Nanometer-Sized Channel Gating by a Self-Assembled Polypeptide Brush

Yoshihiro Ito,*[,†,‡] Yong Soon Park,[§] and Yukio Imanishi[||]

Department of Biological Science and Technology, Faculty of Engineering, The University of Tokushima, Tokushima, 770-8506, Japan, PRESTO, JST, Keihanna Plaza, Seika-cho, Kyoto 619-0237, Japan, Department of Material Chemistry, Graduate School of Engineering, Kyoto University, Kyoto, 606-8501, Japan, and Graduate School of Materials Science, NAIST, Ikoma, 630-0101, Japan

Received August 16, 1999. In Final Form: March 2, 2000

A nanometer-sized signal-responsive gate was fabricated by self-assembly of ionizable polypeptide brushes on a gold-coated porous membrane, and its responsiveness in water was visualized by atomic force microscopy. The gating was regulated by the conformational change of poly(glutamic acid) that undergoes a helix−coil transition in response to pH. As a result, water permeation through the membrane was reversibly regulated by pH. The permeability could be adjusted by varying the length of the polypeptide brush. This type of membrane will be useful for the precise control of substance permeation.

Introduction

A variety of nano- or microstructured materials have been prepared for applications in catalysis and separation and for use as sensors or host compounds for template synthesis of other nanoscopic materials.[1−3] Nanoporous membranes or synthetic transmembrane channels have been designed for mimicking biological functions or for new membrane technology. In addition, environmental stimuli-responsive, or "smart", materials have been designed by graft polymerization on these nanoporous membranes.[4−21] Liquid or solute permeation through these membranes was regulated in response to pH,[4−6,8,10,12−14,16−18] ion concentration,[4] temperature,[9,19] photoirradiation,[11,20] oxidoreduction reaction,[15] and glucose concentration.[7]

However, since these membranes have been constructed by graft polymerization, it was difficult to precisely control the length and density of the graft chain. In the present study, a self-assembly technique, which is one of the simplest and most effective methods to prepare thin films, was employed for fabricating a stimuli-responsive filter. A pH-sensitive polypeptide brush was self-assembled on a nanoporous membrane, and the resultant nanometer-sized channel opened and closed in response to pH, similar to a gate. It is well-known that the conformation of a polyelectrolyte chain is dependent upon environmental conditions such as pH and ionic strength.[22,23] In the region of low pH, a poly(L-glutamic acid) (PLGA) chain is protonated and folded to form an α-helical structure; in the region of high pH, it is deprotonated to form an extended random structure. In the present experiments, the permeation through the porous membrane, the surface of which was covered with the self-assembled polypeptide brush, was sensitively regulated by changing pH because of the direct contact of the polypeptide brush with the environment.

Materials and Methods

Synthesis of Polypeptide Derivative. PLGA carrying a disulfide group at the amino terminal (PLGA-SS) was synthesized as shown in Figure 1. The oxidation of 11-mercaptoundecanoic acid (1.0 g) (Aldrich) was attained by 24-h treatment in DMSO/1 N HCl (100 mL) according to a previous report.[24] The product (216 mg) was dissolved in 15 mL of dimethylformamide (DMF). *O*-(7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (380 mg, HATU, PerSpective Biosystems, Hamburg, Germany) and *N*,*N*-diisopropylethylamine (200 μL, DIEA, Aldrich) were added to the solution at the disulfide:HATU:DIEA molar ratio of 1:2:2.4 (0 °C). After addition of poly(γ-benzyl-L-glutamate) (PBLG, 1.0 g), the solution was stirred for 1 h in an ice bath. PBLG (degree of polymerization (DP): 80 or 480) was purchased from Sigma Chemical Co. The solution was then stirred for 24 h at room temperature. The product, PBLG carrying a terminal disulfide group (PBLG-SS), was isolated by two precipitations with methanol and then dried overnight in vacuo

[†] The University of Tokushima.
[‡] PRESTO, JST.
[§] Kyoto University.
[||] Graduate School of Materials Science, NAIST.

