

PREPARATION OF CHITOSAN/HYDROXYAPATITE GUIDED MEMBRANE USED FOR PERIODONTAL TISSUE REGENERATION*

Kai Zhang^a, Man Zhao^a, Lei Cai^b, Zheng-ke Wang^b, Yong-fu Sun^b and Qiao-ling Hu^{b**}

^a Affiliated Stomatology Hospital of Medicine College, Zhejiang University, Hangzhou 310006, China

^b Institute of Biomedical Macromolecules, Department of Polymer Science and Engineering, Key Laboratory of Macromolecule Synthesis and Functionalization, Ministry of Education, Zhejiang University, Hangzhou 310027, China

Abstract In an effort to develop biomaterials to meet guided tissue regeneration (GTR) standards for periodontal tissue recovery, a homogeneous and transparent chitosan (CS)/hydroxyapatite (HA) membrane with potential applications as GTR barrier in periodontal therapy has been prepared *via in situ* compositing. The membrane has been designed to have a smooth-rough asymmetric structure that meets the demand for GTR. Component and morphology of the membrane are characterized by XRD and SEM. It can be indicated that HA was *in situ* synthesized uniformly in the CS membrane. Mechanical experiments of the membranes with various HA contents show that their tensile strengths are adequate for periodontal therapy. Biological properties of the membrane have been performed by cell toxicity assays, hemolysis tests and animal experiments. Results indicate that the membrane has good biocompatibility and inductive effect for cell growth. Therefore this membrane can be potentially applied as GTR barrier membrane for periodontal tissue regeneration.

Keywords: Chitosan membrane; Hydroxyapatite; *In situ* compositing; Periodontal tissue regeneration.

INTRODUCTION

Tissue engineering has been regarded as an ultimate medical treatment for reconstruction of defective tissues in biomedical engineering fields in recent years^[1]. Guided tissue regeneration (GTR) technique by using barrier membranes has been suggested as a potential modality in periodontal therapy^[2–5]. The purpose of using membrane for GTR was, on the one hand, to prevent apical migration of gingival epithelial cells into bony defect site, while on the other hand to promote growth of progenitor bone and periodontal ligament cells^[6]. Thus, GTR technique required the barrier materials to have good biocompatibility, bio-function and excellent mechanical strength. Nondegradable polytetrafluoroethylene (PTFE) membrane has been used for tissue regeneration, but it is expensive and needs a second operation to take it out. The absorbable collagen membrane also has been widely used in the therapy of human bony defects, but it has inflammatory response to a certain extent^[7, 8]. Poly 3-hydroxybutyrate (PHB) and nano-hydroxyapatite (HA) GTR membrane was manufactured through the air/jet electrospinning process, and could induce the formation of mineral crystal *in vitro*, but it has poor mechanical properties^[9]. And there is few literature reported GTR membranes made of chitosan (CS) and HA with good biocompatibility and excellent mechanical properties.

CS, the partly deacetylated product of chitin, was suggested to be used in orthopedic application to provide

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** Corresponding author: Qiao-ling Hu (胡巧玲), E-mail: huql@zju.edu.cn

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temporary mechanical support for the regeneration of bone owing to its good biocompatible, non-toxic, biodegradable and inherent wound healing characteristics, as well as bioactivity that includes hemostatic, fungistatic, antibacterial, antitumoral, anticholesteremic and the most important osteoconductive properties^[10–13]. CS is a well-known filmogenic material. CS membranes have been investigated in many uses, such as in enzyme immobilization, protein separation and controlled drug-release^[14], etc. In recent years, incorporation of CS membrane with bio-minerals has captured tremendous attention on the preparation of biomaterials. Further modifications are usually done in order to turn CS more multi-functional in tissue regeneration.

