CHAPTER IV
RESULTS AND DICUSSION

4.1 The Effect of Solvent to Surface Topology of PCL Film

The scaffold should have a porous structure for cell seeding and for mass transport of nutrients and metabolic waste removal. It should have a suitable surface for cell attachment. proliferation and differentiation that guides tissue growth.

4.1.1 Surface Morphology of Neat Polycaprolactone Film

Surface morphology of neat polycaprolactone films was prepared in solvent casting technique by dissolving PCL pellet (Mn = 80,000 g/mol) in different solvent systems in order to induce different surface topology of the films. Surface morphology of PCL films are observed by using scanning electron microscope (SEM) shown in Figure 4.1.

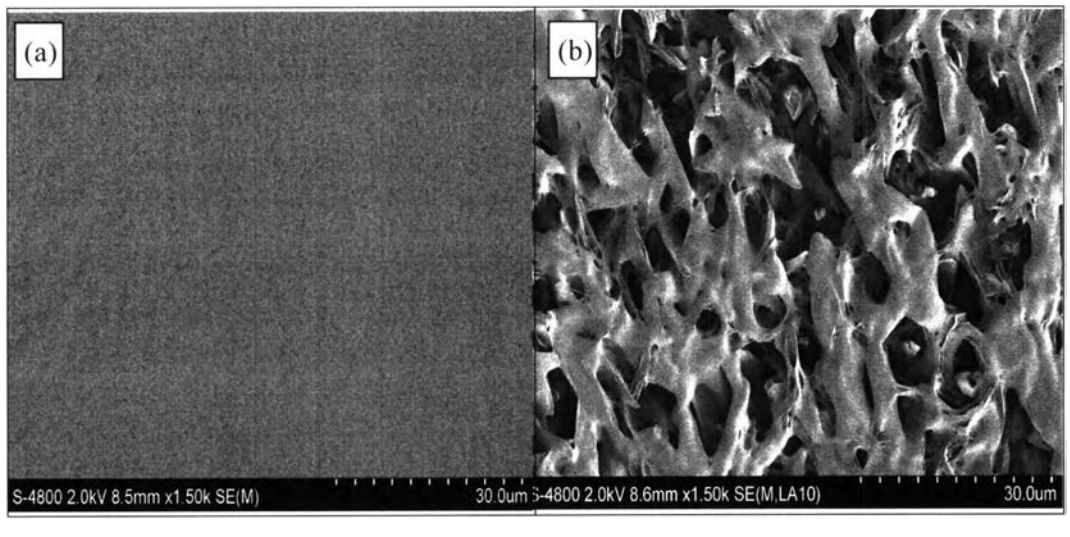


Figure 4.1 SEM images of the surface of PCL film casted from: chloroform (a), and 40:60 (v/v) EtOH:THF (b).

Surface morphology of PCL films in different solvent systems showed in Figure 4.1. In chloroform (used as a good solvent), the solvents molecules can interact with the polymer chains and perform self-avoiding random walk, the

polymer chains can extend to obtain smooth surface, whereas in 40:60 (v/v) EtOH:THF (used as a poor solvent), the polymer chains preferred self-interactions, they will contract in order to avoid the interaction with the solvent molecules, forming porous surface. This effect could be explained that different solubility parameter of solvents induced phase separation and obtained different surface morphology. And the process of phase separation is occurred during solvent evaporation. The solubility parameters of solvents involved in this study were shown in Table 4.1.

Table 4.1 The solubility parameters

Type	**Solubility parameter** ($cal^{1/2} cm^{3/2}$)
Polycaprolactone	9.2
Chloroform	9.21
Ethanol	12.92
THF	9.52

4.1.2 Roughness of PCL Surface

Surface topology of the PCL surface was analyzed by atomic force microscopy (AFM). The AFM analysis of the samples was done for the same size of topography, which was 10 μm in tapping mode. The roughness was described in usual roughness parameters, as the average deviation (R_a) and the root mean square of roughness (R_q) as shown in Table 4.2.

Table 4.2 Value of roughness parameter of the TCPS and PCL films in different solvents casting

Surface	**Roughness (nm)**	
	R_a	**R_q**
TCPS	7.2 ± 1.7	11.5 ± 1.5
Chloroform	140.9 ± 28.4	162.1 ± 31.1
40:60 (v/v) EtOH:THF	432.9 ± 25.4	524.5 ± 26.8

Figure 4.1(a), surface of film casted from chloroform looked flat; however, AFM analysis showed that the root mean square of roughness is 162 nm shown in Figure 4.2 (b). PCL film casted from 40:60 (v/v) EtOH:THF had higher roughness and the value of roughness is 525 nm shown in Figure 4.2 (c). TCPS was used as a control (see Figure 4.2 (a)). The results can conclude that a roughness of the PCL film was increase with the increasing difference in solubility parameter between PCL and the solvents. Solvent-casting film from 40:60 (v/v) EtOH:THF was the highest rough because solubility parameter of ethanol differed from PCL.

