Aesthetic Silver-Doped Octacalcium Phosphate Powders Exhibiting Both Contact Antibacterial Ability and Low Cytotoxicity

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ABSTRACT: Since the introduction of biomaterials, infection has been a serious problem in clinical operations. Although several studies have introduced hybrid materials of calcium phosphate and Ag0 nanoparticles (NPs) that exhibit antibacterial activity, released Ag+ ions and Ag0 NPs are highly cytotoxic and the materials require complex fabrication techniques such as laser irradiation. In this study, we introduce a simple one-pot synthesis method based on crystal-engineering techniques to prepare Ag+-substituted octacalcium phosphate (OCP−Ag) powder that simultaneously exhibits antibacterial activity, little change in color, and low cytotoxicity, thereby overcoming the shortcomings of calcium phosphate as a biomaterial. We used AgNO3-containing (NH4)2HPO4 aqueous solutions as reaction solutions in which Ag+ forms soluble complex [Ag(NH3)2]+ ions that are stable at Ag+ concentrations less than ∼30 mmol/L. Hydrolysis of soluble calcium phosphate in this solution led to pure OCP−Ag when the Ag+ concentration was less than ∼30 mmol/L. Crystallographic analysis showed that Ag+ substituted at the P53O4-conjugated sites and was uniformly distributed. When the concentration of Ag+ in the reaction solution was varied, the Ag+ content of the OCP−Ag could be controlled. The obtained OCP−Ag exhibited little color change or Ag+ release when immersed in various media; however, it exhibited contact antibacterial ability toward resident oral bacteria. The prepared OCP−Ag showed no substantial cytotoxicity toward undifferentiated and differentiated MC3T3-E1 cells in assays. Notably, when the Ag+ content in OCP−Ag was optimized (Ag: ∼1 at %), it simultaneously exhibited contact antibacterial ability, little color change, and low cytotoxicity.

1. INTRODUCTION

As typified by current worldwide efforts toward mitigating the COVID-19 pandemic, preventing infection is an important aspect of the history of clinics, especially those where orthopedic and oral surgeries are performed. Infection has long been regarded as a serious clinical risk and has still not been overcome. Once infection occurs, even simple clinical cases become aggravated and sometimes fatal.1–3 In clinical cases of orthopedic and oral surgery, in particular, infection of implanted bone substitute after an extended period is a severe surgery risk factor that occurs at a rate of several percent of total surgeries.4,5 Although bone substitutes that mainly comprise calcium phosphate can regenerate and reconstruct implanted bone defects, they exhibit no inherent ability to prevent infection. Therefore, when implanted bone substitutes become an infection nest, severe clinical cases result because bone substitutes are closely attached to tissues during implantation and curing.

Attaching antibacterial agents to bone substitutes is one approach to preventing infection. Silver (Ag) has historically been used as an antibacterial agent and exhibits a broad antibacterial spectrum.6–9 Ag exhibits excellent antibacterial activity toward both Gram-positive and Gram-negative bacteria, where it deteriorates substances in their cellular walls, such as peptidoglycan and lipopolysaccharide.10–12 In particular, Ag is known to exhibit excellent antibacterial activity toward Staphylococcus sp. (e.g., methicillin-resistant Staphylococcus aureus (MRSA) and methicillin-resistant Staphylococcus epidermidis (MRSE)), Escherichia sp., and resident oral bacteria such as Streptococcus sp.13–18 Therefore, several Ag-loaded materials have been commercialized as nonresorbable bone substitutes such as coating materials for titanium artificial joints.19,20 However, released Ag0 nanoparticles (NPs) and/or soluble Ag+ salts of Ag-loaded materials both exhibit strong cytotoxicity.21–23 Consequently, no studies related to an Ag-loaded calcium phosphate-based bone substitute with controlled Ag release ability have been reported. In addition, for...
bone substitute production, a simple and easy-to-handle fabrication method is required. During the infection process, bacteria that attach to the surface of the bone substitute lead to implanted bone substitutes becoming infection nests. When Ag is substituted into the unit lattice of calcium phosphate, the Ag-substituted calcium phosphate can potentially exhibit excellent contact antibacterial ability, relatively low cell toxicity, and a weak color change. In fact, Ag-doped materials have already been introduced into biocompatible glass ceramics, resulting in materials that exhibit excellent antibacterial properties and low cytotoxicity. However, to our knowledge, a direct Ag-doping process for pure calcium phosphate has not been reported.

