Studies on the Development of Poly(L-Lactic Acid) Porous Membranes with the Aid of Surfactants

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2017

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Chapter 1 General Introduction

1.1. Poly(L-lactic acid) as sustainable materials

Poly(L-lactic acid) (PLLA), an aliphatic polyester, has been studied as one of the most promising biodegradable plastics [1–4]. Its monomer, L-lactic acid, is synthesized by fermentation of lactic acid bacteria from biomass, which is produced by photosynthesis of plants from carbon dioxide. The polyester can be hydrolyzed in wet and warm conditions and metabolized to carbon dioxide by microorganisms in the natural environment and composting machines (Fig. 1-1). Thus PLLA is considered as one of the sustainable materials that will be alternatives to petroleum-derived plastics in the future. It is used as films and trays for foods, transparent windows for envelopes, mulching films in agriculture, and some parts of electronic appliances and automobiles [5].

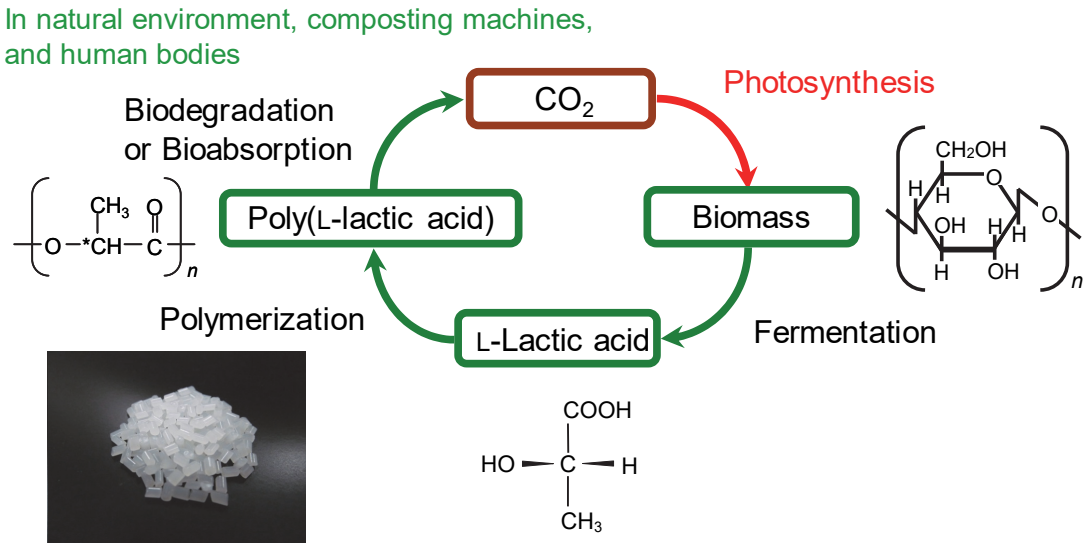

Fig. 1-1 Production and degradation of poly(L-lactic acid).

1.2. PLLA as biomaterials

PLLA is one of the promising bioabsorbable biomaterials. The polyester is also hydrolyzed to L-lactic acid and the monomer is metabolized to carbon dioxide in human body as well as in environment and compost (Fig. 1-1). Due to its bioabsorbability and high strength PLLA is

used as surgical sutures, screws, fixation pins, and plates that are unnecessary to be removed by second surgical procedure in medicine [6]. The bioabsorbable polyester has been receiving much attention in tissue engineering. Many kinds of bioabsorbable scaffolds of PLLA have been prepared by porogen leaching, fused deposition modeling, and phase separation methods [7–11].

1.3. Porous membranes of PLLA

The biodegradable and bioabsorbable characteristics of PLLA has been encouraging the developments of porous membranes of the polymer (Fig. 1-2). Bioabsorbable membranes have been developed in the 1990's as the devices in tissue engineering and drug delivery system by phase separation methods [8,12]. Biodegradable porous membranes for separation and purification processes by phase separation methods after the success of the lower-cost production of the polymer in the late 1990's [13,14].

There are two major phase separation methods for preparation of polymeric porous membranes: thermally induced phase separation (TIPS) and non-solvent-induced phase separation (NIPS) methods. In a TIPS method a polymer solution cast on a mold is cooled to separate the phase of the polymer solution into polymer-rich and polymer-lean phases (liquid-liquid phase separation) [15] (Fig. 1-3) or into connected solid-polymer particles and polymer-lean phases (solid-liquid phase separation) [16]. The polymer-rich phase solidifies after continued cooling in liquid-liquid phase separation. A porous membrane is obtained after the removal of the solvent by a nonsolvent in which the polymer is insoluble but the solvent in the polymer solution is soluble. In a NIPS method a polymer solution is contacted to a nonsolvent to induce the phase separation by extracting the solvent and diffusing nonsolvent molecules into the polymer solution. PLLA microfiltration membranes have been prepared by TIPS methods [13,17,18] and a nonsolvent and thermally induced phase separation method [19] while PLLA ultrafiltration membranes by NIPS methods [14,20]. Microfiltration membranes retain micrometer-size particles such as bacterial cells and let soluble macromolecules, e.g. proteins, permeate while the ultrafiltration membrane retains macromolecules and let low-molecular-weight compounds permeates. PLLA microfiltration membranes are expected be disposed in composts after use in the clarification process of bio and food industries.

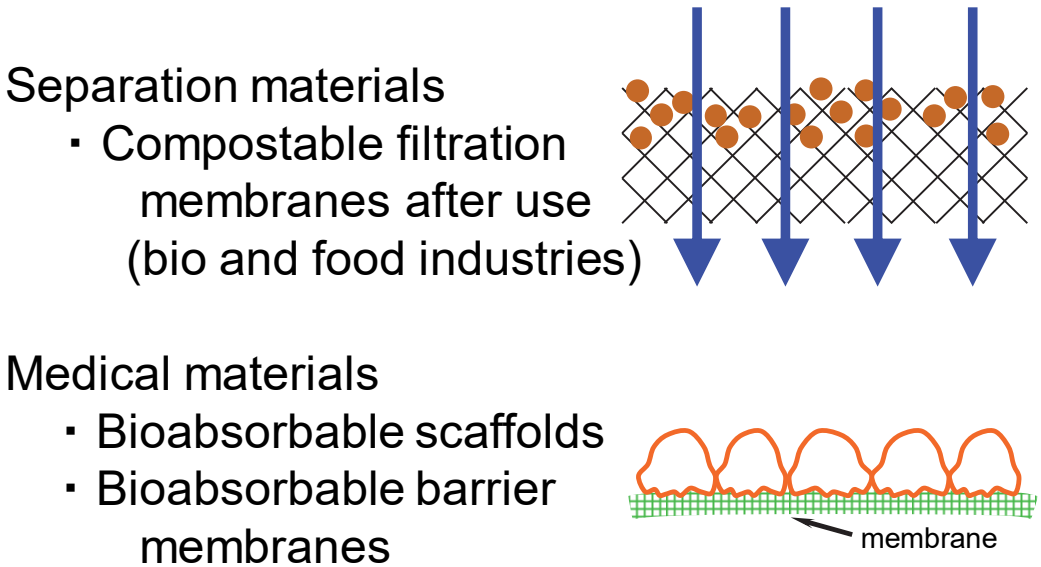

Fig. 1-2 Possible applications of PLLA porous membranes

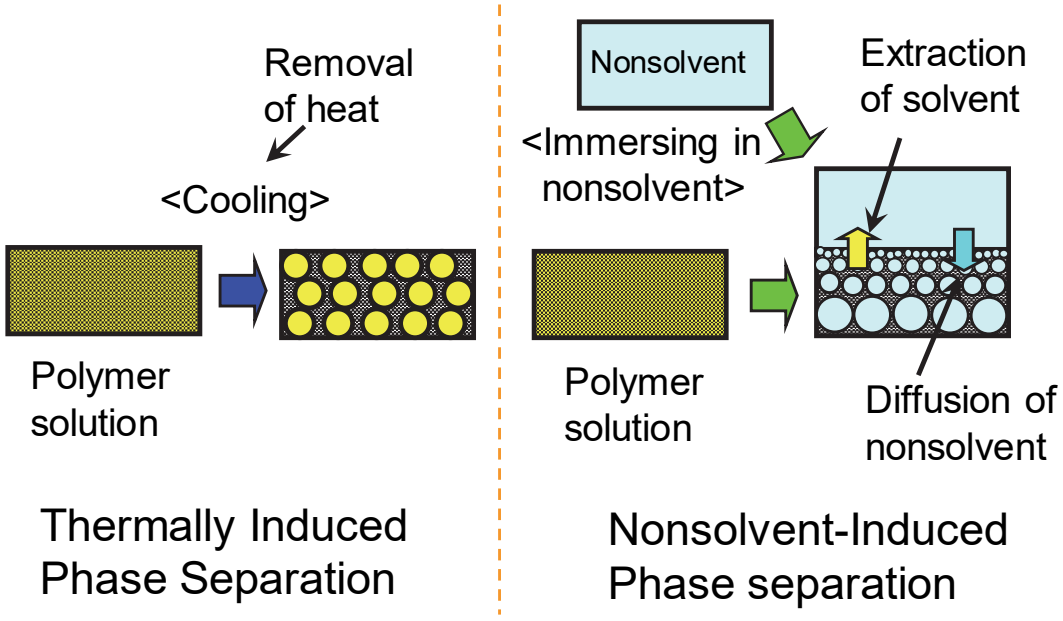

Fig. 1-3 Membrane preparation via thermally induced phase separation (TIPS) and nonsolvent-induced phase separation (NIPS).

1.4. Preparation of porous membranes by NIPS methods with the aid of surfactants

An advantage of NIPS methods over TIPS method is that the membranes can be prepared at a constant temperature. However, preparation of PLLA microfiltration membranes by NIPS methods had not been reported while that of PLLA ultrafiltration membranes were reported when the author started his work in 2011. One of the reasons would be fast extraction of solvent and slow diffusion of nonsolovent in NIPS methods. One of the approaches for solving such problems is the addition of surfactants to polymer solutions to accelerate the diffusion of nonsolvent molecules in the polymer solutions (Fig. 1-4). Surfactants are molecules with both hydrophilic and lipophlic groups and are typically used as solubilizers, detergents, emulsifiers, wetting and spreading agents, and antifoaming agents in accordance with their hydrophilic-lipophilic balance (HLB) values [21]. Among the surfactant class of compounds, polyoxyethylene (20) sorbitan monooleate (Tween 80) (Fig. 1-5) is well known as a reagent to improve the structure of poly(methyl methacrylate) and polyethersulfone membranes [22,23]. The approach is attractive to control the porous structure of the PLLA membranes.

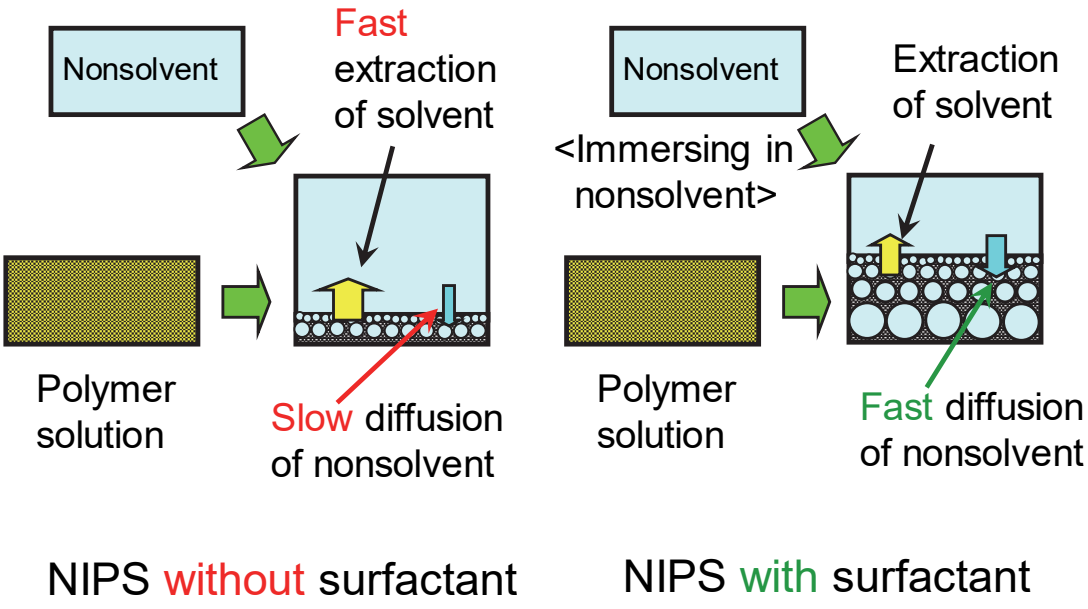

Fig. 1-4 Nonsolvent-induced phase separation with the aid of surfactants.

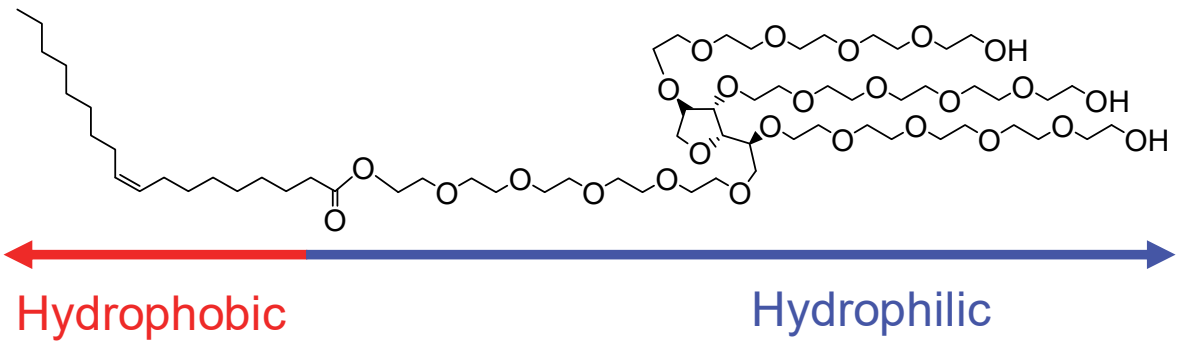

Fig. 1-5 Tween 80, a typical nonionic surfactant.

1.5. Objectives and outline of this thesis

In the author's work PLLA membranes were prepared with the aid of surfactants via NIPS method. The addition of an appreciable amount (10–15%) of surfactants to the polymer solution promotes the diffusion of nonsolvent into the polymer solution when the polymer solution immersed into a nonsolvent bath. The formed porous asymmetric membranes with open finger-like pores have been evaluated as microfiltration membranes and membrane scaffolds for cell culture.

In Chapter 1; the general introduction, the author described the sustainability of biodegradable PLLA, its use as biomaterials, porous membranes of PLLA, and the membrane preparation via a NIPS method with the aid of surfactants.

Nest in Chapter 2, the author prepared the PLLA porous asymmetric microfiltration membrane with different concentrations and kinds of surfactants, and polymer concentrations. The membranes were studied in the view point of the membrane structures and filtration characteristics. The degradability under wet conditions were also examined.

In Chapter 3, PLLA porous membranes with open finger-like pores were evaluated as a scaffolding membrane scaffold. The osteoblast-like cells grown on and in the membrane successfully formed calcium phosphate by osteoinduction.

In the last Chapter 4; the summary of the author's work and future prospects as Concluding Remarks.

1.6. References

[1] A. Torres, S. M. Li, S. Roussos, M. Vert, Poly(lactic acid) Degradation in soil or under controlled conditions, J. Appl. Polym. Sci. 62 (1996) 2295–2302.

[2] J. Lunt, Large-Scale Production, properties and commercial applications of polylactic acid polymers, Polym. Degrad. Stab. 59 (1998) 145–152.

[3] R.A. Gross, B. Kalra, Biodegradable polymers for the environment, Science 297 (2002) 803–807.

[4] E. Castro-Aguirre, F. Iñiguez-Franco, H. Samsudin, X. Fang, R. Auras, Poly(lactic acid)—Mass production, processing, industrial applications, and end of life, Adv. Drug Deliv. Rev. 107 (2016) 333–366.

[5] M. Jamshidian, E. A. Tehrany, M. Imran, M. Jacquot, S. Desobry, Poly-lactic acid: production, applications, nanocomposites, and release studies, Compr. Rev. Food Sci. Food Saf. 9 (2010) 552–571

[6] S. Farah, D. G. Anderson, R. Langer, Physical and mechanical properties of PLA, and their functions in widespread applications — A comprehensive review, Adv. Drug Deliv. Rev. 107 (2016) 367-392.

[7] A.G. Mikos, A.J. Thorsen, L.A. Czerwonka, Y. Bao, R. Langer, D.N. Winslow, J.P. Vacanti, Preparation and characterization of poly (L-lactic acid) foams, Polymer 35 (1994) 1068–1077.

[8] C. Schungens, V. Maquet, C. Grandfils, R. Jérôme and P. Teyssié, Polylactide macroporous biodegradable implants for cell transplantation. II. Preparation of polylactide foams by liquid-liquid phase separation, J. Biomed. Mater. Res., 30 (1996) 449–461.

[9] D.W. Hutmacher, Scaffolds in tissue engineering bone and cartilage, Biomaterials 21 (2000) 2529–2543.

[10] P.X. Ma, R. Zhang, Microtubular architecture of biodegradable polymer scaffolds, J. Biomed. Mater. Res. 56 (2001) 469–477.

[11] L. He, Q. Zuo, Y. Shi, W. Xue Microstructural characteristics and crystallization behaviors of poly(L-lactide) scaffolds by thermally induced phase separation, J. Appl. Polym. Sci. 131 (2014) 39436 (8 pages).

[12] P. van de Witte, H. Esselbrugge, A.M.P. Peters, P.J. Dijkstra, J. Feijen, R.J.J. Groenewegen, J. Smid, J. Olijslager, J.M. Schakenraad, M.J.D. Eenink, A.P. Sam, Formation of porous membranes for drug delivery systems, J. Control. Release 24 (1993) 61–78.

