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Development of fish collagen/bioactive glass/chitosan composite nanofibers as a GTR/GBR membrane for inducing periodontal tissue regeneration

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Abstract

The development of a guided tissue or bone regeneration (GTR/GBR) membrane with excellent performance has been a major challenge in the biomedical field. The present study was designed to prepare a biomimetic electrospun fish collagen/bioactive glass/chitosan (Col/BG/CS) composite nanofiber membrane and determine its structure, mechanical property, antibacterial activity, and biological effects on human periodontal ligament cells (HPDLCs). The effects of this composite membrane on inducing periodontal tissue regeneration were evaluated using a dog class II furcation defect model. It was found that the composite membrane had a biomimetic structure with good hydrophilicity (the contact angle was $12.83 \pm 3^{\circ}$) and a tensile strength of 13.1 ± 0.43 Mpa. Compared to the pure fish collagen membrane, the composite membrane showed some degree of antibacterial activity on *Streptococcus mutans*. The composite membrane not only enhanced the cell viability and osteogenic gene expression of the HPDLCs, but also promoted the expression of RUNX-2 and OPN protein. Further animal experiments confirmed that the composite membrane was able to promote bone regeneration in the furcation defect of dogs. In conclusion, a biomimetic fish Col/BG/CS composite membrane has been developed in the present study, which can induce tissue regeneration with a certain degree antibacterial activity, providing a basis for potential application as a GTR/GBR membrane.

1. Introduction

The functional reconstruction of periodontal tissue defects remains an important challenge in periodontal treatment. Among the treatment modalities, the application of a guide tissue or a bone regeneration (GTR/GBR) membrane is often a necessary clinical treatment. According to the degradation characteristics, the existing GTR/GBR membranes can be classified into two major categories. The critical drawbacks of polytetrafluoroethylene (PTFE)-based nonresorbable membranes relate to the need for secondary surgery [1] and an increased risk of bacterial colonization. Resorbable membranes, such as synthetic polyesters and collagen-based membranes, have shown good biocompatibility and excellent cell affinity, but they have deficiencies, including a lack of sufficient strength and an unpredictable degradation rate [2–4]. It has also been reported that bovine-derived collagen is associated with a higher risk of bovine spongiform encephalopathy (BSE) [5], and mammal collagen may be limited for religious reasons. Therefore, there is an urgent need for the development of inexpensive, safe GTR/GBR membranes with sufficient mechanical properties, a predictable degradation rate, and a structure that mimics closely the native extracellular matrix (ECM). The GTR/GBR membranes also require the ability to induce periodontal tissue regeneration with a certain degree of antibacterial activity, which can effectively prevent infection and the occurrence of peri-implantitis after surgery. Our previous findings indicate that collagen derived from tilapia skin, mainly composed of α-chains (α1 and α2)

and crosslinked chains (β and γ), has an extensive source, contains 19 different amino acids, has good cell affinity and can accelerate rat skin wound healing [6]. However, it is unclear if tilapia collagen has the ability to induce periodontal tissue or bone tissue regeneration and solve the existing problems of the current GTR/GBR membranes by compositing with other materials. It is of great interest and broad significance to study this topic.

So far, there have been two reports about the effects of salmon collagen (SC) gel and hydrolyzed tilapia fish collagen (HFC) on human periodontal ligament fibroblasts (HPdLFs) and human periodontal ligament cells (HPDLCs) [7, 8]. They have found that SC gel and HFC can promote cell growth and the expression of osteogenic-related genes, such as alkaline phosphatase (ALP), type I collagen (COL-I) and osteocalcin (OCN). Nagai *et al* have also demonstrated that the growth rates and the differentiated functions of HPdLFs are at higher levels on the SC gel than on pig collagen gel [7]. This suggests that besides mammal collagen, fish collagen may have the potential to be used for the development of GTR/GBR membranes. It is well known that an ideal GTR/GBR membrane should not only have the ability to induce periodontal tissue or bone tissue regeneration, but also show some degree of antibacterial activity. Several studies have found that bioactive glass (BG) has strong antibacterial activity, good osteogenic ability and angiogenic activity, and has been used for the treatment of long bone infection [9–13]. In addition, chitosan has antibacterial activity against gram-positive and gram-negative strains such as *Streptococcus mutans* (*S. mutans*) and *Porphyromonas gingivalis* (*P. gingivalis*) [14]. If fish collagen can be composited with BG and chitosan by the appropriate technology to fabricate a novel and multifunctional GTR/GBR membrane, it may greatly improve the restoration of periodontal tissue defects and play an important role in tissue regeneration.

The structure of a membrane is essential for its biological function. Compared to gels or other three dimensional matrices, electrospun nanofibrous scaffolds have higher porosity and surface area, and mimic more closely the scales and morphologies of ECM proteins (fibers with diameters ranging from 50 to 500 nm) [15], which can promote cell–cell and cell–matrix interaction. In addition, the efficiency of electrospun scaffolds would be enhanced by incorporating bioactive molecules, offering several unique features required for enabling cell growth, proliferation and differentiation [16].

In the present study, tilapia collagen added with appropriate BG precursor solution and chitosan were used to develop biomimetic fish collagen/bioactive glass/chitosan (Col/BG/CS) composite nanofiber membranes by electrospinning. The effects of the Col/BG/CS composite membrane on the adhesion, proliferation and osteogenic differentiation of HPDLCs were determined. Furthermore, the antibacterial activity of the Col/BG/CS composite membrane on *S. mutans* (one of the main oral bacteria) was investigated. Finally, bilateral class II furcation lesions in dogs were surgically created and used to validate the effects of the Col/BG/CS composite membrane on inducing periodontal tissue regeneration. It was hoped that the present study would provide a basis for future research and develop novel fish Col/BG/CS nanofibers as a new GTR/GBR membrane.

2. Materials and methods

2.1. Preparation of electrospun fish Col/BG/CS composite nanofiber membrane

Tilapia collagen was provided by the Shanghai Fisheries Research Institute and was dissolved in hexafluoroisopropanol (HFIP) (purity > 99.5%, Fluorochem Ltd, UK) solution to obtain an 8% fish collagen solution. Then, 4.0 g P123, 6.7 g tetraethyl orthosilicate (TEOS), 1.4 g $Ca(NO_3)_2{\cdot}4H_2O$, 0.73 g triethyl phosphate (TEP) and 1.0 g 0.5 M HCl were dissolved in 60 g ethanol with vigorous stirring for 24 h to prepare the BG precursor solution (Si/Ca/P = 80:15:5 mole ratio). Chitosan was dissolved in HFIP and trifluoroacetic acid (TFA) solution to obtain an 8% chitosan solution. The polymer solution of fish collagen, BG and chitosan was mixed in a 20:1:1 volume ratio and placed into a syringe; a high voltage (16–18 kV) was then applied to form an electrospinning fish Col/BG/CS composite nanofiber membrane. The flow rate during the electrospinning process was 1.0 ml h^{-1} and the distance from the needle to the aluminum foil collector was 10–15 cm. The membrane was then crosslinked with glutaraldehyde vapor for 24 h and stored in a vacuum oven.

2.2. Characterization of Col/BG/CS membrane

Scanning electron microscopy (SEM) (JEOL, JSM-5600, Japan) was used to observe the morphology of the Col/BG/CS membrane. The chemical structure of tilapia collagen nanofibers was determined by Fourier transform infrared spectroscopy (FTIR) (Nicolet, USA). The weight loss temperature of the Col/BG/CS membrane was determined using a thermogravimetric (TG) analyzer (209F1, Netzsch, Germany). The tensile strength was measured using a universal materials testing machine (H5K-S, Hounsfield, UK). Determination of the contact angle was performed with a contact angle measuring instrument (OCA40, Dataphysics, Germany).

