CHAPTER IV
RESULTS AND DISCUSSION

4.1 Preparation of Poly(ε-caprolactone) Electrospun Fibrous Scaffold

Poly(ε-caprolactone) (PCL; M_n = 80,000 g/mol) electrospun fibrous scaffold was prepared via electrospinning process under fixed conditions as mentioned in the previous chapter. Translucent electrospun PCL fibrous scaffolds with a thickness of (130 ± 5) μm were obtained. Morphological appearance and size of the individual fibers of the scaffolds were examined by JEOL JSM 5200 scanning electron microscopy (SEM) as shown in Figure 4.1(a). At least 100 readings of the fiber diameters from various SEM images were statistically analyzed using SemAphore 4.0 software, from which the arithmetic mean value of the individual fibers within the PCL fibrous scaffolds was determined to be (0.93 ± 0.30) μm. After surface modification of PCL electrospun fibrous scaffold via aminolysis with various 1,6-hexamethylenediamine (HMD) concentration treatments, the morphology of the surface-modified scaffolds was also observed [Figure 4.1 (b,c and d)].

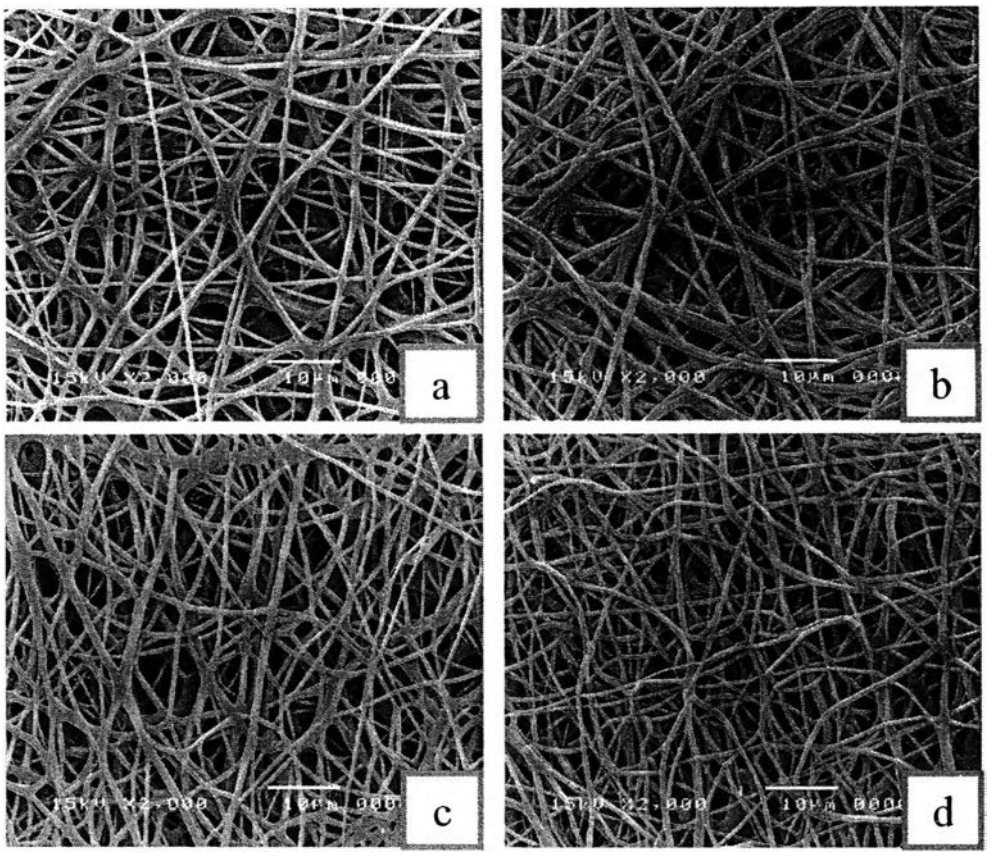


Figure 4.1 Selected SEM image of electrospun PCL fibrous scaffolds (a), aminolyzed PCL fibrous scaffolds which treated with (magnification = 2000x; scale bar = 10 μm) 1,6-hexamethylenediamine at concentration of 0.04 g/ml (b), 0.20 g/ml (c) and 0.40 g/ml (d)

4.2 Characterization of Fibrous Scaffolds

4.2.1 Density, Porosity, and Pore Volume

The density, porosity and pore volume of the unmodified PCL scaffolds and modified PCL scaffolds are shown in Table 4.1. The porosity and pore volume of these scaffolds were in the range of (96 to 97) % and (22 to 29) cm^3/g, respectively. Moreover, the density, porosity percentage and pore volume of all type of the surface-modified scaffolds were not significantly different from the unmodified PCL scaffolds.

Material	density ($x10^{-2}g/cm^3$)	porosity (%)	pore volume (cm^3/g)
PCL pure	4.69 ± 0.44	95.91 ± 0.38	20.62 ± 2.07
aminolyzed PCL	4.25 ± 0.97	96.29 ± 0.85	23.63 ± 5.28
activated PCL	4.14 ± 0.57	96.39 ± 0.50	23.64 ± 3.07
gelatin A immobilized PCL	3.75 ± 0.59	96.72 ± 0.51	26.33 ± 4.52
gelatin B immobilized PCL	3.59 ± 0.88	96.86 ± 0.77	28.15 ± 6.17
BSA immobilized PCL	3.85 ± 0.74	96.64 ± 0.65	25.88 ± 4.97
CBP immobilized PCL	4.58 ± 0.93	96.00 ± 0.81	21.64 ± 4.35

4.2.2 Water Retention Capability

Figure 4.2 demonstrates the water retention capability of these unmodified and modified PCL scaffolds via aminolysis process with different 1,6-hexamethylenediamine (HMD) concentration at 37 °C within 48 hours. In the figure shows the water retention rate increased with increasing HMD concentration treatment.

4.2.3 Degradation Study of PCL Fibrous Scaffolds

In degradation study, the disc shape of the PCL fibrous scaffolds with diameter ~ (100 to 150) μm had been immersed in the 0.1 M phosphate buffer saline (PBS), pH 7.4 at 37 °C within 48 hours. Figure 4.3 shows the degradation rate of the PCL scaffolds at different HMD concentration treatments. The weight loss increased with time and rapidly increased in the early 2 hours and steady stable after 18 hours. Furthermore, the weight loss values increased with increasing HMD concentration treatment.

