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A rapid-acting, long-acting insulin formulation based on a phospholipid complex loaded PHBHHx nanoparticles

Qiang Peng [a], Zhi-Rong Zhang [a], Tao Gong [a], Guo-Qiang Chen [b,**], Xun Sun [a,*]

[a] Key Laboratory of Drug Targeting and Drug Delivery Systems, Ministry of Education, West China School of Pharmacy, Sichuan University, Chengdu 610041, PR China
[b] Department of Biological Science and Biotechnology, School of Life Sciences, Tsinghua University, Beijing 100084, PR China

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ABSTRACT

The application of poly(hydroxybutyrate-co-hydroxyhexanoate) (PHBHHx) for sustained and controlled delivery of hydrophilic insulin was made possible by preparing insulin phospholipid complex loaded biodegradable PHBHHx nanoparticles (INS-PLC-NPs). The INS-PLC-NPs produced by a solvent evaporation method showed a spherical shape with a mean particle size, zeta potential and entrapment efficiency of 186.2 nm, −38.4 mv and 89.73%, respectively. *In vitro* studies demonstrated that only 20% of insulin was released within 31 days with a burst release of 5.42% in the first 8 h. The hypoglycaemic effect in STZ induced diabetic rats lasted for more than 3 days after the subcutaneous injection of INS-PLC-NPs, which significantly prolonged the therapeutic effect compared with the administration of insulin solution. The pharmacological bioavailability (PA) of INS-PLC-NPs relative to insulin solution was over 350%, indicating that the bioavailability of insulin was significantly enhanced by INS-PLC-NPs. Therefore, the INS-PLC-NPs system is promising to serve as a long lasting insulin release formulation, by which the patient compliance can be enhanced significantly. This study also showed that phospholipid complex loaded biodegradable nanoparticles (PLC-NPs) have a great potential to be used as a sustained delivery system for hydrophilic proteins to be encapsulated in hydrophobic polymers.

1. Introduction

The number of diabetic patients has been increasing rapidly during the past decades [1]. Most patients suffered from diabetes will ultimately receive insulin therapies. Recently, more and more research results showed regeneration of pancreatic β-cells in both diabetic patients and animals via different ways including conversion of α-cells to β-cells [2–9], demonstrating some promises for diabetes therapy.

So far subcutaneous injection is still the dominant route for administering insulin although many efforts have been made to explore a better way for an improvement on patient compliance including oral [10–17], intranasal [18–21], or pulmonary delivery [22–26]. Nevertheless, except Exubera no more insulin products administered by those routes are able to make it through the clinical trials [27]. Exubera was the first inhaled insulin formulation introduced to the market several years ago but it failed in the market due to the low acceptance by patients [25]. This failure negatively affects the development of inhaled formulation of insulin.

Diabetic patients have to receive insulin injections at least once a day, which brings patients troubles and pains. A controlled and prolonged release system is needed to reduce the injection frequency and thus increase the patient compliance. The present study was thus focused on delivering insulin through subcutaneous injection with long lasting effect.

Nanoparticle system has been widely used for targeted or sustained delivery of many drug molecules [17,28–36]. In order to achieve a long lasting and controlled release of drugs from nanoparticles, a proper material with biodegradability and biocompatibility is necessary. Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx), one of the members of the microbial polyhydroxyalkanoates (PHA) biopolyester family, has been studied as a bioimplant material for tissue engineering due to its combined properties of adjustable mechanical properties, biocompatibility, biodegradability and non-cytotoxicity of its degraded products [37–47]. Therefore, PHBHHx was used as a biodegradable nanoparticle carrier for the specific and sustained release of hydrophobic drugs [48]. Nevertheless, the hydrophilic insulin cannot be encapsulated into PHBHHx nanoparticles readily due to the strong lipophilic nature of PHBHHx.

* Corresponding author. Tel.: +86 28 85502307; fax: +86 28 85501615.
** Corresponding author. Tel.: +86 10 62783844; fax: +86 10 62794217.
E-mail addresses: chengq@mail.tsinghua.edu.cn (G.-Q. Chen), xunsun22@gmail.com (X. Sun).