2.3. Ionic concentration analysis

A Dulbecco's modified Eagle's medium (DMEM, Hyclone, USA) extract solution of the Col/BG/CS membrane was prepared with a ratio of 6 cm^2 ml^{-1} and incubated at 37 °C for 24 h. The concentrations of

calcium (Ca), phosphorus (P) and silicon (Si) ions were analyzed by an inductively coupled plasma emission spectrometer (ICP-OES, VISTA-PRO, Agilent, USA).

2.4. Cell adhesion and viability assays

The HPDLCs were obtained from the periodontal ligament tissues of premolar teeth for orthodontic reasons. Informed consent was obtained from all patients under a protocol approved by the Ethics Committee of Ninth People's Hospital affiliated to the School of Medicine, Shanghai Jiao Tong University. The isolation and culture of the HPDLCs was the same as in reference [8]. The HPDLCs were seeded on 24-well plates at a density of 1×10^4 cells cm^{-2} containing Col/BG/CS membrane, and cells seeded on cover slips were used as a control. Cells were cultured in DMEM with 10% fetal bovine serum (FBS, Gibco), penicillin (100 U ml^{-1}) and streptomycin (100 mg l^{-1}) (Invitrogen, Carlsbad, CA, USA). After 24 h, SEM was performed to analyze the cell morphology. After 24 h and 72 h, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg ml^{-1}, Sigma Chemical, St Louis, MO, USA) was added and incubated for 4 h, and then dimethyl sulfoxide (DMSO) was added to dissolve the crystals. The OD values at 570 and 630 nm were measured with a microplate reader (Labsystems Dragon Wellscan MK3, Finland).

2.5. Real-time polymerase chain reaction (PCR)

The HPDLCs were seeded on six-well plates containing the Col/BG/CS membrane at a density of 5×10^3 cells cm^{-2} for 3 d and 1×10^3 cells cm^{-2} for 10 d. Cells seeded on cover slips were used as a control. The culture medium was DMEM with 10% FBS, penicillin (100 U ml^{-1}) and streptomycin (100 mg l^{-1}). After being cultured for 3 d and 10 d, the total RNA was isolated via an RNeasy Mini Kit (Qiagen, Germany) and then reverse-transcribed into cDNA by a PrimeScript first strand cDNA synthesis kit (TaKaRa). The expressions of osteogenic differentiation-related genes, such as RUNX-2, ALP, osteopontin (OPN) and OCN were analyzed by a Bio-Rad sequence detection system (MyiQ2, USA) using a real-time PCR (SYBR Premix EX Taq, TaKaRa). The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to normalize the results. The data was expressed as fold changes to control according to the formula $2^{-\Delta\Delta Ct}$. The primers for the selected genes are listed in table 1.

2.6. Western blot

The HPDLCs were seeded on the six-well plates at a density of 1.5×10^4 cells cm^{-2} with the Col/BG/CS membrane, and cells seeded on cover slips were used as a control. After being cultured in DMEM with 10% FBS, penicillin (100 U ml^{-1}) and streptomycin (100 mg l^{-1}) for 3 d, the cells were collected and the protein was extracted by lysing the cells for 30 min in an ice-cold lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate (SDS), Applygen, Beijing, China) containing 1 mM phenylmethylsulphonyl fluoride (PMSF) (Sigma, St Louis, MO, USA) and phosphotase inhibitor cocktail (Sigma, St Louis, MO, USA). The cell lysates (50 μg protein extracts) were loaded onto SDS-polyacrylamide gels (8%–12% separation gels) and then transferred onto nitrocellulose (NC) membranes (Amersham Biosciences, US). The membranes were incubated overnight at 4 °C with primary antibodies anti-RUNX-2 (1:600, a rabbit monoclonal antibody, Bioworld, USA), anti-OPN (1:600, a rabbit monoclonal antibody, Bioworld, USA) and β-actin (1:1000, rabbit polyclonal antibodies, Abcam, UK) at 4 °C. After incubation, the membranes were washed three times in Tris-buffered saline (TBS) containing 0.05% Tween-20 (TBST) and then incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The blots were visualized with an ECL chemiluminescence reagent (Millipore, USA).

Table 1. Primers used in the present study.

Gene/oligo name	Oligo sequence
RUNX-2 forward	AGACCAACAGAGTCAGTGAG
RUNX-2 reverse	TGGTGTCACTGTGCTGAAGA
ALP forward	GGACCATTCCCACGTCTTCAC
ALP reverse	CCTTGTAGCCAGGCCCATTG
OPN forward	CAGTTGTCCCCACAGTAGACAC
OPN reverse	GTGATGTCCTCGTCTGTAGCATC
OCN forward	CAAAGGTGCAGCCTTTGTGTC
OCN reverse	TCACAGTCCGGATTGAGCTCA
GAPDH forward	CTTTGGTATCGTGGAAGGACTC
GAPDH reverse	GTAGAGGCAGGGATGATGTTCT

2.7. Antimicrobial activity assay

S. mutans (UA159) at a density of 1×10^6 colony forming units (CFUs) ml^{-1} was seeded on the Col/BG/CS membrane, and incubated in a sterilized brain heart infusion (BHI) under standard anaerobic conditions (80% N_2, 10% H_2, 10% CO_2, at 37 °C). *S. mutans* seeded on fish collagen nanofibers was used for comparison and *S. mutans* seeded on cover slips was used as a control group ($n = 3$ for each group). After being cultured for 24 h, SEM (JSM-5600LV) was performed to observe the bacterial morphology. In addition, the bacterium suspension was collected and the OD values were determined by a spectrophotometer (UV-160, SHIMADZU) with a wavelength of 600 nm. According to the OD values, the bacterium suspension was diluted with BHI and 100 ul was placed on the AGAR plate for the purpose of making the number of colonies suitable for counting. This was further incubated for 24 h. After that, the number of active bacteria CFUs was counted using an automatic

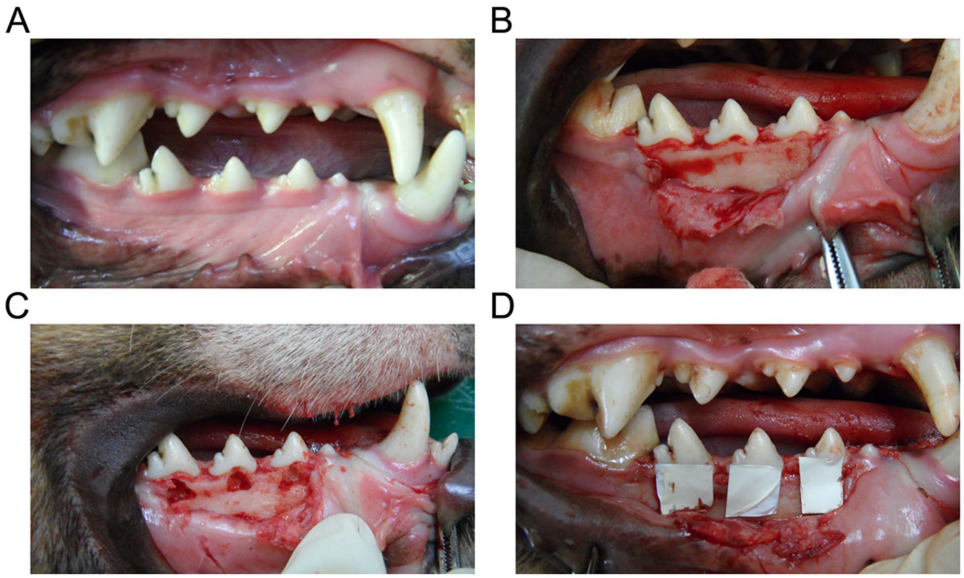

Figure 1. Preparation process of the class II furcation defects in beagles. (A) Before the surgery, (B) gingival flap operation, (C) preparation of defects, (D) the Col/BG/CS membrane cover.

colony counter G: BOX gel doc system with GeneSnap from SynGene software [17].