[13] T. Tanaka, D.R. Lloyd, Formation of poly(L-lactic acid) microfiltration membranes via thermally induced phase separation, J. Membr. Sci. 238 (2004) 65–73.

[14] A. Moriya, T. Maruyama, Y. Ohmukai, T. Sotani, H. Matsuyama, Preparation of poly(lactic acid) hollow fiber membranes via phase separation methods, J. Membr. Sci. 342 (2009) 307–312.

[15] D.R. Lloyd, S.S. Kim, K.E. Kinzer, Microporous membrane formation via thermally induced phase separation. II. Liquid–liquid phase separation, J. Membr. Sci. 64 (1991) 1–11.

[16] D.R. Lloyd, K.E. Kinzer, H.S. Tseng, Microporous membrane formation via thermally induced phase separation. I. Solid–liquid phase separation, J. Membr. Sci. 52 (1990) 239–261.

[17] T. Tanaka, T. Tsuchiya, H. Takahashi, M. Taniguchi, H. Ohara, D.R. Lloyd, Formation of biodegradable polyesters membranes via thermally induce phase separation, J. Chem. Eng. Jpn. 39 (2006) 144–153.

[18] T. Tanaka, M. Ueno, Y. Watanabe, T. Kouya, M. Taniguchi, D.R. Lloyd, Poly(L-lactic acid) microfiltration membrane formation via thermally induced phase separation with drying, J. Chem. Eng. Jpn. 44 (2011) 467–475.

[19] T. Tanaka, T. Nishimoto, K. Tsukamoto, M. Yoshida, T. Kouya, M. Taniguchi, D. R. Lloyd, Formation of depth filter microfiltration membranes of poly(L-lactic acid) via phase separation, J. Membr. Sci. 396 (2012) 101–109.

[20] A. Moriya, P. Shen, Y. Ohmukai, T. Maruyama, H. Matsuyama Reduction of fouling on poly(lactic acid) hollow fiber membranes by blending with poly(lactic acid)–polyethylene glycol–poly(lactic acid) triblock copolymers, J. Membr. Sci. 415–416 (2012) 712–717.

[21] W.C. Griffin, Calculation of HLB values of non-ionic surfactants, J. Soc. Cosmet. Chem. 5 (1954) 249–256.

[22] F.C. Lin, D.M. Wang, C.L. Lai, J.Y. Lai, Effect of surfactants on the structure of PMMA membranes, J. Membr. Sci. 123 (1997) 281–291.

[23] M. Amirilargani, E. Saljoughi, T. Mohammadi, Effects of Tween 80 concentration as a surfactant additive on morphology and permeability of flat sheet polyethersulfone (PES) membranes, Desalination 249 (2009) 837–842.

Chapter 2 Preparation of poly(L-lactic acid) microfiltration membranes by nonsolvent-induced phase separation method with the aid of surfactants

2.1. Introduction

In bioproduction processes, microfiltration membranes are often used to separate cells and cell residues [1]. Microfiltration membranes made from glassfiber [2] or conventional synthetic polymers such as polysulfone [3] have disposal problems after use. In recent years, poly(L-lactic acid) (PLLA) has attracted attention as a key material for sustainable technology because it is produced from biomass and readily degrades in composting processes and in natural environment [4–6]. Consequently, PLLA microfiltration membranes will reduce waste in food and biochemical industries by degrading via composting after use.

PLLA microfiltration membranes have previously been developed by several methods. The first PLLA microfiltration membranes were prepared by the thermally induced phase separation method from PLLA dissolved in 1,4-dioxane containing water. These membranes retained yeast cells (~5 μm in diameter) but allowed bacterial cells (~1 μm in diameter) to permeate [7]. The retention of bacterial cells was improved by inducing controlled evaporation from the surface of the polymer solution before cooling in the thermally induced phase separation procedure [8]. PLLA microfiltration membranes were also prepared from polymer solutions in dimethyl sulfoxide by nonsolvent and thermally induced phase separation methods. Dimethyl sulfoxide was used to dissolve 10 wt-% PLLA at temperatures of 60 °C and higher; however, the polymer solution did not form a membrane but instead formed PLLA particles via the thermally phase separation method. By using a combination of thermally and nonsolvent induced phase separations methods with a polymer solution at 80 °C and a coagulation water bath at 25°C, PLLA membranes that retained bacterial cells were prepared [9].

Although PLLA microfiltration membranes can be prepared by devising complex phase separation conditions, the practical manufacturing of PLLA membranes will be facilitated if simple nonsolvent induced phase separation methods can be developed. This study considers the development of PLLA microfiltration membranes formed via a nonsolvent induced phase separation method with the aid of surfactants. Surfactants are molecules with both hydrophilic and lipophlic groups and are typically used as solubilizers, detergents, emulsifiers, wetting and spreading agents, and antifoaming agents in accordance with their hydrophilic-lipophilic balance (HLB) values [10]. Among the surfactant class of compounds, polyoxyethylene (20)

sorbitan monooleate (Tween 80) is well known as a reagent to improve the structure of poly(methyl methacrylate), polyethersulfone, and poly(vinylidene fluoride) membranes [11–13]. This study reports mainly on the effect of Tween 80 in polymer solutions on the structure and performance of PLLA membranes. The stability and degradability of the membranes at ordinary and composting temperatures (25 and 60 °C) were also examined.

2.2. Experimental Methods

2.2.1. Materials

PLLA was a gift from Toyota Motor Corp. The PLLA properties included weight average molecular weight 1.22×10^5 ($M_w/M_n = 3.0$), optical purity 98.5%, melting point 174.0 °C, and glass transition temperature 59.7 °C. Analytical grade 1,4-dioxane was purchased from Wako Pure Chemical Industries. Seven kinds of surfactants, Tween 80 (polyoxyethylene (20) sorbitan monooleate), Tween 20 (polyoxyethylene (20) sorbitan monolaurate), Tween 40 (polyoxyethylene (20) sorbitan monopalmitate), Tween 60 (polyoxyethylene (20) sorbitan monostearate), polyoxyethylene (20) oleyl ether, Span 80 (sorbitan monooleate), and sodium dodecylsulfate (SDS), were purchased from Wako Pure Chemical Industries. Bovine serum albumin was obtained from Sigma-Aldrich. All chemicals were used without further purification.

2.2.2. Measurement of cloud points

The cloud points were measured by titration of water into PLLA–diluent–water system at 25±2 °C. The polymer was dissolved at 1–15 wt-% in diluent (1,4-dioxane containing 0–15 wt-% of Tween 80) to prepare a 50 g solution in a 100-cm^3 flask with a cork stopper covered with aluminum foil and poly(tetrafluoroethylene) tape and then water was added dropwise. The clouding composition was calculated from the cumulative amount of added water when the polymer solution remained cloudy or a gel formed around water droplet remained after 2 h stirring.

2.2.3. Membrane preparation

PLLA was dissolved in 1,4-dioxane containing a surfactant in a sealed 100 cm^3 flask. All solutions reported in this study were prepared on a wt-% basis. The mixture was first stirred with a PTFE stirring bar and warmed on the stirrer/hot plate at 80 °C for 8 h. The polymer

solution was cast on a glass plate with an 80 mm × 80 mm frame made from PTFE plate. The thickness of the frame was 0.5 mm. After removing excess polymer solution with the edge of another glass plate, the polymer solution on the glass plate was immersed in a coagulation bath of water at 25±2 °C and kept there for 2 h. The resulting membrane was removed from the glass plate, washed with water extensively, and kept in water prior to use.

2.2.4. Filtration experiments

A filtration cell (Amicon model 8010, 4.1 cm^2, Millipore, Bedford, MA) was used without its stirrer for dead-end filtration experiments as reported elsewhere [9]. Water was used to measure the permeation resistance of the membranes. The filtration was mainly performed at a transmembrane pressure of 10 kPa and at 25±2 °C. The membrane resistance, R_m, was calculated using Eqs. (1) and (2),

$$J = dv/dt \quad (1)$$

$$R_m = \Delta P/\mu J \quad (2)$$

where J, v, t, ΔP, and μ are permeation flux, permeation volume per unit filtration area, permeation time, transmembrane pressure, and viscosity of permeate, respectively. The viscosity of water at 25±2 °C was 0.89 mPa s [14].

Microbial cell suspensions of *Lactobacillus plantarum* NBRC15891T (0.7φ × 2.5 μm) were used to examine the retention of bacterial cells by the membranes as reported elsewhere [9]. The bacterium was cultured statistically in a modified MRS medium [15] where fish extract was substituted for meat extract. The culture broth after 17 h cultivation at 30 °C was diluted 10 times in most cases with 0.85 wt-% NaCl solution for filtration experiments. The wet cell concentration was 0.5 kg m^{-3}. The cell leakage was monitored with the absorbance at 660 nm of the initial 30 min permeate (or the initial 10 cm^3 permeate when the filtration finished within 30 min).

Bovine serum albumin (BSA, Sigma) was used to examine the permeation of protein molecules through the membranes. The protein was dissolved at 100 g m^{-3} in 0.1 M sodium-phosphate buffer (pH 6.8). The protein concentration of permeates was determined by BCA Protein Assay Kit (Pierce).

2.2.5. Scanning electron microscopy

The membrane was immersed in liquid nitrogen and then fractured. It was mounted vertically and horizontally on a sample holder. The surface of the sample was coated with gold-palladium using a sputter coater (MSP-1S, Vacuum Device). A scanning electron microscope (TM-1000, Hitachi) with an accelerating voltage of 15 kV was used to examine the membrane cross-sections and surfaces.

2.2.6. Degradation experiments

The change of mechanical strength over time for samples incubated in wet conditions of 25 or 60ºC was measured to evaluate non-enzymatic hydrolysis of the PLLA membranes. The PLLA membranes, the thicknesses of which were 0.25–0.35 mm, were cut into rectangular strips of 50 mm ×10 mm to prepare tensile specimens. The membranes were maintained in wet conditions in sealed glass dishes with wet wiping paper. The membranes were incubated at 25 or 60 °C for a maximum of 28 days.

The mechanical strength was evaluated from breaking elongation by tensile testing. The tensile testing was performed at 25±2 °C under wet conditions with a desk-top tensile testing machine (EZ-S-500N, Shimadzu Corp.). The gauge length and crosshead speed were set at 30 mm and 1.0 mm min^{-1}, respectively. Tensile testing was performed at least three times using the same conditions.

2.3. Results and discussion

2.3.1. Cloud points of PLLA–(1,4-dioxane–Tween 80)–Water system

In the preparation of polymer membranes by phase separation methods, it is important to understand the phase behavior of the polymer solution. Fig. 2-1 shows the composition of the cloud point calculated from data generated by titration of water into PLLA solutions in 1,4-dioxane containing Tween 80. Increasing the Tween 80 concentration decreased the concentration of water necessary for clouding. Tween 80 acted as a nonsolvent in the PLLA-1,4-dioxane mixture. Fig. 2-1 suggests that instantaneous phase separation will occur in the nonsolvent-induced phase separation process at high concentrations of Tween 80.

A binodal curve calculated from the thermally induced phase separation experiments without surfactants [16] is also depicted in Fig. 2-1. The curve is similar to those from the

thermally phase experiments at 25 °C by van de Witte *et al*. [17] and the theoretical calculations at 30 °C by Mannella *et al*. [18]. The binodal curves exist on the right side of the experimental data in this study. The cloud points experimentally determined at 25 °C by Xing *et al.* [19] existed near the bionodal curves. The difference between the experimentally determined cloud points in this study and the previously reported binodal curves are attributed to the fact that the cloud points in Fig. 2-1 do not show the equilibrium state. The gel residue formed around a water droplet was difficult to dissolve in the experiments, suggesting that the mixture did not reach thermodynamic equilibrium. However, the author's experimental data are informative to compare the effect of the surfactant concentration on the phase behavior because the cloud points are judged by the same criteria. Moreover, since membrane formation is a kinetic process, the phase on the surface of the polymer solution in membrane preparation would not reach thermodynamic equilibrium during the solidification as the gel formed around a water droplet was difficult to dissolve. The difficulty in dissolving the gel is attributed to the high optical purity of the PLLA (98.5%) used in this study while that in the study of Xing *et al*. [19] was 95.4%.

The clouding of PLLA-1,4-dioxane-water system was examined in the view points of thermodynamics and kinetics. A mixture containing 9.3 wt-% PLLA, 83.3 wt-% 1,4-dioxane, and 7.4 wt-% water was cloudy and had gels at 25 °C after the preparation of the mixture by adding 4 g of water to a PLLA solution containing 5 g of PLLA and 45 g of 1,4-dioxane. The cloudiness and gels remained after 30-min stirring (Fig. 2-2(a) in Supporting Information. In the supplementary experiments the stirring time was 30 min instead of 2 h in those of Fig. 2-1 because the clouding points had been determined.). The water concentration in the mixture was lower than the binodal curve calculated from the experiments of thermally induced phase separation and higher than the curve in the titration experiments (Fig. 2-1). The mixture became clear after 2-min incubation in a water bath at 80 °C (Fig. 2-2(b)). The clearness remained after it was cooled in a water bath at 25 °C (Fig. 2-2(c)) and then kept in an incubator at 25 °C for 24 h (Fig. 2-2(d)), meaning that the phase of the mixture did not separate thermodynamically. Thus the clouding of polymer solutions partly depended on the kinetics in the mixture containing the PLLA of highly optical purity used in the study.

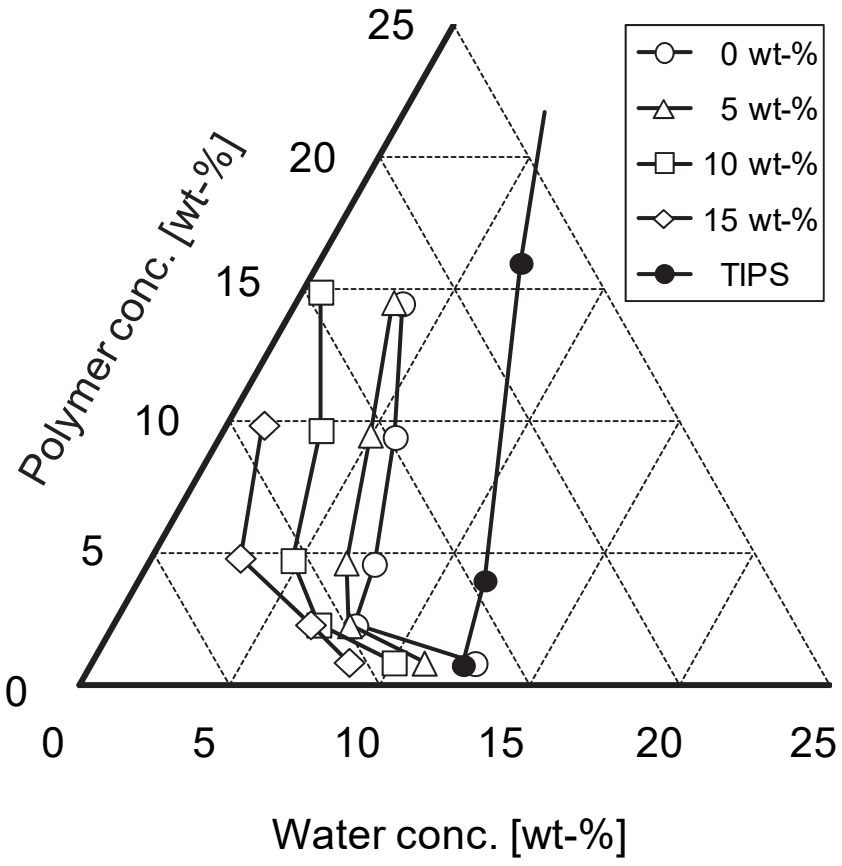

Fig. 2-1 Experimental cloud points of PLLA–(1,4-dioxane–Tween 80)–Water system at difference concentrations of Tween 80 (0–15 wt-%). The closed circles show the phase separation compositions calculated from the data of thermally induced phase separation of PLLA solutions without Tween 80.

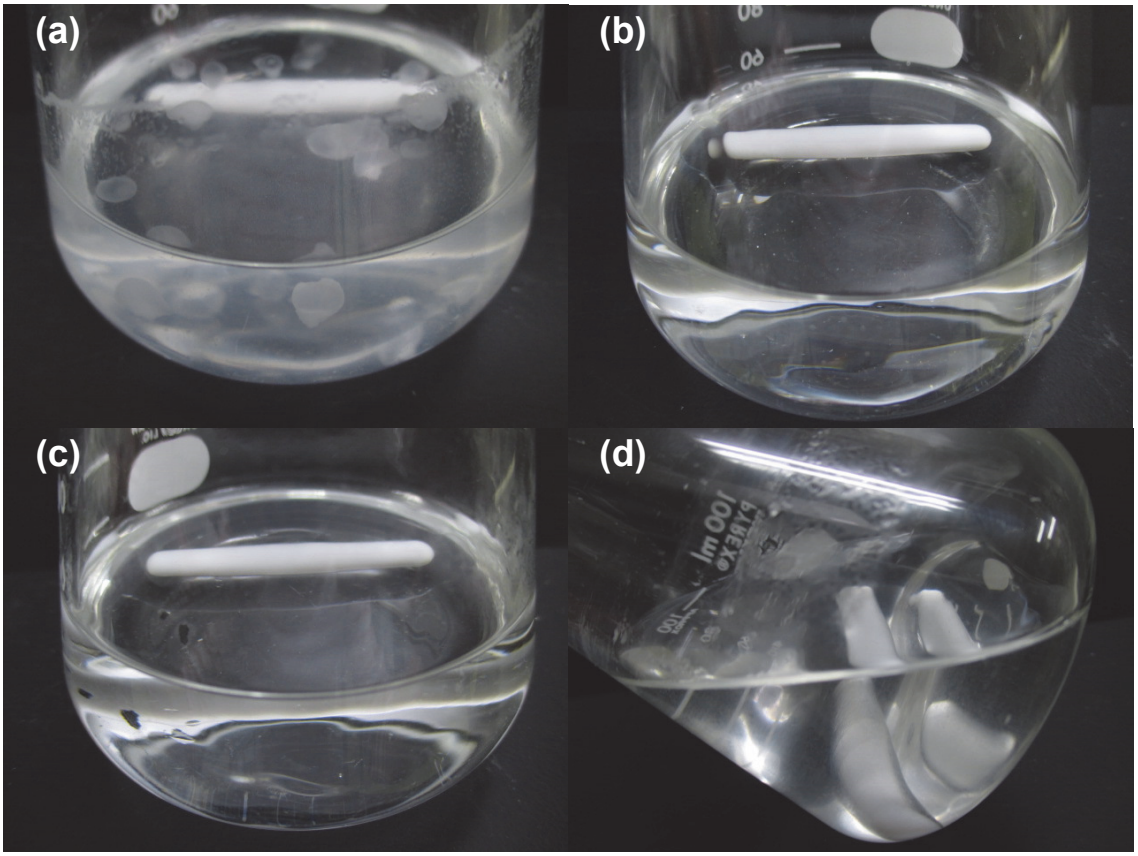

Fig. 2-2 Thermodynamic experiments with a PLLA solution in 1,4-dioxane containing water (PLLA:1,4-dioxane:water = 9.3 wt-%:83.3 wt-%:7.4 wt-%). (a) After 30-min stirring at 25 °C, (b) after 2-min heating at 80 °C, (c) after 30-min cooling at 25 °C, (d) after 24-h incubation at 25 °C.

