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EFFECTS OF SILVER NANOPARTICLES ON *ESCHERICHIA COLI* AND IT'S IMPLICATIONS

BIPASHA GHOSH and D. RAMAMOORTHY*

Department of Ecology and Environmental Sciences, Pondicherry University,
PONDICHERRY, INDIA

ABSTRACT

Effects of silver nanoparticles have been observed on Escherichia coli and its implications have been reported.

Key words: Silver, Nanoparticles.

INRODUCTION

Nanoparticles are very small in size, highly active and have very special characteristics like high surface area to mass ratio which can greatly enhance the different capacities of material made up of it. Thus it enables to manipulate an objective in the atomic level or nanoscale level for it various applications. In addition to high specific surface area, nanoparticles also have unique adsorption properties due to different distributions of reactive surface sites and disordered surface regions. Fundamental electronic, optical, chemical and biological processes are also different at this level. Varying the composition size or surface composition can change the physical and chemical properties of nanoparticles.

Therefore the charactertics of nanoparticles vary based on the following:

(i) Size: It depends upon the material used to produce Nanoparticles – Properties like solubility, transparency, colour, absorption or emission wavelength, conductivity, melting point and catalytic behavior are changed only by the varying particle size.

(ii) Composition: The different set of nanoparticles used will lead to a different physical and chemical behaviour of the material.

(iii) Surface: Properties like adsorptivity, dispersibility catalytic behaviour, conductivity and optical properties alter with different surface properties of the particle.

When it comes to technical application, all these parameters need to be controlled.

*Author for correspondence

Application of nanoparticles

Nanoscience and nanotechnology studies have revised much attention in the last decade. These studies involve a wide spectrum of research area and industrial activities from fundamental science (Physical, Chemistry and Biology) to applied science (Electronic and Material). The uses of nanoscience have a wide range including optical, magnetic, thermal, mechanical, electronic, surface, personal health care, biomedical and environmental applications.

Environmental applications

Nanoparticles have a wide range of application when it comes to environmental pollution and monitoring. Waste water treatment is a very important area where nanoparticles can be used effectively. Many physical as well as chemical agent and methods are widely used in the case of waste water treatment halogen such as chlorine (Cl) and Bromine (Br) are well known and widely used as anti-microbial agents, but the direct use in pure form as bactericide has many problems because of their high toxicity and vapour pressure.

The most common cation in water affecting human and animal health is Ammonium (NH4). In drinking water, ammonia removal is very important due to its extreme toxicity to most fish species, prevent oxygen depletion and algal bloom. It can be replaced with biological acceptable cations like Na^{+}, K^{+}, or Ca^{2+} in the zeolite. During the past few decades, several investigations have been carried out conserving the use of synthetic and natural zeolites, polymer films and metal ions are bactericides for water disinfection. In the area of water purification, nanotechnology offers the possibility of an efficient removal of pollutants and microorganisms.

Today nanoparticles, nanomembranes, nanopowder used for detection and removal of chemical and biological substances including metals (e.g.: Cadmium, Copper, Lead, Mercury, Nickel, Zinc), nutrients (e.g. Phosphate, Ammonia, Nitrate, nitrite), Cyanide, Algae (e.g. Cyanobacterical toxins), viruses, bacteria, parasites. Four class of nanoscale that are being evaluated as functional materials for water purification materials are metal containing nanoparticles, carbonaceous nanomaterials, Zeolites and dendrimers, carbon nanotubes and nanofiber also show some positive result. Nanomaterials reveal good result than other techniques used in water treatment because of its high surface area (surface/ volume ratio). It is suggested that these may be used in future large scale water purification.

Nanocapsules and nanodevices may represent new possibilities for drug delivery, gene therapy, medical diagnostics, antimicrobial activities etc. Silver nanoparticle is used in open wounds and burn treatment[1].

