CHAPTER IV

RESULTS

1. Isolation of the collagen from bovine tendon

The appearance and total protein of the isolated collagen are shown in Table 12 and Figure 13.

Table 13 Appearances and percent of total protein of the isolated collagen.

Characteristics	Isolated collagen
% of Total protein[a)]	71.9%
Appearance	spongy
Color	cream

a) Calculation of total protein is shown in Appendix (A)

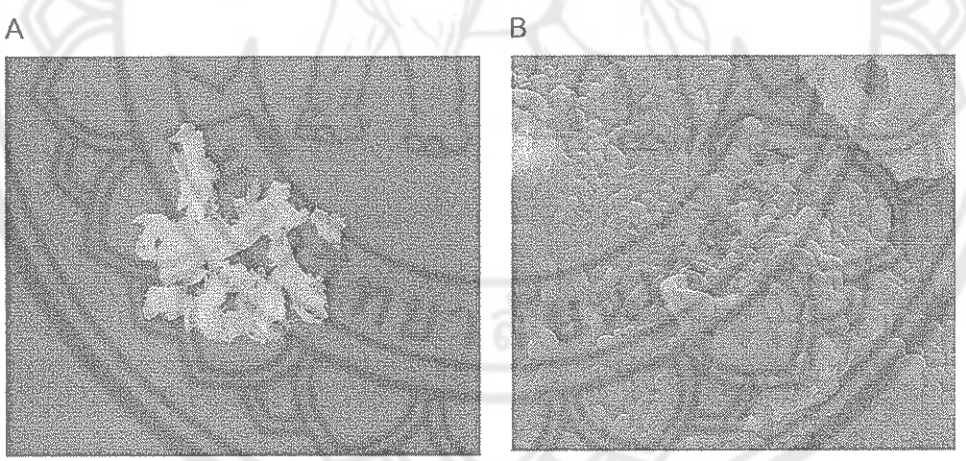


Figure 12 The physical characteristic of isolated bovine tendon collagen: lyophilized collagen (A) and the electron photomicrograph at 3.5 KX (B).

According to FTIR, the chemical characteristic standard collagen and isolated collagen are shown in Figure 13 and 14, respectively. These IR spectra are concluded as followed:

Frequencies (cm^{-1})	Frequencies (cm^{-1}) (Standard Collagen)	Frequencies (cm^{-1}) (Isolated Collagen)	Chemical Charateristic
3080-3330	3286	3285	Amide A and B (N-H-stretching and OH group
1630-1650	1632	1636	Amide I (C=O stretching)
1540-1560	1545	1540	Amide II (N-H bending and C-N stretching)

The obtained results indicated that the spectrum of the isolated collagen was not different to that of the standard type I collagen. However, the peaks height and area of the isolated collagen were different from those of the standard collagen. This may be related to the different amount of protein content in the sample for IR investigation.

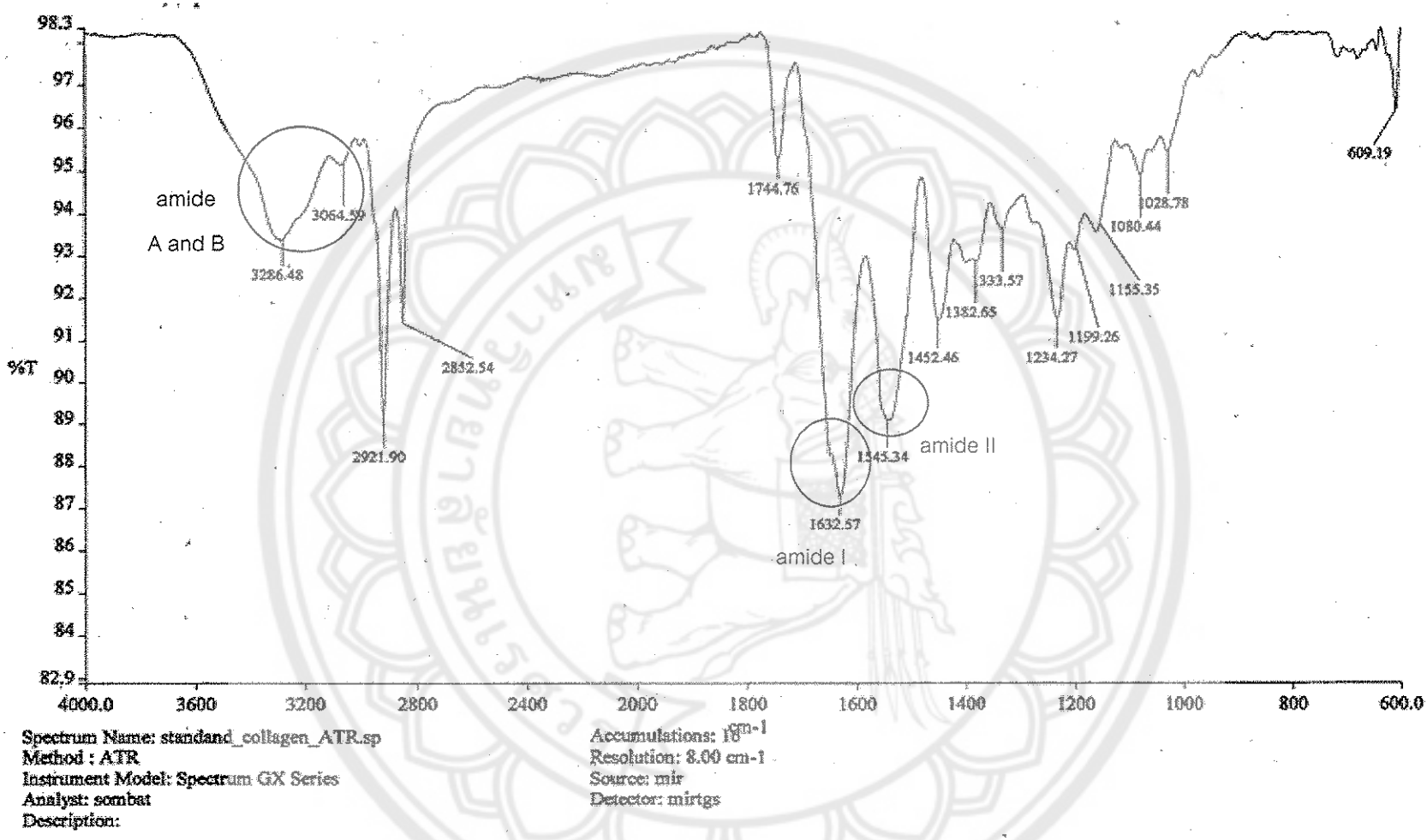


Figure 13 IR spectra of collagen from Sigma-Aldrich.

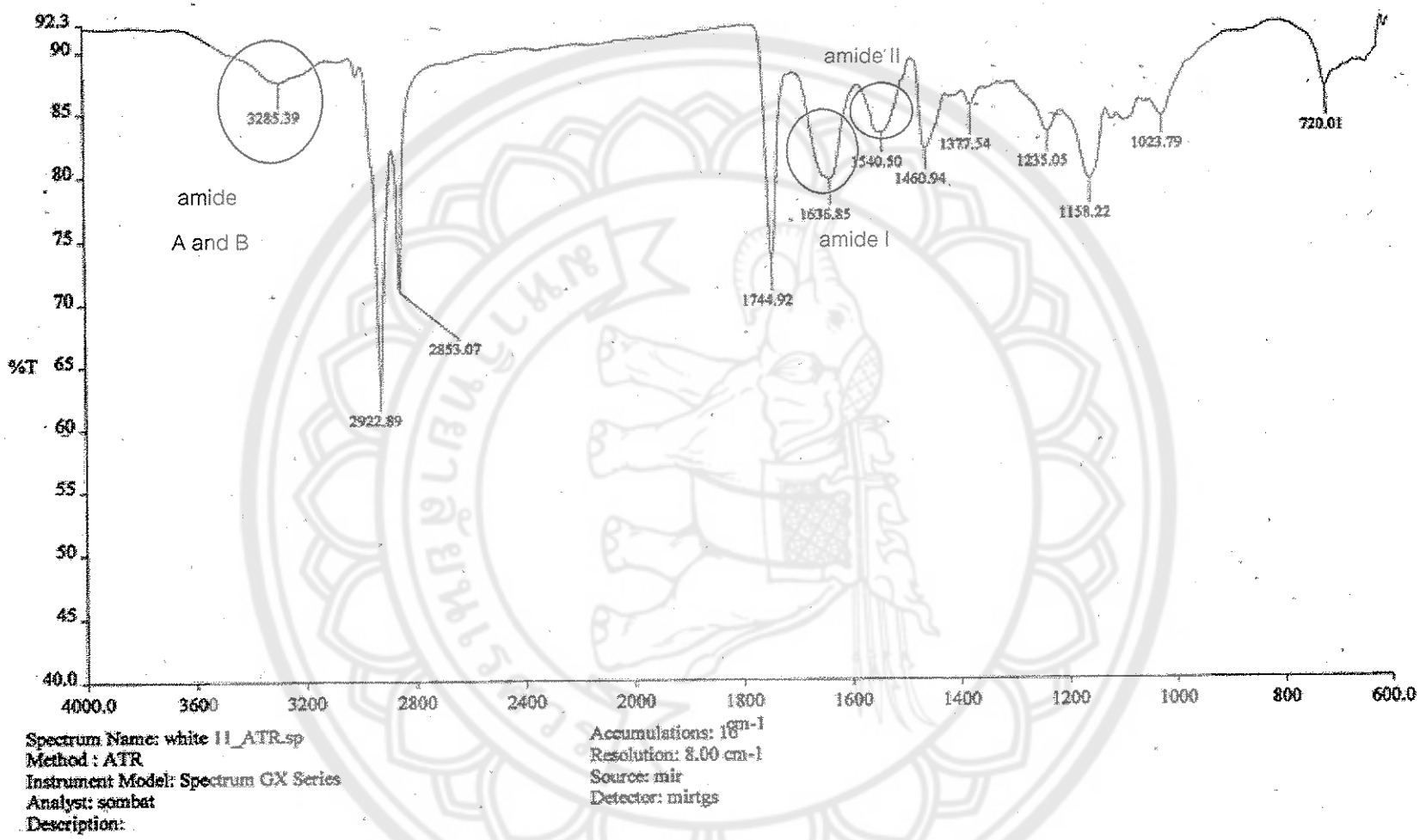


Figure 14 IR spectra of collagen prepared from this study.

Furthermore, the total protein and purification of the isolated collagen were determined by DC assay and SDS-PAGE, respectively, in comparing with the standard collagen. The total protein of the isolated collagen was 71.9%. The result from SDS-PAGE (Figure 15) suggested that the purification of the isolated collagen was quite similar to that of the standard collagen. Both exhibited 3 bands representing two α_2 chains and one α_1 chain.

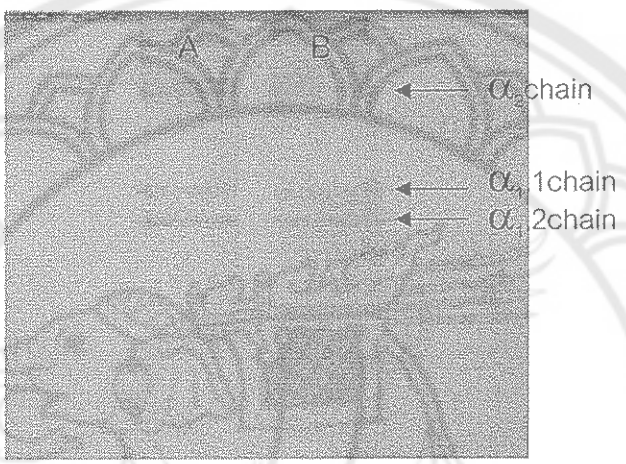


Figure 15 SDS-PAGE of collagen; lane A is the standard collagen, lane B is the isolated collagen.

2. Preparation of matrix

2.1 Preparation of chitosan and PVA matrix

2.1.1 Physical properties of chitosan and PVA matrix

The physical properties of the chitosan and PVA matrix are shown in Table 14 and 15. The examples of the matrix figure are shown in appendix G. Moreover, the surface morphology obtained from SEM is shown in Table 16 and 17. Color, clearness, homogenity, and film formation of the matrix were determined by visualization. After the matrices were formed as a film, they could be peeled off from the glass Petri dish. The color and clearness of chitosan matrix were different among various sources and molecullar weight of chitosan. The shrimp and crab chitosan matrices were less

flexible and brittle whereas the squid chitosan was flexible. The PVA matrices were easy to peel from the Petri dish and more flexible than chitosan matrices.

Table 14 Characteristics of the chitosan matrices.

Formulation	Color	Clearness	Homogenity
Chitosan 0.5% w/v			
- Shrimp MW 30,000	Yellow	++++	++++
- Shrimp MW 100,000	Yellow	++++	++++
- Shrimp MW100,000 -1,000,000	Yellow	++	++++
- Crab MW 100,000 - 1,000,000	Yellow	++	++++
- Squid MW 100,000 - 1,000,000	Light yellow	++++	++++
Chitosan 1% w/v			
- Shrimp MW 30,000	Yellow	++++	++++
- Shrimp MW 100,000	Yellow	++++	++++
- Shrimp MW 100,000 -1,000,000	Yellow	++	++++
- Crab MW 100,000 - 1,000,000	Yellow	+++	++++
- Squid MW 100,000 - 1,000,000	Light yellow	++++	++++
Chitosan 2% w/v			
- Shrimp MW 30,000	Yellow	+++	++++
- Shrimp MW 100,000	Yellow	++++	++++
- Shrimp MW 100,000 -1,000,000	Yellow	+++	++++
- Crab MW 100,000 - 1,000,000	Yellow	++	++++
- Squid MW 100,000 - 1,000,000	Light yellow	++++	++++

Table 14 (cont.).

Formulation	Appearance		
	Color	Clearness	Homogenity
Chitosan 3% w/v			
- Shrimp MW 30,000	Flaming yellow	+++	++++
- Shrimp MW 100,000	Flaming yellow	++++	++++
- Shrimp MW 100,000 -1,000,000	Yellow	+++	++++
- Crab MW 100,000 - 1,000,000	Yellow	+++	++++
- Squid MW 100,000 - 1,000,000	Light yellow	++++	++++
Chitosan 4% w/v			
- Shrimp MW 30,000	Flaming yellow	++	++++
- Shrimp MW 100,000	Flaming yellow	++++	++++
- Shrimp MW 100,000 -1,000,000	Yellow	++++	++++
- Crab MW 100,000 - 1,000,000	Yellow	+++	++++
- Squid MW 100,000 - 1,000,000	Light yellow	++++	++++

Note: the symbols (+) represent appearance. The number of the symbol (+) indicates the degree of the appearance.

Table 15 Characteristics of PVA matrices.

Formulation	Color	Clearness	Homogenity
PVA 0.5% w/v			
- MW 72,000	Colorless	++++	++++
- MW 145,000	Colorless	++++	++++
PVA 1% w/v			
- MW 72,000	Colorless	++++	++++
- MW 145,000	Colorless	++++	++++
PVA 2% w/v			
- MW 72,000	Colorless	++++	++++
- MW 145,000	Colorless	++++	++++

Table 15 (cont.).

Formulation	Color	Clearness	Homogenity
PVA 3% w/v			
- MW 72,000	Colorless	++++	++++
- MW 145,000	Colorless	++++	++++
PVA 4% w/v			
- MW 72,000	Colorless	++++	++++
- MW 145,000	Colorless	++++	++++

Note: the symbols (+) represent appearance. The number of the symbol (+) indicates the degree of the appearance.

Table 16 Surface morphology of chitosan matrix taken by SEM at 3.5KX.

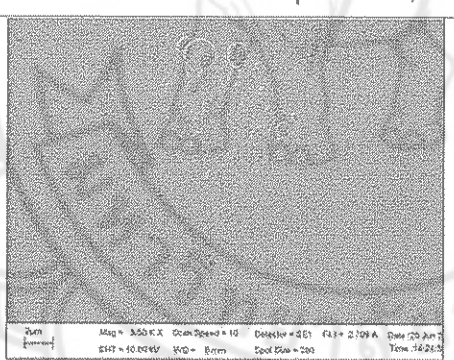
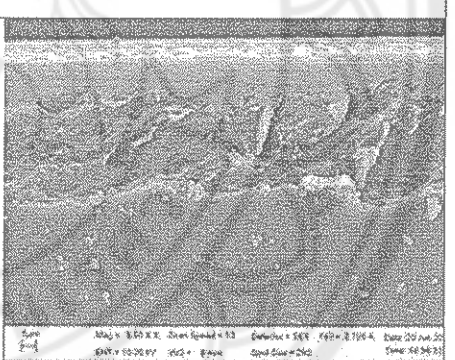
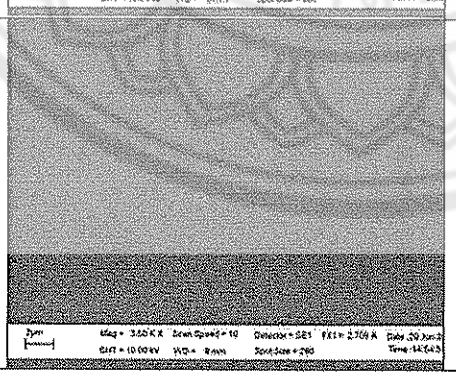
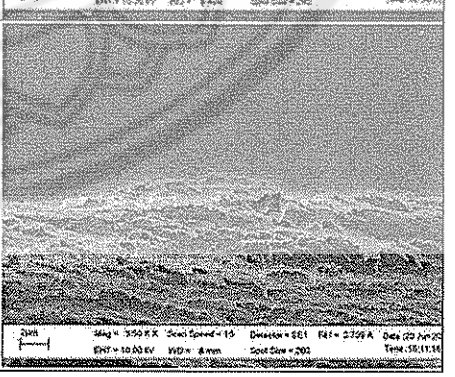
Concentration (% w/v)	Top view	Side view
Shrimp MW 30,000		
0.5		
1		

Table 16 (cont.).

Concentration (% w/v)	Top view	Side view
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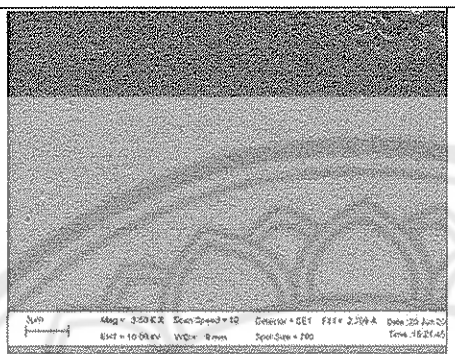
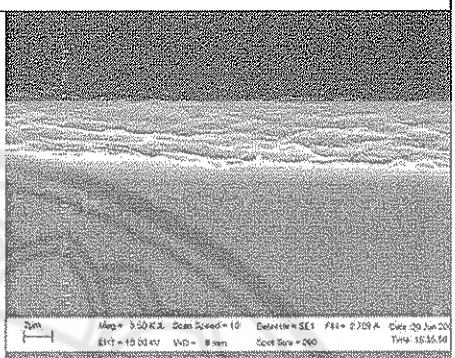
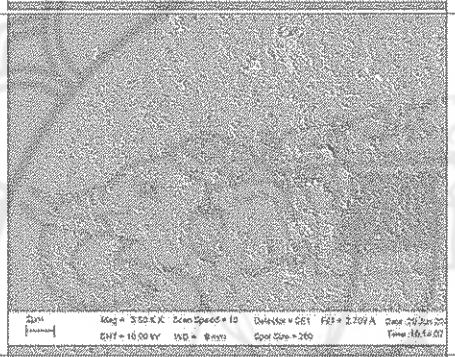
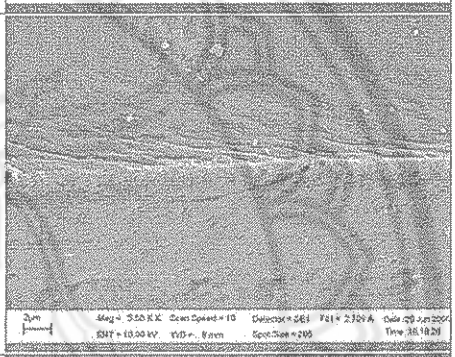
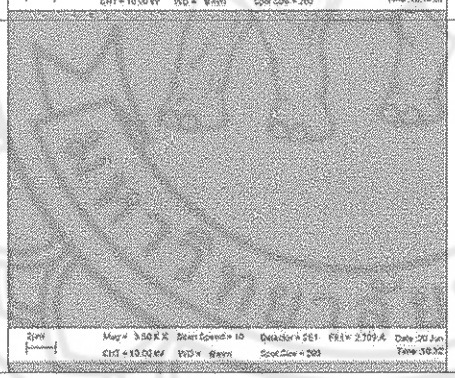
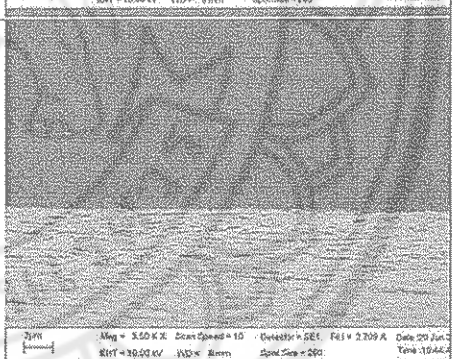
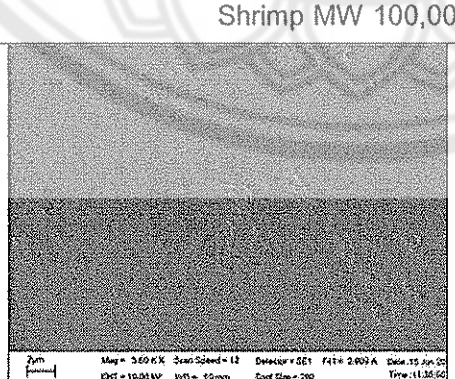
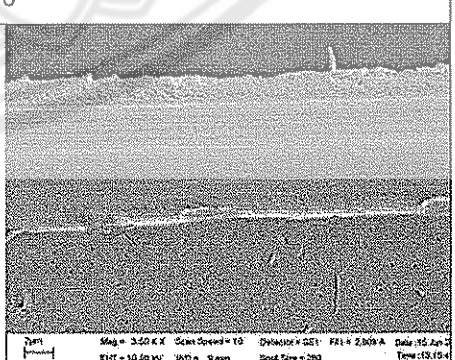
2		
3		
4		
Shrimp MW 100,000		
0.5		

Table 16 (cont.).

