CHAPTER V
DEVELOPMENT OF POROUS HYDROXYAPATITE PARTICLES AS CARRIERS OF PROTEINS IN A POLYCAPROLACTONE SCAFFOLD FOR BONE TISSUE ENGINEERING

5.1 Abstract

This study aimed to develop the porous hydroxyapatite (HAp) particles as a controlled release carrier of proteins was embedded in polycaprolactone (PCL) to fabricate porous HAp-PCL scaffolds. Proteins (Ovalbumin, Gelatin type B, Bovine Serum Albumin and Crude bone protein) had been entrapped within the HAp particles. The porous HAp-PCL scaffolds were prepared by solvent casting/particulate leaching method. Desorption behavior of HAp-PCL scaffolds was investigated by UV-Visible spectrophotometry. The potential for use of HAp-PCL as bone scaffolds was assessed by mouse calvaria derived pre-osteoblastic cells, MC3T3-E1, in terms of indirect cytotoxicity, cell attachment, cell proliferation, alkaline phosphatate (ALP) activity, and mineralization.

(**Key-words**: bone scaffolds; particulate leaching; polycaprolactone; hydroxyapatite)

5.2 Introduction

Modern tissue engineering combines materials science with biotechnology and biology to repair and replace damaged. It has been shown that new tissues can be engineered from living cells and three-dimensional scaffolds. The success of these approaches is largely dependent on the scaffold properties. The ideal scaffold should have the following characteristics: (1) Appropriate porosity, pore size and pore structure to ensure the nutrition of cells, cell attachment and cell proliferation within the scaffold (2) Mechanically strong enough to maintain structural integrity during culture, which is particularly important in load-bearing bone tissues. The material

should be strong enough to withstand physiological stresses, and to transfer loads after implantation. (3) Adequate degradation properties in which the rate of scaffold degradation supports the maintenance of structure support for cellular proliferation and extracellular matrix (ECM). (4) Biocompatible so that the material used to manufacture the scaffold does not evoke an extreme adverse inflammatory or immune response once implanted. Instead, the material should be able to integrate with the host tissue and support cell infiltration, cell attachment for bone formation (5) Appropriate manufacturing process, the manufacturing processes should not affect the material's biocompatibility as a result of residual chemicals involved in these processes (Choong C. *et al.*, 2004).

In this research, three-dimensions HAp-PCL porous scaffolds were prepared by solvent casting/particle leaching method with sodium chloride as a porogen. The inclusion of calcium phosphates (CaPs) in biodegradable polymers enhances the osteoconductive of bone scaffold. Specifically, the inclusion of CaPs in polymer scaffolds enhances its mineral deposition rate, mechanical properties, and protein adsorption, thus improving the overall potential of bone scaffold (Porter, Joshua R *et al.*, 2009).

5.3 Experimental

5.3.1 Materials

- Polycaprolactone (PCL;80,000 g mol^{-1}; Sigma-Aldrich, USA)
- Sodium chloride (Ajax Finechem, Australia)
- Chloroform (Labscan; Asia, Thailand)
- Modified Eagle's medium (MEM; Thermo Scientific, USA)
- Fetal bovine serum (FBS;Sorali, Campo Grande Brazil)
- 1 % L-glutamine (Invitrogen Corp., USA)
- 1 % Lactabumin (Invitrogen Corp., USA)
- 1 % antibiotic (Invitrogen Corp., USA)
- Bicinchoninic acid (BCA; Thermo Scientific, USA)

5.3.2 Preparation of Polycaprolactone (PCL)-Hydroxyapatite (HAp) Scaffold

A solvent casting and salt particulate leaching was used to prepare the scaffold. Firstly, PCL granules were put into a glass bottle with chloroform to make up a PCL solution in the concentration of 14 percentages of polymer weight by volume of the solution (w/v), then the solution was stirred at room temperature for 2 h. Secondly, protein-loaded HAp were added into the glass bottle and they were stirred together. Thirthly, NaCl salt particles with size of 400 μm (PCL/NaCl = 1/30) (w/w) were added into the glass bottle and they were mixed together. And finally, the mixture was packed into the petri-dish with the dimension of 10x10 cm. The mold was then left in the hood for 24 h and immersed in the distill water 1 day for taking the mold out. The materials that come out were immersed in DI water for 2 days, during with time the water was changed approximately every 8 h under the room temperature for the leaching out the salt particles. Then the materials were air-dried for 24 h and freeze-dried overnight to obtain porous scaffolds (Prasansuklap *et al.*, 2008).

5.4 Characterization of Porous Scaffolds

5.4.1 Porosity, Pore Volume and Pore Size

The density of the scaffolds ($\rho_{scaffold}$) is determined by using a Sartorius YDK01, Germany density measurement kit (Buoyancy method) which can be calculated using the following equation.

$$\rho_{scaffold} = \frac{W_a \times \rho_{fl}}{W_a \times W_{fl}}$$

Where W_a is the weight of the scaffold in air, W_{fl} is the weight of the scaffold in water and $\rho_{scaffold}$ is the density of the water (at 25°C, $\rho_{fl} \approx 1$ g/cm^3).

The porosity and pore volume of the scaffolds were calculated using the following equation (Hou, Qingpu *et al.*, 2003)

$$\text{Porosity (\%)} = \left(1 - \frac{\rho_{scaffold}}{\rho_{polymer}}\right) \times 100$$

$$\text{Pore volume} = \frac{1}{\rho_{scaffold}} - \frac{1}{\rho_{polymer}}$$

where $\rho_{scaffold}$ is the apparent density of the porous scaffolds and $\rho_{polymer}$ is the density of the non-porous polymer, compression moulded in the same manner.

Pore size of the scaffold was measured on the SEM micrograph with the UTHSCSA Image Tool version 3.0 software. The average values were calculated from the total 25 pores and accept as the mean pore sizes.

5.4.2 Water Absorption Capability

The dry scaffold scaffolds were weighted and then were immersed in 5 ml of phosphate buffer silane solution (PBS pH 7.4) solution at room temperature for 3 days. Scaffolds were removed from the solution and carefully placed on the glass for 5 seconds to remove the excessive water and weight immediately. The water absorption was calculated using the following equation.

$$\text{Water absorption (\%)} = \frac{(M_{wet} - M_{dry})}{M_{dry}} \times 100$$

Where M_{dry} and M_{wet} are the weight of the scaffold before and after immersion in water, respectively. Five measurements were performed for the calculation of an average water absorption value.

5.4.3 Morphology of Porous Scaffolds

The morphology of the pores size of the porous scaffold were observed by a JEOL JSM-5200 scanning electron microscopy (SEM). The scaffolds were cut with razor blade at the middle of the scaffolds and mounted on stubs. Cross sections of the scaffolds were coated with thin film of gold using JEOL JFC-1100E sputtering devices for 120 second.

