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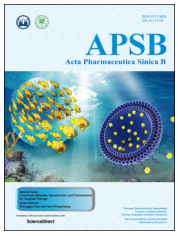

ORIGINAL ARTICLE

Preparation and evaluation of a phospholipid-based injectable gel for the long term delivery of leuprolide acetaterrh

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KEY WORDS

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Abstract A phospholipid-based injectable gel was developed for the sustained delivery of leuprolide acetate (LA). The gel system was prepared using biocompatible materials (SPME), including soya phosphatidyl choline (SPC), medium chain triglyceride (MCT) and ethanol. The system displayed a sol state with low viscosity *in vitro* and underwent *in situ* gelation *in vivo* after subcutaneous injection. An *in vitro* release study was performed using a dialysis setup with different release media containing different percentages of ethanol. The stability of LA in the SPME system was investigated under different temperatures and in the presence of various antioxidants. *In vivo* studies in male rats were performed to elucidate the pharmacokinetic profiles and pharmacodynamic efficacy. A sustained release of LA for 28 days was observed without obvious initial burst *in vivo*. The pharmacodynamic study showed that once-a-month injection of LA-loaded SPME (SPME-LA) led to comparable suppression effects on the serum testosterone level as observed in LA solution except for the onset time. These findings demonstrate excellent potential for this novel SPME system as a sustained release delivery system for LA.

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Abbreviations: LA, leuprolide acetate; LHRH, luteinizing hormone release hormone; MCT, medium chain triglyceride; NMP, *N*-methyl-2-pyrrolidone; SPC, soya phosphatidyl choline; SPME, the injectable gel system (soya phosphatidyl choline, medium chain triglyceride and ethanol); SPME-LA, LA-loaded SPME; VPGs, vesicular phospholipid gels.

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1. Introduction

Prostate cancer is the most commonly diagnosed cancer and accounts for high mortality among men. Routine treatments for prostate cancer include surgical intervention, radiotherapy and chemotherapy. Since prostate cancer cells depend on androgens for proliferation and growth, androgen deprivation remains the most prevalent treatment for prostate cancer. This is especially the case for advanced prostate cancer[1]. Clinically, androgen deprivation therapy includes medical and surgical castration. Notably, the effects of medical castration are comparable to surgical castration with bilateral orchiectomy[2].

Regarding medical castration, luteinizing hormone release hormone (LHRH) agonist analogs are often used for the effective downregulation of LHRH receptors[3], thereby indirectly decreasing testosterone levels. Among various analogs, leuprolide acetate (LA), a potent LHRH agonist, is often used clinically for medical castration. As a water-soluble peptide, LA requires repeated injections to obtain desirable therapeutic effect due to extremely short half-lives *in vivo*[4]. The poor patient compliance resulting from repetitive injections greatly limits this medical use for LA. To solve this problem, various long-term sustained-release preparations, such as microspheres[5–7], implants[8] and *in situ* forming implants[9–11] have been prepared and studied. Among these, *in situ* forming implants based on phase separation show several advantages including ease of preparation and lack of required surgery. After being subcutaneously administered in liquid form, *in situ* forming implants gradually form a solid or semisolid drug delivery depot at the injection site by solvent exchange. Eligard[12], a commercial *in situ* forming implant of LA, shows sustained drug release over one-, three-, four- and six-month periods. Nevertheless, *in situ* forming implants are also associated with potential safety concerns for the presence of high organic solvents in the polymer matrix, such as *N*-methyl-2-pyrrolidone (NMP, about 55%, *w/w*)[13]. Moreover, the high initial burst due to fast phase-transition of the implants *in vivo* remains unresolved[14].

Our group recently developed a novel injectable *in situ* forming gel delivery system. The gel system, herein named SPME, was prepared by mixing soya phosphatidyl choline (SPC), medium chain triglyceride (MCT) and ethanol. SPME has several advantages over poly(lactide-*co*-glycolide) (PLGA)-based *in situ* forming implants, namely minimal amounts of organic solvent, excellent biocompatibility, and significantly less burst effects. Unlike most polymeric *in situ* forming implants, the novel SPME system is based on the phase separation of phospholipids *in vivo*. As the main component of cell membrane, phospholipids are biologically compatible, and soluble in ethanol, yet display poor solubility in water. When administrated in the liquid form, the SPME system undergoes phase separation, thus resulting in the formation of a solid or semisolid gel at the injection site due to the *in situ* water–ethanol exchange between the surrounding body tissues and the SPME system. Phospholipid-based *in situ* forming gel drug delivery systems have been successfully applied to the long term delivery of various drugs, such as doxorubicin[15], bromotetrandrine[16] and exenatide[17]. Previous studies reported that when loaded with octreotide, the gel system showed extended release for 30 days *in vivo*[18].