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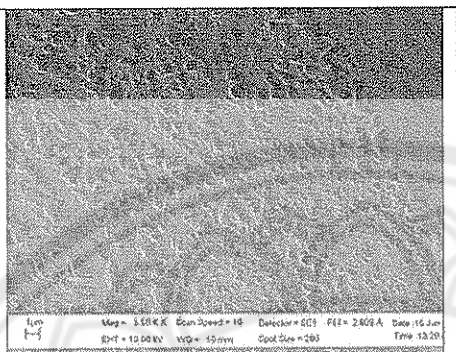
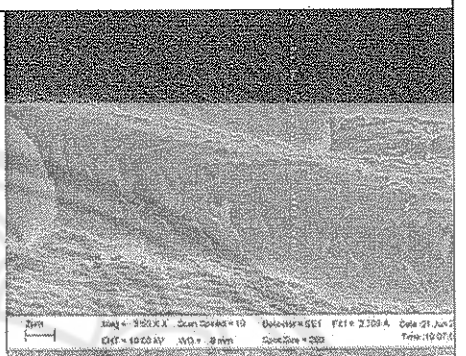
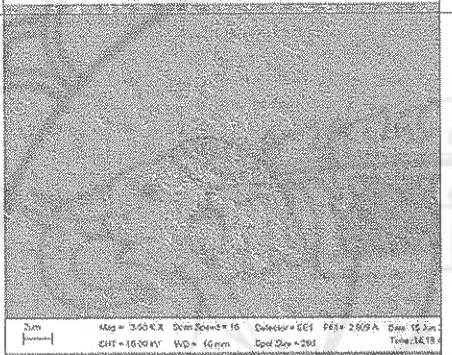
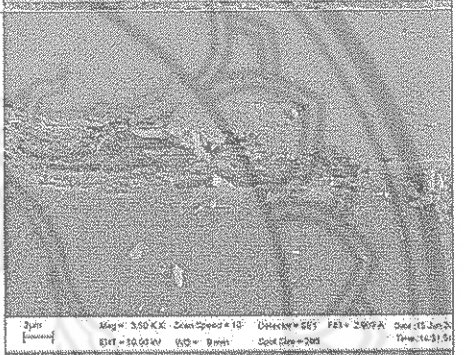
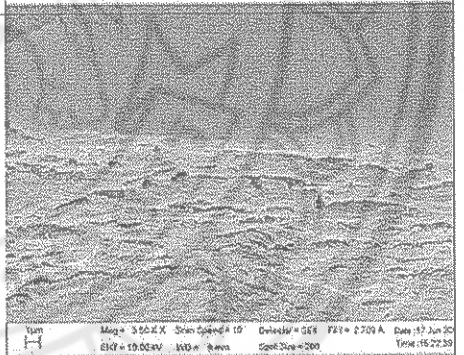
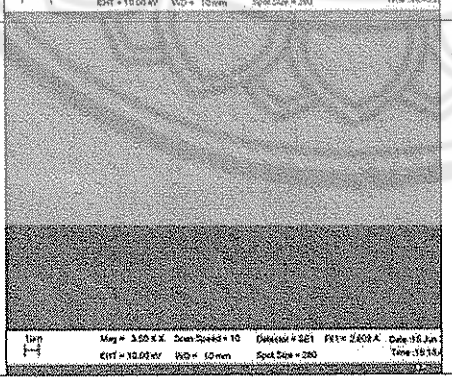
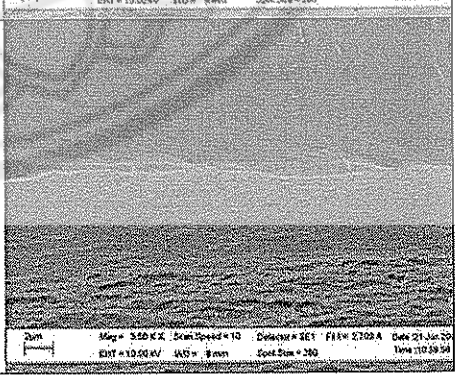
1		
2		
3		
4		

Table 16 (cont.).

Concentration (% w/v)	Top view	Side view
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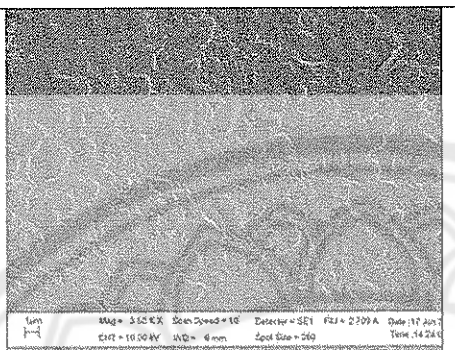
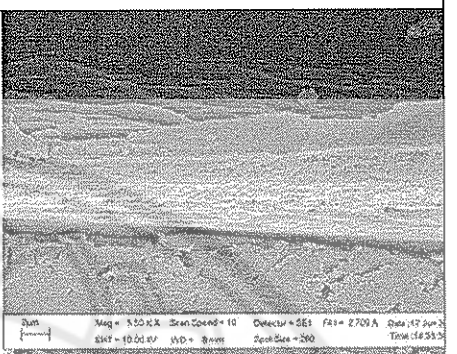
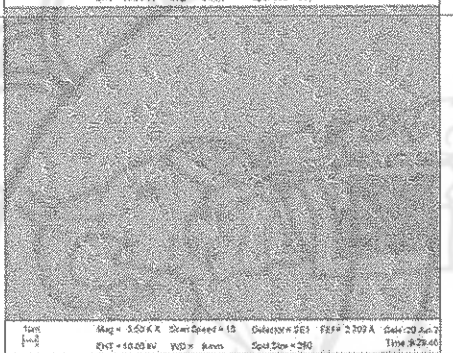
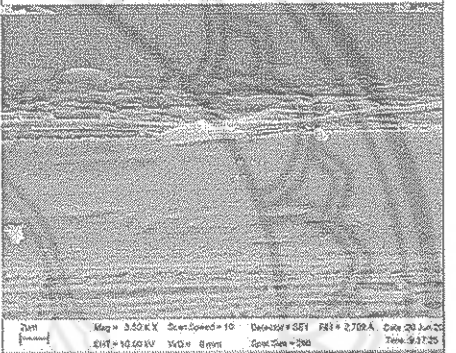
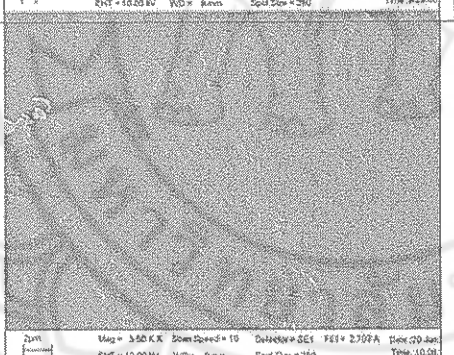
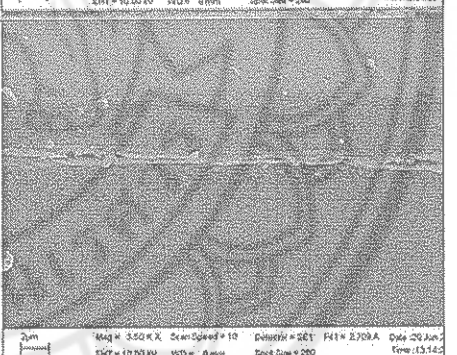
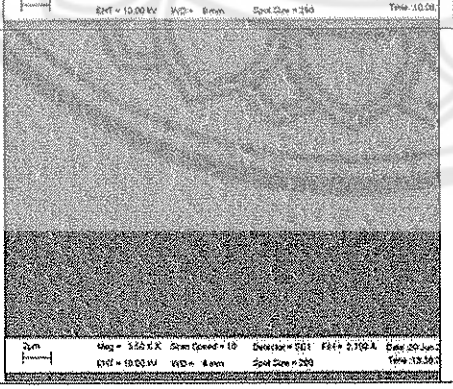
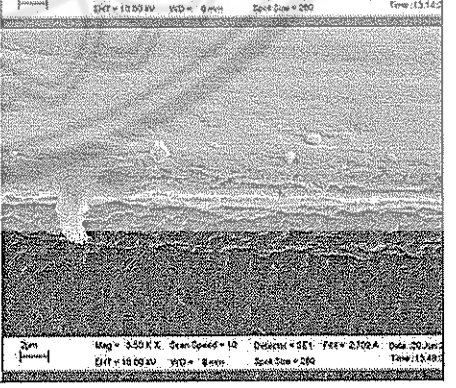
0.5		
1		
2		
3		

Table 16 (cont.).

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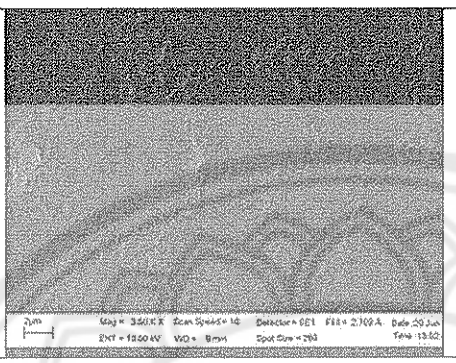
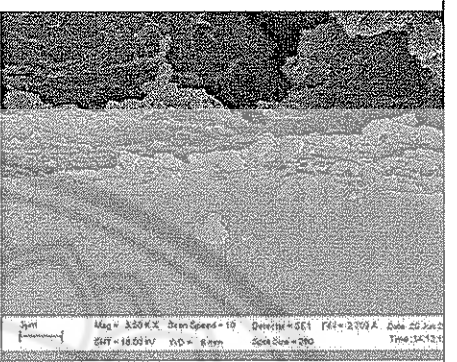
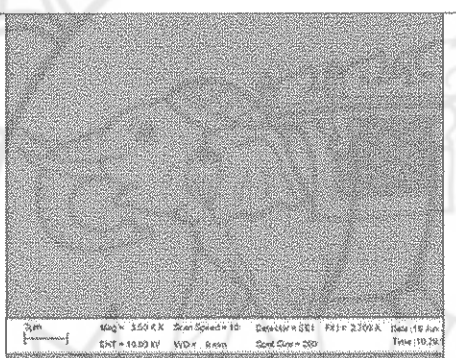
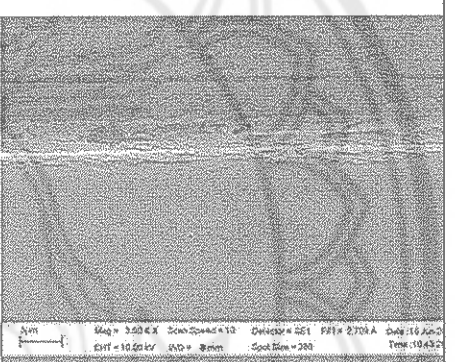
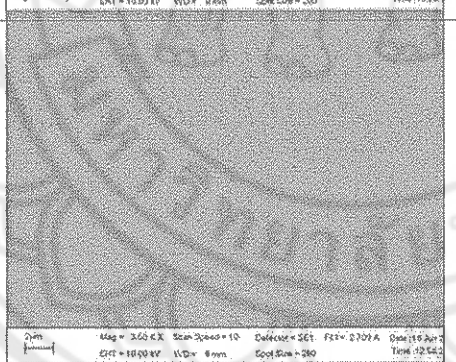
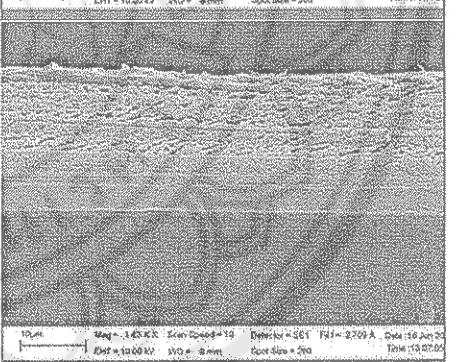
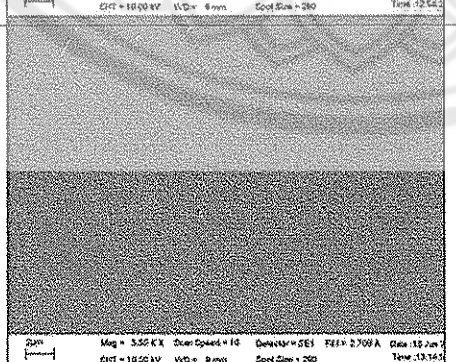
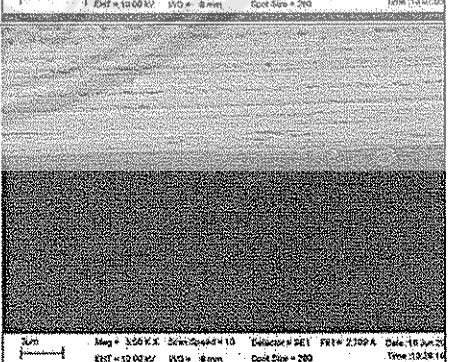
4		
Squid MW 100,000 - 1,000,000		
0.5		
1		
2		

Table 16 (cont.).

Concentration (% w/v)	Top view	Side view
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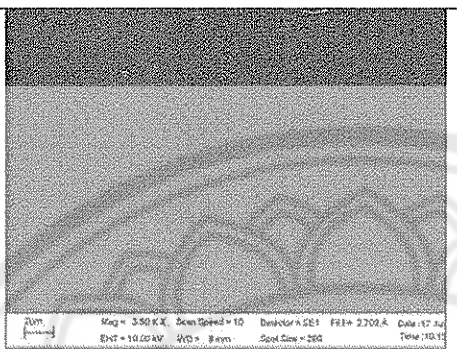
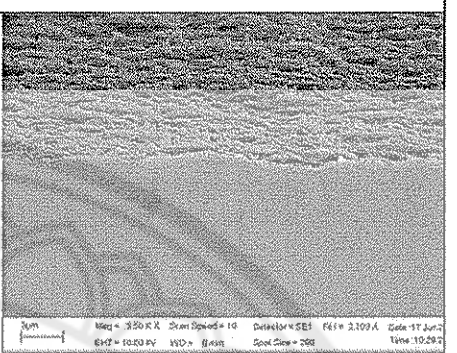
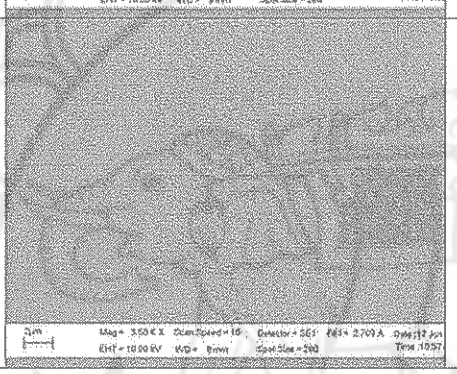
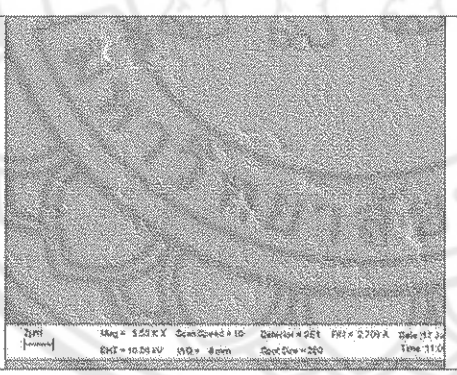
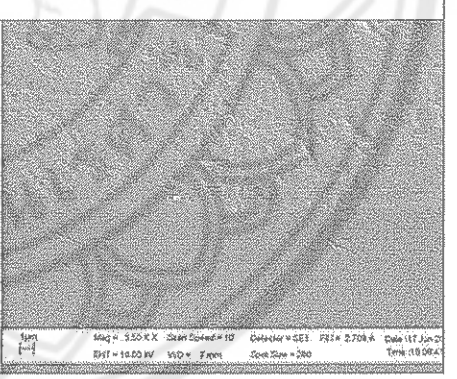
3		
4		
Crab MW 100,000 - 1,000,000		
0.5		

Table 16 (cont.).

Concentration (% w/v)	Top view	Side view
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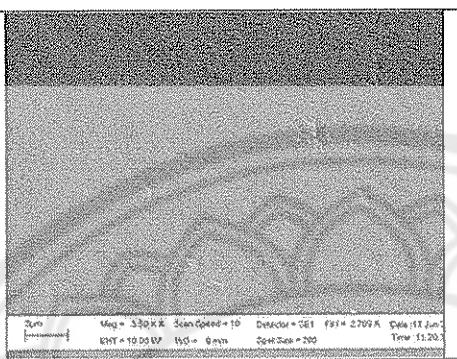
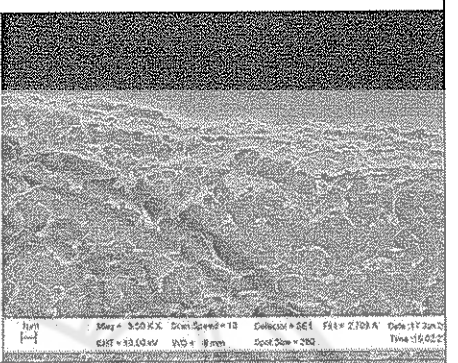
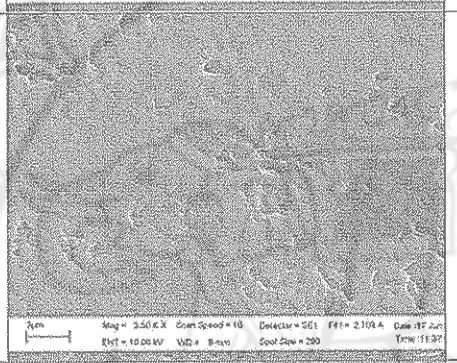
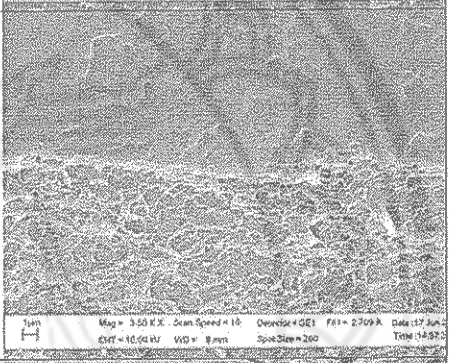
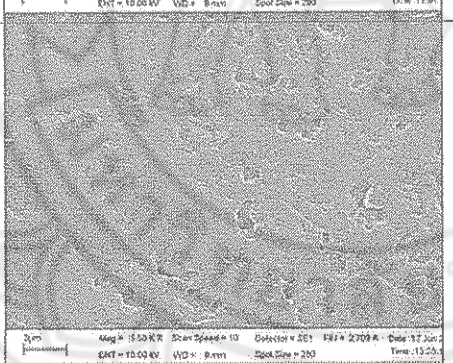
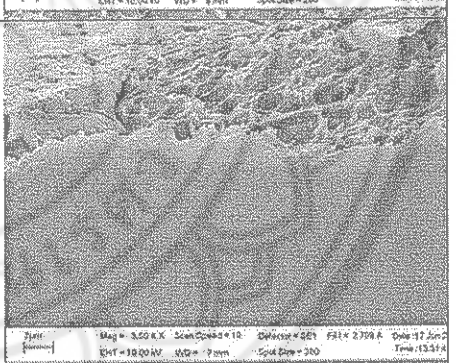
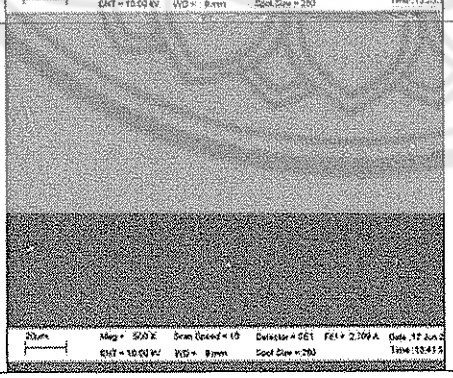
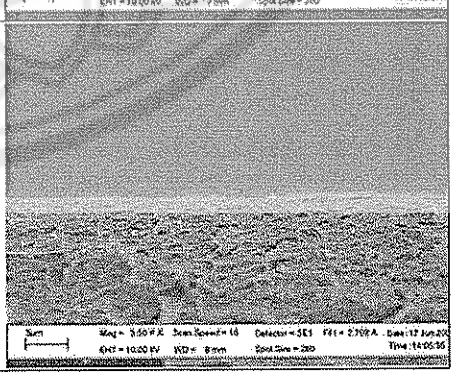
1		
2		
3		
4		

Table 17 Surface morphology of PVA matrix taken by SEM at 3.50 KX.

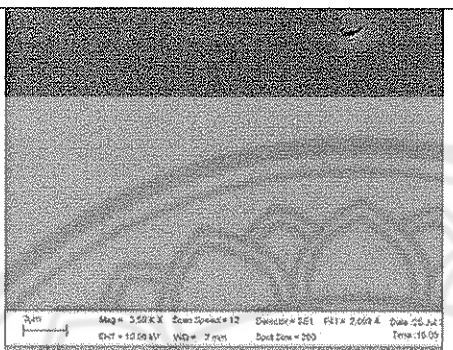
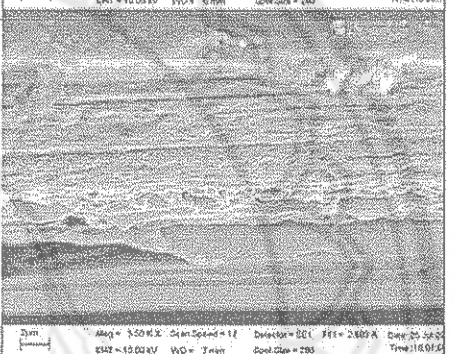
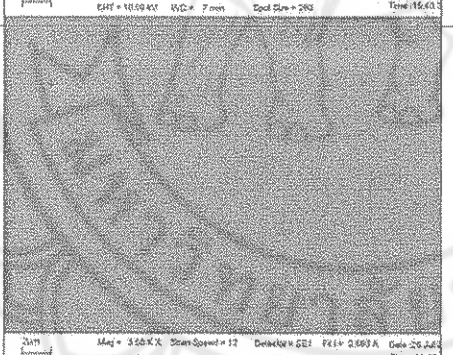
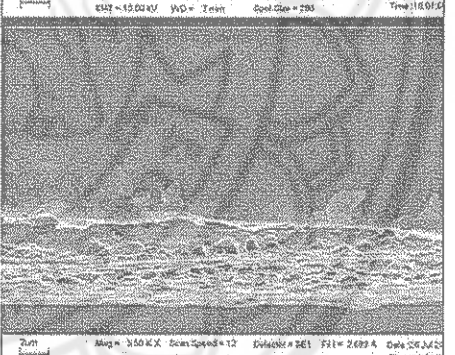
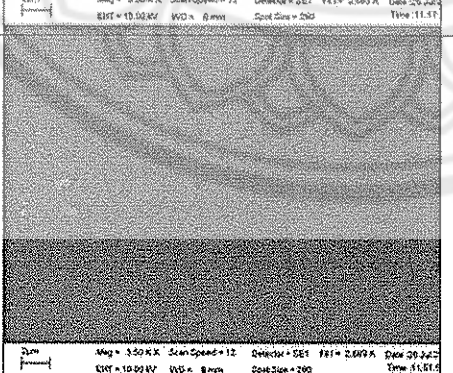
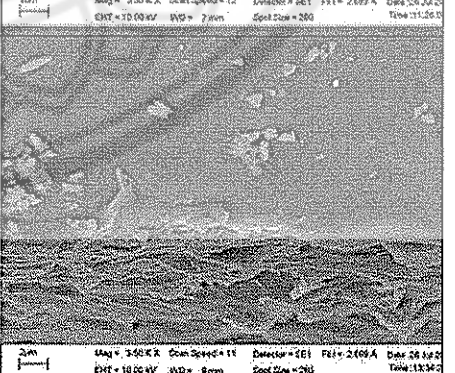
Concentration (% w/v)	Top view	Side view
MW 72,000		
0.5		
1		
2		
3		

Table 17 (cont.).

