In Vitro and *In Vivo* Evaluation of a Once-weekly Formulation of an Antidiabetic Peptide Drug Exenatide in an Injectable Thermogel

LIN YU,[1] KUN LI,[2] XIAOJUN LIU,[2] CHANG CHEN,[1] YONGCHU BAO,[1,2] TIANYUAN CI,[1] QINGHUA CHEN,[1] JIANDONG DING[1,3]

[1]State Key Laboratory of Molecular Engineering of Polymers, Department of Macromolecular Science, Fudan University, Shanghai 200433, China
[2]National Pharmaceutical Engineering Research Center, China State Institute of Pharmaceutical Industry, Shanghai 200437, China
[3]Key Laboratory of Smart Drug Delivery of Ministry of Education, School of Pharmacy, Fudan University, Shanghai 201203, China

Received 8 July 2013; revised 15 August 2013; accepted 3 September 2013

Published online 24 September 2013 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jps.23735

ABSTRACT: An injectable thermogel composed of poly(lactic acid-*co*-glycolic acid)–poly(ethylene glycol)–poly(lactic acid-*co*-glycolic acid) (PLGA–PEG–PLGA) triblock copolymers was evaluated as the matrix of a long-acting drug delivery system of exenatide (EXT), an antidiabetic peptide. The optimal gel formulation containing 2 mg/mL EXT and three pharmaceutical excipients (1.25 wt % zinc acetate, 5 wt % PEG200, and 5 wt % sucrose) was injected subcutaneously, and its pharmacokinetics was investigated. Both *in vitro* and *in vivo* release profiles exhibited a sustained release of EXT over 1 week. After a subcutaneous injection of the EXT formulation into db/db mice, the blood glucose level was maintained in a normal range up to 7 days and meanwhile the growth of body weight was suppressed. The *in vivo* results were consistent with the *in vitro* EXT-release profile. Moreover, twice injections of the gel formulation resulted in the higher blood insulin level and lower plasma concentration of glycosylated hemoglobin compared with twice-daily injections of an EXT solution for 18 days. Histological observations manifested the protection of islet due to administration of the gel formulation. Therefore, the PLGA–PEG–PLGA thermogel provided an excellent candidate for a once-weekly delivery system of EXT, and the optimal EXT formulation not only afforded therapeutic efficacy but also improved patient compliance. © 2013 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 102:4140–4149, 2013

Keywords: hydrogels; peptide delivery; exenatide; type II diabetes; db/db mice; biodegradable polymers; *in vivo*/*in vitro* correlations; pharmacokinetics; pharmacodynamics

INTRODUCTION

Glucagon-like peptide-1 (GLP-1), a hormone of 30 amino-acid residues, can stimulate insulin secretion in a glucose-dependent manner.[1] Because of its glucose-induced insulinotropic effect, GLP-1 controls hyperglycemia of type II diabetic patients without the risk of hypoglycemia. The bottleneck of its clinical application is the extremely short half-life in circulation (<2 min) owing to the rapid degradation by a ubiquitously expressed enzyme, dipeptidyl peptidase IV (DPP-IV).[1,2] This problem is fortunately resolved by the emergence of GLP-1 receptor agonists, which can resist the DPP-IV-induced degradation significantly.[2,3]

Exendin-4, a polypeptide of 39 amino-acid residues, is the most efficient and safe GLP-1 receptor agonist so far.[2,3] Although it shares approximately 53% sequence homology with GLP-1, the site of the proteolytic cleavage by DPP-4 is avoided.[2,3] Glucoregulatory actions of exendin-4 include enhancement of glucose-stimulated insulin secretion, inhibition of gastric emptying, and reduction of appetite. Moreover, exendin-4 can promote β-cell proliferation and consequently improve islet function.[2–5] The potency of the glycemic control of exendin-4 is 5000-fold of that of GLP-1.[3]

Exendin-4 was originally isolated from the salivary gland of the lizard Heloderma Suspectum (Glia monster).[6] Its synthetic product exenatide (EXT) and relevant solution injection (Byetta®) have been approved by both United States Food and Drug Administration (2005) and European Medicines Agency (2006) as a medication in treatment of patients with type II diabetes mellitus. Byetta® is administrated by twice-daily subcutaneous injection. A long-acting microsphere formulation of EXT (Bydureon®) was also developed and approved by both European Union (2011) and United States (2012) as a once-weekly injectable delivery system. Bydureon® shares many advantages such as reduced administration frequency, stable plasma drug level, and better patient compliance.[7,8] Nevertheless, a microsphere formulation often suffers from difficulties such as preparative complication, residual organic solvent, and high cost in sterilization. It is thus helpful for developing more formulation ways of EXT, for instance, a convenient implantation-type formulation.

The present paper concerns a subcutaneously injectable formulation in the form of hydrogels. Hydrogels have been investigated extensively for various biomedical applications.[9–17] Especially, injectable and biodegradable thermogels are promising as an implantation-type sustained-release carrier of bioactive proteins, peptides and other drugs.[18–24] Such a system is a low viscous solution at ambient temperature and undergoes a sol–gel transition upon heating. The drug is easily entrapped by mixing the aqueous polymer solution at room temperature without using any organic solvent. The polymer solution containing drugs is then readily injected using conventional syringe needles, and rapidly gelled at the physiological

Correspondence to: Qinghua Chen (Telephone: +86-021-55514600-135; Fax: +86-021-65420806; E-mail: chen-qh2@163.com); Jiandong Ding (Telephone: +86-021-65643506; Fax: +86-021-65640293; E-mail: jdding1@fudan.edu.cn)

Lin Yu and Kun Li contributed equally to this work.

This article contains supplementary material available from the authors upon request or via the Internet at http://onlinelibrary.wiley.com/.

Journal of Pharmaceutical Sciences, Vol. 102, 4140–4149 (2013)

temperature, resulting in an automatic encapsulation of the drugs. Among all of these systems, the thermogelling triblock copolymer poly(lactic acid-*co*-glycolic acid)–poly(ethylene glycol)–poly(lactic acid-*co*-glycolic acid) (PLGA–PEG–PLGA) may be one of the most promising candidates for the clinical application due to its convenient synthesis, good profile of safety and adjustable properties.[25–30]

Recently, the first case of the thermogel formulation of EXT has been reported by us.[31] A "mixture hydrogel" composed of two PLGA–PEG–PLGA polymers yet with different block lengths was used as the delivery matrix. One polymer is too hydrophobic to dissolve in water, and the other is too hydrophilic to form a hydrogel at any concentration and temperature. Interestingly, the mixture with appropriate compositions could be soluble in water and form a physical hydrogel upon increase of temperature due to a subtle balance between hydrophilicity and hydrophobility when the polymer concentration is higher than the critical gel concentration (CGC).[32,33] By this way, the applicable window of the amphiphilic block copolymers has been broadened to a large extent, and the sol–gel transition temperature T_{gel} could be easily adjusted by mix ratio to satisfy the medical requirements. Meanwhile, to suppress the burst effect of EXT and keep a uniform drug release from the gel, we introduced three excipients, zinc acetate, PEG, and sucrose into the formulation, and found surprisingly that the release profile of this water-soluble peptide could be significantly improved under appropriate conditions due to a synergetic effect.[31] Some key issues of this synergetic "mixture thermogel" still open, for example, more factors to modulate the *in vitro* release profiles, the *in vivo* release and its correlation with the *in vitro* one, the therapeutic efficacy in diabetic mammals. To answer these questions is vital for developing this promising antidiabetic formulation and very meaningful for shedding new insight of pharmaceutics concerning polypeptide drugs and/or physical hydrogels.

