CHAPTER IV

RESULTS AND DISCUSSION

4.1 Non-crosslinked and crosslinked Thai silk fibroin/gelatin scaffolds

Two types of Thai silk fibroin/gelatin scaffolds; non-crosslinked and crosslinked scaffolds using EDC/NHS, fabricated at various weight blending ratios were summarized in table 4.1

Table 4.1 The acronym of non-crosslinked and crosslinked Thai silk fibroin/gelatin scaffolds at various weight blending ratios.

Weight blending ratio of Thai silk fibroin/gelatin	Non-crosslinked scaffolds	Crosslinked scaffolds
0/100	NC 0/100	C 0/100
20/80	NC 20/80	C 20/80
40/60	NC 40/60	C 40/60
50/50	NC 50/50	C 50/50
60/40	NC 60/40	C 60/40
80/20	NC 80/20	C 80/20
100/0	NC 100/0	C 100/0

In this section, physical and biological characteristics of non-crosslinked and crosslinked Thai silk fibroin/gelatin scaffolds were reported and discussed.

4.1.1 Physical characterization

Physical characteristics of non-crosslinked and crosslinked Thai silk fibroin/gelatin scaffolds including morphology, weight loss (%), and compressive modulus in dry and wet conditions were reported.

4.1.1.1 Morphology of scaffolds

Thai silk fibroin/gelatin scaffolds were cross-sectional cut in order to observe the morphology under SEM. Uniform porous structure with smooth surface of non-crosslinked and crosslinked Thai silk fibroin/gelatin scaffolds at various weight blending ratios was noticed as illustrated in Figure 4.1.

For non-crosslinked Thai silk fibroin/gelatin scaffolds (Figure 4.1 (a)-(g)), the pore size of both pure gelatin and Thai silk fibroin scaffolds was approximately 350 μm and 250 μm as shown in Figure 4.1 (a, g), respectively. Interestingly, the pore size of non-crosslinked Thai silk fibroin/gelatin scaffolds decreased as the weight blending ratio of Thai silk fibroin/gelatin was closed to 50/50. The smallest pore size around 100 μm was found in non-crosslinked Thai silk fibroin/gelatin scaffold at the weight blending ratio of 50/50, as presented in Figure 4.1 (d).

For crosslinked Thai silk fibroin and gelatin using EDC/NHS (Figure 4.1 (h)-(n)), it was noticed that crosslinked Thai silk fibroin/gelatin scaffolds at the weight blending ratios of 50/50, 60/40, 80/20, and 100/0 possessed highly porous networks with smaller pore sizes than those of non-crosslinked scaffolds. The result indicated that the crosslinking reaction induced the interactions of silk fibroin and gelatin. Meanwhile, crosslinked Thai silk fibroin/gelatin scaffolds with high gelatin content (60%, 80%, and 100%) had slightly larger pore size than those of non-crosslinked scaffolds. This could be due to the washing step after the chemical crosslinking reaction by EDC/NHS. Crosslinked Thai silk fibroin/gelatin scaffolds were washed with deionized water in order to get rid of byproduct obtained from crosslinking reaction. However the part of non-crosslinked gelatin in scaffolds could possibly be washed out in the washing step.

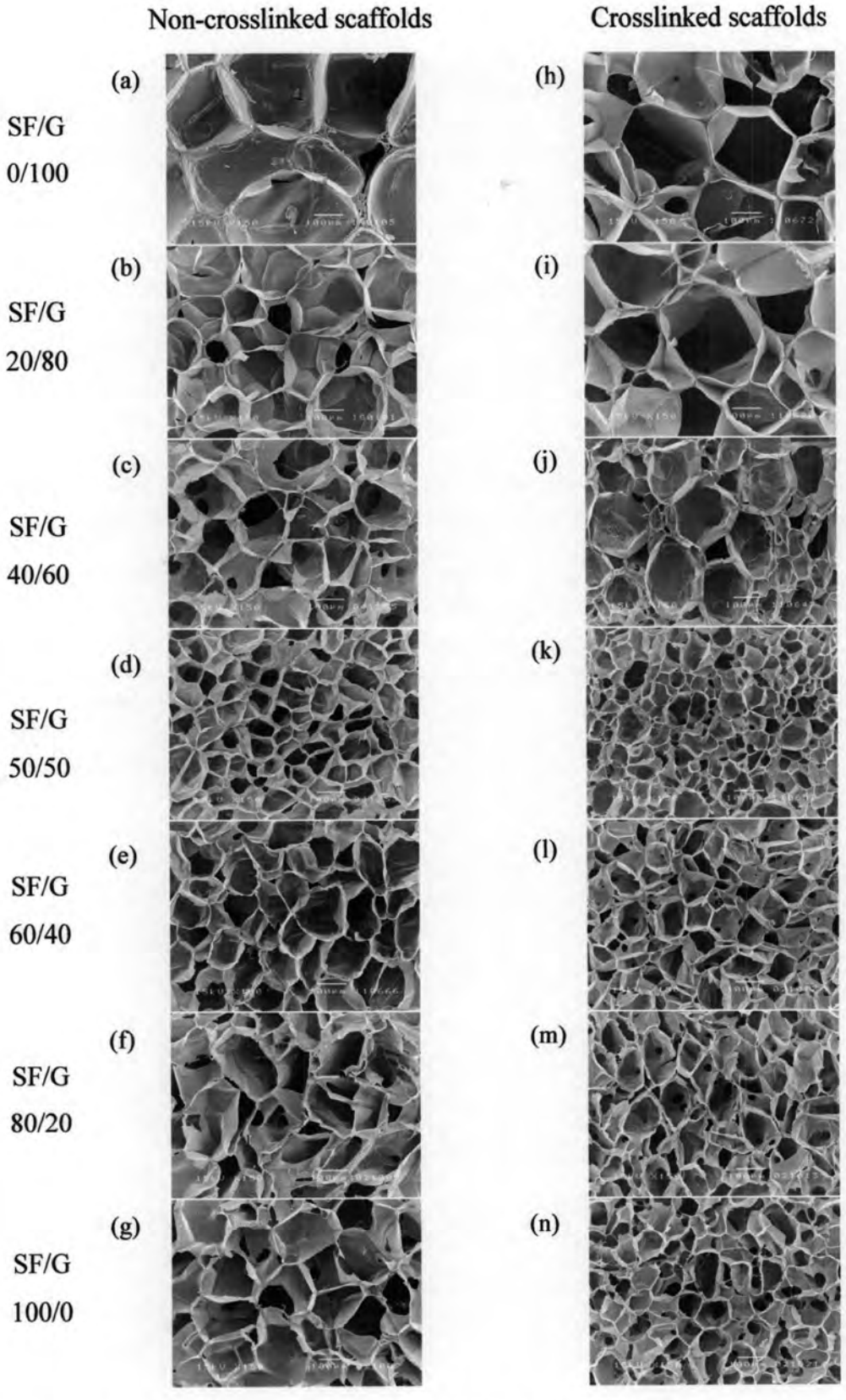


Figure 4.1 SEM micrographs of Thai silk fibroin/gelatin scaffolds (SF/G); non-crosslinked scaffolds (a) 0/100, (b) 20/80, (c) 40/60, (d) 50/50, (e) 60/40, (f) 80/20, (g) 100/0 and crosslinked scaffolds with EDC/NHS (h) 0/100, (i) 20/80, (j) 40/60, (k) 50/50, (l) 60/40, (m) 80/20, (n) 100/0. (— scale bar = 100 μm)

4.1.1.2 Weight loss (%) of scaffolds

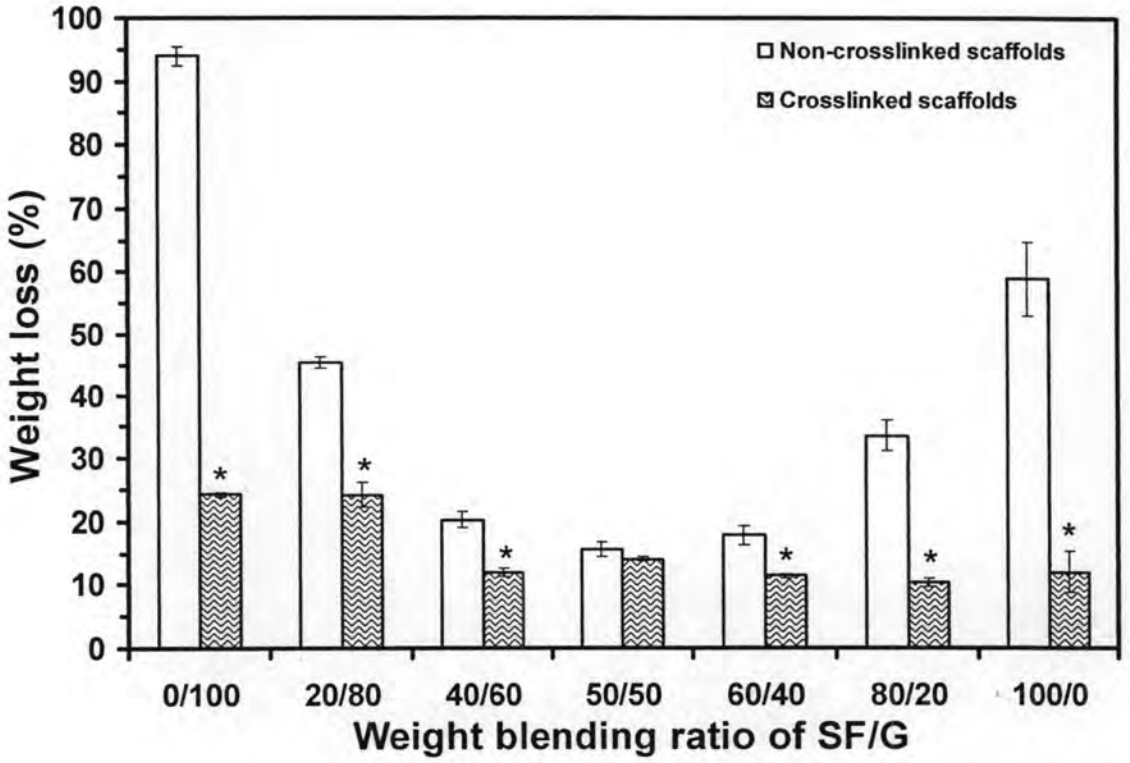


Figure 4.2 Weight loss (%) of non-crosslinked and crosslinked Thai silk fibroin/gelatin scaffolds (SF/G).

* represent the significant difference ($p<0.05$) relative to non-crosslinked scaffolds at each weight blending ratio of SF/G.

Weight loss (%) of non-crosslinked and crosslinked Thai silk fibroin/gelatin scaffolds at various weight blending ratios was presented in Figure 4.2. Considering scaffolds at each constant weight blending ratio, weight loss (%) of crosslinked Thai silk fibroin/gelatin scaffolds was significantly lower than non-crosslinked Thai silk fibroin/gelatin scaffolds except Thai silk fibroin/gelatin scaffold at the weight blending ratio of 50/50. This was a result from the crosslinking reaction by EDC/NHS. EDC reacted with the carboxylic groups of aspartic and glutamic amino acids, and in turn, can interact with the primary amine groups of lysine and hydroxylysine amino acids to form stable amide bonds [34].

For non-crosslinked Thai silk fibroin/gelatin scaffolds, the highest weight loss, around 95%, was found in pure gelatin scaffold, due to the solubility of gelatin. When silk fibroin content was increased, weight loss (%) of non-crosslinked Thai silk fibroin/gelatin scaffolds tended to slightly decrease until the lowest weight loss

around 15% was noticed at the weight blending ratio of Thai silk fibroin/gelatin 50/50. When further increasing in Thai silk fibroin content, the weight loss (%) of non-crosslinked Thai silk fibroin/gelatin scaffolds tended to slightly increase, reaching 60% for pure Thai silk fibroin scaffold. The lowest weight loss of the blended scaffold containing Thai silk fibroin/gelatin 50/50 might be attributed to the electrostatic interactions between silk fibroin and gelatin. The zeta potential, representing the net charge of each blend solution was illustrated in Table 4.2

Table 4.2 Zeta potential (mV) of non-crosslinked Thai silk fibroin/gelatin solution at pH 5.6 (blended solution in DI water) and pH7.4 (blended solution in PBS (-)).