5.4.4 *In Vitro* Release of Proteins from Scaffold

The proteins/HAp-PCL were immersed in 1 ml of the minimum essential medium (MEM; Hyclone, Thermoscientific, USA) in 24 well plate. All samples were incubated in a shaking water bath (70 rpm) at 37 ºC. The amount of proteins releasing from scaffold to the supernatant was measured by BCA protein assay at various times. After the releasing medium (sample solution) was withdrawn 20 µl to mixed with BCA solution, an equal amount of fresh medium was added to maintain a constant volume of the medium. The amount of protein in the sample solution was determined by UV-visible spectroscopy at 562 nm. The MEM used in this study contained plenty of much high molarity inorganic salts comparing with those in PBS such as NaCl 117.24 M, KCl 5.33 M, $CaCl_2$ 1.8 M, NaH_2PO_4-H_2O 1.01 M (Invitrogen, 2009).

5.5 Cell culture

Mouse calvaria-derived, pre-osteoblastic cells (MC3T3-E1) were cultured as monolayers in minimum essential medium with Earle's Balanced Salts (MEM; Hyclone, Thermoscientific, USA), supplemented with 10 % FBS, 1 % L-glutamine and 1 % antibiotic and antimycotic formulation (containing penicillin G sodium, streptomycin sulfate, and amphopericin B (Invitrogen Corp, USA). Cells were cultivated in 5 % CO_2 at 37 ºC in a humidified atmosphere.

5.5.1 Indirect Cytotoxic Study.

An indirect cytotoxic test was conducted on the proteins-loaded hydroxyapatite and PCL-HAp scaffold by use MC3T3-E1. First, extraction medias were prepared by immersing powder of each protein-loaded hydroxyapatite and circular shape of each PCL-HAp scaffold specimens about (15 mm in diameter) in wells of a 24-well culture plate in a 2 % MEM (containing MEM, 2 % FBS, 1 % L-glutamine, 1 % antibiotic and antimycotic formulation) for 24 h. Each of these extraction media was used to evaluate the cytotoxicity of the HAp powder and scaffold. MC3T3-E1 was separately cultured in wells of a 24-well culture plate in serum-containing MEM for 16 h to allow cell attachment on the plate. The cells were then starved with 2 % MEM for 24 h, after which time the medium was replaced

with an extraction medium. After 24, 48, and 72 h of cell culturing in the extraction medium, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was carried out to quantify the amount of via cells.

The MTT assay is based on the reduction of the yellow tetrazolium salt to purple formazan crystals by dehydrogenase enzymes secreted from the mitochondria of metabolically active cells. The amount of purple formazan crystals formed is proportional to the number of viable cells. First, each of culture medium was aspirated and replaced with 400 µl per well of MTT solution at 0.5 mg/ml for a 24-well culture plate. Secondly, the plate was incubated for 30 mins at 37 °C. The solution was then aspired and 900 µl per well of dimethylsulfoxide (DMSO) containing 125 µl per well of glycine buffer (pH=10) is added to dissolve the formazan crystals. Finally, after 10 min of rotary agitation, the absorbance of the DMSO solution at 570 nm was measured using a Thermospectronic Genesis 10 UV-Visible spectrophotometer.

5.5.2 Cell Attachment and Cell Proliferation Study

Mouse calvaria-derived, pre-osteoblastic cells (MC3T3-E1) were allowed to attach on the porous scaffold specimens and empty wells for 2, 4, and 6 h. For the proliferation study, the cells were allowed to attach on the porous scaffold specimens and empty wells for 1, 3, and 5 d. At each time point, a number of the attached and proliferated cells were quantified by MTT assay. Each specimen was rinsed with phosphate buffered saline to remove unattached cells prior to the MTT assay. The morphology of the cells during the attachment and proliferation periods was observed by SEM. At each time point, the culture medium was removed and then the cell-cultured scaffold specimens were rinsed with PBS twice, the cells were then fixed with 3 % glutaraldehyde solution, which was dilute from 50 % glutaraldehyde solution with PBS 500 µl/well. After 30 min, they were rinsed again with PBS. After cell fixation, the specimens were dehydrated in an ethanol solution of varying concentration (30 %, 50 %, 70 % and 90 %, respectively). The specimens were the dried in air. After completely dried, the specimens were mounted on an SEM blass stubs, coated with gold and observed by SEM.

5.5.3 Alkali Phosphate Analysis (ALP)

Mouse calvaria-derived, pre-osteoblastic cells (MC3T3-E1) were cultured on porous scaffolds for 5 and 7 d and in different proteins for 5 and 7 d to observe ALP activity. Each porous specimen was rinsed with PBS after removal of the culture medium. Alkaline lysis buffer (10 mM tris-HCl, 2 mM $MgCl_2$, 0.1 % Triton-X100), pH 10) (1000 µl/well) was added, and the specimen was scrapped and then frozen at -20 °C for at least 30 min prior to the next step. An aqueous solution of 2 mg ml^{-1} p-nitrophenyl phosphate (PNPP; Zymed Laboratories) mixed with 0.1 M aminopropanol (10µl/well) in 2 mM $MgCl_2$ (100 µl/well) having a pH of 10.5 was prepared and added into the specimen. It was then incubated at 37 °C for 2 min. The reaction was stopped by the addition of 0.9 ml/well of 50 mM NaOH, and the extracted solution was transferred to cuvette and placed in the UV-Vis spectrophotometer, from which the absorbance at 410 nm was measured. The amount of ALP was then calculated against a BSA standard curve. To determine the ALP activity, the amount of ALP had to be normalized by the amount of total proteins synthesized. In the protein assay, each specimen was treated in the same manner as in the ALP assay up to the point where it was frozen. After freezing, a bicinchoninic acid (BCA; Thermoscientific, USA) solution was into the specimen. It was subsequently incubated at 37 °C for 5 min. The absorbance of the medium solution was then measured at 562 nm by the UV-Vis spectrophotometer, and the amount of the total proteins was calculated against a BSA standard curve.

5.5.4 Mineralization Analysis

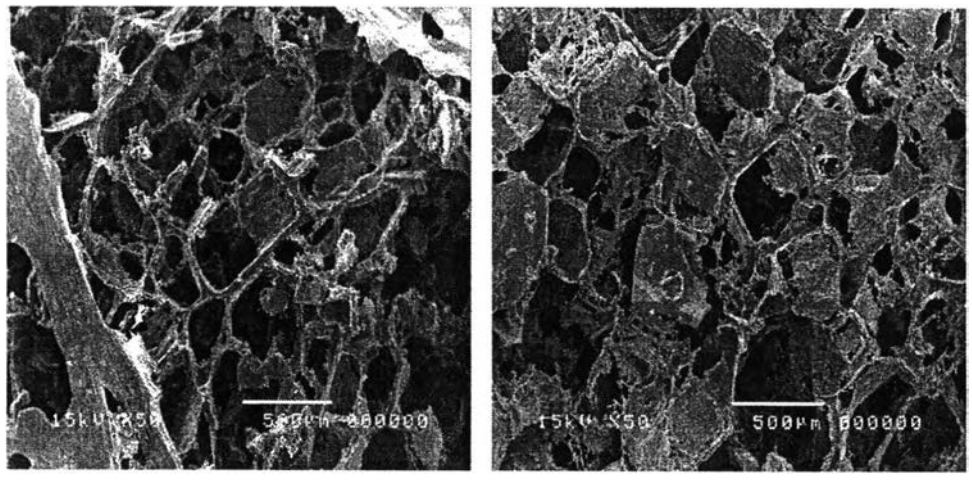
Calcium deposition was quantified by Alizarin Red S staining. MC3T3-E1 were cultured on the porous scaffolds in 24-well plate for 21 days. The cultures in 24-well plate were rinsed with PBS after that the cells were fixed with cold methanol for 10 min and washed with deionized water prior to immersion for 3 min in 370 µl of 1 % Alizarin Red S solution dissolved in 1:100 (v/v) ammonium hydroxide/water mixture for 3 min (pH=3.3). Each stained specimen was washed several times with DI water and air-dried at room temperature. Calcium forms an Alizarin Red S-calcium complex in a chelating process. The stained specimen was

photographed and eluted dye with 10% cetylpyridinium chloride for 20 min. The Alizarin Red concentration was determined by measuring the absorbance at 562 nm.

