CHAPTER IV
RESULTS AND DISCUSSION

4.1 Preparation and Characterization of Porous Scaffolds

Solvent casting and salt particulate leaching process was used to prepare the 5 types of porous scaffolds—polycaprolactone (PCL), poly(1,4-butylene succinate) extended with 1,6-diisocyanatohexane (PBSu-DCH), poly(lactic acid) (PLA), poly(3-hydroxybutyric acid) (PHB), and poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid) (PHBV)— for bone tissue engineering. In this study, Sodium Chloride (NaCl) particles were used as the porogen to generate an open pore structure. The polymer solution and NaCl particles was then thoroughly mixed and cast into either the cylindrical glass molds or Petri-dishes. Pores were connected via the dissolving of NaCl porogen prior to formation of a three-dimensional scaffold, as shown in figure 4.1. In this work, a number of characteristics of the aliphatic polyester scaffolds were studied.

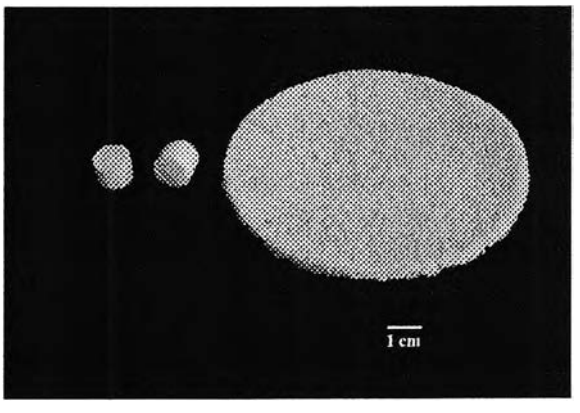


Figure 4.1 The three-dimensional structure of the polycaprolactone porous scaffolds.

4.1.1 Microstructure Observation

Figure 4.2 demonstrates the SEM micrographs of the scaffolds microstructure on the cross sections. For the five types of scaffolds with 30:1 NaCl/polymer weight ratio, they possessed the interconnection of pores which is the important property for transport process— it allows the movement of nutrients and wastes to and from the cells in the scaffolds. The SEM micrographs also showed many square units occurring from the cubic shape of NaCl porogens used in the fabricating process. Among the entire scaffolds, PLA scaffold seemed to be that it showed the best square characteristic whereas the other scaffolds exhibited the lost square characteristic in several parts.

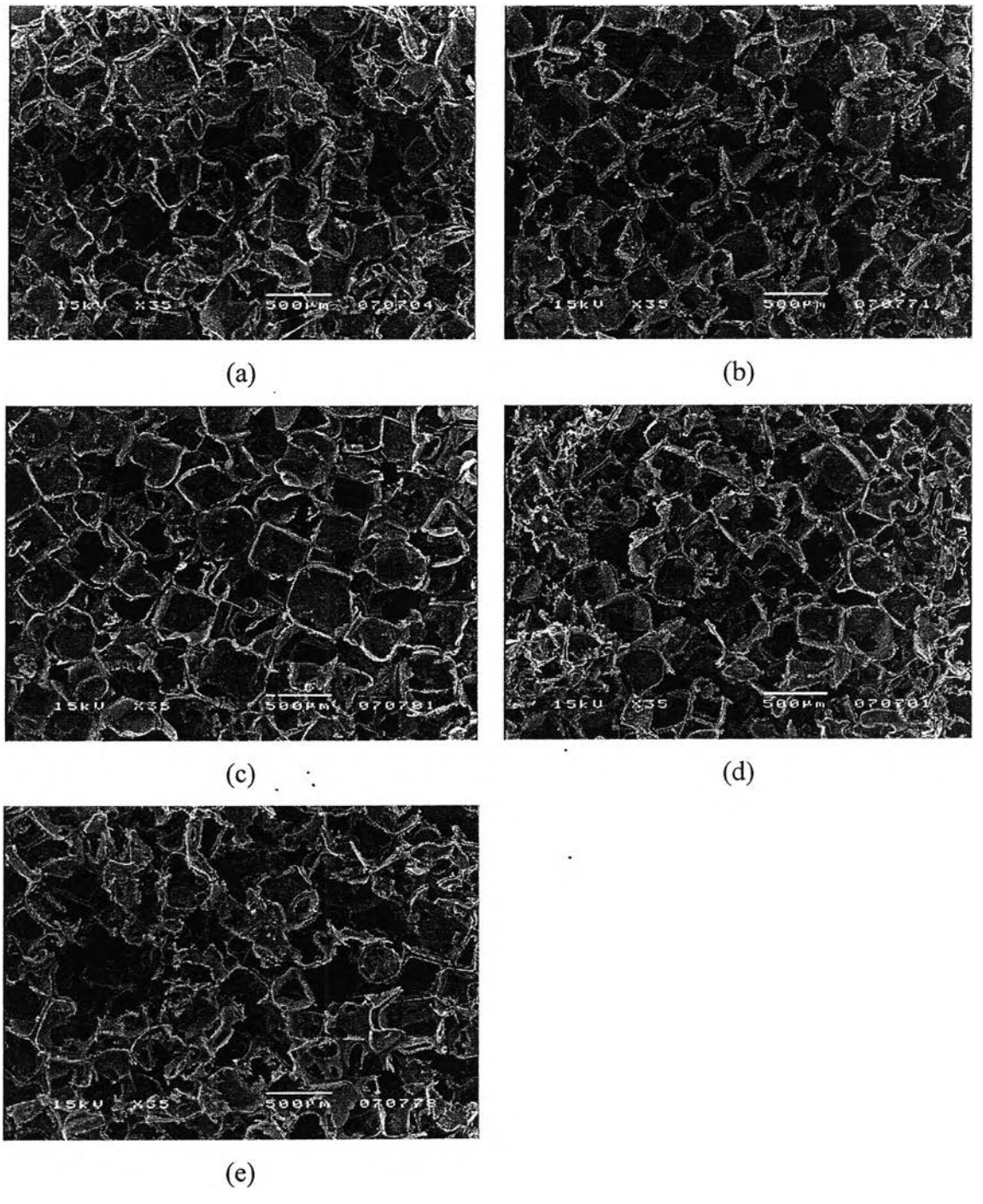


Figure 4.2 SEM images illustrate microstructure of the scaffolds on the cross-sections: (a) PCL, (b) PBSu-DCH, (c) PLA, (d) PHB and (e) PHBV.

