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Potential of an injectable chitosan/starch/β-glycerol phosphate hydrogel for sustaining normal chondrocyte function

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ABSTRACT

An injectable hydrogel for chondrocyte delivery was developed by blending chitosan and starch derived from various sources with β-glycerol phosphate (β-GP) in the expectation that it would retain a liquid state at room temperature and gel at raised temperatures. Rheological investigation indicated that the system consisting of chitosan derived from crab shell and corn starch at 4:1 by weight ratio (1.53%, w/v of total polymers), and 6.0% (w/v) β-GP (C/S/GP system) exhibited the sharpest sol–gel transition at $37 \pm 2\,^{\circ}$C. The C/S/GP hydrogel was gradually degraded by 67% within 56 days in PBS containing 0.02 mg/ml lysozyme. The presence of starch in the system increased the water absorption of the hydrogel when compared to the system without starch. SEM observation revealed to the interior structure of the C/S/GP hydrogel having interconnected pore structure (average pore size 26.4 μm) whereas the pore size of the hydrogel without starch was 19.8 μm. The hydrogel also showed an ability to maintain chondrocyte phenotype as shown by cell morphology and expression of type II collagen mRNA and protein. *In vivo* study revealed that the gel was formed rapidly and localized at the injection site.

1. Introduction

The *in situ*-forming systems have continuously reported in the literature involving biomedical applications including drug delivery, cell encapsulation, and tissue engineering (Peppas, 1997; Rosiak and Yoshii, 1999; Hoffman, 2002). Thermosensitive hydrogels are one example of *in situ*-forming systems where the injectable liquid form can be administered into the desired tissue, organ or body cavity in a minimally invasive manner prior to gelling. The well-known thermosensitive materials are based on either synthetic (Peter et al., 1998; Elisseeff et al., 1999; Guilherme et al., 2004; Rice and Anseth, 2004) or natural polymers (Park et al., 2003; Stevens et al., 2004; Balakrishnan and Jayakrishnan, 2005; Chung et al., 2005). The drawbacks of these polymers are their resistance to metabolism, the need of high polymer concentrations, slow rate of conversion to "semisolid like-gel" and the necessarily high injection temperature.

Chitosan is currently receiving enormous of attention for medical and pharmaceutical application. The addition of polyol salts such as glycerophosphate disodium salt into chitosan solution creates a viscous liquid at room temperatures or below and then converts to a semisolid like-gel as it approaches body temperature (Chenite et al., 2000, 2001; Crompton et al., 2005). However, the chitosan/glycerophosphate system generally exhibits a viscous liquid at high concentration used, slow sol–gel transition and low flexibility of the resultant gel. These properties limit the application of the system as an injectable device for cell delivery and also as a temporary scaffold of soft tissue such as cartilage.

Starch is a natural abundant polymer which is commonly used in pharmaceuticals as a binder, filler and disintegrant. Like chitosan, starch is a biocompatible, biodegradable and non-toxic polymer. In addition, the non-ionic nature of starch makes it compatible with various kinds of polymers including chitosan. Recent studies have reported that the physical properties including flexibility of cross-linked chitosan hydrogel can be improved by blending chitosan with pregelatinized starch (Viyoch et al., 2003, 2005). Additionally, from our preliminary study, the blending of chitosan/β-glycerol phosphate solution with the pregelatinized starch could provide the thermosensitive hydrogels which rapid form gels by visual observation. From these preliminary observations, we considered that this formula might be developed into an application suited in cartilage tissue engineering.

Therefore, the aim of this study was to develop and evaluate the properties of an injectable chitosan/starch/β-glycerol phosphate

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hydrogel for application in cartilage tissue engineering. Chitosan and starch used in this study came from multiple sources and β-glycerol phosphate was used as cross-linking agent. Two parameters have to be considered including ability to maintain the mixture of chitosan and starch solution at nearly neutral pH, and most importantly, ability to perform sharpness of sol–gel transition at $37 \pm 2\,^{\circ}$C. Other properties including enzymatic degradation, water absorption ability and morphology of the developed hydrogel were investigated. The potential of the developed system for application in cartilage tissue engineering was evaluated by the ability of the chitosan/starch/β-glycerol phosphate to maintain chondrocyte phenotype and function.

2. Materials and methods

2.1. Materials

Chitosan (molecular weight of 10^5 to 10^6 Da, degree of deacetylation > 90%) derived from various sources (shrimp shell, crab shell and squid pen) was purchased from Bannawach Bio-Line Co., Ltd., Chonburi, Thailand. Corn starch (amylose:amylopectin, 30.23:69.77) was purchased from Tawan Chemical, Bangkok, Thailand. Tapioca starch (amylose:amylopectin, 30.20:69.80) was supplied by Wide Trade Co. Ltd., Bangkok, Thailand. Rice starch (amylose:amylopectin, 25.83:74.17), β-glycerol phosphate disodium salt pentahydrate (β-GP) and lysozyme from hen egg white were purchased from Fluka Chemie GmbH, Buchs, Switzerland. Trypsin (0.25%)/EDTA (0.01 M) and propidium iodide (PI) were purchased from Sigma–Aldrich Co., St. Louis, Missouri, USA. Collagenase type II from *Clostridium histolyticum* was purchased from Gibco$^{\text{TM}}$, Invitrogen Corporation, California, USA. Penicillin, streptomycin, one-step RT-PCR Taq (Superscript$^{\text{TM}}$ with platinum$^{®}$ Taq), Trizol$^{®}$ reagent, fetal bovine serum (FBS) and the designed primers were purchased from Invitrogen, California, USA. Dulbecco's minimum essential medium (DMEM, low glucose) was purchased from Sigma–Aldrich Chemie GmbH, Steinheim, Germany. Cell proliferation kit II (sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonicacid hydrate, XTT) was purchased from Roche Diagnostics GmbH, Mannheim, Germany. Mouse monoclonal to collagen II and secondary antibodies for type II collagen were purchased from Abcam plc, Cambridge, UK.

2.2. Preparation of chitosan/starch/β-glycerol phosphate (C/S/β-GP) solution

Chitosan (shrimp shell, crab shell or squid pen) and starch (corn, rice or tapioca starch) derived from various sources were used in this study to investigate effect of polymer source on the thermosensitive property. The mixture solution of C/S/β-GP was prepared by blending chitosan solution with pregelatinized starch solution in the presence of β-GP. Firstly, chitosan solution was prepared by dissolving 170 mg of chitosan in 9 ml of 0.1 M HCl. Pregelatinized starch solution was obtained by dissolving 170 mg of starch into 10 ml of deionized water and the resultant starch suspension was then heated until gelatinization. The weight ratio of chitosan to starch was 4:1 and total concentration of the blended polymers was 1.53% (w/v). After cooling 9 ml of the blended chitosan/starch solution, 1 ml of chilled β-GP aqueous solution was carefully added drop by drop. The final concentrations of the β-GP used were in range of 5.0–6.0% (w/v). The mixture was stirred for 10 min to obtain a clear and homogenous liquid solution in a final volume of 10 ml.

