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Huperzine A–phospholipid complex-loaded biodegradable thermosensitive polymer gel for controlled drug release

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ABSTRACT

The huperzine A–phospholipid complex loaded biodegradable thermosensitive PLGA–PEG–PLGA polymer gel was studied as injectable implant system for controlled release of huperzine-A (HA). First, HA molecules were successfully incorporated into the soybean phosphatidylcholine (SP) molecules to form the huperzine-A–soybean phosphatidylcholine complexes (HA–SPC), which was proved by FT-IR, DSC, XRD, solubility study, TEM, etc. The results indicated that hydrogen bonds and electrostatic interaction between HA and SP molecules play an important role in the formation of HA–SPC. Secondly, the HA–SPC was loaded into biodegradable PLGA–PEG–PLGA thermosensitive gel as injectable implant material to control the release of HA. The *in vitro* and *in vivo* drug release behaviors of the prepared products were studied. The *in vitro* release studies demonstrated that the HA–SPC-loaded gel significantly reduced the initial burst of drug release and extended the release period to about 2 weeks. The *in vivo* pharmacokinetics study of HA–SPC-loaded gel in rabbits showed that plasma concentration of HA (2.54–0.15 ng/mL) was detected for nearly 2 weeks from delivery systems upon single subcutaneous injection. What's more, the *in vitro* release pattern correlated well with the *in vivo* pharmacokinetics profile. The present study indicates that HA–SPC loaded PLGA–PEG–PLGA thermal gel may be an attractive candidate vehicle for controlled HA release.

1. Introduction

Alzheimer's disease (AD) is a progressive, neurodegenerative disease characterized by memory loss, language deterioration, impaired visuospatial skills, poor judgment, indifferent attitude, but preserved motor function. Studies focusing on the pathogenetic mechanism have revealed that cholinergic abnormalities are associated with the disturbance of cognitive function in patients with AD and inhibition of the brain acetylcholinesterase (AChE) to increase the synaptic concentration of acetylcholine (ACh) may improve the cognitive dysfunction (Gao et al., 2007). Huperzine-A (HA) is a potent, reversible acetylcholinesterase inhibitor (AChEI), which crosses the blood–brain barrier smoothly, and shows high specificity for acetylcholinesterase (Wang and Tang, 2005; Liang and Tang, 2004). HA has been proved one of the most promising agents for palliative therapy of cognitive deficits in patients with AD (Gao et al., 2007). At present, HA is available in the market as either tablet or capsule, which has to be given 2–3 times per day (Xu et al., 1997; Sun et al., 1999). It is well known that AD is a chronic progressive disease, and patients with AD have decreased memory, so long-term therapy with HA is hard to persist, and over dosing results in adverse effects or underdosing so that a therapeutic level is not reached can also easily occur (Ye et al., 2008). Thus, a sustained release dosage form for long periods of time avoids daily administration, which is the best way to improve patient compliance and secure the therapeutic efficiency.

Hwang and Liu reported a once-a-week transdermal delivery system of HA (Hwang and Liu, 2002). This system can deliver HA constantly into the body through skin and increase the efficacy and convenience for outpatient care of AD patients. But the complication of human skin may impact the release rate, and may vary from one to another, resulting in a variation in plasma concentration and efficacy (Liu et al., 2005). Entrapment of HA in biodegradable poly(lactic-co-glycolicacid) (PLGA) microspheres has also been widely investigated as a technique to produce sustained release formulations. However, microspheres formulations may cause an acute tissue reaction, and possible transient irritation resulting from the presence of particles (Chen and Singh, 2008).

Block co-polymers containing polyethyleneglycol (PEG)–polyester (PET) are gaining importance in controlled release due to their thermogelling, biocompatible and biodegradable properties (Chen et al., 2005; Fang et al., 2009; Bethnia

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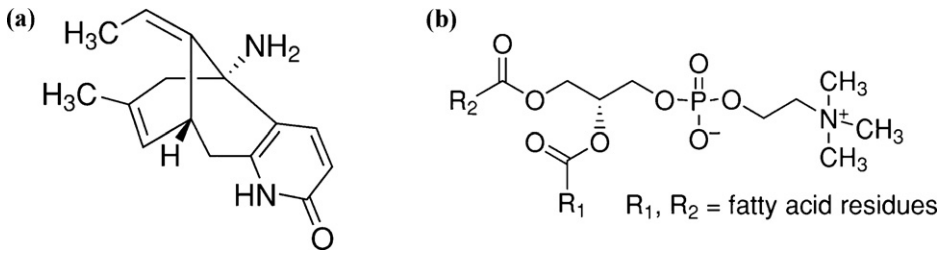

Fig. 1. The molecular structure of HA (a) and SP (b).

et al., 2012). Compared to the widely utilized PLGA, drug delivery systems based on hydrophilic–hydrophobic block copolymers have some unique advantages. Incorporation of hydrophilic blocks in a hydrophobic polymer can modify the degradation rate as well as the permeability of the matrix, leading to release kinetics that can be readily modulated by adjusting the copolymer composition (Chen and Singh, 2008; Wei et al., 2009). Their compositions can be tailored to provide drug delivery over weeks after single injection. The group of Qian (Gong et al., 2009a,b) successfully synthesized the biodegradable, thermosensitive and injectable copolymer (PEG–PCL–PEG; PCL–PEG–PCL) hydrogels. The *in vitro* degradation shows that both the molecular weight of copolymers and the degradation temperature have an influence on the *in vitro* degradation behavior of copolymers. The cytotoxicity study shows that they are biocompatible with low cell cytotoxicity. Different model drugs such as hydrophilic small-molecule drug (VB_{12}), hydrophobic small-molecule drug (honokiol) and protein drug (BSA) are loaded in the gels and the *in vitro* release results indicate that all of those drug could be released from the gels for a longer period. Among the reported block copolymers, PLGA–PEG–PLGA is one of the most widely used polymers as the carrier for the sustained drug release (Duvvuri et al., 2005; Zentner et al., 2001). Its aqueous formulation is a free flowing solution at/below room temperature and forms a hydrogel at body temperature (Pratoomsoot et al., 2008).

