Cucurbit[7]-assisted sustained release of human calcitonin from thermosensitive block copolymer hydrogel

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A R T I C L E   I N F O
Article history:
Received 7 March 2017
Received in revised form 18 April 2017
Accepted 3 May 2017
Available online 4 May 2017

Keywords:
Human calcitonin
Cucurbit[7]
Thermosensitive
Polymer hydrogel
Sustained release

A B S T R A C T
Conventional formulations of human calcitonin (hCT), a peptide drug, normally suffer from limited therapeutic efficacy with low stability and short half-life. We have found that the fibrillation of highly amyloidogenic hCT can be inhibited by cucurbit[7](CB[7]), an amphiphilic small molecule. Meanwhile, a thermogelling copolymer was found to be a suitable candidate for sustained delivery of peptide/protein drugs. Herein, we report a long-term delivery formulation composed of hCT-CB[7] complex and biodegradable thermogel of poly(D.L-lactic acid-co-glycolic acid)-b-poly(ethylene glycol)-b-poly(D.L-lactic acid-co-glycolic acid) (PLGA-PEG-PLGA). A 20 wt% PLGA-PEG-PLGA solution exhibited a temperature-sensitive sol-gel transition at 35°C in phosphate buffer solution and slowly degraded over one month at neutral pH. Both the mass fraction of PLGA-PEG-PLGA copolymer and the complexation of hCT-CB[7] moderated the release process. hCT was sustainedly released over three weeks in the 20 wt% hydrogel with hCT-CB[7] complex at the ratio of 1:10 or 1:25. Further considering its low cytotoxicity, the delivery system is potential for the clinical application of hCT.

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1. Introduction

The use of peptides and proteins as the therapeutic agents has increased considerably in recent years. However, some process characteristics of peptides and proteins such as low bioavailability and chemical instability limit their use for clinic treatment (Chiti and Dobson, 2006; Putney and Burke, 1998; Rakez et al., 2003). For example, human calcitonin (hCT) is a peptide hormone consisting of 32 amino acids. It is involved in the regulation of blood calcium level by inhibiting bone resorption. Particular, hCT has a strong tendency to irreversibly aggregate and form fibrils in aqueous solutions, which make it much inconvenient to use (Chiti and Dobson, 2006; Berger et al., 1988). So many studies have been focused on how to stabilize the conformation of hCT. Our preliminary work has demonstrated that cucurbit[7](CB[7]) can specifically bind to the aromatic groups of hCT (Chinai et al., 2011; Lee et al., 2014) which would inhibit its fibrillation and improve hCT bioactivity in decreasing the blood calcium level of rats. On the other hand, the short half-life of proteins such as hCT also significantly limits their utilization. Thus, multiple injections of peptide/protein drugs are required in clinic use, which results in decreasing patient compliance and increasing side effects (Monica et al., 2002; Singh and Jones, 2014). Therefore various drug delivery systems have been developed for the delivery of peptides and proteins in order to improve therapeutic effects and increase in vivo bioavailability (Smadar et al., 1991; Bromberg and Ron, 1998; Liu and Li, 2016).

Drug delivery systems based on biodegradable polymers have been widely investigated to deliver a variety of peptides and proteins (Lee and Sinko, 2000; Gaylen et al., 2001; Davide et al., 2003; Gahremankhani et al., 2008; Xie and Li, 2017). In particular, thermogelling polymers have been tried to fabricate injectable drug delivery systems with minimally invasive manner, which can dissolve in aqueous media at room temperature and form physically cross-linked thermogels by trigger of temperature (Zhang et al., 2008; Soppimath et al., 2002). Fragile therapeutic agents are easily entrapped into the thermogel by simply mixing them with the polymer aqueous solution at low temperature. Subsequently, the drug-loaded polymer solution can be conveniently injected into a target site of body using a conventional syringe and spontaneously transform into an in situ hydrogel at body temperature, acting as a sustained drug delivery depot.

