CHAPTER IV
RESULTS AND DISCUSSION

4.1 Preparation of Poly(ε-caprolactone) Film Mat

Poly(ε-caprolactone) (PCL; M_n = 80,000 g/mol) film was prepared via solvent casting technique, obtaining the translucent PCL film.

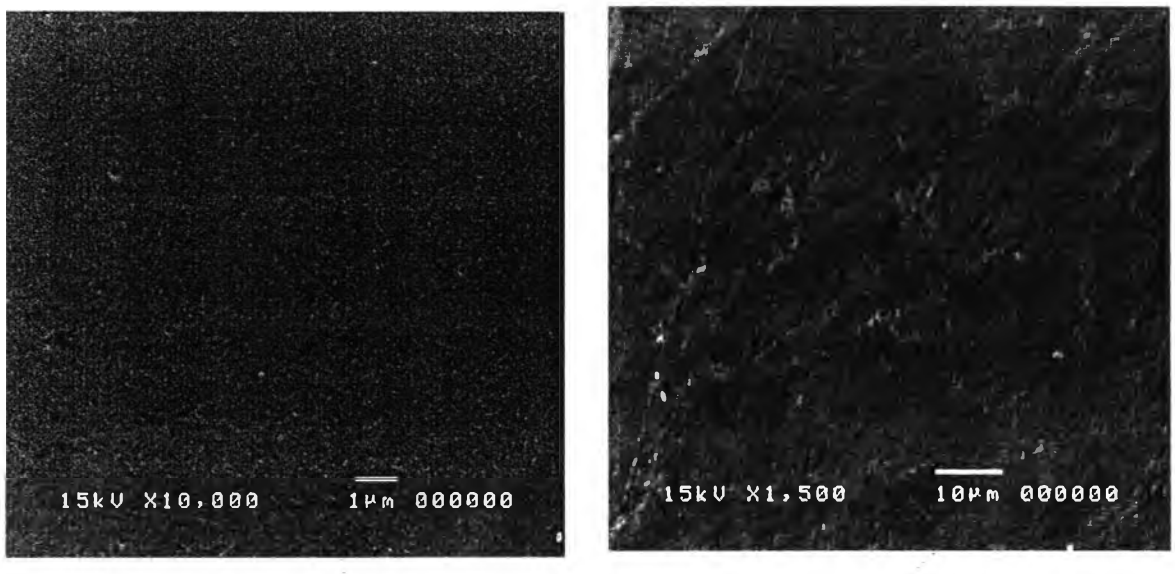


Figure 4.1 Selected SEM image of surface of PCL film (a), and aminolyzed PCL (b).

4.2 Surface Characterizations

4.2.1 Quantification of Amino Groups

Amino groups were covalently introduced onto the surface of the PCL film by reaction with 1,6-hexamethylenediamine (HMD). One amino group (-NH_2) of HMD reacts with an ester group (—COO—) of PCL to form the amide linkage (—CONH—), while another amino group which is unreacted and free can be used as active sites through which biomolecules (i.e. proteins) can be bonded to the surface using *N, N'*-disuccinimidyl carbonate (DSC) as a coupling agent. The chemical pathway for the immobilization of biomolecule on the surface of PCL film is summarized in the Figure 4.2.

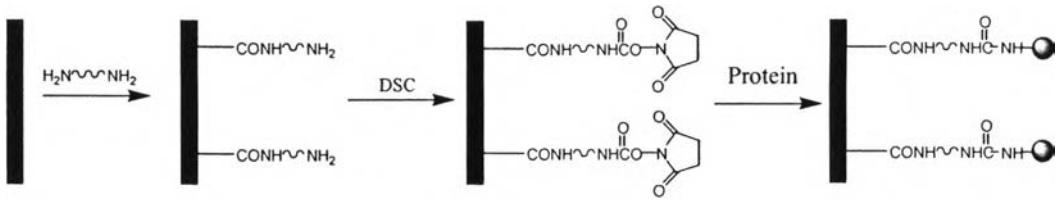


Figure 4.2 The chemical pathway for the immobilization of proteins.

The existence of free amino groups on PCL surface is essential for protein bonding in this modification method. It is important to confirm the existence of amino groups before protein is further introduced. Therefore, ninhydrin is used to confirm and quantify the $-NH_2$ density on the aminolyzed PCL surface. PCL films were aminolyzed in 1.5 M 1,6-hexamethylenediamine/isopropanol solution at room temperature for 8 hours. The NH_2 concentration was evaluated according to the calibration curve obtained with 1,4-dioxane/isopropanol (1:1v/v) solution containing 1,6-hexamethylenediamine of known concentration. Table 4.1 shows that the NH_2 density could not be evaluated on neat PCL because no amino group was found while the NH_2 density on aminolyzed PCL film mat under this condition was $(4.52 \pm 0.04) \times 10^{-8}$. After PCL film mat was activated, no detection of amino group was shown due to the activated reagents reacted with those free amino groups. The NH_2 density was found, once again, after activated film was further immobilized with crude bone protein and bovine serum albumin, which were $(3.91 \pm 0.01) \times 10^{-8}$ and $(3.58 \pm 0.02) \times 10^{-8}$, respectively.

Table 4.1 NH_2 density on the surface of the modified PCL film mats

Sample	NH_2 concentration ($mol \cdot cm^{-2}$)
Neat PCL	-
Aminolyzed PCL[a]	$(4.52 \pm 0.04) \times 10^{-8}$
Activated PCL[b]	-
Crude bone protein-immobilized PCL[c1]	$(3.91 \pm 0.01) \times 10^{-8}$
Bovine serum albumin-immobilized PCL [c2]	$(3.58 \pm 0.02) \times 10^{-8}$

[a]The PCL film mats were immersed in 1.5 M 1,6-hexanediamine/IPA solution at room temperature for 8 hours.