The present study aimed to investigate the application of the SPME system as a long term drug delivery platform for LA. The *in vitro* and *in vivo* release profiles of LA from the SPME system were investigated systematically. Next, the stability of LA in the SPME system was investigated under different temperatures and in the presence of various antioxidants. Finally, *in vivo* studies in male rats were performed to elucidate the pharmacokinetic profiles and pharmacodynamic efficacy of this preparation.

2. Materials and methods

2.1. Materials

Soya phosphatidyl choline (SPC, Lipoid S100) was obtained from Lipoid (Germany, 579010-1140034-04/902). Medium chain triglyceride (MCT) was obtained from Beiya Medical Oil Co., Ltd. (Tieling, China, y110501-3-01). Ethanol was provided by Tianhua (Chengdu, China). Leuprolide acetate (LA) was supplied by Kaijie (Chengdu, China). (±)-α-Tocopherol was obtained from Sigma–Aldrich (St Louis, MO, USA). Glycine was provided by Kemiou (Tianjin, China). All the other chemicals and reagents were of analytical grade.

2.2. Animals

Male Sprague–Dawley (SD) rats (8 weeks old, 300 ± 20 g) were obtained from Experimental Animal Center of Sichuan University (Chengdu, China). During the study, the rats were given free access to food and water *ad libitum*. All animal experiments were performed according to China's Animal Welfare Legislation and the Institutional Animal Care and Use Guidelines of Sichuan University.

2.3. Preparation of SPME-LA and evaluation of viscosity

LA-loaded SPME (SPME-LA) was prepared by mixing SPC, MCT, and 90% (*v/v*) ethanol (70:15:15, *w/w/w*) and stirred for 1 h. For viscosity measurement, dialysis method was applied to simulate the phase separation process. A volume of 20 mL SPME-LA was put into a dialysis bag and then incubated in 4 L of 0.1 mol/L PBS (pH 7.4) under shaking. The Brookfield DV-C rotational viscometer was used to measure the viscosities of SPME-LA before (sol) and after phase separation (gel).

2.4. In vitro release experiment

LA solution (300 μL, 3.75 mg/mL) or SPME-LA (300 μL, equivalent to 3.75 mg/mL LA) was added into dialysis bags and the dialysis bags were placed in 4 mL of different release media containing 0.1 mol/L PBS (pH 7.4) with varying percentages of ethanol (0, 10%, 20% or 30%, *v/v*). The release setup was maintained under shaking at 37 °C. At given time intervals, the entire media was removed and replaced with prewarmed fresh media. The concentration of LA in the release media was analyzed by high performance liquid chromatography (HPLC, Agilent Technologies 1200 series) using UV detection at 220 nm, a Kromasil C18 column (150 mm × 4.6 mm, 5 μm) at 30 °C. The mobile phase consisted of 0.1% trifuroacetic acid and acetonitrile (68:32, *v/v*), and flowed through the column at the rate of 1.0 mL/min. The cumulative released amount of LA was calculated according to the following equation (1) [19]:

$$\text{Cumulative amount released } (\%) = \left(\sum_{t=0}^{t} \frac{M_t}{M_{\text{theoretical}}} \right) \times 100 \quad (1)$$

where M_t represents the amount of LA released at time t, and $M_{theoretical}$ represents the theoretical amount of LA in the formulation.

2.5. Stability study

Different formulations with or without antioxidants were prepared and divided into three groups: group 1, a control group without antioxidants; group 2, containing 0.0001% glycine; group 3, containing 0.2% (±)-α-tocopherol. Afterwards, each ampule was filled with 0.3 mL of formulation and then sealed. The ampules were stored in dark at 4 °C and 37 °C. At predetermined time points, ampules were taken out and restored to room temperature. The drug was extracted from the SPME-LA according to the following procedures: 20 mg of formulation was weighed accurately followed by vortexing with 2.55 mL of 0.1 mol/L PBS (pH 7.4) for 5 min, 450 μL of 5% tritonX-100 solution as demulsifier was then added and centrifuged at 10,000 rpm (3K15 high-speed refrigerated centrifuge, Sigma, German) for 5 min after a brief vortexing. The supernatant was passed through a 0.22 μm membrane filter and 20 μL was injected into the HPLC system for analysis. The remaining percentage amounts of LA at different times were calculated and normalized to value of day 0.

