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Characterizing the release mechanism of donepezil-loaded PLGA microspheres *in vitro* and *in vivo*

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ABSTRACT

Characterizing the release mechanism of microspheres *in vitro* and *in vivo* can establish a more convincing *in vitro–in vivo* correlation. In this paper, long-acting donepezil microspheres were prepared using the emulsion solvent evaporation technique. Subsequently, the release kinetics of the drug, as well as the mechanistic indicators of the release, such as glass transition temperature, mass loss, water uptake, and microsphere swelling, were directly measured and analyzed *in vitro* and *in vivo*. Drug release from microspheres was significantly accelerated *in vivo* compared with incubation *in vitro*. The mass loss data indicated that the microsphere exhibits diffusion and erosion via jointly controlled release both *in vivo* and *in vitro*, but the control of erosion on release is enhanced *in vivo*. The microspheres absorb water more quickly *in vivo* compared with incubation *in vitro*. The study showed that there are some unknown substances or biochemical reactions in the *in vivo* environment that cause the microspheres to absorb water quickly and accelerate the degradation of the polymer.

1. Introduction

Alzheimer's disease is one of the most common neurodegenerative diseases in the elderly. The early clinical manifestation is the loss of memory. As the symptoms worsen, the patient gradually loses language ability and cognitive ability and experiences additional mental illnesses, eventually losing physical function. Inhibition of acetylcholinesterase (AChE) activity can alleviate symptoms, and donepezil can inhibit AChE activity. Currently available on the market is donepezil hydrochloride (DP·HCl) tablets that need to be taken daily. However, it is very difficult for patients with Alzheimer's disease to take the drug on time every day. Oral administration of DP·HCl tablets may cause many adverse reactions, such as diarrhea, muscle cramps, fatigue, nausea, vomiting, and insomnia; therefore, it is very meaningful to develop a prescription for long-term and parenteral administration.

To achieve long-acting release of drugs *in vivo*, in addition to the use of nondegradable implants, the most often used formulation is microspheres, which are prepared using degradable biomaterials such as poly (lactic-*co*-glycolic acid) (PLGA). PLGA-based microspheres have many advantages compared with traditional pharmaceutical dosage forms, such as (i) a long effect time of drug, (ii) the ability to be completely degraded *in vivo*, (iii) good biocompatibility, and (iv) the ability improve the bioavailability of some drugs such proteins [1–4]. Therefore, PLGA-based microspheres are widely used in long-acting, sustained-release drug delivery systems. Using different preparation methods, PLGA-based microspheres can encapsulate different drugs such as small molecules, peptides, and proteins [5–7]. Currently, several PLGA-based microspheres are widely used in clinical practice, such as Vivitrol®, Plenaxis®, Risperdal Consta®, Nutropin®, Lupron®, and Nutropin®.

It is very important to study the mechanism by which drugs are released from microspheres that is beneficial to the design and development of an ideal controlled-release formulation. Many reports have shown that the release of drugs from microspheres is monophase, biphase, or (the most common) triphase [8–10]. In triphase, the first phase is the initial drug burst, followed by the lag phase with a constant release rate, and then a secondary burst with approximately zero-order release kinetics. Drug releases from microspheres in two main ways: diffusion and degradation/erosion [11]. The burst phase is usually considered to be caused by the diffusion of drugs on the surface or in the pore of the microspheres, and the later phases of drug release are controlled by the degradation of PLGA. The following is the recognized process of drug release from the PLGA microspheres: (i) the microspheres absorb water and swell in the release medium, (ii) the polymer matrix is destroyed due to hydrolysis of the polymer chain, (iii) internal pores form in the microspheres, and (iv) diffusion occurs through the microsphere pores [12–16]. Although the mechanism of *in vitro* release of PLGA microspheres has been well studied, it is still undetermined whether the release mechanism *in vivo* is the same as *in vitro*.

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The *in vitro* release of PLGA microspheres is in a determined environment, and even if we can simulate a physiological environment, the release of the drug from the microspheres is still different from the release *in vivo*. Many reports have shown that the drug release rate and PLGA degradation rate of PLGA microspheres *in vivo* are faster than *in vitro* [17,18]. Because the environment *in vivo* is very complicated, when the microspheres are injected into the body, the body will react with a foreign-body response and inflammatory response. The inflammatory reaction causes the surface of the microsphere to be surrounded by macrophages. Furthermore, microspheres are exposed to a variety of endogenous materials, such as enzymes, lipids, and complex microenvironmental hydrodynamics [19,20]. All of the above factors will affect the diffusion of the drug from microspheres and the degradation of the polymer *in vivo*, which will contribute to drug release kinetics *in vivo* that are different from release *in vitro*.

Currently, there are a large number of studies on the release of microspheres *in vivo* that determined the plasma drug concentration and efficacy during release, but few studies have directly measured the release behavior of microspheres and characterized the degradation of PLGA *in vivo* [21–23]. Because of the lack of these data *in vivo*, we cannot accurately establish an *in vitro–in vivo* correlation to predict the *in vivo* release behavior of PLGA microspheres. The environment of microspheres *in vivo* is very complex, and some studies have shown the biological factors, including the foreign-body response, endogenous materials, and unknown microenvironmental hydrodynamics, which may affect drug release from PLGA microspheres, but few studies have confirmed how these biological factors affect the release of drugs. Changing the release temperature, pH, and buffer system may lead to a change in the mechanism of drug release from PLGA microspheres *in vitro* [24,25]. We do not need to study how these biological factors change the drug-release rate, and the most important work is to study what changes have occurred in the mechanism of drug release from microspheres in *in vivo* environments.

