Research Report

Novel development of carbonate apatite-chitosan scaffolds based on lyophilization technique for bone tissue engineering

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ABSTRACT

Background: The natural biopolymer chitosan (Ch) is currently regarded as a candidate for bone tissue engineering. However, Ch is poor for cell adhesion and low bone formation ability. In order to enhance cell adhesion and bone formation ability, combination of Ch with carbonate apatite (CA) was developed. Purpose: The aim of this study was to make carbonate apatite-chitosan scaffolds (CA-ChSs) and evaluate its osteoconductivity in terms of cell proliferation. Methods: Chitosan scaffolds (ChSs) were made by the following procedure. Twenty-five, 50, 100, 200 and 400 mg Ch was dissolved into 5 ml of 2% acetic acid (CH_3COOH), shaked for 15 min and neutralized with 15 ml of 0.1 M sodium hydroxide (NaOH) solution. After centrifugation, Ch gel was packed into the molds then frozen at -80°C for 2h and dried in a freeze dry machine for 24h. The sponges were subjected to UV radiation for 2h. To make CA-ChSs, 200 mg Ch was selected. After neutralization, 50 mg of 0.06 M CA were added into the 200 mg Ch gel. The structure of CA-ChSs was observed by scanning electron microscope (SEM). Mouse osteoblast-like cell (MC3T3-E1) proliferation in these scaffolds was investigated at 1, 7, 14 and 21 days. Results: Three dimensional porous structures of CA-ChSs were clearly observed by SEM. Proliferated cell numbers in CA-ChSs was significantly higher than those in ChSs (control) at each stage (p<0.05). Conclusion: It can be concluded that newly developed CA-ChSs had three-dimensional interconnected porous structure, good handling property and supporting ability of proliferation of osteoblasts. It is suggested that newly developed CA-ChSs could be considered as a scaffolds material for bone tissue enginearing.

Key words: Carbonate apatite, chitosan, scaffolds, bone tissue engineering

ABSTRAK

Latar belakang: Kitosan yang merupakan biopolimer alami dianggap sebagai salah satu kandidat untuk rekayasa jaringan tulang. Namun, kitosan memiliki kelemahan terhadap adhesi sel dan kurang mampu membentuk tulang yang cukup. Untuk meningkatkan adhesi sel dan kemampuan pembentukan tulang, telah dikembangkan suatu scaffolds yang menggabungkan kitosan dengan carbonate apatite (CA). Tujuan: Penelitian ini bertujuan untuk membuat carbonate apatite-chitosan scaffolds (CA-ChSs) serta mengevaluasi osteokonduktivitas CA-ChSs dari sudut pandang proliferasi sel. Metode: Chitosan scaffolds (ChSs) dibuat dengan prosedur berikut ini. Dua puluh lima, 50, 100, 200 dan 400 mg bubuk kitosan dilarutkan dalam 5 ml asam asetat (CH3COOH) 2%, dikocok selama 15 menit dan dinetralkan dengan 15 ml 0,1 M larutan sodium hidroksia (NaOH). Setelah sentrifugasi, gel kitosan dikemas ke dalam cetakan teflon kemudian dibekukan pada suhu -80°C selama 2 jam dan dikeringkan dalam mesin beku kering pada suhu -54°C selama 24 jam. Selanjutnya dilakukan radiasi ultraviolet pada ChSs selama 2 jam. Untuk membuat CA-ChSs, dipilih ChSs yang berisi 200 mg bubuk kitosan. Setelah dinetralisir, 50 mg dari 0.06 M CA ditambahkan ke dalam kitosan gel yang berisi 200 mg bubuk kitosan. Struktur CA-ChSs diamati dengan scanning electron microscope (SEM). Proliferasi mouse osteoblast-like cell (MC3T3-E1) dalam ChSs dan CA-ChSs dievaluasi pada hari ke-1, 7 dan 14. Hasil: CA-ChSs dengan struktur tiga dimensi yang berpori dapat diamati dengan jelas menggunakan SEM. Jumlah pertumbuhan dan perkembangan sel pada CA-ChSs secara signifikan lebih banyak dibandingkan pada ChSs (kontrol) pada setiap tahap pengamatan di hari ke-1, 7, 14 dan 21 (p<0.05). Kesimpulan: Dapat disimpulkan bahwa CA-

ChSs mempunyai struktur tiga dimensi dengan pori-pori yang saling berhubungan satu sama lain dan dapat meningkatkan proliferasi osteoblast. Hal ini menunjukkan bahwa CA-ChSs adalah kandidat untuk rekayasa jaringan tulang.

