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Surface Modification and Fibrovascular Ingrowth of Porous Polyethylene Anophthalmic Implants

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Abstract: The purpose of this study was to determine the effect of surface modification on the fibrovascular ingrowth into porous polyethylene (PE) spheres (Medpor®), which are used as an anophthalmic socket implant material. To make the inert, hydrophobic PE surface hydrophilic, nonporous PE film and porous PE spheres were subjected to plasma treatment and *in situ* acrylic acid (AA) grafting followed by the immobilization of arginine-glycine-aspartic acid (RGD) peptide. The surface-modified PE was evaluated by performing surface analyses and tested for fibroblast adhesion and proliferation *in vitro*. In addition, the porous PE implants were inserted for up to 3 weeks in the abdominal area of rabbits and, after their retrieval, the level of fibrovascular ingrowth within the implants was assessed *in vivo*. As compared to the unmodified PE control, a significant increase in the hydrophilicity of both the AA-grafted (PE-*g*-PAA) and RGD-immobilized PE (PE-*g*-RGD) was observed by the measurement of the water contact angle. The cell adhesion at 72 h was most notable in the PE-*g*-RGD, followed by the PE-*g*-PAA and PE control. There was no significant difference between the two modified surfaces. When the cross-sectional area of tissue ingrowth *in vivo* was evaluated, the area of fibrovascularization was the largest with PE-*g*-RGD. The results of immunostaining of CD31, which is indicative of the degree of vascularization, showed that the RGD-immobilized surface could elicit more widespread fibrovascularization within the porous PE implants. This work demonstrates that the present surface modifications, viz. hydrophilic AA grafting and RGD peptide immobilization, can be very effective in inducing fibrovascular ingrowth into porous PE implants.

Keywords: anophthalmic implant, polyethylene, plasma treatment, acrylic acid, RGD, fibrovascular ingrowth.

Introduction

High-density porous polyethylene (HPPE) has been widely used in anophthalmic socket implants,[1-4] facial bone reconstruction,[5] orbital fracture repair,[6,7] volume augmention,[8] and lower eyelid spacers.[9,10] Once implanted, the socket PE implants need to be well-vascularized, because it is generally accepted that fibrovascular tissue ingrowth would prevent them from migration, infection, and extrusion.[11,12] Moreover, the development of fibrovascularization would reduce the incidence of porous orbital implant exposure, which is the most common complications regarding anophthalmic implant surgery. Currently available materials for porous implants are HPPE (Medpor®, Porex Surgical, GA, USA), synthetic hydroxyapatite (HA) (FCI Ophthalmics Inc., Issy-Les-Moulineaux, France), coralline HA (Bio-Eye, Integrated Orbital Implants Inc., SD, USA), and aluminum oxide (Bioceramic implant, FCI Ophthalmics Inc., Issy-Les-Moulineaux, France). HPPE is an inert, hydrophobic polymer that has been applied to an alloplastic implant in humans since the 1940s.[13] Equipped with interconnected channels through the implant that ranged from 125 to 1,000 μm in size, it is relatively inexpensive, easily processable, biocompatible, and has a high tensile strength, malleability, and biocompatibility.[14-16] The surface is smoother than HA or aluminum oxide, which results in less tissue breakdown by friction. Moreover, sutures can be used through these implants.

In this work, we intended to make a surface-modified PE

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to induce more fibrovascular ingrowth into porous PE implants. To do this, a hydrophilic functional group, acrylic acid (AA) was grafted on the PE surface by using argon plasma treatment, which is an effective way of chemically modifying the surfaces of polymeric materials.[17-20] As a physical method, plasma treatment of polymer affects extremely limited depth from the surface and thus bulk properties of base materials are barely changed. This technique can easily modify a complex shape of 3D structure. The mechanism of plasma-induced surface modification is that once polymer chains are exposed to plasma glow discharge, radicals are generated and they can readily react with monomers to graft hydrophilic functional groups on the surface, such as -OH, -OOH, and -COOH. In addition, a cell-adhesive motif, arginine-glycine-aspartic acid (RGD) peptide was immobilized on the AA-grafted PE surface. RGD is an essential ligand peptide sequence that exists in a variety of extracellular matrix (ECM) for cell-matrix interactions. Many studies have shown that immobilization of RGD can help significantly improve cell adhesion and proliferation on the substrates.[21-23] In our previous studies, AA-grafted or RGD-immobilized poly(L-lactic acid) (PLLA) clearly displayed that fibroblast adhesion and proliferation were substantially improved on the modified substrates.[24-26]

