Guided Tissue Regeneration with Use of β-TCP/Chitosan Composite Membrane

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ABSTRACT: Guided tissue regeneration (GTR) membranes with bioabsorbable 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 have employed chitosan and tricalcium phosphate (β-TCP) as viable membrane materials and evaluated their roles in GTR applications. Three types of β-TCP/chitosan membranes, weight ratio of β-TCP/chitosan 65 : 35, 33 : 67, and 10 : 90, were prepared for three categories: the mechanical strength to create an effective space; the rapid rate to reach hydrolytic equilibrium in phosphate buffer solution; and the ease of clinical manipulations. Consequently, standardized, transosseous, and critical-sized (cavity of 8 mm) skull defects were made in adult rabbits, 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 β-TCP/chitosan membranes in comparison to the control group. The β-TCP/chitosan membranes covered regions showed a clear boundary space between connective tissues and bony tissues. Over all, good cell-occlusion and beneficial osteogensis effects by these bioabsorbable materials toward the wound recovery were indicated. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 112: 3127–3134, 2009

Key words: guided tissue regeneration; chitosan; β-TCP; membrane

INTRODUCTION

Guided tissue regeneration (GTR) techniques have been successfully applied in the treatment of periodontal diseases and provided opportunity for the formation of new bone.[1–3] The techniques to employ membranes as mechanical barriers to create a space around the defects may permit bone regeneration and prevent the epithelial cells from migrating into the bony area. Among the materials used in GTR applications, nonresorbable membranes, such as expanded polytetrafluoroethylene (e-PTFE) membrane, and resorbable membranes, such as collagen membrane, have been successfully applied and are commercially available. Nevertheless, there might exhibit disadvantages in use of e-PTFE membrane, which would require a second surgical procedure to remove it, and of collagen membrane, which might cause localized chronic inflammatory response and rapid degradation behavior.[4,5] Linde and coworkers[6] have found that polylatide/polyglycolide (PLA/PGA) membranes exhibited a valid alternative to e-PTFE membranes with improved bone regeneration in rat models. However, gingival recession, exposure of the device, and surrounding soft tissue inflammation were presented in clinical findings when polyglycolic acid-based membranes were used in GTR techniques.[7,8]

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 would allow migration of bone cells from the surrounding alveolar bone into the targeted area.[9] Ideally, clinical manageability of the barrier membrane is also a necessary consideration during surgical procedures. In addition, effective materials should be safe, nontoxic, nonantigenic, and 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 had been employed to utilize bioresorbable membranes to achieve therapeutic purpose in clinical trials.[10–13] Furthermore, we focused on the development of bioresorbable membrane prepared from chitosan and β-TCP, and attempted to evaluate

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the feasibility of devising composite membranes for GTR applications.

Chitosan [poly(1,4),-β-D-glucopyranosamine], 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.[14,15] Its excellent gel-forming properties and ability to be reshaped into various forms simply by thermally induced separation method strongly enhance 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.[16,17]

Among many commercially available ceramics, β-tricalcium phosphate (β-TCP) is frequently used as bone repairing and replacing materials because of their biocompatibility, bioactivity, mechanical strength, and nontoxicity. Possessing the chemical composition close to the mineral composition of natural bone, calcium phosphate ceramics have been extensively employed as a bone substitute.[18–21] From clinical practices and experiences, β-TCP ceramics can be gradually absorbed that followed by new bone formation yet without compromising the intimacy of bone-implant contact.

We previously reported that the chitosan membranes prepared by thermally induced phase separation method and following treatment with nontoxic NaOH-gelating, $Na_5P_3O_{10}$-, and Na_2SO_3-crosslinking agents exhibited similar properties with chitosan membrane that strengthened by glutaraldehyde as crosslinking agent.[22] In this study, we take advantage of the unique properties of chitosan and β-TCP to prepare a composite membranes system. To retain the good biocompatibility of the two unique materials within the same device, we avoided using any crosslinking agents in the process of preparing β-TCP/chitosan membrane. SEM observation was conducted to examine the morphology of the membrane surface. Also, some basic properties of the β-TCP/chitosan membranes were examined, such as water content (WC) measurements, mechanical strength, and degradation tests. In addition to assessing the physical properties of β-TCP/chitosan membranes, the biological function of the composite membranes was studied by animal test. It is hoped that we may establish the feasibility of the naturally occurring chitosan membrane for GTR in periodontal application.

