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The bone-mimicking effect of calcium phosphate on composite chitosan scaffolds in maxillofacial bone tissue engineering

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Abstract

This research explored a new trend in biomaterials science. The bone-mimicking effect of calcium phosphate on chitosan composite scaffolds was evaluated. Chitosan with 2% calcium phosphate was found to have suitable bone-mimicking performance for maxillofacial bone tissue engineering.

Keywords

Maxillofacial tissue engineering, scaffolds, chitosan, calcium phosphate, bone remodeling, mimicking

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Introduction

Many patients suffer from maxillofacial bone tissue defects.[1] Scaffolds have the ability to induce tissue formation.[2] Scaffolds for the maxillofacial area need to fulfill functions that include activating bone formation and preventing infections during bone formation.[3, 4] Activation of bone formation is the main function of scaffolds.[5]

Composite scaffolds are attractive for bone tissue engineering because they have good ability to support new bone formation.[6] This research examined composite scaffolds for bone formation in maxillofacial bone tissue engineering.

Mimicking is often used in the design of scaffolds that show an ability to induce new tissue formation similar to natural tissue.[7, 8] Mimicking was used to design tissue engineering scaffolds in this research.

Bone remodeling is a lifelong process in natural mature bone tissue that normally has two main minerals: hydroxyapatite and tri-calcium phosphate (CP).[9] Hydroxyapatite is an insoluble component that plays a role in bone formation.[10] Tri-CP is a soluble component that is present in the early stages of bone formation.[11]

CP has been studied for ability to mimic extracellular matrices (ECM) as ex vivo biomaterials for heterotopic ossification.[12] Another previous study found that CP can activate bone formation.[13] Hence, a hydroxyapatite-tri-CP compound was used to create scaffolds in this research.

Due to its positive charges, chitosan functions as a substrate for mineralization that is important for bone remodeling.[14, 15] Chitosan is promising as an antimicrobial material in tissue engineering.[16] Chitosan was fabricated into scaffolds that specifically focused on their function as a substrate for mineralization in this research.

In this research, CP and chitosan were selected to create scaffolds based on bone mimicking. The morphology and

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Table 1. The experimental groups.

Group	Details
Chitosan	Chitosan without calcium phosphate
2% CP	Chitosan with 2% of calcium phosphate (W/V)
4% CP	Chitosan with 4% of calcium phosphate (W/V)
6% CP	Chitosan with 6% of calcium phosphate (W/V)
8% CP	Chitosan with 8% of calcium phosphate (W/V)

CP: calcium phosphate.

performance of those scaffolds were evaluated for their use in maxillofacial tissue engineering.

Materials and methods

Chitosan preparation

Shrimp chitosan powder (Marine Bio Resources Co. Ltd) was dissolved in 0.1 M acetic acid at a ratio of 2% chitosan. After dissolving the chitosan, the solution was stirred for 24 h and then filtered.

Construction of CP with chitosan

CP powder at a ratio of hydroxyapatite to tri-CP of 1:1 was added into the chitosan solutions at concentrations of 2%, 4%, 6%, and 8% (w/v) before stirring. The solutions were injected into 1 M NaOH. Each mixture was rinsed with dH_2O three times to create an adjusted pH-neutral solution to form microfibrils before filtering. The microfibrils were centrifuged at 1500 rpm for 10 min. The microfibril scaffolds were cut into a thickness of 2 mm and a diameter of about 10 mm before freeze drying. The experiment was divided into five groups (Table 1).

Morphology analysis

All scaffolds were studied using scanning electron microscopy (SEM), (Quanta400, FEI, Czech Republic). The samples were pre-coated with gold using a gold sputter coater machine (SPI Supplies, division of Structure Probe Inc., Westchester, PA, USA). The SEM magnifications were 100 x, 500 x, and 30,000 x. The pore size of the scaffolds was evaluated using the Image J program with SEM pictures taken randomly from three areas of each scaffold. The pore size distribution ($n = 25$) was calculated using the QtiPlot program.

