

Biodegradable PLGA Microspheres as a Sustained Release System for a New Luteinizing Hormone-Releasing Hormone (LHRH) Antagonist

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A sustained release poly(DL-lactide-co-glycolide) (PLGA) microsphere delivery system to treat prostate cancer for a luteinizing hormone-releasing hormone (LHRH) antagonists, LXT-101 was prepared and evaluated in the paper. LXT-101 microspheres were prepared from PLGA by three methods: (1) double-emulsion solvent extraction/evaporation technique, (2) single-emulsion solvent extraction/evaporation technique, and (3) S/O/O (solid-in-oil-in-oil) method. The microspheres were investigated on drug loading, particle size, surface morphology and *in vitro* release profiles. An accelerated release approach was also established in order to expedite the evaluation periods. The *in vivo* evaluation of the microspheres was made by monitoring testosterone levels after subcutaneous administration to rats. The LXT-101 PLGA microspheres showed smooth and round surfaces according to a scanning electron microscopic investigation, and average particle size of ca. 30 μm according to laser diffractometry. The drug encapsulation efficiency of microspheres was influenced by LA/GA ratio of PLGA, salt concentrations, solvent mixture and preparation methods. Moreover, LA/GA ratio of PLGA, different preparation methods and different peptide stabilizers affected *in vitro* release of drugs. *In vivo* study, the testosterone levels were suppressed to castration up to 42 d as for the 7.5 mg/kg dose. And *in vivo* performance of LXT-101 microspheres was dose-dependent. The weights of rat sexual organs decreased and histopathological appearance of testes had little changes after 4-month microspheres therapy. This also testified that LXT-101 sustained release microspheres could exert the efficacy to suppress the testosterone level to castration with little toxicity. In conclusion, the PLGA microspheres could be a well sustained release system for LXT-101.

Key words luteinizing hormone-releasing hormone (LHRH) antagonist; poly(DL-lactide-co-glycolide); microspheres; testosterone level

Peptides and proteins are main and important drugs continually along with the progress of genomics and proteomics. However, the unique properties of them, such as high molecular weight, easy degradation (*e.g.* by deamidation, Maillard reaction, oxidation and hydrolysis), instability and low bioavailability, make traditional dosage forms not proper to deliver them.¹⁾ Therefore, modern delivery systems like lipid microparticles,²⁾ nanospheres,³⁾ and microspheres⁴⁾ are applied to carry them though many problems exist. Microspheres prepared from poly(DL-lactide-co-glycolide) (PLGA) polymers have been studied extensively in the last two decades as sustained release dosage forms.^{5–7)} PLGA is biocompatible, and more importantly, the degradation rates of PLGA and the accompanying release of encapsulated drugs can be controlled by the polymer's physicochemical properties such as molecular weight, hydrophilicity, and the ratio of lactide (LA) to glycolide (GA).^{8,9)} Thus, it is possible to obtain the desired drug release from PLGA microspheres by altering the polymer's characteristics. However, typical formulation steps involved in microspheres preparation, such as exposure of molecules to aqueous/organic interface and high shear stress of emulsification, adversely affect the physical and biological properties of the drugs.^{10,11)}

Prostate diseases have profound impact on male health, as illustrated by the fact that prostate cancer (PC) is the most common malignant disease among men in the western world. It is estimated that males in Western countries are at $\approx 10\%$ risk of dying from the disease. The annual incidence of PC continues to increase in most countries, and this malignant disorder is the most common cause of cancer-related death among men in northern Europe. Today, a majority of men are diagnosed with PC in their 60- to 70-ties, of which 40% of

the patients progress to advanced non-curative disease-stages, while it is known that progression from local, organ-confined to advanced disease-stages may require 8 to 12 years, and another 3 to 5 years from metastatic PC to death from the disease.¹²⁾ The introduction of the LHRH analogues and antagonists revolutionized the treatment of advanced PC. No surgery is a potentially important physical and psychological benefit. Moreover, analogues and antagonists of LHRH have a proven and well-established role in the management of PC and aroused growing therapeutic interests recently.^{13,14)} Various applications have been established in the fields of oncology and andrology. LHRH antagonists appear to offer an effective option in the management of PC for suppressing testosterone levels. The major advantage of LHRH antagonists, such as cetrorelix, abarelix, over agonists is that they inhibit the secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH) immediately after application,¹⁵⁾ while agonists and super agonists lead to an undesired flare-up of hormone secretion in the first line before the desired reduction of hormone level is achieved.¹⁶⁾ Moreover, in addition to absence of flare and rapid down regulation, potential advantages of LHRH antagonists versus agonists include no need for combination therapy with an antiandrogen (improved compliance, fewer side effects and minor costs), simplification of management by removing the need to educate patients about antiandrogen use, more targeted therapy (with a potential direct effect on tumor cells) and a more pronounced downregulation of gonadotropin and testosterone. Potential applications of LHRH antagonists can be found in the treatment of infertility, benign prostate hypertrophy and sexual hormone-dependent tumors.^{17,18)} Especially for the latter continuous treatment over long time is usually needed.

