetic rats. J. Control. Release 117, 163–170.

Damge, C., Reis, C.P., Maincent, P., 2008. Nanoparticle strategies for the oral delivery of insulin. Expert Opin. Drug Deliv. 5, 45–68.

Damge, C., Socha, M., Ubrich, N., Maincent, P., 2010. Poly(epsilon-caprolactone)/Eudragit nanoparticles for oral delivery of aspart-insulin in the treatment of diabetes. J. Pharm. Sci. 99, 879–889.

Des Rieux, A., Fievez, V., Garinot, M., Schneider, Y.J., Preat, V., 2006. Nanoparticles as potential oral delivery systems of proteins and vaccines: a mechanistic approach. J. Control. Release 116, 1–27.

Deutel, B., Greindl, M., Thaurer, M., Bernkop-Schnuerch, A., 2008. Novel insulin thiomer nanoparticles: in vivo evaluation of an oral drug delivery system. Biomacromolecules 9, 278–285.

Djenouhat, M., Hamdaoui, O., Chiha, M., Samar, M.H., 2008. Ultrasonication-assisted preparation of water-in-oil emulsions and application to the removal of cationic dyes from water by emulsion liquid membrane. Part 1. Membrane stability. Sep. Sci. Technol. 62, 636–641.

Ferrari, M., 2005. Nanovector therapeutics. Curr. Opin. Chem. Biol. 9, 343–346.

Ferrari, M., Tasciotti, E., 2008. Multistage delivery of active agents. US Patents, US 20080311182A1.

Gaikwad, S.G., Pandit, A.B., 2008. Ultrasound emulsification: effect of ultrasonic and physicochemical properties on dispersed phase volume and droplet size. Ultrason. Sonochem. 15, 554–563.

Galindo-Rodriguez, S.A., Allemann, E., Fessi, H., Doelker, E., 2005. Polymeric nanoparticles for oral delivery of drugs and vaccines: a critical evaluation of in vivo studies. Crit. Rev. Ther. Drug Carrier Syst. 22, 419–463.

Hariharan, S., Bhardwaj, V., Bala, I., Sitterberg, J., Bakowsky, U., Ravi Kumar, M.N.V., 2006. Design of estradiol loaded PLGA nanoparticulate formulations: a potential oral delivery system for hormone therapy. Pharm. Res. 23, 184–195.

Iyer, H., Khedkar, A., Verma, M., 2010. Oral insulin—a review of current status. Diabetes Obes. Metab. 12, 179–185.

Jelvehgari, M., Zakeri, P., Valizadeh, H., Siahi, M.R., Nokhodchi, A., Monajemzadeh, F., Azari, Z., 2009. A pH-sensitive colon targeted oral drug delivery system using insulin nanoparticles employing chitosan with different molecular weights and Eudragit L100-55. Amino Acids 37, 97.

Jintapattanakit, A., Junyaprasert, V.B., Kissel, T., 2009. The role of mucoadhesion of trimethyl chitosan and PEGylated trimethyl chitosan nanocomplexes in insulin uptake. J. Pharm. Sci. 98, 4818–4830.

Jintapattanakit, A., Junyaprasert, V.B., Mao, S., Sitterberg, J., Bakowsky, U., Kissel, T., 2007. Peroral delivery of insulin using chitosan derivatives: a comparative study of polyelectrolyte nanocomplexes and nanoparticles. Int. J. Pharm. 342, 240–249.

Kesisoglou, F., Panmai, S., Wu, Y., 2007. Nanosizing-oral formulation development and biopharmaceutical evaluation. Adv. Drug Deliv. Rev. 59, 631–644.

Khafagy, E.S., Morishita, M., Onuki, Y., Takayama, K., 2007. Current challenges in non-invasive insulin delivery systems: a comparative review. Adv. Drug Deliv. Rev. 59, 1521–1546.

Leong, T.S.H., Wooster, T.J., Kentish, S.E., Ashokkumar, M., 2009. Minimising oil droplet size using ultrasonic emulsification. Ultrason. Sonochem. 16,721–727.

Lin, C.R., Gokhale, R., Trivedi, J.S., Ranada, V., 2004. Recent strategies and methods for improving insulin delivery. Drug Dev. Res. 63, 151–160.

