Synthesis and Characterizations of a Collagen-Rich Biomembrane with Potential for Tissue-Guided Regeneration

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Abstract

Objectives In this study, a collagen-rich biomembrane obtained from porcine intestinal submucosa for application in guided bone regeneration was developed and characterized. Then, its biological and mechanical properties were compared with that of commercial products (GenDerm [Baumer], Lumina-Coat [Critérias], Surgitime PTFE [Bionnovation], and Surgidy Dental F [Technodry]).

Materials and Methods The biomembrane was extracted from porcine intestinal submucosa. Scanning electron microscopy, spectroscopic dispersive energy, glycosaminoglycan quantification, and confocal microscopy by intrinsic fluorescence were used to evaluate the collagen structural patterns of the biomembrane. Mechanical tensile and deformation tests were also performed.

Statistical Analysis The results of the methods used for experimental membrane characterizations were compared with that obtained by the commercial membranes and statistically analyzed (significance of 5%).

Results The collagen-rich biomembrane developed also exhibited a more organized, less porous collagen fibril network, with the presence of glycosaminoglycans. The experimental biomembrane exhibited mechanical properties, tensile strength, and deformation behavior with improved average stress/strain when compared with other commercial membranes tested. Benefits also include a structured, flexible, and bioresorbable characteristics scaffold.

Conclusions The experimental collagen-rich membrane developed presents physical–chemical, molecular, and mechanical characteristics similar to or better than that of the commercial products tested, possibly allowing it to actively participating in the process of bone neoformation.

Keywords ► extracellular matrix  
► porcine intestinal submucosa  
► guided bone regeneration

Introduction

Tissue engineering is based on the study of the manipulation and development of the interactions of molecules, cells, tissues, or organs aiming at the restoration or improvement of impaired tissue function. It is a science of multidisciplinary scope, in which researchers from different areas interact to exchange knowledge and experiences to provide...
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Materials and Methods

Preparation of the Extracellular Matrix

The process of obtaining the biomembranes produced in this study is described in patent document No. PI0805153–4 A2, available for consultation at the Brazilian National Institute of Industrial Property. The refrigerated material was cleaned and sliced in small pieces (15 cm²). The pieces were then washed thoroughly with ultrapure water, obtained by reverse osmosis and then washed in sodium hypochlorite solution (0.1%) for 30 minutes under gentle agitation. Then, the specimens were water-rinsed and mechanically cleaned to remove residues. Next, the specimens underwent a thermochemical treatment by immersing them in an enzymatic solution based on sodium lauryl sulfite proteases for 12 hours under stirring. Then, the specimens were water rinsed with ultrapure water and subjected to another 12 hours treatment with an enzymatic solution, in which the base solution was a combination of sodium lauryl sulfate and lipase. Then, another water rinse with water for injection (WFI) was performed. The biomembranes were then stretched on a regular Teflon surface and then cooled to 3.5°C for 12 hours. The specimens were then placed in an oven at 40°C for 12 hours and at 65°C for another 12 hours with forced ventilation. Finally, the material was subjected to a chemical treatment in which the specimens were immersed in a solution of 1 M HCl, under agitation for 24 hours. At the end of the incubation, the specimens were again washed in WFI ultrapure water and then lyophilized (Liopoot L101, Liobras, Brazil). In the present study, the experimental collagen-rich biomembrane was evaluated and its properties compared with that of four commercial biomembranes were widely used, which were obtained using different synthesis processes. – Table 1 shows the characteristics of all of the membranes included in the present study.

Scanning Electron Microscopy and Energy Dispersive Spectroscopy Test

For scanning electron microscopy (SEM) analysis, the membranes were cut into fragments –9 mm wide and fixed in aluminum support with the aid of an adhesive tape. The membranes were then subjected to the vacuum metallization process.
Table 1 Membranes evaluated in the present study

| Product           | Manufacturer | Composition                        | Tissue type                      | Bioreposable |
|-------------------|--------------|------------------------------------|----------------------------------|--------------|
| GenDerm           | Baumert      | bovine cortical bone membrane      | Bovine bone                      | Yes          |
| Lumina-Coat       | Critérias    | Type I collagen membrane           | Demineralized bovine bone        | Yes          |
| Surgitime PTFE    | Bionnovation  | Polytetrafluoroethylene            | Synthetic origin                 | No           |
| Surgidy Dental F  | Technodry    | Organic matrix of Type I collagen  | Purified and polymerized bovine tissue | Yes          |
| Experimental Membrane |            | Type I collagen; glycosaminoglycan | Porcine intestinal submucosa     | Yes          |