Our hypothesis is that with the aid of cell-adhesive surface properties, fibrovascular ingrowth may be significantly advanced in porous PE sphere implants. To test the hypothesis, PE films and implants were divided into three groups for the experiment, PE grafted with AA (PE-*g*-PAA), PE grafted with AA and subsequently immobilized with RGD peptides (PE-*g*-RGD), and PE control without any surface treatments. The modified surfaces were analyzed using attenuated total reflectance Fourier transform infrared (ATR-FTIR), water contact angles, and electron spectroscopy for chemical analysis (ESCA). While *in vitro* adhesion behaviors of fibroblasts were examined on the different PE film surfaces, *in vivo* animal test of porous PE implants was also carried out for up to 3 weeks.

Experimental

Materials. Non-porous PE film without additives was supplied from Hanhwa Co., Korea. Porous PE sphere (Medpor®, Porex Surgical, GA, USA) was purchased, with an average pore size of 400 μm and 12 mm in diameter. Acrylic acid (AA) and Gly-Arg-Gly-Asp (GRGD) were purchased from Sigma Chem. Co. and Anygen Co., Korea, respectively. Toluidine Blue O and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were purchased from Aldrich Chem. Co. The other chemicals were of reagent grade and used as received.

***In Situ* AA Grafting.** PE films (10 × 20 mm) and porous PE spheres were washed with methyl alcohol and dried in a vacuum oven before being placed in the plasma chamber. The chamber was pumped down to 10 mtorr to remove air, moisture, and residual methyl alcohol on the PE surface. Once argon (Ar) gas plasma treatment of the PE samples was carried out at 200 mtorr at 50 W for 4 min, subsequently, AA was fed into the chamber in a plasma state and this process was lasted for 4 min at 50 W of discharge power. Finally, AA vapor was fed in the chamber at room temperature for 8 min at 200 mtorr. The treated PE was washed with methyl alcohol to remove untreated AA, and then dried at room temperature in a vacuum oven to obtain an AA grafted PE, PE-*g*-PAA. The surface-modified PE specimens were stored in a moisture-free container for further use.

RGD Immobilization. Carboxylic acid groups on the AA-grafted PE surface were activated using a peptide-coupling reagent, EDC. The PE-*g*-PAA films and spheres were immersed in 10 mL of EDC in 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer solution (50 mg EDC; pH 4.6) for 4 h at room temperature with a mild stirring to activate carboxylic acid groups. The activated PE-*g*-PAA was then put in 10 mL of GRGD (4.04 mg) in phosphate-buffered saline (PBS; pH 7.4) solution for 2 h at room temperature with a mild stirring. The modified PE-*g*-GRGD film and sphere were rinsed with PBS solution for 15 min, followed by distilled water for 5 min, and MES buffer solution for 10 min. Finally, they were washed with distilled water for 10 min to obtain an RGD-immobilized PE, PE-*g*-RGD.

Surface Characterization. Detection of functional groups in ATR-FTIR spectra was examined using an IFS 66 spectrometer (Bruker Co., Germany). The atomic compositions on the surface were confirmed by using ESCA. For water contact angle measurements, a water droplet of 5 μL was placed on the PE film surface. Five separate measurements were recorded at different places of the film surface and averaged. The amount of the grafted AA was calculated using Toluidine Blue O method.[27] When the dye solution (0.5 mM; pH 10) was prepared, the grafted film was placed in this solution for 6 h at 30 °C. The films were then removed and thoroughly washed with a sodium hydroxide solution (pH 9) to remove any noncomplex dye adhered to the surface. Once the dye was desorbed from the film in 50% acetic acid, the dye concentration was determined at 633 nm with an UV-VIS spectrophotometer (V-530, Jasco Co., Japan).