The purpose of this paper is to investigate the mechanism of drug release from PLGA microspheres *in vitro* and *in vivo* and to analyze what how the mechanisms of release *in vivo* are different from those in *in vitro* release. To understand the release mechanism of microspheres, the microspheres were first released *in vitro* using the sample-and-separate method, and the microspheres were characterized for water uptake, mass loss, and glass transition temperature (Tg) of PLGA, and the morphology of the microspheres was evaluated by scanning electron microscopy. The same microspheres were administered by subcutaneous injection, and microspheres were recovered from the body at a predetermined time point, to allow for characterizing the recovered microspheres in the same way. Through direct comparison and analysis, we can understand what changes in the mechanism of drug release from the PLGA microsphere occur in the complex environment *in vivo* how these differ from those in *in vitro* release. This kind of analysis will clarify what causes the differences in the release kinetics between drug release *in vitro* and *in vivo*. The findings will be beneficial to the development of the *in vitro* and *in vivo* correlation study of PLGA microspheres and will help in more accurately predicting the release performance *in vivo* by *in vitro release*.

2. Materials and method

2.1. Materials and animals

Donepezil (basic, with a purity of 99.66%) was purchased from Bide Pharmatech Ltd. (Shanghai, China). Poly (d,l-lactide-*co*-glycolide) 75:25 copolymers (PLGA, with an average molecular weight of 47 kDa) was purchased from Jinan Daigang Biomaterial Company, Ltd. (Shandong, China). Dichloromethane and methanol were purchased from Yu wang Reagent Company (Shandong, China). Poly (vinyl alcohol) (PVA; 88% hydrolyzed, MW 20,000–30,000) was purchased from Acros Co, Ltd. All other chemicals and reagents used were of analytical grade and obtained commercially.

Kunming mice (male, 22–25 g) were provided by the Experimental Animal Center of Shenyang Pharmaceutical University. This study was subject to approval by the Animal Ethics Committee of Shenyang Pharmaceutical University (approval SYPU-2018-7-11-108) and also was in compliance with the European Community principles for animal experiments. One week before the study, the animals had free access to food and water.

2.2. Preparation of donepezil-loaded PLGA microspheres

Briefly, a mixture of donepezil and PLGA (with a drug-to-polymer weight ratio of 1:5) dissolved in dichloromethane was used as the oil phase (O). A certain volume and concentration of PVA aqueous solution was used as the aqueous phase (W). Then, the organic phase was injected into the aqueous phase using a syringe with a needle under stirring at 500 rpm to form an oil-in-water emulsion. After 20 min, distilled water was added to the emulsion to avoid the aggregation of microspheres. The emulsion was stirred at room temperature for 4 h to evaporate the dichloromethane. The microspheres were washed three times with distilled water, collected by centrifugation at 4000 rpm for 10 min, then freeze-dried to obtain the final donepezil-loaded PLGA microspheres.

Single-factor studies were carried out preliminarily to investigate the influence on the physical characteristics and parameters of microspheres, for example, the stirring rate, PVA concentration, PLGA concentration, solvent type, water–organic phase volume ratio, and donepezil-to-PLGA weight ratio. Central composite design is one of the most frequently employed design-of-experiment techniques. Using the central composite design, we can obtain the optimal formulation parameters and expected response values.

PVA concentration (C_{PVA}), PLGA concentration (C_{PLGA}), and water–organic phase volume ratio (W/O) were selected as the three independent variables in this study, because they had a significant influence on microspheres according to the single-factor studies. The average particle size of the microspheres, particle size distribution, drug loading, and overall desirability (OD; calculated by the Hassan method [26]) were chosen as the dependent variables. Three-factor and five-level experiments were suitable for optimizing the formulation. The coded value of each variable and the corresponding actual value are listed in Table 1.

2.3. Characterization of donepezil microsphere

2.3.1. Determination of drug loading (DL) and encapsulation efficiency (EE)

The 5-mg donepezil microspheres were transferred into 25-mL volumetric flasks, 2.5 mL of dichloromethane was added into the flask, and the sample was sonicated until the microspheres were dissolved. After that, methanol was added into the flask to a volume of 25 mL. The solution was filtered through a 0.22-μm membrane (Jinlong®, NYLON6, 0.22-μm syringe filters, Tianjin, China), and the donepezil concentration was determined by high-performance liquid chromatography (HPLC). The HPLC system was equipped with an ultraviolet detector (271-nm wavelength for donepezil analysis). A Diamonsil C18 (5 μm, 4.6 × 250 mm, Dikma) analytical column was used with a flow rate of

Table 1
Factors and levels for experiments design.

Factors	Levels				
	−1.68,179	−1	0	1	1.68,179
C_{PVA}	0.5	2.12	4.5	6.88	8.5
C_{PLGA}	5	8.04	12.5	16.96	20
V_W/V_O	10	18.12	30	41.89	50

1 mL/min. Methanol/water containing 1% triethylamine and 0.5% glacial acetic acid (65/35) was used as the mobile phase. The standard curve was A = 47344C + 3780.1 (R^2 = 0.9999), where A is the peak areas and C is the concentration of donepezil. The lower limit of quantification was 180 ng/mL, and the lower limit of detection was 50 ng/mL. DL and EE were calculated by using the following equations:

$$\text{DL}(\%) = (\text{Amount of drug in microspheres/Amount of microspheres}) \times 100 \quad (1)$$

$$\text{EE}(\%) = (\text{Actual drug loading/Theoretical drug loading}) \times 100 \quad (2)$$

$$\text{Yield}(\%) = (\text{Weight of microspheres/Weight of total drug and PLGA}) \times 100 \quad (3)$$

2.3.2. Particle size and morphology

Particle size and particle size distribution of donepezil microspheres were measured by a laser particle size analyzer (Sympatec, HELOS/KF, Germany). Briefly, microspheres were resuspended in PVA solution (1%, w/v) to ensure good dispersion for particle size analysis. The morphology of the microspheres was evaluated by scanning electron microscopy (Hitachi S-3400 N, Japan). The sample was coated on a conductive double-sided tape, which was attached to the copper plate, and then coated under an argon atmosphere. The morphology of the microsphere was observed under suitable magnification.