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To encapsulate hydrophilic drugs into lipophilic or hydrophobic materials, the double emulsion method has been commonly adapted [14,35]. However, the method suffers from very low entrapment efficiency and leakage of the encapsulated drug molecules.

Formation of a drug phospholipid complex was demonstrated to be useful for enhancing the lipophilicity of insulin [17]. Our previous results showed that the phospholipid complex significantly enhanced the lipophilicity of hydrophilic drug molecules, thus facilitating the encapsulation of hydrophilic molecules into hydrophobic nanoparticles with a higher entrapment efficiency [29,33].

So far, no study on application of PHBHHx nanoparticles loaded with hydrophilic drugs such as insulin has been reported. The present study aimed to explore the insulin-phospholipid complex loaded PHBHHx nanoparticles (INS-PLC-NPs) as a sustained drug delivery system to decrease the insulin administration frequency.

2. Materials and methods

2.1. Materials

Phospholipid, namely, soybean lecithin containing 70–97% phosphatidylcholine (PC), was purchased from Shanghai Tai-wei Pharmaceutical Co. Ltd. (Shanghai, China). Pure crystalline porcine insulin was purchased from Xuzhou Wanbang Bio-Chemical Co. Ltd. (Jiangsu, China). Poloxamer188 (F68) was provided by Nanjing Well Chemical Co., Ltd. (Nanjing, China). Sodium deoxycholate (DOC-Na) was supplied by Amresco (Solon, USA). PHBHHx ($M_W = 380{,}000$) containing 11 mol% of R-3-hydroxyhexanoate (HHx) was kindly donated by Lukang Group (Shandong, China). Streptozotocin was purchased from Sigma (St. Louis, USA). All other chemical reagents were of analytical grade or better.

2.2. Preparation of insulin-phospholipid complex (INS-PLC)

INS-PLC was prepared according to a previous study [17] with some modifications. Briefly, insulin and phospholipid, with different molar ratios (1:80, 1:100 and 1:120), respectively, were dissolved in dimethyl sulfoxide (DMSO) containing 5% (v/v) of acetic acid under magnetic stirring at 30 °C. The resultant mixture was continuously stirred for 24 h under the same conditions and subsequently lyophilized for 24 h to remove the solvent. The lyophilized INS-PLC was sealed hermetically and stored at 4 °C until further use.

2.3. Solubility studies of INS-PLC

Solubility studies were performed based on the method reported previously [29]. Briefly, excess of INS-PLC was added in distilled water and n-octanol in sealed glass containers, which were gently shaken on an orbital shaker at 25 °C. Twenty four hr later the samples were centrifuged at 6000 rpm for 10 min. The supernatant was withdrawn and diluted properly before analysis by HPLC. In the meanwhile the solubility of free insulin in distilled water and n-octanol were measured and compared with that of INS-PLC.

2.4. Preparation of INS-PLC loaded nanoparticles (INS-PLC-NPs)

INS-PLC-NPs were prepared based on an emulsion-solvent evaporation method [33]. Briefly, INS-PLC and PHBHHx were dissolved in chloroform with a weight ratio of 1:20 (insulin : PHBHHx) as an organic phase. An aqueous phase was produced by dissolving 0.5% (w/v) of poloxamer188 (F68) and 0.5% (w/v) of sodium deoxycholate (DOC-Na) in distilled water. The organic phase was subsequently mixed with the above aqueous phase at a volume ratio of 1:20, followed by sonication for 6 times (5 s per times) at a power of 400 W. The resulting emulsion was evaporated under vacuum at 30 °C for 15 min to remove the organic solvent.

2.5. Physicochemical characterization of INS-PLC-NPs

The mean particle size, size distribution and zeta potential (ZP) of the resulting INS-PLC-NPs were characterized using dynamic light scattering (DLS) and electrophoretic light scattering (ELS) technology, respectively, with a Zetasizer Nano ZS90 instrument (Malvern Instruments Ltd., U.K.), using water as a dispersant at 25 °C with each cycle of the measurement automatically determined by the instrument system. The particle size was displayed by intensity distribution, and the size distribution was evaluated by polydispersity index (PDI).

