Optimisation of plant mediated synthesis of silver nanoparticles by common weed Plantago major and their antimicrobial properties

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Abstract. Silver nanoparticles synthesized through plant-mediated synthesis have recently gained recognition in the field of biocidal coatings. However, the accurate control of the synthesis using plant extract appears difficult and tends to produce a silver chloride secondary phase. In this study, 2 different methods of synthesis using the same plant extract of Plantago major have been investigated to evaluate their influence on the production of AgCl. In both cases the silver nanoparticles have demonstrated efficient biocidal properties against micro-organisms.

1. Introduction
Ag nanoparticles (NPs) have recently spurred a lot of interest due to their biocidal properties and cost-effectiveness [1]. Ag NPs are now investigated for application as antioxidants [2], antimicrobial agents [3, 4] and biocidal coatings [5]. In fact, increased demand of Ag nanoparticles in biomedical fields calls for non-toxic, cost-effective and eco-friendly synthesis procedures [6]. When applications of nanoparticles extend to areas viz., treatment straw bales for green-housing construction [7], economically-friendly approaches need to be implemented, and green routes for nanoparticle production become more important. Plant mediated synthesis methods appear to be the most suitable ones to meet these criteria [8].

Green or biological synthesis of nanoparticles is classified under the bottom-up approach and is a straight-forward and a simple process. Synthesis involves simultaneous reduction-oxidation reactions catalysed by the microbial enzymes or the plant phytochemicals leading to the stabilization of the nanoparticles further supported by the long chain polymers inside the mixture. Another advantage of green synthesis is a direct functionalization of the surface of the nanoparticle by the plant extract through a synergetic effect [9]. Simple procedures indicate also that plant mediated syntheses are reproducible and therefore applicable worldwide whereupon the production of nanomaterials with local flora is realistic.

Our recent investigations have hence focused on the development of methods for the synthesis of Ag NPs. Therefore, an assessment of antimicrobial activity of greenly synthesized nanoparticles was
A simple weed Plantago major also known as great plantain was chosen for its well-known properties of wound healing activity, anti-inflammatory, analgesic, antioxidant and weak antibacterial properties. Plantago major is also used as an ingredient in the composition of syrup against bronchitis. Plantago major extract was used as the main ingredient for synthesis of silver nanoparticles using two different methods. This paper reports the structural study of the Ag NPs synthesized and the investigation of their biocidal properties.

2. Experimental

2.1. Plant extract preparation

Plantago major plant material was gathered from the humid continental climate in the Baltic region during the growing season. Whole plant without its roots was used. Plants were washed under running tap water. Two different extracts were made. 50g of the plant was dried and 50g was crushed fresh in a mortar. Two liquid media were used for the extraction: ethanol and distilled water. Ethanol extraction was used with the fresh plant material after crushing and distilled water was used with dried plant material (dry mass 6.6g). Ethanol extraction was carried out at room temperature in dark for 24 hours and aqueous extraction under heating at 85ºC in dark for 2 hours. Both extracts were then filtered through Whatman No. 1 filter paper.

2.2. Synthesis

The synthesis of Ag NPs was carried out under two different conditions already used in reported studies. First sample (Type A) was synthesized in a 50ml Erlenmeyer flask placed in a pressure cooker heated at 85ºC for 2 hours to accelerate the speed and increase yield of the synthesis as many studies have suggested [10]. Second sample (Type B) was synthesized in a 50ml Erlenmeyer flask at room temperature under the direct exposure of UV light from the sunlight [11].

(a) Type A particles: Aqueous extraction under thermal conditions

The synthesis was carried out in an Erlenmeyer flask containing 50ml of aqueous plant extract mixed with 50ml of aqueous silver nitrate solution (0.035M). Synthesis was carried out in a pressure cooker at 85ºC for 2 hours. The temperature increase enables a catalytic effect. The translucent green mixture went cloudy after synthesis and settling began within minutes (Figure 1a). Ag NPs were then gathered and washed by repetitive centrifuging at 3500rpm for 5 minutes. During the last step Ag NPs were dried and crushed into fine powder.

(b) Type B particles: Ethanol extraction with UV radiation

The synthesis was carried out in an Erlenmeyer flask containing 50ml of aqueous Plantago major plant extract that was extracted using ethanol before. 50ml of aqueous silver nitrate solution (0.025M) was then added in the flask. The synthesis was carried at room temperature under direct sunlight, where UV radiation had a catalytic effect to the synthesis. The initial intense dark green mixture became red-brown after synthesis and the produced Ag NPs nanoparticles were clearly settling (Figure 1b). Ag NPs were then gathered and washed by repetitive centrifuging at 3500rpm for 5 minutes. During the last step Ag NPs were dried and crushed into fine powder.
Figure 1. Solution of Ag nanoparticles after synthesis (a) type A synthesized in aqueous medium at 85°C, (b) type B synthesized under sunlight.

