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Flower Shaped Silver Nanostructures: An Efficient Bacteria Exterminator

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1. Introduction

Materials in nanoscale range have dominated several areas of engineering science and technology. In the area of biotechnology nano materials have been used increasingly in biomedical analysis, fabrication of biosensors/biointerface, clinical diagnosis and therapy, drug delivery and so on (Cao, 2008; Mirkin et al., 1996; Alivasatos, 2004; Jena and Raj, 2006, 2011; Xiao et al., 2003; Sengupta et al., 2005; Wu et al., 2003; Brigger et al., 2002; Cui et al., 2001; Gao et al., 2004; Rosi and Mirkin, 2005). Particular interest has been focused on nanostructured noble metal particles for biotechnology application because of their biocompatibility, lower toxicity and higher affinity with wide range of biomolecules (Hazarika et al., 2004; Bae et al., 2005). Therefore, different processes have been adopted for tuning the surfaces of metal nanoparticles to explore the selective binding and monitoring of specific targets in biological sensing (Wang, 2005; Wang et al., 2006; Kneipp et al., 2006). Nanoparticles attached to biomolecules act as an artificial receptor and control the cellular processes for various biological applications such as inhibition of enzymatic activity, transcription regulations, etc (De et al., 2008).

The noble metal nanoparticles are known to display plasmon resonance in the visible region (Kreibing and Volmer, 1995). Optical properties of metal nanoparticles originate from the surface plasmon resonance have attracted substantial interest in biotechnology for diagnosis and sensing (Alivasatos, 2004; Gao et al., 2004; Nam et al., 2003; Xiang et al., 2006; Katz and Willner, 2004). The colour change due to the assembly of Au or Ag nanoparticles developed the colorimetric method for many applications. These colorimetric strategies has been utilised to develop DNA detection methods to study the affinity and specificity of DNA-DNA interactions (Mirkin et al., 1996; Nam et al., 2003; Stoeva et al., 2006; Storhoff et al., 1998; Han et al. 2006). The sensing methodology is based on the colour change due to assembly of Au nanoparticle-linked complementary strands formed by the specific binding of targeted strands. Kanaras et al. demonstrated the use of Au nanoparticle DNA interaction for determination of the enzymatic cleavage of DNA (Kanaras et al. 2007). This process is designed to monitor the enzymatic cleavage activity based on the wave length shifting of Au nanoparticles. Jena and Raj have developed an optical method for the sensing of biomedically important polyionic drugs, protamine and heparin based on the

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assembly/disassembly of Au nanoparticles (Jena and Raj, 2008). A colorimetric sensor based on Au nanoparticle-apatamer has been documented for sensing of small molecules and protein (Wang et al., 2008; Liu and Lu, 2006, 2004). Dong and co-workers developed a colorimetric sensor using Au nanoparticle for monitoring α -thrombin based on the specific interaction of aptamer with α -thrombin (Wei et al. 2007).

The wide application of metal nanoparticles in biological process has been motivated to produce nanoparticles of biofriendly in nature. Therefore, synthesis of nanoparticle under green and benign processes were adopted to produce toxic free nanoparticles (Zhang et al., 2007; Mohanpuria et al., 2008; Gill et al., 2007; Goodman et al. 2004; Jin et al. 2007; Singh and Nalwa, 2007; Kannan et al. 2006; Kattumuri et al. 2007). Current nanotechnology research makes great effort to develop bio-friendly methods for production of noble nanostructured materials. For instance, several synthetic methodologies have been explored for the production of metal nanoparticles using vitamins, plant extracts, biomolecules and etc (Nadagauda and Verma, 2006; Shankar et al. 2004; Gardea-Torresdey et al. 1999; Zhang et al., 2006). Shukla et al. explored a green method for synthesis of Au nanoparticles using soybean which are biocompatible in nature (Shukla et al., 2008).

It has been explored that nano-structured materials with novel shapes exhibit more unique physical and chemical properties (Jena et al., 2011). The dramatic enhancement in nanostructure properties has been achieved on tuning the shape in contrast to the size, because different crystal surfaces have different surface atom densities and electronic structures leading to different physical and chemical properties (Jena et al., 2011). Therefore the nanostructured particles with unusual shape possess wider biological and medical applications in comparison with their common spherical counter part. These anisotropic nanomaterials have potential application in signal amplification in bioanalysis and biodiagnosis technology (Grunes et al. 2002; Yu et al. 2003; Turner, 2000; Haes and Van Duyne, 2002). Particle shape has been recognised as an important attribute compared to the size and can be engineered for drug delivery (Mitragotri, 2009). It has been explored that by mimicking the distinctive shapes of bacteria, fungi, and blood cells could improve the ability of nanoparticles to deliver drugs to diseased cells in the body (Mitragotri, 2009). Wijaya et al. have utilized nanoparticles having bone and capsule like shapes for controlled and selective release of drugs (Wijaya et al. 2009). Ray and co-workers have been successfully utilised Au nanorods for screening HIV-1 viral DNA sequencing (Darbha et al., 2008). As far as the particle shape is concerned, very little is explored particularly with respect to structural property relationship of particles and their biological applications. Therefore, the current development is more focused on producing nanosized materials of well-defined morphologies with improved properties.

