CHAPTER IV
RESULTS AND DISCUSSION

4.1 Preparation of Film Mats

PCL and PHBV films were prepared by using solvent casting technique, obtaining the translucent films. Figure 4.1 shows the SEM Micrographs for the surface of original PCL and PHBV films.

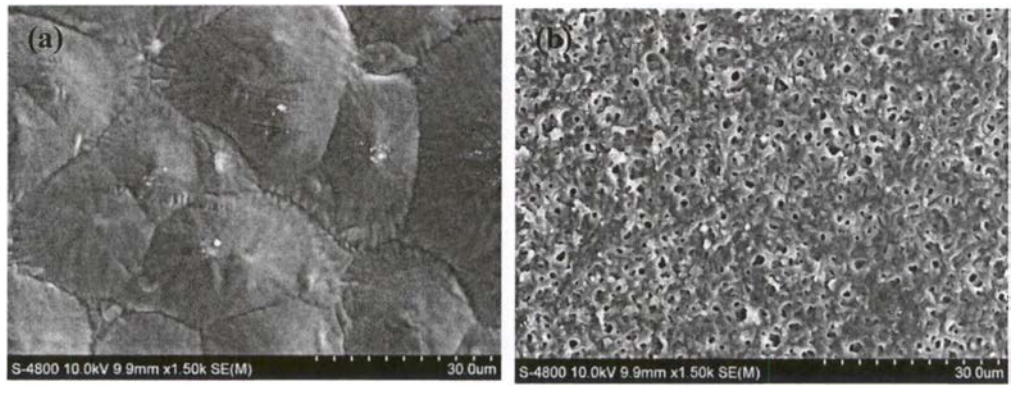


Figure 4.1 SEM micrographs of neat films: (a) PCL; (b) PHBV.

Figure 4.2 shows the Scanning Electron Micrographs of the surface of the blended PCL/ PHBV films in various compositions. From the results, the morphology of PCL films was changed after blending with PHBV. The quantity of porous of blended films increased as PHBV content increased which increased the roughness of surface. The results agreed with Sun and Downes (2009), who produced novel ultrathin polymer-based PCL and PCL/PLA blended films by solvent casting. They pointed out that the porosity of PCL/PLA blended films was changed when compared with PCL and PLA films.

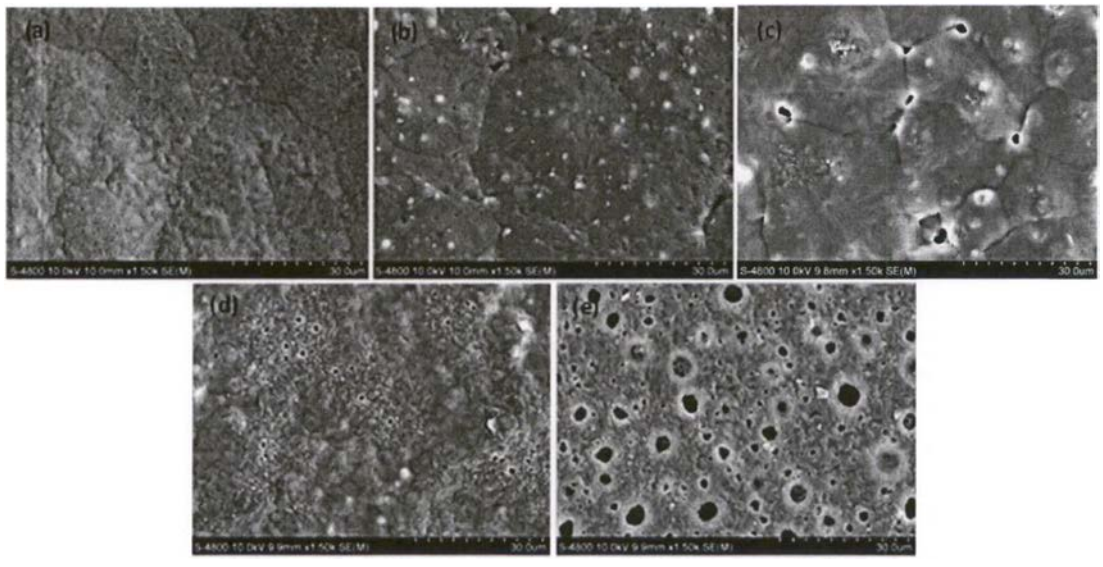


Figure 4.2 SEM micrographs of blended films: (a) PCL/10PHBV; (b) PCL/20PHBV; (c) PCL/30PHBV; (d) PCL/40PHBV; (e) PCL/50PHBV.

AFM images of neat and blended films were showed in figure 4.3 and 4.4, respectively. The 3D images confirmed the results of SEM that blending influenced on surface roughness.

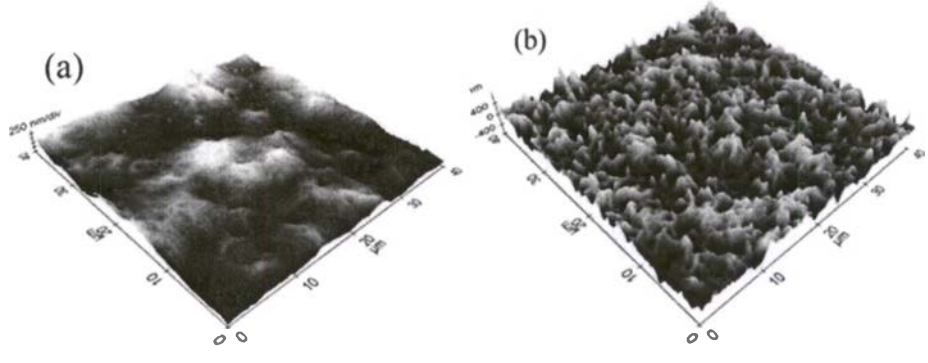


Figure 4.3 AFM images of neat films: (a) neat PCL; (b) neat PHBV.

