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Research Article

Nanolayer Film on Poly(Styrene/Ethylene Glycol Dimethacrylate) High Internal Phase Emulsion Porous Polymer Surface as a Scaffold for Tissue Engineering Application

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Highly open, porous polymer or poly(high internal phase emulsion) (polyHIPE) obtained from polymerized high internal phase emulsions (HIPE) have recently attracted much attention and increasing interest in tissue engineering applications because of their excellent properties. This research is aimed at preparing and developing a process for producing polyHIPE for use as a scaffold in tissue engineering applications. Poly(styrene/ethylene glycol dimethacrylate) (poly(S/EGDMA)) loaded with hydroxyapatite was used to prepare the polyHIPE. Further improvement on hydrophilicity and biological response to tissue fluids of the polyHIPE porous polymer was carried out using a nanolayer coating via the layer-by-layer polyelectrolyte multilayer (PEM) technique. Three types of chemicals were used for coating on the surface of the polyHIPE porous polymer such as poly(sodium 4-styrene sulfonate) (PSS), gelatin (GEL), and alginic acid (ALG). The change in surface properties of the modified poly(S/EGDMA)HIPE was characterized by UV-visible spectroscopy and contact angle measurement. Further assessments consisted of cytotoxicity testing, cell attachment, and proliferation of L929, fibroblast-like cells that were seeded on the surface of the polyHIPE porous polymer. A three-dimensional structure poly(S/EGDMA)HIPE porous polymer with high porosity was successfully prepared. Moreover, it was found that surface modification encouraged poly(S/EGDMA)HIPE with a hydrophilic nanolayer, as observed by the decrease in contact angle degree and cell adhesion of L929 fibroblast-like cells, indicating that the poly(S/EGDMA)HIPE porous polymer was effectively improved by using the layer-by-layer technique. It was revealed by MTT assay that the poly(S/EGDMA)HIPE porous polymer coated with a nanolayer of a polyelectrolyte multilayer led to an enhancement in the amount of cell adhesion and proliferation on the modified poly(S/EGDMA)HIPE porous polymer. Additionally, the most effective polyelectrolyte solution for improving cell adhesion of the poly(S/EGDMA)HIPE was PSS.

1. Introduction

In recent years, tissue engineering has gained more attention for use as a treatment for patients who are suffering from the failure of vital tissues and organs. The most important properties for tissue engineering materials are that they can provide physical support for cell attachment, proliferation, nutrient transport, and new tissue infiltration [1]. Currently, metal and ceramic were chosen in orthopedic surgery but they did not closely match healthy cellular structure with tissue [2]. Many researchers are trying to find more appropriate biomaterials and techniques to make better materials for use in tissue engineering. A variety of materials have been utilized to date as a scaffold in tissue engineering applications in order to create artificial constructs and facilitate cell attachment and proliferation. Several polymers such as

polyester [3], chitosan [4, 5], hyaluronic [6], alginate [7, 8], and gelatin [9, 10] have been employed as scaffold materials or coating materials on a 2D substrate. Several processing techniques have also been developed to obtain suitable scaffold materials, these are electrospinning, solvent casting, and particulate leaching, freeze-drying, high internal phase emulsion (HIPE technique), and the phase separation technique [11–13].

A polyHIPE porous polymer has been developed as a three-dimensional polymer matrix for in vitro tissue engineering applications. As porous support, a poly(styrene/divinylbenzene)HIPE (poly(S/DVB)HIPE) porous polymer has successfully been characterized for its ability to support the growth of cells, and it has good biocompatibility with osteoblasts and solid supports in the in vitro environment [14]. The attractive prospect for further enhancing cell adhesion is improving hydrophilicity of the porous polymer [15]. Numerous reports have indicated that ethylene glycol dimethacrylate (EGDMA) and its derivative have been widely used in the fabrication of scaffolds in tissue engineering applications [16–18]. PolyHIPE with an average pore size between 40 and 100 μm has the potential to be used as a scaffold for bone tissue engineering applications because the osteoblast cells seeded onto a polyHIPE scaffold revealed cellular attachment and proliferation, leading to the support of an osteoblastic phenotype. In addition, a polyHIPE porous polymer produced from biomaterials has also been studied, and a highly porous polyHIPE polymer from poly(ε-caprolactone) (PCL) was prepared by free radical polymerization of a PCL macromonomer [19]. The polyHIPE porous polymer from the PCL macromonomer could act as a substrate for the growth of human fibroblasts. These materials were sufficiently biocompatible to support cell function and growth over a period of 2.5 days. Moreover, a combination of poly(ε-caprolactone) (PCL)/hydroxyapatite (HA) scaffolds exhibited excellent ductility, high strength, and good cellular structure [20]. The results indicated that for a material to be used in tissue engineering applications, there are several criteria that they must possess, and these are biocompatibility, bioactivity, nontoxicity to living cells, and able to support cell growth, adhesion, and proliferation.

In order to enhance the biological response to tissue fluids, various techniques for modifying surface properties are a must to produce suitable materials for tissue engineering applications such as coating, solution casting, and solution and air plasma treatment. To improve the bioactivity and hydrophilicity of a PCL fibrous scaffold, the biopolymer immobilization method as a kind of physical surface modification was applied [21]. The modification of the PCL fibrous substrate with gelatin enhanced the wettability and biocompatibility. The polyHIPE scaffold was also successfully modified by using atmospheric pressure plasma surface modification which led to the enhancement of the hydrophilic properties of the polyHIPE porous polymer surface and improved the interaction between the living cells and the polyHIPE substrate [22]. Nevertheless, the limitation of the atmospheric pressure plasma treatment was also observed, since at high treatment time, 30 min or longer, the specimen was destroyed.

