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Study of a Pingyangmycin delivery system: Zein/Zein–SAIB in situ gels

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Abstract

Venous malformations (VM) are common vascular abnormality, and their management remains difficult. Pingyangmycin hydrochloride (PYM) is a useful sclerosant to treat venous malformations. This study was aimed to evaluate the effect of a new drug delivery system, PYM-loaded Zein/Zein–sucrose acetate isobutyrate (SAIB) in situ gels, in gelling and extending the local release of PYM. It was demonstrated that *in vitro* and *in vivo* release of PYM from the in situ gels could be extended up to 7 and 4 days, respectively. SAIB could significantly cut down the initial burst of PYM from the in situ gels ($P < 0.05$). The possible gel forming and drug release mechanisms were described according to the morphology analysis by atomic force microscopy (AFM), optical microscopy and SEM. The gel forming efficacy and the viscosity of in situ gel solutions were satisfying.

Keywords: Biodegradation; In situ forming; Controlled drug release; Drug delivery; Atomic force microscopy (AFM); Microdialysis

1. Introduction

Pingyangmycin hydrochloride (PYM) is a water-soluble glycopeptide produced by *Streptomyces pingyangensisn*. PYM is chemically similar to bleomycin with antitumor and antiangiogenic effects. As a useful sclerosant, PYM has been used extensively in the Far East for treatment of VM, a common vascular abnormality. Intravenous or intralesional injection of PYM could cause injury and detachment of endothelial cells, thickening of the vessel wall, and narrowing or occlusion of the lumen (Zhao et al., 2004). The main side effects of PYM are pulmonary fibrosis, fever, alopecie, gastrointestinal tract reaction, cutaneous reaction and so on. Currently, the only available formulation of PYM is freeze-dried powders for injection. Since the therapeutic effect of PYM on venous malformation depends on both local drug concentration and exposure time (Kong et al., 2003; Zheng et al., 2003), it is plausible to extend the drug release period by modifying the dosage form for improvement of the drug therapeutic effect of PYM as well as decreasing its side effect.

The injectable biodegradable in situ forming gel system provides various advantages such as depot formation without surgery, ease of drug loading and convenience of dose adjustment (Ganguly and Dash, 2004; Kang and Singh, 2005; Qiao et al., 2005). In the present study, Zein was used as the matrix of the injectable biodegradable gels. Zein is a major storage protein which comprises about 45–50% of the total protein in corn. The molecular structure of Zein is helical wheel conformation in which nine homologous repeating units are arranged in an antiparallel form stabilized by hydrogen bonds (Argos et al., 1982). It has been mainly used as biodegradable films. Zein was also used for preparation of microspheres which exhibited a constant release of drugs (Liu et al., 2005). Earlier study demonstrated that Zein was a promising biomaterial with good biocompatibility for the development of tissue engineering (Dong et al., 2004). In this study, PYM-loaded Zein/Zein–SAIB in situ gel that was originally injected as a liquid and then formed a semi-solid embolic agent which blocked blood flow and sustained the release of PYM.

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2. Materials and methods

2.1. Materials

Zein was purchased from Rixing Pharmaceutical Adjuvant Factory (Gaoyou, China). SAIB was from Aldrich Chemical Co. (Milwaukee, WI, USA). Glycerol formal (GF) was from Acros Organics (Geel, Belgium). PYM was obtained from Taihe Pharmaceutical Co. (Tianjin, China). Trypsin 1:250 (tissue culture grade) was from Sanland-chem International Inc. (Xiamen, China). Sodium 1-pentanesulfonate was from Tedia Company Inc. (Fairfield, OH, USA). All other chemicals and solvents were of analytical grade.

2.2. Preparation of Zein–PYM in situ gel and Zein–SAIB–PYM in situ gel

Typically, 1200 mg of Zein was dissolved in 5 mL of GF, which was left overnight to form a clear solution. For preparation of Zein–PYM in situ gels, 40 mg of PYM (200 mg PYM for *in vivo* study) was added into the above solution and dissolved by stirring. For preparing Zein–SAIB–PYM in situ gel, 500 mg SAIB was dissolved in the Zein–GF solution using ultrasound and then 40 mg PYM (200 mg PYM for *in vivo* study) was added with stirring. After dissolution, this product was preloaded in a syringe.

