Preparation of Glucagon-Like Peptide-1 Loaded PLGA Microspheres: Characterizations, Release Studies and Bioactivities *in Vitro*/*in Vivo*

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The gut hormone glucagon-like peptide-1 (GLP-1) is proposed for treatment of Type II diabetes mellitus. However, the short half life of GLP-1 *in vivo* is a major limitation for its application due to the frequent invasive administrations. To provide a optimal formulation to overcome this limitation, we developed a GLP-1 entrapped microspheres to achieve sustained release GLP-1 for 4-week. GLP-1 was stabilized by GLP-1-zinc complexation with zinc carbonate and encapsulated in poly(D,L-lactic-co-glycolic acid) (PLGA) with S/O/O solvent extraction to obtain GLP-1 loaded PLGA microspheres (MS). The characteristics of MS were evaluated as follows: The surface morphyology was assessed by scanning electron microscopy (SEM); The drug encapsulation efficiency and GLP-1 controlled release profile was tested by HPLC; The sustained release of GLP-1 MS *in vivo* and pharmacological efficacy were studied in normal mice and streptozotocin (STZ)-induced diabetic mice model after subcutaneous administration of GLP-1 MS. GLP-1-zinc complexation significantly reduced initial burst release from 37.2 to 7.5%. The controlled release bioactive GLP-1 *in vitro* was achieved for 4-week period by zinc complexation and addition of $ZnCO_3$. The optimal and complete cumulative release of GLP-1 from MS was increased from 23 to 63% in 28 d by using low MW PLGA (MW 14000). The *in vivo* testing in normal mice and diabetic mice suggest that this zinc-stabilized technique combined with S/O/O method in the presence of water insoluble antacid additive $ZnCO_3$ preserve the biological activity of GLP-1. GLP-1 MS formulation achieved controlled released *in vivo* for 28 d and exhibit sustained long term pharmacological efficacy to decrease blood glucose level in diabetic mice. This GLP-1 MS formulation provides a practical formulation for long-term sustained delivery of GLP-1 to treat Type II diabetes.

Key words glucagon-like peptide-1; poly(D,L-lactic-co-glycolic acid) microsphere; drug release; bioactivity *in vitro*; bioactivity *in vivo*

Glucagon-like peptide-1 [GLP-1(7-36) amide], which is a gut hormone secreted from intestinal L-cells, has been suggested as a therapeutic agent for Type II diabetes[1—3] by stimulating glucose-dependent insulin release.[4] Multiple mechanisms have been reported for GLP-1. For instance, GLP-1 can stimulate insulin gene expression and biosynthesis in β-cells,[5,6] inhibits both glucagon secretion and gastric emptying,[7] and furthermore, enhance insulin sensitivity in diabetic patients and stimulate β-cells formation.[2,8]

The therapeutic advantage of GLP-1 is that it only stimulates insulin secretion in hyperglycemia, while it does not affect normal glucose levels. Unlike insulin, GLP-1 does not cause hypoglycemia. However, the limiting factor for the application of GLP-1 in diabetes treatment is its short half-life *in vivo* ($<$2 min), due to rapid metabolism in plasma by dipeptidylpeptidase IV (DPP IV).[9] DPP IV clears the two N-terminal aminoacid residue from GLP-1. Therefore, GLP-1 has to be continuously infused or repetitively injected in order to achieve therapeutic efficacy.[10] Hence, although GLP-1 is considered as one of the most promising treatment approaches for Type II diabetes, repeated injection for long-term therapy is a major limiting factor in practical use. Therefore, the sustained-release delivery system for GLP-1 over several days or weeks is highly desirable for treatment of Type II diabetes.