2.3.2. Effect of addition of Tween 80 on membrane structure

Fig. 2-3 shows a PLLA membrane prepared from a 10 wt-% PLLA solution in 1,4-dioxane without any surfactants using a square mold of 0.5 mm (500 μm) in depth. The membrane was porous with a dense top surface layer and shrank to one sixth in the direction of thickness although the shrinkage was negligible in the horizontal directions. In addition there were some round defects (200–500 μm in diameter) in the membrane (Fig. 2-3(b)). The defects, which are attributed to dissolved air in the polymer solution, formed reproducibly from the solutions of the same composition although no similar defects were observed in the membranes prepared from the polymer solutions in 1,4-dioxane containing 5–15 wt-% Tween 80, as discussed below. A dense layer near the top surface of a membrane is usually observed in delayed phase separations [20,21]. The thickness shrinkage of the membrane is most likely due to the higher

extraction rate of 1,4-dioxane than that of diffusion of the water in the polymer solution. The shrinkage is discussed below.

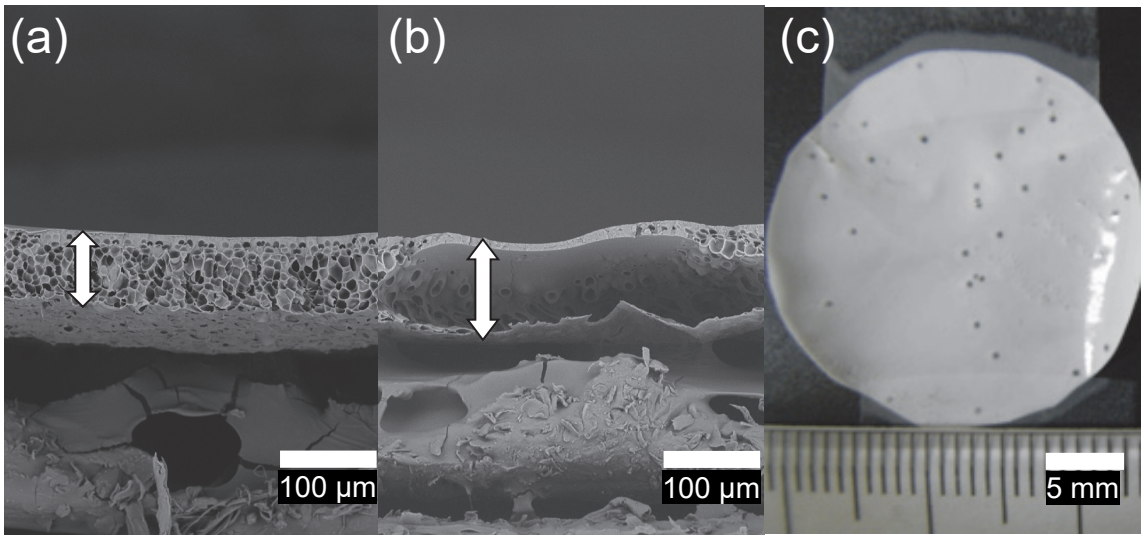

Fig. 2-3 The structures of PLLA membrane prepared from 10 wt-% PLLA solution in 1,4-dioxane without surfactant. (a) Cross-section, (b) cross-section with a defect, and (c) top surface after filtration of water at 10 kPa. Double-headed arrows show membrane thicknesses.

Xing *et al*. [19] reported formation of finger-like structures in a PLLA membrane prepared from a polymer solution in 1,4-dioxane; unfortunately they did not report on membrane thickness decrease nor the properties of the membranes as filtration membranes. They prepared PLLA membranes by casting a 10 wt-% PLLA solution onto a glass plate at 80 °C and then immersing it in a water bath at 25 °C, suggesting that nonsolvent-induced and thermally induced phase separations occurred simultaneously in the polymer solution. The difference in membrane structures between their study and the work reported here is mainly due to the different phase separation methods and partially due to the different clouding properties of the polymers of different optical purities (Fig. 2-1). Formation of finger-like structures in a PLLA membrane has been reported from a polymer solution in dimethylsulfoxide in nonsolvent and thermally induced phase separation elsewhere [9,22].

An attempt was made to form microporous PLLA structures by adding water to the polymer solution. The concentrations of PLLA and water were adjusted to 10 wt-% and 4.5 wt-% to

encourage instantaneous phase separation based on the results in Fig. 2-1. However, the membranes formed in this way shrank in thickness just they had in the experiments without the addition of water (Fig. 2-4(a)). A subsequent attempt to control membrane structures by the addition of surfactants to the polymer solutions led to the membranes shown in Fig. 2-4. Figs. 2-4(b)–(d) show the cross sections of PLLA membrane prepared from 10 wt-% polymer solutions in 1,4-dioxane containing 5–15 wt-% Tween 80. All the membranes were porous and did not contain the defects that were observed in the membrane prepared without Tween 80 shown in Fig. 2-3(b). The membrane thickness increased with the increase in Tween 80 content in the polymer solutions. The membranes were asymmetric and had finger like structures when the membranes were prepared from PLLA solutions in 1,4-dioxane containing 10–15 wt-% Tween 80.

There are two possible reasons for the change in membrane structure. One is the instantaneous demixing due to the decrease in distance between the initial and clouded polymer solutions in the phase diagram (Fig. 2-1) [20,21]. The other possible reason is the increase in diffusion rate of water in the polymer solution by the addition of surfactant. The diffusion rate of water became comparable to the extraction rate of 1,4-dioxane from the polymer solution and thus the membrane thickness remained the depth of mold (500 μm). A similar phenomenon was observed in the formation of PMMA membrane from polymer solutions in acetone containing Tween 80 by immersing the cast solutions in water [11]. Lai *et al*. also showed the addition of *n*-butanol and cyclohexanol into polymer solution causes finger-like structures in poly(4-methyl-1-pentene) (TPX) membranes prepared from polymer solution in cyclohexane although the addition of *n*-propanol remained a sponge-like structure [23]. Sawalha *et al*. reported that the addition of dodecane in polymer solution makes PLLA membrane more porous in the preparation of PLLA solution in dichloromethane with coagulation baths of methanol and water although no finger-like structures formed in their conditions [24].

Figs. 2-5 and 2-6 show the top and bottom surfaces of the PLLA membranes described in Figs. 2-3 and 2-4. The top surfaces were smooth while the bottom surfaces were rough as expected from the cross sections of the asymmetric membranes.

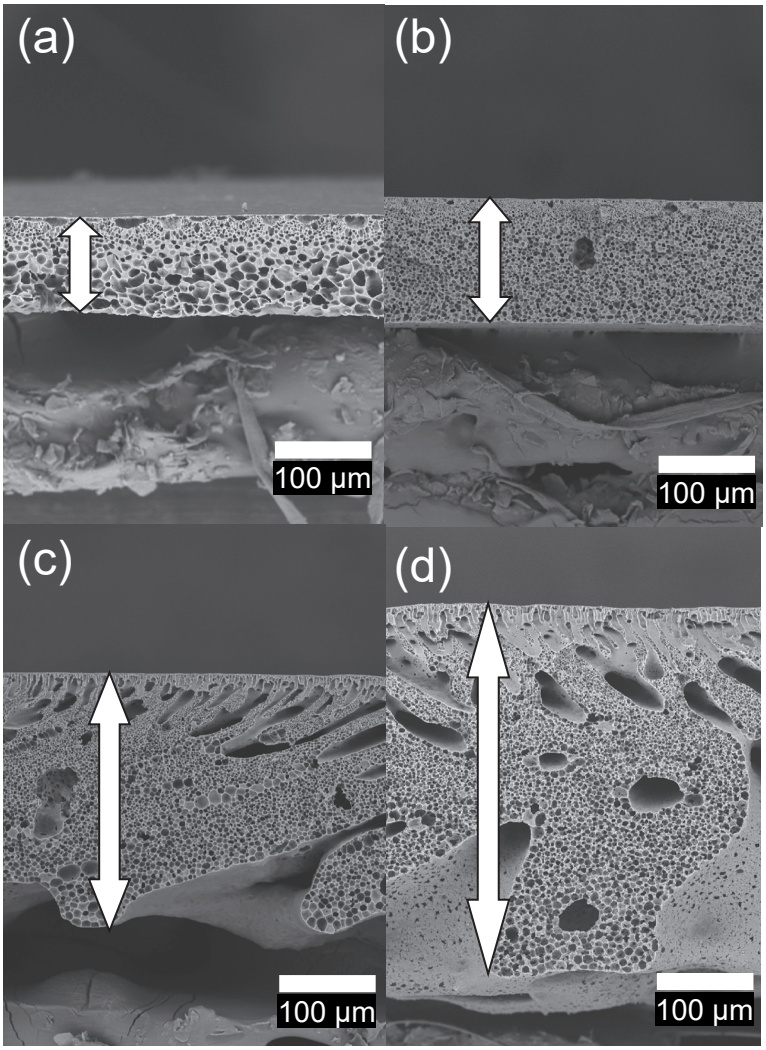

Fig. 2-4 Effect of water and Tween 80 in polymer solutions on the structure of PLLA membranes. Water was added to 1,4-dioxane at 5 wt-% only in the preparation of the membrane (a). Tween 80 concentrations in 1,4-dioxane were 0 wt-% (a), 5 wt-% (b), 10 wt-% (c), and 15 wt-% (d).

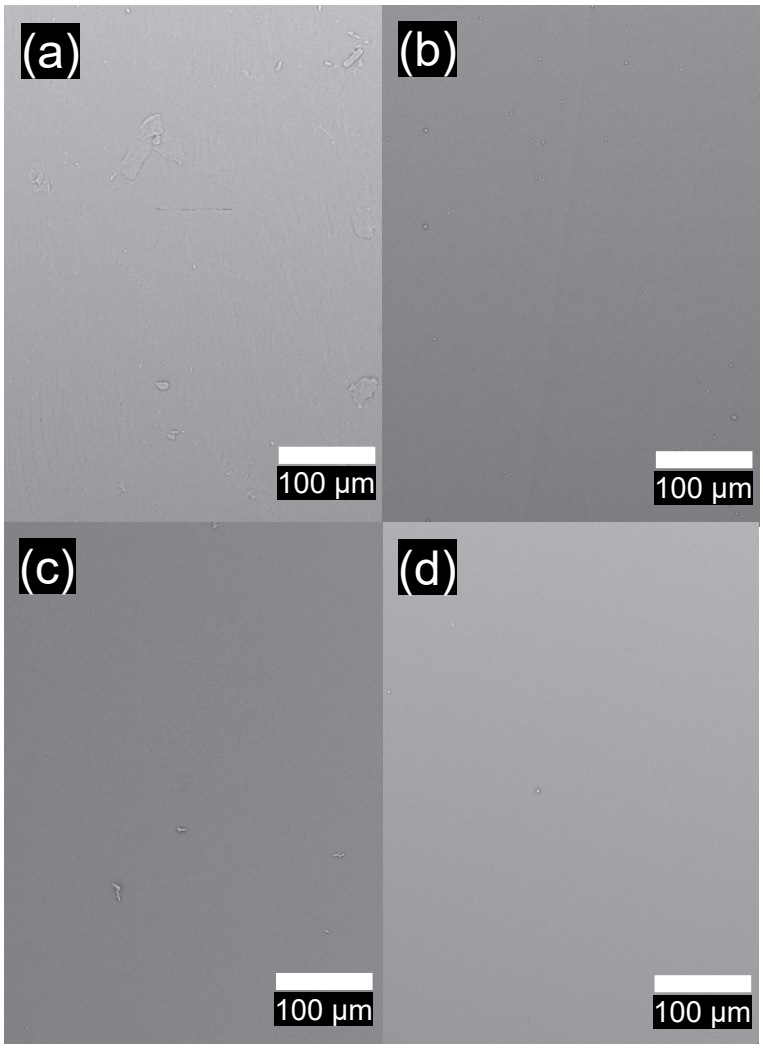

Fig. 2-5 Effect of Tween 80 on the top surface of PLLA membranes. Tween 80 concentrations in 1,4-dioxane were 0 wt-% (a), 5 wt-% (b), 10 wt-% (c), and 15 wt-% (d).

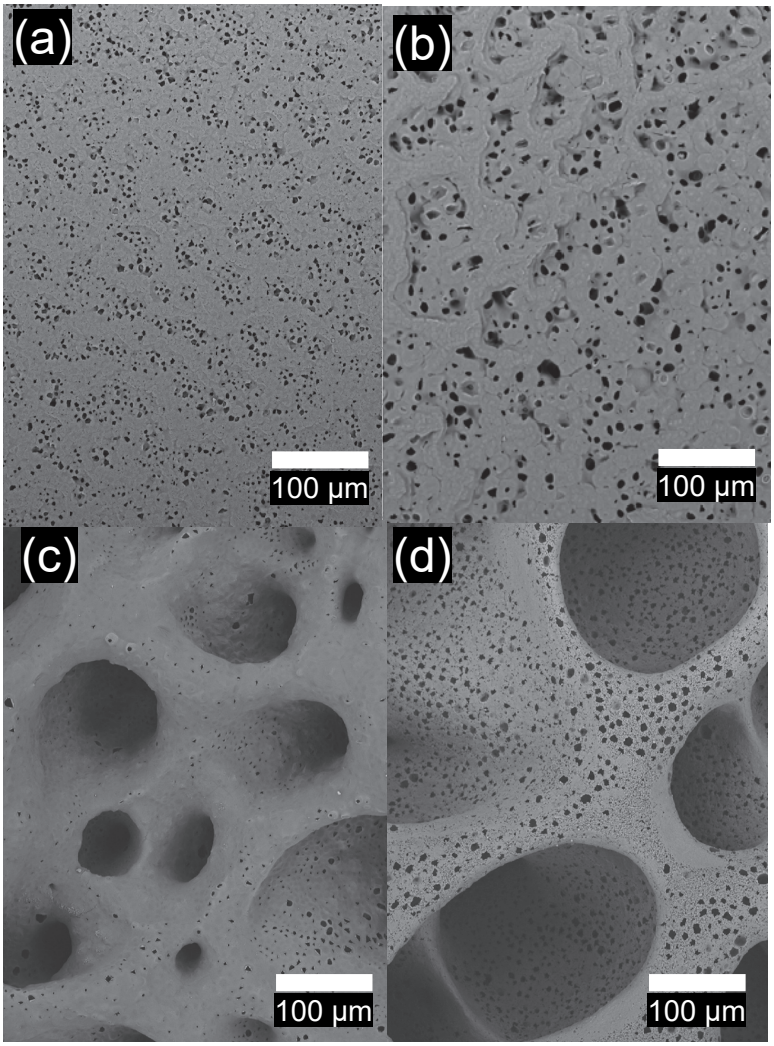

Fig. 2-6 Effect of Tween 80 on the bottom surface of PLLA membranes. Tween 80 concentrations in 1,4-dioxane were 0 wt-% (a), 5 wt-% (b), 10 wt-% (c), and 15 wt-% (d).

The effect of addition of Tween 80 on water intake was examined thermodynamically. A mixture containing 9.3 wt-% PLLA, 8.3 wt-% Tween 80, 75.0 wt-% 1,4-dioxane, and 7.4 wt-% water (the same PLLA and water concentrations as in Fig. 2-2) was cloudy and had gels at 25 °C after the preparation of the solutions by adding 4 g of water to a PLLA solution containing 5 g of PLLA, 4.5 g of Tween 80 and 40.5 g of 1,4-dioxane (Fig. 2-7). The mixture became clear after incubation in a water bath at 80 °C for 4 min (Fig. 2-7(b)). However, the mixture became cloudy again after it was cooled in a water bath at 25 °C (Fig. 2-7(c)). The clouded mixture solidified after 24-h incubation at 25 °C (Fig. 2-7(d)). Comparing the results

shown in Fig. 2-2 (without Tween 80) and Fig. 2-7 (with Tween 80), Tween 80 does not enhance but suppresses the water intake into the polymer solution thermodynamically. Thus the enhancement in water intake during the membrane formation by the addition of Tween 80 will be a kinetic phenomenon.