2.3. Characteristics of hydrogel

2.3.1. Rheological evaluation

The rheological property of the prepared C/S/β-GP was examined with an Advanced Rheometrics Expansion System (Model ARES, Rheometric Scientific Inc., NJ, USA). The measuring system used coquette fixture requiring 8 ml of solution. Samples were covered with mineral oil to prevent water evaporation during the measurement. The values of the strain amplitude were verified to ensure that all measurements were performed within the linear viscoelastic region, such that the elastic modulus (G') and loss modulus (G'') were independent of the strain amplitude. To determine gelation temperature, oscillatory measurement was performed at 6.25 Hz. The temperature was increased at the rate of 1 °C/min between 25 and 45 °C. The gel point was determined as the temperature at the crossover point of G' and G'' (Chenite et al., 2001).

2.3.2. In vitro enzymatic degradation

The mixture solution of the selected system (0.5 ml) was placed in a cylindrical mold and then was incubated at 37 °C for 30 min to obtain cylindrical hydrogels. An individual constructed hydrogel was placed into the phosphate buffer saline (PBS, pH 7.4) or PBS containing 0.02 mg/ml lysozyme at 37 °C for 24 h (Hong et al., 2007). After reaching equilibrium, the sample was weighed (W_0). The same sample was weighed again (W_1) after lyophilization. The sample was taken out and weighed (W_2) on day 1 and day 3 of the first week and every week for a total of 8 weeks and it was subsequently freeze-dried and weighed again (W_3). The study was performed in triplicate. The wet weight remaining ratio (with water) and the dry weight remaining ratio (without water) were calculated using equation as follows:

$$\text{Wet weight remaining ratio}\ (\%) = (W_2/W_0) \times 100$$
$$\text{Dry weight remaining ratio}\ (\%) = (W_3/W_1) \times 100$$

2.3.3. Water absorption ability

Freshly prepared cylindrical gel (0.5 ml of the mixture solution of the selected system) was prepared as mentioned above. An individual sample was weighed and then immersed in 15 ml PBS pH 7.4 contained in vial. The vial was placed in a shaking water bath set at 37 °C and 60 rpm. The swellon gel was carefully removed at predetermined time intervals and weighed after blotting with soft tissue paper to remove surface medium. Measurements were made until hydration reached a constant value. The study was performed in triplicate. The percentage of water absorption of an individual gel was calculated using following equation:

$$\text{Water absorption}\ (\%) = ([W_t\text{-}W_0]/W_0) \times 100$$
where W_t = weight of absorbed gel at time t
and W_0 = initial weight of gel

2.3.4. Morphology

The hydrogels were constructed as cylinders as mentioned above. Scanning electron microscopy (SEM, Model 1455VP, Leo Electron Microscopy, Inc., Cambridge, UK) was used to obtain information on the pore size of such hydrogels. Small pieces of the hydrogel were immersed in liquid nitrogen and then the vitrified samples were cut with a cold knife. The samples were mounted on to aluminum stubs with double-sided adhesive tape and sputter-coated with gold (5 nm thickness). The microstructure of the samples was imaged at 25 kV and 200× magnification.

2.4. Evaluation of effects of hydrogel on chondrocyte function

2.4.1. Isolation of rat chondrocytes

The chondrocytes were isolated from Sprague–Dawley rats (male, 6–8 weeks). The study was done following the guidelines for care and use of experimental animals as laid down by the Institutional Animal Ethics Committee of Naresuan University approval code 50 02 0001. Animals were sacrificed and cartilages were dissected from the hip and knee joints and then digested for 30 min at 37 °C in 0.25% trypsin/0.01 M EDTA, according to the previous study (Tsukazaki et al., 1994) with modifications. The digested materials were collected by centrifugation ($450 \times g$ for 15 min), resuspended in 0.2% type II collagenase prepared in DMEM with 100 U/ml penicillin and 100 μg/ml streptomycin and subsequently incubated for 4 h at 37 °C. Dispersed cells were filtered through a 40 μm mesh to remove debris, recentrifuged ($450 \times g$ for 15 min) and washed twice in PBS to remove residual collagenase.

2.4.2. Cell viability of chondrocytes encapsulated in hydrogel

The mixture solution of the selected hydrogel system was prepared under aseptic condition. The primary chondrocytes of 1×10^6 cells were then suspended in 1 ml of the mixture solution of the hydrogel at 4 °C. Fifty microliters of the cell suspension was transferred to 96-well plate and allowed to gel at 37 °C in a humidified atmosphere containing 5% CO_2 for 20 min. Subsequently, 200 μl of the cultured medium (DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin) was added into each well. The cells encapsulated in the constructed hydrogel (3D condition) were incubated at 37 °C with 5% CO_2 and the cultured medium was changed every 2 days. At a desired period of time (day 1 and day 7), the culture medium was replaced with 200 μl of serum free- and antibiotic-free DMEM. Fifty microliter of XTT labeling mixture was then added to each well. The plate was further incubated at 37 °C with 5% CO_2 for 4 h. Cell viability of the chondrocytes encapsulated was determined by measuring the optical density of the supernatant without hydrogel at 490 nm by using a UV plate reader (Spectra Count, Perkin Elmer, Massachusetts, USA). The blank sample was the cell-free constructed hydrogel. The data were expressed as the optical density and the study was performed in triplicate. The results from 3D hydrogel were compared with cells cultured under monolayer (2D condition) on 96-well tissue culture plate (1×10^5 cells/cm^2).

2.4.3. Morphology of chondrocytes encapsulated in hydrogel

The suspension of chondrocytes in the mixture solution of the hydrogel was prepared, according to the above-mentioned study. In this study, 500 μl of the cell/hydrogel mixture solution was placed into a circular-shape mold and kept to set gel in a humidified, 5% CO_2 incubator at 37 °C for 20 min. The constructed hydrogel encapsulating chondrocytes was then transferred to a 24-well culture plate containing 2 ml of the culture medium. The culture medium was replaced daily during the culture period. Gel constructs without cells were carried out through identical culture conditions as described previously, including fresh media changes daily serving as a control. Culture dishes were shaken slowly on an orbital platform in humidified 5% CO_2 at 37 °C.

After the cultivation to a desired period, chondrocytes encapsulated in the hydrogel matrix were washed with PBS pH 7.4 and then fixed in the buffer containing 2.5% GA for 12 h at 4 °C. This sample was washed with PBS to remove the residual GA and the chondrocytes were fixed with OsO_4 for 2 h and then washed with PBS. The sample was dehydrated through a series of ethanol (30, 50, 70, 90, 100%) for 15 min and followed by soaking in acetone for 2 times with 15 min each. After the sample was dried by the critically point dried system and coated with an ultrathin gold layer, it was observed by SEM.

2.4.4. Expression of type II collagen in chondrocytes encapsulated in hydrogel

To determine whether chondrocytes could function under cultivation in the developed hydrogel, collagen type II (*COL2A1*) expression was used as the marker of chondrocyte-specific gene. Collagen type I (*COL11A2*) was used as a marker for investigating the conversion of the chondrocyte to the fibroblastic phenotype. β-Actin, a housekeeping gene, was used as a control for cDNA integrity.

