Biogenic production of Gold and Silver Nanoparticles using extracts from indigenous Australian plants: Their synthesis, optimisation, characterisation and antibacterial activities

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This thesis is presented for the degree of Doctor of Philosophy of Murdoch University

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Declaration

I declare that this thesis is my own account of my research and contains as its main content, work which has not previously been submitted for a degree at any tertiary education institution.

Monaliben Shah
Dedicated to my husband Nikunj
&
my daughter Freya
Abstract

Today, the primary focus of nanotechnology is the creation of nanometre scale materials, techniques and applications. The main driver for this intense nanotechnology-based research is the unique material properties that occur at the nanometre scale which are significantly different to those at the bulk scale. Importantly, because of their size nanoparticles can act as bridge between bulk materials and molecular structures. Therefore, an important aspect of nanotechnology is the synthesis of nanoparticles of different compositions and the ability to directly control particle parameters such as size, shape and size distribution. Since the material composition and related material parameters will ultimately determine the overall properties of the nanoparticles. Furthermore, there is also a need to develop reliable, sustainable and eco-friendly protocols for manufacturing nanoparticles. In recent years there has been a convergence between biological based technologies, green chemistry and nanotechnology. This convergence has the potential to produce clean technologies that can significantly reduce environmental and human health risks resulting from toxic chemicals and solvents generally used in conventional nanoparticle manufacturing processes. The biogenic synthesis of nanoparticles via plant extracts has the potential to significantly reduce or eliminate the use of hazardous substances.

Worldwide, alternative eco-friendly green chemistry based techniques for biosynthesising metal nanoparticles using various plant species has been actively investigated in recent years. However, finding suitable plant species and developing effective protocols to biosynthesis metal nanoparticles with predetermined physiochemical properties still remains a challenge. Taking up the challenge, this thesis for the first time examines the biogenic properties of three indigenous Australian plant species to produce (Au) gold and (Ag) silver nanoparticles. Plant species studied were Eucalyptus macrocarpa (rose of the west), Xanthorrhoea glauca (grass tree) and Anigozanthos manglesii (red and green kangaroo paw). The thesis is composed of five case studies that deal with the discovery, biosynthesis optimisation & particle characterisation, and identification of Ag nanoparticle/plant antimicrobial properties. The studies reveal all three plant species were effective bio-factories capable of
manufacturing of Ag and Au nanoparticles. Subsequent antimicrobial studies using Ag nanoparticles biosynthesised using Xanthorrhoea glauca and Anigozanthos manglesii were found to be effective agents against bacterial pathogens such as *Deinococcus*, *Escherichia coli* and *Staphylococcus Epidermis*. The research for the first time has demonstrated that three indigenous Australian plant species are capable of producing nanoparticles with unique physiochemical properties and the nanoparticles have the potential to be used in antibacterial pharmaceuticals.
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Doing PhD is not an easy path to step into and one needs constant encouragement and help. A big special thanks to my parent-in-laws, my parents and my husband for their constant support and encouragement throughout this part of my life’s journey.

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Chapter 1 – Introduction

1.1. Background

Today, there is a definite convergence of nanotechnology, green chemistry and biological technologies aiming to create novel materials and new processes to reduce or eliminate the use and production of hazardous substances. The convergence of these three technologies has created the new field of nanobiotechnology which focuses on the creation, manipulation and use of materials at the nanometre scale for advanced biotechnology and biomedical applications via the principles and practice of green chemistry [1]. Studies have shown that nanometre scale materials exhibit physiochemical, electronic, optical and biological properties that significantly differ from their bulk counterparts [2, 3]. It is the extremely small size, shape and size distribution that governs the overall physicochemical and optoelectronic properties of nanoparticles [4]. The International Organisation Standardisation (ISO) defines nanoparticles as materials with all three external dimensions in the size range between 1 nm and 100 nm [5]. In particular, it is the large surface area to volume ratio that leads to the significant differences in properties not seen in the same material at the much larger bulk scale [6, 7]. Differences in material properties identified include biological, catalytic activity, mechanical, melting point optical absorption, thermal and electrical conductivity. The unique properties displayed by various types of nanoparticles is of particular interest for applications including catalysts, chemical sensors, and electronic components to medical imaging, pharmaceutical products and medical treatments [8-12]. Because of the importance of size and shape on the material properties of nanoparticles it is important that their synthesis and associated parameters are effectively controlled during processing to create nanoparticles that fulfil their intended function or application.

