9. EXTRACELLULAR BIOSYNTHESIS OF SILVER NANOPARTICLES BY MARINE INVERTEBRATE (POLYCHAETE) AND ASSESSMENT OF ITS EFFICACY AGAINST HUMAN PATHOGENS

9.1. Introduction

Colloidal particles are receiving augmented attention as an important starting point for the fabrication of micro and nanostructures due to its attractive physical and chemical properties which differ considerably from the bulk phase (Inbakandan et al., 2013). The integration of nanomaterials with biology has led to the development of diagnostic devices, various analytical tools, therapeutic applications, and drug delivery vehicles (Das et al., 2013). Silver nanoparticles (AgNPs) have high reactivity due to the large surface to volume ratio and play a crucial role in inhibiting bacterial growth in aqueous and solid media. For instance, AgNPs has been reported to possess anti-tumour (Lara et al., 2013), antibacterial (Pourali et al., 2013), antifungal (Kim et al., 2009), and anti-viral activity (Lara et al., 2010). The antimicrobial activity of AgNPs are influenced by the dimensions of the particles; usually smaller the particles, greater the antimicrobial effect (Lu et al., 2013). The production of nanoparticles with desired shape and size can be obtained from simple bacteria to highly complex eukaryotes in the reaction mixture (Sharma et al., 2009; Pourali et al., 2013).

However, with the development of new chemical and physical methods for the synthesis of nanoparticles, the concern for environmental contaminations is also increasing. Living organisms have enormous potential for the production of
nanoparticles. The ability of plants and microbes to produce nanoparticles have featured exciting loom towards the escalation of natural nano-factories. This phenomenon has brought an insight among researchers for the development of molecules by using an elegant and ingenious method which is precise and efficient (Sankar et al., 2013). The synthesis of nanoparticles by using biological systems is advantageous over chemical and physical methods as it is a cost effective and environment-friendly which does not require high pressure, energy, temperature and toxic chemicals (Ravindran et al., 2013).

In recent years, many bioactive compounds and nanoparticles have been synthesized from various terrestrial and marine organisms (Bhimba et al., 2010). However, such attempts are curbed mainly to plants and microbes (Asmathunisha and Kathiresan, 2013). There is dearth of information available regarding the synthesis of nanoparticles from marine invertebrates. Polychaetes, the major taxonomic group in estuarine and marine ecosystems occupy a large infaunal habitat and play an important role in bioturbation, transfer of organic materials and nutrients from the overlying water column to the sediment and vice versa (Elayaraja et al., 2010; Singh et al., 2013). Polychaetes are valued by the aquaculture industry as an excellent source of polyunsaturated fatty acids (PUFAs), and they have the potential to supplement fish oil as sources of essential lipid components of feeds (Stabili et al., 2013) and hence they are also entitled as Omega worms (Olive et al., 1992). Therefore, in the present study an attempt was made to address the following questions: (a) Are polychaete extract capable of synthesizing AgNPs? (b) If yes, then what is the size of the nanoparticle? and its further characterizations by analytical tools (c) How efficient are the nanoparticles synthesized from polychaete against human pathogen?. 
9.2. Materials and Methods

9.2.1. Sample collection

Polychaetes were collected during the low tide from sediments of Uppanar estuary (Latitude 11°40'20.29 N; Longitude 79°45'15.79 E) Cuddalore, Tamil Nadu and washed thoroughly with distilled water to remove the unsolicited dirt particles.

9.2.2. Preparation of polychaete extract

The polychaete sample (~10 g) was finely pulverized using mortar and pestle. The extract was made up to 100 ml using Milli-Q water. Then the extract was filtered through Whatman No. 1 filter paper to separate the tissue rubbles and obtain a pure extract.

9.2.3. Synthesis of silver nanoparticles

The polychaete filtrate was used as reducing agent and stabilizer for the synthesis of AgNPs. 10 ml of the filtrate was mixed with 90 ml of 1 mM silver nitrate solution in a 250 ml Erlenmeyer flask and agitated at room temperature in dark. A flask containing 10 ml Milli-Q and 90 ml 1 mM silver nitrate solution was taken as control. The change in colour was visually monitored till the appearance of typical dark brown colour.

