Potentiality of the plant *Pseudotsuga menzietii* to combat implant-related infection in the nanoregime

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Abstract: Today, the most alarming problem in the biomedical arena is bacterial infection at the site of implanted medical devices, prosthetics and sensors. Despite aseptic measures and sterilisation procedures microbial infection poses a major impediment and a big question mark to the utility of biomaterials. Therefore, in this nanoregime, we have attempted to bring forth antimicrobial, self-sterilising silver/chitosan bionanocomposite which can indubitably be used for coating of medical implants and surgical devices. Herbal route employing leaves of the plant *Pseudotsuga menzietii*, the Christmas tree, has been implicated for the formation of silver nanoparticles. For the first time, this plant has been involved for the biosynthesis of nanoparticles and to mitigate the menace of implant associated infection. To investigate the formation of silver nanoparticles and the development of bionanocomposite, several characterisation techniques have been used like UV/visible spectroscopy, transmission electron microscopy, scanning electron microscopy, X-ray diffraction, differential scanning calorimetry, FT-IR spectroscopy, etc. together with antimicrobial analysis. Thus, in a cost effective way we have strived to develop a winning strategy to conquer implant-related infection.

Keywords: bionanocomposite; BNC; biosynthesis; coating; nanotechnology; implant-related infection; nanoparticles.


Biographical notes: Poushpi Dwivedi graduated with BSc (hons.) in Chemistry (2001) and postgraduated with MSc in Chemistry (2003) from Banaras Hindu University, Varanasi, India. She completed her MPhil in Chemistry (2009) from Madurai Kamraj University, Madurai, India and is currently pursuing her PhD from the Chemistry Department, Motilal Nehru National Institute of Technology, Allahabad, India. Her research work involves preparation and characterisation of polymer nanocomposites for biomedical application to mitigate the menace of medical implant-related infection.
1 Introduction

The term 'nanotechnology' first coined by Taniguchi (1974), generally refers to the creation of functional materials, devices and systems through control of matter in the nanometre length scale [1–100 nm], and exploitation of novel phenomena and properties (physical, chemical, biological) at the nanoscale. Though nanoscience is considered to be a modern science has its history dating back to the 9th century. Nanoparticles of gold and silver were used by the artisans of Mesopotamia to generate a glittering effect to pots. The first scientific description of the properties of nanoparticles was provided in 1857 by Michael Faraday in his famous paper ‘Experimental relations of gold (and other metals) to light’. On 29th December 1959, Richard Feynman (1991) gave the first talk on nanotechnology entitled ‘There’s plenty of room at the bottom’. Since then the search has surged ahead and the quest for knowledge deeper into it, to gain a firm hold of this revolutionary technology, will increase ever with the acquisition of it.

Today, silver nanoparticles have got myriads of interesting as well as demanding applications, especially in the nanomedicine landscape. A variety of methods have been developed and studied thus to achieve control over the properties of nanoparticles strongly depended upon their dimensions and structure by Kelly et al. (2003). Recently, biosynthesis of nanoparticles has attracted world wide attention because of the necessity to bring environmentally acceptable, cost-effective and efficient techniques. The synthesis of silver nanoparticles from plant resources is an expanding research area as it minimises the call for toxic reagents and hazardous by products and hitherto investigated by many workers like Shankar et al. (2004), Chandran et al. (2006), Huang et al. (2007), Dubey et al. (2010) and Thakkar et al. (2010). Now it can be unambiguously unanimously said, “There is plenty of room in the garden.”

We report herein, the facile method to reductively prepare silver nanoparticles using silver nitrate and aqueous extract from the plant Pseudotsuga menzietii, the Christmas tree. Study of the effect of variation in concentration of silver nitrate and volume of plant extract on the shape and size of silver nanoparticles is also being elucidated here. It is for the first time when the potentiality of this plant is being explored for the biosynthesis of silver nanoparticles, which after formation is being dispersed in a biopolymer, chitosan
matrix, to develop a bionanocomposite (BNC) material for being used as coating on medical implants, prosthesis and devices.

