Carbohydrate Polymers 88 (2012) 361–368

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Design and evaluation of a highly porous thermosensitive hydrogel with low gelation temperature as a 3D culture system for *Penaeus chinensis* lymphoid cells

Qi Feng Dang[a], Jing Quan Yan[a], Hong Lin[a], Xi Guang Chen[a,*], Cheng Sheng Liu[a,*], Qiu Xia Ji[b], Jing Jing Li[a]

[a] *Ocean University of China, Qingdao 266003, PR China*
[b] *The Affiliated Hospital of Medical College, Qingdao University, Qingdao 266001, PR China*

ARTICLE INFO

Article history:
Received 18 October 2011
Received in revised form 9 November 2011
Accepted 13 December 2011
Available online 22 December 2011

Keywords:
Chitosan
Low gelation temperature
Hydrogel
Biocompatibility
Cell encapsulation

ABSTRACT

The characterizations and biocompatibility of a highly porous chtosan-α,β-glycerophosphate (CS-α,β-GP) thermosensitive hydrogel with low gelation temperature and its feasibility as a 3D culture system for *Penaeus chinensis* lymphoid cells were investigated. A CS-α,β-GP hydrogel that possesses a physiological pH and can form monolithic gels at 25 °C was designed. The properties of the CS-α,β-GP hydrogel were characterized regarding rheological properties, gelation temperature, gelation time, pH, morphology, swelling properties and degradability. This hydrogel showed low BSA adsorption at physiological conditions. The cytotoxicity of the α,β-GP to mouse embryo fibroblasts (MEFs) could be eliminated by washing the CS-α,β-GP hydrogel 3 times with cell culture medium. The *P. chinensis* lymphoid cells cultured on the surface or encapsulated inside of the hydrogel could grow and reproduce. After 20 d culture on the surface or encapsulated inside of the hydrogel, the lymphoid cells were still had good activity and could be subcultured.

1. Introduction

Hydrogels scaffolds based on both natural and synthetic polymers are attractive for potential biomedical applications (Tamura, Furuike, Nair, & Jayakumar, 2011; Wang et al., 2009; Zhang, Li, Xiao, Zheng, Fan, & Zhang, 2011). There are many advantages to having hydrogels as cellular scaffolds. The hydrogels have similar mechanical properties to soft tissue, have low interfacial tension which allows cells to move across the tissue-implant boundary, allow for an aqueous environment for cells and are also porous which make for nutrient and oxygen transport. Both natural and synthetic occurring thermosensitive hydrogels have been deployed in the cell culture scaffolds, and many provide support to cells cultured within the materials (Kraehenbuehl, Ferreira, Zammaretti, Hubbell, & Langer, 2009; Wang & Stegemann, 2010; Zhang et al., 2011).

Among natural biopolymer of interest, chitosan (CS), a hydrophilic cationic copolymer produced by the deacetylation of chitin and composed of glucosamine and acetylglucosamine units, stands out by a unique combination of favourable biological properties such as biocompatibility, biodegradability, and nontoxicity (Muzzarelli, 1997; Rao & Sharma, 1997). Recently, a physical thermosensitive hydrogel system were prepared by neutralizing highly deacetylated semidiluted chitosan solutions with weak base, β-glycerophosphate (β-GP) or α,β-glycerophosphate (α,β-GP) (Chenite et al., 2000; Cho, Heuzey, Bégin, & Carreau, 2005; Dang, Yan, Li, Cheng, Liu, & Chen, 2011; Kim, Nishimoto, Bumgardner, Haggard, Gaber, & Yang, 2010). This system remained in solution at physiological pH and low temperature, while changed into a gel upon heating at physiological temperature, undergoing therefore a heat-induced gelation. Wang and Stegemann (2010) showed that CS-β-GP was potential materials used as cell culture scaffold because of its ability to mimic the extracellular environment and direct cell function.

It is a new idea to use CS-GP hydrogel as a 3D culture system for *Penaeus chinensis* lymphoid cells. Over the past decade, viral diseases have been the major limiting factors in the development of shrimp culture. Although many aspects of crustacean virus diseases have been investigated in the host animals, suitable *in vitro* tissue culture systems are necessary for the development of diagnostic reagents and probes for use in the shrimp aquaculture industry. CS is a polysaccharide derived from the deacetylation of chitin, the main structural component of shrimp or crab shells. The hydrogel prepared with CS and α,β-GP is a type of highly porous hydrogel with controllable gelation temperature (T_{gel}), pore diameter and degradation behavior (Dang et al., 2011). Use the CS-α,β-GP

* Corresponding authors at: College of Marine Life Science, Ocean University of China, 5# Yushan Road, Qingdao 266003, PR China. Tel.: +86 0532 82032586; fax: +86 0532 82032586.
E-mail addresses: xgchen@ouc.edu.cn (X.G. Chen), Liucs@ouc.edu.cn (C.S. Liu).

0144-8617/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2011.12.014

hydrogel as the shrimp 3D cell culture system would be a meaningful research if it is feasible for *P. chinensis* lymphoid cell's primary culture and subculture.

The shrimp cells should be cultured at 25 °C (Lang & Nomura, 2002). According to the previous research, the CS-α,β-GP hydrogel with a higher α,β-GP content had lower T_{gel} (Dang et al., 2011), because the addition of α,β-GP into chitosan solution increased the pH, thereby reducing the electrostatic repulsion between chitosan chains, this reaction, in turn, induces an increase in chitosan interchain hydrogen bonding. As a result, in order to form the gel network at 25 °C, it is necessary to include high concentration of α,β-GP, leading to potential toxicity. However, the concentration of α,β-GP must be limited in order to improve the biocompatibility of this system.

The present study produced a physiologically acceptable pH ranged and room temperature gelling liquid CS-α,β-GP vehicle by changing the solvent strength of CS and the ratio of CS/α,β-GP. The properties of this hydrogel were characterized regarding rheological properties, T_{gel}, pH, surface morphology, and chemical structure. To investigate the biocompatibility of the hydrogel, the tests such as BSA adsorption and cell toxicity experiment were studied. The feasibility of the CS-α,β-GP hydrogel as a 3D culture system for *P. chinensis* lymphoid cells were also investigated, and the viability of lymphoid cells cultured was assessed by calcein-AM incubation.

2. Materials and methods

2.1. Materials

Chitosan (CS), derived from shrimp shell, deacetylation degree 96.5%, dynamic viscosity 140 cps, was freely supplied by Laizhou Haili Biological Product Co., Ltd. (Shandong, China). Bovine Serum Albumin (BSA), Fetal Bovine Serum (FBS), Dulbecco's Modified Eagle' Medium (DMEM), D-Hank's were all obtained from Hyclone (Utah, USA). Trypsin was purchased from Amresco (USA). α,β-glycerophosphate (α,β-GP), lactic acid and polydimethyl siloxane fluid were all analytical grade and obtained from Sigma–Aldrich.

