In Vitro and *in Vivo* Characterization of Huperzine A Loaded Microspheres Made from End-Group Uncapped Poly(*d,l*-lactide acid) and Poly(*d,l*-lactide-co-glycolide acid)

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The purpose of this work was to develop biodegradable microspheres for long term delivery of a potent acetyl cholinesterase inhibitor, huperzine A (Hup-A), which is of interest in the palliative treatment of Alzheimer's disease. Microspheres were successfully prepared with specifically end-group uncapped poly(*d,l*-lactide acid) and poly(*d,l*-lactide-co-glycolide acid) using a simple o/w solvent evaporation method. The morphology, particle size and size distribution, drug loading capacity, drug entrapment efficiency (EE) and *in vitro* drug release were studied in detail. It was found that the terminal group and the inherent viscosity (IV) of the polymers played key role in the drug encapsulation: higher EE was achieved with end-group uncapped and low IV polymers. *In vitro* drug release from microspheres made from the selected three kinds of polymers revealed sustained release of Hup-A without significant burst release. Preliminary pharmacokinetic study following subcutaneous injection of Hup-A loaded microspheres illustrated the sustained release of the drug over 6—8 weeks at clinically relevant doses *in vivo*. The studies demonstrated the feasibility of long term delivery of Hup-A using biodegradable microspheres.

Key words biodegradable microsphere; huperzine A; end-group uncapped poly(*d,l*-lactide acid); end-group uncapped poly(*d,l*-lactide-co-glycolide)

Alzheimer's disease (AD), one of the most common dementia, has become a major public health issue as the proportion of elderly increases in the population. Studies focusing on the pathogenetic mechanism have revealed that cholinergic abnormalities are associated with the disturbance of cognitive function in patients with AD, and inhibition of the brain acetyl cholinesterase to increase the synaptic concentration of acetylcholine may improve the cognitive dysfunction.[1,2] Huperzine A (Hup-A), a novel alkaloid isolated from the Chinese herb *Huperzia serrata* (THUNB.) TREV., has been proven to be one of the most promising agents for palliative treatment of AD, based on its centrally active and long-lasting inhibition of acetyl cholinesterase, high bioavailability and minimal side-effects.[3,4] It is presently approved for human use in China for treatment of AD and also used as supplementary drug in the U.S.A. for correction of memory impairment.[5] The oral bioavailability of Hup-A is high and the half-life is about 288 min,[6] however, chronic medication period was often required to improve the cognitive dysfunction. It is especially hard for patients with this kind of problems to guarantee the therapy. A controlled release dosage form for long periods of time avoids daily administration, and is therefore the best way to improve patient compliance and to secure the therapeutic efficiency.

Biodegradable microspheres have been widely used for an injectable depot formulation of various small molecular weight drugs, peptides and proteins, and poly(*d,l*-lactide acid) (PLA) and poly(*d,l*-lactide-co-glycolide) (PLG) are the most widely used and well-characterized materials for the preparation of biodegradable microspheres.[7—10] There are several ways of preparing microspheres according to the properties of drugs, among which the oil in water (o/w) solvent evaporation method is one of the most conventional and widely used ways. However, it was frequently reported that the method is not suitable for moderately water-soluble and water-soluble drugs due to unacceptable low encapsulation efficiencies.[11,12] In this work specifically end-group uncapped PLA/PLG polymers with relatively low molecular weight were chosen to encapsulate Hup-A, which has a moderate water solubility of at least 850 μg/ml between pH 3.3—10.3 and a higher solubility at the presence of an emulsifier, using o/w solvent evaporation method (Chinese Patent Appl. 200510046872.2), and the EE turned out to be as high as 60% for the best formulation. While with the commonly used end-group capped PLA/PLG polymers, little or no drug content was observed. In this work, *in vitro* and *in vivo* performances of the Hup-A loaded microspheres were investigated.

Experimental

Materials Hup-A and huperzine B (hup-B) were obtained from Wanbang Pharmaceutic Co. Ltd. (Zhejiang, China). Various end-group capped poly(*d,l*-lactide) (PLA) and poly(*d,l*-lactide-co-glycolide) (PLG), and end-group uncapped poly(*d,l*-lactide) (PLA-H) and poly(*d,l*-lactide-co-glycolide) (PLG-H) were purchased from Biodegradable Polymers (Birmingham, U.S.A.), including PLG 50/50 (IV=0.17, 0.59) (A, B), PLG 75/25 (IV=0.25, 0.59) (C, D), PLG-H 75/25 (IV=0.22) (E), PLG-H 85/15 (IV=0.27, 1.3) (F, G) and PLA-H (IV=0.15. 0.45) (H, I). The characteristics of the polymers are summarized in Table 1. Polyvinyl alcohol (17—88) was purchased from Acros Organics (New Jersey, U.S.A.). Dialysis tubes (MWCO 8000—14400) was supplied by Beijing Xiasi Biotech Co. Ltd.