The present study is mainly emphasized on the pharmacokinetics (PK) and pharmacodynamics (PD) studies of the thermogel formulation of EXT, as schematically presented in Figure 1. The total copolymer concentration and drug amount will be examined as a regulator of the *in vitro* release profile of EXT in this study. The *in vivo* release will also be checked for the first time, and a comparison between the *in vivo* and *in vitro* profiles of the formulation with optimal condition will be made via transformation of the transient blood drug concentration into the cumulative drug release *in vivo*. db/db mice of type II diabetes will be employed to assess the therapeutic efficacy for 10 days (a bit longer than one week) upon a single subcutaneous injection of the optimal formulation. In addition, the plasma insulin level, concentration of glycosylated hemoglobin (HbA1c), and histological morphology of pancreatic islet will be examined after twice subcutaneous injections of the long-acting EXT thermogel formulation and compared with that of twice-daily subcutaneous injections of an EXT solution for 18 days.

MATERIALS AND METHODS

Materials

PEG [molecular weight (MW) 200, 1000, and 1500] and tin ethyl hexanoate (stannous octoate, 95%) were obtained from Sigma–Aldrich (USA) . D,L-lactide (LA) and glycolide (GA) were purchased from Purac (The Netherlands). Sucrose and zinc acetate were products of Sinopharm Chemical Reagent Company Ltd. (Shanghai, China). EXT was kindly provided via the Hangzhou Jiuyuan Gene Engineering Company, Ltd. (Hangzhou, China). All of chemicals were used without further purification. Two PLGA–PEG–PLGA triblock copolymers were synthesized as described previously.[31]

Animals

Male Sprague–Dawley (SD) rats were provided from Shanghai Super-B&K Laboratory Animal Corporation Ltd. (Shanghai, China). db/db mice with body weight 50 ± 5 g were purchased from SLAC Laboratory Animal Corporation Ltd. (Shanghai, China). The animals were housed under a 12-h light/dark cycle with free access to food and water during the acclimatization period. All procedures concerning experimental animals obeyed the principles outlined in the Declaration of Helsinki and have been approved by the ethics review board of Novo Nordisk.

Viscosity Measurements of Polymer Solutions and Hydrogels

Aqueous PLGA–PEG–PLGA solutions with 1/1 weight mix ratio of copolymer-1/copolymer-2 were prepared via dissolving polymer in deionized water. The viscosity measurements of aqueous polymer solutions as a function of temperature were

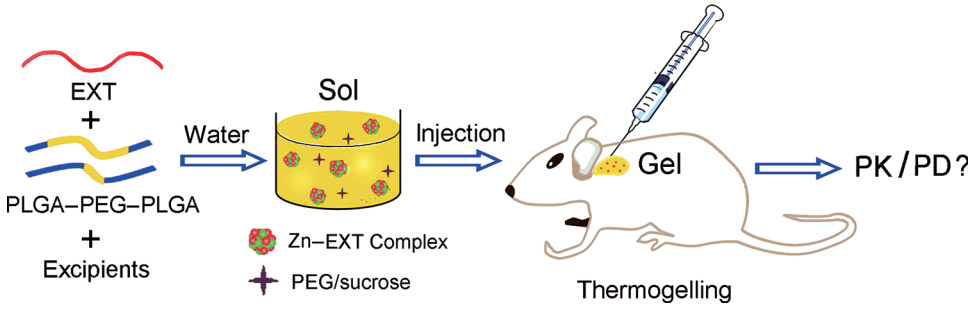

Figure 1. Schematic illustration of the present study of a hydrogel formulation of the peptide drug EXT. EXT with the amino acid sequence NH_2-HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPPPS-$CONH_2$ is soluble in water. Two amphiphilic copolymers composed of one hydrophilic block of PEG and two hydrophobic blocks of PLGA will be used with one copolymer dissolved in water and the other precipitated, yet their mixture is soluble in water and exhibits a sol–gel transition upon increase of temperature. The optimal EXT gel formulation contains three excipients (zinc acetate, PEG200 and sucrose). The formation of Zn-EXT complex is beneficial for reducing the initial burst release and the leaching of PEG200 and sucrose accelerates the late-stage release of EXT. Both PK and PD will be investigated after subcutaneous injections.

carried out on a strain-controlled rheometer (ARES Rheolometric Scientific) using a Couette cell (Couette diameter, 32.0 and 34.0 mm; height 33.3 mm).[34] The measurements were operated with an oscillatory frequency of 10 rad/s and a heating rate of 0.5°C/min between 20°C and 45°C.

In Vivo Gel Formation

The experiments of *in vivo* gel formation and degradation were performed using male SD rats (body weight 180 ± 20 g). The anesthesia of animals was induced via an intramuscular injection of a 3% sodium pentobarbital solution. Then, 400 μL of the 1/1 polymeric mixture system with a total concentration 25 wt % was subcutaneously injected into the neck of rats. At designated time points, the animals were euthanized, and the injection sites were carefully dissected. The photographs of the in situ-formed gels were obtained using a camera.

In Vitro Release Study

The Zn–EXT complex was prepared by adding zinc acetate into an EXT solution. After incubating at 4 °C for 24 h, the sample was freeze-dried. The drug-loading polymer solutions were obtained by mixing the polymer solutions with PEG200, sucrose, and Zn–EXT powder.[31] Each vial (10-mL volume, 14 mm inner diameter) containing 0.5 mL sample was incubated in a water bath at 37°C for 10 min. After the formation of hydrogel, phosphate buffer saline (PBS, pH 7.4, 10 mL) containing 0.02% sodium azide (aseptic agent) was added as the release medium. The shaking rate of the water bath was set at 50 rpm. The remaining gels were collected and the drugs in the gel matrix was determined via high-performance liquid chromatography (Shimadzu SCL-20Avp liquid chromatograph) using a C_4 column (Sepax, Newark, Delaware).[31] Mobile phase A was a mixture of PBS (pH 2.0, 85 mmol) and acetonitrile (95:5, v/v), and mobile phase B was composed of PBS (pH 2.0, 85 mmol) and acetonitrile (61:39, v/v). A linear gradient with 1.0 mL/min flow-rate was run from 35% mobile phase A and 65% mobile phase B to 28% A and 72% B at the initial 0–6 min, and then 28% A and 72% B were maintained for the following 27 min (6–33 min).

Plasma Drug Concentrations in SD Rats

Pharmacokinetics was assessed in 12-week-old SD rats (body weight 475 ± 25 g), which were randomly divided into two groups (three rats per group). The first group (defined as Free EXT) was administrated by a single subcutaneous injection of 0.4 mL EXT (2.0 mg/mL) in acetate buffer solution at pH 4.5 (the pH follows the commercial product Byetta®); the second group (EXT in Gel) was administrated by a single subcutaneous injection of 0.4 mL gel formulation containing 2.0 mg/mL EXT (pH 7.4). The drug dosages in both groups were thus 0.8 mg. At predetermined time points (0.25, 0.5, 1, 1.5, 2, 4, 7.5, 12, and 24 h for the first group; 0.25, 0.5, 1, 1.5, 2, 4, 7.5, 12, 24, 72, 120, 168, 216, and 264 h for the second group), approximately 0.3 mL of blood samples were collected from the tail vein. The rats were provided free access to water during the experiment. After centrifugation at 3000*g* for 10 min, blood serum was obtained and stored at −40°C until the assay. Plasma concentrations of EXT were determined using an Exendin-4 EIA kit (Phoenix Pharmaceuticals, Inc., USA).

