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A modified hydrophobic ion-pairing complex strategy for long-term peptide delivery with high drug encapsulation and reduced burst release from PLGA microspheres

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ABSTRACT

Poor encapsulation and high initial burst were two major obstacles for the water-soluble peptide drug loaded microspheres preparation using the industrial emulsification method. In the present study, we hypothesized that the hydrophobic ion-pairing (HIP) complex strategy with a further healing of the pores within the microspheres may improve drug encapsulation and initial burst release. DSS was chosen as the most suitable one among the three test ion-pairing agents (SDS, DSS and STC) due to its high binding efficiency with drug and reversible dissociation capacity in presence of counter ions. The formation of HIP complex between octreotide acetate and DSS successfully reversed the highly water-soluble nature of the drug. A specific S/O/W method was adopted to encapsulate such drug containing HIP complex. The encapsulation efficiency of the drug was greatly improved compared with the conventional W1/O/W2 method (from 44% to 90%). Under the optimal healing conditions (the healing time 6 h, temperature 40 °C and 4% DEP content), the pores within the microspheres were effectively healed. Initial burst amount of octreotide acetate in S/O/W microspheres decreased to 3.56%. The pore healing effect was further confirmed by the scanning electron microscopy and fluorescence microscopy results. In the process of testing the drug release performance of such new strategy in vitro and in vivo, a more satisfactory single phase release profile with sustained and steady drug release was observed. These results suggested that the modified HIP strategy could be a promising platform for water-soluble peptide encapsulation with high encapsulation efficiency, low initial burst and stable drug release mechanism.

1. Introduction

With the rapid development of the biotechnology and genetic engineering, more and more biomacromolecular drugs especially peptides and proteins have been developed and applied in clinic due to their high therapeutic selectivity and specificity [1–3]. Unfortunately, most of these biotherapeutics do not possess the physicochemical properties of an ideal drug candidate (e.g., lipophilicity and permeability) [4] and suffer from a series of delivery-related problems, such as low bioavailability [5], liable to proteolysis [6], short half-life and poor drug permeability across interstinal mucosa [7,8]. In order to maintain an effective therapeutic drug concentration, repeated administration and frequent injection are necessary. However, frequent dosing leads to poor patient compliance. Hence, long-acting and sustained drug delivery system is urgently needed to be developed.

Biodegradable polymers, especially copolymers of lactic and glycolic acid (PLGA) have been widely used in the peptides and proteins delivery in the past three decades [9]. With one single dose, the efficiency of the encapsulated drug may last several weeks to months [10,11]. Till now, considerable PLGA-based products have been commercially on the market and most of them are peptide loaded microspheres: Lupron Depot® (Leuprolide), Bydureon® (Exenatide), Zoladex® (Goserelin), Sandostatin® LAR Depot (Octreotide) and so on [12–14].

Although polymeric microspheres have made a great success in therapeutic peptides delivery, effectively encapsulating drugs into the polymer matrix and minimizing the initial burst remain a big challenge [15–17]. The water-in-oil-in-water double emulsion technique (W1/O/W2) is commonly used to prepare PLGA microspheres because of the

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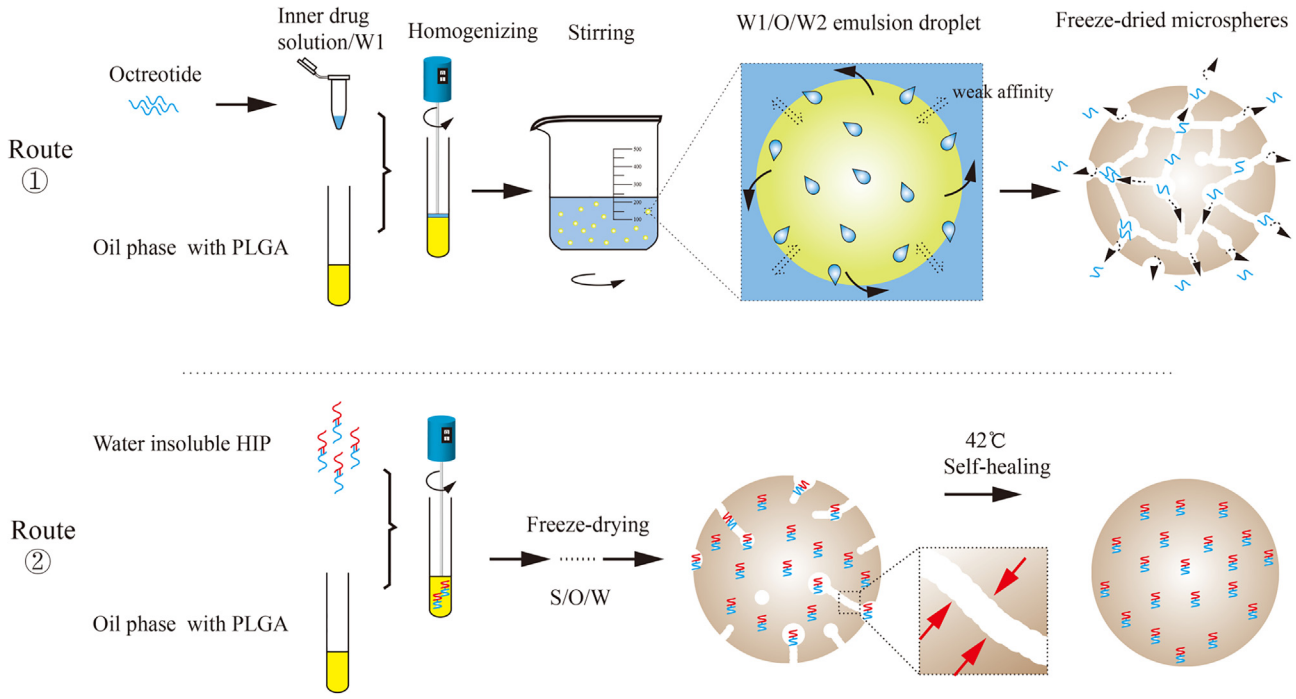

Fig. 1. Schematic diagram of traditional microsphere preparation method (Route ①, W1/O/W2 emulsification method) and the modified HIP complex strategy microsphere preparation method (Route ②, S/O/W emulsification method). Route ①, Drugs in the internal water phase (W1) tended to diffuse to the external water phase (W2). Considerable pores were formed after microsphere preparation. Route ②, The hydrophobic drug containing complex could be effectively encapsulated into the microspheres. Few pores were formed, with further self-healing, the remaining pores were healed.

simplicity of the fabrication procedure and the ability to produce large and reproducible output [18,19]. However, due to the highly water-soluble characteristics of peptide drugs, dissolving of which in the inner water phase (W1) would lead to a significant increase in osmotic pressure and accelerate the water uptake from the external water phase (W2). Under this circumstance, hydrophilic drugs could easily escape from the inner water phase to the outside and distribute over the surface of the emulsion droplet with weak affinity interaction during the microsphere fabrication process. When the drug containing droplets are getting solidified gradually, the mass transfer channels between inner and external water phase and the organic solvent evaporation channels would be left over and form considerable water-filled pores throughout the microspheres, which was responsible for the poor encapsulation and extensively high initial burst (illustrated by Fig. 1, Route ①) [20–22]. Low entrapment of W1/O/W2 method would result in massive drug loss and a remarkable increase in the cost of manufacture. Rapid drug release during the first day leads to severely clinical toxicity with high drug concentration and depletion of drug for long-term release [22].

Considering that emulsion and solvent evaporation method has irreplaceable advantages in industrial PLGA microparticle fabrication, breakthroughs should be made depending on the bioactive agents themselves. Chemical modification of the drugs or encapsulating them in the reverse micelle has achieved a big success in improving the drug encapsulation efficiency and partly inhibiting the initial burst [18]. However, it should be also remarked that the affinity of therapeutic peptides to the target receptor can be also decreased by the chemical methods [23]. Micelle complex is prone to phase transition during microsphere fabrication process and the dissociation mechanism of which in drug release period is not clear yet [24,25].