Metal nanoparticle manufacture can be broadly defined to fall within two generalised approaches. The first is defined as the top down approach and involves a material of interest undergoing size reduction via physical or chemical processes [13, 14]. During the process particle size, shape and surface structure is dependent on the size reduction
technique used. Importantly, size reduction processes tend to introduce surface imperfections that can significantly impact on the overall physicochemical properties of the nanoparticle. In the bottom up approach, nanoparticles are built up from the assembly atoms, molecules and smaller particles/monomers [15]. Unfortunately, many of the conventional chemical and physical processes used to manufacture metal nanoparticles are technically laborious, have low material conversion rates, technically difficult, have high energy requirements and are generally expensive. In general, many of these chemical and physical processes also use hazardous chemicals, produce toxic waste products and are not eco-friendly [16, 17]. Furthermore, processes like chemical precipitation and pyrolysis can result in toxic chemical species being adsorbed onto the surface of the nanoparticle making them unsuitable for clinical and biomedical applications [18]. The use of hazardous chemicals during manufacture and the presence of these toxic substances on the surface of the metal nanoparticles have resulted in significant research efforts into developing clean, reliable, biologically compatible and eco-friendly green synthesis processes [19, 20].

In recent years, biological entities such as actinomycetes [21], bacteria [22], fungus [23], plants [24], viruses [25] and yeast [26] have emerged as an attractive alternative to conventional physical and chemical methods for biologically synthesising metal nanoparticles. Manufacturing via biological entities as efficient biological factories has the potential to deliver new sources of novel nanoparticles that are stable, nontoxic, cost effective, eco-friendly and produced using the principles of green chemistry.
Figure 1. Nanoparticle formation through plant mediated biogenic green synthesis, adapted from [15].

In the case of plant mediated nanoparticle synthesis, the extracts (bark, flower, leaf, etc.) are all eco-friendly, have relatively short production times and have lower cultivation costs compared to other biological entities such as bacteria [27]. Other advantages of using plant extracts is that these offer a straightforward processing route and the ability to be easily scaled up for large-scale nanoparticle production and reduces toxic wastes. Synthesis begins by mixing a sample of plant extract with a metal salt solution. Nanoparticle formation is indicated by a colour change in the reaction mixture and is the result of the metal salt being reduced to form metal ions that accumulate to produce thermodynamically stable nanoparticles as presented in Figure 1 [28]. Growth continues with nanoparticles aggregating to form a variety of morphologies such as cubes, spheres, triangles, hexagons, pentagons, rods and wires [29]. The ultimate size and shape of nanoparticle produced results from competing growth rates between the various crystallographic facets. During nucleation, facet growth rates are influenced by the presence of surface modifying, (capping) agents present in the plant extract and formation parameters such as reactant concentrations, solution pH, reaction time and
temperature. Optimisation of experimental parameters during synthesis results in a significant enhancement of physicochemical properties of the nanoparticle [27, 30].

The Earth is dominated by flora and there exist hundreds of thousands of plant species in the biosphere. Worldwide, many plant species have been investigated for potential bio-factory capabilities towards the synthesis of metal nanoparticles. Studies have shown that the compositional make-up of a plant extract directly influencing the type of nanoparticles produced. With different plant extracts containing different types and concentrations of biochemical reducing agents that directly shape the morphology of the forming nanoparticles [31, 32]. However, current literature reports very few studies of the isolated indigenous Australian plant species being used for the synthesis of metal nanoparticles. Hence, the need for this research project, which undertook the first exploratory investigations into the biogenic synthesis capabilities of three indigenous Australian plant species. The three plants include *Eucalyptus macrocarpa* (rose of the west), *Xanthorrhoea glauca* (grass tree) and *Anigozanthos manglesii* (red and green kangaroo paw). Photographic images of the three plant species are presented in Figure 2 and highlights the diverse nature of the respective plants.