A substantial amount of research has been carried out for the synthesis of Ag nanostructures owing to their wide and potential applications in antibacterial, antimicrobial, SERS and so forth (Wijaya et al., 2009; Braun et al., 2007; Gupta and Silver, 1998; Schultz et al., 2000). The antibacterial activity of Ag nanostructures has been studied and well documented in the literature. The antibacterial activity of silver has been widely exploited in healing the cuts and wounds (Fox Jr. et al., 1974). It has been effectively implemented in surgical procedures to prevent bacterial infections (Bosetti et al., 2002; Alt et al., 2004). The extensive application of Ag nanoparticles as a successful antibacterial agents are due to their effective bacteria extermination capacity even at very low concentrations (Banerjee et al. 2010). It has been

observed that the bacteria and microbes are less likely to build resistance against silver as they usually do against antibiotics (Banerjee et al. 2010). Several studies and explanation has been documented regarding the biocidal activity of Ag nanoparticles. The general perception is that the Ag nanoparticles are prone to bind the sulphur groups of the protein present in bacterial cell wall and open the permeability of the cell membrane and damage the cell wall. Few documents explained that the Ag⁺ ions present on the surface of Ag nanoparticles have the actual biocidal properties for cell destructions. The Ag⁺ ions are capable of entering to the bacterial cells and get reduced to their elemental states. The cellular process attempts to remove these reduced Ag from the cell interior and eventually hampers the cellular process leading to cell death. It has been explored that the Ag nanostructures target the bacterial membrane, destabilize the plasma membrane potential and ultimately damage the bacterial cell. Chattopadhyay and co-workers have established that the small Ag nanoparticles having sizes less than 10 nm make pores on the cell wall and release the cytoplasmic content of the cell and cause cell death (Gogoi, et al., 2006). Morones et al. explored that antibacterial activity depends on the size of Ag nanostructures (Morones et al., 2005). Song and co-workers explored the shape dependent biocidal activity of Ag nanostructures towards gram negative bacterium, *E. coli* (Pal et al. 2007).

Many synthetic processes have been developed for spherical shaped Ag nanoparticles and their promising applications. However, more emphasis has been paid for different shaped Ag nanostructures. Various synthetic protocols have been documented for the production of silver nanoparticles with different shapes (Sun and Xia, 2002a, 2002b ; Jin et al., 2001; Lofton and Singmund, 2005; Pastoriza-Santos and Liz-Marjan, 2002; Bera and Raj, 2010; Nicewarner-Pena et al. 2001; Kim et al. 2004; Yu and Yam, 2004; Im et al. 2005; Hao et al. 2002; Chen et al. 2005, 2002; Jiang et al. 2006; Ducamp-Sanuesa et al. 1992; Sun et al. 2002; Chen and Carroll, 2002; Xue et al. 2008). Xia and co-workers adopted the polyol process to synthesize a variety of different shaped Ag nanostructures by controlling the concentration of capping agent PVP and precursor, Ag NO₃ in solution (Wiley et al. 2006, 2005, 2004; Sun et al., 2003). Templates, polymers and Surfactants have been mainly used to get anisotropic nanostructures. Though these methods are successful to produce well-defined silver nanostructures, the improved chemical and physical properties of these nanostructure particles are hindered by these strong surface protecting agents. Therefore, substantial interest has been focused to explore sterile, nontoxic and environmentally safe protocols for facile synthesis of different shaped silver nanoparticles.

In this investigation, the unique flower shaped Ag nanostructures were synthesized adopting bio-inspired approach and the antibacterial activities investigated. We observed that the antibacterial properties can be improved by shaping the Ag nanostructures from spherical to flower-like morphology.

2. Materials and methods

2.1 Materials

AgNO₃, Rutin hydrate (RT), were obtained from Himedia, India. All other chemicals not mentioned here are of analytical grade and used as received from the suppliers. Carbon coated copper grids were obtained from SPI supplies, USA. All the solutions were prepared with deionised water (18 Ωm) obtained from Millipore system. Glasswares used in this

investigation were well cleaned with freshly prepared aqua regia, then rinsed thoroughly with water and dried prior to use (Caution: aqua regia is a powerful oxidizing agent and it should be handled with extreme care).