HA with the chemical formula $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, its chemical composition and crystallographic data are similar to the inorganic component found in natural bone, has been extensively used for hard tissue replacement and augmentation due to its biocompatibility and osteoconductivity^[15]. HA is generally incorporated in CS in two ways. One is that HA particles are blended directly into CS substrates; another is that HA is precipitated on the surface of CS membranes. Previous work from our lab explored a novel method of *in situ* compositing to synthesize HA within CS matrix, in which the HA particles can be homogeneously dispersed and have better inter-facial interaction forces.

In this research, CS/HA membranes with high-strength were prepared by *in situ* compositing. The membranes also require an asymmetric smooth-rough structure to meet the requirement of GTR. Further *in vivo* tests it was proved that the membrane was a promising candidate biomaterial for guided periodontal tissue regeneration.

EXPERIMENTAL

Materials

Chitosan ($M_n = 5.63 \times 10^5$) was supplied by the Qingdao Haihui Bioengineering Co. Ltd (Qingdao, China) with 91% degree of deacetylation. Calcium nitrate tetrahydrate ($\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$) was purchased from the Chinese Pharmacy Group Shanghai Reagent Company (Shanghai, China). Potassium dihydrogen phosphate (KH_2PO_4), sodium hydroxide (NaOH) and acetic acid (CH_3COOH) were supplied by Hangzhou Xiaoshan Chemical Reagent Corporation (Hangzhou, China).

Preparation of CS/HA Membrane via *in situ* Compositing

A defined amount of $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ and KH_2PO_4 as the HA precursor was added into 100 mL acetic acid aqueous solution with the concentration of 2% (V/V). The ratio of CS/ $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ / KH_2PO_4 was listed in Table 1. CS (3 wt%) was dissolved in the above solution by stirring for at least 2 h. The mixed solution was cast in the mould and dried at 50°C for 10 h. Then the mould with the dried membrane was immersed in 50 g/L NaOH solution for about 5 h to fully neutralize the acetic acid and reacted with HA precursor to form HA. The membrane was then rinsed with water to pH = 7 and dried at 50°C. The thickness of the membranes was (65 ± 5) µm.

Table 1. HA precursor contents in the CS solution

	CS (g)	HA (g)	$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (g)	KH_2PO_4 (g)
1	10	0.5	1.25	0.43
2	10	1.0	2.50	0.86
3	10	1.5	3.75	1.29
4	10	2.0	5.00	1.72

X-ray Diffraction (XRD) Analysis

The crystal of HA was detected with powder XRD (Rigaku D/Max-2550 PC) using $\text{CuK}\alpha$ radiation generated at 40 kV and 50 mA. The sample was scanned from 8° to 80° in 2θ .

Scanning Electron Microscope (SEM) Observations

The morphology of different membranes was observed by field emission scanning electron microscopy (FE-SEM, FEI-SIRION).

Testing of Mechanical Properties

The membranes were retreated in oven at 50°C for 2 h to remove the moisture remaining in the composite. Tensile strength and elongation rate were measured on the universal materials testing machine (Shenzhen Reger Company), the span length was 40 mm, and the extension rate was 2 mm/min.

Cell Toxicity Assays

A CS/HA membrane (100/10, *W/W*) sterilized by ^{60}Co irradiation was cut into square pieces (10 mm $\times$ 10 mm) and placed in a culture plate (100 mm in diameter), added with 1 mL MC3T3-E1 Subclone 14 Cell suspension with a concentration of 10^6 cells/L to allow full attachment of cells to the membrane. After 3 h, 10% FBS DMEM was carefully added on CS membrane. Cultivation was conducted for 48 h at 37°C in 5% CO_2 , fixed with formaldehyde for 24 h, dyed with normal hematoxylin and eosin (H&E), and observed under optical microscope.

