Journal of Applied Polymer Science

Biological Evaluation of Chitosan-Based *In Situ*-Forming Hydrogel with Low Phase Transition Temperature

Qi Feng Dang,[1] Jing Quan Yan,[1] Hong Lin,[1] Cheng Sheng Liu,[1] Xi Guang Chen,[1] Qiu Xia Ji,[2] Jing Li,[1] Ya Liu[1]

[1]Ocean University of China, Qingdao 266003, People's Republic of China

[2]The Affiliated Hospital of Medical College, Qingdao University, Qingdao 266001, People's Republic of China

Correspondence to: C. Sheng Liu (E-mail: liucsouc@126.com or Liucs@ouc.edu.cn) and X.G. Chen (E-mail: xgchen@ouc.edu.cn)

ABSTRACT: The aims of current study were to choose a method for preparing sterile chitosan-α,β-glycerophosphate (CS-α,β-GP) *in situ*-forming hydrogel which had potential applications in tissue engineering and evaluated its biocompatibility and degradation characteristics. The results of sterilization stability tests indicated that sterile formulations could be obtained by ultraviolet irradiation of CS powders, 0.22 μm filtration of α,β-GP and lactic acid solutions, and sterile preparation of CS-α,β-GP formulations. The obtained sterile CS-α,β-GP formulations showed low hemolysis rates and low BSA adsorption at physiological conditions. When injected *in vivo* the CS-α,β-GP sol turned into gel implant *in situ* and could be degraded gradually. A minimal inflammatory reaction which could not be found by macroscopic evaluation was induced after injection and new capillary formation was found around the hydrogel humps, making the CS-α,β-GP hydrogel worthwhile to be considered for tissue engineering and biomedical applications. © 2014 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2015**, *132*, 41594.

KEYWORDS: biocompatibility; biodegradable; biomaterials; functionalization of polymers; gels

Received 2 July 2014; accepted 2 October 2014

DOI: 10.1002/app.41594

INTRODUCTION

In recent years, thermosensitive hydrogels with a sol-gel phase transition properties have attracted increasing interest in a wide range of tissue engineering and biomedical applications.[1–3] There are dozens of polymers, including natural polymers and synthetic polymers, have been used to prepare thermosensitive hydrogels. The thermosensitive hydrogel formulations prepared with most polymers keep a sol phase at high temperature and come into being a gel phase on lowering the temperature. However, an injectable reverse thermosensitive formulations coming into being gel phases on raising temperature were obtained by neutralizing semi-diluted and highly deacetylated chitosan (CS) solution with alkalescent salts such as organic and inorganic phosphates.[4–9]

CS is a well-known linear polysaccharide which has been widely studied in the biomedical and tissue engineering field.[10] The increasing attention to CS from the people inside and outside of academia depends on not only its non-toxicity, biocompatibility, and biodegradability but also its unique cationic property and versatility with various molecules and deacetylation degrees.[11–13] The sodium glycerophosphate (GP), which is used as nutrient additive and flavor for yoghurt in food industry, is an organic weak base and has been proved to improve thermogelling.[5,14,15] CS-GP thermosensitive hydrogel was initially reported by Chenite et al.[4] and has attracted the attentions of more and more research groups.[3,16–20] In our previous studies, we reported a thermosensitive *in situ*-forming CS-α,β-GP hydrogel which characterizations could be regulated by adjusting the CS/α,β-GP ratios, the solvent varieties and the solvent strengths of the CS solutions.[21,22] We also proved the feasibility of this hydrogel as potential 3D culture scaffold for the *penaeus chinensis* lymphoid cells.[23] The CS-α,β-GP hydrogels reported in our previous publications could be designed as injectable *in situ*-forming hydrogel formulations used in tissue engineering and biomedical fields because they remained in solutions (sol phase) at physiological pH and low temperature while changed into semisolids (gel phase) upon heating at body temperature. When designing materials for tissue engineering and biomedical applications it is of utmost importance to characterize them of sterilization stabilities, biocompatibilities and biodegradation behaviors.[24,25]

Sterilization is a very important step in developing *in situ*-forming materials used in tissue engineering and biomedical fields. Sterilization on biomaterials may alter the physical and (or) chemical characterizations, lead to drop or loss of biosafety and functionality of these materials. Although many studies have

been reported in the sterilizations of CS-based systems during the past few decades, little works have been published in the sterilizations of CS-based hydrogels.[26–28] In several previous studies, sterile CS-GP hydrogels were obtained by using regular liquid autoclaving for CS solutions and 0.22 μm filtration for GP solutions.[4,29] Jarry et al.[25] showed that autoclaving of CS solutions induced molecular weight reduction, which leaded to a loss of dynamic viscosities, gelling rates, and mechanical strengths of the CS–GP systems. Jarry, Leroux, Haeck, and Chaput[30] investigated the effects of γ-irradiation on CS and thermogelling CS-β-GP formulations and the results indicated that γ-irradiation was not an appropriate method to sterilize CS solutions because it massively decreased the molecular weight of CS and did not provide a chemically stable sterile product. Thus, an ideal method for preparing sterile CS-GP *in situ*-forming hydrogel is still lacking.

It is a normal physiological reaction for materials used for tissue engineering and biomedical applications undergo host responses when implanted or injected into living tissues. Biocompatibility is usually cleared as the capability of a material to show a suitable host response in a specific application. The quantitative evaluation of protein adsorption and the cytotoxicity of the CS-α,β-GP *in situ*-forming hydrogel have been investigated in one of our previous study and the results indicated this hydrogel had low protein adsorption and cytotoxicity.[23] Nonetheless, more tests such as *in vitro* hemolysis analysis and qualitative evaluation of protein adsorption, and *in vivo* biocompatibility tests should be done, in the interest of ensuring the biocompatibility of the CS-α,β-GP *in situ*-forming hydrogel. Moreover, it is a major step to determine such materials of degradation owing to the fact that they can cause diverse inflammatory reactions.

In this publication, the biological evaluations of CS-α,β-GP *in situ*-forming hydrogel formulations with low phase transition temperature were reported. The effects of autoclaving of CS powders, autoclaving of CS solutions and ultraviolet-irradiation of CS powders on the characterizations of the CS-α,β-GP hydrogel were evaluated to select suitable method to obtain sterile hydrogel formulations. Prior to injection, the CS-α,β-GP hydrogel were tested for hemolysis analysis and qualitative evaluation of protein adsorption to study the *in vitro* biocompatibility. The *in vivo* biocompatibility and degradation were evaluated for 50 days in the adult Kunming Mice.

