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In vitro and *in vivo* study of thymosin alpha1 biodegradable in situ forming poly(lactide-co-glycolide) implants

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ABSTRACT

The purpose of this study was to develop poly(lactide-co-glycolide) (PLGA) based in situ forming implants (ISFI) for controlled release of thymosin alpha 1 (Tα1). The ISFI was prepared by dissolving PLGA in N-methyl-2-pyrrolidone (NMP) or mixtures of NMP and triacetin. Tα1 microparticles, prepared by spray-freeze drying method with chitosan or bovine serum albumin as a protectant, were suspended in PLGA solutions. The effects of Tα1 pre-encapsulation, PLGA molecular weight, PLGA concentration and organic solvents composition on the *in vivo* Tα1 release were evaluated by subcutaneously injecting Tα1-loaded ISFI into Sprague–Dawley Rats. The pharmacological efficacy of Tα1-loaded ISFI was examined using immunosuppressive BALB/c mice induced by cyclophosphamide. The ISFI composed of Tα1 pre-encapsulated with chitosan, higher molecule-weight PLGA at higher concentration and more triacetin showed a lower initial release and a longer sustained release period. The optimal prescription of our study showed a low initial release of 29.3% (24 h), followed by a slow and continuous drug release up to 28 d *in vivo*. An *in vitro* release device was designed to mimic the *in vivo* release of Tα1, and good correlation was observed between the *in vitro* and *in vivo* releases, with the linear correlation coefficient of 0.9899. Tα1-loaded ISFI showed low cytotoxicity as tested by CCK-8 assay. Tα1-loaded ISFI significantly increased the thymic index and spleen index of immunosuppressive mice. These results suggest that the ISFI is a suitable system for controlled release of Tα1.

1. Introduction

Chemotherapy side effects, such as anemia, low white blood cell count, hair loss, nausea and so on, are very common during treatment of cancers, which can sometimes disrupt chemotherapy schedules. Low white blood cell count, a symptom of low body immunity, means increased risk of infection. Therefore, chemotherapeutic agents are often used in combination with immunomodulators. Thymosin alpha1 (Tα1), an acidic polypeptide consisting of 28-amino acids isolated from thymosin fraction V (TF5), has been reported to be an effective immunoenhancer that increases white blood cells (Moody et al., 2002), accelerates restoration of T cell-mediated neutralizing antibody response in immunosuppressive mice (Li et al., 2002), and increases interleukin-2 (IL-2) production and IL-2 receptor expression in human lymphocytes (Knutsen et al., 1999). Tα1 also stimulates haematopoiesis of normal bone-marrow (Paul and Sodhi, 2002) and modulates differentiation and function of dendritic cells (Huang et al., 2004; Yao et al., 2007). Singly used or in combination with cytokines and chemotherapy, Tα1 has been applied in treatment of cancers (Garaci et al., 2000, 2003; Fan et al., 2006), chronic hepatitis (Yang et al., 2008; García-Contreras et al., 2006), reimplantation of avulsed teeth (Loo et al., 2008) and so on. However, Tα1 has a short half-life (less than 3 h) after subcutaneous administration, which requires frequent injection during therapy periods (Rost et al., 1999), so a long-term continuous release delivery system of Tα1 is needed.

Recently, biodegradable polymer based injectable solutions, named "in situ forming implants" (ISFI), have received increasing attention due to several advantages: ease of application, prolonged delivery periods, decreased drug dosage, improved compliance and reduced invasion (Hatefi and Amsden, 2002). Once injected into the body, polymer solutions solidified to form semisolid depots, releasing drugs in a sustained manner. The solidification can be induced by crosslinking (He et al., 2000), solvent-removal (Ravivarapu et al., 2000), a change in temperature (Westhaus and Messersmith, 2001; Kang et al., 2006), a change in pH (Ganguly and Dash, 2004) and so on. Poly(lactide-co-glycolide) (PLGA) is one of the most frequently

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Table 1
Formulation compositions of in situ forming implants.

No.	Tα1		Polymer		Solvent		$Mg(OH)_2$ (mg)
	Amount (mg)	Protectant	Mw	Amount (g)	NMP (ml)	Triacetin (ml)	
1	0.6	–	RG 504H	0.07	0.1	0.1	0.7
2	0.6	BSA	RG 504H	0.07	0.1	0.1	0.7
3	0.6	Chitosan	RG 502H	0.07	0.1	0.1	0.7
4	0.6	Chitosan	RG 503H	0.07	0.1	0.1	0.7
5	0.6	Chitosan	RG 504H	0.06	0.1	0.1	0.6
6	0.6	Chitosan	RG 504H	0.07	0.2	0	0.7
7	0.6	Chitosan	RG 504H	0.07	0.14	0.06	0.7
8	0.6	Chitosan	RG 504H	0.07	0.1	0.1	0.7

used biodegradable and biocompatible polymers as a carrier in sustained drug delivery systems (Luan and Bodmeier, 2006; Rafienia et al., 2009; Zhang et al., 2008). Water insoluble PLGA dissolved in N-methyl-2-pyrrolidone (NMP) or other water-miscible organic solvents precipitates upon injection into aqueous environments (for example, body fluid) because the organic solvents diffuse out and water penetrates into the polymer matrix. Both hydrophilic and hydrophobic drugs can be easily dissolved or suspended in the PLGA solutions and no special chemical or equipment is needed for solidification of ISFI, which is particularly suitable for delivery of protein and peptide drugs.

