CHAPTER IV
SURFACE MODIFICATION OF POLY(STYRENE/ETHYLENE GLYCOL DIMETHACRYLATE) HIPE LOADED WITH HYDROXYAPATITE AS A SCAFFOLD FOR TISSUE ENGINEERING APPLICATION

4.1 Abstract

Poly(High Internal Phase Emulsion) (PolyHIPE) foam is a material that is good candidate for used in tissue engineering application due to its 3D structure and highly porous with interconnected pore. The PolyHIPE was prepared from poly(styrene/ethylene glycol dimethacrylate; 80/20) through high internal phase emulsion polymerization technique and loaded with hydroxyapatite (HA) to improve biocompatibility. In this study, improvement of hydrophilicity of the polyHIPE was carried out by Layer-by-Layer method. Three types of chemicals were used for coating on the surface of polyHIPE such as poly(sodium 4-styrene sulfonate) (PSS), gelatin, and alginic acid. The change of surface properties of the improved polyHIPE was characterized by contact angle measurement. It was found that hydrophilicity of the surface increase after coating as observed by decrease in contact angle degree. The effect of type of coating on cell attachment and cell proliferation was also studied and demonstrated that the PSS coated polyHIPE showed the highest of cell viability, whereas the alginic acid and gelatin coated polyHIPE showed lower the number of living cell than the uncoated polyHIPE. Therefore, the cell attachment and cell proliferation did not only depend on the hydrophilic of surface but also depend on chemical type of coating.

Keywords: High Internal Phase Emulsion; Scaffold; Layer-by-Layer Technique; Tissue Engineering

4.2 Introduction

In recent years, tissue engineering has gained more attention for use as a cure the patients who have failure of vital tissues and organs. The important properties for tissue engineering are they can provide a physical support for cell attachment, proliferation, nutrient transport and new tissue infiltration [1]. Currently, metal and ceramic were chosen in orthopaedic surgery but they are not closely matches healthy cellular structure with tissue [2]. So, many researchers try to find appropriate biomaterials and techniques to make better tissue engineering.

The use of 3D polymer scaffolds has shown increasing potential for tissue engineering. PolyHIPE polymer is the material that is a good candidate for used in tissue engineering application [3] because it has a 3D structure, highly porous with interconnected pore and very low density [4]. PolyHIPE polymer is a porous material prepared through a high internal phase emulsion (HIPE) polymerization route. PolyHIPE can easily be modified by addition of chemicals with the desired properties, e.g. hydroxyapatite to increase cell attachment and penetration into polyHIPE porous foams. There are many studies to investigate the use of polyHIPE polymer as a tissue engineering scaffolds. Akay *et al.* [5] reported the use of polyHIPEs as support structures for cell growth in the patent literature. However, PolyHIPE are usually made from polystyrene (PS) which is a hydrophobic polymer, which is improper for supporting the cell-scaffold interaction and poor adhesion between scaffold and living.

Layer-by-Layer (LbL) technique is one method to modify the surface of polymer by the additional of a functionalizable comonomer onto polymer. Layer-by-Layer (LbL) of polyelectrolyte is an approach for selective surface modification by building up a multilayer ultrathin-film coating of macromolecules. McCarthy [6] reported the surface modification of poly(ethylene terephthalate) (PET) using layer-by-layer deposition. Surface of PET can be modified to contain carboxylate and ammonium functionality. Therefore, it is possible to modify surface of poly HIPE by using LbL technique.

In this study, we describes the synthesis of poly(S/EGDMA)HIPE loaded with hydroxyapatite (HA). Furthermore, we show surface modification by using

layer by layer technique to improve hydrophobic of polyHIPE. We expects that improved poly(S/EGDMA)HIPE can support cell growth and can be another alternative for tissue engineering.

4.3 Experimental

4.3.1 Materials

Styrene (S), ethylene glycol dimethacrylate (EGDMA), sorbitan monooleate (Span80), tetrahydrofuran (THF), polystyrenesulfonate (PSS, MW 70,000), poly(diallyldimethylammonium chloride) (PDADMAC, MW 350,000), gelatin (GEL, type B from bovine skin), and alginic acid (ALG) were purchased from Sigma-Aldrich Chemical. Potassium persulfate ($K_2S_2O_8$) and calcium chloride ($CaCl_2$) were purchased from Fluka Chemie, Thailand. Phosphoric acid (H_3PO_4) was purchased from Merck. Hydroxyapatite (HA) was purchased from MK nano, USA and used as received.

4.3.2 Preparation of Poly(S/EGDMA) HIPE Loaded with Hydroxyapatite

Poly(S/EGDMA) polyHIPE porous foam was prepared by using the high internal phase emulsion technique as described by Akay *et al.* [5]. The oil phase (10% of total volume) contained styrene monomer (S), ethylene glycol dimethacrylate (EGDMA) as a crosslinking agent (S: EGDMA; 4:1 ratio by volume), and sorbitan monooleate (Span80, 2 ml) as a surfactant. While the aqueous phase (90% of the total volume) contained deionized water (88 ml), tetrahydrofuran (THF, 1 ml), potassium persulphate ($K_2S_2O_8$, 0.2 g) as initiator, and calcium chloride ($CaCl_2$, 1 g) as stabilizing salt, hydroxyapatite (HA, 0.45g), and phosphoric acid (H_3PO_4, 1 ml) which is used to dissolve the hydroxyapatite. The aqueous phase was added drop-wise into the oil phase under constant mechanical stirring until all aqueous phase has been added. After emulsification, the resulting emulsion was transferred into glass vials (20 mm internal diameter) and then placed in a water bath for polymerization at 60 °C for 48 hours. After that, all the samples were washed to remove any residual materials. Washing was carried out in isopropanol and then with

water in Soxhlet extractor. All samples were dried in oven at 60 °C until a constant weight was obtained.

