CODEN (USA)-IJPRUR, e-ISSN: 2348-6465

PHS Scientific House
PHARMAHEALTHSCIENCES

International Journal of Pharma Research and Health Sciences

Available online at www.pharmahealthsciences.net

Original Article

Extracellular Biosynthesis of Silver Nanoparticles Using Bacterial Sources and its Pathogenecity Inhibition Assay

Prabhusaran N [1,*], Susethira AR[1], Radhakrishna L[2], Revathi P [2], Jeyaseelan ST [3], Joseph PID [4]

[1]Postgraduate and Research Department of Microbiology, Chennai Medical College Hospital and Research Centre (SRM Group), Tiruchirapalli, India.

[2]Postgraduate and Research Department of Pharmacology, Chennai Medical College Hospital and Research Centre (SRM Group), Tiruchirapalli, India.

[3]Department of Microbiology, Ponniah Ramajayam Institute of Medical Sciences, Kancheepuram, India

[4]Department of Microbiology, Karpaga Vinayaga Institute of Medical Sciences and Research Centre, Madurathagam, Kancheepuram, India.

ARTICLE INFO

Received: 28 Mar 2016
Accepted: 19 Apr 2016

ABSTRACT

Objective: In this investigation, we demonstrated a simple one step method for metal mediated bacterial nanoparticles. **Experimental approach:** The aqueous solution of silver ions was treated with extracellular filtrate of *E. coli* and *Proteus mirabilis* biomass for the synthesis of nanoparticles. The gradual colour change from pale yellow to deep brown is a clear indication for the nanoparticle synthesis. The periodic checking of increase in pH range (alkaline pH) and optical density also determined showed the complete formulation of the structural data of nanoparticles. *In vitro* antibacterial and pathogenicity inhibition were determined. **Findings and Discussion:** This color change showed effective in *E. coli*. Among the bacteria tested, *E. coli* seen to be effective in the production of silver nanoparticles by reaching a pH of 9.6 after 28hours of incubation. The antimicrobial activity by well cutting method showed maximum inhibition of bacterial pathogen including *E. coli, Staphylococcus aureus, Klebsiella pneumoniae* and *Proteus vulgaris, P. mirabilis, Acinetobacter baumanii* species, whereas less inhibition was observed in *Pseudomonas aeruginosa, Salmonella typhi* and *Vibrio cholerae* species. In the case of pathogenicity inhibition study, the interaction of the produced nanoparticles with preconfirmed catalase and coagulase producing *Staphylococcus aureus* showed certain range of inhibition at the concentration of 1.5%. Capsulation of *Klebsiella pneumoniae* and swarming nature of *Proteus* also controlled. **Conclusion:** This showed that the synthesized nanoparticles may have potential action to lose the virulence of the test bacterial pathogen. Further investigation required to analyze whether the nanoparticles have the efficiency to reduce the virulence completely by *in vivo* methods. This analysis may provide the roadmap for the vaccine synthesis for various infections.

Keywords: Bacterial sources, silver nanoparticles, pathogenecity inhibition assay

1. INTRODUCTION

The widespread use and sometimes misuse of antibiotics play a vital role in the emergence of more resistant and virulent strains of microorganism. The notification of rapid spread of multidrug resistant bacterial isolates causing nosocomial infections are the

Corresponding author *
DR PRABHUSARAN N
Postgraduate and Research Department of Microbiology, Chennai Medical College Hospital and Research Centre (SRM Group), Tiruchirapalli, India.
E-mail: leptoprabhu@gmail.com

great concern worldwide.[1] The outbreak observed even in developed countries also due to the *Staphylococcus* infection that resist to various antibiotics leads to human septic shock.[2] In most of the times, even the third generation antibiotics also get failure leads to search or synthesize the novel alternatives.[3]

As alternative, silver has long been recognized as having inhibitory effect on microbes present in medical or industrial process. The most common application of the silver and silver nanoparticles is in medical industry such as topical ointments to prevent infection against burn and open wounds.[4] The synthesis of nanoparticles are unique subset in the field of nanotechnology; thereby it is defined as "any type of particles with atleast one dimension of less than 500 nanometers – probably between 1 to 100nm".[5,6]

Nanoparticles are often crystalline and end up being referred as nanocrystals; the synthesis and characterization of nanoparticles is being an important area of novel drug discovery. The selection of size and shape of nanoparticles provide an efficient control over man of the physical and chemical properties and their potent application in medicine. A new research area "biomimetics" is now getting familiar where the biological systems including yeast, fungi, bacterial and plants are used for the synthesis of nanostructures of biocompatible metals.[7]