The layer-by-layer (LbL) polyelectrolyte multilayer (PEM) surface modification is a simple technique and has been widely used in different areas. The LBL method has received much attention in the field of electrochemistry as a very promising tool for the fabrication of nanostructured films with high organisation at the nanoscale level; this method fabricated films through the specific interactions of organic functional groups for multilayer growth, and the films deposited onto a solid substrate were obtained with high stability [23]. Nanolayer coating via the layer-by-layer (LbL) surface modification technique is one method used for modifying the surface of a polymer by the addition of a functionalizable comonomer onto a polymer. The layer-by-layer (LbL) of polyelectrolyte is an approach for selective surface modification by building up a multilayer ultrathin film (PEM) coating of macromolecules. In 1997, Chen and McCarthy reported the surface modification of poly(-ethylene terephthalate) (PET) using the layer-by-layer deposition [24]. The surface of PET was modified to contain carboxylate and ammonium functionality. The LbL surface modification technique has been used to modify the surface of substrate materials for biomedical applications [25]. The presence of a biologically active multilayer film over the surface of chitosan/poly(ethylene glycol) hydrogel improved antibacterial activity and fibroblast spreading. Additionally, the coating of a polyelectrolyte multilayer thin film on nanofibrous scaffolds improved cell adhesion. It was found that the coated fiber (both poly(diallyldimethylammonium chloride)/poly(sodium 4-styrene sulfonate) (PDADMAC/PSS) and PDADMAC/gelatin) showed higher cell attachment, proliferation, and spreading than the uncoated one [26]. This result suggested that surface modification with a polyelectrolyte multilayer nanocoating is an effective technique for increasing cell adhesion on a polymeric substrate, and it is possible to use the LbL technique to modify the surface of a polyHIPE porous polymer to increase cell adhesion.

The purpose of this study was to improve the interaction between living cells and polyHIPE porous materials. The interaction between the substrate and living cells on hydrophilic materials is better than hydrophobic materials. The application of EGDMA as a comonomer or a crosslinking agent was selected to fabricate a polyHIPE scaffold with improved hydrophilicity, biocompatibility, high water uptake, and low cytotoxicity. The surface modification of polymerized high internal phase emulsion (polyHIPE) porous materials via the nanolayer coating polyelectrolyte multilayer (PEM) technique with superior hydrophilic properties was carried out and studied. Three types of chemicals were coated on the surface of the polyHIPE porous polymer such as poly(sodium 4-styrene sulfonate) (PSS), gelatin (GEL), and alginic acid (ALG). The study of cell adhesion and proliferation of the combination of 3D template poly(S/EGDMA)HIPE and nanolayer was highlighted.

2. Materials and Methods

2.1. Materials. In order to prepare a poly(S/EGDMA)HIPE porous polymer, styrene (S, purity > 99, Fluka Chemie, Switzerland) and ethylene glycol dimethacrylate (EGDMA, Sigma-Aldrich, USA) were used as a monomer and a

comonomer to produce the polyHIPE porous scaffold. The surfactant, which was sorbitan monooleate (Span 80; S80), was purchased from Sigma-Aldrich (USA). Poly(sodium 4-styrene sulfonate) (PSS, MW 70,000), poly(diallyldimethylammonium chloride) (PDADMAC, MW 350,000), gelatin (GEL, type B from bovine skin), and alginic acid (ALG, sodium salt) were purchased from Sigma-Aldrich (USA) and used as a polyelectrolyte solution for the layer-by-layer surface modification. The initiator and stabilizer used in the experiments were potassium persulfate and calcium chloride from Fluka Chemie (Switzerland). Tetrahydrofuran (THF) and phosphoric acid were purchased from Sigma-Aldrich (USA) and Merck (Germany), respectively. Hydroxyapatite (HA) was purchased from MK nano (USA), and all chemicals were used as received.

2.2. Preparation of Poly(S/EGDMA)HIPE Porous Polymer. Poly(S/EGDMA)HIPE porous polymer was prepared by using the high internal phase emulsion (HIPE) technique as described by Akay et al. in 2004 [14]. The oil phase (10% of total volume) contained styrene monomer (S), ethylene glycol dimethacrylate (EGDMA) as a crosslinking agent (S : EGDMA; 80 : 20 ratio by volume), and sorbitan monooleate (Span 80, 2 ml) as a surfactant. The aqueous phase (90% of the total volume) contained deionized water (88 ml), tetrahydrofuran (THF, 1 ml), potassium persulfate ($K_2S_2O_8$, 0.2 g) as an initiator, calcium chloride ($CaCl_2$, 1 g) as a stabilizing salt, hydroxyapatite (HA, 0.45 g), and phosphoric acid (H_3PO_4, 1 ml) which was used to dissolve the hydroxyapatite. In order to improve the capability of porous scaffolds based on a high internal phase emulsion technique for bone tissue engineering applications, hydroxyapatite, which is a naturally occurring mineral form of calcium apatite, was loaded into the polyHIPE system. HA is a promising bone replacement material because of its composition similarity to the human hard tissues. The aqueous phase was added dropwise into the oil phase under constant mechanical stirring until all aqueous phases were 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 a soxhlet extractor. All samples were dried in an oven at 60°C until a constant weight was obtained.