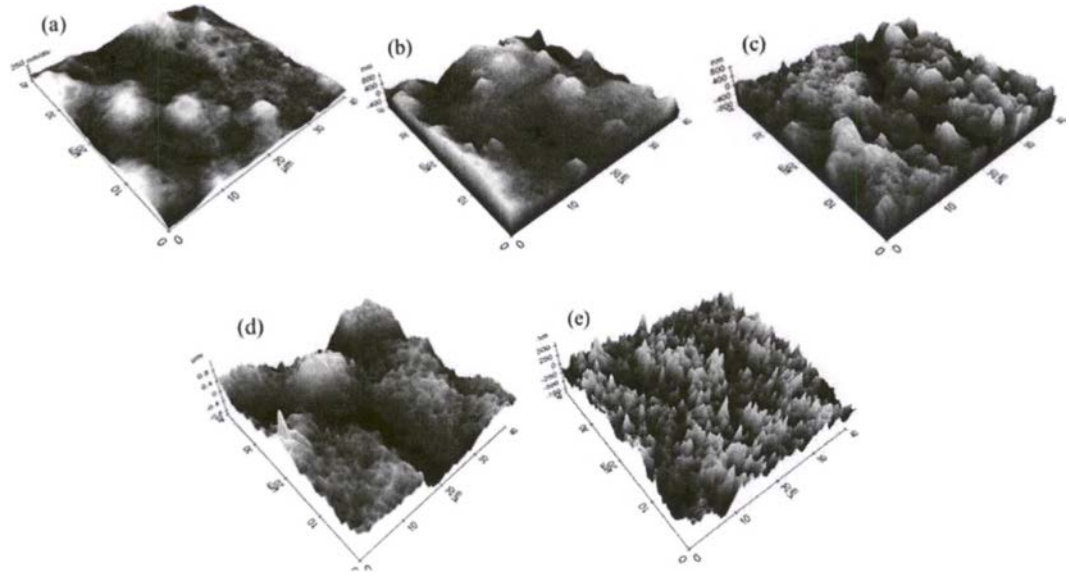


Figure 4.4 AFM images of blended films: (a) PCL/10PHBV; (b) PCL/20PHBV; (c) PCL/30PHBV; (d) PCL/40PHBV; (e) PCL/50PHBV.

For the quantitative analysis, roughness values were investigated by AFM. Arithmetical mean deviation of the profile (R_a), a two-dimension measure of the surface roughness, which is the factor known to influence cell response (Wirth *et al.*, 2007), was in the range of 0.09-0.3μm. This result supported that blending increased the roughness of the surface as PHBV content increased as shown in table 4.1. Moreover, the difference in height (R_z) and root mean square roughness (R_q) had the same trend as R_a.

Table 4.1 Roughness values of neat and blended films by using AFM

Sample	R_a (μm)	R_z(μm)	R_q(μm)
PCL	0.099±0.00	0.312±0.01	0.115±0.00
PCL/10PHBV	0.105±0.01	0.254±0.01	0.138±0.03
PCL/20PHBV	0.124±0.01	0.377±0.02	0.163±0.01
PCL/30PHBV	0.146±0.01	0.576±0.07	0.181±0.02

PCL/40PHBV	0.185±0.01	0.815±0.13	0.244±0.01
PCL/50PHBV	0.238±0.02	0.905±0.13	0.283±0.04
PHBV	0.120±0.01	0.510±0.06	0.143±0.02

4.2 Characterizations

4.2.1 Thermal Behaviors

The thermal behaviors of films were examined by Differential Scanning Calorimeter (DSC). The DSC thermogram showed glass transition temperature (T_g) of neat and blended films as shown in figure 4.5. Glass transition temperature of neat PCL and PHBV films was shown at 60°C and 160°C, respectively. For the blended films, glass transition temperatures were indicated with two peaks which are shown in the same figure.

The result exhibited two composition-independent glass transition temperature close to those of neat components, it showed that PCL and PHBV were completely immiscible.

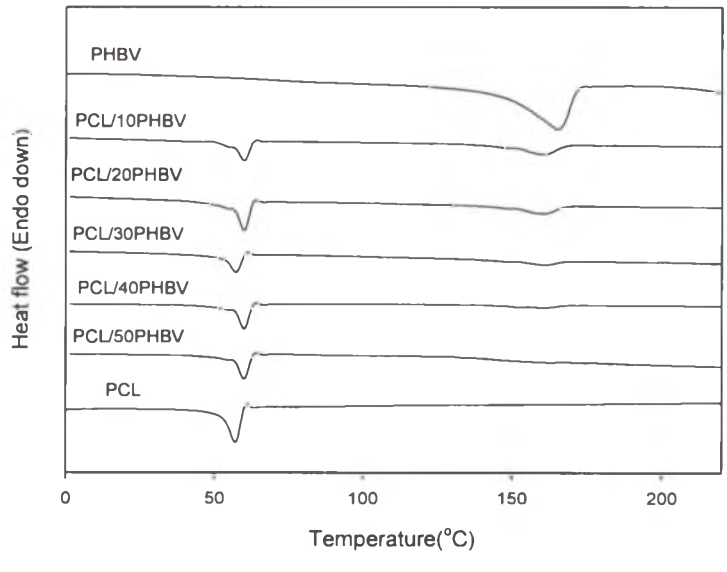


Figure 4.5 DSC thermograms of the obtained film substrates.