[b]The aminolyzed PCL film mats were immersed in 0.1 M DSC solution in the presence of TEA for 1 hour.

[c1, c2] The activated PCL film mats were immersed in 3.0 mg/ml of crude bone protein solutions and bovine serum albumin, respectively, for 24 h followed by the rinsing process.

4.2.2 Surface Wettability

Water contact angle measurement was used to evaluate the surface wettability of the surface of modified and unmodified PCL films. Table 4.2 shows the water contact angle measured by the sessile drop method decreased gradually from 82.9 ± 0.4 to 79.8 ± 0.1 after the films were aminolyzed with 1.5 M of HMD/IPA solution for 8 hours. That is, the introduction of the amino groups on the surface of the PCL film can improve the hydrophilicity of the surface. After the aminolyzed PCL films have been activated with DSC, their surface became more hydrophobic than the neat PCL film. The water contact angle was decreased again after further immobilized the activated films with either crude bone protein or bovine serum albumin. The water drop appearance on the surface of neat PCL, aminolyzed PCL, crude bone protein immobilized PCL and bovine serum albumin immobilized PCL which addition to the above results are shown in Figure 4.2. This means that immobilization of biomolecules can gradually improve the hydrophilicity of the PCL surface.

Table 4.2 The water contact angle of the control and all modified PCL films measured by the sessile drop method

Sample	Contact angle (θ) (Sessile drop)
Neat PCL	82.9 ± 0.4
Aminolyzed PCL[a]	79.8 ± 0.1
Activated PCL[b]	84.9 ± 0.5
Crude bone protein-immobilized PCL[c1]	71.5 ± 0.5
Bovine serum albumin-immobilized PCL [c2]	60.2 ± 0.6

[a]The PCL film mats were immersed in 1.5 M 1,6-hexamethylenediamine/IPA solution at room temperature for 8 hours.

[b]The aminolyzed PCL film mats were immersed in 0.1 M DSC solution in the presence of TEA for 1 hour.

[c1,c2]The activated PCL film mats were immersed in 3.0 mg/ml of crude bone protein solutions and bovine serum albumin, respectively, for 24 h followed by the rinsing process.

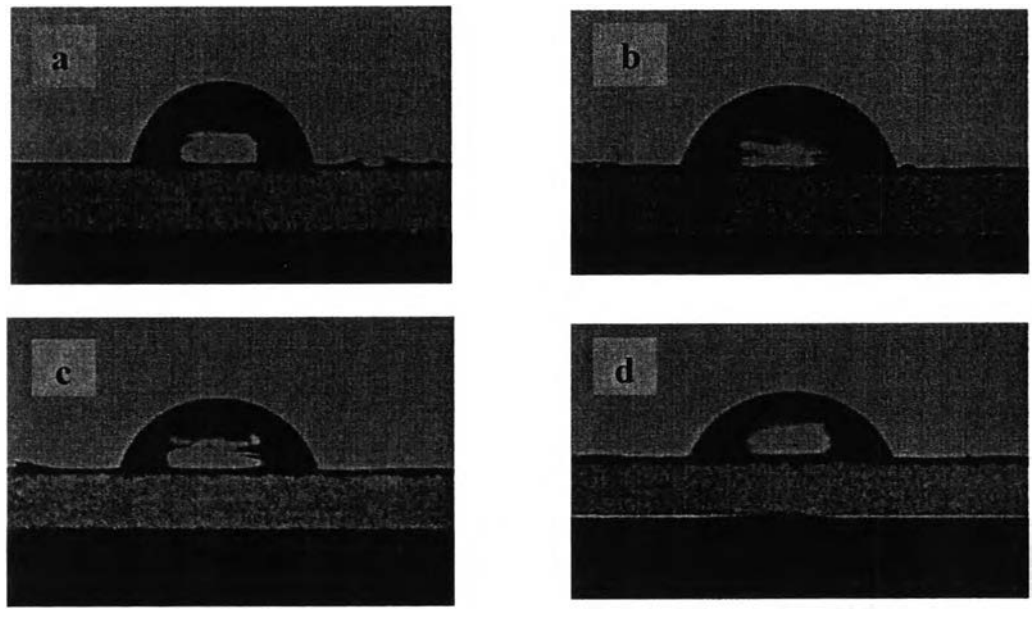


Figure 4.3 Water dropped on the surface of neat PCL (a), aminolyzed PCL (b), 3.0 mg/ml crude bone protein immobilized PCL (c) and 3.0 mg/ml bovine serum albumin (d).

4.2.3 Chemical Analysis of Surface

ATR-FTIR spectra of PCL and modified PCL film mats are shown in Figure 4.4. There was a major absorption peak assigned to the ester carbonyl of PCL appeared at 1755 cm^{-1} found in all types of PCL membranes. C=O stretching and N–H stretching peaks should occur at 1650 cm^{-1} (amide I) and 3300 to 3500 cm^{-1}, respectively, after biomolecules immobilization. Due to the low broad signals at those points were detected, which may be regarded as a result of the extremely low concentration of introduced chemicals which presented within the sampling depth of ATR-FTIR (1-2 μm), X-ray photoelectron spectrometer (XPS) was used to evaluate the element on the unmodified and modified PCL film mats in term of the N_{1s}/C_{1s} ratio.