Weight blending ratio of Thai silk fibroin/gelatin	Zeta potential (mV) at pH 5.6	Zeta potential (mV) at pH 7.4
0/100	2.61 ± 0.22	N/A
20/80	2.90 ± 0.06	N/A
40/60	2.18 ± 0.41	2.05 ± 0.90
50/50	-0.01 ± 0.08	-0.03 ± 0.27
60/40	-0.90 ± 0.18	-1.26 ± 0.67
80/20	-2.54 ± 0.14	N/A
100/0	-4.58 ± 0.09	N/A

Considering the zeta potential of non-crosslinked Thai silk fibroin/gelatin solution at pH 5.6 (blended solution in DI water), the zeta potential of high gelatin containing solution at the weight blending ratios of 0/100, 20/80, and 40/60 was 2.61 ± 0.22, 2.90 ± 0.06, and 2.18 ± 0.41 mV, respectively. These implied positive charges of type A gelatin dominated in blended solution. In contrast, the zeta potential of blended solution with higher silk fibroin content was more negative. These implied negative charges of silk fibroin dominated in blended solution. Noteworthy, the zeta potential of non-crosslinked Thai silk fibroin/gelatin solution at the weight blending ratio of 50/50 was very closed to zero, indicating a balanced charge of silk fibroin and gelatin. When considering the zeta potential of non-crosslinked Thai silk fibroin/gelatin solution at pH 7.4 (blended solution in PBS (-)), the trend of the zeta potential was similar to that in deionized water (pH 5.6). A balanced charge of silk fibroin and gelatin in the solution could result in scaffolds obtained at maximum

electrostatic interactions. Therefore the lowest weight loss of non-crosslinked Thai silk fibroin/gelatin scaffold at the weight blending ratio of 50/50 was observed.

4.1.1.3 Compressive modulus of scaffolds

The compressive modulus of non-crosslinked and crosslinked Thai silk fibroin/gelatin scaffolds at various weight blending ratios in dry condition was illustrated in Figure 4.3 (a). At each constant weight blending ratio, the compressive modulus of crosslinked Thai silk fibroin/gelatin scaffolds was significantly higher than non-crosslinked Thai silk fibroin/gelatin scaffolds by 30-60%. The result revealed that crosslinking reaction promoted the mechanical strength of scaffolds. Following treated with EDC/NHS, the carboxylic groups and primary amine groups on the peptides formed a stable amide bond between the peptide of silk fibroin and gelatin causing an increase in compressive modulus of scaffolds [56]. For non-crosslinked Thai silk fibroin/gelatin scaffolds, it was noticed that the compressive modulus of non-crosslinked Thai silk fibroin/gelatin scaffolds at the weight blending ratios of 40/60, 50/50, and 60/40 was higher than those of 0/100, 20/80, 80/20, and 100/0. This could possibly be the result from suitable electrostatic interaction between silk fibroin and gelatin molecules as previously described in section 4.1.1.2. These results confirmed that the mechanical strength of Thai silk fibroin/gelatin scaffolds depended upon the crosslinking reaction and the weight blending ratio of Thai silk fibroin/gelatin scaffolds.

The compressive modulus of Thai silk fibroin/gelatin scaffolds in wet condition was an important mechanical property for tissue engineering to mimic the condition of scaffolds to use in the human body at pH 7.4. Figure 4.3 (b) depicted compressive modulus of non-crosslinked and crosslinked Thai silk fibroin/gelatin scaffolds at various weight blending ratios in wet condition. The results revealed that the compressive modulus of non-crosslinked and crosslinked Thai silk fibroin/gelatin scaffolds in wet condition ranged from 30 kPa to 100 kPa which is decreased from that in dry condition around 80-90%. Noteworthy, the compressive modulus of non-crosslinked Thai silk fibroin/gelatin scaffolds was slightly higher than crosslinked Thai silk fibroin/gelatin scaffolds. It should be attributed to the larger pore size of non-crosslinked Thai silk fibroin/gelatin scaffolds. They could have great swelling ability and saturation in PBS (-) solution resulting in higher compressive modulus.

For both pure gelatin and Thai silk fibroin scaffolds, the compressive modulus of these two scaffolds could not be measured since the samples were disintegrated when immersed in PBS (-) solution.

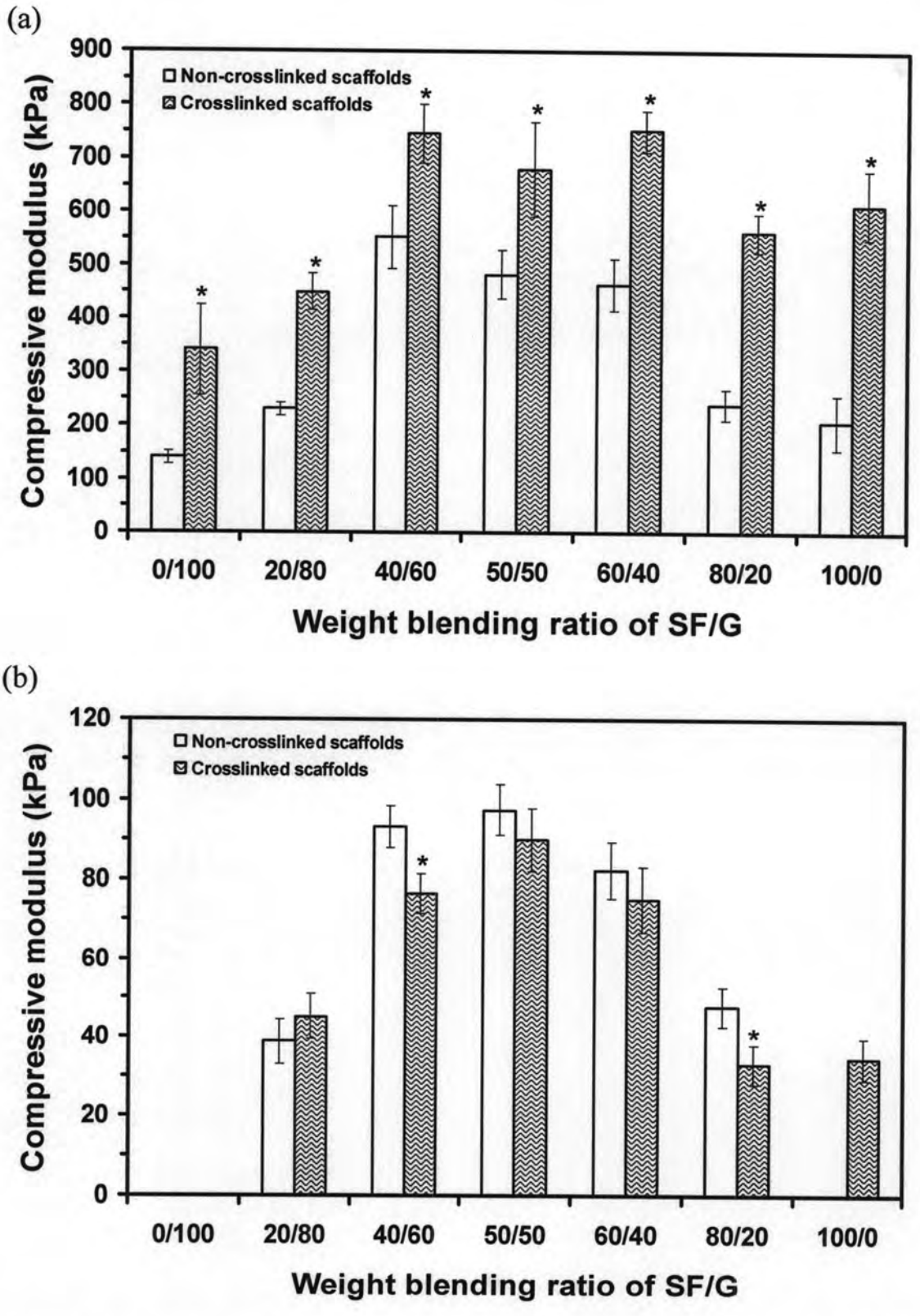


Figure 4.3 Compressive modulus of non-crosslinked and crosslinked Thai silk fibroin/gelatin scaffolds (SF/G) in (a) dry condition and (b) wet condition.

* represent the significant difference ($p<0.05$) relative to non-crosslinked scaffolds at each weight blending ratio of SF/G.

4.1.2 Biological characterization

In this section, biological characteristics of non-crosslinked and crosslinked Thai silk fibroin/gelatin scaffolds were presented and discussed into two parts as follows:

- *In vitro* biodegradability
- *In vitro* biocompatibility

4.1.2.1 *In vitro* biodegradability

To assess the biodegradation behavior of Thai silk fibroin/gelatin scaffolds, we exposed scaffolds to 1 U/ml collagenase at 37°C, pH 7.4 for various periods of time. Weight changes and conformation changes during degradation were evaluated by remaining weight (%) and X-Ray diffraction (XRD), respectively.

4.1.2.1.1 Remaining weight (%)

Figure 4.4-4.6 illustrated the remaining weight (%) of Thai silk fibroin/gelatin scaffolds after incubation in collagenase solution at various periods of time. It was seen that the remaining weight (%) of all scaffolds decreased as the degradation time increased.

Degradation profiles of crosslinked Thai silk fibroin/gelatin scaffolds were observed over the period of 168 h (Figure 4.4). It was found that the remaining weight (%) of pure Thai silk fibroin scaffold hardly changed during the first 24 h and slightly decreased thereafter. After 168 h of incubation in collagenase solution, there was more than 90% remaining weight of pure Thai silk fibroin/gelatin scaffold. This result demonstrated that pure Thai silk fibroin scaffold had slowest biodegradability. In the case of crosslinked Thai silk fibroin/gelatin scaffolds containing from 40% up to 80% of gelatin content, the remaining weight (%) rapidly decreased within the first 12 h. The remained weight of these scaffolds corresponded to the silk fibroin content in the scaffolds. This implied that the loss weight of these scaffolds might be the gelatin portion which was directly digested by collagenase solution [52, 57, 58]. Furthermore, it was observed that pure gelatin scaffold had the remaining weight around 10% in just 15 min after incubation and completely

degraded at the degradation time less than 1 h. This result confirmed that pure gelatin scaffold had fastest biodegradability.

After 24 h of enzymatic degradation, the remaining weight (%) of crosslinked Thai silk fibroin/gelatin scaffolds gradually decreased and began to level off. In general it was noticed that the remaining weight (%) of scaffolds containing high amount of silk fibroin was higher than those with low silk fibroin content. The results revealed that Thai silk fibroin could prolong the biodegradability of Thai silk fibroin/gelatin scaffolds.

As observed that after 168 h (or 7 days) of incubation in collagenase solution, there were more than 50% remaining weight of crosslinked Thai silk fibroin/gelatin scaffolds with high silk fibroin content (80% and 100%). Therefore, these two scaffolds were further incubated in collagenase solution for 7, 14, 21, and 28 days. The result shown in Figure 4.5 demonstrated that the degradation time at 50% remaining weight of crosslinked Thai silk fibroin/gelatin scaffold at the weight blending ratio of 80/20 was around 14 days while only 35% of pure Thai silk fibroin scaffold was degraded in collagenase solution after 28 days.

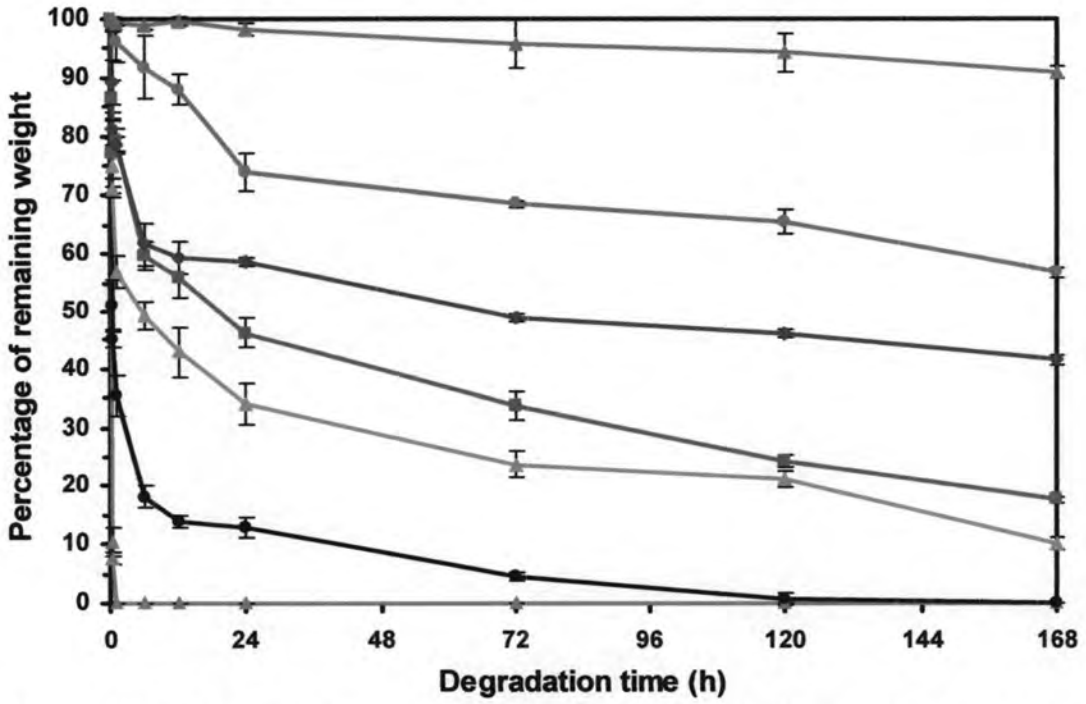


Figure 4.4 The remaining weight (%) of crosslinked Thai silk fibroin/gelatin scaffolds (SF/G) during the enzymatic degradation for 168 h : C 100/0, C 80/20, C 60/40, C 50/50, C 40/60, C 20/80, C 0/100.