2.2 Instrumentation

A variety of characterization equipment was employed in order to study the as-synthesised nanostructured material in a comprehensive way. TEM images were obtained from FEI, TECHNAI G2 transmission electron microscope operating at 200 kV. The specimens were prepared by dropping 2 μ l of colloidal solution onto carbon coated copper grids. The UV-visible spectra of the colloidal solutions were recorded on a Shimadzu UV-1700 spectrophotometer. X-ray diffraction analysis was carried out with X'pert PRO (Pan Analytica) X-ray diffraction unit using Ni filtered CuK α ($\lambda = 1.54 \text{ \AA}$) radiation. X-ray photoelectron spectroscopic analyses were carried out on Kratos Axis 165 X-ray Photoelectron Spectrometer. The X-ray gun was operated at 15 kV and 20 mA and high-resolution spectra were collected using 80 eV pass energy, respectively, with Mg K α 1253.6 eV radiation. The data were obtained with an acquisition time of 121 seconds.

2.3 Synthesis of flower shaped Ag nanostructures (AgNFs)

In a typical synthesis, 10 ml aqueous solution of AgNO₃ (1 mM) was stirred for 2-3 min. and then 0.2 ml of Rutin (15 mM) was added. The stirring was continued for 30 minute and the resulting nanocolloid was stored at 4°C (Jena et al. 2009).

2.4 Synthesis of spherical Ag nanostructures

Citrate stabilized nanoparticles were synthesized according to the previous report (Link and El-Sayed, 1999; Mulvany, 1996) by slight modification. In brief, Ag nanocolloids are prepared by reducing AgNO₃ (1 mM) with sodium citrate (0.3 mM) in aqueous medium at boiling temperature.

2.5 Determination of antibacterial activity

Streptococcus Faecalis (SF), Pseudomonas Aeruginosa (PA) and Escherichia coli (EC) bacterias are used in this investigation. The Stocks were created by passing the original reference organisms once through the Muller-Hinton Broth (MHB) and plating on Muller-Hinton agar (MHA) plates for bacterial organisms. For inoculum preparations, bacteria were sub-cultured in Brain-Heart-Infusion (BHI) at 35 °C for 24 hr. The optical density of each culture was measured at 550 nm. The agar well diffusion method was used to determine the antibacterial activity of as synthesized AgNFs. The media used were Muller-Hinton agar (Himedia) for the bacteria under study. The nutrient agar plates were swabbed with cultured bacteria. A total of 2 mm diameter wells were punched into the agar and filled with 100 μ l of AgNF colloids standard antibiotics (Ciprofloxacin, Gentamicin, Penicillin, and Chloroamphenicol) used as positive control. The plates were incubated at 35 °C for 24 h for bacteria pathogen. The antibacterial activity was calculated by measuring the inhibition zone diameter. The experiments were repeated thrice and the average values of antibacterial activity were calculated.

3. Result and discussions

3.1 Characterization of Ag nanoparticles

The UV-visible absorption spectra of the as-synthesized Ag nanoparticles were recorded. The absorption spectra of Ag nanoparticles shows its plasmon absorption bands at 438 nm with a small shoulder peak at round 600 nm (Fig. 1). It has been explored that the surface plasmon band of metal nanoparticle depends on the shape, size and its surrounding medium. The anisotropic nanoparticles are known to exhibit characteristic bands corresponding to transverse and longitudinal plasmon absorption. This sort of spectra observed for Ag nanoparticles is attributed to the formation of anisotropic nanostructures rather than spherical. In order to confirm for the same, the transmission electron microscope (TEM) measurements has been carried out. Interestingly TEM image shows the formation of flower-like shape (Fig 2). The Ag nano flowers (AgNFs) have an average size in between 40 to 60 nm. Point should be noted here that present report describes a bio-friendly process for rapid synthesis of flower-like Ag nanocrystals without using any template, polymer and surfactant at room temperature. Fig 2 (F) is the selected area electron diffraction (SAED) pattern of the AgNFs; the clear spots and ordered arrangement reveals that the particles are of single crystalline in nature. For further investigation of surface morphology, the structural analyses of AgNFs were carried out by high-resolution TEM measurements. Fig 2 (D) shows the typical HRTEM of a branched AgNFs. The lattice fringe spacing of the AgNFs was measured to be 0.235 nm revealing that the growth of the nanostructure occurs preferentially on (111) planes of a face centred cubic lattice of Ag. A precise investigation and thorough analysis of AgNFs reveals the nanoparticles consist of twin boundaries. The HRTEM measurement thus exposed that the AgNFs are predominant of Ag(111) lattice planes along with the presence of twin boundaries.