2.3. In vitro evaluation of gel forming efficacy and viscosity of in situ gel solutions

There is no appropriate *in vitro* system for evaluation of the efficacy of embolic liquids. In our study, an apparatus described previously (Kazekawa et al., 1997) was modified to evaluate the embolic efficacy of the in situ gel solutions (Fig. 1). Twelve grams of glass beads with a diameter of 2 mm were packed into a 10 mL plastic column. The height of the saline (37 °C) bottle was adjusted to 150 cm, providing a pressure of the same as normal blood pressure, the saline flow-rate through the column was maintained at 0.3 mL s^{-1}. One milliliter of each in situ gel solution was injected into the apparatus near the bottom of column. *In vitro* gel forming efficacy was expressed by the ability of the gel to block the saline flow. The gel forming efficacy of in situ gels made by different amount of Zein was studied and the concentration of Zein in the gel which could stop the saline flow was the minimum concentration of Zein of the preparation.

The viscosity of in situ gel solutions prepared in Section 2.2 and Ethibloc™, a commercial embolic agent, was measured at 37 °C respectively using a NDJ-8S Digital Display Viscometer (Shanghai Precision & Scientific Instrument Co. Ltd., China).

2.4. In vitro release of PYM from in situ gels

One milliliter of each in situ gel solution was injected into the bottom of the apparatus displayed in Fig. 2 with 15 mL PBS (pH 7.4) containing NaN_3 (0.05%, w/v) and trypsin 1:250 (1.0%, w/v). The system was shaken at 60 rpm at 37 °C. The release medium from each sample was periodically removed and replaced with same amount of fresh drug release medium. The concentration of PYM in the samples were analysis by HPLC.

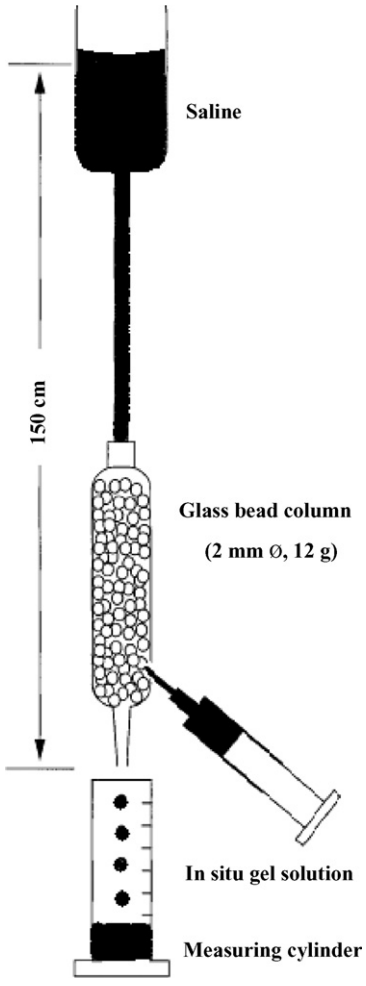

Fig. 1. Schematic representation of the apparatus to evaluate the gel forming efficacy of in situ gel solutions.

2.5. In vivo release of PYM from the in situ gels

To imitate the low blood flow of venous malformation (Fishman and Mulliken, 1993; Jackson et al., 1993) and investigate the local concentration of PYM and its release duration from the in situ gel, the distal part of the rabbit left-ear vein of rabbits (3.5 cm away from the in situ gel injected later) and its visible branches were ligatured (Fig. 3).

Microdialysis sampling technique was used to measure the *in vivo* pharmacokinetic experiment. Concentric vascular microdialysis probes with 10 mm dialysis membrane (MD-2310, Bioanalytical System Inc., USA) were implanted into the edge vein of the rabbit's ear. To study the *in vivo* recovery of microdialysis, 0.1 mL of blank Zein–SAIB in situ gel was injected into the proximal part of the rabbit's left-ear vein (0.5 cm from the tip of the probe) (Fig. 3). Perfusion solutions containing PYM were perfused through the probe at a constant flow-rate (2 μL min^{-1}) using the perfusion pump, respectively. After allowing a 2 h post-surgery stabilization period, the perfusate (C_{perf}) and dialysate