Hemolysis Test

CS membrane and CS/HA membrane (10 mm $\times$ 10 mm) (Fig. 1), sterilized by ^{60}Co irradiation, were added with 10 mL physiological saline for 24 h at 37°C to get leaching liquor. 4 mL fresh heparinized rabbit blood was diluted in 5 mL physiological saline. The test group contained 0.2 mL diluted heparinized rabbit blood mixed with 10 mL leaching liquor of CS or CS/HA membranes while the positive control mixed with 10 mL distilled water and the negative control mixed with 10 mL physiological saline. The mixture of 3 groups was centrifuged respectively with centrifugal acceleration of 750 g for 5 min. Absorbance of the supernatant was measured at 545 nm using a spectrophotometer. The hemolytic rate (HR) can be obtained through the following equation:

$$\text{HR} = \frac{D_t - D_{nc}}{D_{pc} - D_{nc}} \times 100\%$$

where D_t is the absorbance of the test group. D_{pc} and D_{nc} are the absorbance of the positive and negative controls, respectively.

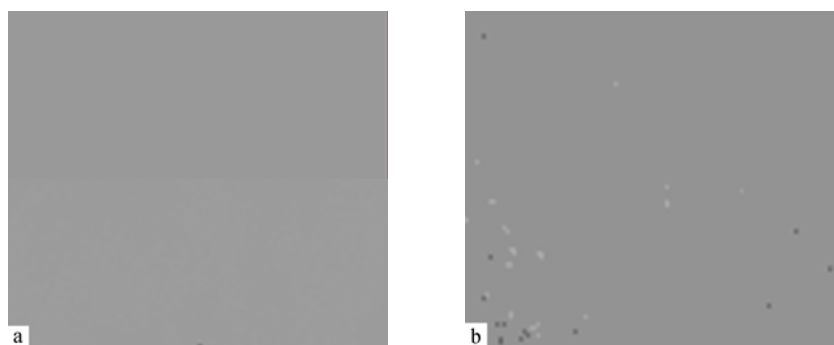


Fig. 1 Photographs of (a) pure CS membrane, (b) CS/HA membrane (100/20, *W/W*)

Animal Experiment

The animal experiment was approved by the Animal Research Ethics Committee of Stomatology Hospital, College of Medicine, Zhejiang University. Adult New Zealand white rabbits were used for the experiments (supplied by Animal Center of College of Medicine, Zhejiang University). A mandibula defect (5.0 mm in diameter) with smooth edges, shown in Fig. 2, was bored in the mandible of the rabbit tail cancellous bone. CS membrane, CS/HA membrane (10 mm $\times$ 10 mm) and blank control group, sterilized by ^{60}Co irradiation, soaked with physiological saline, were used to cover the mandibula defect respectively. The implanted membranes along with their surrounding tissues were retrieved at one and two weeks postoperatively, fixed with formaldehyde for 24 h, decalcified in 0.5 mol/L EDTA, dyed with H&E before observation.

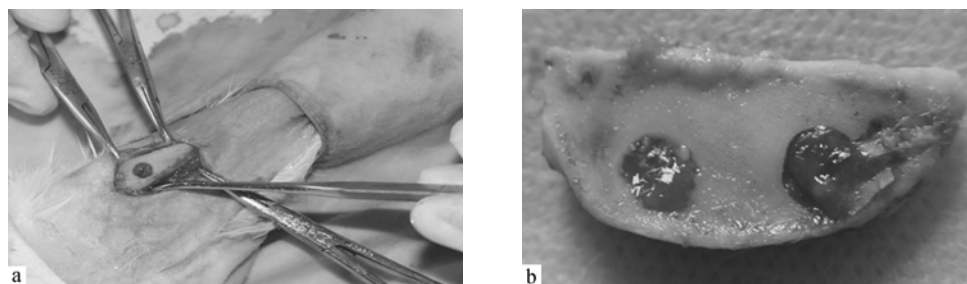


Fig. 2 Photos of (a) animal experiments on the mandible bone of a rabbit (b) a specimen of mandibula defects of a rabbit

