CHAPTER IV
RESULTS AND DISCUSSION

4.1 Preparation of Polymeric Fibrous Scaffolds

Polycaprolactone (PCL; M_n = 80,000 g/mol) electrospun fibrous scaffold were prepared via electrospinning technique under fixed condition as mentioned in previous chapter. Translucent electrospun PCL fibrous scaffolds with a thickness of 150 ± 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 showed in Figure 4.1. 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 fiber diameter within the PCL fiber mats was determined to be 0.50 ± 0.05 μm.

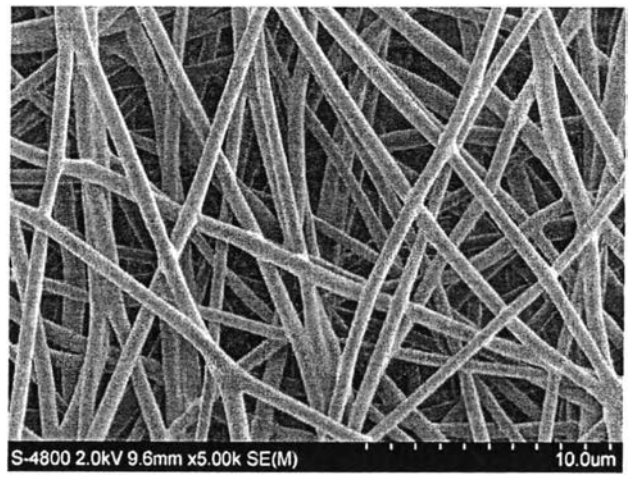


Figure 4.1 Selected SEM image of PCL electrospun fibrous scaffolds (magnification = 5000x; scale bar = 10 μm).

4.2 Scaffold Characterization

4.2.1 Surface Wettability

To further evaluate the effect of aminolysis, surface wettability of the aminolyzed PCL fibrous scaffolds with respect to that of the neat PCL fibrous scaffolds was measured. Besides, water contact angle measurement was also used to evaluate the surface wettability of the BSA-immobilized PCL fibrous scaffolds. Table 4.1 shows the surface wettability of unmodified PCL, aminolyzed PCL, activated PCL and BSA-immobilized PCL (BSA = 3.0 mg/ml) fibrous scaffolds. The water contact angle measured by the sessile drop method decreased slightly from 109.4° to 105.1° after the scaffolds were aminolyzed with 0.2 g/ml of HMD/IPA solution for 2 h. That is, introduction of the amino groups on the surface of the PCL fibrous scaffolds improved the hydrophilicity of the surface. After the aminolyzed PCL fibrous scaffolds have been activated with DSC, their surface became more hydrophobic as evidenced by the water contact angle of 115.0°. Water contact angle decreased importantly when the BSA was immobilized. Figure 4.2 shows that the surface became much more hydrophilic after BSA immobilization. Water contact angle was 94.4° for BSA 3.0 mg/ml immersion. Water contact angle measurements of physisorbed BSA-coated PCL surfaces have contact angle almost the same as the aminolyzed PCL surfaces (103.2° and 105.1°) but much higher values than covalently bonded with protein surfaces. That means activation step is an important step in protein grafting process.

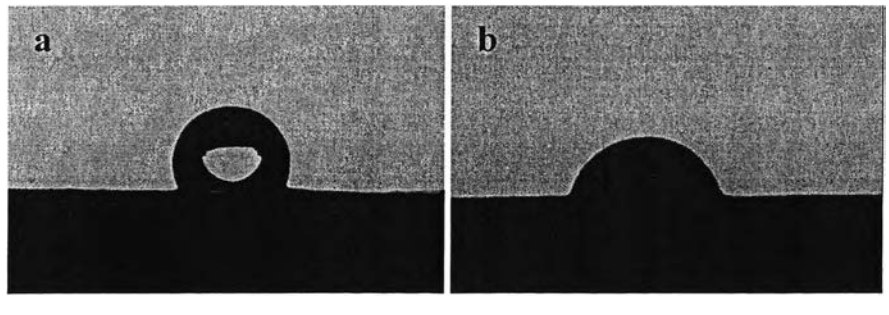


Figure 4.2 Water dropped on the surface of neat PCL fibrous scaffolds (a) and PCL fibrous scaffolds immobilized with 3.0 mg/ml BSA (b).

