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Research Article

IN-VITRO ANTIBACTERIAL ACTIVITY OF GOLD NANOPARTICLES CAPPED WITH POLYSACCHARIDE STABILIZING AGENTS

PREMA. P AND THANGAPANDIYAN. S

Post Graduate and Research Department of Zoology, V.H.N.S.N. College, Virudhunagar – 626001, Tamil Nadu, India.
Email: prema.drprema@gmail.com

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ABSTRACT

The wide use of antibiotics in the treatment of bacterial infections has led to the emergence and spread of resistant strains which would result in the formation of new antimicrobials. In recent scenario, much attention has been paid to metal nanoparticles which exhibit novel chemical and physical properties owing to their extremely small size and high surface area to volume ratio. Hence the present research has been made an attempt to investigate the bactericidal efficacy of gold nanoparticles (AuNPs) on human pathogens. AuNPs were first synthesized by chemical reduction method with Tetrachloroauric acid ($HAuCl_4.3H_2O$) as a metal precursor. Characterization of freshly prepared gold nanoparticles was made using X-ray diffractogram (XRD) and scanning electron microscopy (SEM). Average size of the particles ranged from 8.78-21.96nm. The synthesized particles are spherical in nature. Antibacterial activities of the synthesized Au nanoparticles were assessed by agar well diffusion method. The stabilized AuNPs exhibited excellent antibacterial sensitivity (30mm) to *E. coli*, *K. pneumoniae* and *V. vulnificus* than the other experimental strains used. Two way ANOVA test revealed that the function of AuNPs on bacterial pathogens is statistically significant. From this study, it is concluded that Au nanoparticles with and without stabilizers could act as an effective antibacterial agent and prove as an alternative for the development of new antibacterial drugs to combat resistance problem.

Keywords: Gold nanoparticles, Stabilizing agents, X-ray diffractogram, Scanning electron micrograph, Antibacterial activity.

INTRODUCTION

The worldwide escalation of bacterial resistance to conventional medical practices is a serious threatens for human health. Microorganisms have been developing resistance to many antibiotics due to the indiscriminate use of antimicrobial drugs, increasing clinical problems in the treatment of infections[1]. In view of the increasing incidences of infections with emerging multidrug resistance, there is very little choice left for the physicians to treat such infections[2]. Therefore, there is an immediate need to develop new approaches to handle this problem. One of the promising approaches for overcoming bacterial resistance is the use of metallic nanoparticles[3]. Owing to their small size and higher surface-to-volume ratio, nanoparticles have an enlarged contact area with microorganisms. This feature enhances biological and chemical activity of the nanoparticles with high antibacterial efficacy. Another important property of metallic nanoparticles is their ability to target different bacterial structures[4]. Among the various metallic nanoparticles, gold nanoparticles have wide range of applications in nano-scale devices and technologies due to its chemical inertness and resistance to surface oxidation[5]. Gold nanoparticles also have potential activity against microbial pathogens and it mainly depends on the size and shape of the particles. The coating of aminoglycosidic antibiotics with gold nanoparticles has an antibacterial effect on a range of Gram-positive and Gram-negative bacteria[6]. The enhancement of the biocidal property of gold nanoparticles can be increased by adding antibiotics[7]. The antimicrobial activity of the antibiotic vancomycin was enhanced on coating with gold nanoparticles against vancomycin resistant *Enterococci*[8]. Two fold enhancement of antibacterial activity was observed when chitosan capped gold nanoparticles coupled with ampicillin[9]. Due to the interparticle interaction such as Vander Waals force and magnetic interaction the chemically synthesized particles gets agglomerated. This agglomeration reduces the specific surface area and the interfacial free energy, thereby diminishing particles reactivity. To prevent the agglomeration, the surface of the particles was passivated through the layer of stabilizer. A stabilizer can enhance dispersion and steric hindrance[10]. Carbohydrates can act not only as stabilizer but also as a reducing agent, leading to the formation of noble metal nanoparticles. Such conjugated water soluble nanoparticles can have potential antimicrobial applications[11]. The stabilizers used in this work such as starch, CMC, and chitosan were well known food grade, nontoxic, biodegradable and biocompatible polysaccharides. These biopolymers were widely used in tissue engineering scaffolds and drug delivery applications[12].

The aim of this work was fabrication and characterization of gold nanoparticles with and without toxic-free, biocompatible, natural stabilizing agents and the bactericidal activity was comparatively evaluated.

