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Research paper

In vivo evaluation of solid lipid microparticles and hybrid polymer-lipid microparticles for sustained delivery of leuprolide

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ABSTRACT

This study aims to investigate the potential of solid lipid microparticles (MP) and hybrid polymer-lipid MPs for sustained delivery of a peptide drug, leuprolide. A peptide-phospholipid complex was prepared to increase the compatibility of the peptide with triglyceride (TG) and poly (lactide-co-glycolide) (PLGA). Peptide loaded solid lipid MPs, PLGA MPs, and hybrid MPs were prepared using a spray drying method and characterized in terms of particle size, morphology and encapsulation efficiency. The pharmacokinetics and pharmacodynamics of leuprolide after subcutaneous injection of spray-dried MPs were evaluated in rats. Spray-dried MPs were spherical ranging in size from 4 μm to 10 μm, which are suitable for injection. After subcutaneous administration of reconstituted MPs, leuprolide could be detected in plasma samples of rats for one to two months, depending on the formulation and dose. Sustained release of leuprolide from PLGA MPs and glyceryl tristearate (TG18) MPs was observed over one month, with a chemical castration effect of 25 and 30 days, respectively. The bioavailability of leuprolide from PLGA-TG18 hybrid MPs was approximately four times higher than that from TG18 MP and PLGA MP alone using the same dose of leuprolide (6 mg/kg). Chemical castration in rats was observed over 30 and 60 days after injection of the PLGA-TG18 hybrid MP with a dose of 3 mg/kg and 6 mg/kg leuprolide, respectively. Additionally, a much lower C_{max} was observed for the hybrid MP group. In conclusion, spray-dried PLGA-triglyceride hybrid MPs can be used as better carriers than other MPs for subcutaneous delivery of peptide drugs due to the synergetic effect of lipids and PLGA for sustained drug release from the spray-dried MP.

1. Introduction

Lipid-based formulations have been used for delivery of lipophilic drugs, with several successfully marketed products, due to good biocompatibility and low toxicity of lipids [1,2]. Research in solid lipid excipients has increased greatly in the past two decades and are now considered alternative materials to biodegradable polymers for sustained drug delivery due to the physical and chemical stability and slow degradation rate [3–5]. Protein and peptide drugs, such as insulin and thymocartin, show a sustained release from solid lipid microparticles (MP) or lipid implants lasting from days to months *in vitro* [6–8]. Sustained release of antide from glyceryl monobehenate MP and glyceryl monostearate MP made by cryogenic micronization was also observed *in vivo* over a month [9].

Poly (lactide-co-glycolic acid) (PLGA) MPs have been widely applied in sustained delivery of peptide drugs [10]. Recently, it was reported that incorporating phospholipids in PLGA nanoparticles, using a modified double emulsion method, improved the encapsulation efficiency (EE) of a protein drug from 63% to 90%, meanwhile the burst drug release was reduced from 42% to 24% [11]. Similar results were reported for spray-dried PLGA MPs, the EE of paclitaxel in PLGA MPs was increased from 61% to 84% when phospholipids were added to the formulation, significantly reducing the rate of drug release [12]. Recent reviews of polymer-lipid hybrid particles stated that hybrid particles may be promising for sustained delivery of protein and peptide drugs [13,14]. The composition of excipients in hybrid particles was reported to be one of the key factors affecting drug release profile and drug EE [15].

Protein/peptide loaded PLGA MPs are usually prepared by using the double emulsion method [16], whereas solid lipid MPs are prepared by

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hot melting or spray congealing [17,18]. Particle sizes of triglyceride MPs, prepared by hot melting or spray congealing, ranged from 10 μm to 500 μm [18,19]. Recently, a spray drying method was reported for efficient encapsulating protein into solid lipid MPs using solutions containing both lipid excipients and protein-phospholipid complexes as the feed; the spray-dried solid lipid MPs were found to be smaller than 10 μm [20]. As both solid lipid MPs and PLGA MPs can be used as carriers for sustained drug delivery, it is valuable to investigate whether these excipients have any synergistic effect for drug release regulation. To the best of our knowledge, no study has been reported about *in vivo* comparison of solid lipid MPs and PLGA triglyceride hybrid MPs prepared using the same method.

The aim of the present study was to evaluate potential synergistic effects of lipids and PLGA for sustained drug delivery using leuprolide as a model peptide drug. Leuprolide is a synthetic nonapeptide analog of naturally occurring gonadotropin-releasing hormone. It has a short half-life and is indicated for palliative treatment of advanced prostate cancer. Sustained release of leuprolide from PLGA MPs was observed over one month after intramuscular administration of LUPRON DEPOT (prepared by the double emulsion method) [21]. In the present study, leuprolide-phospholipid complexes were encapsulated in solid lipid MPs, PLGA MPs and PLGA lipid hybrid MPs using the spray drying method reported previously [20]. The potential synergistic effect of lipids and PLGA for sustained drug release was assessed by evaluation of different spray-dried MPs *in vitro* and *in vivo*.