Table 4.1 Water contact angles of the control and modified PCL fibrous scaffolds

Samples	Water contact angle/degree (Sessile drop)
Control PCL	109.4°
Aminolyzed PCL[a]	105.1°
Activate PCL[b]	115.0°
BSA Immobilized PCL[c]	94.4°

[a]The PCL e-spun fiber mats were immersed in 0.2 g/ml HMD/IPA solution for 2 h.

[b]The aminolyzed PCL fibers were immersed in 0.1M DSC solution for 1 h.

[c]The activated PCL fibers were immersed in 3.0 mg/ml BSA solutions for 24 h followed by the rinsing process.

4.2.2 Chemical Analysis of Surface

ATR-FTIR spectra of PCL and modified PCL fibrous scaffolds are showed in Figure 4.6. A major absorption peak assigned to the ester carbonyl of virgin PCL appeared at 1745 cm^{-1} was showed. However, the spectra of all of the modified materials are almost the same as the unmodified PCL. This 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).

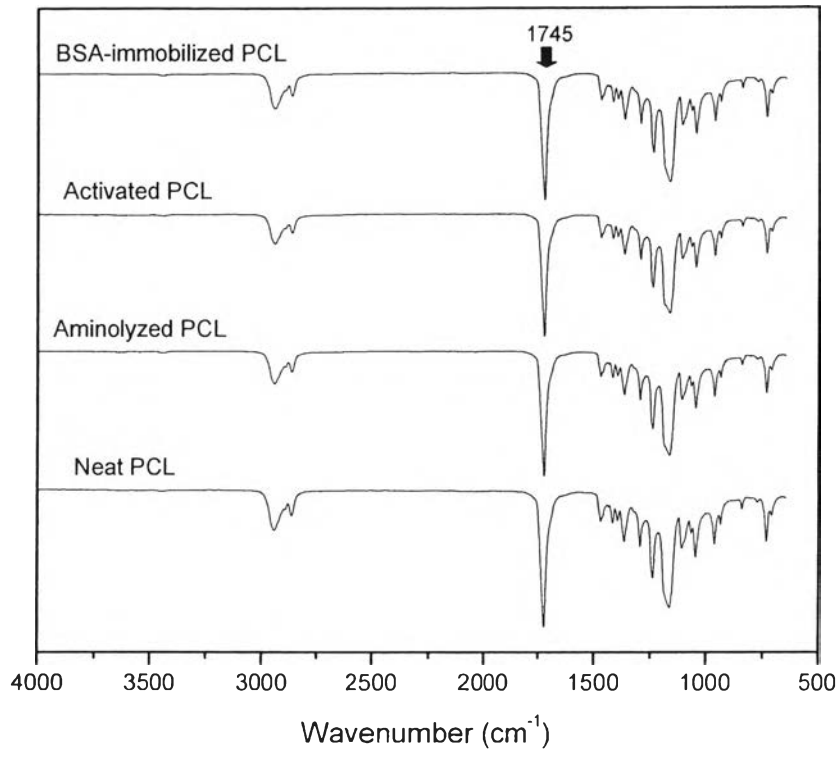


Figure 4.3 ATR-FTIR spectra of neat and modified PCL fibrous scaffolds.

X-ray photoelectron spectrometer (XPS) was applied to confirm the success of aminolysis reaction and immobilization of BSA. It was used to evaluate the N_{1s}/C_{1s} ratio of the unmodified and modified PCL fibrous scaffolds. The N_{1s}/C_{1s} ratio was expected to increase after aminolysis reaction and BSA immobilization.

4.2.3 Elemental Composition of the Surface

The surface alteration after the PCL fibrous scaffolds were modified was studied by XPS. To study the effect of the aminolysis condition on the surface alteration, N_{1s}/C_{1s} ratios as a function of HMD concentration and aminolyzing time were evaluated. XPS analysis of aminolyzed PCL sample surfaces showed the presence of all expected elements, namely nitrogen and carbon. The calculated ratios rose for all aminolyzed samples respect to the non-aminolyzed controls. As showed

in Table 4.2, the N_{1s}/C_{1s} ratio increased with increasing aminolyzing time to reach a maximum value with at about 3 h. After aminolysis reaction, the N_{1s}/C_{1s} ratio reached to 0.0069 because amine groups from HMD were introduced on the surface. As showed in Table 4.3, the N_{1s}/C_{1s} ratio reached to 0.0094 by reaction with DSC. It shows that the nitrogen concentration increased when succinimidyl esters was formed. Finally, the N_{1s}/C_{1s} ratios obviously increase after BSA immobilization due to the large amount of nitrogen atom in biomolecular structure was additionally introduced.