In this study, we developed PLGA based ISFI for sustained delivery of Tα1. Several formulation factors such as Tα1 pre-encapsulation, PLGA molecular weight and PLGA concentration were investigated. Different amounts of triacetin were added to NMP in order to decrease the initial release of Tα1. Tα1 is unstable at acidic pH (especially at pH <2), and therefore insoluble $Mg(OH)_2$ (1%, based on weight of PLGA) was added to protect Tα1 against damage of acidic microenvironments caused by degradation of PLGA (Zhong et al., 2007). An *in vitro* release model was designed to mimic the *in vivo* release profiles of Tα1 from ISFI. The cytotoxicity and pharmacological efficacy of Tα1-loaded ISFI were also evaluated.

2. Materials and methods

2.1. Materials

Poly(lactide-co-glycolide) (PLGA 50:50, RG 502H, Mw = 12 kDa; RG 503H, Mw = 34 kDa; RG 504H, Mw = 48 kDa) was purchased from Boehringer Ingelheim Pharma GmbH and Co. KG (Germany). Thymosin alpha1 was obtained from Hangzhou Sinopep Pharmaceutical Inc. (China). Chitosan (Mw = 20 kDa) was obtained from Golden-Shell Biochemical Co., Ltd (China). Bovine serum albumin (BSA) was purchased from Sino-American Biotechnology Co. (China). N-methyl-2-pyrrolidone (NMP) was kindly provided by International Specialty Products, Inc. (USA). Cyclophosphamide was purchased from Jiangsu Hengrui Medicine Co., Ltd. (China). All other materials were of HPLC or analytical grade.

Male Sprague–Dawley Rats and male BALB/c mice were obtained from Shanghai Slac Laboratory animal Co. Ltd. (China).

2.2. Tα1 pre-encapsulation

In order to be easily suspended in PLGA solutions, Tα1 with or without protectants (chitosan or BSA) was spray-freeze dried to yield uniform microparticles. Briefly, Tα1 was dissolved in deionized water, chitosan solution (4% in 2% acetic acid, w/v) and BSA solution (4% in deionized water, w/v) at 1% (w/v), respectively. The above solutions were sprayed into liquid nitrogen with a Büchi 190 spray dryer (Büchi, Switzerland), and then kept in a deep freezer of −50 °C for 4 h to evaporate liquid nitrogen. The remaining frozen powder was lyophilized with a V55C lyophilizer (Virtis, USA) for 48 h to obtain dried Tα1 microparticles. The morphology of Tα1 microparticles was observed with a scanning electron microscope (SEM) (Tescan, Czech Republic).

2.3. Preparation of Tα1-loaded ISFI

Appropriate amounts of PLGA were dissolved in NMP or mixtures of NMP and triacetin. Tα1 microparticles and $Mg(OH)_2$ micropowders (diameter <50 μm) were suspended in PLGA solutions by homogenization at 5000 rpm for 2 min with a PT3100 homogenizer (Kinematica, Switzerland). Suspensions of eight different formulations were prepared for release experiments of ISFI (Table 1).

2.4. Determination of Tα1 by HPLC method

The concentration of Tα1 was determined by high performance liquid chromatography with a Welch Materials C_{18} column (250 mm × 4.6 mm, 5 μm, 300 Å). The L-2000 HPLC system (Hitachi, Japan) consisted of a quaternary pump L-2130, an autosampler L-2200, a UV-Vis detector L-2420 and a column oven L-2300. The mobile phase consisted of acetonitrile and water (18:82, v/v) containing 0.1% (v/v) trifluoroacetic acid. The column temperature was maintained at 25 °C. The flow rate was set at 1.0 ml/min and the detection wavelength was 205 nm. Sample solution was injected at a volume of 20 μl. A calibration curve was prepared in the Tα1 concentration range of 1–128 μg/ml (r = 0.9999).