2.8. Periodontal defect model in beagle dogs

The experimental protocol was approved by the Animal Care and Experiment Committee of the Ninth People's Hospital affiliated to the School of Medicine, Shanghai Jiao Tong University. The experiments were conducted following the Shanghai Administration Rule of Laboratory Animals. Two healthy, 1-year-old, male beagle dogs were used in the present study. After anesthesia, bilateral class II furcation lesions (5 mm in the apico-occlusal direction and 3 mm in the buccolingual direction, three each side) were surgically created in three mandibular dog premolars (figure 1). Fish Col/BG/CS membranes were applied to the defects on one side ($n = 3$), and those on the other side were not covered as a control; then the gums were sutured. At 30 and 60 d after the operation, the dogs were euthanized and the periodontal tissues were collected for examining the changes to the wound.

2.9. Histopathological observations

At 30 and 60 d after the operation, the wound specimens were collected and fixed in 10% formalin for one week. After fixation, decalcification and dehydration, the specimens were stained with H&E and observed using a microtome (Leica, Tokyo, Japan). The histomorphometric measurements were determined in the stained section using NIH Image J software. The formulas used to calculate new bone formation were as follows: new bone formation = newly formed alveolar bone area/defect area × 100%. Immunohistochemical staining with OCN antibodies (Abcam, Cambridge, UK) was performed to evaluate the osteogenesis ability of the Col/BG/CS nanofibers on day 30 and 60.

2.10. Statistical analysis

The data were expressed as means ± standard deviation (SD) from three independent experiments. The statistical analyses were performed using a Student's t-test and a one-way analysis of variance (ANOVA) with Tukey's post hoc multiple comparison test. Statistical analyses were performed using SPSS 11.0 software. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Characterization of Col/BG/CS membrane

A small amount of BG precursor and chitosan were added to the collagen solution, and then the electrospun Col/BG/CS composite nanofiber membrane was developed. The morphology of the membrane was characterized by SEM, which is shown in figure 2(A), with a fiber diameter of 159 ± 59 nm. The FTIR spectrum (figure 2(B)) of the Col/BG/CS composite nanofibers showed that the characteristic absorption peaks of the amide groups had not changed after crosslinking. The TG image showed that the membrane had good thermal stability (figure 2(C)). The tensile strength was also determined to be 13.1 ± 0.43 Mpa (figure 2(D)), which indicated that the Col/BG/CS membrane had a certain mechanical strength. The contact angle was found to be 12.83 ± 3° (figures 2(E) and (F)), indicating that the Col/BG/CS membrane was highly hydrophilic.

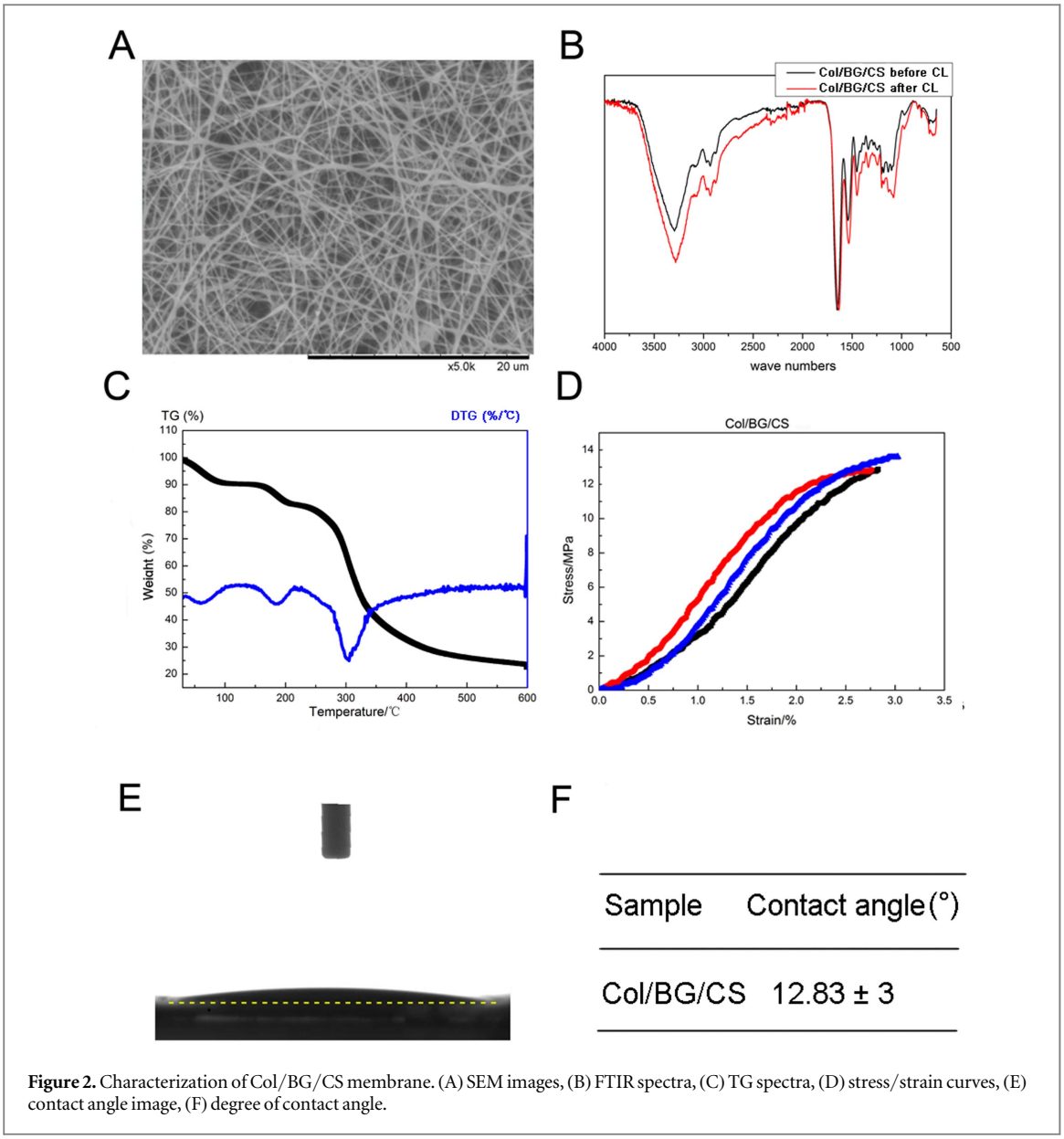

Figure 2. Characterization of Col/BG/CS membrane. (A) SEM images, (B) FTIR spectra, (C) TG spectra, (D) stress/strain curves, (E) contact angle image, (F) degree of contact angle.

Table 2. The ionic concentrations of the extracts (mg l^{-1}).

	Ca	P	Si
Col/BG/CS	65.7 ± 5.16	34.2 ± 1.53	59.1 ± 10.47

3.2. The ionic concentrations of extracts

The ICP results are shown in table 2, calcium (65.7 ± 5.16 mg l^{-1}), phosphorus (34.2 ± 1.53 mg l^{-1}) and silicon ions (59.1 ± 10.47 mg l^{-1}) were released into the culture medium from the Col/BG/CS membrane.

3.3. Cell adhesion and viability of Col/BG/CS membrane

Cell adhesion was observed by SEM. As shown in figures 3(A) and (B), HPDLCs were attached firmly, and spread well on the Col/BG/CS membrane. An MTT assay was used to detect cell viability, and figure 3(C) shows that cell viability was enhanced upon seeding on the Col/BG/CS membrane.

3.4. Osteogenic-related gene expression in HPDLCs

The mRNA expression of osteogenic marker genes is shown in figure 4. The Col/BG/CS membrane promoted the expression of osteogenic-related genes such as RUNX-2, ALP and OPN at 3 and 10 d. At 10 d, it also increased the expression of OCN. These results indicate that the composite membrane has the potential to promote the osteogenic differentiation of HPDLCs.