The morphology of INS-PLC-NPs was observed under a scanning electron microscope (SEM, S4800, Hitachi, Japan) at an accelerating voltage of 3 kV. One drop of the INS-PLC-NPs suspension after proper dilution was placed on a glass surface. After the air-drying process, the sample was coated with gold using an Ion Sputter.

2.6. Entrapment efficiency (EE) studies

The entrapment efficiency of INS-PLC-NPs was studied based on a modified centrifugation method [33]. Briefly, insulin loaded in 1 ml of INS-PLC-NPs was extracted with 5% (w/v) of Triton X-100 under sonication. The resultant suspension was filtered through a membrane (0.22 μm) and an aliquot of the filtrate was injected into HPLC to determine the total added amount of insulin (W_t). In addition, another 1 ml of INS-PLC-NPs was taken and 10% of acetic acid was added to adjust the surface charge of nanoparticles close to zero so that the free drug could be separated easily by centrifugation. The obtained supernatant was injected into HPLC to obtain the amount of free insulin (W_f). Therefore, the entrapment efficiency could be calculated using the following equation:

$$EE = (W_t - W_f)/W_t \times 100\%$$

2.7. Preparation and evaluation of lyophilized INS-PLC-NPs

The lyophilized INS-PLC-NPs were prepared as described below: PEG-4000, as a cryoprotectant was dissolved in INS-PLC-NPs suspension with a concentration of 5% (w/v). The mixture was frozen at −45 °C for more than 6 h and then lyophilized for 24 h using a freeze drier (Thermo Savant ModulyoD-230, USA). The lyophilized products were stored in a sealed desiccator at room temperature.

Evaluation of the lyophilized INS-PLC-NPs was performed by reconstituting the lyophilized products in distilled water. The particle size, PDI, ZP and entrapment efficiency of the reconstituted suspension were evaluated according to the method described above and compared with those before lyophilization.

2.8. In vitro release studies of INS-PLC-NPs

The *in vitro* release of the drug from INS-PLC-NPs was studied using the dialysis method. The dialysis bags with a molecular weight cut off of 50 KD were used to retain INS-PLC-NPs in the bags, allowing the released drug to permeate into the release medium (PBS, pH 7.4). Initially, 1 ml of INS-PLC-NPs was added in a dialysis bag. After tightly bundling the sample loaded bag was soaked in 8 ml of release medium and shaken in a horizontal shaker under controlled conditions (70 rpm, 37 ± 1 °C). At fixed time intervals the release medium was collected and replaced with 8 ml of fresh medium. The collected sample was diluted properly and centrifuged at 4500 rpm for 10 min. The supernatant was stored at −80 °C till further analysis. The content of released insulin was determined by insulin ELISA kit.

2.9. In vivo studies in rats

The healthy male Sprague–Dawley (SD) rats (220–280 g) were purchased from Laboratory Animal Center of Sichuan University (Chengdu, China). All animal experiments were approved by the Institutional Animal Care and Ethic Committee of Sichuan University. The rats were housed in cages (five rats per cage) under controlled conditions of 25 °C and 55% air humidity with free access to water and standard rat chow, the rats were acclimatized for at least 7 days before use.

2.9.1. Induction of diabetes

The healthy SD rats were fasted for more than 12 h with free access to water. Diabetes was induced by a single intraperitoneal injection of streptozotocin dissolved in 0.1 M citrate buffer (pH 4.5) at a dose of 80 mg/kg. Blood samples were taken from caudal vein into the heparinized tubes and centrifuged at 4500 rpm for 5 min to separate plasma. Blood glucose level was measured using a glucose measurement kit (Shanghai Rongsheng Biological Technology Co. Ltd., China) according to the user instructions. The rats with fasting blood glucose level higher than 300 mg/dl were considered as diabetes and used for the following studies.