Table 1. Characteristics of the Polymers

Polymer ID	Polymer type	Terminal group	Inherent viscosity
A	50/50 PLG	Capped end	0.17
B	50/50 PLG	Capped end	0.59
C	75/25 PLG	Capped end	0.25
D	75/25 PLG	Capped end	0.72
E	75/25 PLG	Acid end-group	0.22
F	85/15 PLG	Acid end-group	0.27
G	85/15 PLG	Acid end-group	1.3
H	PLA	Acid end-group	0.15
I	PLA	Acid end-group	0.45

(Beijing, China). Dimethylene chloride (DCM) of HPLC grade was obtained from Shenyang Chemical Reagent Factory (Shenyang, China). All other chemicals were obtained commercially as analytical grade reagents and were used without any further purification.

Microspheres Preparation Hup-A loaded microspheres were prepared according to the well-established solvent evaporation technique, using an o/w emulsion method.[12,13] Briefly, the polymers (40%, w/v) and the drug (4%, w/v) were co-dissolved in 1 ml DCM to form the oil phase. Then the polymer–drug solution was emulsified in 80—120 ml dispersion phase (2% PVA aqueous solution) under vigorous mechanical stirring. Ten minutes later, the stirring rate was slowed down (approximately 600 rpm) to allow the complete evaporation of organic solvent at room temperature (*ca.* 25 °C) and ambient pressure for at least 4 h. The solidified microspheres in the dispersion phase were centrifuged, washed with deionized water, collected on a cellulose acetate filter (0.45 μm) and dried under vacuum at room temperature for at least 48 h before any further study.

Microspheres Characterization Microspheres Morphology: The surface morphology and internal structures of the microspheres were observed with an SHIMADZU Scanning Electron Microscope (SHIMADZU SSX-550, Japan). Microspheres were mounted onto stubs using double-sided tape and coated with gold under an argon atmosphere using a gold sputter module in a high-vacuum evaporator before observation.

Particle Size and Size Distribution: The microspheres were analyzed for their average particle size and size distribution by laser diffractometry using a Beckman Coulter LS32 (Beckman Coulter, U.S.A.) after dispersion in about 120 ml distilled water in the detecting cell. Average particle size was expressed both as the volume mean diameter V_{md} and the number mean diameter N_{md} in microns±S.D.

Drug Content and Encapsulation Efficiency To determine the content of Hup-A in the microspheres, accurately weighed amount of microspheres (about 10 mg) was dissolved with 1 ml acetonitrile in a 25 ml volumetric flask, then 0.1 M HCl was added to volume to precipitate the polymers. The flask was shaken vigorously to complete the precipitation and the amount of Hup-A in the solution was analyzed by HLPC after centrifugation (4000 rpm×5 min) and filtration through a 0.45 μm membrane. The HPLC system consisted of a pump (L-7100, Hitachi, Japan) and a UV detector (L-7420, Hitachi, Japan). A C18 reverse-phase chromatography column (Diamonsil^{TM} 5 μm C18, 250×4.6 mm, Dikma Technologies) was used. The mobile phase was phosphonic acid–triethylamine buffer (pH 3.2)–water (50/50, v/v) and detection was carried out at 310 nm. The injection volume was 20 μl and the flow rate was 1.0 ml/min.

The drug content and EE of the microspheres were calculated as follows:

drug content (% w/w)

=[(amount of Hup-A in the MS)/(weight of the MS)]×100

EE (% w/w)=[(actual drug content)/(nominal drug content)]×100

***In Vitro* Drug Release Study** The *in vitro* drug release from microspheres was performed in phosphate buffer solution, pH 7.4, using the dialysis method. The dialysis tube (MWCO 8000—14400) was soaked in preheated double-distilled water before use, and then 5 ml pre-warmed (37 °C) PBS was poured into the dialysis tube with a weighed amount of microspheres dispersed in it. Both ends of the tube were fixed by clamps. The tube was placed in a conical flask and 50 ml pre-warmed (37 °C) release medium was added. The conical flasks were placed into an air bath thermostatic shaker (ZD-85A, Ronghua Instrument Factory, Jiangsu, China) at 37 °C, and then shaken horizontally at 60 rpm. At fixed time points, the medium in the conical flask was completely removed and replaced with fresh release medium to maintain sink condition and avoid possible contamination. The samples were filtered before analyzed by HPLC method. All experiments were carried out in triplicate.