2. Material and methods

2.1. Materials

PLGA (75/25, RG 753 S, MW around 50 kDa) was purchased from EVONIK (Darmstadt, Germany), PLGA 50/50 (viscosity range 0.55–0.75 dL/g in hexafluoro-isopropanol) was obtained from Durect Corporation (Birmingham, USA). Glyceryl tribehenate (Dynasan D122, TG22) and glyceryl tristearate (Dynasan D118, TG18) were kindly donated by IOI Oleo GmbH (Hamburg, Germany). Soybean phosphatidylcholine (Lipoid S-100, SPC) was purchased from Lipoid GmBH (Ludwigshafen, Germany). Leuprolide (LEU) was bought from Chengdu Kaijie Biopharm Co. (purity > 99%, Chengdu, China). Dichloromethane, dimethyl sulfoxide (DMSO) and acetic acid were obtained from Sigma Aldrich (St. Louis, MO, USA). All other reagents were of analytical grade.

2.2. Preparation of leuprolide phospholipid complexes

Leuprolide-phospholipid complexes were prepared according to a method reported previously with minor modifications [22]. In short, leuprolide and phospholipids, with different weight ratios (Table 1), were mixed with DMSO containing 3% (v/v) of acetic acid under continuous stirring at 37 °C until a clear solution was formed. After freeze drying, the lyophilized leuprolide-phospholipid complexes were sealed and stored at −20 °C for further use.

Table 1
Complexation yield of leuprolide with phospholipid.

Phospholipid: leuprolide (weight ratio)	Complexation yield (%)
1:1	42.3 ± 5.6
2:1	92.1 ± 4.5
5:1	98.3 ± 2.4
10:1	97.2 ± 0.9

Average ± SD, n = 3.

2.3. Quantification of leuprolide phospholipid complexation yield by HPLC

Peptide-phospholipid complexes are soluble in organic solvent [23]. The complexation yield of leuprolide and phospholipids was determined by dissolving freeze-dried leuprolide-phospholipid complex in dichloromethane in a sealed glass tube, which was then gently shaken on an orbital shaker at room temperature for 2 h. The sample was filtered through organic membrane filters (0.45 μm) under vacuum. The concentration of leuprolide-phospholipid complexes in the filtrate was quantified by high performance liquid chromatography (HPLC) (Agilent HPLC system) using a C18 column (4.60 × 100 mm, 5 μm, 300 Å, Waters, USA) at room temperature. A binary solvent system was used at a flow rate of 1 mL/min. Solvent A was 0.1% (v/v) trifluoroacetic acid (TFA) in purified water and solvent B was 0.1% (v/v) TFA in acetonitrile. A gradient method running for 16 min was used; 0–2 min: 26% B, 2–10 min: 26–35% B, 10–10.2 min: 35–100% B, 10.2–13 min: 100% B, 13–13.2 min: 100–26% B, 13.2–16 min: 26% B. Leuprolide was detected at 220 nm. The complexation yield of leuprolide with phospholipid was calculated by the following equation:

$$\text{Complexation yield} = \text{leuprolide measured by HPLC/theoretic leuprolide in the complex} \times 100\%$$

2.4. Preparing microparticles by spray drying

Different MPs were prepared using the spray drying method described previously [20] according to the formulations listed in Table 2. Dichloromethane was used as the organic solvent for preparation of all MPs except TG22 MPs, which were prepared using chloroform. In short, an organic solution containing both excipients and the leuprolide-phospholipid complex was spray-dried using a Buchi 290-Mini Spray Dryer with an inert loop B295 (nozzle diameter, 0.7 mm, Buchi, Flawil, Switzerland) under following conditions: nitrogen flow rate at 35 m^3/h, sample flow of 2 mL/min, atomization gas flow rate of 700 L/h, inlet and outlet temperature was set to 45 °C and 39 °C for the dichloromethane solution, and 55 °C and 45 °C for the chloroform solution, respectively. The MPs were collected from the collection jar and placed under vacuum (0.08 Mbar) overnight to remove residual solvent. The yield of MPs from spray drying process was determined as:

$$\text{Yield of spray drying} = \text{MP collected/total amount} \times 100\%$$

2.5. Particle size analysis

The particle size and size distribution of spray-dried MPs were analyzed by laser diffraction using a Mastersizer 2000 equipped with Hydro 2000S (Malvern Instruments Ltd., UK) in the wet dispersion mode. Wet dispersions were prepared by dispersing the MPs in Tween 80 solution (0.1%), measurements were performed under stirring (1200 rpm) and ultrasound (50% intensity). The obscuration was set between 8% and 12%.

2.6. Quantification of leuprolide encapsulation efficiency

The content of leuprolide in solid lipid MPs and PLGA TG18 hybrid MPs containing 85% or 47% TG18 was measured by HPLC after dissolution of the MPs (10 mg) in 10 mL dichloromethane. The content of leuprolide in PLGA MPs or PLGA TG18 hybrid MPs containing 10% TG18 was measured by dissolution the MPs (20 mg, accurately weighed) in DMSO (2 mL). The leuprolide in the supernatant was quantified by HPLC, after precipitation of excipients by purified water (8 mL) and centrifugation (10,000 rpm, 5 min). The EE was calculated as:

Table 2
Composition and characteristics of spray-dried microparticles.