PD Study of EXT Released from the Thermogel in db/db Mice

The experimental animals, db/db mice were assigned to three groups ($n = 9$ for each group): NaCl [a single subcutaneous injection of 0.9% NaCl solution (NS), 3.75 mL/kg], Free EXT (twice-daily subcutaneous injections of an EXT solution at 10:00 a.m. and 5:00 p.m., 0.375 mg/3.75 mL/kg), and EXT in Gel (a single subcutaneous injection of the hydrogel formulation, 7.5 mg/3.75 mL/kg). The drug dosage in the group of "EXT in Gel" was 20-fold of that of "Free EXT" once, and thus the total dosages in both groups were equal during the 10-day experiment.

The mice were fasted overnight except free access to water. On the first day of this study (defined as D0), 20 μL of blood samples were obtained from the tail vein, and then NS, Free EXT, or EXT gel formulation were subcutaneously administrated. Next, an oral administration of a glucose solution (2 g/10 mL/kg) in db/db mice were performed after the different treatments for 15 min, and blood samples were collected at 30 and 60 min. Blood glucose concentrations of db/db mice were monitored using an AccuCheck Active (Roche Diagnostics, Germany).

On the next 10 days of posttreatment, the animals were fasted from 9:00 a.m. for 4 h, followed by an oral administration of glucose. Blood samples were collected at 0 (before oral administration of glucose), 30 and 60 min after the oral administration of glucose. Body weight was also recorded daily at 10:00 a.m.

After the first treatment for 10 days, the second administration of EXT gel formulation was performed in the group of "EXT in Gel" at the afternoon of day 10. The group of "Free EXT" was accordingly twice-daily injected throughout the entire period. At day 18, blood samples were obtained from the eyes. Plasma insulin levels were measured using an insulin ELISA kit (Mercodia, Sweden), and blood HbA1c concentrations were determined using an analyzer for hemoglobin (Variant II; Bio-Rad, USA).

Moreover, pancreases were isolated from db/db mice. The histological samples were fixed in 10% neutral buffered formalin, embedded in paraffin and cut into slices of thickness 4 μm. The sections were strained with hematoxylin–eosin (HE) and observed under a light microscope for histological examinations.

Statistical Analysis

The peak plasma concentration (C_{max}) and the time to reach the maximum concentration (T_{max}) were determined directly from the data. Noncompartmental PK analyses were performed on the profiles of plasma EXT concentrations versus time using a PK analysis system (Drug and Statistics, DAS2.0).[35] The area under the plasma concentration time curve during the examined period (AUC_{0-t}) was obtained by the sum of areas from time zero to the last day of sampling, which was calculated using the log-trapezoidal approach.[35]

Furthermore, the *in vivo/in vitro* correlation profile was determined via the deconvolution approach.[36] This approach is based on the convolution relation between the response function $R(t)$, input function $I(t)$, and weighting function $W(t)$ written as

$$R(t) = \int_0^t I(\tau)W(t-\tau)\mathrm{d}\tau = I(t)W(t) \quad (1)$$

DOI 10.1002/jps.23735

Here, $R(t) = C_p(t)$, the transient plasma drug concentration at time t for a formulation; I is the *in vivo* dissolution rate, which means here the *in vivo* release rate, namely dA/dt (A is the release amount); and W is calibrated by a so-called unit impulse response, which corresponds to the plasma drug concentration profile upon a unit amount of drug release; "*" denotes convolution. Both response and weighting functions are achieved by experimental measurements. Therefore, the input function could be calculated by deconvolution as

$$I(t) = R(t)//W(t) \tag{2}$$

Then, the *in vivo* cumulative release can be obtained by

$$\textit{In vivo}\ \text{release}(\%) = \frac{\int_0^t I(\tau)\mathrm{d}\tau}{\int_0^\infty I(\tau)\mathrm{d}\tau} \tag{3}$$

It should be indicated that the above relations are based upon the superposition principle, which stands under linear assumptions of dose proportionality and time invariance of the system investigated.

All data were presented as mean ± standard deviation. Groups were compared via least significant difference (LSD) t-tests. A p value less than 0.05 was considered as the criterion of a statistically significant difference between the groups joining in comparison, unless otherwise indicated.

RESULTS

Sol–Gel Transition of the Mixture Hydrogel

Two PLGA–PEG–PLGA triblock copolymers were synthesized and characterized, following our previous protocol.[31] Although both samples did not possess the thermogelling properties, their mixture with an appropriate mix ratio might exhibit a thermoreversible sol–gel transition in water.[31–33] In this study, copoymer-1 with MW 1286–1500–1286 and copolymer-2 with 1508–1000–1508 were synthesized; the molar ratios of LA/GA in the PLGA blocks for both samples were 4/1. The thermogelling ability of the copolymer mixture with weight ratio 1/1 was confirmed. Figure 2 shows the change in viscosity as a function of temperature at three polymer concentrations. The aqueous polymer system exhibited an increase in viscosity with elevating temperature at any polymer concentration. Only with copolymer concentration above CGC (and of course under appropriate copolymer composition and mixture ratio), the sample was able to turn into a semi-solid gel. For instance, the 1/1 mixture system (25 wt %) was a free-flow liquid at room temperature and became viscous with increasing temperature to 26°C. As the temperature was raised to 32°C, the mixture system formed a nonflowing gel. Such a physical hydrogel was kept at the physiological condition.

In Vivo Gel Formation

The *in situ* thermogelling was also confirmed, as demonstrated in Figure 3. After a subcutaneous injection of the 1/1 mixture solution (25 wt %) into the neck of an SD rat, a rapid gelling happened at the injection site (about 30 s), and its integrity was well maintained over 3 weeks. Hence, our mixture hydrogel is potentially used as an injectable and biodegradable depot for sustained drug delivery.

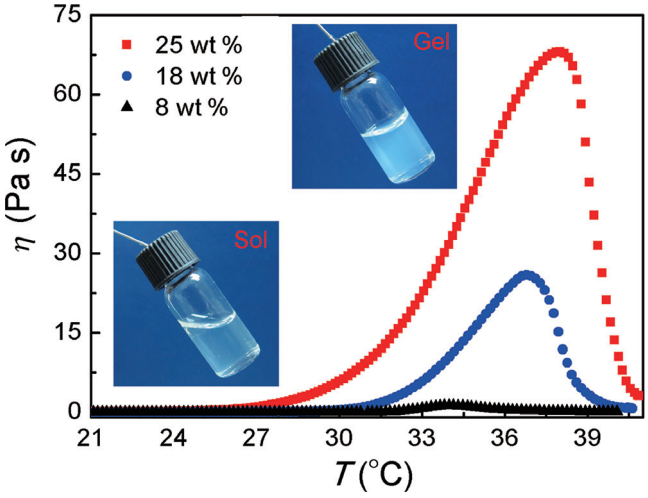

Figure 2. Viscosity of the copolymer aqueous system as a function of temperature at indicated total polymer concentrations with 1:1 weight ratio of copolymer 1 and copolymer 2. The medium is deionized water.

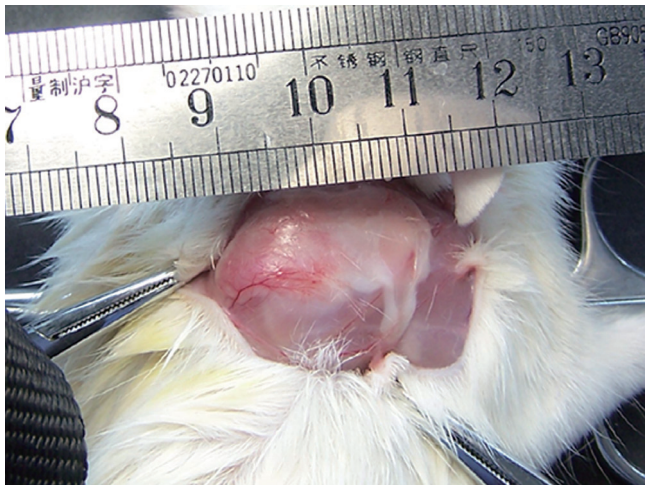

Figure 3. *In situ* gel formation of 1/1 PLGA–PEG–PLGA mixture solution (25 wt %, 0.4 mL) in the neck of an SD rat. The photograph was taken 1 week after the subcutaneous injection.