Swelling properties

The scaffolds were soaked in phosphate-buffered saline (PBS) solution at 37°C, at different times (15, 30, and 60 min). The scaffolds were weighed before and after soaking with PBS to calculate the weight increases following the equation (Ws – Wd)/Wd × 100, where Ws and Wd are the weights of the swollen scaffolds and the dry scaffolds, respectively.[12]

Degradation analysis

The scaffolds in each group were incubated with lysozyme (4 mg/ml of PBS) at 37°C, for different times of 0, 5, 10, 15, and 20 days. The scaffolds from all groups were weighed both before and after soaking to quantify the weight loss and degradation rates.[17]

Cell culturing

MG-63 osteoblast cell lines were cultured on the scaffolds. The cells were maintained in alpha-MEM medium (α-MEM, Gibco™, Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum, 1% penicillin/streptomycin, and 0.1% Fungizone®. In the differentiation stage the cells were induced with osteogenic-supplemented medium (20 mM β-glycerophosphate; 50 μM ascorbic acid; and 100 nM dexamethasone, Sigma-Aldrich).

Cell proliferation, WST-1

Cell proliferation was assessed using a WST-1 Assay Kit (Roche Diagnostics GmbH, Mannheim, Germany). Cell proliferation was performed on days 1, 3, 5, and 7, following the manufacturer's protocol. The scaffolds were washed with PBS and fresh media (850 μl) containing 10% WST-1 reagent. They were incubated for 50 min with the absorbance continually measured at 450 nm using a microplate reader (Biotrak II, UK).

Cell viability

Cell viability was evaluated by fluorescein diacetate staining after days 3 and 5. The scaffolds were removed and the media rinsed with PBS twice. Fresh media was added to the scaffolds, then 5 μl of 5 mg/ml acetone was added and the mixtures incubated at 37°C for 5 min. The scaffolds were then rinsed with PBS and the live cells observed under a confocal microscope.[18]

Alkaline phosphatase activity

Alkaline phosphatase (ALP) activity was measured by Alkaline Phosphatase LiquiColor (Human, Germany) on days 7, 14, and 21. The cells within the scaffolds were lysed with a solution of 1% TritonX in PBS, with three freeze-thaw cycles at −80°C for 1 h and at room temperature for 1 h. The lysed cells in the scaffolds were centrifuged to obtain supernatant solutions for analyses with an ALP kit following the manufacturer's instructions.[19]

Total protein synthesis

The cell lysis solutions were analyzed for protein content on days 7, 14, and 21 following the manufacturer's instructions (Pierce BCA Protein Assay kit, Thermo Scientific, USA). Bovine serum albumin was used to plot the standard curves.

Calcium content

The calcium content was evaluated with a calcium colorimetric assay kit (BioVision). The cell lysis solution was used for calcium detection following the manufacturer's instructions.

Histology analysis

The scaffolds were stained with hematoxylin and eosin (H&E) on days 3 and 5. The cells within the scaffolds were fixed with 4% formaldehyde at 4°C for 24 h after which the scaffolds were placed into paraffin and cut into 5 μm slices. The cut scaffolds were placed on glass slides, stained with H&E, and observed under microscopy.

Statistical analysis

All data are shown as mean ± standard deviation. The samples were measured and statistically compared by one-way analysis of variance and Tukey's honestly significant difference test (SPSS 16.0 software package). A p value < 0.05 was accepted as statistically significant.

Results

Morphology of scaffolds

The chitosan-CP solutions were adjusted with NaOH and examined for fibril bundles (Figure 1(a)) and after rinsing with dH_2O (Figure 1(b)). The chitosan and the 2% CP had loose structures compared to the others (Figures 1(c) and 1(d)). More compact structures were found in 4%, 6%, and 8% CP in scaffolds (Figures 1(e), 1(f), and 1(g)).