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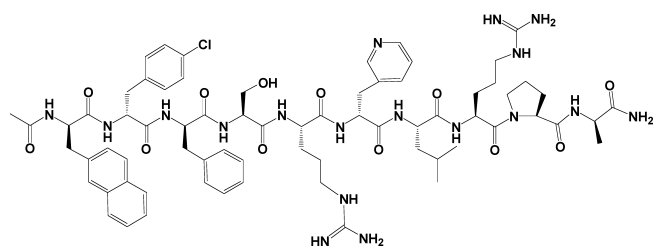


Fig. 1. Structure of LXT-101

A recent review on the role of peptide analogues in the therapy of PC has been published.¹⁹⁾

Nowadays the LHRH antagonists on market include PlenaxisTM and CetrotideTM. PlenaxisTM (abarelix for injectable suspension) is supplied as a white to off-white sterile dry powder which, when mixed with the diluent (0.9% Sodium Chloride Injection), becomes a depot suspension intended for intramuscular injection. CetrotideTM (cetrotirelix acetate for injection) 0.25 or 3 mg is a sterile lyophilized powder intended for subcutaneous injection after reconstitution with sterile water for injection (USP, pH 5–8). CetrotideTM may be administered subcutaneously either once daily (0.25 mg dose) or once (3 mg dose) during the early- to mid-follicular phase.²⁰⁾ As daily injections are undesirable for long-term treatment, incorporation of LHRH antagonists in sustained release formulations was of interest for the PC indication, e.g. liposome based implant,²¹⁾ lipid microparticles²⁾ and microparticles.²²⁾

LXT-101 (Fig. 1) as a decapeptide, molecular weight 1412 Da, is a new antagonist of LHRH and first synthesized by our laboratory (Chinese Patent No. CN90108955.9). In comparison with other antagonists this decapeptide exhibited a lower potential to release histamine, which hampers the use of the first generation of LHRH antagonists. Due to its improved physicochemical properties, LXT-101 is well adapted to clinical use. Though the stability has been increased as compared to natural LHRH, LXT-101 still has to be given parenterally and its short plasma half-life makes daily injections undesirable for long-term treatment. However, development of sustained release biodegradable microsphere injections for peptide delivery will help these agents to realize their full potential as drugs while enhancing patients' compliance and convenience. Moreover, such a formulation was successively developed for LHRH agonists, such as leuprorelin and triptorelin, and would therefore be in line with existing clinical practice, although till now no microspheres formulations for LHRH antagonists have yet reached the market.

The purpose of our investigation was to determine the influence of different process parameters during preparation on LXT-101 PLGA microspheres characteristics, especially surface morphology, encapsulation efficiency, and study *in vitro* release behavior and *in vivo* efficacy.

Experimental

Materials LXT-101, (Ac-D-Nal¹-D-Cpa²-D-Phe³-Ser⁴-Arg⁵-D-Pal⁶-Leu⁷-Arg⁸-Pro⁹-D-Ala¹⁰-NH₂), was synthesized by our laboratory. PLGA (Mw=14 kDa) was synthesized and supplied by Chemical Institute, Nankai University, China. Polyvinyl alcohol (PVA, 88% hydrolyzed, Mw=130 kDa) and all salts (NaCl, KH₂PO₄, K₂HPO₄) were purchased from Beijing chemical reagent Co. All other chemicals were obtained commercially as analytical

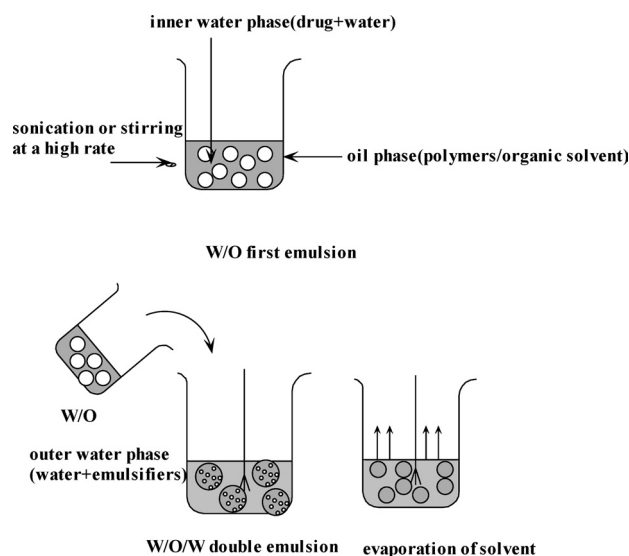


Fig. 2. The Preparation Process of Microspheres Using W/O/W Double Emulsion Solvent Extraction/Evaporation Technique

grade reagents.

Male Sprague-Dawley rats (0.20–0.25 kg) from Laboratory Animal Center of Beijing Institute of Pharmacology and Toxicology (BIPT) were used. Principles in good laboratory animal care were followed and all animal experimentation complied with the requirements of the National Act on the use of experimental animals (People's Republic of China).