![Figure 2](image.png)

**Figure 2.** Photographic images of the three species of plants studied in this project: a) *Eucalyptus macrocarpa*, (rose of the west); b) *Xanthorrhoea glauca*, (grass tree), and c) *Anigozanthos manglesii*, (red and green kangaroo paw) found at the Murdoch University campus.

All three Australian plant species were investigated for their ability to produce metal nanoparticles *via* the synthesis of two noble metal nanoparticles gold (Au) and silver (Ag). Both Au and Ag synthesis were selected because both metal nanoparticles have
attracted significant interest and have been extensively studied due to their unique biological, electrical and optical properties that are derived from their size, shape and surface properties [33-35]. Therefore the results of the present studies could be easily compared to those reported in the literature. Interestingly, both Au and Ag nanoparticles have been investigated for a variety of applications such as biosensors [36], hyperthermia therapy [37], pharmaceuticals [38] and antibacterial drugs [39].

Importantly, the present work also examined the novel antibacterial activity of both Au and Ag nanoparticles against a variety of bacterial pathogens. The medicinal application was an essential aspect of the research since it identified each plant's ability to produce high value therapeutic materials. This is of particular importance since recent studies have clearly shown that antimicrobial resistance to current antibiotic medications is one of the most serious health threats to humanity today (40, 41). Antibiotics are specifically formulated to kill pathogenic bacteria and as a consequence are effective medications for the treatment of various illnesses and diseases. Since their development in the late 1930’s, antibiotics have been successfully used to treat many diseases and illnesses that often killed millions of people worldwide. Understandably, the development of antibiotics is considered to be one of the most important medical advances of the twentieth century. However, in recent years some bacterial pathogens have become resistant and continue to multiply in the presence of therapeutic levels of commonly used antibiotics (42). Worryingly, the number of antibiotic resistant bacterial pathogens are progressively increasing and at the same time the development of new antibiotic medications has steadily declined in recent years. These antibiotic resistant bacterial pathogens are a serious global health threat that can lead to patients experiencing delayed recovery, treatment failure and even loss of life. Crucially some bacterial pathogens are naturally resistant to particular kinds of antibiotic preparations and with time can become resistant due to genetic mutation or obtaining resistance from other bacterium. Thus, the evolution of new resistant bacterial strains over time is a natural phenomenon. Therefore to keep ahead of constantly evolving bacterial strains a major effort is needed to find new sources of antibacterial materials for the development of new antibiotic pharmaceuticals to overcome their resistance [43]. The present work contributes to the worldwide search for new forms of nanometre scale materials with antibacterial properties for potential use as antibiotic agents to control infectious diseases caused by pathogenic bacteria [44].
1.2. Scope of thesis

Although a wide range of nanometre scale materials has been manufactured using a variety of conventional physical and chemical processes, the present work focused on the biogenic synthesis of nanoparticles via eco-friendly green chemistry based principles. As mentioned previously, the literature in this field reports the results of numerous studies into nanoparticle synthesis via plant materials. However, in the case of indigenous Australian plant species there are but a few reports of such synthesis. To address this unsatisfactory situation, this project undertook exploratory studies into nanoparticle synthesis via three indigenous Australian plant species. This line of research was warranted as indigenous plants of Australia have evolved separately because of isolation of continents 50 million years ago. The plants examined were *Eucalyptus macrocarpa* (rose of the west), *Xanthorrhoea glauca* (grass tree) and *Anigozanthos manglesii* (red and green kangaroo paw). The work carried out in this project developed a relatively straightforward room temperature based process that effectively could synthesise both Au and Ag nanoparticles from their respective metal salts. The studies examined the effects of a number of controlling factors such as plant extract concentration, metal salt concentration, reaction time, reaction solution pH and temperature. Also investigated was the influence of controlling parameters on the quality, size and morphology of the synthesized nanoparticles. Finally, the project investigates the antimicrobial properties of the synthesised Au and Ag nanoparticles produced from the various plant species. Research carried out in this project significantly advances knowledge in the field of nanoparticle synthesis via indigenous Australian plant species. The research also provides an insight into the antibacterial properties of the synthesised nanoparticles and their potential to be used in development of new green pharmaceutical products.
1.3. Aims of thesis