2.3. Preparation of PEM Nanolayer by the Layer-by-Layer (LbL) Technique. To improve the hydrophilicity of the poly(S/EGDMA)HIPE porous polymer, a nanolayer coating via the layer-by-layer polyelectrolyte multilayer (PEM) technique was applied as a nanolayer for scaffold specimen modification. This technique was carried out by alternate adsorption between a polycation and a polyanion on the surface of poly(S/EGDMA)HIPE. The poly(S/EGDMA)HIPE porous polymer was cut to a thickness of 1.5 mm and a diameter of 20 mm and dried in a conventional oven at 60°C for 24 hours. Nanolayer coating was performed by injecting a polymer solution through the poly(S/EGDMA)HIPE disk. There were two coatings consisting of the primary coating (containing 4 bilayers of the alternating layers of two polyions, which are PDADMAC(+)/PSS(-)) and the secondary coating (containing 4 bilayers of the alternating layers of two polyions, which are PDADMAC(+)/PSS(-) or ALG(-) or GEL(-)) (see Figure 1). For the primary coating, the PDADMAC solution at pH 7 (10 mM, 10 ml) was manually injected through poly(S/EGDMA)HIPE disks as a positively charged polyelectrolyte for 2 mins and then followed by a PSS solution (10 mM, 10 ml) as a negatively charged polyelectrolyte solution for 2 mins. After each injection with the electrolyte polymer solution, the poly(S/EGDMA)HIPE disks were rinsed three times for 30 s with deionized water to remove the excess polyelectrolyte polymer. This process was repeated until 4 bilayers of the polyelectrolyte polymer were obtained (each bilayer is composed of PDADMAC as positively charged and PSS as negatively charged). The secondary coating was carried out using a similar procedure with different negatively charged polyelectrolyte polymer solutions such as PSS, alginic acid (at pH 5.5), and gelatin (at pH 9).

2.4. Characterization of Poly(S/EGDMA)HIPE Porous Polymer. Physical properties of the polyHIPE porous polymer were investigated, including phase morphology, surface area, and wettability on the polyHIPE surface using SEM (FE-SEM, Hitachi S-4800, Japan), BET (Quantachrome Autosorb 1MP, USA), and contact angle measurement (Krűss model DSA 10, Germany), respectively. A Universal Testing Machine (Lloyd model LRX, USA) was also used for the mechanical testing of the obtained materials.

2.5. Cell Culture. Mouse fibroblast connective tissue (L929) was used in this study to investigate the suitability of the poly(S/EGDMA)HIPE porous polymer as a scaffold in tissue engineering applications. L929 fibroblast-like cells were cultivated 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 $\mu g\,ml^{-1}$ 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 porous polymer was cut into circular disks, which were later sterilized in an autoclave for 1 hour prior to use, and then the specimens were placed in the wells of a 24-well tissue-culture polystyrene plate (TCP; Biokom Systems, Poland). The specimens were pressed with a metal ring in order to prevent the polyHIPE porous polymer specimen from floating on 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); then, they were seeded at a density of 40,000 cells/well on the polyHIPE specimens and TCPS were used as controls.

The cytotoxicity of the poly(S/EGDMA)HIPE porous polymer was evaluated using a method outlined in the standard method (ISO 10993-5) by using L929 fibroblast-like cells. An extracted medium was prepared as described in a previous work [22]. The circular poly(S/EGDMA)HIPE

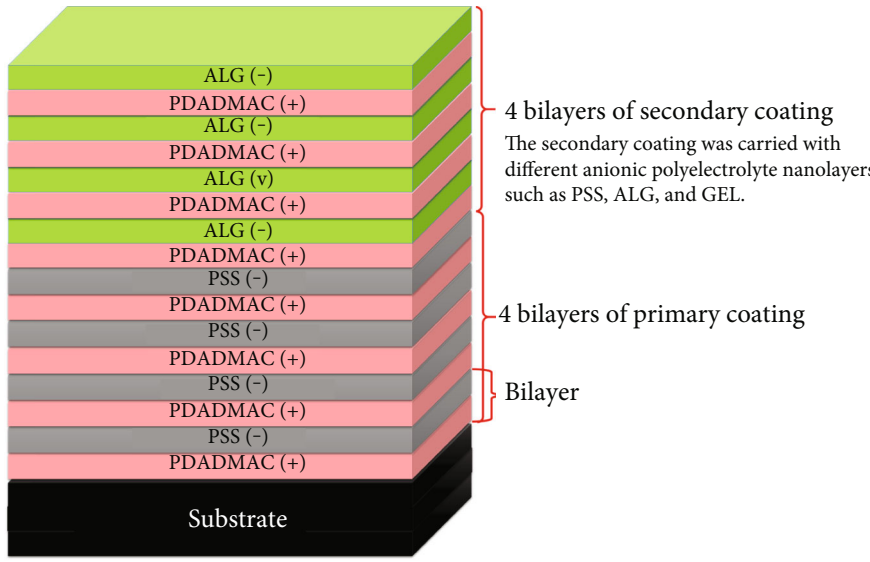

Figure 1: Schematic of the primary coating and the secondary coating of the polyelectrolyte membrane (PEM) nanolayer on a substrate.

specimens were sterilized in an autoclave for 1 hour and placed in a 24-well plate; then, they were 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 culture medium (DMEM) for 48 hours. After incubating for 48 hours, the culture medium was removed and replaced with the as-prepared extraction medium before an additional 24-hour incubation period. The number of living cells was quantified using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; Sigma-Aldrich, USA) assay.