Although the potential of PLGA–PEG–PLGA has been well recognized, especially for drug delivery, there is one problem still existing in these systems as carrier for the water-soluble drugs. It has been reported that the hydrophilic drug tends to partition into the hydrophilic PEG domain of the hydrogel (Qiao et al., 2005). Thus, for the soluble drug in water, release from the gel is often rapidly than that of insoluble drug. Hence, improving the liposolubility of drug is an attractive way to reduce its release rate from the gel. Incorporating an active drug with the phospholipids can increase the solubility of highly lipophilic drug and consequently enhance the bioavailability of drugs (Cui et al., 2006). Also, as a gel usually consists of more than 70% water, the drug molecule can diffuse readily through the gel. Thus, for the water-soluble drug release from the gel is often rapid at first, namely burst release. Hence, adopting a strategy to reduce the burst release of gel is necessary to obtain a fully efficient pharmaceutical gel. To the best of our knowledge, there is no report on the study of HA–SPC loaded in PLGA–PEG–PLGA gel as injectable implant system for controlled release of HA so far.

Therefore, the aim of the present work was to formulate the HA–SPC to improve the drug solubility and simultaneously loaded the HA–SPC into PLGA–PEG–PLGA gel for reducing the burst effect and controlling the HA release. The characterizations of HA–SPC and physicochemical properties of PLGA–PEG–PLGA gel were systematically investigated by the aid of transmission electron microscopy (TEM), Fourier transform infrared spectrophotometry (FT-IR), differential scanning calorimetry (DSC), X-ray diffractogram (XRD), solubilization and viscoelasticity measurements. Moreover, the *in vitro* and *in vivo* drug release behaviors of the prepared products were studied. Both the *in vitro* and *in vivo* release results demonstrated that the HA–SPC-loaded polymer gel could significantly reduced the initial burst of drug release and extended the release period to about 2 weeks. And the *in vitro* release pattern correlated well with the *in vivo* pharmacokinetics profile. The present studies indicate that the HA–SP complexes loaded PLGA–PEG–PLGA thermal gel is an attractive candidate vehicle for controlled release of HA as injectable implant.

2. Experimental

2.1. Materials

The copolymer with PLGA block and PEG block was supplied by Daigang Biotechnology Co. Ltd. (Jinan, China). The average molecular weights of PLGA–PEG–PLGA (2550–1500–2550) was determined by NMR, 2550 represents the molecular weight of PLGA block and 1500 represents the molecular weight of PEG block. Huperzine A (HA, purity > 99.8%) was provided by the Ningbo Traditional Chinese Pharmaceutical Co. Ltd. (Zhejiang, China). Soybean phosphatidylcholine (SP) was purchased from Beijing Biodee Biotechnology Co. Ltd. (Beijing, China). Tetrahydrofuran was bought from Fuyu Fine Chemicals Limited Company (Tianjin, China). Diphenhydramine hydrochloride (purity > 99.4%) was provided by National Institutes for Food and Drug Control (Beijing, China). Double-distilled water was used in all the experiments. All other reagents were of analytical grade. The molecular structures of HA and SP are schematically shown in Fig. 1.

2.2. Preparation and characterization of HA–SPC

2.2.1. Preparation of HA–SPC

The complexes were prepared using solvent evaporation method with HA and SP at an equimolar ratio. In brief, the required amounts of HA and SP were dissolved in tetrahydrofuran in a 50 mL round-bottom flask to react for 2 h at 50 °C. The solution was completely evaporated off under vacuum at 40 °C, and then the dried HA–SPC were gathered and placed in desiccators.

2.2.2. Characterization of the morphology and size of HA–SPC dispersed in aqueous solution

The morphology of HA–SPC in the aqueous solution was observed using a transmission electron microscopy (TEM. JEM-100CXII). After dilution with double distilled water, one drop of the HA–SPC dispersions was adsorbed onto carbon-coated copper grids and allowed to adhere. The excess sample was drawn off with filter paper. A drop of 2.0% (w/w) phosphotungstic acid solution was immediately added and the sample was kept for 20 s for completely dyeing. The sample was dried and finally viewed on a TEM. The size of HA–SPC was determined by photon correlation spectroscopy (PCS) using Delsa™Nano Submicron Particle Size Analyzer (Beckman Coulter Inc., USA). The samples are located in cuvettes at room temperature.

2.2.3. Solubility studies

Solubility studies were carried out according to the reference (Maiti et al., 2007). Briefly, excess amount of HA and HA–SPC was added to 5 mL water or *n*-octanol in a sealed glass container at 25 °C. The liquids were agitated for 48 h and then centrifuged to remove excessive HA or HA–SPC. The obtained solution was filtrated through a 0.45 μm membrane. Then the absorbance of each system was recorded in ultraviolet-visible spectrophotometer (WFZ UV-2102pcs, UNICO).

2.2.4. FT-IR, DSC and XRD characterization of HA–SPC

FT-IR, DSC and XRD were used to characterize the HA–SPC. FT-IR experiments are performing using Bio-Rad Laboratories, Nicolet NEXUS 470 FT-IR Spectrometer. DSC experiments are performed using CDR-4P (Shanghai Precision and Scientific Instrument Co. Ltd., China). The XRD experiments are done on a Rigaku Dmax-rc X-ray diffractometer with Ni filtered Cu Kα radiation.

2.3. Characterization of PLGA–PEG–PLGA copolymer hydrogel

2.3.1. Thermogelling properties

Sol to gel transition temperature of the PLGA–PEG–PLGA copolymers were determined by tube inversion method (Li et al., 1997). A series of different concentration of the PLGA–PEG–PLGA copolymers solutions (15%, 18%, 20%, 23%, 25%, 28%, w/w) were prepared in double-distilled water and 3 mL of the given solution was transferred to the capped glass test tubes. The tubes were then transferred into water bath and heated from 20 to 80 °C. At each 1 °C interval, the tubes were inverted to check the flow properties. Solutions were considered to be in the gel state if no flow was observed for 30 s following tube inversion.

2.3.2. Rheology studies

The rheological properties of PLGA–PEG–PLGA polymer solution with 25% (w/w) concentration were measured using a dynamic shear rheometer with a concentric cylinder measurement cell (Rheostress RS75 HAAKE). The slit between the inner cylinder and the outer cylinder was 3 mm. The solution was transferred to a concentric cylinder for measurement. The plate was equilibrated to the starting temperature of 29 °C and temperature sweep tests were carried out in a range of 29–37 °C. Initially, a strain sweep measurement was made on each sample to define the linear viscoelastic region, then the elastic module (G') and viscous module (G'') were determined. Dynamic frequency spectra were obtained in the linear viscoelastic regime of the samples. In dynamic rheology, the elastic modulus G' is the in-phase component, which provides information about the elastic nature of the material. The viscous modulus G'', on the other hand, is the out-of-phase component, which characterizes the viscous nature of the material (Lee et al., 2005).

2.4. Drug release studies

2.4.1. Preparation of thermosensitive gel loaded with HA–SPC

HA–SPC was added to 25% (w/w) copolymer aqueous solution and homogenized at 4000 rpm to form a homogeneous clear solution at room temperature. The polymer gel was loaded into 1 mL syringe and pushed through 25-gauge needle to investigate its injectability (Molina et al., 2001).