Octacalcium phosphate (OCP: Ca₈(PO₄)₄(HPO₄)₂·5H₂O) exhibits excellent biocompatibility and is therefore attractive as a new bone substitute. Our previous studies related to OCP fabrication have shown that for a cation to be substituted into the OCP unit lattice, its ionic radius should be similar to that of Ca²⁺. Notably, the ionic radius of Ag⁺ (1.00 Å) is very similar to that of Ca²⁺ (1.02 Å), indicating strong potential for the successful substitution of Ag⁺ into the OCP unit lattice. Thus, Ag-substituted OCP could potentially provide contact antibacterial activity without the release of Ag⁺. In this study, we investigated the substitution of Ag⁺ into the OCP unit lattice and evaluated the antibacterial properties and in vitro cytotoxicity of the resultant Ag⁺-substituted materials.

2. RESULTS AND DISCUSSION

2.1. Fabrication of OCP–Ag. In this study, an ion-complexation technique was used for Ag⁺-ion dispersion. Although the Ag⁺ ion is unstable under basic conditions, a Ag⁺–NH₃ complex ion (in this case, [Ag(NH₃)₂]⁺) is stable in basic solution. In fact, at Ag⁺ concentrations less than 30 mmol/L, transparent reaction solutions were obtained, whereas at Ag⁺ concentrations greater than 30 mmol/L, yellowish precipitates were formed. However, when NH₄NO₃ was added to the
reaction solution as a source of additional NH₃, the solution remained transparent even at 100 mmol/L Ag⁺.

In this study, the method used to evaluate how Ag⁺ is substituted into the OCP unit lattice for acquiring contact antibacterial property and reducing cytotoxicity was the same method used in our previous work involving Na⁺ substitution into the OCP unit lattice. The ionic radius and charge of Na⁺ are the same as those of Ag⁺ (Scheme 1). When OCP was fabricated in weak basic solutions containing Na⁺, the Na⁺ substituted into the Ca²⁺ sites conjugated to PS PO₄, which is a root of the HPO₄−OH layer; the relative intensity ratio of I₁(200)/I₁(200) associated with the layer structure of OCP then increased.

Figure 1 shows the X-ray diffraction (XRD) patterns of samples prepared using solutions containing various concentrations of Ag⁺. In the systems, when the Ag⁺ concentration was less than 30 mmol/L, samples consisted only of calcium phosphate as OCP with/without a small amount of hydroxyapatite (HAp). As the Ag⁺ concentration in the treatment solutions was increased, the I₁(200)/I₁(200) relative intensity ratio in the XRD patterns of the products increased (Figure 2). In the systems prepared using a solution with an Ag⁺ concentration greater than 50 mmol/L, XRD peaks of Ag₃PO₄ were observed. Figure 6 shows photographs of the samples. In the cases of low Ag concentrations, the OCP samples exhibited a whitish color, which is the conventional color of calcium phosphates. However, in the case of samples treated with a solution with a concentration greater than 100 mmol/L, the samples showed a substantial yellow coloration. Therefore, we concluded that the yellow color was associated with the formation of Ag₃PO₄. The quantitative analysis of the samples' color, as conducted using color differential meters, was evaluated via color-change testing, as discussed in Section 2.2.

To investigate how the change in sample color occurs, we observed the fine structures of the samples. Figure 7 shows scanning electron microscopy (SEM) micrographs of the samples. When the Ag⁺ concentration in the treatment solution was less than 30 mmol/L, we observed plate-like crystals approximately 5 μm in length, 1 μm in width, and 50 nm in thickness; the shape of these crystals was typical of the shape of OCP crystals and they were closely formed on dicalcium phosphate dihydrate (DCPD: CaHPO₄·2H₂O) in a "pseudomorphic" structure. When the Ag⁺ concentration in the reaction solution was greater than 50 mmol/L, in addition to plate-like crystals, granular crystals with sizes on the order of several nanometers were observed. On the basis of the XRD analysis, the granular crystals formed in solutions with high Ag concentrations were assumed to be Ag₃PO₄. We therefore attributed changes in color to the formation of such Ag₃PO₄ granular crystals.