3.5. Detection of osteogenic protein expression

Western blot analysis was performed to determine whether the Col/BG/CS membrane was able to promote the expression of the osteogenic-related protein. In figure 5, quantitative analysis of the results showed that the Col/BG/CS membrane was able to

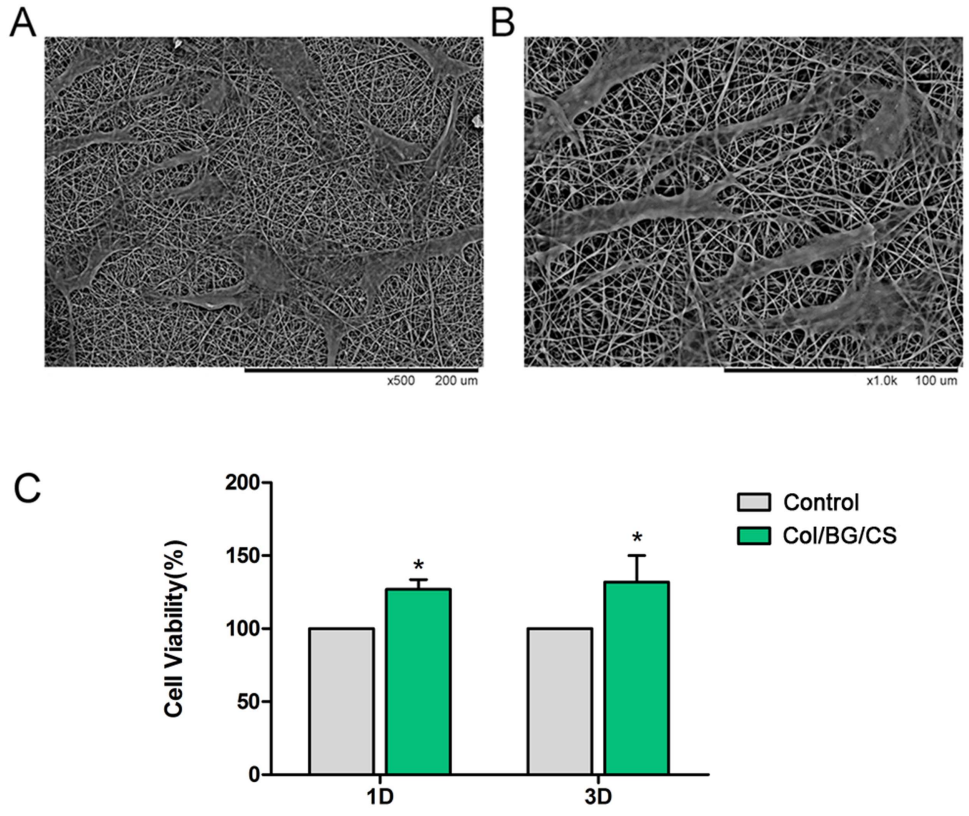

Figure 3. Cell adhesion and viability of HPDLCs cultured on the Col/BG/CS membrane. (A), (B) SEM images of HPDLCs cultured for 1 d; (C) cell viability of HPDLCs cultured for 1 and 3 d. The control group was cultured on cover slips, the data represents the mean ± SD, $n = 5$; $^{*}p < 0.05$ represents a significant difference between the compared groups.

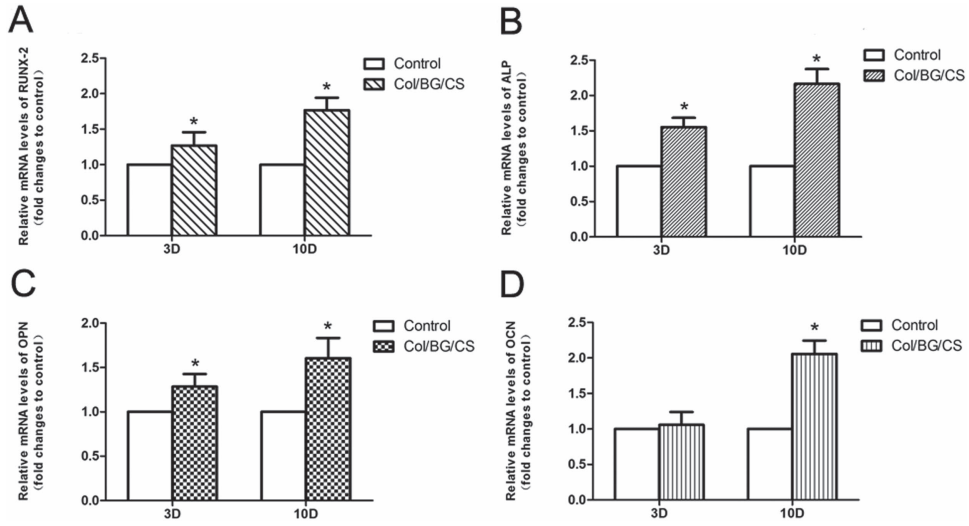

Figure 4. The mRNA expression of the osteogenic differentiation-related genes including (A) RUNX-2, (B) ALP, (C) OPN and (D) OCN in HPDLCs induced by the Col/BG/CS membrane for 3 d and 10 d; the control group was cultured on cover slips. The data represents the mean ± SD, $n = 3$; $^{*}p < 0.05$ represents a significant difference between the compared groups.

promote the expression of RUNX-2 and OPN protein in HPDLCs.

3.6. Antimicrobial activity of Col/BG/CS membrane

To investigate the antibacterial effect, the morphologies of *S. mutans* seeded on the Col/BG/CS membrane were examined by SEM. As shown in figure 6(A), the *S. mutans* cultured on the Col/BG/CS membrane were more dispersed than that of the collagen and control group. Figures 6(B) and (C) also show the number of *S. mutans* seeded on the Col/BG/CS membrane is less than the collagen group and the control group, which indicates

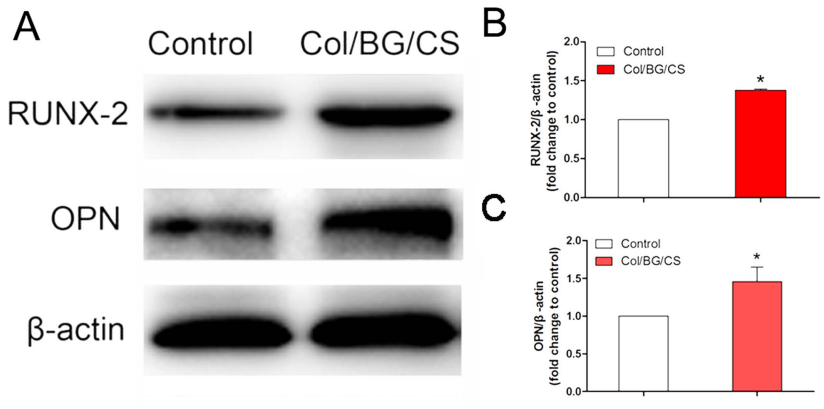

Figure 5. Expression of the osteogenic proteins in HPDLCs cultured on the Col/BG/CS membrane. (A) Osteogenic proteins were detected by western blot for 3 d; (B) semi-quantitative analysis of RUNX-2 and (C) OPN. The data represents the mean ± SD, $n = 3$; $^{*}p < 0.05$ versus the control.

that there was a certain degree of antimicrobial activity on *S. mutans* for the Col/BG/CS membrane.

3.7. Periodontal tissue regeneration in beagle dogs

A dog class II furcation defect model was designed to investigate the capacity of the Col/BG/CS membrane in inducing periodontal tissue regeneration. Figure 7 indicates that compared to the control group, the Col/BG/CS membrane group showed more new bone formation as well as less inflammation and gingival connective tissue at 30 d; the percentage of new bone formation in the Col/BG/CS group and the control group was 30.60% and 10.76%, respectively. At 60 d, the Col/BG/CS membrane group had formed a greater number of bones and very dense connective tissue. The percentage of new bone formation in the Col/BG/CS group and control group was 69.31% and 44.63%, respectively. In addition, immunohistochemical staining also demonstrated the presence of bone matrix proteins (OCN) in the newly formed bone (figure 8). The results indicate that the Col/BG/CS membrane was able to promote bone regeneration in the furcation defect of beagle dogs.