2.2. Optimization of the CS-α,β-GP hydrogel solution

Taking the T_{gel} and pH value of the CS-α,β-GP system as the main evaluation index, the prescription of the CS-α,β-GP hydrogel solution was optimized by changing the CS/α,β-GP ratios and the solvent strength of CS. Clear solutions of CS were prepared by dissolving proper amount of CS in aqueous solutions of lactic acid using a heat collecting magnetic stirrer (DF-1, Beijing Flaming Technology & Trade Co., Ltd. China) at 100 rpm for 8 h at 25 °C, and the finally concentration of the CS was 0.18% (w/v). Then the CS solutions and the 50% (w/v) aqueous α,β-GP solution were chilled to 4 °C for 30 min. The α,β-GP solution was added dropwise to the CS solutions under stirring and the final CS-α,β-GP solutions were stirred for another 30 min. Finally, the resultant CS-α,β-GP hydrogel solutions were stored at 4 °C. The strength of the lactic acid aqueous solutions and the ratio of CS solution and α,β-GP solution (CS/α,β-GP) for each gel were reported in Table 1.

2.3. Characterizations of the CS-α,β-GP hydrogel

2.3.1. Rheological analysis

Rheological measurements were performed by a Physica MCR101 Rheometer (AnTon paar Ltd., Austria) and a circulating environmental system for the control of temperature. CS-α,β-GP solutions (3 ml) were introduced between the concentric cylinders and then covered with polydimethyl siloxane fluid on the surface in order to prevent evaporation during the tests. The effect of the polydimethyl siloxane fluid on the measurements was shown to be negligible. In order to investigate the linear viscoelastic zone, the storage modulus (G'), loss modulus (G'') and phase angle (δ) were measured with the dynamic strain changing from 0.001% to 100%. During the gelation process in non-isothermal conditions, the evolutions of rheological properties (G', G'', and δ) were investigated between 10 °C and 80 °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 T_{gel} of the hydrogel was determined as the crossover of the G' and G'' ($\delta = 45°$) when the G' becomes greater than G'' at a certain temperature.

2.3.2. Gelation time determination

A simple test tube inverting method was used to determine the gelation time (Chung, Go, Bae, Jung, Lee, & Park, 2005; Ganji, Abdekhodaie, & Ramazani, 2007). Each CS-α,β-GP hydrogel solution of 1 ml was added into glass tube (5 ml, with inner diameter of 10 mm) and maintained for 12 h at 4 °C to remove air bubbles. Gelation time was determined as a function of time at constant temperatures of T_{gel} or 37 °C in a water bath. The gelation time was investigated by inverting the glass tubes every 30 s. The sol phase was defined as flowing liquid and the gel phase as non-flowing gel when the CS-α,β-GP system in the test tube was inverted. The time at which the gel did not flow was recorded as the gelation time.

2.3.3. pH value measurement

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

2.3.4. Turbidity of the hydrogel

The turbidity of CS-α,β-GP systems in terms of time at T_{gel} was determined using a UV-1100 spectrophotometer (UV-1100, Shanghai MAPADA Instruments Co., Ltd. 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 pads (Shanghai Kobayashi Daily Chemical Co., Ltd. China) during the sol-to-gel behavior of the hydrogel solutions.

2.3.5. Scanning electron microscopy

CS-α,β-GP hydrogel samples were gelled at T_{gel} and freeze-dried under vacuum for 48 h. Then the surface and interior morphology of the dry CS-α,β-GP porous hydrogels were examined using scanning electron microscopy (SEM) (KYKY-2800B, Scientific Instrument Co., Ltd. Chinese Academy of Sciences, China).

2.4. Swelling and degradation kinetics of the CS-α,β-GP hydrogel

2.4.1. Swelling kinetics

The classical gravimetric method was used to measure the swelling kinetics of the hydrogels (Dang et al., 2011). Hydrogel samples were gelled at T_{gel} and freeze-dried under vacuum for 48 h. The dried gels were immersed in tri-distilled water or PBS 7.2 at 25 °C or 37 °C, and the samples were taken out from water or PBS 7.2 and weighed at predetermined time intervals. The weights of the samples were recorded as the average value of three measurements. The swelling ratio (SR) at time t was defined as the following expression:

$$SR = \frac{Wt - Wd}{Wd} \quad (1)$$

Table 1
The pH value and T_{gel} of CS-α,β-GP hydrogels with different ratio of CS/α,β-GP and different strength of the lactic acid aqueous solution.

CS/α,β-GP (v/v)	Solvent strength (mol/L)			
		0.08	0.09	0.10
8.4:1.6	T_{gel} (°C)	21	26	30
	pH of CS-α,β-GP sol (4 °C)	7.58 ± 0.04	7.43 ± 0.02	7.26 ± 0.02
	pH of CS-α,β-GP gel (37 °C)	7.21 ± 0.03	6.93 ± 0.03	6.77 ± 0.01
8.7:1.3	T_{gel} (°C)	23	28	32
	pH of CS-α,β-GP sol (4 °C)	7.49 ± 0.03	7.37 ± 0.01	7.22 ± 0.04
	pH of CS-α,β-GP gel (37 °C)	7.03 ± 0.02	6.86 ± 0.03	6.71 ± 0.02
9.0:1.0	T_{gel} (°C)	25	31	35
	pH of CS-α,β-GP sol (4 °C)	7.36 ± 0.03	7.25 ± 0.02	7.11 ± 0.02
	pH of CS-α,β-GP gel (37 °C)	6.87 ± 0.04	6.78 ± 0.01	6.69 ± 0.03

Here *W*t was the weights of the hydrogels at time *t* and *W*d was the weights of the dried hydrogels.

2.4.2. Degradation kinetics

CS-α,β-GP hydrogel was immersed in 0.25% (w/v) enzyme solution and incubated at 37 °C. During degradation, changes of weight loss and SR of CS-α,β-GP hydrogel were measured and normalized to its initial values before degradation. The samples were removed from the solution, washed thoroughly with distilled water and then freeze-dried at regular time intervals. The SR at time t was calculated according to Eq. (1) and the degradation was assessed by measuring the weight loss (%), which was defined as the following expression:

$$\text{Weight loss}(\%) = \frac{W_1 - W_2}{W_1} \times 100 \quad (2)$$

Here W_1 and W_2 were the weights of the dry hydrogel before and after degradation respectively.

