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Preparation and characterization of β-tricalcium phosphate co-doped with monovalent and divalent antibacterial metal ions

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Abstract

Ag^+ and Zn^{2+} or Cu^{2+} ions were co-doped with β-tricalcium phosphate (AgZn–TCP and AgCu–TCP), and their substitution models, antimicrobial activities, mechanisms and cytotoxicities were investigated. The lattice constants (*a*-axis and *c*-axis) of AgZn–TCP and AgCu–TCP decreased linearly with the amount of Zn^{2+} or Cu^{2+} ions up to 9.09 mol.%, which indicated that Ag^+ ions were doped at the Ca(4) site and a vacancy in the β-TCP structure, and Zn^{2+} or Cu^{2+} ions were doped at the Ca(5) site. Antibacterial activities of AgZn–TCP and AgCu–TCP on *Escherichia coli* and *Staphylococcus aureus* were higher than those of Ag^+ ions-doped β-TCP (Ag–TCP) and pure β-TCP. These antimicrobial activities suggested that an interaction occurred between bacteria and Ag^+, Zn^{2+} and Cu^{2+} ions eluted from AgZn–TCP and AgCu–TCP and between bacteria and the free radicals generated by antibacterial agents or in bacterial cells. AgZn–TCP and AgCu–TCP can be used over long periods of time with high antimicrobial activity, because the rate at which Ag^+ ions are released from AgZn–TCP and AgCu–TCP is slower than that at which Ag^+ ions are released from Ag–TCP. However, it is necessary to determine the suitable amounts of Ag^+, Zn^{2+} and Cu^{2+} ions in AgZn–TCP and AgCu–TCP by considering both their antimicrobial activities and cytotoxicities, because β-TCP doped with a large amount of these metal ions exhibits cytotoxicity. Furthermore, AgZn–TCP and AgCu–TCP are considered to be promising materials for use in various fields.

Keywords: β-Tricalcium phosphate; Antibacterial metal ions; Solid solution; Free radicals

1. Introduction

Silver, zinc and copper (Ag, Zn and Cu), particularly in the form of free ions, are well known to exhibit strong inhibitory activities and strong antimicrobial effects on various bacteria [1–3]. In addition, these metal ions are superior to organic antimicrobial agents in terms of heat resistance, persistence of antimicrobial effects and safety [4]. However, these metal ions have certain disadvantages in that their ionic states are unstable, and they may have toxic effects when directly ingested. To overcome these disadvantages, extensive research has been conducted to develop antibacterial agents whose metal ions are bound by a substrate so that the stability of ionic states is improved and the metal ions can be released over a long period of time [5]; zeolites [6], hydroxyapatite (HAp) [7], bioglass [8,9], silica [5] and carbon fibers [10] are currently used as substrates, and their antibacterial agents are also used in many fields [11–16].

β-Tricalcium phosphate (β-TCP) has been used as a bone and tooth implant material because of its excellent biocompatibility, osteoinductivity, bioresorbability and safety in living tissues [17,18]. It has been reported previously that monovalent (Li, Na and K), divalent (Mg) and trivalent (Al) metal ions can be easily substituted for Ca^{2+} ions in

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a β-TCP structure; this substitution enhances the mechanical characteristics, improves the thermal stability for the β–α phase transition of TCP, and controls the solubility [19,20]. Therefore, β-TCP is considered to be a promising novel inorganic substrate for antimicrobial metal ions. The substitution model and the antimicrobial activity and mechanism of β-TCP doped with Ag^+ ions (Ag–TCP) have also been demonstrated [21].

In this study, a solid-state reaction was carried out to prepare β-TCP co-doped with monovalent (Ag^+) and divalent (Zn^{2+} or Cu^{2+}) ions as antibacterial metal ions (AgZn–TCP and AgCu–TCP), a substitution model of these metal ions was clarified, and their antimicrobial activities, mechanisms and *in vitro* cytotoxicities were investigated.

2. Materials and methods

2.1. Preparation of Ag–TCP, AgZn–TCP and AgCu–TCP

Ag–TCP, AgZn–TCP and AgCu–TCP were prepared by carrying out a conventional solid-state reaction. Ag–TCP was prepared using $CaHPO_4{\cdot}2H_2O$ (Kishida Chemical Co. Ltd., Osaka, Japan), $CaCO_3$ (Kishida Chemical Co. Ltd.) and $AgNO_3$ (Kishida Chemical Co. Ltd.). $AgNO_3$ was added to the prepared Ag–TCP in order to obtain 0–15 mol.% of Ag^+ ions for Ca^{2+} ions in β-TCP with a molar ratio of $(Ca + 2Ag)/P = 1.50$. AgZn–TCP and AgCu–TCP were prepared using ZnO (Kishida Chemical Co. Ltd.) and CuO (Kishida Chemical Co. Ltd.), respectively, together with the above materials; the amount of $AgNO_3$ added was 9.09 mol.% Ag^+ ions, and the amount of ZnO or CuO was 0–15 mol.% Zn^{2+} or Cu^{2+} ions for Ca^{2+} ions in β-TCP with a molar ratio (Ca + 2Ag + Zn or Cu)/P = 1.50. These raw materials were mixed for 60 min using an agate mortar, and the resulting mixture was calcinated at 1000 °C for 12 h in air. After calcination, the powder was re-mixed and re-calcinated under the same above-mentioned conditions in order to achieve a high degree of crystallinity and complete substitution of the added metal ions.