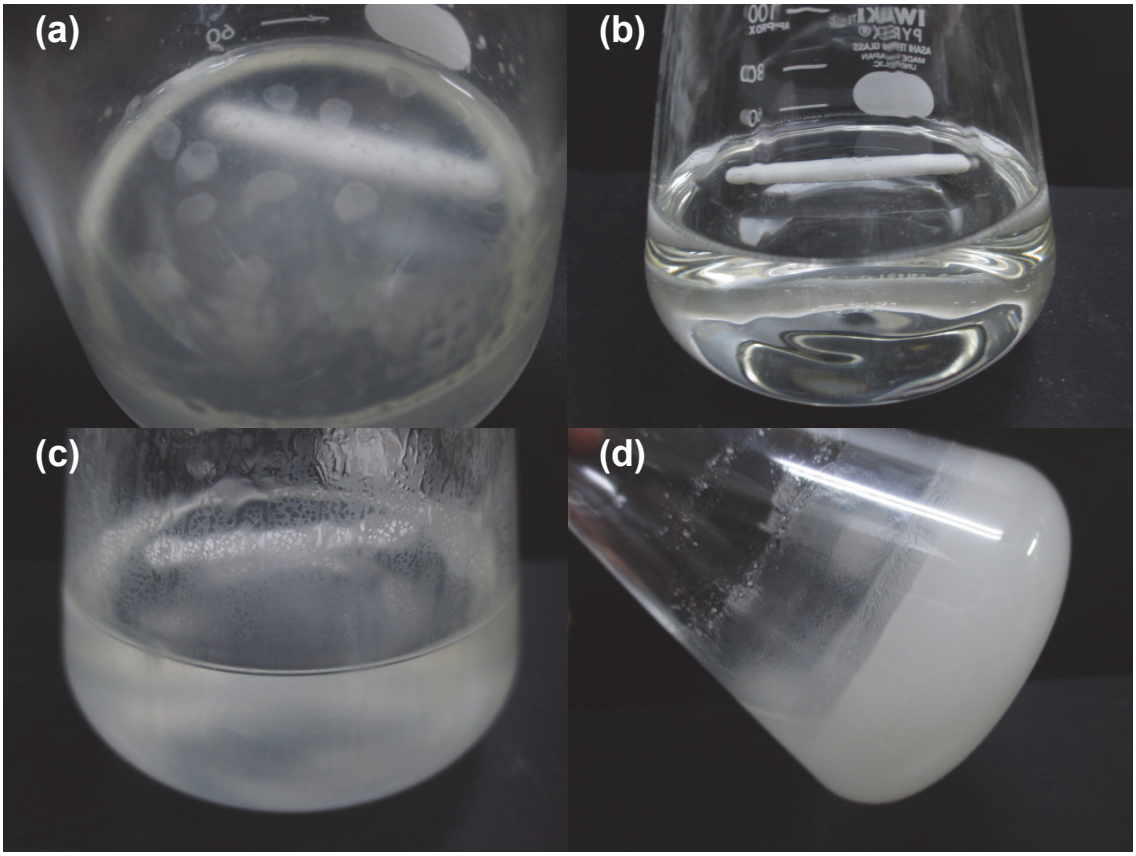

Fig. 2-7 Thermodynamic experiments with a PLLA solution in 1,4-dioxane containing Tween 80 and water (PLLA:1,4-dioxane:Tween 80:water = 9.3 wt-%:75.0 wt-%:8.3 wt-%:7.4 wt-%). (a) After 30-min stirring at 25 °C, (b) after 4-min heating at 80 °C, (c) after 30-min cooling at 25 °C, (d) after 24-h incubation at 25 °C.

2.3.3. Effect of addition of Tween 80 on membrane performance

Fig. 2-8 shows the membrane resistance and the bacterial cell retention in filtration with the PLLA microporous membranes prepared from 10 wt-% polymer solutions in 1,4-dioxane containing different concentrations of Tween 80. The membrane from the polymer solution without Tween 80 (0 wt-%) had low membrane resistance but the membrane after filtration at

10 kPa had many macropores (200–500 μm in diameter) (Fig. 2-3(c)) that seemed to be formed at the defects shown in Fig. 2-3(b). The membrane could not retain bacterial cells. The membrane resistance increased more than 100 times when Tween 80 was added at 5 wt-% in the 1,4-dioxane, suggesting poor connectivity of the microporous structure (Fig. 2-4(b)). Bacterial cell filtration could not be performed because of the high membrane resistance. When the Tween 80 concentration in 1,4-dioxane was 10–15 wt-%, the membrane resistance decreased to 1/100 of that of the membrane prepared from the polymer solution in 1,4-dioxane containing 5 wt-% Tween 80. The lower membrane resistance is due to the finger-like structures that connected the water flow from the top to the bottom of the membrane (Figs. 2-4(c) and (d)). The PLLA membrane prepared with 10 and 15 wt-% of Tween 80 retained 99 and 98% of the *L. plantarum* (lactic acid bacteria) cells (0.7φ × 2.5 μm), respectively.

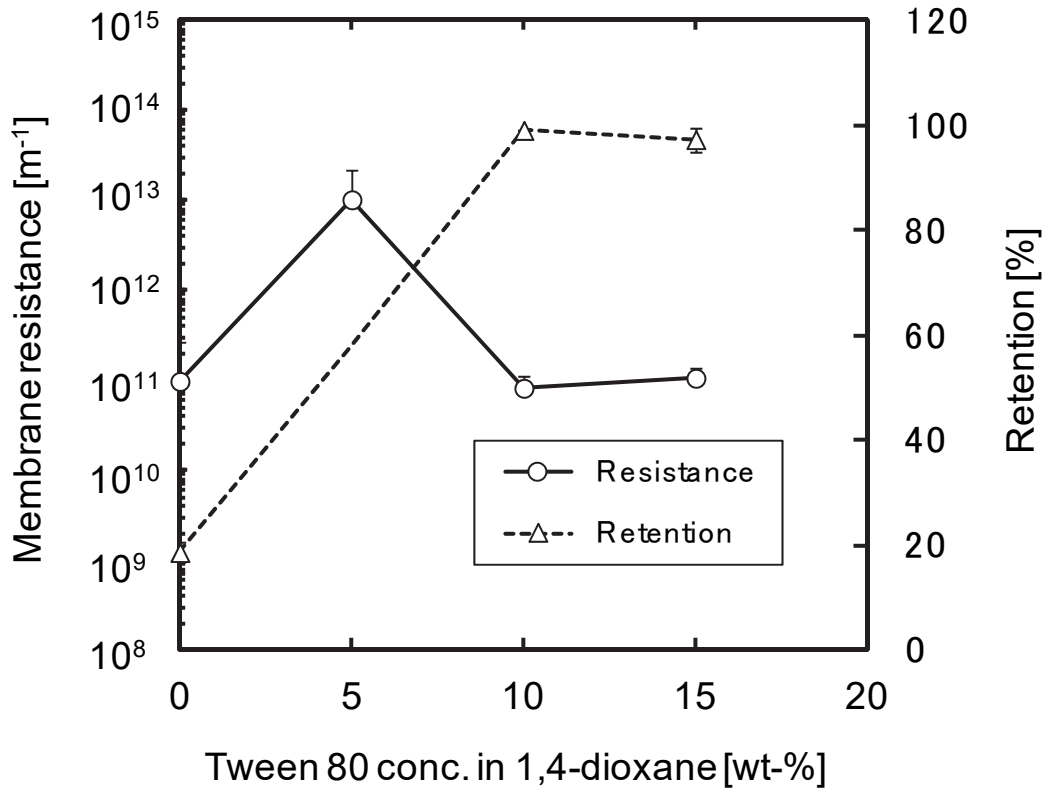

Fig. 2-8 Effect of Tween 80 concentration in 1,4-dioxane on the membrane resistance and the bacterial cell retention of PLLA membranes. Averages and standard deviations from 3–4 experiments are shown.

2.3.4. Effect of kinds of surfactants

The effect of several surfactants, including Tween 80, on the structure and performance of PLLA membranes was examined. Sodium dodecyl sulphate, an ionic surfactant, did not dissolve in 1,4-dioxane at 10 wt-%. Five non-ionic surfactants, Tween 20, Tween 40, Tween 60, polyoxythylene (20) oleyl ether, and Span 80, dissolved in 1,4-dioxane at 10 wt-%. Fig. 2-9 shows the cross-sections of the PLLA membranes prepared from 10 wt-% polymer solutions in 1,4-dioxane containing the non-ionic surfactants at 10 wt-%. The performances of the membranes are shown in Fig. 2-10. The structure of the membranes prepared with Tween 40 and Tween 60 had finger-like structures similarly to that prepared with 10 and 15 wt-% Tween 80 (Figs. 2-4(c) and (d)). The membranes showed low membrane resistance but bacterial cell retention decreased to 96–97%. On the other hand the membranes prepared with 1,4-dixoxane containing 10 wt-% Tween 20 and Span 80 were 150–200 μm in thickness and microporous with low connectivity although the membranes were thicker than that prepared without surfactants. The structures resembled that prepared with 1,4-dioxane containing 5 wt-% Tween 80 (Fig. 2-4(b)). The membrane resistances were too high to filter bacterial suspensions. The structure of the membrane prepared with polyoxyethylene (20) oleyl ether was intermediate between those with Tween 20 and Tween 40. The membrane was microporous and had large macrovoids well-connected with surrounding pores (Fig. 2-9(d)). The membrane showed low resistance and retained nearly 99% of the cells in the filtration of bacterial cells suspensions (Fig. 2-10).

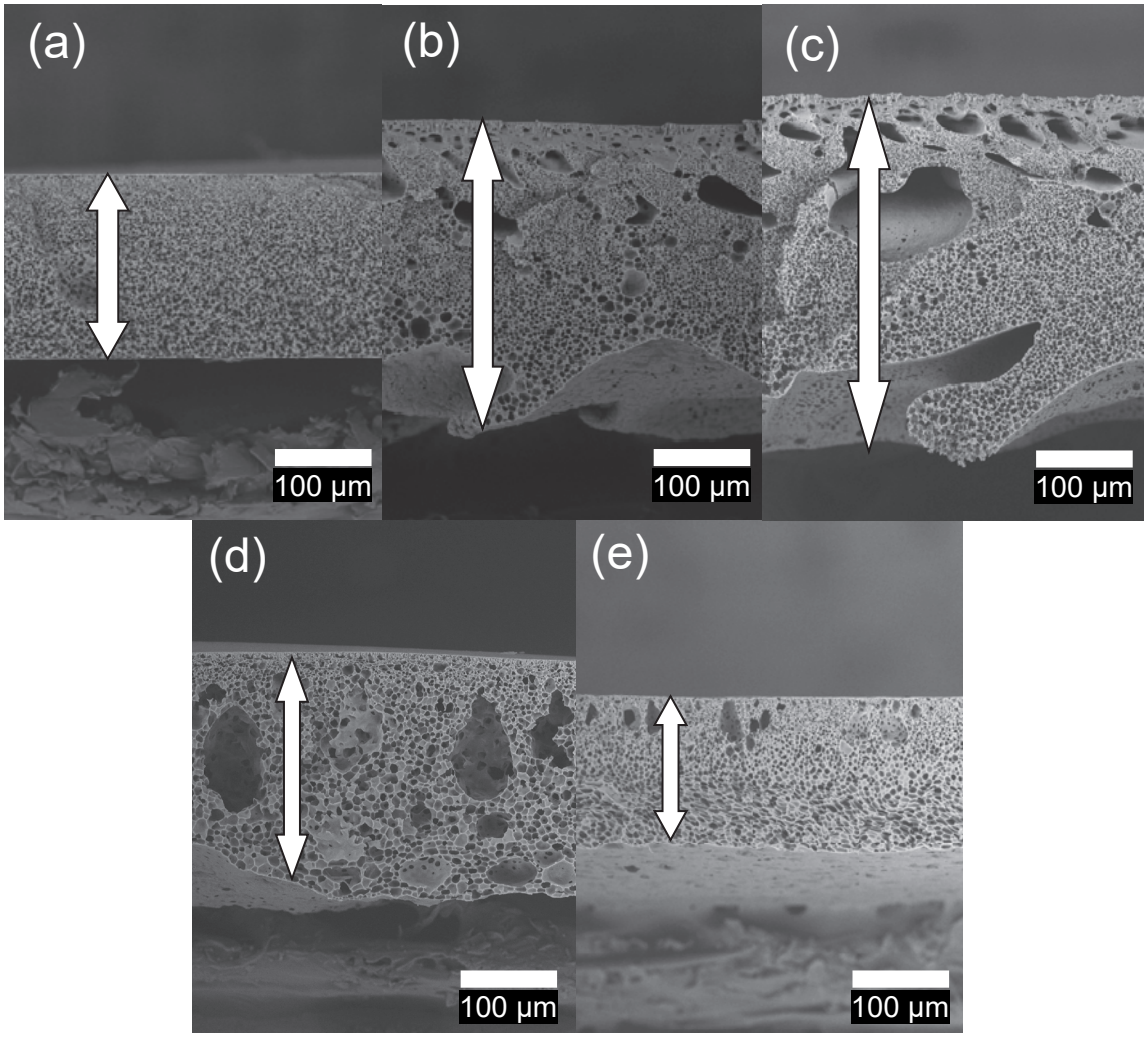

Fig. 2-9 Effect of kinds of surfactants on the structure of PLLA membranes. Tween 20 (a), Tween 40 (b), Tween 60 (c), and polyoxyethylene (20) oleyl ether (d), and Span 80 (e). Double-headed arrows show membrane thicknesses.

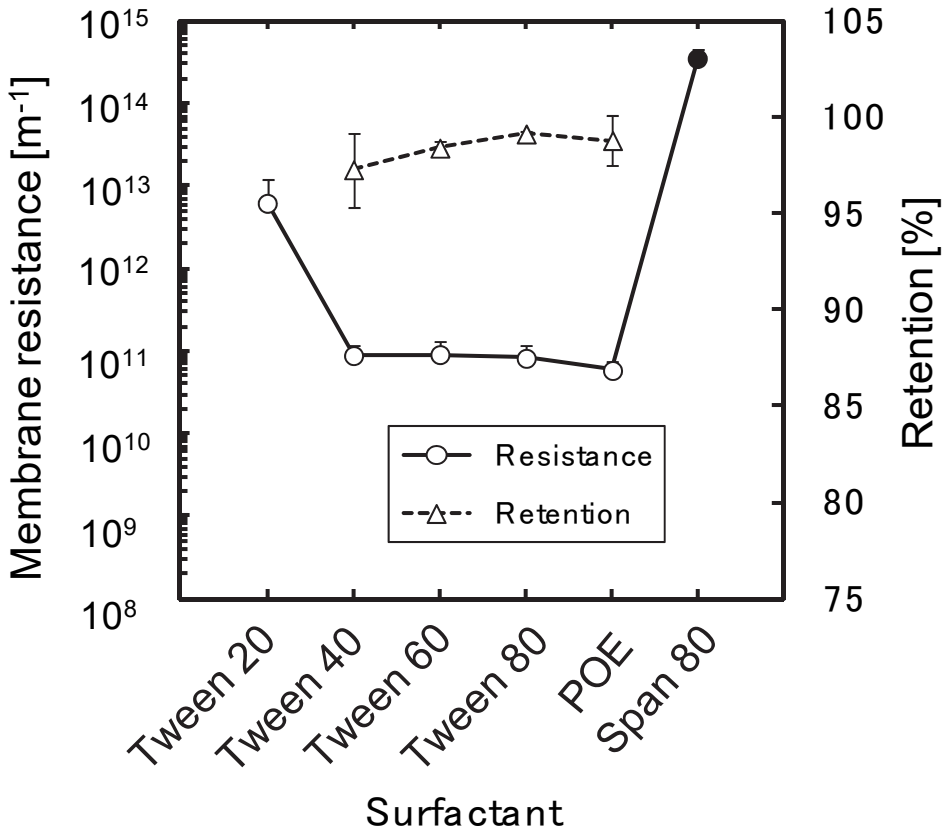

Fig. 2-10 Effect of kinds of surfactants on the performance of PLLA membranes. Surfactant concentrations in 1,4-dioxane are 10 wt-%. POE denotes polyoxyethylene (20) oleyl ether. The closed circle shows that the membrane resistance was estimated to be more than 4×10^{14} m^{-1} because the membrane was impermeable under the filtration conditions. Averages and standard deviations from 3–4 experiments are shown.

The dependence of membrane structure and performance on the HLB values of the surfactant was determined in this study. The HLB values of sodium dodecyl sulphate, Tween 20, Tween 40, Tween 60, Tween 80, polyoxyethylene (20) oleyl ether, and Span 80 were 40, 16.7, 15.6, 14.9, 15.0, 15.3, and 4.3, respectively [10,25]. Higher HLB values show greater hydrophilicity of the surfactant. In the preparation of the PLLA membrane from a PLLA solution in a water bath the diffusion rate of water molecules into the polymer solution should be enhanced to suppress the reduction in membrane thickness as described in Section 2.3.2. The lipophilic surfactant Span 80 did not work well. The ionic surfactant with a high HLB value, sodium dodecyl sulphate, could not be dissolved in 1,4-dioxane. The low performance of Tween 20 is due to the significant extraction of the high hydrophilic surfactant by water in a

coagulation bath although the details should be further examined to prove the hypothesis. The appropriate HLB values to reduce the PLLA membrane resistance were 14.9–15.6 in this study. Lin *et al*. showed the effect of HLB of the surfactant on the structure of PMMA membranes prepared from polymer solutions in acetone by nonsolvent induced phase separation method [11]. They reported that hydrophilic surfactants (Tween 85, Tween 80, Tween 20, and Brij 35; HLB = 11.0–16.9) formed macrovoids in the membrane when water was used as a coagulant while the lipophilic surfactants (Span 85, Span 80, Span 40, and Span 20; HLB = 1.8–8.6) formed macrovoids when *n*-hexane was used as a hydrophobic coagulant. Tween 80 was selected as the surfactant in the following study because of the low membrane resistance and the highest cell retention (Fig. 2-10).