Nanoparticles of silver have thus been studied as a medium for antibiotic delivery, newer antimicrobial agents and synthesizing composites for constructing disinfecting filters and coating materials.[8,9] In recent years, nanoparticles of silver have been found to exhibit interesting antibacterial activities.[10]

Biologically synthesized silver nanoparticles could have many applications including spectrally selective coatings for electrical batteries,[11] optical receptors,[12] catalysts in chemical reactions, biolabelling[13] and antimicrobial properties.[14] There is tremendous excitement in the study of nanoscale matter with respect to their fundamental properties, organization to form superstructures and applications. It certainly does appear that there is plenty of room at the bottom in this fascinating area. With these literature reviews, we designed the objectives to synthesize bacterial induced silver nanoparticles, preliminary characterization, in vitro antimicrobial activity and pathogenicity inhibition assay.

2. MATERIALS AND METHODS

The bacterial biomass including *E. coli* and *Proteus mirabilis* were cultivated in double strength lactose and glucose broth respectively. Mostly for biosynthesis of any metabolites, the growth of bacteria is done in lactose medium but here the inclusion of Proteus mirabilis (lactose non metabolizer) required glucose as substitute for lactose.[3,5]

After acceptable incubation for turbidity formation, the cells were broken using sonicator and the sonicated broth was filtered using sterile 0.22µm pore sized filter papers. The metal solution (10^{-3}M) silver nitrate was prepared and analyzed for metal reaction for 2 hours in dark condition at room temperature. The non color change indicates there is no chemical reaction takes place. Further this concentration may be used for bacterial mediated nanoparticle synthesis.

From the bacterial filterate stock, 10ml of each was dispensed in the sterile reagent flask or screw capped bottles. On that 100ml of 10^{-3}M $AgNo_3$ solution was added, mixed well and finally 2ml of 0.1% lyzozyme solution was also added. This mixture was incubated at room temperature for physical characterization from 0^{th} hour to 28 hours. In the subsequent intervals (0, 1, 2, 4, 8, 16, 24, 28 hours), color change, pH variations and optical density value were recorded.

Further the concentrated nanoparticles were stored incorporated for antimicrobial activity and pathogenicity inhibition assay. The *in vitro*

antibacterial activity of concentrated nano compounds were assessed using battery of bacterial pathogens including *Acinetobacter baumanii, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Proteus mirabilis, P. vulgaris, Salmonella typhi, Staphylococcus aureus* and *Vibrio cholerae* by well cutting method. After incubation at 37°C for 24 hours, the plates were observed for formation of zone of inhibition around the wells. A solution based methodology for determining the antimicrobial activity was also standardized.[15]

For determining pathogenicity inhibition, well grown bacterial colonies from agar stocks were inoculated into the peptone broth. For 5ml of well grown microscopically confirmed bacterial cultures, various concentrations of KB method positive bacterial silver nanoparticles were added and incubated at 37°C for 24 hours. The inhibition of the pathogenicity of *Staphylococcus aureus* (catalase and coagulase), *Klebsiella pneumoniae* (capsular polysaccharide) and *Proteus mirabilis* (swarming nature in agar surface) were determined by standard microbiological procedures. The pathogenicity inhibition was determined for every 2 hours after incubation.

3. RESULTS AND DISCUSSION

It was found that aqueous silver ions when exposed to various bacterial cultures are reduced in solution, thereby leading to the formation of silver nanoparticles. The test biomass were pale yellow in color before addition of silver ions and this changed to brownish to black on the completion of reaction for 28 hours (Table 1). The broth and silver control were also maintained for comparison. The appearance of brown color in solution containing the biomass suggested the formation of silver nanoparticles.[16] Compared to *Proteus mirabilis*, the bacterial strain *E. coli* formed brown color earlier within 8 hours, even after 24 hours also the brown coloration is not formed in the media with *P. mirabilis.*

Table 1: Observation of color change in nanoparticle synthesis

S. No.	Bacterial cultures	Observation of color change versus time in hours							
		0	1	2	4	8	16	24	28
1	*E. coli*	+	++	+++	++++	+++++	+++++	+++++	+++++
2	*Proteus mirabilis*	-	-	+	++	+++	+++	+++	+++

[- (no color change); + (Orange); ++ (Pale red); +++ (red threads); ++++ (dark red) and +++++ (brown)]