(1) Martin C. R. *Science* **1994**, *266*, 1961.
(2) Nanostructured Materials (special issue; Bein, T., Stucky, G. D., Eds.) *Chem. Mater.* **1996**, *8*, 1569.
(3) Frontiers in Materials Science (special issue) *Science* **1997**, *277*, 1213.
(4) Osada, Y.; Honda, K.; Ohta, M. *J. Membr. Sci.* **1986**, *27*, 327.
(5) Okahata, Y.; Noguchi, H.; Seki, T. *Macromolecules* **1987**, *20*, 15.
(6) Iwata, H.; Matsuda, T. *J. Membr. Sci.* **1988**, *38*, 185.
(7) Ito, Y.; Casolaro, M.; Kono, K.; Imanishi, Y. *J. Controlled Release* **1989**, *10*, 195.
(8) Ito, Y.; Kotera, S.; Inaba, M.; Kono, K.; Imanishi, Y. *Polymer* **1990**, *30*, 2157.
(9) Iwata, H.; Oodate, M.; Uyama, Y. Amemiya, H.; Ikada, Y. *J. Membr. Sci.* **1991**, *55*, 119.
(10) Ito, Y.; Inaba, M., Chung, D. J.; Imanishi, Y. *Macromolecules* **1992**, *25*, 7313.
(11) Chung, D. J.; Ito, Y.; Imanishi, Y. *J. Appl. Polym. Sci.* **1994**, *51*, 2027.
(12) Ito, Y.; Park, Y. S.; Imanishi, Y. *Macromol. Rapid Commun.* **1997**, *18*, 221.
(13) Iwata, H.; Hirata, I.; Ikada, Y. *Langmuir* **1997**, *13*, 3063.
(14) Park, Y. S.; Ito, Y.; Imanishi, Y. *Chem. Mater.* **1997**, *9*, 2755.
(15) Ito, Y.; Nishi, S.; Park, Y. S.; Imanishi, Y. *Macromolecules* **1997**, *30*, 5856.
(16) Ito, Y.; Park, Y. S.; Imanishi, Y. *J. Am. Chem. Soc.* **1997**, *119*, 2739.
(17) Ito, Y.; Ochiai, Y.; Park, Y. S.; Imanishi, Y. *J. Am. Chem. Soc.* **1997**, *119*, 1619.
(18) Iwata, H.; Hirata, I.; Ikada, Y. *Macromolecules* **1998**, *31*, 3671.
(19) Park, Y. S.; Ito, Y.; Imanishi, Y. *Langmuir* **1998**, *14*, 910.
(20) Park, Y. S.; Ito, Y.; Imanishi, Y. *Macromolecules* **1998**, *31*, 2606.
(21) Ito, Y. *Kobunshi Ronbunshu* **1998**, *55*, 171.
(22) Holzer, A. *J. Am. Chem. Soc.* **1994**, *116*, 10837.
(23) Spek, E. J.; Gong, Y.; Kallenbach, N. R. *J. Am. Chem. Soc.* **1995**, *117*, 10773.
(24) Tamamura, H.; Otaka, A.; Nakamura, J.; Olubo, K.; Koide, T.; Ikeda, K.; Ibuka, T.; Fujii, N. *Int. J. Peptide Protein Res.* **1995**, *45*, 312.