Lin, Y.H., Chen, C.T., Liang, H.F., Kulkarni, A.R., Lee, P.W., Chen, C.H., Sung, H.W., 2007. Novel nanoparticles for oral insulin delivery via the paracellular pathway. Nanotechnology 18, 1–11.

Lin, Y.H., Sonaje, K., Lin, K.M., Juang, J.H., Mi, F.L., Yang, H.W., Sung, H.W., 2008. Multi-ion-crosslinked nanoparticles with pH-responsive characteristics for oral delivery of protein drugs. J. Control. Release 132, 141–149.

Ma, X., Santiago, N., Chen, Y., Chaudhary, K., Milstein, S., Baughman, R., 1994. Stability study of drug-loaded proteinoid microsphere formulations during freeze-drying. J. Drug Target 2, 9–21.

Nguyen, H.N., Wey, S.P., Juang, J.H., Sonaje, K., Ho, Y.C., Chuang, E.Y., Hsu, C.W., Yen, T.C., Lin, K.J., Sung, H.W., 2011. The glucose-lowering potential of exendin-4 orally delivered via a pH-sensitive nanoparticle vehicle and effects on subsequent insulin secretion in vivo. Biomaterials 32, 2673–2682.

Pan, Y., Li, Y.J., Zhao, H.Y., Zheng, J.M., Xu, H., Wei, G., Hao, J.S., Cui, F.D., 2002. Bioadhesive polysaccharide in protein delivery system: chitosan nanoparticles improve the intestinal absorption of insulin in vivo. Int. J. Pharm. 249, 139–147.

Paulson, S.K., Vaughn, M.B., Jessen, S.M., Lawal, Y., Gresk, C.J., Yan, B., Maziasz, T.J., Cook, C.S., Karim, A., 2001. Pharmacokinetics of celecoxib after oral administration in dogs and humans: effect of food and site of absorption. J. Pharmacol. Exp. Ther. 297, 638–645.

Pillai, O., Panchagnula, R., 2001. Insulin therapies-past, present and future. Drug Discov. Today 6, 1056–1061.

Reis, C., Ribeiro, A., Houng, S., Veiga, F., Neufeld, R., 2007. Nanoparticulate delivery system for insulin: design, characterization and in vitro/in vivo bioactivity. Eur. J. Pharm. Sci. 30, 392–397.

Sajeesh, S., Sharma, C., 2006. Novel pH responsive polymethacrylic acid-chitosan-polyethylene glycol nanoparticles for oral peptide delivery. J. Biomed. Mater. Res. B 76, 298–305.

Sajeesh, S., Vauthier, C., Gueutin, C., Ponchel, G., Sharma, C.P., 2010. Thiol functionalized polymethacrylic acid-based hydrogel microparticles for oral insulin delivery. Acta Biomater. 6, 3072–3080.

Sarmento, B., Ribeiro, A., Veiga, F., Ferreira, D., Neufeld, R., 2007a. Oral bioavailability of insulin contained in polysaccharide nanoparticles. Biomacromolecules 8, 3054–3060.

Sarmento, B., Ribeiro, A., Veiga, F., Sampaio, P., Neufeld, R., Ferreira, D., 2007b. Alginate/chitosan nanoparticles are effective for oral insulin delivery. Pharm. Res. 24, 2198–2206.

Serda, R.E., Godin, B., Blanco, E., Chiappini, C., Ferrari, M., 2010. Multi-stage delivery nano-particle systems for therapeutic applications. Biochim. Biophys. Acta 1810, 317–329.

Sonaje, K., Chen, Y.J., Chen, H.L., Wey, S.P., Juang, J.H., Nguyen, H.N., Hsu, C.W., Lin, K.J., Sung, H.W., 2010a. Enteric-coated capsules filled with freeze-dried chitosan/poly(gamma-glutamic acid) nanoparticles for oral insulin delivery. Biomaterials 31, 3384–3394.