Injectable and biodegradable microspheres (MS) have been widely studied in recent years and have become well established controlled drug delivery systems. Numbers of peptides and recombinant proteins, such as calcitonin (CT), insulin-like growth factor-I (IGF-I), and interferon-alpha (INF-α), have been successfully developed for controlled release system using biodegradable poly(D,L-lactic-co-glycolic acid) (PLGA).[11—13] With one single injection, MS allow long-term sustained delivery of the encapsulated drug over a period of weeks to months. However, it remains challenging to minimize the initial burst of drug release from MS formulation. Significant initial burst release from MS not only fails to achieve long term release, but also results in dangerously high drug concentrations in the body. Several approaches were reported to reduce the initial burst by using high polymer concentration, reducing particle size (for solid in oil in water process) or droplet size of the inner emulsion (for water in oil in water) during encapsulation, and incorporating additives in the MS formulation.[14] However, these approaches still have limitations in reduction of partial burst release, low encapsulation efficiency.[14]

We had prepared GLP-1 microspheres by double emulsion (W/O/W), the burst release of which was high (about 20%), and at the same time their bioactivity decreased.[15] Our primary objective of this paper was to develop GLP-1 MS with reduced initial burst, increased encapsulation efficiency, prolonged cumulative release, and retained bioactivity of GLP-1. We encapsulated water soluble peptide GLP-1 into PLGA MS using metal cation-stabilization technique followed by solid in oil in oil (S/O/O) solvent extraction method. Zinc acetate significantly reduced the 24-h initial burst release of

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GLP-1 by forming zinc compound. The sustained release GLP-1 MS formulation achieved controlled release *in vivo* to achieve biological efficacy over a period of four weeks after single subcutaneous injection.

Experimental

Materials Glucagon-like peptide 1 (GLP-1) was prepared by gene expression and purified by reversed phase HPLC at Department of biochemistry and molecular biology, Second Military Medical University (Shanghai, China). The poly(D,L-lactic-co-glycolic acid) (PLGA, 50 : 50, molecular weight 35 kDa, Resomer® RG503H) was purchased from Boehringer Ingelheim (Germany). The poly(lactic-co-glycolic acid) (PLGA, 50 : 50, molecular weight 14 kDa) was from Birmingham Polymers (U.S.A.). Lecithin (type IV-S) was purchased from Shanghai Taiwei Lecithin Co., Ltd.; Acetonitrile and all other reagents used were of analysis grade (Chemical Reagent Co., Ltd., Shanghai). Male C_{57}BL/6 mice and ICR male mice were purchased from Slaccas laboratory animal Co., Ltd. (Shanghai, China). Glucose injection (SINE Pharmaceutical Co., Ltd.).

Preparation of GLP-1 Microspheres (MS) Peptide Micronization[16,17]: Total of 2 mg of GLP-1 and 10 mg of PEG6000 were dissolved in 1 ml distilled water and lyophilized (Virtis, U.S.A.) at −50 °C. Lyophilization was performed by maintaining the shelf temperature at −20 °C for 3 h and 20 °C for 12 h under a pressure of about 0.02 Torr. Then, the lyophilized powder was dispersed in 1 ml methylene dichloride (DCM) under sonication in water bath. The suspension was centrifuged at 15000 rpm for 5 min (Rutanta460, Hettich Germany). After the supernatant was removed, the residue was redispersed in DCM. This procedure was repeated three times to remove the remaining PEG6000. The final precipitates were dried at 20 °C for 12 h under a pressure of about 0.1 mPa (Binder Germany).

Preparation of Zinc-GLP-1 Compound Microparticles[18,19]: Total of 2 mg GLP-1 was dissolved in distilled water and 2 mM zinc acetate solution was added with GLP-1 to zinc acetate molar ratio of 2 : 1. The pH of zinc-GLP-1 solution was adjusted to pH 6.9. Then, 10 mg PEG6000 was added to above solution, and completely dissolved. The solution was then lyophilized. The lyophilized powders were reconstituted in 1 ml acetonitrile by vortex for 5 min, and centrifuged at 15000 rpm for 5 min to remove supernatant which comprised PEG6000. This procedure was repeated for three times. The residue was placed under vacuum drier at 20 °C for 12 h under a pressure of about 0.1 mPa (Binder Germany).

