CHAPTER VI
PREPARATION OF SILK SERICIN/PVA/CLAY AEROGEL CROSSLINKED BY GLUTARALDEHYDE FOR BIOTECNOLOGY APPLICATION

6.1 ABSTRACT

Silk sericin/PVA/clay aerogel, the three-dimensional (3D) foam-liked material to use as 3D scaffold for tissue engineering was developed via freeze-drying technique. The silk sericin/PVA/clay aerogel was prepared by altering the amounts of silk sericin in 1, 2, 3 and 4 wt% and clay was introduced in 2, 4, 6 and 8 wt%. Glutaraldehyde, in the concentration of 3, 5 and 7µl/ml, was used as a chemical cross-linked agent so as to developed the aerogel which able to maintain the structure in aqueous solution. In addition, the different species of Thai silk cocoon; Nang Noi, Nang Lai, Dok Bua and Luang Pairote were extracted to obtain silk sericin and used to develop aerogel with the purpose of studied the influence of species on the *in vitro* biological test. The morphology of porous silk sericin/PVA/clay aerogel was determined by Field Emission Scanning Electron Microscope (FE-SEM). The mechanical and thermal properties were investigated by Universal Testing Machine in compression mode and Thermogravimetric-Differential Thermal Analyzer (TG-DTA), respectively. With the purpose of applying this aerogel in biotechnological application, the swelling behavior was examined using phosphate buffer saline (pH 7.4) as a swelling media. Additionally, the aerogel was tested for *in vitro* direct contact test and cytotoxicity based on MTT assay to confirmed the possibility to use this material as 3D scaffold for tissue engineering. The freeze-drying technique produced scaffolds with superior modulus, interconnected pores and pore size greater than 100 μm. The mechanical properties could be improved by increasing of clay, silk sericin and cross-linked agent contents. Thermal stability increased when increased silk sericin content but reduced while increased clay contents owing to the oxidation of ferric ion existing in clay galleries. The swelling ratios directly depended on the composition of the aerogels. As enlarging of silk sericin, clay and cross-linked agent contents, the swelling ratio was decreased. The in *vitro* direct

contact test and MTT assay confirmed the possibility to be applied this material as a good candidate for 3D scaffold. The MTT assay suggested the silk sericin contents, glutaraldehyde concentrations and species of silk cocoon were influence on the viability of human gingival fibroblast cell (HGF).

Keywords: Sericin; Silk; Clay aerogel; Scaffold; Tissue engineering

6.2 INTRODUCTION

The periodontitis is an oral disease occurred from the infection of teeth, gums and bone tissues because of plaque, tartar and bacteria that forms constantly on the teeth resulting in the destroying gingival and bone tissue. There are several ways to heal the periodontitis patient, for example, scaling and root planing to remove plaque, tartar and bacteria toxins from deep periodontal pockets, using lasers for periodontal therapy and periodontal surgery. In the case of severe patient, the teeth, gingival and bone tissues are almost destroyed. Thus, the dental implant by using tissue engineering technique should be required to regenerate and replace new tissue in to the periodontal defect site (NIH Publication No. 12-1142 August 2012). The periodontal cell will be seeded onto the artificial 3D scaffold and implanted into the dental defected site in the jaw hence the probable scaffold is the key factor essential for the successful treatment.

The satisfy scaffold should meet several criteria including biocompatibility, interconnected pore, mechanical strength, etc. The suitable porous structure, both in pore size and interconnected pore, is an imperative factor for the regeneration of new tissue. A variety of porous materials have been use for this application in order to support cell growth and cell attachment. One of the potential methods to prepare the porous scaffold is freeze-drying technique because this technique can created the porous structure with interconnected pore without destroying of chemical stability of base materials. The natural protein based polymer such as collagen, chitosan, fibroin and sericin is the commonly considerable base polymer for tissue engineering because of their characteristic which can mimics the cellular surrounding and is

accepted by many cell types (Franks, 1998, Haroun *et al.*, 2009, Mandal *et al.*, 2009, Lungu *et al.*, 2012).

Silk sericin (SS) is the natural glue-like protein contained around 20-30% of silk cocoon which produced from *Bombyx mori* silk worm. In textile production, silk sericin must be removed from silk fiber and leaved in waste water for several tons per year lead to the environmental defilement. Silk sericin can be used in the numerous applications range from supplementary additives for cosmetic to biomedical applications (Zhang *et al.*, 2002). Quite a lot of study used silk sericin for the potential wound dressing due to its hydrophillicity hence it can absorb the exudates from wound and conserve a moisture environment resulting in the acceleration of wound healing (Aramwit *et al.*, 2012). Several studies reported that silk sericin exhibited the efficiency to enhance cell growth, non-cytotoxicity and low immune responses. From this reason, silk sericin could be an appropriated element for cell culture application (Takeuchi *et al.*, 2005, Mandel *et al.*, 2009, Mandel *et al.*, 2011, Lungu *et al*, 2012, Nayak *et al.*, 2012). Silk sericin also has the antioxidant, anti-fungus and anti-bacterial properties which were reported by Sarovart *et al.* They proposed that different species of silk cocoon showed different effect in biological activities which came from a certain dissimilar characteristics like the number of amino acid and their composition. Nevertheless, the usage of silk sericin is limited from its amorphous nature hence the mechanical properties is low. The combination of other organic or inorganic materials is the effective way to expand the application of silk sericin.

Clay aerogel is a low density and highly porous material which possesses characteristics similar to the typical polymeric foams. Clay aerogel generated from the layered silicate clay like Na-bentonite passed though the simple environmental freeze-drying techniques (Bandi 2006). The "house of card" liked structure with interconnected pore is the symbolic morphology of clay aerogel which comes from the alignment of ice crystals during freezing process. Due to this porous structure, many researchers attempted to use this prominent point for a variety of biomedical application. Haroun et al., 2009 and Zheng et al., 2007 studied on the biomimetic 3D scaffold developed from clay aerogel. They proposed that not only the porous

structure and improvement in mechanical properties, clay aerogel also improved cytocompatibility between cell and biocomposite especially osteoblast.

In this study, the silk sericin/PVA/clay aerogels were developed by freeze-drying technique. Silk sericin was extracted from Thai silk cocoons; Nang Noi and freeze-dried to obtain the silk sericin powder. The silk sericin/PVA/clay aerogels were prepared by varied loadings of Na-bentonite and silk sericin. The chemical cross-linked agent, glutaraldehyde (GT), was employed to create the structure able to maintain in aqueous solution. The aerogels were studied on morphology, mechanical and thermal properties using FE-SEM, Universal Testing Machine and TG-DTA, respectively. After that, the silk sericin/PVA/clay aerogel in the composition of clay at 6 wt% was selected in order to prepare the silk sericin/PVA/clay aerogel using different species of Thai silk cocoon (Nang Lai, Dok Bua and Luangn Pairote) so as to study the effect of species on the *in vitro* biological test. The swelling behavior and *in vitro* biological test using human gingival fibroblast cell (HGF) were achieved to study the probability to use this biocomposite as 3D scaffold for tissue engineering in periodontitis.

6.3 EXPERIMENTAL

6.3.1 Raw materials

Silk cocoons (*Bombyx mori*) species; Nang Noi, Nang Lai, Dok Bua and Luang Pairote were obtained from local sericulture in Thailand. Sodium-bentonite was supported from Thai Nippon Co.,Ltd, Thailand : commercial sodium activated bentonite Mac-Gel© (GRADE SAC) with cationic exchange capacity (CEC) of 49.74 meq/100 g clay. Poly(vinyl alcohol) (PVA) was purchased from KURARAY POVAL Co.,Ltd, Japan with average molecular weight at 9000-10000 (characterized by GPC), hydrolyzed at 87-89 mol% and viscosity is 40-48 mPa.s (in 4% aqueous solution at 20°C). Glutaraldehyde (C5H8O2, CAS No.111-30-8) used as cross-linked agent was purchased from Sigma Aldrich Corp., USA with molecular weight 100.12 g/mol and used without further modification.

6.3.2 Preparation of silk sericin powder

20 g of silk cocoons were cut in to small pieces about 5×5 mm^2 and mixed with 300 ml purified water. Silk cocoons were autoclaved under pressure at 120°C for 1 hr. The fibroin was filtered out to obtain the sericin solution. Silk sericin solution was frozen in the glass shells at -40°C for 12 hr. After freezing, the shells were put in to freeze-dryer maintained at -110 °C for 48 hr. Then, freeze-dried silk sericin was grinded in to powder.

6.3.3 Preparation of cross-linked silk sericin/PVA/clay aerogel

Silk sericin with various contents and PVA (5wt%) were dissolved in purified water and heated at 90°C until the polymers were homogeneously dissolved. Bentonite clay was gently added in to the mixture followed by the vigorous stirring for 2 hr. Then, glutaraldehyde was added in to silk sericin/PVA/clay gel precursor under constant stirring followed by another continuous stirring for 1 hr. The mixture was cooled down to the ambient temperature and subjected to the freeze-dried procedure. In details, the cross-linked sericin/PVA/clay gel precursor was frozen in cylindrical glass vials at -40°C for 12 hr and subjected to the freeze-dryer under vacuum. After 48 hr in freeze-dryer, the samples were removed and post cured at 120°C to ensure the maximum curing of the aerogel and removed the residual glutaraldehyde as much as possible.

The samples were coded CxPVA5SSy zGT where x corresponds to clay content (wt%) and y corresponds to silk sericin content (wt%) and z corresponds to glutaraldehyde content. For example, C8PVA5SS1 0.7 GT would compose of clay 8 wt%, PVA 5 wt%, silk sericin 1 wt% and glutaraldehyde 7 µl/ml (0.7 ml in 100 ml of clay gel precursor)

6.3.4 Characterizations

6.3.4.1 Density measurement

The density of the cross-linked silk sericin/PVA/clay aerogels was determined by mass and dimension measurement according to an equation:

$$\rho = M/V \qquad \text{(eq. 6.1)}$$

where ρ is mass density (g/cm^3), M is mass of sample (g) and V is volume of sample (cm^3). Mass and volume was measured using Sartorius BS 224 S analytical balance and digital vernier caliper.

6.3.4.2 Fourier Transform Infrared Spectroscope (FTIR)

The Functional groups of cross-linked silk sericin/PVA/clay aerogel were examined using Thermo Scientific Nicolet 6700 FTIR Spectrometer with attenuated total reflectance mode (ATR/single reflectance diamond). The spectra were recorded over the wavenumber range of 400-4,000 cm^{-1} with the resolution of 4 cm^{-1} and the number of scan at 64.

6.3.4.3 Field Emission Scanning Electron Microscope (FE-SEM)

The morphology of cross-linked silk sericin/PVA/clay aerogel was observed using Hitachi S-4800 Field Emission Scanning Electron Microscope. The samples were coated with platinum under vacuum. FE-SEM micrographs were taken using an accelerator voltage of 2.0 kV with the magnification range between 45-20.0k.

6.3.4.4 Thermogravimetric-Differential Thermal Analyzer (TG-DTA)

Thermal stability of cross-linked silk sericin/PVA/clay aerogels were examined by Perkin-Elmer Pyris Daimond Thermogravimatric Analyzer. The sample was weighted in the range of 5-7 mg and heated at the heating rate of 10 °C/min from 30-900 °C in nitrogen atmosphere with nitrogen flow at 30 ml/min.

6.3.4.5 Compression test

The initial modulus of cross-linked silk sericin/PVA/clay aerogels were investigated by LLOYD Lrx Universal Testing Machine with compression mode using a 500 N load cell at constant crosshead speed of 1 mm/min. The samples were prepared in the cylindrical shape with diameter of ~20 mm and height at least 20 mm. Five samples of each composition were reproduced test. The initial compressive modulus was calculated from the slope of the linear portion of the stress-strain curve.

6.3.4.6 Sulfur analyzer

The sulfur content in silk sericin in all species was determined by LECO®Elemental Analyzer (TruSpec®S). The silk sericin powder was weighted arpund 0.1 g in a ceramic boat. The temperature of furnace was 1,350 °C.

6.3.4.7 Swelling behavior

The swelling behavior of cross-linked aerogels was studied by using conventional gravimetric procedure. Phosphate buffer saline pH 7.4 was used as swelling media and the swelling ratio was observed at room temperature. The dried sample was weighted then immersed in PBS. The swollen gels were withdrawn, wiped to remove the excess water and weighted. The swelling ratio calculated followed an equation:

$$SR = \frac{weight\ of\ swollen\ sample - weight\ of\ dry\ sample}{weight\ of\ dry\ sample} \times 100 \qquad \text{(eq. 6.2)}$$

6.3.4.8 In vitro direct contact test

Fibroblast cells derived from human gingival fibroblast from passage 5 were directly cultured on the sponge (aerogel when immersed in cell culture media). The samples in the size of 3×7 mm^2 were sterilized using UV radiation for 30 minutes. The sterilized samples (sponge) were transfer to culture plates and adhered with dental wax. The solution of cell suspension containing 4×10^4 cells/ml was added to the cultured plates and incubated in the incubator at 37 °C with 5% CO_2 and 100% humidity atmosphere. The cell cultured media was changed once in every two days and the reactivity of human gingival fibroblast cells was studied at 24, 48 and 72 hr. This experiment was repeated using human gingival fibroblast from two human donors. All equipment was sterilized in alcohol before tested.

