Macromolecular Research, Vol. 10, No. 2, pp 67-74 (2002)

Preparation and Characterization of New Immunoprotecting Membrane Coated with Amphiphilic Multiblock Copolymer†

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Received Dec. 18, 2001 ; Revised Feb. 27, 2002

Abstract : New immunoprotecting membranes were prepared by spin coating the amphiphilic random multiblock copolymers of poly(ethylene glycol) (PEG) and poly(tetramethylene ether glycol) (PTMEG) or poly(dimethyl siloxane) (PDMS) on porous Durapore® membrane. The copolymer coating was intended to make a biocompatible, immunoprotecting diffusional barrier and the supporting porous substrate was for mechanical stability and processability. By filling Durapore® membrane pores with water, the penetration of coating solution into the pores was minimized during the spin coating process. A single coating process produced a completely covered thin surface layer (~1 μm in thickness) on the porous substrate membrane. The permselectivity of the coated layer was influenced by PEG block length, polymer composition, and thickness of the coating layer. A composite membrane with the coating layer prepared with PEG 2 K/PTMEG 2 K block copolymer showed that its molecular weight cut-off at day 40 based on dextran was close to the molecular size of IgG (M_w = 150 kDa). However, IgG permeation was detected from protein permeation test, while glucose oxidase (M_w = 186 kDa) was not permeable through the coated membrane.

Keywords : immunoprotecting membrane, biohybrid artificial organ, amphiphilic multiblock copolymer, hydrogel coating layer, spin coating.

Introduction

To treat diseases such as diabetes,[1-4] chronic pain,[3,5] Parkinsons disease,[6,7] and liver failure,[8] biohybrid artificial organs or cell/tissue delivery systems have been proposed. In such systems, an immunoprotecting membrane physically protects the transplanted allogeneic or xenogeneic cells or tissues from the host immune system, particularly from humoral immune components, without using pharmacological immunosuppressants. The polymeric materials used for immunoprotection include acrylic copolymer,[2,3,9-11] AN 69 membrane,[12] alginate and poly(L-lysine) complex,[4] and agarose for either macro- or micro-encapsulation. In order to attain its objects, the membrane biocompatibility, chemical and mechanical stabilities, relevant permselectivity, and processability are prerequisites.[13] In permselectivity, solutes with low molecular weights (i.e. nutrients, metabolites, oxygen, and carbon dioxide) and the bioactive substances of interest (i.e. insulin, dopamine, parathyroid hormone and other bioactive proteins) must freely permeate through the immunoprotecting membrane, whereas antibodies, immunoglobulins, and immunoactive phagocytic cells must be blocked from permeation through the membrane.[14] Although various polymeric membranes have been applied for, the mechanical stability and biocompatibility of the conventional materials often caused implanted artificial organ to fail.

In this study, a new immunoprotecting membrane is proposed for improved mechanical stability and surface biocompatibility, which is composed of a porous ultrafiltration membrane as a substrate for mechanical support and a thin coating layer of amphiphilic multiblock copolymers with urethane linkages for flexible and biocompatible surface. We synthesized poly(ethylene glycol) (PEG)/poly(tetramethylene ether glycol) (PTMEG) or PEG/poly(dimethyl siloxane) (PDMS) multiblock copolymers. PEG[15,16] is a non-toxic and biocompatible polymer and PTMEG[17] and PDMS[18,19] have demonstrated biocompatibility with hydrolytic stability. Furthermore, these copolymers are expected to have appropriate mechanical stability, high elongation capacity, flexibility, and good biocompatibility due to general properties of polyurethanes.[20] In addition, the permselectivity and molecular weight cut-off (MWCO) can be controlled by

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varying PEG block length of the copolymers due to PEG domain in the polymer membrane as a transport channel of solutes.[21,22] The porous substrate membrane (Durapore® membrane) made of poly(vinylidene fluoride) has average pore size of 0.1 μm so that minimal resistance in the transport of cell products can be anticipated. Its good processability was reported because the polymers dissolve in various solvents and demonstrated heat sealability.[23]

