Characterizations of Chitosan-Based Highly Porous Hydrogel—The Effects of the Solvent

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ABSTRACT: In this study, the influences of different solvents on highly porous chitosan (CS)-based hydrogel have been investigated by monitoring the characterizations of the hydrogel. The results determined from rheological analysis and scanning electron microscope showed that all the CS dissolved in different solvents used in this study, when neutralized by α,β-glycerophosphate solution, could transform into a highly porous three-dimensional hydrogel at a certain temperature, and the aperture size and shape of the pores were related to solvent variety and solvent strength. The cytocompatibility and *in vivo* injection experiments demonstrated that the hydrogel prepared with acetic acid, hydrochloric acid, or lactic acid as the solution of CS had low toxicity and high histocompatibility. This study suggested that the gelation temperatures, pH values, turbidity, complex viscosities, and porous structures of the hydrogels for certain experimental applications could be tailored by adjusting the solvent variety and solvent strength of the CS solution.
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Key words: chitosan; hydrogels; gelation; characterizations; solvent

INTRODUCTION

Chitosan (CS) is a biopolymer derived from the deacetylation of chitin, the main structural component of crustacean exoskeletons. It is a pH-dependent cationic copolymer composed of glucosamine and acetylglucosamine.[1] CS-based materials have been played a significant role in biomedical applications[2–4] because of their nontoxicity, biocompatibility, and biodegradability.[5] In particular, CS-based injectable hydrogels with glycerophosphate disodium salt (GP salt) have gained significant attention and were considered as an ideal class of polymeric material for biomedical applications.[6–9]

The CS-GP hydrogel is prepared by neutralizing highly deacetylated semidiluted CS solution with a weak base, β-GP or α,β-GP. This system remains in solution at physiological pH and low temperature, whereas homogeneous gelation of this system can be triggered upon heating.[7,10,11] This temperature-responsive hydrogel has been a attractive candidate for applications related to injectable delivery of biologically active therapeutics, such as growth factors, cells, and medicament.[7,12–14] Successful applications of this system as injectable scaffold in lesions repair and cartilage regeneration were discussed by Hou et al.[15] and Hoemann et al.[16] The properties of CS-GP hydrogels are very important to the success of such applications. Although many studies have been reported in the applications of CS-GP thermosensitive systems during the past few years, little work has been published in their characterizations. Thus, the general mechanisms of gelation of the CS-GP system have been investigated in recent years.[7–9,17–21] Nonetheless, an ideal understanding of CS-GP characteristics is still lacking. Parameters such as concentration, molecular weight, and degree of deacetylation of CS could affect the characteristics of CS-GP hydrogels.[11] Generally, the gelation time decreased with increasing GP concentration, and the reason could be that high GP concentration caused rapid neutralization of protonated amino groups from CS.[22,23] Toward to better understanding of the gelation behavior of CS-α,β-GP system, we

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TABLE I
The Composite Polymer Solutions, Gelation Temperature (T_{gel}), and OD_{600} of the CS-α,β-GP Highly Porous Hydrogel Samples

Samples	Solvent	W_{CS} (g)	V_{H_2O} (mL)	V_{GP} (mL)	V_{acid} (μL)	C_{acid} (mol/L)	T_{gel} (°C)	OD_{600} Sol	OD_{600} Gel
Solvent variety									
CS-MA-α,β-GP	MA	0.27	13.5	1.5	56.0	1.0	39.3	2.492	2.363
CS-AA-α,β-GP	AA	0.27	13.5	1.5	86.3	1.0	47.7	2.652	2.482
CS-PA-α,β-GP	PA	0.27	13.5	1.5	112.5	1.0	44.1	2.559	2.321
CS-HA-α,β-GP	HA	0.27	13.5	1.5	121.2	1.0	41.7	2.559	2.324
CS-LA-α,β-GP	LA	0.27	13.5	1.5	137.8	1.0	35.7	2.339	2.151
Solvent strength									
CS-0.8LA-α,β-GP	LA	0.27	12.6	2.4	110.2	0.8	20.8	0.485	2.480
CS-1.0LA-α,β-GP	LA	0.27	12.6	2.4	137.8	1.0	30.1	1.472	2.312
CS-1.2LA-α,β-GP	LA	0.27	12.6	2.4	165.4	1.2	40.5	2.369	2.267

investigated the changes in gelation temperature, turbidity, and morphology affected by the CS/GP ratios in our previous research.[24]

As the CS-GP thermosensitive systems are essentially designed to be used via parenteral injection or as three-dimensional (3D) cell culture systems, rigorous perception of their characterizations may be helpful to better design of a kind of hydrogel for applications in drug delivery, tissue engineering, and 3D cell culture. In this study, a series of porous CS-α,β-GP hydrogels were prepared by dissolving CS in the solvent with different varieties and strengths. The gelation temperatures and complex viscosities of these hydrogels were recorded by rheological measurements. The effects of the solvents of CS on other characterizations (such as pH values of CS solution, pH values during the gelation process, morphology, turbidly, toxicity, and histocompatibility) of these kinds of porous hydrogels were also investigated.

EXPERIMENTAL

Materials

CS, derived from shrimp shell, dynamic viscosity 140 cps, deacetylation degree 96.5%, was freely supplied by Laizhou Haili Biological Product (Shandong, China). The Kunming mouse and pregnant Kunming mouse were purchased from Qingdao Municipal Institute for Drug Control (Qingdao, China). Fetal Bovine Serum (FBS) and Dulbecco's Modified Eagle Medium (DMEM) were both obtained from Hyclone (Logan, UT). α,β-Glycerophosphate (α,β-GP), formic acid (FA), acetic acid (AA), propionic acid (PA), hydrochloric acid (HA), lactic acid (LA), and polydimethyl siloxane fluid were all of analytical grade (Sigma, St. Louis, MO).