2.3.5. Effect of polymer concentrations

Fig. 2-11 shows the structure of membranes prepared from 5 and 15 wt-% PLLA solutions in 1,4-dioxane containing 10 wt-% Tween 80. The PLLA membrane prepared from a 5 wt-% polymer solution was thin and macroporous (Figs. 2-11(a) and (c)). There were 2–5 μm pores on the flat top surface of the membrane (Fig. 2-11(b)). The membrane prepared from a 15 wt-% polymer solution was thick and had a finger-like structure similar to that from a 10 wt-% solution (Figs. 2-11(d)–(f)). However, the interconnectivity of the macrovoids seemed to be reduced (Fig. 2-11(d)).

The polymer concentration influenced the performance of the membrane (Fig. 2-12). The membrane prepared from a 5 wt-% polymer solution had low resistance and bacterial cell retention. The membrane had pores after filtration as well as that prepared from a 10 wt-% PLLA solution in 1,4-dioxane without any surfactants (Fig. 2-3). On the other hand the membrane resistance increased 10 times by the increase in polymer concentration from 10 to 15 wt-% although the cell retention was 99%. The reduced interconnectivity of the macrovoids (Fig. 2-11(d)) would increase the membrane resistance.

From the results shown above the author has chosen the preparation conditions of PLLA membranes as follows: the surfactant for addition to 1,4-dioxane = Tween 80; the concentration of the surfactant in the diluent = 10 wt-%; the polymer concentration = 10 wt-%. The membrane prepared with these conditions had low permeation resistance (high water permeability) and high retention in the filtration of bacterial cell suspensions. The resistance of the PLLA membrane (1×10^{11} m^{-1}) was comparable with those of the membrane developed for

the retention of bacterial cells before ($0.6–2\times10^{11}$ m^{-1}) [8,9]. However, the membrane preparation method at 25 °C in this study was easy compared with the methods with high temperature, quenching, and/or partial drying of polymer solutions. The retention of bovine serum albumin, a typical protein, was 0% in the filtration of a 100 g m^{-3} solution of the protein with the membrane prepared under the above conditions. The high permeability of the protein molecules and the high retention of bacterial cells indicate that the PLLA membrane functions as a microfiltration membrane.

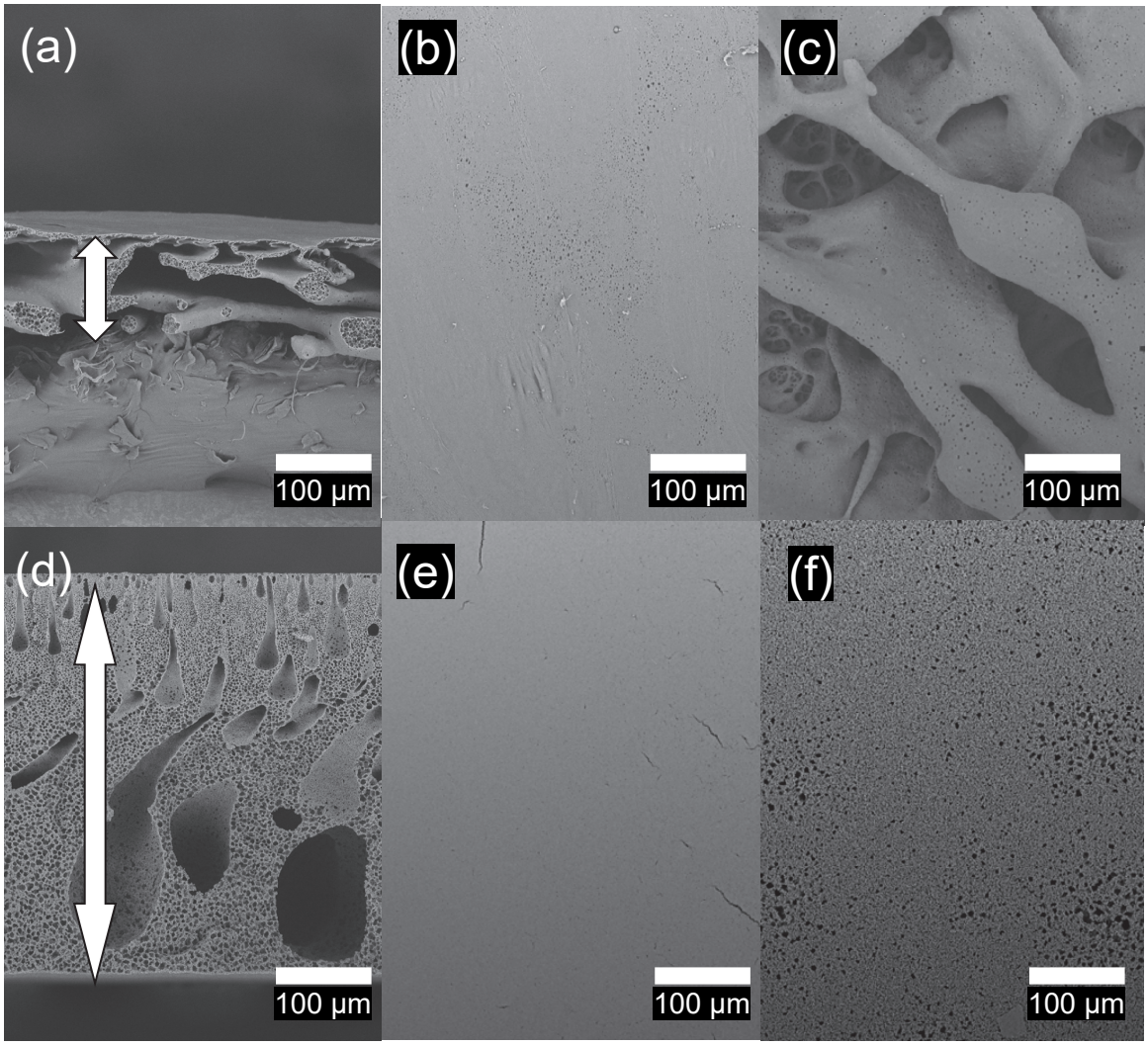

Fig. 2-11 Effect of polymer concentrations on the structure of PLLA membranes. Polymer concentrations were 5 wt-% (a–c) and 15 wt-% (d–f). (a, d) Cross-sections, (b, e) top surfaces, and (c, f) bottom surfaces. Double-headed arrows show membrane thicknesses.

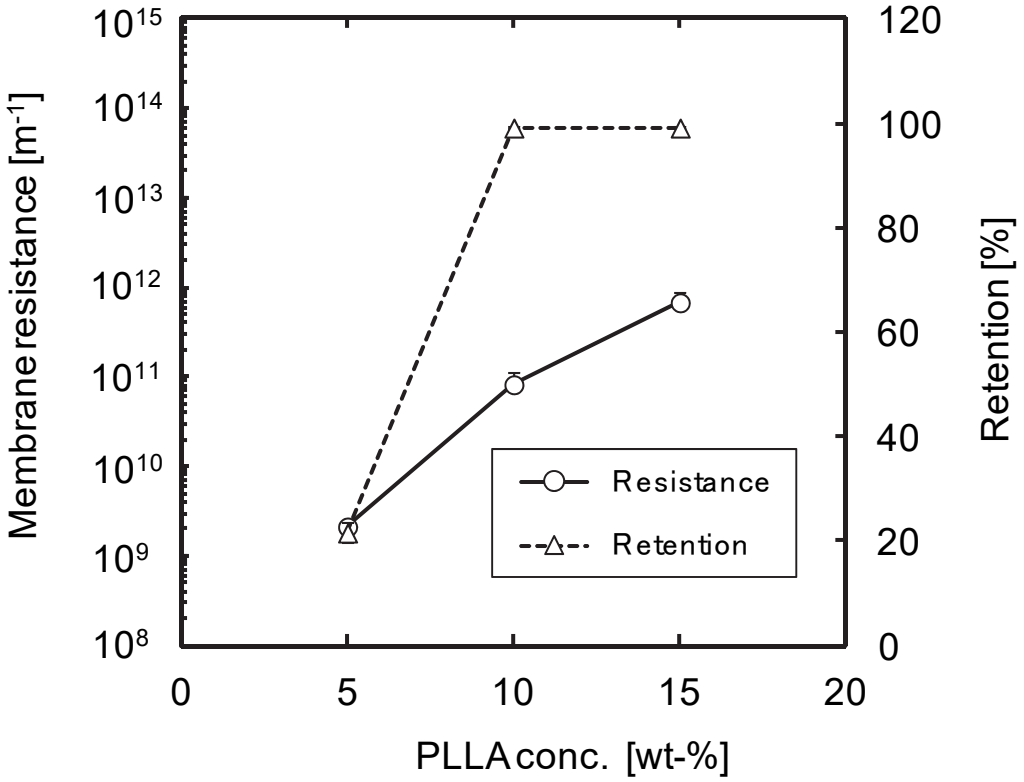

Fig. 2-12 Effect of polymer concentration on the membrane resistance and the bacteria retention of PLLA membranes. Averages and standard deviations from 3–4 experiments are shown.

2.3.6. Filtration characteristics

Figs. 2-13 and 2-14 show the filtration of *L. plantarum* cell suspensions by the PLLA membranes at different conditions (10–100 kPa, 0.5–2.5 kg-wet cells m^{-3}). The filtration rate decreased with an increase in cell concentration and increased with an increase in transmembrane pressure as generally observed in microfiltration [26]. Filter cakes were observed on the membrane after these filtrations. The cell retention was 99% or more at the cell concentrations and transmembrane pressures.

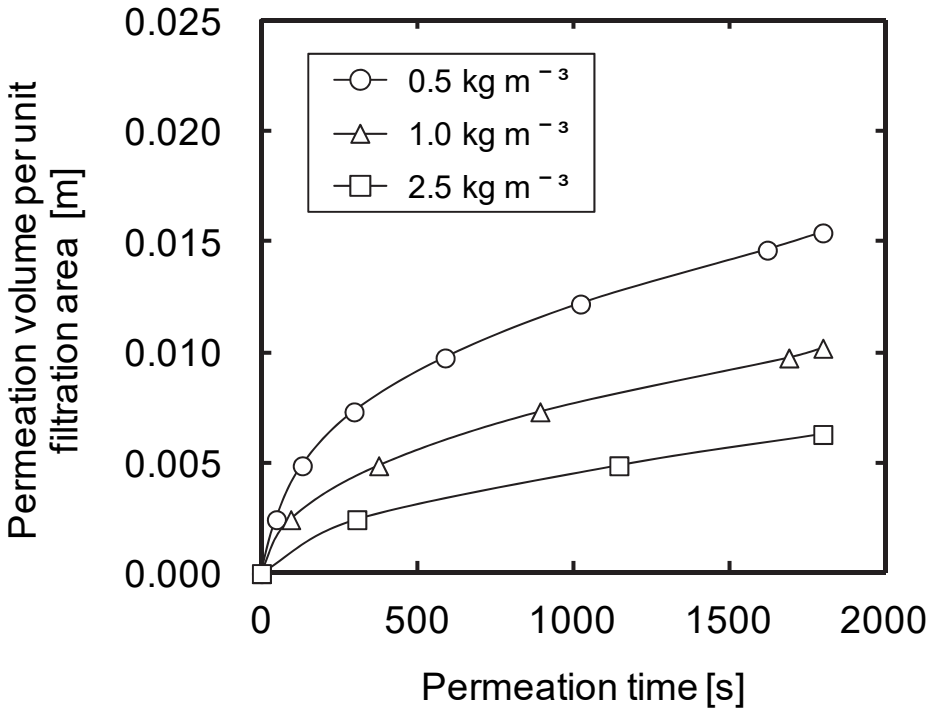

Fig. 2-13 Filtration of bacterial cell suspension at different cell concentrations.

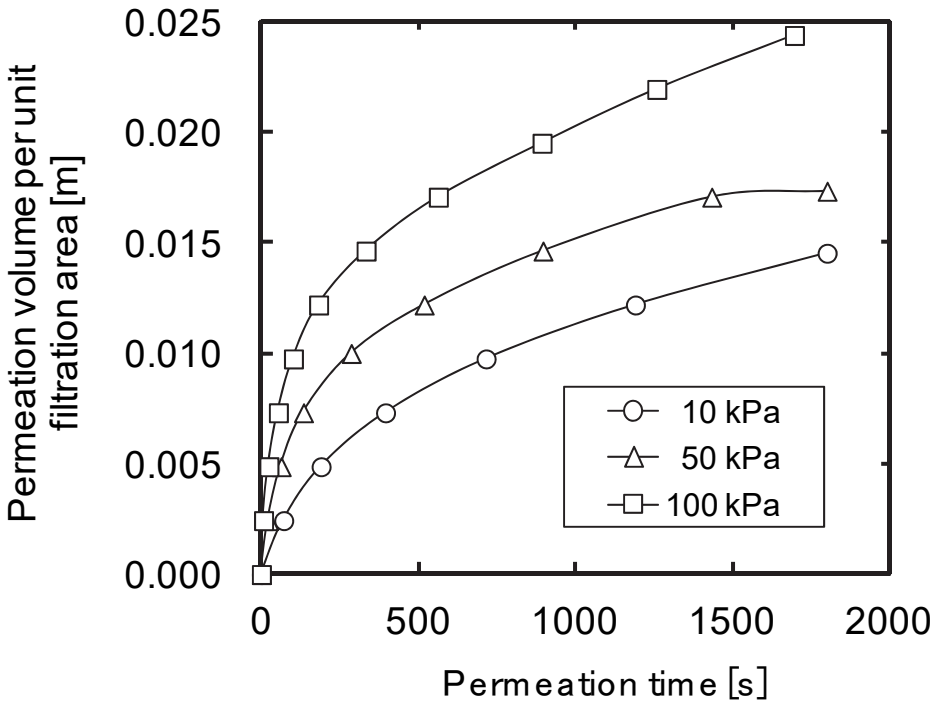

Fig. 2-14 Filtration of bacterial cell suspension at different transmembrane pressures.

2.3.7. Degradability of PLLA membranes

The degradability of the PLLA membrane was investigated by tensile testing. Fig. 2-15 shows the typical stress-strain curves of the membranes. The elongation at break, 0.1, of the PLLA membrane in this study before incubation was lower than that of the membranes prepared by the combined nonsolvent and thermally induced phase separation method (0.3) [9] but higher than that of the membranes prepared by thermally induced phase separation with drying (0.03) [8]. The elongation at break of the membrane decreased to one third of the original one after the incubation at 60 °C for 3 days under wet conditions. Fig. 2-16 shows the time courses of the elongation at break for 28 days at 25 and 60 °C. The elongation at break of the membranes decreased only 20% after 28 days at 25 °C. The mechanical stability was better than the PLLA membrane developed and tested earlier [9]. On the other hand the elongation of membranes incubated at 60 °C decreased promptly. The membranes after incubation 14 days were too fragile to be handled. These results mean that the PLLA membrane is stable at 25 °C even

under wet conditions and degradable at 60 °C under wet conditions even without any microbes. The non-enzymatic degradability will facilitate the degradation after use in composting where the temperature is around 60 °C [27]. The change in membrane performances was also examined at 25 °C in a different set of experiments from those in Fig. 2-16. The membrane resistance increased 3.5 times and the retention of bacteria decreased to 97% after the incubation of 28 days in wet conditions at 25 °C (Fig. 2-17). However, the membrane resistance increased only 2.2 times and the retention of bacteria was 99% after the incubation of 7 days. The results suggest that the PLLA membrane is durable in water during the use of 1–3 days although the membrane should be replaced before the period due to membrane fouling during filtration in biochemical processes. The PLLA membrane is useful as a microfiltration membrane in solid-liquid separation and then disposable bioprocessing [28,29].

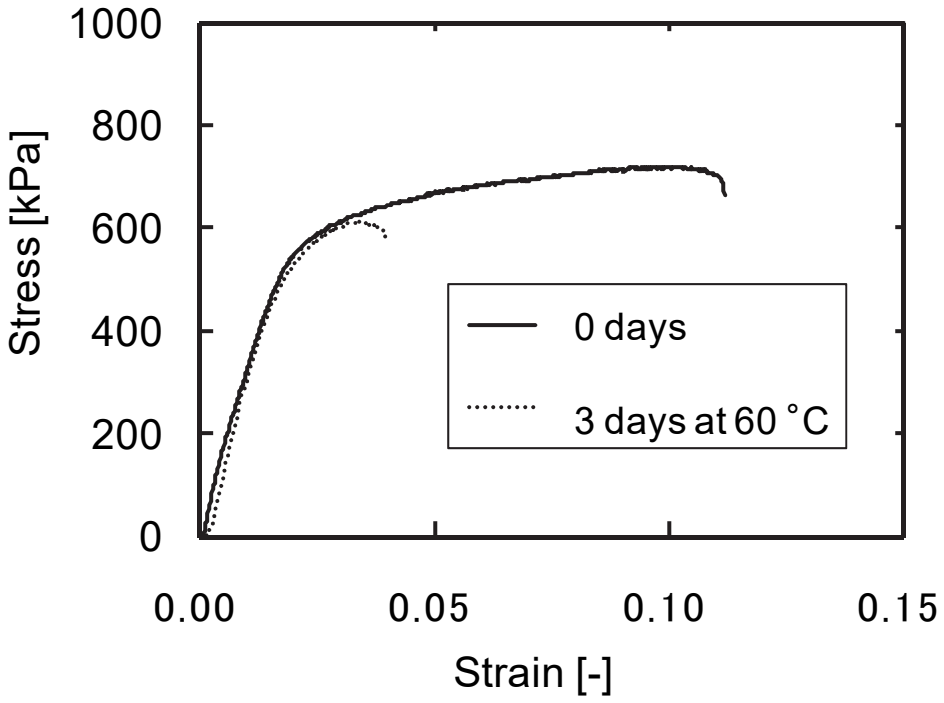

Fig. 2-15 Typical stress-strain curves of PLLA membranes in tensile testing.