***In Vivo* Evaluation** Male Wistar rats (provided by Shenyang Pharmaceutical University Animal Center) weighing *ca.* 300 g were used to evaluate *in vivo* performance of Hup-A loaded microspheres. All animal experiments complied with the requirements of the National Act on the use of experimental animals (People's Republic of China). The animals were maintained under constant environmental conditions (22±1 °C, 50±5% relative humidity). Food and water were available *ad libitum*. Selected microspheres formulations were injected subcutaneously at the back of rats (*n*=6) after reconstitution in a viscous aqueous vehicle (1% carboxymethylcellulose, w/v and 0.5% Tween-80, v/v). Blood samples were collected from the orbital vein at specific time points into heparinized tubes, and plasma was separated immediately by centrifugation and stored at −20 °C until analysis.

Plasma Level Quantification Plasma drug levels were determined by

HPLC analysis as described above. 0.5 ml plasma sample was placed into a centrifuge tube and then 50 μl basifying solution, 10 μl internal standard solution (huperzine B) and 1.5 ml DCM were added. The mixture was vortex-mixed for 5 min, then centrifuged at 4000 rpm for 10 min. The organic phase was then transferred into another centrifuge tube and was dried under N$_2$ in a 40 °C water bath. The residue was re-dissolved by 50 μl PBS, pH7.4, 20 μl of the sample was injected into HPLC system. The limit of detection was 1 ng/ml.

Results and Discussion

The Characteristics of Microspheres Hup-A was encapsulated within microspheres by conventional o/w solvent evaporation method. Three microspheres formulations were successfully prepared with polymer E (PLG-H 75/25, IV=0.22), F (PLG-H 85/15, IV=0.27) and H (PLA-H, IV=0.15). Figure 1 shows SEM pictures of the three microspheres. They appeared to have similar smooth spherical morphologies, however, the cross-section view of the microspheres revealed remarkably different internal structure morphologies. Microspheres made from polymer E and H (E-MS, H-MS) exhibited porous structures while microspheres made from polymer F were quite dense. Moreover, the porous structures of E-MS and H-MS were also quite different. It can be seen that the E-MS had a pronounced nano-porous and reticulated structure, while the pore size of H-MS was much smaller and the internal structure was denser than that of E-MS. The typical explanation of the formation of the pore is that during the solvent evaporation, embryonic methylene chloride droplets containing dissolved polymers were hardened from the surface upon contact with aqueous phase; the polymer solidification of inward was accompanied with in-fluxing of water and out fluxing of solvent which resulted in the formation of a nano-porous and reticulated skeletal backbone structure. The morphology of microspheres depends on the rate of polymer precipitation and solvent removal at the interface. It is generally regarded that the semi-crystalline PLA undergoes crystallization in the bulk internal phase precipitated faster and leaves a more porous structure than the amorphous PLG. In this case we didn't find this kind of relationship. This might due to different properties of end-
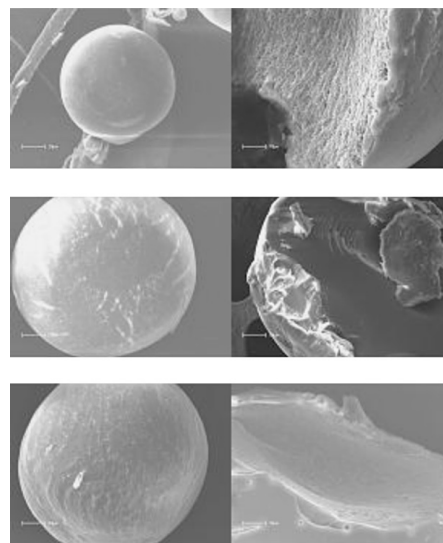

Fig. 1. SEM Micrographs of Microspheres Made from Polymer E, F and H (from Top to Bottom)