2.6. In vivo studies

The *in vivo* studies were evaluated in SD rats (male, 300±20 g). For the pharmacokinetic study, 1 mL of LA solution (3.75 mg/mL) or SPME-LA (equivalent to 3.75 mg/mL LA) was subcutaneously injected into the back of the rats (six animals per group) at a dose of 12.5 mg/kg. Blood samples were collected into the tubes with Na-heparin at 0.25, 0.5, 1, 2, 4, 6, 8, 12 h, 1, 3, 5, 7, 10, 13, 16, 20, 24, and 28 days after administration of SPME-LA, while blood samples were collected at 0.25, 0.5, 1, 2, 4, 6, 8, 12 h, 1, 2, and 3 days after administration of free LA. Plasma was obtained following centrifugation at 4500 rpm for 10 min and stored at –80 °C until further analysis. The LA concentration was measured by a modification of the published method[20]. Acetonitrile (200 μL) was added to 100 μL of plasma, the mixture was vigorously vortexed for 5 min and centrifugated at 15,300 rpm for 5 min. The supernatant was passed through a 0.22 μm membrane filter and 1 μL of filtrate was analyzed by liquid chromatography tandem mass spectrometry (LC–MS/MS). The LC–MS/MS analysis was performed with an Agilent 1200 series system (Agilent Technologies Co., Ltd., USA) equipped with a SL binary pump, SL autosampler, vacuum degasser, and an Agilent triple-quadruple MS. The mobile phase consisted of 0.1% formic acid and acetonitrile (75:25, *v/v*), and flowed through a Diamonsil ODS column (50 mm × 4.6 mm, 1.8 μm) at the rate of 0.4 mL/min. The mass spectrometric analysis was performed under electrospray ionization (ESI). For the quantification of LA, the transition of m/z 605.5→221.1 was adopted under multiple-reaction monitoring (MRM) positive mode. The voltage of fragmentor potential and collision energy were set as 132 and 36 eV, respectively. The flow rate and temperature of nebulizer gas (N_2) were set as 10 mL/min and 350 °C, respectively. The nebulizer is 30 psi, and the capillary is 4000 V.

For pharmacodynamic study, 25 rats were randomly divided into five groups (A, B, C, D, and E) with five animals in each group. Group A, untreated control, received no injection to observe the normal fluctuation of serum testosterone levels. Group B received 1 mL subcutaneous injection of LA saline solution once a day for 35 consecutive days at a dose of 100 μg/kg. The other three groups (C, D, and E) received 1 mL subcutaneous injection of SPME-LA at doses of 0.9, 2.25, and 3.75 mg/rat, corresponding to doses of 3, 7.5, and 12.5 mg/kg, respectively. Blood samples were collected into the tubes at 0, 3, 6 h, and 1, 2, 3, 7, 14, 21, 28 and 35 days after administration, and were centrifuged at 4 °C and 3000 rpm for 10 min to obtain serum which was immediately stored at –80 °C until further analysis of testosterone levels by Testosterone ELISA Kit (Cloud-Clone Corp., China).

2.7. Statistical analysis

All data were expressed as mean±standard deviation (SD). Statistical comparisons between two groups were performed using student's *t*-test. Results in the pharmacokinetic study were analyzed using DAS 3.0 software (Drug and Statistics, Shanghai, China). Results in the pharmacodynamic study were evaluated by one-way ANOVA followed by a Tukey *post hoc* test using GraphPadPrism version 5.0 (GraphPad Software). P values of 0.05 or less were considered statistically significant.

3. Results and discussion

3.1. SPME-LA preparation and viscosity measurement

As shown in Fig. 1A, SPME-LA displayed a yellow, transparent appearance before injection. In this formulation, SPC was used as the core component and 90% (*v/v*) ethanol was used to dissolve the drug LA and SPC, while MCT was used as a combined solvent to reduce the amount of ethanol which might cause skin irritation. In addition, SPC and MCT showed good biocompatibility, and the ethanol concentration was less than 15% (*w/w*), which would not cause severe side effects.

The SPME remained as a solution *in vitro* before injection and rapidly underwent phase separation to form semisolid gels after injection (Fig. 1B). During the phase separation process, a signi-

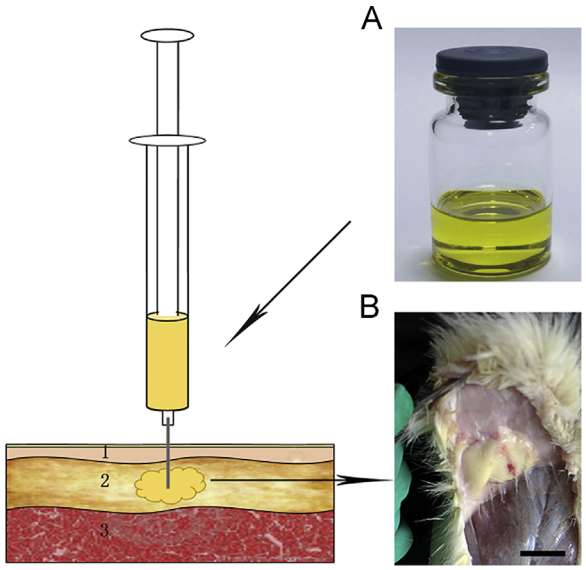

Figure 1 Schematic representation of subcutaneous administration of the *in situ* forming gel ((1) epidemis and dermis; (2) subcutis; and (3) muscles). SPME-LA displays a yellow and transparent appearance *in vitro* (A), and it forms a creamy-yellow gel after subcutaneous administration *in vivo* (B). Scale bar: 1 cm.

ficant change of viscosity was accompanied by this sol-gel separation. As shown in Fig. 2, the initial viscosity of SPME-LA was 251.2 cP, which is below the threshold viscosity of 300 cP for injection[21], indicating a viscosity which was suitable for injection. After separation, the viscosity was approximately 4567 cP, indicating the formation of a semisolid gel.