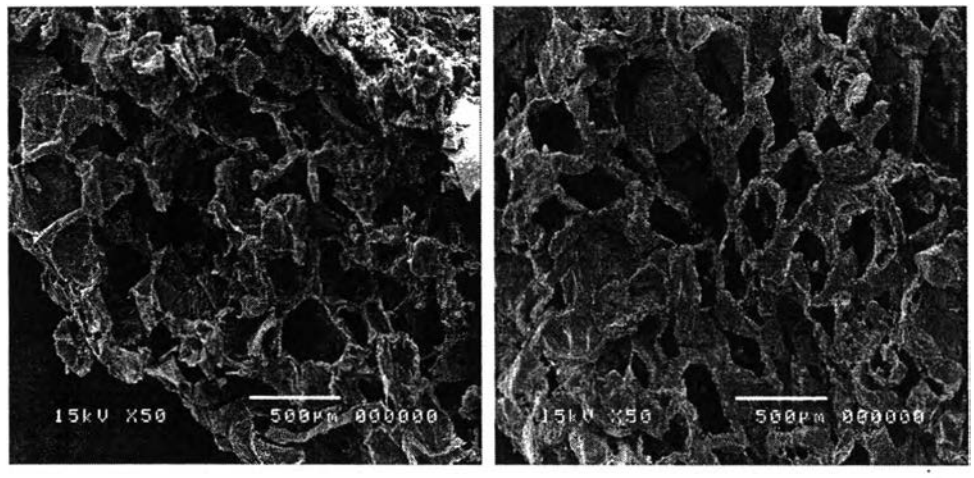
5.6 Results and discussion

5.6.1 Microstructure observation

Figure 5.1 demonstrated the SEM images microstructure of the as-prepared scaffolds when being viewed on the surface and cross sections. For PCL and HAp/PCL scaffold with NaCl : PCL, 30:1 weight ratio. The SEM images of (a) and (c) showed many square units occurring from the shape of NaCl porogens used in the fabricating process. They showed clearly square units due to neat PCL scaffold whereas (b) and (d) exhibited the lost square because HAps embedded in the PCL scaffold.



(a) (b)



(c) (d)

Figure 5.1 SEM images illustrate microstructure of the scaffolds on the surface: (a) PCL, (b) HAp-PCL and on the cross-sections : (c) PCL, (d) HAp-PCL.

5.6.2 Density, Porosity, Pore volume and Pore size

The density, porosity, pore volume and pore size of the scaffold was shown in Table 5.1.

Table 5.1 Density, porosity, pore volume and pore size of PCL scaffold

Scaffolds	Density (g/cm^3)	Porosity (%)	Pore volume (cm^3/g)	Pore size (µm)
PCL	0.1139	90.05	7.91	347.07 ± 42.19

5.6.3 Water Absorption Capability and *In vitro* Degradability

Figure 5.2 illustrates the water absorption capabilities of the PCL scaffolds in 0.1 M PBS at room temperature within 3 days (72 h). The water absorption rate was rapidly increased in the early 24 hours and maintained stable.

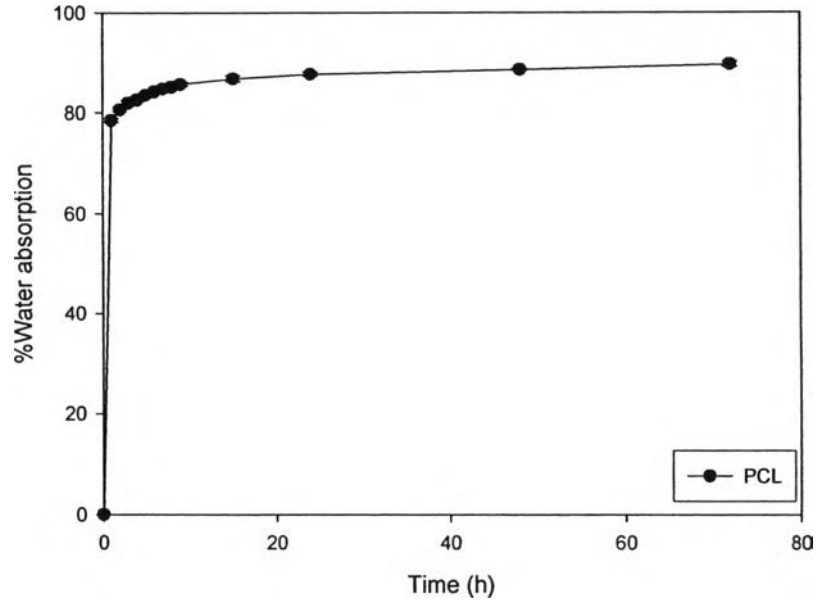


Figure 5.2 Water absorption capability of the PCL scaffolds in 0.1 M PBS at room temperature within 3 days.

5.6.4 *In vitro* OVA, Gelatin type B, BSA, and CBP Release

The release of proteins from HAp-PCL scaffolds were investigated by immersing HAp-PCL scaffolds into *in vitro* culturing environment, 10 % MEM (containing MEM, 10 % FBS, 1 % L-glutamine, 1 % antibiotic). All samples were incubated in a shaking water bath (70 rpm) at 37°C for 21 days. The result shows gelatin and CBP started to before day 7 whereas OVA and BSA release after day 7. The amount of released proteins gradually increased time dependently. This result correspond to the work done by proteins releasing from HAp particles, however, protein release from scaffold spends a longer time due to different media. It is different in ionic strength, in MEM shows higher ionic strength. High ionic strength should promote aggregation of protein and retard release (Hariraksapitak, P., 2009)

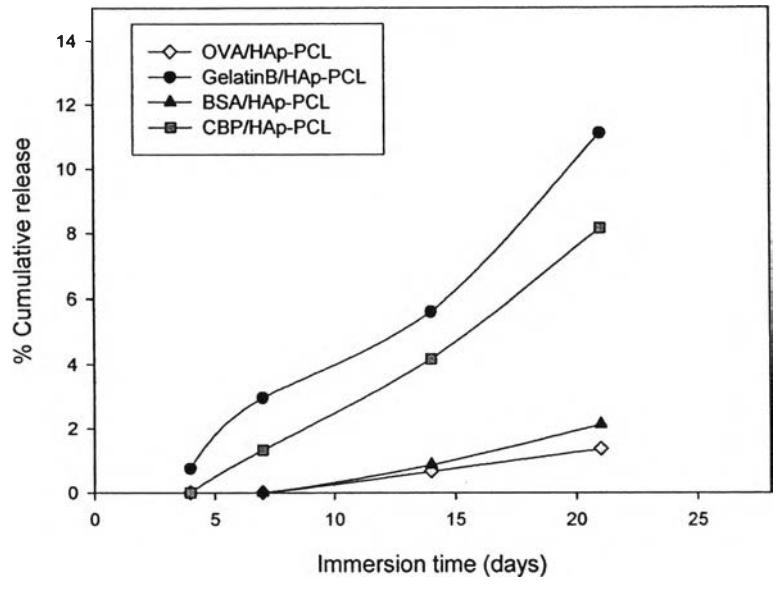


Figure 5.3 Release profile from protein-loaded HAp-PCL scaffold in 10 % MEM.