2.4.2. In vitro release study

The *in vitro* releases of HA solution and HA–SPC-loaded gel was measured in phosphate-buffered saline (PBS) with pH 7.4 containing NaN_3 (0.025%, w/w) at 37 °C. 1 mL polymer solution formulation of HA was injected into a 10 mL test tube and transferred to water bath maintained at 37 °C for 10 min to form a gel. After gelling, 3 mL of PBS containing NaN_3 (0.025%, w/w) was added to the tube. The tube containing gel was kept in a reciprocal shaking water bath maintained at 37 °C with the shaking at 35 rpm. The pure HA without polymer was also studied as controls. At the predetermined time intervals, 1 mL of the release solution was withdrawn from the container. Then, an equal amount of fresh PBS solution was added to replenish the sample to maintain the original volume. The release solution was determined from the absorbance at 307 nm which is a typical absorbance peak of HA by a UV spectrophotometer (WFZ UV-2102pcs, UNICO).

2.4.3. Drug release kinetics

The kinetics of HA release from the products was determined by fitting the curves (% release against time) to distinct models. Data were fitted using Origin 7.5 (Microcal Software, Inc., MA, USA) and the linearised form of each function was evaluated using R regression analysis.

2.5. In vivo release in rabbits

New Zealand White rabbits with 10 weeks old and 1.5–2.0 kg body weight were used in this study. All animal experiments complied with the requirements of the National Act on the use of experimental animals (People's Republic of China). 1 mL thermosensitive polymer formulation containing HA–SPC (1 mg of HA) was injected subcutaneously at the back neck of the rabbits. HA physiological saline solution without polymer was also administered subcutaneously as controls. At designated times, the blood samples were taken and placed into heparinized test tubes. Plasma was immediately separated by centrifugation at 3000 rpm for 10 min. Plasma samples were immediately frozen and maintained at −20 °C until analysis.

The 0.5 mL plasma was adjusted by adding 100 μL of sodium carbonate solution (5%). Diphenhydramine (100 μL) was added as the internal standard. The mixture was extracted with 3 mL ethyl acetate by vortex-mixing for 4 min. The organic layer was separated by centrifugation at 3000 rpm for 10 min, transferred to a clean tube and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue were dissolved in 100 μL mobile phase and vortexed for 1 min. Finally, 20 μL of the sample was injected for HPLC–MS–MS (Agilent G6410 B triple quadrupole mass spectrometer) analysis.

3. Results and discussion

3.1. Morphology and size characterization

TEM was performed to characterize the morphology of HA–SPC dispersed in the aqueous solution, as shown in Fig. 2a. From the TEM image it can be seen that there are two types of structures existing in the HA–SPC dispersed solution. One is the vesicular structure which is fabricated by self-assembly of complexes in the aqueous solution; the other are small complexes with the diameter of about 10 nm. The size and size distribution of the HA–SPC dispersed in the aqueous solution were determined by DLS measurement, shown in Fig. 2b. The mean hydrodynamic diameter (D_h) is about 120 nm with a relatively narrow polydispersity index of 0.189. While no vesicles were formed in the pure HA dispersed solution. This indicates the formation of HA–SPC. Considering the structures of HA and SP it is thought that the hydrogen bonds can be formed within the $-NH_2$ of HA molecules and the $-PO_4^-$ of SP molecules. Simultaneously, the $-NH_2$ of HA molecules are ready to protonate to $-NH_3^+$ in the aqueous solution which can interact with $-PO_4^-$ of SP molecules by static electronic attraction. Thus, the HA–SP complexes are formed mainly by hydrogen bonds and electrostatic attraction. This result is similar with the reports that

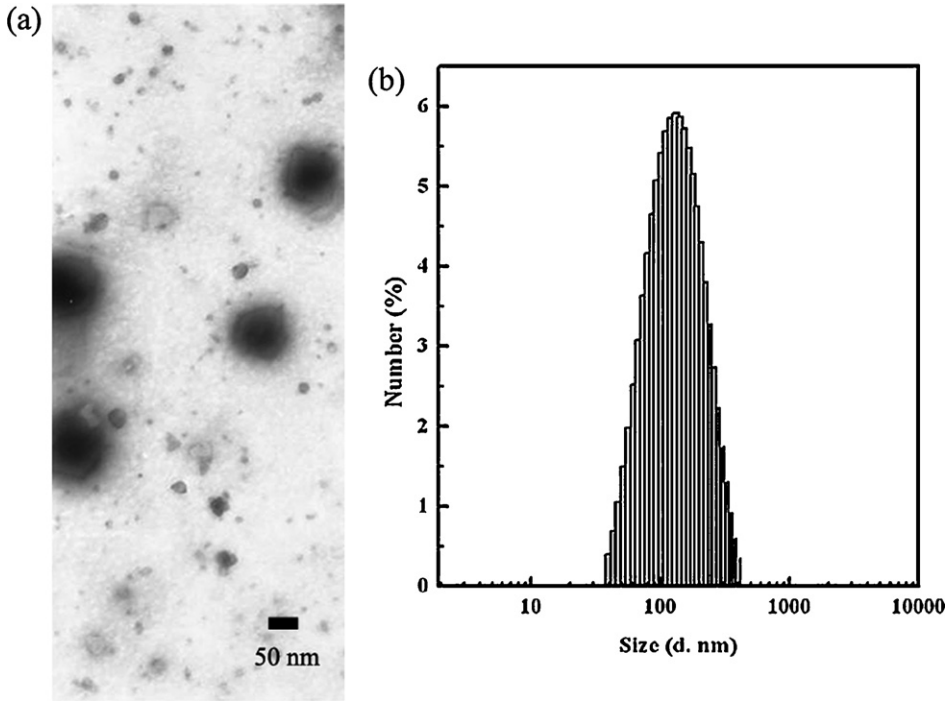

Fig. 2. TEM image of HA–SPC dispersed in distilled water (a) and the size distribution of HA–SPC determined by DLS (b).

SP molecules could form the complexes with insulin and silybin by non-covalent bonds (Cui et al., 2006; Xiao et al., 2006).