3.2. *In vitro release studies*

As shown in Fig. 3, the release of LA from SPME-LA groups showed a sustained release pattern for up to 30 days, whereas free LA showed a 80% cumulative drug release after 8 h. Moreover, the release rate of LA from SPME-LA increased as the content of ethanol increased. About 30% LA was released from SPME-LA in PBS-only media for 28 days. Conversely, approximately 60% LA was released from SPME-LA in the PBS containing 10% (*v*/*v*) ethanol, and more than 90% LA was released in the PBS with 20% and 30% of ethanol (*v*/*v*), which might be attributed to the solubility of SPC in ethanol. Accordingly, SPC dissolved faster in the release media with higher percentages of ethanol, thereby resulting in faster drug release.

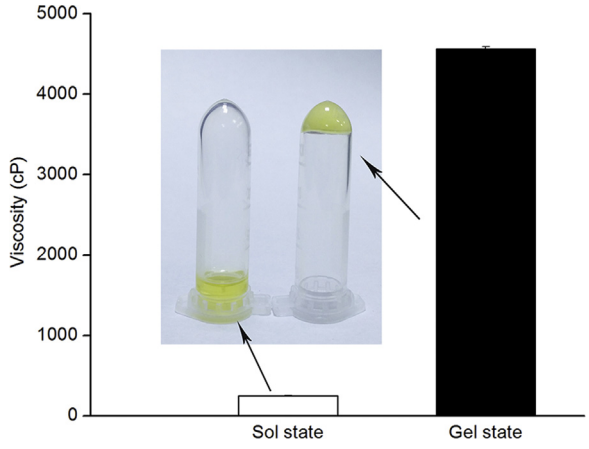

Figure 2 Viscosity of SPME-LA in sol and gel state at 25 °C. Data are presented as mean ± SD, $n = 3$.

To elucidate the release mechanism, various mathematical models were used including zero-order, first-order kinetics, Higuchi, Weibull and Ritger–Peppas models. Correlation coefficient R^2 was chosen to evaluate the fitting degree of an individual model. The Ritger–Peppas model showed the best correlation to experimental data. For the Ritger–Peppas model, the release process is driven by Fickian diffusion ($n < 0.45$), non-Fickian diffusion ($0.45 < n < 0.89$), and matrix erosion ($n > 0.89$)[22]. In our study, the value of n ranged from 0.3999 to 0.4540, indicating that the release of LA process was mainly driven by Fickian diffusion and gradually accompanied by dissolving the gel with higher content of ethanol. Thus, the release of drug increased as the content of ethanol increased. More importantly, no apparent initial burst was observed in any of the release media. A single peak of LA in the HPLC chromatograms was observed indicating that LA remained stable in the release media.

3.3. *Stability study*

Fig. 4 showed the results of storage stability study of LA in the SPME. Antioxidants greatly impacted the stability of LA in the formulation, while temperature showed minimum influence on the stability of LA. Group 1, without antioxidants, was unstable for long-term storage because the remaining percentage of LA after 28 days was about 80% at 4 °C and 37 °C. In contrast, LA in the groups containing antioxidants showed acceptable stability whose content decreased by 10% after 28 days in the presence of 0.0001% glycine or 0.2% tocopherol except for the group 3 at 37 °C whose content decreased by 17%.

SPME was a phospholipid-based phase separation gel in which SPC was used as the core component. A general question encountered in phospholipid-based formulation is the stability of phospholipids, which can easily undergo oxidation under normal storage conditions. Liposomes, mainly composed of phospholipids and cholesterol, are extensively used as a carrier system for chemotherapeutics. However, liposomes often suffer from chemical instability due to phospholipid oxidation[23]. In this study, different kinds of antioxidants were added in the SPME formulation, and a systematic study was conducted to investigate whether the oxidation of SPC would affect the stability of LA. Glycine was used as a water-soluble antioxidant, and (±)-α-tocopherol was

