Research Journal of Biological Sciences 6 (9): 480-486, 2011
ISSN: 1815-8846

An Injectable Hydrogel for Cartilage Engineering: Physiochemical Properties and Biocompatibility with Human Adipose-Derived Stem Cells

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Abstract: The thermosensitive hydrogel for cartilage tissue engineering was developed by blending Chitosan (C) and Starch (S) with β-Glycerol Phosphate (GP). This C/S/GP hydrogel showed its liquid state at room temperature and gel state at raised temperatures. Scanning Electron Microscope (SEM) images revealed interconnected pore structure having pore sizes in the range of 30-120 μm. The material gradually degraded by 44.7 and 57.9% within 8 weeks in Phosphate Buffer Saline (PBS) and PBS containing 1 mg mL^{-1} lysozyme, respectively. The biocompatibility of C/S/GP hydrogel was initially evaluated by cultivating with Adipose-Derived Stem Cells (ADSCs). The SEM observation showed superior structure of the C/S/GP hydrogel with cells compared to hydrogel without cells. Cell adhesion and cell proliferation were also demonstrated by using SEM image and XTT assay, respectively. This study shows that the C/S/GP hydrogel is potentially applicable as an injectable scaffold for further study in cartilage tissue regeneration from ADSCs.

Key words: Adipose-derived stem cells, cartilage tissue engineering, chitosan, injectable hydrogel, thermosensitive hydrogel

INTRODUCTION

Articular cartilage plays role in reducing joint friction at extremities of long bone by covering their apposing surfaces. It is well established that articular cartilage has a poor regenerative capacity when it gets damaged due to the lack of blood supply or lymphatic drainage (Hunziker, 2002). The structure of articular cartilage which has abundant extracellular matrix also prevents migration of progenitor cells to the damaged site (Temenoff and Mikos, 2000). Furthermore, chondrocytes which are the major type of cells found in the cartilage are mature cells that have low mitogenic and metabolic activities. From these reasons, limitation of cartilage repair becomes a challenging problem in clinical treatments of osteoarthritis or other lesions of the articular cartilage.

Osteoarthritis (OA) is the most common arthritic diseases which may result from many causes such as trauma or degeneration. Patients with OA will suffer from joint pain, reduced or disturbed function and variable degrees of inflammation (Hunziker, 2002; Magne *et al*., 2005; Martel-Pelletier and Pelletier, 2010). Therefore, the symptoms can be alleviated with analgesics and Non-Steroidal Anti-Inflammatory Drugs (NSAIDs). However, these medications may not be effective in the advanced stage of knees with OA which usually have full-thickness articular defect. Under this condition, the treatment of choice is usually a replacement arthroplasty. Nevertheless, for smaller focal articular defects, different management options are proposed to enhance healing potential of articular cartilage lesions such as Autologous Chondrocyte Implantation (ACI).

Although, ACI has some advantages for instance pain relief, slow down of disease progression and delayed joint replacement surgery. There are problems from limitation of available source of chondrocytes and the donor site morbidity (O'Driscoll, 1998). Therefore, better strategies for cartilage regeneration are still needed in clinical setting at present. Cartilage tissue engineering is currently a very interesting subject. The concept is to combine varieties of cells and growth factors into biocompatible and biodegradable scaffolds in order to generate complete cartilage tissue (Chung and Burdick, 2008). Scaffold in cartilage engineering can be made from several materials both natural and synthetic polymers (Nesic *et al*., 2006; Vinatier *et al*., 2009) and their structure can be categorized into hydrogels, sponges and meshes (Chung and Burdick, 2008). Hydrogels which are

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polymeric networks and exhibit high water content possess superior properties among them. Firstly, the high water content of hydrogels is close to that found in cartilage and therefore mimics the three-dimensional environment of cells in cartilage (Sontjens *et al*., 2006; Vinatier *et al*., 2009). Secondly, hydrogels can be injected into the defect site without the need of highly invasive procedure. Finally, they will integrate better with the surrounding host cartilage by conforming with the defect (Magne *et al*., 2005). To improve gel retention at the defect site, thermosensitive hydrogel is deemed appropriate, since it would retain its liquid state at room temperature and turn into gel at raised temperatures.