Cell attachment and proliferation on a poly(S/EGDMA)-HIPE porous polymer with and without coating with a PEM nanolayer were studied. L929 fibroblast-like cells at a density of 40,000 cells/well were used. Circular poly(S/EGDMA)-HIPE specimens were placed in a 24-well culture plate with a metal ring and sterilized in an autoclave for 1 hour, washed two times with phosphate-buffered saline (PBS; pH = 7.2), and then with the culture medium (DMEM). Prior to cell seeding, 500 μl of DMEM was added to each well. For a cell attachment study, L929 fibroblast-like cells at a density of 40,000 cells/well were seeded on the poly(S/EGDMA)HIPE samples for 1, 4, and 24 hours. At the required seeding time, the measurement of cell viability was determined by the MTT assay. The proliferation of L929 fibroblast-like cells on a poly(S/EGDMA)HIPE scaffold was determined at different culture periods (4 hours, 1 day, 3 days, and 7 days) then measured again with an MTT assay to determine the changes in the number of viable cells.

The 3-(4,5-dimethylthiazol-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. It employes 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. Firstly, the cell-contained poly(S/EGDMA)HIPE porous polymer was 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 at 37°C for 30 min. After the 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 amount by using a UV-visible spectrophotometer at a wavelength of 570 nm.

The morphology of the poly(S/EGDMA)HIPE porous polymer seeded with L929 fibroblast-like cells was observed using a scanning electron microscope (SEM). All of the poly-HIPE porous polymers were washed twice with PBS, and then cell fixation was carried out with a 3% glutaraldehyde solution (diluted from 50% glutaraldehyde solution with PBS) at 500 ml/well for 30 min. After the fixation, the poly(S/EGDMA)HIPE porous polymer was washed with PBS and dehydrated with ethanol solutions of varying concentrations (i.e., 30, 50, 70, 90, and 100%) for about 2 min at each concentration. After completely drying, the specimens were mounted on copper stubs and coated with gold to observe the cell adhesion on the modified and unmodified poly(S/EGDMA)HIPE porous polymers by SEM.

3. Results and Discussion

3.1. Characterization of Poly(S/EGDMA)HIPE Porous Polymer. The poly(S/EGDMA)HIPE porous polymer loaded with hydroxyapatite was successfully prepared via a high internal phase emulsion polymerization technique. The presence of hydroxyapatite on poly(S/EGDMA)HIPE was confirmed by EDX analysis. The result indicated that the Ca/P ratio of the obtained specimen was 1.45, which was relatively close to the theoretical value of 1.67 [27]. The structure of the poly(S/EGDMA)HIPE porous polymer was confirmed by a SEM image (Figure 2) and showed a three-

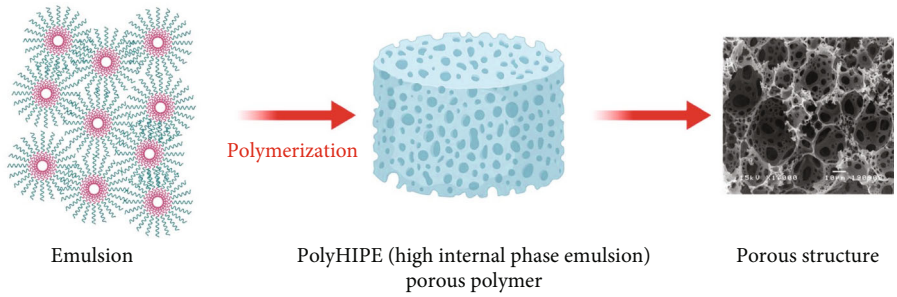

Figure 2: Preparation and SEM images of the poly(S/EGDMA)HIPE porous polymer with an oil and aqueous phase ratio of 80 : 20.

Table 1: Characteristics of the poly(S/EGDMA)HIPE porous polymer.

Properties of poly(S/EGDMA)HIPE porous polymer	
Surface area ($m^2\ g^{-1}$)	11.84
Pore size* (μm)	30-40
Compressive strength (MPa)	0.86 ± 0.02
Compressive modulus (MPa)	17.57 ± 0.86

*Excluding interconnected pore.

dimensional open cellular structure morphology. The average pore size of a spherical cavity was approximated at 30-40 μm (measured from a SEM micrograph by using the SemAfore program and the data did not include an interconnected pore). There were many small interconnected pores on the wall of the large pore which were connected to its neighbors, allowing cells to migrate and proliferate within this structure. This result was consistent with previous results found in the literature on the capability of the polyHIPE porous polymer as a scaffold which indicated that polyHIPE with an average pore size of 40-100 μm was capable of supporting the growth of cells [14].

The surface area, physical properties, and mechanical properties of the polyHIPE porous polymer are shown in Table 1. From N_2 adsorption-desorption results, the BET surface area of the polyHIPE porous polymer was 11.84 $m^2\ g^{-1}$. The mechanical properties of the poly(S/EGDMA)HIPE porous polymer were evaluated through measurements of their Young's modulus and compressive strength, which were 17.57 ± 0.86 MPa and 0.86 ± 0.02 MPa, respectively. From these results, it clearly demonstrated that the 3 dimensional (3D) poly(S/EGDMA)HIPE have high porosity, high interconnectivity, and high structural stability. Therefore, it is likely that the poly(S/EGDMA)HIPE porous polymer loaded with hydroxyapatite would fulfill the requirement of a scaffold material for tissue engineering applications and the preparation was selected for later studies [20].

