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Research Article

Porous Core/Dense Shell PLA Microspheres Embedded with High Drug Loading of Bupivacaine Crystals for Injectable Prolonged Release

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Abstract. Objective of the study was to design an injectable microsphere preparation with high drug loading of bupivacaine for prolonged release and local anesthetic. PLA or PLGA was used as the biodegradable matrix material to fabricate microspheres with the o/w emulsification-solvent evaporation method. The characterization of bupivacaine microspheres was observed by SEM, DSC, and XRPD. The microsphere preparation and extended drug release, as well as the plasma drug concentration and sciatic nerve blockade after injection of the microsphere formulation to rats were investigated. High drug-loading microspheres of more than 70% were successfully obtained with extended drug release over 5 days *in vitro* depending on the type of matrix and the feed ratio of drug to polymer. SEM, DSC, and XRPD results verified a novel microsphere structure characterized as the porous core composed of PLA material and form II bupivacaine crystals and dense shell formed of PLA layer. The mechanism that bupivacaine was dissolved inside the microsphere and diffused across the dense shell was suggested for drug release *in vitro*. The optimized PLA microsphere formulation showed low and steady plasma drug concentration over 5 days and prolonged duration of sensory and motor blockade of sciatic nerve lasted more than 3 days. Results indicated that the porous core-shell structure of PLA microsphere formulation would provide enormous potential as an injectable depot for locally prolonged delivery of bupivacaine and control of postoperative pain.

KEY WORDS: PLA; bupivacaine; microspheres; high drug loading; prolonged release.

INTRODUCTION

Bupivacaine, an amide-type local anesthetic, has been widely used to control postoperative pain due to its rapid and strong onset of anesthetic effect. Because of the well-known short half-life of bupivacaine, novel sustained delivery systems characterized as prolonged drug duration are developed in recent years, including multivesicular liposomes based on the depofoam technology (EXPAREL®, from Pacira Pharmaceuticals, Inc.), *in situ* gel system composed of sucrose acetate isobutyrate and benzyl alcohol in ethanol that formed a viscous gel at the administration site (SABER®, developed by Durect, CO.), and collagen-based intraoperative implant (XaraColl®) (1–3). However, most of these delivery systems are suspended in various stages of clinical trial on account of some reasons. For decades, PLA or PLGA microspheres have been investigated for prolonged release of bupivacaine or lidocaine with great potential of extending local analgesia duration, but great obstacles still must be overcome before application (4,5). The advised dose of bupivacaine HCl injection for postoperative analgesia to healthy adult is 20~40 mg given by intermittent epidural bolus, so the greatest challenge in designing bupivacaine PLA or PLGA microspheres lied in the high dosage of administration and sustained drug release period (3~5 days) was imperatively required (6).

Poly (lactic acid) (PLA) and poly (glycolic acid) (PLGA) are commonly used as carrier materials and solely approved as safe synthetic polymers by FDA (7,8). The hydrophilicity and degradation cycle of PLGA could be controlled by adjusting the molecular weight and the ratio of lactic acid and glycolic acid (9). PLGA has been intensively evaluated and used as tissue engineering material and prolonged drug delivery carrier (10,11). However, up to now, there are only 19 different drugs in PLGA formulations approved by FDA in large part from the lack of a clear molecular understanding

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of PLGA polymers and a mechanistic understanding of PLGA microsphere formation (12). More efforts will be done to take advantage of this drug delivery system, especially in terms of PLGA microspheres in order to carry certain drugs.

Implant depots based on injectable microspheres are one of the most important applications of biodegradable polyesters in drug delivery (13–15), which are successfully applied to the market of pharmaceutical products for intraocular, subcutaneous, intramuscular, and intratumoral injection (16,17). The daily doses of several days or months combined into a single administration of high drug-loading depot is pressing needed, especially for those of relatively low potent drugs (18). The commercial Risperdal Consta® contains around 39% risperidone crystals, based on the net mass of the dry PLGA microspheres, which release the loaded drug following a biphasic *in vitro* release profile with minor initial burst release (19). However, high drug loading is generally related to rapid drug release through the connected micro-channels left in the polymer matrix after drug release. The emulsification-solvent evaporation method is one of the scalable and widely adopted processes in industrial. Recently, advanced techniques with modification mainly to the emulsification process have been introduced to fabricate microspheres with precisely controlled size, special core-shell or anisotropic structure, tunable morphology, and drug release behavior, *e.g.*, membrane-emulsification method based on microfluidic platforms or layer-by-layer assemble (20–22). In order to improve drug loading and extend drug release of PLGA microspheres, novel techniques and modifications to the existing method, *e.g.*, coaxial nozzle spray, are suggested to fabricate core-shell-structured PLGA microspheres (23,24).

The aim of our study is to design injectable bupivacaine microspheres for prolonged drug duration. PLA and PLGA of high molecular weight were chosen as the matrix materials to fabricate microspheres with drug loading more than 70%. Inspired by spherical crystallization technique, bupivacaine crystals were embedded inside microspheres using classic emulsification-solvent evaporation method with proper modifications, for example, increasing the drug feed ratio in the oil phase and changing technological parameters (25). We firstly conceived the precipitated crystals following solvent evaporation could be bound together with PLGA to form "rice ball"-like microspheres, while the optimized formulation unexpectedly turned to be anisotropic and porous core-shell structured microspheres. In order to reveal the potential in prolonged drug delivery, the preparation of microspheres and mechanism of drug release were postulated, and *in vivo* pharmacokinetics and local anesthetic efficacy were evaluated in rats.

MATERIALS AND METHODS

Poly (lactic acid) (IV = 0.91 dl/g, Mw = 80,000 da), Poly (lactic acid) (IV = 2.0 dl/g, Mw = 250,000 da), Poly (lactic-co-glycolic acid) (75/25, IV = 1.6 dl/g, Mw = 200,000 da), and Poly (lactic-co-glycolic acid) (85/15, IV = 2.0 dl/g, Mw = 200,000 da) were kindly gifted by Changchun Sino-Biopolymer Co., Ltd. (Changchun, China). Bupivacaine hydrochloride was purchased from Shanghai San-wei Pharmaceutical Co., Ltd. (Shanghai, China). Polyvinyl alcohol (PVA-217SB) was a kind gift from Kuraray Co., Ltd. (Osaka, Japan). Heparin sodium injection was obtained from Dichroa Biochemical Pharmaceutical Co., Ltd. (Hebei, China). All other chemicals were of analytical or chromatographic grade.