EXPERIMENTAL

Preparation of CS-α,β-GP Hydrogel

CS-α,β-GP hydrogels were prepared according to our previous study.[23] To obtain gel formation at physiologically acceptable pH value and low temperature, a 0.08 mol/L of CS (derived from shrimp shell, deacetylation degree 96.5%, dynamic viscosity 140 cps) solvent strength and a 9.0/1.0 of CS/α,β-GP ratio were chosen.

Sterilization Stability of the CS-α,β-GP Hydrogel

Preparation of Sterile Hydrogel Formulations. Sterile hydrogel formulations were obtained according to the above method (Preparation of CS-α,β-GP hydrogel) in an aseptic environment. All the α,β-GP solutions and lactic acid solutions were sterilized using 0.22 μm filtration. Sterile CS solutions were obtained through the following three patterns. (1) The CS powers were sterilized by autoclaving at 115°C for 10 min, 20 min, 30 min and 121°C for 20 min, respectively. Then the CS solutions were prepared by dissolving CS powers in sterile lactic acid solutions. (2) The CS solutions were prepared firstly and then sterilized by autoclaving at 115°C for 10 min, 20 min, 30 min and 121°C for 20 min, respectively. (3) The sterile CS powers were gained by using ultraviolet ray radiated CS powders for 30 min, 60 min and 120 min at 25°C, respectively. Then the sterile CS solutions were prepared by dissolving CS powers in sterile lactic acid solutions.

Characterizations of CS Solutions and CS-α,β-GP Formulations Before and After Sterilization. The rotational viscosities of the CS solutions (50 mL) before and after sterilization were characterized using a NDJ-8S rotational viscometer (Shanghai Cany Precision Instrument, Shanghai, China) at 25°C. Measuring the rotational viscosities, the second rotating spindle was chosen and submerged in the samples, and the rotation speed was 1.5 rpm. The rotational viscosities (Pa·S) of CS solutions were measured and recorded. Measurements were done in triplicate and the quantitative data were expressed as mean ± standard deviation. The pH values of the CS solutions at 25°C, the CS-α,β-GP sol at 4°C, and the CS-α,β-GP gel at 37°C were measured using a Delta 320 pH Meter (Mettler Toledo, Greifensee, Switzerland). Measurements were done in triplicate and the quantitative data were expressed as mean ± standard deviation. The gelation temperatures (T_{gel}) of the CS-α,β-GP hydrogel formulations were measured in accordance with the method reported in our previous study.[21] The sterilizing effect in killing bacteria of different sterilization methods were measured using spread plate method (Nutrient agar plates), and the colony-forming units were recorded.

Biocompatibility of the CS-α,β-GP Hydrogel

Hemolysis Analysis. The hemolysis tests of the CS-α,β-GP hydrogel formulations and materials used to prepare CS-α,β-GP hydrogel were studied as described by Jumma, Furkert, and Müller[31] and the whole rabbit blood was chosen to evaluate the hemolysis of specimen. Samples of CS-α,β-GP sol and gel were freeze-dried (for 48 h) and pulverized before determined.

In brief, The erythrocyte stock dispersion (60 μl) was added into CS, CS-α,β-GP sol, CS-α,β-GP gel suspensions and α,β-GP solution (3 mL) and incubated under shaking at 100 rpm at 37°C for 1 h, then the suspensions were centrifugated at 750×g for 10 min. The absorbency of the resulting supernatant was measured at 545 nm. The hemolysis rate (HR %) was calculated with 0.9% NaCl as negative control (nc, 0% lysis) and distilled water as positive control (pc, 100% lysis), and was calculated according to the following formula:

$$HR\% = \frac{D_{\text{sample}} - D_{nc}}{D_{pc} - D_{nc}} \quad (1)$$

A mean hemolysis value from three test samples of 5% or less was believed to be acceptable.

Protein Adsorption Assay. For the qualitative assessment of protein adsorption, the suspensions of CS-α,β-GP sol and gel

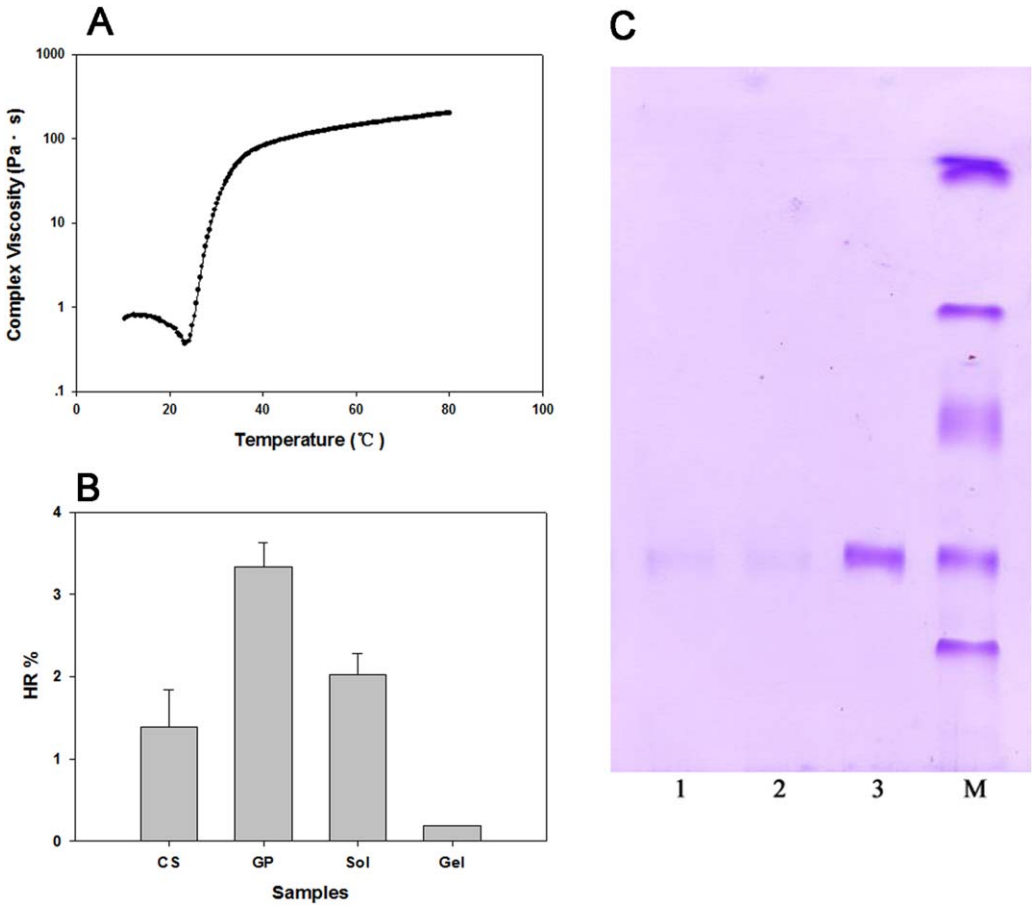

Figure 1. The complex viscosities of the CS-α,β-GP hydrogel (A), HRs of CS, α,β-GP, CS-α,β-GP Sol and CS-α,β-GP Gel samples (B), and SDS-PAGE electrophoresis gel (C: Line1, BSA adsorbed on CS-α,β-GP Sol; Line 2, BSA adsorbed on CS-α,β-GP Gel; Line 3, BSA before adsorption; Line M, Maker). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

were incubated in fresh BSA at 37°C for 9 h, and then was applied to the SDS-PAGE. The SDS-PAGE was performed on an acrylamide gel consisting of 10% separating gel and 5% stacking gel under reducing conditions. The protein was observed by staining with CBB R-250.

