

Original Article

Preparation and characterization of lung-targeting oxymatrine-PLGA microspheres

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Abstract: Oxymatrine (OM), an alkaloid extracted from the Chinese herbs, possesses a variety of pharmacological activities. It has been identified to have the potential to improve lung injury and lung fibrosis. To avoid the fast clearance of OM, we encapsulated OM into poly (DL-lactide-co-glycolide) (PLGA) microspheres (OM-PLGA-MS) and investigated their properties in this study. The microspheres were obtained by using a solvent diffusion method, the mean diameter was $9.55 \pm 0.86 \mu\text{m}$. The optimal formulation was 1:1 of OM:PLGA and 200 ml of 0.5% gelatin according to the result of an orthogonal design. In an *in vitro* study of drug release, the cumulative release percent of OM from OM-PLGA-MS reached $89.13 \pm 1.44\%$ at 36 h, while the cumulative release rate of OM from OM solution approximately reached 92% within 1 h. Both *in vivo* fluorescence imaging observation and OM concentration study suggested that the microspheres delivered OM primarily to lung tissue following administration to mice intravenously. These results suggested that OM-PLGA-MS prepared in this study has well-sustained release and lung-targeted efficacy. It is of potential value in treating lung diseases in animals.

Keywords: Lung-targeted, microspheres, oxymatrine, PLGA, sustained-release

Introduction

Oxymatrine (OM), an alkaloid extracted from the Chinese herbs such as *Sophora flavescens* Ait., *Sophora subprostrata* (shandougen) and *Sophora alopecuroides*, is widely used in China [1]. The chemical structure of OM is shown in **Figure 1**. Basic and clinical research has shown that OM can inhibit inflammation and improve hepatic fibrosis [1, 2]. Recently it was demonstrated that OM possesses the protective effect on acute lung injury and could improve the development of bleomycin-induced lung fibrosis in mice [1, 3]. However, the pharmacokinetics studies indicated there are some main disadvantages with this agent. It has a short half-life, with a $T_{1/2\beta}$ of 63 min in rat and 130 min in human being, respectively [4, 5] and distributes all over the body, predominant in kidney after systemic injection [6]. Moreover, it has inhibitory activities on central nervous systems. OM could induce sedation and hypnotic action in mice after intraperitoneal injection at the dose of 50-200 mg/kg [7, 8]. Owing to

these limitations, lung fibrosis patients have to receive large doses of OM frequently in order to maintain a therapeutic drug concentration. Therefore, a targeted and sustained release drug delivery system is desirable, in order to reduce the dosing frequency, avoid the unwanted off-target effects and improve patient compliance.

Microspheres are currently aroused great attention as effective drug delivery systems to the lungs in recent years. The therapeutic drug entrapped in microparticles with the help of the drug delivery carriers administered intravenously will be distributed to various organs depending on particle size. Particles with mean diameter 5-20 μm are trapped in lung tissues when given intravenously. Furthermore, the carriers can also prolong the time of action to improve therapeutic effect [9].

In the present work, we encapsulated OM in microspheres using the carrier poly (lactic-co-glycolic acid) (PLGA), a biodegradable polymer.

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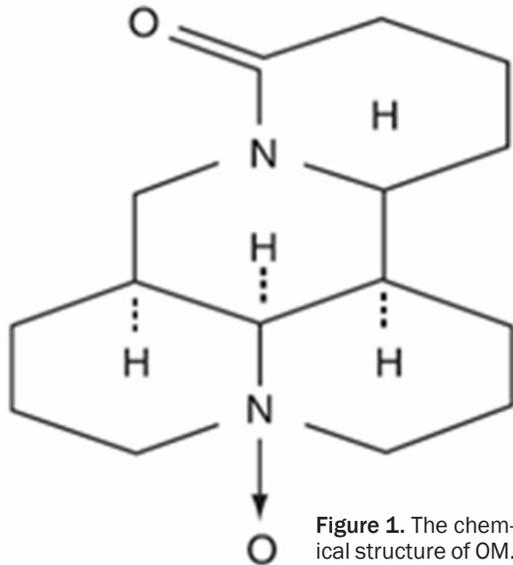


Table 1. Effect of the stirring rate, trichloromethane volume and PLGA content on the percent of microspheres with 10 μm diameter

Test	A	B	C	10 μm MS (%)
1	1500	15	1	55
2	1500	25	2	50
3	1500	35	3	48
4	1700	15	3	68
5	1700	25	1	75
6	1700	35	2	72
7	2000	15	2	55
8	2000	25	3	65
9	2000	35	1	58
K_A	51.000	59.333	62.667	
K_B	71.667	63.333	59.000	
K_C	59.333	59.333	60.333	
R	20.667	4.000	3.667	

A = stirring speed (rpm/min); B = trichloromethane volume (ml); C = PLGA content (g). MS = microspheres.