http://dx.doi.org/10.1016/j.ijpharm.2017.05.006
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Among the thermogel systems, biodegradable and thermogelling triblock copolymer poly(D,L-lactic acid-co-glycolic acid)-b-poly(ethylene glycol)-b-poly(D,L-lactic acid-co-glycolic acid) (PLGA-PEG-PLGA) has become one of the most popular drug delivery carriers due to its expedite synthesis, high productivity, good reproducibility and controllable properties (Yu and Ding, 2008). To date, PLGA-PEG-PLGA thermogels have been suggested as injectable biomaterials for delivery of various drugs, such as protein, peptide and hydrophobic small molecules (Li et al., 2013; Yu et al., 2013). The release behaviour of drug from such a thermogel matrix is mainly dependent on the drug diffusion, the carrier polymer degradation or the combination of the two. Consequently, the polymer molecular weight (MW) and its distribution, polymer concentration, drug loading amount have been used to modulate the drug-release profiles (Chen et al., 2014, 2015; Kang et al., 2006; Tang and Singh, 2009; Zhang et al., 2015).

In this work, an injectable and biodegradable PLGA-PEG-PLGA thermogel will be investigated as a sustained release carrier of hCT. Owing to the good solubility of hCT in water, such a drug-loaded hydrogel system might suffer from a significant burst release at the initial stage. Another crucial problem is the protection of hCT conformation in the hydrogel matrix during the release process. The two problems were eventually resolved by the combination of

![Fig. 1](image-url)  
Fig. 1. (a) $^1$H NMR spectrum of PLGA-PEG-PLGA triblock copolymer in CDCl$_3$; (b) $^{13}$C NMR spectrum of PLGA-PEG-PLGA triblock copolymer in CDCl$_3$. The letters indicate the peak assignments.
adjustment of gel strength and complexation of hCT-CB[7], thus a long-acting delivery system of hCT was developed here.

2. Materials and methods

2.1. Materials

Human calcitonin (hCT) with the sequence of CGNLSTCMLGTYTDFNPQTFPGVAP-NH2 with an intra chain disulfide bridge between Cys1 and Cys7 at N-terminus and a proline amide bridge at C-terminus (99%) was purchased from Shanghai Botai Biological Pharmaceutical Enterprise (Shanghai, China) and stored in a lyophilized state at -20°C before use. Cucurbit[7]uril, PEG of MW 1500 Da and stannous 2-ethylhexanoate Sn(Oct)2 were obtained from Sigma-Aldrich Company Ltd. (USA). Streptomycin, penicillin, heparin sodium, glucose and Dulbecco’s modified Eagle medium (DMEM), cell counting kit-8 (CCK-8), LysoTracker Green DND-26 (LG), Propidium Iodide (PI), fetal bovine serum (FBS, 10%) and 96-well microtiter plate were purchased from Baoxin Biotechnology Co. Ltd. (Chengdu, China). D, L-Lactide (LA) and glycoside (GA) were obtained from Jinan Daigang Biomaterial Co., Ltd (China) and used without further treatment. Ultrapure water produced from a Milli-pore system with a resistivity of 18.25 MΩ × cm was used throughout.

2.2. Synthesis of PLGA-PEG-PLGA triblock copolymer

PLGA-PEG-PLGA triblock copolymer was synthesized via ring-opening polymerization of LA and GA in the presence of PEG. (Ci et al., 2014) Briefly, PEG 1500 (20.05 g) was loaded into a three-neck flask and heated 3 h at 130 °C under vacuum for drying. Then, LA (35.30 g) and GA (11.35 g) were loaded to reactor when the temperature of reactor decreased to 100 °C under argon atmosphere. Next, Sn(Oct)2 (0.06 g) in toluene solution as the catalyst was added, and the flask was under vacuum for 15 min to remove toluene. Subsequently, heating was continued at 150 °C for 12 h under argon protection. After the reaction was completed, the system was kept at 120 °C and linked to a vacuum line for 3 h to

**Fig. 2.** (a) GPC trace of PLGA-PEG-PLGA triblock copolymer (eluent: THF); (b) MALDI-TOF spectra of PLGA-PEG-PLGA triblock copolymer.
eliminate unreacted monomers. In view of that the required sample was insoluble in 80 °C water, the crude copolymer was further washed with deionized water (80 °C) for three times to remove water-soluble low MW polymers. Finally, the residual water in the polymers was removed by freeze drying, and the final production was collected and stored at –20 °C before use.