2.2. Characterization of AgZn–TCP and AgCu–TCP

2.2.1. X-ray diffraction analysis and lattice constants measurements

Phase compositions and lattice constants (*a*-axis and *c*-axis) of the obtained samples were measured using an X-ray powder diffractometer with a rotating anode X-ray tube (RINT-1500, Rigaku Corp., Tokyo, Japan). The lattice constants were refined by a least-squares method using silicon as an external standard.

2.2.2. Antimicrobial tests

Minimum bactericidal concentration (MBC) measurements were performed on *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) to confirm the antimicrobial activity of the prepared samples. *E. coli* (NBRC 3972) and *S. aureus* (NBRC 12732) were obtained from the National Institute of Technology and Evaluation, Tokyo, Japan. The bacteria were cultured on a nutrient agar medium (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) at 37 °C for 20 h, and then their concentration was adjusted to 2.0×10^6 colony-forming units (CFU)·cm^{-3} by diluting them with sterile distilled water. Heat-sterilized samples (64 mg) were added to sterile distilled water (10 cm^3) to achieve a concentration of 6400 μg cm^{-3}; the mixture was then incubated by shaking at 30 °C for 2 h. Dispersion solution (1 cm^3) was added to sterile distilled water (1 cm^3) kept at 30 °C (3200 μg cm^{-3}). Concentrations of the sample solutions (1600–0.01 μg cm^{-3}) were adjusted by repeatedly performing the same operations. One milliliter of the bacterial solution with 2.0×10^6 CFU cm^{-3} was added to each sample solution; subsequently, the resulting solution was shaken at 30 °C for 1 h. After the solution had been shaken, 0.1 cm^3 of the culture solution was spread on the standard method agar plates (Nissui Pharmaceutical Co. Ltd.) and incubated at 37 °C for 48 h. MBC is defined as the concentration at which fewer than five colonies exist.

2.2.3. Dissolution tests of Ag^+, Zn^{2+} and Cu^{2+} ions

Concentrations of Ag^+, Zn^{2+} and Cu^{2+} ions leached from the specimens were evaluated by performing dissolution test. Powders with diameters <75 μm were separated using a sieve, and 2.0 g of the powder was added to saline (40 cm^3); the resulting mixture was shaken at 37 °C for 3 days. Next, the suspended matter was filtered, and the leaching concentrations of Ag^+, Zn^{2+} and Cu^{2+} ions in the filtrate were analyzed using an atomic absorption spectrophotometer (AA-6200, Shimadzu Corp.). Saline was used to ensure the same osmotic pressure *in vivo* and examine the detailed dissolution behavior; it is difficult to determine the dissolution behavior in a solvent containing various metal ions, such as medium and simulated body fluid (SBF).

2.2.4. Electron spin resonance measurements

Electron spin resonance (ESR) measurements were performed by a spin trapping method using a spin trap agent to estimate the concentration of free radicals generated by the photocatalytic reaction of the specimens. The prepared sample (50 mg) was added to a mixed solution of 5,5-dimethyl-pyrroline-*N*-oxide (DMPO, 0.03 cm^3, Sigma, St. Louis, MO) and distilled water (2.97 cm^3), and then the resulting mixture was irradiated with UV light (380 nm) emitted from a 500 W xenon lamp (SX-UI500XQ, Ushio Lighting, Inc., Tokyo, Japan) at 17 mW cm^{-3} for 5 min with stirring. Subsequently, the solution was drawn into a quartz cell, and the ESR spectrum was measured using an ESR spectrometer (JES-FA200, JEOL Ltd., Tokyo, Japan). The ESR spectrum of 1×10^{-4} mol dm^{-3} 4-hydroxy-2,2,6,6-tetramethylpiperdin-1-oxyl (4-hydroxy-TEMPO, Sigma) was measured as a standard sample, and the concentration of generated radicals was calculated by integrating the ESR signal area of the samples and 4-hydroxy-TEMPO.

ID	Title	Pages
1127	Preparation and characterization of β-tricalcium phosphate co-doped with monovalent and divalent antibacterial metal ions	8