5.7 Cell Culture

5.7.1 Cytotoxicity

Indirect cytotoxicity in this studied can be classified into 3 process are indirect cytotoxicity for protein, HAp, and HAp-PCL scaffold. Firstly, an direct cytotoxicity test of proteins were performed in 4 types which are Ovalbumin (OVA), Gelatin type B, Bovine serum albumin (BSA), and Crude bone protein. MC3T3-E1, pre-osteoblast cell lines, in culture of 40,000 cell/well seeded with 10 μg/ml concentration in 2% MEM for 24, 48, and 72 h. The results are shown in Figure 5.4 with %viability of MC3T3-E1 relative to tissue culture polystyrene (TCPS) in all types of proteins show that increasing with cuturing time. The % viability of MC3T3-E1 is more than 80 %, so all types of proteins were no toxicity to cells. Secondly and Thirdly are indirect cytotoxicity test of proteins-loaded HAp and HAp-PCL scaffold. MC3T3-E1 were cultured in 2 % MEM extraction media from proteins-loaded HAp (Figure 5.5) and HAp-PCL scaffold (Figure 5.6) for 24, 48, and

72 h. The % viability of MC3T3-E1 in extraction media of each time are more than 80%, so proteins-loaded HAp and HAp-PCL scaffold were no toxicity to cell.

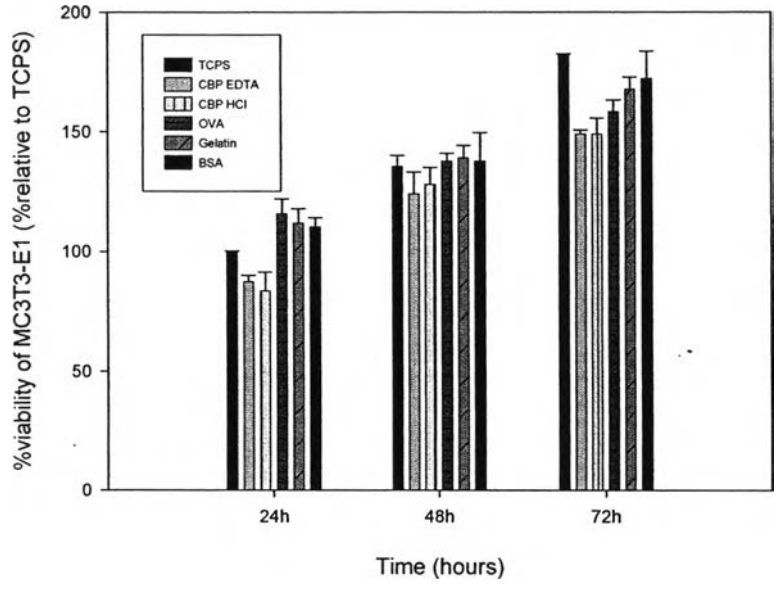


Figure 5.4 Direct cytotoxicity evaluation of proteins based on the viability of MC3T3-E1.

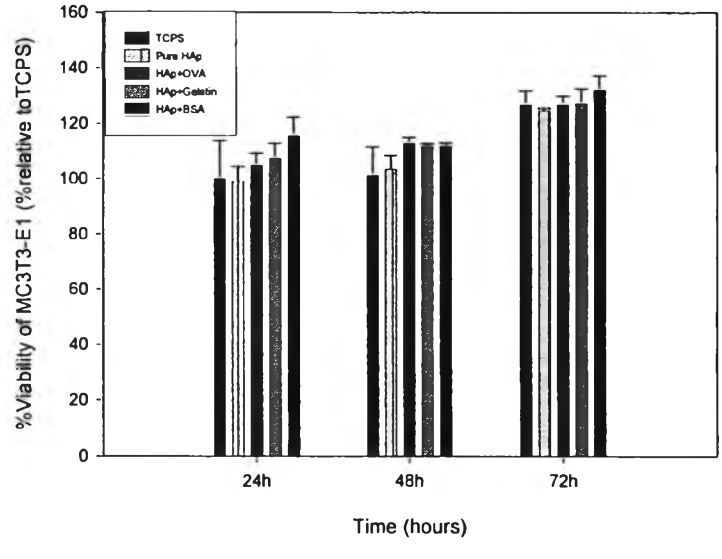


Figure 5.5 Indirect cytotoxicity evaluation of protein-loaded HAp based on the viability of MC3T3-E1.

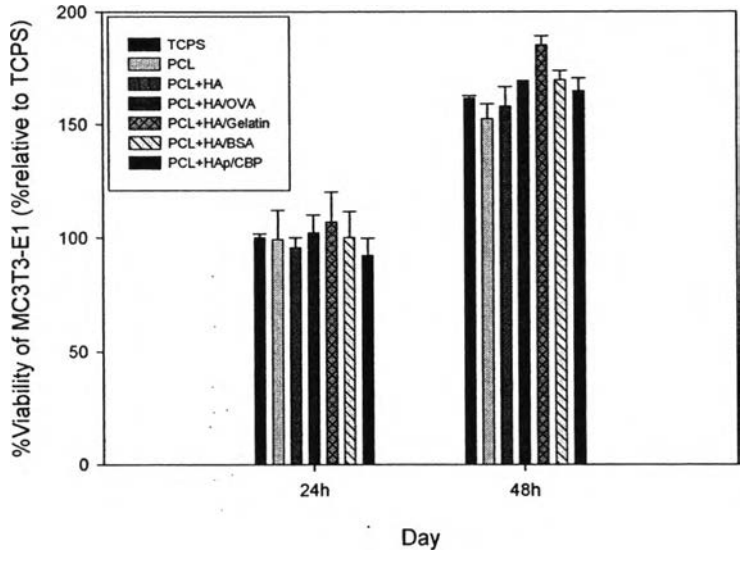


Figure 5.6 Indirect cytotoxicity evaluation of HAp-PCL scaffold based on the viability of MC3T3-E1.

5.7.2 Cell Attachment and Proliferation

Attachment of mouse calvaria-derived, pre-osteoblastic cells (MC3T3-E1) on scaffolding substrate could be quantified by the UV absorbance from MTT assay. Figure 5.7 shows attachment of MC3T3-E1 on the surface of TCPS, PCL, HAp-PCL, OVA/HAp-PCL, Gelatin type B/HAp-PCL, BSA/HAp-PCL, CBP/HAp-PCL at 2, 4, and 6 h after cell seeding. The number of cell attachment on all of the surfaces increase with culturing time. The number of cell attachment on TCPS was the greatest than all of HAp-PCL and proteins-loaded HAp scaffolds at any given time point, in culture of 40,000 cell/well. On TCPS, the number of cell attachment rapidly increased from 100% at 2 h after cell seeding to ~200% at 4 h after cell seeding. Whereas the number of cell attachment on PCL, HAp-PCL and proteins-loaded HAp were lower than TCPS could be because MC3T3-E1 like to attach the smoother surface of TCPS than rough surface of PCL, HAp-PCL and proteins-loaded HAp-PCL scaffold. However, Table 5.2 shows that almost of the formazan crystal still adhere within the scaffolds after using DMSO as eluting agent.