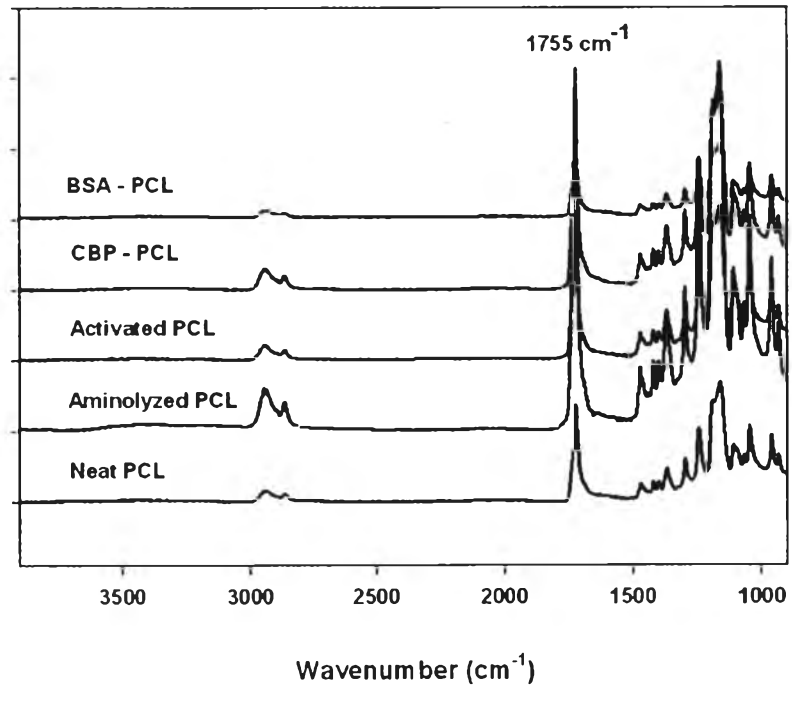


Figure 4.4 ATR-FTIR spectra of neat and modified PCL film mats.

4.2.4 Elemental Composition of the Surface

X-ray photoelectron spectrometer (XPS) was used to study the element on the surface of materials. It is believed that more N_{1s}/C_{1s} ratio could be due to the increasing in NH_2 group concentration. Table 4.3 shows the N_{1s}/C_{1s} ratio in each material, which in addition to the results on ninhydrin analysis method. After

aminolysis of PCL film mat, the N_{1s}/C_{1s} ratio was increased from 0 to 0.0079 because of NH_2 groups were introduced on the surface. Moreover, the increasing in N_{1s}/C_{1s} ratio (~10 fold) was obvious after either crude bone protein or bovine serum albumin immobilization due to the large amount of nitrogen atom in biomolecules structure was additionally introduced.

Table 4.3 N_{1s}/C_{1s} ratios of the control and modified PCL films

Sample	N_{1s}/C_{1s} ratio
control PCL	0.0000
Aminolyzed PCL[a]	0.0079
Activated PCL[b]	0.0066
Crude bone protein Immobilized PCL[c1]	0.0778
Bovine serum albumin Immobilized PCL[c2]	0.0723

[a]The PCL film mats was immersed in 1.5M 1,6-hexanediamine solution at 37 °C for 8 h.
[b]The aminolyzed PCL films were immersed in 0.1 M DSC solution for 1 h.
[c1,c2]The activated PCL films were immersed in 3.0 mg/ml crude bone protein solutions and bovine serum albumin, respectively, for 24 h followed by the rinsing process.

4.3 Biological Characterizations

4.3.1 Indirect Cytotoxicity Evaluation

The potential for use of these film mats as bone tissue engineering was first evaluated by an indirect cytotoxicity with mouse clavaria-derived pre-osteoblastic cells (MC3T3-E1). The extraction media were prepared by immersing samples (i.e. neat, aminolyzed, crude bone protein-immobilized, and bovine serum albumin-immobilized PCL film mats) for 1 and 7 d, respectively. The extraction media were then used to culture MC3T3-E1 for 1 d. The viability of cells that had been cultured in fresh SFM for any given time was used as a basis to obtain the relative viability (Figure 4.5). It was clearly found that cells cultured in media extracted from all materials showed the viability more than 80% at any given time. In addition, the extraction media from all PCL membranes provided slightly higher % viability of cells compared to one that extracted from TCPS on each observed day,

which can be assumned that all materials may have the ability to promote the cell growth. The obtained result can be confirmed that all types of PCL film mats release no substances at levels that toxic or harmful to the cells.

