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Topographic characterization and *in vitro* biofilm adhesion to titanium and polypropylene membranes used for alveolar preservation

Marcela Resende[1] and Elizabeth Ferreira Martinez[2]

[1]*Division of Periodontology, São Leopoldo Mandic Research Institute, Campinas, Brazil*
[2]*Division of Oral Pathology and Cell Biology, São Leopoldo Mandic Research Institute, Campinas, São Paulo, Brazil*

Address for correspondence: Dr. Marcela Resende, Division of Periodontology, Faculdade São Leopoldo Mandic, R. José Rocha Junqueira, 13 Campinas, São Paulo 13045-755, Brazil. E-mail: ceicasaudemr@gmail.com
The work belongs to the Division of Oral Pathology and Cell Biology, São Leopoldo Mandic Research Institute, Campinas, São Paulo, Brazil

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Abstract

Background:

Nonresorbable membranes have been widely used in guided bone regeneration (GBR) procedures in posttooth extraction alveoli. In this context, one of the properties suggested by the GBR technique is that these barriers, when exposed to the oral environment, control or prevent the infiltration of connective and epithelial tissue cells, favoring the proliferation of bone cells inside the alveolus, without the growth of biofilm.

Materials and Methods:

This *in vitro* study evaluated the topographic characteristics and *in vitro* biofilm adhesion on membranes used for alveolar preservation, bone Heal™ and Titanium Seal™. Fragments of these membranes (5 mm × 5 mm) were used for all experiments. The topographical morphology and chemical characterization of the membranes were analyzed by scanning electron microscope and dispersive energy X-ray spectroscopy, respectively. For the *in vitro* biofilm adhesion assay, samples were immersed in *Candida albicans* (American Type Culture Collection [ATCC] 10231) and *Staphylococcus aureus* (ATCC 25923) mixed biofilm for 7 and 14 days. Biofilm formation was measured by quantitative analysis with crystal violet aqueous solution, in a spectrophotometer, with a wavelength of 590 nm.

Results:

The ultrastructural images showed a rough surface for the titanium membrane, without homogeneity in the surface structure, while the polypropylene membrane presented a smoother surface without depressions. The chemical composition of the membranes by Ehlers–Danlos syndrome has identified the presence of copolymer and traces of zinc for the polypropylene membrane; in contrast, the titanium membrane revealed the unique presence of titanium. In addition, there was a decrease in biofilm formation on the surface of the titanium membrane compared to polypropylene ($P < 0.05$), at both evaluated times.

Conclusions:

It can be concluded that despite the greater heterogeneity of the titanium membrane surface, the results showed less biofilm formation on this membrane ($P < 0.05$), which may be indicated in cases of oral cavity exposure.

Keywords: Alveolar preservation, guided bone regeneration, nonabsorbable membrane

INTRODUCTION

After tooth extraction, several techniques have been used in an attempt to minimize bone and tissue remodeling as a result of resorption and volume reduction during alveolar healing.[1,2,3,4,5,6] Most resorption occurs within the first 3 months of healing, which can reduce the buccolingual dimension of the alveolus by up to 50%.[7]

Due to the dimensional limitations of the buccal bone plate, when extraction occurs, resorption at this stage is greater when compared to that of the lingual surface, which may limit the installation of an implant functionally and esthetically.[4]

The use of barriers with prefabricated membranes is more convenient than the use of free gingival graft because it does not require another donor site. It should be biocompatible as a characteristic, thus providing sufficient space for clot formation and cell differentiation, thereby mediating bone tissue formation.[8] The use of an occlusive membrane as an alveolar preservation procedure also prevents particle loss and migration of epithelium and fibroblastic cells from entering the defect area.[9]

With the onset of guided bone regeneration (GBR), the first successful procedure used the expanded polytetrafluoroethylene membrane (e-PTFE), which presented successful applications. This material has become a standard for GBR. It is characterized as a polymer with high stability in biological systems, which resists dissemination by tissues and microorganisms and does not trigger immune reactions.[10,11] Since the successful use of e-PTFE membranes, the results obtained using new materials should always be compared with this one. A disadvantage of the e-PTFE membrane is that it is nonabsorbable and therefore has to be removed during a second surgical procedure.[10] Another disadvantage is the fact that its surgical handling is complex and cannot be exposed to the oral environment at risk of contamination.[12]

Among the alloplastic materials developed, the polypropylene membrane has been widely used in GBR procedures after tooth extraction. This membrane is made up of impermeable, nonresorbable material and when exposed to the oral environment, minimizes infiltration of adjacent tissue cells, favoring the proliferation of bone cells within the alveolus, without the risk of bacterial contamination.[13]