Experimental

Preparation of Coating Materials. The coating materials of PEG/PTMEG and PEG/PDMS multiblock copolymers with urethane linkages (Figure 1) were synthesized as described elsewhere.[24] In brief, PEG (Aldrich; 10 mmol: M_n = 3,400 or 2,000) and PTMEG (Polysciences; 10 mmol: M_n = 2,000) or PDMS (ShinEtsu; 10 mmol: M_n = 1810) were dried *in vacuo* at 80 °C for 4 hrs in order to remove moisture and then dissolved in tetrahydrofuran (Junsei) (10 wt/vol%). Hexamethylene diisocynate (Aldrich; 20 mmol) was added in the solution and dibutyltin dilaurate (Sigma) as a catalyst was dropped. And then, the coupling reaction was proceeded under stirring for one day at room temperature (RT). The synthesized polymer was precipitated by slowly dropping the solution in *n*-hexane and filtrated. The polymer was washed in *n*-hexane and filtrated again. The polymer was further washed twice with distilled water in order to remove unreacted PEG. The final product was dried *in vacuo* at RT for 3 days. The unreacted PEG and PTMEG or PDMS did not appear in gel permeation chromatogram. The synthesized polymers were characterized by proton nuclear magnetic resonance spectroscopy (^{1}H NMR) and GPC.

To prepare coating solutions, the copolymers were dissolved in absolute ethanol (J. T. Baker). The coating solutions were filtrated through 0.45 μm filter in order to remove dust. The polymer concentrations used for coating were listed together with the results of polymer characterization in Table I.

Swelling Study. Swelling test was proceeded at 37 °C for 3 days in phosphate buffer saline (PBS) solution (pH 7.4). The circular specimens of the coating materials with a diameter of 12 mm were used. After soaking in PBS solution for 3 days, the weight-swelling ratio of the polymers was expressed as:

$$\text{Swelling ratio} = \frac{\text{Weight of swollen sample-Weight of dried sample}}{\text{Weight of dried sample}} \quad (1)$$

Preparation of Immunoprotecting Membrane. Before spin coating, Durapore® membranes (about 20 mm× 20 mm) were attached on a glass plate (25 mm× 25 mm) by

