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Long-acting Release Microspheres Containing Novel GLP-1 Analog as an Antidiabetic System

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Abstract

Glucagon-like peptide 1 (GLP-1) has recently received significant attention as an efficacious way to treat diabetes mellitus. However, the short half-life of the peptide limits its clinical application in diabetes. In our previous study, a novel GLP-1 analog (PGLP-1) with a longer half-life was synthesized and evaluated. Herein, we prepared the PGLP-1-loaded poly(D,L-lactide-co-glycolide) microspheres to achieve long-term effects on blood glucose control. The incorporation of zinc ion into the formulation can effectively decrease the initial burst release, and a uniform drug distribution was obtained, in contrast to native PGLP-1 encapsulated microspheres. We demonstrated that the solubility of the drug encapsulated in microspheres played an important role in in vitro release behavior and drug distribution inside the microspheres. The Zn-PGLP-1 microspheres had a prominent acute glucose reduction effect in the healthy mice. A hypoglycemic effect was observed in the streptozotocin (STZ) induced diabetic mice through a 6-week treatment of Zn-PGLP-1-loaded microspheres. Meanwhile, the administration of Zn-PGLP-1 microspheres led to the β-cell protection and stimulation of insulin secretion. The novel GLP-1 analog-loaded sustained microspheres may greatly improve patient compliance along with a desirable safety feature.

Keywords: PLGA microspheres, GLP-1 analogs, In vitro release, Long-acting hypoglycemic efficacy

Introduction

Diabetes mellitus is a global health disease that exhibits major characteristics of hyperglycemia. Currently, throughout the world, more than 400 million people suffer from diabetes.[1] With the increasing number of patients, many attempts have been made to cure the metabolic disorder. In the last few decades, glucagon-like peptide-1 (GLP-1) has become an effective peptide to treat diabetes. GLP-1 is considered as an incretin hormone that can stimulate insulin secretion in response to rising glucose, inhibit the intake of food and protect β-cells from apoptosis.[2] Nevertheless, the short half-life of GLP-1 (approximately 1-2 min) caused by its rapid degradation via dipeptidyl peptidase IV (DPP-IV) limits its clinical application in diabetes. In the previous study, we synthesized a GLP-1 analog with a longer half-life that has the same bioactivity as the endogenous GLP-1(Schematic a).[3]

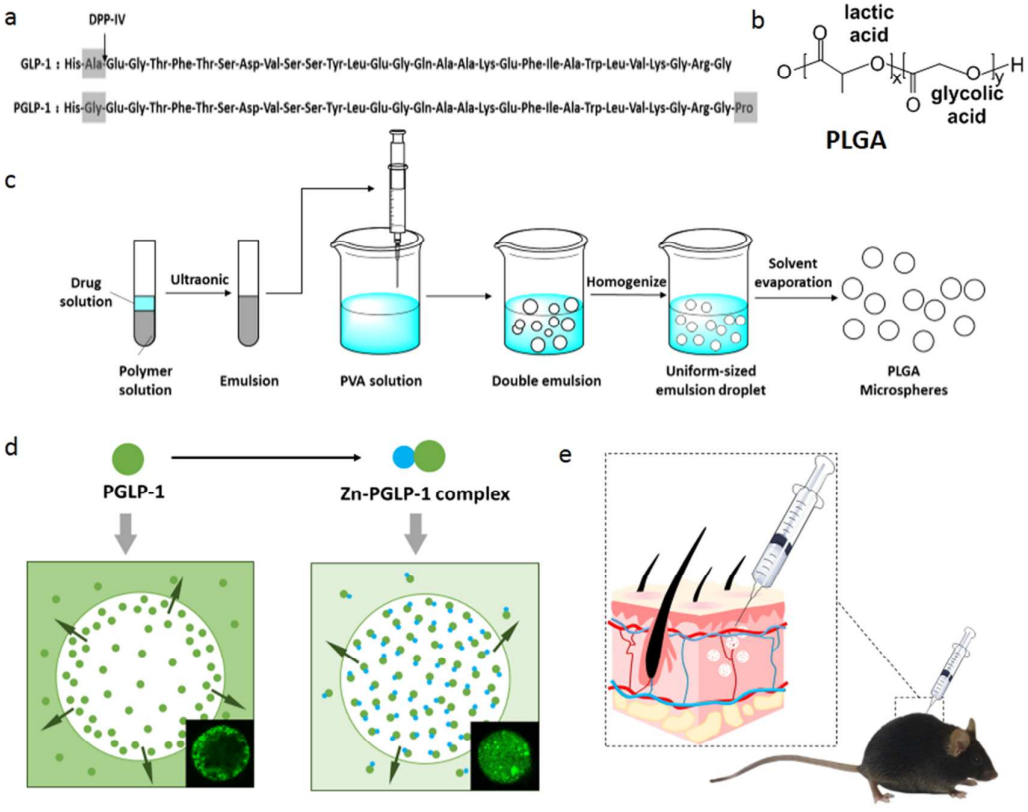
It is notable that repeated injection is needed due to the short half-life of the peptide. The frequent injection decreases patient compliance and affects quality of life. To inhibit the rapid elimination, several approaches have been utilized, such as PEGylation, implanted polymers, complexation, etc.[4] Biodegradable depots with micro-sized particles prepared by poly(D,L-lactide-co-glycolide), also known as PLGA, has shown immense potential for controlled drug delivery. PLGA is approved by the Food and Drug Administration (FDA) as a generally safe polymer with biocompatibility and low toxicity. Moreover, besides the nontoxic nature of the material, PLGA drug delivery devices have many advantages: first, the PLGA matrix is suitable for numerous active pharmaceutical ingredients. In addition to peptides or proteins, vaccines and small molecular drugs can be encapsulated into depots. Second, the release kinetics can be fine-tuned by selecting appropriate PLGA materials. However, the poor reproducibility in large scale production and denaturation of the protein/peptide during the preparation process are major drawbacks. Hence, there is interest in overcoming these limitations.[5]

In vitro release of PLGA formulations, drug diffusion and polymer erosion are the critical factors controlling drug release from polymeric microspheres.[6] It is generally believed that the initial burst release is mainly due to the enrichment of drug near the surface of microsphere.[7] The solubility and distribution of drug are the key factors affecting the initial release.[8,9,10] Solubility of drug not only affects the encapsulation efficiency of the matrix, it also influences the final release behavior.[11] Many studies have investigated the effect of the release pattern in vitro using drugs with different solubility. Doxorubicin hydrochloride and paclitaxel were selected as models of hydrophilic/lipophilic drugs. The release of paclitaxel followed a nearly zero-order kinetics pattern, while in contrast an initial burst release was observed in the doxorubicin hydrochloride release matrix, suggesting that the solubility was the vital factor of the formulation that affected the constant release of drug.[12] As the water-soluble protein/peptide is encapsulated in the microsphere, a homogeneous distribution is expected, which will lead to a minimal initial burst and a maintained continuous release.[13] However, a water-soluble protein/peptide always aggregates near the surface of the microsphere prepared by the water-oil-water (W/O/W) solvent evaporation method.[14] During the fabrication process, the hydrophilic drug tends to diffuse outward, resulting in a non-uniform distribution.[15,16,17] After the rapid release from the surface, a lag release stage is observed. In this period, because of the dense netlike structure inside the matrix, fewer drugs can diffuse out.[18] The slow release of drug is attributed to the increase in the length of the drug diffusion pathways.[19] In this state, the drug release is mainly controlled by the degradation rate of the polymer matrix.[20] Erosion leads to the formation of pores and increases the rate of diffusion.[21] With polymer degradation, the drug trapped inside the matrix slowly diffuses through the water-filled channel.

In general, we are unable to accurately predict in vivo drug release based on the performance in vitro due to the complex physiological environment.[22] Compared to the in vitro release test, the drug release kinetics tends to occur faster in vivo.[23] The lag phase observed in in vitro release disappears

due to the accelerated degradation of the polymer in vivo.[24,25] There are many reasons that may cause the increased release, such as biological lipids, enzymes, endogenous amines and the local pH at the injection site.[24,26,27] Lipids and endogenous amines may plasticize the polymer and enhance chain mobility and enzymes can catalyze hydrolysis.[28] The rapid diffusion caused by erosion of the polymer matrix can lead to a fast release in vivo.