3.2. Determination of Optimum PEM Nanolayer on Poly(S/EGDMA)HIPE. Among the requirements for materials to be used as a scaffold in tissue engineering applications, biocompatibility, low cytotoxicity, good mechanical properties, and superior hydrophilicity of the substrate are the most important requirements determining the biocompatibility of the material with living cells. An increase in the wettability of the materials leads to the promotion of cell growth and the adhesion between the cells and the substrate material. To satisfy these requirements, the applied nanolayer on the poly(S/EGDMA)HIPE porous polymer via the layer-by-layer polyelectrolyte multilayer (PEM) technique was carried out in order to improve the adhesion and the interaction between the poly(S/EGDMA)HIPE porous polymer and the living tissue, which would imply the hydrophilic development of the poly(S/EGDMA)HIPE surface. Poly(diallyldimethylammonium chloride) (PDADMAC) and poly(sodium 4-styrene sulfonate) (PSS) were selected for the primary coating because they were strong electrolytes, with a charge that was independent of pH [26]. The effect of the number of layers modified on the poly(S/EGDMA)HIPE porous polymer is shown in Figure 3. It was found that the absorbance of UV-visible spectroscopy at 613 nm increased when the number of layers applied increased. At 1 to 7 layers, the absorbance dramatically increased with the linear relationship, whereas from 7 to 9 layers there was only a slight increase in the absorbance.

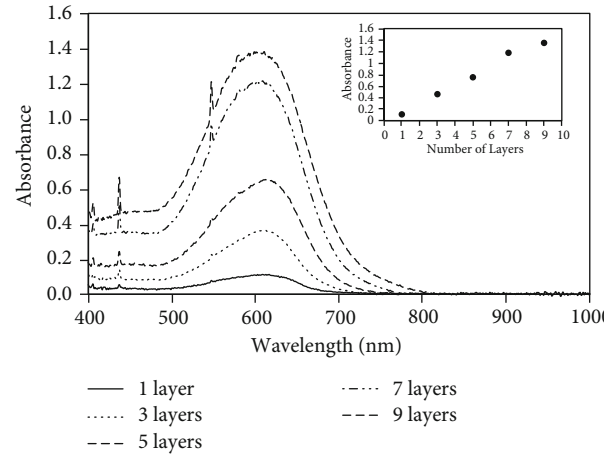

Figure 3: UV-visible spectrum of the poly(S/EGDMA)HIPE surface modified with a PEM nanolayer of PDADMAC/PSS as a function of the number of layers.

The color of indigo dye was used to confirm the effect of the number of layers coated on the poly(S/EGDMA)HIPE. Poly(S/EGDMA)HIPE modified with the PEM nanolayer of PDADMAC (as positively charged) at various layers (1, 3, 5, 7, and 9 layers) with indigo dye on top of the surface is

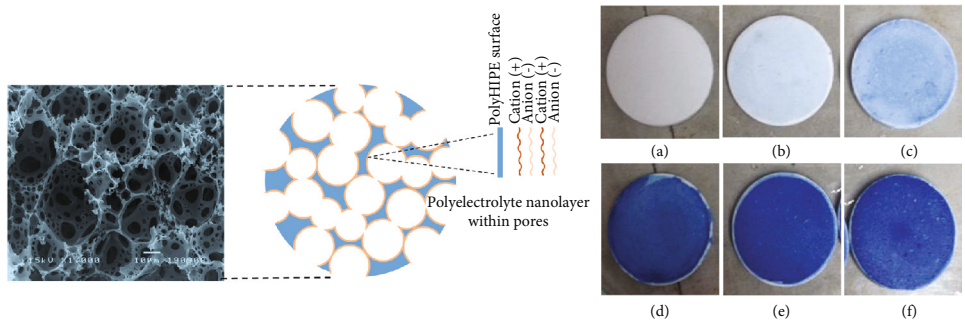

Figure 4: Poly(S/EGDMA)HIPE porous polymer modified surface with a PDADMAC/PSS PEM nanolayer and followed by indigo dye: (a) unmodified, (b) 1 layer, (c) 3 layers, (d) 5 layers, (e) 7 layers, and (f) 9 layers.

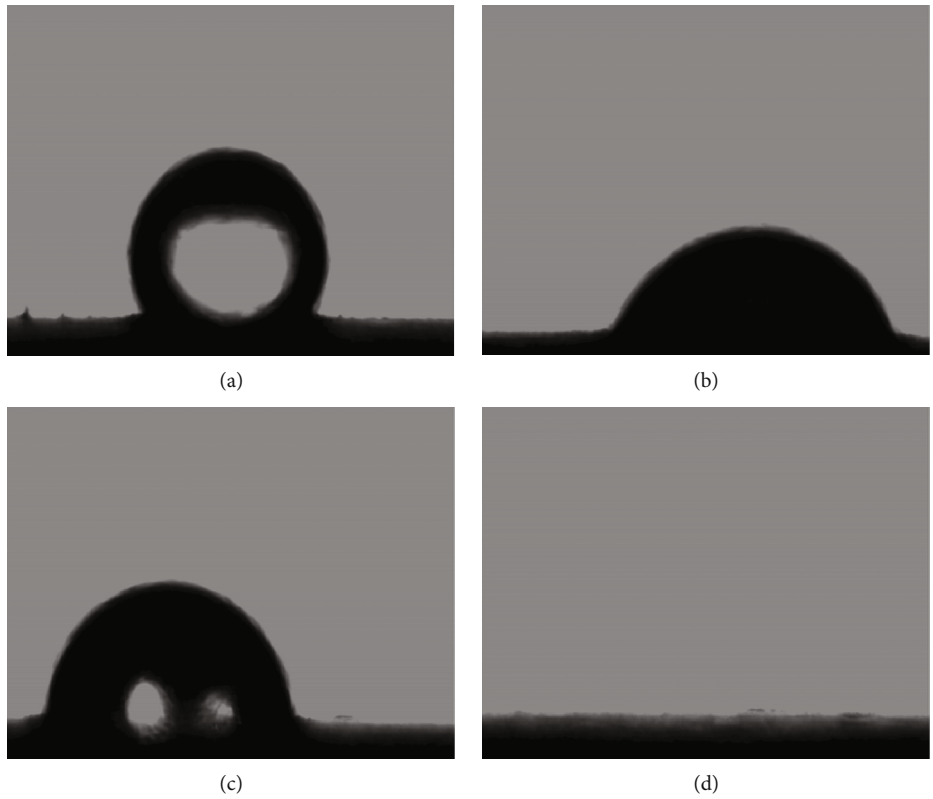

Figure 5: Static water sensible drops on poly(S/EGDMA)HIPE loaded with hydroxyapatite: (a) unmodified, (b) modified with PSS, (c) modified with ALG, and (d) modified with GEL PEM nanolayer.