Formulation	Composition*	Yield (%)	Size (μm)	Span	EE (%)	DL (%)	RS (ppm)
PLGA MP	95:5:1	59.9 ± 3.9	8.4 ± 2.4[a]	3.4 ± 0.8	99.6 ± 2.1[a]	1.00 ± 0.02[a]	N.A
PLGA TG18 hybrid MP	85:10:5:1	56.5 ± 1.7	7.0 ± 1.4[a]	5.6 ± 0.5	87.8 ± 0.1[b]	0.88 ± 0.00[b]	2000 ± 300[a]
PLGA TG18 hybrid MP	47:47:5:1	54.7 ± 1.9	6.7 ± 1.8[a]	4.8 ± 1.3	103.1 ± 3.2[a]	1.03 ± 0.03[a]	300 ± 0[b]
PLGA TG18 hybrid MP	10:85:5:1	54.4 ± 1.1	7.1 ± 0.9[a]	6.0 ± 3.1	98.4 ± 0.2[a]	0.98 ± 0.00[a]	300 ± 0[b]
TG18 MP	95:5:1	51.5 ± 3.0	10.2 ± 0.9[a]	5.3 ± 2.8	106.7 ± 2.3[a]	1.07 ± 0.02[a]	200 ± 0[b]
TG 22 MP	95:5:1	60.7 ± 0.7	3.9 ± 0.2[b]	3.1 ± 0.5	67.8 ± 2.3[c]	0.68 ± 0.02[c]	200 ± 0[b]

MP, microparticles; TG18, glyceride tristearate; TG22, glyceride tribehenate; EE, entrapment efficiency; DL, drug loading, RS, residual solvent. The results are given as Average ± SD (n = 3). N.A: Not analyzed. Values labelled with different letters within the same column are significant different ($p < 0.05$).

* Weight ratio between excipient (PLGA or lipid): phospholipid: leuprolide.

EE=leuprolide extracted from the MP/theoretic leuprolide in the MP × 100%

2.7. Scanning electron microscopy

The morphology of spray-dried MP was observed by scanning electron microscopy (SEM) (Hitachi, TM3030, Japan). The samples were coated with gold under an argon atmosphere for 20 s prior to SEM observation and examined under an accelerating voltage of 5 kV.

2.8. Analysis of solid state using X-ray powder diffraction method

X-ray powder diffraction measurements were performed to elucidate the solid state of lipids and PLGA in spray-dried MPs using a PANalytical X'Pert Pro diffractometer equipped with a PIXcel detector (PANalaytical B.V., Almelo, Netherlands). Measurements were conducted at ambient conditions using Cu Kα radiation at 40 mA and 45 kV, with an angular increment of 0.04°/s and count time of 2 s. Data were collected at 0.05° (2 theta) from 5° to 35° and analyzed by X'Pert highscore plus version 2.2.4 (PANalaytical B.V.).

2.9. Differential scanning calorimetry and thermal gravimetrical analysis

Differential scanning calorimetry (DSC) measurements were carried out to determine the thermal behavior of lipids and the glass transition point of PLGA in spray-dried MPs. Approximately 3 mg of sample was weighed into DSC pans and crimp sealed. Samples were heated from 10 °C to 100 °C at a heating rate of 10 °C/min under nitrogen (20 mL/min). The amount of total residual solvent in MP was determined by thermal gravimetrical analysis (TGA). TGA was performed by heating a weighed amount of sample under a nitrogen atmosphere from 25 °C to 80 °C at a rate of 2 °C/min (Perkin Elmer, Shelton, USA). Both DSC and TGA system were controlled by the software Trios v3.3.1.

2.10. In vitro study of drug release from spray-dried MP

PLGA MPs or PLGA-TG18 hybrid MPs containing 10% TG18 (50 mg) were mixed with 1 mL of release medium (0.03 M PBS, pH 7.4, 0.05% Tween 80, 0.05% sodium azide) in low-protein-binding Eppendorf tubes. The samples were kept at 20 °C under vertical agitation (60 rpm) to prevent particle aggregation and protein/peptide degradation during the long-term release study. During the release study the Eppendorf tubes were centrifuged at 5000 rpm for 2 min and leuprolide in the release medium was extracted and analyzed by HPLC, and fresh release medium (1 mL) was added and MPs were re-suspended to continue the release study for 4 to 5 weeks. For TG22 MPs, TG18 MPs or PLGA-TG18 hybrid MPs containing 47% or 85% TG18, 50 mg of MPs were mixed with 2 mL of the release medium in centrifugal filter tubes (molecular weight cut off 100 kDa, Millipore Amicon® Ultra-4, Darmstadt, Germany) and kept at 20 °C under agitation. Samples were centrifuged at 5000 rpm for 2 min at pre-set times and leuprolide in the filtrated release medium was extracted and analyzed by HPLC, fresh release medium (2 mL) was then added to re-suspend the MP to continue the release study.