3.2. Solubility studies

Solubility studies were conducted to check the solubility of HA after the formation of HA–SP complexes. Table 1 show the solubility of HA and HA–SP complexes in water and *n*-octanol. Each experiment was performed in triplicate and the stand deviation was given. For the pure HA, the data show that it has more liposolubility than water-solubility, that is, HA has much higher solubility (12.1 mg mL^{-1}) in *n*-octanol than that in water (1.41 mg mL^{-1}) and PBS (pH 7.4, 1.15 mg mL^{-1}). The solubility in *n*-octanol is about 9 times of that in the water. When HA molecules are formed complexes with SP molecules the solubilities of HA in the three studied media are all enhanced. While the liposolubility is much more increased than the water-solubility. The solubility of HA in *n*-octanol is increased about 2.5 times while the solubility of HA in water is enhanced about 1.5 times. What's more, the liposolubility of HA in HA–SPC about 14 times of that water-solubility. The higher solubility means the higher bioavailability.

3.3. FT-IR spectra analysis

The FT-IR spectra performed for pure HA, SP and the complexes are shown in Fig. 3. By comparing the FT-IR spectra, there was a significant difference between the pure HA and the complex. The FT-IR spectrum of pure HA shows two typical bands at 3368 cm^{-1} and 3267 cm^{-1} belonging to the N—H primary stretching vibration. However, in the spectrum of the complex, the characteristic absorption peaks of HA are masked by that of SP. The characteristic absorption peak of phosphate group at 1238 cm^{-1} are shifted to lower field in the spectrum of HA–SPC, indicating the formation of hydrogen bonds and the existence of electrostatic interaction between HA molecules and SP molecules. These observations suggest that some weak physical interactions between HA and SP took place during the formation of the complex (Cui et al., 2006).

3.4. DSC measurements

Differential scanning calorimetry (DSC) is a fast and reliable method to study drug-excipient compatibility and provides information about the possible interactions (Maiti et al., 2007). Fig. 4 shows the DSC curves of pure HA (a), SP (b), HA–SPC (c). The DSC result of pure HA shows a endothermic peak at 230 °C, which is in good agreement with the reference report of 232 °C[2], and the first one (~80 °C) might be caused by the phase transition of HA molecules from solid state to liquid state according to the reference (Ruan et al., 2010). The DSC result of pure phospholipid powder shows two endothermic peaks with the temperature of 187 °C and 196 °C, which could be possibly owed to the transition of SP molecules from gel state to liquid crystal state and the melting of carbon–hydrogen chain in phospholipids and changes of isomerous or the crystal. For the HA–SPC, the melting peak of HA disappears, which indicates the drug is amorphous in the complex. The peak at 198 °C is attributed to the melting transition of SP in the complex, which is different from the peak of pure SP. The different melting temperature of SP indicates that the SP in the complex is

Table 1
Solubility of HA and HA–SPC in water and *n*-octanol at 25 °C (*n* = 3).

Samples	Solubility in water (mg/mL)	Solubility in PBS (pH 7.4) (mg/mL)	Solubility in *n*-octylalcohol (mg/mL)
HA	1.408 ± 0.03	1.152 ± 0.06	12.134 ± 0.02
HA–SPC	2.192 ± 0.05	1.766 ± 0.07	30.331 ± 0.03

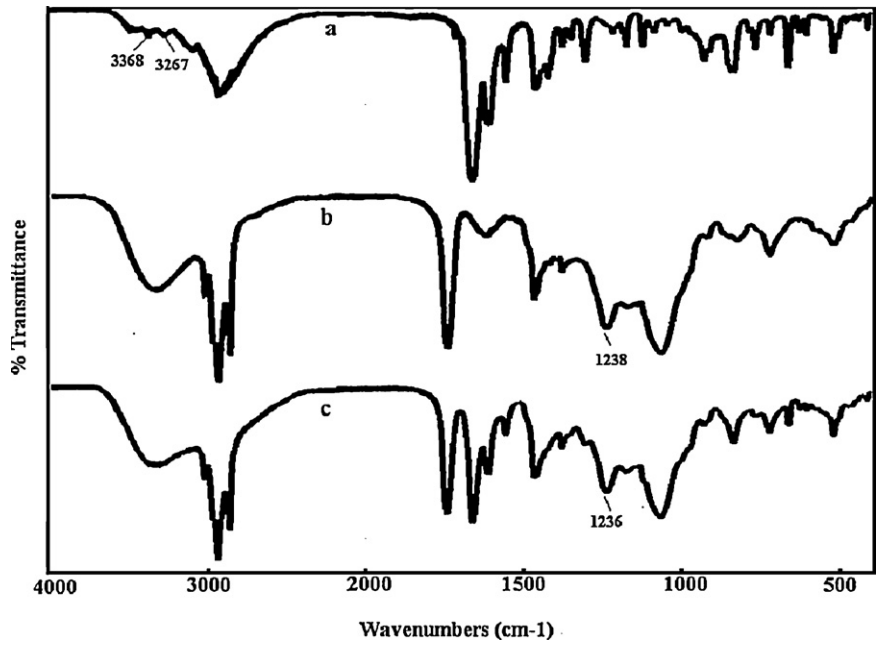

Fig. 3. FT-IR spectra of pure HA (a), pure SP (b), and the HA–SPC (c).

not crystallized as well as the raw one. All the results suggest that some weak interactions, such as the hydrogen bonds or van der Waals force are formed between HA and SP molecules (Xiao et al., 2006).

3.5. XRD measurements

X-ray diffraction (XRD) patterns of the pure HA, pure SP and the HA–SPC are carried out to study the physical state of the samples. As shown in Fig. 5a, HA shows sharp peaks which indicates it is in crystalline state. For the pure SP powder, there only exists a broad XRD peak around $2\theta = 20.1^\circ$ (Fig. 5b), indicating an amorphous structure. The HA–SPC shows only one broad peak at 2θ of 20.8° (Fig. 5c), and the crystalline peaks of HA disappear in the complexes. This suggested that the HA in the phospholipids lipid matrix was in amorphous form (Yue et al., 2010).

