Green Synthesis of AgNPs Stabilized with biowaste and their antimicrobial activities

Nakuleshwar Dut Jasuja¹, Deepak Kumar Gupta², Mohtashim Reza³, Suresh C. Joshi⁴

¹School of Science, Suresh Gyan Vihar University, Mahal, Jagatpura, Jaipur, India.
²Centre for Converging Technologies, University of Rajasthan, Jaipur, India.
³University Science Instrumentation Centre (USIC), University of Rajasthan, Jaipur, India.
⁴Department of Zoology, University of Rajasthan, Jaipur, India.

Submitted: December 21, 2013; Approved: April 17, 2014.

Abstract

In the present study, rapid reduction and stabilization of Ag⁺ ions with different NaOH molar concentration (0.5 mM, 1.0 mM and 1.5 mM) has been carried out in the aqueous solution of silver nitrate by the bio waste peel extract of P.granatum. Generally, chemical methods used for the synthesis of AgNPs are quite toxic, flammable and have adverse effect in medical application but green synthesis is a better option due to eco-friendliness, non-toxicity and safe for human. Stable AgNPs were synthesized by treating 90 mL aqueous solution of 2 mM AgNO₃ with the 5 mL plant peels extract (0.4% w/v) at different NaOH concentration (5 mL). The synthesized AgNPs were characterized by UV-Vis spectroscopy, TEM and SEM. Further, antimicrobial activities of AgNPs were performed on Gram positive i.e. Staphylococcus aureus, Bacillus subtilius and Gram negative i.e. E. coli, Pseudomonas aeruginosa bacteria. The AgNPs synthesized at 1.5 mM NaOH concentration had shown maximum zone of inhibition (ZOI) i.e. 49 / 0.64 mm in E. coli, whereas Pseudomonas aeruginosa, Staphylococcus aureus and Bacillus subtilius had shown 40 / 0.29 mm, 28 / 0.13 and 42 / 0.49 mm ZOI respectively. The MIC value of 30 / 10⁹ g/mL observed for E. coli. Whereas, Staphylococcus aureus, Bacillus subtilius and Pseudomonas aeruginosa had shown 45 / 10⁹ g/mL, 38 / 10⁹ g/mL, 35 / 10⁹ g/mL respectively. The study revealed that AgNPs had shown significant antimicrobial activity as compared to Streptomycin.

Key words: Silver nanoparticles, biowaste, antibacterial activity, MIC, SEM, TEM.

Introduction

Recently, nanoparticles are used in multidisciplinary areas such as biomedicine, biocatalysis, electronics, chemistry and energy due to their extensive applicability. These particles have small size (1-100 nm) and elevated surface area which resulted in increase reactivity, spectacular alteration in optical, electronic and chemical properties which are significantly different from bulk materials (Catauro et al., 2004; Stevanovic et al., 2012; Vijayakumar et al., 2013). Silver nanoparticles (AgNPs) have more concerned as compare to other metallic nanoparticles (MNPs) due to their unique properties like magnetic and optical polarizability, electrical conductivity and antimicrobial activities (Evanoff and Chumanov, 2005). As Inorganic agents (i.e. Ag) have already been used in various medical and industrial processes for an inhibitory effect towards many bacterial strains and microorganisms (Saxena et al., 2010; Jain et al., 2009; Latha and Kannabiran, 2006; Krishnamurthy et al., 2012). AgNPs can be used to destroy microorganisms on textile fabrics (Vivek et al., 2011; White et al., 2012) or they can be employed for water treatment (Binupriya et al., 2010). The capability of pathogenic bacteria to get resistance against antibacterial agents is a tremendous problem in medical practice which limits the efficacy of these drugs (Quelemes et al., 2013). These drawbacks give researchers tremendous opportunities to develop new substances like AgNPs to combat them. Green synthesis of nanoparticles using plants or plant derived extracts is good option over chemical and physical methods because it is rapid, non toxic, eco-friendly, cost effective, don’t require high pressure, temperature, toxic chemicals and compatible for pharmaceutical and biomedical applications (Vivek et al., 2011). Plant-based nanoparticles synthesis has advantages
over other biological methods because of their rapid reaction rate for the synthesis of nanoparticles (White et al., 2012).