2.11. Pharmacokinetic and pharmacodynamic study

The protocol was approved by the Animal Ethical Committee of Sichuan University and adheres to the Principles of Laboratory Animal Care. Male Sprague Dawley rats, weighing 200–250 g on the day of the experiments, were purchased from Chengdu Dashuo Biotechnology co. LTD (Chengdu, China) and maintained on standard chow and water *ad libitum* in the laboratory for at least one week before entering the experiment. Temperature and relative humidity of the laboratory was kept at 25 °C and 50%, respectively. The rats were divided into 6 groups, five rats per group except three rats for the TG22 MP 6 mg/kg group. Details about the formulation and dose of leuprolide are listed in Table 3. Rats were fasted overnight but had free access to water before administration of all formulations. Spray-dried MPs were injected subcutaneously to the back of rat after reconstitution in a suitable vehicle (saline and 0.1% Tween 80). Blood samples were collected into tubes containing heparin sodium via the eye vein at preset time point (2 h, 8 h, 1 d, 2 d, 4 d, 7 d, 10 d, 15 d, 20 d, 25 d, 30 d, 40 d, 50 d, 60 d) after administration. Plasma samples were collected after centrifugation (5000 rpm, 10 min, 5 °C) and stored at −80 °C until analysis.

Table 3
Pharmacokinetic parameters of leuprolide after s.c. administration of spray-dried MP.*

Formulation	Dose (mg/kg)	$AUC_{(0-t)}$ (day*ng/ml)	Dose correlated $AUC_{(0-t)}$ (day*ng/ml)	T_{max} (h)	C_{max} (ng/ml)	Dose correlated C_{max} (ng/ml)	Chemical castration (day)
PLGA MP	6	71.3 ± 10.7[a]	11.8 ± 1.7[a]	2.0 ± 0.0	103.3 ± 44.5[a]	17.2 ± 7.4[a]	25
TG18 MP	6	72.1 ± 10.6[a]	12.0 ± 1.8[a]	2.0 ± 0.0	157.5 ± 39.0[a]	26.3 ± 6.5[a]	30
TG22 MP	4	103.4 ± 26.5[a]	25.9 ± 6.6[b]	2.0 ± 0.0	140.3 ± 49.8[a]	35.1 ± 12.5[a]	30
TG22 MP	6	370.8 ± 32.3[b]	61.8 ± 5.4[c]	3.8 ± 3.3	151.2 ± 58.6[a]	25.2 ± 9.8[a]	60
Hybrid MP	3	112.3 ± 15.2[a]	37.4 ± 5.1[b,d]	2.0 ± 0.0	11.0 ± 3.0[b]	3.6 ± 1.0[b]	30
Hybrid MP	6	271.0 ± 53.6[b]	45.2 ± 8.9[c,d]	2.0 ± 0.0	71.4 ± 35.2[a,b]	11.9 ± 5.9[b]	60

* MP, microparticles; the weight ratio of PLGA to TG18 in the PLGA TG18 hybrid MP was 85:10. The results are presented as mean ± SD (n = 3–5), values labelled with different letters within the same column are significant different ($p < 0.05$).

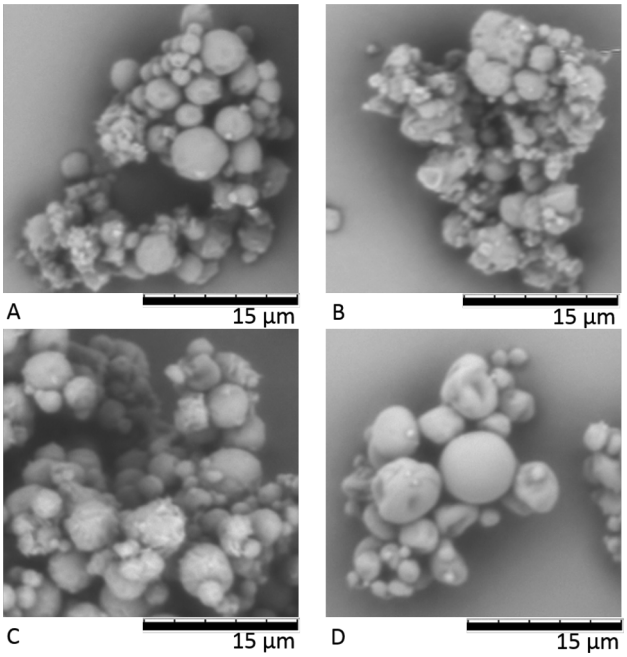

Fig. 1. SEM images of spray-dried microparticles (MP). (A) TG18 MP, (B) TG22 MP, (C) PLGA TG18 hybrid MP with a weight ratio of PLGA:TG18 at 47:47, (D) PLGA MP. Details of the formulations are listed in Table 2.

2.12. Quantification of leuprolide and testosterone in plasma by HPLC-MS/MS

Plasma samples (100 µL) were vigorously mixed with acetonitrile (300 µL) for 5 min and centrifuged at 15,000 rpm for 10 min. The supernatant was filtrated using a 0.22 µm membrane, and leuprolide was analyzed by HPLC-MS/MS (Agilent 1200 series system, triple quadruple MS, Agilent Technologies Co., Ltd., USA). The mobile phase consisted of solvent A: purified water containing 0.1% formic acid and solvent B: acetonitrile. The separation was achieved using a C18 column (3.5 µm, 2.1 × 100 mm, Agilent Eclipse Plus, Agilent technologies, USA). A gradient method running for 8 min with a flow rate of 0.3 mL/min was used; 0–1 min: 10% B, 1–5 min: 10–80% B, 5–5.1 min: 80–10% B, 5.1–8 min: 10% B. Typical injection volume was 5 µL and the retention time of leuprolide was approximately 4.1 min. The mass spectrometric analysis was performed under electrospray ionization, the transition of *m/z* 605.5–221.1 was adopted under multiple-reaction monitoring positive mode. The voltage of fragmentor potential and collision energy were set to 100 eV and 36 eV, respectively. The flow rate and temperature of nitrogen gas were set to 12 mL/min and 350 °C, respectively. The nebulizer gas pressure was 35 psi, and the capillary voltage was 4000 V.