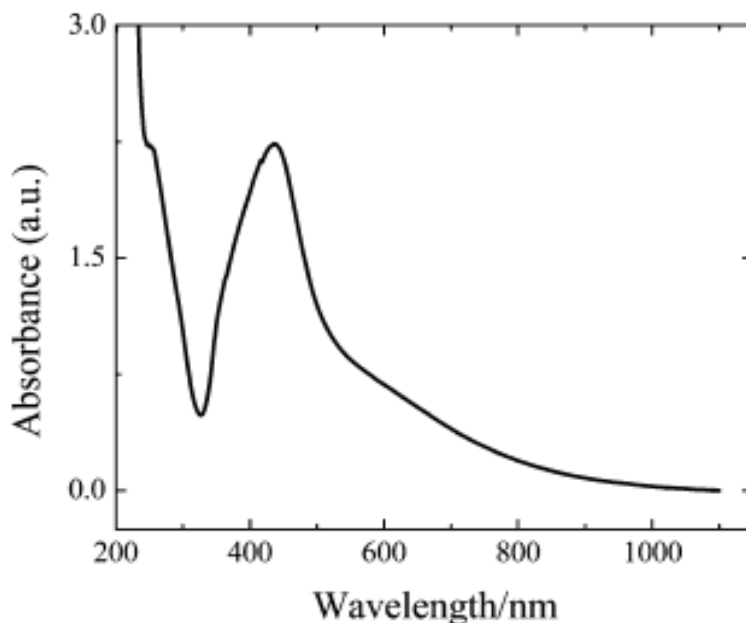


Fig. 1. UV-visible spectrum of Ag nanostructures. Reprinted with permission from (Jena et al., 2009), copyright 2009, American Chemical Society.

