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Optimized preparation of daidzein-loaded chitosan microspheres and *in vivo* evaluation after intramuscular injection in rats

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Abstract

A spherical symmetric design-response surface methodology was applied to optimize the preparation of daidzein-loaded chitosan microspheres by the emulsification/chemical cross-linking technique. The influence of polymer concentration, ratio of drug to polymer, and the stirring speed on the encapsulation efficiency, particle size, particle size distribution, and accumulative drug release percent in microspheres were evaluated. Scan electron microscopy of the optimized microspheres showed spherical particles, loading with drug microcrystal uniformly on the surface of and inside the microspheres. *In vivo* pharmacokinetic characteristics were evaluated after intramuscular injection of the microspheres in rats. The time-resolved fluoroimmunoassay method was used to determine plasma concentrations of daidzein. The data showed that the release of daidzein in the microspheres *in vitro* and *in vivo* almost lasted for 35 days. The bioavailability of daidzein in the microspheres by intramuscular injection increased up to 39% in rats, suggesting that the cross-linked chitosan microspheres are a valuable system for the long-term delivery of isoflavones.

Keywords: Emulsification/chemical cross-linking; Optimization; Daidzein; Bioavailability; Time-resolved fluoroimmunoassay

1. Introduction

Daidzein is a natural isoflavone found in *Leguminosae* and belongs to the family of diphenolic compounds with structural similarities to natural and synthetic estrogens and anti-estrogens. In recent years, it has been reported that daidzein exhibits a variety of beneficial effects on human health (Kurzer and Xu, 1997; Bingham et al., 1998; Setchell and Cassidy, 1999; Clarkson, 2000), including chemoprevention of cardiovascular diseases and cancer as well as an alternative for estrogen replacement therapy (ERT) to prevent and treat osteoporosis (in post-menopausal women with bone loss). Our previous studies have also demonstrated that daidzein can inhibit the growth of cancer cells and prompt the proliferation of osteoblast cells (Li et al., 2001; Zhang et al., 2002; Wang et al., 2002; Jia et al., 2003).

Recently, daidzein preparations and nutritional supplements containing daidzein are widely consumed for their potential health effects. However, the oral bioavailability of daidzein is very poor, which limits its curative effect (Allred et al., 2005; Janning et al., 2000; Qiu et al., 2005; Zheng et al., 2004). The reasons are related to the drug's physicochemical properties which include the low solubility, the low partition coefficient of oil/water, and especially, the strong metabolism that occurs in the intestine and liver (Kulling et al., 2001). Gut transit time is also considered to be a significant determinant of isoflavone bioavailability (Hendrich, 2002). So, parenteral administration may be an attractive alternative to solve the low bioavailability through oral route. Furthermore, daidzein needs to be administrated for long to prevent and treat diseases. Aiming at these conditions, in this study, implantable sustained-release drug delivery system was

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considered to be suitable for daidzein delivery via the parenteral route.

Biodegradable polymers show greatly increasing importance in the development of sustained-release drug delivery system and implantable biomaterials. Among the suitable enzymatically degradable polymers is chitosan, which is a potential useful pharmaceutical material owing to its good biocompatibility and low toxicity (Agnihotri et al., 2004; Sinha et al., 2004). Chitosan is a polymer of glucosamine and *N*-acetylglucosamine derived from the natural polymer chitin. The degradation products of chitosan are nontoxic, nonimmunogenic, and noncarcinogenic. Recently, chitosan microspheres cross-linked with glutaraldehyde were shown to be long-acting biodegradable carriers suitable for controlled delivery of many drugs (Patashnik et al., 1997; Jameela et al., 1998; Prabaharan and Mano, 2005).

In the present study, the preparation of glutaraldehyde crosslinked, daidzein-loaded chitosan microspheres was optimized by a spherical symmetric design-response surface methodology and evaluated on the drug release behaviors *in vitro* and bioavailability *in vivo*. The prepared chitosan microspheres showed good spherical shape, tight matrix density and high drug loading by the micronization technique. The *in vitro* and *in vivo* experiments presented that daidzein in the microspheres released gradually lasting for 35 days and the absolute bioavailability greatly increased by intramuscular injection in rats. The results *in vitro* and *in vivo* suggest that the optimized chitosan microspheres are a useful system for the long-term intramuscular delivery of daidzein.

2. Materials and methods

2.1. Materials

Chitosan (M_w: 100,000) with 85% deacetylation degree was a present from Ocean University of China. The purity of synthesized daidzein (Huike Plants Exploiture Co., Shanxi, China) was 98.79% by weight. Span 80 was purchased from Fluka Chemical Co. (Switzerland). TR-FIA kit for daidzein was purchased from Labmaster Co. (Turku, Finland). Glutaraldehyde (25%) was from Beijing Chemical Co. (China). Acetonitrile was HPLC grade (Burdick & Jackson, Honeywell International Inc., MI, USA). Carboxymethyl cellulose sodium (CMC-Na) purchased from Beijing Chemical Co. (China). All other reagents and solvents used were analytical grade.

2.2. Preparation of the microspheres

The microspheres were prepared using the emulsification/chemical cross-linking technique (Patashnik et al., 1997; Jameela et al., 1998; Prabaharan and Mano, 2005). Chitosan was used as a matrix forming polymer. The polymer was dissolved in 2% acetate acid (w/v). The aqueous phase was an acidic polymer solution suspended with micronized daidzein particles (<5 μm) by ultrasonication. The oil phase consisted of 80 mL liquid paraffin containing 2% Span 80 (w/v). To prepare the microspheres, 4 mL of aqueous phase was dropped into the oil phase using a 6 G needle, which formed a W/O emulsion after 15 min stirring. Then 1 mL of 25% glutaraldehyde solution was added and stirred continuously at 300 rpm for 3 h at room temperature. The formed microspheres were separated by vacuum-induced filtration, washed five times with petroleum ether, distilled water and isopropanol, respectively. The prepared microspheres were dried in a vacuum desiccator for 24 h at room temperature.