***In Vitro* Cell Culture.** Fibroblasts (NIH3T3, ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS, Biowhittectker) and 1% antibiotics (Gibco) at 37 °C in a humidified 5% CO_2 atmosphere. Before cell seeding, all the PE sample of PE control, PE-*g*-PAA, and PE-*g*-RGD were sterilized in UV irradiation for 6 h and then immersed in 70% ethanol for 30 min. After being washed with PBS solution three times, the PE samples were placed on the bottom of the 24-well tissue culture plates. The numbers of the

seeded cells were 2×10^3 and 2×10^4 cells/mL for the non-porous films and porous PE implants, respectively. The cell adhesion and morphology on the different substrates were observed by scanning electron microscope (SEM; S-2500C Hitachi, Japan) and staining with 4,6-diamidino-2-phenylindole (DAPI) after 12, 24, and 72 h culture, respectively. The extent of cell proliferation was assessed using WST-1 assay. The amount of an orange colored product (formazan), soluble in the culture medium is directly proportional to the number of living cells. Once 10 μL WST-1 solution was added to each well and incubated for 4 h, this medium was then transferred to 96-well plate and the absorbance at 450 nm was read.

***In Vivo* Animal Test.** For animal test of the surface-modified porous PE implants, white female albino New Zealand rabbits, each weighing about 3 kg, were used. Under sterile surgical procedure, they were subjected to unilateral incisions on the right abdominal wall along the midline for the insertion of the PE spherical implants between the abdominal muscle layers. Rabbits were anesthetized with intramuscular injections of ketamine (25 mg/kg body weight) into their thighs. When PE control, PE-*g*-PAA, and PE-*g*-RGD were implanted one at a time in a cephalad to caudad position, the overlying muscle layer and skin were closed in separate layers using 4-0 Vicryl sutures. Rabbits were injected locally with 1% lidocaine-epinephrine (1 : 100,000). To block a possible infection, gentamicin sulfate (5 mg/kg/day) was also injected intramuscularly into the thigh before the surgery and for the first 5 days after surgery. After 1, 2, and 3 weeks, postoperatively, implants were harvested from three rabbits per each group of three differently modified implants.

Histology and Immunostaining. The retrieved implants were fixed in 10% formalin solution, embedded in paraffin block, and sectioned in 5 μm thickness. After the sections were stained with hematoxylin and eosin (H&E), they were scanned (Scanjet 4070C, HP, CA, USA) to produce a digital image. A program, ImageJ 1.32j (NIH, MD, USA) was used to calculate the percentage of the cross-sectional area of fibrovascular ingrowth of each porous PE implant at different time points. Sections were also immunostained with CD-31, an endothelial cell marker. Immunostaining was performed using a labeled streptavidin biotin kit (DAKO, Glostrup, Denmark). Anti CD-31 antibody (DAKO, Glostrup, Denmark) was diluted at 1 : 20, and Mayer's hematoxylin was used as a counterstain. The expression of CD-31 was evaluated with the sections at 1 mm below the surface of the PE implant. The number of capillaries expressing CD-31 was counted and the results were averaged.

