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Synthesis of sodium caseinate–calcium carbonate microspheres and their mineralization to bone-like apatite

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ABSTRACT

Phosphoproteins can induce and stabilize calcium carbonate ($CaCO_3$) vaterite, which has desirable features for high reactivity. The purpose of this study was to synthesize bioactive $CaCO_3$ microspheres for bone regeneration. Sodium caseinate (NaCas)-containing $CaCO_3$ microspheres, with the crystal phase of vaterite, were synthesized by fast precipitation in an aqueous solution of $CaCl_2$, Na_2CO_3, and 2 mg/mL of NaCas. The uniform microspheres exhibited rougher surfaces and lower negative charges than $CaCO_3$ particles without NaCas addition. Fourier-transform infrared spectroscopy (FT-IR) of the microspheres showed characteristic peaks or bands corresponding to phosphate and hydroxyl groups. Thermogravimetric analysis (TGA) curves exhibited approximately 5% weight loss below 600 °C due to the decomposition of NaCas. Scanning electron microscope (SEM) images showed lath-like hydroxyapatite (HAp) on the surface after soaking in simulated body fluid (SBF) at 37 °C for 5 and 10 days. Energy dispersive X-ray spectrometry (EDS) revealed that the agglomerates were composed of Ca, C, O, P, Na, and Mg elements, and the Ca/P ratios ranged from 1.53 to 1.56. X-ray diffraction (XRD) patterns exhibited peaks characteristic of hydroxyapatite. The results of this study demonstrated that the addition of NaCas induced the formation of vaterite microspheres which possesses an enhanced apatite formation after soaking in SBF at 37 °C for 5 and 10 days. These NaCas–$CaCO_3$ microspheres may be a potential biomaterial for bone regeneration.

1. Introduction

Calcium carbonate ($CaCO_3$) is one of the most abundant biomaterials in nature, and has been considered as a potential precursor to induce bone-like apatite formation [1,2]. Calcium carbonate has three main crystalline polymorphs (rhombic calcite, needle-like aragonite, and spherical vaterite), two hydrated crystal forms ($CaCO_3 \cdot H_2O$ and $CaCO_3 \cdot 6H_2O$), and one amorphous phase. In aqueous systems at room temperature, the three crystalline polymorphs exhibit decreasing stabilities and increasing solubility limits. Although vaterite is rarely present in nature, it has the advantages of larger surface area, relatively good solubility, better dispersion, lower specific gravity, and the capacity for drug microencapsulation in the field of drug delivery, compared to calcite and aragonite [3,4]. Vaterite can be kinetically stabilized by sodium poly(styrene sulfonate)(PSS) [5], L-aspartic acid [6], glutamic acid, dopamine, and phosphoproteins (e.g., dentin phosphophoryn and casein) [7–10]. Some synthesized vaterite microspheres have exhibited a rough surface composed of nano-scale particles and these have been studied for the application as a drug delivery system [5,11,12]. However, few of the synthesized vaterite polymorphs had osteoinductive bioactivity which is important for bone substitutes. Recently, Kim et al. [8] reported a mussel-inspired route to create carbonated bone apatite from vaterite microspheres, which was stabilized by catechol-containing dopamine. Although dopamine enhances apatite formation from $CaCO_3$, it can cause neurotoxicity by inducing an oxidative stress state and directly interacting with the mitochondrial electron transport system [13,14]. Moreover, dopamine is costly and unfavorable for mass production. Therefore, there is demand for the synthesis of osteoinductive vaterite microspheres that are stabilized by a nontoxic and low-cost protein.

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Casein phosphopeptides (CPPs), a hydrolysis product of sodium caseinate (NaCas) [15], have been proved to enhance calcification and differentiation of bone cells [16]. Sodium caseinate is a low-cost and basic commercial form of bovine milk protein [19], and it can be produced by treating acid-precipitated casein with sodium hydroxide. Sodium caseinate has a higher solubility than casein, and has a supramolecular structure consisting of αs1-casein, αs2-casein, β-casein, and κ-casein [20,21]. In the mammary cells, caseins function by sequestering nanoclusters of calcium phosphate, thus preventing precipitation and calcification of the milk synthesis and transport system [22]. There are several types of phosphoserine residues (e.g., eight in αs1-casein and five in β-casein) and highly repetitive sequences of glutamic acid [23]. The phosphate and carboxylate groups within these phosphoserine residues and glutamic acid have the ability to bind Ca ions [17–21]. In recent years, several papers have investigated the application of NaCas in the fields of controlled drug release [18,19] and bioabsorbable membranes [17,24].

In the present study, we aimed to synthesize vaterite microspheres that exhibited a nanoscale rough surface and in vitro osteoinductive bioactivity, by introducing NaCas during the precipitation process. Sodium caseinate was applied at two different concentrations and compared to a control synthesis performed in the absence of NaCas. Several analytical techniques, including Fourier transform infrared (FT-IR) spectroscopy, X-ray diffraction (XRD), thermogravimetric analysis (TGA), and scanning electron microscopy (SEM) were employed to fully characterize the synthesized products, both with and without the addition of NaCas. The resulting products were added to a simulated body fluid for an extended period of time to test the applicability of the microspheres as a novel application for use as bone substitutes.

