Injectable and Thermosensitive Hydrogel Containing Liraglutide as a Long-Acting Antidiabetic System

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Supporting Information

ABSTRACT: Diabetes, a global epidemic, has become a serious threat to public health. The present study is aimed at constructing an injectable thermosensitive PEG−polyester hydrogel formulation of liraglutide (Lira), a “smart” antidiabetic polypeptide, in the long-acting treatment of type 2 diabetes mellitus. A total of three thermosensitive poly(ε-caprolactone-co-glycolic acid)-poly(ethylene glycol)-poly(ε-caprolactone-co-glycolic acid) (PCGA−PEG−PCGA) triblock copolymers with similar molecular weights but different ε-caprolactone-to-glycolide (CL-to-GA) ratios were synthesized. The polymer aqueous solutions exhibited free-flowing sols at room temperature and formed in situ hydrogels at body temperature. While the different bulk morphologies, stabilities of aqueous solutions, and the varying in vivo persistence time of hydrogels in ICR mice were found among the three copolymers, all of the Lira-loaded gel formulations exhibited a sustained drug release manner in vitro regardless of CL-to-GA ratios. The specimen with a powder form in the bulk state, a stable aqueous solution before heating, and an appropriate degradation rate in vivo was selected as the optimal carrier to evaluate the in vivo efficacy. A single injection of the optimal gel formulation showed a remarkable hypoglycemic efficacy up to 1 week in diabetic db/db mice. Furthermore, three successive administrations of this gel formulation within one month significantly lowered glycosylated hemoglobin and protected islets of db/db mice. As a result, a promising once-weekly delivery system of Lira was developed, which not only afforded long-term glycemic control but also significantly improved patient compliance.

KEYWORDS: liraglutide, type 2 diabetes mellitus, thermosensitive hydrogel, sustained drug release, in vivo degradation, hypoglycemic efficacy

1. INTRODUCTION

Diabetes mellitus, a chronic metabolic disease characterized by hyperglycemia and deterioration of pancreatic β-cell function, seriously challenges global public health. Although many antidiabetic agents such as insulin, sulphonylureas and metformin, can achieve well glycemic control, these drugs still suffer from various side effects and drawbacks like hypoglycemia, body weight gain and β-cell dysfunction. Recently, incretin-based therapies using glucagon-like peptide-1 (GLP-1) analogues or receptor agonists have drawn great attention.2−5 GLP-1 is an incretin hormone of 30 amino acid residues that can stimulate insulin secretion in a glucose-dependent manner and thus control the blood glucose without the risk of hypoglycemia.6 However, the rapid degradation of this hypoglycemic polypeptide via dipeptidyl peptidase IV (DPP-IV) results in a very short half-life (about 2 min) in human body, making it difficult for clinical application.5,6 To overcome this problem, some GLP-1 analogues or receptor agonists have been developed.7,8 Liraglutide (Lira), a novel palmityl-acylated derivative of GLP-1, contains an Arg34Lys substitution and a 16 carbon fatty acid attaching to Lys26. These structural modifications prevent the degradation by DPP-IV and prolong the half-life of Lira to about 13 h in plasma.9,10 Lira maintains the positive physiological activities of GLP-1 including reducing blood glucose, improving β-cell mass and function, decreasing insulin resistance, delaying gastric emptying, and lowering body weight gain.11−13 Particularly, insulin secretion stimulation in the glucose-dependent manner makes Lira with a minimal risk of hypoglycemia and less other side effects.14

The once-daily injection of Lira, also known as Victoza (Novo Nordisk), has been approved by the U.S. Food and Drug Administration (FDA) (2010) for glycemic control of adult patients with type 2 diabetes mellitus (T2DM). Its global sales have rapidly increased to $2700 million in 2015, and it is a promising treatment option for T2DM. Nevertheless, the frequent administrations of Lira negatively affect patients’ compliance. Therefore, a long-acting formulation of Lira with reduced frequency of administration is much desired and meaningful in clinical practice. In fact, it is challenging to achieve a formulation containing a high loading amount of Lira.
block copolymers are one of the most popular materials owing to their good biocompatibility, easy administration, and minimally invasive therapy. Among them, thermosensitive polyester and poly(ethylene glycol) (PEG)−poly(ε-caprolactone-co-glycolic acid) (PCGA−PEG−PCGA) triblock copolymers with similar MWs but different ε-caprolactone-to-glycolide (CL/GA) ratios. Their bulk properties and solution behavior in water were examined and compared. The in vitro release profiles of Lira were checked, and the in vivo degradation of the three hydrogels in ICR mice was also evaluated. Taking together the convenience of construction of Lira delivery system and the matching extent between the drug release profile and the degradation rate of carrier polymer, we confirmed the optimal Lira-loaded gel formulation and further detected its in vivo hypoglycemic efficacy upon a single subcutaneous injection into diabetic db/db mice, as illustrated in Figure 1. Finally, the glycosylated hemoglobin (HbA1c) and pancreatic islet morphology of db/db mice were also examined to assess the long-term glycemic control of animals after multiple subcutaneous injections of the optimal formulation.