2.4. In vitro release study

The sample-and-separate method was used for the *in vitro* release study. About 4 mg of donepezil microspheres were dispersed in 8 mL 0.1 M phosphate-buffered saline solution (pH 7.4, containing 0.1% w/v NaN3) in and Eppendorf tube and incubated at 37 °C with continuous shaking at 100 rpm. At each predetermined time point (at days 1, 2, 3, 4, 5, 6, 7, and 8 and every 2 days until day 42), the suspension of microspheres was centrifuged at 4000 rpm for 5 min, and then 5 mL of supernatant was collected and replaced with 5 mL of fresh buffer. Finally, the quantity of drug released was assayed by HPLC, which was described in section 2.3.1. After preparing the solution of donepezil from 1 μm/mL to 25 μm/mL using release buffer, the concentration of the sample was quantified by the peak area. The standard curve was A = 47597C + 2464.3 (R^2 = 0.9996), where A is the peak areas and C is the concentration of donepezil. The lower limit of quantification was 180 ng/mL, and the lower limit of detection was 50 ng/mL. The microspheres were collected and dried under vacuum for further microsphere degradation study.

2.5. In vitro degradation study

2.5.1. Quantification of microsphere water uptake, mass loss, and swelling of microspheres

The microspheres were collected on the filter at predetermined time points under vacuum filtration conditions. Then, the surface water was removed under vacuum conditions for 1 min, and the weight of the wet microspheres was determined immediately. The wet microspheres were dried at 30 °C under vacuum conditions for 24 h to a constant weight and recorded. Because the microspheres were stacked together, the water between the microspheres was difficult to remove. To eliminate the error, the dried microspheres were suspended in the release medium and taken out immediately; the surface water was removed, and the weight of the wet microspheres was recorded. At a predetermined time point (days 1, 4, 7, 14, 21, 28, 35, and 42), the microspheres were taken out and observed under a microscope. The diameters of about 50 microspheres were measured, and the average value was taken as the average particle diameter.

The water uptake of microspheres at time (t) was calculated by the following equation:

$$\text{Water uptake of microsphere} = W_1 - W_3/W_2 \times 100\% \quad (4)$$

where W_1 is the wet weight of the microsphere, W_2 is the dried weight of the microsphere, and W_3 is the wet weight of the dried microspheres suspended in the release medium and taken out immediately.

The remaining mass was calculated by the following equation:

$$\text{Mass remaining} = W_2/W \times 100\% \quad (5)$$

where W_2 is the weight of the remaining microspheres after released t days and W is the initial weight of the microspheres before release *in vitro*.

The swelling of the microspheres was calculated by the following equation:

$$\text{Swelling of microspheres} = Rt/R_0 \times 100\% \quad (6)$$

where Rt is the average diameter after t day and R_0 is the initial average diameter of microspheres.

2.5.2. Tg of PLGA in microspheres

The Tg of the PLGA at predetermined time points (days 1, 4, 7, 10, 14, 21, 28, 35, and 42) upon *in vitro* release was analyzed by differential scanning calorimetry (Mettler, DSC1, Switzerland). Briefly, the sample was heated at a rate of 10 °C/min from 0 to 100 °C under an inter-atmosphere of nitrogen at a flow rate of 40 mL/min, then the sample was cooled by a cold trap and heated again at a rate of 5 °C/min from 0 to 80 °C.

2.5.3. Morphology of microspheres

During *in vitro* release, the microspheres were collected at each predetermined time point (days 10, 21, and 42), and after lyophilization, the surface morphology was observed by scanning electron microscopy.

2.6. In vivo release study

To study the release behavior of donepezil from microspheres *in vivo*, we measured the residual amount of donepezil *in vivo*. The microsphere suspension was prepared by dispersing 450 mg of microspheres in 9 mL of normal saline containing CMC-Na (0.5, w/v) and T-80 (0.1%). Forty-two Kunming mice were administered the solution by subcutaneous injection, and each animal was injected with 0.3 mL of microsphere suspension (15 mg donepezil microspheres). Six mice were sacrificed at predetermined time points (1, 4, 7, 10, 14, 21, and 28 days after the injection). After peeling the skin off of the mice, the remaining microspheres were carefully removed, washed with water, and dried under a vacuum.

The residual microspheres were placed in a 50-mL volumetric flask and dissolved in 5 mL of dichloromethane. Methanol was added to 50 mL, filtered through a 0.22-μm filter, and analyzed by HPLC as described in section 2.3.1.

$$\text{Cumulative release} = (M - Mt)/M \times 100\% \quad (7)$$

where M is the content of donepezil and Mt is the residual content of donepezil *in vivo*.

