



Full length article

# Physicochemical fabrication of antibacterial calcium phosphate submicrospheres with dispersed silver nanoparticles *via* coprecipitation and photoreduction under laser irradiation

Maki Nakamura<sup>a,\*</sup>, Ayako Oyane<sup>a</sup>, Yoshiki Shimizu<sup>a</sup>, Saori Miyata<sup>b</sup>, Ayumi Saeki<sup>c</sup>, Hirofumi Miyaji<sup>b</sup><sup>a</sup> Nanomaterials Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Central 5, 1-1-1, Higashi, Tsukuba, Ibaraki 305-8565, Japan<sup>b</sup> Department of Periodontology & Endodontology, Hokkaido University Graduate School of Dental Medicine, N13 W7, Kita-ku, Sapporo, Hokkaido 060-8586, Japan<sup>c</sup> Department of Oral Pathobiological Science, Hokkaido University Graduate School of Dental Medicine, N13 W7, Kita-ku, Sapporo, Hokkaido 060-8586, Japan

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## ABSTRACT

We achieved rapid, surfactant-free, and one-pot fabrication of antibacterial calcium phosphate (CaP) submicrospheres containing silver nanoparticles by combining physical laser and chemical coprecipitation processes. In this physicochemical process, weak pulsed laser irradiation (20 min) was performed on a labile CaP reaction mixture supplemented with silver ions as a light-absorbing agent. The silver content in the submicrospheres was controlled for a wide range (Ag/P elemental ratio varied from 0.60 to 62.0) by tuning the initial concentration of silver ions (from 5 to 20 mM) in the CaP reaction mixture. At the silver concentration of 5 mM, we obtained unique nanocomposite particles: CaP submicrospheres (average diameter of approximately 500 nm) containing metallic silver nanoparticles dispersed throughout, as a result of CaP and silver coprecipitation with simultaneous photoreduction of silver ions and spheroidization of the coprecipitates. These CaP submicrospheres containing silver nanoparticles (*ca.* 0.3 mg silver per 1 mg submicrospheres) exhibited antibacterial activity against major pathogenic oral bacteria, *i.e.*, *Streptococcus mutans*, *Aggregatibacter actinomycetemcomitans*, and *Porphyromonas gingivalis*. Moreover, the CaP submicrospheres dissolved and neutralized the acidic environment generated by *Streptococcus mutans*, demonstrating their potential as acid-neutralizing and remineralizing agents. The present process and resulting antibacterial CaP-based submicrospheres are expected to be useful in dental healthcare and infection control.

## Statement of Significance

Nano- and microsized spheres of calcium phosphate (CaP) containing silver nanoparticles have great potential in dental applications. Conventional fabrication processes were time-consuming or weak regarding the size/shape control of the spheres. In this study, we achieved a simple (one-pot), rapid (20-min irradiation), and surfactant-free fabrication of CaP submicrospheres containing silver nanoparticles by pulsed laser irradiation to a mixture of calcium, phosphate, and silver ion solutions. The resulting CaP submicrospheres contained metallic silver nanoparticles dispersed throughout in a sequence of reactions: CaP and silver coprecipitation, laser-induced melting and spheroidization of the coprecipitates, and photoreduction of silver ions. These submicrospheres showed antibacterial activity against oral bacteria and acid-neutralizing property in the bacterial suspension, and hence are worth considering for dental applications.

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## 1. Introduction

Dental caries and periodontal disease are widespread diseases that are caused by pathogenic oral bacteria such as *Streptococcus*

*mutans* (*S. mutans*), *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*), and *Porphyromonas gingivalis* (*P. gingivalis*) [1–3]. These bacteria often proliferate at tiny interstices inside fissures and pits in a tooth and between a tooth and the neighboring tooth, gingiva, and dental prosthetic materials. Nano- and microsized materials having antibacterial activity may be useful for bacterial elimination in such tiny interstices. For

\* Corresponding author.

E-mail address: [ma-ki-nakamura@aist.go.jp](mailto:ma-ki-nakamura@aist.go.jp) (M. Nakamura).

antibacterial materials, a variety of nano- and microsized spheres containing silver nanoparticles have been created, since silver nanoparticles are a relatively safe and biocompatible antibacterial agent [4–6]. As matrices to immobilize silver nanoparticles, artificial materials such as calcium phosphate (CaP), silicon oxide, zinc oxide, and organic polymers have been utilized [7–11]. Among these materials for matrices, CaPs are especially promising for dental therapy because CaPs have a compositional similarity to a human biomineral and can support the remineralization of teeth through degradation [12–14]. It has been reported that CaP spheres containing silver ions and/or silver nanoparticles allow silver release through the degradation of a CaP matrix [7].

Nano- and microsized particles of CaP containing silver nanoparticles have been prepared using conventional chemical and/or physical processes. However, these conventional processes are relatively time-consuming or are weak regarding the size/shape control of the spheres [7,15–18]. For example, Mocanu et al. and Andrade et al. separately fabricated CaP particles and silver nanoparticles *via* chemical precipitation, and integrated two particles in the subsequent step [17,18]. Such a multi-step process is time-consuming; in addition, the resulting composite particles were irregular in shape and size.

We recently developed a simple (one-step) and rapid (short irradiation time) physicochemical process for the fabrication of CaP-based submicrospheres with well-regulated spherical shapes by combining physical laser and chemical precipitation processes [19–21]. In this process, weak pulsed laser irradiation was performed on a labile CaP reaction mixture supplemented with ferric ( $\text{Fe}^{3+}$ ) or ferrous ( $\text{Fe}^{2+}$ ) ions as a light-absorbing agent. These ferric and ferrous ions were incorporated into the initially formed precipitates, and enhanced light absorption, and consequently, melting and spheroidization of the precipitates during laser irradiation. Within only 10–30 min of irradiation, CaP-based submicrospheres with various iron contents were fabricated.