2.3. Characterizations of PLGA-PEG-PLGA

The resulting copolymer was characterized by $^1$H NMR and $^{13}$C NMR measurements in Bruker BioSpin International, AVANCE III HD 400 MHz. CDCl$_3$ was used as the solvent. The MW and MW distribution of copolymer were further determined by gel permeation chromatography (GPC) in 1260 instrument (Agilent). The mobile phase for GPC was tetrahydrofuran (THF) with a flow rate of 1.0 mL/min. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) in Bruker autoflex III smartbeam mass spectrometer were also used to determine the MW of copolymer. Alpha-Cyano-4-hydroxycinnamic acid (CCA) as the matrix were dissolved in tetrahydrofuran (THF) at a concentration of 10 mg/mL.

2.4. Sol-gel transition

The phase diagram of the PLGA-PEG-PLGA copolymer in phosphate buffer saline (PBS) solution (pH = 7.4) was determined using the inverting tube method (Cao et al., 2015; Chen et al., 2016a, 2016b). Firstly, PLGA-PEG-PLGA copolymer aqueous solutions with various concentrations were prepared by dissolving the sample in PBS solution. After storing overnight at 4 °C, 0.5 mL of copolymer solution was transferred into a 2-mL vial. The vials containing samples were immersed in a water bath and allowed to reach equilibrium at each temperature. To evaluate the ability of the system to convert to gel, we raise the temperature of water bath at a rate of 1 °C per step. In case of no visual flow in 30 s after inverting the vials, it was regarded as the gel formation.

2.5. Degradation of thermogel in vitro

The copolymer solutions (20 wt%, 0.5 mL) in PBS was injected into test tubes (the inner diameter was 6 mm) and equilibrated at 4 °C over night to eliminate bubbles. All the tubes were then incubated in a shaking bath with 50 strokes per minute at 37 °C. After equilibrating at 37 °C for 10 min, 9.5 mL of PBS solution containing 0.025% Na$_2$SO$_4$ was added as the medium. The buffer solutions were replaced with fresh ones every 5 days to maintain medium pH. At predetermined time, 3 samples were taken out of the shaking bath and the remaining gels were lyophilized until constant weight. The freeze-dried samples were carefully weighed. A blank sample containing the same volume of PBS was also lyophilized and weighed to deduct the effect of salt weight in the remaining gel. The dried samples were further analysed by GPC.

2.6. Preparation of drug-loaded thermogel system

PLGA-PEG-PLGA copolymer was dissolved in PBS to make 25 wt % polymer solution at 25 °C. hCT-CB[7] complex (hCT: 1.25 mg/mL) at different ratios were also dissolved in PBS to become drug concentrated liquor. Then, hCT or hCT-CB[7] complexes (hCT: CB[7] at molar ratios of 1:10 and 1:25) were added to polymer solution to obtain the drug-loaded hydrogel systems with the indicated polymer concentrations (15 and 20 wt%).

2.7. Transmission electron microscopy (TEM)

Microscopic images of PLGA-PEG-PLGA micelles and PLGA-PEG-PLGA@hCT-CB[7] (hCT: CB[7] at molar ratios of 1:25) were obtained by TEM (Tecni G2 20 TWIN, FEI). Sample solutions (20 µL, 1 wt% copolymer) were dropped on a copper grid coated with a thin carbon film and observed under an accelerating voltage of 200 kV. Samples were dried at room temperature.

2.8. In vitro drug release

2 mL drug-loaded uniform polymer solutions were transferred to 15-mL vials. Then, these delivery systems were incubated in a water bath at 35 °C for 10 min to form stable gel systems loaded with drugs. 10 mL of PBS (pH = 7.4) was added to each vial as release media. These vials were kept in a shaking water bath with 100 strokes per minute at 37 °C for the entire period of study. At each predetermined time (1, 2, 4, 8, 12, 24, 48, 72, 96, 144, 192, 240, 288, 336, 384, 432, 480, 600, 720, 840 h), 2 mL of supernatant solution was withdrawn from each vial, and then added 2 mL fresh buffer. These collected solutions were analysed by UV spectrum. The absorbance at 214 nm was monitored and three measurements were averaged.

