CHAPTER IV
MECHANICAL PHYSICAL AND BIOLOGICAL PROPERTIES OF SCAFFOLDS OF α-CHITIN WHISKERS REINFORCED HYALURONAN-GELATIN NANOCOMPOSITES

4.1 Abstract

Tissue scaffolds made of natural derived polymers present poor mechanical properties which limit their use in regenerating high strength tissues. This study aimed to develop a novel tissue scaffold from α-chitin whiskers reinforced hyaluronan-gelatin nanocomposites by the freeze-drying method. Scaffolds were fabricated with six different weight ratios of α-chitin whiskers to polymer i.e., 0, 2, 5, 10, 20, 30% and were studied for their physical, mechanical, and biological properties. The as-prepared scaffolds exhibited interconnected porous structure with average pore size of 150 μm. At any weight ratio of α-chitin whiskers added, the internal architecture and water absorption capability of the scaffolds were not affected. 2% of α-chitin whiskers strengthened the scaffold by increasing tensile strength twice over the other groups. While 20-30% of α-chitin whiskers improved thermal resistance and biodegradation, scaffolds with 10% of α-chitin whiskers could promote proliferation of SaOS-2 cells the best. In order to enhance mechanical physical or biological properties, scaffolds must be reinforced with a distinct weight ratio of the α-chitin whiskers.

(**Key-words**: bone scaffolds; freeze-drying; α-chitin whisker)

4.2 Introduction

Tissue engineering aims to generate biological substitutes for the lost or defective native tissues. The process of tissue regeneration either in vitro or vivo desires biomaterial scaffold to serve as a template for cell attachment and tissue development (Griffith, 2000; Rezwan *et al.*, 2006). Scaffolds play roles in

determining the transportation of nutrition, metabolites, and regulatory molecules into and out of the cells. With a specified chemistry, scaffolds may also have an important role in signaling (Vunjak-Novakovic, 2006).

In order to achieve iso-morphous tissue replacement, scaffolding material should be biocompatible and biodegradable. Synthetic polymers like polyglycolic, polylactic, or polylactic-glycolic are extensively used due to their chemistries and properties that are able to be tailored such as molecular weight, block structure, mechanical properties, rate of degradation and the crosslinking method (Mathew, 2002; Wu and Ding, 2004; Mercier *et al.*, 2005; Wu and Liao, 2005). Alternatively, natural polymers like alginate, gelatin or collagen are outstandingly biocompatible and biodegradable. They are highly hydrophilic and present certain extracellular-matrix liked structures which provide the naturally replicated environment and eventually enhance cell growth and cell (Alberts *et al.*, 2002; Drury and Mooney, 2003). However, with drawbacks of mechanical property, natural polymer is constrained for the regeneration of a high strength tissue like bone or cartilage.

Since the maintenance or the rate of decline of the mechanical properties of the scaffold may be critical for its efficacy, apart form the modulation of the stress-strain environment at the cellular and tissue levels, improvement of natural polymer made scaffolds have been extensively studied through crosslinking (Lee *et al.*, 2000; Shu *et al.*, 2003; Liu *et al.*, 2005; Segura *et al.*, 2005), blending (Liu *et al.*, 1999; Wong *et al.*, 2001) and modifying chemical structures (Prestwich *et al.*, 1998; Shu *et al.*, 2004). And it is our interest that making a composite of natural polymers might be the solution to achieve a scaffold with desirable properties in term of physical, mechanical and biological aspect. Therefore, scaffolds were fabricated with α-chitin whisker reinforced hyaluronan-gelatin nanocomposite and studied for their pertinent properties.

Hyaluronan is a linear polysaccharide polymer consisting of a repeating disaccharide that is N-acetyl-D-glucosamine and glucuronic acid linked by a β 1-4 glycosidic bond. The disaccharides are linked by β 1-3 bonds to from a high molecular weight hyaluronan chain (Lapcik *et al.*, 1998). Hyaluronan is found in various types of living tissues. It is a member of a group of similar polysaccharides

which have been termed "connective tissue polysaccharides", "mucopoly-saccharides", or "glycosaminoglycans" .

The major advantages of hyaluronan are the size-dependent biological properties which are excellent to be employed in tissue engineering. Hyaluronan carries out many functions in the extracellular matrix like being a cells supporting structure, controls cell adhesion, proliferation, differentiation and cell mobility (Kikuchi *et al.*, 2001). It is also a medium in which the reaction between binding protein, proteglycan and other biomolecules like growth factor occurs (Alberts *et al.*, 2002). Moreover, hyaluronan is a lubricating material between bone joints (Mikos *et al.*, 2004). Blending of hyaluronan with other biomaterials was found to demonstrate better scaffolding properties than either material alone (Oerther *et al.*, 1998; Liu *et al.*, 1999; Oerther *et al.*, 1999; Hoffman, 2002).

Gelatin, the denatured type of collagen, is commonly used in pharmaceutical and medical application due to its biocompatibility and biodegradability. Structurally, gelatin is a heterogeneous mixture of single or multiple stranded polypeptides and their oligomers each of which contains about 300-4,000 amino acid (Tabata and Ikada, 1998; Young *et al.*, 2005; Chaplin, 2007) and comprises glycine, proline and 4-hydroxyproline the most. Gelatin has been widely studied in the field of controlled drug release and tissue engineering. Blending of chitosan gelatin and hyaluronan at a certain proportion was found to resist to enzymatic degradation and promote fibroblasts cell migration and proliferation (Liu *et al.*, 2004). Scaffold made of gelatin-chondroitin-hyaluronan blends was successful in the culture of chondrocytes for 5 weeks in which extracellular matrix and type II collagen were produced (Chang *et al.*, 2003).

Chitin whisker, the fiber of high-purity single crystals, prepared by disrupting and eliminating amorphous regions of chitin under acid hydrolysis (Rinaudo, 2006; Samir *et al.*, 2008), was found to be the effective strengthening filler in many studies. Over 10% by weight of chitin whisker increased significantly the shear modulus of poly(S-co-BuA) (Paillet and Dufresne, 2001) and Young modulus of poly(carpolactone) (Morin and Dufresne, 2002) or soy protein isolate (Lu *et al.*, 2004). Such reinforcement may be ascribed to whisker's highly ordered structure,

high strength of crystallinity, and the intermolecular hydrogen bonding between fillers (Paillet and Dufresne, 2001; Morin and Dufresne, 2002; Samir *et al.*, 2008).

The present contribution aimed to fabricate a novel scaffold from α-chitin whisker reinforced hyaluronan-gelatin nanocomposite which possesses good properties for bone tissue regeneration. The study focused on the correlation between the content of chitin whisker and the ensuing properties of scaffold in term of physical, mechanical and biological features.

4.3 Experimental section

4.3.1 Materials

Chitin powder from crab shells (MW 4x105) was purchased from Fluka (Switzerland). Hyaluronan (MW 1.35x106) was purchased from Coach Industries Inc. (Japan). Gelatin from porcine skin (type A, Bloom no.170-180) was purchased form Fluka (Switzerland). 1-ethy-3-(3-dimethylaminopropyl)carbodiimide (EDC) was purchased form Fluka (Switzerland). Hydrochloric acid (HCl) (37% w/w, AR grade) was purchased from Lab-Scan (Thailand). All other chemical agents were of analytical grade and used without further purification.

4.3.2 Preparation of α-chitin whiskers

α-chitin whiskers were prepared by hydrolyzing chitin powder with 3 N hydrochloric acid at 120°C for 6 h under vigorous stirring and refluxing. The ratio of 3 N HCl to α-chitin powder was 30 mlg^{-1}. After acid hydrolysis, the suspension of chitin whiskers was diluted with distilled water and further centrifuged at 10,000 rpm for 5 min and decanting in triplicate. Then the suspension was transferred to a dialysis bag and dialyzed in running water for 2 h and later in distilled water for 2 days. Dispersion of chitin whiskers was completed by 5-min ultrasonification treatment for every 40 ml aliquot. The solid fraction of the as-prepared whisker suspensions was gravimetrical measured to be 1.43 wt%. It was stored at 4°C until used.

4.3.3 Preparation of scaffolds of α-chitin reinforced hyaluronan-gelatin nanocomposites

The hyaluronan and gelatin powder (1:1 weight ratio) was first mixed and dissolved in deionized water at 50°C to obtain the solution of hyaluronan-gelatin mixture (hereafter, HA-Gel) at a fixed concentration of 2 wt%. The solution was left to cool down to room temperature before various amounts of the as-prepared chitin whiskers suspension were added into the solution at 6 different chitin whisker to HA-Gel weight ratios (i.e., 0, 2, 5, 10, 20, 30% w/w). The mixtures were continuously stirred until homogenized. Afterward, 1 mmol of EDC was added and the mixtures were further stirred for 2 h to crosslink the polymer. A volume of the mixtures was poured into polypropylene dishes to obtain molding samples of two different shapes and dimensions, freezed at -40°C for 24 h and lyophilized at -50°C for 24 h. The obtained cylindrical scaffolds were ~10 and ~2 mm in diameter and height respectively while the disc-shaped scaffolds were ~1 mm thick which were later cut into a desired shape and size for the test of tensile strength. Scaffolds were kept in a desiccator until use.

4.3.4 Characterization

4.3.4.1 Microstructure observation and pore size determination

One cylindrical scaffold was randomly selected from each group of samples. It was cut into pieces along both the longitudinal and the transverse directions. The cut pieces were mounted on copper stubs, coated with gold using a JEOL JFC-1100 sputtering device, and observed for their microscopic structure using JEOL JSM-5200 scanning electron microscopy (SEM). To determine the pore size, 50 pores for each of the cross and the longitudinal sections (i.e., 100 pores in total) were directly measured from the SEM images, using a UTHSCSA Image Tool version 3.0 software. The average values for all of the scaffolds investigated were calculated.

4.3.4.2 Mechanical properties

For the tensile test, specimens with the dimension of 50x5x1 mm^3 were cut form the disc-shaped scaffolds. Tensile strength, Modulus of elasticity and Elongation at break of the scaffolds were determined in their dry state at room

temperature with a Lloyd universal testing machine (LRX-Plus model) using a 10 N load cell. The gauge length was 30 mm and the crosshead speed was 10 $mm.min^{-1}$. The test was carried out in pentuplicate

4.3.4.3 Water absorption capability

The dry cylindrical scaffold specimens were first weighed, and individually immersed in deionized water at room temperature. At a given time point, the specimens were taken out, blotted on a glass plate which was set at ~45° from a horizontal baseline for 5 s to remove excessive water, and immediately weighed. The amount of water absorbed in specimens was determined according to the following equation:

$$\text{Water absorption } (\%) = [(W_w - W_d)/W_w] \times 100,$$

where W_d and W_w are the weight of the specimens before and after submersion in the water, respectively. The experiment was carried out in pentuplicate and measurements were carried out at different time intervals within a period of 24 h.