***In Vivo* Gelation and Biocompatibility Test.** An aqueous sterile CS-α,β-GP sol was prepared and handled by dorsal subcutaneous injections in adult Kunming Mice. Each injection was 0.2 mL in volume and carried out through hypodermic syringe equipped with a gauge 20G1 needle. At certain time intervals after injection, blood was collected via the venous plexus behind eyeball under anesthesia and immediately transferred into pre-chilled MicrotainerR tubes for analysis. After sacrifice of the mice, CS-α,β-GP explants and surrounding tissues were processed for histological study and scanning electron microscopy (SEM) examinations. The animal study followed the NIH guidelines for the care and use of laboratory animals (NIH Publication 85-23 Rev. 1985) and EU Directive 2010/63/EU for animal experiments.

Degradation of the CS-α,β-GP Hydrogel

***In Vitro* Enzymatic Degradation.** The aqueous sol of CS-α,β-GP (2 mL) was placed in a 6-well cell culture plate and allowed to gel at 37°C. Then 4 mL of 0.25% lysozyme solutions (pH 7.0, pH 7.2, and pH 7.4, respectively) were added to the 6-well cell culture plate and incubated at 37°C. *In vitro* degradation of the CS-α,β-GP hydrogel was monitored by capturing images of the discs after 3 h for SEM and 6 h for macro-photograph.

***In Vivo* Degradation.** The *in vivo* injection was conducted just like the injection method mentioned in "*In vivo* gelation and biocompatibility test" above in this manuscript. After dorsal subcutaneous injections of CS-α,β-GP sol in adult Kunming Mice, the resulting gel implants were allowed to develop *in vivo*. At certain time intervals the mice were sacrificed to observe volume loss of the hydrogel and the excised hydrogel was freeze-dried to examine the interior morphology by SEM at given time post-injection.

RESULTS AND DISCUSSION

Preparation of the CS-α,β-GP Hydrogel

The hydrogels prepared with CS and α,β-GP were a type of porous thermosensitive hydrogels with controllable characterizations, such as T_{gel}, pH value, turbidity and complex viscosity.[21,22] When the CS/α,β-GP ratio was 9.0/1.0 and the CS solvent strength was 0.08 mol/L, gel formation was accomplished in 90 s at 25°C and in 30 s at 37°C at physiologically acceptable pH value.[23] As shown in Figure 1(A), the CS-α,β-GP

Table I. The Characterizations of CS Solutions and CS-α,β-GP Formulations Before and After Sterilization

	CS solutions		CS-α,β-GP systems			
Sample	Rotational viscosity (Pa·S)	pH value (25°C)	pH value (Sol, 4°C)	pH value (Gel, 37°C)	T_{gel}	CFU/10 mL
Control	37.36 ± 0.09	5.57 ± 0.03	7.36 ± 0.03	6.87 ± 0.02	25°C	24.3[a]
Steam high-pressure sterilization of CS powers						
115°C 10 min	34.67 ± 0.05	5.26 ± 0.02	7.26 ± 0.03	6.82 ± 0.02	30°C	N[b]
115°C 20 min	32.77 ± 0.08	5.18 ± 0.03	7.18 ± 0.02	6.77 ± 0.03	31°C	N[b]
115°C 30 min	29. 65 ± 0.05	5.05 ± 0.01	7.10 ± 0.02	6.72 ± 0.03	32°C	N[b]
121°C 20 min	22.11 ± 0.07	4.81 ± 0.03	6.91 ± 0.03	6.65 ± 0.02	35°C	N[b]
Steam high-pressure sterilization of CS solutions						
115°C 10 min	27.17 ± 0.11	5.14 ± 0.02	7.16 ± 0.01	6.83 ± 0.02	30°C	N[b]
115°C 20 min	26.83 ± 0.06	5.08 ± 0.01	7.14 ± 0.03	6.76 ± 0.01	32°C	N[b]
115°C 30 min	25.57 ± 0.07	4.97 ± 0.02	7.06 ± 0.02	6.71 ± 0.01	33°C	N[b]
121°C 20 min	19.02 ± 0.10	4.67 ± 0.03	6.81 ± 0.01	6.52 ± 0.02	36°C	N[b]
Ultraviolet sterilization of CS powders						
30 min	37.28 ± 0.07	5.56 ± 0.02	7.34 ± 0.04	6.88 ± 0.03	25°C	7.8[a]
60 min	37.41 ± 0.08	5.57 ± 0.02	7.36 ± 0.01	6.87 ± 0.02	25°C	N[b]
120 min	37.35 ± 0.11	5.55 ± 0.03	7.37 ± 0.02	6.85 ± 0.03	25°C	N[b]

[a] $\times 10^4$
[b] the colony forming units count was 0.

hydrogel had low complex viscosities at low temperature, making it could be easily handled with a micropipette or syringe needle. With the temperature increasing, the complex viscosities of the hydrogel increased rapidly and the system transferred to *in situ* gel.

Sterilization Stability of the CS-α,β-GP Hydrogel

The characterizations of CS solutions and CS-α,β-GP formulations before and after sterilization were shown in Table I. Autoclaving for CSpowders or solutions could kill microorganisms effectively but changed part of the natures of CS. The rotational viscosity of CS solution (0.18%, w/v) was 37.36 ± 0.09 prior to sterilization. After autoclaving, the rotational viscosities of CS solutions were found to decrease with increasing temperature and/or time of the autoclave cycle. The pH values of the CS solutions and CS-α,β-GP formulations were also experienced a major drop during autoclaving of CS powders or solutions. The T_{gel} of CS-α,β-GP hydrogel was 25°C before sterilization and increased obviously after sterilization using autoclaving for CS powders or solutions. All the samples sterilized with autoclaving experienced discoloration [Figure 2(A,B)]. The higher of the temperature and the longer of the time of autoclave cycle, the deeper of the colors of CS powers, CS solutions and CS-α,β-GP gels. Overall, the autoclaving process decreased the rotational viscosities of CS solutions as well as pH values of CS solutions and CS-α,β-GP formulations, enhanced the T_{gel} of the CS-α,β-GP hydrogel. Autoclaving would not seem to be a good alternative for the sterilization of CS powders or CS solutions.