2.7. In vivo degradation study

In vivo degradation studies were performed as described in section 2.5, after the microspheres were separated from the mice at predetermined time points (1, 4, 7, 10, 14, 21, and 28 days after the injection), and the Tg was analyzed by differential scanning calorimetry. The surface morphology and internal structure of the microspheres were observed by scanning electron microscopy.

Table 2
Effect of PLGA on the physical properties of microspheres.

Monomer ratio lactide/glycolide	Molecular weight	Average diameter (μm)	Drug loading (%)
75/25	12,000	41.46 ± 1.55	13.01 ± 0.35
75/25	20,000	45.73 ± 1.83	13.88 ± 0.41
75/25	29,000	51.53 ± 2.31	14.23 ± 0.27
75/25	47,000	70.59 ± 3.21	14.78 ± 0.45
65/35	47,000	64.33 ± 2.87	14.36 ± 0.34
50/50	47,000	55.84 ± 2.46	13.84 ± 0.31

2.8. Statistical analysis

Statistical analysis was performed using SPSS (V19.0). Tg, mass loss, water uptake, swelling of microspheres, and the release kinetics of the drug *in vivo* were compered to incubation *in vitro* using repeated-measures analysis of variance. A value of $P \leq 0.05$ was considered significant.

3. Results and discussion

3.1. Preparation of donepezil-loaded PLGA microspheres

After the single-factor study, we determined the drug-to-polymer weight ratio as 1:5, the emulsification time as 20 min, and the stirring rate as 500 rpm/min. We also studied the effect of different types of PLGA on the particle size and drug loading of microspheres. As can be seen in Table 2, higher drug loading and larger particle size were observed in the microspheres prepared with PLGA with a higher molecular weight. Probably because of the increase in molecular weight, the intrinsic viscosity of the PLGA was higher, the oil phase was not as easily dispersed, and the drug diffusion was reduced. When the ratio of LA/GA is changed from 75/25 to 50/50, the drug loading and particle size of the microspheres become smaller but have little effect. It is likely that PLGA becomes more hydrophilic and the viscosity decreases as the proportion of LA increases; as a result, the drug is more likely to diffuse into the aqueous phase.

The optimal formulation parameters were obtained from response surface analysis using Design Expert software (V10.0.4) with a PVA concentration of 1.005%, PLGA concentration of 11.83%, and W/O of 13.24. The 3D-response surface predicted that the donepezil microspheres prepared by optimal formulation had an average particle size of 72.65 μm, particle size distribution of 0.827, and drug loading of 14.898%.

To validate the model, we prepared five batches of microspheres according to the optimal formulation. The average particle size, particle size distribution, and DL were 71.75 ± 0.35 μm, 0.825 ± 0.007, and 14.88% ± 0.41%, respectively, which were close to the predicted values of equations with SD < 2%. Upon comprehensive consideration, the model is reasonable.

3.2. Characterization of donepezil microsphere

The obtained microspheres show that the loading of donepezil was 14.88% ± 0.41% and the EE was 78% ± 1.2%. The particle size distribution of donepezil microspheres is shown in Fig. 1: the mean particle size was 71.43 μm, and the span value was 0.823. For hydrophobic drugs, after being prepared into the microsphere, the ratio of the surface area and volume decreased as the microspheres became larger in size, leading to a slower release, which is beneficial for the long-acting release of drugs. The morphology of the microspheres under scanning electron microscopy is shown in Fig. 2. The surface of the microspheres was observed to be round and smooth, and no drug crystal or particles adhered to the surface. Many small pores existed on the surface, and the size of the microspheres was uniform.

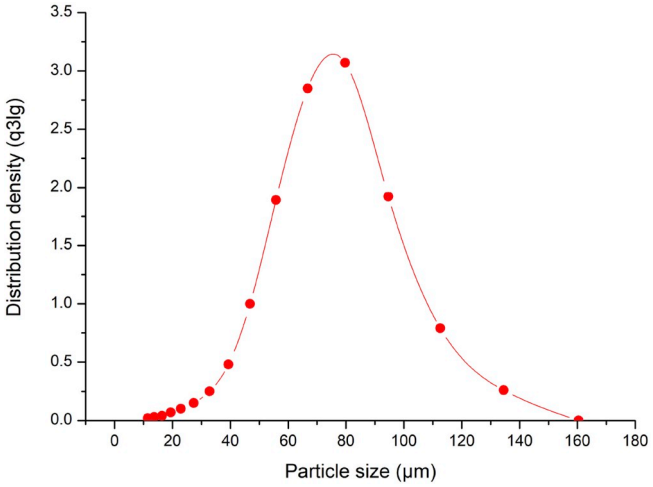

Fig. 1. The particle size distribution of donepezil microspheres.

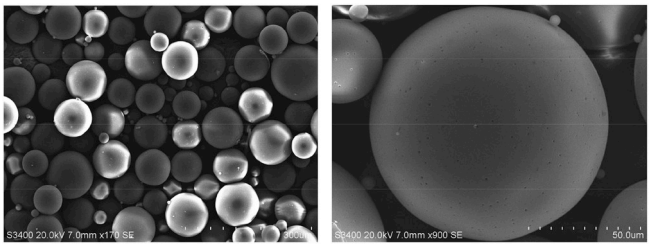

Fig. 2. SEM micrographs of donepezil microspheres.

3.3. Drug release and mechanistic analyses in vitro and in vivo

Donepezil release from microspheres showed a typical triphasic release profile *in vitro*, with a drug initial burst phase of approximately 4%, followed by a lag phase until day 6 ± 1 with a constant release rate, and a secondary burst with approximately zero-order release kinetics. In contrast, we failed to observe a lag phase in the release of donepezil *in vivo*, and the release was much faster than *in vitro* (Fig. 3). The release of donepezil *in vitro* lasted approximately 42 days, but it took only 21 days to release 97% *in vivo*. It is important to understand what changes in the release mechanism contribute to accelerate the release and lag phase and that disappeared *in vivo*.