In this study, a sustained release carrier of a GLP-1 analog using PLGA was established. To avoid the burst release, zinc acetate was added to form a complex with GLP-1, which exhibited low solubility in a neutral solvent. Different release behaviors were observed at pH2.0, pH5.6 (the isoelectric point of the peptide) and pH7.4, which demonstrated that solubility is an important factor controlling the initial release. An optimal hypoglycemic efficacy was investigated in vivo using a streptozotocin-induced diabetes mouse model. The protection of islet and β-cell function by GLP-1 loaded microsphere treatment was also observed.



Scheme 1(a) Amino acid sequences of native GLP-1 and PGLP-1. (b)Structure of poly-D,L-lactide/glycolide. (c) Schemes of microspheres preparation process. (d) Schematic diagram of vitro release and distribution of peptides in different formulations. (e) Long-acting hypoglycemic efficacy in diabetic mice.

2. Materials and Methods

2.1 Materials

PLGA RG502H (poly-D,L-lactide/glycolide 50/50; Mw 17kDa) was purchased from Sigma-Aldrich(St. Louis, Missouri). PGLP-1 peptide was chemically synthesized by GL Biochem (Shanghai, China). PVA (PVA1788, degree of hydrolysis 87%-89%, 75kDa) was provided by Aladdin (Shanghai, China). Streptozotocin (STZ) was purchased from Sigma-Aldrich (St. Louis, Missouri). Fluorescein isothiocyanate (FITC) was provided by Meilun (Dalian, China). Trifluoroacetic acid (TFA), methylene dichloride (DCM) and acetonitrile were supplied by Nanjing Chemical Reagent Co.,Ltd. (Nanjing, China). All other reagents were analytical grade.

Experiment animals

Male C57BL/6J mice (7-8weeks) were obtained from Model Animal Research Center of Nanjing University (Nanjing, China). All of the animals were housed under a controlled temperature of 25 °C with a 12 h light-dark cycle. The mice had free access to food and water. All animals were fed according to the guidelines of the “Principles of Laboratory Animal Care” and approved by the Animal Ethics Committee of China Pharmaceutical University.

2.2Preparation of microspheres

Zn-PGLP-1 complexes were prepared by adding 4 mg PGLP-1 into 1 ml zinc acetate solution (2.5% w/v) and incubated at 4°C for 24 hours. The suspension was lyophilized, and a powder was obtained after 48 hours. The solvent evaporation method was used to prepare the microspheres. Briefly, 4mg PGLP-1 or Zn-PGLP-1 complex was dissolved/suspended into 200μl distilled water. Then, 1ml PLGA methylene dichloride solution (oil phase, 100mg/ml) was added into the inner water phase and sonicated for 120s at 50W to obtain the primary emulsion. Next, the primary emulsion was added to an external aqueous phase containing PVA (2% w/v) and NaCl (2.5% w/v) drop by drop and emulsified by homogenization (T25,IKA,Germany) to form the double emulsion.

Next, the double emulsion was solidified in a PVA solution (0.1% w/v) at room temperature for 4 hours. Finally, the microspheres were collected using centrifugation for 5min at 3000rpm and washed with distilled water three times. The microspheres were obtained after freeze-drying for 48 hours.

2.3Characterization of the microspheres

2.3.1 Drug loading and encapsulation efficiency

First, 5mg microspheres were dissolved in 200μl acetonitrile with sonication for 15 seconds. Then, 800μl 0.01M HCl was added to the solution and the peptide was extracted from the mixed solution. Finally, the sample was filtered by 0.45 μm filters to obtain a clear solution. The concentration of PGLP-1 was determined by high-performance liquid chromatography (Shimadzu, Japan). Water containing 0.05% (v/v) TFA and acetonitrile containing 0.05% (v/v) TFA were used as the mobile phase. The gradient elution procedure was from 80% water phase/20% organic phase to 15% water phase/85% organic phase within 12 minutes. The UV detector was operated at a wavelength of 215 nm, and the flow rate was 1ml/min.

$$\text{Drug loading} = \frac{\text{Mass of drug in microspheres}}{\text{Mass of microspheres}} \times 100\%$$

$$\text{Encapsulation efficiency} = \frac{\text{Drug loading}}{\text{Theoretical drug loading}} \times 100\%$$

2.3.2Size measurement and morphology

The particle size was measured by laser diffraction using a Mastersizer 2000 (Malvern, UK) with sonication. The Span value represented size distribution was calculated using the following equation:

$$\text{Span value} = \frac{D_{v,90\%} - D_{v,10\%}}{D_{v,50\%}}$$

$D_{v,10\%}$ $D_{v,50\%}$ and $D_{v,90\%}$ are volume size diameters at 10%, 50% and 90% of the cumulative volume, respectively.

The detailed morphologies were observed using a field emission scanning electron microscope (FE-SEM, S4800, Hitachi) with a maximum resolution of 1.0 nm. Before the analysis, samples were spread on a metal stub and coated with 10 nm of gold.

2.3.3 Peptide stability

The conformation analysis of the peptide was determined by circular dichroism spectroscopy (Jasco J-810 spectrometer, Japan). From the CD result, we investigated the secondary structure of PGLP-1 and observed the peptide bio-stability. PGLP-1 was extracted from drug-loading microspheres using the same procedure as the abovementioned method, and the native peptide solution was used as a control substance. The detection was performed using a 0.1 cm path cuvette at a scanning speed of 50 nm min^{-1}.

2.3.4 Differential scanning calorimetry and powder X-ray diffraction

The thermal properties of the microspheres were studied using a differential scanning calorimeter (Q2000, TA Instruments). Approximately 5 mg samples were loaded into sealed aluminum pans. The heating curve was recorded from 0°C to 180°C at a heating rate of 10°C/min. The glass transition temperature (T_g) and melting temperature were analyzed by the TA Universal Analysis software.

The crystallinity of peptide in the microspheres was evaluated by using powder X-ray diffraction (PXRD) equipped with Cu Kα radiation (Bruker, Germany). The data were collected over an angular range of 3-40° (2θ) with a continuous scan mode using a step size of 0.02° (2θ) and a step time of 0.3s.

2.4. In vitro drug release

2.4.1 Drug release kinetics

First, 10mg PLGA microspheres were suspended into 1ml release media (phosphate buffer-pH7.4, phosphate buffer-pH5.6 and hydrochloric acid solution-pH2.0) with mild shaking (200rpm) in an air-bath shaker at 37°C. At predetermined time points, the medium was removed by centrifugation and replaced with 1ml fresh medium/buffer. The concentration of the peptide was determined by HPLC. All of the experimental groups were performed in triplicate.

2.4.2 Drug distribution

To monitor the distribution within the microspheres, FITC was used to label the peptide. Briefly, 4mg PGLP-1 and 0.5mg FITC were dissolved into 2ml carbonate buffer (pH9.2) and 0.5ml DMSO, respectively. FITC was added into the peptide solution drop by drop under mild stirring. The resulting solution was incubated for 4 hours at room temperature. Finally, unreacted dye was removed by dialysis. The preparation procedure of the microspheres was performed as described above. The fluorescence of the particles was monitored by confocal laser scanning microscopy (CLSM,Zeiss,LSM700) at an excitation wavelength of 480 nm. 3D reconstitution was used to observe the inner structure (drug distribution) of the microspheres, and the step size was 1 μm along the vertical (z) direction.