Kata kunci: *Carbonate apatite, kitosan, scaffolds, rekayasa jaringan tulang*

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INTRODUCTION

In order to reconstruct damaged bone tissue because of trauma or pathologic disease, tissue engineering is needed as an emerging technology.[1] Various kinds of bone substitutes have been developed to replace bone defect. Autogenous bone graft (autograft) is the most effective bone substitute. However, autograft sometimes has significant limitations coming from donor site morbidity, a limited donor bone supply and an inadequate resorption rate during the healing process.[2,3] These limitations have prompted increasing interest in alternative bone substitute. Allograft as an alternative offers the same characteristics as an autograft, but have several problems such as the risk of disease transmission, immunogenicity, loss of biologic and mechanical properties, and religious concerns.[4,5] Consequently, significant efforts are being made to develop an ideal bone substitute.

Ideally synthetic bone substitutes should be biocompatible, show minimal fibrotic reaction, undergo remodeling and support new bone formation.[4] Scaffolds play an essential role in supporting bone substitute. Recently, significant attention is being given to three-dimensional polymer scaffolds for in vitro study of cell-scaffolds interaction and in vivo study of bone substitute.[6-8] In recent years, one promising tissue engineering strategy has been given to natural polymer chitosan (Ch) because of its properties.

Ch, an amino polysaccharide (poly-1,4-D-glucossamine), is the alkaline deacetylated product of chitin that can be extracted from crustacean. It is biocompatible, biodegradable, renewable, non-toxic, can be fabricated into various forms, has been shown to support the attachment and growth of osteoblasts in vitro and has been widely applied in biomedicine.[9-11] Furthermore, as a foreign object, Ch is not rejected from the body.[9] However, Ch is relatively weak and unstable and swells in solution. Pure Ch scaffolds (ChSs) is easily absorbed and difficult to control absorbance time period.

To make suitable ChSs for bone tissue engineering, current attempts are focused on improving the mechanical strength and biological properties through the incorporation of bio ceramics, such as carbonate apatite (CA). In the field of hard tissue repair and regeneration, CA has been used as a biocompatible and osteoconductive material because of its similarity to inorganic component of hard tissue.[12] It has been reported that the main inorganic content of bone is CA, which contains about 7% carbonate by weight. CA is easier to dissolve because the solubility increased as the content of carbonate in CA increased, and thermodynamically under neutral and basic condition. Therefore CA is expected to become an ideal bone replacement material, which possesses both osseoconductivity and bioresorbability.[13,14]

In this study, CA was chose to combine with Ch as a biodegradable material in order to overcome their mechanical limitation and maximize the beneficial properties of each and create a biodegradable scaffolds.[15] Therefore, the purpose of this study was to make carbonate apatite-chitosan scaffolds (CA-ChSs) based on lyophilization technique and evaluate their microstructure and cell proliferation-conductivity for bone tissue engineering.

MATERIALS AND METHODS

Cylinder ChSs were made by using various amounts of Ch powder provided by YSK (98.7% deacetylation, Yaizu Suisankagaku Industry Co., Ltd., Japan). The procedures were based on previous study.[16] Acetic acid (CH_3COOH) was selected as the solvent for Ch powder and sodium hydroxide (NaOH) was used for neutralization. ChSs were fabricated by the following procedure. First, 25, 50, 100, 200 and 400 mg of Ch powder was dissolved in 5 ml of CH_3COOH at room temperature, shaken for 15 min, neutralized with 5 ml of NaOH solution, and then centrifuged at 1500 rpm for 10 min to prepare Ch gels. The Ch gels were obtained after the removal of excess water and were packed into cylindrical molds (diameter: 5 mm, height: 2 mm). The molds were frozen at -80°C for 2h and transferred into a freeze-drying machine (FD 700D, Tokyo Rikakikai Co., LTD, Tokyo, Japan). Cylindrical ChSs were obtained after drying at -54°C for 24hr and exposure to UV radiation for 2hr.

