CHAPTER III
EXPERIMENTAL

3.1 Materials

3.1.1 Poly(DVB)PolyHIPE Foam

Divinylbenzene (DVB; Merck) was used as monomer to produce polyHIPE materials. Surfactants used in the study were cetyltrimetylammonium bromide (CTAB, Fluka Chemie), sorbitant monooleate (Span80; S80), sorbitant monolaurate (Span20; S20) and dodecylbenzenesulfonic acid sodium salt (DDBSS), all surfactants were purchased from Sigma-Aldrich Chemical. Initiator and stabilizer used in the experiments were potassium persulfate ($K_2S_2O_8$, purity ≥ 98% (RT), Fluka Chemie) and calcium chloride ($CaCl_2$, purity ≥ 97% (KT), Fluka Chemie) respectively. Toluene (T) was employed as a porogenic solvent. Three type of organoclay was prepared in our laboratory from the following procedure.

1) Hybrid organic-inorganic Porous clay heterostructures (HPCH) Na-Bentonite (BTN) (Mac-Gel® GRADE SAC) was converted in to quaternary ammonium cations form by ion exchanged with cetyltrimethylammonium bromide (CTAB). Hybrid organic-inorganic porous clay heterostructures (HPCH) derived from Na-Bentonite were obtained by the surfactant-directed assembly of mesostructured silica within clay layers. The reaction carried out in the presence of intragallery surfactant templates (cetyltrimethylammonium ion and dedecylamine) (Prakobna *et al.*, 2006).

2) Organo-modified bentonite (MOD) derived from Na-Bentonite (BTN) (Mac-Gel® GRADE SAC): To increase d-spacing of organo-modified bentonite, synthesized Na-Bentonite was ion exchanged with quaternary ammonium cations by using Methyl bis(soya amidoethyl)-2-hydroxyethyl quaternary ammonium methyl sulfate (DOAM) as surfactant.

3) Acid treated organo-modified bentonite (AC-MOD) derived from Na-Bentonite (BTN) (Mac-Gel® GRADE SAC): BTN was modified by acid treatment with 3 N HCl solutions at 110°C for 3 h. The clay/acid ratio used in the study was 2% wt/wt. After treatment, the clay was separated and washed with distilled water several times and dries until constant weight was obtained and grind then treated with quaternary alkyl ammonium cations by ion exchange reaction. To increase d-spacing of acid treated bentonite, synthesized Na-bentonite was ion exchanged with quaternary ammonium cations by using Methyl bis(soya amidoethyl)-2-hydroxyethyl quaternary ammonium methyl sulfate (DOAM) as surfactant.

In this study, hybrid organic-inorganic Porous clay heterostructures (HPCH) was prepared in our laboratory. The completely procedure for synthesis and characterization has been described by Prakobna *et al.*, 2006. In addition, the structural characteristics of both MOD and AC-MOD were studied using FT-IR, XRD and XRF.

3.1.2 Poly(S/EGDMA)PolyHIPE Foam Scaffold for Tissue Engineering Application

Styrene (S; Fluka Chemie) and ethylene glycol dimethacrylate (EGDMA; Sigma-Aldrich Chemical) were used as monomers to produce the polyHIPE porous scaffold. The surfactant, which was sorbitant monooleate (Span80; S80), was purchased from Sigma-Aldrich Chemical. The initiator and stabilizer used in the experiments were potassium persulfate ($K_2S_2O_8$, purity ≥ 98% [RT], Fluka Chemie) and calcium chloride ($CaCl_2$, purity ≥ 97% (RT), Fluka Chemie) respectively. Tetrahydrofuran (THF) as a solvent was used as received.