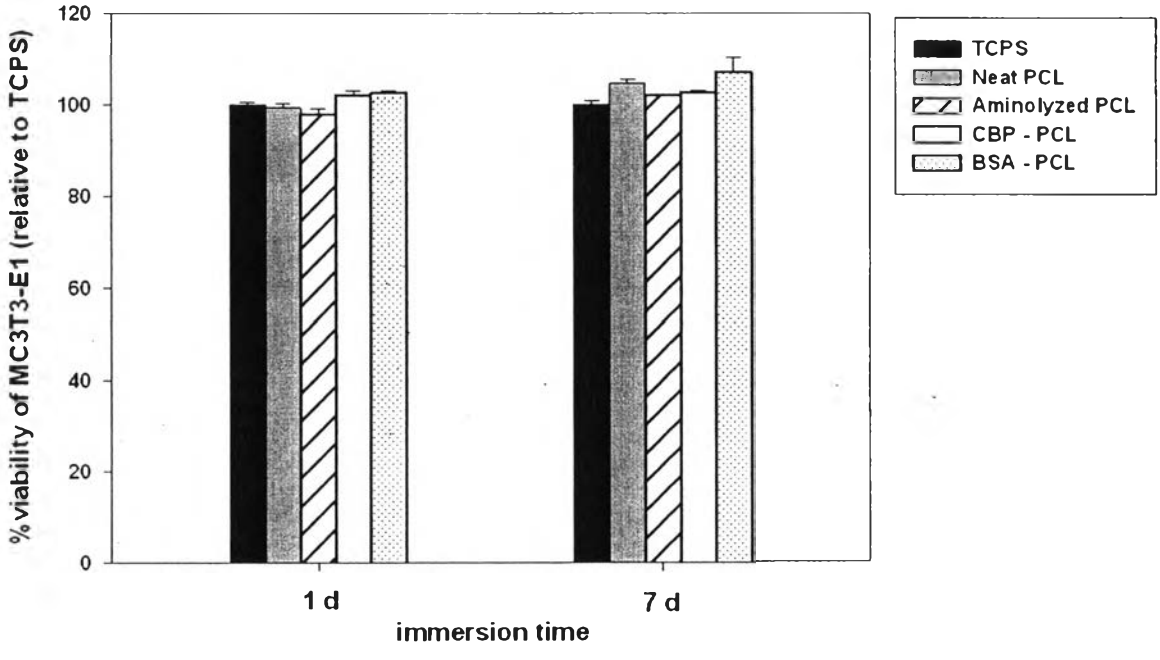


Figure 4.5 Indirect cytotoxicity evaluation of neat and modified PCL based on viability of MC3T3-E1 cultured with SFM extraction media from each materials with the against the viability of the cells that had been cultures with the respective culture media for any given time as a function of the incubation time of the extraction and the culture media of 1 or 7 d. Statistical significance: $^{*}p < 0.05$ compared with control and $^{\#}p < 0.05$ compared to the neat PCL film mats at any given time point.

4.3.2 Cell Attachment and Proliferation

To study cell attachment, 40,000 cells of MC3T3-E1 were cultured on neat(i.e. control), aminolyzed, crude bone protein-immobilized and bovine serum albumin-immobilized PCL film mats and TCPS, which used as a positive control, for 6 h and 24 h, respectively. At each studied time, MTT assay was used to quantify the viable cells. The viability of cells cultured on the surface of neat PCL film mats for 24 h was taken as the basis to obtain the relative viability values shown in the Figure 4.6. From the figure, the viability of cells was increased as the culturing time

increased found in both on TCPS and all types of the film mats. At 6 h after cell seeding, there was no significant difference found in among various types of PCL membranes. However, a number of cells attached on neat film mats were significantly inferior to that on crude bone protein- and bovine serum albumin-immobilized PCL film mats after 24 h cell seeding which could be due to more hydrophilicity achieved on the protein-immobilized PCL surface. Among of various types of PCL film mats, bovine serum albumin-immobilized PCL showed the best support for attachment. The less viability of cells on all types of materials was inferior to those on TCPS at any given time which could be due to less hydrophilicity on the material surface or the surface of material can absorb the stain on their surface, causing some error on evaluation the absorbance.

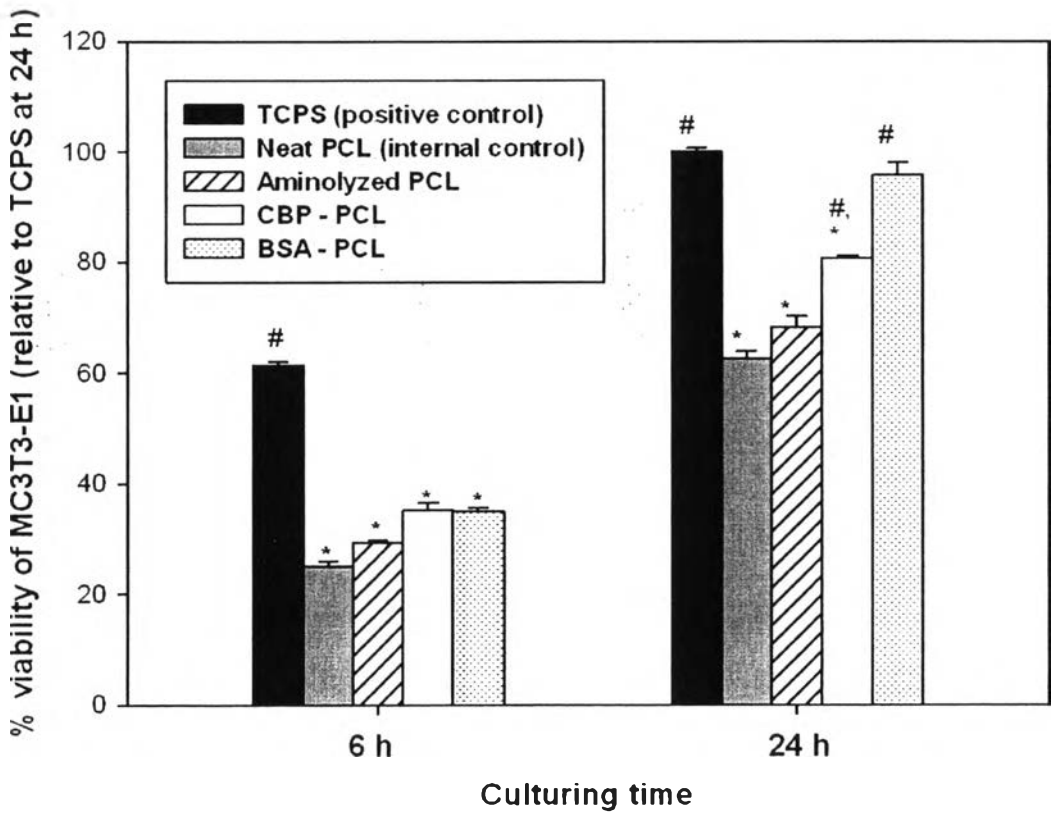


Figure 4.6 Attachment of MC3T3-E1 that had been cultured on the surfaces of TCPS, the neat and the modified PCL film mats for 6 h and 24 h. Statistical significance: $^{*}p < 0.05$ compared with control and $^{\#}p < 0.05$ compared to the neat PCL fibrous scaffolds at any given time point.