Some of the fibril structures appeared as dense clusters (Figure 2(a)) due to the deposition of hydroxyapatite from the CP on the chitosan fibril templates.[20] The pore size analysis showed the 4% CP had the largest pore size of around 437.54 um (Figure 2(p)). The smallest pore size was found in the 6% CP at around 199.27 ± 24 um. Chitosan 2% CP and 8% CP had average pore sizes of 319.75 ± 23, 391.56 ± 22, and 288.45 ± 30 um.

Swelling properties and degradation

The results showed three swelling patterns, high, fair, and poor (Figure 3(a)). Chitosan had high swelling. The 2% CP had fair swelling whereas the 4%, 6%, and 8% CP had poor swelling. The chitosan with CP demonstrated higher degradation than without CP (Figure 3(b)).

Total protein synthesis and cell viability

On day 7, the total protein of the 6% and 8% CP was higher than in the others (Figure 4(a)). The 8% CP had the highest protein synthesis on day 14. The 2% CP showed a cluster of viable cells with an abundance of cells in the 6% and 8% CP (Figure 4(b)).

Cell proliferation and ALP activity

On day 1, the 2% CP showed the highest cell proliferation (Figure 5(a)). On days 3 and 5, the 8% CP had the highest cell proliferation compared to the others. On day 7, the 4% CP had higher cell proliferation than the others. On day 7, the chitosan with CP had higher ALP activity than the chitosan alone (Figure 5(b)).

Calcium content and histology

On day 7, the 8% CP had lower calcium synthesis than the others, which was different than the 2% and 6% CP (Figure 6(a)). On day 3, good cell adhesion and elongation with dense structures were found in the 2%, 4%, 6%, and 8% CP (Figure 6(b)). In the chitosan, the cells formed a globular shape; however, the others formed elongated shapes. On day 5 the scaffolds in all groups showed good cell migration and attachment to the scaffolds.

Discussion

Mimicked biomineralization based on bone remodeling

Chitosan was used as the soft template for biomineralized bone remodeling formations.[21] The mixtures were created with mimicry to ensure fibril formation similar to the ECM of bone.[22] Various CP were deposited on those templates,[23] and the chitosan fibrils with deposition of CP showed similar biomineralization as bone remodeling.[24] An earlier study demonstrated the suitable pore size of bone tissue engineering scaffolds was around 350 to 400 μm.[25] Our scaffolds were within this range and showed pore sizes suitable for bone formation, especially the 2% CP mixture.

Physical performance of the mimicked biomineralized scaffolds

The chitosan with high concentrations of CP mixtures had low swelling. One study found the positive charges of chitosan interacted with the negative charges of the