4.3.4.4 In vitro degradation

The study of in vitro degradation was performed in 3 experiments. At first, cylindrical scaffolds were individually placed in 10 mM phosphate buffer saline solution (PBS; pH 7.4) and left over at room temperature without shaking for 24 h. For the other two tests, cylindrical scaffolds were individually placed either in PBS; pH 7.4 or in bacterial collagenase at a concentration of 373 ng/ml, incubated at 37°C in a shaker bath and shaken at 70 rpm for 24 h. After the given time, all scaffolds were removed form the media, freezed at -40°C for 24 h and lyophilized at -50°C for 24 h. The remaining weight of the dried scaffolds was determined as the weight ratio based on the initial weight of the scaffolds, according to the following equation:

$$\text{Remaining weight } (\%) = W_t/W_o \times 100,$$

where W_o is the initial weight of the scaffolds in their dry state and W_t is the weight of the scaffolds after the degradation assay in their dry state. The experiment was carried out in triplicate.

4.3.4.5 Infrared (IR) spectroscopic analysis

A Thermo Nicolet Nexus® 670 Fourier-transformed infrared spectrophotometer (FT-IR) was used to investigate chemical functionalities of the as-prepared scaffolds by the KBr disk method. One cylindrical scaffold was randomly selected from each group of samples and detected for the FT-IR spectra over 32 scans with a resolution of 4 cm^{-1}.

4.3.4.6 Thermal properties

One cylindrical scaffold was randomly selected from each group of samples and investigated for their thermal properties. Glass transition temperature (Tg) of the samples were detected using the Differential Scanning Calorimeter (DSC7, Perkin-Elmer). Each sample was heated from 25-80°C at a heating rate of 10°Cmin^{-1}. Alternatively, thermal stability and degradation temperature (Td) of the samples were detected using the Thermogravimetric Analyzer (TGA7, Perkin-Elmer). A sample of about 2 mg was placed in a platinum pan and scanned from 30 to 600°C at a heating rate of 10°Cmin^{-1} in nitrogen atmosphere. Data analysis was performed with the TA Instrument Universal Analysis 2000 version 4.2E software (TA Instrument-Waters LLC).

4.3.4.7 Cytotoxicity test

Human osteoblast cell line (SaOS-2) were cultured as a monolayer in α-minimum essential medium (α-MEM; Sigma-Aldrich, USA), supplemented by 10% fetal bovine serum (FBS; Biochrom, Germany), 1% L-glutamine (Invitrogen, USA) and a 1% antibiotic and antimycotic formulation containing penicillin G sodium, streptomycin sulfate, and amphopericin B (Invitrogen Corp.)]. The cells were maintained at 37°C in a 95% humidified atmosphere containing 5% CO_2 and passaged once every 3-4 d.

Only the cylindrical scaffolds containing 0% and 30% of α-chitin whiskers were used in these studies. Cytotoxicity of the as-prepared scaffolds was evaluated by the indirect method, using SaOS-2 as the reference cells. First, the extraction media were prepared by immersing the scaffold specimens in 500 μL of serum-free medium (SFM; containing MEM, 1% L-glutamine, 1% lactabumin, and 1% antibiotic and antimycotic formulation) for 24 h. Each of these extraction media

was later used in the indirect cytotoxicity evaluation. SaOS-2 were cultured in wells of a 24-well culture plate at 4×10^4 cells/well in serum-containing MEM for 16 h to allow the cells to attach on the plate. The cells were then starved with SFM for 24 h, after which time the medium was replaced with an extraction medium. After 24 h of cell culturing in the extraction medium, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was carried out to determine the viability of the cells. The experiment was carried out in quadruplicate.

The MTT assay is based on the reduction of the yellow tetrazolium salt to purple formazan crystals by dehydrogenase enzymes secreted from the mitochondria of metabolically active cells. The amount of purple formazan crystals formed is proportional to the number of viable cells. First, each culture medium was aspirated and replaced with 500 μL/well of MTT solution at 0.5 $mg \cdot mL^{-1}$ for a 24-well culture plate. Secondly, the plate was incubated for 1 h at 37°C. The solution was then aspirated and 1 mL/well of dimethylsulfoxide (DMSO) containing 125 μL/well of glycine buffer (pH 10) was added to dissolve the formazan crystals. Finally, after 5 min of rotary agitation, the absorbance of the DMSO solution at 540 nm was measured using a Thermospectronic Genesis10 UV/Visible spectrophotometer.

4.3.4.8 Cell attachment and proliferation

A primary evaluation for cell attachment was carried out by a direct morphological observation of SaOS-2 that were seeded on the surface of the scaffold specimens. Only the cylindrical scaffolds containing 30% of α-chitin whiskers were used in the study. Specifically, the cylindrical scaffold specimens were put in wells of a 24-well culture plate and sterilized with 1 mL of 70% ethanol for 30 min. They were then washed with sterilized DI water twice and later immersed in α-MEM overnight. The SaOS-2 were then seeded on the surfaces of the specimens at 4×10^4 cells/specimen in a minimum volume of the culture medium and were allowed to attach on the surfaces for 3 h prior to the addition of 1.5 mL/well of the culture medium. The cells were cultivated at 37°C in a humidified atmosphere containing 5% CO_2 for 1 and 7 days, after which time morphology of the attached cells was observed by SEM. After removal of the culture medium, the cell-cultured

scaffold specimens were rinsed with PBS twice and the cells were then fixed with 3% gluteraldehyde solution, which was diluted from 50% glutaraldehyde solution (Electron Microscopy Science, USA) with PBS for 30 min. The specimens were then dehydrated in ethanol solutions of varying concentration (i.e., 30, 50, 70, 90, and 100%, respectively) for ~2 min at each concentration, further dried in 100% hexamethyldisilazane (HMDS; Sigma, USA) for 5 min, and later dried in air after the removal of HMDS. Finally, the specimens were mounted on SEM stubs, coated with gold, and observed by SEM. The examinations were performed on 3 randomly-selected scaffold specimens.

For the study of cell proliferation, the examinations were performed on 4 randomly selected specimens from each group of cylindrical scaffolds. The SaOS-2 were seeded and cultivated with the same procedure as in the study of cell attachment. The number of the proliferated cells was determined by the MTT assays on the 1, 24, 28 and 72 h of cell cultivation.

4.3.5 Statistical analysis

Data were analyzed using the SPSS software version 14.0 for window. Initially, the normal distribution was assessed by the Shapiro-Wilk test. The normal distribution data, representing the homogeneity of the variances, shown by the Levene's test, were then investigated by the one-way analysis of variance (ANOVA) with the Tukey HSD post hoc multiple comparisons. Otherwise, the Dunnett T3 would be applied if the data did not exhibit the homogeneity of the variances. For the data of which the normal distribution was absent but the variance was homogeneous, the Kruskal-Wallis H was applied. To compare the means between 2 data groups, the students' unpaired t-test was used. The significant level was indicated at $p < 0.05$ in any case.

4.4 Results and Discussion

4.4.1 Morphological appearance and sizes of α-chitin whiskers

The as-prepared chitin whiskers exhibited colloidal behavior in water. Protonation of amino groups of chitin in an acidic condition induced positive charges (NH_3^+) to be developed on the surface of chitin whiskers which consecutively

generated electrostatic repulsive force among crystals (Revol and Marchessault, 1993; Li *et al.*, 1996). However, at certain sites where protonation was not completely occurred, the hydrogen bonds between surfaces of chitin whiskers caused attractive force among them. The selected TEM images prepared from a dilute suspension of chitin whiskers (Figure 4.1), therefore, presenting both the individual and the partially aggregated crystallites. All chitin whiskers display the morphology of slender rods with sharp point on both ends. With the average width and length of 255 ± 55.77 and 30.65 ± 5.66 nm respectively, the aspect ratio, the average length to width ratio; L/d, of the as-prepared whiskers was calculated to be about 8. Figure 4.2 illustrated histograms presenting distribution of chitin whiskers' length and width. It is noticeable that the length and width of over 50% of chitin whiskers were between 203-277 and 27-35 nm respectively. These dimensions are in line with the reported values for chitin whiskers obtained from crab shells which were L=100-600, d=4-40 nm (Nair and Dufresne, 2003) and L=100-650, d=10-80 nm (Lu *et al.*, 2004).

4.4.2 Characterization of scaffolds of α-chitin whiskers reinforced Hyaluronan-gelatin nanocomposites

4.4.2.1 Microstructure observation and pore size determination

The as-prepared scaffolds presented the appearance of porous sponges which were light in weight. Their color varied from light to dark corresponded with the increasing amount of chitin whiskers added (Figure 4.3). The selected SEM images of the internal architecture both being viewed on the longitudinal and transversal sections are shown in Figure 4.4. As observed, a well-defined porous structure and the inter-pore connectivity were detected throughout the bulk on both sections which represents a perfect microstructure of a scaffold. For any given type of the scaffolds, there was no significant difference in the microstructure observed along both sections except the one with 30% chitin whiskers, which showed disruption of internal architectures on the longitudinal section. The ranges and average values of pore size of each scaffold specimen are shown in Table 4.1.

Pore size of all groups of scaffold was in the range of ~100-200 μm for both the longitudinal and transversal specimens of which the average value was about 150 μm. The increasing amount of chitin whiskers seems not to have

an effect on the average pore size. With an aim to compare the pore size between those two sections of each scaffold, the One Way ANOVA with Tukey HSD analysis was separately conducted within a single group of scaffold and illustrates the statistically insignificant difference. The analysis of pore sizes corresponds with the morphological appearance from the SEM. However, with the average pore size smaller than 200 μm, transportation of food, air circulation and the ingrowths of novel blood vessels through the as-prepared scaffold could be restricted. Inside the scaffold may lack of sufficient oxygen, the condition of hypoxia, which suits for the cartilage to generate but not for the bone (Boyan *et al.*, 1996; Karageorgiou and Kaplan, 2005). In order to achieve the scaffold with larger pore sizes, before sublimation scaffold should be frozen with a reduced cooling rate comparing to that had been used in this experiment. Since the fast cooling down induced lost of heat for ice crystallization and caused only the formation of tiny ice crystals, scaffolds with small pore size were obtained (Shapiro and Cohen, 1997; Kang *et al.*, 1999).

4.4.2.2 Mechanical properties

Mechanical properties of the as-prepared scaffolds were illustrated with tensile strength, modulus of elasticity and elongation at break as shown in Table 4.2.