The constructed hydrogel encapsulating chondrocytes or chondrocytes on the tissue culture plate were prepared and/or cultured as above-mentioned conditions. After cultivation for a desired period, total RNA was isolated from chondrocytes by using Trizol® Reagent, according to the manufacturer's instruction.

A one-step RT-PCR system was used for reverse transcription of RNA. α1(II)-Collagen, α1(I)-collagen and β-actin cDNA were amplified by PCR (GeneAmp® PCR system 9700, Applied Biosystems, California, USA). β-Actin was incorporated with each gene containing in a single tube. PCR primers were designed as follows (Lee et al., 1997; Gartland et al., 2005): α1(II)-collagen, 5′–3′, 5′-AGT GGA AGA GCG GAG ACT A-3′; 3′–5′, 5′-GAC AGG CCC TAT GTC CAC AC-3′ (579 base pairs): α1(I)-collagen, 5′–3′, 5′-GCC AGC AGA TTG AGA ACA TCC-3′; 3′–5′, 5′-AAC CTT CGC TTC CAT ACT CG-3′ (312 base pairs): β-actin, 5′–3′, 5′-GAC CTT CAA CAC CCC AGC CAT *G*-3′; 3′–5′, 5′-GGG CCG GAC TCA TCG TAC TCC T-3′ (726 base pairs). PCR was conducted in a programmed temperature control system for 40 cycles under the following cycle condition: 15 s at 94 °C for denaturation, 30 s at 46 °C and 51 °C for annealing of α1(II)-collagen and α1(I)-collagen, respectively and 1 min at 68 °C for extension and 5 min at 72 °C for final extension. The PCR products were run on 1% agarose gel.

Collagen type II expression was also examined by using immunofluorescence staining. An individual hydrogel containing chondrocytes was smeared on glass slide. The chondrocytes were fixed with 1 ml of ice-cold methanol for 15–20 min (or until methanol dry) in refrigerator and then permeabilized with 0.1% Triton X-100 in PBS pH 7.4 for 5 min. After the cell samples were blocked with 3% bovine serum albumin in PBS for 30 min, they were incubated with mouse monoclonal to collagen II (1:250) at room temperature in the dark for 2 h. The samples were washed with PSB for 4 times. The specific binding was then visualized by incubating the samples with FITC conjugated goat polyclonal to mouse IgG (1:250) at room temperature with dark illumination for 1 h. After washing with PBS for 4 times, the samples were counterstained with PI diluted in PBS at ratio of 1:200 (v/v) and incubated in at room temperature with dark illumination for 10 min. Finally, the samples were washed with PBS for 4 times before they were photographed under a fluorescence microscope (Nikon Eclipse TE2000-U, Nikon Sankei, Tokyo, Japan). The experiment was performed with cells from different time culturing (7, 14, 21 days).

2.5. Localization of hydrogel after subcutaneous injection

To determine if the C/S/GP could form a gel rapidly and localized to the injection site, 500 μl of the freshly prepared and sterilized solution of the selected system was subsequently administered by dorsal subcutaneous injections through 26 g ½″ in adult male Sprague–Dawley rats ($\sim$250 g) after anesthetizing with carbon dioxide. The gel formation at the injection site was verified visually. After 1 day of injection, rats were sacrificed and the tissues at and around injection area were removed. Such tissues were frozen at −20 °C before gently cross-sectioned by using cryotome (Leica Microsystem Nussloch GmbH, Nussloch, Germany).

Table 1
The hydrogel systems consisting of chitosan (molecular weight of 10^5 to 10^6 Da, degree of deacetylation > 90%), starch and β-GP that provided the gelation temperature of 37 ± 2 °C and pH of 6.8–7.0. Total polymer content and proportion of chitosan to starch used were fixed at 1.53% (w/v) and 4 to 1 weight ratio, respectively.

Source of chitosan	Source of starch		
	Tapioca	Corn	Rice
Shrimp shell	β-GP concentration = 6.0% (w/v)	[a]	β-GP concentration = 6.0% (w/v)
Squid pen	β-GP concentration = 6.0% (w/v)	β-GP concentration = 5.5% (w/v)	β-GP concentration = 5.5% (w/v)
Crab shell	β-GP concentration = 6.0% (w/v)	β-GP concentration = 6.0% (w/v)	[a]

[a] *Note*: The gelation temperature of the system was more than 39 °C at any β-GP concentration used.

2.6. Statistic analysis

Student's *t*-test was used for comparison between the averages of two independent groups, and analysis of variance (ANOVA) was used for multiple comparisons. The *p*-value of less than 0.05 was considered as statistically significance.

3. Results

3.1. Characteristics of hydrogel

In the preliminary study we found that the gelling temperatures in the systems containing 1.53% (w/v) chitosan (from shrimp shell, crab shell or squid pen) and 6.0% (w/v) β-GP were in range of 40–44 °C (data not shown) with slow gel formation. Interestingly, adding starch into chitosan solution could reduce the gelation temperature to 37 ± 2 °C. With the presence of β-GP, the pregelatinized starch solution could be homogeneously mixed with chitosan solution at 4:1 of chitosan and starch weight ratio. However, using higher proportion of starch (3:2 and 2:3 of chitosan:starch) caused the precipitation of the blended polymer solution after adding of β-GP. The data of the systems consisting of chitosan, starch and β-GP which turned into gel state at 37 ± 2° C and exhibited the pH value in range of 6.8–7.0 are summarized in Table 1.

3.1.1. Rheology of the sol–gel transition

The rheological behaviors of the systems that provided the gelling temperature of 37 ± 2 °C and pH of 6.8–7.0 are shown in Fig. 1A–C. The increasing value of the elastic modulus (G') over temperature indicated that the viscous liquid of the polymer solution became a solid hydrogel. The sharpest transitions was observed for the system consisting of 4:1 ratio of chitosan derived from crab shell and corn starch in the present of 6.00% (w/v) of β-GP, as summarized in Table 2. Its liquid state with low viscosity and semisolid gel states are depicted in Fig. 2. Hereafter, this system was replaced

