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Microwave-assisted hydrothermal preparation using adenosine 5′-triphosphate disodium salt as a phosphate source and characterization of zinc-doped amorphous calcium phosphate mesoporous microspheres

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ABSTRACT

Zinc-doped amorphous calcium phosphate (Zn/ACP) mesoporous microspheres have been prepared using $CaCl_2$, $ZnCl_2$ and adenosine 5′-triphosphate disodium salt (ATP) as a biocompatible organic phosphorus source by a microwave-assisted hydrothermal method. Both ATP and Zn^{2+} are reported as the stabilizer for amorphous calcium phosphate (ACP) in aqueous solution. In this study, we have found that ATP is the main factor for stabilizing ACP in aqueous solution and Zn^{2+} ions are doped in ACP for the antibacterial benefit. X-ray powder diffraction (XRD), scanning electron microscopy (SEM), nitrogen adsorption–desorption isotherm and pore size distribution, Fourier transform infrared (FTIR) spectroscopy and thermogravimetry (TG) analysis are used to characterize the as-prepared Zn/ACP mesoporous microspheres. The TG and FTIR results reveal that some residual ATP molecules are adsorbed on the surface of the Zn/ACP mesoporous microspheres. The pH-sensitive Zn^{2+} ion release behavior and good antibacterial activity of Zn/ACP mesosporous microspheres against bacteria *Staphylococcus aureus* and *Escherichia coli* indicate that the as-prepared Zn/ACP mesoporous microspheres are promising for the antibacterial application in the biomedical fields.

1. Introduction

Amorphous calcium phosphate (ACP) is the initial solid phase formed in a highly supersaturated solution containing Ca^{2+} ions and phosphate ions. It was proposed that the $Ca_9(PO_4)_6$ cluster is the transient solution precursor to ACP formation, and that in blood and in body fluids some proportion of the Ca^{2+} and PO_4^{3-} ions is present as ion clusters, possible in the $Ca_9(PO_4)_6$ configuration, which are small enough to pass through biological membranes [1]. ACP generally forms as an unstable precursor, which easily transforms into a stable crystalline phase of calcium phosphates such as hydroxyapatite (HAP) and octacalcium phosphate (OCP) in the absence of any stabilizer in aqueous solution. A number of chemical species have been shown to act as the stabilizers of ACP in aqueous solution. The inorganic stabilizers such as Mg^{2+}, Sr^{2+}, Zn^{2+}, pyrophosphate and tripolyphosphate (TPP) have been reported [2]. The stabilization of ACP by some biomolecules such as adenosine triphosphate (ATP) has also been reported. Blumenthal et al. [3] investigated the a synergistic effect when magnesium and ATP were used together in solution to inhibit the conversion of ACP to crystalline HAP. Recently, this research group reported a microwave-assisted hydrothermal rapid synthesis of highly stable ACP porous microspheres with a relatively uniform size and an average pore diameter of about 11 nm using ATP as a biocompatible organic phosphorous source and stabilizer. The as-prepared ACP porous microspheres had a high stability in the phosphate buffer saline solution for more than 150 h without phase transformation to HAP, and the morphology and size were essentially not changed. The as-prepared ACP porous microspheres were efficient for anticancer drug loading and sustained drug release [4].

The ACP phase is an intermediate phase in the preparation process of other calcium phosphate materials. ACP has a relatively high solubility, excellent bioactivity and biocompatibility. Much attention has been paid to the preparation and applications of ACP materials, particularly in the form of coatings and cements for orthopaedic applications, composites for dental applications and carriers for drug delivery [5–7]. Usually, ACP is used as an additive to adjust the biodegradability, bioactivity and mechanical properties of bone repairing materials. It has been demonstrated that ACP has a better osteoconductivity and biodegradability than those of hydroxyapatite in vivo [8]. ACP may also be a potential remineralizing agent in dental applications. ACP-filled bioactive composites are believed to be effective anti-demineralizing/remineralizing agents for the preservation and repair of tooth structures [9].

In addition to the biocompatibility and bioactivity of biomaterials, the antibacterial ability also plays an important role for their applications. For example, bacterial infection after orthopedic

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implant placement is one of the potentially disastrous complications, which eventually leads to surgical failure, multiple operations, and even sometimes amputation [10]. Therefore, efforts have been devoted to the development of antibacterial biomaterials. Hu et al. [11] prepared zinc incorporated TiO_2 coatings on titanium by plasma electrolytic oxidation to obtain the promising candidates for orthopedic and dental implants with good bacterial inhibition ability and bone-formability. The as-prepared Zn-incorporated TiO_2 coatings could greatly inhibit the growth of both *Staphylococcus aureus* and *Escherichia coli*, and the ability to inhibit bacteria could be improved by increasing the Zn content in the coatings. Kawamura et al. [12] demonstrated that the zinc-releasing calcium phosphate implants could stimulate bone formation in rabbits compared with the calcium phosphate implants without zinc. Gu et al. [13] reported that acidic calcium phosphate-based solutions containing both F^- and Zn^{2+} ions have mineralizing, acid resistance, and antibacterial effects and may be potentially useful as a treatment strategy against dentin caries formation and progression. However, little work has been reported on zinc-containing ACP as an antibacterial biomaterial.