Ongoing research efforts in the biomedical arena have brought forward the hypothesis that silver-containing material can minimise implant associated nosocomial infection which has been confirmed by several in-vitro and in-vivo experiments by Deitch et al. (1987), Gilchrist et al. (1991), Russel and Hugo (1994), Saint et al. (1998), Joyce-Wohrmann et al. (2000), Klueh et al. (2000) and Dowling et al. (2001). Silver nanoparticles having high surface reactivity due to high surface to volume ratio, will release silver ions which is highly antimicrobial with the ability to kill a very broad spectrum of medically relevant bacteria (gram positive and gram negative) as well as fungi (moulds and yeasts). Ionic silver is also oligodynamic, which means that it is antimicrobial at very low doses, as low as about 0.001–0.05 ppm as reported by Berger et al. (1976). Although silver is a heavy metal, at the reference low concentration amounts, it is non-toxic to human cells and therefore very safe according to Williams (1989). Chitosan is used as the matrix which can protect silver nanoparticles from uncontrolled oxidation and stabilise them from agglomeration. Chitosan is a natural biopolymer derived by deacetylation of chitin, a component commonly found in the exoskeleton of crab, shrimp and crawfish and is the second most abundant biopolymer after cellulose. It is composed of poly [β-(1-4)-2-amino-2-deoxy-D-glucopyranose] and has many advantageous features like biocompatibility, biodegradability, non-toxicity, hydrophilicity, together with antimicrobial properties, studied by Balau et al. (2004) and Chengjun and Qinglin (2011).

Therefore, with our resolution to resolve implant-related infection, we have resorted to design via herbal route bactericidal, self-sterilising silver/chitosan BNC as a coating material for biomedical application which when leached out will cause minimal harm to the human body. Thus, our present aim to ameliorate the face of existing biomaterials through self-sterilising surface coatings has been achieved to some extent. This surface modification, in a cost effective way, will render enhanced biocompatibility and tissue integration together with reduced bacterial adhesion as well as hold good psychological effect in the healing process, due to the intervention of our very own Christmas tree.

2 Materials and method

2.1 Materials

Chitosan (degree of deacetylation: 79%, molecular mass: 500,000 g/mol) was purchased from Sea Foods (Cochin), India; AgNO₃ of analytical grade from Thomas Baker (Chemical) Pvt. Ltd. India; acetic acid glacial (extra pure) from Thomas Baker (Chemical) Pvt. Ltd. India. Solutions were prepared using deionised water. Foliage part of the plant *Pseudotsuga menzietii* was collected freshly from the garden.

2.2 Preparation of plant extract

Foliage of *Pseudotsuga menzietii* was washed and air dried. 19 g of clean foliage part were cut into fine pieces and boiled in 100 ml of sterile distilled water in a 500 ml Erlenmeyer flask for 15 min at 100°C. The crude plant extract was filtered using Whatman no. 41 filter paper and stored in closed bottle at 4°C for further use.
2.3 Synthesis of silver nanoparticles

5 ml of the aqueous plant extract was added to 100 ml of 1 mM silver nitrate (AgNO₃) solution and this was marked as (1). Then 10 ml of plant extract was added to another 100 ml of 1 mM silver nitrate (AgNO₃) solution and this was marked as (2). Then another 5 ml of plant extract was added to 100 ml of 2 mM silver nitrate (AgNO₃) solution and this was marked as (3). All the reaction mixtures were kept in closed bottles and incubated at room temperature for stabilisation.

2.4 Development of silver/chitosan BNC

After 48 h all the three reaction mixtures were centrifuged separately at 9,000 rpm for 15 min and the residue (AgNP pellet) was re-dispersed in distilled water. This procedure was repeated three times to isolate Ag nanoparticles from proteins or other bio-organic compounds present. The remnant pellet was dispersed in 15 ml of chitosan solution [2% (w/v) in 1% (v/v) acetic acid] and sonicated for 10 min. Finally, BNC films (1’), (2’), (3’), were prepared by casting the composite solutions on glass slabs (solvent casting) as developed by Mazumder (2002) and dried at room temperature.