Preparation of Microspheres: GLP-1 loaded MS were prepared using the S/O/O solvent extraction technique.[13,20] Briefly, suitable amount of the GLP-1 microparticles or zinc-GLP-1 complex microparticles with or without 3 mg zinc carbonate microparticles (diameter $<5\ \mu$m) was added to a solution of PLGA (100 mg) in 1ml acetonitrile and suspended by sonication in water bath. The suspension was added dropwise to 30 ml of cotton seed oil containing lecithin (30 mg) under mechanical stirring (400 rpm during 2 h) (PW 20.n, IKA, China). Finally, 15 ml of petroleum ether were added to harden the microspheres for 30 min. Microspheres were collected by centrifugation and washed by petroleum ether. The microspheres were vacuum-dried (20 °C, 0.1 mPa) overnight before further characterization.

Determination of GLP-1 Loading and Encapsulation Efficiency of Microspheres GLP-1 was analyzed by reverse-phase high-performance liquid chromatography (RP-HPLC). The column was a Kromasil C-18 reversed-phase column (250×4.6 mm, 5 μm, 300 Å). The mobile phase was water and acetonitrile (64 : 36) containing 0.1% trifluoro acetic acid (TFA). Detection wavelength was 215 nm and the flow rate was 1.0 ml/min.

The GLP-1-loaded MS (10 mg) were dissolved in 0.3 ml DCM in a 1.5 ml Eppendorf tube. Then, 0.7 ml of acetone were added to the tube, vortexed, and centrifuged at 15000 rpm for 5 min. After the supernatant was removed, 1.0 ml of a 3 : 1 acetone : DCM mixture was added. This washing procedure was repeated three times. Finally, the residue was dissolved in isotonic phosphate-buffered saline (PBS, 10 mM, pH=7.4) (zinc-complexed GLP-1 firstly dissolved in small amount of 5 mM EDTA solution) and metered volume to 1 ml. GLP-1 was measured by RP-HPLC. The encapsulation efficiency of GLP-1 was determined by ratio of the actual amount of encapsulated peptide over the amount of peptide which was used in preparation of microspheres (actual loading/nominal loading).

Microspheres Characterization The surface morphology was monitored by scanning electron microscopy (SEM) using Hitachi S6000 microscope (Tokyo, Japan). Particle size distribution was determined using a laser diffraction technique (Coulter LS 230 Particle Sizer, Beckman, U.S.A.).

Residual Organic Solvent in the Microspheres The standard *N*,*N*-dimethylformamide (DMF, SCR, China) solution containing 41 μg/ml acetonitrile was prepared. One hundred milligrams of dried PLGA MS was weighed into 1.0 ml measuring flask and dissolved in 1 ml DMF. The sample was centrifuged at 5000 rpm for 5 min. The supernatant and standard solutions were quantified in triplicate by gas chromatography (Agilent 6890series, Agilent, U.S.A.). The acetonitrile content was compared between the sample solution and the standard solution.

***In Vitro* Release**[21] GLP-1 loaded MS (60 mg) were placed in a 2.0 ml Eppendorf tube and incubated in 0.5 ml of isotonic phosphate-buffered saline (PBS, 50 mM, pH=7.4) containing 0.02% (v/v) Tween-80 and 0.02% (w/v) sodium azide. The tube was placed in a water bath/shaker (100 rpm) and temperature was maintained at 37±0.5 °C. At predetermined intervals, supernatants (0.4 ml) were collected after centrifugation. The released GLP-1 was analyzed by RP-HPLC. At each sampling time, the release removed medium was replaced with fresh buffer in order to maintain the same pH and sink condition. All experiments were performed in triplicate.

Evaluation of the Bioactivity of the GLP-1 in the Microspheres[22] The biological activity of the GLP-1-loaded MS was determined by the *in vivo* animal experiments. The SD rats were purchased from Slaccas laboratory animal Co., Ltd. (Shanghai, China) at 10—12 weeks of age. The animals were fasted for 18 h prior to designed tests to evaluate *in vivo* bioactivity. Briefly, GLP-1 was extracted from GLP-1-loaded MS by previous method. The rats were administrated by an intraperitoneal injection of glucose (18 mmol/kg, $n=6$) alone or in combination with either extracted GLP-1 or standard GLP-1 (0.01 mg/kg, $n=6$). Blood samples were collected at 0, 10, 20, 30 and 50 min from the retrobulbar, intraorbital and capillary plexus with microcentrifuge tubes. Samples were immediately centrifuged, and the serum was separated and stored at −20 °C until analysis. Serum insulin was determined with ELISA (Rat insulin ELISA kits, Mercodia, Sweden).