9.2.4. Characterization of nanoparticles

9.2.4.1. Spectroscopic validation

After 24 hrs of incubation, One ml of sample was withdrawn every six hours and the optical density (OD) was taken at a broad range of wavelengths from 300 to 700 nm using a UV–visible spectrophotometer (UV 2450, Shimadzu) and the graph was plotted based on the OD readings by automated software UV Probe.
9.2.4.2. Atomic force microscopy

In order to further characterize the size and dispersion of the silver nanoparticles the colloidal solution was dried as a thin layer on mica-based glass slide (1mm x 1mm x 1mm) followed by visualization under the atomic force microscope (AFM); Model N9410A Series 5500, Agilent.

9.2.4.3. X-Ray Diffraction pattern

The X-ray diffraction (XRD) measurement of silver nanoparticles was carried out using Cu-Kα radiation source in a wide range of Bragg angle 2θ at a scanning rate of 0.388/min in powder diffractometer (PANalyticalX'per PRO model X-ray diffractometer), at the voltage of 50 kV and current of 30 mA.

9.2.4.4. Scanning electron microscopy (SEM) and energy dispersive X-ray (EDX) analysis

The sample was kept on copper grid stained with uranyl acetate and lead citrate and observed under JEOL-JSM-5610LV Scanning Electron Microscope (SEM). Energy Dispersive X-ray spectroscopy (EDS) analysis was also carried out for the detection of elemental silver by using INCA EDS.

9.2.4.5. Fourier transform infrared spectroscopy (FT-IR) measurements

The freeze-dried AgNPs were pelleted with potassium bromide (KBr) in the ratio of 1:10 and subjected for FTIR spectroscopic measurement (Nicolet IS5, Thermo Scientific). The wavenumber ranged from 450–2500 cm⁻¹ with the resolution of 4cm⁻¹ and were analyzed by subtracting the spectrum of pure KBr.
9.2.5. Assessment of antibacterial activity

Antibacterial activity of the synthesized AgNPs was determined by using the Kirby-Bauer disc diffusion method (Bauer et al., 1966) against five human pathogens namely *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Vibrio parahemolyticus* and *Salmonella typhi*. All the pathogenic bacterial strains were obtained from Raja Muthiah Medical College, Annamalai University, Tamil Nadu, India. Stock cultures were maintained at 4°C on agar slants of nutrient media. Prior to the experiment, pure cultures were sub cultured in Muller Hinton broth and incubated overnight at 37°C. The inoculums suspensions were swabbed uniformly in different petri plates. Filter paper discs saturated with AgNPs was placed aseptically in the plates with the help of sterile forceps and incubated at 37°C. Tetracycline and distill water was taken as positive and negative control respectively. After 24 hrs of incubation the zone of inhibition was observed and measured.
9.3. Results

The successful synthesis of AgNPs by polychaete extract was evident by the formation of dark brown colour. The control (1 mM AgNO₃) solution does not exhibited any color change (Fig. 9.1). The gradual change in colour was observed immediately after two hours of incubation. However, fully dark brown colour appeared only after 72 hours. But, the handling and processing of the extract was less stringent compared to chemical and physical methods. The intermittent UV-Vis spectral observation of the synthesized nanoparticle showed no significant shift in the absorbance intensity as well as absorption maxima indicating the uniform particle size throughout the experiment.

Figure 9.1. Visual observation of colour change in control and synthesized silver nanoparticles
9.3.1. UV-VIS spectra analysis

The UV-Visible spectrophotometric analysis of colloidal reaction mixture of the synthesized AgNPs showed peak at 418-420 nm in the spectrum. The stability of the nanoparticle was also verified. No precipitation was observed in the reaction mixture for the period of six months. The evidence of surface plasmon resonance phenomenon (SPR) is shown in Fig.9. 2.

