Biocidal effect of copper and zinc oxide nanoparticles on human oral microbiome and biofilm formation

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1. Introduction

Nanotherapeutics has lately evoked tremendous interest in controlling the development of oral biofilms by the use of biocidal nanoparticles (NPs). Treatment with conventional antibiotics is considered inadequate, and often leads to chronic oral infections, which compel tooth extractions or implant removal, involving costly restoration or regenerative procedures [1]. Human oral microbiome consists of a complex polymicrobial community, which dwells in specific niches within the oral cavity and forms biofilms (plaques) on teeth, prostheses, and mucosal surfaces. Dental plaques represent a dynamic oral biofilm ecosystem comprising of more than 800 bacterial species [2–5]. Initial colonizers include Streptococcus oralis, Streptococcus sanguinis, and Streptococcus mitis. The co-aggregating partners comprise of Eikenella corrodens, Veillonella atypica and Prevotella intermedia, and the late colonizers are predominantly Aggregatibacter actinomycescomitans, Prevotella intermedia, Treponema denticola and Porphyromonas gingivalis [6]. Oral biofilms are regarded as aetiological agents associated with dental caries and periodontitis [7,8]. An estimated 65–80% of all infections are contemplated to be linked to biofilm formation, as a causal factor for failure of antimicrobial treatments, and thus presents a serious challenge [9–11]. Lesser susceptibility of biofilms to antimicrobial agents, and an increasing trend of multiple drug resistance in bacteria necessitate the investigations on novel alternatives as front-line antimicrobial agents. In this regard, the metal NPs offer the possibility of controlling oral biofilm formation, as the bacteria are less likely to acquire resistance against metal NPs than other conventional antibiotics [12]. Several NPs have been tested earlier for their effects on pure cultures of Streptococcus mutans [13]. A recent study has suggested significant inhibition of biofilm formation of S. oralis (ATCC 35037) and two clinical isolates from periodontitis patients by the soda-lime-glass-nAg [14]. Nevertheless, the effect of metal NPs on oral cavity microbiome is still under exploration. Therefore, in this study, we report the effect of ZnO- and CuO-NPs on oral cavity bacteria and demonstrated the inhibition of biofilm formation on different matrices including glass, polystyrene plates, acrylic dentures and human epithelial cells.

2. Materials and methods

Synthesis and characterization of CuO- and ZnO-NPs: The CuO- and ZnO-NPs were synthesized by the solvo-thermal method [15] and sol–gel method [16], respectively. The NPs were characterized by X-ray diffraction (XRD) and transmission electron microscopy (TEM). The XRD patterns of CuO- and ZnO-NPs powders were acquired by the use of a PANalytical X’Pert X-ray diffractometer (Spectris plc, England) equipped with an Ni filter using Cu Kα (λ=1.54056 Å) radiations, as an X-ray source. For TEM analysis, the dried powders of NPs were suspended in deionized water and sonicated for 15 min at 40 W. A drop of diluted
Fig. 1. TEM and XRD analysis of NPs. Panels A and B: representative TEM images of CuO- and ZnO-NPs, respectively. Panels C and D: XRD profiles of CuO- and ZnO-NPs, respectively.

Fig. 2. Effect of CuO- and ZnO-NPs on bacterial growth, biofilm formation and EPS production. (A) Total CFU counts on NA and MRS media, (B) binding of total oral bacteria to human epithelial cells, (C) and (D) EPS production. Statistical analysis performed by one-way analysis of variance (ANOVA) using Dunnett’s test (Sigma Plot 11.0, USA). *p < 0.005.
suspension was placed onto a carbon-coated copper grid, air-
dried and observed with TEM at 40,000 × (JEM-2100F, JEOL). The particle size distribution and Zeta (ζ) potential were determined by dynamic light scattering (DLS) using ZetaSizer-HT (Malvern, UK).

Effect of ZnO- and CuO-NPs on total oral bacterial population: Slurry from the teeth crown surface of a healthy male was collected using a sterile toothpick and suspended in 1 ml of sterile 1 × PBS. The number of bacterial cells in suspension was adjusted to 5 × 10^8/ml in all the experiments. The cells were treated with ZnO- and CuO-NPs at concentrations of 10, 50, and 100 μg/ml in NA and MRS broths at 37 °C for 16 h. Untreated and treated cells were spread on NA and MRS agar plates, and incubated at 37 °C for 3–5 days. The colony forming units (CFU) were determined and presented as log_{10} CFU/ml.

Assessment of biofilm formation: Biofilm formation on glass and acrylic denture and its inhibition with CuO- and ZnO-NPs were assessed using 0.2% crystal violet (CV) under a light microscope NIKON Eclipse 80i equipped with a Nikon DXM1200C digital camera (Nikon, Japan). Quantitative assessment was performed on polystyrene plate (Nunc, Denmark), and human epithelial (WISH) cells, as described by Burton et al. [17] and Rubens et al. [18], respectively.

Extracellular polysaccharide (EPS) production assay: EPS production was determined as described by Packiavathy et al. [19]. For NPs-induced EPS inhibition, the EPS produced by NPs treated and untreated bacteria was measured spectrophotometrically at 490 nm, and percentage inhibition was determined considering EPS production in untreated bacteria as 100%.