Extraction of Glycosaminoglycans and Agarose Gel Electrophoresis

All of the membranes selected for this study received a perforation at room temperature and were then placed in a 100% acetic solution to remove the lipids. The fragments were then removed from the acetic acid and dried in an oven at 50°C to obtain the ketone powder and the mass evaluated in an analytical balance. The dried powder was subjected to proteolysis by maxatase 4 mg/mL in 0.05 M Tris–HCl pH 8.0 buffer with 1 M NaCl in the proportion of 1 g of ketone powder for every 20 mL of buffer under agitation. This solution was kept under constant stirring for 12 hours at 60°C. After that, 90% trichloroacetic acid was added to the solution until reaching the final 10% concentration for the precipitation of nucleic acids and peptides. The solution was left still, without stirring for 20 minutes at 4°C. After that, the material was centrifuged at 5,000 rpm for 20 minutes at room temperature, and the supernatant part discarded. Then, two volumes of methanol were added for the precipitation of glycosaminoglycans, which was performed at −20°C (freezer) for 24 hours.

Further centrifugation was performed at 5,000 rpm for a further 20 minutes at room temperature and the supernatant discarded again. The precipitated material containing the glycosaminoglycans (GAGs) was oven dried and resuspended in distilled water in the proportion of 5 mg ketonic powder to 10 μL of distilled water. The compounds from the extraction were identified by agarose gel electrophoresis and quantified by densitometry. The identification of sulphated glycosaminoglycans was performed by comparing the electrophoresis migration of the samples with those of known and purified standards. These same standards were used for the quantitative determination of the compounds by means of densitometry at 525 nm. For this purpose, the Quick Scan 2000 Win densitometer (Helena Laboratories - Beaumont, Texas, United States) was used. The patterns used were chondrocyte sulfate, extracted from whale cartilage; dermatan sulfate, extracted from porcine intestinal mucosa and porcine lung heparan sulfate.

Evaluation of the Collagen Structural Pattern by Second Harmonic Generation Confocal Microscopy

Fragments of 10 mm² were taken without any type of treatment to the confocal scanning and laser microscope (Germany), previously configured with the following excitation pattern: Titanium-Sapphire Laser (Ti-S) in pulses that ranged from 100 to 200 fs at a wavelength of 1,600 nm and multiphoton incidence. The images were generated in Z-axis variant planes in sections of 12 μm until the three-dimensional (3D) image could be formed and the collagen visualization pattern was evaluated.

Evaluation of Mechanical Properties of Membranes

The analyses of all the samples were performed in triplicate using a Filizola traction equipment, model BME-20kN, with a load cell of 50 N (5 kgf), resolution of 0.003 N, and the claws separation speed at 20 mm/min.

Statistical Analysis

For the analysis of possible differences among the groups, the analysis of variance test was used followed by the Tukey-Kramer multiple comparisons test (parametric data) and Student’s t-test (significance 5%).

Results and Discussion

Scanning Electron Microscopy and Energy Dispersive Spectroscopy

Fig. 1 illustrates the SEM analysis of the surface of various membranes from different manufacturers. In the morphology of the GenDerm brand membrane (~ Fig. 1 [1A and 2B]), it is possible to notice the typical characteristics of partially mineralized tissue and a coherent histological organization of porcine origin. Presence of large diameter pores, possibly remnants of Haversian canals, and even remnants of structures that appear to be osteocytes, which even after...
When in contact with membranes, undifferentiated mesenchymal cells are expected to repopulate the repair sites giving rise to the periodontal ligament and to the bone tissue. Several studies support the knowledge that collagen promotes adhesion of several cell types, allowing them to remain in vitro for long periods, and stimulating cell proliferation. The structure and composition of the membrane determine the time of degradation, its spatial conformation, and the tissue reactions. If the membrane tends to collapse in the bone defect, this limits the space for bone regeneration. In its initial phase, a membrane’s resistance is determined mainly by the rigidity of the material. From a practical point of view, it should also be able to adapt to the adjacent bone contours. The results showed considerable differences in the membrane architecture and their chemical composition when evaluated by the SEM. The Lumina-Coat membrane has an incredibly porous surface and also a wide variety of pore diameters; this may explain the low mechanical strength noted in the tensile test. A fact that also has to be considered is that this type of structural conformation also becomes more susceptible to degradation, reducing the time of bioabsorption, and with that diminishing its potential use as a physical barrier. The Surgidry Dental F membrane has a surface formed by numerous frames arranged in a disordered manner, with large gaps intermingling the entire structure. This fragility was also detected in the failed test of the material even when subjected to low loads. It is also worth noting that the fragility of the structure entails loss of function as a barrier. GenDerm membrane surface has a more organized structure, but it has large cracks throughout its length. Although it has reached a great resistance in the traction test, with a response similar to a ceramic material, the cracks identified in the material could compromise its function as a protective barrier. Surgitise PTFE membrane has a surface arranged in nonhomogeneous layers interspersed with pores of different diameters. According to the tensile tests, this membrane demonstrated good mechanical resistance. However, this type of material requires a second surgical intervention for its removal, considering that it is a biodegradable membrane. The Experimental Membrane has a surface composed of fibers that are homogeneous and arranged in parallel, similar to that of the collagen fibers.
in the extracellular matrix. This structure is quite organized, which demonstrated excellent performance in the tensile test, and the pores found on its surface are dispersed uniformly.\textsuperscript{30}

