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RESEARCH ARTICLE

SILVER NANOPARTICLES AND THEIR EFFECT ON THE BIOFILM FORMATION IN FOOD BORNE SALMONELLA SPECIES

Sajjad Mohsin Irayyif, Asal Mohseni Sajadi Araghi and *SudhakarMalla

Department of Biotechnology, Centre for Research & PG Studies, Indian Academy Degree College, Bangalore, India

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ABSTRACT

Antibiotic treatment to control majority of infections was slowly becoming a problematic as most of the antibacterial studies were designed for the free planktonic forms. But when it comes to biofilm studies, the antibiotic usage has been restricted due to the formation of thick wall in the biofilms. So for a proper understanding of the physiological mechanism on antibacterial activities, nanoparticles have been used. The food borne pathogens like *Salmonella typhii* and *Salmonella paratyphii* have been used in our study to investigate the effect of silver nanoparticles on them. Our results suggest that there was a significant increase in the percent of inhibition among the two species with the increasing concentration of the nanoparticles. But there was no such effect found between the species. It was found from the results that the rate of percent inhibition was concentration dependent. The inhibition of biofilm formation in *Salmonella typhii* was found to be 0.74± 0.13, 0.69± 0.3, 0.61± 0.23, 0.5± 0.13, 0.42± 0.38, 0.28± 0.29 and 0.17± 0.53 for control, 50µg/ml, 100µg/ml, 200µg/ml, 300µg/ml, 400µg/ml and 500µg/ml respectively. The effect of nanoparticles on the biofilm formation and primary adherence assay was found to be concentration dependent.

INTRODUCTION

Nanotechnology is a multidisciplinary applied field focused on materials whose physical and chemical properties can be controlled at the nanoscale range (1–100 nm) (Sanhai WR, 2008). Nanomaterials and their applications have been greatly increased in the medicine field. With increasing resistance towards the antibiotics and their ease in forming the biofilms, bacteria are slowly evading the medical world. The advances in nanotechnology promise to be of great value for various applications including various medicinal uses, such as therapeutics, diagnosis, or drug delivery (Jain K, 2012).The fast development of antibiotic resistant bacteria in most of the ecosystems has created a great need for the development of new antimicrobial agents. The use of nanomaterials as novel antimicrobial agents can provide novel modes of action (Shilo M, 2012). Nanomaterials exhibit different properties at the nanoscale level than in their bulk scale. The silver or gold particles at the bulk which are not antimicrobial in property might be antimicrobial at their nanoscale level.

Biofilms are usually aggregates of microorganisms (eg, *Pseudomonas* spp., *Escherichia* spp., *Staphylococcus* spp., etc) attached to a substratum in moist environments. The substratum is composed of extracellular polymeric substances produced by microorganisms; the latter have a distorted phenotype with respect to growth rate and gene transcription (Houry A, 2009). The presence of this distorted phenotype can cause a high forbearance to exogenous stress and resistance (up to 1000-fold increase) to antibiotic therapy. Many planned events can predispose bacteria to adhere and form a biofilm (Chambless J, 2006). In overview, biofilm formation is usually initiated by attachment of free-swimming bacteria on a surface that subsequently intersperse with fluid-filled channels. These biofilms are believed to have important role in tolerance to antimicrobial therapy and drug resistance (Chellappa ST, 2013). Thus, it makes it very clear that an understanding of antimicrobial tolerance mechanisms is important to institute novel therapeutic approaches. Most importantly, failure of antimicrobial therapy should not be perceived as a lack of clinical management tools. Conventional antimicrobial agents which are based on antimicrobial susceptibility test results are usually performed with planktonic cells (Vrany J, 1997). Translation of these methods to biofilm is usually difficult and a bad idea due to poor penetration and decreased susceptibility of bacteria. Thus, complementary approaches that are based on surface modifications, use of device applicators and nanomaterialsand phage deliveries are being investigated as a means of prevention and control (Dillen K, 2006).

Biofilm forming capacity in bacterial pathogensis one of the most studied bacterial physiologynowadays as they form a crucial link in pathogenicity (Ramsey MM, 2004; Chellappa ST, 2013, Houry A, 2009). Almost all of the known

Corresponding author:* **SudhakarMalla
Department of Biotechnology, Centre for Research & PG Studies, Indian Academy Degree College, Bangalore, India

pathogenicbacteria of humans, such as *Salmonella, Vibrio cholerae, Pseudomonas aeruginosa*, and pathogenic *E.coli*, are motile. Motility and biofilm forming capacity have been traced out in *Salmonella enteric serovar Typhi* (*S. Typhi*i) (Tang FQ, 2012). It is the defined pathogen of typhoid fever and food borne infections. Salmonella is found to be infecting21.7 million people and literally causing 217,000 deaths annually (Fux C, 2005). Several case studies have investigated risksfor enteric fever; the majority implicate water and foodas important transmission routes (Gelperina S, 2005; Kearns DB. 2010).

