CHAPTER V

RESULTS AND DISCUSSIONS

5.1 Chemical and physical properties of the scaffolds

5.1.1 Preparation of gelatin and collagen/gelatin solutions

Both gelatin types could be easy to dissolve in hot water because they were hydrophilic. In contrast, collagen could only be dissolved in acidic solvent because of its hydrophobicity. To prepare collagen/gelatin scaffolds, collagen and gelatin were allowed to mix in hydrochloric (HCl) solution. In this solution, collagen showed anionic charge, therefore, gelatin was required to have cationic charge. Both gelatin types could have different charges, depending on their pH and pI. Type A gelatin, pH < pI, showed cationic charge while type B gelatin, pH > pI, showed anionic charge as in collagen. Therefore, type A gelatin was chosen to blend with collagen because they were different in charges. With this reason, homogeneous solutions were obtained from the mixing between collagen and type A gelatin.

5.1.2 Morphology of the scaffolds

In general, porosity, pore size and orientation of porous scaffold were indispensable elements of biological activity of biomaterials having open-pored structures. The effects of biomaterials type, solution concentration and blending composition were the interesting points to study.

5.1.2.1 Gelatin scaffolds

The morphology of the gelatin scaffolds seemed to mainly depend upon the gelatin types and solution concentration. Figure 5.1 showed the morphology of the scaffolds prepared from different gelatin types and solution concentrations.

By considering the effect of solution concentration, gelatin scaffolds obtained from low solution concentration showed fiber-like structure while the

scaffolds obtained from high solution concentration showed more membrane-like structure for both gelatin types. For example, a fiber-like structure with a small amount of thin-wall, was found in *0.4A, 0.4B, 0.6A* and *0.6B* scaffolds as presented in Figure 5.1(a), (b), (c) and (d), respectively. At high solution concentration, *0.8A* and *0.8B*, the gelatin scaffolds revealed an interconnected network pore configuration with heterogeneous pores as presented in Figure 5.1(e) and (f). These structures could be explained by the differences in mass content of the scaffolds and the dispersion of ice crystal in the frozen gelatin solutions. At low solution concentration, there was not enough gelatin mass to form a continuous wall in the scaffolds; therefore the continual ice crystals could dispersed without a lot of gelatin obstacle. After lyophilized, a small amount of gelatin walls was found among the fiber texture of gelatin. On the other hand, high solution concentration could form continuous walls of gelatin to block ice crystal formation; therefore the ice crystal could not continuously disperse in the frozen solution. As a result after lyophilization, large and non-uniform pores were found.

By comparing of two gelatin types, *0.4B* and *0.6B* showed more thin-walls among the fibers than *0.4A* and *0.6A* did. The more thin-wall formation in type B gelatin scaffolds might be due to the difference in gelling transition temperature or in producing process of both gelatin types. Type B gelatin might form gel during preparation, and the gel formation represented as the wall, as illustrated in Figure 5.1(b) and (d). At 0.8wt% solution concentration, pores of *0.8B* scaffold were larger than those of *0.8A* and less interconnected. This could be also due to the difference in gelling transition temperature or in producing process affecting the size of ice crystals formed during freezing. In addition, it could be observed that structures of type A gelatin scaffolds were more porous than those of type B. The results suggested that different gelatin types and solution concentrations provided the different morphologies of the scaffolds.

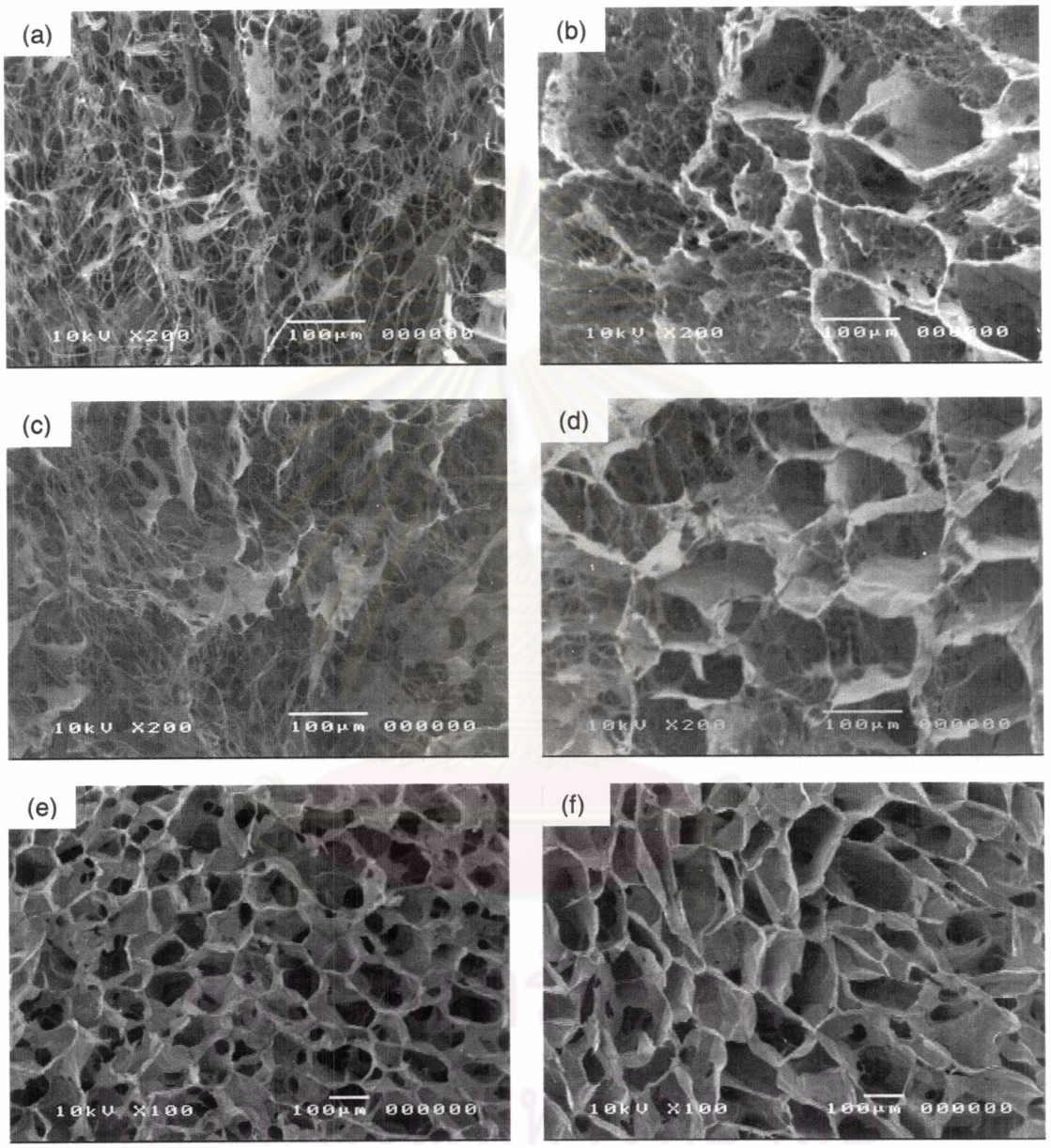

Figure 5.1: SEM micrographs of vertical cross-sections of gelatin scaffolds prepared from various gelatin types and solution concentrations (48 h DHT): (a) *0.4A*, (b) *0.4B*, (c*) 0.6A*, (d) *0.6B*, (e) *0.8A* and (f) *0.8B*.

5.1.2.2 Collagen/gelatin scaffolds

Pore structure of collagen/gelatin scaffolds could be affected by both solution concentration and blending composition. However, the structures of collagen/gelatin scaffolds seemed to be mainly influenced by the solution concentration rather than the blending composition. At the same blending composition but different concentrations, it could be seen that *0.4CG* scaffolds (Figure 5.2(a), (c) and (e)) revealed more fiber-like with interconnected network structure and higher porosity than *0.6CG* scaffolds (Figure 5.2(b), (d) and (f)). The scaffolds showed the interconnected network structure between the collagen representing the membrane-like structure and the gelatin resulting in the fibrous structure. These results could be explained as previously described for gelatin scaffolds. Furthermore, it was found that the pore configuration of all blending compositions of *0.4CG* scaffolds was non-uniform while that of *0.6CG* scaffolds was better regulated, as shown in Figure 5.2(b), (d), (f) and (h).

Comparing the effects of blending composition, the structures became less porous and the pores had more pattern when increasing collagen contents. For example, the *0.6CG10/90* scaffold, with the least collagen content shown in Figure 5.2(b), depicted the poorest pore arrangement while the *0.6CG20/80*, *0.6CG30/70* and *0.6C100* presented better pore arrangement with heterogeneity. Therefore, it could be concluded that collagen mainly affected the porosity and the pore arrangement of the scaffolds.

From the results, the morphologies of collagen/gelatin scaffolds were different due to the difference in both solution concentration and blending composition. The solution concentration affected the presence or absence of fiber texture in the scaffolds. The blending composition resulted in the differences in porosity and pore arrangement of the scaffolds.

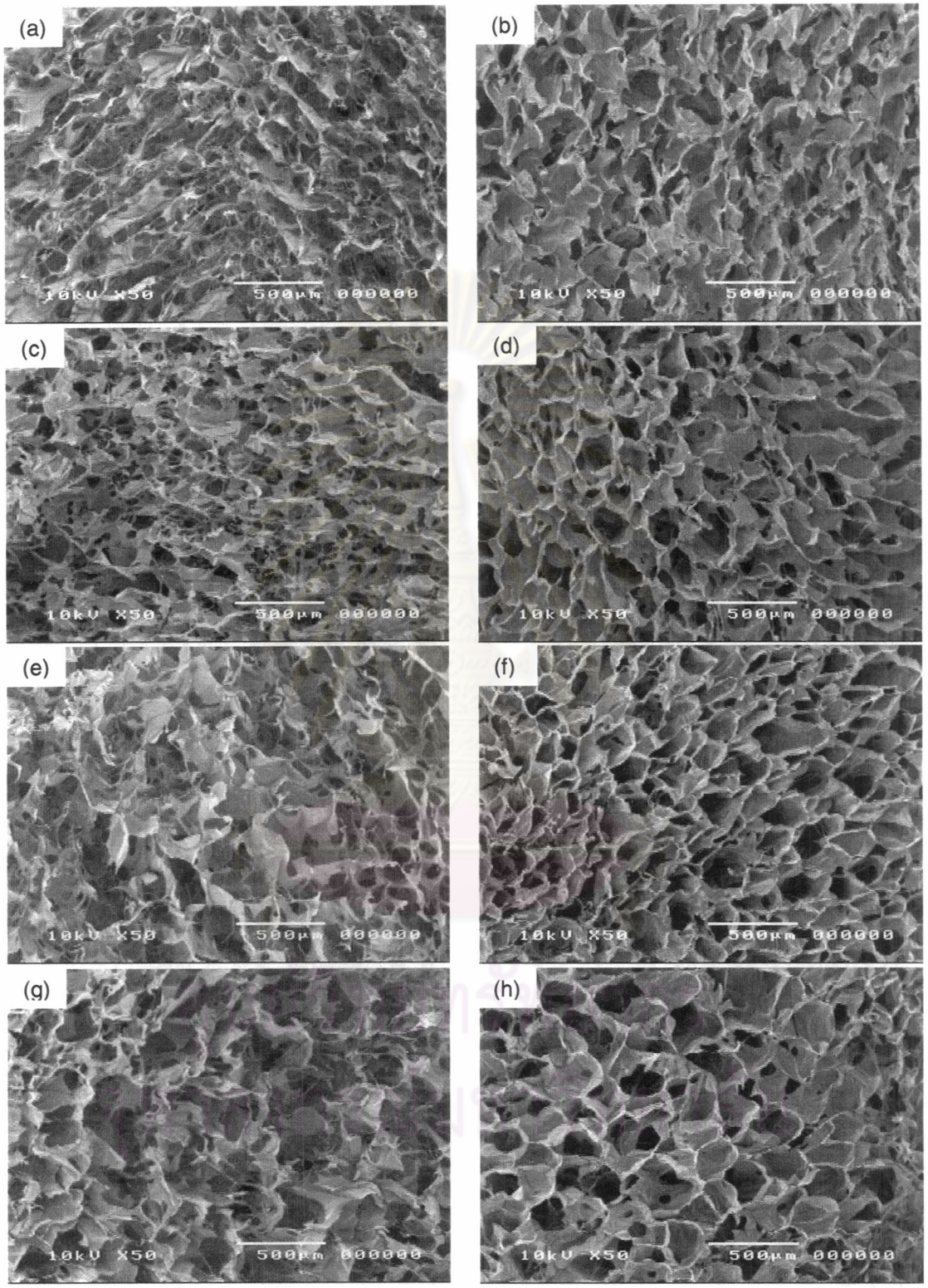

Figure 5.2: SEM micrographs of vertical cross-sections of collagen/gelatin scaffolds prepared from various solution concentrations and blending compositions (48 h DHT): (a) *0.4CG10/90*,(b) *0.6CG10/90*, (c) *0.4CG20/80*, (d) *0.6CG20/80*, (e) *0.4CG30/70*, (f) *0.6CG30/70*, (g) *0.4C100*, (h) *0.6C100*.

