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Antibacterial effect of a fluoride-containing ZnO/CuO nanocomposite

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ABSTRACT

Dental materials that are antimicrobial and acid-resistant can inhibit bacterial colonization and demineralization, thereby preventing caries. Zinc and copper are well-known for their antibacterial effect, as is nanostructured ZnO–CuO composite. Minerals such as fluorine and calcium, can remineralize and demineralize teeth. Therefore, we developed novel fluoride-containing ZnO–CuO (ZCF) nanocomposites; to the best of our knowledge, these are the first nanocomposites of this kind. The fluoride concentrations and antibacterial effects of the ZCF nanocomposites were evaluated.

Nanocomposites comprising zinc and copper (ZC), and zinc, copper, and fluorine (ZCF), were prepared by a simple one-step homogeneous coprecipitation method at a low temperature (80 °C), without the use of organic solvent or surfactant.

The structure and composition of the ZC and ZCF nanocomposites were examined by scanning electron microscopy–energy-dispersive spectroscopy (SEM-EDS). Quantitative analysis of the mass concentration was performed by using ZAF correction methods. The fluorine content in nanocomposites was evaluated by using proton-induced gamma emission (PIGE) at the Takasaki Advanced Radiation Research Institute in Japan. By using 96-well microtiter plates, we analyzed the antibiotic susceptibility of ZC, ZCF, and the control buffer (phosphate-buffered saline) with *Streptococcus mutans* (ATCC 25175).

The SEM images showed that ZC and ZCF nanocomposites were composed of 3D flower-like microstructures with diameters of approximately 1 μm. Environmental SEM-EDS analysis revealed that ZC contained 43.2% Cu, 55.1% Zn, 2.2% F, and 0.1% Cl, whereas ZCF contained 47.5% Cu, 40.5% Zn, 6.7% F, and 5.9% Cl.

Analysis by PIGE showed that ZCF nanocomposite contained 2553.6 ± 199.2 ppm fluorine, whereas no fluoride was detected in ZC. The control buffer enabled bacterial growth to $4 \times 10^7 \pm 9 \times 10^6$ CFU/mL, whereas ZC allowed growth of 12 ± 8 CFU/mL, and ZCF showed no bacterial growth.

Thus, we developed novel fluoride-containing ZnO–CuO nanocomposites, which exhibited antibacterial effects and have the potential for remineralization, thereby demonstrating their potential as multifunctional dental materials.

Abbreviations: ZAF, atomic number, absorption, fluorescence; PIGE, proton-induced gamma emission; SEM-EDS, scanning electron microscopy–energy-dispersive spectroscopy; ZC, zinc and copper; ZCF, zinc, copper, and fluorine

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1. Introduction

The exploration of new dental materials is a research topic currently of great interest because of dental caries, periodontal disease, and halitosis, in relation to both oral health and systemic diseases, such as cardiovascular disease and bacterial endocarditis. Thus, many antibacterial materials have been discovered and applied to clinical use. Notably, dental caries and periodontal problems are among the most prevalent oral diseases worldwide. Acidogenic bacteria, such as *Streptococcus mutans*, are regarded as a contributory factor in the formation of dental caries [1].

Zinc is known to inhibit demineralization of teeth, enhance remineralization of dentin, and prevent dental caries by inhibiting the growth of oral bacteria [2–4]. Matrix metalloproteinases (MMPs), which degrade collagen fibers, require zinc as a co-factor; however, high concentrations of zinc inhibit the activity of MMPs. In addition to protecting collagen from MMPs [5,6], zinc may also influence signaling pathways and stimulate hard tissue mineralization. Furthermore, the presence of zinc may protect seed crystallites on collagen fibrils for later dentin remineralization [7]. Zinc salt has been commonly used clinically, but its effects are limited by poor retention because its levels decrease drastically within 1 h after the application of intraoral treatment [8].