2.5. Biocompatibility of the CS-α,β-GP hydrogel

2.5.1. Protein adsorption assay

For the quantitative evaluation of protein adsorption, the absorbance-amount curve with 5 standard concentrations (1, 2, 3, 4, 5 μg/ml) as the standard curves was defined using a UV-1100 spectrophotometer (Huang, Lü, Ma, & Huang, 2008). The adsorption of Bovine Serum Albumin (BSA) proteins to CS-α,β-GP sol and gel was studied in 0.9% NaCl solution. Samples of CS-α,β-GP sol and gel were freeze-dried (for 48 h) and triturated before used. The suspensions (10 mg/ml) of samples were incubated in fresh BSA (100 μg/ml) at 37 °C for 1 h, 3 h, 6 h, 9 h, 12 h, 15 h, and 24 h respectively, then separated by ultracentrifugation (Beckman Instruments Inc., Palo Alto, CA) at 15,000 × *g* for 15 min at 4 °C. The centrifugal precipitate extensively washed with distilled water (1 ml) three times to remove proteins not firmly adsorbed onto samples. The adsorbed BSA were desorbed from the surface of the CS-α,β-GP sol or gel by PBS containing 1% SDS (Fang, Shi, Pei, Hong, Wu, & Chen, 2006), then the suspension was centrifuged at 15,000 × *g* for 15 min and the 1 ml of supernatant was pipetted out from each tube and mixed with 3 ml Coomassie Brilliant Blue (CBB) G-250 solution and then the absorbencies at 595 nm were measured. The amount of proteins adsorbed on the material surfaces was calculated according to the standard curve.

2.5.2. Cytotoxicity test

2.5.2.1. Preparation of the extracting liquid of the CS-α,β-GP hydrogel. The CS-α,β-GP hydrogel solution was prepared and was added in 6-well flat-bottomed microplates (1.7 ml/well). After gel formation, the gels were divided into 4 groups and washed for 0 (G1), 1 (G2), 2 (G3), 3 (G4) times respectively with Dulbecco's modified eagle medium (DMEM, 8 ml) every 10 min. After that, 8 ml of complete medium (DMEM + 10% FBS) was added in each group and incubated at 25 °C or 37 °C for 12 h. The extracting liquid obtained was filtrated with 0.22 μm filtration. 50% (w/v) of α,β-GP solution was diluted 10 times with distilled water and then took 1.7 ml mixed with 8 ml of DMEM + 10% FBS as the α,β-GP solution used for MTT assay.

2.5.2.2. MTT assay. Cytotoxicity was estimated using the MTT assay *in vitro* in accordance with the method described in our previous study (Zhang, Chen, Liu, & Park, 2009). Mouse embryo fibroblasts (MEFs) which used in the general cytotoxicity test were obtained through primary culture and only cells of 4–7 generations were used in this experiment. Briefly, MEFs at logarithmic growth phase were plated in 96-well flat-bottomed microplates at a density of 5×10^4 cells/well, in DMEM containing 10% FBS. The cells were divided into 6 groups and the medium was replaced after 24 h with different extracting liquid of the CS-α,β-GP hydrogel (200 μl/well). The negative control was blank culture medium. After incubated at 37 °C, 5% CO_2 and 95% relative humidity for 24 h, 200 μl of MTT solution was added to each well and incubated for 4 h. And then, 100 μl of dimethyl sulfoxide (DMSO) was added to each well. The plates were then shaken until the crystals dissolved. Absorbance was determined at 490 nm with a microplate reader (Bio-Rad Model 550, Hercules, CA, USA). The percent of viability was expressed as relative growth rate (RGR %) according to the following equation:

$$\text{RGR}\ (\%) = \frac{D_{\text{sample}}}{D_{\text{control}}} \times 100\% \quad (3)$$

Here D_{sample} and D_{control} were the absorbencies of the tested samples and the negative control at 490 nm respectively.

All reported values were the means of triplicate samples.

2.6. Culture of Penaeus chinensis lymphoid cells

According to the method of Lang and Nomura (2002), the media containing M199 medium plus 20% of fetal bovine serum (FBS), 11.0 g/L of NaCl, 0.4 g/L of KCl, 3.0 g/L of $MgS0_4 \cdot 7H_2O$, 3.3 g/L of $MgCl_2 \cdot 6H_2O$, 0.9 g/L of $CaCl_2 \cdot 2H_2O$, 0.1 g/L of $Na_2HPO_4 \cdot 12H_2O$, 2.38 g/L of HEPES, 0.15 g/L of L-glutamic acid, 0.11 g/L of lactalbumin hydrolysate, 2.2 g/L of $NaHCO_3$, 100 IU/ml of penicillin, and 100 μg/ml of streptomycin was used.

2.6.1. Preparation of the sterile CS-α,β-GP hydrogel

Sterile formulations were obtained by using ultraviolet ray radiated CS powders for 2 h, and using 0.22 μm filtration for α,β-GP and lactic acid solutions. The sterile CS-α,β-GP hydrogel solutions was prepared and 5 ml of CS-α,β-GP sol was plated on the bottom of 25 ml of culture flask and incubated at 25 °C for 5 min. After gel formation, the gels were washed for 3 times with cell culture medium (8 ml) every 10 min. The rest of the CS-α,β-GP sol was stored at 4 °C.

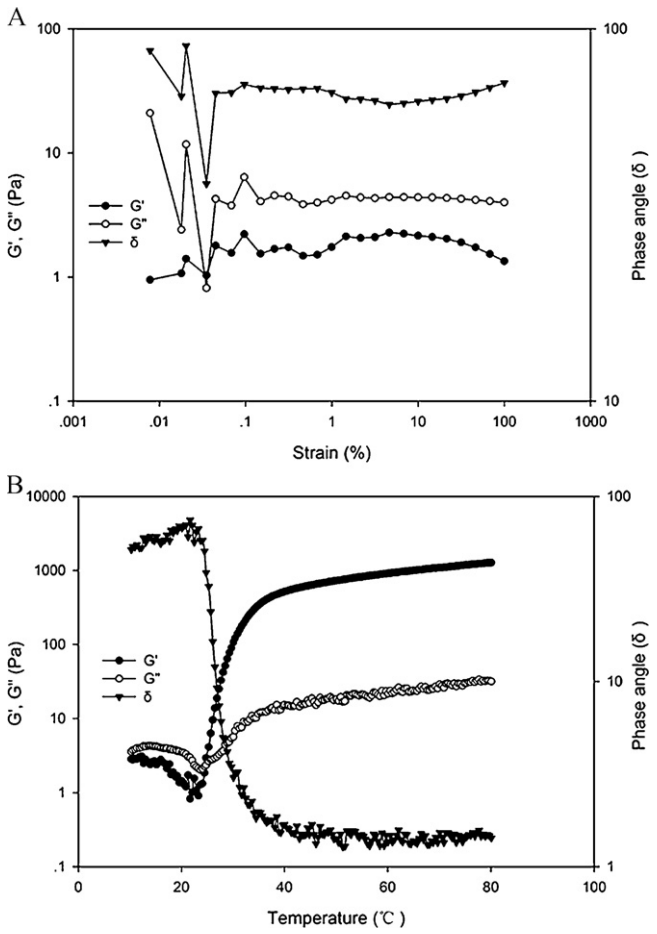

Fig. 1. Rheological evaluation: (A) dynamic strain sweep of the CS-α,β-GP hydrogel; (B) G', G'' and δ of the CS-α,β-GP hydrogel as a function of temperature.