Studies done so far proved that *S.typhi* is frequently associated with the gallstones in asymptomatichuman carriers, in which the bacteria colonises and forms biofilm on the gallstones (Klapper I, 2010). Studies also reported the possible and severe infections andbiofilm forming ability in the food industry. These food industries suffer a huge loss with this pathogen (Murray TS, 2010).

The objective of this study was to study the antibacterial activity of the silver nanopartciles (10nm) towards the food borne pathogens *Salmonella typhii* and *Salmonella paratyphii*. The activity will be confirmed by the antibacterial activity, biofilm studies and primary adherence assays.

MATERIALS AND METHODS

Bacterial strains: The strains of salmonella were procured from the MTCC repository. Two serovars of Salmonella were used for the study for the comparative analysis of their resistance to the nano particles. *Salmonella typii*(MTCC 8767), *Salmonella paratyphii*(MTCC 735).All these cultures were maintained on nutrient agar plates at 4^0C.

Antibacterial Assay: Antimicrobial activity of the silver nanoparticles (10nm) was tested against the two species. Overnight cultures were prepared in Nutrient broth (NB) media by inoculation with a single colony from agar plates and incubated at 37^0C for 12 hrs. Overnight cultures were diluted with fresh NB media to approximately 10^4 colonies forming units (CFU) and used for further assays.

Antibacterial activity using the agar cup plate method: Both the cultures were pour plated onto nutrient agar plates and about 20µl of nanoparticle suspension was added into each well. The antibacterial activity was confirmed first on the plate and later assayed using tube method.

Antibacterial activity using the tube method: Silver nano particles of different concentrations 50µg/ml, 100µg/ml, 200µg/ml, 300µg/ml, 400µg/ml and 500µg/mlwere prepared and used for the antibacterial assay. Eight test tubes filled with 10ml of NB media were used in the study. The first one being negative control without innoculum, and second being the control without treatment. The tubes labelled from 3-8 were used for the treatments. About 10µl of the culture suspension was added to all the tubes labelled from 2-8. The tubes labelled for treatments were added with 20µl of the nanopartcile suspension of varying concentrations (50µg/ml, 100µg/ml, 200µg/ml, 300µg/ml, 400µg/ml and 500µg/ml).The experiment was repeated twice for the confirmation. The percentage inhibition was calculated by using the formula: Percentage Inhibition (%) = [(dc - dt)/dc] x 100, where dc and dt represent OD600 of control and treated sample strains respectively.

Biofilm Cultivation: The two overnight culture suspension of both the species was taken and diluted to a ratio of 1: 200 using Nutrient broth +Glucose solution. The diluted cultures were added to the microtitre plate and incubated at 37^0C for 24 hrs.The wells from 2-7 were added with 20µl of the nanoparticle suspension of varying concentrations as done in the antibacterial assay. The 1^{st} well being the control (without treatment).The plate was incubatedfor overnight at 37^0C. After incubation the wells were washed with 200µl of PBS pH7.4 three times. The plates were then air dried and stained with 2% crystal violet for 15 minutes.The plates were then rinsed under running tap water, air dried and then crystal violet was solubilised in 200µl of ethanol:acetone 80:20. Absorbance was recorded at 590nm.

Primary adherence assay: The cell suspension of both the species were inoculated into respective flasks with Nutrient broth containing 0.5% glucose. One of the flask containing nutrient broth with glucose is labelled as control. The control is used separately for each species. Flasks labelled with 1-7 were treatments with varying concentrations of nanoparticles. The flasks were then incubated at 37^0C overnight. Following incubation, 200µl of the broth with the culture was diluted to an absorbance of 0.1 at 578 nanometres with nutrient Broth containing 0.5% glucose. 10µl of the suspension was added to slides and incubated for two hours at 37^0C.After incubation the slides was washed three times with PBS (pH7.4). The cells were then fixed with glycerine solution and then carried with the gram staining process. Adherent bacterial cells were observed under 40X and mean count was taken for 5 microscopic fields.

RESULTS

Antibacterial Assay: Antimicrobial assay of the silver nanoparticles(10nm) was examined against both the species of Salmonella. A zone of inhibition was found towards both the strains.