For cell proliferation, MC3T3-E1 were also cultures on neat (i.e. control), aminolyzed, crude bone protein-immobilized, bovine serum albumin-immobilized PCL film mats and on TCPS, which used as a positive control, for 1, 2 and 3, respectively. The viability of cells cultured on the surface of neat PCL film mats for 1 d was taken as the basis to obtain the relative viability values. Figure 4.7 shows the viable cells cultured on both TCPS and various types of PCL film mats were increased as increase in the culturing time. Among various types of PCL film mats, protein-immobilization, again, provided better viability of cells. Both crude bone protein-and bovine serum albumin- immobilization provided a significant improvement in cell proliferation on day 3, especially bovine serum albumin which showed the significant improvement since the first day. From the results of both attachment and proliferation can be indicated that immobilization with protein can provide a good support for cells adhesion and proliferation which could be due to the improvement in hydrophilic surface and protein-containing on the surface of substrates.

4.3.3 Cell Morphology

Table 4.4 shows the selected morphology of cells attached on glass, neat, aminolyzed, crude bone protein-immobilized PCL and bovine serum albumin-immobilized PCL film mats at 6 h and 24 h after cell seeding. At 6 h, cells seeded on glass started extending their cytoplasm. In comparison to among PCL membranes at 6 h after, cells seeded on neat and aminolyzed PCL film surfaces were still round while those on protein-immobilized PCL became flat and start spreading. In a closer examination, it was found filopodia around the edge of cell seeded on among various types of PCL membranes which is an evidence of adhesion of the cells. At 24 h, expansion of the cytoplasm of majority of cells on both glass and all types of materials was evident. In comparison with among types of materials, modified PCL illustrated better extension than neat PCL. The results from cell morphology can be concluded that cells prefer to adhere and expansion on the modified surface of PCL.

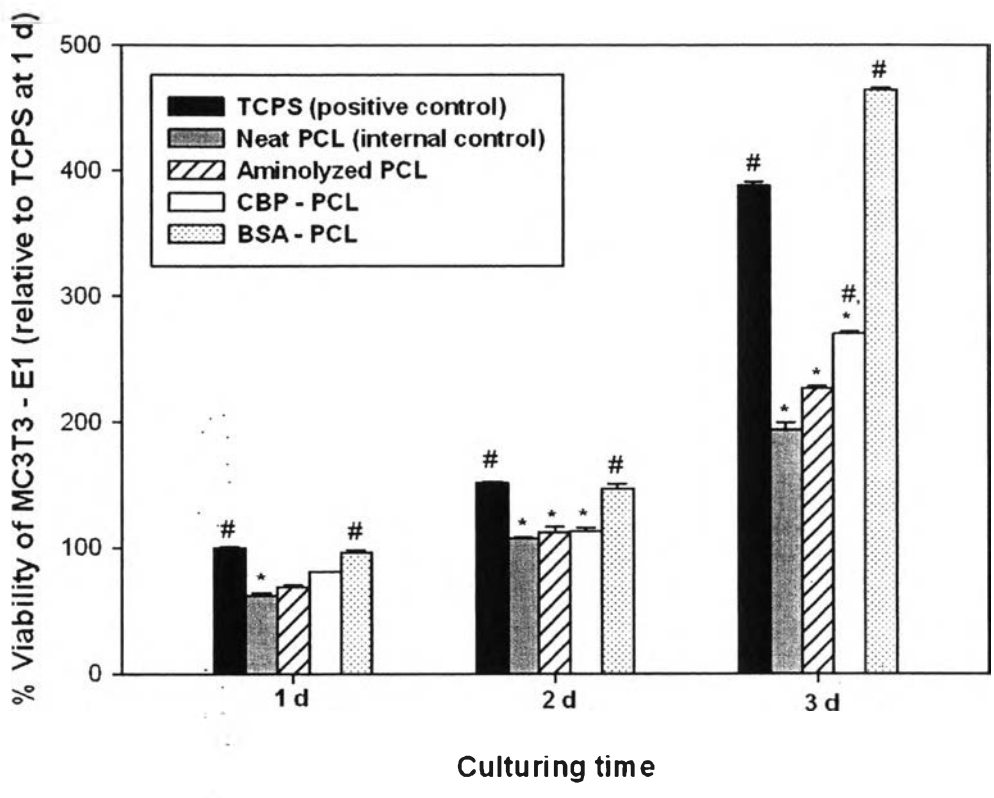


Figure 4.7 Proliferation of MC3T3-E1 that had been cultured on the surfaces of TCPS, the neat and the modified PCL film mats for 1, 2 and 3d. Statistical significance: $^{*}p < 0.05$ compared with control and $^{\#}p < 0.05$ compared to the neat PCL fibrous scaffolds at any given time point.

At 1, 2 and 3 d after cell seeding, the majority of the cells seeded on the surfaces of both TCPS and all types of materials expanded over the limited area (Table 4.5). Cells proliferated on the surface bovine serum albumin present the greatest expansion compared with among various types of materials. Considering the obtained results of attachment, proliferation and morphology, protein-immobilized PCL provides the most positive effect and increase cytocompatibility to the cells. Between two of these protein-immobilization, bovine serum albumin-immobilization is the best support to improve the cell adhesion and cell proliferation which could be due to the existence of protein that can provide more preferable place to the cells.