Ultraviolet-irradiation of CS powders at 25°C for 60 min or 120 min could kill microorganisms completely (Table I). After ultraviolet sterilization for 30 min, 60 min or 120 min of CS powders, the rotational viscosities of CS solutions as well as the pH values of CS solutions and CS-α,β-GP formulations did not show any significant changes (Table I). The pH values range of CS-α,β-GP formulations was 7.37∼6.85, which was a physiologically acceptable pH range according to Cambridge MedChem Consulting, and it would not affect the tissue growth of Kunming Mouse due to the small injection amount. The colors of the samples did not change after ultraviolet sterilization either [Figure 2(C)]. The T_{gel} of CS-α,β-GP hydrogels were not changed during short-time ultraviolet sterilization (within 120 min). Yue, He, Yao, and Wei[32] reported that combined with ultraviolet radiation as an accessory factor, ozone could result in degradation of CS. The rotational viscosities of CS solutions did not change obviously in this research, probably because the short exposure time did not lead to visible degradation of CS. Furthermore, the molecular weight of CS had not significant effects on the T_{gel} of the CS-GP hydrogels,[4] therefore, slightly degradation of CS did not change the T_{gel} of CS-α,β-GP hydrogels obviously. Anyway, ultraviolet sterilization for 120 min of CS powders could kill microorganisms effectively, and did not change the characterizations of CS solutions and CS-α,β-GP hydrogel formulations obviously. Sterile CS-α,β-GP formulations would be obtained by employing ultraviolet ray irradiated CS powders for 120 min, and employing 0.22 μm filtration to filter α,β-GP solution and lactic acid solution in present work.

Biocompatibility of the CS-α,β-GP Hydrogel

Hemolysis Analysis. *In vitro* hemolysis analysis has been always used as a simple and credible way for evaluation of blood biocompatibility of biomaterials.[33] Results obtained from HR % of rabbit blood with CS-α,β-GP formulations were shown in Figure 1(B). It was discovered that HR % was less than 5% at the

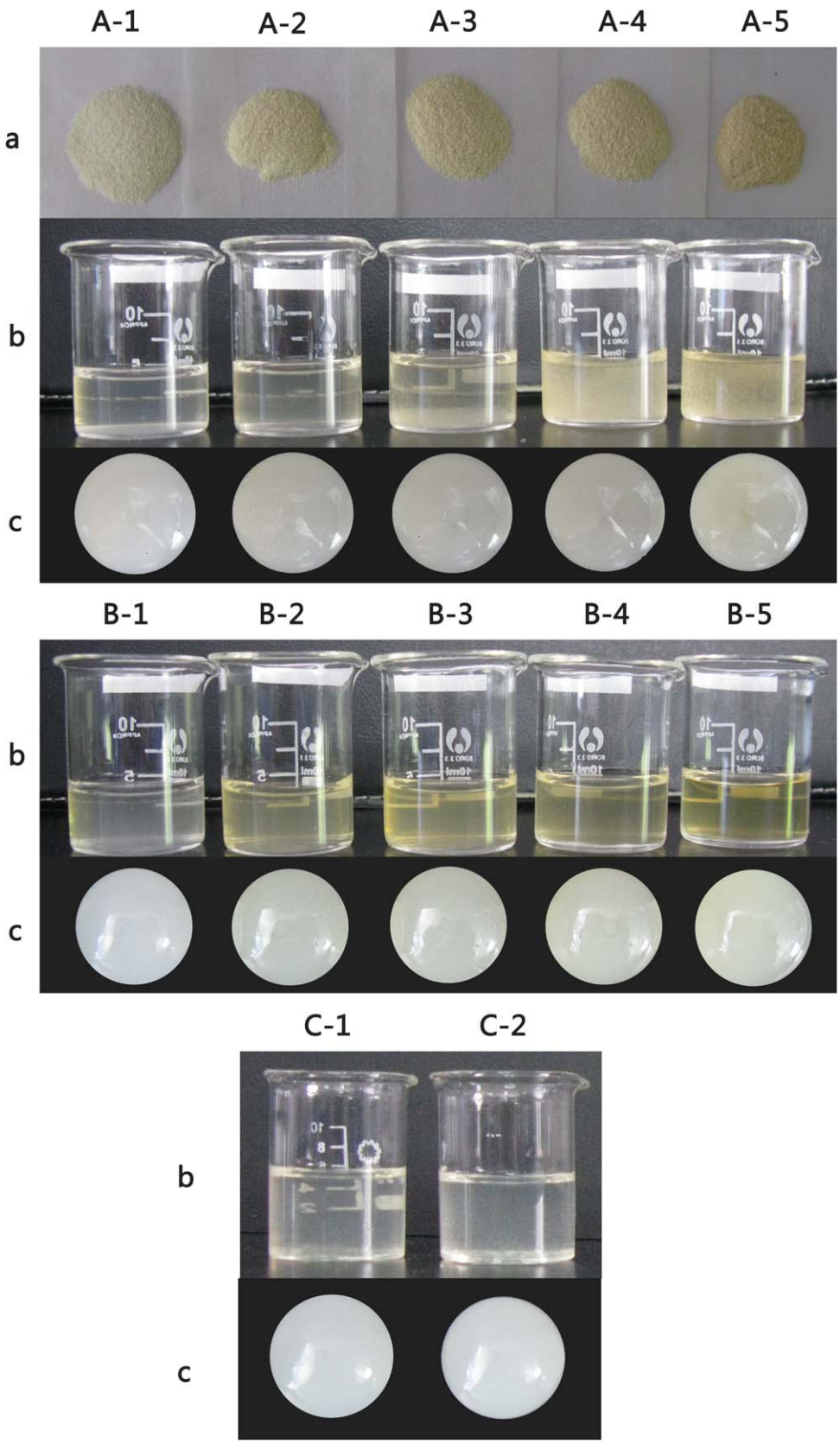

Figure 2. The pictures of CS powder (Row a), CS solutions (Row b) and CS-α,β-GP gel (Row c) before and after sterilization. (Column A-1: before sterilization; Column A-2: autoclaving of CS powders for 10 min at 115°C; Column A-3: autoclaving of CS powders for 20 min at 115°C; Column A-4: autoclaving of CS powders for 30 min at 115°C; Column A-5: autoclaving of CS powders for 20 min at 121°C; Column B-1: before sterilization; Column B-2: autoclaving of CS solution for 10 min at 115°C; Column B-3: autoclaving of CS solution for 20 min at 115°C; Column B-4: autoclaving of CS solution for 30 min at 115°C; Column B-5: autoclaving of CS solution for 20 min at 121°C; Column C-1: before sterilization; Column C-2: ultraviolet ray radiated CS powders for 120 min at 25°C). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