2. MATERIALS AND METHODS

2.1. Materials. PEG with MW of 1500, ε-caprolactone, and stannous 2-ethyl-hexanoate (stannous octoate, 95%) were purchased from Sigma-Aldrich. Glycolide was the product of Purac (Netherlands). Liraglutide was supplied by Chinese Peptide Co., Ltd. (Hangzhou, China). Cyanine5.5 N-hydroxysuccinimide ester (Cy5.5-NHS) was obtained from Lumiprobe. Glycosylated hemoglobin assay kit was supplied by Nanjing Jiancheng Bioengineering Institute (Jiangsu, China). All of the chemicals were used without further purification.

2.2. Experimental Animals. ICR mice (male, 30 ± 2 g) were purchased from SLAC Laboratory Animal Co., Ltd. (Shanghai, China). Diabetic C57BLKS/J db/db mice (male, 6 weeks old) were obtained from Model Animal Research Center of Nanjing University (Jiangsu, China). All the animals were housed in cages under controlled temperature of 22–25 °C with a 12 h light−dark cycle. The mice had free access to standard laboratory Chow diet and tap water. All procedures concerning laboratory animals were in accordance with the approved guidelines of the “Principles of Laboratory Animal Care”
In vivo imaging was performed in ICR mice. The drug was labelled with Cy5.5 NHS at pH 8.4 with a molar ratio of 1:1. Unreacted dye was removed via dialysis, and Cy5.5-labeled Lira (Cy5.5-Lira) was obtained after lyophilization. Then, 0.2 mL of polymer aqueous solution (25 wt %) containing Cy5.5-Lira was subcutaneously injected into the backs of 10 ICR mice. The fluorescence of the whole body and isolated organs including liver, kidney, lung, spleen, and heart were monitored at 0, 2, 4, 7, and 10 days postinjection using an in vivo imaging system (In-Vivo Xtreme, Bruker) with an excitation wavelength of 690 nm and emission wavelength of 790 nm.

2.11. Hypoglycemic Efficacy in Nonfasted db/db Mice. The hypoglycemic efficacy of hydrogel formulation containing Lira was evaluated in nonfasted db/db mice. The polymer concentration of hydrogel was 25 wt %, and the drug-loading amount was 2 mg/mL. A total of 24 male db/db mice aged 8 weeks were randomly divided into three groups \( n = 8 \) per group. On the first day (defined as D0), the mice in the blank gel group received a single subcutaneous injection of polymer hydrogel without drugs at a dosage of 7.5 mL/kg body weight, the Lira/Gel group received a single subcutaneous injection of Lira-loaded gel formulation (15 mg/7.5 mL/kg). While the group of Lira Solution was served as a positive control, the mice were administrated by once-daily subcutaneous injection of Lira solution at a one-tenth dosage of the gel formulation (1 mg/75 mL/kg), and the total dosage of Lira solution was equal to that of Lira-loaded gel formulation during the 10 day experimental period. All of the mice were kept under nonfasted conditions with free access to food and water until the end of experiment. At 0, 4, 8, 12, 24, 48, 72, 96, 120,
Table 1. Composition Parameters of the Copolymers Synthesized in This Study