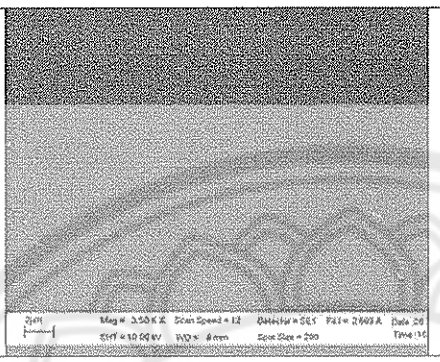
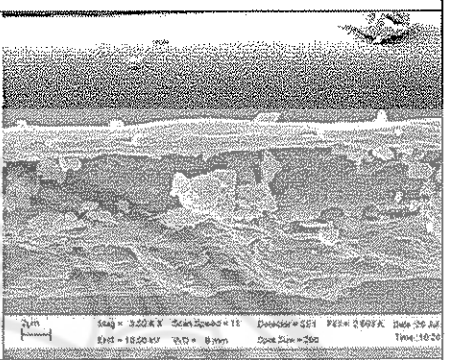
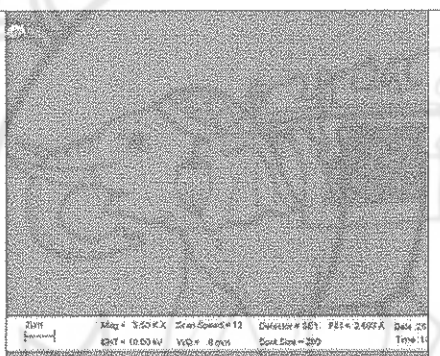
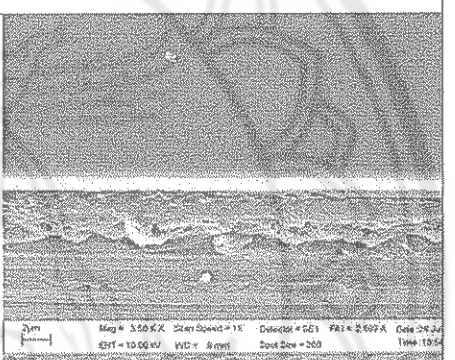
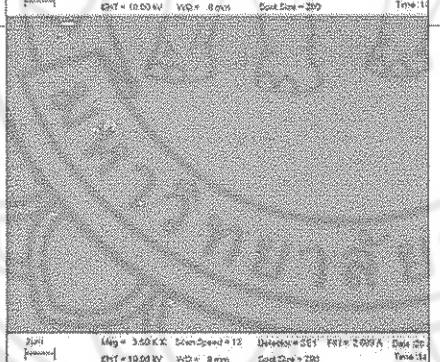
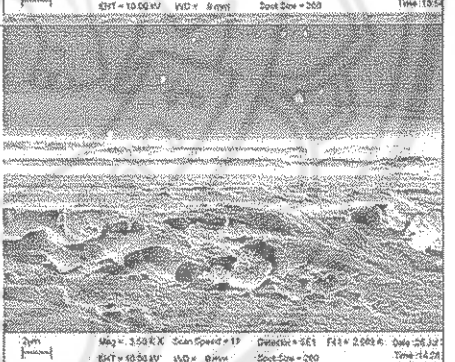
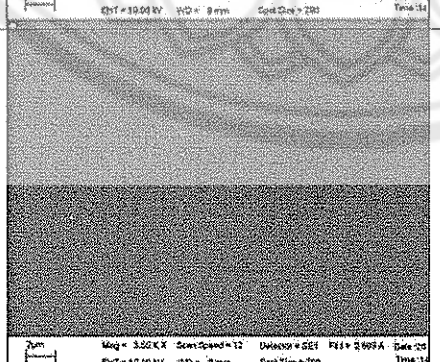
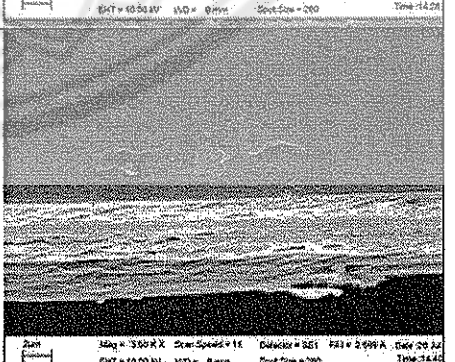
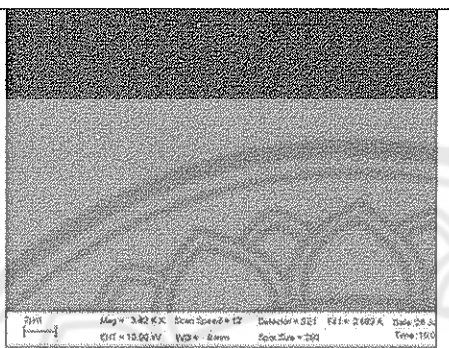
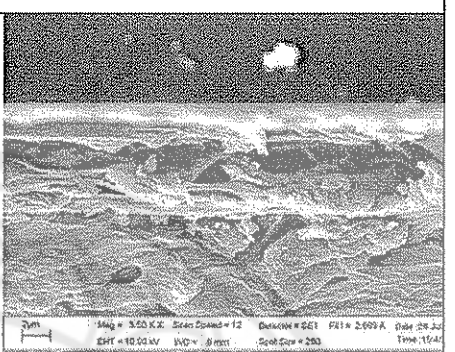
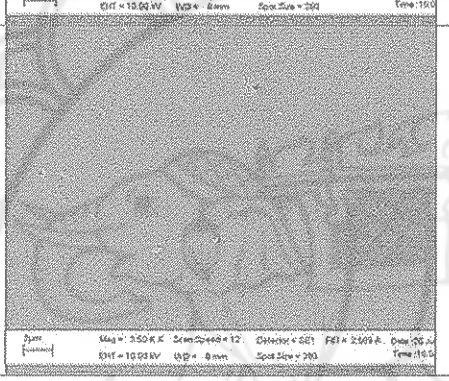
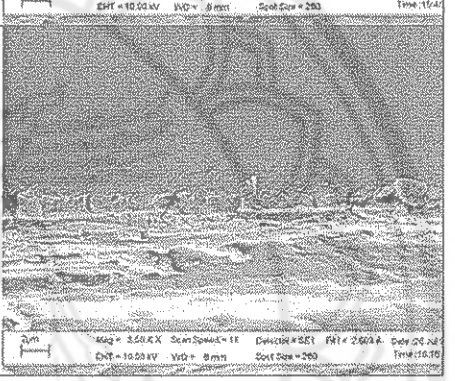
Concentration (% w/v)	Top view	Side view
MW 72,000		
4		
MW 145,000		
0.5		
1		
2		

Table 17 (cont.).

Concentration (% w/v)	Top view	Side view
MW 145,000		
3		
4		

From the surface morphology taken by SEM, it was found that the matrix from shrimp chitosan MW 30,000 at the concentrations of 1% and 4% w/v exhibited higher porosity than that at the other concentrations. The matrices from shrimp chitosan MW 100,000 w/v at the concentrations of 0.5% and 4% w/v also exhitbited higher porosity than that at the other concentrations whereas the matrix from shrimp chitosan MW 100,000 - 1,000,000 and from squid chitosan MW 100,000 - 1,000,000 w/v provided very low porosity. The porosity of the matrix from crab chitosan MW 100,000 - 1,000,000 at the concentrations of 2% and 3% w/v was higher than that at the other concentrations. In addition, the porosity of the PVA matrix was rarely observed by SEM. The matrices with high porosity were selected for further determination of physicochemical property such as mechanical properties.

2.2 Physicochemical properties of chiitosan and PVA matrix

2.2.1 Mechanical properties

The mechanical properties of the matrix were evaluated from the tensile strength and % elongation at break. The results of the mechanical strenght of the matrix from chitosan and PVA are shown in Table 18 and Figure 16-18.

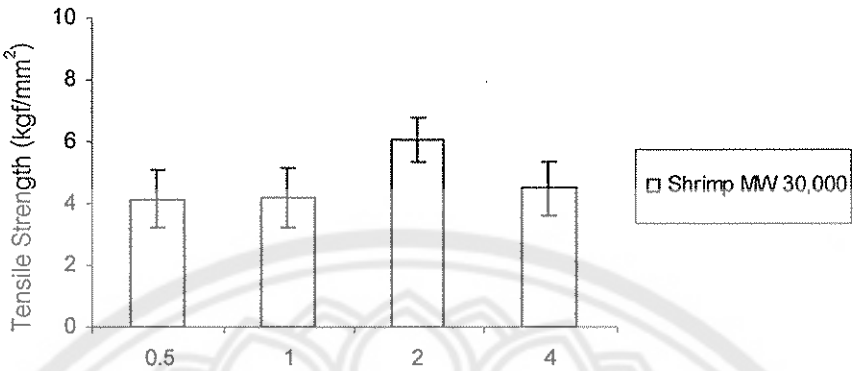
Table 18 Tensile strength and % elongation at break of chitosan matrices.

Chitosan (% w/v)	Patch Number	Thickness (mm)				TS (Kgf/mm^2)	% Elongation at break
		1	2	3	Average		
Shrimp MW 30,000							
0.5	1	0.085	0.105	0.080	0.090	4.03	1.11
	2	0.100	0.115	0.100	0.105	3.28	0.88
	3	0.080	0.105	0.105	0.097	5.12	1.08
	Average					4.14 ± 0.93	1.02 ± 0.13
1	1	0.100	0.085	0.090	0.092	3.48	1.10
	2	0.120	0.080	0.070	0.090	3.82	1.23
	3	0.100	0.080	0.085	0.088	5.28	1.59
	Average					4.19 ± 0.96	1.31 ± 0.25
2	1	0.105	0.120	0.100	0.108	6.82	1.15
	2	0.100	0.100	0.100	0.100	5.37	1.23
	3	0.090	0.090	0.090	0.090	5.98	1.30
	Average					6.06 ± 0.73	1.23 ± 0.08
4	1	0.100	0.105	0.085	0.096	5.16	1.23
	2	0.095	0.080	0.085	0.087	3.52	1.23
	3	0.100	0.100	0.080	0.093	4.85	1.35
	Average					4.51 ± 0.87	1.27 ± 0.07
Shrimp MW 100,000							
0.5	1	0.100	0.090	0.080	0.090	3.55	0.96
	2	0.095	0.085	0.080	0.087	2.67	0.83
	3	0.090	0.080	0.080	0.083	4.97	1.23
	Average					3.73 ± 1.16	1.01 ± 0.20

Table 18 (cont.).

Chitosan (w/v)	Patch Number	Thickness (mm)				TS (Kgf/mm^2)	% Elongation at break
		1	2	3	Average		
Shrimp MW 100,000							
2	1	0.100	0.100	0.100	0.100	4.89	1.19
	2	0.100	0.120	0.100	0.107	4.50	1.05
	3	0.105	0.110	0.105	0.107	4.30	1.12
	Average					4.59 ± 0.30	1.12 ± 0.07
4	1	0.090	0.100	0.100	0.097	5.24	1.16
	2	0.090	0.100	0.100	0.097	6.32	1.41
	3	0.095	0.100	0.100	0.098	4.86	0.90
	Average					5.47 ± 0.76	1.16 ± 0.26
Crab MW 100,000 – 1,000,000							
0.5	1	0.085	0.085	0.080	0.083	5.74	1.04
	2	0.085	0.085	0.080	0.083	4.99	1.11
	3	0.105	0.09	0.105	0.100	4.32	0.94
	Average					5.02 ± 0.71	1.03 ± 0.09
2	1	0.120	0.120	0.120	0.120	4.80	0.95
	2	0.120	0.120	0.120	0.120	5.37	0.96
	3	0.120	0.105	0.100	0.108	4.65	0.99
	Average					4.94 ± 0.38	0.97 ± 0.02
3	1	0.085	0.080	0.080	0.082	6.62	1.30
	2	0.085	0.085	0.085	0.085	4.94	1.35
	3	0.085	0.080	0.080	0.082	5.91	1.30
	Average					6.01 ± 0.56	1.32 ± 0.03

A



B

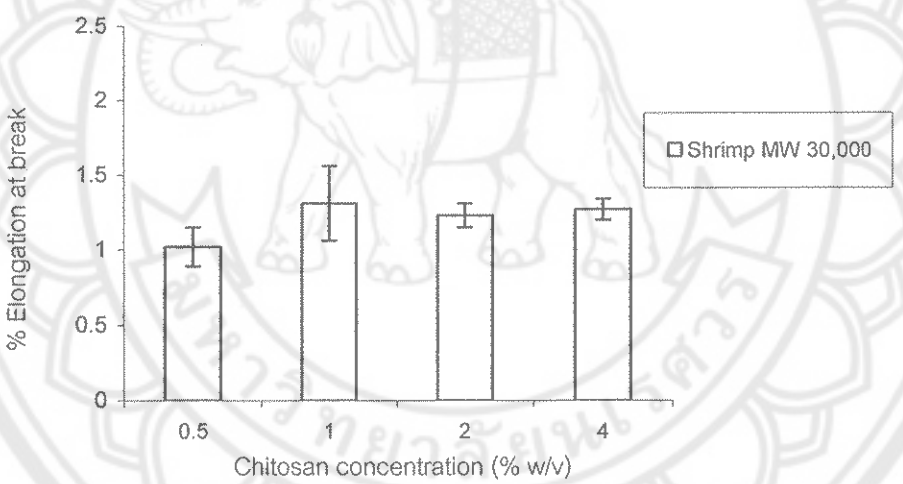
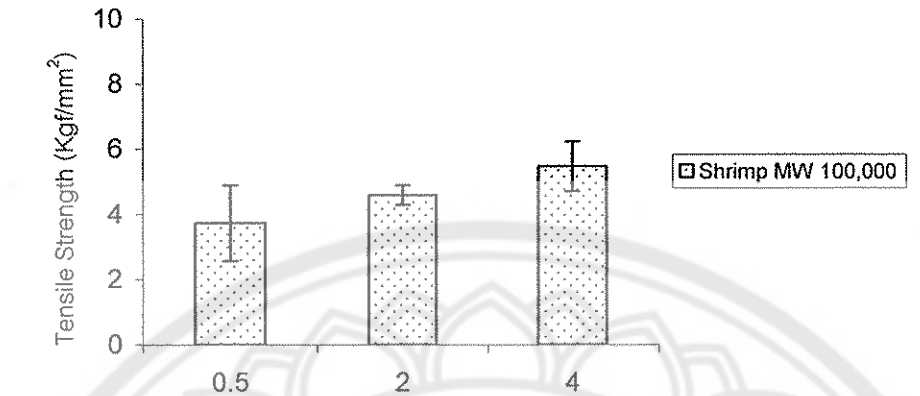


Figure 16 Tensile strength (A) and % elongation at break (B) of the matrix from shrimp chitosan MW 30,000.

A



B

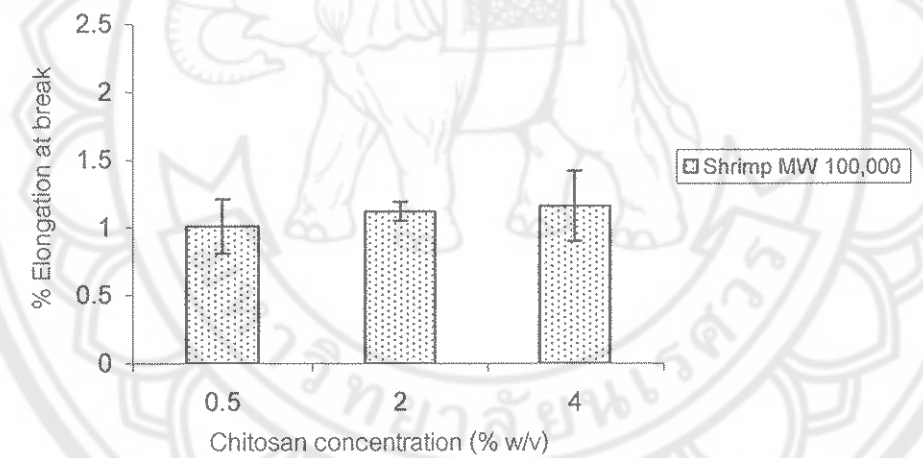
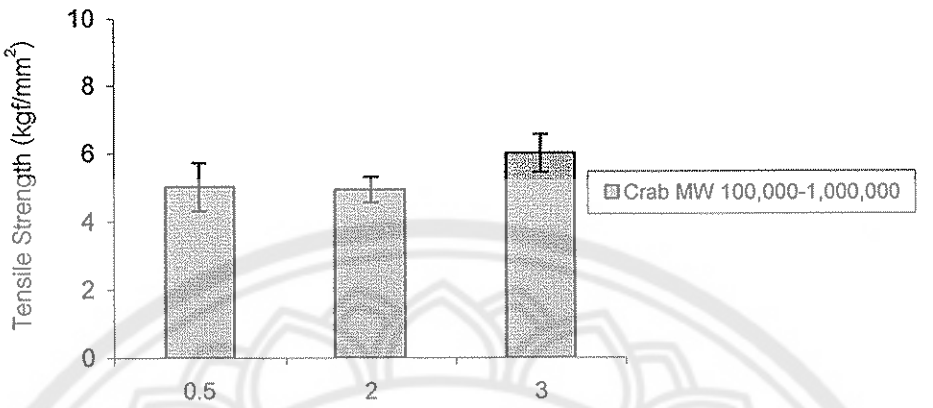


Figure 17 Tensile strength (A) and % elongation at break (B) of the matrix from shrimp chitosan MW 100,000.

A



B

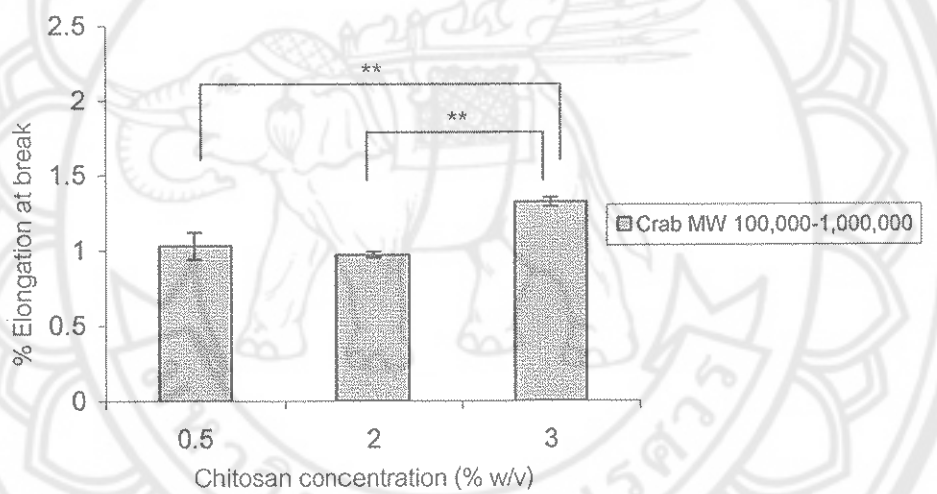


Figure 18 Tensile strength (A) and % elongation at break (B) of the matrix from crab chitosan MW 100,000 - 1,000,000. (** = $p < 0.01$)

The tensile strength and % elongation at break of the chitosan matrix were in the ranges of 4.00-6.00 kgf/mm^2 and 1.00-2.00%, respectively. These matrices tended to brittle due to their low % elongation at break. Tensile strength and % elongation at break of the matrix were not different among types of chitosan or among MW, except the crab chitosan. % Elongation at break of the crab chitosan matrix showed an increasing trend with increasing in the amount of polymer, indicating an increase in the film flexibility.

Since the polymer concentration of the marix from shrimp chitosan MW 30,000 at 1% w/v exhibited % elongation at break tended to increase higher than that from the other concentrations, it was selected for further experiment. The tensile strength and % elongation at break of the matrix from shrimp chitosan MW 100,000 was not significantly different among different concentrations. Therefore, the lowest concentration, 0.5% w/v, was selected. For crab chitosan matrix, the concentration of 3% w/v was selected because the % elongation at break was significantly higher than those from other concentrations.

The tensile strength and % elongation at break of the PVA matrices were in the ranges in 2.0-4.5 kgf/mm^2 and 60-120 %, respectively. It was found that the different of MW had not affected to the mechanical properties of the PVA matrix. In addition, we found that the increases in the concentration of the polymer led to the decrease in the % elongation. In other words, at 0.5% w/v polymer provided the matrix with higher % elongation than at 1%, 2%, 3% and 4% whereas their tensile strength values were not different. This indicated that the flexibility of 0.5% w/v PVA matrix was higher than that of the other concentrations. Hence, PVA at the concentraton of 0.5% w/v was selected for further experiment. From all results, the selected matrices for further studies are shown below.

1. 0.1% w/v shrimp chitosan MW 30,000
2. 0.5% m/v shrimp chitosan MW 100,000
3. 3% w/v crab chitosan MW 100,000 - 1,000,000
4. 0.5% w/v PVA MW 72,000

5. 0.5% w/v PVA MW 145,000

2.3 Preparation of collagen/chitosan matrix

The collagen matrix prepared by casting technique could not be removed from the Petri dish. After adding chitosan into the formulation, the collagen/chitosan matrix was stronger and easier to be peeled off. The physical characteristics of the collagen/chitosan matrix are shown in Table 19-20.

From the results of surface morphology, it was found that the porosity of the collage/shrimp chitosan MW 30,000 and collagen/shrimp chitosan MW 100,000 matrix in ratios 8:2 and 7:3 were higher than that in ratios of 6:4 and 5:5. Therefore, such matrices were selected for further studies. For the collagen/crab chitosan, the ratio 7:3 of collagen: chitosan showed the higher porosity than other ratios. In conclusion, the selected matrices for further studies are listed below.

1. Collagen/Shrimp chitosan MW 30,000 (8:2)
2. Collagen/Shrimp chitosan MW 100,000 (8:2)
3. Collagen/Shrimp chitosan MW 100,000 (7:3)
4. Collagen/Crab chitosan MW 100,000 - 1,000,000 (7:3)

Table 19 Characteristics of the collagen/chitosan matrix in various ratios.

Formulation	Color	Transparency	Homogenity	Flexibility
Collagen/Shrimp chitosan MW 30,000 (1% w/v of total polymer)				
8:2	Yellowish brown	-	+++	++
7:3	Yellowish brown	+	+++	+++
6:4	Yellowish brown	+	++	++++
5:5	Yellowish brown	+	++	++++
Collagen/Shrimp chitosan MW 100,000 (0.5% w/v of total polymer)				
8:2	Yellowish brown	-	++	++
7:3	Yellowish brown	-	++	+++
6:4	Yellowish brown	-	++	+++
5:5	Yellowish brown	-	+++	+++
Collagen/Crab chitosan MW 100,000 - 1,000,000 (3% w/v of total polymer)				
8:2	Brown	+	++++	+++
7:3	Brown	+	++++	++++
6:4	Brown	+	++++	++++
5:5	Brown	+	++++	++++

Note: the symbols (+) and (-) represent appearance and no appearance, respectively. The number of the symbol (+) indicates the degree of the appearance.