2.4.3 Mass remaining and T_g measurement

Plain microspheres (initial weight W_0) were incubated in 1ml PBS (pH7.4) at the same condition as the in vitro study. At 1, 3, 5, 7, 10, 14 and 28 days, the medium was withdrawn and subjected to lyophilization. W_{dry} denotes the total weight of the remaining microsphere. The T_g of the powder was measured by DSC (TA2000).

$$\text{Mass remaining (\%)} = \frac{W_{\text{dry}}}{W_{\text{o}}} * 100\%$$

2.5. Animal experiments

2.5.1 Sustained glucose reduction efficacy

To evaluate the sustained hypoglycemic efficacy in vivo, an intraperitoneal glucose test was performed. Healthy male mice were randomly divided into 3 groups (n=6) and received a subcutaneous injection of plain microspheres, Zn-PGLP-1 microspheres or PGLP-1 solution. For the peptide solution groups, free PGLP-1 at dosage of 0.35mg/5ml/kg was administered daily;[3] the other mice received a single injection of Zn-PGLP-1microspheres (containing 9.8mg/kg PGLP-1) or plain microspheres. The day of the microsphere injection was defined as day 0. At day 1 to day 14,

1.5g/5ml/kg glucose solution was administered by intraperitoneal injection. The mice received glucose after the PGLP-1 solution for 15 min each day. Blood was collected from the tail vein and measured using a blood glucose meter (Omron, Tokyo, Japan) 15-20 min after the glucose was administered.

2.5.2 Pharmacodynamics study in diabetic mice

An STZ-induced mouse model was established by intraperitoneal injection with STZ (50mg/kg) for five consecutive days. On day 7 and day 14 after the last injection, the blood glucose level was measured. Mice with blood glucose concentrations above 11.1mmol/L were accepted for the study. The efficacy of Zn-PGLP-1 microspheres in treating diabetes was evaluated by STZ-induced mice. Thirty-two mice were divided into four groups (n=8): each group was given saline, a PGLP-1 solution (0.35mg/kg), Zn-PGLP-1 MP (equivalent to the dose of twice-daily injections of PGLP-1 for 2 weeks, 0.35 mg/kg ×2 injections ×14 days =9.8mg/kg PGLP-1) or a large-dose PGLP-1 solution (9.8 mg/kgPGLP-1) equivalent to the drug content inside Zn-PGLP-1 MP. For the saline group and the PGLP-1 solution group, mice were subcutaneously injected twice daily, while the MP and large-dose PGLP-1 solution groups received one injection over 2 weeks. The blood glucose measurement was assayed weekly from the tail vein of each mouse after eight hours of fasting. Simultaneously, food intake was evaluated every 2 or 3 days. After a 6-week treatment, the mice were sacrificed and the pancreata and subcutaneous tissues were isolated. Blood samples were collected from the orbit and centrifuged at 8000rpm to obtain serum. Plasma insulin was determined by ELISA kit (Shanghai Lengton Bioscience Co. Ltd., China), and the glycosylated hemoglobin (HbA_{1c}) was evaluated by HbA_{1c} assay kit (Nan Jing Jiancheng Co.Ltd. China).

2.5.3 Histological and immunohistochemistry studies

Pancreata were isolated and fixed in 4% paraformaldehyde overnight. The tissues were embedded in paraffin blocks and cut using a microtome. Sections (7 μm thick) were stained with

hematoxylin and eosin. Insulin immunohistochemistry was performed to determine the function of β-cell protection and insulin secretion improvement. Tissue sections were blocked in PBS containing 3% bovine serum albumin for 30 minutes and incubated with a primary antibody dilution (ratio 1:800) overnight at 4°C. Next, the appropriate secondary antibody was applied onto the slide followed by washing three times in PBS. Finally, the sections were treated with 3,3'-diaminobenzidine (DAB, DAKO, Denmark) and counterstained with hematoxylin. The images were examined with an Olympus microscope (Tokyo, Japan).

2.5.4 Biocompatibility analysis in vivo

To evaluate the safety of Zn-PGLP-1 MP in vivo, the subcutaneous tissue from the injection sites was isolated. The tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Then, they were sectioned at 7 μm and stained with hematoxylin and eosin. An Olympus microscope was used to take photomicrographs of the tissues.

2.5.6 Statistical analysis

Statistical significance was analyzed by unpaired t-test. SPSS statistics version 22 was used to calculate statistical parameters.

3. Results

3.1Characterization of microspheres

3.1.1 Morphology, size and EE/LD

As a sustained drug release device, regular shape, narrow size distribution and high drug loading are required for microspheres. It is believed that the particle size affects the release pattern. The increased device dimension results in increased drug release by accelerating the degradation rate of the polymer.[29] Notably, an initial burst release is observed with the decreasing particle size due to the enlarged surface area.[30] In this study, uniform PLGA microspheres were successfully prepared,

where the median volume diameters of the resultant particles were 23.76μm and 20.63μm for PGLP-1 MP and Zn-PGLP-1 MP, respectively (Table 1). The Span values were small, indicating the narrow size distribution of microspheres (Figure 1C). No significant difference in particle size distribution was observed in the presence or absence of zinc ion. As shown in Figure 1A and 1B, the microspheres of both formulations are spherical in shape and small pores were observed across the surface. However, more pores were observed on the surface of Zn-PGLP-1 MP than PGLP-1MP, which can be attributed to the zinc acetate addition acting as a type of pore-forming agent. These pores on the surface can facilitate water uptake from the medium and provide diffusion channels for drugs.[17] High encapsulation efficiency (EE>90%) and drug loading (LD) were obtained in both Zn-PGLP-1 MP and PGLP-1 MP. The EE of peptides are mainly affected by their solubility in the external aqueous phase and the stability of emulsion.[16] Normally, reducing the solubility of a peptide can lead to a higher EE. Surprisingly, there were no differences in EE between these two formulations using compounds with varied solubility (the Zn-PGLP-1 complex exhibited low solubility in the aqueous medium). This phenomenon may be caused by the addition of salt to the outer water phase, which leads to high osmotic pressure preventing drug diffusion from the inner phase.[31]

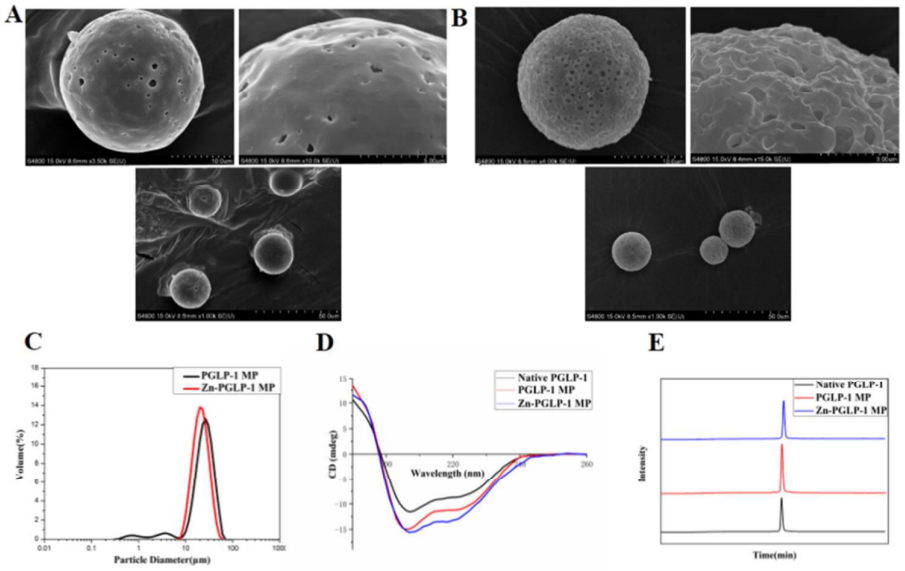


Figure 1. Characterization of PLGA microsphere. (A)SEM images of PGLP-1 microspheres. (B) SEM images of Zn-PGLP-1 microspheres. (C) Size distribution of microspheres. (D) CD spectra and (E) HPLC traces of native and

extracted PGLP-1 peptide.