MATERIALS AND METHODS

Chitosan was purchased from TCI (Tokyo, Japan), with the molecular weight of 3,00,000, deacetylation degree 83%. Tricalcium phosphate (β-TCP), purchased from Merck-Schuchardt (Germany), was classified through a sieve with 0.104 mm opening. Acetic acid was purchased from Sigma (St. Louis, MO). All chemicals used in this study were of reagent grade.

Preparation of chitosan/β-TCP membranes

Chitosan was dissolved in acetic acid (0.1*M*) to prepare a 2% (w/v) chitosan solution. This chitosan solution was filtered and mixed with β-TCP and then stirred for 24 h. Each 19 mL of mixed solution was poured into a 9-cm Petridish and placed in a drying oven at 40°C with proper ventilation for overnight. After drying, the membranes were washed with distilled water and pressed from two sides with polyethylene thin films, and then placed in an oven (40°C) until complete dryness. The β-TCP containing chitosan membranes exhibited ivory-like uniform and opaque texture. All prepared chitosan membranes were stored in desiccators until use.

Water content measurement

The WC of the membrane was determined by swelling the membrane in pH 7.4 of phosphate-buffered saline (PBS) at the 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 WC of the membrane was calculated as:

$$\mathrm{WC} = (W_w - W_d)/W_w \times 100\%$$

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

Mechanical properties measurement

The mechanical properties of the membranes were measured in hydrated condition. The membranes, 1 cm × 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 a MTS Systems (Eden Prairie, USA) at a crosshead speed of 10 mm/min.

In vitro degradation test

The *in vitro* degradation test of the prepared membranes was conducted by incubating the membrane

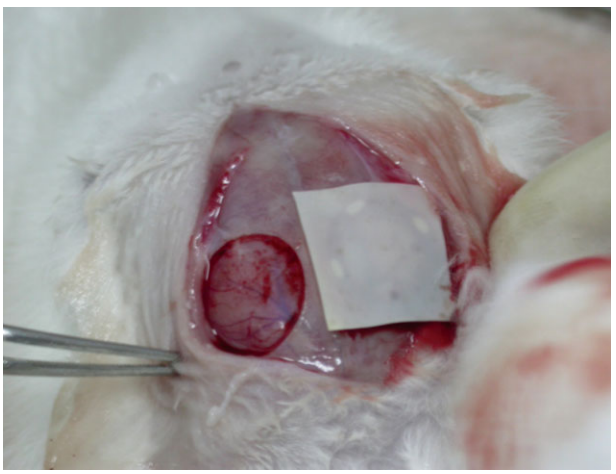

Figure 1 Macroscopic appearance of an experimental site. A bone defect (8 mm in diameter) was created at the skull of a rabbit. A defect on the skull covered with a β-TCP/chitosan membrane (10 mm × 10 mm in area). [Color figure can be viewed in the online issue which is available at www.interscience.wiley.com.]

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 the weight of this membrane 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.

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).

Animals and operation procedure

New Zealand white rabbits between 1.5 and 2 kg, fed with commercial food and RO water, were included in this randomized, blinded study. The rabbits were anesthetized by transabdominal 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 (8 mm in diameter) was generated with a dental round burr (Fig. 1). Before implantation, the membranes were hydrated in physiologic saline to restore their elasticity. The defect was then covered with chitosan membrane (Fig. 1, 10 × 10 mm 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, initial healing periods of 4 weeks were allowed.

Histological preparation and evaluation

After the healing periods, the rabbits 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, then dehydrated in an ascending graded series of ethanol solutions, and afterward 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.