Table 4.2 N_{1s}/C_{1s} ratios as a function of aminolyzing time

Aminolyzing time (min)	N_{1s}/C_{1s} ratio
0	0.0000
10	0.0022
30	0.0040
60	0.0051
120	0.0065
180	0.0069

The aminolysis reaction took place in 0.2 g/ml solution.

Table 4.3 N_{1s}/C_{1s} ratios of the unmodified and modified PCL fibrous scaffolds

Sample	N_{1s}/C_{1s} ratio
Unmodified PCL	0.0000
Aminolyzed PCL[a]	0.0065
Activated PCL[b]	0.0094
BSA Immobilized PCL[c]	0.0179

[a]The PCL e-spun fiber mats were immersed in 0.2 g/ml HMD/IPA solution for 2 h.
[b]The aminolyzed PCL fiber mats were immersed in 0.1 M DSC solution for 1 h.
[c]The activated PCL fiber mats were immersed in BSA solution 3.0 mg/ml for 24 h.

4.2.4 Protein Adsorption Test

The amount of protein adsorbed on the surface PCL fibrous scaffolds were determined based on bicinchoninic acid method by BCA protein assay kit and calculated against the standard curve. Figure 4.4 showed the effect of surface modification to protein adsorption. The neat and activated PCL fibrous scaffolds have more ability to adsorb BSA protein than aminolyzed PCL fiber mats. Due to the ability to rearrange itself of BSA protein, it can form hydrophobic bonding with hydrophobic surface better than that of hydrophilic ones. Hydrophobicity of the aminolyzed PCL fiber mats lead to the small amount of BSA protein which adsorbed on the surface of aminolyzed PCL fiber mats. As above-mentioned, it can be concluded that the amount of protein adsorbed dramatically increased at the initial stage. Then it tended to slightly increased as a function of protein concentration. Thus, the proper value of protein concentration at 3000 μg/ml has been chosen for further analysis due to the most assistance of capability of protein adsorption.

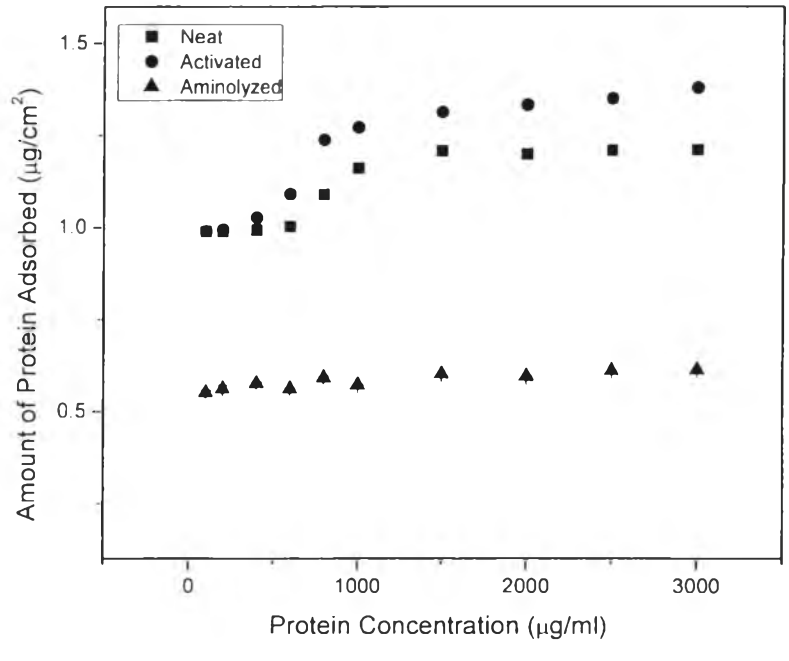


Figure 4.4 The adsorption isotherm of the adsorbed bovine serum albumin on the neat and modified PCL fibrous scaffolds (diameter =1.5 cm).

