Synthesis and in vitro biodegradability of carbonated hydroxyapatite/chitosan composite spheres

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Abstract: Hydroxyapatite (HAp)/biopolymer composites have been extensively applied as bone tissue regenerative materials because of their excellent biocompatibility and osteo-conductivity. However, the low biodegradability of HAp highly limited their practical applications. In this study, the carbonated hydroxyapatite (CHAp)/chitosan composite spheres with the size range of 100-200 μm and 300-400 μm were synthesized from CHAp and chitosan via a water/oil emulsion technique and their microstructure and biodegradability were evaluated. SEM observations showed that the resultant spheres were spherical in shape and had a rough surface. FT-IR spectrum showed that the composite spheres were derived from CHAp and chitosan due to the presence of the characteristic bands of phosphorous groups, carbonate groups and amide groups. XRD patterns showed that the composite spheres maintained the crystalline structure of CHAp crystals after combination of CHAp with chitosan. In vitro biodegradation was performed as the composite spheres were separately soaked in the buffer saline with the pH of 4.5 and 7.4. It was found that the biodegradation of the composite spheres was not affected by the size range of the composite spheres, but highly influenced by the pH value of the buffer saline. The composite spheres little degraded under the neutral condition, while around 25% of the composite spheres degraded after 7 days of incubation under acidic condition. Our results indicate that the present composite spheres are biodegradable and suitable as potential bone regenerative fillers.

1. Introduction
Numerous types of materials have been applied for stimulating bone regeneration, including metallic implants [1], inorganic materials [2], polymers [3], and inorganic-organic hybrid materials [4]. Bone extracellular matrix (ECM) is the nanocomposite of collagen and apatite [5]. The ideal bone regenerative materials should mimic the structure and composition of bone ECM. HAp is the synthetic apatite and shows excellent biocompatibility and osteo-conductivity [6]. HAp and its derivatives have been extensively utilized as drug delivery system [7] and bone tissue regenerative materials [8]. Chitosan is one type of the natural polymers and shows excellent biocompatibility and biodegradability as well as strong anti-bacterial property [9]. HAp/chitosan composites well combine the advantages of both HAp and chitosan and have been extensively utilized for development of bone tissue regenerative materials, including HAp/chitosan composite membranes [10], HAp/chitosan composite scaffolds [11], and HAp/chitosan composite spheres [12]. Compared with other types of HAp/chitosan composites, HAp/chitosan composite spheres are more interesting in the development of bone fillers as they can maximally fill the irregular bone defects without the morphological limitation [13]. However, the low biodegradability of HAp highly limited their practical applications.
Carbonated hydroxyapatite (CHAp) is the carbonated form of HAp and shows the excellent biocompatibility and osteo-conductivity. Compared with the synthetic HAp, CHAp shows much better biodegradability due to the presence of carbonate groups [13]. Especially, CHAp and its derivatives well degrade under the acidic condition created by the osteoclast [14]. Therefore, synthesis of CHAp/chitosan composite spheres would be much more interesting in the development of novel bone regenerative fillers.

In this study, CHAp/chitosan composite spheres were synthesized from CHAp and chitosan via the conventional water/oil emulsion technique and their microstructure and biodegradability were evaluated. In vitro biodegradability was quantitatively investigated by monitoring the weight loss of the composite spheres after immersed in the buffer saline with the pH value of 4.5 and 7.4.

2. Materials and methods

2.1 Synthesis of CHAp

CHAp was synthesized through phase transformation of CaCO3 crystals in the solution of Na2HPO4•12H2O. The same volume of Na2CO3 solution (1.875 mol/L) and CaCl2 solution (1.875 mol/L) was mixed in the flask and kept stirring with a magnetic machine at room temperature to produce CaCO3. After that, the as-synthesized CaCO3 (6.5 g) was added to 100 mL of Na2HPO4•12H2O solution (1 mol/L) and kept stirring at 80°C for transformation to CHAp. After 24 h, the resultant CHAp was separated, dried at 105°C overnight and then ground to powders for the following experiments.

2.2 Synthesis of CHAp/chitosan composite spheres

CHAp/chitosan composite spheres were synthesized via a water/oil emulsion technique [15]. Appropriate amount of chitosan powder was dissolved in the 2%(v/v) acetic acid solution to obtain chitosan 2% (w/v) solution. 1 g of CHAp powder was added to the above chitosan solution and the mixture was sonicated in a sonication bath. After 30 min, the mixture was added to 50 mL of plant oil and kept stirring for 30 min. 3 mL of glutaraldehyde (5%, v/v) was then added to the emulsion system for stabilizing the composite spheres. After 90 min, the composite spheres were separated from the emulsion, washed with acetone and water, and finally freeze-dried. The composite spheres were then sieved into two size ranges: 100-200μm and 300-400μm for the following experiments.

2.3 Characterizations

Size and morphology of the resultant composite spheres were observed with an optical microscope (Axio vert A1, Zeiss), and a scanning electron microscope (SEM, JSM-7100F, Japan). The infrared spectra of the samples were scanned with a Fourier transform infrared spectrophotometer (FT-IR, Bruker, Nicolet 710). The crystalline structure of the samples was identified with an X-ray diffractometer (XRD, Rigaku, Japan).