3.2 Methodology

3.2.1 Poly(DVB)PolyHIPE Preparation for Studing the Effect of Surfactant System and Soxhlet Extraction Time

Poly(DVB)polyHIPE was prepared by polymerization of organic phase as described by Barbetta *et al.*, 2004 and Hainey *et al.*, 1991. Single surfactant (S80) and two different mixtures of surfactant systems which were S80M (Span80, CTAB and DDBSS) and S20M (Span20, CTAB and DDBSS) were studied in this work. Toluene (T) was used as porogenic solvent (S80M_T denotes the poly(DVB)polyHIPE prepared with toluene as porogenic solvent using S80M system as surfactant and S20M_T denotes the poly(DVB)polyHIPE prepared with toluene as porogenic solvent using S20M system as surfactants). By added the aqueous phase (water (90 ml), $K_2S_2O_8$ (0.2 g) and $CaCl_2$ (1.0 g)) drop-wise to an organic mixture (10 ml; contained monomer and porogen (1:1 ratio by volume)) with mechanical stirring to form polyHIPE emulsion. The resulting mixture was placed in a water bath at 60°C for 48 h for polymerization. After that the obtained solid materials was removed from mold and the remaining solvents were extracted with isopropanol by varying Soxhlet extraction time (0, 1, 3, 6, 12, 24, 48 h) before drying in a vacuum oven at 60°C until a constant weight was obtained.

3.2.2 Preparation of Poly(DVB)PolyHIPE Nanocomposites Foam

Poly(DVB)PolyHIPE materials were prepared by using high internal phase emulsion. In the present work, poly(DVB)polyHIPE foam was fabricated and with using three type of organoclay including HPCH, MOD, and AC-MOD as inorganic filler. The continuous phase (10 vol% of total volume) of the emulsion consisted of DVB, toluene (monomer: porogen; 1:1 ratio by volume), Surfactant (S80) and organoclay (0, 1, 3, 5 and 10 wt% relative to monomer). The aqueous phase, which contained water (90 ml), $K_2S_2O_8$ (0.2 g) as initiator and electrolyte; $CaCl_2$ (1.0 g). After all the water has been added, the emulsion was further stirred for 20 min and placed in a glass bottle. Emulsions were capped and put in a water bath at

60°C for 24 h. After polymerization, the cellular materials were removed from the glass bottles and washed by Soxhlet extraction for 6 h with isopropanol. Then the cellular materials were returned to vacuum oven to dry at 60°C until a constant weight was obtained.

3.2.3 Preparation of Poly(S/EGDMA)PolyHIPE Foam Scaffold and Plasma Surface Treatment

Poly(styrene/ethylene glycol dimethacrylate; S/EGDMA)polyHIPE porous foam was prepared by using the high internal phase emulsion technique. The continuous phase (10 vol% of total volume) of the emulsion consisted of a monomer, which is styrene (S), ethylene glycol dimethacrylate (EGDMA) as a crosslinking agent (S: EGDMA; 4:1 ratio by volume), and a surfactant (sorbitant monooleate; Span80 2 ml). The aqueous phase contained water (89 ml), THF (1 ml), $K_2S_2O_8$ (0.2 g) as initiator, and $CaCl_2$ (1.0 g) as electrolyte. To fabricate the polyHIPE emulsion, the aqueous solution was slowly added drop-wise to the organic mixture with mechanical stirring. The resulting materials were polymerized by placing them in a water bath at 60°C for 24 h. After that, the solid materials was extracted with isopropanol for 6 h and then with water for 24 h to remove any residual materials from the pore structure; then they were placed in a vacuum oven at 60°C until a constant weight was obtained.

For plasma surface modification, the apparatus was made in our laboratory (Tasanatanachai *et al.).* A plasma treatment chamber, DBD type, was designed for scaffold-specimen modification. The treatment was done at atmospheric pressure using ambient air as a process gas. A pair of 2.65 x 2.65 cm plane stainless steel plates was used as the electrodes. Both electrodes were covered by PMMA sheets with a thickness of 1 mm. The dried poly(S/EGDMA)polyHIPE foam was subjected to treatment with sinusoidal 8.3 kVrms at frequency of 500 Hz. To investigate the effect of plasma treatment time on the properties and cell viability of polyHIPE scaffold, the time of the plasma exposure was varied at 5, 10, 15, 20, and 30 min prior to sterilization in an autoclave at 110°C for 1 h.

3.3 Equipments

3.3.1 Characterization of Organoclay, Poly(DVB)PolyHIPE Foam and Poly(DVB)polyHIPE Nanocomposites Foam

3.3.1.1 X-ray Diffractometer (XRD)

X-ray diffractometer (XRD) was used to observe the d-value of organoclay i.e. MOD and AC-MOD and to investigate the crystal structure of polyHIPE nanocomposites. X-ray diffraction patterns were measured on a Rigaku Model Dmax 2002 diffractometer with Ni-filtered Cu K_α radiation operated at 40 kV and 30 mA. The powder samples were observed on the 2θ range of 1.8-20 degree with scan speed 2 degree/min and scan step 0.02 degree.

