Guided Tissue Regeneration with Use of CaSO$_4$-Chitosan Composite Membrane

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Abstract

Guided tissue regeneration (GTR) membranes with bioresorbable characteristics have been employed in recent years for periodontal procedures to deflect the growth of gingival tissues away from root surface. They provide an isolated space over regions with defective tissues and allow the relatively slow-growing periodontal ligament fibroblasts to be repopulated over the root surface. In this study, we employed chitosan and CaSO$_4$ composites as one of the viable membrane materials and evaluated their roles in GTR applications. Two types of CaSO$_4$-chitosan composite membranes with molecular weights of chitosan 70 kDa and 300 kDa, respectively, were prepared for study in three categories: mechanical strength to create and sustain an effective space; rapid rate to reach hydrolytic equilibrium in phosphate buffer solution; and ease of clinical manipulations. Consequently, standardized, trans-osseous and critical-sized (cavity of 6 mm) skull defects were made in adult rats, and the defective regions were covered with the specifically prepared chitosan membranes. After 4 weeks of recovering, varying degrees of bone healing were observed beneath the CaSO$_4$-chitosan composite membranes in comparison to the control group. The CaSO$_4$-chitosan composite membrane-covered regions showed a clear boundary space between connective tissues and bony tissues. In summary, good cell-occlusion and beneficial osteogenesis effects by these bioresorbable GTR materials toward the wound recovery were indicated.

Keywords: Guided tissue regeneration (GTR), Chitosan, CaSO$_4$, Membrane

1. Introduction

Guided tissue regeneration (GTR) techniques have been successfully applied in the treatment of periodontal diseases and provided opportunity for formation of new bone [1-3]. The techniques involved procedures employing membranes as mechanical barriers to create a space around the defects that might permit bone regeneration and to prevent the epithelial cells from migrating into the bony area. In general, an effective barrier membrane should have appropriate mechanical strength to occlude the rapid growth of repairing tissues (epithelium and gingival connective tissue) away from the root surface and maintain a protective space over the defect that will allow migration of bone cells from the surrounding alveolar bone into the targeted area. Ideally, clinical manageability of the barrier membrane is also a necessary consideration during surgical procedures. In addition, effective materials should be safe, non-toxic, and non-antigenic ones which induce little or no inflammatory responses around the host tissue. Furthermore, the barrier function must be established and maintained for a period of time long enough for tissue guidance to take effect. As a result, extensive effort has been employed to utilize bio-resorbable membranes to achieve therapeutic purpose in clinical trials [4-7].

Among the bio-resorbable materials used in GTR applications, resorbable membranes such as collagen membranes and polyglycolic acid-based membranes, have been successfully applied and are commercially available. However, there could be disadvantages in the use of collagen membrane because it might cause localized chronic inflammatory response and rapid degradation behavior [8,9]. As for the use of polyglycolic acid-based membrane, there could be problems such as gingival recession, exposure of the device, and inflammation of surrounding soft tissue [10,11].

Chitosan, a natural occurring polysaccharide, is widely present among marine and terrestrial invertebrates and lower forms of the plant kingdom. Highly deacetylated chitosan (e.g., > 85%) exhibits low degradation rate in aqueous media and may last several months, and thus leads to great potential in the development of inexpensive and versatile drug encapsulating...
systems [12,13]. Its excellent gel-forming properties and ability to be re-shaped into various forms simply by thermally induced separation method strongly enhances its potential applications in the biomedical field. Many researchers also found that chitosan has osteoinduction and osteoconduction potentials when used as a bone scaffold material [14,15]. As a biodegradable material, chitosan had been given a lot of attention in biomedical applications. Its blood clotting characteristics made it well accepted as a wound healing agent, skin grafting template and hemostatic agent [16-18]. In general, it is also well-known to promote the formation of scaffolding matrix in tissue regeneration process. On the other hand, among many commercially available ceramics, CaSO₄ is frequently used as bone repairing and replacement material because of its biocompatibility, bioactivity, mechanical strength, and non-toxicity. From clinical experience, CaSO₄ can be gradually absorbed; that is followed by new bone formation yet without compromising the required intimacy of bone-implant contact. Kinetically, CaSO₄ degraded faster than tricalcium phosphates and have shown favorable osteoconduction and resorption properties in animal studies. Also, CaSO₄ can be resorbed and replaced by new remodeled bone completely [19,20]. Consequently, we focused on the development of bio-resorbable membrane prepared from chitosan and CaSO₄, and attempted to evaluate the feasibility of devising composite membranes for GTR applications.