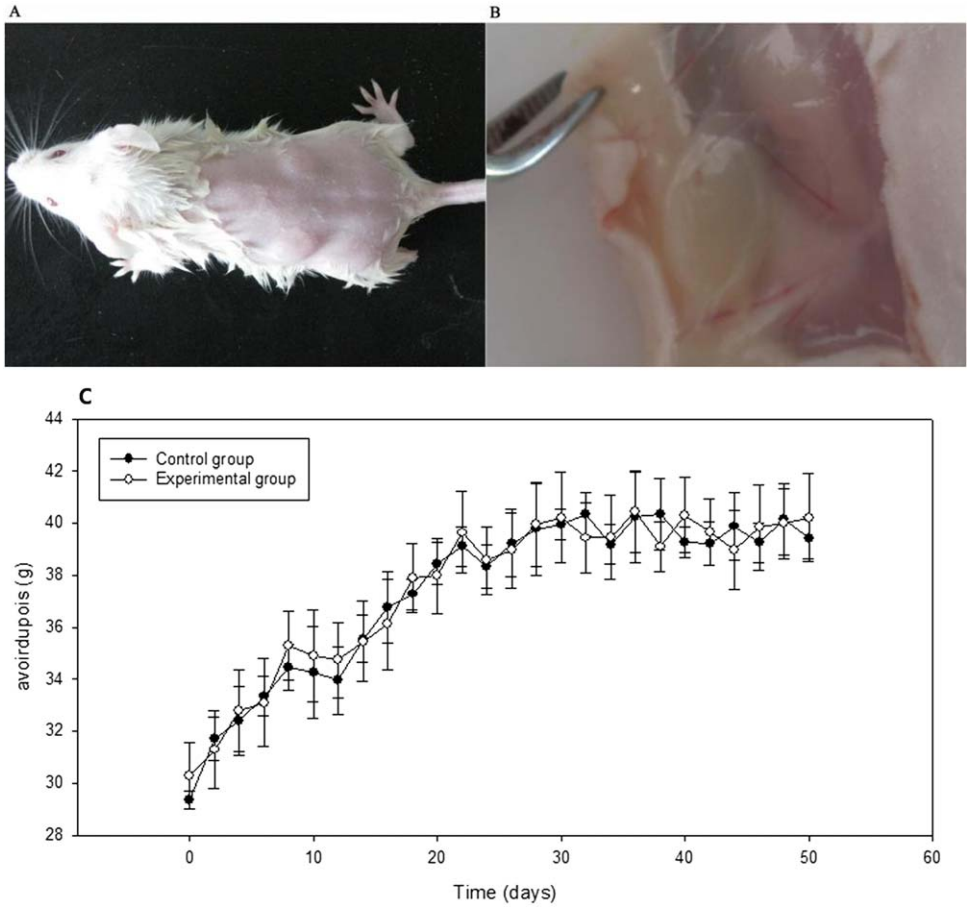

Figure 3. Subcutaneous injection of CS-α,β-GP hydrogels implants in Kunming Mouse. (A: the *in situ* gel humps after injection could be seen obviously; B: the *in situ* gel after anatomy of the mouse; C: the avoirdupois of Kunming Mouse in different implantation time of hydrogels.). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

concentration of 1 mg/mL of CS-α,β-GP formulations, which came well within permissible limit.[34] Thus the CS-α,β-GP sol and CS-α,β-GP gel were confirmed having no hemolysis.

Qualitative Evaluation of Protein Adsorption. When blood was in contact with a foreign material surface, the adsorption of plasma proteins occurs firstly.[33,35] Figure 1(C) showed BSA adsorptions on CS-α,β-GP sol and gel tested using SDS-PAGE. It could be concluded by comparing the BSA on CS-α,β-GP formulations to the BSA [Figure 1(C), Line 3] that the protein adsorption amounts on the CS-α,β-GP sol and gel were relative low. Protein adsorption onto the surface of foreign materials was the first thing that occurred in most *in vivo* implants, and adsorption amount presented the biocompatibility of implant materials. It was generally recognized that less initial protein adsorption commonly proved lower level of subsequent inflammatory and/or thrombotic responses.[36]

***In Vivo* Gelation and Biocompatibility Test.** Sterile thermosensitive CS-α,β-GP aqueous sol could be easily administrated with a micropipette or syringe needle, providing a novel fashion for minimally invasive and site-specific *in situ*-forming implants. Following injection, the CS-α,β-GP sol formed an oval shaped skin protrusion at the injection site as shown in Figure 3(A), indicating that the CS-α,β-GP aqueous sol gelled rapidly due to the increasing temperature of the tissue fluid surrounding the injection site. The gelation times of the hydrogel were 90 s at 25°C and 30 s at 37°C according to our previous study,[23] ensuring that one had enough time to inject the 4°C of CS-α,β-GP sol to dorsal subcutaneous of the mouse. There had no inflammation was found around the CS-α,β-GP gel hump by macroscopic evaluation [Figure 3(B)]. After injection, all the mice of the experimental group survived and had a normal life way. During the observation period, no inflammation was found by macroscopic evaluation, and the avoirdupois of the experimental group did not change obviously compared with the control group [Figure 3(C)].

Abnormal treatment-related values could reflect changes belonging to toxicological effects. These changes could be regarding alterations in a series of *in vivo* analyzable parameters (such as clinical chemistry and hematology data) and tissue morphology (detected by histopathological evaluation).[37] In present work, hematological and histopathological analysis of mice were done to evaluate the safety of CS-α,β-GP hydrogel.

