

Three-Dimensional Printed Biodegradable Polymeric Membranes Used for Guided Bone Regeneration

แผ่นเยื่อแก้วพอลิเมอร์ชนิดสลายตัวได้ที่ผลิตด้วยวิธีการพิมพ์สามมิติเพื่อใช้สำหรับงานชักนำให้กระดูก
คืนสภาพ

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ABSTRACT

Poly(lactic-co-glycolic acid) (PLGA) membranes used for Guided Bone Regeneration were fabricated using three-dimensional printing technique. Physical characteristics and biocompatibility of the membranes made of the PLGA, which consisting of different ratios of lactide (LL) and Glycolide (GL): Group A; 10:90 and Group B; 70:30 were compared. The results demonstrated that mechanical properties and biocompatibility to fibroblasts and osteoblasts of Group B were better than those of Group A ($p < 0.05$). Degradation rate of Group B was slower than that of Group A ($P < 0.05$). In addition, the membranes of Group B produced less acidic environment when compared with Group A. Therefore, the PLGA membrane (LL:GL; 70:30) is suitable for further in vivo and clinical studies.

บทคัดย่อ

การศึกษานี้ได้พัฒนาการผลิตแผ่นเยื่อแก้วพอลิแลคติกโคไกลโคลิกแอซิด ด้วยวิธีการพิมพ์สามมิติ เพื่อใช้สำหรับงานชักนำให้กระดูกคืนสภาพ โดยใช้พอลิแลคติกโคไกลโคลิกแอซิด ในอัตราส่วนของแลคไทด์ ต่อไกลโคไลด์ ในกลุ่ม A; 10:90 และ กลุ่ม B; 70:30 ผลการทดลองพบว่า คุณสมบัติเชิงกล และผลต่อการเจริญเติบโตและความเป็นพิษต่อเซลล์ไฟโบรบลาสต์ และเซลล์ออสติโอบลาสต์ ของกลุ่ม B ดีกว่ากลุ่ม A ($P < 0.05$) อัตราการสลายตัวของกลุ่ม B ช้ากว่ากลุ่ม A ($P < 0.05$) และสร้างสภาวะแวดล้อมที่เป็นกรดน้อยกว่ากลุ่ม A ดังนั้นแผ่นเยื่อแก้วพอลิแลคติกโคไกลโคลิกแอซิดที่มีอัตราส่วนของ แลคไทด์ ต่อ ไกลโคไลด์ 70:30 จึงเหมาะสมที่จะใช้ศึกษาผลในสัตว์ทดลอง และในทางคลินิกต่อไป

Keywords: Poly(lactic-co-glycolic acid), Three-dimensional printing, Membrane

คำสำคัญ: พอลิแลคติกโคไกลโคลิกแอซิด การพิมพ์สามมิติ แผ่นเยื่อแก้ว

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Introduction

Sufficient bone volume is an essential factor for a prognosis of a dental implant. Deficiency of alveolar bone frequently impedes success outcomes of the implant placement. Guided Bone Regeneration (GBR) is a gold standard procedure for increasing volumes of the alveolar bone. In principle, the GBR composes of particulate bone grafts or bone substitutes, which serve as an osteoconductive matrix for enhancing new bone regeneration in the defects and covering membranes, which act as cell exclusion for protecting soft tissue down-growth. In Thailand, almost all of the membranes are imported and expensive such as 13x25 mm of commercial collagen membrane cost 4,577 Baht. Therefore, low-income patients cannot afford the proper treatment using those products. Developing in-house membranes using basic facilities is a good strategy for solving that problem. Regarding the materials, commercial resorbable membranes are commonly made of porcine, bovine, or human collagen. Despite their biocompatibility, main disadvantages of the collagen membranes include antigenicity potential, poor mechanical properties, and too rapid degradation (Lee and Kim, 2014). Alternatively, synthetic polyesters such as polylactic acid (PLA), poly (L-lactic acid) (PLLA), poly (D, L-lactic acid) (PDLLA), polyglycolic acid (PGA), polylactic-co-glycolic acid (PLGA), and polycaprolactone (PCL) have been widely used for fabricating the resorbable membranes due to their good mechanical properties and biocompatibility. Those polymers are approved by the US Food and Drug Administration (FDA) for fabricating several medical devices in clinical use (Song et al., 2018, Makadia and Siegel, 2011). Ideally, the barrier membranes should retain their barrier function at least 16-24 weeks (Hoornaert et al., 2016). It is found that degradation rate of those polymers declines in this order; PLGA, PGA, PLA, PCL, which are in ranges of 1-24 months (Rezwan et al., 2006). PLGA is the copolymer of PLA and PGA, which the manufacturer can control degradation rate by adjusting the percentage of those two polymers (Shin et al., 2006, Zhang et al., 2016). Nowadays, Three-dimensional (3D) printing technologies are widely available and affordable, therefore, several medical and dental products including polymeric membranes used for GBR have been computerized-designed and fabricated using the 3D printers (Kim et al., 2021, Petre et al., 2021). The research team of the Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Prince of Songkhla University has successfully developed the biodegradable PLGA membranes fabricated using the 3D printing technique. In this study, physical characteristics, and biocompatibility of the PLGA membranes, which consisting different ratios of lactide (LL) and Glycolide (GL) were comparatively evaluated in vitro.