3.6. Thermogelling properties of gel

Different amount of copolymer was completely dissolved in distilled water as clear solution for the polymer concentration from 15% (w/w) to 28% (w/w) at 4 °C. These copolymer solutions showed a sol–gel–sol transition with the elevated temperature. The thermosensitive sol–gel transition of PLGA–PEG–PLGA triblock copolymer solutions were summarized in the phase diagram, shown in Fig. 6a. In the studied polymer concentration from 15% (w/w) to 28% (w/w), the triblock copolymer solutions present three physical states: solution, gel, solution (suspension) in the temperature range of 20–70 °C. The sol–gel transition temperature was found to be a function of the polymer concentration. The sol–gel transition temperature slightly decreased with the increase of polymer concentration, while the gel–sol transition temperature markedly increased with the rise of polymer concentration. That is, the temperature range of gel phase becomes wider with

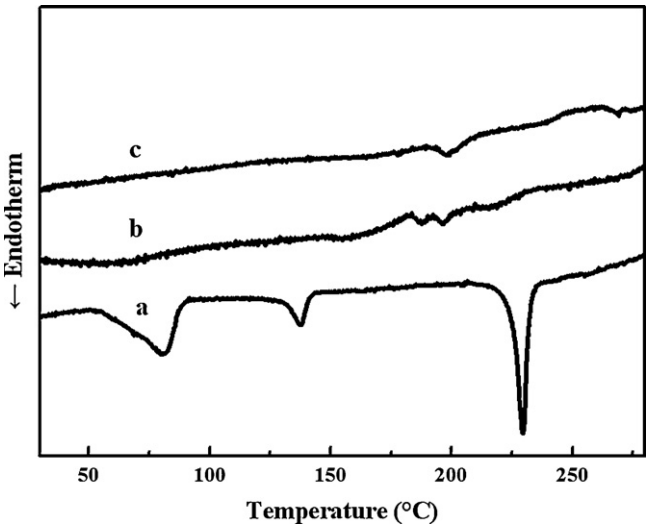

Fig. 4. DSC thermograms of HA (a), SP (b) and HA–SPC (c).

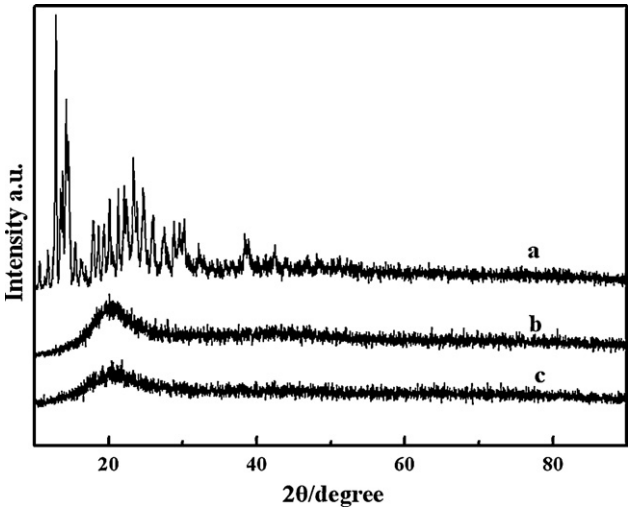

Fig. 5. XRD patterns of HA (a), SP (b) and HA–SPC (c).

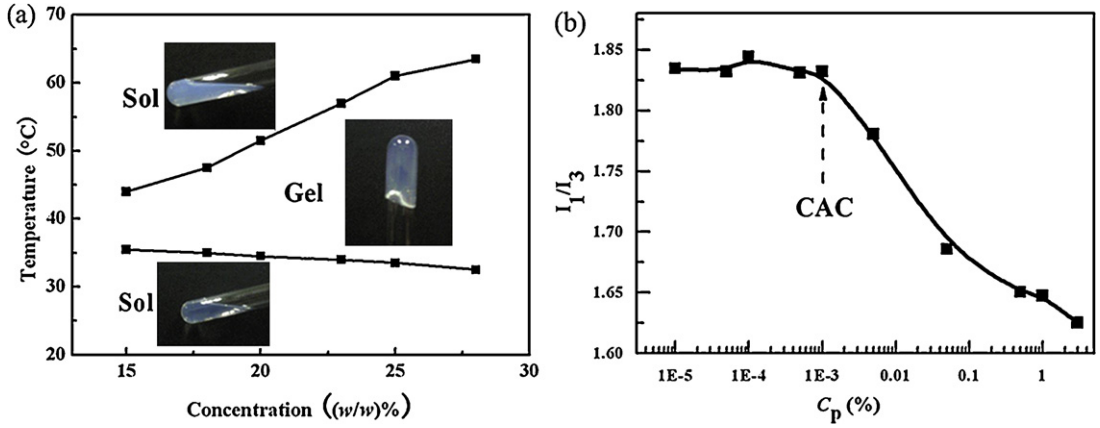

Fig. 6. The phase diagram of PLGA–PEG–PLGA triblock copolymer (a) and the plot of I_1/I_3 *versus* PLEG–PEG–PLGA concentration (%) (b).

the increase of polymer concentration. The gel phase starts from 35.5 °C to 44.0 °C for the polymer concentration of 15%, while it starts from 32.5 °C to 63.5 °C for the polymer concentration of 28%. It has been proved that PLGA–PEG–PLGA copolymer solutions form a gel by packing of the aggregated micelles (Lee et al., 2001). Here the critical agrregate concentration (CAC) of PLGA–PEG–PLGA was determined by pyrene fluorescence probe spectrometry, shown as Fig. 6b. It was shown that I_1/I_3 values are about 1.80 at the beginning, suggesting the pyrene molecules existed in the aqueous solution. Then the I_1/I_3 value starts to decrease at certain concentration which is defined as CAC, about 0.001% (w/w). The fast decrease of I_1/I_3 value indicated that the stronger hydrophobicity of the pyrene molecules located, which suggested the aggregates existed in the solution from this polymer concentration. Increasing polymer concentration means higher aggregate concentration, gelation will be induced by the packing of aggregates, and the fast increase in the numbers of aggregates causes the sol–gel transition at lower temperature. Thus, one can modify polymer concentration to meet specific requirements in drug delivery systems, for example, subcutaneous implantation or injection (Chen et al., 2005).

3.7. Rheology properties of PLGA–PEG–PLGA samples

The angular frequency (ω) dependent of elastic modulus (G') and viscous modulus (G'') for the 25% polymer solution at different temperature are shown in Fig. 7. From the figure it can be seen both G' and G'' have large dependent on the temperature. The plots of G' and G'' *versus* ω at temperature of 29 °C (Fig. 7a) and 31 °C (Fig. 7b) are similar. At these two temperatures, both G' and G'' exhibit a strong dependence of angular frequency. More importantly, at the lower frequency G'' is larger than G', showing very weak elasticity and the viscous dominant property. There is crossover frequency (ω_c) in the two curves. From the reference the relaxation time (τ_r) can be obtained by the formula (Yoshida et al., 2007) of $\tau_r = \omega_c^{-1}$. The relaxation times are 0.131 s and 0.082 s, respectively for the polymer solution at temperature of 29 °C and 31 °C, which also indicates that the elasticity of the sample increases with the rise of temperature. Increasing the temperature to 33 °C, the slopes of G' and G'' on the frequency spectrum become nearly equal, and G' exceeds that of G'', this suggests the transition from sol to gel, which has been reported in many gel systems (Lee et al., 2005; Tung and Dynes, 1982). A further increase in temperature to 37 °C induces a qualitatively different rheological response (Fig. 7d). In this case, G' was higher than that of G''. This indicates elastic behavior, and the lack of frequency dependence implies that the sample does not relax, that is, it has an infinite relaxation time. Thus, the 37 °C sample satisfies the rheological definition of gel (Macosko, 1994). Fig. 7e shows the change in G' and G'' of 25 wt.% polymer solution *versus* temperature. The data are collected under a controlled frequency of 1.0 rad s^{-1} according to the reference (Gong et al., 2009b). From Fig. 7e it can be clearly seen that the G' is smaller than G'' before 32.8 °C, which can be easily understood considering the solution state. After 32.8 °C, the G' is larger than the G'' which indicates that the sample is in the gel state. At 32.8 °C the G' is equal to the G'', which suggests that the sol–gel transition temperature is 32.8 °C.