4.2.2 Chemical Analysis of Surface

The chemical integrity of PCL/PHBV blended films were investigated by ATR-FTIR, shown in figure 4.6 Those of PCL and PHBV counterparts are also shown for comparison. The absorption peak, centered at around 1720 cm^{-1} and belonging to stretching vibrations associated with the crystalline conformation of the carbonyl ester (C=O), was evident in all of the IR spectra. For the blended films, the way in which this peak increased in its intensity with an increase in the total PHBV content is indicative of the increase in the total crystallinity. Referring to Figure 4.6, because all of the characteristic peaks of the neat polymer can be observed in IR spectra of the blended films, the existence of both PCL and PHBV components within the blended films were confirmed.

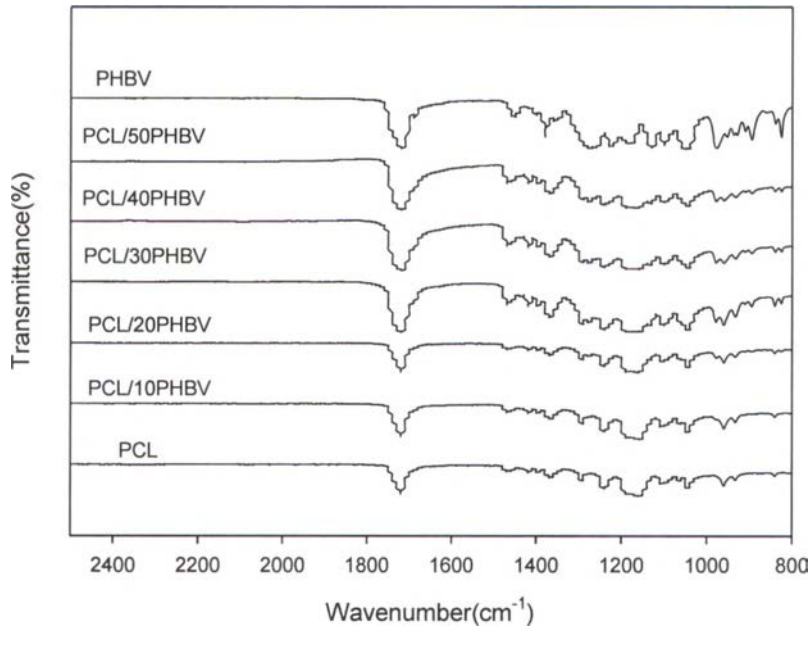


Figure 4.6 ATR-FTIR spectra of neat and blended films.

4.2.3 Degree of Crystallinity

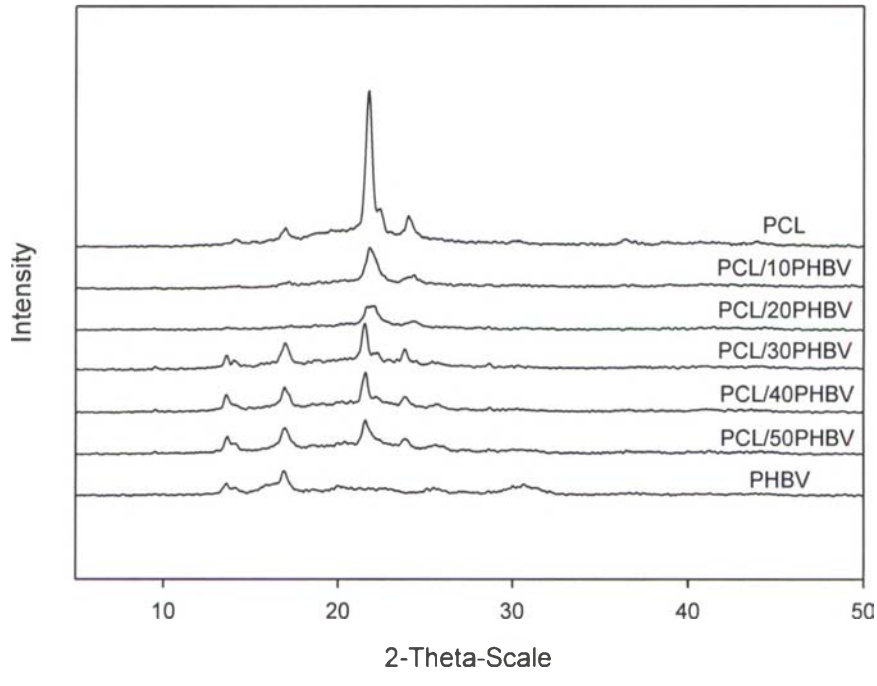


Figure 4.7 XRD pattern from neat and blended films.

The crystalline phases present in the samples were identified by XRD. XRD patterns of the resulting neat and blended films are shown in Figure 4.7. PCL is a semi-crystalline polymer with three diffraction peaks around 16, 21 and 23 (Jiang *et al.*, 2001). On the other hand, PHBV is also semi-crystalline polymer with five diffraction peaks around 13, 16, 19, 25 and 30 (Huang *et al.*, 2009). For blended films, there are no diffraction peaks of other substances and all diffraction peaks are corresponding to PCL and PHBV. Table 4.1 shows the degree of crystallinity obtained from XRD. The degree of crystallinity of PCL and PHBV was 48% and 26%, respectively. It was found that the degree of crystallinity of the blended films was in the range of 18-29%. The results showed that degree of crystallinity range of the blended was lower than degree of crystallinity of pure polymer. Due to the introduction of PHBV reduced the crystallinity

of the resultant polymer blend was reduced from 48% to 20%. Since fluid ingress and subsequent hydrolytic chain scission are restricted in the crystalline regions of polymers, faster resorption of polymer scaffold could be envisaged (Sun and Downes, 2009).