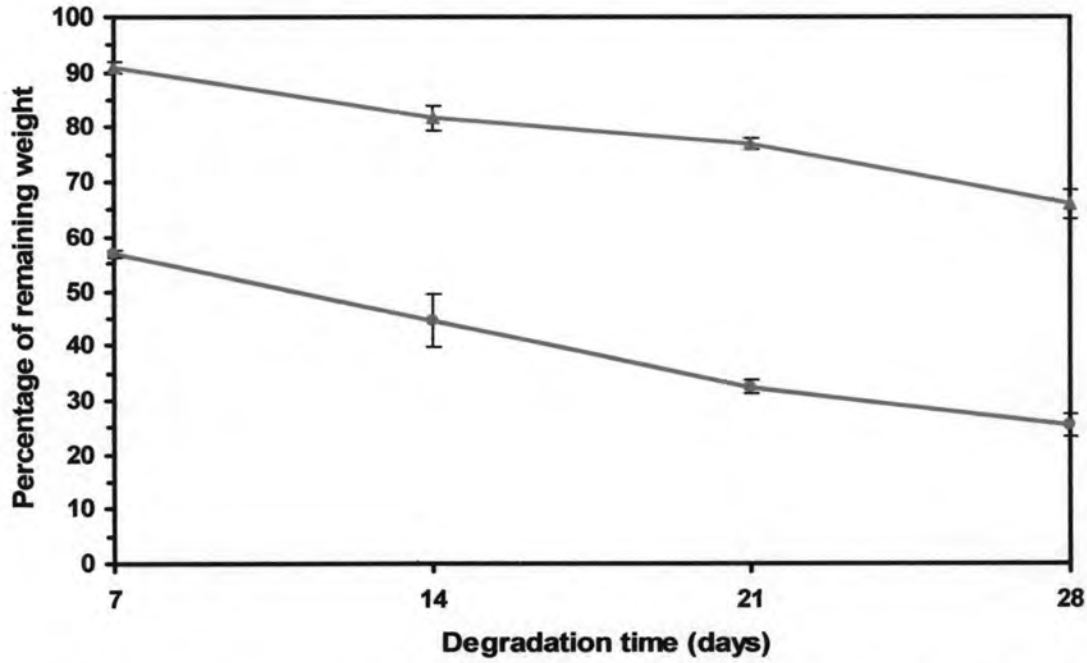


Figure 4.5 The remaining weight (%) of crosslinked Thai silk fibroin/gelatin scaffolds at the weight blending ratios of 80/20 and 100/0 (SF/G) during the enzymatic degradation for 7, 14, 21, and 28 days : C 100/0, C 80/20.

To compare the weight changes of non-crosslinked and crosslinked scaffolds over the degradation time of 168 h, the remaining weight (%) of Thai silk fibroin/gelatin scaffolds at the weight blending ratios of 40/60, 50/50, and 60/40 were investigated as presented in Figure 4.6. At each constant weight blending ratio, the remaining weight (%) of crosslinked Thai silk fibroin/gelatin scaffolds was higher than non-crosslinked Thai silk fibroin/gelatin scaffolds. From the result in Figure 4.6, the degradation time at 50% remaining weight of non-crosslinked and crosslinked Thai silk fibroin/gelatin scaffolds at the weight blending ratios of 40/60, 50/50, and 60/40 could be summarized in Table 4.3. It was obviously noticed that in the case of non-crosslinked scaffolds, the degradation time at 50% remaining weight of Thai silk fibroin/gelatin 40/60, 50/50, and 60/40 scaffolds was 15 min, 1 h, and 6 h, respectively, whereas those of crosslinked scaffolds was 6 h, 24 h, and 72 h, respectively. This showed that the chemical crosslinking by EDC/NHS that formed a stable amide bond between the peptide of silk fibroin and gelatin could delay the biodegradability of crosslinked Thai silk fibroin/gelatin scaffolds [56].

These results confirmed that the biodegradation of Thai silk fibroin/gelatin scaffolds after incubation in collagenase solution depended upon the crosslinking treatment and the weight blending ratio of Thai silk fibroin/gelatin scaffolds.

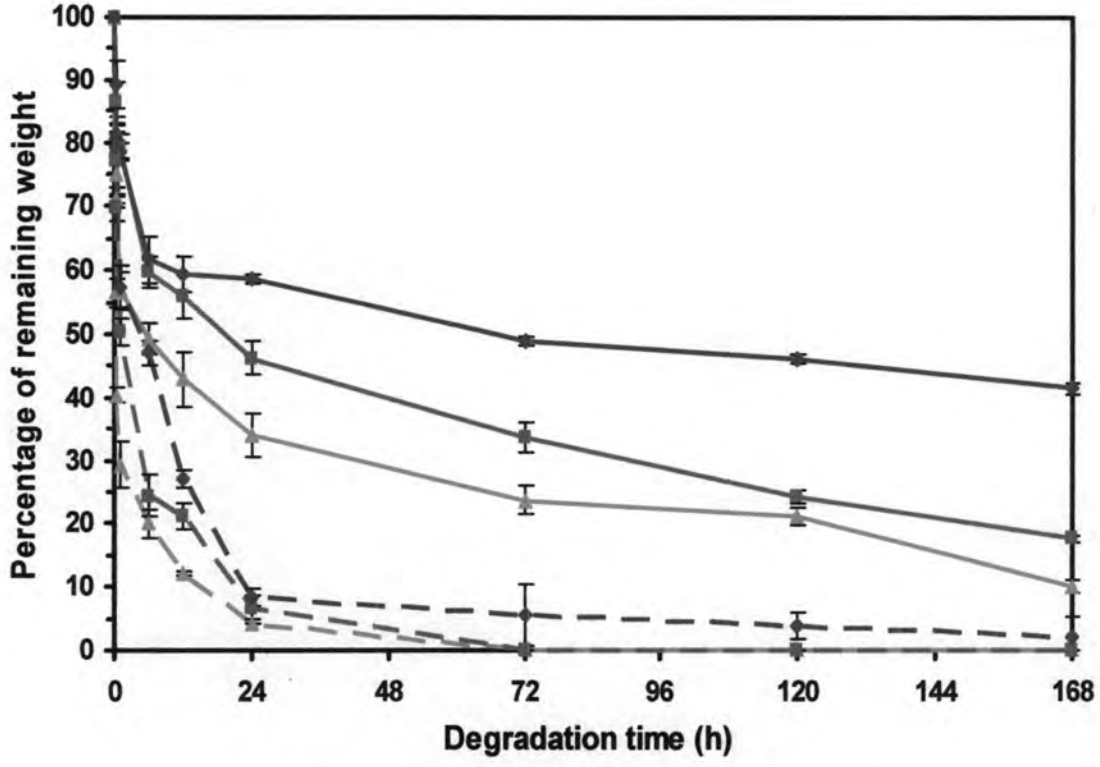


Figure 4.6 The remaining weight (%) of non-crosslinked and crosslinked Thai silk fibroin/gelatin scaffolds at the weight blending ratios of 40/60, 50/50, and 60/40 (SF/G) during the enzymatic degradation for 168 h: —◆— C 60/40, —■— C 50/50, —▲— C 40/60, —◆— NC 60/40, —■— NC 50/50, —▲— NC 40/60.

Table 4.3 Degradation time at 50% remaining weight of non-crosslinked and crosslinked Thai silk fibroin/gelatin scaffolds at the weight blending ratios of 40/60, 50/50, and 60/40.

Weight blending ratio of Thai silkfibroin/gelatin scaffolds	**Degradation time at 50% remaining weight**	
	Non-crosslinked scaffolds	**Crosslinked scaffolds**
40/60	15 min	6 h
50/50	1 h	24 h
60/40	6 h	72 h

4.1.2.1.2 Conformational structure by X-Ray Diffraction (XRD)

In order to verify the conformation changes of Thai silk fibroin/gelatin scaffolds at 50% remaining weight after enzymatic degradation, the non-crosslinked and crosslinked Thai silk fibroin/gelatin scaffolds at the weight blending ratios of 40/60, 50/50, and 60/40 were selected for X-ray diffraction analysis. The results were shown in Figure 4.7-4.10.

- XRD pattern of non-crosslinked Thai silk fibroin/gelatin scaffolds before and after degradation

Figure 4.7 illustrated XRD patterns of non-crosslinked Thai silk fibroin/gelatin scaffolds at the weight blending ratios of 40/60, 50/50, and 60/40 before biodegradation. It was observed that all non-crosslinked Thai silk fibroin/gelatin scaffolds exhibited a broad diffraction peak at around $2\theta=20^{o}$, which indicated an amorphous structure [8, 59]. This implied that the scaffolds prepared by freeze-drying process were amorphous. In addition, non-crosslinked Thai silk fibroin/gelatin scaffolds at the weight blending ratios of 40/60 and 60/40 also possessed a broad diffraction peak at around $2\theta=28.2^{o}$, which belonged to the characteristic of silk I conformation [42, 52].

The conformation changes of non-crosslinked Thai silk fibroin/gelatin scaffolds at the weight blending ratios of 40/60, 50/50, and 60/40 after 50% of degradation in collagenase solution were presented in Figure 4.8. It was noticed that a broad diffraction peak indicated an amorphous structure ($2\theta=20^{o}$) was still observed in non-crosslinked Thai silk fibroin/gelatin scaffolds at the weight blending ratios of 40/60 and 60/40 after degradation.

Noteworthy, the distinct peaks of non-crosslinked Thai silk fibroin/gelatin scaffolds after degradation appeared at around $2\theta=29.2^{o}$, 31.7^{o}, and 39.3^{o} for scaffold containing 40% silk fibroin content, $2\theta=29.2^{o}$ and 39.3^{o} for scaffold containing 50% silk fibroin content, and $2\theta=31.7^{o}$ for scaffold containing 60% silk fibroin content. These three sharp peaks were expected to be artificial peaks originating from the non-protein content. This supposed to be a sodium chloride residue containing in phosphate buffer saline, which used as a solvent for collagenase solution. Also, the

peaks of X-ray diffraction for protein polymers are always broad and the 2 theta value is rarely higher than 30° [60].

- XRD pattern of crosslinked Thai silk fibroin/gelatin scaffolds before and after degradation

The XRD patterns of crosslinked Thai silk fibroin/gelatin scaffolds at the weight blending ratios of 40/60, 50/50, and 60/40 before biodegradation were illustrated in Figure 4.9. It was noticed that crosslinked Thai silk fibroin/gelatin scaffolds at the weight blending ratios of 40/60 and 60/40 showed a broad diffraction peak at around $2\theta=28.2^{\circ}$, which indicated silk I conformation. Meanwhile crosslinked Thai silk fibroin/gelatin scaffold at the weight blending ratio of 50/50 still showed only an amorphous structure ($2\theta=20^{\circ}$). These X-ray profiles were similar to those in non-crosslinked scaffolds (Figure 4.7). The result indicated that the conformation of Thai silk fibroin/gelatin scaffolds was not affected by crosslinking reaction.

The conformation changes of crosslinked Thai silk fibroin/gelatin scaffolds at the weight blending ratios of 40/60, 50/50, and 60/40 after 50% of degradation in collagenase solution were presented in Figure 4.10. All crosslinked Thai silk fibroin/gelatin scaffolds after degradation showed a broad diffraction peak of amorphous structure at $2\theta=20^{\circ}$. In addition, crosslinked Thai silk fibroin/gelatin scaffolds at weight blending ratios of 40/60 and 50/50 also exhibited a dominant peak of the non-protein content at $2\theta=29.2^{\circ}$ similar to those in non-crosslinked scaffolds (Figure 4.8).

It can be concluded from the results that *in vitro* biodegradation in collagenase solution did not clearly affect the conformation changes of Thai silk fibroin/gelatin scaffolds.

