

# RETRACTED ARTICLE: A novel tetrandrine-loaded chitosan microsphere: characterization and in vivo evaluation

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**Abstract:** In this study, novel tetrandrine-loaded chitosan microspheres were prepared by the emulsion cross-linking method. The systems were then characterized for physicochemical properties and in vitro drug release. In addition, the pharmacokinetics and tissue distribution of microspheres were further verified in animal models. Particle-size distribution indicated that the size of microspheres was within the range of 5–15  $\mu\text{m}$ , with a median diameter of 12.4  $\mu\text{m}$ . The drug loading and entrapment efficiency of the formulation were  $34.6\% \pm 12.5\%$  and  $87.3\% \pm 9.7\%$  (mean  $\pm$  SD), respectively. In vitro release showed a typical sustained and long-term drug release behavior. The Higuchi equation was the model that fit best with release data. Maintaining a relatively constant plasma concentration in the long-term drug treatment is an outstanding pharmacokinetic advantage of tetrandrine microspheres in vivo. Moreover, compared with tetrandrine solution, tetrandrine microspheres produced a lower drug concentration in the heart, liver, and kidneys. This indicated that the microspheres used in this study were preferable for targeting lung tissue versus other tissues. No damage to the tissues of the lung was found in histopathological examination.

**Keywords:** tetrandrine, chitosan microspheres, emulsion cross-linking, pharmacokinetics, tissue distribution

## Introduction

Tetrandrine (TED, CAS:518-34-3), the major active constituent of the Chinese herb *Stephania tetrandra* S. Moore, is a bisbenzylisoquinoline alkaloid. Clinically, TED has been found to be effective for the treatment of inflammation,<sup>1,2</sup> pneumosilicosis,<sup>3</sup> and antitumor activity.<sup>4–6</sup> Numerous studies have reported that TED also acts as a nonselective calcium channel blocker<sup>7,8</sup> and calmodulin antagonist.<sup>9</sup> In addition, TED has exhibited excellent pharmacological effects, especially in treating pulmonary hypertension.<sup>10–13</sup> Although it has potentially valuable clinical applications, some problems such as poor solubility contribute to its low and variable oral bioavailability<sup>14</sup> and have greatly inhibited its development.

In recent years, many pharmaceutical methods have been investigated to improve the bioavailability of TED, such as lipid nanocapsules,<sup>3</sup> nanoparticles,<sup>14</sup> ethosomes,<sup>15</sup> and microspheres.<sup>16</sup> Microsphere technology has been widely used in the preparation of sustained formulations in order to maintain targeted concentration in vivo for a sustained period of time.<sup>17</sup> This drug delivery system has emerged as a remedial measure to improve site-specific drug delivery to a considerable extent, since it is nontoxic, well tolerated, and has been applied to improve therapeutic response.<sup>13</sup> Drugs in implant microspheres are absorbed by the injection site and the capillaries of lymph organs, and then enter the systemic circulation to be distributed to the target

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organ to take effect,<sup>14</sup> which can bypass the first pass effect and avoid pre-systemic elimination in the gastrointestinal tract or liver by oral administration.

Chitosan is a kind of polymer with good biocompatibility and the ability to open the intracellular tight junction.<sup>18,19</sup> It has been suggested as a suitable polymeric material for controlling drug release in the form of fibers, membranes, microspheres, and capsules.<sup>20</sup> Chitosan has the most attractive properties with its biodegradability and good biocompatibility and has been widely used in the field of wound healing<sup>21</sup> and drug delivery, tissue engineering and biomedical fields. It is especially used for developing nano/microspheres as a carrier system.

The aim of this study was to prepare TED–chitosan microspheres by the emulsion cross-linking method with glutaraldehyde as the cross-linking agent. The systems were characterized for physicochemical properties and *in vitro* drug release. In addition, the pharmacokinetics and tissue distribution of TED microspheres were further verified in animal models.