In the present study, rapid reduction and stabilization of Ag+ ions with different NaOH molar concentration in the aqueous solution of silver nitrate by the bio waste peel extract of P.granatum reported. Further, the anti-bacterial activity of these biologically synthesized nanoparticles performed against Gram positive (G+) and Gram negative (G-) bacteria.

Experimental

Punica granatum were collected from the National Institute of Ayurveda, Jaipur. Further, plant was identified and registered (Reg. No. RUBL21110) by Herbarium, Department of Botany, University of Rajasthan, Jaipur, India. Punica granatum peels were removed and dried under shade at room temperature for about 10 days. The dried peels were powdered by mechanical grinder and sieved to give particle size 50-150 mm. Powder (34 g) was filled in the thimble and extracted successively with 70% ethanol in soxhlet extractor at 40 °C for 48 h. The extracts were concentrated to dryness using rotary evaporator and used as reducing and capping agent. The stable AgNPs were synthesized by treating 90 mL aqueous solution of AgNO3 (2 mM) with 5 mL filtered (0.45 µm) peels extract (0.4% w/v) and 5 mL NaOH of different molar concentration (0.5 mM, 1.0 mM and 1.5 mM) at room temperature (25 °C) for 20 min (Vasireddy et al., 2012). The obtained solutions were centrifuged at 15,000 rpm for 20 min (Vijayakumar et al., 2013; Gan et al., 2012) subjected to purification and dried for the analysis of the prepared AgNPs. UV-Vis spectral analysis was done between a range of 300-600 nm using a double-beam spectrophotometer (Hitachi, U-3010) with all the samples dispersed in distilled water and kept in a quartz cuvette with a path length of 10 mm (Vasireddy et al., 2012). Scanning electron microscopy (Carl Zeiss EVO® 18 electron microscope) and Transmission electron microscopy (FEI Tecnai T20 TEM System) for the morphological analysis of the prepared AgNPs samples was performed (Vasireddy et al., 2012).

Screening of nanoparticles using disc diffusion method

The antibacterial activities of the synthesized AgNPs were studied against four bacteria, viz. Staphylococcus aureus (G+), Bacillus subtilis (G+), Escherichia coli (G-), and Pseudomonas aeruginosa (G-) by discs diffusion method (Gould, 1952; Rios et al., 1988; Kim et al., 2007). Standard size Whatman No. 1 filter paper discs, 6.0 mm in diameter, sterilized by moist heat at 121 lb in an autoclave for 15 min were used to determine antimicrobial activity of AgNPs (Bhadauria and Kumar, 2012). Muller Hinton Agar (MHA) medium was poured into autoclaved petriplates and allowed to solidify. The homogeneous suspension (100 µL) of test inoculums 1-5 x 10^6 cfu/mL was used for inoculation over the respective agar medium plates. Sterilized filter paper discs were impregnated with 50 µL of AgNPs (100 µg/mL) and placed over the surface of agar plates containing bacterial culture. Negative controls were prepared in the same way but using 50 µL of pure solvent (autoclaved distilled water) on sterile discs. Similarly, The disc of control antibiotics i.e. Streptomycin sulphate (100 g/mL) for antibacterial activity were also aseptically placed over the seeded agar plates for comparison of antibacterial activity of AgNPs. The plates were incubated at 37 °C for 24 h after which the average diameter of the inhibition zone surrounding the disk was measured with a ruler with up to 1 mm resolution. The mean and standard deviation (SD) reported for each type of nanoparticles (0.5 mM, 1.0 mM, 1.05 mM) and with each microbial strain were based on six replicates (Qi et al., 2004). The activity index was calculated on the basis of the size of the inhibition zone by the following formula:

\[
\text{Activity index} = \frac{\text{Inhibition zone of sample (mm)}}{\text{Inhibition zone of standard (mm)}}
\]

Determination of the minimum inhibitory concentration (MIC)