10.1021/la991102+ CCC: $19.00
Published on Web 05/19/2000

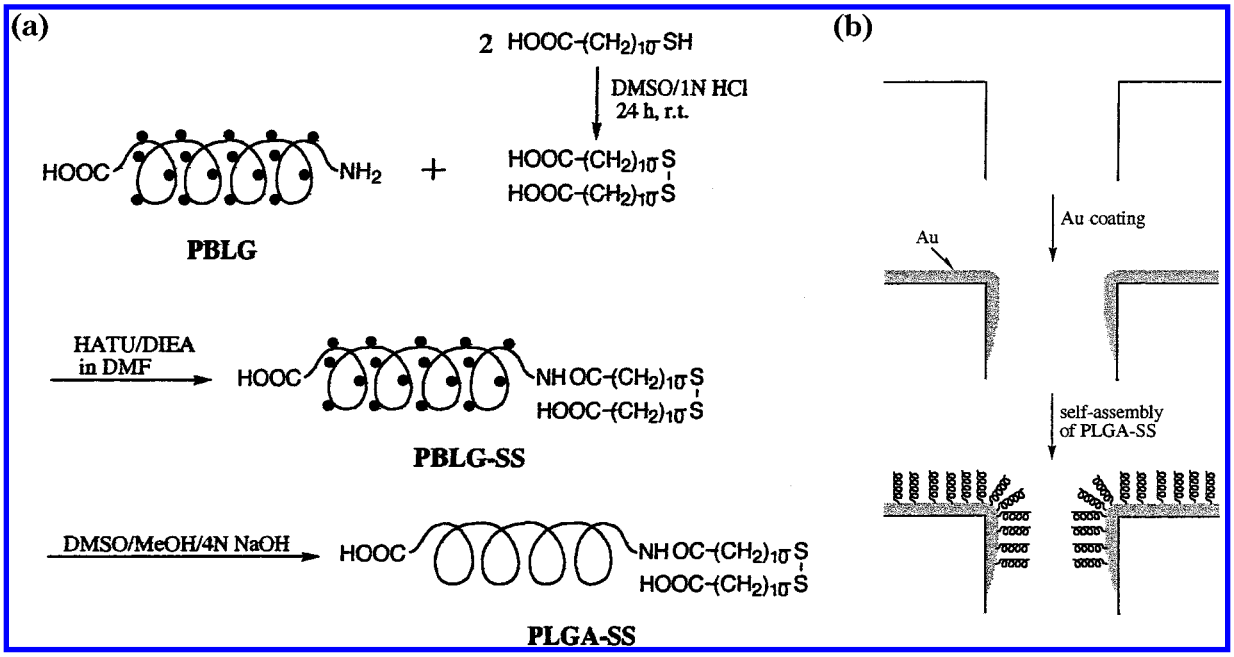

Figure 1. Schematic representation of the synthesis of poly(L-glutamic acid) carrying a terminal disulfide group (a) and self-assembly on an Au-coated porous membrane (b).

at room temperature. To hydrolyze PBLG, 500 mg of PBLG-SS was treated in a dioxane (6 mL)/methanol (2 mL)/4 N NaOH (2 mL) mixture for 2 h at room temperature. The precipitate was dissolved in 500 mL of distilled water and then concentrated to 50 mL by ultrafiltration using an AMICON model 2000 (cutoff mw = 10 000). The concentrated solution was lyophilized to obtain poly(L-glutamic acid) carrying a terminal disulfide group (PLGA-SS).

Self-Assembly. The synthesized polypeptide was self-assembled on the surface of an Au-coated nanoporous membrane. PLGA-SS was assembled on the membrane as follows. Track-etched porous polycarbonate membrane (DuPont Nuclepore membrane; average pore diameter, 200 nm) was coated with platinum and then with gold by an IB-3 ion coater (Eiko Eng. Co., Ibaraki, Japan). The coated membrane was immersed in an aqueous solution of PLGA-SS (2.5 mM, pH = 3.0) for 24 h. The surface-modified membrane was washed with deionized water until the pH of the washing liquid became neutral.

FT-IR Measurement. FT-ATR-IR spectra were measured using a Perkin-Elmer infrared spectrometer equipped with a KRS-5 prism with an incidence angle of 45°. The calibration curve was determined by measuring FT-ATR-IR spectra of known amounts of cast PLGA-SS, which were freeze-dried from an aqueous solution of pH 7.0.

Permeation Experiment. Water permeation through the membrane was investigated using an apparatus described previously.[8,10] The prepared membrane was mounted on an ultrafiltration cell (Toyo Roshi UHP-25) and placed 200 cm below a water reservoir. The reservoir was filled with an aqueous solution and adjusted to different pH values using NaOH and HCl. The aqueous solution was allowed to flow under a constant hydraulic pressure. The permeation rate was calculated by measuring the mass of water permeating through the membrane each minute.

Observation by Atomic Force Microscopy. An atomic microscope Nanoscope IIa (Digital Instruments) equipped with a fluid cell for aqueous solution was utilized to record images.[12] The pH of the aqueous solution was adjusted by the addition of an aqueous solution of NaOH or HCl. For the repulsive mode, commercial Si_3N_4 cantilevers with a nominal force constant of 0.06 N/m were used.

Results and Discussion

Self-Assembly. When the membrane was directly coated with gold, the surface was very rough. Therefore, the membrane was coated first with platinum and second with gold to have a relatively smooth surface. The cross section of coated membrane is shown in Figure 2. The thickness of the coated metal was about 20 nm. The metal layer was formed inside pores less than 600 nm deep. Considering the thickness of the membrane (10 μm), the coated region was located only on the open areas of pores as illustrated in Figure 1b.

The presence of assembled PLGA-SS on the surface of the membrane was confirmed by infrared spectroscopy (Figure 3). At pH 2.0, the FT-ATR-IR spectrum of PLGA-SS-assembled membrane shows amide I (1654 cm^{-1}) and amide II (1547 cm^{-1}) absorption peaks, as well as a carboxylic acid absorption peak (1700 cm^{-1}), which are characteristic of PLGA. At pH 7.0, the absorption peak of carboxylic acid group disappeared and the absorption peak of carboxylate groups overlapped with that of amide I. The absorption peaks of amide I and II appeared at 1658 and 1542 cm^{-1}, respectively, at pH 7.0.

Higashi et al.[25−27] synthesized a Langmuir−Blodgett (LB) film composed of a PLGA derivative and observed the conformational change of PLGA by FT-IR. The absorption peak assigned to the C=O stretching band of side-chain COOH groups of PLGA was observed at 1702 cm^{-1} for the LB film deposited at pH 3.0, while it disappeared for the LB film at pH 9.0 due to deprotonation of the COOH groups. In amide I and II absorption regions, there were obvious differences between LB films prepared at pH 3.0 and 9.0: for the former LB film, absorption peaks characteristic of the α-helix appeared at 1654 and 1550 cm^{-1} whereas the latter LB film gave absorption peaks at 1659 and 1532 cm^{-1} because of its random coil structure, although the minor existence of the β-sheet structure must not be excluded for both films. The IR absorbances of self-assembled PLGA-SS in the present study were almost similar to those of PGLA in LB film.