Preparation of Microspheres Double-Emulsion Solvent Extraction/Evaporation Technique (W/O/W): The microspheres were obtained by double-emulsion solvent evaporation (W/O/W, water-in-oil-in-water), in the presence of PVA as previously described with modifications.²³⁾ The detailed processes were described as the followings and showed in Fig. 2. A 20% (w/v) LXT-101 aqueous solution of 0.15 ml was added into a 35% (w/w) PLGA solution in 0.6 ml methylene chloride, followed by homogenization for 2.5 min with a tissue mixer (Model 985370, Cole Parmer Instrument Co., U.S.A.) at 15000 rpm to form a W/O emulsion. The emulsion was then slowly injected (with a 1 ml syringe and a needle with the diameter 0.5 mm) into a 6% (w/v) aqueous PVA solution of 30 ml and agitated for 2 min at 400 rpm on a GL-4 electromagnet stirrer (Yuhua instrument, Gongyi, China). The resultant W/O/W emulsion was transferred into 2% (w/v) aqueous PVA solutions of 100 ml containing different amount of NaCl. The organic solvent was removed by agitating at room temperature for 3 h till the microspheres were solidified. The hardened white microspheres were centrifuged, washed three times with deionized water, freeze-dried at -50°C , 350×10^{-3} mbar (a LABCONCO freeze-dry system, U.S.A.) for 20 h and stored at 4°C in the desiccators (see Results and Discussion; "Encapsulation Efficiency" and "In Vitro Release").

Single-Emulsion Solvent Extraction/Evaporation Technique (O/W): The LXT-101 loaded microspheres were fabricated by a modified oil-in-water single emulsion solvent evaporation/extraction technique (O/W).²⁴⁾ Prescribed LXT-101 (25 mg/ml) and PLGA (250 mg/ml) were dissolved in methylene chloride/acetic acid (7:3, v/v) of 1.4 ml and the mixture was dropped slowly into 6% (w/v) aqueous PVA solution of 30 ml with 800 rpm agitating on a propeller stirrer, and the additional 2-min agitation was done after that. Then the suspension was transferred into 2% (w/v) aqueous PVA solutions of 100 ml and the organic solvents were removed by agitating at room temperature for 3 h till the microspheres were solidified. The hardened microspheres were centrifuged, washed three times with deionized water, freeze-dried at -50°C , 350×10^{-3} mbar for 20 h and stored at 4°C in the desiccators.

Solid/Oil/Oil Method: As for the microspheres prepared by the Solid/Oil/Oil (S/O/O) method, the drug powder would be micronized firstly. The micronization of LXT-101 using amphiphilic polymers was conducted as described previously with modifications.²⁵⁾ LXT-101 of 10 mg was added into a glass test tube with 1 ml aqueous media containing 90 mg PEG 20000. The mixtures were frozen on a pre-cooled shelf at -50°C in a freeze-dryer (a LGJ-18 freeze-dry system, Beijing Sihuan Scientific Instrument Works, China), and the freezing samples were experienced drying at -20°C for 3 h and 20°C for 12 h in turn. The lyophilized white solid was first dispersed in

methylene chloride of 1 ml to dissolve PEG 20000 and then centrifuged with 14000 rpm for 10 min to remove the amphiphilic polymer. The obtained lyophilized product was dispersed in 0.2 ml of acetonitrile, which resulted in a suspension of LXT-101. Then, 90 mg PLGA were added to the suspension to complete an organic phase. This organic phase was added into 20 ml cottonseed oil containing 1.6% Span 85 (Fisher), and homogenized with a propeller stirrer at 700 rpm for 5 min. The resulting S/O/O emulsion was stirred at 600 rpm for 3 h and then 30 ml petroleum ether (bp 60–90 °C) was added into cottonseed oil to extract acetonitrile. The hardened microspheres were collected by centrifuging and washed with an excess amount of petroleum ether (bp 60–90 °C) for three times, and finally freeze-dried.

Characterization of Microspheres Particle Size Distribution: Particle sizes and its distribution were measured by a laser light scattering technique (a LS800 Particle size analyzer, Omec Instruments, Zhuhai, China). The dried powder samples were suspended in deionized water containing 0.02% (w/v) Tween 20 (Sigma) and then sonicated in water bath for 3 min to disperse the microspheres before measurement. And then the obtained homogeneous suspension was examined to determine the volume mean diameter V_{md} in microns and size distribution.

Surface Morphologic Investigation by Scanning Electronic Microscopy (SEM): The surface morphology of microspheres was examined by a scanning electron microscope (Model JSM-5600LV, JEOL, Tokyo, Japan). The freeze-dried microspheres were mounted onto copper stubs using a double-sided adhesive tape. After vacuum-coated with a thin layer of gold, the specimens were examined by SEM at 15 kV or 25 kV.