It was observed that the pH of the two test bacterial media showed increase in pH from slightly acidic to alkaline. The pH of *E. coli* media was found effective to reach a pH of 9.6 after 28 hours of incubation whereas the pH of *P. mirabilis* increased very slowly from 6.5 to 7.4 after 28 hours of incubation (Figure 1). The controlled pH solution for nanoparticle synthesis in laboratory condition enhances the effective newer nanoparticles which can combat cancer and other infectious diseases.[17,18]

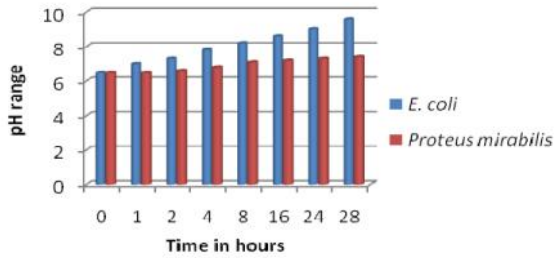

Fig 1: Observation of increase in pH of test bacterial strains

The optical density of the calorimetric assay showed initial value of 0.71 in the case of *E. coli* and gradually increased upto 2.47 in 28 hours of hourly observation. Comparatively, P. mirabilis increased upto 1.62 from initial value of 0.83 (Table 2). Some studies stated that the increase in the optical intensity and density showed the formation of agglomeration of the particles. It is also suggested that there is an observable proof that the agglomeration of molecules increased the color and density.[14]

Table 2: Comparativeness of changes in optical density in nanoparticle synthesis

S. No.	Bacterial cultures	Observation of optical density versus time in hours							
		0	1	2	4	8	16	24	28
1	*E. coli*	0.71	0.93	1.24	1.63	1.94	2.11	2.29	2.47
2	*Proteus mirabilis*	0.83	0.91	0.99	1.12	1.19	1.32	1.49	1.62

The stored nanoparticles were further analyzed for *in vitro* antibacterial activity by Muller-Hinton agar – well cutting method. The results showed maximum inhibition of bacterial pathogens including *E. coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Proteus vulgaris*, *P. mirabilis*, *Acinetobacter baumanii* species, whereas intermediate inhibition was observed in *Pseudomonas aeruginosa* and no inhibition found in *Salmonella typhi* and *Vibrio cholerae* species in *E. coli* mediated silver nanoparticles whereas *P. mirabilis* mediated silver nanoparticles have less effect. Among the concentrations included 1:30 showed very effective antibacterial activity. The detailed antibacterial activity against test bacterial species in various concentrations of the bacterial nanoparticles was depicted in table 3.

Table 3: Comparing effect of bactericidal activity of bacterial silver nanoparticles

Bacterial pathogens	Effect of bacterial based silver nanoparticles (inhibition zone diameter in mm)								Effect of antibiotics (inhibition zone diameter in mm)				
	E. coli nanoparticles				*P. mirabilis* nanoparticles				Antibiotics in 10µg/ disc				
	1:10	1:20	1:30	1:40	1:10	1:20	1:30	1:40	Cef	Cip	Cli	Dox	Sulb
A. baumanii	11	14	19	19	4	6	6	6	11	10	4	10	19
E. coli	8	11	18	20	5	8	9	12	13	21	5	12	14
K. pneumoniae	11	13	16	19	3	3	6	7	10	11	8	10	18
P. mirabilis	10	11	14	17	2	2	5	6	14	13	4	11	21
P. vulgaris	12	15	19	20	2	2	5	6	12	10	4	11	20
P. aeruginosa	4	6	9	11	3	3	5	7	11	20	3	12	11
S. typhi	-	-	2	2	-	-	-	-	19	11	-	8	7
S. aureus	12	15	19	21	3	5	9	11	-	-	21	-	-
V. cholerae	-	-	-	3	-	-	-	-	9	11	-	19	14

[mm - millimeter; Cef - Ceftriaxone; Cip - Ciprofloxacin; Cli - Clindamycin; Dox - Doxycycline; Sulb - Sulbactam)

The pathogenicity inhibition of selected bacterial pathogens were also included in this study, thereby at the concentration of 1:30 itself *Staphylococcus aureus* lost its catalase and coagulase nature in 8 hours of observation. *Klebsiella pneumoniae* lost its capsulation in 16 hours of interactions. The swarming nature of *Proteus* also controlled at the concentration of 1:40 in 8 hours of interaction. The control cultures retain their enzymes, capsules and swarming nature of colonies even after 28 hours (Table 4). An interesting observation is under microscopy, the motile Proteus sp became non motile in 8 hours of exposure to *E. coli* mediated silver nanoparticles. Due to less effect of *P. mirabilis* mediated silver nanoparticles against bacterial battery in determining the sensitivity by plating, it was excluded for determining the pathogenicity inhibition.