Sonaje, K., Lin, K., Wey, S., Lin, C., Yeh, T., Nguyen, H., Hsu, C., Yen, T., Juang, J., Sung, H., 2010b. Biodistribution, pharmacodynamics and pharmacokinetics of insulin analogues in a rat model: oral delivery using pH-responsive nanoparticles vs. subcutaneous injection. Biomaterials 31, 6849–6858.

Sonaje, K., Lin, Y.H., Juang, J.H., Wey, S.P., Chen, C.T., Sung, H.W., 2009. In vivo evaluation of safety and efficacy of self-assembled nanoparticles for oral insulin delivery. Biomaterials 30, 2329–2339.

Stoll, B.R., Batycky, R.P., Leipold, H.R., Milstein, S., Edwards, D.A., 2000a. A theory of molecular absorption from the small intestine. Chem. Eng. Sci. 55, 473–489.

Stoll, B.R., Leipold, H.R., Milstein, S., Edwards, D.A., 2000b. A mechanistic analysis of carrier-mediated oral delivery of protein therapeutics. J. Control. Release 64, 217–228.

Tasciotti, E., Liu, X., Bhavane, R., Plant, K., Leonard, A.D., Price, B.K., Cheng, M.M.C., Decuzzi, P., Tour, J.M., Robertson, F., 2008. Mesoporous silicon particles as a multistage delivery system for imaging and therapeutic applications. Nat. Nanotechnol. 3, 151–157.

Thanou, M., Verhoef, J., Junginger, H., 2001. Oral drug absorption enhancement by chitosan and its derivatives. Adv. Drug Deliv. Rev. 52, 117–126.

Vachon, M.G., Nairn, J.G., 1998. The use of 13C solid state NMR to elucidate physicochemical association in Eudragit(R) RS100 microencapsulated acyl esters of salicylic acid. Eur. J. Pharm. Biopharm. 45, 9–21.

Vandervoort, J., Ludwig, A., 2002. Biocompatible stabilizers in the preparation of PLGA nanoparticles: a factorial design study. Int. J. Pharm. 238, 77–92.

Walstra, P., 1993. Principles of emulsion formation. Chem. Eng. Sci. 48, 333–349.

Wi niewska, M., 2011. The temperature effect on electrokinetic properties of the silica–polyvinyl alcohol (PVA) system. Colloid Polym. Sci. 289, 341–344.

Woitiski, C., Neufeld, R., Veiga, F., Carvalho, R., Figueiredo, I., 2010. Pharmacological effect of orally delivered insulin facilitated by multilayered stable nanoparticles. Eur. J. Pharm. Sci. 41, 556–563.

Woitiski, C.B., Carvalho, R.A., Ribeiro, A.J., Neufeld, R.J., Veiga, F., 2008. Strategies toward the improved oral delivery of insulin nanoparticles via gastrointestinal uptake and translocation. Biodrugs 22, 223–237.

Wong, C., Stylianopoulos, T., Cui, J., Martin, J., Chauhan, V.P., Jiang, W., Popovi, Z., Jain, R.K., Bawendi, M.G., Fukumura, D., 2011. Multistage nanoparticle delivery system for deep penetration into tumor tissue. Proc. Natl. Acad. Sci. U.S.A. 108, 2426–2431.

Wong, T.W., 2010. Design of oral insulin delivery systems. J. Drug Target 18, 79–92.

Wu, Y., Loper, A., Landis, E., Hettrick, L., Novak, L., Lynn, K., Chen, C., Thompson, K., Higgins, R., Batra, U., 2004. The role of biopharmaceutics in the development of a clinical nanoparticle formulation of MK-0869: a Beagle dog model predicts improved bioavailability and diminished food effect on absorption in human. Int. J. Pharm. 285, 135–146.

Xu, X.L., Fu, Y., Hu, H.Y., Duan, Y.R., Zhang, Z.R., 2006. Quantitative determination of insulin entrapment efficiency in triblock copolymeric nanoparticles by high-performance liquid chromatography. J. Pharm. Biomed. Anal. 41, 266–273.

Yin, L.C., Ding, J.Y., He, C.B., Cui, L.M., Tang, C., Yin, C.H., 2009. Drug permeability and mucoadhesion properties of thiolated trimethyl chitosan nanoparticles in oral insulin delivery. Biomaterials 30, 5691–5700.