Results and Discussion

Ocular implants are useful in the replacement of the volume loss after enucleation or evisceration. They are also valuable to improve a cosmetic and psychological rehabilitation for ophthalmic patients. Many biomaterials have been utilized for this purpose, beginning with the application of the hollow glass sphere in 1884 by Mules. Since then, a variety of materials have been developed, i.e., PE, HA, and aluminum oxide. Many studies have revealed that fibrovascular ingrowth into porous anophthalmic orbital implants should be facilitated from the surrounding tissues for successful clinical performance.[3,14,28] The integration between host tissue and implant significantly reduces the chance of postoperative complications, i.e., infection, migration, or extrusion. Therefore, building specific surface properties that would elicit initiation and propagation of fibrovascularization are of importance. This work presented application of surface modification techniques to porous PE implants, resulting in the grafting of AA by plasma treatment and immobilization of RGD peptide. Through the plasma treatment using Ar gas and then *in situ* AA grafting, the hydrophilic functional group could be adopted onto the PE surface and as a result, hydrophilicity obviously improved. Plasma treatment is recognized an effective way of modifying surface in the biomedical research. With appropriate monomers, it can change surface characteristics, such as wettability (hydrophilicity/hydrophobicity), biocompatibility, metal adhesion, dyeability, refractive index, hardness, chemical inertness, and lubricity.[29] Plasma is typically obtained when gases are excited into higher energy states using radiofrequency or microwave radiation, or electrons from a hot filament discharge. High-densities of ionized and excited species change the surface properties of inert materials, without altering their bulk properties.

While the current processes showed little visible changes, the water contact angle of the surface significantly decreased from 105 (PE control) to 50 (PE-*g*-PAA) to 60 degrees (PE-*g*-RGD) after *in situ* AA grafting and further RGD immobilization (Table I). The contact angle is considered a sensitive probe of changes in surface properties, since the angle is largely determined by the nature of surface layer. The degree of hydrophilicity was considerably influenced by varying some parameters of plasma treatment and reaction condition. When the PE spheres were dipped in normal saline solution, half the volume of PE control was exposed to the surface but nearly entire body of PE spheres grafted with either AA or RGD peptides were sank below the surface. It was interesting that water contact angle significantly

Table I. Surface Characteristics of Various PE Film Surfaces

Analysis / Sample	ESCA (atomic %)			Contact Angle (degree)	-COOH Content (mmol/cm^2)
	C_{1s}	O_{1s}	N_{1s}		
PE Control	99.5	0.5	-	105 ± 2.4	-
PE-*g*-PAA	96.5	3.47	-	50 ± 2.1	5.81×10^{-3}
PE-*g*-RGD	95.3	4.11	0.55	60 ± 1.8	-

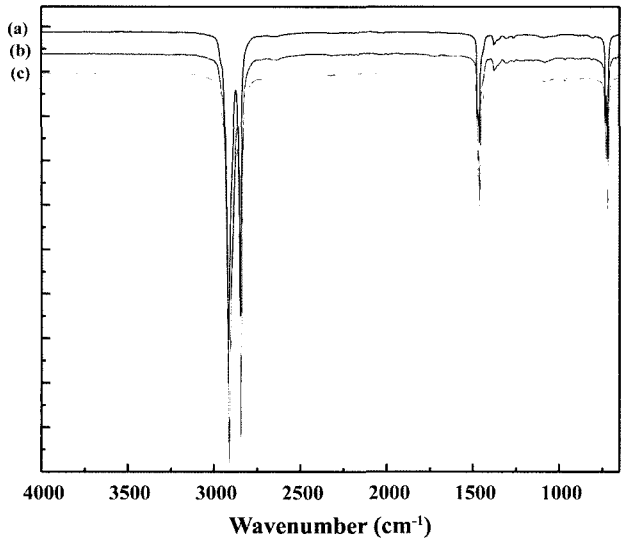

Figure 1. ATR-FTIR spectra for (a) PE control, (b) PE-*g*-PAA, and (c) PE-*g*-RGD films.

