Construction of Cell-Compatible Layer and Culture of Human Umbilical Vascular Endothelial Cells on Porous Polystyrene Membranes

CHANGYOU GAO, AN LI, XIAOSU YI, JIACONG SHEN

Department of Polymer Science and Engineering, Zhejiang University, Hangzhou 310027, People's Republic of China

Received 10 May 2000; accepted 12 August 2000

ABSTRACT: Porous polystyrene (PS) membranes with pore sizes ranged from nanometer scale to several decades' micrometers were fabricated by the thermally induced phase-separation technique, and were characterized by scanning electron microscopy (SEM). Oxidation of PS membranes by concentrated sulfuric acid induced a decrease of the carbon amount on the membrane surfaces accompanying with an increase of the oxygen amount. The hydrophilic surfaces thus constructed proved quite effective to promote endothelial cell adhesion and growth. After culturing for 4 days, a confluent endothelial cells (ECs) layer was observed on nonporous PS membrane treated previously with sulfuric acid for 15 min at 28.5°C. The observation under SEM showed that ECs on the PS membrane surfaces at the fourth day were inversely proportional to the pore size: the smaller the pore size, the larger the amount of ECs on the membrane surfaces. It is supposed that the existence of pores on the membrane surface might generate some disadvantageous resistance to the spreading of cells. © 2001 John Wiley & Sons, Inc. J Appl Polym Sci 81: 3523–3529, 2001

Key words: endothelial cell; tissue engineering; porous polystyrene membrane; thermally induced phase separation

INTRODUCTION

During the last 30 years, much evidence has developed that cultured cells can recognize and respond to substratum surface structure.[1–6] The topography of a material such as grooves/ridges, fibers, cylinders, pores, etc., either deliberately or by accident, has effects on cell adhesion, orientation, movement, growth, and activation,[4,7,8] among which the orientation of cells to topography is one important response, and is frequently studied.[1,2,4–6] Harrison, the first one to detect the importance of substrate topography, cultured various tissues on spider webs and found that cells migrated along the web filaments.[9] This was corroborated by Loeb and Fleisher as well as Weiss.[10,11] Weiss later introduced the term "contact guidance" to describe the cellular orientation along the fibers.[12] Contact guidance on grooved substrata was also observed and demonstrated by Rovensky, Dunn, and some other researchers in several cell types, for example, fibroblasts, epithelial cells, neurons, and macrophages.[1,2] On the other hand, the different effects of topographical materials on cells are considered as one important reason leading to different tissue responses in the macroscopic level, for example, encapsulation, induction of carcinoma, and chronic inflammation of implanted prostheses, even if they

* *Correspondence to:* C. Gao; e-mail: cygao@mail.hz.zj.cn.

Contract grant sponsor: Natural Science Foundation of China; contract grant number: 59803006.

Contract grant sponsor: Major State Basic Research Program of China; contract grant number: G199054305.

Contract grant sponsor: Natural Science Foundation of Zhejiang Province.

Journal of Applied Polymer Science, Vol. 81, 3523–3529 (2001)

are made of the same chemical material.[13] Porous polymer materials are increasingly interesting due to the wide applications as scaffolds for tissue regeneration such as cornea, skin, blood vessel, liver, and pancreas, as well as immune-isolating membrane, etc.[14] Both the surface chemistry and the surface pattern, for instance, pore size and pore distribution of these kind implanted polymer materials or devices have to be specially tailored to obtain suitable tissue compatibility either *in vitro* or *in vivo*.

After the fabrication of polymer regeneration scaffold with desired texture, the scaffold usually has to be further modified so that proper biological responses can be achieved. Several methods such as γ-ray irradiation, photo-oxidation, and grafting, plasma treatments already exist to introduce functional chemical groups like —OH, —SO_3H, —COOH, or —NH_2, etc. or cell growth factors like collagen, gelatin, fibronectin, etc., onto polymer surfaces.[15,16] Many results have proven that these groups or cell growth factors can promote cell adhesion and proliferation both *in vitro* and *in vivo*.[17,18] However, the effectiveness of these modification methods on the three-dimensional scaffolds is uncertain. The reason is that only the superficial surfaces can be exposed into the irradiation; it is imaginable that the reactions mainly occur on the superficial layers. Although in the scaffold bodies there are voids that enable the diffusion or permeation of modifiers, the chemical reactions hardly happen because of the lack of irradiation. In the present article, polystyrene (PS) was selected as the model material to primarily reveal the response of human umbilical vascular endothelial (HUVEC) to the surface porosity. A cell-compatible layer was constructed first through a convenient method by oxidizing PS membranes with concentrated sulfuric acid. By contrast, this method enables the homomodification of the entire porous polymer surfaces including the superficial surface and the surface in the main body. It was shown that the cell shape and proliferation rate of HUVEC onto PS membranes are related to the surface chemistry and porosity.