CS-α,β-GP hydrogel solutions preparation

CS-α,β-GP hydrogel solutions were prepared in the same manner as described in our previous study.[24] The composites of CS-α,β-GP solutions are listed in Table I. Proper amount of CS was dissolved in aqueous acid solutions (FA, AA, PA, HA or LA, 0.1*M*) using a heat-collecting magnetic stirrer (DF-1, Beijing Flaming Technology & Trade, China) at 100 rpm for 8 h at room temperature (25°C) to prepare clear solutions. For the effects of the solvent strength on the characterizations of the CS-α,β-GP hydrogels, the CS was dissolved in 0.8, 1.0, or 1.2*M* of aqueous LA solution. Then, the CS solutions were chilled to 4°C for 30 min. An aqueous α,β-GP solution of 50% (w/v) was prepared with distilled water and chilled along with the CS solutions to 4°C. Then, the α,β-GP solution was added dropwise to the CS solutions under stirring and the final CS-α,β-GP solutions were mixed for another 30 min. Finally, the resultant CS-α,β-GP hydrogel solutions were stored at 4°C.

Rheological measurements of the hydrogels

The rheological measurements were performed by a Physica MCR101 Rheometer (AnTon paar, Austria) and a circulating environmental system for the control of temperature. For the investigations of the viscoelastic properties of the hydrogel samples, the storage modulus (G') and loss modulus (G'') as well as dynamic complex viscosity (η^*) were measured. CS-α,β-GP solutions (3 mL) were introduced between the concentric cylinders and then covered with polydimethyl siloxane fluid on the surface to prevent evaporation during the tests. The effect of the polydimethyl siloxane fluid on the measurements was shown to be negligible. During the gelation process in nonisothermal conditions, the evolution of rheological properties was investigated between 10 and 90°C using a constant heating rate (2°C/min). Small amplitude deformation γ (0.02) and low frequency ω (1.00 rad/s) were applied in order not to disturb the gel formation. The gelation temperature (T_{gel}) of the highly porous hydrogel

was determined as the crossover of the storage modulus (G') and loss modulus (G'').

pH value measurement

The pH values of the CS solutions were measured using a Delta 320 pH Meter (Mettler Toledo, Greifensee, Switzerland) at 25°C. The pH values of the CS-α,β-GP hydrogels were recorded at 4°C before gelation and at T_{gel} after gelation. Temperatures ranging from 4 to 55°C were selected to evaluate the pH values of the hydrogel during the gelation process, and a water bath was selected to control the temperature at a constant heating rate (1°C/min).

The quantitative data were expressed as mean ± standard deviation, and analyzed by analysis of variance (ANOVA), followed by Tukey's *post hoc* tests. A level of significance at $p \leq 0.05$ was used to indicate statistical differences between treatment groups.

Turbidity of the hydrogels

The turbidity of CS-α,β-GP hydrogels in terms of time at T_{gel} was determined using a UV-1100 spectrophotometer (UV-1100, Shanghai MAPADA Instruments, China). The time-dependent absorption at 600 nm was recorded every 10 s at T_{gel} during the gelation process. The temperature was controlled by heat pad (Shanghai Kobayashi Daily Chemical, China) during the sol-to-gel behavior of the hydrogel solutions.

Scanning electron microscopy

Hydrogel samples were gelled at T_{gel} and freeze-dried under vacuum for 48 h to maintain the porous structure without any collapse. Then, the interior morphologies of the dry CS-α,β-GP porous hydrogels were examined using scanning electron microscopy (SEM) (KYKY-2800B, Scientific Instrument, Chinese Academy of Sciences, China).

MTT assay

Mouse embryo fibroblasts (MEFs) were selected for the cytotoxicity study. The CS-α,β-GP hydrogel solutions were prepared and were added in six-well flat-bottomed microplates (1.7 mL/well). After gel formation, 8 mL of complete medium (DMEM + 10% FBS) was added in each group and incubated at 37°C for 12 h. The extracting liquid obtained was sterilized with 0.22 μm of filter. Then, the extracts were diluted with culture medium, and a series of the extract dilutions (100, 50, and 25%) were collected. MEFs used in the general cytotoxicity test were obtained through primary culture and only cells of four to seven generations were used in this experiment. Briefly, MEFs at logarithmic growth phase were seeded into 96-well flat-bottomed microplates at a density of 5×10^4 cells per well, in DMEM containing 10% of FBS. The cells were divided into six groups and the medium was replaced with different extracting liquids of the CS-α,β-GP hydrogel (200 μL/well) after 24 h. The negative control was blank culture medium. After incubated at 37°C, 5% CO_2 and 95% relative humidity for 48 h, 200 μL of MTT solution was added to each well and incubated for additional 4 h. Hundred microliters of dimethyl sulfoxide was subsequently added to each well to dissolve the crystals. Absorbance was determined at 490 nm with a microplate reader (Bio-Rad Model 550, Hercules, CA). The percentage of viability was expressed as relative growth rate (RGR %) according to the following equation

$$\text{RGR } (\%) = \frac{D_{\text{sample}}}{D_{\text{control}}} \times 100\%, \quad (1)$$

where D_{sample} and D_{control} are the absorbancies of the tested samples and the negative control at 490 nm, respectively.

All reported values were the means of triplicate samples.

In vivo injection

A liquid CS-α,β-GP sol was administered by dorsal subcutaneous injections in adult kunming mouse (about 20 g). Sterile formulations were obtained by using ultraviolet ray to radiate CS powders for 2 h, and using 0.22 μm of filtrate for α,β-GP and different kinds of acid solutions. The sterile CS-α,β-GP sol was prepared and to be injected. Each injection was 0.2 mL in volume and performed through hypodermic syringe equipped with a gauge 20G1 needle. After sacrifice of the mouse at a certain time after injection, CS-α,β-GP explants and its surrounding tissues were processed for histological study. The animal study followed the NIH guidelines for the care and use of laboratory animals (NIH Publication 85-23 Rev. 1985).