4. Discussion

It is known that fish processing by-products account for 50%–70% of all original raw materials [18]. Optimal use of these by-products can not only help avoid environmental problems, but can also produce value-added products such as cost-effective fish collagen. The use of fish collagen might also help address patients who decline the use of porcine or bovine collagen for religious reasons. In a previous study, fish collagen was successfully extracted and exhibited good thermal stability with the imino acid content (proline and hydroxyproline) of 190 residues/1000 residues [6], which was close to that of grass carp skin collagen (186 residues/1000 residues) [19]. In the present study, a biomimetic Col/BG/CS composite nanofiber membrane was fabricated by adding BG and chitosan to fish collagen. We evaluated its mechanical strength, antibacterial activity and efficiency in inducing periodontal tissue regeneration and explored its potential as a new generation of GTR/GBR membrane with excellent comprehensive performance.

It is important for the GTR/GBR membrane to have adequate mechanical strength in order to avoid membrane collapse and perform its barrier function. The result shows that the Col/BG/CS membrane has a tensile strength of 13.1 ± 0.43 Mpa (figure 2(D)), which is higher than that of pure fish collagen (6.72 ± 0.44 Mpa) [6], and also comparable with many products such as Resolut LT® (11.7 Mpa) and Bio-Guide® (7.75 Mpa) [15, 20, 21]. The biomechanical property and collagen matrix stability may be enhanced by means of crosslinking and the addition of BG. However, although the stress of the composite Col/BG/CS membrane was increased, the strain is not ideal. It still needs to be improved in the future. Increasing the time during the eletrospinning process and compositing it with a polymer may be beneficial in order to increase the strain of the membrane.

To explore whether the Col/BG/CS membrane has a biological effect on the key cells during the periodontal tissue regeneration process, we discussed the viability of HPDLCs cultured on the membrane *in vitro*. As shown in figure 3, the Col/BG/CS membrane promoted the adhesion and viability of the HPDLCs. Fish collagen may play an important role in this effect, as Song *et al* reported that jellyfish collagen exhibited much higher fibroblast viability than bovine collagen [22]. In addition, a BG precursor solution was selected as an additional component to form electrospun nanofibers with a biomimetic structure, which may also enable the cells to adhere and spread. Many reports indicate that BG nanofibers possess high bioactivity and osteogenic potential *in vitro* [23–25]. Furthermore, fish collagen and chitosan contain carboxyl, hydroxyl and other hydrophilic groups, which enhance the

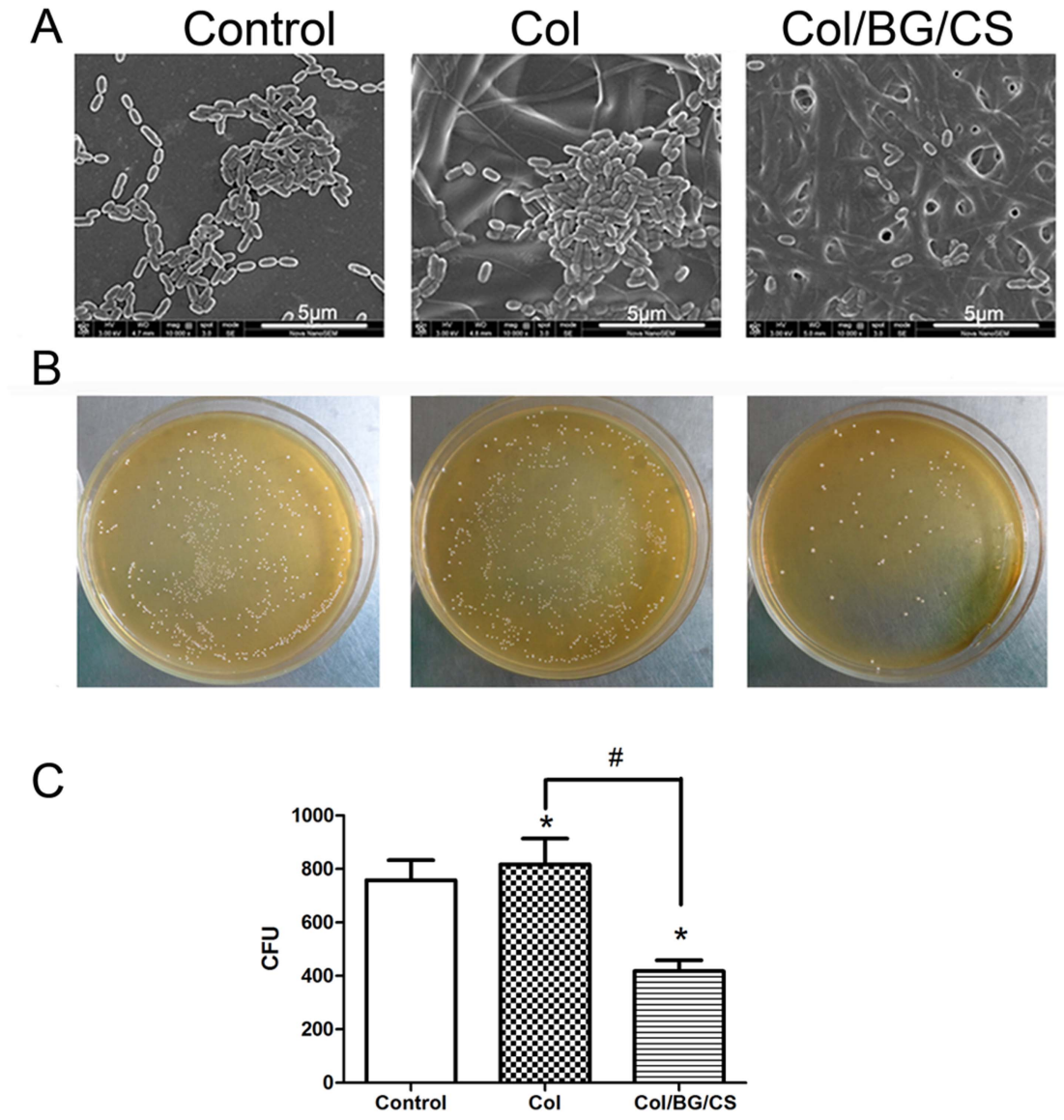

Figure 6. The antibacterial activity of the Col/BG/CS membrane. (A) SEM photographs of *S. mutans* cultured for 1 d; the control group was cultured on cover slips, (B) *S. mutans* cultured on the AGAR for 1 d, (C) the counts of the CFUs; the data represents the mean ± SD, $n = 3$; $^{\#} < 0.05$, $^{*}p < 0.05$ represent a significant difference between the compared groups.

hydrophilicity of the Col/BG/CS membrane. The hydrophilic surface facilitates fibronectin adsorption, resulting in high cell attachment [26].

During the process of inducing periodontal tissue regeneration, it is not enough for the Col/BG/CS membrane to have an effect on HPDLC adhesion and proliferation. If the Col/BG/CS membrane could further induce HPDLC osteogenic differentiation, it would be beneficial for the functional regeneration of the alveolar bone. Therefore, we further investigated the osteogenic differentiation ability of the Col/BG/CS membrane in HPDLCs. As shown in figure 4, the Col/BG/CS membrane promoted the expression of osteogenic-related genes such as RUNX-2, ALP and OPN at 3 and 10 d. OCN is an important late marker of osteogenic differentiation. It was found that the expression of OCN increased significantly at day 10 (figure 4(D)). These results indicate that the composite membrane has the potential to promote the osteogenic differentiation of HPDLCs. This effect may be associated with the fact that fish collagen is rich in amino acids, such as glycine and proline, which have the ability to regulate cell function. In addition, some active ions released from the Col/BG/CS membrane may also play a synergistic role. The ICP results (table 2) show that a certain amount of Ca, P and Si ions are released into the culture medium from the Col/BG/CS membrane. Ca and P ions contribute to form a hydroxyapatite layer, which is the equivalent to the mineral phase of human hard tissues. Silicon ions have a stimulatory effect on osteogenic cell differentiation, and early osteogenesis may be caused by the synthesis and/or stabilization of collagen [27, 28].