As observed, statistic analysis presented the significant increase of the modulus of elasticity as the neat scaffold was reinforced with chitin whiskers from 2-30%. Nevertheless, an increase in the content of the chitin whiskers did not markedly affect to the modulus of elasticity as there was no corresponding tendency to be observed. The profile of the entire modulus of elasticity was in agreement with the percentage elongation at break. The neat scaffold has a significantly high percentage elongation at break comparing with those of the chitin whiskers reinforced samples which were not significantly different among each others. Considering the tensile strength, scaffold reinforced with 2% chitin whiskers demonstrated the significantly highest tensile strength whereas the others presented quite the same values as the neat scaffold. The result was in consistent with the study of Sriupayo et al. in 2005 which found that 2.96% of chitin whisker provided the highest tensile strength of the chitosan composite film. Reinforcing with 7.4, 14.8, 22.2 and 29.6% of chitin whisker reduced the tensile strength but at the insignificant

level comparing among each others (Sriupayo *et al.*, 2005). On the evidence available, modulus of elasticity and tensile strength of the HA-Gel scaffold reinforced with 2% chitin whiskers in this study were 1,000 and 100 times lower than those of bone which are 16.4 GPa, and 117.4 GPa respectively (Currey, 2004). Therefore, the mechanical property of the as-prepared scaffold still is a drawback and need to be further improved.

The result of this study illustrated that reinforcing with 2% chitin whiskers obviously decreased the elasticity and increased the tensile strength of the HA-Gel scaffold. However, the increasing amount of chitin whiskers up to 30% did not clearly improve mechanical properties of the scaffold. It is explicable that properties of the selected materials themselves and porosity of scaffold are the two factors responsible for the scaffolds' mechanical properties. Since the internal architectures and porosity among groups of the as-prepared scaffolds were insignificantly different, the obtained scaffolds' mechanical properties truly manifested the properties of the as-prepared chitin whisker reinforced HA-Gel composite.

4.4.2.3 Water absorption capability and in vitro degradability

The ability of the as-prepared scaffolds in absorbing water in a basic condition at room temperature within 24 h is graphically shown in Figure 4.5. All groups of the scaffolds demonstrated comparable water absorption within the first 60 m which was over 95% of the total weight of the wet scaffolds and was as high as a superabsobent (Park and Park, 1994). The result of this study was similar to that of Park et al. in 2002 in a collagen-hyaluronan sponge (Park *et al.*, 2002). Such high water absorption capability was a characteristic of hydrogel material like hyaluronan and gelatin which are very hydrophilic (Drury and Mooney, 2003). The scaffold, therefore, becomes a hydrated environment which could enhance the process of tissue regeneration by protecting cells and cellular products like peptide or DNA. Moreover, the hydrated environment also functions as a route for the nutrition to transport in and out of the cells (Hoffman, 2002).

From Figure 4.6, since all groups of the scaffolds presented similar water absorption values and profiles, i.e., water absorption gradually decreased for a longer submersion time, reinforcing the HA-Gel scaffold with chitin

whiskers hardly affect the ability of water absorption. This may be due to the interconnectivity of scaffolds porous microstructure, together with their hydrophilicity, encourages capillary reaction and absorbs a high quantity of water at a fast rate (Park and Park, 1994). Consequently, the peak of water absorption was observed within the first 15 m in all types of the scaffolds.

From the other standpoint, the high water absorption weakens the scaffold and accelerates degradation (Hoffman, 2002). The degradation of hydrogel occurs by hydrolysis, dissolution and enzymatic cleavage (Drury and Mooney, 2003). This study investigated the hydrolytic and dissolution degradation of the scaffolds in PBS over a submersion period of 24 h in both a static condition at room temperature and a dynamic condition, i.e., shaking at 70 rpm at 37 °C. The enzymatic degradation was also investigated in 373 ng/ml of bacterial collagenase which modeled the concentration of tissue collagenase in the synovial fluid of the patients with osteoarthritis (Maeda *et al.*, 1995; Holland *et al.*, 2005). The investigation was done in a dynamic condition by shaking the specimens at 70 rpm, at 37°C for 24h and results are shown in Figure 4.6.

Figure 4.6 shows the remaining weight of the scaffolds after having been immerged in PBS or collagenase for 24 h. As observed, the remaining weights were about 58-76% of the original when scaffolds were submerged in the static condition at room temperature without any remarkably relationship to the amount of the chitin whiskers. The hydrolytic and/or dissolution degradation, therefore, occurred within the first 24 h.

The study of degradation in PBS in the dynamic condition shows dissimilar results. Scaffolds' remaining weight was lesser than that in the static condition at the same amount of chitin whiskers. Thermal degradation of the hyaluronan molecules might occur at 37°C so that the remaining weights were only about 33-60% of the original (Lapcik *et al.*, 1998). Reinforcing with 20-30% of chitin whiskers significantly enhanced the resistance to such degradation comparing with the neat scaffold. Though there is a direct relationship between the remaining weight and amount of chitin whiskers, statistic analysis among the scaffolds with 5, 10, 20 and 30% chitin whiskers does not show any significantly difference.

In collagenase, degradation of the scaffolds were apparently much accelerated as the remaining weight of scaffolds after degrading was further decreased to be about 11-52% of the original. In addition to scaffolds agitation and the thermal degradation of the hyaluronan molecules, the scission of gelatin peptide bonds at glycine subunit by the collagenase was the main factor that hastened scaffold degradation (Park *et al.*, 2002). Over 5% by weight of the chitin whiskers significantly improved scaffold stability in the collagenase. The considerable reason was the crosslinking between chitin whiskers and the HA-Gel molecules which hinder collagenase to approach the site of HA-Gel chain scission (Vizarova *et al.*, 1995).

4.4.2.4 Infrared (IR) spectroscopic analysis

Figure 4.7 illustrates FT-IR spectra over the wave number range of 3500-500 cm^{-1} of the as-prepared scaffolds and the corresponding substrates. The absorption peak at 1656, 1547, 1450 and 1237 cm^{-1} characterizing amide bond (CO-NH) are observed in the spectrum of gelatin (Magoshi *et al.*, 1979; Coates, 2000; Park *et al.*, 2002), whereas those at 1412 and 1076 cm^{-1} representing carboxylate salts (symmetric stretching) and ester bond respectively are observed in the spectrum of hyaluronan of which repeating units are glucuronic acid and acetylglucosamine. The spectrum of chitin whisker is similar to that of the Hyaluronan since they both are polysaccharide. However, being crystallized structures, the chitin whisker presents the sharper and higher intensity spectra (Rinaudo, 2006) particularly at 1076 (ester bond), 1378 (carboxylate salts) 3267 and 3446 cm^{-1} (hydroxyl group) (Coates, 2000).

The chitin whisker additionally shows the characteristic amide I peak at which divided into 1621 and 1656 cm^{-1} and the amide II peak at 1556 cm^{-1} (Magoshi *et al.*, 1979; Nair and Dufresne, 2003). The 1621 cm^{-1} is the specific spectrum peak found in α-chitin whisker only, whiles the 1656 cm^{-1} representing the stretching of hydrogen bond between carbonyl group (CO) and the neighboring amine group (NH) of the intra-sheet chain which is also found for the amide bond of protein (Rinaudo, 2006).

For the crosslinked HA-Gelatin scaffold without chitin whisker added, the peak at 1650, 1550, 1412, and 1076 cm^{-1} are observed.

Crosslinking with the EDC generates both the amide and ester bond in the structure of the compound (Prestwich, 2001). The amide bond was formed between the carboxyl group of the gelatin and/or of the glucuronic acid in hyaluronan and amino group of the gelatin. The ester bond was established between such carboxyl group and the hydroxyl group of gelatin and/or of hyaluronan (Tomihata and Ikada, 1997; Sannino *et al.*, 2005). The mentioned reaction also occurred with the chitin whiskers as the higher intensity of the peak at 1378 and 1076 cm^{-1} is observed and the absorption shoulder at 3267 cm^{-1} is remarkably noticed when the content of chitin whisker is higher.

4.4.2.5 Thermal properties

The TGA thermograms of the as-prepared scaffolds are shown in Figure 8. All samples exhibited an identical profile of the weight change along the range of the testing temperature. The correspondingly specific temperature for a certain value of weight loss and the residual weight are determined and shown in Table 4.3, from which the characteristic temperatures for weight losses of 5%, 25% and 50% are T-5%, T-25% and T-50% respectively. As observed, at the temperature below 200°C, weight losses of all samples are lesser than 5% without any significant difference among groups. At the temperature around 210-230 °C, weight loss abruptly increases and reaches a highest rate at 245-250°C as signified by the presenting peaks of the corresponding derivative curves (not shown). When the temperature comes up around 260-360°C, weight losses are remarkably detected for their discrepancy among samples and are inversely related to the increasing amount of chitin whisker. Furthermore, considering specifically at the 25% and 50% weight loss, the temperature used for the samples reinforced with 10-30% chitin whiskers are higher than those with 2-5% chitin whiskers. Thermal stability of the as-prepared scaffolds is, therefore, more pronounced in the scaffold reinforced with higher content of chitin whiskers. However, the remarkable thermal stability promoted by chitin whiskers is clearly observed in a certain range of temperature only, which is 260-360°C, as the residual weight of all samples detected at 550°C are comparable.

The DSC curves of all samples run in Nitrogen gas are shown in Figure 4.9. The Tg of the neat HA-Gel scaffold is at about 35.75°C while Tg of

the others increase up to 39.29°C as the amount of chitin whiskers increases. With a Tg lower than 37°C, the physical status of the as-prepared scaffold could be altered when being used in human body. The observed Tg may be the reason why the scaffolds with 5-30% chitin whiskers exhibited the superior resistance to biodegradation in comparison with the 0-2% chitin whiskers scaffolds. Apart from the influence on the weight loss of scaffold at the high temperatures, reinforcing with chitin whiskers is also has an effect on the Tg of the HA-Gel scaffold in the similar way.

4.4.2.6 Cytotoxicity test, cell attachment and proliferation

Cytotoxicity of the as-prepared scaffolds was assessed by observing the relative viability of SaOS-2 that were cultured with the extraction media from the scaffolds containing 0 and 30% chitin whiskers against that of the cells that were cultured with SFM for 24 h. The results are shown in Table 4.4 as the absorbance of formazan in DMSO solution at 540 nm, which is directly proportional to the number of viable cells.

Evidently, the viability of the cells that were cultured with either the extraction media from the scaffold with 0% chitin whisker or SFM was statistically the same, a result indicating that the neat HA-Gel scaffolds are biocompatible to the bone cells. Nevertheless, viability of bone cells was found to significantly reduce when being cultured with the extraction media form 30% chitin whiskers scaffold. The increase of chitin whiskers tends to have an adverse effect on cell viability. Further evaluation for the potential use of the scaffolding materials was, therefore, carried out with the test for cell attachment and cell proliferation by direct seeding the bone cells on the scaffolds.