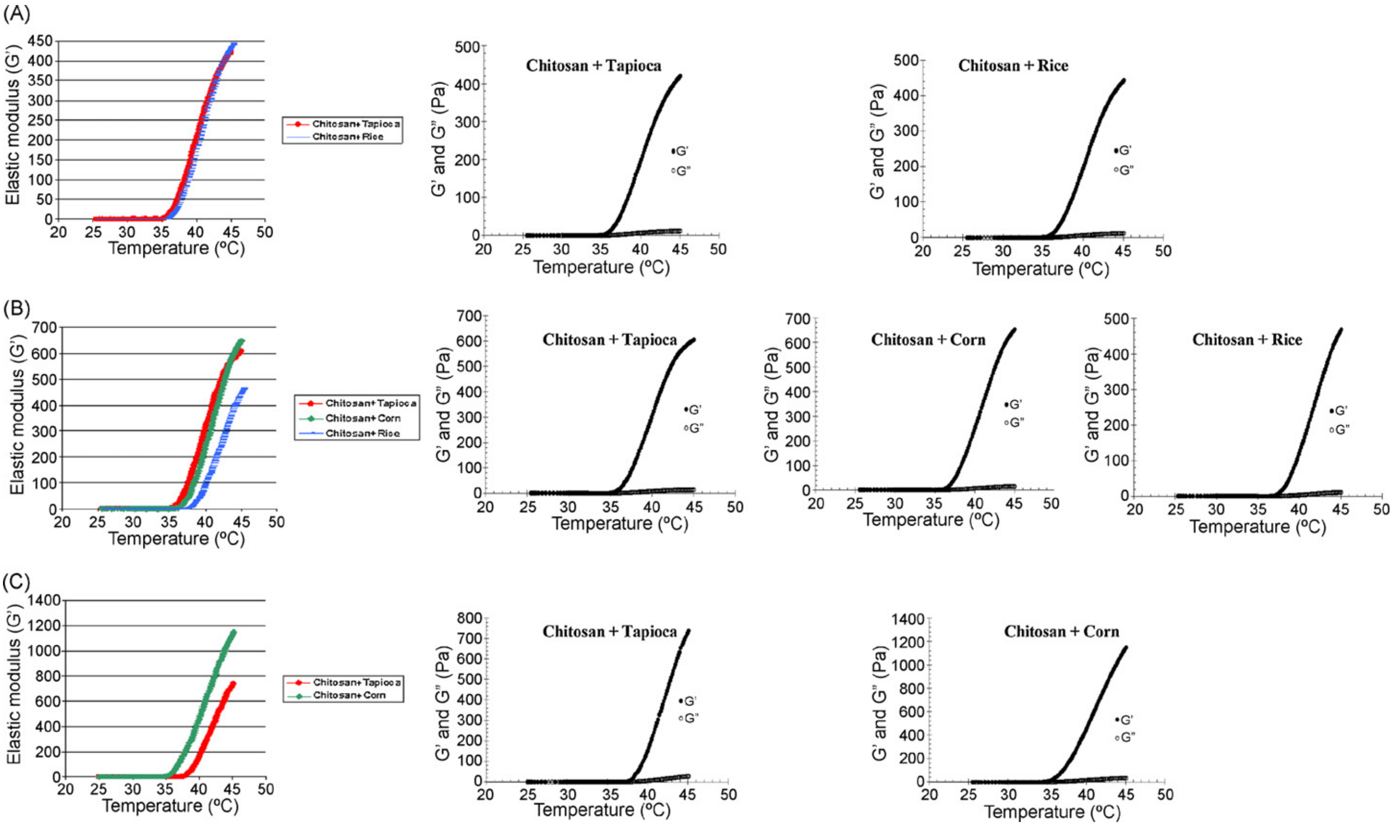

Fig. 1. The relation between elastic modulus (G') and temperature of the developed hydrogel systems that provided the gelation temperature of 37 ± 2 °C and pH of 6.8–7.0. All systems consisted of 1.53% (w/v) of total polymers. Chitosan derived from shrimp shell (A), squid pen (B) or crab shell (C) was blended with tapioca (●), corn (♣) or rice (▬) starch at the weight ratio of chitosan and starch of 4:1 by using β-GP as cross-linking agent. The concentrations of β-GP used are shown in Table 1. The plot of storage (elastic) modulus G' and loss (viscous) modulus G'' versus temperature of each system is also represented in this figure. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

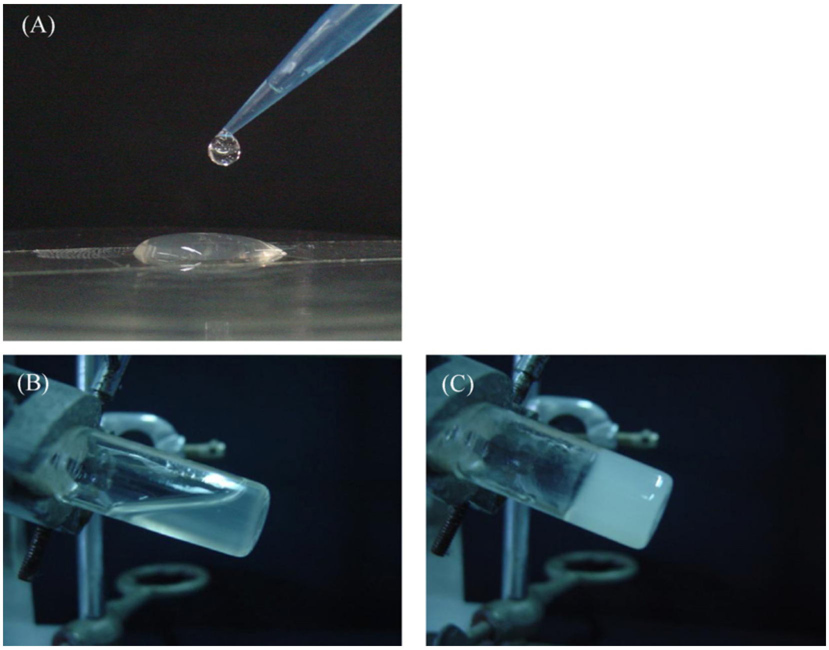

Fig. 2. The appearance of liquid state with low viscosity at room temperature (A and B) and gel state at 37 °C (C) of the C/S/GP hydrogel system. This hydrogel consisted of 1.53% (w/v) of total polymers. Chitosan derived from crab shell was blended with corn starch at the weight ratio of chitosan and starch of 4:1 by using 6.0% (w/v) of β-GP as cross-linking agent.

by the term of C/S/GP hydrogel and therefore, was subsequently selected for further study of its properties and for determining its potential in cartilage tissue engineering applications.

3.1.2. *In vitro enzymatic degradation*

In this study, lysozyme (0.02 mg/ml in PBS) was used to test degradation rate of the C/S/GP hydrogel. Fig. 3A shows the decline in wet weight with *in vitro* enzymatic degradation of the constructed hydrogel compared to PBS alone. Additionally, we also compared our C/S/GP hydrogel with the C/GP hydrogel to assess the effect of incorporating starch into the hydrogel. From the results, the degradation rates with lysozyme of the C/S/GP hydrogel slightly changed during the first 2 weeks and then gradually and linearly decreased as time increase from 100% to about 67% by the 56th day. In contrast without lysozyme, the percentage of wet weight remaining ratio decreased slowly from 100% to about 88%. For the C/GP hydrogel, its percentage of wet weight remaining ratio decreased slowly from 100% to about 79% at the 56th day in lysozyme treatment whereas that decreased from 100% to about 88% over the study period in PBS solution.