The hydrophobic ion-pairing (HIP) complex is a physically reversible combination that has emerged as a promising strategy for proteins and peptides delivery [26–29]. The formation of HIP is a pretreatment of the drugs through electrostatic interactions between the ionized groups of the drug molecules and the oppositely charged groups of surfactant or polyelectrolytes before encapsulation [30]. This process will greatly reduce the water-soluble nature of the drugs and thereby increasing the encapsulation efficiency with a specific solid-in-oil-in-water (S/O/W) microspheres preparation technique. In the present study, we want to investigate the reversing effect of a HIP-based, modified strategy on the low encapsulation and high initial burst of peptides loaded PLGA microspheres. Octreotide acetate was chosen as the model peptide, which has been widely reported to be poorly encapsulated and with high initial burst in the previous study (encapsulation efficiency could even be as low as 21% [20], initial burst accounted for 10–80% of the total drug loading [31,32]). We intended to improve the drug encapsulation and reduce its initial burst through the following specific processes: Step (1) reduce the highly water-solubility nature of octreotide acetate using the HIP strategy and then encapsulate this hydrophobic dosage form of drug by S/O/W method. It would greatly improve the entrapment of the octreotide. Moreover, the initial burst may also be partly decreased because the HIP strategy will not induce an osmotic gradient between the emulsion and the external water phase so that the porosity of the prepared microsphere will not be so high. Step (2) “healing” the remaining pore within the microspheres using a popular “self-healing” method further enhances the inhibition of burst release. “Self-healing” is a unique phenomenon of the polymer, in which the damaged structures (e.g., dents, crevice and pores) are healed (repaired) through spontaneously rearrangement of the polymer chains [33–36]. In the initial stage, peptide mainly release from the interconnected pores within the microspheres [37]. Sealing off the pore-diffusion pathway would lead to cessation of the initial burst of the peptide. The whole hypothesis was illustrated by Fig. 1 (Route ②). Various ion-pairing agents were prepared and characterized then encapsulated into the microspheres using S/O/W technique. The formed microspheres were “healed” in different temperatures with the addition of plasticizer (diethyl phthalate (DEP)). In vitro and in vivo evaluation was carried out about this novel strategy (S/O/W + “self-healing”) on the improvement of microsphere encapsulation and initial burst compared with the traditional method (W1/O/W2), including surface and cross-sectional morphology, porosity, permeability of microspheres, in

vitro and in vivo release. Following such modified HIP strategy, the encapsulation of octreotide acetate was significantly improved (around 90%) and the initial burst amount of drug was highly inhibited (around 3%). Furthermore, a steady drug release profile was observed in vitro and in vivo. With the obtained knowledge, we hope to provide a solid basis for the efficient delivery of the water-soluble biopharmaceuticals in the future.

2. Materials and methods

2.1. Materials

Octreotide acetate (Oct, H_2N-D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-ol; Mw: 1139.35) was purchased from Nanjing Senbeijia Biological Technology Inc. (Jiangsu, China). PLGA Resomer RG 503H (Mw: 2.4–3.8 kDa, free carboxylic acid) was supplied by Evonik Specialty Chemicals (Shanghai, China). Sodium dodecyl sulfate (SDS, Mw: 288.38), dextran sulfate sodium (DSS, Mw: 40,000), sodium taurocholate (STC, Mw: 537.68) and diethyl phthalate (DEP) were procured from Aladdin's Reagent Co., Ltd. (Shanghai, China). Poly (vinyl alcohol) (PVA, Mw: 9,000–10,000, 80% hydrolyzed), trifluoroacetic acid (TFA) and methylene chloride were purchased from Sigma-Aldrich (Shanghai, China). All chemicals were of analytical grade as received commercially.

2.2. Preparation and characterization of the ion-paring complex

2.2.1. Preparation of HIP complex

HIP complex was prepared by a simple mixing of the aqueous solutions containing octreotide acetate and ion-pairing agent [30,38,39]. Three ion-pairing agents were investigated named sodium dodecyl sulfate (SDS), dextran sulfate sodium (DSS) and sodium taurocholate (STC). Briefly, octreotide acetate and ion-paring agents were dissolved in citrate buffer (10 mM) separately. Then the ion-pairing agent solution was added to the drug solution dropwise (octreotide acetate: 1 mg/ml, ion-pairing agent: predetermined proportion with the drug). After 5 h, the water insoluble complex was precipitated and separated by centrifugation at 10,000 rpm for 10 min. The free (uncomplexed) octreotide acetate remaining in the supernatant was analyzed by high performance liquid chromatography (HPLC). Specific chromatographic conditions will be detailed in the following experiments. The resulting complex was freeze-dried for 48 h (Freezing Dryer LGJ-10C, Four-ring Science Instrument Plant Beijing Inc.) and stored in a desiccator at 4 °C before further investigation. Bind efficiency of the drug to the ion-pairing agent was determined by an indirect method as followed. Each sample was analyzed in triplicate.

$$\text{Bind efficiency} = (1 - \text{amount of drug in the supernatant}) / \text{initial added amount of drug} \times \%$$

2.2.2. Effect of preparation parameters on the binding efficiency

HIP complexes were prepared with different mole ratio of ion-pairing agents to drug to get the predetermined charge ratio. These mole ratios represent the addition amount of ion-pairing agent into the octreotide acetate solution. The pH and temperature effect on the bind efficiency were also studied. The pH of the citrate buffer was adjusted with hydrochloric acid or sodium hydroxide (0.05 M) to achieve a series of pHs from 2 to 7. The temperatures of the reaction environment were set as 25, 37 and 42 °C separately. Once the water insoluble complex formed, it was separated by centrifugation at 10,000 rpm for 10 min. The bind efficiency was calculated following the aforementioned HPLC method (Section 2.2.1). Each sample was analyzed in triplicate.

2.2.3. Aqueous solubility of the HIP complex

Approximately 1 mg octreotide acetate equivalent HIP complex was added into a 10 ml polyethylene tube containing 5 ml deionized water. Then it was put in an orbital shaker (Shaker THZ-103B, Shanghai Bluepard Instrument) under continuous shaking at 300 rpm. After 24 h' incubation, the solution was centrifuged at 10,000 rpm for 10 min. The amount of the water-soluble drug in the supernatant was determined by the HPLC method (Section 2.2.1). Each sample was analyzed in triplicate.

2.2.4. Dissociation of HIP complex

Dissociation of HIP complex was investigated under different ionic strengths in phosphate buffer saline (7.74 mM Na_2HPO_4, 2.26 mM NaH_2PO_4 and 3 mM KCl, pH 7.4), which contained various amount of NaCl (10, 25, 50, 100 and 200 mM). About 5 mg octreotide acetate equivalent HIP complex was put in 10 ml dissociative medium under continuous shaking at 300 rpm. After 3 h' incubation, the solution was centrifuged at 10,000 rpm for 10 min. Amount of dissociated octreotide acetate in supernatant was quantitated by the HPLC method (Section 2.2.1). Each sample was analyzed in triplicate.

2.2.5. Particle size measurement

The particle size of the HIP complex was measured using a Zeta Sizer (Zeta Sizer Nano-ZS90, Malvern Instruments Ltd., UK). Samples were suspended in deionized water and tipsonicated for 1 min before analysis.

2.3. Preparation of microsphere

2.3.1. Preparation of microsphere by S/O/W method with or without self-healing

HIP complex containing octreotide acetate was prepared using the optimal ion-pairing agent and preparation parameters (e.g., mole ratio or pH) based on the above experiments. A solid-in-oil-in-water (S/O/W) emulsion solvent extraction/evaporation method was used for the HIP complex loaded microsphere formulation. Briefly, 600 mg PLGA was dissolved in 2 ml of methylene chloride. In the case of self-healing microsphere preparation, 0–10% hydrophobic plasticizer (DEP, w/w relative to the polymer material) was dissolved in the methylene chloride together with the PLGA. 60 mg octreotide acetate equivalent HIP complex was added into the polymer solution. Then the suspension was homogenized at the speed of 11400 rpm for 2 min in the ice bath using a T10 digital basic ULTRA-TURRAX (IKA, Germany). The first solid-in-oil emulsion (S/O) was stabilized by addition of 2 ml of 1% PVA solution and was vigorously vortexed for 30 s. The formed emulsion was immediately transferred into a 100 ml 0.5% PVA aqueous solution under stirring at 350 rpm to evaporate the methylene chloride. For self-healing microsphere preparation, the temperature (25–42 °C) and time (6–24 h) during the methylene chloride evaporation stage were studied to effectively heal the pores. In the case of evaluating the pore healing effect, small amount of water-soluble fluorescent dye (coumarin, 0.5 mg, Mw 350.44) was added into the 100 ml 0.5% PVA solution before the pore healing experiment. After several hours, the microspheres were washed repeatedly with deionized water and were sieved to obtain uniform particle size (45–90 μm, Test Sieve, Endecotts ASTM E11). Finally, the microspheres were lyophilized for 48 h (Freezing Dryer LGJ-10C, Four-ring Science Instrument Plant Beijing Inc.) to dry the microspheres and stored in a desiccator at 4 °C before further investigation.

2.3.2. Preparation of microsphere by W1/O/W2 method with or without self-healing

Octreotide acetate was also encapsulated into the PLGA microspheres by the conventional W1/O/W2 emulsion solvent evaporation method as a comparison. Since octreotide acetate was highly water-soluble, it was dissolved in 100 μl internal water phase before added to

the polymer solution (oil phase). Then 600 mg PLGA and hydrophobic plasticizer (0–10%) were dissolved together in methylene chloride. The following emulsification and solvent evaporation processes including the self-healing microsphere preparation were same to the S/O/W method (Section 2.3.1).

2.4. Determination of drug loading and entrapment efficiency

Approximately 5 mg freeze-dried microsphere (prepared by S/O/W or W1/O/W2) was dissolved in 2 ml DMSO. Sample was filtrated through a 0.45 μm syringe filter. The concentration of octreotide acetate was determined using high performance liquid chromatography (HPLC). HPLC conditions: octreotide acetate was analyzed by reversed phase (RP)-HPLC using a Kromasil C-18 column (4.6 mm × 250 mm) and separation of the peptide was accomplished using a gradient elution method: mobile phase A: 0.1% (v/v) TFA in acetonitrile and B: 0.1% (v/v) TFA in water. The linear gradient of mobile phase A changed from 25% to 35% over 15 min with a flow rate of 1.0 ml/min. UV detection was set at 215 nm. Each sample was analyzed in triplicate.