4.1.2 Density, Porosity, Pore volume and Pore size

The density, porosity, pore volume and pore size of the scaffolds were shown in table 4.1. Although, the entire polyesters were prepared in the same manner, the porosity and pore volume illustrated statistically differences between each group. However, the porosity and pore volume of these scaffolds were in the range of 93-96 % and 14-18 cm^3/g, respectively. Moreover, the data did not present any differences of pore size between each group and they were in the range of those of the porogens (400-500 μm).

Table 4.1 Density, percentage of porosity, pore volume and pore size of the scaffolds

Scaffolds	Density** (g/cm^3)	Porosity** (%)	Pore volume* (cm^3/g)	Pore size* (μm)
PCL	0.070^{a}	93.88 ± 0.38^{a}	14.15 ± 0.83^{a}	416.31 ± 46.21^{a}
PBSu-DCH	$0.067^{a,b}$	$94.82 \pm 0.81^{a,b}$	$14.97 \pm 1.95^{a,b}$	426.25 ± 42.77^{a}
PLA	0.059^{b}	95.32 ± 0.25^{b}	$16.98 \pm 1.13^{b,c}$	428.44 ± 59.58^{a}
PHB	0.058^{b}	94.82 ± 0.11^{b}	$17.03 \pm 0.37^{b,c}$	431.10 ± 58.08^{a}
PHBV	0.057^{b}	94.59 ± 0.08^{a}	17.40 ± 0.58^{c}	446.48 ± 68.39^{a}

a,b,c are significantly different at $p < 0.05$ for an individual feature; *one way ANOVA with Tukey HSD. **one way ANOVA with Dunnett T3, n = 5 for porosity and pore volume, n = 25 for pore size.

4.1.3 Water Absorption Capability

Figure 4.3 illustrates the water absorption capabilities of the 5 types of polyester scaffolds in 0.1 M PBS at room temperature within 4 days. All groups showed a similar profile of the water absorption, the water absorption rate increased rapidly in the early 24 hours and maintaining stable. However after a couple of weeks, they showed a gradual decrease of the water absorption causing by the degradation of the scaffolds. Moreover, the water absorption capabilities of the scaffolds were compared statistically at 24 hours; it found that the PHBV scaffolds exhibited the highest level of water absorption compared to the others.

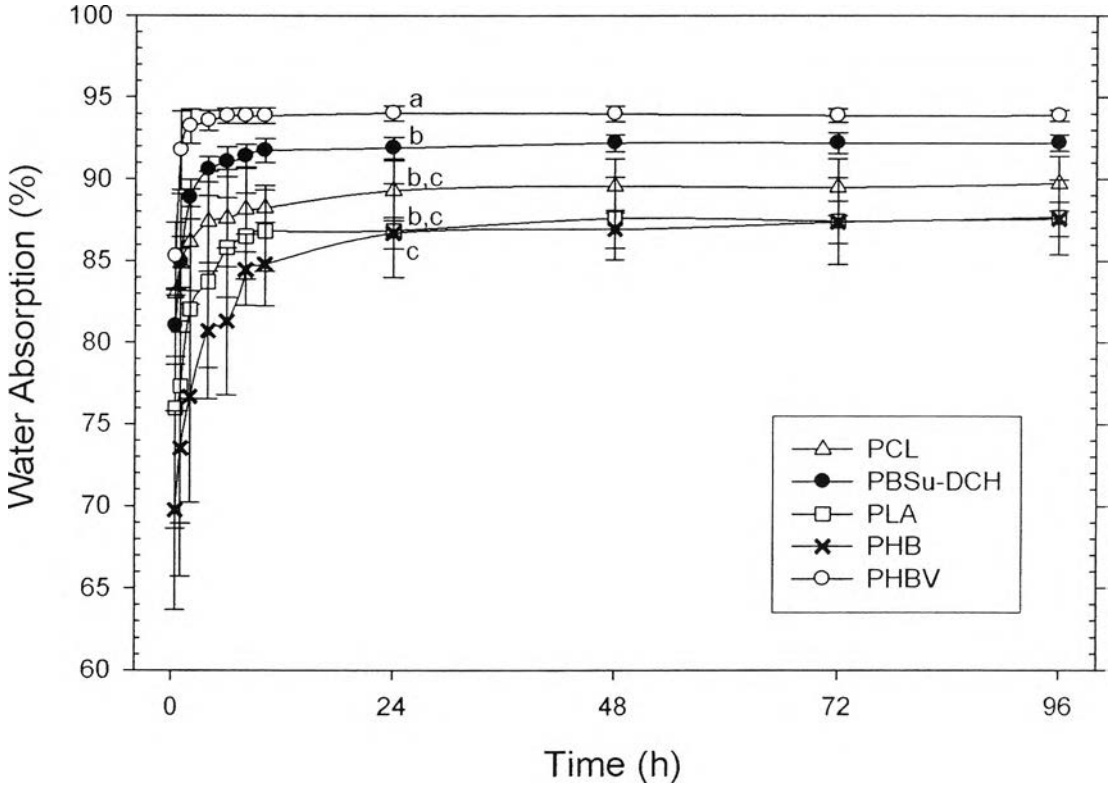


Figure 4.3 Water absorption capability of the scaffolds in 0.1 M PBS at room temperature within 4 days. a,b,c are significantly different at $p < 0.05$; One way ANOVA with Dunnett T3.