Preparation of Bupivacaine PLA or PLGA Microspheres (BUP-MSs)

Bupivacaine free base was obtained by alkalifying bupivacaine hydrochloride with ammonium hydroxide as described in the supporting information. BUP-MSs were prepared using o/w emulsification-solvent evaporation method. Briefly, PLA or PLGA and BUP were dissolved in dichloromethane (DCM), and the organic phase was then poured into 10 mL 1% PVA aqueous solution, and the mixture was homogenized with a high-speed disperser (ULTRA-TURRAX® T18 digital, IKA Werke GmbH & Co., Staufen, Germany) at a speed of 8000 rpm to form o/w emulsion. The emulsion was transferred into 140 mL 1% PVA aqueous solution, and the organic solvent was then removed using the rotary evaporator at 40 °C under vacuum. BUP-MSs were collected by filtration, rinsed with purified water and freeze-dried in a freeze dryer (FD-1, LABFREEZ Instruments Co., Ltd., China).

Characterization of BUP-MSs

The size of BUP-MSs was measured using an optical microscope (BA300 Pol, Motic Ins., Xiamen, China). The morphology of BUP-MSs was observed using a scanning electron microscope (S-3400, Hitachi High Technologies, Kyoto, Japan) after sputtering coat with gold. The differential scanning calorimeter (DSC) curves were measured using DSC1 equipment (Mettler-Toledo AG, Switzerland) equipped with a refrigerated cooling system, and the data was processed using STARe SW 9.30 software. Samples were hermetically sealed in aluminum pans, and then heated from 30 to 300 °C at a rate of 20 °C/min under nitrogen atmosphere. X-ray powder diffraction (XRPD) patterns were performed on a X-ray powder diffractometer (XRD-6000, Shimadzu, Japan) from 5 to 60° at a step of 5°/min.

Drug Loading (DL) of BUP-MSs

Ten milligrams of BUP-MSs were placed in a 25 mL volumetric flask, added 20 mL methanol, ultrasonic treated for 30 min, and added methanol to the volume. The supernatant was separated after centrifuging and analyzed using Uv-Vis spectrophotometer (UV5100, WAYEE, China) at 263 nm to determine the concentration of BUP. The drug loading was calculated as below:

$$\mathrm{DL},\ \% = \frac{\text{Amount of drug embeded in MSs}}{\text{Mass of MSs}} \times 100\% \qquad (1)$$

In Vitro Drug Release

Ten milligrams of BUP-MSs were suspended in 25 mL release media (PBS pH 7.4) and incubated in a shaking bath (ZHWY 110X30, Zhicheng Instrument Co., Shanghai, China) at 37 ± 0.5 °C and 100 rpm/min. At predetermined time intervals, after centrifugation at 2000 rpm/min for 10 min,

4 mL PBS was withdrawn and replaced with the same volume of fresh media. The amount of released drug was determined by UV-vis spectrophotometer at 263 nm. During the drug release test, samples were withdrawn and observed using an optical microscope under normal and polarized light at the predetermined time intervals (26–28).

Plasma Bupivacaine Concentration

Male Sprague-Dawley rats weighing from 200 to 250 g were divided into two groups: group A (control group) and group B (test group). The rats of group A were administered with a single dose injection of 0.5 mL bupivacaine hydrochloride injection (30 mg/kg equivalent to bupivacaine) at the right sciatic nerve, and the rats of group B were administrated with 0.5 mL BUP-MSs suspension (Formulation No.10, 150 mg/kg equivalent to bupivacaine) in normal saline containing 0.2% CMC-Na. Control group rats were detected the plasma bupivacaine concentration in the time of 1 h, 2 h, 4 h, 6 h, 12 h, and 24 h after administration, and the test group rats were monitored the plasma concentration at the time intervals of 4 h, 8 h, 12 h, 24 h, 48 h, 72 h, 96 h, and 120 h. Blood samples were collected at predetermined time intervals from orbit vein into heparinized polypropylene tubes, and centrifuged at 4000 r/min for 10 min, and the supernatant plasma samples were stored in – 20 °C until further analysis. Plasma samples were extracted as follows: alkalized by adding of 1 M sodium hydroxide, extracted with heptane-ethyl acetate mixture solvents (90:10, v/v) for 3 times, combined, and removed the supernatant organic solvent under nitrogen stream; the residue was reconstituted with 0.05 M sulfuric acid and then buffered with 0.2 M sodium acetate. Finally, the plasma concentration of bupivacaine was determined by HPLC at 228 nm (LC-10A, Shimadzu, Kyoto, Japan) equipped with a C8 reverse phase column (Diamonsil® C8 (2), 5 μm, 250 mm × 4.6 mm). The mobile phase was 0.01 M sodium dihydrogen phosphate and acetonitrile (78:28 v/v, pH 2.1) at a flow rate of 1.0 mL/min.

Sciatic Nerve Blockade Test

Male Sprague-Dawley rats weighing from 200 to 250 g were divided into four groups: group A (blank control), group B (positive control), group C (low dose of BUP-MSs), and group D (high dose of BUP-MSs). The administration method was introduced in briefly: samples were injected through introduced posteromedial to the greater trochanter and advanced in an anteromedial direction until bone contacted. Group A was injected with 0.5 ml normal saline, group B was injected with bupivacaine hydrochloride (dosage was 30 mg/kg), group C and group D were received BUP-MSs equivalent to 50 mg/kg and 150 mg/kg. At predetermined time intervals, the four group rats were detected the effect of sciatic nerve blockade including sensory and motor blockade.

To investigate sensory blockade by the hotplate test, the right hind paw of rat was exposed to a hotplate device at 56 °C, which represented a strong stimulus and the most clearly distinguisher full sensory blockade from milder analgesic effect. The sensory block duration was defined as the mean duration time for which the latency of rats was greater than or equal to 7 s. The time defined thermal latency until the rat withdrew its paw was recorded with using 12 s as a cutoff latency. If the paw remained on the hotplate over 12 s without self-withdrawal, then it was removed to avoid thermal injury or hyperalgesia. In the test, only the single hind leg injected with the formulation was placed on the hotplate, and the contralateral leg on the wooden block at room temperature. No case of righting reflex, visible convulsion, death, or evidence of systemic anesthesia was observed.