The production procedures of CA-ChSs were based on previous study.[16] Firstly, 200 mg Ch powder was dissolved in 5 ml CH_3COOH at room temperature, shaken for 15 min and neutralized with 15 ml NaOH solution to obtain Ch gels. 50 mg of CA was then homogenously mixed with Ch gels and centrifuged at 1500 rpm for 10 min to prepare CA-Ch gels. After the removal of excess water, CA-Ch gels were transferred into cylindrical molds (diameter: 5 mm, height: 2 mm), frozen at -80°C for 2hr and transferred into the freeze-drying machine. Cylindrical CA-ChSs were obtained after drying at -54°C for 24hr and exposure to UV radiation for 2hr.
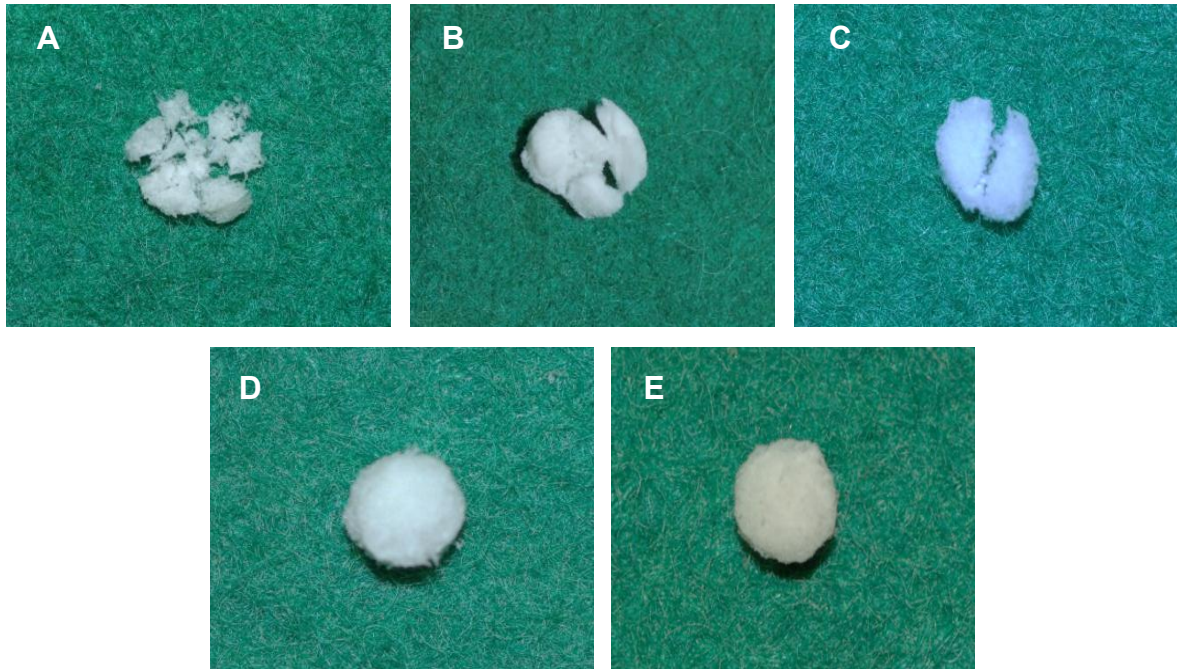
Figure 1. Macroscopical shape of ChSs prepared with different amount of Ch powders (Ø: 5.0 mm). Ch powder used (mg) A: 25; B: 50; C: 100; D: 200; E: 400. A, B and C were brittle. On the other hand, D and E showed good handling property.
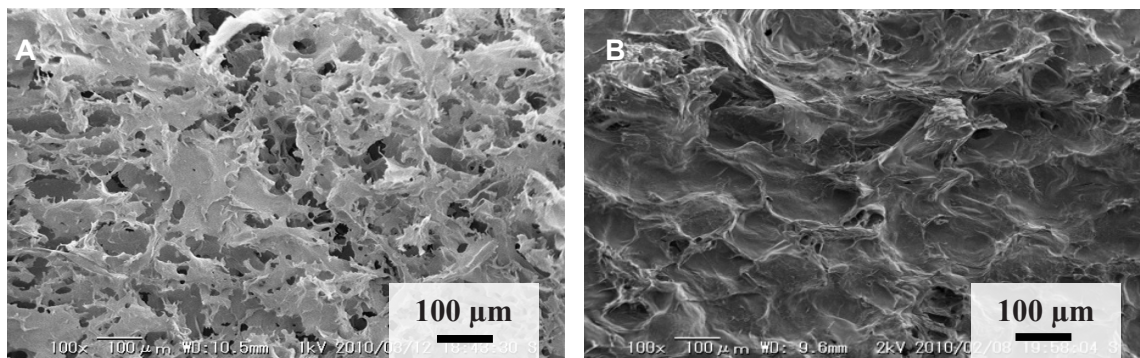