2.4 In vitro biodegradability

In vitro biodegradability was performed through soaking the composite spheres in the buffer saline with different pH values. 30 mg of the composite spheres with two size ranges: 100-200μm and 300-400μm were soaked in 4 mL of either phosphate buffer saline (pH=7.4) or the sodium acetate/acetic acid buffer solution (pH=4.5). After 1, 3, 5, and 7 days, the composite spheres were collected from the buffer solution by centrifugation at 5000 rpm, freeze-dried, and weighed on an electronic balance. The degradation rate of the composite spheres was calculated according to the following equation.

\[
\text{Degradation rate (\%)} = \left( \frac{W_o - W_s}{W_o} \right) \times 100\%
\]

Where Wo and Ws are the weight of the composite spheres before and after soaked in the buffer saline.
3. Results and discussion

3.1 Morphology and structure of CHAp/chitosan composite spheres

HAp/chitosan composite spheres have been synthesized as the aqueous mixture of HAp and chitosan were emulsified in the oil phase via the emulsion technique [12]. However, synthesis of CHAp/chitosan composite spheres has little been reported. As HAp and CHAp had the similar structure and composition, it is reasonable that the HAp/chitosan composite spheres were also synthesized by means of the emulsion method. As our expected, the composite spheres were indeed synthesized by means of the emulsion technique. The composite spheres were spherical in morphology and had a smooth surface (Figure 1a). Their size was ranged from 150 to 500 μm. The high magnification of SEM image further showed that the composite spheres had a rough surface (Figure 1b) and constructed by the 1-2μm primary nanoparticles (Figure 1c).

Figure 1 shows XRD patterns of (a) CHAp and (b) CHAp/chitosan composite spheres. CHAp presented the typical diffraction bands at 26° and 32°[15]. In general, the former was assigned to the reflection plane of (002), while the latter was assigned to the reflection plane of (211). A similar XRD pattern was observed between CHAp and CHAp/chitosan composite spheres, indicating that the composite spheres maintained the crystalline structure of CHAp. The combination of chitosan has no significant effect on the crystalline structure of CHAp.

![Figure 1](image_url) Figure 1 (a) Optical microscopy image and (b, c) SEM images of CHAp/CS composite spheres.

Figure 2 shows XRD patterns of (a) CHAp and (b) CHAp/chitosan composite spheres. CHAp presented the typical bands at 567cm⁻¹, 604cm⁻¹, and 1040cm⁻¹, which were assigned to PO₄³⁻ groups. The peaks at 1471, 1413, and 873 cm⁻¹ were assigned to B-type CO₃²⁻. After combination of CHAp and chitosan, the composite spheres also gave the similar peaks at 1631 cm⁻¹.

![Figure 2](image_url) Figure 2 XRD patterns of (a) the pristine CHAp and (b) CHAp/chitosan composite spheres.

Figure 3 shows FT-IR spectra of (a) chitosan, (b) CHAp, and (c) CHAp/chitosan composite spheres. In the case of Chitosan, the adsorption bands at 1631 cm⁻¹ and 1600 cm⁻¹ were assigned to amide I and amide II groups, respectively. CHAp has the typical bands at 567 cm⁻¹, 604 cm⁻¹, and 1040 cm⁻¹, which were assigned to PO₄³⁻ groups. The peaks at 1471, 1413, and 873 cm⁻¹ were assigned to B-type CO₃²⁻. After combination of CHAp and chitosan, the composite spheres also gave the similar peaks at 1631 cm⁻¹.
cm⁻¹, 1040 cm⁻¹, and 873 cm⁻¹, indicating that the successful fabrication of CHAp/chitosan composite spheres.

![FT-IR spectra](image)

**Figure 3** FT-IR spectra of chitosan, CHAp, and CHAp/chitosan composite spheres.

### 3.2 In vitro biodegradation

Biodegradation is one of the important parameters of the bone regenerative materials and the biodegradable materials could be in vivo degraded during the formation of new bone tissue. It is necessary to evaluate the biodegradation rate of the as-synthesized composite spheres. It has been demonstrated that the degradation of implants is correlated with the acidic condition created by the osteoclast [14]. Therefore, it is necessary to evaluate the biodegradation of the composite spheres under acidic condition.

Figure 4 shows the biodegradation rate of the composite spheres after soaked in the different types of buffer saline solution. Under neutral condition, despite the difference in size range, the as-synthesized composite spheres little degraded and their degradation rates were less than 4% with increasing the incubation time up to 7 days. In contrast, the size range of the composite spheres has no significant effect on the degradation rate. Under acidic condition, the as-synthesized composite spheres degraded. After 7 days of incubation, the degradation rate of the as-synthesized composite spheres was around 25%. In contrast, the size range of the composite spheres has no significant effect on the degradation rate.
Figure 4 Biodegradation rate of the composite spheres with size ranges: 100-200μm and 300-400μm after soaked in the neutral and acidic buffer solution up to 7 days.

Figure 5 SEM images of the composite spheres after soaked in the acidic buffer solution for (a) 3 and (b) 7 days (Arrows indicate the broken spheres).

To further confirm the biodegradation behavior, size and morphology of the resultant composite spheres after soaked in the acidic buffer solution for 3 days and 7 days were observed under SEM machine. A significant change in size and morphology of some composite spheres was observed with increasing the soaking time. After 3 days, the composite spheres little degraded (Figure 5a) and a small part was broken (arrows). After 7 days, the composite spheres strongly degraded (Figure 5b) and some spheres were completely broken (Figure 5, Inset). This result was consistent with the result from Figure 4. It is understandable that the composite spheres strongly degraded under the acetic condition due to the presence of the carbonate groups (Figure 4). Additionally, chitosan has the pKa value around 6.3 [16]. Under the acidic condition, the protonation of amino groups in the chitosan molecular chains would improve the dissolution of the chitosan in the aqueous solution. Such effect would also contribute to the degradation of the composite spheres.

4. Conclusions
In this study, the CHAp/chitosan composite spheres with the size range of 100-200 μm and 300-400...
μm were successfully synthesized via an emulsion technique. Their biodegradation was highly influenced by the pH value of the buffer saline. The composite spheres little degraded under the neutral condition (pH=7.4) and well degraded under acidic condition (pH=4.5).

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