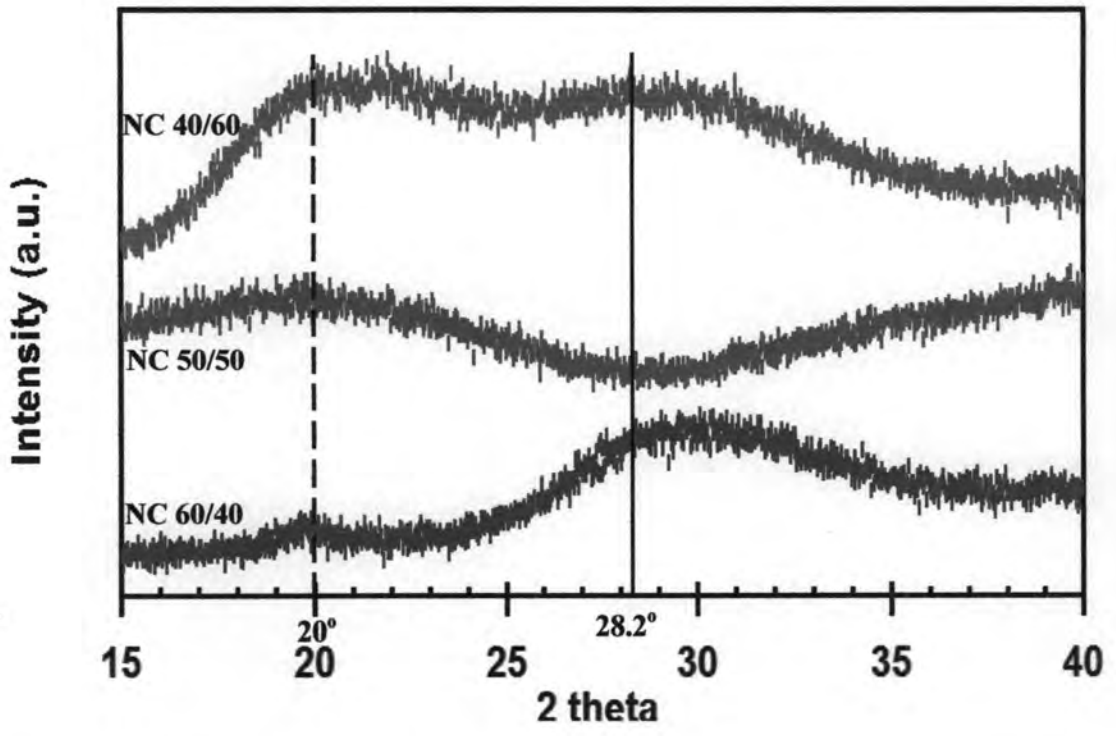


Figure 4.7 XRD patterns of non-crosslinked Thai silk fibroin/gelatin scaffolds at the weight blending ratios of 40/60, 50/50, and 60/40 (SF/G) before biodegradation.

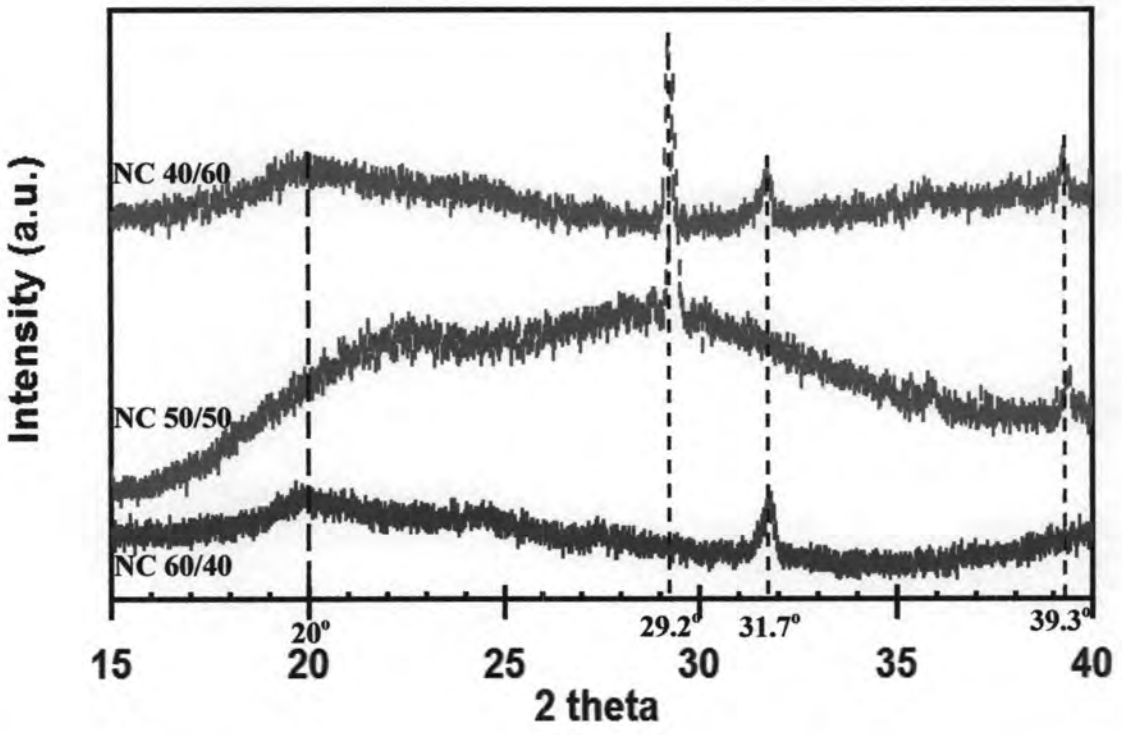


Figure 4.8 XRD patterns of non-crosslinked Thai silk fibroin/gelatin scaffolds at the weight blending ratios of 40/60, 50/50, and 60/40 (SF/G) after biodegradation in collagenase solution.

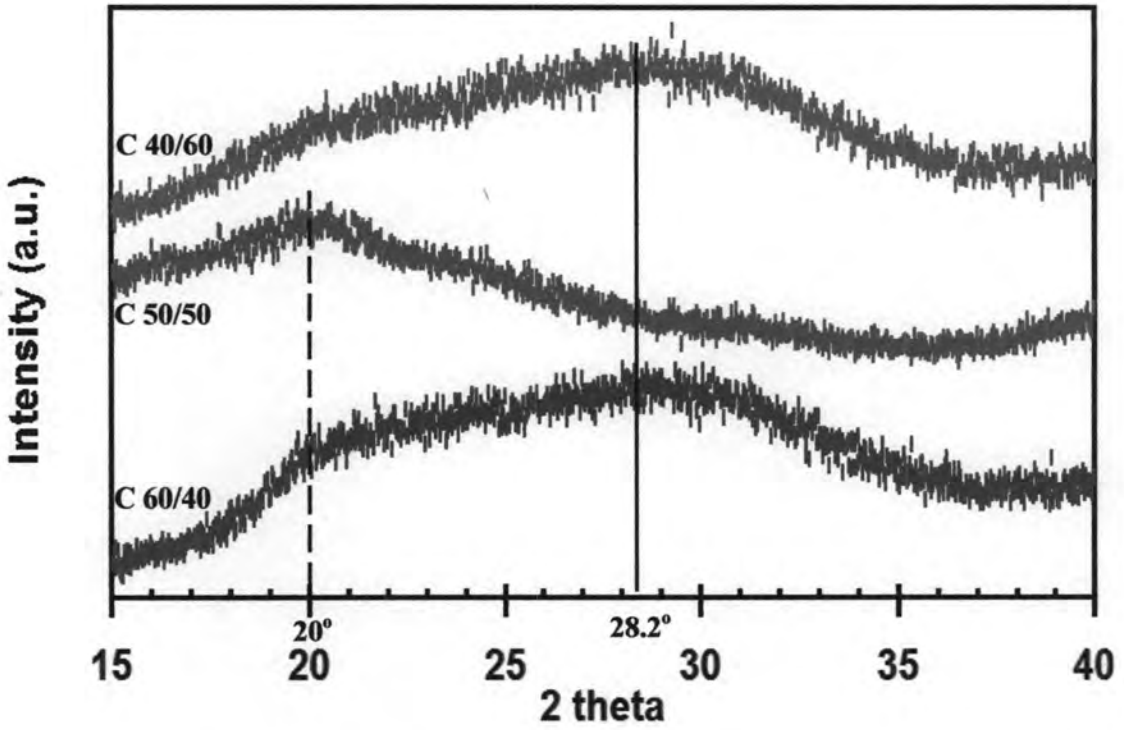


Figure 4.9 XRD patterns of crosslinked Thai silk fibroin/gelatin scaffolds at the weight blending ratios of 40/60, 50/50, and 60/40 (SF/G) before biodegradation.

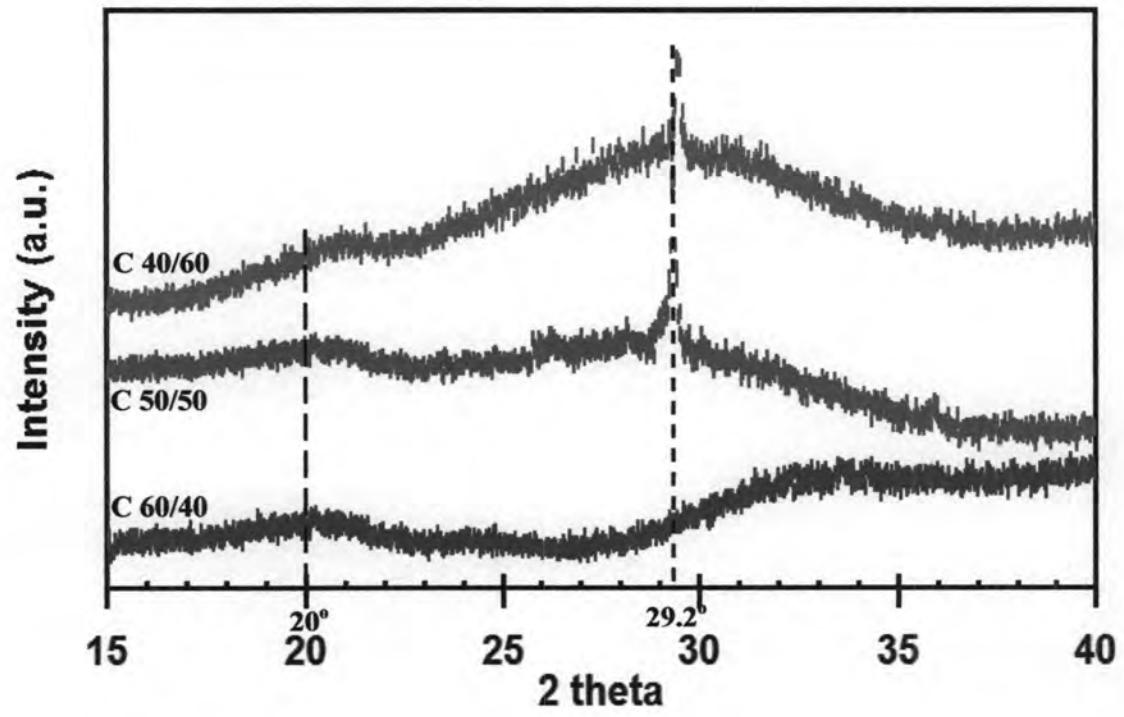


Figure 4.10 XRD patterns of crosslinked Thai silk fibroin/gelatin scaffolds at the weight blending ratios of 40/60, 50/50, and 60/40 (SF/G) after biodegradation in collagenase solution.

4.1.2.2 *In vitro* biocompatibility using bone marrow-derived stem cells (MSCs)

The non-crosslinked and crosslinked Thai silk fibroin/gelatin scaffolds at the weight blending ratios of 40/60, 50/50, and 60/40 were selected for *in vitro* biocompatibility tests since they showed great compressive strength. In addition, these non-crosslinked Thai silk fibroin/gelatin scaffolds could maintain the structural integrity without crosslinking agents used so it was interest to compare the biocompatibility of non-crosslinked and crosslinked Thai silk fibroin/gelatin scaffolds. Bone marrow-derived stem cells (MSCs) isolated from 3 weeks old female wistar rats were cultured on these scaffolds. Cell attachment and proliferation by DNA assay and SEM observation were assessed.