Thermosensitive hydrogels can be synthesized from several polymers including chitosan (Klouda and Mikos, 2008). The thermosensitive chitosan-based hydrogel is developed by adding polyol salts such as β-Glycerophosphate (GP) into the Chitosan (C) solution (Chenite *et al*., 2000).

However, the C/GP system generally exhibits gel state at high concentration with slow sol-gel transition and low flexibility of the resultant gel. These properties limit its applicability as an injectable device and for cell delivery. Ngoenkam *et al*. (2010) developed an injectable hydrogel that consisted of Chitosan (C), gelatinized Starch (S) and β-GP. They found that the C/S/GP system exhibited the sol-gel transition at 37±2°C with low viscosity in liquid state and less gelation time. Furthermore, the C/S/GP hydrogel also showed ability to sustain normal chondrocyte functions. There are many drawbacks in using chondrocytes as a cell source in tissue engineering, the use of mesenchymal stem cells is then acknowledged as a better solution.

Mesenchymal Stem Cells (MSCs) are adult stem cells traditionally found in the bone marrow. However, they can also be isolated from other tissues including adipose tissue. Although, Bone Marrow-derived Stem Cells (BMSCs) are normally used as standard source of MSCs, Adipose Tissue-Derived Stem Cells (ADSCs) received more attention. Currently, these are actively researched in clinical trials for treatment in a variety of diseases.

Many advantages of ADSCs over BMSCs have been reported. ADSCs can be maintained *in vitro* for extended periods of time (up to passage 15) with stable population doublings and low senescence levels and can retain their chondrogenic differentiation potential up to 175 days (Zuk *et al*., 2001). Besides, lipoaspiration technique to obtain ADSCs from subcutaneous fat tissue is a minimally invasive procedure and the yield of MSCs in adipose tissue is >500 folds found in bone marrow (Fraser *et al*., 2006; Mizuno, 2009). There have been many reports since 2001, confirming chondrogenic differentiation potential of ADSCs both *in vivo* and *in vitro* (Zuk *et al*., 2001; Erickson *et al*., 2002; Strem *et al*., 2005; Schaffler and Buchler, 2007; Cui *et al*., 2009; Im *et al*., 2011).

ADSCs are therefore used as the cell source for cartilage tissue engineering instead of BMSCs in this study. The objective is to investigate the physical properties of the chitosan-based thermosensitive hydrogel and its biocompatibility with ADSCs.

MATERIALS AND METHODS

Raw materials used in the preparation were commercial products obtained from various sources. Chitosan (molecular weight of 10^5-10^6 Da, degree of deacetylation >90%) derived from crab shell was purchased from Bannawach Bio-Line Co., Ltd., Chonburi, Thailand. Corn starch (amylase: amylopectin, 30.23:69.77) was purchased from Tawan Chemical, Bangkok, Thailand. β-glycerol phosphate disodium salt and lysozyme from hen egg white were purchased from Fluka Chemie GmbH, Buchs, Switzerland. StemPro® Human Adipose Derived Stem Cells (hADSCs) were purchased from Invitrogen, New York, USA. Cell proliferation kit II (sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate, XTT) was purchased from Roche Diagnostics GmbH, Mannheim, Germany. Other materials for cell culture were purchased from Sigma-Alrich, Missouri, USA.

Preparation of thermosensitive hydrogel: The thermosensitive hydrogel was prepared by blending chitosan solution with pregelatinized starch solution in the presence of β-GP as previously reported (Ngoenkam *et al*., 2010). In brief, chitosan solution was prepared by dissolving 170 mg chitosan in 10 mL of 0.1 M HCl. Pregelatinized starch solution was obtained by dissolving 170 mg starch into 10 mL of deionized water and the resultant starch suspension was subsequently heated until gelatinization. Before mixing, both chitosan solution and pregelatinized starch solution were sterilized by autoclave and then blended together at the ratio of 4:1 (v/v).