Table 1 Characterization of microspheres (diameters, encapsulation efficacy and drug loading)

	Volume mean diameter(μm)			Span	Drug loading (%)	Encapsulation efficiency (%)
	$D_{v,10\%}$	$D_{v,50\%}$	$D_{v,90\%}$			
PGLP-1 MP	11.45	23.03	38.23	1.16	3.58±0.15	93.24±3.91
Zn-PGLP-1 MP	11.84	19.35	31.26	1.00	3.66±0.20	95.30±5.21

3.1.2 Bio-stability of peptide

PLGA microspheres are widely used to delivery peptides/proteins. It is important to highlight that an irreversible inactivation occurs during the preparation and release process of microspheres.[32] When drug is encapsulated into microspheres, the protein/peptide is exposed to a large organic/water interface and high shear force leading to drug inactivation. Peptide absorption at the PLGA surface and an acidic microenvironment induced by polymer hydrolysis are also potential sources of peptide/protein aggregation.[33] Compared to proteins, peptides lack a complicated tertiary structure, so circular dichroism spectroscopy (CD) and HPLC are effective ways to determine the bio-stability of the drug.[34,68] As shown in Figure1D, no significant difference was observed between the extracted peptide in either formulation (Zn-PGLP-1 MP and PGLP-1 MP) and native PGLP-1, indicating a minimal effect on structural characteristics during the emulsion process. The peaks at 208 and 222nm were assigned to α-helix and β-sheet structures of the peptide.[35,36] From the HPLC result (Figure 1E), the retention time did not change compared with the native PGLP-1 solution and no degradation peak was detected. From the aforementioned discussion, we can conclude that the peptide maintained its sequence integrity and secondary structure.[36]

3.1.3 Thermal analysis of microspheres

The PXRD results are shown in Figure2A. PXRD was used to study the crystallinity of the peptide encapsulated in PLGA microspheres.[37] A halo pattern was observed in the PXRD pattern of PLGA. In contrast, the raw peptide exhibited sharp crystalline diffraction peaks. For the peptide-loaded microspheres, no significant diffraction was detected, indicating that the peptide

existed as the amorphous form or tiny crystals which might be difficult to detect by powder X-ray diffraction.[38,39]

The results of DSC analysis are shown in Figure2B. The DSC trace of crystalline PGLP-1 revealed a narrow endothermic peak at 166.3°C, which was attributed to the melting of the peptide. A single T_g was characterized in PLGA raw materials. DSC traces of the peptide encapsulated microspheres showed only a single T_g without any melting event, suggesting no crystalline material left in the microspheres. The DSC results were consistent with the PXRD patterns of Zn-PGLP-1 MP and PGLP-1 MP, where only halo patterns were observed to support the amorphous state of materials. Moreover, the T_g did not change in the presence or absence of drug. Thus, there is no plasticizing effect of the free peptide regarding the PLGA polymer.[40,41]

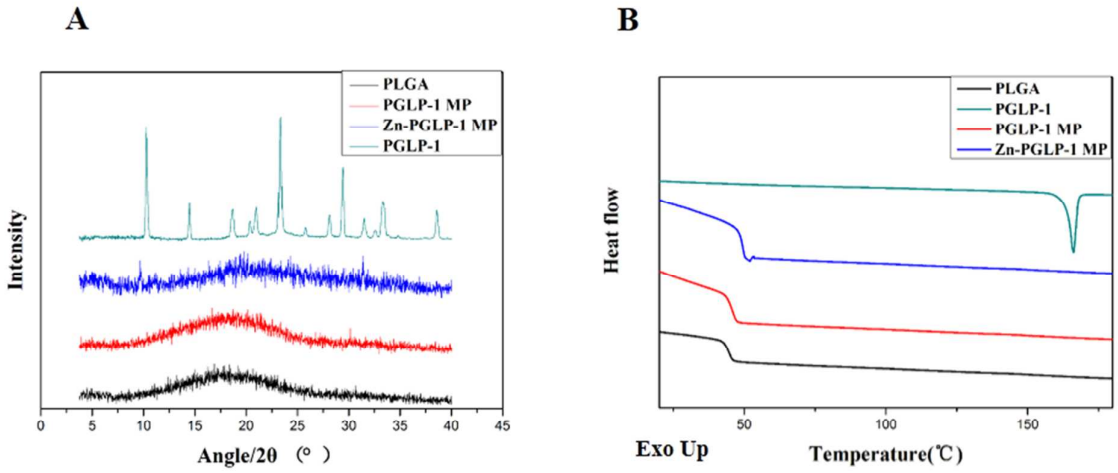


Figure 2. Physical properties of microspheres. (A) PXRD pattern and (B) DSC traces of PGLP-1, PLGA material, PGLP-1 microsphere and Zn-PGLP-1 microsphere.

3.2The effects of release in vitro

3.2.1Release behavior in vitro

The release behavior of the microspheres was determined at different pH conditions, including 2.0(acidic environment), 5.6(isoelectric point) and 7.4(physiologic environment). As shown in Figure 3B, an obvious burst release of PGLP-1 MP was observed in the pH 7.4 medium. In contrast, the same formulation showed only a 9% drug release in the pH 5.6 buffer followed by a slow release rate.

This phenomenon can be attributed to the different solubility of the peptide in various pH media. At the isoelectric point, the peptide exhibited the lowest solubility. The low solubility led to a slow dissolution rate. In the pH 7.4 buffer, the drug can easily dissolve and diffuse throughout the surface pores.[42] However, after a 35% release in the pH 2.0 medium, an approximate linear release was obtained due to the catalysis effect of PLGA erosion in the acidic environment.[43]

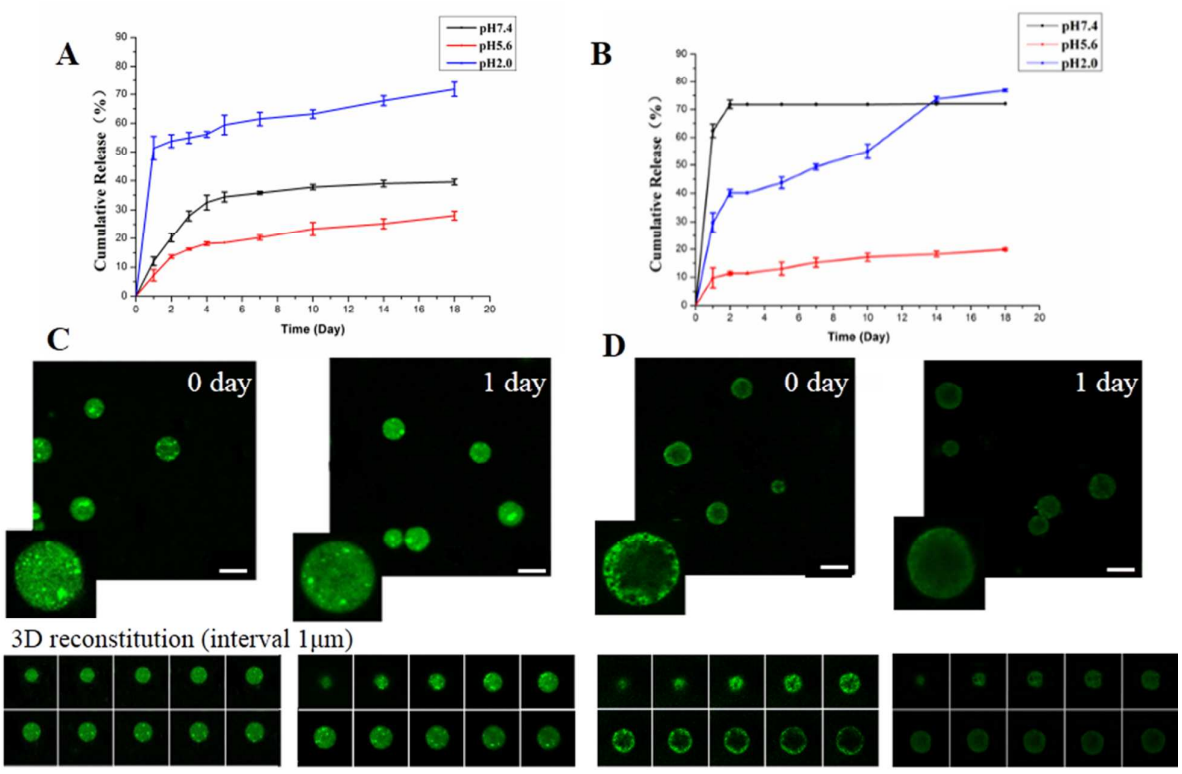


Figure 3. In vitro release profiles of PGLP-1 from (A) Zn-PGLP-1 microspheres and (B) PGLP-1 microspheres under pH 2.0, pH5.6 and pH7.4. CLSM images and 3D reconstitution (1 μm step size along the vertical (*z*) direction) of (C) Zn-PGLP-1 microspheres and (D) PGLP-1 microspheres incubated in pH7.4 buffer at day 0 and day 1. Scale bar: 20μm

