Original article

Development of chitosan/nanosized apatite composites for bone cements

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Background: Calcium phosphate cements (CPC) is a promising materials for bone defect repair. Nanosized apatite or calcium orthophosphate has a better bioactivity than coarser crystals. Chitosan is produced commercially from chitin that is the structural element in the exoskeleton of crustaceans such as crabs and shrimp. The mixing of nanosized apatite and chitosan may provide the consistency cement, improving mechanical properties of the set bone cement.

Objective: Develop nanosized apatite powder with chitosan for bone composite cement.

Materials and method: Nanosized apatite was synthesized by chemical method at low temperature and used as the single-component for bone cement. The nanosized apatite powder was characterized using X-ray diffraction method, Fourier transform infrared spectroscopy, and transmission electron microscopy. CPCs were developed based on chitosan/nanosized apatite and calcium sulfate hemihydrate. The compressive strength of the set cement was measured after one to four weeks. The phase composition and the morphology of the set cements were investigated.

Results: Calcium sulfate hemihydrate was effective in increasing the compressive strength after setting in a simulated body fluid for seven days. The compressive strength of chitosan/nanosized apatite composite was about 18 MPa after soaking.

Conclusion: The workability and setting time of this composite were suitable to handling for bone cement. These composite cements had a significant clinical advantage for substitution of the regenerated bone.

Keywords: Apatite, bone cements, calcium phosphate cement, chitosan, composites, nanosized apatite

Self-setting calcium phosphate cements (CPC) are materials consisting of a liquid and one or more solid compounds of calcium and/or phosphate salts. They can be molded during the operation or simply injected into the bone defect, showing a similarity with the mineral phase of bone [1].

Nanosized apatite or calcium orthophosphate has a better bioactivity than coarser crystals [2-4]. Several apatite phases can be formed, including calcium-deficient hydroxyapatite, oxy-hydroxyapatite, and carbonate substituted hydroxyapatite. The major substitute in biological apatite is carbonate. In bone mineral, it occurs at levels, typically of several weight percents (wt%) [5]. The biological apatites are always calcium-deficient with poor crystallinity.

Chitosan is a linear polysaccharide composed of randomly distributed β -(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine. It is produced commercially from chitin that is the structural element in the exoskeleton of crustaceans such as crabs and shrimp [6].

When chitosan is added to the liquid component, we can obtain the chewing-gum like consistency cement [7]. Therefore, the mixing of nanosized apatite and chitosan may provide the consistency cement and improve mechanical properties of the set bone cement. Up to date, several studies have been performed on the combination of chitosan with other bioactive inorganic ceramics for enhancement of tissue regenerative efficacy and osteoconductivity [8-11]. However, no study has been made on chitosan and nanosized apatite for bone composite cement.

In this study, we developed single-component bone cement from nanosized apatite and calcium

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sulfate hemihydrate (CSH) as an additive with chitosan. We investigated the effects of the liquid/powder ratios on the properties of the present composite cement, and examined the bioactivity and biodegradability using in vitro testing.

Materials and methods

Synthesis of nanosized apatite powder

Chitosan (biomedical grade, 90±5% degree of deacetylation) was supplied by Bannawach Bio-line Co (Chonburi, Thailand). Citric acid and alpha-calcium sulfate hemihydrate were purchased from Merck (New Jersey, USA), and Siam Gypsum Plaster (Bangkok, Thailand), respectively. Nanosized apatite was prepared according to the method by Zoulgami et al. [12]. The mixture of ethanolic solution of $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$ (Sigma) and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (Riedel-de Haan) was prepared at 75–80°C as a precursor of the cement powder. The reaction took place at pH 9–10 under stirring for 90 minutes [13]. The obtained gel was washed with the de-ionized water many times. The dry gels were heated in air at 200–500°C for 10 hours. The white powders were characterized by X-ray diffraction (XRD) (Siemens D500, Siemens AG, Germany, Cu-K α radiation) and Fourier transform infrared spectroscopy (FT-IR). The morphology of the synthesized powder was observed using transmission electron microscopy (TEM).

Cement preparation

The calcium phosphate cement was obtained by mixing a powder and a liquid component. The powders consisted of the nanosized apatite and alpha CSH (50 wt%). The liquid components were solutions of chitosan (3 wt%) in citric acid. The ratio of liquid to powder (L/P ratio) was changed from 0.3 to 0.8 ml/g to examine its effect of on the properties of the composite cement. This paste was blended using mortar and pestle, and firmly packed by hand into the mold. Upon setting, the sample ends were filed for flush with the mold. Then, the samples were carefully removed and cured in 100% humidity at 37°C for 7–28 days.

