Antibacterial efficacy of triple-layered poly(lactic-co-glycolic acid)/nanoapatite/lauric acid guided bone regeneration membrane on periodontal bacteria

Nur Najihah SAARANI1, Kalitheerta JAMUNA-THEVI1,2, Neelam SHAHAB3, Hendra HERMAWAN4 and Syafiqah SAIDIN5

1 Faculty of Biosciences & Medical Engineering, Universiti Teknologi Malaysia, 81310 UTM Johor Bahru, Malaysia
2 Advanced Materials Research Center, SIRIM Berhad, 09000 Kulim, Kedah, Malaysia
3 Industrial Biotechnology Research Center, SIRIM Berhad, 40700 Shah Alam, Selangor, Malaysia
4 Department of Mining, Metallurgical and Materials Engineering & CHU de Québec Research Center, Laval University, Quebec City, G1V 0A6, Canada

Corresponding authors, Syafiqah SAIDIN; E-mail: syafiqahsaidin@biomedical.utm.my; syafiqahsaidin@gmail.com, Hendra HERMAWAN; E-mail: hendra.hermawan@gmn.ulaval.ca; hendra.hermawan@gmail.com

A guided bone regeneration (GBR) membrane has been extensively used in the repair and regeneration of damaged periodontal tissues. One of the main challenges of GBR restoration is bacterial colonization on the membrane, constitutes to premature membrane degradation. Therefore, the purpose of this study was to investigate the antibacterial efficacy of triple-layered GBR membrane composed of poly(lactic-co-glycolic acid) (PLGA), nanoapatite (NAP) and lauric acid (LA) with two types of Gram-negative periodontal bacteria, Fusobacterium nucleatum and Porphyromonas gingivalis through a disc diffusion and bacterial count tests. The membranes exhibited a pattern of growth inhibition and killing effect against both bacteria. The increase in LA concentration tended to increase the bactericidal activities which indicated by higher diameter of inhibition zone and higher antibacterial percentage. It is shown that the incorporation of LA into the GBR membrane has retarded the growth and proliferation of Gram-negative periodontal bacteria for the treatment of periodontal disease.

Keywords: Antibacterial, Guided bone regeneration, Lauric acid, Periodontal bacteria

INTRODUCTION

Guided bone regeneration (GBR) membrane is used in a surgical therapy for the treatment of infected mandible and alveolar bone defects caused by periodontal diseases1,2. The membrane is positioned to prevent the down-growth of fibroblastic cells into an intraosseous wound during healing, while allowing slow migration of osseous cells into the defective area, resulting in direct bone tissues regeneration3. Currently, synthetic resorbable polymers are widely used as GBR membranes due to their degradation ability that allow periodontal regeneration3,4. Polymers such as poly(lactic acid) (PLA), poly(glycolic acid) (PGA), poly(lactic-co-glycolic acid) (PLGA), poly(caprolactone) (PCL) and their co-polymers or tissue-derived collagen are usually become the base component of the membranes3,5,6. It has been approved by European authorities and Food and Drug Administration (FDA) for the treatment of periodontal disease, covering bone defects, empty sockets, dental pre- and peri-implant surgeries5. However, excessive accumulation of acidic degradation products has been reported which will leads to unwanted inflammatory reactions6.

One main factor that contributes to unsuccessful tissue regeneration by GBR membrane treatment is the action of periodontal bacteria such as Porphyromonas gingivalis (P. gingivalis) and Actinobacillus actinomycetemcomitans7. The proliferation of these bacteria should be controlled on the site of GBR membrane restoration to ensure successful tissue migration, growth and proliferation8. Several antibiotics such as tetracycline hydrochloride and metronidazole benzoate have been used extensively to overcome this problem5,6. However, in a view to prevent antibiotic resistance, alternative antibacterial agents are of interest to be incorporated into the GBR membrane9,10. Lauric acid (LA) is a typical free fatty acids found in human sebum and natural resources such as coconut palm and milk11,12. Its biocompatibility and strong antibacterial property has attracted attention of researchers to explore the antibacterial effect of LA against variety of microorganisms12,13. However, the effectiveness of LA against periodontal bacteria and its potential to be used in GBR membrane is still unknown.

Other than the antibacterial property, it is also preferable for the membrane to bioactive for rapid osteoblast proliferation and migration, so that the inner surface will be populated with osteoblast cells, leading to acceleration of bone formation9. Nanoapatite (NAP) is commonly used as a nanofiller due to its excellent bioactive property with the resemblance to inorganic constituent of bone14,15. Its chemical composition, Ca10(PO4)6(OH)2, and rough morphological structure are the main factors that contribute to the acceleration of osteoblast cells attachment and proliferation15,16. Therefore, in this study, the GBR membranes obtained

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from Advanced Materials Research Center, SIRIM Berhad, Malaysia, were composed of PLGA, NAp and LA to provide an optimal characteristic of GBR membrane.