4.2.5 Mechanical Properties

The ultimate tensile strength, elongation at break and Young's modulus are determined by a universal testing machine. Samples with different aminolyzing time were evaluated. Table 4.4 gives the data obtained.

Table 4.4 Mechanical properties of aminolyzed PCL fibrous scaffolds prepared at different time

Aminolyzing Time (min)	Ultimate Tensile Strength (MPa)	Elongation at Break (%)	Young's Modulus (MPa)
0	2.205 ± 0.073	323.135 ± 13.960	4.715 ± 0.107
10	2.082 ± 0.239	317.812 ± 21.303	4.701 ± 0.246
30	2.101 ± 0.152	317.970 ± 19.306	4.676 ± 0.469
60	1.995 ± 0.151	316.858 ± 16.702	4.650 ± 0.175
120	1.990 ± 0.157	316.769 ± 13.675	4.612 ± 0.343
180	1.982 ± 0.249	310.101 ± 10.570	4.604 ± 0.210

The values are the average of 5 tests performed on each specimen. Statistical significance: $^{*}p < 0.05$ compared with neat PCL fiber mats (0 min) at any given time point.

Although the aminolysis reaction caused the polymer chain to break, Table 4.4 illustrates that there is no significant change of calculation on ultimate tensile strength, elongation at break and Young's modulus of the fiber mats probably because the individual fiber diameters of samples were large in this level of HMD concentration. Thus, the aminolyzing time was not the main factor that could change the mechanical properties of PCL fibrous scaffolds in this case. However, the fibrous scaffolds with 2 h. of aminolysis were selected to do further experiments for comparing the results to previous work.

4.3 Degradation Study

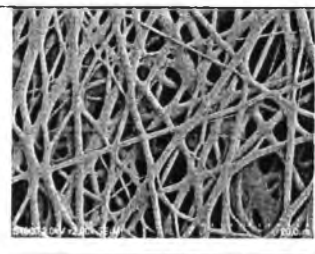
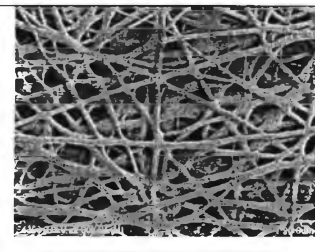
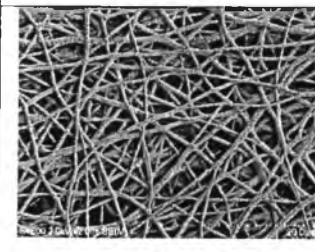
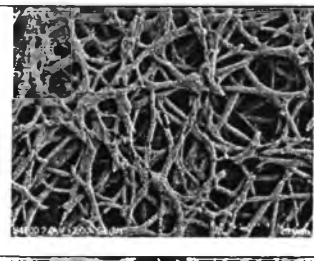
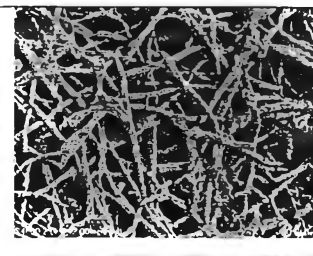
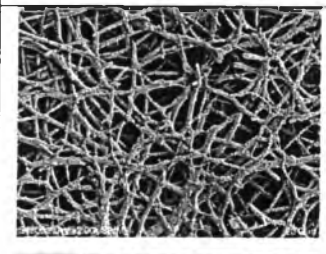
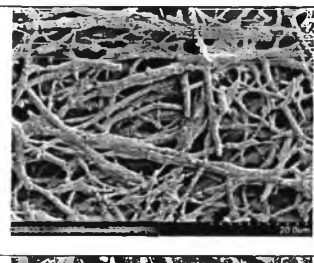
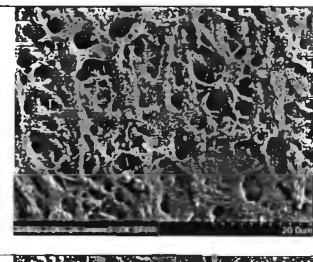
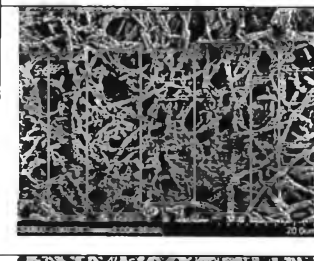
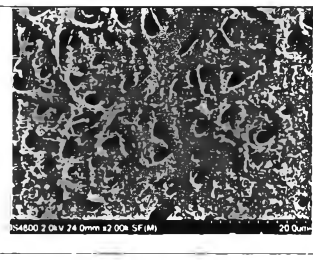
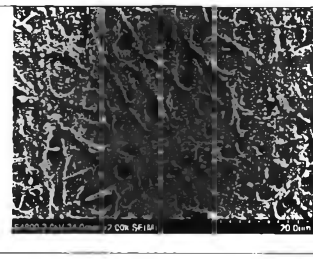
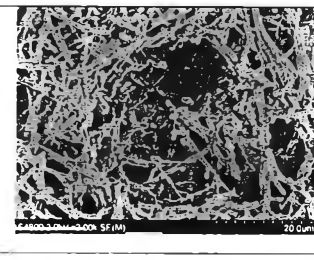
In degradation study, the disc shapes of the fibrous scaffolds had been immersed in the 0.1 M PBS pH 7.4 containing with and without the enzyme lipase from *Pseudomonas sp.* at 37 °C for 30 days. The buffer solution was replaced every 84 h. so the enzyme activities were maintained at a desired level throughout the experiment.