Intraperitoneal Glucose Tolerance Test[23,24] Mice were fasted for 18 h before the experiment. The GLP-1-loaded or blank MS were suspended in an aqueous solution containing 1% carboxymethylcellulose sodium, 5% mannitol and 0.1% Tween-80. The GLP-1-loaded or blank MS suspension (GLP-1 dosage is 6.2 mg/kg, blank MS is 10 mg, $n=6$) were injected subcutaneouly to Male C_{57}BL/6 mice. After 4 h the mice were injected intraperitoneally with 10 mmol/kg of glucose. The blood was collected from the retrobulbar, intraorbital and capillary plexus at $t=0$, 10, 20, 30, 50, 80 min for immediate measurement of glycaemia using a blood glucose analyzer (Leapon, Beijing, China). This process was repeated at 4th, 9th, 14th, 19th, 24th, 28th day after administration.

Evaluation of the Bioactivity of the GLP-1 Microspheres in Diabetes Mice[22,25] The experiment was conducted using mices with streptozotocin (STZ)-induced diabetes. Briefly, the ICR male mice weighing 20—25 g were injected intraperitoneally with 160 mg/kg of STZ dissolved in 50 mM citric acid–citrate Na buffer (pH=4.0). At 3rd day the blood samples were collected from the retrobulbar, intraorbital and capillary plexus. Mice with blood glucose levels higher than 15 mmol/l were used for the following experiments. The experimental mice were given a single subcutaneous injection of GLP-1-loaded or blank MS suspension (GLP-1 dosage is 6.2 mg/kg, blank MS is 10 mg, $n=6$). Blood samples were collected before and after treatment to measure the blood glucose. All samples were taken at 10:00 in the morning everyday until the 28th day. Food was withdrawn at 18 h prior to blood samples collection. The blood glucose level was determined immediately using a blood glucose analyzer after blood samples collection.

Result and Discussion

Characterization of Microspheres and Residual Organic Solvent The particle diameters of MS were in the range from 33.4 to 45.7 μm as reported in Table 1. No significant difference in particle size distribution was observed using different molecular weight of polymers, with or without zinc-compound formation, or in the presence or absence of $ZnCO_3$. However, examination of the surface morphology revealed that there are less microparticles and pores on the surface of MS with formation of zinc-compound and addition of $ZnCO_3$ microparticles (Fig. 1, batch A and C).

The encapsulation efficiency of the different batch of GLP-1 loaded MS was higher than 90% (Table 1). In contrast, the low encapsulation efficiency (<60%) was observed with S/O/W solvent evaporation technique (data not shown) since peptide is easily dispersed into the water phase. Since

Table 1. Particle Size and Encapsulation Efficiency of PLGA MS Containing GLP-1 Formulation

Batch	Theory drug loading (%)	Mean MW	Zinc acetate	$ZnCO_3$	Diameter size (μm)	Encapsulation efficiency (%)
A	1.24	35000	−	−	36.6±4.3	94.2±3.2
B	1.24	35000	+	+	45.7±6.7	93.0±2.5
C	1.44	14000	−	−	33.4±4.8	92.7±3.6
D	1.44	14000	+	+	41.2±3.5	93.5±2.3