EXPERIMENTAL

Preparation of PS Membranes

Polystyrene (PS), with a molecular weight ranging from 200,000 to 300,000, was obtained from the Yanshan Chemical Company of China, and was purified twice by dissolving in chloroform and then precipitating in methanol. Nonporous (transparent) PS membrane was acquired by casting PS/chloroform solution onto glass plate at $\sim$ 25°C.

The thermally induced phase separation (TIPS) technique was employed to fabricate porous PS membranes. The process was already described before.[20] In brief, the purified PS was dissolved in cyclohexane in a glass container bearing a stainless steel membrane-forming platform as the cover at 50°C to form a uniform solution (0.1 g/mL), degassed, and inverted upside down to form a uniform solution layer on the stainless steel platform. Then the mold was put onto a brass cold plate that had been cooled to the desired temperature by liquid nitrogen. When the membrane was totally solidified the glass mold was removed and the stainless steel platform with the PS membrane was transferred into a desiccator to remove cyclohexane by freeze drying.

For the aim of the cell culture, the membranes were cut into round pieces with a diameter of 6 mm, which are slightly smaller than that of the 96-well culture plate (φ = 6.4 mm); for the aim of the surface modification study, the membranes were cut into 15 $\times$ 15 mm pieces.

Oxidation of PS Membranes with Concentrated Sulfuric Acid

Porous and nonporous PS membranes were treated by concentrated sulfuric acid at 28.5°C for various times as designed. After rinsing with water until neutral and incubated in 3-distilled water for 24 h, the membranes were transferred into ethanol and then dried at room temperature.

Endothelial Cells Culture

The oxidized membranes for cell culture were sterilized with 75% ethanol, and incubated twice in sterilized PBS for 24 h, then were transferred into a culture plate. The endothelial cells (ECs) were isolated from human umbilical cord veins of a new-born baby through digestion with 0.1% collagenase (type I, Sigma) for 15 to 20 min at $\sim$ 25°C, then were routinely cultured in the wells that were prelaid PS membranes, as well as in the wells of tissue culture polystyrene (TCPS) (Nunc, Denmark) as a control. The endothelial cells were maintained in a humidified atmosphere of 5% CO_2 in air at 37°C, in a serum-containing culture medium, which consisted of 20% little calf serum and 80% RPMI1640 (Gibco, containing L-glutamine), supplemented with 100 U/mL of penicillin and 100 μg/mL of streptomycin. After incuba-

tion for 24 h the culture medium was changed and then changed every 2 days. The cultured ECs were fixed with 2.5% glutaraldehyde for 30 min, and then stained the cell nuclei with Giemsa (The Third Chemical Co. of Shanghai, China) for 5 to 10 min.

Characterization

For the measurement of water wettability of modified PS membranes, nonporous PS membranes were employed and adhered onto glass substrates. The static contact angles (θ) of distilled water on the PS membrane surfaces were measured by the sessile drop method, employing a JY-82 apparatus. For each sample, 10 to 12 separate drops were examined, and the average contact angles were recorded.

XPS spectra were recorded on an ESCALAB Mark II Spectrometer by using $Al_{K\alpha}$ exciting radiation. The basic pressure in the measurement chamber was kept around 1×10^{-8} Torr. The value of 285.0 eV of the hydrocarbon C_{1S} core level was used as a calibration for the absolute energy scale. Overlapping peaks were resolved into their individual components by means of the software dedicated in the XPS Spectrometer.

The cultured ECs on various PS membranes were fixed and stained as described above. The PS membranes were taken off the culture plate and put onto glass slides to observe the morphology of cultured ECs by means of an Inverted Microscope (LOIC). For scanning electron microscope (SEM) observation, the PS membranes with or without cultured ECs were exposed to the vapor of gold in high vacuum, then were observed by StereoScan 600.