EXPERIMENTAL

Materials and methods

Preparation of bare silver nanoparticles

Particles prepared according to Siewach and Sen (2005) has been synthesized by

employing a unique, physical, top-down approach of EEW technique. In the EEW technique, a wire is exploded on a plate of the same material by passing current of 1010 ampere/meter sq. in a time of 6 to 10 m/sec. Flow of current through the wire plates leads to the heating of wire plates at point of contact followed by melting. The melted metal at the point of contact is further heated by the ever increasing current density which leads to evaporation of material and subsequent plasma formation. The plasma is contained by the self induced magnetic field. When the vapour pressure of plasma overwhelms self induced magnetic field exploration occurs and the plasma products are dispersed in the medium with high speed. Silver nanoparticles augmentation is synthesized by fragmentation of the parent material, in water medium which simultaneously caps and collect the particle[2].

Test for bactericidal activity of bare silver nanoparticles

The bactericidal activity of silver nanoparticles was checked by determining MIC (minimum inhibitory concentration) and MKC (Minimum killing concentration). MIC of silver nano particle was conducted by a simple serial dilution spreading method. The biological experiments were conducted by serial dilution of each treated bacterial sample for 106 times of Luria-Bertani (LB) broth tubes. The tubes were then incubated at 35 ± 50^{o}C for 24 hours and were observed for the presence and level of visible growth of pre-treated *E. coli* DH 5α cells with 10, 20, 30, 40, 50, 80 and 100 µg/mL of bared silver nano particle in the LB medium. In this study varying concentrations of silver nanoparticle, as mentioned above were used to determine the MIC in LB media. Concentrations of the anti-microbial test compound below the MIC fail to inhibit microbial growth. *E. coli* DH 5α bacterial cells were grown overnight in the presence of cellular nanoparticles. Growth in the sample was determined by observing the turbidity of the culture by measuring optical density (OD) at 595 nm. The minimum concentration of Ag-nanoparticle, at which no visual turbidity could be observed, represented the MIC of Ag-nanoparticle.

Time dependent treatment of bacterial strains

The silver nanoparticle was suspended in triple distilled water to perform the time dependent anti-bacterial study of Ag-nanoparticle with the bacterial strain. Bacterial cells were centrifuged at 6000 rpm for 20 minutes; pellets homogenized in 1ml PBS buffer and were treated with 1.0 mL of each concentration (10, 20, 30, 40, 50, 80 and 100 µg/mL) of bared Ag-nanoparticle for 30 minutes with varying time interval for each concentration. Ag-nanoparticles suspension was homogenized by ultrasonic cleaner. Each treated bacterial culture was serially diluted until 10^{6} dilution factor, spread 100 µL from each in LB agar plates. The plates were then incubated at 37^{o}C for 24 hours and number of colonies grown on agar plate was observed.

Growth pattern for combined treatment of nanoparticles and ultrasonic irradiation

The inocule *of E. coli* DH 5α bacterial cells were prepared by growing test strain in LB medium at 37˚C until approximately 106-108 CFU reached cells were centrifuged and pellet mixed in same amount of PBS buffer. 10 mL of each sample were treated with 1.0 ml of 10, 20, 30, 40, 50, 80 and 100 µg/mL homogenized Ag- nanoparticles; all these samples were exposed for 5 and 15 minutes at 35 KHz ultrasound frequency after addition of Ag-nanoparticle. 24 plates

were prepared containing 20 mL LB broth, 100 µL 106 times diluted, all pre-treated DH 5α culture spread over it. After 30 minutes all treated culture samples were poured in Petriplates with 20 mL of LB agar medium and incubated these petriplates at 17˚C for 24 hours. Colony numbers were counted after 24 hours of bacterial growth on LB agar petriplates. All assays were carried out in duplicates in an effort to eliminate errors during procedure.

Cytoplasmic leakage analysis for Ag-nanoparticle treated cells

Nucleic acid leakage test

Ag-nanoparticles treated cells were centrifuged pellet discarded and supernatant used to study protein and Nucleic acid leakage analysis. The amount of nucleic acid (NA) released in the ultrasonic Ag-nanoparticle treated cells was measured at 260 nm using UV spectrophotometer. For preliminary identification nucleic acid leakage test ninhydrin test showed positive result.

Protein leakage test

If bacterial cells burst, intracellular materials will be released outside. The amount of protein present in intracellular material of bacterial cells will also be released. If all the conditions are same and concentration of the leaked protein is higher intracellular fluid of a sample, implies that cells disruption rate is higher.