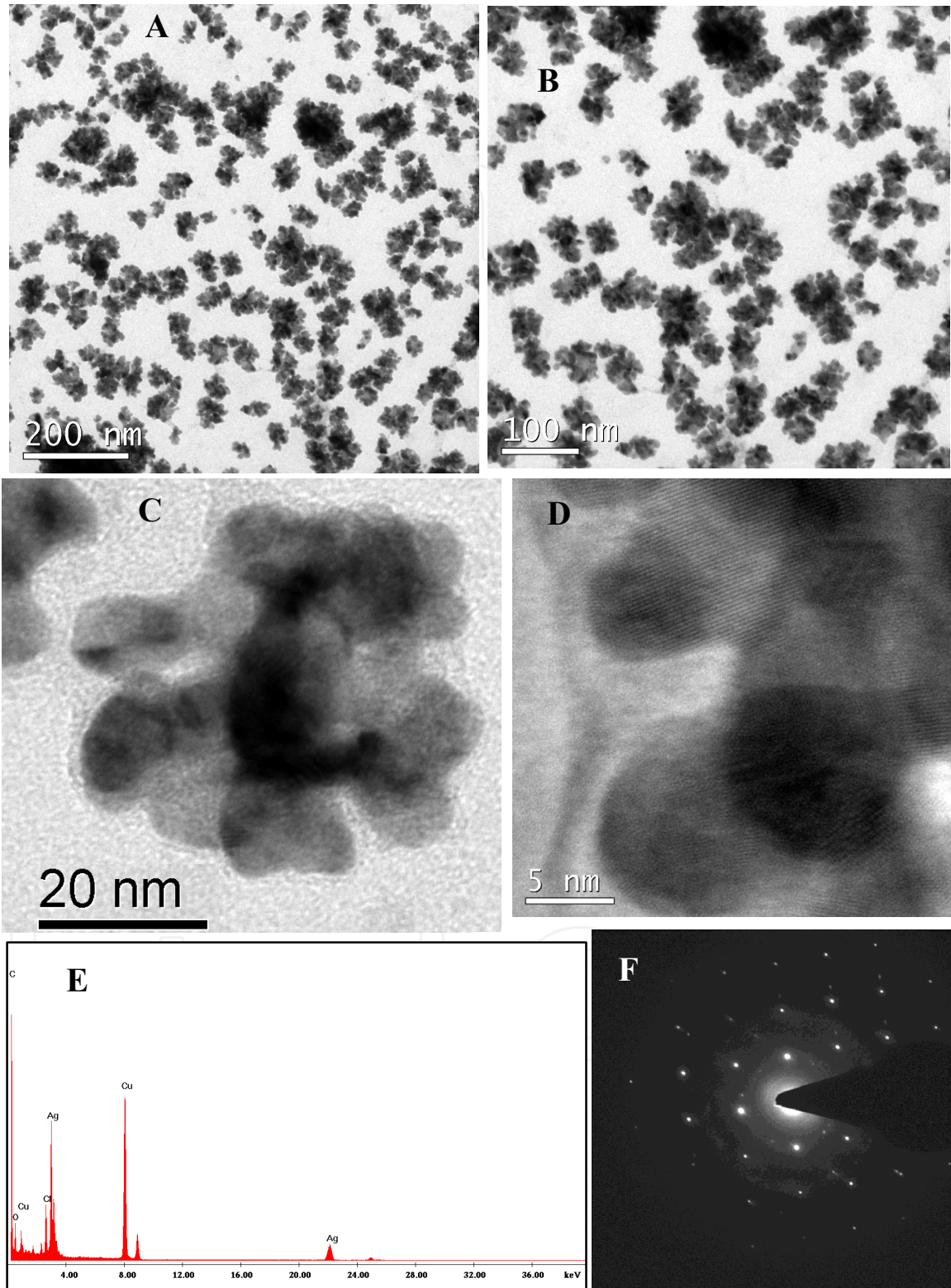


Fig. 2. TEM images of AgNFs induced by rutin

The energy dispersive spectrum confirmed that the AgNFs consist of only Ag (Fig. 2E). Interestingly, the XRD spectrum of AgNFs shows only one peak corresponding to Ag(111) (data not shown). Further the XPS characterization is made to confirm the elemental status. The XPS analysis clearly shows that the prepared AgNFs consist of elemental Ag (data not shown).

The growth of nanostructured particles is highly perceptible to the concentration of rutin. A facile and fruitful synthesis of AgNFs has been achieved at room temperature after manipulating and controlling the concentrations of rutin. By slightly changing the concentration of rutin, nanostructured particles with different morphology were obtained. First, we have examined the influence of concentration of rutin at a fixed concentration of the precursor (1 mM). The TEM images of Ag nanoparticles at different concentrations of rutin have measured (Fig. 3A-C). At higher concentration of rutin, we observed spherical nanoparticles. But at low concentration of rutin we obtained anisotropic nanoparticles. These results indicate the shape and morphology of the nanostructured particles greatly depends on the concentration of rutin. The growth of nanostructured particles is highly sensitive to the concentration of precursor. When the concentration of Ag^+ increases, we obtained nanoparticles with different morphology (Fig. 3D, E). Thus, it is cleared that the size and shape evolution of Ag nanoparticles are highly sensitive to the concentration of precursor as well as reducing/capping agent at normal conditions.

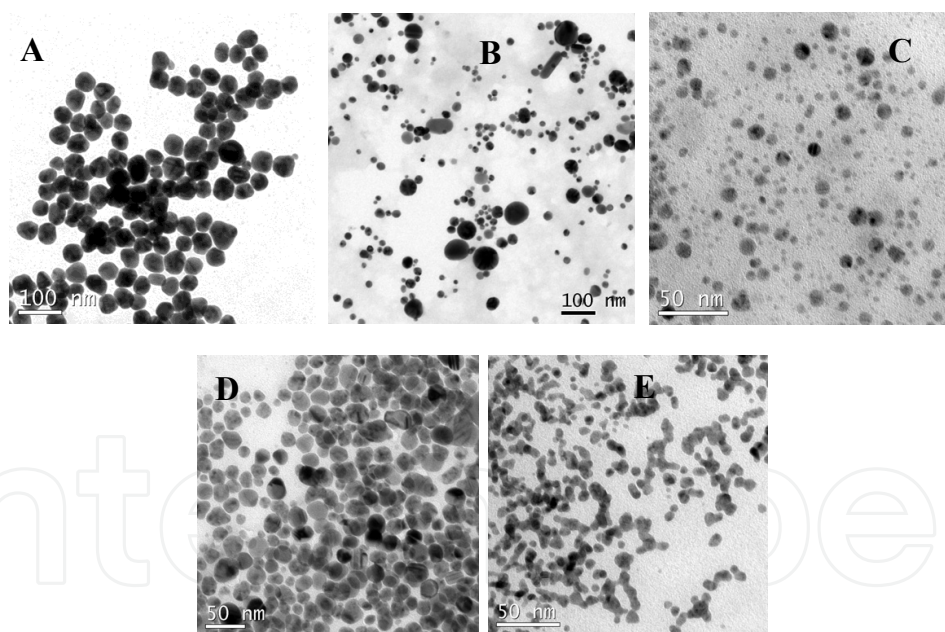


Fig. 3. TEM images of rutin-induced formation of Ag nanoparticles: A-C at fixed concentration (1mM) of Ag^+ ; [rutin] (A) 3, (B) 1, and (C) 0.5 mM and D, E at fixed concentration (0.3 mM) of rutin. [Ag^+]: (A) 0.5 mM and (B) 2mM. Reprinted with permission from (Jena et al., 2009), copyright 2009, American Chemical Society.

The mechanism for the formation of flower shaped silver nanoparticles, were traced out by TEM measurements at different intervals of time over the entire period of formation of nanoparticles. It has been found that at initial stage of the reaction, small spherical shaped nanoparticles were formed and in due course of time, these small nanoparticles led to

formation of initial anisotropic nanoparticles of short branches. Thereafter, a further nucleation and assembly process took place for the crystals to grow into a final flower shaped nanoparticles with many branches (Fig. 4).