RESULTS

Morphology of β-TCP/chitosan membrane

In Figure 2 are SEM micrographs, which show the characteristic morphological aspects of various chitosan membranes. All chitosan membranes produced by thermal-induced phase separation demonstrated a dense morphological structure. Various degrees of roughness on the surfaces of the β-TCP/chitosan membranes were visible on the chitosan membrane with different contents of β-TCP. From the SEM observations, it revealed that the surface roughness of β-TCP/chitosan membranes increased with increase of β-TCP content. Contrarily, the pure chitosan membrane showed to have a smoother surface when compared with all β-TCP/chitosan membranes. Figure 3 showed the surface morphologies of the β-TCP/membranes after 60 days of shaking. SEM examinations revealed that these β-TCP/membranes still exhibited rough surfaces and with good integrity, albeit that there were signs of degradation on the surface. It probably was attributed to that β-TCP 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.

Basic properties of β-TCP/chitosan membrane

When considering the development of GTR barrier materials, the basic bulk and mechanical properties must be noted. Besides, the appropriate degradation rate of material also has to fit the requirement of tissue regeneration. Interestingly, β-TCP/chitosan membranes became more elastic as indicated by

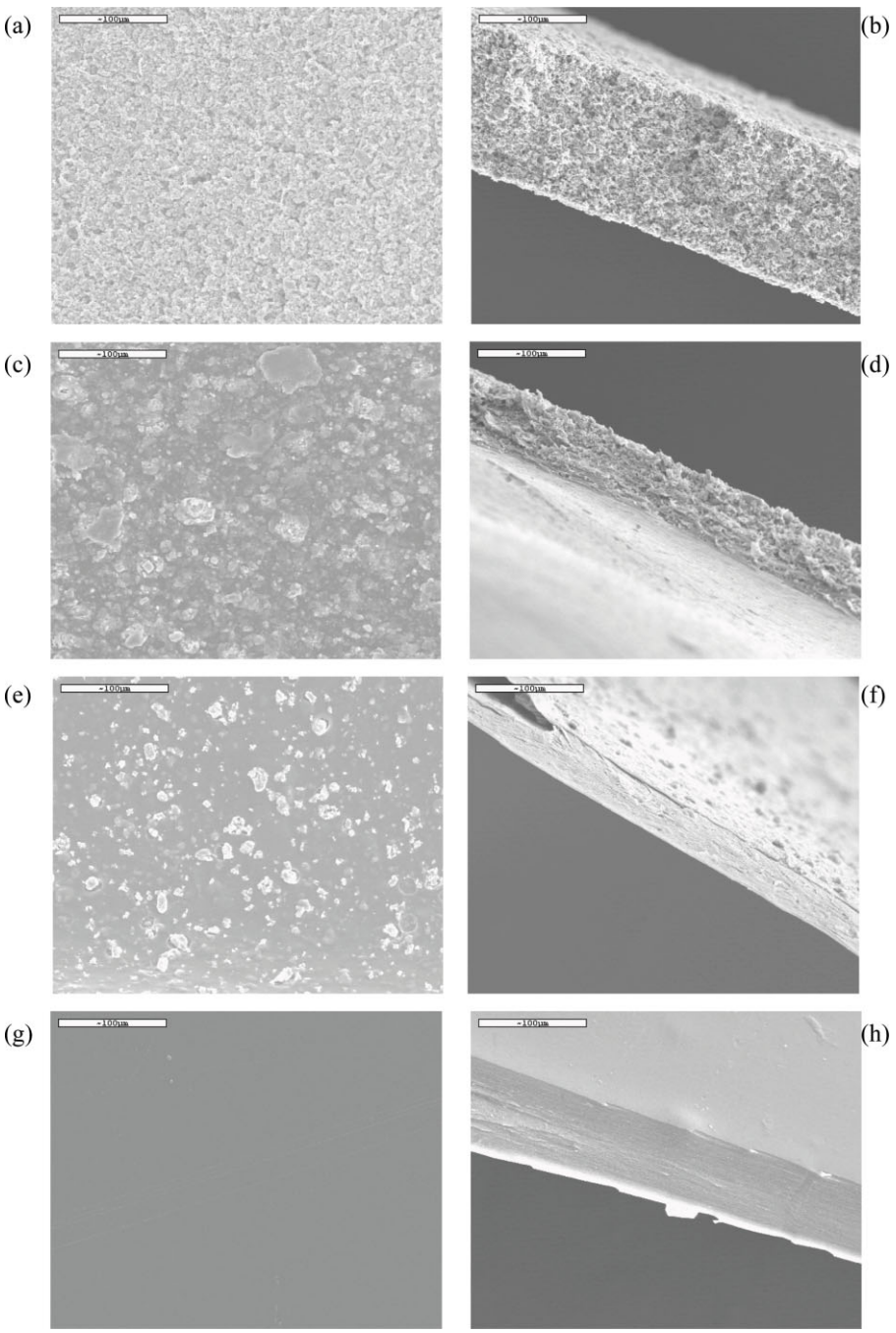

Figure 2 SEM micrographs of the membrane: (a, b) a β-TCP/chitosan (65 : 35) membrane; (c, d) a β-TCP/chitosan (33 : 67) membrane; (e, f) a β-TCP/chitosan (10 : 90) membrane; and (g, h) a plain chitosan membrane.

higher ultimate length. However, β-TCP would slightly affect Young's modulus of the membranes (as shown in Table I). The Young's modulus of the membrane decreased from 15.0 (pure chirosan) to 13.0 MPa β-TCP/chitosan (65/35). Figure 4 showed the mechanical properties of the membranes in the period of 60-day shaking. The Young's Modulus of β-TCP/chitosan membranes was insignificantly changed when compared with the unshaken one. It revealed that these membranes still stayed intact and retained the mechanical strength after 60 days of shaking. In clinical practice, when materials are used as tissue regeneration barrier membranes, they are generally required to maintain certain barrier functions for 4–6 weeks to secure the successful restoration of periodontal tissues.[23] From mechanical strength tests, it demonstrated that these membranes have had appropriate mechanical strength for the requirement of GTR. Furthermore, β-TCP/chitosan (65 : 35) membrane degraded most slowly when compared with the other membranes in 60-day shaking test. As shown in Figure 5, the degradation of β-