EXT Release *In Vitro* for 9 Days

In our recent report, an optimal thermogel formulation containing 2 mg/mL EXT was obtained via addition of three excipients (1.25 wt % zinc acetate, 5 wt % PEG200, and 5 wt % sucrose) in the 1/1 mixture hydrogel.[31] In the present study, the effects of polymer concentration and drug amount on release profiles were further investigated. As the polymer concentration decreased from 25 to 18 wt %, the initial burst release increased significantly (Fig. 4a). The burst release from the 18 wt % gel may be explained via its low viscosity, as shown in Figure 2. This feature suggests that the control of the initial burst depends on not only the synergistic effect of above mentioned excipients but also the polymer concentration. The drug concentration influenced the release profiles as well (Fig. 4b). Both the initial burst and cumulative release amounts decreased with increasing drug concentration.

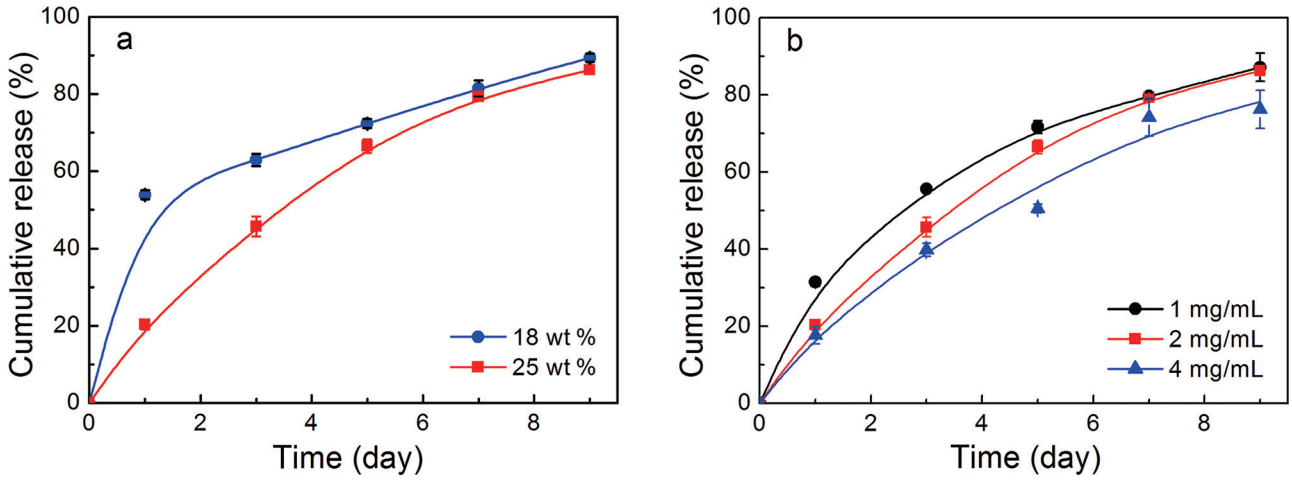

Figure 4. *In vitro* release profiles of EXT from the 1/1 mixture hydrogel containing three additives (1.25 wt % zinc acetate, 5 wt % PEG200, and 5 wt % sucrose) at (a) two indicated total copolymer concentrations but a fixed drug concentration (2 mg/mL), and (b) three indicated drug concentrations but a fixed copolymer concentration (25 wt %). The lines are used just for the guides of the eyes.

Tests of SD Rats to Monitor Drug Concentrations in Plasma for 11 Days

Pharmacokinetics studies of the hydrogel formulation of EXT were performed in SD rats and reported for the first time in this paper. The plasma concentrations of EXT were detected at a series of time points after a single subcutaneous injection of the optimal EXT hydrogel formulation/Free EXT. A steady-state drug level was maintained up to day 9 and dropped at day 11 for the optimal EXT formulation, as shown in Figure 5. In contrast, the duration of drug was kept only for a few hours in the case of Free EXT.

On the basis of the profiles, we calculated the relevant PK parameters such as C_{max} and half elimination time $t_{1/2z}$. The results are listed in Tables S1 and S2 in Supporting Information. The C_{max} of EXT gel formulation was 53 ± 5 ng/mL, significantly lower than that of Free EXT (248 ± 21 ng/mL); $t_{1/2z}$ of EXT gel formulation read 34.3 ± 5.6 h, 16-fold of that of Free EXT (2.1 ± 0.4 h). A significant sustained release of the peptide drug EXT has thus been achieved *in vivo* by using our thermogel.

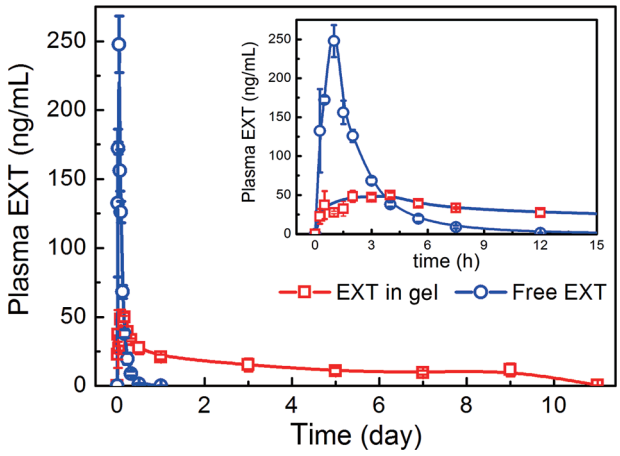

Figure 5. Plasma EXT concentrations in SD rats treated with the optimal EXT hydrogel formulation or Free EXT. The results are presented as mean ± standard deviation (n = 3). The lines are used just for the guides of the eyes.

Tests of db/db Mice to Detect Blood Glucose Concentrations and Body Weights for 10 Days

We further employed hyperglycemic db/db mice as a model of type II diabetes to check PD of the hydrogel formulation of EXT. The db/db mice exhibited high blood glucose levels within the entire period of *in vivo* tests, suggesting the stability of the animal model. After an oral gavage of glucose, the blood glucose concentrations of mice were further elevated. The concentrations were lowered rapidly after injecting either Free EXT or the hydrogel formation of EXT. The data in Figure 6 about the blood glucose levels of db/db mice were analyzed by LSD t tests, and the resultant p values are listed in Supplementary Table S3. There was a significant difference between the EXT gel formulation (or Free EXT) and the control group, as shown in Figure 6 and Tables S3. The blood glucose levels were kept steady for one week after just a single subcutaneous injection in the group of the hydrogel formulation.