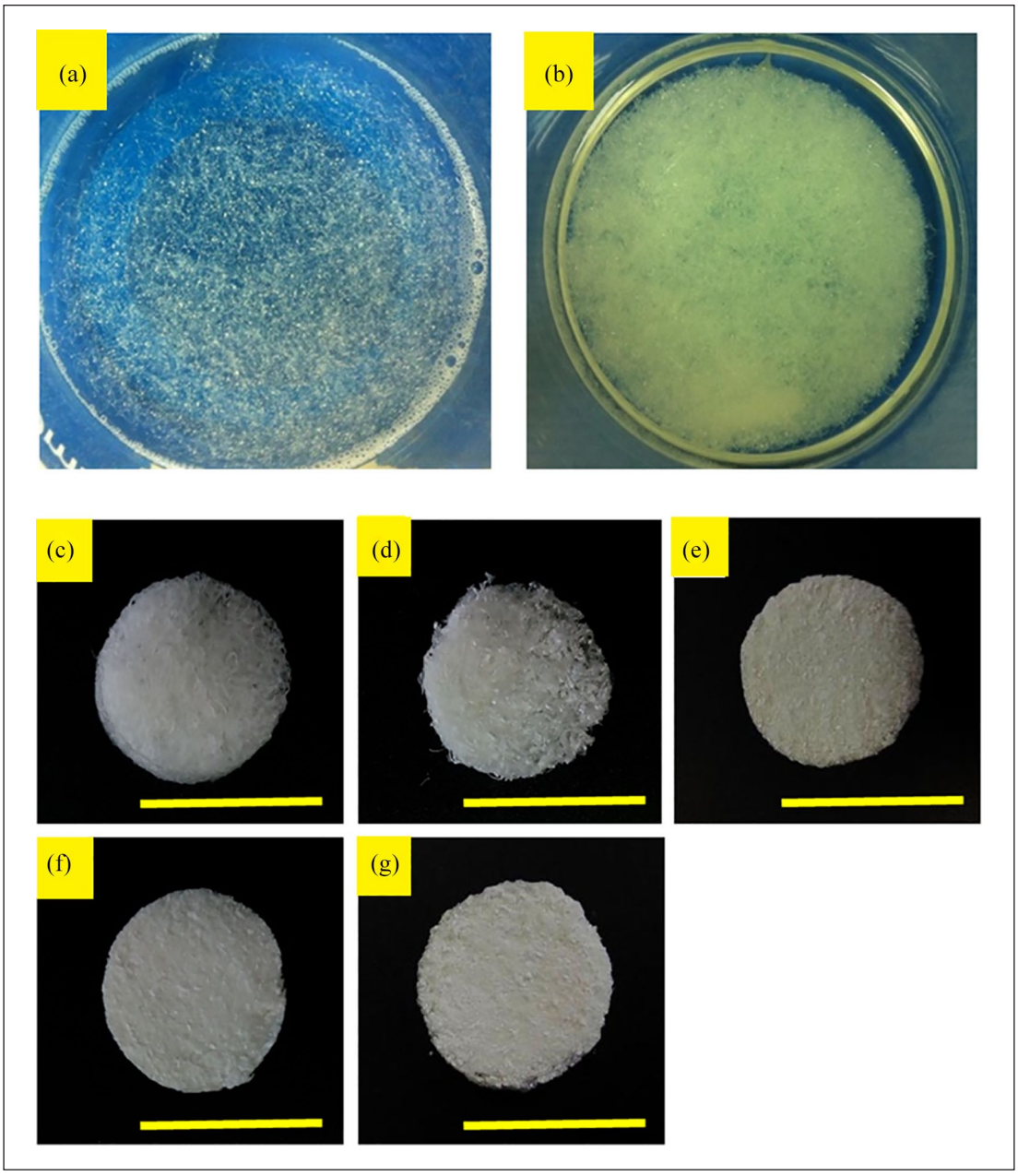

Figure 1. The chitosan/calcium phosphate (CP) solution adjusted with NaOH (a) and after rinsing with dH_2O (b). All groups of scaffolds: (c) chitosan, (d) 2% CP, (e) 4% CP, (f) 6% CP, and (g) 8% CP. Scale bar 10 mm.

filler particles,[26] which in turn reduced the swelling properties. The largest concentration of 8% caused the CP to become dense cluster aggregations, which caused a reduction in degradation.[27] Chitosan composite scaffolds with hydroxyapatite particles in the range of 2% had suitable swelling and degradation for new tissue formation.[28] Our research showed similar results because our 2% CP had an optimal physical performance balance of fair swelling and low degradation, which is a combination suitable for tissue formation.

Biological performance of the mimicked biomineralized scaffolds

CPs are involved in the inducement of cell proliferation that is related to the enhancement of cell adhesion.[29] Chitosan with the various CP mixtures performed strongly in terms of enhancing cell adhesion and proliferation.[30] CP has the ability to enhance biomarkers for ALP activity and calcium content.[31] In our study, the scaffolds of chitosan with 2% CP showed the greatest