To decrease the initial release in the physiologic environment (pH 7.4) for clinical application, zinc ion was used to form the water-insoluble Zn-PGLP-1 complex which was further encapsulated into the PLGA matrix (Figure 3A).[4] The release rate was reduced on the first day in the pH 7.4 buffer while maintaining a rapid release behavior over 5 days. Zn-PGLP-1 complex encapsulated microspheres exhibited a constant release with the highest burst at approximately 51.3% at pH2.0. The lowest initial release was obtained at pH5.6. To better understand the cause of the different release behavior, the solubility of the Zn-PGLP-1 complex in different pH values was measured

(Table S1). The results show that the Zn-peptide complex is extremely soluble at pH2.0, whereas low solubility is observed at pH5.6 and pH7.4. The solubility of the Zn-peptide complex at pH7.4 was 1.6-fold higher than those at pH5.6. It is important to highlight that our solubility results (pH2.0>pH7.4> pH5.6) are consistent with the behavior of initial release of microspheres at different pH conditions (pH2.0>pH7.4>pH5.6). This aforementioned discussion concluded that the initial release was controlled by the solubility of the encapsulated compound.[44] The release profile of PGLP-1 MP in pH 7.4 buffer exhibits plateaus after an initial burst, which can be attributed to drug exhaustion near the surface, as demonstrated by the CLSM results (Figure 3D). For microspheres with a slow initial release, the peptide located in the outermost layer exhibited a sustained release due to slow dissolution rates in the certain media.[45]

3.2.2 Drug distribution

The drug distribution is another key factor controlling drug release, and CLSM is a visualized approach to reflect drug distribution within the microspheres.[46] The drug distributions of Zn-PGLP-1 MP and PGLP-1 MP within the polymeric matrix were remarkably different. As shown in Figure3D, a bright outer ring of PGLP-1 MP was observed with a darker core, suggesting that a large portion of the peptides was near the surface. In contrast, Zn-PGLP-1 MP showed uniform fluorescence distribution (Figure3C). During the emulsion and solidification steps, the hydrophilic peptide molecules tended to migrate from the inner domains toward the exterior of the microsphere and the water-insoluble Zn-peptide complex was located uniformly within the polymer matrix due to the slow diffusion rate.[7] To further evaluate how drug distribution affects the release pattern, the following experiment was performed. The newly prepared microspheres with FITC-labeled peptide were suspended in the pH7.4 buffer. After one day, the samples were collected and investigated by CLSM. Zn-PGLP-1 MP showed no obvious change compared to the fresh microspheres, exhibiting the lower initial drug release. However, for PGLP-1 MP, due to the high solubility of PGLP-1 at

pH7.4, the peptide located on the surface easily diffused out into the release medium, resulting in the significant diminishing of fluorescence after incubation. These results strongly support the speculation that the solubility of the drug can significantly influence its distribution within PLGA microspheres.

Based on the in vitro release results, we conclude that drug solubility and distribution inside the polymer matrix are critical for the release kinetics at the initial stage.[8,9] By modulating drug solubility, a desired release kinetics could be achieved, and a formulation with homogeneous drug distribution could be in favor of sustained release.[47]

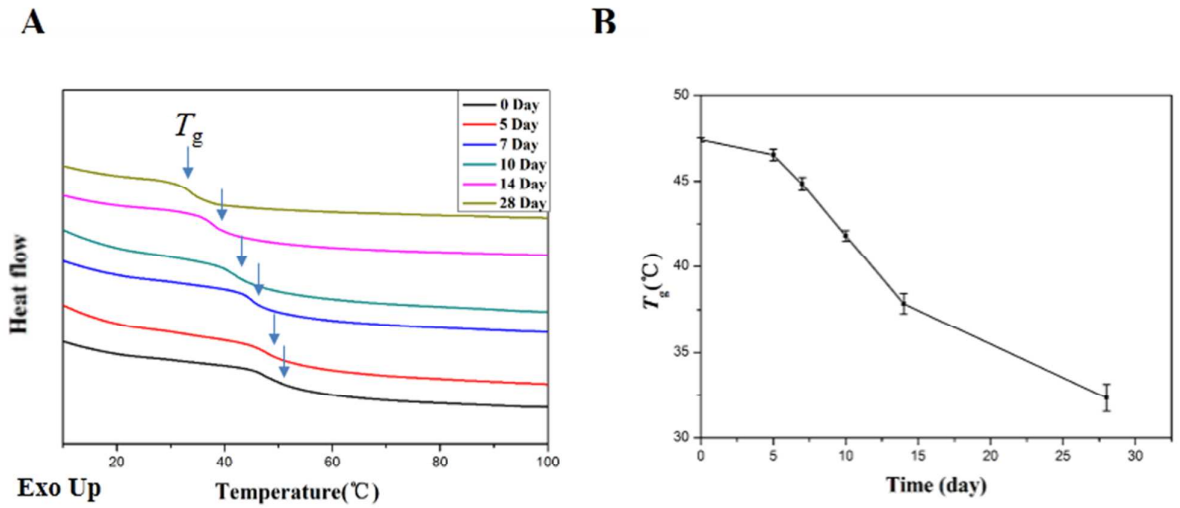


Figure 4. Polymer degradation behaviors of PLGA microspheres upon exposure to pH7.4 phosphate buffer: (A) DSC traces. (B) Changes of glass transition temperature (T_g).

3.2.3 Degradation in vitro release

After rapid release in the first few days, a lag-time was observed. During the slow release stage, polymer degradation becomes important to control drug release.[21] Based on previous results, the PGLP-1/Zn-peptide complex appeared to have no plasticizing effect on the polymer. In this circumstance, under the specific physiological conditions, the plain MP was used for the degradation study to represent the drug-loaded MP. As shown in Figure4B, raw PLGA powder exhibited a T_g at 47.1°C. Time to time (different days) experimental T_g tended to decrease, and this decreasing of T_g

might be caused by polymer chain hydrolysis (Figure 4A).[48,49] In the first five days, the decrease of T_g was not as pronounced. However, as the incubation progressed, polymer degradation accelerated, which was mainly attributed to an autocatalytic effect on PLGA.[50] Polymer erosion produced a water-soluble acid oligomer by ester bond cleavage.[51,52] With increasing the incubation time, acidity accumulated inside the matrix and the microclimate pH steeply decreased.[33] The acidic microclimate pH in turn accelerated polymer degradation, causing a rapid decrease on the T_g of microspheres.[53] A porous structure formed inside the microspheres, and the drug slowly diffused throughout these channels.[40]

3.3 In vivo study of hypoglycemic efficacy and mechanism

3.3.1 Sustained efficacy of hypoglycemia in vivo

To determine the sustained hypoglycemic effect of Zn-PGLP-1 MP, the acute glucose reduction test was performed in normal C57BL/6J mice (Figure 5A). The blood glucose level (BGL) can increase immediately after intraperitoneal glucose injection.[54] GLP-1 analogs exhibit significant hypoglycemic efficacy against increasing blood glucose levels.[3] As shown in Figure 5B, the group injected with blank MP was considered the negative control and a rapid glucose increase was observed. The mice that received free PGLP-1 suppressed the high glucose level after administration of glucose for 20 min and reached a BGL value of approximately 10mmol/L. Based on these results, we concluded that PGLP-1, a novel GLP-1 analog, can reduce glucose levels. Moreover, a noticeable BGL decline was observed in the MP group and a normoglycemia state was maintained, which was similar to the BGL value of mice treated with free PGLP-1 over 14 days, indicating that the Zn-PGLP-1 MP formulation had a 2-week-long hypoglycemic effect. Mice injected with blank MP showed a negligible influence on BGL increase, whereas mice treated with Zn-PGLP-1 MP and free PGLP-1 could decrease the acute increase of BGL. Importantly, an equivalent blood glucose level decreasing effect of drug-loaded MP was obtained compared with daily peptide administration over 2

weeks.