6.3.4.9 MTT assay

Cell viability and mitochondria activities were studied by MTT assay. The aerogel was cut in to round shape with the dimension around 4 mm and sterilized for 30 minutes using UV radiation. The samples were immersed in cell cultured media at 37 °C for 24 hr. Human gingival fibroblast cells were seeded in to the 24-wellplate with the concentration of 4×10^4 cells/ml. The cultured plates were incubated in the incubator at 37 °C under 5 % CO_2 atmosphere at 100% humanity. After 24 hr of incubation, the cell cultured media was removed and replaced with the cell cultured media obtained from the immersion of sponges. The cell cultured plates were incubated for a second time at 37 °C in the incubator for 48 hr. Then, the cell

cultured media was removed and replaced by 50 µl of MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and 300 µl of DMEM without phenol red, followed by incubation for 4 hr at 37 °C. The MTT solution was removed and added 1 ml of dimethyl sulfoxide in order to dissolved formazan crystals. The optical density (OD) was measured by spectrophotometer to compare the OD valued between cell cultured media from samples and control. The experiment was carried twice using human gingival fibroblast cells from two human donor contributions. All equipment was sterilized in alcohol before tested.

6.4 RESULTS AND DISCUSSION

6.4.1 Appearance of cross-linked silk sericin/PVA/clay aerogels

In order to simplify the silk sercin/PVA/clay aerogel in biotechnological field like the scaffold for tissue engineering, the aerogels should be maintained their shape and structure while immersed in aqueous cell suspension or human body fluid for a period of time. The uncross-linked silk sericin/PVA/clay aerogels were losing their structure when they were in aqueous solution because silk sericin is the hydrophilic protein that can dissolve in water due to the high content of polar amino acid and its random coil structure (Kunda *et al.*, 2008, Zhang, 2002). Furthermore, PVA is well known as the hydrophilic biocompatible synthetic polymer because of the pendant hydroxyl groups in the repeating units hence it can be dissolved in the water. In this study, several cross-linked agents were employed to create the cross-linked network of silk sericin and PVA in order to reduce the hydrophillicity of the raw materials, assisted the aerogels to stabilize their shape and retarded the biodegradation of scaffolds. After that, the most suitable cross-linked agent, which made the aerogel could keep up the structure in the aqueous solution for a long period of time was selected to apply as the chemical cross-linked agent in all experiment.

Figure 6.1, 6.2 and 6.3 show the silk sericin/PVA/clay aerogels with variety of cross-linked agents. The aerogels cross-linked by poly(ethylene glycol) diglycidyl ether (PEG-DE) exhibited the most foam like materials compared with

other cross-linked agents. By using tetraethyl orthosilicate (TEOS) mixed with bentonite clay so as to introduce the stiffness construction which came from the silica xerogel followed by cross-linked with PEG-DE gave the superior hardness aerogels. Both of those two cross-linked agents provided the aerogels which not appropriated for scaffold application since the aerogels could not maintained their shape when immersed in phosphate buffer saline (PBS) pH 7.4 and water for a long period of time. The aerogels cross-linked by glutaraldehyde (GT) showed the highly porous aerogels with modulate hardness. In addition, GT cross-linked aerogels could sustain their shape for more than 2 weeks in PBS and water. Glyceraldehyde (GC) was also gave the same results as GT, but the aerogels could sustain their shape in the fluid just for an hour due to the low reactivity of cross-linked agent itself. From this point, glutaraldehyde was selected to be used as the effective cross-linked agent in all study.

After post curing at 120°C, the physical appearance of GT cross-linked silk sericin/PVA/clay aerogels were changed from pale yellow to brownish yellow because the cross-linked reaction between the carbonyl group of GT, amino groups in silk sericin and pendant hydroxyl groups in PVA resulting in the cross-linked network which changed in the chemical characteristic of protein (Pojanavaraphan *et al.*, 2010A, Nayak *et al.*, 2012).

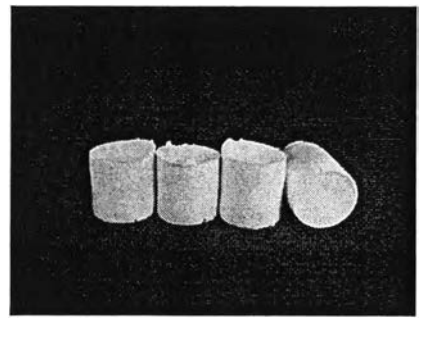

Figure 6.1 Silk sericin/PVA/clay aerogels cross-linked by PEG-DE.

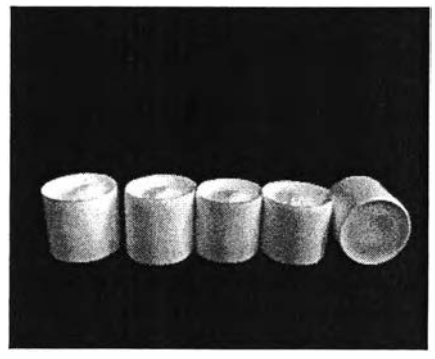

Figure 6.2 Silk sericin/PVA/clay-TEOS aerogels cross-linked by PEG-DE.

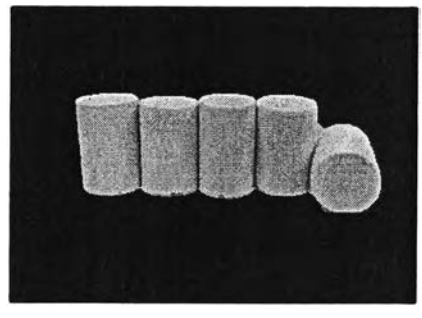

Figure 6.3 Silk sericin/PVA/clay aerogels cross-linked by glutaraldehyde.

6.4.2 Chemical analysis of cross-linked silk sericin/PVA/clay aerogel

Glutaraldehyde is the effective chemical cross-linked agent used to crosslink protein because of the two reactive groups of dicarbonyl compound which can react with amino groups (Pojanvaraphan *et al.*, 2010A). Furthermore, GT usually used as cross-linked agent for hydroxyl groups containing compounds like PVA hydrogel. The reaction took place via carbonyl groups of GT and hydroxyl groups of PVA (Mandel *et al.*, 2011). In silk sericin/PVA/clay aerogel system, the ability to stabilize its shape in aqueous solution came from both intramolecular and intermolecular cross-links. The cross-linked reaction by GT can be occurred between silk sericin or PVA itself, silk sericin and PVA, silk sericin and silanol groups of clay and also between PVA and silanol groups of clay. The proposed cross-linked mechanisms of silk sericin and PVA by GT are shown in Figure 6.4, 6.5 and 6.6, respectively. The cross-linked reaction of GT and silk sericin almost occurred via

amino acid lysine residue due to the highest reactivity of the reaction between aldehyde compound and protein was found in lysine followed by tyrosine, histidine, cystieine, proline, serine, glycine, and arginine, respectively (Migneault *et al.*, 2004).

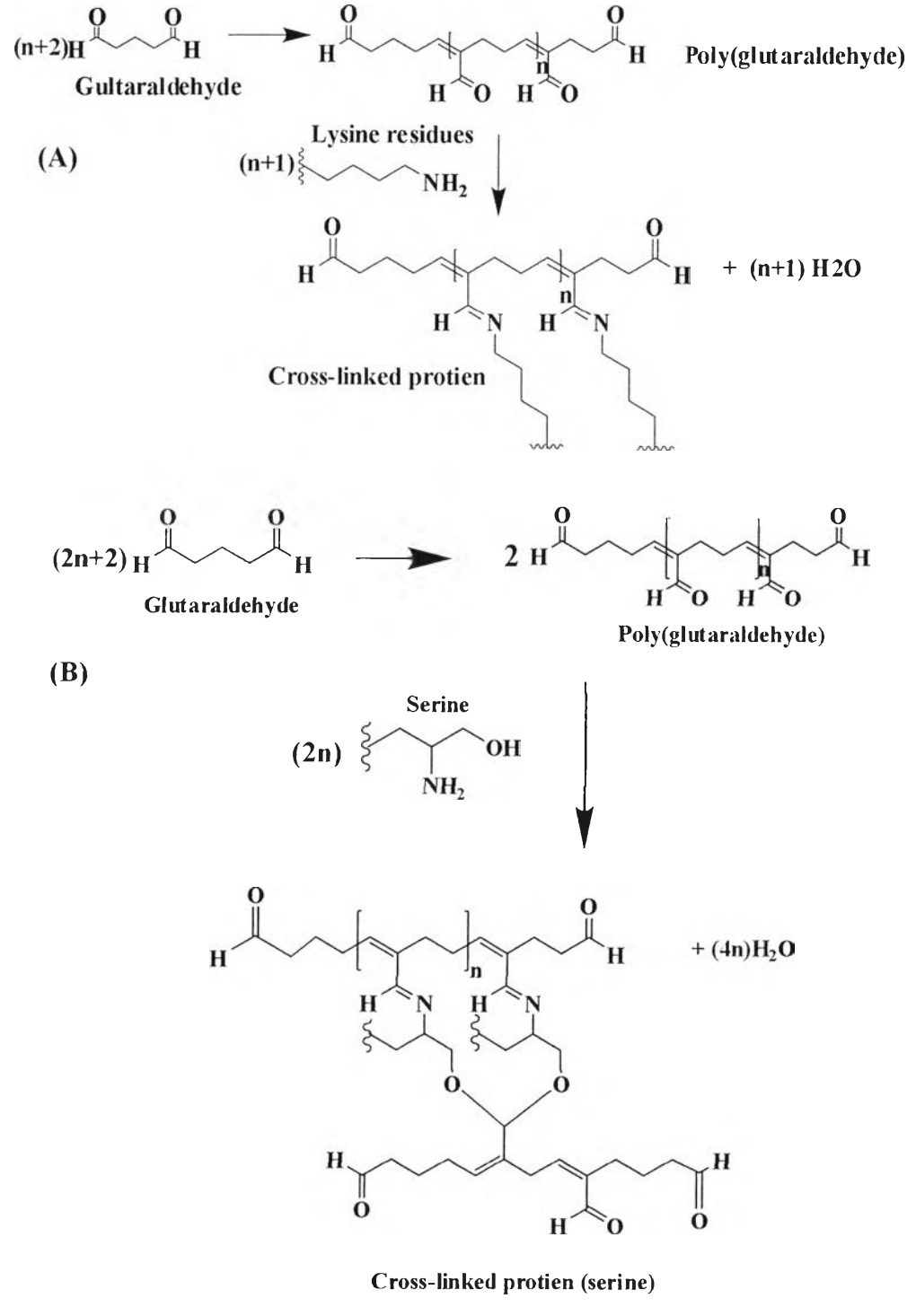

Figure 6.4 Purposed cross-linked mechanism of silk sericin protein by glutaraldehyde (A) Lysine and (B) Serine.

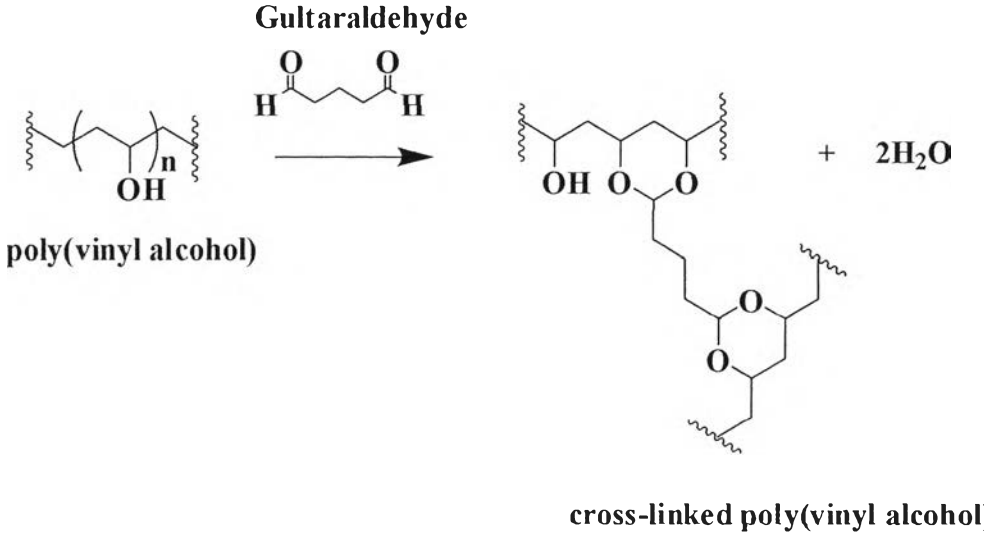

Figure 6.5 Purposed cross-linked mechanism of poly(vinyl alchohol) by glutaraldehyde.

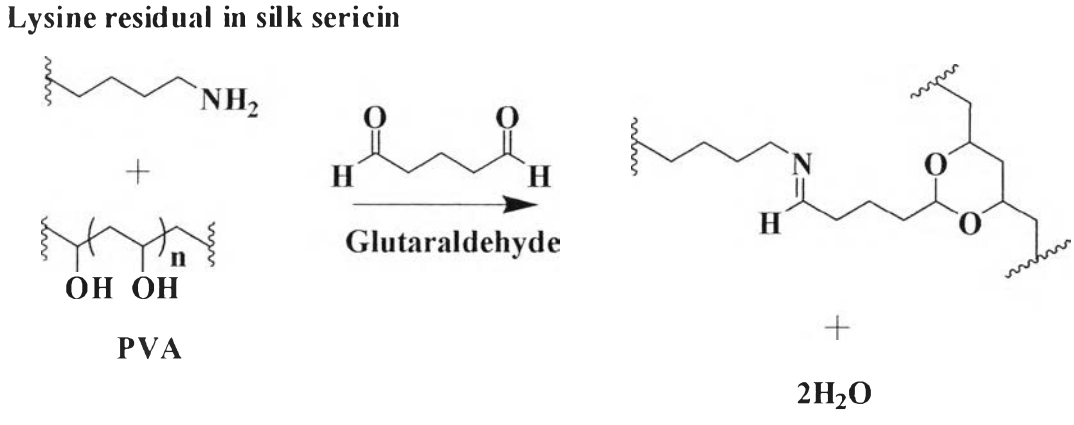

Figure 6.6 Purposed cross-linked mechanism between silk sericin protein and poly(vinyl alchohol) by glutaraldehyde.