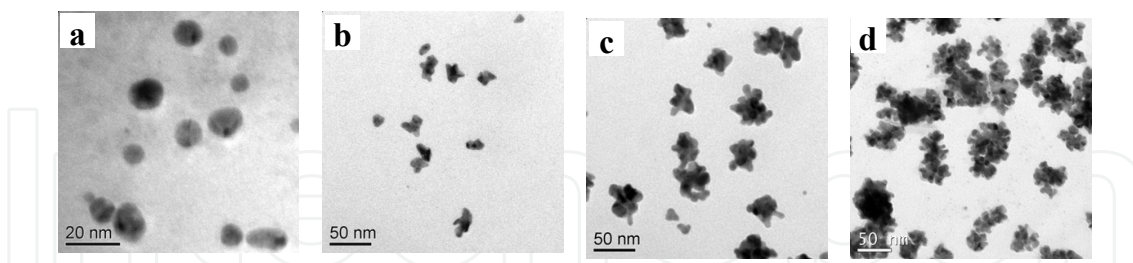


Fig. 4. TEM measurements showing the growth stages of AgNFs at different time intervals of the reaction. (a) 5 min (b) 10 min and (c) 20 min and (d) 30 min.

The formation of AgNFs is attributed to the reduction of Ag^+ by rutin. It is well documented in the literature that rutin has the tendency to donate electrons by carbonilation of $-\text{OH}$ groups at 3', 4' positions favouring a stable quinonic resonant structure (Fig 5A inset). Thus the formation of elemental Ag^0 is possible due to transfer of electrons from rutin to Ag^+ ions. The UV-visible spectra of rutin shows three absorption peak X, Y, and Z at 398, 320 and 270 nm respectively (Fig. 4 A (a)). The absorption peak X corresponds to B ring position and the intensity of the peak is due to the $-\text{OH}$ groups at 3', 4' positions. It was observed that the intensity of peak at X diminishes on subsequent oxidation of rutin to its stable quinonic structure. As a matter of fact, He et al. have shown similar pattern on spectro-electrochemical oxidation of rutin (He et al 2007). In order to confirm the above fact, absorption spectra of supernatant of Ag nanoparticles were recorded. Surprisingly, a decrease in the intensity of X band with slight increase in the intensity of Y and Z bands were observed (Fig. 4Ab). This observation is attributed to the oxidation of rutin by carbonilation of $-\text{OH}$ groups at 3', 4' positions to form a stable quinonic resonant structure simultaneously reducing Ag^+ to Ag^0 which undergoes nucleation in due course to produce Ag nanostructures (fig. 5 B).

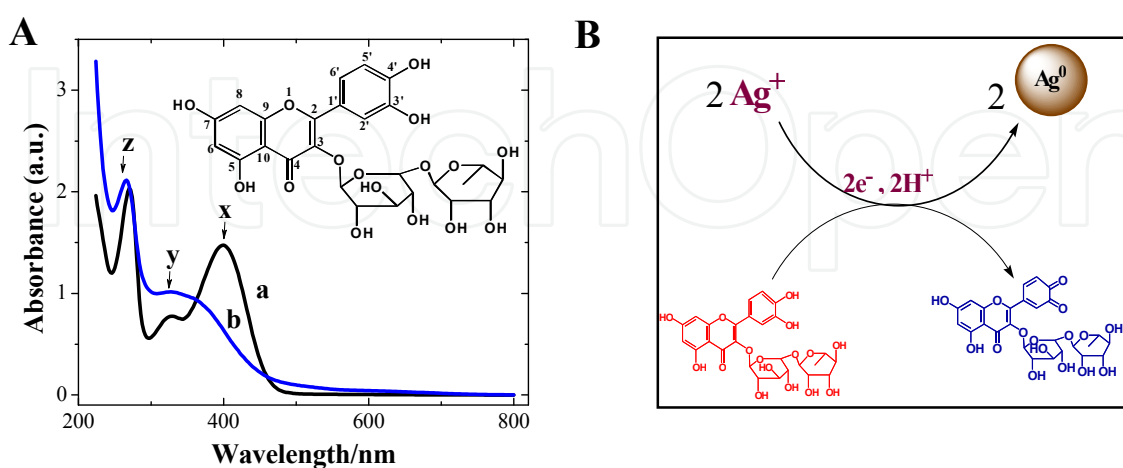


Fig. 5. (A) UV-visible spectra of 0.3 mM rutin (a) and its oxidation product (b). Inset shows the structure of rutin. (B) Scheme showing the possible mechanism towards reduction of Ag^+ by rutin.