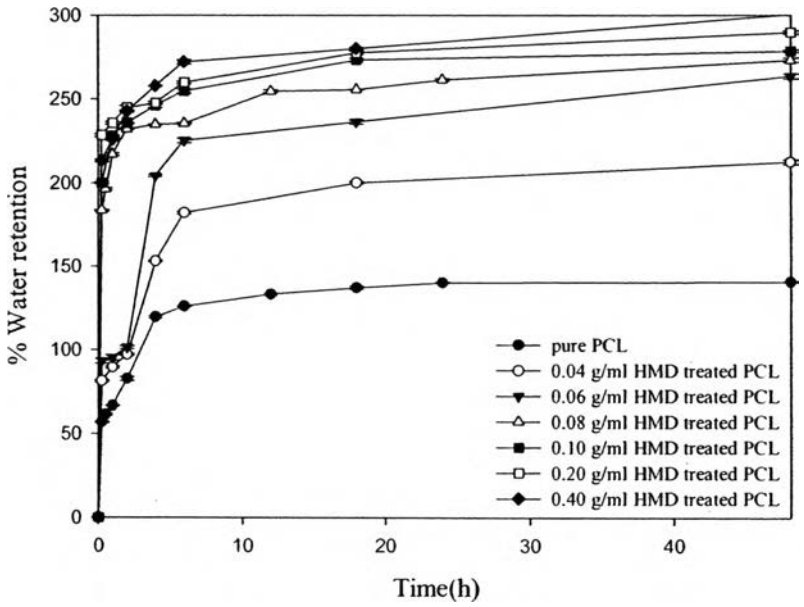


Figure 4.2 Water retention capability of these unmodified and modified PCL scaffolds via aminolysis process with different 1,6-hexamethylenediamine(HMD) concentration at 37 °C within 48 hours.

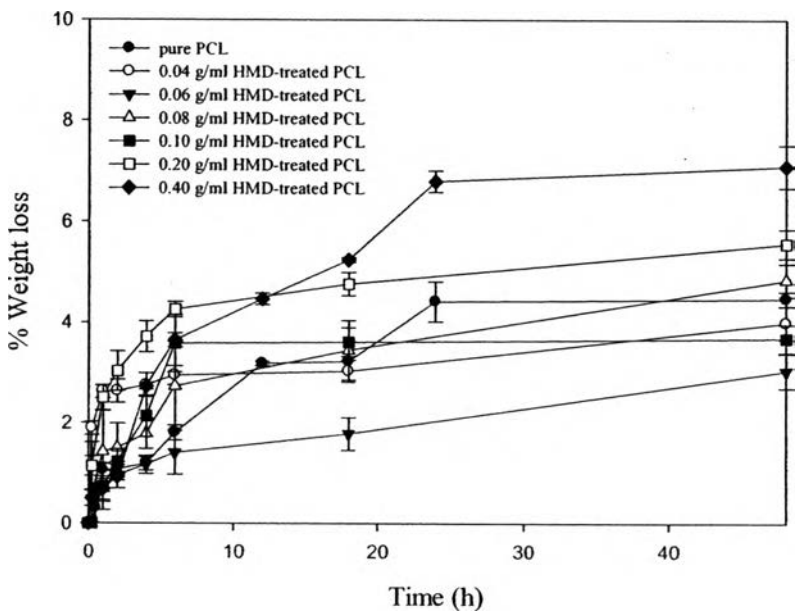


Figure 4.3 The degradation rate of the PCL scaffolds at different HMD concentration treatments.

4.3 Surface Characterization

4.3.1 Quantification of Amino Groups

Amino groups can be covalently introduced onto the surface of the electrospun PCL fibrous scaffolds 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. However, two-step procedure was carried out to avoid aggregation,. The attached amino groups had been first activated with DSC which *N*-hydroxysuccinimide was lost from the reaction and the as-formed succinimidyl esters were later reacted with respective biomolecules, *N*-hydroxysuccinimide again being cleaved from the reaction. The chemical pathway for the immobilization of biomolecule on the surface of electrospun PCL fibrous scaffolds is summerized in the Figure 4.4.

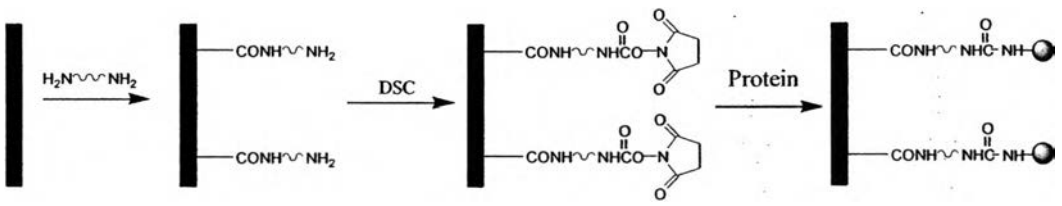


Figure 4.4 The chemical pathway for the immobilization of proteins.

The existence of free amino groups (NH_2) 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. The NH_2 density on electrospun PCL fibrous scaffold surfaces is relative to the concentration of 1,6-hexamethylenediamine, aminolyzing time, temperature, and so on. Figure 4.5 shows that NH_2 density increased with increasing 1,6-hexamethylenediamine concentration. However, when the concentration of 1,6-

hexamethylenediamine is greater than 0.20 g/ml, the structural morphology of the electrospun PCL fiber scaffolds became worse (as shown in Figure 4.1 d). To maintain structural morphology of the electrospun PCL fiber scaffolds for practical applications, PCL electrospun fibrous scaffolds were aminolyzed in a 0.20 g/ml 1,6-hexamethylenediamine/isopropanol solution at evaluated temperature for 2 hours. According to the calibration curve obtained with 1,4-dioxane–isopropanol (1:1, v/v) solution containing 1,6-hexamethylenediamine of known concentration, the NH_2 density on PCL electrospun fibrous scaffolds aminolyzed under these conditions was 1.37×10^{-7} mol/cm^2.

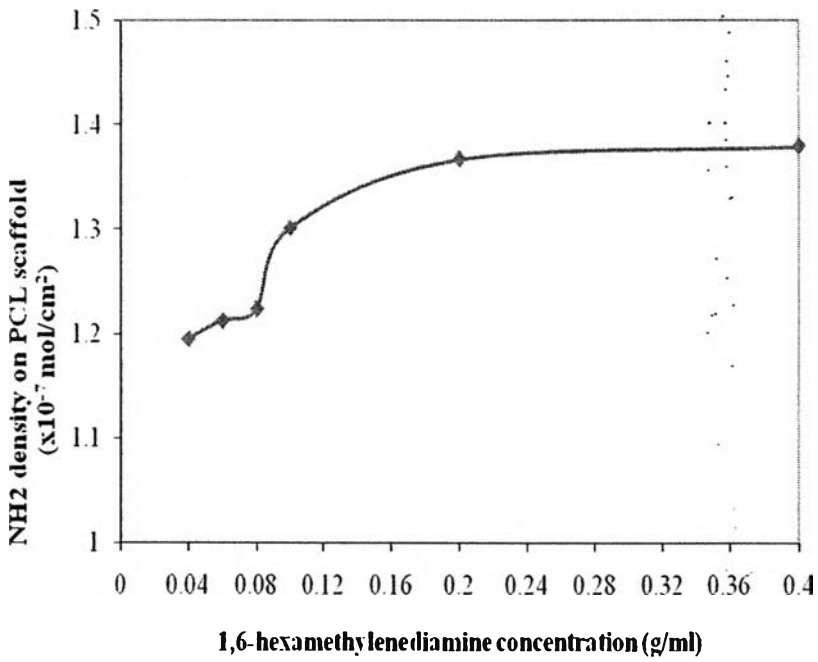


Figure 4.5 NH_2 density on PCL electrospun fibrous scaffolds as a function of concentration of 1,6-hexamethylenediamine/isopropanol solution. The PCL scaffold was aminolyzed for 2 hours.