4.3.4 Alkaline Phosphatase (ALP) Activity

Alkaline phosphatase is a membrane bound enzyme and its activity is used as an osteoblastic differentiation marker, as produced only by cells showing mineralized ECM. The ALP activity of MC3T3-E1 was conducted on TCPS (i.e. controls), neat PCL, aminolyzed PCL, crude bone protein- and bovine serum albumin-immobilized PCL for 3, 5 and 7 days in culture. Figure 4.8 illustrates the amount of ALP synthesized by the cells that were cultured on TCPS and all of the film mats increased with the initial increase in time in culture along day 3^{rd} and 5^{th}, which reached a maximum level on day 5^{th}, and then decreased with a further increase in culturing day on day 7^{th}. In comparison to all of those control and materials, bovine serum albumin-immobilized PCL film mats exhibited the high ALP activity of MC3T3-E1 at any given culturing time; however, there is no significantly difference on ALP activity found in all types of materials.

For the decrease in the ALP activity after day 5 for the cells grown on both TCPS and all the types of substrates, with a further increase in the culturing time can be due to cells synthesized extremely amount of proteins which related to cellular process which may switch to further step (i.e. mineralization) (Choi *et al.*, 1996) since ALP is not an exclusive protein synthesized by osteoblasts as it is also found in tissues of such organs as kidneys, small intestines, and placenta. Hence, results of ALP could not be used as the only one marker to confirm the differentiation of osteoblastic cells (Tsukamoto *et al.*, 1992). In order to clarify the effect of substrates on the differentiation of osteoblasts are currently determined by long term experiments (i.e. mineralization).

Table 4.4 Selected SEM images of cultured specimens, i.e., glass (as a control), neat PCL, aminolyzed PCL, crude bone protein-immobilized PCL and bovine serum albumin-immobilized PCL film mats at various time points after MC3T3-E1 were seeded on their surfaces (magnification = 1,500X; scale bar = 10 μm)

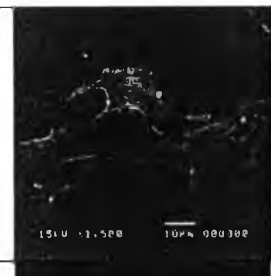
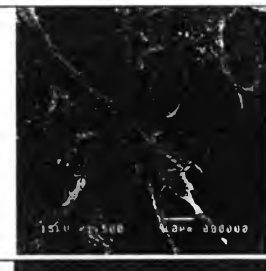
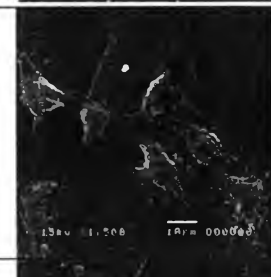
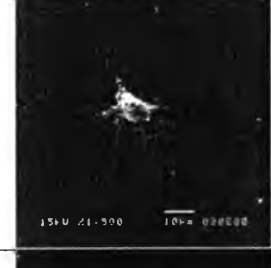
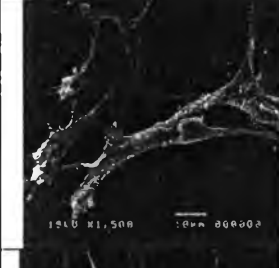
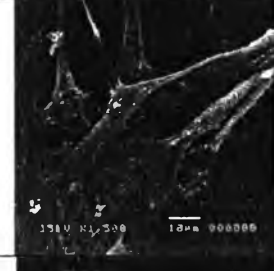
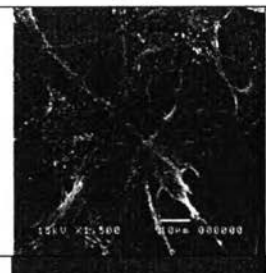
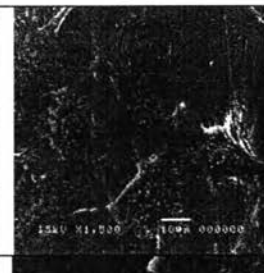
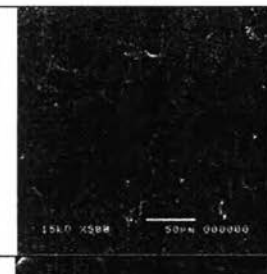
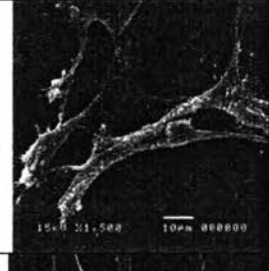
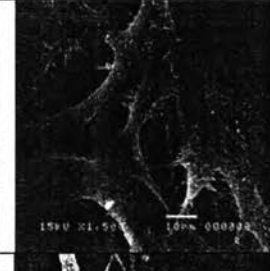
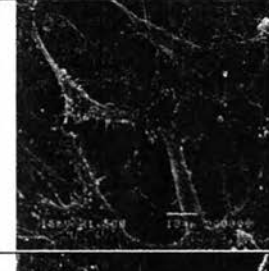
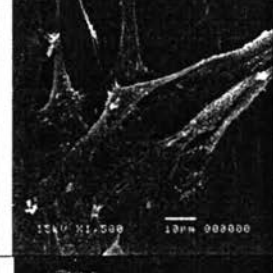
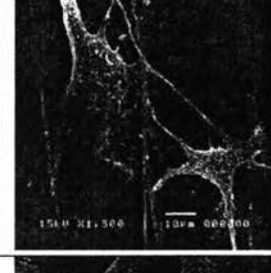
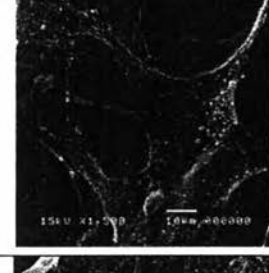
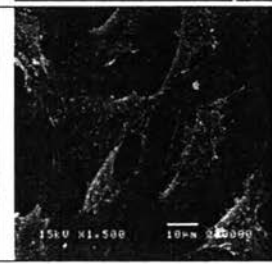
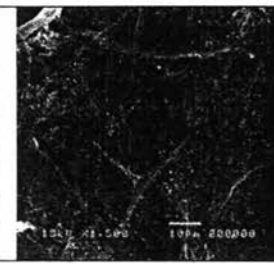
Material	Magnification = 1,500X Scale bar = 10 μm	
	6 h	24 h
Glass (control)		
Neat PCL		
Aminolyzed PCL		
CBP immobilized PCL		
BSA immobilized PCL		