This project presents the research results of a series of exploratory studies that investigated the biogenic synthesis of Au and Ag nanoparticles via three indigenous Australian plant species and the subsequent identification of particle antibacterial properties. The project is constructed around three aims: 1) discovery; 2) synthesis optimisation & particle characterisation, and 3) identification of particle/plant antimicrobial properties. Each aim is addressed by a number of individual case studies that allow for a more detailed investigation into the various aspects of nanoparticle synthesis, particle properties and particle/plant antimicrobial properties. Chapters three and four contain the case studies, with chapter three focusing more on particle synthesis and characterisation, while chapter four focuses more on particle/plant antibacterial properties. In summary, the three aims are:

Aim 1  Exploratory studies into the biogenic synthesis of Au and Ag nanoparticles using extracts taken from indigenous Australian plant species. This aim is addressed in Chapter three by Cases Studies 1, 2 and 3.

Aim 2  Synthesis process optimisation and advanced characterisation techniques to determine the various physiochemical properties of the manufactured Au and Ag nanoparticles. This aim is addressed in the Case studies in Chapters three and four.

Aim 3  Identify antibacterial properties of the synthesised Ag nanoparticles produced by the respective indigenous Australian plant species. This aim is addressed specifically in Chapter four by Cases Studies 4 and 5.

The overall structure of this thesis is composed of five chapters that are specifically designed to address the above-mentioned aims, and elucidate the current state of research in this field. The thesis’s relevance is founded in finding new plant-mediated sources for the synthesis of noble metal nanoparticles. The present chapter has given a brief background that outlines the scope of the thesis and the specific aims of the research project. Chapter 2 is composed of two major review articles that examine the
current use of biological entities to synthesise metal nanoparticles. The first review article focuses on recent trends in developing sustainable eco-friendly technologies for the production of metal nanoparticles using the principles of green chemistry. In particular, it focuses on the use of plants and their respective extracts (bark, flower, leaf, etc.) to be used as bio-factories for the manufacture of metal nanoparticles. The second review article focuses on the largely unexplored field of marine plants for the synthesis of nanoparticles. The second review is of particular importance since there is relatively few reports in the literature reporting the synthesis of nanoparticles via marine based plants such as algae and sea grasses. Chapter three reports the results of a number of exploratory studies investigating the synthesis of Au and Ag nanoparticles via indigenous Australian plant species. While chapter 4 presents, for the first time, reports the results of Au and Ag nanoparticle synthesis, characterisation and antibacterial studies of *Xanthorrhoea glauca* (grass tree), *Anigozanthos manglesii* (red and green kangaroo paw). Chapter five summarises the research work carried out, discusses the results of the research and the implications of the findings. The chapter concludes with suggestions for future research to be undertaken to further investigate the biogenic properties of indigenous Australian plant species for the synthesis of metal nanoparticles and their potential medicinal applications.

References


5. International Organisation for Standardisation (ISO); Terminology and definitions for nano-objects: Nanoparticle, nano-fibre and nano-plate, ISO/TS 27687, 2008 (E); Geneva, Switzerland.