Our study showed that the OM-loaded PLGA microspheres (OM-PLGA-MS) demonstrated sustained release efficacy *in vitro*. And it was found that the microspheres accumulated mainly in the lungs after intravenous injection.

Materials and methods

Materials

The following materials were used: OM (purity > 98% by high-performance liquid chromatogra-

phy, Shanxi Hui ke Botanical Development Co. Ltd, Xian, China), PLGA (75:25, Mw = 20,000, Jinan Daigang Biomaterial Co. Ltd, Jinan, China). Gelatin (Mw = 50000-60000, Shandong zibo Ochang Gelatin Market Co. Ltd., Zibo, China). Cy7 monofunctional N-hydroxysuccinimide ester (Cy7-NHS, Beijing Fanbo Biochemicals Co., Ltd, Beijing, China). All other reagents were of analytical grade.

Microsphere preparation

OM-PLGA-MS were prepared by the solvent diffusion method as previously described by Hu et al [10] with slight modifications. Briefly, according to the formulas listed in **Table 1**, 1 g of OM and 1g PLGA were dissolved in 25 mL dichloromethane with ultrasonic for 0.5h using a sonicator (Kunshan KQ-50B, China) at 50 W. Then the mixture was poured into 200 mL 0.5% (w/v) gelatin solution and homogenized at a rate of 1700 rpm for 10 min, followed by stirring at 900 rpm/min for 2-3 hours. The resultant dispersion was centrifuged at 4,000 rpm, 15 min for separation of the microspheres. The obtained sediments were washed three times with purified water, and collected by filtration, then lyophilized for 30 min at -80°C , finally subjected to freeze drying for 48 hours. PLGA microspheres (PLGA-MS) without the addition of OM were also prepared as a control using the same procedure.

To study the tissue distribution of the microspheres, Cy7-NHS contained OM-PLGA-MS were prepared with the above methods except that 1:1 of OM:PLGA was replaced with 1:1:1 of CY7-NHS:OM:PLGA.

The concentration of gelatin, the volume of gelatin solution and the ratio of OM to PLGA were investigated for their effects on OM loading. Orthogonal design methods for selecting the optimal formula and process parameters were applied.

Microsphere morphology and size distribution assay

The microspheres were observed by scanning electron microscopy (SEM, Tokyo, Japan) at 15 kV, after gold-sputtering the microspheres. Particle size distribution and measurement were carried out using optical microscopy [10].

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OM loading efficacy

The OM loading, was determined as previously described [4]. Briefly, OM was extracted from the microspheres with acetonitrile, then the extract was filtered through a 0.45 µm syringe filter (Millipore), and the concentration of OM was determined by high performance liquid chromatography (HPLC, Shimadzu LC-20AD, Japan) (mobile phase = acetonitrile/alcohol/3% phosphoric acid solution (85/10/5, v/v/v), flow rate = 1 mL/min). The detective wave is 220 nm. OM loading was expressed as mg drug/100 mg of OM-PLGA-MS (% , w/w).

The stability of OM-PLGA-MS

The microsphere powders were kept at 3~5°C for 6 months and periodically tested for the microsphere stability.

In vitro drug release

One milliliter of OM-loaded PLGA microparticles suspension in 0.85% NaCl solution was placed into a dialysis tube (MWCO 3500), and the end sealed dialysis tube was immersed fully in 50 mL of 0.85% NaCl solution. The OM-PLGA-MS dispersion was incubated at 37±1°C under magnetic stirring at 50 rpm. All of the release media were removed and replaced with fresh media, and the OM concentration was determined by HPLC [11]. All release experiments were performed as triplicates. The results of all measurements were used to calculate cumulative drug release. The release behavior of OM from microspheres were analyzed with Higuchi model [12].