5.1.3 Crosslinking degree

Crosslinking via physical or chemical methods was often applied to prevent a rapid degradation of gelatin-based biomaterials during *in vivo* application. Dehydrothermal (DHT) crosslinking was a physical method which was applied to stabilize scaffold and to prevent scaffold collapse while no cytotoxic reagents were introduced. Stabilization was believed to be due to the formation of interchain crosslinks as a result of condensation reactions either by esterification or amide formation. After DHT treatment, porous scaffold morphologies were retained, as in non-treated scaffolds. DHT treatment of gelatin-based scaffolds resulted in a decrease in the free amino group content and in the PBS solution adsorption and an increase in the compressive modulus. In this study, gelatin and collagen/gelatin scaffolds were crosslinked by DHT treatment at the temperature of 140°C under vacuum. DHT crosslinking generated chemical bonding between NH of amino groups and H of carboxyl groups due to thermal dehydration. The decreasing of free amino groups could be detected by TNBS reaction. The results were represented as the increasing of crosslinking degree(%) which could be explained by the decreasing of free amino group content using β-alanine as a standard [60].

5.1.3.1 Gelatin scaffolds

Figure 5.3 presented the crosslinking degree of gelatin scaffolds prepared from various gelatin types, solution concentrations and DHT treatment times. The results from TNBS showed that the extent of crosslinking of gelatin scaffolds was a function of DHT treatment time and gelatin type but showed no significant difference between various gelatin concentrations. With the increasing DHT treatment time from 24 to 72 h, the crosslinking degree increased from about 28% to 41% for type A gelatin scaffolds and from about 15% to 34% for type B gelatin scaffolds.

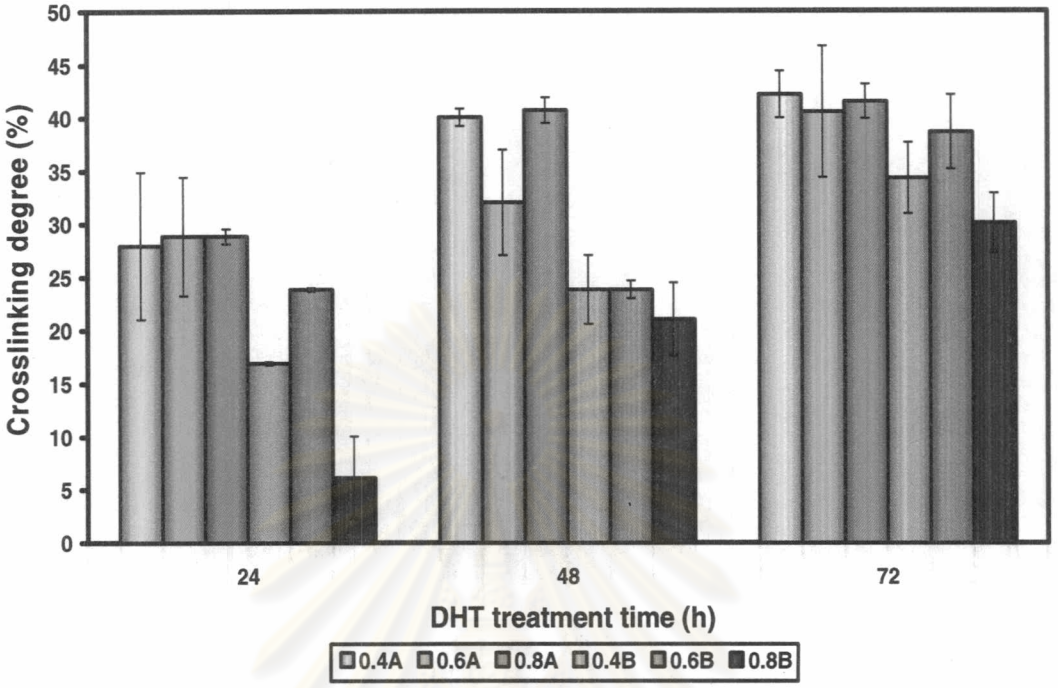

Figure 5.3: Crosslinking degree of gelatin scaffolds prepared from various gelatin types, concentrations and DHT treatment times.

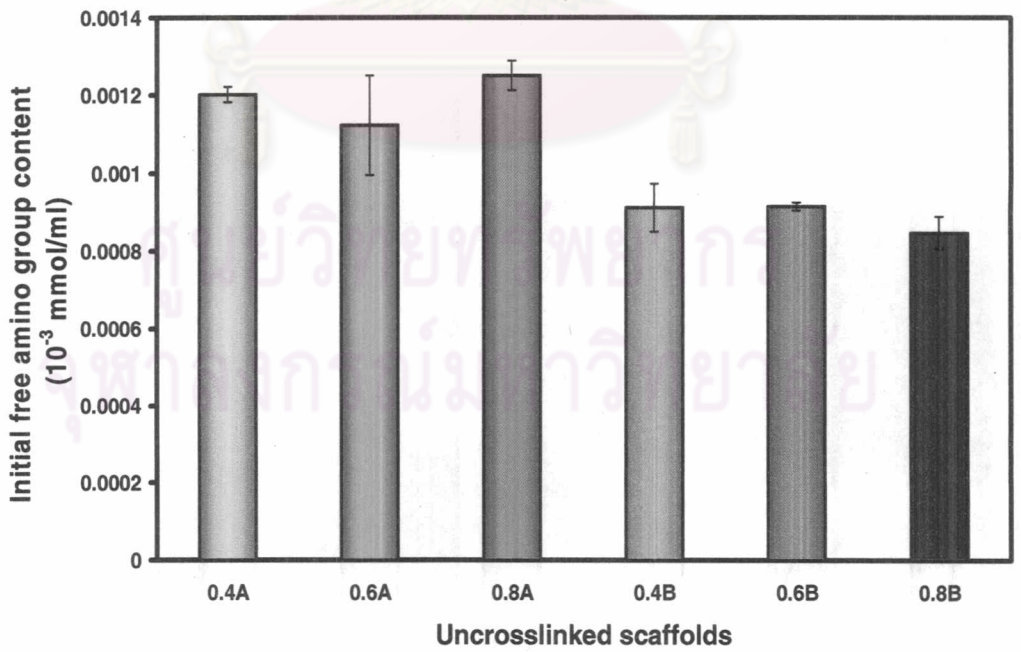

Figure 5.4: Initial free amino group content in gelatin scaffolds prepared from various gelatin types and concentrations.

The difference in crosslinking degree between scaffolds from two gelatin types was the result of the difference in initial free amino group content which could be affected by source and producing process of gelatin. Collagen from various connective tissues had different amino group contents[33]. Because type A gelatin was derived from porcine collagen while type B gelatin was derived from bovine collagen, amino group contents in both gelatin types were different. In addition, the different producing process of two gelatin types, acid process and alkaline process, could bring to the difference in free amino group contents. The amount of initial free amino group in each gelatin scaffold could be seen obviously in Figure 5.4. Before the DHT treatment, type B gelatin scaffolds showed less initial free amino group contents than type A gelatin scaffolds. The less amount of initial free amino group contents resulted in the less crosslinking in type B gelatin. On the other hand, type A gelatin originally had more free amino group contents so that there were abundant amino groups to link each other, leading to the higher crosslinking degrees. Furthermore, the difference in crosslinking degree may be due to a difference in molecular structure of both gelatin types. The results corresponded with a recent study by Tabata et al. [23] reporting that type A gelatin could be crosslinked more than type B gelatin could. They proved that DHT crosslinking could occur only if the amino and carboxyl groups were close to each other, which meant that the gelatin molecules were closer to each other because the transition temperature from random coil to helix conformation is higher. Therefore, the more crosslinking degree of type A gelatin might be due to the closer of its molecules than that of type B gelatin.

Along the increasing in DHT treatment time from 24 to 72 h, crosslinking degree of type B gelatin scaffolds gradually increased. Unlike type B gelatin, crosslinking degree of type A gelatin scaffolds increased significantly when the DHT treatment time increased from 24 to 48 h but they showed a little increasing after 48 h DHT. Therefore, it was adequate for type A gelatin scaffolds to be treated only 48 h DHT crosslinking.

5.1.3.2 Collagen/gelatin scaffolds

For collagen/gelatin scaffolds, DHT treatment was performed only for 24 and 48 h, because the results from gelatin scaffolds has revealed that there was no significant increasing of crosslinking degree after 48 h DHT treatment for type A gelatin. The results on crosslinking degree of collagen/gelatin scaffolds obtained from 0.4wt% and 0.6wt% solution concentrations, as illustrated in Figure 5.5 and 5.7, respectively, showed that the extent of crosslinking was a function of solution concentration, blending composition and DHT treatment time.

The comparison between two solution concentrations, 0.4wt% and 0.6wt%, indicated that the crosslinking degree increased from about 29% to 34% for collagen/gelatin scaffolds prepared from 0.4wt% solution concentration (*0.4CG10/90, 0.4CG20/80* and *0.4CG30/70*) and from about 33% to 45% for collagen/gelatin scaffolds prepared from 0.6wt% solution concentration (*0.6CG10/90, 0.6CG20/80* and *0.6CG30/70*) when increasing DHT treatment time from 24 to 48 h. The results represented that collagen/gelatin scaffolds prepared from 0.6wt% solution concentration could be crosslinked more than the ones from 0.4wt%. As discussed in the results of gelatin scaffolds that the initial free amino group contents of collagen/gelatin scaffolds prepared from 0.6wt% solution concentration which were higher than those from 0.4wt%, as presented in Figure 5.6 and 5.8, resulted in higher possibility to crosslink to each other.

Considering pure and blended scaffolds, pure collagen scaffolds (*0.4C100* and *0.6C100*) were crosslinked less than other collagen/gelatin scaffolds. This could be caused by the lowest initial free amino group contents in pure collagen, as showed in Figure 5.6 and 5.8. Another reason could be the structure of collagen. Collagen was composed of three chains, twisted together in a tight triple helix. The tight triple helix of collagen might hinder the crosslinking. For blended scaffolds, the scaffolds containing high gelatin content showed high crosslinking degree because gelatin had more free amino groups than collagen. However, it was interesting to note that collagen/gelatin scaffolds obtained from 0.6wt% solution concentration could be crosslinked more than pure gelatin scaffold from the same solution concentration. This might imply that the interaction between gelatin and collagen in the blend would

give some open sites of free amino groups, leading to a more opportunity for crosslinking.

Also, the results revealed that the higher crosslinking degree of the scaffolds along the rising of DHT treatment time from 24 to 48 h was independent to solution concentration or blending composition.

In conclusion, the crosslinking degree of scaffolds depended on solution concentration, blending composition and DHT treatment time. Moreover, high collagen-containing scaffolds showed lower crosslinking degree than low collagen-containing scaffolds because of the limiting of its structure and the fewer initial free amino group contents.

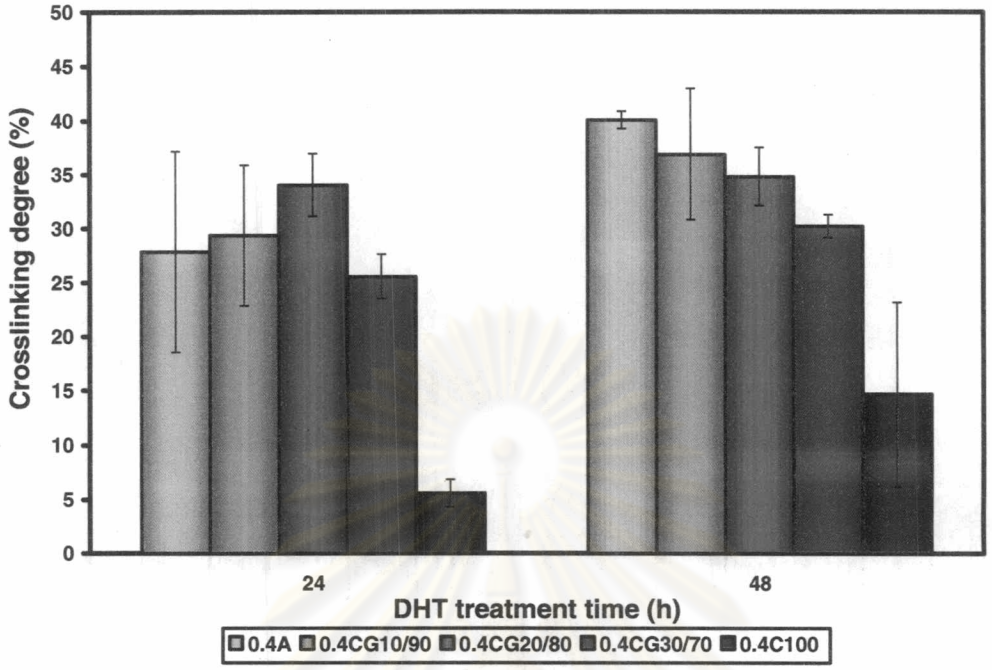

Figure 5.5: Crosslinking degree of collagen/gelatin scaffolds prepared from 0.4wt% solution concentration with various blending compositions and DHT treatment times.