decreased after Ar plasma treatment alone without the AA grafting. However, after two weeks, the angle returned back to the original level before the treatment. It seemed that though hydrophilic components were initially exposed on the surface, they might be unstable and thus have a short half-life. When the content of the grafted AA in PE films was determined by the colorimetric method of Toluidine Blue O assay, the concentration of carboxylic acid was 5.81 $\times 10^{-3}$ mmol/cm^2 in the PE-*g*-PAA. No trace of carboxylic acid was found in the untreated PE control. From the results of ATR-FTIR, the specific peak at 1750 cm^{-1}, an indicative of carboxylic acid was barely distinguishable among three different specimens (Figure 1). However, the altered surface chemical compositions were further identified by ESCA survey scans (Table I). PE control, PE-*g*-PAA, and PE-*g*-RGD showed three unique peaks, corresponding to C_{1s} (binding energy, 285 eV), N_{1s} (399 eV), and O_{1s} (532 eV). The modified PE surfaces reserved higher atomic percent of oxygen than the PE control. The atomic percent of oxygen content (0.5%) on the PE control changed after AA grafting (3.47%) and then RGD immobilization (4.11%). In addition, the nitrogen peak was only spotted on the PE-*g*-RGD surface, indicating the presence of the immobilized RGD peptide.

Along with the improved hydrophilicity, positive effect of

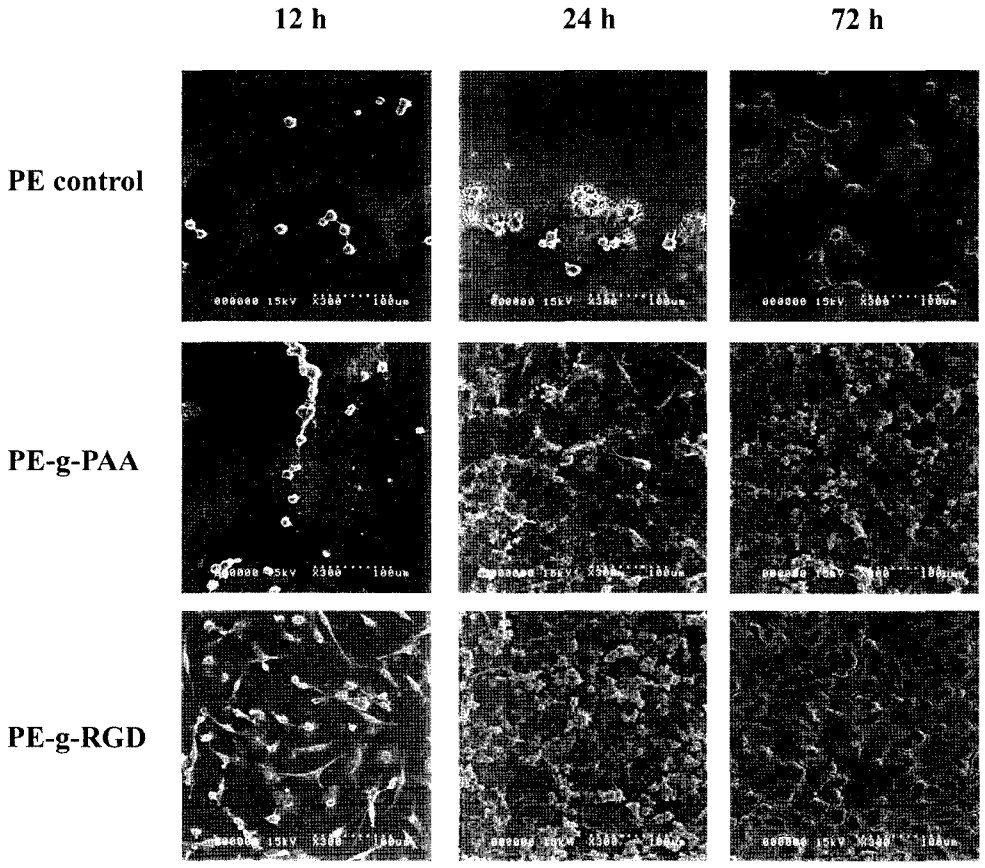

Figure 2. SEM observation of fibroblast adhesion to different surfaces of PE control, PE-*g*-PAA, and PE-*g*-RGD films at 12, 24, and 72 h, respectively.