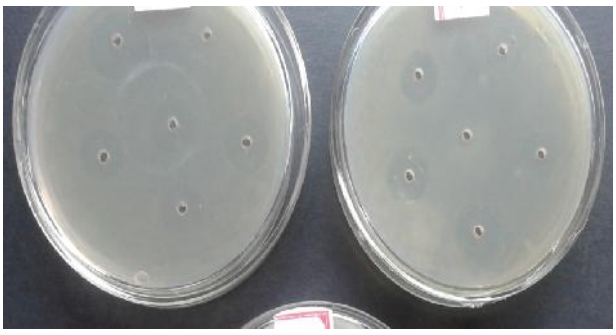

Fig 1 Figure showing the plates with zone of inhibition. Left: *Salomella typhii*, Right: *Salmonella paratyphii*.

The results suggested that silver nanopartciles exhibits bactericidal property *in-vitro* i.e. the growth of microorganisms

was inhibited in its presence as shown in figure. The percent inhibition for *Salmonella typhii* was found to be 2.90, 13.43, 12.07, 17.65, 26.19 and 29.03 for control, 50µg/ml, 100µg/ml, 200µg/ml, 300µg/ml, 400µg/ml and 500µg/ml respectively. The percent inhibition for *Salmonella paratyphii* was found to be 1.39 ± 0.43, 8.45± 0.41, 12.31± 0.52, 28.07 ± 0.23, 39.02± 0.17 and 48.00 ± 0.23 for control, 50µg/ml, 100µg/ml, 200µg/ml, 300µg/ml, 400µg/ml and 500µg/ml respectively.

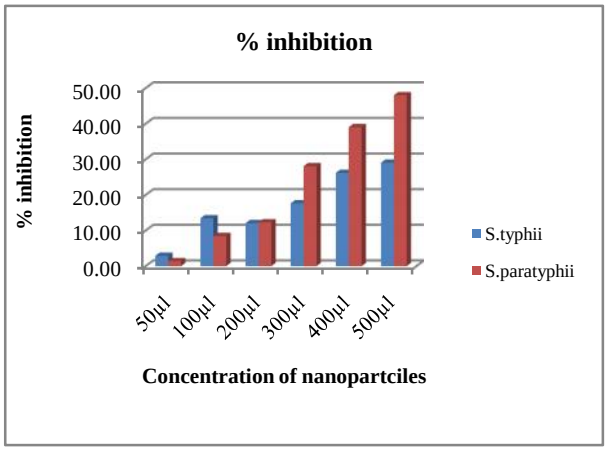

Fig 2 Graph showing the percent inhibitory effect of silver nanoparticles on the growth of Salmonella species. All the results were average of triplicates.

A two way ANOVA between the bacterial cultures *Salmonella typhii* and *Salmonella paratyphii* and treatments (different concentrations) was conducted to compare the effect of nanopartciles on the percent inhibition. All effects were statistically significant at the 0.05 significance level. There was a significant effect of different concentrations of silver nanopartciles on the growth of the species remembered at the p<0.05 level. Both the species showed significance to the treatment [F(5,5) =8.742636, p =0.01636]. There was no significant effect observed between the species towards the treatment. Our results suggest that there was a significant increase in the percent of inhibition among the two species with the increasing concentration of the nanopartciles. But there was no such effect found between the species. It was found from the results that the rate of percent inhibition was concentration dependent.

Biofilm Cultivation: The biofilm assay as done was also found significant to the treatment. Both the species responded to the treatment. The response was more shown by the *Salmonella paratyphii* the formation of biofilm was found to be inhibited by the treatment and the inhibition was also concentration dependent. The inhibition of biofilm formation in *Salmonella typhii* was found to be 0.74± 0.13, 0.69± 0.3, 0.61± 0.23, 0.5± 0.13, 0.42± 0.38, 0.28± 0.29 and 0.17± 0.53 for control, 50µg/ml, 100µg/ml, 200µg/ml, 300µg/ml, 400µg/ml and 500µg/ml respectively. The formation of biofilm andits inhibition in relation to the control in *Salmonella paratyphii* was found to be 0.82 ± 0.34, 0.77± 0.42, 0.65± 0.33, 0.52± 0.14, 0.46± 0.24, 0.31± 0.14 and 0.14± 0.54 for control, 50µg/ml, 100µg/ml, 200µg/ml, 300µg/ml, 400µg/ml and 500µg/ml respectively.

A two way ANOVA between the biofilm formation and nanoparticle treatment was conducted to compare the effect of treatment on biofilm formation in both the species.All effects were statistically significant at the 0.05 significance level. There was a significant effect of treatment on biofilm formation in both the species remembered at the p<0.05 level.