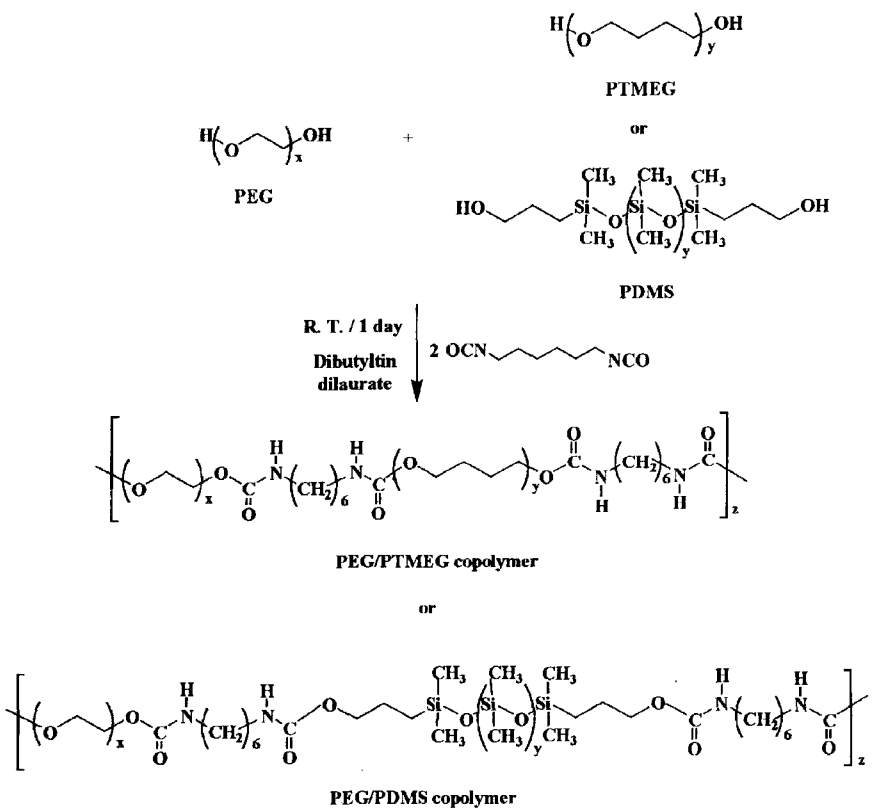


Figure 1. Synthetic schemes of PEG/PTMEG and PEG/PDMS multiblock copolymer with urethane linkages.

Table I. Characteristics of Amphiphilic Multiblock Copolymers Used in This Study

Polymer	Sample Code	M_w[a]	M_n[a]	PDI[c]	PEG wt%[d]	Block Ratio (PEG/PTMEG or PEG/PDMS)	Concentration of Coating Solution in Ethanol
PEG3400/ PTMEG2000	PE34PT20	58,400	46,300	1.26	57.97	0.95	7 wt/vol%
PEG2000/ PTMEG2000	PE20PT20	66,200	51,500	1.29	44.96	0.96	7 wt/vol%
PEG3400/ PDMS1810	PE34PD20	62,400	50,700	1.23	65.98	1.23	3 wt/vol%
PEG2000/ PDMS1810	PE20PD20	65,300	54,100	1.21	52.33	1.18	4 wt/vol%

[a]Weight average molecular weight (M_w) was measured by GPC.
[b]Number average molecular weight (M_n) was measured by GPC.
[c]PDI : Polydispersity index.
[d]PEG wt% was measured by ^{1}H NMR.

Scotch™ tape and were then soaked with distilled water for 3 hrs in order to minimize the penetration of polymer solution through the pores during the coating process. The wet membrane was placed on the chuck of a spin coater (Laurell: WS-400-6NPP/Lite), and a polymer solution of 300 μL was dropped on center of the membrane. It spun at 10,000 rpm for 30 seconds. The coated membrane was dried at RT for at least two days. In order to further remove the residual solvent, the coated membranes were dried again *in vacuo* at RT for one day.

Morphology Study. The surface and cross-sectional morphologies of Durapore® membrane and the coated membranes (PE20PT20-D, PE34PT20-D, PE20PD20-D, and PE34PD20-D) were examined by a scanning electron microscope (SEM; JEOL, JSM-5800). Before evaluation of surface morphology, the samples were dried *in vacuo* for one day. For preparation of cross-section of the coated membrane, it was frozen in liquid nitrogen and fractured. These samples were dried *in vacuo* for one day. The dried samples were gold-coated by a gold sputter coater (SPI supplier, SPI-MODULE™). Surface and cross-section morphologies were observed at 10 kV of electron velocity.

Permselectivity Study. The permeability test of Durapore® membrane and the coated membranes for fluorescein isothiocynate-labeled dextrans (FITC-dextrans) (Sigma; MW 12 kDa, 19.5 kDa, 42 kDa, and 77 kDa) was performed at 37 °C. Before setting the membrane between two diffusion half-cells, the membrane was soaked to swell in PBS (pH 7.4 at 37 °C) for three hours. After swelling, the membrane was clamped between the two diffusion half-cells with tight sealing. The diameter of the effective circular diffusional area was 1 cm. PBS (6 mL) was filled in each half-cell, and this set was maintained for three hours. Additional blank PBS (1 mL) was poured in the receiver cell and FITC-dextran solution (1 mL) was added in the donor cell. The initial concentration of FITC-dextrans was 1 mg/mL for Durapore® membrane and 4 mg/mL for the coated membranes. The both diffusion half-cells were stirred at 750 rpm to avoid boundary layer effect. At predetermined time intervals, the receiver-side solution of 0.1 mL was sampled, and then the same volume of fresh PBS solution was added in the receiver cell. The concentration of the samples was determined by a microplate reader (Molecular Devices: Spectra MAX 340) at an excitation wavelength of 485 nm and an emission wavelength of 515 nm. Permeability (P) was calculated by Equation (2).[25]