4.3.2 Surface Wettability

To further evaluate the effect of aminolysis, surface wettability of the modified PCL fibrous scaffolds was measured. Therefore,water contact angle measurement was used to evaluate the surface wettability of the surface modified PCL fibrous scaffolds. Table 4.2 shows the water contact angle measured by the

sessile drop method decreased gradually from 123.8° to 118.0° after the scaffolds were aminolyzed with 0.04 g/mL of HMD/IPA solution for 2 hours and slightly decreased with increasing HMD concentration. That is, the introduction of the amino groups on the surface of the PCL fibrous scaffolds improved the hydrophilicity of the surface. Figure 4.6 and 4.7 show that the surface became more hydrophilic after aminolysis and protein immobilization. After the aminolyzed PCL fibrous scaffolds have been activated with DSC, their surface became more hydrophobic than the neat PCL scaffold and water contact angle also decreased after the proteins were bonded. The water drop appearance on the surface of neat PCL, gelatin type-A immobilized PCL, BSA immobilized PCL and CBP immobilized PCL are shown in Figure 4.7. CBP immobilized PCL scaffold has largest decrease of contact angle as compared with the neat PCL scaffold (control). This means that immobilization of gelatin crude bone protein can most improve the hydrophilicity of the surface.

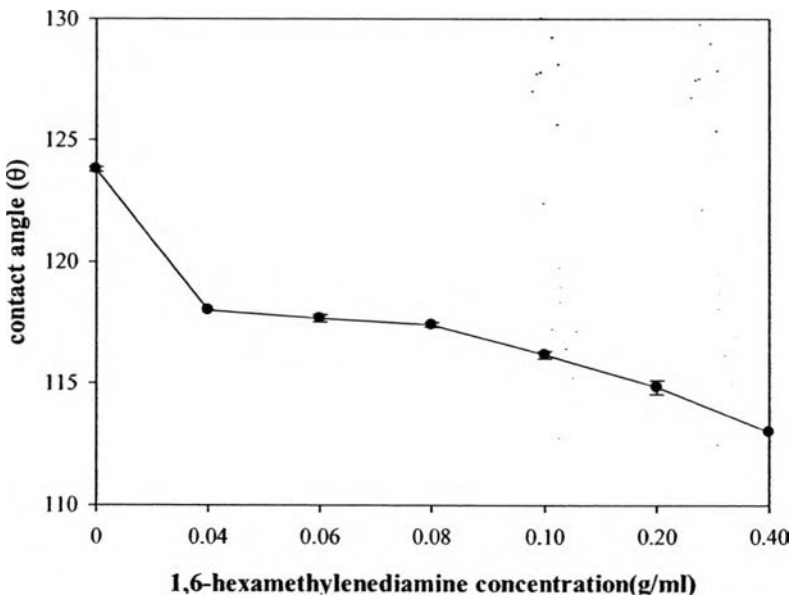


Figure 4.6 Water contact angles of the control and aminolyzed PCL scaffolds at different HMD concentration treatments measured by the sessile drop method.

Table 4.1 The water contact angle of the control and all modified PCL fibrous scaffolds measured by the sessile drop method

Material	Contact angle(θ)
Neat PCL (control)	123.7 ± 0.1
Aminolyzed PCL[a]	115.3 ± 0.2
Activated PCL[b]	118.7 ± 0.3
Gelatin type-A immobilized PCL[c1]	95.5 ± 1.1
Gelatin type-Bimmobilized PCL [c2]	94.4 ± 2.1
BSA immobilized PCL [c3]	97.5 ± 2.0
CBP immobilized PCL [c4]	48.5 ± 1.8

[a]The PCL electrospun fibrous scaffolds was immersed in 0.20 g/ml 1,6-hexamethylenediamine solution at 30 °C for 2 hours.

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

[c1.c2.c3.c4] The activated PCL scaffolds were immersed in 3.0 mg/mL gelatin type-A, gelatin type-B, BSA, and CBP solutions, respectively, for 24 h followed by the rinsing process.

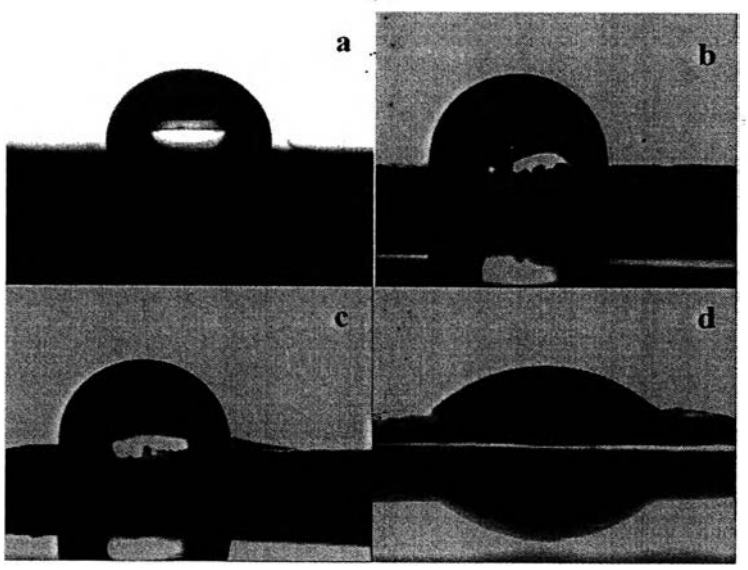


Figure 4.7 Water dropped on the surface of neat PCL fibrous scaffold (a), and PCL fibrous scaffold immobilized with 3.0 mg/ml gelatin type-A (b), 3.0 mg/ml bovine serum albumin (c) and 3.0 mg/ml crude bone protein (d).

4.3.3 Chemical Analysis of Surface

ATR-FTIR spectra of PCL and modified PCL fibrous scaffolds are shown in Figure 4.8 and 4.9 There was a major absorption peak assigned to the ester carbonyl of neat PCL appeared at 1755 cm^{-1}.

The low broad signals from N–H stretching of NH_2 at ~ (3300 to 3500) cm^{-1} or carbonyl stretching of amide group at 1650 cm^{-1} were observed on those surface modified PCL. After biomolecule immobilization, C=O stretching peak occurs at 1650 cm^{-1} (amide I) and N–H bending peak at 1550 cm^{-1} (amide II) and 1350 cm^{-1} (amide III) evidently appeared in the spectra of all biomolecule-immobilized PCL scaffolds. Moreover, the higher broad peak in the range of 3000 to 3600 cm^{-1} corresponding to N–H stretching of NH_2 in gelatin type-A, gelatin type-B, BSA and CBP was observed indicating that gelatin type-A, gelatin type-B, BSA and CBP have been successfully immobilized on the amino-containing PCL scaffolds. However, the signal of FTIR spectrum were low, resulting from the extremely low concentration of NH_2 present within the sampling depth of ATR-FTIR (1 to 2 μm).