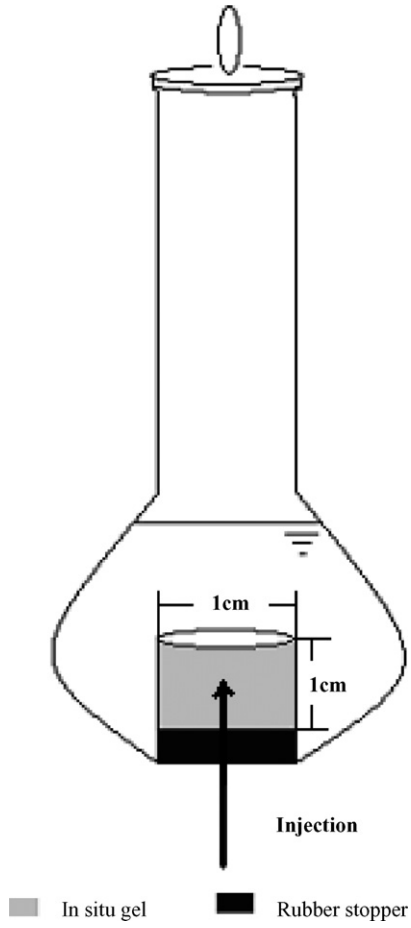

Fig. 2. Schematic representation of the apparatus to evaluate the *in vitro* release of in situ gels.

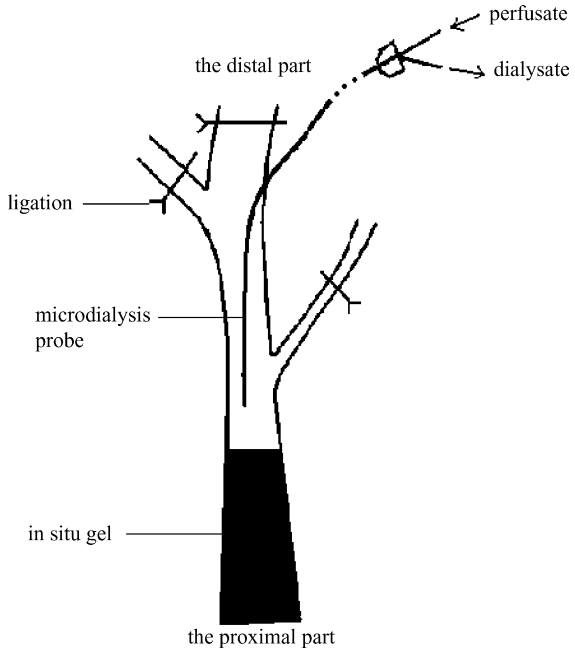

Fig. 3. Schematic diagram of the position of the microdialysis probe inserted into the rabbit ear vein toward in situ gel. The distal part of the rabbit ear vein (3.5 cm away from the in situ gel) and its visible branches were ligatured.

(C_{dial}) concentrations of PYM were determined by HPLC (Gao et al., 2006). The relative loss of PYM during retrodialysis (L_{retro}) or *in vivo* recovery ($R_{in\ vivo}$) by dialysis was then calculated as follows (Gao et al., 2006):

$$L_{retro} = R_{in\ vivo} = \frac{C_{perf} - C_{dial}}{C_{perf}}$$

For *in vivo* drug release experiment, PYM solution, PYM–Zein and PYM–Zein–SAIB in situ gels (0.1 mL, containing 4 mg of PYM, respectively) were injected respectively into the proximal part of the rabbit's left-ear vein (0.5 cm from the tip of the microdialysis probe). At each time-point microdialysis sample was collected for 30 min and the 20 μL of the dialysate was injected into analyzed HPLC for analysis of PYM.

2.6. Morphology analysis

All AFM (atomic force microscopy) images were collected using a CSPM4000 scanning probe microscope system (Benyuan Nano-Instruments Co. Ltd., Beijing, China). A μMasch NSC21/Al BS cantilever (MicroMasch, Russia) with a force constant of 17.5 N m^{-1} was used.

Samples of Zein for AFM were prepared as following: 10 μL of Zein–GF solutions (2 and 100 μg mL^{-1}) were deposited onto a newly cleaved mica surface and incubated in water at 37 °C for 2 min, respectively. The samples were then imaged by AFM in the tapping mode and the size of Zein globules was measured by AFM and analyzed with software Imager 4.20 (Benyuan Nano-Instruments Co. Ltd., Beijing, China).

Sample of gel for AFM was prepared as following: Zein and Zein–SAIB in situ gel solutions prepared in Section 2.2 were respectively injected into water of 37 °C to form gels. Because of the strong adherence ability of the gel, it could tightly adhere to the mica which was put into the water before the injection. The gel was then imaged by AFM in the tapping mode in water.

Gel samples for Motic DMBA400 Digital Biological Microscope (Micro-Optic Industrial Group Co. Ltd., Xiamen, China) were prepared as following: Zein in situ gel solutions prepared in Section 2.2 were injected into water to form gels and the resultant gels were frozen and thin-sectioned in planes perpendicular to the gel surface and stained with hematoxylin and eosin (H&E).