## Materials and methods

### Chemicals and reagents

TED with a purity of 93% was purchased from Hao-xuan Biotechnology Co. Ltd (Xian, People's Republic of China). Chitosan with a deacetylation degree of 90% was obtained from Zhejiang Jingke Biopharm Co. Ltd (Zhejiang, People's Republic of China). Glutaraldehyde, Span 80 and dichloromethane were purchased from Sinopharm, Shanghai, People's Republic of China. All of the reagents were of high-performance liquid chromatography (HPLC) grade (Sigma-Aldrich Co., St Louis, MO, USA), containing acetonitrile and methanol. Other reagents were of analytical grade. Experiments were carried out using purified water from the Milli-Q system (microporous; Millipore Corporation, Billerica, MA, USA).

### Microsphere preparation

TED-loaded chitosan microspheres were prepared by the emulsion cross-linking method.<sup>22,23</sup> In short, TED (20 mg) and chitosan (115 mg) were added to 5 mL of dichloromethane. After complete dissolution, the solution was slowly added to the solution of 1% Span 80, and then the mixed solution was emulsified with a propeller agitator at 50× *g* for 15 minutes.

Then, 25% glutaraldehyde solution was slowly added to the emulsion system and cross-linked for 2 hours until the microspheres were coagulated. Microspheres were filtered through a 20 μm sieve and then washed with deionized water three times and dried in a vacuum dryer for 48 hours.

### Particle-size analysis

Particle-size distribution of TED-loaded chitosan microspheres was measured by the laser diffraction method. Microspheres were then dispersed in 100 mL of deionized water with a laser particle-size analyzer (AimSizer 2011; AimSizer Scientific, Shen yang, People's Republic of China). The zeta potential of the microspheres was measured using a Zetasizer Nano ZS analyzer (Malvern Instruments, Malvern, UK).

### Scanning electron microscopy

Samples were dispersed in a double-sided adhesive tape fixed on aluminum–carbon tape stub and then sputtered onto a gold film to make them conducting. Scanning electron microscopy images were taken using the JSM-6010 electron microscope (Tokyo, Japan), and the acceleration voltage of the primary electron was 15 kV. Images captured from the collection of secondary electrons were obtained at a working distance of 11 mm.

### Drug loading and entrapment efficiency

TED-loaded chitosan microspheres (10 mg) were added into 100 mL anhydrous alcohol and heated with reflux condensation at 60°C for 1 hour under magnetic stirring (15× *g*).<sup>24</sup> After cooling down to room temperature and centrifugation, the amount of TED released in the solution was analyzed by means of HPLC analysis. Drug loading (DL%) and entrapment efficiency (EE%) of drug-loaded microspheres were calculated according to equations (1) and (2):<sup>25</sup>

$$DL\% = \frac{W_M}{W_P + W_M} \times 100 \quad (1)$$

$$EE\% = \frac{W_M}{W_F} \times 100 \quad (2)$$

where  $W_P$ ,  $W_M$ , and  $W_F$  represent the weight of initial throwing in polymer, the weight of drug incorporated into microspheres, and the weight of initial throwing in drug, respectively.

### In vitro release

Properties of *in vitro* release of TED from microspheres were investigated in an aqueous release medium phosphate-buffered saline (PBS, pH 7.4) by a dialysis method. Briefly, TED-loaded chitosan microspheres (20 mg) were transferred to a dialysis bag with a molecular weight cutoff between 8,000 and 14,000 kDa. The dialysis bags were soaked in deionized water for 12 hours before use. Each bag was introduced into

an Erlenmeyer flask filled with 100 mL dialysis medium and shaken at  $5\times g$  at  $37^{\circ}\text{C}$ . At fixed time intervals (0.5, 1, 2, 3, 4, 6, 8, 10, 12, 14, 16, and 24 hours), 2 mL of the sample was taken and replaced by 2 mL of fresh medium. The filtrate was analyzed using the HPLC method, as described in HPLC analysis section.