Scanning transmission electron microscopy-energy-dispersive X-ray (STEM-EDX) mapping enabled us to observe the atomic distribution in the samples. As shown in Figure 8, Ag⁺ content of the samples controlled the crystallographic tendencies of the OCP. We subsequently measured the Ag content of the samples with Ag concentrations below the concentration at which residual Ag₃PO₄ formed using inductively coupled plasma atomic emission spectroscopy (ICP–AES); the results are shown in Figure 4. With increasing Ag⁺ concentration in the treatment solutions, the Ag content of the samples increased linearly until Ag₃PO₄ formed, at which point the Ag content of the samples increased markedly.

On the basis of previous bulk analyses, we considered that OCP–Ag could be formed in the same manner as OCP–Na. In subsequent discussions, to clarify the effect of the Ag⁺ content in OCP–Ag, we refer to the OCP–Ag prepared using a AgNO₃ solution of concentration N mmol/L as OCP–Ag(Na). We refer to the sample OCP–Ag(Ag0) as OCP-law.

As a further bulk evaluation method, X-ray photoelectron spectroscopy (XPS) was used to evaluate the chemical states of some of the samples. We analyzed two OCP samples (OCP–Ag (OCP–Ag(Ag20)) and OCP–Na as the reference) embedded in resin because the fabricated materials were in the powdered form (note that XPS measurements require high-vacuum conditions (~1.0 × 10⁻⁶ Pa)). OCP might decompose upon loss of crystal water during a thermal treatment, leading to collapse of the OCP lattice. However, because XPS measurements are not otherwise possible, we conducted XPS measurements under such high-vacuum conditions. Figure 5 shows the XPS spectra of samples. The peaks of Ca 2p and P 2p of OCP–Ag are shifted slightly toward lower binding energies compared with those of OCP–Na. The peaks of Ag 3d, which indicate the charge of the Ag doped into the OCP unit lattice, are consistent with coexisting Ag²⁺ and Ag⁺ chemical states.

The color of the OCP–Ag samples was subsequently observed. Figure 6 shows photographs of the samples. In the cases of low Ag concentrations, the OCP samples exhibited a whitish color, which is the conventional color of calcium phosphates. However, in the case of samples treated with a solution with a concentration greater than 100 mmol/L, the samples showed a substantial yellow coloration. Therefore, we concluded that the yellow color was associated with the formation of Ag₃PO₄. The quantitative analysis of the samples' color, as conducted using color differential meters, was evaluated via color-change testing, as discussed in Section 2.2.
was uniformly distributed in the plate-like crystals, along with the other atoms associated with calcium phosphate. This phenomenon is consistent with the bulk elemental analysis results.

2.2. Evaluation of the Color Change and Ag⁺-Releasing Behavior of OCP−Ag. As a further study, we evaluated the change in color of OCP−Ag as a function of its Ag⁺ content. Figure 9 shows the reconstructed color of samples before immersion and after immersion in each investigated medium, as measured using a color meter. In the cases of OCP−Ag without Ag₃PO₄, the color of the samples after immersion in various media changed little compared with that of calcium phosphate without Ag⁺. By contrast, samples containing Ag₃PO₄ exhibited a substantial color change after immersion.

As an additional evaluation method, ICP−AES was used to analyze the Ag content in the media after the samples were immersed to measure the Ag⁺ released from the samples. Notably, the medium and temperature conditions of immersion testing were the same as those used in the in vitro cell and bacterial culture experiments; however, for immersion testing, a quicker shaking process (200 rpm) was also employed to avoid precipitation of the sample. Therefore, we considered the experimental conditions of immersion testing to be stricter than those for the in vitro cell and bacterial culture experiments. Table 1 shows the Ag content of each medium after sample immersion. Except in the case of samples containing Ag₃PO₄, the Ag contents were near the detection limit of our ICP−AES instrument (~1 ppm). We also evaluated the fine structures of OCP−Ag samples after immersion to confirm whether insoluble Ag salts such as AgCl formed. We hardly detected any significant morphological change in the OCP−Ag samples except for some slight dissolution. We concluded that a small amount of Ag⁺ was released from the OCP−Ag into the media.