4.1.2.2.1 MSCs initial attachment and proliferation tests

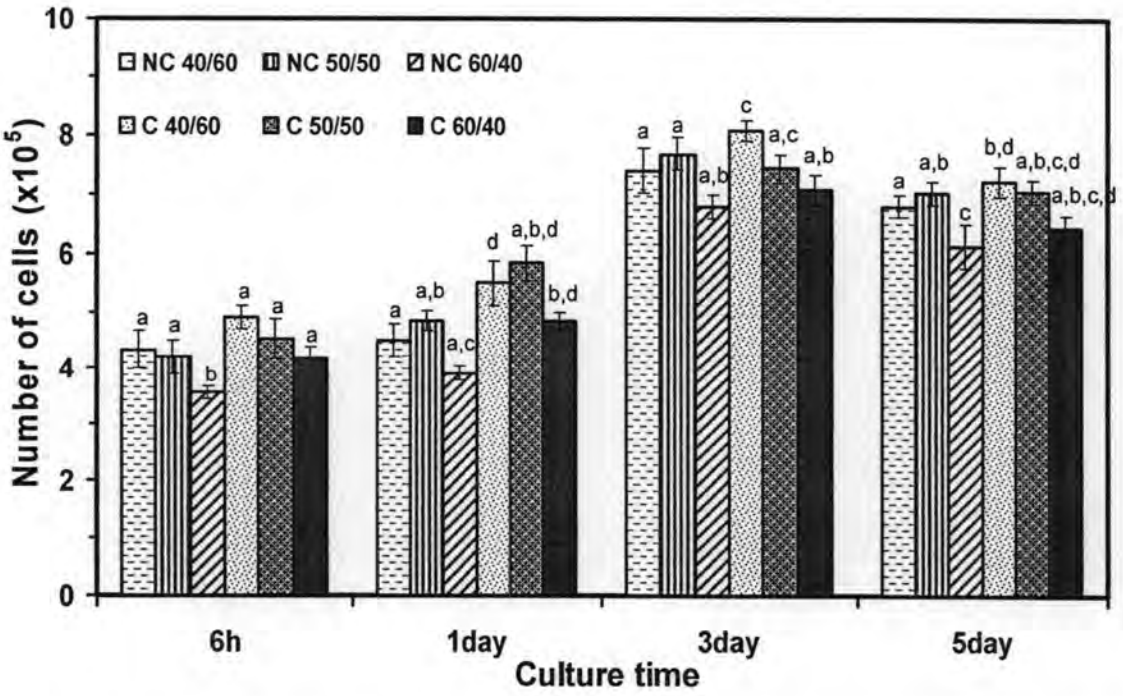


Figure 4.11 Number of MSCs attached and proliferated on non-crosslinked and crosslinked Thai silk fibroin/gelatin scaffolds at the weight blending ratios of 40/60, 50/50, and 60/40 (SF/G) under proliferating medium for 6 h, 1, 3, and 5 days, determined by DNA assay (seeding: 5×10^5 cells/scaffold).

a, b, c, d represent the significant difference ($p<0.05$) within each culture time.

(The results with the same alphabet indicate that they are not significantly different)

MSCs (5×10^5 cells per scaffold) were seeded on non-crosslinked and crosslinked Thai silk fibroin/gelatin scaffolds at the weight blending ratios of 40/60, 50/50, and 60/40 to evaluate cell attachment and proliferation when cultured under proliferating medium (α-MEM, 15% FBS, 100 U/ml penicillin/streptomycin). Figure 4.11 presented the number of cells determined by DNA assay after 6 h, 1, 3, and 5 days of the culture. After 6 h of seeding, it could be noticed that there were no significant differences between the cells adhered on all Thai silk fibroin/gelatin scaffolds except non-crosslinked Thai silk fibroin/gelatin scaffold at the weight blending ratio of 60/40. After 1 day of the culture, non-crosslinked and crosslinked Thai silk fibroin/gelatin scaffolds at the weight blending ratios of 50/50 showed the highest number of MSCs compared to other non-crosslinked and crosslinked scaffolds, respectively. After 3 days of the culture, there were more MSCs on each type of Thai silk fibroin/gelatin scaffolds compared to those at 6 h of the culture. In addition, the number of cells of non-crosslinked Thai silk fibroin/gelatin scaffolds at the weight blending ratios of 40/60, 50/50, and 60/40 was around 1.9, 2.0, and 2.2 times of those after 6 h of seeding, whereas in the case of crosslinked Thai silk fibroin/gelatin scaffolds, the number of cells was 1.8, 1.8, and 1.9 times of those after 6 h of seeding, respectively. After 5 days of the culture, it was noticed that the number of cells on all Thai silk fibroin/gelatin scaffolds tended to slightly decrease. This might be the reason of mass transfer limit in static culture. The transfer of oxygen and nutrient supply to cells as well as metabolic waste drainage from cells could be obstructed causing cell death. Moreover, it was reported that the neutral charge of these scaffolds was not favorable to the attachment and proliferation of cells due to the heterodimeric transmembrane glycoprotein such as integrins presented on cell surface that carries negative charges [50, 61].

The results of MSCs initial attachment and proliferation implied that, blended Thai silk fibroin/gelatin scaffolds could support attachment and proliferation of MSCs, regardless of crosslinking and the weight blending ratio of Thai silk fibroin and gelatin. Also, blended Thai silk fibroin/gelatin scaffolds containing high amount of gelatin tended to promote cell proliferation better than those with low gelatin content. This could be the result of arginine-glycine-aspartic (RGD) sequence contained in gelatin that was reported to promote cell adhesion and migration [62].

4.1.2.2.2 MSCs morphological observation

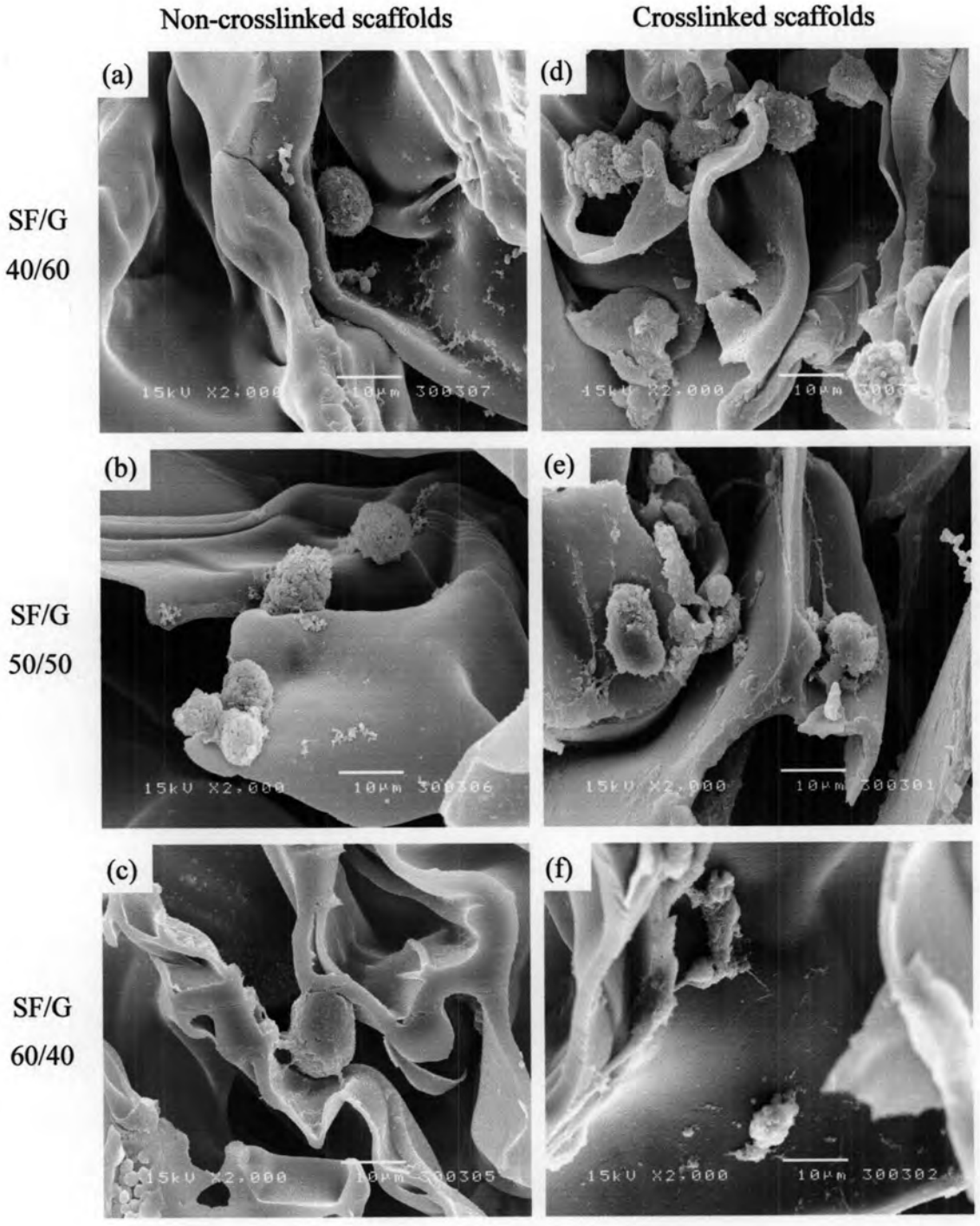


Figure 4.12 Morphology of MSCs cultured under proliferating medium for 5 days on Thai silk fibroin/gelatin scaffolds (SF/G); non-crosslinked scaffolds (a) 40/60, (b) 50/50, (c) 60/40 and crosslinked scaffolds (d) 40/60, (e) 50/50, (f) 60/40 (seeding: 5×10^5 cells/scaffold).

The morphology of MSCs cultured on non-crosslinked and crosslinked Thai silk fibroin/gelatin scaffolds at the weight blending ratios of 40/60, 50/50, and 60/40 under proliferating medium for 5 days were depicted in Figure 4.12. It was observed that morphology of MSCs proliferated on all Thai silk fibroin/gelatin scaffolds was round-shaped and no filamentous actin, except morphology of MSCs on crosslinked Thai silk fibroin/gelatin scaffold at the weight blending ratio of 50/50 that showed a sign of filamentous actin as seen in Figure 4.12 (e). This cell morphology implied that MSCs might be slightly more active on crosslinked Thai silk fibroin/gelatin scaffold at the weight blending ratio of 50/50. In addition, the cells observed on Thai silk fibroin/gelatin scaffold at the weight blending ratio of 60/40 seemed to be less than those on Thai silk fibroin/gelatin scaffolds at the weight blending ratios of 40/60 and 50/50. This corresponded to the results on the number of cells determined by DNA assay. This phenomenon could demonstrate a difference in the ability of cells to proliferate on the surface of Thai silk fibroin/gelatin scaffolds containing high and low gelatin contents.

The results on *in vitro* cell culture suggested that MSCs preferred to attach and proliferate on scaffolds containing high amount of gelatin. Together with the great physical and mechanical properties of non-crosslinked Thai silk fibroin/gelatin scaffold at the weight blending ratio of 50/50 observed without the use of EDC/NHS crosslinking agent, this scaffold was therefore selected to investigate the effects of the incorporation of hydroxyapatite particles.

4.2 Homogenized Thai silk fibroin/gelatin scaffolds with and without hydroxyapatite incorporation

After *in vitro* biocompatibility tests, the number of cells and morphology of cultured cells on MSCs-seeded scaffolds was reported and discussed. Therefore, non-crosslinked Thai silk fibroin/gelatin solution at the weight blending ratio of 50/50 was selected as a protein-based solution to mix with sieved hydroxyapatite particles at the weight blending ratio of 30/70 organic (Thai silk fibroin and gelatin)/inorganic (hydroxyapatite) parts using homogenization method.

In this section, physical and biological characteristics of homogenized Thai silk fibroin/gelatin scaffolds with and without hydroxyapatite incorporation were investigated and compared.

4.2.1 Morphology of scaffolds

The homogenized Thai silk fibroin/gelatin scaffolds with and without hydroxyapatite incorporation were vertically cut in order to observe the inner structure from cross-sectioned plane under SEM from the top (air-exposed side) through the bottom (plate-exposed side) of scaffolds as presented in Figure 4.13.

Non-uniform porous structure with small and large pores of homogenized Thai silk fibroin/gelatin scaffold without hydroxyapatite incorporation was noticed, as shown in Figure 4.14. Considering at the wall of homogenized Thai silk fibroin/gelatin scaffold without hydroxyapatite incorporation, a non-smooth surface with the distribution of small pores was exhibited. Owing to high speed used to mix the solution and high freezing rate in this scaffold preparation, small bubble could be formed and embedded in the wall of scaffold. Furthermore, at the bottom region of homogenized Thai silk fibroin/gelatin scaffold without hydroxyapatite incorporation, a closed-pore with a lot of small fibers inside these pores was noticed, as presented in Figure 4.14 (c). This implied that, heat and shear force that occurred from high speed homogenization resulted in an occurrence of silk fibroin fibers.