The lowest concentration of AgNPs that exhibits antibacterial activity was quantified by modified tube dilution method (Qi et al., 2004). The MIC was determined based on batch cultures containing varying concentration of AgNPs in suspension (20-100 µg/mL) (Ruparelia et al., 2008). Muller Hinton broth media were poured into a 16-by 125-mm glass tubes and autoclaved. A standard suspension of test bacterium (~0.5 McFarland standard), was prepared for bacterial inoculums (1-5 x 10^6 cfu/mL). The Ag-NPs solutions of different pH (9-11) and concentration of test bacterium (~0.5 McFarland standard), was prepared for bacterial inoculums (1-5 x 10^6 cfu/mL). The Ag-NPs solutions of different pH (9-11) and concentration were diluted with Mueller-Hinton broth and inoculated with the tested bacterial suspension. The tubes were then incubated to determine the MIC. The high rotary shaking speed was selected to minimize aggregation and settlement of the nanoparticles over the incubation period. Lower rpm setting during incubation may cause underestimation of the antimicrobial activity of the nanoparticles (Ruparelia et al., 2008). All the experiments were carried out in triplicate. The average bacterial growth was measured as increase in absorbance at 600 nm determined using a spectrophotometer (Thermo Spectronic, Helios Epsilon, USA). The experiments included a positive control (tubes containing nanoparticles and Mueller-Hinton broth, devoid of inoculum) and a negative control (tubes containing inoculum and Mueller-Hinton broth, devoid of nanoparticles). The negative controls indicated the microbial growth profile in the absence of nanoparticles. Afterwards, the growth in all tubes at different concentrations of AgNPs was compared.
with that of the nanoparticles-free control in order to determine inhibition after 24 and 48 hours of incubation.

**Statistical analysis**

Statistical analysis was carried out by SPSS version 16.0 software. The result express as arithmetic mean ± SD.

**Results and Discussion**

In the present study, the AgNO₃ solution immediately turned dark brown after the addition of P. garnatum peel extract as a reducing and stabilizing agent in all of the samples of different NaOH molar concentrations, which shows the formation of AgNPs (Figure 1). The oxidation reaction of phenol groups (Figure 2 a-d) in peel extract was responsible for the reduction of silver ions (Wang et al., 2007; Soundarajan et al., 2012). It is observed that addition of 0.5 mM (I) NaOH showed broadening of the surface plasmon resonance (SPR) peak at 406 nm. Whereas, addition of 1.0 mM (II) NaOH shifted the absorption peak at 401 nm and 1.5 mM (III) NaOH resulted in a blue shift of λmax to 395 nm (Figure 3). Study revealed that increase in NaOH concentration may accelerate the nucleation process which increases the absorption intensity and the shifting of absorption peaks may be due to decrease in the particle size of Ag-NPs (Vasireddy et al., 2012). Further, the phenolic groups of flavonoids and glycosides (Figure 2 a-d) of P. granatum peels (Van Elswijk et al., 2004; Jasuja et al., 2012) act as a reducing agent may be ionized at higher molar NaOH concentration which leads rapid reduction reaction and synthesized spherical particles of AgNPs. The mechanistic reaction of the formation of AgNPs is expressed in Figure 4.

The electrons moves freely in conduction band and valence band which lie very close to each other in Metal NPs i.e. AgNPs. The collective oscillations of electrons (Plasmon) generate surface plasmon resonance (SPR) absorption band (Taleb et al., 1998; Noginov et al., 2007; Link and El-Sayed, 2003; Kreibig and Vollmer, 1995) occurring due to the resonance with the incident light wave (Nath et al., 2007). The electric field of an incident wave induces a polarization of these electrons with respect to much heavier ionic core of AgNPs (Das et al., 2009). UV-Visible wave induces a polarization of the loosely bound surface electron due to low penetration depth (approximate 50 nm). As a result the net charge difference take place which acts as a restoring force. This creates a dipolar oscillation of all the electrons with the same phase (Inbakandan et al., 2010). A strong absorption takes place when the frequency of the electromagnetic field becomes resonant with the coherent electron motion, which may be the origin of dark brown colour. Due to the localized SPR, Metal NPs Shows strong absorption peak while bulk metal particles shows propagating SPR. This absorption strongly depends on the particle size, dielectric medium and chemical surroundings (Noginov et al., 2007; Link and El-Sayed, 2003; Umashankari et al., 2012). The UV/Vis absorption spectra of the silver nanoparticles dispersed in water is shown in the Figure 1. When the size of particles is smaller than the average free path of the electrons (52 nm for silver metal (Abdullin et al., 1998; Henglein, 1998), silver dielectric function modifies which leads to an increased Plasmon bandwidth with decreasing size of particle (Baset et al., 2011).  