The mice were also weighed, with the results shown in Figure 7. The body weight increased in the control group. There was no significant difference in body weight gain between Free EXT and the control group, as shown in Tables S4. In contrast, a significant decrease in body weight compared with the control group or Free EXT was observed after administration of the EXT gel formation. Especially, a significant decline in body weight of near 10% was seen in db/db mice receiving the hydrogel formulation of EXT compared with the saline-treated control, indicating that the sustained release of EXT delayed gastric emptying and reduced appetite. This phenomenon was consistent with the pharmacology of EXT as reviewed in the literature,[3] and a similar finding was also observed in the microsphere formulation of EXT.[37]

Tests of db/db Mice to Detect Blood Insulin and HbA1c Concentrations after 18 Days and the Histological Observations Afterward

The plasma concentration of insulin in hyperglycemic db/db mice increased significantly after continuously twice-daily

DOI 10.1002/jps.23735

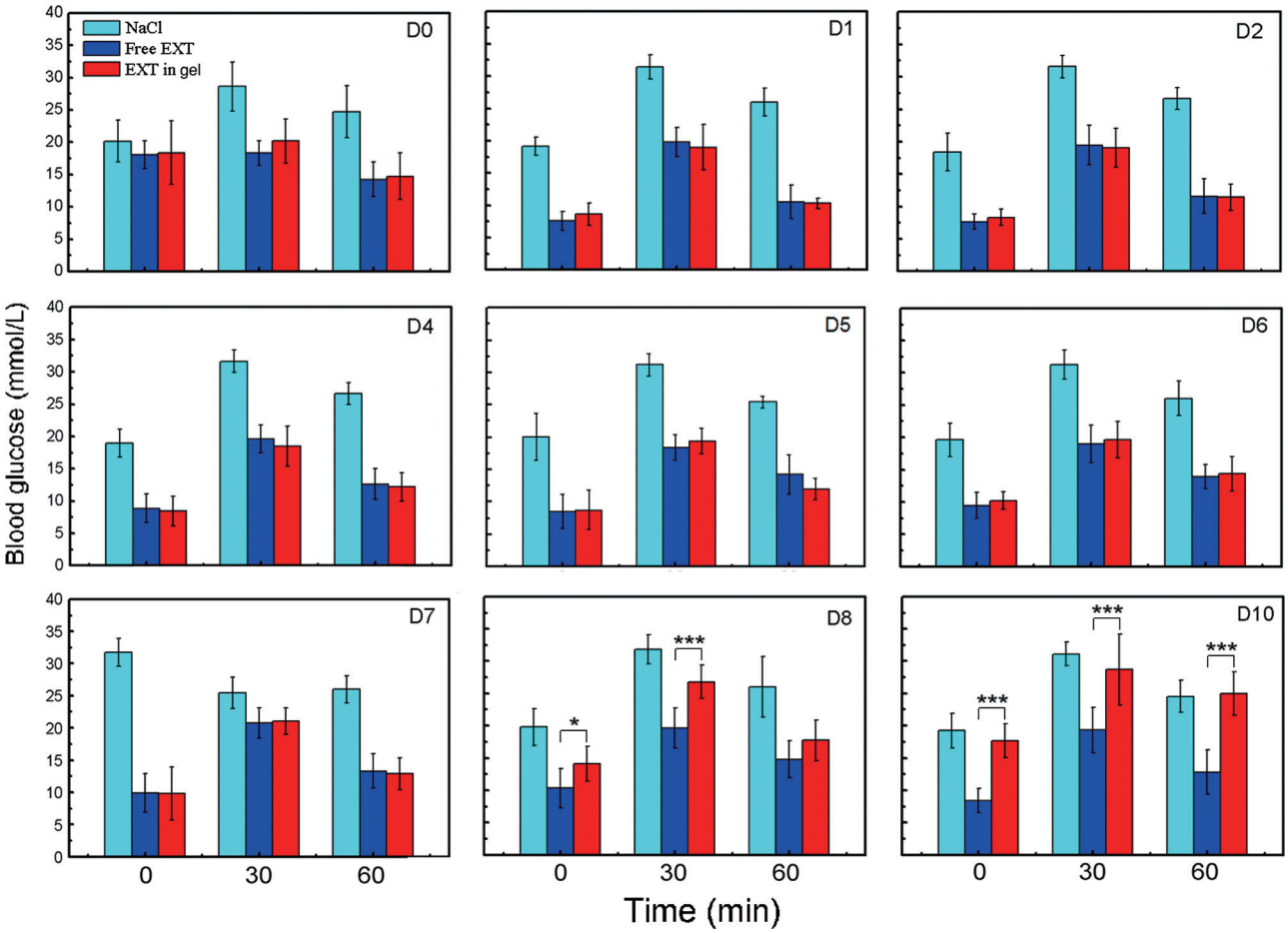

Figure 6. Blood glucose levels in db/db mice treated with saline solution, Free EXT or the EXT gel formulation. The caption of "D" denotes the time (day) after treatment, and the first injection of the EXT formulation was denoted as day 0. The oral gavage of glucose in mice was once-daily performed. The three data with respect to time 0 at D0 reflect the basic blood glucose concentration, namely, the limosis blood glucose level without any treatment. From days 1–10, time 0 in the horizontal coordinate indicate actually the limosis blood glucose levels immediately before glucose garage in mice that experienced 4 h of limosis before once-daily garage of glucose. The time points of 30 and 60 min started at finishing glucose gavage, which lasted for about 10 s as usual. Free EXT was administered by twice-daily subcutaneous injections at an EXT dose of 0.375 mg/3.75 mL/kg for each injection. The hydrogel formulation of EXT was administrated by a single subcutaneous injection at an EXT dose of 7.5 mg/3.75 mL/kg. $n = 9$ for each group. Significant differences between the groups of "Free EXT" and "EXT in Gel" are specifically marked: "*", $p < 0.05$; "***", $p < 0.001$.

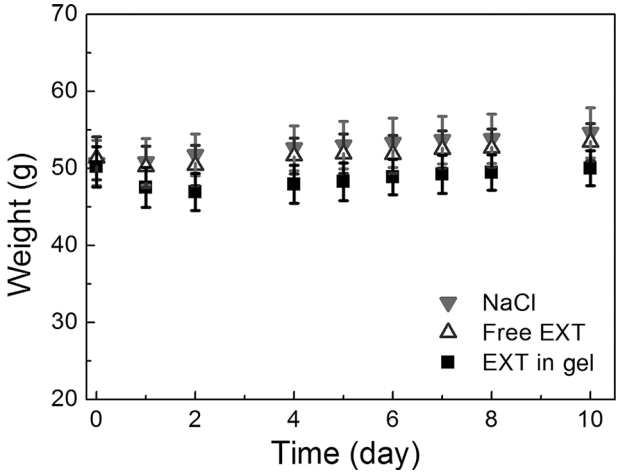

Figure 7. Change in body weight of db/db mice after indicated treatments. $n = 9$ for each group. There is a significant difference between the groups of "NaCl" (or "Free EXT") and "EXT in Gel" at days 1–10.

injections of Free EXT for 18 days compared with the saline-treated control. A significant increase in insulin concentration was also observed in the group of the hydrogel formation, as shown in Figure 8 and Table S5. Here, a higher insulin level was determined via injections of the hydrogel formulation of EXT just twice during the 18 days. Therefore, the sustained release of EXT from our mixture hydrogel promoted secretion of insulin to a large degree.

HbA1c concentrations were detected as well. HbA1c comes from non-enzymatic irreversible glycation of Hb and represents an indirect indicator of cumulative blood glucose concentrations. Consequently, the level of HbA1c is regarded as a more convincing index of the glycemic control than the blood glucose concentration, which fluctuates highly.[37] As shown in Figure 8 and Table S5, administration of Free EXT had no significant influence on HbA1c compared with the saline control. In contrast, a significant decrease in HbA1c concentration in plasma was observed in db/db mice receiving the EXT formulation for 18 days. This finding strengthens that the sustain-released EXT offers a beneficial effect on sustained glycemic control.