<table>
<thead>
<tr>
<th>sample</th>
<th>composition</th>
<th>$M_n$</th>
<th>CL/GA (mol/mol)$^a$</th>
<th>$M_w/b$</th>
<th>($M_w/M_n$)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>PCL—PEG—PCL</td>
<td>1900–1500–1900</td>
<td>–</td>
<td>8190</td>
<td>1.38</td>
</tr>
<tr>
<td>P2</td>
<td>PCGA—PEG—PCGA</td>
<td>1845–1500–1845</td>
<td>5:1</td>
<td>7640</td>
<td>1.35</td>
</tr>
<tr>
<td>P3</td>
<td>PCGA—PEG—PCGA</td>
<td>1850–1500–1850</td>
<td>1:1</td>
<td>7230</td>
<td>1.28</td>
</tr>
</tbody>
</table>

“The $M_n$ of the central block PEG was provided by Sigma-Aldrich. The $M_n$ of polyester block and molar ratios of CL and GA units were calculated by $^1$H NMR. $^b$Measured by GPC, relative to polystyrene standards.

144, 168, 192, 216, and 240 h post-administration, a drop of blood was obtained from the tail vein of each mouse to measure the blood glucose level using a blood glucose meter (AccuCheck Active, Roche Diagnostics). Subsequently, on day 10 and day 20 after the first administration of gel formulation, the mice in the blank gel and Lira/Gel groups received twice injections of blank gel or Lira-loaded gel formulation, respectively. Correspondingly, the mice in the group of Lira Solution received a once-daily injection of Lira solution in the next 20 days. In addition, body weights of all animals were measured every day at 9 a.m.

On day 30, blood samples of all the mice were collected from the eyes. Hba1c levels in plasma were determined by a glycosylated hemoglobin assay kit.

2.12. Histological and Immunohistochemistry Studies. After the animals were sacrificed by euthanasia, the pancreases of db/db mice were isolated and then fixed in 10% neutral buffered formalin. After being embedded in paraffin, the pancreatic tissues were sectioned by 4 μm and stained with HE for histological observation. Insulin immunohistochemistry was also performed to measure the proliferation of β cells in the pancreas.36,37 The tissues embedded in paraffin were sectioned by 4 μm. Endogenous peroxidase blocking process was performed after a 10 min pretreatment at room temperature. First, paraffin sections were treated with rabbit anti-insulin antibody (Cell Signaling Technology, Inc.) diluted at a ratio of 1/800 as the primary antibody at 4 °C overnight and then washed three times using PBS. Next, biotin-labeled secondary antibody (Maixin Biotech, China) was added, and the samples were incubated for 10 min at room temperature and then washed three times using PBS. Finally, sections were stained with 3,3′-diaminobenzidine (DAB, Maixin Biotech, China) and counter-stained with hematoxylin. Photomicrographs of all the slices were captured by a light microscope (ZEISS, Axiosvert 200).

2.13. Statistical Analysis. All of the results were expressed as mean ± standard deviation (SD). Comparisons between groups were analyzed by Student t test, and a p value of less than 0.05 was considered as a significant difference, unless otherwise indicated.

3. RESULTS

3.1. Synthesis and Characterization of Triblock Copolymers. The triblock copolymers were synthesized via ring-opening polymerization of CL and GA in the presence of PEG, catalyzed by stannous octoate. The obtained samples were characterized by $^1$H NMR and GPC. According to the previous protocols,36,57 the number-average MWs ($M_n$) and CL-to-GA ratios of the copolymers were determined via $^1$H NMR spectra, as shown in Figure S1. Herein, PCL—PEG—PCL triblock copolymer without GA segments was named as P1, while PCGA—PEG—PCGA triblock copolymers with CL-to-GA ratios of 5:1 and 1:1 were referred to as P2 and P3 samples, respectively. Their weight-average MWs ($M_w$) and MW distributions were further confirmed by GPC analysis, and a unimodal pattern with polydispersity index ($M_w/M_n$) of less than 1.40 was observed in the GPC traces for all the samples, as presented in Figure S2, suggesting that the polymerization was successfully performed and the desired products were synthesized. The detailed compositions of copolymers in this study are summarized in Table 1.

