Preparation of Thermoplastic Poly(L-Lactic Acid) Membranes for Guided Bone Regeneration

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Purpose: The objective of this study was to evaluate the feasibility of application of thermoplastic poly-L-lactic acid (PLLA) membranes for guided bone regeneration in rabbit parietal bone. Materials and Methods: PLLA membranes with a molecular weight of 100,000 (PLLA-100,000) and a molecular weight of 380,000 (PLLA-380,000) were dissolved in chloroform to prepare concentrations of 8% by weight and 4% by weight, respectively. The compression strength, temperature, and time to prepare each formulation were measured. Moreover, the pH was noted and cytotoxicity of the membrane was determined by monotetrazolium assay. In vivo experiments were performed to measure the volume of newly formed bone tissue in hematoxylin and eosin–stained tissue sections 4 and 12 weeks after implantation. Results: The membrane prepared from PLLA-380,000 showed excellent thermoplasticity at 75°C to 80°C and the compressive strength was equal to that of titanium mesh, in contrast to that of PLLA-100,000 and poly(lactic acid-co-glycolic acid). There was a significant change in the pH of an aqueous solution in which the PLLA-380,000 membrane was placed, but there was no cytotoxic activity. The membrane made of PLLA-380,000 induced new bone formation in a dome shape without any membrane deformation. Conclusion: Thermoplastic PLLA membrane shows promise for guided bone regeneration in vertical bone augmentation. Int J Oral Maxillofac Implants 2013;28:973–981. doi: 10.11607/jomi.2729

Key words: bone augmentation, dental implants, guided bone regeneration, membrane, poly(L-lactic acid), thermoplasticity

Surgical treatment, including dental implant therapy, has been successfully performed to restore dental occlusion and masticatory function. However, when there is significant resorption and atrophy of the alveolar bone, it is difficult or impossible to place implants. Thus, a variety of bone transplant materials have been developed and used for alveolar ridge augmentation. Autologous bone1,2 and synthetic apatites3–5 have been the most commonly used graft materials because they act as a scaffold for cell proliferation and differentiation. However, since gingival epithelial or connective tissue often invades the site before new bone formation, regeneration of bone tissue is inhibited. The technique of guided bone regeneration (GBR) was developed to solve this problem. When biomaterial membranes are used under the periosteum, the invasion of epithelium and connective tissue is physically suppressed, while in some cases osteogenic cells infiltrate under the membranes, proliferate, and differentiate to form the bone tissue.6,7 Resorbable membranes made of collagen8 and poly(lactic acid-co-glycolic acid) (PLGA)9 and nonresorbable ones containing e-poly(tetrafluoroethylene) (e-PTFE)10,11 have been applied clinically and have demonstrated therapeutically acceptable characteristics.12 This three-dimensional formation of new bone tissue is clinically required to increase the dimensions of the alveolar ridge. Granulated bone graft materials are often used in clinical cases. Various GBR membranes have been applied, but they have often failed to form bone tissue as expected because of poor mechanical strength. A GBR membrane should therefore be mechanically reinforced to prevent shape deformation.
by occlusal forces. Titanium meshes have been used to improve the mechanical characteristics; however, they do not always have a barrier function to prevent invasion of soft tissue, and shape formation is technically difficult.\(^{13}\) For example, a thin titanium frame can be incorporated into collagen or e-PTFE membranes and used as a composite barrier membrane. However, this does not provide sufficient mechanical strength, and the shape of the newly formed bone is clinically inadequate.\(^{8}\) Therefore, membranes have been developed that can prevent soft tissue invasion, exhibit thermoplasticity applicable to irregularly shaped bone defects, and possess sufficient mechanical strength to resist functional pressure.

Poly-L-lactic acid (PLLA) is a polymer of L-lactic acid that is a metabolite in the body; it degrades physiologically to generate water and carbon dioxide in the tricarbonic acid cycle.\(^{14}\) It has been widely used for surgical plates and screws for bone fixation in orthopedic\(^{15,16}\) and oral and maxillofacial cases.\(^{17,18}\) Moreover, since its glass transition temperature is low, PLLA can be readily formed into the shape required during surgery by simple immersion in hot water.\(^{19}\) In addition, PLLA has been used clinically as a drug delivery carrier.\(^{20-22}\) On the other hand, PLLA itself is not suitable for cell adhesion because of its poor potential to promote cell proliferation.\(^{23}\) However, if the material were used in GBR membranes, this poor cytocompatibility might prove to be beneficial by inhibiting invasion of soft tissue.

