

1 **Antibacterial effect of a fluoride-containing ZnO/CuO nanocomposite**

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1     **Abstract**

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

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

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

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

1 40.5% Zn, 6.7% F, and 5.9% Cl.

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

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

8

9 Keywords: nanocomposites, antibacterial compounds, remineralization, bacterial growth, dental  
10 materials

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

## 1     **Introduction**

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

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

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

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

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

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

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

1

## 2 **Material and Methods**

### 3 *2.1 Sample preparation*

4 ZnO-CuO nanocomposites (ZC) were prepared as in a previous study (19). In particular, 2.0 mmol  
5 ZnCl<sub>2</sub>, 1.0 mmol CuSO<sub>4</sub>·5H<sub>2</sub>O, and 10.0 mmol NaOH were dissolved in 40 mL distilled water under  
6 stirring. To synthesize fluoride-containing ZnO-CuO nanocomposite (ZCF), 2.0 mmol ZnCl<sub>2</sub>, 1.0  
7 mmol CuSO<sub>4</sub>·5H<sub>2</sub>O, 6.0 mmol NaF, and 10.0 mmol NaOH were dissolved in 40 mL distilled water  
8 under stirring. The mixed solution was maintained at 80°C for 12 hours, and subsequently cooled to  
9 room temperature naturally. The products were separated by centrifugation at 96 RCF for 5 minutes,  
10 washed with distilled water and absolute alcohol several times to remove possible residues, and then  
11 dried at 80°C for 12 hours.

12

### 13 *2.2 Scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDS).*

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

1

## 2 *2.3 Fluoride concentration by PIGE and PIXE techniques*

3 Fluorine was analyzed by using a proton-induced gamma-ray emission (PIGE) technique at the Takasaki Ion  
4 Accelerators for Advanced Radiation Application (TIARA) (11). Micro-PIGE/PIXE analysis was performed  
5 as previously described (20). In brief, a 3.0-MeV proton beam was emitted from an ion microbeam apparatus.  
6 Each sample was placed on titanium and set within the window at the end of the microbeam system. The beam  
7 spot was approximately 1  $\mu\text{m}$  in diameter, and the beam current was approximately 100 pA. The proton beam  
8 in ambient air bombarded the sample. The maximum scanned area was 1000  $\mu\text{m}^2$ . A nuclear reaction (i.e.,  $^{19}\text{F}$   
9  $(p,\alpha\gamma)^{16}\text{O}$ ) was used to measure the fluorine concentration; the generated gamma rays were detected with an  
10 81-cm<sup>2</sup> sodium iodide detector, which was placed 5 mm behind the sample. Fluorine concentrations were  
11 measured by micro-PIXE, which was simultaneously performed with a silicon-lithium detector in the vacuum.  
12 The beam intensity was monitored by the X-ray yield from a copper foil for quantitative elemental analysis;  
13 quantitative analysis of trace elements in PIGE/PIXE is based on the counts of characteristic gamma/X-rays  
14 discharged from specimens. Quantitative analyses of fluorine were performed as previously described(11). For  
15 quantitative analysis, the beam intensity was monitored with the X-ray yield from a copper foil by switching  
16 the beam onto the foil for 3 s every 30 s.

17

## 18 *2.4 Antibacterial effect analysis*

### 19 2.4.1 Bacterial Cultures and Growth Conditions

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



1

## 2 2.4.2 Antibacterial Test

3 Overnight cultures of test bacteria were diluted and adjusted in fresh media to  $5 \times 10^5$  cells/mL; 500  $\mu$ L of the  
4 suspension was placed in each well of a 96-well plate. Samples of ZC and ZCF (500  $\mu$ L at 1.0 mg/mL) and  
5 sterile phosphate-buffered saline control solution (500  $\mu$ L) were added to the 96-well plated and incubated for  
6 24 hours at 37°C. Subsequently, 100  $\mu$ L of bacterial suspension from each group (ZC, ZCF, and Control) was  
7 used to inoculate an agar plate, which was then incubated under aerobic conditions at 37°C for 5 days. The  
8 numbers of viable *S. mutans* colonies were counted after the incubation period. The bacterial growth of each  
9 group was analyzed by using the Games-Howell test ( $p < 0.05$ ).