Cement testing and materials characterization

Cylindrical specimens (6.0 mm diameter and 12.0 mm height) were molded to evaluate the compressive strength development. Compressive strength test of the cylindrical specimens were measured using an

Instron machine (Instron 5560, Marlin, USA) at a crosshead speed of 1 mm/minute. The cement samples were analyzed in the phase using XRD, and measured in the setting times using the Gilmore needle method [14]. After mixing and molding cement pastes, the initial and the final setting time were determined when the 113 g and 453.6 g mass loaded to a needle, respectively.

Bioactivity and biodegradability testing in vitro

To evaluate the bioactivity of the cements in vitro, the samples were incubated in a simulated body fluid (SBF) [15]. Samples were molded into the cylindrical specimens, placed into the SBF, and incubated at 37°C for 1–28 days. The SBF was changed every three days. After incubated for 1–28 days, the compressive strength was measured (n=4) using the Instron machine (crosshead speed: 1 mm/minute). The phase analysis was recorded by XRD. The surface morphology of the samples was observed by scanning electron microscopy (SEM).

For evaluation the biodegradability in vitro of the cements, the samples were molded in the cylindrical specimens and incubated in the SBF at 37°C for 7, 14, 21, and 28 days. The concentrations of Ca- and P-ions in SBF were measured using inductively coupled plasma mass spectrometry (Perkin Elmer, Model Optima 3300 RL, Massachusetts, USA).

Results

Figures 1 and 2 show XRD and FT-IR analysis of the synthesized apatite powder with different heat-treatments, respectively. In FT-IR spectra, the broad peaks between 2700 and 3700 /cm are attributed to OH stretch of apatite and NH stretch of NH_4^+ . The bending mode of H_2O is observed at 1635/cm. However, it is difficult to identify the weak band at 1454/cm where it can be expected to substitute by ammonium (NH_4^+) group or carbonate (CO_3^{2-}) group in the structure [1]. We note that after heat-treated up to 300 and 400°C, the weak band at 1454/cm has disappeared in b and c.

Figure 3 shows the synthesized apatite powder after heat-treatment at 200–500°C were agglomerated powders of nanosized apatite. Interestingly, the synthesized powder was crystallized apatite with nanosized particles. Thus, the nanosized apatite for this cement was synthesized by the chemical method and heat-treatment at 300°C.

Figure 1. X-ray diffraction (XRD) patterns of the nanosized apatite powders after heat-treatment at (a) 200°C, (b) 300°C, (c) 400°C and (d) 500°C for 10 hours. (▲ = apatite)

Figure 2. Fourier transform infrared spectroscopic (FT-IR) spectra of nanosized apatite powders after heat-treatment at 200°C (a), 300°C (b), 400°C (c) and 500°C (d) for 10 hours

Figure 3. Transmission electron microscopic images of nanosized apatite powders after heat-treatment at 200°C (a), 300°C (b) and 400°C (c) for 10 hours

Effects of L/P ratios

Figure 4 shows the compressive strength of the cements with chitosan (3 wt%) in citric acid in for various values of L/P ratios after incubation in 100% humidity for one and seven days. Interestingly, the cement with L/P ratio of 0.4 ml/g had the highest compressive strength, but it did not have paste consistency and was more difficult to handle. Therefore, the cement with L/P ratio of 0.5 ml/g could be molded into the desired shape with the compressive strength of 11.5 MPa seven days after incubation.

Seven days after incubation, the phase of calcium sulfate dihydrate (CSD) appeared in the samples with L/P ratios more than 0.3 mL/g. With L/P ratio of 0.3 mL/g, the liquid content was too little for the

cement setting and conversion from CSH to CSD. By increasing the L/P ratios, the CSH was converted into the CSD as the setting reaction of plaster. The compressive strength of the cements increased seven days after incubation due to the conversion of CSH. The high L/P ratios could create the high porosity in the cement.

Table 1 summarizes the setting times of the cements for L/P ratios of 0.3–0.8 ml/g. We note that the setting times increased with increase in L/P ratios. The initial and final setting times of the cement for L/P ratio of 0.5 ml/g were 5.30 and 28.15 minutes, respectively. These levels were suitable for medical application.