**SEM and TEM analysis**

Figures 5 (A) and (B) showed the SEM and typical bright-field TEM micrographs of the synthesized AgNPs. The micrographs of AgNPs found polydisperse and mostly spherical in shape. In some places, Agglomeration of AgNPs may be due to possible sedimentation at a later time. The average size estimated was 15 nm for AgNPs. It is reported earlier that proteins can bind to nanoparticles either through free amine groups and therefore, stabilization of the AgNPs by protein is a possibility (Daniel and Astruc, 2004; Kawser et al., 2009; Shahverdi et al., 2007; Chien et al., 2007; Ahmad and Sharma, 2012).

**Antibacterial activity**

The study demonstrated the synergistic activity of AgNPs against gram-positive and gram-negative bacteria. The maximum inhibitory effects of AgNPs observed when prepared with higher NaOH (1.5 mM) molar concentration. The study revealed that AgNPs (50 μg/mL) had shown significant inhibitory effect against E.coli and Bacillus subtilis i.e. 49 ± 0.64 and 42 ± 0.49 mm when compared with Streptomycin (100 μg/mL) i.e. 43 ± 0.52 and 40 ± 0.31 mm respectively Figure 6 (a-d) and Table 1.

The MIC value of 30 μg Ag/mL was observed in E. coli. Whereas, Staphylococcus aureus, Bacillus subtilis and Pseudomonas aeruginosa had shown 45 μg/mL,
38 g/mL, 35 μg/mL MIC respectively (Table 2). AgNPs had shown more than 1 active index for E. coli and Bacillus subtilius when compared with standard drug (Figure 7).

The screening results indicated that AgNPs disc (50 μg/mL) were more active against gram-negative bacteria i.e. Escherichia coli with a mean zone of inhibition 49 ± 0.64 mm (Table 1). This may be due to the differences in the cell wall of gram-positive and gram negative bacteria. The cell wall of gram positive bacteria is wider than the gram-negative bacteria (Thiel et al., 2007; Martinez-Castañon et al., 2008; Kim et al., 2007). Gram negative bacteria have a layer of lipopolysaccharide (LPS) which surrounded by a thin layer of peptidoglycan (7-8 nm) (Kreibig and Vollmer, 1995). The overall charge of bacterial cells at biological pH values is negative because of excess number of carboxylic groups, which upon dissociation make the cell surface negative (Raffi et al., 2008). Weak positive charges present on silver nanoparticles (Schultz et al., 2000) are attracted towards negative charges on the LPS. Moreover, Excess formation of free radicals may attack LPS which lead to a breakdown of membrane function. Increased permeability of the cell membrane or leakage of cell contents could be caused by Reactive Oxygen Species (ROS) (Mendis et al., 2005). This also leads morphological changes of bacterial cells and growth inhibition (Amro et al., 2000; Danilczuk et al., 2006; Sondi and Salopek-Sondi, 2004). It is logical to state that binding of the nanoparticles to the bacteria depends on the surface area available for interaction. Nanoparticles have larger surface area available for interaction which enhances bactericidal effect than the large sized particles (Raffi et al., 2008) eg. Inorganic substances or antibiotics; hence AgNPs exhibits more toxicity to the microorganism (Baker et al., 2005).

Figure 2 - (a-d) Flavonoids and their glycosides from P. granatum peels (Van Elswijk et al., 2004; Jasuja et al., 2012).

Figure 3 - UV-Visible spectra of AgNPs prepared at different NaOH (0.5 mM, 1.0 mM, and 1.5 mM) molar concentrations.