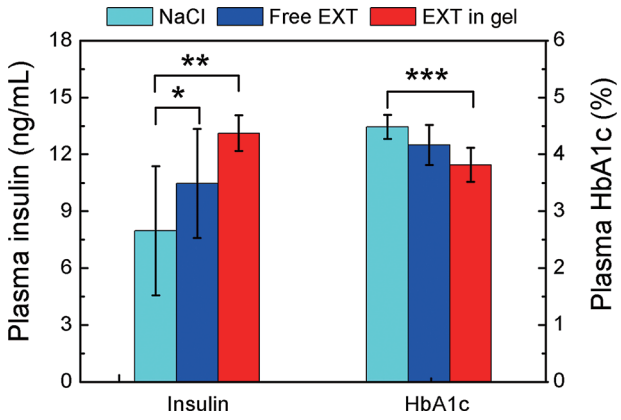

Figure 8. Insulin and HbA1c concentrations in plasma of db/db mice upon indicated treatments for 18 days. Free EXT was administered via twice-daily subcutaneous injections at an EXT dose of 0.375 mg/3.75 mL/kg for each injection; the hydrogel formulation of EXT was subcutaneously injected twice on day 0 and 10 at an EXT dose of 7.5 mg/3.75 mL/kg. $n = 9$ for each group. "*", $p < 0.05$; "**", $p < 0.01$; "***", $p < 0.001$.

After sacrificing the mice at day 18, we carried out the histological examinations of pancreatic sections. Normal mice usually exhibit round or elliptical pancreatic islets with a distinct outline and tight organization between islet cells. In contrast, islets of the saline-treated db/db mice presented a deranged morphology combined with loss of islet volume and cellular homogeneity. Moreover, cellular necrosis, broadening gap between islet cells and hyperplasia of fibrous tissue were observed (Fig. 9a). Compared with the control group, the volume of islets enlarged and the amount of islet cells increased in db/db mice receiving Free EXT or the EXT hydrogel formulation (Figs. 9b and 9c). Therefore, the histological observations are consistent with the PD of EXT by confirming the promotion of proliferation of pancreatic islet cells and the preservation of the islet structure in db/db mice.

DISCUSSION

Diabetes mellitus has, despite a chronic metabolic disease, been one of the most dangerous epidemics in the world. EXT is the first GLP-1 receptor agonist to be approved to treat type II diabetes mellitus clinically. Unlike the glucose-independent agents such as insulin and sulfonylureas, EXT can, as a glucoregulatory agent, stimulate insulin secretion during either euglycemia or hyperglycemia; and its insulinotropic action rapidly reduces when the blood level of glucose is lower than 3 mmol/L.[3] Consequently, even if EXT is continuously released, the risk of hypoglycemia is quite low and the drug safety is rather high.

In this study, the PLGA–PEG–PLGA triblock copolymer hydrogel was used as the reservoir of EXT. The thermogelation of the amphiphilic copolymers in water occurs via formation of a percolated micelle network.[32,38,39] The *in situ* formed gel can maintain integrity up to several weeks at the target site after a single subcutaneous injection (Fig. 3). The thermogel is quite unique due to the spontaneous formation of gel at the body temperature free of any chemical reaction, as schematically presented in Figure 1.

An optimal thermogel formulation of EXT was prepared by mixing the drug (2 mg/mL) and the three excipients (1.25 wt% zinc acetate, 5 wt% PEG200 and 5 wt% sucrose) with the aqueous polymer solution at ambient temperature. The copolymer aqueous system containing drug and excipients was still a free-flowing solution at low temperatures ($< T_{gel} < T_{body}$) and turned into a gel at the body temperature. Because of the low viscosity at the sol state, a subcutaneous injection of our copolymer system is feasible using common syringe needles, such as a 23-gauge one. The *in vitro* release experiment confirmed that the optimal EXT hydrogel formulation showed a sustained release profile up to 9 days (Fig. 4). Such an excellent result was attributed to the synergistic effect of three excipients. The formation of insoluble Zn–EXT nanoparticles via the complex of zinc ion with the anions in amino acids of EXT delayed the initial burst, and the porogen effect of hydrophilic PEG200 and sucrose promoted the uniform and complete drug release.[31]

The *in vivo* PD was confirmed in db/db mice model. As displayed in Figure 6 and Table S3, both the fasting and

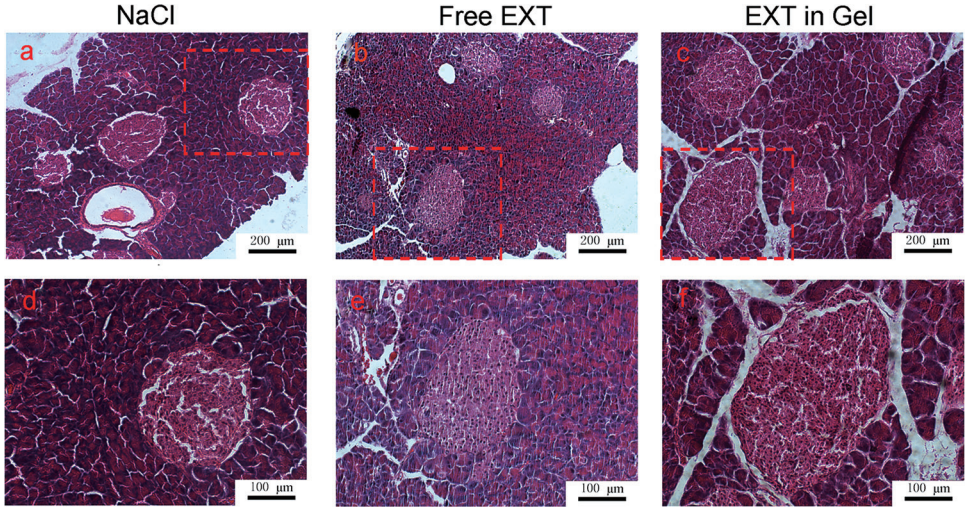

Figure 9. HE-strained histological sections of pancreas islets isolated from indicated db/db mice groups after treatment for 18 days. (a) db/db mice treated with NaCl; (b) db/db mice treated with Free EXT; (c) db/db mice treated with the EXT hydrogel formulation. (d), (e), and (f) are magnified images of indicated regions in (a), (b), and (c), respectively.

DOI 10.1002/jps.23735

post-glucose-loaded blood glucose concentrations were significantly decreased. During the 10 days of examinations, a single subcutaneous administration of the hydrogel formulation successfully achieved a normal glucose level up to 1 week. This mammal test has convinced the bioactivity of EXT released from the thermogel matrix and the effectiveness of our EXT formulation to control fasting and postprandial blood glucose excursions in type II diabetic animals in a sustained release manner.

Meanwhile, the growth of body weight was effectively suppressed after administration of the EXT formulation (Fig. 7). This result is attributed to the anorectic effects of EXT because the drug can act on hypothalamus to increase satiety and slow gastric emptying.[3] This feature is beneficial especially for patients of type II diabetes along with obesity.

The levels of insulin and HbA1c in db/db mice were detected as well. As the interval of reliable detection of HbA1c concentration via instrument usually requires one month for human and two weeks for mouse,[40] we prolonged the blood tests to day 18, and thus the second injection of the hydrogel formulation of EXT was carried out at the afternoon of day 10 following the above-mentioned 10-day db/db mice experiments. Figure 8 and Table S5 present that the plasma level of insulin remarkably increased and the blood concentration of HbA1c decreased significantly after twice injections of the EXT formulation in db/db mice. In contrast, the increase in insulin level was relatively low, and the HbA1c concentration did not change significantly after even twice-daily injections of Free EXT for 18 days. Hence, the efficacy of long-term injections of the EXT hydrogel formulation is also superior to that receiving twice-daily administration of Free EXT for the same time period. Moreover, our histological observations affirmed the increasing proliferation of islet cells and protection of islet function following treatment with the EXT formulation.