The membranes were comprised of three stacked layers (L1, L2 and L3) as shown in Fig. 1: L1 acts as the outermost layer that interfaces the gingival; L2 acts as the intermediate layer; and L3 acts as the inner layer that interfaces the crestal bone. The construction of these three layers was based on the work by Jamuna-Thevi et al. where the L1 was incorporated with PLGA, the highest proportion of LA and the lowest proportion of NAp to accommodate the layer with an optimum antibacterial effect. While the L3 was incorporated with PLGA, the highest proportion of NAp and without LA to equip the layer with an optimum bioactive property. The antibacterial efficacy of the triple-layered GBR membrane was then identified with two types of Gram-negative periodontal bacteria, *Fusobacterium nucleatum* (*F. nucleatum*) and *P. gingivalis* through a disc diffusion test.

**Table 1** Composition of triple-layered GBR membranes

<table>
<thead>
<tr>
<th>Sample</th>
<th>L1 Composition (wt%)</th>
<th>L2 Composition (wt%)</th>
<th>L3 Composition (wt%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>PLGA:20 NAp:— LA:—</td>
<td>PLGA:9 NAp:— LA:—</td>
<td>PLGA:9 NAp:— LA:—</td>
</tr>
<tr>
<td>1 wt% LA</td>
<td>PLGA:20 NAp:10 LA:1</td>
<td>PLGA:9 NAp:20 LA:1</td>
<td>PLGA:9 NAp:30 LA:—</td>
</tr>
</tbody>
</table>

**MATERIALS AND METHODS**

**Sample preparation**

Samples of triple-layered GBR membrane were obtained from Advanced Materials Research Center, SIRIM Berhad, Malaysia. It was fabricated via solvent casting and thermally induced phase separation/solvent leaching technique as described in the previous work. Three stacked layers (L1, L2 and L3) as shown in Fig. 1(b) were prepared by incorporating PLGA (LA:GA 85:15, Durect LACTEL Absorbable Polymers, Cupertino, CA, USA), LA (98% purity, Sigma Aldrich, St. Louis, MO, USA) and NAp powders (composition: 40.08 wt% Ca, 18.58 wt% P, 1.84 wt% Na, 1.46 wt% Mg, 0.06 wt% K and 4.80 wt% CO₃²⁻) at 9–20, 1–3 and 10–100 wt%, respectively. Table 1 shows composition of the triple-layered GBR membranes. The GBR membranes were then punched into 6 mm diameter of disc shape. The discs were disinfected in 70% ethanol for 20 s and dried in a closed sterile petri dish. Samples of triple-layered pure PLGA membrane without NAp and LA were also fabricated and mentioned as control.

**Preparation of saliva impregnated GBR membrane disc**

In this antibacterial study, saliva impregnated membranes have been prepared and tested, to identify whether saliva could impact on different antibacterial reactions of the triple-layered GBR membranes. Saliva has a direct antibacterial activity and possesses antifungal and antiviral properties. The impregnation of a sample with saliva is crucial to mimic the actual oral physiological environment. Unstimulated saliva from a healthy donor was collected between 9.00 am to 11.00 am and stored in an ice-chilled test tube. A single donor was selected to minimize variation in saliva composition that may arise between individual. The donor was asked to gargle with distilled water to reduce bacterial contamination prior to collection.

The collected saliva was centrifuged at 10,000 rpm for 30 min. Its suspension was collected and sterilized by filtration. Then, 100 µL of the sterilized saliva was pipetted on each sterilized GBR membrane disc for the impregnation process. The impregnated discs were then dried in a closed sterile petri dish. The discs were disinfected in 70% ethanol for 20 s and dried in a closed sterile petri dish. Samples of triple-layered pure PLGA membrane without NAp and LA were also fabricated and mentioned as control.
finally dried in a laminar flow chamber.

**Disc diffusion test**

Antibacterial efficacy of the triple-layered GBR membranes was investigated against *F. nucleatum* (ATCC 25586) and *P. gingivalis* (ATCC 33277). The supplement media of vitamin K-hemin solution was prepared by mixing vitamin K (1 mg/mL) and hemin (5 mg/mL) at a ratio of 1:100. It was wrapped with an aluminum foil and stored in a dark chamber. The bacteria were cultured on brucella blood agar (Sigma Aldrich) supplemented with vitamin K-hemin solution media in an anaerobic chamber at 37°C for 3 to 10 days. The grown colonies were sub-cultured on fresh brucella blood agar and stored at 4°C in a gas jar supplied with an anaerobic gas pack up to 4 days.