Protein leakage analysis of Ag-nanoparticle treated cell was performed by Bradford assay of protein concentration determination. Supernatant was collected after centrifugation of the Ag-nanoparticle treated cells at 6000 rpm for 20 minutes. For each sample 200µl of supernatant was mixed in 800 µL of Bradford reagent. Optical density was measured after 10 minutes of incubation in dark at 595 nm.

Atomic adsorption analysis for K^+ level in extracellular fluid

If the Ag- nanoparticles disrupt the bacterial cell, the intercellular concentration of K^+ will increase in extracellular fluid as concentration of nanoparticle increases. Active culture of *E. coli* DH 5α cells were treated with 25, 50, 100, 200, 300, 400, 500 & 600 µL of Ag-nanoparticle solution for 12 hours. Treated samples were centrifuged for 15 minutes at 5000rpm, supernatant stored for determination of K^+ by Atomic Absorption spectrophotometer (AAS). Multivalent ions standard is used for this assay.

Effect of Ag-nanoparticle on outer membrane

SDS (Sodium dodecyl sulphate) detergent which destroys the bacterial cells by disrupting the cell membrane. If bacterial cells lose membrane stability due to any stress effect SDS will disrupt these cells more effectively than the normal cells. Effect of Ag-nanopartcle on the outer membrane was checked by the SDS treatment of pre-treated cells of *E.coli* by Ag-nanoparticles. Active *E. coli* culture was mixed in PBS buffer (O.D. – 0.1) containing 35 µg/mL concentration of Ag-nano particle for 30 minutes. Cells were centrifuged and a pellet mixed in same volume of PBS. SDS 0.15% was mixed in Ag-nano treated cell. After every two minutes time interval OD was measured.

RESULTS AND DISCUSSION

Effect of bare Ag-nanoparticles on the bactericidal activity

The growth of *E. coli* began to be inhibited at 10 µg/mL concentration of nanoparticle. This is the minimum concentration of nanoparticle when the growth of *E. coli* DH5α cells were inhibited known as Minimum Inhibitory Concentration (MIC) of nanoparticle (Table 1).

Table 1: Table showing the colony count data of Ag-nanoparticles treated *E.coli* DH5α cells

Ag-nanoparticles (µg/mL)	Treatment time with Ag-nanoparticles (No. of colonies)				
	15 min	30 min	2 hrs	6 hrs	12 hrs
0	250	247	247	257	240
10	240	228	1200	105	39
20	180	132	82	42	9
30	160	81	59	24	0
40	120	70	25	11	0
50	90	65	10	6	2
60	85	46	2	4	3
70	60	25	3	3	0
80	20	15	2	0	2
90	2	3	0	0	0
100	2	2	2	0	0

Time dependent treatment of bacterial strains

LB agar plates incorporating increasing concentration of Ag nanoparticles were inoculated with 0.1 OD of bacterial culture. In *E. coli* DH5α the numbers of colonies decrease as concentration increased and at 50 µg/mL concentration, 60% inhibition was observed, when bacterial cells were treated for 30 minutes. When treatment was increased from 30 minutes to 4 hours 10 µg/L Ag nano particle was sufficient to inhibit 60% of bacterial growth (Fig. 2) and 50 µg/L was sufficient to cause 100% inhibition. It was possible only with 10 µg/L Ag-nano particle if the treatment time was 12 hours (Tables 1 and 2).

Growth curve analysis of *E. coli* DH5α

The effect of Ag-nanoparticle on the growth of *E. coli* DH5α was studied and time dependent changes in bacterial growth were monitored by measuring OD at 595 nm. The OD at 595 nm is due to scattering light by the bacterial cells, is a function of bacterial cell density and thus correlates with growth of colonies. It is evident from the table that Ag-nanoparticle at the concentration oh 35 µg/L was inhibited growth of *E. coli* whereas the concentration 10 µg/L was marginal. The control sample (in absence of Ag-nanoparticle) shows no growth inhibitions.