2.9.2. Hypoglycaemic effect of INS-PLC-NPs after subcutaneous injection

The diabetic rats were fasted for 12 h with free access to water. They were divided into three groups (five rats for each group). As a negative control, group one was subcutaneously injected with physical saline. Group two, serving as a positive control was treated by subcutaneous injection of insulin solution at a dose of 1 IU/kg. Group three was given INS-PLC-NPs at a dose of 4 IU/kg. At predetermined time intervals blood samples were collected, processed and measured in the same way described above. The change of the blood glucose level (percentage to the initial value before injection) was presented by a blood glucose level-time curve. The area above the curve (AAC) was used to calculate the pharmacological bioavailability (PA) according to the following equation.

$$PA = (AAC_{NPs}/Does_{NPs})/(AAC_{ins}/Does_{ins}) \times 100\%$$

where the subscript "NPs" represented the formulation of INS-PLC-NPs and "ins" represented the formulation of insulin solution.

In addition, starting from 24 h post-injection, rat chow was provided after sampling every time and removed again 12 h before the next sampling time. The water was supplied at all times.

2.10. Statistical analysis

The data were expressed as mean ± s.d. (standard deviation). For comparison between groups a one-way analysis of variance (ANOVA) was used. The difference between two groups was considered to be statistically significant when the p value was less than 0.05.

3. Results

3.1. Preparation and solubility studies of insulin-phospholipid complex (INS-PLC)

It is clear that the solubility of insulin in water or n-octyl alcohol (n-octanol) was significantly enhanced after the formation of PLC (Table 1), more insulin was solubilized with the enhanced proportion of phospholipid in the complex. Compared with free insulin, the insulin solubility in water was enhanced by 19-fold for INS-PLC (1:80), 22-fold for INS-PLC (1:100) and 34-fold for INS-PLC (1:120), respectively. The solubility in n-octanol was enhanced by 237-fold for INS-PLC (1:80), 271-fold for INS-PLC (1:100) and 455-fold for INS-PLC (1:120) (Table 1). All these results indicate that the lipophilicity of insulin was significantly enhanced by the formation of INS-PLC.

3.2. Preparation and characterization of insulin-phospholipid complex loaded nanoparticles (INS-PLC-NPs)

The mean particle size, polydispersity index (PDI), zeta potential (ZP) and entrapment efficiency of INS-PLC-NPs containing different INS-PLC were characterized (Table 2). The size and PDI fluctuated slightly among the three types of INS-PLC-NPs, while the zeta potential was significantly higher for INS-PLC-NPs containing INS-PLC (1:120). Importantly, the entrapment efficiency of INS-PLC-NPs was significantly enhanced with increased amount of phospholipid in INS-PLC, which was 87% for the INS-PLC-NPs containing INS-PLC (1:120) compared with 53% and 76% for the INS-PLC-NPs containing INS-PLC (1:80) and INS-PLC (1:100), respectively (Table 2). Therefore, only the INS-PLC-NPs containing INS-PLC (1:120) was used for the subsequent studies as they exhibited a smaller size with the highest entrapment efficiency. Accordingly, the phrase INS-PLC-NPs only represented the nanoparticles containing INS-PLC (1:120) in the following studies unless it was stated otherwise. The INS-PLC-NPs observed by SEM were mostly spherical in shape and well distributed in size (Fig. 1).

3.3. Preparation and evaluation of lyophilized INS-PLC-NPs

The lyophilized INS-PLC-NPs appeared as a white powder form, they could be quickly re-dispersed in distilled water to form a homogeneous nanoparticles suspension within 10 s under gently shaking, similar to the freshly prepared INS-PLC-NPs. After re-dispersion in distilled water, the mean particle size of the lyophilized NPs became larger, with their PDI also higher than that of their values prior to lyophilization, yet without substantial differences in their zeta potential or entrapment efficiency (Table 3). These results indicated that the lyophilization process did not lead to a significant change on properties of INS-PLC-NPs before and after their lyophilization.

Table 1
Solubility of insulin and insulin phospholipids complex (INS-PLC) in water and n-octyl alcohol at 25 °C.

Sample	Solubility in water	Solubility in alcohol
Free insulin	59.12 ± 5.37 μg/ml	24.96 ± 2.09 μg/ml
INS-PLC (1:80)	1.15 ± 0.04 mg/ml	5.91 ± 0.17 mg/ml
INS-PLC (1:100)	1.29 ± 0.06 mg/ml	6.76 ± 0.19 mg/ml
INS-PLC (1:120)	1.99 ± 0.09 mg/ml	11.35 ± 0.46 mg/ml

Values are mean ± s.d. ($n = 3$).