The white blood count (WBC), red blood count (RBC), platelet count (PLT), hemoglobin (HGB), mean corpuscular volume

Table II. Baseline Hematologic Values of Kunming Mice at Different Injection Time of Hydrogels

Blood routine	WBC ($\times 10^9$/L)	RBC ($\times 10^{12}$/L)	HGB (g/L)	MCV (fL)	MCH (pg)	MCHC (g/L)	PLT ($\times 10^9$/L)
Control	4.85 ± 0.19	9.06 ± 0.22	148 ± 6	52.17 ± 1.68	16.4 ± 0.37	315.0 ± 15.9	702 ± 57
1 d	3.11 ± 0.79	8.59 ± 0.21	132 ± 5	52.10 ± 0.7	15.4 ± 0.2	295.0 ± 0.5	553 ± 96
5 d	6.63 ± 2.6	9.32 ± 0.77	145 ± 14	50.00 ± 1.36	15.6 ± 0.22	313.0 ± 11.5	450 ± 80
10 d	4 ± 0.88	9.14 ± 0.25	151 ± 11	46.20 ± 1.11	16.5 ± 0.31	356.0 ± 10.7	390 ± 55
15 d	3.78 ± 1.05	9.65 ± 0.09	153 ± 6	47.70 ± 1.32	15.9 ± 0.75	334.0 ± 7.8	821 ± 65
20 d	3.57 ± 0.46	8.67 ± 0.80	142 ± 8	49.47 ± 2.57	16.5 ± 0.57	334.0 ± 9.62	613 ± 33
25 d	4.22 ± 1.09	9.39 ± 0.17	145 ± 11	52.16 ± 1.41	16.4 ± 0.31	331.0 ± 8.33	711 ± 44
30 d	4.93 ± 0.16	9.17 ± 0.12	142 ± 3	52.30 ± 2.0	16.7 ± 0.46	326.0 ± 19.1	723 ± 23
35 d	3.81 ± 0.58	8.84 ± 0.36	151 ± 6	49.78 ± 1.54	16.5 ± 0.77	319.0 ± 12.2	685 ± 67
40 d	4.97 ± 0.36	8.94 ± 0.47	148 ± 4	51.57 ± 1.37	16.6 ± 0.57	302.0 ± 13.6	741 ± 13
45 d	4.48 ± 0.91	9.03 ± 0.29	144 ± 9	48.74 ± 1.39	16.1 ± 0.39	328.0 ± 14.4	711 ± 59
50 d	4.84 ± 0.20	9.12 ± 0.18	153 ± 3	53.07 ± 2.31	16.5 ± 0.16	299.0 ± 12.2	733 ± 42

WBC: white blood count; RBC: red blood count; HGB: hemoglobin; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpusular hemoglobin concentration; PLT: platelet count.

(MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) of the mice were examined at 1 d, 5 d, 10 d, 15 d, 20 d, 25 d, 30 d, 35 d, 40 d, 45 d, and 50 d post-injection and the results were shown in Table II. The results from the experimental group were compared with that of control group. It could be seen that the levels of WBC, RBC, PLT, HGB, MCV, MCH, and MCHC were all in the normal range, indicating that the injection of CS-α,β-GP hydrogel did not produce obvious effects on the hematopoiesis of the mice.

The inflammatory response at different times after injection could be characterized using hematoxylin-eosin (HE) staining. The injected hydrogels and the surrounding tissues were carefully harvested using scalpel and tweezers after the mice were sacrificed at 1 d, 5 d, 10 d, 15 d, 20 d, 25 d, 30 d, 35 d, 40 d, 45 d, and 50 d post-injection for histopathological analysis. Figure 4 showed the representative histology photomicrographs of the injection site at different time point. After injection, some polymorphonuclear leukocytes, lymphocytes, plasma cells, and monocytes were observed on the tissues around the injection site. Nonetheless, the inflammatory response could not be found by macroscopic evaluation, indicating a minimal inflammatory response was induced after injection of the CS-α,β-GP hydrogel. The inflammatory responses were the universally known foreign body reactions as the normal responses to biocompatible and biodegradable materials.[38] The shape, size, physical properties, and chemical properties of the biomaterials might be responsible for variations in the intensity and time duration of the inflammatory processes. During the first 2 weeks following injection, there were more inflammatory cells presented in the surrounding tissues than that at 20 d, 25 d, 30 d, and 35 d post-injection. The leukocyte in blood migrated into the injection sites because of the sudden intrusion of foreign body and the mechanical injury created by injection, leading the increase of the inflammatory cells. At 45 d post-injection, an increase of the inflammatory response was noted. This phenomenon was inferred to be caused by the breakdown into fragments of the CS-α,β-GP hydrogel. The formation of fragments, usually of much smaller size and larger contact area with tissue fluid than the original hydrogel, triggered a tissue response which was predominantly macrophages and the continuing degradation of the biomaterials occurs quickly under these conditions.

From the 5th day, new capillary formation was found around the hydrogel humps. Figure 5 showed the representative tissue response which leaded to neoangiogenesis at 5 d, 15 d, 35 d, and 45 d post-injection, indicated healing of the injection sites. This was the first report on the CS-α,β-GP hydrogel could elicit new capillary formation. The results suggested that the CS-α,β-GP hydrogel had good tissue compatibility.

Degradation of the CS-α,β-GP Hydrogel

***In Vitro* Enzymatic Degradation.** The degradation properties were important prognosticator of the *in vivo* metabolic processes involving implant materials, needed for the applications of the hydrogels in biomedical and tissue engineering fields. The effects of lysozyme on CS-α,β-GP hydrogel degradation were shown in Figure 6. After 6 h degradation, the hydrogels were degraded into small fragments [Figure 6(A)]. In the range of pH7.0–7.4, the degradation rates increased gradually with the increase of pH value. Figure 6(B) showed the internal structures of the CS-α,β-GP hydrogel before [Figure 6(B1)] and after [Figure 6(B2)] 3 h degradation in 0.25% (w/v) lysozyme solution at 37°C. After 3 h degradation, the hydrogel resulted in lager pore diameters than before degradation and this might in virtue of the mass released from the gel matrix. With time extension, the hydrogel became very fragile and disintegrated as small fragments [Figure 6(A)] because of the degradation of the hydrogel networks. This phenomenon could be illustrated by the *in vitro* degradation kinetics of the hydrogel which had been reported in our previous research.[21]

***In Vivo* Degradation.** The volume of CS-α,β-GP hydrogels progressively decreased post-injection, indicating the hydrogels were continuously degraded. The degradability of the CS-α,β-GP