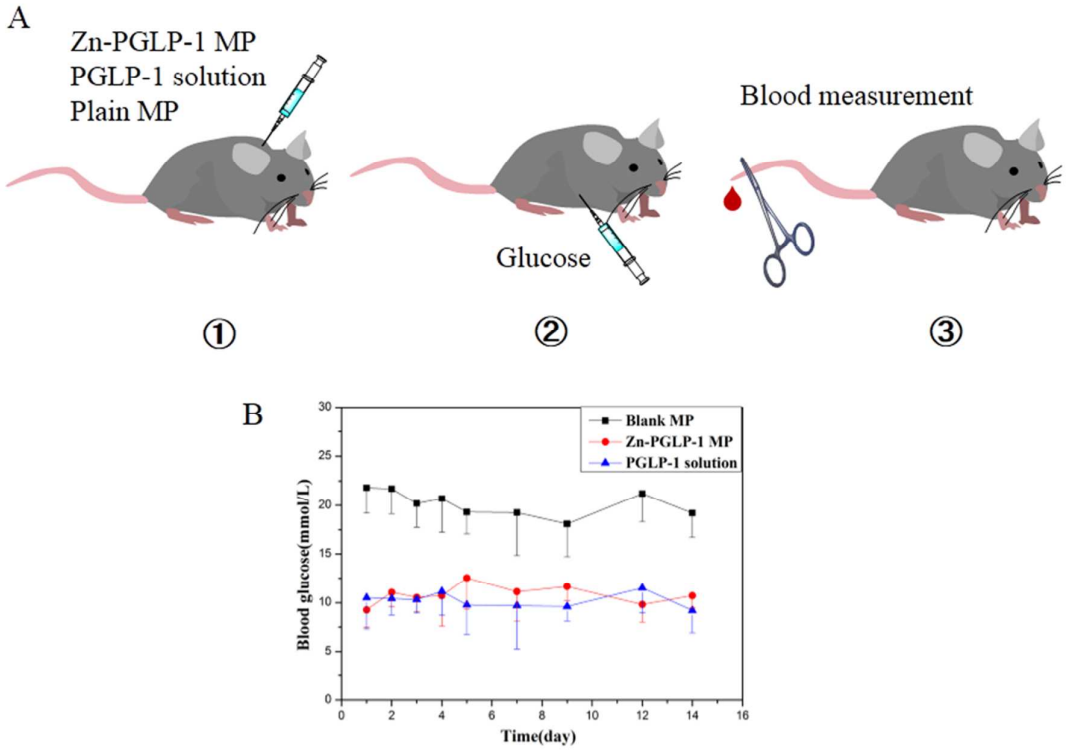


Figure 5. Sustained hypoglycemic efficacy of Zn-PGLP-1 MP in healthy mice. (A) Schematic of experiment. (B) Acute hypoglycemic effect of healthy mice treated with blank MP, Zn-PGLP-1 MP and peptide solution. (Single injection for the groups of Zn-PGLP-1 MP and blank MP; daily injection for the group of PGLP-1 solution).

Due to the in vitro release results of Zn-PGLP-1 MP in pH7.4 medium, in which a rapid and continuous release in the first five days followed by a lag release phase was observed, it was surprising that the drug-loaded MP could achieve a 2-week hypoglycemic effect in mice. There are two explanations that could account for this phenomenon. One reason is that the novel PGLP-1 exhibits glucose reduction efficacy at a low dose.[3] Another reason is the poor correlation between in vitro and in vivo data (IVIVC).[55] For biodegradable implants, it is common that the release profile in vitro cannot predict the profile in vivo accurately.[23] Normally, a faster release rate is obtained in vivo than in vitro.[25] For the in vitro test, lower drug release rates have been reported due to aggregation of the microspheres in the release medium.[56] For in vivo studies, the complicated physiological environment at the site of administration can cause accelerated release.[57] The adequate fluid volume available in subcutaneous tissue can remove drug released from microspheres immediately.[24]

Biodegradable polymer hydrolysis can be catalyzed by enzymes in the physiological environment,[27] and biological lipids can plasticize the polymer to increase the chain mobility, leading to rapid drug diffusion.[26]

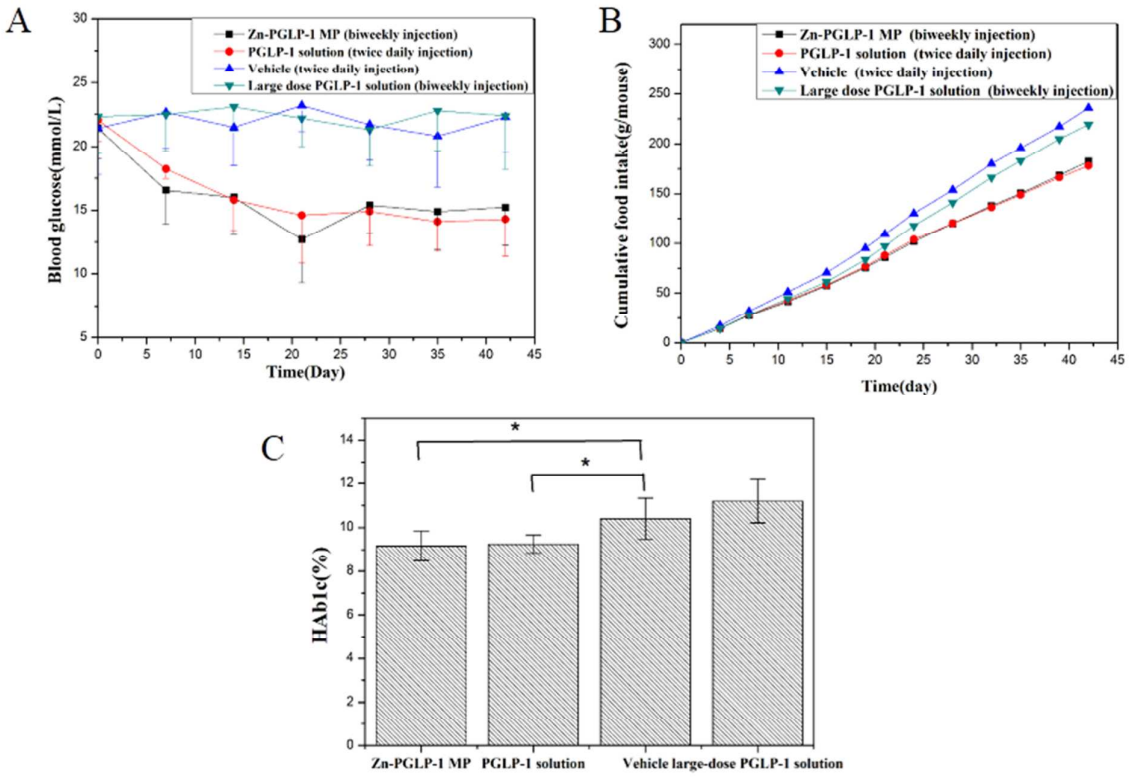


Figure 6. The pharmacological study of PGLP-1 loaded microspheres. (A) Blood glucose level of fasted mice treated with the different formulations. (B) Cumulative food intake of diabetes mice after subcutaneous injection of different formulation. (C) HbA_{1c} level of diabetes mice after 6 weeks treatment. *P<0.05

3.3.2 Pharmacodynamics study in diabetic mice

Due to the better release behavior of Zn-PGLP-1 MP compared with native PGLP-1 MP in vitro, the Zn ion addition formulation was used to treat STZ-induced diabetic mice. The mice were divided into four groups. Three administrations of Zn-PGLP-1 MP were given on day 1, day 15 and day 29. As shown in Figure 6A, the mice receiving MP and twice-daily injections showed an obvious hypoglycemic efficacy in weekly blood glucose measurements. There was no significant difference in BGL between the daily treatment groups and the long-term microspheres group. The BGL maintained a downward trend throughout the experimental period whereas the mice treated with vehicle maintained high glucose levels. Surprisingly, the large dose peptide injection group had a

negative hypoglycemic effect on diabetic mice due to the short half-life of PGLP-1. In the literature, a GLP-1 receptor agonist can delay gastric emptying and reduce appetite.[3] In this study, energy intake was monitored every 2 or 3 days. The cumulative food intake was shown in Figure 6B. Zn-PGLP-1 MP and PGLP-1 solution treatment reduced the cumulative energy intake compared to the saline group and biweekly injection of the large dose peptide solution had little effect on inhibiting food intake. In summary, the Zn-PGLP-1 MP treatment provide equivalent efficacy as frequent solution injection. HbA_{1c} was determined by assay kit, and the percentage was compared to the vehicle group (Figure 6C). The results revealed that the HbA_{1c} level of groups receiving the sustained formulation and PGLP-1 solution was significantly reduced compared with the vehicle group, indicating better long-term blood glucose control.