Table 4.2 The degree of crystallinity by using XRD

Sample	**Degree of Crystallinity (%)**
PCL	47.24
PCL/10PHBV	28.31
PCL/20PHBV	18.55
PCL/30PHBV	18.58
PCL/40PHBV	18.80
PCL/50PHBV	20.14
PHBV	26.01

4.2.4 Surface Wettability

Water contact angle measurement was used to evaluate the surface wettability of the surface of blended films. Table 4.3 shows no significant of the water contact angle of the films by the sessile drop method.

Table 4.3 The water contact angle of the neat and blended films measure

Sample	Contact angle (θ)
PCL	71.1 ± 0.05
PCL/10PHBV	72.0 ± 0.45
PCL/20PHBV	72.4 ± 0.34
PCL/30PHBV	73.7 ± 0.41
PCL/40PHBV	72.1 ± 0.83
PCL/50PHBV	72.2 ± 0.23
PHBV	72.8 ± 0.13

After coating the film substrate with the bovine serum albumin protein, the water contact angles were slightly increased which is shown in Figure 4.8. From the result, protein coating can gradually improve the hydrophilicity of the film substrates.

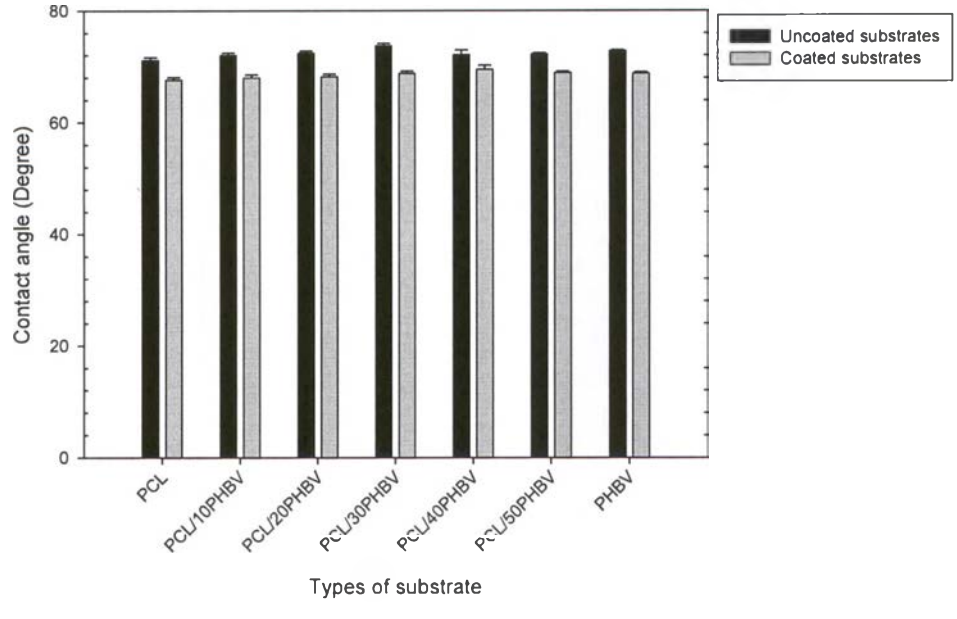


Figure 4.8 Contact angles of neat and blended films.

4.3 Protein Adsorption

The amount of adsorped protein of each substrate was shown in figure 4.9 by using BCATM protein assays. From the results, a trend can be observed between amount of adsorped protein and increasing PHBV content in the blend. From SEM and AFM images, the pore size and surface roughness increased with higher PHBV content and could lead to higher amount of adsorped protein. The investigation agrees with a research done by Woo et al., who fabricated nanofibrous scaffolds by porogen leaching and phase separation, demonstrated that the scaffolds with nanofibrous pore walls enhance protein adsorption more than scaffold with solid pore walls.

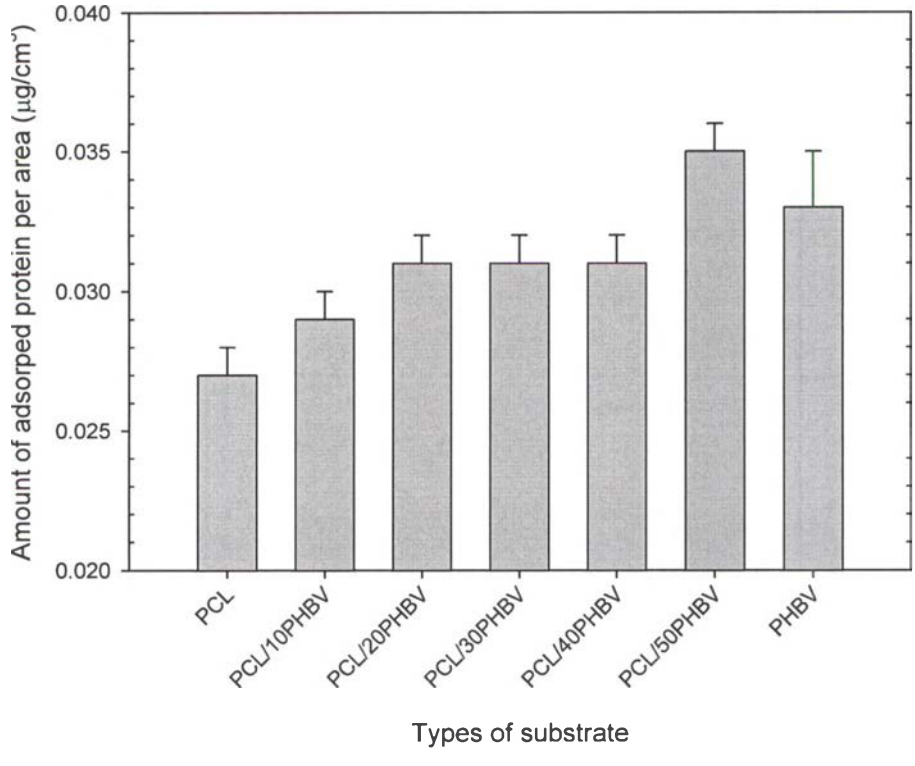


Figure 4.9 Protein adsorption of different types of substrates.