Table 4: Bacterial pathogenecity inhibitory effects of *E. coli* Ag NPs

Concentration of *E. coli* Ag NPs	Bacteria & its pathogenecity nature	Bacterial pathogenecity inhibitory effects of *E. coli* Ag NPs in hours							
		0	1	2	4	8	16	24	28
1:10	St. cat	-	-	-	-	-	-	-	+
	St. coa	-	-	-	-	-	-	-	+
	Pr. swa	-	-	-	-	-	-	-	+
	Kl. cap	-	-	-	-	-	-	-	+
1:20	St. cat	-	-	-	-	-	-	-	+
	St. coa	-	-	-	-	-	-	-	+
	Pr. swa	-	-	-	-	-	-	-	+
	Kl. cap	-	-	-	-	-	-	-	+
1:30	St. cat	-	-	-	-	+	+	+	+
	St. coa	-	-	-	-	+	+	+	+
	Pr. swa	-	-	-	-	-	-	-	+
	Kl. cap	-	-	-	-	-	+	+	+
1:40	St. cat	-	-	-	-	+	+	+	+
	St. coa	-	-	-	-	+	+	+	+
	Pr. swa	-	-	-	-	+	+	+	+
	Kl. cap	-	-	-	-	-	+	+	+

[*E. coli* Ag NPs (*Escherichia coli* silver nanoparticles); St. cat (*Staphylococcus* catalase); St. coa (*Staphylococcus* coagulase); Pr. swa (*Proteus* swarming); Kl. cap (*Klebsiella* capsule); - (no inhibition) and + (inhibited)]

In this study, the biological synthesis of bacterial mediated silver nanoparticles was carried where the reduction processes of silver ions by test bacteria were found to be different. In most of the studies, it was identified that silver nanoparticles were exclusively formed extracellularly [9, 11, 19] and also the time duration take place to form gold nanoparticles after 1 day of addition of chloroaurate ions, while the silver nanoparticles were formed after 7 days.[7, 20] In this study the inclusion of silver only mainly due to the cost effectiveness and user friendly nature. Compare with other studies, we also believe that the nanoparticles were stabilized by the surface-active molecules released into the solution by bacteria.

There is a saying rightly called "silver moiety with two functions" – one is inducing the organism to synthesize nanoparticles at lower concentration, another is the induction of cell death at higher concentration. The synthesis of bacterial mediated silver nanoparticles including *E. coli* with 1-100nm sized[21] and *P. mirabilis*

with 10-20 nm sized[22] which had been reported to synthesize silver nanoparticles are characterized but not much reported about potent antimicrobials .

For current utilization, pharmaceutical and biomedical sectors are facing the challenges of continuous increase in the multidrug-resistant human pathogenic microbes. Systemic patients were infected by the MDR pathogens leading to the high infection rates. Thus this study forward and induce more interest on nanotechnology and framing the work to use nanoweapon against clinical multidrug resistant pathogens. Our present study is on silver nanoparticles mediated by *E. coli* and *P. mirabilis*, and its efficacy against bacterial pathogens act as a therapeutic agent to overcome of the antibiotic resistance especially in nosocomial infection pathogens. The biosynthesized silver nanoparticles using *E. coli* proved excellent antibacterial activity. The antibacterial activity is well demonstrated with agar well diffusion and pathogenecity inhibition. Later silver nanoparticles are characterized with color change, pH and optical density in controlled manner. Thus, it is proven from this study that the Ag-NPs mediated by *E. coli* seems to be promising and effective antibacterial agent against the multidrug resistant strains of bacteria.