Table 2: Showing the OD of the growing culture of both the treated and non-treated *E. coli* DH5α strain

Incubation time (hrs)	Control	O.D. at 595 nm of *E. coli* cell treated with Ag-nanoparticles		
		10 µg/mL	25 µg/mL	35 µg/mL
1	0.084	0.0792	0.0582	0.0282
2	0.0863	0.0784	0.0581	0.0223
3	0.0921	0.0788	0.0585	0.0201
4	0.1096	0.0785	0.059	0.0232
5	0.2302	0.0804	0.0685	0.0228
6	0.3797	0.0845	0.0718	0.0325
7	0.4381	0.0979	0.1279	0.0462
8	0.469	0.2018	0.2012	0.0591
9	0.4712	0.3271	0.2675	0.0661
10	0.4724	0.3575	0.2923	0.0731
11	0.4833	0.3702	0.3138	0.0925
12	0.4831	0.3924	0.3215	0.1624
13	0.4833	0.3975	0.331	0.1998
14	0.4832	0.3978	0.3315	0.2202
15	0.4721	0.3977	0.3319	0.2252
16	0.4692	0.3965	0.3318	0.2301
17	0.442	0.3942	0.3282	0.2309

Growth pattern for combined treatment of nanopariticle and ultrasonic irradiation

Ultrasound is defined as acoustic energy or sound waves with frequency above 20 KHz. Ultrasonication are commonly thought to be detrimental to cell growth. Ultrasonic increases transport of small molecules in a liquid solution by increasing the convection in the solution otherwise stagnant or relatively slow moving fluid (Table 3).

It is evident that the OD of the growing culture decreased when the cells were treated with both ultrasound 35 KHz and Ag-nano particle. When the treatment of time of ultrasound was increased, a growth of bacterial cell decreased. The cells treated for 15 minutes had lesser OD than 5 minutes ultrasound treatment. It has been reported that ultrasound permit the transport of Ag-nanoparticle from LB medium to bacterial cell. Ultrasound facilitates the entry of Ag-nanoparticle and shows enhanced anti-bacterial properties. It can be concluded from these investigations that not only binding of nanoparticle to the outer membrane is responsible for biocidal effect but also caused due to binding of Ag-nano particle with some intra-cellular

elements. Ultrasound increases the convection of liquid by atleast two mechanisms. The first is Acoustic streaming flow in which the momentum from propagating sound waves is directly transferred to the liquid causing the liquid to flow in the direction of sound propagation. Thus any amount of ultrasound in liquid proceeds additional convection transport from acoustic streaming.

Table 3: Showing the optical density of the culture combined with Ag-nanoparticles and ultrasound (35 KHz)

Ag-nanoparticles (µg/mL)	Ag-Nanoparticles treatment	Ag-Nanoparticles/Ultrasonic treatment (O.D. 595 nm)	
		5 min	15 min
0	0.525	0.522	0.522
15	0.38	0.368	0.298
25	0.168	0.142	0.116
35	0.097	0.087	0.069
45	0.0675	0.053	0.031
55	0.021	0.018	0.011
65	0.003	0.000	0.000
75	0.005	0.002	0.001

The second and more notable example of enhancing convection is known as micro streaming and is preceded by cavitating gas bubbles to expand and shrink which in turn creates shear flow around the oscillating bubbles.

Cytoplasm leakage analysis for Ag-nanoparticle treated cells

Ag-nanoparticle injured cells have often being reported to release ninhydrin positive material purine and pyrimidine into a suspension. Nucleic acid and its relative compounds such as pyrimidine and purine are well known to absorb UV light at 260 nm range. The presence of these materials in a suspension indicates damage to the cell at the membrane level (Table 4).

The amount of nucleic acid release into suspension was analysed by measuring the absorbance upto 50 nm. The amount of protein released in the suspension of the Ag-nanoparticle treated cell was estimated by Bradford Assay (Table 5). The amount of Nucleic acid and protein released form the cell increased as the amount of Ag-nanoparticle increased. These results indicated that most of the nanoparticles treated cells were ghost cells from which intracellular material were released into the cell suspension.