Differential Scanning Calorimetry (DSC): Glass transition temperatures (T_g) of the polymers and the drug-loaded microspheres were measured with a differential scanning calorimeter (DSC823, METTLER TOLEDO, Switzerland). The samples of 3 mg were crimped into aluminum pans, and subjected to a heat/cool/heat cycle between –40 °C and 100 °C. Heating and cooling rates were 10 °C/min and a steady stream of nitrogen gas was supplied at 20 ml/min. T_g were calculated from the second heating cycle by analysis software supplied by the instrument manufacture.

Peptide Determination: The microspheres of 3 mg were dispersed in 0.5 ml acetonitrile to dissolve PLGA. Water of 4.5 ml was added into the solution and agitated for 2 min to extract the peptide. The peptide was assayed in the aqueous phase by HPLC method. The HPLC system consisted of a pump (L-7110, Hitachi, Japan) and a UV detector (L-7420, Hitachi, Japan). A C18 reverse-phase chromatography column (Kromasil™ 100A, 250 × 4.6 mm, Dikma Technologies, China) was used. The mobile phase was 48% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid, 0.2% (v/v) triethylamine and detection was carried out at 215 nm. The injection volume was 20 μ l and the flow rate was 1.0 ml/min.

The encapsulation efficiency of the microspheres was calculated as the following equal formula.

$$\text{encapsulation efficiency (\% w/w)} = \frac{\text{(actual drug content)}}{\text{(nominal drug content)}} \times 100$$

In Vitro Release of Microspheres A 2-ml phosphate buffer solution (10 mM, pH=7.4) containing 0.02% Tween 80 and 0.02% Tween 20 (Amresco, US) was added into a 5-ml tube, and then the drug-loaded microspheres of 10 mg were added in the tube and suspended thoroughly. The tube was placed in a 50 °C water bath and shaken at 160 rpm speed horizontally. At different time intervals, the tube was centrifuged at 3500 rpm for 10 min and a 2-ml supernatant was removed to be determined and the equal volume of fresh phosphate buffer was re-added. The released LXT-101 was assayed by spectrophotometry on a fluorescence spectrometer (F4500, Hitachi, Japan) with Ex (excitation wavelength) 280 nm and Em (emission wavelength) 335 nm. The tests were carried out in triplicates.

In Vivo Study Male Sprague–Dawley rats ($n=5$) weighing 0.20–0.25 kg were used to evaluate *in vivo* performance of LXT-101 microspheres prepared by the W/O/W technique. The microspheres were injected subcutaneously with different doses of LXT-101 PLGA microspheres at the left leg after reconstitution with a suitable medium (0.5% carboxymethylcellulose and 5% mannitol, w/v). The rats that were only administered with the equal volume of the blank reconstituted medium were as controls. 0.5 ml blood samples were collected from the ophthalmic venous plexus into heparinized Eppendorf tubes at designed time points. The samples were centrifuged and blood plasma was collected which stored at –20 °C until analysis.

The blood plasma testosterone concentrations of blank or after administration were assayed using an immunochemiluminescence assay with the specific Access Immunoassay System (Beckmann Coulter). The testosterone detection limit was 0.08 ng/ml, and the intra- and inter-assay coefficients of

variation were 10 and 9%, respectively.

Histological Assessment and Weight Changes of Sexual Organs The microspheres prepared by the W/O/W technique were subcutaneously injected to the rats on the back with the dose of 15 mg/kg. At 4-month after administration, the sexual organs of rats were isolated including testis, prostate glands and seminal vesicles after all the rats were ethically sacrificed. Testis were rinsed with a saline solution, fixed with 10% neutral carbonate buffered formaldehyde, embedded in paraffin using an embedding center, and cut into slices. The slices were stained with hematoxylin and eosin and observed under a light microscope (CK30/CK40 Olympus, Japan). The three kinds of glands were weighed. The rats that were only administered with the equal volume of the blank reconstituted medium were as controls.

Results and Discussion

Microsphere Morphology For microspheres prepared with a W/O/W technique, morphological investigations by SEM showed the spherical shape as well as the internal sporadic porosity due to solvent evaporation prior to SEM. As already noticed by others,²⁶⁾ the surface structure was the consequence of the W/O/W preparation process. The microspheres prepared using the W/O/W technique were prepared from a clear homogeneous solution of polymer and drug, and the drug would like to be molecularly distributed in the PLGA matrix. However, the appearances of microspheres prepared by an O/W technique were relatively different. The surface was smooth, compact and exempt of pores. This could be related to the lack of the internal aqueous phase during the O/W technique. As also observed in previous studies,²⁶⁾ the volume of the internal aqueous phase influences the microstructure (porosity) of the microspheres. With the increase of the internal aqueous phase a more porous matrix structure would be observed. But the internal structure could not be deduced from SEM since no broken spheres were present. As for microspheres produced by a S/O/O method, they were less spherical compared with microspheres produced by the two methods above. And there were some concaves on the surface. This might be explained by the better stabilization of water/oil interfaces due to the presence of PVA. For the absence of PVA as the emulsifier and stabilizer in the outer phase during the course of the S/O/O emulsion, small air bubbles might be collapsed and therefore induce some concaves on the surface (Fig. 3).