3.3.3 Histological and immunohistochemistry examination

The protection of islet and β-cell function by GLP-1 treatment has been demonstrated in many studies.[58,59] HE staining and immunohistochemistry are effective approaches to evaluate the effect of PGLP-1 loaded microspheres on islet protection and insulin secretion. The pancreatic islets isolated from STZ-induced mice were stained by hematoxylin and eosin to analyze islet morphology. As shown in Figure 7A-a, the islet structure remained intact with a round or elliptical shape in the Zn-PGLP-1 MP and PGLP-1 solution groups, whereas an irregular islet shape was observed in the vehicle group.[59] This result revealed that sustained treatment with the peptide solution and Zn-PGLP-1 loaded MP maintained the integrity of islet morphology, suggesting a protective effect on islet cells. To better detect insulin secretion from β-cells, pancreatic islets were immunostained (Figure 7A-b). The brown color of DAB was indicative of an insulin-positive signal, indirectly reflecting the active β-cell number. Compared to the vehicle group, the peptide group and MP group exhibited a deeper color and larger positive signal area. Through the distribution of the brown color, a tight organization was observed between β-cells in the Zn-PGLP-1 MP group. However, the vehicle

group did not show a compact β-cell distribution within the islet area. Combined with the HE results, we concluded that treatment with PGLP-1 can improve β-cell viability and inhibit islet cell apoptosis.[60,61] Administration of Zn-PGLP-1 microspheres similarly protected the islets compared to frequent PGLP-1 solution injection.

Some studies have reported that GLP-1 analogs have the insulinotropic function, which can promote insulin secretion and decrease blood glucose levels.[62] STZ-induced mice were chosen because they exhibit β-cell damage and insulin shortage.[3] Sustained GLP-1 analog treatment can stimulate β-cell proliferation.[63] With increasing β-cell activity, insulin produced from β-cells also promotes lower blood glucose levels. The plasma insulin detected also supported this conclusion. As shown in Figure 7B, the plasma insulin levels of the groups treated with Zn-PGLP-1 MP and twice-daily PGLP-1 solution increased compared with the negative vehicle group. Moreover, there was a statistically significant difference ($P<0.05$) between the MP and normal saline groups and there was no significant difference between the MP and PGLP-1 solution treatment, indicating that the sustained release microspheres showed the same efficacy in promoting insulin secretion as the twice-daily PGLP-1 solution injection.

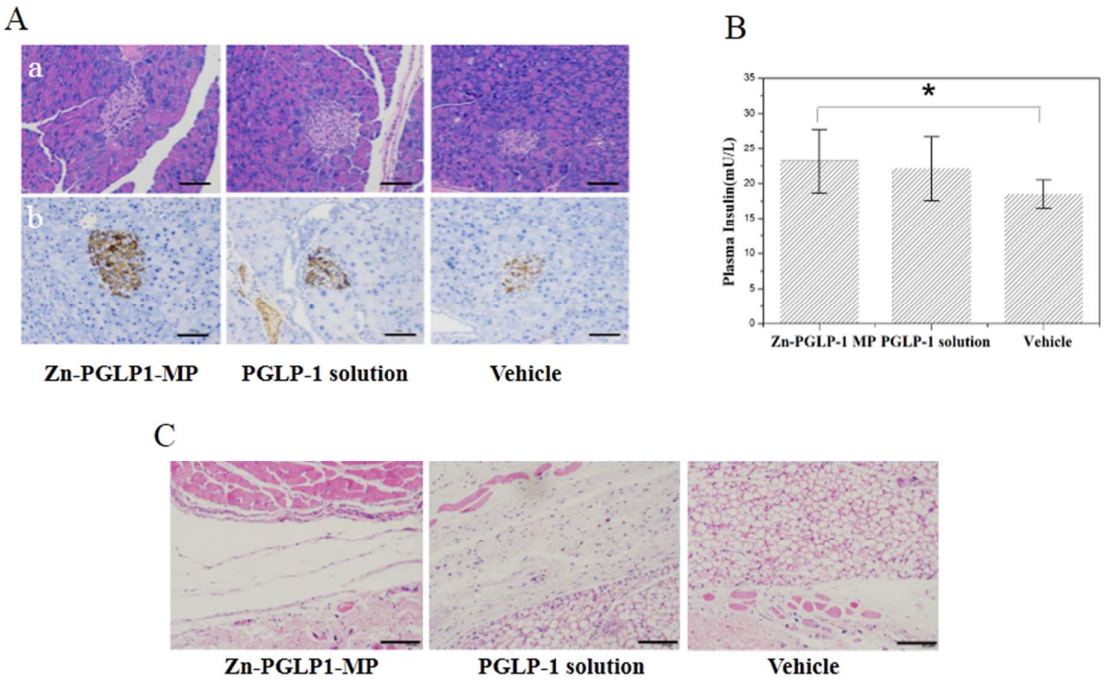


Figure 7. Pancreas morphology in STZ-induced hyperglycemic mice. After 6 week of subcutaneous treatment with Zn-PGLP-1 MP, PGLP-1 solution and vehicle, the pancreas and subcutaneous tissue of injection site were collect for histologic analyses. (A) HE staining and Insulin-immunostained of pancreas. Scale bar 100μm. (B) Plasma insulin level of mice. Scale bar 100μm. (C) HE staining of skin and muscle sections from the injection site. Scale bar 50μm. * P<0.05

3.3.4 Biocompatibility

To assess the biocompatibility of PLGA microspheres, subcutaneous tissue of the injection site was isolated and stained with hematoxylin and eosin. Although the FDA has approved PLGA as a safe material in clinical application, some studies reported an inflammatory response due to acidic oligomers degraded from PLGA.[30,59] Thus, the biocompatibility of the injection was evaluated. As shown in FigureS3, no skin protrusion at the injection sites was found after a six-week treatment in the long-term MP and peptide solution treatment groups. A mild inflammatory response was observed in the microspheres group with some monocyte and inflammatory cell infiltration (Figure 7C). For subcutaneous implants, biomaterials often lead to acute inflammatory reactions.[64] PLGA degradation produced an acidic microclimate in the subcutaneous tissue, which caused a foreign body reaction, but the effect was limited and would disappear over time.[59] However, tissue-injected PGLP-1solution and vehicle groups showed no obvious histopathological changes, indicating that frequent injection of the peptide solution did not damage the tissue around the injection site. In summary, Zn-PGLP-1 MP can be considered as a biocompatible and safe implant.

4. Discussion

4.1Microspheres with high EE/LD and minimal peptide bioactivity loss

To avoid the rapid elimination of PGLP-1 by DPP-IV, a long-acting GLP-1analog was designed through changing the amino acid sequence at restriction enzyme cut sites (Schematic 1A). As shown in Schematic 1B, the double emulsion method was used to prepare the microspheres, and regular spherical microspheres with higher encapsulation efficacy and drug loading were successfully

prepared. The pores on the surface provided channels for rapid release of peptide. PGLP-1 encapsulated in the microspheres was amorphous as evidenced by DSC and PXRD results. In addition, circular dichroism spectra confirmed that the release of peptide from MP exhibited no change in secondary structure compared with native PGLP-1 and no degradation peak was detected through HPLC analysis, indicating minimal bioactivity loss during the microsphere preparation.