$$-\frac{V}{2A}\ln\left(1-\frac{2C_t}{C_o}\right) = Pt \tag{2}$$

where V is the volume of half-cell, A the effective area, t the time, C_t the concentration of permeated solute in receiver cell at time t, and C_0 the concentration of solute in donor cell at time 0.

Protein Permeation Study. The permeation test for FITC-immunoglobulin G (FITC-IgG, Sigma), FITC-insulin, FITC -bovine serum albumin (FITC-BSA), glucose oxidase (GOD, Sigma) was carried out by the same procedure as described before. Before testing, FITC-insulin and FITC-BSA were prepared by conjugating the proteins with FITC.[26] The concentration of FITC-proteins in the receiver cell was determined by a microplate reader at 485 and 515 nm. For GOD, the concentration was evaluated by UV/VIS spectrometer (Varian Cary 1E system) with He/Ne laser source at 280 nm and finally determined by gel electrophoresis (BioRad) with protein markers (molecular weights from 29 to 200 kDa, Gibco).

Results and Discussion

Swelling. For PEG/PTMEG or PEG/PDMS multiblock copolymer, the swelling increased with increasing PEG block length or PEG wt% (Table II). For the copolymers with the same PEG block length but different hydrophobic blocks,

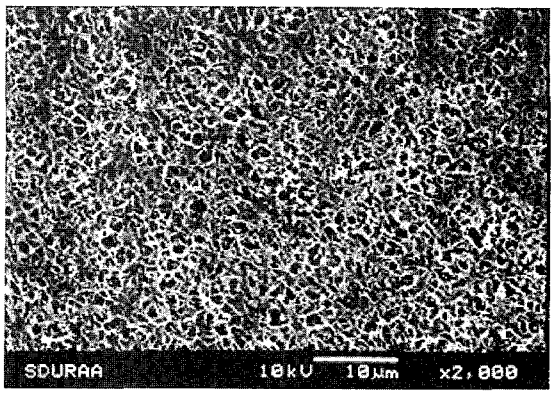
Table II. Swelling Ratio of the Coating Materials

Sample Code	Swelling Ratio
PE34PT20	2.77 ± 0.07 (n = 18)
PE20PT20	1.31 ± 0.04 (n = 27)
PE34PD20	2.14 ± 0.05 (n = 12)
PE20PD20	1.30 ± 0.02 (n = 19)

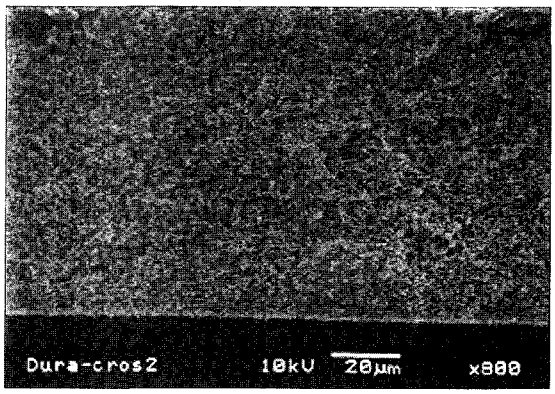
the swelling ratios of PEG/PTMEG multiblock copolymers were higher than those of PEG/PDMS multiblock copolymers because PTMEG possesses less hydrophobic nature than PDMS. However, in case of PE20PT20 and PE20 PD20, no significant difference in swelling ratio was found. In overall, PEG wt%, PEG block lengths and hydrophobic blocks influenced the swelling.