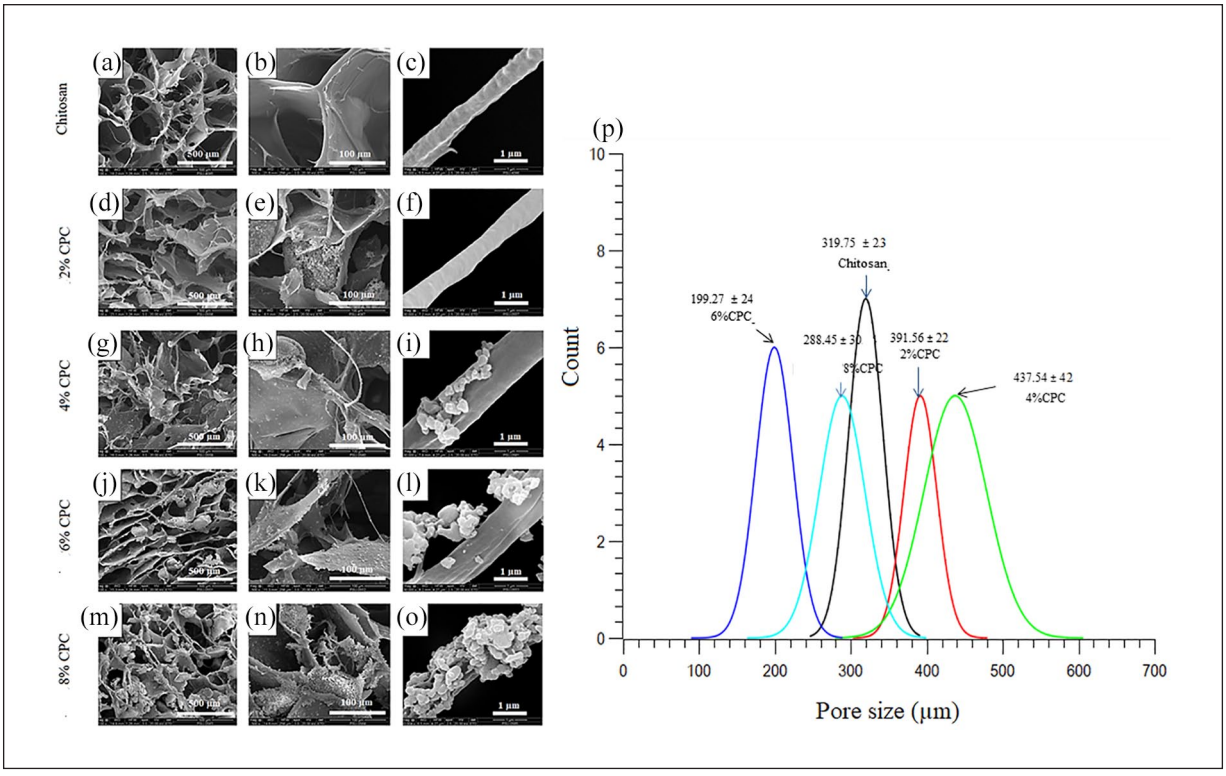

Figure 2. Morphology of scaffolds: (a), (b), (c) chitosan, (d), (e), (f) 2% calcium phosphate (CP), (g), (h), (i) 4% CP, (j), (k), (l) 6% CP, (m), (n), (o) 8% CP, and (p) pore size distribution.
Magnification of (a), (d), (g), (j), (m) images 100 x, (b), (e), (h), (k), (n) images 500 x, and (c), (f), (i), (l), (o) images 30,000 x.