illustrated in Figure 4. It is clearly shown that the intensity of the color increased as the number of layers increased. The homogenous coating was achieved at 7 layers of PDADMAC (positively charged) and was used to modify the poly(S/EGDMA)HIPE; the indigo dye was found to evenly coat on the entire surface of poly(S/EGDMA)HIPE. Therefore, the primary coating of 7 layers of PDADMAC was chosen to modify poly(S/EGDMA)HIPE in this study. For the secondary coating, PSS, alginic acid, and gelatin were used to functionalize the modified poly(S/EGDMA)-HIPE at pH 7, 5.5, and 9. Also, the poly(S/EGDMA)HIPE surface modified with a polyelectrolyte solution exhibited an insignificant phase morphology change.

3.3. Effect of PEM Nanolayer on Wettability of Poly(S/EGDMA)HIPE Porous Polymer. The static contact angle of distilled water was used to investigate the effect of different negatively charge polyelectrolyte solutions of the secondary coating on the wettability of the poly(S/EGDMA)-HIPE surface. After the PEM nanolayer was applied on poly(S/EGDMA)HIPE, improving wettability was observed on the poly(S/EGDMA)HIPE modified with the PEM nanolayer and the water droplet contact angle on the surface was decreased from 122° to 55°, 72°, and 0° for PSS, ALG, and GEL, respectively (Figure 5). This is because of the additional hydrophilic functional group on the poly(S/EGDMA)HIPE surface. Therefore, it clearly demonstrated that the PEM

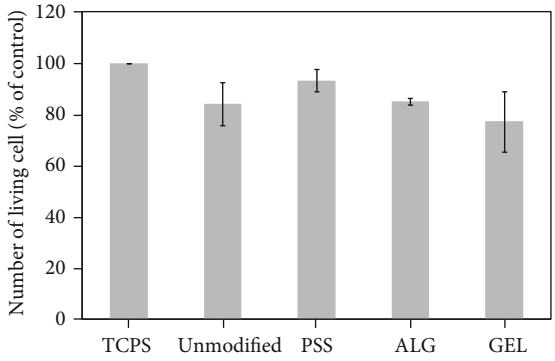

Figure 6: Cytotoxicity of the poly(S/EGDMA)HIPE porous polymer: unmodified (before) and polyHIPE modified with PEM nanolayer (after).

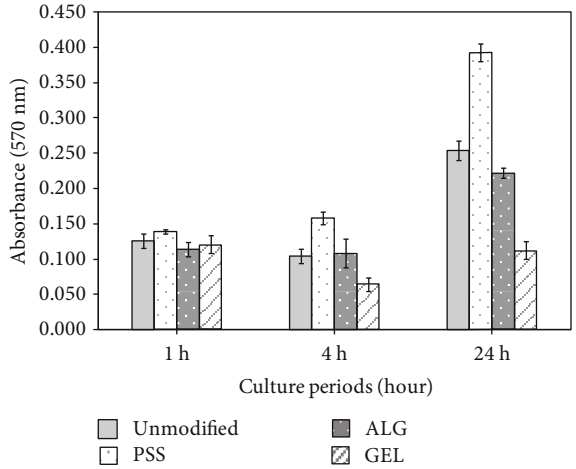

Figure 7: Cell attachment of L929 fibroblast-like cells on the unmodified polyHIPE and the modified poly(S/EGDMA)HIPE with PEM nanolayer, after 1, 4, and 24 hour(s) of cell culture periods.

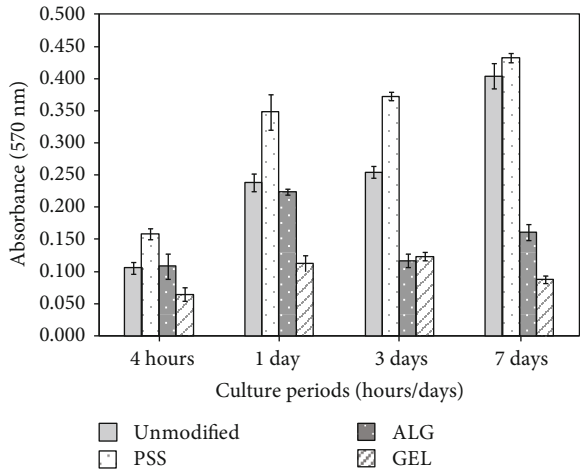

Figure 8: Cell proliferation of L929 fibroblast-like cells on the unmodified polyHIPE and the modified poly(S/EGDMA)HIPE with PEM nanolayer, after 4 hours, and 1, 3, and 7 day(s) of cell culture periods.

nanolayer coating is an effective technique for improving the hydrophilicity of the poly(S/EGDMA)HIPE surface.