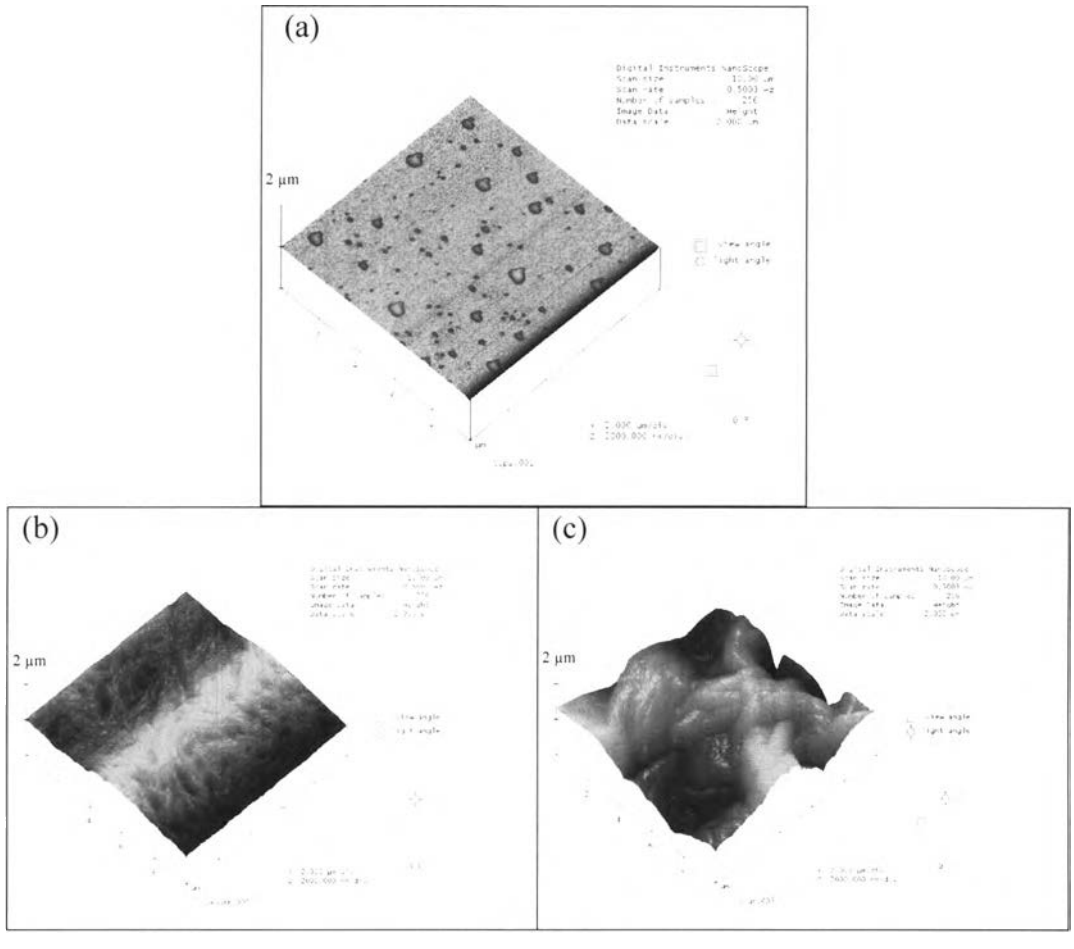


Figure 4.2 AFM pictures of TCPS (a) and the surfaces of PCL films casted from: chloroform (b), and 40:60 (v/v) EtOH:THF (c).

4.1.3 Surface Wettability

The contact angle is a measure of the ability of a liquid to spread on a surface. Measurement gives information about the affinity of a liquid to a solid surface. Surface wettability of PCL film was measured by water contact angle measurement using sessile drop method. The value of contact angle corresponds to wettability. Hydrophobic surface provides highly contact angle ($\theta > 90^{o}$), if the water drop can spread on the surface and obtain low contact angle ($\theta < 90^{o}$), it is hydrophilic surface.

Mixing the nonsolvent ethanol with a good solvent tetrahydrofuran to dissolve polycaprolactone, it has higher contact angle than film casted from chloroform because of the porous surface. The water drop on the surface casted from 40:60 (v/v) EtOH:THF cannot spread on the surface as well as casted from chloroform do. According to the water drop on the hydrophobic surface, the fraction of air in the pores in contact with the water drop is large, and this reason describes for increase in water contact angle (shown in Figure 4.3).

Surface	Solvents	
	Chloroform	40:60 (v/v) EtOH:THF
Neat PCL	77 ± 2^{o} 	88 ± 2^{o} 

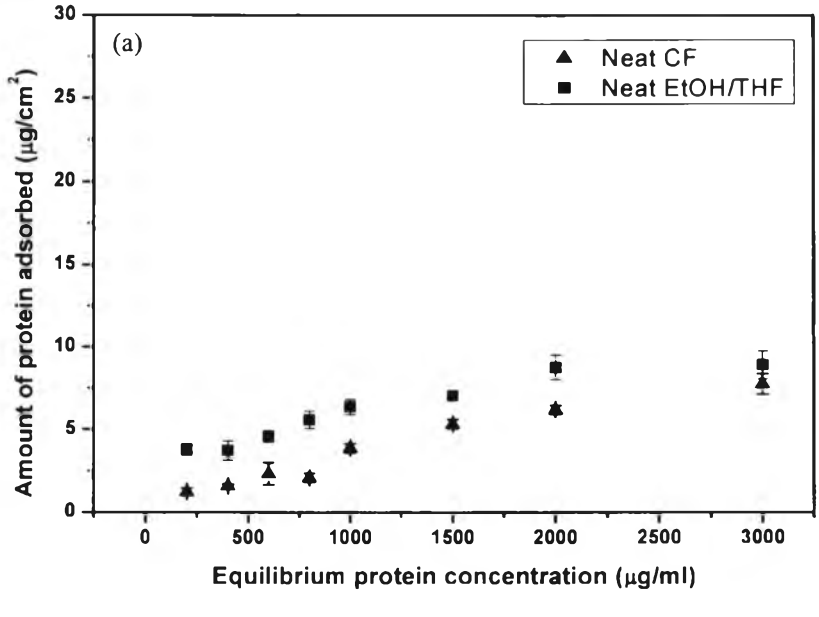
Figure 4.3 Water drop and value of contact angle on the surface of PCL films casted from chloroform and EtOH/THF.

4.2 The Effect of PCL Surface Topography to Protein Adsorption

4.2.1 Protein Adsorption Test

The calibration curve of BSA was used as the standards to calculate and to determine the amount of protein adsorbed on the surface of PCL film was determined based on bicinchoninic acid method by using BCA protein assay kit. The adsorption isotherm of the adsorbed bovine serum albumin was shown in Figure 4.4.

The adsorption isotherm of bovine serum albumin on the neat and modified PCL films displayed that the amount of protein adsorbed increased with the increasing protein concentration and almost saturated at the protein concentration around 3000 μg/ml. The surface casted from 40:60 EtOH:THF had higher amount of protein adsorbed than from chloroform. These results can be concluded that BSA protein preferred to adsorb on the rough surface (shown in Figure 4.4 (a) and (b)).