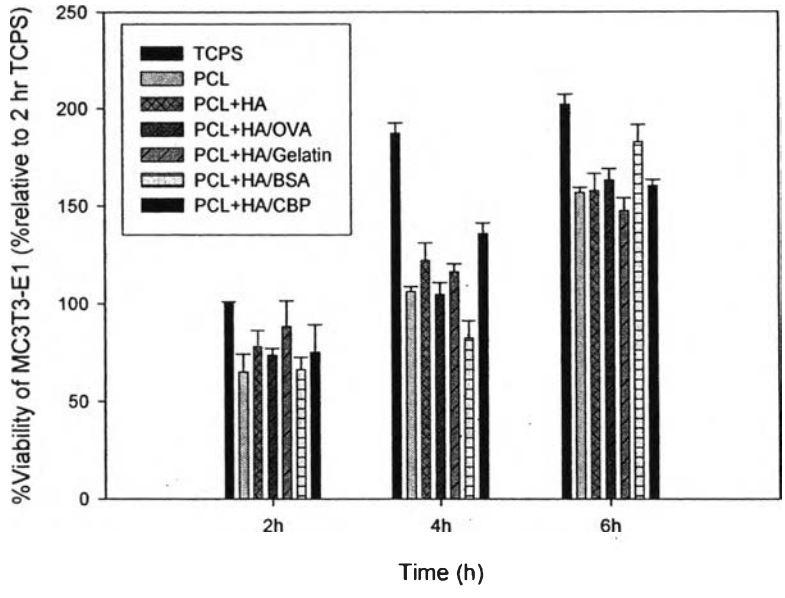
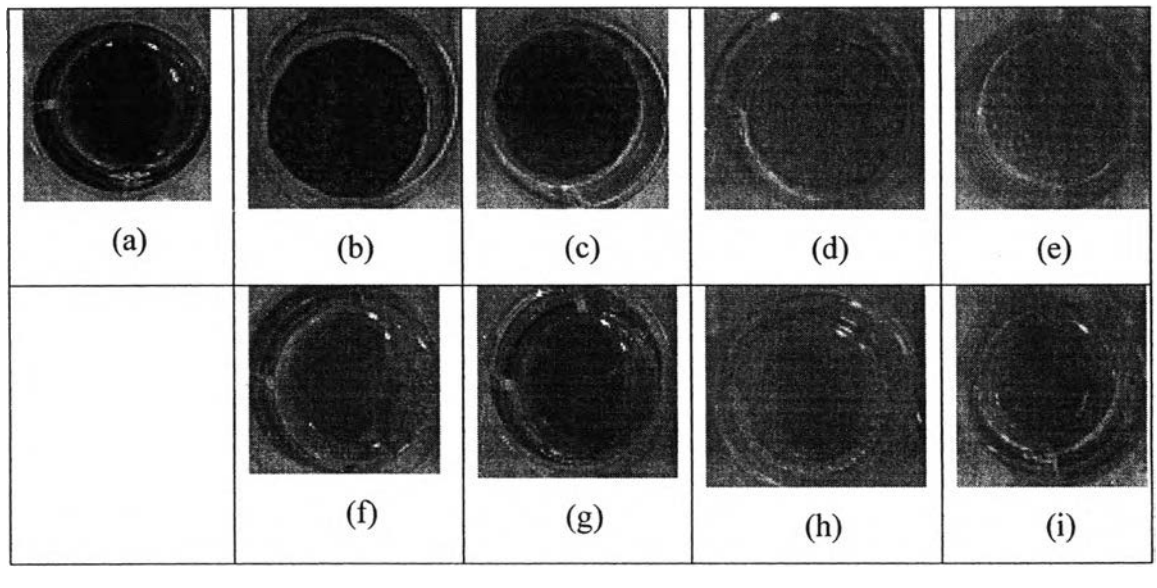


Figure 5.7 Attachment of MC3T3-E1 on control TCPS, PCL, HAp/OVA-PCL, HAp/gelatin B-PCL, and HAp/BSA-PCL

Table 5.2 Formazan crystal within TCPS, PCL, HAp-PCL scaffold were eluted with DMSO after cell seeding 6 h. (a) Eluted formazan from TCPS. (b), (c) Formazan still adhere in scaffold. (f), (g) Eluted formazan from scaffold. (d), (e) scaffold without cells inside. (h), (i) Eluted scaffolds without cells



Proliferation of mouse calvaria-derived, pre-osteoblastic cells (MC3T3-E1) on scaffolding substrate could be quantified by the UV absorbance from MTT assay. Figure 5.8 shows proliferation of MC3T3-E1 on the surface of TCPS, PCL, HAp-PCL, OVA/HAp-PCL, Gelatin type B/HAp-PCL, BSA/HAp-PCL, CBP/HAp-PCL at 1, 2, and 3 d after cell seeding in culture of 40,000 cell/well. The number of cell prolieration on all of the surfaces at any given time point were constant. The number of cell proliferation on TCPS was greater than all of PCL, HAp-PCL, and proteins-loaded HAp-PCL scaffolds at any given time point. On PCL, HAp-PCL and proteins-loaded HAp-PCL scaffold, the number of cell proliferation was only 60%. Whereas the number of cell attachment on HAp-PCL and proteins-loaded HAp were lower than TCPS could be because MC3T3-E1 like to proliferate the smoother surface of TCPS than rough surface of PCL, HAp-PCL and proteins-loaded HAp-PCL scaffold. The difference between the number of cell proliferation to PCL, HAp-PCL, and protein-loaded HAp-PCL scaffold are not clear because of no effect of proteins release from HAp. However, Table 5.3 shows that almost of the formazan crystal still adhere within the scaffolds after using DMSO as eluting agent.