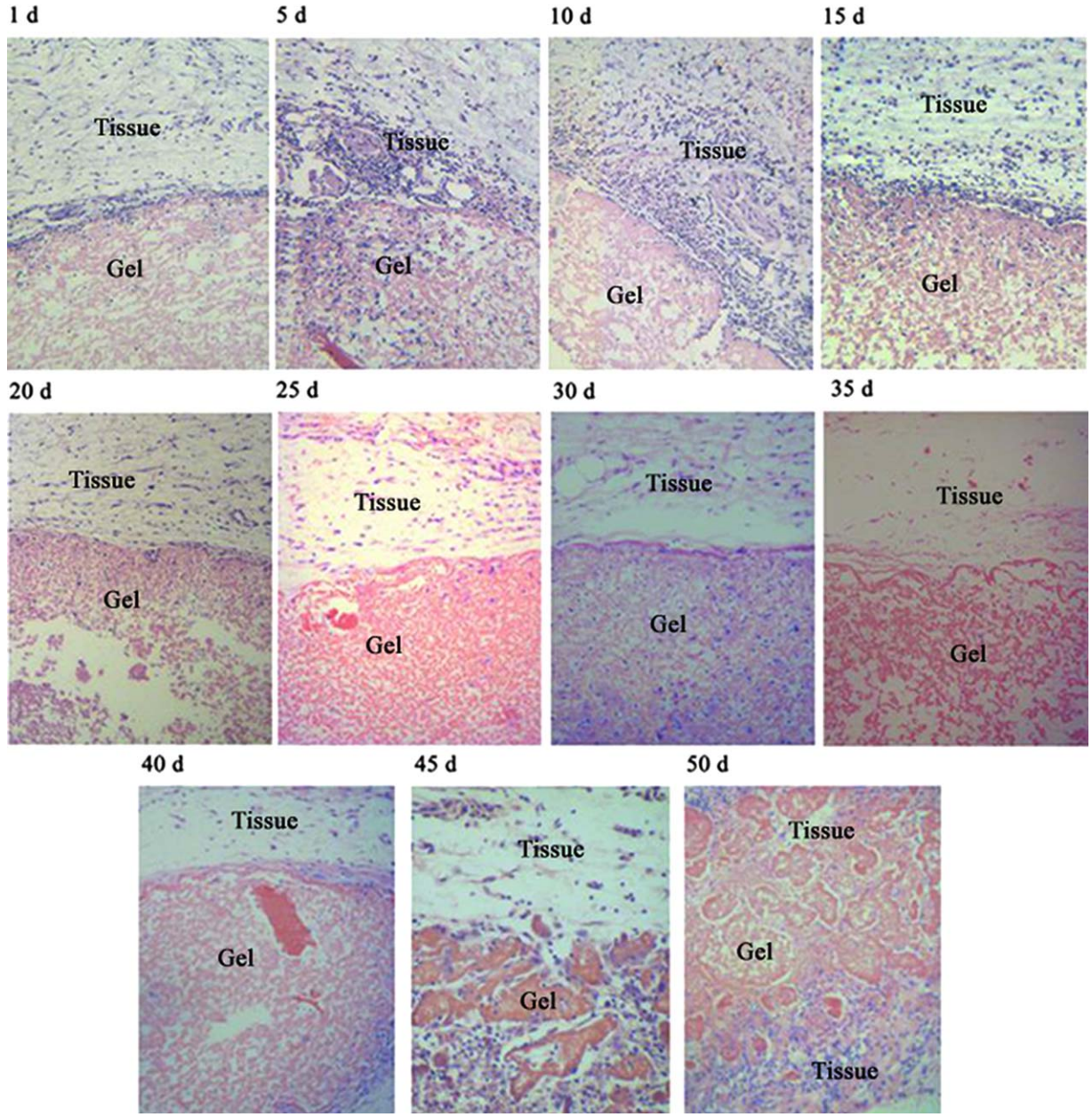

Figure 4. Photomicrographs of the inflammatory reaction after subcutaneous injection of the CS-α,β-GP *in situ* hydrogel in Kunming Mice. (MAG = ×100). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

hydrogel *in vivo* also could be observed clearly by the HE staining (Figure 4). The edges of the hydrogel humps could be observed degraded clearly from the 20 d post injection. With time extension, the hydrogels degraded continuously and became many small fragments surrounding few macrophages at 45 d post injection. There only a very small amount of CS-α,β-GP gel pieces were left at 50 d post injection and the gel could be degraded completely after 60 d injection. The SEM images of the CS-α,β-GP hydrogel after 5 d and 20 d injection were shown in Figure 7. It was shown that the pores of the hydrogel external layer became flat and thin after injection due to the skin pressure and limited space under the skin, while the pores shape deep inside of the hydrogel changed little. After injection, the morphology of the hydrogel changed progressively with time and finally degraded. At 5 d post injection, the CS-α,β-GP hydrogel still had a intact configuration. The internal appearance showed degraded at 20 d post injection, and the leucocyte and fibroblast were observed infiltrated into the gel inside. Infiltration of leucocyte and fibroblast accelerated the degradation process of the injection humps. The results suggested that the CS-α,β-GP hydrogel had good tissue compatibility and could be degraded substantially *in vivo*. These properties offer great potential for the utilization of CS-α,β-GP hydrogels in tissue engineering and other related biomedical fields.