Motor blockade test was performed at each time point to examine the rats' ability to hop and place weight on the hind leg. Duration of motor blockade is defined as the mean time for return to a motor score of 2 on the 1–4 scale. All the data was expressed as mean ± standard deviation, and comparisons between groups were performed using the *t* test.

Animal Care

Male Sprague-Dawley rats were obtained from the Experimental Animal Center of Shenyang Pharmaceutical University. The rats were kept on 12 h:12 h of light:dark cycles and fed with standard food and water *ad libitum*. All the pharmacokinetic and pharmacodynamic studies involving animals used were conducted in accordance with the Ethical Guidelines on Animal Experiments of Shenyang Pharmaceutical University (Shenyang, China).

RESULTS

Preparation and Characterization of BUP-MSs

Bupivacaine microspheres were prepared through altering the type of PLA or PLGA, the feed ratio of drug to polymer, homogenization speed, and other additives added, which were optimized according to morphologic observation shown in Table I and Fig. 1. SEM images indicated that the surface of BUP-MSs using PLGA (Formulation No.1, 2) and low molecular weight PLA (Formulation No.3) as the matrix material was covered by clusters of needle-like bupivacaine crystals that would lead to obvious initial burst release. Other formulations showed smooth surface, or less to no crystal clusters adhere to the surface of microspheres. Replacement of DCM with ethyl acetate (Formulation No.6) and addition of benzyl alcohol in the organic phase (Formulation No.12) resulted in the coarse or irregularly shaped microspheres. Altering the homogenization speed and addition of polysorbate-80 into the outer aqueous phase did not affect the surface morphology, but changed the particle sizes partly, which may provide possible ways to tailor the size of microspheres (Formulation No.4, 5, 7). Increasing the feed ratio of drug to polymer could widely increase the content of drug embedded (Formulation No.8~11). The surface smoothness of PLA microspheres was dependent on the feed ratio of drug to polymer. However, it should be noted that the irregular shape and coarse surface related to the very high feed ratio of drug to polymer may inevitably leading to the poor flowability of microspheres and the poor needle-through property (Formulation No.11).

DSC thermograms in Fig. 2a showed the metastable crystal of bupivacaine base (Tm = 102 °C) was embedded inside the microspheres when the feed ratio of drug to polymer was 70% and 80%, Tm = 105 °C when the feed ratio was 90%; however, the form of bupivacaine free base or the mixture of blank microspheres and bupivacaine was stable

Table I. Formulations and the Properties of the BUP-MSs

No.	PLGA, mg	BUP, mg	DCM, ml	Ethyl acetate, ml	Tween80, mg	Benzyl alcohol, ml	Rotation speed, r/min	Diameter, μm	DL, %	Shape
1	40 (PLGA 75/25, IV = 1.6)	160	0.7	–	–	–	6000	43.5 ± 11.3	74.1	Rough
2	40 (PLGA 85/15, IV = 2.0)	160	0.8	–	–	–	8000	30.5 ± 5.7	68.9	Rough
3	40 (PLA, IV = 0.91)	160	0.5	–	–	–	6000	24.2 ± 7.8	68.6	Rough
4	40 (PLA, IV = 2.0)	160	0.8	–	–	–	6000	38.7 ± 8.2	73.8	Smooth
5	40 (PLA, IV = 2.0)	160	0.8	–	–	–	8000	39.6 ± 9.8	75.9	Smooth
6	40 (PLA, IV = 2.0)	160	–	1.2	–	–	8000	32.8 ± 6.8	68.2	Rough
7	40 (PLA, IV = 2.0)	160	0.8	–	60	–	10,000	28.3 ± 10.8	70.3	Smooth
8	60 (PLA, IV = 2.0)	140	1.1	–	60	–	8000	30.5 ± 9.7	68.9	Smooth
9	50 (PLA, IV = 2.0)	150	1.0	–	60	–	8000	29.3 ± 9.6	71.0	Smooth
10	40 (PLA, IV = 2.0)	160	0.8	–	60	–	8000	32.0 ± 10.6	71.4	Smooth
11	20 (PLA, IV = 2.0)	180	0.5	–	60	–	8000	28.7 ± 10.9	76.4	Rough
12	40 (PLA, IV = 2.0)	160	0.7	–	60	0.1	8000	45.1 ± 17.6	79.9	Rough