The amount of the assembled PLGA (DP: 80) or PLGA (DP: 480) was calculated from the IR absorbance. The

(25) Higashi, N.; Sunada, M.; Niwa, M. *Langmuir* **1995**, *11*, 1864.
(26) Higashi, N.; Saitou, M.; Mihara, T.; Niwa, M. *J. Chem. Soc., Chem. Commun.* **1995**, 2119.
(27) Higashi, N.; Yamamoto, T.; Yokoyama, K.; Niwa, M. *Macromolecules* **1995**, *28*, 2585.

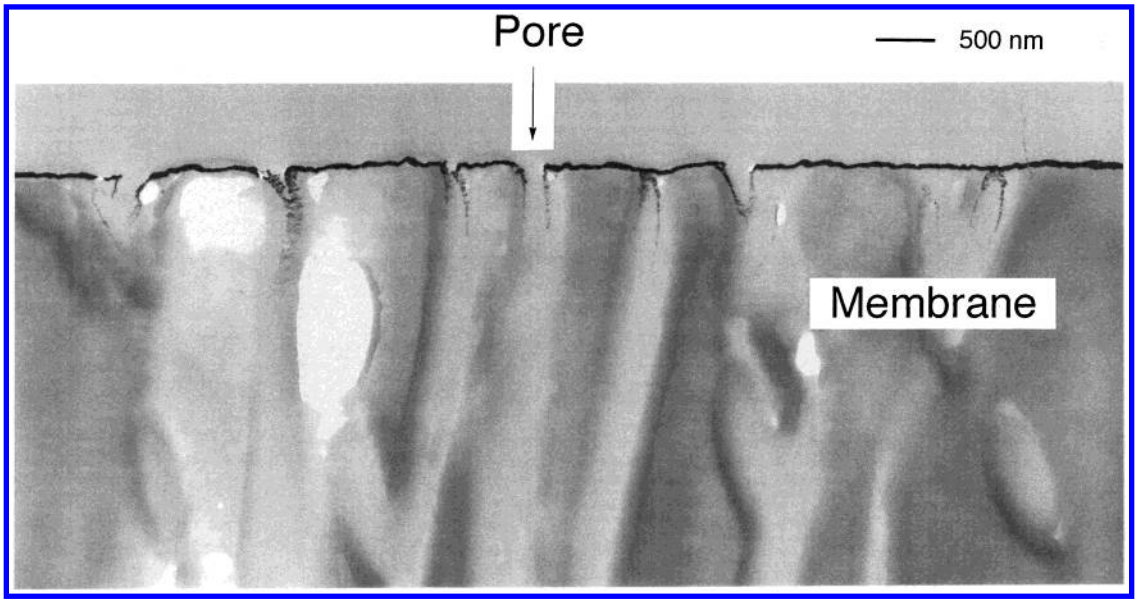

Figure 2. Electron micrograph (40 000×) of cross section of platinum- and gold-coated porous membrane.