Copper is also regarded as an inhibitor of MMPs in human dentin [9]. Copper nanoparticles have been applied to various fields, including biomedical equipment and devices [10]. In addition, zinc-copper oxide (Zn-CuO) nanocomposites reportedly exhibit weaker embryotoxicity, compared with zinc oxide (ZnO) and CuO [9]. Therefore, Zn-CuO nanocomposites may constitute safer antibacterial metal nanocomposites.

Furthermore, sodium fluoride is known to inhibit demineralization and enhance remineralization of enamel and dentin [11–14]. Application of fluorine has been shown to inhibit the effects of MMP-2 [15]; therefore, fluoride compounds can inhibit both demineralization of dentin and breakdown of collagen fibers and degradation of dentin, suggesting they are advantageous for both remineralization and organic collagen stability. Therefore, development of fluorine-containing metal nanocomposites is a promising endeavor.

The use of adjunctive methods, such as mouthwashes, is useful for the prevention of plaque accumulation [16]. Routine mouth rinses, including chlorhexidine, exhibit antibacterial effects, but may cause staining of the tooth surface and mucosal irritation. Therefore, an alternative antimicrobial agent with minimal side effects seems necessary.

Nanotechnology has been introduced to the field of dental materials in recent years, and nanoparticles have been inserted into the structure of dental composites [17,18]. Therefore, cytotoxic properties of nanoparticles require further research. Moreover, bioavailability and stability of nanoparticles as therapeutic delivery systems must be investigated, along with discoloration effects and cosmetic changes that have been observed during use of some nanoparticles.

Thus far, there have been no studies regarding development of fluoride-containing nanoparticles that include determinations of the antimicrobial effects of nanoparticles. The present study aimed to develop and characterize new fluorine-containing nanoparticles, and to investigate the bactericidal and bacteriostatic effects of colloidal solutions containing ZnO, CuO, and fluorine nanoparticles on *S. mutans*.

2. Material and methods

2.1. Sample preparation

ZnO-CuO nanocomposites (ZC) were prepared as in a previous study [19]. In particular, 2.0 mmol $ZnCl_2$, 1.0 mmol $CuSO_45H_2O$, and 10.0 mmol NaOH were dissolved in 40 mL distilled water under stirring. To synthesize fluoride-containing ZnO-CuO nanocomposite (ZCF), 2.0 mmol $ZnCl_2$, 1.0 mmol $CuSO_4{\cdot}5H_2O$, 6.0 mmol NaF, and 10.0 mmol NaOH were dissolved in 40 mL distilled water under stirring. The mixed solution was maintained at 80 °C for 12 h, and subsequently cooled to room temperature naturally. The products were separated by centrifugation at 96 RCF for 5 min, washed with distilled water and absolute alcohol several times to remove possible residues, and then dried at 80 °C for 12 h.

2.2. Scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDS)

Samples in absolute ethanol were mounted on an aluminum stub with uncoated carbon tape for SEM and EDS. SEM was used to analyze particle morphology and size distribution at ×45 K and ×110 K magnification by using an S-4800 (Hitachi, Tokyo, Japan) scanning electron microscope at 5 kV. EDS spectra of samples, 100 μm × 100 μm area, were recorded by using a S-2380 N (Hitachi) scanning microscope system with a Genesis G4000 (EDAX Japan) detector at 15 kV. The resulting X-Ray spectra were used for identification of the minerals and particle compositions. The elemental analysis % weight of samples was determined by applying the ZAF correction method.