RESULTS AND DISCUSSION

Cells divide and proliferate *in vitro* only if they can adhere on a substratum with proper surface properties like wettability and charge. The hydrophobicity or poor wettability of unmodified PS is disadvantageous for cell adhesion and growth.[15,16] Therefore, surface modification of the PS membrane to construct a cell-compatible layer previously is required.[19] Concentrated sulfuric acid was used as an oxidant to oxidize PS membranes. It has been already proven that some hydrophilic groups like hydroxyl, carbonyl, carboxylic, and sulfonate groups could be introduced onto the membrane surface.[21] These groups, on the one hand, could modify the poor wettability of the PS surface, and on the other hand, have the ability to accelerate cell adhesion and growth.[15,16,19]

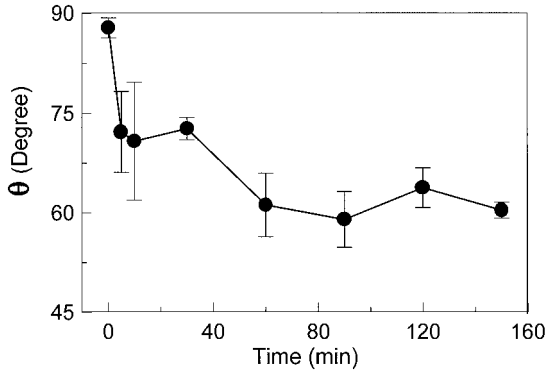

Figure 1 The water contact angles (θ) on nonporous polystyrene membrane surfaces as a function of the oxidation time in concentrated sulfuric acid.

The water contact angle (θ), as a function of oxidation time, is shown in Figure 1. The 90° contact angle demonstrates less wettable property of the unmodified PS surface. θ decreased rapidly to around 70° after oxidizing at 28.5°C for 5 min, and decreased further to 60° when oxidized for 60 min and then kept unchanged for up to 150 min. It was also observed that a uniform water layer was easily formed on the treated PS membrane. These results prove that concentrated sulfuric acid is quite effective to modify the PS membrane to improve its surface wettability.

The binding energy and relative element amounts from XPS measurement are listed in Table I. The total amounts of the carbon element on surfaces decreased while oxygen increased along with the prolonged oxidation time, while the introduction of the sulfonyl group became apparent only for the long-time oxidation. The peak-resolving results of C_{1S} demonstrate that only hydroxyl group was introduced onto the surface; no carbonyl or carboxylic groups could be detected from Figure 2. From the results in Figure 1 and Table I, one knows that the amount of hydroxyl group introduced onto the PS membrane surfaces is limited, but it is already large enough to modify the surface wettability.

ECs were separated and seeded on the PS membranes oxidized previously for various times at a density of 6.4×10^4 cells/cm^2, and were cultured for 4 days. The fixed and stained cells were observed under an inverted microscope. The results showed that ECs could adhere on the PS membrane surfaces either oxidized or not, but the cell amount and morphology were different, as shown in Figure 3. Cell adhesion and prolifera-

Table I The Binding Energy (eV), Peak-Resolving Data of C_{1S}, and Relative Element Amount (%) of PS Membranes Oxidized by Concentrated Sulfuric Acid for Different Times

T (min)	CH (%)	C—OH (%)	Total C_{1S} (%)	O_{1S} (%)	S_{2P} (%)
0	285.0 (85.2)	286.8 (9.7)	(94.9)	533.3 (5.1)	—
5	285.0 (80.6)	286.4 (14.1)	(94.7)	532.6 (4.7)	169.5 (0.6)
30	285.0 (79.6)	286.4 (14.3)	(93.9)	532.6 (5.7)	169.4 (0.4)
60	285.0 (81.9)	286.7 (10.9)	(92.9)	532.7 (6.4)	169.1 (0.7)
150	285.0 (77.4)	286.4 (14.5)	(91.9)	532.7 (7.1)	169.1 (1.0)