Having high rigidity and elastic memory, the polypropylene membrane remains stable only with the positioning of the buccal and palatal/lingual tissue flaps, sutured without tension or approach, requiring no screw fixation. It can be removed from 7 to 10 days of its placement, in a period of effective use.[13,14]

A new waterproof titanium membrane is available on the national market and is proposed for use in GBR of alveoli after tooth extraction, indicated for exposure to the oral environment.[15,16] The electrochemical substrata modification alters the crystalline structure of titanium oxide layer into an anatase configuration, which has a potential application in medicine due its antimicrobial effect.[17,18]

In literature, there are no studies that prove the effectiveness of these membranes for alveolar preservation, to prevent contamination with biofilm, when exposed to the oral environment. In this context, the aims of the present study were to assess *in vitro*, the topographic and constitutional characteristics, as well as the inhibition of a mixed biofilm formation in membranes used for alveolar preservation, with different compositions, one made up of polypropylene (Bone Heal™) and the other of titanium (Surgitime Titanium Seal™).

MATERIALS AND METHODS

Thirty-six titanium membrane fragments Surgitime Titanium Seal™ (Bionnovation, SP, Brazil) and 36 polypropylene membrane fragments from Bone Heal™ (Bone Heal, SP, Brazil), both measuring 5 mm × 5 mm, were used [Table 1]. For the scanning electron microscopy and chemical composition analyses, nine fragments of each membrane were used. For the quantitative analysis of biofilm adhesion, 18 membranes of each composition were used, for both periods of evaluation (7 and 14 days).

Topographic characterization

The ultrastructural morphology and chemical composition (Ehlers–Danlos syndrome [EDS]) of the different membrane surfaces were evaluated, as described below.

Scanning electron microscopy

Fragments of polypropylene (n = 3, BoneHeal™) and titanium (n = 3, Titanium Seal™) were used. The samples were observed by a scanning electron microscope (SEM), Jeol, JSM-6610 (Jeol, Tokyo, Japan), to observe the topography and morphology of the membranes. The images were obtained at ×5000 and ×10,000, performed at the high-resolution microscopy multi-lab (LabMic) of the Federal University of Goías.

Chemical composition analysis (Ehlers–Danlos syndrome)

For chemical characterization and analysis of membrane surfaces, a dispersive energy X-ray spectroscopy was used.

A microscope attached to the SEM was used and by electron excitation, the X-ray energy was measured by the SEM, Jeol, JSM-6610 equipped with EDS ThermoScientific NSS Spectral Imaging (MA, USA), representing the atomic composition of the material.

In all analyses, three random points with 10.0 kV voltage acceleration and ×500 magnification were used.

Quantitative analysis of biofilm adhesion

Standard strains of *Staphylococcus aureus* (American Type Culture Collection [ATCC] 25923) and *Candida albicans* (ATCC 10231) microorganisms were purchased from the (ATCC, VA, USA). From lyophilized cultures, stationary phase cultures were prepared following the instructions recommended in the certificate accompanying the culture. The primary culture was preserved in order to maintain its morphological, physiological, or genetic characteristics and its complete viability during the storage period.

For the preparation of the cultures for the experiments, a tube from the frozen primary stock was withdrawn for reactivation. Thawing was performed in an ice bath, and the material was immediately transferred to a 10-ml brain–heart infusion broth containing 20% glycerol and Triptic Soy Broth (TSB) (Accumedia Manufacturers, Lansing, Michigan, USA), respectively, for *S. aureus* and *C. albicans*. The tubes were cultured in an aerobic greenhouse (ECB 1.2, Odontobrás, Curitiba, PR, Brazil) for 24 h at 36°C ± 1°C.

The specimens were then gently washed individually three times in 2 mL of phosphate-buffered saline (PBS) (Hawser; Douglas, 1994) to ensure the removal of cells not adhered to the specimens (Kuhn *et al.*, 2002). The specimens were then reinserted in 2 mL of TSB and incubated in a 37°C greenhouse with 24-h shaking (Kumamoto, 2008; Pereira-Cenci *et al.*, 2008) for biofilm development.

After growth, *C. albicans* was inoculated in Petri dishes containing TSB agar and incubated at 37°C under constant agitation, in order to prevent the accumulation of fungal cells in a single area by deposition. The cultures were constantly verified through morphology analysis. In addition, the cultures were frequently checked for possible contamination. After 48 h, *S. aureus* was added to the agar surface at a concentration of 1×10^8 colony-forming unit/ml (nephelometric scale no. 1 McFarland, Nefelobac, Probac do Brasil Ltda., Sao Paulo, Brazil) to form a mixed biofilm. The cultures will be maintained for 7 or 14 days, and biofilm formation was measured.