4.2 Degradation Study of Porous Scaffolds

In degradation study, the cylindrical shapes of the porous 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 13 weeks. The buffer solution was replaced every 84 hours therefore the enzyme activities were maintained at a desired level throughout the experiment. Figure 4.4 shows the shape changes of the scaffolds after immersion in lipase/PBS with incubation time. It shows that the PBS-DCH scaffold demonstrated a largest shape change, while the PHBV scaffold almost maintain the three-dimensional structure until the end of 9 weeks.

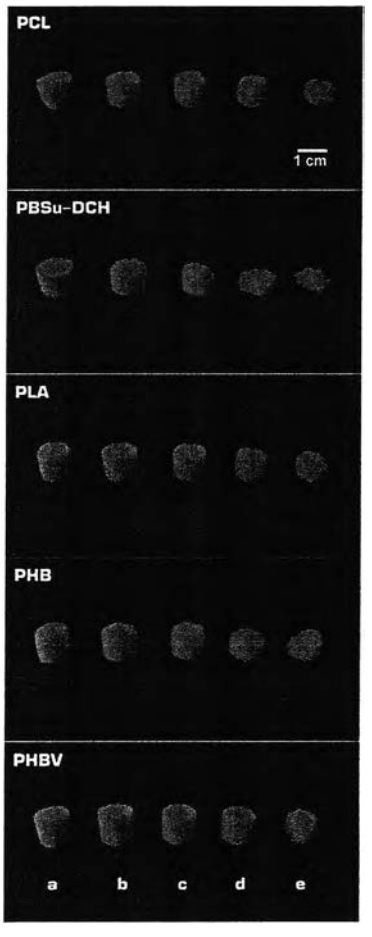
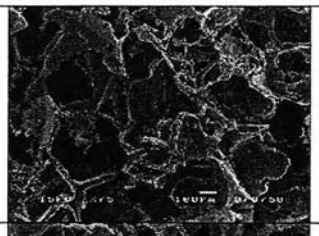
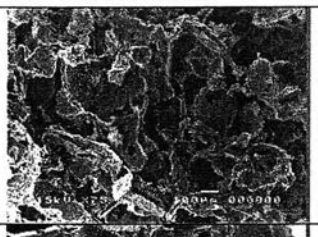
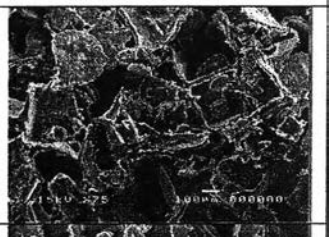
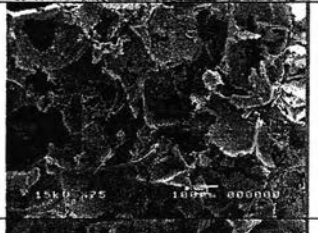
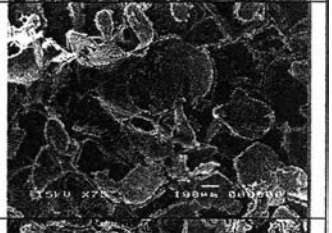
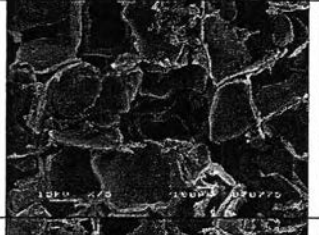
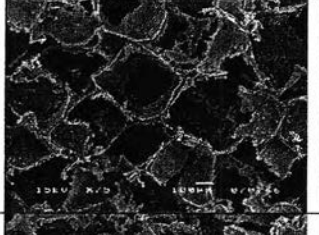
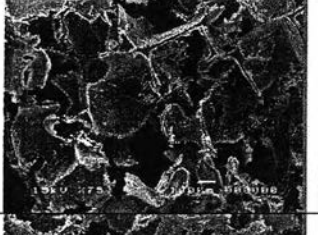
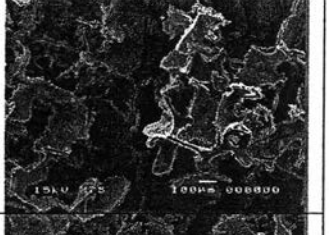
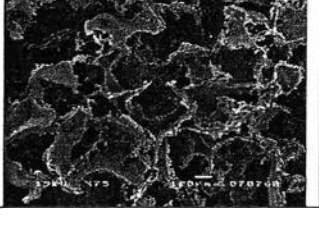
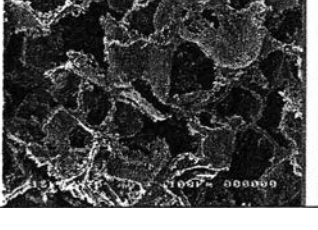
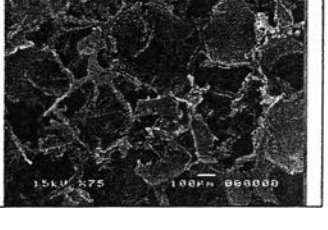


Figure 4.4 Change of shape and dimension of PCL, PBSu-DCH, PLA, PHB and PHBV scaffolds after immersion in lipase/PBS with incubation time; (a) before immersion in lipase/PBS, (b) 1 week, (c) 5 week, (d) 9 week, (e) 13 week.