The type of preparation methods did not influence the size of the microspheres significantly. In all cases, the average size of microspheres was between 30 and 50 μ m (volume mean diameter V_{md}) (Fig. 4), which was suitable for intramuscular or subcutaneous injections, and a unimodal narrow size distribution (span, uniformity).

Thermal Behavior of Drug-Loaded Microspheres DSC thermograms were processed and T_g values were calculated for both the polymers and microspheres. PLGA 75/25 polymer has a T_g value of 38 °C whereas drug-loaded microspheres exhibited considerably higher T_g values of 42 °C. This result corresponded with Okada²⁸⁾ and Shameem *et al.*²⁹⁾ observed that T_g increased from 1 to 8 °C with loading of peptide. This increase might be ascribed to ionic interaction between the basic amino acids of the drug and the terminal carboxylic anions of the polymer.²⁸⁾ And this interaction between the polymer and the peptide would like to increase the rigidity of the polymer macromolecule and was consistent with the hydrophobicity of the peptide, which might increase the T_g .

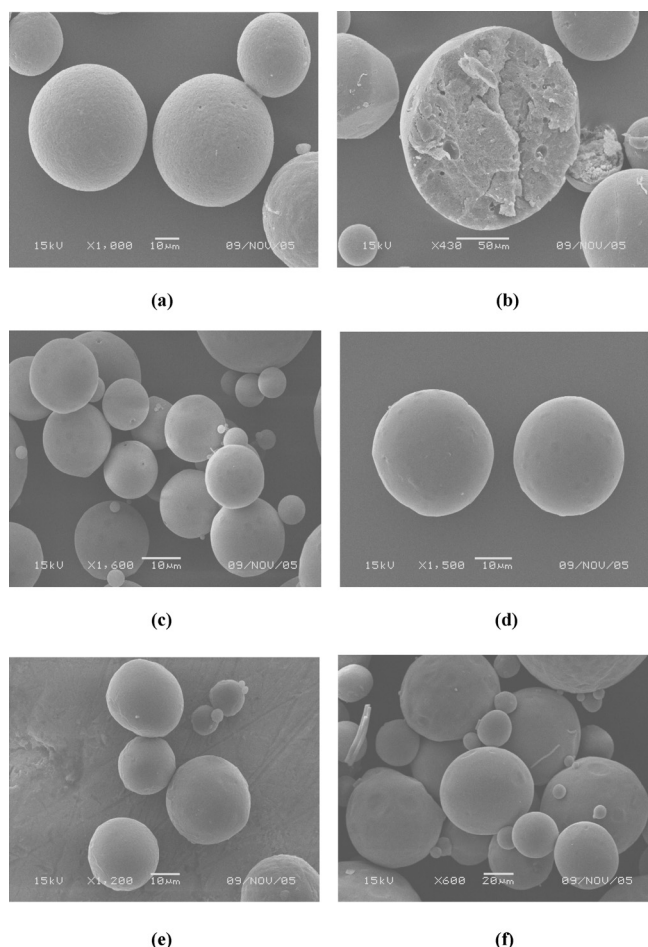


Fig. 3. Scanning Electron Microscopy Pictures of Microspheres Prepared by Different Methods: Double-Emulsion Solvent Extraction/Evaporation Technique (W/O/W) (a, b), Single-Emulsion Solvent Extraction/Evaporation Technique (O/W) (c, d), S/O/O Methods (e, f)

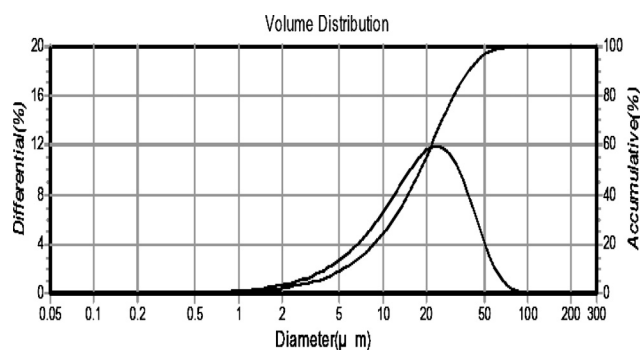


Fig. 4. Particle Size Distribution of LXT-101 Microspheres

Encapsulation Efficiency Effect of LA/GA Ratio: Encapsulation efficiency is an important index to evaluate drug-loaded microspheres as it is much more economical with higher encapsulation efficiency. A W/O/W technique is mostly used for the encapsulation of water-soluble drugs and therefore is the main method of choice for the peptide LXT-101. One objective of this study was therefore to investigate the important factors that influenced the encapsulation efficiencies during the W/O/W technique. First, the encapsulation efficiency was highly dependent on the LA/GA ratio of PLGA (Table 1). The encapsulation efficiency reduced sig-

Table 1. Effect of LA/GA Ratio, Salt Concentrations in Outer Aqueous Phase and Solvent Mixture on Encapsulation Efficiency of the Drug-Loaded Microspheres