4.3.3 Layer-by-Layer (LbL) Surface Modification

To increase hydrophilicity of poly(S/EGDMA) HIPE by using the layer-by-layer polyelectrolyte multilayers (PEM) technique. Poly(S/EGDMA) HIPE were cut to 1.5 mm thick and 20 diameter. PEM coating was performed by injected polymer solution through poly(S/EGDMA) HIPE disk. There are two coating consisted of the primary coating and the secondary coating. For primary coating, PDADMAC solution (10 mM, 10 ml) was manually injected through polyHIPE disks as positively charged polyelectrolyte for 2 min and then follow by PSS solution (10 mM, 10 ml) as negatively charged polyelectrolyte for 2 min. After each was injected in the electrolyte, the polyHIPE disks were rinsed for 30 s three times with deionized water to remove excess polyelectrolyte. This process was repeated until seven layers of polyelectrolyte had been obtained. The secondary coating was carried out using the similar procedure but different negatively charged polyelectrolyte such as alginic acid and gelatin were used.

4.3.4 Characterization of Poly(S/EGDMA) HIPE Loaded with Hydroxyapatite

4.3.4.1 Fourier Transform Infrared Spectroscopy (FT-IR)

To confirm the existence of hydroxyapatite, PSS, alginatic acid and gelatin on surface of the polyHIPE, the Atteuated Total Reflectance (ATR) technique was used.

4.3.4.2 Scanning Electron Microscope (SEM) and EDX

To investigate the phase morphology of the polyHIPE by using Scanning electron microscopy(FE-SEM, Hitachi S-4800). The specimens were coated with platinum under vacuum before testing and viewed by using accelerating voltage of 10kV.

The SEM/EDX studies were performed generally for the material identification and dispersion. In this case, hydroxyapatite load poly(S/EGDMA) HIPE scaffold was identified. All in the glove box filled with Ar

gas (99.999%) and coupled to the SEM/EDX chamber, SEM microphotographs and EDX spectra were recorded.

4.3.4.3 Surface Area Measurement

N_2 adsorption-desorption isotherms were obtained at -196°C on a Quantachrome Autosorb-1MP. Samples were degassed at 110°C during 12 hours in a vacuum furnace prior to analysis. Surface areas were calculated using the BET equation.

4.3.4.4 Contact Angle Measurement

-The static contact angle measurement was performed using a Krüss (model DSA 10) contact angle measuring instrument at ambient temperature to prove the wettability change of the layer-by-layer coated surface poly(S/EGDMA) HIPE scaffold. A 10 μL sessile droplet of de-ionized water was then vertically dropped with a micro-syringe onto the foam surface. The contact angles were using the drop shape analyzer program and were then averaged.

4.3.4.5 Mechanical Properties

Lloyd Universal testing machine was used to measure mechanical properties of all samples in compression mode, according to ASTM D822. Test specimens in a cylinder shape 2.54 cm in diameter × 2.54 cm in height were prepared. A speed of 0.127 cm/min and 500 N load cells were used for all measurements. The value of the compression stress and the Young's modulus were determined from an average of five samples.

4.3.5 Cell Culture

Mouse fibroblast connective tissue (L929) was used in this study in order to investigate the ability of the poly(S/EGDMA) polyHIPE foam to act as a scaffold in tissue engineering applications. L929 fibroblast-like cells were grown in Dulbecco' s modified Eagle' s medium (DEME: Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (FBS, BIOCHROM AG), together with 100 U ml-1 penicillin (GIBCO) and 100 μg/ml streptomycin (GIBCO). The medium was replaced every 3 days and the cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Each polyHIPE foam scaffold was cut into circular discs (about 15 mm in diameter and 1 mm thick), which were later sterilized in an

autoclave for 1 h prior to use and then the disc specimens were placed in the wells of a 24-well tissue-culture polystyrene plate (TCPS; Biokom Systems, Poland). The specimens were pressed with a metal ring (about 12 mm in diameters) in order to prevent the polyHIPE foam specimen from floating in the culture medium, and subsequently they were immersed in 500 µl of the culture medium overnight before cell seeding. The L929 fibroblast-like cells from the culture plate were trypsinized with 0.25% trypsin containing 1 mM EDTA (GIBCO) and were counted by a hemacytometer (Hausser Scientific, USA). They were then seeded at a density of 40,00 cells/well on the polyHIPE specimens and TCPS were used as controls.

4.3.5.1 Cytotoxicity Test

Evaluation of the cytotoxicity of the poly(S/EGDMA) polyHIPE foam using L929 fibroblast-like cells was done based on the stand method (ISO 10993-5). To prepare an extracted medium, circular polyHIPE specimens were sterilized in an autoclave for 1 h and placed in a 24-well plate, then washed 3 times with a serum free medium (SFM) before further incubating at 37 °C in a fresh culture medium for 24 hours. L929 fibroblast-like cells were seeded in the wells of a 24-well plate at a density of 40,000 cells/well with serum-containing DMEM for 48 h. After that, the DMEM was removed and replaced with the poly(S/EGDMA) polyHIPE foam extraction medium before an additional 24-hour incubation period. The measurement of cell viability was done using a 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT; Sigma Aldrich, USA) assay.