The scale bars represent 20 μm and 10 μm for the appearance (right) and section (left) graphs, respectively.
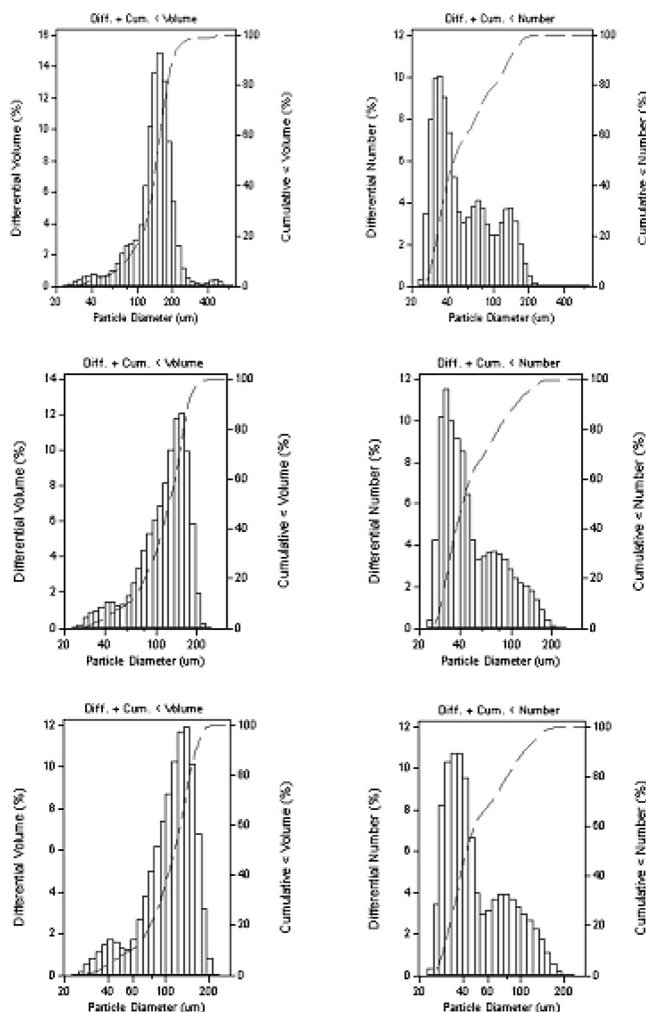
Fig. 2. Volume and Number Mean Particle Size Distribution and Accumulative Curves of E-MS, F-MS and H-MS (from Top to Bottom)

Fig. 3. Drug Encapsulation Efficiency of Microspheres Made from Different Polymers (Mean±S.D., $n=3$)
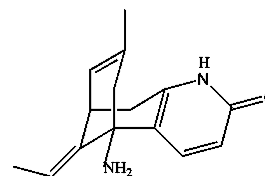
Fig. 4. Molecular Structure of Huperzine A

group uncapped polymers from commonly used end-group capped polymer. More work should be done on the formation of different morphologies of the E-MS, F-MS and H-MS, thus its effect on the drug release.

The V_{md} and N_{md} of E-MS, F-MS and H-MS ($n=6$) were $142.8\pm57.98\,\mu$m and $64.13\pm42.92\,\mu$m, $120.3\pm42.42\,\mu$m and $55.83\pm33.36\,\mu$m, and $113.7\pm38.93\,\mu$m and $56.42\pm32.09\,\mu$m, respectively (Fig. 2). There were no noticeable differences in particle size among the three different microspheres having different polymer compositions. The maximum particle size was all below $250\,\mu$m which was suitable for subcutaneous injections.