Figure 4.10 and 4.11 shows selected SEM images of SaOS-2 that had been seeded on the surfaces of the 30% chitin whiskers scaffolds for 1 and 7 day respectively. Apparently, cells attached and adhered well on the surface of the scaffolds within 24 h, with an evidence of cytoplasmic expansion (filoprodia) of the cells over the surfaces (Figure 4.10, white arrow). The observed cell appearance exhibits the well initial response of bone cell to the as-prepared scaffold which

influences the further processes of cell proliferation and differentiation (Anselme, 2000).

After 7 days of cultivation, cell morphology obviously changed to be flattened and fused to the scaffolds (Figure 4.11, white arrow) indicating the perfect adaptation of bone cells by adhering and spreading onto the substrate (Alberts *et al.*, 2002). Though the cytotoxicity test of the 30% chitin whiskers scaffolds revealed the significantly poor result, the observation for bone cells attachment presented their biocompatibility and ability to promote the growth of bone cells.

With an aim to investigate the effect of chitin whiskers on bone cells proliferation, this study performed the MTT test at the 1, 24, 48 and 72 h of the direct seeding SaOS-2 cultivation. The result shown in Figure 12 demonstrated that proliferation of bone cells on the tissue culture plate was much higher than those on the as-prepared scaffold, particularly at 48 and 72 h. The portion of hyaluronan in the HA-Gel blend scaffolds as high as 50% might restrict cell adhesion or proliferation (Stern *et al.*, 2006). The study of Liu et al. in 2004 (Liu *et al.*, 2004) found that the HA-Gel-Chitosan film containing only 0.31% of hyaluronan (wt/wt, HA/film) significantly promoted fibroblast cell attachment, migration and proliferation over that with 1.56% or 3.11% of hyaluronan. In order to establish much better positive environment for cells growth, the proportion of hyaluronan in the as-prepared scaffold must be reduced.

However, considering of cell proliferation among groups of the scaffolds, a tendency of the increased cell proliferation was found at the longer period of cell cultivation in all groups. Specifically observed, the HA-Gel scaffolds reinforced with 10% chitin whiskers exhibited a significantly higher cell number than the others which were rather comparable at an individual time point of investigation. Form the biocompatibility standpoint, therefore, reinforcement of the HA-Gel composites scaffold with chitin whiskers should be set at about 10% by weight of a dry scaffold.

4.5 Conclusion

The chitin whiskers reinforced HA-Gel nanocomposites scaffolds were successfully fabricated by the freeze-drying method. Characteristics of the as-prepared scaffolds could be regulated through the amount of the incorporating chitin whiskers so that the optimal balance among their mechanical, physical and biological properties could be achieved. High portion of chitin whiskers was found to enhance thermal stability and the resistance to biodegradation, whereas the rather low portion of chitin whiskers encourage tensile strength and compatibility for cell attachment and proliferation. However, none of any chitin whiskers portion was found to have an obvious effect on the morphology or internal architecture of the obtained scaffolds. Reinforcing with chitin whiskers, therefore, has some restriction for the simultaneous improvement of the harmonized properties of the obtained nanocomposites scaffolds. The distinct weight ratio of chitin whiskers seems to individually enhance physical, mechanical or biological properties. Though, the scaffolds of α-chitin whiskers reinforced HA-Gel nonocomposites is not a proper bone scaffold used in the high stress bearing area. However, they can be a candidate of bone scaffold used in the low stress bearing area like the socket of dental root.

4.6 Acknowledgements

The authors acknowledged full support received form the Ratchadaphiseksomphot Endowment Fund, Chulalongkorn University and partial support received from (a) the National Center of Excellence for Petroleum, Petrochemicals, and Advanced Materials (NCE-PPAM), and (b) the Petroleum and Petrochemical College (PPC), Chulalongkorn University.

4.7 References

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P. (2002). Molecular biology of the cell (4 ed.). New York: Garland Science.

Anselme, K. (2000) Osteoblast adhesion on biomaterials. Biomaterials, 21(7), 667-681.

Boyan, B.D., Hummert, T.W., Dean, D.D., and Schwartz, Z. (1996) Role of material srufaces in regulating bone and cartilage cell response. Biomaterials, 17(2), 137-146.

Chang, C.H., Liu, H.C., Lin, C.C., Chou, C.H., and Lin, F.H. (2003) Gelatin-chondroitin-hyaluronan-tri-copolymer scaffold for cartilage tissue engineering. Biomaterials, 24(26), 4853-4858.

Chaplin, M. "Gelatin." Water structure and science 25 June 2008. 24 September 2008 <http://www.lsbu.ac.uk/water/hygel.html>

Coates, J. 2000. Interpretation of infrared spectra, A practical approach. In R. A. Meyers (Ed.), Encyclopedia of Analytical Chemistry: 10815-10837. Chichester: John Wiley & Sons Ltd.

Currey, J. (2004) Incompatible mechanical properties in compact bone. Journal of Theoretical Biology, 231(4), 569-580.

Drury, J.L. and Mooney, D.J. (2003) Hydrogels for tissue engineering: scaffold desing variables and applications. Biomaterials, 24(24), 4337-4351.

Griffith, L.G. (2000) Polymeric Biomaterials. Acta Materialia, 48(1), 263-277.

Hoffman, A.S. (2002) Hydrogels for biomedical applications. Advanced Drug Delivery Reviews, 54(1), 3-12.

Holland, T.A., Tabata, Y., and Mikos, A.G. (2005) Dual growth factor delivery from degradable oligo(poly(ethylene glycol)fumurated) hydrogel scaffolds for cartilage tissue engineering. Journal of Controlled Release, 101(1-3), 111-125.

Kang, H.-W., Tabata, Y., and Ikada, Y. (1999) Fabrication of porous gelatin scaffolds for tissue engineering. Biomaterials, 20(14), 1339-1344.

Karageorgiou, V. and Kaplan, D. (2005) Porosity of 3D biomaterial scaffolds and osteogenesis. Biomaterials, 26(27), 5474-5491.

Kikuchi, T., Yamada, H., and Fujikawa, K. (2001) Effects of high molecular weight hyaluronan on the distribtution and movement of proteoglycan around chondrocytes cultured in alginate beads. Osteoarthritis and Cartilage, 9(4), 351-356.

Lapcik, L.J., Lapcik, L., Smedt, S.D., Demeester, J., and Chabrecek, P. (1998) Hyaluronan: Preparation, structure properties and applications. Chemical reviews, 98(8), 2663-2684.

Lee, K.Y., Rowley, J.A., Eiselt, P., Moy, E.M., Bouhadir, K.H., and Mooney, D.J. (2000) Controlling mechanical and swelling properties of Alginate hydrogels independently by cross-linker type and cross-linking density. Macromolecules, 33(11), 4291-4294.

Li, J., Revol, J.-F., Naranjo, E., and Marchessault, R.H. (1996) Effect of electrostatic interaction on phase separation behaviour of chitin crystallite suspensions. International Journal of Biological Macromolecules, 18(3), 177-187.

Liu, H., Yin, Y., Yao, K., Ma, D., Cui, L., and Cao, Y. (2004) Influence of the concentrations of hyaluronic acid on the properties and biocompatibility of Cs-Gel-HA membranes. Biomaterials, 25(17), 3523-3530.

Liu, L.S., Thompson, A.Y., Heidaran, M.A., Poser, J.W., and Spiro, R.C. (1999) An osteoconductive collagen/hyaluronate matrix for bone regeneration. Biomaterials, 20(12), 1097-1108.

Liu, Y., Shu, X.Z., and Prestwich, G.D. (2005) Biocompatibility and stability of disulfide-crosslinked hyaluronan films. Biomaterials, 26(23), 4737-4746.

Lu, Y., Weng, L., and Zhang, L. (2004) Morphology and properties of soy protein isolate thermoplastics reinforced with chitin whisker. Biomacromolecules, 5(3), 1046-1051.

Maeda, S., Sawai, T., Uzuki, M., Takahashi, Y., Omoto, H., Seki, M., and Sakurai, M. (1995) Determination of interstitial collagenase (MMP-1) in patients with rheumatoid arthritis. Annals of the Rheumatic Diseases, 54(12), 970-975.

Magoshi, J., Mizuide, M., and Magoshi, Y. (1979) Physical properties and struture of silk. VI. Conformational changes in silk fibroin induced by immersion in water at 2 to 1300C. Journal of Polymer Science: Polymer Physics Edition, 17(3), 515-520.

Mathew, H.W.T. 2002. Polymer for tissue engineering scaffold. In S. Dumitriu (Ed.), Polymeric Biomaterials: 168. New York: Marcel Dekker Inc.

Mercier, N.R., Costantino, H.R., Tracyc, M.A., and Bonassar, L.J. (2005) Poly(lactide-co-glycolide) microspheres as a moldable scaffoldnext term for cartilage tissue engineering. Biomaterials, 26(14), 1945-1952.

Mikos, A.G., Lu, L., Temenoff, J.S., and Tessmar, J.K. 2004. Synthetic bioresorbable polymer scaffolds. In J. E. Lemons (Ed.), Biomaterials science: An introduction to materials in medicine, 2 ed., Vol. 1: 735-749. California: Elsevier Inc.

Morin, A. and Dufresne, A. (2002) Nanocomposites of chitin whiskers from Riftia tubes and poly(caprolactone). Macromolecules, 35(6), 2190-2199.

Nair, K.G. and Dufresne, A. (2003) Crab shell chitin whiskers reinforced natural rubber nanocomposites. 1. Processing and swelling behavior. Biomacromolecules, 4(3), 657-665.

Oerther, S., Gall, H.L., Payan, E., Franc, o.L., Presle, N., Hubert, P., Dexheimer, J., 1, P.N., and Franc, o.L. (1998) Hyaluronate-alginate gel as a novel biomaterial: Mechanical properties and formation mechanism. Biotechnology and Bioengineering, 63(2), 206-215.

Oerther, S., Payana, E., Franc, o.L., Nathalie Preslea, Hubert, P., Muller, S., Netter, P., and Franc, o.L. (1999) Hyaluronatenext term-alginate combination for the preparation of new biomaterials: investigation of the behaviour in aqueous solutions. Biochimica et Biophysica Acta, 1426(1), 185-194.

Paillet, M. and Dufresne, A. (2001) Chitin whisker reinforced thermoplastic nanocomposites. Macromolecules, 34(19), 6527-6530.

Park, H. and Park, K. (1994). Hydrogels in Bioapplications. Paper presented at the Hydrogels and biodegradable polymers for bioapplications, Washington DC America.

Park, S.-N., Park, J.-C., Kim, H.O., Song, M.J., and Suh, H. (2002) Characterization of porous collagen/hyaluronic acid scaffold modified by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide cross-linking. Biomaterials, 23(4), 1205-1212.