tion were found on the unmodified PS membrane, but most of the cells were observed as polygonal shapes with pseudopods attached to the membrane surface [Fig. 3(a)]. Together with the few numbers of cells and the lack of cytoplasm, the results suggested that the unmodified PS membrane are no benefit for ECs growth, probably due to the fact that adhesion between cells and substratum is not proper. Thus, it could be concluded that the unmodified PS membrane is disadvantageous for supporting ECs growth. The PS membranes oxidized for 5 to 60 min, however, represented a strong ability to facilitate ECs growth, as shown in Figure 3(b). A large number of ECs were obtained after 7 days of culture. They aligned as an elongated shape with abundant cytoplasm, and a confluent cells layer had formed on some areas. Through comparison of Figure 3(a) and (b), one can easily conclude that oxidized membranes have a positive effect on acceleration of ECs growth. The cell culture results on PS membranes oxidized previously by sulfuric acid for over 60 min showed that these surfaces are disadvantageous for ECs growth (images were not shown). The shrunken cell morphology and the smaller cell volumes suggested that necrosis or deterioration probably had occurred during the cell culture process. It is not well understood so far, probably because some nondetectable groups that are harmful to ECs were introduced with oxidation for over 60 min at 28.5°C. The same result was acquired when the experiment was repeated with a seeding density of 9.6×10^4 cells/cm^2 and culturing time of 5 days. The result shows that the

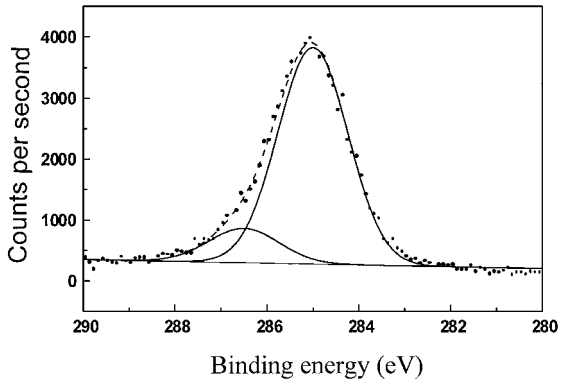

Figure 2 Peak-resolving of C_{1S} of PS membrane oxidized by concentrated sulfuric acid for 150 min. The black dot (●) indicates the experimental data, and the dashed line (—) is the theoretical curve.

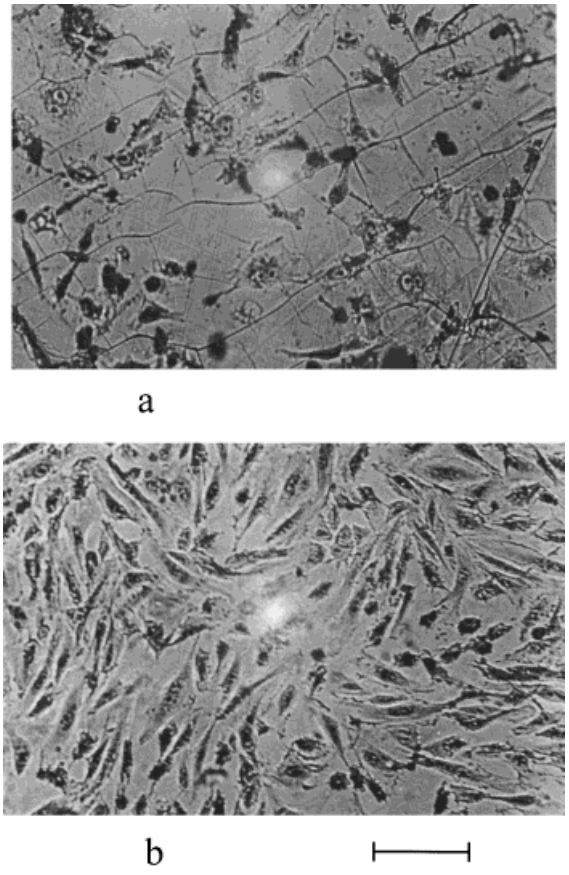

Figure 3 Inverted microscope photographs of ECs cultured on nonporous PS membranes on the fourth day: (a) control, (b) oxidized by concentrated sulfuric acid for 15 min at 28.5°C. Original seeding density is 6.4×10^4 cells/cm^2. Scale bar 100 μm.