Objectives of the study

1. To fabricate PLGA membranes using 3D printing process.
2. To evaluate their properties in terms of
 - Morphologies and microstructures
 - Surface wettability
 - Mechanical properties using tensile test

- Degradability including percentages of weight loss and pH change
 - Biocompatibility using cytotoxicity test and cell proliferation for osteoblasts and fibroblasts
3. To compare those properties of the PLGA membranes between the ratios of LL:GL=10:90 and 70:30

Methodology

Study groups

The study groups were divided into **Group A**: the membranes fabricated from PLGA (LL:GL=10:90) and **Group B**: PLGA (LL:GL=70:30).

Fabrication of the biodegradable membranes

In brief, the medical grade PLGA includes PLGA (LL: GL=10:90) (PURASORB PLG 1017, Corbion, Netherlands) and PLGA (LL:GL=70:30) (CMU-Bioplasorb PLG, Thailand). The materials of both groups were melted in the melting chambers of the melting-extruding machine, then extruded through the nozzle tip the filaments (Figure 1). The filaments were stocked for the 3D printing procedure.

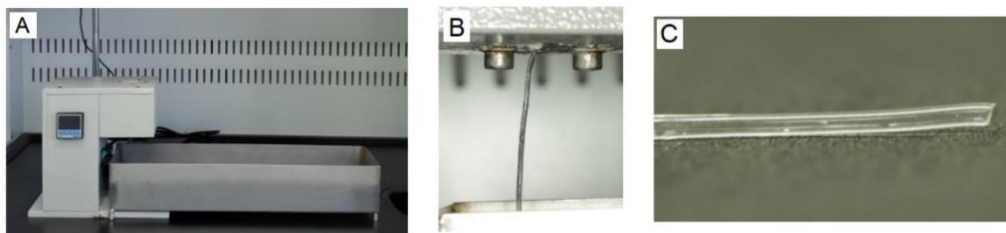


Figure 1 A: The melting-extruding machine. B: The melted PLGA is extruded through the nozzle tip of the machine. C: The stocked PLGA filament.

The PLGA membranes of both groups were fabricated using the 3D printer (RAISE3D E2, Raise 3D Technologies Inc., USA). The arranging pattern of the membranes were designed using the software program (ideaMaker version 4.1.1, Raise 3D Technologies Inc., USA) to be a layer height of 0.05 mm and eight layers per printing time (Figure 2).

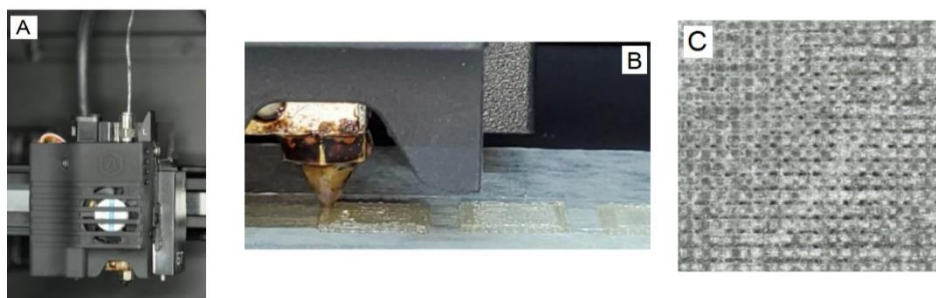


Figure 2 A: The PLGA filament was loaded into the print-head of the printer machine. B: the membrane was printed through the nozzle tip followed the designed pattern. C: The architecture of the membrane.