2.5. In vivo release of Tα1-loaded ISFI

Male Sprague–Dawley rats (200–220 g) were maintained in the room kept at 25 °C with free access to food and water. They were randomly divided into eight groups and subcutaneously injected with 0.2 ml suspensions of different formulations (Table 1) containing 0.6 mg Tα1, respectively. At predetermined time points, three rats of each group were sacrificed, and the implants were retrieved and freeze dried. Dried implants were dissolved in 2 ml acetonitrile. After centrifugation at 15,000 rpm for 5 min, the supernatant was discarded. The dissolution and centrifugation procedures were carried out for three times. The residue was treated with an Eppendorf 5301 concentrator (Eppendorf, Germany) to evaporate remaining acetonitrile and then redissolved in deionized water or 2% acetic acid (for chitosan microparticles). After centrifugation at 15,000 rpm for 5 min, the supernatant was used for determination of Tα1 by HPLC method. The cumulative release of Tα1 was calculated according to residual Tα1 content in solidified implants.

2.6. In vitro release of Tα1-loaded ISFI

In vitro release of ISFI was conducted in a home-made cylinder with a diameter of 8 mm (Fig. 1), which was made of 0.45 μm

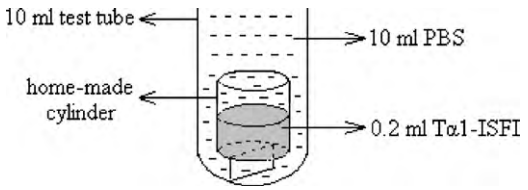

Fig. 1. Schematic presentation of the *in vitro* release device.

PVDF microporous membrane. About 0.2 ml suspensions of formulation 8 in Table 1 were injected into cylinders. The cylinder was immersed in 10 ml of phosphate buffered saline (PBS, pH 7.4) in a glass vial and shaken in an SHZ-88 water bath oscillator (Taicang Laboratorial Equipment Factory, China) at 37 °C. 0.1% (w/v) NaN_3 was added to PBS as a preservative. At predetermined time points, solidified implants were retrieved and disposed as same as the *in vivo* experiment. All experiments were carried out in triplicate. The linear correlation coefficient between the *in vitro* and *in vivo* release of formulation 8 was calculated. Structures of the *in vitro* and *in vivo* solidified implants (24 h after injection) of formulation 8 were observed by SEM.

2.7. In vitro cytotoxicity assay of Tα1-loaded ISFI

About 0.2 ml suspension of formulation 8 was injected into a home-made cylinder, and then it was immersed in 2 ml of isosmotic PBS (pH 7.4) and shaken in an SHZ-88 water bath oscillator at 37 °C. At 0 h, 24 h, 72 h and 168 h, release medium was replaced with fresh PBS and samples of release medium were collected after an incubation period of 1 h. Samples were filtered through a sterile 0.22 μm microporous membrane and diluted with isosmotic PBS (pH 7.4) to a series of concentrations (100%, 50%, 25% and 12.5%).

In vitro cytotoxicity of formulation 8 was evaluated using Cell Counting Kit-8 (CCK-8) (Dojindo Laboratory, Japan) (Königs et al., 2007). 2-(2-Methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium in CCK-8 can be deoxidized by viable cells to form yellow formazan dye. Experiment design was guided by the CCK-8 manufacturer's instructions. Briefly, NIH 3T3 cells were plated into a 96-well plate at a density of 10^4 cells/well in 100 μl DMEM which contained 10% newborn calf serum. After 24 h incubation at 37 °C under 5% CO_2, cell culture medium was replaced with 150 μl fresh culture medium. 50 μl release medium dilution was added and PBS was used as control. After another 24 h, 10 μl CCK-8 solution was added to each well, followed by incubation of the microplates for 4 h. Absorbance was measured at 450 nm using a Multiskan MK3 microplate reader (Thermo Labsystems, USA) with a reference wavelength of 630 nm. The experiments were carried out in triplicate.

2.8. Immunosuppressive mice model

Male BALB/c mice of 18–20 g were treated intraperitoneally with 50 mg/kg body weight of cyclophosphamide on days 0, 7, 14, 21 and 28. Thirty-two mice were randomly divided into four groups. Two negative controls were group A (0.9% NaCl) and group B (ISFI of formulation 8 without Tα1). Group C was Tα1 solution (750 μg/ml). The experimental group D was Tα1-loaded ISFI of formulation 8. Mice in group B and D were injected subcutaneously with 0.1 ml suspension on day 0, and mice in group A and C were injected with 0.1 ml 0.9% NaCl and 0.1 ml Tα1 solution on days 0, 7, 14 and 21. On day 28, all the mice were sacrificed. Their thymuses and spleens were weighed and the immune organ indexes were calculated as follows:

$$\text{immune organ index} = \frac{\text{weight of immune organ (mg)}}{\text{weight of mouse (g)}}$$

2.9. Statistical analysis

Statistical comparisons were made using Student's *t*-test. The probability value of less than 0.05 was considered as significant.

2.10. Ethics

The protocol of the animal experimental procedures was approved by the Animal Experimentation Ethics Committee of Second Military Medical University.

