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# Reinforced chitosan membranes by microspheres for guided bone regeneration

Di Huang[a,b,1], Lulu Niu[a,c,1], Jian Li[a], Jingjing Du[a,*], Yan Wei[a], Yinchun Hu[a], Xiaojie Lian[a], Weiyi Chen[b], Kaiqun Wang[a,*]

[a] Department of Biomedical Engineering, Research Center for Nano-biomaterials & Regenerative Medicine, College of Mechanics, Taiyuan University of Technology, Taiyuan 030024, PR China

[b] Institute of Applied Mechanics & Biomedical Engineering, Shanxi Key Laboratory of Material Strength & Structural Impact, Taiyuan University of Technology, Taiyuan 030024, PR China

[c] Research Center for Nano-Biomaterials, Analytical & Testing Center, Sichuan University, Chengdu 610064, PR China

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ABSTRACT

In order to improve the osteogenic activity and mechanical strength of the guided bone regeneration (GBR) membrane for repairing bone defect, nano-hydroxyapatite/chitosan (nHA/CS) composite microspheres were prepared through *in situ* biomimetic method, then composite microspheres were incorporated into CS membrane. The morphologies and mechanical properties of the composite membranes were investigated through scanning electronic microscopy (SEM) and universal mechanical testing machine. The results show that the *in situ* biomimetic nHA/CS microspheres were embedded in CS membrane and were integrated tightly with CS matrix. The mechanical properties of GBR membranes containing *in situ* nHA/CS microspheres is significantly higher than that of membranes containing pure CS microspheres and blending nHA/CS microspheres. Its elongation rate at break reaches 5.61 ± 0.95%. The elastic modulus and strength of the GBR membranes can reach 766.27 ± 20.68 and 43.32 ± 0.95 MPa, respectively. Further, The work-of-fracture of the membranes with *in situ* microspheres approaches 2.71 ± 0.25 $J/m^2$, which is about 3 times of the pure CS membrane. The cell culture results display that the GBR membranes containing *in situ* biomimetic nHA/CS microspheres exhibit good cytocompatibility.

## 1. Introduction

The regeneration and repair of bone defect has become an important subject in the research of regenerative medicine at the present time (Vladescu et al., 2016). A large number of studies show that guided bone regeneration (GBR) membrane technology is an effective method to repair bone defects (Kharaziha et al., 2013; Xue et al., 2014; Al-Kattan et al., 2017). GBR membrane can give full play of space maintenance and bone guidance in the defect area. Biodegradable polymer materials (such as chitosan (CS), collagen, poly (lactic acid) (PLA)) have good biological effects and has become the main direction of GBR membrane research (Xue et al., 2014; Norowski et al., 2015; Ma et al., 2016; Hengjie et al., 2017).

In the field of existing biodegradable GBR membrane materials, CS have attracted wide attention because of its good biocompatibility, controllable degradation rate and suitable structures (Ma et al., 2014, 2016; Norowski et al., 2015). Numerous studies have found that CS has excellent capacity to form microspheres, membranes and fibers, which make it significantly better than the other absorbable membrane materials for designing packaging structures (Sivakumar et al., 2002; Yang et al., 2009; Hengjie et al., 2017). Furthermore, CS is readily soluble in various acidic solvents and exhibits a positive charge, while it has similar structure to glycosaminoglycans, which provides the suitable environment for the cells to be able to effectively accomplish their biological functions such as inducing bone tissue regeneration and promoting drug absorption (Huang et al., 2011, 2012).

However, the single CS membrane has poor mechanical properties and is insufficient to effectively support bone regeneration (Pourhaghgouy et al., 2016). CS membrane could not fully play the role of space maintaining and bone guiding in the bone defect area, thus limiting its application in bone regeneration (Mohammadi et al., 2016; Tamburaci and Tihminlioglu, 2017). It is viable to load hydroxyapatite-based microspheres in CS membranes to improve the mechanical and bone conductive properties of GBR membranes. Studies have shown

* Corresponding authors.
*E-mail addresses:* dujingjing198539@163.com (J. Du), wangkaiqun@tyut.edu.cn (K. Wang).
[1] These authors contribute equally to this work.

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that nano-hydroxyapatite/chitosan (nHA/CS) composite microspheres has excellent biological activity and osteoconduction (Sivakumar et al., 2002; Inanç et al., 2007; Li et al., 2014), which make it possible to enhance CS membrane and improve the bioactivity of the composite conducts to meet the clinical applications.

According to the conducted studies, so far no work has been done on incorporating *in situ* nHA/CS composite microspheres into CS GBR membranes. In the current research, CS microspheres and both nHA/CS composite microspheres prepared by *in situ* biomimetic and blending method have been incorporated into CS membranes. The mechanical properties of GBR membranes are expected to be reinforced by loading *in situ* nHA/CS composite microspheres. Meanwhile, it is prospected that the cytocompatibility of GBR membranes could be improved by incorporation of *in situ* nHA/CS composite microspheres. Such a new type of GBR composite membrane with better biological function and mechanical properties will provide a good prospect for further research and development in GBR membrane.

## 2. Materials and methods

### 2.1. Materials

Chitosan (CS) with a molecular weight of about 300,000 and a degree of N-deacetylation of 95% was purchased from Haidebei Biotechnology Co., Ltd. (Jinan, China). Ice-acetic acid was purchased from Qibang Chemical Co., Ltd. (Tianjin, China). $Ca(NO_3)_2{\cdot}4H_2O$, $Na_3PO_4{\cdot}12H_2O$, span 80, liquid paraffin, glutaraldehyde (50% aqueous solution, biological grade), petroleum ether (boiling point 30–60 °C), Isopropyl alcohol and NaOH were purchased from Guangfu Chemical Co., Ltd. (Tianjin, China). All reagents were of analytical grade.