MATERIALS AND METHODS

Materials

Tetrachloroauric acid ($HAuCl_4.3H_2O$), Trisodium citrate ($Na_3C_6H_5O_7.2H_2O$), Sodium borohydride ($NaBH_4$), Starch, Carboxymethyl Cellulose (CMC), Chitosan, Acetic acid were purchased from Himedia (P) Ltd, Mumbai, were used as starting materials without further purification. Milli-Q water was used for the fabrication of nanoparticles throughout the experiment.

Methods

Preparation of aqueous dispersion of AuNPs (AuNPs)

Gold nanoparticles were prepared by reducing $HAuCl_4.3H_2O$ with trisodium citrate[6]. In this method, $HAuCl_4.3H_2O$ (0.5ml, 1mM) solution was heated to boiling. To this solution, 0.5 ml of 0.01 M trisodium citrate was added. During this process, solution was mixed vigorously. After the addition of trisodium citrate, the previous yellow colored solution of $HAuCl_4.3H_2O$ suddenly became transparent and colorless which is due to the formation of citric acid from trisodium citrate. Ultimately, the solution color changed to black and after then slowly to wine red indicates the formation of gold nanoparticles.

Mechanism of reaction could be expressed as follows:

$2HAuCl_4 + 3C_6H_8O_7$ (citric acid) $\rightarrow 2Au + 3C_5H_6O_5$ (3-ketoglutaric acid) $+ 8HCl + 3CO_2\uparrow$

Preparation of aqueous dispersion of starch capped AuNPs (AuNPs/S)

According to Tan *et al*.[13] 600 µl of 0.05 M $HAuCl_4.3H_2O$ aqueous solution was added to 200ml of 0.2 wt % starch aqueous solution in the reaction vessel, followed by constant stirring. The mixture was kept at 110˚C for 12 hours to obtain colloidal AuNPs stabilized by starch.

Preparation of aqueous dispersion of CMC capped AuNPs (AuNPs/CMC)

Procedure for the synthesis of CMC stabilized AuNPs was same according to the description of Tan *et al*.[13] instead of starch; CMC was used as a stabilizer.

Preparation of aqueous dispersion of chitosan capped AuNPs (AuNPs/CH)

Chitosan stabilized AuNPs was prepared following the method of Seoudi and Said[14]. The detailed synthetic procedure was as follows: a completely dissolved chitosan solution, 0.04g (20mg/ml) in 1% acetic acid solution was prepared first; due to the poor solubility of chitosan, the mixture was vortexed to completely dissolve it. The solution was filtered through 30µm millipore syringe filters to remove any impurities before use. 20ml of aqueous solution of $HAuCl_4$ (10 mM) was added to 40ml chitosan solution under magnetic stirring for 2hours and 8ml of freshly prepared $NaBH_4$ (0.1 M) was added drop by drop. The solution turned brown immediately after addition of $NaBH_4$; stirring was continued until a transparent wine-red solution obtained.

All the above obtained gold colloids were centrifuged at 10000 rpm for 30min. The resultant solution was washed three times in Milli-Q water and once in ethanol solution. The supernatant solution was discarded and particles were dried overnight in hot air oven at 60˚C. The dried particles were taken for further analysis.

CHARACTERIZATION OF GOLD NANOPARTICLES

Visual inspection

The reduction of metal ions was roughly monitored by visual inspection of the solution by color change.

X-ray diffractogram

The crystallographic analysis of the sample was performed by powder X-ray diffraction. The X-ray diffraction patterns of synthesized gold nanoparticles were recorded with an X'pert PROPAN analytical instrument operated at 40 kV and a current of 30 mA with Cu α radiation (λ=1.54060 A˚). A continuous scan mode was used to collect 2θ data from 10.02˚ to 79.92˚. The diffraction intensities were compared with the standard JCPDS files. The information of the particle size was obtained from the full width at half maximum (FWHM) of the diffracted beam. Crystalline nature of the nanoparticles was calculated from the line broadening of X-ray diffraction peak according to the Debye-Scherer's formula[15].

$$D = k\lambda / \beta \cos \theta,$$

Where D is the thickness of the nanocrystal, 'k' constant, 'λ' wavelength of X-rays, 'β' width at half maxima of reflection at Bragg's angle 2θ, 'θ' Bragg's angle.

scanning electron microscopy

Surface morphology and size distribution of the particles were observed using Scanning Electron Microscope. For SEM micrograph, the solid samples were sprinkled on the adhesive carbon tape which is supported on a metallic disk. The sample surface images were taken at different magnifications using the JEOL (SU 1510) operated at an accelerating voltage of 5kV and magnification x10k.