3.2. Thermal Properties and Aqueous Solution Stabilities of Copolymers. The differential scanning calorimetry (DSC) thermograms of copolymers with varying CL-to-GA ratios are shown in Figure 2a. Crystalline P1 copolymer presented a bimodal endothermic peak at 46 and 52 °C in the heating curve, which was assigned to the melting of PCL constituent followed by the melting of recrystallized PCL domain.31,42 Meanwhile, a weak melting peak at 24 °C in the heating curve was also observed, which were attributed to the PEG segment. The cooling process presented two exothermic peaks at 4 and 24 °C, which came from the crystallization of PEG and PCL, respectively. These findings were well coincided with similar PCL—PEG—PCL copolymers reported elsewhere.41,42 The incorporation of GA segment into PCL blocks greatly hindered the crystallization of PCL. Consequently, shifts of melting and crystallization peaks to lower temperature were observed in the P2 system. For example, the double melting peak of PCL segment shifted to 26 and 35 °C in the heating curve. Also, the crystallization peak of PCL was located at 2 °C, while that of PEG appeared at −23 °C in the cooling curve. In the case of P3 copolymer, neither melting peak nor crystallization peak was detected in both the heating and cooling cycles, suggesting the amorphous state of this copolymer. Nevertheless, a remarkable glass transition at −47 °C was observed during the heating process of P3. The detailed DSC data including $T_m$, $T_g$, $\Delta H_m$, $\Delta H_g$, and $T_f$ are summarized in Table S1. We also applied PEG1500 as a control. From the DSC data of PEG1500, P1, and P2, we further found that the introduction of PCL segments resulted in the decrease of $T_m$ and $T_f$ of PEG in P1 and P2 systems. The reason is that earlier crystallization of the PCL segment freezes the whole structure and hinders the crystallization process of PEG block.41

The XRD measurements were also performed to confirm the crystalline behavior of the three copolymers. As shown in Figure 2b, the XRD pattern of P1 copolymer exhibited two strong diffraction peaks at 21.4° and 23.8°, which came from the crystallization of the PCL segments. Meanwhile, a weak diffraction peak at 19.4° was also observed, which was
attributed to the crystallization of the PEG domain.\textsuperscript{33} While P2 copolymer presented a similar pattern of P1, the peak intensities were lower than those of P1, reflecting the decrease of crystallization due to the incorporation of GA units. No crystallization peak was found in P3 system, demonstrating its amorphous state. These features are well consistent with the DSC results.

The morphologies of the three copolymers in the bulk state are presented in Figure 3a. Both P1 and P2 specimens exhibited powder forms, enabling convenient preparation of their aqueous solutions. In contrast, P3 just showed a sticky paste state in bulk, which was difficult to weigh, transfer, and handle, and continuous stirring over 1 day was required to dissolve the viscous polymers in water. Figure 3b displays their aqueous solutions with polymer concentration of 25 wt %. After being stored in a refrigerator at 4 °C overnight, the copolymer aqueous solution of P1 spontaneously formed an opaque gel because of crystallization, as presented in Figure 3c. In comparison, P2 and P3 solutions remained the free-flow sol states, even after several days of storage.

3.3. Sol–Gel Transitions of Copolymers in Water. The three specimens were dissolved in water at ambient temperature and formed in situ hydrogels as the temperature increased. Figure 4 shows their phase diagrams in PBS (pH 7.4). P1 system exhibited a smaller critical gelation concentration (CGC) (about 6 wt %) compared with those of P2 and P3 (about 12 wt %). The formation of a percolation micellar network via micellar aggregation was responsible for the temperature-induced gelation of polyester–PEG–polyester copolymers in water.\textsuperscript{35} The micellar reorganization and aggregation of the PCGA–PEG–PCGA system with the incorporation of GA segment may become difficult compared with the PCL–PEG–PCL system. As a result, a higher CGC was needed for the PCGA–PEG–PCGA hydrogel. In contrast, the sol–gel transition temperatures of P1 copolymer solutions were higher than those of P2 and P3. For example, the sol–gel transition temperatures of P1, P2, and P3 copolymer solutions at the same concentration of 25 wt % were 36, 30, and 28 °C, respectively. This feature indicates that the sol–gel transitions of polyester–PEG–polyester systems may be influenced by many factors, such as molecular structure, hydrophobicity and crystallization tendency of polyester block, etc. The intrinsic reasons for differences in sol–gel transition temperatures among the three systems with different polyester compositions are still open, and further studies are required. Luckily, the gel regions covered body temperature (37 °C) for all the three copolymer/water systems, indicating that these injectable thermosensitive hydrogels with proper concentrations are suitable for biomedical applications.

