

Synthesis of hybrid copper-chitosan nanoparticles with antibacterial activity against cariogenic *Streptococcus mutans*

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Hybrid nanoparticles (CuChNP) comprising of copper nanoparticles with a chitosan shell were synthesized. Antimicrobial properties of CuChNP were assessed against *Streptococcus mutans* (*S. mutans*), one of the main bacterium that causes tooth decay. Antibacterial activity of CuChNP against *S. mutans* was comparable to that of oral antimicrobial agents, such as chlorhexidine, and cetylpyridinium chloride. Particularly, CuChNP exhibited superior capacity to prevent the *S. mutans* growth on human tooth surface as well as disrupt and kill the bacterial cells in an established dental biofilm. Chitosan may interact with both tooth hydroxyapatite and bacterial cell wall, which improves the adherence of copper to the tooth surface and potentiates their anti-biofilm action. The antimicrobial properties exhibited by CuChNP could be useful for the future development of more effective treatments for the control of dental plaque biofilms.

Keywords: Copper nanoparticles, Chitosan, *Streptococcus mutans*, Dental caries

INTRODUCTION

Metallic nanoparticles with unique nanoscale surface properties appear as a new generation of antimicrobials for biomedical applications. Copper nanoparticles (CuNPs) exhibit antimicrobial activity against several microorganisms, including bacteria, fungi and algae^{1,2}. Although biological mechanism of bacterial cell killing by the CuNPs is not yet fully understood, the most recent investigations suggest that their activity is due to nascent ions generated from the oxidation of the nanoparticles when they are in the vicinity of cells or organic medium components³. The finding indicates that 'particle-specific' effect rather than 'ion-specific' one is responsible for the CuNP action⁴. The antimicrobial activity of CuNPs has been studied against different pathogenic microorganisms, however, no studies have been reported about their activity against cariogenic bacteria. Dental caries is one of the most prevalent infectious disease worldwide⁵ caused by teeth demineralization by acid-producing bacteria that form the dental plaque⁶. Due to the complex composition and organization of biofilm, dental plaque bacteria are particularly resistant to oral antimicrobial agents.

Potential applications of metallic nanoparticles in dentistry require that nanoparticles interact well with oral tissues, in order to produce a sustained antibacterial effect on the time. Biopolymers like chitosan have demonstrated to have excellent adhesive properties to the oral mucosa as well antimicrobial action against bacteria⁷. The combination of CuNPs with chitosan could improve the adhesivity, bioactivity

and biocompatibility of the metallic nanoparticles. Several works have reported the preparation of antimicrobial bulk nanocomposites produced by the incorporation of copper and silver nanoparticles into chitosan matrices⁸⁻¹⁰. In the current study, the preparation of hybrid copper-chitosan particles with nanometric dimensions is presented. Antibacterial properties of the nanoparticles are investigated against the cariogenic *Streptococcus mutans* through traditional antibacterial parameters and by using an *in vitro* biofilm model on a tooth surface.

MATERIALS AND METHODS

Synthesis of nanoparticles

Neat CuNP powder was synthesized by using a procedure optimized in our lab¹¹. Briefly, 0.242 g of starch was dissolved in 96.8 mL of ascorbic acid 10% for 1 min in a microwave (600 W). Then, 3.14 mL of copper acetate 0.2 M solution was added and heated in the microwave for 1 min in two series of 30 s each. This procedure was used to obtain 100 mL of 400 ppm CuNP suspension. The nanoparticles were separated by centrifugation (12,000 rpm) and washed by three centrifugation-redispersion cycles with distilled water. The precipitate was then freeze dried to obtain a fine CuNP powder.

Hybrid nanoparticles (CuChNP) were synthesized by dissolving 0.5 g of high molecular weight chitosan (310,000–375,000 Da, Sigma-Aldrich, St. Louis, MO, USA) into 100 mL of 1% lactic acid solution (pH adjusted to 4.7). Four hundred milligrams of CuNP powder was added, and the suspension was sonicated. Afterward, 25 mL of 0.25% sodium polymetaphosphate solution

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was added to 75 mL of the CuNP-chitosan suspension under magnetic stirring to rapidly produce a CuChNP colloidal suspension. The nanoparticles were separated by centrifugation (12,000 rpm). The precipitate was then freeze dried to obtain a CuChNP powder.