Table 2
Characterization of INS-PLC-NPs prepared using different INS-PLC.

INS-PLC-NPs[a]	Size (nm)	PDI	Zeta potential (mv)	EE (%)
INS-PLC (1:80)	223.5 ± 6.2	0.158 ± 0.013	−26.45 ± 1.94	53.20 ± 2.07
INS-PLC (1:100)	202.4 ± 5.3	0.123 ± 0.011	−25.76 ± 2.67	76.62 ± 4.02
INS-PLC (1:120)	182.4 ± 5.3	0.151 ± 0.019	−36.93 ± 3.91	87.19 ± 3.26

Values are mean ± s.d. ($n = 3$).
[a] The INS-PLC-NPs were prepared by mixing the organic phase containing INS-PLC with the aqueous phase, followed by sonication and evaporation.

3.4. In vitro release studies of INS-PLC-NPs

The *in vitro* release of INS-PLC-NPs was studied using the dynamic dialysis method (Fig. 2). It was clear that the *in vitro* release rate of insulin from INS-PLC-NPs was very slow, only 20% of insulin was released within 31 days. The release profile can be divided into two phases: the first one considered as the initial burst release phase lasting 8 h, allowed insulin to be released at a relatively fast rate. During the 8 h burst release, only 5.42% of insulin encapsulated was released. The second release phase starting after the first 8 h was considered as the sustained release phase, during which over 14% of the loaded insulin was released at a relatively low rate (Fig. 2).

3.5. Hypoglycemic effect of INS-PLC-NPs after subcutaneous injection

The hypoglycaemic effect of insulin solution, physical saline and INS-PLC-NPs following subcutaneous injection were studied and compared, respectively (Fig. 3). No hypoglycaemic effect following subcutaneous injection of physical saline was observed. As expected the blood glucose level (BGL) decreased significantly soon after the subcutaneous injection of insulin solution (1 IU/kg). The BGL decreased to 70% (this value is considered as the threshold of

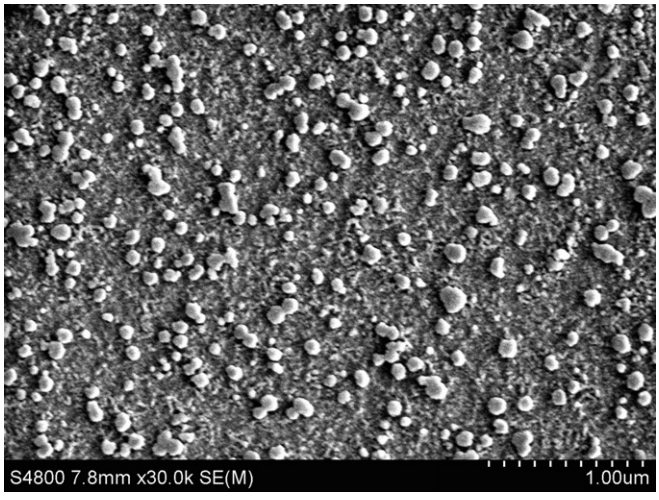

Fig. 1. SEM image of insulin phospholipid complex loaed PHBHHx nanoparticles (INS-PLC-NPs). Scale bar: 1.00 μm; accelerating voltage: 3 kV.

Table 3
Characterization of INS-PLC-NPs before and after lyophilization.