Gel samples for SEM (TM1000, Hitachi High-Technologies Co., Tokyo, Japan) were prepared as following: Zein and Zein–SAIB in situ gel solutions prepared in Section 2.2 were respectively injected into water of 37 °C to form gels. The gel sample was then taken out of the water and imaged by SEM.

2.7. Statistical analysis

Significance between the mean values was calculated using ANOVA one-way analysis (Origin 7.0 SRO, Northampton, MA, USA). Probability values <0.05 were considered significant.

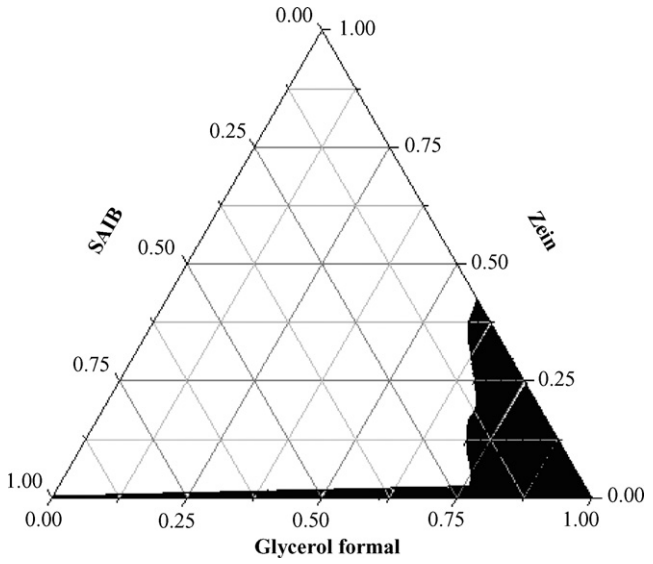

Fig. 4. Solubility phase diagram with various glycerol formal/Zein/SAIB weight ratios. The region of stable solution was presented in the phase diagrams as blackened area.

3. Results and discussion

3.1. Preparation of Zein–PYM in situ gel and Zein–SAIB–PYM in situ gel

Glycerol formal (GF) is an organic solvent often used in pharmacology due to its high solubility. Although GF has little toxicity, the amount used in the in situ gels was well below the established adverse effects level (Tipton and Dunn, 2000; Mottu et al., 2001). In the present study, GF was used as solvent. The solubility ternary phase diagram of glycerol formal:Zein:SAIB with various weight ratios was displayed in Fig. 4. The region of stable solution was presented in the phase diagram as blackened area.

3.2. In vitro evaluation of gel forming efficacy and viscosity of in situ gel solutions

With the increase of Zein concentration, the embolic efficacy of the in situ gel increased with an increased concentration of Zein. However, the concentration of Zein could not be infinitely high due to the higher viscosity of the solution with higher concentration of Zein. The viscosity should be appropriate for the ease of injection through a thin catheter. Thus, considering both the embolic efficacy and the viscosity of the in situ gel solution, we chose a concentration of Zein (as shown in Section 2.2) which was slightly higher than the minimum concentration of Zein that could stop the saline flow effectively. The viscosity of the in situ gel solutions was compare with the clinical used embolic liquids, Ethibloc™. The viscosity of the in situ gel solutions was no higher than Ethibloc™ (Table 1). Thus, they seemed to be suitable for embolization.

Table 1
Viscosity of three preparations at 37 °C ($n = 3$)

Preparation	Viscosity[a] (mPa s)
Ethibloc™	200 ± 5
Zein in situ gel solution[b]	188 ± 2
Zein–SAIB in situ gel solution[c]	193 ± 4

[a] Viscosity data were expressed as mean ± S.D.
[b] The ratios of Zein:GF was 24:100 (g mL^{-1}).
[c] The ratios of Zein:SAIB:GF was 24:10:100 (g g^{-1} mL^{-1}).

3.3. Release of PYM from in situ gels

3.3.1. In vitro release of PYM from in situ gels

Due to the lack of a system to degrade the Zein *in vitro*, measurement of *in vitro* sustained release was not possible in conventional release medium. To evaluate the sustained release phase and effects of SAIB on the overall release, trypsin 1:250 (1.0%, w/v) (Leo et al., 1999) was added in the release buffer to simulate the degraded rate of Zein *in vivo* (Górriz et al., 1998). At the same time, no interference of trypsin on the stability and the analysis of PYM was observed.

