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Silver nanoparticles with antimicrobial activities against *Streptococcus mutans* and their cytotoxic effect

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ABSTRACT

Microbial resistance represents a challenge for the scientific community to develop new bioactive compounds. The goal of this research was to evaluate the antimicrobial activity of silver nanoparticles (AgNPs) against a clinical isolate of *Streptococcus mutans*, antibiofilm activity against mature *S. mutans* biofilms and the compatibility with human fibroblasts. The antimicrobial activity of AgNPs against the planktonic clinical isolate was size and concentration dependent, with smaller AgNPs having a lower minimum inhibitory concentration. A reduction of 2.3 log in the number of colony-forming units of *S. mutans* was observed when biofilms grown in a CDC reactor were exposed to 100 ppm of AgNPs of 9.5 ± 1.1 nm. However, AgNPs at high concentrations (>10 ppm) showed a cytotoxic effect upon human dermal fibroblasts. AgNPs effectively inhibited the growth of a planktonic *S. mutans* clinical isolate and killed established *S. mutans* biofilms, which suggests that AgNPs could be used for prevention and treatment of dental caries. Further research and development are necessary to translate this technology into therapeutic and preventive strategies.

1. Introduction

Biofilms are microbial consortia embedded in self-produced exopolymer matrices composed of mainly exopolysaccharides (EPS). Microbes living in these matrices benefit from nutrient and water supplies [1], improved lateral gene transfer [2] and protection against adverse environmental insults, such as desiccation and chemicals, including detergents, disinfectants, and antibiotics [3,4]. Biofilms can be also reservoirs for pathogenic organisms and sources of disease outbreaks. For instance, biofilms are implicated in otitis media [5], otolaryngologic infections [6], osteomyelitis [7], bacterial endocarditis, cystic fibrosis [8], non-healing wounds [9] and oral biofilm [10].

Dental caries and periodontal diseases are widespread diseases, both of which are highly prevalent in industrialized societies, and are rising in developing countries [11]. Dental caries and periodontal diseases result from a complex interaction of environmental triggers, the resident microorganisms and the host. If the composition of individual's resident oral microorganisms is out of balance, dental disease can occur [12]. Even at the earliest times of initial colonization, flowing saliva bathes both cleaned surfaces and already attached cells with a variety of species suspended in saliva. A highly selective mechanism of coaggregation between species is involved in the development of multispecies communities. The primary initial colonizers are streptococci and some *Actinomyces*, and early colonizing veillonellae coaggregate with streptococci and *Actinomyces* [13]. Although culture independent sequencing has illuminated the complexity of the human oral microbiome, *Streptococcus mutans* remains widely regarded as a primary etiological agent in caries [14]. This facultative anaerobic, Gram positive, bacterium excels in the complex environment of the oral cavity where stresses including low pH and an oxidative environment are coupled to variable salivary flow and carbohydrate supply. The physiologic adaptations of *S. mutans* to these pressures provide a competitive advantage versus non-cariogenic commensals that underpin the establishment and progression of caries [15].

Treatment of an infection after the biofilm is established is frequently futile with current remedies. Often, the only solution is the physical removal of the biofilm or implant, which is costly and traumatic to the patient [16]. The control of oral biofilms depends in part on the use of chemical actives that kill or remove plaque. Actives that kill microorganisms presumably reduce bacterial virulence and retard the rate of plaque accumulation. The penetration of such actives into the microbial biofilm is a fundamental requirement for their efficacy [17].

In recent years the application of nanoparticles in various fields has been expanded considerably. Nanoparticles have been successfully

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used in medical and pharmaceutical nanoengineering for the delivery of therapeutic agents [18], in chronic disease diagnostics, and in sensors [19]. AgNPs are efficient non-specific antimicrobial agents against planktonic forms of a broad spectrum of bacterial and fungal species [20,21]. Their antimicrobial activities are attributed to the unique physicochemical characteristics of AgNPs, such as the high ratio of surface area to mass, high reactivity, and nanometer sizes, which confer them to a major advantage for the development of alternative products against multi-drug resistant microorganisms [22].

Knowledge of nanoparticle diffusivity is a parameter necessary to understand the mobility, aggregation, and toxicity of these composites. The diffusion of nanoparticles may be hindered by: (i) the porous structure of the biofilm; (ii) the local accumulation of nanoparticles by cells, non-diffusing macromolecules, or the polysaccharide matrix; and (iii) the adsorption of the solute to freely diffusing species, abiotic particles, or gas bubbles [23]. Due to the protection offered by the biofilm matrix to the diffusion of antibiotic agents within the exopolymer matrix, the antimicrobial activity of AgNPs was tested against both planktonic bacteria and biofilms formed under high fluid shear conditions using a bioreactor. Results presented in this study show that AgNPs were able to inhibit the growth of an *S. mutans* clinical isolate and also kill *S. mutans* inhabiting the biofilm matrix, suggesting that AgNPs could be used for the treatment of dental caries.