2.3. Experimental design

Preliminary experiments indicated that the variables, such as polymer concentration, drug to polymer ratio, and stirring speed during emulsification, were the main factors that affect the particle size, size distribution, drug-loading, and the release rates of the chitosan microspheres. Thus, a spherical symmetric design-response surface methodology was used to systemically investigate the effects of these three critical formulation variables on the medium particle diameter (particle size), span (particle size distribution), drug loading (%, w/w) and accumulative release percent (%, w/w) at 24 h ($Q_{24\,h}$) of the prepared microspheres. The details of the design are outlined in Table 1. For each factor, the experimental range was selected, based on the results of preliminary experiments and taking into consideration of the feasibility of preparing the microspheres at the extreme values. All the formulations in these experiments were prepared in duplicates.

2.4. Particle size snalysis of the microspheres

Particle size was determined by a laser particle counter (Malvern Mastersizer Microplus, Malvern Instruments Ltd., UK). Briefly, proper amounts of dry microspheres were mixed with distilled water and suspended completely by ultrasonication (10 s). The suspension was then placed in the laser particle counter. The medium particle size (D_{50}, the particle size when cumulative value is 50% by volume in the particle

Table 1
Independent variables and their correspondent values for the optimization of microspheres preparation using the spherical symmetric design-response surface methodology

Variables	Levels				
	$-\sqrt{3}$	−1	0	1	$\sqrt{3}$
X_1 (chitosan concentration, %)	1	1.85	3	4.15	5
X_2 (ratio of drug to polymer, w:w)	0.13:1	0.5:1	1:1	1.5:1	1.87:1
X_3 (stirring speed, rpm)	400	570	800	1030	1200

size cumulative distribution profile) and particle size distribution (span = $(D_{90} - D_{10})/D_{50}$, D_{90} or D_{10}, the particle size when cumulative value is 90% by volume or 10% by volume in the particle size cumulative distribution profile) were measured for three times.

2.5. Morphological characteristics of the microspheres

A few of the microspheres were spread on an aluminium flat and coated with thin gold under vacuum. Scanning electron microscopy (SEM) (Model S-450, Hitachi, Tokyo, Japan) was used to record the size, shape surface characteristics and the internal structure of the blank microspheres and the drug loaded microspheres.

2.6. Drug loading of the microspheres

The drug content in the microspheres was measured using HPLC. Proper amounts of dry microspheres were mechanically ground in a mortar with a pestle. About 10 mg of the microspheres were placed into a 50 mL tube with a cap and 20 mL methanol was added. After the mixture in the tube was broken up by ultrasonication for 30 min (400 W), the tube was capped and shaked for 24 h at room temperature. Finally, the mixture was transferred into a 25 mL flask and diluted to 25 mL with methanol. The suspension was filtered through a 0.45 μm membrane. The concentration of drug in the filtered solution was measured at wavelength of 250 nm by HPLC (Waters, 600 controller, 996 photodiode array detector, 717 plus autosampler, USA) using a C_{18} column (250 mm × 4.6 mm, 5 μm, Kromasil). In briefly, the mobile phase consisted of methanol–acetonitrile–1% acetic acid by weight (50:10:40, v/v/v) and the flow rate was 0.8 mL/min. The volume of 10 μL was injected directly into the HPLC.

2.7. In vitro drug release

Generally, the dialysis bag method is applied in the *in vitro* release experiment of implantable sustained-release drug delivery system. In our study, the drug release in the designed fifteen formulations was carried out using the paddle method (Zhou et al., 2002) in order to reduce experimental time and optimize formulation rapidly. Briefly, the dissolution medium consisted of 200 mL phosphate buffer solution (PBS, pH 7.4), containing 0.1% HP-β-CD (w/v) at 37 ± 0.5 °C. The HP-β-CD was added to increase the solubility of daidzein in the medium and obtain the sink condition. About 10 mg of the microspheres were loaded in each release experiment. The rotational speed was set at 50 rpm. Samples (3 mL) were taken through a 0.45 μm membrane filter at designated times. The withdrawn samples were replaced with fresh dissolution medium. Samples were diluted to the appropriate concentration with fresh dissolution medium, and the UV absorbance was measured at 250 nm (Model 752 Ultraviolet Spectrophotometer, Shanghai Instruments Co., China). The amount of daidzein released at each time point was calculated using a standard curve of daidzein in PBS (pH 7.4) containing 0.1% HP-β-CD. The accumulative release percent of daidzein at 24 h (Q_{24}) was used to evaluate the release rate.

The drug crystal release experiment and the drug release in the optimized microspheres experiment were evaluated by the dialysis bag method (Zhou et al., 2002). About 1 mg of drug crystal or 10 mg of the microspheres were suspended in 5 mL of PBS (pH 7.4) and placed into a cellulose dialysis bag with the molecular weight cutoff 12–14 kDa. The tied dialysis bag was put into 250 mL of PBS (pH 7.4) at 37 ± 0.5 °C, and the stirring speed was set at 50 rpm. The medium was replaced completely by the fresh medium every 3 days. Samples were withdrawn the same as those used in the paddle method. The content of daidzein in the samples was determined by HPLC as mentioned in Section 2.6, but the injection volume was changed to 50 μL. The accumulative release percent of daidzein was calculated.

2.8. In vivo degradation and release

The *in vivo* degradation study was performed for the optimized daidzein-loaded chitosan microspheres. About 10 mg of the microspheres were suspended in 0.2 mL of CMC-Na solution and intramuscularly injected into the legs of 36 female ICR mice (8 weeks old, 6 groups), which were purchased from Beijing Vital Animal Co. (Beijing, China). The mice were sacrificed at an appropriate time point to determine the remaining microspheres. The muscle tissue around the injection site (about 0.3 g) was collected from and stored at −70 °C until analysis.