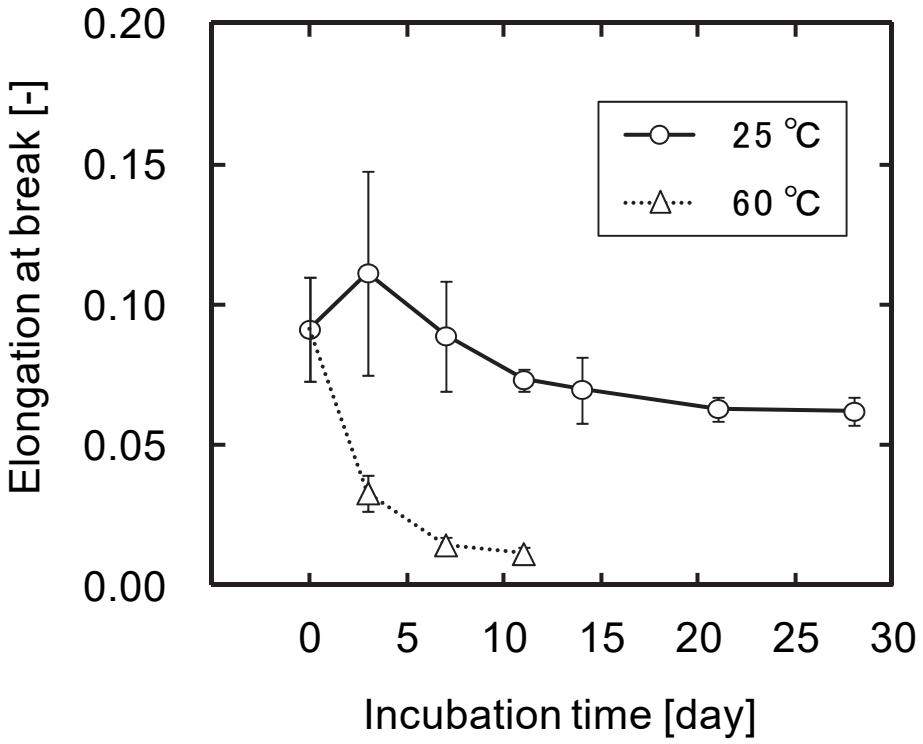

Fig. 2-16 Change in elongation at break of PLLA membranes at 25 and 60 °C in wet conditions.

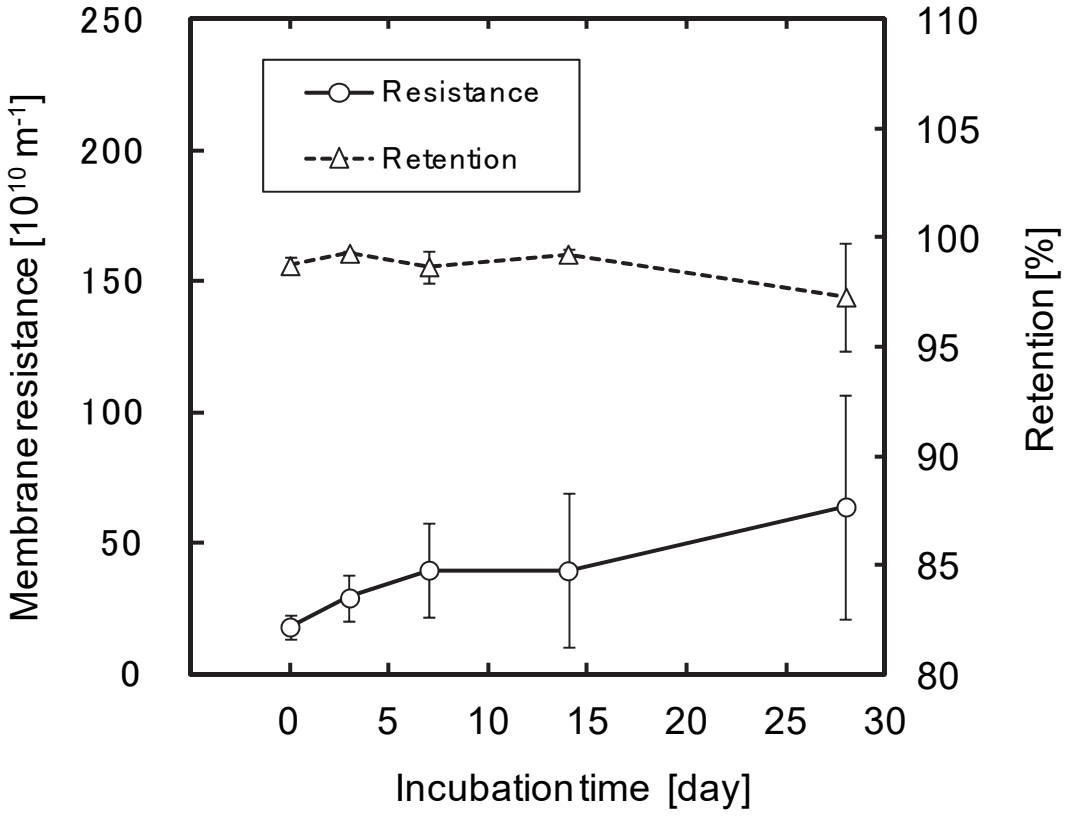

Fig. 2-17 Change in membrane resistance and bacterial cell retention of PLLA membranes at 25 °C in wet conditions. The membranes were prepared from 10 wt-% PLLA solution in 1,4-dioxane containing 10 wt-% Tween 80.

2.4. Conclusions

A microfiltration membrane of PLLA has been developed using nonsolvent-induced phase separation method with the aid of surfactants at 25 °C. Tween 80 was the best surfactant to prepare a microfiltration membrane among the surfactants examined in this study. The surfactant facilitated the instantaneous phase separation and the diffusion of water molecules in polymer solutions in a water bath. The PLLA membrane had asymmetric finger-like structures and showed low membrane resistance, high retention of bacterial cells, and high permeability of protein molecules when the membrane was prepared from a 10 wt-% PLLA solution in 1,4-dioxane containing 10 wt-% Tween 80. The membrane was mechanically stable at 25 °C but degradable at 60 °C in wet conditions. The PLLA membrane would be applied as a compostable microfiltration membrane.

2.5. References

[1] R. van Reis, A. Zydney, Bioprocess membrane technology, J. Membr. Sci. 297 (2007) 16–50.

[2] G.R. Bolton, D. LaCasse, M.J. Lazzara, R. Kuriyel, The fiber-coating model of biopharmaceutical depth filtration, AIChE J. 51 (2005) 2978–2987.

[3] M. Yoshida, M. Taniguchi, T. Tanaka, Microfiltration of bacterial cell suspension with an asymmetric depth filter, Kagaku Kogaku Ronbunshu 36 (2010) 494–500.

[4] R.A. Gross, B. Kalra, Biodegradable polymers for the environment, Science 297 (2002) 803–807.

[5] J. Lunt, Large-scale production, properties and commercial applications of polylactic acid polymers, Polym. Degrad. Stab. 59 (1998) 145–152.

[6] A. Torres, S.M. Li, S. Roussos, M. Vert, Poly(lactic acid) Degradation in soil or under controlled conditions, J. Appl. Polym. Sci. 62 (1996) 2295–2302.

[7] T. Tanaka, D.R. Lloyd, Formation of poly(L-lactic acid) microfiltration membranes via thermally induced phase separation, J. Membr. Sci. 238 (2004) 65–73.

[8] T. Tanaka, M. Ueno, Y. Watanabe, T. Kouya, M. Taniguchi, D.R. Lloyd, Poly(L-lactic acid) microfiltration membrane formation via thermally induced phase separation with drying, J. Chem. Eng. Jpn. 44 (2011) 467–475.

[9] T. Tanaka, T. Nishimoto, K. Tsukamoto, M. Yoshida, T. Kouya, M. Taniguchi, D.R. Lloyd, Formation of depth filter microfiltration membranes of poly(L-lactic acid) via phase separation, J. Membr. Sci. 396 (2012) 101–109.

[10] W.C. Griffin, Calculation of HLB values of non-ionic surfactants, J. Soc. Cosmet. Chem. 5 (1954) 249–256.

[11] F.C. Lin, D.M. Wang, C.L. Lai, J.Y. Lai, Effect of surfactants on the structure of PMMA membranes, J. Membr. Sci. 123 (1997) 281–291.

[12] M. Amirilargani, E. Saljoughi, T. Mohammadi, Effects of Tween 80 concentration as a surfactant additive on morphology and permeability of flat sheet polyethersulfone (PES) membranes, Desalination 249 (2009) 837–842.

[13] P.Y. Zhang, H. Yang, Z.L. Xu, Preparation of polyvinylidene fluoride (PVDF) membranes via nonsolvent induced phase separation process using a Tween 80 and H_2O mixture as an additive, Ind. Eng. Chem. Res. 51 (2012) 4388−4396.

[14] W.M. Haynes (Eds.), CRC Handbook of Chemistry and Physics, 93rd ed., CRC Press, Boca Ration, 2012, p. 6-1.

[15] J.C. de Man, M. Rogosa, M.E. Sharpe, A medium for cultivation of lactobacilli, J. Appl. Bacteriol. 23 (1960) 130–135.

[16] T. Tanaka, T. Tsuchiya, H. Takahashi, M. Taniguchi, H. Ohara, D.R. Lloyd, Formation of biodegradable polyesters membranes via thermally induce phase separation, J. Chem. Eng. Jpn. 39 (2006) 144–153.

[17] P. van de Witte, P.J. Dijkstra, J.W.A. van den Berg, J. Feijen, Phase behavior of polylactides in solvent-nonsolvent mixtures, J. Polym. Sci. Part B. Polym. Phys. 34 (1996) 2553–2568

[18] G.A. Mannella, V. La Carrubba, V. Brucato, I.C. Sanchez, Lattice fluid model generalized for specific interactions: an application to ternary polymer solutions, Fluid Phase Equilib. 312 (2011) 60–65.

[19] Q. Xing, X. Dong, R. Li, H. Yang, C.C. Han, D. Wang, Morphology and performances of PLLA-based membranes by phase separation, Polymer 54 (2013) 5965-5073.

[20] M. Mulder, Basic Principles of Membrane Technology, 2nd ed., Kluer Academic Publishiers, Dordrecht, 1996, pp. 123–129.

[21] G.R. Guillen, Y. Pan, M. Li, E.M.V. Hoek, Preparation and characterization of membranes formed by nonsolvent induced phase separation: A review, Ind. Eng. Chem. Res. 50 (2011) 3798–3817.

[22] A. Moriya, T. Maruyama, Y. Ohmukai, T. Sotani, H. Matsuyama, Preparation of poly(lactic acid) hollow fiber membranes via phase separation methods, J. Membr. Sci. 342 (2009) 307–312.

[23] J.Y. Lai, F.C. Lin, C.C. Wang, D.M. Wang, Effect of nonsolvent additives on the porosity and morphology of asymmetric TPX membranes, J. Membr. Sci. 118 (1996) 49–61.

[24] H. Sawalha, K. Shroën, R. Boom, Polylactide films formed by immersion precipitation: effects of additives, nonsolvent, and temperature, J. Appl. Polym. Sci. 104 (2007) 959-971

[25] T. Tagami, M.J. Ernsting, S.D. Li, Optimization of a novel and improved thermosensitive liposome formulated with DPPC and a Brij surfactant using a robust in vitro system, J. Control. Release 154 (2011) 290-297.

[26] M. Mulder, Basic Principles of Membrane Technology, 2nd ed., Kluer Academic Publishers, Dordrecht, 1996, pp. 447–451.

[27] H. Zhang and T. Matsuto, Mass and element balance in food waste composting facilities, Waste Manag. 30 (2010) 1477–1485.

[28] G. Rao, A. Moreira, K. Brorson, Disposable bioprocessing: the future has arrived, Biotechnol. Bioeng. 102 (2008) 348–356.

[29] S.M. Cramer, M.A. Holstein, Downstream bioprocessing: recent advances and future promise, Curr. Opin. Chem. Eng. 1 (2011) 27–37.

Chapter 3 Preparation of a Poly(L-Lactic Acid) Membrane Scaffold with Open Finger-Like Pores Prepared by a Nonsolvent-Induced Phase Separation Method with the Aid of a Surfactant

3.1. Introduction

Tissue engineering will likely become one of the key technologies to support human health in the near future. Three of the major components of tissue engineering comprise cells, signal molecules, and scaffolds [1,2]; of these, scaffolds have been developed for the purpose of implanting adhesive cells. However, recent developments in cell sheet technology through the use of thermo-responsive culture dishes suggests that the necessity for such scaffolds during tissue engineering may be reduced, although complex culture procedures are required to effect vascularization in these multilayer cell systems [3]. In comparison, vascular-like connected pores in scaffolds represent effective measures for the support of cell cultures used for tissue engineering implants.

Scaffolds are generally prepared from bioabsorbable polymers as they degrade during and subsequent to tissue regeneration. Among them, poly(L-lactic acid) (PLLA) exhibits good tensile strength and is used accordingly in orthopedic surgeries [4,5]. In the early years of scaffold development, porogens such as leachable salt crystals were used to prepare porous PLLA membranes for cell transplantation [6]. The thermally induced phase separation (TIPS) method has also been used for the preparation of PLLA porous scaffolds [7-9]. PLLA porous scaffolds prepared by the TIPS method are usually uniform and contain cellular pores smaller than 30 μm. However, large cells such as human osteoblast-like cells (20 × 50 μm or larger) [10] are difficult to grow in these pores. Thus, the pore size on the inlet side should be larger than 100 μm to allow cells to grow in the pores and those on the opposite side should be smaller than 10 μm to retain the cells in the scaffold prior to cell adhesion.

Recently PLLA microporous membranes with finger-like pores (Fig. 3-1) have been developed using a nonsolvent-induced phase separation (NIPS) method with the aid of surfactants [11] in a manner similar to that used for the preparation of poly(methyl methacrylate) membranes [12]. Surfactants, which exhibit hydrophilic-lipophilic balance (HLB) values of 14.9–15.6, e.g. polyoxyethylene (20) sorbitan monooleate (Tween 80, HLB = 15.0), enhance the diffusion of water molecules in the polymer solution leading to the formation of membranes with “open” finger-like pores (Fig. 3-1(b)). Similar open finger-like pores were observed in the PLLA membranes prepared by using Tween 40 (HLB = 15.6),

Tween 60 (HLB = 14.9) and polyoxyethylene (20) oleyl ether (HLB = 15.3) while they were not formed in the membranes prepared by using sodium dodecyl sulfate (HLB = 40), Tween 20 (HLB = 16.7) and Span 80 (HLB = 4.3) [11]. The balance between hydrophilicity and lipophilicity (HLB 14.9–15.6) of surfactants is critical for the diffusion of water molecules in PLLA–1,4-dioxane solutions to form the open finger-like pores. The membranes formed in the study using the NIPS method exhibited pores larger than 100 μm on the side of the glass plate of the mold and pores smaller than 0.7 μm on the side of water. The structures differed from those of the PLLA membranes with finger-like pores prepared via the NIPS method with *N*-methyl-2-pyrrolidone and poly(ethylene oxide) by Gao et al., wherein the pore did not reach the glass plate ("closed" finger-like pores, Fig. 3-1(a)) [13]. The open finger-like pores formed with Tween 80 are expected to function as vascular-like connected pores in the PLLA membrane when the membranes are used as scaffolds (Fig. 3-1(c)). In this study a PLLA membrane scaffold with open finger-like pores was developed for the growth of human osteoblast-like cells to facilitate the development of tissue engineering systems for bone reconstruction. The osteoinduction of the cells grown in the membrane was also examined.

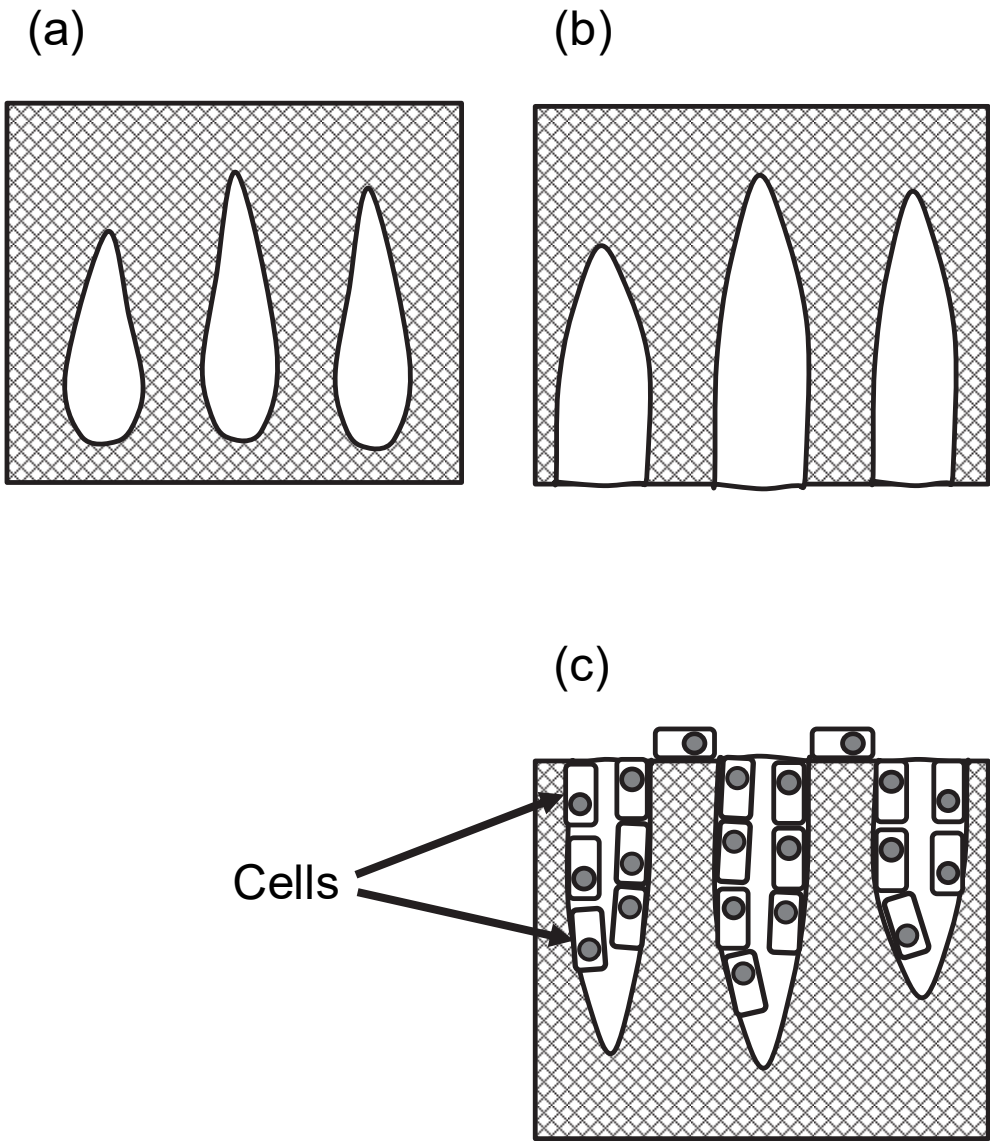

Fig. 3-1 Schematic cross sections of membranes with closed (a) and open (b) finger-like pores, and a membrane scaffold with open finger-like pores (c).