A well-appreciated concern for the release of in situ gel was shape. Since the systems were applied as liquids, they could flow during the application period and at least for part of the time during the gelling transition. As the result, the systems might have different surface area for a set injection size due to difference in shape. It would be expected that larger surface area would lead to faster drug release. To eliminate the influence of shape and get a better reproducibility of *in vitro* release, we adopted an *in vitro* release installation as illustrated in Fig. 2, which could unify the gels to a certain shape and make the result comparable.

As demonstrated in Fig. 5A, the PYM release from the Zein in situ gels could be extended up to 7 days. There was an initial burst within the first 2 days (Fig. 5B). But the initial burst was much lower than that of other in situ gel drug delivery systems for water-soluble drugs (Okumu et al., 2002; Ganguly and Dash, 2004). The possible explanation for this phenomenon would be described in Section 3.4.

Usually, relatively more polymer was added to minimize the initial burst, which unavoidably led to high viscosity. SAIB, a small molecule of mixed ester of sucrose, was employed in the present study, which only resulted in slightly increase of viscosity (Table 1). However, the initial burst of PYM from the in situ gels was significantly cut down ($P < 0.05$) (Fig. 5B). The possible mechanism would be described in Section 3.4.

3.3.2. In vivo release of PYM from in situ gels

Since the therapeutic effect of PYM on venous malformation depended on both local drug concentration and exposure time, it was critical for the evaluation of this formulation to know the local concentration and the release duration of PYM from this in situ gel. In the present study, microdialysis was adopted to measure the local drug release from the gels. Microdialysis is a powerful sampling technique for studying biochemical events in the extracellular fluid, and it has been used for sampling of various tissues and fluids (Anand et al., 2004; Bagger and Bechgaard, 2004; Leveque et al., 2004; Wang et al., 2006). Because the microdialysis technique involved no biological fluid

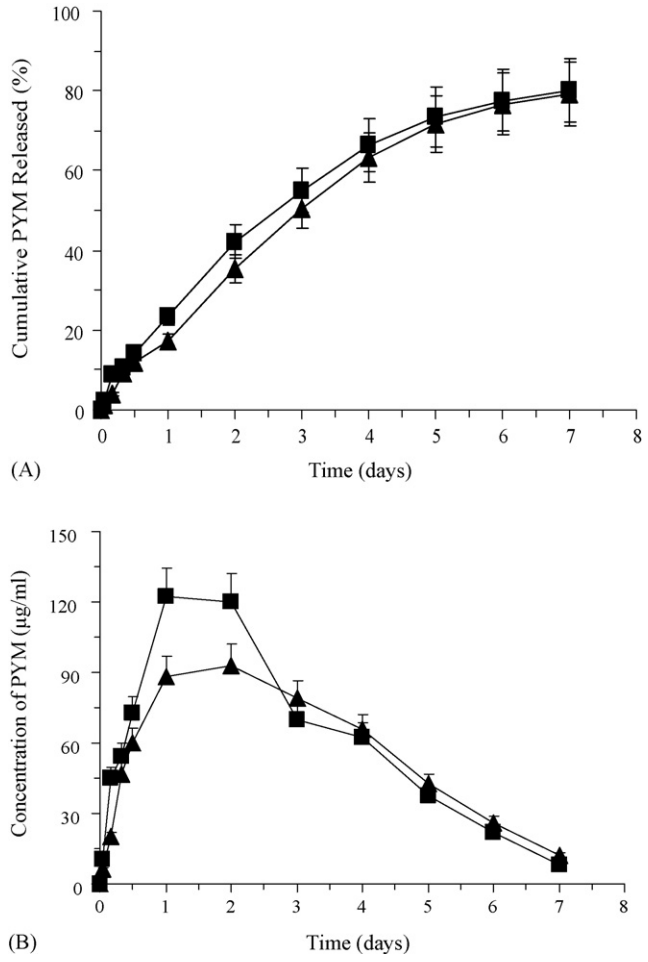

Fig. 5. *In vitro* release profile of PYM from Zein (▲) and Zein–SAIB (■) in situ gels in PBS, pH 7.4, 37 °C. Each point represents the mean ± S.D. ($n = 4$). (A) The cumulative release profile and (B) the PYM concentration–time profile.

loss from the body, higher temporal resolution of the sampling interval and continuous sampling over long periods could be achieved.