The FTIR spectra of uncross-linked and cross-linked silk sericin/PVA/clay aerogels are shown in Figure 6.7. The spectra showed the characteristic peaks of silk sericin, PVA and bentonite. The characteristic peak of silk sericin presented around 1530 cm^{-1} (amide II), 1240 cm^{-1} (amide III) and 1740 cm^{-1} (amide IV)(Nayak *et al.*, 2012). The peaks around 1750 cm^{-1} corresponded to acetate groups remaining in PVA chain owing to the partial hydrolyzed of poly(vinyl

acetate) to poly(vinyl alcohol) (Mansur *et al.*, 2009). The Na-bentonite presented the characteristic peaks around 1040 and 920 cm^{-1} corresponded to Si-O stretching and Al-OH bending (Pojanavaraphan *et al.*, 2010A). The FTIR resulted showed almost similarly spectra in both uncross-linked and cross-linked sample hence this technique cannot used to confirm the cross-linked reaction between silk sericin, PVA and clay bentonite.

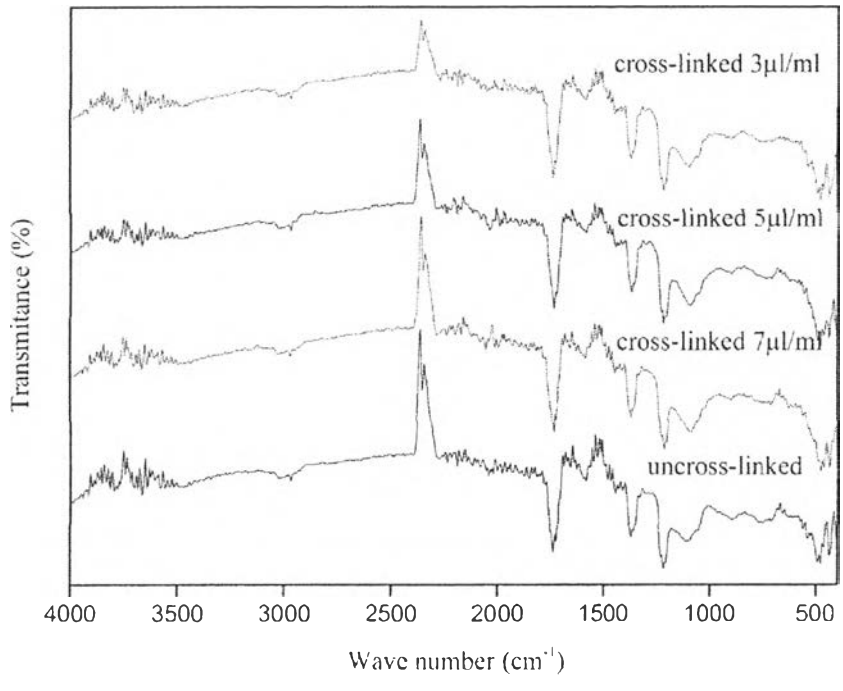

Figure 6.7 FTIR spectra of uncross-linked and cross-linked silk sericin/PVA/clay aerogel.

6.4.3 Morphology of cross-linked silk sericin/PVA/clay aerogels

The FE-SEM micrographs of silk sericin/PVA/clay aerogels cross-linked by glutaraldehyde (GT) with different silk sericin and clay contents are shown in Figure 6.8 and 6.9. The GT cross-linked silk sericin/PVA/clay aerogels exhibited the lamella morphology related to uncross-linked aerogels. The decreasing of clay loading (8 to 2 wt%) resulting in the decreasing of the distance between each layer and porosity because each layer was highly covered with thick and dense network of silk sericin and PVA. Thus, each layers appeared to be thicker and connected by

webs of these two polymers (Gawryla et al., 2008). By decreasing silk sericin from 4 to 1 wt%, the structure changed from co-connected lamella structure to separate lamella structure. The network of cross-linked silk sericin and PVA on the layer of clay is shown in Figure 6.10.

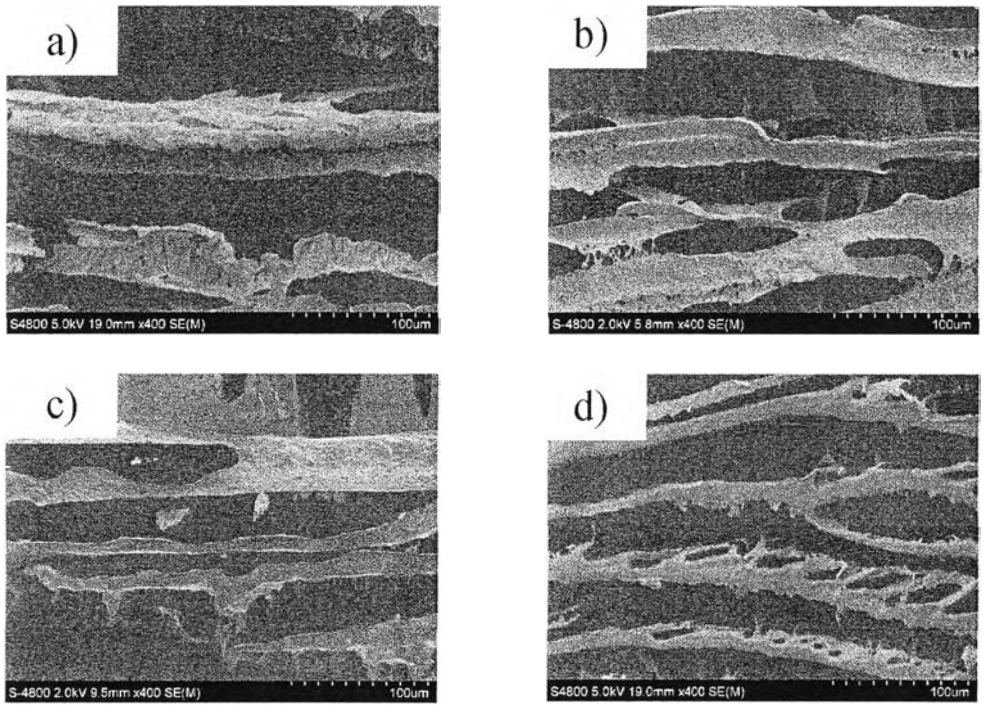

Figure 6.8 FE-SEM micrograph of silk sericin/PVA/clay aerogels cross-linked by glutaraldehyde: (a) C2PVA5SS1 7GT, (b) C4PVA5SS1 0.7GT, (c) C6PVA5SS1 0.7GT and (d) C8PVA5SS1 0.7GT.

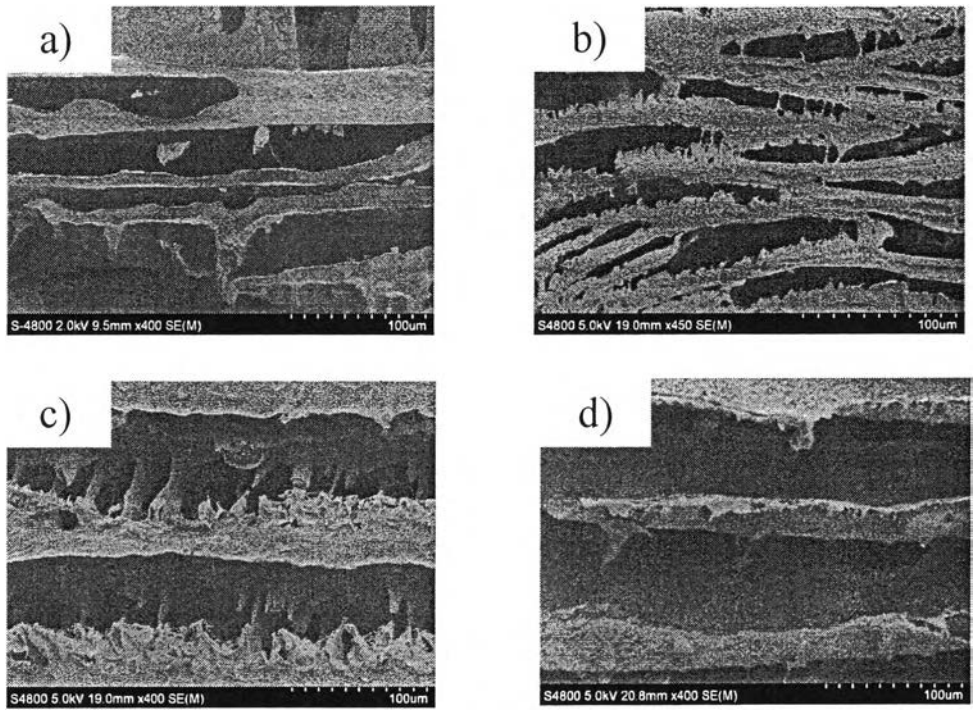

Figure 6.9 FE-SEM micrograph of silk sericin/PVA/clay aerogels cross-linked by glutaraldehyde: (a) C6PVA5SS1 0.7GT, (b) C6PVA5SS2 0.7GT, (c) C6PVA5SS3 0.7GT and (d) C6PVA5SS4 0.7GT.

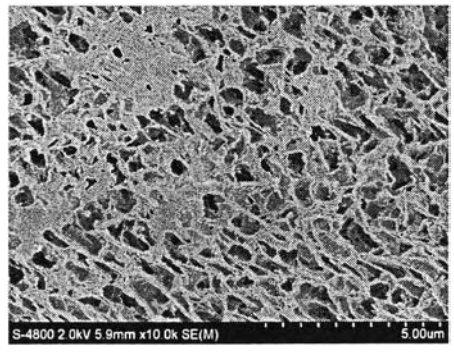

Figure 6.10 FE-SEM micrographs of cross-linked silk sericin/PVA on the surface of clay templates of C6PVA5SS1 0.7GT.

The lamella structure (house of cards structure) with interconnected pore of GT cross-linked silk sericin/PVA/clay aerogels were appropriated for many medical applications like 3D scaffold for tissue engineering. Beside the morphology of the clay aerogels, the bentonite clay also introduced the stiffness structure for the

3D scaffold and improved the biocompatibility between the scaffold composites and cells such as osteoblasts (Haroun *et al.*, 2009, Lui *et al.*, 2011). The efficiency to enhance cell growth of the silk sericin is the other important motive to apply this aerogel into the scaffold for tissue engineering.

6.4.4 Thermogravimetric-differential thermal analyzer

The thermal stability of GT cross-linked silk sericin/PVA/clay aerogels were observed by thermogravimetric analysis. Figure 6.11 - 6.14 show the TGA and DTG thermograms of cross-linked silk sericin/PVA/clay aerogels using 7 µl/ml of GT with various clay and sericin loadings. The GT cross-linked aerogels showed two steps of weight loss. Firstly, the degradation detected up to 100 °C corresponded to the evaporation of water in bulk samples (~1-2 %weight loss) followed by the second step around 270 °C was associated with the decomposition of silk sericin and PVA around 260 °C and 280 °C, respectively. By increasing of clay loading the onset and peak degradation temperatures decreased owing to the oxidation of ferric ion effect (Fe^{3+}) (Morlat *et al,*. 2004, Pojanavaraphan *et al.*, 2010). The onset and peak degradation temperatures of cross-linked silk sericin/PVA/clay aerogels significantly increase when the silk sericin loading increased. This result suggested the anti-oxidation property of silk sericin that can be limited the oxidation of ferric ion. The increasing number of hydrogen bonds between silk sericin, PVA molecules and silanol groups of clay bentonite was the another important factor to promote the thermal stability of the aerogels (Teramoto *et al*, 2007). Table 6.1 summarizes the thermal stability of cross-linked silk sericin/PVA/clay aerogel with different silk seicin and clay content at 7 µl/ml of GT. The aerogels using the concentrations of glutaraldehyde at 5 µl/ml and 3 µl/ml were exhibited the same thermal behavior as silk sericin/PVA/clay aerogel with 7 µl/ml of GT.

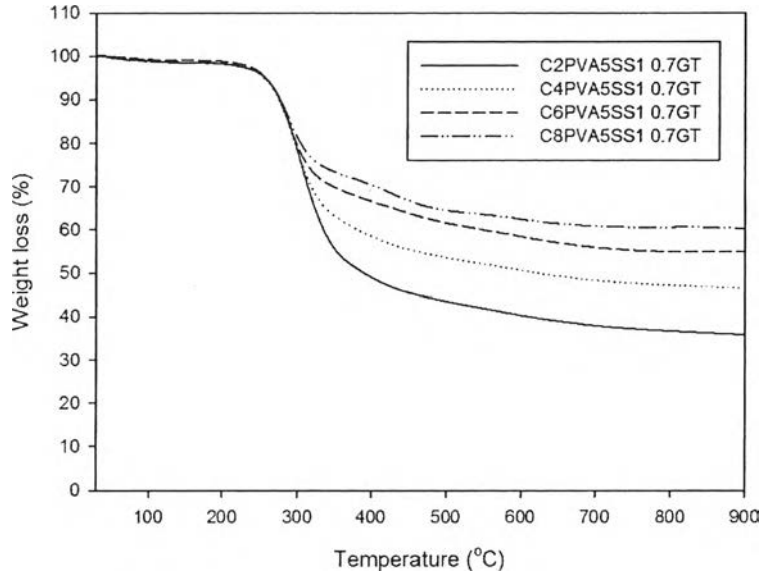

Figure 6.11 TGA thermograms of cross-linked silk sericin/PVA/clay aerogels with various clay contents.