The first purpose of this study was to fabricate CaP submicrospheres containing silver nanoparticles using silver ions ( $\text{Ag}^+$ ) as a light-absorbing agent. We considered that other metal ions besides ferric and ferrous ions should also be effective as a light-absorbing agent for the fabrication of CaP-based submicrospheres, if the metal ions can be incorporated into the initial CaP precipitates and absorb sufficient laser light energy to induce melting and spheroidization of the precipitates. Silver ions are known to be converted into metallic silver ( $\text{Ag}^0$ ) by photoreduction under appropriate conditions [22,23]. Therefore, we hypothesized that by adding silver ions as a light-absorbing agent to the reaction mixture, CaP submicrospheres containing metallic silver nanoparticles should be able to be fabricated. The second purpose of this study was to demonstrate the antibacterial activity of the resulting CaP-based submicrospheres. We therefore performed an antibacterial assay for the resulting submicrospheres using three major pathogenic oral bacteria, *S. mutans*, *A. actinomycetemcomitans*, and *P. gingivalis*.

## 2. Materials and methods

### 2.1. Pulsed laser irradiation of the CaP reaction mixture supplemented with silver ions

For the calcium, phosphate, and silver ion source solutions, aqueous  $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$  (Wako Pure Chemical Industries, Ltd., Japan; 200 mM),  $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$  (Nacal Tesque, Inc., Japan; 200 mM) and  $\text{AgNO}_3$  (Wako Pure Chemical Industries, Ltd.; 200 mM) were prepared, respectively, by dissolving the reagent grade chemicals into ultrapure water. In a cylindrical glass vessel, a reaction mixture (4 mL) was prepared by mixing and stirring the

three ion source solutions. The final concentrations of calcium, phosphate, and silver ions in the reaction mixture were 16.7 mM, 10.0 mM, and 0–20 mM (0, 0.5, 1.0, 2.0, 5.0, 10.0, and 20.0 mM), respectively.

Pulsed laser irradiation was immediately applied to the freshly prepared reaction mixture under constant stirring using the output of the third harmonic (355 nm) of an Nd:YAG laser (Quanta-Ray LAB-150-30, Spectra-Physics, USA) operated at 30 Hz with a fluence of 200 mJ/pulse/cm<sup>2</sup> (output power of 3 W). The laser beam had an output diameter of 8 mm and was irradiated without focusing. After irradiation for 20 min, the product was collected using centrifugation (6000 rpm (3700g), 10 min), and washed with ultrapure water (as-collected products). The as-collected products were dispersed again in ultrapure water, finally centrifuged three times for 10 min at 1000 rpm (103g), and the sediment was collected as the final product. For only the products from the reaction mixture with 5.0 mM silver ions, before the final centrifugation, irregularly-shaped microprecipitates were removed using slower pre-centrifugation (three times for 5 min at 500 rpm (26g)).

### 2.2. Characterization of the products

The morphology, chemical composition, and crystalline structure of the final products were examined using scanning electron microscopy (SEM; Model XL30, FEI, USA and S-4800, Hitachi High-technologies Co., Japan), energy dispersive X-ray spectroscopy (EDX; Genesis 2000, EDAX, USA and EMAX x-act, HORIBA, Ltd., Japan), and thin-film X-ray diffractometry (XRD; M18X, MAC Science, Japan) with  $\text{CuK}\alpha$  X-rays, respectively. The micro and crystalline structure of the final products were investigated using transmission electron microscopy and diffraction (TEM and TED; JEM-2010, JEOL, Japan). The final products were mounted on a silicon substrate before SEM, EDX, and XRD analyses, and on a thin carbon-coated copper grid before TEM and TED analyses. The hydrodynamic diameter of the final products dispersed in ultrapure water was measured by dynamic light scattering (DLS) with a particle size analyzer (Zetasizer Nano-ZS, Malvern, UK).

The contents of Ca, P, and Ag contained in the final products were determined using chemical analysis as follows. First, the products were dried under reduced pressure and dissolved in a concentrated solution of nitric acid (Wako Pure Chemical Industries, Ltd.; 60–61%, 1 mL). After dilution with water (9 mL), Ca, P, and Ag in these solutions were quantified using inductively coupled plasma-atomic emission spectrometry (ICP-AES; ULTIMA2, HORIBA, Ltd.).

### 2.3. Cross-sectional analysis of the products using TEM

For the selected final product, cross-sectional analysis was performed using a TEM (Tecnai Osiris, FEI) operated at 200 kV, equipped with EDX and a high-angle annular dark-field scanning transmission electron microscopy system (HAADF-STEM) with a probe diameter of  $\sim 1$  nm, to further investigate the microstructure and the elemental distribution inside one submicrosphere. A cross-sectional specimen was prepared using conventional mechanical thinning followed by argon ion milling as detailed below. The dried final products embedded in an epoxy resin were cured between two silicon substrates, and this was cut into some cross-sectional slabs, mechanically thinned to  $\sim 100$   $\mu\text{m}$ , and then dimpled to  $\sim 30$   $\mu\text{m}$  in the center of the specimen. Final thinning was then performed using argon ion milling in a Precision Ion Polishing System (PIPS; Model 691, Gatan, USA). The incident beam angle and voltage were  $\pm 3^\circ$  and 2.0–5.5 kV, respectively, and a specimen cooling system using a liquid nitrogen stage was applied during ion milling.

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