Table 20 Surface morphology of collagen/chitosan matrix taken by SEM at 3.50 KX.

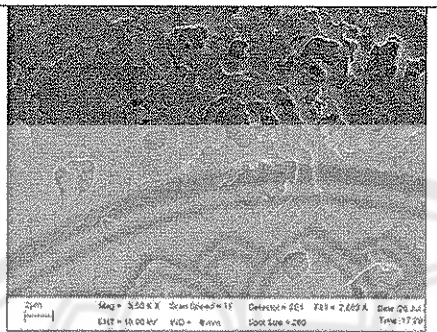
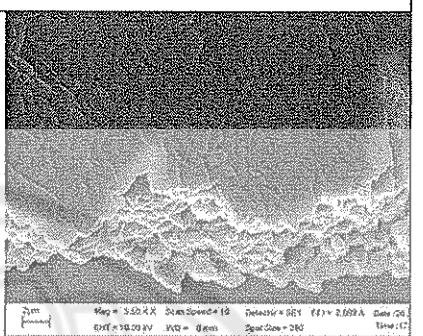
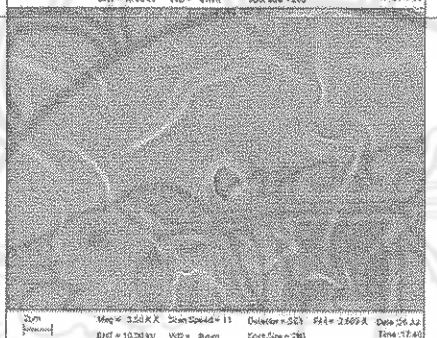
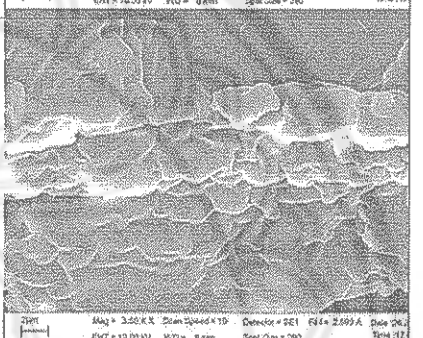
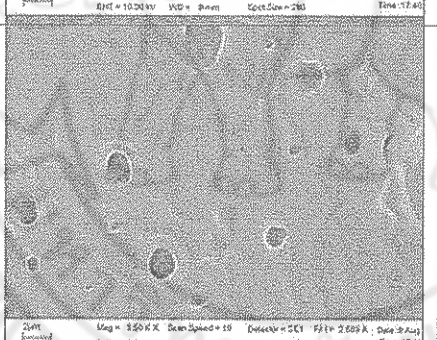
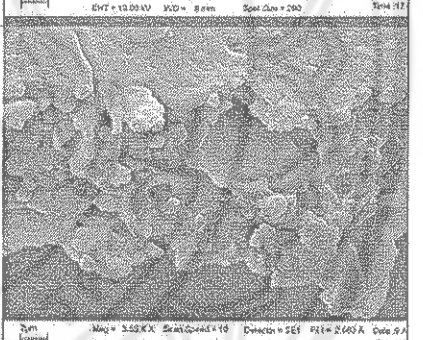
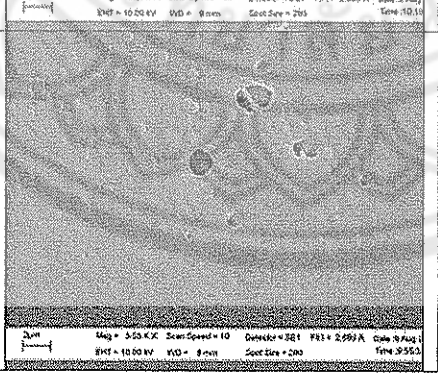
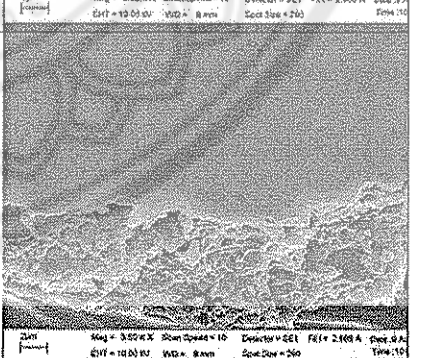
Collagen/Chitosan	Top view	Side view
Collagen/Shrimp chitosan MW 30,000 (1% w/v of total polymer)		
8:2		
7:3		
6:4		
5:5		

Table 20 (cont.).

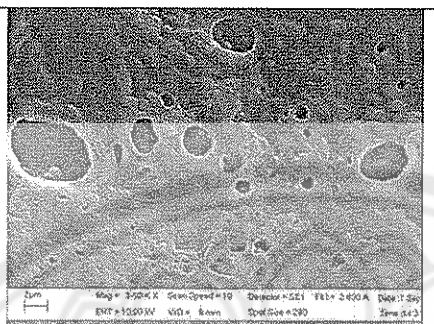
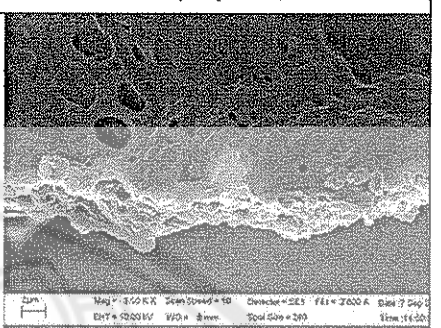
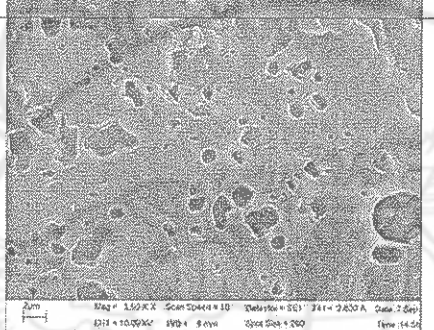
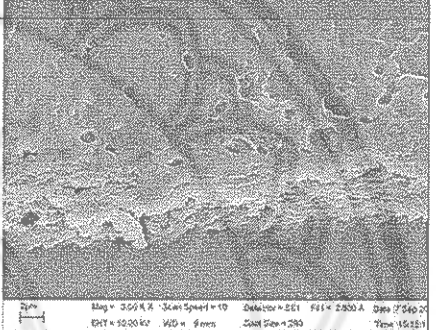
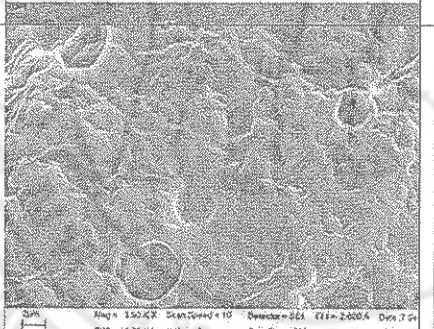
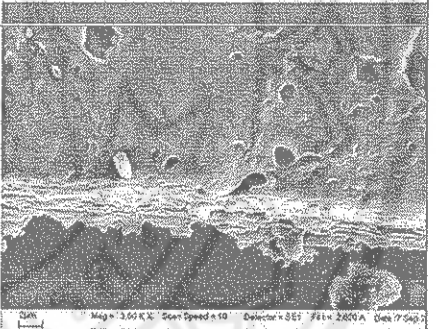
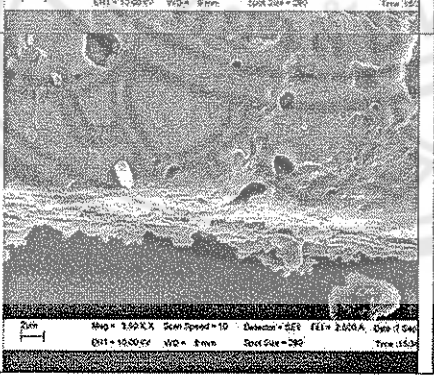
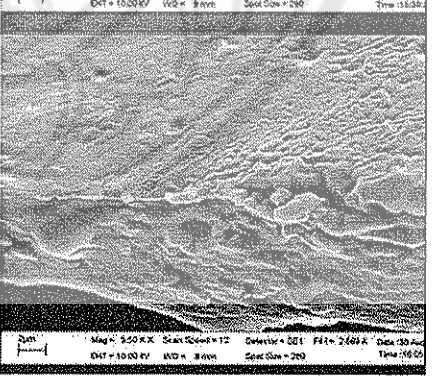
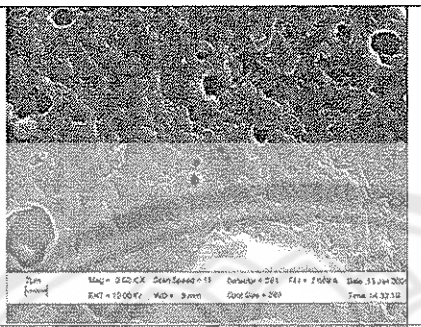
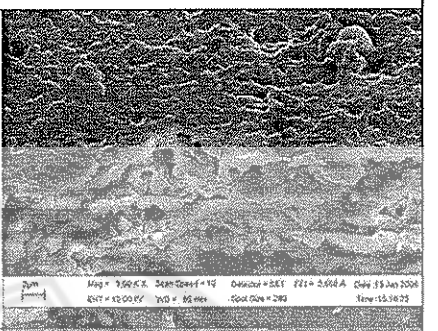
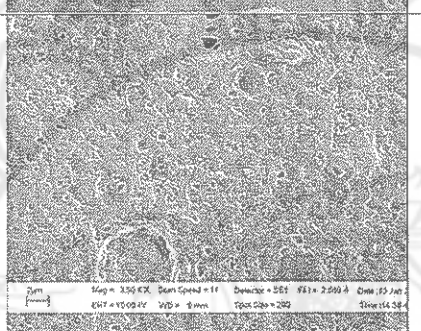
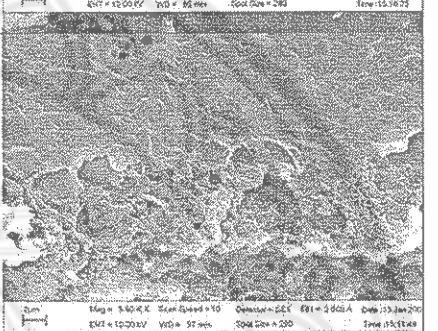
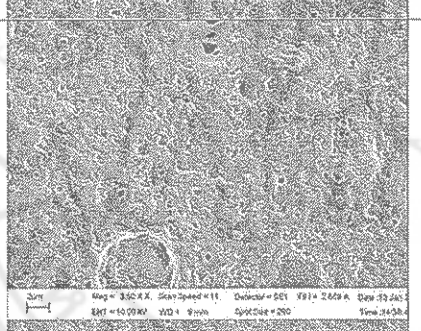
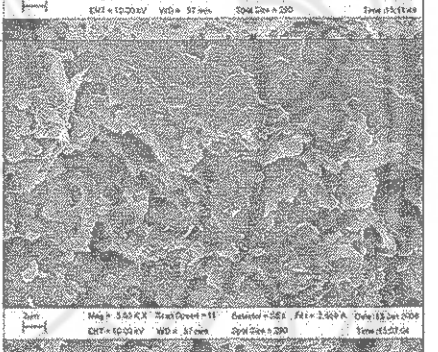
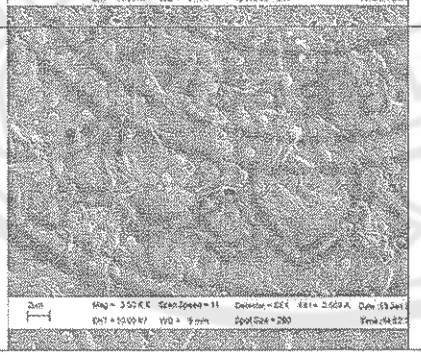
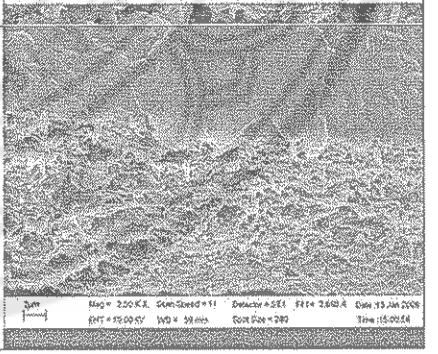
Collagen/chitosan	Top view	Side view
Collagen/Shrimp chitosan MW 100,000 (0.5% w/v of total polymer)		
8:2		
7:3		
6:4		
5:5		

Table 20 (cont.).

Collagen/Chitosan	Top view	Side view
Collagen/Crab chitosan MW 100,000 - 1,000,000 (3% w/v of total polymer)		
8:2		
7:3		
6:4		
5:5		

2.4 Preparation of collagen/chitosan crossliked with glutaldehyde (GA) or β-glycerolphosphate (GP)

2.4.1 Physical charateristics

The color of collagen/chitosan matrices crosslinked with GA and prepared from casting technique were yellowish brown or brown. GP crosslinking provided yellowish, yellowish brown or cream matrix. The matrix crosslinked with GA or GP were homogeneous and flexible. The examples of matrix prepared from collagen/chitosan crosslinked with GA or GP is shown in appendix F. Their physical characteristics are shown in Table 21 and 22. The obtained matrices were easy to be peeled off from the glass Petri dish. However, the transparency of the obtained matrix was poor.

It is found that the addition of crosslinkg agent into the formulation tended to increase the strength and the flexibility, as comparing to the formula without the crosslinking agent.

Table 21 Physical characteristics of collagen/chitosan matrix crosslinked with GA.

Formulation	Color	Transparency	Homogenity	Flexibility
Collagen/Shrimp chitosan MW 30,000 8:2 (1% w/v of total polymer)				
0.05% GA	Yellowish brown	+	++++	++
0.10% GA	Yellowish brown	+	+++	+++
0.15% GA	Yellowish brown	+	+++	++++
Collagen/Shrimp chitosan MW 100,000 8:2 (0.5% w/v of total polymer)				
0.05% GA	Brown	++	+++	+++
0.10% GA	Brown	++	+++	+++
0.15% GA	Brown	++	+++	+++
Collagen/Shrimp chitosan MW 100,000 7:3 (0.5% w/v of total polymer)				
0.05% GA	Brown	++	++++	++++
0.10% GA	Brown	++	++++	++++
0.15% GA	Brown	++	++++	++++
Collagen/Crab chitosan MW 100,000 -1,000,000 7:3 (3% w/v of total polymer)				
0.05% GA	Brown	+	++++	++++
0.10% GA	Brown	+	++++	++++
0.15% GA	Brown	+	++++	++++

Note: the symbols (+) and (-) represent appearance and no appearance, respectively.

The number of the symbol (+) indicates the degree of the appearance.

Table 22 Physical characteristics of the collagen/chitosan matrix crosslinked with GP.

Formulation	Color	Transparency	Homogenity	Flexibility
Collagen/Shrimp chitosan MW 30,000 8:2 (1% w/v of total polymer)				
0.5% GP	Yellowish	+	++++	+++
1.0% GP	Yellowish	+	++++	+++
1.5% GP	Yellowish	+	++++	+++
Collagen/Shrimp chitosan MW 100,000 8:2 (0.5% w/v of total polymer)				
0.5% GP	Cream	++	+++	+++
1.0% GP	Cream	++	+++	+++
1.5% GP	Cream	++	+++	+++
Collagen/Shrimp chitosan MW 100,000 7:3 (0.5% w/v of total polymer)				
0.5% GP	Cream	++	++++	++++
1.0% GP	Cream	++	++++	++++
1.5% GP	Cream	++	++++	++++
Collagen/Crab chitosan MW 100,000 - 1,000,000 7:3 (3% w/v of total polymer)				
0.5% GP	Yellowish brown	-	++++	+++
1.0% GP	Yellowish brown	-	++++	+++
1.5% GP	Yellowish brown	-	++++	+++

Note: the symbols (+) and (-) represent appearance and no appearance, respectively.

The number of the symbol (+) indicates the degree of the appearance.

Table 23 Surface morphology of collagen/shrimp chitosan MW 30,000 in ratio of 8 to 2 and crosslinked with GA (3.50 KX).

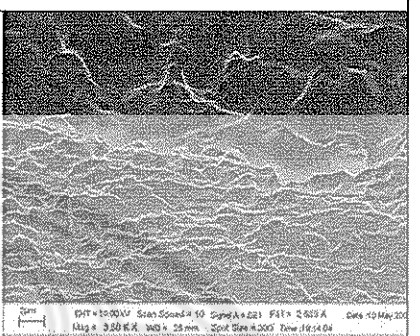
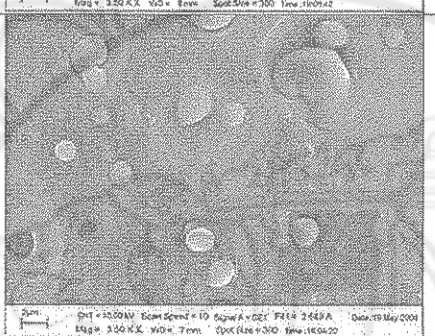
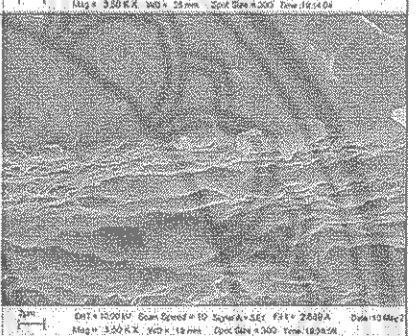
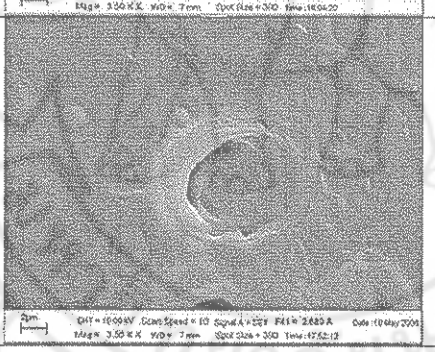
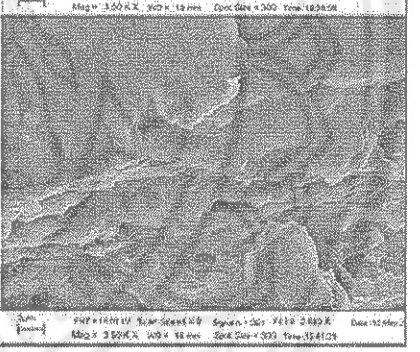
Collagen/Chitosan	Top view	Side view
0.05% GA		
0.1% GA		
0.15% GA		

Table 24 Surface morphology of collagen/shrimp chitosan MW 100,000 in ratio of 8 to 2 and crosslinked with GA (3.50 KX).

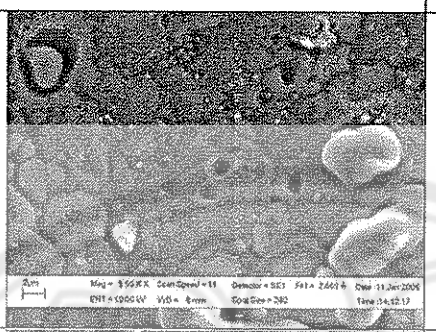
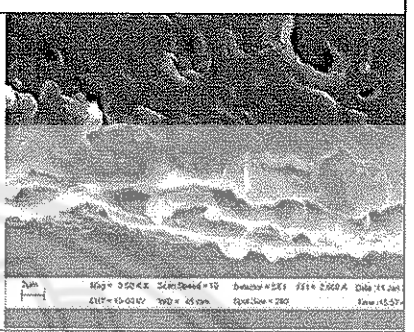
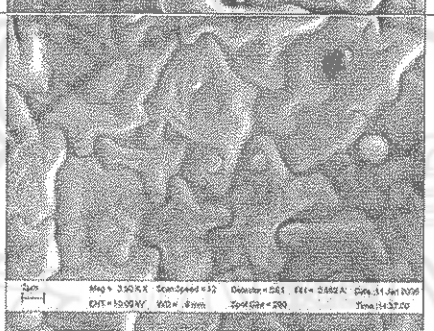
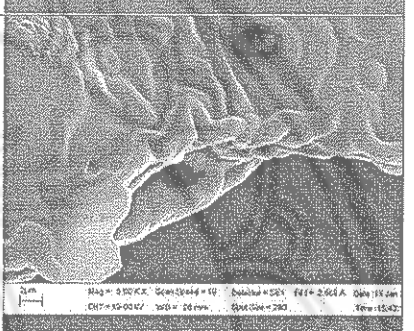
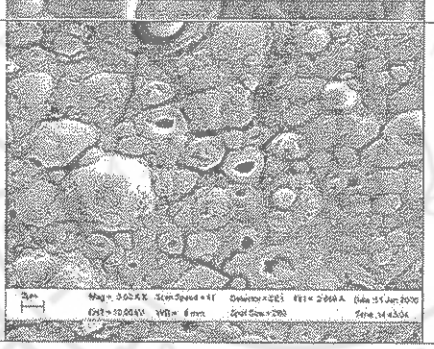
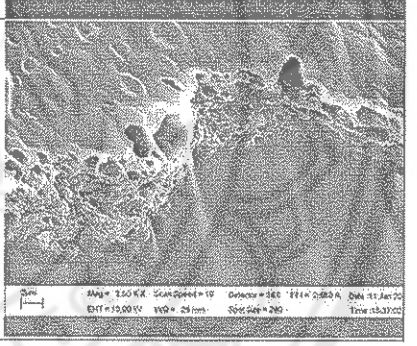
Collagen/Chitosan	Top view	Side view
0.05% GA		
0.1% GA		
0.15% GA		

Table 25 Surface morphology of collagen/shrimp chitosan MW 100,000 in ratio of 7 to 3 and crosslinked with GA (3.50 KX).