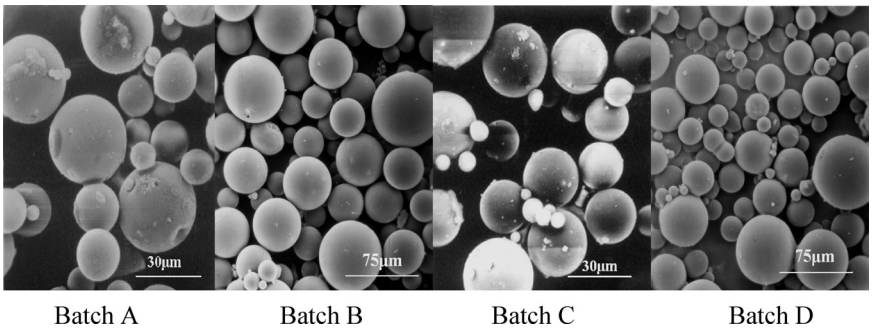

Fig. 1. Scanning Electron Micrography of the Surface Morphology of GLP-1 MS

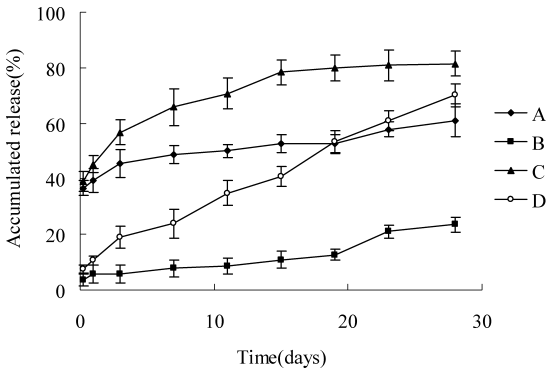

Fig. 2. Percent of Accumulative GLP-1 Released from MS in Different Formulations

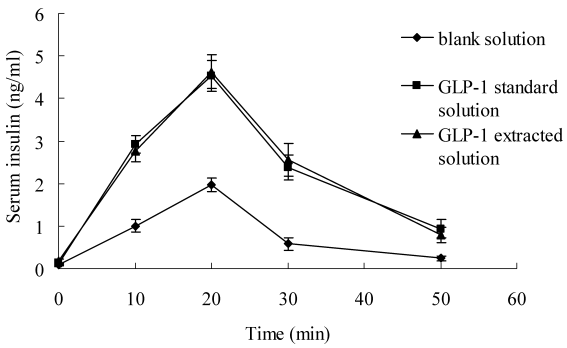

Fig. 3. The Activity of Encapsulated GLP-1 for Stimulating Insulin Secretion

organic solvent residues in MS may influence the properties of drug loaded MS and it is also a safety concern, the organic solvent levels must be controlled at minimal levels. The data showed that acetonitrile residued in GLP-1 MS was in the low ppm range (33 ppm).

***In Vitro* Release** To select the best formulation with lower burst release and optimal accumulative release profile, we conduct the *in vitro* GLP-1 release experiment. GLP-1 release profile from the PLGA 50 : 50 (MW＝35000) MS without Zinc compound showed a burst release of approximately 37% of the total dose during the first 6 h, followed by a continuous release of additional 25% of the total dose until 28th day (Fig. 2, batch A). The MS with Zinc compound exhibited 3% burst release of total peptide encapsulated dose, but the continuous release rate kept very slow with only 23% of peptide release until 28th day (Fig. 2, batch B). In comparison, the GLP-1 loaded MS with or without Zinc acetate and $ZnCO_3$ using low molecular weight PLGA (MW＝14000) released the entrapped peptide with a burst release of approximately 7.5% and 39%, followed by the additional release of peptide of 63% and 42% within 28 d, respectively (batch D, C). These data suggest that the burst release can be decreased with addition of Zinc acetate and $ZnCO_3$ microparticles, while the total cumulative release can be increased by using the PLGA polymer with low molecular weight. Therefore, the batch D formulation with a lower initial burst and a higher cumulative release was selected for *in vivo* experiment. It has been reported that zinc complexation provided a means to stabilize the peptide physically and chemically by reducing its solubility.[26,27] In our experiment, GLP-1 microparticles can completely be dissolved in 1 ml distilled water, while almost no zinc–GLP-1 compound microparticles can be dissolved. It indicates that Zn ion and GLP-1 may form complex with low solubility of GLP-1. Uncomplexed GLP-1 is extremely soluble in water, thus, the plentiful GLP-1 accreted on the surface of MS is quickly released from hydrophobic surface or released *via* pores and water channels. From refs. 28, 29, the burst release of insulin or IGF-I from PLGA MS was diminished due to addition of ZnO or $ZnCO_3$, the zinc compound can decrease the hydrophilicity of microspheres surface and water channels within microspheres. The initial release may decrease due to a reduction of microspheres hydration. This is the reason for the burst release without zinc compound. This low burst release of GLP-1 from MS with zinc is mostly due to the low solubility and