2.9. Cell viability test

The cytotoxicity of PLGA-PEG-PLGA was evaluated via CCK-8 assay. L929 cells were cultured to the third generation. Cells were seeded in a 96-well plate at an initial density of 10$^4$ cells/well and cultured in 100 µL minimum essential medium DMEM, supplemented with 10% heat-inactivated FBS, 100 units/mL of penicillin and 100 µg/mL of streptomycin at 37 °C, under an atmosphere of 5% CO$_2$ and 95% relative humidity for 24 h. Then, 100 µL fresh culture media containing serial dilutions of polymer, hCT-CB[7] complex and mixed delivery system (filtration is applied to eliminate bacteria) was added to each well incubated for 24 h. After that, cells were incubated with 20 µL CCK-8 for 4 h. The CCK-8 assay was performed by a microplate reader at 450 nm to measure the optical density (OD) values.

In vitro cytotoxicity of PLGA-PEG-PLGA and hCT-CB[7] complex were also examined by Olympus IX71. Alive and dead cells were stained with LG and PI, respectively. First, solutions of hCT-CB[7] complex, PLGA-PEG-PLGA with or without hCT-CB[7] were separately filtered through 0.45 µm filters for further usage. L929 cells in culture media were grown for 24 h in 6-well cell culture plates (2$^{10^3}$ cells/well). Then, the culture media was replaced by fresh systems mentioned above. After 24 h, the culture media was removed and the cells were washed three times with PBS. Next, cells were stained by LG and PI. Finally, the cover slips

### Table 1

Molecular Weight and Composition of Synthesized PLGA-PEG-PLGA Triblock Copolymer.

<table>
<thead>
<tr>
<th>Copolymer</th>
<th>Block length $^a$</th>
<th>LA/GA (mol/mol) $^a$</th>
<th>$M_0$ $^b$</th>
<th>$M_n/M_0$ $^b$</th>
<th>Morphology</th>
<th>Solution stability $^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLGA-PEG-PLGA</td>
<td>1880–1500–1880</td>
<td>2.5/1</td>
<td>7480</td>
<td>1.27</td>
<td>Sticky paste</td>
<td>Yes</td>
</tr>
</tbody>
</table>

$^a$ PEG number-average $M_n$ was provided by Aldrich, block length of PLGA and molar ratio LA/GA was calculated from $^1$H NMR.  
$^b$ Weight-average molecular weight (MW) over $M_n$ of PLGA-PEG-PLGA triblock copolymer obtained from GPC.  
$^c$ Stability of solution means that copolymer do not be precipitated in PBS solution at room temperature.
were placed on a glass microscope slide and the prepared samples were subjected for fluorescence images.

3. Results and discussion

3.1. Synthesis and characterizations of PLGA-PEG-PLGA triblock copolymer

The PLGA-PEG-PLGA triblock copolymer was synthesized by typical ring-opening polymerization of LA and GA using PEG as a macromolecular initiator in the presence of the catalyst, Sn(Oct)$_2$. Fig. 1a displays the $^1$H NMR spectrum of the PLGA-PEG-PLGA triblock copolymer in CDCl$_3$. The proton signal peaks appearing at 3.65 ppm (-CH$_2$CH$_2$O-), 4.8 ppm (-CH$_2$COO-) and 5.25 ppm (-CH(CH$_3$)COO-) were used to calculate the number-average MW and LA/GA molar ratio. $^{13}$C NMR spectrum of the product of PLGA-PEG-PLGA copolymer showed the peaks at 60.7, 69.0 and 70.4 ppm for the carbon skeleton (Fig. 1b). In order to further determine MW and MW distribution, GPC analysis was performed and the GPC trace of the copolymer exhibited a unimodal manner with a molar mass dispersity $(M_w/M_n)$ of 1.27 (Fig. 2a). The sample were also measured via MALDI-TOF (Fig. 2b), which confirms the formation of PLGA-PEG-PLGA triblock copolymer. The detailed results of characterization of the copolymer used in this study are summarized in Table 1. Considering the gel strength, degradation rate and sol-gel phase transition temperature for drug delivery, the PLGA-PEG-PLGA used in this work has a block length of 1880-1500-1880, i.e., the PEG is 1500 (repeating unit: 34) and PLGA is 1880 (repeating unit of LA and GA are 27.9 and 11.1, respectively), after testing a series of ratios in our preliminary study.