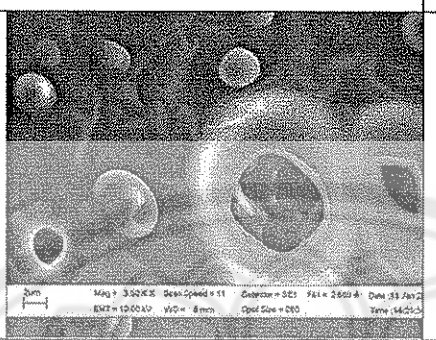
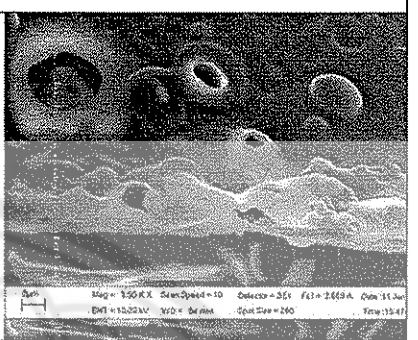
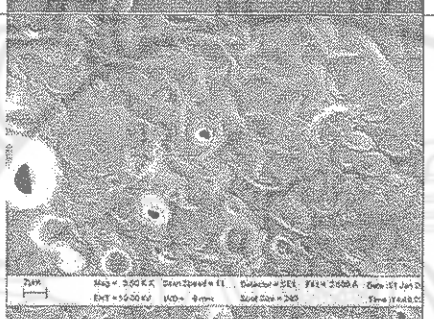
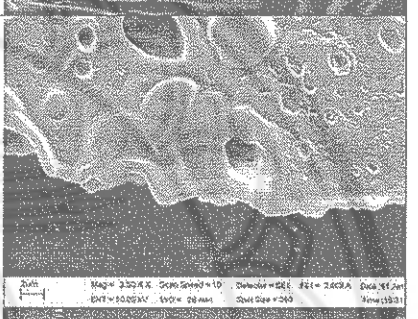
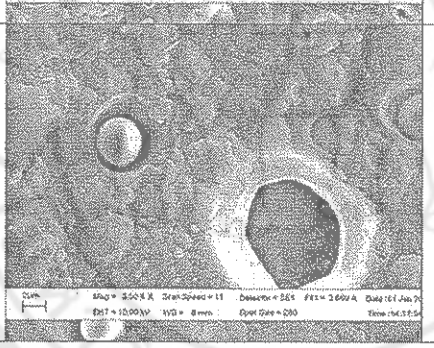
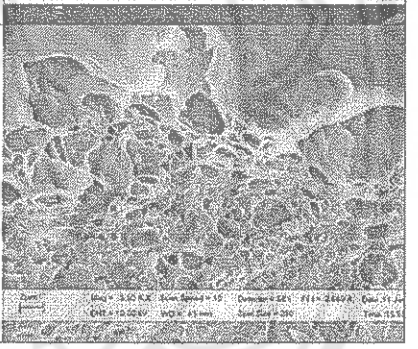
Collagen/Chitosan	Top view	Side view
0.05% GA		
0.1% GA		
0.15% GA		

Table 26 Surface morphology of collagen/crab chitosan MW 100,000 - 1,000,000 in ratio of 7 to 3 and crosslinked with GA (3.50 KX).

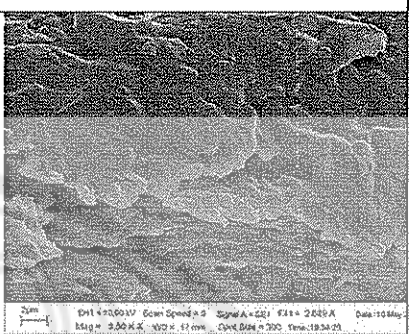
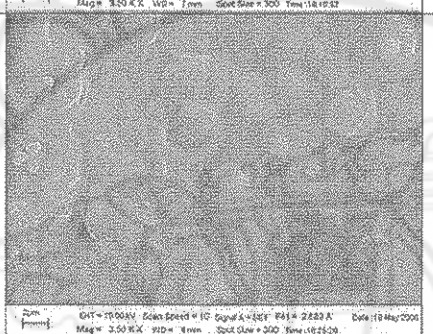
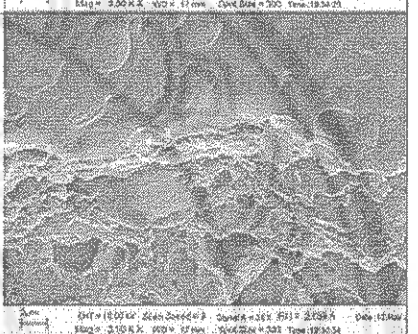
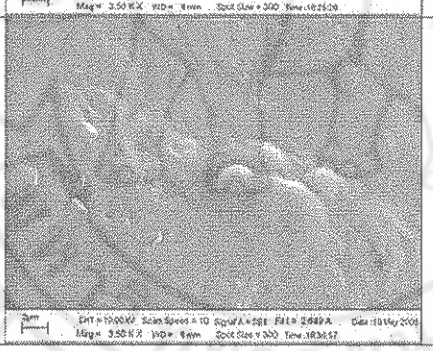
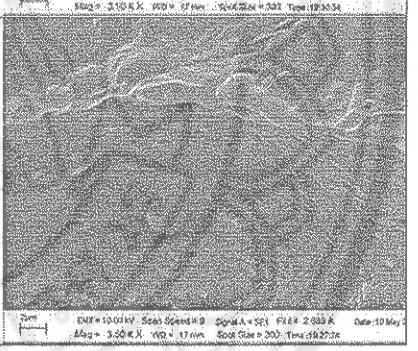
Collagen/Chitosan	Top view	Side view
0.05% GA		
0.1% GA		
0.15% GA		

Table 27 Surface morphology of collagen/shrimp chitosan MW 30,000 in ratio of 8 to 2 and crosslinked with GP (3.50 KX).

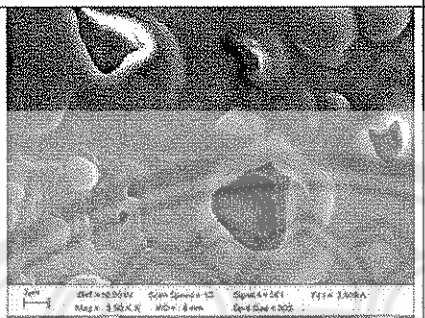
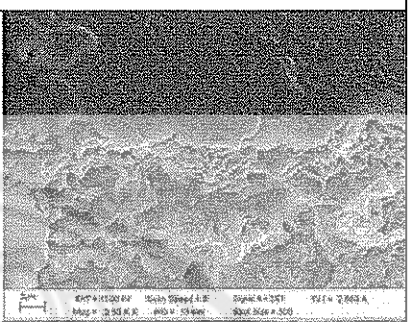
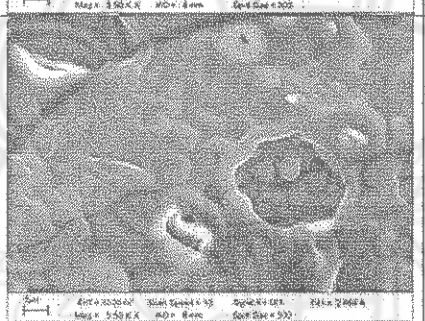
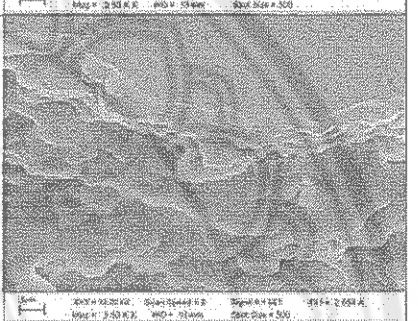
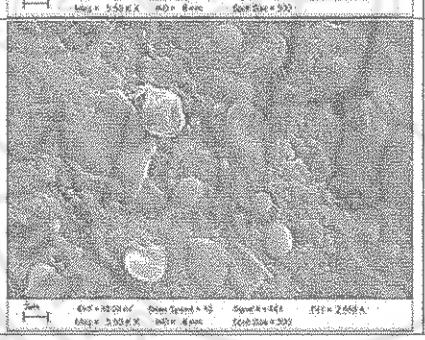
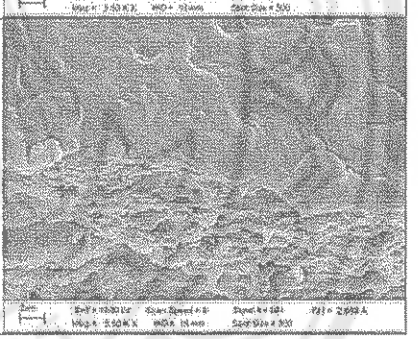
Collagen/Chitosan	Top view	Side view
0.5% GP		
1.0% GP		
1.5% GP		

Table 28 Surface morphology of the collagen/shrimp chitosan MW 100,000 in ratio of 8 to 2 and crosslinked with GP (3.50 KX).

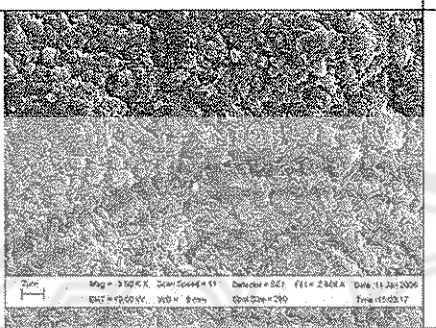
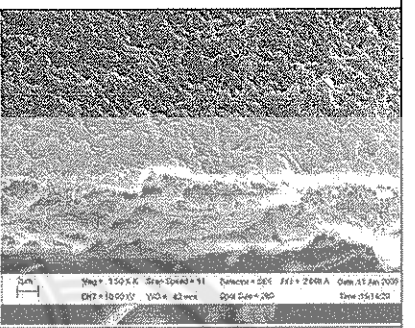
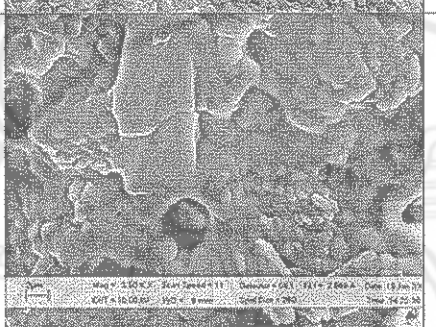
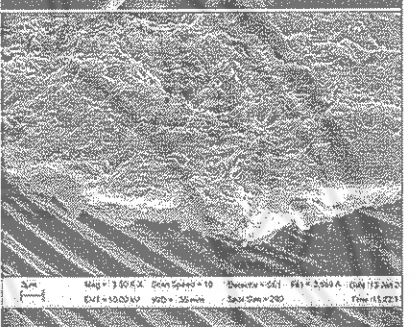
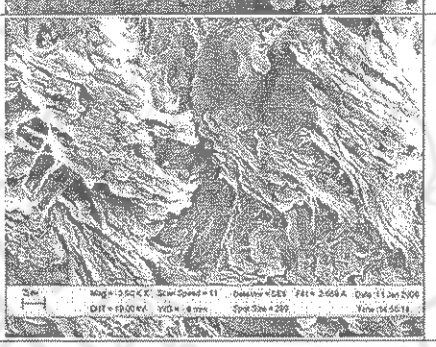
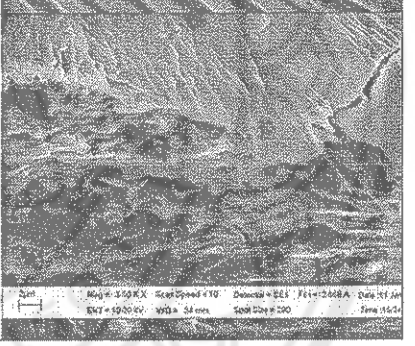
Collagen/Chitosan	Top view	Side view
0.5% GP		
1.0% GP		
1.5% GP		

Table 29 Surface morphology of the collagen/shrimp chitosan MW 100,000 in ratio of 7 to 3 and crosslinked with GP (3.50 KX).

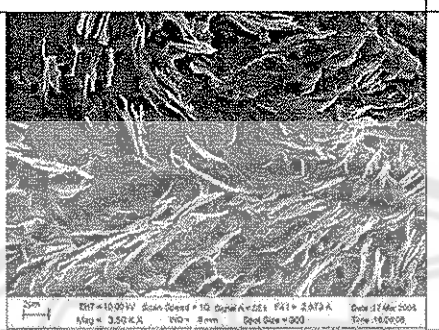
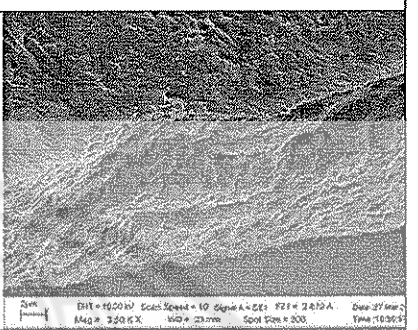
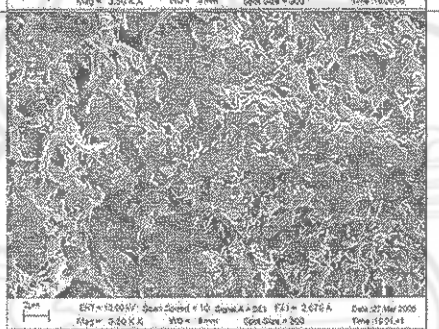
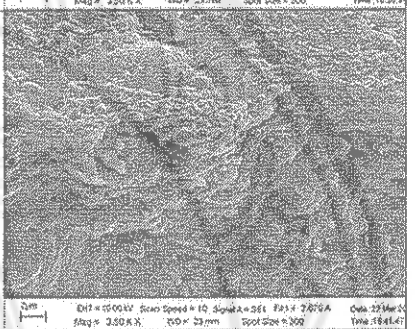
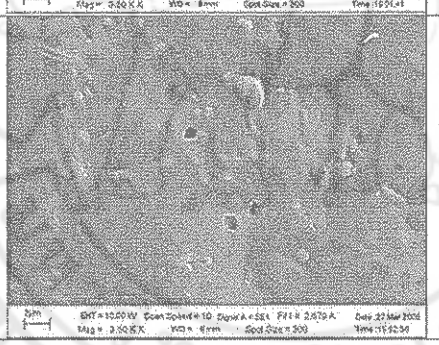
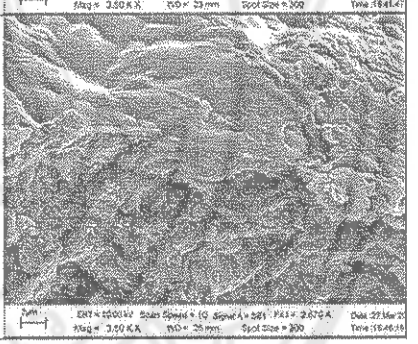
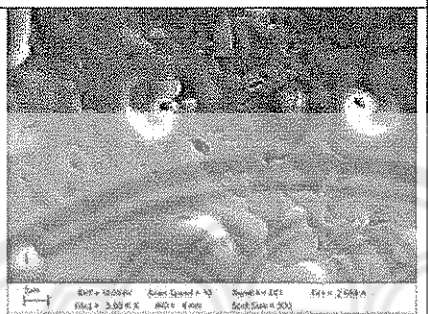
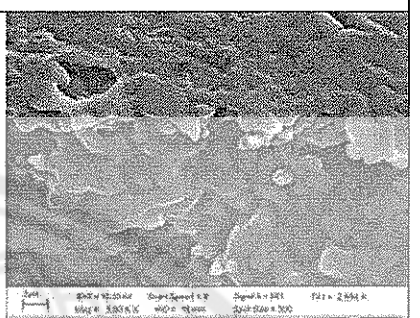
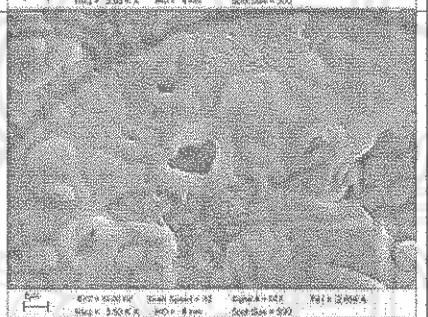
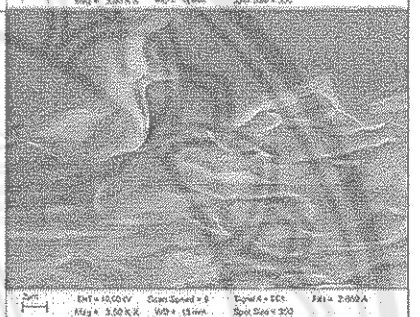
Collagen/Chitosan	Top view	Side view
0.5% GP		
1.0% GP		
1.5% GP		

Table 30 Surface morphology of the collagen/crab chitosan MW 100,000 - 1,000,000 in ratio of 7 to 3 and crosslinked with GP (3.50 KX).

Collagen/Chitosan	Top view	Side view
0.5% GP		
1.0% GP		
1.5% GP		

The Tables 23-30 show the surface morphology of the matrix crosslinked with GA or GP. After crosslinking, the porosity of matrix prepared form collagen/shrimp chitosan MW 30,000 in ratio of 8 to 2 and collagen/shirimp chitosan MW 100,000 in ratio of 8 to 2 and 7 to 3 crosslinked with GA in an amount of 0.15% by weight of total polymer were higher than those crosslinked with GA in other amounts. For collagen/crab chitosan MW 100,000 - 1,000,000 matrix, although this matrix showed the low porosity on top view, the porosity of side view tended to higher than others. Crosslinking with 0.1% GA provided the collagen/crab chitosan matrix with higher porosity than crosslinking with 0.05% and 0.15% GA.

All obtained results indicated that the lower porosity of the matrices crosslinked with GP, as comparing to those crosslinked with GA. Focusing on GP crosslinking, crosslinking of the colagen/shrimp chitosan MW 30,000 (8:2) and collagen/crab chitosan MW 100,000 - 1,000,000 (7:3) with GP in an amount of 0.5% by weight of total polymer provided the matrices with higher porosity than the other formulas. In the case of GP crosslinking, the matrix prepared from collagen/shrimp chitosan MW 100,000 provide the matrix with low porosity and sheet stack like. However, the matrix prepared from collagen/shrimp chitosan MW 100,000 in ratio of 8:2 and 7:3 crosslinked with 1.0% of GP provided higher porosities than the other formulas of the collagen/shrimp chitosan MW 100,000.

According to the porosity of the matrix, the selected formulations for further studies are as follow:

1. Collagen/Shrimp chitosan MW 30,000 (8:2) + 0.15% GA
2. Collagen/Shrimp chitosan MW 100,000 (8:2) + 0.15% GA
3. Collagen/Shrimp chitosan MW 100,000 (7:3) + 0.15% GA
4. Collagen/Crab chitosan MW 100,000 - 1,000,000 (7:3) + 0.1% GA
5. Collagen/Shrimp chitosan MW 30,000 (8:2) + 0.5% GP
6. Collagen/Shrimp chitosan MW 100,000 (8:2) + 1% GP
7. Collagen/Shrimp chitosan MW 100,000 (7:3) + 1% GP
8. Collagen/Crab chitosan MW 100,000 - 1,000,000 (7:3) + 0.5% GP

2.4.2 Physicochemical properties of prepared matrix

The strength and flexibility of the obtained matrix were determined by the tensile strength and % elongation at break using the tensometer. The thickness of the matrix was 60±10 μm. The measurment condition was similar as the previous mention. The tensile strength and % elongation of the obtained matrix are shown in Table 31-32 and Figure 19.

The tensile strength and % elongation at break of the matrix prepared from collagen/crab chitosan matrix crosslinked with GA were significantly higher than those of the other matrices. Focusing on the matrices crosslinked with GP, the matrix

prepared from collagen/shrimp chitosan MW 100,000 (8:2) exhibited the highest tensile strength and % elongation at break.

Table 31 Tensile strength and % elongation at break of collagen/chitosan matrix crosslinked with GA.

Ratio	Patch no.	Thickness (mm)				TS (kgf/mm^2)	%Elongation at break
		1	2	3	Average		
Collagen/Shrimp chitosan MW 30,000 + 0.15% GA							
8:2	1	0.07	0.07	0.07	0.07	1.074	0.310
	2	0.07	0.07	0.07	0.07	0.671	0.730
	3	0.07	0.07	0.07	0.07	-	-
	Average					0.873±0.285	0.520±0.297
Collagen/Shrimp chitosan MW 100,000 + 0.15% GA							
8:2	1	0.06	0.06	0.06	0.06	0.083	0.880
	2	0.06	0.06	0.06	0.06	0.671	0.400
	3	0.06	0.06	0.06	0.06	0.224	0.530
	Average					0.326±0.307	0.603±0.248
7:3	1	0.06	0.06	0.06	0.06	0.895	0.076
	2	0.06	0.06	0.06	0.06	0.358	0.660
	3	0.06	0.06	0.06	0.06	0.224	0.330
	Average					0.492±0.355	0.355±0.293
Collagen/Crab chitosan MW 100,000 - 1,000,000 + 0.5% GA							
7:3	1	0.06	0.06	0.06	0.06	8.456	2.1
	2	0.06	0.06	0.06	0.06	8.38	3.04
	3	0.06	0.06	0.06	0.06	8.632	2
	Average					8.489±0.129	2.38±0.573

Table 32 Tensile strength and %elongation at break of the collagen/chitosan matrix crosslinked with GP.