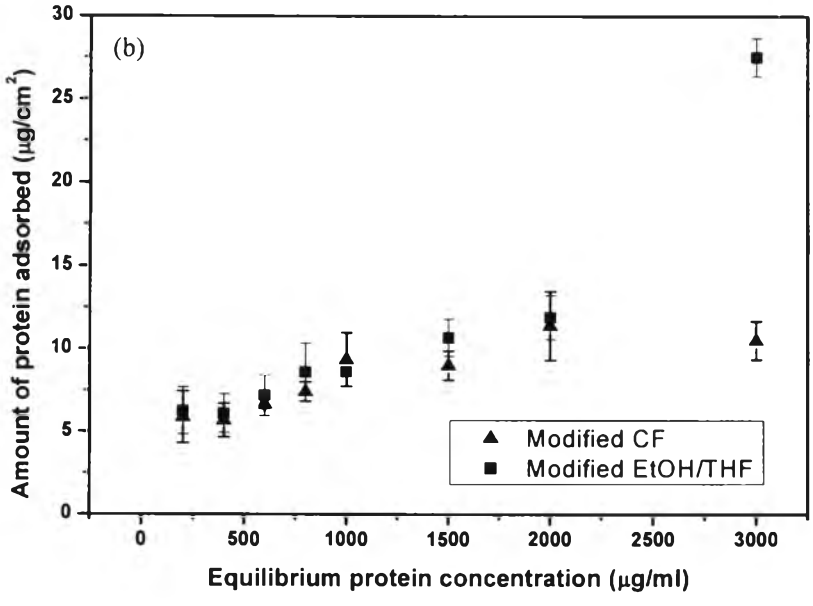


Figure 4.4 The adsorption isotherm of the adsorbed bovine serum albumin : neat PCL (a), and modified PCL films (b) (diameter = 1.5 cm) casted from different solvent systems.

The adsorption isotherms of neat surface compared with modified surface were shown in Figure 4.5 (a) and (b). PCL films were modified surface via aminolysis. HMD/IPA solutions were used to aminolyzed the scaffolds. The amino groups on aminolyzed the scaffolds were activated using DSC/DMSO before immobilized protein on the surface. Neat PCL had a slightly lower hydrophobicity on the surface than activated PCL (shown in Figure 4.8), therefore; modified PCL films had more adsorbed the amount of protein adsorbed than neat PCL films (shown in Figure 4.5). This effect is occurred because of hydrophobic interaction. Bovine serum albumin (BSA) is a flexible protein. It can change conformation to rearrange the inner hydrophobic groups react with the hydrophobic surface of the scaffolds. Therefore, the interaction between BSA and the hydrophobic surface is stronger than on hydrophilic surface. Furthermore, Figure 4.5 (b) showed the highest amount of protein adsorbed at about 3,000 μg/mL of equilibrium protein concentration compared with neat surface. Because rough PCL film had high surface area and modified surface helped adsorb higher the amount of protein.

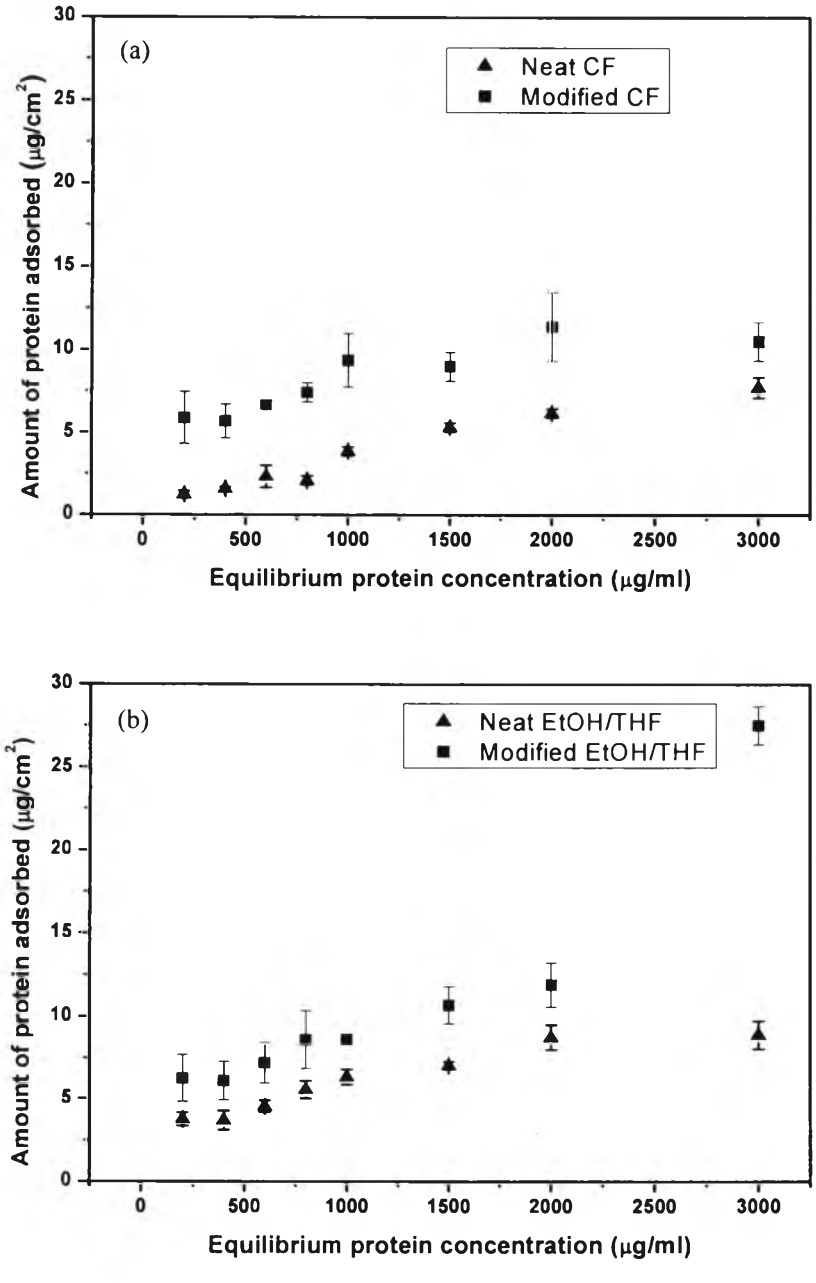


Figure 4.5 The adsorption isotherms of the adsorbed bovine serum albumin : neat and modified PCL films casted from chloroform (a), and neat and modified PCL films casted EtOH/THF (b) (diameter = 1.5 cm).