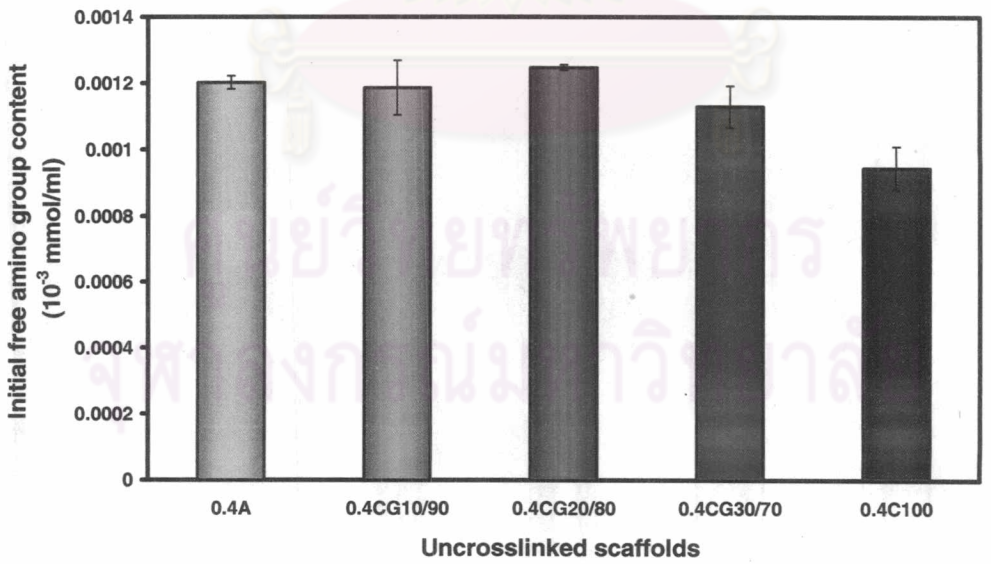

Figure 5.6: Initial free amino group content in collagen/gelatin scaffolds prepared from 0.4wt% solution concentration with various blending compositions and DHT treatment times.

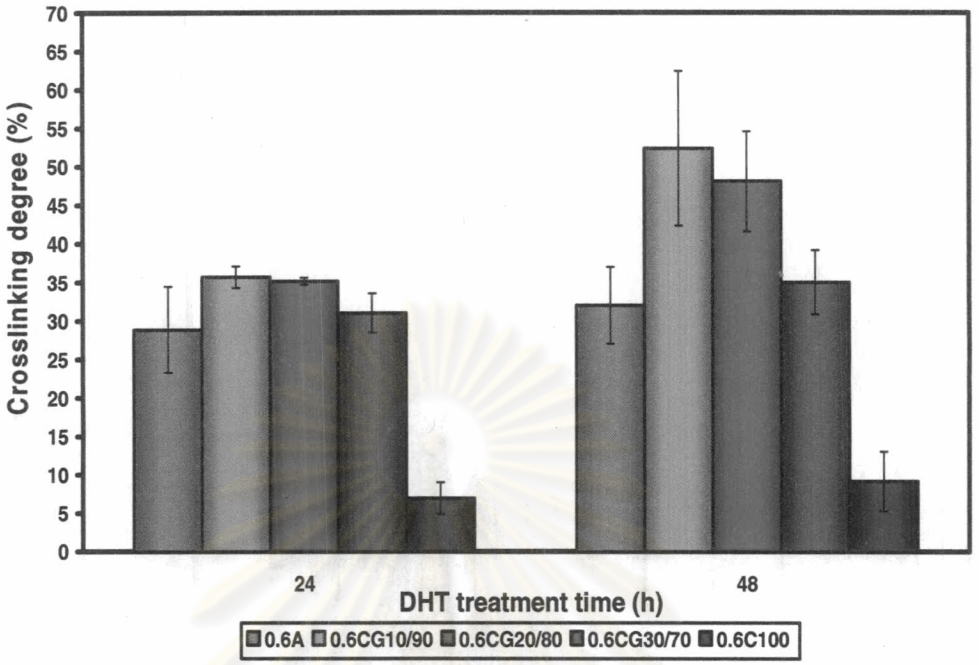

Figure 5.7: Crosslinking degree of collagen/gelatin scaffolds prepared from 0.6wt% solution concentration with various blending compositions and DHT treatment times.

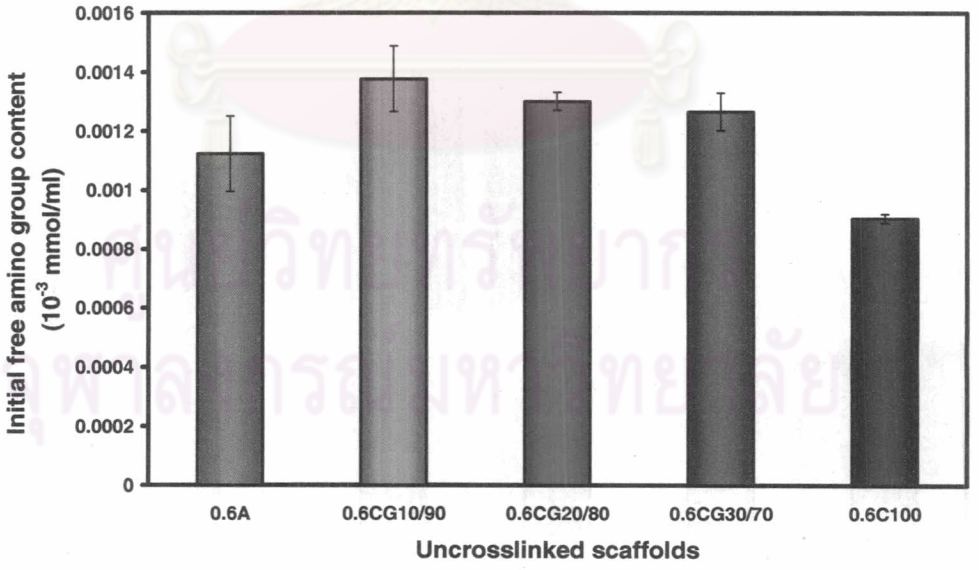

Figure 5.8: Initial free amino group content in collagen/gelatin scaffolds prepared from 0.6wt% solution concentration with various blending compositions and DHT treatment times.

5.1.4 Mechanical properties of the scaffolds

One of the most important properties of the scaffolds was the mechanical strength. To maintain the scaffold when used as a skin substitute, the scaffolds had to be strong enough in order to support extensive vasculatures, the lymphatic system, nerve bundles and other structure in the skin. Therefore, the scaffolds should have appropriate compressive modulus to absorb forces when they are implanted into the wounds.

5.1.4.1 Gelatin scaffolds

Compressive modulus of gelatin scaffolds, elucidated in Figure 5.9, was remarkedly affected by the solution concentration rather than gelatin type and DHT treatment time.

By comparing the solution concentrations of both gelatin types, it could be obviously observed that the compressive modulus was increased as increasing the solution concentration. Particularly for gelatin scaffolds obtained from 0.8wt% solution concentration of which the compressive modulus was about 4 times higher than those from 0.6 wt% and about 16 times higher than those from 0.4wt%. These results ensured that gelatin scaffolds obtained from 0.8wt% solution concentration possessed mechanical integrity. However, flexibility was required in scaffolds to be used as a skin substitute so that scaffolds from 0.4wt% and 0.6wt% solution concentrations were more suitable in this case.

Considering the effects of gelatin type and DHT treatment time, there was no significant difference between two gelatin types and among three DHT treatment time periods. This might be suggested that gelatin type and crosslinking time hardly affected the compressive modulus of the scaffolds.

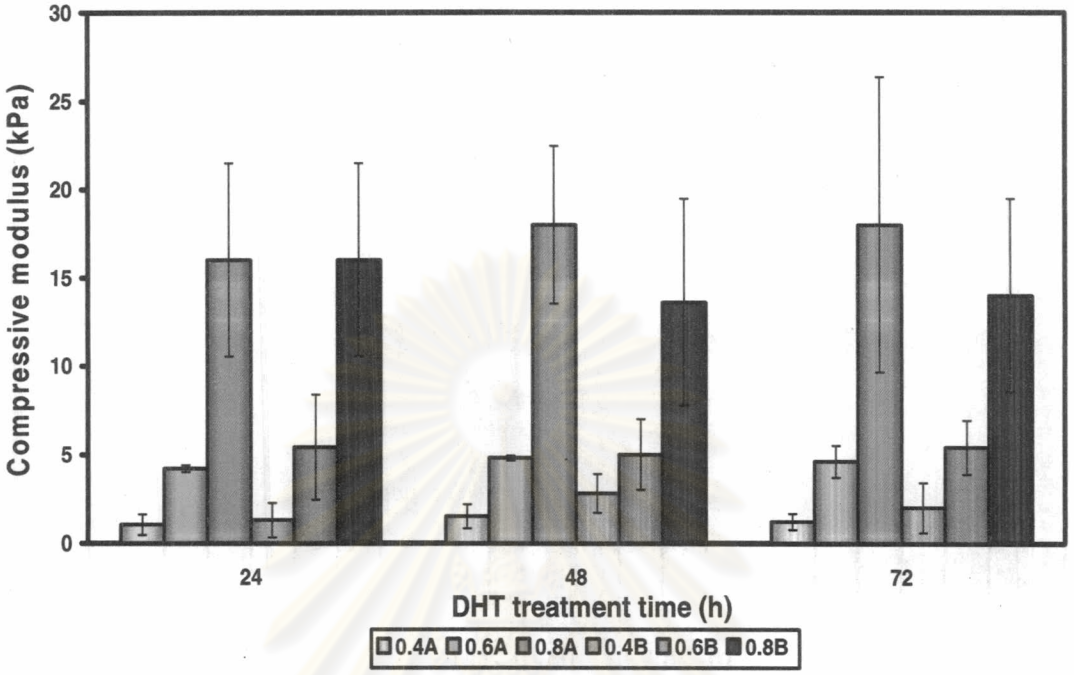

Figure 5.9: Compressive modulus of gelatin scaffolds prepared from various gelatin types, concentrations and DHT treatment times.

5.1.4.2 Collagen/gelatin scaffolds

Compressive modulus of collagen/gelatin scaffolds prepared from 0.4wt% and 0.6wt% solution concentrations, illustrated in Figure 5.10 and 5.11, were influenced by solution concentration, blending composition and DHT treatment time.

Comparing between 0.4wt% and 0.6wt% solution concentrations as presented in Figure 5.10 and 5.11, respectively, it was clear that the scaffolds prepared from high solution concentration had more compressive modulus than those from low solution concentration.

Blending composition had an influence on the compressive modulus. With the increasing of the collagen content, the scaffolds showed higher compressive modulus for both solution concentrations, 0.4wt% in Figure 5.10 and 0.6wt% in Figure 5.11. The compressive modulus of gelatin scaffolds was lowest while that of collagen scaffolds was highest. This was reflected from the structure of both biomaterials. Collagen structure was triple-helix while gelatin structure was random-coil. Random-coil structure of gelatin provided low mechanical property. To enhance the mechanical property of gelatin scaffolds, collagen was allowed to blend with gelatin. This was evidenced that the scaffolds with high collagen content, such as *0.4CG30/70* and *0.6CG30/70*, showed higher compressive modulus than the scaffolds with low collagen content, such as *0.4CG10/90* and *0.6CG10/90*. Moreover, high compressive modulus of high collagen content scaffolds could be attributed to the well-regulated pore arrangement, which could support more compressive force.

In addition, increasing in DHT treatment time could improve the mechanical property of the scaffolds as longer DHT treatment time allowed more crosslinking within the structure of the scaffolds. As a result, the more crosslinked scaffolds could resist more compressive force.

The results indicated that both solution concentration and blending composition represented the significant effects on the compressive modulus due to the natural structures of gelatin and collagen, as well as the pore arrangement of the scaffolds.

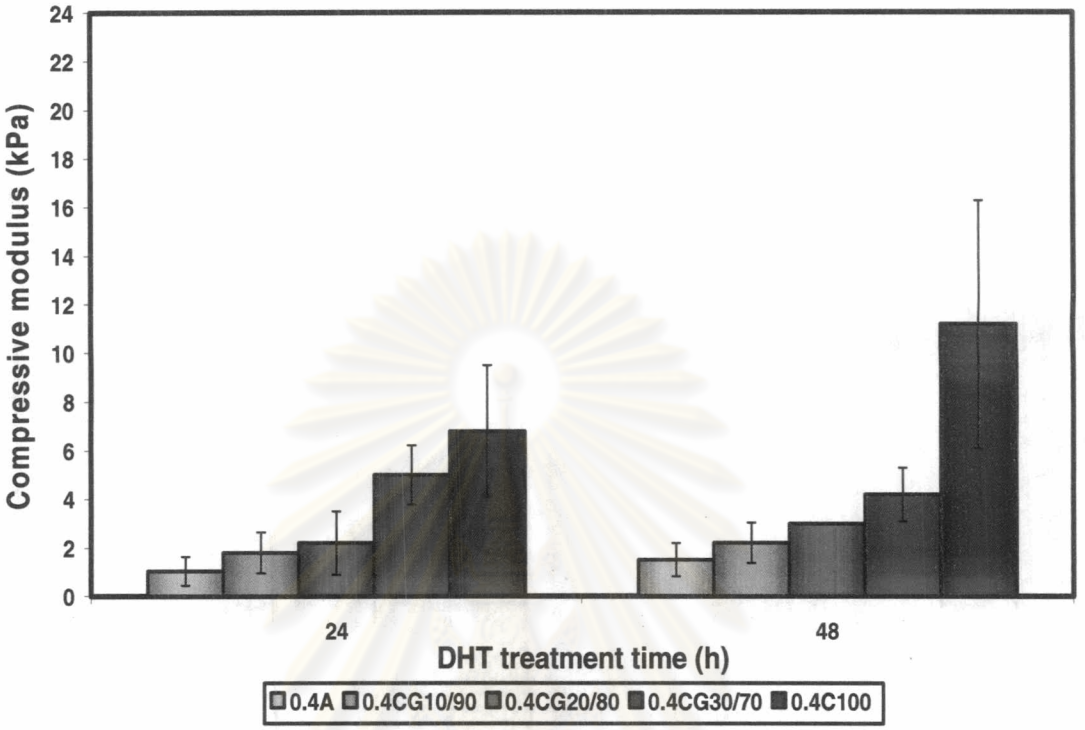

Figure 5.10: Compressive modulus of collagen/gelatin scaffolds prepared from 0.4wt% solution concentration with various blending compositions and DHT treatment times.