Another necessary animal test is the detection of time-dependent drug concentration, the key study of *in vivo* PK. In Figure 5, the elevated plasma level of EXT remained over 1 week after a single subcutaneous injection of the EXT hydrogel formulation into SD rats. Although Figure 5 for *in vivo* PK appears ahead of those data of *in vivo* PD, the data of this figure were obtained actually later than those of Figures 6–9 in our experiments because we were faced with experimental difficulties of *in vivo* PK. An ideal animal model for the *in vivo* PK seemed db/db mice in this study. However, approximately 0.3 mL blood is usually required to precisely detect drug level in plasma, whereas the whole blood available for a mouse is about 2.0 mL. Also, many time points must be detected to obtain a complete PK profile. That is why we eventually chose db/db mice, a very sensitive animal model for type II diabetic tests to examine *in vivo* PD, and SD rats, a model with much more blood to check *in vivo* PK quantitatively. Of course, an alternative choice is to use directly Zucker diabetic fatty rats.[37,41] Fortunately, our results demonstrated that the reduced blood level of glucose in db/db mice was consistent with the increased plasma concentration of EXT in SD rats for the same time period. It is worthy of pointing out that the number of SD rats in each group ($n = 3$) is relatively small in the present study of PK and more animals are usually required in an *in vivo* study (as $n = 9$ in our db/db mice experiments for PD).

We also tried to compare the *in vivo* and *in vitro* PK results on the basis of the data in Figures 4 and 5. The straight comparison is not available because the plasma drug concentration is not equal to the release amount of EXT from the hydrogel after a subcutaneous injection into the neck. Eventually, we employed a deconvolution method to calculate the *in vivo* drug release rate via Eq. 2. Here, the transient drug concentration in blood (data in Fig. 5 for the group of "EXT in Gel") was employed as the response function, and the data in Figure 5 for the group of "Free EXT" divided by the total drug dosage (0.8 mg) as the weighting function. Then we used Eq. 3 to calculate the cumulative drug release *in vivo*, with the results displayed in Figure 10a. Similar to the *in vitro* release profile, a sustained release profile *in vivo* was achieved. The 100% release was due to almost zero (undetectable) plasma drug concentration at the late stage in Figure 5. Our results demonstrated a very good correlation between *in vitro* release and *in vivo* PK, as shown in Figure 10b. Hence, the *in vitro* release profile, the *in vivo* PK, and the *in vivo* PD are consistent with each other in our studies quiet well.

CONCLUSIONS

The thermogelling system composed of two PLGA–PEG–PLGA triblock copolymers and three additives (zinc acetate, PEG200, and sucrose) were used as the drug depot of the water-soluble peptide EXT. The polymer–drug solution presented good

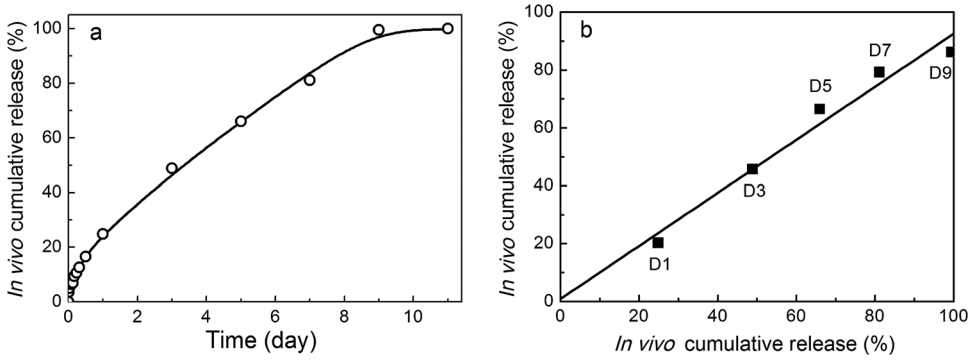

Figure 10. (a) The *in vivo* release profile of the hydrogel formulation of EXT with 2 mg/mL EXT, 25 wt % total copolymer, and three additives (1.25 wt % zinc acetate, 5 wt % PEG200, and 5 wt % sucrose) calculated according to the deconvolution method based on the response and weighting functions via Eqs. 2 and 3. (b) The corresponding *in vitro*/*in vivo* correlation profile of the hydrogel formulation of EXT at indicated days. Data were linearly fitted with a squared coefficient 0.96.

injectability at low temperatures, and the spontaneous thermogelation occurred at the body temperature. The physical gel kept its integrity at subcutaneous sites at least for one week. The *in vitro* release profile was adjusted by copolymer concentration and drug amount. The sustained release of EXT from the gel matrix after a single subcutaneous injection improved glucose tolerance and controlled both fasting and postprandial blood glucose excursions in type-II diabetic db/db mice for 1 week. The body weight gain of mice was suppressed as well. Moreover, the treatment with the EXT thermogel formulation twice during 18 days promoted insulin secretion, decreased HbA1c concentration, alleviated histological morphologic damage of pancreatic islets, and protected islet in db/db mice. We also tried to transform the time-dependent plasma drug concentrations into the *in vivo* drug release rates in the injection site, and the *in vivo* PK tests agree with the *in vitro* release tests well. As a result, it is feasible to use our optimal mixture thermogel formulation of EXT as a once-weekly delivery system, which has the potential to increase therapeutic efficacy and improve patient compliance significantly.

ACKNOWLEDGMENTS

The group was supported by NSF of China (Grants No. 51273217, No. 91127028, and No. 21034002), Chinese Ministry of Science and Technology (973 Programs No. 2009CB930000 and No. 2011CB606203), and Science and Technology Developing Foundation of Shanghai (Grant No. 12JC1402600).