2.5 Coating

Coating of medical implants, such as Foley’s catheter tube composed of latex was done by dipping the biomaterial in the composite material of silver reinforced chitosan matrix (dip coating), and then by air drying. Stainless steel rod and stainless steel screw were also coated by pouring the composite material on them (solvent casting technique) and air dried. Facile and less time consuming methods of coating were adopted for preliminary and basic studies.

2.6 Characterisation of silver nanoparticles

The reduction of Ag⁺ to Ag⁰ was monitored by measuring the UV-Vis. spectrum of each reaction mixture (having AgNO₃ solution + leaf extract) after 48 h when complete stabilisation and no further colour transformation was observed. The spectra of plant extract alone and AgNO₃ solution were also taken. The UV-visible spectra were recorded using UV-visible spectrophotometer (Shimadzu UV – 2,450) from 200 to 800 nm. Deionised water was used as blank. Transmission electron microscopy (TEM) samples of the silver nanoparticles synthesised were prepared by placing drops of the product solution onto carbon-coated copper grids and allowing the solvent to evaporate. TEM measurements were performed on the (HR TEM TECNAI 20 G²) instrument operated at an accelerating voltage of 200 kV. TEM images were taken for the study of shape and size of the silver nanoparticles. Analysis of the nanoparticle size was done further with the help of (NANOTECH) particle size analyser instrument. Histograms with the particle size distribution were recorded for the purpose.
2.7 Characterisation of the silver/chitosan BNC

Silver/chitosan BNC was coated with a thin layer of graphite and examined in a scanning electron microscope (SEM) (JEOL JXA 8100) for studying the morphological nature. The structure and physical properties were studied using X-ray diffraction (XRD, Philips, Xpert, Cu Kα) at a scanning speed of 2° min. Differential scanning calorimetry (DSC) assessment was done (using Mettler Toledo DSC 25) to investigate the thermal and other physical properties. FTIR spectra were recorded over the range of (500–4,000) cm⁻¹ with (with FTLA 2000 ABB) to assess the chemical properties.

2.8 Antimicrobial activity test

The BNCs were assayed for antimicrobial activity against *Pseudomonas aeruginosa* (Gram negative), and *Staphylococcus aureus* (Gram positive). Disc diffusion method as explained by Cruickshank (1968) was used to find out the standard zone of inhibition (ZOI). Antibacterial test was done against (1’), (2’), (3’) BNC films; biomaterials Foley’s catheter (disc shaped pieces 6 mm in diameter) coated with (1’), (2’), (3’) BNC material and medical grade stainless steel rod (30 × 12 mm) coated with BNC (1’). The films were cut into disc shape having 5 mm diameter, sterilised by UV radiation for 30 min and placed on different cultured agar plates. Muller Hinton Agar was used as culture media and inoculated with 300 μl of bacterial organism containing broth. These plates containing the bacterial and silver nanocomposite films were incubated at 37°C for two days. The plates were then examined for evidence of zones of inhibition, which appear as a clear area around the discs. The diameter of such zones of inhibition was measured using a metre ruler.

3 Results and discussion

3.1 Effect of variation in concentration of metal precursor and volume of plant extract on the biosynthesis of silver nanoparticles