3.2. Phase diagram of thermogelling block copolymer in PBS

First, we investigated the occurrence of the sol-gel transition of PLGA-PEG-PLGA triblock copolymer aqueous solution. With an increase of temperature, we found that the polymer/water system was able to convert from a low viscous fluid to a translucent, semisolid thermogel within 1 min at a specific temperature, at which point a physically cross-linked network was formed. When the thermogel was put at low temperature, it turned into a clear solution again, indicating its thermoreversible property. Fig. 3a displays the phase diagram of PLGA-PEG-PLGA triblock copolymer
aqueous solutions using temperature and concentration as coordinates. In the examined physiologically important temperature range of 10–55 °C, the polymer/water system underwent three physical states: sol, gel, sol (suspension) as the temperature increased. Both the critical gel concentration (CGC) and sol-gel transition temperature could be detected by using the phase diagram. For example, the CGC was about 4 wt%. Also, the sol-gel transition temperatures of 20 wt% and 15 wt% PLGA-PEG-PLGA copolymer solutions in PBS were 35 °C and 36 °C, respectively. The sol-gel transition temperature in the case is below body temperature, thus this system is suitable for biomedical applications. So we selected the PLGA-PEG-PLGA thermogel at the concentration of 15 and 20 wt% for subsequent experiments.

The temperature-induced physical gelation remained after adding of hCT-CB[7] complex. For example, 20 wt% copolymer solution containing hCT-CB[7] (1:25) showed a typical sol-gel transformation phenomenon as presented in Fig. 3b and c. The gelation happened in about 1 min after raising temperature to 35 °C. All of hCT-CB[7] complex were thus spontaneously entrapped into this hydrogel matrix.

3.3. In vitro degradation of thermogel

Biodegradability is vital for biomaterials. The degradation of PLGA-PEG-PLGA thermogel mainly depends on the hydrolysis of PLGA segments (Yu et al., 2010). In this study, we examined the degradation of 20 wt% PLGA-PEG-PLGA thermogel in PBS at 37 °C. The gels gradually swelled and the initial translucent gel state turned into the opaque one following degradation.

Some remaining gels were taken out and lyophilized at the designated time points. The dried samples were weighed to analyse the mass loss and MW change during this degradation process. This time-dependent weight change of the sample is shown in Fig. 4a. The PLGA-PEG-PLGA hydrogel showed a gradually weight decrease and nearly 70% mass loss occurred within 35 days of in vitro degradation. The GPC trace of the triblock copolymer before degradation was unimodal (Fig. 4b) and the single-peak profile maintained before 20 days, follow by exhibiting a bimodal manner. Meanwhile, a gradual decrease of MW was observed by an increase of the retention time as the hydrolysis proceeded.

3.4. Microscopic images of PLGA-PEG-PLGA and PLGA-PEG-PLGA@hCT-CB[7]

Amphiphilic copolymers tend to self-assemble into micelles in aqueous solutions. TEM image shows typical micelles formed at a concentration of 1 wt% PLGA-PEG-PLGA copolymer in water, as demonstrated in Fig. 5a. The self-assembly micelles were spherical in shape with diameters in this image are approximately 20 nm. Incubation of hCT-CB[7] complex in aqueous solution led to
nanoparticles with 20–30 nm in diameter. As illustrated in Fig. 5b, the particles of PLGA-PEG-PLGA@hCT-CB[7] increased slightly.

### 3.5. In vitro release of hCT from PLGA-PEG-PLGA hydrogel

2 mL drug-containing uniform polymer solutions were transformed into stable hydrogels at 37 °C. The loading capacity of hCT-CB[7] complexes (hCT: CB[7] at molar ratios of 1:10 and 1:25) in PLGA-PEG-PLGA copolymer (20 wt%) were 0.22% and 0.48%, respectively, while calculating the total amount of hydrogel as the carrier. Meanwhile, since hCT-CB[7] complex and PLGA-PEG-PLGA copolymer were both completely dissolved in PBS buffer, with the increase of temperature, the system was able to convert from a clear solution to a stable thermogel, thus all hCT-CB[7] complex can be encapsulated in the thermogel.