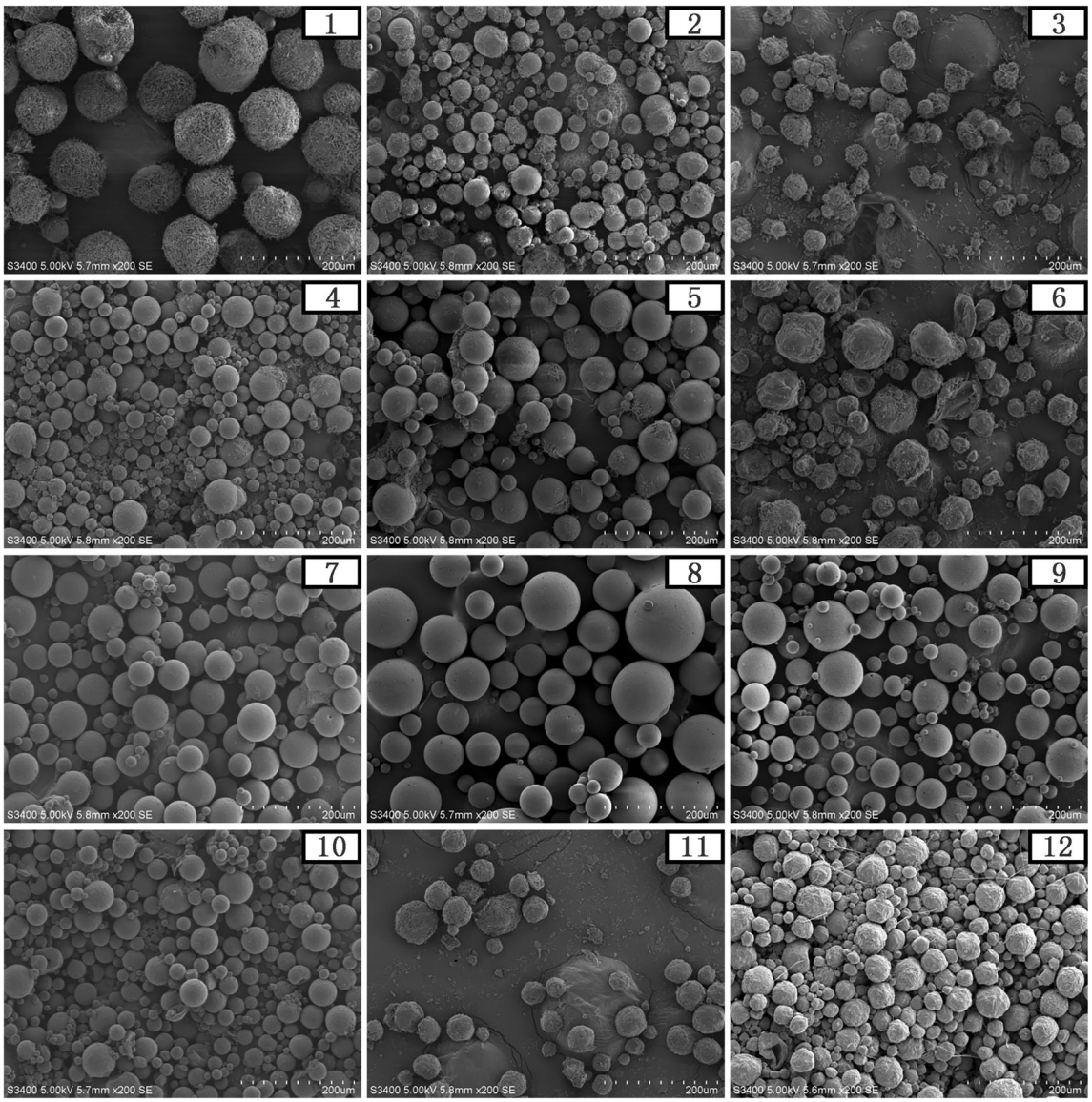

Fig. 1. SEM images of BUP-MSs

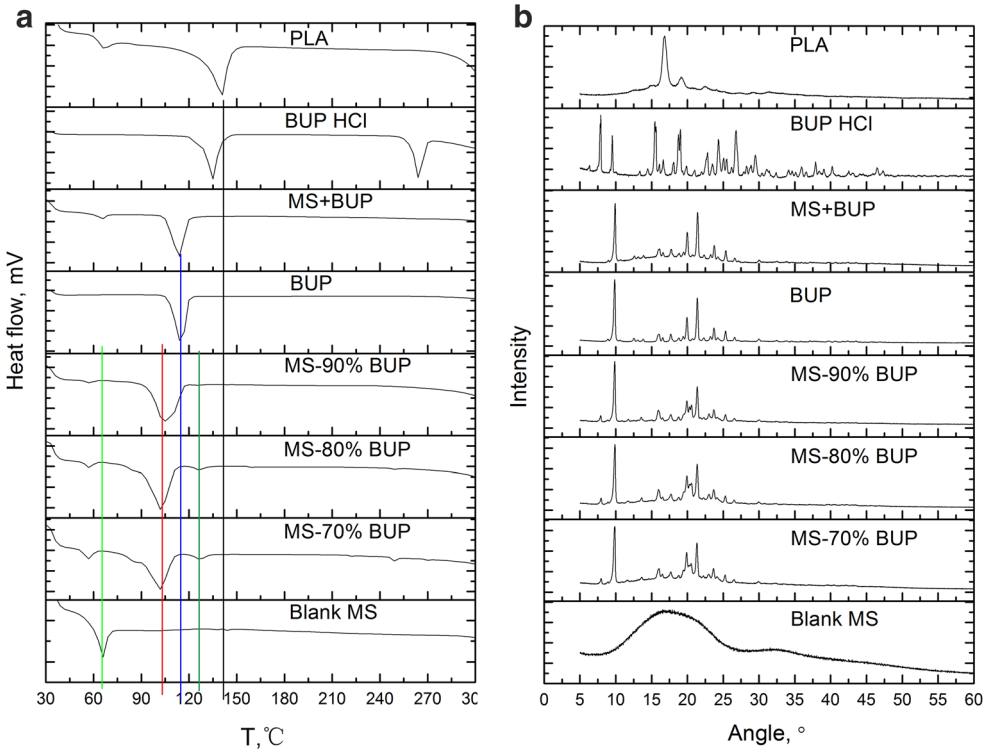

Fig. 2. DSC thermograms (**a**) and XRPD patterns (**b**) of PLA, BUP, their physical mixture, and BUP-MSs of different drug loading

crystal (Tm = 114 °C). The XRPD patterns in Fig. 2b also demonstrated different crystal peaks of the embedded bupivacaine compared to that of bupivacaine base. The DSC and XRPD results of both the blank microspheres and the mixture of blank microspheres and bupivacaine indicated that the amorphous PLA formed the matrix of microspheres, through comparing the DSC curves and XRPD patterns of polymer PLA.

In Vitro Drug Release

In vitro drug release test of BUP-MSs was performed to verify the effects of the formulation composition and feed ratio of drug to polymer (Fig. 3). PLGA microspheres showed no prolonged drug release behavior with about 80% of the embedded drug releasing within 1 day (Formulation No.1, 2), and higher ratio of glycolic acid and lower molecular weight (Formulation No.1) corresponded to even faster drug release. The initial burst release due to the drug crystals adhered to the surface of microspheres may give another possible explanation to the rapid release of PLGA microspheres. Compared to PLGA microspheres, PLA microspheres of the same feed ratio of drug to polymer (80/20) showed prolonged drug release over 5 days (Formulation No.1, 4, 12). The relatively slower drug release rate of formulation No.12 could be attributed to its larger particle size, and benzyl alcohol incorporated in this formulation was not expected to affect drug release obviously. Figure 3b showed the PLA microspheres release profiles of different feed ratios of drug to polymer, which indicated a directly dependent manner of drug release rate to the feed ratio. BUP-MSs of lower feed