2.3. Antibacterial Evaluation of OCP−Ag. The aforementioned experiments indicated that OCP−Ag fabricated from suitable Ag-containing solutions exhibited only a slight color change and released only a small amount of Ag⁺ when immersed in various media. These phenomena suggest that Ag⁺ was tightly substituted into the OCP unit lattice in OCP−Ag. We therefore investigated the antibacterial properties of OCP−Ag to evaluate its suitability for use as a biomaterial. In this study, we selected three pathogens: S. aureus, Escherichia coli, and Streptococcus mutans.

Figure 10 shows the results of proliferation tests of each bacterium culture medium in the presence of OCP−Ag samples. With increasing Ag⁺ content of the OCP−Ag, the turbidity of the medium markedly decreased.

In addition, fine structures of OCP−Ag samples after bacteria proliferation were observed by SEM to evaluate the
adhesive antibacterial ability of OCP−Ag (Figure 11). In the cases of Ag-free samples, each bacterium proliferated under agitation and adhered onto the OCP−Ag powder surface while maintaining its unique shape. However, in the cases of Ag-containing samples, partial attachment of bacteria was observed. In addition, the shape of each bacterium was sometimes collapsed. In the case of OCP−Ag with higher Ag+ contents (medium-transparent conditions), bacteria on samples were scarcely observed.

Colony counts provided a quantitative measure of bacterium proliferation. Figure 12 shows the agar plates of cultured samples and each bacterium (a−c) and the corresponding logarithmic colony-forming unit (log CFU) calculated from agar plates cultured with each sample and each bacterium (d). In the cases of S. mutans, the LogCFU decreased linearly with increasing Ag+ content in the OCP−Ag. In the case of E. coli and S. aureus, colony counts indicated that OCP−Ag samples with a low Ag+ content exhibited weak antibacterial activity, whereas antibacterial activity increased substantially above a threshold Ag+ content. Furthermore, the threshold Ag+ content in OCP−Ag, especially for E. coli, corresponded to a slight color change in the medium-immersion tests.

2.4. Cytotoxicity Evaluation of OCP−Ag. Although the fabricated OCP−Ag exhibited both excellent antibacterial activity and slight color changes depending on the Ag content, for the OCP−Ag to be used as a biomaterial, its cytotoxicity should be evaluated. Notably, we employed NP materials in cytotoxicity evaluations. Indeed, nanoparticulate calcium phosphates exhibit cytotoxic properties toward osteoblast cells. Nevertheless, NPs of samples were suitable for evaluation because they were easy to uptake into cells. Figure 13 shows the cell viabilities of undifferentiated MC3T3-E1 cells and differentiated MC3T3-E1 cells measured by WST-1 assay. We used the WST-1 assay for evaluation instead of the MTT assay because the WST-1 assay is better suited to evaluating NPs because the formed formazan is soluble. In the case of undifferentiated MC3T3-E1 cells, except in the case of OCP−Ag(Ag500), which contained Ag3PO4 as a residual material, the viabilities of undifferentiated MC3T3-E1 cells ranged from 75 to 125% of that of the control and were similar to that of HAp. No relation between the Ag content of the samples and the viability of cells was evident, except in the case of OCP−Ag(Ag500). In the case of differentiated MC3T3-E1
cells, the cell viabilities gradually decreased with increasing Ag content in the OCP−Ag, especially in low-dose cases (10 μg/mL). Nevertheless, the viabilities of OCP−Ag were greater than or the same as that of HAp. Therefore, the cytotoxicity of OCP−Ag toward undifferentiated MC3T3-E1 cells and differentiated MC3T3-E1 cells was the same as that of HAp.