Because genes need to be transcribed and translated into protein to exert biological function, we then focused on whether the Col/BG/CS membrane could promote the expression of the osteogenic-related protein. As shown in figure 5, the Col/BG/CS membrane

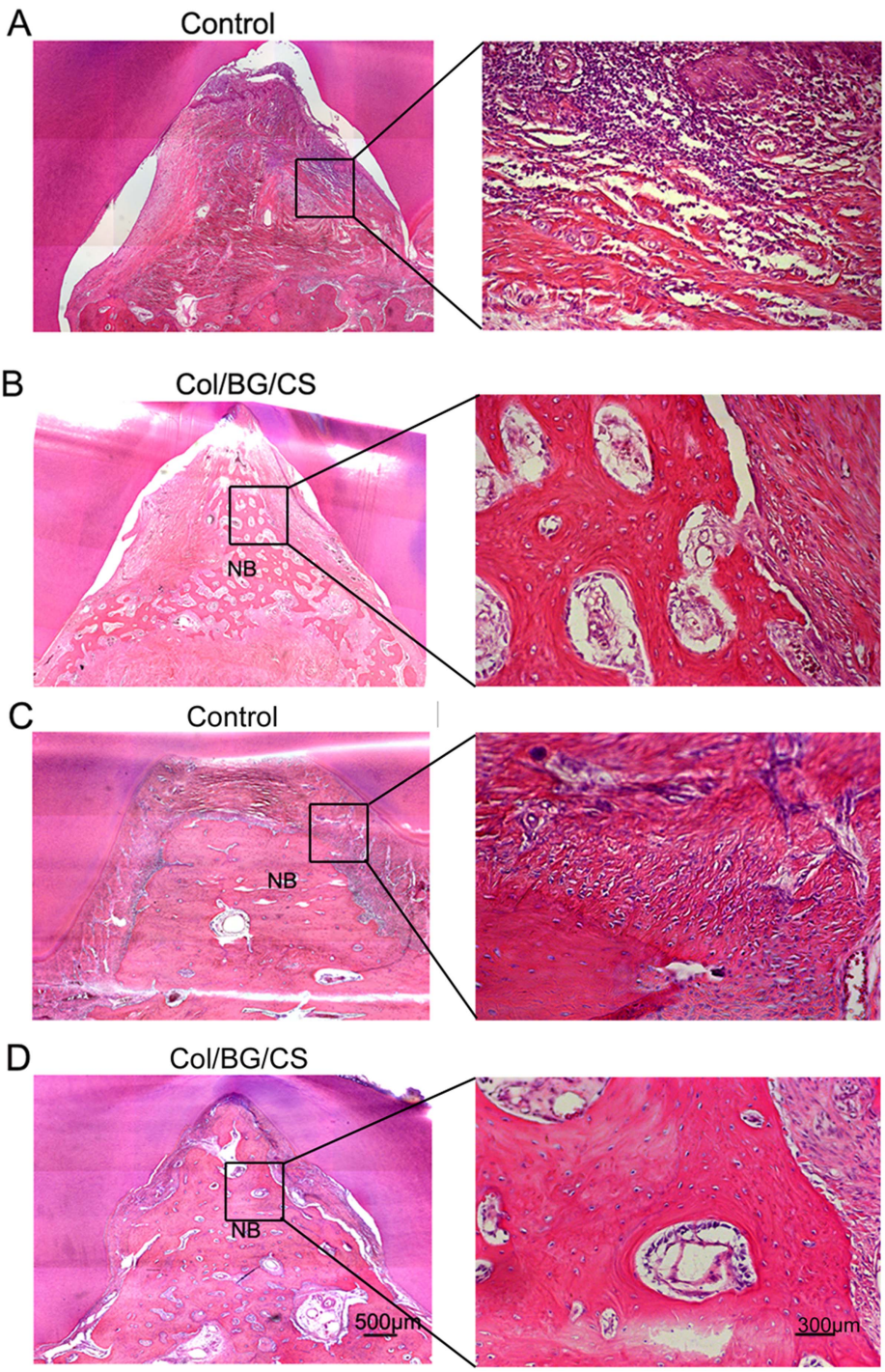

Figure 7. The mesiodistal H&E staining of a class II furcation defect at 30 d (A), (B) and 60 d (C), (D); post surgery of control group (A), (C) and the Col/BG/CS membrane group (B), (D). The control group was not covered with a membrane. NB: new bone.

induced the protein expression of RUNX-2 and OPN in the HPDLCs. As a key transcription factor required for osteogenic differentiation, RUNX-2 can activate downstream osteoblast-related gene expression. OPN is a major component of the bone matrix and is thought to be responsible for cell attachment to the ECM [29]. Our results indicate that the Col/BG/CS membrane may promote osteogenic differentiation in HPDLCs.

As we all know, the oral cavity is usually an environment that displays the existence of various bacteria, and the presence of bacteria, to a certain extent, can interfere with the function of the GTR/GBR membrane. Therefore, if the Col/BG/CS membrane can both improve the osteogenic ability and exert some antibacterial activity, it would have greater potential for clinical application. As shown in figure 6, although the Col/BG/CS membrane only contains a small amount of BG and CS, it still inhibits the adhesion and proliferation of *S. mutans* compared with the pure fish collagen membrane. We speculate that this antibacterial activity may be related to the structure of

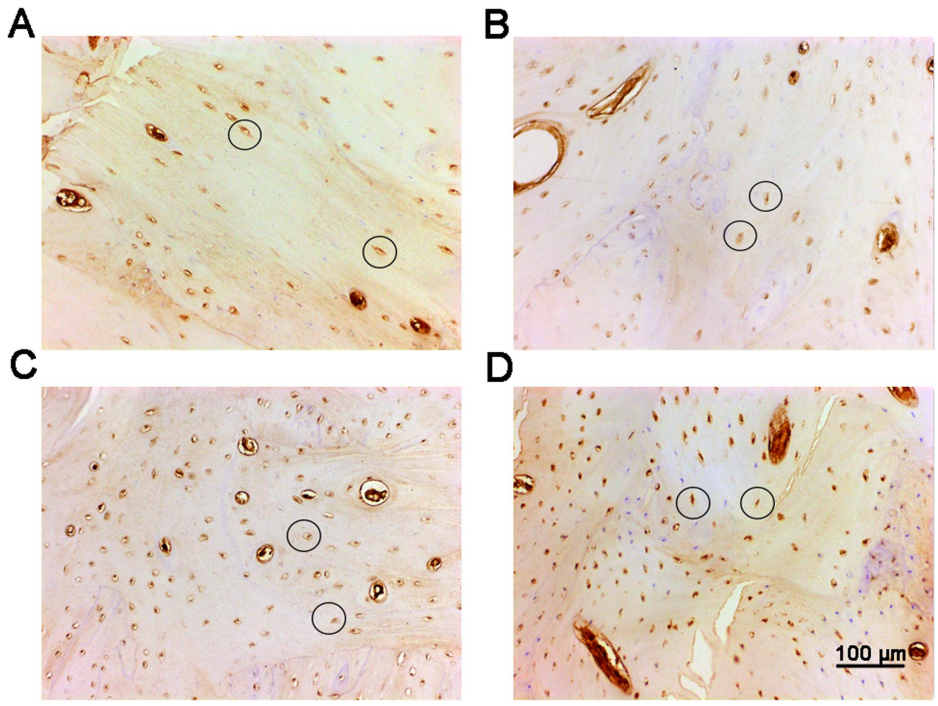

Figure 8. Immunostaining (OCN) of class II furcation defect at 30 d (A), (B) and 60 d (C), (D); post surgery of control group (A), (C) and the Col/BG/CS membrane group (B), (D). The control group was not covered with a membrane. Osteocytes are positive for OCN (brown stain).