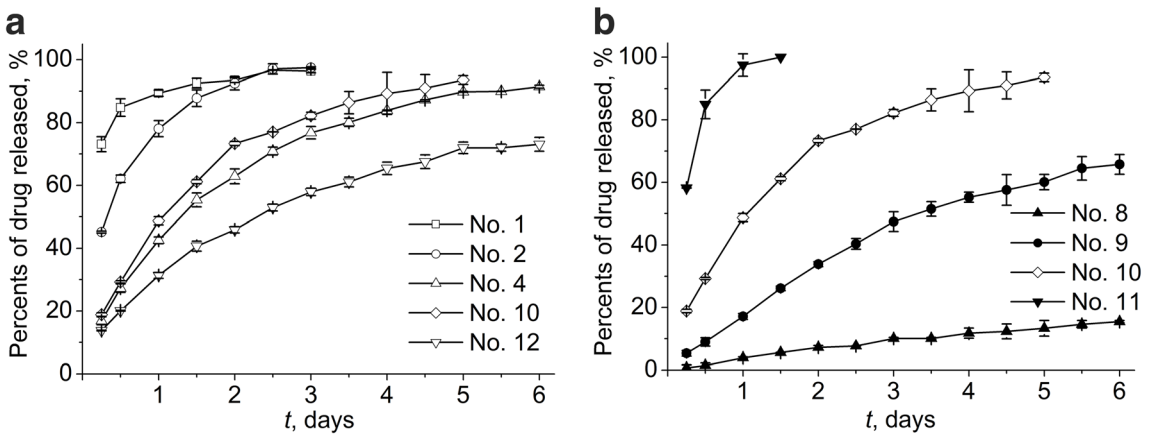

Fig. 3. *In vitro* drug release profiles of BUP-MSs. **a** Effect of the compositions of the formulation. **b** Effect of drug loaded

ratio (70/30, Formulation No.8) released less than 20% of the total loaded drug in 7 days, while the feed ratios of 75/25 and 80/20 (Formulation No.9, 10) displayed steady and prolonged release profiles over 7 days and 5 days, respectively. The BUP-MSs of higher feed ratio (90/10, Formulation No.11) released bupivacaine completely within 1 day. Ritger-Peppas empirical equation, Higuchi equation, and first-order equation were introduced to fit the drug release kinetics. Results showed that bupivacaine release from BUP-MSs occurred in accordance with the first-order equation and could be expressed by the following equation: $\ln(1\text{-}Q) = -0.5237\ t$ ($r^2 = 99.72$).

The bright field of the optical microscopic images was corresponded to the bupivacaine crystals rich area at different release time intervals observed by polarized microscope in Fig. 4. These images clearly illustrated almost overall distribution of crystals inside the microspheres and the crystals dissolved process over the extended period of release time. For the BUP-MSs of lower feed ratio of drug to polymer (70/30), crystals could be observed obviously even after 120 h of drug release. Crystals almost disappeared after release for 72 h as to the BUP-MSs of feed ratio of drug to polymer (80/20). On the contrary, for the BUP-MSs of higher feed ratio (90/10), bupivacaine crystals were completely dissolved within 24 h. Lower feed ratio microspheres had higher PLA content, which consequently led to low diffusivity of matrix and slow dissolving rate of bupivacaine crystals. The gradually inward withdrawal of the bright field displayed the erosion or dissolution process of drug crystals with release time, until all crystallized drug dissolved.

As the high molecular weight and more hydrophobic PLA was relatively resistant to degradation both *in vitro* and *in vivo*, but almost intact microsphere (regular shape, but relatively rough surface after drug release) was left after 7 days *in vitro* drug release or even after 5 days *in vivo* test (Fig. 5a). Although we previously believed that connected channels or porous structures of the microsphere matrix would occur after drug release from microspheres, an interesting discovery of only non-porous surface morphology was observed. The result reminded us of any special microscopic structure of microspheres instead of once believed homogeneous microsphere matrix composed of bupivacaine crystals and PLA. The SEM image of the microsphere cross-section clearly showed porous inner matrix structure surrounded by a dense shell composed of continuous thin polymer layer (Fig. 5a (d) and Fig. 5b).

Plasma Bupivacaine Level and Sciatic Nerve Blockade After Administration to Rats

Plasma bupivacaine level *versus* time curves after sciatic nerve injection of bupivacaine hydrochloride injection and BUP-MSs (Formulation No.10) were shown in Fig. 6. Plasma bupivacaine concentration of the control group rapidly decreased below 10 ng/mL at about 4 h after administration. The test group showed steady and low plasma bupivacaine concentration fluctuating in the range of 1~10 ng/mL for 5 days. Table II reported other parameters of the plasma bupivacaine level.

The sciatic nerve blockade test of BUP-MSs also showed that the rats of group A received normal saline (blank control) manifested no sensory or motor blockade, while the