Table 4.5 Selected SEM images of cultured specimens, i.e., glass (i.e., control), neat PCL, aminolyzed PCL, and crude bone protein- and bovine serum albumin-immobilized PCL film mats at various time points after MC3T3-E1 were seeded on their surfaces (magnification = 1,500X; scale bar = 10 μm)

Material	Magnification = 1,500X Scale bar = 10 μm		
	1 d	2 d	3 d
Glass (control)			
Neat PCL			
Aminolyzed PCL			
CBP immobilized PCL			
BSA immobilized PCL			

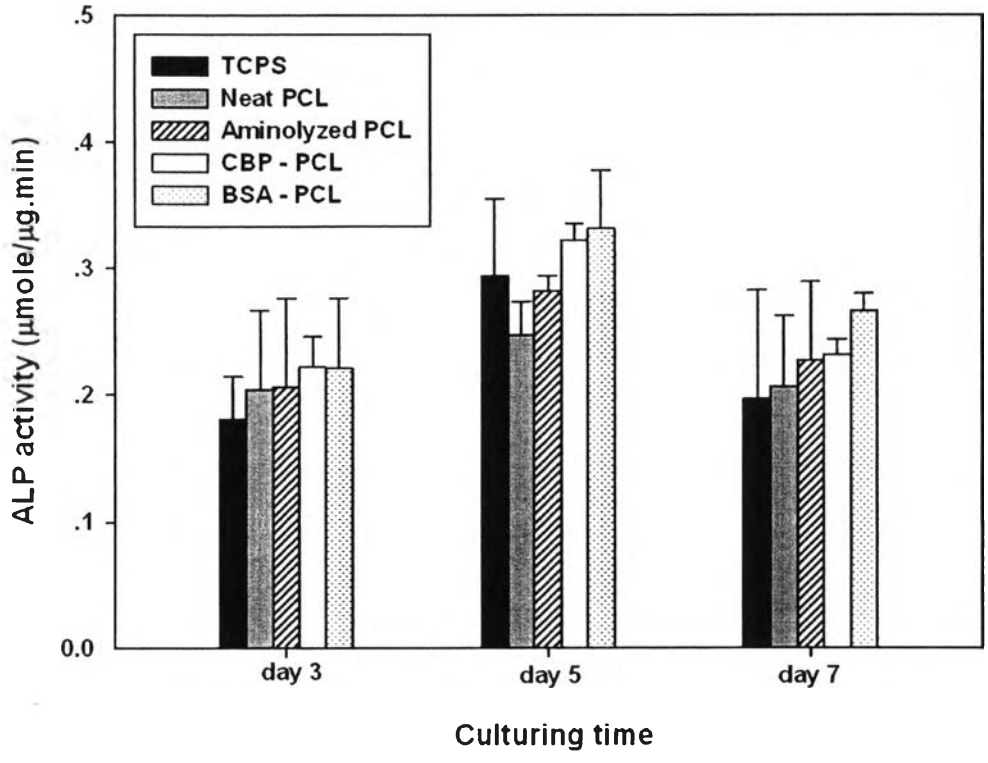


Figure 4.8 Alkaline phosphatase activity (ALP) of MC3T3-E1 that were cultured on the surfaces of TCPS, the neat and the modified PCL film mats for 3, 5 and 7 d. Statistical significance: $^{*}p < 0.05$ compared with control and $^{\#}p < 0.05$ compared to the neat PCL fibrous scaffolds at any given time point

4.3.5 Mineralization

Mineralization is the process through which an organic substance becomes impregnated by inorganic substances. To evaluate an ability of MC3T3-E1 on those TCPS (i.e. control) and materials was Alizarin Red-S was used as a dye solution to quantify the amount of present calcium salts. The strain was extracted with 10 % cetylpyridinium chloride in 10 mM sodium phosphate and the absorbance of collected dye was read at wavelength 570 nm in spectrophotometer (A Thermo Spectronic Genesis10 UV-visible spectrophotometer). The absorbance is relative to the quantity of deposited minerals on the materials as shown in Figure 4.9. All various types of PCL membranes provided higher amount of mineral deposition than TCPS did and among of these, bovine serum albumin was the highest mineral deposition. The images of materials seeded with MC3T3-E1 for 21 days and stained with Alizarin Red-S illustrated in Figure 4.10 confirmed the above data where high

intensities of stained minerals were observed on all type of modified PCL film mats. Marked mineral deposition was achieved with bovine serum albumin-immobilized PCL film, followed by crude bone protein-immobilized PCL.