We previously reported that the chitosan membranes prepared by thermally induced phase separation method followed by treatment with non-toxic NaOH-gelating, Na₂P₂O₁₀ and Na₂S₀₄ cross-linking agents exhibited similar properties with chitosan membrane that had been strengthened by glutaraldehyde as a cross-linking agent [12,15,21]. In this study, we took advantage of the unique properties of chitosan and CaSO₄ to prepare a composite membranes system. To retain the good biocompatibility of these two unique materials within the same device, we used NaOH as the gelating agent in the process of preparing CaSO₄-chitosan composite membrane. SEM observation was conducted to examine the morphology of the membrane surface. Also, some fundamental properties of the CaSO₄-chitosan composite membranes were examined, such as water content, mechanical strength and degradation. In addition to assess the physical properties of CaSO₄-chitosan composite membranes, the biological function of the composite membranes was studied by animal test. We wish to establish the feasibility of composite membranes made of the two naturally occurring biomaterials, CaSO₄ and chitosan, for GTR in periodontal application.

2. Materials and methods

Chitosan with molecular weight of 300,000 and deacetylation degree of 83% was purchased from TCI (Tokyo, Japan). Chitosan with molecular weight of 70,000 and deacetylation degree of 75% was purchased from Sigma (St. Louis, MO). CaSO₄ (Merck-Schuchardt, Germany) was classified through a sieve with 0.104 mm openings. Acetic acid was purchased from Sigma (St. Louis, MO). All chemicals used in this study were of reagent grade.

2.1 Preparation of CaSO₄-chitosan composite membranes

Chitosan was dissolved in acetic acid (0.1M) to prepare a 2% (w/v) chitosan solution. This chitosan solution was filtered and mixed with CaSO₄, weight of ratio CaSO₄/chitosan 65:35, and then stirred 24 hours. Each 19 ml of mixed solution was poured into a 9-cm Petri-dish and placed in a drying oven at 40°C with proper ventilation for overnight. After drying, the membranes were immersed in 0.1N NaOH 4 hours, and then washed with distilled water and pressed from two sides with polyethylene (PE) thin films, and then placed in oven (40°C) until completely dry. The chitosan-containing CaSO₄ membranes exhibited ivory-like uniform and opaque texture. All prepared chitosan membranes were stored in desiccators until use.

2.2 Water content measurement

The water content (W.C.) of the membrane was determined by swelling the membrane in pH7.4 of phosphate buffered saline (PBS) at room temperature. After the membrane reached equilibrium state, the wet membrane was blotted with filter paper to remove the water adhered on the surface. The water content of the membrane was calculated as:

\[ W.C. = \frac{(W_w - W_d)}{W_w} \times 100\% \]  

where \( W_w \) and \( W_d \) are the weights of wet and dry membrane, respectively. The experiment was conducted 3 times and a mean and standard deviation were calculated.

2.3 Mechanical property measurement

The mechanical properties of the membranes were measured in hydrated condition. The membranes, 1 cm \( \times \) 6 cm, were hydrated in 0.1 M pH 7.4 phosphate buffer before being subjected to mechanical testing. The tensile strength measurements of the membranes were charted up to the point where they were broken. The mechanical parameters of these chitosan membranes were calculated and recorded automatically by using an MTS Systems (Eden Prairie, USA) at a crosshead speed of 10 mm/min.

2.4 In vitro degradation test

The in vitro degradation tests of the prepared membranes were conducted by incubating the membrane in 10 ml of pH 7.4 PBS on a shaker set at 40 rpm and 37°C. At predetermined time intervals, the membrane was taken out of the incubation medium, washed with distilled water, dried and its weight was measured. Another fresh 10 ml PBS was added into the vial for continuum degradation test. The degradation profiles were expressed as the accumulated weight losses of the membrane.

2.5 SEM observation

The surface microstructure of membranes was examined by scanning electron microscope (SEM). Before SEM observation, all samples were dried, sputter-coated with gold, and examined under a scanning electron microscope (JEOL, JSM-5300, Japan).
2.6 Animals and operation procedure

SD rats weighing about 200 g, fed with commercial food and RO water, were included in this randomized, blinded study. The rats were anesthetized by trans-abdominal injection of Zoletil 50 (mixture of Tiletamine and Zolezepam, 1:1) (0.2 ml/200 g). Following the injection, the skull was shaved and the surfaces at surrounding sides of the skull were exposed via full-thickness incision. A man-made defect (6 mm in diameter) was generated with a dental round burr (Figure 1(a)). Prior to implantation, the membranes were hydrated in physiologic saline to restore their elasticity. The defect was then covered with the membranes (Figures 1(b-e), 10 × 10 mm² in area, 0.1 mm in thickness). In the control group, the bone defect was not covered with any chitosan membrane. The wounds were then carefully sutured. For each animal with membrane and control, an initial healing period of 4 weeks was allowed.