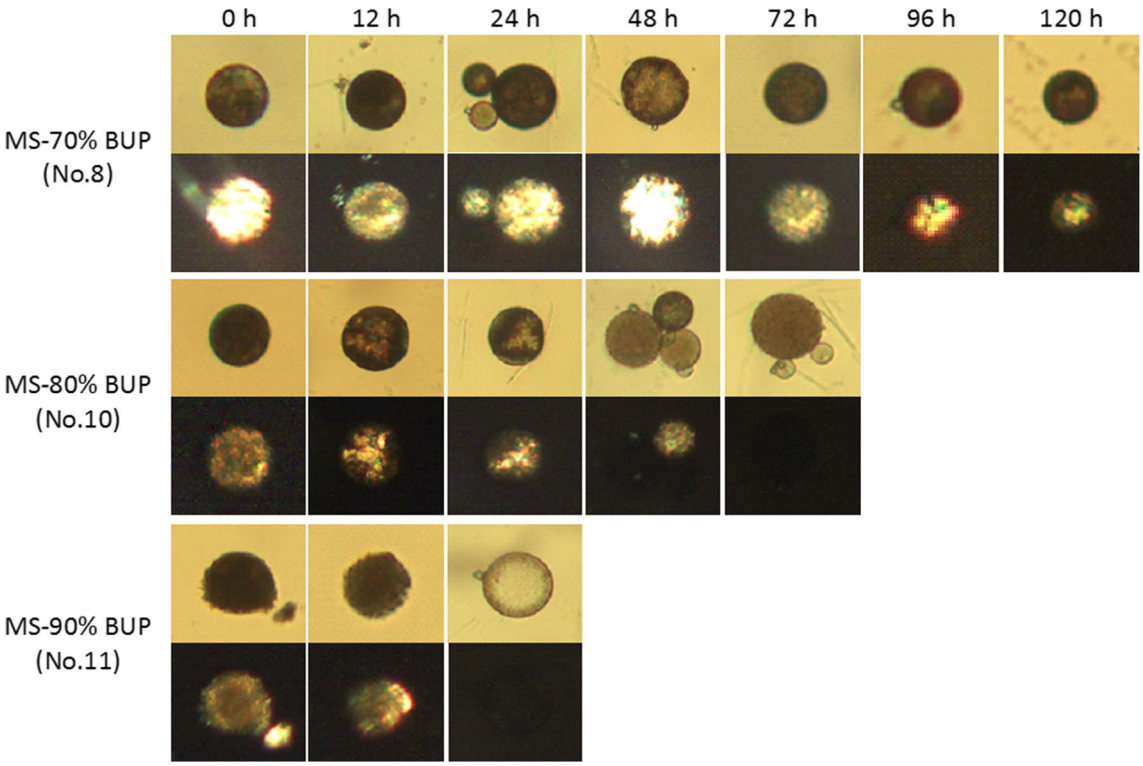

Fig. 4. Optical microscopic images of the BUP-MSs at different time of drug release observed under normal light (top) and polarized light (bottom), respectively

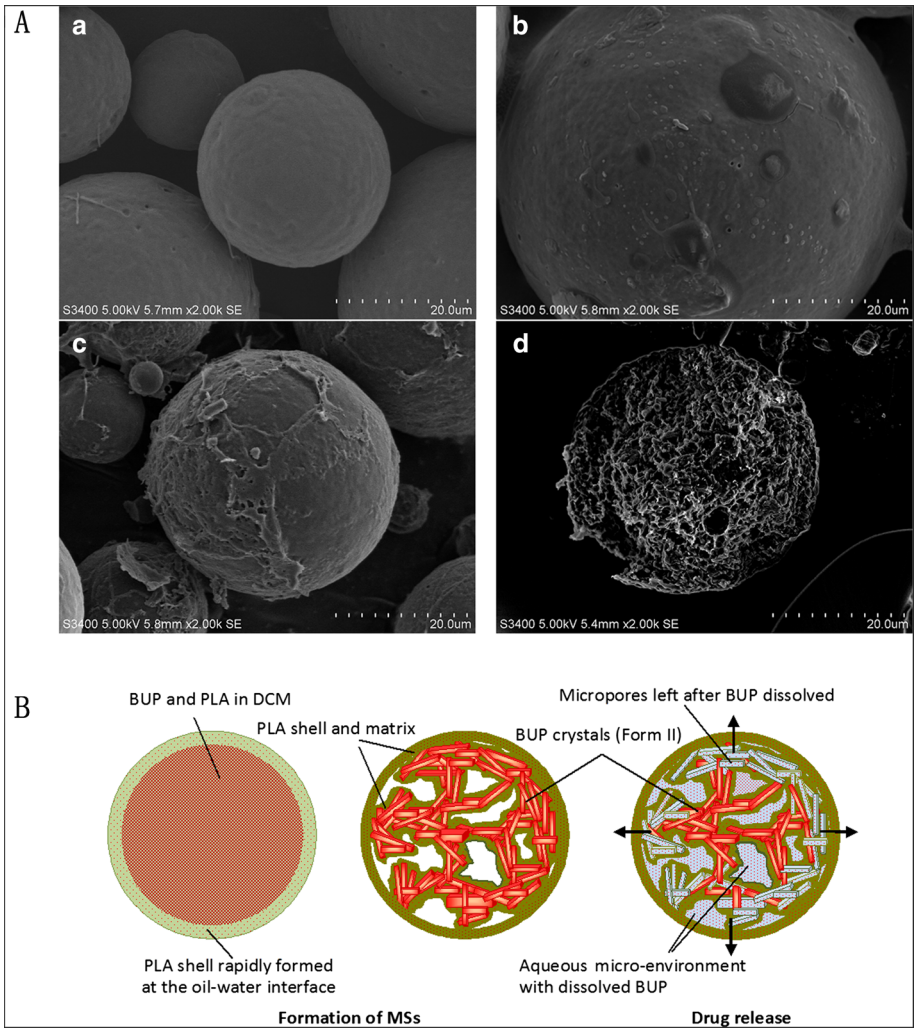

Fig. 5. A SEM images of the surface morphologies of the intact microspheres (*a*), and microspheres after *in vitro* drug release (*b*), *in vivo* test (*c*), and the cross section of the microspheres before release *in vitro* (Formulation No.10) (*d*). **B** An illustration to the possible porous core-dense shell structure and drug release mechanism of BUP-MSs

rats of group B (positive control) and group C (low dose of BUP-MSs) both recovered to normal state from sciatic nerve blockade state within 12 h. However, the rats of group D (high dose of BUP-MSs) produced prolonged duration of anesthetic action with significant sensory and motor block duration lasted more than 3 days compared to group A and group B (Fig. 7).

DISCUSSION

An ideal microparticulate depot system should bear various merits, including regularly spherical shape and uniform size, acceptable amount of drug loading, low initial burst, and predictable release duration period, which were mainly determined by the formulation composition, molecular weight of polyester and fabrication techniques. Leuprorelin microspheres prepared by w/o/w double emulsion-solvent evaporation process were characterized as structure of poly cores and high peptide concentration, which could provide different drug release durations from 1 month to 3 months depending on the type and molecular weight of PLGA (29,30). Besides formulation compositions, various fabrication techniques and newly emerging devices, processing parameters, such as evaporation rate of organic solvent, also play an important role in drug loading, morphology, drug distribution, initial burst, and extended release behavior of microspheres fabricated through the emulsification-solvent evaporation method (31).