Tissue distribution of microspheres

Tissue concentration of OM: Pathogen-free BALB/c mice of either sex, weighing between 20 and 22 g were obtained from the Laboratory Animal Center of Third Military Medical University (Chongqing, China). All animals were divided into two groups. The control group was treated with OM solution (dissolved in 0.85% NaCl solution), and the other group was administered with OM-PLGA-MS suspension (dispersed in 0.85% NaCl solution). Each formulation was given intravenously at a dose of 20 mg/kg OM. Mice were sacrificed at 30 min, 2 h, 24 h and 72 h after administration. When the mice were sacrificed, the tissues of interest (heart, liver, spleen, lung and kidney) were col-

lected after being washed using 0.85% NaCl solution and dried with tissue paper to remove excess fluid. The above described animal experiments were approved by the Animal Ethics Committee of Third Military Medical University.

The isolated tissues were weighed accurately. Added acetonitrile (weight: volume = 1 g:9 ml for heart, spleen and lung tissues; 1 g:3 ml for kidney tissue; 1 g:2 ml for liver tissue), which included 200 ng/ml finasteride (internal standard, IS), and then homogenized. Following centrifugation of the homogenate at 12000 rpm/min, 100 µl supernatant was collected. Mixed with 400 µl distilled water and then OM concentration was determined by Agilent 1290 UHPLC-Agilent 6460 mass spectrometry (LS-MS) [13].

The fragmentor voltage is 135 and the ionization mode is ESI for both OM and IS. But the collision energy was 35 for OM, and 20 for finasteride, respectively. The calibration curves used were as follows: $y = 0.003x + 0.007$, $r = 0.999$ (for heart tissue); $y = 0.003x + 0.013$, $r = 0.999$ (for liver tissue); $y = 0.003x + 0.014$, $r = 0.993$ (for spleen tissue); $y = 0.003x + 0.011$, $r = 0.993$ (for lung tissue); $y = 0.003x + 0.005$, $r = 0.997$ (for kidney tissue).

The blood samples were handled with the same method. In brief, 100 µl plasma mixed with 200 µl acetonitrile including 200 ng/ml finasteride, followed by centrifugation and then assayed with LS-MS. The calibration curve for plasma sample is $y = 0.002x + 0.003$, $r = 0.999$.

Organ distribution imaging of microparticles:

To determine the organ distribution of our microspheres, a fluorescent agent Cy7-NHS was used to mark the in situ microsphere deposition [14]. BALB/c mice weighing between 20 and 22 g were administered with Cy7-NHS-loaded, OM-PLGA-MS microparticles intravenously. Then the animals were sacrificed at 0.5, 2, 24, 72 h post-injection of the particle. The organs of interest (heart, liver, spleen, lung and kidney) were collected. The fluorescence was monitored and imaged (Cy7: λ_{ex} , 747 nm; λ_{em} , 774 nm) using In-Vivo Imaging system FX Pro (Kodak company, USA).

Statistical analysis

All data were presented as the mean ± standard deviation. Comparisons among three or

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Table 2. Effects of different formulas on drug loading capacity

Formulas	A	B	C	Drug loading capacity (%)
MS1	0.5	150	0.5:1.0	3.77
MS 2	0.5	200	1.0:1.0	4.08
MS 3	0.5	250	1.0:1.5	3.41
MS 4	1.0	150	1.0:1.5	3.70
MS 5	1.0	200	0.5:1.0	3.76
MS 6	1.0	250	1.0:1.0	3.39
MS 7	1.5	150	1.0:1.0	3.44
MS 8	1.5	200	1.0:1.5	3.51
MS 9	1.5	250	0.5:1.0	3.89
K_A	3.753	3.637	3.557	
K_B	3.617	3.783	3.890	
K_C	3.613	3.563	3.537	
R	0.140	0.220	0.353	

A = Gelatin concentration (w/V); B = Gelatin volume (ml); C = OM:PLGA Ratio (g/g).

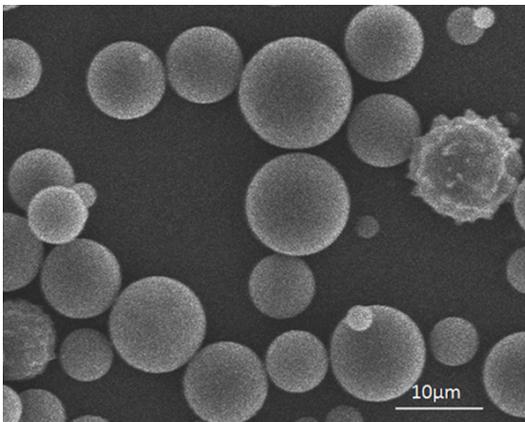


Figure 2. Morphology of OM-loaded microspheres observed by scanning electron microscopy. The scale bar represents 10 μm .

more groups were performed by analysis of variance (ANOVA) followed by post hoc test. Student's *t*-test was performed for comparison between two groups. All analyses of data were performed using SPSS version 12.0 software package (Chicago, IL, USA). Differences were considered to be statistically significant at $P < 0.05$.