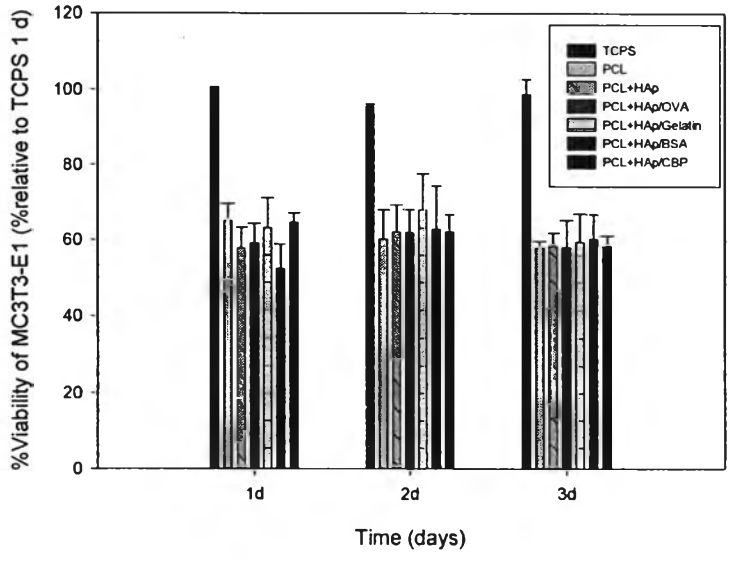
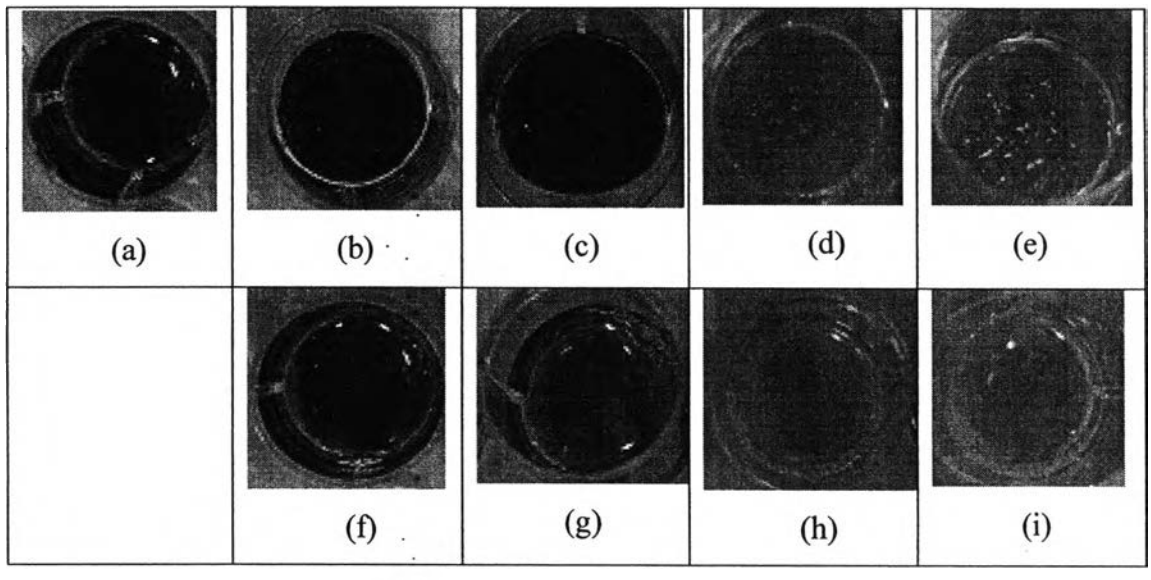


Figure 5.8 Proliferation of MC3T3-E1 that had been seeded or cultured on the surfaces of TCPS, PCL, HAp-PCL and proteins-loaded HAp-PCL scaffolds at 2 and 3 d.

Table 5.3 Formazan crystals within scaffolds after using eluting dye (DMSO) after cell seeding 1 d. (a) Eluted formazan with DMSO from TCPS. (b), (c) Formazan crystal still adhere in scaffolds. (f), (g) eluted formazan crystal with DMSO from scaffolds. (d), (e) scaffold without cells inside. (h), (i) eluted DMSO from scaffolds without cells.



5.7.3 Cell Morphological

Table 5.4 shows selected the attachment SEM images of MCT3T-E1 (magnification = 2000X; scale bar = 10 μm) at 2, 4, and 6 h. Based on the initial 40,000 cells/well of cells seeded, the majority of the cells on the glass surface and porous scaffolds were still in the round shape at 2 h and started to extend their cytoplasm at 4 h. After cell seeding, expansion of the cytoplasm of the majority of the cells was evident at 6 h. Table 5.5 shows that the selected proliferation SEM images of MC3T3-E1 (magnification = 2000X; scale bar = 10 μm) were cultured on the surfaces of glass, PCL, HAp-PCL, and proteins-loaded HAp scaffolds. The use of glass as the control instead of TCPS was due to the ease of taking the samples to SEM observation. These images provided snap shots in time that revealed the morphology of the cells and interaction between the cells and the tested surfaces. The expansion of the cells was evident with depend on the increasing time at 1, 2, and 3 days. All proteins within the HAp show the same results due to at that time the proteins were no affect to the cells.

Table 5.4 Selected SEM images of MC3T3-E1 after seeding on TCPS, PCL, OVA/HAp-PCL, Gelatin type B/HAp-PCL, BSA/HAp-PCL, and CBP/HAp-PCL scaffolds at 2, 4, and 6 h (magnification = 2,000X; scale bar = 10 μm)

