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DOCTORAL THESIS

Plant Mediated Synthesis of Silver-Based Nanoparticles and Their Use as Antimicrobial Agent in Environmentally-Friendly Applications

Siim Küünal

TALLINNA TEHNIKAÜLIKOOL
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TALLINN UNIVERSITY OF TECHNOLOGY
School of Engineering
Department of Mechanical and Industrial Engineering
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Supervisor: Prof. Erwan Rauwel
Estonian University of Life Sciences
Institute of Technology, Chair of Biosystems Engineering
Fr. R. Kreutzwaldi 56, Tartu 51006, ESTONIA

Co-supervisor: Prof. Protima Rauwel
Estonian University of Life Sciences
Institute of Technology, Head of Chair of Energy Application Engineering
Fr. R. Kreutzwaldi 56, Tartu 51006, ESTONIA

Co-supervisor: Prof. Irina Hussainova
Tallinn University of Technology
School of Engineering
Department of Mechanical and Industrial Engineering
Ehitajate tee 5, Tallinn 19086, ESTONIA

Opponents: Dr. Gregory Guisbiers, Asisstant Professor
Department of Physics and Astronomy
University of Arkansas at Little Rock
2801 S. University Ave, Little Rock, AR 72204, USA

Dr. Nemeshwaree Behary, Associate Professor
ENSAIT, GEMTEX, F-59100 Roubaix, FRANCE

Defence of the thesis: 28/01/2022, Tallinn

Declaration:
Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for doctoral or equivalent academic degree.

Siim Küünal

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signature

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Taime ekstrakti abil sünteesitud hõbedal põhinevad nanoosakesed ning nende kasutus antimikroobse vahendina keskkonnasõbralikes rakendustes

SIIM KÜÜNAL

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Contents

List of original publications that constitute the thesis

The thesis is based on the following academic publications, which are referred to in the text by Roman numerals I to VII:

I. Küünal, S., Visnapuu, M.; Volubujeva, O.; Soares Rosario, M.; Rauwel, P.; Rauwel, E. (2019). Optimization of plant mediated synthesis of silver nanoparticles by common weed Plantago major and their antimicrobial properties. IOP Conference Series: Materials Science and Engineering, 1−8. DOI: 10.1088/1757-899X/613/1/012003

II. Küünal, S., Rauwel, P., Rauwel, E. (2018). Plant extract mediated synthesis of nanomaterials applied to antibacterial and antifungal coating applications. In: A. Barhoum (Ed.). Handbook of nanoparticles and architectural nanostructured materials (1−12). DOI: 10.1016/B978-0-323-51254-1.00014-2

III. Küünal, S.; Kutti, S.; Rauwel, P.; Guha, M.; Wragg, D.; Rauwel, E. (2016). Biocidal Properties Study of Silver Nanoparticles Used for Application in Green Housing. *International Nano Letters*, 6 (3), 191−197. DOI: 10.1007/s40089-016-0186-7

IV. Küünal, S.; Kutti, S.; Rauwel, P.; Wragg, D.; Hussainova, I.; Rauwel, E. (2016). New methodology for the antifungal testing of surfactant-free silver metal nanoparticles for applications in green housing. In: Hussainova, I.; Veinthal, R. (Ed.). *Engineering Materials and Tribology* (133−138). Trans Tech Publications Ltd. (Key Engineering Materials; 674). DOI: 10.4028/www.scientific.net/KEM.674.133

V. Küünal, Siim; Kutti, Sander; Guha, Mithu; Rauwel, Protima; Wragg, David; Nurk, Gunnar; Rauwel, Erwan (2015). Silver Nanoparticles Study for Application in Green Housing. *ECS Transactions*, 64 (47), 15−24. DOI: 10.1149/06447.0015ecst

VI. Rauwel, E.; Simón-Gracia L.; Guha M.; Rauwel, P.; Kuunal, S.; Wragg, D. (2017). Silver metal nanoparticles study for biomedical and green house applications. IOP conf. Series: Materials Science and Engineering 175, 012011. DOI: 10.1088/1757-899X/175/1/012011

VII. Rauwel, P.; Küünal, S.; Ferdov, S.; Rauwel, E. (2015). A Review on the green synthesis of silver nanoparticles and their morphologies studied via TEM. Advances in Materials Science and Engineering_Hindawi, Volume, Article ID 682749, 1–9. DOI: 10.1155/2015/682749

Copies of these articles are included in Appendix.

Author's contributions to the publications

I. First author. Original idea and study design and methods. Synthesis. Characterization preparation. Antimicrobial assays. Results interpretation. Manuscript preparation.

II. First author. Original idea. Data interpretation. Manuscript preparation.

III. First author. Original idea and study design and methods. Antimicrobial assays. Results interpretation. Manuscript preparation.

IV. First author. Original idea and study design and methods. Antimicrobial assays. Results interpretation. Manuscript preparation.

V. First author. Study design and methods. Antimicrobial assays. Results interpretation. Manuscript preparation.

VI. Fifth author. Data collection and manuscript preparation.

VII. Second author. Original idea. Manuscript preparation.

Other publications and conference presentations

Küünal S.*, Visnapuu, M.; Volobujeva, O.; Rosario Soares, M.; Rauwel, P.; Rauwel, E. (2019). Plant mediated syntheses of silver nanoparticles using common weed (Plantago Major L.). 10th International conference Biosystems Engineering 2019, Tartu, Estonia, 8-10th May. Tartu: Estonian University of Life Sciences.

Rauwel, E.*; Rauwel, P.; **Küünal, S**.; Volobugeva, O.; Ivask, A.; Galeckas, A.; Ducroquet, F.; Wragg, D. (2017). Hybrid nanocomposites and ultrastable metal nanoparticles studied for the development of applications in nanomedicine, water purification and energy harvesting. EMN Europe Meetings, Energy Materials Nanotechnology, May 9th to 13th, 2017 San Sebastian, Spain. San Sebastian: OAHOST, Open-Access Publication and Conference Management, 1.

Rauwel, P.; Behr, A.; **Küünal, S.***; Volobujeva, O.; Sõukand, Ü.; Rauwel, E. (2017). Sand based nanocomposite applied to heavy metal ions extraction from polluted water. International IX Oil Shale Conference 2017, "Oil shale industry in circular economy", Ida-Viru, Estonia, 15-16 November. Oil Shale Competence Centre (OSCC).

Rauwel, E.*; Rauwel, P.; **Küünal, S**.; Guha, M.; Gracia, L.; Wragg, D. (2016). Stable metal nanoparticles study for biomedical and green housing applications. The 4th International Conference on Competitive Materials and Technology Processes, Miskolc-Lillafüred in Hungary, 03-07 October 2016. IOP Publishing Ltd, 1.

Küünal, Siim*; Kutti, Sander; Rauwel, Protima; Guha, Mithu; Wragg, David; Hussainova, Irina; Rauwel, Erwan. (2015). Study of silver metal nanoparticles used as antifungal coating for green housing applications. 24th International Baltic Conference in cooperation with IFHTSE ENGINEERING MATERIALS & TRIBOLOGY BALTMATTRIB 2015, Tallinn, ESTONIA. TTÜ kirjastus.

Rauwel, Erwan*; **Küünal, Siim**; Rauwel, Protima; Guha, Mithu; Kutti, Sander; Wragg, David. (2014). Ultrastable Surfactant Free Silver Nanoparticles Study for Application in Green Housing and Cancer Treatment. 226th Meeting of the Electrochemical Society, Cancun.

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Abbreviations

Ag MNP	Silver metal nanoparticles
Ag@AgCl	Silver and silver chloride nanoparticles
AMR	Antimicrobial resistance
CHNS	Elemental analysis of carbon-hydrogen-nitrogen-sulfur
DNA	Deoxyribonucleic acid molecule
DTA	Differential thermal analysis
FWHM	Width at half maximum height (XRD pattern peak)
GHG	Green-house gas (emission)
HEK	Human Embryonic Kidney cell
HRSEM	High resolution scanning electron microscope
HRTEM	High resolution transmission electron microscopy
JCPDS	XRD powder diffraction database International Centre for Diffraction
MBC	Minimum bactericidal concentration
MDR	Multi-drugs resistant
MIC	Minimum inhibitory concentration
MTT	Colorimetric assay for assessing cell metabolic activity
NADH	Nicotinamide adenine dinucleotide (activated carrier molecule in cellular respiration)
PBS	Phosphate-buffered saline (buffer solution to maintain constant pH)
PCA	Plate Count Agar
pH	Potential of hydrogen
SEM	Scanning electron microscopy
spp.	Species
STEM	Scanning transmission electron microscope
TGA	Thermogravimetric analysis
TEM	Transmission electron microscopy
TOPAS	Profile fitting based software for quantitative phase analysis, microstructure analysis and crystal structure analysis (XRD)
XPS	X-ray photoelectron spectroscopy
XRD	X-ray diffraction

1 Introduction

In the 20th century or post-industrial era, our society has been gradually moving towards more sustainable and environmentally friendly approaches in all fields of development [1]. We are constantly in a search of better, more sustainable and nature-preserving ways to carry out our everyday activities. Environmental problems such as a lack of potable water, good air quality, climate and ecosystem changes, as well as resource depletion are all serious issues that need to be dealt with urgently [1]. In particular, the construction sector is energy intensive; a high amount of energy is consumed during the construction material life cycle [2,3]. In order to offset these high-energy requirements, the focus has shifted to a green and smart housing in recent years. The utilization of natural environmentally friendly and cost-effective building materials, is the basic idea behind green housing. In addition, the indoor climate quality is a key factor in choosing environmentally friendly options. For instance, straw is an agricultural waste by-product that is not often considered as an alternative to conventional building materials. However, its composition is very similar to wood, which makes it a sustainable material for building construction; additionally, straw is a cost-effective resource obtained from crops. When built correctly, straw bale houses provide healthy, comfortable, cost-effective and natural living environments. However, since straw is a naturally biodegradable material, problems related to microorganism development are common [2-4] and require eco-friendly methods to combat them. Furthermore, in order to maintain the eco-friendliness of the entire process, alternate methods to treat natural materials in order to make them more resistant are required, instead of conventional toxic chemical repellents. These include technologies, such as antifungal coatings that fight antimicrobial resistance (AMR) among others [5].

During the past two decades, scientific research groups have been extensively investigated nanomaterials, as they offer unique properties and ways to improve our everyday lives [6,7]. Noble metal nanoparticles are very promising candidates for the development of new generation of antimicrobials [8]. Presently, conventional antibiotics are losing their effect on common pathogens due to AMR [9]. On the other hand, silver compounds have proven to be efficient antimicrobials for centuries [10]. For nano-size silver, its antimicrobial properties are enhanced due to multiple factors i.e., shape, size, ion release and possible uptake, which make them more efficient for battling fungi and bacteria including multi-drug resistant (MDR) bacteria. Although, only a few remedies can be considered absolutely ‘safe’, the cytotoxicity of silver nanoparticles is significantly lower in humans compared to prokaryotic organisms [11].

Conventional synthesis methods for silver metal nanoparticles (Ag MNP), such as physio-chemical methods are usually not cost effective. They require specific equipment, skillsets and in some cases even toxic reagents. Moreover, many synthesis processes are usually energy consuming and produce in most cases hazardous by-products [12]. Therefore, the development of more environmental-friendly and cost-effective approaches is needed and biological synthesis routes appear to be the most promising solution in that regard [13]. Plant-mediated syntheses applied to Ag MNP production are more particularly investigated because they offer simple procedures and a wide variety of alternatives in terms of plant materials [12]. In addition, by combining the plant extract’s intrinsic properties and the biocidal properties exhibited by Ag MNP, it is possible to obtain a synergistic effect, enhancing the overall antimicrobial properties of the bio-based nanoparticles. This thesis highlights that the same plant can provide

different types of nanoparticles depending on how the plant's active molecule is extracted. In the end, a cost-effective and environmental-friendly production cycle of Ag MNP will be the key to its sustainability, thereby justifying its applicability to green housing protection or antimicrobial coatings.

2 State of the Art

2.1 Green housing and straw bale construction

The construction sector accounts for over one third of the total energy consumption and green-house gas emissions (GHG) [14]. More specifically, nearly 10 % of the total energy used in the world is associated with the life cycle of the value chain of the construction materials i.e., from construction to destruction [14,15]. Harvesting, mining or collection of raw materials followed by their processing and production record extremely high-energy consumption. This cycle, if not effectively controlled, will affect markedly our natural habitats. Natural non-renewable resources are limited and high-energy consumption for their production will further affect the on-going energy crisis. Consequently, this will also engender adverse effects on the sustainability of related processes and products stemming out of them. The environmental policies target several sectors; in all cases, the environmental requirements for a more efficient utilization of natural resources have to be upheld. This suggests that by-products of processes have to find a second-life, or waste has to be valorized as a resource in order to ensure sustainability of processes.

Green housing has been conceptualized for the construction sector, emphasizing on minimal life-cycle costs and effects [16]. This sector is growing annually, as ecological construction materials help in reducing the impact of building construction on the environment [17]. Straw bale construction meets all the requirements to boost the sustainability of the construction sector as well as guarantee appropriate indoor climates for residents. Straw is a waste for the farmers as only a small fraction of it is needed to maintain soil quality and feed livestock. Surplus of straw needs to be either eliminated or utilized in other sectors. Burning straw waste appears to be the most obvious choice; however, it releases a huge amount of GHG into the atmosphere leaving a large carbon footprint. The utilization of straw as construction material will help in limiting GHG and keep carbon entrapped. One hectare of land is enough for generating straw waste usable for the construction of an average home every year [18].

Straw bale as a construction material has many benefits, such as renewability, abundance, cost-effectiveness along with good thermal and sound insulation properties [19]. However, if the material is not prepared or stored correctly, or if cracks or fissures appear in the construction wall, then the natural hygroscopic properties of straw turn it into a suitable environment for the development of microorganism colonies and more particularly for fungi. Once humidity impregnates straw, it becomes difficult to stop microorganism proliferation. In fact, typical fungi can convert carbohydrates into water through their respiration process, which means that once settled, molds no longer require external humidity for self-sustenance [20]. Additionally, preventing mold growth is more efficient than combatting it once it has infested the straw bales.

Another problem is the fungi reproduction and proliferation mechanisms. Fungi reproduce through fungal spores, which are reproduction units for all molds. These spores are very resilient and harmful for human health. Even after the death of a colony and its eradication, spores are still present in the surroundings. Moreover, these spores can induce allergies, mycoses, inflammation[21] and damage to organs, such as lungs, heart, liver [22].

In Estonia, rye straw is the most popular material for construction among crop waste, where specific genera of bacteria and fungi are found. More specifically typical molds

include genera of *Cladosporium*, *Aspergillus*, *Penicillium*, *Alternaria*, *Fusarium*, *Helminthosporium*, *Mucor* and *Rhizopus*. In case of bacteria, *Streptomyces* and *Pseudomonas* spp. are predominant [23,24].

The mold growth on organic material can be prevented by using repellents, which are usually toxic chemicals such as boric acid or quaternary ammonium compounds [25,26]. These chemicals are used to treat construction timber, which has as a similar consistency to straw (cellulose, hemicelluloses and lignin) and therefore share similar colonizing bacteria and mold problems. Manifestly, if an eco-friendly material is treated with harmful substances, it decreases the eco-friendliness of the process. In addition, the aforementioned chemicals are volatile with relatively long lifetimes during which they can be released into the environment [25]. Ag MNP on the other hand have been broadly tested and implemented as a possible new generation of antimicrobials exhibiting specific toxicity against microorganisms (bacteria and fungi) with lower effects on higher organisms [27-30]. Moreover, Ag MNP do not degrade over time, implying that they can be employed as long-lasting repellents compared to conventional chemicals that can undergo compositional changes over time and therefore need regular renewals [31].

2.2 Silver and silver chloride nanoparticles

Silver nanoparticles have been a research focus for years, placing them in the forefront for commercial applications of nanomaterials such as antimicrobial coating, silver nanoparticle ink, antimicrobial fabrics, drug delivery and medical device coatings [32-35] Only carbon-based nanomaterials have surpassed the number of references in scientific databases compared to silver nanomaterials[36]. This is mainly due to the fact that silver nanomaterials have a very wide range of applications due to their optical [37], catalytic [38], electrical [39] and antimicrobial properties [40] that are dependent on their physical characteristics (size, shape, surface…) [41].

Bottom-up approaches are the main procedures for nanoparticle syntheses and include various chemical methods that involve the use of different reducing agents such as citrates, borohydrides, polyvinylpyrrolidone or other toxic inorganic substances [42-44]. One could argue that nanoparticles synthesized via these routes are not appropriate for green technology (environmentally friendly) purposes. Therefore, research in the field of nanoparticle synthesis directed towards more environmental-friendly alternatives has been the focus of several efforts during the last decades [12]. One of these alternatives is plant mediated synthesis, which fits into the category of green and sustainable approaches for synthesis. Silver nanoparticles and silver in general have prominent antimicrobial properties [12]. Considering that silver nanoparticles are applied as antimicrobials, it would therefore be more rational to develop cost-effective and non-toxic routes of production. It is likely that greenly synthesized silver nanoparticles will be increasingly solicited in several applications and more particularly in antimicrobial applications when synthesized with plant extracts. Silver and silver-based nanoparticles (such as silver halide nanoparticles) have been under investigation in numerous studies revealing their applicability in burn and wound treatment, dental materials, sunscreen lotions, water treatment, textile fabrics and construction materials, owing to their effective antimicrobial properties [4,13,45,46].

Silver-based compounds also exhibit photosensitivity [47]. In particular silver chloride has been widely investigated and used as a photo-catalyst [48]. In addition, when coupled with pure silver metal, the photo-catalytic features of Ag@AgCl nanoparticles make them good candidates for water remediation applications [49]. These two-phased

nanoparticles are highly efficient for the degradation of toxic organic pollutants (e.g. recalcitrant dyes, polyhalogenated organics and pharmaceuticals) [50] under visible light excitation [48]. These properties make Ag@AgCl nanoparticles also promising nanomaterials for solar energy harvesting [51]. Furthermore, the secondary phase of silver chloride along with the silver metal nanoparticles appears to be a more efficient antimicrobial agent [52,53].

2.3 Silver nanoparticles as antimicrobial protection for green housing

There are many important advantages in using straw as a main construction material near agricultural lands. These include, cost-effectiveness along with effective sound and thermal insulation properties [54].

However, the main problem with straw and other organic construction materials is that it is a fertile ground for microbial and mold growth. Straw is highly hygroscopic and therefore, capable of creating enough humidity for mold to proliferate [2]. Therefore, organic construction materials such as wood, straw or reed are at risk of bio-deterioration due to these microorganisms. In nature, decomposition and recycling of organic matter is a critically important task carried out by different kind of molds. However, in housing, their presence brings physical and aesthetic damage to ecological construction materials along with health problems to the house residents [55].

There are very few common-mold protection agents exclusively for straw bales; however, due to the similarities with wood in terms of composition, protection agents used for timber suit straw equally well. For example, chemical compounds such as boron-based fungicides (i.e., boric acid), copper-based agents such as chromated copper arsenate, triazoles and different ammonium chloride compounds are presently applied. However, these chemicals are environmentally harmful and therefore, not recommended for green construction (several of them have been banned over the years) [56,57].

Houses built with straw bale are still a very niche topic as of today. Presently, mold protection agents are mainly available for the wood sector. The main approach is presently based on restricting water availability to fungi in order to prevent colonization by these microorganisms. More specifically, controlling moisture is usually carried out with hydrophobic agents such as plant oils, resins and waxes. Another approach is to use biocidal agents that are incorporated into or on the building material itself. The utilization of less harmful competing microorganisms to prevent the proliferation of more harmful molds is also being investigated. Plant materials that contain antifungal agents or molecules can also be used for the protection of natural construction materials. In fact, these plant materials contain chemical compounds such as alkaloids, flavonoids, phenols, terpenes, quinones that protect the plant from these pathogens in nature. Plant extracts are promising solution for the protection of biodegradable materials. Plant active molecules against microorganisms, separately and combined with nanoparticles appear to be one of the most promising solutions for the protection of ecological construction materials. In addition, this solution is eco-friendly and cost-effective [32,56,58,59]. In this work, the nanoparticles were synthesized in accordance with 12 green chemistry principles [60,61] by using plant extracts that exhibit antimicrobial and/or antifungal properties on their own [62].

The Green chemistry principles that are related to this work are enumerated below [60,61]:

- Design less hazardous chemical syntheses: Design syntheses to use and generate substances with little or no toxicity to humans and the environment.
- Design safer chemicals and products: Design chemical products that are fully effective yet have negligible or zero toxicity.
- Use safer solvents and reaction conditions: Avoid using solvents, separation agents or other auxiliary chemicals or use more benign versions.
- Increase energy efficiency: Run chemical reactions at room temperature and pressure whenever possible.
- Use renewable feedstocks: Use starting materials (also known as feedstocks) that are renewable. The source of renewable feedstock is often agricultural products or the wastes of other processes; the source of non-renewable feedstock is often fossil fuels (petroleum, natural gas, or coal) or mining operations.
- Use catalysts, not stoichiometric reagents: Minimize waste by using catalytic reactions. Catalysts are effective in small amounts and can carry out a single reaction many times. They are preferable to stoichiometric reagents, which are used in excess and carry out a reaction only once.

2.4 Study of environmentally friendly synthesized silver nanoparticles as an alternative for antimicrobials applied in sustainable areas

2.4.1 "Green routes" for nanoparticle synthesis

Although, research in nanotechnology towards green production routes is in the early stages, the development of this branch of science is growing rapidly. As mentioned, the synthesis of nanoparticles using plant extract or micro-organisms like bacteria, algae, yeast or fungi is a step towards the twelve principles of green chemistry developed by Paul Anastas and John Warner in 1998 [61]. The cultivation of microorganisms and the utilization of these microorganisms for the production of nanoparticles is usually more complicated and less cost-effective than the utilization of plant extracts [12]. The difficulty in cell culture maintenance and the nanoparticle harvesting using microorganisms therefore make plant extract utilization more attractive, especially for larger scale production. For example, using plant mass such as dried grass, which is considered as waste, is generally less expensive and simpler than cultivating bacterial or fungal strains [13,63].

2.4.2 Plant mediated synthesis

The collection of plants and the extraction of active biomolecules for nanoparticle production is a relatively straight-forward and simple process. Plant extracts consist of various biomolecules (e.g. NADH-dependent reductase, terpenoids, sugars, alkaloids, flavonoids, phenols, tannins and proteins) that either have a reducing potential of metal ions and/or stabilizing effect on particle growth after nucleation [12]. Biomolecules that are involved in the reduction and stabilization processes vary and reports are available on many different potential compounds such as vitamins, enzymes, organic acids, proteins and lipids among others [12]. Usually the active molecules possess hydroxyl, carbonyl, amine or methoxide functional groups [12]. The plant material is not the only parameter that plays a role in producing nanoparticles of specific structure, size or shape. The synthesis conditions such as temperature, sunlight exposure, pH, mechanical stirring of the dispersion and time can also affect the size, structure and chemical properties of the synthesized nanoparticles. In addition, the growth condition can promote the

presence of secondary phases during the synthesis and the ratio between the phases may depend on the growth conditions. This dependence is highlighted in the discussion part of this thesis and has been one of the focuses of these investigations. In this thesis, we highlight that the method selected to prepare the plant extract can also affect the structural properties and significantly promote the formation of a secondary phase during the synthesis of the Ag MNP.

Plant mediated syntheses are recognized as environmental-friendly, inexpensive and facile up-scalable methods of synthesis. Plants are available all over the world in every climate, which makes plant extracts abundantly available and cost-effective for the production of nanoparticles. The simplicity and availability of precursor materials are major advantages for the synthesis of nanoparticles using plant extracts. However, controlling the synthesized nanoparticles shape, size and composition using plant extract mediated syntheses is more challenging compared to chemical routes due to complex composition of the plant extracts themselves with multiple organic molecules. One of the advantages of plant extracts is the functionalization of the nanoparticle's external surface (coating) with active plant molecules. The combination of the intrinsic properties of the nanoparticles with the specific properties of the plant molecules creates a synergistic effect. This synergistic effect is all the more important for plant-based synthesis of nanoparticles employed as antimicrobials. In this study, Ag MNP is a renowned antimicrobial and Plantago major extract used for their synthesis is also a potent antimicrobial; together they create a synergistic antimicrobial effect [53,64].

2.4.2.1 Plantago Major

Plantago Major, also called "the great plantain", is one of the most widely distributed plants owing to its hardiness as it can grow on disturbed soils and withstand successive aggression such as being trampled. The great plantain is considered as a weed and the leaves are accredited for their disinfectant and wound healing properties [65]. In many parts of the world such as Scandinavia and Baltics, the great plantain has prominent use in folk medicine due to its healing properties. Even the translated name in Swedish and Norwegian for plantain is related to healing[65]. Plantago major's antioxidant, anticancer and antimicrobial properties have been linked to high amounts of biomolecules predominantly phenolic acids, flavonoids, terpenoids and tannins [62,66,67]. The Plantago major extract contains biomolecules that exhibit antimicrobial properties and the utilization of its extracts creates a biomolecule coating on the nanoparticle surface during the synthesis process. This results in the production of a synergistic effect against microorganisms through the combination of intrinsic biocidal properties of Ag MNP and the antimicrobial properties of the biomolecule coating the nanoparticle surface (Figure 1). Therefore, the eco-friendly-route produced nanoparticles are more efficient towards battling pathogenic microorganisms. Several studies report similar results, i.e. plant mediated synthesized nanoparticles exhibit more enhanced biocidal properties than their counterparts synthesized using conventional chemical methods devoid of surface-coated active antimicrobial-molecules [64].

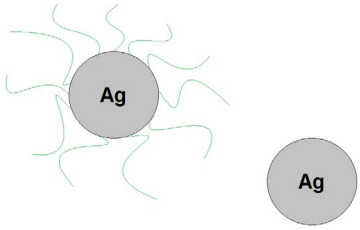

Figure 1. A schematic image of Ag MNP with and without surface coating from the green synthesis.

2.5 Preliminary results from early research

Preliminary work highlighted the potential of using silver nanoparticles synthesized via non-hydrolytic sol-gel methods using silver acetate precursors as microbial and fungal repellents [4]. Ag MNP studied in this preliminary research were surfactant-free with a very narrow size-distribution and had significant effect on the bacteria and fungi that dominantly grow on straw bales. These findings were the basis of this current thesis, which is the continuation of this study in order to gain a better insight on the antimicrobial and antifungal properties of these Ag MNP.

The coating of straw with these nanoparticles was more particularly studied in addition to the limiting factors of such protective treatment methods. As a second step in the current thesis, the possibility of using a more cost-effective and more environmental-friendly routes to produce these nanoparticles was investigated, as Ag MNP revealed to be good candidates for preventing microbial and fungal growth on different surfaces [4].

2.6 Objectives and claims of the study

Eco-friendly and degradable construction materials are an efficient way to reduce the energy consumption in the construction sector. Nevertheless, these materials are prone to microorganism infestations. Combatting microbial proliferation requires additional environmentally compatible methods in order to maintain the sustainability of the value chain. The overall objective of the study is the development of eco-friendly antimicrobial nanomaterials viz. Ag nanoparticles. It also consisted of designing a unique methodology to eradicate common fungi and bacteria. One of the aims of the study was to optimize the biosynthesis of silver nanoparticles using the selected plant extract in order to produce most efficient silver nanoparticles against fungal proliferation. More specifically, the goals of the work were:

- Identification of common microorganisms invading ecological construction material (straw).
- Testing novel silver nanoparticles against common bacteria and fungi as an antimicrobial agent.
- Developing a method for testing silver nanoparticles against microorganisms on real construction materials.
- Synthesizing “green” silver nanoparticles via a suitable ecologically friendly method.

- Analyzing and optimizing the precursor extract preparation and synthesis routes for specific nanoparticles.
- Evaluating the applicability of green nanoparticles i.e., different silver/silver chloride nanoparticles as repellents on various microorganism.

Novelty of this work lies in the possibility of controlling the size and phase ratio (Ag / AgCl) of the nanoparticles and the Ag MNP / AgCl NP ratio using the same plant material with plant mediated synthesis but by altering synthesis conditions and extract preparation methods.

3 Structure of the study

The schematic structure of the thesis work is presented in Figure 2 emphasizing on the published papers and their contribution in achieving the objectives of this study:

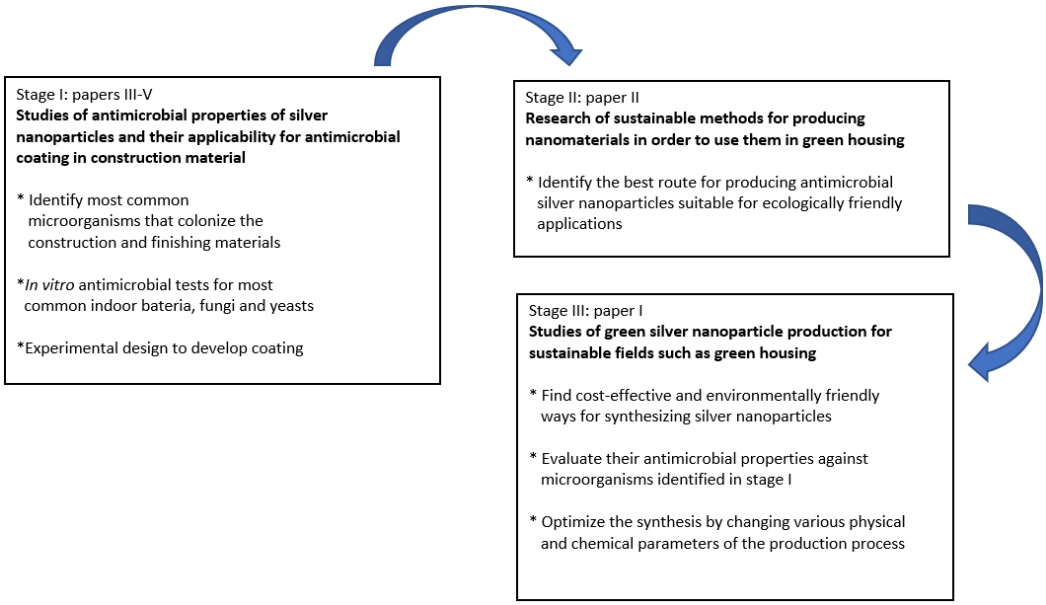

Figure 2. Structure of the research study.

4 Materials and methods

4.1 Stage one: Applicability of surfactant free silver nanoparticles in green housing as an antimicrobial agent

The preliminary results from the authors Master's thesis showed that surfactant free Ag MNP exhibit biocidal properties that make them potential candidates for antimicrobial and antifungal agents for building materials. Articles III-V focus on the study of the biocidal properties of these Ag MNP against microorganisms and possibilities of testing on straw bales in different conditions. The full description of materials and methods can be found in articles III-V [2,3,4].

4.1.1 Stage one silver nanoparticle synthesis

The synthesis of Ag MNP was carried out in a glovebox and is based on non-hydrolytic sol-gel method developed by Prof. Erwan Rauwel. Silver acetate (99%, Aldrich) precursor mixed in benzylamine was used for the synthesis of the Ag MNP. The mixture was transferred into a sealed stainless-steel autoclave inside the glovebox. Then, the autoclave was put into a furnace at 200^{o}C for 48 hours. Due to the autoclave configuration, it was not possible to analyze the gas by-products, and no equipment available could analyze the liquid by product after reaction. After synthesis, the resulting suspensions were centrifuged and the precipitates thoroughly washed with ethanol and dichloromethane. See paper III-V [2,3] and [68].

4.1.2 Characterization

X-ray diffraction (XRD) study was performed using a Bruker D8 equipped with a LynxEye detector. Copper radiation (kα1=1.54056Å) was selected using a Ge (1 1 1) monochromator. Depending on the sample the crystallite size was calculated manually using Scherrer method and Origin software or using full profile Scherrer methods in TOPAS, with a fundamental parameters peak shape.

Thermogravimetric analyses (TGA) were performed with Rheometric Scientific STA 1500 TGA instrument. The Ag MNP samples were heated from room temperature to 800^{o}C with a heating rate of 5^{o}C/min to study their thermal stability against oxidation under flowing air atmosphere, and to check the presence of surfactant or organic molecule on their surface.

Elemental analysis carbon-hydrogen-nitrogen-sulfur (CHNS) was performed with Leco Truspec Micro CHNS Analyzer model 630–200-200 at temperature of 1075ºC. Nitrogen was measured by thermal conductivity and carbon; hydrogen and sulfur were measured by infrared absorption.

Transmission electron microscopy (TEM) studies were carried out using two different microscopes; a probe corrected Titan G2 80-200 kV operating at 200 kV and disposing a point-to-point resolution of 0.8 Å in STEM mode, and the morphology and size distribution were studied with a JEOL 2010 LaB_6 filament TEM providing point-to-point resolution of 1.94 Å at 200kV acceleration voltage was used to perform.

Scanning electron microscopy (SEM) study was carried out using a high-resolution scanning electron microscope (HR-SEM) Zeiss Merlin equipped with an energy dispersive

X-ray spectrometer (Bruker EDX-XFlash6/30 detector) to study the shape and size of the clusters, and the morphology of the synthesized Ag and AgCl nanoparticles.

X-ray photoelectron spectroscopy (XPS) studies were performed using a Kratos Analytical Axis UltraDLD photoelectron spectrometer equipped with Al Kα X-ray source.

4.1.3 Stage on antimicrobial studies

The Identification of the studied microorganisms was carried out after collecting them from the straw bale samples that were stored outside. Pieces of straw were then taken and printed in previously prepared sterile selective agar plates. For bacteria, the agar medium consisted of distilled water, 1.5% of agar, 0.5% of tryptone soy, 0.5% sodium chloride and 0.08% cycloheximide dissolved in ethanol previously. For fungal agar, the medium was composed of 1.5% of agar, 2% of malt extract and 0.02% of chloramphenicol. The prepared media were heated at 120^{o}C for 60 min. After cooling at room temperature, the straws were printed in the Petri dishes. All samples were placed in an oven that produces warm and humid conditions to enable the complete growth of the cultures, required for future identification. Bacteria and fungi that grew in the Petri dishes were taken and heat fixed on microscope slides for identification. In the case of bacteria, Gram's Method was used [69], where bacteria were stained and subsequently classified as Gram-negative or Gram-positive bacterial strains. In the case of fungi, fungal cells were stained using 5% "Rose Bengal" solution and the identification was carried out using a microscope and based on online databases and Bergey's manual [70].

Antimicrobial assays were conducted against common bacteria that were initially identified. The tests were done against bacteria and fungi (i.e., *Aspergillus* spp.) in separated petri dishes similarly to the ones used for their identification, but biocidal tests were also performed all together in non-selective plate count agar (PCA). The study of biocidal properties was done in Petri dishes via the droplet test and the well-diffusion method. Micro broth dilution assays were also used for the biocidal tests against fungi. Finally, in situ straw bale covering tests were performed, and for this purpose, a new methodology was developed to evaluate the protection level of Ag MNP against microorganism that can develop on straw surface [2,3]. The full description of the methods can be found in articles III-V [2,3]. During the first stage of the study, the following methods were used:

Droplet tests were carried out to the isolated cultures found on the rye straw. Silver nanoparticle dispersion drops with several concentrations were dripped onto developed and undeveloped bacterial and fungal colonies in Petri dishes and control was made via visual evaluation (full description in the article V) [2-4], after fungal growth in the petri dishes.

Broth dilution assay tests were chosen to evaluate the minimal inhibitory concentration and minimum fungicidal concentration for common mold *Aspergillus* spp. To determine these concentrations, liquid sample from each flask was seeded onto agar plates to confirm viability of the culture treated with Ag MNP (full description in the article III and IV) [2,3] .

Outdoor tests were performed. Straw samples were decorated with silver nanoparticles and then stored outdoors mimicking the storage conditions or realistic scenario of construction material stored in construction site. The methodology of straw decoration with Ag nanoparticles is fully described in article IV [2,3]. Figure 3 shows the schematic image of system used to spread Ag NPs on the surface of the straw. Straw samples were immersed inside the vessel where silver nanoparticles were dispersed in

ethanol solution using a magnetic stirrer. Ethanol was selected because of its volatility that make the sample easy to dry after coating. After outdoor stay, straws taken from the decorated straw samples were printed on agar plates to evaluate the growth of fungi and bacteria. Plates were stored in an oven used to incubate microbial organisms visually compared the growth of microorganisms in the petri dishes between treated straw and untreated straw.

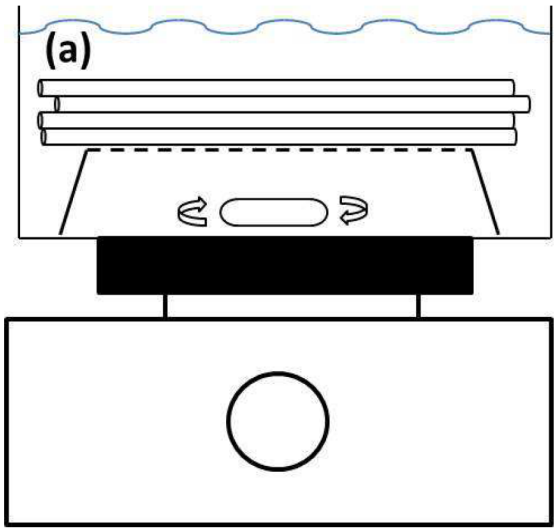

*Figure 3. A schematic image of vessel system where straw was decorated with silver nanoparticles. The figure is adopted from **Article IV** with permission [2].*

4.1.4 Toxicity study against human cells

The toxicity of different concentrations of Ag MNP was studied against Human Embryonic Kidney (HEK293) cells via MTT assay (M). HEK293 cells were seeded on day 0 at a density of 1000 per well in 96-well microtiter plates. On day 1, different concentrations of Ag MNP were added. After 24 hours of incubation, the media containing Ag MNP was removed from the plate in order to ensure that no nanoparticles remain in the solution in order to avoid overlap or hinder MTT assay. After 24 hours, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well (0.5 mg/ml; Sigma Aldrich) and plates were maintained at 37°C for 2 h. The medium was then discarded, and DMSO was added to each well to lyse the cells. Absorbance was measured at 450 nm using a multiwall spectrophotometer (Tecan, microplate reader). All MTT assay were performed in triplicate and repeated twice.

4.2 Stage two specific materials and methods: Developing new plant-mediated synthesis method for Ag nanoparticle production and optimization of the synthesis

The first stage of the thesis demonstrated that Ag MNP can be used effectively as antimicrobial repellents and against microorganisms like fungi, that develop on straw bales. Therefore, research designs incorporating environmental-friendly methods of synthesis would conform more to the field of green housing. The focus of this thesis was to investigate the possibility of using bio-synthesized Ag MNP as biocidal coating to

protect the straw bales that are used in green house construction. Therefore, a reliable, cost effective and eco-friendly way for the production of Ag MNP has been developed during the thesis. The principles of green chemistry were applied to the synthesis[60]. The full description of materials and methods used for synthesis can be found in Papers I-II [53,64] and in addition, they are briefly described below.

4.2.1 Plant selection

Plant mediated synthesis of nanoparticles was chosen for its novelty, simplicity and suitability for green housing. Main principles for the plant selection were that it should be common and abundant with known antimicrobial properties. Weeds and large-scale cultured plants are therefore the best candidates. In that regard, the plantain (Plantago major), a common weed was a good option and it is already used in syrups for soothing the throat and the respiratory tracts. An additional goal was to investigate how different conditions can affect the nanoparticle production using the same plant material.

4.2.2 Plantago major extract preparation

Several routes were investigated to collect Plantago major extract from fresh leaves of the plant. The plant extract is known to contain bioactive molecules that act simultaneously as reducing, stabilizing and capping agents.

Type A extract: Heat extraction method. The first route for the extraction of Plantago major active molecules was to crush it with a mortar and pestle and put the crushed plant materials into a glass container with distilled water. The distilled water and plant material were then heated up to 85^{o}C in a pressure cooker for an hour. Afterwards, the solution containing the extract was filtered with Whatman No 1 filter paper and the extract was ready for synthesis of Ag NP.