Results and discussion

The optimal formulation

It is demonstrated that the *in vivo* deposition of microspheres depends largely on the particle

size. Following intravenous injection, microspheres of 7 μm or more in diameter are rapidly entrapped in the lungs by the simple process of mechanical entrapment, thereby achieving passive lung targeting [15]. We first used an orthogonal design to optimize the stirring speed (A), the trichloromethane volume (B) and PLGA content (C) by assaying and calculating the percent of microspheres of 10 μm diameter. As shown in **Table 1**, the statistical range was $R_A > R_B > R_C$, therefore, we can assume that the differences of 10 μm microspheres ratio mainly depend on the stirring speed, then the concentrations of trichloromethane and finally the PLGA concentration. According to **Table 1**, the initial optimal experimental condition should be 1700 rpm of stirring speed, 25 ml trichloromethane and 1 g PLGA.

The statistic range analysis showed that $R_C > R_B > R_A$ (**Table 2**). So the changes of the drug loading capacity mainly depend on OM:PLGA Ratio. The results shown in **Table 2** indicated that the optimal formulation was 1:1 of OM:PLGA and 200 ml of 0.5% gelatin. Microspheres prepared using the optimal experimental conditions were spherical with an average drug loading of $3.59 \pm 0.33\%$.

Analysis of appearance, size distribution and stability of OM-PLGA-MS

OM-loaded PLGA microspheres SEM photograph was shown in **Figure 2**. These microspheres possess a spherical shape, smooth surfaces. During storage at 3~5°C for 6 months, the surface morphology showed no notable changes. Moreover, the particle size was $9.55 \pm 0.86 \mu\text{m}$, and 83% of the microspheres were within the size range of 5~20 μm , which implied that the microspheres in this study meet the requirement of the lung targeting particles.

In vitro release characteristics of OM-PLGA-MS

In vitro release profiles of OM in microspheres (OM-MS) and OM solution were monitored and shown in **Figure 3**. Comparing two release profiles, the great difference was obvious between them.

OM release from microspheres was biphasic (**Figure 3**), consisting of a burst phase (up to 0.5 h), sustained release (0.5~36 hours). The burst release within the first 0.5 hour was

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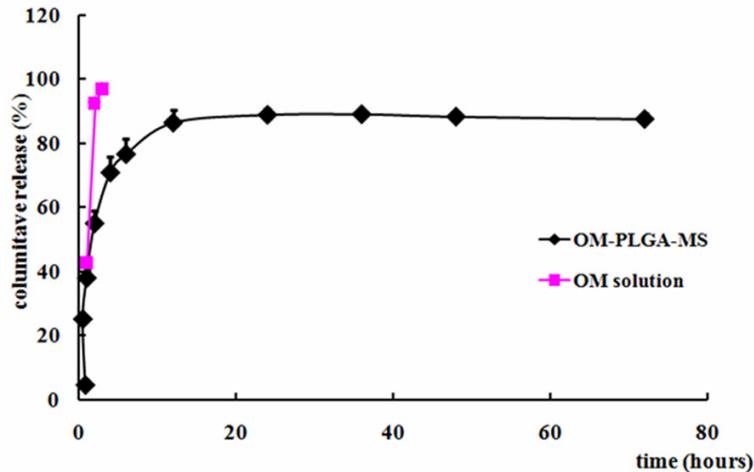


Figure 3. *In vitro* cumulative release of OM from the OM-PLGA-MS or the OM solution. Data as mean \pm S.D., n = 3.

25.14 \pm 1.42%, which may reflect the significant amount of OM adsorbed on or incorporated near the surface of the microspheres. In clinical practice, it will bring about fast effect to the patients [16]. During this second period OM was released from microsphere in a steady and gradual way. At 36 h, the cumulative release amount of OM reached 89.13 \pm 1.44% of total loadings.

In contrast, the release of OM from the OM solution was very fast. Only 1 h, the cumulative release rate approximately reached 92% (Figure 3). The data obtained from *in vitro* release studies fitting to the Higuchi model showed a good correlation (Figure 3, $r = 0.9966$) [12].