Sample	Mean size (nm)	PDI	ZP (mv)	EE (%)
Before lyophilization	186.2 ± 6.7	0.160 ± 0.014	−38.4 ± 6.4	89.73 ± 3.77
After lyophilization	238.8 ± 9.8	0.218 ± 0.026	−37.7 ± 1.5	91.27 ± 4.53

Values are mean ± s.d. ($n = 3$). Lyophilization conditions: pre-freezing at −45 °C for more than 6 h, followed by lyophilization for 24 h.

therapeutic effect [23]) at ~1 h post-injection and reached the minimum (40.51%) at 2 h, followed with a rapid increase to 70% again at ~5.5 h. As such, the period of time, during which the BGL is lower than 70% (PT-70%) is only 4.5 h (Table 4). Subsequently, the BGL returned to the basal level (89.47%) at 8 h and maintained there till the end of this study. This result indicates that insulin solution is a rapid and short-acting formulation. The BGL also decreased significantly soon after the subcutaneous injection of INS-PLC-NPs (4 IU/kg). However, it reached 70% at ~0.5 h post-injection and the minimum glucose level (16.66%) obtained also at 2 h was significantly lower than that after subcutaneous injection of insulin solution ($^{*}p < 0.05$). Importantly, after reaching the minimum value the BGL did not increase to 70% until ~83.5 h post-injection with significant difference to insulin solution group ($^{*}p < 0.05$, $^{**}p < 0.001$). As such, the PT-70% of INS-PLC-NPs is about 83 h, which is significantly longer than that of insulin solution (Table 4). Following the long-term hypoglycaemic effect, the BGL returned to the basal level (101.15%) at 96 h. This result demonstrates that INS-PLC-NPs belong to a rapid-acting and long-acting formulation. In addition, the pharmacological availability (PA) of INS-PLC-NPs relative to insulin solution is 350.29% (Table 4), indicating that the bioavailability of insulin was significantly enhanced by INS-PLC-NPs.

4. Discussion

It is a challenge to entrap hydrophilic insulin directly in PHBHHx nanoparticles due to the strong lipophilicity of this material. To improve the affinity between insulin and PHBHHx, insulin phospholipid complex (INS-PLC) was prepared. The lipophilicity of insulin was significantly enhanced by the formation of phospholipid complex (Table 1), which facilitated the entrapment of insulin in PHBHHx nanoparticles, leading to the high encapsulation efficiency of insulin in the INS-PLC-NPs system. Additionally, sodium deoxycholate as an ionic surfactant contributed to the high entrapment efficiency during the preparation of INS-PLC-NPs. Without sodium deoxycholate INS-PLC-NPs could not be produced even under intensive sonication. As an anionic surfactant, sodium deoxycholate is able to increase the negative surface charge presented as zeta potential of the nanoparticles, allowing the nanoparticle system to maintain its stability via electrical repulsion among the nanoparticles, and thus preventing particle aggregation and leakage of the drug from nanoparticles.

The complete separation of free drug from nanoparticles is the prerequisite for the accurate measurement of entrapment efficiency. The separation of free drug from INS-PLC-NPs was difficult in this study using conventional methods due to the small particle size (~200 nm) and high zeta potential (~−40 mv) of the nanoparticles. Therefore, the entrapment efficiency was determined using a modified centrifugation method described in our previous study [33]. Considering the instability of insulin in the strong acid, 10% acetic acid was employed in this present study.

Sugars with small molecular weight including sucrose [49], maltose [33] and mannose [50] are most commonly used as cryoprotectants in the lyophilization process. In the present study, however, PEG-4000 was chosen as the cryoprotectant to avoid interferences of the sugars in the diabetes therapy process, resulting in satisfactory stability of INS-PLC-NPs after the lyophilization (Table 3). Although the lyophilized INS-PLC-NPs became a bit larger after reconstitution in distilled water possibly due to the partial aggregation among particles, no drug leakage from INS-PLC-NPs following lyophilization was observed as indicated by the stable entrapment efficiency.

Most studies showed the significantly faster *in vitro* release rate including the initial burst release of insulin loaded nanoparticles than that of the present INS-PLC-NPs [17,28,31]. Cui and colleagues showed that ~70% of total insulin including free insulin and SPC-combined insulin was released at 8 h in simulated intestinal medium at pH 6.8 [17]. The relatively fast release of insulin possibly due to a relatively large amount of insulin adsorbed on the surface of nanoparticles, is beneficial to oral delivery as the gastrointestinal transit time is limited. In another study [31], ~60% of insulin was released from DS/Chit nanoparticles when soaked in simulated intestinal fluids at pH 6.8 for 4 h. This faster release of insulin is likely due to the higher hydrophilicity and faster biodegradability of DS/Chit than that of PHBHHx. In the present study, the strong hydrophobicity and very slow degradability of PHBHHx resulted in the slower release of insulin from INS-PLC-NPs with a quite low burst release (Fig. 2), which possibly met the requirement of long term *in vivo* release of insulin. The degradation of PHBHHx