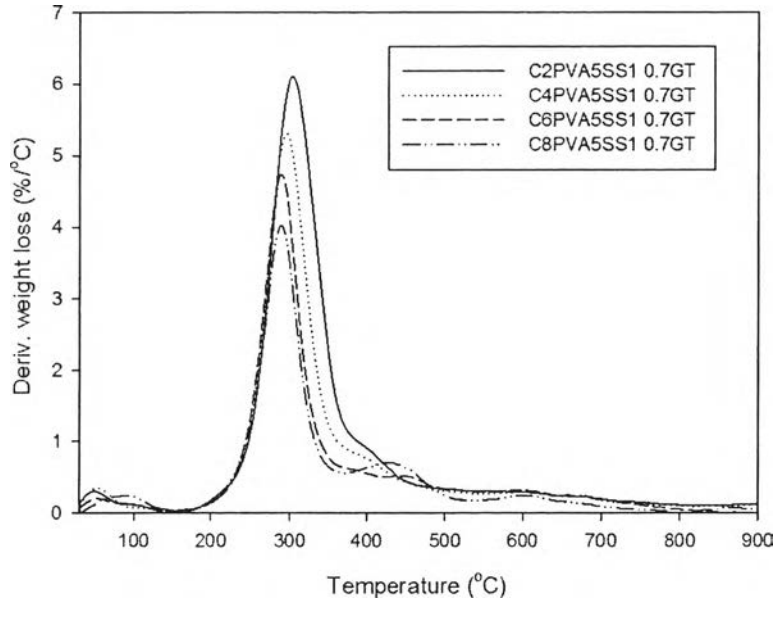

Figure 6.12 DTG thermograms of cross-linked silk sericin/PVA/clay aerogels with various clay contents

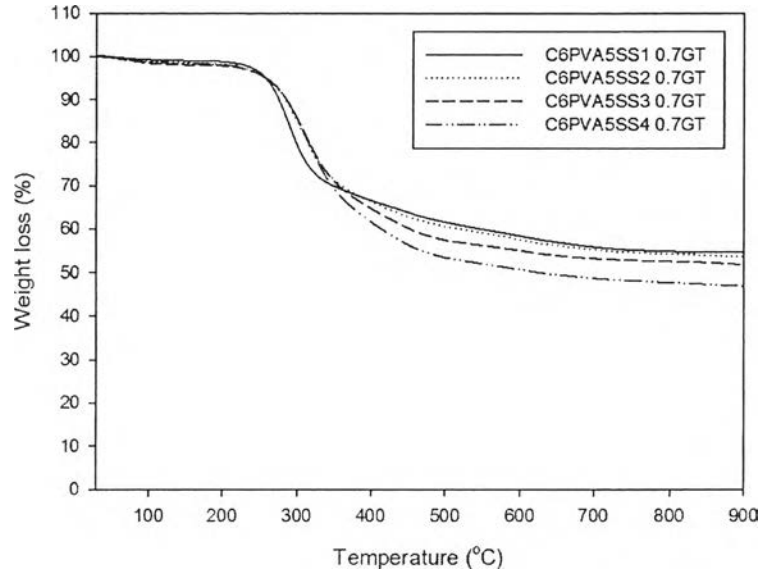

Figure 6.13 TGA thermograms of cross-linked silk sericin/PVA/clay aerogels with various silk sericin content.

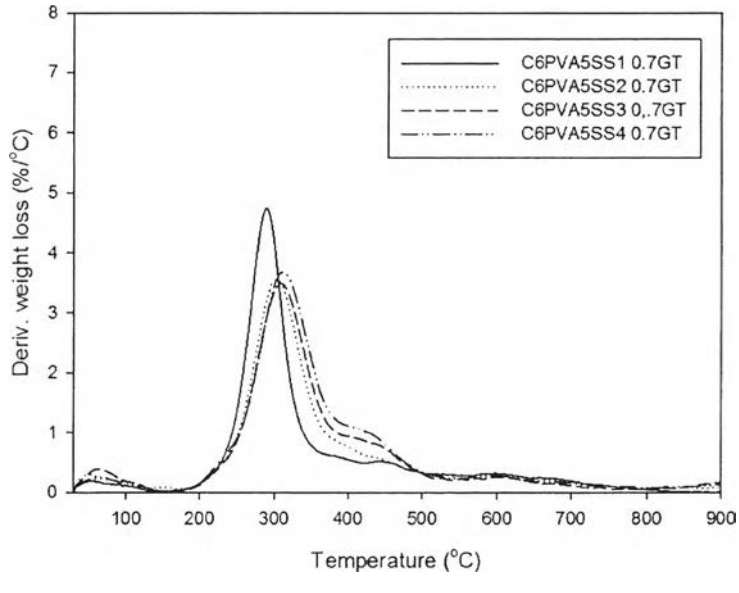

Figure 6.14 DTG thermograms of cross-linked silk sericin/PVA/clay aerogels with various silk sericin contents.

Table 6.1 Thermal stability of cross-linked silk sericin/PVA/clay aerogels with various clay contents

Samples	T_d onset (°C)	Peak decomposition (°C)	Char residual (%)	Moisture content (%)
C2PVA5SS1 0.7GT	266.9	302.9	37.2	1.1
C4PVA5SS1 0.7GT	261.4	294.4	47.9	1.3
C6PVA5SS1 0.7GT	257.4	289	55.9	1.1
C8PVA5SS1 0.7GT	256.9	288.5	61.9	1.3

Table 6.2 Thermal stability of cross-linked silk sericin/PVA/clay aerogels with various silk sericin contents

Samples	T_d onset (°C)	Peak decomposition (°C)	Char residual (%)	Moisture content (%)
C6PVA5SS1 0.7GT	257.4	289	55.9	1.1
C6PVA5SS2 0.7GT	262.9	301.8	55	1.4
C6PVA5SS3 0.7GT	264.7	306.4	53.2	1.9
C6PVA5SS4 0.7GT	265.3	311.2	48.4	1.7

Cross-linked by glutaraldehyde showed the slight decreased in thermal stability compared with uncross-linked aerogel. Owing to the formation of covalent bond formed from the cross-linked reaction between silk sericin, PVA and silanol group of bentonite decreased the strong hydrogen bond between silk sericin and PVA chains. This effect was obviously observed in the aerogel with high content silk

sericin. The concentration of GT was not significant effect on the thermal stability of silk sericin/PVA/clay aerogel. Fig 6.15 and 6.16 show the TGA thermograms of silk sericin/PVA/clay aerogel both uncross-linked and cross-linked with various concentrations of GT. At higher concentration of GT, the thermal stability tended to be slightly decreased due to the formation of cross-linked network of silk sericin and PVA which linked together by covalent bond were increased resulted in the powerfully decreased in strong hydrogen bond between silk sericin and PVA chain. Table 6.3 compared thermal behavior of uncross-linked and cross-linked silk sericin/PVA/clay aerogel at with different concentrations of GT.

Almost TGA thermograms of GT cross-linked silk sericin/PVA/clay aerogel showed less water absorption content suggesting less polar side group due to cross-linked reaction. The higher residue content of GT cross-linked aerogel was than those of the uncross-linked ones. This better thermal stability of the cross-linked aerogels supported the success of crosslinking reactions taken placed in PVA and sericin by glutaraldehyde.

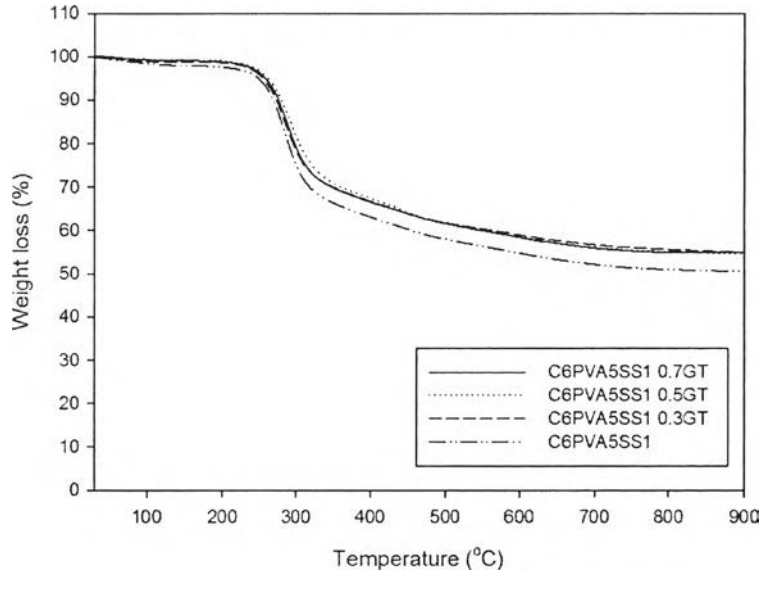

Figure 6.15 TGA thermograms of uncross-linked and cross-linked silk sericin/PVA/clay aerogels with various glutaraldehyde contents.

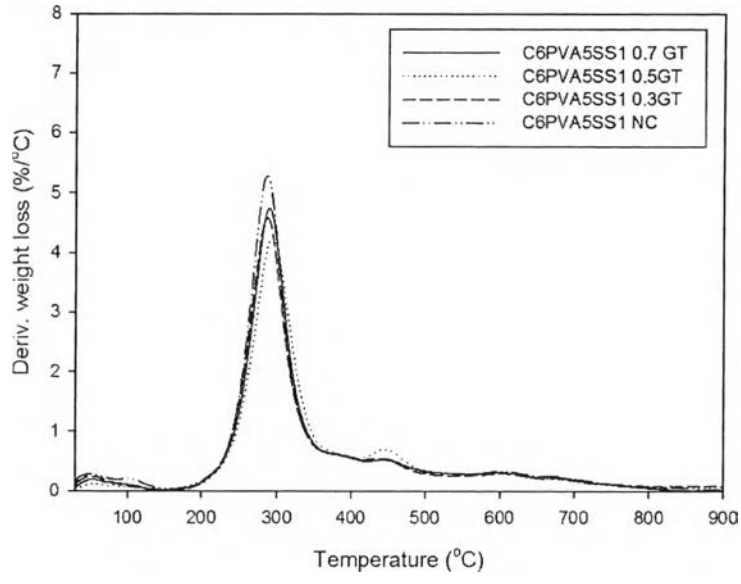

Figure 6.16 DTG thermograms of uncross-linked and cross-linked silk sericin/PVA/clay aerogels with various glutaraldehyde contents.

Table 6.3 Thermal stability of uncross-linked and cross-linked silk sericin/PVA/clay aerogels with various glutaraldehyde contents

Samples	T_d onset (°C)	Peak decomposition T (°C)	Char residual (%)	Water loss (%)
C6PVA5SS1	255.9	285.7	52.6	1.9
C6PVA5SS1 0.3GT	254	285.3	56.2	1.3
C6PVA5SS1 0.5GT	258.5	292.9	55.3	0.7
C6PVA5SS1 0.7GT	257.4	289	55.9	1.1
C6PVA5SS2	266.6	305.8	52.0	1.6
C6PVA5SS2 0.3GT	263.1	302.5	52.1	1.2
C6PVA5SS2 0.5GT	260.3	299.8	50.5	1.1
C6PVA5SS2 0.7GT	262.9	301.8	55	1.4
C6PVA5SS3	273.1	317.6	51.3	1.7
C6PVA5SS3 0.3GT	266	308.4	49.9	1.9
C6PVA5SS3 0.5GT	261.6	307.9	53	1.2
C6PVA5SS30.7GT	264.7	306.4	53.2	1.9
C6PVA5SS4	275.2	319.3	50.70	1.5
C6PVA5SS4 0.3GT	268.7	313.3	47	1.7
C6PVA5SS4 0.5GT	269	313.6	47.2	1.7
C6PVA5SS4 0.7GT	265.3	311.2	48.4	1.7

6.4.5 Mechanical properties of cross-linked silk sericin/PVA/clay aerogels

The compressive stress-strain curves of GT cross-linked silk sericin/PVA/clay aerogels are shown in Figures 6.17 and 6.18. The mechanical properties of GT cross-linked silk sericin/PVA/clay aerogels are summarized in Table 6.4 The GT cross-linked silk sericin/PVA/clay aerogels had the stress-strain curves followed the pattern of conventional rigid form like behavior. At low strain (initial loading), aerogels showed the linear-elastic deformation. Increasing

compression load, the horizontal plateau was observed followed by the densification region where all porous were collapsed (Pojanavaraphan *et al.*, 2008, Pojanavaraphan *et al.*, 2010). The GT cross-linked aerogels exhibited non-elastic materials which the shape of the aerogels were not recovery after compression testing. The bentonite clay and silk sericin contents were strongly affected to the mechanical properties of the aerogels; increasing clay contents (2-6 wt%), the initial modulus dramatically increased from 580 up to 6963 kPa because of the high reinforcing efficiency of clay bentonite excepted in the case of aerogels with 8 wt% of clay, the initial modulus and others mechanical properties were slightly fallen down. From the preparation, 8 wt% of clay produced very high viscous clay gel dispersion and difficult to process resulting in inhomogeneous dispersion of the aqueous clay gel precursor and the structure defects such as air bubbles was observed. Similarly to clay content, the increasing silk sericin contents greatly improved the mechanical properties of the aerogels since the large amounts of silk sericin enlarged the interpenetrating co-continuous networks (Alhassan *et al.*, 2010) and fulfilled the cavities between clay layer lead to the denser materials.