Aqueous solution of β-GP, sterilized with 0.2 micron filter was carefully added to the chitosan/starch solution drop by drop to obtain the final concentration of 6.0% (w/v). The mixture was further stirred for 10 min and then incubated at 37°C for 20 min to exhibit the sol-gel transition of hydrogel or semi-solid hydrogel.

Characterization of thermosensitive hydrogel

Morphology: The morphology of semi-solid hydrogel was observed by using Scanning Electron Microscope (SEM,

Model 1455VP, Leo Electron Microscopy, Inc., Cambridge, UK). Small pieces of the hydrogel were lyophilized and then cut with a razor. The samples were mounted on to aluminium stubs and sputter-coated with gold (5 nm thickness). Micrographs were taken at 10 kV and 200× magnification.

***In vitro* enzymatic degradation:** The percent remaining of the semi-solid hydrogel after incubating in lysozyme solution (1 mg mL^{-1}) was used as the indicator of enzymatic degradation. In brief, the hydrogel was placed into the PBS (pH 7.4) at 37°C for 24 h or until equilibrium. The equilibrated hydrogel was weighed to give a wet weight (W_0) and then further incubated in PBS or PBS containing 1 mg mL^{-1} lysozyme.

The remaining weight (W_1) of samples was determined on day 3 of the 1st week and every week for 8 weeks. The percent of weight remaining was calculated using the equation:

$$\text{Weight remaining}(\%) = \left(\frac{W_1}{W_0}\right) \times 100$$

Biocompatibility of hADSCs and thermosensitive hydrogel: Cell compatibility was assessed in order to determine the quality of thermosensitive hydrogel for tissue engineering.

hADSCs expansion: hADSCs were outgrown and expanded in MesenPRO RS™ medium. When cells reached a confluence of 80-90%, they were trypsinized and subcultured. hADSCs in passage 5-7 were used in this study.

Morphology of hADSCs entrapped in thermosensitive hydrogel: The hydrogel solution was prepared under aseptic condition. hADSCs of 1×10^6 cells were mixed in 1 mL of the hydrogel solution. About 50 μL of the cell/hydrogel mixture were placed into a cylinder-shape mold (ϕ = 1 cm) and kept in a humidified, 5% CO_2 incubator at 37°C for 20 min to allow the semi-solid gel setting and then transferred to a 24 well plate containing 2 mL of the culture medium (High Glucose Dulbecco's Modified Eagle's Medium (DMEM-HG) supplemented with 10% Fetal Bovine Serum (FBS) and 1% antibiotics). After 1 week, the sample was washed with PBS and then fixed in 2.5% phosphate-buffered glutaraldehyde solution for 12 h at 4°C. After washing with PBS to remove the residual glutaraldehyde, the sample was lyophilized and then mounted on aluminium supports and sputter-coated with gold for SEM examination.

hADSCs viability and proliferation in thermosensitive hydrogel: The assay is based on the cleavage of the XTT to form a formazan dye by viable cells. The hydrogel solution was prepared under aseptic condition. hADSCs of 60,000 cells were mixed in 1 mL of the hydrogel solution. About 50 μL of the cell suspension was transferred to 96 well plate and allowed to undergo semi-solid gel setting at 37°C in humidified atmosphere containing 5% CO_2 for 20 min. Subsequently, 200 μL of the culture medium (DMEM-HG supplemented with 10% FBS and 1% antibiotics) was added into each well. The hADSCs entrapped in hydrogel were incubated at 37°C with 5% CO_2 and the culture medium was changed every 3 days. At a desired period of time (day 1 and 4 of the 1st week and every week for 2 weeks), the culture medium was replaced with 100 μL DMEM-HG and 50 μL of 1 mg mL^{-1} XTT solution. The plate was further incubated at 37°C with 5% CO_2 for 4 h. The viable cells were determined by measuring the optical density of the supernatant at 450 nm by a microplate reader. The data was expressed as the optical density and the study was performed in triplicate.