Drug loading and encapsulation efficiency was determined as:

$$\text{Drug loading} = (\text{weight of drug loaded/weight of microspheres}) \times 100\%.$$

$$\text{Encapsulation efficiency} = (\text{experimental drug loading/theoretical drug loading}) \times 100\%$$

2.5. Porosity analysis

The porosity of the microsphere treated with or without self-healing was determined by Mercury Porosimeter AutoPore IV 9500 (Micromeritics Instrument Corp.,). Pressure was increased from ambient pressure to 60,000 psi. Each sample was analyzed in triplicate.

2.6. The glass transition temperature

The glass transition temperature (Tg) of the microspheres was characterized by differential scanning calorimetry (DSC 25, TA instruments). The samples were purged with pure and dry nitrogen and subjected to a first heating cycle, ramp from 0–80 °C at 10 °C/min, isothermal for 2 min, and cooled to 0 °C at a rate of 100 °C/min. Then to a second cycle, ramp from 0–80 °C at 10 °C/min. Tg was determined as the glass transition midpoint in the reversing signal.

2.7. Fourier Transform Infrared Spectroscopy

A Fourier Transform Infrared Spectroscopy (FTIR) was used to investigate the bonding interactions: (a) between octreotide acetate and ion-pairing agent; (b) between octreotide acetate and polymer material (Spectrum 100; PerkinElmer, Inc., Waltham, MA). Samples were weighed and mixed perfectly with KBr to form a uniform mixture. A small amount of the powder was compressed into a thin semi-transparent pellet for FTIR analysis. The IR spectrum of the pellet was recorded in the 4000–400 cm^{-1} region at a resolution of 4 cm^{-1} and 50 scans per sample.

2.8. Scanning electron microscopy

The surface and cross-sectional morphology of microspheres and HIP complex after freeze-drying was characterized using scanning electron microscopy (SEM). To observe the cross-sectional morphology, a frozen section method was adopted [18,40]. Briefly, microspheres were dispersed in a mixed aqueous solution containing 15% gelatin, 5% glycerin and 80% water. This solution was preheated to 37 °C and kept in that condition for 4 h to allow the occupation of the pore space of the microspheres with the aqueous medium. Then it was frozen immediately with liquid nitrogen. The frozen cube was sectioned with a freezing microtome at −20 °C to achieve thin slices about 8 μm thick (Leica CM 1950, Leica Biosystem Germany). Microspheres and HIP complex were coated with 40 nm of gold using a sputter coater for 150 s. Secondary electron micrographs were taken by a NPE 218 SEM (Nova Nano SEM, FEI, USA).

2.9. Fluorescence microscopy

To evaluate the effect of microsphere pore self-healing, experiment on the diffusion/escape of the fluorescent substance from the single microsphere was carried out using a 96-well plate monitored by Olympus microscopy (IX 51, Japan). Small amount of coumarin containing microspheres (fluorescent dye, Mw 350.44) treated with or without self-healing were put into each well with addition of 250 μl PBST (pH 7.4). The plate was kept at 37 °C in the orbital shaker under continuous shaking at 50 rpm. In order to minimize water evaporation, the plates were closed and covered with parafilm. At predetermined time points, pictures were captured using an Olympus camera (DP 71, Japan) to record the diffusion of the fluorescent substance.

2.10. In vitro release test

In vitro drug release kinetics of peptide encapsulated in PLGA microsphere was examined in PBST release medium (7.74 mM Na_2HPO_4, 2.26 mM NaH_2PO_4, 137 mM NaCl and 3 mM KCl, 0.02 wt% Tween 80, pH 7.4). Approximate 20 mg microspheres were placed into a 10 ml polyethylene tube containing 1 ml PBST. Samples were incubated in an orbital shaker under 37 °C at 300 rpm. Release medium was replaced once a week. At predetermined time points, microspheres were collected, washed and lyophilized. Cumulative drug release content was determined by HPLC. The drug released in the first day was taken as the initial burst release amount. Each sample was analyzed in triplicate.

2.11. In vivo drug release

Sprague-Dawley Male Rats weighing about 300 g were used for in vivo drug release characteristics from the microspheres. Briefly, SD rats were randomly assigned to cages and treated with the prepared microspheres (by S/O/W or W1/O/W2) with or without self-healing (n = 6). The microspheres were reconstituted with Sterile Water for Injection (SWFI). Rats were injected behind the neck region with a dose of 3 mg octreotide acetate equivalent microspheres. At predetermined time points, blood samples were collected and centrifuged at 10,000 rpm for 10 min. Then the serum was separated and put in a 1.5 ml polypropylene tube by storage at −20 °C.

Serum drug concentrations were determined using a specific octreotide acetate ELISA kit (Caobenyuan Biotechnology Co Ltd, Nanjing, China). It allowed for quantification of the peptide within the range of 0.1–8.1 ng/ml. Samples were diluted to the appropriate concentration before determination. A test microplate was coated with octreotide conjugated antigen. Octreotide in the serum would compete with the precoated octreotide conjugated antigen in the microplate for anti-octreotide antibody markers. Drug concentration was determined at 450 nm microplate reader (Infinite® F50 microplate reader).

Sprague-Dawley Male Rats were purchased from Yangzhou University Medical (Yangzhou, China). The animal study protocol was in accordance with the Guidelines for Care and Use of Laboratory Animals of Nanjing Tech University and experiments were approved by the Animal Ethics Committee of Jiangsu Center for Safety Evaluation of Drugs.

2.12. Statistical analysis

Results were analyzed using student's *t* test and GraphPad Prism 5 software and presented as means of three replicates ± standard

deviation (SD). Statistical comparisons were assessed using an unpaired *t*-test and one-way ANOVA. Differences with a value of $P < 0.05$ were considered to be statistically significant. SPSS 11.0 software was used for the analysis.

3. Results and discussion

3.1. Preparation and characterization of HIP complex

In order to reverse the high hydrophilic nature of peptide drug and improve the poor partition of which to hydrophobic polymer matrix, the water insoluble HIP complex of peptide was prepared. Three ion-pairing agents were selected to form HIP complex with the drug named sodium dodecyl sulfate (SDS), dextran sulfate sodium (DSS), sodium taurocholate (STC). Octreotide acetate had two pKa (7 and 10.15), which was owing to amine of lysine and guanidine group of terminal arginine [38]. At pH < 7, these amine groups would be protonated and positively charged ($-NH_3^+$). The pKa of all of the three ion-pairing agents was < 2 due to the sulfonic acid group [30]. At pH > 2, sulfonic acid groups will ionize to produce $-SO_3^-$. When drug and ion-pairing agent solutions were mixed together at a certain pH range (2 < pH < 7), water insoluble HIP complex would be rapidly formed through ionic interactions between oppositely charged amine and sulfonate group.

Hydrophobic ion pairing complexation was optimized with respect to mole ratio of ion-pairing agent to the drug, pH and temperature of the reaction medium. Firstly, effect of mole ratio of ion-pairing agent to drug was evaluated in citrate buffer (pH 4.0, 37 °C, Fig. 2A). Since the formation of HIP complex was a process of charge neutralization, the charge ratio was taken to represent the change of mole ratio. There was a simple conversion between these two parameters. For example, the mole ratio of SDS/STC: octreotide increased from 0.2:1–16:1, which was equal to the charge ratio from 0.1:18:1. Similarly, the mole ratio of DSS: octreotide acetate changed from 0.00078: 1–0.0624:1, which was equal to the charge ratio from 0.1:1–8:1. A significant rise in bind efficiency was observed in all of the three HIP complexes with the charge ratio increased (Fig. 2 A). For SDS and DSS, the bind efficiency both reached a maximum at the charge ratio of 1:1 to the drug (96.39% and 92.60 separately). However, further increase of the charge ratio of ion-pairing to drug did not improve the bind efficiency but resulted in some decline. Similar phenomenon has been widely reported in other researches [38]. This was due to the self-aggregation of the ion-pairing agents into micelles, which subsequently promoted the dissolution of the HIP complex. For STC, the bind efficient was always low (< 30%) although it made some increase as the charge ratio of STC: Oct increased. This was responsible for the relatively less hydrophobic nature of STC compared to the other two ion-pairing agents.

pH effect of the release medium on the bind efficiency was investigated at the charge ratio of 1: 1 (ion-pairing agent: octreotide acetate, 37 °C, Fig. 2 B). For SDS and DSS, both the bind efficiencies of these two HIP complexes (SDS/Oct and DSS/Oct) were high within the studied pH range (2 < pH < 7). It confirmed the success combination of the ion-pairing agent and octreotide acetate and considerable water insoluble HIP complexes were formed under such pH environment. At pH 4, a maximum bind efficiency was achieved in SDS/Oct and DSS/Oct HIP complexes. Above or below this pH condition, the bind efficiencies of these two formulations both slightly decreased. This was related to the ionization of the ion-pairing agent and the drug. As the pH increased from 4 (4–7), it was getting more and more difficult for the protonation of the amino group. Similarly, at pH < 4, the deprotonation of the sulfate group was also gradually inhibited. Hence, pH 4 was proved to be the optimal condition for the complexation reaction. For STC, due to the hydrophilic nature, the bind efficiency of this HIP complex was low and achieved a maximum at pH 3 (35.16%).