### 2.2. Preparation of CS microspheres

0.428 g CS powder was dissolved in 2 wt% ice-acetic acid to obtain a 4 wt% CS solution. 124.742 g liquid paraffin and 3.858 g span80 were added into a 250 mL conical flask and the mixture was stirred mechanically for 0.5 h to form an oil phase. Then CS solution was slowly added into the oil phase with constant stirring and the mixture was kept stirring at 37 °C for 3 h to obtain a homogeneous W/O emulsion. Then, 0.5 mL 50% glutaraldehyde aqueous solution was gradually incorporated to the emulsion and kept stirring for a further 3 h for cross-linking process. Finally, the products were separated by precipitation and washed consecutively with petroleum ether and isopropyl alcohol. CS microspheres were obtained after dried in an oven for 12 h at 50 °C.

### 2.3. Preparation of nHA/CS composite microspheres through blending method

nHA slurry was prepared through wet chemical precipitation as reference described (Huang et al., 2014a, 2014b). nHA slurry and 4 wt % CS ice-acetic solution were mixed together to prepare nHA/CS composite solution. 124.742 g Liquid paraffin and 3.858 g span80 were added into a flask and stirred mechanically for 0.5 h. Then nHA/CS composite solution was slowly added into the oil phase with constant stirring and the mixture was kept stirring at 37 °C for 3 h. Then, 0.5 mL 50% glutaraldehyde aqueous solution was gradually incorporated to the emulsion and kept stirring for a further 12 h at 37 °C for cross-linking process. Finally, the mixture of products was precipitated and the precipitation was washed consecutively with petroleum ether and isopropyl alcohol. The nHA/CS composite microspheres, thus, were obtained after dried in an oven for a 12 h at 50 °C.

### 2.4. Preparation of nHA/CS composite microspheres through in situ biomimetic method

0.428 g CS powder was dissolved in 2 wt% ice-acetic to give a 4 wt% CS solution. 1.007 g $Ca(NO_3)_2{\cdot}4H_2O$ was dissolved in the above polymer solution and stirred 0.5 h to obtain a homogeneous solution. The mixed solution was added slowly to 124.742 g Liquid paraffin containing 3.858 g span80 under constant stirring. The mixture was kept stirring at 37 °C for 3 h to obtain a homogeneous W/O emulsion. Afterwards, 0.5 mL 50% glutaraldehyde aqueous solution was gradually added to the emulsion and the stirring continued for a further 0.5 h at 37 °C for cross-linking process. Then, NaOH solution was added into the solution to adjust pH value to 8. Then, 0.972 g $Na_3PO_4$ solution was added slowly to the W/O emulsion, and kept stirring for 12 h. Finally, the stabilized composite microspheres were washed consecutively with petroleum ether and isopropyl alcohol and then were dried in an oven at 50 °C for 12 h.

### 2.5. Preparation of CS GBR membrane containing microspheres

CS membrane containing microspheres was prepared through a solution casting method. 0.428 g CS power was dissolved in 2 wt% ice-acetic acid to obtain a 4 wt% CS solution. Then, 0.043 g different microspheres were dispersed in the 4 wt% CS solution, and the mixture was stirred gently for 0.5 h. The mixed solution was set aside for about 4 h at room temperature to remove air bubbles, and then the mixture was casted it on the glass plate, dried at room temperature for 24 h until the solvent evaporated, resulting in a film containing microspheres. Afterward, the films were treated with 0.1 mol/L NaOH solution to neutralize the ice-acetic. Then, the samples were peeled off the glass mould and followed by washing repeatedly with deionized water to pH = 7.0. Finally, samples were set aside on filter paper to dry for 48 h at room temperature.

### 2.6. Characterization

#### 2.6.1. Morphologies

The morphologies of pure CS microspheres, nHA/CS composite microspheres through blending method and *in situ* biomimetic method, CS GBR membranes containing different microspheres were observed by scanning electric microscopy (SEM, Tescan MIRA3, Czech; Jeol JSM-7100F, Japan). Prior to examination, each specimen was coated with gold.

#### 2.6.2. Mechanical properties

CS membranes containing different microspheres were tailored into dumbbell specimens of 30 mm in gauge length, 5 mm in width and 0.1 mm in thickness for the measurement of tensile strength. Mechanical properties were performed on a universal mechanical testing machine (500 N, Instron 5544, US) with a cross-head speed of 5 mm/min. The test conditions: the temperature is 24 °C and the relative humidity is 20%. After testing, the fracture surface of the specimens was analysed by SEM.

The ultimate elastic modulus of the composite GBR membranes was calculated according the slope of stress-strain curve. And the tensile strength is the stress of fractures of samples. The elongation rate ($\delta$) was calculated and recorded on the basis of the following formula: $\delta = \Delta l/l \times 100\%$, where $l$ is gauge length (mm), $\Delta l$ is the variation of $l$.

A three-point flexural test with a span of 10 mm was used to fracture the specimens at a cross-head speed of 0.5 mm/min on the universal mechanical testing machine (5 N, Instron 5544, US) (Xu et al., 2000). The specimens were cut into the size of $0.1 \times 6 \times 25\ mm^3$. The work-of-fracture (or the energy per volume) was evaluated from the load-displacement curve:

Work-of-fracture (or the energy per volume): $WOF = A/(bh)$

Where $A$ is the area under the load-displacement curve, which is the work done by the applied load to deflect and fracture the specimen, $b$ is the specimen width and $h$ is the specimen thickness. For all the specimens, the test was stopped at a maximum cross-head displacement of 3 mm for a consistent calculation of the WOF values.