The dry weight remaining ratio of the C/S/GP hydrogel showed a similar changing tendency as shown in Fig. 3B which decreased from 100% to about 52% and 82% in lysozyme and in PBS solution, respectively. When compared to the C/S/GP hydrogel, the C/GP hydrogel showed a lower decrease in weight remaining ratio (from 100% to about 74% and 82% over the study period in lysozyme and in

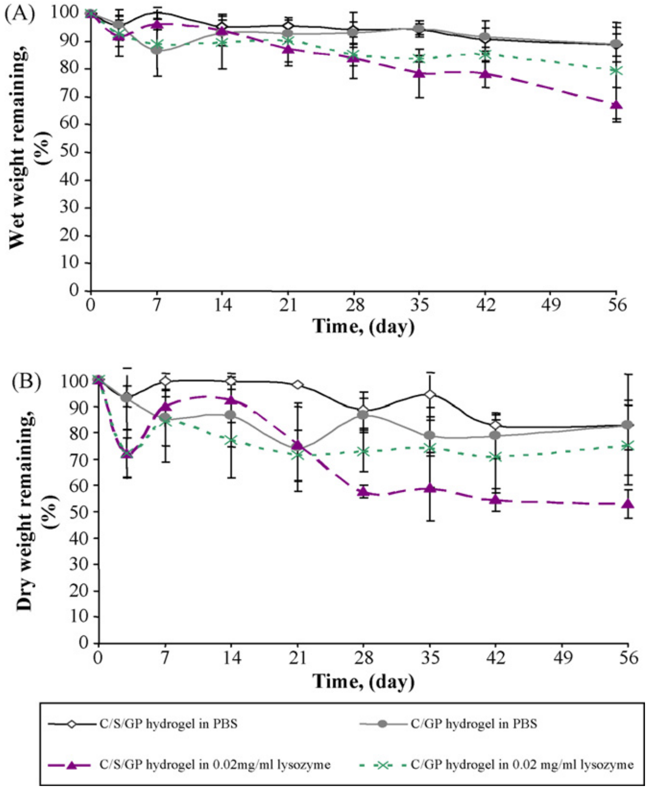

Fig. 3. The percentages of wet weight remaining ratio (A) and dry weight remaining ratio (B) of the C/S/GP hydrogel incubated in PBS (pH 7.4) (—◇—) or in lysozyme (0.02 mg/ml in PBS pH 7.4) (—▲—) and of the C/GP hydrogel incubated in PBS (pH 7.4) (—●—) or in lysozyme (0.02 mg/ml in PBS pH 7.4) (- -×- -) at 37 °C as a function of time. Each point represents mean ± SD ($n = 3$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 2
The slope or sharpness of transition calculated from the elastic modulus (G') of the hydrogel systems consisting of chitosan (molecular weight of 10^5 to 10^6 Da, degree of deacetylation > 90%), starch and β-GP. The concentrations of polymers and β-GP used were similar to those presenting in Table 1.

Source of chitosan	Slope or sharpness of transition		
	Tapioca starch	Corn	Rice
Shrimp shell	37.2	–	39.7
Squid pen	51.8	60.4	49.0
Crab shell	98.3	115.8	–

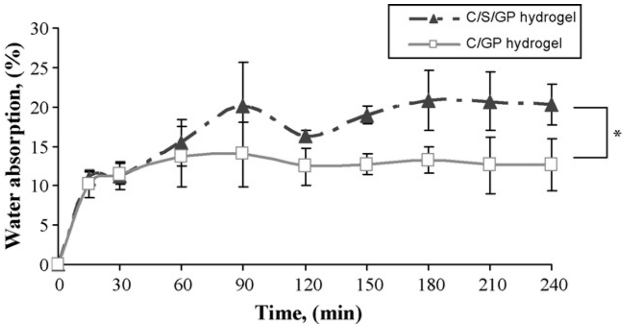

Fig. 4. The percentages of water absorption of the C/S/GP (▲) and the C/GP hydrogel (□) incubated in PBS (pH 7.4). Each point represents mean ± SD ($n = 3$). Two-way ANOVA showed a significant difference in percent absorption between two types of hydrogels (*$p < 0.05$).

PBS solution, respectively). Results from the study suggested that the degradation rate of the C/S/GP hydrogel tended to be higher than that of the C/GP hydrogel, especially in lysozyme condition.

3.1.3. Water absorption ability

Fig. 4 shows the percentage of water absorption of the C/S/GP hydrogel. We found that the hydrogel absorbed water rapidly at the initial and then gradually reached equilibrium. This phenomenon was also found in the C/GP hydrogel. However, a significant different ($p < 0.05$) in percent absorption between two types of hydrogel was found. The highest percentage of water absorption of the C/S/GP hydrogel was 20.79% whereas that of the C/GP hydrogel was 13.23%.

3.1.4. Hydrogel morphology

The structure and pores size of the cross-sectional area of the developed C/S/GP hydrogel were examined by SEM (Fig. 5A). Corn starch was revealed to have a significant effect on the structure of the hydrogel since it provided porous, honey-like structures with interconnected pores hydrogel. The pore size of the C/S/GP hydrogel averaged 26.4 μm ($n = 40$) whereas the C/GP hydrogel (consisting of 1.53%, w/v of chitosan derived from crab shell and 6.0%, w/v of β-GP) averaged 19.8 μm ($n = 40$) (Fig. 5B). However, it is important to note that for SEM analysis hydrogel must be completely dried and that the freeze-drying process generally creates a scaffold with limited interconnected pores which may not represent the hydrated porosity.

3.2. Effects of hydrogel on chondrocyte function

In this study, chondrocytes were isolated from enzymatic digestion of articular cartilages dissected from hip and knee joint of rats. The viability of such chondrocytes encapsulated in the C/S/GP hydrogel disks (three-dimensional condition, 3D culture, Fig. 7E) was observed as comparison to that of the chondrocytes cultured on the tissue culture plate (two-dimensional condition, 2D culture). According to XTT assay, the values of the optical density at 490 nm of the formazan dye derived from chondrocytes at day 1 and day 7 of cultivation are shown in Fig. 6. Such values correspond to cell viability which, at least partially, proportionally relate to cell number. We found that, in comparison to day 1, the optical density value of cells cultured under both conditions (2D and 3D cultures) significantly increased ($p < 0.01$) at day 7 of cultivation. However, the density of 2D cultured cells was significantly higher ($p < 0.05$, 2-way ANOVA) than that cultured under 3D. The results imply that the proliferation of cells under 2D condition is higher than that of cells under 3D condition.

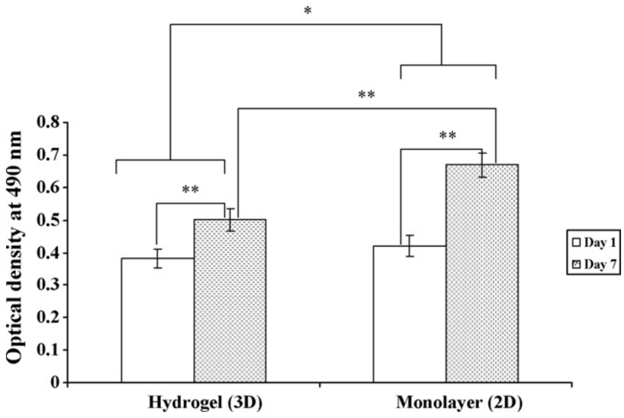

Fig. 6. The optical density at 490 nm of formazan dye derived from chondrocytes cultured on tissue culture plate (monolayer, 2D) or encapsulated in the C/S/GP hydrogel disks (hydrogel, 3D) at day 1 and day 7 of cultivation. Each bar represents as mean ± SD ($n = 3$). Student's *t*-test showed a significant difference in averaged optical density value between day 1 and day 7 of the same cultivation condition (**$p < 0.01$) and between two culture conditions (at day 7, **$p < 0.01$). Two-way ANOVA showed a significant difference in optical density between two culture conditions (*$p < 0.05$).