The complexation reaction was also carried out under different temperatures (25, 37 and 42 °C) to evaluate the temperature effect on the reaction degree and efficiency. As shown in Fig. 2 C, there was no significant difference of the achieved bind efficiency under different temperatures ($P > 0.1$, result was compared with 25 °C for each group, data not shown).

At the optimal mole ratio (charge ratio, 1:1), reaction pH (4) and temperature (25 °C), the HIP complex of octreotide acetate was formed. We then characterized the properties of the lyophilized HIP complex. The purpose of addition of the oppositely charged ion-pairing agent was to reduce the water solubility of the hydrophilic octreotide acetate. The reduction effect was determined by measuring the water solubility of the formed HIP complex. As shown in Fig. 2D, for SDS/Oct and DSS/Oct HIP complexes, the concentration of octreotide in the supernatant was 4.73 and 5.32 μg/ml separately (only 2.4% and 2.7% of the corresponding HIP dissociated and dissolved in the water). It meant that the formation of HIP between octreotide and SDS or DSS greatly reduced the water solubility of the drug. However, for STC/Oct HIP complex, due to hydrophilic nature of STC, although the complex was formed, the resulting complex was still somewhat water-soluble (82.10 μg/ml, about 41.0% of STC/Oct HIP complex dissolved).

The HIP complex was a physically reversible combination driven by ionic interactions. For successful application of it, the HIP complex should be able to dissociate in the presence of counter ions without inhibiting or blocking drug release. To investigate the dissociation of the HIP complex in physiological medium, a series of concentrations of NaCl in simulated physiological medium were taken (10–200 mM NaCl in phosphate buffer, 7.74 mM Na_2HPO_4, 2.26 mM NaH_2PO_4 and 3 mM KCl, Fig. 2E). The HIP complexes incubated with purified water acted as a negative control (absence of counter ions). For DSS/Oct complex, without presence of counter ions, less than 3% complex dissociated. Low dissociation of DSS/Oct in purified water (low water solubility as stated above) was the guarantee of its high encapsulation and good distribution into the polymer matrix during microsphere preparation process. When incubated with phosphate buffer, with the concentration of NaCl increased (10, 25, 50, 100 and 200 mM), the dissociation degree of the DSS/Oct was getting higher and higher (43.5%, 75.2%, 96.3%, 96.9 and 97.5%). Up to 50 mM NaCl, the DSS/Oct complex was almost completely dissociated. Hence, under normal physiological conditions (137 mM NaCl in phosphate buffer), the DSS/Oct complex could easily dissociate without inhibiting drug release. However, for SDS/Oct complex, a depressed dissociation was observed. Even when the concentration of NaCl was increased to 200 mM, the dissociation of the complex was still less than 20%. Similar low dissociation of SDS complex has been reported in other researches [30,41], which was ascribed to the presence of additional hydrophobic interactions between hydrophobic amino acids of octreotide and long hydrophobic chain of SDS except to the electrostatic interactions [30]. For STC/Oct, the complex could highly dissociate even in the purified water (> 40%) due to its high water solubility nature. With about 25 mM NaCl, the STC/Oct complex was completely dissociated.

Generally, all of the three test HIP complexes showed a similar size distribution (Fig. 2 F, average sizes of SDS/Oct, DSS/Oct and STC/Oct were 1.10, 1.39 and 2.02 μm separately). For STC/Oct complex, the addition of STC didn't efficiently reverse the high hydrophilic nature of octreotide. For SDS/Oct complex, the difficulty in its dissociation inhibited and blocked drug release. Based on the solubility and dissociation results, DSS/Oct complex was chosen for the further study (Fig. S1 and S2) and the subsequent microsphere preparation by S/O/W method.

3.2. Preparation of microspheres

In order to compare the effects of traditional microsphere preparation method and HIP strategy on the encapsulation and initial burst release of octreotide acetate, the drug was incorporated into the microspheres using the conventional W1/O/W2 and the modified S/O/W emulsification methods separately. During the preparation process, the

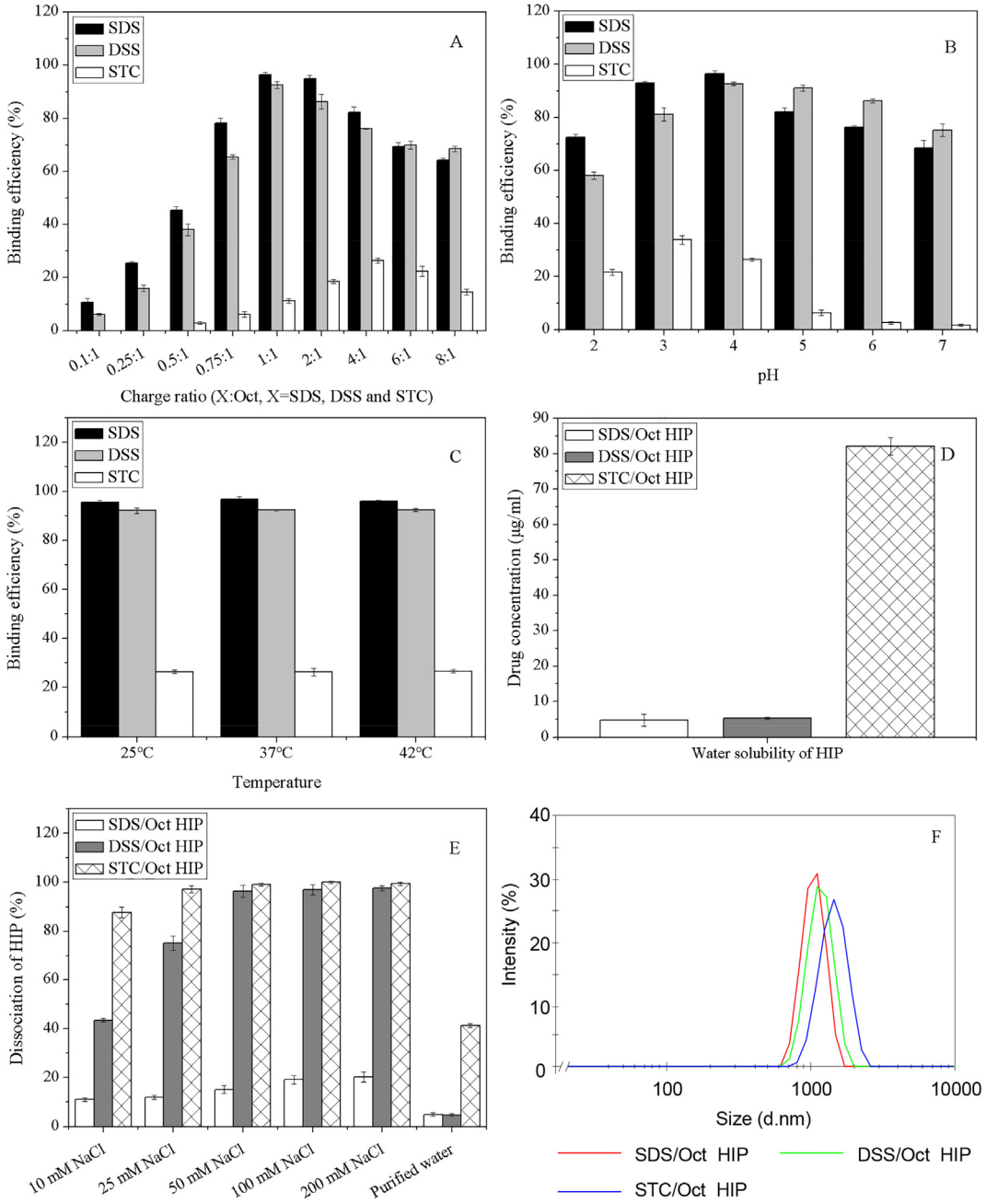

Fig. 2. Effects of charge ratio, pH and temperature on the binding efficiency between octreotide acetate and the three ion-pairing agents (SDS, DSS and STC) were shown in Fig. 2A–C. The water solubility, dissociation and size distribution of the three HIP complexes (SDS/Oct, DSS/Oct and STC/Oct HIP) were shown in Fig. 2D–F.

self-healing experiment was also carried out to investigate the effects of which on the pore healing and initial burst of drug. The test formulations were divided into four groups as shown in Table 1: microspheres were prepared by conventional W1/O/W2 method without self-healing (WM), with self-healing (WMH), prepared by S/O/W method without self-healing (SM) and with self-healing (SMH). The optimization of the healing parameters and the corresponding results were detailed in Group 2 (WMH 2 ~ WMH 2-11) and Group 4 (SMH 4 ~ SMH 4-11).