RESULTS AND DISCUSSION

Gel fabrication

Totally, eight kinds of different CS-α,β-GP porous hydrogels were obtained in this article as summarized in Table I. For the investigations of the effects of solvent variety on the characterizations of the hydrogels, the CS-FA-α,β-GP, CS-AA-α,β-GP, CS-PA-α,β-GP, CS-HA-α,β-GP, and CS-LA-α,β-GP hydrogels were prepared by dissolving CS in FA, AA, PA, HA,

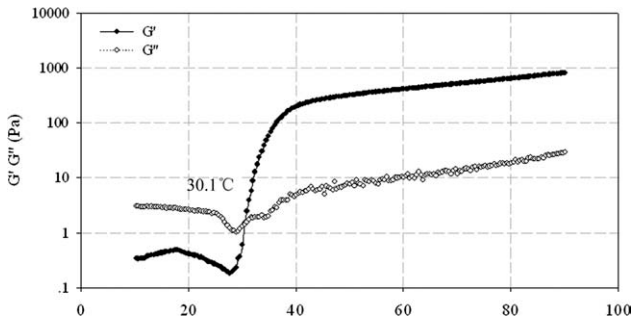

Figure 1 Loss modulus (G'') and storage modulus (G') of CS-1.0LA-α,β-GP hydrogel solutions as a function of temperature. The crossover point is indicative of gel formation as G' becomes greater than G'' in a certain temperature.

and LA (0.1*M*) aqueous solutions, respectively, and these hydrogels were initiated using 5% (g/mL) of α,β-GP. The CS-0.8LA-α,β-GP, CS-1.0LA-α,β-GP, and CS-1.2LA-α,β-GP were obtained by dissolving CS in 0.8, 1.0, and 1.2*M* of aqueous LA acid solutions, respectively, and these hydrogels were formed using 8% (g/mL) of α,β-GP as an initiator.

Effects of solvent variety and solvent strength of CS solution on gelation temperature

We have obtained eight types of CS-α,β-GP porous hydrogels differing in solvent variety and solvent strength of the CS solution. During the gelation process, the evolution of the rheological properties was investigated as a function of temperature by oscillatory shear measurements. Figure 1 shows the temperature dependence of G' and G'' used to determine T_{gel} of CS-1.0LA-α,β-GP hydrogel solution. The G' reflected the solid-like component of the rheological behavior, which was thus low at solution stage but increased drastically at the gelation point. The gel point was determined as the crossover point of the G' and G'' when the G' raised rapidly, and the gelation temperature (T_{gel}) was determined as the temperature at gel point, and this method had been shown to give an appropriate value of the T_{gel}.[6,18,25] The T_{gel} of the eight types of hydrogels could be changed by changing the solvent variety of CS solution as well as by varying the strength of the solvent.

Table I lists the T_{gel} of the hydrogels with different solvent varieties of the CS solution. Although the solvent strengths of the CS solutions were all 0.1*M*, the differences in acid varieties accounted for the differences in T_{gel} at the same solution concentration. At the same acid concentration, the CS-LA-α,β-GP hydrogel exhibited the lowest T_{gel} (35.7°C), whereas the CS-AA-α,β-GP hydrogel, with the AA as the solvent of the CS solution had the highest T_{gel} (47.7°C).

The results for the effects of the solvent strength on the T_{gel} of the hydrogel are also summarized in Table I. Higher concentration of LA led to higher T_{gel}. The T_{gel} of the hydrogel with 0.8*M* LA as solvent of CS (CS-0.8LA-α,β-GP) was 20.8°C, whereas the T_{gel} of the hydrogel with 1.2*M* LA as solvent (CS-1.2LA-α,β-GP) was 40.5°C. Therefore, the solvent strength of CS solution was one important factor for CS-α,β-GP hydrogel formation. The results demonstrated that a appropriate kind of acid and a relative lower solvent strength were both necessary for ideal T_{gel} of CS-α,β-GP porous hydrogels.

Effects of the solvent variety and solvent strength of CS solution on pH value

For experiments examining the effects of the solvent variety on the pH values, the pH values of the CS solutions and the CS-α,β-GP hydrogels during the gelation process were recorded (Fig. 2). Before the addition of α,β-GP [Fig. 2(A)], pH values of the CS solutions ranged from 4.98 to 5.40 with different solvent varieties. After the α,β-GP addition and before gelation [Fig. 2(B)], pH values of the tested samples ranged from 6.99 to 7.14, and compared with the CS solutions, the disparity of pH values among different solvents was smaller. After gelation [Fig. 2(C)], the pH values decreased in all the samples with different solvent varieties. Figure 2(E) shows a curve that indicates the pH changes of CS-LA-α,β-GP hydrogel, which was monitored as a function of temperature. The pH values decreased with increasing temperature in the sol-to-gel behavior. Figure 2(D) shows the changes in pH during the gelation process before vs. after gel formation. A distinct drop in pH occurred in all the test samples. The hydrogel with higher T_{gel} had a bigger pH decreased than other samples. For instance, the CS-AA-α,β-GP hydrogel which had a highest T_{gel} (47.7°C) among the samples with different solvent varieties had the biggest pH decreased (0.68).

The effects of the solvent strength on the pH values of the hydrogels are shown in Figure 3. Before the addition of α,β-GP [Fig. 3(A)], pH values ranged from 4.19 to 5.86 with higher pH values at lower solvent strength. The addition of α,β-GP could increase the pH values before gelation, and then the pH decreased with increasing temperature during the gelation process. The changes in pH values of the hydrogels during sol-to-gel behavior are shown in Figure 3(B); the hydrogel with a lower solvent concentration had a lower pH drop.

Solubility of CS in aqueous solutions was attained via protonation of its amine groups in acidic environments. Once dissolved, CS remained in solution up to a pH in the vicinity of 6.2. Neutralization