The tissue samples were triturated into powder under liquid nitrogen, to which 20 mL of methanol and 3 mL of genistein solution (1 mg/mL, as an internal standard) were added. The sample treatment and analytical processes as those described in Section 2.6. The standard curve was prepared from the samples of the mice sacrificed at time zero following the injecting known amount of microspheres. The ratio of the remaining amount of daidzein in the microspheres to the injected amount was the percent drug release from the microspheres *in vivo*.

The microspheres recovered from the *in vivo* degradation study were observed with a scanning electron microscope in the same manner as described above in Section 2.5. Their photomicrographs were taken for the microspheres recovered at 7 and 35 day after intramuscular injection.

2.9. Bioavailability study

Wistar rats (10 female, 200–210 g) were provided by Beijing Vital Animal Co. (Beijing, China). Animals were housed in a room with controlled temperature and humidity, and allowed to freely access to food (isoflavones free) and water. They were fasted overnight before the experiments. The five rats were administrated with daidzein solution (40 mg/mL, DMSO) via the sublingual vein injection at a single dose of 20 mg/kg. Blood samples were collected from the suborbital vein at 0, 0.083 (5 min), 0.17 (10 min), 0.5, 1.0, 2.0, 4.0, 8.0, 12.0, 24.0 and 48.0 h after administration. Another five rats were administrated with daidzein-loaded chitosan microspheres suspension via the intramuscular injection in back legs at a single dose of 150 mg/kg. Blood samples were collected from the subor-

bital vein at 0, 0.042 (1 h), 0.167 (4 h), 0.5, 1, 2, 3, 7, 11, 15, 19, 23, 27, 31, 35 and 39 day after administration. Samples were centrifuged at $3000 \times g$ for 5 min. Plasma was transferred into microcentrifuge tubes and stored at −20 °C until analysis.

The determination of daidzein in rat plasma was carried out using the TR-FIA kit. After samples were treated with hydrolysis and extraction, the values of fluorescence were measured by a time-resolved fluorometry Victor^3 1420 multilabel counter (PerkinElmer, USA) according to the protocol recommended by the manufacturer. The final data was calculated according to the standard curve and dilution factor.

Pharmacokinetic parameters were calculated with 3p97 program (a practical pharmacokinetic program, developed by the Chinese Society of Mathematical Pharmacology). The absolute bioavailability of the daidzein-loaded chitosan microspheres to the intravenous administration of daidzein solution was calculated through comparing those two AUC using the following equation:

$$F\,(\%) = \frac{(\mathrm{AUC}/D)_A}{(\mathrm{AUC}/D)_B} \times 100$$

where F is the absolute bioavailability, AUC the area under the plasma concentration–time curve, D the dose administered, A the intramuscular injection daidzein-loaded chitosan microspheres and B the intravenous administration of daidzein solution.

2.10. Data analysis

In the optimization by the spherical symmetric design-response surface methodology, statistical software STATISTICA Version 6.0 was applied to the response surface regression analysis. Differences were considered significant at $p \leq 0.05$ using Student's t-test.

3. Results

3.1. Optimization of formulation

The spherical symmetric design-response surface methodology constitutes an alternative approach because it offers the possibility of investigating a high number of variables at different levels, performing only a limited number of experiments (Chacón et al., 1996; Wu and Cui, 2000). The variables in Table 1 were chosen taking into account those in our preliminary experiments and other studies dealing with microparticles preparation using the emulsification/chemical cross-linking method (Wang et al., 1996).

Table 2 shows the experimental results concerning the tested variables on drug-loaded percent, medium diameter, span and accumulative release percent at 24 h. The four regressor values ranged from 1.89 to 37.46% by weight, 26.59 to 56.38 μm, 0.868 to 1.917, 32.45 to 92.77% by weight. A mathematical relationship between factors and parameters was generated by response surface regression analysis in statistical software STATISTICA version 6.0. The three-dimensional response surface plots for the most statistical significant variables on the evaluated regressors are shown in Fig. 1. The response surface diagrams shows that the higher the chitosan concentration the larger the particle size, the smaller the span and the slower the release rate. On the other hand, the higher the stirring rate resulted in the smaller the particle size, the larger the span and the slower the release rate. However, both of the polymer concentration and the stirring rate had no significant effect on the drug-loaded percent. Furthermore, the drug-loading significantly increased with the increasing drug to polymer ratio, indicating that the ratio significantly affected the drug-loading.

The optimized variables showed a good fit to the second-order polynomial equation, with correlation coefficient (r) of 0.9988, 0.9812, 0.9842, 0.8282, respectively. After model simplification with backward stepwise solution, the r value decreased

Table 2
Response values of different variables for the optimization of microspheres preparation using the spherical symmetric design-response surface methodology

Formulation	Orthogonal values			Response values		
	X_1	X_1	X_1	Drug-loaded percent (%)	Medium size (μm)	Span	Accumulative release percent at 24 h (%)
1	−1	−1	−1	11.69	35.95	1.870	88.60
2	−1	−1	1	8.510	30.77	1.902	80.54
3	−1	1	−1	35.78	39.85	1.660	57.15
4	−1	1	1	33.97	31.88	1.917	55.35
5	1	−1	−1	16.68	50.20	0.947	85.16
6	1	−1	1	13.53	33.49	1.483	92.77
7	1	1	−1	34.51	56.38	1.030	57.79
8	1	1	1	34.77	37.03	1.551	63.72
9	$-\sqrt{3}$	0	0	20.91	26.59	1.818	58.77
10	$\sqrt{3}$	0	0	25.69	44.8	0.868	32.45
11	0	$-\sqrt{3}$	0	1.890	35.41	1.613	83.90
12	0	$\sqrt{3}$	0	37.46	33.3	1.911	62.09
13	0	0	$-\sqrt{3}$	27.96	51.95	1.259	38.62
14	0	0	$\sqrt{3}$	25.13	30.74	1.615	69.91
15	0	0	0	25.42	33.51	1.479	48.12