2.6.2. Surface culture

For the primary cultures, lymphoid tissues fragments were inoculated into 25 ml of culture flask or on the CS-α,β-GP gel surface and fed with the media mentioned above. For the subcultures, lymphoid cells formed monolayer with in 7 days on the bottom of the culture flask, subculture was conducted with the method of Wang, Liang, Wang, Chen, Zhang, & Sun (2001). The subcultures of lymphoid cells cultured on the surface of the gel were conducted after 20 days of primary culture. The mechanical properties of the

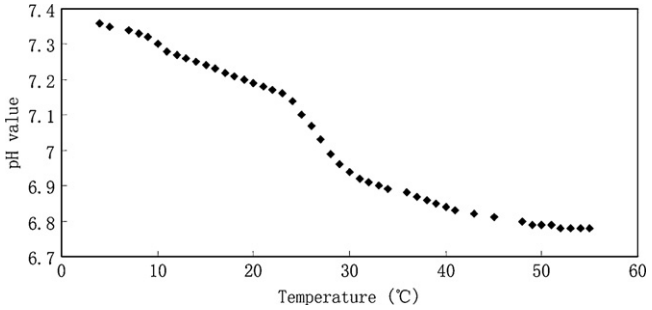

Fig. 2. The pH value of CS-α,β-GP system as a function of temperature.

CS-α,β-GP gel were infirm after 20 days of primary culture and could be fragmented by slight shake. The fragments of the gels were inoculated into 25 ml of culture flask or on the CS-α,β-GP gel surface and cultured with 5% CO_2 at 25 °C in CO_2 incubator.

2.6.3. 3D culture

For the primary cultures, lymphoid tissues fragments were suspended in CS-α,β-GP sol and the mixture was poured into the bottom of the culture flask, allowed to gel at 25 °C. The gels were washed for 3 times with cell culture medium (8 ml) every 10 min and fed with the cell culture medium, then were incubated with 5% of CO_2 at 25 °C in CO_2 incubator. For the subcultures, fragments of the gels were inoculated on the bottom of the culture flask, or on the surface of the gel, or encapsulated in the gel after 20 days primary culture, then incubated with 5% CO_2 at 25 °C in CO_2 incubator.

2.7. Viability assessment

The cell viability assessment was conducted after 7 days culture. Constructs were washed in sterile D-Hank's three times for 10 min and then incubated for 1 h at room temperature with 4 μM calcein-AM (SITC Logistics Co., Ltd, Japan) in D-Hank's. After incubation, constructs were washed again for fluorescence microscopy.

2.8. Statistical analysis

All of the quantitative data were expressed as mean ± standard deviation. Statistically significant differences ($p < 0.05$) between the groups were measured with one-way ANOVA using SPSS13.0 software (SPSS, USA).

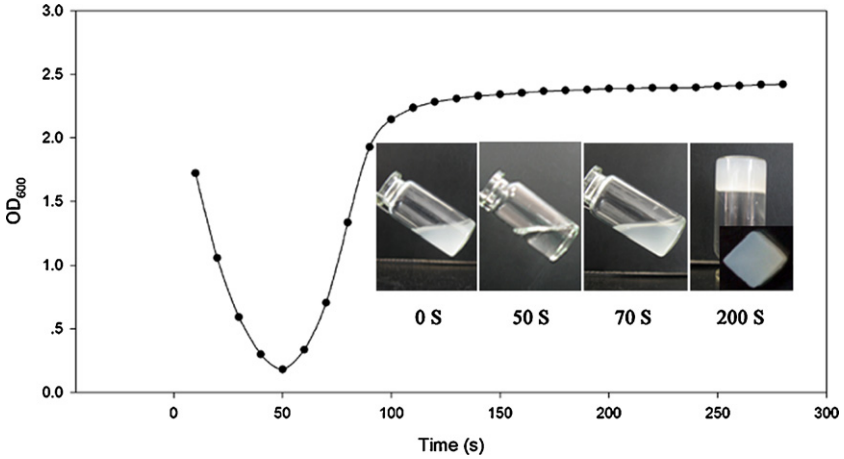

Fig. 3. Dynamics curves of absorbency at 600 nm of CS-α,β-GP hydrogel during sol-to-gel behavior at T_{gel}. Insets are pictures of hydrogel at 0 s, 50 s, 70 s and 200 s during the gelation progress.

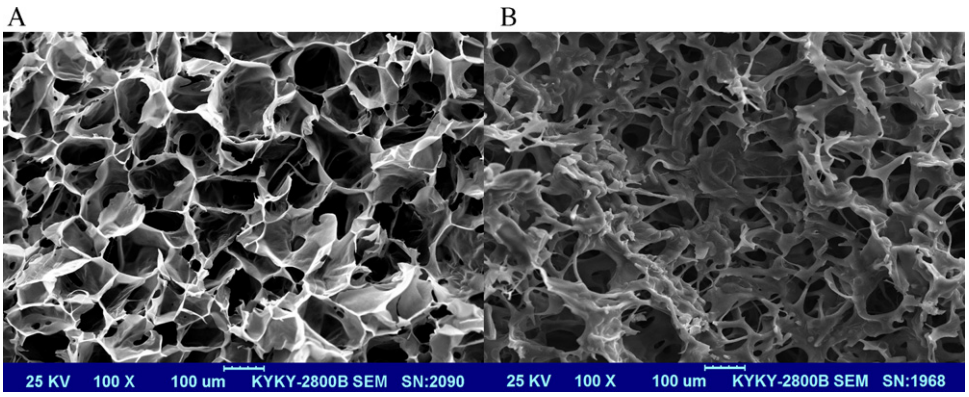

Fig. 4. SEM images of CS-α,β-GP hydrogel: (A) transverse surface and (B) surface.