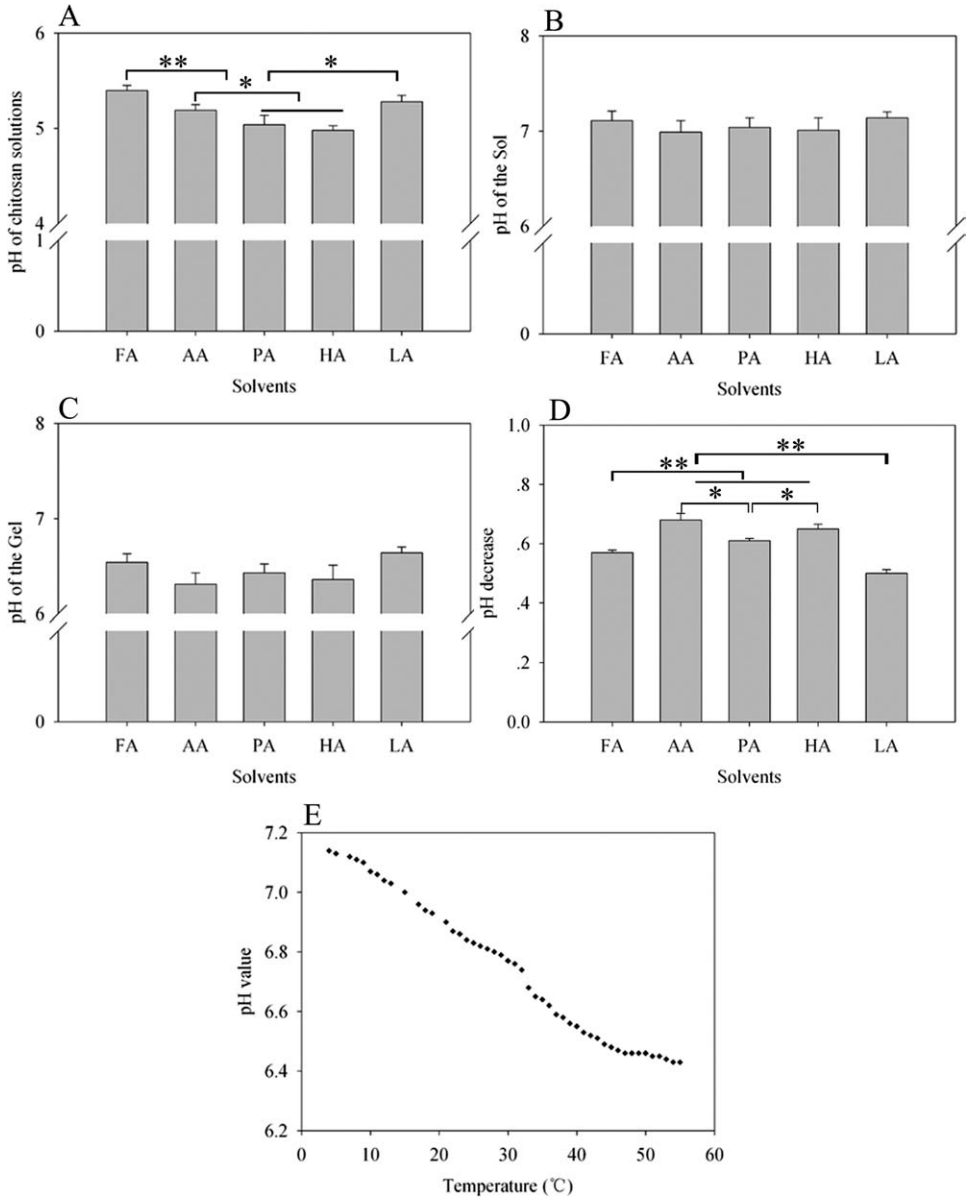

Figure 2 Effects of solvent variety of CS solution on pH values of hydrogels. (A) pH of the CS solutions before α,β-GP addition. (B) pH of the CS-α,β-GP hydrogels after α,β-GP addition but before gelation at 4°C. (C) pH of the CS-α,β-GP hydrogels after gelation at T_{gel}. (D) Chart of pH decreases after gelation. (E) The curve that the pH changes of CS-LA-α,β-GP hydrogel which monitored as a function of temperature ($n = 3$, *, statistically significant difference, $^*p \leq 0.05$, $^{**}p \leq 0.01$).

of CS aqueous solutions to a pH exceeding 6.2 systematically led to the formation of a hydrated gel-like precipitate. After the α,β-GP was added to the CS solution, the primary action of α,β-GP was the rapid neutralization of protonated amino groups from CS. At the same acid concentration, the pH changed by changing the acid variety. For the same acid, higher solvent strength led to lower pH at constant CS concentration. More acid in the CS solution needed more α,β-GP solution for neutralization, but higher α,β-GP concentration could decrease the cell metabolic activity in tissue engineering applications.[14,26] Therefore, as a prerequisite, to ensure the completely dissolution of CS, the concentration of acid should be controlled as low as possible. During the gelation process, the ionic strength increased at elevated temperature,[6,7,18] and therefore the pH decreased during the sol-to-gel behavior [Figs. 2(C–E) and 3]. The results indicated that the pH during all the process of gelation should be considered to design an ideal hydrogel for different experimental applications.

Effects of different solvent variety and solvent strength of CS solution on complex viscosity

The complex viscosity (η*) changes resulting from the different solvent varieties and solvent strengths

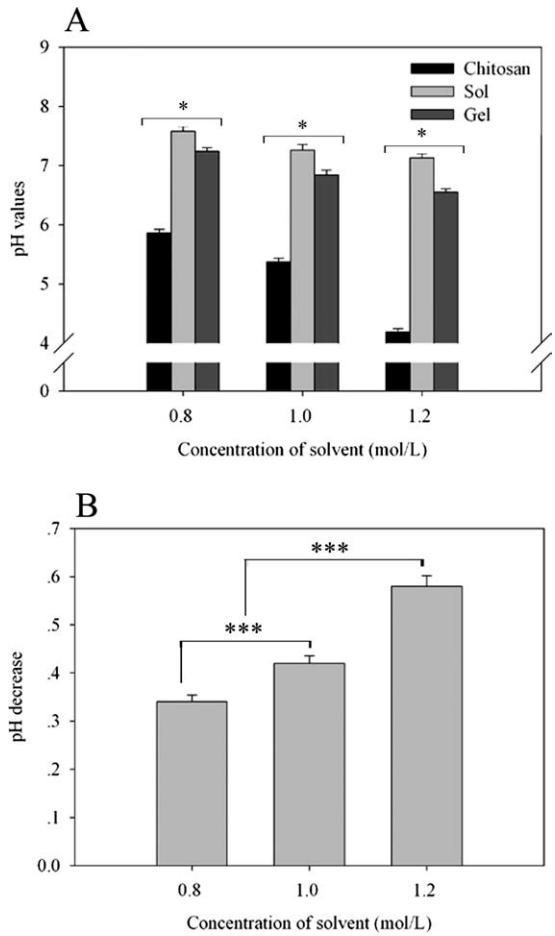

Figure 3 Effects of solvent strength of CS solution on pH values of hydrogels. (A) pH of the CS solutions before α,β-GP addition, sol at 4°C and gel at T_{gel}. (B) Chart of pH decreases after gelation. *, Statistically significant differences existing among the groups ($p \leq 0.05$) and ***, statistically significant differences existing between different solvent strength samples ($p \leq 0.001$).

of CS solution are shown in Figure 4. Along with temperature increasing, three regions were defined according to the mechanical properties: region 1: a slowly decreasing behavior, region 2: a fast increasing behavior, and region 3: a slowly increasing behavior. The complex viscosity of the system could be operated by changing the solvent variety and solvent strength of CS solutions (Fig. 4).