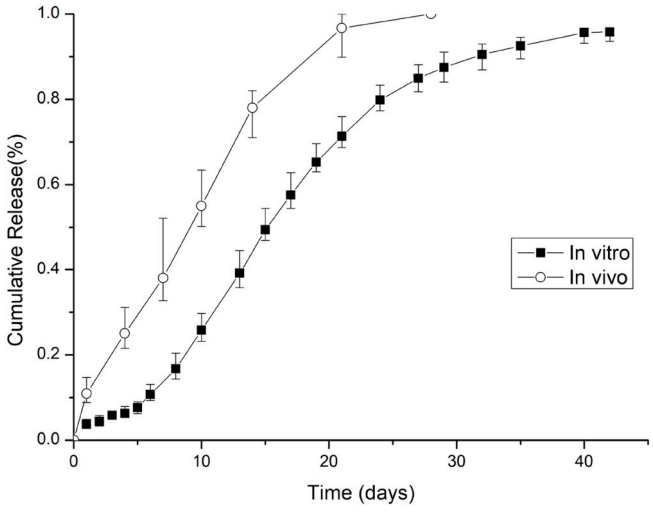

Fig. 3. Donepezil release from PLGA microspheres *in vitro* and *in vivo*. Each point represents the mean ± SD, n = 3–4. $P < 0.001$.

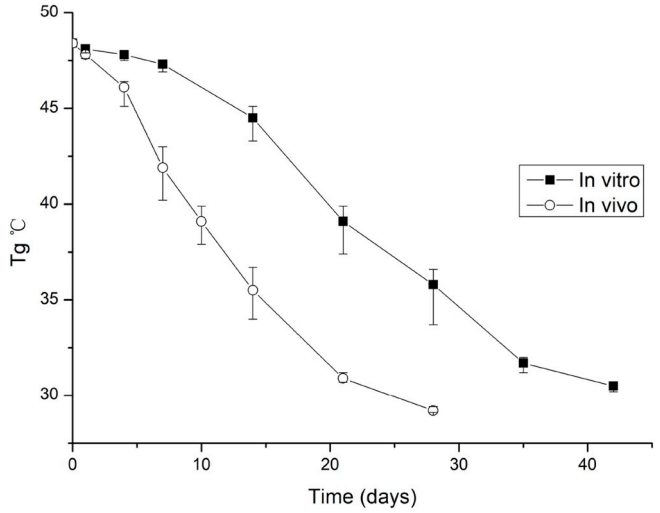

Fig. 4. *In vitro* and *in vivo* microspheres T_g values change with time. Each point represents the mean ± SD, n = 3–4. P < 0.001.

By investigating some important parameters including PLGA degradation, water uptake, swelling rate, erosion of microsphere, and morphological changes, we analyzed the difference in mechanisms in *in vitro* and *in vivo release*. The degree of degradation of PLGA can be measured indirectly. It has been reported that the Tg of a polymer depends on the molecular structure of the polymer [27,28]. Therefore, the degradation of PLGA can be described by the Tg value. As seen in Fig. 4, the Tg value from 48.4 °C to 30 °C takes about 40 days *in vitro* but only 20 days *in vivo*. At the same time, it can be seen that the Tg value decreases slowly in the initial stage, but the Tg value decreases rapidly *in vivo*. The result of the change of the Tg value indirectly indicates that the hydrolysis rate of PLGA *in vivo* is significantly faster than that *in vitro*, especially in the initial stage of degradation.

Erosion is an important mechanism affecting the release of drug from polymer. If erosion is a major mechanism and donepezil is uniformly distributed in the polymer PLGA, as the polymer loses mass, the drug would release at a similar rate [15,17]. As show in Fig. 5, the rate of erosion of microspheres *in vivo* is significantly faster than *in vitro*. In the first 10 days of release *in vitro*, the mass loss of the polymer does not change much; after 14 days of release, the polymer lost only 8.42% of mass. When the drug is completely released, the mass loss of the polymer is 50.746%. In contrast, the microspheres were released *in vivo*

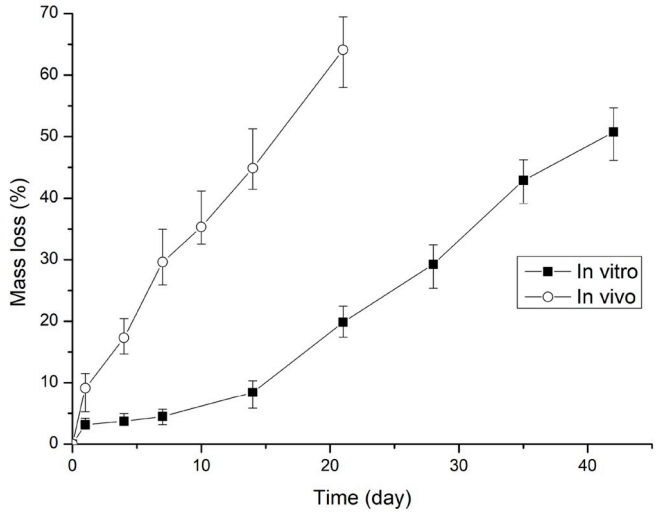

Fig. 5. *In vitro* and *in vivo* mass loss of donepezil microspheres. Each point represents the mean ± SD, n = 3–4. P < 0.001.