The aforementioned biological evaluations indicate that when OCP−Ag is prepared using a solution containing approximately 10 mmol/L Ag⁺ via a hydrolysis process of DCPD, the obtained OCP−Ag exhibits properties that render it suitable as a bone substitute, as indicated by its antibacterial activity toward resident oral bacteria, low cytotoxicity, and slight color change. An advantage of our OCP−Ag fabrication method is that the final Ag⁺ content of the OCP−Ag can be easily controlled by varying the Ag⁺ concentration of the treatment solution. In this study, we fabricated powder-formed OCP−Ag exhibiting both high contact antibacterial ability and low cytotoxicity. Thus, the hybridization process OCP−Ag and a suitable binder would enable its application on antibacterial

Figure 7. SEM micrographs of the treated samples. (a,b) [Ag⁺] = 0 mmol/L (OCP-law), (c,d) [Ag⁺] = 10 mmol/L, (e,f) [Ag⁺] = 20 mmol/L, (g,h) [Ag⁺] = 50 mmol/L, and (i,j) [Ag⁺] = 500 mmol/L.

Figure 8. STEM-EDX mapping images of samples treated in a solution with [Ag⁺] = 20 mmol/L: (a) bright-field (BF) image, (b) Ca, (c) P, and (d) Ag.

Figure 9. Color changes of OCP−Ag before and after immersion into media.

Table 1. Ag Content in Media after Immersion Testing (ppm)

| Sample               | PBS | α-MEM | Bouillon |
|----------------------|-----|-------|----------|
| medium               | 0.00| 0.20  | 0.00     |
| OCP-law              | 0.00| 0.00  | 0.00     |
| OCP-Ag(Ag1)          | 0.00| 0.42  | 0.02     |
| OCP-Ag(Ag5)          | 0.00| 1.02  | 0.04     |
| OCP-Ag(Ag10)         | 0.00| 0.94  | 1.12     |
| OCP-Ag(Ag20)         | 0.00| 0.64  | 1.99     |
| OCP-Ag(Ag30)         | 0.00| 0.62  | 1.62     |
| OCP-Ag(Ag50)         | 0.90| 0.62  | 8.12     |
| OCP-Ag(Ag500)        | 2.72| 0.58  | 29.1     |
| OCP-Na               | 0.00| 0.00  | 0.92     |
| HAp                  | 0.00| 0.00  | 0.16     |
arrows indicate bacterial bodies.

Figure 10. Photographs of medium suspensions containing OCP–Ag after bacteria culture testing: (a) E. coli, (b) S. aureus, and (c) S. mutans.

equipment not only biomaterials but also scaffolds, building materials, wallpapers, and furniture.

Although the literature includes some previous studies in which calcium phosphate–Ag composites have been used as antibacterial agents for biomaterials, these previous approaches suffer several disadvantages. For example, they require an unstable medium during fabrication, involve advanced laser techniques, or result in the formation of Ag\(^{0}\) NPs on/in calcium phosphate.\(^{45}\) Notably, our method involves a simple fabrication process using popular reagents and does not require advanced equipment. In addition, OCP is a metastable phase of calcium phosphate and can be easily converted into other calcium phosphate phases such as HAp, carbonate apatite, and β-tricalcium phosphate (β-TCP).\(^{46–49}\) Therefore, various calcium phosphates containing Ag could be introduced as antibacterial agents.

3. CONCLUSIONS

In this study, we demonstrated a one-pot synthesis of Ag\(^{+}\)-substituted OCP through a hydrolysis process of DCPD, which is a widely commercialized and highly bio-compatible material, using solutions containing complexed Ag\(^{+}\) ions. The Ag\(^{+}\) content of the fabricated OCP–Ag could be controlled by simply varying the Ag\(^{+}\) concentration of the reaction solution. Ag\(^{+}\) tightly substituted into the OCP crystal structure at the P5 \(\beta\) PO\(_4\)-conjugated sites. Biological evaluations showed that the OCP–Ag exhibits excellent antibacterial activity toward resident oral bacteria and low cytotoxicity toward fibroblast and osteoblast cells.