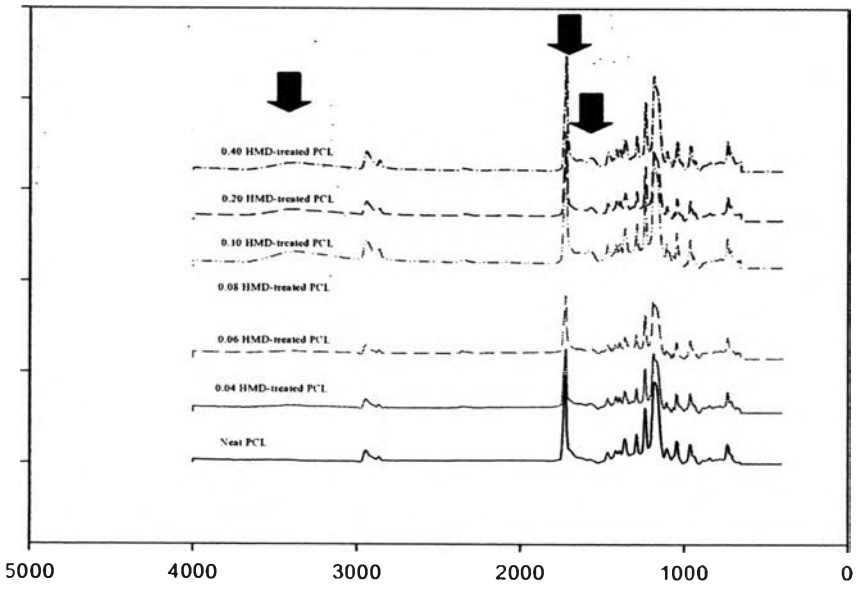


Figure 4.8 ATR-FTIR spectra of neat and aminolyzed PCL fibrous scaffolds with different HMD concentration treatments.

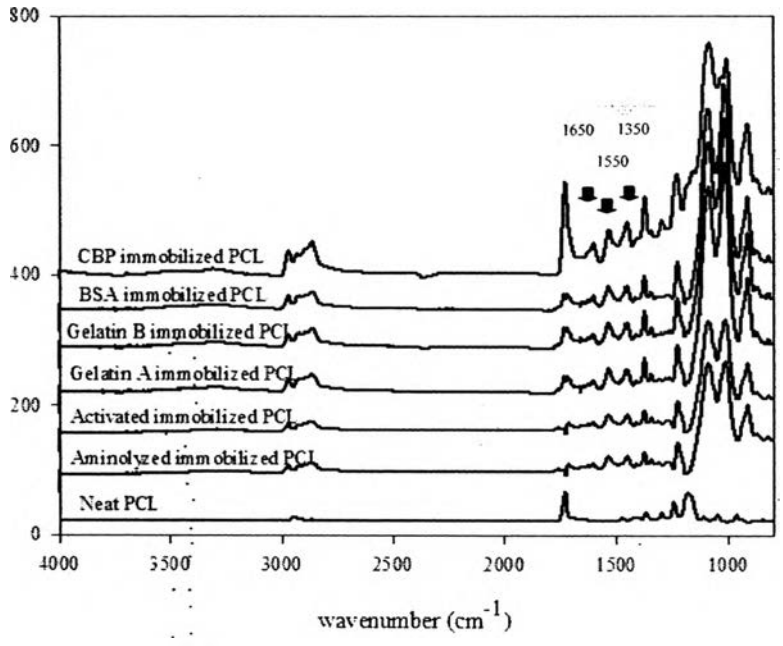


Figure 4.9 ATR-FTIR spectra of neat and modified PCL fibrous scaffolds.

4.3.4 Elemental Composition of the Surface

The surface of modified PCL fibrous scaffolds were further determined the elemental composition by using X-ray Photoelectron Spectrometer (XPS). To study the effect of the aminolysis condition on the surface, N_{1s}/C_{1s} ratios as a function of HMD concentration treatment were evaluated. Table 4.4 shows that the more diamine concentration treated, the more N_{1s}/C_{1s} ratio observed due to the increasing in NH_2 groups. After aminolysis, the N_{1s}/C_{1s} ratio was increased from 0 to 0.0168 because NH_2 groups were introduced on the PCL surface. Figure 4.10 demonstrated that N_{1s} peak appeared after immobilization with proteins (gelatin type-A , gelatin type-B , bovine serum albumin, and crude bone protein) due to the large amount of nitrogen atom in proteins structure was introduced.

Table 4.2 N_{1s}/C_{1s} ratios as a function of 1,6-hexanediamine concentration

1,6-hexamethylenediamine concentration (g/ml)	N_{1s}/C_{1s} ratio
0.04	0.0063
0.06	0.0079
0.08	0.0163
0.10	0.0169
0.20	0.0170
0.40	0.0170

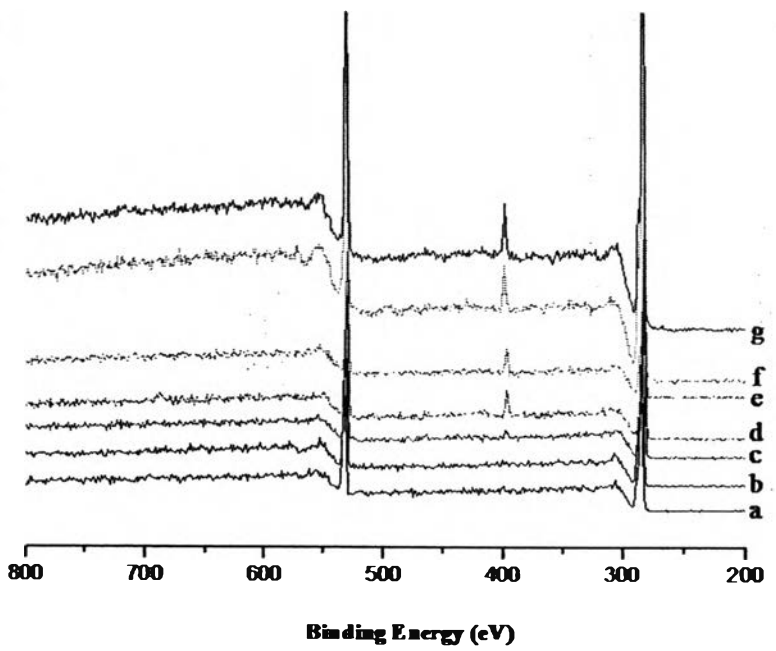


Figure 4.10 The survey XPS spectra of (a) neat PCL, (b) aminolyzed PCL with 0.20 g/ml HMD treatment, (c) activated PCL, (d) PCL immobilized with 3 mg/ml gelatin type-A solution, (e) 3 mg/ml gelatin type-B solution, (f) 3 mg/ml bovine serum albumin solution, and (g) 3 mg/ml crude bone protein solution.