Prestwich, G.D., Marecak, D.M., Marecek, J.F., Vercruysse, K.P., and Ziebell, M.R. (1998) Controlled chemical modification of hyaluronic acid: Synthesis,

applications, and biodegradation of hydrazide dervatives. Journal of Controlled Release, 53(1-3), 93-103.

Prestwich, G.D. "Biomaterials form chemically-modified hyaluronan." GlycoForum/GlycoScience/Science of Hyaluronan 26 February 2001. 15 May 2008 <http://www.glycoforum.gr.jp/science/hyaluronan/HA18/HA18E.html>

Revol, J.F. and Marchessault, R.H. (1993) In vitro chiral nematic ordering chitin crystallites. International Journal of Biological Macromolecules, 15(6), 329-335.

Rezwan, K., Chen, Q.Z., Blanker, J.J., and Boccaccini, A.R. (2006) Biodegradable and bioactive porous polymer/inorganic composite scaffolds for bone tissue engineering. Biomaterials, 27(18), 3413-3431.

Rinaudo, M. (2006) Chitin and chitosan: Properties and applications. Progress in Polymer Science, 31(7), 603-632.

Samir, M.A.S.A., Alloin, F., and Dufresne, A. (2008) Review of recent research into cellulosic whiskers, their properties and their application in nanocomposite field. Biomacromolecules, 6(2), 612-626.

Sannino, A., Papada, S., Madaghiele, M., Maffezzoli, A., Ambrosio, L., and Nicolais, L. (2005) Crosslinking of cellulose derivatives and hyaluronic acid with water-soluble carbodiimide. Polymer, 46(25), 11206-11212.

Segura, T., Anderson, B.C., Chung, P.H., Webber, R.E., Shull, K.R., and Shea, L.D. (2005) Crosslinked hyaluronic acid hydrogels: A strategy to functionalize and pattern. Biomaterials, 26(4), 359-371.

Shapiro, L. and Cohen, S. (1997) Novel alginate sponges for cell culture and transplantation. Biomaterials, 18(8), 583-590.

Shu, X.Z., Liu, Y., Palumbo, F., and Prestwich, G.D. (2003) Disulfide-crosslinked hyaluronan-gelatin hydrogel films: A covalent mimic of the extracellular matrix for in vitro cell growth. Biomaterials, 24(21), 3825-3834.

Shu, X.Z., Liu, Y., Palumbo, F.S., Luo, Y., and D, P.G. (2004) In situ crosslinkable hyaluronan hydrogels for tissue engineering. Biomaterials, 25(7-8), 1339-1348.

Sriupayo, J., Supaphol, P., Blackwell, J., and Rujiravanit, R. (2005) Preparation and characterization of a-chitin whisker-reinforced chitosan nanocomposite films with or without heat treatment. Carbohydrate Polymers, 62(2), 130-136.

Stern, R., Asari, A.A., and Sugahara, K.N. (2006) Hyaluronan fragments: An information-rich system. European Journal of Cell Biology, 85(8), 699-715.

Tabata, Y. and Ikada, Y. (1998) Protein release from gelatin matrices. Advanced Drug Delivery Reviews, 31(3), 287-301.

Tomihata, K. and Ikada, Y. (1997) Cross-linking of hyaluronic and with water-soluble carbodiimide. Journal of Biomedical Materials Research, 37(2), 243-251.

Vizarova, K., Bakos, D., Rehakova, M., Perikova, M., Panakova, E., and Koller, J. (1995) Modification of layered atelocollagen: enzyamatic degradation and cytotoxicity evaluation. Biomaterials, 16(16), 1217-1221.

Vunjak-Novakovic, G. 2006. Tissue Engineering: Basic Considerations. In R. I. Freshney (Ed.), Culture of Cells for Tissue Engineering: 132-155. Hoboken, New Jersey: A John Wiley & Sons, Inc.

Wong, M., Siegrist, M., Wang, X., and Hunziker, E. (2001) Development of mechanically stable alginate/chondrocyte constructs: Effects of guluronic acid content and matrix systhesis. Journal of Orthopaedic Research, 19(3), 493-499.

Wu, C.-S. and Liao, H.-T. (2005) A new biodegradable blends prepared from polylactide and hyaluronic acid. Polymer, 46(23), 10017-10026.

Wu, L. and Ding, J. (2004) In vitro degradation of three-dimensional porous poly(D,L-lactide-co-glycolide) scaffold for tissue engineering. Biomaterials, 25(27), 5821-5830.

Young, S., Wong, M., Tabata, Y., and Mikos, A.G. (2005) Gelatin as a delivery vehicle for the controlled release of bioactive molecules. Journal of Controlled Release, 109(1-3), 256-274.

Table 4.1 Pores size of the α-chitin whiskers reinforced HA-Gel scaffolds determined from the transversal and longitudinal sections. [a,b,c,d,e,f] compared only between sections of an individual specimen at $p < 0.05$; One-Way ANOVA with Tukey HSD, $n = 50$.

Specimen	Modulus of elasticity (MPa)	Elongation at break (%)	Tensile strength (MPa)
0% CW	00.988 ± 0.099[a]	53.476 ± 10.688[a]	0.518 ± 0.064[a]
2% CW	14.096 ± 2.228[b]	28.346 ± 6.163[b]	1.028 ± 0.089[b]
5% CW	19.958 ± 3.640[b]	06.357 ± 1.418[c]	0.532 ± 0.153[a,c]
10% CW	12.456 ± 2.000[b]	11.632 ± 4.154[c,d]	0.478 ± 0.190[a,c]
20% CW	07.103 ± 0.931[c]	16.070 ± 0.955[b,d]	0.465 ± 0.058[a]
30% CW	11.963 ± 0.472[b]	16.030 ± 3.396[b,d]	0.722 ± 0.081[c]

Table 4.2 Mechanical properties of the α-chitin whiskers reinforced HA-Gel scaffolds. [a,b,c,d] compared among groups of specimen for a single property at $p < 0.05$; One-Way ANOVA with Dunnett T3, $n = 5$.

Specimen	Pore size (μm)			
	Transversal sectioning		Longitudinal sectioning	
	Range (Min-Max)	Average (Mean ± SD)	Range (Min-Max)	Average (Mean ± SD)
0% CW	92.34 – 208.76	140.91 ± 21.82[a]	112.80 – 231.37	165.76 ± 27.68[aa]
2% CW	114.42 – 181.98	139.38 ± 16.01[b]	108.37 – 182.09	145.44 ± 15.31[b]
5% CW	104.12 – 224.47	153.15 ± 23.92[c]	110.59 – 171.08	142.70 ± 13.84[c]
10% CW	118.01 – 202.32	158.86 ± 19.08[d]	104.27 – 205.35	162.76 ± 23.63[d]
20% CW	113.48 – 200.48	151.44 ± 19.29[e]	105.77 – 180.96	143.85 ± 17.19[e]
30% CW	108.48 – 184.36	155.41 ± 15.67[f]	97.22 – 231.27	148.99 ± 25.09[f]

Table 4.3 Temperature (°C) at 5, 25 and 50% weight loss (T-5%, T-25% and T-50% respectively) of the α-chitin whiskers reinforced HA-Gel scaffolds and the residual weight at 550 °C.

Specimen	T-5% (°C)	T-25% (°C)	T-50% (°C)	Residue at 550 °C (wt.%)
0% CW	200.64	264.86	362.57	40.41
2% CW	212.61	269.02	368.00	39.64
5% CW	194.44	268.43	374.95	39.56
10% CW	213.28	274.18	387.80	42.54
20% CW	203.27	278.48	385.04	40.00
30% CW	214.43	290.66	390.45	41.11

Table 4.4 Indirect cytotoxicity evaluation of the as-prepared 0%CW and 30%CW HA-Gel scaffolds using human osteoblast cell line (SaOS-2). *p < 0.05; One-Way ANOVA Tukey HSD, n = 4.

Sample	Absorbance @ 570 nm		
	Control	0%CW	30% CW
1	0.575	0.524	0.465
2	0.508	0.536	0.410
3	0.537	0.487	0.409
4	0.540	0.569	0.428
Average	0.540 ± 0.027	0.529 ± 0.034	0.428 ± 0.026*

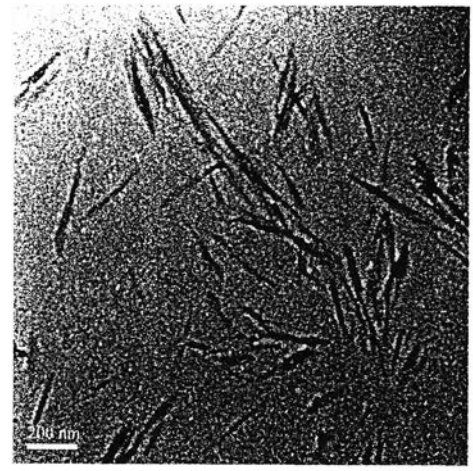
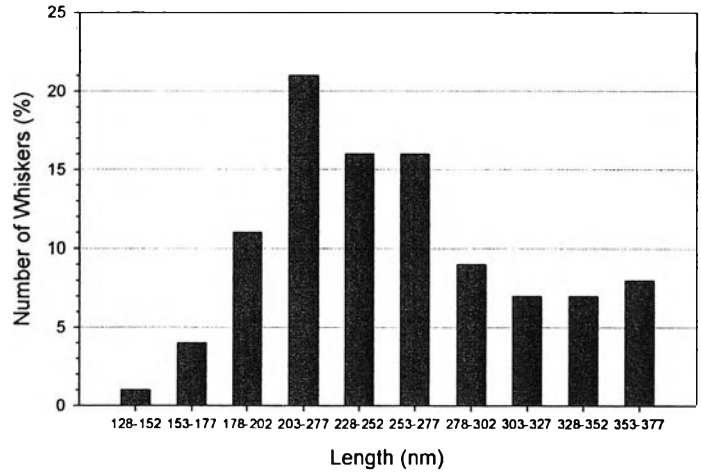
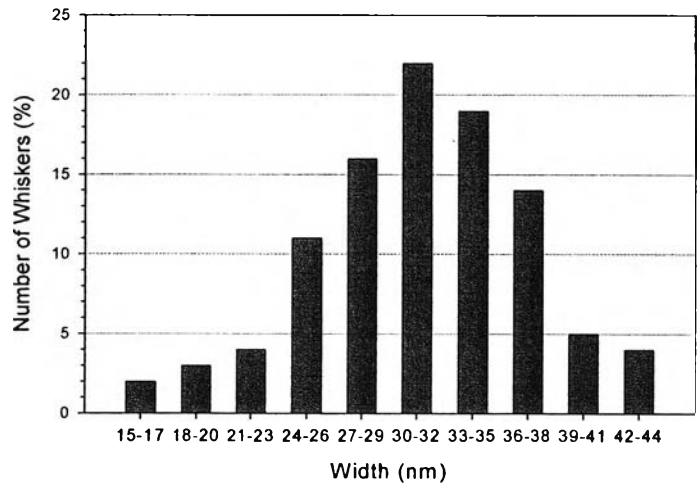


Figure 4.1 Selected TEM images presenting the as-prepared chitin whiskers.