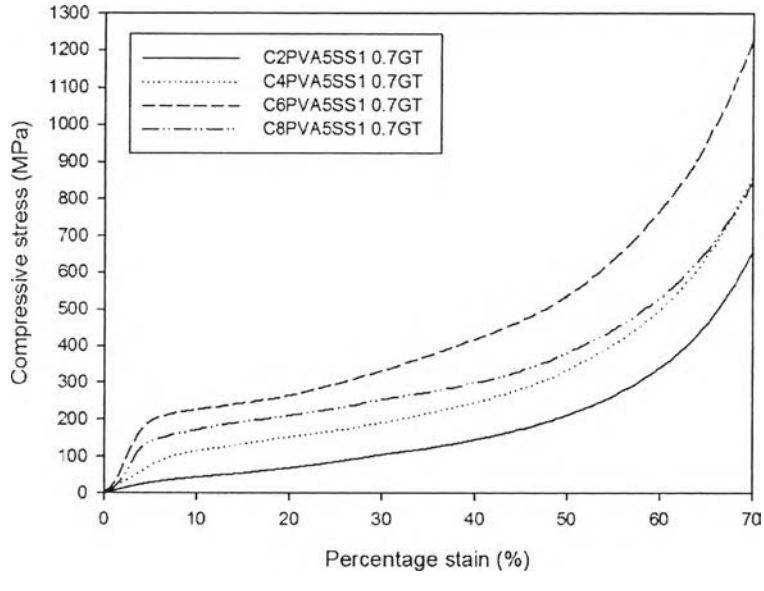

Figure 6.17 Stress-strain curves of cross-linked silk sericin/PVA/clay aerogels with various clay contents

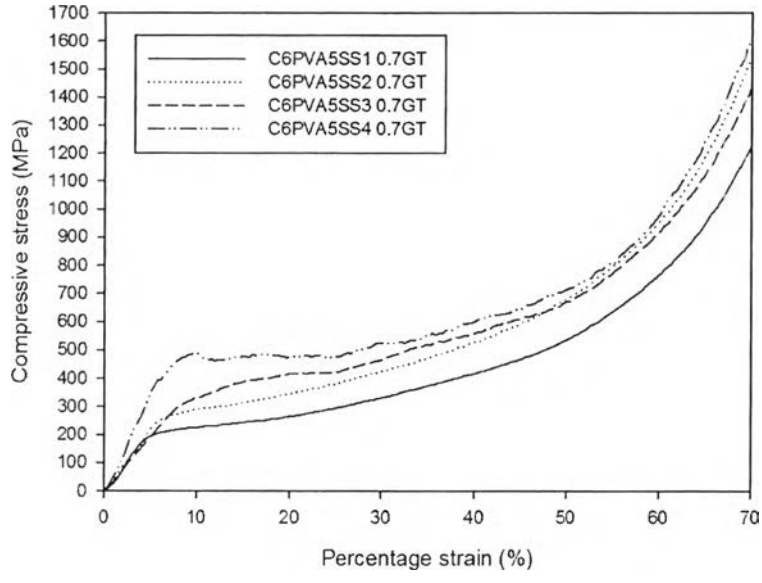

Figure 6.18 Stress-strain curves of silk sericin/PVA/clay aerogels with various silk sericin contents

Table 6.4 Mechanical properties of cross-linked silk sericin/PVA/clay aerogels at 7 µl/ml of GT with various silk sericin and clay contents

Samples	Initial modulus (kPa)	Young's Modulus (kPa)	Stiffness (kN/m^2)
C2PVA5SS1 0.7GT	580±40	4460±513	49±5.5
C4PVA5SS1 0.7GT	1497±126	6200±328	64±4.1
C6PVA5SS1 0.7GT	2943±309	6330±780	68±7.6
C8PVA5SS1 0.7GT	2656±373	4775±224	56±3.6
C6PVA5SS2 0.7GT	3673±103	7706±875	67±4.7
C6PVA5SS3 0.7GT	4671±710	7904±431	81±6.3
C6PVA5SS4 0.7GT	6963±666	14579±1427	109±16.8

Cross-linked by GT improved the mechanical properties of the silk sericin/PVA/clay aerogel. When compared between uncross-linked and GT cross-linked silk sericin/PVA/clay aerogels, GT cross-linked approximately 50%

improvements in initial modulus. According to Pojanavaraphan *et al.*, 2010A, the cross-linked network can dissipated energy more efficiently under apply load than uncross-linked. Moreover, cross-linked silk sericin exhibit high content of β-sheet structure hence the mechanical properties can be improved (Tao *et al.*, 2004). The concentrations of glutaraldehde were influence on the mechanical properties. As increasing of glutaraldehyde concentration, the mechanical properties of the aerogels were increased owing to the higher covalent bonds formed from cross-linked reaction resulting in the large amount of network structure. But in the case of aerogels cross-linked by 7 µl/ml of GT, the initial modulus was slightly dropped. When the cross-linked networks were formed, viscosity of the clay gel precursor was increased resulting in bubbles defect in the sample hence the mechanical properties was fallen down. The stress-strain curves and initial modulus compared between uncross-linked and cross-linked silk sericin/PVA/clay aerogels at composition of C6PVA5SS1 with different concentrations of GT are shown in Figure 6.19 and Table 6.4, respectively.

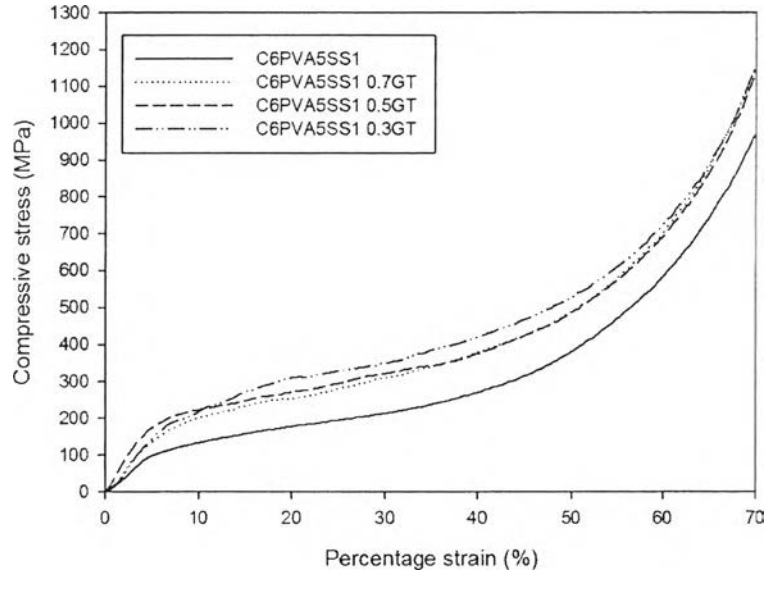

Figure 6.19 Stress-strain curves of silk sericin/PVA/clay aerogels at the composition of C6PVA5SS1 with uncross-linked and cross-linked with 3, 5 and 7 µl/ml of glutaraldehyde.

Table 6.5 Mechanical properties of cross-linked silk sericin/PVA/clay aerogel with and without glutaraldehyde

Samples	Initial modulus (kPa)	Young's Modulus (kPa)	Stiffness (kN/m^2)
C6PVA5SS1	1869±320	5965±674	65±6.4
C6PVA5SS1 0.3GT	3296±469	7192±335	77±4.5
C6PVA5SS1 0.5GT	3958±536	7104±812	68±7.8
C6PVA5SS1 0.7GT	2943±308	6329±780	68±7.6

6.4.6 Swelling behavior

In order to use silk sericin/PVA/clay aerogel for tissue engineering, scaffold should be able to conserve the 3D shape and porous while being immersed in an aqueous cell suspension or human body fluid for a period of time. The swelling ratio (SR) is an important index for the application of scaffold and is related to the composition of a biocomposite, pore size and interconnection of pores (Haroun *et al.*, 2009, Lungu *et al.*, 2012). The swelling ratio of cross-linked silk sericin/PVA/clay aerogel was in the range between 800-1300% depended on the composition and cross-linked agent concentration. The high swelling ratio of the aerogel corresponded to the high porosity with interconnected pore and large pore size which was confirmed by FE-SEM micrographs.

Figure 6.20, 6.21 and 6.22 presented the SR of cross-linked silk sericin/PVA/clay aerogel plotted as a function of time with different clay loadings. The results suggested the SR increased with time and the initial SR of the aerogel increased rapidly and reached equilibrium in a short period of time. Clay loading had powerfully influenced on the swelling behavior of the aerogels. While the clay loading increased, the SR decreased because the clay bentonite sheets limited the interaction and absorption between silk sericin, PVA and swelling media (Haroun *et al.*, 2009, Pojanavaraphan *et al.*, 2008). In the case of 2 wt% of clay (1 wt% of silk sericin), the aerogels exhibited the maximum swelling ratio around 1200-1600% but the maximum swelling ratio was decreased to 900-1100% when the clay loading

raised up to 8 wt% of clay. The swelling behavior of C2PVA5SS1 0.3GT could not observed and was not reported in Figure 6.20. Due to the samples from C2PVA5SS1 0.3GT were unstable in PBS buffer solution because the ratio of polymer and cross-linked agent was too high hence the concentration of GT was not enough to cross-link all of polymer chains lead to the low cross-linked density.

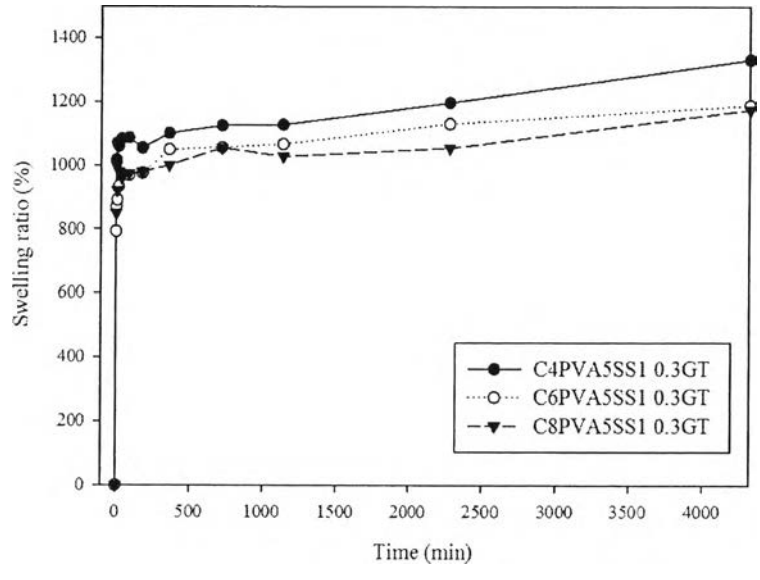

Figure 6.20 Swelling ratio of cross-linked silk sericin/PVA/clay aerogels plotted as a function of time at the composition of silk sericin 1 wt%, PVA 5 wt% and GT 3 μl/ml with different clay loading.

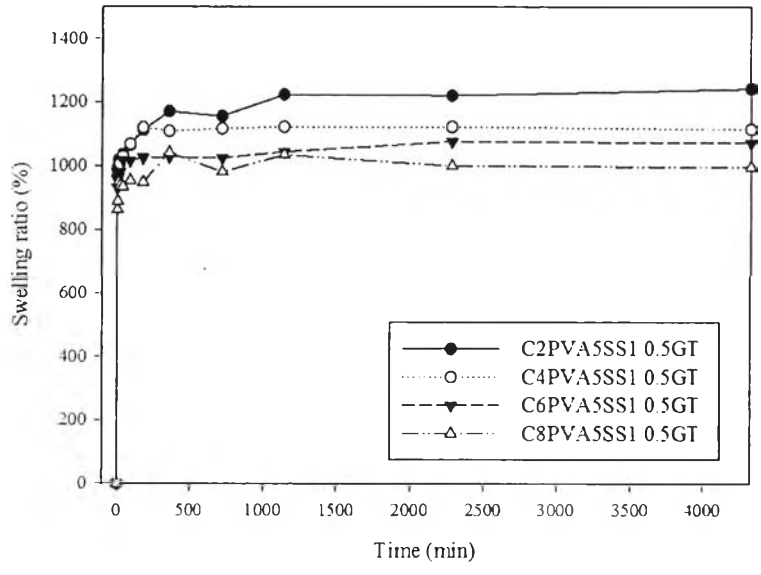

Figure 6.21 Swelling ratio of cross-linked silk sericin/PVA/clay aerogels plotted as a function of time at the composition of silk sericin 1 wt%, PVA 5 wt% and GT 5 μl/ml with different clay loading

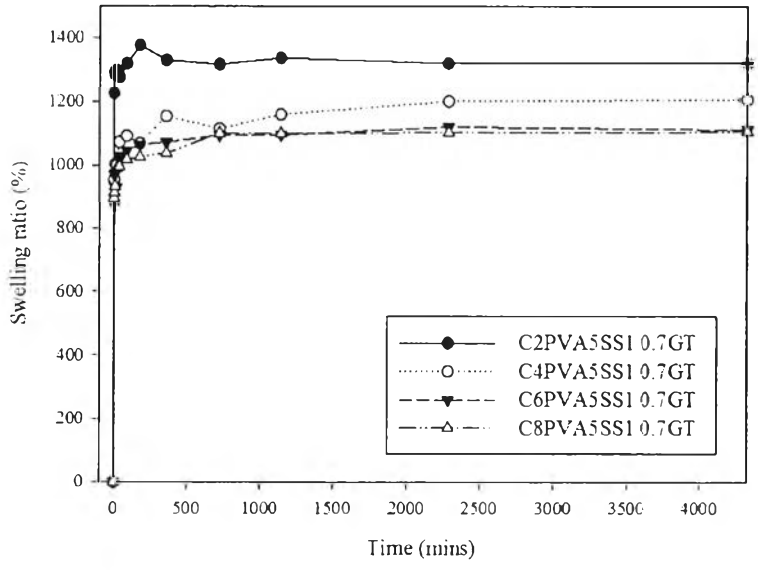

Figure 6.22 Swelling ratio of cross-linked silk sericin/PVA/clay aerogels plotted as a function of time at the composition of silk sericin 1 wt%, PVA 5 wt% and GT 7 μl/ml with different clay loading.