2.3. Characterization
X-ray diffraction (XRD) patterns were collected using a Panalytical Empyrean diffractometer with a Cu Kα1 radiation source ($\lambda = 0.15406$ nm). The particle size was determined using Scherer’s formula [12] and quantitative analysis of two phases of Ag@AgCl nanoparticles was also carried out [13]. The shape and size of the synthesized nanoparticles were examined by a high-resolution scanning electron microscope (HR-SEM) Zeiss Merlin with Bruker XFlash 6 EDS detector. The morphology and size distribution of Ag NPs were also studied by transmission electron microscope with a JEOL 2010 LaB$_6$ filament providing a point to point resolution of 1.94 Å at 200kV acceleration voltage.

2.4. Antimicrobial assays
(a) Antifungal assay on S. Cerevisiae
The methodology of antifungal assays was conducted according to the method described here [14, 15]. Common yeast S. Cerevisiae was chosen as a target organism to evaluate the antifungal effect of the Ag and AgCl nanoparticles synthesized by Plantago major plant extracts.
(b) Antibacterial assay on E. Coli and S. Aureus
The antibacterial testing was carried out by the international standard ISO 20776-1 “Reference method for testing the in-vitro activity of antimicrobial agents against rapidly growing aerobic bacteria involved in infectious diseases” [16]. Target organisms were chosen E. Coli as Gram-negative bacteria and S. Aureus as Gram-Positive bacteria to evaluate the antibacterial effect of the silver and silver chloride nanoparticles synthesised by Plantago major plant extracts.

3. Results and Discussion
3.1. Structural characterizations
The XRD patterns of Ag NPs synthesized with Plantago major highlight the presence of 2 phases for both syntheses (Figure 2a and 2b). The typical XRD pattern of cubic Ag metal nanoparticles is visible on both XRD patterns and the characteristic diffraction peaks at 38.15°, 44.30°, 64.52°, 77.42° correspond to 111, 200, 220 and 311 reflections respectively (ICDD file no. 00-087-0718). A secondary phase that corresponds to the presence of AgCl structure is also visible on the XRD patterns. Typical XRD peaks at 27.88°, 32.26°, 46.25°, 54.85°, 57.50°, 67.51°, 74.45°, 77.40° that correspond to 111, 200, 220, 311, 222, 400, 331, 420 and 422 reflections are clearly identified (ICDD file no. 00-001-1013). AgCl is a well-known secondary phases commonly produced when using plant extracts mediated
synthesis [17]. However, this secondary phase is usually neglected in published reports. Here, the XRD pattern shows that synthesis method and conditions highly influence Ag/AgCl secondary phase ratio.

Figure 2. XRD patterns of Ag NPs synthesized (a) in aqueous medium at 85°C (type A), and (b) under sunlight (type B). XRD peaks of Ag NPs and AgCl NPs are indexed in black and green respectively.

Figure 3. Type A and type B nanoparticles weight and volume percentages based on the XRD data.

XRD data was also used to calculate the weight and volume percentages of the phases present in the nanoparticles [13]. The results are presented in table 1. In the case of aqueous synthesis combined with thermal heating (85°C) (type A), the amount of AgCl secondary phase is lower (figure 3). Ag is the main phase. Form XRD pattern figure 2a and the Debye-Scherrer method, an average nanoparticles size of 25nm and 37nm for Ag NPs and AgCl NPs was calculated respectively. The Ag NPs are smaller than AgCl NPs. In the case of type B (UV sunlight exposure), the amount of AgCl phase is more important and is the dominant phase (figure 3). Debye-Scherrer method was applied to XRD pattern figure 2b and 2 average diameters of 8.5nm and 19nm was calculated for Ag NPs and AgCl NPs respectively. Ag NPs synthesized at room temperature and sunlight exposure are smaller than those synthesized in a pressure cooker at a higher temperature of 85°C. However, the sunlight exposure and biomolecule extracted using ethanol promoted the synthesis of AgCl phase that becomes the primary phase. This
study shows that it is possible to promote the synthesis of AgCl under specific synthesis conditions and the proportion of both phases can be controlled.

Figure 4. SEM micrographs of Ag NPs and AgCl NPs synthesized (a) in aqueous medium at 85°C (type A), and (b) under sunlight (type B).