The initial concentration of silver nitrate solutions and the volume of plant extract added to them were also the parameters studied in the biosynthesis of silver nanoparticles. The colour transformation of silver nitrate solutions after the addition of aqueous plant extract, from water colour to brown indicated the reduction of Ag⁺ to Ag⁰ and the formation of silver nanoparticles. The slight differences in the colour of the different reaction mixtures (Figure 1) as a function of varying concentration and volume of aqueous extract shows that the shape and size of the nanoparticles produced using this technique could be controlled through these adjustments.
The colour development was rapid in reaction mixture (1), slightly slower and lighter in reaction mixture (2), and too fast as well as darker in reaction mixture (3). The inference drawn from this observation was that, reaction mixture (1) has optimum ratio of Ag\(^+\) to plant extract required for the formation of silver nanoparticles having optimum size. In reaction mixture (2) increased volume of plant extract delayed the process of nanoparticle formation hence at one point of time the colour observed was lighter perhaps due to lesser nanoparticles formed or smaller size of silver nanoparticles; this also implies, due to increased volume of aqueous extract added, the total volume of the reaction mixture also increases therein the concentration of Ag\(^+\) present to be reduced to Ag\(^0\) per unit volume also decreases. Thus, there is reduced collision of the agents in the plant biomass, inducing reduction of Ag\(^+\) to Ag\(^0\), with the silver ions. Reaction mixture (3) showed fast colour transformation, almost twice the rate of reaction mixture (1), because the concentration of Ag\(^+\) to be reduced to Ag\(^0\) was higher, double the concentration, therefore, faster bio-reduction and biosynthesis of silver nanoparticles; the colour was deeper due to more number of silver nanoparticles formed and larger size due to clustering. AgNO\(_3\) solution and aqueous plant extract individually showed no change in colour with time.

### 3.2 UV-visible spectral studies

The UV-visible absorption spectra recorded from the nanoparticle suspensions of the three reaction mixtures, (1), (2), (3), after 48 h of reaction and complete stabilisation with no further colour transformation, are shown in Figure 2. In all the three cases, a surface plasmon resonance (SPR) band absorption peak appears between 430–480 nm, which is characteristic of silver nanoparticles. The peak position of reaction mixture (1) and (2) occurs at 440 nm where as the band peak for (3) appears at 446 nm. Spectral analysis after the development of the three silver/chitosan BNCs (1’), (2’), (3’), also exhibited similar absorbance peak as their aqueous suspensions of the nanoparticles. The absorption spectrum of aqueous AgNO\(_3\) only solution was at about 220 nm, chitosan itself is transparent in the UV-visible region. The spectrum of leaf extract rises at ~400 nm without any maxima or minima.
According to Mie’s theory, only a single SPR band peak is expected in the absorption spectra of spherical nanoparticles, whereas anisotropic nanostructures or aggregates of spherical nanoparticles could give rise to two or more SPR bands depending upon the shape of the particles as studied by Kelly et al. (2003). In the present investigation, the suspensions showed a single SPR band revealing spherical shape of silver nanoparticles. The absorption peak of silver nanoparticles due to SPR shifts towards longer wavelength with increasing particle size as reported by Sosa et al. (2003).

Figure 3  TEM images and SAED pattern of silver nanoparticles displaying their crystal nature, in (a) of nanoparticle suspension 1, (b) of nanoparticle suspension 2, (c) of nanoparticle suspension 3; TEM micrographs; (d), (e), (f), images of nanoparticle suspension 1 at different magnifications; (g), (h), (i), images of nanoparticle suspension 2; (j), (k), images of nanoparticle suspension 3 at different magnifications
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different magnifications; (g), (h), (i), images of nanoparticle suspension 2; (j), (k),
images of nanoparticle suspension 3 at different magnifications (continued)
3.3 TEM observations

The morphology of silver nanoparticles synthesised by the intervention of our coveted Christmas tree was observed by TEM. Figure 3 shows TEM images of silver nanoparticles formed by varying the volume of foliage extract and concentration of silver precursor. TEM observations revealed that silver nanoparticles formed are chiefly spherical but are irregular in shape and non-uniform in size. There is also increase in particle size with increase in the concentration of the metal precursor as displayed by nanoparticle suspension (3) and with enhancement in the plant biomass added as shown by suspension (2). Nanoparticle suspension (1) shows optimum size. Most of the particles showed interparticle interactions, which may have been due to the peripheral complexation of capped biomolecules. The ring-like diffraction pattern indicated that the particles were crystalline in nature. This finding was reflected in the approximately circular nature of the selected area electron diffraction (SAED) spots in Figures 3(a), 3(b) and 3(c).