3.3.1.2 Fourier Transform Infrared Spectroscopy (FT-IR)

The incorporation of organic group into silicate network by ion exchanged with quaternary ammonium cations was investigated by using FTIR. The FT-IR spectra of organoclay was obtained using a Nicolet Nexus 670 FT-IR spectrometer in the frequency range of 4000-400 cm^{-1} with 32 scans at a resolution of 2 cm^{-1}. KBr pellet technique was applied in the preparation of powder samples.

3.3.1.3 X-ray Fluorescence (XRF)

Chemical compositions of organoclay i.e. MOD and AC-MOD were obtained using Oxford Model ED2000 X-ray tube with silver as a filter, operate at voltage 35 kV.

3.3.1.4 Surface Area Analyzer (SAA)

N_2 adsorption–desorption isotherms were carried out at -196°C on a Quantachrome Autosorb I using a BET model. The samples were degassed at 100°C for 12 hours in a vacuum furnace prior to analysis. Surface areas were calculated using the BET equation (see equation 1) and pore volumes were

determined by the t-plot method of De Boer (Gregg *et al.*, 1982). The resulting BET equation is expressed by equation (1):

$$\frac{1}{W\left(\frac{P_0}{P}-1\right)} = \frac{1}{W_m C} + \frac{C-1}{W_m C}\cdot\frac{P}{P_0} \tag{1}$$

where W is the weight of gas adsorbed at a relative pressure (P/P_0), W_m is the weight of adsorbate constituting a monolayer of surface coverage and C, a constant that is related to the heat of adsorption. Equation (1) is an adsorption isotherm and can be plotted as a straight line with $1/W[(P_0/P)-1]$ and P/P_0 according to the experimental results. A linear relationship between $1/W[(P_0/P)-1]$ on the y-axis and P/P_0 on the x-axis is required to obtain the quantity of nitrogen absorbed. The slope A and intercept I were used to determine the quantity of nitrogen adsorbed in the monolayer (W_m) and used to calculate the total surface area. The following equations were used:

$$A = \frac{C-1}{W_m C} \tag{2}$$

$$I = \frac{1}{W_m C} \tag{3}$$

$$W_m = \frac{1}{A+I} \tag{4}$$

the weight of a monolayer W_m was obtained from equation (4) by combining equation (2) and (3). A total surface (S) area was evaluated by the following equation:

$$S = \frac{W_m N A_{cs}}{M} \tag{5}$$

where N is Avogadro's number (6.023×10^{23} molecules/mol), M is the molecular weight of the adsorbate and the cross sectional area (A_{cs}) for N_2 at 77 K (-196°C) is 16.2 $Å^2$.

3.3.1.5 Morphology

Scanning electron microscopy was done with a JEOL scanning electron microscope (MP 152001 Model), operating at 15 kV and 1000×, 1500×, and 15000× magnification. All polyHIPE specimens were coated with gold under vacuum before analysis. TEM micrographs was taken on a Tecnai G2 Sphera electron microscope with an accelerating voltage of 80 kV to observe the pore structure and secondary pore in the cell wall of poly(DVB)polyHIPE foam. Micrographs were recorded at magnifications of 3,500×, 7,800×, 80,000×, and 150,000× magnifications. TEM samples were prepared by embedding polyHIPE in a support resin and sectioning on an ultrathinmicrotome. The thin sections were supported on 300 mesh copper grids.

3.3.1.6 Thermogravimetric Analysis (TGA)

PolyHIPE foam was determined by thermogravimetric analysis (TGA) using a Perkin-Elmer Pyris Diamond TG-DTA instrument under N_2 flow of 100 ml/min. The heating process was conducted from 30-800°C at a rate 10°C/min. The degradation temperature was determined at a weight loss 50% from the weight loss vs. temperature curve.