## Pharmacokinetic study

Twelve Sprague–Dawley rats were used to study the effect of microsphere formulation on the pharmacokinetics of TED after intravenous administration. The animals were kept in a well-ventilated room. The temperature was maintained at  $23^{\circ}\text{C}\pm 2^{\circ}\text{C}$  and relative humidity of  $50\%\pm 10\%$ . Throughout the study, the animals had free access to water. This study was approved by our institutional animal care and use committee at Zhongshan Hospital and performed in accordance with institutional guidelines.

The rats were randomly divided into two groups and given a single dose of 8 mg/kg of TED-loaded chitosan microspheres and TED injection by tail intravenous administration, respectively. Blood samples (0.5 mL) were collected into heparinized tubes from the orbital vein at 0.25, 0.5, 1, 1.5, 2, 4, 6, 8, 10, 12, and 24 hours after intravenous administration. The blood was immediately processed for plasma by centrifugation at  $4000\times g$  for 10 minutes. Plasma samples were immediately centrifuged at  $4000\times g$  for 10 minutes and frozen at  $-70^{\circ}\text{C}$  until analysis.

## Tissue distribution

A total of 48 Kunming mice (20–22 g) were equally divided into two groups in the study of tissue distribution. They were given TED-loaded chitosan microspheres and TED injection, respectively, at a dose of 15 mg/kg via the tail vein. At each time point, the mice were sacrificed at 0.5, 4, 10, and 24 hours, and tissue samples were collected from the heart, liver, spleen, lung, and kidney. All of the tissue specimens were rinsed with sterile physiological saline and immediately stored at  $-70^{\circ}\text{C}$  for later analysis. After the experiment, the lungs were pressed between filter pads, weighed, and then fixed in 10% neutral formalin using standard techniques and stained with hematoxylin and eosin for histopathological examination. All the tissue samples were examined and graded under light microscope with  $500\times$  magnification. Saline was used as the control.

## HPLC analysis

The HPLC system consisted of a mobile phase delivery pump (LC-10 AD; Shimadzu, Kyoto, Japan) and a ultraviolet–visible

(UV–vis) detector (SPD-10A; Shimadzu). HPLC separation was achieved using a Dikma C18 column ( $4.6\times 250$  mm,  $5\ \mu\text{m}$ ) maintained at  $30^{\circ}\text{C}$ . The wavelength of the ultraviolet detector was set at 210 nm. Water and ethanol (55:45, v/v) were used as the mobile phase at a flow rate of 1 mL/min.

Two hundred microliters of the plasma sample were transferred to a 10 mL plastic test tube together with 10  $\mu\text{L}$  of internal standard solution (0.1 mg/mL tetrahydropalmatine). After vortex shook for 30 seconds, 0.2 mL NaOH (1 mol/L), 0.5 mL acetonitrile, and 5 mL chloroform were added, and the mixture was vortexed for 2 minutes. After centrifugation for 10 minutes ( $600\times g$ ), the supernatant was quantitatively kept in the water bath ( $45^{\circ}\text{C}$ ) to allow the evaporation of the upper organic layer. The residue was reconstituted in 100  $\mu\text{L}$  of the mobile phase and then vortexed for 1 minute. Then, 20  $\mu\text{L}$  of the supernatant was injected into HPLC after centrifugation at  $600\times g$  for 10 minutes.

Tissue samples were homogenized in a mixed solution of 200  $\mu\text{L}$  PBS (pH=7.4). Ten microliters of IS solution (0.1 mg/mL tetrahydropalmatine) were added to 200  $\mu\text{L}$  of tissue samples and then vortexed for 1 minute. The preparation method was consistent with that of the plasma sample.