Factors	Encapsulation efficiency (%)
LA/GA ratio ^{a)}	
40 : 60	55.8
50 : 50	62.9
60 : 40	71.2
75 : 25	74.7
Salt concentrations (% w/v)	
2	46.3
5	65.5
8	70.1
10	72.3
Solvent mixture % (w/v)	
Ethyl acetate : methylene chloride ^{b)}	
100 : 0	47.3
75 : 25	51.2
50 : 50	58.5
0 : 100	63.5

a) Ratio of mole. b) Ratio of volume.

nificantly from 74.7% with LA/GA (75:25, mol/mol) to 55.8% with LA/GA (40:60, mol/mol). This may be due to that with the increase of GA ratio, the hydrophilicity of the polymer increase and enhance the chances of hydrophilic LXT-101 diffusing into the outer aqueous phase. However, the encapsulation efficiency of LA/GA (75:25, mol/mol) and LA/GA (60:40, mol/mol) microspheres had no significant difference, which might be related to the hydrophilicity of the two different PLGA was close.

Effect of Salt Concentrations: In the W/O/W method, the influence of salt concentrations on the encapsulation efficiency of microspheres was also evaluated. It was shown that salt concentrations had little influences on encapsulation efficiency when salt concentrations were above 8%, but when the salt concentrations were below 8%, the encapsulation efficiency tended to decrease with the decrease of salt concentrations in the outer aqueous phase, which might be attributed to the osmotic pressure gradient between the internal and external aqueous phase.²⁷⁾ In general, high encapsulation efficiencies were obtained with a higher electrolyte concentration in the external phase (Table 1).

Effect of Different Solvents: As an alternative to the widely used but toxic methylene chloride, ethyl acetate has been evaluated as a solvent for the formation of microspheres. As shown in Table 1, when ethyl acetate was partly or fully used as the solvent to fabricate microspheres, the difference in the encapsulation efficiency was negligible. However, the morphology changes of microspheres were obvious. The microspheres prepared fully by ethyl acetate were not smooth when compared with those prepared fully by methylene chloride and had much more porous on the surface. This might be caused by that ethyl acetate was miscible with water (8.7% wt% ethyl acetate in water), and would be rapidly extracted from the polymer phase in the W/O/W emulsion, leading to fast precipitation of the matrix polymer and the formation of porous microspheres. (Fig. 5). Porous surface definitely increased the chance for the drug to diffuse out since the diffusion channel and water uptake.

Effect of Different Preparation Methods: The different preparation methods also influence the encapsulation effi-

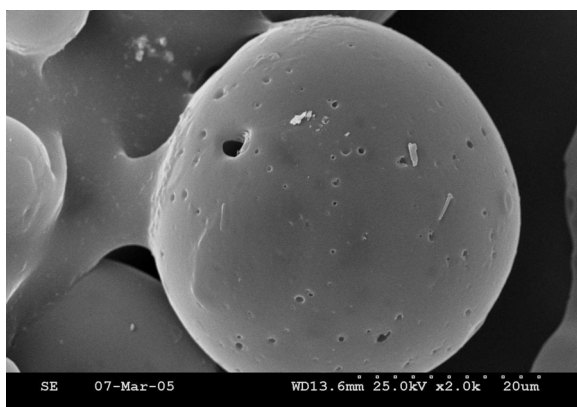


Fig. 5. The Scanning Electron Microscopy Picture of Microspheres Prepared with Ethyl Acetate as Solvent

ciency. Among the three preparation methods, the W/O/W method had the highest entrapment efficiency (79.0%) and the differences between the other two methods (S/O/O and O/W techniques) were negligible (58.9, 62.6% respectively). As for the S/O/O technique, solid drug particles were suspended in the organic polymer solution. The step of dissolving the drug into the internal aqueous phase was eliminated. Thus drug particles might be easily migrate to the surface of the droplet, and subsequently into the outer oil phase. However, the internal drug phase of the microspheres prepared by the O/W technique consisted of drug suspension without visible particles and not of a drug solution as LXT-101 was not completely dissolved in that polymer solution. This probably led to some drug loss during the preparation of microspheres by the O/W technique.

In Vitro Release Selection of Investigating *in Vitro* Release Conditions: Many factors would like to influence the drug release of PLGA microspheres such as the preparation techniques, the ratio of the copolymers, *etc.*³⁰ The burst effect of the microspheres attracts much attention because it may result in the severe adverse action or economically waste. In most cases, the burst release was attributed to the release of the drug dispersed on the surface of the microspheres. So the burst release was connected closely with those factors that influenced the drug dispersion in microspheres and the porosity caused by an organic solvent evaporation.

It usually takes a long time to evaluate *in vitro* release profiles of microspheres (usually above 30 d) and it's essential to establish an accelerated release approach to shorten the evaluation periods. For all accelerated conditions, release was faster at temperatures above the T_g of the host polymer.³¹ Peptide release from drug-loaded microspheres at 50 °C within 30 h correlated well with the real-time release at 37 °C within 30 d by optimization of release conditions such as temperature and surfactant concentration (Fig. 6). This result may be applied to *in vitro* release profiles of other drug-loaded microspheres in order to expedite the evaluation periods.