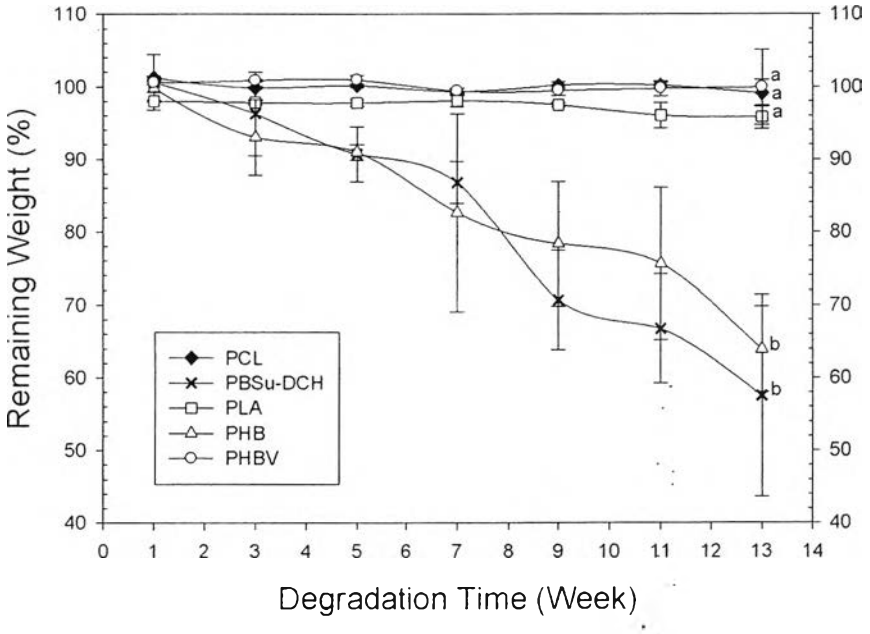
Morphological changes of the porous scaffolds after degrading in lipase/PBS solution for 13 weeks were observed by SEM, as shown in table 4.2. The scaffolds still maintained their pores shape and size in the early 5 weeks. In the ninth week of enzymatic degradation, many circular perforations were found on the wall of the scaffolds and their pore shapes were damaged in some areas. This phenomenon might be occurred by degradation in the amorphous regions; the breakage of the molecular chains connecting between the crystalline domains, resulting in defects of scaffolds (Chu *et al.*, 1997). The deformation of the microstructure was clearly observed in the thirteenth week.

Table 4.2 SEM images illustrate microstructure of the scaffolds after degrading in lipase/PBS solution for 5, 9 and 13 weeks on the cross-sections

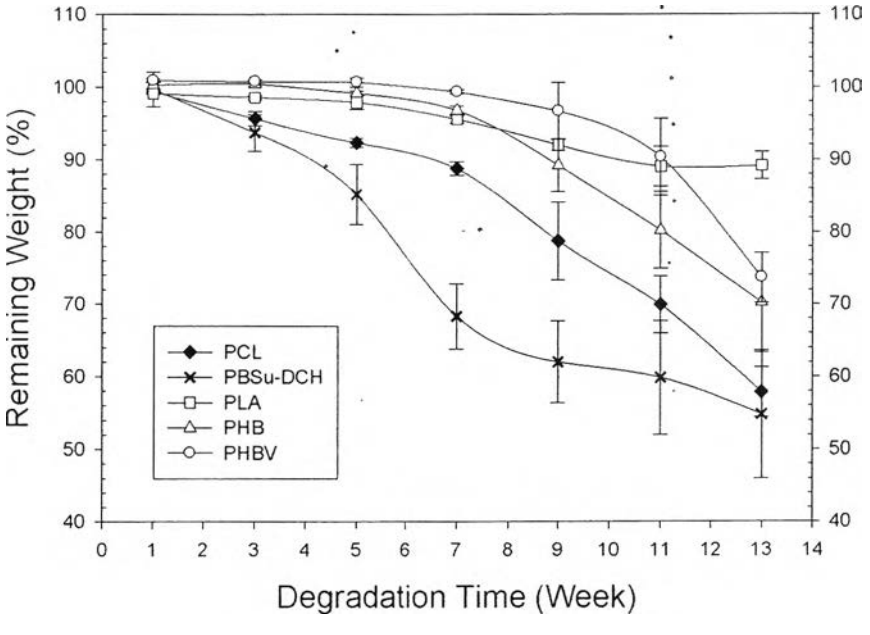
Scaffolds	5 weeks	9 weeks	13 weeks
PCL			
PBSu-DCH			
PLA			
PHB			
PHBV			

4.2.1 Weight Remaining After Degradation

Figure 4.5 shows the remaining weight of the scaffolds after degrading in the absence and presence of the enzyme lipase in 0.1 M PBS pH 7.4 at 37°C for 13 weeks. In the absence of lipase (figure 4.5(a)), the PCL, PLA and PHBV scaffolds did not show the change of remaining weight until the end of 13 weeks, whereas the remaining weight of PBSu-DCH and PHB scaffolds was rapidly significant drop and remaining weights were only 57.5% and 63.9%, respectively. It can be said that polyester having longer methylene chain (PBSu-DCH) was readily degraded (Tsutsumi *et al.*, 2003) and the PBSu-DCH scaffold also showed high water uptake which is another factor in the hydrolytic degradation due to water could lead to swelling of the polymer and thus facilitate degradation. Although, the chemical structure of PHB and PHBV is quite similar, the PHBV scaffold exhibited less degradation compared to PHB scaffolds. This might cause by molecular weight of PHBV was higher than PHB about 2 times so the PHBV scaffold showed more resistant to hydrolytic degradation. Obviously, the degradation rate of PBSu-DCH and PHB scaffolds was comparable; they showed the degradation rate of 3.7 and 2.8 % per week, respectively. In the presence of lipase (figure 4.5(b)), it was clearly observed that the remaining weight of PCL and PHBV scaffolds was much decreasing compare to the absence of lipase condition; the percentage of decreasing weight were 41.5 and 27.2, respectively. However, PHBV scaffold almost maintain remaining weight until the end of 9 weeks and degraded rapidly. The activity of this enzyme lipase exhibited a little effect to PBSu-DCH, PLA and PHB scaffolds. Conformably, PLA film was not degraded in the several lipase solutions at 37°C, pH 7.0. However, PLA film could be degraded completely at 55°C, pH 8.5 with lipase from *Alcaligenes sp.* during 20 days (Hoshino *et al., 2002*). Additionally, the degradation profiles of PCL and PHB were similar; they revealed the gradual decreasing of weight in early 7 weeks and dramatically drop of weight after 7 weeks, whereas the degradation rate of PHBV scaffold tented to decrease rapidly in the eleventh week. The biodegradability of these polyester scaffolds in lipase solution can be rank as follows: PBSu-DCH > PCL > PHB > PHBV > PLA.