In order to study the *in vivo* concentration of PYM, it was necessary to determine the recovery of the dialysis probe. In this study the retrodialysis technique was used. According to the definition of recovery, the following equation was obtained: $C_d - C_p = -0.466C_p + 0.6673$ ($r^2 = 0.9983$). The *in vivo* recovery of PYM was 46.6 ± 3.1% ($n = 4$). Fig. 6 showed

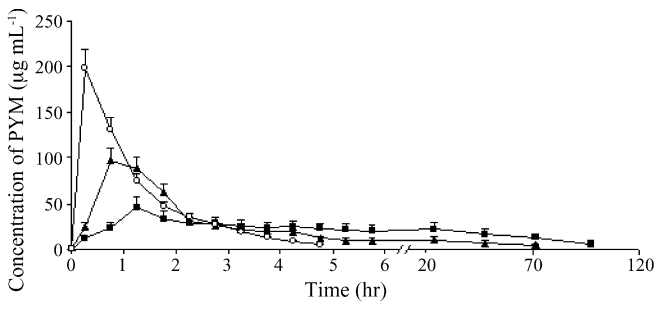

Fig. 6. PYM concentration–time profile obtained from microdialysis following a 4 mg dose in conscious rabbits. (◯) Normal saline, (▲) Zein in situ gel, and (■) Zein–SAIB ($n = 4$).

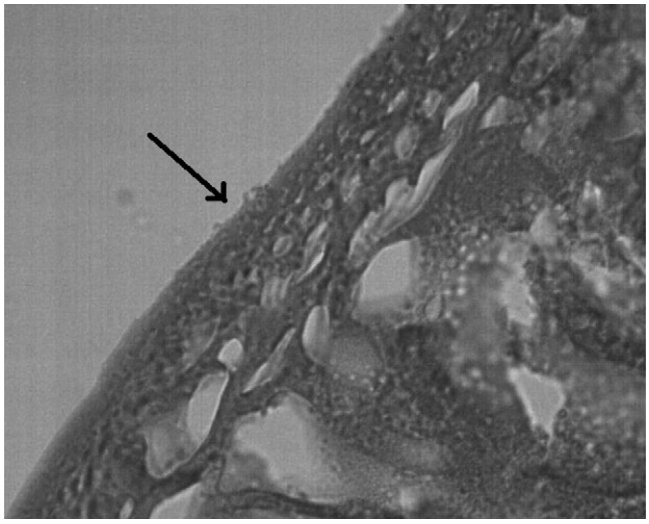

Fig. 7. H&E stained optical photomicrograph showing the diffusion barrier of the Zein in situ gel (as shown by the arrow) (original magnification, 1500×).

the time-course of local concentration of PYM corrected by *in vivo* recovery in rabbit blood after administrated PYM solution and PYM-loaded in situ gels. Comparing to the PYM solution, the in situ gel could sustain the drug release and prolonged its local retention. *In vivo* releases of the in situ gels of PYM–Zein and PYM–Zein–SAIB were up to 3 and 4 days, respectively.

The *in vivo* drug release profile was somewhat different from that of the *in vitro* ones. This difference might come from the difference of release buffer, surface areas of gels, and amount of PYM and gel. However, both the *in vitro* and *in vivo* studies demonstrated that PYM–Zein/PYM–Zein–SAIB in situ gel could extend the release of PYM and SAIB could significantly cut down the initial burst release of PYM from the in situ gels ($P < 0.05$).

3.4. Morphology analysis and possible gel forming mechanism

The optical photomicrograph showing the diffusion barrier and SEM images of the Zein in situ gel were shown in Figs. 7 and 8, respectively. It was obvious that the gel porosity at the surface was much lower than that of the internal part of the gel. This diffusion barrier (skin) around the depot after contacting with the aqueous buffer would be helpful to modulate the initial burst of PYM. And the forming of this "skin" might be the result of the rapid immigration of Zein with the quick diffusion of GF toward release buffer at the beginning of the gel forming, and relatively more Zein precipitated on the surface of the gel. With the diffusion rate of GF stepping down, the immigration of Zein would not be remarkable. So the internal part of the gel would be more porous than the outer part of the gel.

Recently, AFM was found to be a helpful instrument to evaluate the surface character of the nanoparticles (Jiang et al., 2005). In this study, AFM was employed to study the surface property of Zein globules and in situ forming gels. Fig. 9A was one of a