The morphology and size of silver nanoparticles were also studied by SEM. The SEM micrographs in figure 4 give an overview of nanoparticles. The spherical morphology and agglomeration of the particles are clearly visible. Also the size of the Ag NPs appears larger on the SEM micrographs than the size calculated from the XRD patterns. This certainly means that visible nanoparticles correspond to the agglomeration of smaller ones. However, SEM study confirm the XRD results and the Ag NPS synthesized in aqueous medium (Type A) are bigger with a diameter slightly lower than 100 nm (figure 4a) than Ag NPs synthesized in ethanol medium (The type B) that exhibit a diameter ranging from 50 to 70nm (figure 4b). In addition, it is not possible to discriminate between Ag NPs and AgCl NPs.

Figure 5. TEM micrographs of Ag NPs and AgCl NPs synthesized (a) in aqueous medium at 85°C (type A), and (b) under sunlight (type B).
3.2. TEM study

The morphology of Ag nanoparticles was also studied by TEM. Transmission electron micrographs in figure 5 give an overview of the Ag nanoparticles. TEM study confirms the bigger size of Ag NPs synthesized in aqueous medium and thermal heating (type A) (figure 5a). Bigger agglomerates are also visible demonstrating a lower control during the synthesis. TEM micrograph in figure 5b shows a narrower size distribution of the nanoparticles and confirm the average size of 10-20nm.

3.3. Biocidal study

The specific toxicity of silver and silver chloride nanoparticles against microorganisms is already well documented [3, 17]. Bacteria such as Staphylococcus aureus and Escherichia coli and yeasts such as Saccharomyces cerevisiae are very commonly used for testing the antimicrobial properties of metal nanoparticles [18]. Therefore, a similar antimicrobial study was performed. Antibacterial tests were carried out according to the ISO 20776-1 standard showed efficient antibacterial properties (table 1). The minimum inhibitory concentration (MIC) that corresponds to the lowest quantity of Ag NPs to prevent the visible growth of bacteria and the minimum bactericidal concentration (MBC) that corresponds to the lowest quantity of Ag NPs required to kill all bacteria were measured.

| Table 1. Antibacterial assay performed on Ag NPs and AgCl NPs synthesized in aqueous medium at 85ºC (type A) and under sunlight (type B). |
|---------------------------------------------------------------|
| **Type of nanoparticles** | **MIC ug/mL** | **MBC ug/mL** |
| E. coli | Type A Ag/AgCl nanoparticles | 1.6 | 3.2 |
| Type B Ag/AgCl nanoparticles | 1.6 | 3.2 |
| S. aureus | Type A Ag/AgCl nanoparticles | 0.8 | 0.8 |
| Type B Ag/AgCl nanoparticles | 0.8 | 0.8 |

This investigation shows that there is no difference between two different samples of nanoparticles (type A and type B) and both samples exhibit strong antibacterial properties whatever the Ag/AgCl ratio. Ag NPs and AgCl NPS proportion does not seems to modify the antimicrobial properties and the biocidal properties are high.

Antifungal tests with S. Cerevisiae are shown in figure 5. These tests did not show either any difference between the two samples (type A and type B). AgNO₃ has been used as a control and by itself shows anti-microbial efficiency. Nevertheless, both plant mediated silver nanoparticles had better biocidal properties against bacteria than yeasts. The minimum bactericidal concentration (MBC) with bacteria is 10 times higher than with S. Cerevisiae (Figure 6).

According to the methodology described by Suppi et al., [14]; antifungal properties against S. Cerevisiae are observed from a concentration of silver nanoparticles of 30µg/mL.
4. Conclusions

In summary, we have investigated 2 different methods of plant extract mediated-synthesis using Plantago major extract and studied the structural properties of the silver nanoparticles. We observed the presence of AgCl secondary phase and the proportion of AgCl in the sample is strongly dependant on the method of synthesis. It is possible to reduce the amount of AgCl through thermal heating. On the other hand, direct UV sunlight exposure at room temperature promotes the synthesis of AgCl nanoparticles. All the nanoparticles are spherical, but synthesis in aqueous medium under thermal heating produces bigger nanoparticles that tend to agglomerate. The study of the biocidal properties shows that the antibacterial and antifungal properties are not dependent on the Ag NPs and AgCl proportion. In all the cases silver nanoparticles exhibit efficient biocidal properties against bacteria and fungi, with higher toxicity against bacteria. Finally, plantago major extract mediated synthesis appears to be an efficient and cost-effective method for the synthesis of antimicrobial silver nanoparticles.

Acknowledgement

This research has been supported by the European Regional Development Fund project EQUITANT (F180175TIBT). LMGP, MINATEC is acknowledged for access to their transmission electron microscope.

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