In this study, various thermoplastic membranes for GBR were prepared from PLLA of different molecular weights. The mechanical strength of the membranes and the cell proliferation on their surfaces were examined. Following application of PLLA membranes onto the parietal bone of rabbits, bone formation was evaluated histologically.

**MATERIALS AND METHODS**

**Materials**

PLLA with a molecular weight (Mw) of 100,000 (PLLA-100,000) (LACTEL) and PLLA with a Mw of 380,000 (PLLA-380,000) (Boehringer Ingelheim) were used as the membrane materials. A PLGA membrane (GC Membrane) was purchased from GC, and a titanium mesh (Jeli Ti mesh, pore size: 1.48 to 1.51 µm) was acquired from ProSeed.

**Preparation of PLLA Membranes and Observation of Surface Morphology**

The PLLA-100,000 and PLLA-380,000 samples were dissolved in chloroform (Nakaraitesque) to obtain concentrations of 8% and 4% by weight, respectively. Various volumes of PLLA chloroform solution were added to 33-mm-diameter glass petri dishes and left at room temperature overnight to allow the chloroform to completely evaporate and form membranes of different thicknesses. The membranes were removed and stored after being sterilized with ethylene oxide gas. The surface morphology of PLLA and PLGA membranes was observed with a scanning electron microscope (SEM) (S-4000, Hitachi High-Technologies).

**Compression Testing**

PLLA and PLGA membranes (12 × 30 mm) were immersed in hot water at 70°C to 80°C, molded into a half-pipe shape, and then left at room temperature for fixation. Titanium meshes of similar shape were also prepared, and then all membranes and meshes were fixed on a wood block with pins (Fig 1). Compression tests were performed with a versatile examination machine (AG-5000B, Shimadzu) at a crosshead speed of 1 mm/min. The thickness of PLLA membranes with compressive strength equal to that of titanium meshes was determined. Compression tests were performed independently on three samples of each type of membrane.

**Temperature and Duration of PLLA Membrane Formation**

The compression strength of the PLLA membranes corresponded to that of titanium meshes with thicknesses of 0.5 and 0.3 mm for PLLA-100,000 and PLLA-380,000, respectively. The former and latter membranes were soaked in warm water at 70°C to 75°C and 75°C to 80°C and pressurized to obtain the half-pipe shape shown in Fig 1. The setting temperature and time until the half-pipe shape was achieved were measured and recorded.

**pH Testing**

The PLLA and PLGA membranes (15 mg) were immersed in Hank's balanced salt solution (Sigma-Aldrich) at 37°C. The pH of the solution was measured with a pH meter (pH/ION/COND Meter F-55, Horiba) at 1, 3, 6, and 12 hours and at 1, 3, 5, 7, 10, 14, 21, 28, and 35 days. The pH testing was performed independently on four samples at each sampling point. As a control, Hank's balanced salt solution was used alone.

**Cell Culture on the Membranes**

The PLLA and PLGA membranes (4 × 4 mm) were placed in each well of a 96-well culture plate (no. 655185, CELLSTAR, Greiner Bio-one). Gingival epithelium (GE) cells (RIKEN BioResource Center) were suspended in Eagle's minimum essential medium supplemented with 10% (by weight) fetal calf serum, 0.12% (by weight) sodium bicarbonate, and 100 units/mL...
mixed penicillin-streptomycin. The GE cell suspension (100 µL, 1,000 cells/cm²) was added to each well and cultured at 30°C for 1, 3, and 7 days in 95% air and 5% carbon dioxide. To examine cell adhesion, the membrane was removed 1 day after culture and thoroughly washed twice with phosphate-buffered saline solution (pH 7.4); this was followed by morphological observation. The membrane was fixed with 2.0% (by volume) glutaraldehyde to view cells adhered to the membrane with SEM (S-4000, Hitachi). The number of cells that proliferated on the membrane was determined by the conventional thiazolyl blue tetrazolium bromide (monotetrazolium [MTT]) assay (Dojindo). Briefly, 10 µL of the MTT stock solution was added to each well, followed by incubation at 37°C for 4 hours. The absorbance of the solution was measured at a wavelength of 450 nm on a microplate reader (VERSAmax, Molecular Devices).

**Animal Experiments**

PLLA and PLGA membranes were formed into a dome shape (outside diameter 11.5 mm, inside diameter 7.5 mm, height 2.0 mm) with a molding apparatus (Saigoku Machine Factory). To evaluate changes in the membranes over time, the parietal bone of New Zealand white rabbits (20 weeks old, 3 to 5 kg body weight; Shimizu Laboratory Supply) was used.