Drug Content and Encapsulation Efficiency Several end-group capped or uncapped polymers with different inherent viscosity, *i.e.*, different molecular weight, were employed to encapsulate the drug with same preparation conditions. Figure 3 shows the EE of Hup-A in microspheres made from different polymers with the optimized formulation. Due to microspheres agglomeration during the centrifugation and drying process, discrete microspheres could not be obtained from polymer A (PLG 50/50, IV=0.17). We attributed this to the low polymers L/G ratio (50/50) and relative low inherent viscosity (0.17) which lend it the fast hydration property. The other three end-group capped polymers, B (PLG 50/50,
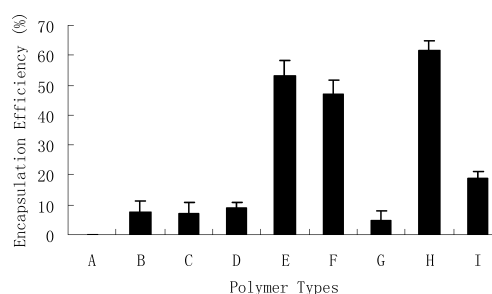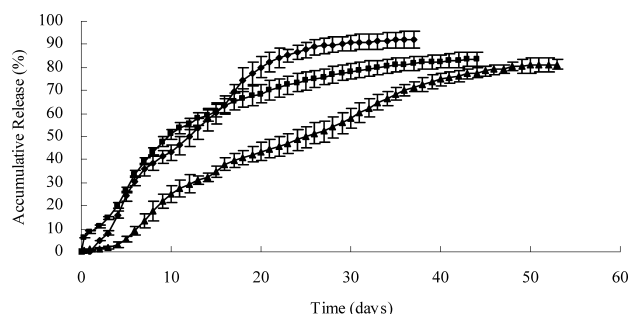
IV=0.59), C (PLG 75/25, IV=0.25) and D (PLG 75/25, 0.59), all resulted in negligible drug content (<1%) and unacceptable low EE. Much higher drug content and EE were obtained with the end-group uncapped polymers except polymer G (PLG-H, IV=1.3). Moreover, the drug content and EE of F-MS (PLG-H 85/15, IV=0.27) and H-MS (PLA-H, IV=0.15) were higher than that of G-MS (PLG-H 85/15, IV=1.3) and I-MS (PLA-H, IV=0.45), respectively. The results indicated that the state of the terminal groups and the inherent viscosity (*i.e.* MW) of the polymers were two critical factors in the drug encapsulation. Hup-A was readily to be encapsulated into the end-group uncapped polymers and higher EE was achieved with end-group uncapped polymers with lower inherent viscosity. Since Hup-A is an alkaloid and apparently bears positive charge at ionized state, it was suspected that ionic interaction between basic amino residue of the drug and uncapped carboxylic acid end group in the uncapped polymers played a key role in the drug encapsulation (The molecular structure of Hup-A is shown in Fig. 4.). This speculation was in accordance with several previous reports that successfully encapsulated water soluble basic drugs using solvent evaporation technique.[14—17]

According to Okada *et al.*[16,17] and Heya *et al.*,[15] the polymer molecules were arranged around the drug cores in a similar way to surfactant molecules in a micelle due to the ionic interaction between the basic amino acids of the drug and the terminal carboxylic anions of the polymer, forming a rigid structure of the microspheres. Thus, a barrier against diffusion of the hydrophilic drug was created by the hydrophobic long alkyl chains of the polymer and the drug was effectively entrapped in the polymers during the in-water drying process.

O/w solvent evaporation method has been widely reported to be only suitable for water insoluble drugs, but this was not necessarily true. With the proper selection of polymers (polymer E, F and H), Hup-A, a moderate water soluble drug (water solubility of at least $850\,\mu$g/ml between pH 3.5—10.3) were successfully encapsulated into biodegradable microspheres. For the optimized formulation, the drug content was $5.36\pm0.28\%$, $4.53\pm0.43\%$ and $6.16\pm0.32\%$ for E-MS,

Table 2.   Drug Encapsulation Efficiency in Different Polymers

Polymer ID	Mean particle size (μm)	Drug content (%)	EE (%)
A	—	—	—
B	64.81±40.98	0.72±0.37	7.35±3.78
C	57.23±38.68	0.68±0.36	7.01±3.71
D	73.99±33.86	0.87±0.21	8.79±2.12
E	64.13±42.92	5.36±0.28	53.04±5.16
F	55.83±33.36	4.53±0.43	47.08±4.38
G	113.99±31.86	0.43±0.32	4.57±3.40
H	56.42±32.09	6.14±0.32	61.43±3.52
I	63.12±24.52	1.88±0.21	18.99±2.12

Fig. 6.   Plasma Levels of Hup-A in Rats after Subcutaneous Injection of 3 mg Drug Contained in Each of the Three Different Microspheres Formulations: End-Group Uncapped PLG 75/25, IV=0.22, Drug Content 5.30% (E-MS, ◆); End-Group Uncapped PLG 85/15, IV=0.27, Drug Content 4.63% (F-MS, ▲); End-Group Uncapped PLA, IV=0.15, Drug Content 6.12% (H-MS, ■) (Mean±S.D., n=6)

Fig. 5.   *In Vitro* Drug Release Profiles from E-MS (◆), F-MS (▲) and H-MS (■) (Mean±S.D., n=3)

F-MS and H-MS (n=6), respectively. Best EE was between 45—65% for the three kinds of polymer as shown in Table 2. However, EE of *ca.* 45—65% was still not satisfactory; further work has to be done.