Chapter 2 - Literature review

2.1. Overview and author contributions

Advances in nanotechnology have delivered many new tools, processes and characterization techniques that have made it possible to engineer materials and structures at the molecular scale. The size, shape and surface chemistry of materials produced at the nanometre scale have a profound influence on the physiochemical, optical and electronic properties when compared to the properties of their conventional bulk counterparts. For example, gold (Au) nanoparticles have unique chemical and physical properties that gives them immense potential as delivery platforms for pharmaceuticals in a variety of therapeutic applications [1, 2] and as imaging agents in a number of diagnostic imaging protocols [3]. While current research into the medicinal use of (Ag) nanoparticles has shown microbial cell membrane damage via particle size, shape, surface chemistry interactions and biological sorption [4, 5]. Because of the attractive antibacterial properties displayed by Ag nanoparticles they have been widely used in a variety of products such as cosmetic products, detergents, shampoos, medical and pharmaceutical preparations [6]. It is due to the attractive and unique material properties of metal nanoparticles that makes them a prime candidate for future application in antimicrobials, pharmaceuticals, biomedical protocols, diagnostic imaging systems and environmental remediation projects.

Contemporary physical and chemical production methods are extensively used for the manufacture of nanoparticles. Unfortunately, during processing many of these techniques use toxic chemicals and solvents that make these processes unfriendly to the environment and also leave undesirable capping coatings on the nanoparticles which must be removed before application. Therefore, to avoid the disadvantages of conventional processing methods current research has focused on developing new green chemistry based procedures that are eco-friendly and produce clean metal nanoparticles. Recent research has shown that biological systems such as bacteria, fungi, yeast, and plant extracts can be used to varying degrees to produce metal nanoparticles. The
advantage of producing metal nanoparticles via biological entities is that it accomplishes the principles and practice of green chemistry as defined by Anastas and Warner, who define green chemistry as the design of chemical products and processes to reduce or eliminate the use and generation of hazardous substances [7]. The objective of Chapter 2 was to review current literature in the field of biogenic synthesis of metal nanoparticles via entities such as actinomycetes algae, bacteria, fungi, viruses, yeasts and plants. Chapter 2 is composed of two extensive review articles that examine the use of biological entities to synthesise metal nanoparticles without the use of harsh, toxic and expensive chemicals commonly used in conventional physical and chemical processes. A recent trend in developing sustainable eco-friendly technologies for producing metal nanoparticles using the principles of green chemistry is discussed. In the first review article, the first part of the review briefly surveys the use of microorganisms to produce metal nanoparticles. The second and more extensive part discusses the role of plants and their respective extracts (bark, flower, leaf, etc.) to be used as bio-factories for the manufacture of metal nanoparticles.

During the literature searches for the first review article it became very clear that there were relatively few research articles and no reviews discussing the biogenic synthesis of metal nanoparticles using marine plants. To date, the marine environment that covers approximately 70% of the earth’s surface is largely unexplored and is home to many forms of marine algae and sea grasses. A marine alga, generally known as seaweeds are a largely unknown research field in terms of nanoparticle synthesis and offers some unique opportunities for exploration and developing new green chemistry based biogenic processes. To highlight and promote this new and exciting research area a second review article was prepared from the currently available literature in the field. At the time of preparing this thesis there was no nanoparticle synthesis via marine plant reviews in the literature and the present review is believed to be the first in this field.

The author contributions in the reviews consisted of G.E.J. Poinern acting as principal supervisor who designed the overall concept of the review papers in conjunction with M. Shah. In the first review, M. Shah was first author and significantly contributed to the content of the paper. All text, tables and images were developed and written by M. Shah under the technical guidance of D. Fawcett and S. Sharma. M. Shah was assisted by G.E.J. Poinern, S. Sharma, and D. Fawcett in over-coming some of the various
technical difficulties encountered during manuscript preparation and with the editorial changes to the manuscript as recommended by reviewers. In the second review, M. Shah was second author working closely with D. Fawcett to research, develop and write the manuscript. J. Verduin, G.E.J. Poinern, and S. Sharma provided technical assistance during the development of both reviews and manuscript submission. All authors provided feedback during the preparation of both reviews which was coordinated by M. Shah.