The EDS evaluation allowed the identification of part of the chemical composition of each one of the membranes tested. The results show little significant differences in the chemical composition of the membranes, with emphasis only on the high concentration of fluoride, in the form of fluoride ion (F\textsuperscript{-}), on the Surgitime PTFE membrane. Data are expressed as the percentage by weight, not considering the carbon and nitrogen atoms, which, due to the low atomic number, are not accurately quantified. Table 2 presents the averages of the results obtained in the analyses of all of the membranes studied. The results of the chemical analysis by ESD showed the presence of chemical elements and proportions of these different elements among the membranes analyzed. Elements such as magnesium (Mg\textsuperscript{2+}), fluorine (F\textsuperscript{-}), potassium (K\textsuperscript{+}), and chloride (Cl\textsuperscript{-}) were detected (\textbullet{} Table 2). These findings should be interpreted with caution as the results are expressed in percentages of the chemical element in relation to the total sample weight, and it does not consider carbon and nitrogen ions, the main components of the collagen molecules. The actual percentage of the other elements may be much lower. Even so, the presence of elements in varying proportions, such as chlorine and potassium, is an interesting finding and may constitute a contaminant derived from the processes of membrane fabrication.

Quantification of Glycosaminoglycans

\textbullet{} Fig. 2 illustrates the results concerning the presence of sulfated glycosaminoglycans in the composition of the membranes evaluated. It is noticeable that the Experimental Membrane was the only membrane to present 0.4 mg/mg of dermatan sulfate sample, while the other membranes, perhaps due to the structural difference found in their extracellular membranes, presented no detectable levels of glycosaminoglycans in the composition.

Structural Evaluation of Collagen Using Confocal Microscopy by Intrinsic Fluorescence

As described previously in methods, the collagen molecule, when structured (in its native form), is capable of emitting fluorescence. \textbullet{} Fig. 3 shows in 3D how collagen is distributed on membranes. It should be highlighted that the Surgitime PTFE membrane, which is the only one of synthetic origin and therefore has no collagen fibrils in its structure. In this way, only the collagen membranes were evaluated. Supporting the data obtained by SEM, GenDerm membrane obtained from porcine bone cortical (\textbullet{} Fig. 3A) presents a different pattern of collagen distribution, clearly exhibiting regions with larger pores are located in the same areas in which the collagen fibrils are absent. The collagen filaments of LuminCoat membrane (\textbullet{} Fig. 3B) presented larger diameters also presenting regions with larger pores. \textbullet{} Fig. 3C shows the structural collagen pattern of the Experimental Membrane, which presented a profile of dense distribution of collagen, with fewer detectable pores. \textbullet{} Fig. 3D demonstrated that the Surgidy Dental F membrane has thinner collagen filaments when compared with other membranes. In this way, this membrane presents a more regular surface, exhibiting lower fluorescence peaks when three-dimensionally analyzed using confocal microscopy. Also, it is possible to observe that the pore regions are equivalent to those observed in the Experimental Membrane.

Mechanical Properties of the Membranes

Some basic characteristics are necessary so that a membrane can be used in guided bone regeneration, which may include biocompatibility, cellular occlusion capacity, adaptation to surgical space (malleability), ease of handling by the surgeon, and mechanical resistance.\textsuperscript{31} The tensile strength test is the quickest and simplest way to evaluate the mechanical properties of the materials, being performed by traction of a test piece until its rupture.\textsuperscript{32} The tensile force is produced in the material when two forces in opposite directions are applied.