4. METHODS AND MATERIALS

4.1. Fabrication of Ag-Substituted OCP (OCP–Ag). All reagents were purchased from FUJIFILM Wako Chemical Corp., Japan. \((\text{NH}_4)\text{H}_2\text{PO}_4\) and AgNO\(_3\) were codissolved in distilled water to form solutions of 1.0 mol/L \((\text{NH}_4)\text{H}_2\text{PO}_4\) with 0–500 mmol/L AgNO\(_3\).

A 2.39 g sample of DCPD (14 mmol) was immersed in 20 mL of the solutions at 60 °C for 1 day. The initial and final pH values of the solutions were measured at room temperature using a pH electrode (LAQUA TouPH 9615S-10D electrode connected to a D-72 pH meter, Horiba Co., Kyoto, Japan). The treated samples were washed with distilled water several times to remove the residual solutions and then placed in a drying oven at 40 °C overnight.

4.2. Characterization. The crystallographic properties of the samples were characterized by XRD (MiniFlex600, Rigaku Co., Japan) at an acceleration voltage of 40 kV and a current of 15 mA. The diffraction angle was continuously scanned over the range 3° ≤ 2\(θ\) ≤ 70° at a scanning rate of 2°/min for characterization and over the range 2° ≤ 2\(θ\) ≤ 12° at a scanning rate of 0.8°/min for crystallographic parameter analysis. The integral intensities of each peak (\(I_x\); \(x =\) each peak) in the XRD patterns of the samples were calculated via quantitative analysis using the PDFX2 software (Rigaku Co., Japan). The relative rate of the yield in each sample was calculated from the \(d_{(100)}\) and \(d_{(200)}\) spacings of the OCP unit lattice at around 4.7 and −9.4° in the XRD patterns, respectively. The proportion of each phase (\(R_x\); \(x =\) phase or intensity of peaks) in the samples was calculated as

\[
R_x = \frac{C I_x}{I_{\Sigma}}
\]

where \(C\) is the phase or diffraction coefficient (here, \(C = 1.00\)).

The chemical bonding structure of the samples was characterized via FT-IR spectroscopy (Nicolet Nexus670, Thermo Fisher Scientific Co., USA) using a triglyceride sulfate detector (64 scans, 2 cm\(^{-1}\) resolution) with a GeSe attenuated total reflection prism. The spectra were recorded under an air atmosphere.

Ag contents and chemical compositions (Ca and P (PO\(_4\))) of the samples were measured by ICP–AES (S1100VDV, Agilent Technology Co., Japan) after the samples were dissolved in 2% HNO\(_3\). The chemical states of samples were...
characterized using an X-ray photoelectron spectrometer (XPS: K-Alpha, Thermo Fisher Scientific Co., USA) equipped with a monochromatic X-ray source (Al Kα) operated at 12 kV and 6 mA; the pressure in the sample chamber was $\sim 1.4 \times 10^{-6}$ Pa. The spot size of the incident beam was set to $400 \mu$m.

The binding energies were normalized to the C 1s peak at 284.80 eV. Before measurements, the samples were embedded into light-curing resin that consisted of 90% hydroxyethyl methacrylate (HEMA, FUJIFILM Wako Pure Chem Inc., Japan) and 10% diurethane dimethacrylate (UDMA, Sigma-Aldrich Co., USA) with camphorquinone and 4-(dimethylamino)benzoic acid ethyl ester (Sigma-Aldrich Co., USA) as initiator agents.

The fine structure of the samples was observed by field-emission SEM (FE-SEM, JSM-6700F, JEOL Co., Japan) at an acceleration voltage of 3 kV after the samples were sputter-coated with Os. The fine atomic distribution of the samples was observed by STEM−EDX (JEM-ARM200F, JEOL Co., Japan) at an acceleration voltage of 200 kV.