Ratio	Patch no.	Thickness (mm)				TS (kgf/mm^2)	%Elongation at break
		1	2	3	Average		
Collagen/Shrimp chitosan MW 30,000 + 0.5% GP							
8:2	1	0.06	0.06	0.06	0.06	0.877	0.207
	2	0.06	0.06	0.06	0.06	0.7495	0.137
	3	0.06	0.06	0.06	0.06	0.774	0.128
	Average					0.800±0.068	0.157±0.043
Collagen/Shrimp chitosan MW 100,000 + 1% GP							
8:2	1	0.06	0.06	0.06	0.06	0.895	0.7
	2	0.06	0.06	0.06	0.06	0.447	0.54
	3	0.06	0.06	0.06	0.06	0.5817	0.54
	Average					0.641±0.229	0.59±0.092
7:3	1	0.06	0.06	0.06	0.06	1.119	1.09
	2	0.06	0.06	0.06	0.06	0.8053	0.06
	3	0.06	0.06	0.06	0.06	3.132	0.95
	Average					1.685±1.263	0.7±0.559
Collagen/Crab chitosan MW 100,000 - 1,000,000 + 0.5% GP							
7:3	1	0.06	0.06	0.06	0.06	0.7718	0.136
	2	0.06	0.06	0.06	0.06	0.7427	0.199
	3	0.06	0.06	0.06	0.06	0.7495	0.1652
	Average					0.755±0.015	0.167±0.032

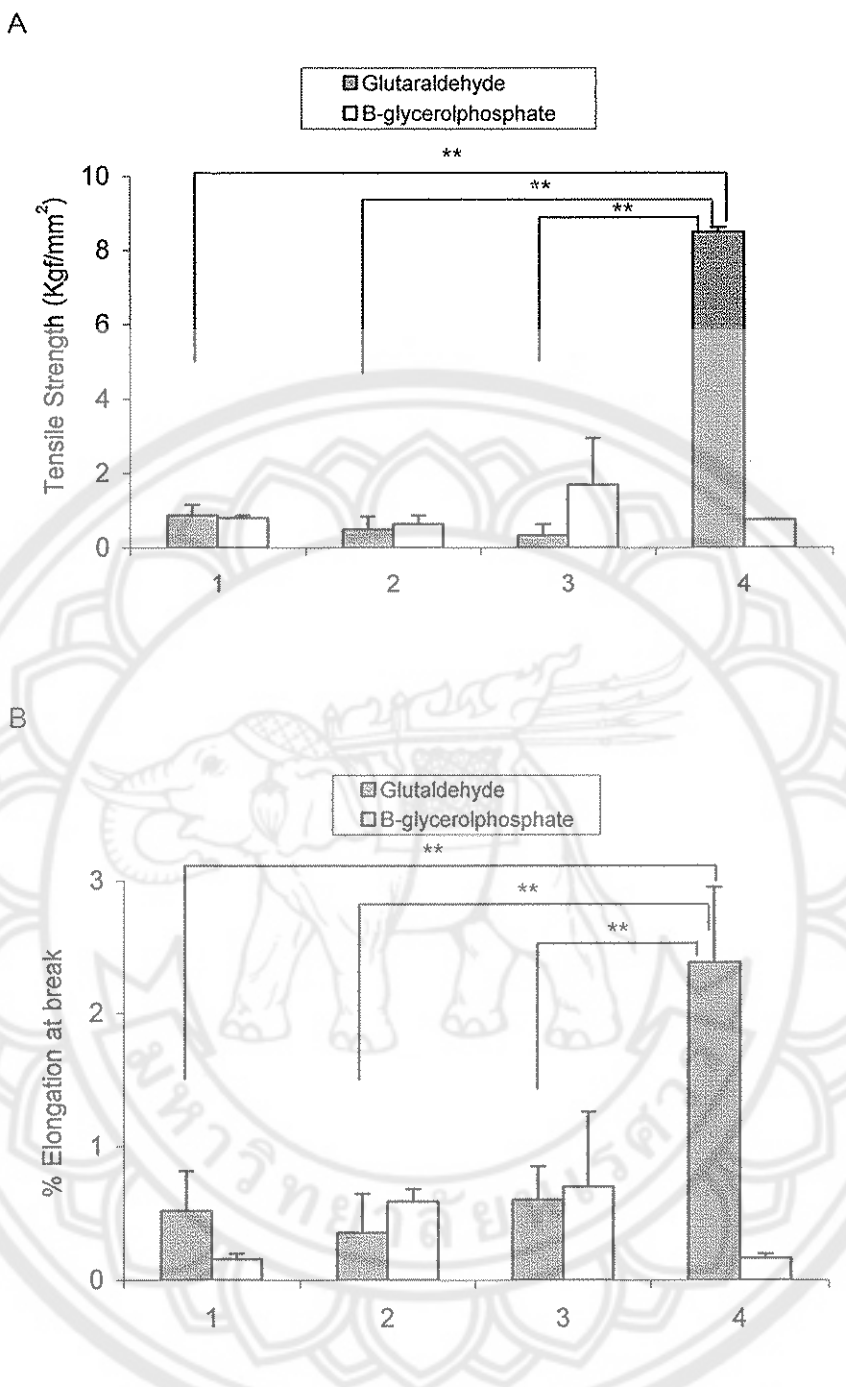


Figure19 Mechanical characteristics of the collagen/chitosan matrices: tensile strength (A) and %elongation at break (B). Number of 1, 2, 3 and 4 on x axis represent the matrix prepared from collagen/shrimp chitosan MW 30,000 (8:2), collagen/shrimp chitosan MW 100,000 (8:2), collagen/shrimp chitosan MW 100,000 (7:3), collagen/crab chitosan MW 100,000 - 1,000,000 (7:3), respectively. (** = $p < 0.01$)

2.5 Preparation of collagen/PVA matrix

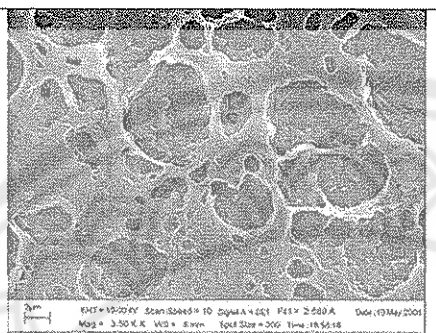
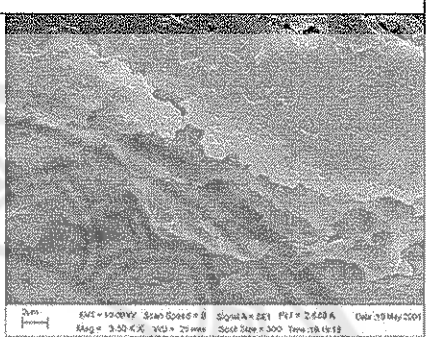
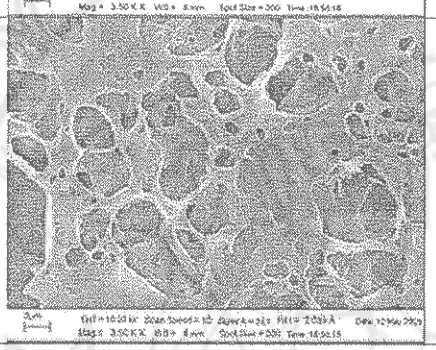
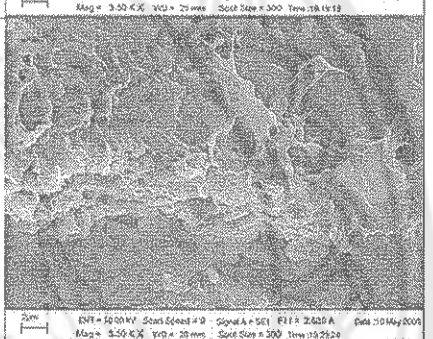
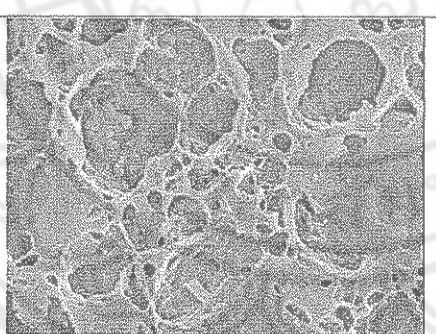
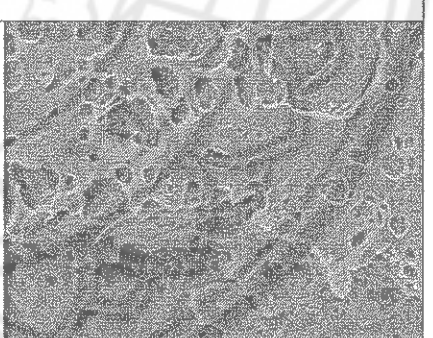
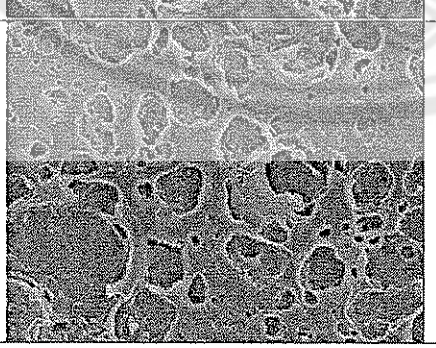
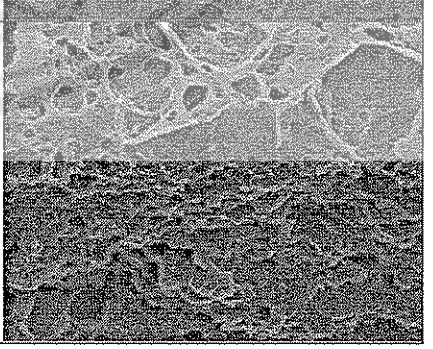
2.5.1 Physical characteristics of collagen/PVA matrix

When collagen was blended with PVA, it was found that the strength and the flexibility obviously improved. Therefore the crosslinking agent was not necessary to used. Increasing PVA in the formulation could increase the flexibility of the collagen matrix. On other hand, when the amount of PVA was high, the flexibility was also high. However, the difference in MW of the PVA had no effect on the physical properties and flexibility of the collagen/PVA matrix. The examples of collagen/PVA matrices are shown in appendix F. The physical charateristics of the collagen/PVA matrix are shown in Table 33-34. All of the matrices prepared from collagen/PVA provided the matrix with high porosity. The ratio of 8 to 2 collagen and PVA matrix with the both MW (72,000 and 145,000) were selected for further studies. The matrices obtained from the blending collagen to PVA (MW 72,000 and 145,000) in ratio of 8 to 2 were selected for further study, according to their high flexibility.

Table 33 Physical characteristics of collagen/PVA matrix.

Formulation	Color	Transparency	Homogenity	Flexibility
Collagen/PVA MW 72,000 (0.5% w/v of total polymer)				
9:1	Yellowish brown	+	+++	++
8:2	Yellowish brown	+	++++	++++
Collagen/PVA MW 145,000 (0.5% w/v of total polymer)				
9:1	Yellowish brown	+	+++	++
8:2	Yellowish brown	+	++++	+++

Table 34 Surface morphology of collagen/PVA at the concentration of 0.5% by weight of total polymer.

Ratio of Collagen/PVA	Top view	Side view
MW 72,000		
9:1		
8:2		
MW 145,000		
9:1		
8:2		

2.5.2 Physicochemical properties of colalgen/PVA matrix

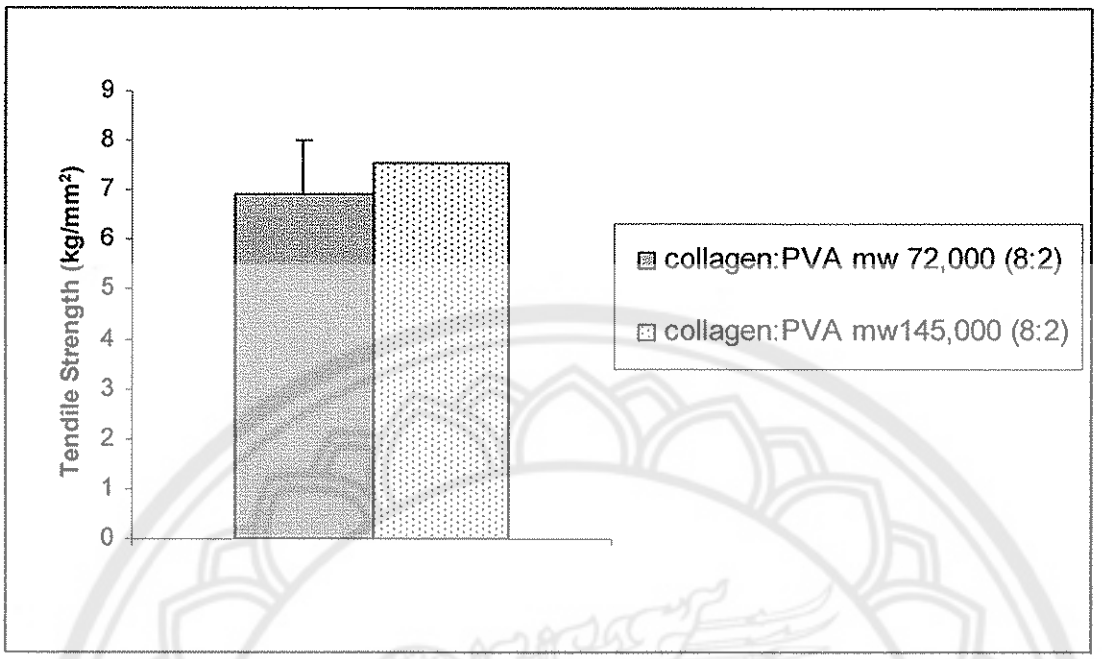
The thickness of the collagen/PVA matrices was 60±20 μm. The experiment condition was similar to the previous mention. Table 35 and Figure 20 show the tensile strength and % elongation of the collagen/PVA matrix.

At the same ratios of collagen/PVA, the molecular weight of PVA had affected the tensile strength and % elongation of the collagen/PVA. In other words, PVA with higher molecular weight (145,000) provided the matrix with higher strength than the lower molecular weight (72,000). However, such difference was not significant.

Table 35 Tensile strength and % elongation at break of collagen/PVA matrix.

Ratio	Patch no.	Thickness (mm)				TS (kgf/mm^2)	%Elongation at break
		1	2	3	Average		
Collagen/PVA MW 72,000							
8:2	1	0.08	0.08	0.08	0.08	5.688	1.96
	2	0.06	0.06	0.06	0.06	7.495	1.76
	3	0.06	0.06	0.06	0.06	7.583	1.90
	Average					6.922±1.070	1.873±0.103
Collagen/PVA MW 145,000							
8:2	1	0.06	0.06	0.06	0.06	7.562	2.00
	2	0.06	0.06	0.06	0.06	7.538	1.91
	3	0.06	0.06	0.06	0.06	7.538	1.96
	Average					7.546±0.014	1.957±0.045

A



B

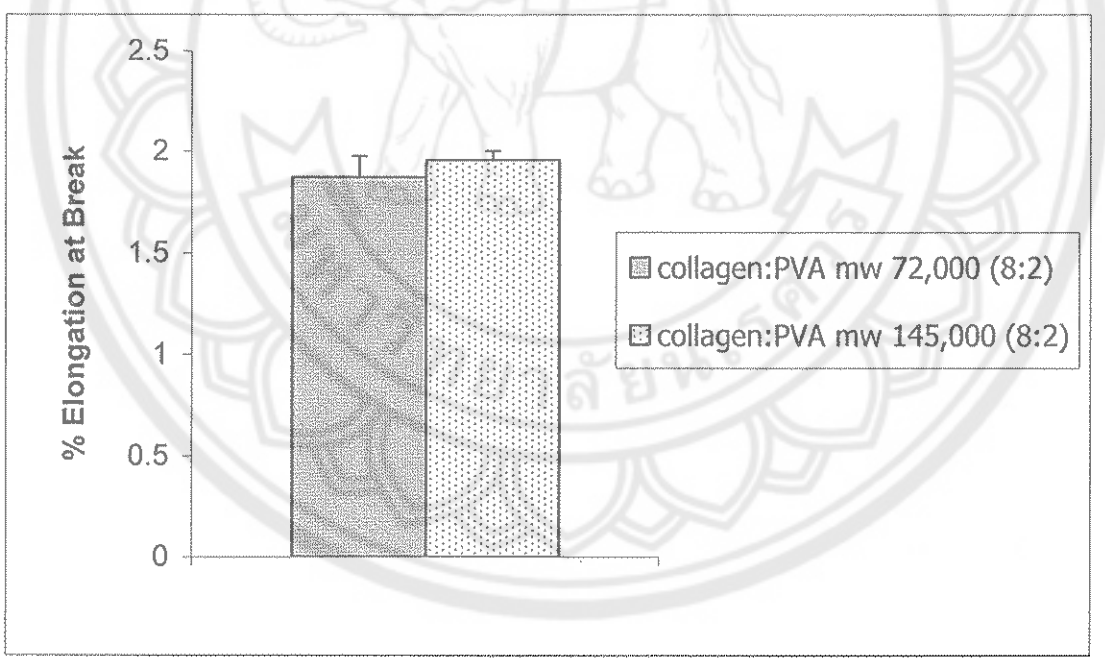


Figure 20 Mechanical properties of the collagen/PVA matrix: tensile strength (A) and % elongation at break (B).

2.6 Swelling and biodegradation properties of matrix

The following matrices were investigated for swelling and biodegradation properties.

1. Collagen/Shrimp MW 30,000 chitosan 8:2 (1% w/v of total polymer) + 0.15% GA
2. Collagen/Shrimp MW 100,000 chitosan 8:2 (0.5% w/v of total polymer) + 0.15% GA
3. Collagen/Shrimp MW 100,000 chitosan 7:3 (0.5% w/v of total polymer) + 0.15% GA
4. Collagen/Crab MW 100,000 - 1,000,000 chitosan 7:3 (3% w/v of total polymer) + 0.1% GA
5. Collagen/Shrimp chitosan MW 30,000 8:2 (1% w/v of total polymer) + 0.5% GP
6. Collagen/Shrimp chitosan MW 100,000 8:2 (0.5% w/v of total polymer) + 1% GP
7. Collagen/Shrimp chitosan MW 100,000 7:3 (0.5% w/v of total polymer) + 1% GP
8. Collagen/Crab chitosan MW 100,000 - 1,000,000 7:3 (3% w/v of total polymer) + 0.5% GP
9. Collagen/PVA MW 72,000 (8:2)
10. Colagen/PVA MW 145,000 (8:2)

The results of the swelling property are shown in Table 36 and Figure 21 and 22.

Table 36 Swelling property of matrix.

Formulation	Size (mm^2)				
	0 hr	1 hr	2 hr	3 hr	4 hr
1. Collagen/Shrimp	7.071	9.412	9.412	9.412	-
MW 30,000 (8:2)	12.375	18.014	18.014	18.014	-
+ 0.15% GA	10.607	16.844	18.329	18.329	-
Average	10.02±2.7	14.76±4.7	15.25±5.1	15.25±5.1	
2. Collagen/Shrimp	8.839	12.867	12.867	-	-
MW 100,000 (8:2)	8.839	12.867	12.867	-	-
+ 0.15% GA	8.839	15.274	15.274	-	-
Average	8.84±0	13.67±1.4	13.67±1.4		
3. Collagen/Shrimp	15.911	21.177	21.177	21.177	
MW 100,000 (7:3)	12.375	15.019	18.014	18.014	
+ 0.15% GA	14.143	17.164	18.824	18.824	
Average	14.14±1.8	17.79±3.1	19.34±1.6	19.34±1.6	
4. Collagen/Crab MW	15.911	27.494	27.494	-	-
100,000 - 1,000,000	15.400	27.573	27.573	-	-
(7:3) + 0.1% GA	15.400	27.573	27.573	-	-
Average	15.57±0.3	27.55±0.1	27.55±0.1		
5. Collagen/shrimp	11.250	11.250	11.250	12.800	11.625
MW 30,000 (8:2)	11.250	12.013	12.013	12.013	12.800
+ 0.5% GP	11.250	12.013	12.013	12.013	12.013
Average	11.25±0	11.76±0.4	11.76±0.4	12.28±0.4	12.15±0.6
6. Collagen/Shrimp	9.000	9.000	9.610	10.560	10.560
MW 100,000 (8:2)	9.000	9.000	9.610	10.240	10.240
+ 1% GP	9.000	9.300	9.920	10.560	10.560
Average	9.00±0	9.10±0.2	9.71±0.2	10.45±0.2	10.45±0.2

Table 36 (cont.).

Formulation	Size (mm^2)				
	0 hr	1 hr	2 hr	3 hr	4 hr
7. Collagen/Shrimp MW 100,000 (7:3) + 1% GP	9.000	9.300	9.920	10.240	10.240
	9.000	9.000	9.610	10.560	10.560
	9.000	9.300	9.920	10.240	10.240
Average	9.00±0	9.20±0.2	9.82±0.2	10.35±0.2	10.35±0.2
8. Collagen/Crab MW 100,000 - 1,000,000 (7:3) + 0.5% GP	11.250	12.400	12.400	11.625	11.625
	11.250	12.013	12.013	12.800	12.800
	11.250	11.625	11.625	12.013	12.013
Average	11.25±0	12.01±0.4	12.01±0.4	12.15±0.6	12.15±0.6
9. Collagen/PVA MW 72,000 (8:2)	7.071	12.219	rupture	-	-
	7.071	12.219	rupture	-	-
	7.071	12.219	rupture	-	-
Average	7.071±0	12.219±0	-		
10. Collagen/PVA MW 145,000 (8:2)	12.375	25.150	rupture	-	-
	10.607	21.557	rupture	-	-
	12.375	25.150	27.188	29.333	29.333
Average	11.79±1.0	23.95±2.0	27.188	29.333	29.333
11. Collagen/Shrimp MW 100,000 (8:2)	10.607	rupture	-	-	-
	10.607	rupture	-	-	-
	10.607	rupture	-	-	-
Average	10.607±0				
12. Collagen/Shrimp MW 100,000 (7:3)	12.375	rupture	-	-	-
	12.375	rupture	-	-	-
	12.375	rupture	-	-	-
Average	12.375±0				

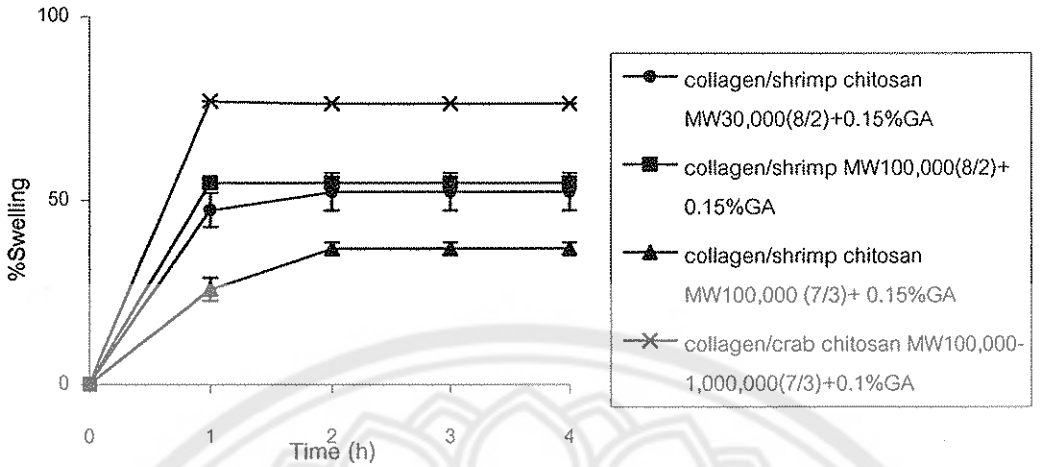


Figure 21 Percent swelling of the matrices crosslinked with GA in PBS (pH 7.4).

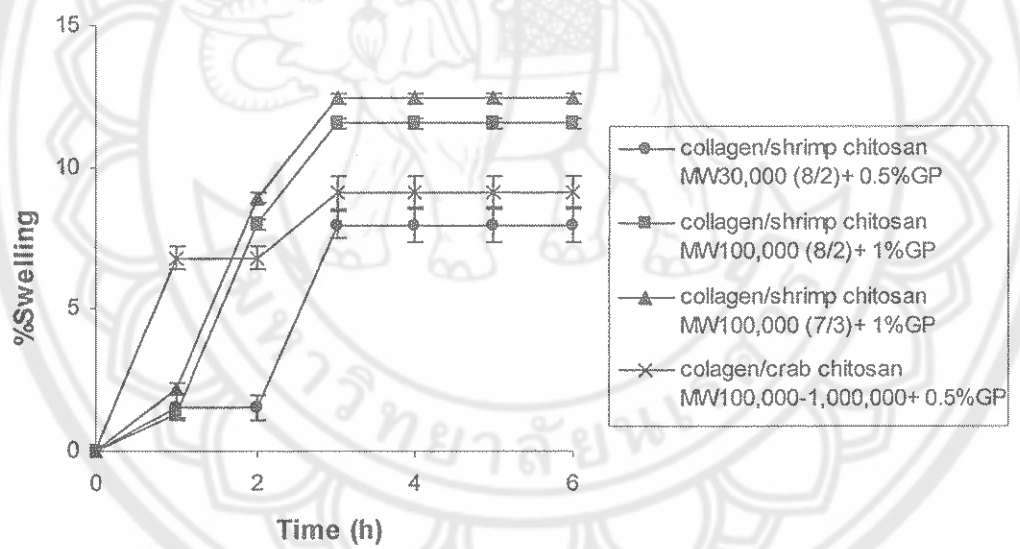


Figure 22 Percent swelling of the matrices crosslinked with GP in PBS (pH 7.4).