3.2 Antibacterial application of AgNFs

The antibacterial efficiency of AgNFs was examined against three representative microorganisms, *Pseudomonas Aeruginosa* (gram negative), *S. Faecalis* (gram positive), and *Escherichia Coli* (Gram-negative) of clinical interest. It is well known that *E. coli* is the most characterized bacterium and can cause gastroenteritis, urinary tract infections and neonatal meningitis; *P. Aeruginosa* causes chronic respiratory infections in individuals with cystic fibrosis and cancer; and *S. Faecalis* can cause endocarditic as well as bladder prostate and epididymal infections. Fig. 6X demonstrates the antibacterial activity of AgNFs that shows a clear zone of inhibition after 24 h incubation of the plate at 35 °C. The antibacterial efficacies of AgNFs are compared with the standard antibiotics (Fig. 6Y). Most diagnostically, the AgNFs found to show their potential antibacterial activity against *Pseudomonas Aeruginosa*, *S. Faecalis* and *E. coli*. Further, a certain amount of colloidal AgNFs was added into the bacterial broth medium and incubated for 24 h. It was found that the coagulation of the bacterial medium was absolutely converted to a transparent medium (Fig. 6Z). Hence, it harmonizes the dramatic antibacterial activity of colloid AgNFs.

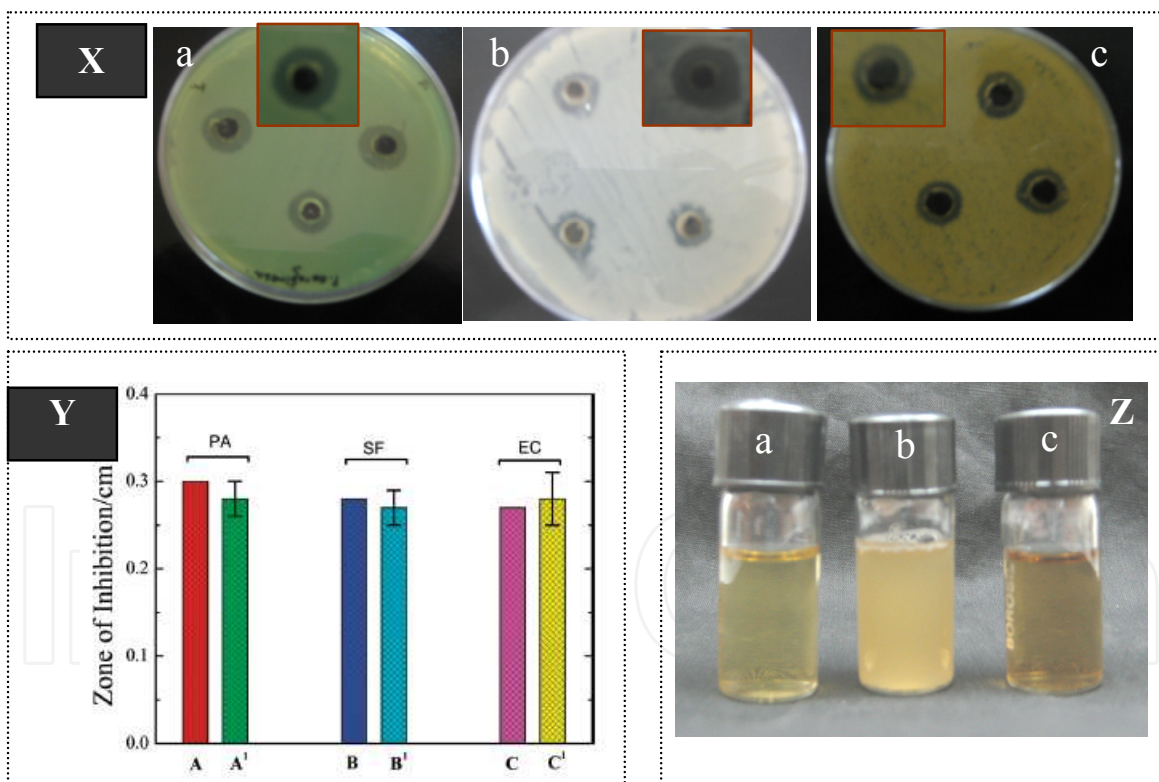


Fig. 6. (X) Diffusion disc showing the antibacterial activity of AgNFs towards (a) *P. Aeruginosa* (b) *S. Faecalis* and (c) *E. Coli*. (Y) Plots showing the antibacterial activity of AgNFs (A', B', C') with respect to the standard antibiotics, Gentamicin (A, C) and Penicillin (B). PA: *Pseudomonas Aeruginosa*; SF: *S. Faecalis*; EC: *Escherichia Coli*. (Z) Photographic image showing the antibacterial activity of AgNFs (A) growth medium, (Z) after growth of bacteria (*E. Coli*) and (c) bacteria solution in presence of AgNFs after 24 h. Reprinted with permission from (Jena et al., 2009), copyright 2009, American Chemical Society.