Nanoparticle characterization

NPs were analyzed by scanning electron microscopy (SEM, Jeol JSM-IT300LV, JEOL USA, Peabody, MA, USA) equipped with energy dispersive X-ray detector (Aztec EDS system, Oxford Instruments, Abingdon, UK). Particle size distribution was estimated by using the analysis tools of the microscope software. CuChNP were also characterized by attenuated total reflectance with Fourier transform infrared spectroscopy (ATR-FTIR) on an Agilent Cary 630 Agilent Technologies FTIR-ATR (Agilent Technologies, Santa Clara, CA, USA). Thermogravimetric (TG) analysis (from 10–900°C) of CuChNP was done in a Netzsch TG 209F1 Libra® (Netzsch Group, Selb, Germany) at a heating rate of 10°C/min in air.

Minimum inhibitory and minimum bactericidal concentration assessment

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the NPs was tested against *S. mutans*, (ATCC 25175) in planktonic state. The NPs concentration causing bactericidal effect was selected based on absence of colonies on the agar plate.

Antibacterial activity of nanoparticles against S. mutans in biofilm state on tooth surfaces

The use of human extracted third molars (caries-free, and without cracks and structural defects in coronal portion) was approved by the Ethics Committee of the Faculty of Dentistry, University of Chile (approval number: 2012/13). Immediately after extraction the teeth were thoroughly cleaned using curettes and stored in 0.1% thymol solution at 4°C until used for the study. Enamel blocks (6×4×2 mm) were prepared using a water-cooled low speed diamond saw (Isomet, Buehler, Lake Bluff, IL, USA), and the surface cleaned for approximately 5 s with a slurry of flour of pumice using a rubber cup. Tooth enamel surfaces were immersed in magnetic stirred suspensions of CuNP, CuChNP, traditional oral antimicrobial agents (CHX: 0.12% chlorhexidine, CPC: 0.07% cetylpyridinium chloride) or distilled water as negative control. The treated surfaces were then incubated in a 0.5 McFarland bacterial suspension for 48 h. Afterward, the surfaces were washed with a 0.88 wt% NaCl solution and 1% Tween 80 to remove the bacteria grown on the surface. Samples of 100 µL were taken from the bacterial suspension, diluted and plated in BHI agar. After 48 h of incubation at 37°C, the colonies were counted and the colony forming units per mL (CFUs) were calculated.

In a second experiment, *S. mutans* biofilms previously grown on enamel for 48 h, were immersed in the NPs suspension. The remaining biofilm was removed with 1% Tween 80, and CFU counting was

performed following the procedure above described. In both experiments, the biofilm/enamel surfaces were also analyzed by SEM.

Statistical analysis

Statistical analysis was performed with IBM SPSS Statistics software (Microsoft SPSS, SPSS, Chicago, IL, USA). Since MIC and MBC data did not meet the assumption of normal distribution (Shapiro-Wilk test), non-parametric Mann Whitney test was used. Data obtained from the antibacterial activity test was analyzed by 1-way analysis of variance. A *post-hoc* Bonferroni was used for multiple comparisons. For all comparison $p < 0.05$ was considered as significant.

RESULTS

Nanoparticle characterization

Particle size of the synthesized CuChNP as determined by analysis of the SEM micrographs was estimated to be around 131 (±36) nm (Figs. 1a, b). EDX elemental mapping of an individual CuChNP particle showed that its structure is constituted by copper, oxygen, and carbon, with copper atoms more densely distributed in the particle core zone (Figs. 1c, d). The polymeric organic fraction in the CuChNP determined by TG analysis was found to be around 19 wt%. FTIR-ATR analysis of CuChNP (Fig. 2) revealed a decrease in the intensities of the N–H amide/amine bending (~1,578 cm⁻¹) and O–H/N–H stretching bands (~3,303 cm⁻¹) of chitosan structure as compared with the neat chitosan polymer¹². In the case of neat CuNP, the peak around 2,100 cm⁻¹ has been ascribed to the presence of atmospheric CO₂¹³.

MIC and MBC measurement

The MIC and MBC values of CuNP and CuChNP against cariogenic *S. mutans* are presented in Table 1. The MIC and MBC values of CuNP on *S. mutans* were lower than those of CuChNP, however the differences between the two groups were not statistically significant ($p > 0.05$).

Antibacterial activity of nanoparticles against S. mutans in tooth surfaces.