The percent swelling of collagen/chitosan matrix crosslinked with GA was in the range of 36-76%, and the time of equilibrium swelling was about 2-3 hr. The percent swelling of collagen/chitosan matrices crosslinked with GA was lower than that of the collagen/PVA matrices. Especially in PVA MW 145,000, the percent swelling was too high (150%) and equilibrium time was also long (4 hr). In the case of PVA MW 72,000,

the result was investigated for only one hour because this sample rapidly swelled and finally ruptured. Therefore, its percent swelling could not be evaluated. The collagen/chitosan uncrosslinked matrix could not be evaluated also because it rapidly swelled and ruptured, this indicated its weakness.

In GP crosslinking, percent of swelling was in the range of 8-13%. Regarding the same MW of chitosan, increasing an amount of collagen increased the percent of swelling of the matrix. In addition, the MW of chitosan had affected on the percent swelling. The percent swelling of the matrix preapared from the high MW of chitosan was higher than that of the low MW. However, these differences were not significant. When compared the swelling between GA and GP crosslinking, the percent swelling of GA crosslinking were significantly higher than that of the GP crosslinking.

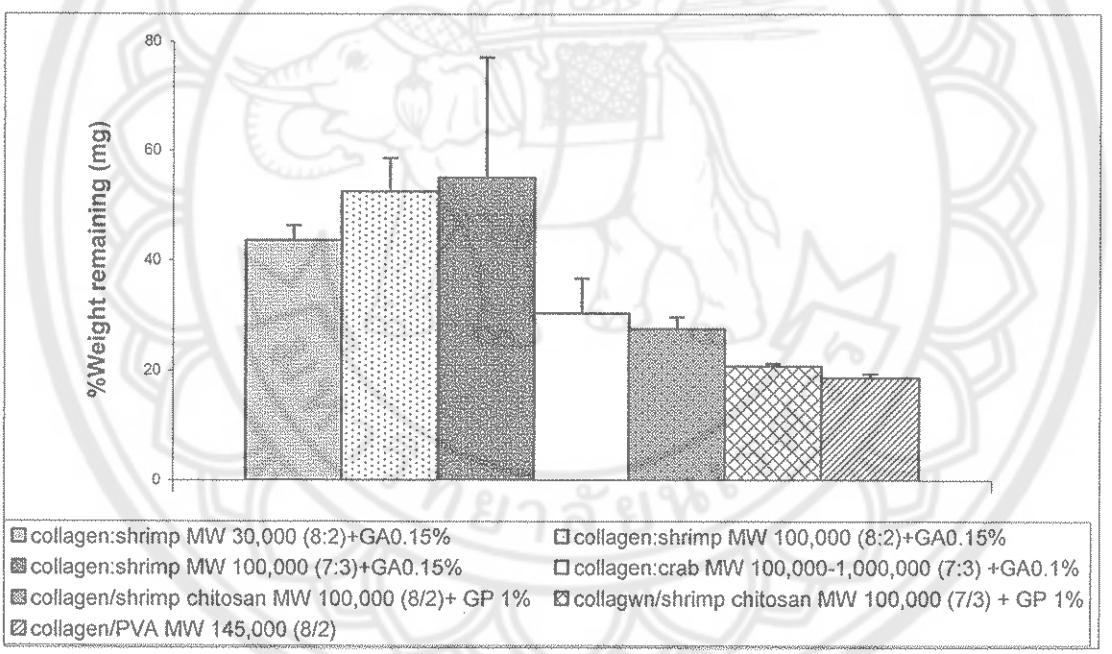


Figure 23 Percent remaining weight of the matrix after degraded with collagenase for 1 month (Appendix B).

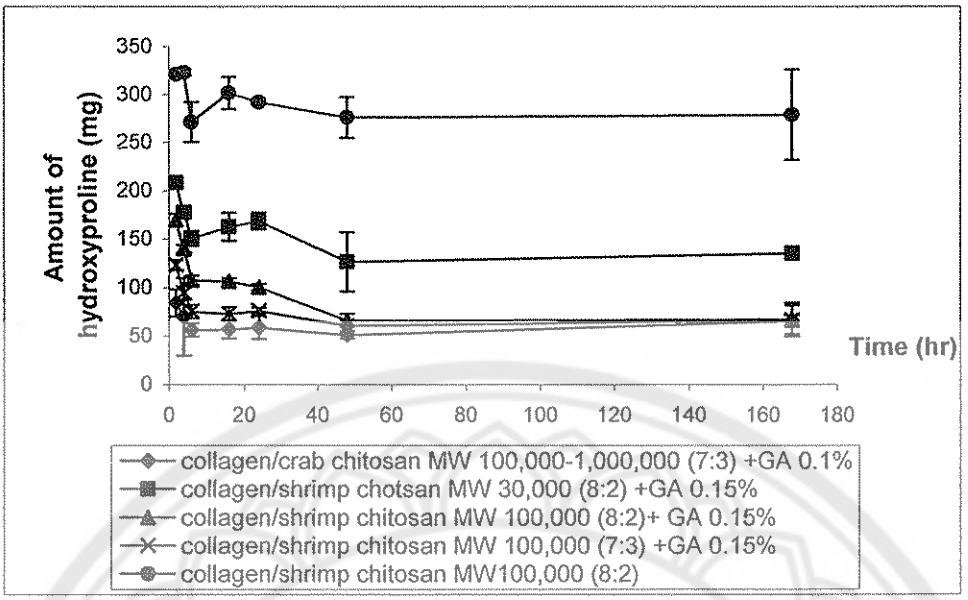


Figure 24 Amount of hydroxyproline released from the collagen/chitosan matrices crosslinked with GA after degraded by collagenase for 1 week (Appendix C).

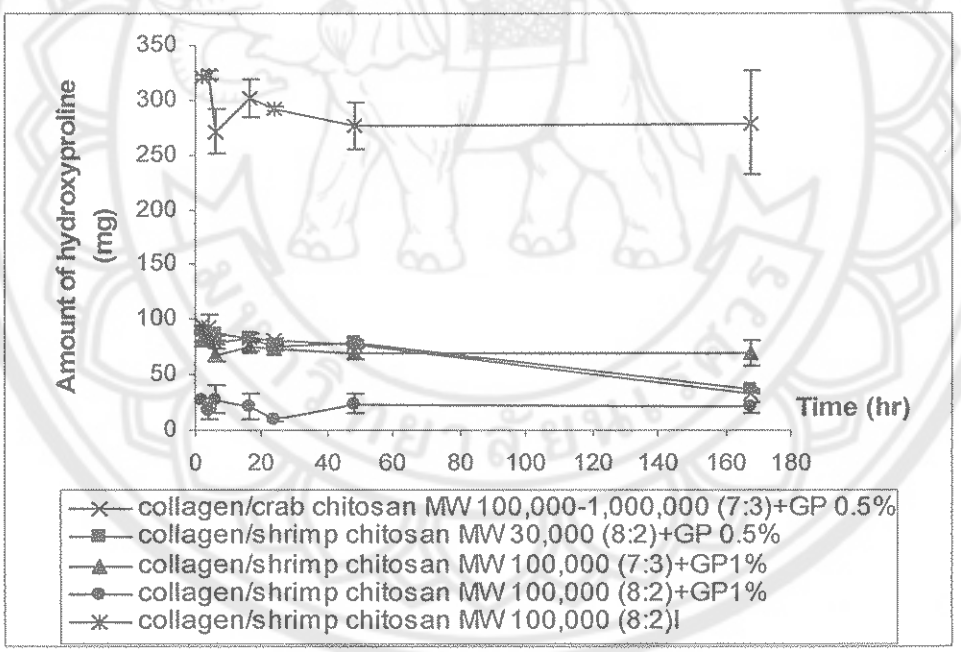


Figure 25 Amount of hydroxyproline released from the collagen/chitosan matrices crosslinked with GP after degraded by collagenase for 1 week (Appendix C).

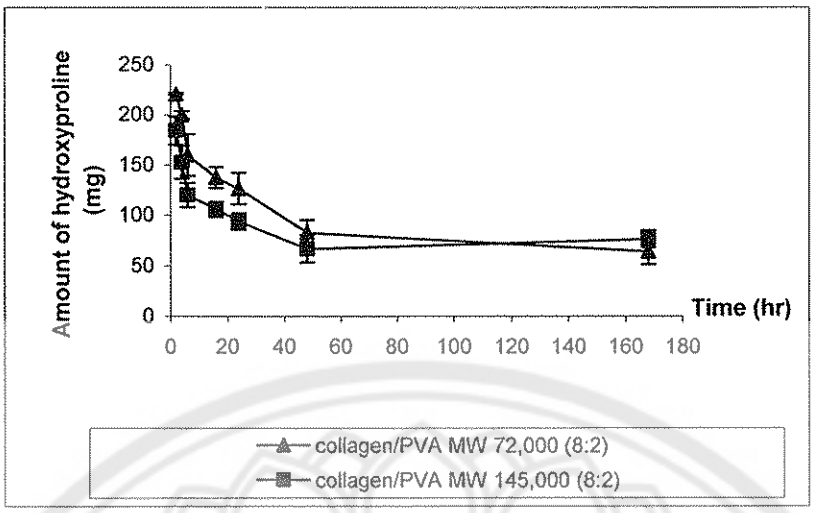


Figure 26 Amount of hydroxyproline release from the collagen/PVA matrices after degraded by collagenase for 1 week (Appendix C).

Figure 23 shows percent of remaining weight of the matrices after degraded by collagenase. During 1-month degradation period, the matrices crosslinked with GA were degraded more slowly than the uncrosslinked matrices. It was also found that when the molecullar weight of the chitosan was increased, the percent remaining weight tended to increase. Moreover, increasing the amount of chitosan could decrease the degradation by collagenase. Almost matrices crosslinked with GP was completely degraded by collagenase after 1 month except the matrix prepared from collagen/shrimp chitosan MW 100,000 (8:2 and 7:3), which remained at 28 and 21%, respectively. In the case of PVA, the PVA with MW 145,000 provided the matrix with slower degradation than that with MW 72,000.

When the matrix was degraded by collagenase, the hydroxyproline (the major component of collagen molecule) was released. Therefore, the hydroxyproline released was measured to confirm the result of the degradation of the matrix (Figure 24-26). The result of hydroxyproline released of the matrix correlated to the result of the remaining weight. Crosslinking with GA and GP decreased the release of the hydroxyproline compared with the uncrosslinked matrix. For the collagen/PVA matrix, the PVA with high MW (145,000) released the hydroxyproline slower than the low MW (72,000).

2.7 Cytotoxicity test

2.7.1 Cytocompatibility test

Since the matrix composed of MW 72,000 showed rapidly rupture after immesed in the PBS, it was not selected in this study. Therefore, the matrices used in the cytocompatibility test are as follow:

1) Collagen/Shrimp MW 30,000 chitosan (8:2) + 0.15% GA
2) Collagen/Shrimp MW 100,000 chitosan (8:2) + 0.15% GA
3) Collagen/Shrimp MW 100,000 chitosan (7:3) + 0.15% GA
4) Collagen/Crab MW 100,000 - 1,000,000 (7:3)+ 0.1% GA
5) Collagen/Shrimp chitosan MW 30,000 (8:2) + 0.5% GP
6) Collagen/Shrimp chitosan MW 100,000 (8:2) + 1% GP
7) Collagen/Shrimp chitosan MW 100,000 (7:3) + 1% GP
8) Collagen/Crab chitosan MW 100,000 - 1,000,000 (7:3) + 0.5% GP
9) Colagen/PVA MW 145,000 (8:2)

Table 37 shows the photographs of HaCaT cells observed through the light microscpoe at 5 days after deposition of the matrix on the confluent cells. All of the test matrices no cytotoxicity towards cutaneous cells could be observed *in vitro*. After a prolonged direct contact of the matrices with HaCaT cells, neither cell death nor growth disorder was observed.

Table 37 Cell morphology of HaCaT after 5 days of matrix deposition taken by a light microscopy at 20x.

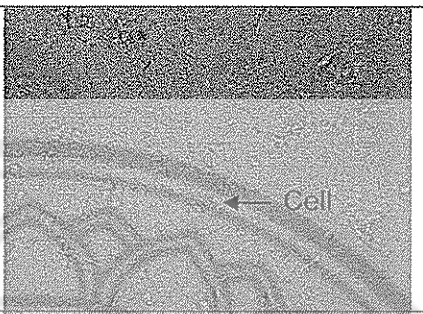
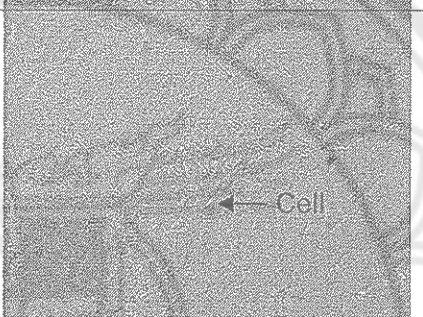
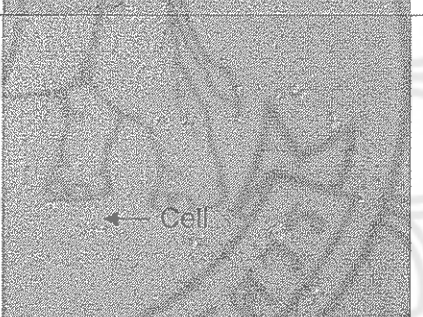
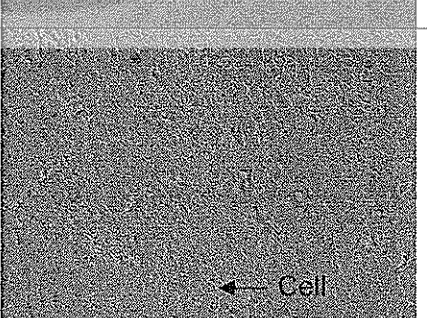
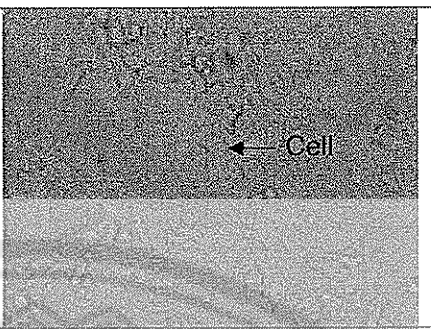
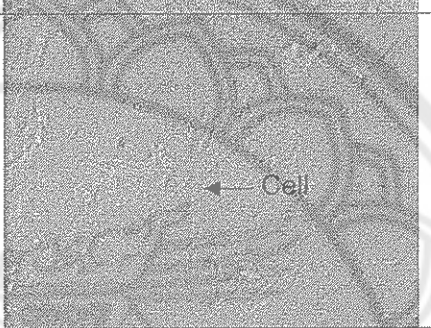
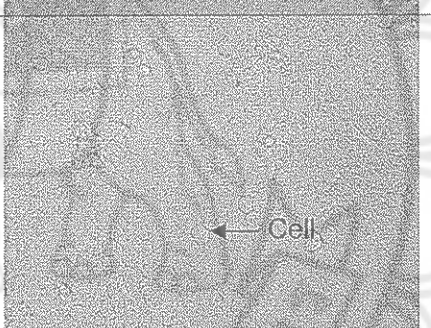
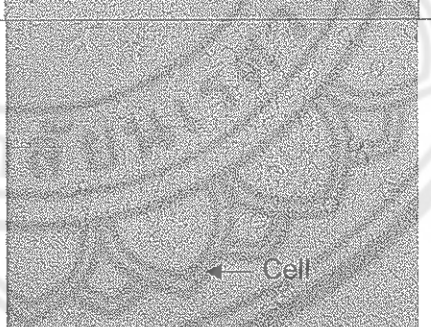
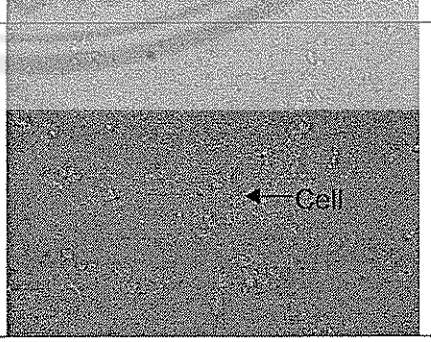
Matrix	Morphology
Control (without the matrix deposition)	
Collagen/Shrimp chitosan MW 30,000 (8:2) + 0.15% GA	
Collagen/Shrimp chitsan MW 100,000 (8:2) + 0.15% GA	
Collagen/Shrimp Chitosan MW 100,000 (7:3) + 0.15% GA	
Collagen/Crab chitosan MW 100,000-1,000,000 (7:3) + 0.15% GA	

Table 37 (cont.).

Matrix	Morphology
Collagen/Shrimp chitosan MW 100,000 (8:2) + 1% GP	
Collagen/Shrimp chitosan MW 100,000 (7:3) + 1% GP	
Collagen/Shrimp chitosan MW 30,000 (8:2) + 1% GP	
Collagen/Crab chitosan MW 100,000-1,000,000 (7:3) + 0.15% GA	
Collagen/PVA 145,000	

2.7.2 Cell adhesion test

Table 38 and Figure 27 show the result of the XTT assay carried out 3 hours after HaCaT cells had seeded on each matrix. The data are present as the percent of cell adhesion on the various matrices. The amount of viable cells adhesion on the plastic plate was adjusted as 100%. The matrices which showed the percent adhesion more than 70% were collagen/shrimp MW 30,000 (8:2) chitosan + 0.15% GA, collagen/crab MW 100,000 - 1,000,000 (7:3) + 0.1% GA and collagen/PVA MW 145,000 (8:2). Moreover, the highest percent cell adhesion was obtained from the matrix prepared from collagen/shrimp MW 30,000 (8:2) chitosan + 0.15% GA. The other matrices exhibited the percent adhesion of less than 50%.

Table 38 Percent of adhesion of HaCaT on the matrix after 3 hr culture.

Matrix	% Adhesion ± SD
Control	100.00
1. Collagen/Shrimp MW 30,000 (8:2) + 0.15% GA	92.54±0.068
2. Collagen/Shrimp MW 100,000 (8:2) + 0.15% GA	69.119±0.124
3. Collagen/Shrimp MW 100,000 (7:3) + 0.15% GA	39.055±0.071
4. Collagen/Crab MW 100,000 - 1,000,000 (7:3) + 0.1% GA	77.62±0.043
5. Collagen/Shrimp MW 30,000 (8:2) + 0.15% GP	34.24±0.199
6. Collagen/Shrimp MW 100,000 (8:2) + 0.15% GP	20.62±0.068
7. Collagen/Shrimp MW 100,000 (7:3) + 0.15% GP	27.61±0.081
8. Collagen/Crab MW 100,000 - 1,000,000 (7:3) + 0.1% GP	41.76±0.100
9. Collagen/PVA MW 145,000 (8:2)	73.43±0.032

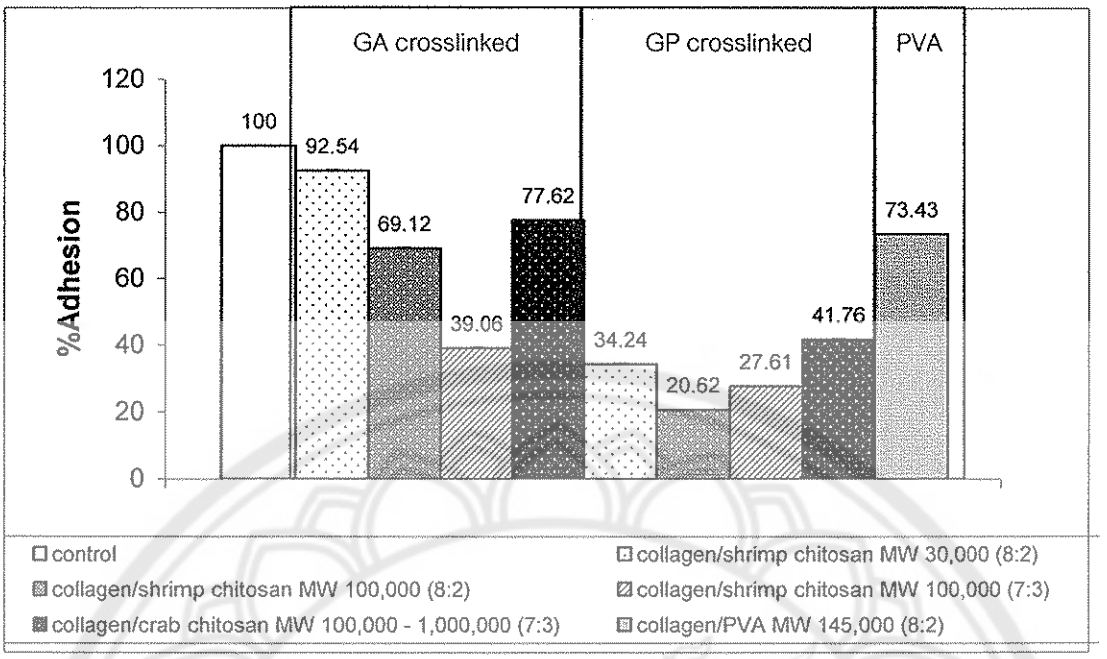


Figure 27 Percent cell adhesion on the matrix compared with that on plastic plate after 3 hr cuture.

2.7.3 Cell proliferation test

Table 39 shows light microscopy photographs of HaCaT cells on the matrices after 5 days of cultivation as well as control culture on the plastic plate. On day 5, the XTT test was performed to quantify the cell viability. The result of percent proliferation is shown in Table 40 and Figure 28. From these results, it was found that the matrix from collagen/crab chitosan MW 100,000 - 1,000,000 (7:3) + 0.1% GA allowed cells proliferation better than the other matrices and the plastic plate. On the contrary, the cell proliferation on the matrix from collagen/PVA MW 145,000 (8:2) decreased when compared with the cell proliferation on the plastic plate.

Table 39 Cell proliferation on the matrix after 5 days culture taken by a light microscope at 20x.

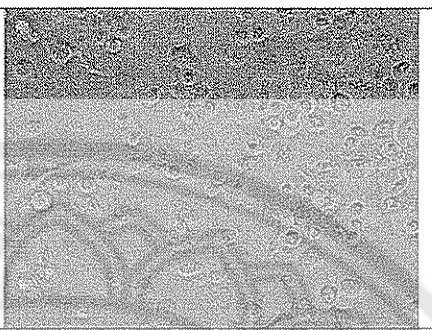
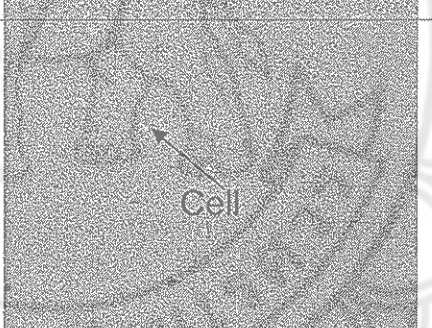
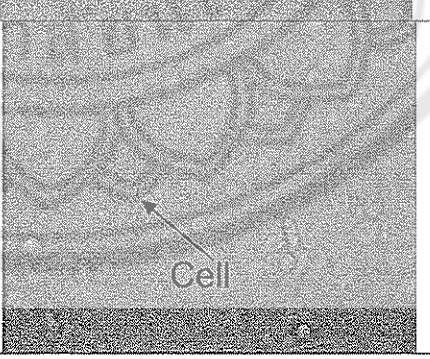
Matrix	Day 5
HaCaT on plastic plate	
Matrix without cell	
Collagen/Shrimp chitosan MW 30,000 (8:2) + 0.15% GA	
Collagen/Shrimp chitosan MW 100,000 (8:2) + 0.15% GA	

Table 39 (cont.).