Atomic absorption analysis for K^+ level in extra cellular fluid

Membrane potential maintenance is an important factor for the survival and growth of bacteria. Membrane potential of *E.coli* is largely maintained by a high intracellular k^+ ion

concentration mediated by inward influx. After the viable time period incubation of *E.coli* DH5α cells with Ag-nano particle, it was found that the extracellular K^+ ions increased with increasing incubation period of bacterial cell in Ag-nanoparticle environment. Ag-nanoparticles induced the massive loss of intracellular K^+ ions.

The K^+ ion concentration in extracellular fluid indicates the extent of damage of bacterial cells by Ag-nanoparticles. Increasing concentration of K^+ indicates continuously increasing rate of cell damage with increasing the incubation time of bacterial cells in Ag-nanoparticle environment.

Table 4: Showing OD of the diffusion of nucleic acid material from the cells treated with Ag-nanoparticles, measured at 260 nm

Ag-Nanoparticles (µg/mL)	O.D. at 260 nm of Ag-nanoparticles treated cell suspension	
	Control (*E. coli*)	20 µg/mL *(E. coli)*
5	0.005	0.004
15	0.021	0.031
35	0.041	0.062
45	0.053	0.059
55	0.059	0.07
65	0.062	0.092
75	0.075	0.102
95	0.099	0.136
115	0.121	0.162
200	0.215	0.272

Table 5: Showing the amount of protein leakage (µg/mL) of Ag-nanoparticles treated cells of *E. coli*

Ag-Nanopartcles (µg/mL)	*E. coli* 1 hr	*E. coli* 4 hrs
10	58.643	74.118
30	65.233	121.002
50	73.848	120.989
100	118.242	166.876

Membrane destabilization assay

Sensitivity of the Ag-nanoparticles injured cells was measured by their capacity to be lysed by Sodium-dodecyl sulphate (SDS). SDS is an anionic detergent used for cell lysis.

Treated cells were mixed with PBS buffer. Those cells in which membrane deformed after Ag-Nanoparticle treatment showed increased lasting effect of cells. Ag-nanoparticles treated cells in PBS buffer were incubated at 37^{o}C with shaking at 120 rpm in the presence of 0.1% SDS and the cell density was measured at 600 nm.

The OD of Ag-nanoparticle treated cells was dramatically reduced within 30 minutes of incubation in the presence of SDS but it did not decrease significantly in the absence of SDS. In untreated cell suspension, no significant difference in OD was observed after 30 minutes, either in presence of Ag-nanoparticle or in absence of nanoparticle. The result validates the fact that most of the cells inactivated by the Ag-nano particles remain unlysed in cell suspension in the absence of SDS and these are highly sensitive to lysis by SDS. It is clear from tables that treatment of nanoparticle destabilized the outer membrane. It is elucidated that the Ag-nanoparticle can destruct outer membrane compound such as porins and lipopolysaccharides. It is predicted that the Ag-nanoparticles initially bind with outer membrane but this particles can enter the cells at the higher concentrations.[3]

Table 6: Showing the OD measured at 595 nm of the membrane destabilization of both treated and non-treated cells of *E. coli* DH5α

Time (min)	Control	SDS-Treatment of *E. coli* cells (OD 595 nm)
0	0.153	0.156
2	0.149	0.153
4	0.156	0.15
6	0.151	0.142
8	0.158	0.136
10	0.156	0.128
12	0.148	0.082
14	0.156	0.083
16	0.15	0.069
18	0.153	0.065
20	0.149	0.062
22	0.156	0.06
24	0.151	0.051
26	0.158	0.052
28	0.156	0.052
30	0.148	0.05

CONCLUSION

The following conclusions can be made based on the study

- The effect of silver nanoparticle was time and dose dependent.
- The biocidal effect on bacterial cells treated with both ultrasonic and Ag-nanoparticle was more pronounced than the bacterial cells treated only with nanoparticles.
- Ag-Nanoparticles treated *E.coli* DH5α cells were disrupted quickly with sodium-dodecyl sulphate (SDS) than the non-treated cells.

Therefore, Ag nanoparticles can be used for developing nanofilter membrane for water treatment due to its biocidal effect against bacterial population.

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