RESULTS AND DISCUSSION

Mechanism of Preparing CS/HA Membranes by in situ Compositing

The solubility of CS depends largely on the pH value of environments because of the existence of amino groups. The free amino group of CS was protonated to $\text{CS}-\text{NH}_3^+$ at $\text{pH} = 4.2$ ^[16, 17]. Viala *et al.*^[18] established that the presence of calcium and phosphate ions in the solution allowed the soluble form of CS to exist below $\text{pH} 6.7$, while pH of HA formation was more than 10. So calcium and phosphate ions existed in the form of ions in the resulting solution and the CS was precipitated in the presence of HA formation simultaneously when the pH of the environment was more than 10.

Component Determination by XRD

The XRD pattern of CS/HA membrane *via in situ* method is shown in Fig. 3. The wide peak appeared at $2\theta = 20.9^\circ$ was typically assigned to CS^[19]. The sharp diffraction characteristic peaks appeared at $2\theta = 26.64^\circ$, 32.72° and 39.86° were the typical peaks of HA^[16]. Therefore the calcium phosphate *in situ* synthesized existing in the matrix is exactly HA.

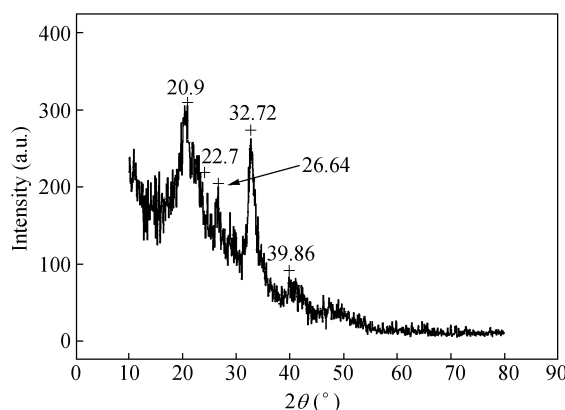


Fig. 3 XRD pattern of CS/HA (100/10, W/W) membrane prepared by *in situ* compositing

Morphology of CS/HA Membranes

SEM photographs of CS/HA membranes are shown in Fig. 4. The back surface of the membrane (Fig. 4a) indicated that it was quite smooth. The front rough surface of the membrane (Fig. 4b and Fig. 4c) showed that the HA particles were synthesized homogeneously on the front of membranes. Cell attachment and proliferation were surface roughness sensitive and increased as the roughness increased^[20], so the smooth-rough structure of the membrane may meet the requirement of GTR. The rough side with HA particles serves to promote growth of progenitor bone and periodontal ligament cells while the smooth side plays an important role in preventing apical migration of gingival epithelial cells into bony defect sites.

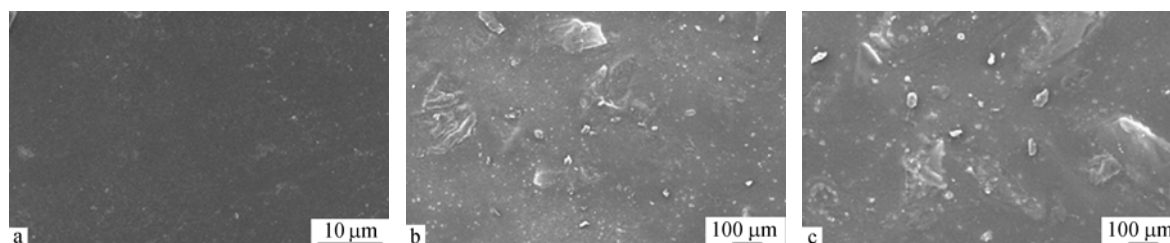


Fig. 4 SEM photographs of CS/HA (100/10, *W/W*) membranes, (a) the back smooth surface ($\times 5000$), (b) the front rough surface ($\times 100$) and (c) the front rough surface ($\times 200$)

Mechanical Properties of CS/HA Membranes

The mechanical properties of the CS/HA membranes are shown in Fig. 5. The results indicated that the tensile strength and elongation ratio slowly decreased with the increasing of HA content in the CS matrix. This could be explained by the interaction forces between HA particles and CS matrix that are weaker than the forces within CS membrane itself. Fortunately, the reduction of the mechanical properties was slight. Even the group with the lowest mechanical properties (HA content is 20%) can adequately meet the demand for GTR.