Morphologies and microstructures

Surface morphologies of the PLGA membranes size 10 x 10 mm were evaluated using a scanning electron microscope (SEM, Hitachi, SU3900, Japan) (n=3). Image J software was used to measure struts and pore size of the membranes

Mechanical properties

Tensile tests

The 50 x 10 mm PLGA membrane was immersed in phosphate buffer saline (PBS) for 3 hours prior to the experiment. The specimen in wet stage was secured at initial distance of 30 mm between the grips of the universal machine (Lloyd, Bognor Regis, West Sussex, UK) (n=5/group) (Figure 3). Tensile force was applied at a cross-head speed of 3 mm/min until the specimen was broken. Tensile strength, elastic modulus and elongation at break was recorded.

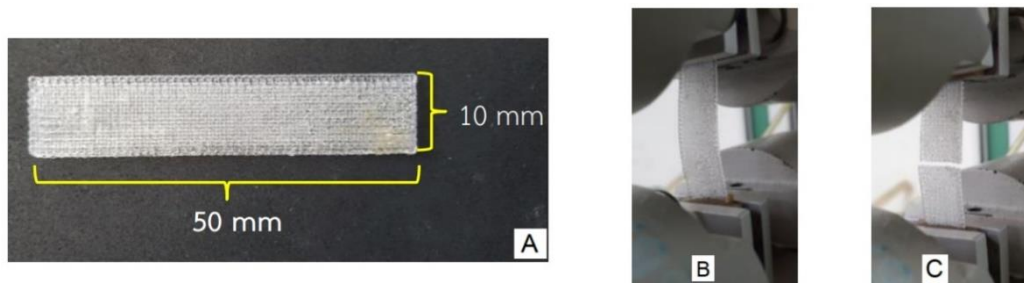


Figure 3 The setting of the tensile test. A: Shape of the specimens. B: The membrane was secured between the grips of the universal machine. C: Tensile force was applied until the specimen was broken.

Surface wettability

The surface wettability of the specimens was analyzed by an optical contact angle analyzer (Dataphysics, OCA25, Germany). The sessile drop method was used to measure the contact angle by depositing 5 μ l ultrapure water on the upper and lower surfaces of the PLGA membrane size 10 x 10 mm (n=3/surface/group). The lower surface was the surface which contact the plate of the printer.

Degradability

The 10x10 mm PLGA membranes were immersed in Simulated Body Fluid (SBF) over 120 days for assessment of their degradation behavior in terms of weight loss (%)(n=5/group/time point) and pH change (n=5/group). SBF was prepared in accordance with Kokubo et al (KoKubo et al., 2003).48 consisting of ion concentrations nearly equal to those of human blood plasma and buffered at pH 7.4 at 37°C. For pH observation, the control group was pure SBF. Prior to the experiment, pH of the SBF was measured for baseline using the pH meter (SevenCompact, Mettler Toledo, Switzerland), then the specimens was immersed in 4 mL of SBF/tube in 50 ml centrifuge tubes (Corning, USA) and incubated at a constant temperature of 37°C. The pH of the solution was measured at days 1, 3, 7, 14 and 21. For the weight loss observation, each specimen was weighted (Wd0) using an analytical balance (Satorius,

Germany) before immersing in 4 mL of SBF/well in 12-well tissue culture plates (Costar, Corning, USA) then incubated at a constant temperature of 37°C and added SBF every 10 days. The specimens were collected at days 15, 30, 60, 90 and 120 thereafter for measuring their weight loss. At each time point, the specimens were removed from the solution, each specimen was rinse with distilled water and freeze-dried in the freeze drying and vacuum concentration equipment (LaboGene ApS, Denmark) for 3 h, its dried weight (Wdt) was measured. Weight loss of the specimen was calculated using the following equation; %Weight loss = $100 \times (Wd0 - Wdt)/Wd0$. The morphologies of the membranes at each time point were evaluated using SEM (n=2/time point).

Biocompatibility

Murine osteoblasts (MC3T3-E1 cell line, ATCC, USA) and Murine fibroblast (L929 cell line, ATCC, USA) were grown in the proliferation medium [α -MEM medium (Gibco, USA) supplemented with 10 % fetal bovine serum (FBS, Gibco, USA) and antibiotic (10,000 unit/ml penicillin, 10 mg/ml streptomycin and 250 μ g/ml amphotericin B) (Gibco, USA)]. The cells in passage 3-5 were used for the experiments. Prior to the experiments, the PLGA membranes size 10x10 mm were sterilized using ethylene oxide gas at 2 weeks prior to the experiments.