for 1 day, and the polymer mass loss was 9.13%. When the drug release is 96.7% in 21 days, the polymer mass lost is 64.12%. We analyzed quantitatively the relationship between dissolution and release by performing nonlinear regression analysis of release and mass loss. There were two important parameters: $t_{50,release}$ (the time it takes for the drug to release 50%) and $t_{50,erosion}$ (the time it takes for the polymer to lose 50% of its mass). Values of $t_{50,release}/t_{50,erosion}$ varied from 0 to 1. The larger the value of $t_{50,release}/t_{50,erosion}$, the more significant the erosion control of drug release from PLGA microspheres. In this study, the time taken for the release of 50% of donepezil from the microspheres *in vitro* was 15.1 ± 0.6 days, and the time taken to reach 50% mass loss was 42 ± 4 days: $t_{50,release}/t_{50,erosion}$ = 0.3595. *In vivo* research shows the time taken to reach 50% release was 9.25 ± 1.5 days, and the time taken to reach 50% mass loss was 15.85 ± 3.1 days: $t_{50,release}/t_{50,erosion}$ = 0.5836. We can see that the ratio of $t_{50,release}/t_{50,erosion}$ *in vivo* is significantly higher than observed *in vitro*. Although the rate of release and erosion are all accelerated *in vivo*, the erosion rate is faster than drug release at the same time scale, indicating that there are some factors *in vivo* that change the release mechanism, and drug release from microspheres is more dependent on erosion. Amy C. Doty et al. [17]. also conducted a similar study in which two different types of PLGA were used to prepare microspheres. Both formulations exhibited erosion-controlled release *in vitro*, but only microspheres prepared with a low-molecular-weight, free acid–terminated PLGA exhibited the same release mechanism *in vivo*. Microspheres prepared with a higher-molecular-weight, ester-terminated PLGA exhibited an osmotically induced/pore diffusion mechanism *in vivo*.

Water uptake is also an important factor affecting the release of drug by multiple processes. Previous authors have described the initial degradation of PLGA as being caused by the absorption of water in the microspheres, and the polymer chain is hydrolyzed, followed by ester bond cleavage and a subsequent decrease in Mw [29]. On the other hand, the secondary burst phase has been suggested to be associated with water uptake and to form pore networks on the surface of and inside the microspheres. There are many small pores on the surface of the microsphere before it is immersed in the release medium. In the initial stage of release, the pores may be too small to transport the drug outside the microspheres. When the microspheres absorb water, they form more pore-transport networks on the surface of and inside the microspheres to accelerate drug transport [30,31]. After the microspheres absorb water, the polymer inside the microsphere begins to hydrolyze first. The degradation production and dissolved drug continuously increase the osmotic pressure inside the microspheres, which is helpful for drug transport, as the erosion of the polymer produces more pores, thus increasing the rate of drug release [32]. As show in Fig. 6, after the microspheres began to release *in vitro*, the water uptake increases sharply in 1 day, and then the amount of water absorption is no longer increased substantially until after 10 days of incubation. The water absorption of microspheres *in vivo* has changed significantly compared with incubation *in vitro*. At the beginning of release, the water content was dramatically increased, and more water content at each time point was observed *in vivo* relative to incubation *in vitro*. When the drug was completely released, the content of water absorbed by the microspheres *in vivo* is greater than during release *in vitro*. The increase in water uptake in the microspheres accelerated the hydrolysis of polymer PLGA and subsequent erosion, and at the same time, the microspheres absorb more water *in vivo* than incubation *in vitro*, consistent with the mass loss data described above. Keiji Hirota et al. [15]. Prepared leuprolide-loaded PLGA microspheres, which were incubated in different release media. Data related to drug release kinetics, water absorption, mass loss, and PLGA molecular weight were recorded. Only when the microspheres are incubated in a buffer medium of pH 7.4, with the increase of water absorption, does the molecular weight of PLGA decrease, and the release of drug increases. In other release environments, this correlation will change.

Further investigation into the mechanisms causing accelerated

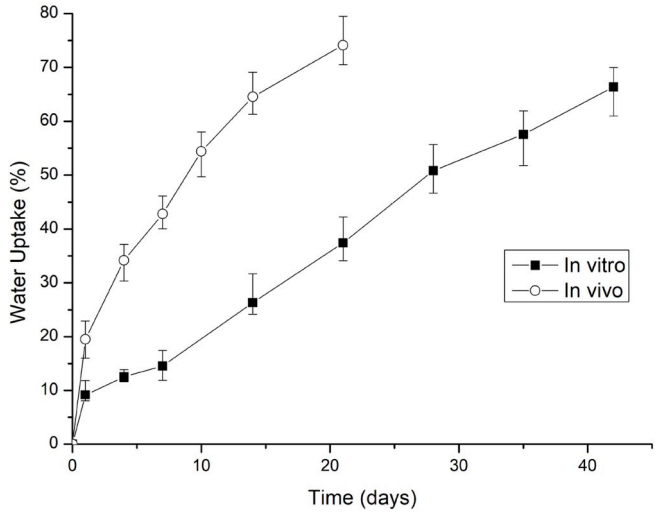

Fig. 6. *In vitro* and *in vivo* water uptake of donepezil microspheres. Each point represents the mean ± SD, n = 3–4. P < 0.001.