For the formulations prepared by W1/O/W2 method (Group 1 and Group 2), the encapsulation efficiency of the thirteen formulations in these two groups all showed a low level (around 44%), which was due to the rapid escape of the water-soluble drug to the external water phase during microsphere formulation process. There was no significant difference in the encapsulation efficiency between any of the twelve formulations in Group 2 (WMH 2 ~ WMH 2-11) with the formulation without self-healing in Group 1 (WM 1). It indicated that the self-healing process (temperature, time and plasticizer) didn't affect the encapsulation of octreotide acetate in the PLGA microspheres. In Group 2, the specific self-healing conditions were investigated with respect to the self-healing time, healing temperature and amount of plasticizer. With the self-healing time increased from 6 to 24 h (WMH 2-1 ~ WMH 2–3), the porosity of the microspheres slightly decreased (75.26–70.86%). The initial burst amount of drug from microspheres was also decreased (from 32.15% to 30.38%) due to the slight closure of the pores within the microspheres. When the self-healing time was 12 h, the porosity of the microspheres has decreased from 75.26% to 71.76%. Further prolonging the healing time didn't significantly reduce the porosity of the microspheres (from 71.76% to 70.86%). Hence, 12 h

Table 1
Microspheres prepared by the conventional W1/O/W2 (Group 1 and Group 2) and the modified S/O/W emulsification methods (Group 3 and Group 4) were healed under different self-healing conditions (0–12 h, 25–42 °C and 2–10% DEP content).

Group	Formulation	Self-healing temperature (°C)	Self-healing time (h)	DEP loading (w/w %)	Drug loading (w/w %) Theoretical loading	Actual loading	Encapsulation efficiency (%)	Porosity (%)	Initial burst (%)	Tg (°C)
1	WM 1	–	–	–	9.09	4.04 ± 0.29	44.48 ± 3.19	81.47 ± 1.70	32.99 ± 2.93	45.48 ± 0.97
2	WMH 2	40	12	4	8.77	4.16 ± 0.45	47.40 ± 5.18	39.91 ± 4.28	21.03 ± 2.32	34.47 ± 1.83
	WMH 2–1	40	6	0	9.09	4.13 ± 0.12	45.47 ± 1.28	75.26 ± 4.32	32.15 ± 2.98	44.71 ± 2.11
	WMH 2–2	40	12	0	9.09	3.93 ± 0.54	43.20 ± 5.97	71.76 ± 2.30	31.38 ± 1.20	46.19 ± 2.69
	WMH 2–3	40	24	0	9.09	4.03 ± 0.49	44.33 ± 5.36	70.86 ± 1.87	30.38 ± 1.47	45.72 ± 3.27
	WMH 2–4	25	12	0	9.09	4.22 ± 0.21	46.42 ± 2.29	80.56 ± 2.04	32.73 ± 2.91	46.50 ± 0.74
	WMH 2–5	37	12	0	9.09	4.17 ± 0.43	45.91 ± 4.77	78.30 ± 1.72	32.49 ± 4.39	47.12 ± 1.02
	WMH 2–6	40	12	0	9.09	3.68 ± 0.46	40.48 ± 5.01	72.42 ± 3.19	31.50 ± 2.29	44.93 ± 1.25
	WMH 2–7	42	12	0	9.09	4.30 ± 0.52	47.27 ± 5.68	64.13 ± 3.68	28.75 ± 2.15	44.84 ± 1.25
	WMH 2–8	40	12	0	9.09	4.22 ± 0.47	46.46 ± 5.12	71.03 ± 4.01	32.08 ± 1.87	44.80 ± 0.92
	WMH 2–9	40	12	2	8.93	3.82 ± 0.36	42.74 ± 4.05	59.34 ± 3.67	26.66 ± 3.75	40.01 ± 2.77
	WMH 2–10	40	12	4	8.77	3.91 ± 0.40	44.62 ± 4.60	41.27 ± 0.90	20.70 ± 2.10	34.24 ± 1.81
	WMH 2–11	40	12	10	8.33	3.49 ± 0.34	41.86 ± 4.06	27.19 ± 3.91	18.70 ± 4.40	27.31 ± 0.72
3	SM 3	–	–	–	8.87	8.16 ± 0.07	92.00 ± 0.79**	43.18 ± 3.25	16.81 ± 1.46	43.96 ± 0.65
4	SMH 4	40	12	4	8.57	7.86 ± 0.17	91.75 ± 2.00	10.97 ± 1.98	3.56 ± 1.17	33.34 ± 3.54
	SMH 4–1	40	6	0	8.87	7.81 ± 0.40	88.05 ± 4.52	42.84 ± 3.90	18.09 ± 1.79	45.61 ± 1.31
	SMH 4–2	40	12	0	8.87	7.65 ± 0.31	86.28 ± 3.50	39.09 ± 1.38	15.52 ± 1.10	44.95 ± 1.31
	SMH 4–3	40	24	0	8.87	8.25 ± 0.34	93.05 ± 3.89	39.36 ± 1.56	15.16 ± 1.23	46.19 ± 0.32
	SMH 4–4	25	12	0	8.87	7.88 ± 0.75	88.88 ± 8.43	43.31 ± 4.63	17.03 ± 2.36	44.33 ± 0.30
	SMH 4–5	37	12	0	8.87	7.89 ± 0.33	88.95 ± 3.72	42.34 ± 4.11	16.62 ± 2.23	45.51 ± 2.59
	SMH 4–6	40	12	0	8.87	8.17 ± 0.17	92.11 ± 1.96	40.06 ± 2.76	16.04 ± 2.08	44.89 ± 0.84
	SMH 4–7	42	12	0	8.87	8.09 ± 0.56	91.17 ± 6.31	30.45 ± 2.59	12.54 ± 1.50	44.95 ± 1.07
	SMH 4–8	40	12	0	8.87	7.91 ± 0.31	89.14 ± 3.48	39.53 ± 0.55	15.74 ± 1.57	46.48 ± 0.93
	SMH 4–9	40	12	2	8.72	8.28 ± 0.23	95.00 ± 2.63	25.66 ± 3.04	9.36 ± 1.17	38.85 ± 2.15
	SMH 4–10	40	12	4	8.57	7.76 ± 0.43	90.51 ± 5.02	12.57 ± 5.32	4.57 ± 2.07	34.08 ± 1.04
	SMH 4–11	40	12	10	8.15	7.4 ± 0.19	90.80 ± 2.27	10.71 ± 3.70	3.75 ± 1.74	25.90 ± 0.98

T-test was carried out on the encapsulation efficiency between Group 1 and Group 2: compared with formulation without self-healing (Group 1, WM 1); between Group 3 and Group 4: compared with formulation without self-healing (Group 3, SM 3); between Group 1 and Group 3: compared with Group 1 (WM 1), P* < 0.05, P** < 0.01.

self-healing was selected. The self-healing temperature was then investigated (WMH 2-4 ~ WMH 2–7). The porosity of these four formulations decreased gradually from 80.56% to 64.13% with the healing temperature increased. Initial burst was also getting some decrease (from 32.73% to 28.75%). Although a relatively good healing effect was achieved under 42 °C, such temperature was a little high for the healing of the following plasticizer containing microspheres. Microspheres containing small amount of plasticizer were prone to aggregation and adhesion during the healing experiment under 42 °C (Fig. S3). Therefore, 40 °C was the optimal healing temperature. Finally, the effect of plasticizer on the self-healing of microspheres was investigated (WMH 2-8 ~ WMH 2-11). It was worthy of noting that the porosity and initial burst of drug were greatly reduced with addition of several amount of plasticizers (DEP, 0–10% w/w relative to the polymer material). The porosity of microspheres decreased from 71.03% to 27.19% and the initial release of drug was from 32.08% to 18.70%. This was due to the glass transition temperature (Tg) depression with the incorporation of several amount of plasticizers in the microspheres. Without presence of plasticizer, the Tg of the formulations was almost unchanged (around 45 °C, WMH 2-1 ~ WMH 2-8). When the plasticizer was added in (0–10%, WMH 2-8 ~ WMH 2-11), the Tg decreased gradually (44.80, 40.01, 34.24 and 27.31 °C separately). Although at 10% plasticizer content, both of the porosity and the initial burst of the microspheres were very low, this amount of additive could lead to the over-plasticization and aggregation/adhesion of microspheres (Fig. S4). Therefore, 4% plasticizer content was chosen.

For the formulations prepared by S/O/W method (Group 3 and Group 4, around 90%), the encapsulation efficiency of which was greatly improved compared with the formulations prepared by conventional W1/O/W2 emulsification method (Group 1 and Group 2, around 44%). The porosity or initial burst also decreased significantly (e.g., initial burst of Group 1 and Group 2 was 32.99% and 16.81% separately). In the self-healing experiment, with the extension of the healing time and increase of healing temperature or the added amount of plasticizer, the pores showed a similar healing trend like the formulations prepared by W1/O/W2 method discussed above. Generally, with addition of 4% plasticizer and after 12 h healing at 40 °C, the best healing effect was achieved (SMH 4, porosity and initial burst amount were 10.97% and 3.56% separately).

WM 1 in Group 1, WMH 2 in Group 2, SM 3 in Group 3 and SMH 4 in Group 4 were formulations without self-healing or with the best self-healing, which were prepared by W1/O/W2 or S/O/W methods separately. In order to further evaluate the microspheres prepared by the modified hydrophobic ion-pairing complex strategy, these four formulations were chosen for the following study.