2.2. Publications

Review Article 1

Review Article 2
Derek Fawcett, Monaliben Shah, Jennifer Verduin, Shashi Sharma and Gérrard Eddy Jai Poinern. Biogenic Synthesis of Metal and Metal Oxide Nanoparticles via Marine Alga and Sea Grasses - Submitted

2.3. Chapter summary

Chapter 2 consisted of two extensive reviews that examined and discussed current literature in the use of biological entities to produce metallic nanoparticles. The reviews highlight the ability of various biological entities to produce metal nanoparticles. Importantly, nanoparticles synthesised via plant extracts were found to be stable, nontoxic, and eco-friendly. However, much of this field remains largely unknown due to the diversity of biological entities. Plant extracts have the potential to produce nanoparticles with a specific size, shape and composition. In particular, their synthesis is inexpensive, easily scaled up and environment-friendly. Biogenic synthesis of nanoparticles via plants have the potential to be used in a wide variety of applications such as the delivery of therapeutic drugs, antibacterial agents in bandages and optoelectronics and sensors [8-11]. However, there are some unresolved issues
involving particle size and shape consistency, process reproducibility and formation mechanisms. Thus, both reviews have highlighted the need for further research into understanding plant/biomolecule dependent mechanisms involved in reducing, capping and stabilizing the nanoparticles to prevent agglomeration. Importantly, the literature surveys presented in this chapter have also revealed that very few indigenous Australian plant species have been investigated for potential metal nanoparticle synthesis. Hence the need for this research project investigating the potential of using indigenous Australian plant species to biologically synthesise metal nanoparticles.

References


Green Synthesis of Metallic Nanoparticles via Biological Entities

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Abstract: Nanotechnology is the creation, manipulation and use of materials at the nanometre size scale (1 to 100 nm). At this size scale there are significant differences in many material properties that are normally not seen in the same materials at larger scales. Although nanoscale materials can be produced using a variety of traditional physical and chemical processes, it is now possible to biologically synthesize materials via environment-friendly green chemistry based techniques. In recent years, the convergence between nanotechnology and biology has created the new field of nanobiotechnology that incorporates the use of biological entities such as actinomycetes algae, bacteria, fungi, viruses, yeasts, and plants in a number of biochemical and biophysical processes. The biological synthesis via nanobiotechnology processes have a significant potential to boost nanoparticles production without the use of harsh, toxic, and expensive chemicals commonly used in conventional physical and chemical processes. The aim of this review is to provide an overview of recent trends in synthesizing nanoparticles via biological entities and their potential applications.

Keywords: green chemistry; biological synthesis; nanoparticles

1. Introduction

In recent years, the convergence of nanometre size scale technologies and biological technologies has created the new field of nanobiotechnology. This relatively new field is focused on the creation, manipulation, and use of materials at the nanometre scale for advanced biotechnology [1]. At the forefront of this field is the synthesis of nanometre size scale particles via biological entities. Nanoparticles are of great interest due to their novel physicochemical, magnetic, and optoelectronic properties that are governed by their size, shape, and size distribution [2–6]. It is predominantly the nanoparticles’ extremely small size and large surface area to volume ratio that leads to the significant differences in properties (e.g., biological, catalytic activity, mechanical properties, melting point optical absorption, thermal and electrical conductivity) not seen in the same material at larger scales in their bulk form [7]. Because of these unique physicochemical and optoelectronic properties, nanoparticles are of particular interest for a number of applications ranging from as catalysts, chemical sensors, electronic components, medical diagnostic imaging, pharmaceutical products, and medical treatment protocols. For example, metallic nanoparticles of noble metals such as gold, silver, platinum, and palladium have been widely used in products ranging from cosmetic to medical and
pharmaceuticals. Gold nanoparticles have been extensively used in biomedical applications [8–10], separation sciences [11], disease diagnostics [12], and pharmaceuticals [13, 14]. Silver nanoparticles have been found to possess both anti-bacterial and anti-inflammatory properties that can promote faster wound healing. Because of these advantageous properties, silver nanoparticles have been integrated into commercially available wound dressings, pharmaceutical preparations, and medical implant coatings [15–20]. Platinum nanoparticles have been widely used in biomedical applications in either pure form or alloyed with other nanoparticles [21] and palladium nanoparticles in catalysis and electro-catalysis applications [22–24], chemical sensors [25], optoelectronics [26], and anti-bacterial applications [27]. In addition, non-noble metallic nanoparticles such as iron [28,29], copper [30], zinc oxide [31], and selenium [32] have also been used in medical treatments, cosmetic formulations, and anti-bacterial applications.