## Statistical analysis

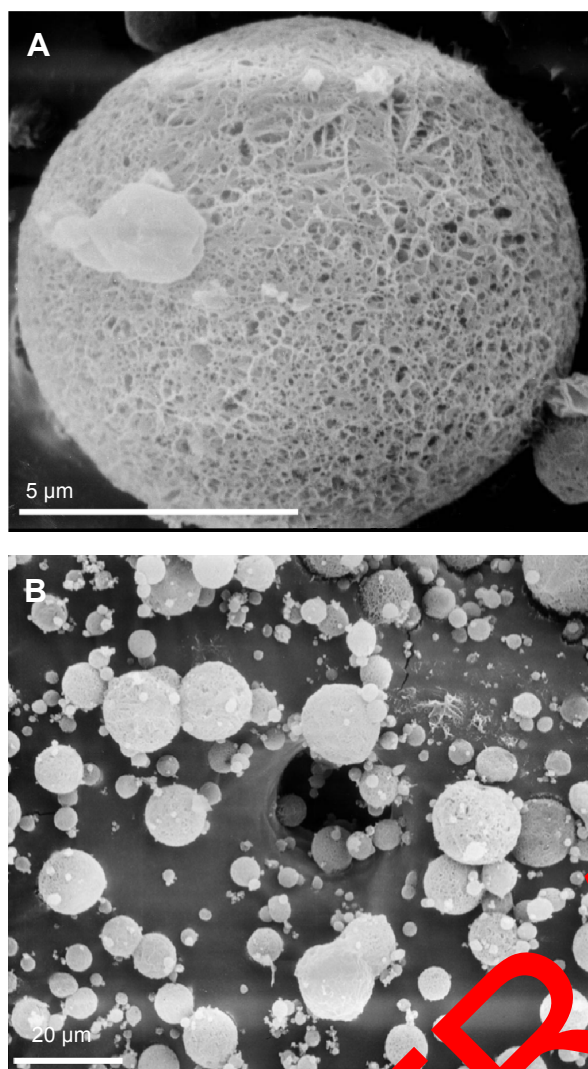
Data were collected in a Microsoft Excel 2013 worksheet, and the results were presented as mean  $\pm$  standard deviation (SD). A Student's *t*-test was performed to determine the statistical significance between experimental groups. A *P*-value  $< 0.05$  was considered to be statistically significant.

## Results and discussion

### Preparation and characterization

The method described in this article appeared to be suitable for the preparation of TED-loaded chitosan microspheres. There are many advantages of the emulsion cross-linking method, for example, it can be done under ambient temperature with constant stirring<sup>26</sup> and no special equipment is needed.<sup>27</sup> In additional, the chemical cross-linking method uses glutaraldehyde as the cross-linking agent to induce the rigidity of microspheres. The influence of process parameters, such as stirring speed, mixing time, and temperature, was analyzed. It was observed that the shape, size, and entrapment efficiency of formulations were also influenced by these variables. Span 80 was used for the purpose of wetting chitosan.

Particle-size distribution analysis indicated that the size of the microspheres was within the range of 7–15  $\mu\text{m}$  with a median diameter of 12.4  $\mu\text{m}$ . The DL% and entrapment



**Figure 1** Scanning electron microscope photographs of TED-loaded chitosan microspheres.

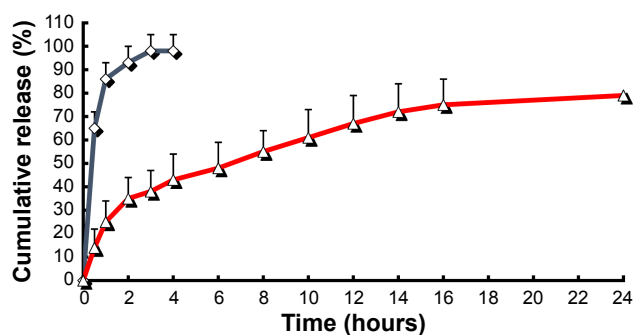
**Note:** (A) Single microsphere and (B) overview of microspheres; magnification 2000 $\times$ .

**Abbreviation:** TED, tetrandrine.

efficiency of the formulation were  $34.6\% \pm 12.5\%$  and  $87.3\% \pm 9.7\%$ , respectively. The microspheres were negatively charged with zeta potentials of  $-15.7$  mV. As shown in Figure 1, TED-loaded chitosan microspheres displayed a smooth surface, spherical shape, uniform particle-size distribution, and slight adhesion between microspheres.