Antibacterial Assay

Bacterial Culture

The following bacterial pathogens namely *Staphylococcus aureus*, *Streptococcus epidermis, Bacillus cereus, Vibrio vulnificus*, *Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae* and *Flexibacter* sp. were procured from the Microbial Type Culture Collection (MTCC), Chandigarh, India. All the cultures were grown on nutrient agar plates and maintained in the nutrient agar slants at 4˚C. Overnight culture in the nutrient broth was used for the present experimental study.

Evaluation of antibacterial activity

The antibacterial activity of gold nanoparticles with and without stabilizers against the test strains was examined by agar well diffusion method. Pure cultures of each bacterial strain were sub-cultured in nutrient broth for 24hours at 37˚C. After 24 hours, the inoculum was spread with sterile cotton swab on Mueller Hinton agar plates. Wells of 6 mm diameter were made using sterile cork borer and 50µl of nanoparticles suspension were poured onto each well in all plates. The plates were incubated overnight at 37˚C and results were recorded by measuring the diameter of inhibition zone (mm).

RESULTS AND DISCUSSION

Visual inspection

Appearance of wine red color colloids indicated the formation of gold nanoparticles (**Figure 1**). The formation of color in the reaction solution arises from excitation of surface plasmon vibration in the metal nanoparticles. Grace and Pandian [6] reported that the formation of wine red colloids is the characteristic of gold nanoparticles.

Fig. 1: Colloidal solution of gold nanoparticles

X-ray diffractogram

X-ray diffraction patterns recorded for gold nanoparticles prepared in this present study were depicted in **Figure 2**. The intensive diffraction peaks at 2θ value of 38.38˚, 38.12˚, 38˚, and 38.24˚ corresponding to (111), (200) and (220) lattice plane of face centered cubic (fcc) form for unstabilized AuNPs and stabilized AuNPs respectively. In the obtained spectrum, the Bragg's peak position and their intensities were compared with the standard JCPDS files (No. 4-0784). The size of the particles was found to be 21.96nm, 17.56 nm(starch), 8.78 nm(CMC), and 17.57nm (chitosan). Similar characteristic peaks and corresponding lattice planes were observed by some other researchers[16,17].

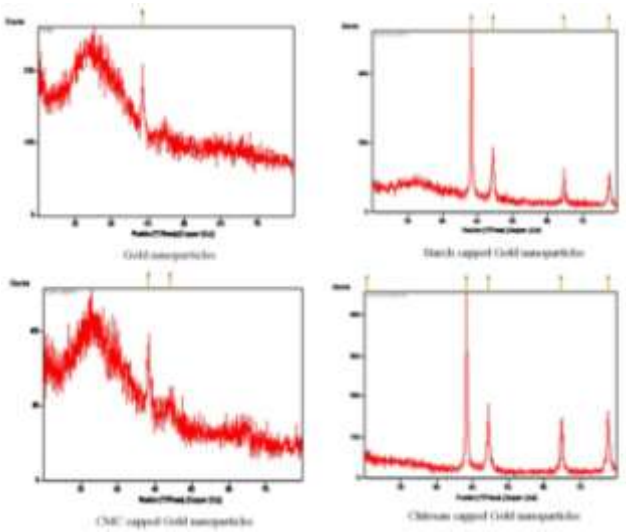

Fig. 2: X-ray diffraction pattern of gold nanoparticles with and without capping agents

Scanning electron micrograpy

The morphology of the synthesized gold nanoparticles was observed under scanning electron microscopy. The micrograph reveals that the particles are spherical in nature (**Figure 3**) and it did not appear as discrete one but form much larger dendritic flocs. The aggregation is attributed due to the Vander Waals forces and magnetic interactions among the particles. This SEM results coincides with the earlier report of Sobczak-Kupiec *et al*.[18].

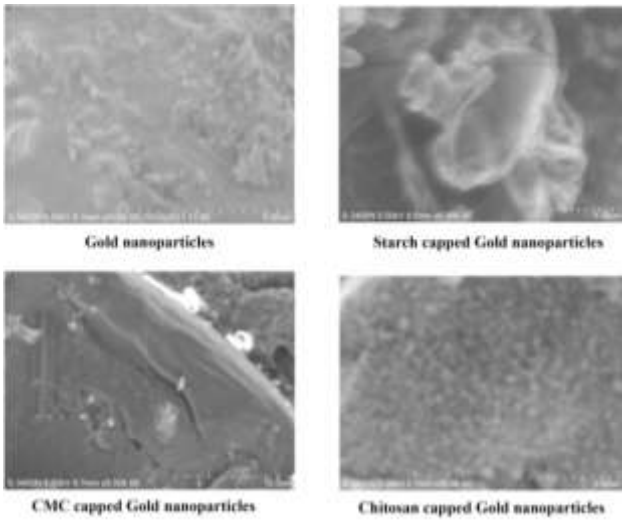

Fig. 3: Scanning electron micrograph of gold nanoparticles with and without capping agents