The morphology of chondrocytes was further observed by SEM (Fig. 7). Freshly-isolated cells appeared elliptical or rounded shape (Fig. 7A and B). For 5 days on culture plate (2D condition), the majority of cells attached to the polystyrene surface exhibited a fibroblast-like morphology (Fig. 7C and D), and the cells population rapidly expanded. In contrast, when the chondrocytes were encapsulated in the disk-shaped of the C/S/GP hydrogel (Fig. 7E) or cultured under 3D condition, a spherical morphology still appeared after cultivation for 14 days (Fig. 7F and G).

To show if chondrocytes encapsulated in the C/S/GP hydrogel disk could maintain their functional phenotype, the mRNA expression of type II collagen observed. The agarose gel electrophoresis of the RT-PCR products of this study are shown in Fig. 8. The chondrocytes cultured under 2D condition for 7 days did not express type II collagen mRNA. Additionally, such chondrocytes expressed high

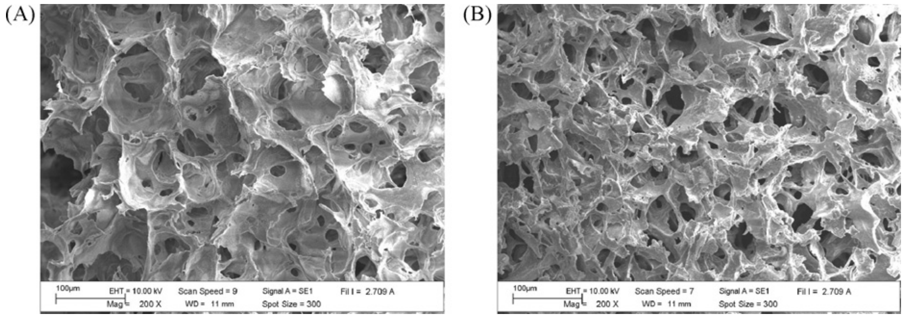

Fig. 5. SEM images of the cross-section of the C/S/GP hydrogel (A) and the C/GP hydrogel (B). The C/GP hydrogel system consisted of 1.53% (w/v) of chitosan derived from crab shell and 6.0% (w/v) of β-GP.

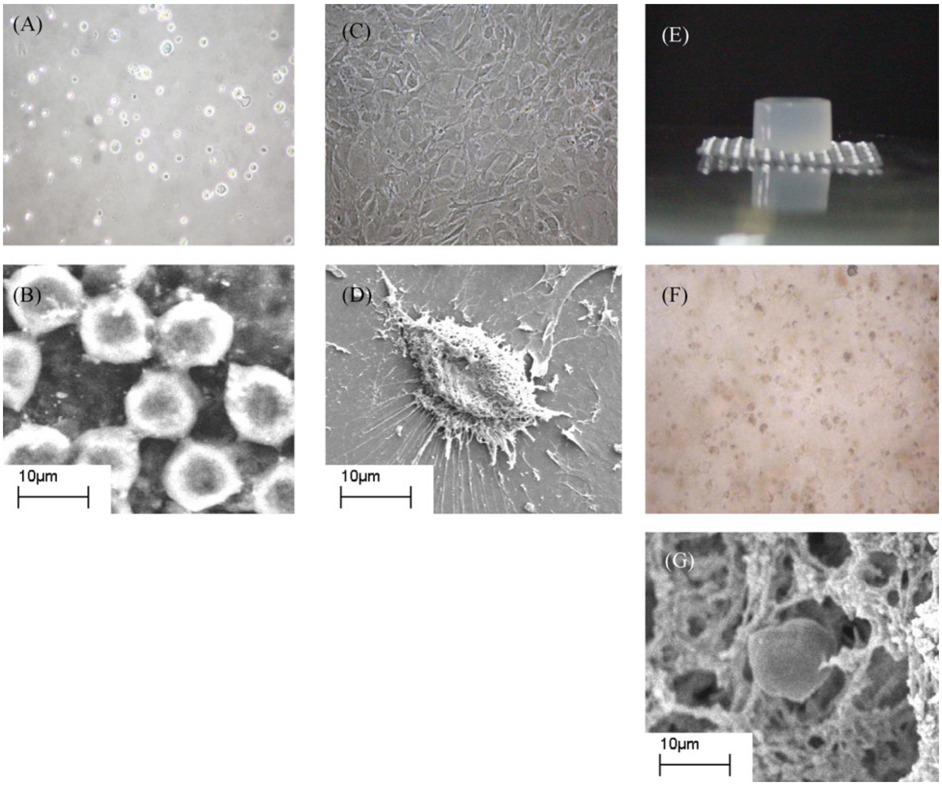

Fig. 7. Cell morphology imaged by inverted microscopy at magnification of 20× and by SEM of the freshly-isolated-chondrocytes (A and B), chondrocytes cultured on the tissue culture plate (2D culture) for 5 days (C and D) and chondrocytes encapsulated in the C/S/GP hydrogel disk (3D culture, E) for 14 days (F and G).

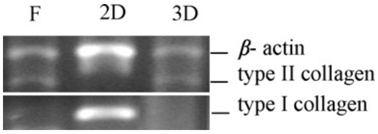

Fig. 8. RT-PCR analysis of type II collagen mRNA expression. Total RNA was purified from the freshly-isolated rat chondrocytes (F), from cultured on the tissue culture plate (2D) for 7 days and from chondrocytes encapsulated in the C/S/GP hydrogel disk (3D) for 14 days.

level of type I collagen mRNA, suggesting a loss of chondrocyte phenotype. In contrast to chondrocytes cultured in plastic plate, chondrocyte cells after 3D culture within the constructed hydrogel for 14 days showed the expression of type II collagen mRNA. The 3D culture of chondrocytes also showed a marked downregulation of type I collagen expression as comparison to 2D culture. In addition, according to immunofluorescence study, intracellular and extracellular localization of type II collagen could observe in chondrocytes cultured within the constructed C/S/GP hydrogel for 7, 14 and 21 days (Fig. 9). These results suggest that the C/S/GP hydrogel could maintain a chondrocyte-like phenotype of chondrocytes.

3.3. *Localization of hydrogel after subcutaneous injection*

In this study, we investigated the formation of the hydrogel after subcutaneous injection. The formation of gel network was observed within 10 min after subcutaneous injection on the back of Sprague–Dawley rat. As shown in Fig. 10, cross-sectioned tissues on the injected site also indicated the localization of the formed hydrogel. This suggests that *in situ* gel formation was sufficiently fast to confine the network to the injection site.