During this thesis, several different routes for the preparation of the Plantago major extract were investigated. Fresh and dry plant material were both investigated with ethanol or with distilled water and temperatures ranging from room temperature to 85°C. All these routes are summarized in the 'Results' section (Table 7).

Type B extract: In the second approach, pure ethanol was used to prepare the plant extract solution from fresh plant material. It is also known as herbal tincture. It is well known that alkaloids and plant resins dissolve better in ethanol than water. Ethanol can dissolve both polar and non-polar biomolecules to some extent [71]. Ethanol extraction was carried out in dark conditions to avoid UV-light modification of the extracted biomolecule. The plant material was soaked in ethanol for 24h. The solution containing the soaked plant was then filtered with Whatman No. 1 filter paper to eliminate the solid parts.

Unfortunately, the Plantago Major extract obtained using this method was not sufficiently active (reducing/stabilizing properties) to produce suitable Ag MNP when mixed with silver nitrate precursor ($AgNO_3$), even with UV-assisted activation or heating.

For this reason, the ethanol solvent was evaporated inside the furnace at an average temperature of 50ºC and the remaining solute left in the container was then dissolved in distilled water to prepare an active solution that would react with $AgNO_3$ precursor. Following the dissolution of Plantago major solute in distilled water, the resulting solution was filtered again with Whatman No 1 paper filter and the plant extract was ready to use for synthesis.

The two main procedures described above were more specifically used during the thesis, as they appeared to be the best extraction procedures of plant based active molecules for the synthesis of high-quality Ag MNP.

4.2.3 Plantago major mediated synthesis of Ag MNP

Plantago major extract type A and Plantago major plant extract type B (described above) were used in this study to synthesize Ag@AgCl NP. Two different catalytic methods were tested:

1. Thermal energy via temperature increases with Plantago major type A extract.
2. UV light radiation with Plantago major type B extract.

The work was carried out under the hypothesis that completely different synthesis methods result in distinct nanoparticles. The goal was to study how the activation energy can influence the Ag MNP's physical and chemical properties during the synthesis process using similar Ag precursors and plant extract ratios.

Type A synthesis: The first method of Ag@AgCl NP synthesis was conducted using Plantago major extract type A (plant material boiled in distilled water). The synthesis of Ag@AgCl NP was carried out in Erlenmeyer flask 50 mL of distilled water containing 0.025 M of silver nitrate (prepared with 212.3 mg of $AgNO_3$) was mixed with 50mL of Plantago major extract type A (50:50 ratio). The synthesis was performed in dark conditions (inside a pressure cooker) at approximately 85°C for 1 hour. When the precursor solution of $AgNO_3$ was added to the plant extract, the mixture turned immediately translucent and a clear sediment formed at the bottom of the flask. In type A synthesis, thermal energy (85°C) is used to catalyze and promote the reduction of silver ion during the reaction synthesis. Ag NP of different shape, size and properties than for type B synthesis were expected. The obtained silver nanopowder was washed 3 times successively, first with ethanol and after three rounds with distilled water. Between each rinsing step, the nanopowder was centrifuged in a 15 mL tube at 3500 rpm for 5 minutes. The final product was dried in an oven at 70°C for 1 day to remove any residual water and ethanol traces. After drying and complete solvent evaporation (ethanol and water), a pellet was formed. The pellet was then grinded into fine powder with a mortar and pestle before further characterization.

In summary, the Plantago major type A extract was used in dark conditions under thermal at 85°C and resulted in biosynthesized "type A" Ag@AgCl NP. More specifically the sample was named "GSW003".

Type B synthesis: The synthesis of Ag@AgCl NP using plant extract 1 (ethanol-based plant extract) was carried out in Erlenmeyer flask at room temperature under direct sunlight exposure after mixing the different precursors. Sunlight is also composed of UV radiations that are high energy electromagnetic radiation ranging from 3 to 4 eV [72]. Solar light can then promote the synthesis of Ag/AgCl MNP through high-energy UV exposure owing to its catalytic effect (electrons generated react with cations of Ag^+) [73,74]. Under UV light exposure, the reaction takes place instantly with the formation of visible sediments at the bottom of the Erlenmeyer. In this synthesis, 50 mL of distilled water containing 0.025 M of silver nitrate (prepared with 212.3 mg of $AgNO_3$) was mixed with 50mL of Plantago major extract 1 (50:50 ratio). Under the natural UV light exposure, the dark green mixture turned immediately red-brown with the formation of visible sediments. AgCl is known to turn dark under sunlight exposure; hence, the subsequent

color change confirms its formation. The obtained silver nanopowder was washed, dried and grinded similarly to type A. In summary, the sunlight-induced method was used with Plantago major extract type B to biosynthesize "type B" Ag@AgCl nanoparticles. Sample was named "GS003".

4.2.4 Stage two antimicrobial studies

The antimicrobial property of biosynthesized Ag nanopowder was studied in vitro. The antibacterial tests were conducted following the ISO 20776-1:2006 standard protocol "Clinical laboratory testing and in vitro diagnostic test systems – Susceptibility testing of infectious agents and evaluation of performance of antimicrobial susceptibility test devices -- Part 1: Reference method for testing the in vitro activity of antimicrobial agents against rapidly growing aerobic bacteria involved in infectious diseases in order to acquire better comparison with the research done in the field." To assess the antifungal properties, yeast Saccharomyces was selected as fungal reference model organism. Antifungal tests were carried out according to the test developed by Suppi et al., and Kasemets et al. [75,76]. The complete description of the antimicrobial studies can be found in paper I [53,64].

4.2.5 Characterization of biosynthesized Ag nanoparticles

All the synthesized green Ag nanopowder samples were characterized by XRD in cooperation with CICECO laboratory at the University of Aveiro in Portugal (see section 4.1.2). The XRD patterns obtained from XRD measurements gave insights on the material crystallinity, phase, electron density and crystallite size. The latter can be determined using the Scherrer equation (Eq.1) for nanoparticles exhibiting spherical shape. Nevertheless, it can also be used to qualitatively estimate the average size of nanoparticles within a synthesis irrespective of their shapes. However, for more precise morphology and size distribution of nanoparticles, more adapted techniques exist.

$$L = \frac{K\lambda}{\beta cos\theta} \quad (1)$$

where:

- L is the average size of the crystallite, which is smaller or equal to the grain size;
- K is a constant and depending on a crystallite shape has a typical value of 0.9. 0.94 for spherical crystallites with cubic symmetry;
- λ is the X-ray wavelength in nanometers (here λ = 0.15406 nm);
- β measured in radians is the diffraction pattern peak width at half maximum height (FWHM);
- θ is the Bragg angle[77].

XRD diffraction patterns obtained from XRD measurements were compared with the powder diffraction database (JCPDS) to identify the materials, their crystalline structure and the presence of secondary phases. In fact, XRD studies highlighted the presence of AgCl nanoparticles as a secondary phase in the synthesized silver nanopowder samples.

4.2.6 Quantitative analysis of powder mixtures

The XRD patterns of biosynthesized Ag nanopowder produced using Plantago Major extract and silver nitrate precursor show that produced Ag nanopowders are composed of two phases; a mixture of cubic silver metal nanoparticles and cubic silver chloride nanoparticles. Subsequently, the proportions of the two different phases present were first estimated by the method described in the book of Y. Waseda et al., "X-Ray

Diffraction Crystallography: Introduction, Examples and Solved Problems" [78] because TOPAS was not available initially, Ag/AgCl ratio was therefore calculated manually. This part describes the methodology applied to estimate Ag/AgCl ratio manually. Quantitative X-ray diffraction analysis is based on the fact, that crystalline substance concentration in mixtures is related to the intensity of the diffraction pattern. However, the relation is not always linear; the diffraction peaks corresponding to specific planes of the structure should be analyzed and separated from other phases.

Ag and AgCl exhibit similar structure (i.e., face centered cubic (fcc)), but with different unit cell size, and AgCl exhibits fcc NaCl structure type with ionic bonding. In case of Ag and AgCl the first plane is (111).

Volume fraction calculation of two phases by the direct comparison method:

$$\frac{I_{AgCl}}{I_{Ag}} = \frac{R_{AgCl} * C_{AgCl}}{R_{Ag} * C_{Ag}} \quad (2)$$

$$\frac{C_{Ag}}{C_{AgCl}} = \frac{I_{Ag} * R_{AgCl}}{I_{AgCl} * R_{Ag}} \quad (3)$$

Peak intensities (I_x) are taken from the XRD data and R constant is calculated as follows:

$$R = [|F|^2 * p * \left(\frac{1 + \cos^2 2\theta}{2sin^2\theta * cos\theta}\right)]\frac{e^{-2M_T}}{\Omega^2} \quad (4)$$

1. First step would be to determine the *F* component, which is the structure factor. It describes the relationship between the crystal structure and intensity of the diffracted X-rays from each plane.

(111) plane: When *h, k*, and *l* are unmixed and (*h* + *k* + *l*) is odd number.

$$F = 4(f_{Na} - f_{Cl}) \implies |F|^2 = 16(f_{Na} - f_{Cl})^2$$

F_{111} = $16(f_{Na} - f_{Cl})$ decrease in intensity due to $(f_{Na} - f_{Cl})$

(200) plane: when *h*, *k* and *l* are unmixed and (*h* + *k* + *l*) is even number

$$F = 4(f_{Na} + f_{Cl}) \implies |F|^2 = 16(f_{Na} + f_{Cl})^2$$

F_{200} = $16(f_{Na} + f_{Cl})$ increase in intensity due to $(f_{Na} + f_{Cl})$

The atomic scattering factor f_x is taken from table1 by considering the $\frac{\sin\theta}{\lambda}$ values.

For example, the $\frac{\sin\theta}{\lambda}$ values for Ag (111) plane are $\frac{\sin 19.07}{1.542495}$ = $\frac{0.3267}{1.54}$ = 0.2118

According to the table below, the f_{Ag} value is 36.26

Table 1. Atomic scattering factors as a function of sin θ /λ [78]

$\frac{\sin\theta}{\lambda}$ (Å^{-1})	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
Ag	47	42.8	36.9	31.5	26.9	23.8	21.3	19.0	17.1	15.3
Cl	17	14.6	11.3	9.25	8.05	7.25	6.5	5.75	5.05	4.4

Therefore *F*= 4(f_{Ag}) and $|F|^2$=16*36.26^2= 21 036.6

2. Next component is *p*, which is a multiplicity factor and these values for crystalline powder samples are given here:

Table 2. Multiplicity factors for crystalline powder samples [78]

Cubic		*hkl*	*hkk*	*hk0*	*hh*0	*hhh*	*h*00		
		48*	24	24*	12	8	6		
Hexagonal		*hk·l*	*hh·l*	*h*0·*l*	*hk·*0	*hh·*0	*h*0·0	00·*l*	
		24*	12	12	12*	6	6	2	
Trigonal	Ref. to rhombohedral axes	*hkl*	*k̄kh*	*hkk*	*hk*0	*k̄hh*	*hhh*	*hh*0	*h*00
		12*	12*	6	12*	6	*2*	6	6
	Ref. to hexagonal axes	*hk·l*	*hh·l*	*h*0·*l*	*hk·*0	*hh·*0	*h*00	00·*l*	
		12*	12*	6	12*	6	6	2	
Tetragonal		*hkl*	*hhl*	*hh*0	*hk*0	*h*0*l*	*h*00	00*l*	
		16*	8	4	8*	7	4	2	
Ortho-rhombic		*hkl*	*hk*0	*h*00	0*k*0	00*l*	*h*0*l*	0*kl*	
		8	4	2	2	2	4	4	
Monoclinic	Orthogonal axis b	*hkl*	*hk*0	0*kl*	*h*0*l*	*h*00	0*k*0	00*l*	
		4	4	4	2	2	2	2	
Triclinic		*hkl*	*hk*0	0*kl*	*h*0*l*	*h*00	0*k*0	00*l*	
		2	2	2	2	2	2	2	

For (111) plane, the value is 8.

3. Then the next component $\left(\frac{1+\cos^2 2\theta}{2sin^2\theta*cos\theta}\right)$ has to be determined. It describes the Lorentz-polarization factor (LP).

For Ag (111) plane, the LP is $\left(\frac{1+\cos^2 38.14}{2sin^2 19.07*cos 19.07}\right)$= $\frac{1.618588}{0.2017793}$= 8.0216

4. Last component is $\frac{e^{-2M_T}}{\Omega^2}$, where Ω is volume of unit cell and e^{-2M_T} is the temperature factor.

Volume unit cell (Ω) of Ag with fcc structure is given by the lattice parameter a = 0.409 nm. And volume is a^3 = 0.068418 nm^3.

The temperature factor has also to be taken into consideration because atoms in a crystal vibrate around their mean positions and the displacement is dependent on the temperature. At room temperature the displacement is about 5%. Therefore, this results in a reduction in the intensity of the reflections in the XRD patterns. Deybe-Waller factor is therefore used, e^{-2M_T}, where the quantity of M is calculated. However in practice function M_T is not calculated, but the coefficient BT is taken, which is an estimation of measured intensity data at different temperatures.

$$M_T = 8\pi^2(u^2)\left(\frac{\sin\theta}{\lambda}\right)^2 = B_T\left(\frac{\sin\theta}{\lambda}\right)^2 \quad (5)$$

$$B_T = \frac{6h^2}{mk_B}\frac{T}{\Theta^2}\left\{\varphi(x) + \frac{x}{4}\right\} \quad (6)$$

Here the T is absolute temperature, m is the mass of vibrating atom, h and k_B are the Planck and Boltzmann constants, and Θ is the Deybe characteristic temperature.

First part of the equation can be simplified as follows:

$$\frac{6h^2}{mk_B} = \frac{6N_A h^2}{Mk_B} = \frac{6*(0{,}6022*10^{24})*10^3*(6{,}626*10^{-34})^2}{M*(1{,}3806*10^{-23})*10^{-20}} = \frac{1{,}15*10^4}{M}$$

$$\left\{\varphi(x) + \frac{x}{4}\right\}$$

For another component x needs to be found;

$$\text{i.e } x = \frac{\Theta}{T} \tag{7}$$

Θ is the Debye temperature from the table below

Table 3 Atomic weight, density, Debye temperature and mass absorption coefficients (cm^2/g) for elements [78]

Characte-ritic radiation	Wavelength (Å)	41 Niobium	42 Molybdenum	43 Technetium	44 Ruthenium	45 Rhodium	46 Palladium	47 Silver	48 Cadmium
	Atomic weight	92.9064	95.94	[99]	101.07	102.9055	106.42	107.8682	112.411
	Density	8.58	10.22	11.50	12.36	12.42	12.00	10.50	8.65
	Θ(K)	275	450		600	480	274	225	209
Cr *Ka*	2.2910	4.16E+02	4.42E+02	4.74E+02	5.01E+02	5.36E+02	5.63E+02	6.02E+02	6.26E+02
Cr Kb_1	2.0849	3.25E+02	3.45E+02	3.70E+02	3.92E+02	4.20E+02	4.41E+02	4.72E+02	4.90E+02
Fe *Ka*	1.9374	2.67E+02	2.84E+02	3.05E+02	3.23E+02	3.46E+02	3.63E+02	3.89E+02	4.05E+02
Fe Kb_1	1.7566	2.05E+02	2.19E+02	2.35E+02	2.49E+02	2.67E+02	2.81E+02	3.01E+02	3.13E+02
Co *Ka*	1.7903	2.16E+02	2.30E+02	2.47E+02	2.62E+02	2.80E+02	2.95E+02	3.16E+02	3.29E+02
Co Kb_1	1.6208	1.66E+02	1.76E+02	1.90E+02	2.01E+02	2.16E+02	2.27E+02	2.43E+02	2.53E+02
Cu *Ka*	1.5418	1.45E+02	1.54E+02	1.66E+02	1.76E+02	1.89E+02	1.99E+02	2.13E+02	2.22E+02
Cu Kb_1	1.3922	1.10E+02	1.17E+02	1.26E+02	1.34E+02	1.44E+02	1.51E+02	1.63E+02	1.69E+02
Mo *Ka*	0.7107	1.77E+01	1.88E+01	2.04E+01	2.17E+01	2.33E+01	2.47E+01	2.65E+01	2.78E+01
Mo Kb_1	0.6323	8.10E+01	1.38E+01	1.49E+01	1.58E+01	1.70E+01	1.80E+01	1.94E+01	2.02E+01

Θ: Debye temperature, Unit of density: Mg/m^3.

Characte-ritic radiation	Wavelength (Å)	17 Chlorine	18 Argon	19 Potassium	20 Calcium	21 Scandium	22 Titanium	23 Vanadium	24 Chromium
	Atomic weight	35.4527	39.948	39.0983	40.078	44.9559	47.867	50.9415	51.9961
	Density	3.214E-03	1.663E-03	0.862	1.53	2.99	4.51	6.09	7.19
	Θ(K)		92	91	230	360	420	380	630
Cr *Ka*	2.2910	3.16E+02	3.42E+02	4.21E+02	4.90E+02	5.16E+02	5.90E+02	7.47E+01	8.68E+01
Cr Kb_1	2.0849	2.44E+02	2.66E+02	3.28E+02	3.82E+02	4.03E+02	4.44E+02	4.79E+02	6.70E+01
Fe *Ka*	1.9374	2.00E+02	2.18E+02	2.70E+02	3.14E+02	3.32E+02	3.58E+02	3.99E+02	4.92E+02
Fe Kb_1	1.7566	1.52E+02	1.67E+02	2.07E+02	2.42E+02	2.56E+02	2.77E+02	3.09E+02	3.85E+02
Co *Ka*	1.7903	1.61E+02	1.76E+02	2.18E+02	2.55E+02	2.69E+02	2.91E+02	3.25E+02	4.08E+02
Co Kb_1	1.6208	1.22E+02	1.34E+02	1.66E+02	1.95E+02	2.06E+02	2.27E+02	2.50E+02	2.93E+02
Cu *Ka*	1.5418	1.06E+02	1.16E+02	1.45E+02	1.70E+02	1.80E+02	2.00E+02	2.19E+02	2.47E+02
Cu Kb_1	1.3922	7.95E+01	8.75E+01	1.09E+02	1.29E+02	1.37E+02	1.52E+02	1.66E+02	1.85E+02
Mo *Ka*	0.7107	1.15E+01	1.28E+01	1.62E+01	1.93E+01	2.08E+01	2.34E+01	2.60E+01	2.99E+01
Mo Kb_1	0.6323	8.20E+00	9.14E+00	1.16E+01	1.38E+01	1.49E+01	1.68E+01	1.87E+01	2.15E+01

Θ: Debye temperature, Unit of density: Mg/m^3.

The value of x determines the ϕ(x) according to the table 4 here:

$$\varphi(x) = \frac{1}{x}\int_0^x \frac{\xi}{e^{\xi} - 1} d\xi \tag{8}$$

Where, x = $\frac{\Theta}{T}$, Θ: Debye temperature (7)

Table 4. Values of φ(x) as a function of x [78]

x	.0	.1	.2	.3	.4	.5	.6	.7	.8	.9
0	1.000	0.975	0.951	0.928	0.904	0.882	0.860	0.839	0.818	0.797
1	0.778	0.758	0.739	0.721	0.703	0.686	0.669	0.653	0.637	0.622
2	0.607	0.592	0.578	0.565	0.552	0.539	0.526	0.514	0.503	0.491
3	0.480	0.470	0.460	0.450	0.440	0.431	0.422	0.413	0.404	0.396
4	0.388	0.380	0.373	0.366	0.359	0.352	0.345	0.339	0.333	0.327
5	0.321	0.315	0.310	0.304	0.299	0.294	0.289	0.285	0.280	0.276
6	0.271	0.267	0.263	0.259	0.255	0.251	0.248	0.244	0.241	0.237

For x >7, ϕ(x) values are approximately 1.642/x.

In case of Ag, x is $\frac{225}{293}$ = 0.767918 (225 is Debye temperature and 293 is room temperature). ϕ(x) is therefore 0.8247.

Therefore, all together the B_T value is,

$$B_T = \frac{1.15 * 10^4}{M} * \frac{T}{\Theta^2} * \left(\varphi(x) + \frac{x}{4}\right) \quad (6)$$

$$\Rightarrow B_T = \frac{1.15*10^4}{107.87} * \frac{293}{225^2} * \left(0.8247 + \frac{0.766918}{4}\right)$$

= 106.6098*0.00578765*1.0164295 = 0.62716

$$e^{-2M_T} = e^{-2B_T\left(\frac{sin\theta^2}{\lambda}\right)}$$

$$\Rightarrow e^{-2B_T\left(\frac{sin\theta^2}{\lambda}\right)} = e^{-2*0.62716*\frac{sin19.07^2}{1.542495}} = e^{-0.08679} = 0.9168696$$

So, all together:

$$R = \left[|F|^2 * p * \left(\frac{1 + \cos^2 2\theta}{2sin^2\theta * cos\theta}\right)\right]\frac{e^{-2M_T}}{\Omega^2} \quad (4)$$

$$\Rightarrow R = [21\,036.6 * 8 * (8.0216)] * \frac{0.91668696}{0.068418^2} = 264\,366\,098.\,618\,918$$

Then, R value is calculated with both phases in the same plane. The volume fraction can be found:

$$\frac{C_{Ag}}{C_{AgCl}} = \frac{I_{Ag} * R_{AgCl}}{I_{AgCl} * R_{Ag}} \quad (3)$$

$$where, C_{Ag} + C_{AgCl} = 1$$

Note: Volume fractions for powder samples that were examined by the XRD are shown in the Table 8 in the "Results section".

5 Results

The first part of the thesis summarizes the results obtained during the first stage and published in articles III-VI [2,3]. Certain results of these publications are highlighted here to complete the description of the research or emphasize on certain aspects of this research. The second part describes in detail the results obtained with the biosynthesized Ag nanopowder. This part also includes unpublished results. These results complete the discussion and outcomes of the thesis. They also highlight aspects that need to be further investigated in order to gain a deeper understanding of the biocidal properties of the biosynthesized Ag nanopowders.

5.1 Antimicrobial evaluation of silver nanoparticles against common fungi and bacteria colonizing ecological building materials – Stage one study

The antimicrobial properties of silver nanoparticles synthesized by non-aqueous sol-gel routes were first studied (articles III – VI) [2,3]. The physical and chemical properties were investigated along with the evaluation of their toxicity. The biocidal properties of these Ag MNP were studied against fungi and bacteria *in vitro* and also *ex vitro* with outdoor tests on straw bales. For outdoor testing, we needed to develop a methodology that enabled us to evaluate the efficiency of Ag MNP as micro-organism repellent (article IV) [2,3].

5.1.1 Characteristics of silver nanoparticles synthesized via non-aqueous sol-gel method

The synthesis of silver nanoparticles by non-aqueous sol-gel routes using acetate precursor showed a very good yield of 95.15 ± 5%. During the synthesis, 482 mg of silver acetate precursor mixed with benzylamine solvent enabled the production of 296.7 mg of surfactant-free Ag MNP for a theoretical weight of 311.4 mg for production yield of 100%. The production yield could be estimated with accuracy because CHN and TGA studies highlighted that Ag MNP are surfactant free and only carbon originating from air contamination could be detected. CHN measured a carbon content of 0.072 weight% (see below). XRD pattern of the produced Ag MNP showed a high crystallinity with sharp and intense diffraction peaks, and Ag MNP exhibit a face-centered cubic structure (JCPDS File No 87-0720) (Figure 4). The Scherrer method was applied to the XRD pattern, and an average crystallite size of 58 nm was estimated.

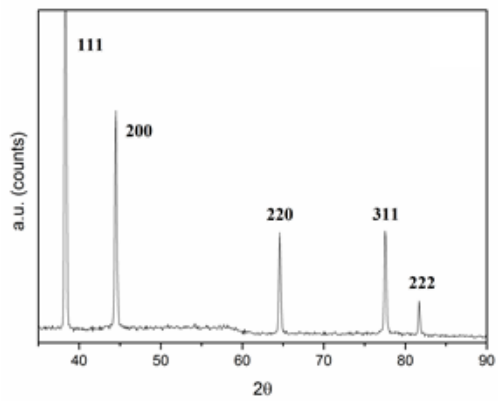

*Figure 4. XRD pattern of Ag MNP synthesized by non-aqueous sol-gel method using silver acetate precursor and benzylamine. The figure is adopted from **Article IV** with permission [2].*

HRTEM study demonstrated that the nanoparticles are spherical with a size ranging from 5 nm to 20 nm (see figure 5a), which validates the utilization of the Scherrer equation for the estimation of Ag MNP size (article VI)[79]. A Slightly larger nanoparticle of 10 nm is also visible on the HRTEM image of figure 5b oriented along the <110> zone axis of the Fm-3m cubic structure with a lattice parameter of 0.4 Å [2,3]. TEM study highlights the natural agglomeration of the Ag MNP and the simultaneous presence of larger Ag MNP, which would explain the higher nanoparticle size estimation from the Scherrer equation. The agglomeration tendency is certainly promoted due to the fact that these Ag MNP are surfactant free (article VI). Their natural agglomeration tends to decrease their surface energy[80].

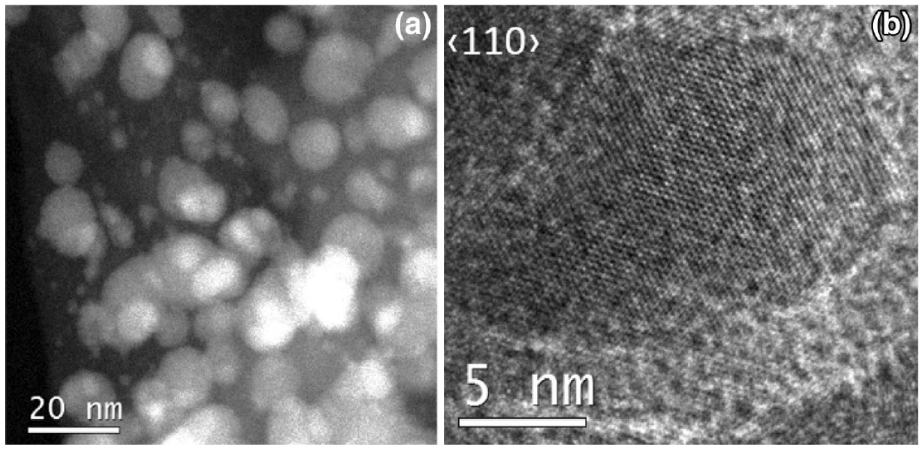

*Figure 5. (a) Scanning transmission electron microscope (STEM) image overview of Ag nanoparticles dispersed on a carbon grid, (b) HRTEM image of Ag nanoparticle oriented along [110] zone axis. The figure is adopted from **Article III** with permission [3].*

TGA was performed in order to evaluate silver nanoparticle stability against oxidation and estimate the presence of organic species adsorbed on the Ag MNP surfaces after synthesis. TGA measurements are shown in figure 6 and no weight loss nor gain during the whole measurement from room temperature to 800ºC is observed. This confirms that the produced Ag MNP are surfactant-free and stable under air even at very high

temperatures. **Differential thermal analysis (DTA)** shows a slight decrease of the heat capacity (-0.4mW/mg) with the increase of the temperature that could be attributed to a probable aggregation of the Ag MNP together during the heat treatment.

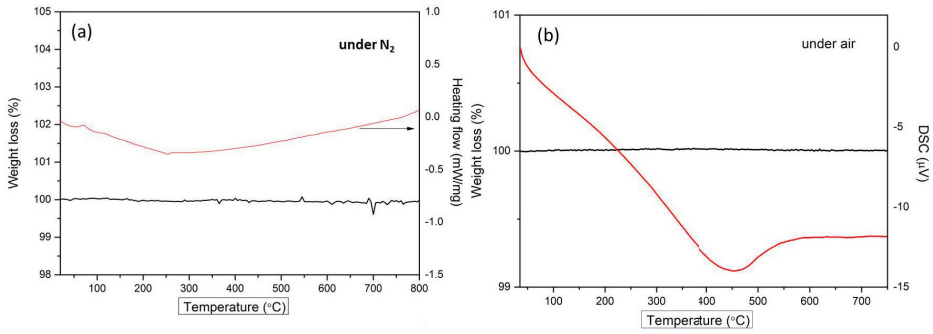

*Figure 6. TGA (black line) and DTA (red line) performed on Ag MNP (a) under N_2 and (b) under air. The figure is adopted from **Article III** with permission [3].*

Carbon-hydrogen-nitrogen-sulfur (CHNS) measurements were performed to evaluate the cleanliness of the Ag MNP and to detect possible remaining organic species on their surfaces. After analyzing 2.032 mg of Ag MNP, 0.072 weight % of carbon and 0.016 weight % of nitrogen was measured; neither hydrogen nor sulfur was detected. This analysis suggests that carbon and nitrogen detected are mainly adsorbed from air.

XPS measurements were performed on surfactant-free Ag MNP to study the nature of their surface and confirm their purity [79]. XPS study was performed on Ag MNP samples that were stored for 6 months in the powder form under air ambient. Figure 7 presents the XPS survey spectra, showing only binding energy peak from Ag metal [81,82] and no visible peak of nitrogen (see inset) or oxygen is visible confirming the CHNS measures. The carbon binding energy peak can be attributed to probable air contamination that cannot be avoided. Due to the nanosize of the Ag MNP and the high surface to volume ratio, any oxidation of the Ag MNP surface would have been detected by XPS analysis. The position of the Auger peak corresponds here to metallic silver. In addition, the Ag $3d_{5/2}$ photoelectron peak corresponds to metal Ag (368.3 eV), thus confirming the purity and the metallic nature of the synthesized Ag MNP.

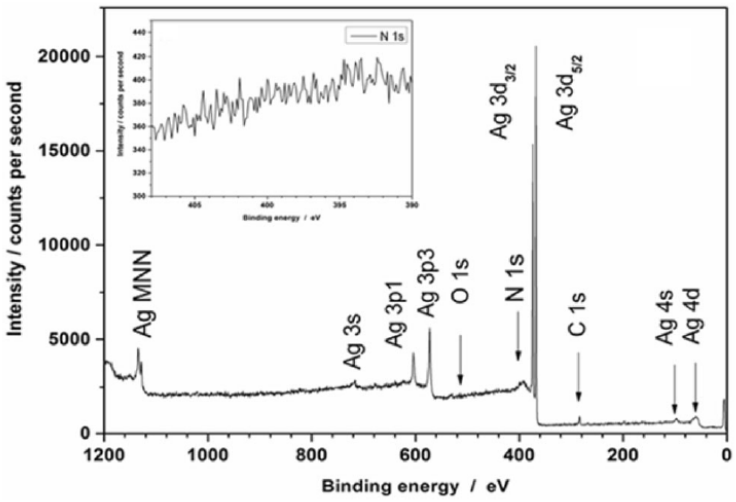

*Figure 7. XPS survey of Ag MNP, inset shows the absence of N binding energy peak. The figure is adopted from **Article III** with permission [3].*

5.1.2 Antimicrobial study of Ag MNP synthesized via non-aqueous sol-gel routes

5.1.2.1 Identification of fungi and bacteria

On the rye (*Secale cereale*) straw several microorganisms were identified. Rye straw was selected as a source for the identification of microorganisms and testing the antimicrobial properties of Ag MNP because this species of crop is commonly used in green housing in certain regions [83].

Results of Gram-staining and microscopy studies revealed that genera of Gram-positive *Streptomyces* and Gram-negative *Pseudomonas* were predominantly colonizing the straw. In case of fungi, *Cladosporium*, *Penicillium* and *Aspergillus* genera were identified on untreated straw samples (article V, Table 5, Figures 7 and 8) [4]. The antimicrobial properties were then investigated against 2 different bacterial strains: *Streptomyces, Pseudomonas,* and 3 different fungal strains: *Cladosporium*, *Penicillium* and *Aspergillus*.

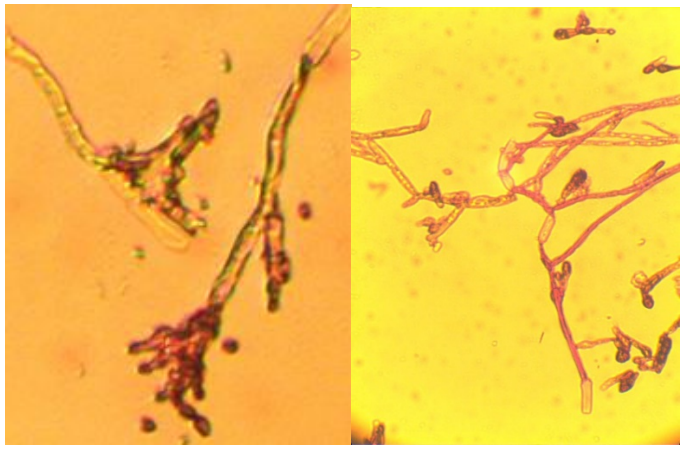

*Figure 8. Photo of (a) Penicillium spp and (b) Cladosporium spp. The figure is adopted from **Article III** with permission [3].*

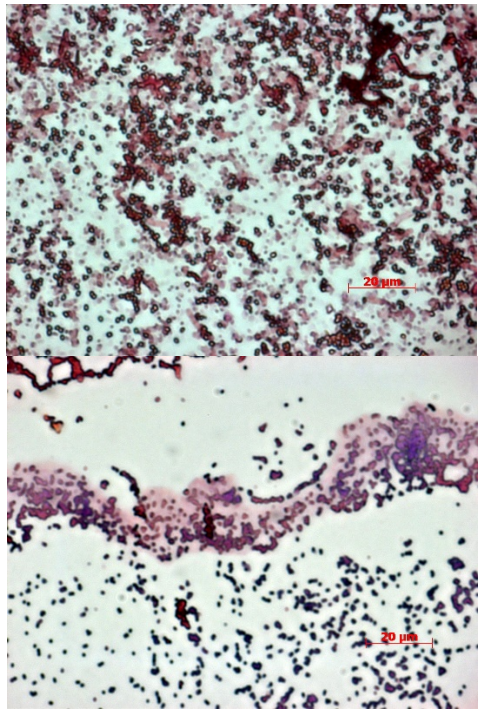

Figure 9. Photo of Gram-negative Pseudomonas spp and Gram-positive Streptomyces spp bacteria.

Preliminary work (some extent published in article V) [4] showed that Ag MNP inhibit the growth of the whole spectrum of microorganisms when applied on the surface of the straw (Table 5). These findings confirm the preliminary hypothesis that Ag MNP can prevent the proliferation of fungi on straw surfaces, and it motivated the continuation of the investigations with bio-synthesized Ag MNP.

Table 5. Dominant microorganisms found and identified in studied straw bales [4].

	Untreated wet straw	**Straw with ethanol**	**Straw with ethanol silver nanoparticle dispersion 100mg/L**
Genera of fungi	*Aspergillus, Penicillium, Cladosporium*	*Aspergillus*	No fungal activity
Genera of bacteria	*Streptomyces, Pseudomonas*	*Pseudomonas*	*Streptomyces, Pseudomonas*

5.1.2.2 Biocidal study

The first stage of the research was carried out according to the information gathered during preliminary study (article V) [4].

Tests in petri dishes were initially performed using the droplet method on isolated cell cultures. Ag MNP were dispersed in water and ethanol with different concentrations. Before adding the droplet in the petri dish, the Eppendorf tube was agitated for 30

seconds in order to obtain a homogeneous dispersion of the nanomaterials in the solution and an amount of solution was taken immediately in order to prevent the nanoparticles from settling down at the bottom of the tube. Since Ag MNP are surfactant-free, they do not form colloids in water or ethanol, so they immediately drop down to the bottom of the tube. A droplet of solution was added to the petri dish in order to verify inhibitory effects on nutritious agar medium under ideal conditions for isolated selected cultures. In paper V [4], we showed that typical straw colonizing mold *Aspergillus* spp. was affected by Ag MNP dispersed in ethanol and distilled water dispersions with a concentration of 50 and 100 mg/L (figure 10a). Similarly, antibacterial tests performed on agar plates containing Streptomyces spp. showed a clear inhibition zone for an Ag MNP concentration of 100mg/L in ethanol (figure 10b).

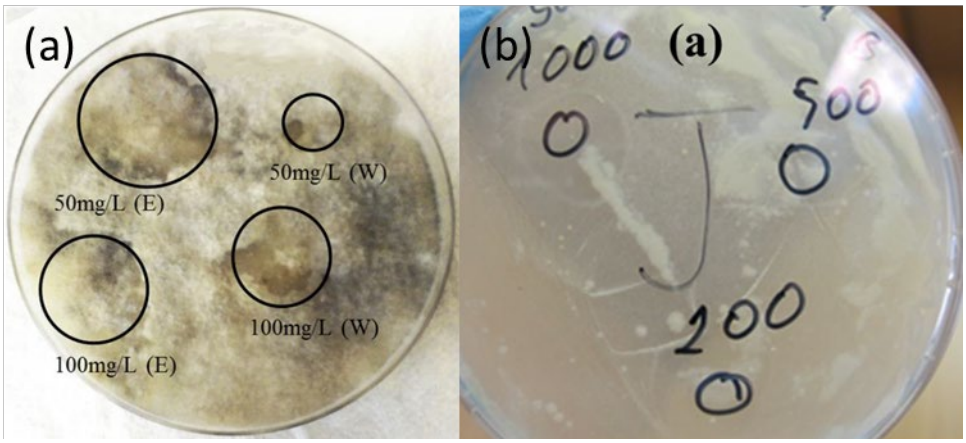

Figure 10. (a) Antifungal tests performed on Aspergillus spp. with Ag MNP dispersed in water (W) and ethanol (E), (b) antibacterial tests performed on Streptomyces spp with Ag MNP dispersed in ethanol and showing inhibition zone for 100mg/L (marked as 1000). The figure is adopted from ***Article V*** *with permission [4].*

Fungi and bacteria cultured in favorable conditions (warm and humid on highly nutritious agar medium) were affected by the presence of Ag MNP. In order to evaluate the minimum concentration needed to completely prevent the proliferation of colonies, the testing medium had to be changed so that nanoparticles would distribute evenly across the medium, which is more difficult on agar plates. In order to evaluate the biocidal properties of Ag MNP against fungi thoroughly, the liquid broth media and isolated *Aspergillus* culture was selected for the tests.

The test was based on broth dilution assays, where different concentrations of Ag MNP were added and dispersed into the liquid broth medium that contained the ingredients for *Aspergillus* spp. growth and proliferation (See full description in the article IV) [2]. Figure 11 shows the photos of reference and liquid broth media that contain the four different concentrations of Ag MNP (i.e., 100mg/L, 400mg/L, 800mg/L and 1000mg/L). The color difference is mainly due to the higher concentration of Ag MNP in the solution. Figure 11 shows that the development of *Aspergillus* spp. is visible in all samples for the various Ag MNP concentrations used. The poor effect against the development of *Aspergillus* spp. fungi was attributed to the reaction between amino acids present in the broth liquid medium that have chains of sulfur groups and the metallic surface of Ag MNP that is surfactant-free. In fact, sulfur groups have a good

affinity to the silver metal surface and the biocidal properties of the Ag MNP appear to have been shielded by the bonding of these sulfur groups on the surface of the Ag MNP whereupon, preventing any silver ion release or direct contact with *Aspergillus* spp.

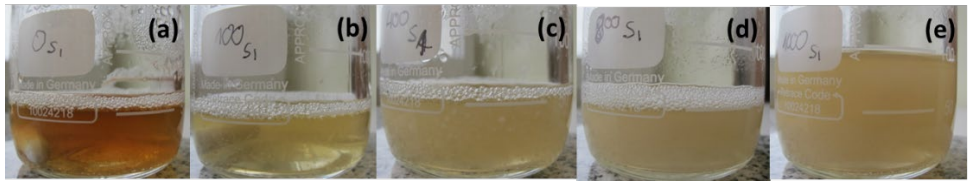

Figure 11. Micro broth dilution assays test on Aspergillus spp. (a) reference (b) 100mg/L (c) 400mg/L (d) 800mg/L (e) 1000mg/L of Ag MNPs. The figure is adopted from ***Article IV*** *with permission [2].*

Even though high concentrations (1g/L) had some slight effect on the isolated fungal culture (Figure 11e) this study shows that these nanomaterials cannot be used in liquid medium that contains organic molecules that exhibit an affinity to surfactant-free Ag MNP. It is then important to either functionalize the Ag MNP or synthesize the Ag MNP using another method to avoid any bonding with the organic molecule present in the liquid broth medium. To study the biocidal properties on straw itself, a new testing methodology was further developed in order to directly test on the building material itself in more real environments (outdoor tests).