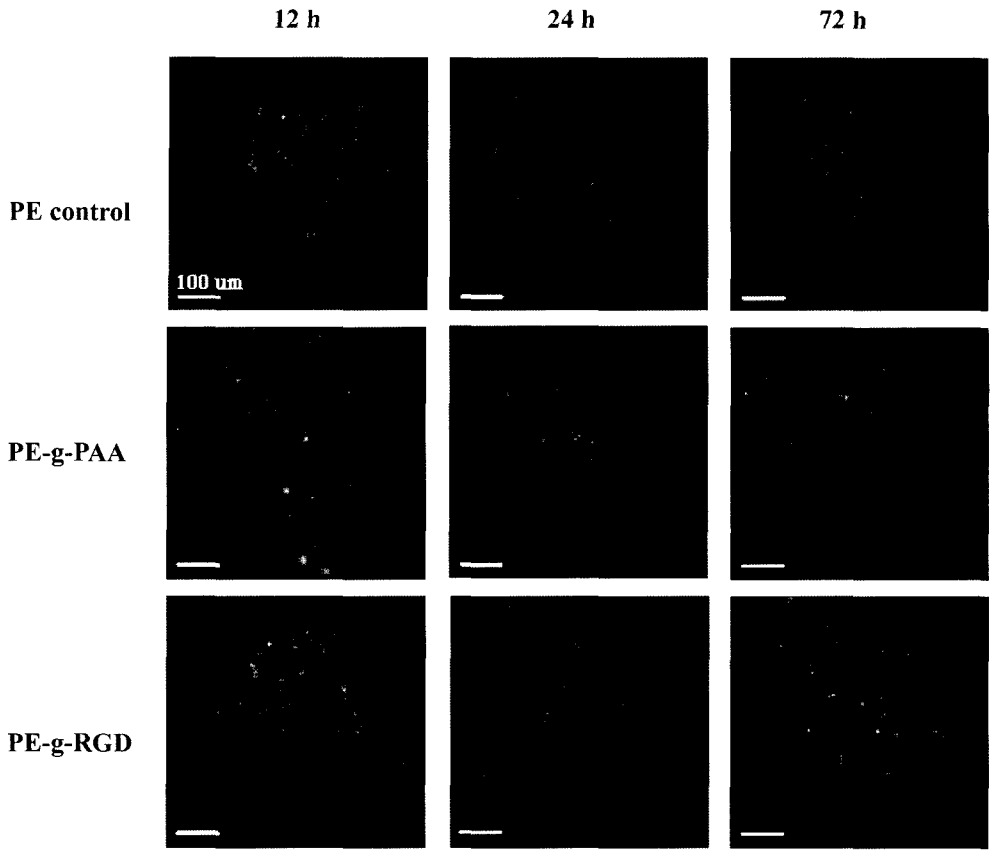

Figure 3. DAPI staining of fibroblast adhesion to different surfaces of PE control, PE-*g*-PAA, and PE-*g*-RGD films at 12, 24, and 72 h, respectively.