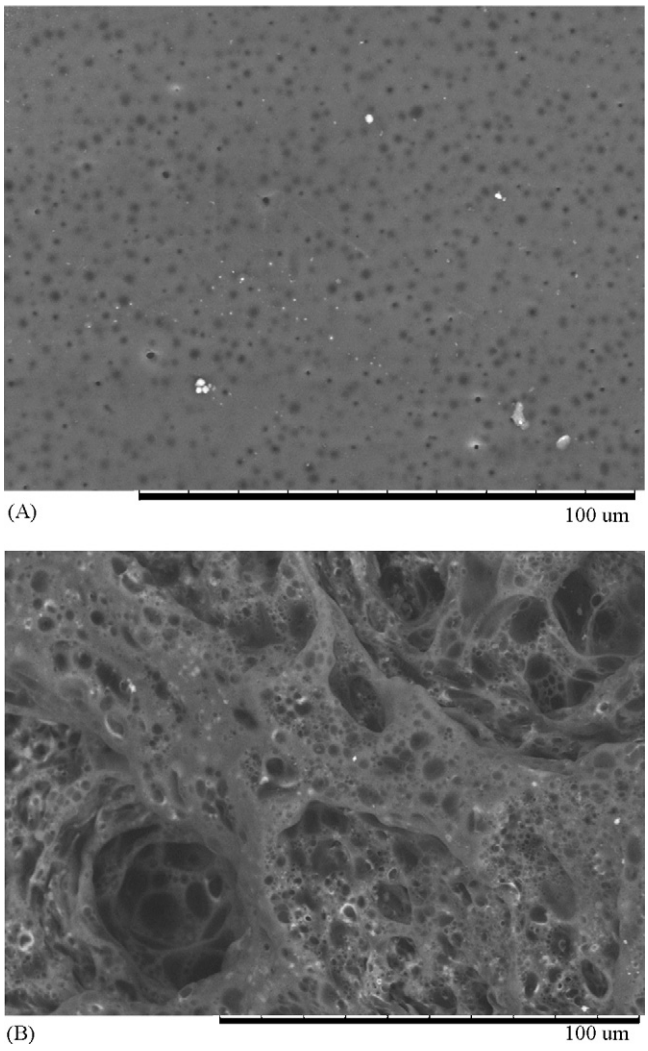

Fig. 8. SEM (TM-1000, Hitachi Ltd., Japan) image of Zein in situ gel. (A) Surface and (B) internal part.

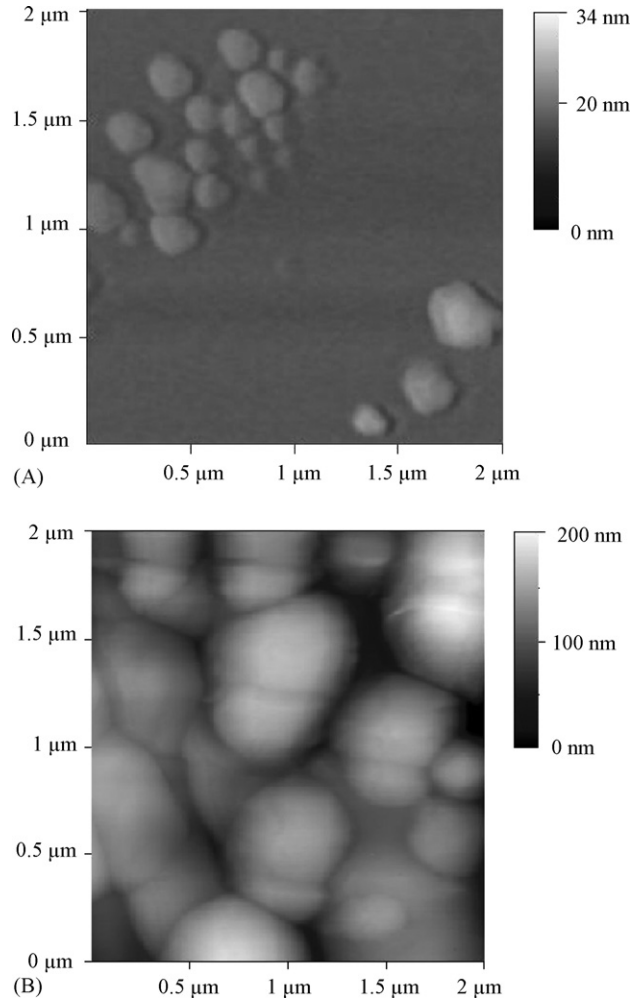

Fig. 9. AFM image of globules of Zein proteins prepared with Zein solution. (A) 2 μg mL^{-1} and (B) 100 μg mL^{-1}.