Morphology. The surface of Durapore® membrane showed porous structure with rough morphology and its cross-sectional morphology presented complex tortuosity between two surfaces of the membrane (Figure 2). However, the membranes coated with the multiblock copolymers demonstrated a smooth surface with partial penetration of the coating layer into the substrate pores (Figures 3 and 4). When we compare the published results, the surface morphology of the coated membranes seems smoother than those of membranes of PVA,[27] polyamide 4,6,[28] AN69,[29,30] and PU-PDMS,[31] the better biocompatibility for the membrane is expected.

Filling pores with water made the surface smooth and this produced fully covered surface with a single coating step.

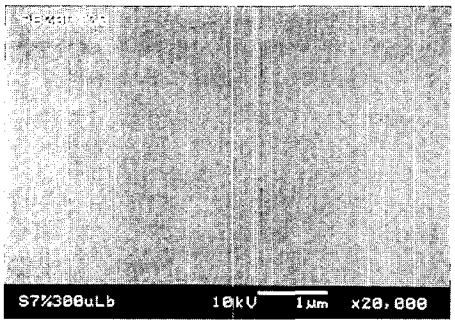


(a) Surface

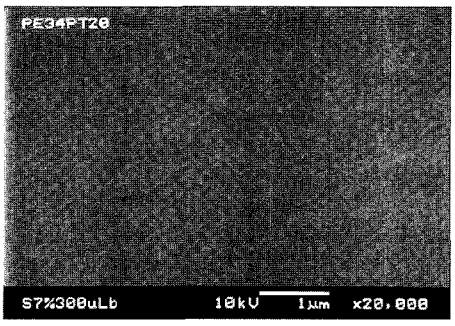


(b) Cross-section

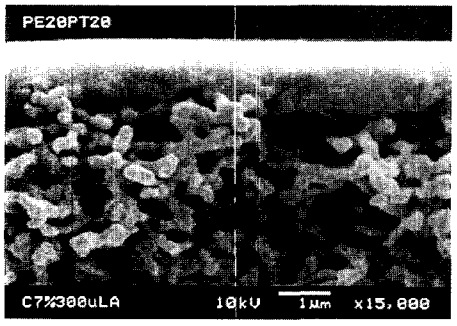
Figure 2. Surface and cross-sectional morphology of Durapore® membrane.