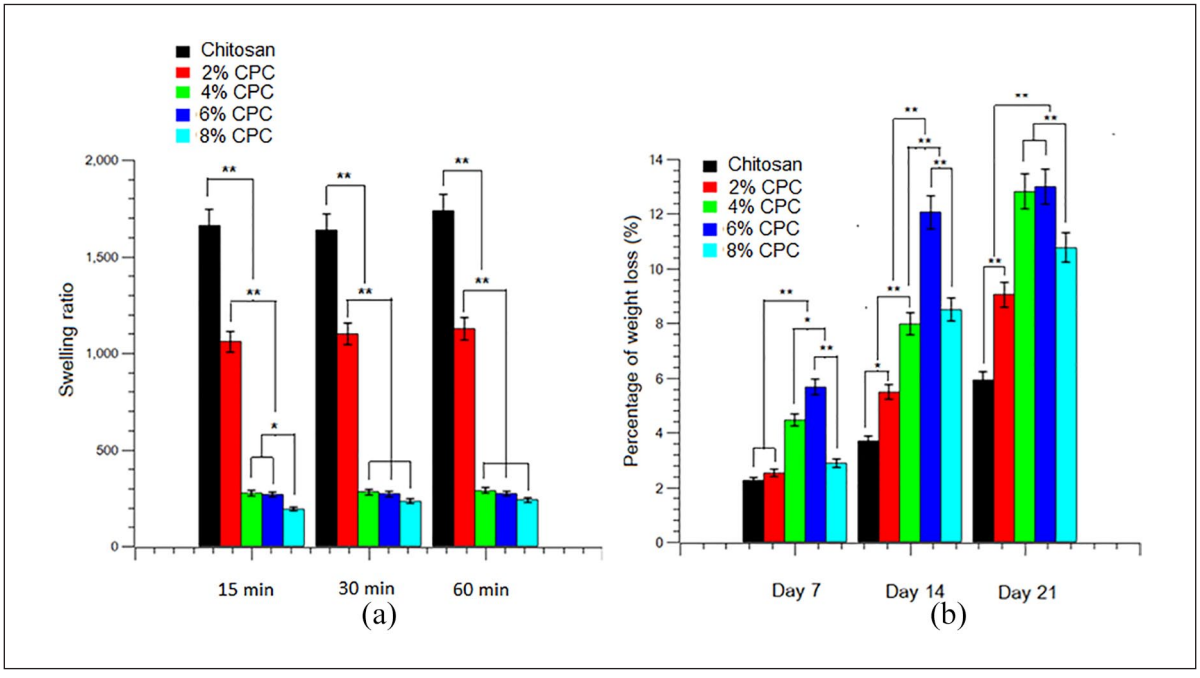

Figure 3. (a) The swelling ratios and (b) degradation.