The plasma testosterone level was measured using the same sample and a similar method described above. The mobile phase for separation of testosterone consists of 55% solvent A and 45% solvent B, and the run time for each injection was 3 min; the retention time of testosterone was approximately 2.4 min. The transition of *m/z* 289.4–109.1 was adopted under multiple-reaction monitoring positive mode. The voltage of fragmentor potential and collision energy were set to 135 eV and 24 eV, respectively.

2.13. Data analysis

PK parameters were determined by non-compartmental analysis: observed maximum concentration (C_{max}), time of observed maximum concentration (t_{max}) and area under the leuprolide plasma concentration-time curve (AUC) from time zero to time of last quantifiable concentration were calculated using Drug and Statistics software (DAS version 3.2.5, Drug Clinical Research Center of Shanghai University of T.C.M., Shanghai, China). Individual concentration-time data were used to determine C_{max} and t_{max}, and the AUC was calculated by the linear trapezoidal rule. The results were also presented after dose correlation. The AUC and C_{max} were analyzed with analyses of variance (ANOVA) using GraphPad Prism (GraphPad Software, San Diego.), the difference was considered to be statistically significant when the p-value was less than 0.05. The data were expressed as mean ± standard deviation (SD).

3. Results and discussion

3.1. Formation of leuprolide-phospholipid complexes

To increase the compatibility of hydrophilic peptide drugs with hydrophobic excipients, leuprolide-phospholipid complexes were prepared. Although there have been no studies of leuprolide-phospholipid complex formation reported previously, studies of other peptide drugs showed that peptide-phospholipid complexes were formed by hydrogen bonding and Van der Waals' forces between the hydrophilic peptide and lipophilic phospholipids [23,24]. In the present study, the weight ratio between phospholipid and leuprolide was found to be critical in the formation of the complex. When the weight ratio of phospholipid to peptide increased from 1:1 to 5:1, the complexation yield increased from 42% to 98% (Table 1). No further increase of the complexation yield was observed when the ratio was increased to 10:1 (Table 1), therefore, a weight ratio of 5:1 phospholipid to leuprolide was chosen for further investigation.

3.2. Physicochemical properties of spray-dried microparticles

Leuprolide-loaded MPs were prepared according to the formulations listed in Table 2. The yield of spray-dried MP was around 60%, similar to that reported previously using the same model of spray drying machine [20,25]. The size of MP measured using laser diffraction was found to be approximately 10 µm, (except for TG22 MP (Table 2)). However, the MPs appeared smaller in the SEM analysis, probably due to aggregation of the MPs in aqueous medium for the laser diffraction measurement. The shape of spray-dried MPs was spherical and the surface of the solid lipid MPs and PLGA MPs was smooth (Fig. 1). With the increase of TG18 content in PLGA-TG18 hybrid MPs, the surface of the particles gradually changed from smooth to coarse, possibly caused by crystallization of lipids on the surface of the MP (Supplement Fig. S1).

Leuprolide was efficiently encapsulated in the spray-dried MP, apart from the TG22 MP (Table 2). Similar results were reported in a previous study for the encapsulation of insulin in solid lipid MPs using spray drying, i.e. the encapsulation efficiency of insulin in TG22 MPs was lower than that for TG18 MPs [20]. The wall materials for spray drying may affect encapsulation of active components in MP [26]. The relative low EE (68%) of leuprolide in TG22 could be a combination of fast solidification of the lipids during the spray drying process and the strong hydrophobic interactions between the very long chain fatty acyl groups in the TG22 MPs, leading to limited space for drug molecules [20].

According to the International Council for Harmonisation (ICH) guideline, dichloromethane is a class 2 residual solvent with a concentration limit of 600 ppm [27]. Gas chromatography is normally used for quantification of residual solvent [28], but thermo-gravimetric analysis (TGA) has also been used to determine the amount of residual solvent in pharmaceutic products [29]. All testes MPs met the requirement of the ICH guidelines, except for PLGA MPs (PLGA 50:50, data not shown) and PLGA-lipid hybrid MPs containing 85% PLGA (Table 2). One reason could be due to the viscosity of the lipid solution, which was lower than that of the PLGA solution, leading to fast evaporation of the organic solvent during the spray drying process and low