4.3.1 Surface Analysis of PCL fibrous scaffolds

Morphological changes of the fibrous scaffolds after degrading in lipase/PBS solution for 30 days were observed by SEM, as showed in Table 4.4. The structure of fibrous scaffolds continue destroyed since day 5 of degradation until the end of the experiment while the degradation mode of these fiber mats were slight difference. In the first 5 days of degradation, all electrospun fibers seemed to be breaking down due to the influence of enzymatic degradation because the fiber diameter still being the same except the breakage that taking place throughout the surface. By 15 days of degradation, the neat and activated PCL fibers showed slight swelling, the diameter of those fibers were greater than that of BSA-immobilized ones. The activated fibers seemed to be partially melted, and then adhered to one another because the degradable temperature that be used (37°C) was closed to the crystalline point of polycaprolactone (33-36°C) so it was possible to fused and rearrange itself to form the new crystal. By 30 days of degradation, the neat and activated PCL fibers mats tended to be film, the pore structure seemed to be disappeared. For BSA-immobilized fibers, the fracture of fibers became worse. The fibers start breaking into the smaller ones without swelling. A great number of the broken ends and the small holes were noticed as well as the surface of the fibers became rougher, this indicated that the superficial erosion was taking place. (Ana *et al.*, 2008). Moreover, the BSA-immobilized fiber mats became fragile and brittle as a function of degradation time. The breakage of BSA-immobilized fibers can be clarified in terms of the rigidity of crystalline regions. The bulky groups of protein made the polymer chains be difficult to move resulting in rigidity of polymer chain.

As soon as there was a weak point on the surface of fibers, they tended to be easily broken and the broken ends were more sensitive to hydrolytic attack on account of vulnerability to degradation medium over a period of degradation time. As showed in the Table 4.4, the degradation rate of the neat and activated fibrous scaffolds tended to be higher than that of the BSA-immobilized fibrous scaffolds probably because the lipase was easier to react with ester bonds of uncoated protein fibers than that of coated protein ones.

Table 4.5 SEM analysis of degraded electrospun fibrous scaffolds in lipase/PBS solution for 0, 5, 15, 30 days (magnification = 2000x; scale bar = 20μm)

Degradation time (days)	Magnification = 2000x; scale bar 20 μm.		
	Neat	Activated	BSA-immobilized
0			
5			
15			
30			

4.3.2 Weight Remaining After Degradation

Figure 4.5a shows the mass loss as a function of degradation time in the absence of the enzyme lipase. The mass loss for all of samples did not show the change of weight until the end of 30 days, whereas the mass loss in the presence of enzyme lipase as showed in Figure 4.5b shows that in first 15 days of degradation the weight loss of all samples were obviously increased, after that the rate of mass loss was level off till the end of degradation time for neat and activated fiber mats probably because the surface area of samples was decrease as evidenced by SEM morphology that resulting in decreasing of the lipase that can attack the samples. In case of BSA-immobilized fibers, the weight loss seemed to be linearly increased as a function of degradation time due to the ability of BSA in stabilized the enzyme activity and the surface area of samples reduced systematically.