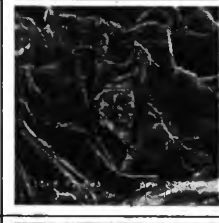
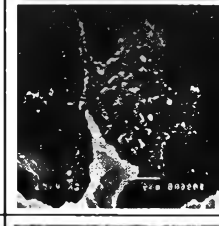
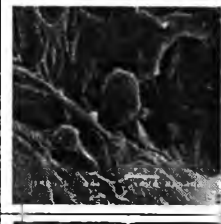
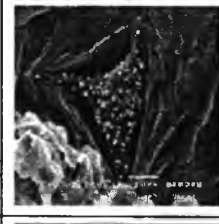
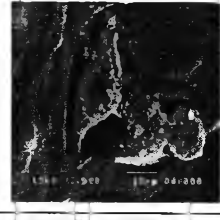
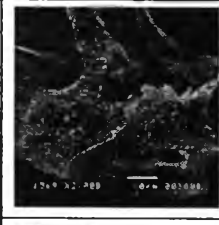
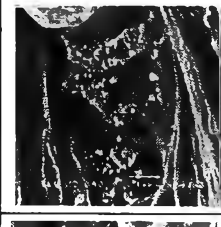
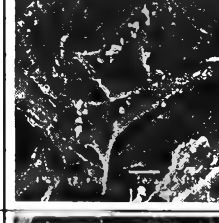
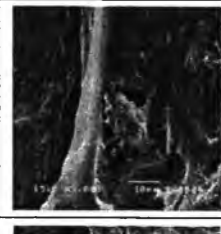
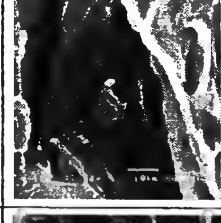
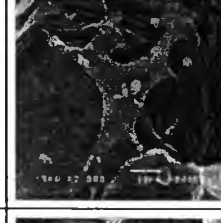
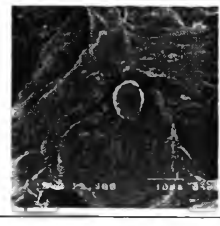
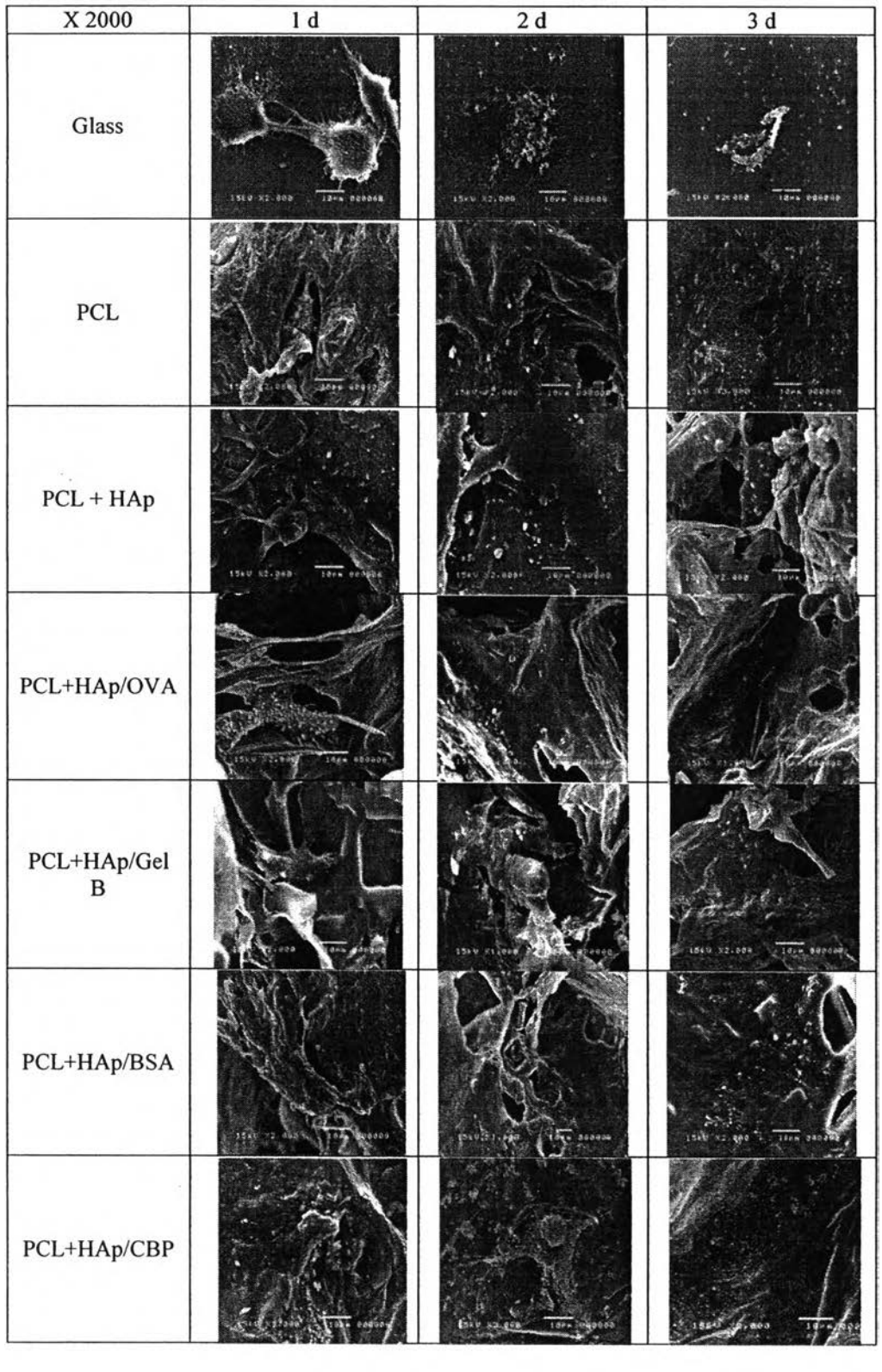
	2 h	4 h	6 h
Glass			
PCL			
PCL + HAp			
PCL+HAp/OVA			
PCL+HAp/Gel B			
PCL+HAp/BSA			
PCL+HAp/CBP			

Table 5.5 Selected SEM images of MC3T3-E1 after seeding on TCPS, PCL, HAp-PCL, OVA/HAp-PCL, Gelatin type B/HAp-PCL, and BSA/HAp-PCL scaffolds at 1, 2, and 3 days (magnification = 2,000X; scale bar = 10 μm)



5.7.4 Alkaline phosphatase (ALP) activity

Alkaline phosphatase is an indicator of osteoblast phenotype activity. It was determined after culturing the cells seeded onto the scaffold. The ALP activity of MC3T3-E1 on TCPS, PCL, HAp-PCL, OVA/HAp-PCL, Gelatin type B/HAp-PCL, BSA/HAp-PCL and CBP/HAp-PCL were monitored at 3 and 7 days in culture. In Figure 5.9 at 3 days shows the maximum level of ALP activity and at 7 days, ALP activity was decreased. In actually, ALP activity of PCL, HAp-PCL, OVA/HAp-PCL, Gelatin type B/HAp-PCL, BSA/HAp-PCL and CBP/HAp-PCL scaffold should be the same result of cell proliferation due to at that time the proteins were no effect to the cell, however, they were slightly fluctuated because PCL, HAp-PCL, OVA/HAp-PCL, Gelatin type B/HAp-PCL, BSA/HAp-PCL and CBP/HAp-PCL scaffolds were porous materials, so the cells could go through their scaffolds and could not extract the cells completely. In Figure 5.9, The ALP activity of MC3T3-E1 in OVA, Gelatin type B, BSA, and CBP with 100 μg/ml concentrations in 2%MEM was monitored at 5 and 7 days in culture. At 5 days shows the maximum level of ALP activity and at 7 days, ALP activity was decreased due to cellular process switching to mineralization. This result correspond to the work done by ALP activity of MC3T3-E1 on substrates.

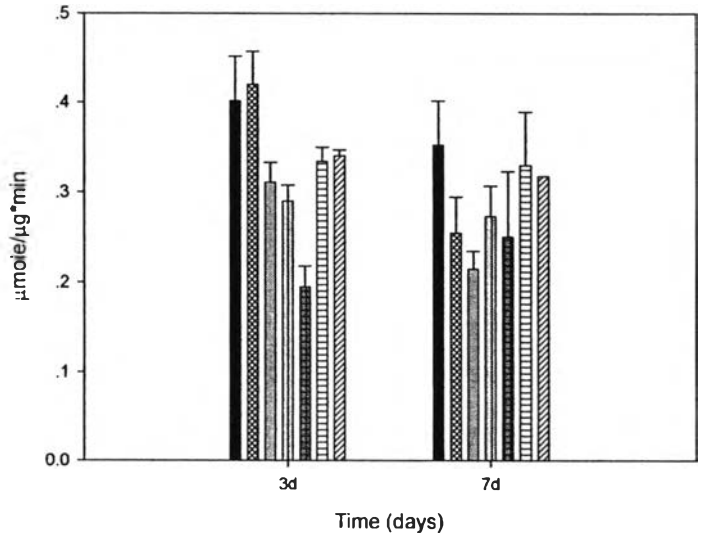


Figure 5.9 ALP activity of MC3T3-E1 cultured on TCP, PCL, HAp-PCL, OVA/HAp-PCL, Gelatin B/HAp-PCL, BSA/HAp-PCL and CBP/HAp-PCL porous scaffolds after 3 and 7days in culture.