### In vitro drug release

The in vitro drug release curve (cumulative release versus time) of TED-loaded chitosan microspheres is demonstrated in Figure 2. A very fast release behavior of TED was observed in the injection group, while a sustained cumulative release rate of TED in microspheres formulation was observed. In the injection group, more than 85% of the drug



**Figure 2** In vitro release of TED-loaded chitosan microspheres in PBS (pH 7.4) by a dialysis method.

**Note:** Black line: TED injection; red line: TED-loaded chitosan microspheres.

**Abbreviations:** TED, tetrandrine; PBS, phosphate buffered saline.

was released in the first hour of the dissolution process in PBS, and  $\sim 100\%$  of TED was released in the first 2 hours. In contrast, only 35% of TED was released from microspheres in the first 2 hours.

In the following 2 hours, the microspheres entered the slow release period, and cumulatively up to  $\sim 79\%$  was released by the end of the observation (24 hours). During the process, the release of microspheres exhibited two distinct phases: the first was the rapid release during the first 2 hours, which may be related to the release of the drug adsorbed onto the surface of microspheres (because the drug was only physically entrapped in the microspheres instead of chemically reacting with the polymer). In the second stage, the release rate slowed down, thus showing typical sustained and long-term drug release behavior. The driving force for TED dissolution is the concentration difference of TED in chitosan matrix, and TED is released by the interior of the swollen chitosan matrix through a more convoluted pathway.<sup>28</sup>

The aforementioned release data were fitted with different mathematical models, and the following equations were obtained, as shown in Table 1. Obviously, Higuchi equation was the best fitting model among the three because it had the highest correlation coefficient  $R$ , which may show that diffusion is the main mechanism for TED in vitro release.<sup>29</sup>

**Table 1** Dissolution kinetic parameters of TED from microspheres

Model	Formulations	
	Equation	Correlation coefficient (R)
Zero-order equation	$Q=3.241t-1.281$	0.9231
First-order equation	$\ln(1-Q)=-2.162t+0.261$	0.9435
Higuchi equation	$Q=7.281t_{1/2}+1.982$	0.9926
Weibull equation	$\ln(1/(1-Q))=2.217\ln t+0.721$	0.9832

**Abbreviations:** TED, tetrandrine. In, natural logarithm;  $1-Q$ , 100%-cumulative release amount;  $\ln t$ , natural logarithm (time).



## Analysis method validation

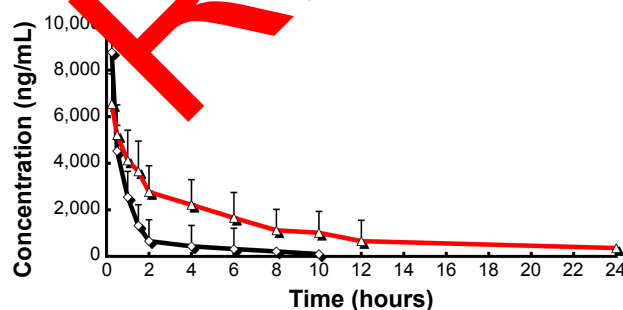
The linear range of the assay for plasma and tissues of animal was 0.2–10 µg/mL, and the standard curve of TED was  $Y=21.54C+0.271$  ( $r=0.9991$ ). The intraday and interday assay variability for all the samples did not exceed 12% and 15%, respectively. The method recoveries were in the range of 91%–102%. The detection limits and quantitation limits of liquid chromatography analysis were both determined to be 200 ng/mL for in vivo plasma/tissue samples. No interfering peaks were observed in any of the chromatograms. It is noteworthy that the HPLC technique, although simple, is an effective method to analyze TED in the microsphere system.

## Pharmacokinetic

Figure 3 shows the mean plasma drug concentration versus time curves corresponding to the intravenous administration of TED injection (8 mg/kg) and TED-loaded chitosan microspheres (8 mg/kg). As is shown in Figure 3, after the single injection of TED, the plasma drug concentration quickly reached maximum ( $8672.2\pm 1012.6$  ng/mL) in 15 minutes, and then decreased rapidly and left 10% of the peak concentration in plasma ( $C_{max}$ ) value after 2 hours. This implied that a rapid in vivo elimination of TED existed in rats. In the case of intravenous administration, the in vivo curve of microspheres was smoother than that of the TED injection group.