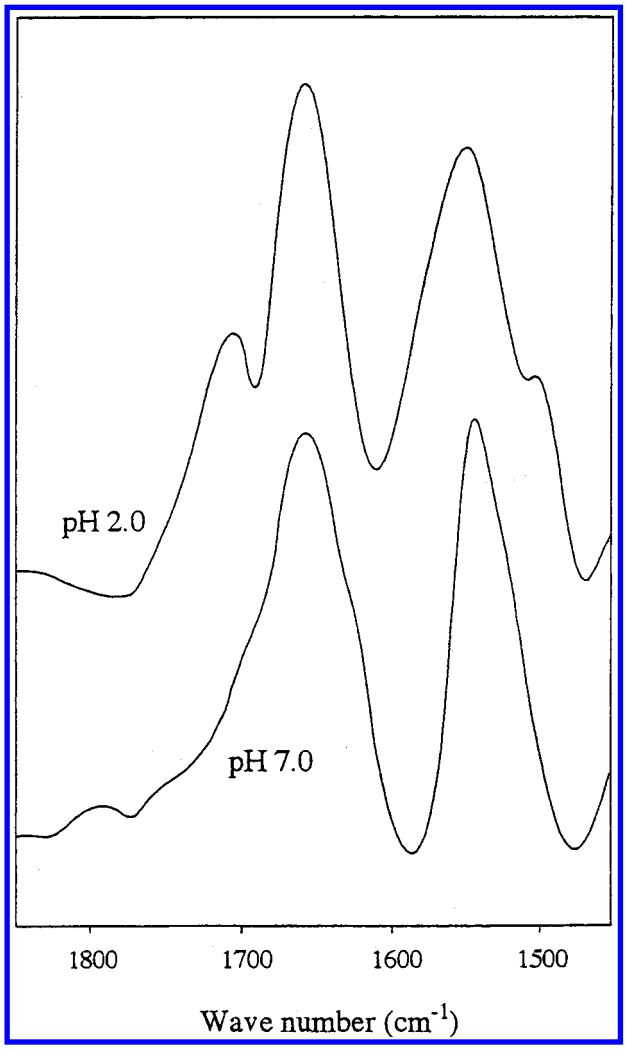

Figure 3. FT-ATR-IR spectra of assembled PLGA (DP: 480), which was freeze-dried from an aqueous solution of pH 2.0 and 7.0.

former was 6×10^{-11} mol/cm^2, and the latter, 4×10^{-11} mol/cm^2. Higashi et al.[26] demonstrated that the mean area of one PLGA (DP: 56) derivative in LB film was 0.6 nm^2 under maximum surface pressure, which meant that the amount of PLGA was 2.5×10^{-10} mol/cm^2. This result suggested that the assembled PLGA-SS formed a relatively dense layer in the present study, although the density of assembled layer is less than that of PLGA in the LB film.

Enriquez et al.[28] reported the self-assembly of a hydrophobic polypeptide, poly(γ-benzyl L-glutamate), carrying lipoic acid, on gold. They found that a self-assembled monolayer was formed wherein the α-helix conformation was retained and the polypeptides were preferentially bound to gold through the disulfide moiety. The terminal bond to the substrate results in a nonplanar distribution of the helical axes (i.e., on average tilted up from the gold surface).

Water Permeation. The rate of water permeation through a nonassembled membrane was independent of pH. The rate of water permeation through the grafted membrane was dependent upon pH, with high permeation at low pH but low permeation at close to neutral pH (Figure 4). It is expected that in the region of low pH, the PLGA chain is protonated and folded to form an α-helical structure, and it lies on the surface via hydrophobic interaction. On the other hand, in the region of high pH, it is deprotonated to form an extended, random structure, and extended to the solution. This type of conformational change of the polypeptide chain may affect the porosity of the membrane, leading to the pH-dependent permeability of water. The inflection point (pH 4.5−5) of the water permeation rate was almost the same as the isoelectric point of poly(glutamic acid) (DP: 620), i.e., 4.58.[29]

The pH dependence varied with the length of the polypeptide chain. pH sensitivity was less when PLGA-SS was long (DP: 480). The long PLGA-SS was considered to cover the pore so extensively that water permeation and pH sensitivity were suppressed.

Assuming that the permeation fluid is expressed by the Hagen−Poiseuille equation, the permeation rate J is expressed as follows

$$J = n\pi R^4 AP/8\eta d$$

(28) Enriquez, E. P.; Gray, K. H.; Guarisco, V. F.; Linton, R. W.; Mar, K. D.; Samulski, E. T. *J. Vac. Sci. Technol.* **1992**, *A10*, 2775.
(29) Olander, D. S.; Holzer, A. *J. Am. Chem. Soc.* **1968**, *90*, 4549.

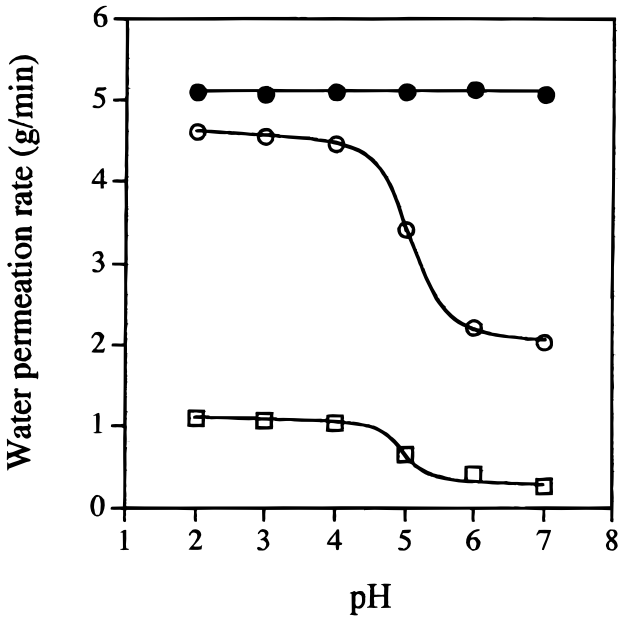

Figure 4. pH dependence of water permeation through (●) nonassembled, (○) PLGA-SS (DP: 80), and (□) PLGA-SS (DP: 480) self-assembled porous membranes.