(a)



(b)

Figure 4.5 Remaining weight of the scaffolds after 13 weeks 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.

4.2.2 Compressive Modulus

Mechanical property of the porous scaffolds was presented in the term of compressive modulus. The table 4.3 and table 4.4 demonstrate the compressive modulus of degraded scaffolds in PBS containing with and without lipase, respectively. The compressive modulus of the scaffolds before degrading study was found to decrease as follow: PLA > PHBV ≈ PHB > PBSu-DCH > PCL. Moreover, it found that the compressive modulus of degraded scaffolds decreased with increasing of degradation time. Apparently, the existence of lipase could accelerate the decreasing of compressive modulus of the PCL scaffold, as shown in figure 4.6. This result quite conformed to the alteration of the remaining weight.

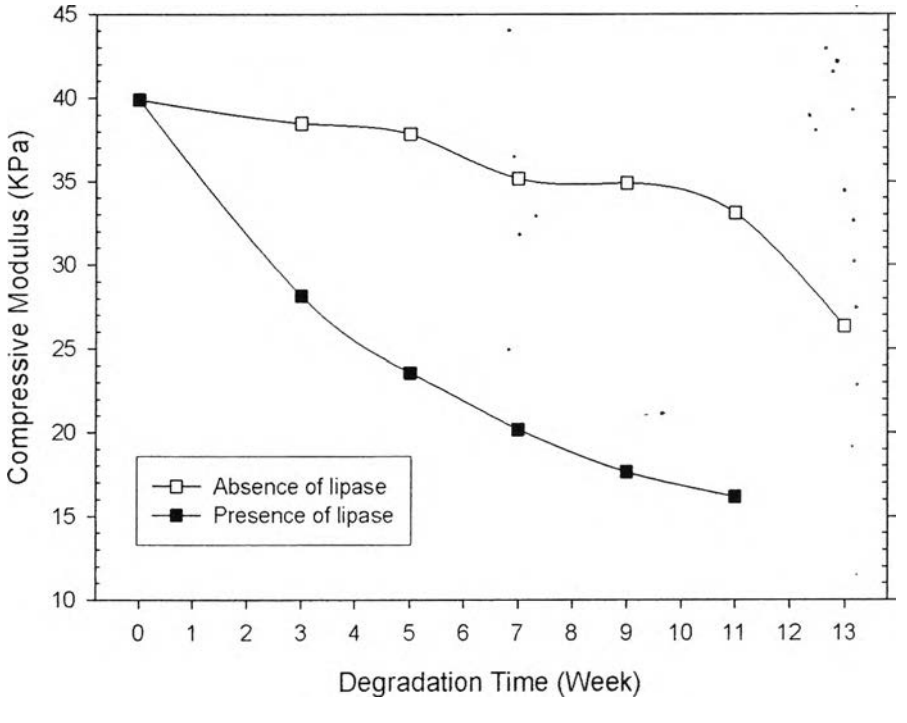


Figure 4.6 Compressive modulus of the degraded PCL scaffold in PBS containing with and without lipase.

Table 4.3 Compressive modulus of degraded scaffolds in PBS solution

Time (week)	Compressive Modulus (KPa)				
	PCL	PBSu-DCH	PLA	PHB	PHBV
0	39.90 ± 5.55	67.43 ± 8.90	164.94 ± 10.89	98.83 ± 14.76	109.38 ± 11.42
1	Nd	Nd	157.44 ± 14.81	42.22 ± 9.55	103.30 ± 10.90
3	38.49 ± 4.51	53.11 ± 9.00	152.44 ± 16.33	36.12 ± 5.40	98.53 ± 15.57
5	37.84 ± 4.52	32.91 ± 7.32	152.22 ± 7.15	35.54 ± 4.93	92.82 ± 17.19
7	35.18 ± 3.01	27.71 ± 8.30	152.06 ± 9.59	34.86 ± 8.92	88.12 ± 21.90
9	34.90 ± 8.30	*	149.24 ± 13.50	33.65 ± 8.04	86.08 ± 21.87
11	33.09 ± 7.50	*	134.56 ± 12.36	20.07 ± 3.33	84.89 ± 9.24
13	26.33 ± 3.51	*	123.25 ± 16.13	*	71.51 ± 6.66

Table 4.4 Compressive modulus of degraded scaffolds in lipase/PBS solution

Time (week)	Compressive Modulus (KPa)				
	PCL	PBSu-DCH	PLA	PHB	PHBV
0	39.90 ± 5.55	67.43 ± 8.90	164.94 ± 10.89	98.83 ± 14.76	109.38 ± 11.42
1	Nd	Nd	160.68 ± 17.95	93.10 ± 16.42	90.57 ± 5.30
3	28.15 ± 7.18	34.18 ± 4.07	120.15 ± 14.95	89.26 ± 13.47	89.00 ± 5.98
5	23.58 ± 4.29	22.33 ± 5.37	118.72 ± 15.19	85.48 ± 16.95	88.94 ± 12.41
7	20.19 ± 5.29	*	114.02 ± 21.33	66.70 ± 26.42	87.16 ± 10.20
9	17.63 ± 5.90	*	104.44 ± 9.44	61.65 ± 12.22	75.02 ± 10.09
11	16.15 ± 3.07	*	104.29 ± 14.57	59.16 ± 6.52	63.11 ± 7.16
13	*	*	103.58 ± 15.30	55.14 ± 6.22	56.34 ± 11.95

Nd : not determined

The asterisk means that it can't be determined because it lost of cylindrical shape.

4.2.3 Thermal Properties

Thermal properties of the porous scaffolds were investigated by 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 4.5 illustrates the thermal properties of PCL, PBSu-DCH, PLA, PHB and PHBV porous scaffolds before degradation study.