The animals were anesthetized with an intramuscular injection of droperidol (0.25 mg/kg), an intravenous injection of pentobarbital (20 mg/kg), and inhalation of isoflurane (2%) by vol. Lidocaine (1.8 mL) was also administered as a local anesthetic. The skin and periosteum were incised and elevated, the surface of the parietal bone was exposed, and the membrane was fixed to the bone with bonding material (Aron Alpha A Sankyo, Daiichi Sankyo) for surgery (Fig 2). Each experimental group consisted of three rabbits. The soft tissue was sutured with interrupted 4-0 Vicryl (Ethicon) and the skin was closed with 3-0 silk sutures. After surgery, the rabbits were kept in cages and given their usual regimen of food and water.

At 4 and 12 weeks, the animals were sacrificed by intravenous administration of sodium pentobarbital, while PLLA and PLGA membranes were resected together with the parietal bone and fixed in 10% (by weight) neutral phosphate-buffered formalin solution to assess bone regeneration. Bone specimens were placed in 10% (by volume) neutral phosphate-buffered formalin solution, decalcified with 10% formic acid for 4 and 12 weeks, and processed for paraffin embedding. Sections (5 µm thick) were prepared and stained with hematoxylin and eosin. To measure the volume of newly formed bone tissue, the stained sections obtained at 12 weeks after implantation were viewed using the computer program ImageJ (US National Institutes of Health). The stained sections were not visible at 4 weeks, so samples from this time point were not measured. The scale for measurement was the area of newly formed bone tissue in the dome from the cranial surface. All animal experiments were performed in accordance with the Guidelines of Animal Experimentation of Kyoto University.

**Statistical Analysis**

All results were expressed as means ± standard deviations. For statistical analysis, the Tukey-Kramer post hoc test for multiple comparisons was used, and differences were considered to be significant at $P < .05$. 

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**Fig 1** Schematic illustration of compression test for the membrane.

**Fig 2** Sites in the parietal bone of rabbits to evaluate bone formation with the different membranes. A = PLLA-100,000 membrane; B = PLLA-380,000 membrane; C = PLGA membrane.
RESULTS

Surface Properties of the Membranes
SEM images of the PLLA and PLGA membrane surfaces are shown in Fig 3. The PLLA-380,000 membranes were translucent and the surface was smooth, whereas the PLLA-100,000 membranes had a rough surface with a wavelike structure. The PLGA membranes exhibited a rough surface with a microporous network structure.

Compressive Strength
The compressive strength values relative to membrane thickness are shown in Table 1. The titanium meshes had a thickness of 0.5 mm and a mean compressive strength of 72.8 ± 4.23 kPa. PLLA-100,000 and PLLA-380,000 membranes with thicknesses of 0.55 ± 0.05 mm and 0.30 ± 0.05 mm, respectively, exhibited a compressive strength roughly equal to that of the titanium meshes (69.2 ± 6.10 kPa and 69.7 ± 0.91 kPa, respectively). The PLGA membranes had a thickness of 0.25 mm and a mean compressive strength of 9.93 ± 0.56 kPa.

PLL A Membrane Formation Characteristics
The temperatures and times required to form the PLLA membranes are shown in Table 2. The time required to form the membrane in hot water at 75°C to 80°C was 3 minutes for PLLA-100,000 and 1 minute for PLLA-380,000.

pH Values
The changes in pH of Hank’s balanced salt solution containing PLLA membranes are shown in Fig 4. The pH of the PLLA-380,000 membrane solution did not change dramatically over the 35 days of the experiment. In contrast, the pH of the PLLA-100,000 membrane solution decreased gradually during the initial 3 days, dropped rapidly until 28 days, and decreased dramatically until 35 days, for a final pH of 5.58 ± 0.08. The pH of the PLGA membrane solution decreased within 6 hours and was 5.65 ± 0.05 at 35 days. However, there were no changes in the appearance of each membrane.

Results of Cell Culture
The SEM micrographs of GE cells on PLLA and PLGA membranes 1 day after culture are shown in Fig 5. Many GE cells had adhered to the PLGA membrane, whereas no cell adhesion was observed on the two PLLA membranes.
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The proliferation of cells on all membranes is shown in Fig 6. Fewer cells proliferated on the two types of PLLA membranes than on the PLGA membranes. The number of cells that had proliferated after 7 days was about 82,000 cells/cm² on the PLGA membrane, in contrast to about 32,000 and 29,000 cells/cm² on the PLLA-100,000 and PLLA-380,000 membranes, respectively. No significant difference in the number of proliferated cells between the two types of PLLA was observed.