4.4 Biological Characterizations

4.4.1. Indirect Cytotoxicity Evaluation

The potential for using these films as substrates was first evaluated by an indirect cytotoxicity with mouse calvaria derived pre-osteoblastic cells (MC3T3-E1). The extraction media was prepared by immersing each sample for 1, 3 and 7 days, respectively. Then, the extraction media were used to culture MC3T3-E1 for 1 day. 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 4.10 showed viability of MC3T3-E1 cultured on uncoated films and coated films, respectively. It was clearly found that cells cultured in extraction media from all materials maintained the viability more than 80% at any given time. The obtained results indicated that all types of films do not release any substances at levels that are toxic or harmful to the cells.

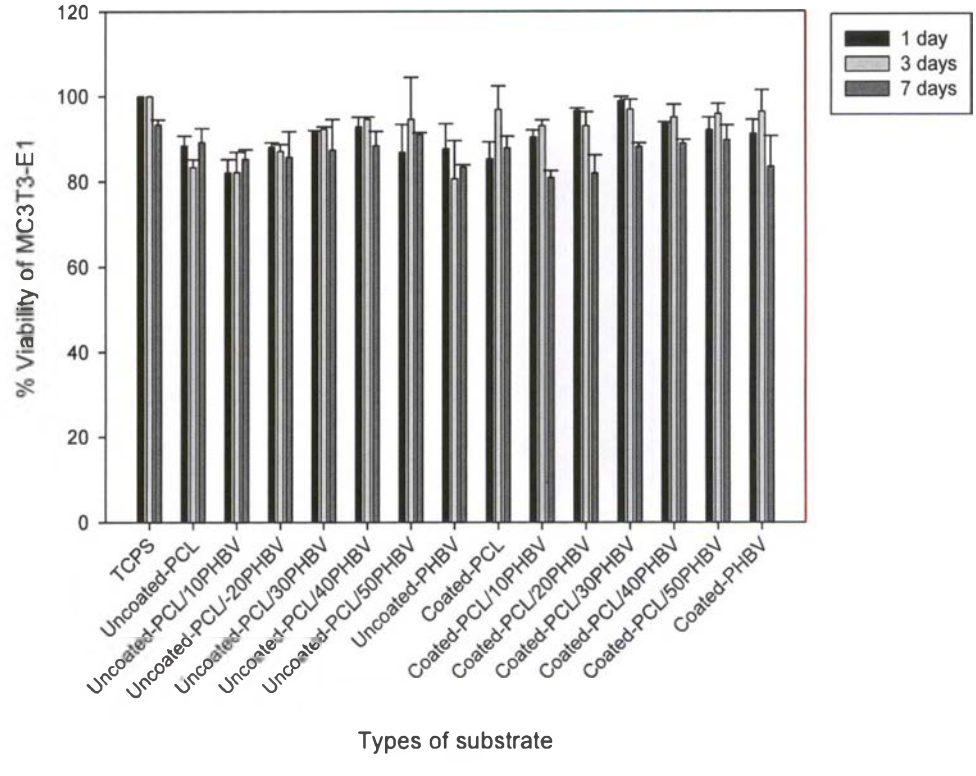


Figure 4.10 Indirect cytotoxicity evaluation of MC3T3-E1 cultured on TCPS, uncoated and coated films. MC3T3-E1 were cultured with SFM extraction media from each materials after immersed with materials for 1, 3 or 7 days and the cells viability was monitored by MTT assay compared to the viability of the cells that had been cultures with SFM media for 1 days. Statistical significance: *$p < 0.05$ compared with control and #$p < 0.05$ compared to the Uncoated-PCL film mats at any given time point.

4.4.2 Cell attachment and Proliferation

Quantitative analysis for the adherence of MC3T3-E1 after having been seeded on the surface of all types of substrates and TCPS, which used as positive control, for 4 and 16 hours was shown in Figure 4.11. At each studied time, MTT assay was used to quantify the viable cells. The viability of cells cultured on the surface of TCPS for 24 hours was taken as basis to obtain the relative viability values. The attachment of cells on materials have been studied in the past (Mattanavee et al, 2009) and primarily study in this work of neat PCL has confirmed that neat PCL is non-favorable for cell attachment. Moreover, blending PCL with PHBV did not improve its ability since the statistical analysis of the data showed that the uncoated substrates still had significantly lower cell attachment/viability than TCPS as shown in figure 4.11.

Interestingly, the attachment of cells was improved after the material surfaces has been modifying by coating with bovine serum albumin. However, there was no significant different found in among various types of coated substrates.