4. REFERENCES

1. Phua J, Koh Y, Du B. Management of severe sepsis in patients admitted to Asian intensive care units: prospective cohort study. BMJ 2011; 342: 3245-3251.
2. Kumar A, Roberts D, Wood K. Duration of hypertension before initiation of effective antimicrobial therapy is the critical determinant of survival in human septic shock. Crit Care Med 2006; 34: 1589-1596.
3. Prabhu N, Joseph PID, Chinnaswamy P. Fungus mediated silver nanoparticle synthesis and its leptospiricidal action. Trends Biotech 2009; 2: 12-17.
4. Devendra J, Hemant KD, Sumita KSL, Kothari. Synthesis of plant mediated silver nanoparticles using papaya fruit extract and evaluation of their antimicrobial activities. Dig J Nanomat Biostruct 2009; 4: 723-727.
5. Maribel GG, Jean D, Stephan G. Synthesis of silver nanoparticles by chemical reduction method and their antibacterial activity. Int J Chem Biomol Engg 2009; 2: 104-111.
6. Branes CA, Elsaesser A, Arkusz J, Smok K, Palus J, Lesniak A, Salvati A, Hanrahan JP, Jong WHD, Dziubaltowska E, Stepnik M, Rydzynski K, George M, Lynch I, Dawson KA, Vyvyan CH. Reproducible comet assay of amorphous silica nanoparticles detects no genotoxicity. Nano Lett 2008; 8: 3069-3074.
7. Upendra KP, Preeti SS, Anchal S. Bioinspired synthesis of silver nanoparticles. Dig J Nanomat Biostruc 2009; 4: 159-166.
8. Siddhartha S, Bera T, Arnab R, Gajendra PS, Ramachandra R, Debabrata D. Characterization of enhanced antibacterial effects of novel silver nanoparticles. Nanotechnol 2007; 18: 103-111.
9. Saifuddin N, Wong CW, Yasumira ANN. Rapid biosynthesis of silver nanoparticles using culture supernatant of bacteria with microwave irradiation. J Chem 2009; 6: 61-70.
10. Jose RM, Jose LE, Alejandra C, Katherine H, Juan BK, Jose TR, Miguel JY. The bactericidal effect of silver nanoparticles. Nanotech 2005; 16: 2346-2353.
11. Klaus JT, Joerger R, Olsson E, Granqvist CG. Bacteria as workers in the living factory. Proc Trends Biotechnol 2001; 19: 15-21.

12. Schultz S, Smith DR, Mock JJ, Schultz DA. Nanophotonics with surface Plasmon. Proc Natl Acad Sci 2000; 97: 996-999.
13. Hayat MA. Optics and biophotonics of nanoparticle. Chem Phys 1989; 90: 51-56.
14. Prabhu N, Divya TR, Yamuna G. Synthesis of silver phyto nanoparticles and their antibacterial efficacy. Dig J Nanomat Biostruc 2010; 5; 185-189.
15. Jakub M, Ewa M, Anna W, Arkadiusz S. Antimicrobial potential of commercial silver nanoparticles and the characterization of their physical properties toward probiotic bacteria isolated from fermented milk products. J Env Sci Hlth 2016; 51: 222-229.
16. Sastry M, Ahmed A, Khan MI, Kumar R. Biosynthesis of metal nanoparticles using fungi and actinomycete. Curr Sci 2003; 85: 162-170.
17. Prabhu N, Revathi N. Fungal silver nanoparticles: preparation and its pH characterization. Trenz in Biotech 2009; 4: 27-29.
18. Lim HA, Mishra A, Yun SI. Effect of pH on the extracellular synthesis of gold and silver nanoparticles by Saccharomyces cerevisiae. J Nanosci Nanotechnol 2011; 11: 518-522.
19. Vijayakumar M, Priya K, Nancy FT, Noorlidah A, Ahmed ABA. Biosynthesis. Characterization and antibacterial effect of plant mediated silver nanoparticles using Artemisia nilagirica. Ind Crops Prod 2013; 41: 235-240.
20. Reddy AS, Chen CY, Chen CC, Jean JS, Chen HR, Tseng MJ, Fan CW, Wang JC. Biological synthesis of gold and silver nanoparticles mediated by the bacteria Bacillus subtilis. J Nanosci Nanotechnol 2010; 10: 6567-6574.
21. Gurunathan S, Kalishwaralal K, Vaidyanathan R, Venkatraman D, Pandian SRK, Muniyandi J, Hariharan N, Eom SH. Biosynthesis, purification and characterization of silver nanoparticles using Escherichia coli. Coll Surf B 2009; 74: 328-335.
22. Samadi N, Golkaran D, Eslamifar A, Jamalifar H, Mohseni FA. Intra/ extracellular biosynthesis of silver nanoparticles by an autochthonous strain of Proteus mirabilis isolated from photographic waste. J Biomed Nanotechnol 2009; 5: 247-253.

Conflict of Interest: None

Source of Funding: Nil