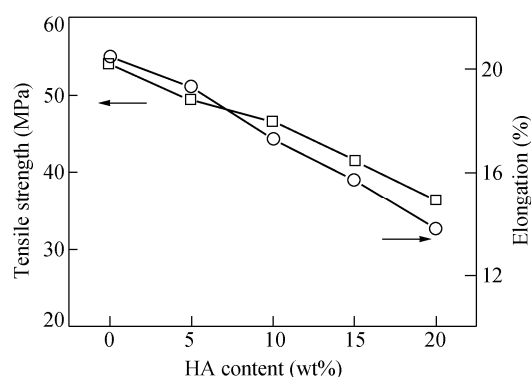


Fig. 5 The tensile strength and elongation rate of CS/HA membranes with different HA contents

Cell Toxicity Assays

Cell toxicity assays were performed after 48 h cultivation using an optical microscope. As can be seen in Fig. 6, MC3T3-E1 Subclone 14 cells *in vitro* showed that no cell toxicity was observed on the CS/HA (100/10, *W/W*) membrane with a concentration of 10^6 cells/L.

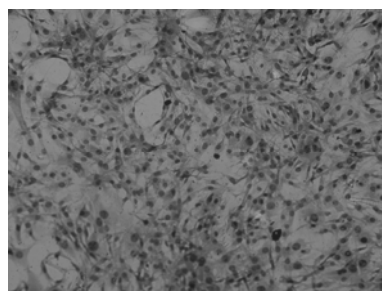


Fig. 6 Photographs of cell proliferation by optical microscope

Hemolysis Tests

Hemolysis tests were conducted in three parallel experiments A, B and C. The results in Table 2 showed that materials from both two groups have no obvious hemolysis reaction. The hemolytic rate *in vitro* met the requirement of hemolysis test standard ($< 5\%$) for medical materials.

Table 2. Hemolytic rate of pure CS membrane and HA/CS membrane

Group	A	B	C
Pure CS membrane	0.1%	0.0%	0.7%
HA/CS = (100/10, <i>W/W</i>) membrane	0.0%	0.1%	0.5%

Animal Experiments

In this study, the *in vivo* biocompatibility and osteoconductivity of CS/HA membranes were assessed in a rabbit model *via* mandibula defects. It was found in all the groups a little inflammatory reaction surrounding the tissue. The osteoid was formed after one week in the blank control group, together with proliferated fibrous tissue. The newly-formed bone tissue was found after two weeks in Fig. 7(d) with plenty of fibrous connective tissues. Figures 7(b) and 7(e) showed that the fibrous tissue, osteoblast, trabecular bone and mature bone tissue were in orderly arrays, mixed with parts of osteoclast and paltry bone tissue, which indicated the bone tissues were forming and reconstructing simultaneously. It was shown in Fig. 7(c) and Fig. 7(f) of CS/HA membrane group that the newly-formed bone tissue and osteoblast were abundant in the osteoid matrix, as well as many orderly-arranged trabecular bone. So the results of the three groups of tests explained the active formation of new bones in the defected areas. The CS/HA membrane effectively accelerated the regeneration of defected areas. More arranged mature bone tissue and less soft tissue and connective tissue were grown compared with other groups.