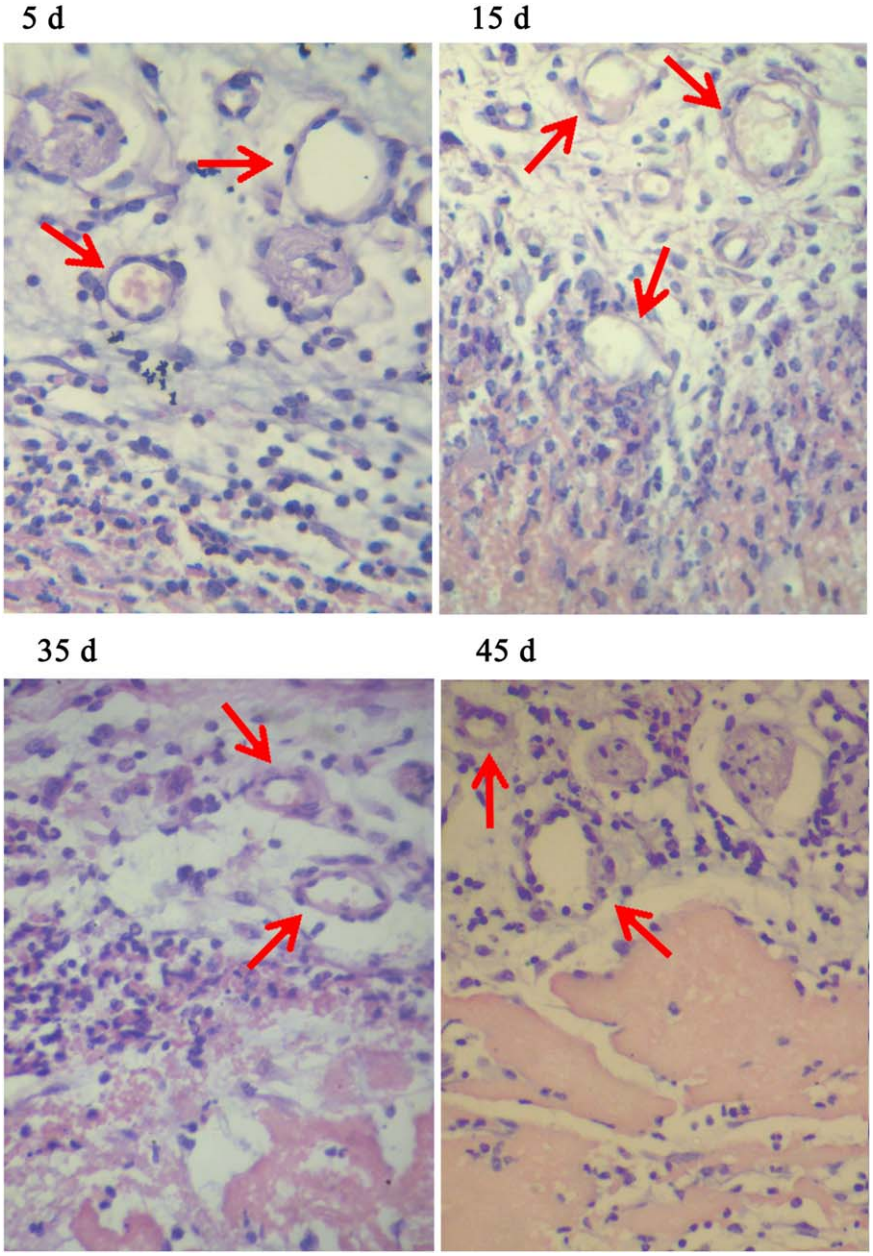

Figure 5. Photomicrographs of the representative tissue response which leads to neoangiogenesis after subcutaneous injection of the CS-α,β-GP *in situ* hydrogel in Kunming Mice. (MAG = ×400). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

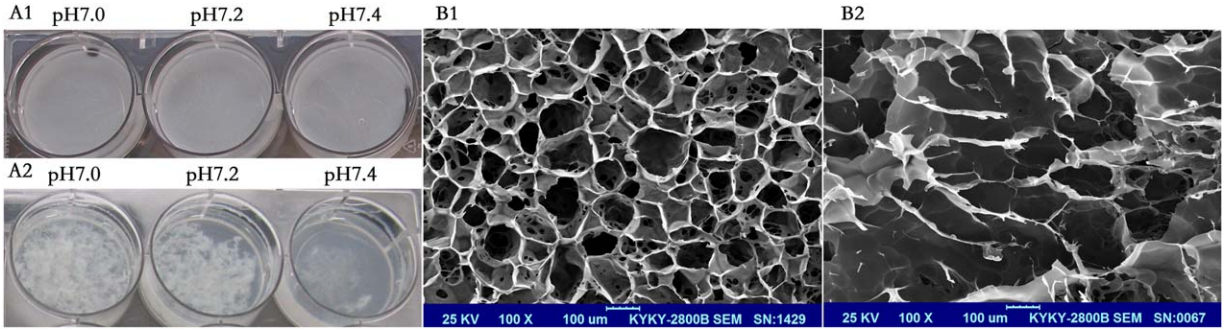

Figure 6. Degradation characteristics of CS-α,β-GP *in situ* hydrogel in lysozyme. (A1: Photograph of hydrogel before degradation; A2: Photograph of hydrogel after degradation for 6 h at 37°C; B1: SEM images of hydrogel before degradation; B2: SEM images of hydrogel after degradation for 3 h at 37°C). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

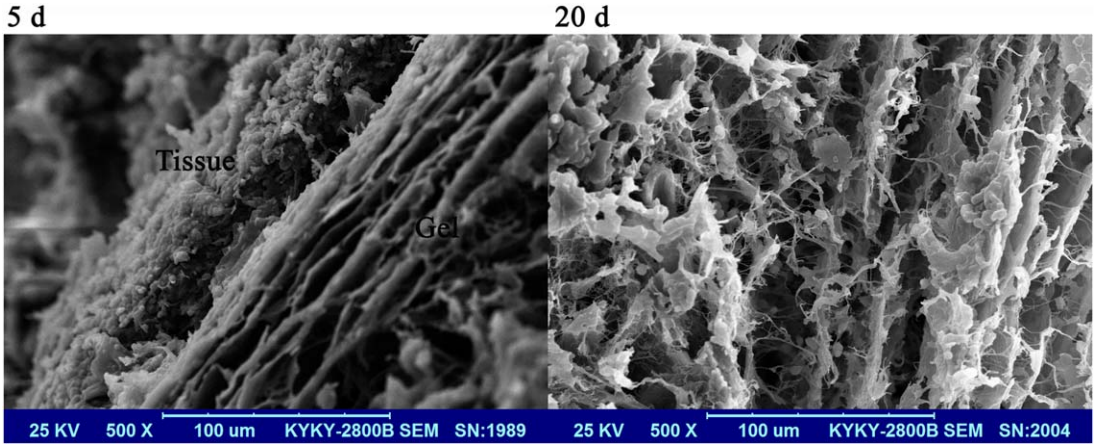

Figure 7. SEM images of *in situ* gel at 5 d and 20 d post injection. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

CONCLUSIONS

Autoclaving for CS powders or CS solutions would alter the characterizations of CS, leading the property changes of CS-α,β-GP formulations. Ultraviolet sterilization for 120 min of CS powders could kill microorganisms effectively, and did not change the characterizations of CS and CS-α,β-GP formulations obviously. Sterile CS-α,β-GP formulations could be obtained by employing ultraviolet ray irradiated CS powders for 120 min, and employing 0.22 μm filtration to filter α,β-GP solution and lactic acid solution. *In vitro* biocompatibility study showed the CS-α,β-GP hydrogel had good hemocompatibility and low protein adsorption. Thermosensitive CS-α,β-GP aqueous sol could be easily administered through injections and formed an oval shaped skin protrusion at the injection site. After injection, all the mice of the experimental group survived and had a normal life way. The results of hematological and histopathological evaluation showed that the CS-α,β-GP hydrogel had negligible effects on hematopoiesis and no toxicity to the body. The CS-α,β-GP hydrogel could be degraded substantially *in vivo* after 60 d injection. These results demonstrated that such a thermosensitive hydrogel would have potential applications in tissue engineering and other related biomedical fields.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (NSFC, 31400812), the Fundamental Research Funds for the Central Universities (201213006), and the Shandong Province Natural Science Foundation (ZR2011CZ003).

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