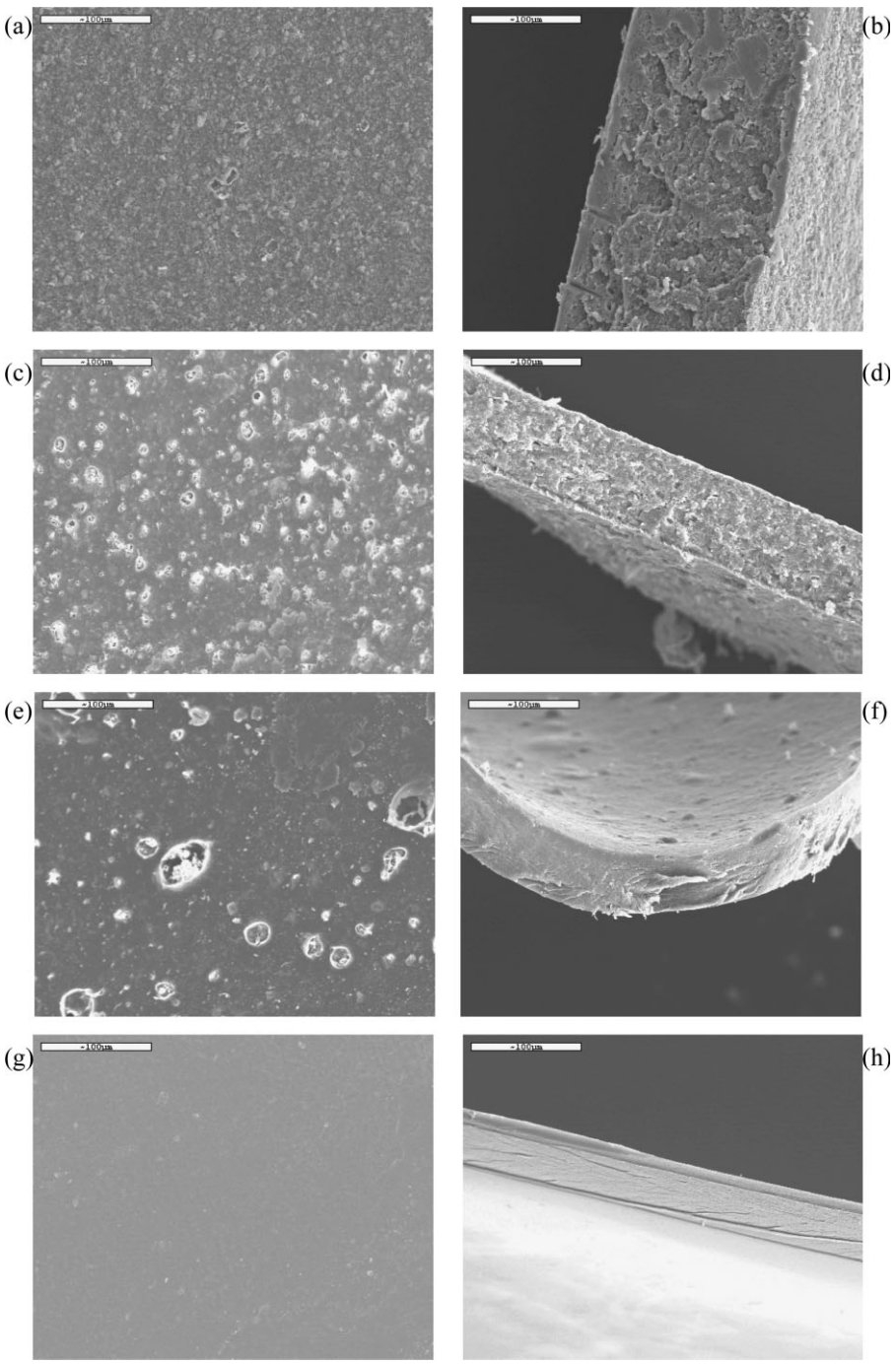

Figure 3 SEM micrographs of the membrane: (a, b) a β-TCP/chitosan (65 : 35) membrane; (c, d) a β-TCP/chitosan (33 : 67) membrane; (e, f) a β-TCP/chitosan (10 : 90) membrane; and (g, h) a plain chitosan membrane after 60 days of shaking.

TABLE I
Physical Properties of β-TCP/Chitosan Composite Membranes

β-TCP/Chitosan	Equilibrium water content at 24 h (%)	Young's modulus (MPa)[a]	Ultimate elongation (mm)	Ultimate strength (Pa)
65 : 35	33.2 ± 1.4	13.0 ± 1.0	20.8 ± 1.4	290.0 ± 34.8
33 : 67	48.4 ± 4.7	10.1 ± 2.1	17.8 ± 5.7	182.9 ± 47.1
10 : 90	48.5 ± 2.7	11.4 ± 1.1	15.9 ± 3.6	190.0 ± 42.9
Pure chitosan	50.3 ± 1.7	15.0 ± 1.9	10.9 ± 3.2	169.3 ± 64.7

Average ± SD, $n = 4$.
[a] Chitosan membranes were hydrated in pH 7.4 PBS before experiment run.