Table 4.5 Thermal characteristics and apparent crystallinity of PCL, PBSu-DCH, PLA, PHB and PHBV porous scaffolds before degradation study

Scaffolds	T_g[a] (°C)	$T_{m,o}$[a] (°C)	$T_{m,s}$[a] (°C)	$T_{m,c}$[a] (°C)	ΔH_f^0 ($J.g^{-1}$)	ΔH_f[a] ($J.g^{-1}$)	χ_c[b] (%)
PCL	-60	65.7	56.9	30.79	139.3	79.56	57.1
PBSu-DCH	-28	115.2	114.3	75.2	110.5	96.16	87.0
PLA	62.5	149.7	149.6	62.92	203.4	20.43	10.0
PHB	-	175.5	144.8	61.5	146.6	93.13	63.5
PHBV	-	164.7	145.9	50.1	146.6	104.5	71.3

[a] These data were determined by DSC: $T_{m,o}$, apparent melting peak temperature of the original samples, obtained from first heating (HEAT1); $T_{m,c}$, apparent crystallization peak temperature of the samples, obtained from cooling (COOL); $T_{m,s}$, apparent melting peak temperature of the samples subjected to second heating (HEAT2), obtained from second heating and ΔH_f, apparent enthalpy of fusion, obtained from first heating.

[b] χ_c, apparent degree of crystallinity, calculated from $\frac{\Delta H_f}{\Delta H_f^0} \times 100$.

ΔH_f^0 is the enthalpy of fusion of 100% crystalline polymer. The ΔH_f^0 of PCL was reported to be 139.3 $J.g^{-1}$ (Jenkins *et al.*, 2003). The theoretical value for a perfectly crystalline PBS (110.5 $J.g^{-1}$), which was determined on the group contribution basis (Nikolic *et al.*, 2001). Ma *et al.* reported the ΔH_f^0 of PLA (203.4 $J.g^{-1}$). The enthalpy of fusion of 100% crystalline polymer for PHB and PHBV was reported by Sombatmankhong *et al.* in 2006 (146.6 $J.g^{-1}$).

Table 4.5 illustrates the crystallinity of PCL, PBSu-DCH, PLA, PHB and PHBV porous scaffolds before degradation study. Although the PBSu-DCH scaffold exhibited the highest crystallinity, it dose not show the best degrading resistance. This phenomenon should be caused by the glass temperature of PBSu-DCH scaffold is quite low (-28°C). Hence, the polymeric chain can move independently at room temperature resulting in easy to degrade. Interestingly, lipases cannot degrade the optically active polymers with high T_m such as PLA (Tokiwa *et al.*, 2004).

Table 4.6 Change of apparent crystallinity and melting temperature of PCL, PBSu-DCH, PLA, PHB and PHBV porous scaffolds after immersion in lipase/PBS for 13 weeks

Scaffolds	Week 0		Week 13	
	$T_{m.o}$ (°C)	χ_c (%)	$T_{m.o}$ (°C)	χ_c (%)
PCL	65.7	57.1	68.1	57.2
PBSu-DCH	115.2	87.0	115.3	87.2
PLA	149.7	10.0	148.0	14.4
PHB	175.5	63.5	173.4	64.1
PHBV	164.7	71.3	164.4	71.5

The degradation of semicrystalline polymers begins mostly from the amorphous regions, where the segments of the macromolecules pack more loosely and can be more easily attacked by water molecules. Consequently, the amorphous regions are preferentially removed, leading to the relative increase of the crystallinity of the remained material. On the other hand, the breakage of the segments leads to a decrease in molecular entanglement and an increase in chain mobility. The chains could then reorganize themselves to a more orderly macromolecular arrangement (Gong *et al.*, 2007). Therefore, the crystallinity of the PCL, PBSu-DCH, PLA, PHB and PHBV scaffolds after degradation was apparently increased (Table 4.6). Although the crystallinity of the degrade PLA, PHB and PHBV scaffolds was improved, the melting temperature of these scaffolds shifted to lower temperatures.

This might be caused by the crystallites become more disordered. A smaller molecular weight is of benefit to molecular movement too (Tsuji *et al.*, 2000).

4.3 Cytotoxicity

An indirect cytotoxicity test was conducted on the 5 types of porous scaffolds by use L929 cell lines. In order to use these porous scaffolds as potential bone scaffolds, it was mandatory to test the materials with L929 just to comply with the ISO10993-5 standard test method. Figure 4.7 shows the absorbance obtained from an MTT assay of the cells which were cultured with the extraction media in comparison with those cultured with serum free medium (that is, the control) for 24 hours. The average absorbance values of PCL scaffold exhibited much greater average absorbance values in comparison with that of the control, while PBSu-DCH scaffold was not significantly different. On the other hand PLA, PHB and PHBV scaffolds exhibited a slightly lower average absorbance value, in comparison with that of the control. All of the obtain results indicated that PCL and PBSu-DCH scaffolds posed as good candidates to be used as bone scaffolds.

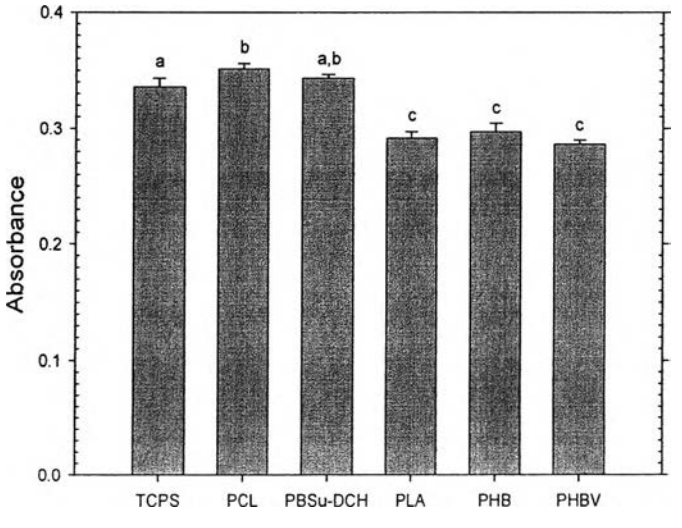


Figure 4.7 Indirect cytotoxic evaluation of porous scaffolds based on the viability of mouse fibroblasts (L929).