Cytotoxicity test

The cells were seeded on the 24-well plates (Costar, Corning, USA) at 2×10^4 cell/well and 1 ml of the proliferation medium was added (n=5/cell type/group/time point). The plates were incubated in 5% CO₂ at 37°C for 4 h for allowing cell attachment. Afterwards, the membrane was be immersed into the medium of each well and secured as close as the bottom surface of the well that they could direct contact to the cells, and the cells were cultivated in 5% CO₂ at 37°C. At 24 h, 48h and 72 h thereafter, the membranes were removed, and viability of the cells was qualitatively evaluated using PrestoBlue (Thermo Fisher Scientific, USA). The absorbance of each well was read at 600 nm using a microplate reader (Thermo Fisher Scientific, Finland).

Cell proliferation

The cells were seeded on the membranes at 2×10^4 cells/membrane in 24-well plates (Costar, Corning, USA). The plates was incubated in 5% CO₂ at 37°C for 4 h for allowing cell attachment on the membrane. Afterwards, the membrane was transfer to fresh 24-well plates then cultured in 1 ml of the proliferation medium for 14 days (n=5/cell type/group). At day 1, 7 and 14 after seeding, amounts of the viable cells were measured using PrestoBlue reagent. The absorbance of each well was read at 600 nm using a microplate reader (Thermo Fisher Scientific, Finland). At each time point after the measurement, the cells-membrane constructs of each well in the 24-well plate were wash several time using PBS and refreshed with 1 ml of the proliferation medium. The culture was continued for the next testing.

SEM

The cells were seeded on the membranes at 2×10^4 cells/membrane in 24-well plates (Costar, Corning, USA) and cultured as previously described (n=2/cell type/group/time point). At day 1, 7 and 14

after seeding, the cell-membrane constructs were fixed in 10% formalin for 15 min. After that, freeze-dried in the freeze drying and vacuum concentration equipment (LaboGene ApS, Denmark) for 2 hours, then coated with gold-palladium. Cell characteristics of the constructs were examined via SEM.

Statistical analysis

The data was analyzed using statistical analysis software. The morphologies of the membranes and the microscopic features of cells in the constructs were assessed descriptively. Independence T-test and One-way Analysis of Variance (ANOVA) followed by Tukey HSD was applied to compare the differences of the quantitative parameters among the experiment groups and the time points. If the data was not normal distribution or homogeneity of variance was violated, Kruskal-Wallis test was applied. The level of statistical significance was set at a $p < 0.05$.

Results

Morphologies and microstructures

The SEM image shows the smooth surface of PLGA membrane. Shapes of the pores were almost round, the average pore's size was $232 \pm 23 \mu\text{m}$, width of the struts was $170 \pm 17 \mu\text{m}$, width of the struts that connected each layer was $307 \pm 21 \mu\text{m}$ and 23% porosity (Figure 4A, B). Cross-sectional aspect shows that each layer connected by melting material, thickness of membrane was $0.65 \pm 0.02 \text{ mm}$.

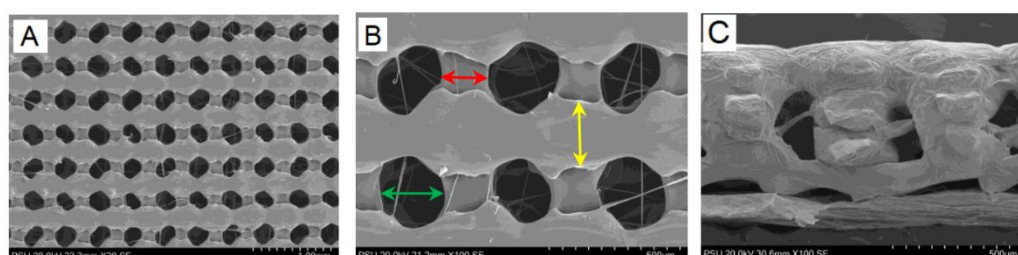


Figure 4 The SEM image of PLGA membrane A: surface of the membrane using magnification 30x. B: surface of the membrane using magnification 100x, the green arrow identified pore's size, the red arrow identified width of the struts, the yellow arrow identified width of the struts that connected each layer. C: cross-sectional of the membrane using magnification 100x.

Table 1 Summary of the mechanical properties of the membrane. (*=significant difference, $p < 0.05$)

Group	Tensile strength (MPa)	Strain at maximum load (%)	Young's modulus (MPa)
A	1.64 ± 0.29	10.45 ± 1.06	40.50 ± 6.66
B	2.12 ± 0.62	2.49 ± 0.73	99.92 ± 12.36
p-value	0.049*	0.000*	0.000*

Tensile tests

The mechanical properties of the membrane represent in table 1. Tensile strength and young's modulus of group B were higher than those of group A, whereas % strain at maximum load of group A was higher. Statistically significant differences between groups were detected.