4.3.5.2 Cell Attachment and Cell Proliferation

For cell attachment and proliferation was study as described by Pakeyangkoon *et al.* [4]. L929 fibroblast-like cells at a density of 40,000 cells/well were used. Circular polyHIPE foam specimens (15 mm in diameter and 1 mm thick) were placed in a 24-well culture plate with a metal ring. All polyHIPE foam samples were sterilized in an autoclave for 1 h, washed two times with phosphate buffer saline (PBS) and then with the culture medium (DMEM). Before cell seeding, 500 µl of the DMEM was added to each well of the 24-well culture plates. L929 fibroblast-like cells, at a density of 40,000 cells/well, were seeded on the polyHIPE foam samples and culture plate as control at 1, 4, and 24 h for the cell attachment study. Each time point, the cell attachment number was determined by

MTT assay. The proliferation of L929 fibroblast-like cells was determined at different culture periods (4 h , 1 day , 3 days, and 7 days) then measured again with MTT assay to determine the changes in the number of viable cells. In addition, the effect of plasma surface modification and treatment time on the cell attachment of the poly(S/EGDMA) polyHIPE foam was also investigated. In this part, L929 fibroblast-like cells, at a density of 40,000 cells/well, were seeded on the polyHIPE foam and on the culture plate as control for 1 day. Determination of the amount of cell attachment was also done using MTT assay.

4.3.5.3 MTT Assay

A 3-(4,5-dimethykthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT: Sigma Aldrich, USA) assay is a quantitative method and standard colorimetric assay (an assay which measures changes in color) for the measurement of cell viability and growth. The reduction of yellow tetrazolium salt in metabolically active cells to form an insoluble purple formazan crystal product by the dehydrogenase enzymes secreted from the mitochondria of viable cells. This assay can also be used to determine the cytotoxicity of potential medicinal agents and other toxic materials. Firstly, the cell-contained polyHIPE foam washed two times with PBS to remove any unattached cells, and then a 300 µl MTT stock solution (5 mg/mL in medium without phenol red) was added to each well and incubated 37 °C for 30 min. After incubation of the cells with the MTT solution, a buffer solution containing dimethylsulfoxide (DMSO: 900 µl / well) and glycine buffer (100 µl/well) was placed in each well in order to extract the purple formazan crystal and determine their by using microplate reader at a wavelength of 570 nm.

4.3.5.4 Morphological Observation of Cell Culture

The morphology of the L929 fibroblast-like cells containing poly(S/EGDMA) polyHIPE foam was observed using a scanning electron microscope (SEM). All of the polyHIPE foam was washed twice with PBS, and then cell fixation was done with a 3% glutaraldehyde solution (diluted from 50% glutaradehyde solution with PBS) at 500 ml/well for 30 min. After the fixation, the polyHIPE foam was washed with PBS and dehydrated with ethanol solutions of varying concentration (i.e. 30, 50, 70, 90, and 100%) for about 2 min at each concentration. After being dried completely, the specimens were mounted on copper

stubs, and coated with gold to observe the cell adhesion on the polyHIPE foam by SEM.

4.4 Results and Discussion

4.4.1 Characterization of Poly(S/EGDMA) HIPE Loaded with Hydroxyapatite

Poly(S/EGDMA) HIPE porous foam loaded with hydroxyapatite was successfully prepared via the high internal phase emulsion polymerization technique. The presence of hydroxyapatite on the polyHIPE was confirmed by EDX analysis. The result indicated that the Ca/P ratio is 1.45 which is relatively close to the theoretical value of 1.67 [7]. The structure of the polyHIPE porous foam was confirmed by SEM image (Figure 4.1) and showed three-dimensional open cellular structure morphology. The average pore size was approximated 60 μm. There was small interconnected pore in each pore that is connected to its neighbors, which cells can migrate and proliferate within this structure.

For physical properties and mechanical properties of the polyHIPE porous foam are shown in Table 4.1. According to the N_2 adsorption-desorption results, BET surface area was 11.84 m^2/g. The mechanical properties of the polyHIPE porous foams were evaluated through measurements of their Young's modulus and compressive strength, were 17.74 and 0.86 MPa, respectively.

From these results, it clearly demonstrated that the polyHIPE have high porosity, highly interconnectivity, and high structural stability. Therefore, it is likely that the polyHIPE loaded with hydroxyapatite can be fulfill requirement of scaffold material for tissue engineering applications and the preparation was selected for further studies [8].

4.4.2 Determination of Optimum Layers on Poly(S/EGDMA) HIPE Loaded with Hydroxyapatite

To increase hydrophilicity of the substrate is the most important of scaffold material requirement in order to promote the adhesion between cells and the substrate material. Layer – by – layer (LbL) technique was used to satisfy this

requirement. PDAD and PSS were selected as primary coatings because they were strong electrolyte, which charge was independent of pH. The effect of the number of layers coated on the polyHIPE is shown in Figure 4.2. It was found that absorbance of UV spectroscopy increased when the number of layers increased. At 1 to 7 layers, the absorbance dramatically increased with linear relationship, whereas 7 to 9 layers slightly increased. The color of indigo dye was used to confirm the effect of the number of layers coated on the polyHIPE (Figure 4.3). It showed that increasing in color as number of layer increased and the homogenous coating. Only 7 layers of PDAD/PSS modified the polyHIPE, indigo dye coated covers all of surface of the polyHIPE. Therefore, primary coating chose PDAD/PSS modified polyHIPE for 7 layers in this study. For secondary coating, PSS, alginic acid, and gelatin were used for finally functionalization.