Figure 3 shows the NPs capacity to inhibit the growth or to reduce the number of viable bacteria in a *S. mutans* biofilm established onto enamel surface. The hybrid CuChNP showed greater capacity for both to prevent the biofilm formation and to reduce viable bacteria in an established biofilm than CuNP. In addition, antibacterial effect exhibited by CuChNP was comparable to that of traditional oral antimicrobial agents (CHX and CPC). SEM images in Fig. 4, show that the organization and quantity of bacterial biofilm is notably altered by effect of the NPs, particularly after CuChNP treatment. EDX elemental analysis (Figs. 4-b3, c3) revealed a greater copper atomic density on the biofilm/tooth surface treated with CuChNP in comparison to that exposed to CuNP.

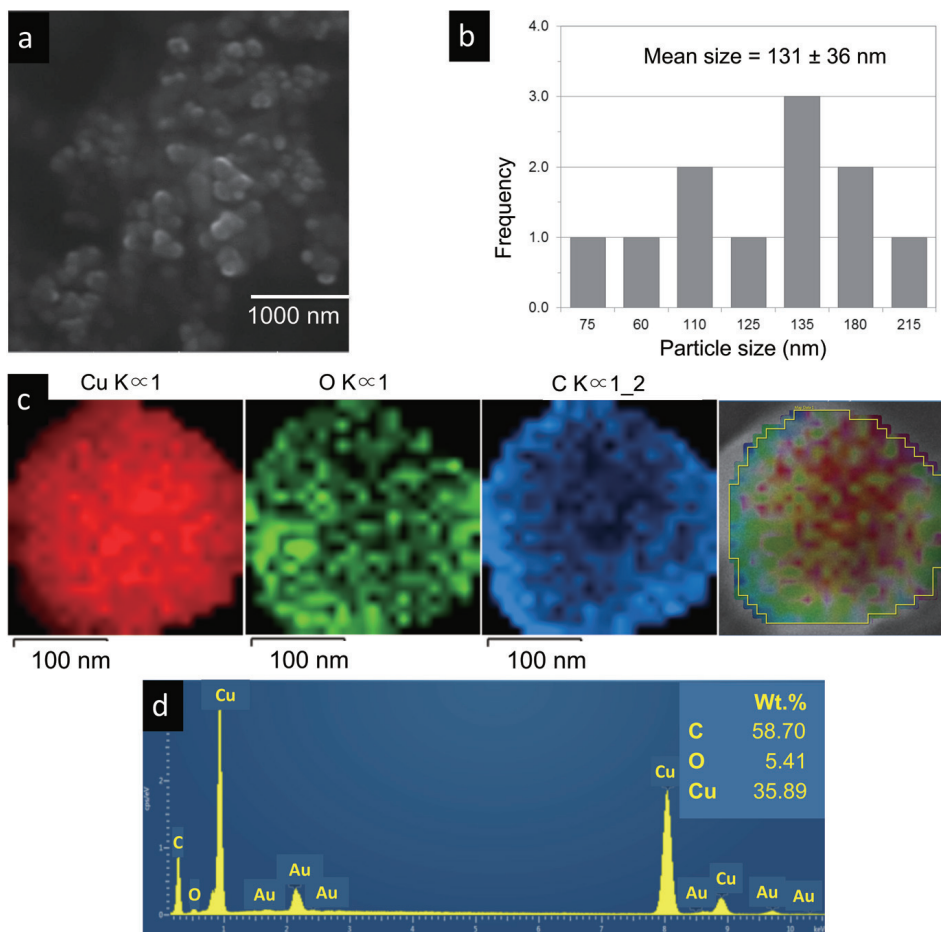


Fig. 1 SEM image (a), particle size distribution (b), and EDX elemental mapping (c, d) of CuChNP ($n=11$).