3.4 Nanoparticle size determination

Analysis of nanoparticles through particle size analyser also emphasises the fact that when concentration of Ag⁺ is increased there is increased reduction of Ag⁺ to Ag⁰ simultaneously therefore larger particles are formed, together with agglomeration. Similarly, when the volume of plant extract is enhanced, the plant biomass responsible for reduction of Ag⁺ is enhanced, hence enhanced reduction of Ag⁺ to Ag⁰ on the whole and therefore, larger particle size due to clustering but the number of nanoparticles formed per unit volume is lesser. Details of the determination are vividly shown through the particle size distribution graph in Figure 4.
Figure 4  Histograms of particle size distribution, (a) of nanoparticle suspension 1 (b) of nanoparticle suspension 2 (c) of nanoparticle suspension 3 (see online version for colours)
3.5 Characterisation of the silver/chitosan BNCs

Furthermore, the three silver/chitosan BNCs (1’), (2’) and (3’), developed from the corresponding nanoparticle suspensions were studied for their morphological characteristics through scanning electron microscopy (SEM). Images in Figures 5(a), 5(b) and 5(c) show nanoparticles well dispersed and distributed in the chitosan biopolymer matrix with minimum aggregation. The interaction, between the lone pair of electrons present at the amine group of chitosan and the partial positive charge developed at the surface of the silver nanoparticles due to electron drift, effectively stabilises the silver nanoparticles and prevents them from agglomeration. The BNC suspensions were able to be coated uniformly over the surface of medical grade implants used inside the human body, e.g., Foley’s catheter, stainless steel rod and stainless steel screw; together with imparting smooth surface modification of the biomaterials; vividly elucidated through the SEM micrographs in Figures 5(d) to 5(l).

Figure 5  SEM images, (a), (b), (c) are micrographs of BNC films 1’, 2’, 3’, respectively; (d) image of uncoated Foley’s catheter; (e), (f), (g) micrographs of coated Foley’s catheter with BNCs 1’, 2’, 3’, respectively; (h) image of uncoated stainless steel screw; (i) image showing uncoated as well as coated part of the stainless steel screw with BNC 3’; (j) micrograph of the coated stainless steel screw with BNC 3’; (k) uncoated stainless steel rod; (l) micrograph of the stainless steel rod coated with BNC 1
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Figure 6  DSC spectra showing thermal decomposition, (a) spectrum of biopolymer chitosan film only (b) spectrum of silver/chitosan BNC film developed from suspension 1 (c) spectrum of silver/chitosan BNC film developed from suspension 3.
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The enthalpy change of the BNC films with respect to temperature and time was investigated through DSC and is shown in Figure 6. The BNC film (1’) melts at ~106°C and film (3’) at ~80°C undergoing further endothermic decomposition. XRD pattern denotes that the BNC prepared has an amorphous nature (Figure 7). In the FTIR spectra of the BNCs in Figure 8, the peak at ~3,500 is more pronounced corresponding to the axial OH group of the chitosan molecule. The bending vibrations between 1,600 and 1,000 cm⁻¹ intensify in (a), (b), and (c) of Figure 8, indicating possible interaction between silver nanoparticles and amino group of chitosan.

**Figure 7** XRD spectrum of silver/chitosan BNC film (1’) (see online version for colours)

![XRD spectrum of silver/chitosan BNC film (1')](image)

**Figure 8** FTIR spectra [transmission/Wavenumber(cm⁻¹)] of (a) silver/chitosan BNC film 1, (b) silver/chitosan BNC film 2, (c) silver/chitosan BNC film 3, (d) chitosan film
Figure 8  FTIR spectra [transmission/Wavenumber(cm^-1)] of (a) silver/chitosan BNC film 1, (b) silver/chitosan BNC film 2, (c) silver/chitosan BNC film 3, (d) chitosan film (continued)
3.6 Antimicrobial activity test