The grown colonies following the incubation period were harvested and dispensed in 5 mL of brucella broth. Turbidity of the suspension was adjusted to an equivalent bacterial concentration of $1 \times 10^8$ cfu/mL using a spectrophotometer (Varian, Cary 50, Agilent, CA, USA) at an optical density of 550 nm. The sterilized membrane discs, with and without the impregnation of saliva, were then firmly placed on brucella blood agar which had been smeared with 100 µL of $1 \times 10^8$ cfu/mL bacteria. The agar plates were incubated anaerobically at 37°C for 3 days. After 3 days, diameter of the inhibition zones was measured using a ruler.

**Bacterial count test**

In order to validate the disc diffusion data, which has a limitation in the effect of sample solubility and diffusion rate in an agar medium, a quantitative bacterial count analysis was also performed. The bacterial count test was conducted according to ASTM E2180-07 standard. One milliliter of $1 \times 10^8$ cfu/mL bacteria was inoculated into 9 mL of agar slurry. Then, 0.1 mL of the inoculated slurry was pipetted on top of each sterilized membrane disc and swirled lightly to cover the whole membrane surface. The inoculated membrane discs were then anaerobically incubated at 37°C for 24 h. After 24 h, the discs were washed with 9.9 mL of sterile phosphate buffered saline (PBS). The washing solution of 1 mL was transferred into 9 mL of sterile trypton water and it was serially diluted. Each dilution suspension (100 µL) was then dispensed onto brucella blood agar. The suspension was spread firmly on the agar and incubated at 37°C for 10 days, anaerobically. After incubation, colonies formed on the agar were counted to calculate colony forming unit (CFU) using the following standard equation:

$$\text{CFU} = \frac{\text{Number of colonies} \times \text{Dilution factor}}{\text{Volume inoculated}}$$  \hspace{1cm} (1)

The CFU values were further used to calculate the antibacterial percentage using following percentage equation:

$$\text{Antibacterial percentage} (%) = \frac{\text{CFU}_{\text{control}} - \text{CFU}_{\text{test}}}{\text{CFU}_{\text{control}}} \times 100$$ \hspace{1cm} (2)

where CFU$_{\text{control}}$ is the CFU value of control sample and CFU$_{\text{test}}$ is the CFU value of test sample.

**Statistical analysis**

The triplicate antibacterial data were expressed in mean±standard deviation. Significance differences between formulation of the antibacterial GBR membranes were determined through an analysis of variance (ANOVA) using SPSS v.16.0 (SPSS, Chicago, IL, USA) statistical software at 95% confidence level ($p<0.05$). A statistical *post-hoc* analysis was also conducted through Tukey’s multiple comparison test to evaluate differences between pair of data.

**RESULTS**

**Disc diffusion analysis**

Figure 2 shows an obvious inhibition zones around the triple-layered GBR membranes (1, 2 and 3 wt% LA) toward both periodontal bacteria compared to the control (pure PLGA) discs. The ranges of inhibition zones were differed significantly for all samples which subjected to *P. gingivalis* and all samples which subjected to *F. nucleatum* as presented in Table 2. These results indicate that the addition of LA into the GBR membranes gave a notable effect on the antibacterial property of the membrane. Besides, the diameter of the inhibition zones increases with the increasing of LA concentration (Table 2). Interestingly, *P. gingivalis* was found to be more susceptible to the antibacterial effect of the GBR membrane compared to *F. nucleatum*.

The *post-hoc* statistical analyses demonstrated that the discs with different concentration of LA produced significant inhibition zones except for 2 and 3 wt% LA membrane discs and 2 and 3 wt% LA saliva impregnated membrane discs on *F. nucleatum*. The 2 wt% LA membranes were enough to inhibit the growth of *F. nucleatum* as increasing the LA content did not contribute to higher range of inhibition zones, significantly. Furthermore, the *post-hoc* analyses also revealed that there was no significant different between the inhibition zones of membrane discs and the saliva impregnated membrane discs, either on *P. gingivalis* or *F. nucleatum* for the similar content of LA concentration. The antibacterial effect of saliva was not strong enough to further inhibit bacterial growth, while in a formulation point of view, the saliva did not interrupt the release of antibacterial agent from the GBR membranes.