4.2.2 Surface Chemical Analysis

ATR-FTIR spectra of neat PCL casting from different solvents were shown in Figure 4.6. The ester carbonyl of PCL could be shown at 1724 cm^{-1} is the major absorption peak. The different solvents were used to prepare PCL films and provided the same spectra. This result concluded that both solvent systems to obtain PCL films were completely evaporated solvent.

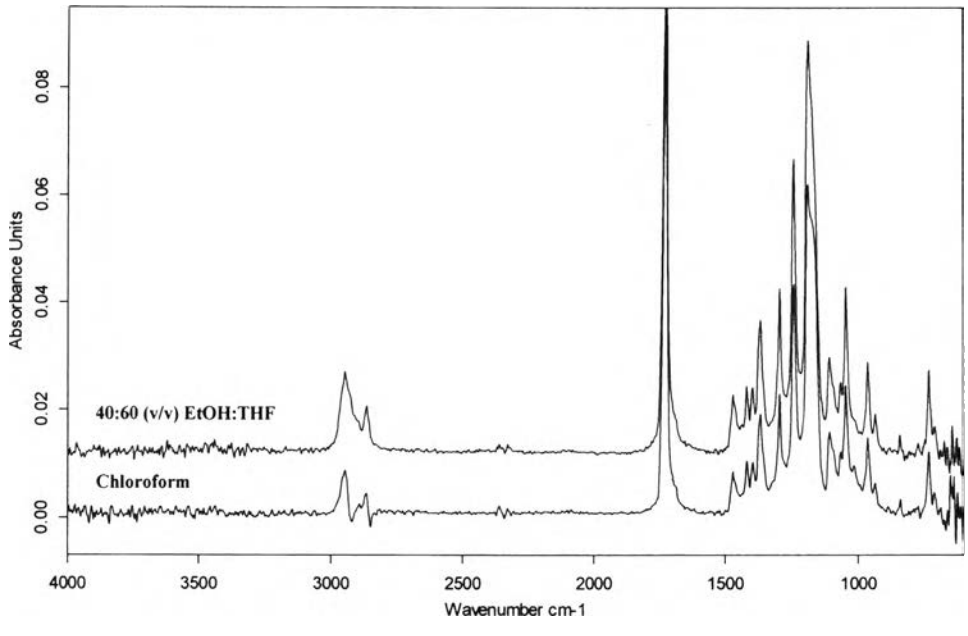


Figure 4.6 ATR-FTIR spectra of neat PCL films casted from chloroform and 40:60 (v/v) EtOH:THF.

The functional groups of PCL are characterized by infrared spectroscopic analysis. After adsorbed proteins on the surface of PCL, FTIR spectra of the samples (adsorbed collagen, pre-adsorbed BSA (200, 1500 and 3000 μg/mL) and adsorbed BSA) showed C=O stretching at 1650 cm^{-1} and N-H stretching at 3300-3500 cm^{-1}. Furthermore, PCL film casted from 40:60 (v/v) EtOH:THF should be clear higher peaks at 1650 and 3330-3500 cm^{-1} than film casted from chloroform. According to amount of protein can be adsorbed on the rough surface.

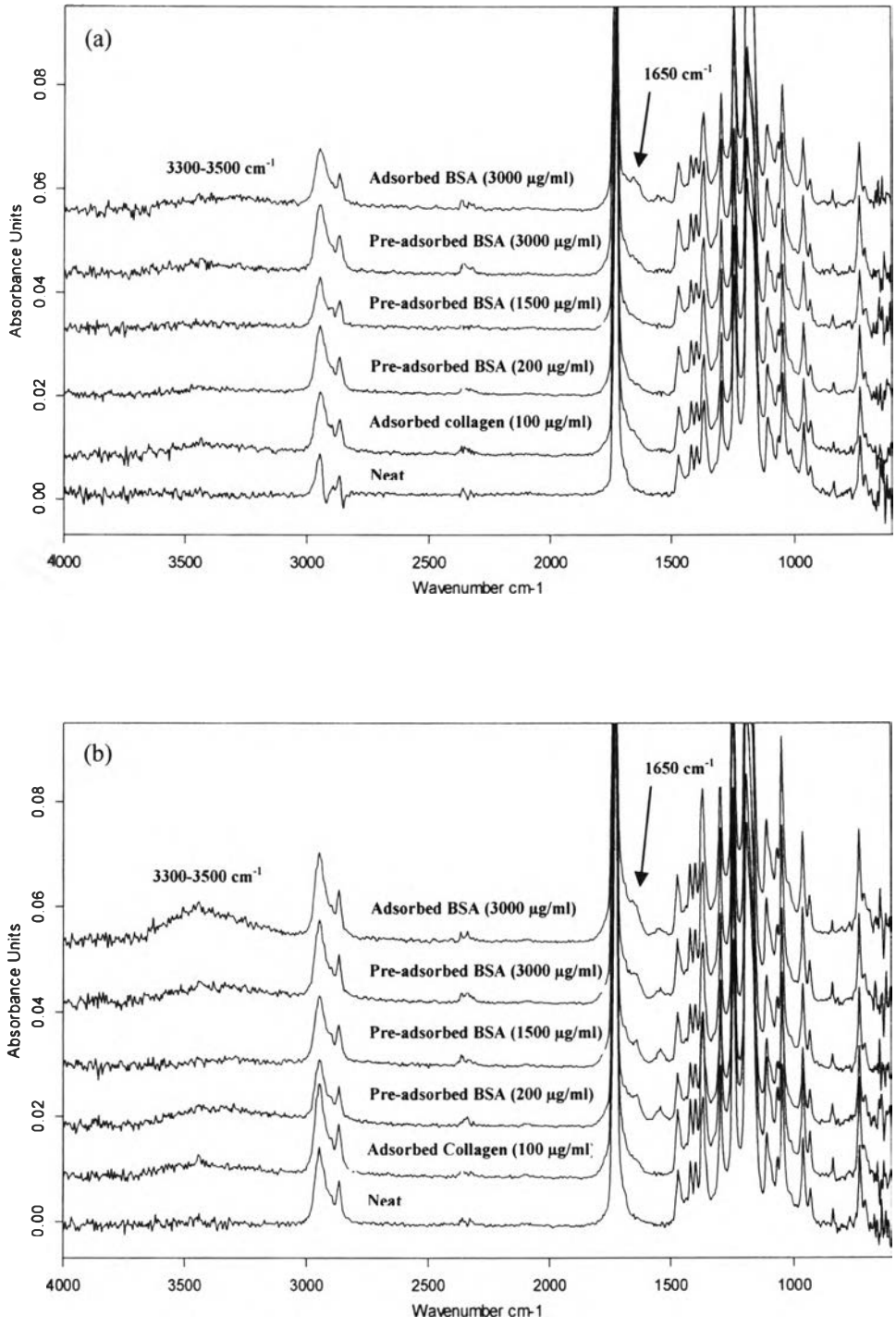


Figure 4.7 ATR-FTIR spectra of neat PCL films casted from chloroform (a) and 40:60 (v/v) EtOH:THF (b); on different proteins adsorbed films.