![UV-VIS spectra analysis](image)

**Figure 9.2.** UV-VIS absorption spectra of silver nanoparticles synthesized from polychaete extract

9.3.2. Atomic force microscopic analysis

The atomic force microscopy (AFM) results displayed the surface morphology of the polydispersed AgNPs. The particle size of the AgNPs ranged from 40 to 90 nm. The topographical image of AgNPs in particular bright spots indicated that they are agglomerated and formed distinct nanoparticles mostly spherical in shape (Figures 9.3. (a) and 3(b)).
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Figure 9.3. AFM topography of synthesized silver nanoparticles by the polychaete extract

9.3.3. SEM and EDS analysis

Scanning electron microscopy provided further insight into the morphology and size details of the synthesized nanoparticles. The SEM image showed relatively spherical and triangular shaped nanoparticle with diameter ranging from 40-90 nm (Fig. 9.4a). This further substantiated the result of AFM analysis. The SEM image also showed polydispersed nanoparticles on the surface. Energy dispersive X-ray analysis (EDX) analysis further confirmed the presence of elemental silver signals of the silver nanoparticles (Fig. 9.4b).
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Figure 9.4. (a) SEM image and 4(b) Energy dispersive X-ray Spectroscopy (EDS) analysis confirming the presence of elemental silver

The scale bar corresponds to 1 µm.

9.3.4. X-ray diffraction study

The XRD pattern of the synthesized nanoparticles corresponds to that of silver nanoparticles. The XRD pattern shows four intense peaks in the whole spectrum of 2θ values ranging from 30 to 80. Comparative analysis of the XRD spectrum with the standard confirmed that the extracellularly synthesized silver particles were in the form of nanocrystals, evident by the peaks at 2θ values of 38.25°, 46.37°, 64.60° and 77.62°, corresponding to 111, 200, 220 and 311 planes for silver, respectively (Fig. 9.5).
9.3.5. FTIR analysis and possible mechanism for the synthesis of AgNPs

To identify the possible biomolecules responsible for efficient stabilization of the silver nanoparticles in polychaete extract was ascertained by FTIR spectral studies. The spectra revealed the presence of prominent peaks at 3345, 2922, 1670, 1384, 1088 and 1037 cm$^{-1}$ corresponding to different functional groups viz. NH, OH, C≡N, N-O, C=C, C=N and C=CH$_2$ in synthesized nanoparticles, analogous to the well-known signatures in the infrared region of the electromagnetic spectrum (Fig. 9.6). The possible chemical reaction which might be responsible for the synthesis of AgNPs are illustrated in Fig. 9.7.
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**Figure 9.6.** FTIR spectrum (transmittance mode) of the polychaete extract after synthesis of silver nanoparticles

**Figure 9.7.** Possible mechanism of reaction for the synthesis of silver nanoparticles by the active molecules

(A) 2,4-di-tert-butylphenol, (B) Glycidyl hexadecyl ether, (C) Stigmasterol and (D) 9-Hexadecenoic acid
9.3.6. Antibacterial Activity

Antimicrobial effects of the synthesized silver nanoparticles were evaluated against five human pathogens (Fig. 9.8). The maximum antibacterial activity was observed against *Staphylococcus aureus* which showed 13 mm diameter zone of inhibition followed by *Escherichia coli* (10 mm), *Pseudomonas aeruginosa* (10 mm) and *Salmonella typhi* (9 mm). The lowest was noticed against *Vibrio parahaemolyticus* (8 mm). However from Fig. 9.7 it is quite evident that the positive control (tetracycline) also showed better zone of inhibition ranging from 12-14 mm.

![Antibacterial activity of silver nanoparticle against human pathogens.](image)

**Figure 9.8.** Antibacterial activity of silver nanoparticle against human pathogens. Blue cone represents silver nanoparticles (AgNPs), whereas positive control (tetracycline) is depicted by red cone.

Here, SA stands for *Staphylococcus aureus*; EA- *Escherichia coli*; PA- *Pseudomonas aeruginosa*; VP- *Vibrio parahaemolyticus* and SA- *Salmonella typhi*.
9.4. Discussion

Owing to the applicability of silver nanoparticles in wide sectors, its demand is increasing at an overwhelming rate which has resulted in increased production. Researchers are continuously developing newer methods for synthesis of highly monodisperse silver nanoparticles which are efficient in terms of synthesis rate as well as energy usage. Biological methods have emerged as an alternative to the conventional methods for synthesis of nanoparticles. The appearance of dark brown is a clear indication of the formation of silver nanoparticles formed in the reaction mixture (Kathiresan et al., 2009). Marine source has recently been explored for the synthesis of gold nanoparticles from sponge, *Acanthella elongata* (Inbakandan et al., 2013). However, present report is the first attempt on testing the efficacy of marine polychaete for the synthesis of silver nanoparticle. Synthesis of inorganic nanoparticles by biological systems makes nanoparticles more biocompatible and environmentally benign (Govindaraju et al., 2010) and cost effective (Roy and Barik, 2010).