For this reason, the decoration of straw samples for building material applications followed by outdoor storage in order to facilitate colonization of bacteria and fungi were investigated. The straw samples were prepared and decorated with Ag MNP through the method fully described in article IV [2]. The small straw bale samples were immersed into the vessel that contained two different concentrations of Ag MNP homogeneously spread in the vessel using a magnetic stirrer. The amount of Ag MNP was calculated as a function of the weight of the straw immersed in the solution. Two ratios of 2.5 mg/g and 1 mg/g were more particularly investigated. Treated straw bales were stored outdoors for two weeks: then, straw samples were taken from each bale and imprinted in petri dishes in order to check the presence of living organisms that possibly developed on their surface. Figure 12 shows the photos of the petri dishes 72 hours after imprint.

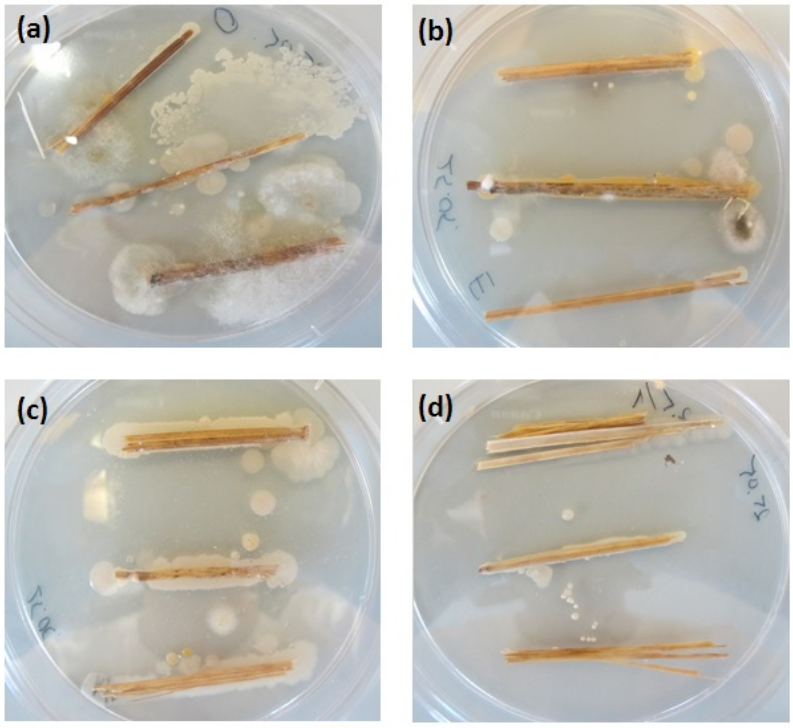

*Figure 12. Straw samples that were first stored for 2 weeks outdoor and then imprinted in petri dishes for 72 hours at 32°C on PCA plate: (a) no treatment; (b) ethanol; (c) 1mg/g; (d) 2.5mg/g. The figure is adopted from **Article III** with permission [3].*

The tests showed that the straws were partially protected, even during outdoor storage. The method we developed showed reliability in terms of testing the Ag MNP with a visible repelling effect *in situ*. Photographs of the petri dishes in figure 12 indicate antifungal and antibacterial properties of the nanoparticle coating added on the straw surface. Bacteria seem less affected by the coating than fungi, mostly because more bacterial colonies are present than fungal colonies (structurally distinguishable from flatter bacteria). In reality, molds tend to dominate over bacteria and are therefore more of a threat to house residents [2,3]. The higher antimicrobial activity against fungi appears to be an asset for such applications.

5.1.3 Toxicity tests of surfactant-free pure silver nanoparticles

For applications in green housing construction, it is important to also check the possible toxicity of the nanomaterials (here, Ag MNP) against human cells. In order to evaluate their toxicity and confirm their possible use as repellents against common microorganisms on building materials, toxicity tests against human cells were also performed. These tests can confirm the utilization of Ag MNP for applications in which direct contact with people will produce no adverse effects.

The cytotoxicity of Ag MNP was studied on HEK (human embryonic kidney) cells. Different concentrations of Ag MNP dispersed in PBS solution were studied by MTT assay. Figure 13 shows MTT assays performed on Ag MNP solutions of concentration ranging from 5 µg/L to 200 mg/L. Figure 13 shows the mean ± SEM (standard error of mean) of duplicate measurements of a representative sample of three independent experiments. This toxicity study towards HEK cells shows that mortality rate is over 50% only for very

high concentrations of Ag MNP (200 mg/L). Ag MNP exhibit no toxicity for 0.5 mg/L (Table 6) and considerably low toxicity up to 100 mg/L, which makes them a suitable nanomaterial for antimicrobial and antifungal agents in green housing [2,3].

Table 6. Result of MTT assay test [2,3].

Concentration of Ag MNPs	0	5 μg/L	50 μg/L	0.5 mg/L	5 mg/L	50 mg/L	100 mg/L	200 mg/L
Cells survivability	0.4680	0.4420	0.4167	0.4197	0.3627	0.3313	0,2457	0.1427

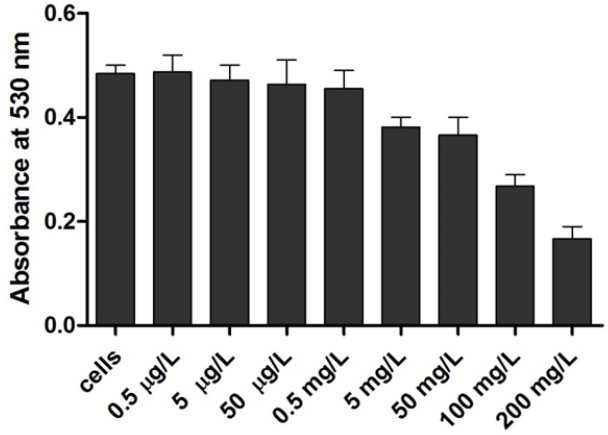

*Figure 13. Toxicity test of Ag MNP against HEK293 cells. The figure is adopted from **Article III** with permission [3].*

5.2 Developing new plant-mediated synthesis methods for Ag/AgCl nanoparticle production and optimization of the synthesis – Stage two study

5.2.1 Design of simpler and more environmentally friendly antimicrobial nanoparticle syntheses

Green housing by definition is linked to the pragmatism of sustainability. Therefore, when developing antimicrobial nano-coatings for building materials, the type of precursor for the nanomaterial synthesis is of utmost importance. Additionally, the choice of the plant should be motivated by its availability and abundance in its geographical location. Straw Bale buildings treated with silver nanoparticles produced from mango peel [84] in North-Eastern Europe region cannot be considered as a sustainable option. However, if the plant is common in the area of construction, is either a weed or leftover product, then it is ideal for the implementation of this technology. Considering this, various plant extracts were examined to meet these criteria. Plantago major is a common plantain in Estonia and, it is considered as a weed. Therefore, it was a good candidate for the production of nanomaterials.

Two main methods of Ag MNP bio-synthesis were developed through optimization. Figure 14 shows a visual comparison between two types of Ag MNP synthesized using 2

different methods. Firstly, heat-induced synthesis using Plantago major extract (extracted from the fresh plant material by boiling it for 30 minutes) with silver nitrate precursor without any exposure to UV radiation (Type A) was carried out. The second method is based on UV-mediated synthesis using Plantago major extract that was extracted from the fresh plant with the help of ethanol and later dissolved in distilled water and mixed with silver nitrate. The resulting solution was then exposed to UV-irradiation to promote the Ag MNP synthesis (Type B).

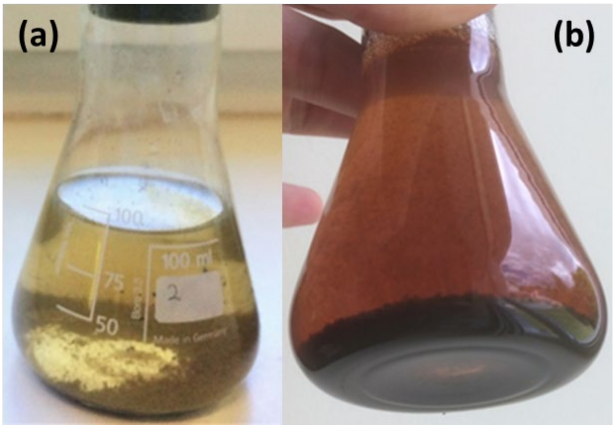

Figure 14. Solution of Ag nanoparticles after synthesis (a) type A synthesized in aqueous medium at 85ºC, (b) type B synthesized under sunlight. The figure is adopted from ***Article I*** *with permission [53].*

5.2.2 Characterization of Ag MNP synthesized using Plantago major extract

The XRD patterns in figure 15 show the typical face centered cubic (fcc) silver metal structure and they highlight the presence of silver chloride (AgCl) as secondary phase. Cubic Ag metal nanoparticle structure exhibits diffraction peaks at 38.15º, 44.30º, 64.52º, 77.42º that correspond to 111, 200, 220 and 311 reflections respectively (JCPDS card number 04-0783). The diffraction peaks at 27.88º, 32.26º, 46.25º, 54.85º, 57.50º, 67.51º, 74.45º, 77.40º correspond to 111, 200, 220, 311, 222, 400, 331, 420 and 422 reflections of the cubic silver chloride structure (JCPDS card number 31-1238)[85]. In the case of bio-synthesized nanoparticles, the presence of both Ag MNP and AgCl NP were clearly identified from the XRD patterns.

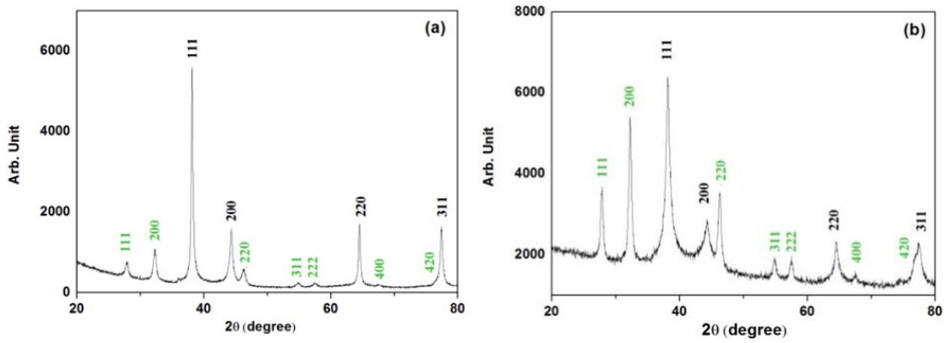

Figure 15. XRD patterns of Ag NP synthesized (a) in aqueous medium at 85ºC (type A – GSW003), and (b) under sunlight (type B – GS003). XRD peaks of Ag MNP and AgCl NP are indexed in black and green respectively. The figure is adopted from ***Article I*** *with permission [53].*

The XRD patterns in figure 15 show that synthesis methods and conditions highly influence Ag/AgCl secondary phase ratio and size of nanomaterials. The difference in peak intensities and broadness is clear. XRD patterns were treated in order to calculate weight and volume percentages of both Ag MNP and AgCl NP phases (quantitative analysis). XRD study showed that type A synthesis conditions favor the production of silver metal nanoparticles; whereas, type B synthesis method promotes the synthesis of silver chloride phase in larger amounts. However, with both syntheses, the amount of each phase is significant and neither of the phases can be neglected. The amount of each phase is reported in the figure 16. It can also be seen that the method of synthesis also influences the size of the produced nanoparticles. The average size of Ag and AgCl nanoparticles were estimated using Scherrer equation applied to 111, 200, 220 and 311 diffraction peaks.

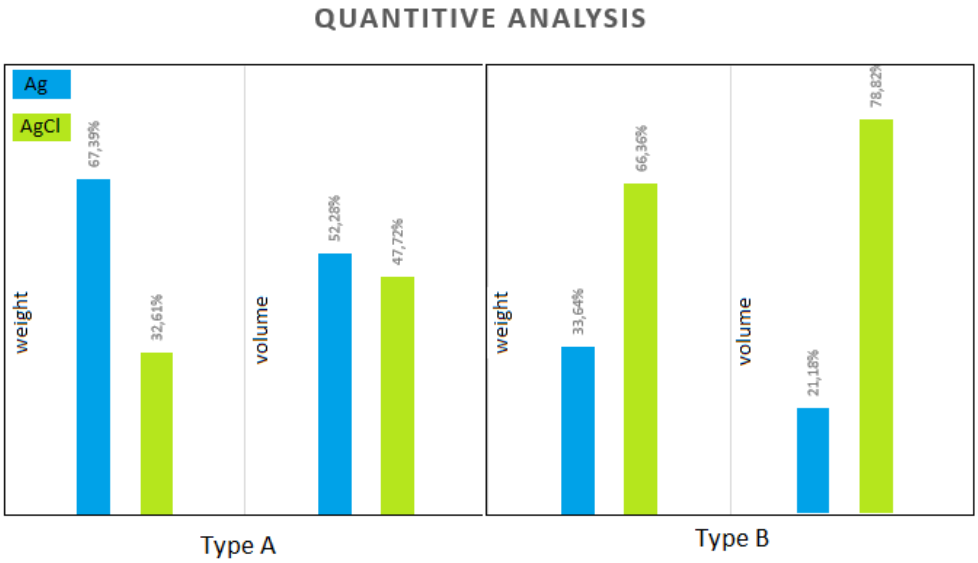

*Figure 16. Type A and type B Ag/AgCl nanoparticles weight and volume percentages based on the XRD data. The figure is adopted from **Article I** with permission [53].*

The morphology of the nanoparticles produced using the two types of synthesis (A) and (B) were studied by SEM (Figure 17) and the micrographs show spherical morphology with agglomeration of the nanoparticles. SEM study shows that Type A exhibit larger agglomerates (100 nm) than Type B where agglomerate sizes range from 50 to 70 nm of diameter. XRD also highlighted that Type B nanoparticles are smaller than Type A, which certainly explains the bigger agglomerates for Type A.

TEM study was also performed and confirms the larger size of Type A nanoparticles and the larger agglomerates that could be observed with SEM (Figure 18). TEM study illustrates that Type B nanoparticles are smaller and exhibit narrower size distribution with an average size of 10-20nm. TEM study therefore revealed a narrower size distribution of Type B nanoparticles compared to Type A nanoparticles. However, the TEM study could not discriminate Ag MNP from AgCl nanoparticles (Figure 18).

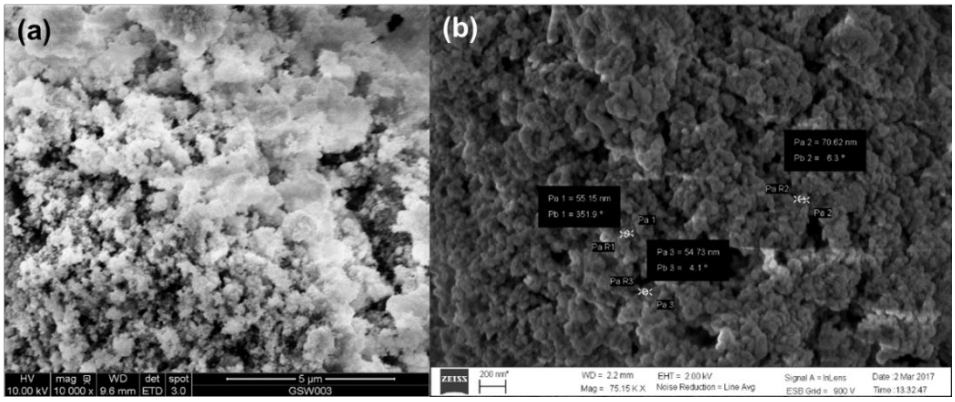

*Figure 17. SEM micrographs of Ag MNP and AgCl NP synthesized (a) in aqueous medium at 85ºC (Type A - GSW003), and (b) under sunlight irradiation (Type B – GS003). The figure is adopted from **Article IV** with permission [53].*

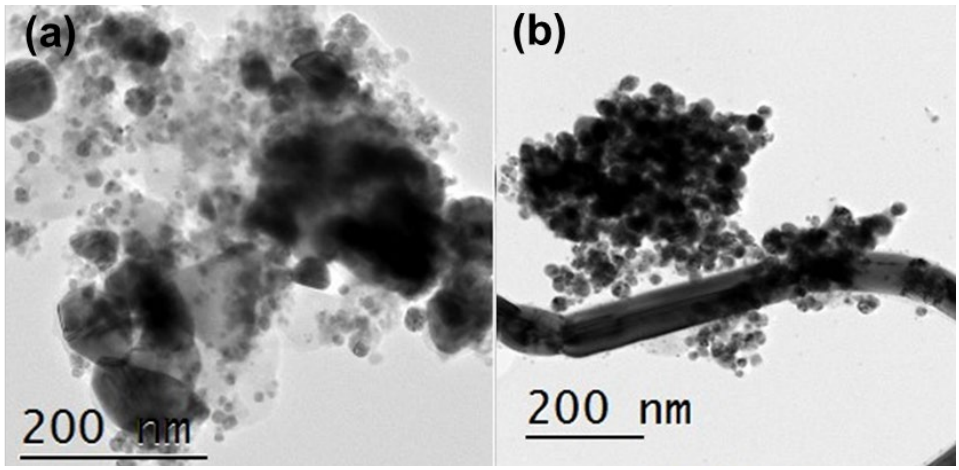

*Figure 18. TEM micrographs of Ag MNP and AgCl NP synthesized (a) in aqueous medium at 85ºC (Type A – GSW003), and (b) under sunlight (Type B – GS003). The figure is adopted from **Article IV** with permission[53].*

Nevertheless, XRD patterns from figure 15 show that XRD peaks from AgCl structure are broader than XRD peaks from Ag MNP structure. If we consider from TEM study that all nanoparticles are spherical, XRD patterns highlight that AgCl are smaller than Ag MNP. The smallest nanoparticles from the TEM images can then be identified as AgCl nanoparticles.

5.2.3 Detailed comparison of different Plantago major extract induced syntheses

Scherrer equation is usually applied to polycrystalline samples and thin films to evaluate crystallite size, however it can also be applied to estimate the average size of spherical nanoparticles with a good accuracy. Scherrer equation was applied to all the XRD patterns in order to compare their estimated size[86]. Even though several samples had Ag with varying nanoparticle sizes, the general tendency of size increase or decrease can be obtained by applying the Scherrer equation to higher order crystal planes. 9/10 samples particle size was measured also with SEM or/and TEM to get more accurate information.

Table 7. Different synthesis routes with Plantago Major plant extract. GS - Green synthesis based on ethanol extraction. GSW - Green synthesis based on boiled plant extract (85°C).

Sample	Plantago Major extract information	Synthesis information	Characterization information
GS001	Type B extraction. 24g fresh plant material.	Type B synthesis. 50ml extract + 50ml nitrate solution. Instant synthesis at room temp.	AgCl phase dominant. XRD: 20.7(10.1) nm. SEM: 26-50 nm
GS002	Type B extraction. 24g fresh plant material.	Type A synthesis. 50ml extract + 50ml nitrate solution. Duration 1h at 70°C.	AgCl phase dominant. XRD:23.1(11.7) nm. SEM: 40-80nm.
GS003	Type B extraction. 24g fresh plant material.	Type B synthesis. 50ml extract + 50ml nitrate solution. Duration 2h at room temp.	AgCl phase dominant. XRD:13.1(5.8) nm. SEM:55-70nm. TEM: 5-40 nm.
GS004	Type B extraction. 24g fresh plant material.	Type A synthesis. 50ml extract + 50ml nitrate solution. Duration 1h at 100°C.	AgCl phase dominant. XRD:14.2(5.5) nm, Intensity lower. SEM: 33-43nm.
GS005	Type B extraction. 26g fresh plant material.	Type B synthesis. 50ml extract + 50ml nitrate solution. Instant synthesis at room temp.	AgCl phase dominant. SEM: 16-27nm
GS0062	Type B extraction. 24g fresh plant material.	Type A synthesis. 100 ml extract + solid silver nitrate (same weight). Duration of 1 h at 85°C.	AgCl phase dominant. XRD: 47.4 (10.8) nm. SEM - micrometer range agglomerates
GS0064	Type B extraction. 24g fresh plant material.	Type B synthesis. 100 ml extract + solid silver nitrate (same weight). Instant synthesis at room temp.	AgCl phase dominant, XRD: 40.4 (20.2) nm. SEM - micrometer range agglomerates
GSW003	Type A extraction. 50g fresh plant material.	Type A synthesis. 100 ml extract + solid silver nitrate (same weight). Duration of 1 h at 85°C.	Ag phase dominant. XRD. 32.1 (8.2)) nm. SEM: 100 nm. TEM: 5-100 nm.
GSW004	Type A extraction. 6.56g dried plant material (from 50g fresh).	Type A synthesis. 100ml extract + solid silver nitrate (same weight). Duration of 1 h at 85°C.	AgCl phase dominant. XRD. 24.5 (6.9) nm. SEM: 50-200 nm. TEM 10-200 nm
GSW005	Type A extraction. 14.76g dried plant material (from 100g fresh).	Type A synthesis. 100ml extract + solid silver nitrate (same weight). Duration of 1h at 85°C. Production yield stayed the same.	AgCl phase dominant. XRD. 23.8 (12.8) nm.

Type A extraction - Thermal extraction
Type B extraction - Ethanol extraction
Type A synthesis - Heat induced synthesis
Type B synthesis - Sunlight induced synthesis
Sample used in antimicrobial assay

Table 8. Sample description obtained from XRD study (estimates).

Sample	Weight ratio (Ag/AgCl)	Ag nanoparticle size (nm)	AgCl nanoparticle size (nm)
GS001	22/78	9,54	28,14
GS002	21/79	10,13	31,70
GS003	34/66	8,61	18,98
GS004	21/79	7,72	17,47
GS062	12/88	48,83	46,56
GS064	21/79	10,37	50,38
GSW003	67/33	24,96	37,16
GSW004	27/73	25,13	24,07
GSW005	25/75	14,63	29,45

Determination of Ag/AgCl ratio and nanoparticles' average size for syntheses using ethanol extraction:

GS001/GS003 – sunlight induced ethanol extraction synthesis (Table 7 and 8).

- GS001 weight ratio 22.20 % / 77.80 %; Size 9.54 / 28.14 nm
- GS003 weight ratio 33.64 % / 66.36 %; Size 8.62 / 18.98 nm

Plant-extract prepared using ethanol extraction mainly promotes the synthesis of AgCl phase. It is also clearly visible that produced Ag nanoparticles are of smaller size. This means that the plant-extract preparation method most probably contains a higher amount of chlorine that will promote AgCl formation. The Ag/AgCl ratio also shows that the duration of activation under sunlight (i.e., time during which the precursors are exposed to sunlight) is also an essential factor that promotes the formation of the silver metal phase in the mixture. In fact, a longer synthesis under sunlight promotes silver nanoparticle phase. In addition, the average size of the AgCl nanoparticles also decreased for longer synthesis durations (i.e. from few seconds to few hours under sunlight). It could indicate that the increased UV-radiation is capable of degrading AgCl and consequently, Ag nanoparticles are formed.

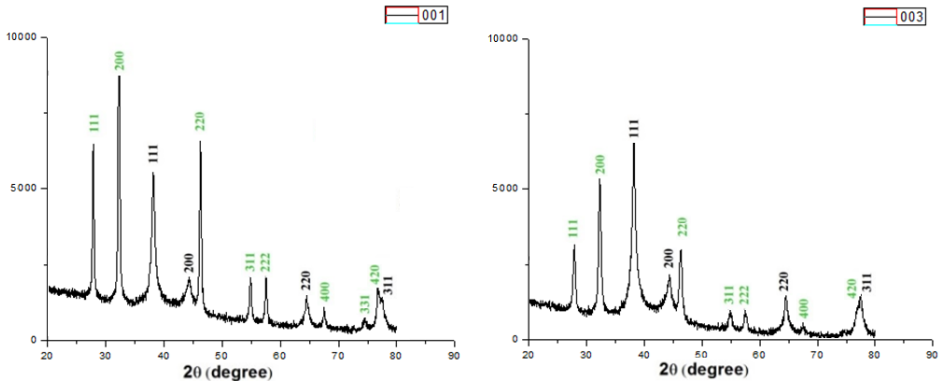

Figure 19. GS001 and GS003 XRD diffraction patterns (Table 7 and 8).

GS002/GS004 – ethanol extraction in pressure cooker synthesis (70-100°C) (Table 7 and 8)

- GS002 weight ratio 20.99 % / 79.01 %; Size 10.13 / 31.70 nm
- GS004 weight ratio 20.86 % / 79.14 %; Size 7.72 / 17.47 nm

Experiments demonstrate that thermal energy activation (heating in pressure cooker) instead of sunlight (UV) still promotes the synthesis of AgCl phase over Ag metal nanoparticles. It confirms the probable presence of chlorine being a key factor that promotes the formation of AgCl, even if using ethanol-assisted plant extracts as precursor. Similarly, to type A, Ag metal nanoparticles are of smaller sizes than AgCl nanoparticles. In these experiments, we studied the influence of synthesis temperature; in fact, an increase in temperature, increases reaction kinetics. One observed that higher temperatures induce the formation of smaller nanoparticles for both Ag and AgCl nanoparticles. The nanoparticle size decreases from 10.13(min)/31.70(max) nm to 7.72(min)/17.47 (max) nm when temperature increases from 70 to 100°C. The reason could be that quicker reaction kinetics promote the formation of several nucleation sites that can grow rather quickly and hinder the coagulation process. The coagulation of the nanoparticles could be also prevented by the presence of plant extracts that act as stabilizers/surfactants. Conversely, at lower temperatures, the growth process is sufficiently slow and encourages the formation of larger nanoparticles.

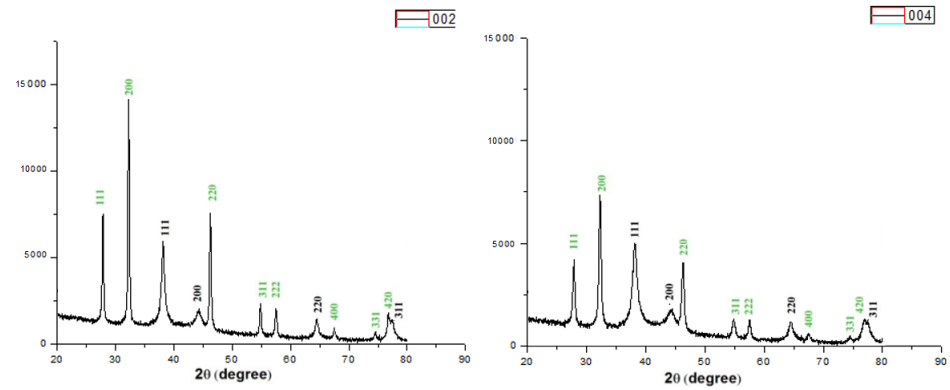

Figure 20. GS002 and GS004 XRD diffraction patterns (Table 7 and 8).

GS062 and GS064 (light/dark) ethanol extraction (control synthesis) (Table 7 and 8)

- GS0062 heat induced (without sunlight) weight ratio 11.60% / 88.40%; Size 48.83 / 46.56 nm
- GS0064 sunlight induced weight ratio 20.82% / 79.18 %; Size 10.37 / 50.38 nm

This control synthesis was made from identical plant material and extract (ethanol extraction) in order to understand the effect of activation energy (UV vs heat) on Ag/AgCl ratio and nanoparticle size. These experiments highlight that UV radiation is more favorable for the formation of pure silver nanoparticles than thermal energy, as also suggested in the literature [74]. However, in both cases (heat and UV), the main phase that is produced is AgCl. These experiments also confirm that UV radiation (sunlight) promotes the growth of smaller Ag nanoparticles for the same plant extract. However, in the case of AgCl growth, the nanoparticles have the same size. It suggests that the formation mechanisms of Ag and AgCl are most probably different, i.e., two different growth mechanisms plausibly occur simultaneously.

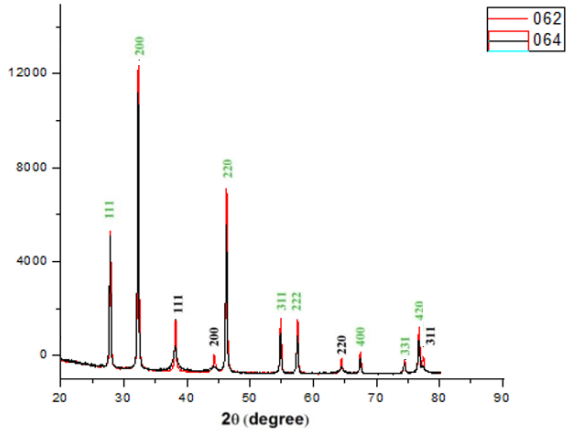

Figure 21. Overlapping XRD diffraction patterns of GS062 and GS064 syntheses (Table 7 and 8).

Determination of Ag/AgCl ratio and nanoparticles' average size for thermal extraction (boiled) and heat induced syntheses (with fresh plant leaf extract) (Table 7 and 8).

- GSW003 weight ratio 67.39% / 32.61%; Size 24.96 / 37.16 nm

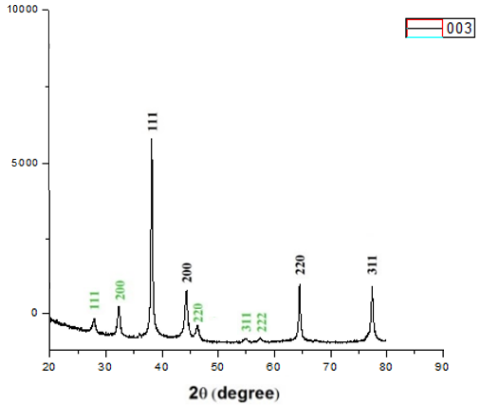

Figure 22. XRD diffraction pattern for the sample GSW003. (Table 7 and 8).

The only nanopowder sample that contains more Ag nanoparticles than AgCl nanoparticles was the sample grown using plant extract prepared with boiled fresh plant. It suggests that plant extract prepared by this method contains a lower amount of chlorine and the formation of Ag nanoparticles is then promoted against AgCl. The sample is composed of 67.39 % of Ag metal nanoparticles. It therefore implies that ethanol promotes a higher extraction of chlorine from the plant parts during the preparation of the extract. Similarly, preparing plant extract by boiling dried plant parts also promotes a higher chlorine content in the plant extract. This shows that the plant extract preparation has also an important influence on the synthesis itself. Reports on green synthesis method using plant extract usually do not investigate the influence of plant extract preparation. The focus of the study is to understand the effect of the different parts of the plant used for preparing extracts on the nanoparticle precipitation. The utilization of plant extract prepared with boiled fresh leaves plant also promotes the formation of larger Ag nanoparticles compared to the other syntheses, but their size remains under 25 nm, which is reasonable for the targeted application. It most probably indicates a slower reaction process that promotes the growth of bigger Ag nanoparticles. The size of Ag and AgCl nanoparticles are 24.96 (min) nm / 37.16 (max) nm respectively, compared to 10 (min) nm/30 (max) nm for the ethanol extracted syntheses.

Determination of Ag/AgCl ratio and nanoparticles' average size for syntheses using boiled dried plant extract and thermal energy (pressure cooker):

- GSW004 weight ratio 26.78% / 73.22%; Size 25.13 / 24.07 nm
- GSW005 weight ratio 25.00% / 75.00%; Size: 14.63 / 29.45 nm

In the case of heat activated plant-extract preparation using dried plant, the main phase synthesized is AgCl. These experiments show that plant extract prepared in this way certainly contains more chlorine than fresh plant parts. In these syntheses, the higher amount chlorine present in the plant extract again most probably promotes the formation of AgCl. It means that to promote the formation of Ag nanoparticles, it is important to use fresh plant during the plant extract preparation. It suggests that chlorine is more stable in fresh plant parts than dried ones and remains captured within the solid part of the plant during the boiling process. To promote the formation of AgCl nanoparticles, dried plant should be used for the preparation of the plant extract. During the experiments, different amounts of plant for the extract (concentration) were used. It can be seen from the experiments that a higher concentration only affects the size of Ag nanoparticles and does not change Ag/AgCl ratio. Ag nanoparticles size decreases with the increase of plant extract concentration from 25.13 nm (50 g of fresh plant before drying) to 14.63 nm (100 g of fresh plant before drying).

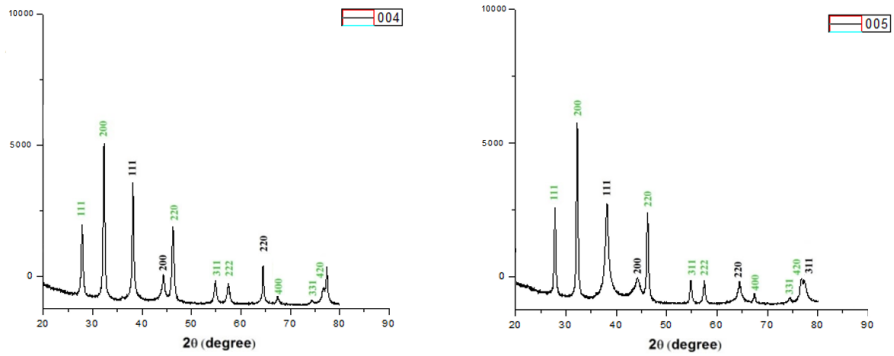

Figure 23. XRD diffraction patterns for the samples GSW004 and GSW005. (Table 7 and 8).

5.2.4 Summary of silver nanoparticle syntheses using Plantago major extract

These experiments showed that the plant extract preparation method strongly influences the size of the produced nanoparticles and the Ag/AgCl ratio. The main parameter for promoting the Ag metal phase is the use of fresh Plantago Major plant in distilled water for the preparation of the plant extract that will be used for the bio-synthesis of nanoparticles. Thereafter, thermal energy should be used for the synthesis of Ag nanoparticles at a temperature of 85°C. In fact, ethanol extracted plant extract combined with sunlight activation, as well as plant extract prepared using dried plant with distilled water combined with heating, both promote the formation of the AgCl phase due to a higher content of chlorine in the plant extract itself.

The comparison of nanoparticles produced using sunlight or thermal energy shows that the utilization of thermal energy produces larger nanoparticles in general. However, increasing the temperature from 70°C to 100°C stimulates the production of smaller nanoparticles. Nevertheless, due to the utilization of water as solvent, it was not possible to apply a higher temperature than 100°C during the synthesis.

Compared to thermal energy activation, sunlight activation promotes the synthesis of smaller Ag nanoparticles (~10 nm of diameter). In all syntheses performed, AgCl nanoparticles were bigger than Ag nanoparticles (~20-50 nm) and were more abundant. The amount of AgCl was only significantly reduced in the case of plant extract prepared by boiling fresh plant parts. To understand the influence of the sunlight activation we compared with different illumination durations ranging from a couple of seconds to 2 hours. In fact, UV radiation from sunlight boosts Ag nanoparticle reduction on the surface of larger AgCl particles with time. Some studies reported that Ag@AgCl nanocomposites can also form due to the sensitization of AgCl nanoparticle surface through high-energy UV light exposure that promotes Ag shell formation [74].

5.2.5 Antimicrobial study of green silver/silver chloride nanoparticles

The samples are composed of a mixture of both Ag MNP and AgCl nanoparticles with different ratios. It was not possible to separate both types of nanomaterials from each other. Therefore, we could only evaluate the antimicrobial properties of the mixture of the nanomaterials for all samples knowing the Ag/AgCl ratio. Silver and silver chloride nanoparticles antimicrobial properties studies are well documented [30,87]. However, in some studies the presence of AgCl as secondary phase was completely neglected by the authors for unknown reasons. Nevertheless, in our investigations, we have considered the presence of both phases, as they are intrinsic to the biosynthesis method itself. It is noteworthy that AgCl NP also exhibit antimicrobial properties. This is the reason why the amount (i.e., Ag/AgCl ratio) was estimated for all samples as they may have an effect on the efficiency of the antimicrobial properties. This comparison could highlight which phase exhibits the highest antimicrobial properties, as it has never been clearly investigated before. For this reason, GSW003 (Type A) and GS003 (Type B) samples have been selected with an Ag/AgCl ratio of (67/33) and (34/66), respectively.

To compare these results with literature, model organisms were chosen for the assays. Antibacterial tests were carried out according to the ISO 20776-1 standard protocol and results of both types of silver / silver chloride nanoparticles are presented in Table 9.

Table 9. Antibacterial assay performed on Ag NPs and AgCl NPs synthesized under sunlight (type A – GSW003) and in aqueous medium at 85ºC (type B – GS003). [53]

	Type of nanoparticles	MIC µg/mL	MBC µg/mL
E. coli	Type A Ag/AgCl nanoparticles	1.6	3.1
	Type B Ag/AgCl nanoparticles	1.6	3.1
S. aureus	Type A Ag/AgCl nanoparticles	0.8	0.8
	Type B Ag/AgCl nanoparticles	0.8	0.8

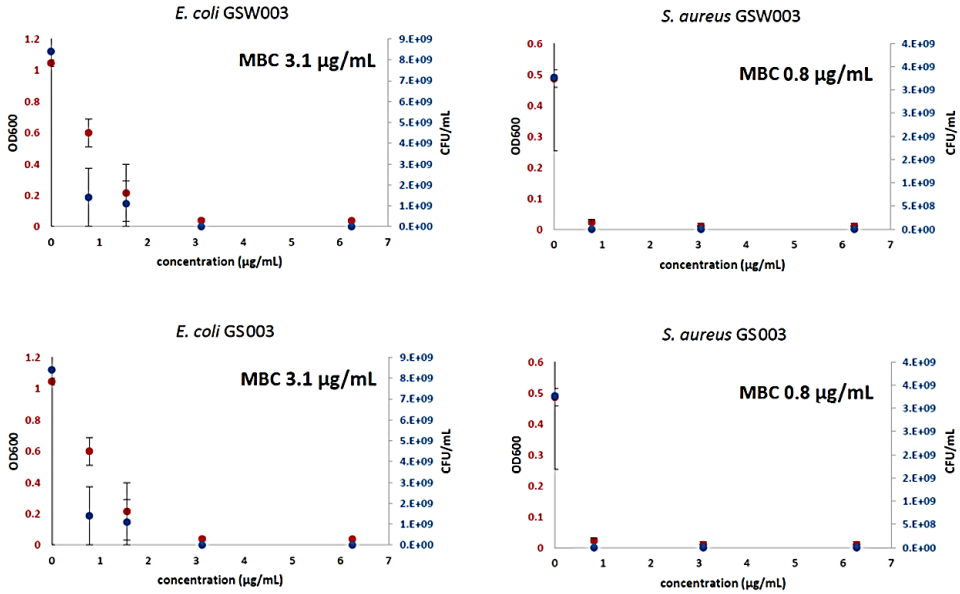

Figure 24. GSW003 and GS003 test results (ISP 20776-1 standard protocol).

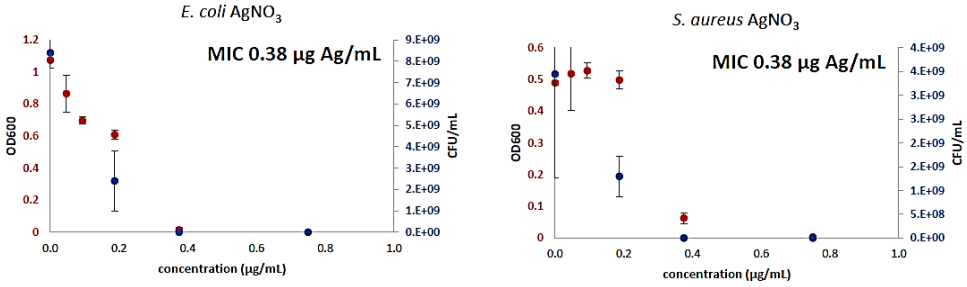

Figure 25. Control with $AgNO_3$ against Gram-positive S. aureus and Gram-negative E. Coli MIC value matched with MBC value.