electrospun nanofibers, and the function of BG and chitosan. First, electrospun nanofibers have the advantage of a small pore size, which can effectively protect the wound from bacterial infection [30]. Second, the antibacterial action of BG is affected by its chemical composition and the dissolution conditions in its surroundings [31]. Since the Col/BG/CS membrane can release a certain amount of calcium, phosphorus and silicon ions to the culture medium, the released ions may affect the osmotic pressure of the environment and inhibit bacteria growth [32–34]. Third, the antibacteria mechanism of chitosan is speculated to involve the interaction between chitosan and the outer membrane of microorganisms [35]. Abdel-Rahman *et al* report that chitosan-containing nonwoven cotton fabric possesses high antibacterial activity against *Escherichia coli* and *S. aureus*, which may be related to the chemical interaction between positively charged chitosan and negatively charged microbial cell membranes [36].

Finally, in order to demonstrate the effect of the Col/BG/CS membrane as a GTR/GBR membrane *in vivo*, a dog class II furcation defect model was designed to investigate the capacity of the Col/BG/CS membrane in inducing periodontal tissue regeneration. The experimental model is commonly used in much of the literature, as Chantarawaratit *et al* reported that acemannan significantly accelerates new alveolar bone, cementum and periodontal ligament formation in class II furcation defects [37]. Reis *et al* reported that rigid membranes made of 25% or 35% hydroxyapatite and polyhydroxybutyrate partially improved the regeneration of class II furcation defects in dogs, but an inflammatory infiltrate was also observed in the dense connective tissue [38]. Figures 7 and 8 show that in our study, the percentage of new bone formation in the Col/BG/CS group was higher than that of the control group at 30 and 60 d, and bone matrix protein (OCN) was presented in the newly formed bone. This indicates that the Col/BG/CS membrane can accelerate new bone formation. Although new periodontal ligament and cementum formation were not observed at 30 and 60 d, we still found that gingival connective tissue did not grow into the defect by the application of the Col/BG/CS membrane, and the inflammatory cells were less than that of the control group (figure 7). This indicates that compared to the control group, the Col/BG/CS membrane group not only played a role as an effective mechanical barrier, preventing the gingival connective tissue growing without causing significant inflammatory reaction, but also increased bone formation and mineralization, which was comparable with other reports and provides great potential for clinical application. The possible mechanisms of the Col/BG/CS membrane for inducing periodontal tissue regeneration include the combined effects of collagen, BG and chitosan, as illustrated in figure 9. In this study, the electrospun Col/BG/CS membrane shows the potential to be used as a GTR/GBR membrane for inducing periodontal tissue regeneration. It also has the possibility of being used in other biomedical fields, such as wound dressings, vascular scaffolds and so on.

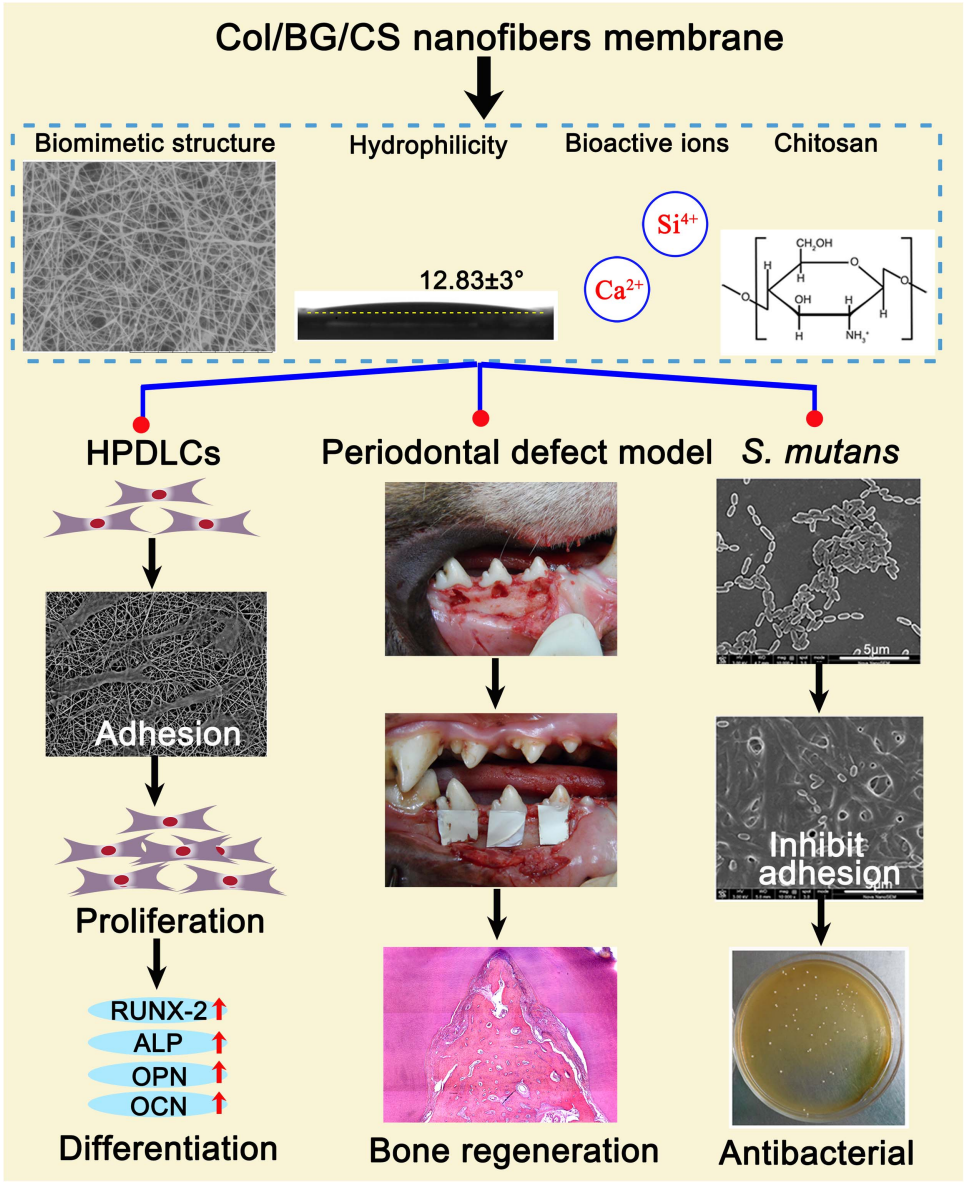

Figure 9. A schematic graph of the Col/BG/CS nanofiber membrane in inducing periodontal tissue regeneration.

5. Conclusion

In the present study, a biomimetic fish Col/BG/CS nanofiber membrane was successfully developed. The composite membrane showed a certain degree of antibacterial activity against *S. mutans* and was able to promote adhesion, viability and osteogenic differentiation of HPDLCs. Animal experiments further showed that the Col/BG/CS membrane was able to promote bone regeneration in the furcation defect of dogs. These biological effects were presumably associated with the biomimetic structure, hydrophilicity and composition of the Col/BG/CS membrane. In this study, a multifunctional biomimetic Col/BG/CS composite membrane was developed that has the ability to induce periodontal tissue regeneration and a certain degree of antimicrobial activity, providing the potential for clinical application as a GTR/GBR membrane.