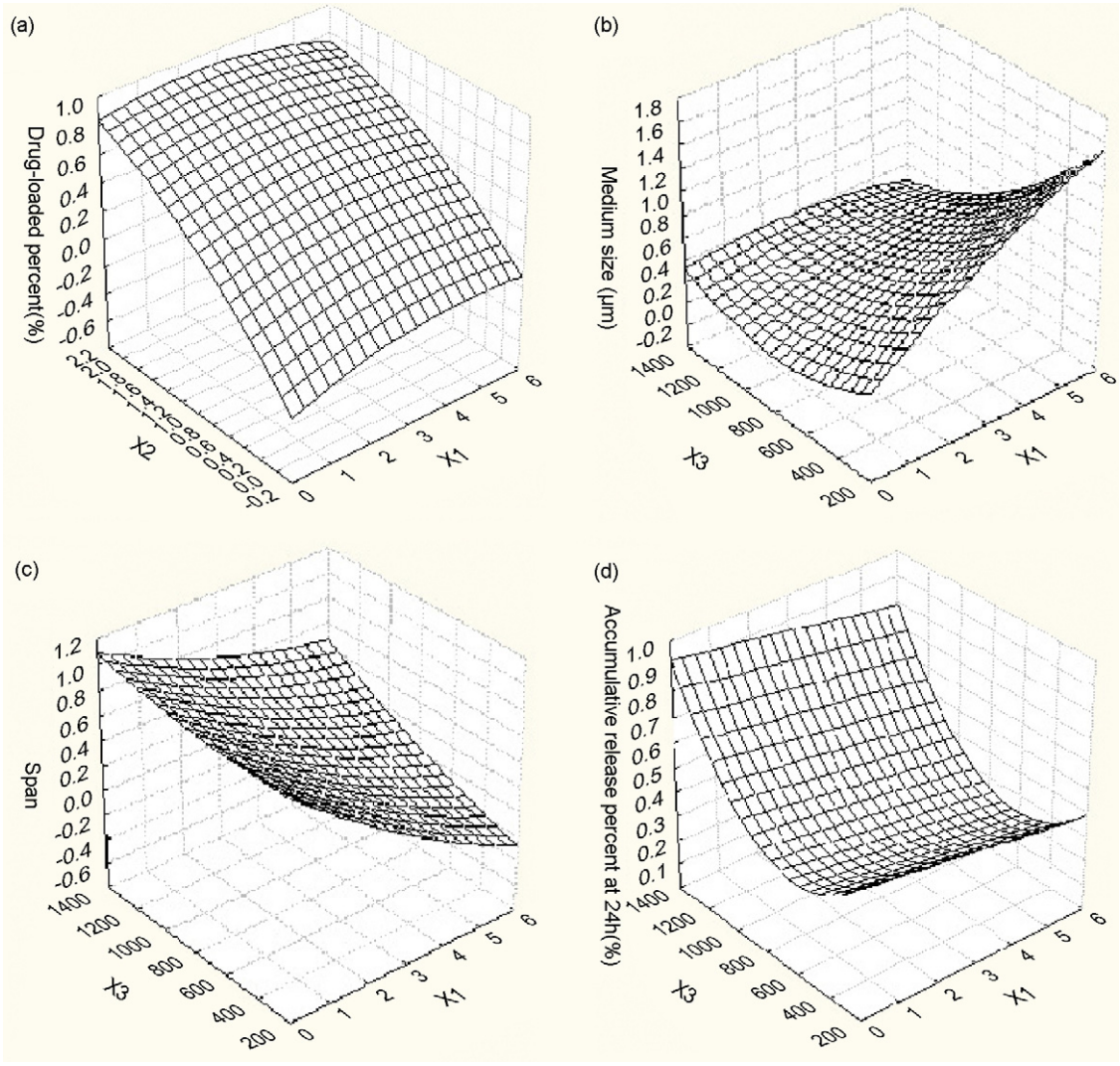

Fig. 1. Three-dimensional response surface plots showing the variation in the (a) drug-loaded percent, (b) medium diameter, (c) span and (d) accumulative release percent at 24 h with changes in polymer concentration (X_1), ratio of drug to polymer (X_2) and stirring speed (X_3).

slightly to 0.9974, 0.9699, 0.9706, 0.8239, respectively. The lack-of-fit was not significant at 95% confidence level. All the remaining parameters were significant at $p \leq 0.05$. The statistical analysis of the results generated the following polynomial equations:

$$\text{Drug-loaded percent}\,(\%) = -16.6021 + 7.5144X_1 - 0.6885X_1^2 + 45.1088X_2 - 8.4284X_2^2 - 0.004X_3 - 2.2783X_1X_2$$

$$\text{Medium size}\,(\mu\text{m}) = 45.929 + 13.014\,X_1 - 0.0634X_3 + 0.00004X_3^2 - 0.01083X_1X_3$$

$$\text{Span} = 2.5625 - 0.4191X_1 - 0.8749X_2 + 0.4712X_2^2 + 0.000215X_1X_3$$

$$Q_{24}\,(\%) = 141.4966 - 1.7314X_1 - 99.1348X_2 + 38.8234X_2^2 - 0.0826X_3 + 0.0001X_3^2$$

where X_1, X_2 and X_3 represent the orthogonalized values of the polymer concentration, drug to polymer ratio and stirring rate, respectively.

Since the fitting results indicated that the smallest span and the slowest release rate were obtained at the lowest levels of the polymer concentration and stirring rate, the chitosan concentration and stirring rate in the optimized formulation was selected as 5% by weight and 400 rpm, respectively. The predicted range of drug to polymer ratio was 0.75–1.2. Table 3 shows that the experimental values of the two batches prepared in the optimum range were very close to the predicted values, with low percentage bias, suggesting that the optimized formulation was reliable and reasonable.