The influence of silk sericin content on SR was measured as shown in Figure 6.23, 6.24 and 6.25. The SR significantly decreased with the increasing of silk

sericin contents. The interconnected pore and pore size were the key factor to enhanced water uptake of porous materials. As increasing silk sericin content from 1 to 4 wt%, the pore size was reduced because silk sericin was fulfilled the space in the pore lead to the denser material and the distance between layer was decreased hence the water uptake was restricted and water molecules was slowly penetrated in to inner core of the aerogel (Mandal *et al.*, 2009, Lungu *et al.*, 2012). At 1 wt% of sericin (6 wt% clay), the maximum swelling ratio was in the range 1000-1200%, increasing silk sericin content up to 4 wt%, the maximum swelling ratio was reduced to 800-900%.

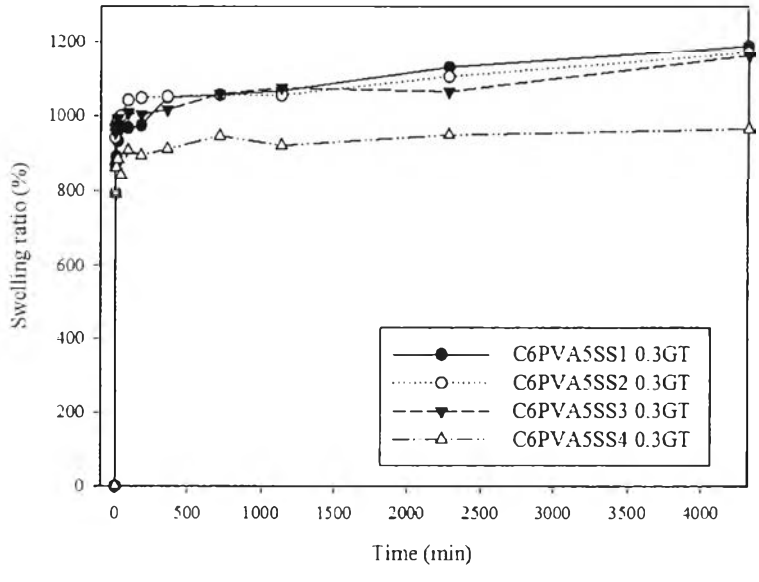

Figure 6.23 Swelling ratio of cross-linked silk sericin/PVA/clay aerogels plotted as a function of time at the composition of clay 6 wt%, PVA 5 wt% and GT 3 μl/ml with different silk sericin contents.

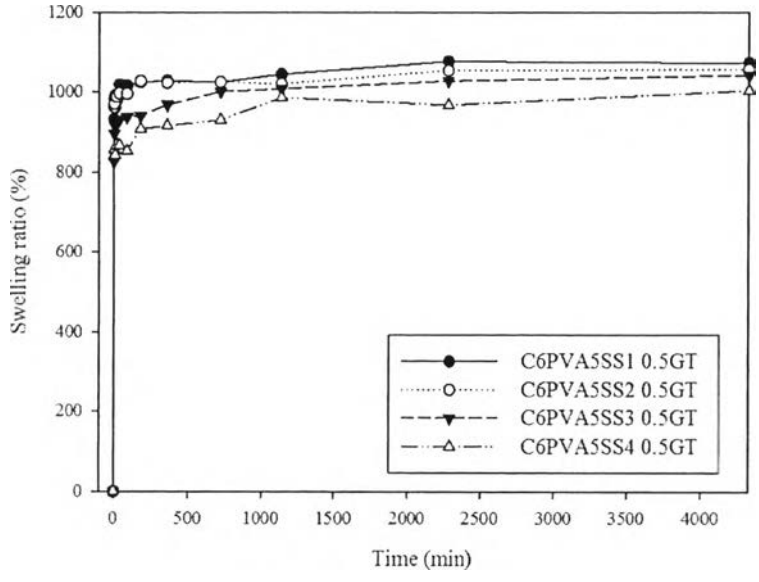

Figure 6.24 Swelling ratio of cross-linked silk sericin/PVA/clay aerogels plotted as a function of time at the composition of clay 6 wt%, PVA 5 wt% and GT 5 μl/ml with different silk sericin contents.

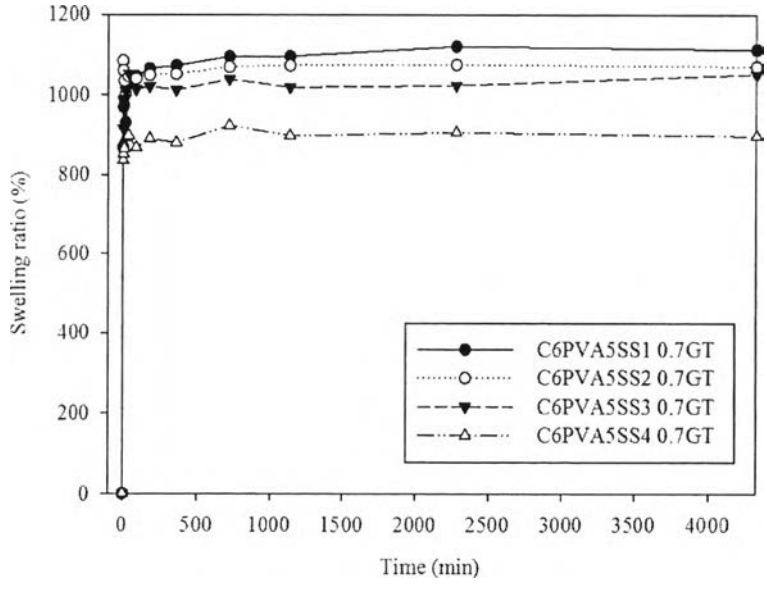

Figure 6.25 Swelling ratio of cross-linked silk sericin/PVA/clay aerogels plotted as a function of time at the composition of clay 6 wt%, PVA 5 wt% and GT 7 μl/ml with different silk sericin contents.

The effect of GT concentration on SR is shown in Fig 6.26. The SR was decreased with the increasing of GT concentrations. The highest of maximum

swelling ratio was observed in aerogel with 3µl/ml of GT and the swelling ratio of this composition tended to be higher or the swollen gel was not reached the equilibrium after 3 days of the test. The network of silk sericin and PVA formed from the chemical cross-linked by GT was hold the clay bundles and limited the swelling of silk sericin and PVA themselves. At high GT concentration, the degree of cross-linked was higher than small GT concentration lead to the reducing in swelling ratio. The maximum swelling ratio of aerogel with 7 µl/ml of GT was slightly higher than aerogels with 5 µl/ml because during the cross-linked reaction taken placed; the clay gel precursor was higher viscous owing to the formation of covalent bond between GT, silk sericin, PVA and silanol groups of clay lead to the bubble defect generated inside the structure of the aerogel. The bubbles defect could be preserved the solution inside the cavities resulting in the higher water uptake hence the swelling ratio was increased.

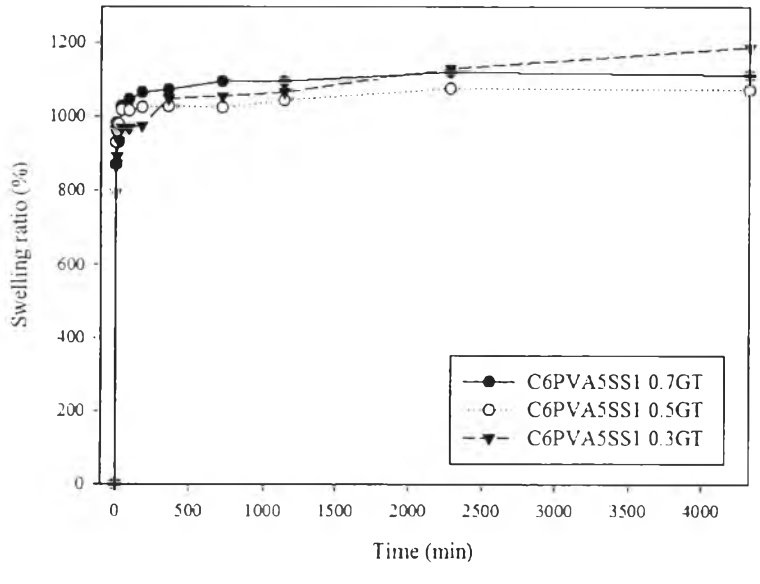

Figure 6.26 Swelling ratio of cross-linked silk sericin/PVA/clay aerogels plotted as a function of time at the composition of C6PVA5SS1 with different glutaraldehyde concentrations.

The species of silk sericin were also affected on the swelling ratio of the silk sericin/PVA/clay aerogel. The swelling ratio of silk sericin/PVA/clay aerogel

as function of time with different species is shown in Figure 6.27 The highest maximum swelling ratio was discovered in Luang Pairote species (~1200%) and the lowest was found in Nang Lai species (~1000%). Because of Luang Pairote had the highest polar amino acid serine and aspartic acid content when compared with other species hence Luang Pairote could be absorbed water more than others resulting in the high swelling ratio. In contrast, Nang Lai had the lower amino acid serine than others therefore water uptake was reduced. The amino acid composition in each species was showed in Table 4.3 in chapter 4.

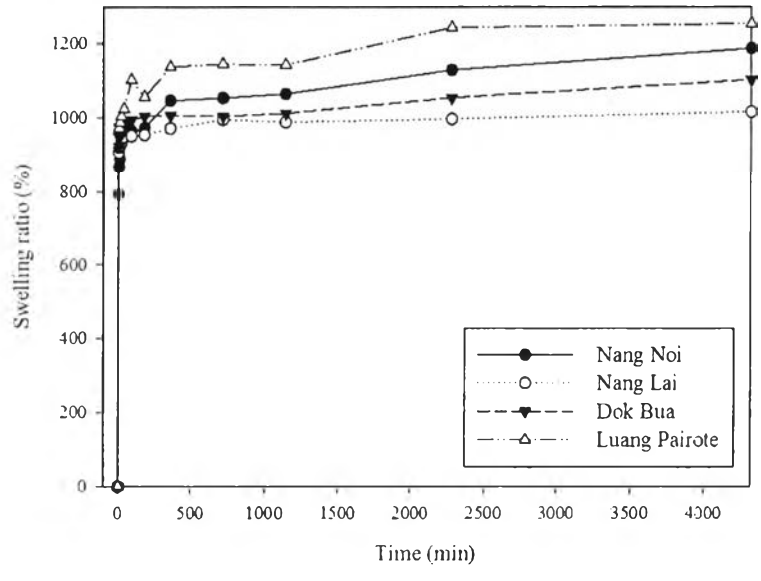

Figure 6.27 Swelling ratio of cross-linked silk sericin/PVA/clay aerogels plotted as a function of time at the composition of C6PVA5SS1 with different species of Thai silk cocoon.

6.4.7 *In vitro* biological tests

With the purpose of using silk sericin/PVA/clay aerogels as a scaffold for periodontal disease, the direct contact test and MTT assay were used to confirm the non-cytotoxicity of the aerogel. The human gingival fibroblasts (HGF) from the passage 5 which obtained from two human donor were cultured on the sponge silk sericin/PVA/clay aerogel in the composition: C6PVA5SS1 0.3GT, C6PVA5SS2 0.3GT, C6PVA5SS3 0.3GT, C6PVA5SS4 0.3GT, C6PVA5SS1 0.5GT and C6PVA5SS1 0.7GT in order to investigated the effect of silk sericin content and GT

concentration to the cell cytotoxicity and cell viability. From this point, the word "sponge" was referred to silk sericin/PVA/clay aerogel after immersed in cell culture media.

6.4.8.1 Direct contact test

Direct contact test is the qualitative test was carried out so as to study the interaction between cells and scaffold and the formation of cell cluster to support the biocompatibility and non-cytotoxicity by directly cultured cells on to the scaffold. Figure 6.28 shows the optical photographs presented the interaction between HGF and sponge in different silk sericin contents. The results indicated the positive attachment and growth of HGF on the sponge after 48 hr and 72 hr of the test. Within 72 hr, the outside layer surface of the sponge was abundantly covered by cell clusters (fiber shape) and confluent cells (round shape), demonstrating that the silk sericin/PVA/clay aerogels in all contents of silk sericin were not direct toxic to HGF.