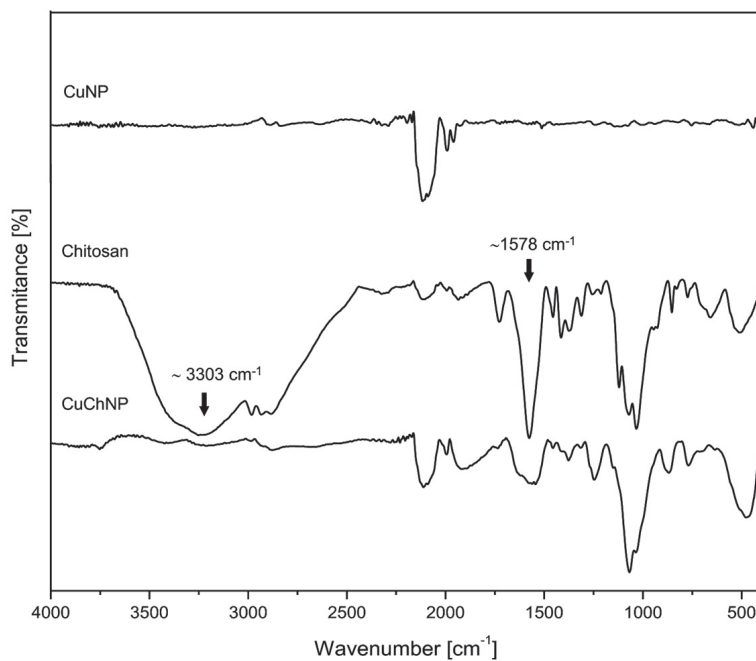


Fig. 2 FTIR-ATR analysis of CuChNP and chitosan polymer.

Table 1 MIC and MBC of CuNP and CuChNP nanoparticles against *Streptococcus mutans*

Nanoparticle	MIC ($\mu\text{g/mL}^{-1}$)	MBC ($\mu\text{g/mL}^{-1}$)
CuNP	30	40
CuChNP	35	60
<i>p</i>	0.114	0.057

(Data expressed in median, $n=4$)

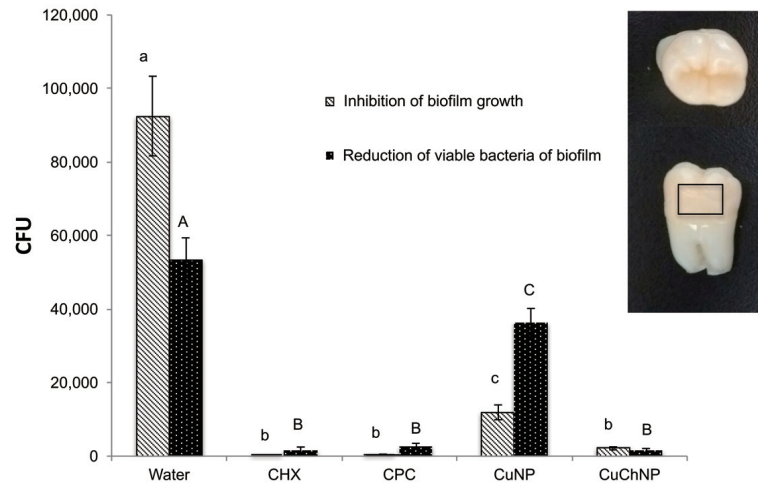


Fig. 3 Antibacterial activity of CuChNP compared to CuNP, traditional oral antimicrobials and negative control (water) to inhibit the growth and to reduce the number of viable bacteria of *S. mutans* biofilm on tooth enamel surface (for inhibition of biofilm growth matching lower-case letters, and for reduction of viable bacteria of biofilm matching upper-case letters indicate values with no statistically significant difference ($p>0.05$)). The insert shows surface area of tooth enamel selected for biofilm growth ($n=6$).