3.4. Cytotoxicity Test. The cytotoxicity evaluation based on the viability of L929 fibroblast-like cells was carried out by an indirect method. The number of living cells after incubation for 24 hours was reported as the percentage of the controls (tissue culture plates (TCPs)). The linear relationship between the absorbance of the MTT solution and the number of living cells indicated that both the unmodified and PEM-nanolayer-modified poly(S/EGDMA)HIPE porous polymers exhibited noncytotoxicity for the fibroblast-like cells (L929). This is in agreement with the results of our earlier study. From the result, it showed that the number of living cells after culturing in the poly(S/EGDMA)HIPE porous polymer extraction medium solutions for 24 hours was higher than 70% of the controls for both the unmodified poly(S/EGDMA)HIPE and the modified poly(S/EGDMA)HIPE. The number of living cells was found to be about 84%, 93%, 85%, and 77% for the unmodified poly(S/EGDMA)HIPE, the poly(S/EGDMA)HIPE modified with PSS, ALG, and GEL PEM nanolayer, respectively (see Figure 6). For the poly(S/EGDMA)HIPE modified with the GEL PEM nanolayer, the results indicated that the living cells was about 77% when compared with controls, which was relatively close to the standard value (70%). Therefore, we were able to conclude that there were no toxic products leached from the poly(S/EGDMA)HIPE porous polymer to the monolayer of the L929 fibroblast-like cells.

3.5. Cell Attachment and Proliferation. For cell attachment, L929 fibroblast-like cells were seeded on the poly(S/EGDMA)HIPE porous polymer for 1, 4, and 24 hour (s). At each time point, the number of L929 fibroblast-like cells was quantified by its relative absorbance, as shown in Figure 7. The amount of living cells attached on the poly(S/EGDMA)HIPE substrate relates directly to the absorbance determined by the MTT assay. The result demonstrated that at a longer incubation time, the number of cell attachments on the poly(S/EGDMA)HIPE substrate was higher. At all seeding times, the PSS-modified poly(S/EGDMA)HIPE porous polymer showed a higher level of cell attachment than other poly(S/EGDMA)-HIPE porous polymers. This was probably due to the sharp decrease in water contact angle from 122° to 55° (Figure 5(b)), which was due to the improved surface hydrophilicity, and consequently enhanced the attachment between living cells and the modified poly(S/EGDMA)-HIPE substrate [25]. For the poly(S/EGDMA)HIPE modified with ALG and GEL, the cell attachment was less than the unmodified poly(S/EGDMA)HIPE (control). The proliferation of L929 fibroblast-like cells was signified by the viability of the cells at 4 hours, 1 day, 3 days, and 7 days after seeding cells on the unmodified and modified poly(S/EGDMA)HIPE. Figure 8 shows the relationship between the absorbance and culture time, which indicated that the number of viable cells increased with an increase in the time in culture. The PSS-modified poly(S/EGDMA)HIPE showed