3.3. FTIR analysis

To investigate whether the HIP complex between the drug (Oct) and DSS was successfully formed, a FTIR experiment was adopted. Two sharp absorption peaks were shown in the pure drug (1653 and 1529 cm^{-1}, Fig. 3A), which were ascribed to the stretching vibration of carbonyl group (C═O) on the peptide bond and a superposition vibration effect of nitrogen-hydrogen (N—H) bond and carbon-nitrogen bond (C—N). For DSS, a series of characteristic peaks of sulphate groups were observed: S—O—S (804 cm^{-1}), symmetric and asymmetric stretching vibration of SOO− (984 and 1227 cm^{-1}). When the drug and ion-pairing agent were physically mixed, all the characteristic absorption peaks of the two substances could be easily observed in the spectrum of their mixture. However, when they formed HIP complex, the vibration of the aforementioned peaks was highly diminished and even disappeared. The attenuation of the observed peaks confirmed the formation of the HIP complex.

After microspheres fabrication using the W1/O/W2 and the S/O/W

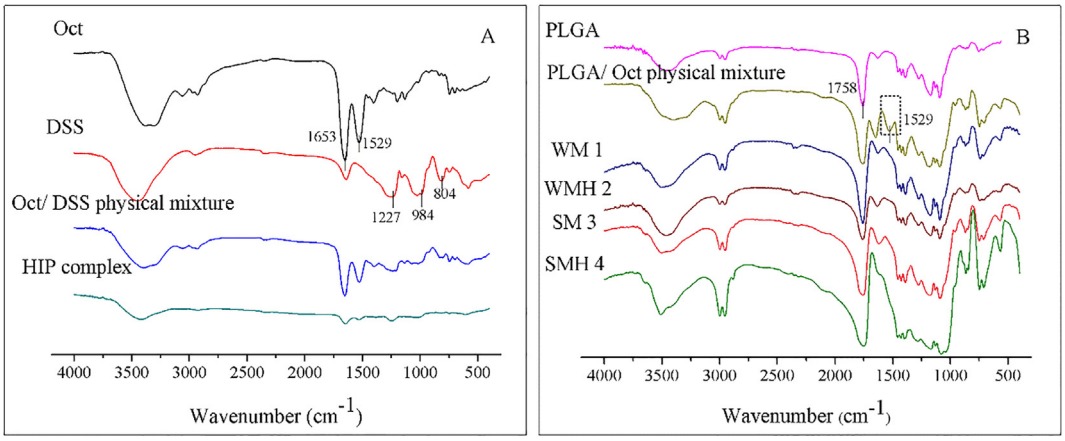

Fig. 3. Fourier Transform Infrared Spectroscopy of the substances before (A) and after (B) microspheres preparation.

emulsification methods, the interaction between the drug and PLGA was also investigated. As shown in Fig. 3B, the typical vibrations of PLGA were observed including the sharp characteristic peak of carboxyl group (COO—, 1758 cm^{-1}) and stretching vibration peaks of carbon-hydrogen bond (C—H, 2999 ~ 2952 cm^{-1}). It was found that the band of peptide was so close with the PLGA characteristic peak (Fig. 3A and B, Oct and PLGA). When the polymer was physically mixed with octreotide acetate, most peaks of the mixture were consistent with PLGA (aforementioned sharp peak of COO— and peaks of C—H, 1758 cm^{-1} and 2999–2952 cm^{-1} separately). Besides that, an obvious peak of drug was also observed (one of the sharp peak of octreotide acetate, 1529 cm^{-1}), which was circled by the dotted box. It indicated that all the characteristic peaks of the drug and polymer were reflected in the spectrum of their mixture. Hence, there was no bonding interaction existed within these two components. However, when the drug was encapsulated into the microspheres using W1/O/W2 and the S/O/W emulsification methods (WM 1, WMH 2, SM 3 and SMH 4), the characteristic peak at 1529 cm^{-1} representing the drug disappeared. It suggested that octreotide acetate in all of the four formulations prepared by W1/O/W2 and the S/O/W methods was effectively distributed into the PLGA matrix by molecular form. Similar study has been reported in previous study [18].

3.4. Scanning electron microscopy

The surface and internal morphology of the microspheres prepared by W1/O/W2 and S/O/W emulsification methods with or without self-healing were visualized using the scanning electron microscopy (SEM, Fig. 4). From the overall view of the microspheres, it can be seen that the four test formulations (WM 1, WMH 2, SM 3 and SMH 4) all showed a spherical shape and uniform particle size. Partial view of WM 1 showed that there was considerable amount of pores on the outer surface of the microspheres. Whereas few pores were observed on the microsphere surface prepared by HIP complex strategy (SM 3). When these two types of microspheres were dissected, distinguished pore differences were also similarly observed. Microspheres prepared by conventional W1/O/W2 method (WM 1) showed a porous internal structure with large spherical cavities and channels while microspheres prepared by S/O/W showed a few flat channels (SM 3). The large cavities found in WM 1 were related to the osmotic pressure difference formed between the internal high concentration drug solution (W1) and the external water phase (W2) of the W1/O/W2 method. This difference in osmotic pressure will result in great mass transfer between internal and external water phase. A large amount of drug was lost from the emulsion droplet along the osmotic gradient while the water in the outer phase diffused into the emulsion droplet against the osmotic gradient. This further explained the reason for the low drug encapsulation efficiency of formulations prepared by W1/O/W2 method. When the water was removed during the solvent evaporation or microsphere lyophilization process, large cavities and channels were left.

For formulations with self-healing, the microsphere surface got much smoother (overall morphology, comparison between WMH 2 and WM 1, comparison between SMH 4 to SM 3). No adhesion or agglomeration of microspheres was observed, which indicated that the selected healing conditions were mild and didn't lead to an overplasticization of the microspheres. Under such healing conditions, a good healing effect was achieved. Most of the surface pores of the microspheres were healed (partial morphology). The inner large cavities or channels also showed different degrees of healing (section morphology). The best healing effect was achieved in microspheres prepared by the S/O/W method (SMH 4). Barely visible pores could be observed inside or outside of SMH 4, whereas there were still some cavities left behind in WMH 2. Incomplete healing of the internal cavities/channels of WMH 2 was probably ascribed to the high initial value (too many and too large cavities and channels initially).

3.5. Fluorescence microscopy

To evaluate the pore healing effect, small amount of coumarin was added into the external water phase before the healing experiment was carried out. Coumarin, a commonly used water-soluble fluorescent dye, when excited by the blue light, it will fluoresce green. During the long term healing period (12 h), the dye would diffuse and penetrate into the microspheres through the interconnected water-filled pores within the microspheres. With the gradual healing of the pores, the dyes would be trapped into the microspheres. 12 h later, the microspheres were collected and washed to remove the free dyes from the microsphere surface. Then these microspheres were put into a 96-well plate with fresh release medium (PBST, pH 7.4). The diffusion/escape of the fluorescent dye from the single microsphere was monitored by Olympus microscopy (IX 51, Japan). Pore healing effect of the microspheres was determined by the amount of fluorescent dyes lost from the microspheres, which could be observed by the degree to which the microsphere fluorescence weakened with time. This method allowed monitoring the real-time diffusion of the dyes from the same microspheres, offering highly valuable insight into the underlying mass transfer process [42].

For WM 1, a strong green fluorescence was observed at 0 h (Fig. 5), which meant that a large amount of dyes has penetrated into the microspheres through the numerous interconnected water-filled pores within such formulation. However, without self-healing, the dyes diffused out gradually from the pores and the green fluorescence weakened or even became invisible with time when the microspheres were put into the blank release medium (PBST, pH 7.4). Similar strong green