The minimum inhibitory concentration (MIC) corresponds to the lowest quantity of nanoparticles needed to prevent the visible growth of bacteria and colonies. The minimum bactericidal concentration (MBC) corresponds to the lowest quantity of nanoparticles required to kill all bacteria. Both samples showed equally potent antibacterial properties for 2 different ratios. No deduction could be made about the Ag/AgCl ratio influence and it suggests that both nanomaterials exhibit very similar antimicrobial properties when synthesized using Plantago major extract. The control tests made with $AgNO_3$ show that the antimicrobial properties of the bio-synthesized nanoparticles are lower than $AgNO_3$. However, a longer lasting effect can be expected from the utilization of bio-synthesized silver nanoparticles due to a higher stability of the silver nanomaterials (Ag and AgCl) compared to $AgNO_3$ on surfaces.

Antifungal tests against yeast model organism *S. Cerevisi*ae spp. were performed to evaluate the antifungal properties of the bio-synthesized Ag NP and are shown in Figure 26. Methodology was adapted from the Suppi et al. [75]. From this antifungal test, the inhibiting concentration for isolated fungal culture starts with 10mg/L and killing concentration from 30 mg/L.

Culture densities were measured with UV-spectrometer and the values are indicated in the left column. More specifically, values of 1.20, 1.17 and 1.19 correspond to the UV-Vis cell density at 600 nanometers (OD600 nm). If the value is 1, then the medium contains 1.3 x 10^7 cells / mL. Susceptibility to nanoparticles is clearly dependent on the number of the cells in the culture medium. Numbers of cells that are tested originate from the methodology described Suppi et al. [75]. $AgNO_3$ was used as reference as it is known to kill *S. Cerevisiae* spp. with a concentration of 100 mg/L. The comparison shows that Type A and Type B nanoparticles exhibit similar antifungal properties, despite having a different Ag/AgCl ratio. The antifungal properties need further investigation on other fungal strains in order to better understand the influence of AgCl on the antifungal properties of silver nanomaterials synthesized under different conditions.

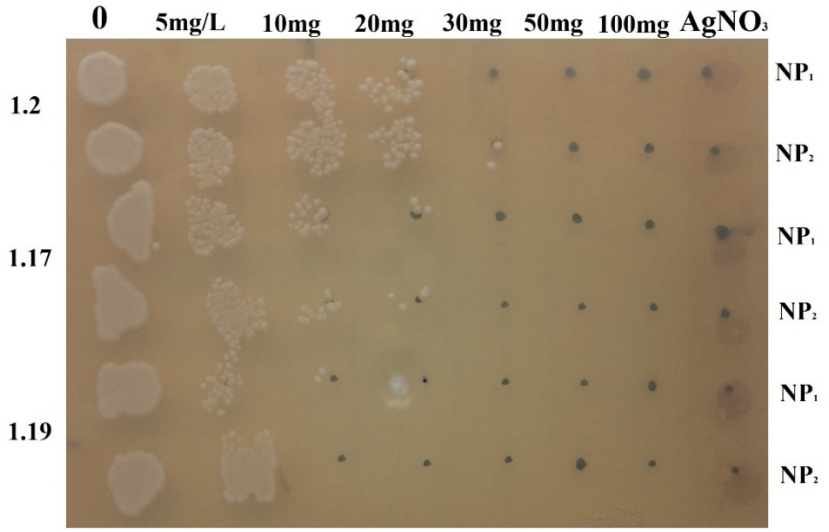

*Figure 26. Antifungal test with S. Cerevisiae with type A – GSW003 (NP1) and type B – GS003 (NP2). The figure is adopted from **Article I** with permission [53].*

6 Discussion

The antimicrobial properties of Ag MNP synthesized using non-aqueous sol-gel routes were first investigated (articles III – V) [2,3,4]. Ag MNP were applied as repellent on individual straws and straw bales, and their biocidal properties against both fungi and bacteria through *in vitro* and outdoor tests where demonstrated. For the outdoor tests, a new methodology (articles IV) [2] to study the antimicrobial and antifungal properties in real conditions had to be developed. More specifically, the aim of these tests was to show that it is possible to hinder the microbial and fungal activity on indoor finished materials used for ecological construction such as straw bales, even in the hardest conditions. Therefore, initially most common bacteria, yeasts and fungi were gathered from the straw intended for construction material. The rye (*Secale cereale*) straw was selected due to the fact that this crop is one of the most commonly used straw in green housing [83] in Estonia. Following the positive outcome of the study, investigations with bio-synthesized Ag MNP were then assessed. The utilization of plant extracts as a medium to synthesize Ag MNP that exhibit similar or higher antimicrobial properties due to a synergistic effect originating from a combination of the intrinsic properties of the Ag MNP with the properties of the plant extract itself used for the synthesis. Plantago major was selected for its availability in Estonia and its known antimicrobial properties [53,64]. After the successful syntheses of Ag MNP, the antimicrobial assays were carried out again to evaluate the potential of the bio-synthesized nanoparticles as an antimicrobial repellent for green housing (article I) [53] or other similar applications.

The antimicrobial mechanism exhibited by Ag MNP is most probably a combination of multiple effects on microorganisms that can occur simultaneously or separately. The main identified mechanisms are (i) silver ions release, (ii) direct contact due to the large surface to volume ratio between Ag MNP and bacteria that damages the bacterial membrane; and (iii) uptake of the Ag MNP by the microorganism. When Ag MNP encounter the microorganism cell membrane, they tend to disturb their permeability and respiration in some cases. The MNP's positive charge is very strong can interaction with the cell wall, which will damage the wall and increase the permeability of the cells, increasing the penetration of nanoparticles into the bacteria (cellular intake). As an outcome, functions inside internal functions of the cell are disrupted. Simultaneously, reactive oxygen species (ROS) (O^{2-}, OH^{-}, H_2O_2, O_2) are generated that are toxic to bacteria and can damage the DNA replication and respiratory chain of the cell, resulting in death of the microorganism. These ROS species can also be produced outside the cells (ion diffusion) and penetrate indirectly the bacteria through their cell walls. Considering Ag MNP that were used in the first stage research (articles III-V) [2,3,4] were surfactant free and pure, the most probable biocidal mechanism is the direct contact with the microorganism combined with silver ion release. Ion release is certainly the most efficient as it will induce the denaturation of the proteins inside the microorganism through the production of reactive oxygen species; the blockage of DNA functions, the alteration of enzymatic processes and damage to the cellular membrane. The investigations (articles III to V) [2,3,4] showed that fungi seem more affected by pure Ag MNP compared to bacteria. However, due to the chitin cell wall absent in bacteria, fungi should exhibit a better resistance to nanoparticle biocidal agents. However, bacteria have the ability to grow a biofilm around the colony in aggressive environments (outdoor tests) that will protect them from external threats. The biofilm protects them from direct contact and Ag ions will diffuse in lower quantities or with more difficulty

into the biofilm. Therefore, the bacteria were able to protect themselves from the surfactant-free Ag MNP. This hypothesis was confirmed by the *in vitro* tests. In petri dishes, there is no bacterial stress response because of the lack of competition over food supplies with other microorganisms. Therefore, the biocidal test in petri dishes showed that Ag MNP affects the proliferation of bacteria more efficiently than in outdoor tests (articles III-V) [2,3,4].

Fungal colonies need less favourable conditions to spread and therefore tend to dominate over bacteria *ex vitro*, or in real conditions. In damp buildings, health problems are mainly caused by moulds. The main issue is that they remain nocuous even after their death. The fungal spores persist after eradication of the colonies and on their release, growth of new colonies is observed. These fungal spores themselves are noxious and cause health problems. Moreover, fungi do not need external humidity to survive as their life cycle itself provides the necessary humidity for their growth. Consequently, it is more judicious to prevent fungal proliferation than combat existing colonies using different chemicals. To that end, the scientific outcomes from articles III to V demonstrate the potential of Ag MNP as protective coating on construction materials [2,3,4].

In vitro laboratory testing revealed some challenges that appear to be specific to metal nanoparticles, when applied in antimicrobial testing. For isolated cultures, one of the most reliable methods is to use micro broth dilution assays [88]. However, paper IV results showed that in nutritious fungal media (2% of malt extract and 0.02% of chloramphenicol) surfactant-free Ag MNP interact with the organic molecules that compose the liquid broth medium, most likely with sulfur groups of amino acids in malt extract due to a high affinity of Ag metallic surfaces to sulfur groups [2]. Similarly, in agar plates, if droplet tests or well diffusion is used, the porous agar that contains similar organic molecules can hinder the effect of Ag MNP and reduce their antimicrobial and antifungal properties. Therefore, directly treating the straw proved to be the most accurate method, as the straw could be later printed on the agar to study the Ag MNP hindrance capability on the microorganism growth. Furthermore, treated straw can be tested in harsh environmental conditions mimicking the actual storing conditions of the construction material, or the condition after wall damage that can induce the proliferation of microorganism in straw bales used for the house insulation. The study has shown that in the case of ideal growth conditions or in laboratory conditions (high nutrient availability and warm humid environment), a higher concentration of the antimicrobial agent is needed to hinder the growth of both fungi and bacteria. Additionally, an important aspect to consider here is the visual representation of the results using the treated straw. Fungi in fact can also spread through conidia and spores, therefore the spread on the agar plate can be misleading, as the straw surface can be clean; however, around the straw the growth is visible. Therefore, the surface of the material itself has to be observed carefully in order draw accurate conclusions on the effect of the nanoparticles. Also, the area where the nanoparticle repellent is applied should be cleaned (see figure 4 of Appendixes in article V) [4].

Ag MNP perform well as suitable agents in antimicrobial repellents for green housing when coated homogeneously on the targeted surface, such as straw, providing a complete protection. If the antimicrobial agent is applied to the protected surface by spraying, the dispersion has to be extremely homogenously spread out. During the thesis, one observation was that Ag MNP in water or ethanol or some other solvent tend to agglomerate and sediment, as illustrated in papers I to V [2,3,4,53]. However, if the material that needs to be protected/coated is inside a container, where the nanoparticles

are in suspension and free to circulate, the contact time with the active nanomaterial increases and the coating is more homogeneous regardless of their agglomeration. Test results in article IV showed that (in smaller scale, where straw bale samples were approximately 10 times smaller than the standard straw bale used for construction) the methodology using a magnetic stirrer could be effectively implemented for the protection of the material [2]. The two presumed modes of microorganisms infecting straw are first, during storage, if they are not properly protected from rain or humid ground. Second, after the building is already constructed moisture enters through cracks in the wall. The experiment was designed according to the first hypothesis, but can be extrapolated to the second hypothesis. Treated straw bale samples were stored outside to mimic real-life conditions. After several tests, one assessed that such a method could be applied as a basis for developing a standard protocol for this type of testing. These tests were combined with standard antimicrobial and antifungal tests in a lab environment enabling us to compare our results with literature reports, as our methodology is the first of its kind.

To justify the use of nanoparticles for protecting ecological building materials as an alternative to the conventional preparation of silver nanoparticles through chemical routes had to be examined. As mentioned in the thesis conventional synthesis methods require several chemicals and specific conditions for proper synthesis. Chemical synthesis usually induces the production of toxic by-products and some of these can remain attached on the surface of the nanoparticles. Green synthesis principles have been established in 1998 by Anastas and Warner[60], and bio-synthesis using plant extract applies most of these principles. For example, using plant extracts of locally available plants to reduce silver ions into pure metal nanoparticles without producing toxic by-products makes it also cost-effective. The plant extracts should consist of reducing agents, as well as stabilizing and capping agents. One additional advantage is the possible synergy that can be obtained during the synthesis. The biosynthesized metal nanoparticles will be functionalized during the synthesis through the attachment of active biomolecules from the plant extract, and their intrinsic properties are combined with those of the synthesized nanomaterial, which will enhance the properties of the biosynthesized nanoparticles compared to conventionally produced nanoparticles. The economic aspects are also interesting, as plant extracts are cost-effective to produce and the production process using plant extracts can be easily scaled up due to its simplicity compared to conventional alternatives. [12] The additional experiments suggest that the Plantago Major extract can indeed be reused and no significant drop in yield after initial synthesis of Ag MNP was witnessed.

As stated above the Plantago major or the Great Plantain was selected for its availability in Estonia and as its well-known antimicrobial properties. At the time these experiments were carried out, no research group in the world had published a report on this plant for silver nanoparticle synthesis. During the thesis, several options were tested for the biosynthesis of the Ag MNP. After the synthesis of Ag MNP via Plantago major was successful, the optimization of the synthesis itself was focused on obtaining Ag MNP with the highest antimicrobial properties and understanding the influence of the method of synthesis on the ratio Ag/AgCl in the sample. The aim of the investigations was to focus on the synthesis itself with one selected plant and investigate the effect of plant extract preparation methods on the properties of the synthesized Ag MNP. In addition, different energy sources for catalyzing the chemical reactions were studied (i.e., thermal energy and UV light from sunlight). For this reason, no further plants after the great plantain

were investigated more profoundly; even though, some of them showed potential for nanoparticle bio-synthesis. For example, plant extracts prepared from nettles (Urtica dioica and Lamium album) showed the possibility to produce nanoparticles that were visually very similar to the ones produced with the Great Plantain. In addition, other plants were tested for the biosynthesis of Ag MNP, such as birch leaves, Norway maple leaves, red clover, yarrow and dandelion plants, but these syntheses did not produce the desired outcomes.

Our investigations showed that the utilization of Plantago major plant extract produces two different nanoparticles: Ag and AgCl. This is predominantly the case in plant extract mediated silver nanoparticle syntheses. In many reports, the presence of AgCl nanomaterials in biosynthesized Ag MNP is neglected for unknown reasons. The presence AgCl nanomaterials in the XRD pattern is usually considered as either a crystallization of bioorganic phase on the surface of the Ag MNP. Additionally, this secondary phase in the diffraction pattern is simply being ignored as a casual error (figure 27). This is especially the case when the peak intensities are low and AgCl nanoparticles are smaller than Ag MNP. Usually, AgCl nanoparticles are present in the Ag MNP samples synthesized using plant extract due to the presence of chlorine in this extract, more specifically if the extract comes from the plant leaves. AgCl nanoparticles are easy to synthesize and they can be produced using $AgNO_3$ and KCl [89]. Similarly to Ag MNP, AgCl nanoparticles exhibit biocidal properties and studies report their possible use in antimicrobial membranes [90]. AgCl nanomaterials have also been investigated for the development of wound dressing [91] due to its antimicrobial properties through its slow release of silver ions [92]. It is now well established that green synthesis using plant extract can induce the production of AgCl nanoparticles as secondary phases (figure 27). However, the improved biocidal effect of AgCl NPs against microorganisms through a synergistic combination with Ag has to be further investigated and demonstrated. This research is presently under investigation by the same research group that contributed to this thesis.

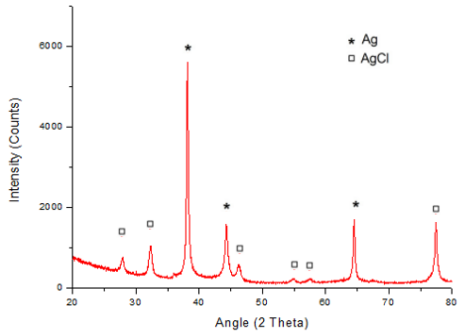

Figure 27. XRD of a typical plant mediated synthesis of silver and silver chloride nanoparticles.

In this current study, AgCl nanoparticle secondary phase is clearly visible in all samples, but are more predominant in Type-B synthesis (ethanol extracted plant + sunlight induced synthesis). In almost all samples, XRD peaks of Ag fcc structure have lower intensity and a broader full width at half maximum (FWHM) compared to XRD peaks of AgCl structure. It highlights that Ag metal nanoparticles are of smaller size than AgCl nanoparticles, which confirms the results published in reports using other plant extracts [49,93]. As stated in paper I, depending on the method of producing the plant extract and synthesis conditions, the ratio of Ag MNP and AgCl nanoparticles is different [53].

It was found that the same plant extract can produce nanoparticles of different sizes with tunable Ag MNP/AgCl NP ratio depending on the synthesis conditions and plant extract preparation. This outcome can be of interest in the field of green synthesis using plant extracts if specific characteristics or Ag/AgCl NP ratios are needed. The investigations of this research showed that it is possible to promote the synthesis of one phase over the other.

Ag nanoparticles and their intrinsic properties are well studied; however, AgCl nanoparticles have several properties themselves that make them unique and valuable in different applications. Ag@AgCl are mainly known as plasmonic photocatalysts that have in addition to antimicrobial properties also promising applications in water remediation as this composite material can decompose hazardous organic compounds [74]. The antimicrobial as well as decomposing effect can occur under visible light due to the surface plasmon resonance of silver nanoparticle component of these composites. Due to the very low solubility of silver halides in general (AgCl 1.77×10^{-10}) this material is durable in aquatic environments, therefore also safer for habitats compared to pure silver [74]. This possible application in the field of water purification needs to be further investigated due to the eco-friendly method of production and the possibility to scale-up the Ag nanoparticle production [12].

This research shows that Plantago major can be used to produce nanoparticles that are effective against several microorganisms colonizing ecological construction and finishing materials. However, no distinguishable difference in antimicrobial effect was noted on comparing nanopowders with Ag as the main phase and AgCl as the main phase. Therefore, it suggests that there may be not just one main active agent in Ag@AgCl nanoparticles that contributes to antimicrobial properties, but rather a potential synergy between Ag, AgCl and Plantago major molecules. In all the cases silver nanoparticles (Ag/AgCl mixture) exhibit efficient biocidal properties against bacteria and fungi, with higher toxicity against bacteria. For the sol-gel synthesized silver nanoparticles, the hypothesis was that the main anti-microbial mechanism of Ag MNP on the microorganism is the direct contact with the microorganism combined with silver ion release. However, with plant-mediated nanoparticles, plant molecules on the surface could also contribute to the antimicrobial properties. Since results of two very different Ag/AgCl nanoparticles were so similar against bacteria and fungi, it substantiates that Plantago major being the common element can itself play a significant role in the antimicrobial mechanism.

7 Conclusions

Green housing is an expanding topic that is becoming more and more relevant in the present as an energy efficient solution. It is only a matter of time until public interest grows, thereupon turning green materials into one of the most important aspects when constructing homes or producing items. Since the entire value chain has to be considered along with a life-cycle analysis, the origin of the raw materials therefore is of capital interest in order to reply to the sustainability of the processes involved. Therefore, researchers, scientists and engineers are encouraged to prioritize their raw materials and production methods in order to promote the Green Industry initiative of the EU Green Deal. Green housing as well as greenly synthesized nanoparticles both transpire sustainability.

Results of this study indicate and confirm that silver and silver halide nanoparticles indeed are suitable candidates for protecting construction materials and finishing materials from proliferation of microorganisms. Moreover, plant mediated synthesis is an optimal route for producing antimicrobial nanoparticles used in ecologically friendly fields such as green housing and other sustainable engineering areas due to the environmentally friendly origin of these nanoparticles.

Based on the research outcomes, the following conclusions can be drawn:

- Straw as an ecologically friendly construction material is a suitable environment for many common bacteria and fungi including Gram-positive bacteria *Streptomyces* spp., Gram-negative bacteria *Pseudomonas* spp., and fungal strains like *Cladosporium* spp., *Penicillium* spp. and *Aspergillus* spp and more.

- Surfactant free silver nanoparticles demonstrate antimicrobial properties when tested *in vitro*. However, the culture medium containing amino acids with sulfur groups had high affinity with the pure silver nanoparticle surfaces that shield their antimicrobial properties. It was therefore necessary to develop a more accurate testing method.

- Method for testing silver nanoparticles against microorganisms on real construction materials was developed to evaluate the protective effect of antimicrobial silver nanoparticles on straw. After 20 days outdoor, the coated straws were then printed on the universal PCA plates to evaluate the microbial development and hindering effect was visible on the coated straw with a lower development of microorganism (starting from the concentrations of 1 mg/ g).

- "Green" silver nanoparticles synthesis via a suitable ecologically friendly method was developed and optimized for producing different nanoparticles with the same reagents (e.g., plant extract and silver nitrate) by altering the plant extract preparation and synthesis conditions.

- Biosynthesized silver metal and silver chloride nanoparticles demonstrated antimicrobial properties against model organisms. A concentration of 30 µg/mL of nanoparticle was sufficient to prevent the development of *Saccharomyces cerevisiae* spp. Lower concentration of 3.1 µg/mL and 0.8 µg/mL of nanoparticle were sufficient to kill *Escherichia coli* spp. (Gram-negative) and *Staphylococcus aureus* spp. (Gram-positive) bacteria, respectively. It demonstrated the efficiency of the protective coating if applied to straw bales used as construction materials in green housing construction.

The main novelty of this work lies in the possibility of controlling the size and phase ratio (Ag / AgCl) of the nanoparticles and the Ag MNP / AgCl NP ratio using the same plant material with plant mediated synthesis but by altering synthesis conditions and extract preparation methods. It was shown that not only the choice of plant plays a role in synthesizing different nanoparticles, but also the synthesis conditions. In addition, the method of extraction of the biomolecules from the same plant affects the nanoparticle properties.

8 Future work and challenges

In order to apply biosynthesized green nanoparticles in green housing, routes for producing high amounts of nanoparticles should be up-scalable. The synthesis of nanoparticles at the laboratory scale should be carried out for one proper sized straw bale via industrially viable methods. This would suggest that the production of nanoparticle-based repellents can be subsequently scaled up for larger amounts of straw bales (>100) corresponding to a small building. Therefore, the production scalability is an important topic that needs to be investigated and developed further. The advantage of biosynthesis using plant extract is their simplicity that makes them easy to scale-up (article II) [12]. In addition, the reuse of plant material in order to make several syntheses was briefly accosted in this thesis, however this too has to be investigated further and confirmed. The re-use of materials may also promote the formation of the other minority phases (Ag or AgCl).

These investigations highlighted that the presence of AgCl or Ag phases in Ag@AgCl nanoparticles can be promoted using the same plant but different plant extract preparation methods and synthesis parameters, which however needs further and more profound investigations with other extracts. In addition, the role and contribution of the two different phases (Ag and AgCl) in antimicrobial properties is still unclear; it needs to be further investigated. Our research group is presently investigating the role of AgCl in the antibacterial properties using other plant materials for the preparation of plant extracts in order to identify the influence of the different phases on the antimicrobial properties.

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Abstract

Plant mediated synthesis of silver-based nanoparticles and their use as antimicrobial agent in environmentally-friendly applications

Energy efficiency is an important topic related to the construction sector as it is estimated that as high as 40% of the total energy consumed by the economy is directly related to housing development. To counteract this energy intensive value chain, the development of eco-friendly housing is gaining momentum. Green housing requires the use of renewable and abundant raw materials offering low environmental impact and ensuring sustainability of the entire value chain. For example, straw bale is a promising construction material for green housing, as the material is an agricultural by-product or waste. However, the organic content and porous surfaces of straw make them vulnerable to microorganisms that colonize it. Therefore, urgent and eco-friendly solutions for antimicrobial protection are required that can be expanded to all ecological construction and finishing materials. In that regard, using greenly synthesized silver-based nanoparticles, which demonstrate high potential for such applications, is a novel idea.

Silver has been known for its antimicrobial properties for centuries and silver-based nanoparticles have been implemented broadly as antimicrobial agents in last decades. Nevertheless, their effects on common molds and bacteria of building materials have to be tested to ensure their applicability. This PhD work investigates firstly the effect of silver nanoparticles against such microorganisms, as well as against non-isolated and isolated cultures in laboratory conditions.

Based on the significant effects on the tested cultures, the focus of this work gradually turns to the development of the green synthesis routes of silver nanoparticles and their incorporation into green housing applications. Amongst all the methods, plant mediated synthesis stood out as the most suitable to implement in the field of eco-construction. Several plants contain biomolecules that reduce silver ions from the silver nitrate precursor, as well as stabilize precipitated nanoparticles. Particular emphasis was put on the development of the silver and silver chloride nanoparticle synthesis using common weed *Plantago Major*.

The novelty of the work lies in the role of the plant-extract preparation method and different silver nanoparticle synthesis conditions. It was demonstrated that the same plant material and precursor salt can be used to promote silver or silver chloride nanoparticle formation within synthesized Ag@AgCl nanopowders. Different characteristics of the silver nanoparticles were measured and compared. They were also tested against microorganisms for potential use as antimicrobials which showed antifungal and antibacterial effect on model organisms. Potential coating originating from the plant extract made the effect on the microorganisms similarly efficient.

Kokkuvõte

Taime ekstrakti abil sünteesitud hõbedal põhinevad nanoosakesed ning nende kasutus antimikroobse vahendina keskkonnasõbralikes rakendustes

Kasvava energianõudluse ja kasvuhoonegaaside emissioonide tõttu on energiatõhusus kerkinud oluliseks märksõnaks. Ainuüksi ehitussektori energiavajadus moodustab hinnanguliselt 40% kogu maailma energiavajadusest. Vastukaaluks on hakatud otsima lahendusi juurutamaks ehituses energiasäästliku mõtteviisi, sealhulgas on aasta-aastalt hoogustunud ka keskkonnasõbraliku ökoehituse arendamine. Üks suundadest on ökoloogiliselt sõbralike ehitusmaterjalide nagu seda on põllumajandustegevusest üle jääv põhk kasutamine. Samas on põhuehituses kasutatav põhk soodne kasvupinnas bakteritele ja hallituseentele ning seetõttu on vajadus ökoloogiliselt sõbralikule tõrjevahendile nii põhule kui ka üldiselt ehitus- ning viimistlusmaterjalidele. Rohesünteesitud hõbedapõhised nanoosakesed liigituvad eelmainitud kategooriasse.

Hõbeda antimikroobsed omadused on teada juba sajandeid, samuti on viimase kahekümne aasta jooksul laialdaselt uuritud hõbeda nanoosakesi ning nende mõju bakteritele ja seentele. Selleks viidi läbi uuringud testimaks puhtaid hõbenanoosakesi levinud mikroorganismide peal, mida põhult enim leida. Doktoritöö esimene faas keskendus antimikroobsetele katsetele bakterite ja seenete vastu, mis eraldati põhult. Katsed viidi läbi nii materjali pinnal, bakterite ja seente peal eraldi söötmetel Petri tassides kui ka isoleeritud kultuuridel laboratoorsetes oludes.

Katsetuste tulemused andsid aluse doktoritöö fokusseerimisele ökoloogiliselt sõbralike hõbenanoosakeste sünteesivõimaluste arendamisele, et neid sarnaselt kasutada ökomaterjalide kaitsmisel. Nn rohesünteesivõimaluste seast oli taimeekstraktide kasutus hõbenanoosakeste sünteesiks hõbenitraadist üks arvestatavamaid oma lihtsuse ning skaleeritavuse tõttu. Kuna kasutati ka umbrohuna levinud teelehte (Plantago major), siis oli protsess lisaks ka ökonoomne. Mitmed laialt levinud umbrohuks peetavad taimed olid võimelised osakesi sünteesima, kuid edasi arendati teelehe sünteesiprotsessi.

Doktoritöö uudsus seisneb selles, et mitte ainult taime valik ei mõjuta sünteesitud nanoosakeste parameetreid ja koostist, vaid ka sama taime erinevad ekstrakti valmistusviisid ning nanoosakeste sünteesi parameetrid. Sõltuvalt sellest, kuidas teeleheekstrakti valmistati ning millisel viisil süntees läbi viidi, suudeti kontrollida nanoosakeste koostist – kas peamine faas oli hõbe või hõbekloriid. Mõlemat tüüpi hõbedapõhiseid nanoosakesi katsetati edukalt ka mudelorganismide korral, et hinnata nende sobivust antimikroobse vahendina kasutamiseks.

Appendix

Publication I

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

S Küünal[1], M Visnapuu[2,3], O Volubujeva[1], M Soares Rosario[4] and P Rauwel[1,5], E Rauwel[1,5]

[1]Faculty of Engineering, TalTech, Akadeemia tee 15, 12618 Tallinn (Estonia)
[2]Institute of Physics, University of Tartu, W. Ostwaldi Str 1, 50411, Tartu (Estonia)
[3]National Institute of Chemical Physics and Biophysics, 12618, Tallinn (Estonia)
[4]CICECO- Aveiro Institute of Materials, University of Aveiro (Portugal)
[5]Institute of Technology, Estonian Univ. of Life Science, 51074 Tartu (Estonia)

Email: erwan.rauwel@emu.ee

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

conducted. 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 synthesize of silver nanoparticles using 2 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 24hours 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.

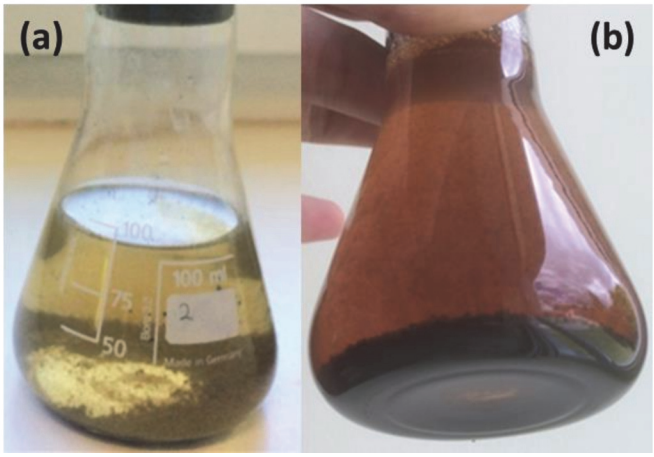

Figure 1. Solution of Ag nanoparticles after synthesis (a) type A synthesized in aqueous medium at 85ºC, (b) type B synthesized under sunlight.

2.3. Characterization

X-ray diffraction (XRD) patterns were collected using a Panalytical Empyrean diffractometer with a Cu $K_{\alpha 1}$ radiation source (λ = 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 38.15º, 44.30º, 64.52º, 77.42º 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 27.88º, 32.26º, 46.25º, 54.85º, 57.50º, 67.51º, 74.45º, 77.40º 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.

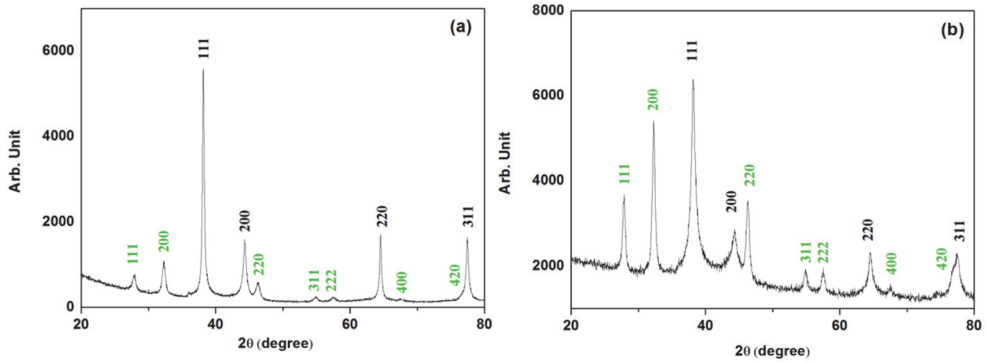

Figure 2. XRD patterns of Ag NPs synthesized (a) in aqueous medium at 85ºC (type A), and (b) under sunlight (type B). XRD peaks of Ag NPs and AgCl NPs are indexed in black and green respectively.

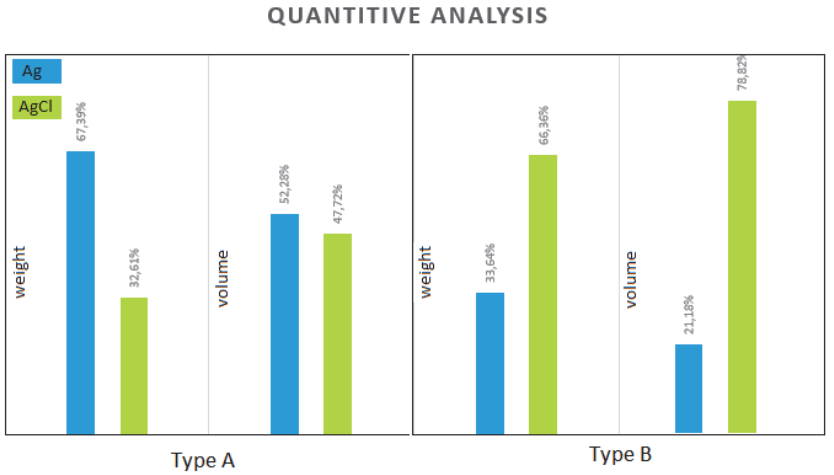

Figure 3. Type A and type B nanoparticles weight and volume percentages based on the XRD data.

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 (85ºC) (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 85ºC. 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.

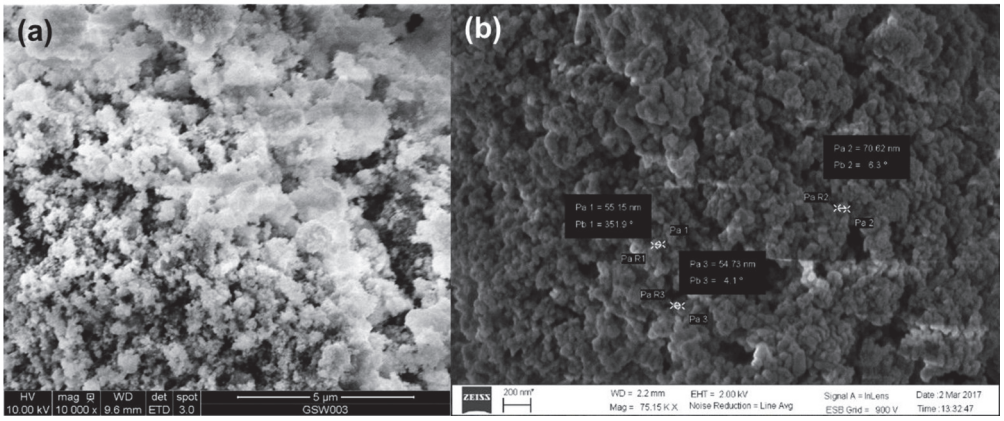

Figure 4. SEM micrographs of Ag NPs and AgCl NPs synthesized (a) in aqueous medium at 85ºC (type A), and (b) under sunlight (type B).

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.

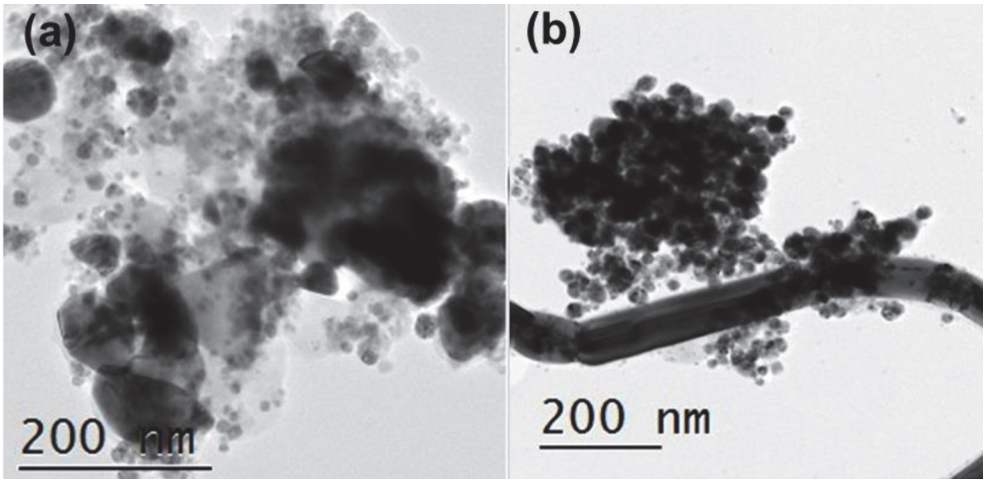

Figure 5. TEM micrographs of Ag NPs and AgCl NPs synthesized (a) in aqueous medium at 85ºC (type A), and (b) under sunlight (type B).

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 antibacterial 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). $AgNO_3$ 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.

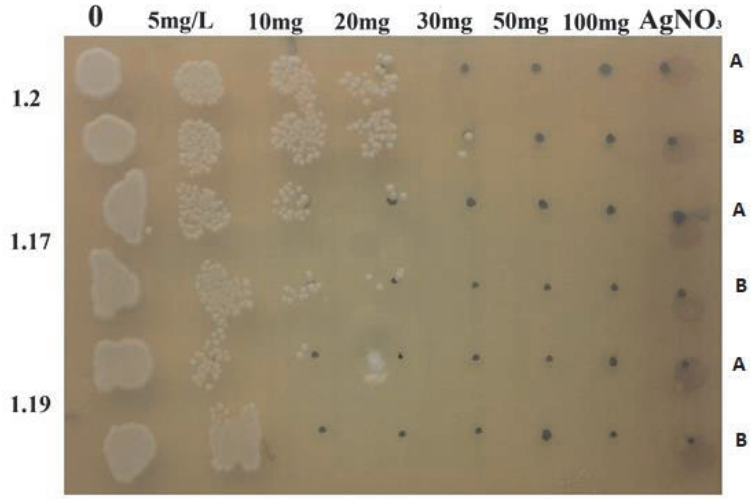

Figure 6. Antifungal tests result with silver nanoparticles synthesized in aqueous medium at 85ºC (type A), under sunlight (type B) on yeast *S. Cerevisiae*. Y-axis shows the optical density measured at 600nm by UV-Vis spectrometer, which indicates the number of cells in the tested media. X-axis shows the concentration of silver nanoparticles in the solution.

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.

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Chapter 14

Plant extract mediated synthesis of nanoparticles

Siim Küünal, Protima Rauwel, Erwan Rauwel
Tallinn University of Technology, Tartu, Estonia

1 INTRODUCTION

Metals, metal oxides, and their salts have been used as antimicrobial coatings for thousands of years. Documented use of silver, copper, zinc, and mercury salts dates back to ancient times, to the reign of Geeks, Romans, and even ancient Egyptians. For ages, medical practitioners have applied metal salts to clean wounds and have used silver threads as sutures. Silver and silver salts were the main agents for active wound healing and their use can be dated back to as early as the middle of the last century [1]. Therefore, mankind has already benefited from the discovery of antimicrobial properties of different metals and their compounds and is still relevant in today's context.

Starting in the 1940s, especially during the World War II, antibiotics became an essential part of healing processes [2]. However, bacterial infections have again become a threat recently, mainly due to their overuse. Antibiotic resistance has widely spread across the globe and many "superbugs" threaten in-patients and out-patients [3]. We are slowly but surely entering a pre-antibiotic era as a resort to combat large spectrum antibiotic resistant pathogens [2].

Nanotechnology as a research field has been gaining momentum during the past few decades, as nanomaterials possess a wide range of enhanced properties compared to their bulky counterparts. Hence the implementation of nanomaterials becomes more and more relevant in the field of antimicrobial coatings. The use of natural antibacterial materials, such as many metals and metal oxides, with nanoscale dimensions, enables more efficient interactions with microorganisms due to the increased surface to volume ratio.

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Nanoparticles can then act as long-lasting biocides with low volatility and high thermal stability providing characteristic bactericidal and bacteriostatic effects through several mechanisms [4,5].

Currently, nanoparticles exhibiting biocidal properties are in demand for being incorporated into several products. Hence the production of nanoparticles is constantly increasing. All kinds of synthesis methods are used, such as chemical [6], electrochemical [7], radiation [8], photochemical [9], pulsed laser [10], Langmuir/Blodgett [11], and biological techniques [12]. However with increased demand for nanomaterials, production has to be scaled up with adapted synthesis methods. Synthesis methods in general are expensive and detrimental to the environment due to the hazardous chemicals involved. Moreover, the energy consumption during production has to be curbed to increase cost-effectiveness and eco-friendliness of synthesis procedures in order to make them commercially viable. This would require all complex, expensive, multi-step preparation methods to be replaced with a single step synthesis at ambient conditions (e.g. room temperature and normal pressure) with green reagents [5].