4.4.3 Effect of LbL Surface Modification on Chemical Composition, and Wettability of Poly(S/EGDMA) HIPE Loaded with Hydroxyapatite

The chemical change to the polyHIPE after surface modification was characterized using the Attenuated Total Reflectance Infrared (ATR-IR) Spectroscopy. The ATR-IR spectra of the unmodified polyHIPE and the modified polyHIPE are shown in Figure 4.4. In the spectra of PSS, the broad peak around 3100-3700 cm^{-1} correspond to stretching vibration of H_2O and the peak at 1178 and 1124 cm^{-1} belong to the $-SO_3$ asymmetric stretching vibrations [9], when compared with the functional group on the unmodified polyHIPE. For ALG and GEL modified polyHIPE, the peak was relatively similar with the peak of PSS modified polyHIPE. However, the results showed the broad peak at 3400-3500 cm^{-1} that corresponded to the carboxyl groups and N-H stretching for ALG and GEL, respectively.

The static contact angle of distilled water was used to investigate the effect of different secondary coating on wettability of the polyHIPE surface. After modified polyHIPE with LbL technique, the droplet of water can spread on the polyHIPE surface and the contact angle decreased from 122° to 55°, 72°, and 0° for PSS, ALG, and GEL, respectively (Figure 4.5). This is because of hydrophilic functional group addition on the polyHIPE surface. Therefore, it clearly

demonstrated that LbL surface modification technique is an effective technique for increasing hydrophilic of the polyHIPE.

4.4.4 Cytotoxicity Test

The cytotoxicity evaluation, based on the viability of L929 fibroblast cells by indirect method. The number of living cells after incubation 24 hours was reported as percentage of controls. The result showed that the number of living cells was higher than 70% of the controls for both the unmodified polyHIPE and the modified polyHIPE (Figure 4.6). For GEL modified polyHIPE, the results indicated that the living cells 68.47% when compared with controls, which relatively close with the standard value (70%). Therefore, we can conclude that there were no toxic products leached from the polyHIPE that assessed initially prepared scaffold.

4.4.5 Cell Attachment and Proliferation

For cell attachment, the number of L929 fibroblast cells was quantified by its relative absorbance, as shown in Figure 4.7. The result demonstrates that at longer incubation times, the number of cell attachment on the polyHIPE was higher. The PSS modified polyHIPE shows the level of cell attachment higher than another polyHIPE. This is because of the water contact angle sharply decreased from 122° to 55° (Figure 4.5 (b)), which improved surface hydrophilicity, which consequently enhances the attachment between living cells and polyHIPE substrate. For polyHIPE that modified with ALG and GEL, the cell attachment was less than the unmodified polyHIPE.

The proliferation of L929 fibroblast cells were signified by the viability of the cells at 4 h, 1 day, 3 days, and 7 days after seeding cells on the unmodified and modified polyHIPE. Figure 4.8 shows the relationship between the absorbance and culture time, which indicated that the number of viable cells increased with increasing the time in culture. The PSS modified polyHIPE showed highest the level of proliferation, that resulting from the surface improvement. An amount of cell on PSS modified polyHIPE increased up to 138% when compare with unmodified the polyHIPE. For ALG and GEL modified polyHIPE showed the cell proliferation was less than the unmodified polyHIPE. This may be because ALG and

GEL is a weak electrolyte, which easy to form crosslink with inorganic salt in culture medium, resulting in culture medium contamination. So, L929 fibroblast cells can't grow and finally die.

4.4.6 Cell Morphology

The morphology of L929 fibroblast-like cells on the polyHIPE was observed using scanning electron microscope (SEM), as shown in Figure 4.9. It is seen that the number of living cells on the PSS modified polyHIPE (Figure 4.8 (d-f)) was greater than the unmodified polyHIPE, and the number of living cells occupied on polyHIPE increased with increasing incubation time. For the ALG and GEL modified polyHIPE, the number of living cells was less than the unmodified polyHIPE. There was small flake cover the surface of the ALG polyHIPE, which inhibit the proliferation of cells. The GEL modified polyHIPE, there was small fibril from cells, and this may be occurring from mechanism of cell and resulting in cell died.

4.5 Conclusions

Poly(S/EGDMA) HIPE loaded with hydroxyapatite porous foams can be fabricated via the high internal phase emulsion polymerization technique. The polyHIPE porous foams can be fulfilling of the requirements for the ideal scaffolds such as having high porosity, highly interconnectivity, and high structural stability. The LbL surface modification was successfully used to enhance the hydrophilic properties of the polyHIPE surface. The unmodified polyHIPE and the modified polyHIPE exhibited non-toxic for L929 fibroblast cells. The PSS modified polyHIPE showed the highest efficiency of attachment of the L929 fibroblast cells and an amount of cell increased up to 138% when compare with unmodified the polyHIPE. Moreover, the PSS modified polyHIPE exhibited the highest efficiency of proliferation of the L929 fibroblast cells. Therefore, PSS modified polyHIPE was suitable for using in tissue engineering application.