3.3 Comparative bacterial inhibition studies

A Comparative bacterial inhibition studies were made with different Ag nanoparticles synthesized using rutin and citrate. The citrate stabilized nanoparticles have an average size of 30-40 nm. The antibacterial efficacies of four different nanoparticles were investigated against *S. Faecalis* bacterium (Fig. 7). As it can be seen (Fig. 7A), the inhibition zone is high in case of AgNFs as compared to other nanoparticles. So it can be inferred that the shape might have played a significant role for their potential antibacterial activity. The effective antibacterial activity of AgNFs can be ascribed to its higher tendency to react with sulphur and phosphorous containing compounds in the bacterial cell leading to bacterial death. It is also worthwhile to mention that the AgNF has higher antibacterial property which makes it a promising candidate material for clinical and industrial applications.

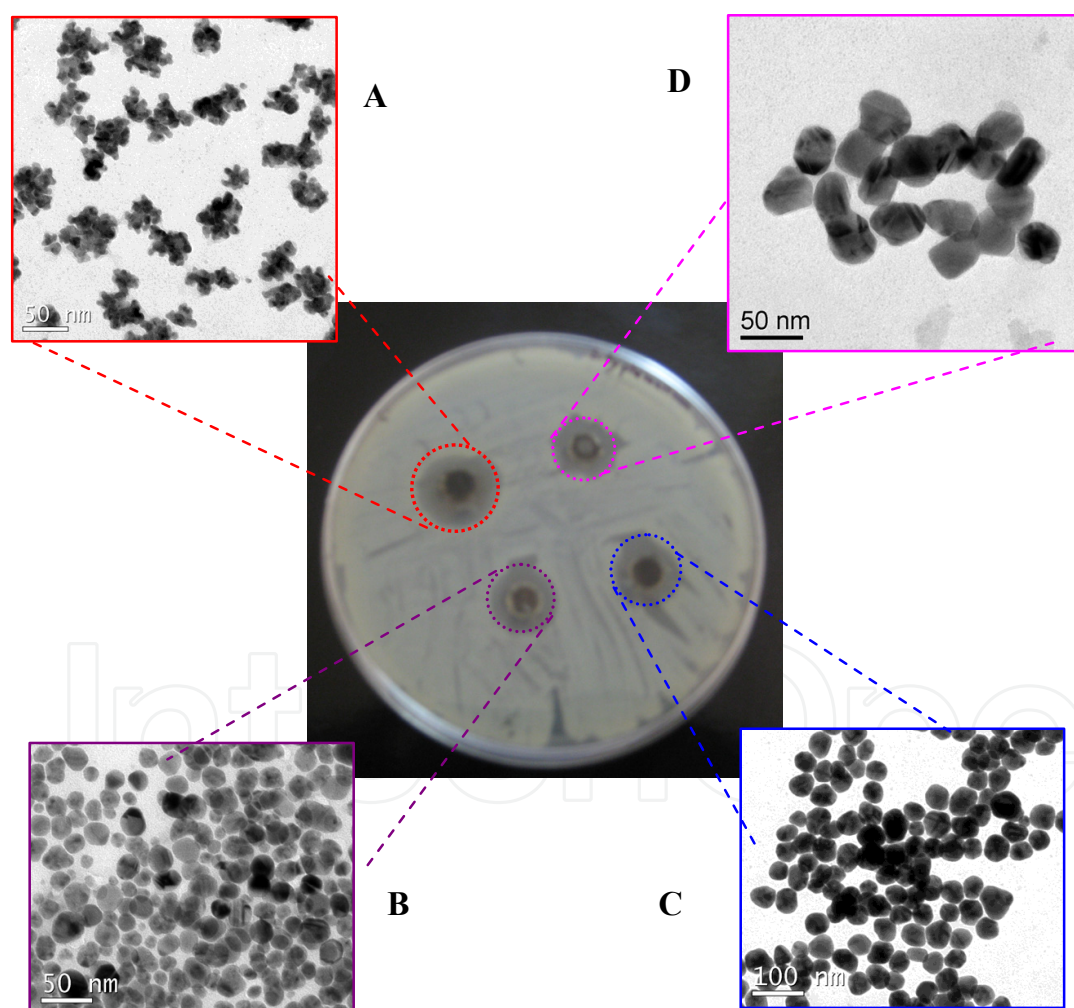


Fig. 7. Diffusion disc showing the antibacterial efficacies of four different shapes of nanoparticles induced by rutin (A, B, C) and citrate (D) against *S. Faecalis* bacterium. Reprinted with permission from (Jena et al., 2009), copyright 2009, American Chemical Society.

4. Conclusion

We have developed a bioinspired and environmental friendly procedure for rapid room temperature synthesis of flower like Ag nanoparticles circumventing the extra controls and additives. The as prepared AgNFs are potentially capable of attracting diverse biological/clinical applications. The AgNFs shows antibacterial activity towards E. Coli, P. Aeruginosa and S. Faecalis bacterium that are of clinical interest. The AgNFs show an improved potential antibacterial activity compared to its spherical counterparts. The shape of present Ag nanostructure plays a vital role to tune the improved antibacterial properties.

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