Surface wettability

Figure 5A shows that the contact angle values between group A and B were not significantly different, thus wettability of the surfaces of the membranes of both groups was similar. The contact angle values of top and bottom of the membranes of group A were 101.79 ± 1.07 and 103.40 ± 1.61 respectively. The contact angle values of top and bottom of the membranes of group B were 101.44 ± 0.98 and 101.98 ± 1.37 respectively. Figures 5B-E represent that the obtuse of the contact angles, the hydrophobic behavior they have.

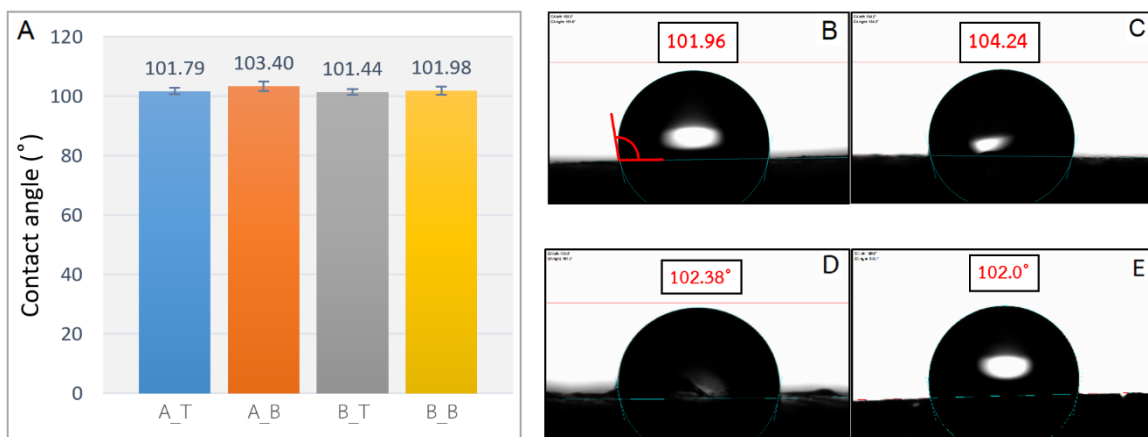


Figure 5 A: The bar chart demonstrates the contact angle values of the PLGA membranes (A_T; Top of the membrane group A, A_B; Bottom of the membrane group A, B_T; Top of the membrane group B, B_B; Bottom of the membrane group B), (* $p < 0.05$). B, C, D and E: The optical images of water contact angles of A_T, A_B, B_T and B_B, respectively.

Degradation

The degradation profiles of the PLGA membranes are demonstrated in figure 6A. Over 90 days, the membranes of both groups remarkably degraded with time and no difference between groups was detected. Average percentages of weight loss of groups A and B over 120 days were 75.26 ± 2.89 and $72.24 \pm 1.82\%$, respectively. At day 15 and day 30, the degradation rates of group B were statistically significant slower than those of group A, the percentages of weight loss of groups A and B at day 15 were 23.02 ± 2.08 and $4.82 \pm 0.47\%$, respectively and at day 30 were 38.04 ± 3.78 and $27.22 \pm 2.11\%$, respectively. In figure 7, the SEM images demonstrate that the PLGA membranes of groups B had slight erosion in some areas on the first 30 days and afterwards, erosion gradually increased. Erosive areas and crack lines on the surfaces of the membranes were obviously seen on day 120. The membranes of

group A lost their integrity on day 14 that shown in figure 10C and 11C. Therefore the specimens of group A could not be processed for SEM.

The changes of pH of SBF, which containing the PLGA membranes are demonstrated in figure 6B. It was found that the pH of group B slightly changed over 21 days. In contrast, the pH of group A decreased gradually during the first 7 days and dropped rapidly thereafter until day 21. The final pH of group A was 3.05, group B was 6.59 and SBF alone was 7.37.