(a)



(b)

Figure 4.2 Histogram illustrating distribution of the as-prepared chitin whiskers' length (a) and width (b)

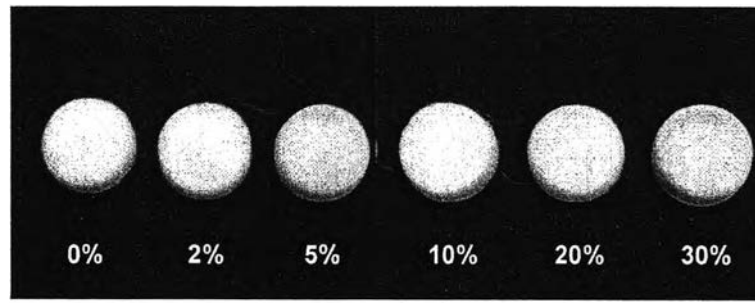


Figure 4.3 The α-chitin whiskers reinforced HA-Gel nanocomposites scaffolds (The numbers symbolize wt% of chitin whiskers)

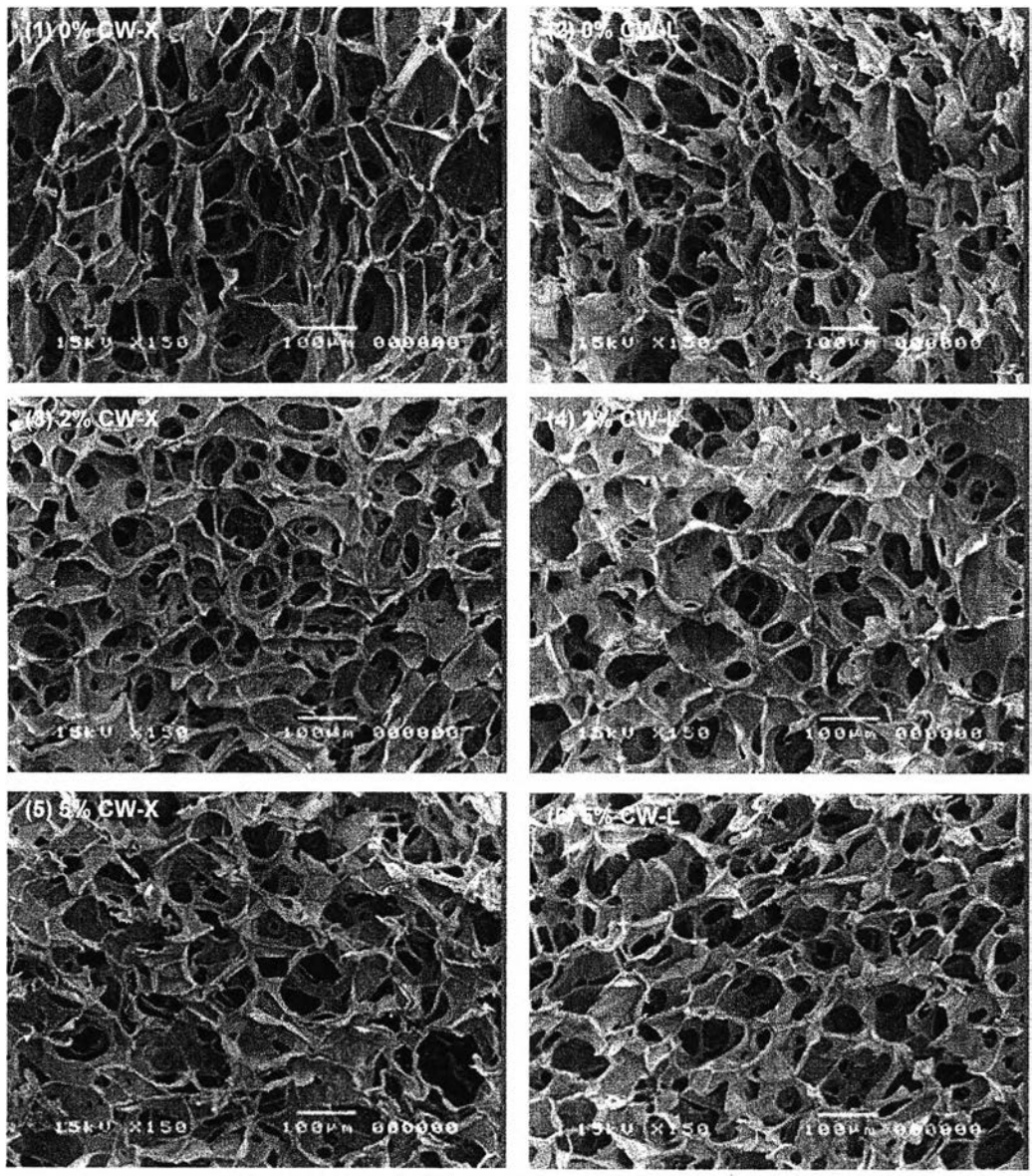


Figure 4.4-1 SEM images illustrating internal structure of the α-chitin whiskers reinforced HA-Gel scaffolds viewed on the transversal (1,3,5) and longitudinal section (2,4,6): (1,2) 0% CW, (3,4) 2% CW, (5,6) 5% CW.

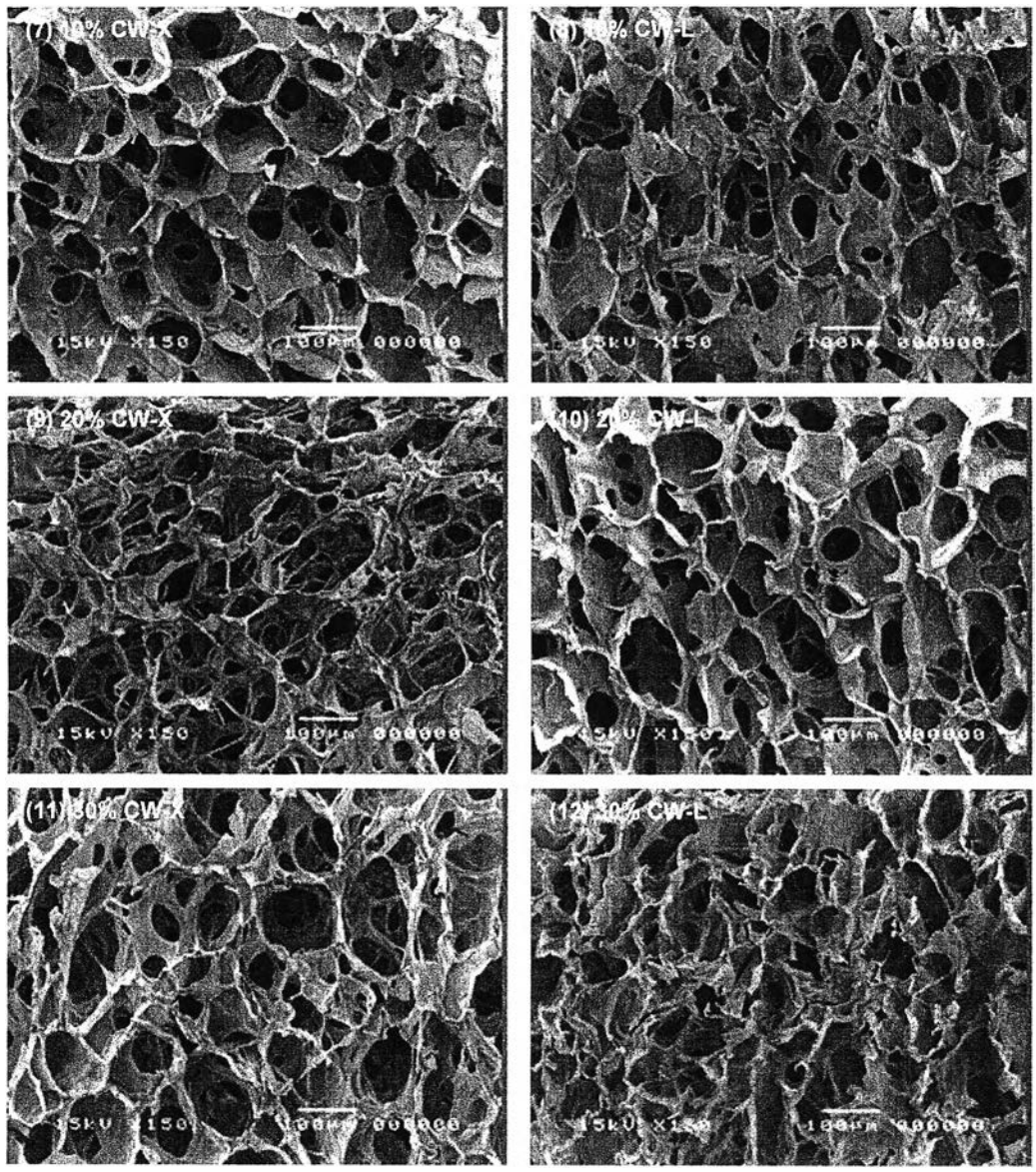


Figure 4.4-2 SEM images illustrating internal structure of the HA-Gel scaffolds viewed on the transversal (7,9,11) and longitudinal section (8,10,12): (7,8) 10% CW, (9,10) 20% CW, (11,12) 30% CW.

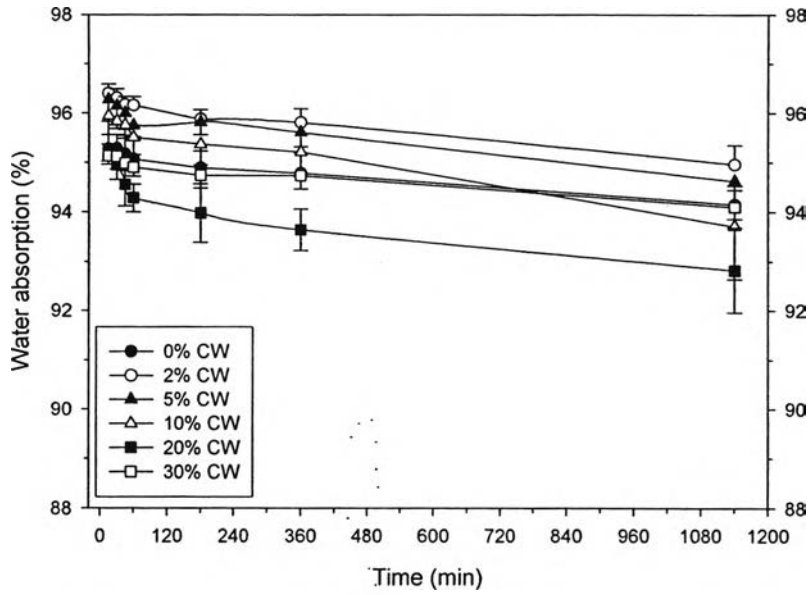


Figure 4.5 Water absorption capability of the α-chitin whiskers reinforced HA-Gel scaffolds, at room temperature.