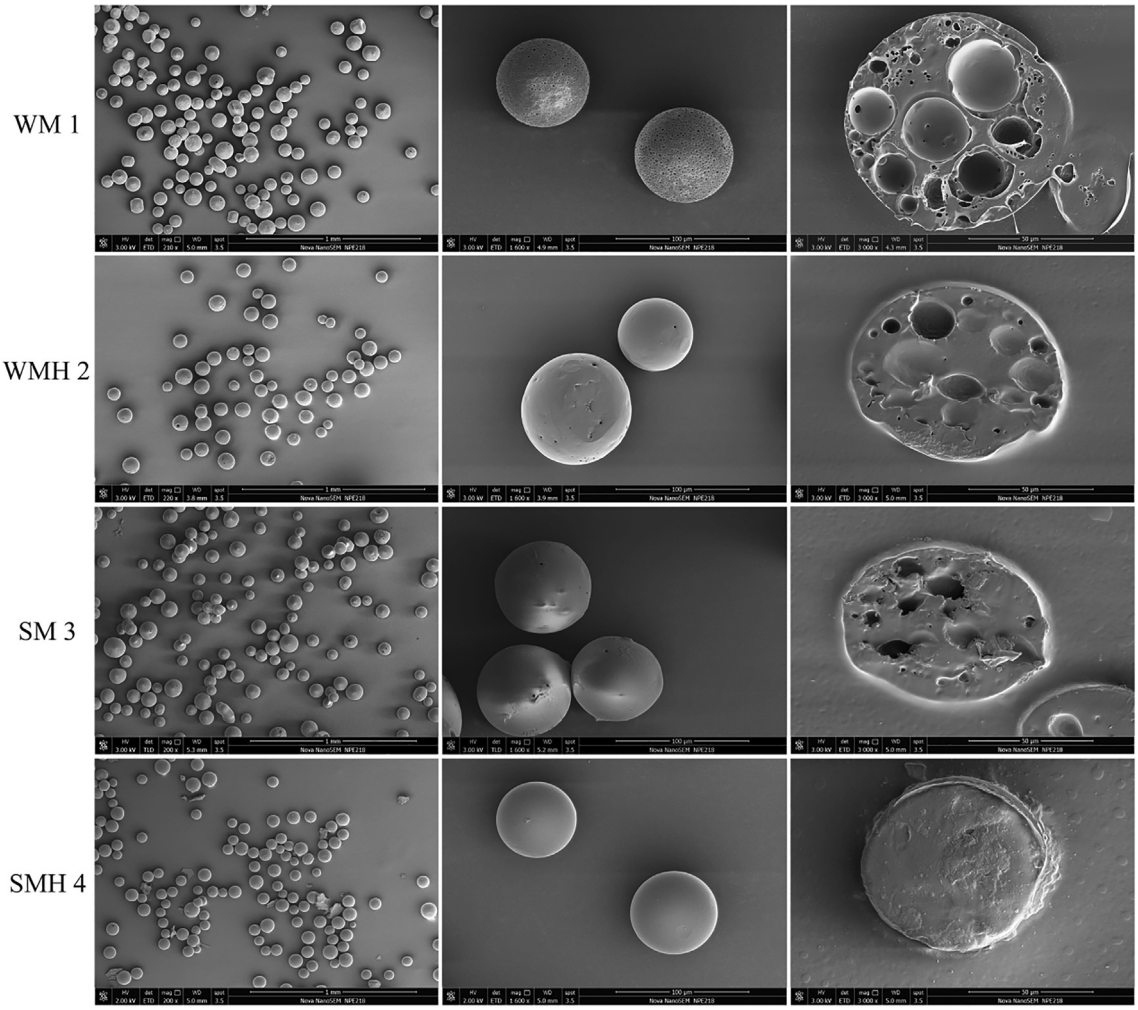

Fig. 4. Surface and internal morphology of the microspheres prepared by the W1/O/W2 or S/O/W method with or without self-healing (WM 1, WMH 2, SM 3 and SMH 4).

fluorescence was observed in WMH 2 at 0 h. With the incubation proceeding, the fluorescence was still strong with slightly weakened. It indicated that a good pore healing effect was achieved after self-healing experiment. The slightly weakened fluorescence was due to the incomplete healing in this formulation as described above (SEM). For SM 3 and SMH 4, a weak fluorescence ring was observed on the surface of the two formulations at 0 h. It was because very few pores were formed in the microspheres prepared by S/O/W and the few pores were not connected to each other. Hence, the dyes didn't penetrate into the microspheres. This result was consistent with the SEM observation. Without healing, the fluorescence on the microsphere surface of SM 3 was getting weak with time. However, for SMH 4, after pore healing experiment, the fluorescence of it was unchanged, which confirmed that a good healing effect was achieved.

3.6. In vitro drug release

The in vitro release profiles of the four test formulations prepared by W1/O/W2 and S/O/W with or without self-healing were characterized (WM 1, WMH 2, SM 3 and SMH 4). As shown in Fig. 6, after 35 day's incubation, the drugs in all of the four formulations were completely released. For both of these two preparation methods, with self-healing, the initial burst amounts were highly reduced (from 32.99% to 21.03%, 16.81% to 3.56% for W1/O/W2 and S/O/W separately), which has been discussed above. Two types of release patterns were observed (①: A and B, ②: C and D). For formulations prepared by W1/O/W2 method (WM 1 and WMH 2), the drug release behaviors followed a typically triphasic release profile (initial burst, lag phase and a secondary apparent zero-order phase) [43,44]. This release pattern was fitted to non-mechanistic sigmoidal curve fitting model with high fitting coefficients 0.9979 and 0.9977 (Fig. 6, the red fitting line) [45,46]. The high initial burst during the first day' incubation was due to the pore-related and surface-bound drugs, which was a diffusion-controlled release stage (the first stage). After burst release stage, a lag phase was followed. During this period, drug was rarely released (the second stage). When the polymer degraded rapidly, the drug release followed an apparent zero order release kinetics, which was an erosion-controlled stage (the third stage). The rate constants for WM 1 and WMH 2 in the secondary apparent-zero order phases were calculated and found similar (4.79 and 4.19 day^{-1} separately). It suggested that the healing experiment didn't affect drug release behavior.

On the other hand, for formulations prepared by S/O/W method (SM 3 and SMH 4), the drug release pattern was completely different. Undesired high initial burst and lag phase stage were not observed. Both of the two formulations showed a more satisfactory single phase release profile with sustained and steady drug release (C and D), which was fitted to zero-order kinetics with fitting coefficients 0.9872 and 0.9911. The possible causes resulting in such release mechanism were as followed: (1) the formation of hydrophobic ion pairs improved the compatibility between the drug and the polymer carrier; (2) the low porosity of the formulations prepared by S/O/W method greatly inhibited the diffusion-controlled release; (3) the solid HIP complex

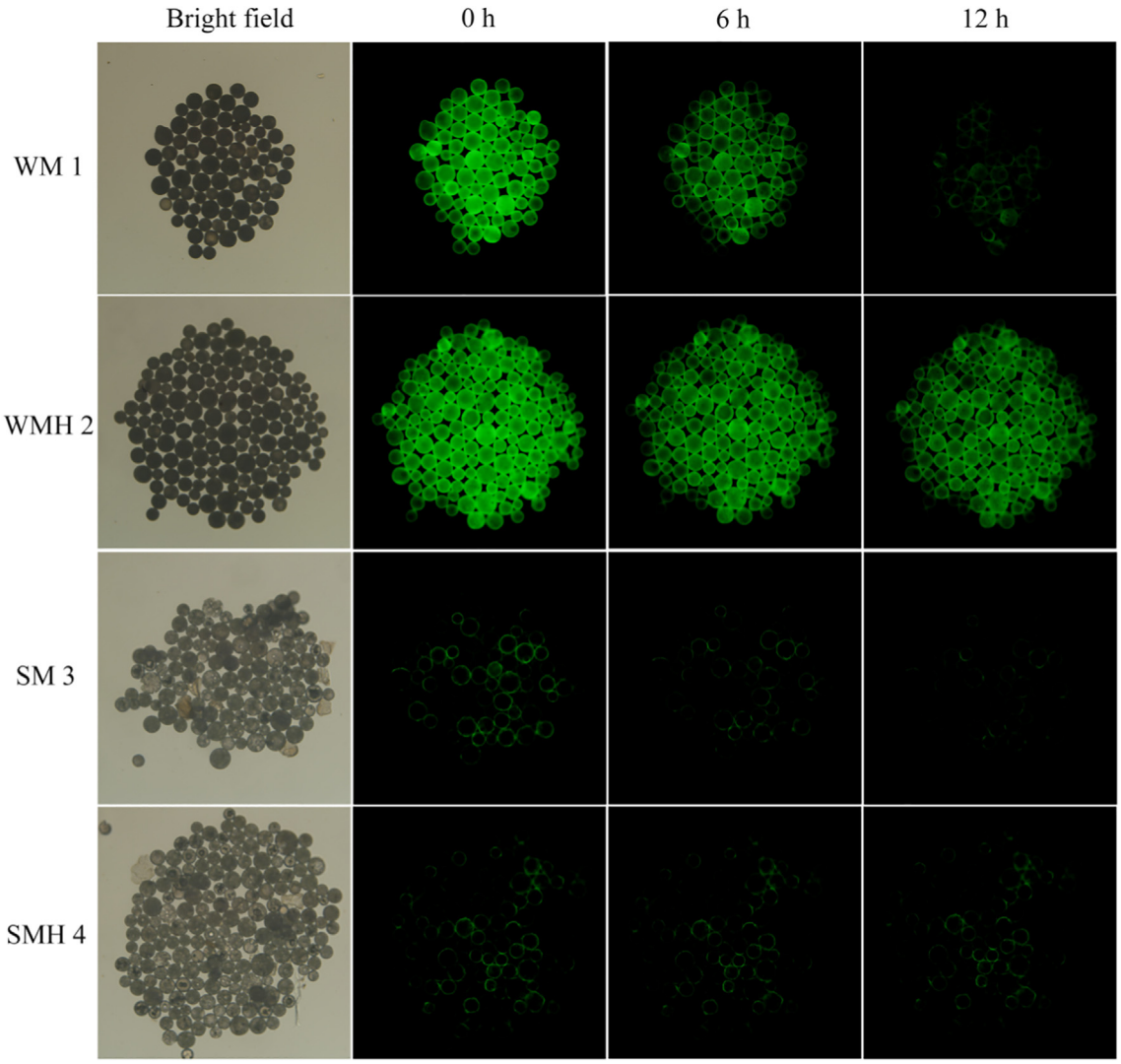

Fig. 5. The diffusion/escape of the fluorescent dye from the single microsphere prepared by the W1/O/W2 or S/O/W method with or without self-healing (WM 1, WMH 2, SM 3 and SMH 4).

within the microspheres will not induce an increase in the osmotic pressure so that the water uptake capacity of the microspheres decreased.