Figure 2. SEM Images of ChSs prepared with different amount of Ch powders. Ch powder used (mg) A: 200; B: 400. (A) shows three-dimensional structure with many pores. On the other hand, (B) shows three-dimensional structure with few pores.

Macroscopically images of ChSs and CA-ChSs were obtained by a digital camera (60D, Canon, Japan). The microscopic structure and porosity of the ChSs and CA-ChSs were analyzed using a scanning electron microscope (SEM) (3D Microscope VE-8800, Keyence, Japan).

MC3T3-E1 cells were cultivated on a tissue culture polystyrene dish using DMEM supplemented with 10% fetal bovine serum (FBS), 100 U penicillin, 0.1 mg/ml streptomycin and 2 mM L-glutamine. The cells were sub cultured every 7 days for two times.

MC3T3-E1 cells were harvested by trypsinization and suspended in DMEM containing 10% FBS, 100 U penicillin, 0.1 mg/ml streptomycin and 2 mM L-glutamine. Then, cell suspensions (20 µl) were added to ChSs and CA-ChSs placed in each well of a 24-well tissue culture plate to the density of 2×10^4 cells. After 2hr, 980 µl of the medium was added to each well. The cells were incubated in an incubator under 5% CO_2 at 37°C for 1, 7, 14 and 21 days. The culture medium was changed every 3 days. The number of living cells was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assays using Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). ChSs and CA-ChSs were washed with PBS and incubated for 2h in 500 µl of culture medium containing 50 µl MTT reagents in an incubator under 5% CO_2 at 37 °C. The supernatant medium (110 µl) was transferred into a 96-well culture plate and the absorbance was measured using a micro plate reader (550, Biorad) at 450 nm wavelength.

Data of cell proliferation were statistically analyzed using SPSS® software (version 12.0). To evaluate the differences in cell proliferation among experimental groups,

one-way analysis of variance (ANOVA) with Tukey's post hoc test ($p<0.05$) was performed and data are presented as the mean ± standard deviation.

RESULTS

Macroscopic images of ChSs are shown in Figure 1. ChSs prepared with 25, 50 or 100 mg Ch powders were brittle and could not maintain their own shapes (Figure 1A, B, C). In contrast, ChSs prepared with 200 and 400 mg Ch powders could maintain their shapes with good handling properties (Figure 1D, E). As shown by SEM images (Figure 2A), ChSs prepared with 200 mg Ch powder showed three-dimensional structure with many pores. On the other hand, ChSs prepared with 400 mg Ch powder exhibited three-dimensional structure with few pores (Figure 2B).

This result suggests ChSs prepared with 200 mg Ch powder was a possible candidate. However, pure ChSs was easily absorbed and difficult to control absorbance time period. As shown in Fig. 3 we could observe three-dimensional structure with many pores both of ChSs and CA-ChSs. The pore size of the scaffolds was approximately 60 μm. In general, CA-ChSs prepared with 50 mg CA showed three-dimensional structure and attachment of CA powder (Figure 3B).