Figure 6.29 presents the optical photographs of the interaction between HGF and sponge with various GT concentrations. The resulted revealed that the range of GT concentrations between 3-7 µl/ml were not exhibited cytotoxicity to HGF due to the fully covered of HGF around the sponges surface was observed. In the case of sponge with 3 µl/ml of GT, the fragmentation of sponge was observed owing to the low concentration of cross-linked agent resulted in inferior cross-linked density.

The silk sericin/PVA/clay aerogel with different species of Thai silk cocoon was tested on the cytotoxicity using direct contact test and the results as shown in Figure 6.30. The result suggested that all species of Thai silk sericin exhibited cytocompatibility with HGF. As mentioned earlier, the cytocompatibility could be observed by the growth and the attachment of HGF around the surface of sponge in all species of Thai silk cocoon. Within 48 hr, the growth of HGF was observed around the scaffold and the density of HGF was dramatically increased in 72 hr.

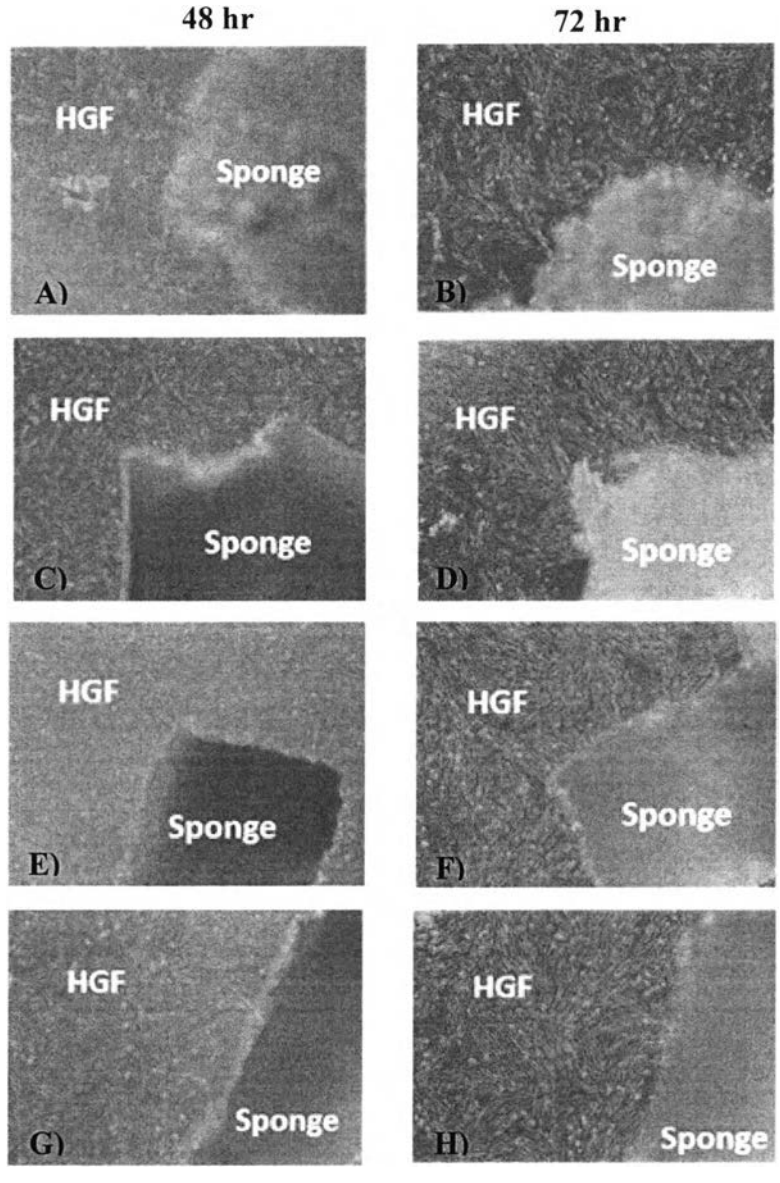

Figure 6.28 Optical photograph were taken by inverted phase contrast microscope presented the interaction between HGF and sponge with different silk sericin contents in 48 and 72 hr ; A) C6PVA5SS1 0.3GT at 48 hr., B) C6PVA5SS1 0.3GT at 72 hr., C) C6PVA5SS2 0.3GT at 48 hr., D) C6PVA5SS2 0.3GT at 72 hr., E) C6PVA5SS3 0.3GT at 48 hr., F) C6PVA5SS3 0.3GT at 72 hr., G) C6PVA5SS4 0.3GT at 48 hr. and H) C6PVA5SS4 0.3GT at 72 hr.

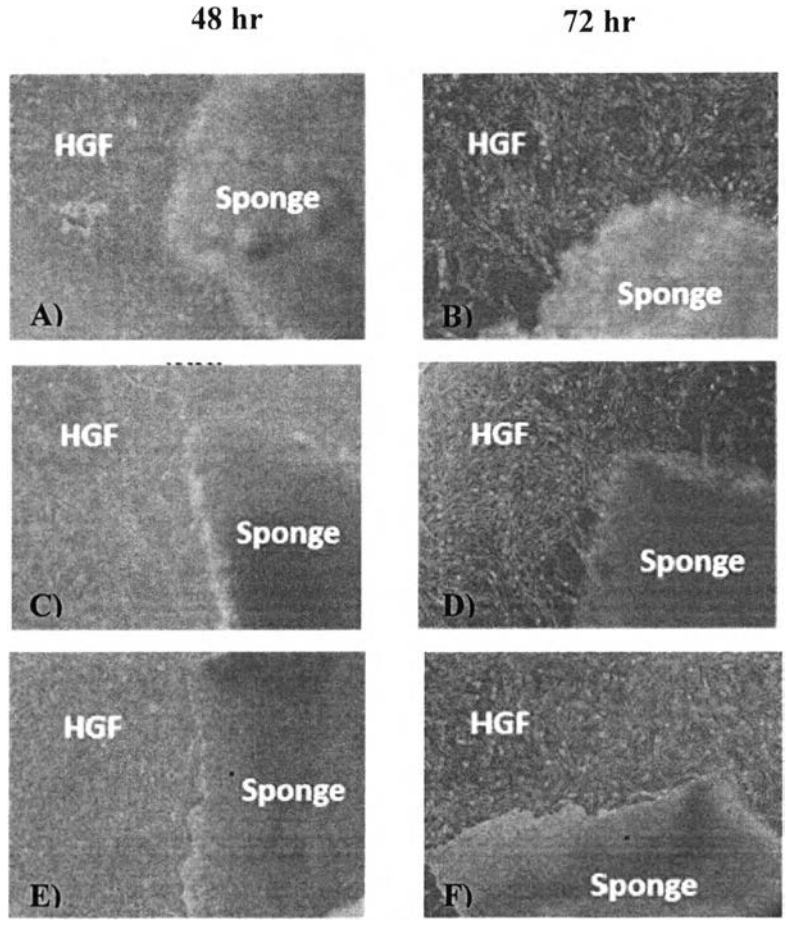

Figure 6.29 Optical photographs were taken by inverted phase contrast microscope presented the interaction between HGF and sponge with different glutaraldehyde concentration in 48 and 72 hr ; A) C6PVA5SS1 0.3GT at 48 hr., B) C6PVA5SS1 0.3GT at 72 hr., C) C6PVA5SS1 0.5GT at 48 hr., D) C6PVA5SS1 0.5GT at 72 hr., E) C6PVA5SS1 0.7GT at 48 hr. and F) C6PVA5SS1 0.7GT at 72 hr.

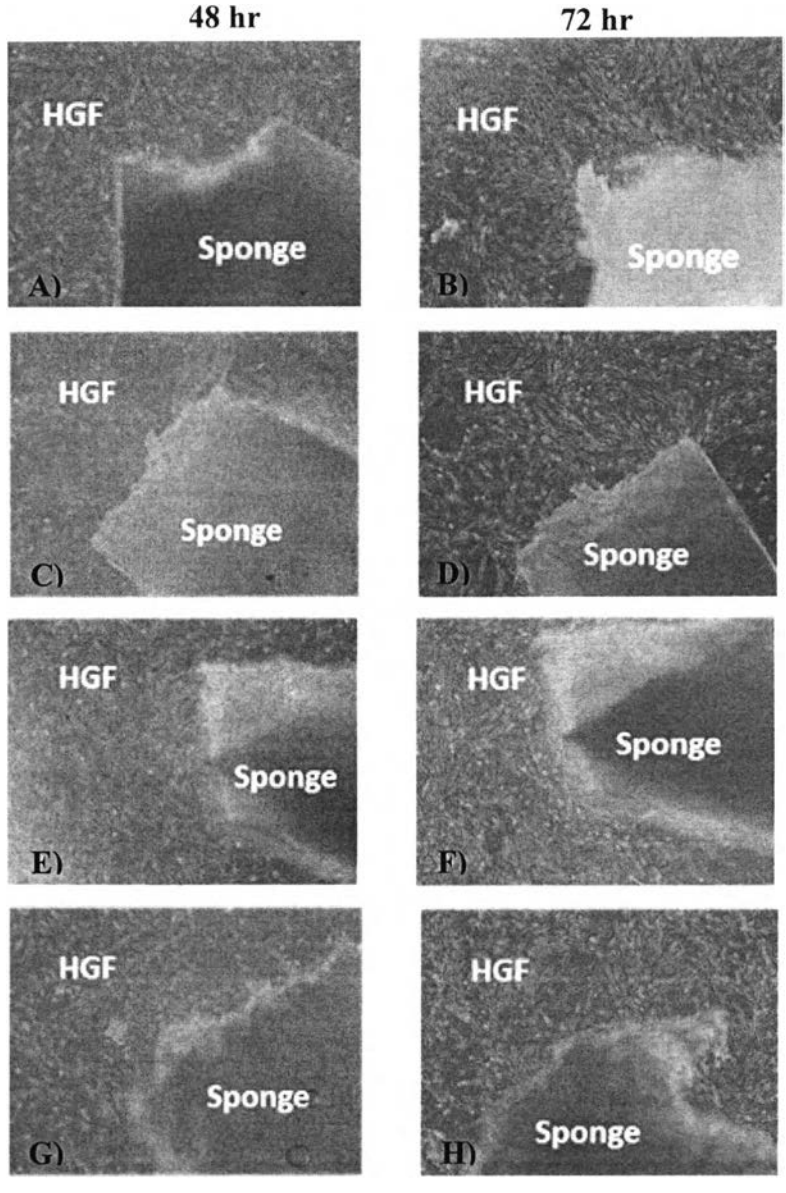

Figure 6.30 Optical photographs were taken by inverted phase contrast microscope presented the interaction between HGF and sponge with different species of Thai silk cocoon at the composition of C6PVA5SS1 0.3GT in 48 and 72 hr ; A) Nang Noi species at 48 hr., B) Nang Noi species at 72 hr., C) Nang Lai species at 48 hr., D) Nang Lai species at 72 hr., E) Dok Bua species at 48 hr., F) Dok Bua species at 72 hr., G) Luang Pairote species at 48 hr. and H) Luang Pairote species at 72 hr.

For the second donor contributions, the same resulted was observed as shown in Appendix D. From direct contact test, the results indicated that

the silk sericin/PVA/clay aerogel can be a good candidate for the 3D scaffold for periodontal disease because the sponge from silk sericin/PVA/clay aerogel presented the positive results (non-cytotoxicity and cytocompatibility) with human gingival fibroblasts which are the one kind of periodontitis cells.

6.4.8.2 MTT assay

MTT assay is the quantitative test used to studied cell viability and mitochondria activities in order to confirm the biocompatibility and cytotoxicity by indirect assay. The viability of cell and mitochondria activities were determined by the formation of formazan crystals and measured by using the optical density of the cell cultured media of samples compared with cell culture media of control.

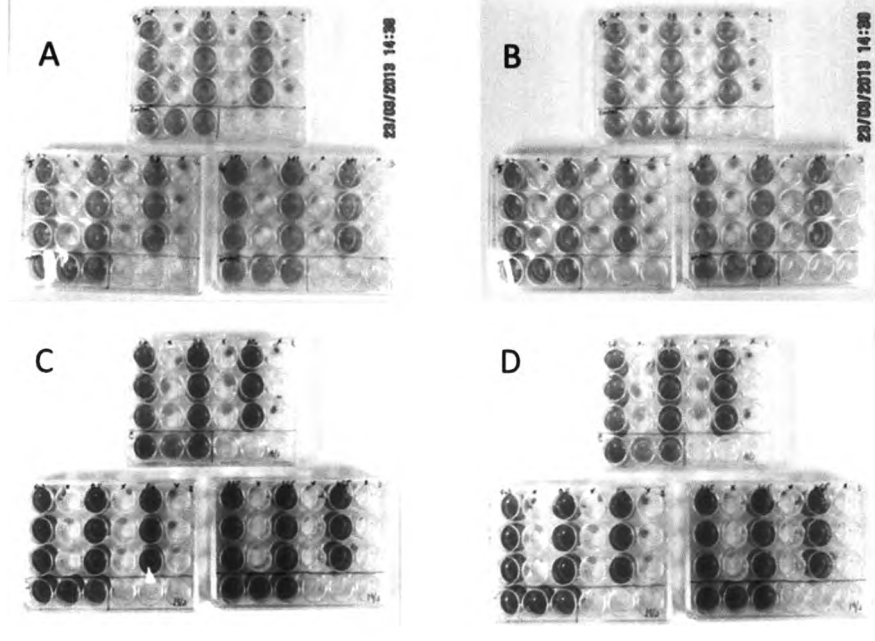

Figure 6.31 The MTT assay results: A and B showed the 24-wellplated of cell cultured by cell culture media from the releasing of sponge at 37°C for 24 hr., C and D showed the 24-wellplates of cell cultured after reacting with MTT solution for 48 hr.