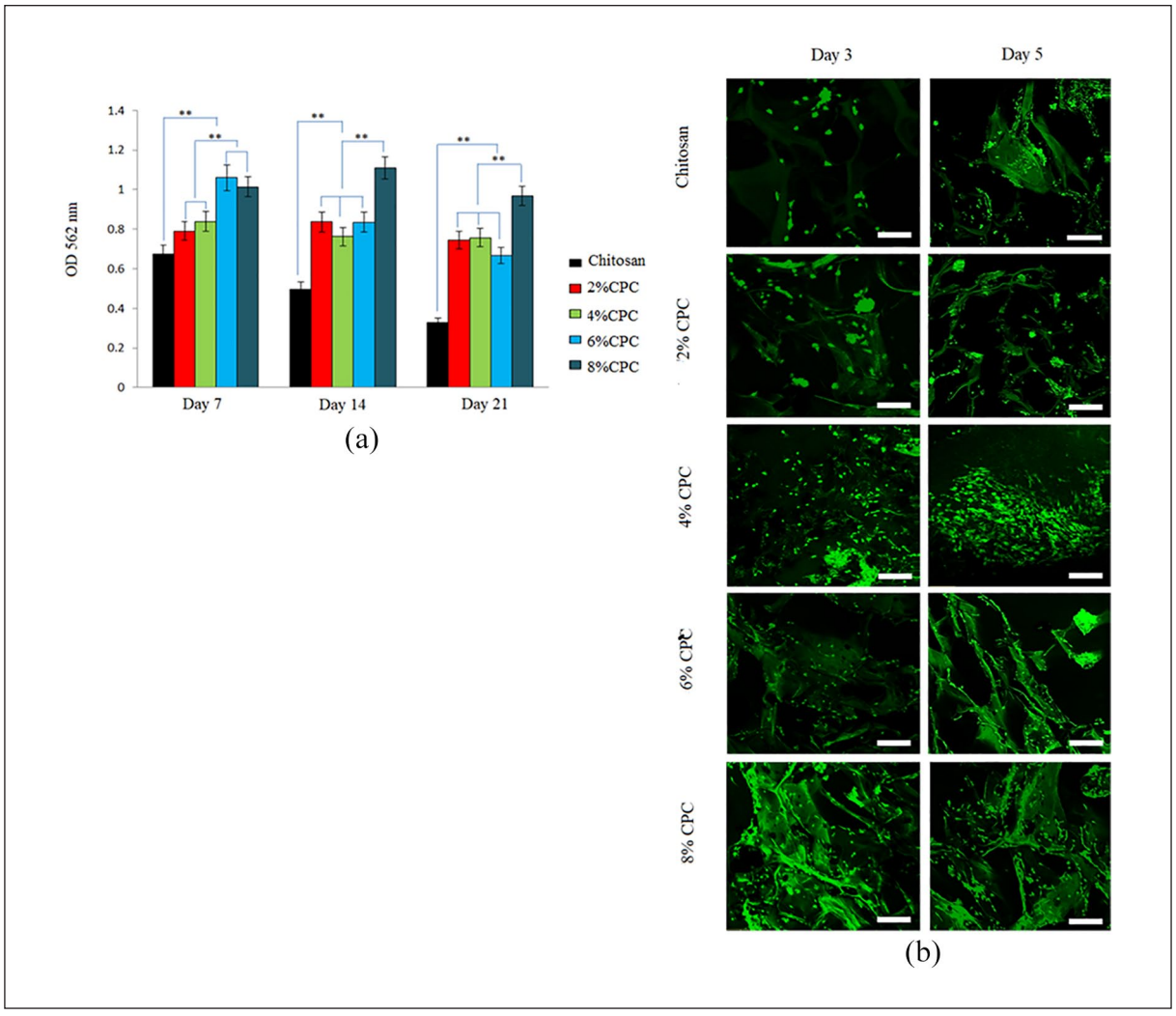

Figure 4. (a) The protein synthesis at days 7, 14, and 21, (b) the cell viability in the scaffolds, at days 3 and 5. Scale bar 200 μm.

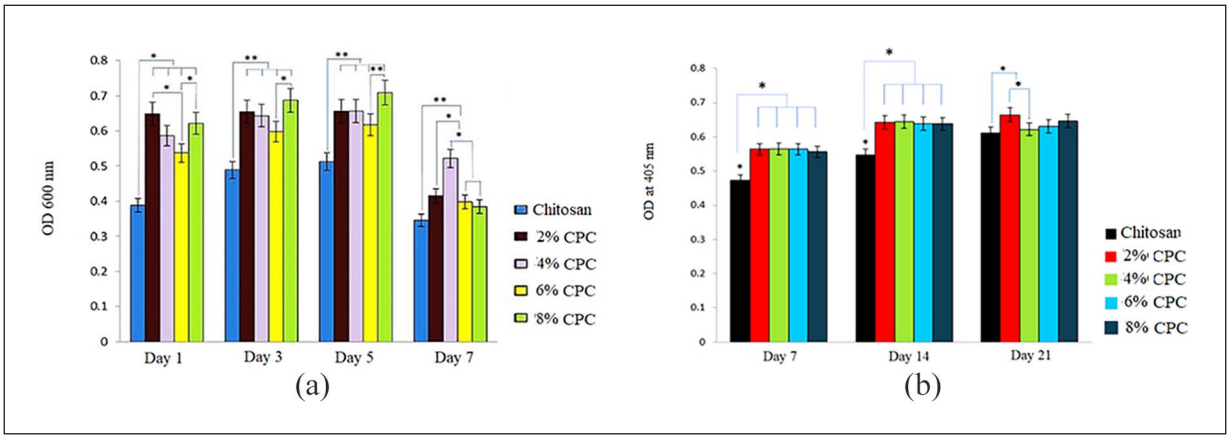

Figure 5. (a) Cell proliferation on the scaffolds on days 1, 3, 5, and 7 and (b) the alkaline phosphatase (ALP) activity in scaffolds at days 7, 14, and 21.