The results of cell proliferation assay on the ChSs and CA-ChSs are shown in Figure 4. The absorbance values for CA-ChSs containing 50 mg of CA were significantly higher than those of ChSs at all-time points (day 1: ChSs: 0.364 ± 0.02, CA-ChSs: 0.378 ± 0.04; day 7: ChSs: 0.548 ± 0.06, CA-ChSs: 0.594 ± 0.07; day 14: ChSs: 0.503 ± 0.05, CA-ChSs: 0.631 ± 0.08; day 21: ChSs: 0.720 ± 0.09, CA-ChSs: 0.814 ± 1.01) ($p < 0.05$).
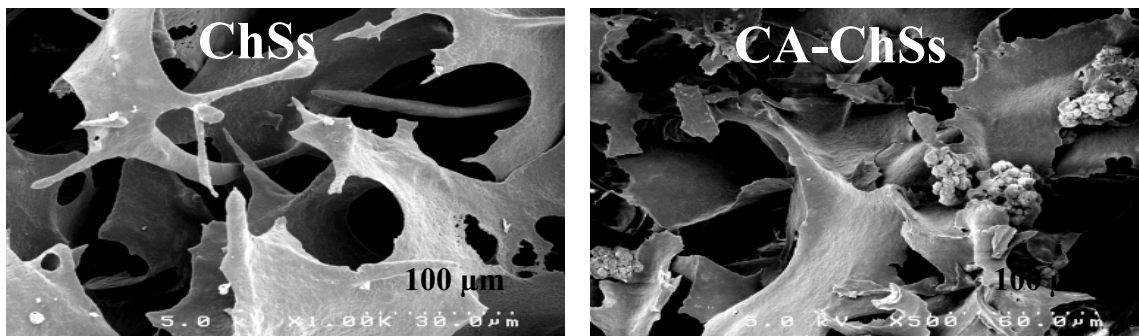

Figure 3. SEM Images of ChSs prepared with 200 mg Ch powder and CA-ChSs prepared with 50 mg CA powder.
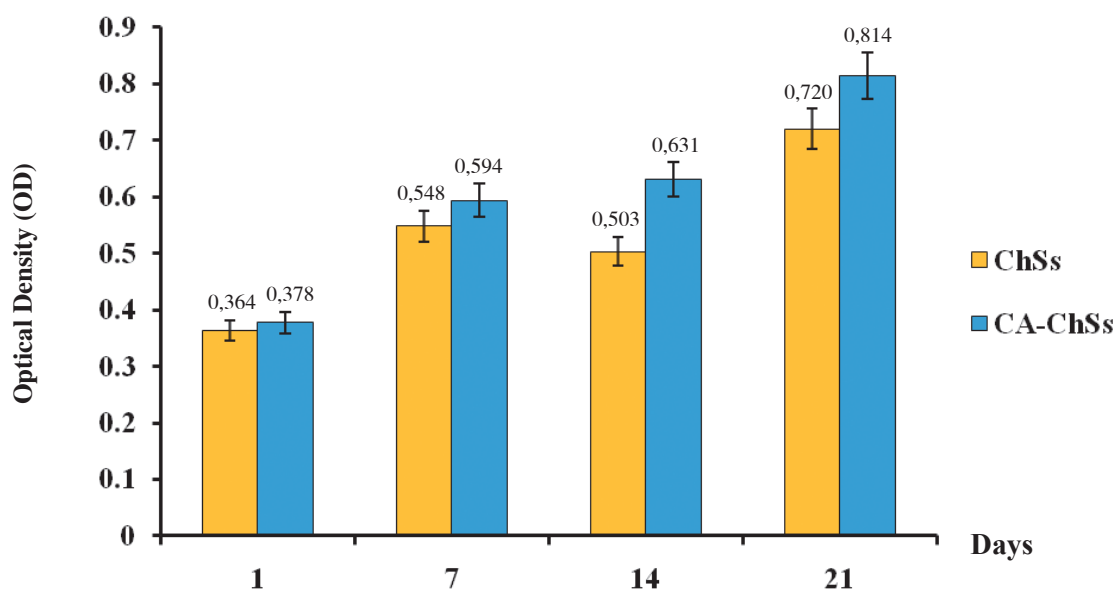

Figure 4. Cell proliferation on ChSs and CA-ChSs, analyzed by the MTT assay. CA-ChSs prepared with 50 mg CA powder showed an increasing tendency in the absorbance values on the first day of observation until day 21 ($p<0.05$) (n=6).

DISCUSSION

In bone tissue engineering, scaffolds play an important role in supporting bone regeneration. To date, many attempts have been made to develop three-dimensional scaffolds that provide necessary support as artificial extracellular matrices, allowing cells to proliferate, differentiate and maintain their functions.[1,10] Prerequisites of scaffolds include non-toxicity, controllable biodegradability, suitable microstructure and appropriate mechanical properties.[1,2,8] Additionally, they must be capable of promoting cell adhesion and retaining their functions.[5]

In the present study, combination of Ch with CA was developed. Firstly, to decide optimal concentration of Ch powder. ChSs with several amounts of Ch powder were evaluated by SEM in terms of porosity. Based on SEM images, ChSs prepared with 200 mg Ch powder had a good handling property and three-dimensional porous structure with pore size was approximately 50–200 μm. These findings may mean space-making ability of this ChSs. It was suggested that an optimal content of Ch powder was 200 mg.