Figure 4(A) shows the complex viscosity affected by the solvent variety. At 10°C before gelation, the complex viscosity values of the hydrogels from high to low in turn were CS-HA-α,β-GP, CS-AA-α,β-GP, CS-LA-α,β-GP, CS-PA-α,β-GP, and CS-FA-α,β-GP. Along with the temperature increasing, the complex viscosity of the CS-LA-α,β-GP sample increased rapidly and entered into region 2 at 35.7°C (the lowest temperature among the samples), whereas the CS-AA-α,β-GP sample entered into region 2 at 47.7°C (the highest temperature among the samples). At region 3 after gelation, the differences among the complex viscosities of the CS-AA-α,β-GP, CS-LA-α,β-GP, and CS-HA-α,β-GP samples were not obvious, whereas the complex viscosities of CS-FA-α,β-GP and CS-PA-α,β-GP samples were similar.

The complex viscosities affected by solvent strength of CS solution are shown in Figure 4(B). The results showed that the increase of the solvent strength could increase the temperature of the hydrogel entered region 2. At region 3 after gelation, the complex viscosities increased with the increase of the solvent strength.

Before gelation, the complex viscosities of all the hydrogels in this study were low enough to be easily handled with a micropipette and syringe (data not shown). The complex viscosities increased rapidly when the temperature reached the gelation point (region 2) and the increasing speed was fast enough to be used as *in situ* forming hydrogels for tissue repair. The complex viscosities of the hydrogels entered into region 2 at different temperatures for different solvent varieties [Fig. 4(A)] and solvent strengths [Fig. 4(B)]. The results demonstrated that the complex viscosities and gelation temperatures of the CS-α,β-GP hydrogels could be easily manipulated by changing the solvent variety and solvent strength of the CS solutions.

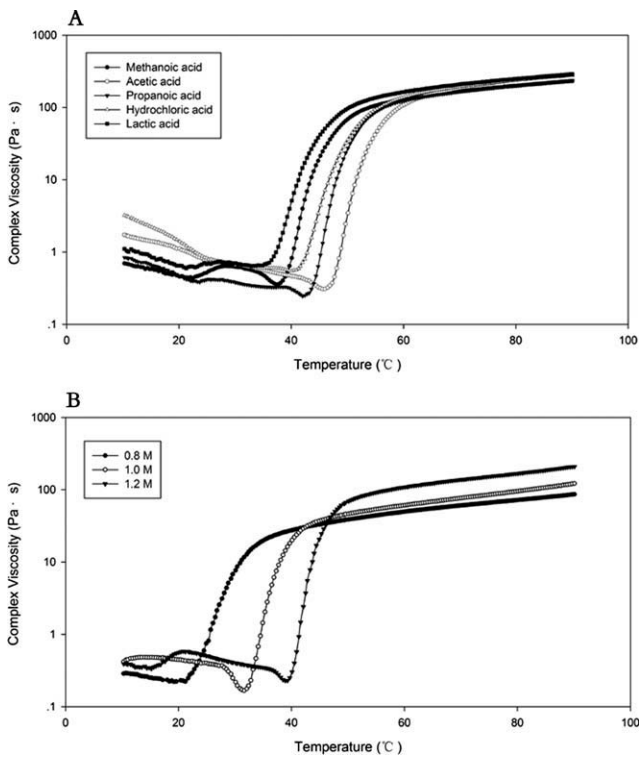

Figure 4 The complex viscosity (η*) changes of the hydrogels resulting from the different solvent varieties (A) and solvent strengths (B) of CS solutions.

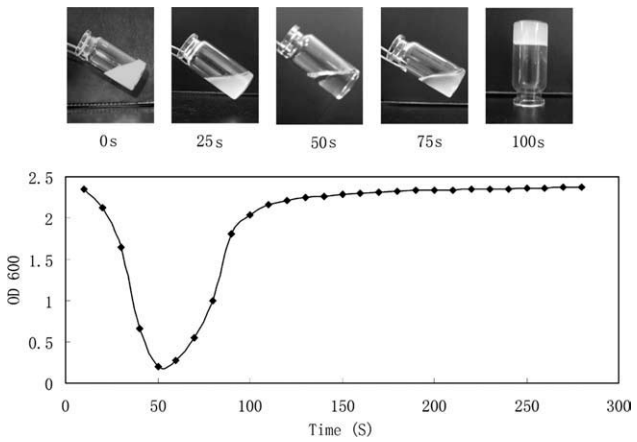

Figure 5 The turbidity changes of CS-LA-α,β-GP hydrogel sample in the function of time at T_{gel}. Insets are pictures of CS-LA-α,β-GP hydrogel at 0, 25, 50, 75, and 100 s during the gelation process.

Effect of solvent variety and solvent strength of CS solution on turbidity

The effects of solvent variety and solvent strength of CS solution on turbidity are summarized in Table I. The results indicated that the absorbancy at 600 nm (OD_{600}) of the sol state and gel state of the hydrogel could be changed by changing the solvent variety and solvent strength of the CS solution. The OD_{600} of the sol state increased but the OD_{600} of the gel state decreased along with increasing of the solvent strength.