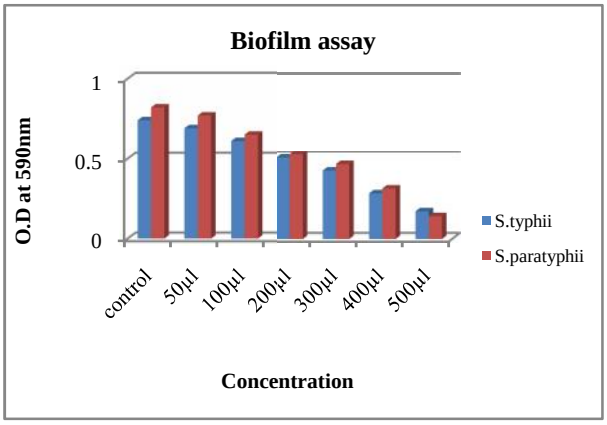

Fig 3 Graph showing the O.D values of biofilm cultivation assay of bacterial cultures. All the values are the averages of triplicates. The O.D values were noted after solubilisation of the stained sample with ethanol solution following an incubation time of 24 hours.

Both the cultures showed significance to the nanopartcile treatment [F(6,6) =146.424, p =3.0893E-06] and the biofilm formation [F(1,6)= 6.7826, p=0.0404]. Our results suggest that there was a significant inhibition in the rate of biofilm formation in both the species at increasing concentrations of the nanopartciles. *Salmonella paratyhii* showed more rate of inhibition than *typhii.*

Primary adherence assay: Our results suggest that there was a significant decrease in the adherence of the bacterial cells of both the species at different concentrations of particles. Itwas found that the adherence of cells was less at high concentration of the nanopartciles. And both the species responded similar for the adherence effect. The cell count was found to abruptly decrease in both the species. The inhibition in the cell count for *Salmonella typhii* was found to be 231, 228, 215, 197, 174, 161 and 134 for control, 50µg/ml, 100µg/ml, 200µg/ml, 300µg/ml, 400µg/ml and 500µg/ml respectively. The inhibition in the cell count for *Salmonella paratyphii* was found to be 253, 246, 237, 211, 185, 155 and 128 for control, 50µg/ml, 100µg/ml, 200µg/ml, 300µg/ml, 400µg/ml and 500µg/ml respectively.

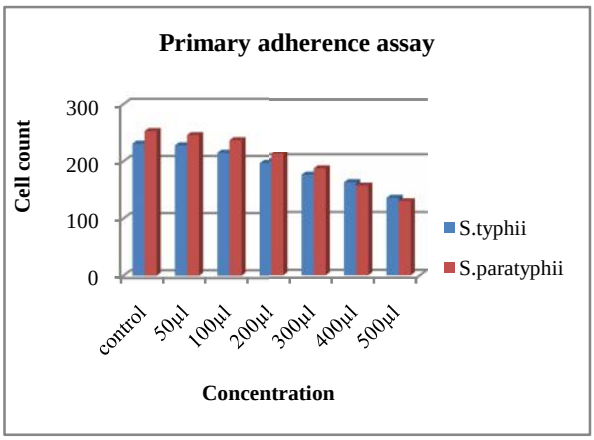

Fig 4 Graph showing the cell count of primary adherence assay of bacterial species. All the values are the averages of triplicates. The cells were incubated for 2 hours, then counted after Gram staining.

A two way ANOVA between the treatments and bacterial cultures was conducted to compare the adherence of bacterial cells at different concentrations of nanoparticles in both the cultures. All effects were statistically significant at the 0.05 significance level. There was a significant effect adherence of bacterial cells of both species at different concentrations of nanopartciles remembered at the $p<0.05$ level. Cultures showed significance to the treatments [$F(6,6) = 48.7694$, $p = 7.87E-05$] but there was no significant effect found between the adherence effect among the species.

CONCLUSION

Nanotechnology has been promising and fulfilled a lot of solutions to the problems in the field of medicine. The emerging food borne infections are becoming trouble some for the food industry. The silver nanopartciles at 10nm size were found to be effective towards the food borne pathogens *Salmonella*. Both the species of Salmonella (*Salmonella typhii and Salmonella paratyphii*) showed response to the nanopartciles. The study on the antibacterial activity, biofilm formation and their primary adherence capacity all proved of the role of the nanoparticles as antimicrobial agents. Moreover, a keen study observed was the response was more towards *Salmonella paratyphii* than *Salmonella typhii*. The treatment was done at different concentrations and the response was purely concentration dependent. Though the particles seem to be effective as antimicrobial, we cannot merely ignore the particle concentration, which can be harmful to the environment as well. Further optimization is needed to minimize the concentration of the particles and henceforth use them as safe antimicrobials.

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