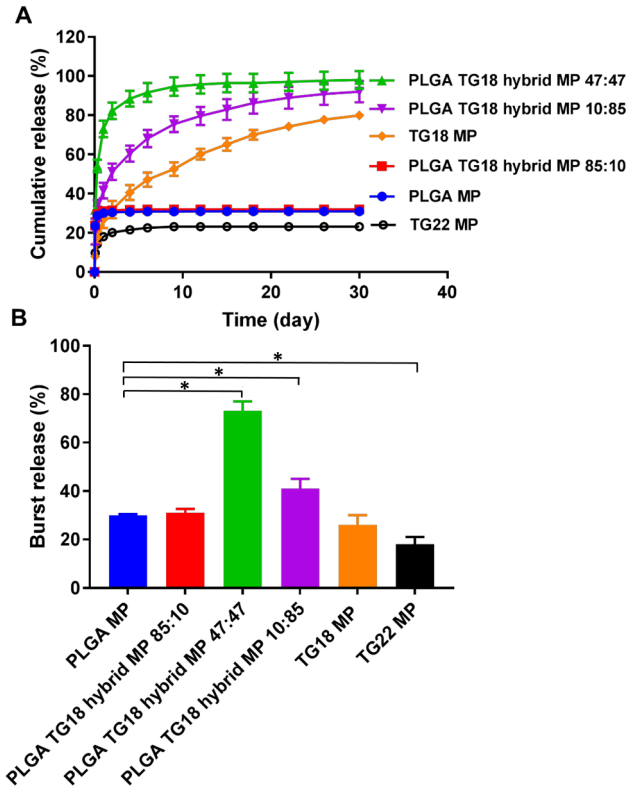

Fig. 2. (A) Release profile of leuprolide from spray-dried MPs *in vitro*, (B) Burst release of leuprolide from spray-dried MP. *p < 0.05 vs. PLGA MP (n = 3).

level of residual solvent in the spray-dried lipid MPs. The results suggest that solid lipid MPs and PLGA-lipid hybrid MPs with lower PLGA content are superior to PLGA MPs for large-scale production, as there is no further requirement for removing residual solvent for these MPs.

Lipid crystallinity and polymorphic transformation of lipids may affect drug release and product stability. In order to investigate this, the solid state of lipids in the spray-dried MPs were characterized by XRPD and DSC. The results showed that TG18 was partially transformed from β to α form in the TG18 MPs and PLGA TG18 hybrid MPs during the spray drying process, whereas TG22 maintained an α conformation in the TG22 MPs (Supplement Figs. S2 and S3). A previous study of insulin-loaded spray-dried MPs showed that no change in lipid solid state in TG22 MPs was observed after storage at room temperature over one year, suggesting solid lipid MPs are stable [20].

3.3. In vitro release of leuprolide from spray-dried MP

In vitro experiments of the release profiles of leuprolide from all spray-dried MPs showed an initial burst release in the first 24 h, followed by different release rates depending on composition (Fig. 2A). Sustained release of leuprolide from the TG18 MPs was observed with approximately 80% of the drug released over 30 days. Drug release from TG22 MPs was much slower than that from TG18 MPs. A strong correlation between protein release from triglyceride microspheres and buffer intrusion was reported preciously and a mechanism of water penetration into triglyceride microspheres followed by drug diffusion out of the matrices was proposed for protein release [30,31]. Similar results were observed for the release of proteins from cylindrical lipid matrices by confocal microscopy and the mechanism of protein release from solid lipid matrices was proposed to be diffusion controlled [30,31]. The mechanism of peptide release from these spray-dried MPs could be similar; the increased hydrophobicity of TG22 would reduce the rate of water penetration into spray-dried MPs, leading to slower drug release rate compared to TG18 MPs.

The drug release profile of PLGA lipid hybrid MPs was strongly correlated to the ratio of PLGA and lipids. Hybrid MPs with 10% of TG18 or 10% TG22 had almost the same release profile for the peptide compared to PLGA MPs (Fig. 2A and supplement Fig. S4). Similar results were obtained when the hybrid MPs contained 15% or 25% of TG18 (data not shown), suggesting that the release of leuprolide may be primarily controlled by PLGA degradation *in vitro* when the MPs contain a relatively small proportion of lipids. Addition of PLGA (10%) to solid lipid formulations accelerated the release of leuprolide from the MPs. Around 90% and 60% of the peptide was released over 30 days from PLGA TG18 and PLGA TG22 hybrid MPs, respectively (Fig. 2A and supplement Fig. S4). Even though the polymorphic form of the lipids in the spray-dried MP was not affected by addition of 10% PLGA in the hybrid MP (Supplement Figs. S2 and S3), the presence of PLGA could still disrupt the ordered packing of crystalline lipids and improve the wetting of lipid matrices, leading to increased intrusion of the medium and accelerated drug release.

Composition of the spray-dried MPs was found to have a strong effect on the extent of leuprolide burst release. Burst release of the peptide from TG22 MPs in the first 24 h was significantly lower than that from PLGA MPs (Fig. 2B). The ratio between PLGA and lipid in the hybrid MPs also affected the level of burst drug release. Around 80% of leuprolide was released in the first 24 h from PLGA TG18 hybrid MPs containing equal amount of TG18 and PLGA, suggesting that PLGA and TG18 are not compatible at a weight ratio of 1:1. The incompatibility of TG18 and PLGA in the hybrid MPs is most likely caused by the different solidification rates of TG18 and PLGA during the spray drying process and the heterogeneity of TG18 and PLGA blocks in the hybrid MPs. Similar results were observed for leuprolide burst release from PLGA TG22 hybrid MPs when the weight ratio of PLGA and TG22 was 1:1 (Supplement Fig. S4). Further studies on the distribution of lipids and polymer in the hybrid MPs will be useful to gain a better understanding of the drug release mechanism from hybrid MPs.