3.2. Experimental methods

3.2.1. Materials

PLLA was a gift from Toyota Motor Corp. (Japan). The PLLA properties included weight-average molecular weight 1.22×10^5 ($M_w/M_n = 3.0$), optical purity 98.5%, melting point 174.0 °C, and glass transition temperature 59.7 °C. Analytical grade 1,4-dioxane, Tween 80 (polyoxyethylene (20) sorbitan monooleate), kanamycin, dexamethasone, glutaraldehyde, ethanol, *t*-butanol, 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), and L-ascorbic acid phosphate magnesium salt *n*-hydrate were

purchased from Wako Pure Chemical Industries (Japan). Dulbecco's modified Eagle's medium (DMEM) and trypsin were obtained from Sigma-Aldrich (USA). β-Glycerophosphate disodium salt pentahydrate was a product of Calbiochem (USA). Fetal bovine serum (FBS) was purchased from Equitech-Bio (USA). All chemicals were used without further purification.

3.2.2. Preparation of PLLA membrane scaffolds

PLLA membranes were prepared via the NIPS method similar to as reported elsewhere [11]. Typically, 5.00 g PLLA was dissolved in 38.25 g 1,4-dioxane containing 6.75 g Tween 80 in a sealed 100 cm^3 flask. The mixture was first stirred with a polytetrafluoroethylene (PTFE) stirring bar and warmed on the stirrer/hot plate at 80 °C for 8 h. The polymer solution was cast on a glass plate with an 80 × 80 mm frame (thickness 1.0 mm) made from a PTFE plate. After removing excess polymer solution with the edge of another glass plate, the polymer solution on the glass plate was immersed in a coagulation water bath at 25±2 °C for 2 h. The resulting membrane was removed from the glass plate, washed extensively with water, and stored in water until use. For use as scaffolds, the membrane was cut into 15 × 15 mm pieces, sterilized in 70% ethanol at 4 °C for over 3 days, and washed with sterile water in a laminar flow hood to remove the ethanol.

3.2.3. Cell culture in the PLLA membrane scaffolds

In this study, human osteoblast-like Saos-2 cells [14,15] were used to evaluate the membrane as a scaffold for cell growth. The cells were maintained in 60-mm culture dishes (Nunc 150288, Thermo Fisher Scientific, USA) with 5 mL growth medium (DMEM supplemented with 10% FBS and 0.01% kanamycin) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. After the recovery of cells from dish culture by trypsin, Saos-2 cells in 0.1 mL growth medium were seeded at a density of 1.0×10^4 cells per 15 × 15 mm scaffold (= 44 cells/mm^2) on the indented side of the membrane. Five milliliter growth medium was exchanged with fresh medium every 7 days for the first 14 days and every 3 or 4 days for the next 14 days with and without osteoinduction. To induce osteoblast differentiation, 50 μg/mL L-ascorbic acid 2-phosphate magnesium salt *n*-hydrate, 10 nM dexamethasone, and 5 mM β-glycerophosphate were added to the medium after 14-day culture without the inducers.

3.2.4. Scanning electron microscopy (SEM)

The prepared membrane scaffolds and the cells grown thereon were observed using SEM as described elsewhere [8]. Briefly, the cells grown on the membranes were fixed for 2 h with 2.5% glutaraldehyde in phosphate buffered saline (PBS). The samples were then dehydrated through a series of ethanol solutions (70–100%) and *t*-butanol, and then freeze-dried. To examine the cross-sections, the membrane scaffolds were freeze-fractured in liquid nitrogen. The surfaces of the samples were coated with gold-palladium using a sputter coater (MSP-1S; Vacuum Device, Japan) for SEM examination (TM-1000; Hitachi, Japan) at an accelerating voltage of 15 kV.

3.2.5. MTT staining

To detect the living cells in the membrane scaffold, 1 mL MTT solution (5 mg/mL) was added to the culture dish and incubated for 2 h, after which the membrane scaffold was rinsed twice with PBS. Following staining with purple formazan, the samples were observed using a USB microscope (DigiScope II v2, Chronos, Taiwan) under epi-illumination; then, the formazan was extracted in 5 mL DMSO. The absorbance of the extracted formazan was measured at a wavelength of 570 nm using a spectrophotometer (UV-1600; Shimadzu, Japan). The absorbance of the formazan solution from Saos-2 cells grown on the culture dishes and the cell density [cells per mm^2] as assessed under a phase contrast microscope (CKX31, Olympus, Japan) were measured to calculate the cell densities per unit apparent area on the membrane scaffolds.

3.2.6. Electron dispersive X-ray spectroscopy

The freeze-dried and gold-palladium sputter-coated scaffolds were analyzed using SEM with an electron dispersive X-ray spectroscopy (EDS) detector (JCM-6000-JED-2300; JEOL, Japan) at an acceleration voltage of 15 kV. The data obtained therefrom provided the elemental composition from the surface to a depth of 1 μm under the conditions recommended by the manufacturer. The osteoinduction data were tested using a one-tailed Welch's *t*-test with Microsoft Excel 2016 software (USA) ($n = 3$).

3.3. Results and discussion

3.3.1. Preparation of PLLA microporous membranes with open finger-like pores

The addition of Tween 80 to the PLLA solution in 1,4-dioxane is an effective means to reduce the shrinkage in thickness that may occur during the preparation of PLLA membranes via the NIPS method [11]. However, the membrane thickness was approximately 250 μm (0.25 mm) when the membranes were prepared from a 10 wt-% PLLA solution in 1,4-dioxane containing 10 wt-% Tween 80 using a 0.5-mm-deep mold. In comparison, the size of a Saos-2 cell, a widely used cell type for a human osteoblastic model, is approximately 20 × 50 μm in a T25 flask [10]. Thus, the membranes in this study were prepared with a 1.0-mm-deep mold to increase the membrane thickness to effect an enlargement of the internal area for cell adhesion per unit apparent area of the membrane scaffolds. Figs. 3-2(a) and 3-2(d) show the cross section and the side in contact with a glass plate, respectively, during the preparation of PLLA membranes prepared from 10 wt-% PLLA solution in 1,4-dioxane containing 10 wt-% Tween 80. The thickness of the membrane was observed to be approximately 500 μm, as expected. However, the development of finger-like structure did not occur. Thus, different conditions for scaffold preparation were examined to identify those capable of forming open finger-like pores.

The author found that a reduction of the polymer concentration in the preparation to 5 wt-% was effective in forming 100–200 μm open pores on the side where the polymer solution was in contact with the glass plate of the mold (Fig. 3-2(e)). However, the internal porous structure did not develop (Fig. 3-2(b)). Conversely, an increase in Tween 80 concentration to 15 wt-% yielded development of open finger-like pores in PLLA membrane scaffolds (Figs. 3-2(c) and 3-2(f)). The increase in surfactant concentration is thought to accelerate the diffusion of water molecules in the polymer solution. Furthermore, no clear PLLA solutions were obtained at 25 °C when the concentrations of PLLA and Tween 80 were 10 wt-% and wt-20%, respectively, as expected from the phase diagram reported elsewhere [11]. Thus, the PLLA membranes with open finger-like pores and 500 μm thickness prepared from a 10 wt-% PLLA solution in 1,4-dioxane containing 15 wt-% Tween 80 were used in this study. Further note that the surface that was in contact with water in the preparation was smooth, as shown in Fig. 3-2(c). The author therefore expects that the internal structure of these generated membranes will retain the cells when they are inoculated on the “indented side” where the polymer solution contacts the glass plate and the finger-like pores are open (Fig. 3-1(c)).

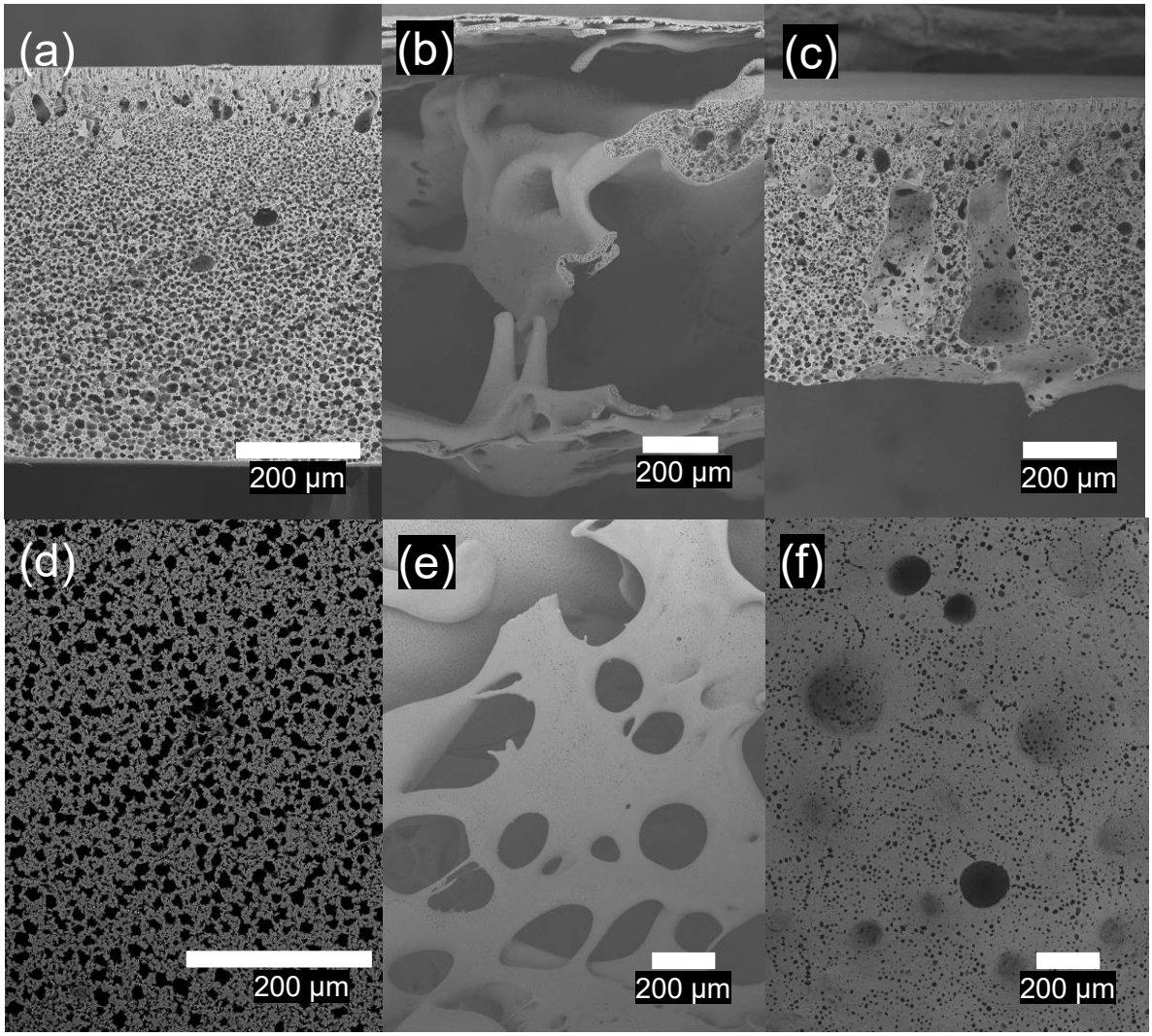

Fig. 3-2 Effect of polymer and surfactant concentrations on the structure of PLLA membranes. (a–c) Cross-sections; (d–f) the surface in contact with a glass plate during the preparation. Polymer concentrations were 10 wt-% (a, c, d, f) and 5 wt-% (b, e). Surfactant concentrations were 10 wt-% (a, b, d, e) and 15 wt-% (c, f).

3.3.2. Growth of osteoblast-like cells in PLLA membrane scaffolds

Fig. 3-3 shows the optical micrograph of the PLLA membrane scaffold and the Saos-2 cells stained with MTT. The cells were inoculated on the indented side of the scaffold with open finger-like pores. The purple pigment indicates the formazan produced from MTT by viable cells. The density of the pigment increased in the pores during cultivation (Figs. 3-3(a)–3-3(d)). In addition, it was observed that the cells grew both on the membrane surface and inside of the pores.

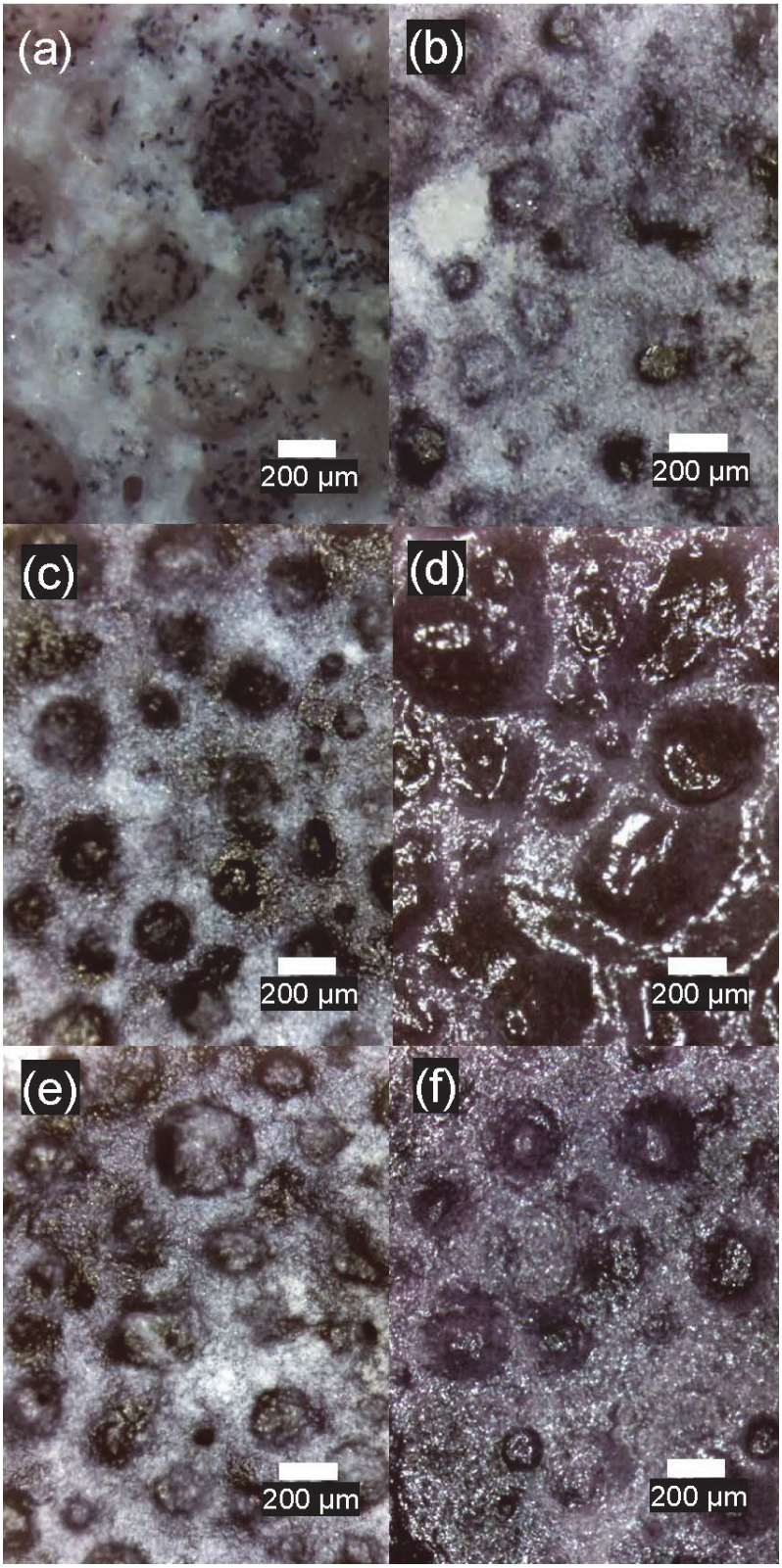

Fig. 3-3 PLLA membrane scaffolds and Saos-2 cells stained by MTT. (a–d) Cells cultured without osteoinduction for 7 (a), 14 (b), 21 (c), and 28 days (d); (e, f) cells cultured without osteoinduction for 14 days and then cultured with osteoinduction for 7 (e) or 14 (f) days.