Effect of LA/GA Ratio on *in Vitro* Release: The *in vitro* release properties of the microspheres were influenced by different LA/GA ratio of PLGA (Fig. 7). LXT-101 release from PLGA microspheres was governed by an asymptotic profile in which up to 87% (40/60, mol/mol), 64% (50/50, mol/mol), 60% (60/40, mol/mol) of the peptide was released

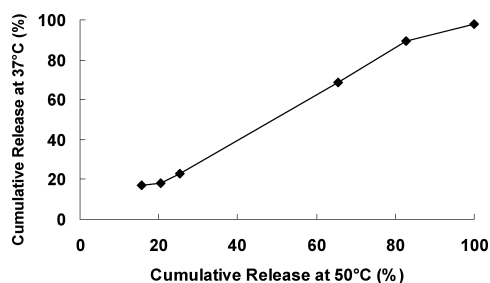


Fig. 6. Correlation between *in Vitro* Release at 50 °C and 37 °C of LXT-101 Microspheres

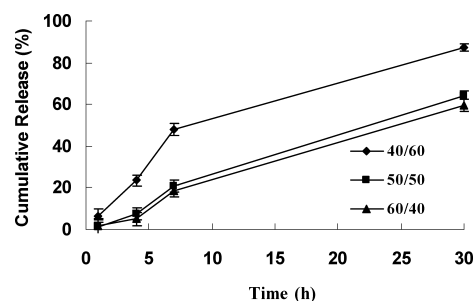


Fig. 7. *In Vitro* Release Profiles of LXT-101 Microspheres Prepared by PLGA with Different LA/GA Ratios

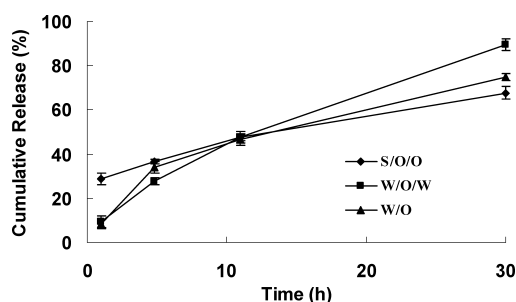


Fig. 8. *In Vitro* Release Profiles of LXT-101 Microspheres Prepared by Different Preparation Methods

within 30 h. Incomplete release after this time was perhaps due to the formation of LXT-101 aggregates during the encapsulation procedure. The results hereinbefore could be explained by the following reasons. At the beginning of the drug release, the release profile was mainly dominated by permeation mechanism. Along with the introducing of the GA content, the hydrophobicity of the materials was decreased markedly. It makes the drug diffusion through the microsphere's wall much easier.

Effect of Different Preparation Methods on *in Vitro* Release: Figure 8 indicated the release profiles of microspheres prepared by different methods. Comparing with the initial release of the microspheres prepared by W/O/W and O/W methods, that of the microspheres made by a S/O/O method was significantly high (28.2%). For the microspheres prepared by the O/W method, the lowest initial release (8.51%) was observed, the differences of the initial release between W/O/W and O/W methods were slight (9.25% and 8.51%, respectively). This might be explained by that there were much more dry drug powder attached or dispersed on the surface or underneath the surface of the microspheres prepared by the S/O/O method.

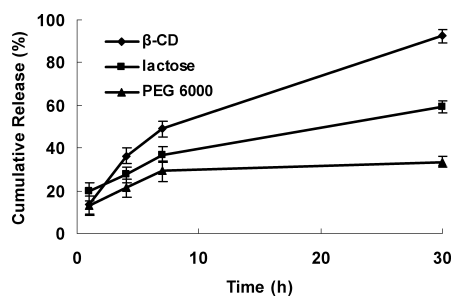


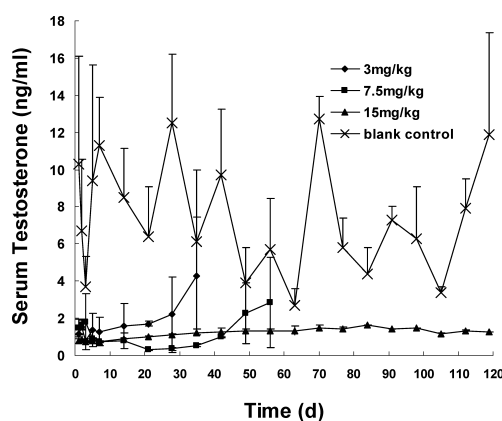
Fig. 9. *In Vitro* Release Profiles of LXT-101 Microspheres Prepared with Different Stabilizers

Effect of the Stabilizers on *in Vitro* Release: In order to control the drug's stability and release activity, some stabilizers are added into the formulations, for example, gelatin, amino acids, proteins and cyclodextrin. And these stabilizers also have influences on the release profiles of microspheres. Figure 9 shows the release profiles of microspheres with β -cyclodextrin (β -CD), lactose and polyethylene glycol 6000 (PEG 6000). The drug release rate was decreased markedly when PEG 6000 were added into the formulations. This may be due to that there is stronger force between hydrophilic PEG 6000 and LXT-101. However, the microspheres prepared with β -CD have smooth appearance release profiles. The initial burst was 13.5% and up to 90% of the peptides were released within 30 h (Fig. 9).