3. Results and discussion

3.1. In vivo release of Tα1 from Tα1-loaded ISFI

3.1.1. Influence of Tα1 pre-encapsulation on in vivo Tα1 release

In vivo release of Tα1 from formulations 1, 2 and 8 is shown in Fig. 2. Pure Tα1 group had a high initial release (24 h, 43.3%), followed by a continuous release up to 14 days. In contrast, for Tα1/chitosan microparticles, the ISFI showed a much lower initial release (29.3%) and a longer continuous release period (28 days). Pre-encapsulation with BSA did not have much effect on the initial release of Tα1 (44.2%) but increased the continuous release period to 28 days. The different roles between chitosan and BSA are mainly due to their solubility: both BSA and Tα1 can be easily dissolved in neutral water and they will diffuse out of implants quickly during polymer precipitation, but chitosan will partially prevent Tα1 escaping from polymer matrix because of its poor solubility. Morphologies of microparticles also contributed to the difference of Tα1 release: Tα1/chitosan microparticles were spherical and dense, with a diameter of approximate 30 μm (Fig. 3A and B), while Tα1/BSA microparticles were loose and irregular (Fig. 3C and D), which made it easier for Tα1 to diffuse out. Another possible reason is that in neutral environment Tα1 with negative charges may interact with cationic chitosan but not with anionic BSA. In addition, both of the two kinds of microparticles were porous, and therefore neither of them decreased the initial release of Tα1 to less than 10%.

3.1.2. Influence of PLGA molecular weight on in vivo Tα1 release

In vivo release of Tα1 from formulation 3, 4 and 8 is shown in Fig. 4. Tα1 release from ISFI made of RG 502H or RG 503H showed much higher initial releases (74.6%, 77.3%) and much shorter release periods (7 and 14 days), compared with that of RG 504H. RG 502H and RG 503H have lower molecular weights and trend to give lower solution viscosities, so organic solvents as

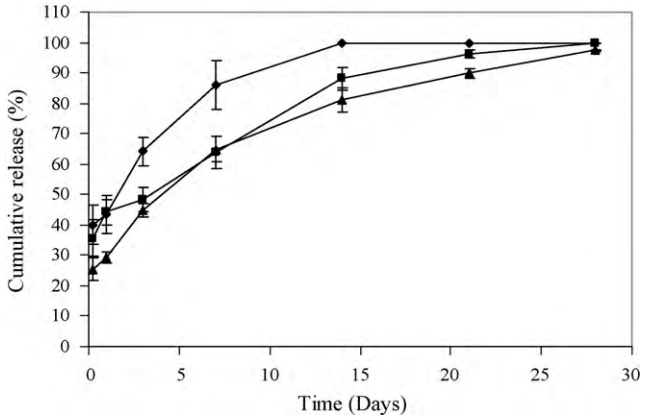

Fig. 2. *In vivo* release of Tα1-loaded ISFI with microparticles pre-encapsulated by different protectants. *Key*: (♦) no protectant; (■) BSA; (▲) chitosan (mean ± S.D., $n = 3$).