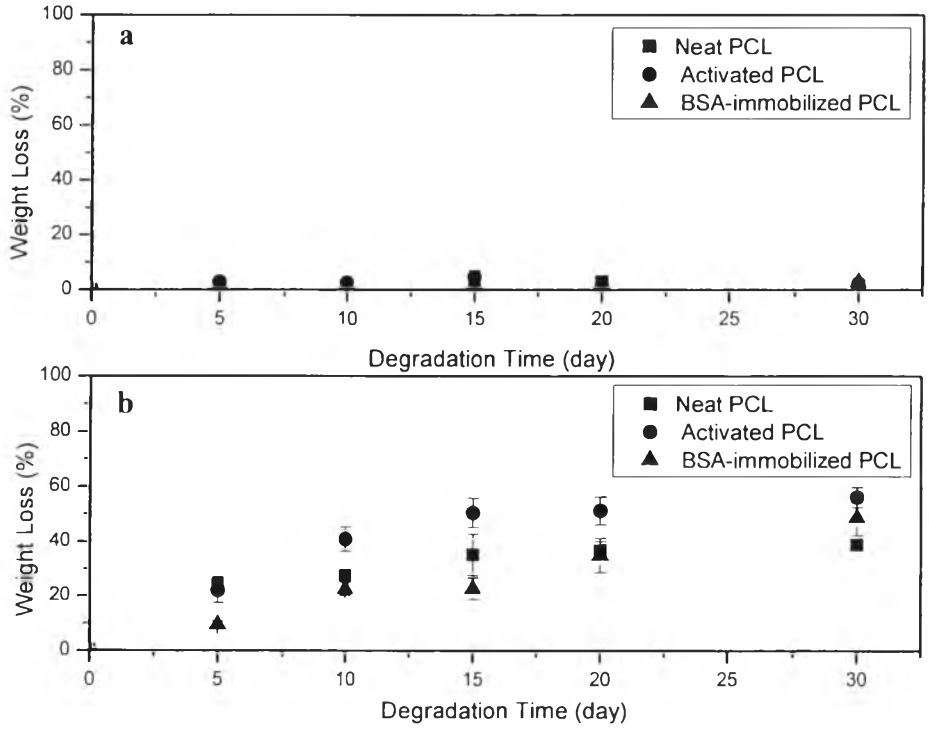


Figure 4.5 Degradation profile of the scaffolds after 30 days degradation in 0.1 M PBS containing (a) without lipase, (b) with lipase. a,b is significantly different at $p < 0.05$ for one way ANOVA with Dunnett T3.

4.3.3 Thermal Properties

Thermal properties of the porous scaffolds were investigated by a Mettler Toledo differential scanning calorimeter (DSC). Each cycle in DSC included 3 steps of HEAT1, COOL and HEAT2. The objective of first heating run (HEAT1) is to observe the melting behavior of the original crystalline entity of the sample, cooling (COOL) is to observe the ability of the sample to crystallize when it was subjected to a constant cooling scan and second heating (HEAT2) is to observe the melting behavior of the crystalline entity of the sample which was formed during the cooling scan.

Table 4.6 Thermal characteristics and apparent crystallinity of PCL fibrous scaffolds after degradation study

		Neat	Activated	BSA-immobilized
Day 0	$T_{m,o}$ (°C)	60.61	60.59	61.00
	X_c (%)	43.51	43.50	43.79
Day 5	$T_{m,o}$ (°C)	60.66	60.12	60.98
	X_c (%)	43.54	43.16	43.78
Day 10	$T_{m,o}$ (°C)	60.74	60.94	62.76
	X_c (%)	43.60	43.75	45.05
Day 15	$T_{m,o}$ (°C)	61.14	60.31	60.74
	X_c (%)	43.89	43.30	43.60
Day 20	$T_{m,o}$ (°C)	59.13	58.13	59.56
	X_c (%)	42.44	41.73	42.76
Day 30	$T_{m,o}$ (°C)	59.74	59.34	58.85
	X_c (%)	42.89	42.60	42.25

Table 4.6 illustrates the thermal properties of PCL fibrous scaffolds after degradation study. Since the degradation experiment acted as the desired condition that means these samples were crystallized under the same condition so the first heating scan was particularly observed.