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References

[1] Kasaj A, Reichert C, Gotz H, Rohrig B, Smeets R and Willershausen B 2008 *In vitro* evaluation of various bioabsorbable and nonresorbable barrier membranes for guided tissue regeneration *Head Face Med.* **4** 22

[2] Zhang E *et al* 2016 Electrospun PDLLA/PLGA composite membranes for potential application in guided tissue regeneration *Mater. Sci. Eng.* **58** 278–85

[3] Behring J, Junker R, Walboomers X F, Chessnut B and Jansen J A 2008 Toward guided tissue and bone regeneration: morphology, attachment, proliferation, and migration of cells

cultured on collagen barrier membranes. A systematic review *Odontology/Soc. Nippon Dental Univ.* **96** 1–11
[4] Bottino M C and Thomas V 2015 Membranes for periodontal regeneration—a materials perspective *Front. Oral Biol.* **17** 90–100
[5] Carruthers J and Carruthers A 2002 Mad cows, prions, and wrinkles *Arch. Dermatol.* **138** 667–70
[6] Zhou T *et al* 2016 Electrospun tilapia collagen nanofibers accelerating wound healing via inducing keratinocytes proliferation and differentiation *Colloids Surf.* **143** 415–22
[7] Nagai N *et al* 2007 *In vitro* growth and differentiated activities of human periodontal ligament fibroblasts cultured on salmon collagen gel *J. Biomed. Mater. Res.* A **82** 395–402
[8] Liu C and Sun J 2015 Hydrolyzed tilapia fish collagen induces osteogenic differentiation of human periodontal ligament cells *Biomed. Mater.* **10** 065020
[9] Aurégan J-C and Bégué T 2015 Bioactive glass for long bone infection: a systematic review *Injury* **46** S3–7
[10] Hu S, Chang J, Liu M and Ning C 2009 Study on antibacterial effect of 45S5 bioglass *J. Mater. Sci., Mater. Med.* **20** 281–6
[11] Lepparanta O *et al* 2008 Antibacterial effect of bioactive glasses on clinically important anaerobic bacteria *in vitro J. Mater. Sci., Mater. Med.* **19** 547–51
[12] Day R M 2005 Bioactive glass stimulates the secretion of angiogenic growth factors and angiogenesis *in vitro Tissue Eng.* **11** 768–77
[13] Hench L L and Jones J R R 2015 Bioactive glasses: frontiers and challenges *Front. Bioeng. Biotechnol.* **3** 194
[14] Ji Q X, Zhong de Y, Lu R, Zhang W Q, Deng J and Chen X G 2009 *In vitro* evaluation of the biomedical properties of chitosan and quaternized chitosan for dental applications *Carbohydr. Res.* **344** 1297–302
[15] Bottino M C *et al* 2012 Recent advances in the development of GTR/GBR membranes for periodontal regeneration—a materials perspective *Dent. Mater.* **28** 703–21
[16] Babitha S, Rachita L, Karthikeyan K, Shoba E, Janani I, Poornima B and Purna Sai K 2017 Electrospun protein nanofibers in healthcare: a review *Int. J. Pharm.* **523** 52–90
[17] Yu Y, Jin G, Xue Y, Wang D, Liu X and Sun J 2017 Multifunctions of dual Zn/Mg ion co-implanted titanium on osteogenesis, angiogenesis and bacteria inhibition for dental implants *Acta Biomaterialia.* **49** 590–603
[18] Liu D, Nikoo M, Boran G, Zhou P and Regenstein J M 2015 Collagen and gelatin *Annu. Rev. Food Sci. Technol.* **6** 527–57
[19] Zhang Y, Liu W, Li G, Shi B, Miao Y and Wu X 2007 Isolation and partial characterization of pepsin-soluble collagen from the skin of grass carp (*Ctenopharyngodon idella*) *Food Chem.* **103** 906–12
[20] Milella E, Ramires P, Brescia E, La Sala G, Di Paola L and Bruno V 2001 Physicochemical, mechanical, and biological properties of commercial membranes for GTR *J. Biomed. Mater. Res.* **58** 427–35
[21] Coïc M, Placet V, Jacquet E and Meyer C 2010 Mechanical properties of collagen membranes used in guided bone regeneration: a comparative study of three models *Rev. Stomatol. Chir. Maxillofac.* **111** 286–90
[22] Song E, Yeon Kim S, Chun T, Byun H J and Lee Y M 2006 Collagen scaffolds derived from a marine source and their biocompatibility *Biomaterials* **27** 2951–61
[23] Kim H W, Kim H E and Knowles J C 2006 Production and potential of bioactive glass nanofibers as a next-generation biomaterial *Adv. Funct. Mater.* **16** 1529–35
[24] Deliormanli A M 2015 Preparation, *in vitro* mineralization and osteoblast cell response of electrospun 13–93 bioactive glass nanofibers *Mater. Sci. Eng.* **53** 262–71
[25] Deliormanlı A M 2015 Preparation and *in vitro* characterization of electrospun 45S5 bioactive glass nanofibers *Ceram. Int.* **41** 417–25
[26] Wei J *et al* 2009 Influence of surface wettability on competitive protein adsorption and initial attachment of osteoblasts *Biomed. Mater.* **4** 045002
[27] Vahabzadeh S, Roy M and Bose S 2015 Effects of silicon on osteoclast cell mediated degradation, *in vivo* osteogenesis and vasculogenesis of brushite cement *J. Mater. Chem.* **3** 8973–82
[28] Obata A and Kasuga T 2009 Stimulation of human mesenchymal stem cells and osteoblasts activities *in vitro* on silicon-releasable scaffolds *J. Biomed. Mater. Res.* A **91A** 11–7
[29] Tsai S W, Hsu F Y and Chen P L 2008 Beads of collagen-nanohydroxyapatite composites prepared by a biomimetic process and the effects of their surface texture on cellular behavior in MG63 osteoblast-like cells *Acta. Biomaterialia.* **4** 1332–41
[30] Zahedi P, Rezaeian I, Ranaei-Siadat S O, Jafari S H and Supaphol P 2010 A review on wound dressings with an emphasis on electrospun nanofibrous polymeric bandages *Polymer. Adv. Tech.* **21** 77–95
[31] Mortazavi V, Nahrkhalaji M M, Fathi M H, Mousavi S B and Esfahani B N 2010 Antibacterial effects of sol-gel-derived bioactive glass nanoparticle on aerobic bacteria *J. Biomed. Mater. Res.* A **94** 160–8
[32] Drago L *et al* 2014 *In vitro* antibiofilm activity of bioactive glass S53P4 *Future Microbiol.* **9** 593–601
[33] Coraca-Huber D C, Fille M, Hausdorfer J, Putzer D and Nogler M 2014 Efficacy of antibacterial bioactive glass S53P4 against *S.aureus* biofilms grown on titanium discs *in vitro J. Orthop. Res.* **32** 175–7
[34] Andersson O H, Rosenqvist J and Karlsson K H 1993 Dissolution, leaching, and Al_2O_3 enrichment at the surface of bioactive glasses studied by solution analysis *J. Biomed. Mater. Res.* **27** 941–8
[35] Xu C, Lei C, Meng L, Wang C and Song Y 2012 Chitosan as a barrier membrane material in periodontal tissue regeneration *J. Biomed. Mater. Res. B* **100** 1435–43
[36] Abdel-Rahman R M *et al* 2016 Wound dressing based on chitosan/hyaluronan/nonwoven fabrics: Preparation, characterization and medical applications *Int. J. Biol. Macromolec.* **89** 725–36
[37] Chantarawaratit P, Sangvanich P, Banlunara W, Soontornvipart K and Thunyakitpisal P 2014 Acemannan sponges stimulate alveolar bone, cementum and periodontal ligament regeneration in a canine class II furcation defect model *J. Periodontal. Res.* **49** 164–78
[38] Reis E C *et al* 2013 Guided tissue regeneration using rigid absorbable membranes in the dog model of chronic furcation defect *Acta Odontol. Scand.* **71** 372–80