series showing the appearance of Zein. Many Zein globules of non-uniform size were observed after preparing the sample as described above. The apparent diameter (the apparent width at half vertical height) of Zein globules was mainly between 100 and 300 nm, and the apparent height is between 5 and 30 nm. The diameters and heights of Zein globules (Fig. 9B) of samples with higher Zein concentration were obviously greater than that of aforementioned sample. The apparent diameter of the Zein globules was mainly between 150 and 650 nm, and the apparent height is between 50 and 170 nm. The formation of these larger globules might be due to the higher degrees of protein aggregation than that of aforementioned sample as shown in Fig. 9B. From this point of view, gel forming ability of Zein might be explained as following: the molecular structure of Zein was helical wheel conformation in which nine homologous repeating units were arranged in an anti-parallel form stabilized by hydrogen bonds (Argos et al., 1982). After the Zein in situ gel solution was injected into PBS, the solvent composition at the aqueous interface changed as the GF diffused away from the depot and water infused in, causing the dissolved Zein to precipitate. Thus the concentration of Zein at the aqueous interface increased. When the concentration of Zein reached a certain value, the proteins agglomerated, and hydrogen bonding, disulfide bonding and hydrophobic interactions between protein molecules occurred which maintained the meshwork and led to the formation of the Zein gel. The appearance of Zein gel imaged by AFM showed that the Zein globules aggregated tightly and formed a rough membrane at the aqueous interface (Fig. 10A). The gel surface with SAIB appeared smoother and more perfect as comparing with the one without SAIB (Fig. 10B). The roughness average (R_a) value was calculated from the roughness profile determined by AFM. The R_a of the gel surface decreased from the original 10.10 to 3.69 nm with the addition of SAIB. That might be why SAIB could cut down the initial burst release of PYM from the in situ gels. SAIB is a sucrose derivative, fully esterified at a ratio of six isobutyrate groups to two acetate groups. It is soluble in most organic solvents but insoluble in water. Its major application areas were inks, coatings, paper production, beverages and controlled-release formulations for pharmaceuticals (Okumu et al., 2002). SAIB also could be employed as modifier and extender leading to higher solid by extending the film-forming polymer. Flowabil-

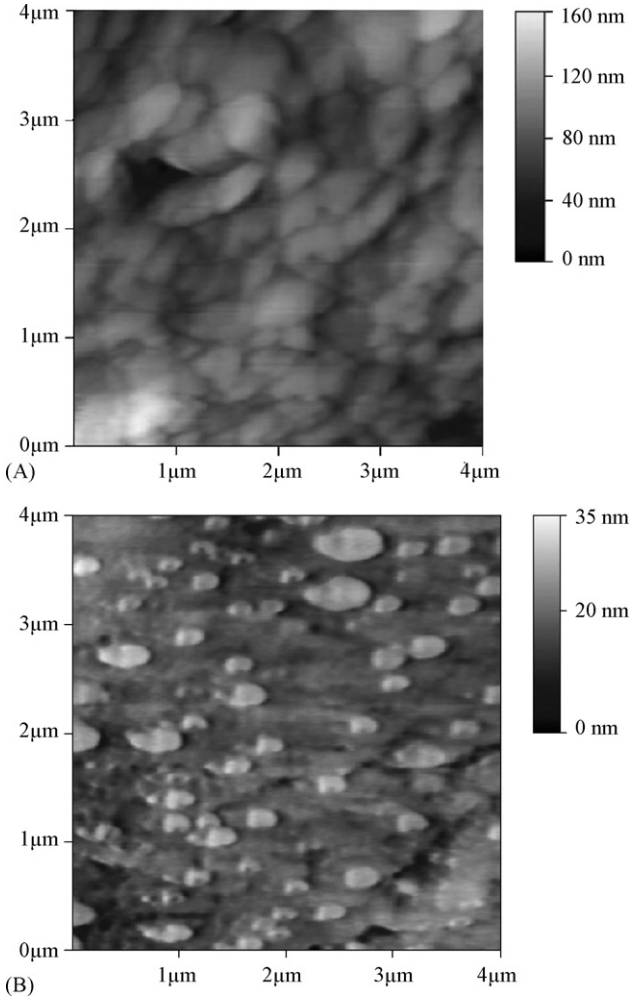

Fig. 10. AFM image of surface of in situ gels. (A) Zein in situ gel and (B) Zein–SAIB in situ gel.

ity of the polymer was often improved and surface defects of the film could be reduced. In this study, SAIB was employed as a modifier and plasticizer in the gel forming process of Zein.

4. Conclusions

The present study described the evaluation of Zein/Zein–SAIB based in situ gels for extended release of PYM to scleroses venous malformations. SAIB could significantly cut down the initial burst of PYM from the in situ gels. Gel forming efficacy and the viscosity of the present in situ gel solutions were satisfying. These results suggested that the PYG-loaded injectable Zein/Zein–SAIB based in situ gels was a promising formulation for sclerotherapy of venous malformations.

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