Figure 5 shows the turbidity changes of CS-LA-α,β-GP samples in the function of time at T_{gel}. As shown in Figure 5, the OD_{600} of CS-LA-α,β-GP hydrogel at T_{gel} was time dependent. The dynamics curve of turbidity decreased rapidly along with the extending of time till 50 s, and then it underwent a rapidly increased part following a slowly increased part. The insets in Figure 5 demonstrate the appearance of the CS-LA-α,β-GP hydrogel at 0, 25, 50, 75, and 100 s during sol-to-gel behavior at T_{gel}. At 0 s, the hydrogel sample was milk-white and in the sol state, when the time expanded to 50 s, the hydrogel became transparent and also in the sol state; along with the extending of time, the OD_{600} increased and the CS-LA-α,β-GP hydrogel became gel state. When the samples were shifted from a 4°C environment to a higher temperature environment (T_{gel}), the temperature of the hydrogels was 4°C at 0 s, and then it increased with extended time until achieved balance with surrounding environment. The reduced solubility and increased hydrogen bond of CS at low temperature at 0 s tended to form physical junctions, resulting in the increase of system turbidity. With the temperature increasing along with the time extending from 0 to 50 s, the weaker hydrogen bond between CS skeleton and water molecules could make CS molecules unfold freely, and thus leading to the decrease in turbidity.[7,18] When the time extended from 50 to 100 s, the temperature raised to T_{gel} and the hydrophobic interaction between CS skeleton and possible ion bridge increased, and this resulted in the gel formation, the turbidity of system increased again.[18,27]

Effects of solvent variety and solvent strength of CS solution on morphology of the hydrogel

The porous structure of the hydrogels was studied by SEM. SEM images of the inside of the hydrogels prepared with different solvent varieties and solvent strengths are shown in Figure 6, and the structure of the matrix clearly changed with solvent variety [Fig. 6(A–E)] and solvent strength [Fig. 6(F–H)]. The comparison of the SEM pictures of the hydrogels with solvent variety [Fig. 6(A–E)] showed the different porous structures. The interior morphology of the CS-FA-α,β-GP [Fig. 6(A)], CS-AA-α,β-GP [Fig. 6(B)], CS-HA-α,β-GP [Fig. 6(D)], and CS-LA-α,β-GP [Fig. 6(E)] hydrogels demonstrated open, interconnected highly porous structure, whereas the CS-PA-α,β-GP hydrogel [Fig. 6(C)] showed a flake and porous structure. Compared with the CS-FA-α,β-GP, CS-AA-α,β-GP, and CS-HA-α,β-GP samples, the CS-LA-α,β-GP sample had a much bigger pore diameter and a more compact and glosser framework. The results of the effects of solvent strength on morphology of the hydrogels are shown in Figure 6(F–H). The results indicated that the higher of the solvent strength was, the smaller of the pore diameter and the glosser of the framework of the hydrogel was. This might be because of the reason that the hydrogel prepared with higher solvent strength had higher T_{gel}. As the temperature increased, the chain flexibility and compactness of polyelectrolyte molecules such as CS in solution increased, causing the reduction of the molecular volume. Therefore, the hydrogel prepared with higher solvent strength had smaller pore diameter.[28]

MTT assay

The MTT assay was generally accepted as a routine method for establishing cytotoxicity of biomaterials. The MTT reagent is a yellow tetrazolium salt which produces a dark-blue formazan crystal when incubated with viable cells. Therefore, the level of the reduction of MTT into formazan will reflect the level of the live cells metabolism. To prove the safety of CS-α,β-GP hydrogel as a biomaterial, the effects of the CS-α,β-GP hydrogel on the viability of MEFs were determined using the MTT assay (Fig. 7). Figure 7(A) shows the effects of the CS solvent variety