Figure 4.15 depicted SEM micrographs of homogenized Thai silk fibroin/gelatin scaffold with hydroxyapatite incorporation. It was found that hydroxyapatite granules were homogeneously localized in the walls of scaffold. The

particle size of these hydroxyapatite particles (less than 10 μm) was much smaller than the sieved size (100-212 μm). This implied that original hydroxyapatite particles excessively broke into small pieces during homogenization process. Considering the inner structure of homogenized Thai silk fibroin/gelatin scaffold with hydroxyapatite incorporation, homogeneous porous structure with the pore size around 100 μm was noticed at the middle and bottom regions of scaffold, as presented in Figure 4.15 (b)-(c). While at the top region of scaffold, presented in Figure 4.15 (a), non-uniform porous structure with closed-pore on the surface was formed.

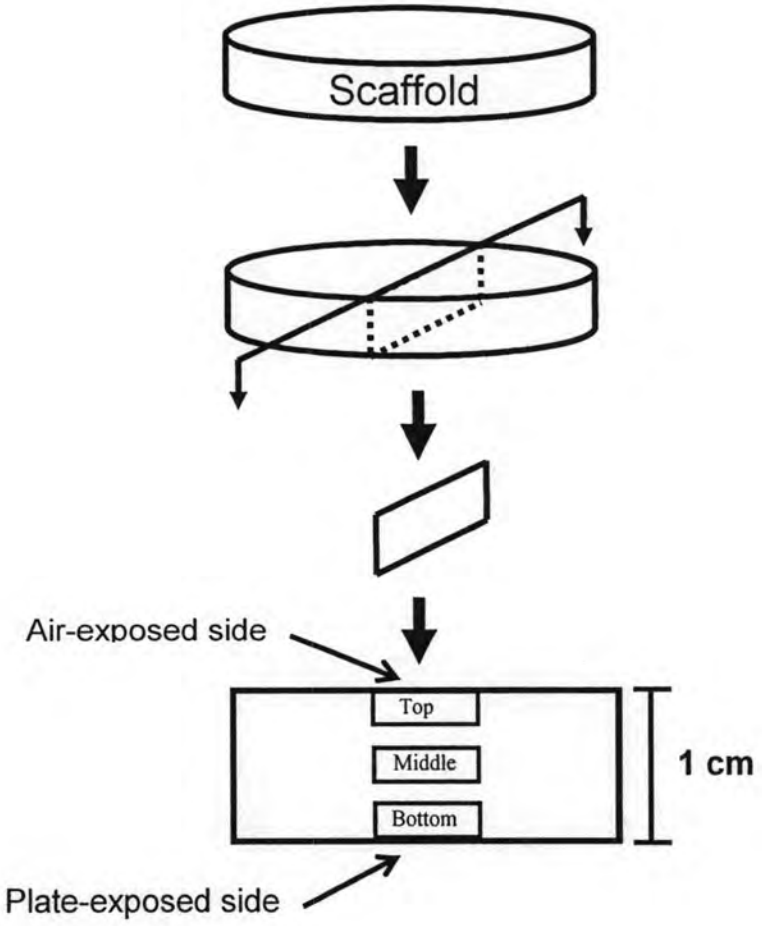


Figure 4.13 Schematic diagram of cross-sectional plane from the top (air-exposed side) through the bottom (plate-exposed side) of scaffolds.

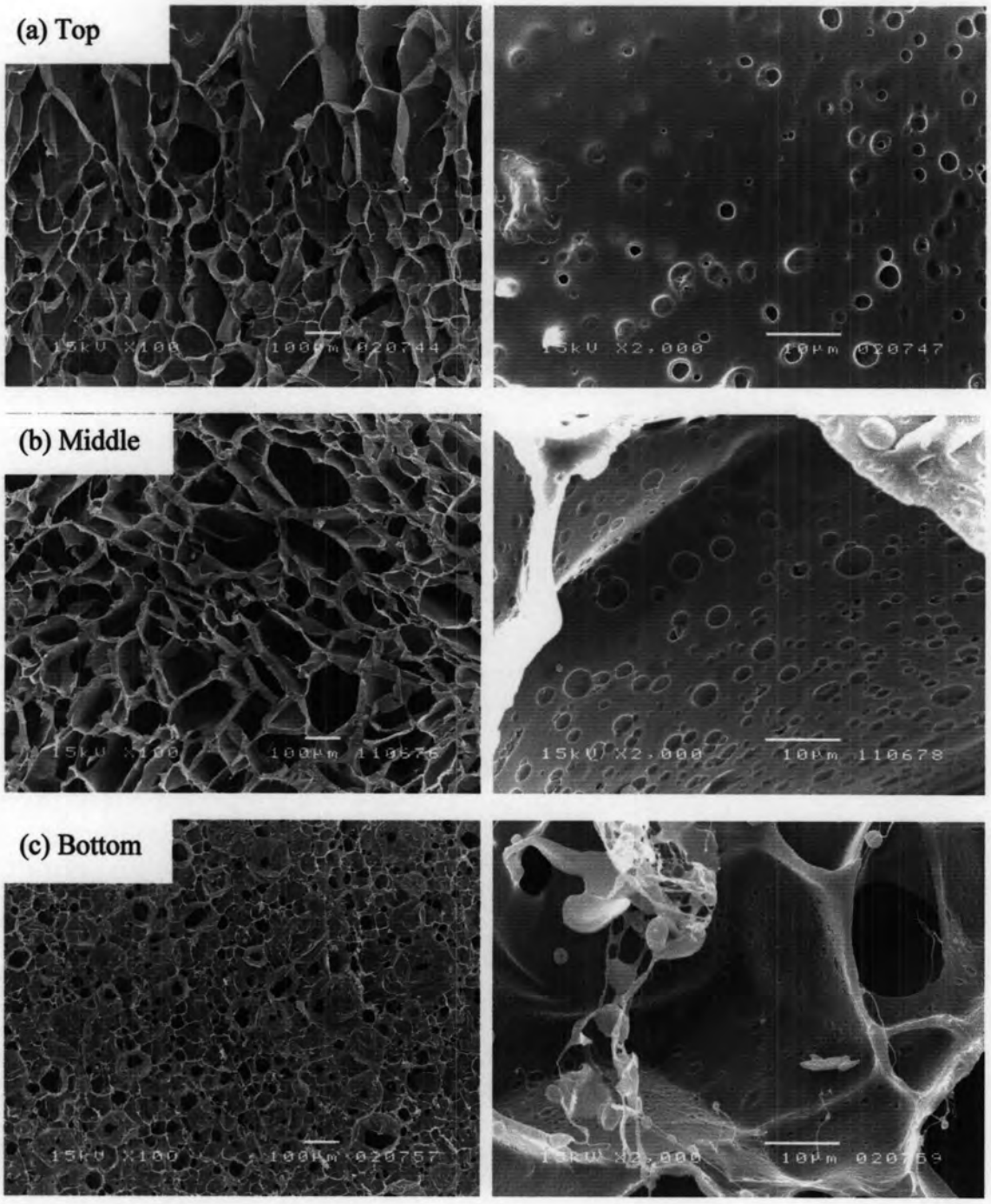


Figure 4.14 SEM micrographs of homogenized Thai silk fibroin/gelatin 50/50 scaffold without hydroxyapatite incorporation at (a) top, (b) middle, and (c) bottom positions of the scaffold.

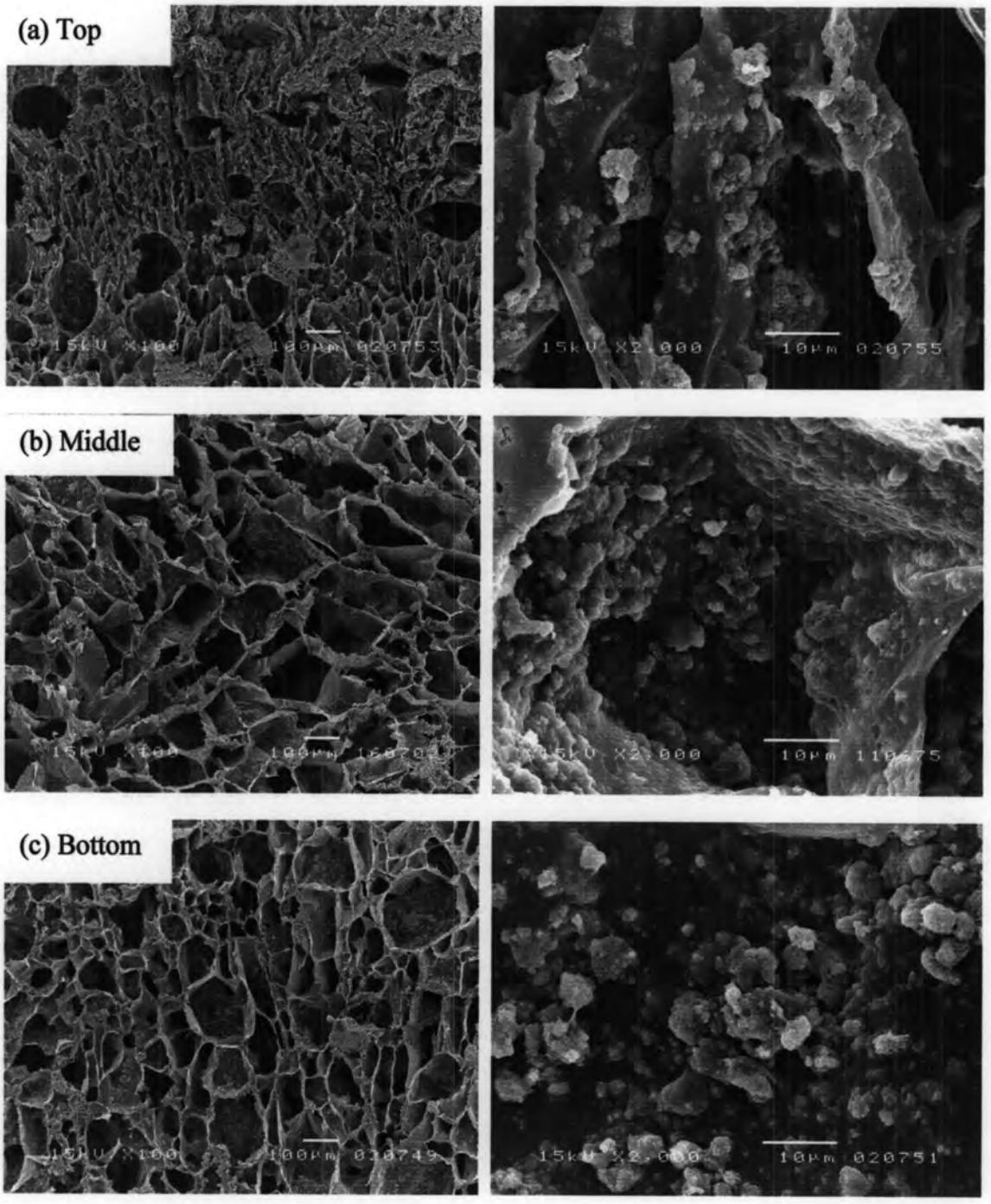


Figure 4.15 SEM micrographs of homogenized Thai silk fibroin/gelatin 50/50 scaffold with hydroxyapatite incorporation at (a) top, (b) middle, and (c) bottom positions of the scaffold.

4.2.2 *In vitro* biocompatibility using bone marrow-derived stem cells (MSCs)

In this section, non-crosslinked Thai silk fibroin/gelatin scaffold at the weight blending ratio of 50/50 was used to incorporate hydroxyapatite particles. In order to effectively distribute hydroxyapatite particles in the blended solution, high speed homogenization was employed instead of normal stirring. *In vitro* biocompatibility of homogenized Thai silk fibroin/gelatin scaffolds with and without hydroxyapatite incorporation was presented and discussed into two parts as follows:

- MSCs initial attachment and proliferation tests by DNA assay
- Osteogenic differentiation test by ALP activity and calcium content

4.2.2.1 MSCs initial attachment and proliferation tests

MSCs (5×10^5 cells per scaffold) were seeded on homogenized Thai silk fibroin/gelatin scaffolds with and without hydroxyapatite incorporation to evaluate cell attachment and proliferation when cultured under proliferating medium (α-MEM, 15% FBS, 100 U/ml penicillin/streptomycin). Figure 4.16 illustrated the number of cells determined by DNA assay after 6 h, 1, 3, and 5 days of the culture. It was observed that at 6 h after seeding, the number of attached cells on homogenized Thai silk fibroin/gelatin scaffolds with and without hydroxyapatite incorporation was around 3×10^5 and 4.3×10^5 cells per scaffold, respectively. After that the number of proliferated cells on both scaffolds along 5-day culture period was lower than the number of cells initially attached at 6 h, indicating the death of some cells on these scaffolds. It supposed to be the result of mass transfer limit causing from static culture used in this study. In addition, the high hydrophobicity of these homogenized scaffolds was noticed during the first day of the culture. This resulted in low swelling ability of scaffolds, possibly causing cell detachment and death after 6 h of agitation seeding. Another reason could be the toxicity of chloroform residue in scaffolds that was used to sustain air bubble during homogenization.