Biomass of bacteria, fungi and algae, living plants, and plant extracts are the main precursors for producing ecologically friendly nanoparticles [13]. However, the simplest and most effective route for green synthesis to date is with plant extracts. In fact, plant extracts have great reducing potential, they are simple to collect, cost effective, healthier to handle and non-contaminating, and therefore have little or no environmental impact [4,14].

2 PLANT EXTRACT MEDIATED SYNTHESIS OF NANOPARTICLES

Antimicrobial nanoparticles are not only in demand in several environmentally important areas, such as renewable energies and environmental remediation but also in biomedical devices and overall medicine. All aforementioned fields already have many products available on the market, which contain nanoparticle-impregnated materials [15]. In such fields, where sustainability and safety play a key role, green nanotechnology would be the preferred direction to go towards. Even if the nanoparticles are effectively implemented in the above-mentioned areas, the possible release of hazardous reducing and stabilizing agents or by-products to the environment can nullify the benefits of the material itself.

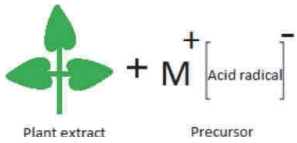
In research, the term “green nanotechnology” has raised a great amount of interest over recent years. Green synthesis using plant extract is a part of a branch of Chemistry called “Green Chemistry” which is the design of

chemical products and processes that reduce or eliminate the use and/or generation of hazardous substances [16]. The significant demand for green nanoparticles has initiated a comprehensive investigation to find most efficient ways to produce environmentally friendly nanoparticles [17] and there is a clear emerging trend in nanoparticle synthesis that uses plant extracts for the development of new applications [18]. Thus, green and rapid nanoparticle synthesis methods using plant extracts have drawn attention due to their simplicity, inherent safety, and affordability [19]. Furthermore, in the beginning of the year 2017, a self-search revealed a total of 2451 research publications on the Scopus database using "plant synthesis nanoparticles" as keywords, implying that plant extracts are far more easily produced, handled, and used as precursors compared to bacterial or fungal biomass mediated synthesis methods [12]. Compared to the latter two methods, plant extracts are easier to scale-up at an industrial level.

Plant extracts used to produce metal and metal oxide nanoparticles are extracted from a variety of plant parts such as the leaf, fruit, stem, root or tuber, bark powder, latex, fruit peel, and seeds [13]. Biomolecules contained in such plant parts are responsible for rapid reduction of metal salts along with capping and stabilizing them. Generally, plant mediated nanoparticles can have a variety of different shapes and sizes ranging from spherical, triangular, cubic, or rod-like. Depending on the shape, these nanoparticles will have different effects on microorganisms [20]. Recent reports show that the biocidal effect is linked to the size of the nanoparticle, where smaller size is more effective due to better contact with or up taking from the microorganisms [21]. In addition, the biomolecules involved with synthesis do not only reduce the metal salt, but can also functionalize the surface of the nanoparticles, which induces synergistic effects for antimicrobial or cancer treatment applications. In fact, the functionalization can improve biocidal properties against microorganisms or cancer cells, and can also decrease the toxicity to higher organisms including human cells [22]. Therefore one-step synthesized nanoparticles can in fact offer better biocompatibility with important benefits to future medicine.

2.1 Plant extract reduction mechanism

Research on plant mediated nanoparticle synthesis has been rather comprehensive in recent years namely due to the availability of raw materials. Nanoparticles can be produced using extracts made from all kinds of different plant parts. It has been more than a decade since the first reports of plant mediated nanoparticle synthesis has been published [23,24] although the number of publications on the topic keeps rising annually, the exact synthesis mechanisms are still far from being completely understood [14]. Essentially, the formation of nanoparticles consists of two important steps: first is



■ **FIGURE 14.1 A simple schematic of plant mediated synthesis process, where metal precursor reacts with a plant extract, usually at room temperature.**

the nucleation followed by particle growth in the second step, and requires reducing and stabilizing organic molecules. Furthermore, several conditions like the concentration of precursor and extract, pH, temperature, contact time, and sunlight radiation can affect the rate and yield of production and other characteristics of produced nanoparticles [25]. Fig. 14.1 is a simple schematic example of the reactants in a plant mediated synthesis approach, where a plant extract reacts with a precursor containing a metal salt. Typical precursors involve nitrates, sulphates, chlorides and other compounds.

More clarity on the plant extract reduction mechanism will soon be available, as presently, plant mediated nano-factories have become rather popular. Thus more and more investigations and hypotheses can be drawn and insights can be given on the mechanistic aspects of nanoparticle syntheses.

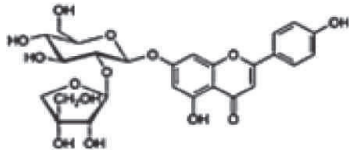
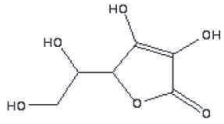
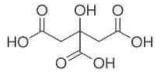
As already mentioned, biosynthesis exploits the reducing and stabilizing potential of biomolecules extracted from plants. It is a chemically complex process, with a wide variety of plant extracts involved in the bio-reduction of metal ions [26]. Plant extracts that have been reported to be successful in nanoparticle growth contain wide array of compounds: vitamins, enzymes, proteins, organic acids, polysaccharides, lipids, and more. The phytochemical screening has revealed that metabolites present in plants belong to chemical groups of terpenoids, flavonoids, phenols, alkaloids, proteins, and carbohydrates. Hydroxyl, carbonyl, amine, and methoxide are the main functional groups responsible for reacting with the precursor and also found in the aforementioned compounds (Table 14.1) [26]. More specifically, some of the main compounds documented as responsible for nanoparticle formation are provided in Table 14.2 [14].

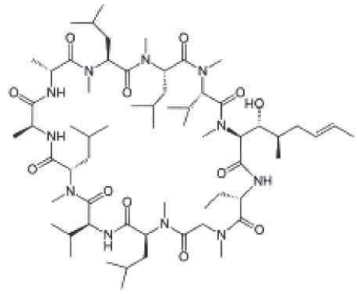
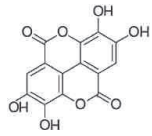
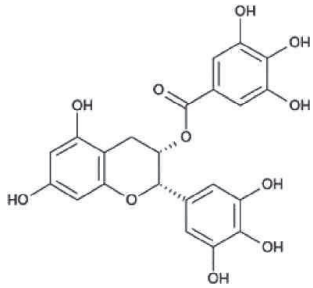
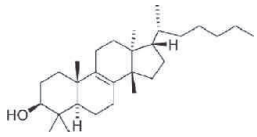
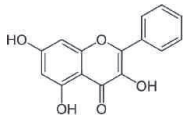
Numerous studies on plant-mediated synthesis of nanoparticles exist along with identification of reactive aggregates. Nevertheless, very few studies have investigated biological synthesis using isolated compounds [14]. In the example of apiin (Table 14.2), which was isolated from Henna (*Lawsonia inermis*), silver and gold nanoparticles were successfully synthesized [27]. Carbonyl groups (Table 14.1) bind to the metal ions through electrostatic interaction and thus initiate the reduction process. Elsewhere, phyllanthin (Table 14.2) was extracted from Leafflower (*Phyllanthus amarus*) leaves and used for the biosynthesis of silver and gold nanoparticles [28]. The interaction of the methoxide group (Table 14.1) and metal ions resulted in nanoparticle synthesis. Another study with flavonoids (Table 14.2) showed that chemicals isolated from propolis extract could give a more homogeneous size distribution of nanoparticles compared to the extract itself [29]. These results indicate that many compounds with different reducing properties, usually the case with plant extracts, can complicate the synthesis process, thereby affecting the simplicity of the synthesis and the size distribution of nanoparticles.

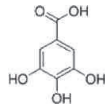
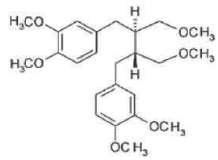
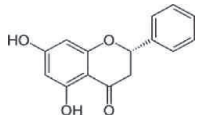
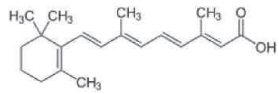
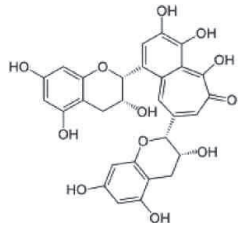
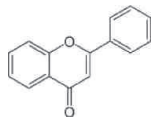
Table 14.1 Main Functional Groups Available in Plant Extracts and Reacting With Precursors

Functional Group	Formula
Hydroxyl group	
Carbonyl group	
Amine groups	
Methoxy group	

Table 14.2 Specific Compounds Identified and Their Functional Groups Highlighted

Compound	Formula
Apiin	
Ascorbic acid	
Citric acid	

Compound	Formula
Cyclic peptide	
Ellagic acid	
Epicatechin gallate	
Euphol	
Galangin	

Compound	Formula
Gallic acid	
Phyllanthin	
Pinocembrin	
Retinoic acid	
Sorbic acid	
Theaflavin	
Flavonoids: have distinctive 2 phenyl rings and 1 heterocyclic ring	

(Source: from Ref. [14]).

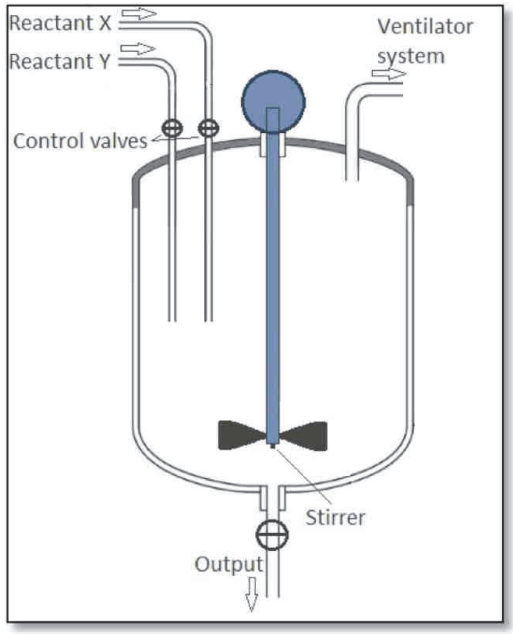
In addition to the biomolecules and precursor interactions, there are, as mentioned, several variables to consider. It is easy to improve reaction by changing the mixture content ratio or contact time, also by controlling the temperature or sunlight exposure (i.e., ultraviolet (UV) exposure). However, one major influence can also react to pH and its ability to alter the electrical charges of chemicals inside the extract-precursor mixture. The change of electrical charges may affect the capping and stabilizing capacity of biomolecules involved and thereby affect the growth of nanoparticles [22]. It is one promising route to get favorable shapes and sizes, as pH is used to achieve greater stability in the mixture.

2.2 Mass production

Environmentally friendly methods are preferred for nanoparticle synthesis and greenly produced nanoparticles should fully replace commercially available nanoparticles produced via conventional methods. Therefore, green nanoparticle synthesis production needs to be scaled up. Methods for mass production must be developed and in every step, sustainability has to be considered.

As mentioned before, nanoparticle synthesis depends on multiple conditions and parameters, such as temperature, reagent quantities and their ratio, UV exposure, the pH of the mixture, and pressure. It is more complicated to fully control all the variables as in the case of synthesis, with beakers on large hotplates and condensers compared to more advanced batch reactors (Fig. 14.2), which are mainly used for scaling up the production of nanoparticles. In such systems, the reactants are introduced in a controlled manner with the help of valves heated to the required temperatures. The stirrer helps in obtaining a homogeneous solution, which then reacts. Gaseous by-products are vented out through the ventilation system and the precipitated nanoparticles are emptied out with a bottom fitted tube, in turn fitted with a valve. However, discrepancies inside such reactors exist, along with differences between batches. Furthermore, non-uniformity of different conditions results in broad particle size distributions and unnecessary energy consumption [22].

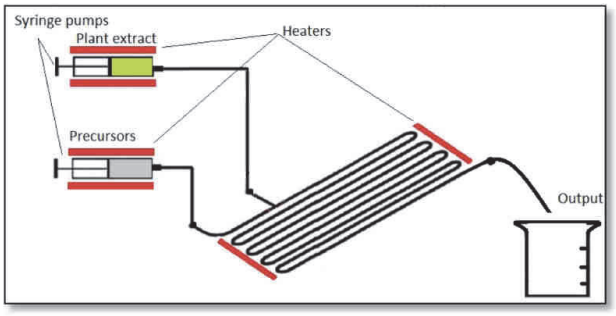
Consequently, this brings the microfluidic reactor systems (Fig. 14.3) into the spotlight, as conditions can be relatively well controlled during the synthesis. In such microsystems, pumps, typically syringe pumps, generate pressure driven flow in the micro-channels, and then the precursor and plant extract solutions are manipulated inside these devices. Flow movement, temperature, and mixing ratio are controlled by electric/magnetic fields or channel geometrical design. Controlling the flow rate, input rate,



■ **FIGURE 14.2 Batch system used for large-scale production of nanoparticles.**

temperature, and mixing and reacting time size-controlled nanoparticles will be formed. Designed valves and channels help to get rid of the by-products and the output would consist typically of unrinsed nanoparticle colloids. The large surface to volume ratio and confined space of the microchannel reaction chambers, where diameters are under a millimeter in size, and enable temperature control, pressure, concentration, and flow rates [30]. Microfluidic reactors enable the nucleation to occur more quickly with efficient mixing and as mentioned, allows reagents to be added and downstreamed simultaneously in a very controlled manner [31].

The development of large-scale green nanoparticle synthesis is an important aspect to make our environment cleaner. Plant mediated syntheses are usually carried out at an ambient temperature and pressure and production brings about no toxic by-products. The latter implies that even simpler continuous flow microsystems can be built, as without toxic by-products, the cleaning process is not an issue either.



■ FIGURE 14.3 Schematic of the microfluidic reactor.

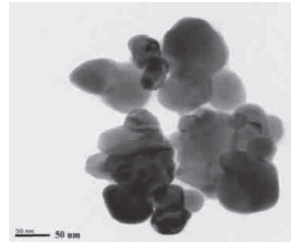
3 METAL OXIDE NANOPARTICLES

Several metal oxides have been known to have antimicrobial properties. For example, Calamine lotion consists of a combination of zinc oxide and ferric oxide, both of which are known to possess some antibacterial properties [32]. The first use of this metal oxide containing lotion dates back to 1500 BC [33]. Nowadays, Calamine is mainly used for sunburns, but as the size of the materials decrease down to the nanoscale regime, new unique features emerge or properties are amplified. Also, metal oxide nanoparticle antibacterial efficacy improves with the increase in their surface to volume ratio. The following list of antimicrobial metal oxide nanoparticles have the most potential for plant mediated production and subsequent coating applications.

3.1 Zinc oxide nanoparticles

Zinc oxide and its nanoparticles have a whole range of different applications such as semiconductors, UV absorbers, electroluminescent material, and antibacterial material among others [34]. Compared to all metal oxide nanoparticles, ZnO has one of the highest antibacterial effects [32] and is already integrated into various consumer products, such as antimicrobial food packaging [35,36]. Similarly to others, ZnO nanoparticles have size-dependent antimicrobial effects and a diameter under 30 nm seem to be the most effective against bacteria [4]. Also, the surface charge is an important factor, as a positively charged surface area is more effective against Gram positive and Gram negative bacteria [4].

Few studies have also shown the potential of plant extracts to produce greener zinc oxide nanoparticles [31,37]. The precursor is usually zinc nitrate, acetate, or chloride that is mixed with plant extracts [38–40]. ZnO nanoparticles produced by one such green synthesis method are shown in Fig. 14.4, which also illustrates nanoparticles of various sizes ranging from 20 to 100 nm. Greenly produced zinc oxide nanoparticles are also effective against Methicillin-resistant *Staphylococcus aureus* [41]. In addition to the antimicrobial effect, plant mediated ZnO nanoparticles have shown also anticancer [42] and antifungal [38] properties. Even though, ZnO nanoparticles can be toxic to humans [43, 44], biosynthesized nanoparticles appear safer [45]. This opens ways to other possible applications such as coatings in fabrics, as in the case of plant mediated synthesized zinc oxide nanoparticles incorporated in cotton [46].



■ **FIGURE 14.4 TEM image of greenly synthesized ZnO-NPs (*Ocimum basilicum* L. var. *purpurascens* Benth.-Lamiaceae leaf extract).** *(Source: from Ref. [47]. Copyright License Number 4035320773068).*

3.2 Magnesium oxide nanoparticles

Magnesium oxide has various applications, such as an adsorbent for toxic chemical agents [48], photocatalyst [49], refractory [49], and so on. Magnesium oxide nanoparticles also possess antibacterial properties [50]. Comparing Gram positive and Gram negative bacteria, MgO nanoparticles were shown to be more effective against Gram positive *S. aureus* bacteria [48]. Elsewhere its effects were comparable with Gram negative *E. coli* as well [51]. Moreover, green-synthesized magnesium oxide nanoparticles were found to possess anticancer [52] and antioxidant properties [53]. Same nanoparticles showed photocatalytic activity both under UV irradiation and sunlight, which opens more possible application areas of these ecologically friendly MgO nanoparticles [52]. The most common plant-mediated synthesis of the magnesium oxide nanoparticles employs magnesium nitrate as a precursor [54], similar to other greenly produced nanoparticles. Moreover, magnesium oxide nanoparticles can be incorporated into fabrics to effectively protect them [48].

3.3 Titanium dioxide nanoparticles

TiO_2 or titanium dioxide or titania is the most stable photocatalyst with prominent optical properties. Titanium dioxide nanoparticles are used in various fields such as cosmetics, medicine, and different coatings and coloring [55]. Titanium dioxide nanoparticles have more particularly been integrated in self-cleaning windows [56]. Also, titanium dioxide nanoparticles manifest efficient antibacterial properties against Gram negative and Gram positive bacteria, also its antioxidant characteristics are outstanding [57]. Greenly produced titanium dioxide nanoparticles have also been reported to possess antifungal [58] and antiparasitic properties [59]. During the plant-mediated syntheses, the main precursor used is metatitanic acid [55,57]. Titania nanoparticles have

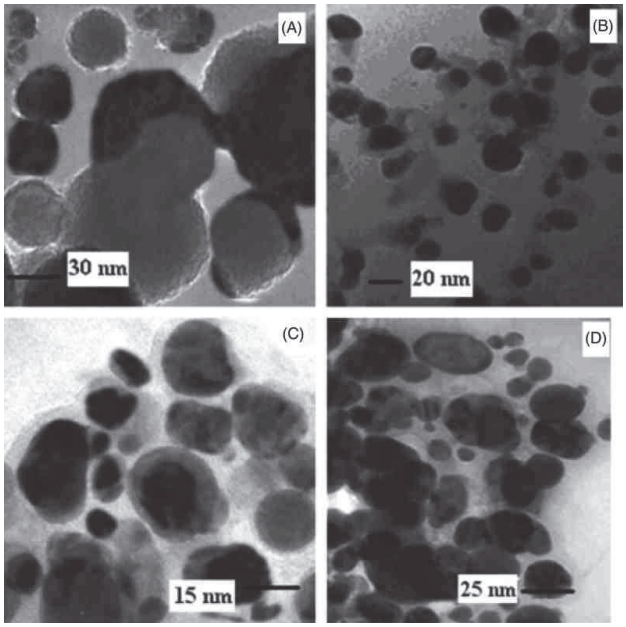
been used for coatings for quite some time already [60], and therefore plant mediated TiO_2 nanoparticles with enhanced properties can offer a promising alternative for coating applications in environmentally important areas. Moreover, the photoactive antifouling effect of titanium dioxide is prominent, but a less known feature is its antibacterial effect, even in dark conditions, which shows the high potential of these metal oxide nanoparticles [61].

3.4 Copper (II/IV) oxide nanoparticles

Copper oxide nanoparticles have been used as batteries, catalysts, gas sensors, high temperature superconductors, and tools for solar energy conversion among others [62]. Also, copper oxide has a well-documented antimicrobial effect [63] with efficiency against bacteria, yeast, and fungi [62]. Because of its biocidal and anti-viral properties, these nanoparticles are used as anti-microbial coatings in textiles, wound dressings [64], and plastics [65]. Furthermore, copper oxide nanoparticles have shown anticancer properties [62]. However, many tests suggest that CuO nanoparticles are more toxic compared to other available metal oxide nanoparticles [66]. Nevertheless, plant molecules functionalizing their surfaces tend to reduce toxicity against normal cells. Main precursors for plant mediated copper oxide nanoparticle synthesis are copper sulfate [62] and copper (II) nitrate trihydrate [63]. In Fig. 14.5, CuO nanoparticles were synthesized with *Aloe vera* leaf extract (ALE). In all the images of Fig. 14.5, the size distribution of the nanoparticles is quite large. Nevertheless, a sample synthesized with 10% ALE appeared to provide the most homogenous size distribution with sizes around 20 nm. Conventional copper oxide nanoparticles have been incorporated into different fabrics such as cotton and polyester fibers [67]. For commercial applications plant mediated CuO nanoparticles should be preferred as they are more stable with less ion release and possess less toxicity compared to conventionally engineered nanoparticles [63].

3.5 Iron oxide nanoparticles

The most used iron oxide nanoparticles include magnetite (Fe_3O_4), hematite, or iron (III) oxide (Fe_2O_3) and the less available iron (II) oxide (FeO). Magnetite (Fe_3O_4) nanoparticles are used in their biomedical field due to their magnetic properties and biocompatibility and more particularly for their superparamagnetic properties. These magnetite nanoparticles are known as superparamagnetic iron oxide nanoparticles and are more particularly studied for drug delivery [68] and hyperthermia therapy [69,70]. Magnetite nanoparticles can generate reactive oxygen species (ROS), which kill pathogens, making them promising candidates for also being used as antimicrobial agents [71]. Greenly synthesized magnetite



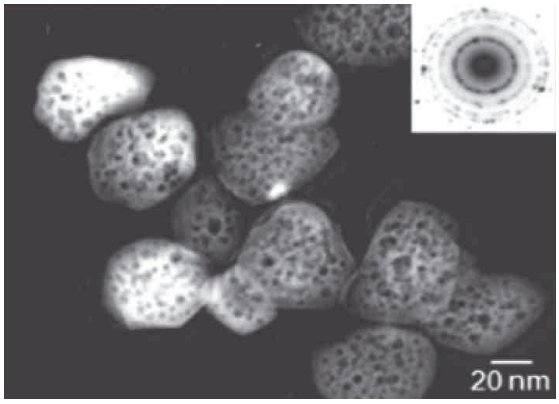
■ **FIGURE 14.5 TEM images of Aloe Vera leaf extract (ALE) mediated CuO nanoparticles.** (A) 0% ALE, (B) 10% ALE, (C) 25% ALE, and (D) 50% ALE. *(Source: from Ref. [65]. Copyright License Number 4035350460846).*

nanoparticles are reported to be effective against Gram negative *Pseudomonas aeruginosa*. In plant-mediated synthesis, the precursor used is usually ferric chloride [72].

In the case of hematite (Fe_2O_3), the nanoparticles are also mainly used in biomedical fields as they possess magnetic properties, and wastewater treatment is more particularly studied. In plant-mediated synthesis the precursor, for hematite synthesis is usually iron sulphate [73].

FeO, on the other hand, is used mainly for its photocatalytic properties in environmental remediation and biomedicine [74]. Plant mediated FeO nanoparticles are synthesized from iron (III) chloride and they exhibit antioxidant properties [74].

Several iron oxide nanoparticles have also been reported to have anticancer properties [75]. One such synthesis is provided in Fig. 14.6, where rather



■ **FIGURE 14.6 TEM image of α-Fe_2O_3 NPs synthesized by green tea leaves.** *(Source: from Ref. [76]. Creative Commons Attribution License).*

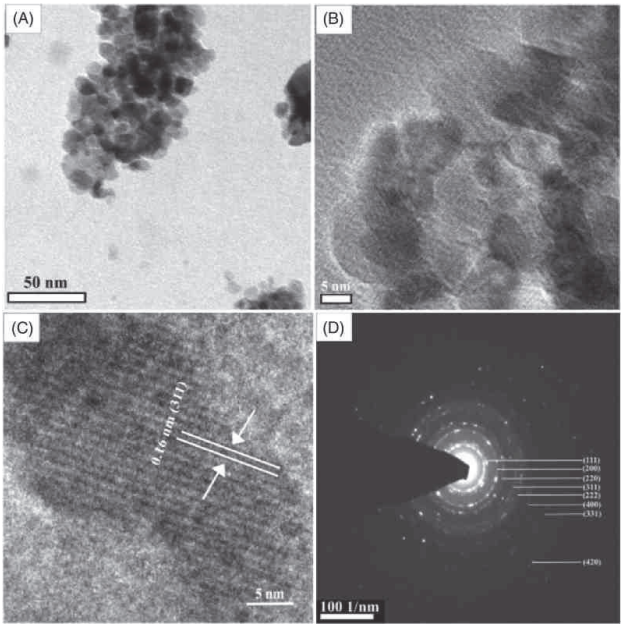
evenly sized α-Fe_2O_3 nanoparticles are produced with green tea extracts. The average size of the nanoparticles produced is around 40 nm. However, the shapes of these nanoparticles tend to vary and they moreover depict superficial porosity. For their unique properties, conventionally synthesized iron oxide nanoparticles have been used as antimicrobial coatings [34], and as there are several reports of plant mediated synthesis of iron oxide nanoparticles, they appear as a good option for functionalized biocidal coatings.

3.6 Calcium oxide nanoparticles

CaO nanoparticles are implemented mainly in the field of detection and diagnostics, therapeutics, catalysis, microelectronics, and antimicrobial applications. To our knowledge, only one plant-mediated synthesis of CaO nanoparticles has been carried out and calcium chloride was used as precursor. Results showed excellent activity against common Gram negative and Gram positive pathogens [77]. Green synthesized calcium oxide nanoparticles have demonstrated good potential for medical applications such as wound healing, where they could be incorporated into fabrics as an antibacterial coating.

3.7 Cerium oxide nanoparticles

Cerium oxide (CeO_2) is amongst the most used rare earth compounds [78]. In fact CeO_2 nanoparticles are most commonly used in the biomedical field as catalysts [79] and antioxidants [78]. Few plant-mediated syntheses have



■ **FIGURE 14.7** (A–C) TEM images of CeO_2 NPs and (D) selected area electron diffraction. *(Copyright License: 4057030945540).*

been conducted, where $CeCl_3$ [80] and cerium (III) nitrate hexahydrate [78] were used as precursors. Antimicrobial tests show good results against Gram negative and Gram positive bacteria [80, 81]. Although Ce_2O might be considered a toxic substance [82], greenly synthesized cerium oxide nanoparticles show less or no toxicity [83]. Considering these two findings, Ce_2O nanoparticles show potential for antibacterial coatings in several substrates. In Fig. 14.7 spherical cerium oxide nanoparticles that were synthesized by *Gloriosa superba* L. leaf extract, have an average size of 5 nm [80].

4 ANTIMICROBIAL METAL NANOPARTICLES

Nanoparticles have unique properties compared to bulk in general. In fact, nanoscale materials have a large fraction of surface atoms with reduced defects and high surface energy, which assigns them interesting properties and opens up many applications for metal nanoparticles [84]. Compared

to metal oxide nanoparticles, pure metal nanoparticles are even more well known for their antimicrobial, antiparasitic, antiviral, antioxidant, and anticancer properties [84,85]. They have been incorporated into several products in different fields such as wound dressings, medical devices, cosmetics, bone cements, surgical implants, pharmaceuticals, food packaging, and antibacterial clothes like socks, sprays, and creams [84,86]. This is especially evident with noble metal nanoparticles, which have been the main focus of new generation of antimicrobial investigations [84].

4.1 Noble metal nanoparticles

Noble metals are the most documented metals in human history due to their stability. Dated back through the history of human civilization, there has been noted use of copper, gold and silver [87]. Even today their popularity has not decreased and new applications emerge from all directions. Their unique optical and antimicrobial properties and high resistance to oxidation have made noble metal nanoparticles interesting for scientific investigation and application development [88]. Due to recent findings, noble metal nanoparticles, such as gold, silver, and copper nanoparticles, are widely applied to consumer goods in direct contact with humans, therefore a growing need to develop environmentally friendly processes of nanoparticle synthesis with non-toxic chemicals is the need of the hour [89]. There are several reports of plant mediated noble metal nanoparticles and their antimicrobial properties, however more than half of all publications address silver nanoparticles synthesis [14].

4.1.1 *Silver nanoparticles*

Silver nanoparticles are the most commercialized nanoparticles due to their exceptional physico-chemical properties, such as their optical, catalytic, electric, and of course antimicrobial properties [90]. Due to enhanced antimicrobial activity and low toxicity against human cells, silver nanoparticles as antimicrobials are widely exploited in fields such as cosmetics, textiles, household appliances, water purification systems, molecular imaging, wound dressings, diagnosis and treatment of cardiovascular diseases, cancer treatment [91,92], and drug delivery [90,93,94]. Other metal nanoparticles can show comparable effectiveness against some microorganisms, but all in all, silver is reported to be the most efficient against a wide range of microorganisms [95]. Therefore, whenever antimicrobial properties are needed, silver nanoparticles are the metal of choice.

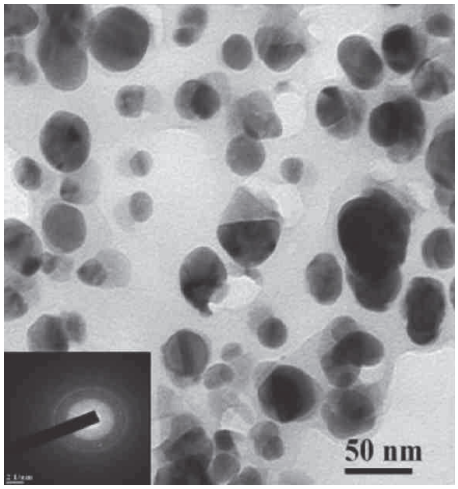
In addition to the antimicrobial properties, silver is also known to possess antiparasitic, antiviral, antioxidant, and anticancer properties and to have a potential for development as a novel therapeutic agent [14,96–98].

Compared to the standard antioxidant, ascorbic acid, silver nanoparticles showed greater antioxidant activity in terms of increased reducing power [14]. Although free radical scavenging was slightly lower than with ascorbic acid, it still shows silver nanoparticle potential in the given field.

Silver nanoparticle antimicrobial and antiviral coatings are mainly used in medicine, for example in plastic catheters, where bacterial biofilm formation could be life-threatening [99]. Other than in medicine where safe contact surfaces are required, silver nanoparticles can also be utilized as a novel biocide to coat household appliances like the fridge or building materials. After all, people spend most of their time indoors and excessive humidity promotes mold growth. Hence, silver nanoparticles are used as coatings on gypsum panels [100] or ecologically friendly building blocks [101,102]. Even silver nanoparticle incorporated paints are used to achieve indoor microbe-free walls [5]. This surfacing is more specifically studied for application in hospitals in which multi-drug resistant bacteria are developing. All those applications can benefit from implementing greenly synthesized silver nanoparticles as they are safer for humans and also reduce the ecological footprint.

The most common plant mediated synthesis of silver nanoparticles uses silver nitrate as a precursor, which is then added to the plant extract solution. For energy conservation purposes, ambient conditions such as room temperature and normal pressure are preferred. The list of plants used is extensive and new plants to reduce silver ions are discovered daily. In Fig. 14.8 a TEM image of silver nanoparticles synthesized with $AgNO_3$ and latex is provided. The larger particles tend to harbor defects such as stacking faults, while the small nanoparticles appear to be defect free.

Environmentally friendly and simple plant mediated synthesis of silver nanoparticles enables the production of nanoparticles that are applicable in various areas, such as coatings where ecological safety and low toxicity to human cells are required. For example, antimicrobial cream that consists of plant extract derived silver nanoparticles shows excellent antimicrobial activity against common pathogens [103]. Moreover, several studies state that biosynthesized silver nanoparticles have been incorporated into different fabrics such as cotton [104], nonwoven materials [105], and leather [106]. Green-silver nanoparticle impregnated fabrics are used as wound and burn dressings [104,107]. Even after several washes, biosynthesized silver nanoparticles are retained in anti-bacterial fabrics and maintain their anti-bacterial effect [104]. The steadfast anchoring of these metal nanoparticles is a prerequisite in order to avoid their release into the environment. Biogenic silver nanoparticles are also great alternatives for fungicides and



■ **FIGURE 14.8 Transmission electron micrograph of silver nanoparticles.** Synthesized from 5×10^{-3} M $AgNO_3$ solution and 3% latex at 85°C for 4 h. Inset shows the SAED pattern of polycrystalline silver particles. *(Source: from Ref. [111]. Copyright License Number: 4061300784723).*

antifungal coatings [108]. They can be applied to make indoor environments safer for residents by coating the inner decor, as mentioned before.

In terms of biocidal coatings, nanosilver is very long lasting, as it will not be used up or dissipated over time, nor will it be lost during high temperature material processing [109]. Plant mediated silver nanoparticle coatings have additional advantages over conventional nanoparticles due to active organic molecules being present in various plant extracts. Many plants possess antimicrobial activity separately as well, and biomolecules responsible can attach to the surface of the nanoparticle and functionalize nanoparticle surfaces, which results in synergistic effects [110]. Also, molecules that stabilize silver nanoparticles can act as an anchor, attaching to cell membranes of bacteria [110]. These features can give a significant advantage to plant extract derived silver nanoparticles and promote their applicability in antimicrobial coatings.

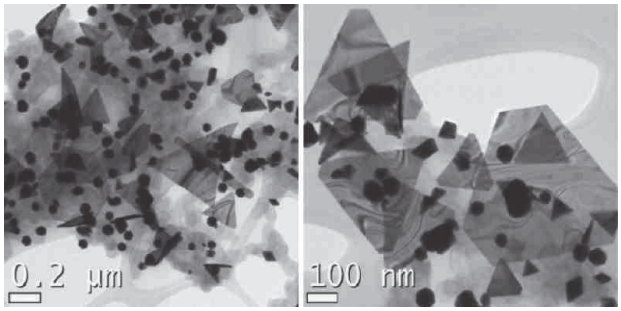
4.1.2 *Gold nanoparticles*

Gold nanoparticles have a broad spectrum of application areas including medicine, food industry, water purification, and biological applications

[112]. In particular, gold nanoparticles are applied to drug-delivery, photo-thermal therapy, imaging, sensing, catalysis, and even antimicrobials [112,113]. The actual list of applications of gold nanoparticles is much longer due to their unique properties. Gold nanoparticles are known to be biocompatible but conventional reduction methods can leave some toxic chemical species on the surface, which may compromise their advantages [114]. Therefore, greenly synthesized gold nanoparticles have much more potential in different fields.

Gold nanoparticles exhibit natural biocidal properties and although their use as antibacterial agents is not so prominent compared to silver nanoparticles, they still have significant antibacterial effects on several pathogens [113,115]. Moreover, green-synthesized gold nanoparticles have also shown their antioxidant [116] and antifungal activity [112,117]. Fungi such as eukaryotes possess more resistant cell boundaries compared to bacteria [101]. The efficacy of gold nanoparticles is therefore manifested in their manifold biocidal effects.

Plant mediated synthesis of gold nanoparticles is usually carried out with chloroauric acid ($HAuCl_4$) as a precursor, which is then added to the given plant extract to be reduced into elemental gold [118]. Greenly synthesized gold nanoparticles using plants have low cytotoxicity compared to chemically synthesized gold nanoparticles. These results refer to several shapes and sizes ranging from spherical to triangular and 10 to 300 nm respectively [119]. Fig. 14.9 shows gold nanoparticles with variable size and shapes have been obtained from alkaline pear fruit extract. Plant extract derived gold



■ **FIGURE 14.9 HR-TEM micrographs of the gold nanoparticles obtained from alkaline pear fruit extract.** *(Source: from Ref. [123]. Copyright License Number 4061301024698).*

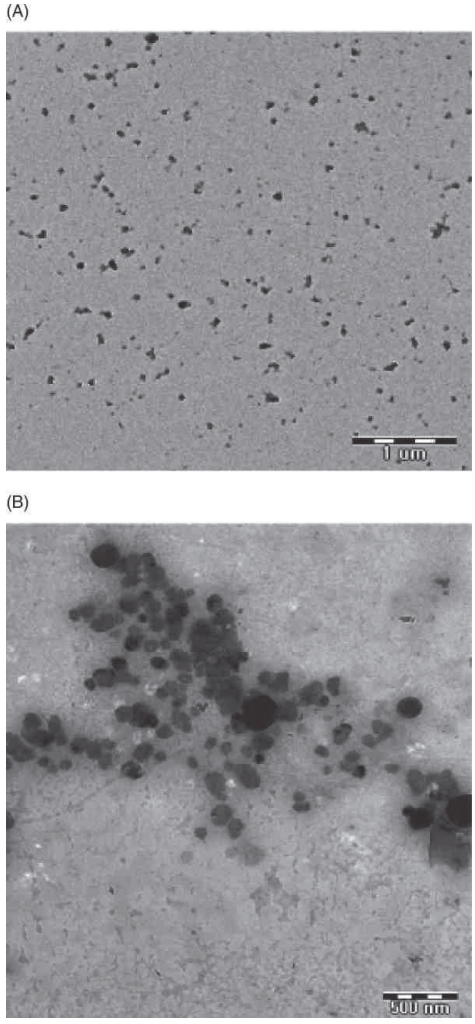
nanoparticles are used to coat acrylic glass [120] and window glass [121], and they also have potential as coatings in food packaging materials due to active bacterial mold protection [112] and antioxidant activity [116]. In the same way, plant mediated gold nanoparticles can be used to cover different fabrics such as cotton, silk, and leather to achieve antibacterial properties [122]. Recent reports have shown an enhanced antibacterial activity of green-synthesized gold nanoparticles due to biomolecules originating from plant extracts [122].

4.1.3 *Copper nanoparticles*

Amongst the noble metals, copper is definitely the most reactive and toxic one [15,124]. Copper is approximately six times cheaper than silver and 24 times cheaper than gold, and Cu nanoparticles exhibit efficient biocidal properties [125]. Some reports have demonstrated that copper nanoparticles can show even greater antibacterial effects than silver nanoparticles [126]. These biocidal properties and other available application areas such as catalysis, anti-fouling, anti-microbial, and energy applications have made scientists investigate for ways to reduce copper nanoparticle toxicity and make them safer to implement in those areas [127,128]. Copper nanoparticles are already used in wound dressing and clothing [129].

Although known to possess efficient antimicrobial properties [21] copper metal nanoparticles are very sensitive to oxygen and are easily oxidized under air, which decreases their efficiency against bacteria [130]. In addition, this sensitivity to oxygen makes them pyrophoric and dangerous to handle under air. Plant mediated synthesis of copper nanoparticles can offer a protection against oxidation as well as toxicity against human cells, when nontoxic capping is provided by biomolecules from plant extracts [15,131]. Therefore biosynthesized copper nanoparticles have great potential for medical applications and coatings and sometimes can possess better anti-bacterial properties compared to conventional copper nanoparticles [132]. The most common precursor salts used for green syntheses of copper nanoparticles include copper sulphates and copper nitrates [21,131]. In Fig. 14.10, Cu nanoparticles produced via magnolia Kobus leaf extract, showing particle size variation as a result of the extract concentration.

Plant mediated copper nanoparticles are reported to be anti-fungal, antiviral, anticancer, anti-parasitic, antioxidant, and are efficient pesticides [131,133]. Due to their lower cost and safer properties, plant derived copper nanoparticle coatings have many possible applications for consumer products in direct contact with humans such as antimicrobial latex foams in mattresses [132] or medical textiles in scrubs and masks [134].