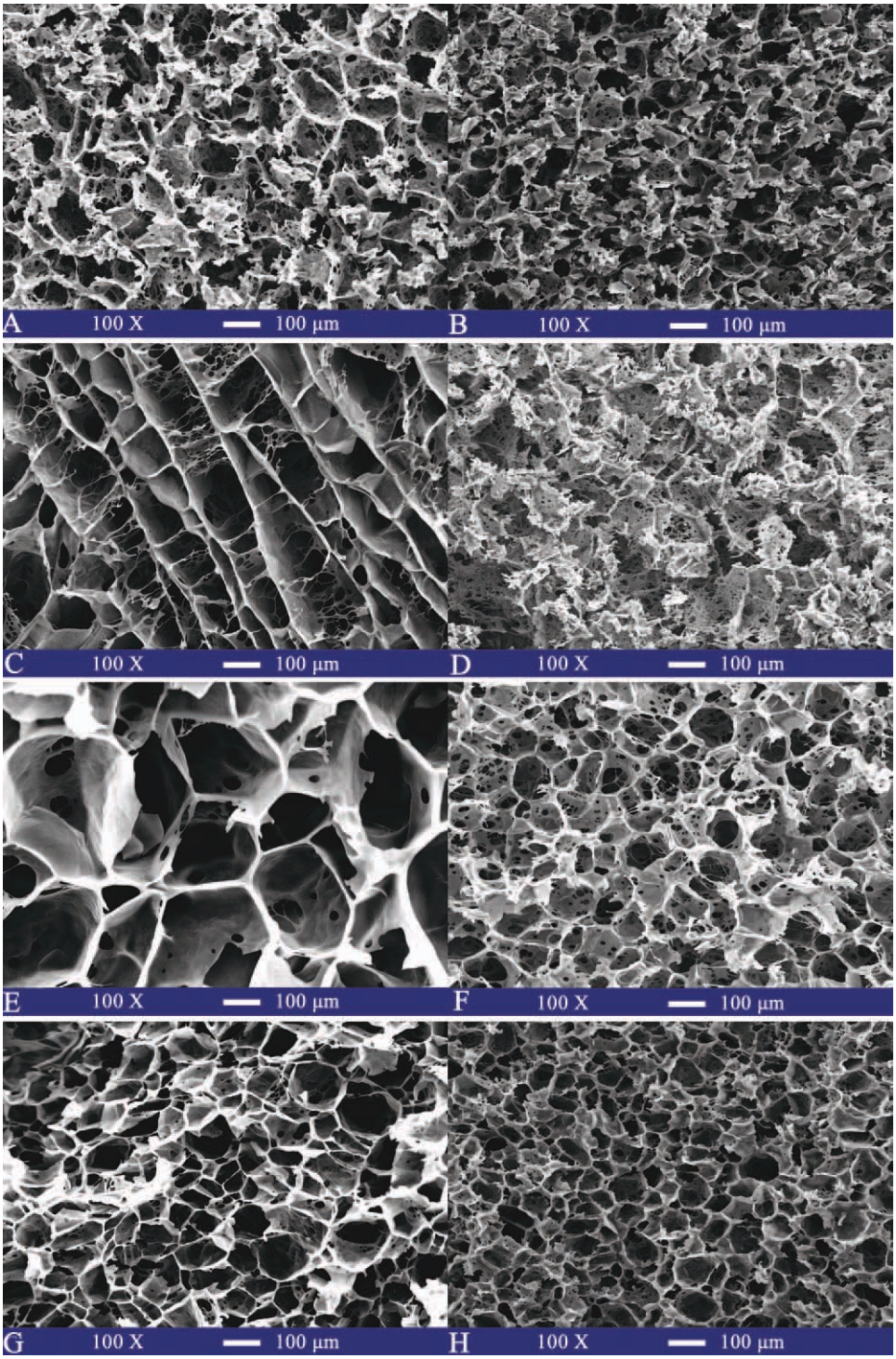

Figure 6 SEM images of the hydrogels. (A) CS-FA-α,β-GP hydrogel, (B) CS-AA-α,β-GP hydrogel, (C) CS-PA-α,β-GP hydrogel, (D) CS-HA-α,β-GP hydrogel, (E) CS-LA-α,β-GP hydrogel, (F) CS-0.8LA-α,β-GP hydrogel, (G) CS-1.0LA-α,β-GP hydrogel, and (H) CS-1.2LA-α,β-GP hydrogel. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

used to prepare hydrogel on the RGR % at different concentrations of hydrogel extracts. Incubation of the MEFs with 25% of extracting solutions resulted in a RGR % above 80%, indicating a low toxicity of the extracts. The RGR % decreased obviously as the concentration of the extracts increased. When the concentration of the extracts solutions increased to 100%, the RGR % for CS-FA-α,β-GP and CS-PA-α,β-GP were <75%, whereas the RGR % for CS-AA-α,β-GP, CS-HA-α,β-GP, and CS-LA-α,β-GP were >80%,

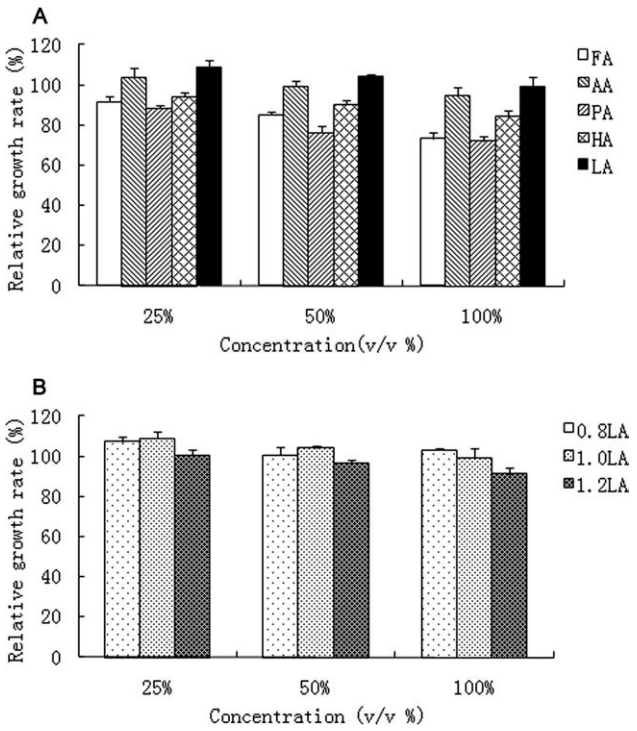

Figure 7 *In vitro* cytotoxicity of different extracting solutions of CS-α,β-GP hydrogel prepared with different solvent varieties (A) and different solvent strengths (B).

indicating that the CS-FA-α,β-GP and CS-PA-α,β-GP hydrogel had higher cytotoxicity. Figure 7(B) shows the RGR % affected by the extracts of the hydrogel prepared with different solvent strengths. The RGR % increased slowly as the solvent strength decreased and the RGR % for all the samples were >95%, indicating a low toxicity of the extracts.

The cytocompatibility of hydrogel materials is an important consideration for the applications, because the materials must give no harm when interacting with the body. To prove the safety of CS-α,β-GP hydrogel as a biomaterial, the effects of the eight kinds of hydrogel extracts on the MEFs viability were investigated. As shown in the MTT assay results, RGRs % for CS-AA-α,β-GP, CS-HA-α,β-GP, and CS-LA-α,β-GP were >80%, showing a low toxicity of the extracts.

In vivo biocompatibility test

Thermosensitive CS-α,β-GP aqueous sol could be easily handled with a micropipette and syringe needle, opening new method for minimally invasive and site-specific *in situ* forming implants. Following injection, the CS-LA-α,β-GP, CS-0.8LA-α,β-GP, and CS-1.0LA-α,β-GP sol could form an oval skin protrusion at the injection site, indicating that these aqueous solutions gelled rapidly owing to the increasing temperature of the tissue fluid surrounding the injection site. The CS-MA-α,β-GP, CS-AA-α,β-GP, CS-PA-α,β-GP, CS-HA-α,β-GP, and CS-1.2LA-α,β-GP sol could not form gel because of their high T_{gel}. All the mice of the experimental group survived and had a normal lifeway after injection. During the observation period, no inflammation was found in the CS-AA-α,β-GP, CS-HA-α,β-GP, CS-LA-α,β-GP, CS-0.8LA-α,β-GP, CS-1.0LA-α,β-GP, and CS-1.2LA-α,β-GP injection groups, and the avoirdupois of the mouse, compared with the control group, did not change obviously (data not shown). The CS-MA-α,β-GP and CS-PA-α,β-GP injection groups showed mild inflammatory response, indicating that the MA and PA were not suitable solvents of CS to prepare injectable hydrogel.