Antibacterial activity

The effectiveness of gold nanoparticles with and without stabilizing agents was tested against both Gram positive and Gram negative bacteria such as *Staphylococcus aureus*, *Streptococcus epidermis, Bacillus cereus, Vibrio vulnificus*, *Pseudomonas aeruginosa, Escherichia coli, Flexibacter* sp. and *Klebsiella pneumoniae*. Gold nanoparticles exhibited highest antibacterial sensitivity to all the test strains used in this study (**Figure 4**). The maximum inhibition zone (30mm) was obtained against *E. coli*, *K. pneumoniae* and *V. vulnificus* for chitosan capped gold nanoparticles which are followed by CMC and starch (**Table 1**). The results obtained from the present experiment revealed that the stabilized gold nanaoparticles offered maximum bacterial susceptibility than unstabilized AuNPs. This enhancement is due to the large surface area and high penetrating power of the gold nanoparticles. Grace and Pandian[6] investigated that the gold nanoparticles have a great bactericidal effect and it possess well-developed surface chemistry, chemical stability and appropriate smaller size which make them easier to interact with the microorganisms. In specifically, the nanoparticles bind to the building elements of the outer membrane causing structural changes, degradation and finally cell death. Percentage fold increase of gold nanoparticles in the presence of various stabilizers is given in **Tables 2-4**. *Staphylococcus aureus* (114% for starch, 171.43% for CMC and 257.14% for chitosan) showed highest fold increase. Among the stabilized nanoparticles, chitosan stabilized gold nanopaticles exhibited better bactericidal effect. Chitosan is a mucoadhesive, modified natural carbohydrate polymer prepared by the partial N-deacetylation of chitin and it is positively charged under acidic environment due to its free amino groups. The electrostatic attractive forces between the positively charged amino groups of chitosan and the negatively charged $AuCl_4^-$ ions drive the nanoparticle formation and lend the nanoparticles high stability. Each gold nanoparticles is surrounded by a number of stabilizer molecules which prevent the agglomeration, reduced surface area and interfacial free energy of the nanoparticles, thereby the particles reactivity is maintained in constant state[11]. This makes the particles easily interact with outer membrane components of the cell and makes significant changes and damage on their surfaces leading to cell death. Chwalibog *et al.*[19]reported that the interaction between gold nanoparticles and *Staphylococcus aureus* were trapped by biofilm and the substance released by cells causing distortion of the cell wall. Gold nanoparticles closely bind to surface of the microorganisms causing visible damage to the cells, and it can minimize the treatment durations and side effects of drugs[20]. Gold nanoparticles generate holes in the cell wall, resulting in the leakage of cell contents leads to death, in another way it can binds to the DNA of bacteria and inhibit the DNA transcription[21]. Two-way ANOVA test revealed that the bactericidal effect of gold nanoparticles with and without stabilizing agents against bacterial pathogens is statistically significant ($F = 3.51$; $P<0.05$; **Table 5**).

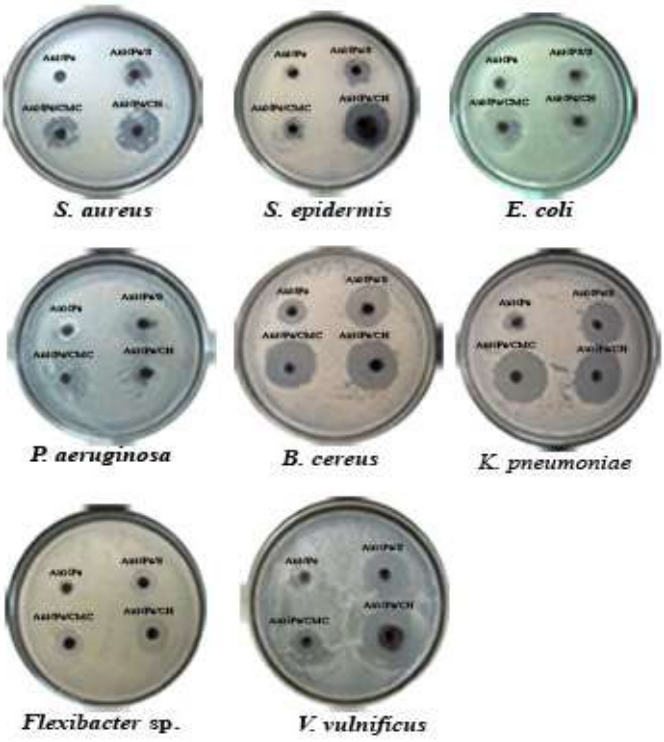

Fig. 4: Antibacterial activity of gold nanoparticles with and without capping agents