In this work, zinc-loaded amorphous calcium phosphate (Zn/ACP) mesoporous microspheres have been prepared by a simple, low-cost and surfactant-free microwave-assisted hydrothermal method for antibacterial benefit. The microwave-assisted hydrothermal method combines the advantages of rapid microwave volumetric heating and pressurized hydrothermal process, thus has advantages such as rapid heating and high reaction rate, which reduces the preparation time often by orders of magnitude, often in minutes compared with several days by the conventional hydrothermal preparation, leading to very high efficiency and energy saving [14,15]. The Zn/ACP mesoporous microspheres have been prepared by a rapid microwave-assisted hydrothermal method for a very short period of time (only 10 min). In this work, adenosine 5′-triphosphate disodium salt (ATP) is chosen as both a biocompatible organic phosphorus source and a stabilizer for the ACP phase. Zn^{2+} ion is chosen as an antibacterial agent because zinc is nontoxic, stable and essential for mammalian growth and normal development [16]. The morphology, composition, stability and growth mechanism of Zn/ACP mesoporous microspheres are explored. As shown in Scheme 1, ATP biomolecules hydrolyze to produce PO_4^{3-} ions in aqueous solution which react with Ca^{2+} ions and Zn^{2+} ions to form Zn/ACP clusters, then these clusters aggregate and grow into Zn/ACP mesoporous microspheres under microwave hydrothermal conditions. The pH-sensitive Zn^{2+} ion release and antibacterial activity against bacteria *S. aureus* and *E. coli* indicate that as-prepared zinc-loaded Zn/ACP mesoporous microspheres are promising for the antibacterial benefit in the biomedical fields, such as implant materials and oral care composites, where good biocompatibility, antimicrobial and high safety are required.

2. Experimental

2.1. Preparation of Zn/ACP mesoporous microspheres

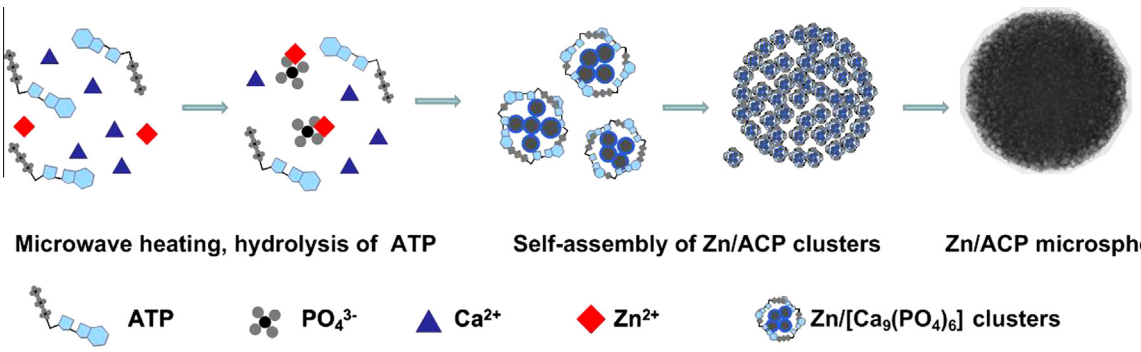
In a typical experiment, 0.1110 g $CaCl_2$ and 0.0136 g $ZnCl_2$ were dissolved in 20 mL deionized water, then 19 mL aqueous solution containing 0.1000, 0.2200 or 0.3000 g adenosine 5′-triphosphate disodium salt (ATP) was added to the above solution under magnetic stirring at room temperature. The pH value of above solution was maintained at 6.8 by slow addition of dilute ammonia aqueous solution. The resulting solution was transferred into a 60 mL autoclave, sealed and microwave-heated in a microwave oven (MDS-6, Sineo, China) to 110 °C and maintained at this temperature for 10 min. It took about 2 min to reach the temperature of 110 °C under microwave irradiation at a frequency of 2.45 GHz and a powder of 1000 W. After cooling down to room temperature, the product was separated by centrifugation, washed with deionized water and ethanol, and dried at 60 °C. The zinc-free calcium phosphate microspheres as a control sample were synthesized in the absence of Zn^{2+} ions under the same conditions as the above sample. The additional control sample HAP was prepared using $(NH_4)_2HPO_4$ as a phosphorus source without ATP in the presence of Zn^{2+} ions.

2.2. pH-activated Zn^{2+} ion release from Zn/ACP mesoporous microspheres

pH-activated release of Zn^{2+} ions from Zn/ACP mesoporous microspheres was performed as follows: the powder (0.0500 g) of Zn/ACP mesoporous microspheres were dispersed in 50 mL deionized water with a pH 2.00 ± 0.05, 4.00 ± 0.05 and 7.00 ± 0.05, respectively. The pH value of the solutions was maintained at 2.00 ± 0.05, 4.00 ± 0.05 or 7.00 ± 0.05 during the Zn^{2+} ion release process by addition of 0.5 M HCl. 1 mL supernatant was withdrawn and diluted for inductively coupled plasma (ICP) analysis at given time intervals.

2.3. Antibacterial activity tests of Zn/ACP mesoporous microspheres

S. aureus (*S. aureus*, ATCC 25923) and *E. coli* (*E. coli* ATCC 25922) were used in the antibacterial tests. The aqueous suspensions of *S. aureus* and *E. coli* were diluted at a final concentration of 2×10^6 colony forming units (cfu)/mL. The bacterial suspension (2 mL) was added into 15 mL centrifuge tube with various amounts of Zn/ACP powder and oscillated for 20 s. The concentrations of Zn/ACP mesoporous microspheres in the bacterial suspensions were in the range of 1000–16000 ppm. After incubation for 24 h at 37 °C in the incubator, the bacterial suspensions were spread on trypticase soy broth (TSB) agar plates. The colonies formed on the agar were counted after the incubation at 37 °C for 24 h. The



Scheme 1. Schematic diagram for the formation of Zn/ACP mesoporous microspheres synthesized by a rapid microwave-assisted hydrothermal method using ATP as an organic phosphorus source and a stabilizer.