The plasma concentration was close to  $C_{max}$  of  $6435.7\pm 1129.2$  ng/mL at 15 minutes. Then, the drug concentration gradually decreased and became stable at a value of approximately 3,000 ng/mL from hour 1 to hour 2, following that, the plasma drug concentration dropped slowly to less than 341 ng/mL at 24 hours.

The first high plasma drug concentration of TED in the earlier hour was caused by the initial rapid drug release from



**Figure 3** Mean plasma TED concentration in rats after intravenous administration of two formulations ( $n=6$ , dose =8 mg/kg).

**Note:** Red line: TED-loaded chitosan microspheres; black line: TED injection.

**Abbreviation:** TED, tetrandrine.

**Table 2** Pharmacokinetic parameters of the two formulations

Parameter	Formulations	
	Injection	Microspheres
$t_{1/2}$ (h)	0.21±0.08	3.21±0.87*
$C_{max}$ (ng/mL)	8,672.2±1,012.6	6,435.7±1,129.2*
$AUC_{0-t}$ (ng·h/mL)	8,738.6±1,029.3	29,637.8±2,254.6*
$AUC_{0-\infty}$ (ng·h/mL)	9,872.2±1,121.5	32,128.3±2,435.7*
MRT (h)	2.76±0.79	342.26±31.76*
CL (L/h)	37.13±2.72	1.55±0.43*

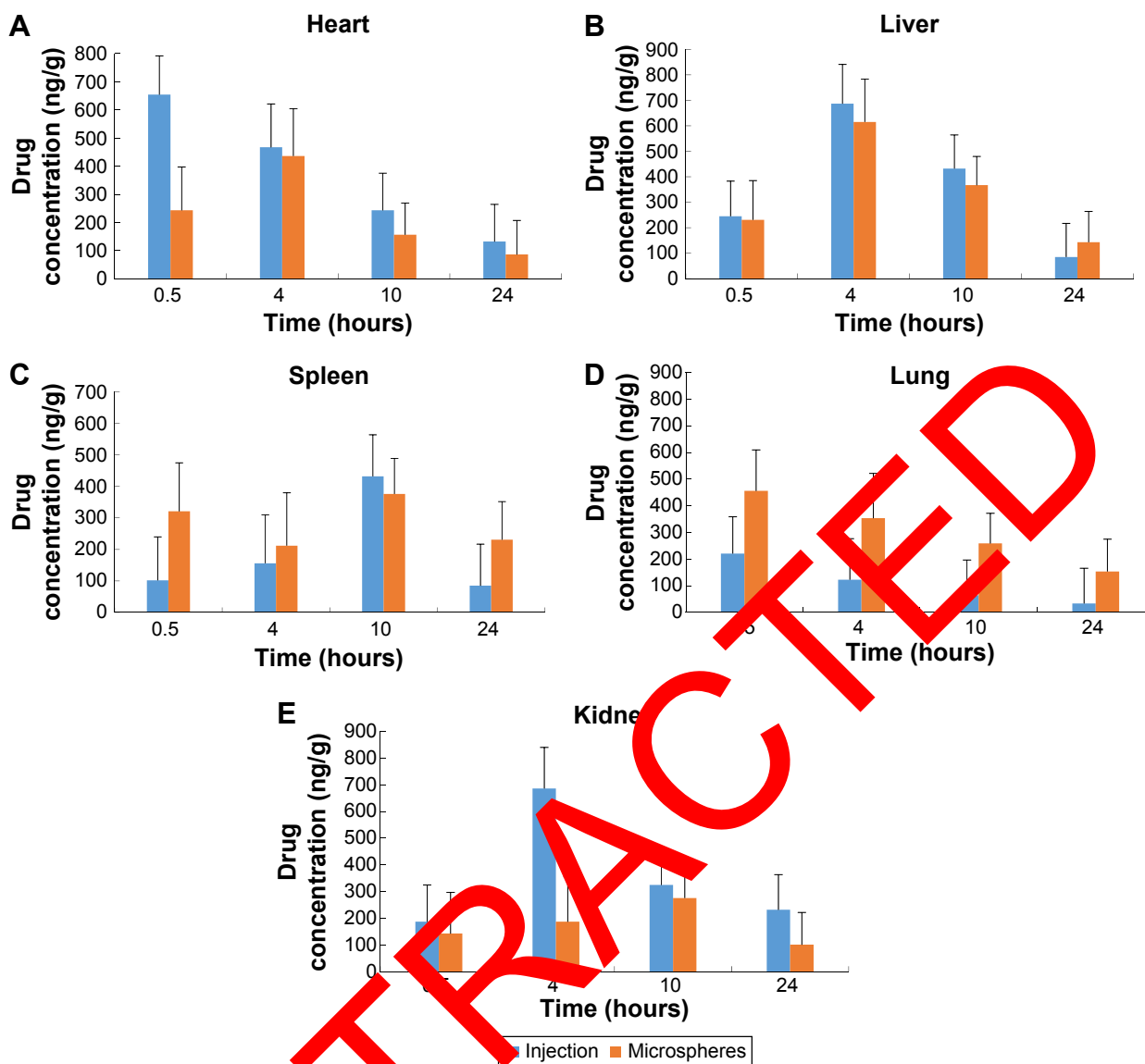
**Note:** \* $P<0.05$ : TED microspheres vs TED injection.