The effect of silk sericin contents on indirect cytotoxicity was evaluated and the results as shown in Table 6.5 and Figure 6.32. The optical density indicated that the increasing of silk sericin contents resulted in the insignificantly

decreased in optical density when compared with control. In details; at low silk sericin contents (1-2 wt%), the viability of HGF was similarly to control but at higher silk sericin contents (3-4 wt%), the viability of HGF tended to be reduced but not significant. The same results were observed in the HGF from second human donor as shown in Appendix D. The decrease in cell viability and mitochondria activities came from the high leaching of uncross-linked silk sericin protein to the culture media in the initial phases of test (Mandel *et al.*, 2009, Nayak *et al.*, 2012)

Table 6.6 The optical density obtained from MTT assay of the first human donor as a function of silk sericin contents

Sample	OD1	OD2	OD3	Avg. OD	Ratio of avg. OD/ Control OD
C6PVA5SS1 GT 0.3	0.855	1.074	1.008	0.979	0.99
Control	0.940	1.033	0.983	0.985	1
C6PVA5SS 2 GT0.3	1.302	1.258	1.280	1.28	0.99
C6PVA5SS 3 GT0.3	1.250	1.192	1.227	1.22	0.95
C6PVA5SS 4 GT0.3	1.225	1.227	1.191	1.21	0.94
Control	1.290	1.289	1.294	1.29	1

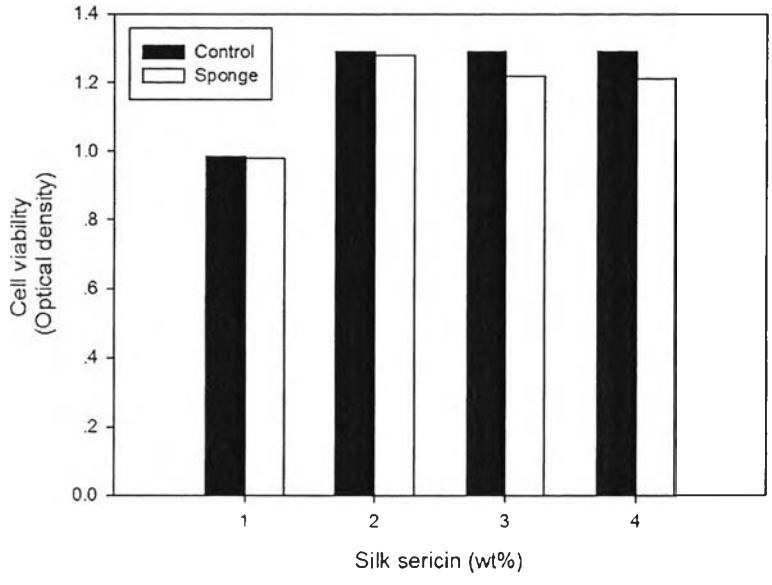

Figure 6.32 MTT assay of HGF on different content of silk sericin compared with control.

The effect of GT concentrations on the viability of cells were reported as optical density as shown in Table 6.6 and Figure 6.33. As increasing of GT content, the OD tended to be higher. Owing to the high number of covalent formed from cross-linked reaction in high GT concentration resulting in the higher stability of sponge in cell cultured media hence the fragmentation of sponge was not discovered and low silk sericin and PVA were leached out from the sponge. Additionally, the MTT assay results could be confirmed that the GT concentrations up to 7 µl/ml was not exhibited cytotoxicity to HGF for silk sericin/PVA/clay aerogel system. The results were almost similarly in the HGF obtained from second human donor. Glutaraldehyde was usually chosen as cross-linked agent for protein owing to the high reactivity of di-carbonyls groups. It is well know that, the excess of glutaraldehyde showed the toxicity to all kind of cells because of the unreacted of GT was leached out from the scaffold (Pojanavaraphan *et al.*, 2010, Rattanaruangsrikul *et al.*, 2009). In the silk sericin/PVA/clay aerogel system, the highest of GT concentration (7 µl/ml) showed the slight increase of cell viability. This results can confirmed that the post cured at 120°C can be used as the effective way in order to remove unreacted GT from the aerogel.

Table 6.7 The optical density obtained from MTT assay of the first human volunteer as a function of GT concentrations

Sample	OD1	OD2	OD3	Avg. OD	Ratio of avg. OD/ Control OD
C6PVA5SS1 0.3 GT	0.855	1.074	1.008	0.979	0.99
C6PVA5SS1 0.5 GT	1.127	1.077	1.093	1.099	1.12
C6PVA5SS1 0.7 GT	1.176	1.107	1.133	1.139	1.16
Control	0.940	1.033	0.983	0.985	1

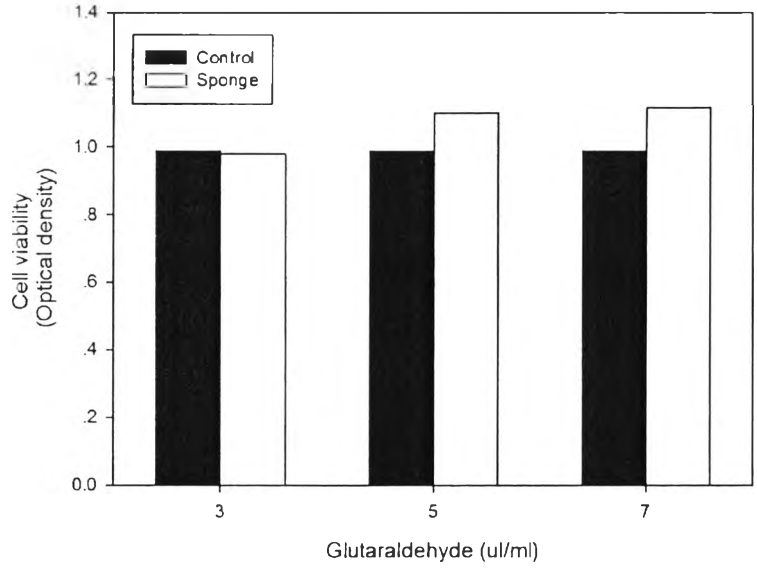

Figure 6.33 MTT assay of HGF on different concentration of glutaraldehyde compared with control.

The effect of species of silk cocoon on cell viability and mitochondria activities was studied and shown in Table 6.7 and Figure 6.34. The species of Thai silk cocoon had an influent on the cell viability observed by the slight increase in OD of the sponge compared with control. As compared with control at same composition of clay, silk sericin and glutaraldehyde, the cell viability and mitochondria activities was increased in the order of Nang Lai >Dok Bua >Nang

Noi>Luang Pairote. In details; Nang Lai and Dok Bua tended to promote cell viability, Nang Noi was not promoted but not inhibited cell viability but Luang Pairote slightly inhibited cell viability when compared with the control. The results proposed that it has dissimilarly characteristics of silk sericin which affected on the biological activities. Aramwit *et al*, 2009 and Sen *et al*., 2007 suggested that the ability to promote wound healing of silk sericin was significantly depended on the amino acid composition. The sulfur containing amino acid like methionine and cysteine play an important role on the ability to enhance cell growth. The species of cocoon which its sericin contained higher methionine composition presented the higher wound healing efficiency, promote cell growth (fibroblast) and promote collagen synthesis in bone, skin, ligament and newly healed wound. Methionine and cysteine are well known for their ability of accelerating the healing rate of wounds in injured rats which was reported by Williamson *et al*. (1952). However, the mechanisms that could explain the effects of methionine on healing and cell growth were still unclear (Aramwit *et al*., 2009). From Chapter 4 (Table 4.3), all species exhibited the slightly dissimilar in amino acid composition. Table 4.3 showed the amino acid composition only 15 amino acid owing to the others 3 amino acid including methionine and cysteine had a very small content. From the calculation, silk sericin from Nang Noi, Nang Lai and Dok Bua showed higher content of the other 3 amino acid than Luang Pairote. It can be concluded that Luang Pairote species (8.18%) has the slightly lower methionine and cysteine which were the important factor to promote cell growth, than other species (Nang Noi 9.89%, Nang Lai 11.42% and Dok Bua 8.28%).

To confirm the sulfur contents contained in four different species of Thai silk sericin, the element analyzer was employed to determine the percentage of sulfur in each species. Table 6.10 reported the sulfur contents in four different species of Thai silk sericin. The resulted suggested that silk sericin from Luang Pairote species contained the lowest of sulfur content compared with others. On the other hand, silk sericin from Nang Lai species exhibited the highest of sulfur content. This results related to the cell viability from MTT assay which silk sericin from Luang Pairote presented the lowest cell viability compared with others owing to the lowest sulfur content.

However, the results of both direct contact test and MTT assay can be varied depended on the host of HGF cells.

Table 6.8 The optical density obtained from MTT assay of the first human volunteer as a function of different species of silk sericin

Sample	OD1	OD2	OD3	Avg. OD	Ratio of avg. OD/ Control OD
C6PVA5SS1 0.3GT NN	0.855	1.074	1.008	0.979	0.99
Control	0.940	1.033	0.983	0.985	1
C6PVA5SS1 0.3GT NL	0.934	0.955	0.972	0.954	1.24
C6PVA5SS1 0.3GT DB	0.971	0.972	0.870	0.938	1.22
C6PVA5SS1 0.3GT LP	0.734	0.731	0.649	0.705	0.92
Control	0.797	0.720	0.783	0.767	1

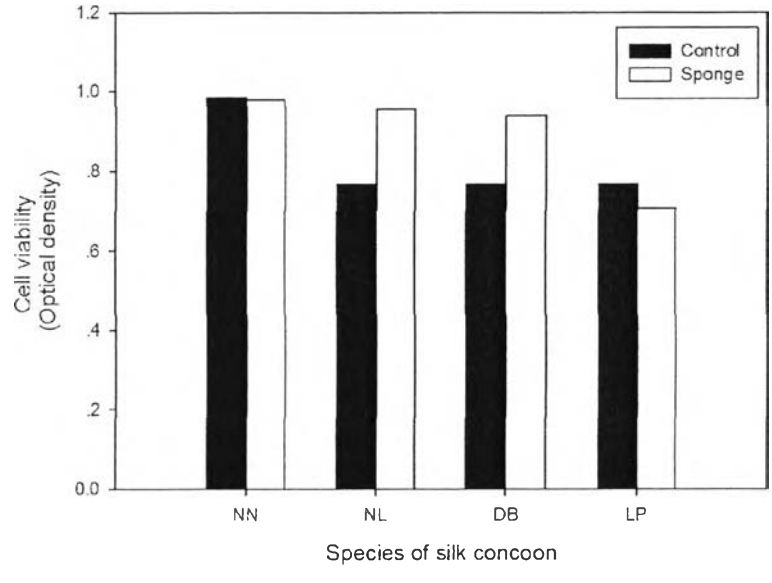

Figure 6.34 MTT assay of HGF on different species of Thai silk cocoon compared with control; NN = Nang Noi, NL =Nang Lai, DB =Dok Bua and LP =Luang Pairote.

Table 6.9 The percentage of sulfur contained in four different species of Thai silk sericin

Species of silk sericin	Sulfur content (%)
Nang Noi	0.65±0.300
Nang Lai	0.79±0.007
Dok Bua	0.64±0.150
Luang Pairote	0.55±0.158

6.5 CONCLUSIONS

The biomimetic 3D scaffold was firstly developed from silk sericin, PVA and clay in the form of 3D clay aerogel using freeze-drying techniques. The aerogel presented the lamella structure, interconnected pore with high porosity and pore size was greater than 100 μm. Clay content was powerfully affected to the properties of the aerogels. The increasing of clay loading improved the mechanical properties of the aerogels but reduced the thermal stability of the aerogel due to the existing of ferric ion in the bentonite galleries. At 8 %wt clay, the clay loading was excess leading to the inhomogeneous structure with more power to accelerate decomposition causing the inferior mechanical and thermal properties. Silk sericin also had an influence on the mechanical and thermal properties; increasing of silk sericin increased in mechanical properties and thermal stability of the aerogel. The significantly diminished of swelling ratio was verified as the clay and silk sericin was increased and also as the concentration of glutaraldehyde was raised. This aerogel has potential to be used as 3D scaffold for dental implant in periodontal disease due to the suitable pore size and biotechnological activities. The *in vitro* biological tests, including *in vitro* direct contact test and MTT assay using human gingival fibroblast cell (HGF) suggested the cytocompatibility and cell viability of the aerogel was comparable to control. The cell viability tended to decrease while increased the silk sericin contents. The glutaldehyde concentration in the range between 3-7 μl/ml was

not toxic to HGF cell and the cell viability slightly increased when glutaraldehyde concentration was enlarged. The species of Thai silk had the significant affected to cell viability. Nang Lai species had the highest capability to enhanced cell growth and Luang Pairote species had the lowest potential owing to the lowest contents of sulfur containing amino acids (methionine and cysteine).

6.5 ACKNOWLEDGEMENTS

The authors appreciate to thank the Center of Excellence on Petrochemical and Materials Technology and the government budget 2012 for the financial support.

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