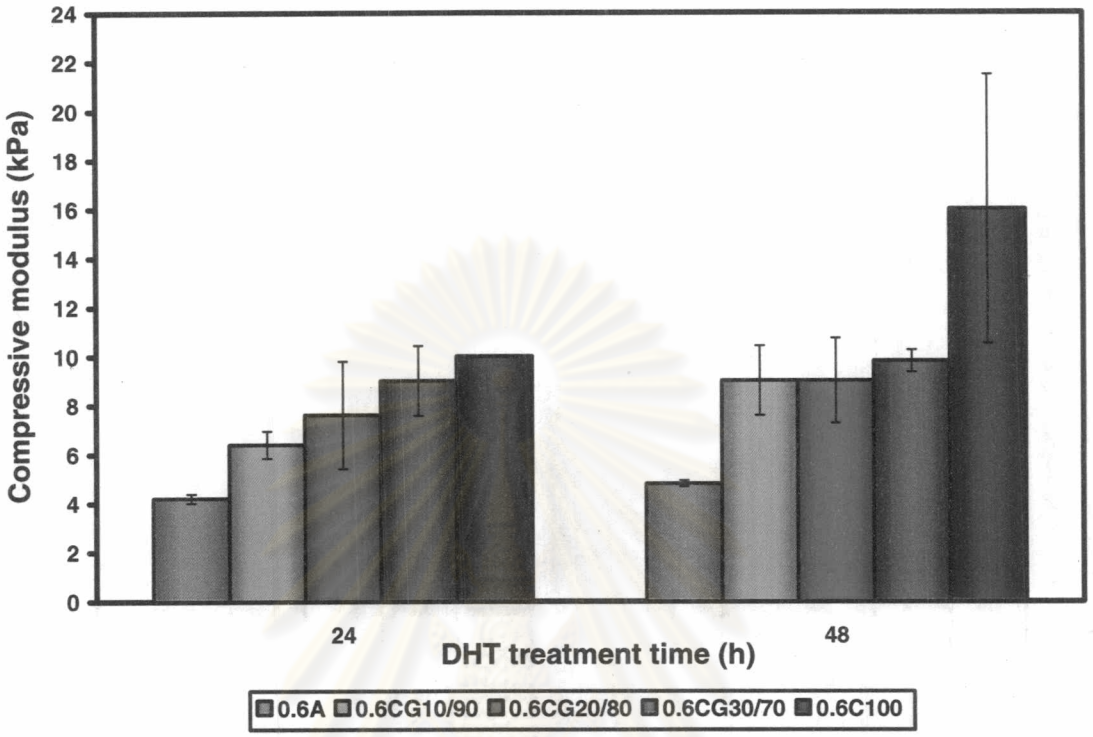

Figure 5.11: Compressive modulus of collagen/gelatin scaffolds prepared from 0.6wt% solution concentration with various blending compositions and DHT treatment times.

5.1.5 PBS solution adsorption

The swelling ability of scaffold plays an important role during the *in vitro* culture. The degree of swelling of the scaffold determined through this investigation was calculated by applying conventional Flory-Huggins swelling formula [61]. When the scaffolds were capable of swelling, it allowed the pore sizes to increase in diameter thus facilitating the cells not only to just attach but also to penetrate inside the scaffolds and grow in a three-dimensional fashion, during *in vitro* culture studies.

In this study, the swelling ratios of the scaffolds at 5^{th} and 24^{th} h were investigated since they were the time for the initially cell attachment and equilibrium swelling, respectively. The tests were performed using PBS (pH 7.4) solution at 37°C, which was the human body temperature. All conditions were the same as the conditions allowing in *in vitro* culture test.

5.1.5.1 Gelatin scaffolds

Swelling properties of gelatin scaffolds depended on many factors such as gelatin type, solution concentration, DHT treatment time and swelling time. Among these factors, solution concentration and gelatin type had more significant effects than DHT treatment time and swelling time. Figure 5.12 and 5.13 illustrated the swelling ratios of gelatin scaffolds prepared from various gelatin types, solution concentrations and DHT treatment times at 5^{th} and 24^{th} h of swelling, respectively

From Figure 5.12 and 5.13, the high gelatin content scaffolds could swell in PBS solution better than the low gelatin content scaffolds. This was because of the water-binding characteristic of gelatin. Gelatin was known to be able to adsorb water more than 10 times of its dry weight; therefore scaffolds with high gelatin mass could absolutely absorb more PBS solution.

Comparing of two gelatin types, type B gelatin scaffolds could adsorb more PBS solution than type A gelatin. This was due to many reasons. First, type B gelatin had less free amino group content, as indicated in the results of crosslinking degree. The less free amino group content meant the lower of crosslinking degree and the stronger swelling ability of the scaffolds [62]. Second, swelling properties could relate to the morphology of the scaffolds. SEM photographs of gelatin scaffolds, as

demonstrated in Figure 5.1, supported that thin-walls found in *0.4B-48* and *0.6B-48* scaffolds could retain more PBS solution within the scaffolds comparing to fiber-like structure found in *0.4A-48* and *0.6A-48* scaffolds. Last, pH of type B gelatin (pH 5-6) was not much different from pH of PBS solution (pH 7.4) so they were well-combining.

Furthermore, swelling ratios of gelatin scaffolds was decreased with an increasing in DHT treatment time. The results could be explained that the more crosslinking occurred at longer DHT treatment time led to the lower swelling ability. In addition, the swelling ratios of gelatin scaffolds at 5^{th} h of swelling test was slightly lower than that at 24^{th} h. This indicated that the swelling ability of the scaffolds at 5^{th} h did not yet reach their equilibrium states.

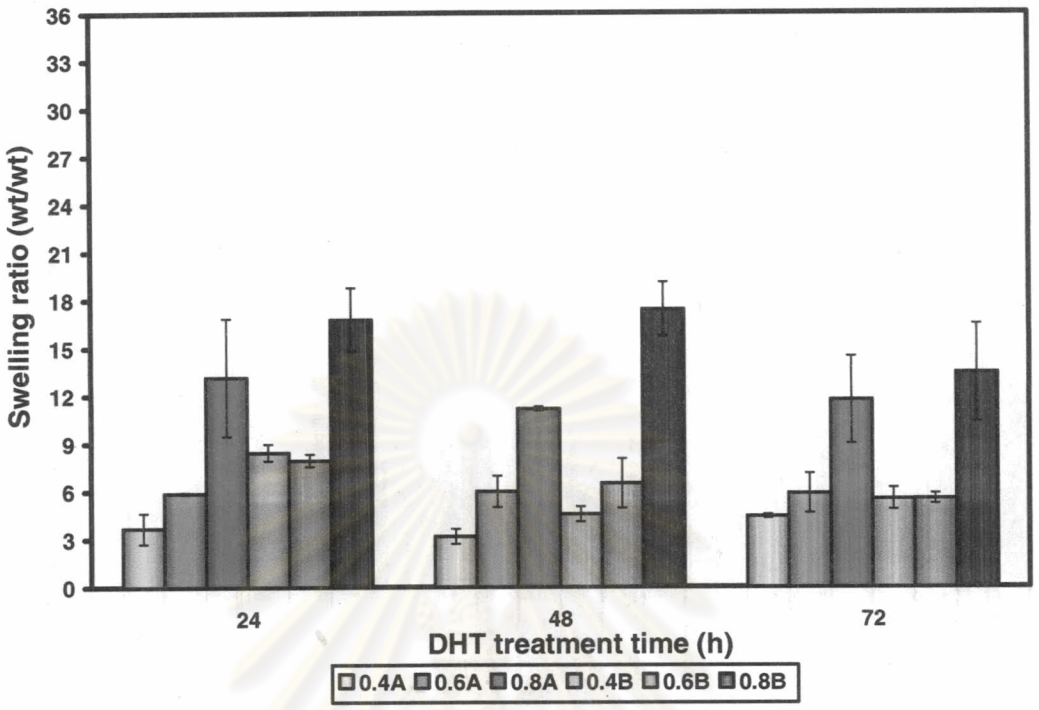

Figure 5.12: Swelling ratio of gelatin scaffolds prepared from various gelatin types, concentrations and DHT treatment times at 5th h of swelling.

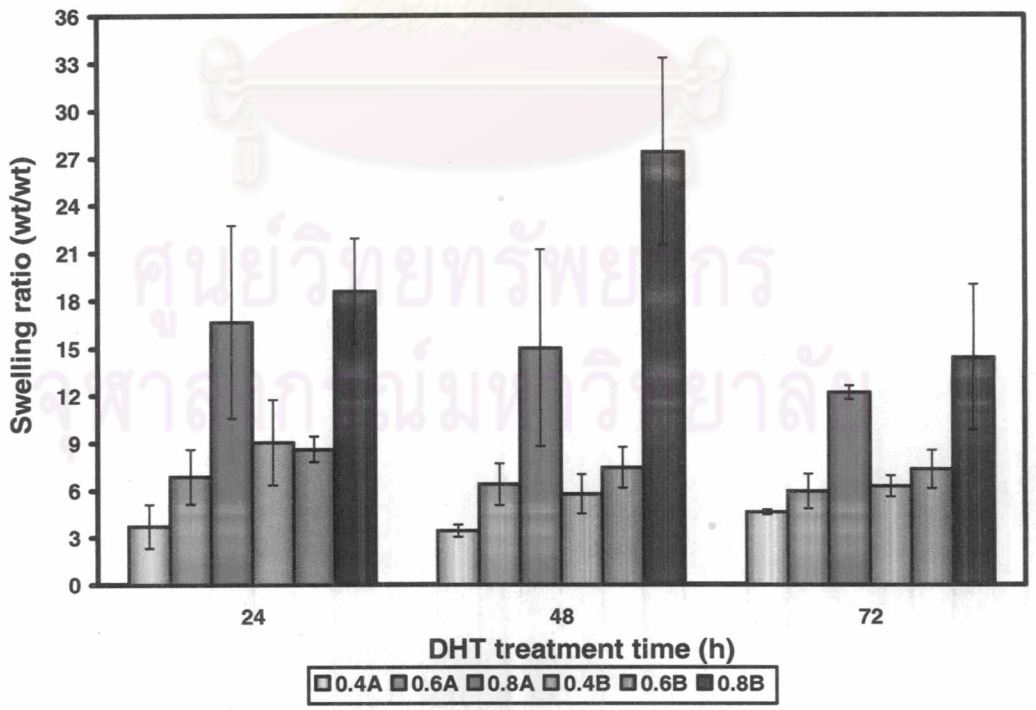

Figure 5.13: Swelling ratio of gelatin scaffolds prepared from various gelatin types, concentrations and DHT treatment times at 24th h of swelling.

5.1.5.2 Collagen/gelatin scaffolds

Swelling ability of collagen/gelatin scaffolds was influenced by solution concentration, blending composition, DHT treatment time and swelling time. Figure 5.14 to 5.17 showed the swelling ratios of collagen/gelatin scaffolds obtained from 0.4wt% and 0.6wt% solution concentrations with various blending compositions, DHT treatment times and swelling times.

The swelling ratios at 5^{th} and 24^{th} h of swelling of collagen/gelatin scaffolds prepared from 0.4wt% solution concentration, as depicted in Figure 5.14 and 5.15, respectively, showed that collagen blending improved swelling ability of pure gelatin scaffolds although collagen was hydrophobic. This implied that there were other factors that strongly affect the swelling ability of collagen/gelatin scaffolds. It could be seen from SEM photographs in Figure 5.2 that morphology of collagen/gelatin scaffolds had much less porosity than that of gelatin scaffold, as showed in Figure 5.1. Therefore, collagen/gelatin scaffolds could retain more PBS solution. The swelling abilities were consistent to the morphology and mechanical property. For scaffolds obtained from 0.6wt% solution concentration, as shown in Figure 5.16 and 5.17, the swelling ability of gelatin scaffold overcame that of collagen/gelatin scaffolds. It could be explained that the hydrophilic property of gelatin could compensate the disadvantage of its morphology.

The scaffolds also showed a slight decreasing in swelling property as increasing DHT treatment time, same as in the case of gelatin scaffolds. A slightly higher swelling ratios of collagen/gelatin scaffolds at 24^{th} h of testing was also observed comparing to those at 5^{th} h.

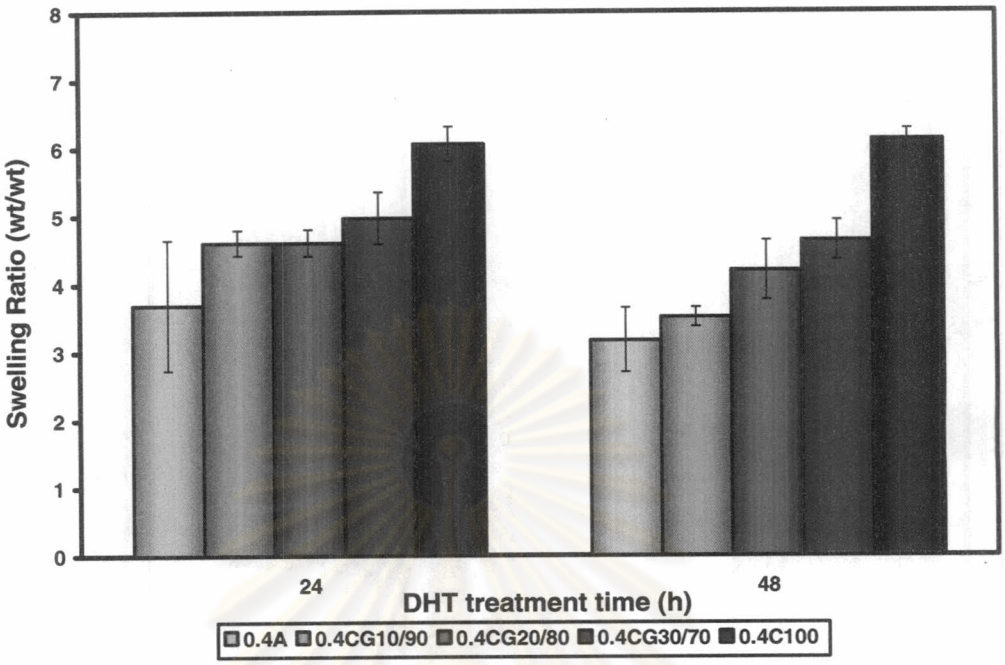

Figure 5.14: Swelling ratio of collagen/gelatin scaffolds prepared from 0.4wt% solution concentration with various blending compositions and DHT treatment times at 5th h of swelling.