**Abbreviations:** AUC, area under the curve; MRT, mean residence time; CL, clearance; TED, tetrandrine;  $t_{1/2}$ , elimination half-life;  $C_{max}$ , the peak concentration in plasma.

the microspheres. The later higher drug level compared to the TED injection may be associated with the in vivo drug release of the microspheres. The pharmacokinetic parameters of the two formulations are listed in Table 2. Compared with the drug injection group, the values of the time of maximal concentration in plasma ( $T_{max}$ ), area under the curve (AUC), elimination half-life ( $t_{1/2}$ ), and mean residence time of the microsphere group were significantly higher. These data suggest that the prepared TED microspheres demonstrated a release rate as slow as we previously expected. Maintenance of a relatively constant plasma concentration in long-term drug therapy is a superior pharmacokinetic advantage of the TED microspheres.

## Tissue distribution

The tissue concentrations of TED after intravenous administration of TED-loaded chitosan microspheres and TED injection are shown in Figure 4. The AUCs of different deliveries in each tissue or plasma were calculated, and the targeting parameters are shown in Table 3. The uptake by the reticuloendothelial system organs, especially the spleen and lung, was observed to be higher with TED-loaded chitosan microspheres than TED injection. TED-loaded chitosan microspheres showed the largest value of AUC for the lungs. High concentration of drugs in the lung is mainly due to the physical capture of microspheres in the pulmonary vascular network. Similar behaviors have been widely reported in the literatures for nanostructure lipid carriers.<sup>30</sup> The TED-loaded microspheres produced a lower drug concentration in the heart, liver, and kidney than the TED solution. This indicated that the microspheres used in this study were preferable to target lungs than other tissues. The lung was histopathologically examined for any damage to the tissues. Microphotographs of the lung were taken following incubation with microsphere formulations for more than 24 hours (Figure 5). Saline was used as the control group. Signs such



**Figure 4** Concentrations of TED in mice tissues after intravenous administration of TED-loaded chitosan microspheres and TED injection (n=6).

**Note:** Results for (A) heart, (B) liver, (C) spleen, (D) lung, (E) and kidney tissues.

**Abbreviation:** TED, tetrandrine.

as the appearance of epithelial necrosis and sloughing of epithelial cells were not detected.