2.3. Fluoride concentration by PIGE and PIXE techniques

Fluorine was analyzed by using a proton-induced gamma-ray emission (PIGE) technique at the Takasaki Ion Accelerators for Advanced Radiation Application (TIARA) [11]. Micro-PIGE/PIXE analysis was performed as previously described [20]. In brief, a 3.0-MeV proton beam was emitted from an ion microbeam apparatus. Each sample was placed on titanium and set within the window at the end of the microbeam system. The beam spot was approximately 1 μm in diameter, and the beam current was approximately 100 pA. The proton beam in ambient air bombarded the sample. The maximum scanned area was 1000 $μm^2$. A nuclear reaction (i.e., 19F (p,αγ)160) was used to measure the fluorine concentration; the generated gamma rays were detected with an 81-cm^2 sodium iodide detector, which was placed 5 mm behind the sample. Fluorine concentrations were measured by micro-PIXE, which was simultaneously performed with a silicon-lithium detector in the vacuum. The beam intensity was monitored by the X-ray yield from a copper foil for quantitative elemental analysis; quantitative analysis of trace elements in PIGE/PIXE is based on the counts of characteristic gamma/X-rays discharged from specimens. Quantitative analyses of fluorine were performed as previously described [11]. For quantitative analysis, the beam intensity was monitored with the X-ray yield from a copper foil by switching the beam onto the foil for 3 s every 30 s.

2.4. Antibacterial effect analysis

2.4.1. Bacterial cultures and growth conditions

In all experiments, *S. mutans* (ATCC 25175) was grown anaerobically at 37 °C in brain heart infusion medium.

2.4.2. Antibacterial test

Overnight cultures of test bacteria were diluted and adjusted in fresh media to 5×10^5 cells/mL; 500 μL of the suspension was placed in each well of a 96-well plate. Samples of ZC and ZCF (500 μL at 1.0 mg/mL) and sterile phosphate-buffered saline control solution (500 μL) were added to the 96-well plated and incubated for 24 h at 37 °C. Subsequently, 100 μL of bacterial suspension from each group (ZC, ZCF, and Control) was used to inoculate an agar plate, which was then incubated under aerobic conditions at 37 °C for 5 days. The numbers of viable *S. mutans* colonies were counted after the incubation period. The bacterial growth of each group was analyzed by using the Games-Howell test ($p < 0.05$).