3. Results and discussion

Synthesis and characterization of ZnO- and CuO-NPs, zeta potential and particle size distribution: Fig. 1A and B shows the typical TEM images of CuO- and ZnO-NPs, respectively. The CuO-NPs were mostly spherical, and the ZnO-NPs were polygonal in shape with smooth surfaces and average sizes of 40 nm and 35 nm, respectively. The XRD pattern of CuO-NPs in Fig. 1C indicates the peaks of CuO-NPs, indexed to the monoclinic crystal system CuO (C2/c space group, JCPDS Card no. 45-0937). Similarly, the peaks of ZnO-NPs at 2θ = 32.06°, 34.66°, 36.54°, 47.82°, 56.89° and 63.16° were assigned to (100), (002), (101), (102), (110) and (103), respectively and suggested a polycrystalline wurtzite structure (Zincite, JCPDS 5-0664). The crystallite sizes based on Scherrer's equation [20] were determined to be 39.87 nm and 35.23 nm for CuO- and ZnO-NPs, respectively.

Biocidal effect of CuO- and ZnO-NPs on bacterial viability: Treatment of oral bacterial population with CuO- and ZnO-NPs resulted in significant decrease in total bacterial counts on both the NA and MRS agar plates (Fig. 2A). At a concentration of 10 μg/ml of ZnO- and CuO-NPs, the CFU counts on NA plates were decreased by 30% and 66%, while on MRS agar, the reduction was 35% and 59%, respectively. Exposure at a higher concentration

![Fig. 3. Inhibition of biofilm formation by ZnO- (A1) and CuO-NPs (A2) on the surface of glass (magnification 40 ×); and acrylic denture (B).](image-url)
of 50 μg/ml significantly affected the cell viability, resulting in 82–92% reduction in the survival of treated bacteria as compared to the untreated control. Thus, the data in Fig. 2A suggested that the reduction of planktonic cells was significant only for NPs concentrations > 50 μg/ml. Also, the biodegradation rate of CuO–NPs was significantly higher than that of the ZnO-NPs, which is consistent with the results obtained with the biofilm formation (Fig. 2C). The EC50 values of CuO- and ZnO-NPs were estimated to be 22.5 μg/ml and 70.5 μg/ml on NA plates vis-à-vis 25 μg/ml and 66 μg/ml on MRS agar, respectively. [Hall-Stoodley et al. 10] reported the inhibition of oral bacteria Actinomyces viscosus and S. mutans with ZnO whisker in the range between 78 and 312.5 μg/ml. The EC50 value of nano-Cu has been reported to be 78 μg/ml on Vibrio fischeri [21]. Indeed, the susceptibility to conventional antimicrobial agents varies greatly from organism to organism; however, our results demonstrated the strong biocidal effects of ZnO- and CuO-NPs on oral bacterial community.

**NPs-induced inhibition of biofilm formation:** Effects of CuO- and ZnO-NPs on binding of oral bacteria to a monolayer of human epithelial cells are shown in Fig. 2B. The binding was not affected at lower concentration range up to 10 μg/ml of NPs. However, it was significantly decreased (p < 0.001) with CuO-NPs at concentrations above 50 μg/ml. Also, the biofilm formation on polystyrene surface was significantly inhibited at both 50 and 100 μg/ml of CuO- and ZnO-NPs (Fig. 2C). A recent study demonstrated ZnO- and CuO-NPs-induced inhibition of S. mutans on artificial teeth and glass surface [22]. Our results also exhibited complete inhibition of biofilm formation at 100 μg/ml of ZnO- and CuO-NPs on the glass (Fig. 3A1 and A2) and acrylic denture (Fig. 3B) surfaces. Over all, the results suggest greater toxic effects of CuO-NPs vis-à-vis ZnO-NPs, which could be attributed to (i) differences in shape of CuO-NPs (spherical) and ZnO-NPs (polyhedral), and (ii) higher ζ-potential of 20.8 for Cu-NPs and 16.4 for ZnO-NPs. It is suggested that more cationic NPs exhibit greater toxicity associated with cell wall disruption and higher membrane permeability.

**NPs-induced inhibition of EPS production:** Fig. 2D shows the NPs concentration dependent reduction in EPS production. About 27% and 18% inhibition occurred at 10 μg/ml of ZnO- and CuO-NPs, respectively. The extent of EPS inhibition increased remarkably to 59% and 61% at concentration of 50 μg/ml NPs. Thus, NPs-induced biofilm inhibition is attributed to repressed EPS synthesis. Earlier studies suggested the role of glucosyltransferase in the synthesis of polysaccharide and demonstrated its inhibition by metal ions [23].

4. **Conclusions**

In the study, the CuO- and ZnO-NPs exhibited significant inhibitory activity on oral bacteria and biofilm formation. The results substantiated the potential of these NPs as antimicrobial agents, and elucidated their effective control on oral biofilm formation, as a novel approach in preventing dental infections. Further studies are warranted to ascertain the sensitivity of specific dental plaque colonizers to different types of biocidal NPs and understand the mechanism of biofilm inhibition, for developing effective strategies for dental hygiene.

**Appendix A. Supporting information**

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.matlet.2013.01.085.

**References**