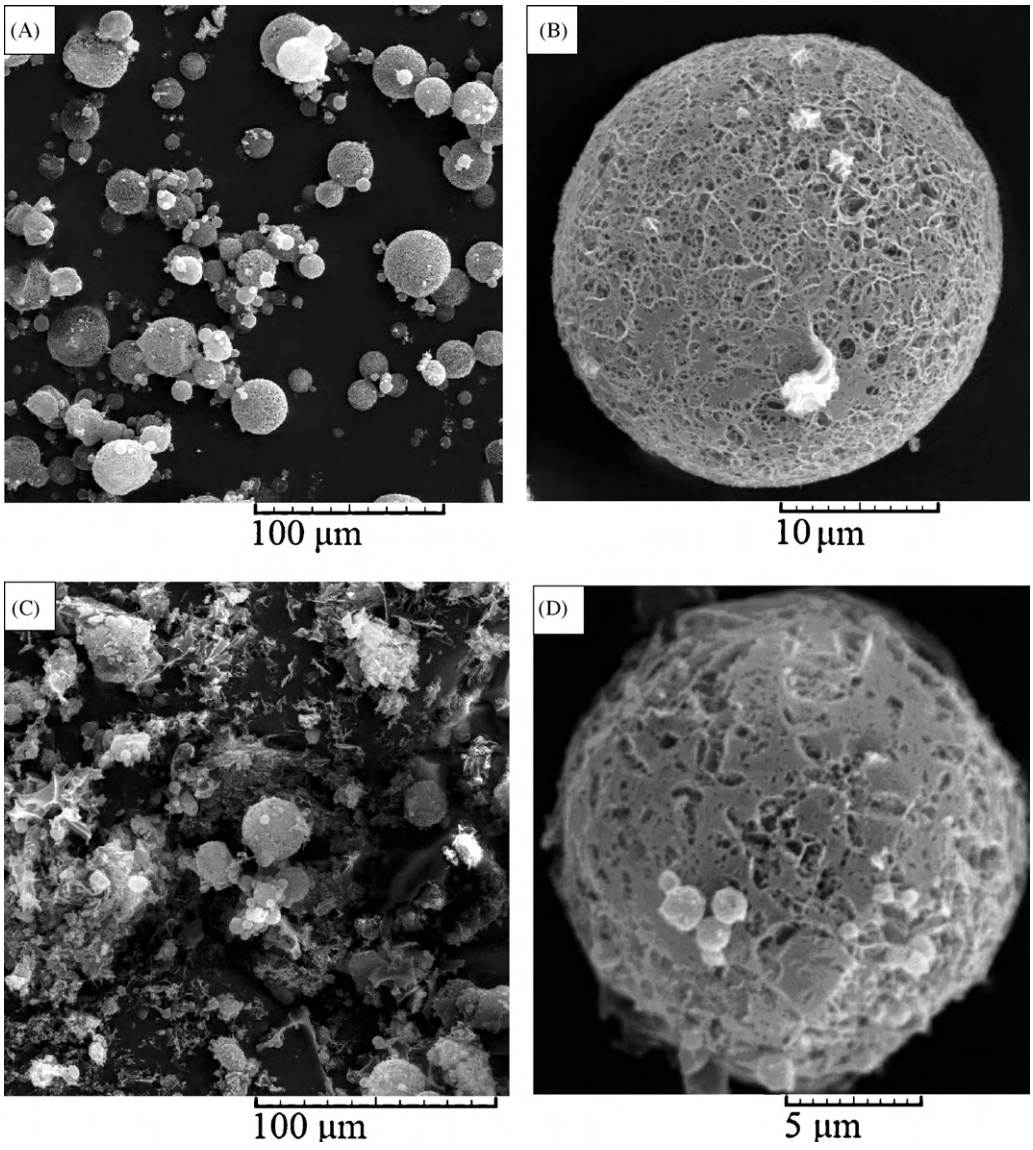

Fig. 3. Scanning electron micrographs of Tα1 microparticles encapsulated by chitosan (A and B) and BSA (C and D).

well as Tα1 can diffuse out of polymer matrix more quickly when contacted with aqueous environments, leading to a faster polymer precipitation and a much higher initial release. Another important factor is the higher porosity of solidified ISFI prepared with lower molecule-weight PLGA due to faster solidification, which further increases the diffusion rate of Tα1 into body fluid (Astaneh et al., 2006). However, RG 503H didn't show better properties than RG 502H in controlling the initial release of Tα1, caused by the cellular and spongy-like structure of solidified ISFI prepared with RG 503H (Astaneh et al., 2009). Due to its high molecular weight, RG 504H was expected to lose mass slower as it reached the critical molecular weight when it became soluble in the body fluid, resulting in a longer release period (Ravivarapu et al., 2000).

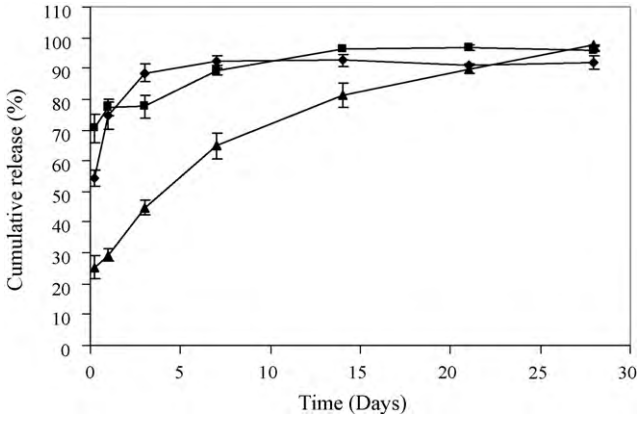

Fig. 4. *In vivo* release of Tα1 from ISFI prepared with PLGA of different molecular weights. *Key*: (♦) RG 502H; (■) RG 503H; (▲) RG 504H (mean ± S.D., $n = 3$).

3.1.3. Influence of PLGA concentration on in vivo Tα1 release

In vivo release of Tα1 from formulation 5 and 8 is shown in Fig. 5. The lower concentration of RG 504H led to a much higher initial release of Tα1 (52.3%), but it didn't change the trends of the continuous release period, indicating that it only accelerated diffusion of organic solvents during solidification process of ISFI. Increasing the polymer concentration means a multitude of effects, including lower system diffusivities, thicker skins and increased solution hydrophobicity, all of which may result in the slower phase inversion. The slower phase separation rate, as well as the slower water influx rate and the morphological transition to a less porous structure of solidified ISFI, contributes to the slower initial release of