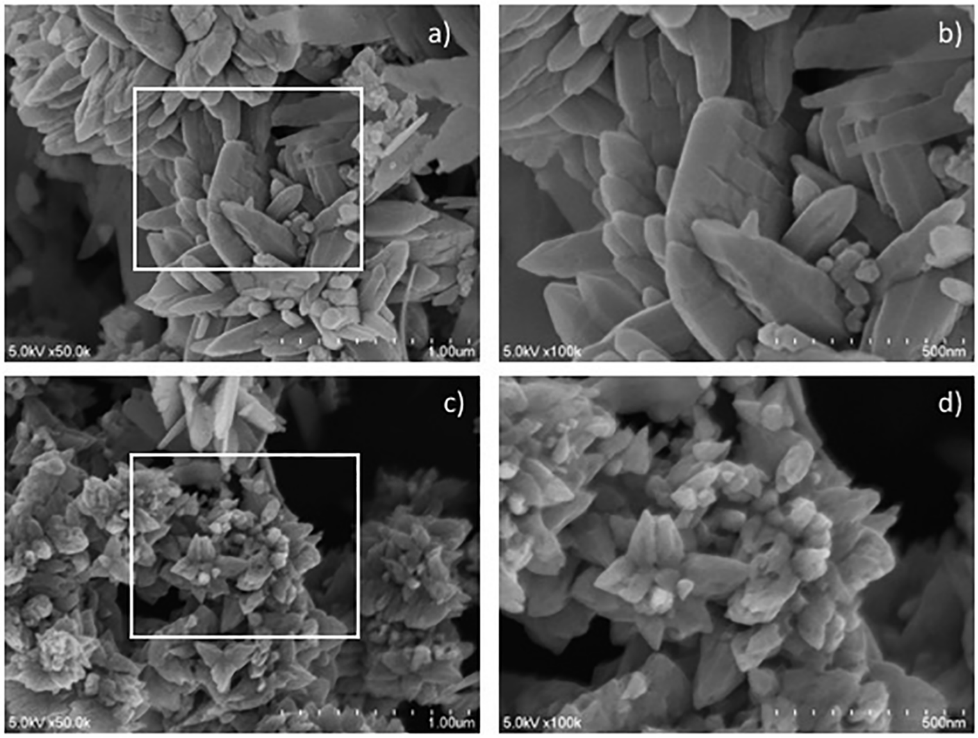

Fig. 1. SEM images of prepared (a, b) ZnO–CuO nanocomposite (ZC) and (c, d) fluorine-containing ZnO–CuO nanocomposite (ZCF).

3. Results

The morphological and elemental compositions of ZC and ZCF were revealed through SEM and EDX analysis. Fig. 1 shows SEM micrographs of ZC and ZCF, which indicated that the particles were firmly attached together and agglomerated. Each of the ZC nanoparticles was approximately 500 nm in size and exhibited a rugged rod-like structure (Fig. 1a and b). In contrast, ZCF nanoparticles were approximately 100 nm in size and exhibited square crystal structures (Fig. 1c and d). A fluorine peak was observed only in ZCF nanoparticles (Fig. 2).

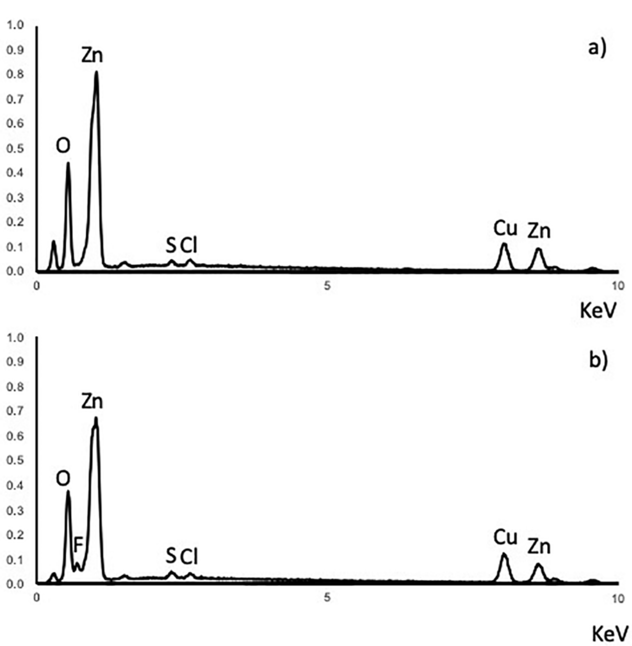

Fig. 2. EDS spectra of the ZnO–CuO nanocomposite (ZC) and fluorine-containing ZnO–CuO nanocomposite (ZCF).

Quantitative analysis by the ZAF method showed that the weight ratios of ZC were Zn: 43.2 ± 1.5%; Cu: 55.1 ± 2.1%; F: 2.2 ± 0.7% and Cl: 0.1 ± 0.1%, while those of ZCF were Zn: 40.6 ± 1.7%; Cu: 47.5 ± 1.0%; F: 6.7 ± 0.8%, and Cl: 5.9 ± 1.5%. Mineral mapping of ZC by PIXE analysis showed relatively uniform spreading of zinc and copper (Fig. 3); PIGE detected a fluorine signal, but quantitative analysis showed 9.0 ± 6.7 ppm fluorine. Mineral mapping of ZCF by PIXE analysis showed heterogeneous spreading of zinc and copper; fluorine quantitative analysis by PIGE showed 2553.6 ± 199.2 ppm fluorine.

We examined the activity of ZC and ZCF on *S. mutans* growth in suspension. Bacterial cultures of *S. mutans* were incubated with ZC and ZCF over a period of 24 h. The control group demonstrated $4.1 \times 10^7 \pm 8.4 \times 10^6$ CFUs/mL of *S. mutans*. Both ZC and ZCF nanocomposites (1.0 mg/mL concentrations) showed antibacterial effects: incubation with ZC resulted in 12.0 ± 7.5 CFUs/mL of bacterial growth, whereas incubation with ZCF resulted in no growth (Fig. 4).