4.4 Biological Characterizations

4.4.1 Cytotoxicity

4.4.1.1 Direct Cytotoxicity

Direct cytotoxicity test is a method to evaluate the toxic effect of proteins directly to cells. The effect was investigated with mouse calvaria-derived pre-osteoblastic cells (MC3T3-E1), based on the initial 40,000 cells/well seeded. MC3T3-E1 were cultured in 24-well culture plate to allow cell growth. The test was conducted by adding gelatin type-A, gelatin type-B, BSA protein and crude bone protein extracts in 2% serum-containing MEM diluted with 7-day extraction medium in seeded cells as mentioned above and cultured for 1, 2, and 3 d. Figure 4.11 shows that the viability of MC3T3-E1, cultured with 2% serum-containing MEM extraction media adding with all types of proteins for 1, 2 and 3 days, was increased with increasing the culturing time in the respective media. The viability of cells (relative with control TCP) of all material is more than 80 %. All of the obtained results clearly suggested that all types of proteins, released no substances at levels that were harmful to cells.

4.4.1.2 Indirect Cytotoxicity

The potential use of these fibrous scaffolds as bone scaffolds was first evaluated by an indirect cytotoxicity test with mouse calvaria-derived pre-osteoblastic cells (MC3T3-E1), based on the initial 40,000 cells/well seeded. The test was carried out on TCP (control), the neat PCL, aminolyzed PCL, activated PCL, gelatin type-A, gelatin type-B, BSA and CBP immobilized PCL fibrous scaffolds. MC3T3-E1 were cultured in a 24-well culture plate in 2 % serum-containing MEM extraction medium from all type of fibrous scaffolds for 1, 2, and 3 days. Figure 4.12 shows that the viability of MC3T3-E1, cultured with 2 % serum-containing MEM diluted with 1-day extraction media prepared by all types of modified scaffolds, was increased with increasing the culturing time in the respective media and the viability of cells on all types of material measured by MTT assay was more than 80 %. All of the obtained results clearly suggested that all types of the PCL fibrous scaffolds, released no substances at levels that were harmful to cells.

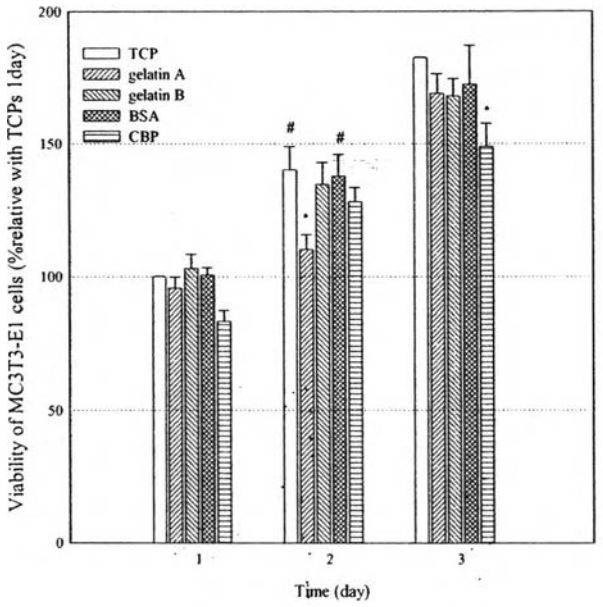


Figure 4.11 The viability of MC3T3-E1, cultured with 2% serum-containing MEM extraction media adding with all types of proteins for 1, 2 and 3 days. Statistical significance: $*p < 0.05$ compared with control and $^{\#}p < 0.05$ compared to the neat PCL fibrous scaffolds at any given time point.

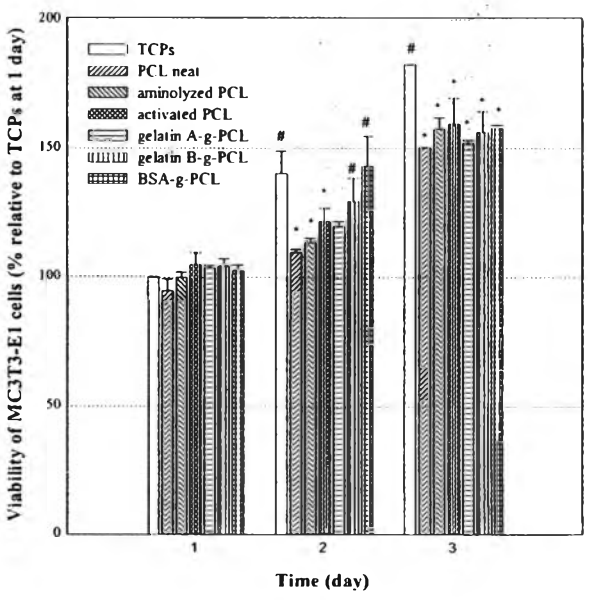


Figure 4.12 The viability of MC3T3-E1, cultured with 2% serum-containing MEM extraction media from all type of PCL fibrous scaffolds for 1, 2, and 3 days relative to TCPS at 1 day Statistical significance: $*p < 0.05$ compared with control and $^{\#}p < 0.05$ compared to the neat PCL fibrous scaffolds at any given time point.

4.4.2 Cell Attachment and Proliferation

The potential for use of the neat and the modified PCL fibrous scaffolds was further evaluated by determination their ability to support both the adhesion and the proliferation of MC3T3-E1. The viability of the cells that had been cultured on the surface of TCPS for 2 h was taken as the basis to arrive at the relative viability shown in a Figure. Figure 4.13 shows the attachment of MC3T3-E1 on the surfaces of TCPS, neat PCL, aminolyzed PCL, activated PCL, and protein-immobilized PCL at 2, 4, and 6 h after cell seeding in terms of viability. On TCPS, the number of the attached cells increased rapidly from ~100% at 2 h after cell seeding to ~257% at 6 h after cell seeding, based on the initial number of cells seeded (40,000 cells/well). While, the number of cells attached on these fibrous scaffolds was lower in comparison with that on TCPS at any given time point. There was the most viability of cells on crude bone protein-immobilized PCL fibrous scaffold among various types of the modified fibrous scaffolds. This obtained result suggested that the cells prefer to adhere on CBP-immobilized PCL fibrous scaffold rather than other modified scaffolds. However, The lesser viability of cells in the attachment period on various types of the fibrous scaffolds in comparison with that on TCPS could be due to the lesser number of cells that were able to attach on the rough surface of the fibrous scaffolds in comparison with the smoother and hydrophilic surface of TCPS.

Figure 4.14 shows the proliferation of MC3T3-E1 on the surfaces of TCPS, neat PCL, aminolyzed PCL, activated PCL and protein-immobilized PCL on day 1, 2, and 3 after cell culture in terms of viability (%relative to TCPS at day1). On TCPS, the number of cells increased from ~100% at 1 day after cell culture to ~133 % at 3 days after cell culture, based on the initial 40,000 cells/well seeded. In comparison with that on TCPS, the viability of the cells cultured on various types of PCL fibrous scaffolds were significantly higher at any given time point. The viability of cells proliferated on these fibrous scaffolds, at day 3, was higher than TCPS of about (3 to 4) fold for neat PCL, aminolyzed PCL, and activated PCL and about 8 fold for all type of protein-immobilized PCL. The improvement was achieved with the protein-immobilized PCL fibrous scaffolds on day 3 after cell culture. The

greater number of cells in the proliferation period on all types of fibrous scaffolds could be because of high surface area to volume and high porosity of the electrospun fibrous scaffolds through which the cells were able to penetrate into the scaffolds. Among the various modified PCL fibrous scaffolds, the BSA-immobilized PCL fibrous scaffolds provided the most significant improvement in the ability to support the proliferation of the cells which could be due to the protein-containing and more hydrophilic surface of the substrate.