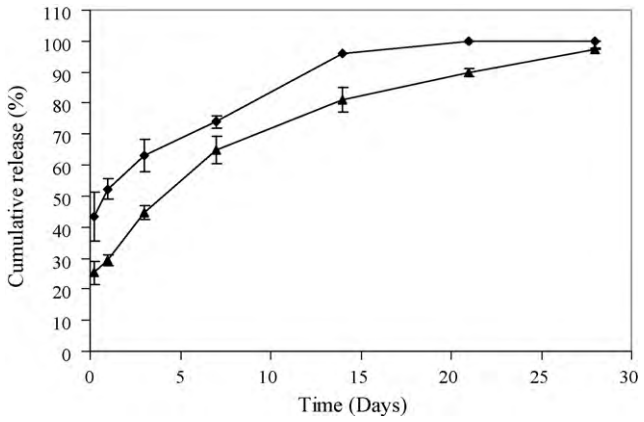

Fig. 5. *In vivo* release of Tα1 from ISFI prepared with RG 504H of different concentrations. *Key*: (♦) 0.30 g/ml; (▲) 0.35 g/ml (mean ± S.D., $n = 3$).

Tα1 (Graham et al., 1999). From the 2nd week to the 4th week, the polymer matrix gradually collapsed and the cumulative amounts of released Tα1 became close.

3.1.4. Influence of triacetin on in vivo Tα1 release

In vivo release of Tα1 from formulations 6, 7 and 8 is shown in Fig. 6. In this study, different amounts of triacetin were added to NMP (0:10, 3:7, 5:5, v/v) to dissolve PLGA. Addition of triacetin in NMP/PLGA solution significantly decreased the initial release of Tα1 from 76.9% to 56.2% and 29.3%. It has been reported that ISFI with low solvent/water affinity undergo much slower phase inversion, leading to a less porous, more fluid, two-phase structure (Brodbeck et al., 1999; Kang and Singh, 2005). A fast phase inversion of ISFI was observed upon injection into aqueous environments for water-miscible NMP while a much slower phase inversion was found for hydrophobic triacetin. For mixed solvents of NMP and triacetin, NMP diffuses out of the polymer matrix quickly resulting in a solidified exterior of ISFI and hydrophobic triacetin trends to be detained in polymer matrix during solidification, which prevents exchanges of liquids and diffusion of Tα1. The exchange of triacetin with water is presumed to last for up to 2 weeks (Brodbeck et al., 1999), and slower phase inversion of polymer leads to denser structures of solidified implants and slower release of Tα1.

3.2. In vitro release of Tα1 from Tα1-loaded ISFI

In vitro release of Tα1 from formulation 8 in home-made cylinders is shown in Fig. 7. A sustained release of Tα1 with a relative low initial release (15.7%) was observed. The ISFI released 89.5% of Tα1 up to 28 days at a little slower rate than that of the *in vivo* release. The morphologies of formed implants *in vitro* and *in vivo* were studied through SEM. As shown in Fig. 8, the *in vivo* formed implants (A and B) were porous and uniform, while the *in vitro* formed implants (C) showed obvious shell/core structure: the outer layer of the *in vitro* formed implants (D) was as porous as the *in vivo* implants but the core (E) was dense with few channels. When ISFI is subcutaneously injected, organic solvents diffuse out quickly from tablet-like polymer matrix, but body fluid is not so sufficient as to penetrate into the matrix in time to make it hardened, so continuous loss of organic solvents results in channels in formed implants. However, ISFI *in vitro* contacts with sufficient water immediately after immersed in PBS and its outer layer solidifies in a short time, which prevents diffusion out of internal organic solvents. After initial solidification, internal organic solvents slowly diffuse out through channels of outer layer and dense core forms. Tα1 encapsulated in the core cannot leak out until degradation of polymer matrix, and this may explain the difference between the *in vitro* and *in vivo* release.

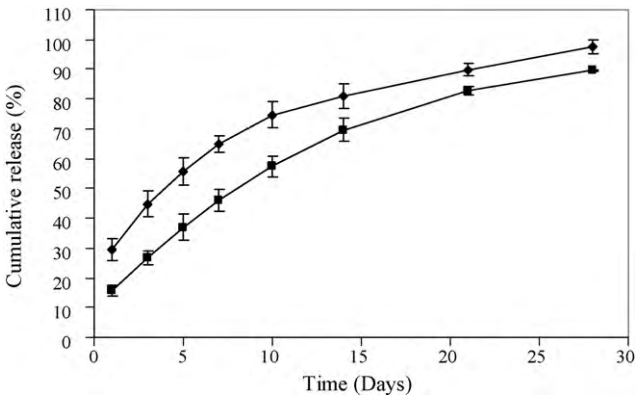

Fig. 7. Tα1 release from ISFI of formulation 8 *in vitro* (■) and *in vivo* (♦) (mean ± S.D., $n = 3$).