Table 3
Comparison of the observed and predicted values in the microspheres prepared under predicted optimum conditions

Response variable	Predicted value	Observed value	Bias (%)
Drug-loaded percent (%)	30.48	31.04	1.84
Medium size (μm)	70.40	72.91	3.57
Span	0.526	0.553	5.13
Release percent at 24 h (%)	52.74	49.95	5.29

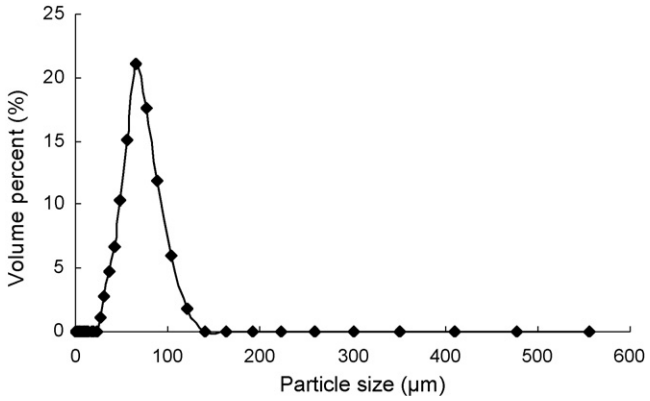

Fig. 2. Particle size distribution of the optimized chitosan microspheres.

3.2. Characteristics of optimized daidzein-loaded chitosan microspheres

The curve of particle size distribution of optimized daidzein-loaded microspheres is given in Fig. 2. The medium particle diameter was 72.91 μm and the span was 0.553. Fig. 3 shows the scanning electron micrography photos of blank microspheres and optimized daidzein-loaded microspheres. The result presented that the blank microshperes had a smooth surface (Fig. 3a) but the optimized daidzein-loaded microspheres had much drug microcrystal on the smooth surface (Fig. 3b). The cross section of the daidzein-loaded microspheres (Fig. 3d) clearly showed that the drug was dispersed uniformly inside the microspheres matrix, compared with the cross section of the blank microspheres (Fig. 3c).

HPLC assay showed that the daidzein loading in the optimized microspheres was 31.04% by weight. The *in vitro* accumulative release percent of daidzein from the optimized microspheres was 49.95% at 24 h by paddle method (Fig. 4a) and 93.13% at 35 day by dialysis bag method (Fig. 4b). The *in vitro* accumulative release profile of daidzein crystal by the dialysis bag was presented in Fig. 4c. Compared with the paddle method, it seemed that the burst effect at the initial stage was not shown clearly in the dialysis bag method. It may be explained that daidzein in the microspheres was the microcrystal form which had a very low dissolution rate in the release medium, following the zero order release characteristics (Polakovic et al., 1999; Zhou et al., 2002).

3.3. In vivo degradation and release

The *in vivo* release of daidzein in the chitosan microspheres was measured by HPLC. The linear calibration curve obtained

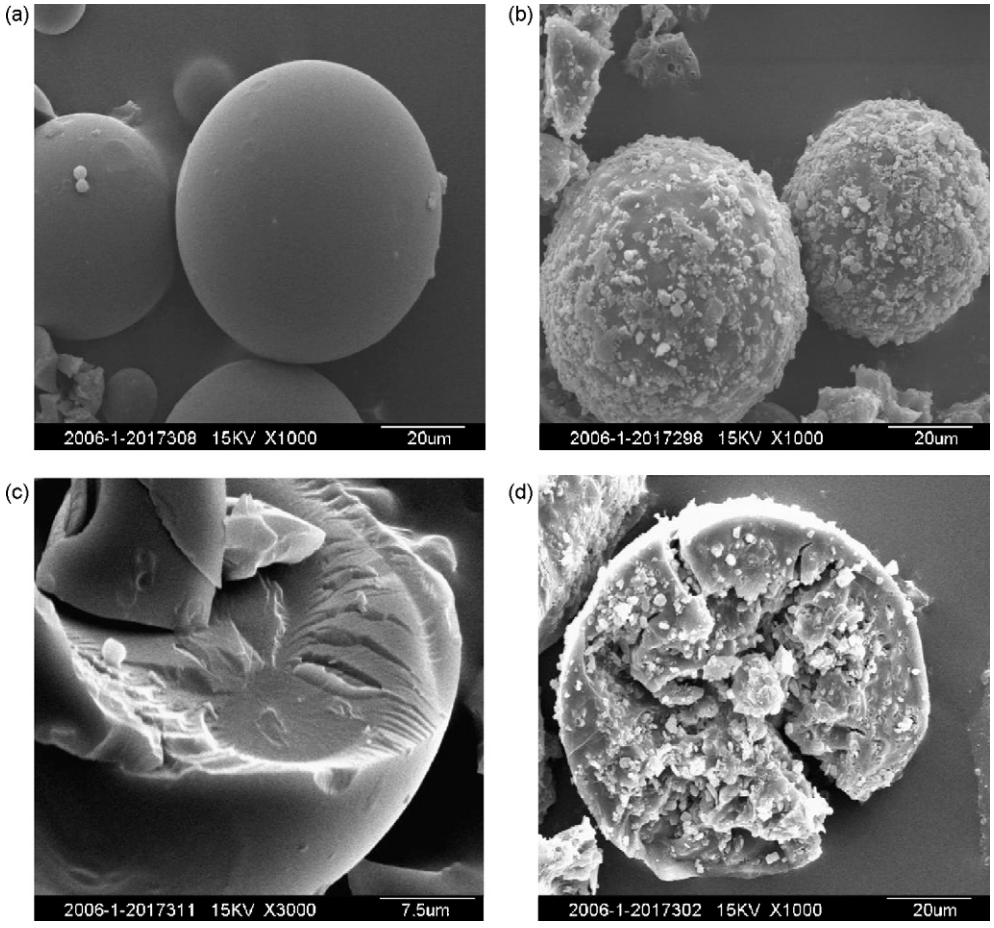

Fig. 3. Scanning electron microphotographs of: (a) blank microspheres (×1000); (b) surface of drug-loaded microspheres (×1000); (c) cross-section of blank microspheres (×3000); (d) cross-section of drug-loaded microspheres (×1000).