Statistical analysis: Student's t-test was used for comparison between the averages of two independent groups. Analysis of Variance (ANOVA) was used for multiple comparisons. The $p<0.05$ was considered as statistically significance.

RESULTS

Thermosensitive hydrogel morphology: The morphology of the hydrogel was shown in Fig. 1a-c. The hydrogel solution appeared in low viscosity fluid at room temperature (Fig. 1a). After being heated at 37°C for 20 min, the liquid solution turned into a semi-solid hydrogel (Fig. 1b). The structure of the cross-section of the semi-solid hydrogel was examined by SEM (Fig. 1c) to possess porous, honeycomb-like structures with interconnectivity. The pore size was in the range of 30-120 μm.

***In vitro* enzymatic degradation:** The degradation rate of semi-solid hydrogel in the presence or absence of lysozyme was evaluated by determining the remaining gel weight as a function of time (Fig. 2). During 8 weeks of incubation, remaining weights of hydrogel in both lysozyme and PBS solution gradually decreased. Although, degradation was faster in lysozyme solution than in PBS ($p<0.001$) there was still 42.1±1.3% remaining weight at the end at week 8.

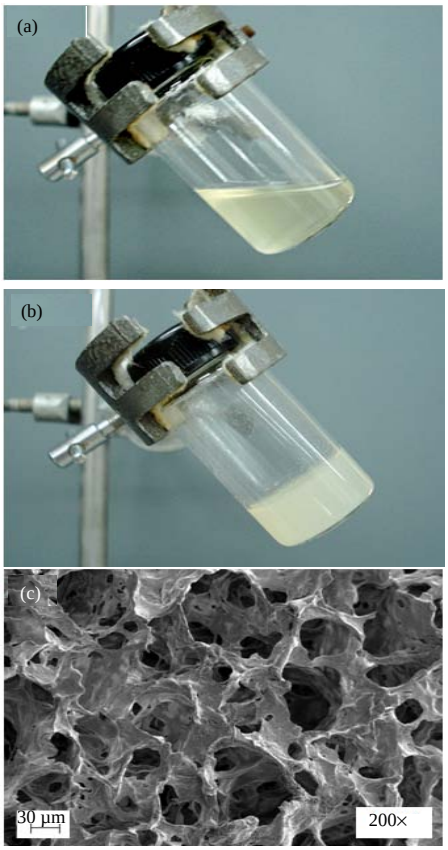

Fig. 1: Hydrogel morphology, appears as a liquid state at: a) room temperature; b) gel state at 37°C and c) SEM image of the cross-section of the semi-solid hydrogel at magnification of 200×

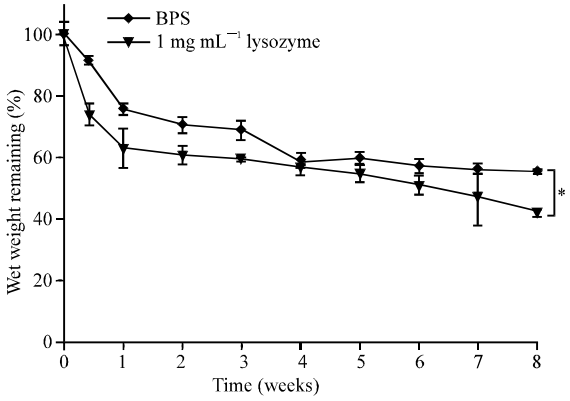

Fig. 2: The percentages of remaining weight of the semi-solid hydrogel incubated in PBS (pH 7.4) (black circle) or in lysozyme (1 mg mL^{-1} in PBS, pH 7.4) (white triangle) at 37°C as a function of time. Each point represents mean±SD (n = 3). Two-way ANOVA showed a significant difference in percent remaining weight between two treatments (*p<0.001)