4.6 Acknowledgements

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4.7 References

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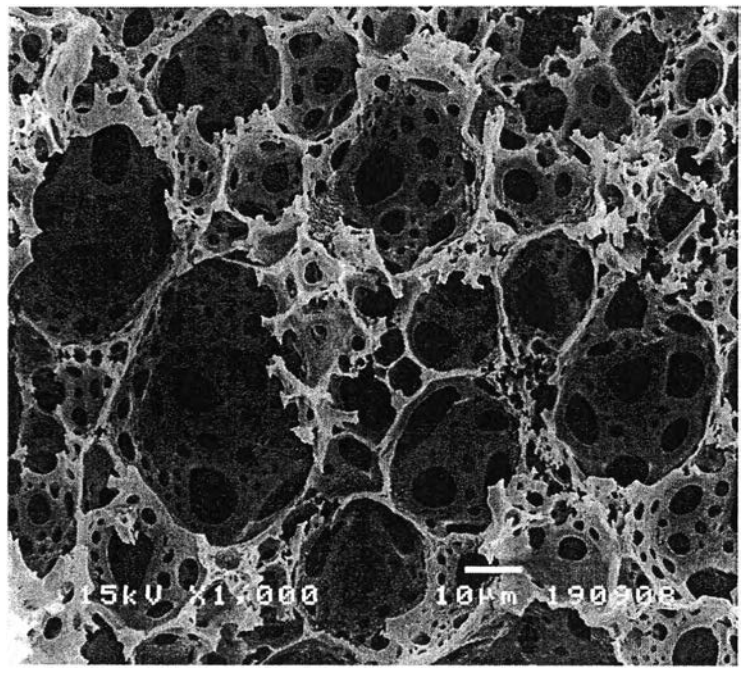

Figure 4.1 SEM micrographs of poly(S/EGDMA) HIPE loaded with hydroxyapatite.

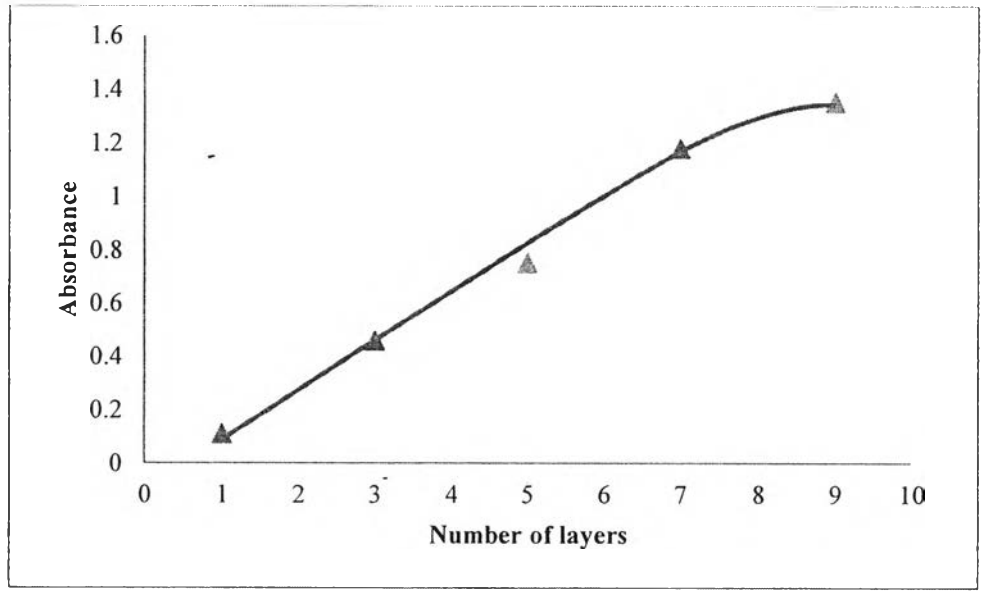

Figure 4.2 Absorbance as a function of the number of layers for PDAD/PSS modified on poly(S/EGDMA) HIPE loaded with hydroxyapatite.

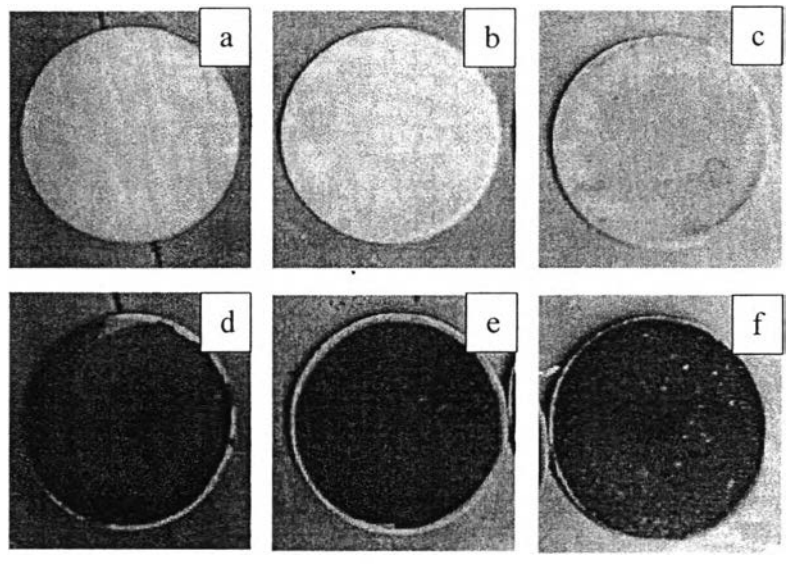

Figure 4.3 Photograph of poly(S/ EGDMA)HIPE loaded with hydroxyapatite modified surface with PDAD/PSS and followed by indigo dye; (a) unmodified, (b) 1 layer, (c) 3 layers, (d) 5 layers, (e) 7 layers, and (f) 9 layers.