Fig. 3-4 shows the SEM images of a cross section of the membrane scaffold and cells incubated for 21 days. The Saos-2 cells grew on the surface of the scaffold and in the open finger-like pores to which the cells adhered via pseudopods. Fig. 3-5 shows the density of cells in the PLLA membranes as calculated from the absorbance of the formazan solutions extracted with DMSO from the scaffolds and cells (Fig. 3-3). The cell density increased to 5000–6000 cells/mm^2 after 14–28 days of culture at which time the culture reached stationary phase. In comparison, the maximum cell density of Saos-2 cells has been reported to be 2000–3000 cells/mm^2 on culture flasks and dishes [11,15]. Therefore, the cells grew 2–3 times higher per unit apparent area in the PLLA membrane scaffold than in monolayer cultures. Although the internal structure might be further improved by optimizing the preparation conditions, the PLLA membrane with open finger-like pores therefore represents a potential candidate to function as a scaffold to support the implantation of osteogenic cells in bone tissue engineering.

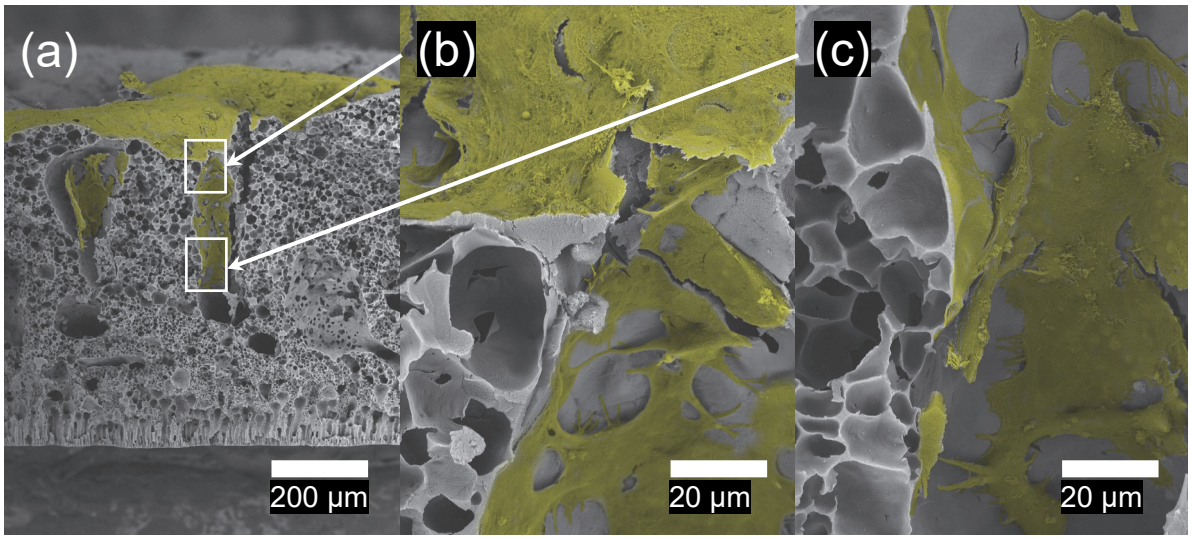

Fig. 3-4 SEM images of a cross section of the PLLA membrane scaffolds with Saos-2 cells grown for 21 days without osteoinduction. Saos-2 cells are colored in yellow. (a) Overview; (b) a representative part near the scaffold surface; (c) a representative middle part of the scaffold.

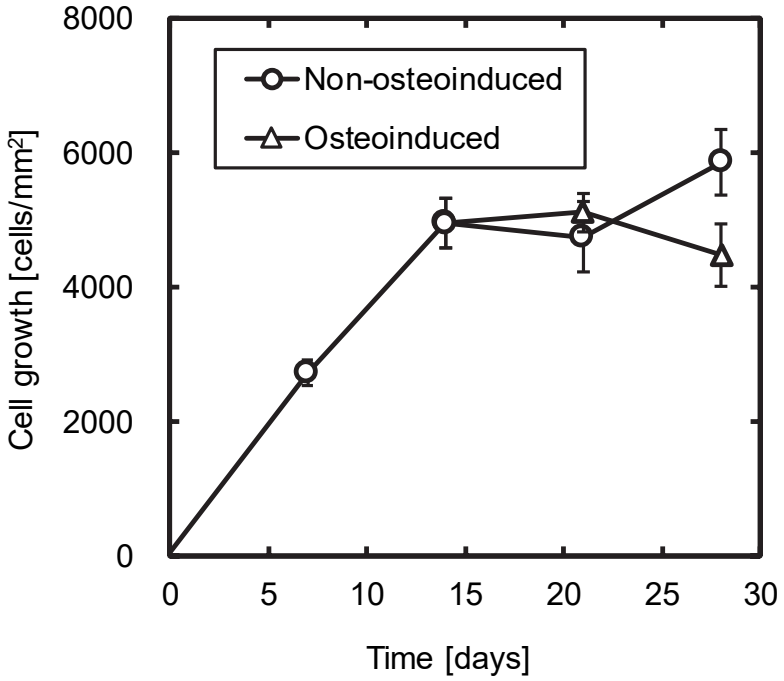

Fig. 3-5 Growth of Saos-2 cells on and within the PLLA membrane.

3.3.3. Osteoinduction of Saos-2 cells in the PLLA membrane scaffold

Human osteoblast-like cells are often used in osteoinduction experiments [16,17]. In this study the PLLA membrane was examined by EDS although the elemental analysis was limited to the depth of 1 μm. L-Ascorbic acid 2-phosphate, dexamethasone, and β-glycerophosphate were used as inducers after 14 days of culture without osteoinduction. The cell growth was comparable or slightly repressed compared to that without osteoinduction (Figs. 3-3 and 3-5). Fig. 3-6 shows the mass percentage of calcium to the total mass of calcium, carbon, nitrogen, and oxygen during the 28-day culture with and without osteoinduction. Note that hydrogen was not detected by EDS and the peaks of phosphorus did not separate from that of the gold sputter-coated on the samples. The content of carbon was 35–45% during the culture with and without osteoinduction. After 21–28 days of culture without osteoinduction, the calcium content slightly increased (<1%), whereas the calcium content was increased five times by osteoinduction (3–5%). The p values in one-tailed Welch's t-tests ($n = 3$) were 0.067 and 0.034 on 21 and 28 days, respectively. Thus the osteoinduction by L-ascorbic acid 2-phosphate, dexamethasone, and β-glycerophosphate significantly increased the calcium content in the

culture of Saos-2 cells after 28 days ($p < 0.05$), although the content slightly increased in the stationary phase of the control culture. An accumulation of insoluble calcium compounds suggests that the osteoblast-like cells on the PLLA membrane were successfully osteoinduced by the mixture of the three chemical inducers to produce calcium phosphates such as hydroxyapatite.

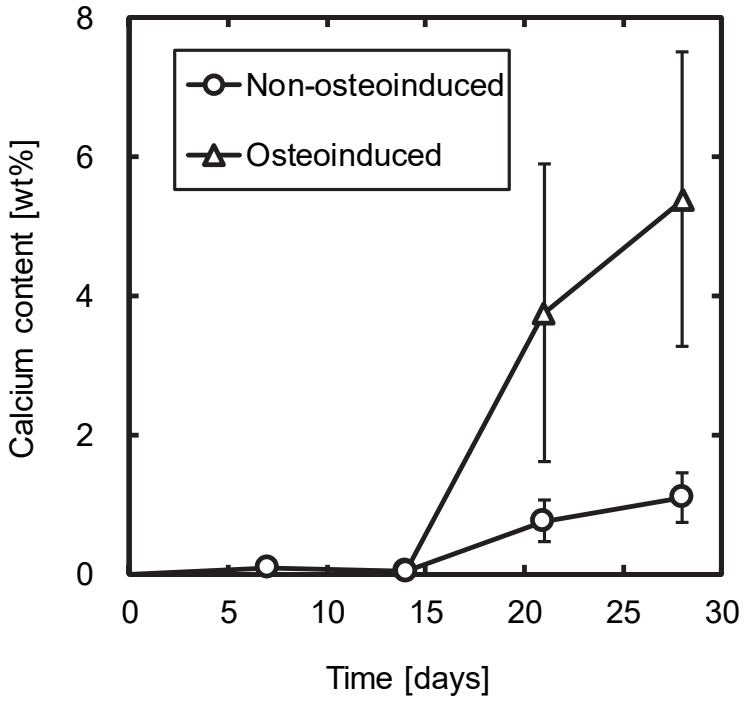

Fig. 3-6 Change in calcium content of the surface of PLLA membranes with Saos-2 cells with/without osteoinduction.

In some reports of tissue regeneration of periodontal bone defects, primary cultured periosteal tissues have been applied as a barrier membrane and source of osteogenic cells induced from periosteal cells [18]. Notably, cultured periosteal tissues spontaneously form cell-multilayers and are successfully osteoinduced on PLLA microporous supports prepared via TIPS method [8]. In addition to these primary tissue cultures, Ma and Zhang reported that MC3T3-E1 cells from mouse were osteoinduced in a PLLA scaffold with microtubular-architecture prepared by TIPS method [19]. Thus, PLLA scaffolds represent promising candidates for bone tissue regeneration wherein osteoprogenitor cells can be

osteoinduced. Recently, stem cells banks containing cells with different human leucocyte antigen profiles have been generated for use in tissue regeneration [20,21]. For effective tissue regeneration, such stem cells should first be properly differentiated in scaffolds prior to implantation. The author's findings regarding the osteoinduction of osteoblast-like cells grown in the PLLA membrane scaffold further suggest that these scaffolds are suitable for implantation after cell differentiation.

3.4. Conclusions

A PLLA microporous membrane scaffold with open finger-like pores was prepared via the NIPS method with the assistance of Tween 80. Notably, the surfactant concentration was critical to form the membrane structure. Osteoblast-like cells grew in the membrane scaffold and the cell numbers per unit apparent area of the membrane scaffold reached 2–3 times those achieved in monolayer cultures. In addition, the osteoblast-like cells grown in the membrane scaffold were successfully osteoinduced to deposit calcium compounds. Thus, the PLLA membrane scaffold with open finger-like pores will likely be useful for the implantation of cells as well as cell multilayers in bone tissue engineering.

3.5. References

[1] R. Langer, J.P. Vacanti, Tissue engineering, Science 260 (1993) 920–926.

[2] F.J. O'Brien, Biomaterials & scaffolds for tissue engineering, Mater. Today 14 (2011) 88–95.

[3] T. Kikuchi, T. Shimizu, M. Wada, M. Yamato, T. Okano, Automatic fabrication of 3-dimensional tissues using cell sheet manipulator technique, Biomaterials 35 (2014) 2428–2435.

[4] D.W. Hutmacher, Scaffolds in tissue engineering bone and cartilage, Biomaterials 21 (2000) 2529–2543.

[5] L.S. Nair, CT Laurencin, Biodegradable polymers as biomaterials, Progr. Polym. Sci. 32 (2007) 762–798.

[6] A.G. Mikos, A.J. Thorsen, L.A. Czerwonka, Y. Bao, R. Langer, D.N. Winslow, J.P. Vacanti, Preparation and characterization of poly (L-lactic acid) foams, Polymer 35 (1994) 1068–1077.

[7] T. Tanaka, S. Eguchi, H. Saitoh, M. Taniguchi, D.R. Lloyd, Microporous foams of polymer blends of poly(L-lactic acid) and poly(ε-caprolactone) Desalination 234 (2008) 175–183.

[8] T. Kawase, T. Tanaka, T. Nishimoto, K. Okuda, M. Nagata, D.M. Burns, H. Yoshie, Improved adhesion of human cultured periosteal sheets to a porous poly(L-lactic acid) membrane scaffold without the aid of exogenous adhesion biomolecules, J. Biomed. Mater. Res. Part A 98A (2011) 100–113.

[9] L. He, Q. Zuo, Y. Shi, W. Xue, Microstructural characteristics and crystallization behaviors of poly(L-lactide) scaffolds by thermally induced phase separation, J. Appl. Polym. Sci. 131 (2014) 39436.

[10] C. Pautke, M. Schieker, T. Tischer, A. Kolk, P. Neth, W. Mutschler, S. Milz, Characterization of Osteosarcoma Cell Lines MG-63, Saos-2 and U-2 OS in Comparison to Human Osteoblasts, Anticancer Res. 24 (2004) 3743–3748.

[11] H. Minbu, A. Ochiai, T. Kawase, M. Taniguchi, D.R. Lloyd, T. Tanaka, Preparation of poly(L-lacticacid) microfiltration membranes by a nonsolvent-induced phase separation method with the aid of surfactants, J. Membr. Sci. 479 (2015) 85–94.

[12] F.C. Lin, D.M. Wang, C.L. Lai, J.Y. Lai, Effect of surfactants on the structure of PMMA membranes, J. Membr. Sci. 123 (1997) 281–291.

[13] A. Gao, F. Liu, H. Shi, L. Xue, Controllable transition from finger-like pores to inter-connected pores of PLLA membranes, J. Membr. Sci. 478 (2014) 96–104.

[14] J. Fogh, J.M. Fogh, T. Orfeo, One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice, J. Natl. Cancer Inst. 59 (1977) 221–226.

[15] S.B. Rodan, Y. Imai, M.A. Thiede, G. Wesolowski, D. Thompson, Z. Bar-Shavit, S. Shull, K. Mann, G.A. Rodan, Characterization of a human osteosarcoma cell line (Saos-2) with osteoblastic properties, Cancer Res. 47 (1987) 4961–4966.

[16] D.J. McQuillan, M.D. Richardson, J.F. Bateman, Matrix deposition by a calcifying human osteogenic sarcoma cell line (SAOS-2), Bone 16 (1995) 415–426.

[17] M. Prideaux, A.R. Wijenayaka, D.D. Kumarasinghe, R.T. Ormsby, A. Evdokiou, D.M. Findlay, G.J. Atkins, SaOS2 Osteosarcoma cells as an in vitro model for studying the transition of human osteoblasts to osteocytes, Calcif. Tissue Int. 95 (2014) 183–193.

[18] K. Yamamiya, K. Okuda, T. Kawase, K. Hata, L.F. Wolff, H. Yoshie, Tissue-engineered cultured periosteum used with platelet-rich plasma and hydroxyapatite in treating human osseous defects, J. Periodontol. 79 (2008) 811–818.

[19] P.X. Ma, R. Zhang, Microtubular architecture of biodegradable polymer scaffolds, J. Biomed. Mater. Res. 56 (2001) 469–477.

[20] C.J. Taylor, E.M. Bolton, J.A. Bradley, Immunological considerations for embryonic and induced pluripotent stem cell banking, Phil. Trans. R. Soc. Lond. B Biol. Sci. 366 (2011) 2312–2322.

[21] C.J. Taylor, S. Peacock, A.N. Chaudhry, J.A. Bradley, E.M. Bolton, Generating an iPSC bank for HLA-matched tissue transplantation based on known donor and recipient HLA types, Cell Stem Cell 11 (2012) 147–152.

Chapter 4 Concluding Remarks

The author's work in the thesis are summarized as follows:

1) Preparation of poly(L-lactic acid) microfiltration membranes by a nonsolvent-induced phase separation method with the aid of surfactants.

The author prepared an asymmetric microfiltration membrane of PLLA by a NIPS method with the aid of Twee80, a nonionic surfactant. This porous membrane was prepared by adding the surfactant; Tween 80. The membrane was prepared a PLLA solution at 10 wt-% of polymer concentration and at 10 wt-% of the surfactant concentration in 1,4-dioxane solvent with water as a nonsolvent. The membrane thus prepared showed a low filtration resistance, a high bacterial cell retention of 99%, and high protein permeability of 100%. The degradability of the membrane under wet condition was also shown by tensile testing.

2) Preparation of a poly(L-lactic acid) membrane scaffold with open finger-like pores prepared by a nonsolvent-induced phase separation method with the aid of a surfactant.

A membrane scaffold of PLLA for cell culture in regenerative medicine was developed. Osteoblast-like cells; Saos-2 cells, were grown on the membrane with open finger-like pores. The cells continued to proliferate for 3 weeks to reach 2-3 times higher on the culture flasks and dishes in the literature. The cells grown on and in the membrane formed calcium compounds by osteoinduction. Thus, the PLLA membrane is a candidate of membrane scaffold in bone tissue engineering.

The author developed PLLA membranes with high performance with the aid of surfactants. The membranes will be useful as compostable biomass membranes in sustainable industries and membrane scaffolds in regenerative medicine.

List of Publications

[1] Hiromi Minbu, Akihito Ochiai, Tomoyuki Kawase, Masayuki Taniguchi, Douglas R. Lloyd, Takaaki Tanaka, Preparation of poly(L-lactic acid) microfiltration membranes by nonsolvent-induced phase separation method with the aid of surfactants Journal of Membrane Science 479 (2015) 85–94. (Chapter 2 in this thesis)

[2] Hiromi Minbu, Tomoyuki Kawase, Akihito Ochiai, Masayuki Taniguchi, Takaaki Tanaka, Preparation of a poly(L-lactic acid) membrane scaffold with open finger-like pores prepared by a nonsolvent-induced phase separation method with the aid of a surfactant, MEMBRANE 41 (2016) 304–310. (Chapter 3 in this thesis)

Acknowledgements

The studies in the thesis have been carried out in the Course of Materials Science and Technology of Doctor Program in Advanced Materials Science and Technology of Graduate School of Science and Technology, Niigata University.

The author wishes to express his sincere gratitude to Professor Takaaki Tanaka with whose guidance and advice the author conducted the works in the thesis.

The author gratefully appreciates to Professor Masayuki Taniguchi, Professor Isao Kimura, and Associate Professor Motofumi Ohki of Niigata University for their kind and valuable guidance and suggestions to summarize the thesis.

Acknowledgement is made to Professor Emeritus Douglas R. Lloyd of The University of Texas at Austin, and Associate Professor Tomoyuki Kawase and Assistant Professor Akihito Ochiai of Niigata University for their helpful suggestions.

The author tenders his gratitude to Toyota Motors Co. for the kind gift of samples.

Finally, thanks are due to the members of Laboratory of Biofucutional Materials, Department of Materials Science and Technology, Niigata University, for their helpful discussions and kind supports.

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Doctoral Program in Advanced Materials Science and Technology
Graduate School of Science and Technology
Niigata University

February 2017