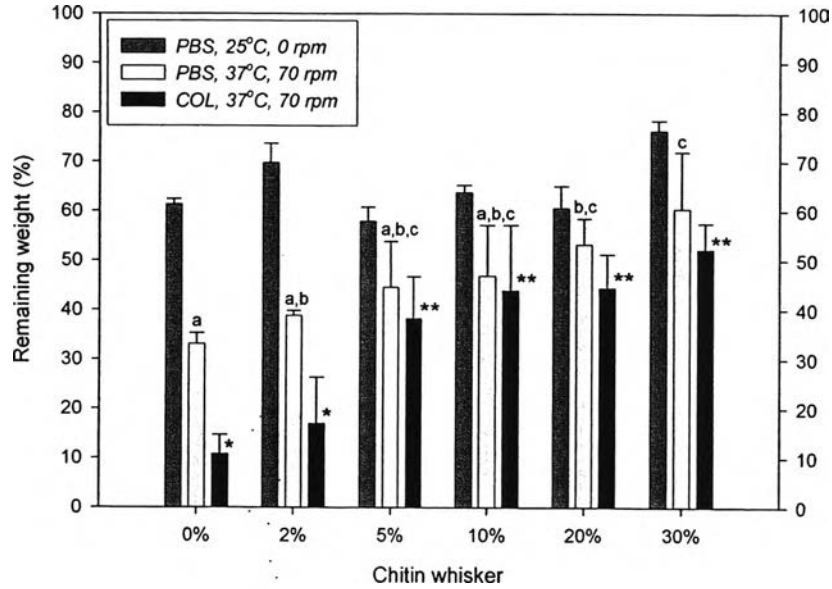


Figure 4.6 Remaining weight of the α-chitin whiskers reinforced HA-Gel scaffolds after having been immerged in PBS or collagenase in various condition for 24 h. ^{a,b,c,} * at $p < 0.05$; One-Way ANOVA with Tukey HSD, $n = 4$.

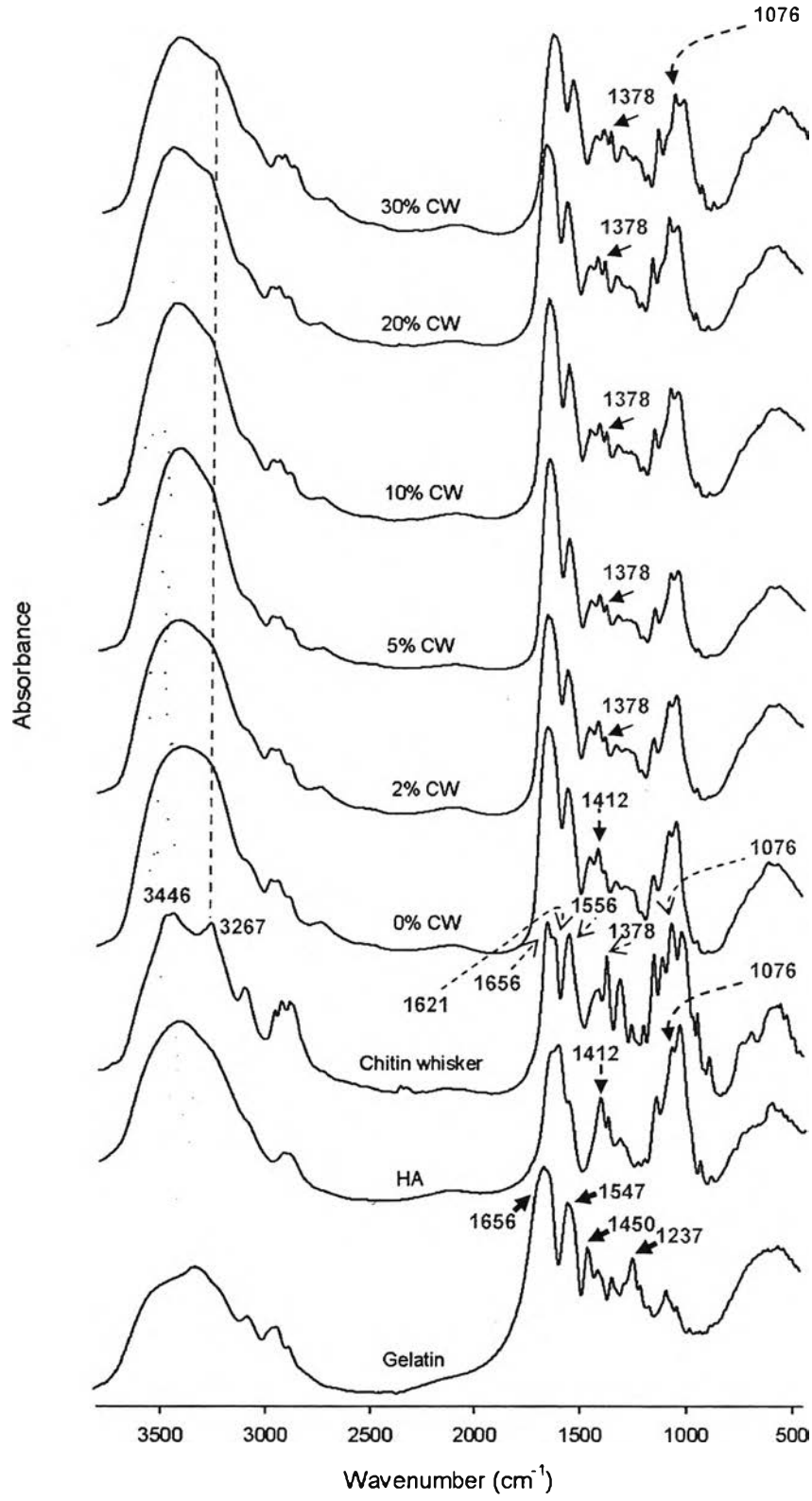


Figure 4.7 Infrared spectra of the gelatin, HA, α-chitin whiskers, and the α-chitin whiskers reinforced HA-Gel nanocomposites

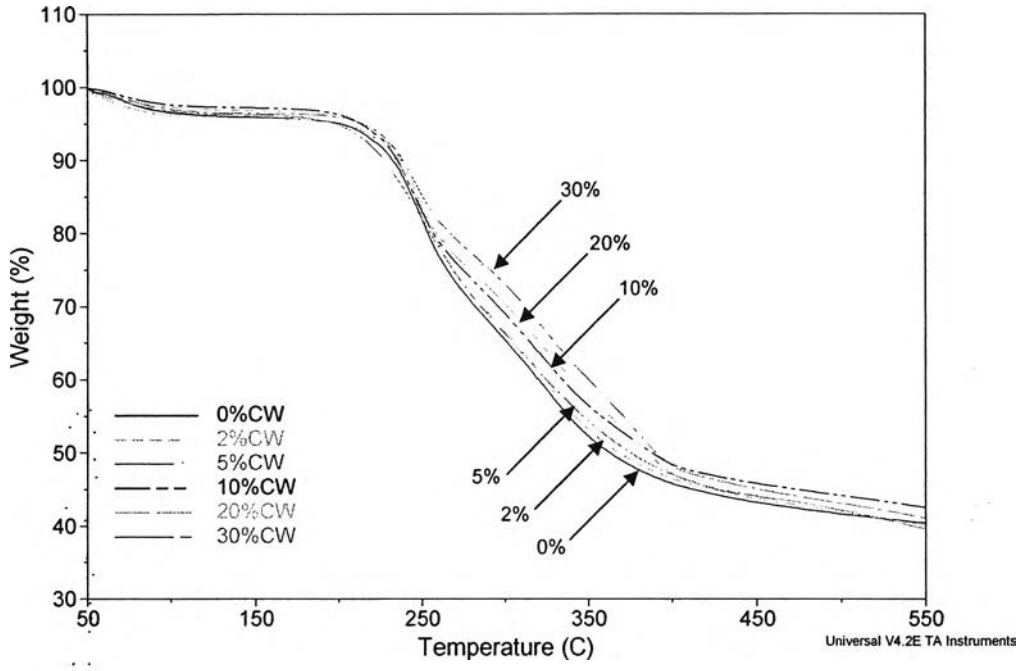


Figure 4.8 TGA thermograms of the α-chitin whiskers reinforced HA-Gel scaffolds containing different whisker contents, at 30-600°C, rate of temperature increase 10°C/min, under N2 gas.

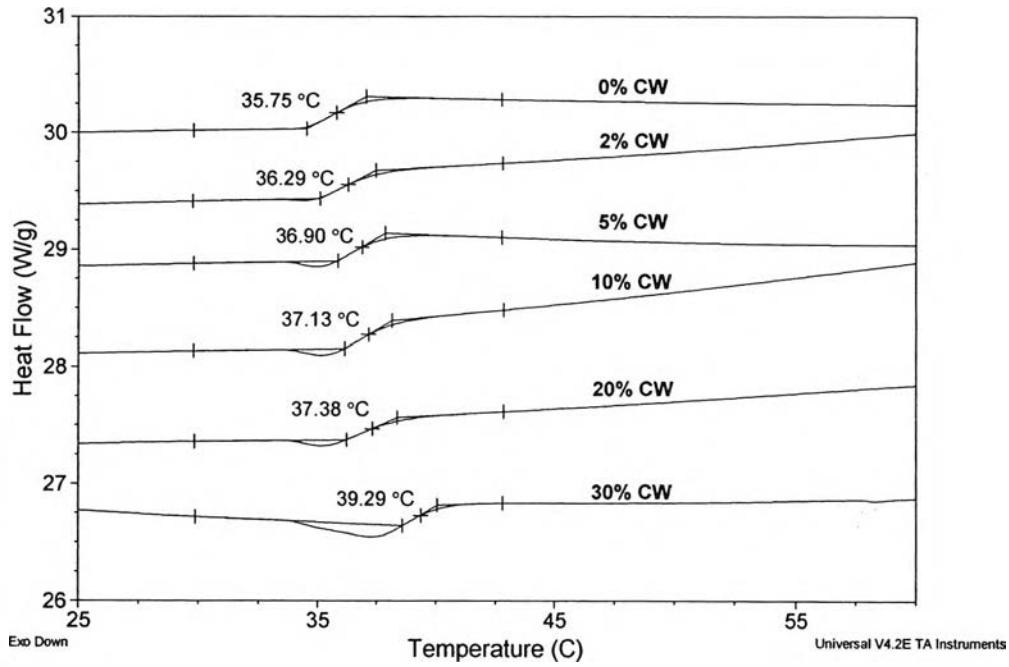


Figure 4.9 DSC thermograms of the α-chitin whiskers reinforced HA-Gel scaffolds containing different whisker contents, at 25-60°C, rate of temperature increase 10°C/min, under N2 gas.