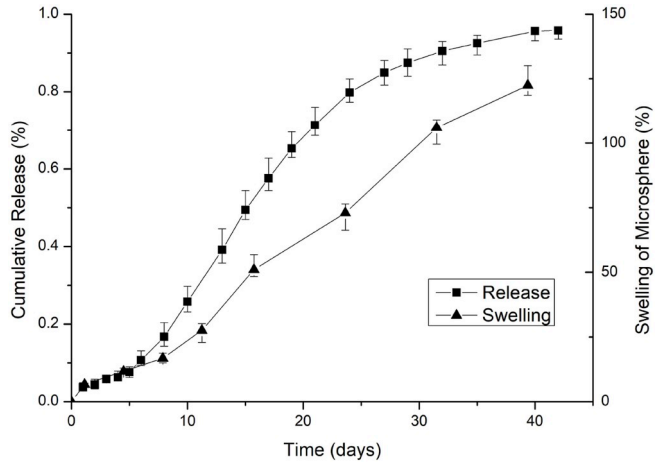

Fig. 7. Relationship between *in vitro* donepezil release kinetics and the swelling kinetics of PLGA microspheres. Each point represents the mean ± SD, n = 3–4.

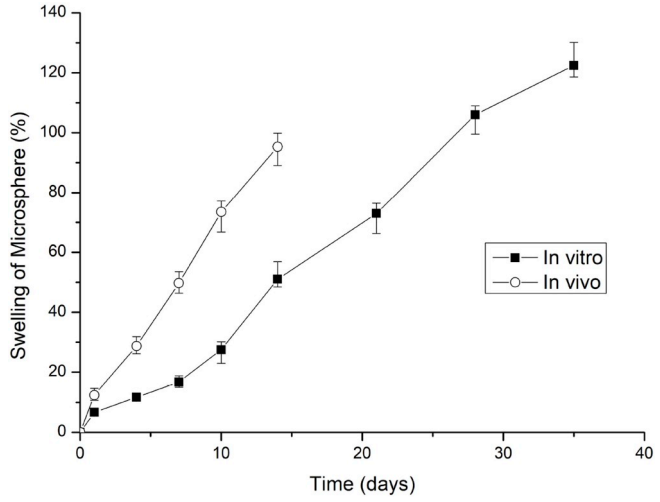

Fig. 8. *In vitro* and *in vivo* swelling of donepezil microspheres. Each point represents the mean ± SD, n = 3–4. P < 0.001.

donepezil release *in vivo* included measuring the size change of microspheres over the time course of release. As show in Fig. 7 (the *in vitro* donepezil release kinetics and the swelling kinetics of PLGA microspheres), in the first 5–7 days, the volume of the microspheres did not change much, and then the microspheres began to swell continuously. As can be seen in the figure, the time at which the microspheres' size changes dramatically is consistent with the onset of the third drug release phase (secondary burst). This phenomenon may be due to the high degree of polymer matrixing in the initial stage of incubation, preventing the swelling of the microspheres. However, when the microspheres absorb water, the molecular chains of the polymer begin to hydrolyze, and the degree of entanglement of the polymer chains is reduced, which fails to resist microsphere swelling. Once the microspheres begin to swell, more water is attracted into the microspheres, and so the drug molecules are easy to transport [33]. In other words, the degree of swelling of the microspheres determines the mobility of the drug molecules in the polymers, which determines the release rate of the drug. The swelling kinetics of the microspheres *in vivo* is completely different from that incubated *in vitro* (Fig. 8). The microspheres swell more rapidly (the size expands 12.37% *in vivo* for 1 day vs 6.65% *in vitro*), and the period in which the particle size of the microsphere remains unchanged disappears compared with release *in vitro*. The release kinetics and swelling kinetics of microspheres are consistent both *in vivo* and *in vitro*. In a study by H. Gasmi et al. [14], the authors prepared eight different drug loadings of ketoprofen PLGA microsphere formulations and three different particle size PLGA microsphere formulations, which were separately incubated in phosphate buffer at pH 7.4 (containing 0.02% Tween 80). They found that the microsphere size remained approximately constant for the first 7 days and then increased significantly, independent of the drug loading and initial size. This study found similar results, in that the onset of microsphere swelling was consistent with the onset of the third (secondary burst) drug release phase. In addition, changes in particle size were accompanied by morphological changes: the surface of the particles was initially smooth, but became more irregular over time. In another study, Amélie Gaignaux et al. [29]. Prepared clonidine-loaded PLGA microspheres, and the *in vitro* release kinetics of the drug showed only burst and second burst phases without a lag phase. Interestingly, the particle size of the microspheres continues to increase upon incubation *in vitro*, and there is no period in which the particle size remains constant.

As we can see in Fig. 9, the morphology of the microspheres after 7 days of *in vitro* release shows that the number of pores on the surface of the microspheres increases and the pore size becomes larger. After a period of release, as shown in Fig. 10, many microspheres were broken. It can be seen from the broken fault plane that the degradation of the polymer inside the microsphere is more serious than at the surface. The surface depression of the unbroken microspheres also proves that the degradation of the microspheres is from the inside to the outside. The degradation of microspheres *in vivo* is also from the inside to the outside, but the degradation rate is significantly faster than *in vitro*.

Donepezil releases from the microspheres by diffusion and degradation of the PLGA. In the early stage of *in vitro* release, the degradation rate of the PLGA is very slow. Almost only donepezil on the surface and in the internal pores of the microspheres dissolve and diffuse into the release medium. As the water absorption of the microspheres increases, the high-degree polymer matrix becomes easily destroyed, accelerating the degradation of the PLGA, and donepezil is rapidly released from the microspheres. In contrast, we failed to observe a lag phase in the release of donepezil *in vivo*, and the release was much faster than *in vitro*. By characterizing the Tg, mass loss, water uptake, swelling of microspheres *in vivo*, we found that PLGA degraded rapidly from the beginning *in vivo*, resulting in faster release of donepezil from the microspheres than incubation *in vitro*.