3.4. In Vivo Degradation of Hydrogels. In vivo degradation of all the three thermosensitive hydrogels was examined after subcutaneous injection into ICR mice. Some representative images are shown in Figure 5. The copolymer aqueous solutions spontaneously transformed into in situ physical hydrogels within half a minute after injection. The opaque gels with spherical morphology were observed at day 1 postinjection, and the size of the three gels gradually decreased following the hydrolysis of polyester segments. P1 gel maintained its integrity over 56 days, indicating the very slow degradation rate; P2 gel was eliminated from the mice for no more than 4 weeks; P3 gel exhibited the fastest degradation rate and disappeared within 3 weeks. These results indicate that the in vivo degradation rate of hydrogels may be modulated by the GA contents in the copolymers.

The inflammatory response of tissues surrounded the hydrogels at different time points was also evaluated by histological observation. Figure 6 shows the optical micrographs of HE-stained slices of surrounding connective and muscular tissues after subcutaneous injection of P2 hydrogel into ICR mice. Similar to other thermosensitive PEG–polyester hydrogels,\textsuperscript{41–46} P2 hydrogel induced an acute inflammation response in the first week and a number of inflammatory cells such as lymphocytes and macrophage infused into the surrounding tissue (Figure 6b,c). As the time went on, the acute inflammation gradually turned into a mild chronic inflammation, as evidenced by significant reduction of inflammatory cells at 3 weeks postinjection (Figure 6e), and almost no inflammatory cell was observed after the complete degradation of hydrogel at day 28 (Figure 6f), suggesting that the affected region was restored to the normal state. Besides, no sign of tissue necrosis, hemorrhaging, hyperemia, or edema was observed at the administration site during the whole period of examination. These features demonstrated that these PEG–
polyester hydrogels have acceptable biocompatibility as implantable biomaterials.

3.5. In Vitro Release of Lira. In vitro release profiles of Lira from the hydrogels were investigated, and the results are presented in Figure 7. All the three Lira-loaded gel systems exhibited a similar sustained release manner up to 10 days, and the cumulative release amounts reached about 70−80%. The release data were further fitted via the Higuchi equation, \( Q = kt^{1/2} \) \( (Q < 0.6) \), where \( Q \) is the cumulative release amount, \( t \) is release time, and \( k \) is a constant.47 All of the release data well matched with the Higuchi equation with \( R^2 > 0.99 \) (Table S2), indicating that the diffusion-controlled mechanism governed the release of drug from these hydrogel matrices.

3.6. In Vivo Imaging of Lira from the P2 Hydrogel System. In vivo fluorescence imaging of Lira was carried out to monitor the release behavior of Lira from hydrogel in ICR mice. Considering both the convenience of administration and the matched degradation rate, we selected Lira-loaded P2 hydrogel formulation as the optimal drug delivery system. The drug was first labeled by Cy5.5, and then the P2 polymer solution containing Cy5.5-Lira was subcutaneously injected into the backs of ICR mice. As shown in Figure 8a, the fluorescence of Cy5.5-Lira was clearly observed at injection site, and the intensity gradually diminished within the next 10 days, reflecting the sustained release of drug out of the gel matrix. As is well-known, the widely distributed endogenous enzymes, like DPP-IV, in multiple organs and tissues are involved in the in vivo degradation of Lira after subcutaneous injection.4,48 Hence, ex vivo imaging of the major organs was also performed to track the distribution of degradation products of Lira. The fluorescence was observed in both livers and kidneys, as shown in Figure 8b, which was accounted for the degradation segments of released Cy5.5-Lira, and they were further eliminated by glomerular filtration.

3.7. In Vivo Hypoglycemic Efficacy. Hypoglycemic efficacy of Lira-loaded P2 gel formulation was evaluated in nonfasted diabetic db/db mice. The mice receiving a single injection of blank gel without drug maintained high blood-glucose levels during the whole experimental period. According to Figure 9, glucose-lowering effects were achieved by once-

![Figure 5](image-url) In vivo degradation behavior of the indicated thermosensitive hydrogels in ICR mice. The photographs were taken at predetermined time after subcutaneous injections of the indicated gels. “D” denotes “day”.

![Figure 6](image-url) Optical micrographs of HE-stained slices of surrounding tissues: (a) normal control and at (b) 1 day, (c) 7 days, (d) 14 days, (e) 21 days, and (f) 28 days after subcutaneous injection of P2 hydrogel (25 wt %) into ICR mice. S: skin tissue; M: muscle tissue; F: fibrous connective tissue; G: hydrogel. Scale bar: 500 μm.