4.2.3 Surface Wettability

The contact angle is a measure of the ability of a liquid to spread on a surface. Measurement gives information about the affinity of a liquid to a solid surface. Surface wettability of PCL film was measured by water contact angle measurement using sessile drop method. The value of contact angle corresponds to wettability. Hydrophobic surface provides highly contact angle ($\theta > 90^{o}$), if the droplet can spread on the surface and obtain low contact angle ($\theta < 90^{o}$), it is hydrophilic surface.

Surface wettability of PCL is high hydrophobicity but it can be improved via aminolysis and activation of amino groups. Neat PCL is high the water contact angle. After surface was improved via aminolysis by HMD/IPA, the water contact angle showed decrease. Then an activation of amino groups on the surface was reacted by 0.1 M DSC/DMSO to increase the water contact angle and showed more hydrophobicity. The water contact angle was decrease again after proteins adsorption on the surface and it provided lower the water contact angle. Therefore, this result indicated that the biomolecules of proteins can be improved hydrophilicity of PCL films (see in Figure 4.8).

For the PCL film casted from 40:60 (v/v) EtOH:THF had higher contact angle than casted from chloroform because of the porous surface. The water droplet on the surface casted from 40:60 (v/v) EtOH:THF cannot spread on the surface as well as casted from chloroform do. In Figure 4.8 showed the water droplet on the surface of PCL films casted from different solvents and different modified-surface.

Surface	Solvents	
	Chloroform	40:60 (v/v) EtOH:THF
Neat PCL	77 ± 2° 	88 ± 2° 
Aminolyzed PCL	76 ± 2° 	83 ± 2° 
Activated PCL	78 ± 1° 	88 ± 2° 
Adsorbed collagen (100µg/ml)	61 ± 1° 	49 ± 3° 
Pre-adsorbed BSA(200µg/ml)	59 ± 1° 	77 ± 2° 
Pre-adsorbed BSA(1500µg/ml)	55 ± 2° 	69 ± 2° 
Pre-adsorbed BSA(3000µg/ml)	34 ± 2° 	54 ± 1° 
Adsorbed BSA(3000µg/ml)	34 ± 1° 	43 ± 1° 

Figure 4.8 Water drop and value of contact angle on the surface of PCL films casted from chloroform and EtOH/THF on different proteins adsorbed films.

4.3 Biological Characterization

4.3.1 Indirect Cytotoxicity Evaluation

The cytotoxicity evaluation is the important test because it determines the viable cells. It indicated that all the materials did not toxic released substances. The specimens were immersed in serum free media (SFM) for 1, 3 and 7 d in order to extract released the product from the samples. Then, the extraction media used to culture MC3T3-E1 for 1 d. MTT is a method used to determine viability of cells. The result indicated that the viability of cells is higher than 80% relative to control (TCPS). Furthermore, the adsorbed surface of different proteins films obtained a slightly higher percent viability of cells than the control for 3 and 7 d, it explained that the surface-immobilized different proteins films could support to cell growth (see in Figure 4.9 and 4.10).

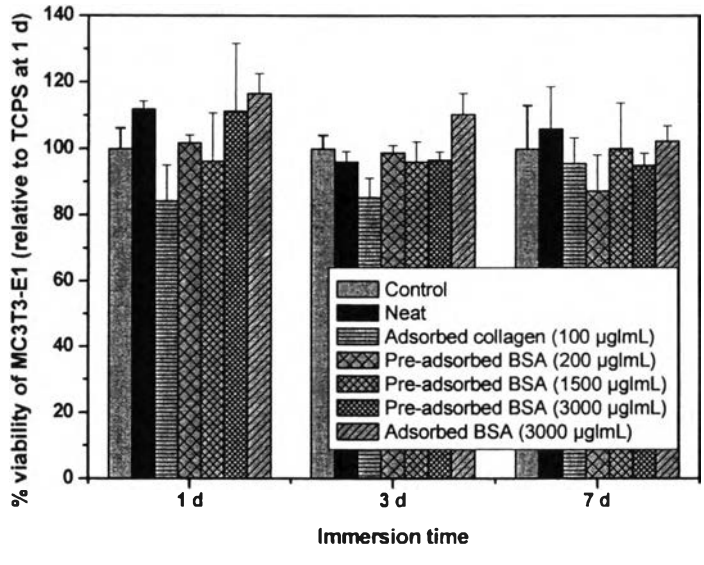


Figure 4.9 The percent viability of MC3T3-E1 on the control (TCPS) and the extract solution of different proteins adsorbed films casted from chloroform.

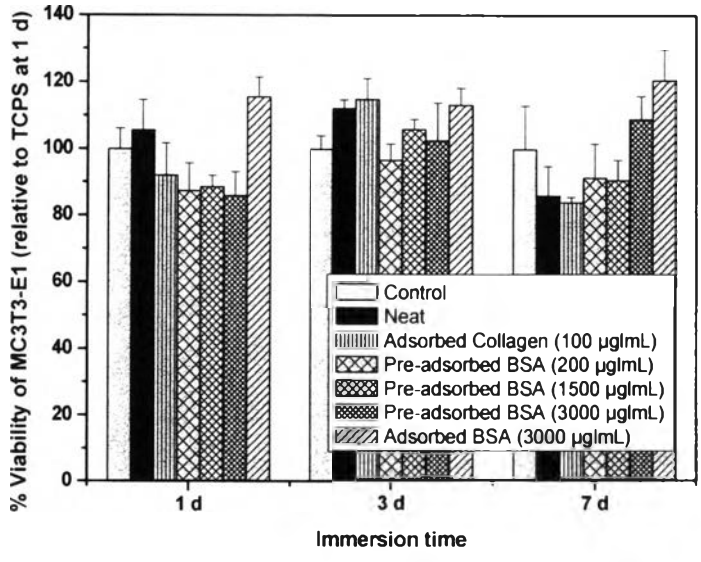


Figure 4.10 The percent viability of MC3T3-E1 on the control (TCPS) and the extract solution of different proteins adsorbed films casted from 40:60 EtOH/THF.