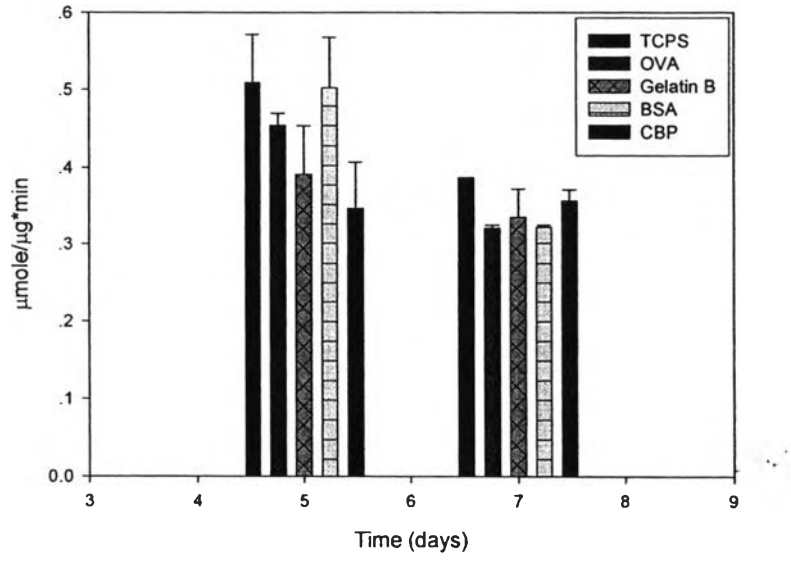
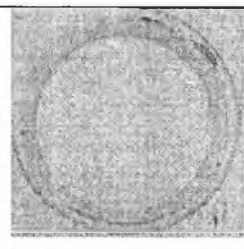
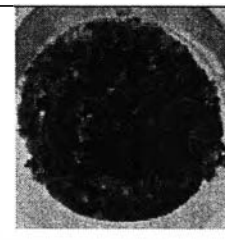
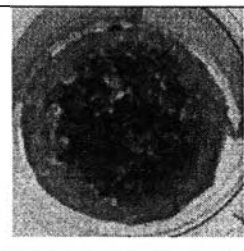
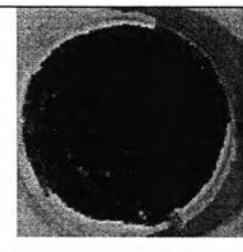
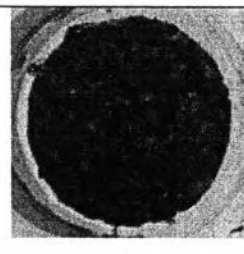
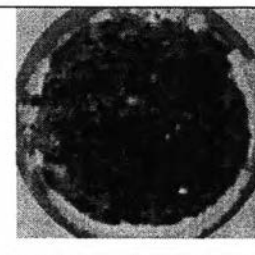
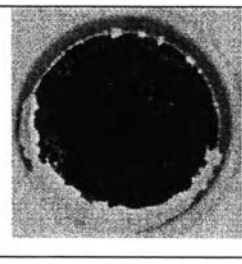
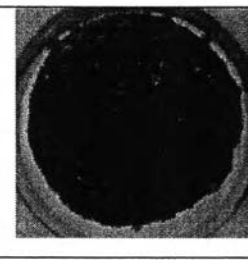


Figure 5.10 ALP activity of MC3T3-E1 cultured with 100 µg/ml in 2%MEM, OVA, Gelatin type B, BSA and CBP after 5 and 7 days in culture.

5.7.5 Mineralization

Mineralization refers to the process where an organic substrance is converted to an inorganic substrance. Alizalin Red S staining was used to characterize the bone formation of MC3T3-E1. Table 5.6, and Figure 5.11 show photographic images of Alizarin Red S staining of cells cultured on the different surfaces on 21 days. In the presence of calcium, the staining product such as an Alizarin red S-calcium chelating product appeared red. The results showed that PCL, HAp-PCL, and proteins-loaded-HAp scaffold were significantly higher than that on TCPS. Calcium content from MC3T3-E1 culture on CBP/HAp-PCL scaffold was the most positive staining.

Table 5.6 Alizarin Red S staining for mineralization assessment of MC3T3-E1 on 21 days after being cultured on the surfaces of neat PCL, HAp-PCL, HAp/OVA-PCL, HAp/Gelatin-PCL, HAp/BSA-PCL, and HAp/CBP-PCL

Substrate	21 d.		21 d.
TCPS		HAp/OVA-PCL	
Neat PCL		HAp/GelB-PCL	
Control		HAp/BSA-PCL	
HAp-PCL		HAp/CBP-PCL	

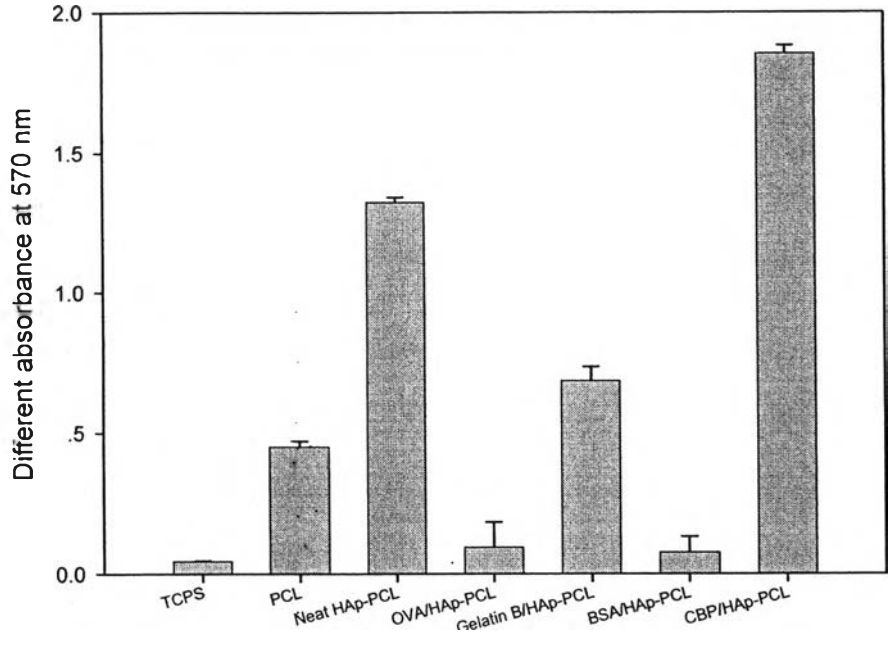


Figure 5.11 Different absorbance of alizalin red S from MC3T3-E1 on TCPS, PCL, HAp-PCL, proteins-loaded HAp-PCL at 21 days.

5.7 Conclusions

The amount of HAp in the PCL scaffold was 40% (w/w, based on the amount of PCL). The direct and indirect cytotoxicity evaluation of the proteins, HAp and protein-loaded HAp-PCL scaffold with MC3T3-E1 indicated that non-toxic. Potential for using of proteins-loaded HAp-PCL scaffolds was assessed in terms of cell attachment, cell proliferation, and ALP activity were no effect of proteins release from HAp. Alkaline phosphatate (ALP) activity at 7 days showed decreasing of the ALP activity due to cellular process switching to mineralization. According to mineralization or calcium deposition of cell for 21 days, the CBP/HAp-PCL scaffold was better than HAp/PCL, PCL, Gelatin/HAp-PCL, OVA/HAp-PCL, BSA/HAp-PCL, TCPS respectively.

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5.10 References

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