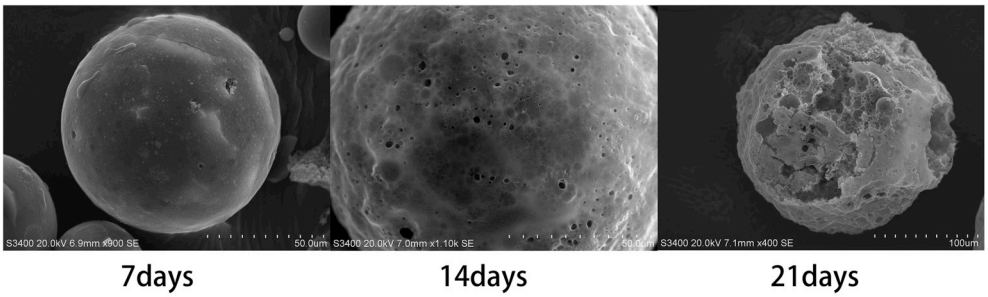

Fig. 9. Pores change on the surface of microspheres in different time periods *in vitro*.

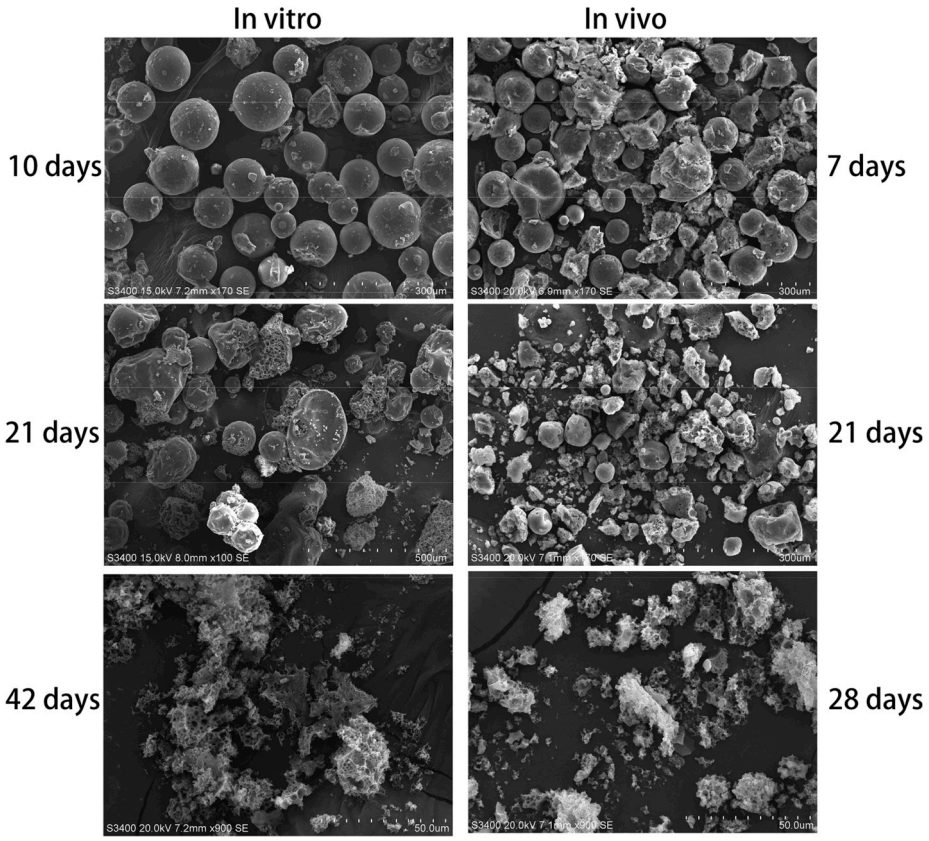

Fig. 10. Morphology of microspheres in different time periods *in vitro* and *in vivo*.

4. Conclusions

By retrieving the microspheres from mice at a predetermined time point, we can directly measure the content of drug released and characterize the release mechanism of the microspheres. The results showed that the degradation rate of PLGA, mass loss, water uptake, and swelling of microspheres *in vivo* all increased compared with incubation *in vitro*. At the same time, scanning electron microscopy showed that the microspheres degraded from the inside to the surface both *in vitro* and *in vivo*. From drug release kinetics and mass loss kinetics, we can see that the microsphere exhibits a diffusion and erosion jointly controlled release both *in vivo* and *in vitro*, but faster rate of mass loss relative to the release kinetics indicates that the control of erosion on release is enhanced *in vivo* (the ratio of $t_{50,release}/t_{50,erosion}$ *in vivo* is significantly higher). In analyzing the results of the study, which include the release kinetics of the drug and characterization data of the microspheres, the rate of water uptake is the main reason for the difference in the mechanism of release *in vitro* and *in vivo*. This study indicated that there are some unknown substances and biochemical reactions *in vivo* that contribute to the ability of PLGA microspheres to absorb water quickly. In future research, we should focus on finding the cause of this phenomenon in the *in vivo* environment. Ultimately, we can simulate a similar environment to the body and use the basis of the same release mechanism to predict the release behavior of PLGA microspheres *in vivo*.

Conflicts of interest

We declare that we have no conflicts of interest to this work.

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