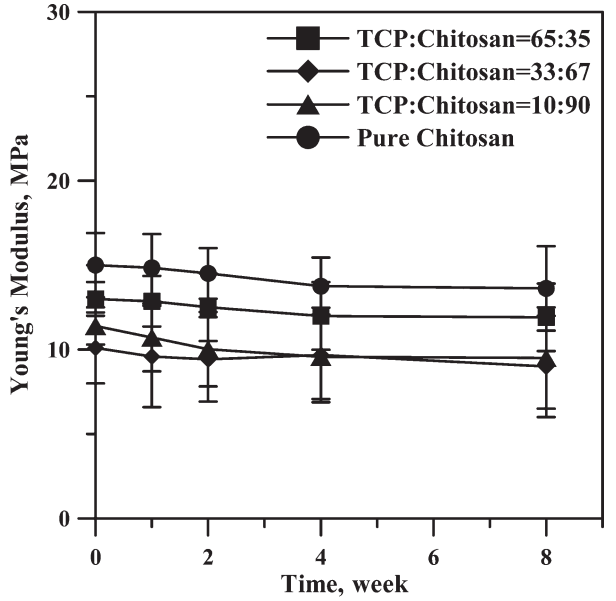

Figure 4 Young's modulus changes of β-TCP/chitosan membranes and pure chitosan membranes as a function of time.

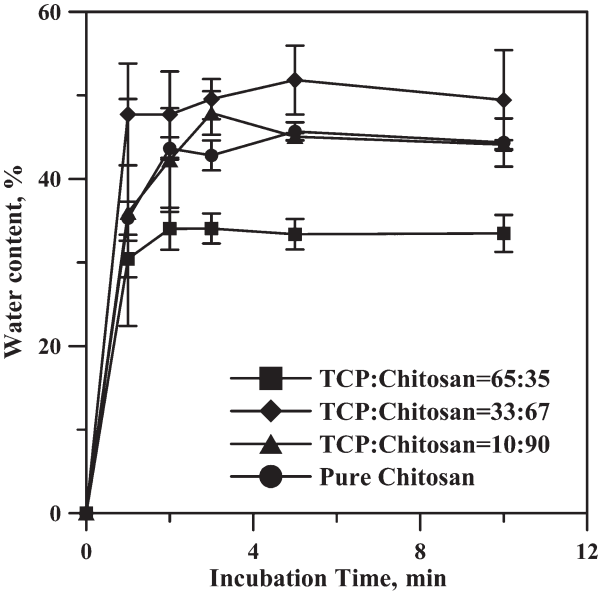

Figure 6 Water contact profile of the membranes in pH 7.4 PBS solution in 10-min period of experiment.

TCP/chitosan membranes increased with the decrease of the content of β-TCP. All three β-TCP/chitosan membranes degraded about 5–10% of initial weight after 60-day shaking test. 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 min period of the experiment, as shown in Figure 6 of water contact profiles. This rapid swelling phenomenon would be beneficial to the clinical manageability and surgical procedures. The bulk properties of prepared β-TCP/chitosan membrane were summarized in Table I. From all above results, it indicated that the β-TCP/chitosan membranes prepared in this study fulfilled the requirement of bioresorbable membrane used for GTR.

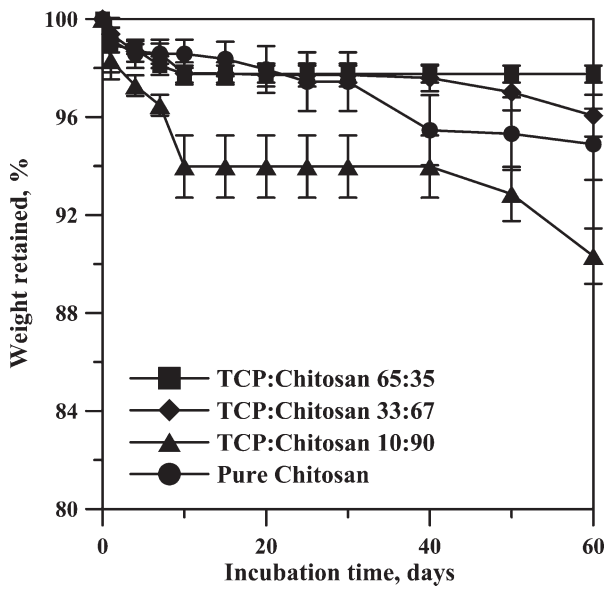

Figure 5 The degradation profile of the membranes in pH 7.4 PBS solution at 37°C for 60-day shaking.