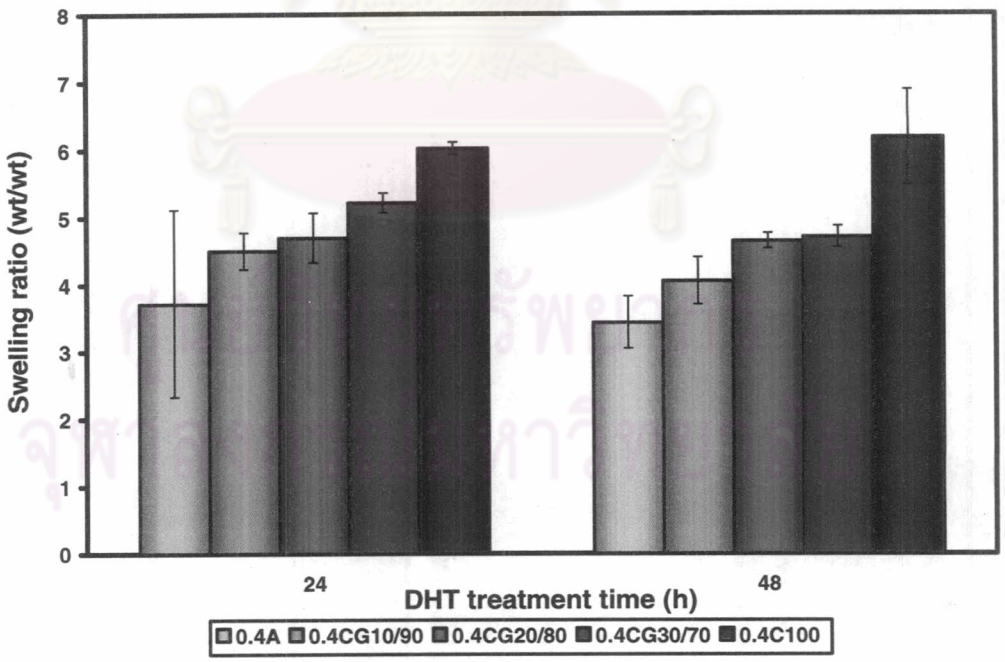

Figure 5.15: Swelling ratio of collagen/gelatin scaffolds prepared from 0.4wt% solution concentration with various blending compositions and DHT treatment times at 24th h of swelling.

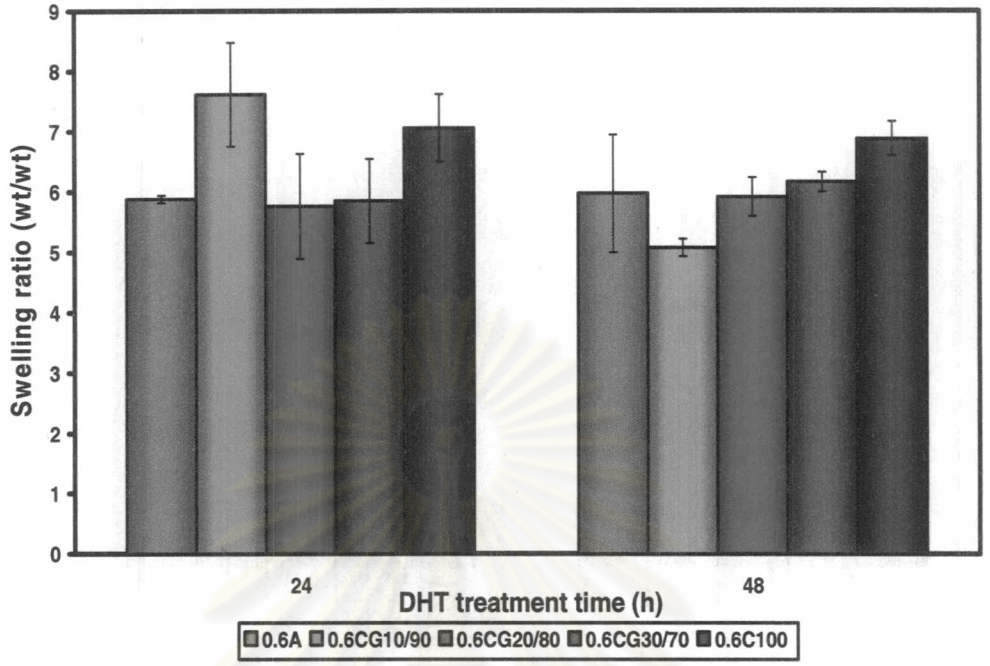

Figure 5.16: Swelling ratio of collagen/gelatin scaffolds prepared from 0.6wt% solution concentration with various blending compositions and DHT treatment times at 5th h of swelling.

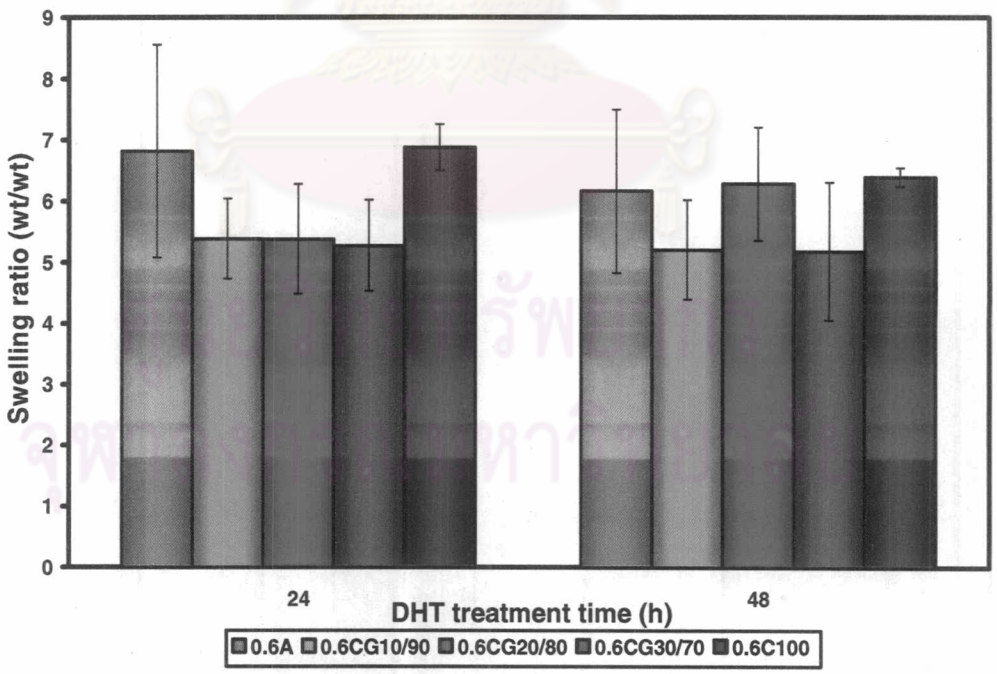

Figure 5.17: Swelling ratio of collagen/gelatin scaffolds prepared from 0.6wt% solution concentration with various blending compositions and DHT treatment times at 24th h of swelling.

5.2 Biological properties of the scaffolds

5.2.1 *In vitro* biodegradation behavior

Gelatin, collagen/gelatin and collagen scaffolds prepared from both 0.4wt% and 0.6wt% solution concentrations and crosslinked for 24 and 48 h were selected to test *in vitro* biodegradation. As lysozyme naturally presented in body fluid, the *in vitro* degradation behavior of scaffolds in lysozyme solution at 37°C was investigated. From the test, pure gelatin scaffolds from both gelatin types degraded rapidly in lysozyme solution because of its random coil structure and hydrophilicity. Hence, only degradation behavior of collagen-containing scaffolds crosslinked at various DHT treatment times was reported.

Figure 5.18 and 5.19 presented the remaining weight of pure collagen and collagen/gelatin scaffolds prepared from 0.4wt% and 0.6wt% solution concentration, respectively. Remaining weight of pure collagen and collagen/gelatin scaffolds freeze dried from 0.4wt% solution concentration, as illustrated in Figure 5.18, could be divided into two groups which were 24 h and 48 h DHT treated scaffolds. The scaffolds freeze dried from 0.4wt% solution concentration crosslinked for 24 h (*0.4CG10/90-24, 0.4CG20/80-24, 0.4CG30/70-24* and *0.4C100-24)* could completely degrade within a week while the ones crosslinked for 48 h (*0.4CG10/90-48, 0.4CG20/80-48, 0.4CG30/70-48* and *0.4C100-48)* degraded within two weeks. Similar to the scaffolds from 0.4wt% solution concentration, the scaffolds from 0.6wt% solution concentration crosslinked for 24 h (*0.6CG10/90-24, 0.6CG20/80-24, 0.6CG30/70-24* and *0.6C100-24)* degraded within a week while the ones crosslinked for 48 h (*0.6CG10/90-48, 0.6CG20/80-48, 0.6CG30/70-48* and *0.6C100-48)* could remain up to three weeks.

Collagen content in the scaffolds had a pronounced effect on the degradation rate. The scaffolds with higher collagen content could last longer than the scaffolds with lower collagen content when tested in lysozyme solution. This could be explained by the structure of collagen and gelatin. The triple helix structure led collagen more difficult to degrade because there was less opportunity for molecules to contact the enzyme. Moreover, collagen was hydrophobic so it was difficult to dissolve in enzyme solution. In contrast, random coil structure of gelatin was much

easier to contact enzyme and degrade. The degradation rate of scaffolds was consistent to the morphology of the scaffolds, as showed in Figure 5.2. Scaffolds with higher collagen content showed less porosity, which reduced surface area of scaffolds to contact enzyme.

Considering the effect of DHT treatment time, it was as expected that the scaffolds crosslinked for 48 h resulted in a slower degradability comparing to the scaffolds crosslinked for 24 h. For example, the scaffolds prepared from 0.4wt% solution concentration crosslinked for 48 h could completely degraded within two weeks while the ones crosslinked for 24 h degraded within only a week.

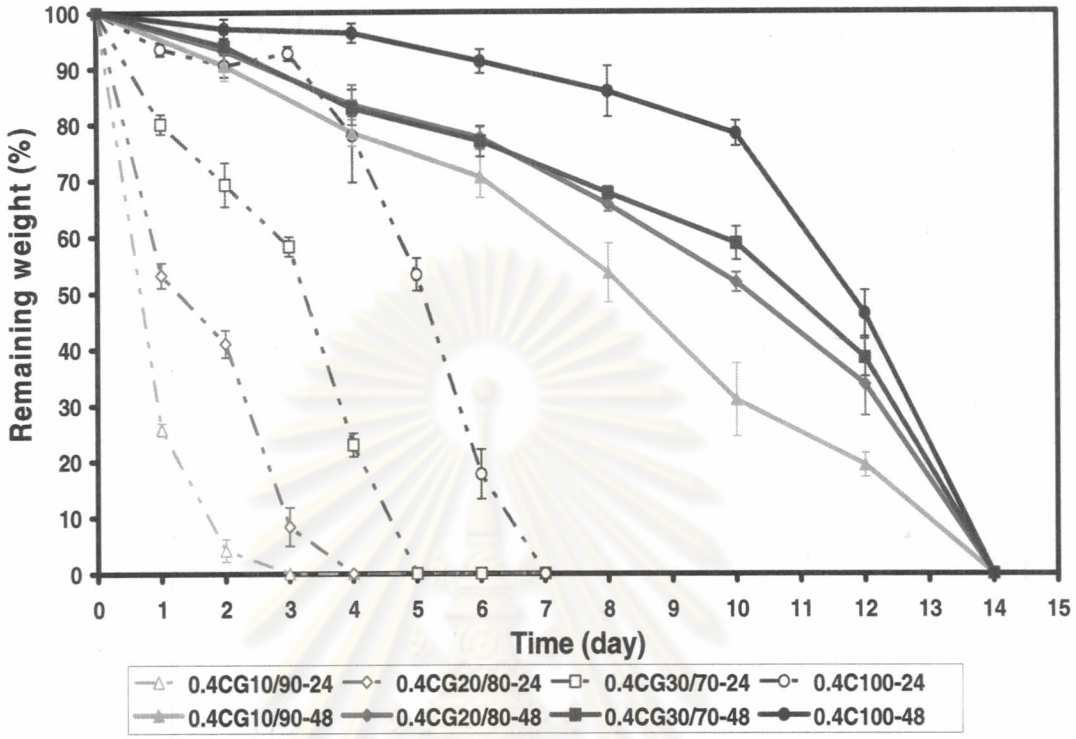

Figure 5.18: Remaining weight of collagen and collagen/gelatin scaffolds prepared from 0.4% solution concentration with various blending compositions and DHT treatment times in lysozyme solution at 37°C.