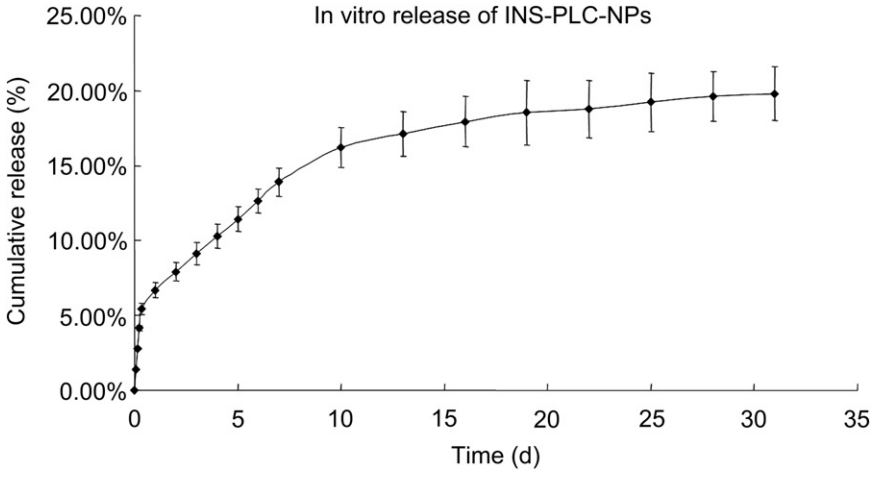

Fig. 2. *In vitro* release profile of INS-PLC-NPs in PBS (pH 7.4) at 37 °C under shaking speed of 70 rpm. Each data presented as mean ± s.d. ($n = 3$).

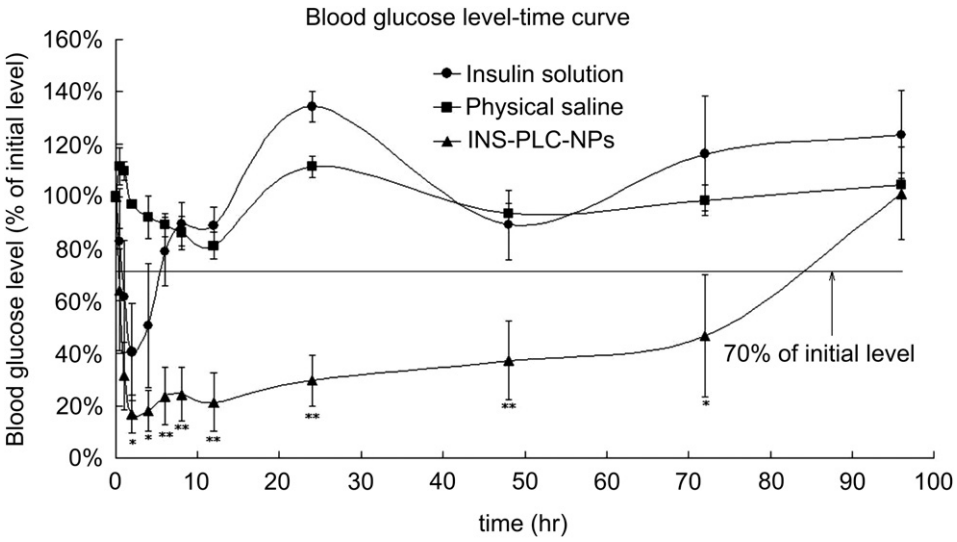

Fig. 3. Blood glucose level-time curve after subcutaneous injection of insulin solution (●) at a dose of 1 IU/kg, physical saline (■) and INS-PLC-NPs (▲) at a dose of 4 IU/kg to diabetic male Sprague–Dawley (SD) rats (220–280 g). Each data presented as mean ± s.d. ($n = 5$). Statistically significant difference between INS-PLC-NPs and insulin solution: $^{*}p < 0.05$, $^{**}p < 0.001$.