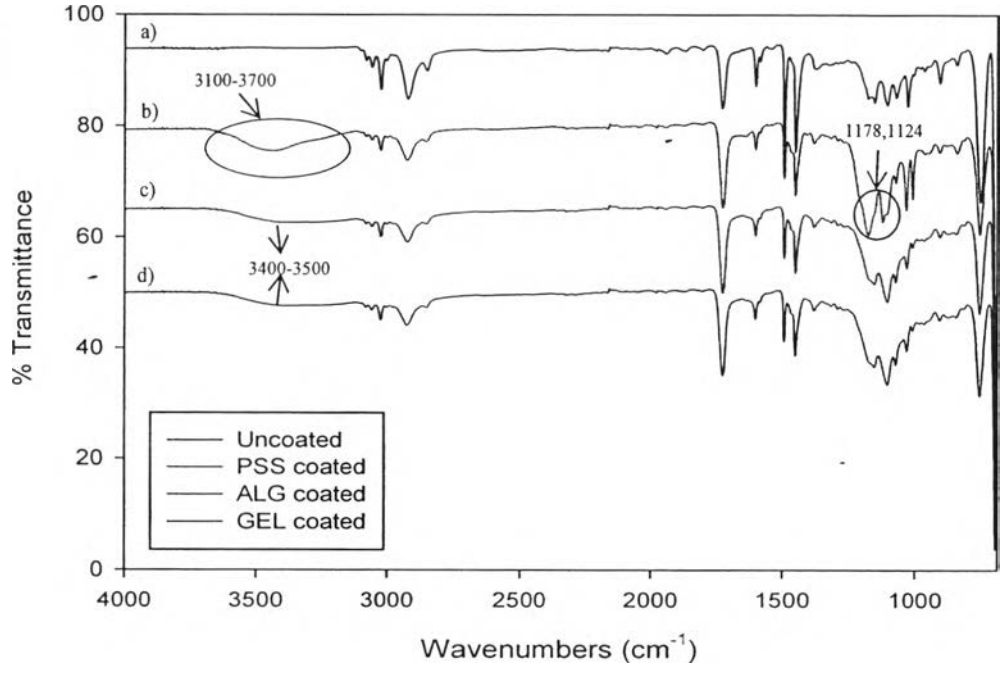

Figure 4.4 ATR-IR spectra of poly(S/ EGDMA)HIPE loaded with hydroxyapatite; a) unmodified, b) modified with PSS, c) modified with ALG, and d) modified with GEL.

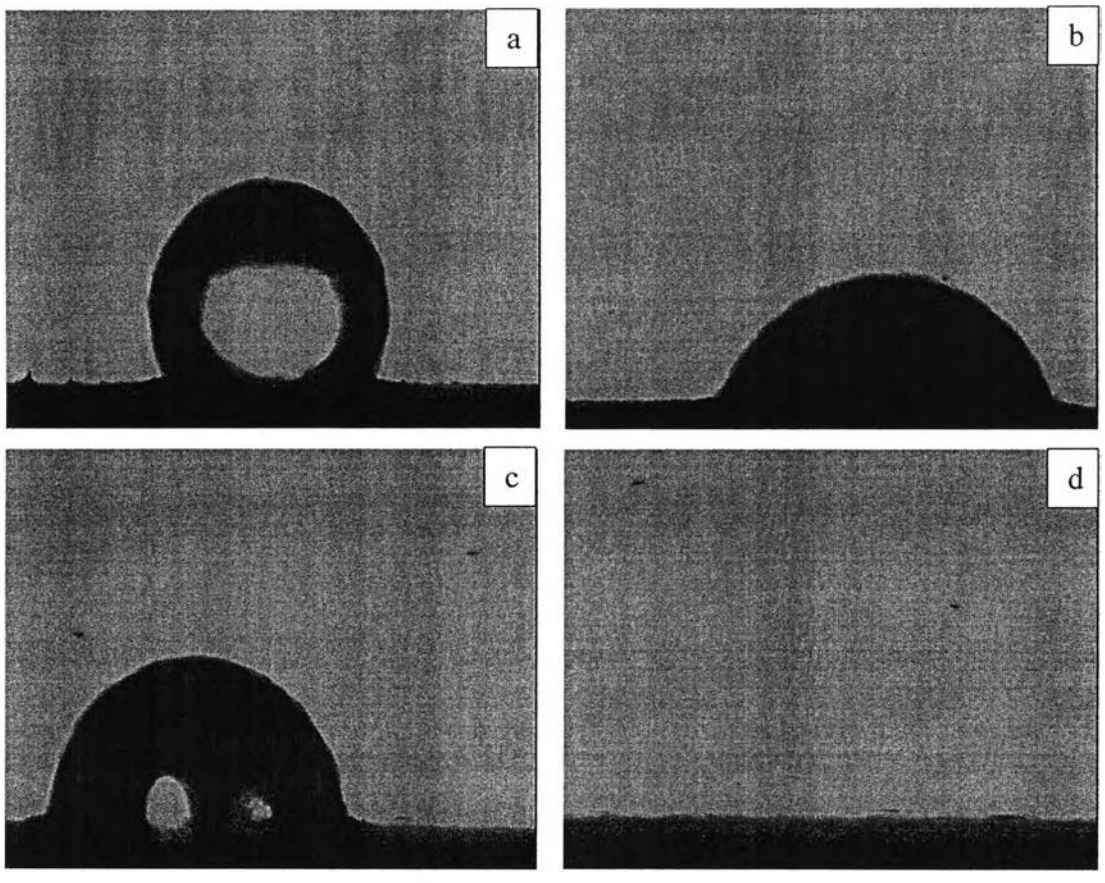

Figure 4.5 Static water sensible drops on poly(S/EGDMA)HIPE loaded with hydroxyapatite; a) unmodified, b) modified with PSS, c) modified with ALG, and d) modified with GEL.