4.3. Color-Change Testing of OCP−Ag. Four hundred milligrams of the prepared OCP−Ag powders with different Ag contents was immersed into each investigated medium of phosphate-buffered saline (PBS: FUJIFILM Wako Pure Chem Inc., Japan), minimal essential medium with no phenol red ($\alpha$-MEM: Thermo Fisher Scientific Co., USA), and brain heart infusion broth (bouillon medium) (PEARLCORE: Eiken Chem Ind. Co., Japan). The resultant mixtures were shaken on a thermostatic shaker (BioShaker BR-23FP, Taitec Co., Japan) at 200 rpm and 40 °C for 3 days.

The treated media were centrifuged and subsequently filtered through a 220 nm syringe filter to remove particles. The filtered media were then diluted with 2% HNO$_3$ for subsequent measurement of their Ag content by ICP−AES.

The treated OCP−Ag samples were centrifuged and then washed with distilled water and dried using the same procedure used to prepare the original OCP−Ag samples. The color of the OCP−Ag samples was quantitatively measured using a colorimeter (ZE-2000, Nippon Denshoku Ind. Co., Japan) as a reflecting method.

4.4. Antibacterial Testing. The bacterial evaluation was approved by the AIST microbial experiment ethical committee (approval no. B2019-110 and, B2019-113). The bacterial strains S. aureus NBRC100910$^T$, E. coli NBRC102203$^T$, and S. mutans NBRC13955$^T$ were purchased from the NITE Biological Resource Center (NBRC), National Institute of Technology and Evaluation (NITE, Japan) as type culture strains. The dried bacterial stocks were thawed and cultured in bouillon medium.

Figure 12. (a) Agar plates after bacteria culture testing. (b) log CFU values calculated from colony-count results. Red: S. mutans; blue: E. coli; and green: S. aureus. **: $p < 0.05$ toward the results of the OCP-law (Ag-0) sample.

Figure 13. Cell viabilities of (a) undifferentiated MC3T3-E1 cells and (b) differentiated MC3T3-E1 cells cultured with OCP−Ag. Red: 100 and blue: 10 μg/mL. **, *: $p < 0.05$ toward the control and HAp cases, respectively.
Approximately, $1.0 \times 10^6$ of each bacterium were dispersed into 4 mL of OCP–Ag bimetallic-medium suspensions (0.1 g/mL) in L-shaped glass tubes. The vessels with samples were settled in a shaking incubator at 37 °C and at a shaking rate of 100 rpm for 24 h. The treated samples were separated into reagents and precipitates. The precipitates were washed with PBS several times to remove residual reagents and then soaked in 10% formalin neutral buffer solution (FUJIFILM Wako Pure Chem Inc., Japan) overnight at 4 °C. After incubation, the solutions were poured into a sequence of ethanol solutions with concentrations starting from 70% and allowed to incubate in each solution for several hours. The poured materials were then placed on a sample stage and observed by FE-SEM in the same manner described in Section 4.2.

The reagents of cultured bacteria were diluted with PBS and then 0.2 mL aliquots of the diluted reagents were dispersed onto agar plates for colony-number evaluation. The treated agar plates were incubated at 37 °C for 24 h.

4.5. Cytotoxicity Evaluation. Mouse calvarium-derived fibroblast-like (MC3T3-E1) cells were purchased from the RIKEN BioResource Center, Japan. The MC3T3-E1 cells were cultured in α-MEM (Thermo Fisher Scientific Inc., USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS: HyClone Laboratories, GE Healthcare, USA), 100 units/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B (antibiotics mixture: Nacalai Tesque Inc., Japan). The MC3T3-E1 cells were differentiated into osteoblast cells by treatment with ascorbic acid, hydrocortisone, and β-glycerophosphate using an osteoblast-inducer reagent (for animal cells) (Takara Bio Inc., Japan).

To measure cell viability, 10^5 cells were seeded in a 96-well plate ($n = 3$). They were incubated for 24 h and then the culture medium was removed. Subsequently, differentiation medium with OCP–Ag samples was applied at a concentration of 10 and 100 μg/mL, and the cells were incubated for an additional 1 (undifferentiated MC3T3-E1 cells) or 7 days (differentiated MC3T3-E1). The cell viability was determined as mitochondrial activity. The WST-1 assay was conducted in vitro (di

The authors declare no competing financial interest.

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