2.7 Histological preparation and evaluation

After the healing periods, the rats were sacrificed by injecting an overdose of KCl into the heart. The skull tissue containing bone defects was removed by a larger-size dental trephine burr. The specimens of center of defect were fixed in 10% neutral-buffered formalin, decalcified in 10% formic acid and then dehydrated in an ascending graded series of ethanol solutions, and afterwards, embedded in paraffin. A series of 5-μm transverse sections encompassing the entire bone defect specimen were prepared and stained with hematoxylin-eosin and then subjected to light microscopic observation.

3. Results

3.1 Morphology of CaSO₄-chitosan composite membrane

As indicated in Figure 2, the SEM micrographs showed the characteristic morphological aspects of various chitosan membranes. All chitosan membranes produced by thermally
induced phase separation demonstrated a dense morphological structure. The roughness was visible on the CaSO$_4$-chitosan composite membranes. Contrarily, the pure chitosan membrane showed a smoother surface as compared with all CaSO$_4$-chitosan composite membranes. Furthermore, with elemental analysis using EDX (Figure 3), calcium could be detected on the surfaces of the composite membranes. Interestingly, the calcium ratio of the CaSO$_4$-chitosan (300 kDa) composite membrane surface was higher than that of CaSO$_4$-chitosan (70 kDa). It manifested that CaSO$_4$ could be “float” more on the surface of the membrane when using 300 kDa chitosan as the source of binding material.

3.2 Basic properties of CaSO$_4$-chitosan composite membrane

When considering the development of guided tissue regeneration barrier materials, the basic bulk and mechanical properties ought to be reviewed. In addition, the appropriate degradation rate of a material has to be evaluated to assess whether it meets the requirements and diversities of tissue regeneration procedures. Interestingly, CaSO$_4$-chitosan composite membranes have become very desirable elastomers to biomedical engineers, as indicated by the wider range of physical characteristics, especially with higher ultimate length. As expected, the presence of CaSO$_4$ would affect Young’s modulus of the membranes in this study, as shown in Table 1. The Young’s modulus of the membrane increased from 15.0 MPa (for pure chitosan) to 18.9 MPa CaSO$_4$-chitosan composite membrane.

Moreover, as shown in Figure 4, the CaSO$_4$-chitosan composite membranes showed a more rapid mass loss than the pure chitosan membranes in 30-day shaking test. All CaSO$_4$-chitosan composite membranes degraded to about 30%–50% of initial weight after 30-day shaking test. For the composite membranes, the degradation behavior showed a typical biphasic profile. There was an initial rush of degradation during the first 6 days of the study. This rapid degradation period probably represented the release of feebly entrapped and surface-associated CaSO$_4$. After the first 6 days, the composite membranes showed slower degradation rates. However, the degradation rates were slightly different between the composite membranes. The degradation of CaSO$_4$-chitosan composite membranes increased with the decrease of the molecular weight of chitosan, probably due to CaSO$_4$ on the CaSO$_4$-chitosan (300 kDa) composite membrane surface being richer than that of CaSO$_4$-chitosan (70 kDa). As a result, in the first 6 days of degradation, the calcium ion was released faster from the membrane surface. Overall speaking, the CaSO$_4$-chitosan (300 kDa) composite membranes showed a more rapid mass loss. In other words, the degradation result corresponded roughly to the EDX analytical result.

Figure 5 shows the surface morphologies of the CaSO$_4$-chitosan composite membranes after 30 days of shaking. SEM examinations revealed that these CaSO$_4$-chitosan composite membranes still exhibited rough
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Figure 5. SEM micrographs of the membrane: (a-b) a chitosan (70 kDa) membrane, (c-d) a chitosan (300 kDa) membrane membrane, (e-f) CaSO$_4$-Chitosan (70 kDa) composite membrane, and (g-h) CaSO$_4$-Chitosan (300 kDa) composite membrane after 30 days of shaking.

Figure 6. Water contact profile of the membranes in pH 7.4 PBS solution in 10-minute period of experiment.

surfaces and had good integrity, albeit with signs of degradation on the surface. CaSO$_4$ was steadily released and dissolved from the membranes while, concurrently, chitosan was degraded only gradually. Relatively speaking, the surface of neat chitosan retained an intact and smooth surface morphology as before. As a guided regeneration barrier material, the appropriate degradation rate of material ought to be managed to fit into the schedule of remodeling of tissue regeneration. Although, the resorption process could be further facilitated by enzyme digestion in real applications, we suggested that the membranes prepared in this study could reasonably meet the degradation requirement of bioresorbable membrane used for GTR from these in vitro observations.