The inflammatory response after injection could also be characterized using hematoxylin–eosin (HE) staining. The injected hydrogels were carefully removed using tweezers after the mice (the CS-LA-α,β-GP, CS-0.8LA-α,β-GP, and CS-1.0LA-α,β-GP groups) were sacrificed in 1 and 10 days after injection. Figure 8 shows the histological response of the implantation of CS-α,β-GP hydrogel. At 1 day post-injection, a few lymphocytes and macrophages were observed in the tissues among all the three groups [Fig. 8(A1–C1)]. After 10 days injection, the degree of inflammation was much milder and neutrophil infiltration was significantly alleviated [Fig. 8(A2–C2)]. The results suggested that the CS-LA-α,β-GP, CS-0.8LA-α,β-GP, and CS-1.0LA-α,β-GP hydrogel had good tissue compatibility and could be designed as injectable hydrogels.

The results of the present study demonstrated that the characterizations of the CS-α,β-GP hydrogel could be regulated by adjusting the solvent variety and solvent strength of CS solution. The characterizations of the CS-α,β-GP hydrogel could also be regulated by changing the CS/α,β-GP ratio and this has been reported in our previous study.[24] The requirements for the characterizations (such as T_{gel}, pH value during the gelation process, complex viscosity, porous structure, etc.) of the hydrogel were different according to the different applications of the hydrogel. For example, the hydrogel designed for tissue engineering should have a T_{gel} lower than 37°C, whereas the hydrogel designed as 3D culture scaffold for shrimp cell should have a T_{gel} between 20 and 25°C. The present study gave a method to design a hydrogel with appropriate characterizations meeting the application requirements by changing the solvent variety and solvent strength of CS solution. As the process of the hydrogel formation was simple and usually performed under mild conditions, we believe that such a highly porous hydrogels will have potential applications in tissue engineering and other related biomedical fields.

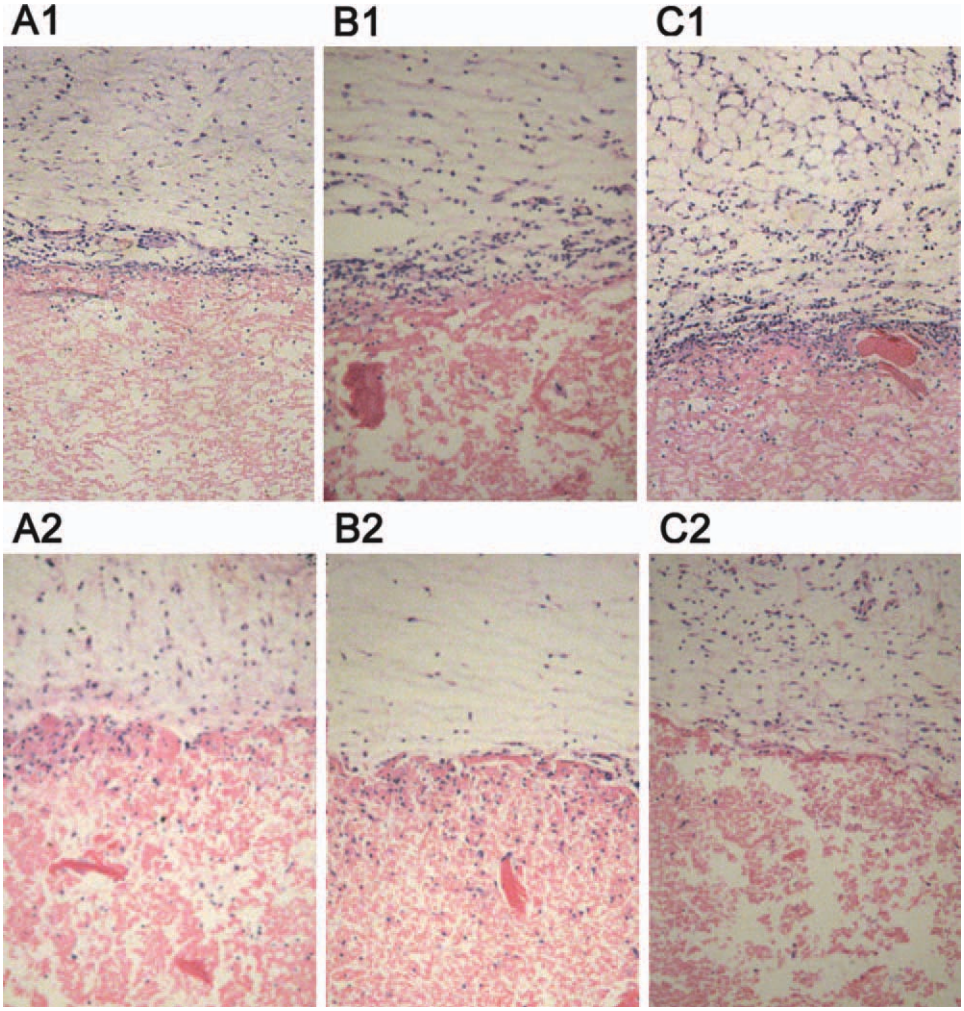

Figure 8 Light microscopy investigation of the inflammatory reaction after subcutaneous injection of the CS-α,β-GP hydrogel in mouse. (A1: 1 day after injection of CS-LA-α,β-GP hydrogel; B1: 1 day after injection of CS-0.8LA-α,β-GP hydrogel; C1: 1 day after injection of CS-1.0LA-α,β-GP hydrogel; A2: 10 days after injection of CS-LA-α,β-GP hydrogel; B2: 10 days after injection of CS-0.8LA-α,β-GP hydrogel; C2: 10 days after injection of CS-1.0LA-α,β-GP hydrogel). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

CONCLUSION

In the present study, a series of CS-α,β-GP hydrogels were prepared by dissolving CS in different solvents. The SEM images showed that all the hydrogels prepared in this study demonstrated highly porous structure. The comparison of the SEM pictures showed that the hydrogels with different solvent varieties and solvent strengths had different pore diameters and framework morphologies. The gelation temperatures, complex viscosities, pH values, turbidity, and porous structures of the hydrogels prepared in this study could be changed by changing the solvent variety and solvent strength of the CS solutions. The hydrogel prepared with the CS dissolving in LA had lower T_{gel} and could form an oval skin protrusion at the injection site when injected into dorsal subcutaneous in adult kunming mouse. All the hydrogels prepared with AA, HA, or LA as the solution of CS had good biocompatibility. These studies indicated that the thermosensitive highly porous hydrogels for different experimental applications could be designed by adjusting the solvent variety and solvent strength of the CS solution.

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