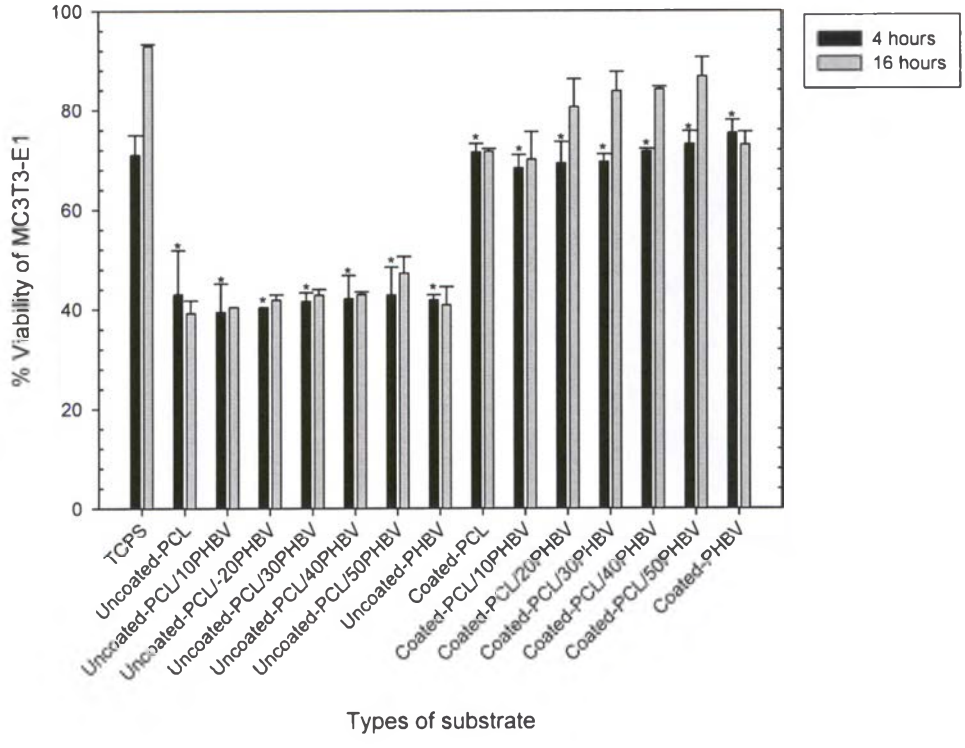


Figure 4.11 Attachment of MC3T3-E1 that was seeded onto the surfaces of TCPS and various uncoated and coated types of substrates as function of the cell seeding time. Statistical significance: $^{*}p < 0.05$ compared with control and $^{\#}p < 0.05$ compared to the uncoated-PCL film mats at any given time point.

To examine the effect of materials on cell proliferation, a quantitative analysis has been performed to determine the cell number after cultured for 1, 2, and 3 days as shown in Figure 4.12. The number of cells cultured on the uncoated substrates increased with an increase in PHBV content. Nevertheless, it remained lower compared to the cell cultured on TCPS.

After coating the surface of substrates, the number of cells on coated-substrates was slightly greater than TCPS. Among the modified materials, cells cultured

on PCL/50PHBV showed the significantly greater cell number compared to both TCPS and coated-PCL on days 2 and 3 as shown in the figure.

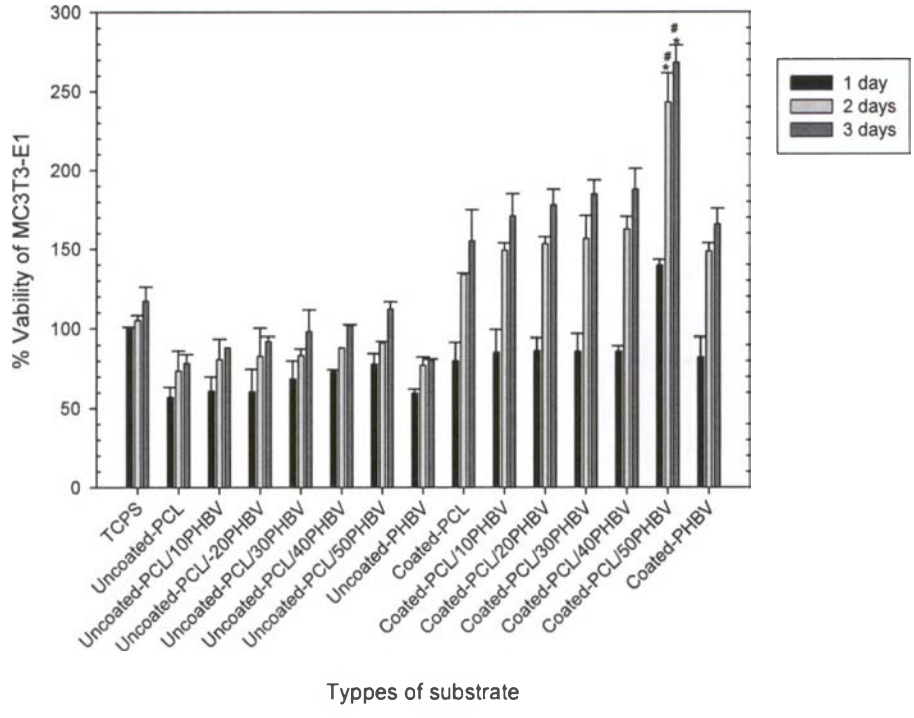


Figure 4.12 Proliferation of MC3T3-E1 that was seeded onto the surfaces of TCPS and various coated types of substrates as function of the cell seeding time. Statistical significance: $^{*}p < 0.05$ compared with control and $^{\#}p < 0.05$ compared to the coated-PCL film mats at any given time point.

The comparison of osteoblast attachment and proliferation on these various types of substrate showed some differences. Since, it is evident that osteoblast react differently when culture on different substrate roughness (Bigerell et al., 2002). As mentioned earlier, blending affected the roughness of the surface, with increasing PHBV content increased. Results showed that the attachment and proliferation of cells increased slightly as surface roughness increased without any significant difference. The greatest number of cells was found on the surface of PCL/50PHBV which has the

highest values of Ra around 0.238 μm. Results supported the effect of surface roughness on cell attachment: the higher roughness, the greater the cell number. The results are in agreement with that of Hatano et al. who studied the effects of surface roughness in promoting osteoblastic differentiation using tissue culture polystyrene as substrate; the rough surface plastic cover strips were prepared by hand grinding their surfaces with waterproof grinding paper coated with SiC particles. The smooth surface plastic cover strips were not abraded.