Table 1: Antibacterial activity of gold nanoparticles with and without stabilizing agents against selected human bacterial pathogens

Pathogens	Zone of inhibition (mm)			
	AuNPs	AuNPs/S	AuNPs/CMC	AuNPs/CH
Staphylococcus aureus	7	15	19	25
Streptococcus epidermis	11	20	21	26
Escherichia coli	16	26	28	30
Pseudomonas aeruginosa	10	20	25	29
Bacillus cereus	15	22	27	28
Klebsiella pneumoniae	15	23	30	30
Flexibacter sp.	11	13	20	23
Vibrio vulnificus	13	24	20	30

Table 2: Percentage fold increase of gold nanoparticles (AuNPs) and starch capped gold nanoparticles (AuNPs/S) against selected human bacterial pathogens

Pathogens	Zone of inhibition (mm)		Increased zone size (mm)	Percentage of fold increase F= ((b-a)/a)*100
	AuNPs (a)	AuNPs/S (b)		
Staphylococcus aureus	7	15	8	114.29
Streptococcus epidermis	11	20	9	81.82
Escherichia coli	16	26	10	62.50
Pseudomonas aeruginosa	10	20	10	100
Bacillus cereus	15	22	7	46.66
Klebsiella pneumoniae	15	23	8	53.33
Flexibacter sp.	11	13	2	18.18
Vibrio vulnificus	13	24	11	84.62

Table 3: Percentage fold increase of gold nanoparticles (AuNPs) and CMC capped gold nanoparticles (AuNPs/CMC) against selected human bacterial pathogens

Pathogens	Zone of inhibition (mm)		Increased zone size (mm)	Percentage of fold increase F= ((b-a)/a)*100
	AuNPs (a)	AuNPs/CMC (b)		
Staphylococcus aureus	7	19	12	171.43
Streptococcus epidermis	11	21	10	90.91
Escherichia coli	16	28	12	75
Pseudomonas aeruginosa	10	25	15	150
Bacillus cereus	15	27	12	80
Klebsiella pneumoniae	15	30	15	100
Flexibacter sp.	11	20	9	81.82
Vibrio vulnificus	13	20	7	53.85

Table 4: Percentage fold increase of gold nanoparticles (AuNPs) and chitosan capped gold nanoparticles (AuNPs/CH) against selected human bacterial pathogens

Pathogens	Zone of inhibition (mm)		Increased zone size (mm)	Percentage of fold increase F= ((b-a)/a)*100
	AuNPs (a)	AuNPs/CH (b)		
Staphylococcus aureus	7	25	18	257.14
Streptococcus epidermis	11	26	15	136.36
Escherichia coli	16	30	14	87.50
Pseudomonas aeruginosa	10	29	19	190.00
Bacillus cereus	15	28	13	86.67
Klebsiella pneumoniae	15	30	15	100.00
Flexibacter sp.	11	23	12	109.09
Vibrio vulnificus	13	30	17	130.77

Table 5: Two-way analysis of variance for bactericidal effect of gold nanoparticles against selected human bacterial pathogens

Sources of variation	SS	DF	MS	'F' Value	P
Total variance	1197.66	31	-	-	
Variance between AuNPs	1026.76	7	146.68	3.51	<0.05
Variance between bacterial pathogens	293.50	3	97.83	2.34	<0.05
Error variance	877.40	21	41.78	-	

SS - Sum of Squares; DF – Degrees of Freedom; MS – Mean Square.

Note: $P < 0.05$ is statistically significant

CONCLUSION

Nanobiotechnology is an upcoming and developing field with potential application for human welfare owing to its small size and volume ratio to fight against antibiotic resistant pathogens. The synthesized particle size was calculated by XRD, and its morphology was observed through SEM. From our results it is proved that chemically synthesized and stabilized gold nanoparticles are

effective against various bacterial strains. Using biopolymers for stabilization make the particles as effective and biocompatible. Hence it is concluded that the stabilized gold nanoparticles may be suitable for the formulation of new types of bactericidal materials equivalent to the antibiotics against microbial infections.

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