4.3.2 Cell Attachment and Proliferation

The 40,000 cells of MC3T3-E1 were cultured to attach specimens and TCPS used as a control for 4 hrs, 1 d and 3 d. At each time point, the percent viability of cells was determined by MTT assay. Figure 4.11 and 4.12 concluded that the number of cells increased with the cell seeding time. The surface-protein adsorbed films casted from chloroform were lower the percent viability of cells than control (Figure 4.11). In contrast, the surface-protein adsorbed films from casted 40:60 EtOH/THF were higher the percent viability of cells than control at 3 d seeding time (Figure 4.12) because the surface casted from 40:60 EtOH/THF was roughness and more adsorbed protein than the surface casted from chloroform. Furthermore, At 3 d, the BSA- adsorbed films casted from 40:60 EtOH/THF was the highest percent viability of cells. This result concluded that the pre-adsorbed BSA (200, 1500 and 3000 µg/mL) and adsorbed collagen (100 µg/mL) systems on the surface did not promote cells growth as well as adsorbed BSA (3000 µg/mL) system did.

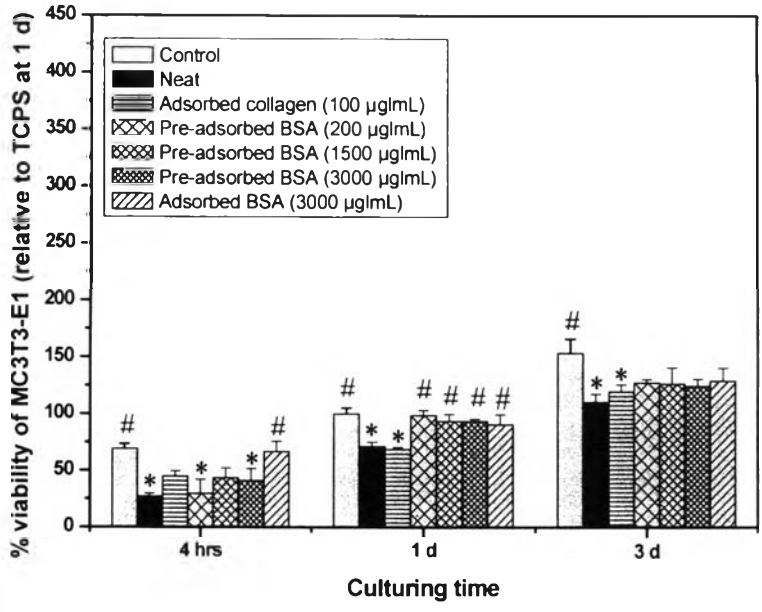


Figure 4.11 The percent viability of MC3T3-E1 on the control (TCPS) and the different proteins adsorbed films casted from chloroform as a function with seeding time. *Significance at $p < 0.05$ with respect to control. #Significance at $p < 0.05$ with respect to the neat substrate of each solvent at any given time point.

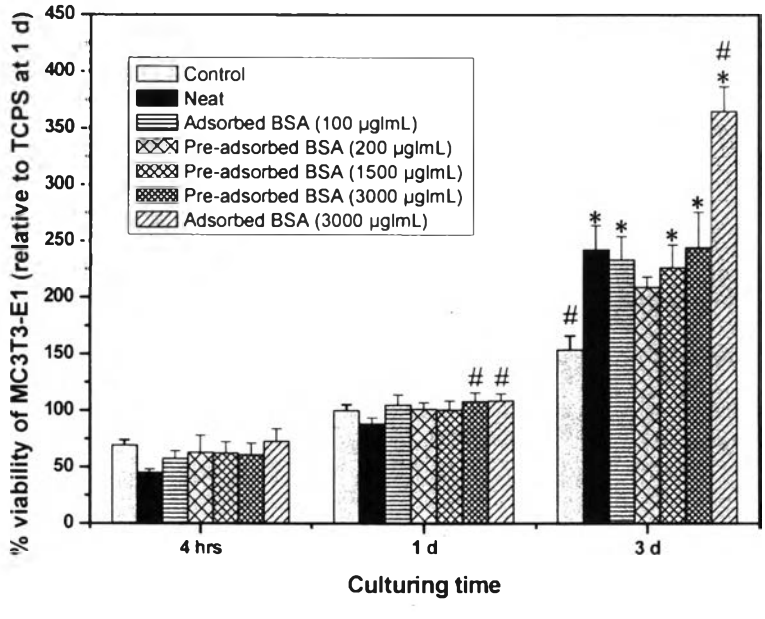
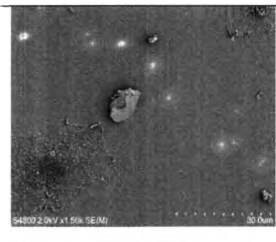
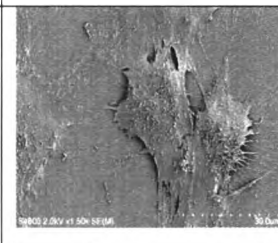
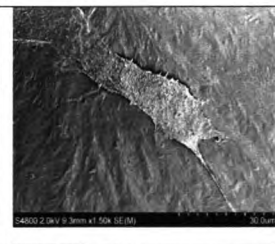
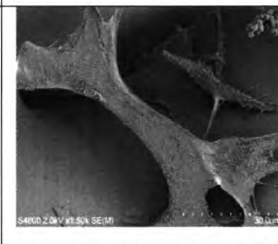
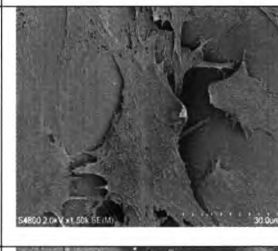
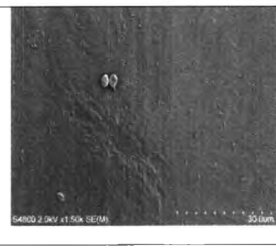
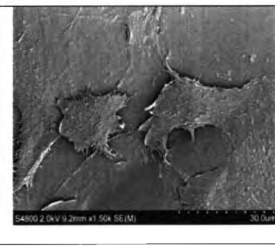
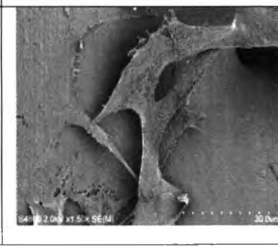


Figure 4.12 The percent viability of MC3T3-E1 on the control (TCPS) and the different proteins adsorbed films casted from 40:60 EtOH/THF as a function with seeding time. *Significance at $p < 0.05$ with respect to control. #Significance at $p < 0.05$ with respect to the neat substrate of each solvent at any given time point.