4. Discussion

In this study, β-GP was used with the expectation of the transformation of pH-gelling chitosan solutions into thermally sensitive pH-dependent gel-forming aqueous solutions. Chitosan solution can be adjusted to pH values between 6.7 and 6.8 *via* the addition of β-GP. This system can maintain the chitosan in liquid state at the temperature in range of 4–25 °C and change to gel state when raised to 35–45 °C. The ability to maintain liquid state at pH near to neutrality and the gelling mechanism of the chitosan and β-GP system have been reported in the past (Back et al., 1979; Ruel-Gariepy et al., 2000; Chenite et al., 2001; Cho et al., 2006). In acidic conditions, the development of electrostatic repulsion between chitosan chains results from a positively charged ammonium groups (NH_3^+) resided on chitosan chain. Adding β-GP causes the neutralization of positively charged ammonium groups by the negatively charged phosphate groups presenting in β-GP, consequently resulting in decreasing electrostatic repulsions. In addition, glycerol moieties of β-GP can interact with water and then promote the protective hydration of chitosan chains and keep the polymer chains stretched freely in solution at low temperature. Raising the temperature would increase the internal energy and break the hydrogen bonds between chitosan and water. Therefore, the water molecules bound to chitosan chains are released and move with freedom. The movement of free water molecules allows the chitosan chains to become closer, and hydrophobic interaction between the polymer chains is manifest as gelation.

From our preliminary study, we found that the system consisting of 1.53% (w/v) of chitosan (molecular weight of 10^5 to 10^6 Da, degree of deacetylation > 90%) derived from crab shell and 6.0% (w/v) of β-GP provided the phase transition around 40–44 °C. In addition, such hydrogel exhibited high viscosity at liquid state and slow sol–gel transition. Thus this system may be unsuited to the delivery of bioactive materials. Interestingly, decreasing content of

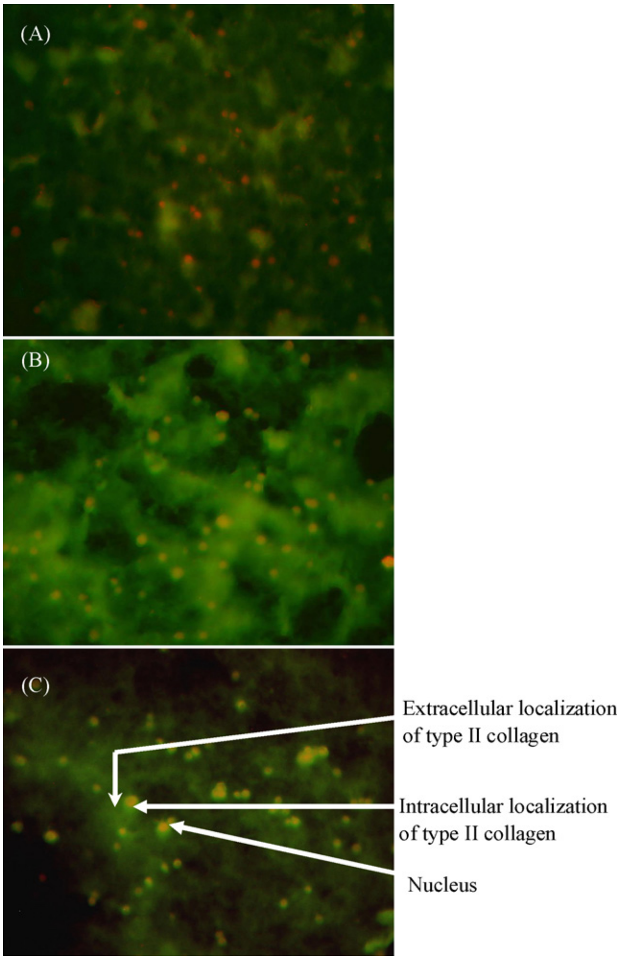

Fig. 9. Immunofluorescence staining of chondrocytes encapsulated in the C/S/GP hydrogel disk (3D culture) for 7 (A), 14 (B) and 21 (C) days. Chondrocytes were fixed, permeabilized, and labeled with FITC conjugated goat polyclonal to mouse IgG and counterstained with propridium iodide to visualize the nuclei.

chitosan together with adding pregelatinized starch provided the system with lower viscosity in the liquid state and faster gelling at lower temperature. This phenomenon indicated that addition of starch further promoted chitosan–chitosan hydrophobic interactions which are expected to play a major role in the gelation of chitosan solution with the presence of β-GP, and such phenomenon might be explained by physical and chemical interaction between chitosan and starch. For chemical interaction, adding of pregelatinized starch into the chitosan solution could propose that the hydrogen bondings occurred between –OH of starch and $-NH_2$, $-NH_3^+$ or –OH of chitosan (Xu et al., 2005; Wang et al., 2007). After adding the β-GP solution, phosphate moieties would ionically interact with ammonium groups presented on chitosan chains. However, comparing with system without starch (C/GP), the availability of ammonium groups on chitosan chains in the system with starch (C/S/GP) might be decreased because the ammonium groups had partly bonded through hydrogen bond with the hydroxyl groups of starch. The reduction of ammonium charge density on the chitosan chains was likely to reduce interchain electrostatic repulsion (Chenite et al., 2001) and/or reduce the ratio of the $-NH_3^+$ of chitosan and glycerol moiety of β-GP. Such reduction then allowed the lower thermal energy to remove glycerol moieties between chitosan chain. Therefore, the gelation of the mixture solution of the C/S/GP system required a lower gelling temperature when compared to the C/GP system.

In addition, the effect of low amounts of starch could possibly be explained in the terms of physical interaction. Starch might act as steric hindrance molecules that interfered with the bonding between phosphate moieties of β-GP and $-NH_3^+$ of chitosan. This, again, was likely to reduce interchain electrostatic repulsion of chitosan. Therefore, in C/S/GP system, the glycerol moiety could move out easily from the chitosan chain by a lower raising temperature.

From the rheological study, the hydrogel consisting of the mixture of chitosan derived from crab shell, corn starch and β-GP provided a sharp phase transition. The explanation might be the source of chitosan. Chitosan derived from crab or shrimp shell is α-chitosan chain arranging in an anti-parallel direction. This arrangement causes hydrogel with the sharpness of transition. On the contrary, squid chitosan is β-chitosan chain arranging in a parallel direction which caused a looser network forming of hydrogels (Peesan et al., 2003; Rinaudo, 2006) and a lower the value of sharpness of transition. When comparing between chitosan derived from crab and shrimp shell, although these two sources of chitosan are α-chitosan chain, but the rheological study showed different result in the value of sharpness of transition. The reason of this difference is needed to further clarification.

From our study, the increasing the proportion of starch (3:2 and 2:3 of chitosan:starch ratio) resulted in the precipitation of the blended polymers solution. This precipitation might be due to the interference of starch on the glycerol moiety of GP for coating on the chitosan chains. Thus chain to chain aggregation of non-coated chitosan chains easily occurred.