Noteworthy, the number of proliferated cells on homogenized Thai silk fibroin/gelatin scaffolds with and without hydroxyapatite incorporation at 5 days of the culture tended to be slightly increased, comparing to the number of cells at 3 days

of the culture. It was possibly due to the toxicity of scaffolds that occurred from chloroform residue was washed out during the changing medium at the first 3 days of the culture. Morphology of MSCs cultured under proliferating medium for 5 days on both scaffolds, shown in Figure 4.17, was round-shaped. Slightly more cells on the surface of homogenized Thai silk fibroin/gelatin scaffold with hydroxyapatite incorporation noticed corresponded to the number of cells determined from DNA assay.

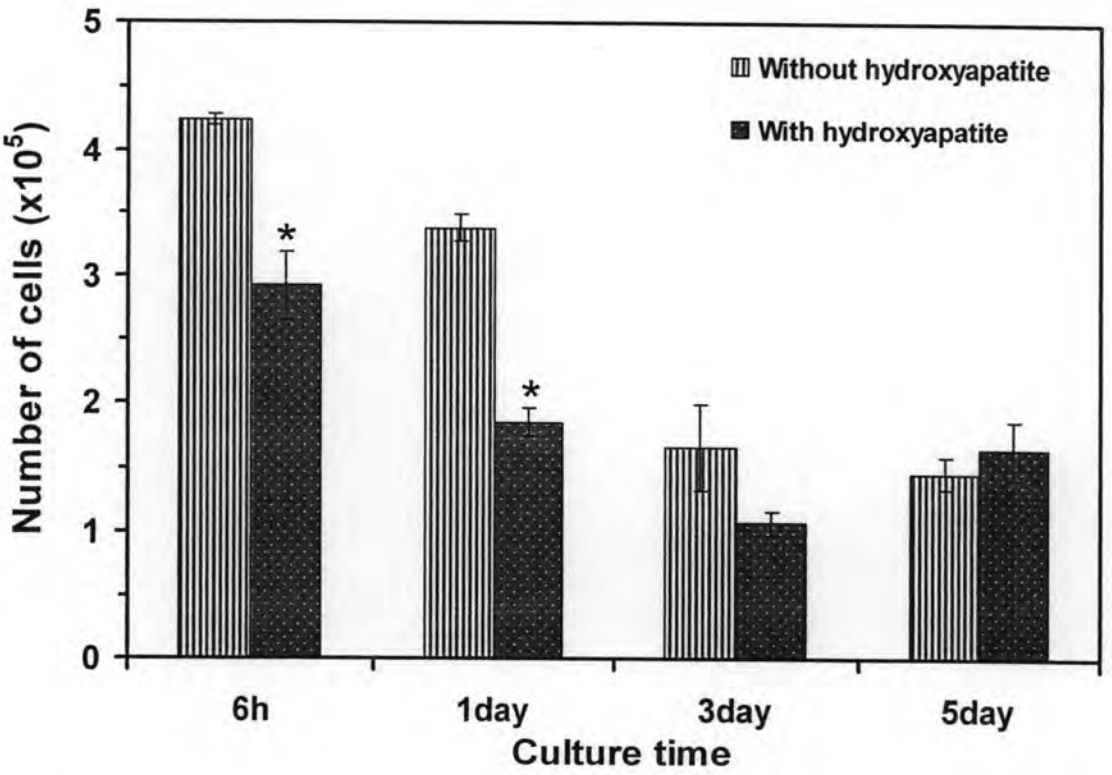


Figure 4.16 Number of MSCs attached and proliferated on homogenized Thai silk fibroin/gelatin 50/50 scaffolds with and without hydroxyapatite incorporation under proliferating medium for 6 h, 1, 3, and 5 days, determined by DNA assay (seeding: 5×10^5 cells/scaffold).

* represent the significant difference ($p<0.05$) relative to homogenized Thai silk fibroin/gelatin scaffold without hydroxyapatite incorporation at each culture time.

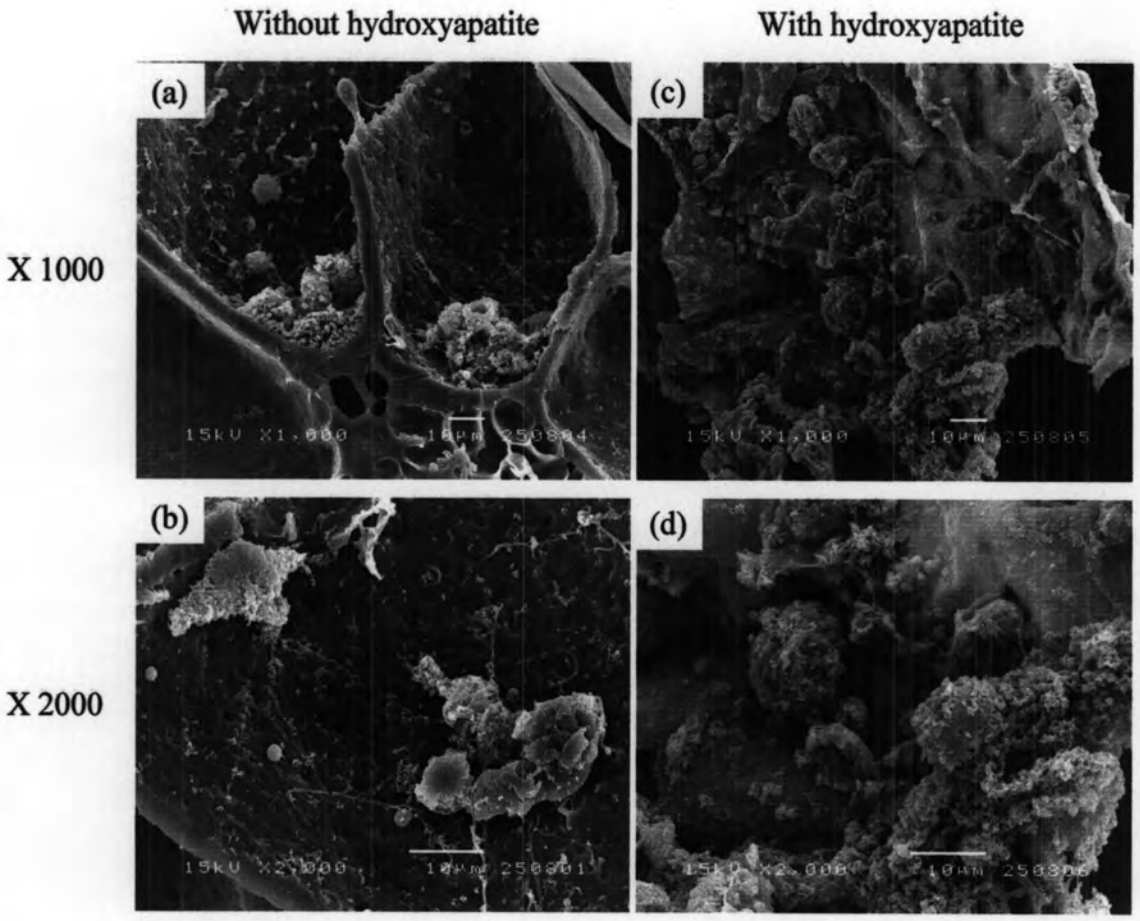


Figure 4.17 Morphology of MSCs cultured under proliferating medium for 5 days on (a, b) homogenized Thai silk fibroin/gelatin 50/50 scaffold without hydroxyapatite incorporation and (c, d) homogenized Thai silk fibroin/gelatin 50/50 scaffold with hydroxyapatite incorporation (seeding: 5×10^5 cells/scaffold).

4.2.2.2 Osteogenic differentiation test

MSCs (1×10^6 cells per scaffold) were cultured on homogenized Thai silk fibroin/gelatin scaffolds with and without hydroxyapatite incorporation under proliferating medium (α-MEM, 15% FBS, 100 U/ml penicillin/streptomycin) for 1 day. After that, the medium was changed into osteogenic medium (α-MEM, 10% FBS, 10 mM β-glycerophosphate, 50 μg/ml L-ascorbic acid, and 10 nM dexamethasone). MSCs were cultured on these scaffolds under osteogenic medium for 7, 14, 21, and 28 days.

The number of cells on homogenized Thai silk fibroin/gelatin scaffolds with and without hydroxyapatite incorporation under the culture with osteogenic medium, analyzed by DNA assay, was presented in Figure 4.18. It was found that at 7 day after seeding, the number of cells on homogenized Thai silk fibroin/gelatin scaffolds with and without hydroxyapatite incorporation was around 1.6×10^5 and 1.5×10^5 cells per scaffold, respectively. After 7-day culture period, the number of cells tended to be slightly increased along the further culture till 28-day period. At 21-day and 28-day culture period, the number of proliferated cells on homogenized Thai silk fibroin/gelatin scaffold with hydroxyapatite incorporation under osteogenic medium was significantly higher than those on scaffold without hydroxyapatite incorporation. It was interesting that homogenized Thai silk fibroin/gelatin scaffold with hydroxyapatite incorporation could promote proliferation of MSCs under osteogenic medium. This result corresponded to the result on morphology of MSCs cultured under osteogenic medium for 28 days on both scaffolds (Figure 4.19). More cells on the surface of homogenized Thai silk fibroin/gelatin scaffold with hydroxyapatite incorporation were noticed, comparing to those on scaffold without hydroxyapatite particles. This could be due to the roughness of the scaffold surface induced by hydroxyapatite particles which was able to promote the adhesion and proliferation of cells [50, 63]. Another reason could be the scaffold shrinkage along 28 days of cultured period. The shrinkage of homogenized Thai silk fibroin/gelatin scaffold with hydroxyapatite incorporation was noticed to be slightly less than scaffold without hydroxyapatite, indicating more surface area for the proliferation of cells along culture period. Hiraoka *et.al.* [64] have studied the fabrication and biocompatibility of collagen sponge reinforced with poly(glycolic acid) fiber. They suggested that

shrinkage of sponge after cell seeding was suppressed by fiber incorporation. It is possible that shrinkage suppression resulted in the superior cell attachment and proliferation. In addition, Han *et.al.* [63] have studied the biomimetic chitosan–nanohydroxyapatite composite scaffolds for bone tissue engineering. They found the favorable biological response of pre-osteoblast (MC3T3-E1) on chitosan–nanohydroxyapatite scaffolds, including improved cell adhesion, higher proliferation, and well spreading morphology, comparing to pure chitosan scaffold.

ALP activity of MSCs cultured on homogenized Thai silk fibroin/gelatin scaffolds with and without hydroxyapatite incorporation under osteogenic medium for 7, 14, 21, and 28 days was elucidated in Figure 4.20 (a). It was observed that the highest ALP activity was found at 7-day of osteogenic culture. After 14-day culture period, ALP activity of MSCs cultured on both scaffolds tended to slightly decrease with no significant different along the further culture till 28 days. Arpornmaeklong *et.al.* [65] have studied growth and differentiation of mouse osteoblasts on chitosan/collagen scaffolds. They suggested that increase in ALP activity indicating early osteoblastic differentiation stage can be seen after 4 days, with activity peaking at 7-10 days and then slowly decreasing.

Calcium content of MSCs cultured on homogenized Thai silk fibroin/gelatin scaffolds with and without hydroxyapatite incorporation under osteogenic medium for 7, 14, 21, and 28 days was shown in Figure 4.20 (b). Significantly higher calcium content was found in the case of homogenized Thai silk fibroin/gelatin scaffold with hydroxyapatite incorporation within the early periods of osteogenic culture (7-day and 14-day culture period). This could be the result from the osteoinductive of hydroxyapatite. However, it was interesting that, for longer osteogenic culture (21-day and 28-day culture period), calcium content of MSCs cultured on both scaffolds was similar. This revealed the osteoconductivity of Thai silk fibroin/gelatin scaffolds.

