Antimicrobial Properties and Synthesis of Tricalcium Phosphate Doped with Alkali Metal and Silver Ions

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ABSTRACT

In order to prepare an inorganic antimicrobial agent with a biocompatibility, we have attempted synthesis of beta-tricalcium phosphate (β-TCP) doped with silver using a solid-state reaction method, and have investigated the solubility and the bactericidal activity to Escherichia coli or Staphylococcus aureus of the obtained products. β-TCP solid solutions were formed by reaction of the mixture of 3-TCP and silver ion. The Ca²⁺ ion in β-TCP was replaced with silver ion of up to 10 mol%. It was considered that Ca²⁺ ion and vacancy located Ca(4) site in β-TCP structure were displaced by two silver ions. The products doped with silver ion showed a high bactericidal activity and their antimicrobial effects increased with increase of the amount of silver ion. In order to change the bactericidal activity of β-TCP doped with silver ion, further more, alkali metal ion was added to β-TCP together with silver ion. As the result, alkali metal ion also replaced Ca²⁺ ion, and formed the substituted solid solution that is similar to silver ion. The bactericidal activity of β-TCP doped with silver and potassium ions was increased and that of β-TCP doped with silver and lithium ions was decreased. This depended on the solubility of the obtained β-TCP solid solution. By means of ESR evaluation using a spin-trap reagent, we found that superoxide radicals was generated from β-TCP doped with silver ion by UV irradiation, and considered that β-TCP doped with silver ion has both an antimicrobial effect that was generated by photo irradiation and an antimicrobial effect that was caused by the dissolved silver ion.

INTRODUCTION

Calcium phosphate materials such as hydroxyapatite (HAp) and beta-type tricalcium phosphate (β-TCP) are used as the bioceramics for bone repair because they have a good biocompatibility. Especially, β-TCP is known as bio-active materials having a high solubility under in vivo. On the other hand, the materials containing silver ion are used as an antimicrobial agent. In particular, zirconium phosphate and titanium phosphate containing silver ion have been reported in phosphate materials. We attempted the preparation of an antimicrobial agent with both a biocompatibility and an antimicrobial characteristic, and probed the reactivity of silver ion to some calcium phosphates. Silver ion did not respond to HAp. However, silver ion respond to β-TCP and β-TCP solid solution that was substituted by silver ion was formed.

In this study, the substituted limit of the solid solution replaced Ca²⁺ ion in β-TCP by silver ion was investigated and the solubility and the antimicrobial characteristic of the obtained Ag-containing β-TCP solid solution. In order to change the solubility and the antimicrobial characteristic of Ag-containing β-TCP solid solution, further more, formation of the β-TCP solid solution that was replaced with alkali metal ions such as lithium, sodium and potassium ions in addition to silver ion was investigated.

EXPERIMENTAL

For the purpose of preparation of β-TCP doped with alkali metal and silver ions, Ca₃P₂O₇, CaO, AgO and
alkali metal carbonate (Li$_2$CO$_3$, Na$_2$CO$_3$, or K$_2$CO$_3$) as the starting materials were mixed at the mixing conditions of additive amount of silver or alkali metal ions of 0-10\text{mol\%} to the total amount of Ca$^{2+}$ ion in $\beta$-TCP and the molar ratio of ($\text{Ca}+\text{Ag}+\text{A}$)/$\text{P}$ = 1.50 ($\text{A}$: Li, Na, or K) and then the mixture was heated at 1000 °C for 5 h in air.

Crystalline phases of the obtained products were identified by X-ray diffraction (RAD-2C, Rigaku, Japan). The lattice parameters of $\beta$-TCP and the obtained solid solution were determined by X-ray diffractometer with rotating anode X-ray tube (RINT-1500, Rigaku, Japan) using Si powder as an internal standard.

The bacteria used in this study were *Escherichia coli* (E-coli, IFO 3972) as a typical Gram-negative bacterium and *Staphylococcus aureus* (S.aureus, IFO 12372) as a typical Gram-positive bacterium. Each of the bacterium was pre-cultured at 37°C for 18 hours in agar medium. The concentration of bacteria was diluted approximately $1.0 \times 10^8$ CFU/cm$^3$ with Muller Hinton Bouillon (MHB) culture medium. The obtained product was homogeneously dispersed in a sterilized test tube. The suspension was diluted with sterilized water from 6400 ppm to 25 ppm respectively and then was added with bacteria suspension of 0.1 cm$^3$. The test tubes contained bacteria and the product were cultured at 37 °C for 24 hours in a shaking incubator. The growth of the bacteria was observed after the culture. MIC value was represented as a minimum concentration of the product that was checked the growth of the bacteria.

Superoxide radicals were detected by means of a spin-trap method. 5,5-Dimethyl-Pyrroline-N-Oxide (DMPO) was used as a spin-trap reagent. The obtained product was immersed in the mixed solution of 2 cm$^3$ water and 20 mm$^3$ DMPO. ESR measurement was performed by FA-200 (JEOL, Japan). ESR spectra were measured after the suspension was irradiated UV using Xe lamp (SX-UI 500XQ, Ushio, Japan, 500 W, UV intensity is 17 mW/cm$^2$).

**RESULTS AND DISCUSSION**

The $\beta$-TCP doped with various amount of silver ion was prepared by a solid-state reaction method. X-ray diffraction patterns of the obtained products were shown in Fig. 1. X-ray diffraction patterns of the products obtained by adding silver ion of 2 - 8 mol \% were similar to that of $\beta$-TCP and the diffraction peaks shifted to high angle with increase of amount of silver ion. Furthermore, X-ray diffraction pattern of the products that was added silver ion of 10 mol \% was a mixture of $\beta$-TCP (●) and Ag$_3$PO$_4$ (■).