![Figure 7](image-url) In vitro release profiles of Lira from the indicated hydrogels. Copolymer concentration was 25 wt %, and the drug-loading amount was 2 mg/mL. Data are presented as mean ± SD \( n = 3 \) for each group.
The daily administration of Lira solution in mice. The blood glucose levels of mice were also effectively lowered after a single subcutaneous administration of the Lira-loaded gel formulation. Its hypoglycemic efficacy was not significantly different from that achieved by once-daily injection of Lira solution in the first 7 days. Meanwhile, a lower blood glucose level was observed even at day 10 postinjection compared with that of the blank gel group.

The mice were also weighed everyday morning. As shown in Figure 10, the body weights of mice in the blank gel group steadily increased over time-scale. In contrast, the body weights of mice greatly decreased after the administration of Lira solution or Lira-loaded gel formulation. Compared with the group of Lira solution, a relatively more-remarkable reduction of body weights was observed in the group of Lira/Gel formulation during the whole experimental period, indicating that the more-steady blood drug concentration achieved by the sustained release of Lira provided a higher efficiency of gastric emptying delay and appetite reduction.

3.8. Long-Term Glycemic Control. To evaluate the long-term glycemic control of the Lira-loaded gel formulation in model animals, two subsequent administrations of Lira-loaded gel formulation were carried out on day 10 and day 20 after the first treatment. After 1 month, the mice were sacrificed, and their blood was collected from their eyes. The HbA1c levels in the plasma was detected by a glycosylated hemoglobin assay kit. HbA1c comes from irreversible glycation of hemoglobin and reflects a total condition of pilot of the plasma glucose over a long time. Hence, HbA1c is acknowledged as a reliable index for the long-term glycemic control.50 In this study, compared with levels in the blank gel group, a significant decrease of HbA1c was observed in those mice who received the treatment of Lira solution or Lira-loaded gel formulation (Figure 11). Meanwhile, there was no significant difference of HbA1c between the Lira solution and Lira/Gel formulation groups, indicating that the efficacy of the Lira-loaded gel formulation is equal to that of a once-daily injection of Lira solution in long-term glycemic control.

3.9. Histological and Immunohistochemistry Examination. To examine the effects of the Lira-loaded gel formulation on the islet morphology and β-cell function, HE staining and immunocytochemical staining of pancreatic tissues isolated from animals were performed. As shown in Figure 12a, the islet of mice in the blank gel group exhibited boundary definition loss and degeneration, whereas the morphology of islet of mice receiving the treatment with Lira solution or with the Lira-loaded gel formulation was normal, which presented a round or elliptical shape with a distinct boundary and tight organization between islet cells, suggesting the sustained proliferation of β cells following administration of Lira. In addition, the mice pancreatic tissues were immunostained for...
insulin to detect β cells. Representative section images are presented in Figure 12b. The insulin-positive signals with brown color of DAB means the insulin secretion in islets and thus directly reflect the existence of islet β cells.39,40 The DAB-stained intensity in the blank gel group was weak, indicating the loss of insulin-producing β cells in the pancreas islet. In contrast, the staining intensities in both the Lira solution and the Lira/Gel groups were greatly higher than that in the blank gel group, implying the prevention of β cells loss in pancreas islet after the treatment of Lira. Combining with the results of HE staining and insulin immunostaining, we confirmed that both the Lira-loaded gel formulation and Lira solution can protect the pancreases and improve the pancreatic function of db/db mice.

4. DISCUSSION

Lira, a fatty acid derivative of GLP-1, is designed for once-daily injection in treatment of T2DM because of its half-life of 13 h.9,10 For the reduction of injection frequency and improvement of patients’ compliance, developing long-acting Lira formulations is very meaningful and important in clinics but challenging in pharmaceutics and material science. It is well-known that high-dose administration of antidiabetic agents such as insulin may cause severe hypoglycemic shock and even death. Unlike insulin, Lira is capable of stimulating insulin secretion in a blood-glucose-dependent manner, and this feature makes it suitable for sustained delivery to achieve long-acting glycemic control without the hypoglycemic risk.