10

## 11 **3. Results**

12 The morphological and elemental compositions of ZC and ZCF were revealed through SEM and EDX analysis.  
13 Fig. 1 shows SEM micrographs of ZC and ZCF, which indicated that the particles were firmly attached  
14 together and agglomerated. Each of the ZC nanoparticles was approximately 500 nm in size and exhibited a  
15 rugged rod-like structure (Fig. 1a and b). In contrast, ZCF nanoparticles were approximately 100 nm in size  
16 and exhibited square crystal structures (Fig. 1c and d). A fluorine peak was observed only in ZCF nanoparticles  
17 (Fig. 2). Quantitative analysis by the ZAF method showed that the weight ratios of ZC were Zn:  $43.2 \pm 1.5\%$ ;  
18 Cu:  $55.1 \pm 2.1\%$ ; F:  $2.2 \pm 0.7\%$  and Cl:  $0.1 \pm 0.1\%$ , while those of ZCF were Zn:  $40.6 \pm 1.7\%$ ; Cu:  $47.5 \pm 1.0\%$ ;  
19 F:  $6.7 \pm 0.8\%$ , and Cl:  $5.9 \pm 1.5\%$ . Mineral mapping of ZC by PIXE analysis showed relatively uniform spreading  
20 of zinc and copper (Fig. 3); PIGE detected a fluorine signal, but quantitative analysis showed  $9.0 \pm 6.7$  ppm

1 fluorine. Mineral mapping of ZCF by PIXE analysis showed heterogeneous spreading of zinc and copper;  
2 fluorine quantitative analysis by PIGE showed  $2553.6 \pm 199.2$  ppm fluorine.

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

8

#### 9 **4. Discussion**

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

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

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

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

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

18 It is assumed that the mechanism of action of copper nanoparticles is similar to that of silver nanoparticles.  
19 Copper ions adhere to DNA molecules and form crosslinks within and between nucleic acid chains, thus  
20 disrupting the helical structure of the DNA. Moreover, copper ions impair the biochemical processes of

1 bacterial cells. Combinations of silver and copper nanoparticles may provide a complete bactericidal effect  
2 against mixed bacterial populations (29).

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

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

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

16

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19

20 Declarations of interest: none.

1 Figure captions

2 Figure 1

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

5

6 Figure 2

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

8

9 Figure 3

10 Elemental PIXE map (zinc and copper) and PIGE map (fluorine). White dots in the maps (250  $\mu\text{m}$   $\times$  250  $\mu\text{m}$   
11 area) represent PIXE or PIGE signals from zinc, copper, and fluorine, respectively.

12

13 Figure 4

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

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1   References

- 2   [1] J.D. Featherstone. The science and practice of caries prevention. *J. Am. Dent. Assoc.* 131 (2000) 887–899.  
3   <https://doi.org/10.14219/jada.archive.2000.0307>
- 4   [2] R. Osorio, I. Cabello, M. Toledano. Bioactivity of zinc-doped dental adhesives. *J. Dent.* 42 (2014)  
5   403–412. <https://doi.org/10.1016/j.jdent.2013.12.006>
- 6   [3] Y.M. Kim, D.H. Kim, C.W. Song, S.Y. Yoon, S.Y. Kim, H.S. Na, J. Chung, Y.I. Kim, Y.H. Kwon.  
7   Antibacterial and remineralization effects of orthodontic bonding agents containing bioactive glass. *Korean J.*  
8   *Orthod.* 48 (2018) 163–171. <https://doi.org/10.4041/kjod.2018.48.3.163>
- 9   [4] D. Boyd, H. Li, D.A. Tanner, M.R. Towler, J.G. Wall. The antibacterial effects of zinc ion migration from  
10   zinc-based glass polyalkenoate cements. *J. Mater. Sci. Mater. Med.* 17 (2006) 489–494.  
11   <https://doi.org/10.1007/s10856-006-8930-6>
- 12   [5] A. Hoppe, N.S. Güldal, A.R. Boccaccini. A review of the biological response to ionic dissolution products  
13   from bioactive glasses and glass-ceramics. *Biomaterials* 32 (2011) 2757–2774.  
14   <https://doi.org/10.1016/j.biomaterials.2011.01.004>
- 15   [6] A. Smith, B. Scheven, Y. Takahashi, J. Ferracane, R. Shelton, P. Cooper. Dentine as a bioactive  
16   extracellular matrix. *Arch. Oral Biol.* 57 (2012) 109–121. <https://doi.org/10.1016/j.archoralbio.2011.07.008>
- 17   [7] M. Toledano, S. Sauro, I. Cabello, T. Watson, R. Osorio. A Zn-doped etch-and-rinse adhesive may  
18   improve the mechanical properties and the integrity at the bonded-dentin interface. *Dent. Mater.* 29 (2013)  
19   e142–e152. <https://doi.org/10.1016/j.dental.2013.04.024>
- 20   [8] R.J. Lynch. Zinc in the mouth, its interactions with dental enamel and possible effects on caries; a review