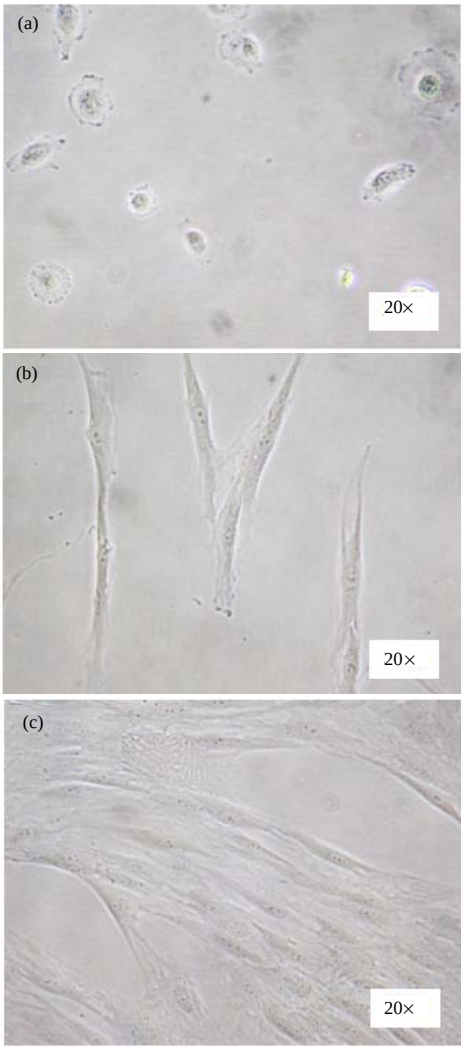

Fig. 3: Cell morphology imaged by inverted microscopy at magnification of 20× of hADSCs at: a) day 0; b) day 1 and c) day 7 of the culture time

hADSCs morphology: During the culture, hADSCs showing rounded-shaped cells before attaching to the plastic surface (Fig. 3a). After that the cells showed flat, spindle-shaped, fibroblast-like morphology (Fig. 3b). They reached around 60% confluence after 7 days of culture (Fig. 3c) and reached 80-90% confluence within 10-14 days.

Morphology of hADSCs entrapped in thermosensitive hydrogel: By using SEM, the morphology of hADSCs entrapped in the semi-solid hydrogel was evaluated. After 7 days of incubation, the acellular hydrogel showed degraded appearance (Fig. 4a) compared to the hADSCs seeded hydrogel (Fig. 4b). Cell attachment to the hydrogel was shown in Fig. 4c.

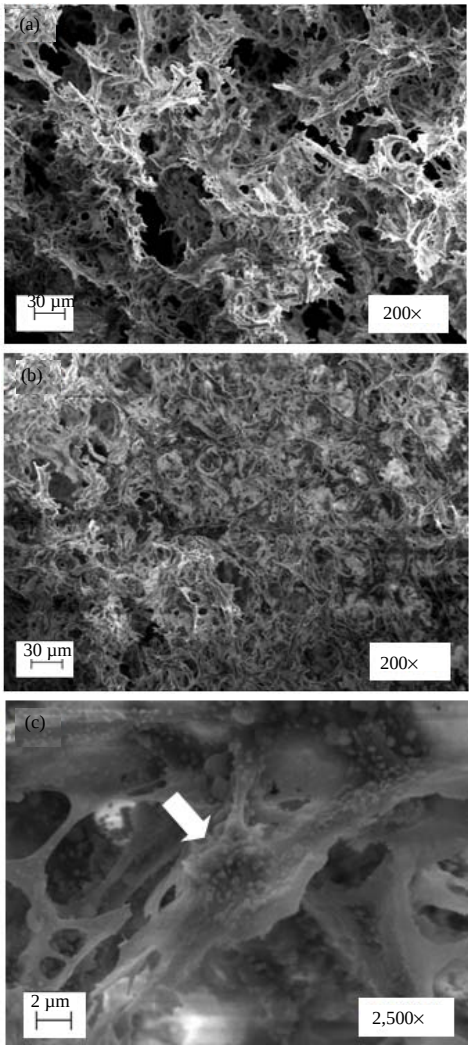

Fig. 4: SEM Photographs of: a) acellular hydrogel morphology and b, c) hADSCs/hydrogel morphology after 7 days of incubation (The white arrow indicates hADSCs attached to hydrogel)