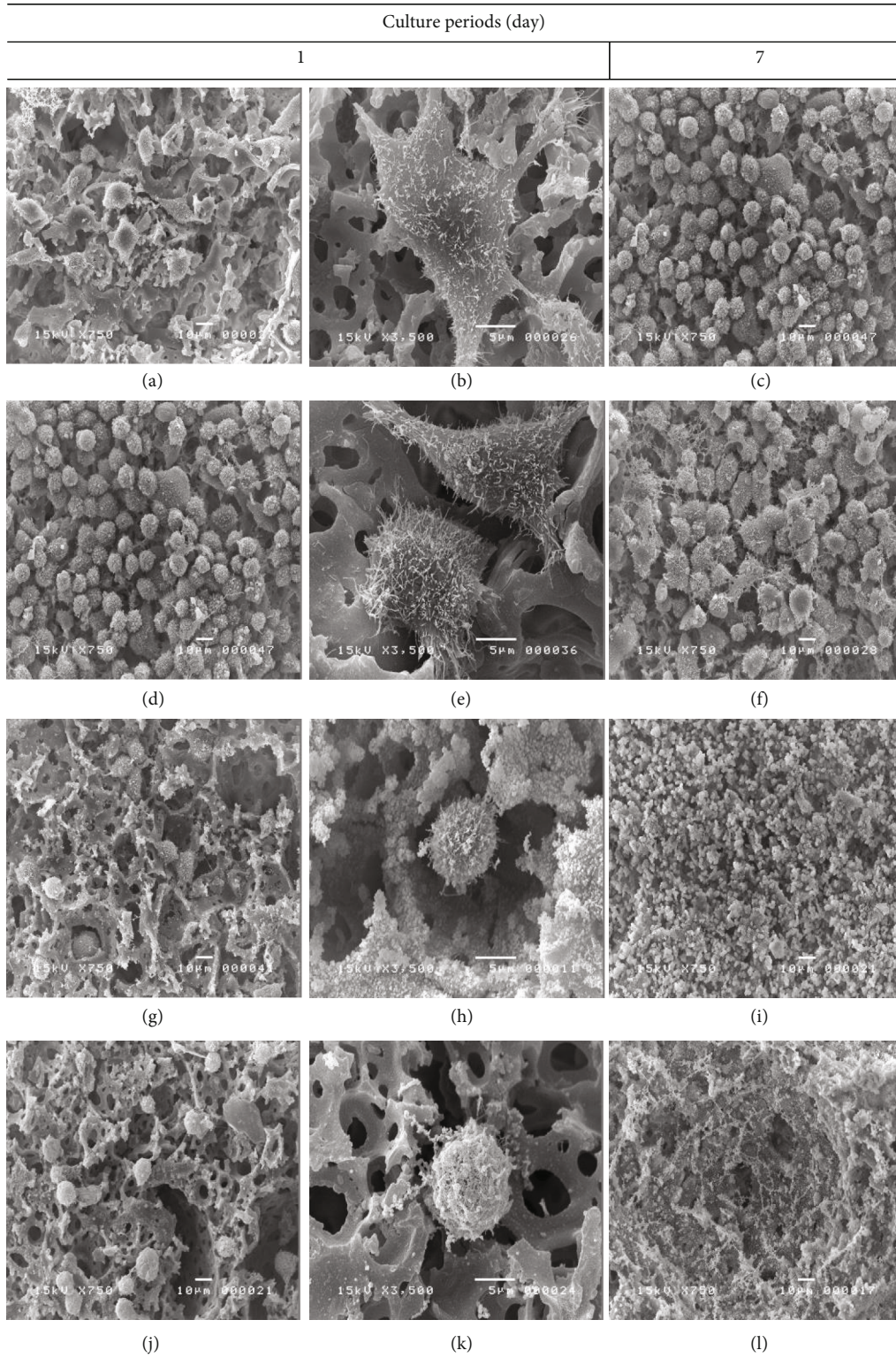

Figure 9: SEM image of the L929 fibroblast-like cells on poly(S/EGDMA)HIPE porous polymer: (a–c) unmodified, (d–f) modified with PSS, (g–i) modified with ALG, and (j–l) modified with GEL.

the highest level of proliferation because PSS is a strong electrolyte and it can provide a stable film with good adhesion on the poly(S/EGDMA)HIPE substrate that results from the surface improvement [26]. The amount of cells on the PSS-modified poly(S/EGDMA)HIPE was increased up to 60% when compared with the unmodified polyHIPE porous polymer (control). On the other hand, at 7 days of culturing, the proliferation of L929 fibroblast-like cells on the PSS-modified polyHIPE polymer was slightly better than that on the unmodified polyHIPE polymer. This situation could be

due to the L929 cells that were already allowed to fully attach on the polyHIPE surface and some of them have penetrated into the polyHIPE 3D structure (inside the porous structure). It was difficult to extract them out from the porous structure. So, the determination of the number of cell viability by using the MTT assay shows the slightly different value between the modified and unmodified polyHIPE porous polymer at 7 days of culturing. In contrast, for poly(S/EGDMA)HIPE modified with ALG and GEL, results showed that cell proliferation was less than the unmodified poly(S/EGDMA)HIPE. This may be because ALG and GEL are weak electrolytes. Additionally, the secondary coating with ALG and GEL PEM nanolayer was typically constructed at pH 5.5 and 9, respectively. They provided an unstable layer on the surface of the poly(S/EGDMA)HIPE substrate, which was easy to dissolve in the culture medium, resulting in the culture medium being contaminated and altering the pH balance; thus, L929 fibroblast cells were not able to grow and eventually died.

The morphology of L929 fibroblast-like cells after they were cultured on the poly(S/EGDMA)HIPE porous polymer for a period of 1 and 7 day(s) was observed using a scanning electron microscope, as shown in Figure 9. It was observed that the number of living cells on the PSS-modified poly(S/EGDMA)HIPE (Figures 9(d)–9(f)) was greater than the unmodified poly(S/EGDMA)HIPE, and the number of living cells found on poly(S/EGDMA)HIPE was increased with an increase in the incubation time from 1 day to 7 days. After seeding, the L929 fibroblast-like cells were observed on the poly(S/EGDMA)HIPE surface in round and filopodia shapes for both the unmodified and PSS-modified poly(S/EGDMA)HIPE. However, the L929 fibroblast-like cells cultured on the PSS-modified poly(S/EGDMA)HIPE substrate was more expanded and more spread out compared to the unmodified substrate. For the ALG- and GEL-modified poly(S/EGDMA)HIPE, the number of living cells was less than that found on the unmodified poly(S/EGDMA)HIPE. The appearance of L929 fibroblast-like cells for both ALG- and GEL-modified poly(S/EGDMA)HIPE was not similar to unmodified and PSS-modified poly(S/EGDMA)HIPE. There were some small flakes covering the surface and small fibrils from cells of the poly(S/EGDMA)HIPE modified with ALG and GEL PEM nanolayer, respectively, which inhibited the proliferation of cells and resulting in cell death.

4. Conclusions

Poly(S/EGDMA)HIPE loaded with hydroxyapatite porous polymers were fabricated via the high internal phase emulsion polymerization technique. The poly(S/EGDMA)HIPE porous polymer fulfilled the requirements for the ideal scaffolds such as having high porosity, highly interconnectivity, and high structural stability. The PEM nanolayer via the layer-by-layer technique was successfully applied on the surface of poly(S/EGDMA)HIPE to enhance the hydrophilic properties and wettability. The unmodified poly(S/EGDMA)HIPE and the PEM-nanolayer-modified poly(S/EGDMA)HIPE exhibited nontoxicity for L929 fibroblast-like cells. Furthermore, cell attachment and cell proliferation did not only depend on the hydrophilic properties of the surface but also depended on the chemical type of coating. The PSS-modified poly(S/EGDMA)HIPE showed the highest efficiency of cell attachment and cell proliferation of the L929 fibroblast-like cells, and the amount of cells increased up to 60% when compared with unmodified poly(S/EGDMA)-HIPE. This could be due to PSS being a strong polyelectrolyte, which provided a more stable layer, and the strong adhesion on the poly(S/EGDMA)HIPE substrate. Therefore, PSS-modified poly(S/EGDMA)HIPE could be a good candidate for use in tissue engineering applications with the exception of ALG- and GEL-modified poly(S/EGDMA)HIPE which showed a lower number of the living cells than the unmodified poly(S/EGDMA)HIPE.

Data Availability

Any additional data can be made available upon request from the corresponding author.

Conflicts of Interest

We have no conflicts of interest to disclose. We declare that this work is original and has not been published elsewhere nor is it currently under consideration for publication elsewhere.

Authors' Contributions

All authors have approved the manuscript and agree with its submission to the Journal of Nanomaterials.

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