![Fig.1 X-ray diffraction patterns of the products obtained by changing amount of Ag$^+$ ion.](image)

![Fig.2 Lattice parameter of $\beta$-TCP doped with various amount of Ag$^+$ ion.](image)
We have examined on the lattice parameters of the obtained products and the relation between lattice parameters of the obtained products and additive amount of Ag⁺ ion was shown in Fig. 2. The a-axis length of the obtained products did not change but the c-axis length of the obtained products linearly decreased up to 10 mol% Ag⁺ ion. It was found from these results that Ca²⁺ ion in β-TCP was replaced by silver ion of up to approximately 10 mol%. And it was considered that Ca²⁺ ion and vacancy located Ca(4) site in β-TCP structure were displaced by two silver ions.

In order to change a bactericidal activity of β-TCP doped with silver ion, further more, alkali metal ion was added to β-TCP together with silver ion. We have synthesized β-TCP doped with silver and alkali metal ions by changing an additive amount and a kind of alkali metal ion, and have examined on lattice parameters of the obtained products. The relation between lattice parameters of the obtained products and amount of alkali metal ion was shown in Fig. 3. The additive amount of Ag⁺ ion fixed at 4 mol%. In case of additive lithium ion, the c-axis length of the obtained products did not change but the a-axis length of the obtained products decreased up to 6 mol% of additive lithium ion. And in case of additive sodium ion, the a-axis length did not change but the c-axis length decreased up to 6 mol% of additive sodium ion. Furthermore, in case of additive potassium ion, the a-axis length increased and the c-axis length decreased up to 6 mol% of additive potassium ion. It was found that Ca²⁺ ion in β-TCP doped with silver ion (additive amount of 4 mol%) was displaced by 6 mol% of all alkali metal ion that was used in this work and it was considered that alkali metal ion substituted at the Ca²⁺ ion site that was substituted by silver ion.

Next, the water solubility of β-TCP doped with silver and various alkali metal ions was evaluated by immersing the products (sample weight: 1.0g) in water of 100 cm³ at 25 °C. Water solubility of the product with lithium ion was decreased but that of product with potassium ion was increased. From thus result, it was found that water solubility of β-TCP doped with silver ion was changed by variation of a kind of the doped alkali metal ions.

Final, we have examined the bactericidal characteristics of β-TCP doped with silver and alkali metal ions that was prepared in this study. The experimental results of bactericidal characteristics using MIC method were shown in Table 1. The MIC value of β-TCP doped with silver ion decreased to 800 ppm with increase of amount of silver ion. The obtained β-TCP doped with silver ion showed a high bactericidal activity. In case of
doping various alkali metal ions, further more, bactericidal activity of β-TCP doped with silver and potassium ions increased and that of β-TCP doped with silver and lithium ion decreased. It was considered that the reason for a high bactericidal activity of the antimicrobial agent is mainly that silver ion dissolves from β-TCP doped with silver ion into water little by little.

The superoxide radical generated from β-TCP doped with silver ion by UV irradiation was evaluated using ESR measurement. DMPO(5,5-Dimethyl-Pyrroline-N-Oxide) was used as a spin trap reagent. ESR spectrum of the superoxide radical generated from β-TCP doped with silver ion by UV irradiation was shown in Fig.5. The formation of DMPO-OH adduct was observed by ESR measurement. From this result, it was found that hydroxyl radical(OH\textsuperscript{-}) was generated from β-TCP doped with silver ion by UV irradiation. We defined that β-TCP doped with silver ion has both an antimicrobial effect that was generated by photo irradiation and an antimicrobial effect that was caused by the dissolved silver ion in this study. Hence β-TCP doped with silver and alkali metal ions is useful as a novel antimicrobial material.

**Table 1** The evaluation of bactericidal characteristics of β-Ca\textsubscript{3}(PO\textsubscript{4})\textsubscript{2} doped with alkali metal and silver ions using MIC method.

<table>
<thead>
<tr>
<th>Ion Type</th>
<th>Conc. (ppm)</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag 2 mol%</td>
<td>3200 ppm</td>
<td>800 ppm</td>
</tr>
<tr>
<td>Ag 6 mol%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ag 10 mol%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Li 6 mol%</td>
<td>1600 ppm</td>
<td>800 ppm</td>
</tr>
</tbody>
</table>

**SUMMARY**

In order to prepare an inorganic antimicrobial agent with a biocompatibility, we have attempted synthesis of beta-tricalcium phosphate (β-TCP) doped with silver using a solid-state reaction method, and have investigated the solubility and the bactericidal activity to *Escherichia coli* or *Staphylococcus aureus* of the obtained products. β-TCP solid solutions formed by solid-state reaction of the mixture of β-TCP and silver ion (and alkali metal ion). The Ca\textsuperscript{2+} ion in β-TCP was replaced by silver and alkali metal ions of up to 10 mol%. β-TCP doped with silver ion showed a high bactericidal activity. We defined that β-TCP doped with silver ion has both an antimicrobial effect that was generated by photo irradiation and an antimicrobial effect that was caused by the dissolved silver ion. Hence β-TCP doped with silver and alkali metal ions is useful as a novel antimicrobial material.

**REFERENCES**