Both of the *in vitro* and *in vivo* release of Tα1 from ISFI followed Higuchi kinetics with correlation coefficients of 0.9948 and 0.9839. The linear correlation coefficient between the *in vitro* and *in vivo* release was 0.9899, demonstrating the good availability of the home-made cylinder as a model to investigate the *in vivo* release profiles of ISFI.

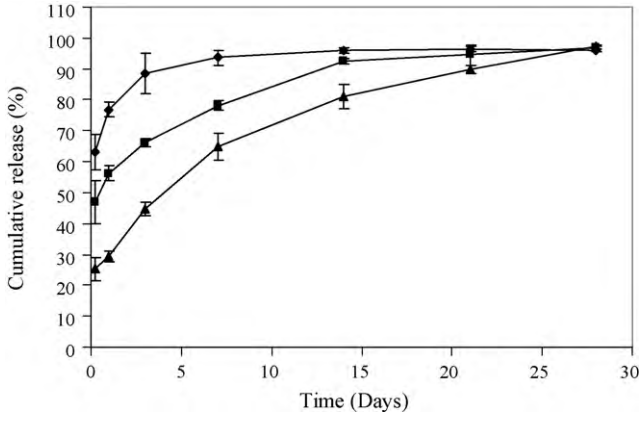

Fig. 6. *In vivo* release of Tα1 from ISFI prepared with different concentrations of triacetin. *Key*: (♦) 0 ml/ml; (■) 0.3 ml/ml; (▲) 0.5 ml/ml (mean ± S.D., $n = 3$).

3.3. In vitro cytotoxicity assay of Tα1-loaded ISFI

The result of the *in vitro* cytotoxicity assay is shown in Fig. 9. A decrease in viability of NIH 3T3 cells was observed for the high concentration samples (100% and 50%) of 1 h, but the decrease did not exceed 20% when compared with the PBS group, and all the other *in vitro* release medium-treated groups were statistically insignificant compared with PBS treatment, indicating low cytotoxicity of the ISFI.

3.4. Effect of Tα1-loaded ISFI on immunosuppressive mice

Table 2 shows the pharmacological results obtained after different treatments for 28 days. Thymus and spleen are considered to be the most important immune organs of body, reflecting conditions of the body immune system. Cyclophosphamide is a commonly used immunosuppressive agent for inducing reduction of spleen index or thymic index of model animal (Bujalance et al., 2007; Chen et al., 2007). Compared with the other three groups, Tα1-loaded ISFI significantly increased the average thymic index of immunosuppressive mice ($p < 0.05$). The average spleen index of mice treated