3. Results and discussion

3.1. Optimization of the CS-α,β-GP hydrogel

Taking the T_{gel} and pH as the main evaluation indexes, the prescription of the CS-α,β-GP hydrogel was optimized by changing the CS/α,β-GP ratio and CS solvent strength, and the results were shown in Table 1. When the CS solvent strength was 0.08 mol/L and the CS/α,β-GP ratio was 9.0/1.0, gel formation was accomplished at physiologically acceptable pH value and 25 °C.

The hydrogels prepared with CS and α,β-GP were a type of highly porous hydrogels with controllable T_{gel} and pH value. Recently, CS-GP hydrogels have been studied to encapsulate cells and proved that CS-GP was potential materials used as cell culture scaffold because of its ability to mimic the extracellular environment and direct cell function (Roughley, Hoemann, DesRosiers, Mwale, Antoniou, & Alini, 2006; Wang & Stegemann, 2010). CS is a polysaccharide derived from the deacetylation of chitin, the main structural component of shrimp or crab shells. Using the CS-based hydrogel as the shrimp 3D cell culture system would be a mean-

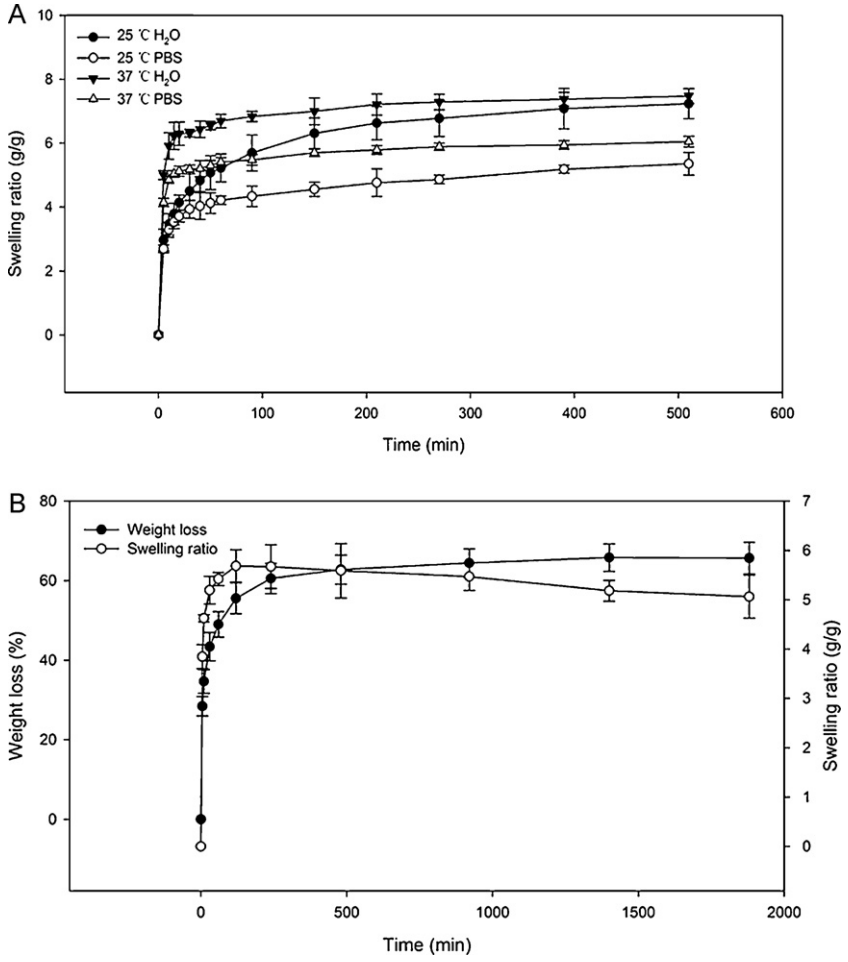

Fig. 5. Swelling kinetics (A) and degradation kinetics (B) of CS-α,β-GP hydrogel.

ingful research if it feasible for shrimp cell's primary culture and subculture. This study developed a thermosensitive highly porous CS-α,β-GP hydrogel which possess a physiological pH and can form monolithic gels at 25 °C, meeting the initial conditions as 3D culture system of marine animal cells (Lang & Nomura, 2002).

3.2. Characterizations of the CS-α,β-GP hydrogel

3.2.1. Rheological analysis

In order to determine the linear viscoelastic zone, the G', G'' and δ were measured with the dynamic strain changing from 0.001% to 100% (Fig. 1A). With increasing dynamic strain from 0.1% to 100%, the G', G'' and δ of the CS-α,β-GP hydrogel had no obvious change, showing a linear viscoelastic zone. The small strain of 2% was chosen to apply to the rheological analysis during the gelation process in order not to disturb the gel formation.

The temperature dependences of G', G'' and δ used to determine T_{gel} of the CS-α,β-GP hydrogel were shown in Fig. 1B. The G' reflected the solid-like component of the rheological behavior, which was thus low at sol stage but increased drastically at the T_{gel}. The T_{gel} was determined as the temperature at crossover of the G' and G'' ($\delta = 45°$) when the G' raised rapidly, and this method had been shown to give an appropriate value of the T_{gel} (Cho et al., 2005). With increasing temperature from 10 °C to 80 °C, the CS-α,β-GP hydrogel studied had a rapid increase of the G', and the δ reached 45°where $G' = G''$ at which the temperature was 25 °C. The greater growth rate of the G' compared with the G'' indicated that development of the gel structure contributed to the increase in elasticity.

3.2.2. Gelation time

Since the CS-α,β-GP hydrogel was essentially designed to be used as a 3D cell culture system, the gelation time was a key factor to the success for this applications. The gelation times of the CS-α,β-GP hydrogel at T_{gel} (25 °C) and 37 °C were investigated with a test tube inverting method. The gel was formed in 90 s at 25 °C and formed in 30 s at 37 °C and these results demonstrated the CS-α,β-GP hydrogel prepared in this paper could form a gel rapidly when the temperature reached the gelation point.

3.2.3. pH value

The changes in pH values of the CS-α,β-GP hydrogel during sol-to-gel behavior were given in Fig. 2. The pH values decreased from 7.36 to 6.87 with increasing temperature from 4 °C to 37 °C during the gelation process. Upon temperature increase, three regions were defined according to the decrease of pH value: following a slowly decreased region and a fast decreased region; the pH value entered a slowly decreased region again. The results demonstrated the CS-α,β-GP hydrogel prepared in this paper had a physiologically acceptable pH range while the temperature increased from 4 °C to 37 °C.