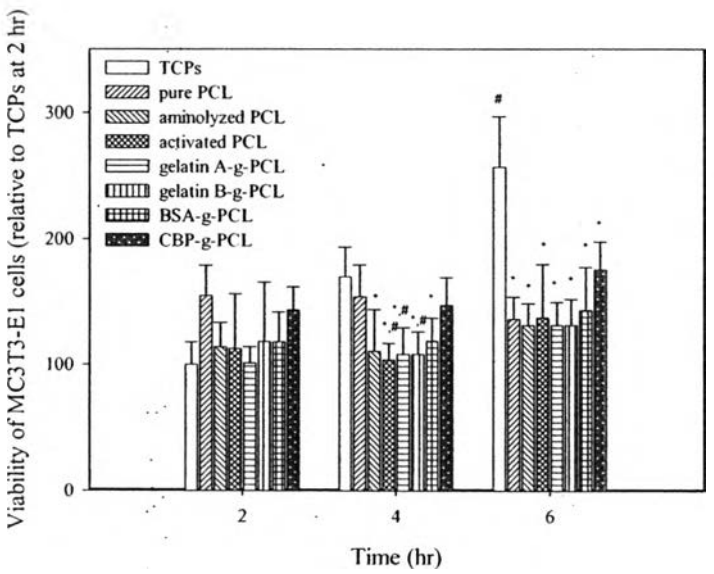


Figure 4.13 Attachment of MC3T3-E1 that had been seeded or cultured on the surfaces of TCPS and the neat and the modified PCL fibrous scaffolds for 2, 4, and 6 h. Statistical significance: $^{*}p < 0.05$ compared with control and $^{\#}p < 0.05$ compared to the neat PCL fibrous scaffolds at any given time point.

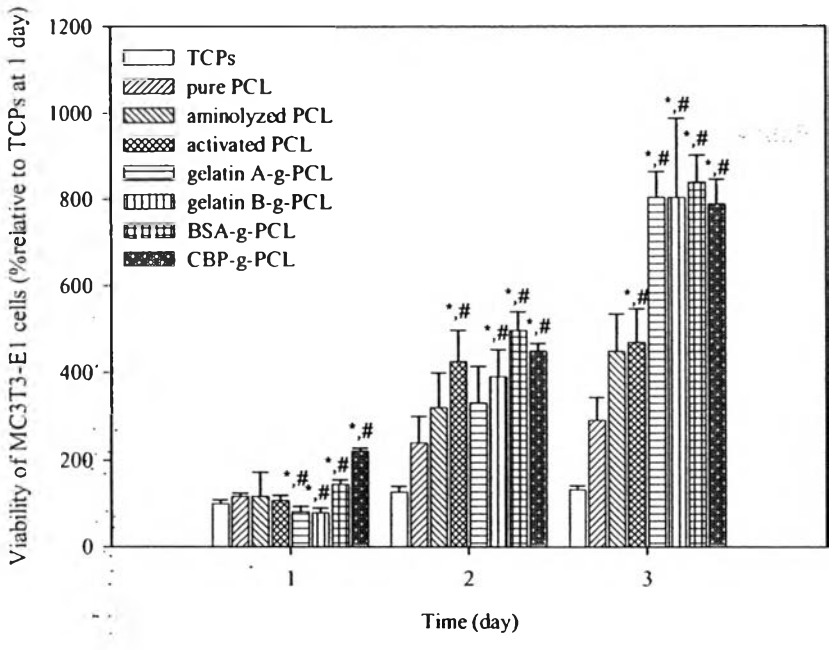


Figure 4.14 Proliferation of MC3T3-E1 that had been seeded or cultured on the surfaces of TCPS and the neat and the modified PCL fibrous scaffolds for 1, 2, and 3 days. Statistical significance: $^{*}p < 0.05$ compared with control and $^{\#}p < 0.05$ compared to the neat PCL fibrous scaffolds at any given time point.

4.4.3 Cell Morphology

Table 4.6 and 4.7 show selected SEM image (magnification = 2000X; scale bar = 10 μm) of MC3T3-E1 that were cultured on the surfaces of neat PCL, activated PCL and gelatin type-A, gelatin type-B, bovine serum albumin, and crude bone-immobilized PCL at different time points. These images provided snap shots in time that revealed the morphology of the cells and interaction between the cells and the tested surfaces. At 2 hours after cell seeding, based on the initial 40,000 cells/well seeded, the morphology of cells on almost all types of the modified PCL scaffolds became round. Exceptionally, the morphology of cells on BSA-immobilized and CBP-immobilized started to extend their cytoplasm and became ellipse. At 4 h after cell seeding, the majority of the cells on all types of modified PCL scaffolds extended their cytoplasm, an evidence of the ability of the cells to attach on the modified surface. At 6 h after cell seeding, expansion of the cytoplasm

of the majority of the cells was evident. The majority of MC3T3-E1 that had been seeded on the surfaces of unmodified PCL fibrous scaffolds for 4 h was remained round, but a closer examination around the edge of the cells revealed an evidence of filopodia. The majority of the cells were evidently expanded after 6 h of cell seeding. On the other hand, the majority of the cells seeded on the surfaces of various types of modified PCL fibrous scaffolds showed an evidence of the extension of their cytoplasm on the fibrous surface even at 4 h after cell seeding. These results suggested that the cells prefer the fibrous surfaces of modified PCL over that of the unmodified. At 1,2, and 3 days after cell seeding, the majority of the cells seeded on the surfaces of all types of modified PCL fibrous scaffolds expanded over the area of the scaffolds which was the most expansion on the surface of BSA-immobilized and CBP-immobilized PCL fibrous scaffolds. From the attachment, proliferation and cell morphology results, we can suggest that the cell prefer to grow and expand on BSA-immobilized and CBP-immobilized PCL fibrous scaffolds over other materials.