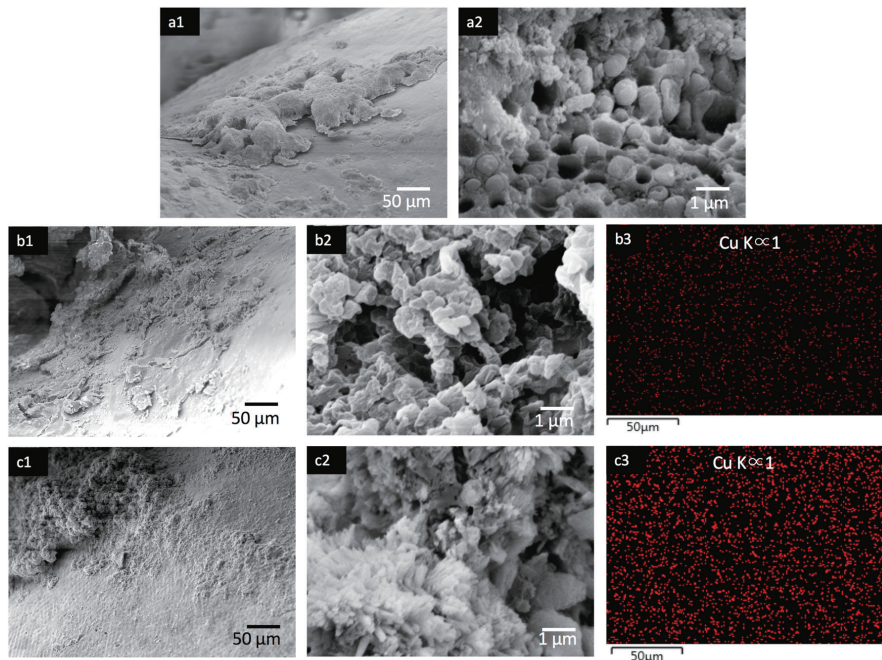


Fig. 4 SEM images of biofilm of *S. mutans* treated with distilled water (a1, a2), neat CuNP (b1, b2) and CuChNP (c1, c2). EDX elemental mapping showing copper distribution on the dental/biofilm surface treated with neat CuNP (b3) and CuChNP (c3).

DISCUSSION

In this study, hybrid copper-chitosan particles with nanometric dimensions were synthesized. When CuChNPs antibacterial properties were compared against planktonic *S. mutans*, CuNPs proved to be stronger. However, when a *S. mutans* biofilm model grown on enamel surfaces was used, the hybrid nanoparticles showed greater capacity to prevent the formation and disturb the biofilm, than CuNPs. Furthermore, this capability was equivalent to that of traditional oral antimicrobial agents.

The CuChNPs presented a nanometric particle size of around 131 nm with a hybrid structure composed of a copper core surrounded by a chitosan shell. FTIR analysis revealed that the intensities of the N–H amide/amine bending and O–H/N–H stretching bands of chitosan structure¹²⁾ appear decreased in the CuChNP spectrum, which suggests covalent interactions between the metallic copper surface and the chitosan functional groups^{14,15)}. It has been also reported that copper oxide nanoparticles dispersed into a chitosan solution shift the FTIR peaks of the polymer¹⁶⁾, adding further evidence about the formation of chemical interactions in the copper-chitosan interface and confirming the hybrid nature of the nanoparticle.

CuChNP presented greater MIC and MBC values than CuNPs, which can be attributed to the presence of chitosan in the hybrid structure. Although chitosan has also bactericidal properties, the MIC values of chitosan for *S. mutans* have been reported to range from 500 to 2,000 $\mu\text{g}/\text{mL}^{-1}$,^{17,18)}. Despite of this, MIC value of CuChNP against *S. mutans* is comparable to that reported for silver nanoparticles (50–222 $\mu\text{g}/\text{mL}^{-1}$) or traditional oral antibacterials such as chlorhexidine (1,250 $\mu\text{g}/\text{mL}^{-1}$)^{19,20)}. It is even more interesting to note, that CuChNP exhibited superior antibacterial on *S. mutans* in biofilm state grown on a tooth surface. This result can be explained by the improved adhesive properties of the hybrid particle. Chitosan contained in CuChNP may decrease the adhesion of *S. mutans* and increase the adherence of copper to the enamel. It has been reported that chitosan is capable of interfering with *S. mutans* adhesion and primary biofilm formation²¹⁻²³⁾. Chitosan is also known as a bioadhesive polymer²⁴⁾ that may interact with the negatively charged hydroxyapatite surface of enamel as well as with the negative cell wall of biofilm bacteria²⁵⁾. These kinds of interactions would be responsible of the higher copper retention on the biofilm/enamel surface (EDX mapping) and the improved anti-biofilm action exhibited by CuChNP. In addition, bactericidal activity of CuChNPs is consequence of the bacterial cell damage provoked by the oxidative stress induced by copper. Moreover, CuNP forms complex with organic components of cellular medium that promotes the generation of reactive oxygen species (ROS), which causes membrane lipid per-oxidation and chromosomal DNA degradation⁴⁾. Metallic nanoparticles may also inhibit the activity of glucosyltransferase (GTF)²⁶⁾, enzyme that produces glucans that participate in the

adhesion of *S. mutans* to the tooth and favor the bacterial aggregation within a biofilm²⁷⁾.

The results of this work demonstrate that antibacterial action of CuChNP prevent the growth, disrupt and kill the bacterial cells in cariogenic biofilm grown on tooth surface. The antimicrobial properties exhibited by CuChNP could be useful for the future development of more effective treatments for the control of dental plaque biofilms.

CONCLUSIONS

Nanoparticles with a copper-chitosan hybrid structure were synthesized. Chitosan improves the adherence of copper to the tooth surface, which potentiates the bactericidal action and anti-biofilm properties of the hybrid nanoparticle against *S. mutans*.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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