Their results demonstrated that the surface roughness itself caused increases in osteoblastic proliferation and differentiation in cell cultures.

As far as biomaterials interactions with proteins are concerned, adsorped proteins play an equally important role in cell attachment. After coating the surface of the substrates with bioactive proteins, the number of attached cells was higher in every type of substrates. This result showed that coating with bovine serum albumin caused a dramatic increase in attachment and proliferation and confirmed the hypothesis that adsorped proteins promoted cell attachment and proliferation. In addition, the amount of protein adsorption showed that the substrates with higher surface roughness adsorbed more proteins than substrates with lower surface roughness. Therefore, the higher the amount of adsorbed proteins on the surface of scaffolds, the better it might be in promoting cell attachment (Leong et al., 2008). Due to PCL/50PHBV blended films had the highest protein adsorption capacity and also had the highest number of viable cells. This investigation also in agreement with a previous work done by Leong et al., who prepared solvent-cast film, solid fibers and porous fibers scaffolds using PLA as materials. They found that the fibrous nature of the electrospun scaffolds promoted cell attachment as compared with the solvent-cast film, and the nanoporous fiber scaffolds further enhance this interaction. Such observations can be attributed to the differences in surface morphology and protein adsorption capacity of the three different types of scaffolds.

In summary, the blended substrates coated with bioactive proteins promoted better cell attachment and proliferation than neat PCL and PHBV substrates,

or even blended substrates without coating. Therefore, two parameters that promoted cells growth in this study were surface topology and adsorbed proteins.

4.4.3 Cell Morphology

Selected SEM images illustrate the morphologies of MC3T3-E1 that had been cultured on the surface of glass and all type of uncoated and coated substrates for various time interval are shown in Table 4.4, Table 4.5 and Table 4.6, respectively. At 4 hours after cell seeding, the cells on all type of uncoated substrates and glass surfaces were still round while those on all type of coated substrates became flat and start spreading. After 1 and 3 days, the cells could cover all the surface substrates. However, cells showed a better coverage on coated substrates compared to the uncoated one. On the basis of the results, coated-PCL/50PHBV was clearly the best among the investigated substrates for supporting the growth of MC3T3-E1.

Table 4.4 Representative SEM images of MC3T3-E1 that were seeded/cultured on the surface of a glass substrate at difference time points

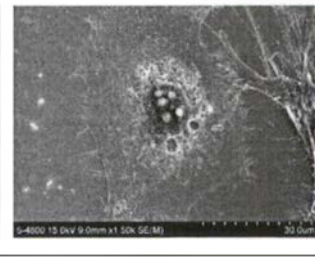
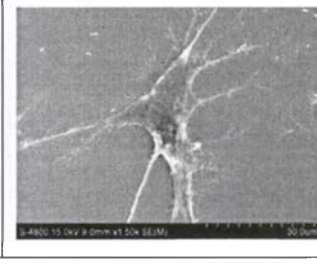
Material	**Magnification = 1,500X** **Scale bar = 10 μm**		
	4 hours	**1 day**	**3 days**
Glass (control)			

Table 4.5 Representative SEM images of MC3T3-E1 those were seeded on the surface of various uncoated substrates at difference time points.