Table 4.3 Selected SEM images of cultured specimens, i.e., glass (i.e., control), neat PCL, aminolyzed PCL, activated PCL, and protein-immobilized PCL fibrous scaffolds at various time points after MC3T3-E1 were seeded on their surfaces (magnification = 2,000X; scale bar = 10 μm)

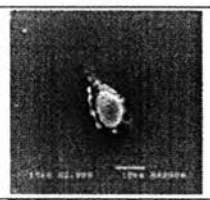
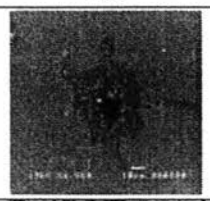
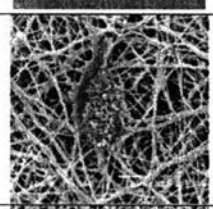
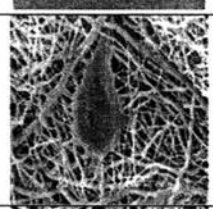
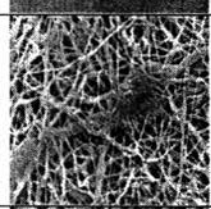
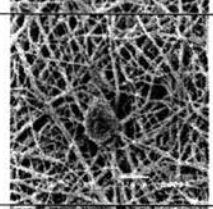
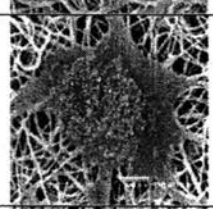
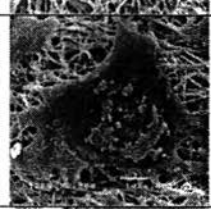
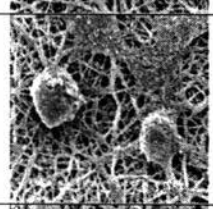
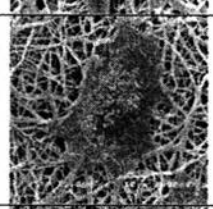
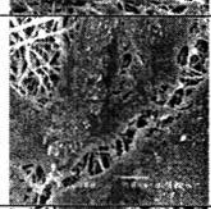
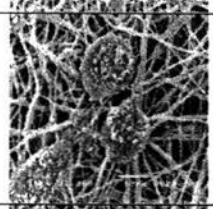
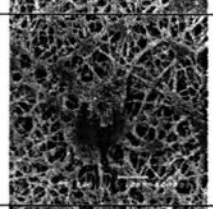
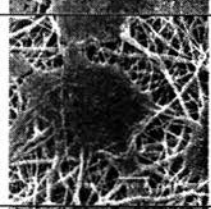
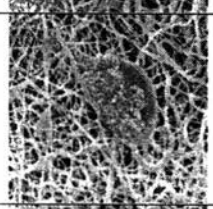
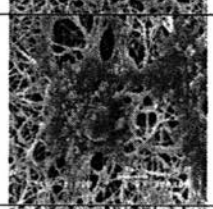
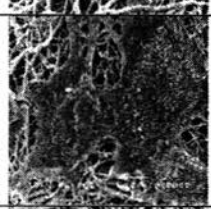
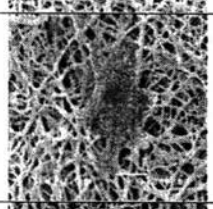
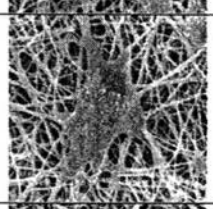
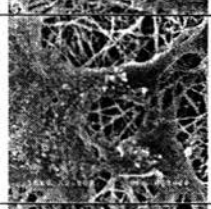
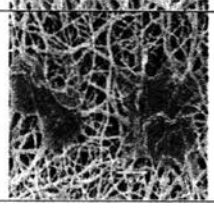
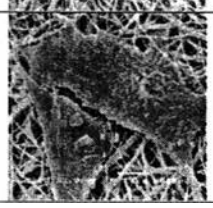
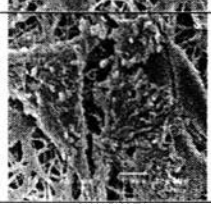
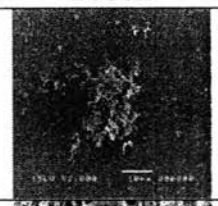
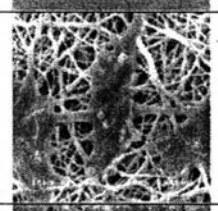
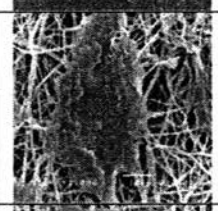
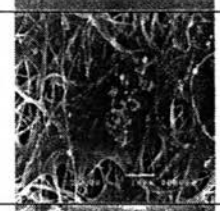
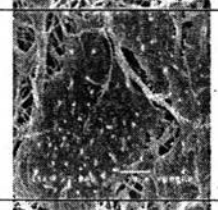
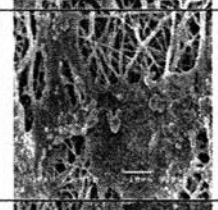
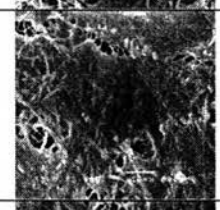
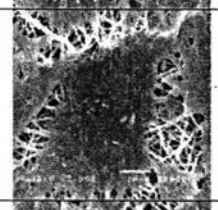
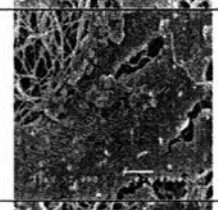
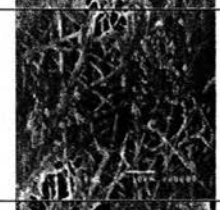
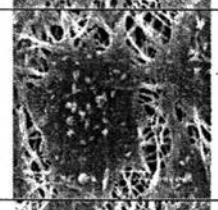
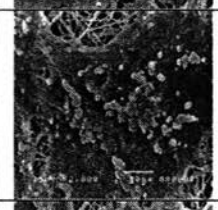
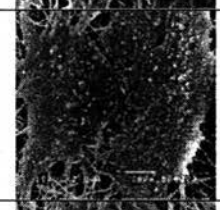
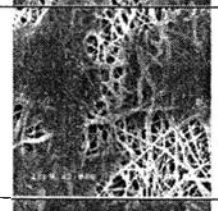
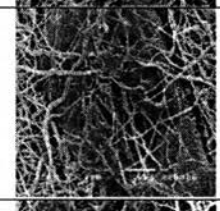
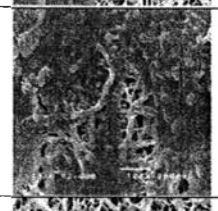
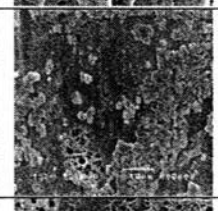
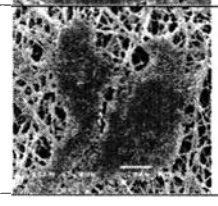
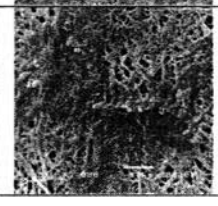
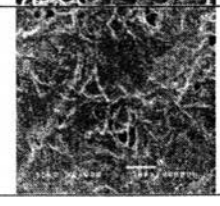
Material	2 h	4 h	6 h
Glass (control)			
Neat PCL			
Aminolyzed PCL			
Activated PCL			
Gelatin A immobilized PCL			
Gelatin B immobilized PCL			
BSA immobilized PCL			
CBP immobilized PCL			

Table 4.4 Selected SEM images of cultured specimens, i.e., glass (i.e., control), neat PCL, aminolyzed PCL, activated PCL, and protein-immobilized PCL fibrous scaffolds at various time points after MC3T3-E1 were seeded on their surfaces (magnification = 2,000X; scale bar = 10 μm)