- 1 of the literature. *Int. Dent. J.* 61 (2011) 46–54. <https://doi.org/10.1111/j.1875-595x.2011.00049.x>
- 2 [9] A. De Souza, R. Gerlach, S. Line. Inhibition of human gingival gelatinases (MMP-2 and MMP-9) by  
3 metal salts. *Dent. Mater.* 16 (2000) 103–108. [https://doi.org/10.1016/s0109-5641\(99\)00084-6](https://doi.org/10.1016/s0109-5641(99)00084-6)
- 4 [10] R.J. Pinto, S. Daina, P. Sadocco, C. Pascoal Neto, T. Trindade. Antibacterial activity of nanocomposites  
5 of copper and cellulose. *Biomed. Res. Int.* 2013 (2013) 280512. <https://doi.org/10.1155/2013/280512>
- 6 [11] H. Komatsu, H. Yamamoto, Y. Matsuda, T. Kijimura, M. Kinugawa, K. Okuyama, M. Nomachi, K.  
7 Yasuda, T. Satoh, S. Oikawa. Fluorine analysis of human enamel around fluoride-containing materials under  
8 different pH-cycling by mu-PIGE/PIXE system. *Nucl. Instrum. Meth. B* 269 (2011) 2274–2277.  
9 <https://doi.org/10.1016/j.nimb.2009.03.081>
- 10 [12] M. Yasuhiro, O. Katsushi, Y. Hiroko, K. Hisanori, K. Masashi, S. Takahiro, H. Naoki, O. Saiko, K.  
11 Chiharu, S. Hidehiko. Fluorine uptake into the human enamel surface from fluoride-containing sealing  
12 materials during cariogenic pH cycling. *Nucl. Instrum. Meth. Phys. Res. Sect. B.* 348 (2015) 156–159.  
13 <https://doi.org/10.1016/j.nimb.2015.01.062>
- 14 [13] M. Fukuyama, C. Kawamoto, P. Saikaew, Y. Matsuda, R.M. Carvalho, D. Selimovic, H. Sano. Effect of  
15 topical fluoride application on enamel after in-office bleaching, as evaluated using a novel hardness tester  
16 and a transverse microradiography method. *Eur. J. Oral Sci.* 125 (2017) 471–478.  
17 <https://doi.org/10.1111/eos.12386>
- 18 [14] S. Ushimura, K. Nakamura, Y. Matsuda, H. Minamikawa, S. Abe, Y. Yawaka. Assessment of the  
19 inhibitory effects of fissure sealants on the demineralization of primary teeth using an automatic pH-cycling  
20 system. *Dent. Mater. J.* 35 (2016) 316–324. <https://doi.org/10.4012/dmj.2015-297>

- 1 [15] M.T. Kato, A. Bolanho, B.L. Zarella, T. Salo, L. Tjaderhane, M.A. Buzalaf. Sodium fluoride inhibits  
2 MMP-2 and MMP-9. *J. Dent. Res.* 93 (2014) 74–77. <https://doi.org/10.1177/0022034513511820>
- 3 [16] J.L. Wennstrom, G. Dahlen, K. Grondahl, L. Heijl. Periodic subgingival antimicrobial irrigation of  
4 periodontal pockets. II. Microbiological and radiographical observations. *J. Clin. Periodontol.* 14 (1987)  
5 573–580. <https://doi.org/10.1111/j.1600-051x.1987.tb01518.x>
- 6 [17] B. Aydin Sevinç, L. Hanley. Antibacterial activity of dental composites containing zinc oxide  
7 nanoparticles. *J. Biomed. Mater. Res. Part B.* 94 (2010) 22–31. <https://doi.org/10.1002/jbm.b.31620>
- 8 [18] F. Heravi, M. Ramezani, M. Poosti, M. Hosseini, A. Shajiei, F. Ahrari. In vitro cytotoxicity assessment  
9 of an orthodontic composite containing titanium-dioxide nano-particles. *J. Dent. Res. Dent. Clinics Dent.*  
10 *Prospects* 7 (2013) 192. <https://doi.org/10.5681/joddd.2013.031>
- 11 [19] B. Li, Y. Wang. Facile synthesis and photocatalytic activity of ZnO–CuO nanocomposite. *Superlattices*  
12 *Microstructures.* 47 (2010) 615–623. <https://doi.org/10.1016/j.spmi.2010.02.005>
- 13 [20] T. Sakai, T. Kamiya, M. Oikawa, T. Sato, A. Tanaka, K. Ishii. JAERI Takasaki in-air micro-PIXE system  
14 for various applications. *Nucl. Instrum. Meth. B.* 190 (2002) 271–275.  
15 [https://doi.org/10.1016/s0168-583x\(02\)00469-x](https://doi.org/10.1016/s0168-583x(02)00469-x)
- 16 [21] M. Eshed, J. Lellouche, S. Matalon, A. Gedanken, E. Banin. Sonochemical coatings of ZnO and CuO  
17 nanoparticles inhibit *Streptococcus mutans* biofilm formation on teeth model. *Langmuir.* 28 (2012)  
18 12288–12295. <https://doi.org/10.1021/la301432a>
- 19 [22] Y. Takahashi, S. Imazato, A.V. Kaneshiro, S. Ebisu, J.E. Frencken, F.R. Tay. Antibacterial effects and  
20 physical properties of glass-ionomer cements containing chlorhexidine for the ART approach. *Dent. Mater.*