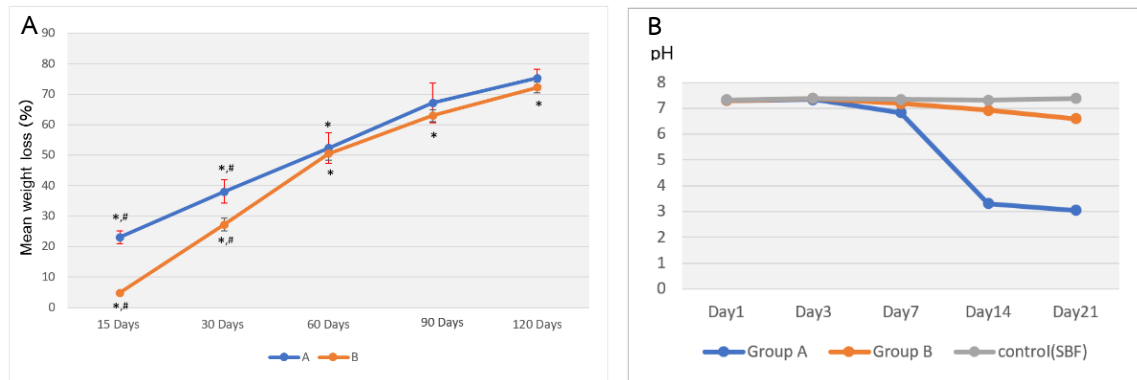


Figure 6 A: The graph demonstrates the degradation of PLGA membranes over 120 days. (# p<0.05 compared between the groups at each time point, * p<0.05 compared among the time points), B: The graph demonstrates profiles of the pH changes of SBF over 21 days.

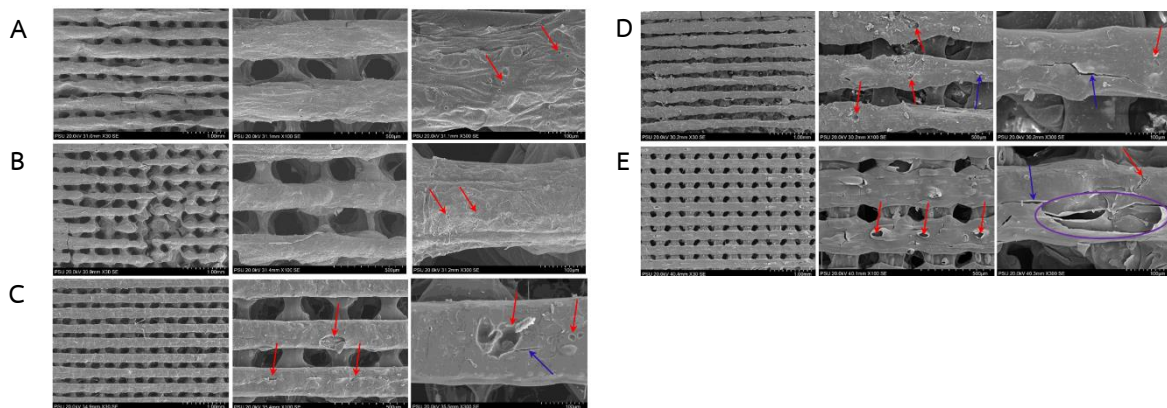


Figure 7 The SEM images of the PLGA membrane of group B; A: on day 15, B: on day 30, C: on day 60, D: on day 90 and E: on day 120. The red arrows indicate the erosive areas and blue arrow indicates the crack line. The purple circle shows the large defect.

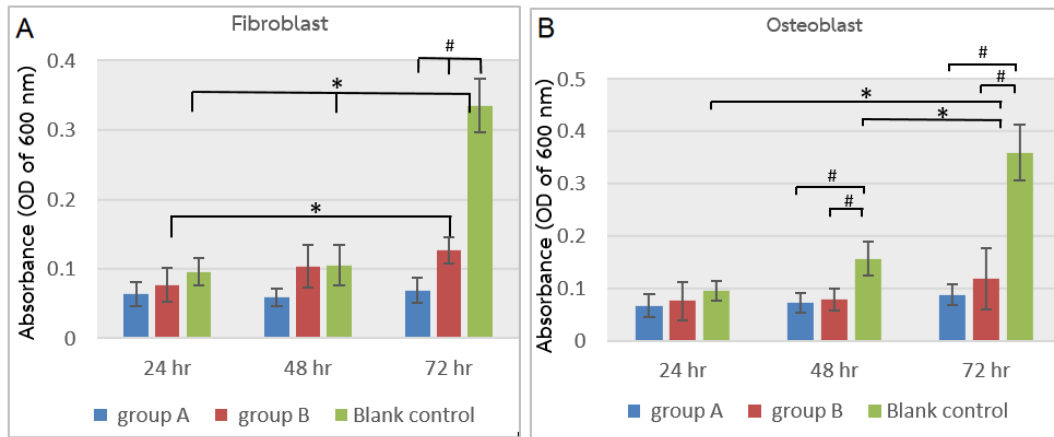


Figure 8 A: The graph demonstrates the cytotoxicity result using fibroblasts. B: The graph demonstrates the cytotoxicity using osteoblasts (# p<0.05 compared among the groups, * p<0.05 compared among the time points)

Cytotoxicity test

Figure 8A demonstrates the cytotoxicity result using fibroblast. The amounts of cells of group B increased with time, whereas those of group A slightly increased. However, the viable cells of both groups were less than those of the control group. Figure 8B, using osteoblast demonstrates that the amounts of cells of both groups slightly increased, and they were still less than the control. Group.