After biofilm formation (7 and 14 days), the samples destined for quantitative analysis were gently washed three times in 2 mL PBS and dried at an ambient temperature for 1 h. Then, the biofilm was fixed using 2-mL methanol 99% for 15 min, remaining in a refrigerator at 15°C during this period. The excess of methanol was removed with a micropipette, and the samples were exposed to ambient temperature for complete evaporation of the fixative. For biofilm staining, the samples were transferred to a new well containing 2 mL of 0.02% crystal violet aqueous solution. Twenty minutes were waited for the complete diffusion of the pigment to the biofilm. Then, the samples were gently washed three times in 2 mL of sterile Milli-Q water to remove nondiffused pigment. Subsequently, the samples were transferred to a new well where 2-mL 95% methanol was added for biofilm discoloration and supernatant release. The discoloration process supernatant was collected, and 100 µl of the solution was transferred to a 96-well plate, in triplicate, for each analysis time. Then, the absorbance of the solution was quantified by the Epoch spectrophotometer (Bio Tek, USA), using a wavelength of 590 nm so that only the value of the crystal violet released from the discoloration process was quantified, and the absorbance value of 100 µl of methanol 95% was also measured and used as the reference value. Thus, results were obtained by subtracting the value of blank from supernatant. The experiments were repeated three times under the same conditions to ensure accuracy.

Statistical analysis

The statistical analyses were conducted at a 5% significance level on SPSS 20 (SPSS Inc., Chicago, IL, USA). Analysis between groups was performed using one-way (analysis of variance), followed by the Tukey's test.

RESULTS

Topographic characterization

Representative images of the morphological analyses of the studied membranes are shown in Figure 1.

For the Bone Heal™ membrane, a dense structure with some irregularities on its surface represented by protrusions [Figure 1a and b] was observed. For the Titanium Seal™ membrane, a dense surface was observed with nano depressions [Figure 1c and d].

The characterization of the chemical composition of the membranes by EDS is shown in Figure 2. For Bone Heal™ membrane, there is a predominant presence of copolymer (Co) and traces of Zn (zinc). The Titanium Seal™ membrane revealed the unique presence of titanium (Ti).

Quantitative analysis of biofilm formation

The results of biofilm formation are expressed in Table 2. A decrease in biofilm formation is observed on the surface of the Titanium Seal™ when compared to Bone Heal™ membranes ($P < 0.05$), at both of the evaluated times. No differences were observed in the amount of biofilm at 7 and 14 days for each membrane ($P > 0.05$).

DISCUSSION

Procedures aiming the preservation of alveolar bone and soft tissue contour after extraction are of great clinical interest.[19] In this context, due to the lack of closure tissue aboard the alveolus,[20] membranes were designed that can remain intentionally exposed to the oral cavity, sealing and protecting the alveolus, so that it can regenerate while maintaining its volume.[21,22,23]

In this context, the present study compared two membranes used for alveolar preservation after tooth extraction, which, despite sharing similar clinical indications, consist of different materials. Bone Heal™ is composed of polypropylene, and Titanium Seal™ is a Grade 1 titanium membrane; both are impermeable and may be exposed to the oral cavity according to the manufacturers' indications.

The characteristics of a material may influence cell recruitment, but also biofilm accumulation.[24] In order to better indicate the membranes, the topographic characteristics were evaluated considering the ultrastructural morphology. The results showed that the Bone Heal™ membrane had a dense surface with small protrusions, while Titanium Seal™ had a dense surface, but with nano depressions. The EDS test confirmed that the Bone Heal™ membrane consists predominantly of copolymer, polypropylene, and traces of zinc, whereas Titanium Seal™, which is titanium only, is considered a dense metallic barrier.

Given these results, it can be considered that both membranes are dense barriers, fulfilling the purpose of generating a framework to maintain the alveolar walls, preventing soft-tissue penetration and promoting clot stability. Dense membranes are an effective and predictable alternative for the treatment of major bone defects, easy removal, and preservation of keratinized tissue, and when exposed, they do not compromise the regeneration and vascularization qualities of the region.[25] However, most nonresorbable membranes have a significant complication associated with increased vertical bone crest, lack of soft tissue for primary closure, oral exposure, and subsequent bacterial colonization, requiring premature removal, with consequent loss of bone graft.[26] Despite these disadvantages, there is not a membrane with all the ideal characteristics. In addition, resorbable membranes have as a disadvantage the resorption time, which needs to be strictly controlled to allow a new bone formation, besides the effect of its degradation, mainly via hydrolysis, which can create an acidic environment, impairing the regeneration of bone tissue.[27]