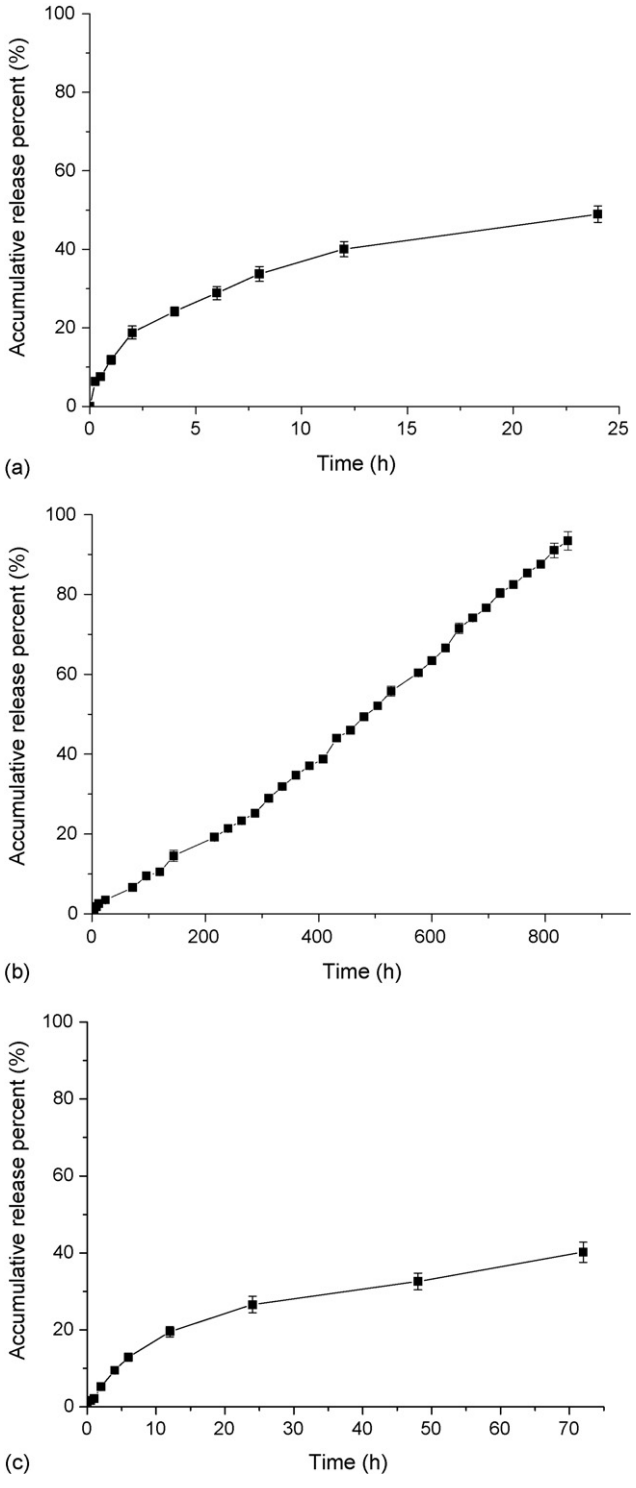

Fig. 4. *In vitro* release profiles of the optimized daidzein-loaded chitosan microspheres using the (a) paddle method and (b) dialysis bag method and daidzein crystal (c) by the dialysis bag method.

with peak-area ratio (y) of daidzein to internal standard versus drug concentration (x) was $y = 112.76x - 0.877$. The correlation coefficient was 0.9993. The *in vivo* release of the chitosan microspheres was shown in Fig. 5. After 3 days of incubation, drug release from the microspheres was 9.54% by weight of the loaded amounts. The drug was released uniformly in the following days and was completely released at 35 day. The *in vitro* (using the dialysis bag method) and *in vivo* data of drug release showed a good correlation and the dialysis bag method was verified to be chosen as the method of choice for the *in vitro* release of drug from microspheres experiments.

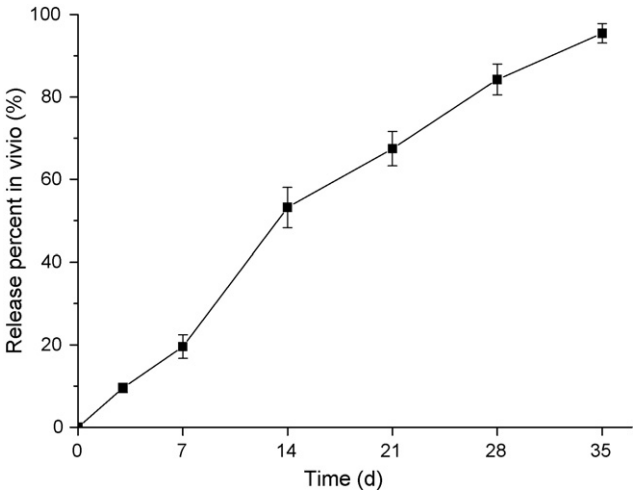

Fig. 5. *In vivo* release profile of the optimized daidzein-loaded chitosan microspheres after intramuscular injection administration in mice ($n = 6$).

After intramuscular injection, the morphology of the chitosan microspheres varied in a time-dependent pattern. At 7 day (Fig. 6a), the microspheres remained in spherical shape but the smooth surface became rough with some shallow cavities, indicating that degradation occurred at the out layer of the microspheres. At 35 day (Fig. 6b), the microspheres were degraded into a loose and porous structure and partially into fragments, at which most of the drug were released completely.

3.4. Bioavailability evaluation

TR-FIA is a simple, specific, rapid and sensitive method for the determination of daidzein in plasma with high precision and accuracy and has been successfully applied to the determination of daidzein in biological samples (Uehara et al., 2000; Wang et al., 2000). Calibration curves for the plasma assay developed with the fluorescence value ratio of standard daidzein solution to blank assay buffer (Y) versus the logarithmic value of drug concentrations (X) were found to be linear over the concentration range of 0.13–61.02 ng/mL. The linear regression equation of the calibration curve was $Y = 0.68717–0.33165X$, and the related coefficient was 0.9981.