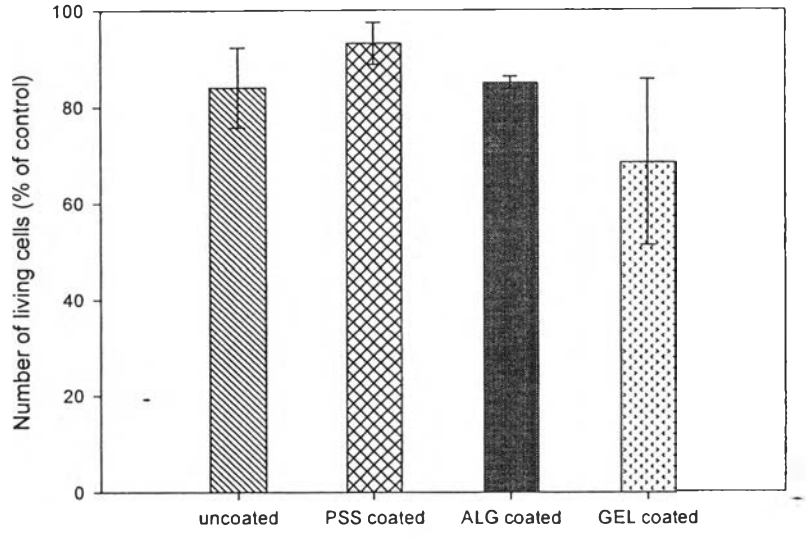

Figure 4.6 Cytotoxicity of the poly(S/EGDMA)HIPE foam of before (unmodified) and after (modified) LbL surface modification.

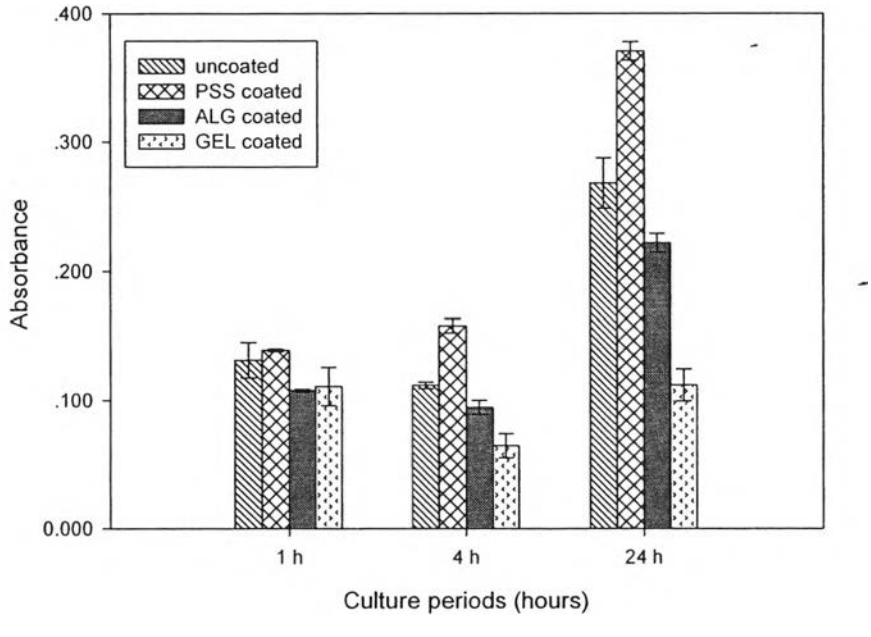

Figure 4.7 Cell attachment of L929 fibroblast-like cells on the poly(S/EGDMA) HIPE foam in modified and unmodified with LbL surface modification, after 1, 4, and 24 h of cell culture periods.