3.8. In vitro release studies

HA–SPC was dispersed in 25% (w/w) PLGA–PEG–PLGA copolymer solution, forming a homogeneous clear solution under the mild stirring. The formulation easily passed through the 25-gauge needle at room temperature. Injectability was first examined because it is a critical factor for drug delivery systems, as compared with implants that require surgery (Molina et al., 2001).

The *in vitro* release of HA and HA–SPC from gels made of the PLGA–PEG–PLGA triblock copolymers were investigated, respectively. As shown in Fig. 8, the HA (without gels) was rapidly dissolved and released, the release amount reached nearly 100% within 1 day. For the HA-loaded PLGA–PEG–PLGA gel, approximately 34% of the drug is released within 1 day. The amount of HA in day 1 released samples is treated as burst release. This is normally considered to be due to surface located drug and sequential drug release may be due to diffusion of drugs from polymers. However, in the HA–SPC system, approximately 19% of HA was released within the first day from gel. The slower release rate of the drug from the gel may be caused by the following reasons. First, the hydrogen bonds and static electric interaction between HA and SP molecules make HA molecules more difficult to diffuse out the gel; Secondly, the hydrophobicity of HA molecule is increased by the formation of HA–SPC, which leads HA molecules to intercalating into the hydrophobic PLGA domain of the hydrogel (Qiao et al., 2005). Thirdly, the size of the complex is larger than that of the free drug molecule, rendering them unable to move through the gel freely, and this can retard the drug release rate from gel (Brohede et al., 2005). Therefore, HA–SPC-loaded gel can effectively slow down the drug release. After 13 days, approximately 70% of HA was released from HA–SPC-loaded gel. The *in vitro* release result clearly reveals that these HA–SPC-loaded gel could be a suitable polymeric carrier for controlled release of HA.

3.9. Drug release kinetics studies

Mathematical models such as first-order, Higuchi's and Ritger–Peppas models were usually used to describe the kinetics of

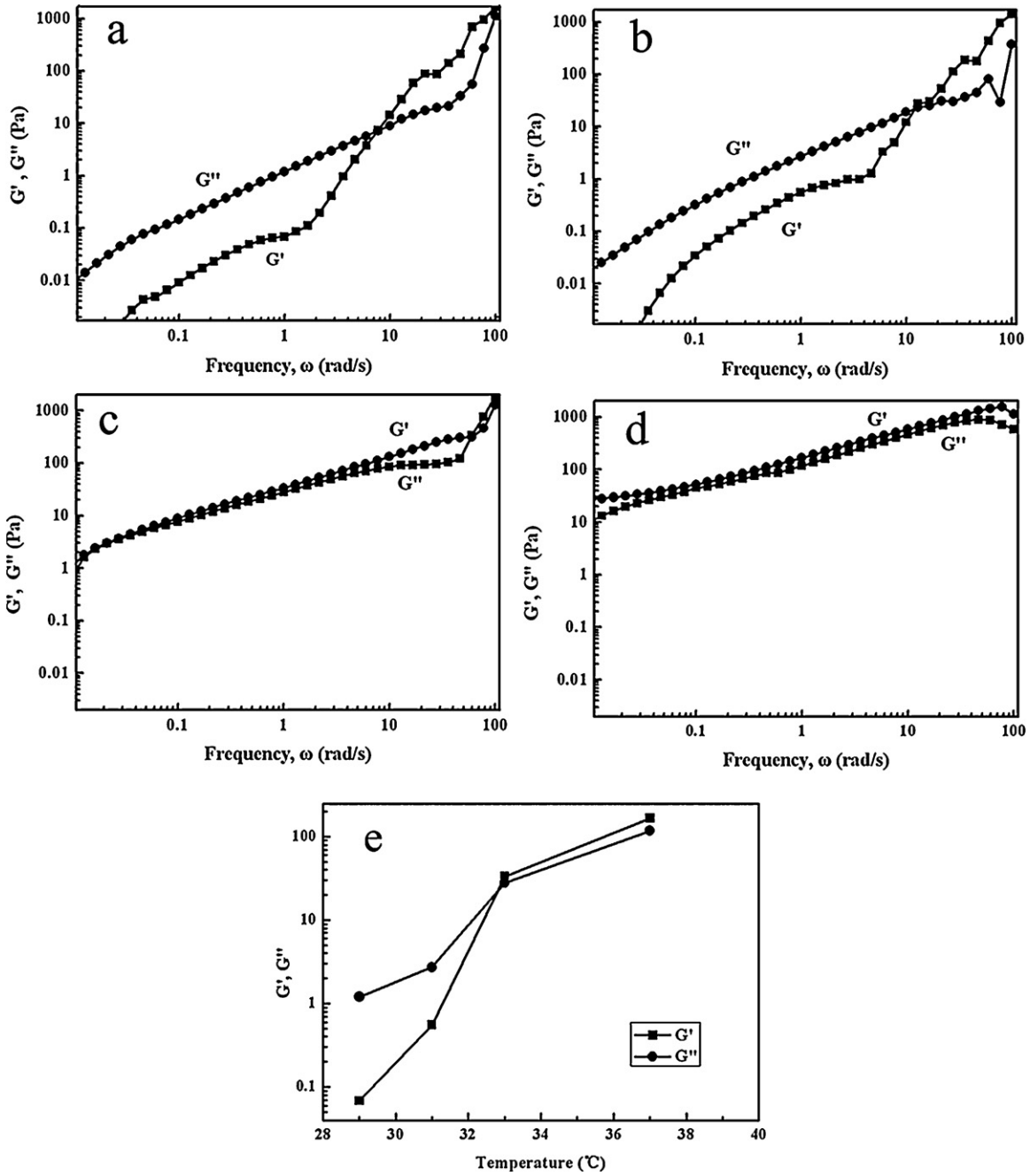

Fig. 7. Elastic modulus (G') and viscous modulus (G'') as function of angular frequency (ω) for PLGA–PEG–PLGA (25%, w/w) copolymer at different temperatures: (a) 29 °C, (b) 31 °C, (c) 33 °C, (d) 37 °C and (e) G' and G'' of copolymer solution as function of temperature.

the drug release from the test formulation. The criterion for selecting the most appropriate model was based on a goodness-of-fit test (Bamba et al., 1979).