According to *in vitro* release results, OM-PLGA-MS prepared in our study had a well-controlled release efficacy.

OM concentrations in tissues

OM is widely used in China in clinical practice. However, the pharmacokinetics studies indicated this agent has a short half-life [6]. To overcome these shortcomings, avoid the unwanted off-target effects and improve patient compliance, targeting OM to lung tissues was an optimal approach. Based on *in vitro* observations, the size of the microspheres we prepared met the requirement for lung targeting. To test the distribution of OM from the microspheres *in vivo*, the drug concentrations in various tissues were assayed following intravenous administration of the OM-PLGA-MS or OM solution to mice. Wang has reported

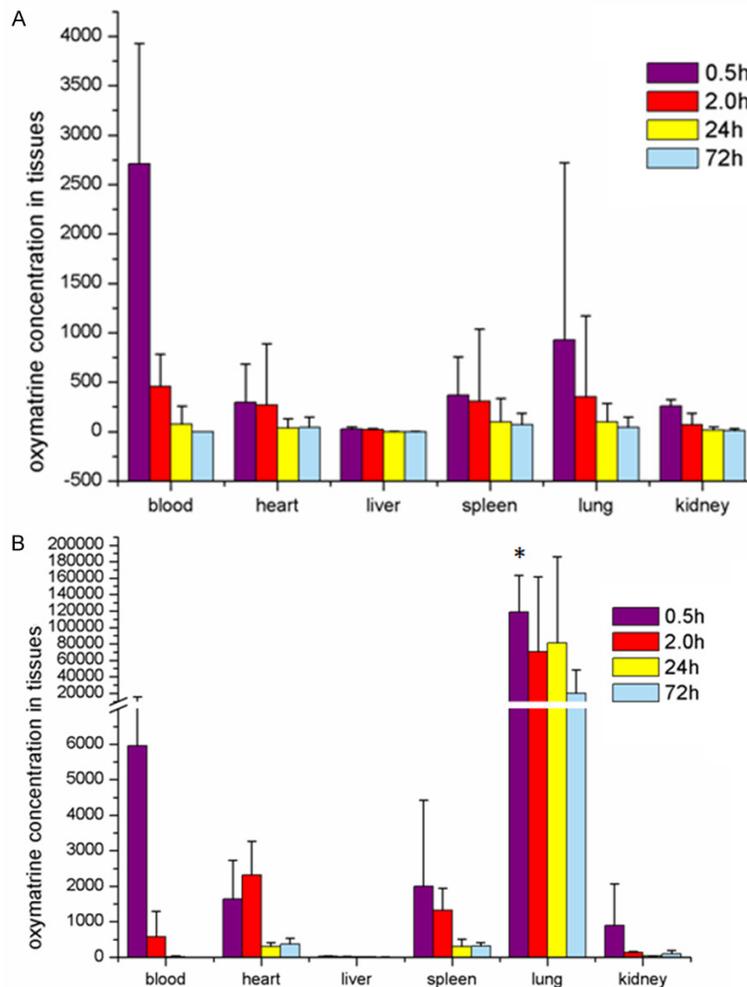


Figure 4. Concentration of OM in tissues (ng/g) or blood (ng/mL) at different time following intravenous injection to mice. A. OM solution; B. OM-PLGA-MS. * $P < 0.01$ vs OM concentrations in other tissues at 0.5 h. n = 4.

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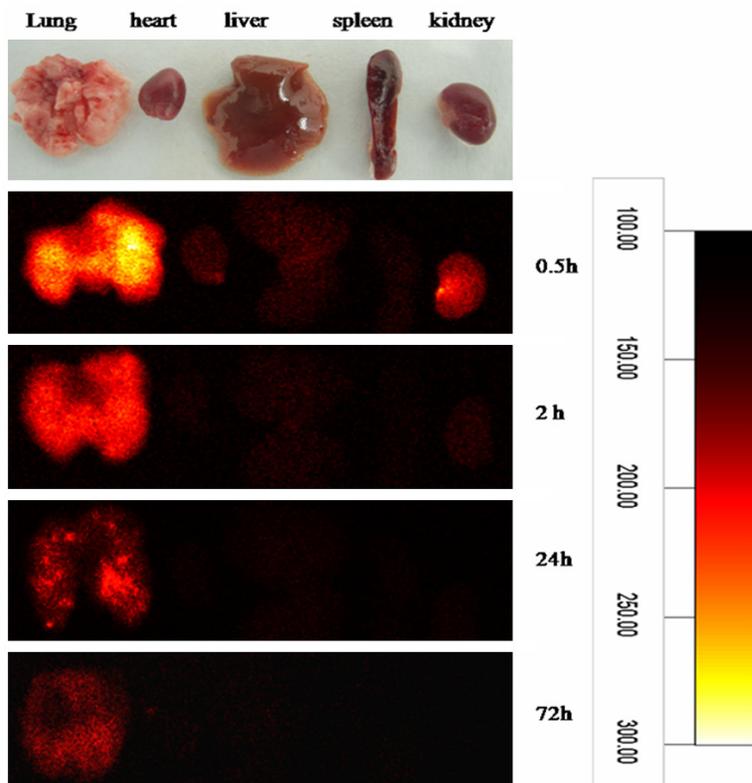