4.4 Cell Attachment and Proliferation

Attachment of human osteoblastic cells (SaOS-2) on a scaffolding substrate could be quantified by the UV absorbance from MTT assay. Figure 4.8 shows attachment of SaOS-2 on TCPS (i.e., controls), PCL, PBSu-DCH, PLA, PHB and PHBV porous scaffolds. Clearly, the attachments of SaOS-2 on the 5 types of porous scaffolds were significantly better than that on the TCPS during the early 4 hours in culture. However, the attachment of SaOS-2 on PBSu-DCH and PCL scaffolds were greater than that on PLA, PHB and PHBV porous scaffolds. After 4 hour, the number of cells attached on the PCL, PBSu-DCH scaffolds and TCPS still increased significantly, while it was rarely increasing to that on PLA and PHB scaffolds. Interestingly, the attachment of SaOS-2 on the PHBV scaffold was practically equivalent to that on TCPS at 22 hours in culture. On the other hand, the number of cells attached on the porous scaffolds at 22 hours in culture can be ranked as follows: PBSu-DCH > PCL > PHBV ≈ PHB ≈ PLA

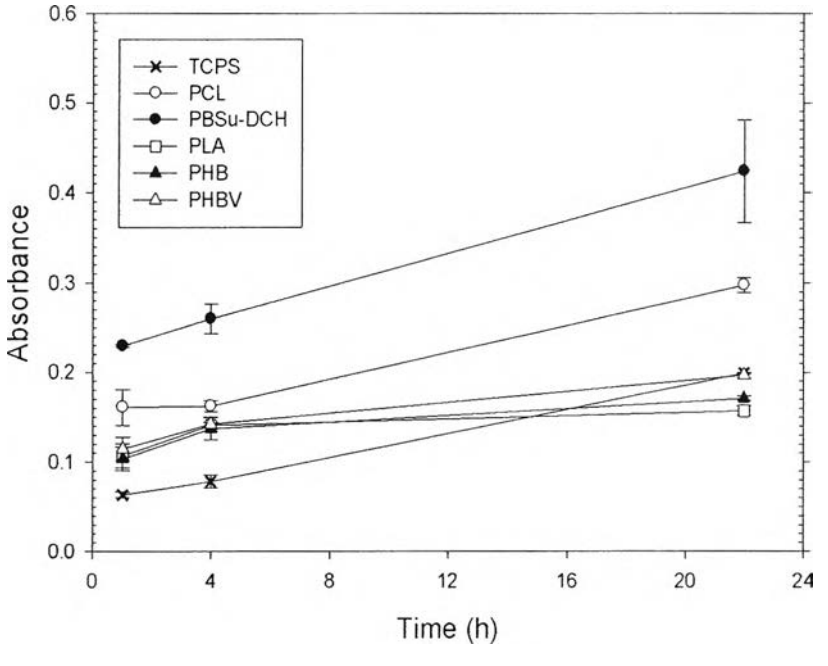


Figure 4.8 Attachment of SaOS-2 on TCPS, PCL, PBSu-DCH, PLA, PHB and PHBV porous scaffolds as a function of time in culture.

Figure 4.9 illustrates proliferation of SaOS-2 on TCPS, PCL, PBSu-DCH, PLA, PHB and PHBV porous scaffolds at day 1, 2, and 3. Apparently, the proliferation of SaOS-2 on the PBSu-DCH and PCL scaffolds was significantly better than that on the other scaffolds and TCPS at day 1. Although, all porous scaffolds exhibited high proliferation rate between day 1 and 2, they showed a decrease in the proliferation rate during day 2 and 3. However, the proliferation rate of SaOS-2 on TCPS was very high, the absorbance from MTT assay of TCPS substrate was practically equivalent to that on PCL scaffold at day 2 and equivalent to that on PBS at day 3 in culture, respectively.