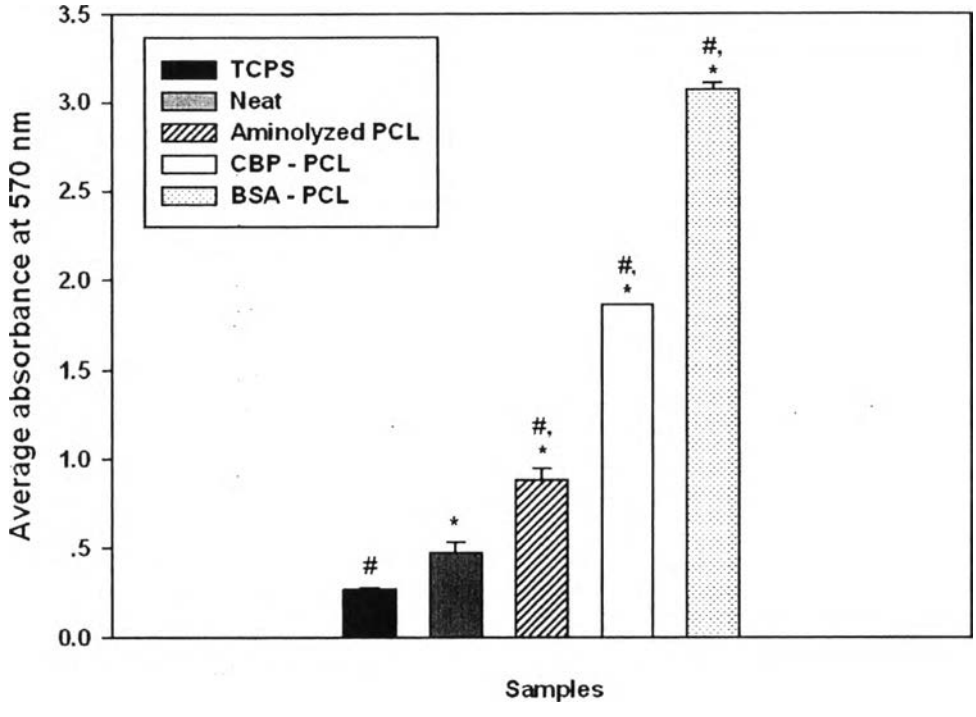


Figure 4.9 Quantification of mineral deposition in MC3T3-E1 at 21 d by the method of Alizarin Red-S staining measured the absorbance by UV-Vis spectrometer at 570 nm. Statistical significance: $^{*}p < 0.05$ compared with control, $^{\#}p < 0.05$ compared to the neat PCL at any given time point.

Considering comprehensive results, introduction of protein on modified PCL can provide positive effects for bone cells; moreover, bovine serum albumin-immobilization illustrates the best results in all experiments, followed by those that had been immobilized with crude bone protein. The improvement in those abilities of cells on protein-immobilization may be regard to the increase in wettability and compatibility on the surface of protein-modified PCL. The greater results showed in bovine serum albumin in comparison to crude bone protein may regard to lesser purify of synthesis method of crude bone protein or function of bovine serum albumin which acts as protein carrier so protein may absorb on its surface quite better and provide more preferable place for the cells. The other point

may due to the appropriate binding sites along the bovine serum albumin molecules provide more adsorption of protein extracted from the cells or even the protein in culture medium, since bovine serum albumin is a single polypeptide chain while crude bone protein may provide less suitable binding site for other protein absorbed.

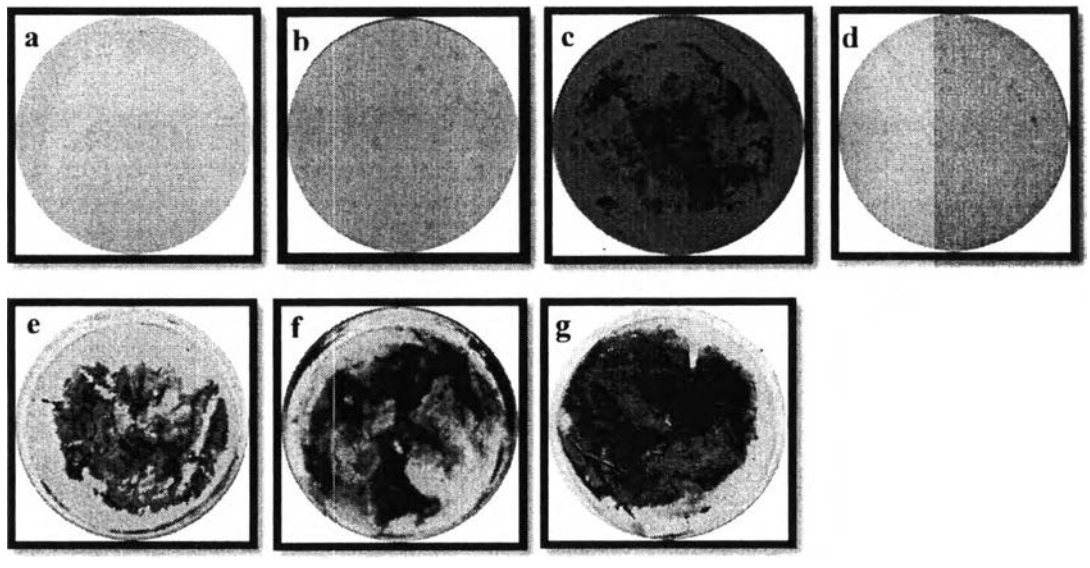


Figure 4.10 Image of Alizarin Red-S staining for the mineralization in MC3T3-E1 cells for 21 d: TCPS without (a) and with (b) cells, neat PCL without (c) and with (d) cells, aminolyzed PCL (e) crude bone protein (f), and bovine serum albumin (g) immobilized PCL film mats.

All of the obtained results can be used to support that immobilization of bovine serum albumin or crude bone protein is an attractive method for using as bone tissue engineering.