The mean plasma concentration–time profile of daidzein after intravenous injection is shown in Fig. 7. Daidzein concentration in plasma decreased rapidly in the first hour. The distribution half-life was very short, but the elimination half-life was longer, which showed that daidzein was rapidly transported to tissues or organs from blood, then cleared slowly from blood. The plasma concentration–time data were best fitted to a two-compartment model after intravenous administration and the pharmacokinetic parameters were summarized in Table 4.

Fig. 8 shows the mean plasma concentration–time profile of daidzein after intramuscular injection of the daidzein-loaded chi-

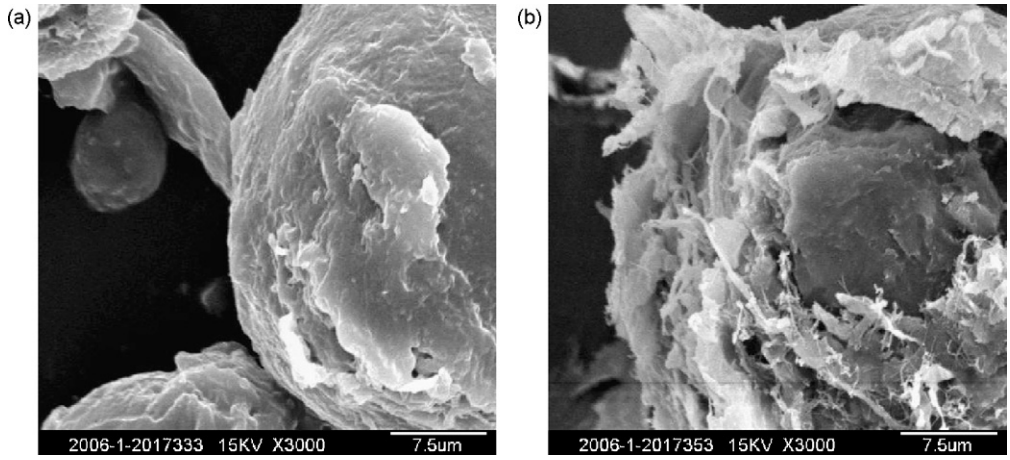

Fig. 6. Scanning electron microphotographs of chitosan microspheres retrieved at (a) 7 day (×3000) and (b) 35 day (×3000) after intramuscular injection in mice.

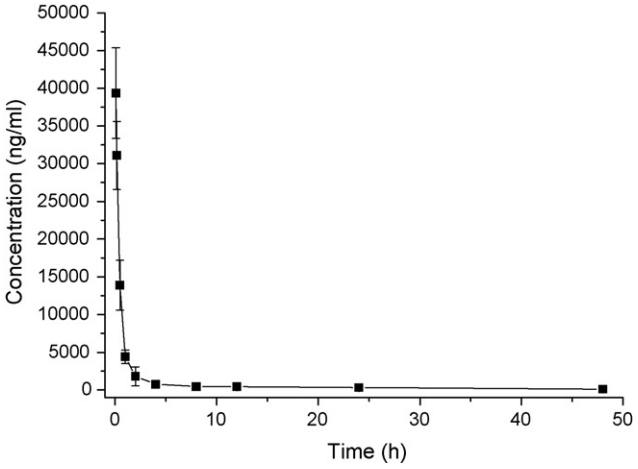

Fig. 7. The mean plasma concentration–time profile of daidzein after intravenous injection administration of the daidzein solution ($n = 5$).

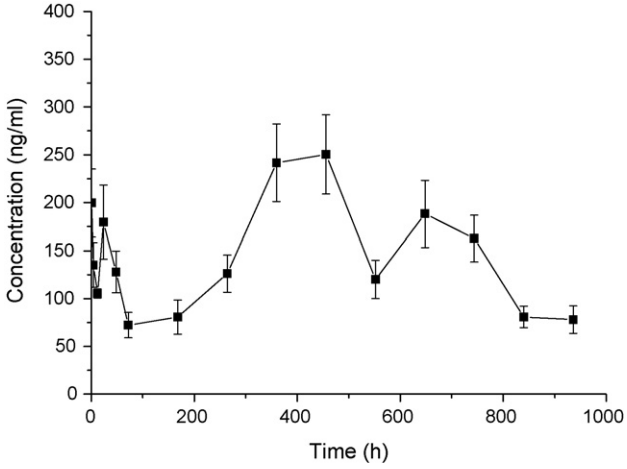

Fig. 8. The mean plasma concentration–time profile of daidzein after intramuscular injection administration of the chitosan microspheres ($n = 5$).

tosan microspheres. The total daidzein concentration showed fluctuation and lasted for about 35 days in plasma. The absolute bioavailability (F) of the daidzein-loaded chitosan microspheres was 39.02%. It has been reported that the absolute bioavailability of daidzein solution at doses of 10 and 100 mg/kg are 9.7 and 2.2%, respectively, after oral administration in rats (Janning et al., 2000). Another study also shows that the absolute bioavailability of daidzein solution and daidzein suspension are 12.8 and 6.1%, respectively, after oral administration in rats at a dose of 20 mg/kg (Qiu et al., 2005). Compared with the low absolute bioavailability of oral formulations, our result indicated that intramuscular injection of the daidzein-loaded chitosan microspheres significantly improved the bioavailability of daidzein over orally administered daidzein.