It has been reported that drug release from the hydrogel occurs by two principal mechanisms: (1) drug molecules diffusion from the hydrogel during the initial release phase and (2) release of drug by the erosion of the hydrogel matrix during the later release phase (Pratoomsoot et al., 2008). The HA release data from the hydrogel was well fitted to Higuchi equation ($Q = a + bt^{1/2}$) in the first stage and the Ritger–Peppas equation (Ritger and Peppas, 1987) ($\ln Q = a + n \ln t$) in the late stage, as shown in Table 2. Our analyses illustrate that the first-stage drug release from the hydrogel was basically diffusion-controlled. In the late stage the release data

Table 2
Kinetic assessment of release data fitted by Higuchi and Peppas equations.

	c (%, w/w)	Drug loading (%)	n	R^a
Copolymer[b]	25	0.5	–	0.977
Formulation[b]	25	0.5	–	0.995
Copolymer[c]	25	0.5	0.511	0.996
Formulation[c]	25	0.5	0.524	0.994

[a] Squared correlation coefficient.
[b] Fitted by Higuchi equation ($Q < 0.6$). Here, Q is the fraction of drug released at time t.
[c] Fitted by Ritger–Peppas equation ($Q > 0.6$).

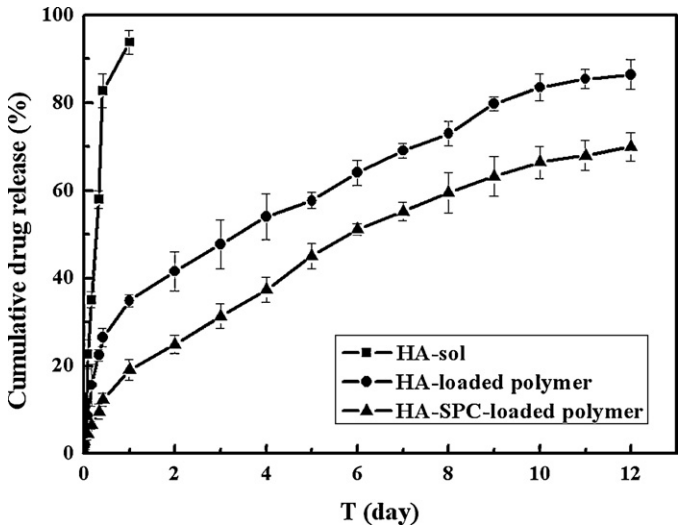

Fig. 8. *In vitro* release of HA (free and SP-complexed) from the polymer gels. The copolymer concentration was fixed at 25% (w/w) and the drug loading was 0.5% (w/w).

of different release systems were well fitted to the Ritger–Peppas equation. The drug release from swellable matrices is usually complex. Although some processes may be distinctly classified as either diffusion or erosion controlled, most of the drug releases from the gel are governed by both mechanisms. Analysis of the experimental data using the Ritger–Peppas equation, and interpretation of the release exponents (n), provides a better understanding of the mechanisms controlling release in the late stage. The value of $n \leq 0.5$ indicates Fickian Diffusion, while $0.5 < n < 1$ indicates Anomalous Transport (Singh et al., 2006). As shown in Table 2, the release exponent were 0.511 and 0.524 for HA-loaded polymer gel and HA–SPC-loaded polymer gel fitted by Ritger–Peppas equation, respectively. This value of release exponent indicates an anomalous (non-Fickian) transport for the hydrogel, suggesting that the mechanism of the sustained drug release in the late stage must be a combination of diffusion and degradation.

3.10. In vivo release of HA in rabbits

The concentration of the HA in the plasma samples was detected with a validated HPLC–MS–MS method (Wang et al., 2004). Fig. 9 showed the profiles of mean drug concentration in plasma *versus* time after subcutaneous administration of different formulations in New Zealand White rabbits. A subcutaneous administration of HA solution resulted in its maximum concentration at 0.5 h and a gradual decrease to nearly zero over the 12-h period (Fig. 9a). In contrast, constant plasma levels of HA (free and SP-complexed) were detected for nearly 2 weeks from the HA-loaded and HA–SPC-loaded polymer formulation, which indicate constant rate of HA release *in vivo* upon single subcutaneous injection (Fig. 9b). The plasma concentration of HA (4.36 ng/mL for HA-loaded gels and 2.54 ng/mL for HA–SPC-loaded gels) were different after being given. It can be concluded that HA–SPC-loaded gel can effectively slow down the burst release, which will reduce toxic effects. The data for pharmacokinetics in rabbits were analyzed with DAS 2.1.1, and pharmacokinetic parameters were shown in Table 3. It can be seen from the figure that the HA–SPC-loaded gel resulted in lower maximum plasma concentration (C_{max} = 2.54) compared with the HA solution (C_{max} = 7.15) and HA-loaded gel (C_{max} = 4.36). When compared with subcutaneously administering HA solution, the $t_{1/2}$ of HA was increased obviously by using the thermosensitive polymer gel. Simultaneously, the mean residence time ($MRT_{0-\infty}$) of