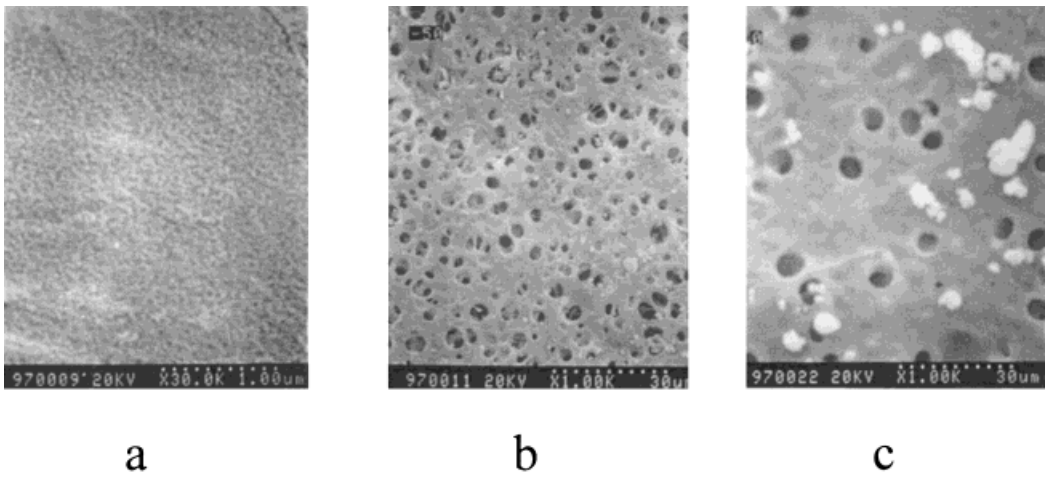

Figure 4 Scanning electron microscope images of porous PS membranes prepared by the thermally induced phase separation technique. (a), (b) bottom (PS-50B) and top (PS-50T) surfaces of membranes obtained on a −50°C cold plate, respectively; and (c) top surface (PS-0T) obtained on a 0°C cold plate.

hydroxyl groups, the main groups introduced during the oxidation for 60 min, are beneficial for ECs growth. The effectiveness of hydroxyl groups on acceleration of ECs growth was further confirmed by grafting of 2-hydroxylethyl acrylate onto a polyetherurethane membrane surface.[17]

The surface morphology of the PS membranes employed for the ECs culture is shown in Figure 4. Figure 4(a)–(c) represent porous PS membranes obtained on −50°C (bottom surface), −50°C (top surface), and 0°C (top surface) coldplates with mean pore sizes of several decades of nanometers, 2.7 ± 0.1 μm and 5.6 ± 0.2 μm in diameter, and were named as PS-50B, PS-50T, and PS-0T, respectively. It has to be indicated that there existed some larger pores with a diameter of 40–50 μm homogeneously distributed on the PS-0T surface (images were not shown). Spinodal decomposition in TIPS results in bicontinuous phases, the polymer-rich phase, and the polymer-poor phase. Upon solvent removal either by freeze drying or solvent extracting, the polymer-rich phase forms a continuous porous membrane with the homogenous surface morphology. At a fixed polymer concentration, the pore size of the polymer membrane thus obtained is inversely proportional to the quenching rate; the higher the quenching rate, the smaller the pore size. Due to the longer coarsening time of the top surface, the pore size on the top surface is usually larger than the bottom surface, which directly contacts the stainless steel platform.[15]

According to the above results, the porous PS membranes fabricated by the TIPS technique were treated by concentrated sulfuric acid at 28.5°C for 15 min. ECs were then seeded on these porous membranes at a density of 2×10^5 cells/cm^2, and were cultured for 4 days. The direct observation of the ECs morphology cultured on various substrata, revealed that the surface morphology of PS membranes, mainly the pore size, did have an influence on ECs proliferation rate, as could be inferred from the SEM images shown in Figure 5.

The ECs found on the PS membrane surfaces on the fourth day were inversely proportional to the pore size; the smaller the pore size, the larger amount of ECs existed on the membrane surfaces. As a control, the modified nonporous PS membrane [Fig. 5(a) and (b)] showed a comparable result on supporting ECs growth as TCPS that further confirmed the effectiveness of the surface modification technique. The mono-EC could not be distinguished from the SEM images. A confluent endothelial monolayer had formed. The inverted microscope observation revealed that the entire substratum was covered by the cobblestone-like ECs with smaller cell size. When ECs were cultured on a porous PS membrane with a pore size in nano scale, for example, on PS-50B, a slightly less form of the confluent cell layer was observed, as shown in Figure 5(c) and (d). With still lager pores, for example, in the case of PS-50T and PS-0T, it was found that the porous membrane surfaces were not completely covered by the cultured ECs, as shown in Figure 5(e)–(h). The existence of cell colonies implied that the cell growth rate should be lower on these porous sur-