Focusing on the *in vitro* degradation, lysozyme was used to evaluate the enzymatic degradation of the developed C/S/GP hydrogel. Generally, lysozyme is present in certain human body fluids (Varum et al., 1997). The lysozyme in cartilage is derived from the surrounding fluids which come in contact with the matrix, in the hypertrophic zone of epiphysial cartilage. As the developed hydrogel was mainly consisted of chitosan, we assumed that the weight loss of the C/S/GP and C/GP hydrogels in lysozyme solution was mainly by breakage of the glycosidic bonds of the chitosan molecules. We found that the degradation rate of the C/S/GP hydrogel tended to be higher than that of the C/GP hydrogel. This phenomenon might possibly result from the formation of relatively looser polymeric network of the C/S/GP hydrogel, as confirmed by SEM. As the looser polymeric network and larger pore size increased accessability of lysozyme through the hydrogel matrix, resulting in a higher degradation rate.

Water absorption is an important parameter in the development of hydrogels as the degree of hydration directly influences their surface properties, mechanical properties, permeability of nutrients and hence cell growth. The results obtained from the study indicated that the hydrogel consisting of chitosan, starch and β-GP (C/S/GP) had greater water absorption than that of the hydrogel consisting of chitosan and β-GP (C/GP). This could imply a hydrophilic property of starch in allowing water absorption (Wang et al., 2007). Again, the blending of corn starch into the hydrogel system resulted in the formation of the relatively looser hydrogels which provided the larger pore size of the gel network for water penetration and hence water absorption of the C/S/GP hydrogel.

The effect of the C/S/GP hydrogel on chondrocytes was investigated in this study. The monolayer culture conditions of chondrocytes are well known to undergo a gradual dedifferentiation characterized by a change in their spherical shape into a fibroblastic appearance (Glowacki et al., 1983) and downregulation of type II collagen expression, but up-regulation of type I collagen expression (Schnabel et al., 2002). However, dedifferentiation can be minimized by using 3D culture scaffolds. Of these, hydrogels are among the most promising alternatives since these materials can provide a temporary support structure during the repair process. As a consequence of these data, we investigated whether our

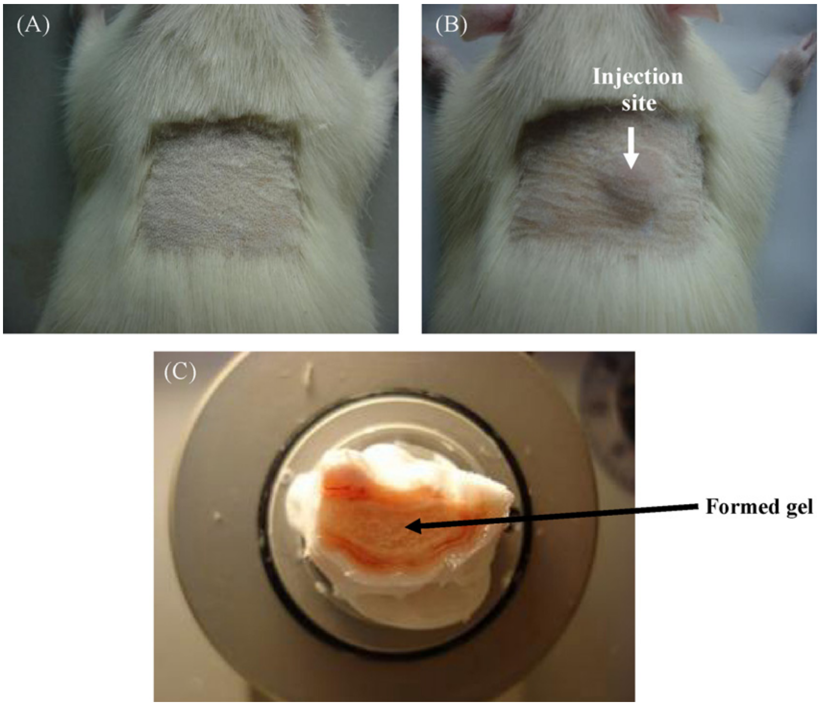

Fig. 10. *In vivo* formation of gel after subcutaneous injection of the C/S/GP hydrogel on the back of Sprague–Dawley rat: before (A) and after 1 day (B) of administration. Cryotome sectioning (C) showed localization of the gel to the injection site.

developed hydrogels could maintain a chondrocyte-specific gene by examining expression of transcripts coding for collagen (type I and II). From XTT assay, chondrocytes seeded and cultured on the C/S/GP hydrogels showed lower proliferation after cultivating for 7 days. However, the inverted microscopy and SEM observation showed that the chondrocytes cultured on the developed (C/S/GP) hydrogel maintained the spherical morphology. The spherical morphology of chondrocyte would limit the rate of mitosis and/or proliferation (Buckwalter and Mankin, 1998). For the chondrocytes cultured on the plastic plate, a rounded-polygonal shaped of freshly-isolated rat chondrocytes became gradually elongated and fibroblastic in appearance during cultivation, which confirmed by light microscopy and SEM observation. This cell characteristic could rapidly expand, resulting in higher number of cell population.

Freshly-isolated cells expressed type II collagen which could be confirmed by RT-PCR analysis. After monolayer cultivation for 7 days, chondrocyte cells cultured in plastic plate (2D condition) expressed high level of type I collagen transcripts. This suggested that these cells lost of chondrocyte phenotype. Interestingly, when chondrocytes were cultured within the constructed C/S/GP hydrogel (3D condition) for 14 days, they could maintain original phenotype by expression of type II collagen which was similar to those observed in primary cell. Additionally, immunofluorescence staining of chondrocytes cultured within the constructed hydrogel exhibited the expression of type II collagen protein at both intracellular and extracellular after cultivation for 7, 14 and 21 days. It seems therefore that the developed C/S/GP hydrogel exhibited a convenient 3D vehicle for stabilizing chondrocyte cells into a normal phenotype and function.

The ability to rapid gel formation after injection through a needle is important for an injectable hydrogel system because slow gelation causes delocalized gel formation due to diffusion of the gel precursors away from the injection site (Gupta et al., 2006). Our study *in vivo* indicated that the gel was rapidly formed and localized to the injection site. This can minimize undesired leakage of the biomaterials to be encapsulated to the surrounding tissues and compromise the therapeutic outcome.

5. Conclusions

This study reports the potential of thermosensitive hydrogels prepared from chitosan derived from crab shell, corn starch and β-GP for application in cartilage tissue engineering. The system showed the ability to permit the raising of pH of chitosan solution to neutral values and provide sharp transitions from the liquid state to semisolid like-gel upon heating to $37 \pm 2\,^{\circ}$C. We found that low amounts of starch in the chitosan system (chitosan to starch, 4 to 1) could reduce the gelation temperature of the system to close to $37\,^{\circ}$C. In addition, the presence of starch in the system could increase the water absorption and average pores size of the hydrogels. The hydrogel could degrade in 0.02 mg/ml lysozyme with slow rate during 56 days of incubation. Importantly, hydrogel we developed showed rapid formation and localization at the injection site and had a potential to stabilize chondrocytes which was characterized by the expression of type II collagen mRNA and protein. This feature makes the C/S/GP hydrogel potentially applicable as an injectable scaffold for cartilage tissue regeneration.

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