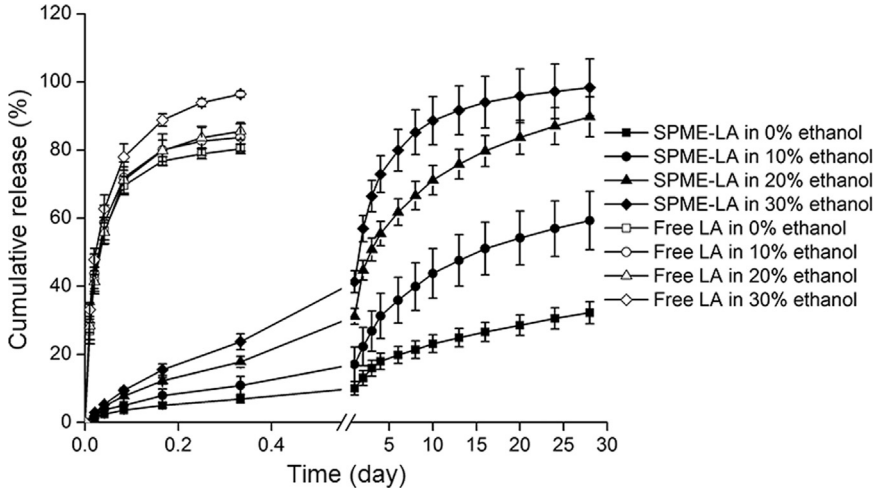

Figure 3 *In vitro* release of LA from SPME-LA or LA solution in PBS (pH 7.4) containing 0, 10%, 20% and 30% (*v*/*v*) of ethanol at 37 °C. Data are presented as mean ± SD, $n = 3$.

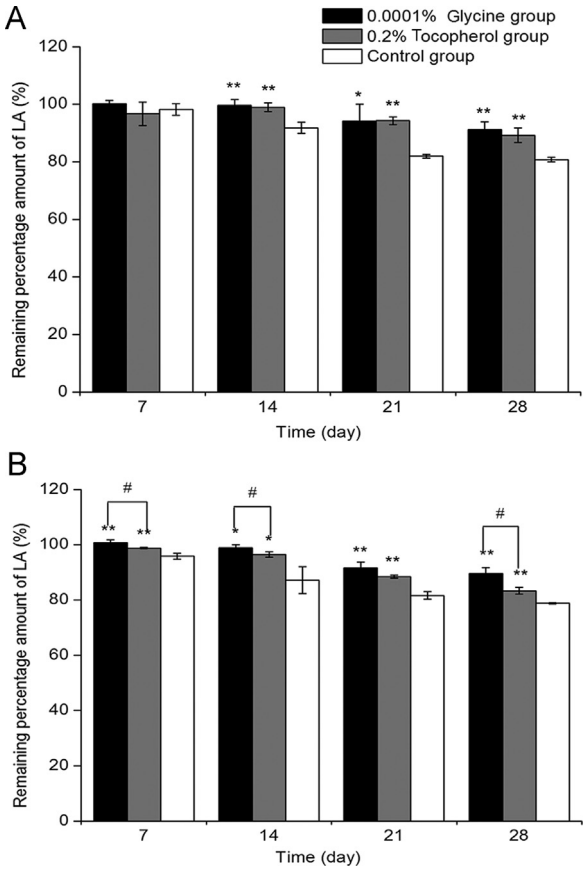

Figure 4 Storage stability of LA in SPME with or without antioxidants at 4 °C (A) and 37 °C (B). SPME-LA without antioxidants was served as control. $^{*}P<0.05$, $^{**}P<0.01$ *vs.* control group at the same time. $P<0.05$ *vs.* 0.2% tocopherol group at the same time. Data are presented as mean ± SD, $n=3$.

used as an oil-soluble antioxidant. The stability of LA showed significant differences between control group and the other two groups from day 14 to day 28 both at 4 °C and 37 °C ($P < 0.05$). More obviously, the presence of glycine showed better antioxidant effect than (±)-α-tocopherol at 37 °C, implying that SPC oxidation might affect the stability of LA in the SPME-LA. Hence, antioxidants are necessary to maintain the stability of the gel formulation, and filling with nitrogen was also critical to long-term storage. Further study is required to investigate other approaches for maintaining stability during long-term storage.

3.4. *In vivo studies*

Plasma levels of LA in rats after subcutaneous injection of LA solution and SPME-LA are shown in Fig. 5. After subcutaneous administration of LA solution, LA concentrations increased quickly and reached a maximum value of 2783.91 ng/mL within 1 h, followed by a rapid clearance. In contrast, the maximum concentration of LA was 227.64 ng/mL after injection of SPME-LA, which then gradually decreased but maintained above the minimal therapeutic concentration for up to 28 days. The $t_{1/2}$ values of the SPME-LA and LA solution were 139.02 ± 97.57 and 18.35 ± 9.01 h, respectively. A paired *t*-test showed that pharmacokinetic parameters between free LA and SPME-LA had significant differences (Table 1).

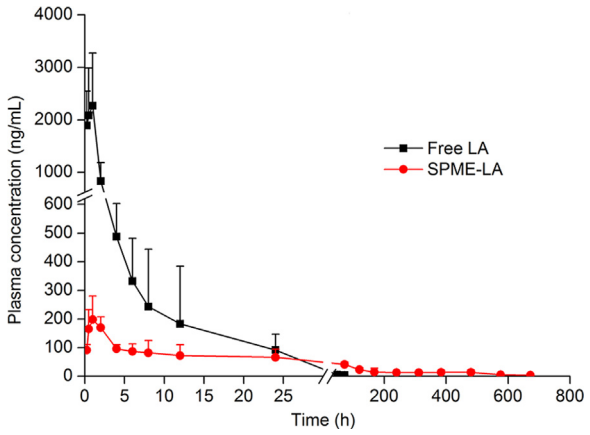

Figure 5 Plasma concentration of LA after a single subcutaneous injection of free LA and SPME-LA at a dose of 12.5 mg/kg in rats. Data are presented as mean ± SD, $n=6$.