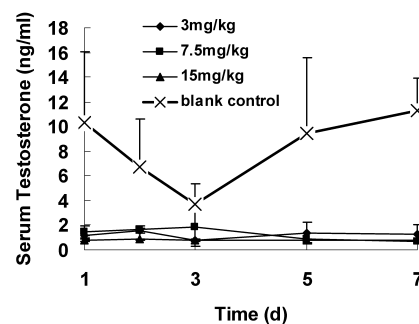
Testosterone Suppression *in Vivo* The advantage of the antagonists over the agonists/superagonists is that they prevent the characteristic stimulatory phase of the pituitary-gonad axis leading to an initial acute increase of hormonal secretion (surge) which may worsen disease symptoms (flare), *e.g.* in the case of PC.³²⁾ Figure 10 shows testosterone levels in rats after a single subcutaneous administration of the peptide-containing PLGA microspheres. As for the 7.5 mg/kg dose, the testosterone levels were suppressed to 1.68 ng/ml at 8 h, 0.91 ng/ml at 5 d and the levels remained near this level up to 42 d. Performance of the LXT-101 microspheres was dose dependent and improved efficacy both in onset and duration of action. Onset of inhibition was faster and duration of action was prolonged with increasing the dose. Distinction between the different doses of the microspheres was confirmed, in the order 15 mg/kg (119-d testosterone inhibition) > 7.5 mg/kg (42-d testosterone inhibition) > 3 mg/kg (14-d testosterone inhibition). At 1 d, the testosterone level of 15 mg/kg dose (0.80 ng/ml) was lower than 7.5 and 3 mg/kg (1.49, 1.15 ng/ml respectively) (Fig. 10).

Histological Assessment and Weight Changes of Sexual Organs in Rats The testis section from a rat treated with the LXT-101 microspheres showed slight atrophy of seminiferous tubules and tubular degeneration. However many tubules still had a normal appearance (Fig. 11).

Testis, seminal vesicle and prostate weights were significantly decreased after subcutaneous administration for 4 months, compared with the control. It demonstrated that LXT-101 sustained release microspheres could treat PC by suppressing the testosterone level to castration (Table 2). As serum testosterone concentrations were reduced more rapidly, it is anticipated that tumor and prostate volume also decreased faster.



(A)



(B)

Fig. 10. *In Vivo* Serum Testosterone Concentrations in Rats after a Single Subcutaneous Administration with Different Doses (A) and the Figure below with Magnification from 1 to 7 d (B)

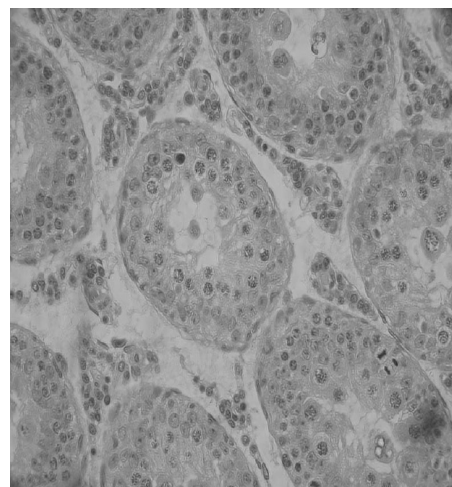


Fig. 11. Histopathological Appearance of Testes, H&E, Magnification, 400 \times

Table 2. Weight Changes of Sexual Organs in Rats after Subcutaneous Administration for 4 Months

Group (n=6)	Control	LXT-101
Testis (g) ^{b)}	1.685 \pm 0.201 ^{a)}	0.180 \pm 0.028**
Seminal vesicle (g) ^{b)}	1.604 \pm 0.249	0.083 \pm 0.012**
Prostate (g) ^{b)}	0.717 \pm 0.173	0.187 \pm 0.054*

** $p < 0.01$ significant difference from control. * $p < 0.05$ significant difference from control. a) Mean \pm S.D. b) Weight of unilateral tissues.

Conclusions

Microspheres prepared from polymers as a new drug delivery systems have been studied extensively in the last years and enlarge the fields of pharmaceutical research. A new LHRH antagonist, LXT-101 of the paper is a successful example of the microspheres. In this work, we: (1) successfully prepared LXT-101 PLGA microspheres with different LA/GA ratio and three preparation methods with a relatively high encapsulation efficiency, (2) established an accelerated method to expedite the evaluation periods of *in vitro* release, (3) showed a well efficacy in rats after single subcutaneous administration. Much more microspheres including LXT-101 will be prepared from more peptides on the basis of this paper. It can be predicted that the microspheres will be applied to anti-cancer and gene therapy much more extensively.

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