Effects of thermosensitive hydrogel on hADSCs viability and proliferation: The biocompatibility of hADSCs and thermosensitive hydrogel was determined using XTT assay. Values of the Optical Density (OD) correspond to cell viability which is at least partially, proportionally related to cell number. In comparison to day 1 of cultivation, the OD value of cells in hydrogel significantly increased ($p<0.05$) at day 4 and 7. In addition, the OD value was significantly higher ($p<0.001$) in day 14 compared to day 1 (Fig. 5). The OD value of cells cultured in hydrogel that increased as a function of time indicated that the thermosensitive hydrogel showed no toxicity and could support hADSCs proliferation.

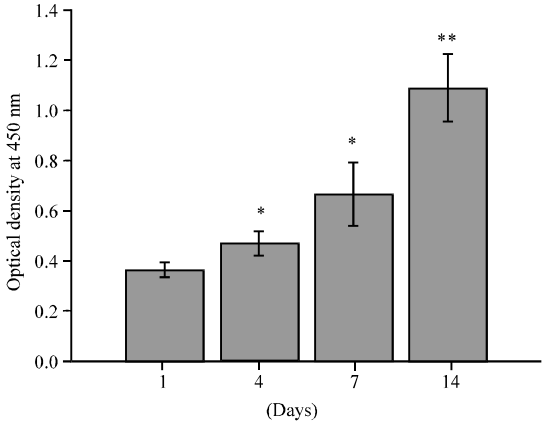

Fig. 5: The optical density at 450 nm of formazan dye derived from hADSCs entrapped in the hydrogel (Cell density = 60,000 cells mL^{-1}) at day 1, 4, 7 and 14 of cultivation. Each bar represents mean±SD (n = 3). Student's t-test showed a significant difference in day 4 and 7 (*$p<0.05$) and day 14 (**$p<0.001$) as compared to day 1

DISCUSSION

Owing to its avascular nature, the articular cartilage has very little capacity for spontaneous healing after damage. Although, many repair techniques have been proposed over the past four decades, none has successfully regenerated the long-lasting hyaline cartilage to replace the damaged tissue (Suh and Matthew, 2000). Recently, several tissue engineering techniques have been developed, including *ex vivo* cultivation of chondrocytes in biocompatible and biodegradable scaffold before being transplanted to the lesion. However, due to the limited number of donor cells available and the need of surgery, cultivation of adult mesenchymal stem cells (e.g., ADSCs) has attracted significant attention.

The scaffold is an essential factor in tissue engineering acting as a special cell-carrier material. It usually serves at least to allow cell attachment and migration to deliver and retain cells and biochemical factors or to enable diffusion of vital cell nutrients and expressed products. Different natural or synthetic and biodegradable or biocompatible materials have been investigated. Chitosan is one of the biomaterials that fulfills those criteria and has been widely used as a natural scaffold. Additionally, its structure is similar to natural occurring Glycosaminoglycans (GAGs) found in normal cartilage (Suh and Matthew, 2000). GAGs play an important role in regulating expression of the chondrocyte phenotype and in supporting chondrogenesis (Buckwalter and Mankin, 1998; Temenoff and Mikos,

2000). Therefore, application of chitosan as a component of the cartilage scaffold biomaterial is a reasonable approach to enhance chondrogenesis. Injectability is also important in clinical usages.