Figure 4 - Schematic diagram of reduction reaction of AgNO₃ by peel extracts to form AgNPs.
Figure 5 - (a) Scanning electron micrograph of AgNPs synthesized by green methods (b) Transmission Electron Microscopy (TEM) image of AgNPs (scale bar 100 nm).

Figure 6 - (a-d) Antibacterial Activities of Streptomycin sulphate disc (1 mg/10 mL) on (a) Staphylococcus aureus (b) Bacillus subtilius (c) E. coli (d) Pseudomonas aeruginosa. (e-h) Antibacterial Activities of AgNPs (60 μg/10 mL) on (e) Staphylococcus aureus (f) Bacillus subtilius (g) E. coli (h) Pseudomonas aeruginosa.

Table 1 - Antibacterial activity of Streptomycin (100 μg/mL) and AgNPs (100 μg/mL) against bacterial species tested by disc diffusion assay.

| Sr. No. | Name of organism          | Agar-well diffusion (Zone of Inhibition in mm) | Streptomycin   |
|---------|---------------------------|------------------------------------------------|----------------|
|         |                           | AgNPs (50 μg/mL)                            |                |
|         |                           | (0.5 mM NaOH) | (1.0 mM NaOH) | (1.5 mM NaOH) | (100 μg/mL)  |
| 1       | *Staphylococcus aureus*   | 26 ± 0.33 | 26 ± 0.45 | 28 ± 0.13 | 28 ± 0.22 |
| 2       | *Bacillus subtilius*      | 40 ± 0.64 | 41 ± 0.81 | 42 ± 0.49 | 40 ± 0.31 |
| 3       | *E. coli*                 | 45 ± 0.55 | 46 ± 0.21 | 49 ± 0.64 | 43 ± 0.52 |
| 4       | *Pseudomonas aeruginosa*  | 39 ± 0.73 | 40 ± 0.12 | 40 ± 0.29 | 42 ± 0.11 |

Values are mean zone of inhibition (mm) ± S.D of three replicates.
Conversely, the cell wall in gram-positive bacteria is composed of a thick layer (about 20-80 nm) of peptidoglycan, consisting of linear polysaccharide chains cross-linked by short peptides to form a three dimensional rigid structure (Wiley et al., 2006). The rigidity and extended cross-linking not only provide the cell walls with fewer anchoring sites for the silver nanoparticles but also make them difficult to penetrate. Earlier studies also revealed that silver species release Ag+ ions which interact with the thiol groups of bacterial proteins, may retard or change the replication of DNA (Marini et al., 2007; Martinez-Castanon et al., 2008). Somehow, it may be the reason that the G+ Bacillus subtilius also inhibited by AgNPs significantly when compared with control antibiotics.

Conclusions

It is concluded that the extract of P. granatum are capable of producing stable AgNPs by reduction of aqueous Ag+ ions in to Ag0. This green chemistry approach toward the synthesis of AgNPs has various advantages i.e rapid reduction, economic viability etc. Applications of such eco-friendly nanoparticles in bactericidal, wound healing, medical and electronic applications, makes this method poten-
tially exciting for the large-scale synthesis of other inorganic nanomaterials (Ankanna et al., 2010) e.g. Au, Fe, Zn, Cu, Graphenes etc. The increase in zone of inhibition reported in this study was dependent on the concentration of nanoparticles due to higher NaOH molar concentration. Attachment of nanoparticles by cell wall of bacteria would be due to negative charges and specific functional groups on the bacterial surface. AgNPs after penetration into the bacterial cell may disturb the rigidity of cell wall or lipo-polysaccharides membrane, inactivate their transport system, enzymes functioning, generate H2O2 which resulted in bacterial death. The silver nanoparticles synthesized via green route are highly toxic to G-ve and somehow for G+ve bacteria can be used in medical applications (Singh et al., 2010).

Acknowledgments

The authors are sincerely thankful to Mr. Sunil Sharma, Chancellor and Dr. Sudhanshu Sharma, Chief Mentor of Suresh Gyan Vihar University for providing a platform for this research. The authors also appreciation vows to USIC, University of Rajasthan, Jaipur, India for providing SEM and TEM facilities.

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