In the past decade, injectable thermosensitive PEG–polyester copolymers hydrogels have been extensively suggested as promising carriers for the delivery of various protein and polypeptide drugs in the treatment of different diseases.45,47,50,51 In this study, a series of thermosensitive PCGA–PEG–PCGA triblock copolymers with different CL-to-GA ratios were designed and synthesized and then utilized to encapsulate and deliver Lira. To achieve an ideal delivery system of Lira, the following three points should be taken into account and satisfied: (1) it is easy to construct and handle such a delivery system; (2) the delivery system can realize a sustained drug release; (3) the carrier polymer has an appropriate degradation rate and thus matches with the release period of drugs to avoid the accumulation of polymer residues after repeated administrations.

Easy-to-Handle Carrier Polymers and Proper in Vivo Persistence Time of Hydrogels. Among the thermosensitive polymers synthesized by us, PCL–PEG–PCL triblock copolymers (P1) presented a powder form in bulk. However, its aqueous solution was unstable at room temperature or lower and spontaneously formed an opaque gel within a few of hours due to the crystallization of PCL segments. In addition, the crystallization of PCL blocks led to a very low degradation rate of the hydrogel in vivo, as displayed in Figure 5. The incorporation of GA segments effectively hindered the regular crystallization of PCL blocks as evidenced by DSC and XRD analysis (Figure 2). In the case of P3 with a high GA content, neither the melting/crystallization peak in DSC nor the diffraction peak in XRD was observed, indicating that the polymer lost the ability of crystallization and just exhibited an amorphous state. As a result, bulk P3 just showed a sticky paste, resulting in the difficulty in handling, weighing, and transferring. P2 copolymer not only kept a powder form in bulk but also exhibited a stable solution state after dissolution in water (Figure 3), which was convenient to handle and store. This feature was attributed to an adequate crystallization ability of the triblock copolymer achieved by the appropriate CL-to-GA ratio. Meanwhile, the degradation of these polymer hydrogels mainly depends on the hydrolysis of polyester segments, and the introduction of GA units significantly accelerated the degradation of hydrogels in vivo due to both the destruction of crystallization of PCL blocks and the higher hydrolysis rate of GA segments.12 Consequently, the in vivo persistence time of P2 gel reduced to 3–4 weeks, while the in vivo integrity of P3 system was not more than 3 weeks (Figure 5). Their final degradation fragments are glycolic acid, 1,6-hydroxyacaproic acid, and nondegradable PEG, which are nontoxic and easily cleared from body.45,46,53,54

Convenient Loading and Sustained Release of Drugs. All of the polymer/water systems underwent sol–gel transitions as the temperature increased and formed nonflowing physical hydrogels at body temperature (Figure 4), indicating that they can be used as minimally invasive injectable carriers for sustained drug delivery. Meanwhile, the feature of sol–gel transition enabled convenient encapsulation of Lira at low temperature without any loss, and this process was free of any organic solvent. These advantages were beneficial in avoiding the degradation or denaturation of loaded Lira.

In vitro release tests demonstrated that all the Lira-loaded gel formulations exhibited a relatively slow and consistent release rate of drug during the 10 day examination period, and no significant difference among them was observed (Figure 7). The similar release profiles revealed that the introduction of GA...
into PCL block had no significant influence on the drug release behavior. Furthermore, we confirmed that diffusion-controlled mechanism governed the release of Lira from the hydrogel depot. Such a sustained drug release manner was attributed to the hydrophobic interaction of the drug and carrier polymers due to the amphiphilic nature of both Lira polypeptide and polyester−PEG−polyester copolymers, or, to be more precise, the hydrophobic interaction between C16 side chain of Lira and polyester segments of carrier polymers resulted in the sustained release of drug from the hydrogel matrix. In contrast, hydrophilic polypeptides, such as insulin and exenatide, just displayed a high burst effect and a short release duration from the similar thermosensitive hydrogels owing to absence of such a hydrophobic interaction between drugs and carriers.47,50,55 It is worth pointing out that this question is still open, and thus, further studies are needed to provide more direct evidence. In addition, dynamic rheological measurement demonstrated that the introduction of Lira into P2 aqueous solution had no obvious influence on the sol−gel transition temperature of hydrogel system and its injectability yet resulted in a certain degree of increase in both storage modulus G′ and complex viscosity η of the hydrogel formulation, as displayed in Figure S3.