The release profile of hCT was monitored by UV absorption at 214 nm. The maximum absorption wavelengths of hCT and hCT-CB[7] complex are very similar (Fig. 6a), which indicates that the introduction of CB[7] has little effect on hCT conformation. In Fig. 6b, the release of drug from thermogel is plotted against time. The influence of PLGA-PEG-PLGA copolymer concentration and different complexation ratios of hCT-CB[7] on the release profile was examined and compared. A low burst release effect was observed in the 15 wt% thermogel system loaded with hCT due to the good solubility of drug in water. Increasing the carrier polymer concentration resulted in a decrease in the drug release rate, which was attributed to the stronger gel strength with higher polymer concentration. The complexation of hCT-CB[7] also effectively delayed the drug diffusion from the gel matrix, however, no significant difference was observed between the two complexation ratios of hCT-CB[7]. In particular, the 20 wt% thermogel system containing hCT-CB[7] complex exhibited a sustained release manner over three weeks. Therefore, both the barrier of thermo-responsive copolymer and the complexation of hCT-CB[7] play important roles in adjusting hCT release rate, and a desired release profile was achieved by their combination. As compared with the data of other commercially available drug-loaded thermogel systems such as Pluronic F127 (composed of block copolymer of PEG and poly(propyl glycol)), which also exhibits sol-gel transition upon heating but release almost all drugs within 2 days (Chen et al., 2016a, 2016b), the PLGA-PEG-PLGA copolymer reported in this work can form stable gel system and release drug for much longer time.

### 3.6. In vitro cytotoxicity measurement

In order to confirm the biocompatibility of this delivery system, standard CCK-8 assay was carried out to evaluate the cell cytotoxicity (Fig. 7a). PLGA-PEG-PLGA copolymer did not show obvious cytotoxicity even at a concentration up to 5 mg/mL, which demonstrates its good biocompatibility for drug delivery. Meanwhile, the drug-loaded PLGA-PEG-PLGA@hCT-CB[7] (hCT-CB[7] = 1:25) were also evaluated at the same concentration range. The cell viability of PLGA-PEG-PLGA@hCT-CB[7] was higher than 91% even at the highest concentration, indicating its high
biocompatibility as well. Furthermore, the cell viability against hCT-CB[7] at ratio of 1:25 were tested and a slightly increased cell viability was observed as a function of drug concentration (Fig. 7b). So, the copolymer, hCT-CB[7] and PLGA-PEG-PLGA@hCT-CB[7] (hCT-CB[7] = 1:25) all exhibited good biocompatibility with low cell cytotoxicity.

To further evaluate the in vitro cytotoxicity of this PLGA-PEG-PLGA@hCT-CB[7] (hCT-CB[7] = 1:25), we performed alive/dead cells stain with fluorescent dye. LG is cell-permeant acid stains that show a large fluorescence enhancement upon bind lysosome. The PI dye can be used to stain dead eukaryotic cells. A combined use of these two dyes can indicate the number and proportion of alive/dead cells (Fig. 8). Cytotoxicity of L929 cells was compared to the blank control in which cells have been cultured without delivery system in the culture medium (almost no dead cells). It only showed sporadic dead cells even at the highest concentration (5 mg/mL) of PLGA-PEG-PLGA copolymer and PLGA-PEG-PLGA@hCT-CB[7] (hCT-CB[7] = 1:25). As for cells treated with hCT-CB[7] complex (hCT: 0.5 mg/mL), we can hardly see dead cells. These findings are well coincided with the results of CCK-8 measurements.

4. Conclusions

This paper reports a biodegradable and biocompatible PLGA-PEG-PLGA thermogel system loaded with hCT-CB[7] complex for sustained drug delivery. The drug-loaded thermogel system was a low-viscous sol at room temperature and spontaneously turned into a non-flowing gel at body temperature. Both PLGA-PEG-PLGA copolymer concentration and different ratios of hCT-CB[7] complex influence this release process. When we used 20 wt% hydrogel loaded with hCT-CB[7] complex at the ratio of 1:25, the system exhibited good stability and hCT was released in a sustained manner for approximately 35 days, without a significant initial burst and with almost complete release at neutral pH. The CCK-8 assay and alive/dead cells test confirm these delivery systems were of low cytotoxicity. This current combination strategy might also be applied to control the release of other peptide/protein drugs.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (21534008 and 21474019), and State Key Project of Research and Development (grant No. 2016YFC1100300).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at 10.1016/j.ijpharm.2017.05.006.

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