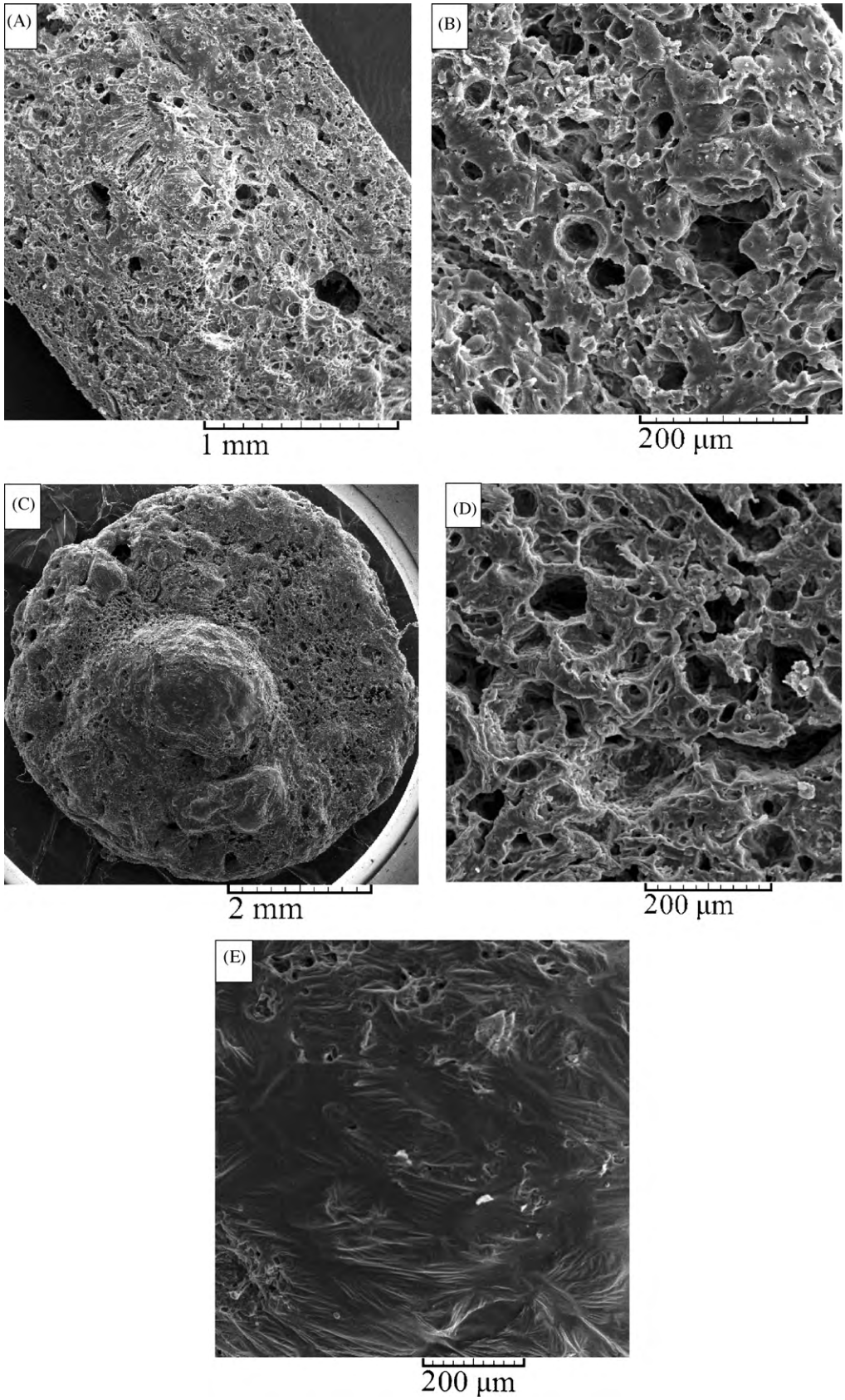

Fig. 8. Scanning electron micrographs of formed ISFI of formulation 8: (A and B) cross section of *in vivo* formed implant; (C) cross section of *in vitro* formed implant; (D) outer layer of *in vitro* formed implant; (E) core of *in vitro* formed implant.

Table 2
Immune organ indexes of four groups of mice after treatments for 28 days ($n = 8$).

	Groups			
	A	B	C	D
Thymic index (mg/g)	0.761 ± 0.055	0.809 ± 0.055	0.773 ± 0.776	$0.974 \pm 0.094^{a,b,c}$
Spleen index (mg/g)	4.344 ± 0.566	4.651 ± 0.795	4.726 ± 0.629	5.122 ± 0.708^{a}

[a] $p < 0.05$ vs. 0.9% NaCl as control.
[b] $p < 0.05$ vs. blank implant as control.
[c] $p < 0.05$ vs. Tα1 solution.

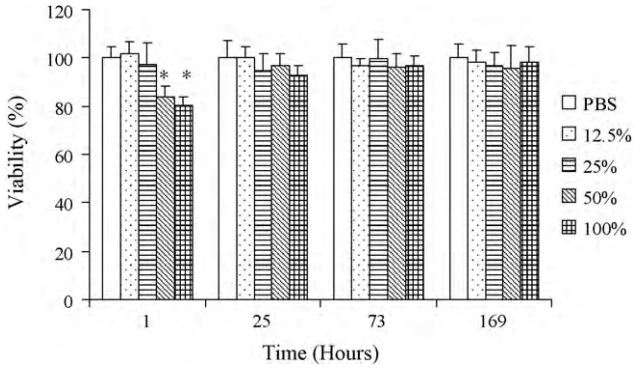

Fig. 9. Cytotoxicity of ISFI determined by CCK-8 assay (expressed as % viability). $^{*}p < 0.05$, $n = 3$, compared with PBS group.

with Tα1-loaded ISFI was significantly larger than that of mice treated with 0.9% NaCl ($p < 0.05$). The experimental data demonstrated the good efficacy of Tα1-loaded ISFI in enhancement of body immunity.

4. Conclusions

In this study, we describe the possibility of in situ forming implant as a carrier for extending the circulation half-life of the model peptide compound Tα1, which is currently restricted in clinic due to its short plasma half-life. The formulation of Tα1-loaded biodegradable ISFI with PLGA and several influencing factors on its release behavior were studied and evaluated. The *in vitro* and *in vivo* release was sustained for at least 4 weeks for the ISFI containing Tα1 pre-encapsulated with chitosan. Higher concentration of PLGA with higher molecule weight and addition of triacetin for the Tα1-loaded ISFI showed a less initial release and a longer sustained release period. In comparison with BSA, chitosan is a more suitable material for pre-encapsulation of Tα1 for ISFI formulation. An *in vitro* release model was designed to mimic the *in vivo* release of ISFI, and the good correlation was observed between the *in vitro* and *in vivo* release with the linear correlation coefficient of 0.9899. *In vitro* cytotoxicity assay proved ISFI to be a safe delivery system for sustained release of drugs. Tα1-loaded ISFI significantly increased the average thymic index and spleen index of immunosuppressive mice. All the results indicated that the ISFI formulation of protein/chitosan microparticles, mixed organic solvents, and an insoluble base may be a useful approach in the development of PLGA based ISFI for other therapeutic proteins.

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