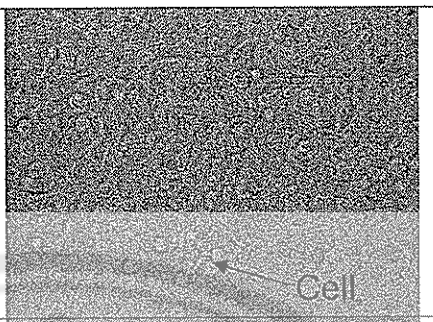
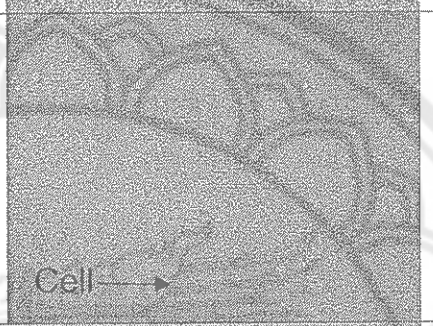
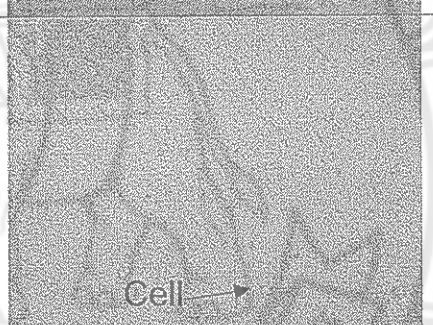
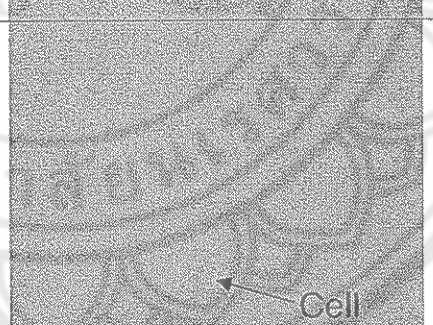
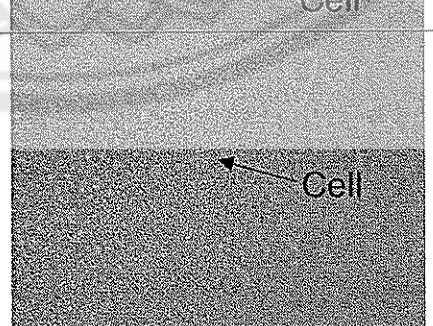
Matrix	Day 5
Collagen/Shrimp chitosan MW 100,000 (7:3) + 0.15% GA	
Collagen/Crab chitosan MW 100,000 - 1,000,000 (7:3) + 0.1% GA	
Collagen/Shrimp chitosan MW 30,000 (8:2) + 0.5% GP	
Collagen/Shrimp chitosan MW 100,000 (8:2) + 1% GP	
Collagen/Shrimp chitosan MW 100,000 (7:3) + 1% GP	

Table 39 (cont.)

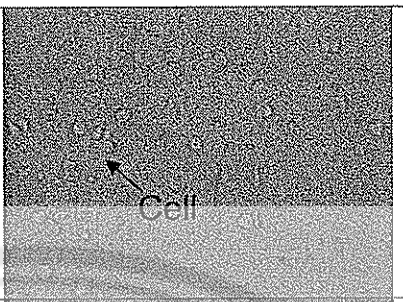
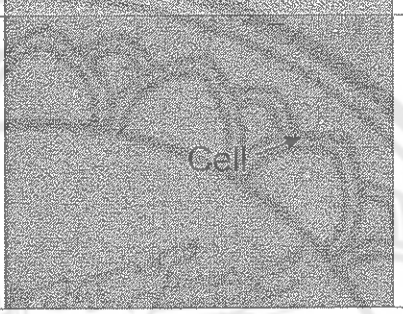
Matrix	Day 5
Collagen/Crab chitosan MW 100,000 - 1,000,000 (7:3) + 0.5% GP	
Collagen/PVA MW 145,000	

Table 40 Percent proliferation of HaCaT cell on the matrix after 5 days culture.

Matrix	% Proliferation ±SD
Control	100.00
1. Collagen/Shrimp chitosan MW 30,000 (8:2) + 0.15% GA	86.63±0.154
2. Collagen/Shrimp chitosan MW 100,000 (8:2) + 0.15% GA	89.61±0.104
3. Collagen/Shrimp chitosan MW 100,000 (7:3) + 0.15% GA	88.07±0.038
4. Collagen/Crab chitosan MW 100,000 - 1,000,000 (7:3) + 0.1% GA	133.36±0.260
5. Collagen/Shrimp chitosan MW 30,000 (8:2) + 0.5% GP	48.03±0.180
6. Collagen/Shrimp chitosan MW 100,000 (8:2) + 1% GP	57.72±0.024
7. Collagen/Shrimp chitosan MW 100,000 (7:3) + 1% GP	36.51±0.074
8. Collagen/Crab chitosan MW 100,000 - 1,000,000 (7:3) + 0.5% GP	78.07±0.168
9. Collagen/PVA mw 145,000 (8:2)	48.87±0.153

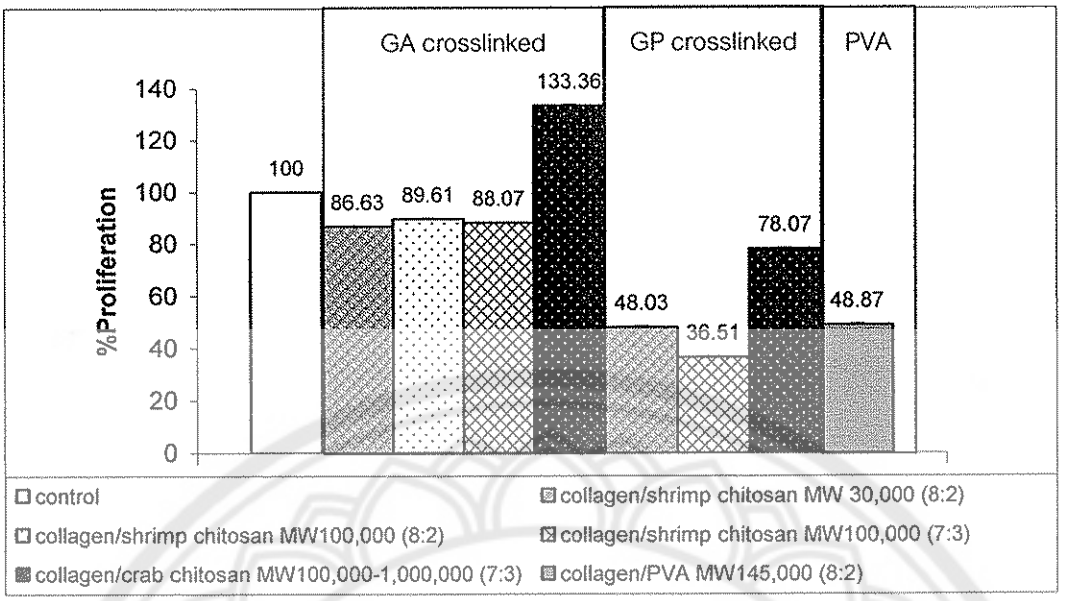


Figure 28 Percent of cell proliferation on the matrix compared with that on plastic plate after 5 days cuture.

2.7.4 Morphology of cell adhesion on the matrix

After cultivation, SEM of HaCaT proliferation on the matrix prepared from collagen/crab chitosn MW 100,000 - 1,000,000 (7:3) crosslinked with 0.1% GA (Figure 29) depicted their morphology. Because the pore size of the matrix was small (less than 15μm), cells were stand on the surface (Figure 29A) and side (Figure 29C) of matrix instead of getting into the pore. Most of the HaCaT cells grew and proliferated with the flat shape like monolayer. However, some of their morphology was spherical on the edge of pore (Figure 29B). In addition, Figure 29B revealed the HaCaT on this matrix could produce adhesion protein to adhere on the surface of the matrix.

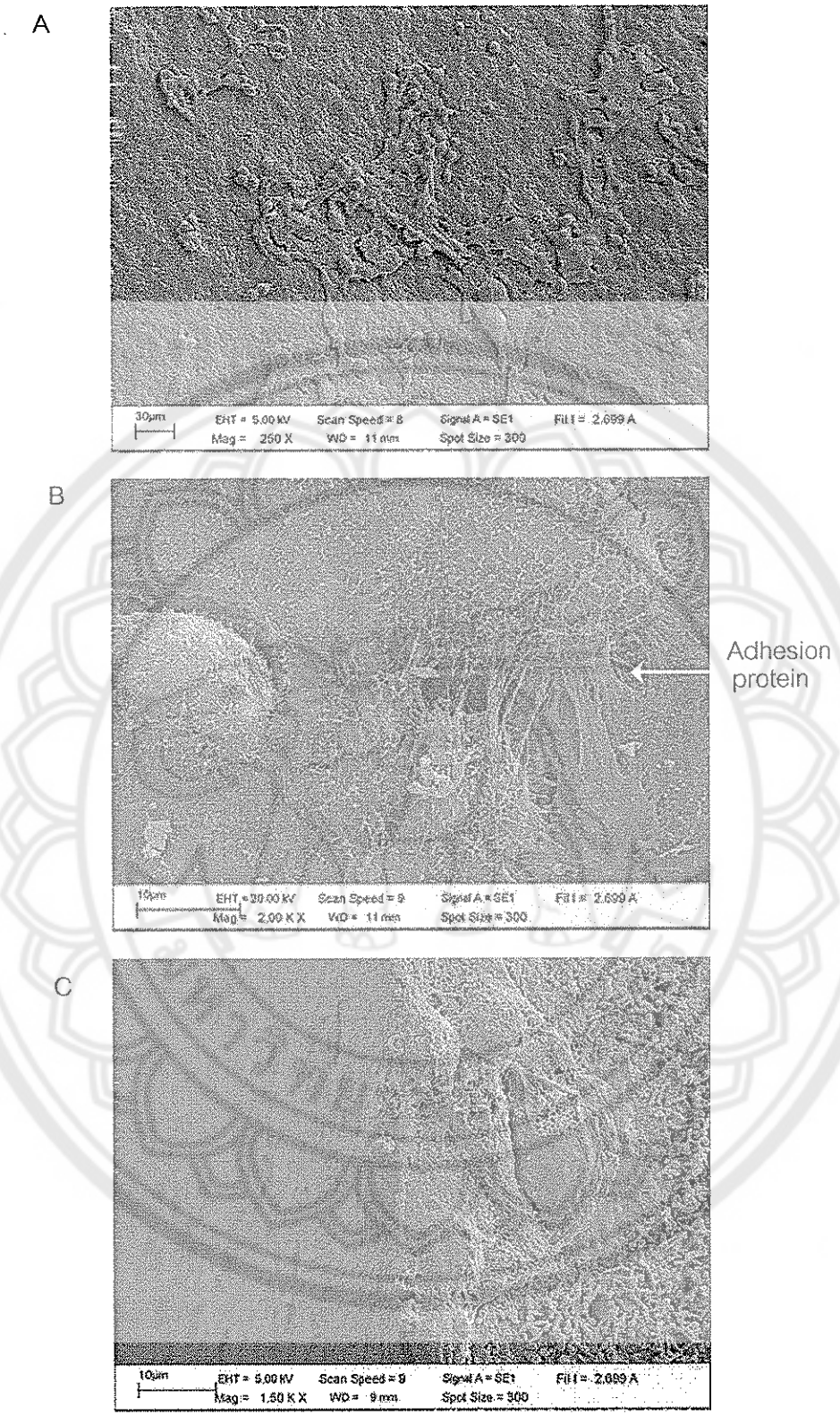


Figure 29 Morphology of HaCaT on the matrix prepared from collagen/crab chitosan MW 100,000 -1,000,000 (7:3) + GA 0.1%: top view at 250X (A), top view at 2KX (B) and side view at 1.5KX (C).

2.7.5 Chemical characterization of the matrix

The spectra from figure 30 indicated that the matrix did not contain free aldehyde molecule, which is toxic component. The aldehyde peaks (aldehyde Iand II in Figure 30D) were not shown in the spectrum of the matrix crosslinked with GA. This refer to the complete crosslink of the GA and polymer chains. The aldehyde groups of glutaldehyde (-COH) formed covalent imine bonds with the amino groups ($-NH_2$) of chitosan and collagen. Figure 30E shows that the peak at 1655 is the imine bond of such crosslink.

Frequencies (cm^{-1})	Frequencies (cm^{-1}) of Matrix	Chemical Charateristic
2860-2800	2850	Aldehyde I
2760-2700	2751	(C-H aldehyde stretching)
1740-1690	1714	Aldehyde II
		(C=O aldehyde stretching)
1690-1640	1655	Imine
		(C=N stretching)

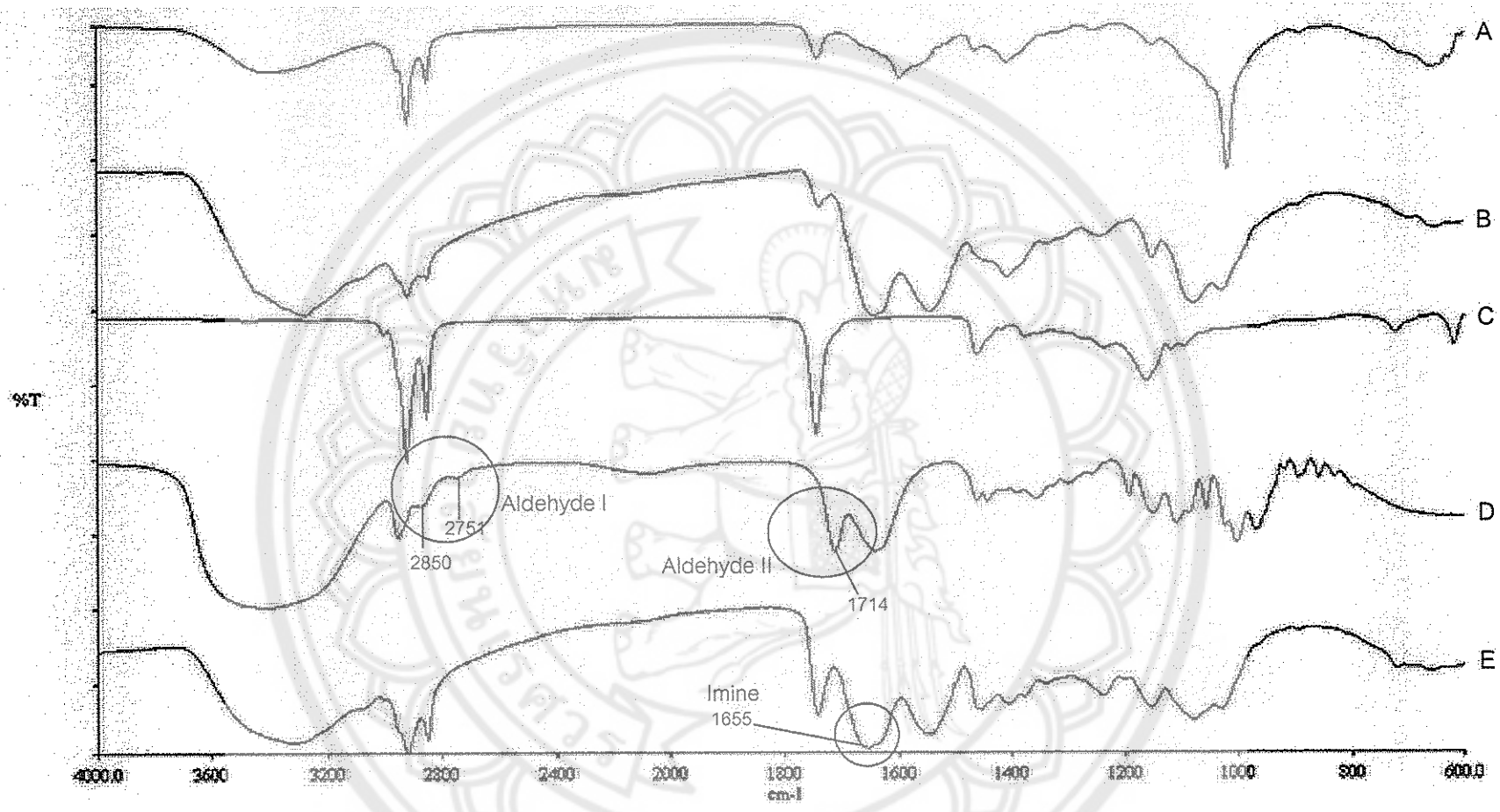


Figure 30 FTIR spectra of the matrix: shrimp chitosan MW 100,000 (A), collagen (B), collagen/shrimp MW 100,000 in ratio of 8 to 2 (C), collagen/shrimp MW (8:2) crosslinked with 0.15% GA (E) and spectrum of the glutaraldehyde solution (D).

2.7.6 Rate of cells proliferation on the matrix

According to XTT assay, the amount of UV absorption at 490 nm of the formazan dye is shown in Figure 31. This amount proportional realated to amount of cell viability after cultured on collagen/crab chitosan MW 100,000 - 1,000,000 (7:3) crosslinked with 0.1% GA matrix. It was interesting that the proliferation rate of HaCaT on such matrix after 5 days of culture (Table 41) was significantly higer than the proliferation on plastic plate, although the initial time of proliferation was lower. This indicated that this matrix could promote cell proliferation.

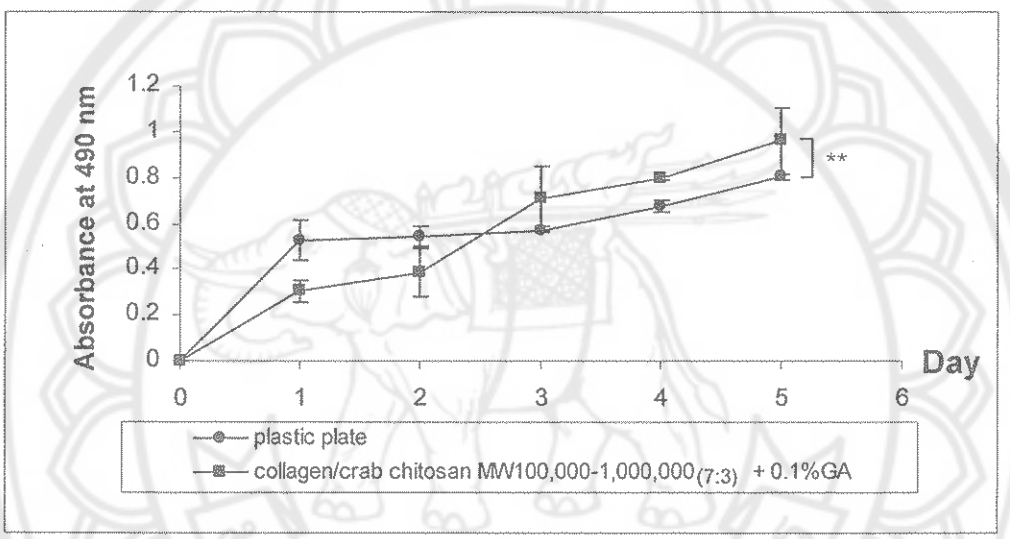


Figure 31 Absorbance of HaCaT cells proliferation on the surface of matrix compared with that on plastic plate (** = $p < 0.01$).

Table 41 Rate of HaCaT cells proliferation on the surface of matrix and plastic plate.

Incubation time (Day)	Proliferation rate (Absorbance/Day)*	
	Matrix	Plastic plate
0 - 2	0.194	0.270
2 - 3	0.323	0.033
3 - 5	0.125	0.115

* Calculation of proliferation rate is shown in appendix G.

2.7.7 Characterization of keratinocyte growth on the developed matrix

Figure 32 shows result of the expression pattern of KGFR and keratins in cell cutured in the matrix was compared to that on the plastic plate. GAPDH was used as a house keeping gene for internal control. The investigated keratin 4-14 gene of HaCaT on the matrix prepared from collagen/crab chitosan MW 100,000 - 1,000,000 (7:3) crosslinked with GA 0.1% was appeared and constantly expressed after cultivation for 2 months. For KGFR gene, it also expressed in the similar pattern between cultivation on plastic plate and matrix.

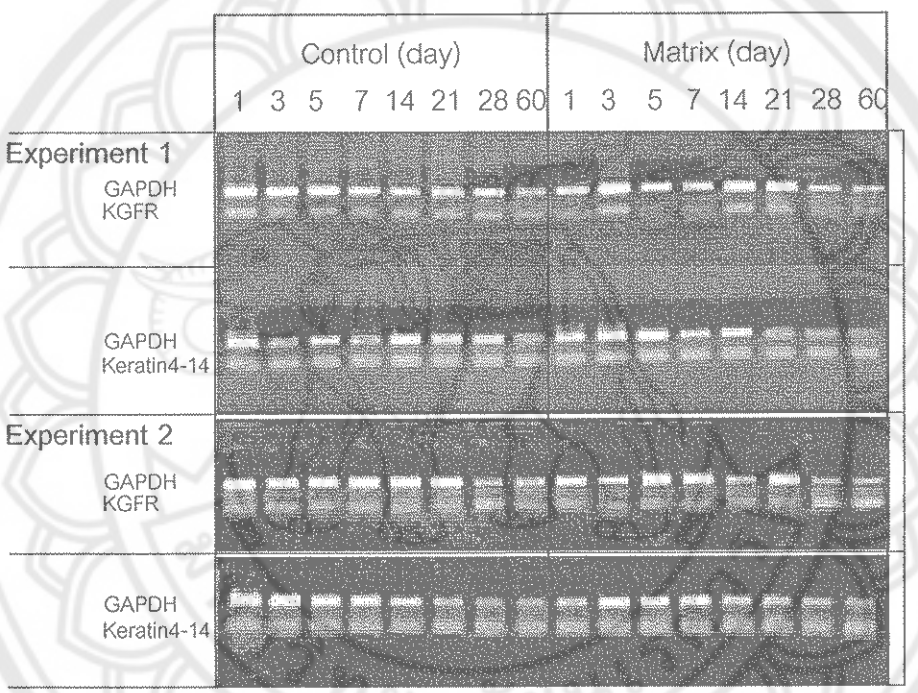


Figure 32 Expression pattern of KGFR and keratins in cell cutured in the matrix was compared to that on the plastic plate.