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

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Here, we prepared a poly(L–lactic acid) (PLLA) microporous membrane with open finger–like pores as a scaffold for tissue engineering by a nonsolvent–induced phase separation method with the aid of a surfactant. The increase of surfactant content in the polymer solution caused the mold (glass plate) side of the membrane to adopt an indented structure with open finger–like pores to enhance the internal surface area. Osteoblast–like cells inoculated on the indented side grew on and in the PLLA membrane scaffold to reach 2 ~ 3 times higher cell density per unit apparent area compared to that attained in monolayer cultures. The cells on the membrane deposited calcium compounds by osteoinduction with ascorbic acid 2–phosphate, dexamethasone, and β–glycerophosphate. The PLLA membrane with open finger–like pores will likely be useful as scaffolds to support the implantation of osteogenic cells in bone tissue engineering.

Keywords: Poly(L–lactic acid) / Scaffold / Nonsolvent–induced phase separation / Osteoblast–like cells / Osteoinduction

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. 1) have been developed using a nonsolvent-induced phase separation (NIPS) method with the aid of surfactants \(^1\) in a manner similar to that used for the preparation of poly(methyl methacrylate) membranes \(^10\). Surfactants, which exhibit hydrophilic-lipophilic balance (HLB) values of 14.9~15.6, e.g. polyoxyethylene (20) sorbitan monoooleate (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. 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 polyoxyethyylene(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\(\text{--}1,4\text{--}dioxane\) 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 \(N\text{--}methyl\text{--}2\text{--}pyrrolidone\) and poly(ethylene oxide) by Gao et al., wherein the pore did not reach the glass plate (“closed” finger-like pores, Fig. 1(a)) \(^15\). 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. 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.

2. Experimental

2.1 Materials

PLLA was a gift from Toyota Motor Corp. (Japan). The PLLA properties included weight-average molecular weight \(1.22 \times 10^5\) \((M_w/M_n = 3.0)\), 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 monoooleate), kanamycin, dexamethasone, glutaraldehyde, ethanol, \(t\)-butanol, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), and \(L\text{--}\)ascorbic acid phosphate magnesium salt \(n\text{--}hydrate\) were purchased from Wako Pure Chemical Industries (Japan). Dulbecco’s modified Eagle’s medium (DMEM) and trypsin were obtained from Sigma–Aldrich (USA). \(\beta\text{--}\)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.

2.2 Preparation of PLLA membrane scaffolds

PLLA membranes were prepared via the NIPS method similar to as reported elsewhere \(^15\). 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.

2.3 Cell culture in the PLLA membrane scaffolds

In this study, human osteoblast–like Saos–2 cells 10, 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% CO2. 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 × 10⁴ cells per 15 × 15 mm scaffold (≈ 44 cells/mm²) 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.

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.

### 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 mm²] 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.

### 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. Results and discussion

#### 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. However, the membrane thickness was approximately 250 µm (0.25 mm) when the membranes were prepared from a 10% PLLA solution in 1,4-dioxane containing 10% 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. 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. Figures 2(a) and 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% PLLA solution.

![Fig. 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.](image-url)
in 1,4-dioxane containing 10% Tween 80. The thickness of the membrane was observed to be approximately 500 \(\mu\)m, 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.

We found that a reduction of the polymer concentration in the preparation to 5% was effective in forming 100 \(\sim\) 200 \(\mu\)m open pores on the side where the polymer solution was in contact with the glass plate of the mold (Fig. 2(e)). However, the internal porous structure did not develop (Fig. 2(b)). Conversely, an increase in Tween 80 concentration to 15% yielded development of open finger-like pores in PLLA membrane scaffolds (Figs. 2(c) and 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% and 20%, respectively, as expected from the phase diagram reported elsewhere. Thus, the PLLA membranes with open finger-like pores and 500 \(\mu\)m thickness prepared from a 10% PLLA solution in 1,4-dioxane containing 15% Tween 80 were used in this study. We further note that the surface that was in contact with water in the preparation was smooth, as shown in Fig. 2(c). We therefore expect 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. 1(c)).

### 3.2 Growth of osteoblast-like cells in PLLA membrane scaffolds

Fig. 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 (Fig. 3(a)–3(d)). In addition, it was observed that the cells grew both on the membrane surface and inside of the pores. Fig. 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. 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). The cell density increased to 5000 \(\sim\) 6000 cells/mm\(^2\) after 14 \(\sim\) 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 \(\sim\) 3000 cells/mm\(^2\) on culture flasks and dishes. Therefore, the cells grew 2 \(\sim\) 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.
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 \(\mu\)m. L–Ascorbic acid 2–phosphate, dexamethasone, and \(\beta\)–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 and 5). Fig. 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. We 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 \(\beta\)–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.

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. Our 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.
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.

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