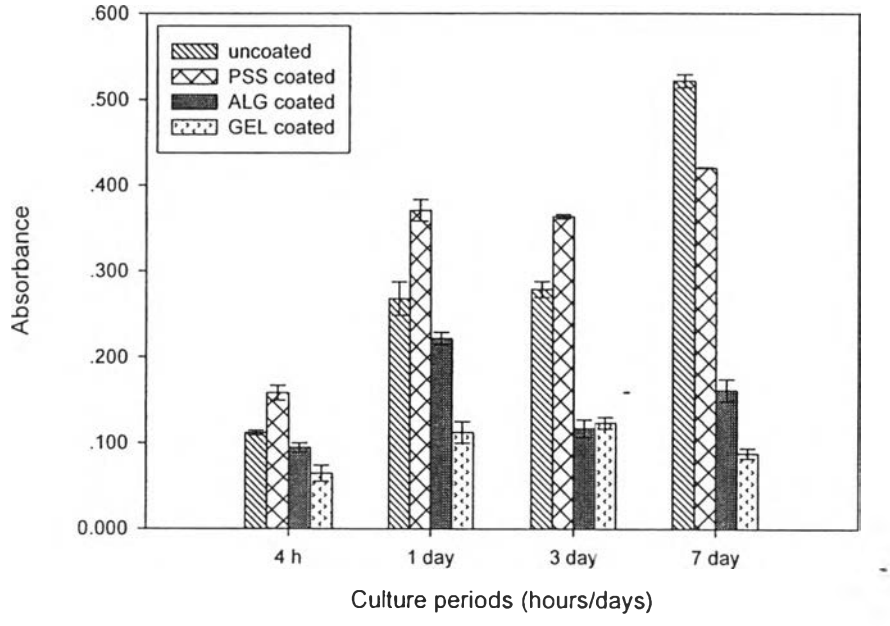

Figure 4.8 Cell proliferation of L929 fibroblast-like cells on the poly(S/EGDMA) HIPE foam in modified and unmodified with LbL surface modification, after 4 h, and 1, 3, and 7 days of cell culture periods.

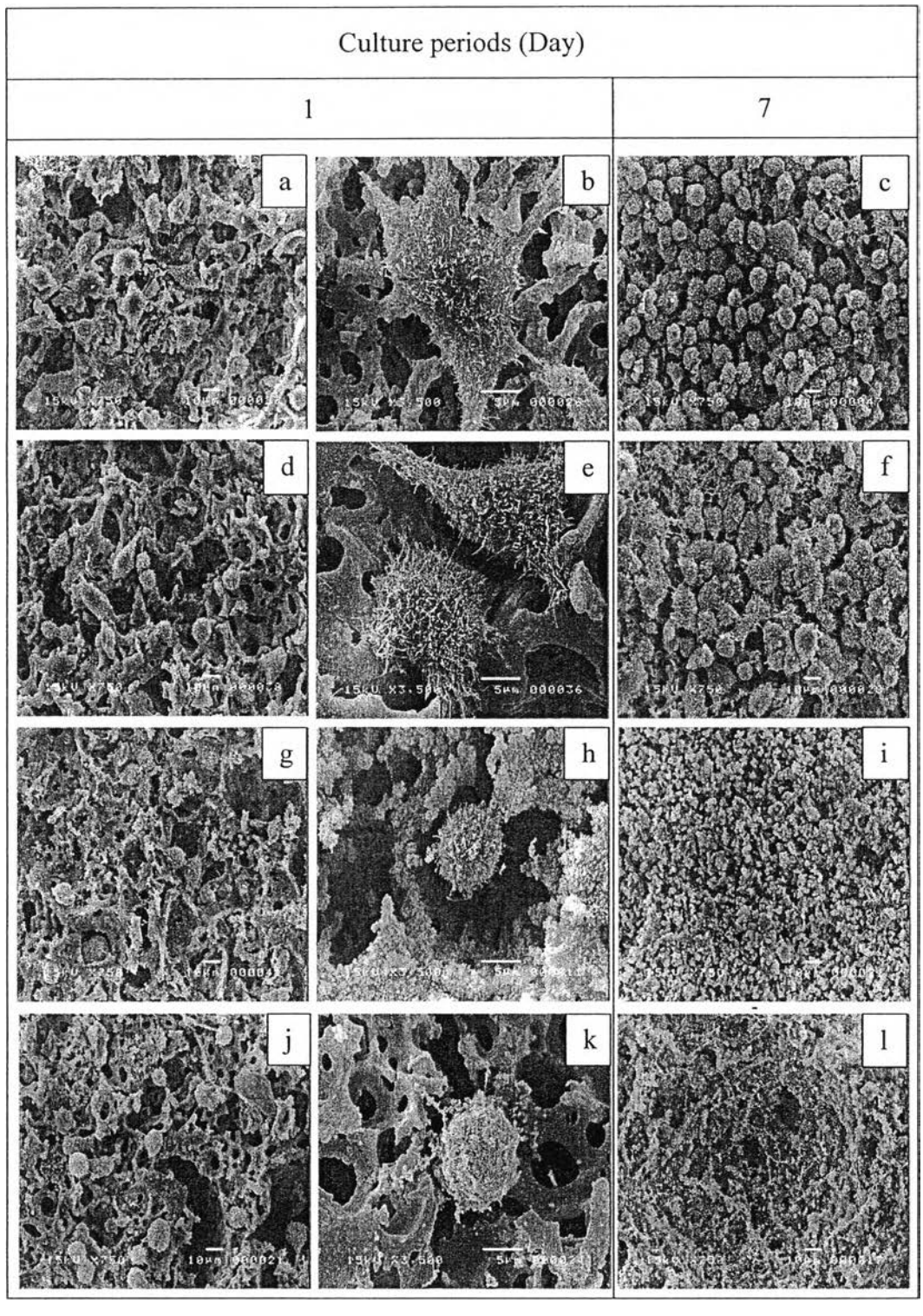

Figure 4.9 SEM image of the L929 fibroblast-like cells on poly(S/EGDMA)HIPE loaded with hydroxyapatite; a-c) unmodified, d-f) modified with PSS, g-i) modified with ALG, and j-l) modified with GEL.

Table 4.1 Characteristics of the poly(S/EGDMA) HIPE load with hydroxyapatite porous foam

Properties	**Poly(S/EGDMA)HIPE load with hydroxyapatite porous foam**
Surface area (m^2/g)	11.84
Pore size (μm)	40 - 60
Compressive strength (MPa)	0.86 ± 0.02
Young's modulus (MPa)	17.74 ± 0.58