Analysis of the first heat cycle for all samples during the study was quiet similar in both melting temperature (Fig. 4.6) and crystallinity (Table 4.6). The non-dependence on crystallinity indicates that degradation occurred in both phases, amorphous and crystalline, at the same time.

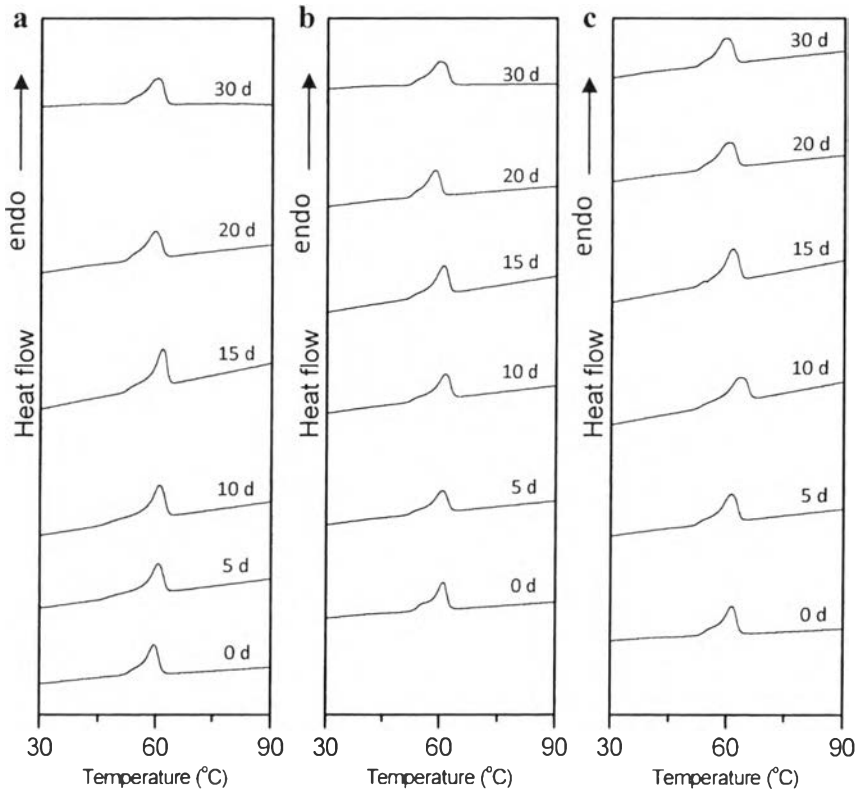


Figure 4.6 DSC traces of the first heat cycle showing the variation in melting point (^{o}C) and Enthalpy of fusion for samples of ; (a) neat PCL (b) aminolyzed PCL and (c) BSA-immobilized PCL fibrous scaffolds over time.

4.3.4 pH Measurements

pH changes of the aqueous buffer (Figure 4.7) over degradation time were determined for all the tested samples in order to verify the release of the acidic oligomers from the PCL fibrous scaffolds. In first 15 days, the pH values fluctuated in narrow range then greatly decreased until the end of degradation. During all the incubation time (30 days), the pH of the degradation buffer for all type of fiber mats tended to decreased lower than the initial pH (7.4). The pH reduction observed may be due to the dissolution of acidic oligomers, formed during the decomposition of fiber mats, in the degradation buffer.