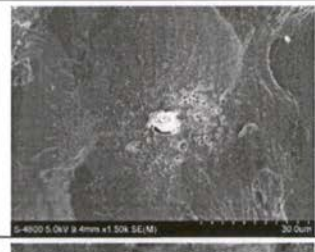
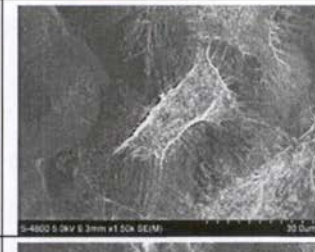
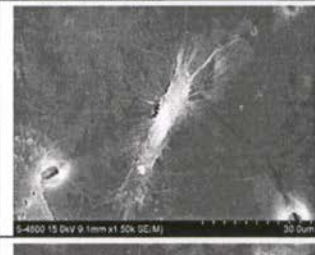
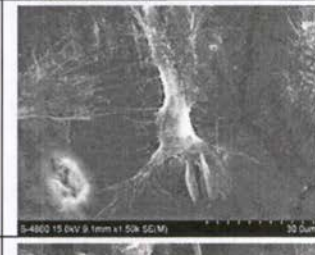
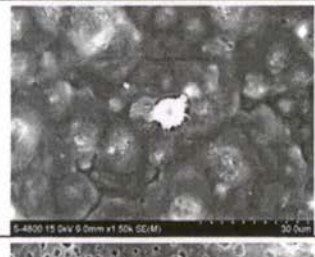
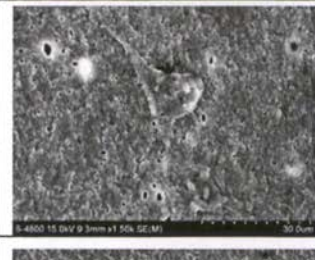
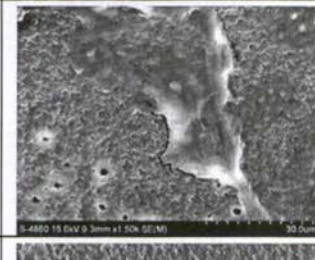
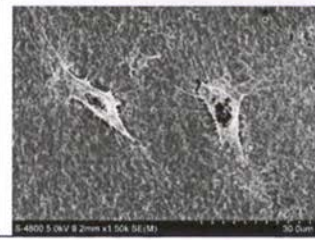
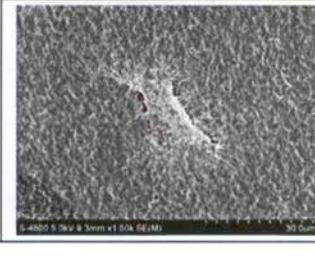
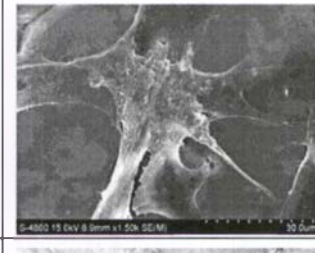
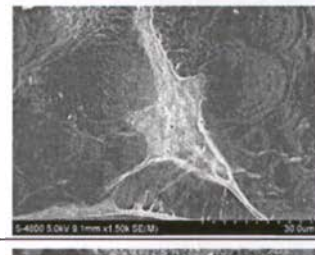
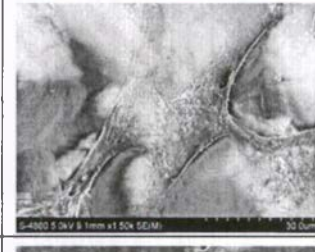
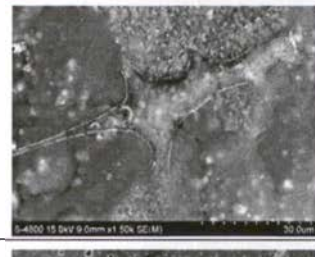
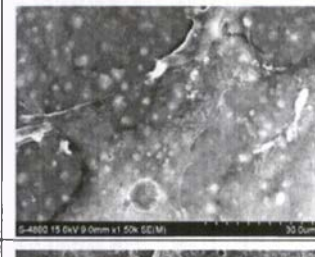
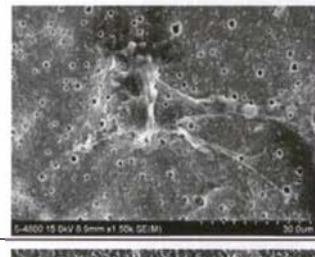
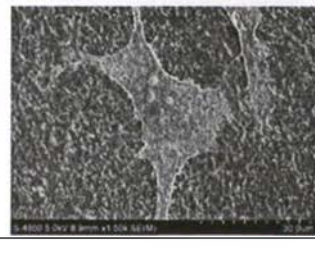
Material	Magnification = 1,500X Scale bar = 10 μm		
	4 hours	1 day	3 days
Uncoated PCL			
Uncoated PCL/10PHBV			
Uncoated PCL/30PHBV			
Uncoated PCL/50PHBV			
Uncoated PHBV			

Table 4.6 Representative SEM images of MC3T3-E1 those were cultured on the surface of various coated substrates at difference time points

Material	Magnification = 1,500X Scale bar = 10 μm		
	4 hours	**1 day**	**3 days**
Coated PCL			
Coated PCL/10PHBV			
Coated PCL/30PHBV			
Coated PCL/50PHBV			
Coated PHBV			

To confirm the results from SEM, cytoskeleton distribution pattern of cells was examined by actin staining. The actin filaments are the flexible polymer in the cells that controlled cell crawling (attachment) on the surface of substrates. Fluorescence microscope (FM) images showed the pattern of actin of MC3T3-E1 cells seeded/cultured on the surface of glass and all type of uncoated and coated substrates for various time 4 hours and 1 day interval are shown in Table 4.7, and Table 4.8, respectively. After 4 hours, the pattern of actin of cells on surface of both uncoated and coated substrates support that they were not spreaded. At day 1, cells cultured on the less surface roughness substrates showed lesser degree of spreading compared to the cells cultured on higher surface roughness. It is possible that the binding of cells on the substrate surface is mediated by proteins that are adsorbed on the polymeric substrate (Gomez-Tejedo et al., 2011). At day 1, cells showed fully spread.

From SEM and FM images, the substrates, which have high surface roughness and porosity, was favorable to the attachment and proliferation of MCT3T-E1.

Table 4.7 Representative FM images of MC3T3-E1 those were seeded/cultured on the surface of a glass coated substrates at difference time points

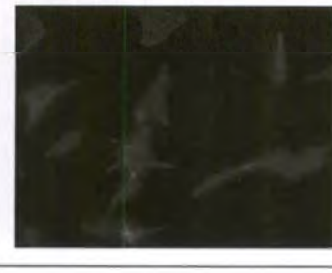
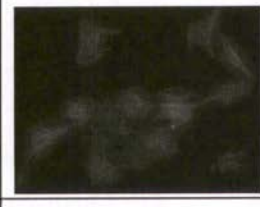
Material	**Magnification = 1,500X** **Scale bar = 10 μm**	
	4 hours	**1 day**
Glass (control)		

Table 4.8 Representative FM images of MC3T3-E1 those were seeded/cultured on the surface of various uncoated and coated substrates at difference time points

Material	Uncoated substrates		Coated substrates	
	4 hours	1 day	4 hours	1 day
PCL				
PCL/10PHBV				
PCL/30PHBV				
PCL/50PHBV				
PHBV				

4.4.4 Mineralization

The ability to promote bone formation is the most important characteristic of a bone scaffold. This ability can be monitored by in vitro calcification assay. Cultured of MC3T3-E1 on TCPS and all types of cast film substrates for 21 days in osteogenic medium (complete medium plus ascorbic acid, beta glycerophosphate and dexamethasone). Calcium deposition will be detected by Alizarin Red S staining. However, the proper differentiation and induction of in vitro calcification of MC3T3-E1 could be observed up to passage 25. Since the cells that were used in this study was in passage 23-26, the calcification results was not clear and the lower passage of cells is required to repeat the experiments.