The most key factor deciding the morphology and drug loading of bupivacaine microspheres was the type and molecular weight or intrinsic viscosity of PLA or PLGA used. Another important factor was the rate of organic solvent removal and polymer precipitation also known as solidification rate. The homopolymer of levo-rotamer lactide (PLLA) of high viscosity (IV = 2.0 dl/g) was found to be more effective in embedding high content of bupivacaine and rapid removal of the organic solvent performed by vacuum evaporation at elevated temperature (40 °C). The high intrinsic viscosity polymer would increase the solidification rate, which may hinder bupivacaine diffusion from the dispersion droplet in organic solvent phase outward to outer water phase, and then inhibit the concomitant crystallization

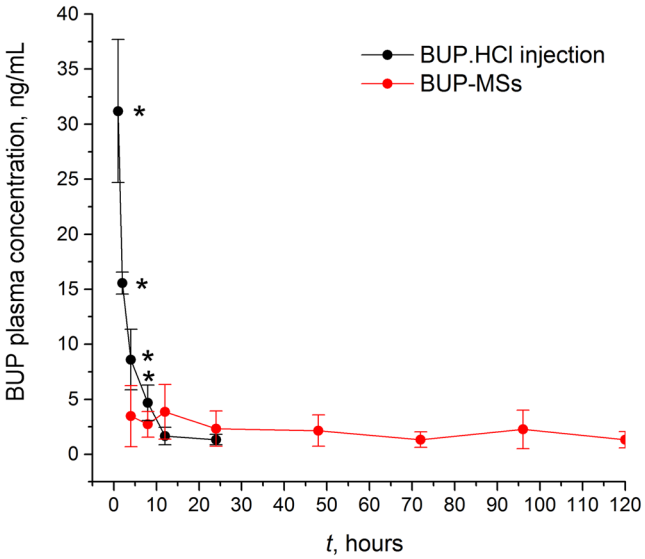

Fig. 6. Plasma BUP concentrations in rats received (-●-) bupivacaine hydrochloride injection (equivalent to 30 mg/kg BUP) and (-●-BUP-MSs (equivalent to 150 mg/kg BUP), respectively ($n = 6$). ($^*p < 0.05$ *vs* BUP-MSs)

of bupivacaine on the surface of microspheres. Another advantage of high molecular weight PLA lied in its higher glass transition temperature, thus less fuse tendency of the microspheres (5). We found that no evidence of aggregation phenomenon of the bupivacaine loaded in PLA microspheres was observed even after storage at −20°C for 9 months. Considering the high dosage and convenience in administration of bupivacaine, a high drug loading of microspheres at least 50% will be reasonable to inject for exerting extended local anesthetic effect lasting over 3~5 days (27). In order to gain high drug loading of microspheres, the feed ratio of drug to polymer in the formulation was normally higher than 7/3 (w/w) in this study.

During the formation of microspheres, PLA firstly tended to precipitate out with the rapid evaporation of DCM, and then a solidified polymer shell layer came into being at the beginning of solidification stage at the interface between the dispersion droplets and the outer aqueous phase, which may inhibit the further leakage of drug and thus lead to higher amount of drug retention inside the microspheres. In the core of the droplets, the precipitated bupivacaine crystals and amorphous PLA adhered together to form the inner matrix that was filled with pores left after complete removal of the residual DCM (Fig. 5b). The special core-shell structured microspheres were totally different to the co-precipitate and homogeneous structured microspheres (26).

Table II. Other Parameters of the Plasma Bupivacaine Level

Parameters	BUP HCL injection	BUP-MSs
Compartment model	One	Two
$t_{1/2}$, h	3.18	114.41
T_{max}, h	1.00	12.00
C_{max}, ng/mL	31.19	3.866
AUC	114.08	474.40

Core-shell structured microspheres can be fabricated with various methods, *e.g.*, Xu *et al.* obtained the microspheres using slow biodegradation matrix and low diffusivity polymer shell that provided an alternative way to optimize drug release pattern (23).

Due to the high drug feed ratio of drug to polymer in most of our test formulations, it was certain that the water insoluble bupivacaine base precipitated and presented in the crystal state inside the solidified microspheres by the polarized microscope, DSC and XRPD analysis. Bupivacaine base has two kinds of crystal forms with melting points of Tm = 114 °C (Form I) and Tm = 102 °C (Form II), respectively (32,33). Bupivacaine was in a metastable crystal state inside the microspheres prepared by emulsification-solvent evaporation method in our test. The DSC results could showed the melting point of bupivacaine free base loaded in microspheres may be closely related to the feed ratio of drug to polymer. In the same time, we found that a weak endothermic peak at about 126 °C that may be attributed to the melting point of PLA plasticized by bupivacaine (the melting point of pure PLA is about 142 °C) in the DSC curves of bupivacaine microspheres (34).

As is well-known, the release patterns of PLGA microspheres generally follow the polymer degradation-controlled mechanism or the polymer degradation–drug diffusion-controlled mechanism (35). In the case of the special microsphere structure of porous inner matrix and dense shell layer, the possible dissolution–diffusion-controlled drug release mechanism of the BUP-MSs was postulated. During drug release process, release media firstly diffused into the porous inner matrix and aqueous micro-compartments began to form, and then bupivacaine crystals gradually dissolved and maintained saturation over an extended period of time, and finally the drug molecules diffused out across the PLA shell layer driven by the concentration gradient (Fig. 5b). Due to the relatively slow degradation rate of PLA, the PLA shell layer kept integrate without occurring obvious crevices, which functioned as an effective diffusion barrier during the *in vitro* release period. The duration of drug release process depended on the amount of drug loaded and the diffusivity of the polymer shell, while higher feed ratio of drug to polymer may result to decreased glass transition temperature and intactness of the PLA shell, resulting in higher diffusivity of the release media and drug molecule (36). If this mechanism was reasonable, a zero-ordered release profile would be expected for a single microsphere or monodispersed microspheres before all the bupivacaine crystals disappeared. As to the heterodispersed microspheres with a wide size distribution, the combined release of each single microsphere would certainly result to a non-linear drug release kinetics.

Bupivacaine belonged to local anesthetic drug and is applied to postoperative analgesia widely. Therefore, analgesic experiment was chosen to investigate the pharmacodynamic characteristics of bupivacaine microspheres. Sciatic nerve blockade was usually used to relieve the pain in the lower extremity. Sciatic nerve blockade test in rats with sensory blockade and motor blockade as the indexes was used to perform the pharmacodynamic evaluation of BUP-MSs including analgesic effect and action time. We obtained the optimal bupivacaine microsphere preparation (Formulation No.10) through formulation screening and drug release study *in vitro*.