In addition to biocompatibility characteristics, membranes for alveolar regeneration and preservation should offer mechanical strength for framework formation and biological corrosion, be nonpyrogenic, and allow efficient cell blockage.[21] In this sense, for Titanium Seal™, the 0.4-mm-thick laminated titanium sheets, besides these characteristics, have high osteophilia, which distinguishes them from plastic barriers, such as Bone Heal™, besides causing little injury to the soft tissues.[15] Despite this, polypropylene membranes offer many advantages, such as the indication of intentional exposure of the membrane to the oral environment, where the flaps should be kept apart; the need not to use other biomaterials inside the alveolus; the impermeability without suffering dimensional changes during the period of stay in the surgical bed and nonadherence to the tissues; and blood adsorption promoted by the internal surface.[13,28,29]

It is unquestionable that the presence of blood clot is an essential factor for the success of both hard- and soft-tissue grafts.[28] Following this principle, Salomão and Siqueira[28] have shown that isolating an alveolus with a polypropylene barrier from the rest of the connective and/or mucosal tissues, the blood clot itself will be responsible for bone regeneration and the three-dimensional maintenance of the edentulous

edge, thus providing a much more predictable rehabilitation. Although this technique is indicated whenever there is bone loss in dental extraction and as long as there are no systemic contraindications, the choice of surgical technique logically depends on the clinical conditions evaluated.[13]

There is no doubt that intentional exposure of a barrier to the oral environment raises the risk of contamination and the onset of inflammatory process, with consequent graft resorption, bone loss, and even implant loss.[30] Given this problem, the present study evaluated *in vitro* the accumulation of mixed biofilm of *C. albicans* and *S. aureus* on the membrane surfaces studied, in view of its indication by the manufacturer.

Despite the inhomogeneous topographic characteristics for both membranes, there was a lower biofilm formation on Titanium Seal™ compared to Bone Heal™ ($P < 0.05$), especially in the first 7 days. These findings are interesting given that the main events for tissue regeneration occur during the first 7–14 days.[31]

The lower formation of biofilm on the Titanium Seal™ membrane may be related to its anodized surface, whose titanium presents electrochemical treatment, converting amorphous titanium oxide into anatase. TiO_2 crystallized in the form of anatase is responsible for some physical chemical surface characteristics, such as an increase in hydrophilicity as well as conductor properties,[32] which may be suitable for cell colonization, improving recruitment and adhesion of osteoblasts and fibroblasts.[16,24,33]

Additionally, the anatase crystalline structure has been used in some medical devices due to its antimicrobial activity,[17,18,34,35] which *in vivo* might represent a decrease in salivary pellicle formation and consequently, the biofilm formation.

Associated with the antimicrobial effect, the nano depressions found on the Titanium Seal™ membrane may have promoted an increase in roughness, which in fact may clinically contribute to greater clot stability and consequent adhesion of progenitor cells, promoting tissue repair.

Based on these results, it was verified that the anodized titanium membrane and the polypropylene membrane have topographic characteristics that allow its indication for alveolar preservation. However, the titanium membrane presented less biofilm formation at the time of evaluation of the present *in vitro* study, suggesting its better indication of exposure to the oral environment. Future *in vivo* researches in patients are recommended in order to verify biofilm accumulation and consequent tissue involvement for both membranes.

CONCLUSIONS

Considering all the present data collected, the results indicate that despite the morphological characteristics with surface irregularities for both membranes, the anodized titanium membrane showed less biofilm formation compared to the polypropylene one, which may be better indicated in cases of oral cavity exposure.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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Figures and Tables

Table 1

Technical specifications of the membranes used.

Membrane	Manufacturer	Composition	Batch
Surgitime Titanium Seal™	Bionnovation	Titanium degree I (ASTM F-67)	059927
Bone Heal®	Bone Heal	polypropylene	68848

Figure 1

Representative image of Bone Heal™ (a and b) and Titanium Seal™ (c and d) membranes, by scanning electron microscopy. The presence of surface irregularities (arrows), with protrusions in the Bone Heal™ (a and b) and depressions in the Titanium Seal™ (c and d) membranes, is observed. Bars: a and c = ×5000; b and d = ×10,000

Figure 2

Analysis of the chemical composition of the Bone Heal™ (a) and Titanium SealTM (b) membranes, at 3 random points

Table 2

Average (SD) of biofilm absorbance grown on Bone Heal® and Titanium Seal® membranes at 7 and 14 days

	7 d	14 d
Bone Heal™	0.253 (0.083) Aa	0.203 (0.019) Aa
Titanium Seal™	0.123 (0.046) Bb	0.159 (0.017) Bb

Different lowercase letters represent statistical differences for studied membranes, at each time (columns). Different capital letters represent statistical differences for each studied membrane at different times (lines). Significance level was 5%