3.2.4. Turbidity of the hydrogel

The turbidity changes of CS-α,β-GP hydrogel as the function of time at T_{gel} were shown in Fig. 3. The dynamics curve of turbidity decreased rapidly along with time extension till 50 s, and then it undergo a rapidly increased part following a slowly increased part. The insets in Fig. 3 showed the appearance of the CS-α,β-GP hydrogel at 0 s, 50 s, 70 s, and 200 s during gelation progress at T_{gel}. At 0 s, the hydrogel was milk-white and in the sol state, when the time extended to 50 s, the hydrogel became transparent and also in the sol state, with the time extending from 50 s to 70 s, the hydrogel became milk-white again and its viscosity augment, and at 200 s, the appearance of the hydrogel was opaque and in the gel state.

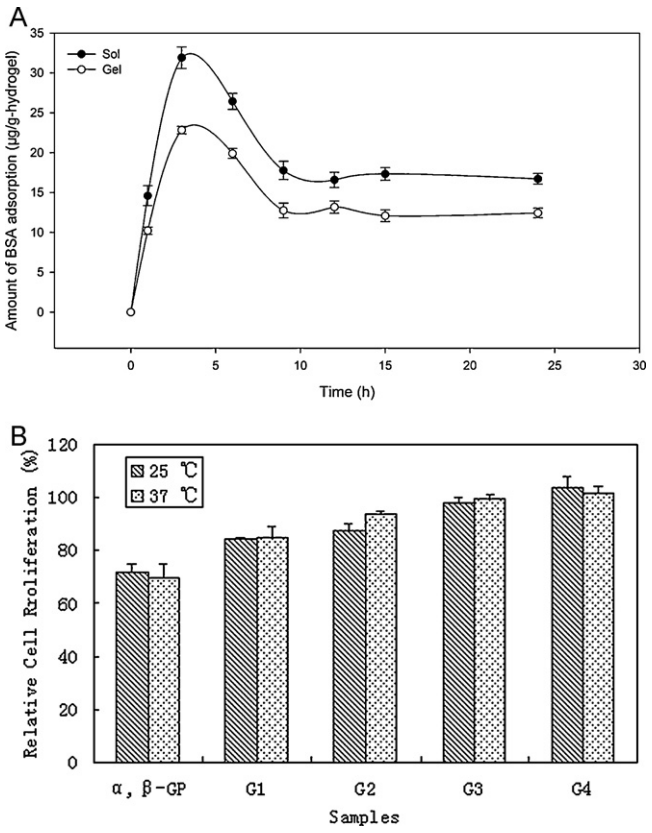

Fig. 6. Biocompatibility of the CS-α,β-GP hydrogel: (A) BSA adsorption profiles of CS-α,β-GP hydrogel in sol state and gel state and (B) *In vitro* cytotoxicity of different extracting solutions of CS-α,β-GP hydrogel and α,β-GP solution in MEFs.

3.2.5. Morphology observation

The surface and internal morphology of the CS-α,β-GP hydrogel studied by means of SEM were shown in Fig. 4. The surface and interior morphology of the hydrogel displayed a highly porous structure, and the pores formed an interconnecting "open-cell" structure, resembling other natural macromolecular hydrogel system structures (Tan, Chu, Payne, & Marra, 2009). The pore diameter of the CS-α,β-GP hydrogel was in the range of 50–150 μm (Fig. 4A).

3.3. Swelling and degradation kinetics of the CS-α,β-GP hydrogel

The swelling kinetics of the CS-α,β-GP hydrogel in distilled water and PBS 7.2 at 25 °C or 37 °C was shown in Fig. 5A and the hydrogel reached a high swelling equilibrium in a reasonably short time in this research. The typical weight loss and SR curve in Fig. 5B suggested CS-α,β-GP hydrogel prepared in this study should be degradable in aqueous media by a bulk erosion process (Dang et al., 2011).

3.4. Biocompatibility of the CS-α,β-GP hydrogel

3.4.1. Protein adsorption assay

To examine the specific interactions of protein with the CS-α,β-GP hydrogel, adsorption for BSA was studied and the results were shown in Fig. 6A. Fig. 6A showed a low BSA adsorption of CS-α,β-GP hydrogel in sol state and gel state. It could be seen from the results the BSA adsorption increased with time extending in the first 3 h, and then, it decreased in the after 6 h and reached stable in the following hours, showing a wavelike BSA adsorptions.

Protein adsorption is an important indicator for biocompatibility. It was demonstrated that the CS-α,β-GP sol and gel materials had a little BSA adsorption at physiological conditions. Proteins