Results of Animal Experiments
The photographs of PLLA and PLGA membranes placed on the parietal bone of rabbits at 12 weeks after implantation are shown in Fig 7. Some cracks had formed in the PLLA-100,000 membrane, and the dome shape of the PLGA membranes had collapsed after 4 weeks (data not shown). The dome shape of the PLLA-100,000 membrane was destroyed by degradation, whereas the PLGA membrane had completely

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Degraded 12 weeks later. However, the PLLA-380,000 membrane maintained the dome shape without degradation, even at 12 weeks postoperative. New bone formation on the surface of the cortical parietal bone 4 weeks after implantation of the membranes is shown in Fig 8. In the case of the PLLA-380,000 membranes, new bone had formed and matured, revealing a cortical bone–like structure 12 weeks later. The new dome-shaped bone was formed inside the membrane. In contrast, for the PLLA-100,000 and PLGA membranes, the bone formation was insufficient, and the bone shape was not guided by the membrane.

The volume of newly formed bone tissue seen at 12 weeks after implantation is shown in Fig 9. The volume of bone formed showed significant differences; PLLA-380,000 showed the greatest volume, followed by PLLA-100,000 and PLGA. The PLGA membranes were insufficient.

Fig 7 (Left) Photographs of membranes on the parietal bone of rabbits 12 weeks after treatment with membranes. A = PLLA-100,000; B = PLLA-380,000; C = PLGA.

Fig 8 (Below) Hematoxylin and eosin–stained histologic specimens of tissue formation on the parietal bone of rabbits at 4 weeks and 12 weeks after placement of PLLA-100,000, PLLA-380,000, and PLGA membranes. Arrows indicate the parietal bone line. Bar = 2.0 mm.

Fig 9 The volume of new bone tissue formed on the parietal bone of rabbits 12 weeks after treatment with PLLA-100,000, PLLA-380,000, and PLGA membranes. *P < .05, significantly different between the two groups.
DISCUSSION

Extensive research has been conducted on guided tissue regeneration since the first clinical application of membranes to periodontal tissues in 1982. GBR was designed based on the principle of guided tissue regeneration and was first applied for the regeneration of osseous tissues in 1989. Since the development of GBR membranes, many researchers have investigated their application. The role of GBR membranes is to maintain the space necessary for natural induction of bone regeneration and prevent the invasion of soft tissue. However, it is difficult in the clinical situation to maintain the three-dimensional space in the body that is necessary to guide the regeneration of bone in the expected shape and volume. This is because the strength of GBR membranes prepared from collagen, e-PTFE, and PLGA is inadequate. Therefore, titanium meshes and frames have been used for alveolar ridge augmentation when three-dimensional bone outgrowth is required. However, titanium is a non-degradable material, and long-term retention in the body often causes mechanical stimulation; also, the release of metal ions is sometimes clinically unacceptable. In addition, the presence of titanium hinders diagnostic imaging because of its radiopacity.

PLLA is a thermoplastic and biodegradable polymer. It is degraded by simple hydrolysis in the body to finally generate nontoxic water and carbon dioxide. PLLA has been clinically applied in the form of various surgical biomaterials, such as mini-plates for bone joints and screws for bone fixation in orthopedic and head and cervical surgical areas. The good biocompatibility, thermoplasticity, and mechanical strength of PLLA have been proven though clinical application. Based on these known factors, in this study, membranes were prepared from two types of PLLA with different molecular weights to determine the mechanical property that would be suitable for GBR application.

The mechanical strength required for the PLLA membrane would be a compressive strength equal to that of the 5-mm-thick titanium mesh that has been used clinically for GBR. The compression test demonstrated that membrane thicknesses of 0.55 ± 0.05 mm and 0.3 ± 0.05 mm for PLLA-100,000 and PLLA-380,000 membranes, respectively, had comparable compressive strengths. The PLLA-380,000 membrane was thinner than the titanium mesh. It is understandable that handling of the membrane improved considerably in terms of shape formation and local tissue fit. Titanium mesh that is 1.0 mm thick is commercially available, but in clinical practice, this mesh is difficult to handle, and local irritation after implantation may occur.