Histological observations

Varying degrees of bone healing were observed beneath the β-TCP/chitosan membranes in comparison to the control group. Typically, Figure 7 showed a 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 its natural form or space, and thus destroyed the wholesome process of new bone growth [Fig. 7(d)]. Interestingly, in the experimental groups (i.e., defect covered with β-TCP/chitosan (65 : 35), β-TCP/chitosan (33 : 67), and β-TCP/chitosan (10 : 90) membranes), the connective tissue cells proliferated only limited on their original sites and prevented the connective tissue cells from intruding into the space of the bone defect. [Fig. 7(a–c)]. Sequentially speaking, the bone defect was allowed with the space and

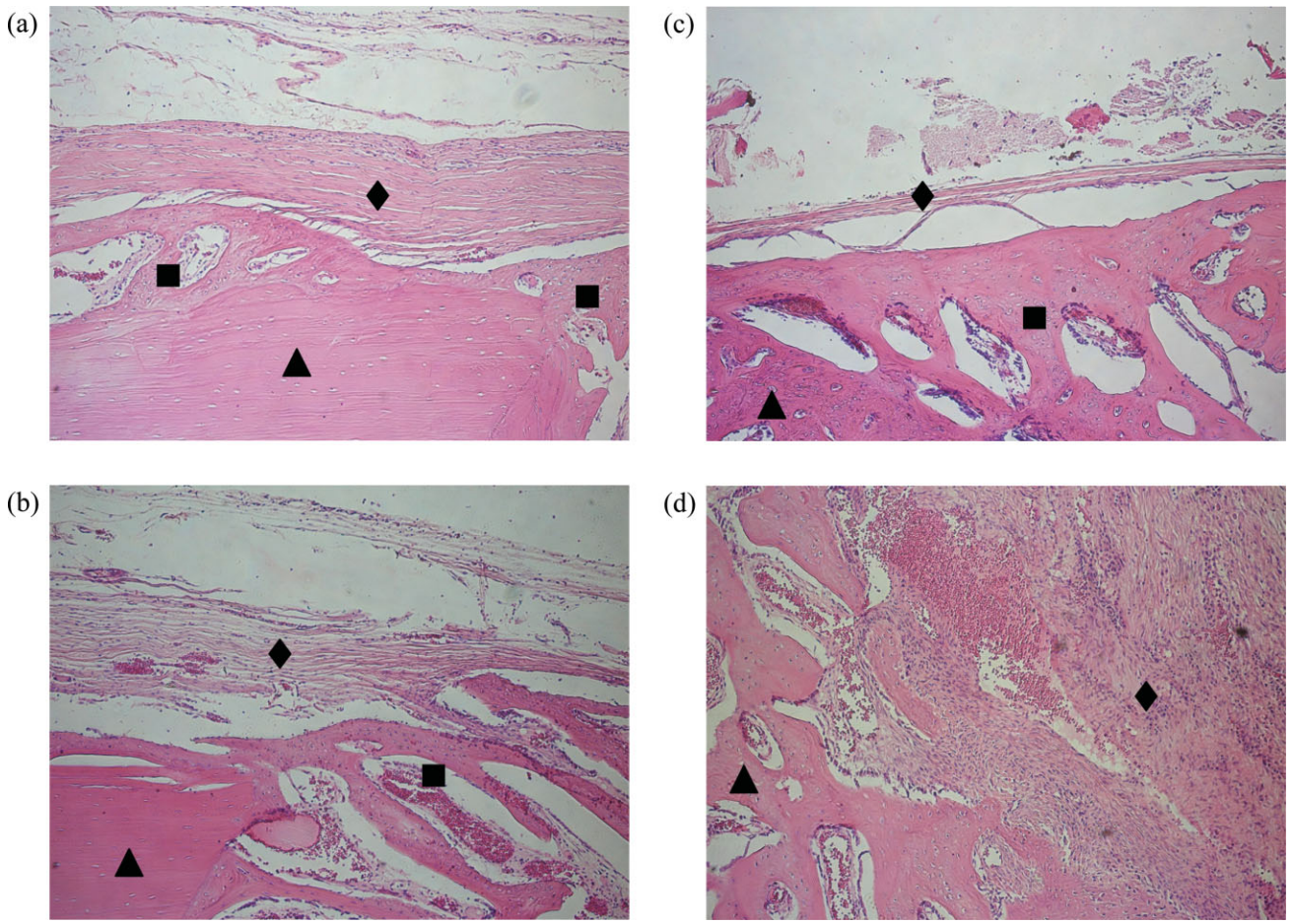

Figure 7 Histological section of the skull portion of a rat undergoing GTR procedure 4 weeks after surgery; (a) control, without chitosan membrane coverage, (b) with β-TCP/chitosan (65 : 35) membrane coverage, (c) with β-TCP/chitosan (33 : 67) membrane coverage, and (d) with β-TCP/chitosan (10 : 90) membrane coverage. (◆), fibrous tissue; (■), new bone; and (▲), bone. Hematoxylin and eosin stain; original magnification ×100. [Color figure can be viewed in the online issue which is available at www.interscience.wiley.com.]

critical healing time to be repaired by newly yet slowly formed bone that proliferated in regions partially or fully protected and separated by the β-TCP/chitosan membrane. This process practically prevented any of connective tissue from invading into the bone defect. Furthermore, no obvious inflammatory response was observed around the chitosan membranes 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 to be healed at this isolated area (when compare with the control).

The preliminary results demonstrated that these prepared β-TCP/chitosan membranes in this study could successfully isolate the bone defect from ingress of connective tissue cells and provide with the space where bony tissue cells could grow later. There are other advantages including, the easy gel-forming properties, and being inexpensive, biodegradable, and osteoinductive. Full utilization of the characteristics of chitosan could afford itself to become a promising material in GTR applications.

DISCUSSION