Here n, A, R, P, η, and d represent the number of pores per unit area, surface area of membrane, pore radius, applied pressure, viscosity of flowing liquid, and membrane thickness, respectively. The thickness of the assembled PLGA-SS was estimated by the following equation, assuming no significant change of membrane thickness.

$$J_{pH=7}/J_{pH=2} = (R_{pH=7}/R_{pH=2})^4$$

The thickness of the assembled polypeptide (DP: 80) was calculated to be 1.5 or 16 nm at pH 2.0 and pH 7.0, respectively. In the case of DP = 480, it was calculated to be 25 or 42 nm, respectively. Although this estimation is very rough, the values qualitatively represent the pore size.

The permeation rate also depended on the ionic strength (Figure 5). pH dependence decreased with increasing ionic strength. In the high pH region, permeability was strongly dependent on the ionic strength. The high concentration of ions is thought to moderate charge-to-charge interactions of the polypeptide brush, leading to conformational change. A similar effect of ionic concentration on polypeptide conformation was discussed previously.[30]

The change in permeability of the PLGA-SS-assembled membrane in response to pH change occurred within a few minutes (Figure 6). PLGA brushes were directly contacted to media of different pH values; this situation allowed the brushes to respond quickly to the environmental change. The permeation control in response to pH change (pH 3 and 7) could be repeated 10 times without any significant changes.

It was previously demonstrated that membranes composed of polypeptides carrying carboxylic groups allowed the higher rate of permeation of an aqueous solution in the region of high pH.[31,32] This behavior was explained by the "through polymer" mechanism. In this mechanism,

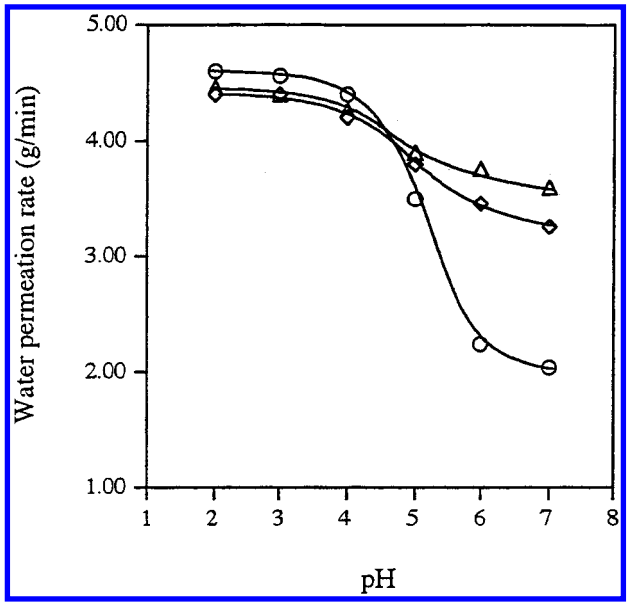

Figure 5. pH-dependent permeation of water having different ionic strengths through the porous membrane with self-assembled PLGA-SS brush. Ionic strengths were 0 (○), 0.1 (◇), and 1.0 (△).

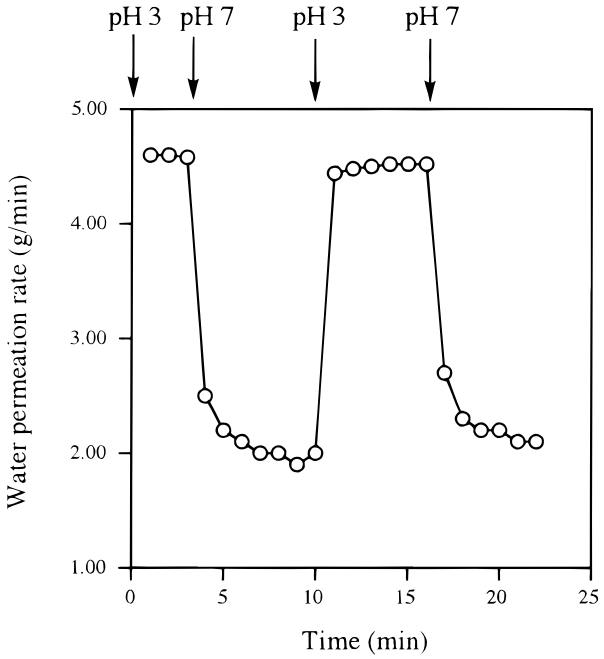

Figure 6. Time course of permeation rate of water through the porous membrane with self-assembled PLGA-SS brush in response to pH change with time.

the diffusion of permeating species through the interstices of swollen, ionized polypeptide chains was slow due to the diffusion limitation.