3.3.1.7 Differential Scanning Calorimetry (DSC)

The glass transition temperature of polyHIPE foam was determined using a Perkin-Elmer DSC 7 instrument. The sample was first heated from 30°C to 250°C and cooled down at a rate of 10°C/min under a N_2 atmosphere with a flow rate of 10 ml/min. The sample was then reheated to 250°C at the same rate.

3.3.1.8 Universal Testing Machine (LLOYD)

A Lloyd Universal Testing Machine was used to measure the 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 length 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.

3.3.1.9 Adsorption of Carbon dioxide (CO_2)

Adsorption of CO_2 was carried at room temperature using gas adsorption unit, which was made in laboratory of Department of chemical technology, Faculty of science, Chulalongkorn University. Samples were cut into small pieces weigh about 1–2 g and outgases at 100°C prior to measurement. The samples were loaded into sample tube 2×25 cm. CO_2 3 mL/min and He 17 mL/min were flowed through the sample at room temperature. The amount of CO_2 gas adsorbed by the polyHIPE foam was determined by gas chromatography, column used Shimadzu 2014 and the detector was FID type using Helium (He) as the carrier gas.

3.3.2 Characterization of Poly(S/EGDMA)PolyHIPE Scaffold for Tissue Engineering Applications

3.3.2.1 Physical Characterization of Poly(S/EGDMA)PolyHIPE Foam Scaffold

Phase Morphology

The scanning electron microscopy was performed with a JEOL scanning electron microscope (MP 152001 Model), operating at 15 kV with 35×, 500×, 1000×, 2000×, and 3500× magnification in order to investigate the phase morphology of the poly(S/EGDMA)polyHIPE foam. All specimens were sputter-coated with gold under vacuum before analysis.

Surface Area Measurement

N_2 adsorption–desorption isotherms were obtained at -196°C using a BET model Quantachrome, Autosorb I. The samples were degassed at 100°C for 12 h in a vacuum furnace prior to analysis. The surface areas were calculated using the BET equation.

Fourier Transform Infrared Spectroscopy (FT-IR)

In order to analyze the functional groups presented on the virgin polyHIPE foam surface and on the atmospheric air-plasma treated one, the Diffuse Reflectance Infra-red Fourier Transform (DRIFT) technique was used. The FT-IR spectra of the untreated and plasma-treated poly(S/EGDMA)polyHIPE foam were collected over a wave number range of 4,000–500 cm^{-1} on a Nicolet Nexus 670 FT-IR spectrometer with 32 scans at a resolution of 2 cm^{-1}.

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 plasma-treated surface of the PS scaffold. After plasma exposure, the DBD-treated foam was kept in an ambient environment for about 15 min, which was set as a standard time for the contact angle measurement in this work. When the assigned interval time was reached, 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 measured using the drop shape analyzer program and were then averaged.

Mechanical Properties

The mechanical properties of all the samples were measured in compression mode, according to ASTM D822. Cylindrical test specimens (2.54 cm in diameter × 2.54 cm in length) were prepared. A speed of 0.127 cm/min and a 2.5 KN load cell were used for all measurements.

3.3.2.2 Cell Culture of Poly(S/EGDMA)PolyHIPE Foam

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 (DMEM; 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 40,000 cells/well on the polyHIPE specimens and TCPS were used as controls.

Cytotoxicity Test

Evaluation of the cytotoxicity of the poly(S/EGDMA)polyHIPE foam using L929 fibroblast-like cells was done based on the standard 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.

Cell Attachment and Proliferation

For the cell attachment and proliferation study of the poly(S/EGDMA)polyHIPE foam, L929 fibroblast-like cells at a density of 40,000 cells/well were used. Circular polyHIPE foam specimens 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.

Morphological Observation of Cell Culture

The morphology of the L929 fibroblast-like cells containing poly(S/EGDMA)polyHIPE foam was observed using SEM. All of the polyHIPE foam was washed twice with PBS, and then cell fixation was done with a 3% glutaraldehyde solution (diluted from a 50% glutaraldehyde 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.

Statistical Analysis.

Statistical analysis of the data obtained from the cell culturing, including the effect of plasma treatment on the cell culture, cell attachment, and proliferation, were achieved using the SPSS software program. One-way ANOVA and student's *t*-test were used to determine the statistical significance when the P value is less than 0.05 (n = 3 for each experiment).