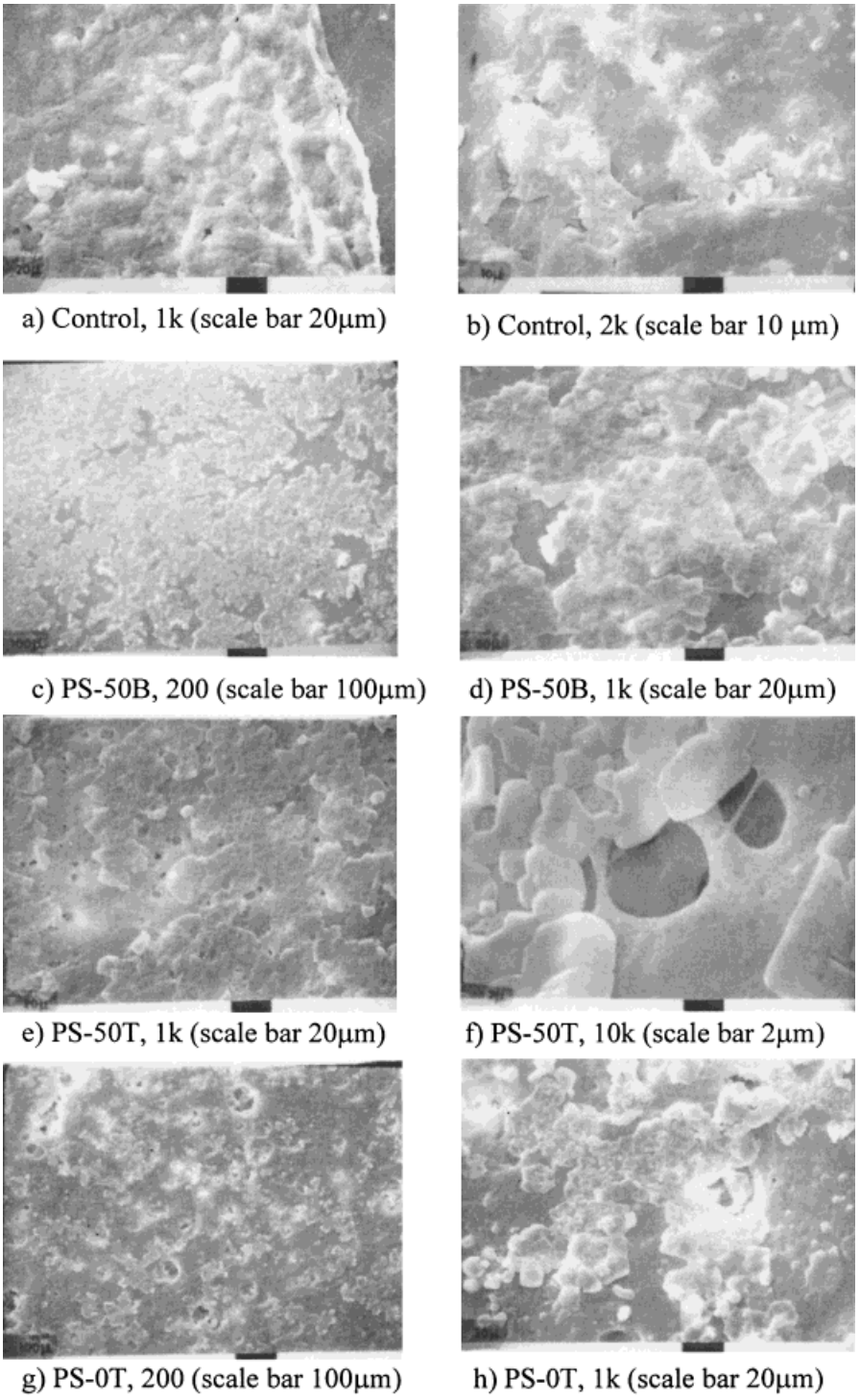

Figure 5 Scanning electron microscope images of ECs cultured on PS membranes with variable pore sizes as shown in Figure 4, which were previously treated by sulfuric acid for 15 min at 28.5°C. The original seeding density is 2×10^5 cells/cm^2. Images were taken on the fourth day.

faces. It is worth noting that ECs could also grow over a pore that is comparable with or larger than the cell size, as could be seen from Figure 5(f) and Figure 5(h), respectively.