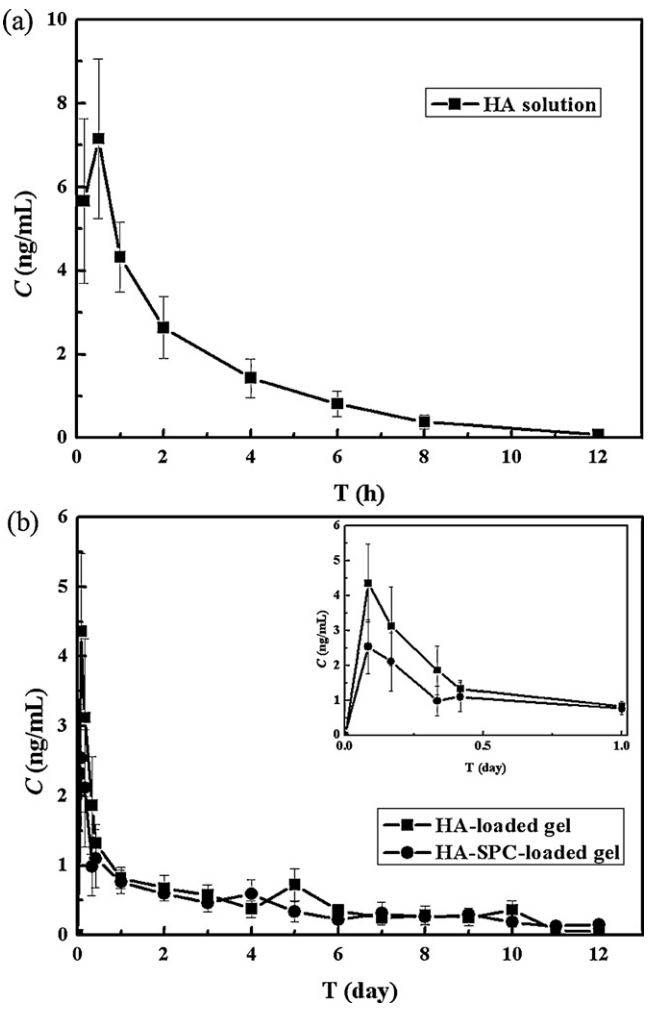

Fig. 9. *In vivo* release of HA in rabbits after administration of different formulation. (a) HA solution; (b) HA and HA–SPC-loaded gels (small panel is the *in vivo* release of HA after administration within 1 day).

HA–SPC-loaded polymer gel formulations (129 h) was 46 times longer than that of HA solution (2.76 h). These results clearly showed that the HA–SPC loaded gel exhibited good controlled-release properties *in vivo* within the 12 days.

It is usual to evaluate an *in vitro*/*in vivo* correlation to establish the *in vitro* test as a surrogate for *in vivo* studies, which may reduce the number of bioequivalence studies performed during the initial approval process as well as with certain scale-up and post-approval changes (Frick et al., 1998). The *in vitro* and *in vivo* HA release correlation was studied, the later was plotted as cumulative area under plasma HA curve normalized as percent of the total area under the curve. As shown in Fig. 10, the *in vitro* release rate of HA correlated rather well with the estimated *in vivo* release (R = 0.9921 for HA-loaded gel and R = 0.9960 for HA–SPC-loaded gel, n = 12, $p < 0.0001$).

Table 3
Pharmacokinetic parameters after administration of HA solution, HA-loaded gels and HA–SPC-loaded gels in rabbits (mean ± SD, n = 3).

Parameters	HA solution	HA-loaded gels	HA–SPC-loaded gels
C_{max} (ng/mL)	7.15 ± 1.90	4.36 ± 1.12	2.54 ± 0.77
$t_{1/2}$ (h)	2.15 ± 0.18	36.2 ± 23.2	69.3 ± 26.9
MRT_{0-inf} (h)	2.76 ± 0.16	89.8 ± 2.03	129 ± 43.1
AUC_{0-inf} (ng h/mL)	18.3 ± 4.41	154 ± 41.86	141 ± 25.1

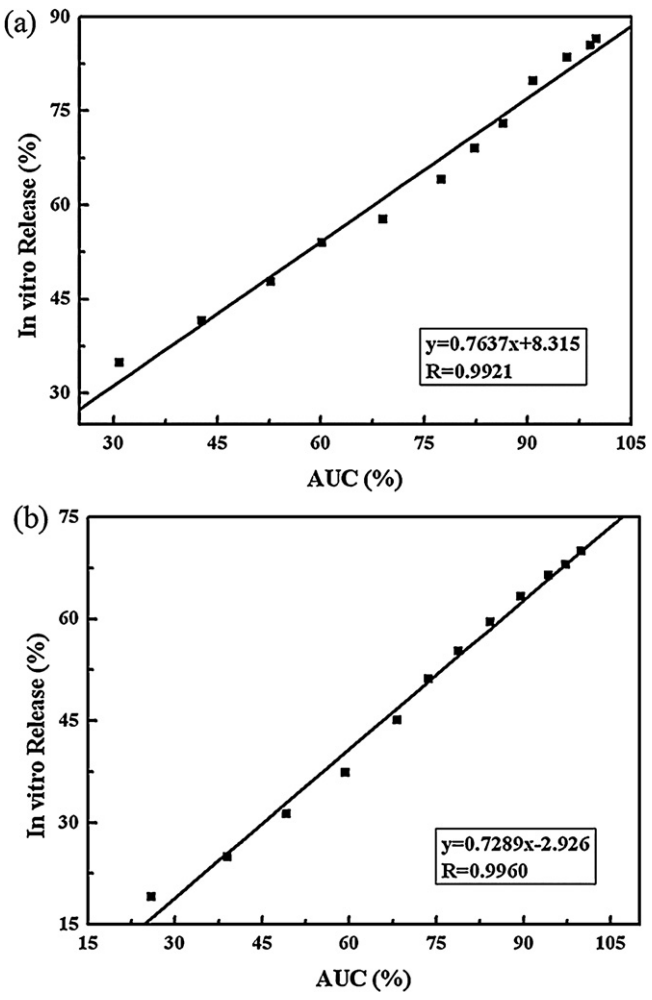

Fig. 10. *In vitro–in vivo* correlation plot of HA-loaded gels (a) and HA–SPC-loaded gels (b).

4. Conclusions

In this study, we report a novel formulation of HA–SPC loaded in PLGA–PEG–PLGA thermal gel which has been prepared with the desired content of drug and investigate on its utility in controlled drug release. The formulation can be used to deliver HA following simple hypodermic or intramuscular injection without the need for implant removal because of its biodegradable nature. We prepared HA–SPC by a very simple and reproducible method. The complexes formation of HA–SPC was proved by FT-IR, DSC, XRD technique and the results showed that HA molecules were successfully incorporated into the SP molecules by hydrogen bond and electrostatic attractions. The water-solubility and liposolubility of HA have been markedly enhanced by formation of HA–SPC. The biodegradable and biocompatibile triblock copolymer of PLGA–PEG–PLGA were used to prepare the injectable HA–SPC-loaded thermosensitive gel for drug delivery. The *in vitro* and *in vivo* release results indicated that the prepared HA–SPC-loaded gel could control the delivery of HA for about 2 weeks. *In vivo* experiment showed that, after intramuscular injection, the plasma concentration of HA reached a max at 2 h, then fell gradually to a level of 0.15 ng/mL from the gel after 2 weeks. Simultaneously, the *in vitro* release rate of HA correlated very well with the estimated *in vivo* release. Thus, the present studies indicate that HA–SPC loaded polymer gel is a good injectable delivery system for long controlled release for AD.

Acknowledgements

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