Figure 5. NIR fluorescence real-time images of OM-PLGA-MS distribution in lung, heart, liver, spleen and kidney tissues at different time after intravenous injection of OM-PLGA-MS loaded with cy7-NHS.

that OM dissolved in saline solution predominately distributes in kidney tissues after injection [6]. Unlike his results, our studies indicated that the concentrations of OM were a litter higher in both the lung and kidney tissues than those in other tissues (**Figure 4A**, $P > 0.05$). In contrast, the OM-PLGA-MS delivered OM primarily to the lung after intravenous injection, and the concentration of OM in the lung was as about 20-fold as in blood and 70-fold higher than that of other organs at 0.5 h, significantly higher than that in other tissues ($P < 0.01$, **Figure 4B**).

OM-PLGA-MS distribution imaging of mice tissues

To get further evidence about OM-PLGA-MS distribution in mice tissues, near-infrared (NIR) fluorescence imaging system was used. The fluorescence signals in deep organs were weakened because of optical impedance by soft tissues [17]. So little fluorescence in the heart, liver, spleen, lung and kidney were observed

from the outside. And then we observed the fluorescence in excised organs post-administration at the indicated time points. NIR results suggested that that the microspheres showed highest retention in lung among the 5 selected organs from 0.5 to 72 h i.v., which indicates that the microparticles were predominately retained in lungs. Additionally, the peak fluorescent intensity was observed at 0.5 h, and decreased gradually from 2 h to 72 h, which was consistence with the above results about the drug concentration studies (**Figure 5**).

Based on the observations, we successfully prepared OM-PLGA-MS by the solvent diffusion method, which showed both obvious lung-targeted and sustained drug release characteristics.

Due to short half-life and rapid clearance *in vivo*, the effective concentration of OM in the lung tissues could not maintain for a long period. So a large dose of OM was needed to produce a relatively satisfactory result. That may result in the unwanted off-target effects and poor compliance of patients and then reduce the efficacy.

To improve patients compliance many kinds of OM sustained-release drug delivery system have been developed, such as the sustained-release tablets, capsules, liposomes and so on [18-20]. However, there has no studies related with the preparation of lung-targeting and long-lasting OM loading system.

There have been investigated various strategies like polymeric nanoparticles, microspheres, liposomes, and solid lipid nanoparticles to deliver therapeutic agents to lungs in a sustained manner. Among them, passive lung-targeted drug delivery systems via intravenous administration had been widely used for decades. There have been reported microsphere carriers used for targeting drug delivery

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to the lung for the treatment of lung tuberculosis, tumors, and pneumonia through intravenous administration in recent years [21-23]. In the present study, we designed and prepared the OM loaded microspheres. After giving the particles to mice, no obvious changes about diet or drinking were observed in mice following injection. And no somnolence or lassitude was found in the OM-PLGA-MS treated mice. It implied that OM-PLGA-MS may avoid the off-targets side effects of OM and it was valuable to investigate the effects of the particles on lung fibrosis in animal models in further study.

Conclusions

In this study, OM-loaded PLGA microspheres were prepared by using the solvent diffusion method combined with an orthogonal design. The release study showed a sustained release characteristic of the microspheres for about 36 h *in vitro*. Further *in vivo* experiments evidenced that the microspheres has satisfactory lung-targeted property. Based on our experimental results, we conclude that OM-loaded PLGA microspheres may be of potential value in treating lung injury and lung fibrosis in animals.

Acknowledgements

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Disclosure of conflict of interest

None.

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