Silver/chitosan BNC films (1’), (2’), (3’), having the bioreduced silver and biomaterials coated with the BNC (1”), (2”), (3”), (S), were assayed for antimicrobial activity against *Pseudomonas aeruginosa* (Gram negative) and *Staphylococcus aureus* (Gram positive), which cause majority of the biomedical implant-related infection. Disc diffusion method was adapted to find out the standard ZOI. Details of the result obtained are shown in Figure 9 and listed in Table 1. It has been observed in the present study that the effect was well pronounced against gram negative bacteria which contains only a thin peptidoglycan layer of 2–3 nm between the cytoplasmic membrane and the outer membrane, and even against gram positive bacteria which lack the outer membrane but has a peptidoglycan layer of about 30 nm thickness as was described by Shrivastava et al. (2007). BNC (1’) was most effective in inhibiting the microbes as it was dispersed with the silver nanoparticles from suspension (1) having optimum size of the particles.

*Figure 9* Photographs taken after 48 h of incubation, showing the antibacterial activity through ZOI; silver/chitosan BNC films (1’), (2’), (3’); coated biomaterials (catheter disc pieces) (1”), (2”), (3”) with silver/chitosan BNCs (1’), (2’), (3’) respectively; stainless steel rod (S) coated with silver/chitosan BNC (1’); against *Pseudomonas aeruginosa* and *Staphylococcus aureus* (see online version for colours)
The antimicrobial activity of silver has been known since ages when silver vessels were used for storing to prevent spoilage. Hippocrates recognised the role of silver in the prevention of disease and clinicians have accepted it for over 100 years or more. The mode of action has been studied in the last few decades until recently with the advent of nanotechnology and silver nanoparticles by Lansdown (2002). Metallic silver when exposed to aqueous environment releases silver ions (Ag⁺) which binds with the thiol groups of certain amino acids and inhibits the enzymes of respiratory cycle and also interferes with the DNA replication of the micro-organism. Smaller sized particle has larger surface to volume ratio and thus more effective. According to Shrivastava et al. (2007), the main mechanism through which silver nanoparticles manifested antibacterial properties was by anchoring to and penetrating the bacterial cell wall, and modulating cellular signalling by dephosphorylating putative key peptide substrates on tyrosine residues. Finally, causes cell lysis by rupturing the cytoplasmic membrane.

<table>
<thead>
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<th>Name of the micro-organism</th>
<th>BNC film (1’)</th>
<th>BNC film (2’)</th>
<th>BNC film (3’)</th>
<th>Catheter coated with BNC 1’ – (1”)</th>
<th>Catheter coated with BNC 2’ – (2”)</th>
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<td><em>Pseudomonas aeruginosa</em></td>
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<td>16</td>
<td>17</td>
<td>25</td>
<td>21</td>
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<tr>
<td><em>Staphylococcus aureus</em></td>
<td>25</td>
<td>15</td>
<td>16</td>
<td>21</td>
<td>12</td>
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Note: *NA represents not applicable (test not done). **Nil represents no ZOI was observed.

4 Conclusions

Thus, this first attempt to synthesise silver/chitosan BNC through herbal route via the intervention of the plant *Pseudotsuga menzietii*, shows significant potentiality to mitigate the menace of medical implant associated infection. Bio-reduction of silver ions with plant extract provides a facile path for the production of silver nanoparticles, avoiding the use of obnoxious reducing agents which persistently adhere to the surface of the nanostructures, rendering them hazardous to be handled as well as to be applied. Plant proteins and other organic matter are the principle biomolecules involved in the biosynthesis. The size and the percentage of the particles produced can be easily controlled, according to the requirement, by the initial concentration of the metal precursor and volume of the plant biomass.

Taking into advantage, the plenty of room in the garden and at the bottom; this biodegradable, bactericidal, self-sterilising, biocompatible material holds sure shot
potency to ameliorate the face of existing biomaterials through surface modification in a cost effective way. It provides a winning strategy to combat implant-related infection.

References