Current attempts are focused on improving the osteoconductivity and bone formation ability of ChSs through the incorporation of calcium phosphate, such as hydroxyapatite (HA), β-tricalcium phosphate (β-TCP) and carbonate apatite (CA).[11,16] HA and β-TCP have drawbacks such as their degradation or dissolution rates are difficult to be predicted. Highly sintered HA is non-degradable. However, it has been reported that interconnected porous calcium hydroxyapatite (IP/CHA) composites had a systematic arrangement of uniform pores and almost all pores were interconnected and it had a good bone formation ability in vivo study. On the other hand, crystallinity of β-TCP is too low, so it degrades too fast. In some cases, this material absorbs before obtaining enough new bone formation. Among these calcium phosphates, CA has been reported to have a proper absorbance time and good bone formation ability. However, CA also has limitations for use as a scaffold because of difficulties in shaping and designing for bone tissue engineering.[12-14,17,18] In this study, Ch as a biodegradable material and CA was chose to combine with Ch in order to get a proper absorbance time period and enhance bone formation ability.

The characteristics of scaffolds depend on the fabrication method. Several techniques have been developed to fabricate scaffolds, including solvent-casting technique, sol-gel technique and lyophilization technique.[19,20] However, it has been reported that in the solvent-casting technique, the existence of a pyrogen salt harmfully affects cells because of the loss of water-soluble biomolecules and the induction of non-uniform deformation.[21,22] In the sol-gel technique, difficulties have been reported for the control of the precise pore structure and the separate completion of hydrolysis and condensation.[19,21] In this study, a lyophilization technique was adopted to fabricate scaffolds, because this technique is simpler and easier than other methods previously described and by using lyophilization technique, porous properties of CA-ChSs were provided by ice removal and varied freezing rate.[16]

Nowdays, one promising tissue engineering strategy is to use three-dimensional porous structure and biodegradable scaffolds to facilitate bone tissue regeneration. In this study, CA-ChSs were fabricated with favorable three-dimensional porous structures and had a good handling property with approximately 50–200 μm pore sizes. As scaffolds for tissue engineering should have porous structures with pore size ranging from 40 to 300 μm to allow tissue ingrowth and migration of vascular tissues.[23,24] These three-dimensional materials are suitable for cell and vascularization including growth factors.

When considering to use CA-ChSs for clinical applications, such as bone augmentation at buccal defect of implant treatment or to fill bone socket after tooth extraction, CA-ChSs must be sterilized prior to use. Regarding sterilization, gamma irradiation, steam autoclaving, ethylene oxide and radio frequency glow discharge plasma sterilization methods have been used. Because, CA-ChSs is an organic polymer composite, it is considered that gamma irradiation is appropriate for CA-ChSs sterilization in order to maintain chemical structure of this material.

After the development of an adequate porous structure, the choice of reliable source of cells has an influence on the success of tissue engineering. In this study, MC3T3-E1 cells were cultured on CA-ChSs for cell proliferation measurement. For bone tissue engineering applications, osteoblasts are commonly used to confirm responsibility for the bone formation.[25,26]

From the findings of cell proliferation assays, it was shown that by combining ChSs with CA powder, higher cell proliferation ability was observed compared with that of only ChSs. It might be suggested that the addition of CA powder into ChSs serves to increase surface area of the materials which is available for cells to adhere. Furthermore, CA-ChSs prepared from 200 mg Ch powder with 50 mg CA powder showed significantly higher cell proliferation ability than those of other groups. It was suggested that it had a favorable three-dimensional porous structure and adequate surface area of the material for bone tissue engineering.

Further histological study in bone regeneration on CA-ChSs should be warranted to ensure the possibility of scaffolds of CA-ChSs. It can be concluded that newly developed CA-ChSs had three-dimensional interconnected porous structure, good handling property and supporting ability of proliferation of osteoblasts. Based on the limited results of this study, it is suggested that newly developed CA-ChSs may be a possible scaffolds material for bone tissue engineering.

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