3.4. Pharmacokinetic and pharmacodynamic results

The mean plasma concentration versus time profiles of leuprolide after subcutaneous administration of different MPs are shown in Fig. 3 and the pharmacokinetic parameters are listed in Table 3. During the first 2 h after dosing, the concentration of leuprolide increases rapidly, followed by continuous decrease in all groups. Sustained release of leuprolide was observed for all spray-dried MPs. The concentration of leuprolide could be quantified in plasma samples over 30–60 days, depending on the formulation (Fig. 3). Leuprolide is an agonist of the luteinizing hormone-releasing hormone and can reduce testosterone production via a negative feedback loop [32]. The baseline level of

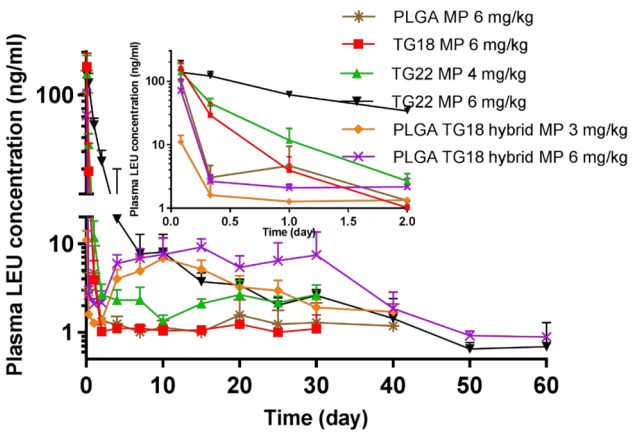

Fig. 3. Plasma concentration of leuprolide (LEU) over 2 months after s.c. administration of spray-dried microparticles (MP) to rats (n = 3–5). The weight ratio of PLGA to TG18 in PLGA TG18 hybrid MPs was 85:10.

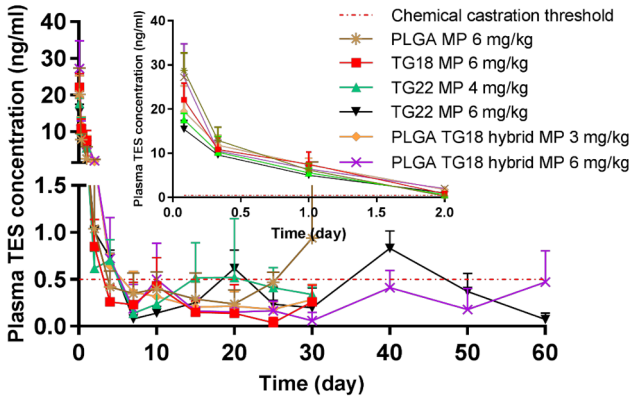

Fig. 4. Plasma concentration of testosterone (TES) after s.c. administration of spray-dried microparticles (MP) to rats (n = 3–5). The dotted line represents the chemical castration threshold. The weight ratio of PLGA to TG18 in PLGA TG18 hybrid MP is 85:10.

plasma testosterone in rats was found to be 1.83 ± 0.51 ng/ml (n = 8). As expected, plasma testosterone increased after subcutaneous administration of different MP, followed by a drop to the castration level (0.5 ng/ml), illustrating the pharmacodynamic effect (Fig. 4).

Solid lipid MPs containing TG18 showed similar PK and PD profile as PLGA MPs. Sustained release of leuprolide from PLGA MPs was observed over 40 days (Fig. 3), accompanied by a chemical castration in rats for 25 days (Fig. 4). This result is in similar to a previous report of an *in vivo* study of degarelix-loaded PLGA MPs prepared using different methods [33], confirming that spray-dried PLGA MPs in the present study have similar behavior as previously reported PLGA MPs for sustained drug delivery. Spray-dried TG18 MPs could maintain the chemical castration effect in rats for 30 days, slightly longer than PLGA MPs (Fig. 4).

Comparing the two different solid lipid microparticles, TG22 MPs were better carriers for leuprolide than TG18 MPs. The AUC of TG22 MPs showed a greater than a five-fold increase when compared to TG18 MPs with the same dose of leuprolide (6 mg/kg). There was no significant difference between C_{max} and t_{max} for the groups; however, TG22 MPs provided longer chemical castration effects over 60 days (Table 3). When the dose of leuprolide was reduced to 4 mg/kg, the chemical castration effect of TG22 MPs could be observed over 30 days and the AUC of TG22 MPs was still larger than that of TG18 MPs (6 mg/kg).

The AUC of PLGA TG18 hybrid MPs was approximately four times larger than that of TG18 MPs and PLGA MPs containing the same dose of leuprolide (6 mg/kg), whereas the C_{max} of the hybrid MPs was significantly lower than that of the other groups (Table 3). A lower C_{max} generally prevents/reduces the side effects of drugs [34]. The observed reduction of C_{max} may allow the safe administration of larger dose of peptide drugs, further prolonging the duration of therapeutic effectiveness. Chemical castration in rats was observed over 30 and 60 days after injection of the PLGA TG18 hybrid MPs with a dose of 3 mg/kg and 6 mg/kg leuprolide, respectively (Fig. 4,Table 3). When the dose of leuprolide was reduced to 3 mg/kg for the group of PLGA TG18 hybrid MPs, the AUC was still significantly higher than that of PLGA MPs and TG18 MPs (6 mg/kg) (Table 3), demonstrating the superior of the hybrid MP over the other spray-dried MP for sustained delivery of the peptide drug.