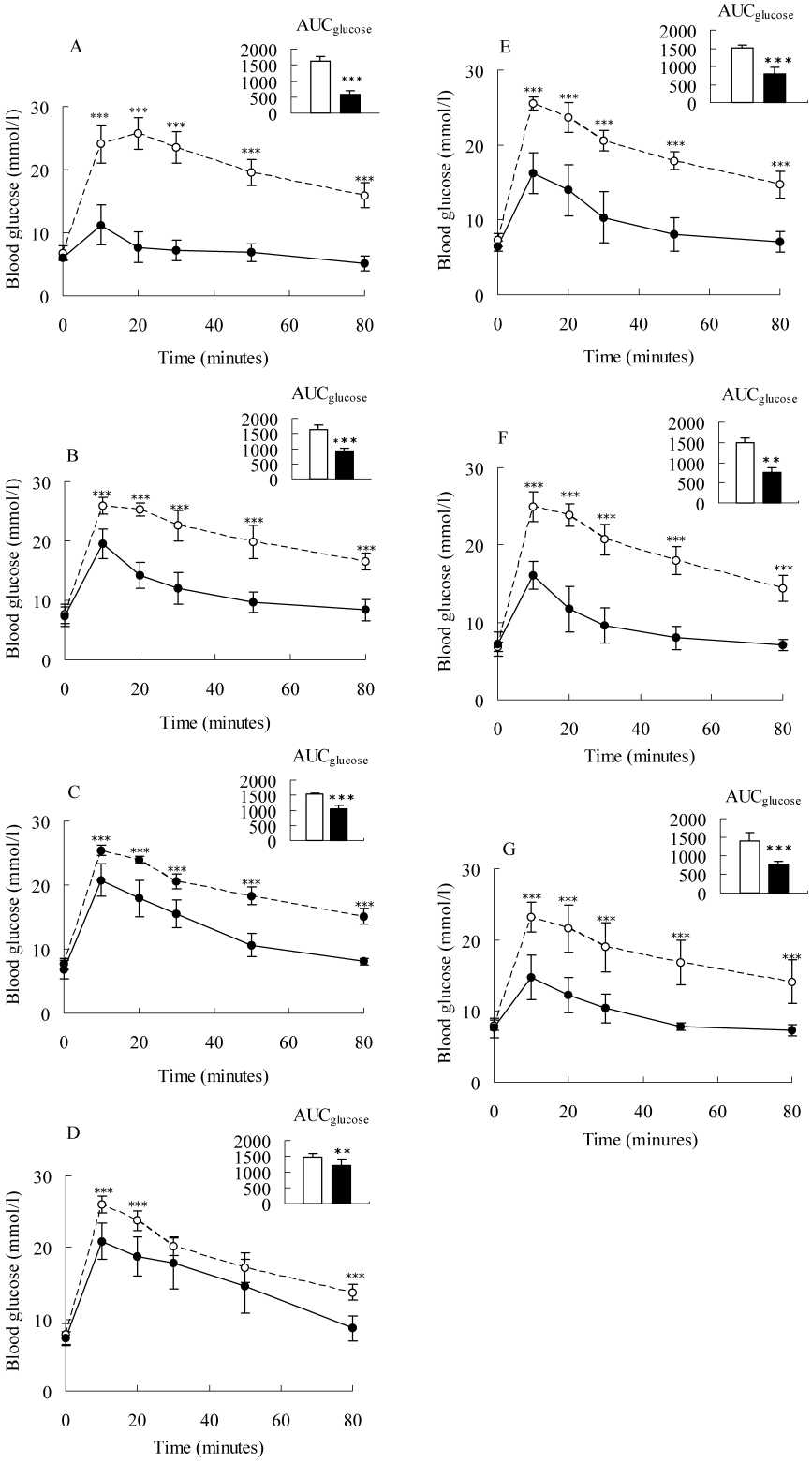

Fig. 4. Blood Glucose Levels *vs.* Time in Mice after Subcutaneously Injected GLP-1 Loaded MS (●, Solid Line, 6.2 mg/kg, $n=6$) or 10 mg Blank MS (○, Dotted Line, $n=6$)