Silver nanoparticles exhibit new optical properties, which are observed neither in molecules nor in bulk metals. The synthesized nanoparticle exhibited peak at 418 - 420 nm in the spectrum. This band appears due to the surface plasmon-oscillation modes of conduction electrons which are coupled through the surface to external electromagnetic fields (Stepanov, 2005). Electrons are limited to specific vibrations modes by the particle’s size and shape. Therefore, metallic nanoparticles have characteristic optical absorption spectrums in the UV-Vis region (Wang et al., 2007). Many plants, bacteria as well as fungal species have
been used for silver nanoparticle synthesis (Mandal et al., 2006). But most of them have been reported to accumulate AgNPs intracellularly. Intracellular synthesis always takes longer reaction times and also demands subsequent extraction and recovery steps (Shankar et al., 2004). On the contrary, present study reports the successful extracellular biosynthesis of AgNPs from marine polychaetes with longer reaction time (72 hours) for complete synthesis. The exact reasons and mechanisms need to be explored in this particular species.

AFM analysis showed the particle size of the AgNPs ranged from 40 to 90 nm. The topographical image indicated agglomeration of the nanoparticle which could be attributed to the fact that AgNPs tend to form aggregates on the surface during deposition (Sileikaite, et al., 2006). Scanning electron microscopy image showed spherical and triangular shaped polydispersed nanoparticles. The shape and size of the metal nanoparticles considerably change the optical and electronic properties (Vijayakumar et al., 2013). The XRD patterns clearly revealed the crystalline nature of the biosynthesized silver nanoparticles. The peaks corresponding to the $2\theta = 38.25^\circ$ (111), 46.37$^\circ$ (200) and 64.60$^\circ$ (220) and 77.62$^\circ$ (311) of the sample respects the Bragg’s model of diffraction given by Joint Committee on Powder Diffraction Standards (JCPDS, file nos. 04-0783 and 84-0713). FT-IR has become an important tool in understanding the involvement of functional groups in relation between metal particles and biomolecules. The amide group corresponds to the presence of enzymes which are further prerequisite for the reduction synthesis and stabilization of the metal ions (Asmathunisha et al., 2010). The presence of other functional groups such as OH, N-O, C=O, C-N and C=CH$_2$ may have an effective role in the synthesis of AgNPs. The probable
chemical reactions attributed that the synthesis and stabilization of AgNPs is mainly achieved by the phenolic, ether, sterols and fatty acids present in the extract. Higher content of lipid, fatty acid and glycosaminoglycan (GAG) has been reported from the polychaete species, *Sabella spallanzanii* (Stabili *et al.*, 2013; Singh *et al.*, 2013).

The synthesized nanoparticle showed better antibacterial activity against all the five human pathogens evident by zone of inhibition ranging from 8 to 13 mm diameter. The maximum antibacterial activity was observed against *Staphylococcus aureus* and least against *Vibrio parahemolyticus*. Previous study on antibacterial activity of the crude methanolic extract from polychaete has shown maximum inhibition (8 mm) against *S. aureus* (Elayaraja *et al.*, 2010). Though silver nanoparticles find use in many antibacterial applications, the mechanism of action on microbes is still obscure. However, there are various proposed mechanisms involved in cell lysis and growth inhibition which shows that the inhibition is due to ionic binding of the AgNPs on the surface of the bacteria which creates a great intensity of the proton motive force (Sharma *et al.*, 2009). Moreover, the small size of these particles facilitates the penetration through cell membranes and affects intracellular processes (Ravindran *et al.*, 2013). Silver nanoparticles pretense to have strong bactericidal activity against gram-negative and gram-positive bacteria including multidrug resistant strains (Rai *et al.*, 2012).

The synthesized silver nanoparticle showed comparatively good antibacterial activity against all the tested human bacterial pathogens. This study advocates that, not only plants and microbes, but marine invertebrates do have potential for synthesizing nanoparticles by a cost effective and eco-friendly approach.