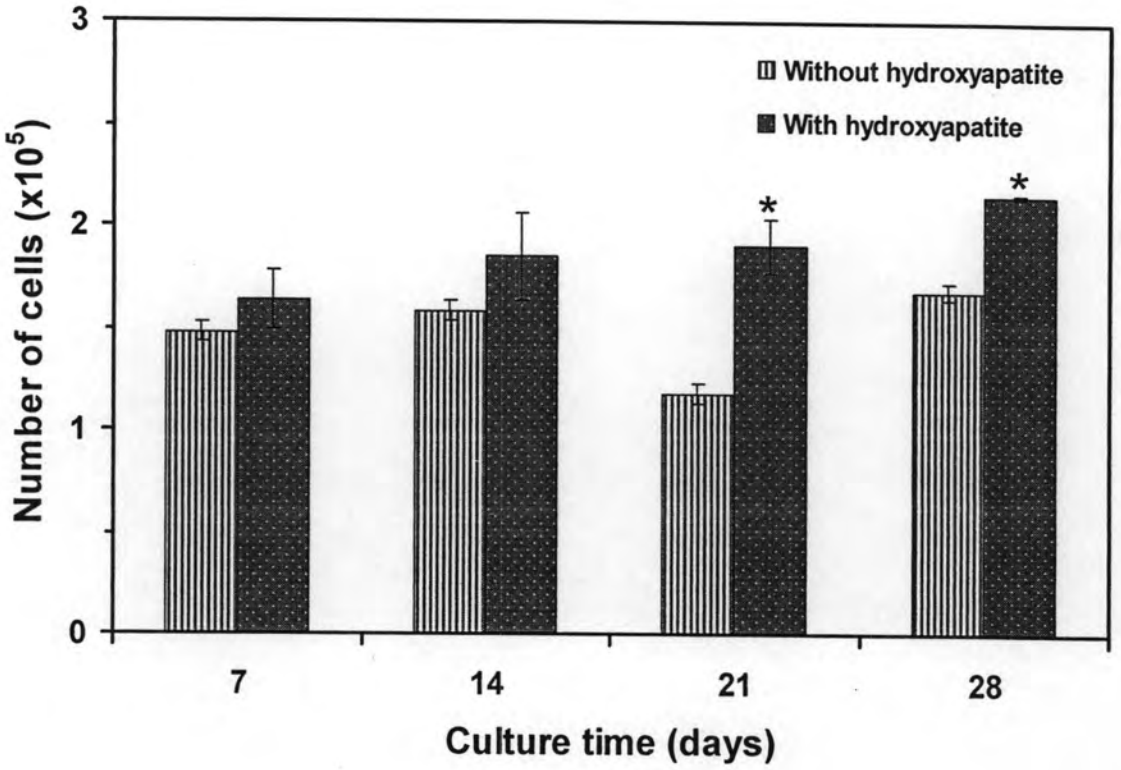


Figure 4.18 Number of MSCs cultured on homogenized Thai silk fibroin/gelatin 50/50 scaffolds with and without hydroxyapatite incorporation under osteogenic medium for 7, 14, 21, and 28 days, determined by DNA assay (seeding: 1×10^6 cells/scaffold).

* represent the significant difference ($p<0.05$) relative to homogenized Thai silk fibroin/gelatin scaffold without hydroxyapatite incorporation at each culture time.

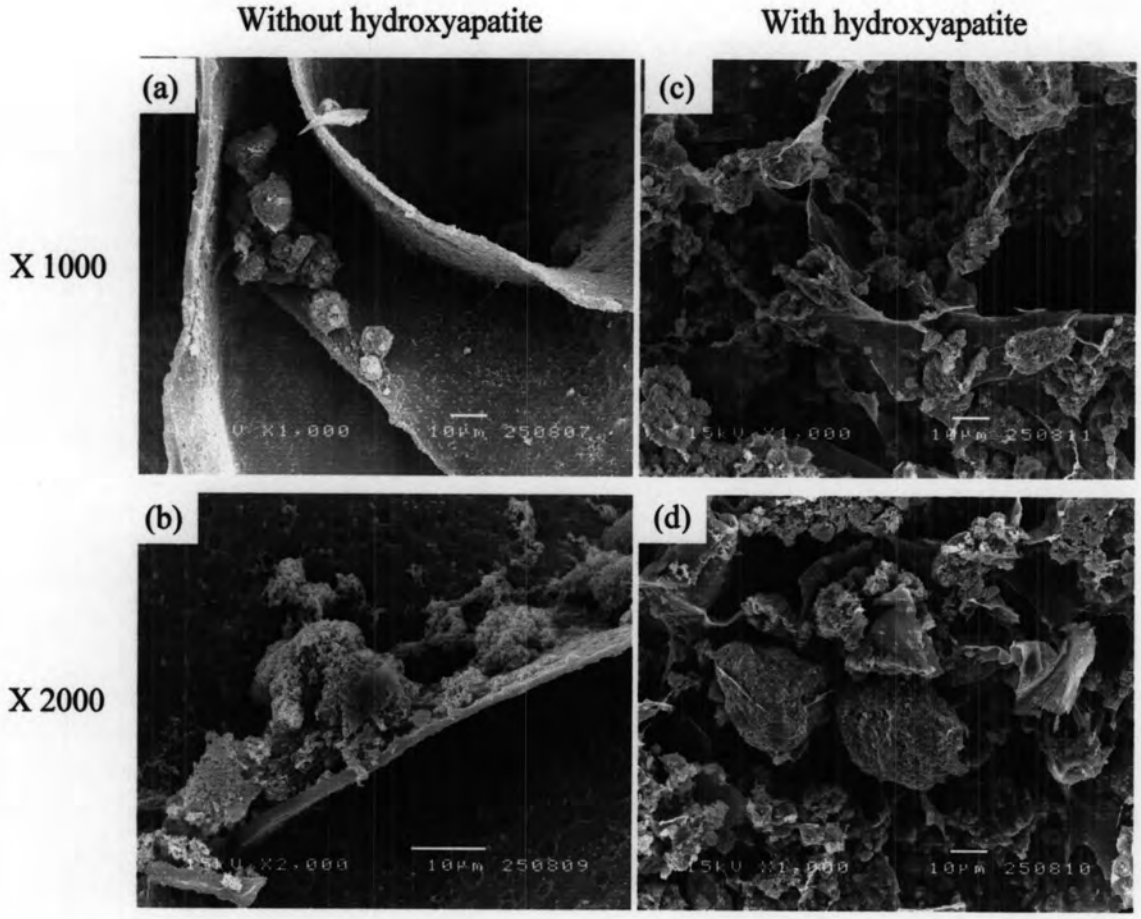
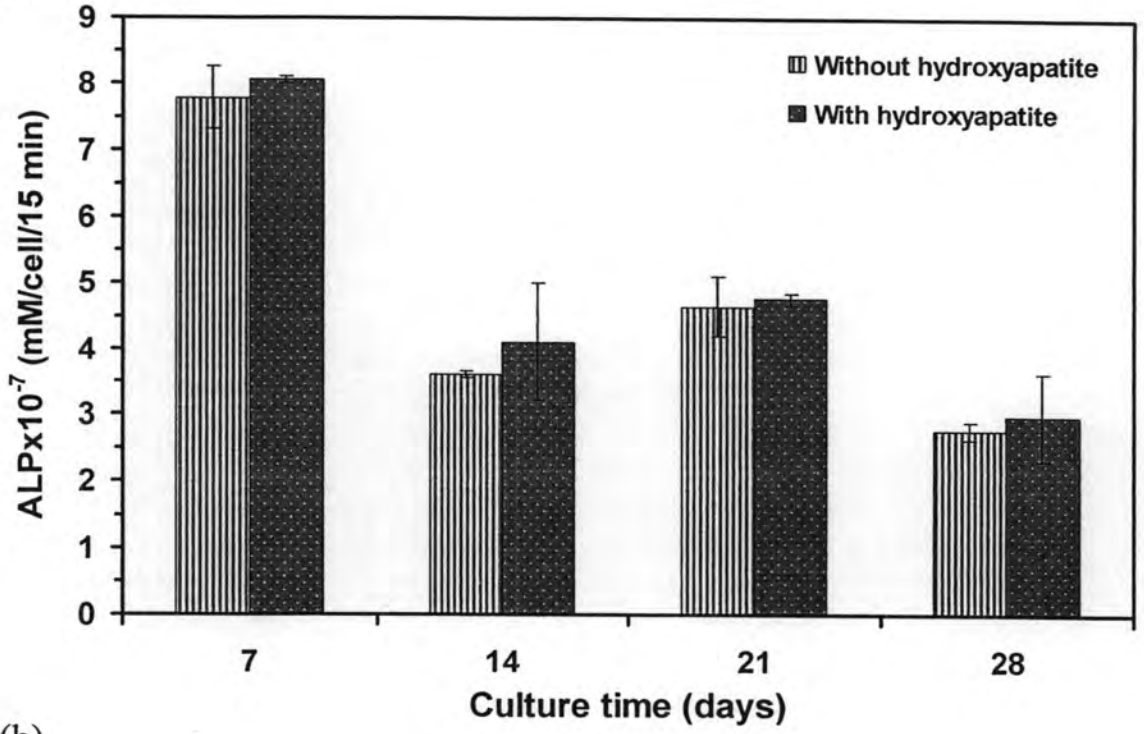


Figure 4.19 Morphology of MSCs cultured under osteogenic medium for 28 days on (a, b) homogenized Thai silk fibroin/gelatin 50/50 scaffold without hydroxyapatite incorporation and (c, d) homogenized Thai silk fibroin/gelatin 50/50 scaffold with hydroxyapatite incorporation (seeding: 1×10^6 cells/scaffold).

(a)



(b)

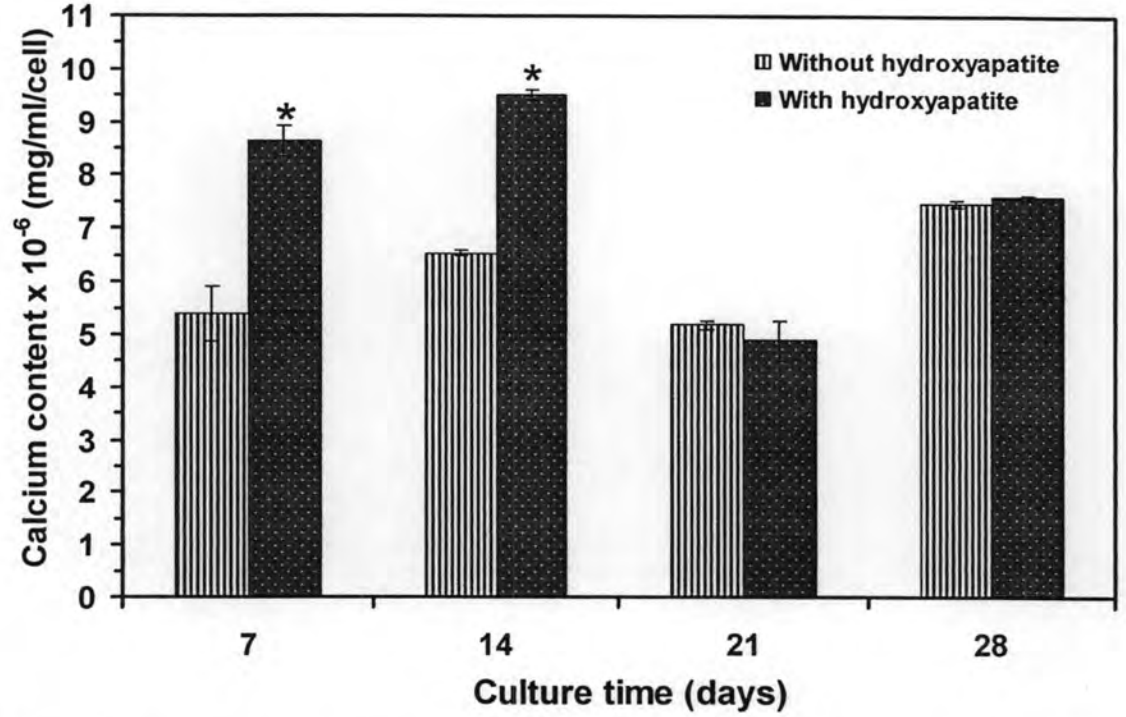


Figure 4.20 (a) ALP activity and (b) calcium content of MSCs cultured on homogenized Thai silk fibroin/gelatin 50/50 scaffolds with and without hydroxyapatite incorporation under osteogenic medium for 7, 14, 21, and 28 days (seeding: 1×10^6 cells/scaffold).

* represent the significant difference ($p<0.05$) relative to homogenized Thai silk fibroin/gelatin scaffold without hydroxyapatite incorporation at each culture time.