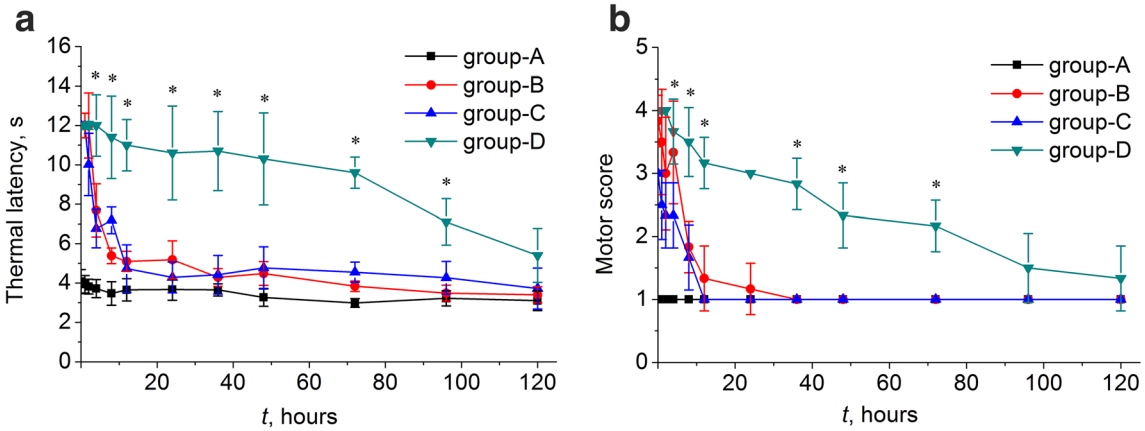

Fig. 7. Sciatic nerve block of BUP-MSs in rats ($n = 6$). **a** Sensory blockade. **b** Motor blockade. (*$p < 0.05$ *vs* group A)

Literature suggested that rat sensory blockade time was longest when the injection administration of BUP-MSs to rat was up to 150 mg/kg equivalent to bupivacaine (37). In this study, BUP-MSs administration group could be divided into high dose of BUP-MSs (150 mg/kg) and low dose of BUP-MSs (50 mg/kg) to investigate the analgesic effect of BUP-MSs. And the control group included blank control and positive control, injected with saline and bupivacaine hydrochloride, respectively. The administration dose of high group (150 mg/kg equivalent to bupivacaine) was five times as the positive control group (30 mg/kg equivalent to bupivacaine) because of the bupivacaine microspheres prolonged release for 5 days. Therefore, pharmacodynamic test rats would be divided into four groups: group A (blank control), group B (positive control), group C (low dose of BUP-MSs), and group D (high dose of BUP-MSs). As regards pharmacokinetics study, BUP-MSs administration group only needed to detect the plasma bupivacaine concentration level of rats injected with the high dose of BUP-MSs (150 mg/kg equivalent to bupivacaine), and the control group was the positive control administrated with bupivacaine hydrochloride (30 mg/kg equivalent to bupivacaine). So the test rats were divided into two groups: group A (control group) and group B (test group).

For general PLGA microspheres, the drug release *in vivo* was generally faster than *in vitro* due to the accelerated polymer degradation caused by enzyme or other physiological conditions (38). However, the phenomenon of lagging drug release *in vivo* test compared to release *in vitro*, which was not usually observed in other literatures. A possible explanation was that the bupivacaine microspheres were not obviously biodegraded within the *in vivo* test period, and the drug release was partly diffusion controlled by the intact microsphere shell (Fig. 5a(c)). As the limited amount of tissue fluid in the local region of sciatic nerve and the low diffusion of drug in the peripheral tissue adjacent to the injected microspheres, the drug release *in vivo* may be obviously slower than that *in vitro* (39). After the ending of the sciatic nerve blockade test, rats were sacrificed and the BUP-MSs were recovered from the injection site, and the polarized microscopic images of the BUP-MSs showed obvious crystals inside microspheres even after administration to rats for 5 days. The residue amount of drug inside the microspheres was determined to be still about 10~15% after 5 days *in vivo* compared to complete release in PBS pH 7.4 *in vitro*. Such results also reminded the necessity and importance of tailoring more accurate rate of drug release and establishing more reliable correlation of *in vitro*/*in vivo*.

The microspheres loading high content of bupivacaine were designed for injectable prolonged release in this study. *In vitro* release, 80% of the drug released from the microspheres exhibited a sustained drug release behavior in 5 days. However, the bupivacaine microspheres were used to postoperative analgesia, which meant investigating local analgesic effect of in rats test. The sciatic nerve blockade test with sensory blockade and motor blockade as the indexes was used to study the local analgesic effect of BUP-MSs. To avoid the cardiovascular side effect of bupivacaine, the plasma bupivacaine concentration must be monitored during the administration of BUP-MSs to rats. Hence, there was a great connection between *in vitro* and *in vivo* release.

For local anesthetic, repeated administration of bupivacaine is required due to its short half-life and action duration, which may lead to cardiovascular and central nervous system toxicity related to high plasma drug concentration (40). The BUP-MSs were expected with low plasma bupivacaine concentration, especially when high dose of BUP-MSs in our study was administrated for purpose of the extended action duration, which could significantly reduce the toxicity of bupivacaine. The plasma concentration of the low and high group at all the time points were far below the threshold for central nervous system toxicity in human or in rat (41).

In this study, through utilizing the low solubility and quick precipitation of high molecular weight PLA at the oil/water interface, we obtained porous core-shell microspheres of high drug loading by the commonly used an o/w emulsion-solvent evaporation method. Ultimately, the optimized microsphere formulation was composed of the poly cores and dense shell serving as the main barrier to retard drug release and minimize initial burst release *in vitro* and *in vivo* of rats.

CONCLUSION

Biodegradable PLA microspheres loading bupivacaine free base were fabricated with the well-known emulsification-solvent evaporation method. Novel heterogeneous structured microspheres with porous cores composed of bupivacaine

form II crystals and dense polymer shell were obtained by using the high molecular weight PLLA as the matrix material and adjusting the preparation parameters including high feed ratio of drug to polymer and fast solvent evaporation under vacuum. The microspheres were characterized by high bupivacaine loading of more than 70% and extended drug release over several days. The formation of the robust PLA shell was postulated to play an important role in retaining drug inside the microspheres and maintaining their sustained release behavior both *in vitro* and *in vivo*. *In vivo* tests indicated plasma bupivacaine concentration was steady and low over 5 days, and the phenomenon of prolonged sensory and motor blockade duration of sciatic nerve was discovered after injection of bupivacaine microsphere formulation. No obvious side effects or other evidences of systemic anesthetic happened during *in vivo* tests.

Our present results indicated a great potential of the PLA microsphere formulation for prolonged delivery of bupivacaine and extended duration of locally regional anesthesia or analgesia. The understanding to the formation of microspheres and the extended drug release mechanism also provided a feasible strategy for injectable and prolonged delivery of other water insoluble actives especially when high drug loading was required.

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