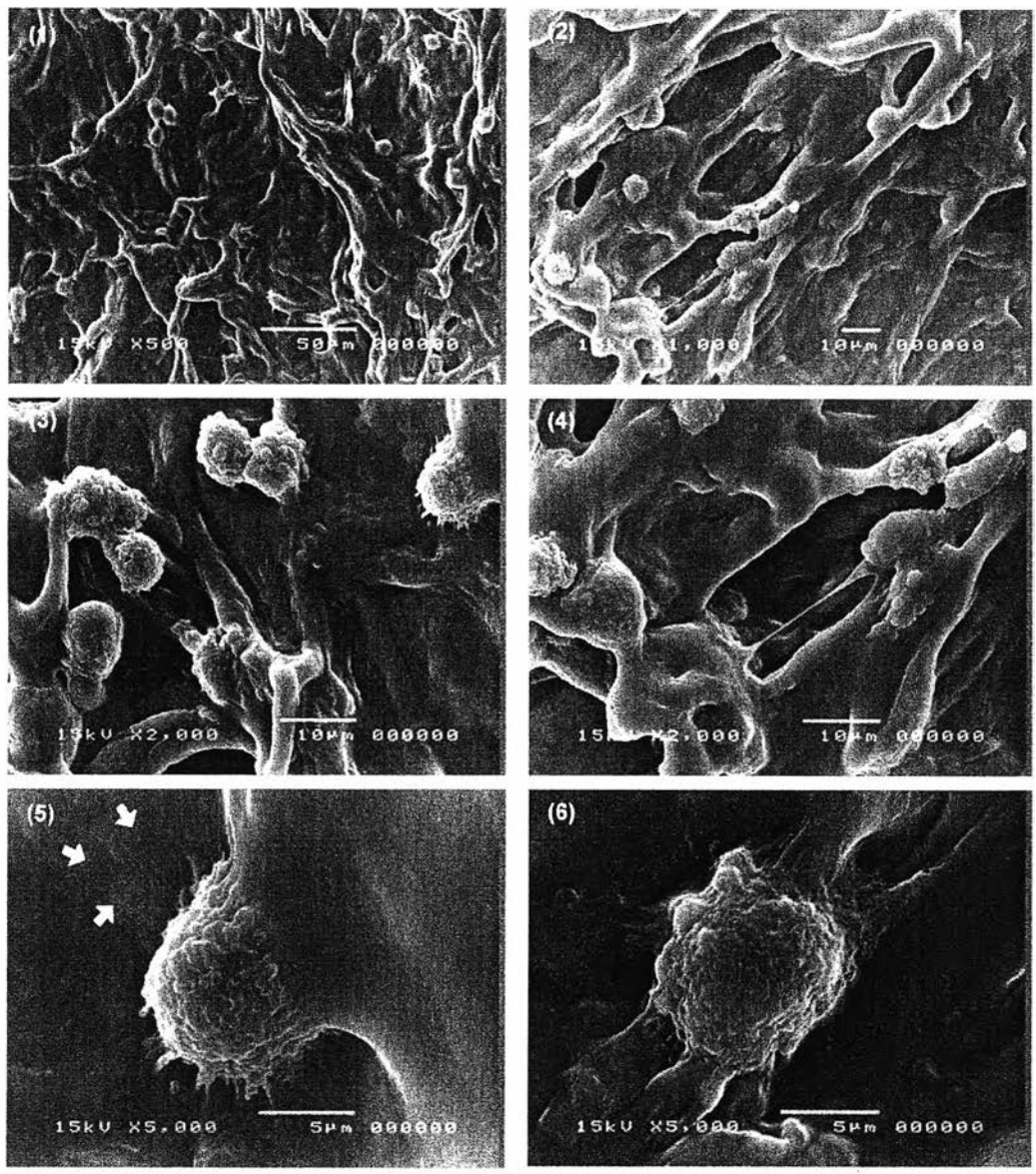


Figure 4.10 Selected SEM images illustrating morphology of SaOS-2 that were seeded on the surface of the 30%CW HA-Gel scaffolds for 24 h. White arrows show the cytoplasmic processes of the cells.

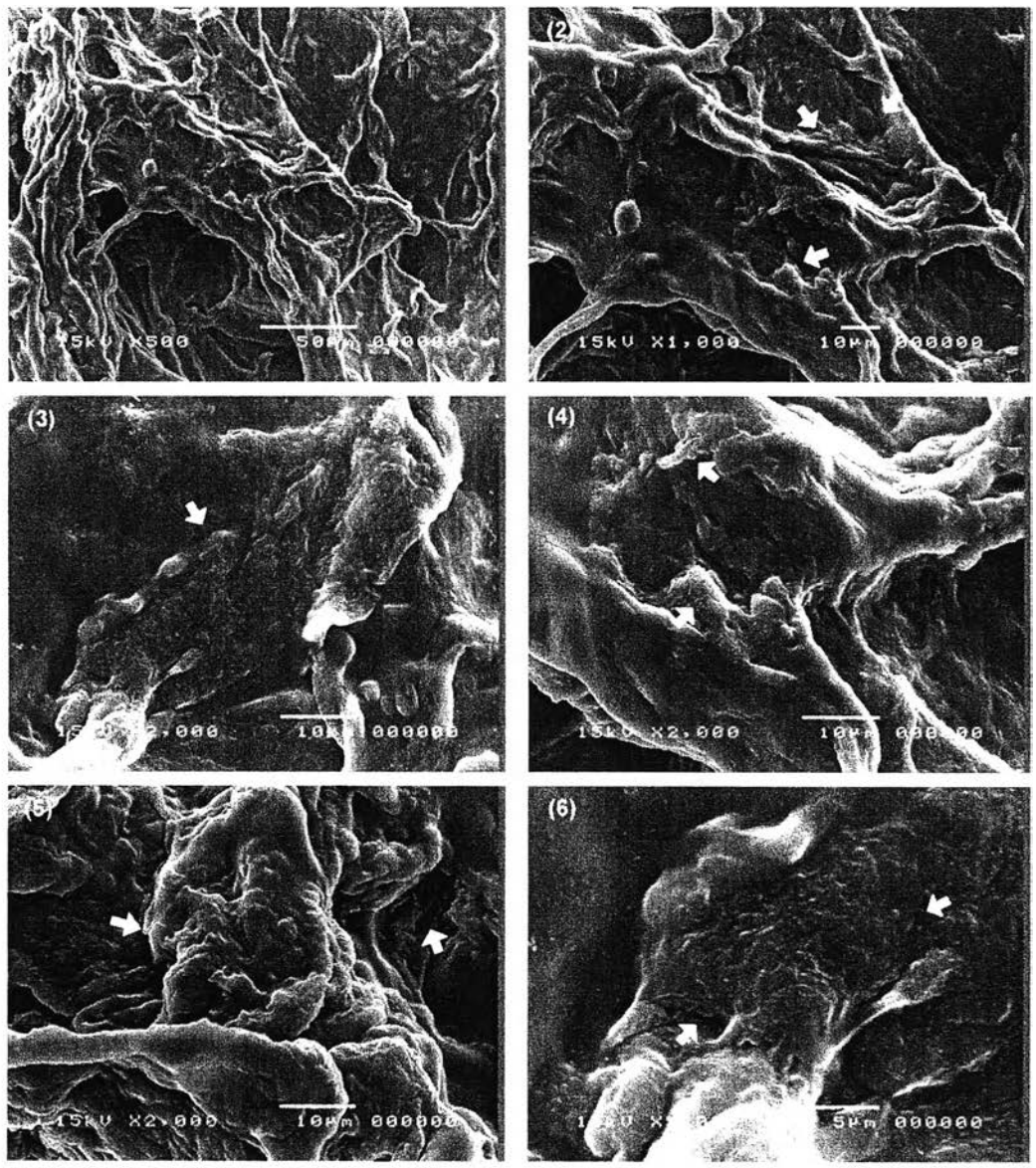


Figure 4.11 Selected SEM images illustrating morphology of SaOS-2 that were seeded on the surface of the 30%CW HA-Gel scaffolds for 7 days. White arrows show cells that fuse into the scaffolds.

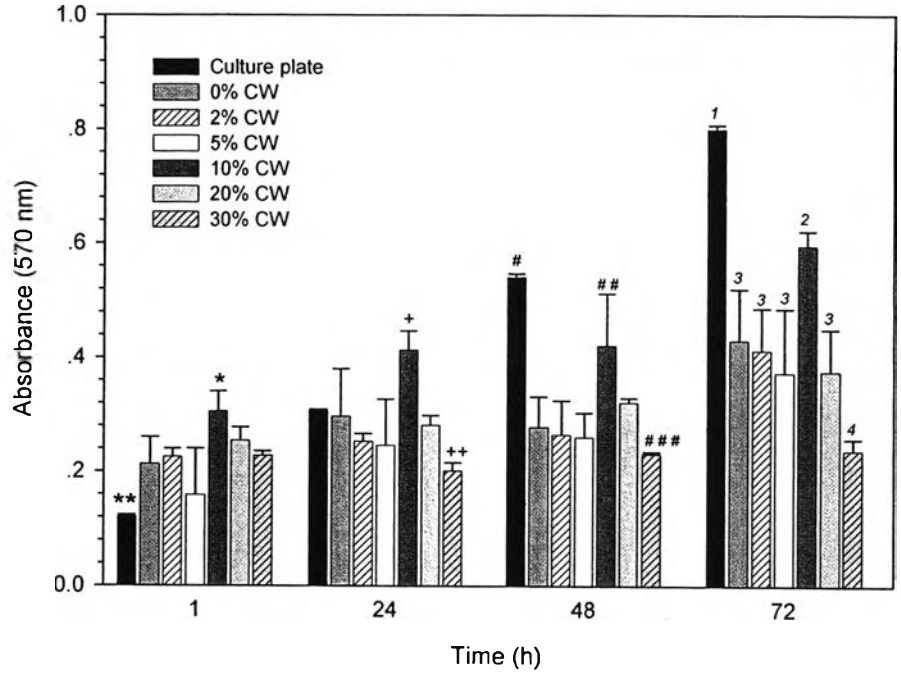


Figure 4.12 Evaluation of the SaOS-2 proliferation on the as-prepared 30%CW HA-Gel scaffolds $^{*, +, \#, 1}$ $p < 0.05$; One-Way ANOVA Tukey HSD, n = 4.