- 1 22 (2006) 647–652. <https://doi.org/10.1016/j.dental.2005.08.003>
- 2 [23] H. Komatsu, H. Yamamoto, M. Nomachi, K. Yasuda, Y. Matsuda, Y. Murata, T. Kijimura, H. Sano, T.  
3 Sakai, T. Kamiya. Fluorine uptake into human enamel around a fluoride-containing dental material during  
4 cariogenic pH cycling. *Nucl. Instrum. Meth. Phys. Res. Sect. B.* 260 (2007) 201–206.  
5 <https://doi.org/10.1016/j.nimb.2007.02.068>
- 6 [24] I.D. Mandel. Antimicrobial mouthrinses: overview and update. *J. Am. Dent. Assoc.* 125 (1994) 2S–10S.  
7 [https://doi.org/10.1016/s0002-8177\(94\)14001-x](https://doi.org/10.1016/s0002-8177(94)14001-x)
- 8 [25] S.B. Mossad, M.L. Macknin, S.V. Mendendorp, P. Mason. Zinc gluconate lozenges for treating the  
9 common cold: a randomized, double-blind, placebo-controlled study. *Ann. Intern. Med.* 125 (1996) 81–88.  
10 <https://doi.org/10.7326/0003-4819-125-2-199607150-00001>
- 11 [26] W. Opsahl, H. Zeronian, M. Ellison, D. Lewis, R.B. Rucker, R.S. Riggins. Role of copper in collagen  
12 cross-linking and its influence on selected mechanical properties of chick bone and tendon. *J. Nutrition* 112  
13 (1982) 708–716. <https://doi.org/10.1093/jn/112.4.708>
- 14 [27] J.F. Hernández-Sierra, F. Ruiz, D.C.C. Pena, F. Martínez-Gutiérrez, A.E. Martínez, A.D.J.P. Guillén, H.  
15 Tapia-Pérez, G.M. Castañón. The antimicrobial sensitivity of *Streptococcus mutans* to nanoparticles of silver,  
16 zinc oxide, and gold. *Nanomedicine Nanotechnol. Biol. Med.* 4 (2008) 237–240.  
17 <https://doi.org/10.1016/j.nano.2008.04.005>
- 18 [28] H. Lee, S.Y. Yeo, S.H. Jeong. Antibacterial effect of nanosized silver colloidal solution on textile fabrics.  
19 *J. Mater. Sci.* 38 (2003) 2199–2204. <https://doi.org/10.1023/A:1023736416361>
- 20 [29] J.P. Ruparelia, A.K. Chatterjee, S.P. Duttagupta, S. Mukherji. Strain specificity in antimicrobial activity

1 of silver and copper nanoparticles. *Acta Biomaterialia* 4 (2008) 707–716.

2 <https://doi.org/10.1016/j.actbio.2007.11.006>

3 [30] K.Y. Yoon, J.H. Byeon, J.H. Park, J. Hwang. Susceptibility constants of *Escherichia coli* and *Bacillus*

4 *subtilis* to silver and copper nanoparticles. *Science Total Environ.* 373 (2007) 572–575.

5 <https://doi.org/10.1016/j.scitotenv.2006.11.007>