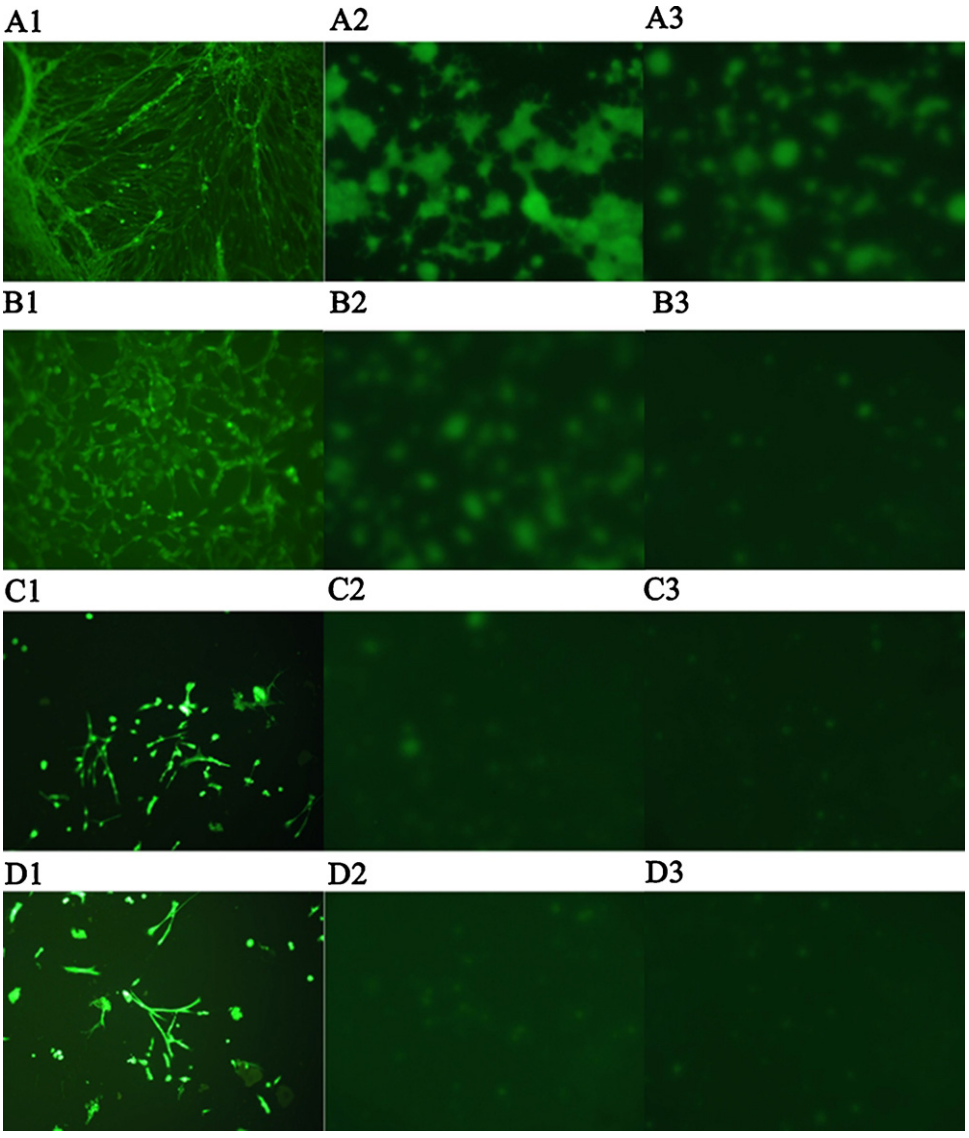

Fig. 7. Photomicrographs showing viability of *Penaeus chinensis* lymphoid cells continuous cultured 7 d. (A) Primary cultured cells (A1, on the bottom of the culture flask; A2, on the surface of the hydrogel; A3, encapsulated inside of the hydrogel); (B) subcultured cells which primary cultured on the bottom of the culture flask (B1, on the bottom of the culture flask; B2, on the surface of the hydrogel; B3, encapsulated inside of the hydrogel); (C) subcultured cells which primary cultured on the surface of the hydrogel (C1, on the bottom of the culture flask; C2, on the surface of the hydrogel; C3, encapsulated inside of the hydrogel); D, subcultured cells which primary cultured encapsulated inside of the hydrogel (D1, on the bottom of the culture flask; D2, on the surface of the hydrogel; D3, encapsulated inside of the hydrogel).

adsorption onto the surface of a biomaterial is the initial event that takes place in most *in vivo* settings, and adsorption amount decides the biocompatibility of biomaterials. It was considered that less preliminary protein adsorption always demonstrated lower level of subsequent inflammatory and/or thrombotic responses (Hoemann et al., 2007). Furthermore, the wave adsorption of the CS-α,β-GP sol and gel materials under physiological conditions indicated the combination of the BSA and CS-α,β-GP materials was physical adsorption. The physical adsorption does not change the configuration of adhesion proteins, which indicated CS-α,β-GP hydrogel in sol and gel state had good biocompatibility.

3.4.2. MTT assay

The MTT assay is generally accepted as a routine method for establishing cytotoxicity of biomaterials. In order to further 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. 6B). Incubation of the MEFs with extracting solutions of G1 at 25 °C or 37 °C for 24 h resulted in a RGR % above 80%, indicating a low-toxicity of the extracts. The RGR % increased as the hydrogel washing times with cell culture medium increased regardless of the temperature. There were no significant difference between the absorbance of the negative control and the wells treated with extracts of the hydrogels washing for 3 times with DMEM (RGR % reached 100%), showing a non-toxicity of the extracts.

Incubation of the MEFs with α,β-GP solutions had a obvious effect on the metabolic activity of cells, relative to extracts of CS-α,β-GP hydrogels. The MEFs incubated with α,β-GP solutions had a RGR % lower than 75%, indicating α,β-GP had cytotoxicity. The cytotoxicity reduced when the α,β-GP reacted with CS and generated CS-α,β-GP hydrogel. The cytotoxicity of the α,β-GP could be removed by washing the CS-α,β-GP hydrogel 3 times with DMEM.

The cytocompatibility of hydrogel materials is an important consideration for cell encapsulation, since cell viability must be maintained during gel fabrication. As shown in the MTT assay results, α,β-GP solution obviously affected MEFs viability,

indicating the toxic effect of the α,β-GP (Morin et al., 2004). The present study produced a physiologically acceptable pH ranged and 25 °C gelling liquid CS-α,β-GP hydrogel by decreasing the solvent strength of CS and increasing the ratio of CS/α,β-GP, which minimized the amount of α,β-GP to decrease toxicity of the hydrogel. The previous study demonstrated that the toxic effect of the GP was dependent on exposure time and exposure for 0.5 h was not cytotoxic (Wang & Stegemann, 2010), suggesting that timely removal of excess of α,β-GP after gel formation was sufficient to maintain cytocompatibility. Our results revealed that the toxic effect of the α,β-GP could be eliminated by washing the CS-α,β-GP hydrogel 3 times with cell culture medium.

3.4.3. Culture of Penaeus chinensis lymphoid cells

Vital staining of *P. chinensis* lymphoid cells cultured on the bottom of the culture flask, on the surface of the CS-α,β-GP hydrogel and encapsulated inside of the hydrogel, shown in Fig. 7, revealed that the lymphoid cells cultured on the surface or encapsulated inside of the hydrogel could grow and reproduce. Lymphoid cells primary cultured or subcultured on the bottom of the culture flask fell into two morphological classes: fibroblast-like cells, which showed elongated and showed parallel orientation, and epithelioid cells, which looked cuboidal or polygonal (Fig. 7A1, B1, C1, D1). The differences between lymphoid cells cultured on the surface or encapsulated inside of the hydrogel compared to the lymphoid cells cultured on the bottom of the culture flask were the morphology of the cells cultured on the surface or encapsulated inside of the hydrogel could not be distinguished. The lymphoid cells cultured on the surface (Fig. 7A2, B2, C2, D2) or encapsulated inside (Fig. 7C1, C2, C3, C4) of the hydrogel showed non-uniform sizes and shapes, indicating that the cells located in different depths within the hydrogel and grew in different directions.

After 7 days primary culture on the bottom of the culture flask, the lymphoid cells formed monolayer and then subcultured on the bottom of the culture flask, or on the surface or encapsulated inside of the hydrogel, shown in Fig. 7B, indicated the lymphoid cells maintained in subculture still could grow and reproduce. After 20 days primary culture on the bottom of the culture flask, the lymphoid cells activity decreased and could not survive in the subculture. After 20 days culture on the surface or encapsulated inside of the hydrogel, the lymphoid cells were still had good activity, and could be subcultured (Fig. 7C, D).