3.7. In vivo drug release

To further investigate the actual drug release behaviors in vivo, the blood samples of rats were collected and analyzed using a specific octreotide acetate ELISA kit (Caobenyuan Biotechnology Co Ltd, Nanjing, China). The pharmacokinetic curve was shown in Fig. 7 and the pharmacokinetic parameters were shown in Table 2. The range of safe and effective therapeutic concentrations of octreotide in vivo was 2–25 ng/ml (Fig. 7, dotted lines) [47–51].

For formulations prepared by W1/O/W2 method (WM 1 and WMH 2), the C_{max} was achieved in 1 h (T_{max}) after administration, which was due to high initial burst amount of the peptide (Fig. 7A and B, Table 2, 54.52 and 30.54 ng/ml separately). Excessive drug release during the initial stage would cause certain adverse reactions such as localised pain, biliary sludge and thyroid dysfunction etc. [52]. 3 h later, the drug concentration dropped to undetectable level (Fig. 7B). After about 3 days, the drug was detected again in the blood and the concentration of which was increasing to around 11 ng/ml in the following 15 days. Then drug concentration dropped again till undetectable during the clearance phase. A remarkable triphasic release profile was observed in these two formulations. It was consistent with the in vitro drug release results. For SM 3 and SMH 4, before the clearance phase, drug concentration in the blood remained relatively steady except for some initial burst in SM 3 due to lack of self-healing (20.56 ng/ml). Such steady drug concentration was rationalized by the single phase zero-order release kinetics confirmed in vitro.

In summary, it has been further confirmed in vivo that the modified HIP strategy with self-healing not only highly inhibited the initial burst, which avoided potential risks of overdose at the initial stage and guaranteed sufficient drug release at the prolonged stage, but it did also achieve an improved sustained drug release behavior. This was responsible for the safe and stable blood drug concentration during treatment. Hence the bioavailability of the octreotide acetate within the microspheres prepared by such strategy was significantly increased (the $AUC_{0-\infty}$ of WM 1 and SMH 4 was 178.14 and 289.07 separately).

4. Conclusion

The formation of hydrophobic ion-pairing (HIP) complex between octreotide acetate and dextran sulfate sodium (DSS) successfully reversed the highly water-soluble nature of the drug. Octreotide acetate was encapsulated into the microspheres in the form of hydrophobic

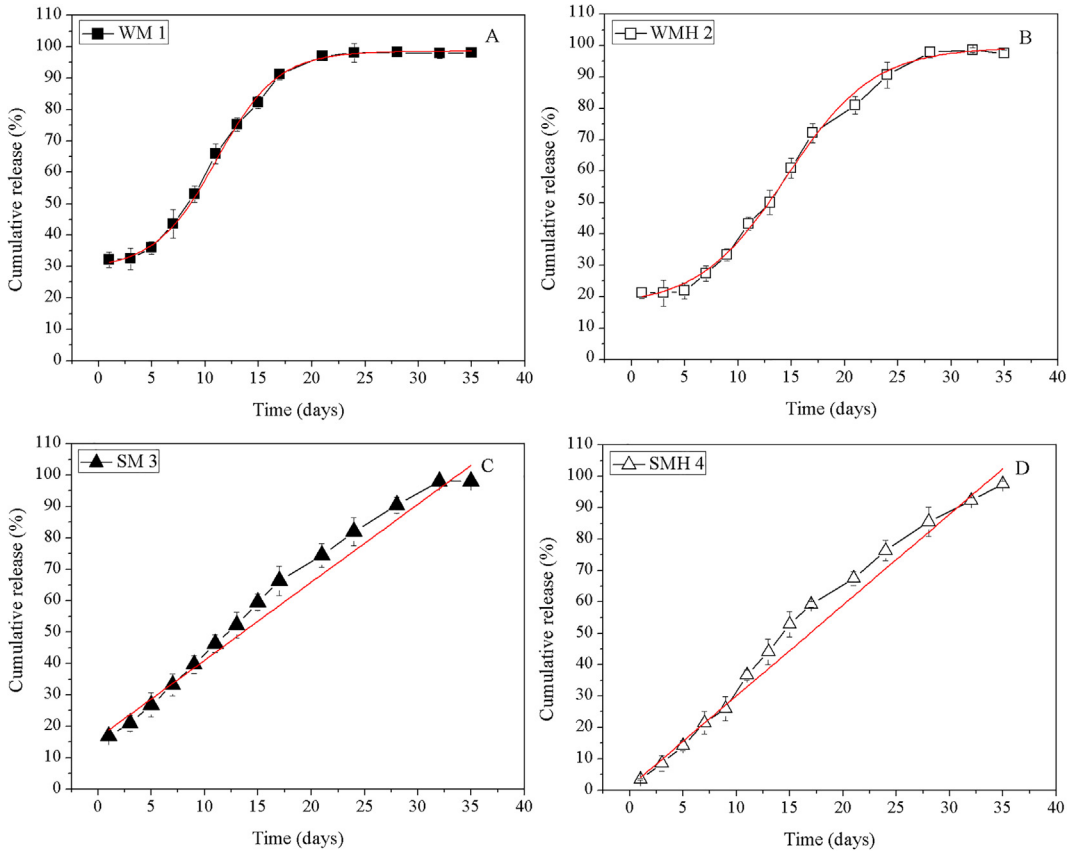

Fig. 6. In vitro drug release profiles of microspheres prepared by the W1/O/W2 or S/O/W method with or without self-healing (WM 1, WMH 2, SM 3 and SMH 4). The added red lines were fitted trend lines. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

complex using S/O/W method. The encapsulation efficiency of the drug was greatly improved compared with the conventional W1/O/W2 method (from 44% to 90%). With further self-healing, the surface pores and inner cavities or channels of HIP microspheres were effectively healed. Sealing off the drug-diffusion pathway leaded to cessation of the initial burst.

In the process of testing the in vitro release performance, a more satisfactory single phase release profile with sustained and steady drug release was observed in such HIP microspheres. This steady drug release pattern was responsible for the safe and stable blood drug concentration and high bioavailability in vivo. In summary, the modified HIP strategy could be a promising platform for highly water-soluble peptide encapsulation with high encapsulation efficiency, low initial burst and stable drug release mechanism.

Declaration of Competing Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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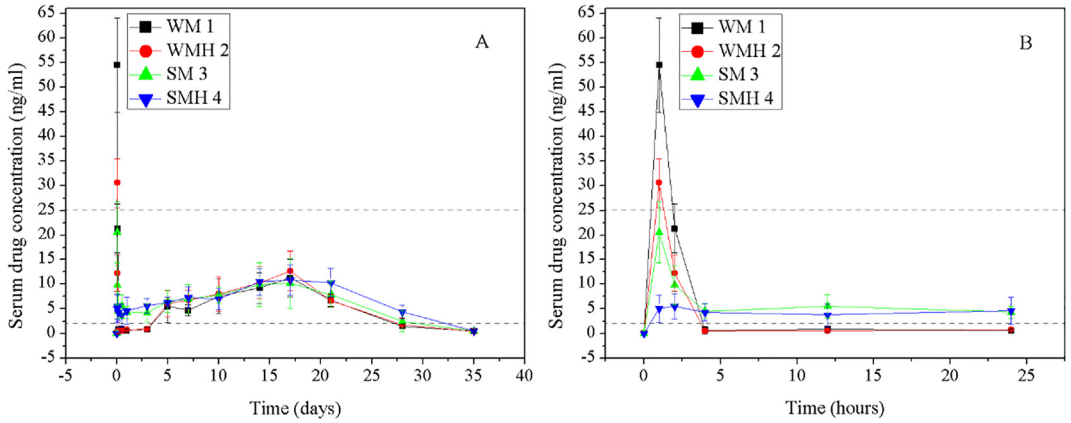

Fig. 7. In vivo drug release profiles of microspheres prepared by the W1/O/W2 or S/O/W method with or without self-healing (WM 1, WMH 2, SM 3 and SMH 4; Fig. 7A: 0–35 d, Fig. 7B: the first 24 h).

Table 2
Pharmacokinetic parameters of microspheres prepared by the W1/O/W2 or S/O/W method with or without self-healing (WM 1, WMH 2, SM 3 and SMH 4).

Parameters	WM 1	WMH 2	SM 3	SMH 4
$AUC_{0\text{-}35}$	176.70 ± 30.25	193.72 ± 60.99	209.20 ± 68.51	239.19 ± 50.00
$AUC_{0\text{-}\infty}$	178.14 ± 29.93	196.45 ± 64.29	213.45 ± 69.31	289.07 ± 58.59*
MRT0-35 (day)	15.15 ± 0.92	14.97 ± 0.65	14.31 ± 1.49	15.70 ± 0.33
$MRT_{0\text{-}\infty}$ (day)	15.50 ± 0.88	15.27 ± 0.82	14.56 ± 0.86	14.31 ± 1.97
$T_{1/2}$ (day)	3.12 ± 0.20	3.67 ± 0.66	3.81 ± 1.65	11.20 ± 7.83
C_{max} (ng/ml)	54.52 ± 9.62	30.54 ± 4.97*	20.56 ± 6.19**	12.35 ± 1.04**
T_{max} (day)	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	17.33 ± 3.51**

The pharmacokinetic parameters of WMH 2, SM 3 and SMH 4 were compared with WM 1 separately. P* < 0.05, P** < 0.01.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejpb.2019.09.022.

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