Figure 7 shows the atomic force microscopic image of the PLGA-SS (DP: 480)-assembled pore in water. The pore size was large at pH 3.0 and small at pH 7.0. The size change roughly corresponded to the folding or extension of self-assembled PLGA-SS. The statistical analysis of pore size distribution also demonstrated the same tendency (Figure 7c)

Previously, we grafted a polypeptide chain on a porous membrane by *N*-carboxyanhydride polymerization.[17] Al-

(30) Kono, K.; Kimura, S.; Imanishi, Y. *Biochemistry* **1990**, *29*, 3631.
(31) Maeda, M.; Kimura, M.; Hareyama, Y.; Inoue, S. *J. Am. Chem. Soc.* **1984**, *106*, 250. (b) Chung, D.-W.; Higuchi, S.; Maeda, M.; Inoue, S. *J. Am. Chem. Soc.* **1984**, *106*, 5823. (c) Aoyama, M. Youda, A.; Watanabe, J.; Inoue, S. *Macromolecules* **1990**, *23*, 1458.

(32) Kinoshita, T.; Iwata, T.; Takezawa, A.; Tsujita, Y. *Colloid Polym. Sci.* **1983**, *261*, 933. (b) Kinoshita, T.; Kakiuchi, T.; Takizawa, A.; Tsujita, Y.; Oya, M.; Iizuka, Y.; Iwatsuki, M. *Macromolecules* **1994**, *27*, 1389.