2. Material and methods

2.1. Synthesis of AgNPs

Silver nanoparticles with spherical and pseudospherical shapes with three different sizes were synthesized. All preparations started with a 0.01 M $AgNO_3$ solution placed in a 250 ml reaction vessel. Under magnetic stirring, 10 ml of deionized water containing gallic acid (0.1 g for 9.3 and 78.7 nm samples and 0.5 g for 21.3 nm sample) was added to 100 ml of silver nitrate solution. After the addition of gallic acid the pH value of the solution was immediately adjusted (for the 9.5 nm sample the pH was raised to 11 with NaOH 1.0 M and for the 25.9 nm sample, pH was raised to 10 with NH_4OH). For the 78.7 nm sample, after the addition of gallic acid, the mixture was irradiated with UV light (254 nm, 15 W) for 30 min (pH was not modified). After that, the solution was heated for 30 min at 80 °C [24]. Immediately after the synthesis and in order to purify the silver nanoparticles, the obtained dispersions were dialyzed using a dialysis membrane (12 kDa molecular weight) for 48 h.

2.2. Characterization of the AgNPs

AgNPs were characterized by Dynamic Light Scattering (DLS), the hydrodynamic diameter and zeta potential were determined in triplicate by using a Malvern Zetasizer Nano ZS (Instruments Worcestershire, United Kingdom) operating with a He–Ne laser at a wavelength of 633 nm, and a detection angle of 90°; all samples were analyzed for 60 s at 25 °C. To confirm shape, each sample was diluted with deionized water and 50 μl of each suspension was placed on a copper grid for Transmission Electron Microscopy (TEM). All samples were analyzed by Transmission Electron Microscopy (JEOL JEM-1230, Tokyo, Japan) at an accelerating voltage of 100 kV.

2.3. Patients

The study included saliva from subjects with primary dentition, all residents of the city of San Luis Potosí, Mexico. Subjects were recruited by the department of Pediatric Dentistry of the Advanced General Dentistry Program. The children's parents completed a standardized health questionnaire that included oral pediatric information and assessments, as well as the recently used antibiotics. A voluntary informed consent was obtained from parents prior to the clinical examination, the research was done according to the Helsinki Declaration of 1975, and approved by the Masters Degree in Dental Science with Specialization in Advanced General Dentistry Program at San Luis Potosí University, Mexico. Pediatric samples of patients with active caries without any repair were considered. The inclusion criteria were children aged between 3 and 6 years. Exclusion criteria included those children who received antibiotics during the last three months preceding the survey.

2.4. Sample collection

Paraffin-stimulated whole saliva from children was sampled over a 5 min period in a sterilized propylene tube; this process was carried out consistently in the morning (9–10 am) to minimize the circadian rhythm effects, 2 h after the previous meal. Saliva samples were dispersed by sonication for 10 s and 10-fold dilutions were prepared in saline solution (0.9% NaCl), 100 μl of each dilution was spread by spiral bacteria plate (in duplicate) onto Mitis-salivarius agar (MSA) supplemented with 20% sucrose and 1% potassium tellurite. The plates were incubated in an atmosphere of 10% CO_2 at 37 °C for 24 hours (h); then plates were incubated for 24 h at room temperature. A stereoscopic microscope (Olympus, SD-ILK, Japan) was used to verify the presence of colony forming units (CFU) resembling *S. mutans*.

2.5. Bacterial culture

Microorganism strains were maintained in solidified broth using 1.5% agar trypticase soy plate supplemented with 5% sheep blood, cultures were placed in a humidified atmosphere supplemented with 5% CO_2 for 24 h in stagnant condition. The clinical strain was identified using a VITEK® system (data not shown).

2.6. Antimicrobial test

The microdilution method for estimation of minimum inhibitory concentration (MIC) values was carried out to evaluate the antimicrobial activity. The MIC values were determined on 96-well microdilution plates and according to published protocols [25]. MICs were determined by incubating *S. mutans* in 96-well microplates in a humidified atmosphere supplemented with 5% CO_2 for 24 h. Microorganisms were exposed to serial dilutions of the nanoparticles, and the end points were determined when no turbidity in the well was observed. The antibacterial activities of the nanoparticles were compared to oxacillin. The turbidity background from the nanoparticles was subtracted from the final reading. All assays were carried out in triplicate.

2.7. Biofilm formation on coupons

Anti-biofilm activity of the 9.5 nm AgNPs was evaluated on high shear biofilm grown in the CDC Biofilm Reactor (CDC-BR) (model CBR 90, Biosurface Technologies Corporation, Bozeman, MT) [30] according to published protocols [26]. Briefly, the CDC-BR consists of a 1 L vessel with eight polypropylene coupon holders, which can accommodate three sample coupons (0.5 inch diameter) in each of the eight holders. Liquid growth medium enters through the top of the vessel and exits via a side-arm discharge port. A magnetic stir bar incorporating a mixing blade provides fluid mixing and surface shear creating a turbulent flow (Reynolds number between 800 and 1850). For these calculations, the bulk fluid was assumed to possess the properties of water at 20 °C when growing a *S. mutans* biofilm.

The reactor was filled with 400 ml of 1% strength TSB and inoculated with 1 ml of an overnight culture of *S. mutans*. The reactor was maintained in a batch mode (mixed, no flow) for 24 h at 37 °C. At the end of the first 24 h flow (1% strength TSB) was started and maintained for another 24 h.