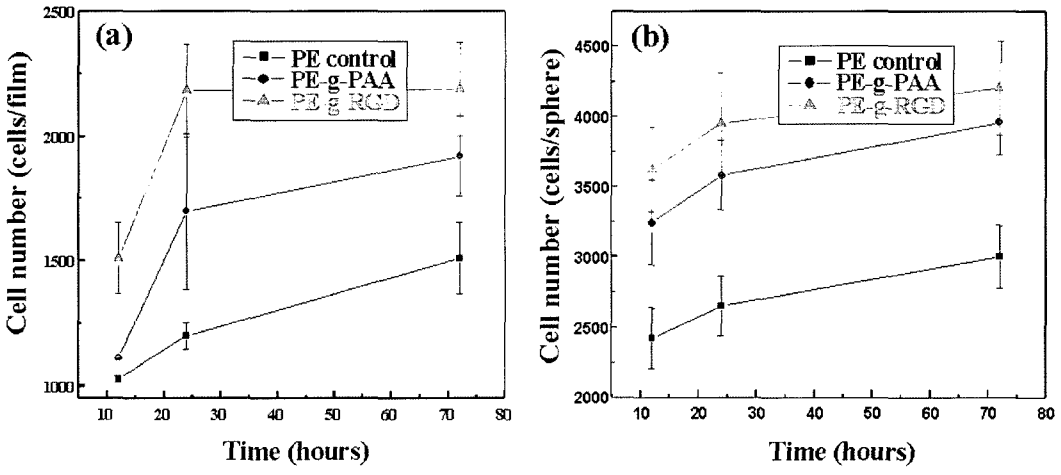

Figure 4. Quantitative measurement of fibroblast cellularity on (a) PE films and (b) PE spheres at 12, 24, and 72 h, respectively.

the surface modifications was noticed during fibroblast culture, in which PE-*g*-RGD film was especially better in cell adhesion and proliferation than PE control (Figure 2). As shown in the SEM images, fibroblasts were actively proliferating on the modified PE surface with time. DAPI staining also presented the similar result (Figure 3). Quantitative measurement of the cell numbers, WST-1 assay supported the results of SEM and DAPI staining, with much higher cellularity in the PE-*g*-RGD film, followed by PE-*g*-PAA and PE control (Figure 4). Fibroblast adhesion, spreading, and growth were notably improved with moderately hydrophilized poly(L-lactide-*co*-glycolide) (PLGA) surfaces with

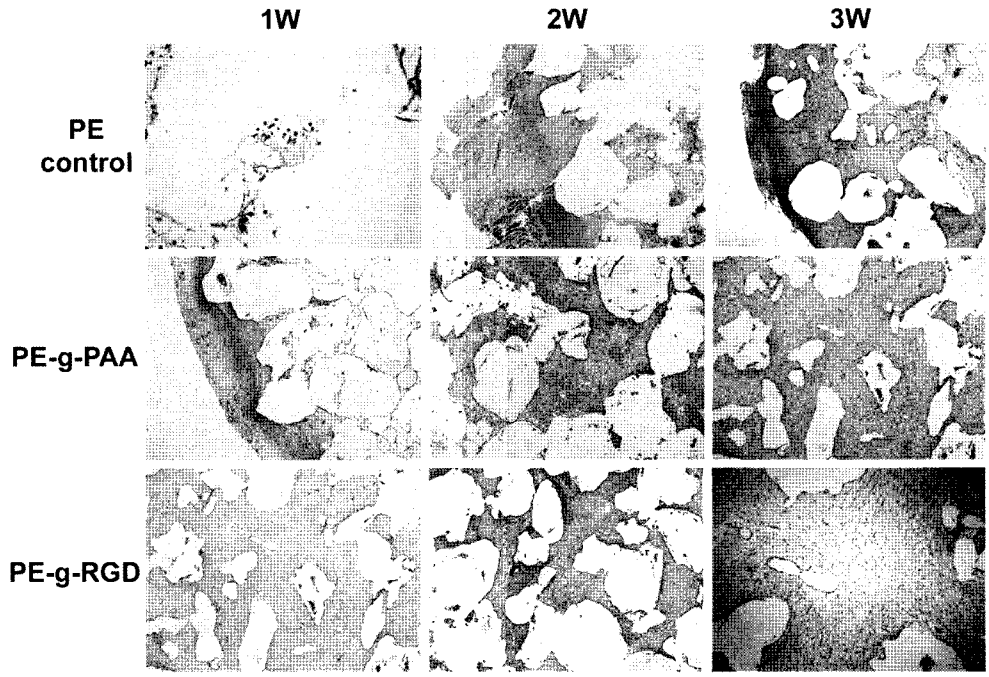

Figure 5. H&E staining of the retrieved porous PE implants of PE control, PE-*g*-PAA, and PE-*g*-RGD, respectively. Each was implanted *in vivo* for 1, 2, and 3 weeks, respectively (× 200).