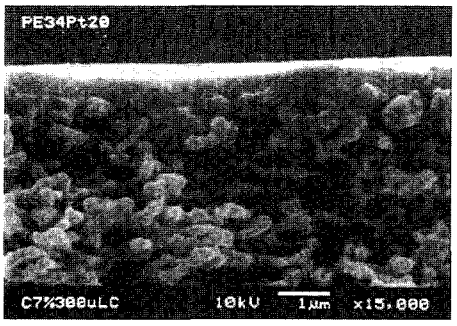
(a) Surface of PE20PT20-D



(b) Surface of PE34PT20-D

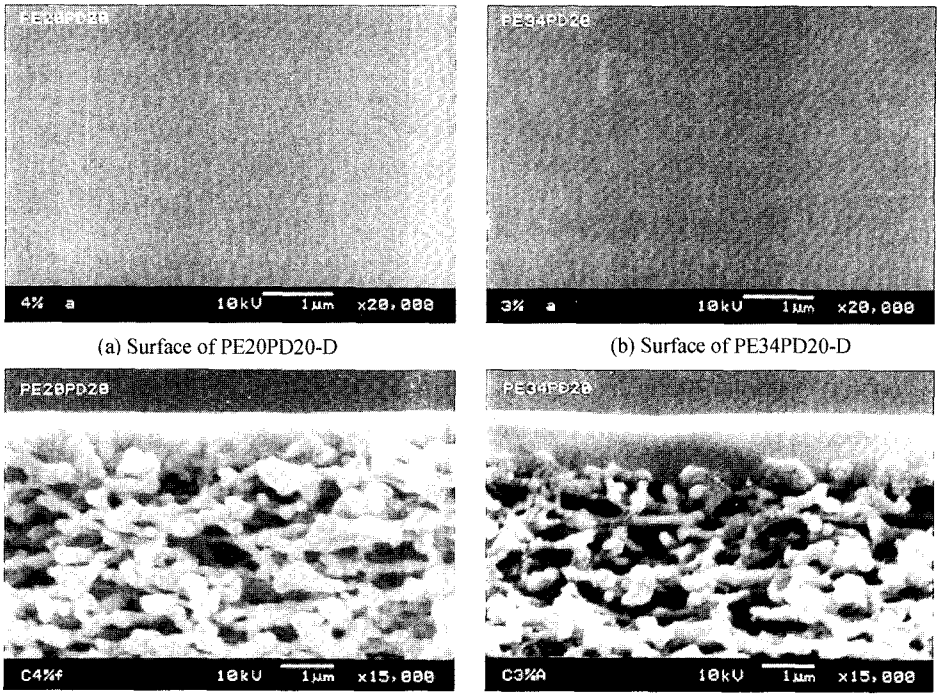


(c) Cross-section of PE20PT20-D



(d) Cross-section of PE34PT20-D

Figure 3. Surface and cross-sectional morphologies of coated membranes (PE20PT20-D and PE34PT20-D).



(a) Surface of PE20PD20-D (b) Surface of PE34PD20-D

(c) Cross-section of PE20PD20-D (d) Cross-section of PE34PD20-D

Figure 4. Surface and cross-sectional morphologies of coated membranes (PE20PD20-D and PE34PD20-D).

Without water filling, repeated coatings were required for the complete coverage of Durapore® surface. When the alcoholic polymer solution contacted the aqueous phase, the polymer gradually precipitated because the polymers were not soluble in pure water. This process may allow partial penetration of the solution into the water-filled pores and eventually cover the surface. From the cross-sectional morphology of the coated membranes, it was found that the thickness of the coating layer and the degree of penetration varied. This may be due to the differences in viscosity of the polymer solution. The thickness of the top coating layer (unpenetrated and penetrated portion), obtained from SEM pictures, in a dried state was 0.89 ± 0.04 μm (n = 9) for PE34PT20-D, 1.07 ± 0.09 μm (n = 9) for PE20PT20-D, 0.88 ± 0.06 μm (n = 6) for PE34PD20-D, and 0.66 ± 0.10 μm (n = 7) for PE20PD20-D. Although the coated membrane showed partially penetrated, interlocked structure between two membrane structures, the delamination of the coating layer from PE34PD20 and PE34PT20 was observed when these membranes were soaked in PBS for 10 days and exposed to weak shear stress. This may be caused by high swelling capacity of the coating materials. However, with moderate swelling of PE20PT20-D and PE20PD20-D, no delamination was found with the same treatment. Because the delamination of the coating layer from the immunoprotecting membrane will cause a failure of the membrane in the body, the physical locking mechanism by penetration is of importnace in this context.

Permselectivity. Permselectivity is one of the most important factors for an immunoprotecting membrane to prevent the transpanted cells or tissues in the body from humoral immune contact. At first, the permselectivity to the membranes was characterized with model solutes of FITC-dextrans with various molecular weights.