Many aspects of technical development of bioabsorbable membrane materials in GTR applications are focusing on the rigidity and degradation rate, and with special emphasis on the ease of clinical manageability. Lots of efforts have been spent on improving the biological function of barrier membrane.[17,24,25] For example, surfaces coated with alginate were found to resist cell adhesion. In this study, we studied two attractive biomaterials—β-TCP and chitosan, which are in abundance and inexpensive, and possess excellent biocompatible and biodegradable characteristics, in the applications for GTR. Consequently, we report here in details a simple phase-induced separation method to prepare a series of β-TCP/chitosan barrier membranes. Briefly, β-TCP was annexed to chitosan matrix to prepare the

composite membranes with high mechanical strength. Following the process, β-TCP also altered morphological structure and degradation behavior of the chitosan membranes. These changes probably are attributed to that β-TCP could bind with chitosan through ionic bonding, and subsequently crosslinked 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 β-TCP/chitosan membranes were not dissolved completely even after 24 h of shaking (not shown), when a neat chitosan membrane would be readily dissolved.

Subsequently, we applied these β-TCP/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 Fig. 7(a–c)) than control [Fig. 7(d)]. Among the chitosan membranes tested, the defect covered with β-TCP/chitosan membranes had higher percentage of new bone formation. On the contrary, in case of the control group, the connective tissue might grew preemptively into the bone defect area, and that caused the bony cells, with much slower growth rate, not being able to grow back into its original space or form. Another important factor for the success of GTR techniques was 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 4–6 weeks 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. On the basis of the results observed, it could be concluded that the β-TCP/chitosan membranes prepared in this study appeared to be of great promise for application in GTR in general. However, to assess and explore the full potential of these materials in physiologically more demanding periodontal applications for GTR, we shall need to perform extensive and in-depth studies of animal models that would be more closely parallel to the human anatomy, such as in the porcine oral cavities.

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