REFERENCES

1. Holst JJ. 2002. Therapy of type 2 diabetes mellitus based on the actions of glucagon-like peptide-1. Diabetes Metab Res Rev 18(6):430–441.
2. Drucker DJ, Nauck MA. 2006. The incretin system: Glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. Lancet 368(9548):1696–1705.
3. Nielsen LL, Young AA, Parkes DG. 2004. Pharmacology of exenatide (synthetic exendin-4): A potential therapeutic for improved glycemic control of type 2 diabetes. Regul Pept 117(2):77–88.
4. Kim PH, Lee M, Kim SW. 2012. Delivery of two-step transcription amplification exendin-4 plasmid system with arginine-grafted bioreducible polymer in type 2 diabetes animal model. J Control Release 162(1):9–18.
5. Li X, Li L, Wang X, Ren Y, Zhou T, Lu W. 2012. Application of model-based methods to characterize exenatide-loaded double-walled microspheres: In vivo release, pharmacokinetic/pharmacodynamic model, and in vitro and in vivo correlation. J Pharm Sci 101(10):3946–3961.
6. Eng J, Kleinman WA, Singh L, Singh G, Raufman JP. 1992. Isolation and characterization of exendin-4, an exendin-3 analogue from Heloderma suspectum venom. J Biol Chem 267(11):7402–7405.
7. DeYoung MB, MacConell L, Sarin V, Trautmann M, Herbert P. 2011. Encapsulation of exenatide in poly-(D, L-lactide-Co-glycolide) microspheres produced an investigational long-acting once-weekly formulation for type 2 diabetes. Diabetes Tech Therap 13(11):1145–1154.
8. Bergenstal RM, Li Y, Porter TKB, Weaver C, Han J. 2013. Exenatide once weekly improved glycaemic control, cardiometabolic risk factors and a composite index of an HbA1c < 7%, without weight gain or hypoglycaemia, over 52 weeks. Diabetes Obes Metab 15(3):264–271.
9. Loh XJ, Peh P, Liao S, Sng C, Li J. 2010. Controlled drug release from biodegradable thermoresponsive physical hydrogel nanofibers. J Control Release 143(2):175–182.
10. Pescosolido L, Vermonden T, Malda J, Censi R, Dhert WJA, Alhaique F, Hennink WE, Matricardi P. 2011. In situ forming IPN hydrogels of calcium alginate and dextran-HEMA for biomedical applications. Acta Biomater 7(4):1627–1633.
11. Vermonden T, Censi R, Hennink WE. 2012. Hydrogels for protein delivery. Chem Rev 112:2853–2888.
12. Teixeira LSM, Bijl S, Pully VV, Otto C, Rong J, Feijen J, van Blitterswijk CA, Dijkstra PJ, Karperien M. 2012. Self-attaching and cell-attracting in-situ forming dextran–tyramine conjugates hydrogels for arthroscopic cartilage repair. Biomaterials 33(11):3164–3174.
13. Ko DY, Shinde UP, Yeon B, Jeong B. 2013. Recent progress of in situ formed gels for biomedical applications. Prog Polym Sci 38(3–4):672–701.
14. Ham AS, Ugaonkar SR, Shi L, Buckheit KW, Lakougna H, Nagaraja U, Gwozdz G, Goldman L, Kiser PF, Buckheit RW, Jr. 2012. Development of a combination microbicide gel formulation containing IQP-0528 and tenofovir for the prevention of HIV infection. J Pharm Sci 101(4):1423–1435.
15. Corrente F, Paolicelli P, Matricardi P, Tita B, Vitali F, Casadei MA. 2012. Novel pH-sensitive physical hydrogels of carboxymethyl scleroglucan. J Pharm Sci 101(1):256–267.
16. Koop HS, Da-Lozzo EJ, de Freitas RA, Franco CRC, Mitchell DA, Silveira JLM. 2012. Rheological characterization of a xanthan–galactomannan hydrogel loaded with lipophilic substances. J Pharm Sci 101(7):2457–2467.
17. Vardar E, Vert M, Coudane J, Hasirci V, Hasirci N. 2012. Porous agarose-based semi-IPN hydrogels: Characterization and cell affinity studies. J Biomater Sci Polym Ed 23(18):2273–2286.
18. Jeong B, Bae YH, Lee DS, Kim SW. 1997. Biodegradable block copolymers as injectable drug-delivery systems. Nature 388(6645):860–862.
19. Ruel-Gariepy E, Leroux JC. 2004. In situ-forming hydrogels—Review of temperature-sensitive systems. Eur J Pharm Biopharm 58(2):409–426.
20. Yu L, Ding JD. 2008. Injectable hydrogels as unique biomedical materials. Chem Soc Rev 37:1473–1481.
21. Huynh CT, Nguyen MK, Lee DS. 2011. Injectable block copolymer hydrogels: Achievements and future challenges for biomedical applications. Macromolecules 44(17):6629–6636.
22. Il Kim J, Kim B, Chun C, Lee SH, Song SC. 2012. MRI-monitored long-term therapeutic hydrogel system for brain tumors without surgical resection. Biomaterials 33(19):4836–4842.
23. Cho JK, Park JW, Song SC. 2012. Injectable and biodegradable poly(organophosphazene) gel containing silibinin: Its physicochemical properties and anticancer activity. J Pharm Sci 101(7):2382–2391.
24. Oak M, Singh J. 2012. Controlled delivery of basal level of insulin from chitosan–zinc–insulin-complex-loaded thermosensitive copolymer. J Pharm Sci 101(3):1079–1096.
25. Zentner GM, Rathi R, Shih C, McRea JC, Seo MH, Oh H, Rhee BG, Mestecky J, Moldoveanu Z, Morgan M, Weitman S. 2001. Biodegradable block copolymers for delivery of proteins and water-insoluble drugs. J Control Release 72(1–3):203–215.
26. Shim MS, Lee HT, Shim WS, Park I, Lee H, Chang T, Kim SW, Lee DS. 2002. Poly(D,L-lactic acid-co-glycolic acid)-b-poly(ethylene glycol)-b-poly (D,L-lactic acid-co-glycolic acid) triblock copolymer and thermoreversible phase transition in water. J Biomed Mater Res 61(2):188–196.
27. Choi S, Baudys M, Kim SW. 2004. Control of blood glucose by novel GLP-1 delivery using biodegradable triblock copolymer of PLGA–PEG–PLGA in type 2 diabetic rats. Pharm Res 21(5):827–831.
28. Yu L, Chang GT, Zhang H, Ding JD. 2008. Injectable block copolymer hydrogels for sustained release of a PEGylated drug. Int J Pharm 348:95–106.
29. Chang GT, Ci TY, Yu L, Ding JD. 2011. Enhancement of the fraction of the active form of an antitumor drug topotecan via an injectable hydrogel. J Control Release 156:21–27.
30. Yu L, Zhang Z, Ding JD. 2011. Influence of LA and GA sequence in the PLGA block on the properties of thermogelling PLGA–PEG–PLGA block copolymers. Biomacromolecules 12:1290–1297.
31. Li K, Yu L, Liu XJ, Chen C, Chen QH, Ding JD. 2013. A long-acting formulation of a polypeptide drug exenatide in treatment of diabetes

using an injectable block copolymer hydrogel. Biomaterials 34:2834–2842.

32. Yu L, Zhang Z, Zhang H, Ding JD. 2009. Mixing a sol and a precipitate of block copolymers with different block ratios leads to an injectable hydrogel. Biomacromolecules 10(6):1547–1553.

33. Yu L, Zhang Z, Zhang H, Ding JD. 2010. Biodegradability and biocompatibility of thermoreversible hydrogels formed from mixing a sol and a precipitate of block copolymers in water. Biomacromolecules 11(8):2169–2178.

34. Yu L, Zhang H, Ding JD. 2006. A subtle end-group effect on macroscopic physical gelation of triblock copolymer aqueous solutions. Angew Chem Int Ed 45(14):2232–2235.

35. Gibaldi M. 1984. Biopharmaceutics and clinical pharmacokinetics. Philadelphia: Lea & Febiger, pp 17–28.

36. Gaynor C, Dunne A, Davis J. 2008. A comparison of the prediction accuracy of two IVIVC modelling techniques. J Pharm Sci 97(8):3422–3432.

37. Kwak HH, Shim WS, Hwang S, Son MK, Kim YJ, Kim TH, Yoon ZH, Youn HJ, Lee GI, Kang SH, Shim CK. 2009. Pharmacokinetics and efficacy of a biweekly dosage formulation of exenatide in Zucker Diabetic Fatty (ZDF) rats. Pharm Res 26(11):2504–2512.

38. Yu L, Chang GT, Zhang H, Ding JD. 2007. Temperature-induced spontaneous sol–gel transitions of poly(D,L-lactic acid-co-glycolic acid)-b-poly(ethylene glycol)-b-poly(D,L-lactic acid-co-glycolic acid) triblock copolymers and their end-capped derivatives in water. J Polym Sci Part A: Polym Chem 45:1122–1133.

39. Zhang H, Yu L, Ding JD. 2008. Roles of hydrophilic homopolymers on the hydrophobic-association-induced physical gelling of amphiphilic block copolymers in water. Macromolecules 41(17):6493–6499.

40. Chen D, Liao J, Li N, Zhou C, Liu Q, Wang G, Zhang R, Zhang S, Lin L, Chen K, Xie X, Nan F, Young AA, Wang MW. 2007. A nonpeptidic agonist of glucagon-like peptide 1 receptors with efficacy in diabetic db/db mice. Proc Natl Acad Sci USA 104(3):943–948.

41. Huynh DP, Im GJ, Chae SY, Lee KC, Lee DS. 2009. Controlled release of insulin from pH/temperature-sensitive injectable pentablock copolymer hydrogel. J Control Release 137(1):20–24.