Cell proliferation

Figure 9 demonstrates the proliferation of the cells on day 1, 7, and 14. For fibroblasts, the amounts of cells of group B remarkably increased with time, whereas the amounts of the cells of group A increased on day 7 but decreased thereafter. For osteoblasts, there was not statistically different between both groups on the first 7 days. On day 14, the number of cells of group B was statistically significantly higher than that of group A.

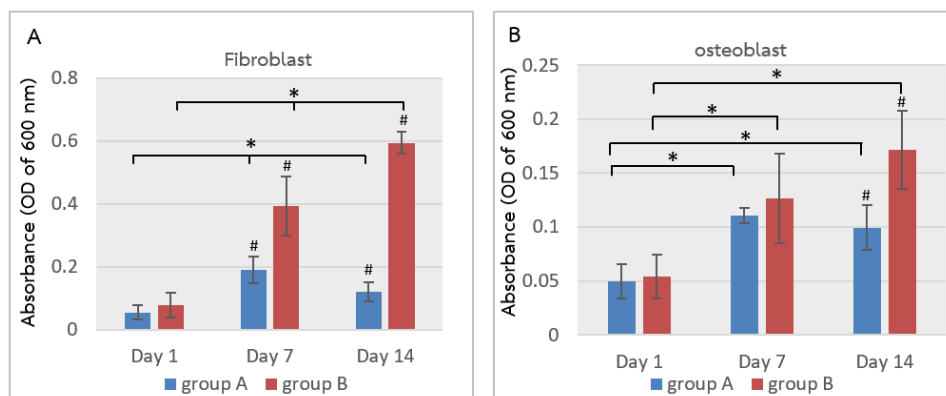


Figure 9 The bar graphs of the cell proliferation on the PLGA membranes. A: for fibroblasts and B for osteoblasts (# p<0.05 compared between groups, * p<0.05 compared among the time points).

The SEM images demonstrate that the fibroblasts could attach and grow well on the surfaces of membranes of both groups on day 1 and day 7. On day 14, the cells of group B continued growing in multi-layers, whereas cells of group A remarkably decreased (Figure 10 and 11).

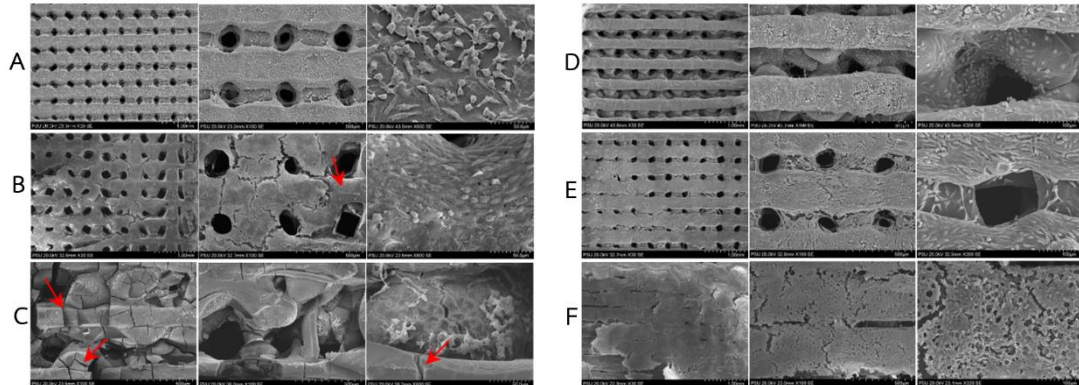


Figure 10 The SEM images of the fibroblasts proliferated on the PLGA membranes in group A (A, B, C) and group B (D, E, F). A and D: day 1, B and E: day 7, C and F: day 14. The red arrows indicate the crack lines.