![Table 2](https://example.com/table2.png)

**Table 2** EDS analyses of the membranes

|                | C\textsuperscript{2+} | N\textsuperscript{1+} | O\textsuperscript{2-} | K\textsuperscript{+} | Cl\textsuperscript{-} | Mg\textsuperscript{2+} | Na\textsuperscript{+} | F\textsuperscript{-} | Ca\textsuperscript{2+} |
|----------------|------------------------|------------------------|------------------------|-----------------------|------------------------|------------------------|-----------------------|------------------------|------------------------|
| GenDerm        | 50.8                   | 23.6                   | 25.6                   |                       |                        |                        |                       |                        |                        |
| Lumina-Coat    | 44.8                   | 25.4                   | 27.5                   | 2.4                   |                        |                        |                       |                        |                        |
| Surgitime PTFE | 26.9                   |                        |                        |                       |                        |                        |                       |                        |                        |
| Surgidy Dental F | 47.6                   | 23.7                   | 24.7                   | 2.3                   | 0.9                    | 1.0                    |                       |                        |                        |
| Experimental Membrane | 48.0                   | 21.1                   | 27.5                   | 0.6                   | 0.3                    | 0.7                    |                       |                        |                        |

Abbreviation: EDS, spectroscopic dispersive energy.

Values obtained from the average result, of the three-point analysis of each sample, and expressed as a percentage of the total weight, excluding carbon and nitrogen atoms.
in the same line of application to elongate the material, and the tensile strength comes from the attractive molecular forces that tend to hinder the separation of the material. In the present study, the maximum tensile strength of the membranes until their rupture was evaluated; that is, it clinically represents the membranes’ ability to absorb the physiological and external loads imposed at the implant site. This information is essential, considering that clinically speaking the implant site may be subject to a wide variety of experimented loads. Figs. 4 and 5 display the results of the membranes studied in this study.

GenDerm membrane presented a mechanical resistance significantly superior to that of others, with a tension value of 19 MPa, against less than 3 MPa of the other materials, but it was the most brittle, which according to the manufacturer it needs at least 5 minutes to moisturize and become more flexible and safer for handling. The GenDerm sample obtained an average deformation of 10 MPa, the others above 20 MPa, except the Surgidry Dental F material that presented a deformation of 18 MPa. Surgitime PTFE membrane presented a deformation of 36.7%, and had the third best mechanical property, with 3 MPa of tension. The Experimental Membrane developed in the present study presented the best tension x deformation commitment, with a tension of 6.2 MPa, the 2nd best, and the greatest deformation, around 50 MPa. Lumina-Coat and Surgidry Dental F materials showed lower tension of rupture (0.4 and 0.1 MPa, respectively), and a deformation of 20 MPa. By analyzing the stress/strain graph, important information can be obtained regarding the elasticity, plasticity, stiffness, rupture, and energy that tissue can absorb before its rupture. The linear region of the curve corresponds to the elastic phase, where the deformation...
increases linearly with the force applied, and the material will deform only while the load is being applied to it, returning to its original size when the load is removed.\textsuperscript{33} The nonlinear region corresponds to the plastic phase of the membranes in which the tissue becomes permanently deformed and it is not able to recover its initial length after the external force stopped.\textsuperscript{15,36} In the elastic region, the Experimental Membrane developed here presented the higher deformation and that presented higher tensile strength at the limit of the elasticity. In the plastic region, the Experimental Membrane presented the second highest tensile strength means, with mechanical characteristics with the highest average stress/strain when compared to the other membranes tested.

The tissue biocompatibility of the Experimental Membrane was also evaluated in male Wistar rats, which received subcutaneous implants in evaluation times up to 84 days (data not shown). At the final evaluation time, areas with chronic inflammation, light to mild fibroplasia, and also mild to moderate fibrosis were observed in both sides of the Experimental Membrane, similar to that of found in the control group (\textit{Lumina-Coat}). These characteristics somehow complemented the results demonstrating that the experimental collagen-rich biomembrane developed here has a potential for application in guided bone regeneration.

\section*{Conclusion}

Taken together, the results of the present study allow to conclude that the Experimental Membrane developed has physical–chemical and molecular characteristics similar to or better than that of the commercial products tested. The experimental collagen-rich biomembrane derived from porcine small intestinal submucosa demonstrated the potential for tissue-guided regeneration in dentistry with qualities for using as a physical barrier. It is also advantageous to be considered an extracellular matrix for being rich in macromolecules such as glycosaminoglycans, which actively participate in the process of bone neof ormation in the niche in areas of cell growth, proliferation, and differentiation.

\section*{Conflict of Interest}

None declared.

\section*{Acknowledgments}

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