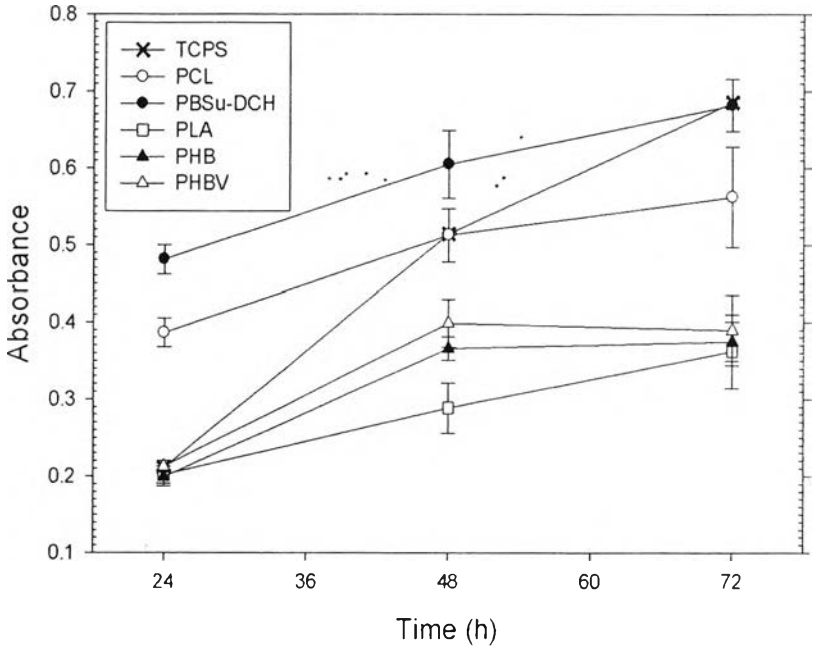
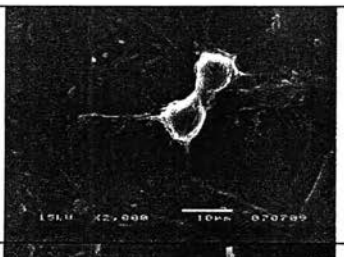
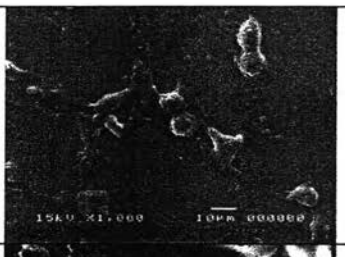
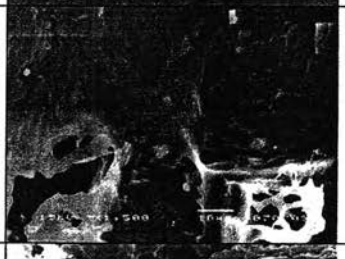
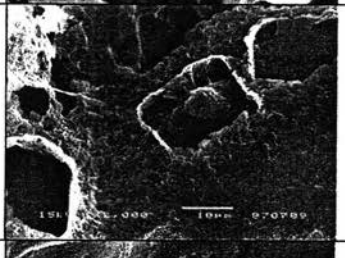
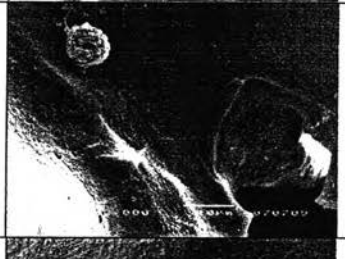
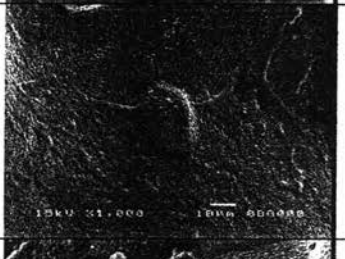
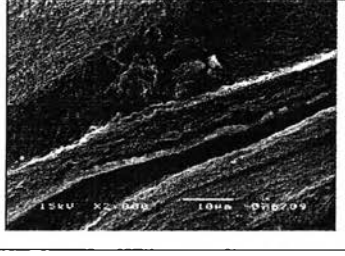


Figure 4.9 Proliferation of SaOS-2 on TCPS, PCL, PBSu-DCH, PLA, PHB and PHBV porous scaffolds as a function of time in culture.

4.5 Morphological Observation of Cultured Cells

Table 4.7 shows the SEM micrographs of SaOS-2 cultured on the PCL, PBSu-DCH, PLA, PHB, PHBV porous scaffolds and glass substrate for 1 and 7 days. These images confirm that the phenotype of SaOS-2 was maintained after they were seeded on different types of substrates. At the first day, the cells that were cultured on PCL, PBSu-DCH, PLA scaffolds and the glass substrate illustrated broad lamellipodia around the cells to help attach themselves on the surface of substrate, while those cultured on PHB and PHBV porous scaffolds were still in round shape. At longer times (7 days), the cells on PCL, PBSu-DCH, PLA, PHBV scaffolds and the glass substrate expanded even more. On PHB scaffold, SaOS-2 revealed a rounded cell shape due to reduced spreading. The filamentous organization of the actin cytoskeleton was impaired (Nebe *et al.*, 2001). The obtained results suggest that all porous scaffolds promote the attachment of SaOS-2 and help maintain the integrity of the cells during culture. Especially for cells that were cultured on PBSu-DCH scaffold, they adhered well and spread extensively their lamellipodia over the surface.

Table 4.7 Selected SEM images SaOS-2 cultured on PCL, PBSu-DCH, PLA, PHB and PHBV porous scaffolds and glass (control) at 1 and 7 days in culture.

Substrate	Time in culture	
	1 day (2000x)	7 day (1000x)
Glass		
PCL		
PBSu-DCH		
PLA		
PHB		
PHBV		

4.6 Alkaline Phosphatase (ALP) Activity

After cell attachment and proliferation studies, the ability for porous scaffolds to support differentiation of SaOS-2 was investigated. Among the various biological functions of osteoblasts, secretion of alkaline phosphatase (ALP) is an important indicator determining the activity of the cells on the scaffold. Figure 4.10 exhibits the ALP activity of SaOS-2 cultured on PCL, PBSu-DCH, PLA, PHB and PHBV porous scaffolds after 5 days in culture. The ALP activity of cells attached on the porous scaffolds at 5 days in culture can be ranked as follows: PBSu-DCH > PCL > PHBV > PLA > PHB. This result quite conformed to the alteration of the cell attachment and proliferation.

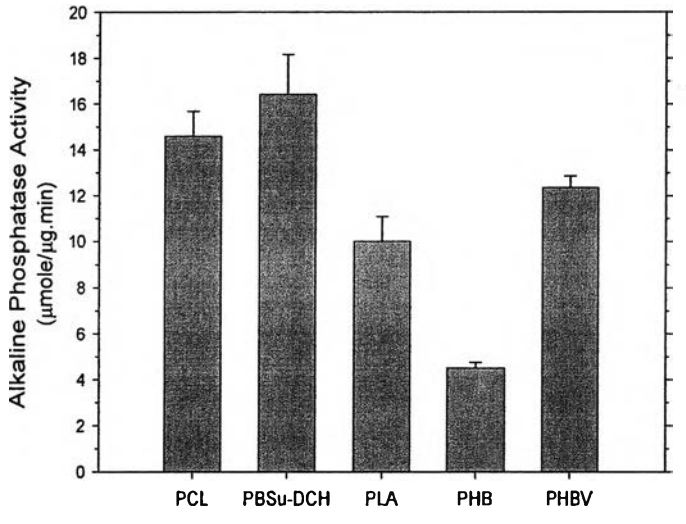


Figure 4.10 ALP activity of SaOS-2 cultured on PCL, PBSu-DCH, PLA, PHB and PHBV porous scaffolds after 5 days in culture.