Means±S.E. of $AUC_{glucose}$ (under concentration curve) within 80 min are shown in the inset. (A) Four hours after MS administration, the mice were injected 10 mmol/kg glucose intraperitoneally. (B—G) The mice were injected 10 mmol/kg glucose intraperitoneally after the administration of MS at 4th day (B), 9th day (C), 14th day (D), 19th day (E), 24th day (F), 28th day (G). ** $p<0.01$, *** $p<0.001$.

less microparticles absorbed on the surface of MS.

In addition, the release rate of GLP-1 peptide from PLGA MS depends greatly on the degradation of the polymer and the surface pores of the MS which are linked to the inner water channels. It has been reported that the biodegradation time of PLGA with the same composition mainly depends on different molecular weights.[30] The high molecular weight polymers lose their mechanical strength slower than that of

low molecular weight ones.[28] Therefore, the degradation time for polymers with high molecular weight is longer than ones with low molecular weight. Indeed, our *in vitro* release experiment confirmed these finding. For instance, the undegraded MS with high molecular polymer was observed in release medium after 28 d, while MS with low molecular weight polymer is degraded completely. These results indicate that complete cumulative release of GLP-1 from formulation D is due to the fast degradation of polymer.

It has been reported that addition of excess zinc carbonate (>10%) may lead to high solids loading and a very high surface area to volume ratio, resulting in an increase of the initial release.[29] Thus, we select less than 10% of zinc carbonate as additive to adjust initial burst release. According to our research, the purpose of reducing burst release and increasing successive release can be reached by changing amount of Zinc acetate and $ZnCO_3$ and changing type of PLGA respectively.

Evaluation of the Bioactivity of the GLP-1 in Microspheres In general, organic solvent such as acetonitrile are known to induce reversible or irreversible denaturation of macromolecule especially in the presence of water-organic solvent interface.[31] In addition, the microencapsulation process, such as sonication, shear strength, or liquid–liquid/solid–liquid interface, may influence integrity of peptide. Our S/O/O solvent extracted technique offers the advantage for GLP-1 formulation with a moderate condition to avoid heat, intensive agitation, and water-organic solution interface. The folding microparticles were directly dispersed into acetonitrile in MS manufacturing process.

To test whether the formulation process would cause any activity loss for GLP-1 peptide, we extract the GLP-1 from the formulation to monitor its biological activity. The bioactivity evaluation test showed that the GLP-1 in the MS formulation process maintained the activity for stimulating insulin secretion compared to the standard GLP-1 control ($p>0.05$) as shown in Fig. 3.

Intraperitoneal Glucose Tolerance Test To test the controlled release and biological activity of MS *in vivo*, we performed the intraperitoneal (i.p.) glucose tolerance test in normal mice. Figure 4 shows that blood glucose levels after subcutaneously injection of GLP-1 loaded MS. In control group (Blank MS), injection (i.p.) of 10 mmol/l glucose significantly increased blood glucose level from 8 to 26 mmol/l, and the glucose level sustained at 10 mmol/l after 80 min. However, the treatment group with GLP-1 MS maintained the blood glucose level at 8—9 mmol/l range within this 80 min. In the treatment group, the blood glucose level only slightly increased to 11 mmol/l at 10 min after i.p. injection of 10 mmol/l glucose. The areas under curve for glucose ($AUC_{glucose}$) of GLP-1 loaded MS group was lower remarkably than that of control group ($p<0.001$). These data indicate that GLP-1 MS formulation offer significant therapeutic efficacy to control the blood glucose levels.