The permeabilities of FITC-dextrans to the coated membranes decreased with increasing the molecular weight of FITC-dextran (Table III). From a general rule, it is expected that the permeability increases with an increase of PEG block length because the PEG blocks or domains play a role as transport channels of water soluble solutes in the amphiphilic multiblock copolymers.[21,22] Although this general relationship holds for the copolymers containing PTMEG, it is intersting to note that the permeabilities of dextrans through PE20PD20-D was higher than those through PE34PD20-D. From this result, the permeability through the coated membrane may be influenced by complex factors of PEG block length, PEG wt%, the thickness in PBS solution, swelling ratio, the degree of penetration of the coating layer, and possible phase mixing effect. This could make one difficult to establish a proper relationship of membrane parameters with the permeability. In particular, the solid interlocking layer, unless degraded, is restricted from swelling by physicallly embeding in the rigid substrate pore structure, thus being a possible rate limiting in permeation. This restriction may also cause stress on one side of the top layer, limiting free swelling of the membranes.

Semipermeable membrane performance is characterized

Table III. Permeabilities of FITC-dextrans to Membranes

M_w of FITC Dextran (Da)	Permeability (cm/sec)				
	PE34PT20-D	PE20PT20-D	PE34PD20-D	PE20PD20-D	Durapore®
12,000	$6.00(\pm 1.25)\times 10^{-6}$ (n=4)	$4.15(\pm 0.32)\times 10^{-6}$ (n=3)	$2.46(\pm 0.17)\times 10^{-6}$ (n=6)	$2.36(\pm 0.19)\times 10^{-6}$ (n=6)	$3.60(\pm 0.23)\times 10^{-5}$ (n=5)
19,500	$3.84(\pm 0.28)\times 10^{-6}$ (n=4)	$1.30(\pm 0.67)\times 10^{-6}$ (n=6)	$9.33(\pm 0.71)\times 10^{-7}$ (n=6)	$1.19(\pm 0.63)\times 10^{-6}$ (n=5)	$2.82(\pm 0.23)\times 10^{-5}$ (n=3)
42,000	$5.59(\pm 0.96)\times 10^{-7}$ (n=4)	$3.25(\pm 1.34)\times 10^{-7}$ (n=4)	$3.49(\pm 0.98)\times 10^{-7}$ (n=5)	$3.75(\pm 0.38)\times 10^{-7}$ (n=6)	$1.17(\pm 0.35)\times 10^{-5}$ (n=5)
77,000	$2.22(\pm 0.46)\times 10^{-7}$ (n=4)	$2.15(\pm 0.82)\times 10^{-7}$ (n=4)	$2.69(\pm 0.45)\times 10^{-7}$ (n=3)	$3.14(\pm 1.10)\times 10^{-7}$ (n=6)	$9.10(\pm 2.99)\times 10^{-6}$ (n=6)

by the molecular weight at which 90% of the solute will be retained (prevented from permeating) by the membrane. This value is called the Molecular Weight Cut-Off (MWCO) and defined by Equation (3). In the diffusion study, because the amount of a solute in the receiver cell increases with time, MWCO is time-dependent. The estimated MWCOs based on dextran permeation of the coated membranes as a function of time were presented in Table IV. For immunoprotection, the permeability of IgG is of importance. Thus, the MWCO at day 20, 30, and 40 was evaluated from the permeabilities of FITC-dextrans of various molecular weights. The selected time intervals were based on the half-life of human IgG (20 days).[32] The complete loss of IgG activity is expected after about 40 days. For PTMEG containing copolymers, the MWCO of the coated membranes increased with increasing PEG block length, but not for PDMS containing copolymers due to previously discussed various factors. In the literature, it was reported that the molecular size of IgG is similar to that of 44 kDa dextran.[33] Thus, the extent of rejection of IgG to the membranes can be approximated from the rejection of 44 kDa dextran. Under this assumption, PE20PT20-D membrane as an immunoprotecting membrane would be appropriate because its MWCO for 44 kDa dextran at day 40 is close to the molecular size of IgG.

