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Novel gallium-doped amorphous calcium phosphate nanoparticles: Preparation, application and structure study

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ABSTRACT

Novel gallium-doped amorphous calcium phosphate (ACP) nanoparticles with different (Ga + Ca)/P molar ratio (1.8–3.0) were synthesized using sol-gel method. The antibacterial effects of the gallium-doped ACP samples were tested using disk diffusion assays, against *Pseudomonas aeruginosa*. The results showed that the gallium-doped ACP samples have obvious effect of inhibiting the growth of *Pseudomonas aeruginosa* and could have a long term antibacterial properties. The local structures of these amorphous calcium phosphate nanoparticles were studied by ^{31}P, ^{71}Ga single pulse, double-resonance and ^{31}P homonuclear dipolar recoupling solid state NMR techniques. The solid state NMR studies indicate that gallium-doped ACP nanoparticles are consisted of $Ga_x(OH)_yO_z$ and calcium phosphate clusters. Gallium-doped ACP nanoparticles could be promising complex materials with the properties of antibacterial and biological mineralization.

1. Introduction

In recent years, antibiotic bacteria are increasing due to heavy antibiotic use and person-to-person spread of bacteria. To discover new antimicrobial agents is extremely urgent [1]. Antibacterial materials have drawn much attention because they can eliminate bacterial cross-infection, especially the inorganic antibacterial materials with advantages of good chemical stability, wide antibacterial scope, long-effectiveness, little drug-resistance and high safety to human bodies [2,3]. The antibacterial ion plays an important role in inorganic antibacterial materials. Many metal ions have antibacterial properties, such as Ag^{+}, Cu^{2+}, Zn^{2+}, Ni^{3+}, Bi^{3+} et al.; among which, Ag^{+}, Cu^{2+} and Zn^{2+} have been widely used [4–6]. Gallium is a trivalent metal that shares certain chemical characteristics with Fe^{3+}, such as chemical valence, ionic radius [7]. An important property of gallium is its high affinity binding to transferrin, the iron transport protein in the circulation [8]. But different with iron, gallium cannot be reduced under physiological conditions [9]. Iron metabolism is a key factor in vulnerability of infecting bacteria as they require Fe for growth and the functioning of key enzymes, such as those involved in DNA synthesis, electron transport and oxidative stress defenses [10,11]. Hence, gallium disrupts cellular iron homeostasis. The gallium-induced block in cellular iron uptake, coupled with a direct effect of intracellular gallium, leads to inhibition of bacterial growth and reproduction. Gallium nitrate has been proved to have anticancer effects by clinical trials [7] and approved by FDA to treat hypercalcemia of malignancy [12].

Nowadays, gallium nitrate has recently emerged as a new generation antibacterial agents that may be useful in treating and preventing localized infections. However, gallium nitrate has little biological activity, low bioavailability that requires long-term intravenous injection [13]. However, excessive amounts of Ga^{3+} could lead to a series of side effects, such as diarrhea, nausea, hypocalcemia, renal toxicity and so on [14], which limits the application of gallium nitrate in biomedical field.

Chemically durable materials, that can slowly release gallium ions for long periods, prolong its duration of action, would be considered desirable materials for medical applications [15]. Calcium phosphate nanoparticles are such materials.

Calcium phosphate nanoparticles have gained increasing interest in recent years because of their high biocompatibility, biodegradability and bioactivity, which is due to the fact that calcium phosphates are the main inorganic mineral of mammalian bone and teeth [16–19]. To date, only a few of studies have reported the preparation of gallium-doped calcium phosphates, which mainly based on crystalline calcium phosphates, such as hydroxyapatite (HA), tricalcium phosphate (TCP) or brushite [11,13,20,21]. Compared with other crystalline calcium phosphates, amorphous calcium phosphates have following advantages: (1) ACP has better osteoconductivity and artificial bone cell adhesion properties than hydroxyapatite [22]; (2) the biodegradation rate of ACP is higher than tricalcium phosphate, and can change the component to adjust certain characteristics [23]; (3) ACP play a vital role in the process of biomineralization of bone: Firstly, it is a precursor

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phase during the formation of other crystalline phases [24]; Secondly, ACP is employed as a temporary storage for bone apatite as Ca and P sources, effectively prevent rickets [25]; Thirdly, the amorphous phase can also be found in the stabilized form for the mechanical purposes as the biomineral itself, due to its isotropic characteristic [26]. Thus, ACP was widely used in biological mineralization and biomedical field, such as bone and tooth repair and replacement, drug delivery, gene transfection and diagnostic imaging [27–29].

In this study, gallium as antibacterial element and ACP as carrier and biological mineralization component were devised to develop a novel antibacterial and biological mineralization gallium (Ga^{3+})-doped ACP complex material. The Ga^{3+}-doped ACP nanoparticles were prepared using sol-gel method. The rate of degradation and the antibacterial effect of the material were investigated. The structures of ACP were studied by multiple solid state NMR techniques. The local structures of phosphorus in ACP were studied by ^{31}P single pulse spectra and ^{31}P CT-DRENAR-BABA-xy16. The coordination number of Ga^{3+} was studied by Hahn echo experiments. The connectivity between Ga^{3+} and P^{5+} ions was studied by $^{31}P\{^{71}Ga\}$ REAPDOR experiments. The antibacterial experiment and the NMR results indicate that this material has potential antibacterial and mineralization complex effect.

2. Experimental

2.1. Synthesis

$Ca(NO_3)_2 \cdot 4H_2O$, $(NH_4)_2HPO_4$ and $Ga(NO_3)_3 \cdot 6H_2O$ were purchased from Sigma-Aldrich. PEG400 was purchased from Shanghai Runjie Chemical Reagent Co. All the chemicals were of analytical grade, and used as received without further purification.