**Bacterial count analysis**

Bacterial count analysis was conducted on the GBR membranes without the impregnation of saliva due to indistinct data of inhibition zone produced by the saliva impregnated membranes. Figure 3 shows antibacterial percentage of GBR membranes on *P. gingivalis* and *F. nucleatum*. The triple-layered GBR membranes displayed a great potential in killing and retarding periodontal bacterial growth, significantly. After 10 days of incubation, the 2 and 3 wt% LA membranes exhibited high antibacterial activities against *P. gingivalis* and
Table 2 Diameter of inhibition zones produced by triple-layered GBR membranes with different composition of LA on P. gingivalis and F. nucleatum bacterial species (mean±standard deviation)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Membrane disc (mm)</th>
<th>Saliva impregnated membrane disc (mm)</th>
<th>P. gingivalis</th>
<th>F. nucleatum</th>
<th>F. nucleatum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P. gingivalis</td>
<td>F. nucleatum</td>
<td>P. gingivalis</td>
<td>F. nucleatum</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>1 wt% LA</td>
<td>10.50±1.80</td>
<td>8.43±1.50</td>
<td>10.67±1.52</td>
<td>8.77±0.68</td>
<td></td>
</tr>
<tr>
<td>2 wt% LA</td>
<td>12.70±0.58</td>
<td>11.10±1.65</td>
<td>13.00±1.00</td>
<td>11.67±1.53</td>
<td></td>
</tr>
<tr>
<td>3 wt% LA</td>
<td>15.70±0.58</td>
<td>13.00±1.73</td>
<td>16.33±0.58</td>
<td>13.57±1.25</td>
<td></td>
</tr>
</tbody>
</table>

In particular, the 3 wt% LA membranes killed up to 73% of P. gingivalis and 72% of F. nucleatum. These data were in accordance to the post-hoc statistical analyses where there was a significant different between all samples (1, 2 and 3 wt% LA) in killing P. gingivalis but only 2 and 3 wt% LA have a significant different in killing F. nucleatum. Therefore, the addition of LA, up to 3 wt% is necessary for the long term antibacterial effect of the GBR membranes against both periodontal bacteria.

**DISCUSSION**

A GBR membrane has been increasingly used for repair and regeneration of both structure and function of damaged periodontal tissues. In this study, LA was incorporated into a GBR membrane to accommodate
the membrane with an antibacterial property. The highest composition of LA (3 wt%) has produced the largest inhibition zone and the highest antibacterial percentage compared to the control, 1 and 2 wt% LA GBR membranes.

The antibacterial mechanism of LA is associated to the straight chain saturated fatty acid (C12)\textsuperscript{23,24}. The carboxyl group of LA molecules are also contributing to the antibacterial effect of this compound as reduction of carboxyl group to aldehyde/alcohol or transformation into amide/amine group will increase the bacteriostatic property of LA\textsuperscript{20}. Zhao et al.\textsuperscript{13} stated that another antibacterial mechanism involves in killing the bacteria is anchor to the composition of LA which deactivates enzyme activity and denatures bacterial protein, thus leading to bacterial apoptosis.

In this study, it was proved that the LA has a wide inhibition spectrum to strict anaerobic Gram-negative bacteria. Although these results confirmed the incorporation of LA into the GBR membrane has retarded the growth of \textit{P. gingivalis} and \textit{F. nucleatum}, further studies on the feasibility of the membrane against other periodontal relevant bacteria (e.g.: \textit{Actinobacillus mycetecomitans}, \textit{Aggregatibacter actinomyctecomitans}, \textit{Bacteroides forsythus (Tannerella forsythensis)}) need to be investigated. Theoretically, the LA incorporated GBR membrane would easily disrupt and kill Gram-positive periodontal bacteria due to absence of an outer membrane which can prevent the influx of foreign molecules\textsuperscript{24}.

Clinically, oral administration of antibiotics such as metronidazole benzoate (MET) as an adjunctive treatment for periodontal diseases has reduced the presence of pathogens in gingival fluid and periodontal pocket\textsuperscript{20}. Furthermore, a PLGA membrane loaded with MET fabricated by a solvent casting method showed a significant improvement on periodontal regeneration\textsuperscript{23,24}. The membranes with 2 and 3 wt% LA produced long term antibacterial activities against both periodontal bacteria, significantly. Therefore, the incorporation of LA as an antibacterial agent is crucial to sustain the antibacterial property of the GBR membrane, thus preventing inflammatory reaction for successful gingival and bone tissues regeneration.

CONCLUSION

Antibacterial efficacy of the triple-layered PLGA/NAP/LA GBR membranes were identified against Gram-negative periodontal bacteria, \textit{P. gingivalis} and \textit{F. nucleatum}. The membranes with 2 and 3 wt% LA produced long term antibacterial activities against both periodontal bacteria, significantly. Therefore, the incorporation of LA as an antibacterial agent is crucial to sustain the antibacterial property of the GBR membrane, thus preventing inflammatory reaction for successful gingival and bone tissues regeneration.

ACKNOWLEDGMENTS

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REFERENCES

15. Nordin JA, Prajitno DH, Saidin S, Nur H, Hermawan H.