Table 1 Pharmacokinetic parameters of leuprolide acetate (LA) after subcutaneous injections of free LA and SPME-LA in rats.

Parameter	Dose (12.5 mg/kg)[a]	
	Free LA	SPME-LA
C_{max} (ng/mL)	2783.87 ± 1283.04	227.64 ± 66.68**
T_{max} (h)	0.50 ± 0.27	1.25 ± 0.61*
AUC_{0-t} (ng · h/mL)	9729.48 ± 2695.70	12733.03 ± 1467.57*
$AUC_{0-\infty}$ (ng · h/mL)	9838.24 ± 2651.47	13564.31 ± 1806.84*
$t_{1/2}$ (h)	18.35 ± 9.02	139.02 ± 97.57*

[a]Data are presented as mean ± SD, $n=6$.
$^{*}P<0.05$.
$^{**}P<0.01$ *vs.* free LA.

SPME-LA showed a significantly lower C_{max} value than that found for the LA solution, indicating that the initial burst of SPME-LA was much lower than that of LA solution. According to literature, the initial burst of PLGA/NMP system is caused by the fast phase-transition[14]. However, for the SPME system, the initial burst may be attributable to the diffusion of ethanol during phase separation. The percentage of ethanol in the pregel solution was limited, thereby causing a less significant initial burst. On the other hand, the $t_{1/2}$ value of the SPME was much longer than that of the LA solution, indicating a sustained release of LA from SPME-LA.

For pharmacodynamic studies, testosterone levels have been regarded as an important pharmacological index for LHRH agonist therapy to monitor castration levels. In this study, testosterone levels were monitored for 35 days, and the serum testosterone level of each group was compared with the chemical castration threshold (0.5 ng/mL). Fig. 6 presents the fluctuations of the mean level of serum testosterone in rats of five groups. Group A, an untreated control, showed the natural fluctuation of serum testosterone levels which were greater than castration levels within 35 days. In contrast, the mean levels of serum testosterone of all LA-treated groups demonstrated a transient surge immediately after the initial administration of LA, and were reduced slowly or rapidly during the treatment of LA. This is likely to be related to the

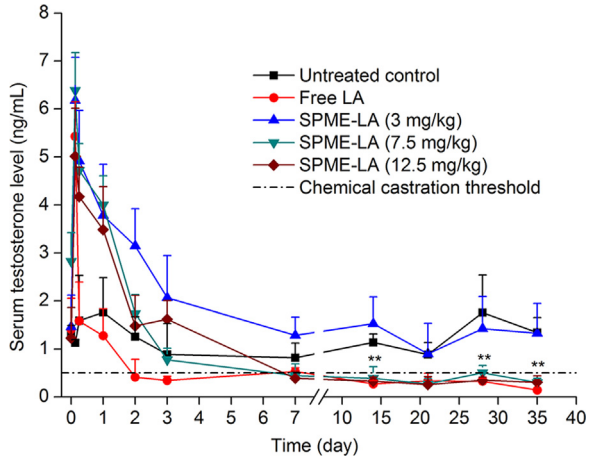

Figure 6 Serum testosterone levels in rats after subcutaneous administration of LA for 35 days. Untreated control (■); Free LA, a consecutive injection at a dose of 100 μg /kg/d for 35 days (●); SPME-LA, a single injection at a dose of 3 mg/kg (▲), 7.5 mg/kg (▼), 12.5 mg/kg (◆). The dash dotted line represents the chemical castration threshold (0.5 ng/mL). $^{**}P<0.01$ *vs.* untreated control. Data are presented as mean ± SD, $n=5$.

mechanism of LHRH agonists[24], which stimulate gonadotropin sections to produce luteinizing hormone to directly act on testis to produce testosterone. However, LHRH receptors are down-regulated because of the sustained release of LA, and as a result, the production of luteinizing hormone is reduced which then decrease the production of testosterone. For group B, the mean level of serum testosterone in rats increased from 0.14 to 0.54 ng/mL three hours after the initial subcutaneous injection of LA solution. Afterwards, the mean level of serum testosterone decreased rapidly to the castration level on day 2, and maintained the inhibitory effect until day 35. This result further implied that a sustained release of LA was required to achieve and keep the castration level of testosterone.