nanoparticles is primarily based on the hydrolysis of PHBHHx. The insulin can be released from nanoparticles (NPs) mainly through two approaches. 1) The insulin adsorbed on the surface of NPs is released via desorption and leads to the initial burst release; 2) The insulin existing in the inner space of NPs is released by diffusion through the NPs skeleton, or following the degradation of the skeleton, which are both dependent on the degradation of PHBHHx. Therefore, the hydrolysis rate of PHBHHx has substantial effect on the release profile. The faster the PHBHHx is hydrolyzed, the faster the NPs skeleton is damaged, resulting in a faster release of insulin.

In contrast to the slow *in vitro* release behaviour, the *in vivo* release rate of insulin was significantly enhanced due to the faster biodegradation of PHBHHx. A variety of degradative enzymes and the dynamic internal environment in the living body may contribute to the faster degradation of PHBHHx *in vivo*. Although the *in vivo* release profile was shorter than that of the *in vitro* one, the result of maintaining the efficacy of insulin for more than 3 days following a single subcutaneous injection of INS-PLC-NPs was exciting. It is better than other reported insulin loaded nanoparticles systems regarding sustained and controlled release of insulin after a single subcutaneous injection [51,52].

Four reasons may contribute to the rapid and durable efficacy of INS-PLC-NPs (Fig. 3). Firstly, some free insulin not separated from the nanoparticles prior to administration contributed to the rapid decrease of glucose level. Secondly, the burst release, although very low, also led to the rapid and remarkable decrease of glucose level at the initial stage. Thirdly, the high entrapment efficiency of INS-PLC-NPs and relative slow biodegradation of PHBHHx resulted into the slow release of loaded insulin, and should be the main reason for the long-term efficacy. Finally, the loaded insulin could also be partially released in the form of INS-PLC [17], which may delay the absorption of insulin due to the larger molecular size.

Table 4
Pharmacodynamic parameters after subcutaneous injection of various formulations ($n = 5$).

Parameters[a]	Physical saline	Insulin solution	INS-PLC-NPs
Dose (IU/kg)	–	1	4
BGL_{min} (%)	81.15	40.51	16.66
T_{min} (h)	12	2	2
PT-70% (h)	0	1–5	0.5–79.5
$AAC_{0-96\ h}$ (h)	–	3.86	54.08
PA (%)	–	100	350.29

[a] BGL_{min} represents the minimum blood glucose level. T_{min} represents the time to reach BGL_{min}. PT-70% represents the period of time, during which the BGL was lower than 70%. $AAC_{0-96\ h}$ represents the area above the curve during 0–96 h. PA (%) represents the relative pharmacological availability.

5. Conclusions

Insulin phospholipid complex loaded biodegradable nanoparticles (INS-PLC-NPs) produced by a solvent evaporation method showed a small particle size, spherical shape and high insulin entrapment efficiency. Lyophilized INS-PLC-NPs with 5% (w/v) of PEG-4000 serving as a cryoprotectant presented similar properties compared with the freshly prepared INS-PLC-NPs suspension. Insulin was released *in vitro* from INS-PLC-NPs at a slow rate and with a low burst release. More importantly, the hypoglycaemic effect of INS-PLC-NPs in diabetic rats was found to occur rapidly and last for more than 3 days following a single subcutaneous injection, indicating that the administration frequency could be reduced substantially by INS-PLC-NPs. Additionally, the bioavailability of insulin was significantly enhanced by INS-PLC-NPs (PA = 350.29%) compared with insulin solution. In conclusion, beside its application in tissue engineering, PHBHHx can also serve as a promising biodegradable material for sustained and controlled delivery of hydrophilic drugs using the PLC-NPs system developed in this study. Meanwhile, INS-PLC-NPs have a great potential to serve as a novel rapid and long-acting insulin release formulation, based on which the patient compliance could be significantly improved by INS-PLC-NPs due to the substantial decrease of the injection frequency.

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