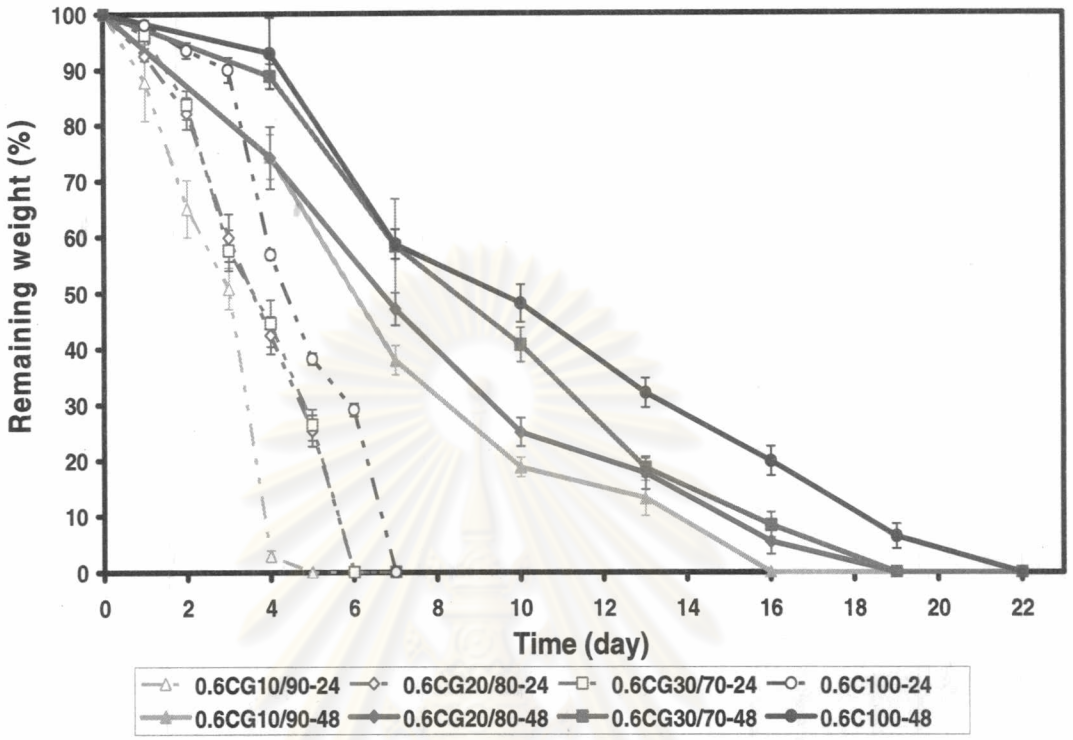

Figure 5.19: Remaining weight of collagen and collagen/gelatin scaffolds prepared from 0.6% solution concentration with various blending compositions and DHT treatment times in lysozyme solution at 37°C.

5.2.2 *in vitro* cell adhesion and cell proliferation

For cell culture, the appropriate scaffolds were selected to test their biological properties. Table 5.1 revealed the statistic significant of each parameter considered for selecting the scaffolds. There were many criteria for selecting the scaffolds. First of all, scaffolds should provide porous morphology which promoted cell penetration. The SEM photographs of gelatin scaffolds in Figure 5.1 have revealed that the scaffold structures prepared from 0.4wt% and 0.6wt% solution concentration had more porosity than those from 0.8wt%. Same as gelatin scaffolds, collagen/gelatin scaffolds obtained from both solution concentrations were selected because of their appropriate structures, seen in Figure 5.2. Swelling property was the second criterion for selecting the scaffolds. Scaffolds, which were able to swell, could enhance cell attachment and penetration. All blended collagen/gelatin scaffolds prepared from both 0.4wt% and 0.6wt% solution concentration showed no significant difference in swelling ability so all of them were selected. Third, biodegradability of the scaffolds was another factor which was important when the scaffolds were used as skin substitute. The results from Figure 5.18 and 5.19 indicated that collagen/gelatin scaffolds with various solution concentrations, blending compositions and DHT treatment times provided a variety of degradation rate. A variety of degradation rate could be useful for a variety of wound healing. Lastly, the scaffolds should have appropriate compressive modulus to absorb forces when they are implanted into the wounds. With this criterion, all scaffolds previously selected from other criteria had the acceptable mechanical property. In addition, only scaffolds crosslinked for 48 h were used for the test of biological properties because the crosslinking degree was not significantly increased after 48 h DHT. Furthermore, the crosslinking degree might not have much effect on the cell response.

In this case, pure type A gelatin and pure collagen scaffolds freeze dried from both solution concentrations were selected to serve as the negative and positive controls, respectively. In addition, pure type B gelatin scaffolds were tested to compare the difference in biological properties with type A gelatin. Thus 12 experiments for *in vitro* cell culture were summarized, as listed in Table 5.2.

Table 5.1: The effect of each variable on the properties of scaffolds.

Parameters	Morphology	Swelling property	Degradation rate	Compressive modulus	Crosslinking degree
Gelatin type	**	*	-	*	***
Solution Concentration	***	***	**	***	*
Blending composition	*	**	*	**	**
DHT treatment time	-	*	***	*	***

Note: - no effect, * slight effect, ** significant effect, *** strong effect

Table 5.2: Experiments for *in vitro* cell culture

Scaffold type		**48 h DHT treatment**
Concentration	**Blending composition of collagen/gelatin**	
0.4 wt%	0/100 (type A gelatin)	*0.4A-48*
	0/100 (type B gelatin)	*0.4B-48*
	10/90	*0.4CG10/90-48*
	20/80	*0.4CG20/80-48*
	30/70	*0.4CG30/70-48*
	100/0	*0.4C100-48*
0.6 wt%	0/100 (type A gelatin)	*0.6A-48*
	0/100 (type B gelatin)	*0.6B-48*
	10/90	*0.6CG10/90-48*
	20/80	*0.6CG20/80-48*
	30/70	*0.6CG30/70-48*
	100/0	*0.6C100-48*

To assess cell adhesion, L929 fibroblasts (60,000 cells per scaffold) were seeded on the scaffolds. Figure 5.20 represented cell adhesion property of different scaffolds in term of the number of fibroblast cells attached on each scaffolds at 5 h after the culture. It should be pointed out that there was no significant difference in the cell adhesion property among these scaffolds except the gelatin scaffold prepared from 0.4wt% solution concentration, *0.4A* ($p < 0.05$). The adhesion of *0.4A* scaffold was significantly better than the others because of its porous morphology as can be seen in Figure 5.1. However, the physical properties, compressive modulus and swelling property of *0.4A* were not good. Especially, the biodegradation rate of *0.4A* scaffold was too fast to be used as a skin substitute. Therefore, it could be concluded that the porosity was the essential property induced the cell to penetrate into the scaffolds even if the scaffolds showed poor physical properties.

When considering the collagen/gelatin scaffolds, the results showed that cell adhesion on these scaffolds were similar to that on the collagen (control) scaffolds ($p > 0.05$). It was suggested that no significant difference for initial cell attachment on all collagen/gelatin scaffolds comparing to pure collagen scaffolds.

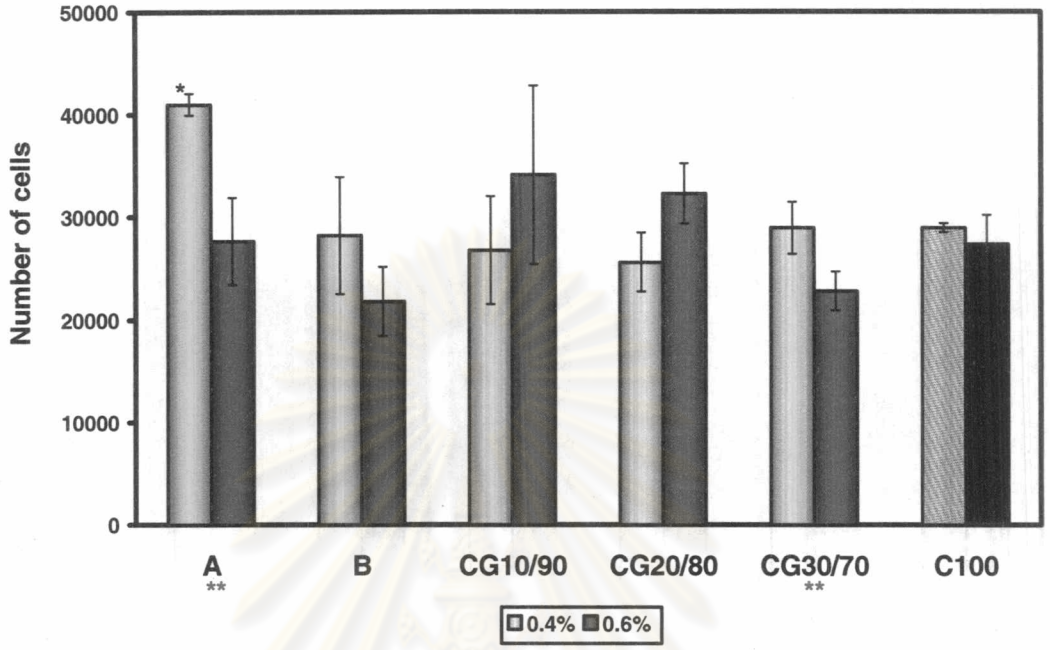

Figure 5.20: L929 cell adhesion on different scaffolds at 5 h after the culture (n = 3, * $p < 0.05$ compared with controlled collagen scaffolds at the same solution concentration, ** $p < 0.05$ compared between two solution concentrations of the same scaffolds).

L929 fibroblasts (30,000 cells per scaffold) were seeded on the selected scaffolds to evaluate cell proliferation. Figure 5.21(a) and (b) illustrated the number of fibroblast cells proliferated on each scaffolds at 24 and 48 h after the culture, respectively. The results showed that the proliferation occurred on all scaffolds. The number of cells on the scaffolds at 48 h was doubled from that at 24 h.

At 24 h after the culture, the number of cells between two solution concentrations of each type was relatively similar. There was no significant difference of the number of proliferated cells on the scaffolds compared with the collagen (control) scaffolds at the same solution concentration ($p > 0.05$).

At 48 h after the culture, there was still no significant difference of the number of proliferated cells on the scaffolds compared with the collagen (control) scaffolds at the same solution concentration, despite the number of cells was doubled. In contrast, the difference in the number of cells between two solution concentrations were found in all scaffold types, especially in type A gelatin and *CG20/80* scaffolds ($p < 0.05$). The reason of this difference was also the morphology of the scaffolds, as previously explained in the case of cell adhesion. It was obvious that the scaffolds obtained from 0.4wt% solution concentration had more porosity than the scaffolds obtained from 0.6wt% solution concentration. Therefore, the scaffolds from 0.4wt% solution concentration could allow the cells to penetrate and proliferate even though they had less mass of biomaterial (gelatin and collagen) for cells to attach. By considering other physical properties such as compressive modulus and swelling property, it was found that these properties had no effect on the cell proliferation. Compressive modulus and swelling ratio of the scaffolds prepared from 0.4wt% solution concentration were less than those from 0.6wt%. However, the porous morphology of scaffolds from 0.4wt% solution concentration could compensate the disadvantages of their physical properties. Therefore, morphology of the scaffolds was the most important factor for cell proliferation property.

By considering only collagen/gelatin scaffolds which were the goal of this study, it could be concluded that all collagen/gelatin scaffolds could induce the cell proliferation as good as the collagen (control) scaffolds could.

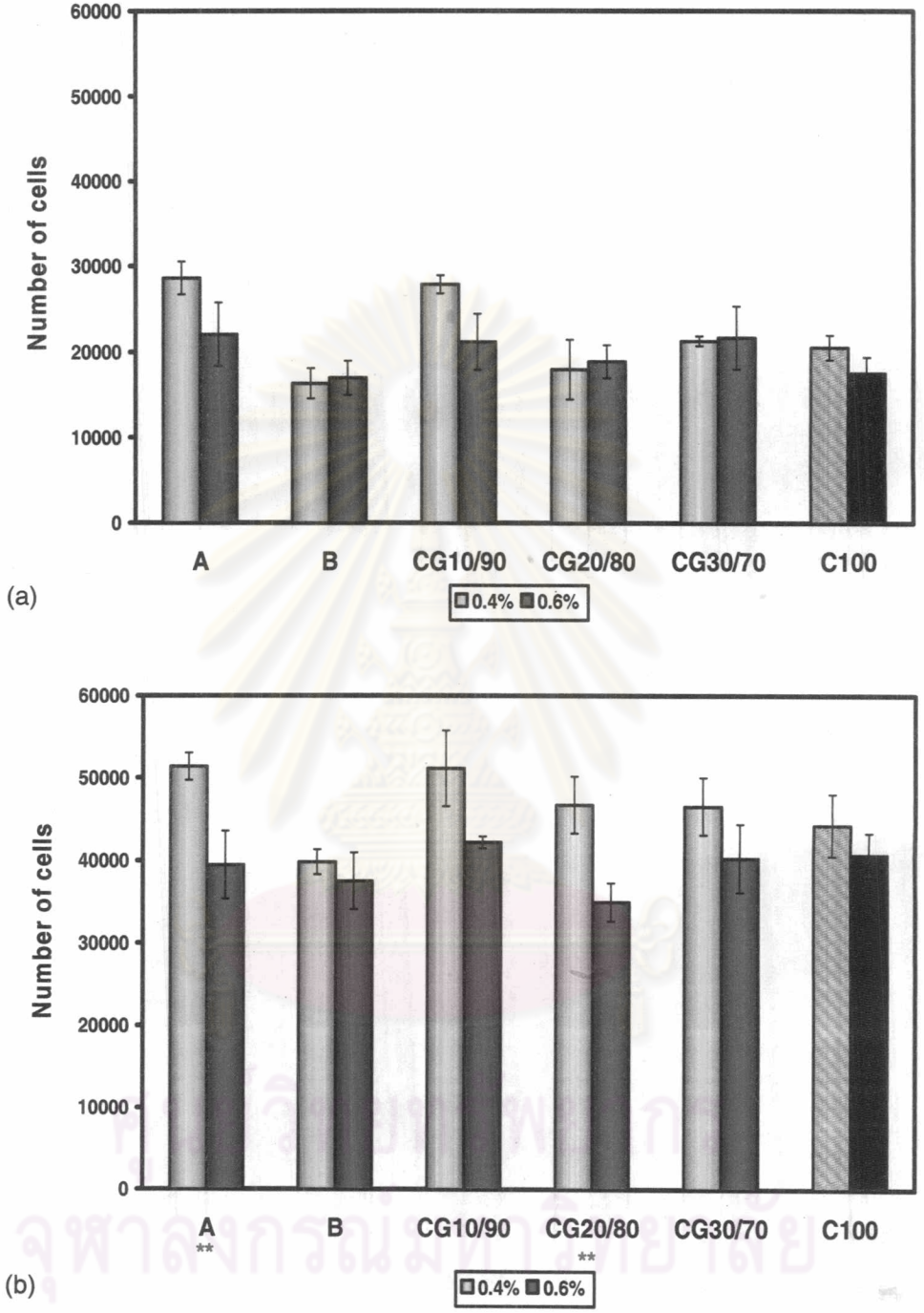

Figure 5.21: L929 cell proliferation on different scaffolds in 10%DMEM at each time after the culture (a) 24 h, (b) 48 h (n = 3, * $p < 0.05$ compared with controlled collagen scaffolds at the same solution concentration, ** $p < 0.05$ compared between two solution concentrations of the same type scaffolds).