The other three groups received different doses of SPME-LA for an effective dose study. Rats in group C which received a single injection of SPME-LA at a dose of 3 mg/kg were insufficient to suppress the serum testosterone level to the castration level. Conversely, the serum testosterone level in rats receiving a dose greater than 3 mg/kg showed a different pattern over treatment. The mean serum testosterone concentration of rats in groups D and E increased from 0.29 and 0.12 ng/mL to 0.64 and 0.50 ng/mL three hours after the subcutaneous injection of SPME-LA at doses of 7.5 and 12.5 mg/kg, respectively. Afterwards, the serum testosterone level of rats in groups D and E decreased slowly to the castration level on day 7, and maintained the therapeutic level until day 35. There were no significant differences between LA solution and SPME-LA groups (B and D, B and E, $P>0.05$) during the therapeutic period from day 7 to day 35. Moreover, groups D and E showed a similar testosterone suppression effect without any significant difference ($P>0.05$). No apparent weight loss or skin irritation was observed in any of the treated rats during the whole study. Therefore, the minimal effective dose using SPME-LA is probably greater than 3 mg/kg and less than 7.5 mg/kg.

Consistent with the *in vitro* release study, LA solution exhibited the fastest release and resulted in the shortest onset time after daily subcutaneous administration. In contrast, SPME-LA achieved sustained drug release for 28 consecutive days *in vitro* and resulted in a persistent suppressive effect of testosterone release *in vivo* except for the group C which received an injection of SPME-LA at a dose of 3 mg/kg. In this study, the dose of 3 mg/kg was designed to correspond to the dose of 100 μg/kg/d at steady state for 30 days. However, 3 mg/kg was proven unable to suppress the serum testosterone level to castration levels, possibly attributable to the dose loss. For example, the dose loss for the once-a-month injectable microspheres of LA was nearly 25% which was mostly caused by the initial burst and the residual at the injection site[25]. As for the SPME-LA system, one of the reasons causing dose loss is possibly owing to the amount of gel remaining at the injection site. As discussed for *in vitro* release study, only 30% LA released from SPME-LA in the PBS-only media for 28 days indicating that the majority of LA remained in the dialysis bag. Moreover, the rats were sacrificed and dissected at day 35, and SPME-LA remaining at the injection site was observed. Therefore, the amount of LA remaining at the injection site might explain the dose loss. As discussed above, since phospholipids are the core component of the SPME-LA, phospholipid degradation determines the release rate of drug. For example, in macromolecule-loaded vesicular phospholipid gels (VPGs), the drug release is governed by the erosion of phospholipids matrix, and as a result, higher lipid concentrations retard the erosion process[26]. In this paper, the high phospholipids concentration (70%) with low viscosity was chosen in order to achieve a longer sustained release of drug, substantiated by the pharmacokinetic and pharmacodynamic studies. Based on the literature, the volume of pitavastatin-loaded phospholipid gel decreased gradually over time, indicating the phospholipid-based gel is biodegradable[27]. Therefore, advantages such as the ease of preparation, biodegradability and sustained release properties demonstrate the potential of the SPME system as a new and effective sustained delivery system for LA.

4. Conclusions

A phospholipid-based injectable gel was developed for the long-term delivery of LA in this study. Both *in vitro* and *in vivo* studies showed a sustained release of LA from SPME system for 30 consecutive days. A preformulation and stability study showed that the presence of antioxidants enhanced the stability of LA during storage. Moreover, serum testosterone levels in male rats decreased to castration levels on day 7, and maintained the therapeutic effect for up to 35 days after a single injection of SPME-LA. Thus, the injectable SPME gel system represents a promising platform for the sustained delivery of LA to achieve long-term effects *in vivo*.

References

1. Mottet N, Bellmunt J, Bolla M, Joniau S, Mason M, Matveev V, et al. EAU guidelines on prostate cancer. Part II: treatment of advanced, relapsing, and castration resistant prostate cancer. *Eur Urol* 2011;**59**:572–83.
2. Arimura A, Schally AV. Physiological and clinical studies with natural and synthetic luteinizing hormone-releasing hormone (LH-RH). *Med J Osaka Univ* 1972;**23**:77–100.
3. Cox MC, Scripture CD, Figg WD. Leuprolide acetate given by a subcutaneous extended-release injection: less of a pain? *Expert Rev Anticancer Ther* 2005;**5**:605–11.