2. Materials and methods

2.1. Materials

Sodium caseinate (NaCas, C8654) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Analytical grade sodium carbonate (Na_2CO_3) and calcium chloride ($CaCl_2$) were purchased from Guangzhou Chemical Reagent Co. (Guangzhou, Guangdong, China) and used without further purification. Simulated body fluid (SBF) was prepared according to the recipe of Kokubo and Takadama [25]. The composition of the 1 × SBF solution was as follows: Na^+, 142.0 mM; K^+, 5.0 mM; Mg^{2+}, 1.5 mM; Ca^{2+}, 2.5 mM; Cl^-, 103.0 mM; HCO_3^-, 4.2 mM; HPO_4^{2-}, 1.0 mM; and SO_4^{2-}, 0.5 mM.

2.2. Fabrication of $CaCO_3$ microspheres and their mineralization to apatite

Calcium carbonate crystals were prepared by fast precipitation in an aqueous solution at room temperature. Various concentrations of NaCas (0, 0.2, and 2 mg/mL) were dissolved in a 15 mM Na_2CO_3 solution in one beaker, while 15 mM $CaCl_2$ was dissolved in a separate beaker without NaCas. The $CaCO_3$ precipitates were prepared by quickly mixing equal volumes of $CaCl_2$ and Na_2CO_3 solutions under vigorous stirring (500 rpm) for 15 min. The precipitates were centrifuged and rinsed with distilled water three times. The resulting precipitates were then dried under vacuum and stored at room temperature before characterization. For analysis of in vitro osteoinductive bioactivity, the synthesized particles were soaked in 1 × SBF and incubated at 37 °C for 5 and 10 days. The incubated particles were washed with distilled water, centrifuged, and dried under vacuum overnight before characterization. The three groups of crystals synthesized with different concentrations of NaCas are described in Table 1.

Table 1
Percentage of vaterite for the products was calculated from XRD data.

Samples	Calcite (%)	Vaterite (%)
CAS-0	99	1
CAS-0.2	26	74
CAS-2	11	89

2.3. Characterization

The surface morphology and mineralization of the synthesized particles were observed with a thermal field emission environmental scanning electron microscope (FEG-SEM, Quanta 400F, Oxford, UK) with an energy dispersive X-ray spectrometer (EDS) attachment, working at 20 kV and 10 μA. Before SEM observation, the particles were deposited onto aluminum stubs and coated by Au sputtering. The zeta potential of the $CaCO_3$ particles was measured with a Zetasizer Nano-ZS90 (Malvern, UK). The specific surface area of the $CaCO_3$ particles was analyzed by the Brunauer–Emmett–Teller (BET) method of nitrogen adsorption/desorption at −196 °C with an ASAP2010 surface area analyzer (Micromeritics Instrument, USA). The particle size distribution was measured with laser diffractometry (Mastersizer 2000E, Malvern, UK). Phase characterization was performed by X-ray powder diffraction (Inel CPS 120 diffractometer, $\lambda = 1.78897$ Å) using monochromatic Cu Kα radiation at a scanning rate of 3°/min. Rietveld quantitative X-ray diffraction analysis was performed using the GSAS suite of programs [26]. The presence of NaCas within the synthesized particles and hydroxyapatite formation after in vitro mineralization were further confirmed by transmission FT-IR spectroscopy (VERTEX 70, Bruker Optics, Germany) and thermogravimetric analysis curves (TGA, TG 209, Netzch, Germany). TGA analysis was performed within a temperature range of 25–900 °C and with a rate of increasing temperature of 10 °C/min. The concentration of NaCas in the $CaCO_3$ particles was analyzed with a BCA protein determination kit (Thermo Scientific Pierce, Rockford, IL, USA). Briefly, the $CaCO_3$ particles were dissolved in 2 mM EDTA (ethylenediaminetetraacetic acid) at a molar ratio of 1:1, and the resulting solutions were analyzed using the kit according to the manufacturer's protocol.

3. Results

According to the SEM images, $CaCO_3$ particles synthesized in the absence of NaCas (CAS-0) exhibited a typical rhombohedral morphology (Fig. 1A, D). The addition of 0.2 mg/mL NaCas (CAS-0.2) induced the formation of a small amount of spherical particles, which exhibited the outlines of rhombohedrons (Fig. 1B, E). In contrast, the crystals prepared in the presence of 2 mg/mL NaCas (CAS-2) were predominantly uniform microspheres (Fig. 1C). The magnified image shows that these microspheres had rough surfaces which were composed of nanoparticles 50–100 nm in size (Fig. 1F).

The crystal phase of the synthesized $CaCO_3$ particles was further characterized by XRD analysis. In the absence of NaCas (CAS-0), the XRD peaks were characteristic of a calcite structure (PCPDS # 83−0578), supporting the pure calcite phase of the precipitates (Fig. 2A). The coexistence of peaks for calcite and vaterite (JCPDS # 74-1867) with the major peaks for vaterite was observed for CAS-0.2 and CAS-2 (Fig. 2B, C), indicating that NaCas induced $CaCO_3$ crystallization of vaterite phase [3,5]. The calculated quantitative results are shown in Table 1. Calcite was observed to be the predominant crystal phase (99%) in the absence of NaCas, whereas vaterite became the main crystal phase (74%) in