Material	1 day 2000x	2 day 2000x	3 day 2000x
Glass(control)			
Neat PCL			
Aminolyzed PCL			
Activated PCL			
Gelatin A immobilized PCL			
Gelatin B immobilized PCL			
BSA immobilized PCL			
CBP immobilized PCL			

4.4.4 Alkaline Phosphatase Activity (ALP)

The ability for these PCL fibrous scaffolds to support differentiation, in addition to attachment and proliferation, of cultured cells is another important purpose suggesting potential for use of the scaffold. Alkaline phosphatase is used as an osteoblastic differentiation marker, as it is produced only by cells showing mineralized ECM. The ALP activity of MC3T3-E1 on TCPS (i.e. controls), neat PCL, aminolyzed PCL, activated PCL, and protein-immobilized PCL were evaluated at 3 and 7 days in culture. Figure 4.15 apparently shows the amount of ALP synthesized by the cells that were cultured on TCPS and all of the fibrous scaffolds increased with the initial increase in time in culture. In comparison with other substrates, BSA immobilized PCL fibrous scaffolds exhibited the highest ALP activity of MC3T3-E1 which is close to that of the activated PCL and TCPS at day 7 of cell culturing time. From the obtained results, it was suggested that BSA-immobilized PCL fibrous scaffold was the best among the fibrous scaffolds that promoted both proliferation and differentiation of MC3T3-E1. However, additional long term experiments in order to clarify the effect of scaffolds on the differentiation of osteoblasts are currently determined.

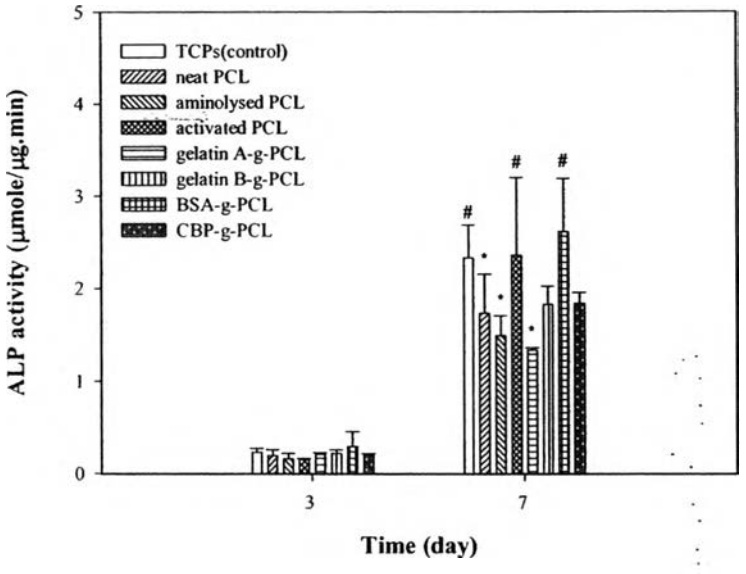


Figure 4.15 Alkaline phosphatase activity (ALP) of MC3T3-E1 that were cultured on the surfaces of TCPS and the neat and the modified PCL fibrous scaffolds for 3 and 7 d. Statistical significance: $^{*}p < 0.05$ compared with control and $^{\#}p < 0.05$ compared to the neat PCL fibrous scaffolds at any given time point

4.4.5 Mineralization

Mineralization was quantified by Alizarin Red-S which is a dye that binds selectively calcium salts. The strain was extracted with 10 % cetylpyridinium chloride in 10 mM sodium phosphate for 1 hour 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 4.16. The images of scaffolds seeded with MC3T3-E1 for 21 days and stained with Alizarin Red-S illustrated in Figure 4.17 confirmed that the above data where high intensity of stained minerals were observed on all type of immobilized PCL scaffolds. and. The CBP-immobilized PCL scaffolds showed the highest mineral deposition compared with control, neat PCL, and other modified materials. This result can be used to support that the crude bone protein immobilization is an attractive method to be used for fabrication of further developed fibrous scaffolds for bone tissue engineering.

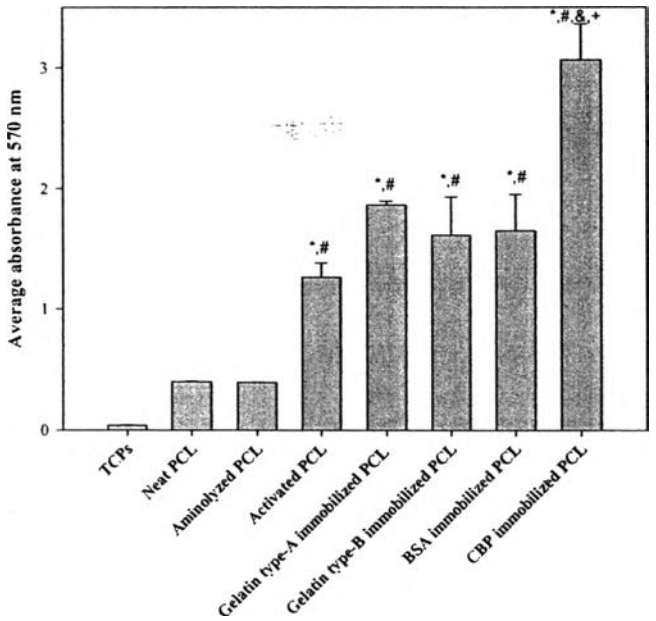


Figure 4.16 Quantification of mineral deposition in MC3T3-E1 at 21 d by the method of Alizarin Red-S staining measured the absorbance by UV-vis spectrometer at 570 nm. Statistical significance: $^{*}p < 0.01$ compared with control, $^{\#}p < 0.01$ compared to the neat PCL, $^{\&}p < 0.01$ compared with gelatin type-A immobilized PCL, and $^{+}p < 0.01$ compared with gelatin type-B immobilized PCL fibrous scaffolds at any given time point.

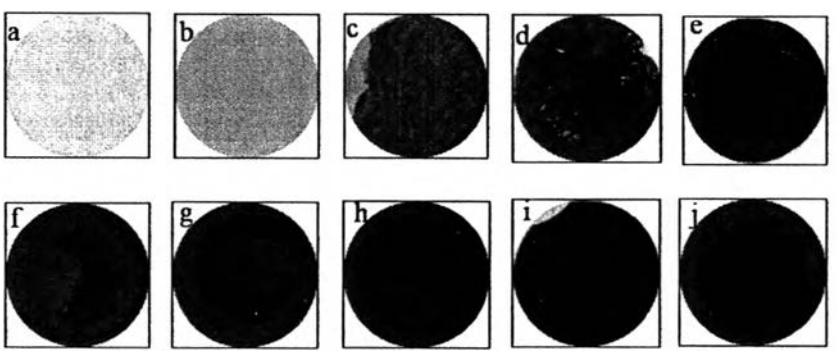


Figure 4.17 Image of Alizarin Red-S staining for the mineralization in MC3T3-E1 cells for 21 d: TCPS without (a) and with (b) cells , neat PCL without (c) and with (d) cells, aminolyzed (e) and activated PCL (f),and gelatin type-A (g), gelatin type-B (h), bovine serum albumin (i) and crude bone protein (j) immobilized PCL fibrous scaffold.