4. Okada H. One-and three-month release injectable microspheres of the LH-RH superagonist leuprorelin acetate. *Adv Drug Deliv Rev* 1977;**28**:43–70.
5. Okada H, Inoue Y, Heya T, Ueno H, Ogawa Y, Toguchi H. Pharmacokinetics of once-a-month injectable microspheres of leuprolide acetate. *Pharm Res* 1991;**8**:787–91.
6. Okada H, Doken Y, Ogawa Y, Toguchi H. Preparation of three-month depot injectable microspheres of leuprorelin acetate using biodegradable polymers. *Pharm Res* 1994;**11**:1143–7.
7. Woo BH, Na KH, Dani BA, Jiang G, Thanoo BC, DeLuca PP. *In vitro* characterization and *in vivo* testosterone suppression of 6-month release poly (D,L-lactide) leuprolide microspheres. *Pharm Res* 2002;**19**:546–50.
8. Cukierski MJ, Johnson PA, Beck JC. Chronic (60-week) toxicity study of DUROS leuprolide implants in dogs. *Int J Toxicol* 2001;**20**:369–81.
9. Ravivarapu HB, Moyer KL, Dunn RL. Sustained activity and release of leuprolide acetate from an *in situ* forming polymeric implant. *AAPS PharmSciTech* 2000;**1**:1–8.
10. Singh S, Singh J. Phase-sensitive polymer-based controlled delivery systems of leuprolide acetate: *in vitro* release, biocompatibility, and *in vivo* absorption in rabbits. *Int J Pharm* 2007;**328**:42–8.
11. Luan XS, Bodmeier R. Influence of the poly (lactide-*co*-glycolide) type on the leuprolide release from *in situ* forming microparticle systems. *J Control Release* 2006;**110**:266–72.
12. Sartor O. Eligard: leuprolide acetate in a novel sustained-release delivery system. *Urology* 2003;**61**:25–31.
13. Ravivarapu HB, Moyer KL, Dunn RL. Parameters affecting the efficacy of a sustained release polymeric implant of leuprolide. *Int J Pharm* 2000;**194**:181–91.
14. Parent M, Nouvel C, Koerber M, Sapin A, Maincent P, Boudier A. PLGA *in situ* implants formed by phase inversion: critical physicochemical parameters to modulate drug release. *J Control Release* 2013;**172**:292–304.
15. Wu WQ, Chen H, Shan FY, Zhou J, Sun X, Zhang L, et al. A novel doxorubicin-loaded *in situ* forming gel based high concentration of phospholipid for intratumoral drug delivery. *Mol Pharm* 2014;**11**:3378–85.
16. Luo JW, Zhang T, Zhang Q, Cao X, Zeng X, Fu Y, et al. A novel injectable phospholipid gel co-loaded with doxorubicin and bromotetrandrine for resistant breast cancer treatment by intratumoral injection. *Colloids Surf B Biointerfaces* 2015;**140**:538–47.
17. Zhang Y, Zhong Y, Hu M, Xiang NX, Fu Y, Gong T, et al. *In vitro* and *in vivo* sustained release of exenatide from vesicular phospholipid gels for type II diabetes. *Drug Dev Ind Pharm* 2015;**42**:1042–9.
18. Zhang T, Peng Q, San FY, Luo JW, Wang MX, Wu WQ, et al. A high-efficiency, low-toxicity, phospholipids-based phase separation gel for long-term delivery of peptides. *Biomaterials* 2015;**45**:1–9.
19. Ito Y, Ochii Y, Fukushima K, Sugioka N, Takada K. Three-layered microcapsules as a long-term sustained release injection preparation. *Int J Pharm* 2010;**384**:53–9.
20. Sofianos ZD, Katsila T, Kostomitsopoulos N, Balafas V, Matsoukas J, Tselios T, et al. *In vivo* evaluation and *in vitro* metabolism of leuprolide in mice-mass spectrometry-based biomarker measurement for efficacy and toxicity. *J Mass Spectrom* 2008;**43**:1381–92.
21. Xuan JJ, Balakrishnan P, Oh DH, Yeo WH, Park SM, Yong CS, et al. Rheological characterization and *in vivo* evaluation of thermosensitive poloxamer-based hydrogel for intramuscular injection of piroxicam. *Int J Pharm* 2010;**395**:317–23.
22. Siepmann J, Peppas NA. Modeling of drug release from delivery systems based on hydroxypropyl methylcellulose (HPMC). *Adv Drug Deliv Rev* 2012;**64**:163–74.
23. Crommelin DJA, Grit M, Talsma H, Zuidam NJ. Liposomes as carriers for drugs and antigens: approaches to preserve their long term stability. *Drug Dev Ind Pharm* 1994;**20**:547–56.
24. Sharifi N, Gulley JL, Dahut WL. Androgen deprivation therapy for prostate cancer. *J Am Med Assoc* 2005;**294**:238–44.
25. Okada H, Heya T, Ogawa Y, Schimamoto T. One-month release injectable microcapsules of a luteinizing hormone-releasing hormone agonist (leuprolide acetate) for treating experimental endometriosis in rats. *J Pharmacol Exp Ther* 1988;**244**:744–50.
26. Tian W, Schulze S, Brandl M, Winter G. Vesicular phospholipid gel-based depot formulations for pharmaceutical proteins: development and *in vitro* evaluation. *J Control Release* 2010;**142**:319–25.
27. Xiang NX, Zhou X, He XY, Zhang Y, Zhang JJ, Zhang ZR, et al. An injectable gel platform for the prolonged therapeutic effect of pitavastatin in the management of hyperlipidemia. *J Pharm Sci* 2016;**105**:1148–55.