Chenite *et al*. (2000) showed that chitosan solution could be transformed from liquid state to semi-solid (gel) state when the temperature was increased to 37°C by blending with β-Glycerophosphate (β-GP). Ngoenkam *et al*. (2010) also showed that adding of 1.7% pregelatinized starch into the C/GP system could decrease the viscosity in the liquid state and shorten the gelation time. With low viscosity in liquid state, cells will be homogenously mixed and easily injected into the defect. The less gelation time also prevents flowing out of the hydrogel from the defect. In addition, porosity, biodegradability and biocompatibility are important properties for using as a scaffold (Hunziker, 2002).

High porosity and adequate pore size facilitate cell proliferation and diffusion of nutrients. The pore structure also strongly influences cell growth and mechanical properties. Therefore, porosity and interconnectivity of the scaffold are required properties in applications of various tissue engineering. Porous chitosan-based thermosensitive hydrogel (C/S/GP) is shown in Fig. 1c. It is formed by freezing and lyophilizing process. The water content in the hydrogel is frozen during the freezing process. Then, the ice crystal subsequently removed by lyophilization generates a porous material. The SEM image shows a homogeneous porous structure. The pore size ranging from 30-120 μm allows space both for cell attaching and permeating of nutrients and wastes.

Biodegradability is another essential factor. The scaffold should preferably be absorbed by surrounding tissues without the need of surgical removal. The rate at which degradation occurs has to coincide as much as possible with the rate of tissue formation. This means while cells are fabricating their own natural matrix around themselves, the scaffold can provide structural integrity within the body and will eventually break down leaving the neotissue. In neocartilage regeneration, it requires the scaffold support for at least 8 weeks to complete the defect (Im *et al*., 2011). Figure 2 shows that the remaining weight of the chitosan/starch hydrogel can sufficiently support the structure of the engineered tissue during neocartilage formation. Even with lysozyme, the remaining weight is still around 40% after 8 weeks. The gradual degrading property as a function of time also supports the remodeling process. This result was also supported by the previous study (Nair *et al*., 2011).

As for the biocompatibility; besides performing as a substrate to deliver the cells, the scaffold should not elicit any undesirable effects in those cells. Cell adhesion to biomaterials is also an important prerequisite for cell proliferation and differentiation. Compared to synthetic polymers, natural polymers show superiority by direct cell-scaffold interactions (Chung and Burdick, 2008). Chitosan is a biosynthetic derivative of chitin, the primary structural polymer in arthropod exoskeletons (Klouda and Mikos, 2008).

Structurally, chitosan is a copolymer of glucosamine and N-acetyl glucosamine with >50% of degrees of acetylation (Rinaudo, 2006). The potential of chitosan as a biomaterial is based on its cationic nature and high charge density in solution. Madihally and Matthew (1999) reported that the cationic nature of chitosan allowed for electrostatic interactions with negatively charged species. These ionic interactions may serve as a mechanism for retaining and recruiting cells, growth factors and cytokines within a tissue scaffold. Consequently, *in vitro* cytotoxicity of a biomaterial is the initial step in a biocompatibility study. In the present study, cell adhesion and XTT assay were respectively used as indicators for qualitative and quantitative analyses.

Figure 4 shows cell morphologies and cell-material interactions on the polymer surface. This demonstrates that the C/S/GP hydrogel possesses the cell adhesion property. Furthermore, hADSCs can also proliferate well in the C/S/GP hydrogel according to the XTT assay (Fig. 5).

The number of cells increased significantly over days 4 and 7 or 14 as compared to day 1 of the culture. Similarly, Ji *et al*. (2010) reported that cell proliferation in a chitosan-based hydrogel was significantly increased during 5 days of the culture.

CONCLUSION

In this study, the results demonstrate proper physical properties of the thermosensitive C/S/GP hydrogel and its possibility as a scaffold for tissue engineering. Further investigations are now in progress to evaluate potential application of the C/S/GP hydrogel as a biomaterial in cartilage tissue engineering.

ACKNOWLEDGEMENT

This study was supported by grants from the Faculty of Medicine of Khon Kaen University in Thailand.

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