Taken together with the convenience of administration of carrier polymers and the in vivo integrity maintenance of gels, we finally selected P2 copolymer as the optimal carrier polymer to perform the in vivo evaluation while minimizing the number of animals used. Lira was labeled by Cy5.5 fluorescent dye and then loaded into P2 gel to noninvasively track the in vivo release of drug from the optimal Lira formulation. The in vivo imaging in mice showed that the sustained release of drug from P2 gel lasted for more than 10 days (Figure 8), which was coincidence with the in vitro release profile.

**Long-Term Glycemic Control, Effective Body Weight Reduction, and Enhanced Pancreatic Function.** The mutant mouse, C57BLKS/J db/db, is able to develop spontaneously diabetes, and its phenotypes are similar to those of T2DM patients.36 Thus, we choose this animal model for the in vivo evaluation of hypoglycemic efficacy. After a single administration in diabetic db/db mice, the Lira-loaded P2 gel formulation achieved a week-long glycemic control in normal levels (Figure 9). Subsequently, the blood glucose of mice in the Lira/Gel group gradually rebounded to the high level, but it was still significantly lower than that of the blank gel group on day 10 postinjection. These features were consistent with both the in vitro release profile and the in vivo imaging results and attributed to the continuous liberation of drug from the gel by diffusion. The change in body weight of mice over time was also recorded (Figure 10). The moderate reduction of body weight in db/db mice was achieved by the administration of Lira solution or the gel formulation, which was very helpful for the T2DM patients with obesity. In fact, the once-daily injection of Lira (Saxenda) has been approved by FDA in 2014 for body-weight management.37 Therefore, our Lira-loaded gel formulation may also serve as a promising long-acting formulation for antiobesity. Moreover, the sustained in vivo hypoglycemic efficacy and effective body weight reduction further confirmed that released Lira was bioactive and did not suffer from the degradation or denaturation in the gel matrix.

HbA1c comes from irreversible glycation of hemoglobin and is not influenced by temporary blood glucose fluctuation. Thus, it has been acknowledged as a golden standard for monitoring of average glycemic control over the previous several months.58 Considering that the interval of reliable detection of HbA1c usually requires at least one month in humans, we prolonged the experimental duration to 30 days through successive administrations of two more gel formulations to mice. The results showed that the HbA1c levels in both the groups of Lira solution and Lira/Gel were significantly lower than that of the blank gel group, inflecting the efficient glycemic control of mice within the one-month experimental period. Meanwhile, there was no significant difference of HbA1c between the two treatment groups (Figure 11). These findings indicate that our long-acting formulation can achieve a comparable long-term glycemic control with once-daily administration of Lira solution, whereas it is more convenient for patients with greatly reduced frequency of injection.

It is well-known that β-cell function progressively deteriorates in patients with T2DM.59 Therefore, it is very important to preserve β-cell function in pancreatic islets in treatment of T2DM. We performed histological observations to evaluate the β-cell function after administration of our Lira formulation for one month (Figure 12). The results of histological examinations showed that the treatment of Lira-loaded gel formulation not only promoted the proliferation of islet cells but also improved the β-cell function in db/db mice. This feature coincides well with the pharmacology of Lira as reviewed in the previous literature.11,13

5. CONCLUSIONS

In this study, we successfully developed an expected once-weekly injectable hydrogel formulation of Lira. A total of three thermosensitive PCGA−PEG−PCGA triblock copolymers with different CL-to-GA ratios were designed and synthesized. The PCGA−PEG−PCGA copolymer with a CL-to-GA ratio of 5:1 exhibited a powder form in bulk and maintained a stable aqueous solution before heating, which is easy to handle and store. Meanwhile, the proper degradation rate in vivo of this gel is able to meet with the requirement of repeated administrations in treatment of T2DM patients. A single injection of such an optimal Lira-loaded hydrogel formulation showed a remarkably hypoglycemic efficacy, up to 1 week in diabetic db/db mice. Furthermore, multiple administrations of this formulation significantly lowered HbA1c and protected islets of db/db mice. Hence, a once-weekly injectable hydrogel formulation of antidiabetic liraglutide with great improvement of patient compliance was achieved, and this formulation has great potential for repeated administrations in T2DM patients in a long-term glycemic control.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.6b09415

1H NMR spectra and GPC chromatograms of copolymers, rheological studies of the copolymer aqueous solutions, thermal properties of the copolymers measured by DSC, and in vitro release kinetic assessment. (PDF).

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