■ **FIGURE 14.10 TEM images of copper nanoparticles.** Formed with (A) 15% and (B) 20% *Magnolia kobus* leaf broth in 1 mmol/L $CuSO_4 \cdot 5H_2O$ solution at 95°C. *(Source: from Ref. [132]. Reproduced with permission from John Wiley & Sons).*

4.1.4 *Bimetallic nanoparticles*

The synergistic effect of plant extract derived silver and gold bimetallic nanoparticles has been reported [13]. Recent investigations have shown that biologically synthesized bimetallic nanoparticles consisting of a gold core and silver shell exhibit efficient antibacterial response to biofilm control of Gram negative and Gram positive bacteria [135]. Bimetallic nanoparticles combining a silver core and a gold shell exhibit higher biofilm inhibition of bacteria compared to single metal nanoparticles [13]. These findings are very promising and urge scientists to combine different metal and metal oxide nanoparticles for achieving the desired effects.

4.2 Antimicrobial mechanism of metallic nanoparticle coating

The exact antimicrobial mechanism of metallic nanoparticles is still to be determined, however it most likely consists of several effects on microorganisms that occur simultaneously [136]. Many metals respond to humidity and are hence ionized through partial oxidation [137]. Metal ion exposure has well known antimicrobial effects. It can bind to tissue proteins, bacterial cell walls, and nuclear membranes, leading to cell modification and lysis [138]. When metallic ions can get inside a bacterial cell, they tend to bind with DNA and RNA molecules and denature them, which in turn disrupts their replication and kills them [137]. For example, silver ions prevent phosphate uptake by replacing them and initiating an efflux of vital compounds such as glutamine, mannitol, proline, and succinic acid [139].

Apart from ions, metal nanoparticles themselves possess a threat to microbes as well. Large surface to volume ratio and surface defects can make metallic nanoparticles more reactive and interaction with bacteria cells becomes more efficient [140]. A size under 30 nm is stated to be the most capable for anchoring to bacterial cells and thereafter infiltrating them [4,140]. As a result, bacterial membrane potentials and absorptivity are disrupted, leading to the death of the microbe [140].

Another key factor for metallic nanoparticle antimicrobial efficiency, apart from their small size, is their surface charge or zeta potential [4]. Nanoparticles with positive surface charge tend to be more efficient in anti-microbial assays [4]. With few exceptions, both Gram positive and Gram negative bacteria generally possess an overall negative charge of their cell wall due to the teichoic acids and lipopolysaccharides respectively [141]. The electrostatic interaction of a negatively charged bacterial surface draws metallic nanoparticles with positive zeta potential to the bacteria membrane and thereon other modes of action continue to alter microbial existence [4].

Another important factor of metallic nanoparticle toxicity against microorganisms is their ability to generate ROS [125]. It is mainly because several metallic ions can accept and donate single electron generating radicals [124]. ROS as radicals can damage lipids and proteins including DNA, which leads to cell death [125,142]. Moreover, the respiratory chain of the cell will be damaged after free radicals alter the adenosine triphosphate (ATP) production [140].

In order to have an efficient antibacterial effect, nanoparticles first have to establish the connection with the cell walls of bacteria. However, bacteria set up natural barriers, such as biofilm formation, when their environment becomes undesirable or when the bacterial colony is under stress. Biofilm is a highly hydrated slime layer that contains mainly polysaccharides. It protects a bacterial colony from outside harm such as harsh temperature or antibiotic substances. A colony that becomes more insensitive to outside effectors tends to become more difficult to destroy [1]. In vitro assays are usually carried out in ideal conditions for a colony, which implies that no stress genes are activated and antimicrobial agents such as metal nanoparticles are therefore more efficient in such conditions. It is therefore easier to prevent bacterial growth compared to killing a well-established colony. Consequently, the positive zeta potential, which is efficient in anti-microbial assays, becomes an undesirable property in the case of nanoparticle coatings. In contrast, negative surface charge helps to minimize the interaction of negatively charged bacteria and the coated surfaces and is therefore preferred [4].

On the other hand, fungal colonies do not usually have the ability to grow biofilms around them. Thus metallic antifungal nanoparticles can easily latch onto their cells. Nevertheless, fungal cells have a hard cell wall; in general, eukaryotic cells are more difficult to penetrate compared to prokaryotic cells, like in the case of bacteria [143]. Nevertheless, if a metallic nanoparticle manages to get inside via endocytosis, for example [144,145], it could directly affect the mitochondria, which are very similar to bacterial cells [146]. It analogously has electron transport, ATP synthesis, and proton motive force, which all can be affected by metallic nanoparticles and their ions [136,147].

5 EXAMPLES OF APPLICATIONS USING PLANT EXTRACT MEDIATED SYNTHESIS

Bactericidal nanoparticle coatings, which are achieved by simple green synthesis methods, have a huge potential in environmentally friendly applications and skin-to-surface contact areas [5]. Eco-friendly plant mediated synthesis protocols for metallic nanoparticles ensure that no toxic chemical

Table 14.3 Coating and Applications of Various Plant Extracts

Plant Mediated Nanoparticle/ Average Size (nm)	Plant Used	Coating Application	Reference
Ag/70–110	*Withania somnifera*	Antimicrobial cream	[103]
Ag/50–100	*Azadirachta indica* (Neem)	Cotton fabric	[107]
Ag/20	*Eucalyptus citriodora*, *Ficus bengalensis*	Cotton fabric	[104]
Ag/7–27	Mango peel	Nonwoven fabric	[105]
Ag/12–16	Vegetable oil	Paint	[5]
Ag/10–20	*Syzygium cumini*, *Bauhinia purpurea*, *Cymbopogon*	PMMA glass[a]	[120]
Ag/10–20	*Erigeron annuus*	Cotton and leather fabrics	[106]
Ag/8–30	*Citrus limon* leaves	Cotton, silk	[149]
Au/10–100	*Syzygium cumini*, *Bauhinia purpurea*, *Cymbopogon*	PMMA glass[a]	[120]
Au/7–3000	*Madhuca longifolia*	Glass[a]	[121]
Au/62	*Abelmoschus esculentus*	Antimicrobial packaging[a]	[112]
Au/12–21	Lignin-containing unbleached softwood pulp	Antioxidant package	[116]
Au	*Ginkgo biloba* Linn leaf	Cotton, silk, and leather fabrics	[122]
ZnO/100	*Pongamia pinnata*	Cotton fabric	[46]
MgO/27	*Emblica officinalis*	Cotton fabric	[48]

[a] *Not designed as antimicrobial coating, but has antimicrobial potential.*

species are absorbed on the nanoparticle surface, which is essential for implementing them in medicine [148]. Although antimicrobial coatings from plant-derived nanoparticles are not so common yet, there are still several cases reported, as discussed earlier (Table 14.3).

6 ADVANTAGES AND DRAWBACKS OF PLANT EXTRACT MEDIATED SYNTHESIS

For the nanoparticle synthesis to take place, three components are required: precursor, reducing agent such as sodium borohydride, and capping or stabilizing agent such as polyvinyl alcohol [150]. Moreover, depending on the chemicals used, serious toxicity towards humans and the environment may be experienced. Therefore, a whole new environmentally friendly approach with sustainable synthesis protocols has to be implemented. The main advantage of plant extract mediated synthesis is the guarantee that no toxic residual products are left on the particles. This is a primordial condition when humans directly consume them via creams or clothes, for example, where even trace amounts of toxic residues inhibit safe applications [88]. Also, the costs of chemical and physical synthesis routes are generally much higher considering the fixed costs of apparatus and reagents. The amount of

secondary products generated is also significant, as it has to be disposed of correctly and safely [151]. Compared to other green methods such as microorganism-mediated syntheses, plant extract mediated synthesis protocols do not include microorganism isolation, identification, growth optimization, culture preparation, and maintenance, which are all complex and challenging procedures [150]. Additionally, plant-mediated green syntheses are generally faster, resulting in shorter production times, which indicate better options for up-scaling, as it is a more straightforward method for green nanoparticle production [24,89,135]. There are, however, still obstacles to overcome compared to more conventional nanoparticle synthesis methods. A main limitation has been the control over sizes and shapes of greenly produced nanoparticles. Shapes and sizes are mostly predetermined by different phyto-chemical compositions with predefined molecule sizes found in the plant. Even the compositions of similar plants grown in different geographical areas or harvested in different seasons can bring about differences in the active components. This, in turn, would affect the size and shape of the nanoparticles precipitated. The latter would engender a drop in their market value as commercial nanoparticles are usually finely tuned. Therefore, finding the right applications and market for plant-mediated nanoparticles is sometimes more challenging [150]. Also, compared to chemical methods, plant extracts contain a large number of active ingredients. Therefore, the isolation and purification of the greenly synthesized nanoparticles from plant material can be a challenge [152]. Still, the positive aspects outweigh the negative ones, which further motivates researchers to refine their plant-mediated synthesis methods.

Mechanisms of nanoparticle formation are mainly associated with biomolecules found in plants, as already discussed. More specifically, these are macromolecules that act as a capping agent and also possess a reducing potential of metal salts. There really is not a universal understanding of the exact mechanism of nanoparticle formation through plant extracts, but there are several reports, which state a whole range of phytochemicals and biomolecules responsible for reduction and capping, such as NADH-dependent reductase, terpenoids, sugars, alkaloids, flavonoids, phenols, tannins, and proteins [153,154]. Almost all these methods mention substances contained in hydroxyl groups (-OH), which are considered one of the main functional groups involved with reduction and formation of different nanoparticles [150]. Further exploration and understanding is required in order to control green synthesis methods in the precipitation of inorganic nanoparticles. For the moment, other than the non-toxicity and synergetic effect of green synthesized nanoparticles, the size and shape distribution of such obtained nanoparticles has to be more controlled. In that regard, the

chemical synthesis route has many years of advances and continues to be the main reason why co-workers prefer it.

7 SUMMARY

Methods using plant extracts as precursors for the production of nanoparticles is environmentally friendly, inexpensive, and easily up-scalable. Scientific literature contains great number of studies that show that the plant extract based syntheses are rapid compared to other syntheses, and can provide nanoparticles with controlled morphology and size. Even though most of the green synthesis reports have been about metal nanoparticle production, more and more studies are performed to apply plant mediated synthesis methods to the production of metal oxide nanoparticle synthesis. Considering that the existing variety of plants is immense, plant systems that serve as biological nanoparticle factories can be set up almost everywhere in the world. In every climate, specific plants exist that have reducing potential and offer additional features depending on their application areas.

The importance of green and clean technologies in science and industry is significant, because we live in an era where mankind should be more attentive to his surrounding environment rather than making progress on its behalf. The plant extract based synthesis methods follow green chemistry procedures and therefore the potential of plants for nanoparticle synthesis has to be investigated and implemented. However, there are several challenges to overcome before it can practically replace conventional synthesis methods. There are still issues about biomolecules involved with reducing and capping processes and their interaction with each other. Consequently, controlling the size, shape, crystallinity and composition of greenly synthesized nanoparticles can be a challenge. Biosynthesis mechanisms can vary quite a lot depending on the plant systems. Also, active biomolecules can differ under certain conditions, although precursors remain the same. Another important factor to consider is the stability of biosynthesized nanoparticles. As mentioned earlier, greenly synthesized nanoparticles are often functionalized by attachment of biomolecules from plants. It is important to ensure that nanoparticles remain stable during their storage with no changes in their composition inside or on the surface before using them in practical applications. It is also important that the functionalized surface is not concealing the intrinsic properties of the green nanoparticles.

A large variety of metal and metal oxide nanoparticles with different shapes and sizes have shown efficient anti-microbial effects on various human pathogens. Considerable amounts have been synthesized by using environmentally friendly routes such as plant extract mediated syntheses. Synergistic effects

of nanoparticles and plant molecules attached to their surfaces are inherently safe for use in medicine and human contact areas as well as improved antimicrobial effects depending on the nature of the given plant. Green metal and metal oxide nanoparticles and their coatings offer an alternative to current medicine in the coming conventional antibiotic "free" future.

ACKNOWLEDGMENTS

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Publication III

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ORIGINAL ARTICLE

Biocidal properties study of silver nanoparticles used for application in green housing

Siim Küünal[1] · Sander Kutti[1] · Protima Rauwel[2] · Mithu Guha[3] · David Wragg[4] · Erwan Rauwel[1]

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Abstract We report on the study of surfactant-free silver nanoparticles synthesized using non-hydrolytic sol–gel methods for applications in straw bale constructions. Micro-organism infestation in green constructions is of concern as their proliferation tends to induce health problems. We demonstrate the biocidal properties of these Ag nanoparticles and their efficacy against fungi. Outdoor tests with Ag nanoparticles have demonstrated the effective protection of straw against micro-organisms. Indoor tests using broth liquid are compared with a method of testing we recently developed where the possible nature of the biocidal properties of the silver nanoparticles are further probed. In contrast to the commonly reported results, this study shows that Ag nanoparticles synthesized using non-hydrolytic sol–gel methods have antifungal properties against common fungi in outdoor conditions which demonstrate high potential in related applications.

Keywords Silver nanoparticles · Sol–gel · Antifungal properties · Green housing · Toxicity

✉ Erwan Rauwel
erwan.rauwel@ttu.ee

[1] Tartu College, Tallinn University of Technology, 51008 Tartu, Estonia

[2] Institute of Physics, University of Tartu, Riia, Tartu, Estonia

[3] Department of General and Molecular Pathology, Faculty of Medicine, University of Tartu, Tartu, Estonia

[4] Department of Chemistry and SMN, University of Oslo, Oslo, Norway

Introduction

During the last decades, silver metal nanoparticles (MNPs) have spurred a lot of interest due to their conductive, optical [1], catalytic [2], and more particularly biocidal properties [3, 4]. In fact, among noble metals, Ag MNPs possess the most efficient biocidal properties with a very broad bactericidal and fungicidal activity spectrum. This specificity makes them the best candidate for developing applications in emerging areas like medicine [5], and a good candidate for applications in the ecological constructions. The importance of green housing is growing every year as ecological materials reduce the construction life cycle energy used and therefore, the environmental impact of the building construction [6]. Straw bales appear to be an ecologically friendly choice for construction due to their low cost, good acoustic [7], and thermal insulation. Nevertheless, their durability and hygroscopic properties make them suitable environments for many harmful microorganisms that can cause many diseases including fungi like *Stachybotrys atra* that appear to be a more important threat than bacteria [8].

The present day solutions rely on chemical products like boric acid or biotol/ammonium chloride compounds that present some drawbacks [9]. All routes to produce Ag MNPs have their advantages, but there are some drawbacks such as cost, scalability, pollution, size, and size distribution. Presently, a lot of effort has been put into the development of "green synthesis" methods [10]. In fact, noble metal nanoparticles synthesis based on the utilization of fungi or plant extracts has spurred a lot of investigation over the world. This paper presents investigations on the antifungal properties of Ag MNPs synthesized by facile and cheap sol–gel synthesis used as a protective agent in houses built using eco-friendly materials.

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Materials and methods

Synthesis of the silver metal nanoparticles

Ag MNPs were synthesized using non-hydrolytic sol–gel methods described elsewhere [11–13]. Silver acetate (99 %, Aldrich) precursor along with benzylamine solvent was used for the synthesis of the nanoparticles. The resulted mixture was transferred into a stainless steel autoclave and was carefully sealed. Thereafter, the autoclave was taken out of the glovebox and heated in an oven at 200 °C for 48 h. The resulting suspensions were centrifuged and the precipitates were thoroughly washed with ethanol and subsequently dried in air at 70 °C.

Characterization of the nanoparticles

X-ray diffraction (XRD) data were collected using a Bruker D8 Discover instrument equipped with a LynxEye detector. Cu ($k\alpha 1 = 1.54056$ Å) radiation selected by a Ge (111) monochromator was used. The crystallite size was calculated from the XRD data using full profile Scherrer methods in TOPAS, with a fundamental parameters peak shape. A Pawley fit based on the lattice parameter for cubic silver was used. The thermal history of the silver nanoparticles was studied under air from room temperature to 800 °C using a Rheometric Scientific STA 1500 Thermogravimetric analysis (TGA) instrument. Transmission electron microscopy (TEM) studies were carried out on a probe corrected Titan G2 80–200 kV operating at 200 kV. The point to point resolutions in TEM and STEM modes are 2.4 and 0.9 Å. X-ray photoelectron spectroscopy (XPS) analysis was carried out on a Kratos Analytical Axis UltraDLD photoelectron spectrometer equipped with Al K_α X-ray source. Liquid broth conditions and agar plate preparation can be found in [9].

Liquid broth tests

Different concentrations ranging from 100 mg/L to 1 g/L of Ag NPs were crushed and dispersed into the liquid broth medium composed of maltose and Chloramphenicol. Then after *Aspergillus* spp. was added to the mixture. Continuous magnetic stirring was performed for 24 h, i.e., during the whole experiment to homogenize the solution of dispersed Ag MNPs.

Coating of the straw bales

Ethanol solutions containing different silver nanoparticle concentrations were prepared by dispersion of the Ag nanoparticles (10 and 40 mg/L) in pure ethanol. The dispersion was improved using a magnetic stirring for 2 days combined with stay in an ultrasonic bath at 35 °C for 30 min to disperse them homogeneously in the solution. The straw bales were dipped for 1 h into the ethanol solution after dispersion and then taken from the vessel for drying under air. The use of a magnetic stirrer enables homogeneous decoration of the straw with Ag MNPs. Then, the straw bale samples were placed into aggressive outdoor conditions for 18 days covered from direct rainfall and sunlight. The samples were then cut and printed into agar plates for 72 h under 32 °C for microorganism staining and identification.

Identification

For identification, the microorganisms were heat fixed on microscope slides. The fungal cells were stained using 5 % bengal red solution. To acquire better visualization, all of the fungal slides were stained three times. After drying of the slides, the identification was carried out via microscopy using online databases. To check whether bacteria were Gram-positive or Gram-negative, the bacteria were stained using Gram‘s method as described by the manufacturer (Sigma–Aldrich).

Toxicity study

Different concentrations of nanoparticles were studied via MTT assay (M). Human embryonic kidney (HEK) cells [14] were seeded on day 0 at a density of 1000 per well in 96-well microtiter plates. On day 1, silver nanoparticle at different concentration was added. After 24 h incubation, the medium containing nanoparticles was removed from the plate to make sure that no nanoparticles remain in the solution and avoid overlap or hinder MTT assay. After 24 h, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well (0.5 mg/mL; Sigma–Aldrich), and plates were maintained at 37 °C for 2 h. The medium was then discarded, and DMSO was added to each well to lyse the cells. Absorbance was measured at 450 nm using a multiwall spectrophotometer (Tecan, microplate reader). All MTT assay were repeated twice.

Discussion

The utilization of 482 mg of silver acetate precursor enabled the production of 296.7 mg of pure Ag MNPs giving an average reaction yield of 95.15 ± 5 % that was calculated with five syntheses. This shows that the reaction process is very efficient and economically interesting. Figure 1 shows a typical XRD pattern of the prepared Ag nanoparticles. It can be clearly seen from the XRD pattern

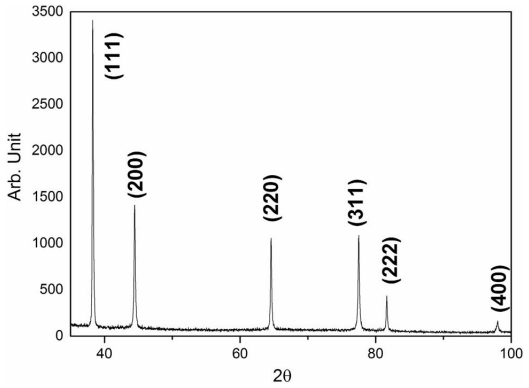

Fig. 1 XRD pattern of cubic silver nanoparticles

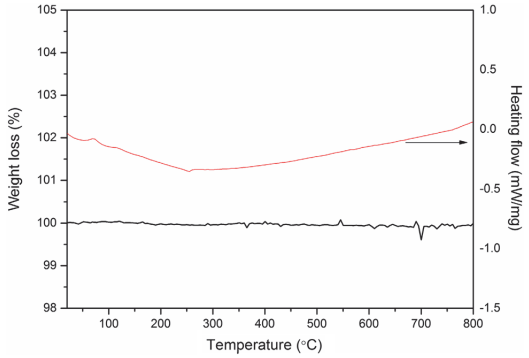

Fig. 2 TGA and DTA performed under N_2 of silver nanoparticles

that the Ag NPs are single phase nature and only exhibit the characteristic diffraction peaks of the Ag metal cubic structure (JCPDS File No 87-0720). No secondary phase of silver oxide structure was detected by XRD and the high intensity of the XRD peaks indicates that the Ag NPs are highly crystalline.

Thermogravimetric analysis was performed on the Ag nanoparticles to evaluate their purity and estimate the amount of organic species adsorbed on the silver nanoparticle surface and their resistance against oxidation under air. Typical thermogram in Fig. 2, does not show any weight loss or gain during the whole measurement till 800 °C, suggesting the absence of organic species on the surface (surfactant-free) and the high stability against oxidation. Differential thermal analysis (DTA) shows a very slight decrease of the heat capacity (−0.4 mW/mg) with the increase of the temperature that could be attributed to a probable aggregation of the Ag MNPs together.

The morphology and size distribution of Ag nanoparticles were also studied by (S)TEM. The HAADF-STEM electron micrograph in Fig. 3a gives an overview of the nanoparticles. The size distribution of the nanoparticles lies within a range of 5–20 nm. A few larger particles were also observed with sizes of around 50 nm. The HRTEM image in Fig. 3b is of a 10 nm crystalline Ag nanoparticle oriented along the [110] zone axis of the Fm-3 m cubic structure with a lattice parameter of 0.4 Å.

XPS measurements were performed on Ag MNPs to confirm their purity and study the nature of their surface. XPS study was performed 6 months after the synthesis of the Ag NPs that were stored in the powder form under ambient air. Figure 4 presents the XPS survey spectra, showing only binding energy peak from Ag metal [15] and no visible peak of nitrogen (see inset) or oxygen. The carbon binding energy peak is certainly due to air contamination that cannot be avoided. Due to the nanosize of the Ag MNPs and the high surface to volume ratio, any oxidation of the Ag MNPs surface would be detected by XPS analysis. The position of the Auger peak corresponds here to metallic silver. In addition, the Ag 3d5/2 photoelectron peak corresponds to metal Ag (368.3 eV), thus confirming the purity and the metal nature of Ag MNPs. The XPS study then confirm the purely metallic nature of these Ag NPs and their high stability against oxidation.

Two methods to test the biocidal properties of Ag were used: broth liquid solution and decoration of the straw with Ag MNPs dispersed in ethanol solutions at different concentrations. The antifungal study based on broth dilution assay on *Aspergillus* spp. showed that this method was not an adapted one as the Ag MNPs are surfactant-free. In fact, the broth medium contains amino acids that contain several chains of sulfur groups and these groups are able to bond on Ag MNPs surface and then passivate them, neutralizing their biocidal properties. For this reason we have developed a second method that consists of immersing the straw samples into a vessel filled with a solution containing Ag MNPs dispersed in ethanol for 1 h under magnetic stirring and then taken out for drying under air. A ratio of 1 mg and 2.5 mg of Ag MNPs for 1 g of straw were dispersed in ethanol. Previous tests showed that concentration of 100 mg/L of Ag NPs was sufficient to observe a biocidal effect [11]. Two reference samples where straw samples dipped into pure ethanol or water were also prepared. The four samples were covered and contaminated by placing them in outdoor conditions for 21 days. Covering them ensured protection from direct sunlight and rainfall during the month of May which most probably also promoted the rapid development of microorganisms. After 3 weeks, some straws were taken from each straw bale and deposited directly onto the agar plates [11] and printed into agar plates for 72 h at 25 °C for microorganisms staining and identification.

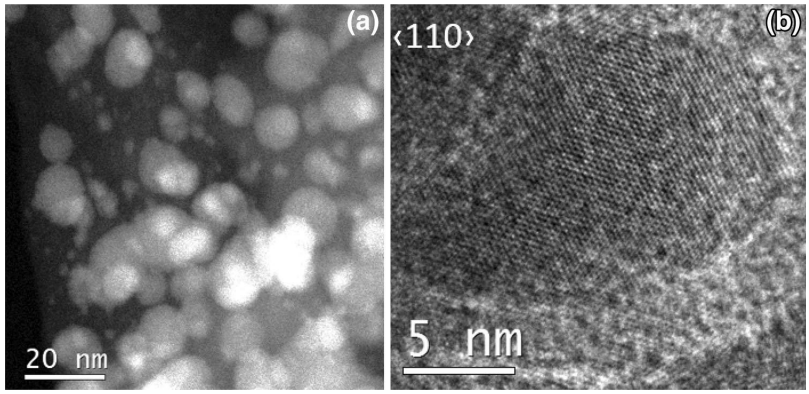

Fig. 3 **a** STEM image overview of Ag nanoparticles dispersed on a carbon grid. **b** HRTEM image of Ag nanoparticle oriented along [110] zone axis

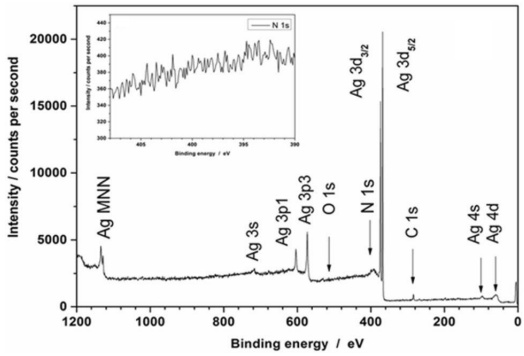

Fig. 4 XPS survey of Ag MNPs, inset shows the absence of N binding energy peak

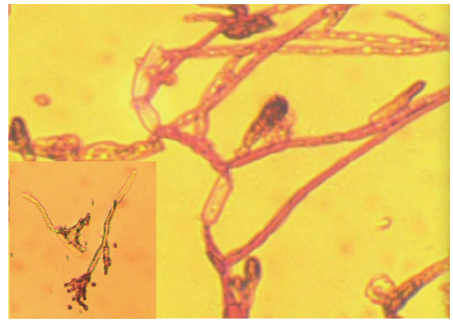

Fig. 5 *Penicillium* hyphae and conidiophore (*small picture*) isolated from untreated straw

From the first observation, bacteria were present on all straw samples due to the highly nutritious agar which promoted the growth of bacterial colonies; Gram-positive and Gram-negative bacteria were equally represented and no specific effect on one particular bacterial group was witnessed (Fig. 5). However, the agar plates which contained straws treated with silver nanoparticles, showed differences in the straw surface (color and smoothness) compared to untreated counterparts (Fig. 5c, d). Untreated straw displayed color change due to the direct growth of colonies on the straw surface. Additionally, treated samples did not contain any visible fungal activity that was observed in the case of untreated and only ethanol-treated straw. In fact, fungi need less favorable conditions including low moisture content to grow in houses [15]; therefore, the absence of fungi in this study was rewarding.

Penicillum spp. and *Cladosporium* spp. were clearly identified on untreated and ethanol-treated straw (Fig. 5).These fungi species are known to produce hazardous mycotoxins, which compromise the health of house residents due to their allergenic and probably even carcinogenic properties [16, 17]. Moreover, some fungal spores retain their toxicity even after their death, indicating that threat to health is persistent once mold has grown on the building material. The difference is clearly displayed in Fig. 6, where straw bale samples treated with ethanol solution containing 1 and 2.5 mg of Ag MNPs per gram of dry straw showed less microorganism growth on the straw surface and complete absence of fungal growth on the plates.

The antimicrobial mechanism of silver nanoparticles is not yet fully understood; however, experimental data suggest a combination of multiple effects on microorganisms that can occur simultaneously or separately [16–21]. In addition, the experimental evidence also shows that silver nanoparticles are normally more effective against bacteria than fungi [22]. Fungi are eukaryotes and have more resistant cell boundaries viz., a cell wall, which consists of hard substance called chitin. Compared to bacteria, fungal cells should in principle be less affected by silver nanoparticles. However, for the series of outdoor tests, we

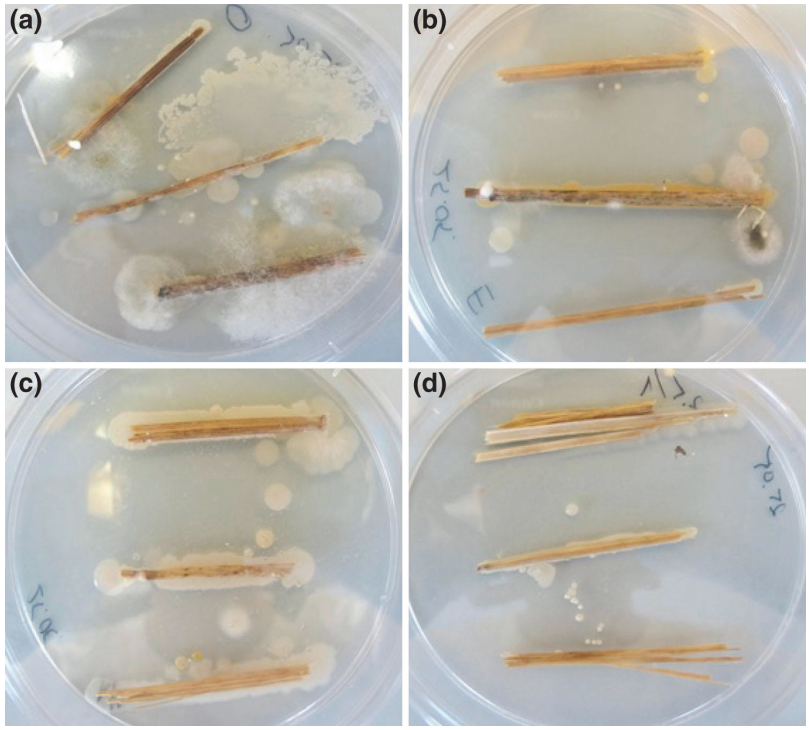

Fig. 6 Straw printed to the plate count agar (PCA) after 72 h at 25 °C, **a** untreated straw, **b** straw soaked with ethanol, **c** straw treated with 1 mg of silver nanoparticles per gram of dry straw, **d** straw treated with 2.5 mg of silver nanoparticles per gram of dry straw

observed that typical bacteria that colonize straw seem to be less sensitive to the Ag MNPs than the typical straw-colonizing fungi.

Due to the metallic nature of the surface of the nanoparticles and the absence of oxidized silver on their surface, the most probable origin of the biocidal properties is through direct contact. In fact, no Ag cation could be released from a purely metallic surface. Nevertheless, one explanation of this phenomenon implies that bacterial colonies have the capacity to grow a protective biofilm around them, when facing aggressive environment. This biofilm is usually not produced in agar plates as they tend to be in a highly favorable environment. This biofilm is not easily penetrable and, therefore, can provide protection to colonies against Ag MNPs. On the flip side, the biofilm also self-limits the growth and expansion of the bacterial colonies. This is further manifested by the clean surfaces of the treated straw, not affected by bio-organisms. The latter demonstrates that Ag nanoparticles can inhibit the growth of biofilm protected colonies by confining them to only certain areas and curbing their proliferation. Furthermore, the absence of color change on the straw treated with silver nanoparticles supports the claim that silver nanoparticles specifically inhibit fungal development. Absence of fungal colonies on the agar plates, which contained silver

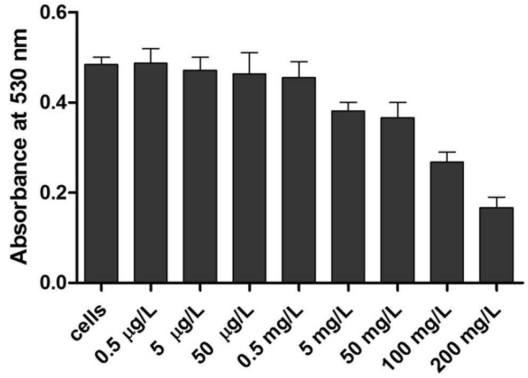

Fig. 7 Toxicity test of silver metal nanoparticles on human HEK cells

nanoparticle-treated straw, can be explained by the fact that these fungal colonies cannot generate protective biofilm layers around them. Contrary to the results of our experiment, in real life environmental stress conditions fungi tend to dominate over bacterial colonies because they need less suitable conditions to proliferate [15]. As a result, fungi are more problematic within the walls of residential buildings. This study shows that Ag MNPs dispersed in ethanol can

Table 1 Result of MTT assay test

Concentration of Ag MNPs	0	5 µg/L	50 µg/L	0.5 mg/L	5 mg/L	50 mg/L	100 mg/L	200 mg/L
Cells survivability	0.4680	0.4420	0.4167	0.4197	0.3627	0.3313	0.2457	0.1427

be a promising ecological option for the protection of straw bales that are used in eco-housing and leading a healthy lifestyle and taking into account that the cost of treatment Ag treatment is similar to conventional chemical treatment in straw bales.

Before using these silver nanoparticles as antibacterial and antifungal treatment for the protection of straw bales, it is necessary to study the toxicity of these Ag MNPs against human cells. HEK cells are very commonly used for testing the toxicity of metal nanoparticles [23]. Therefore, the cytotoxicity of these Ag MNPs was investigated on HEK cells. Different concentrations of Ag MNPs dispersed in PBS solution were studied by MTT assay. Figure 7 shows MTT assay performed on Ag MNPs solutions of concentration ranging from 10 to 200 mg/L. Figure 7 shows the mean ± SEM of duplicate measurements of a representative sample of three independent experiments. This toxicity study toward HEK cells shows that mortality rate is over 50 % only for very high concentration of silver nanoparticles (200 mg/L). For a lower concentration, the Ag MNPs are not toxic to human cells (Table 1), which makes them a suitable material for antimicrobial and antifungal treatment applications.

Conclusion

This study supports the fact that the origin of the biocidal properties of the Ag MNPs produced by non-hydrolytic sol–gel method is related to direct contact of the Ag MNPs with the micro-organisms. In fact, these Ag MNPs are surfactant-free and their surface is metallic preventing ion release in the medium. In addition, fungal species were more affected than bacterial colonies that can produce protective biofilm. These results suggest that it is possible to apply Ag MNPs in ecological straw bale construction as a protective agent. MTT assay toxicity showed that these Ag NPs are only toxic for high concentrations. Unlike several chemical repellents, which are toxic to human health and degrade over time, Ag MNPs represent a cheaper, harmless, and more permanent solution for eco-friendly house development.

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Publication IV

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New methodology for the antifungal testing of surfactant-free silver metal nanoparticles for applications in green housing

Siim Küünal[1,a], Sander Kutti[1], Protima Rauwel[2], David Wragg[3], Irina Hussainova[4,5] and Erwan Rauwel[1,b*]

[1] Tartu College, Tallinn University of Technology, Puiestee 78, 51008 Tartu, Estonia
[2] Institute. of Physics, University of Tartu, Ravila 14c, 51010 Tartu, Estonia
[3] Univ. of Oslo, Dpt. of Chemistry and SMN, N-0315 Oslo, Norway
[4] ITMO University, Kronverksky 49, St. Petersburg, 197101, Russian Federation
[5] Dept. of Mat. Eng., Tallinn University of Technology, Ehitajate tee 5, 19086 Tallinn, Estonia

[a]siim.kuunal@ttu.ee, [b]erwan.rauwel@ttu.ee*

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Abstract

This work presents a method that enables the cheap production of surfactant-free stable metal nanoparticles in air. The silver nanoparticles are good candidates for new applications in green housing. Recently, we demonstrated usage of air-stable silver MNPs as biocidal coatings instead of chemical agents for protection of straw bales used in a green housing construction. Application of the silver nanoparticles as antifungal agents needs a method that does not neutralize their biocidal properties as a result of the testing method. In this study, we discuss the usual methods that are used for the antibacterial property testing and the development of a method that allows demonstrating the antifungal properties of metal nanoparticles.

Introduction

Silver nanomaterials are the second most referenced nanomaterials after carbon and the leader for commercial applications among all metals. Silver nanomaterials have a wide range of applications due to their optical [1], catalytic [2], electrical [3] and antimicrobial properties [4] that are highly dependent on their shape, size and structure [5]. Silver metal nanoparticles (Ag MNPs) have proved to be one of the most effective antimicrobial agents among the different types of nanomaterials owing to its biocidal properties against bacteria, viruses and other eukaryotes. In addition, the low toxicity of Ag NPs against human cells has also been demonstrated [6]. Numerous studies are being conducted in order to exploit these properties viz., disinfection of medical tools, burn and wound treatment, dental materials, coatings for stainless steel materials and textile fabrics; water treatment, sunscreen lotions [7, 8] and also in ecologically friendly construction as relatively harmless repellents [9].

The housing construction field is beginning to put more and more emphasis on occupant health and is aiming at decreasing environmental pollution. Statistically, approximately 10% of the total energy used worldwide is related to the construction materials [10]. Therefore the importance of the materials that require minimal processing and have little embodied energy is becoming increasingly apparent. Straw is a natural and renewable material. A crop farmer needs only a very small amount of straw to maintain the quality of the agricultural soil and all the excess is discarded as waste. Straw can be compacted into bales and stacked up to form walls in the construction of a building. Theoretically only a few acres of land are sufficient for the annual production of straw in order to construct a modest home [11], which makes it a promising resource in the green housing industry. However, the main drawback comes from the hygroscopic properties of straw that can lead to mould growth within the bale walls. Several moulds like *Stachybotrys atra* are known to present risks to the health [12],[13]. In suitable humid conditions, moulds can proliferate and it is then difficult to stop their propagation[14].

Conventional fungicides include chemical compounds such as boric acid, ammonium chloride compounds and other environmentally harmful and toxic agents. These chemicals are used to treat construction timber. This calls for implementation of a greener alternative, more adapted to green construction. In addition, chemical compounds have variable life times [15]. The utilization of Ag MNPs appears to be an ecologically friendly alternative that presents safer aspects and lower degradability over time. The present method of synthesis is cheaper than the cost of chemical treatment. Ag MNPs have already been broadly implemented as a new generation of antimicrobial solution [16-19].

During the last decade, many reports on the study of Ag MNPs antibacterial properties have been published. However, the antifungal properties of Ag MNPs have not stirred much interest. In this paper, the effects of surfactant-free stable Ag MNPs on microorganisms and more specifically on fungi that appear as the most important threat for straws were investigated.

The usual *modus operandi* for antibacterial properties testing is based on the use of petri dishes or agar plates that provide all necessary nutrients to the micro-organism to grow in the best conditions and can be combined with disk-diffusion schemes to investigate the antibacterial properties of antibiotics. Mueller-Hinton agar culture medium is commonly used for antibiotic testing [20]. The susceptibility of the biocidal properties of Ag MNPs can also be estimated via micro broth dilution assays of the Clinical and Laboratory Standards Institute[21]. However, because Ag MNPs are surfactant free, these methods cannot be directly applied to the antifungal study and it was necessary to develop a new method of testing.

Materials and methods

Synthesis and method. Ag MNPs were prepared by non-hydrolytic sol-gel method [22, 23] using Ag acetate precursor (99%, Aldrich) and have been described in a recent paper published elsewhere [9]. The prepared Ag MNPs in ethanol solutions (10 mg/L and 40 mg/L) were poured into two vessels, which contained straw bales samples (10 g). A description of the method is provided figs. 1. The use of a magnetic stirrer enables homogeneous decoration of the straw with Ag MNPs. The straw samples were dipped into the solution for 1 hour and then taken from the vessel for drying under air. Then the straw bales samples were placed into more aggressive outdoor conditions for 18 days covered from direct rainfall and sunlight. The samples were then cut and printed into agar plates for 72 hours under 32˚C for microorganism staining and identification.