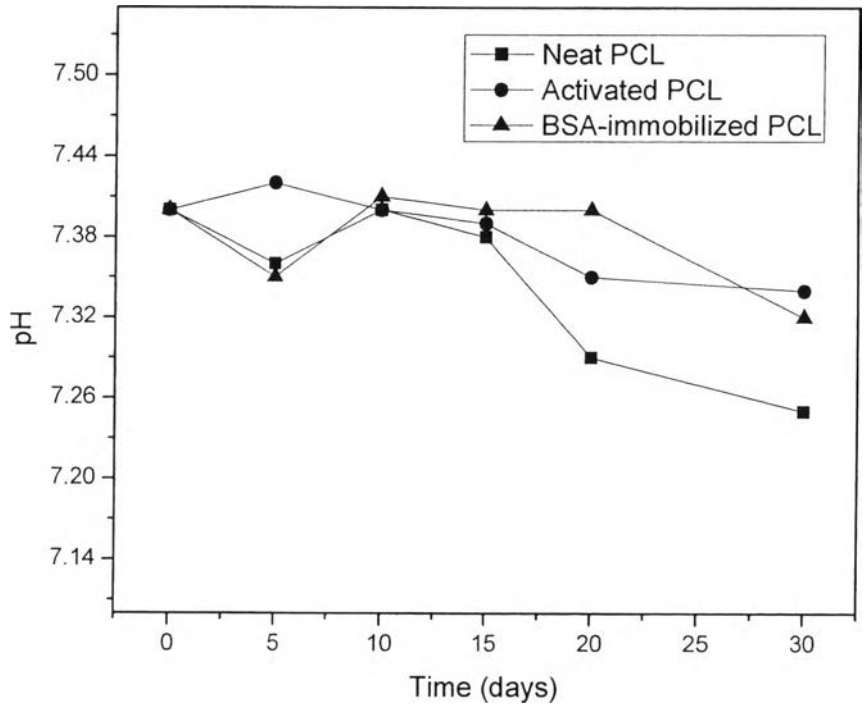


Figure 4.7 pH values of the degradation buffer during the degradation of the PCL fibrous scaffolds.

4.4 Biological Characterizations

4.4.1 Cytotoxicity

The potential used for these fiber mats as bone scaffolds was first assessed by an indirect cytotoxicity evaluation with mouse calvaria-derived pre-osteoblastic cells (MC3T3-E1), based on the initial 40,000 cells/cm^2 of cells seeded. Indiect cytotoxicity test was conducted on neat, aminolyzed, activated, BSA-immobilized PCL fibrous scaffolds. Figure 4.8 shows %viability obtained from MTT assay of MC3T3-E1 which were cultured with the 1,3,7 day-extraction media in comparison with those cultured with SFM (i.e. control).

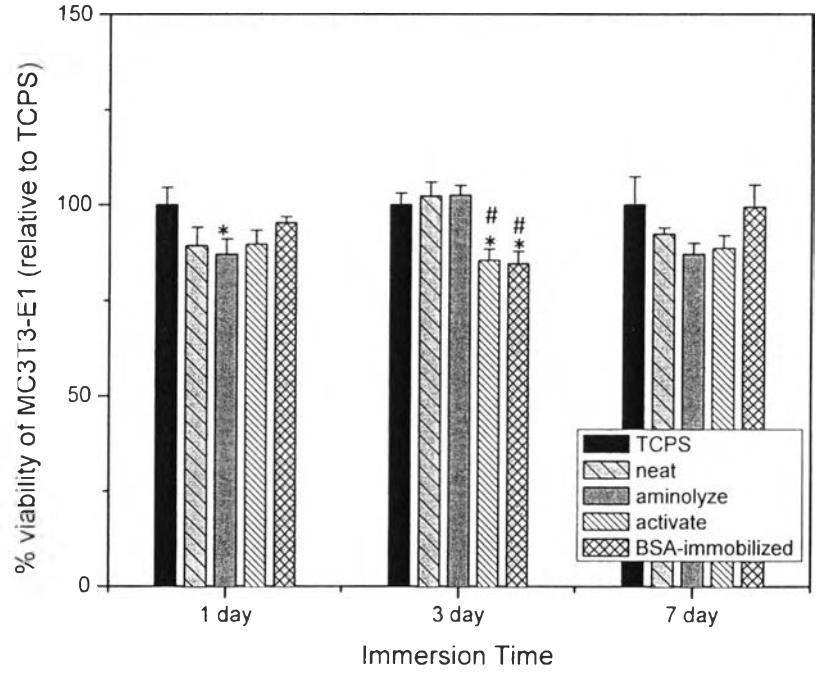


Figure 4.8 Indirect cytotoxic evaluation of neat, aminolyzed, activated, BSA immobilized PCL fibrous scaffolds based on viability of pre-osteoblast (MC3T3-E1) that had been cultured with the respective culture media for 1 d as a function of the incubation time of the extraction and the culture media of 1,3,7 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.

The viability of the cells that had been cultured with SFM at any given time point was taken as the basis to arrive at the relative viability showed in the Figure 4.8. Apparently, the viability of MC3T3-E1 for all types of modified PCL fibrous scaffolds exhibited slightly lower but not less than 80% compared to that of the control (100%), meaning that none of the toxic substances was released from both modified and unmodified PCL fibrous that harmful to cells.