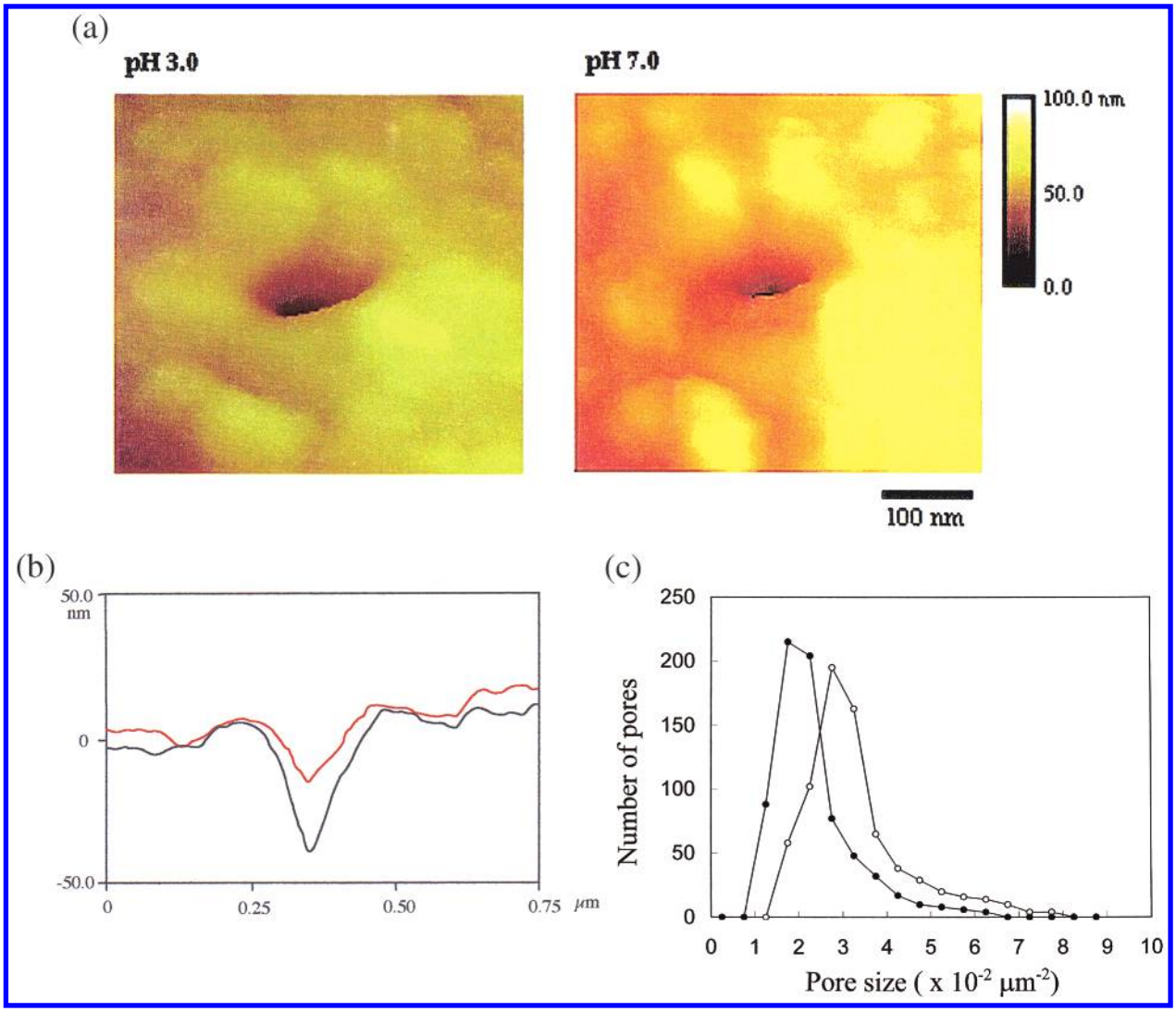

Figure 7. (a) Atomic force micrographs (AFM) of the PLGA-SS (mw: 17 400) self-assembled porous membrane at pH 3.0 and pH 7.0. (b) Cross-sectional plot of pore depth at pH 3.0 (black line) and pH 7.0 (red line). (c) The pore size distribution at pH 3.0 (○) and 7.0 (●). The areas of pores (approximately 700) per 20 μm × 20 μm AFM image were calculated as numbers of pixels and then converted to μm^2.

though similar pH-dependent water permeation was observed in that membrane and in the present membrane, the magnitude of permeation change was smaller in that membrane than in the present one. In addition, it is difficult to regulate the length and density of graft chains by the grafting method. The present study showed that grafted chains of known length could be assembled and that the conformational change of the grafted chains reversibly regulated the pore size of the membrane. Recently Kaetsu et al.[33] and Santini et al.[34] reported controlled-release microchips. They constructed polymer or silicon microchips that can provide controlled release of single or multiple chemical substances on demand by microfabrication technology. Polyelectrolyte hydrogel layers or thin anode membrane were used as the gates of fine holes of the microreservoir. The present fabrication method will be useful for construction of such gates.

Although it was well reported that the responsive permeability membranes are promising for controlled release, few such delivery systems have been tested in clinics to demonstrate that the responsive release is truly advantageous over the conventional controlled release.[35] One main reason may be that the human body can tolerate only small fluctuations from the norm, and the disease signals or external stimuli would normally be small in magnitude. On the other hand, the pH range of fluids in the gastrointestinal tract is wide. This may provide environmental stimuli suitable for responsive release using pH-sensitive membranes. In addition, the pH responsive system will become a glucose-sensitive insulin-releasing system by coupling with glucose oxidase. When the membrane in the present study is employed, the coupled enzyme produces gluconic acid in response to glucose concentration and lowers the environmental pH, and thus the system releases insulin as the system reported previously.[7]

Multistimuli-responsive membranes will also offer intriguing possibilities for future applications. A hydrogel showing responsive swelling to both pH and temperature was used in a condition where both phenomena are coupled such as the site of a blood clot,[36] a tumor,[37] or inflam-

(33) Kaetsu, I.; Uchida, K.; Ueta, S.; Sutani, K.; Doi, A. *Drug Delivery Syst.* **1996**, *11*, 317. (b) Kaetsu, I.; Uchida, K.; Shindo, H.; Gomi, S.; Sutani, K. *Rad. Phys. Chem.* **1999**, *55*, 193.

(34) Santini, J. T., Jr.; Cima, M. J.; Langer, R. *Nature* **1999**, *397*, 335.

(35) Peng, T. Ph.D. thesis at the University of Toronto 1999.

(36) Brazel, C. S.; Peppas, N. A. *Macromolecules* **1995**, *28*, 8016.

mation.[38] A pulsatile local delivery system, based on pH- and temperture-sensitive hydrogels, was proposed and might be applied for treatment of coronary thrombosis or stroke patients.[39] However, for the medical uses, in addition to these device designs, stimuli responsiveness under biological conditions should be investigated, because various inorganic and organic substances adsorb on the devices and reduce the sensitivity. Suppression of the substances adsorption is the practical issue that has to be overcome from the standpoint of surface chemistry.

Acknowledgment. Y. Ito is grateful for the financial support of the Japan Securities Scholarship Foundation, the Asahi Glass Science Foundation, and the Ministry of Education, Science, Sports, and Culture of Japan (11167254).

LA991102+

(37) Ferraretto, A.; Sonnino, S.; Soria, M. R.; Masserii M. *Chem. Phys. Lipids* **1996**, *82*, 133.
(38) Park, J. B.; Lakes, R. S. In *Biomaterials: an introduction*, Plenum Press: New York, 1992; p 223.
(39) Brazel, C. S.; Peppas, N. A. *J. Controlled Release* **1996**, *39*, 57.