Action of silver nanoparticles toward biological systems: cytotoxicity evaluation using hen´s egg test and inhibition of *Streptococcus mutans* biofilm formation

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Abstract

The present study aimed to evaluate the cytotoxicity and bactericidal properties of silver nanoparticle containing colloids and their ability to inhibit *Streptococcus mutans* biofilm formation on dental enamel. Colloid cytotoxicity was evaluated using the hen´s egg test by following signs of vascular changes on the chorioallantoic membrane (HET-CAM). Bactericidal properties and inhibition of *S. mutans* biofilm formation were determined using a parallel flow cell system and a dichromatic fluorescence stain.

Synthesis of silver nanoparticles in aqueous solution was carried out by chemical reduction of silver nitrate ($AgNO_3$) with sodium borohydride ($NaBH_4$) in presence of chitosan biopolymer as stabilizing agent, as described by Wei, D., et al. [1]. This route leads to small seeds (AgNP1). Three other formulations were obtained by adding, subsequently, different $AgNO_3$ amounts. The average particle sizes are 8.7±3.1nm (AgNP1), 15.0±7.9nm (AgNP2), 31.8±10.4nm (AgNP4), 43.2±14.3nm (AgNPs5). AgNP1 and AgNP2 present, essentially, spherical particles, while AgNP4 and AgNP5 present many particles with different shapes (plates, triangles). Absorption spectra of all samples and a TEM image of AgNP5 are presented in Figure 1.

Streptococcus mutans AU 159 was obtained from the Laboratory of Microbiology, UNICAMP, São Paulo, Brazil. A stock culture was maintained in brain heart infusion (BHI) broth, at -70 ºC. The inoculum used in the experimental assays was obtained from overnight cultures grown (for 18 h) in BHI broth at 37 °C. Bacteria were washed three times in 0.9% saline solution, and re-suspended to provide a bacterial inoculum of approximately 5 x 108 colony forming units (CFU/mL), optical density (OD) at 660 nm was 1.5. A Coupon Evaluation Flow Cell was used to cultivate the *S. mutans* biofilms under dynamic flow to replicate oral environmental conditions. Biofilm formation was assayed using a method described by Kumada et al [2].

HET-CAM tests were performed according to the methodology described by Steiling *et al.* [3]. All assays were repeated 5 times. Membranes were observed for 5 minutes for signs of vasoconstriction, hemorrhage and coagulation. The time (in seconds) at which these processes were recorded and applied in the following equation [4]:

$$IS = \frac{[(301 - hae) * 5] + [(303 - lys) * 7] + [(301 - coa) * 9]}{300}$$

, where *hae*, *lys*, and, *coa* refer to hemorrhage, lysis and, coagulation, respectively. The potential for irritation (irritation score - IS) is related to the following scale: 0.0 – 0.9 denotes no irritation; 1.4 – 4.9, slight irritation; 5 – 8.9, moderate irritation and; 9 – 21, severe irritation.

The viability of *S. mutans* in biofilm formation on dental enamel in a dynamic flow cell system measured by fluorescence analyses and cytotoxicity evaluated for the development of irritant vasoconstriction, haemorrhage and coagulation endpoints are shown in Table 1.

The chorioallantoic membrane has been proposed as a model for a living membrane because it has a functional vasculature. The acute effects induced by a test substance on the small blood vessels and proteins of this soft tissue membrane can be considered to be similar to the rabbit eye test, while offering the advantages of being more universally acceptable as a non-animal test and being completed more rapidly [5]. Several studies have been conducted to evaluate the feasibility of using HET-CAM as a complete replacement for the in vivo rabbit ocular test. This test has several advantages, including its simplicity, rapidity, sensitivity, ease of performance and relative low cost [6,7]. Figure 2 present representative images for a non-irritating (AgNP1) and an irritating samples (AgNP4).

Samples AgNP1, AgNP2 and, AgNP5 presented good inhibition to biofilm formation and no detected cytotoxicity. It is not clear, yet, the reason why sample AgNP4 behave differently. In

conclusion, silver colloids, as formulated here, present a better potential to be used as anticaries agents than silver diamine fluoride which is, actually, used clinically.

Table 1. S. mutans viability in biofilm formation on dental enamel in a dynamic flow cell system and cittotoxicity evaluation by HET-CAM.

Sample	*S. mutans* viability (%)	Irritation score (IS)
AgNP1	0.0	0.0±0.0
AgNP2	0.0	0.0±0.0
AgNP4	41.3	1.94 ± 0.20
AgNP5	0.0	0.0±0.0
diamine silver fluoride	36.5	1.42±0.35
saline solution	35.4	-
1% sodium lauryl sulfate	-	17.74 ± 0.4

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Figures

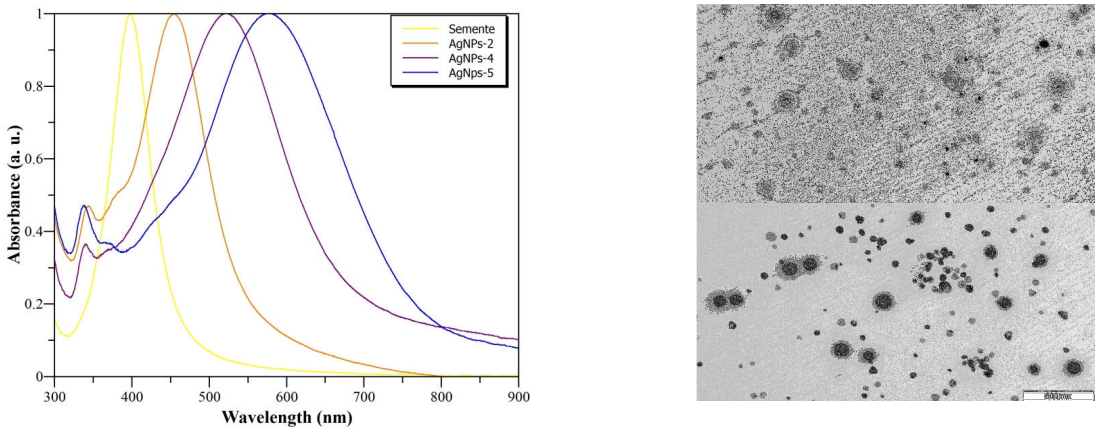

Figure 1. (left) Normalized absorption spectra for silver colloids; (yellow) seeds, AgNPs-1; (orange) AgNPs-2; (purple) AgNPs-4 and; (blue) AgNPs-5. (right) TEM image for AgNPs-5 sample.

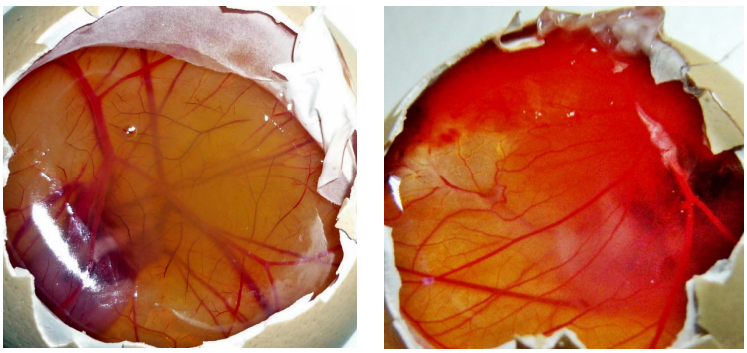

Figure 2. HET-CAM test. (left) AgNPs-1 sample, non-irritating; (right) AgNPs-4 sample, slightly irritant, shows hemorrhage.