Table 4
Pharmacokinetic parameters for daidzein in rats (mean ± S.D., $n = 5$) after a single intravenous dose of daidzein at 20 mg/kg

Parameter	Value	Parameter	Value
α (h^{-1})	2.33 ± 0.38	V_c (L/kg)	0.46 ± 0.07
β (h^{-1})	0.0366 ± 0.0015	K_{21} (h)	0.073 ± 0.003
A (μg/mL)	43.30 ± 6.01	K_{10} (h)	1.17 ± 0.20
B (μg/mL)	0.71 ± 0.10	K_{12} (h)	1.13 ± 0.20
$t_{1/2(\alpha)}$ (h)	0.30 ± 0.05	AUC (μg h/mL)	38.09 ± 5.01
$t_{1/2(\beta)}$ (h)	18.94 ± 0.78	Cl(s) (L h/kg)	0.53 ± 0.07

4. Discussion

In recent years, it is reported that the chitosan microspheres crosslinked by glutaraldehyde are long acting biodegradable carriers suitable for controlled delivery of many drugs (Patashnik et al., 1997; Jameela et al., 1998; Prabaharan and Mano, 2005). In our study, the emulsification/chemical cross-linking method was used to prepare the daidzein-loaded chitosan microspheres. The preparing process was composed of two steps, W/O emulsification and chemical crosslink with glutaraldehyde. In the emulsification step, daidzein, a needle crystal, was micronized to decrease the particle size of the drug powder and load much more drug powder into the emulsion droplets (up to 31.04%). The emulsion droplets were followed by the step of chemical crosslink with glutaraldehyde. The result showed that the prepared microspheres showed high drug-loaded percent (up to 31.04%) and good spherical shape (Fig. 3 and Table 3). The formed chitosan microspheres by chemical crosslink showed

high matrix density and good morphology (Wang et al., 1996; Jameela et al., 1998).

In the experimental design of the spherical symmetric design-response surface methodology, the preliminary studies were investigated in detail. Chitosan concentration from 1 to 5% by weight was chosen in the optimization experiments because preliminary study showed that the formed microspheres at chitosan concentration less than 1% were not well separated (coagulation). On the other hand, at a concentration of 5% or more, it was difficult to prepare the microspheres, due to the enhanced viscosity of the polymer solution. When chitosan concentration increased, the particle size decreased, and the release rate also decreased (Fig. 1). This effect has been reported previously (Wang et al., 1996; Yoshino et al., 2003) which may be attributed to the greater viscosity in the higher concentration of chitosan solution and the enhancement of matrix density in the microspheres. Furthermore, in the design, the levels of ratio of drug to polymer were chosen based on the feasibility of preparation of the microspheres. The minimum ratio and the maximum ratio were 0.13:1 and 1.87:1, respectively. The result showed that high ratio of drug to polymer significantly increased drug loading of the microspheres (Fig. 1a). It may be proposed that high of drug to polymer ratio enhanced the density of drug in the emulsion droplet to increase the drug content. Furthermore, the three-dimensional response surface plots (Fig. 1) show the effect of stirring rate on the microspheres. The high stirring rate decreased the size of emulsion droplet but decreased its uniformity, which therefore led to a great span (Wang et al., 1996; Patashnik et al., 1997; Jameela et al., 1998; Prabaharan and Mano, 2005).

Fig. 3 also clearly shows that the micronized daidzein microcrystal (particle size <5 μm) were uniformly dispersed over the surface of the microspheres and incorporated into the matrix. In the *in vitro* drug release experiment by the dialysis bag method, no clear burst release of drug was observed and zero-order release profile was obtained (Fig. 4b), but the different release profile was found in the paddle method (Fig. 4a). The release profile *in vitro* by the dialysis bag method had good correlation to the release results *in vivo* by measuring the residue amount of drug in mouse (Fig. 5) and plasma drug concentration profile in the bioavailability experiment by intramuscular injection (Fig. 8). The reasons for long sustained release were considered to be the small volume of tissue fluid and slow rate of blood stream in muscle as well as the low solubility of daidzein in release medium, resulting in the low release rate *in vitro* and *in vivo*. Recently, there are some debates about whether drugs on the surface of microspheres need to be cleared thoroughly or not. It is suggested that part of drugs on the surface of microspheres help to get the effective concentration of drug rapidly *in vivo* and the inner drugs in the microspheres keep sustained release continuously. Our result of the plasma drug concentration profile also presented the characteristics.

The *in vivo* process of isoflavones containing daidzein has been reported previously (Janning et al., 2000; Kulling et al., 2001; Setchell et al., 2003; Qiu et al., 2005). After oral administration, daidzein is subjected to glucuronidation at its 7-hydroxyl group and sulfatation at its 4′-hydroxyl group. Glucuronide conjugate and sulfate conjugates are its main metabolites. Many researchers have reported that the process *in vivo* of isoflavones after oral administration is very complex and the main reasons for the low bioavailability in human and animals involve extensive metabolism in liver, enterohepatic circulation, gut microflora metabolism, etc. (Hendrich 2002; Rowland et al., 2003; Setchell et al., 2003). Therefore, parenteral administration of daidzein is considered as a possible alternative to reduce metabolism and enhance bioavailability (Jameela et al., 1998; Pandey and Khuller, 2004; Sinha et al., 2004; Varde and Pack, 2004). In our study, the sustained-release daidzein-loaded microspheres were prepared successfully by optimization and administrated by intramuscular injection in rats. The concentrations of daidzein in plasma were determined using a time-resolved fluoroimmunoassay kit. Compared with HPLC/UV, LC/MS/MS (Janning et al., 2000; Qiu et al., 2005), etc., the time-resolved fluoroimmunoassay assay method was very rapid, sensitive and convenient. The *in vitro* and *in vivo* data (Figs. 4b, 5 and 7) showed that the release of daidzein in the chitosan microspheres was slow and lasted for a long time. Compared with oral administration, the absolute bioavailability of daidzein increased in the microspheres by intramuscular injection route, primarily because of the absence of first-pass metabolism.

5. Conclusion

The daidzein-loaded chitosan microspheres were optimized using the spherical symmetric design-response surface methodology by fitting a second-order model to the response data. The experimental values of the microspheres prepared under the optimum conditions were mostly close to the predicted values, with low percentage bias. The release of drug in the microspheres *in vitro* and *in vivo* sustained longtime for 35 days by optimization. Our results concluded that the intramuscular injection of the chitosan microspheres improved the bioavailability of daidzein significantly and the glutaraldehyde crosslinked chitosan microspheres were long acting biodegradable carriers suitable for parenteral delivery of isoflavones.

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