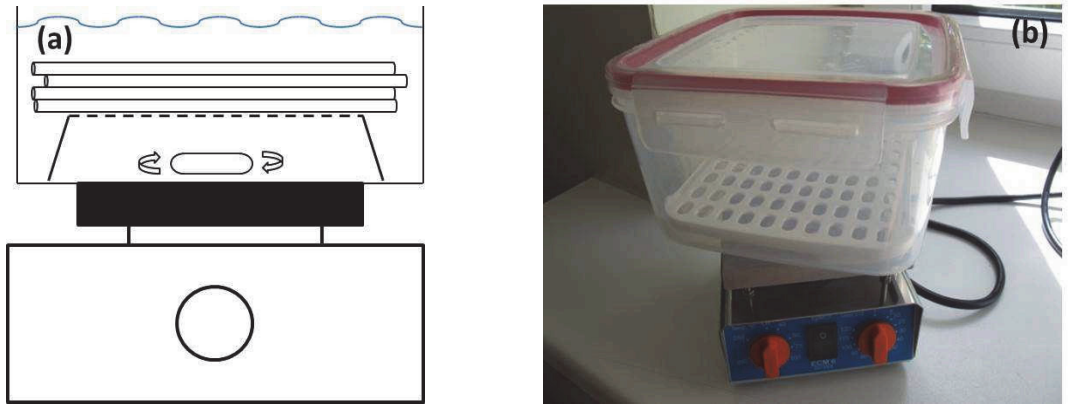

Figure 1. (a) Scheme and (b) photo of system of Ag NPs dispersion on the surface of the straw.

Characterization. Ag NPs were characterized by X-ray diffraction (XRD) and transmission electron microscopy (HRTEM). XRD patterns were obtained using a Bruker D8 Discover instrument equipped with a LynxEye detector. Cu ($k_{\alpha 1}$=1.54056) radiation selected by a Ge (1 1 1) monochromator was used. Transmission electron microscopy (TEM) studies were carried out on a probe corrected Titan G2 80-200 operating at 200 kV and disposing a point to point resolution of 0.8 Å in STEM mode

New protocol for straw bale test. Implementing metal NPs as antimicrobial agent on the straw bales is a novel idea. This suggests that a new protocol has to be developed for testing the metal nanoparticles antifungal and antibacterial properties and how to sufficiently cover straw bales.

Discussion

Structural properties. XRD pattern on fig. 2a shows that Ag MNPs are highly crystalline with the face-centered cubic structure (JCPDS File No 87-0720). From the XRD pattern, the Scherrer method applied to the 111 and 200 reflections estimated an average crystallite size of 58 nm. The HRSTEM study showed that Ag NPs are spherical with a size distribution ranging from 2 to 10 nm, but several bigger Ag NPs of 50 nm were also observed, suggesting that the value obtained by Scherrer calculation is not misleading. Thermogravimetric analysis did not show any weight loss, confirming the absence of organic species on the surface of the Ag MNPs [9].

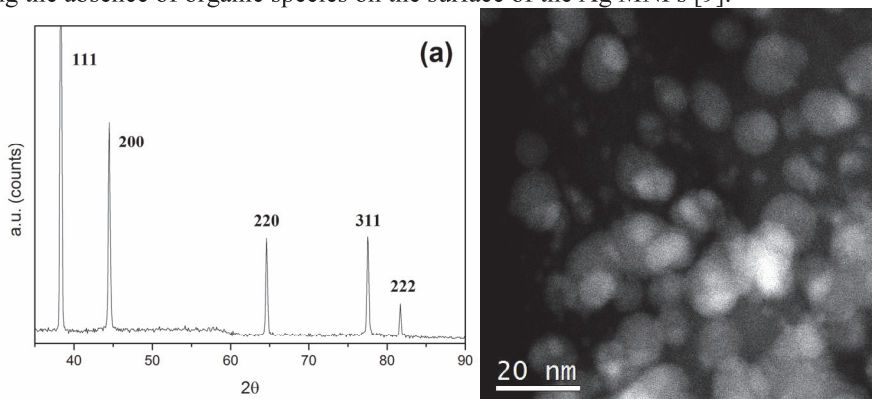

Figure 2. (a) XRD pattern and (b) HAADF-STEM image of silver nanoparticles**.**

The antifungal properties of silver nanoparticles. In a first approach, test was based on broth dilution assay on *Aspergillus* spp. Different concentrations of Ag NPs were first crushed and dispersed into the liquid broth medium composited of maltose and Chloramphenicol. Then after *Aspergillus* spp. was added to the mixture. Continuous magnetic stirring was performed for 24 hours, i.e. during the whole experiment to homogenize the solution of dispersed Ag NPs. No visible antifungal property was clearly observed during this test and *Aspergillus* spp. developed in the broth medium (Fig. 3). The color difference between liquid broth containing no silver nanoparticles (Fig.3a) and all other mixtures (Fig.3 b-e) indicates that Ag NPs reacted with liquid broth medium. However, for the highest concentration of Ag MNPs (1g/L) a visible decrease of the fungi development was observed, but did not totally prevent its development (Fig. 3e). From this experiment we concluded that it is not possible to test the antifungal properties of the Ag NPs via this method. In fact, the culture medium consists of many amino acids that contain several chains of sulphur groups. TGA and x-ray photoelectron spectroscopy (*not shown here*) have demonstrated that Ag NPs are not oxidized and have no organic species on their surface, which make them very sensitive to sulphur groups that attach on their metallic surface and neutralize their biocidal properties. So, the liquid broth media, which ideally can be used to test antimicrobial inhibition or mortality rate, reacts with the Ag NPs. However, from this study, antifungal property was demonstrated for high Ag NPs concentration (1g/L). To study the biocidal properties a specific method adapted to straw coating and pure surfactant-free Ag MNPs was necessary to develop.

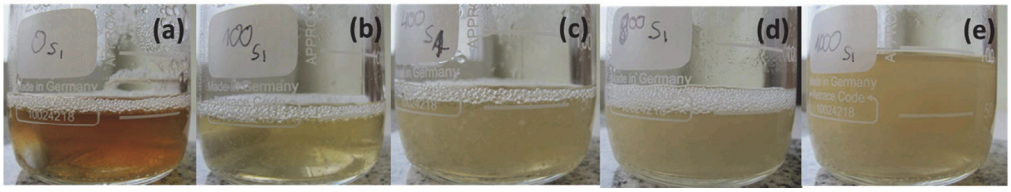

Figure 3. Micro broth dilution assays test on *Aspergillus* spp. (a) reference (b) 100mg/L (c) 400mg/L (d) 800mg/L (e) 1000mg/L of Ag MNPs.

Several tests on agar plates showed that lower concentrations of silver nanoparticles cannot prevent the micro-organism growth under the ideal conditions for fungi and bacteria (32°C and highly nutritious agar culture medium). It appears that Ag MNP concentrations, which were sufficient in real life conditions, were not efficient due to the diffusion into agar plate. Agar is a porous polymer and during the pre-treatment of the plates, Ag MNPs could easily have diffused into the agar medium. Therefore, during their cultivation, micro-organisms encounter less Ag MNPs, which promotes their colonization.

Antimicrobial tests under outdoor conditions. The test method is based on coating the straw using a solution of ethanol containing different Ag MNPs concentrations. For testing against specific fungi, treated straw bales can be contaminated directly with species, which is most relevant for research. However from the green housing aspect, direct outdoor storage of bales gives a better overview. Treated straw was protected from direct sunlight and rain and was in direct contact with the soil that contained a wide range of microorganisms.

In this paper, an 18 day outdoor test of was conducted. Four representative straw-bale samples were prepared. Two of the samples were treated with a solution of containing 2.5mg and 1mg of Ag MNPs per 1g of straw. One sample was treated with pure ethanol that was used as a solvent for first two experiments. The last sample was use as a reference and remained untreated. After 18 days of outdoor storage during the month of May, which should promote the development of microorganisms, straw samples were then taken from each bale and printed on the universal plate count agar (PCA) plate. The agar plates were then stored for 72 hours at 32˚C.

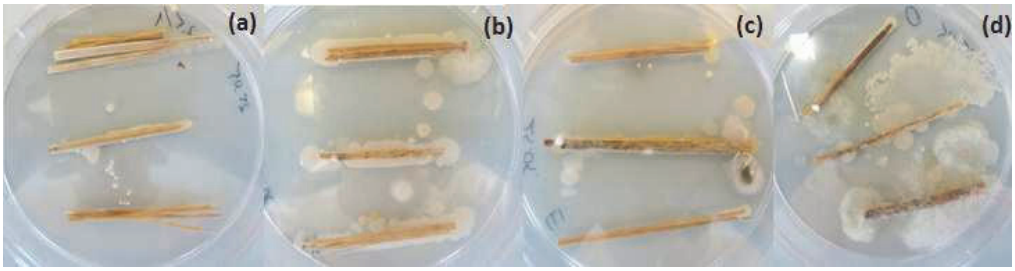

Figure 3. Straw samples after 72 hours at 32°C on PCA plate: (a) 2.5mg/g; (b) 1mg/g; (c) ethanol; (d) no treatment.

Universal PCA agar medium was used to obtain all the microbial activity from the straw bales. The current paper focuses more on fungi, which tend to be a bigger threat in households [12]. Bacteria will be discussed in future publications. Untreated straw (Fig. 3d) showed a wide spectrum of micro-organism colonies that develop in such a climate. Similarly to the untreated straw, the sample treated with ethanol also showed bacteria and fungi development (Fig. 3c). A significant difference came from the treated samples. No fungal activity was observed, i.e. absence of filamentous mycelium from the agar plates was noticed. However, straw treated with the lowest Ag MNPs concentration (1mg/g) showed some bacterial growth (Fig. 3b). The samples from the straw bale treated with 2.5mg/g of Ag MNPs showed a visibly lower microbial activity than the rest of the samples (Fig. b-d), demonstrating a sufficient concentration for application in antifungal treatment.

One important aspect of this method is that only fungal colonies that are growing directly from the straw can be taken into account. In fact, fungi spread using spores that can move away from the protected straw, and then develop on highly nutritious media. In this study we observed that the treated straw was not attacked by micro-organisms and further strengthens the efficiency of this new methodology. On the other hand the micro-organisms spread out to the rest of the agar medium and formed colonies.

Conclusion

The utilization of Ag MNPs as antifungal repellent in straw bale construction materials was investigated. Structural studies of Ag MNPs demonstrate that they are spherical, stable under air and surfactant free. A new protocol was developed to efficiently cover the straw with Ag MNPs. The biocidal properties of the Ag MNPs were studied in harsh environmental conditions. Problems of in vitro testing were pointed out and antifungal properties were demonstrated. This study showed that tests in outdoor conditions are far more efficient than indoor in vitro tests. Problems lie in the fact, that in ideal growth conditions (high nutrient availability and warm humid environment) Ag MNPs cannot hinder microbial growth in the same concentrations than on straw. We also highlighted the fact that liquid media containing amino acids cannot be used for surfactant free Ag MNPs antifungal properties testing. However, in real working conditions Ag MNPs work at low concentrations and offer antifungal properties with ethanol solution containing 1mg of silver nanoparticles per 1g of straw. Mould growth, which has been demonstrated as the most significant drawback to widespread applications of straw bales as building materials, can be restricted also with more environmentally friendly methods rather than hazardous chemicals. Other investigations to further consolidate the study are under way and will be the topic of future publications.

Acknowledgments

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Publication V

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Silver Nanoparticles Study for Application in Green Housing

S. Küünal[a], S. Kutti[a], M. Guha[b], P. Rauwel[c,d], D. Wragg[e], G. Nurk[f] and E. Rauwel[a,*]
[a]Tartu College, Tallinn University of Technology, 51008 Tartu, Estonia
[b]Univ. of Tartu, Faculty of Medicine, Dpt. of General and Molecular Pathology, Tartu, Estonia
[c]Dept. of Physics, Univ. of Oslo, Sem Saelandsvei 24, Oslo, Norway
[d]Institute of Physics, Univ. of Tartu, Ria, Tartu, Estonia
[e]Univ. of Oslo, Dpt. of Chemistry and SMN, Oslo, Norway
[f]Institute of Chemistry, Univ. of Tartu,Ravila 14a Str., Tartu, Estonia
[*]erwan.rauwel@ttu.ee

Abstract

We report on antibacterial and antifungal properties study of silver nanoparticles in applications relating to straw bale construction. Humidity related growth of microorganisms in green constructions is of concern as it can induce health problems among the house residents and also can cause lower durability of ecologically friendly construction materials. We provide a comparative analysis of the effects of silver nanoparticles on most common house-colonizing fungi and bacteria. Outdoor tests demonstrated an efficiency of the silver nanoparticles for fighting micro-organisms. Indoor tests in petri dishes were then performed and compared with outdoor tests and the possible nature of the biocidal properties of the silver nanoparticles is probed. In contrast to the commonly reported results, we observe that antifungal properties of silver nanoparticles against common fungi in outdoor conditions show great potential in related applications.

Introduction

The interest of noble metal nanoparticles such as silver dates back from centuries, when artisans added noble metal nanoparticles to glass and ceramics causing them to glitter and scatter light. Nowadays the emphasis is mainly on their conductive, optical [1], catalytic [2] and antibacterial properties. In fact, among noble metals, silver nanoparticles possess the most efficient antibacterial properties with a very broad bactericidal and fungicidal activity spectrum. Silver nanoparticles were recently used in the development of disinfecting medical devices, antibacterial burn wound bandage, water filter, antibacterial clothes, etc. This specificity makes them the best candidate for developing applications in emerging areas like medicine [3], but also a good candidate for applications in the ecological constructions as antimicrobial repellents. The importance of green housing is growing every year as people become more aware about the large energy consumption in the civil engineering sector and the irreversible effects it could have on our environment. The utilisation of ecological materials reduces the construction life cycle energy used and therefore, the environmental impact of the building construction [4]. Therefore, the choice of the main construction material should be based on the location of the intended building construction and straw that is an agricultural by-product, is generally in the vicinity of construction sites. Although, it is considered as a waste material, straw bales contain little embodied energy making them ecologically friendly choice for construction [5]. In addition to its low cost, a sufficient

amount of straw provides good sound [6] and thermal insulation properties. The main issues with ecologically green building materials such as straw bales are their questionable durability and the fact that they are suitable environments for many harmful microorganisms if humidity is captured within walls. Several fungi and bacteria commonly found indoors can induce many diseases.Straw bales can provide food for decay fungi, wide-spread, long term fungal activity can destroy a straw bale home. In addition to decay of the straw, decay fungi are a concern because mold fungi release spores. High concentrations of mold spores in indoor air can cause health problems in infants or the elderly, lung disease and allergies [7].

Present day solutions propose chemical products like boric acid, sodium hypochlorite or Biotol. These chemicals are used to treat construction timber due to the similar compactness as straw and similar types colonizing bacteria and moulds. However, these chemical agents do not meet the eco-friendly criteria due to their toxicity, volatility and most importantly the health risks to humans and animals. In addition, less harmful agents such as boric acid suffer from low efficiency and those presenting high antifungal and antibacterial properties contain ammonium and chloride compounds, which are too toxic for applications in eco- construction [8]. A recent report show that silver coating appears to be the most effective antibacterial agent compared to the other available solutions [9]. Many chemical and physical methods were reported for the synthesis of silver nanoparticles. Both routes have their advantages, but also their drawbacks that can be related to cost, scalability, pollution, size distribution, etc. More recently, a lot of efforts were put into the development of “green synthesis”, but particles size distributions are still to be controlled by these methods [10]. Implementing silver nanoparticles can provide the impact needed for ecologically suitable criteria. Many recent studies have demonstrated the efficient antifungal and antibacterial properties of silver nanoparticles against common bacteria and fungi, which tend to proliferate in damp buildings [11-13]. This study presents the investigations on the antimicrobial properties of silver nanoparticles used as a protective agent against microorganisms that can develop into houses built using eco-friendly materials.

Experimental

Synthesis

The synthesis of Ag NPs was carried out under air and is based on non-hydrolytic sol-gel method developed elsewhere [14, 15]. Silver acetate (99%, Aldrich) precursor along with benzylamine solvent was used for the synthesis of the nanoparticles. The resulted mixture was transferred into a stainless steel autoclave and was carefully sealed. Thereafter, the autoclave was taken out of the glovebox and heated in a furnace at 200℃ for 48 hours. The resulting suspensions were centrifuged and the precipitates thoroughly washed with ethanol and subsequently dried in air at 70℃.

Characterization

The silver nanoparticles were characterized by the thermogravimetric analyses (TGA), X-ray diffraction (XRD) and transmission electron microscopy (TEM). The thermal analysis of silver nanoparticles was carried out using Netzsch STA 449 F3 Jupiter instrument with a heating rate of 5^{o}C/min under flowing air atmosphere. The samples were heated from room temperature to 800^{o}C. XRD patterns were obtained using a Bruker D8 Discover instrument equipped with a LynxEye detector. Cu (kα1=1.54056) radiation selected by a Ge (1 1 1) monochromator was used. Crystallite size analysis from the XRD data was carried out using

full profile Scherrer methods in TOPAS, with a fundamental parameters peak shape. A Pawley fit using the lattice parameter for cubic silver was used. Transmission electron microscopy (TEM) studies were carried out on a JEM2010F operating at 200 kV and disposing a point to point resolution of 1.9Å and a probe corrected Titan G2 80-200 kV operating at 200 kV.

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Straw bale sample preparation and test

Ethanol solutions containing different silver nanoparticle concentrations were prepared. 20mg, 50mg and 100 mg of Ag nanoparticles were dispersed in 1L of pure ethanol and also in 1L of distillate water for the indoor tests. The dispersion of the Ag nanoparticles was improved by using a magnetic stirring for 2 days combined with stay in an ultrasonic bath at 35 ℃ for 30mins in order to homogeneously disperse them in the solution. For the outdoor tests, the silver nanoparticles in the solution containing 100mg/L of nanopowder were spread on the 70 x 250 x 50 mm straw bale. For comparison the other straw bale was left untreated and a third straw bale sample was immersed in pure ethanol. After ethanol evaporation, the three samples were placed into outdoor conditions for 14 days covered from direct sunlight and rainfall. After outdoor storage, some pieces of straw were taken from the bale samples and then printed in agar plates for 96 hours under 25^{o}C for microorganisms staining and identification. Bacteria and fungi were cultivated in two different types of specific media. The fungal media consisted of 15g of agar, 20g of malt extract, 0.2g of chloramphenicol and 1000ml of deionized water. The bacterial media consisted of 15g of agar, 40ml of tryptic soy broth, 0.8g of cycloheximide in 40ml of ethanol, 960ml of deionized water. All types of growth media were autoclaved at 120^{o}C and poured to Petri dishes to dry over-night. The cultures were grown on the petri dishes at 25^{o}C. All chemicals and reagents (Fluka) were purchased from HNK Analüüsitehnika

Staining and identification

For staining, the microorganisms were heat fixed on microscope slides. The bacteria were stained firstly using Gram`s method as described by the manufacturer (Sigma-Aldrich). The fungal slides were stained using 5% bengal red solution. All of the fungal slides were stained three times for better visualization. After drying of the slides the identification was carried out via microscopy. The bacteria were identified using Bergey`s Manual. The fungi were identified using online databases.

Toxicity

Different concentrations of nanoparticles were studied via MTT assay (M). HEK cells were seeded on day 0 at a density of 1000 per well in 96-well microtiter plates. On day 1, silver nanoparticle at different concentration was added and incubation was continued for 24 hours. After 24 hours, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well (0.5 mg/ml; Sigma Aldrich), and plates were maintained at 37°C for 2 h. The medium was then discarded, and DMSO was added to each well to lyse the cells. Absorbance was measured at 450 nm using a multiwall spectrophotometer (Tecan, microplate reader).

Results and discussion

Structural properties

A typical XRD pattern of the prepared silver nanoparticles is presented in the figure 1 and shows that silver nanoparticles are highly crystalline with a cubic structure. The XRD pattern shows diffraction peaks at 38.30, 44.50, 64.60, 77.50, 81.70, 98.00, 110.60 and 115° which correspond to 111, 200, 220, 311, 222, 400, 331 and 420 planes respectively and these are characteristic of the face-centered cubic structure of silver (JCPDS File No 87-0720). From the XRD pattern an average crystallite size of 58 nm was calculated using Scherrer method.

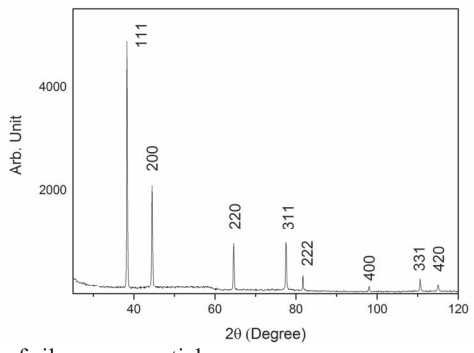

Figure 1. XRD pattern of silver nanoparticles.

TGA was performed on the silver nanoparticles to evaluate their purity and estimate the amount of organic species adsorbed on the nanoparticle surface. Typical TGA measurement is shown in figure 2 and do not show any weight loss or gain during the whole measurement till 800ºC. This demonstrates the high purity of the silver nanoparticles as in the case of organic adsorption on their surface, a weight loss would be observed. The stability of the nanoparticles is also demonstrated as a weight increase would be observed in the case of the oxidation of the silver nanoparticles. TGA shows that the synthesized silver nanoparticles are surfactant free. A slight decrease of the heat capacity (-12µV) is visible with the increase of the temperature. This increase is certainly related to the agglomeration and probably the fusion of the nanoparticles together.

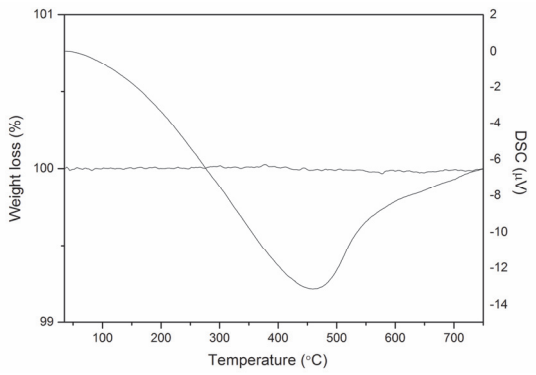

Figure 2. TGA and DTA performed under N_2 of silver nanoparticles.

The electron micrograph of spherical silver nanoparticles is shown in figure 3. Before dispersing it on a carbon coated copper grid for TEM observations, the solution had to be sonicated at a temperature of 40℃ for 15 min. When the sample was observed with sonication times of 1 min at ambient water temperature the particles were agglomerated and size estimation proved to be difficult. STEM images revealed that particles are easily dispersed in ethanol and surfactant-free. Most of the silver nanoparticles are approximately 10 nm in diameter with a few particles of around 2nm in size.

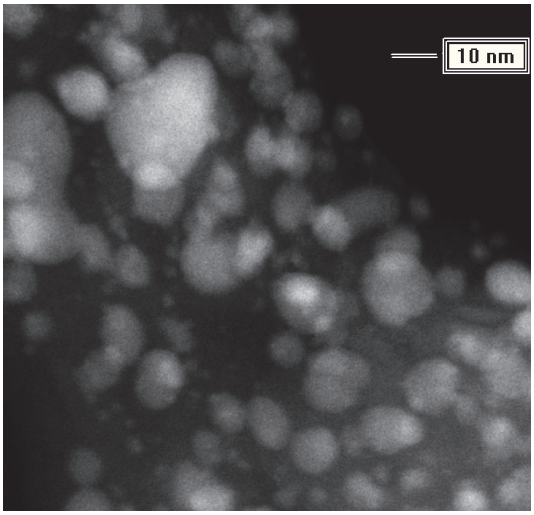

Figure 3. HRTEM of silver nanoparticles dispersed on a carbon grid.

Test results

For outdoor conditions, three straw-bale representative samples were prepared for this study. The three samples were stored outside for 14 days during the month of May that should promote a rapid development of microorganisms. After 14 days, some straws were taken from each straw bale samples and directly deposited in agar plates. Microorganisms were then isolated and identified from the straw specimen.

As expected, the whole spectrum of micro-organisms that can colonize the straw under outdoor conditions in the current climate and environment was present on the untreated straw bales (See figure 4a and table 1). The most dominant bacteria were *Streptomyces* and *Pseudomonas,* whose species tend developing in water damaged buildings and can be a serious threat to human health causing respiratory problems among others [16, 17]. In addition, the bacteria from the highly diverse phylum *Firmicutes* were found on all three samples. Three families of fungi were also identified*: Aspergillus spp*, *Penicillum spp* and *Cladosporium spp*. Some of *Aspergillus* and *Penicillum* are serious threat to human health as they produce hazardous mycotoxins that are carcinogenic and can damage the immune system, liver, kidneys and are harmful to the foetus [18, 19]. In addition, their fungal spores retain their toxicity even after the death. Many *Cladosporium* spp. fungi can induce health problems such as allergies, mycoses and inflammation. These fungi possess airborne conidia, which make the spreading of the potential pathogens simpler [20].

In the case of the sample treated with alcohol, the microorganism development was slower, but colonization by bacteria and fungi was observed. The pure ethanol treated straw bale

sample also contained many species of microorganisms (See figure 4b and table 1). However, the growth of several fungal colonies such as *Penicillum* spp., *Cladosporium* spp. and bacterial colonies of *Streptomyces* spp. were hindered. We noticed that in that case, mainly one type of fungus and bacteria, develop on the straw. This is possibly due to the fact that ethanol kills all microorganisms before the storage and then promotes the development of the microorganisms that can develop fast. In addition, both untreated straw and straw treated with ethanol suffered from colour change (See figures 4a and 4b).

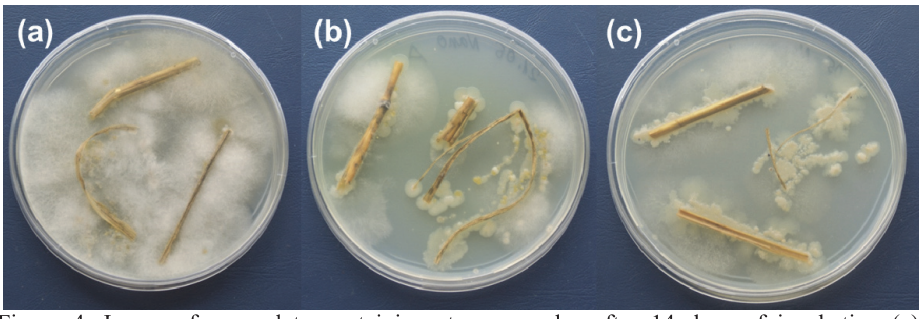

Figure 4. Image of agar plate containing straw samples after 14 days of incubation (a) untreated straw (b) straw treated with ethanol, (c) straw treated with silver nanoparticles.

The result on straw bale sample treated with an ethanol solution containing 100 mg/L of silver nanoparticles (fig.4b) was very different from the 2 other samples. First of all, no trace of fungi was detected on these samples. Moreover, we observe that silver nanoparticle coating prevents completely the development of fungi on this sample. However, some Gram-negative and Gram-positive bacteria were detected in the straw bale sample treated with silver nanoparticles (See figure 4c and table 1), but these colonies were not directly visible on the straw to the naked eyes like it was the case for straw bales treated with pure ethanol with no visible colour change(figure 4c).

TABLE I. Dominant microorganisms found in straw bale.

	Untreated wet straw	**Straw with ethanol**	**Straw with ethanol silver nanoparticle solution 100mg/L**
Genera of fungi	*Aspergillus, Penicillum, Cladosporium*	*Aspergillus*	No fungal activity
Genera of bacteria	*Streptomyces, Pseudomonas*	*Pseudomonas*	*Streptomyces, Pseudomonas*

To have a better understanding of the outdoor results and confirm them more specifically with bacteria, the biocidal properties of the silver nanoparticles were tested in indoor conditions. Moreover, literature results communicate that most of antibacterial tests are performed in indoor conditions, which are in fact not representative of real conditions in our case. Our experimental conditions therefore allowed to compare both test results.

A previous study on the identification of microorganisms that develop on rye straw and along with outdoor tests, enable us to isolate bacteria species in the indoor tests and petri dishes were then prepared with typical microorganisms that usually colonize the straw in outdoor conditions. In order to test also different silver nanoparticles concentrations, ethanol solution and water solution containing different silver nanoparticles concentrations (20mg/L, 50mg/L and 100mg/L) were prepared and droplets of 100µL were deposited on marked places in the agar plates to test the biocidal properties of these solutions. After 4 days of incubation at 25°C, the resulting inhibition of both bacteria and fungi was observed.

The first observations show different efficiencies against bacteria and some of them were more resistant to low silver nanoparticle concentrations. However, we observed that 100mg/L was sufficient to prevent the development of *Streptomyces* bacteria in the agar plates (Figure 5a). Silver nanoparticle antimicrobial properties are well-known and experimental data suggests a combination of multiple effects on microorganisms that occur separately or simultaneously [20-25]. The influence of the concentration of the silver nanoparticles on the different species of bacteria is presently under study and will not be developed in this paper. In the case of fungi grown on agar plates, similar results to the ones obtain with outdoor tests were observed. All concentrations were sufficient to prevent the Aspergillus *spp* fungi growth, even in the case of silver nanoparticles dispersed in distillate water (Figure 5b). These results confirm the efficiency of the solutions containing silver nanoparticles against fungi development.

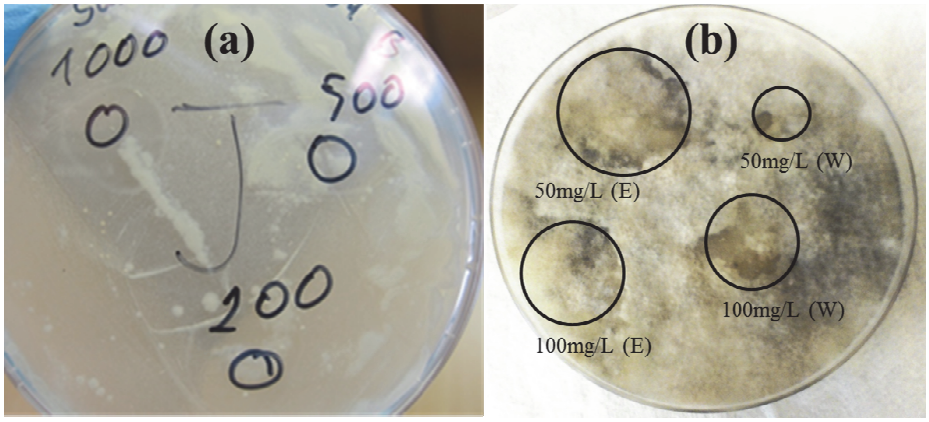

Figure 5. Image of agar plate containing (a) *Streptomyces* Bacteria showing the inhibition zone of the area treated with the ethanol solution containing 100mg/LM/L of silver nanoparticles and (b) Aspergillus *spp* fungi showing the inhibition zone of the area treated with the ethanol (E) and water (W) solution containing 100mg/L and 50mg/L of silver nanoparticles.

Discussion

In the literature the experimental data suggests, that silver nanoparticles are more effective against bacteria than fungi [26]. In fact, fungi are more resistant due to their chitin cell wall, absent in the case of bacteria. However, for the outdoor tests, we observed that bacteria seem to be less sensitive to the silver nanoparticles than the fungi. This can be explained by the fact that in stressful environments, due to the constant microbial competition in straw with other microorganisms the bacterial colonies grow protective biofilms around them. This kind of

biofilm represents an efficient protection for the bacteria against the silver nanoparticles and in consequence, the silver nanoparticles cannot affect the colonies. It has been estimated that biofilms can tolerate antimicrobial agents (disinfectants, antibiotics, surfactants) at concentrations of 10-1000-times that needed to inactivate genetically equivalent planktonic bacteria [27]. Usually, in the case of in-vitro tests, the bacterial stress response is not observed due to the lack of competition over food supplies with other organisms and in such environments, the silver nanoparticles can affect the proliferation of bacteria more efficiently. On the other hand, certain fungi cannot generate protective biofilm layers around them. This therefore allows the silver nanoparticle ethanol solution to produce enough aggressive environmental stress to inhibit the fungal development. This hypothesis is reinforced by the absence of colour change on the straw treated with silver nanoparticles in ethanol solution. This demonstrates that even if colonies are able to develop under biofilm protection, they are limited to the size of the biofilm and colonies cannot spread all over the straw. The silver nanoparticle coating then acts as a confining coating that suppresses complete bacteria and fungi development.
Since the fungi have a tendency to dominate over bacterial colonies in the environment and need less suitable conditions to proliferate, they can be more problematic within the walls of residential buildings. Considering the latter, straw bales treated with silver nanoparticle ethanol solutions can be a promising ecological option for a more ecological and healthy living environment.

Toxicity

To apply silver nanoparticles as antibacterial and antifungal treatment in green housing, it is necessary to study the toxicity of these nano-objects for human cells. The cytotoxicity of the synthesized silver nanoparticles in HEK (human embryonic kidney) cells was then investigated. Different concentrations of nanoparticles, from 10mg/L to 200mg/L were studied by MTT assay. This study shows that the silver nanoparticles used for this study do not present toxicity to human cells. The toxicity study shows that only from a concentration in solution of 100mg/L, the silver metal nanoparticles show some toxicity effects for HEK cells with an approximately 50% rate of mortality, which makes them a suitable material for such applications.

Conclusion

In summary, we report on the synthesis of spherical silver nanoparticles with average diameter of 3 nm for application in green house construction using straw bales. The structural properties study of these nanoparticles show that they are spherical with an average diameter of 3 nm, surfactant free and stable under air. The biocidal properties of these silver nanoparticles were studied in outdoor conditions and they demonstrate high antifungal properties and an ability to confine the bacterial colonies that can develop on straw materials. This study showed that a concentration of 100mg/L was sufficient to avoid the fungal and bacterial development under outdoor conditions. The indoor test showed that under stress, bacterial colonies are able to develop, but remain contained in the protective biofilm that they produce for their development. The use of silver nanoparticles for straw bales treatment appears to be a promising ecological solution. Even after the treated straw bales finish their life cycle, the possibility of recycling the Silver nanoparticles is more economical and ecological compared to other methods. Further results on this topic will be published in future works.

Acknowledgments

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Publication VI

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Silver metal nanoparticles study for biomedical and green house applications

E Rauwel[1], L Simón-Gracia[2], M Guha[3], P Rauwel[1], S Kuunal[1], D Wragg[4]

[1]Tartu College, Tallinn Univ. of Technol., 51008 Tartu, Estonia
[2]Laboratory of Cancer Biology, Institute of Biomedicine, Centre of Excellence for Translational Medicine, University of Tartu, Ravila 14b, 50411 Tartu, Estonia.
[3]Fac. of Medicine, Dept. of Gen. and Mol. Pathology, Univ. of Tartu, Tartu, Estonia
[4]Univ. of Oslo, Dpt. of Chemistry and SMN, 0316 Oslo, Norway

Email: erwan.rauwel@ttu.ee

Abstract. Metallic nanoparticles (MNP) with diameters ranging from 2 to 100nm have received extensive attention during the past decades due to their many potential applications. This paper presents a structural and cytotoxicity study of silver metal nanoparticles targeted towards biomedical applications. Spherical Ag MNPs of diameter from 20 to 50 nm have been synthesized. The encapsulation of Ag MNPs inside pH-sensitive polymersomes has been also studied for the development of biomedical applications. A cytotoxicity study of the Ag MNPs against primary prostatic cancer cell line (PPC-1) has demonstrated a high mortality rate for concentrations ranging from 100 to 200mg/L. The paper will discuss the potential for therapeutic treatments of these Ag MNPs.

1. Introduction

Noble metallic nanoparticles (MNPs) with diameter ranging from 2 to 100 nm have received extensive attention during the past decades due to their many potential applications in fields such as catalysis, biomedicine and cancer therapy. The biocidal properties of Ag MNPs have been known for eons, therefore making the study of Ag MNPs even more attractive for biomedical applications[1]. In fact, among noble metals, silver nanoparticles possess the most efficient antibacterial properties with a very broad bactericidal and fungicidal activity spectrum and have been used for the development of new generation of antibacterial burn wound bandage, water filter, antibacterial clothing, etc. In addition, Ag MNPs have a combination of unique physico-chemical properties like chemical stability, catalytic activity, high thermal and electrical conductivity and non-linear optical properties which also make them very appealing for the development of ink and microelectronic applications. The encapsulation of MNPs into biologically friendly water soluble polymer with an extremely low cytotoxicity is a prerequisite for the development of biomedical applications in nanomedicine. In fact, the development of functionalized, nontoxic and biocompatible nanoparticles has focused a lot of interest for applications in cancer diagnostic, drug delivery and anticancer drugs [2] during the last two decades.

The biocidal properties of these Ag NPs have recently been studied against fungi and bacteria that develop on straw in order to protect straw bales that are used in green housing construction [3, 4]. This study has demonstrated the toxicity of these Ag NPs against bacteria, but fungi appear to be a more serious threat, and these Ag NPs need to be combined with another metal or chemical group to enhance their efficiency. In this paper we have studied the toxicity of these Ag MNPs against human cells and cancer cells via MTT assay. The encapsulation of MNPs into polymer with an extremely low cytotoxicity is a prerequisite for the development of biomedical application in nanomedicine. pH-sensitive polymersomes are specially convenient for intracellular delivery, because they are stable vesicles at physiological pH and release the cargo at acidic pH inside the endosomes, releasing the cargo in the cytosol [5]. For these reasons, the encapsulation of these Ag MNPs inside pH-sensitive polymersomes has also been studied for the development of biomedical applications.

2. Experimental

2.1. Synthesis

The synthesis of Ag NPs was carried out under air and is based on non-hydrolytic sol-gel method developed elsewhere [6, 7] and has been reported elsewhere [3]. The Ag NPs were encapsulated inside pH-sensitive polymersomes composed by the co-polymer $POEGMA_{20}$-$PDPA_{90}$ (poly oligo(ethylene glycol) methacrylate co-poly(2-(diisopropylamino)ethyl methacrylate) [8] following the protocol described by Chen et al. [9]. Briefly, the co-polymer dissolved in DMF (dimethylformamide) was mixed with the Ag NPs dissolved in methanol and dodecanethiol. Then water was added slowly to form the polymeric vesicles. The organic solvent was removed by dialysis and the sample was purified by centrifugation.

2.2. Characterization

XRD data were collected using a Bruker D8 Discover instrument equipped with a LynxEye detector. Cu (ka1=1.54056) radiation selected by a Ge (1 1 1) monochromator was used. Crystallite size analysis from the XRD data was carried out using full profile Scherrer methods in TOPAS, with a fundamental parameters peak shape. Transmission electron microscopy (TEM) studies were carried out on a probe corrected Titan G2 80-200 kV operating at 200 kV in STEM mode and a Tecnai 10, Philips (Netherlands) to assess the size, surface topology and morphology of Ag NPs and assembled Ag-polymeric nanoparticles, respectively. X-ray photoelectron spectroscopy (XPS) analysis was carried out on a Kratos Analytical Axis UltraDLD photoelectron spectrometer equipped with Al Ka X-ray source.

2.3. Cytotoxicity assays

Different concentrations of nanoparticles were studied via MTT assay (M). Primary prostatic cancer cell line (PPC-1) were seeded on day 0 at a density of 1000 per well in 96-well microtiter plates. On day 1, silver nanoparticles at different concentrations were added and incubation was continued for 48 hours. After 48 hours, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well (0.5 mg/ml; Sigma Aldrich), and plates were maintained at 37°C for 2 h. The medium was then discarded, and DMSO was added to each well to lyse the cells. Absorbance was measured using a multiwall spectrophotometer (Tecan, microplate reader). All MTT assay were repeated twice.

3. Results and Discussion

3.1. Structural characterizations

The XRD pattern of the prepared Ag MNPs (figure 1a) shows that silver nanoparticles are highly crystalline with the face-centred cubic structure of silver metal (JCPDS File No 87-0720) and no secondary phase of silver oxide structure is visible. From the XRD pattern an average crystallite size of 58 nm was calculated using the Scherrer method.

The morphology and size of Ag MNPs were also studied by (S)TEM. The HAADF-STEM electron micrograph of spherical silver nanoparticles is shown in figure 1b and gives an overview of the nanoparticles. Before dispersing it on a carbon coated copper grid for TEM observations, the solution had to be sonicated at room temperature for 2 min. STEM images revealed that particles are easily dispersed in ethanol and surfactant-free. The silver nanoparticle diameter ranges from 20 to 50 nm with a few bigger particles of around 100nm in size suggesting that the average crystallite size suggested by XRD is not misleading.