4. Conclusions

This study developed a physiologically acceptable pH ranged and 25 °C gelling CS-α,β-GP hydrogel by changing the solvent strength of CS solution and the CS/α,β-GP ratio. The surface and interior morphology of the hydrogel displayed a highly porous structure, and the pores diameters of the CS-α,β-GP hydrogel were in the range of 50–150 μm. The gelation times of this hydrogel were 90 s at 25 °C and 30 s at 37 °C. The *in vitro* study showed the CS-α,β-GP hydrogel had low protein adsorption and cytotoxicity. The cytotoxicity of the α,β-GP to MEFs could be eliminated by washing the CS-α,β-GP hydrogel 3 times with cell culture medium. After 20 days culture on the surface or encapsulated inside of the hydrogel, the lymphoid cells were still had good activity, and could subculture on the surface or encapsulated inside of the hydrogel. The results demonstrated that the hydrogel prepared in the present paper was the potential 3D culture scaffold for the *P. chinensis* lymphoid cells.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (NSFC, 81071274 & 40876065), International S&T Cooperation Program of China (ISTCP, 2011DFA31270) and the Fundamental Research Funds for the Central Universities (841111042, 201213006).

References

Chenite, A., Chaput, C., Wang, D., Combes, C., Buschmann, M. D., Hoemann, C. D., et al. (2000). Novel injectable neutral solutions of chitosan form biodegradable gels in situ. *Biomaterials*, *21*, 2155–2161.

Cho, J., Heuzey, M. C., Bégin, A., & Carreau, P. J. (2005). Physical gelation of chitosan in the presence of β-glycerophosphate: The effect of temperature. *Biomacromolecules*, *6*, 3267–3275.

Chung, H. J., Go, D. H., Bae, J. W., Jung, I. K., Lee, J. W., & Park, K. D. (2005). Synthesis and characterization of Pluronic® grafted chitosan copolymer as a novel injectable biomaterial. *Current Applied Physics*, *5*, 485–488.

Dang, Q. F., Yan, J. Q., Li, J. J., Cheng, X. J., Liu, C. S., & Chen, X. G. (2011). Controlled gelation temperature, pore diameter and degradation of a highly porous chitosan-based hydrogel. *Carbohydrate Polymers*, *83*, 171–178.

Fang, C., Shi, B., Pei, Y. Y., Hong, M. H., Wu, J., & Chen, H. Z. (2006). In vivo tumor targeting of tumor necrosis factor-α-loaded stealth nanoparticles: Effect of MePEG molecular weight and particle size. *European Journal of Pharmaceutical Sciences*, *27*, 27–36.

Ganji, F., Abdekhodaie, M. J., & Ramazani, A. S. A. (2007). Gelation time and degradation rate of chitosan-based injectable hydrogel. *Journal of Sol–Gel Science and Technology*, *42*, 47–53.

Hoemann, C. D., Chenite, A., Sun, J., Hurtig, M., Serreqi, A., Buschmann, M. D., et al. (2007). Cytocompatible gel formation of chitosan–glycerol phosphate solutions supplemented with hydroxyl ethyl cellulose is due to the presence of glyoxal. *Journal of Biomedical Materials Research*, *56*, 521–529.

Huang, Y., Lü, X., Ma, J., & Huang, N. (2008). In vitro investigation of protein adsorption and platelet adhesion on inorganic biomaterial surfaces. *Applied Surface Science*, *255*, 257–259.

Kim, S., Nishimoto, S. K., Bumgardner, J. D., Haggard, W. O., Gaber, M. W., & Yang, Y. (2010). A chitosan/β-glycerophosphate thermo-sensitive gel for the delivery of ellagic acid for the treatment of brain cancer. *Biomaterials*, *31*, 4157–4166.

Kraehenbuehl, T. P., Ferreira, L. S., Zammaretti, P., Hubbell, J. A., & Langer, R. (2009). Cell-responsive hydrogel for encapsulation of vascular cells. *Biomaterials*, *30*, 4318–4324.

Lang, G. H., & Nomura, N. (2002). Masatoshi Matsumura, Growth by cell division in shrimp (*Penaeus japonicus*) cell culture. *Aquaculture*, *213*, 73–83.

Morin, C., Hitchcock, A. P., Cornelius, R. M., Brash, J. L., Urquhart, S. G., Scholl, A., et al. (2004). Selective adsorption of protein on polymer surfaces studied by soft X-ray photoemission electron microscopy. *Journal of Electron Spectroscopy and Related Phenomena*, *137/140*, 785–794.

Muzzarelli, R. A. A. (1997). Human enzymatic activities related to the therapeutic administration of chitin derivatives. *Cell and Molecular Life Sciences*, *53*, 131–140.

Rao, S. B., & Sharma, C. P. (1997). Use of chitosan as a biomaterial: Studies on its safety and hemostatic potential. *Journal Biomedical Materials Research*, *34*, 21–28.

Roughley, P., Hoemann, C., DesRosiers, E., Mwale, F., Antoniou, J., & Alini, M. (2006). The potential of chitosan-based gels containing intervertebral disc cells for nucleus pulposus supplementation. *Biomaterials*, *27*, 388–396.

Tamura, H., Furuike, T., Nair, S. V., & Jayakumar, R. (2011). Biomedical applications of chitin hydrogel membranes and scaffolds. *Carbohydrate Polymers*, *84*, 820–824.

Tan, H., Chu, C. R., Payne, K. A., & Marra, K. G. (2009). Injectable in situ forming biodegradable chitosan-hyaluronic acid based hydrogels for cartilage tissue engineering. *Biomaterials*, *30*, 2499–2506.

Wang, L., & Stegemann, J. P. (2010). Thermogelling chitosan and collagen composite hydrogels initiated with β-glycerophosphate for bone tissue engineering. *Biomaterials*, *31*, 3976–3985.

Wang, T., Jiang, X. J., Tang, Q. Z., Li, X. Y., Lin, T., Wu, D. Q., et al. (2009). Bone marrow stem cells implantation with a-cyclodextrin/MPEG–PCL–MPEG hydrogel improves cardiac function after myocardial infarction. *Acta Biomaterialia*, *5*, 2939–2944.

Wang, W. N., Liang, H., Wang, A. L., Chen, T., Zhang, S. E., & Sun, R. Y. (2001). Effect of pH and Zn^{2+} on subcultured muscle cells from *Macrobrachium nipponense*. *Methods in Cell Science*, *22*, 277–284.

Zhang, J., Chen, X. G., Liu, C. S., & Park, H. J. (2009). Investigation of polymeric amphiphilic nanoparticles as antitumor drug carriers. *Journal of Materials Science: Materials in Medicine*, *20*, 991–999.

Zhang, L., Li, K., Xiao, W., Zheng, L., Xiao, Y., Fan, H., et al. (2011). Preparation of collagen-chondroitin sulfate-hyaluronic acid hybrid hydrogel scaffolds and cell compatibility in vitro. *Carbohydrate polymers*, *84*, 118–125.