Gallium-doped ACP nanoparticles were synthesized through a typical reaction process: $(NH_4)_2HPO_4$ was dissolved in 20 ml deionized water to form 0.15 M phosphate solution. $Ca(NO_3)_2 \cdot 4H_2O$ and $Ga(NO_3)_3 \cdot 6H_2O$ were dissolved in 20 ml deionized water to form mixed solution. The raw materials were designed with a (Ca + Ga)/P molar ratio from 1.8 to 3.0 and a fixed Ga/P molar ratio of 0.665. The pH values of the above solutions were adjusted to the range within 9–9.5 with concentrated ammonia. A certain amount of PEG400 was added into the mixed solution containing $Ca(NO_3)_2 \cdot 4H_2O$ and $Ga(NO_3)_3 \cdot 6H_2O$, to reach PEG/(Ca + Ga) molar ratio of 6. The mixture was stirred for 15 min to form a homogeneous solution. Phosphate solution was added into the homogeneous solution under stirring and the pH value was maintained within the 9.5–10 range by addition of ammonia. The resulting mixture was agitated for another 60 min at room temperature. Finally, the obtained nanoparticles were collected by centrifugation and washed several times with ethanol and deionized water to remove any residual ions and solvents. The precipitates were freeze-dried without addition of any further cryoprotectant.

2.2. Characterized by XRD and SEM

X-ray powder diffraction (XRD) analysis was performed using Cu Ka radiation and a Ni filter in a Philips PW-1840 diffractometer operating at 40 kV and 40 mA. The scanning electron microscopy (SEM) micrographs were recorded on a JEOL JSM-6700F field emission scanning electron microscope. For SEM observation, the obtained powders were dispersed in the absolute ethanol and dropt on the silicon wafer.

2.3. Degradation study

Degradation analyses were conducted for five gallium-doped ACP samples. 0.04 g of Ga^{3+}-doped ACP nanoparticles with different (Ca + Ga)/P molar ratio were placed in dialysis bags with a molecular weight cutoff of 3500, placed in plastic containers, respectively, filled with 40 ml deionized water (pH 7 ± 0.5). All these samples were incubated in a constant temperature vibrator at 37 °C under agitation on a shaker plate (100 rpm) for different time periods. At various time points (24, 48, 72 and 96 h), the samples were taken out of their respective containers and withdrawn release medium (8 ml) for ion release measurement. All the samples were placed into a fresh 40 ml deionized water and placed back into the 37 °C incubator.

2.4. Ion release measurements

Ion release measurement of the degrading medium was conducted at each time point (24, 48, 72 and 96 h). The medium was analyzed for Ga, P and Ca ion using ICP-MS (Inductively coupled plasma mass spectrometry).

2.5. Inhibition of microbial growth by gallium-doped ACP nanoparticles

The antibacterial effect of Ga^{3+}-doped ACP nanoparticles was measured based on disk-diffusion methodology (BSAC Disk Diffusion Method for Antimicrobial Susceptibility Testing, Version 4, 2005). Briefly, Isosensitest agar (Oxoid, Basingstoke, UK) plates were inoculated with a standardized culture of *Pseudomonas aeruginosa*. All Ga^{3+}-doped ACP nanoparticles were pressed into small pieces with a diameter of about 13 mm with the same quality, and then placed on the inoculated plates. Filter papers with diameter of 13 mm as fictitious samples, which didn't contain any gallium, were placed on the plates as blank samples for negative control. These plates were then incubated overnight in air at 37 °C. The diameters of any zones that had formed around the Ga^{3+}-doped ACP samples were measured using calipers.

2.6. Solid state NMR analysis

All the solid-state magic angle spinning (MAS) NMR experiments were performed on a Bruker Avance 500 spectrometer corresponding to a magnetic field strength of 11.7 T, using 4 mm MAS probe at a spinning rate of 12 KHz.

^{31}P MAS NMR experiments were measured at a resonance frequency of 202.45 MHz. ^{31}P MAS NMR spectra were acquired using a pulse length of 4.55 μs (90° flip angle) and a long recycle delay of 120 s. ^{31}P chemical shifts were calibrated to 85% H_3PO_4 solution, but $NH_4H_2PO_4$ was used as secondary reference compound, with the chemical shift being set to 1.1 ppm. ^{71}Ga MAS NMR spectra were recorded at 152.5 MHz using a Hahn echo sequence with a 180° pulse of 9.6 μs and a relaxation delay of 0.2 s. Chemical shifts were referenced relative to 1 M $Ga(NO_3)_3$ solution.

To probe the spatial correlation between ^{71}Ga and ^{31}P nuclei, $^{31}P\{^{71}Ga\}$ rotational echo adiabatic passage double resonance

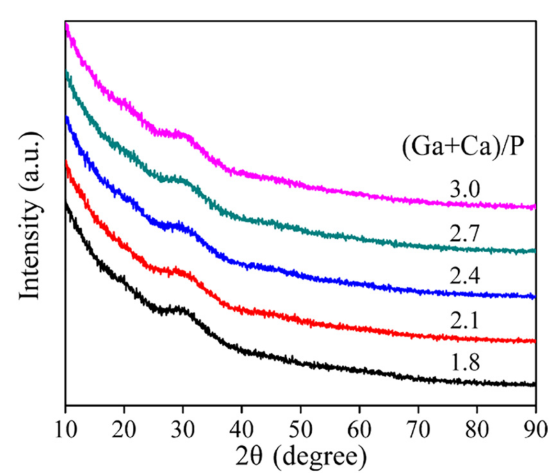

Fig. 1. XRD patterns of Ga^{3+}-doped ACP samples synthesized in the presence of PEG at room temperature with the initial (Ca + Ga)/P atomic ratio from 1.8 to 3.0.