4. Discussion

Within the limitations of this study, our results showed that both ZC and ZCF nanoparticles were less than 500 nm in size, and both showed antibacterial effects. A previous study reported inhibition of *S. mutans* biofilm formation on teeth by sonochemical coating with ZnO and CuO nanoparticles [21]. However, high concentrations of ZnO and CuO nanoparticles (1.0 mg/mL) did not result in the growth inhibition of *S. mutans*. The procedure used to make nanocomposites in this experiment was based on a previous study [19], but we modified the mixing method and time. Consequently, the nanocomposites were smaller.

Fluorine is one of the most critical elements for dental research and clinical treatment. Therefore, we aimed to develop new nanocomposites that included fluorine; by first adding sodium fluoride, we generated new fluoride-containing nanocomposites. Of the known dental materials, glass ionomer cement, which releases fluorine, is well-known as an antibacterial material [22]. ZCF includes almost 3000 ppm fluorine; this is higher than the concentration of dentifrice, which inhibits MMP activity [15].

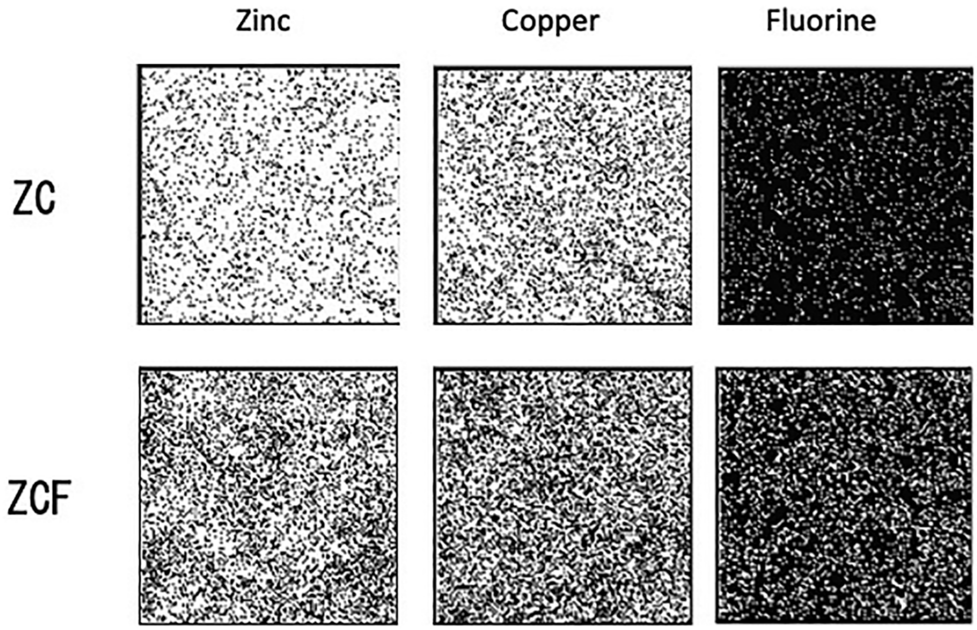

Fig. 3. Elemental PIXE map (zinc and copper) and PIGE map (fluorine). White dots in the maps (250 µm × 250 µm area) represent PIXE or PIGE signals from zinc, copper, and fluorine, respectively.