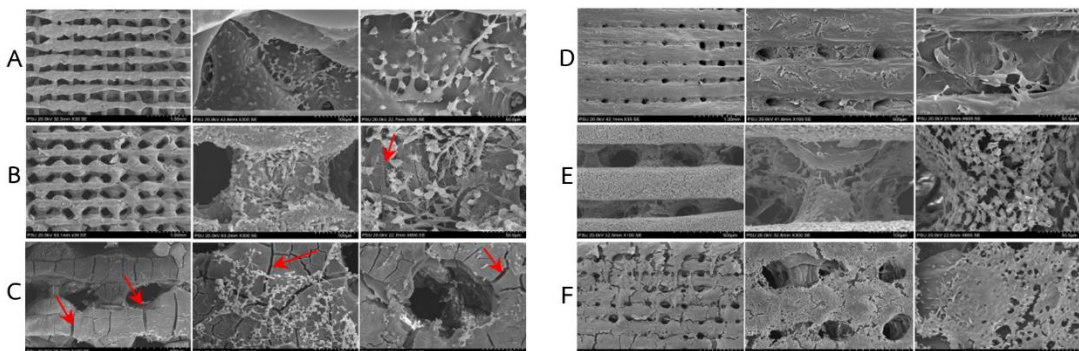


Figure 11 The SEM image of osteoblast proliferated on the PLGA membranes in group A (A, B, C) and group B (D, E, F). A and D: day 1, B and E: day 7, C and F: day 14. The red arrows indicate the crack lines.

Discussion

In this study, the PLGA membranes were fabricated using the 3D printing technique. By using the in-house melt-extruding technique and the device, both ratios of PLGA could be processed to be the filaments which had average diameter of 1.7 mm. This diameter is the standard size of the polymeric filament used for general 3D printers. Therefore, the stock filaments can be applied for fabrication in large batches, and they can be used with other high-end printers that especially designed for fabricating medical devices. The architectures of the membranes were designed using the computerized design-software, therefore, the structures of the membranes such as pore size and thickness can be easily customized prior to the fabrication. In this study, the membranes were designed to have micro-pores

which had the average pore size of 232 ± 23 μm on both sides for allowing vessel regeneration that is essential for initial bone healing process (Gutta et al., 2009). The PLGA materials used in this study were medical grade that are suitable for fabricating the membranes for clinical use. The properties of the PLGA membranes which made from the different ratios of lactide (LL) and Glycolide (GL) of the PLGA were comparatively evaluated. Regarding the results, the tensile strength of the PLGA membranes of group A and B was 1.64 ± 0.29 MPa and 2.12 ± 0.62 MPa respectively. When compared to that of the commercial collagen membrane in wet stage such as BioGide (1.68 ± 0.54 MPa) and Ossix Plus (1.2 ± 0.14 MPa) (Brosh et al., 2019), it implied that our PLGA (70:30) membranes had better mechanical strength. Therefore, they would maintain underneath spaces without collapsing during bone healing process better than the commercial collagen membranes. For the degradation behaviors, the membranes of both groups degraded more than 50% within 60 days and more than 70% within 90 days. This seemed to be commensurate with bone healing process (Roden, 2010). It is known that the degradation rates of PLGA depend on the different composition ratios of lactide and Glycolide. If it has more LL, the degradation is slower. Initially, it was hypothesized that the PLGA (10:90) membrane was categorized to be the rapid resorbable membrane which is comparable to the collagen membrane (Owens and Yukna, 2001, Moses et al., 2008). Whereas the PLGA (70:30) membrane is the slow resorbable membrane. The results demonstrated that the PLGA (10:90) membranes and the PLGA (70:30) membranes had high contact angles which implied that they had hydrophilic behaviors. Nevertheless, the PLGA (10: 90) membranes obviously loss their integrity on day 15 when compared with the PLGA (70: 30) membranes. In addition, they produced more acidic environment after day 7 that would be unsuitable for viable of cells. This condition was clearly demonstrated in the cell viability tests. The number of viable cells in the medium which immersed with the PLGA (10: 90) membranes was reduced when compared with those of the PLGA (70:30) membranes. Correspondingly, the growth of the cells on the PLGA (10: 90) membranes was decreased after day 7, whereas the cells could grow well on the PLGA (70:30) membranes until day 14 of the observation period.

Conclusion

The PLGA membranes were successfully fabricated using the 3D printing technology. The membranes made from PLGA (LL: GL 70: 30) were suitable for GBR in terms of good mechanical properties, degradability and biocompatibility. However, their biocompatibility and degradation behavior should be re-evaluated and proved in animal models prior to clinical trials.

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