4.2 Drug distribution and polymer degradation controlled in-vitro release

Drug release from microspheres always occurs in two stages.[38] The first stage (diffusion control) occurs as follows: when microspheres contact with aqueous fluid, water penetrates into the system and dissolves drugs so that the compound can easily diffuse through the pores on the surface.[65] During this period, the release rate is controlled by diffusion.[7] Based on the mechanism, a hypothesis has be proposed that the solubility of drug and the distribution inside MP are vital factors that influence the initial release.[15] In our study, water-soluble PGLP-1 and poorly water-soluble Zn-PGLP-1 complex were used to test this hypothesis. The results of CLSM revealed that PGLP-1 congregated near the surface of microspheres whereas uniformed peptide distribution was observed in Zn-PGLP-1 MP. The final initial release of PGLP-1 MP was 6-fold higher than Zn-PGLP-1 MP in pH7.4 medium, due to the varied solubility of the encapsulated compounds and different drug distributions as mentioned above. Stage two (erosion control) occurs as follows: ester hydrolysis of the polymer leads to decreased its molecular weight over time. The reduced T_g is attributed to a shortened polymer chain length. Along with polymer degradation over time, a porous structure forms inside the microspheres and the drug trapped in the inner core diffuses out through these channels.[53] In this study, the continuous decrease of T_g analyzed by DSC supports the phenomenon of polymer degradation. For both formulations (PGLP-1 MP and Zn-PGLP-1 MP) incubated at pH2.0, after an initial release, a rapid linear release was observed, caused by catalysis of hydrolytic proton processes.[29] It is well known that ester hydrolysis of PLGA accelerates in an acidic environment.[66]

During this period, the rate of release is determined by the degradation speed,[67] accounting for the different release behaviors in various pH media.

4.3 Long-acting hypoglycemic efficacy, protection of pancreatic function and improved insulin level

The acute glucose reduction test was performed in healthy mice to evaluate the sustained glucose control of Zn-PGLP-1 MP in vivo. After intraperitoneal injection of glucose, the blood glucose level (BGL) of mice increased immediately in the blank microspheres group. However, the BLG of the Zn-PGLP-1 MP group rebounded to a normal level within 20 minutes and maintained hypoglycemic efficacy for 2 weeks. These features further confirmed that the peptide released from MP maintained its bioactivity and two weeks of glycemic control was achieved.

The pharmacodynamics evaluation was tested using an STZ-induced mouse model. Biweekly microsphere injections exhibited the same hypoglycemic efficacy as the administration of the PGLP-1 solution. Additionally, there was no significant difference in BGL between the two treatment groups. Therefore, Zn-PGLP-1 loaded microspheres can be utilized as a long-acting formulation to treat diabetes with a decreasing number of injections.

HbA_1c is considered the gold standard to determine long-term glycemic control and can reflect the blood glucose for 1 to 2 months.[52,59] Compared to the negative control group, the $HbA_{1c}$was notably different in the Zn-PGLP-1 MP and PGLP-1 solution groups. This result was consistent with the BGL measurement. Although the blood glucose and HbA_{1c} did not rebound to normal levels, a significant decrease was observed in the sustained formulation and PGLP-1 treatment groups, reflecting the effective hypoglycemic action of microspheres.

To evaluate the β-cell function after long-term PGLP-1 treatment, the pancreatic tissues were stained with HE and immunostained with an insulin antibody. A compact pancreatic islet with a regular shape was observed in the Zn-PGLP-1 MP group. The insulin-positive signals of the

sustained PGLP-1 treatment groups were much stronger than the normal saline group, and this phenomenon was attributed to the efficacies of a GLP-1R agonist in β-cell protection and insulin secretion stimulation.[3,59] The effects of the GLP-1 analog were also demonstrated via the plasma insulin results, where the insulin levels were higher in the MP group and PGLP-1 solution groups, suggesting increased insulin production from residual β-cells. Overall, the improved β-cell function was monitored through the six-week treatment of the long-term formulation.

Conclusion

In conclusion, we successfully prepared biweekly injectable microspheres encapsulating an innovative GLP-1 analog (PGLP-1) which maintained the hypoglycemic efficacy of the original GLP-1 with a longer half-life. Zinc acetate was added to the PGLP-1 solution during the preparation to form a water-insoluble Zn-PGLP-1 complex. The release behavior of microspheres at different pH conditions was found to be strongly dependent on the solubility of encapsulated peptides. The Zn-PGLP-1 complex encapsulated microspheres exhibited the uniform peptide distribution with a steady release profile in the physiological environment, whereas the soluble native PGLP-1peptides were enriched at the surface of microspheres resulting in an undesirable initial burst release. After a 6-week treatment of Zn-PGLP-1loaded microspheres, a remarkable hypoglycemic effect was observed in the STZ-induced mice model. Furthermore, the administration of long-acting microspheres significantly lowered glycosylated hemoglobin and increased plasma insulin as a result of β-cell protection.

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Notes

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: xxxxxx

Mass losing of plain microspheres in phosphate buffer, histological and immunohistochemistry study of the group treated with large-dose PGLP-1 solution, pictures of mice dorsum and relevant skins at the administration site, energy-dispersive X-ray spectroscopy (EDS) analysis of Zn-PGLP-1 complex, and a table of solubility of Zn-PGLP-1 complex under different pH conditions.

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Table of Contents Graphic

Long-acting release microspheres containing novel GLP-1 analog as an antidiabetic system

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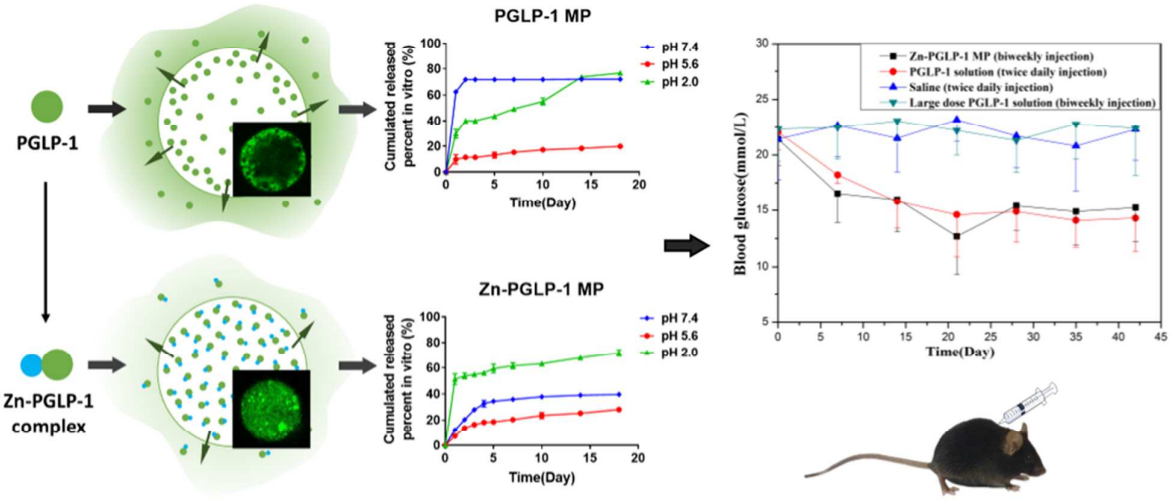
PGLP-1
Zn-PGLP-1
complex
PGLP-1 MP
Zn-PGLP-1 MP
Cumulated released percent in vitro (%)
Time(Day)
pH 7.4
pH 5.6
pH 2.0
Blood glucose(mmol/L)
Zn-PGLP-1 MP (biweekly injection)
PGLP-1 solution (twice daily injection)
Saline (twice daily injection)
Large dose PGLP-1 solution (biweekly injection)