Another important characteristic of these chitosan membranes is that they could reach steady hydration equilibrium state within the 10-minute period of the experiment, as seen in Figure 6 showing water content profiles. This rapid swelling phenomenon would be beneficial to the clinical manageability and surgical procedures. The bulk properties of prepared CaSO$_4$-chitosan composite membrane are summarized in Table 1. The results indicated that the CaSO$_4$-chitosan composite membranes prepared in this study fulfilled the requirements of bioresorbable membrane employed as GTR material. In clinical practice, when materials are used as tissue regeneration barrier membranes, they are generally required to maintain certain barrier functions for 4 to 6 weeks in order to secure the successful restoration of periodontal tissues. The mechanical strength test results indicated that these membranes had appropriate mechanical strength for the requirement of GTR.

3.3 Histological observations

Varying degrees of bone healing were observed beneath the CaSO$_4$-chitosan composite membranes in comparison to the control group. Figure 7 shows a typical transverse section of experimental bone defect 4 weeks after surgery. In the control group, the connective tissue grew into the bone defect area and prevented the bony cells from growing back to their natural form or space and thus destroyed the healthy process of new bone growth (Figure 7(a)). Interestingly, in the experimental groups (i.e., defect covered with CaSO$_4$-chitosan (300 kDa), and CaSO$_4$-chitosan (70 kDa) composite membranes), the connective tissue cells had only limited proliferation on their original sites and prevented the connective tissue cells from intruding into the space of the bone defect (Figures 7(b)-(e)). Sequentially speaking, the bone defect was allowed with the space and critical healing time to be repaired by newly yet slowly formed bone that proliferated in regions partially or fully protected and separated by the CaSO$_4$-chitosan composite membrane. This process practically prevented any connective tissue from invading into the bone defect. Furthermore, no obvious inflammatory response was observed around the chitosan membrane at this initial healing stage. However, the bone healing seemed to be slowed down while the chitosan membrane still occupied and saved the space exclusively for the bone tissue to be healed in this isolated area (as compared to the control).
The preliminary results demonstrated that these prepared CaSO₄-chitosan composite membranes in this study could successfully isolate the bone defect from ingress of connective tissue cells and provide a space where bony tissue cells could grow into later. There are other advantages, including the readily gel-forming properties, and being easily available in pure form, biodegradable and osteoinductive. Full utilization of the characteristics of chitosan could allow it to become a promising material in GTR applications.

4. Discussion

Many aspects of technical development of bioresorbable membrane materials for GTR applications are focusing on their rigidity and degradation rate, and with special emphasis on the ease of clinical manageability. Lots of effort has been spent on improving the biological function of barrier membrane. For example, surfaces coated with alginate were found to resist cell adhesion. In this study, we brought together two attractive biomaterials, CaSO₄ and chitosan, which are in abundance and inexpensive, and possess excellent biocompatibility and biodegradability, in applications for GTR. Consequently, we have presented here a simple phase-induced separation method to prepare a series of CaSO₄-chitosan barrier membranes. Briefly, CaSO₄ was annexed to chitosan matrix to prepare the composite membranes with high mechanical strength. Following the process, CaSO₄ also altered morphological structure and degradation behavior of the chitosan membranes. These changes probably are attributed the ability of CaSO₄ to bind with chitosan through ionic bonding, and subsequently, to cross-link to the different parts of chitosan polymer chains. To verify this postulation, we tested the membranes in a medium of 0.1 N acetic acid. We observed that CaSO₄-chitosan membranes were not dissolved completely even after 24-hour of shaking (not shown), whereas a neat chitosan membrane would be readily dissolved.

Subsequently, we applied these CaSO₄-chitosan composite membranes to the animal GTR study models.
Based on the findings from the histological evaluation, all three prepared chitosan membranes apparently exhibited better membrane integrity in bone defect healing after 4 weeks and provided good cell separation ability (see Figures 7(b)-7(e)) compared with controls (Figure 7(a)). Among the chitosan membranes tested, the defects covered with CaSO₄-chitosan composite membranes had higher percentage of new bone formation. On the contrary, in the case of the control group, the connective tissue might have grown preemptively into the bone defect area, and that caused the bony cells, with much slower growth rate, to not be able to grow back into their originally designated space and form. Another important factor for the success of GTR techniques is that the barrier material ought to withstand a period long enough for the bony tissue to reach sufficient healing stages. In clinical practice, the tissue regeneration barrier membranes are generally required to maintain their barrier functions for 4 to 6 weeks, with ample mechanical strength left, in order to secure the restoration of periodontal tissues. As observed from Figure 5, it could be concluded that these chitosan membranes were apparently suited for GTR in biodegradable characteristics.

5. Conclusion

On the basis of the results observed, it could be concluded that the CaSO₄-chitosan composite membranes prepared in this study appeared to be of great promise for applications in GTR in general. However, to assess and need to perform extensive and in-depth studies of animal models that more closely resemble to the human anatomy, such as in the porcine oral cavities.

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References