## Conclusion

In this study, novel TED-loaded chitosan microspheres were prepared by the emulsion cross-linking method, and

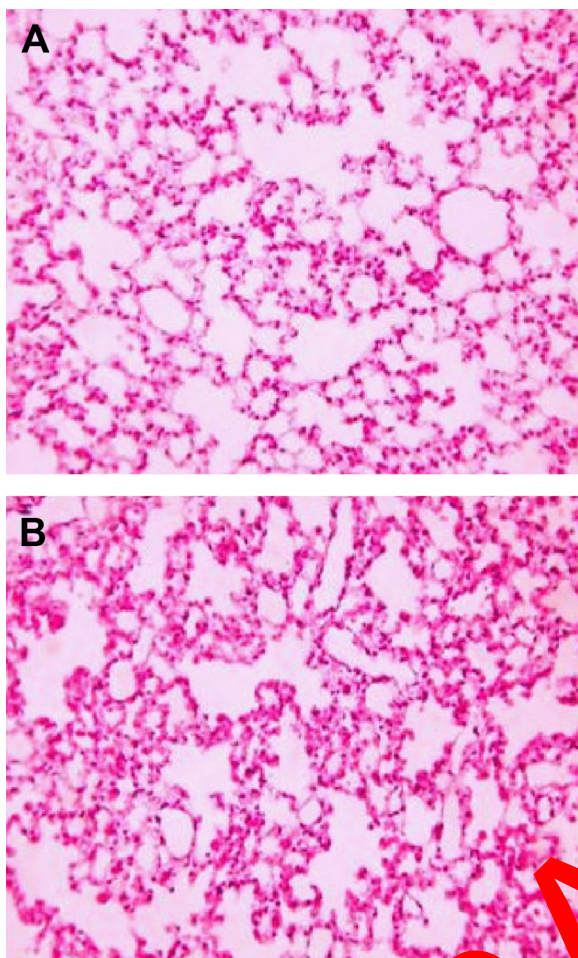
then the systems were characterized for physicochemical properties and in vitro drug release. In addition, the pharmacokinetics and tissue distribution of microspheres were further verified in animal models. Particle-size distribution indicated that the size of the microspheres was within the range of 7–15  $\mu\text{m}$  with a median diameter of 12.4  $\mu\text{m}$ . The

**Table 3** AUC<sub>0–24 h</sub> of TED in the heart, liver, spleen, lung, and kidney after IV administration of TED-loaded chitosan microspheres and TED injection to mice (n=6)

Formulation	Heart	Liver	Spleen	Lung	Kidney
TED injection (ng·h/g)	6,716.7±728.1	8,607.3±898.1	5,835.7±543.1	1,859.2±192.5	8,464.5±836.3
TED microspheres (ng·h/g)	4,658.2±426.2	7,996.3±825.4	6,945.8±714.2	6,147.4±598.3	4,605.2±443.6
Ratio <sup>#</sup>	0.69*	0.93	1.19	3.31*	0.54*

**Notes:** <sup>#</sup>The ratio for the formulation was AUC (TED microspheres)/AUC (TED injection); \**P*<0.05: microspheres vs injection.

**Abbreviations:** TED, tetrandrine; IV, intravenous; AUC, area under the curve; h, hours.



**Figure 5** Images of H&E staining of tissue sections of mice after IV injection of TED-loaded chitosan microspheres and saline.

**Note:** (A) Saline and (B) TED-loaded chitosan microspheres; magnification:  $\times 400$ .  
**Abbreviations:** H&E, hematoxylin and eosin; IV, intravenous; TED, tetrandrine.

DL% and entrapment efficiency of the formulation were  $34.6\% \pm 12.5\%$  and  $71.3\% \pm 9.7\%$ , respectively. In vitro release showed a typical sustained and long-term drug release behavior. Higuchi equation was the best fitting model with release data. Maintaining a relatively constant plasma concentration in long-term drug treatment is an outstanding pharmacokinetic advantage for TED microspheres in vivo. Moreover, TED microspheres produced a lower drug concentration in the heart, liver, and kidneys than TED solution. This indicated that the microspheres used in this study were preferable to target lungs than other tissues. No damage to the tissues of the lungs was found in the histopathological examination.

## Acknowledgments

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## Disclosure

The authors report no conflicts of interest in this work.

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