***In Vitro* Release**   The *in vitro* release profiles of Hup-A loaded E, F and H-MS are shown in Fig. 5. The *in vitro* drug release profiles didn't exhibit triphasic patterns which were typical for bulk-eroding monolithic-controlled-release systems as previously reported by Bhardwaj *et al.* and Kim *et al.*[18,19] There were only moderate burst release effect of less than 10% within the first 24 h with E and H-MS and no obvious burst release was observed with F-MS. The burst release was attributed to the rapid leaching of the drug through pores and channels of E-MS and H-MS. Extended releases can be seen during the 5—6-week incubation period and more than 80% of the loaded drug was release at the end of incubation. The two formulations with porous inter structures (E-MS and H-MS) demonstrates a much faster release than the third formulation (F-MS). However, the release trends cannot be explained solely by morphological factors in the microspheres. The different drug release profiles can also be related to copolymer composition and molecular weight of the polymer. The microspheres prepared from polymer with higher glycolide content and lower molecular weight degrades faster than microspheres prepared with lower glycolide content and higher molecular weight. The weight of the microspheres at the end of drug release (after drying) was about 30%, 56% and 46% of the initial release weight of E-MS, F-MS and H-MS, respectively. The weight loss proportions of the three microspheres were in accordance with the overall drug release rate: drug release from E-MS was the fastest and drug release from F-MS was the most slowly and constant.

In most reports, the *in vitro* release experiments were performed by incubation of microspheres in certain volume of
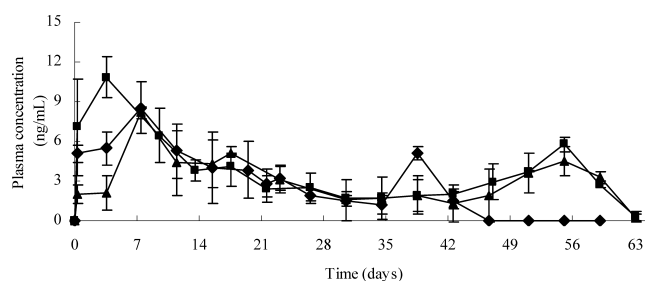
release medium, aliquot of samples were withdrew after centrifugation and/or filtration during which undesired loss of microspheres and disturbance of physical characteristics of microspheres are unavoidable. There is also a pH reduction problem due to the polymer degradation. Therefore, we chose the dialysis method to study the *in vitro* drug release to avoid the above problems.[20] According to Woo *et al.*,[21] the *in vitro* release profile obtained by the dialysis method permitted better correlation with *in vivo* release. Dialysis membrane with a MWCO of 8000—14400 was chosen for the fast equilibrium of drug (MW 242.32) between the inside and outside of the membrane.

***In-Vivo* Hup-A Level**   For pharmacokinetic study, E-MS, F-MS and H-MS were injected subcutaneously to each group of rats (n=6) and Hup-A plasma concentrations were monitored for 9 weeks. The common feature of the plasma level profiles was an initial increase within 8 h after injection and reached a C_{max} within about 1 week (Fig. 6). An extended plasma level was followed. The drug levels remained above *ca.* 2 ng/ml for 6 weeks for the E-MS and more than 8 weeks for the other two formulations. All the three formulations exhibited an increased drug level in the last 10-d of release, forming a second burst release, which might due to the collapse of microspheres *in vivo*. The *in vivo* drug release extended for a little longer period of time than that of *in vitro*. We attributed it to the relatively good drug water solubility and the bulk aqueous solution surrounding the microspheres during *in vitro* release.

Conclusion

The objective of this work was to develop degradable microspheres for delivering huperzine-A at significant plasma levels over a prolonged period of time. In this work, we: (1) successfully encapsulated huperzine-A in specifically end-group uncapped PLA/PLG polymers using a simple o/w solvent evaporation method with a relatively high encapsulation efficiency, (2) demonstrated *in vitro* and *in vivo* drug release from microspheres made from different types of polymers, and (3) achieved significant drug plasma levels in rats over 6—8 weeks after single subcutaneous administration.

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