Table IV. MWCO of the Coated Membranes at Various Time Periods

	20 days	30 days	40 days
PE34PT20-D	44,500 Da	54,500 Da	68,000 Da
PE20PT20-D	31,000 Da	38,000 Da	45,000 Da
PE34PD20-D	26,500 Da	36,500 Da	57,000 Da
PE20PD20-D	28,500 Da	42,000 Da	> 77,000 Da

$$R=\frac{\text{Feed Concentration}(C_{d,0})-\text{Permeate Concentration}(C_{r,t})}{\text{Feed Concentration}(C_{d,0})}\times 100 \quad (3)$$

where $C_{d,o}$ is the contentration of solute in the donor cell at $t = 0$ and $C_{r,t}$ is the solute concentration in the receiver cell at time t.

Protein Permeation. The permeabilities of proteins to PE20PT20-D and PE20PD20-D were represented in Table V. The permeabilities decreased with an increase of molecular weight as expected again. For GOD, the permeation was not detected by gel electrophoresis with Coomassie staining

Table V. Permeabilities of Proteins to Membranes

Protein	M_w (Da)	Stokes Diameter[29-31] (nm)	Permeability (cm/sec)			
			PE20PT20-D	PE20PD20-D	Durapore®[36*]	D in Water (cm^2/sec)[30,37]
FITC-Insulin	6,000	2.34 ~ 2.56	$1.97(\pm 0.27)\times 10^{-5}$ (n=4)	$4.39(\pm 0.41)\times 10^{-5}$ (n=4)	5.39×10^{-5}	2.0×10^{-6}
FITC-BSA	67,000	7.21	$7.29(\pm 3.19)\times 10^{-7}$ (n=3)	$7.41(\pm 1.21)\times 10^{-7}$ (n=4)	2.64×10^{-5}	9.64×10^{-7}
FITC-IgG	150,000	10.26	$6.61(\pm 2.68)\times 10^{-7}$ (n=3)	$6.69(\pm 0.34)\times 10^{-7}$ (n=3)	1.55×10^{-5}	6.29×10^{-7}
GOD	186,000	10.4	ND	ND	NT	-

*Estimated by $D_{37}=D_{25}\frac{310\eta_{25}}{298\eta_{37}}$ from data at 25 °C viscosities at 37 and 25 °C are 0.6916 cp and 0.8903 cp, respectively).[34]

ND: not detectable by Coomassie staining, NT: not tested.

method. Thus, the MWCO of the membrane for protein may locate between 155 to 186 kDa. However, for IgG the experimentally measured permeability was higher than that estimated from the result of 42 kDa dextran and unlikely GOD, it permeated through both membranes. This permeability may be caused by the molecular shape of IgG that is not spherical. Burczark *et al.* described that a membrane with a mesh size as low as 9.0 nm may not be effective as a barrier for IgG although its stokes diameter is about 10 nm.[35] When compared to other studies, the diffusivities of IgG to two membranes (PE20PT20-D and PE20PD20-D) represented about 4.9 times and 15.4 times lower than those to 5% agarose microbead[31] and PVA hydrogel membrane,[35] respectively. Yang *et al.* reported no permeation of IgG to Durapore® membrane coated with regenerated cellulose[23] and Dionne *et al.* reported that mass transfer coefficient of IgG was 3.59×10^{-8} cm/sec.[37]

Conclusions

By filling pores of Durapore® membrane with water which is not a good solvent for the amphiphilic multiblock copolymers synthesized in this study, the single spin coating of alcoholic solutions of the copolymers on the porous substrate produced a smooth thin surface with complete coverage and controlled degree of penetration of the coating into the pores. The permeabilities of dextrans of various molecular weights and proteins are influenced by complex factors such as the nature of hydrophobic block, PEG length, and the extent of penetration of the coating layer in addition to the conventional factors of molecular size of penetrant and degree of swelling. The MWCO of the composite membrane covered with the copolymers containing PEG 2000 was about the size of glucose oxidase (186 kDa). With further optimization of the copolymers with a shorter PEG than 2,000 Da, the composite membrane may offer a more practical immunoptotecting membrane for biohydrid artifical organs with mechanical stability, biocompatibility, and controlled permeability.

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