To test the controlled release of GLP-1 from MS formulation and the sustained pharmacological effect, we also test the blood glucose level for i.p. injection of 10 mmol/l glucose after 4—28 d administration of GLP-1 MS formulation. The data revealed that the blood glucose levels in GLP-1 MS treatment group returned to normal glucose levels within 80 min, while that of control group was significantly higher than normal glucose levels in 80 min ($p<0.001$). Although the $AUC_{glucose}$ of GLP-1 MS treatment group was lower than that of control group from 4—28 d, the $AUC_{glucose}$ in GLP-1 MS group was higher at the 4th, 9th, and 14th day (921.1±106.5, 1039.7±125.4, and 1195.8±216.7 mmol/l per 80 min) compared to the $AUC_{glucose}$ in the 19th, 24th, and 28th day (797.1±186.0, 764.6±112.8, and 767.8±93.3 mmol/l per 80 min). The blood glucose concentration increased at 4th, 9th, and 14th day may be due to the plateau phase of drug release at the corresponding time. These data strongly suggest that GLP-1 MS formulation can achieve controlled release profile *in vivo* to increase the glucose tolerance within 28 d.

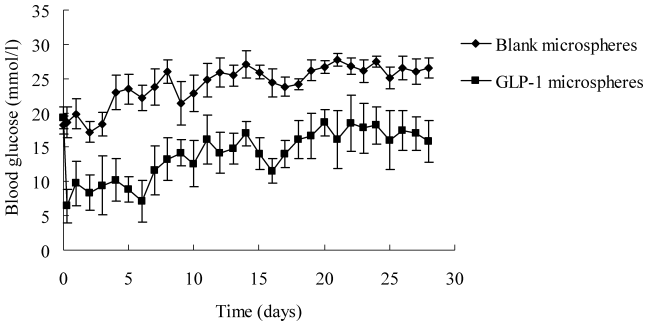

Fig. 5. Blood Glucose Concentration Profile after Intraperitoneally Administration of Blank or GLP-1 Loaded MS to Diabetic Mice ($n=6$)

Evaluation of the Bioactivity of the GLP-1 Loaded MS in Diabetes Mice After selecting one formulation from *in vitro* and *in vivo* data, we also test the controlled release and pharmacological efficacy of GLP-1 MS formulation D in diabetic model. Figure 5 showed the blood glucose profiles after subcutaneous administration of the GLP-1 MS (batch D) and control blank MS to diabetic mice. In this diabetic mice model, the blood glucose level is 20 mmol/l. Within 6 h after GLP-1 MS administration, a rapid and immediate decrease in the blood glucose level was observed. This indicates that the initial release rate of GLP-1 is adequate for its pharmacological efficacy. In first week after GLP-1 MS administration, glucose level is maintained at a lower concentration level. The blood glucose concentration had fluctuation in the 2nd to 4th week after GLP-1 MS administration, but the glucose level was significantly lower than that of blank control group (>25 mmol/l) in the 3rd and 4th week. These data strongly indicate that the GLP-1 MS formulation provides adequate initial release and controlled GLP-1 release *in vivo* to maintain the blood glucose levels.

Conclusion

In summary, GLP-1 entrapped-microspheres (MS) was prepared by using zinc compound stabilizing technique followed by solid in oil in oil (S/O/O) solvent extraction method. The zinc compound and $ZnCO_3$ in MS reduced the initial burst release of GLP-1. The controlled release bioactive GLP-1 *in vitro* was achieved for 4-week period by zinc complexation and addition of $ZnCO_3$. The optimal and complete cumulative release of GLP-1 is achieved by using low molecular PLGA polymer. The *in vivo* testing in normal mice and diabetic mice suggests that this zinc-stabilized technique combined with S/O/O method in the presence of water insoluable antacid additive $ZnCO_3$ preserves the biological ac-

tivity of GLP-1. GLP-1 MS formulation achieved controlled release *in vivo* for 28 d and exhibited sustained long term pharmacological efficacy to decrease blood glucose level in diabetic mice. This GLP-1 MS formulation provides a practical formulation for long-term sustained delivery of glucagon-like peptide-1 (GLP-1) to treat Type II diabetes.

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