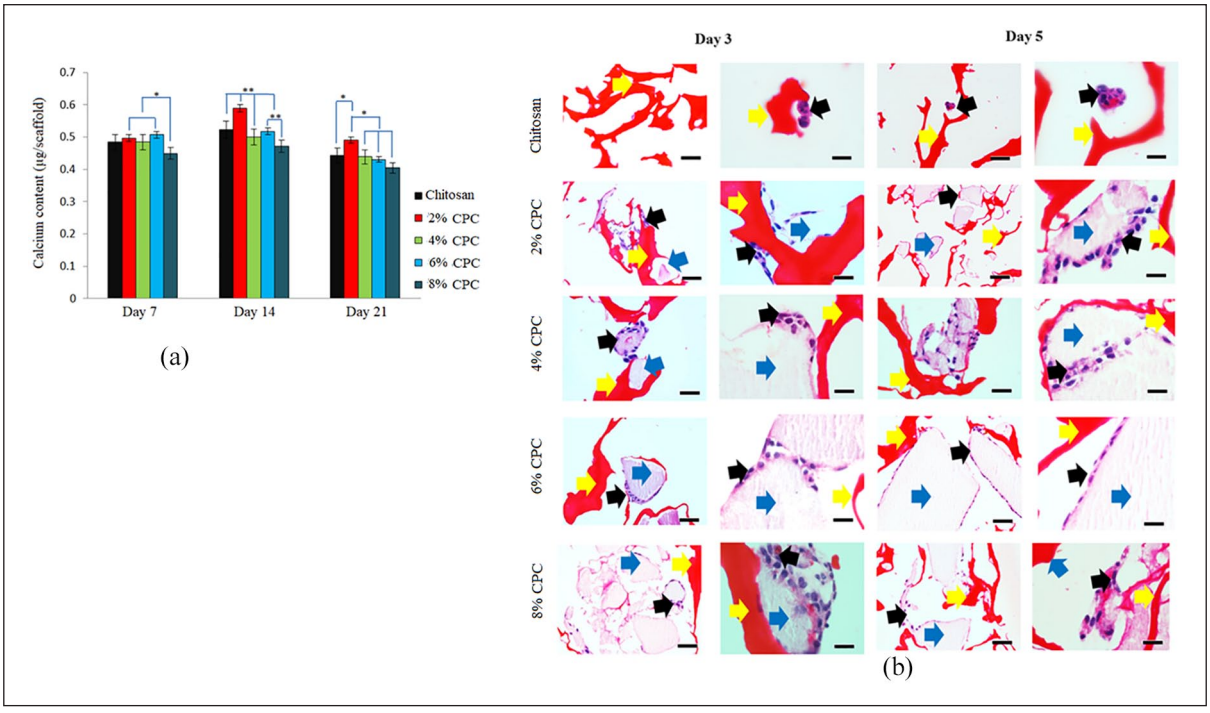

Figure 6. (a) The calcium content analysis in scaffolds at different time points, 7, 14, and 21 days; (b) histology of cultured cells in the scaffolds, at different time points, at 3 and 5 days. The yellow, blue, and black arrows denote chitosan, calcium phosphate (CP) compounds, and cells, respectively.
Scale bar 100 μm.

suitability for bone formation because they had the unique biomarkers of ALP activity and calcium content. Those biomarkers are indicators for the potential of bone formation.[32]

Conclusion

In this research, chitosan composite with various concentrations of CP was fabricated into mimicked biomineralized scaffolds based on bone remodeling. The morphology of the scaffolds was similar to biomineralization. The scaffolds of chitosan with the 2% CP compound had optimal physical and biological performance for bone formation. We conclude that mimicked biomineralized scaffolds based on bone remodeling are promising for maxillofacial tissue engineering.

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Declaration of Conflicting Interests

The authors declare that there is no conflict of interest.

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