The warming temperature and time to form the PLLA membrane into the required shape were determined. Since the glass transition temperature of PLLA is 55°C to 60°C, a higher temperature is needed for this purpose. Therefore, the water temperatures for the respective membranes were set at 70°C to 75°C and 75°C to 80°C. The PLLA-380,000 membrane was formed into the required shape by immersion in water at 75°C to 80°C for 1 minute. On the other hand, immersion for 3 minutes was required for the PLLA-100,000 membrane. This is because the PLLA-100,000 membrane was thicker than the PLLA-380,000 membrane. The hot water (75°C to 80°C) that is required for preparation of the membrane can be made available chair side. A thermoplastic PLLA membrane, which can be readily formed by immersion for 1 to 3 minutes, will be clinically acceptable. The membrane hardens gradually after removal from the hot water, and it is possible to fine-tune the shape of the membrane during handling.

The PLLA-380,000 membrane did not contribute to decreases in the pH, in contrast to the PLGA and PLLA-100,000 membranes. Local pH is an important factor in stabilizing and maintaining bone conduction in vivo. It is reported that adhesion, movement, and proliferation of cells are suppressed at an environmental pH of 5.0. The bone resorption activity of osteoclasts increases if the extracellular pH decreases to an acidic range. It is possible that PLGA and PLLA generate acidic substances upon degradation, resulting in a decrease in local pH. When the pH is lowered to 5.5, as it was over 35 days for the PLGA and PLLA-100,000, tissue damage may occur. In this preliminary experiment, no significant difference in the weight change of the three groups was observed at 7 days. In addition, because at 7 days all pH values remained above 7.0 (Fig 4), it is thought that this factor may have no influence on cell proliferation.

The culture experiments with GE cells demonstrated less initial cell adhesion for the PLLA membranes compared with the PLGA membrane. One of the reasons may be the easy adhesion of epithelial cells onto the surface of the PLGA membrane, which has a microporous structure. The surface property also affects cell adhesion and proliferation. It has been demonstrated that the cell adhesion to PLLA materials is low and the proliferative activity is poor because of surface hydrophobicity. At 4 and 12 weeks after surgery, the animals were sacrificed, similar to previous reports. No cytotoxic activity was observed for either type of PLLA membrane. It is highly conceivable that the low cell adhesion on the PLLA membrane may translate in the clinical situation to less soft tissue invasion, meaning that the eventual removal of the membrane from the body might be easier. In the animal experiment, a crack was observed in the PLLA-100,000 membrane, and the dome form of the PLGA membrane had collapsed by 4 weeks after implantation. The differences...
in the characteristics of the membranes may be a result of the faster degradation of the PLLA-100,000 and PLGA membranes.

The small volume of new bone formed under the PLGA membrane had resolved almost completely after 12 weeks, and the PLLA-100,000 membrane did not contribute to new bone formation. The maintenance of dome-shaped new bone 12 weeks after implantation of the PLLA-380,000 membrane is most likely a result of its slow degradation. Membranes have been implanted into parietal bone defects in rats and rabbits to evaluate their capacity for bone formation. However, since the bone defect model may not always be suitable to evaluate the change in the strength of the membrane for in vivo bone formation, it is not a good model to test the structural integrity of the GBR membrane. Therefore, in this study, an overlaying model was selected. In this study, a 0.3-mm-thick PLLA-380,000 membrane showed strength equal to that of the 5.0-mm titanium mesh. This membrane is thermoelastic and can be readily molded by hand into the required shape. In addition, the feasibility of its application as a GBR membrane was experimentally confirmed by the potential for vertical bone growth. However, it has been reported that, with degradation of PLLA, crystallization increases to harden the membrane, while long-term retention may sometimes induce an inflammatory reaction in the surrounding tissue. Long-term examinations of PLLA-induced inflammation should be conducted before its clinical application.

CONCLUSION

The membrane prepared from poly-L-lactic acid (PLLA) with a molecular weight of 380,000 (PLLA-380,000) showed excellent thermoplasticity at 75°C to 80°C and the compressive strength was equal to that of a titanium mesh, in contrast to the strength of a PLLA membrane with a molecular weight of 100,000 (PLLA-100,000) and poly(lactic acid-co-glycolic acid) membranes. The pH of a solution did not change when the PLLA-380,000 membrane was immersed in it, and cytotoxic activity was not observed. When the dome-shaped membranes were applied to the parietal bone of rabbits, they induced formation of new, dome-shaped bone tissue inside the membrane. The thermoplastic PLLA membrane appears to be promising for vertical bone augmentation.

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