4.3.3 Cell Morphology Observation

Quantitative analysis for attachment and proliferation was determined by MTT assay. The cell morphology can be observed by SEM for seeding time at 4 hrs, 1d and 3 d showed in Table 4.3 and 4.4.

Table 4.3 SEM images of MC3T3-E1 that were cultured on the surfaces of control (glass) and the different proteins adsorbed films casted from chloroform as a function with seeding time (magnification = 1500X).

Solvents used casting film	**Seeding time**		
	4 hrs	**1 d**	**3 d**
Control (glass)			
Neat			
Adsorbed collagen (100 μg/mL)			
Pre-adsorbed BSA (200 μg/mL)			

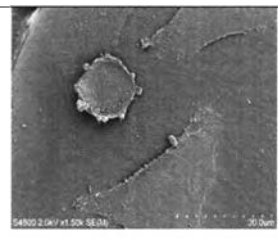
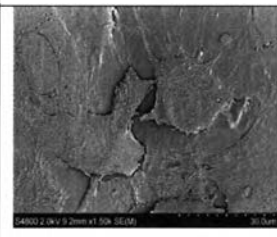
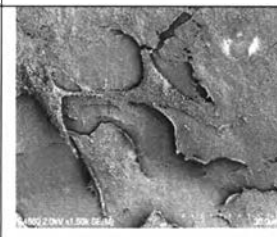
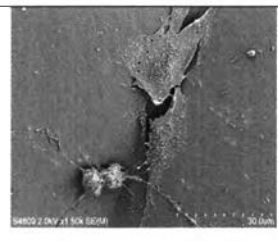
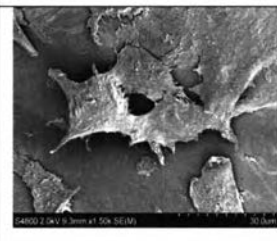
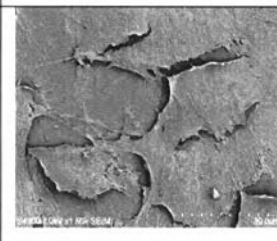
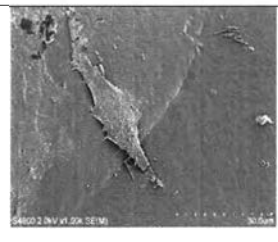
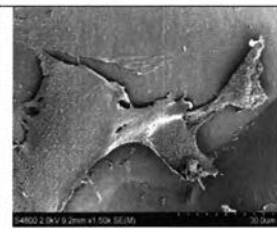
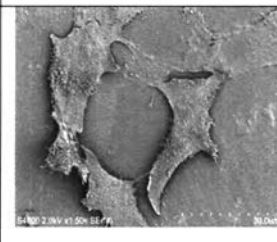
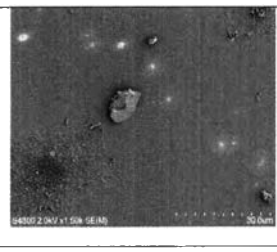
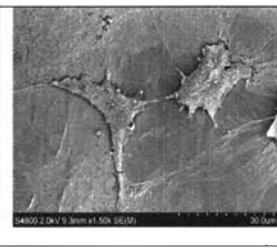
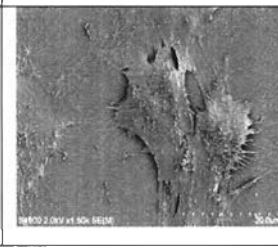
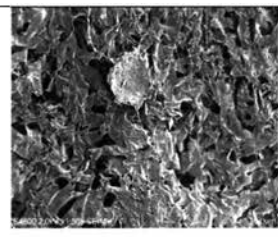
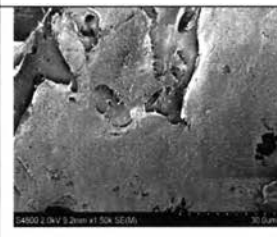
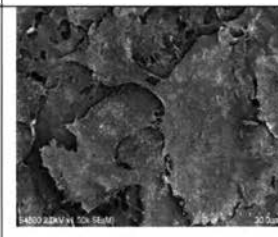
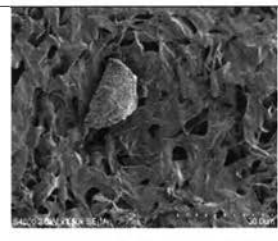
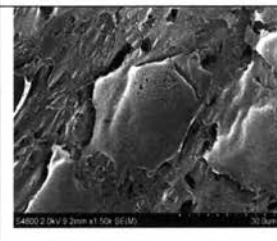
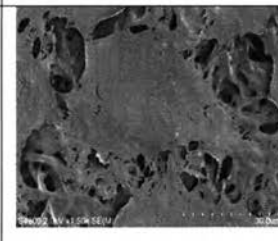
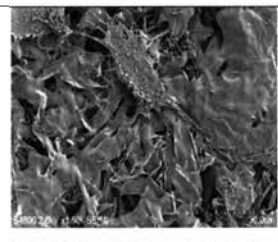
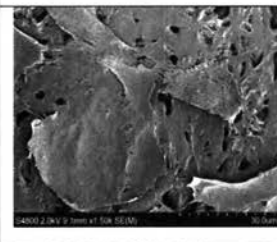
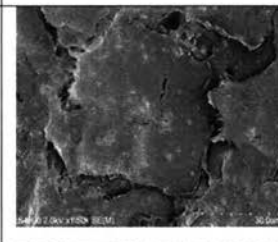
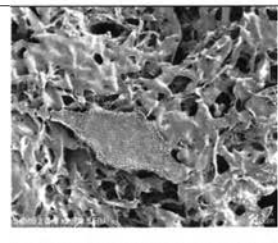
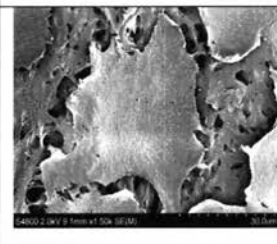
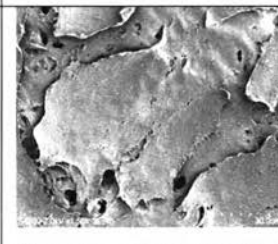
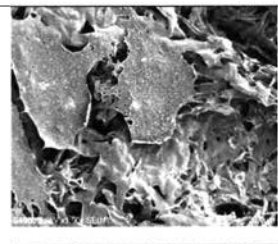
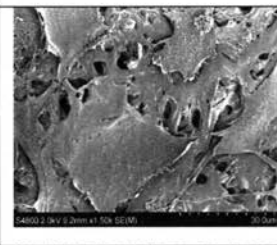
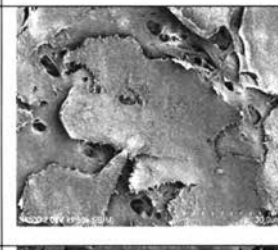
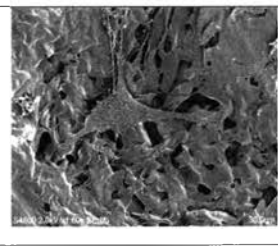
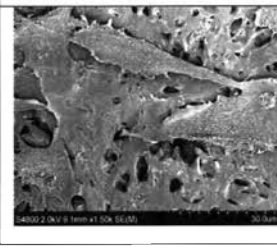
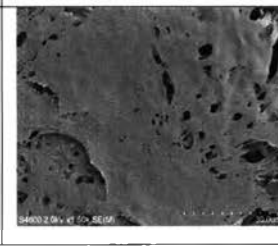
Solvents used casting film	Seeding time		
	4 hrs	1 d	3 d
Pre-adsorbed BSA (1500 μg/mL)			
Pre-adsorbed BSA (3000 μg/mL)			
Adsorbed BSA (3000 μg/mL)			