Serum containing a variety of nutrition for cells could stimulate the cell proliferation and sometimes lead to the overestimated number of cells. The cell proliferation of scaffolds in 10%DMEM containing serum would not only be the influence of scaffolds but also the influence of nutrition in serum. To remove the effect of serum on cell proliferation, serum free medium (SFM) was allowed to test as the procedure in the case of 10%DMEM. The effect of the scaffolds on cell proliferation could then be observed. Figure 5.22 represented cell proliferation property in SFM.

Without the effect of serum, *0.4A* and *0.4CG30/70* scaffolds showed dominant cell proliferation ($p < 0.05$) at 24 h after the culture while the others showed no significant difference when compared with the collagen (control) scaffolds at the same solution concentration. The comparison of cell proliferation of scaffolds prepared from two solution concentrations revealed that only type A gelatin scaffolds showed a significant difference. The dominant proliferation of *0.4A* scaffold was found over *0.6A* scaffold.

At 48 h after the culture, the increased number of cells was not as much as the proliferation in 10%DMEM containing serum. By comparing with collagen (control) scaffolds at the same solution concentration, there were some differences among them. For scaffolds prepared from 0.4wt% solution concentration, the number of cells of only *CG10/90* scaffold was significant higher than that of the control ($p < 0.05$). For scaffolds prepared from 0.6wt% solution concentration, the number of cells of type B gelatin and *CG20/80* scaffolds was significant higher than that of the control ($p < 0.05$). By considering the cell proliferation in both 10%DMEM with and without serum, it could be assumed that the *CG10/90* scaffold prepared from 0.4wt% solution concentration was the most suitable composition. In contrast, the most suitable composition for scaffold obtained from 0.6wt% solution concentration was *CG20/80* scaffold. However, none of the scaffolds showed significant lower number of cells than the control.

The comparison between two solution concentrations showed that the number of cells of *0.4CG10/90* and *0.4C100* scaffolds were significant higher than that of *0.6CG10/90* and *0.6C100*, respectively. The other scaffolds obtained from 0.4wt% solution concentration also showed a trend of slightly higher number of cells than that

from 0.6wt%. The reason was due to the difference in their morphology as indicated previously in the cell proliferation in 10%DMEM containing serum.

By considering only collagen/gelatin scaffolds, the conclusion was the same as that of the proliferation in 10%DMEM containing serum. All collagen/gelatin scaffolds could induce the cell proliferation as good as the collagen (control) scaffolds. The results proved that gelatin could be used to partly replace collagen for scaffold fabrication. Resulting blended scaffolds such as *CG20/80* still possessed comparable properties, especially the cell proliferation, to those of collagen scaffolds. Therefore, a large amount of collagen used in scaffold fabrication could be reduced leading to a much lower cost of biomaterials used.

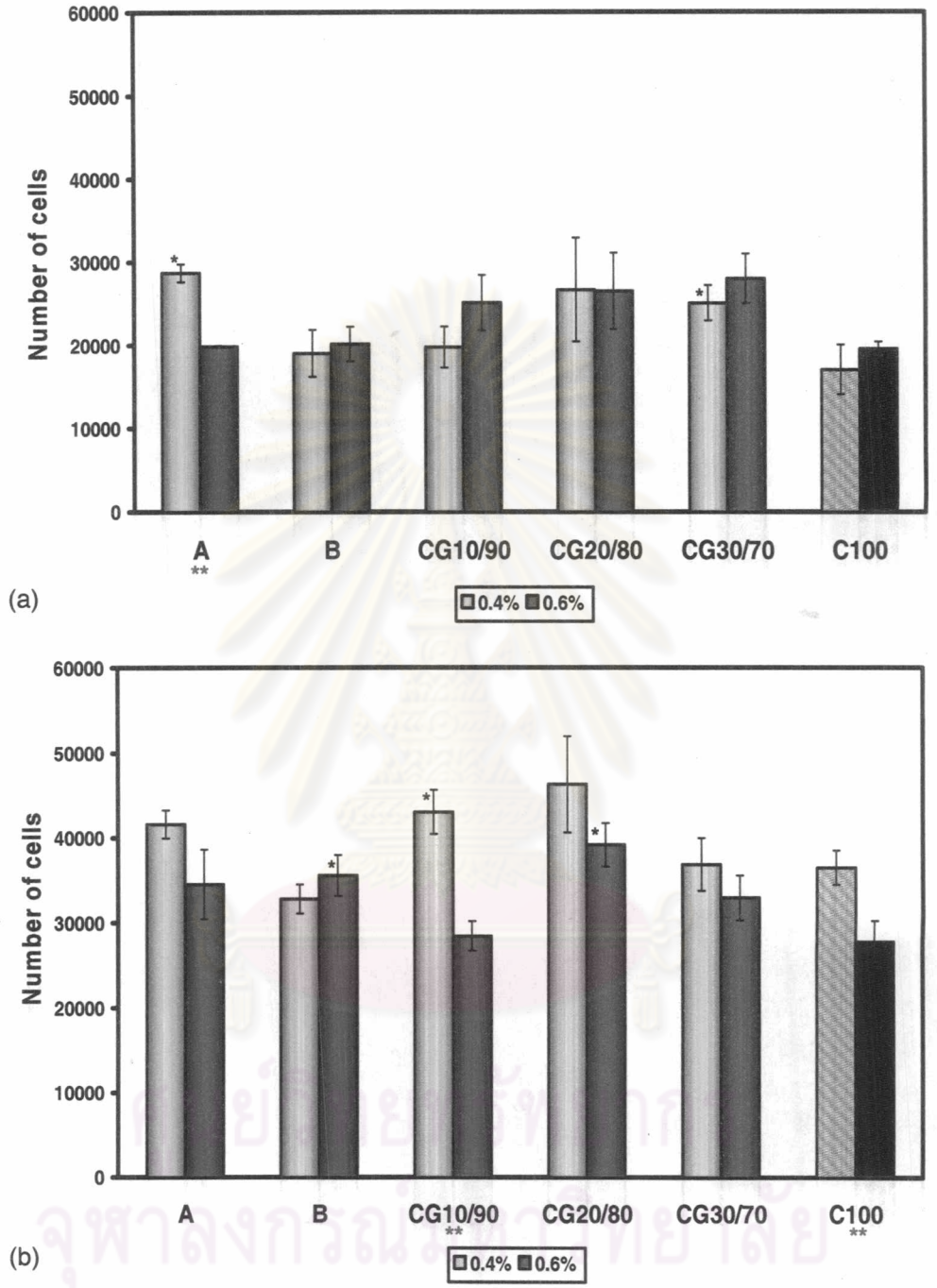

Figure 5.22: L929 cell proliferation on different scaffolds in SFM at each time after the culture (a) 24 h, (b) 48 h (n = 3, * $p < 0.05$ compared with controlled collagen scaffolds at the same solution concentration, ** $p < 0.05$ compared between two solution concentrations of the same type scaffolds).

5.2.3 L929 cells spreading area observation

L929 cells spreading were observed from the cell seeding side down to the plate-exposed side, as described in section 4.3.9. Figure 5.23, 5.24, 5.25 and 5.26 showed the penetration of cells on 0.4CG20/80, 0.4C100, 0.6CG20/80 and 0.6C100 scaffolds, respectively.

For 0.4CG20/80 and 0.4C100 scaffolds, L929 cells were found throughout the thickness of scaffolds (position 1 to 4), as shown in Figure 5.23 and 5.24, respectively. On the contrary, cells were rarely found at position 4 for 0.6CG20/80 and 0.6C100 scaffolds, as shown in Figure 5.25 and 5.26, respectively. The less of cell penetration of 0.6CG20/80 and 0.6C100 scaffolds than that of 0.4CG20/80 and 0.4C100 scaffolds was due to the less porous morphology. The denser 0.6CG20/80 and 0.6C100 scaffolds probably hinder the penetration of cells from the seeding side, especially from position 3 to 4. This cell penetration could explain the number of cell presented in Figure 5.21. Numbers of cells of 0.4CG20/80 and 0.4C100 scaffolds were more than those of 0.6CG20/80 and 0.6C100 scaffolds because of the reason described above.

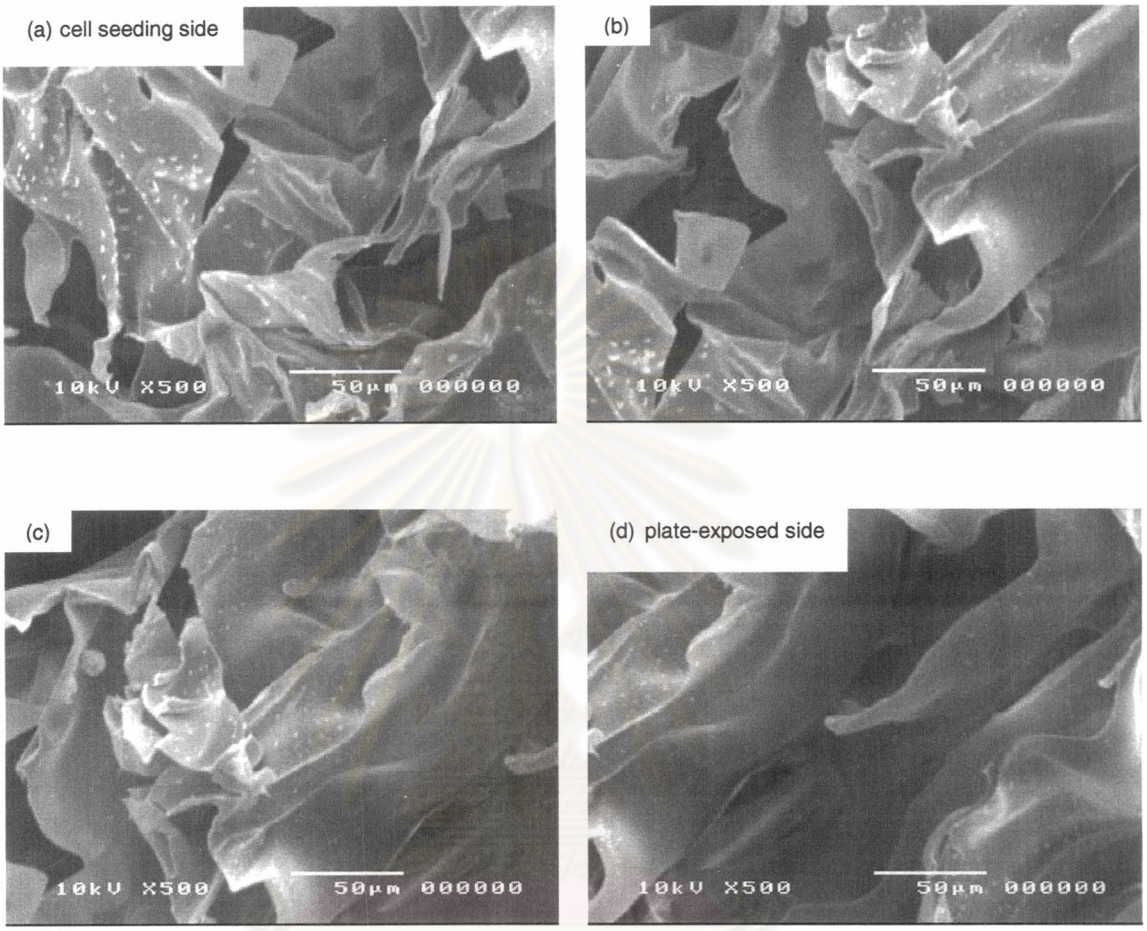

Figure 5.23: SEM micrographs of cross-sectional plane of 0.4CG20/80 scaffolds at position (a) 1(cell seeding side), (b) 2, (c) 3, and (d) 4 (plate-exposed side).