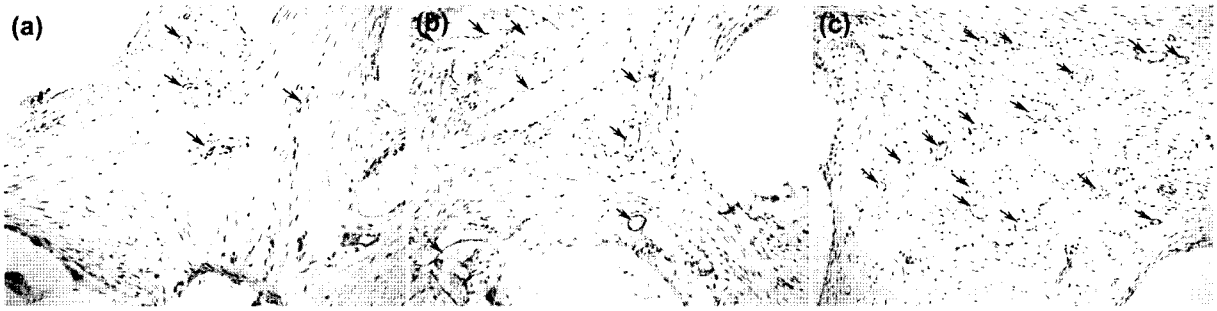

Figure 6. Immunostaining of CD-31: (a) PE control, (b) PE-*g*-PAA, and (c) PE-*g*-RGD. Upon the positive staining of endothelial cells, capillary vessels are marked in arrows (× 200).

water contact angle of 55 degrees.[30] It is also found that more endothelial cells were attached to the regions of moderate hydrophilicity in the corona discharge treated PE and maximum adhesion was observed at the contact angles of mid 50s.[31] Moreover, the immobilized RGD peptide appeared to have contributed to the improvement of cell attachment and expansion, as documented in the early studies.[32,33]

From *in vivo* rabbit test of PE implants, H&E staining of the retrieved specimens at 1 week displayed a budding of new fibrous tissue at the periphery of porous PE implant (Figure 5). As compared to PE control and PE-*g*-PAA, significantly improved formations of dense collagen and fibrous capsule were observed with time in the PE-*g*-RGD sphere. The areas of fibrovascular ingrowth into porous PE spheres were negligible in all the groups at 1 week, postoperatively. However, fibrovascularization was substantially progressed after 2 and 3 weeks of implantation: 15, 20, and 24% at 2 weeks and 42, 65, and 73% at 3 weeks for PE control, PE-*g*-PAA, and PE-*g*-RGD, respectively. As the role of RGD was indicated in the cell attachment on the film, the PE-*g*-RGD sphere implants could lead to the greatest fibrovascularization *in vivo*. The number of CD-31 positive staining was proportional to the cells adhered to the PE specimens (Figure 6). CD-31 is a platelet endothelial cell adhesion molecule of the IgG family, and has been a sensitive marker for vascular endothelial cells specifically, but not for lymphatic endothelium.[34] This marker is supposed to reveal the cellularity as well as distribution of endothelial cells in the fibrovascularized tissue grown on the implant. The numbers of CD-31 positive vessels were found to be significantly greater in the AA-grafted or RGD-immobilized PE implants, especially more in the PE-*g*-RGD.

Conclusions

This study demonstrated that inert, hydrophobic porous PE sphere could be successfully surface-modified with AA grafting and immobilization of RGD peptide. This was confirmed from various surface analyses, such as ESCA, measurement of contact angle, -COOH, and RGD concentrations. The surface-modified PE sphere implants, especially PE-*g*-RGD exhibited much better performance of fibroblast attachment and proliferation *in vitro* than PE control. *In vivo* fibrovascular ingrowth and CD-31 expression of the surface-modified PE implants also supported the same results as witnessed *in vitro*. The present surface modification methods may thus be useful and effective in reducing the rate of extrusion and infection of porous PE anophthalmic socket implants.

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