3.5. Discussion

PLGA MPs are usually prepared by solvent evaporation, whereas solid lipid MPs are prepared by hot melting or spray congealing method. In the present study, MPs with different excipients were prepared by spray-drying organic solution containing both hydrophobic excipients and peptide-phospholipid complexes in order to elucidate the effect of excipient on drug release from the MPs. Solid lipid MPs, i.e. TG18 and TG22, were prepared to elucidate the effect of lipids on drug release from the MPs. Additionally, PLGA TG18 hybrid MPs were prepared to investigate the effect of mixtures of PLGA and solid lipid on release of the peptide from spray-dried MPs. PLGA MPs were prepared as a reference group. Pharmacokinetic and pharmacodynamic results in rats demonstrated that both the composition of excipients and the dose in the MP affected drug release and function of leuprolide-loaded MPs.

The present *in vivo* study shows that TG22 MPs were better than TG18 MPs and PLGA MPs with higher bioavailability and a slower release of leuprolide, as well as a longer duration of effect on reducing the level of testosterone (Table 3). The results agree with previously proposed drug release mechanisms, i.e. water penetration into triglyceride matrices followed by drug diffusion out of the matrices [30,31]; TG22 is more hydrophobic than TG18, leading to slower water intrusion and a slower rate of drug release from TG22 MPs *in vivo*. Although the *in vitro* study demonstrated that the peptide was released slowly from TG22 MPs, it was difficult to observe further drug release from TG22 MPs after a few days (Fig. 2), indicating the disadvantage of the method for the drug release study. A Subcutaneous Injection Site Simulator (Scissor) was reported recently by Kinnunen et al. as an *in vitro* method to evaluate the fate of subcutaneously administered biopharmaceuticals; they found a good correlation between *in vitro* drug release and *in vivo* bioavailability of monoclonal antibodies [35]. We are interested in evaluating the release of leuprolide from our spray-dried MPs using such a model with a dialysis-based injection chamber to investigate potential *in vitro in vivo* correlations in the future.

PLGA TG18 hybrid MPs were demonstrated to be the best carriers for leuprolide among the spray-dried MPs *in vivo*. The superior function of the hybrid MPs with low initial drug concentrations and long drug release periods (Table 3) suggests a synergistic effect of PLGA and triglyceride for sustained delivery of peptide drugs *in vivo*. The degradation mechanism of PLGA is well studied and characterized as bulk degradation and hydrolysis of the polymer backbone [36]. The aforementioned protein release from solid lipid matrices is primarily controlled by diffusion of water and drug molecules in and out of drug carriers [36]. An *in vivo* study of triglyceride implants in a rabbit model also showed erosion behavior of lipids at body temperature [37]. The co-existance of PLGA and solid lipids in the spray-dried MPs may alter the inner structure of the particles, leading to different drug release profiles from hybrid MPs. No distinct crystalline peaks of triglyceride were observed from the diffractogram and thermogram of hybrid MPs (Supplement material Figs. S2 and S3), suggesting that the triglycerides were amorphous or that the lipid molecules were well distributed amongst the PLGA chains. The methyl group of PLA may interact with fatty acid chains via van der Waal forces, leading to a slower degradation of PLGA *in vivo*. The proposed molecular interaction between PLGA and triglycerides agrees well with the observed *in vivo* results, i.e. slower drug release and lower C_{max} together with a prolonged pharmacodynamic effect of leuprolide from the hybrid MPs (Table 3). Further studies on the molecular interactions between PLGA and triglycerides in spray-dried MPs is required. It will not only provide a better understanding of the synergistic effect of PLGA and lipids in the sustained release of peptide drugs, but also aid in determining good carriers for sustained delivery of protein drugs.

4. Conclusion

Leuprolide loaded solid lipid MPs and PLGA lipid hybrid MPs were successfully prepared by spray drying solutions of hydrophobic excipients and leuprolide-phospholipid complexes. Spray-dried MPs were spherical and the particle size found to be suitable for injection. Sustained release of leuprolide from the MPs was confirmed by elevated concentrations of plasma leuprolide from one to two months and accompanied by reduced levels of testosterone in the plasma of rats. Solid

lipid MPs showed better or similar effects compared to PLGA MPs *in vivo*, depending on the chain-length of acyl groups in the lipids. Longer chain lipids in the MPs resulted in prolonged drug release and suppression of testosterone. The addition of 10% triglyceride in PLGA formulations reduced the rate of drug release from the hybrid MPs, probably due to slower degradation of PLGA caused by molecular interactions between PLGA and triglycerides. Additionally, a much lower C_{max} was observed in rats after subcutaneous injection of hybrid MPs, demonstrating a synergistic effect of PLGA and triglycerides for sustained drug delivery. In conclusion, PLGA lipid hybrid MPs are promising carriers for sustained delivery of peptide drugs.

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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.2019.07.010.

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