The reasonable explanation would be that the existence of pores on the surfaces generated some disadvantageous resistance to the spreading of cells. It could be imagined that cells adhered and divided on the plane area between the pores initially. When they divided and spread towards the outside, it was unavoidable to encounter the pores, as it is known already that ECs can proliferate *in vitro* only if they adhere on a substratum. Yet the area occupied by a pore is empty, and there is nothing to support the cells to continue spreading outside. In the case that the pore is large enough, for example, comparable with the cell size as shown in Figure 5(f), the cells on the edge of the pore may stop dividing; thus, the proliferation rate would be slowed down. The covering of a pore by ECs, which is far larger than EC, for example, the 40–50 μm in diameter in Figure 5(h), probably was achieved by cell spreading from around the area to the pore center. The thermally induced phase separation process usually does not generate penetrative pores with sharp edges; the pores often slant into the membrane body, and there are filaments passing through the pores [Fig. 4(b)]. In this special case, the pore is more like a "pit" than a "hole" [Fig. 4(c)]. The slanting area on the edge of the pore could act as a supporting "bridge" that induces the cells to grow over the pore to form a concave cell colony shape, as shown in Figure 5(h). The sediment of some substance like proteins in the culture medium into the pore may further facilitate this spreading process.

CONCLUSIONS

Concentrated sulfuric acid oxidation was proven to be effective to introduce hydroxyl groups onto PS membrane surfaces, which resulted in a steady decreasing of water contact angle along with the oxidation time. The PS membranes treated at 28.5°C for 5 to 60 min displayed cell compatible property and could be used as substrata to support endothelial cell growth. PS membranes with various pore sizes ranged from nonporous to several decades' micrometers, which were fabricated by thermally induced phase separation, were employed to study the cell response to the surface morphology *in vitro*. It was observed that ECs on the PS membrane surfaces at the fourth day were inversely proportional to the pore size; the smaller the pore size, the larger amount of ECs existing on the membrane surfaces. It is supposed that the existence of pores on membrane surface might generate some disadvantageous resistance to the spreading of cells.[23]

This project was supported by the Natural Science Foundation of China (59803006), the Major State Basic Research Program of China (G1999054305), and the Natural Science Foundation of Zhejiang Province.

REFERENCES

1. Rovensky, Y. A.; Slavnaja, I. L.; Vasiliev, J. M. Exp Cell Res 1971, 65, 193.
2. Dunn, G. A.; Brown, A. F. J Cell Sci 1986, 83, 313.
3. Meyle, J.; Wolburg, H.; von Recum, A. F. J Biomater Appl 1993, 7, 362.
4. Curtis, A.; Wiliinson, C. Biomaterials 1997, 18, 1573.
5. Brunette, D. M. Exp Cell Res 1986, 164, 11.
6. Clark, P.; Connolly, P.; Curtis, A. S. G.; Dow, J. A. T.; Wilkinson, C. D. W. Development 1990, 108, 635.
7. Curtis, A. S. G.; Clark, P. Crit Rev Biocomp 1992, 5, 343.
8. Richter, E.; Fuhr, G.; Müller, T.; Shirley, S.; Rogaschewski, S.; Reimer, K.; Dell, C. J Mater Sci Mater Med 1986, 7, 85.
9. Harrison, R. G. Science 1911, 34, 279.
10. Loeb, L.; Fleisher, M. S. J Med Res 1917, 37, 75.
11. Weiss, P. J Exp Zool 1934, 68, 393.
12. Weiss, P. J Exp Zool 1945, 100, 353.
13. Ratner, B. D. Trends Polym Sci 1994, 2, 402.
14. Langer, R.; Vacanti, J. P. Science 1993, 260, 920.
15. Hoffman, A. S. Adv Polym Sci 1984, 57, 141.
16. Ikada, Y. Radial Phys Chem 1992, 39, 509.
17. Lee, J. H.; Park, J. W.; Lee, H. B. Biomaterials 1991, 12, 443.
18. Bruck, S. D. Int J Artif Organs 1990, 13, 469.
19. Klein-Soyer, C.; Hemmendinger, S.; Cazenave, J.-P. Biomaterials 1989, 10, 85.
20. Gao, C. Y.; Li, A.; Feng, L. X.; Yi, X. S.; Shen, J. C. Polym Int 2000, 49, 323.
21. Gao, C. Y.; Zheng, D. F.; Yang, B.; Wei, Q.; Shen, J. C. Chem J Chin Univ 1995, 16, 147.
22. Guan, J. J.; Gao, C. Y.; Feng, L. X.; Shen, J. C. J Biomater Sci Polym Ed, 2000, 11, 523.
23. Maroi, Y.; Sigot-Luizard, M. F.; Guidoin, R. ASAIO J 1999, 45, 272.