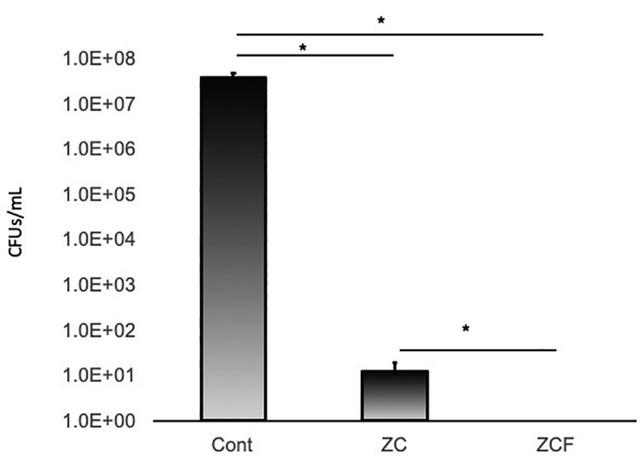

Fig. 4. Numbers of viable bacteria after the incubation period. Asterisk indicates significantly different at $P < 0.05$ by the Games-Howell test. The bacterial growth-inhibiting effects significantly differed among ZC, ZCF, and Control groups; notably, ZCF did not allow any growth of *Streptococcus mutans*.

Comparing fluorine analysis between PIGE and EDS, PIGE is more sensitive, because the background of the EDS method is high in lower energy areas related to light elements, such as fluorine and boron. We have continuously optimized the quantitative analysis of fluorine [23], so the difference between EDS and PIGE data is based on the detection limit of EDS analysis. Therefore, light elements, such as fluorine and boron, should be analyzed with the PIGE technique.

Zinc is already used as a commercial dental product because of its antibacterial effects. Some mouthwashes include zinc chloride [24]. Additionally, zinc gluconate was reported to significantly reduce the duration of symptoms of the common cold [25]. Among many therapeutic ions, copper is considered a potent inhibitor of MMPs in human dentin [9]. Copper is required crosslink collagen in bone [26], and the structure of dentin is similar to bone. To protect dentin structure, treatments should inhibit bacterial activity, protect collagen structure, prohibit demineralization, and enhance remineralization. Therefore, each of the above elements should be included in dental materials.

Antibacterial properties of some nanoparticles, such as silver and gold, have been verified in previous studies [27] and different mechanisms have been proposed to contribute to their effects. Silver nanoparticles inhibit enzymes of the cell respiratory cycle and damage deoxyribonucleic acid (DNA) synthesis [28]. Hernández-Sierra et al. [27] indicated that silver nanoparticles inhibit the growth of *S. mutans* at lower concentrations, compared to zinc and gold, and should therefore more effectively inhibit dental caries.

It is assumed that the mechanism of action of copper nanoparticles is similar to that of silver nanoparticles. Copper ions adhere to DNA molecules and form crosslinks within and between nucleic acid chains, thus disrupting the helical structure of the DNA. Moreover, copper ions impair the biochemical processes of bacterial cells. Combinations of silver and copper nanoparticles may provide a complete bactericidal effect against mixed bacterial populations [29].

The copper/zinc ratio of ZC is 1.28, while that of ZCF is 1.17; ZCF showed stronger antibacterial effects than ZC. Malka et al. reported that CuO has a stronger antibacterial effect than ZnO, suggesting that copper has an antibacterial impact equal to that of silver [30]. However, our results showed that ZCF, which has a lower copper concentration, has a stronger antibacterial effect than ZC. These results indicate that each element has a different antibacterial effect. Therefore, it is critical to determine the correlation of elements with biological effects, such as antibacterial activity, enzyme inhibition, and biomineralization.

It should be noted that complete simulation of the oral cavity is not possible in laboratory conditions. The incubator cannot completely recapitulate the mouth temperature. Furthermore, antibacterial agents constantly contact bacterial microorganisms in culture media, but the contents of mouthwashes are diluted and neutralized immediately in the oral cavity.

In conclusion, we developed novel fluoride-containing ZnO–CuO nanocomposites, which exhibit antibacterial effects and have the potential for remineralization through the inclusion of fluorine. Therefore, they show potential to serve as multifunctional dental materials.

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Declaration of Competing Interest

None.

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