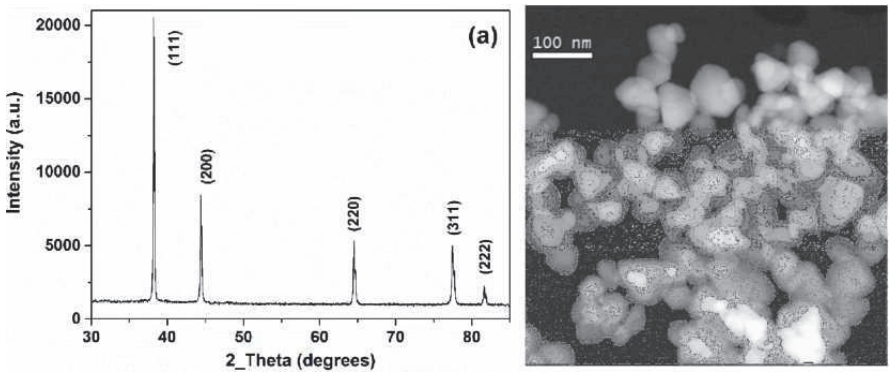

Figure 1. (a) XRD pattern of Ag metal nanoparticles, (b) STEM image overview of Ag MNP

The biocidal properties of these Ag MNPs have recently been reported [10], however, it has also been observed that the culture medium consisting of many amino acids that contain Sulphur groups shielded these biocidal properties due to the attachment of these molecules on the surface of the metal Ag NPs [4]. The metallic character of the surface promotes the bonding of Sulphur groups on the surface of Ag MNPs. To prevent such an attachment and shielding, the possibility of encapsulating these Ag MNPs with diameters around 10-50 nm inside pH-sensitive polymersomes composed by the co-polymer POEGMA-PDPA (poly oligo (ethyleneglycol) methacrylate co-poly(2-(diisopropylamino)ethyl methacrylate) [8] was studied. The formation of the hybrid metallic/polymeric nanoparticles was then investigated using TEM.

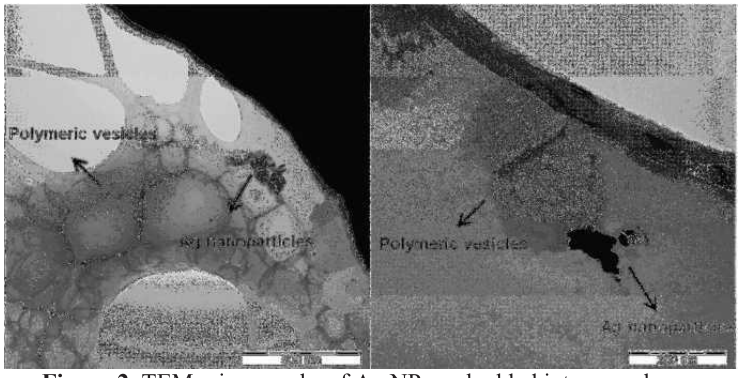

Figure 2. TEM micrographs of Ag NPs embedded into co-polymer POEGMA-PDPA vesicles. The Ag NPs are attached on the surface of the polymeric vesicles

TEM study shows that Ag MNPs are mainly localized outside of the polymer shell (Fig 9) and the Ag MNPs agglomerate on the surface of the vesicle. This study shows that the Ag MNPs need either to be functionalized to promote their encapsulation inside the polymer shell or a specific polymer containing sulfur or thiol groups should be used. We plan to test a surfactant that contains thiol groups that can attach on the metallic surface of the Ag MNPs. The encapsulation of the metal nanoparticles appears to be very important to enhance the efficiency of the metal nanoparticles for cancer therapy. The encapsulation inside other types of polymersome or inside gelatin shell will also be tested.

In order to confirm the nature of the surface of the Ag NPS, XPS measurements have been performed [3]. This study was performed 6 months after the synthesis and only binding energy peak from Ag metal were detected. Figure 3 provides the XPS spectra obtained for Ag 3d5/2, the

photoelectron peak corresponds to metal Ag and confirms the metallic nature of the surface of the Ag NPs. No photoelectron peak corresponding to silver oxide was detected. Due to the high surface to volume ration present in nanoparticles, any oxidation of the Ag NPs surface would have been detected by XPS.

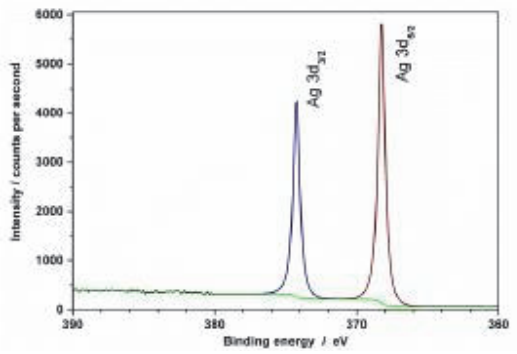

Figure 3. XPS spectra obtain on Ag NPs

3.2. Toxicity study

The toxicity of these Ag MNPs on Human Embryonic kidney (HEK 293) cells[11] as already been tested showing a moderate toxicity [3]. HEK cells are very commonly used for testing the toxicity of metal nanoparticles and the toxicity study has shown that mortality rate is over 50 % only for a concentration of silver nanoparticles of high concentration, which makes them a suitable material for biomedical application. Therefore, a similar cytotoxicity study has been performed on cancer cells and more particularly on the primary prostatic cancer cell line (PPC-1).

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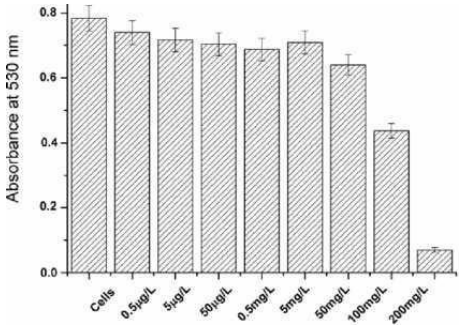

Figure 3. Toxicity test of silver metal nanoparticles on PPC-1 cells

Figure 3 shows MTT assays performed on Ag MNPs solutions of concentration ranging from 0.5 mg/L to 200 mg/L. Figure 3 shows the mean ± SEM of duplicate measurements of a representative sample of three independent experiments. This toxicity study on PPC-1 cells shows that mortality rate is over 44 % for a concentration of silver nanoparticles of 100 mg/L and shows a higher mortality rate (over 91 %) for a concentration of 200 mg/L. These results demonstrate that these Ag MNPs are potential candidates in the treatment of cancer, but their functionalization is needed.

4. Conclusions

In summary, we have reported on the synthesis of spherical silver nanoparticles with average diameters ranging from 20 to 50 nm for biomedical applications. The biocidal properties of these Ag MNPs have recently been demonstrated and the study of their encapsulation into pH-sensitive polymersomes was then performed for their potential use in cancer therapy. It has been shown that stabilisation of the metallic surface of the Ag MNPs requires the use of specific polymersomes that contain Sulphur or thiol groups. The cytotoxicity study shows that at a concentration of 100mg/L the mortality rate is over 44 % and increased to 91 % for a concentration of 200mg/L (200mg/mL). The synthesized Ag MNPs exhibit noteworthy cytotoxicity and show highly effective apoptotic activity against primary prostatic cancer cell line (PPC-1). These Ag MNPs therefore have high potential in therapeutic treatments and may be helpful for the development of anticancer therapeutics via their encapsulation into the appropriate polymersome.

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Review Article

A Review on the Green Synthesis of Silver Nanoparticles and Their Morphologies Studied via TEM

Protima Rauwel,[1] Siim Küünal,[2] Stanislav Ferdov,[3] and Erwan Rauwel[2]

[1] *Department of Physics, University of Tartu, Riia 142, 51014 Tartu, Estonia*
[2] *Tartu College, Tallinn University of Technology, Puiestee 78, 51008 Tartu, Estonia*
[3] *Department of Physics, University of Minho, 4800-058 Guimarães, Portugal*

Correspondence should be addressed to Erwan Rauwel; erwan.rauwel@ttu.ee

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Silver has been recognized as a nontoxic, safe inorganic antibacterial/antifungal agent used for centuries. Silver demonstrates a very high potential in a wide range of biological applications, more particularly in the form of nanoparticles. Environmentally friendly synthesis methods are becoming more and more popular in chemistry and chemical technologies and the need for ecological methods of synthesis is increasing; the aim is to reduce polluting reaction by-products. Another important advantage of green synthesis methods lies in its cost-effectiveness and in the abundance of raw materials. During the last five years, many efforts were put into developing new greener and cheaper methods for the synthesis of nanoparticles. The cost decrease and less harmful synthesis methods have been the motivation in comparison to other synthesis techniques where harmful reductive organic species produce hazardous by-products. This environment-friendly aspect has now become a major social issue and is instrumental in combatting environmental pollution through reduction or elimination of hazardous materials. This review describes a brief overview of the research on green synthesis of silver metal nanoparticles and the influence of the method on their size and morphology.

1. Introduction

For over centuries, silver based compounds were used as nontoxic inorganic antibacterial agents owing to their biocidal properties in many applications such as wood preservatives, water purification in hospitals, in wound or burn dressing, and so forth. In fact, silver ions and their related compounds have low toxicity toward animal cells but present a high toxicity to microorganisms like bacteria and fungi. The recent advances in the field of nanoparticle synthesis have a strong impact in many scientific areas and the synthesis of silver nanoparticles has also followed this tendency. These unique properties of nanomaterials have spurred numerous investigations and applications in electronics, nanomedicine, biomaterials, energy, and food. In fact, silver based compounds are much cheaper than gold based one; moreover, silver nanoparticles are now considered as an important class of nanomaterials. They are presently mainly used as catalyst [1] or antibacterial/antifungal agents [2].

Environmentally friendly synthesis methods are becoming more and more popular in chemistry and chemical technologies. This trend has several origins, including the need for greener methods counteracting the higher costs and higher energy requirements of physical and chemical processes. For this reason, scientists search for cheaper methods of synthesis. The other reason is that conventional methods for nanoparticle synthesis usually require harmful reductants such as sodium borohydride or hydrazine and many steps in the synthesis procedure including heat treatments, often producing hazardous by-products. In order to reduce the environmental impact of nanoparticle synthesis, greener routes have been investigated for over a decade. The principles of green chemistry were presented by Anastas and Warner who developed 12 principles that eloquently describe green chemistry [3]. Green chemistry should aim at thwarting waste, minimizing energy use, employing renewable materials, and applying methods that minimize risk. The three main concepts for the preparation of nanoparticles

in a green synthesis approach are the choice of the solvent medium (preferably water), an environmentally friendly reducing agent, and a nontoxic material for the stabilization of the nanoparticles [4].

To be energy efficient, the synthesis processes should be carried out close to ambient temperature and pressure and under neutral pH. The biological systems then appear as the most suitable factory for reaching such natural chemistry conditions. It is well known that many microorganisms can provide inorganic materials either intra- or extracellularly [5] and it was found that some of these microorganisms can be used as ecofriendly nanofactories for the production of nanomaterials, more particularly for the production of silver metal nanoparticles (Ag NPs).

Many approaches were investigated, and microorganisms such as bacteria, yeasts, fungi, and algae were used in the biosynthesis of metal nanoparticles. More recently, the utilization of plants for the production of metal nanoparticles has spurred numerous investigations in the field of green synthesis. The aim of this review is to provide a brief overview of the current research on the green synthesis of silver nanoparticles and describe how the different methods of synthesis can affect the size and the morphologies of the silver metal nanoparticles.

2. Green Synthesis Methods

2.1. Green Synthesis Using Bacteria as a Medium. Bacteria are known to produce inorganic materials either intra- or extracellularly. This makes them potential biofactories for the synthesis of nanoparticles like gold and silver. Silver is well known for its biocidal properties; however, some bacteria are known to be silver resistant [6] and can accumulate silver on the cell wall to as much as 25% of their dry weight biomass, thus suggesting their use in industrial recovery of silver from ore materials [7]. Therefore, the use of prokaryotic bacteria as nanofactories was first studied. First noble metal nanoparticle synthesis, using bacteria, was done using silver resistant bacterial strains *Pseudomonas stutzeri* AG259, which were cultured in high concentrations of silver nitrates. It was demonstrated that the cells accumulate silver in large quantities and the majority of the silver was deposited in the form of particles of 200 nanometers of diameter [8]. Significant results were observed when bacteria *Proteus mirabilis* PTCC 1710 were used for producing silver nanoparticles. It was found that depending on the type of "broth" used during the incubation of bacteria, extracellular or intracellular synthesis can be promoted. This kind of selection makes bacteria-based green synthesis flexible, inexpensive, and a suitable method for large-scale production [9]. It is important to point out that bacteria continued to grow after the synthesis of silver nanoparticles. However, the main drawback of using bacteria as nanofactories is the slow synthesis rate and the limited number of sizes and shapes available compared to the conventional chemical methods of synthesis. For this reason, fungi-based nanofactories and chemical reaction involving plant based materials were investigated (see Table 1) [10].

2.2. Green Synthesis Using Fungi as Medium. Similar to bacteria, due to their tolerance and metal bioaccumulation ability, high binding capacity, and intracellular uptake, fungi have been of interest in biological production of the metallic nanoparticles [11]. Compared to bacteria, fungi are simpler to handle in a laboratory process. The mechanism of nanoparticle production using fungi is different; fungi secrete large amounts of enzymes which are used to reduce silver ions that induce the formation of the metal nanoparticles [12].

The first synthesis involving fungus-mediated approaches for the metal nanoparticle synthesis was performed in the beginning of the 20th century, and Ag NPs with diameter of 25 ± 12 nm were synthesized using fungus *Verticillium* [13, 14]. In previous studies involving bacteria, bacteria *Pseudomonas stutzeri* AG259 isolated from silver mines were able to produce Ag NPs of well-defined size and distinct morphology within the periplasmic space of the bacteria [8]. Synthesis using *Verticillium* takes the green approach even further. During the exposure of the fungus to $AgNO_3$ solution, the reduction of ions and the formation of Ag NPs take place. Nanoparticles were approximately 25 nm in diameter presenting a rather good monodispersity and spherical morphology. Contrary to bacteria, Ag NPs were formed below the surface of the fungal cells [13]. This result differs from the work of Klaus et al. 1999, where particle morphologies synthesized using bacteria ranged from spherical, triangular to hexagonal. The mechanism of nanoparticle formation was then studied and the main hypothesis is that, in the case of fungi-based synthesis, the NPs are formed on the surface of the mycelia and not in the solution. It was then suggested that in the first step Ag^+ ions are adsorbed on the surface of the fungal cells due to electrostatic interaction between negatively charged carboxylate groups in enzymes present in the cell wall of mycelia and positively charged Ag ions. Finally, the silver ions are then reduced by the enzymes present in cell wall, leading to the formation of silver nuclei [13]. The shift from bacteria to fungi as a means of developing natural nanofactories offers the advantages of simpler downstream processing and handling of the biomass. Compared to bacteria, fungi are known to secrete much higher amounts of proteins, which tends to significantly increase the productivity of this biosynthetic approach; moreover, fungi could be used for the production of large amounts of metal nanoparticles.

The first report involving extracellular synthesis of silver nanoparticles using eukaryotic systems such as fungi was reported by Ahmad et al. 2003. They showed that secreted enzymes are responsible in the reduction process [15]. Before this report, all the fungi based biosyntheses were intracellular. Extracellular synthesis is advantageous as the synthesized nanoparticles will not bind to the biomass [16, 17] and it is therefore possible to extend this approach for the biosynthesis of nanomaterials over a range of chemical compositions, such as oxides, nitrides, and so forth.

When compared to other classes of microorganisms, their ecofriendliness and simplicity during handling lead to increasing the use of fungi in green synthesis. For example, fungus like white rot fungus is nonpathogenic and this contributes to the mass production of Ag NPs [18]. Another

Table 1: Size dependent synthesis of silver nanoparticles via eco-friendly synthesis routes.

Type	Diameter	References
Bacteria		
Pseudomonas stutzeri AG259	200 nm	[8]
Proteus mirabilis PTCC 1710	10–20 nm	[9]
Fungi		
Verticillium	25 nm ± 12 nm	[13, 14]
Fusarium oxysporum	5–15 nm	[15]
Cladosporium cladosporioides	10–100 nm	[16]
Fusarium oxysporum	20–50 nm	[17]
Aspergillus fumigatus	5–25 nm	[19]
A. flavus	5–30 nm	[20]
Yeast		
MKY3	2–5 nm	[23]
Baker's yeast, *Saccharomyces cerevisiae*	60–80 nm	[24]
Algae		
Sargassum wightii	8–12 nm	[25]
Plant extracts		
Alfalfa sprouts	2–20 nm	[26]
Geranium leafs	14–46 nm	[27]
Chrysanthemum indicum L.	38–72 nm	[38]
Acacia leucophloea	17–29 nm	[39]
Ganoderma neojaponicum Imazeki	5 nm	[40]
Colletotrichum sp.	20–40 nm	[41]
Caffeine and tea extract	60 nm	[42]
Gelatin and glucose	3.68 nm and 5.28 nm	[43]
Thevetia peruviana (latex)	10–60 nm	[45]
Elaeagnus latifolia	30–50 nm	[46]
Leptadenia reticulata	50–70 nm	[47]
Olive extract (1 mL)	30 nm	[48]
Olive extract (5 mL)	15 nm	[48]
Terminalia chebula fruit extract	100 nm	[49]
Tinospora cordifolia	17–29 nm	[50]

important factor for choosing the method of synthesis is the reaction rate. First report of rapid synthesis using fungi was using *Aspergillus fumigatus* that allowed obtaining monodispersed Ag NPs within 10 minutes [19]. In addition, one of the most common molds *Aspergillus fumigatus* was used to make Ag NPs in a matter of minutes, when silver ions entered into contact with the cell filtrate. These investigations were clear examples describing suitability and the potential of using fungi for mass production of nanoparticles. More recently, Ag NPs were synthesized using *A. Flavus* fungi to be combined with antibiotics to enhance the biocidal efficiency against multidrug-resistant bacteria. This study demonstrated the efficiency of antibiotics combined with Ag NPs [20].

Similar to fungi, yeasts were also widely investigated for silver nanoparticle synthesis [12, 21, 22]. Silver-tolerant yeast strain MKY3 was first used for extracellular synthesis. The outcome of the synthesis was satisfying due to simplicity of the separation of the nanoparticles when using differential thawing [23]. After that, several studies followed but until recently synthesis has never been carried out by commercial baker's yeast available in grocery stores. All the aggravating steps of cultivation of the yeast were avoided, thus making the process much simpler [24].

Similar to moving from prokaryotes to eukaryotes green synthesis, the utilization of eukaryotic autotrophs widened the possibilities of green synthesis. For example, using marine algae *Sargassum wightii* allowed obtaining very stable nanoparticles compared to other biological methods [25].

2.3. Green Synthesis Using Plants and Plant Extract as a Medium. One of the first approaches of using plants as a source for the synthesis of metallic nanoparticles was with alfalfa sprouts [26], which was the first report on the formation of Ag NPs using a living plant system. Alfalfa roots have the capability of absorbing Ag from agar medium and transferring them into the shoots of the plant in the same oxidation state. In the shoots, these Ag atoms arranged themselves to form nanoparticles by joining themselves and forming larger arrangements. In comparison to bacteria and fungi, green synthesis using plants appears to be faster and the first investigations demonstrate that synthesis procedures are able to produce quite rapidly Ag NPs. Shankar et al. showed that using *Geranium* leaf takes around nine hours reaching 90% reaction compared to the 24 to 124 hours necessary for other reactions reported earlier [27]. Therefore, the use of plant extracts in green synthesis has spurred numerous investigations and studies up till now. It was demonstrated that the production of metal nanoparticles using plant extracts could be completed in the metal salt solution within minutes at room temperature, depending on the nature of the plant extract. After the choice of the plant extract, the main affecting parameters are the concentration of the extract, the metal salt, the temperature, the pH, and the contact time [28].

In addition to the synthesis parameters, the main issue is the choice of the plant from which the extract could be used. The advantages of using plants for the synthesis of nanoparticles are that the plants are easily available and safe to handle and possess a large variety of active agents that can promote the reduction of silver ions. Most of the plant parts like leaves, roots, latex, bark, stem, and seeds are being used for nanoparticle synthesis [10]. The most important point is the active agent contained in these parts which makes the reduction and stabilization possible. Ecofriendly plant extracts contain biomolecules, which act as both reducing and capping agents that form stable and shape-controlled nanoparticles. Main compounds which affect the reduction and the capping of the nanoparticles are biomolecules such as phenolics, terpenoids, polysaccharides, flavones, alkaloids, proteins, enzymes, amino acids, and alcoholic compounds. However, quinol and chlorophyll pigments, linalool, methyl chavicol, eugenol, caffeine, theophylline, ascorbic acid, and

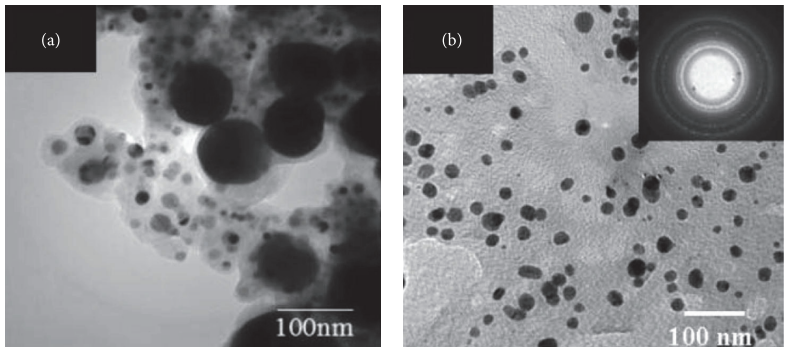

Figure 1: Ag NPs from coffee (a) and tea (b) extract [42]. Copyright license number: 3482980509111.

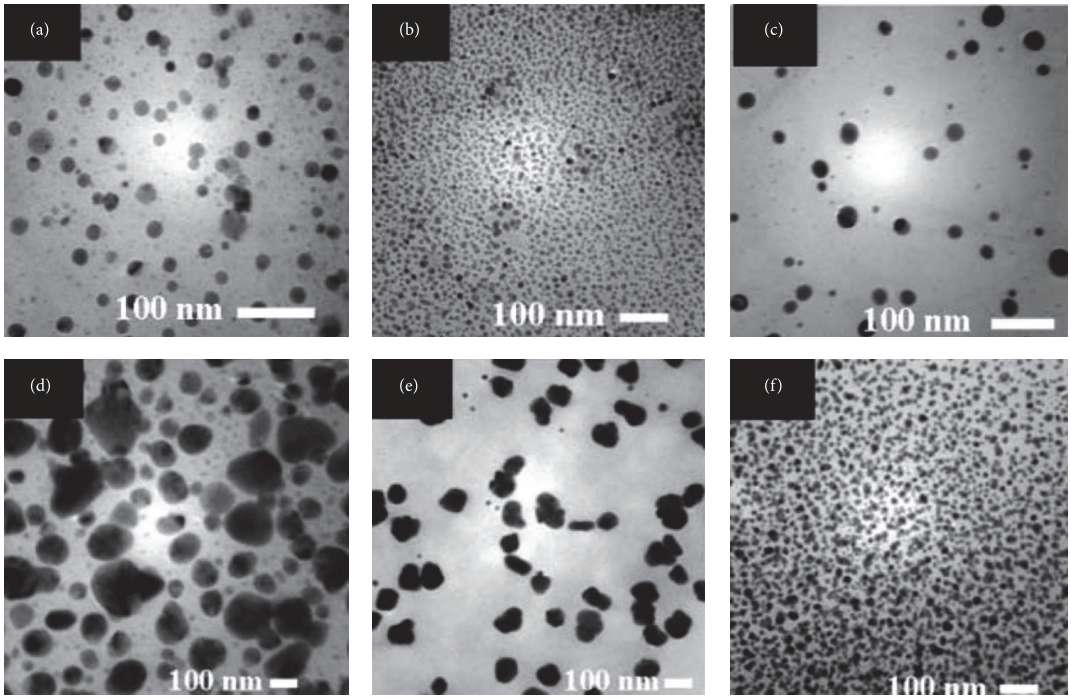

Figure 2: TEM image of silver nanoparticles synthesized using (a) Bigelow tea, (b) Folgers coffee, (c) Lipton tea, (d) Luzianne tea, (e) Sanka coffee, and (f) Starbucks coffee extract at room temperature in one step without using any hazardous reducing chemicals or nondegradable capping agents [42]. Copyright license number: 3482980509111.

other vitamins have also been reported [29–36]. The nontoxic phytochemicals including aforementioned flavonoids and phenols have unique chemical power to reduce and also effectively wrap nanoparticles, thus preventing their agglomeration. Phenolic compounds possess hydroxyl and carboxyl groups, which are able to bind to metals [37].

Most of the Ag NPs synthesized via green synthesis are investigated for biomedicine and more particularly as antibacterial agent or for cancer treatment. Recent reports showed that it was possible using *Chrysanthemum indicum* L. [38] or *Acacia leucophloea* extract [39] to synthesize Ag NPs of diameter ranging from 38–72 nm to 17–29 nm, respectively. Both samples demonstrated very good antibacterial properties. In the same manner, *Ganoderma neojaponicum Imazeki* was used for the synthesis of Ag NPs as potential cytotoxic agents against breast cancer cells [40]. As these methods

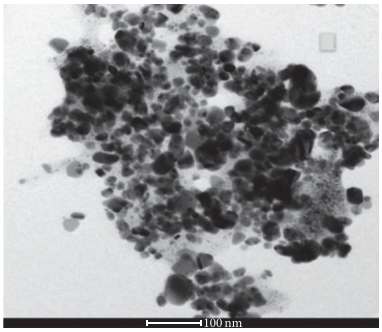

Figure 3: Representative TEM image of Ag NPs synthesized at room temperature using latex extract from the stems of fresh fruits of *Thevetia peruviana* [45] (reproduced with permission from IOP).

involving green chemistry are being more and more explored and scientists are starting to combine different options together, it was recently reported that the symbiotic biological systems such as *Geranium* leaf combined with endophytic fungus *Colletotrichum* sp. can synergize the outcome of the reaction. In fact, plants contain biomolecules which are able to stabilize unstable particles whereas fungi secrete enzymes for reduction [41].

3. Morphological Characterization via Transmission Electron Microscopy

The size distribution of nanoparticles in general is an important issue as nanoparticles exhibit different physical and chemical properties depending on their shape and size. Synthesis methods that generate uniformly sized and shaped nanoparticles are therefore being pursued. Transmission electron microscopy (TEM) is therefore one of the most adapted techniques to study the size and shape of the nanoparticles and provide their distribution. It is important to note that the majority of the TEM studies were performed on plant extracted green synthesis of silver nanoparticles.

Many different plant extracts have been used to prepare silver nanoparticles in the aim of producing Ag NPs presenting different capping layer molecules and presenting different morphologies. These studies in TEM have shown that the presence of a capping layer in plant mediated synthesis of Ag NPs, where the plant extract acts as capping layers, shapes the nanoparticle during its growth. It also has an effect on the size distribution of these nanoparticles. The use of medicinal plants in the synthesis of Ag NPs is not only used for size and shape control but also used to provide properties to the Ag NPs along with the antimicrobial properties of the plant. In this section, special emphasis is given to the Ag NPs morphology and size distribution as a result of synthesis parameters using TEM.

Everyday products such as tea and coffee can be used to produce Ag NPs, namely, tea leaves and coffee beans. Nadagouda et al. have explained that the caffeine and polyphenol in tea and coffee help in forming complexes with metal ions and reducing them to the corresponding metal [42]. They have used commercially available coffee and tea extract mixed with $AgNO_3$. TEM samples were prepared by diluting the nanoparticles in water. The TEM images provide monodispersed nanoparticles in each case, indicating that the polyphenols act not only as a reducing agent but also as a capping agent and therefore restrict their growth to 60 nm (Figure 1). In Figure 1, the TEM images provide size distribution of Ag NPs and indicate that tea extract reduced Ag NPs present a small particle size distribution compared to the caffeine reduced ones (Figure 1(b)). By using different types of tea and coffee extract the authors have shown that the size of the nanoparticles can be further varied (Figure 2).

Other edible products such as gelatin and glucose have also been employed by Darroudi et al. to modify the Ag NPs size and shape [43]. The synthesis was carried out by dissolving gelatin in water and adding silver solution. A small amount of glucose was also added and the solution was heat treated at different temperatures, that is, 20, 40, and 60°C. Synthesis with and without the presence of glucose was performed. The particle size showed a decrease when the temperature was increased and when glucose was absent. At 60°C, the nanoparticles had an average size of 3.68 nm and 5.28 nm. This is attributed to the rate of reduction reaction of $AgNO_3$.

In another study, ecofriendly honey was used as a reducing agent and replaced synthetic reducing agents such as hydrazine and dimethyl formamide which are not totally environmentally safe. In this study, the particle size depended on the concentration of honey and the pH of the aqueous solution [44]. Another encounter where Rupiahsi et al. used latex in the green synthesis of Ag NPs has also been reported [45]. Milky white latex was extracted from the stems of fresh fruits of *Thevetia peruviana* and filtered out. This was then mixed with $AgNO_3$. The TEM images of the particles thus obtained show spherical particles with a wide size distribution with 75% of the nanoparticles presenting particle size between 10 and 30 nm (Figure 3). Less than 10% of the nanoparticles were under 10 nm in size and between 50 and 60 nm. Most of the particles in the TEM image are polydispersed despite latex being used as a capping and reducing agent; the nanoparticles were nevertheless spherically shaped. However, other nanoparticle shapes of Ag NPs are also possible with other green synthesis methods as explained below.

Another plant mediated synthesis of Ag nanoparticles includes *Elaeagnus latifolia* a native evergreen shrub to Asia. In the work of Phanjom et al. the nanoparticles were precipitated after a room temperature reaction of the leaf extract with $AgNO_3$ and provided a size distribution of Ag NPs between 30 and 50 nm (Figure 4) [46]. However, the TEM contrasts exhibit the presence of defects such as stacking faults or twinning in them (Figure 4). The authors also suggest the formation of an organic capping layer on the surface of these nanoparticles thereby explaining the high level of monodispersion of the nanoparticles.

Swamy et al. have employed *Leptadenia reticulata* which is a medicinal plant native to the Indian subcontinent [47]. It has been actively used to alleviate symptoms of

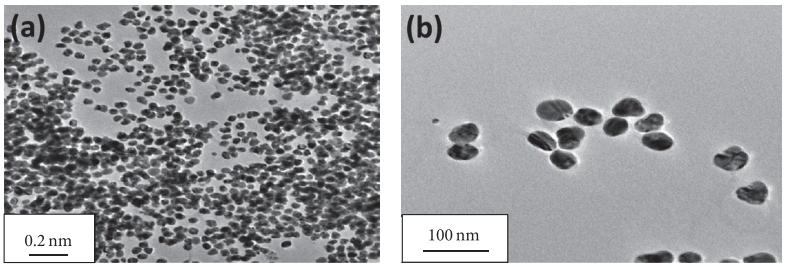

Figure 4: TEM images of Ag NPs synthesized with *Elaeagnus Latifolia* as a reducing agent: (a) overview of Ag NPs and (b) higher magnification image of the Ag NPs showing defects [46] (reproduced with permission from the journal).

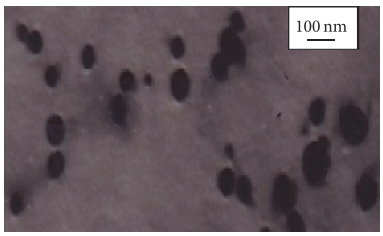

Figure 5: Ag NPs synthesized using *Leptadenia reticulata* extract [47] (reproduced with permission from Springer).

hematopoiesis, emaciation, cough, dyspnoea, fever, burning sensation, and night blindness. Extracts of this plant are also used to cure skin disorders. For the synthesis, once again $AgNO_3$ was mixed with the leaf extract in 1 : 9 proportions. The nanoparticles obtained were stored in ionized water and frozen for further study. For TEM study these nanoparticles were suspended on a carbon coated copper grid. The particles appear oblong with sizes between 50 and 70 nm (Figure 5).

Olive leaf extract with $AgNO_3$ has been used by Khalil et al. for the synthesis of Ag NPs and their antibacterial properties have been evaluated [48]. It is well known that olive leaf is effective against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli*. Their studies focused first of all on the effect of concentration of the leaf extract on the particle size. In this context they observed a decrease in the particles size with increase in the olive leaf extract concentration during reaction as indicated in Figure 6. The authors have calculated that for the 1 mL concentration average particle size was 30 nm (Figure 6) while for 5 mL of olive leaf extract concentration the particle size was less than 15 nm (Figure 6).

Moreover, the effect of pH of the reaction solution on the particles size was also evaluated and they noticed a clear decrease in particle size with increase in pH. Here Ag NPs with 2 different pH values (Figure 7) were studied with TEM and smaller particle sizes were obtained for higher pH (Figure 7). It is therefore possible to vary the pH and the extraction concentration and tailor the size of the NPs.

Terminalia chebula fruit extract has also been used by Kiran Kumar et al. for the production of silver nanoparticles [49]. In their reaction Ag_2SO_4 was mixed with the extraction liquid compared to other methods presented here where $AgNO_3$ was used.

The nanoparticles produced by this method showed sharp facets. All the nanoparticles were under 100 nm in size. The shape anisotropy is believed to be due to lack of protective biomolecules which assists in the homogeneity of the shape during growth, thus forcing them to attain thermodynamic stability by acquiring shapes such as triangles and hexagons. The monodispersed aspect of these nanoparticles was again attributed to the capping layer of polyphenols which are known to reduce Ag^{+2} to Ag^{+0} and the oxidized polyphenol binds to the Ag NPs via –C=O bonds and also simultaneously stabilizes them.

Other plant mediated Ag NPs synthesis involves *Tinospora Cordifolia* by Anuj and Ishnava [50]. Here they used the stem extract to obtain a suspension that was then mixed with $AgNO_3$. The nanoparticles produced in their study were spherical except for larger particles that presented nonuniform shapes. They also noticed a fine film on the TEM grid which corresponded to the capping layer produced from the *Tinospora Cordifolia* extract (Figure 8).

More recently, it was shown that Ag NPs synthesized using *Acacia Leucophloea* extract present a spherical morphology with a diameter ranging from 17 to 29 nm. The authors observed an enhancement of the antibacterial activity of Ag NPs synthesized using this method [39].

4. Conclusion

During the last decades, many efforts were put into the development of new green synthesis methods. Living organisms have huge potential for the production of nanomaterials that can be applied to many fields and more specifically to biomedicine. Organisms ranging from simple bacteria to highly complex eukaryotes can all be used for the production of nanoobjects with the desired size and shape. Prokaryotes are the simplest forms of biomass and therefore are easy to manipulate genetically to make them produce more desired substances for synthesis. However, the cultivation of the bacteria and large scale production remains problematic

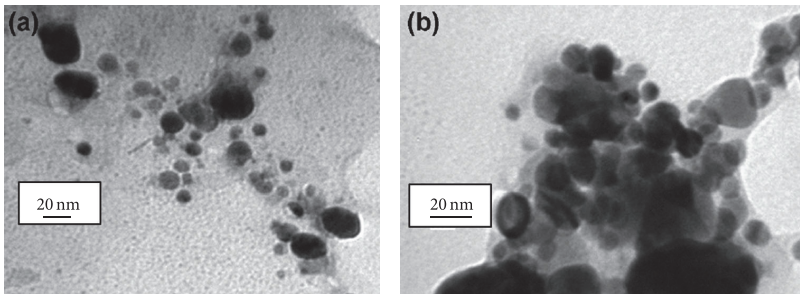

Figure 6: TEM micrographs with (a) 1 mL and (b) 5 mL olive leaf extract [48] (reproduced with permission from the journal).

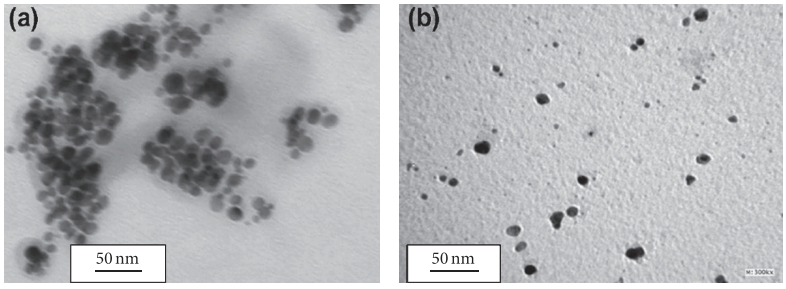

Figure 7: TEM micrograph of the silver nanoparticles [48]: (a) at pH 3, nm and (b) at pH 8 (reproduced with permission from the journal).

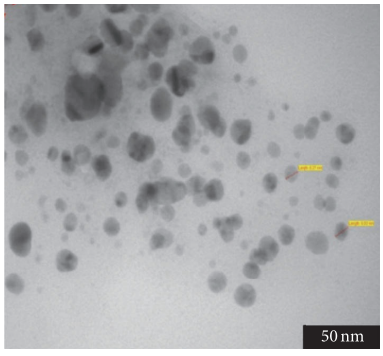

Figure 8: Ag NPs synthesized using *Tinospora Cordifolia* extract as a reducing agent [50] (reproduced with permission from the journal).

compared to others. Therefore, as a first approach, bacteria were studied as first nanofactories for the production of noble metal nanoparticles. However, the low synthesis rate and the limited number of size and shape distributions available oriented the investigations to the use of fungi and algae.

Fungi present a suitable option for large-scale green nanoproduction. They are easy to handle during downstream processing and they secrete large amounts of enzymes needed in the reduction. They also present filamentous tolerance towards metals, high binding capacity, and intracellular uptake. Nevertheless, the genetic manipulation to overexpress specific enzymes in order to intensify synthesis is much more difficult among eukaryotes.

More recently, many investigations were carried out on the possible use of plant extracts and the number of research papers published in this field has increased exponentially during the last 2 years due to their easy availability, environmental friendliness, and cost-effectiveness. Moreover, plants contain the most effective compounds for synthesis and therefore enhance the synthesis rate. Size and shape distribution of the nanoparticles as obtained from TEM studies shows that many factors affect their morphologies including the nature of the plant extract, the pH of the solution, and the temperature of the reaction. Nevertheless, obtaining uniform size and shape distribution of Ag NPs remains a subject of investigation.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Curriculum Vitae

Personal data

Name:	Siim Küünal
Date of birth:	10th of June 1990
Place of birth:	Tartu
Citizenship:	Estonian

Contact data

E-mail:	siim.kuunal@taltech.ee

Education

2014-2022	Tallinn University of Technology	PhD
2009-2014	Tallinn University of Technology	MSc
2006-2009	Hugo Treffner Gymnasium	Secondary Education

Language competence

Estonian	Native language
English	High level (C1)
Russian	Basic level (A2)
Finnish	Basic level (A2)

Professional employment

01.01.2017-…	Tallinn University of Technology, School of Engineering, Tartu college	Invited lecturer
01.02.2018- …	Diotech OÜ	Sales and project manager

Elulookirjeldus

Isikuandmed

Nimi:	Siim Küünal
Sünniaeg:	10.06.1990
Sünnikoht:	Tartu
Kodakondsus:	Estonian

Kontaktandmed

E-posti aadress:	siim.kuunal@taltech.ee

Hariduskäik

2014-2022	Tallinna Tehnikaülikool	PhD
2009-2014	Tallinna Tehnikaülikool	MSc
2006-2009	Hugo Treffner Gümnaasium	keskharidus

Keelteoskus

Estonian	emakeel
Inglise keel	Kõrgtase (C1)
Vene keel	Algtase (A2)
Soome keel	Algtase (A2)

Teenistuskäik

01.01.2017-…	Tallinna Tehnikaülikool, Inseneriteaduskond, Tartu kolledž	külalislektor
01.02.2018- …	Diotech OÜ	müügi- ja projektijuht

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