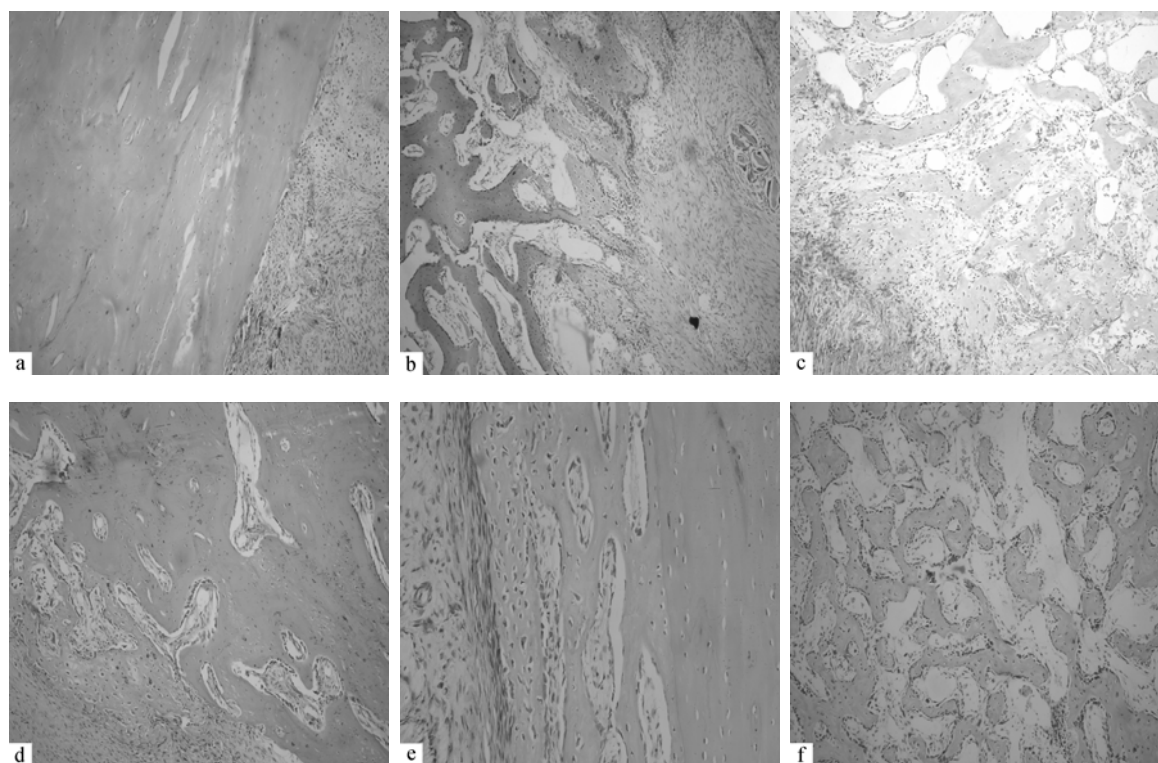


Fig. 7 Micrographs of the tissues implanted with (a) blank control group, (b) pure CS membrane, (c) CS/HA (100/10, *W/W*) for one week, (d) blank control group, (e) pure CS membrane, (f) CS/HA (100/10, *W/W*) for two weeks

CONCLUSIONS

Through the new method of the *in situ* compositing of CS/HA membranes, the inorganic fillings — HA precursor could disperse homogeneously in the CS matrix to ensure the gradual release of HA which played a significant role in the process of the bone fracture recovery. The good mechanical properties of the membrane were capable of sustaining the burden *in vivo*. The asymmetric smooth-rough structure could prevent apical

migration of gingival epithelial cells into bony defect site while promoting growth of progenitor bone and periodontal ligament cells, which met the demand for GTR. The membranes have excellent biocompatibility, according to the cell toxicity assays, hemolysis tests and animal experiments. The mandibula defects regenerate better and more quickly with the use of CS/HA membrane than the pure CS membrane and blank control group. Moreover, the concentration of HA in the membrane could be altered in accordance with the different situations. Therefore we can expect that this membrane as a promising applicable candidate in periodontal therapy for guided tissue regeneration.

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