Table 4.4 SEM images of MC3T3-E1 that were cultured on the surfaces of control (glass) and the different proteins adsorbed films casted from 40:60 EtOH/THF as a function with seeding time (magnification = 1500X).

Solvents used casting film	Seeding time		
	4 hrs	1 d	3 d
Control (glass)			

Solvents used casting film	Seeding time		
	4 hrs	1 d	3 d
Neat			
Adsorbed collagen (100 μg/mL)			
Pre-adsorbed BSA (200 μg/mL)			
Pre-adsorbed BSA (1500 μg/mL)			
Pre-adsorbed BSA (3000 μg/mL)			
Adsorbed BSA (3000 μg/mL)			

SEM images showed the morphologies of MC3T3-E1 that had been cultured on the glass and all types of films. At 4 hrs after cell seeding, the cells on almost types of films casted from chloroform were still round but the cells on immobilization BSA materials were slight expansion cytoplasm. At 1 d and 2 d after cell seeding, the cells can proliferate on all types of films. Furthermore, the effect of topology affected to cell growth on the films (Table 4.4 and 4.5), the cells were higher proliferation on the films casted from 40:60 EtOH/THF than casted from chloroform.

4.3.4 Alkaline Phosphatase (ALP) Activity

Alkaline phosphatase is an enzyme produced by osteoblasts that is used as a maker for osteogenic differentiation. Alkaline phosphatase activity from MC3T3-E1 that was cultured on the surfaces of control (TCPS) and the different proteins adsorbed films casted from chloroform and 40:60 EtOH/THF at seeding time for 7 days is shown in Figure 4.13. The cells were cultured on the film casted from 40:60 (v/v) EtOH/THF provided slightly higher the cell growth than film casted from chloroform because of the effect of topology. In the different proteins-adsorbed on surface, the cells preferred slightly growth on the BSA-adsorbed surface than other protein systems. On the other hand, the ALP activities of the cells that had been grown on the polycaprolactone films casted from chloroform and 40:60 (v/v) EtOH/THF were lower values in significance compared with TCPS. Because differentiation would begin as soon as the proliferation rate starts to decrease.

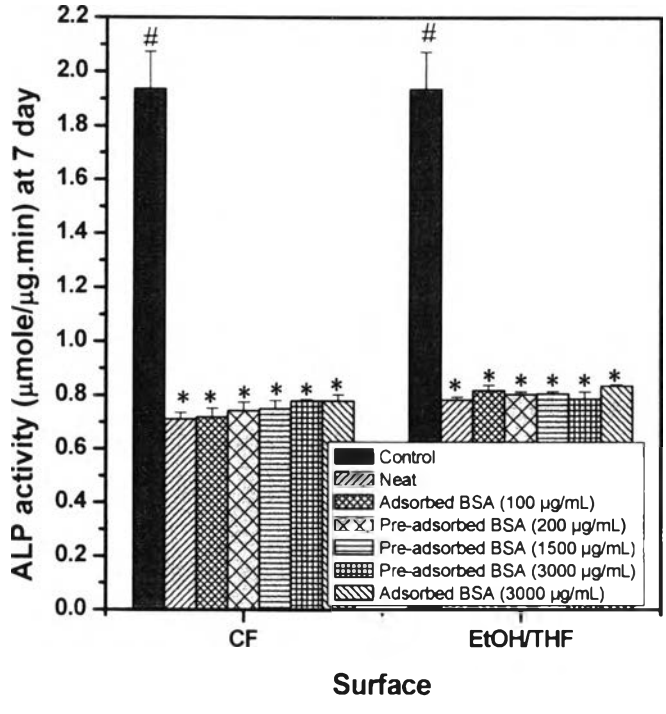


Figure 4.13 ALP activities of MC3T3-E1 that was cultured on the surfaces of the control (TCPS) and the different proteins adsorbed films casted from chloroform and 40:60 EtOH/THF. *Significance at $p < 0.05$ with respect to control. #Significance at $p < 0.05$ with respect to the neat, substrate of each solvent seeding time at 7 d.