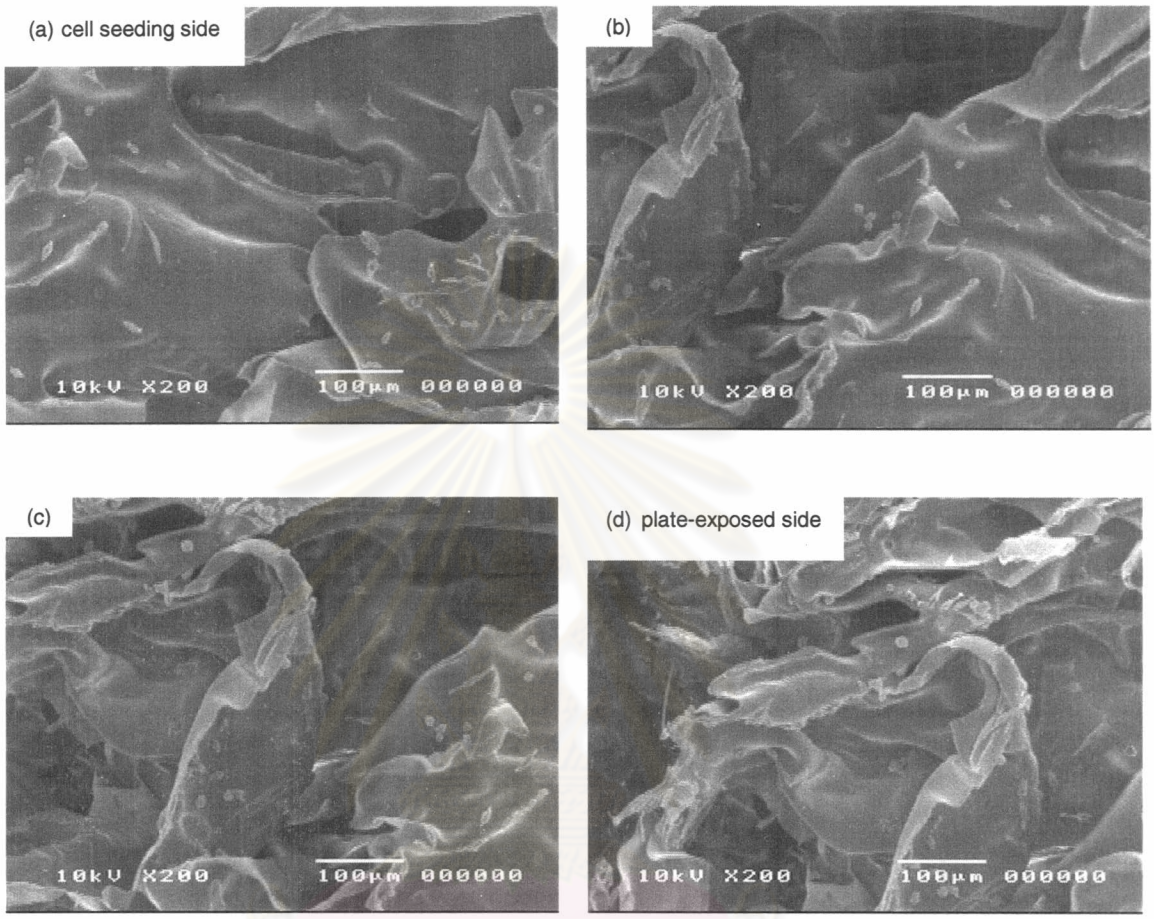

Figure 5.24: SEM micrographs of cross-sectional plane of 0.4C100 scaffolds at position (a) 1 (cell seeding side), (b) 2, (c) 3, and (d) 4 (plate-exposed side).

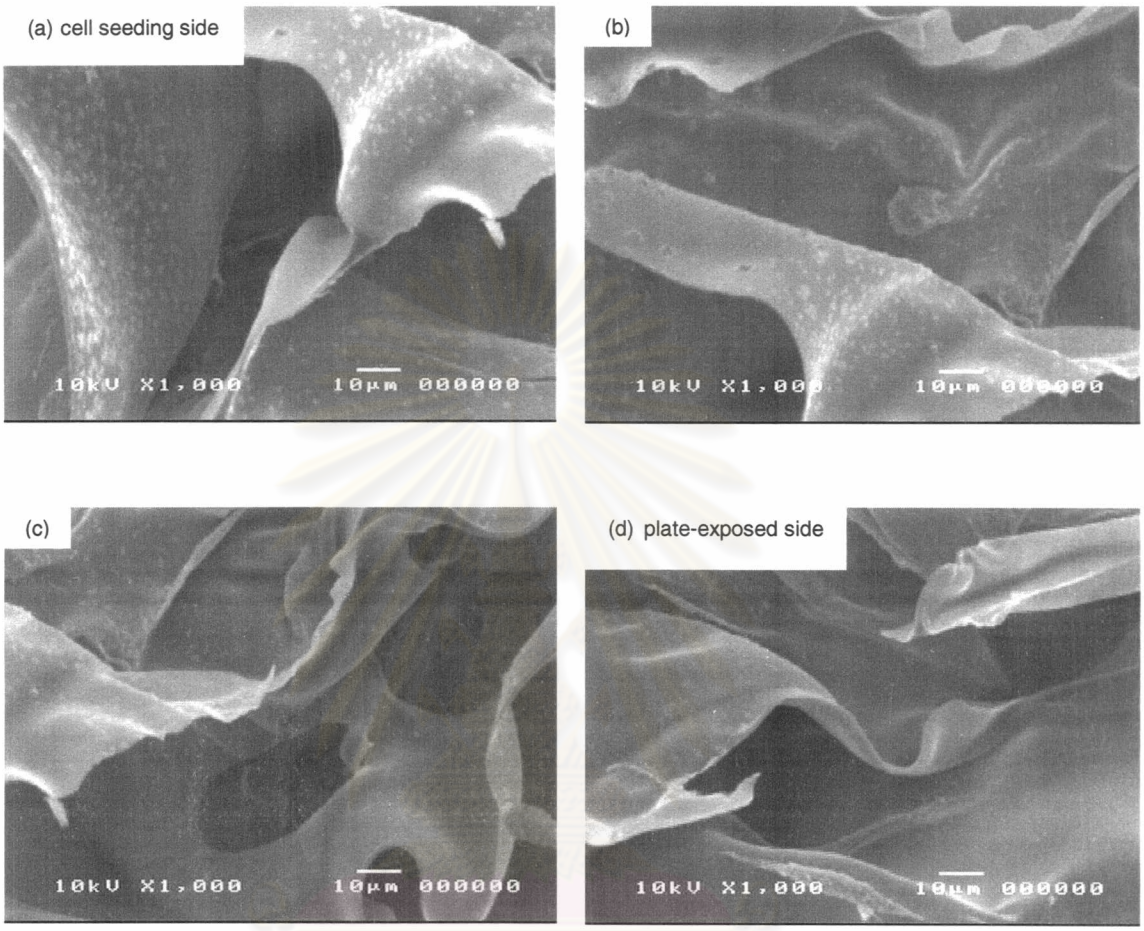

Figure 5.25: SEM micrographs of cross-sectional plane of 0.6CG20/80 scaffolds at position (a) 1 (cell seeding side), (b) 2, (c) 3, and (d) 4 (plate-exposed side).

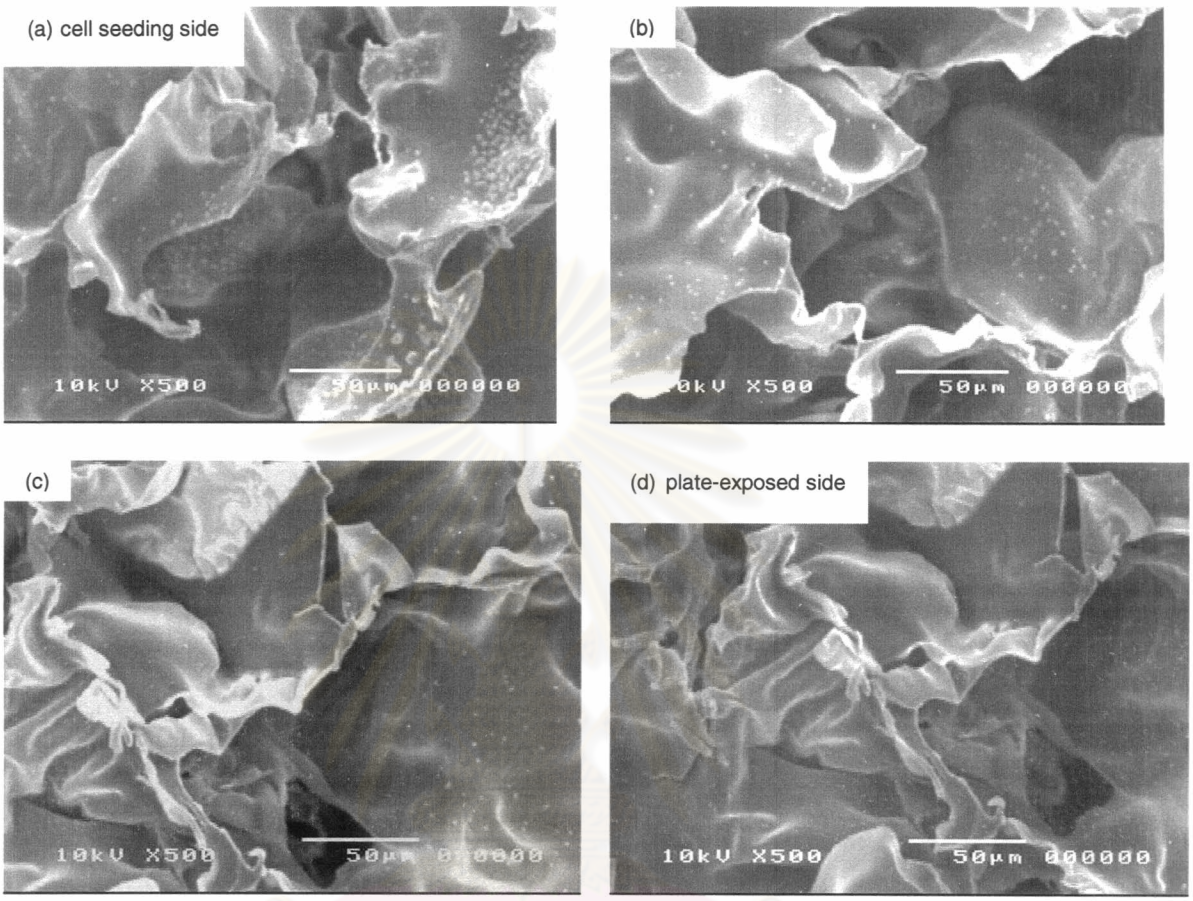

Figure 5.26: SEM micrographs of cross-sectional plane of 0.6C100 scaffolds at position (a) 1 (cell seeding side), (b) 2, (c) 3, and (d) 4 (plate-exposed side).

5.3 Cost of scaffolds

Collagen, which was widely used as material for skin substitutes, was relatively expensive. The aim of this study was to substitute a portion of collagen scaffolds with a much cheaper material, gelatin. The approximate costs of both materials were compared in Table 5.3.

Table 5.3: Cost of collagen and gelatin

Material	Approximate cost (per unit)
Gelatin (lab grade)	2,160 baht/kg
Collagen	30,000 baht/kg of 0.6wt% solution

From the results of physical and biological properties, it was seen obviously that the collagen/gelatin scaffolds could be used instead of the collagen scaffolds without the limiting of cell response. Comparing with C100 scaffolds, CG30/70, CG20/80 and CG10/90 scaffolds reduced amount of used collagen by 70%, 80% and 90% respectively. Thus, collagen/gelatin scaffolds led to much lower cost of material used. Table 5.4 illustrated the approximated cost of collagen in scaffolds fabrication. Because gelatin was very cheap compared with collagen, cost of gelatin would be neglected in this calculation. The calculation, as presented in Appendix C, was on the basis of equal amount of product. The costs of different scaffolds per sheet ($10x10x0.5\ cm^3$) were reported in Table 5.4.

Table 5.4: Cost of collagen in collagen/gelatin scaffolds ($10x10x0.5\ cm^3$)

Solution concentration	Scaffold	Cost of scaffold * (baht)	Saving cost ** (baht)	Saving cost (%)
0.4 wt%	C100	990	0	0
	CG30/70	297	**693**	**70**
	CG20/80	198	**792**	**80**
	CG10/90	99	**891**	**90**
0.6 wt%	C100	1485	0	0
	CG30/70	446	**1040**	**70**
	CG20/80	297	**1188**	**80**
	CG10/90	149	**1337**	**90**

* Material (collagen) cost only

** Saving cost compared to collagen scaffolds

In conclusion, the costs of collagen/gelatin scaffolds were much lower than the costs of pure collagen scaffold while their physical and biological properties were comparable to those of collagen scaffolds.

5.4 Chondroitin-6-sulfate/collagen/gelatin scaffold

As previously described in section 2.1 that ECM structure in natural dermis composes of type I collagen crosslinked with glycosaminoglycan (GAG), such as chondritin-6-sulfate (CS). To improve biological properties of scaffolds to be more like the natural ECM, CS was allowed to blend with gelatin and collagen to produce CS/collagen/gelatin scaffolds with various blending composition. From the blending, it was found that CS was cation, like type A gelatin. Blending together of CS, gelatin and collagen could not form homogeneous solution because there was repelling between CS and gelatin. In this case, suspensions of CS/collagen/gelatin were obtained. The suspensions of CS/collagen/gelatin were unable to cast uniform scaffolds although they were immediately frozen in liquid nitrogen. Non-homogeneous scaffolds could not be employed for the *in vitro* cell culture. As a result, CS might not be a suitable GAG for collagen/gelatin scaffolds.