Figure 4. Compressive strength of chitosan/nanosized apatite cements as a function of the liquid/powder (L/P) ratios after curing in 100% humidity at 37°C for one and seven days

Table 1. Initial and final setting times of the chitosan/nanosized apatite cements for various L/P ratios

Liquid/powder (L/P ratio) (ml/g)	Initial setting time: I (minute)±30 seconds	Final setting time: F (minutes)±30 seconds
0.3	3.45	24.00
0.4	5.15	27.30
0.5	5.30	28.15
0.6	6.00	30.00
0.7	10.15	45.15
0.8	15.00	>60

Bioactivity and biodegradability of the cements in SBF

Figure 5 shows the compressive strength of the cement with chitosan (3 wt%) after incubation in SBF at 37°C for L/P ratio of 0.5 mL/g. Interestingly, the compressive strength increased to 18.4 MPa seven days after incubation until it became a constant up to 28 days.

In XRD patterns, CSD peak appeared in the sample seven days after incubation. However, peak of CSD decreased within 14 days and disappeared within 21 days, while peak of HA appeared within seven days. This indicated that the compressive strength increased due to the formation of CSD in the cement seven days after incubation. Fourteen days after incubation, the compressive strength reduced slightly with degrading in CSD in vitro, while HA

formed in the cement within seven days.

Figure 6 shows images of nanosized apatite cements after soaking the nanosized apatite cement in SBF for 1-28 days. As shown in a and b, the surfaces of the cement were composed of flake-shaped and needle-shaped CSH crystals. In c-e, the flake-shaped crystals on the surface disappeared, and many micropores interconnected inside the sample. Some small flake-shapes crystals were coated and isolated on the surface. The CSH crystals formed on the surface and inside the cement after incubation for a day and increased amounts within seven days. However, the CSH crystals degraded in vitro to make the interconnected micropores. The HA formation increased to reinforce the cement and maintained the level of compressive strength 14 days after incubation.

Figure 5. Compressive strength of chitosan/nanosized apatite cements as a function of the period of time after soaking in stimulated body fluid (SBF)

Figure 6. Scanning electron microscopic images of chitosan/nanosized apatite cements after soaking the chitosan/nanosized apatite cement in SBF for 1-28 days (a-e).

Figure 7 shows the concentrations of Ca-, P-ions and pH in the SBF during 7-28 days. The concentration of Ca-ion in the initial state was low and increased with the incubation time, while the concentration of P-ion was not significantly different in the incubation time. The pH of the SBF was 7.42 at the initial state and dropped to 7.23 and 7.22 after 14 and 28 days, respectively.

Discussion

Self-setting calcium phosphate cements (CPC), materials consisting of a liquid and one or more solid compounds of calcium and/or phosphate salts, have the advantage over calcium phosphate bioceramics that they can be molded during the operation or simply injected into the bone defect. Four types of CPC are known, depending on the type of calcium phosphate formed during setting, that can be either dicalcium phosphate dihydrate ($\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ or DCPD), calcium-deficient hydroxyapatite ($\text{Ca}_9(\text{HPO}_4)(\text{PO}_4)_5\text{OH}$ or CDHA), hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ or HA), or amorphous calcium phosphate (ACP). Based on bone cement components, their mixtures consisted of a major percentage of inorganic phases with organic/polymer. The mixing of many inorganic components need many steps in their synthesis route. They are time and energy consuming for multi-

component bone cements.

In this study, a chitosan/nanosized apatite composite was developed for bone cements. With three wt% chitosan in citric acid, nanosized apatite composite with 50 wt% alpha-calcium sulfate hemihydrate had compressive strength of 18 MPa after soaking in SBF for seven days. Calcium sulfate dihydrate formed in the initial state and degraded slowly from 14 days after incubation as can be seen the concentration of Ca ion in the SBF increased. Additional, HA formed slowly on the surface of the cement as the concentration of P ion gradually decreased. A biodegradable material, as a scaffold for tissue engineering, should degrade at a rate that ideally matches to tissue formation [16]. Degradation of scaffold enables the release of bioactive agents and the formation of extracellular matrix in desirable in some cases. On the other hand, a slowly degrading material can maintain its structural and mechanical properties over longer periods of time.

In conclusion, the compressive strength of chitosan/nanosized apatite cement can be improved by the chelate reaction between chitosan in citric acid and calcium in apatite powder and the conversion of plaster for reinforcement of the cements. Self-setting of chitosan/nanosized apatite cement with alpha plaster can be considered for a scaffold for tissue engineering.

Figure 7. Relationship between pH and concentration of Ca- and P-ions with the period of time after soaking the chitosan/nanosized apatite cement in SBF

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