Due to the increased demand for various metallic and non-metallic nanoparticles over the past two decades, a wide range of physical and chemical techniques have been developed to produce nanoparticles of different sizes, shapes, and compositions. Traditionally, nanoparticles have been synthesized and stabilised via physical and chemical techniques. The physical approach includes techniques such as laser ablation [33], lithography [34] and high-energy irradiation [35]. While the chemical approach uses techniques such as: chemical reduction, electrochemistry, and photochemical reduction [36–40]. Studies have shown that during the synthesis process, size, shape, stability, and physicochemical properties of the nanoparticles are strongly influenced by a variety of factors. These factors include process parameters (temperature, concentrations, etc.), process kinetics involving the interplay between the metal ion precursors and the reducing agent, and adsorption kinetics involving the stabilizing agent and the nanoparticles [41,42]. Consequently, designing a process that effectively controls the size, shape, stability, and physicochemical properties is currently at the forefront of research into nanoparticle synthesis [43,44]. Conventional synthesis of nanoparticles can involve expensive chemical and physical processes that often use toxic materials with potential hazards such as environmental toxicity, cytotoxicity, and carcinogenicity [45]. The toxicity problems arise from the hazardous substances, such as organic solvents, reducing agents, and stabilizers that are used to prevent unwanted agglomeration of the colloids. In addition, some nanoparticles have also been found to be toxic due to factors such as composition, size, shape, and surface chemistry. As a result, the presence of these toxic formation agents on the synthesized nanoparticles and potentially the nanoparticles themselves has prevented their clinical and biomedical application. Importantly, all these factors can be potentially controlled via biological mediated production. As a result, there is currently widespread interest in developing clean, reliable, biologically compatible, benign, and environment-friendly green processes to synthesize nanoparticles [44,46].

In recent years, biological synthesis has emerged as an attractive alternative to traditional synthesis methods for producing nanoparticles. Biosynthesis involves using an environment-friendly green chemistry based approach that employs unicellular and multicellular biological entities such as actinomycetes [47,48], bacteria [49–53], fungus [54–57], plants [58,59], viruses [60,61], and yeast [62–64]. Synthesising nanoparticles via biological entities acting as biological factories offers a clean, nontoxic and environment-friendly method of synthesising nanoparticles with a wide range of sizes, shapes, compositions, and physicochemical properties [65]. Another interesting feature of many biological entities is their ability to act as templates in the synthesis, assembly and organisation of nanometre scale materials to fabricate well-defined micro and macro scale structures. For example, viruses have been used to assemble gold and iron oxide nanoparticles to form microstructures [66], bacteriophages have also been used to form intricate nanometre and micrometre scale structures [67–69] and phage based assemblies of liposomes have been used in targeted drug delivery procedures [70–73]. Comparing the above-mentioned biological identities and their potential to become efficient biological factories, synthesizing nanoparticles via biological entities, is a relatively straight forward and advantageous approach [74,75]. In comparison with microorganisms, the plant approach is more advantageous since it does not need any special, complex, and multi-step
procedures such as isolation, culture preparation, and culture maintenance. Furthermore, synthesis in plants tends to be faster than microorganisms, is more cost-effective and is relatively easy to scale up for the production of large quantities of nanoparticles [74,76–79]. The aim of this review is to present a brief overview of the techniques used to characterise nanoparticles, microbial routes for synthesising metal and metal oxide nanoparticles, use of plants extracts for synthesis of nanoparticles, factors influencing the synthesis process, possible mechanisms involved in nanoparticle formation and growth, and potential applications of nanoparticles synthesised using natural biological factories found in plants.

2. Characterisation Techniques

To date, there are numerous techniques for synthesizing nanoparticles. However, these techniques fall into two broad approaches and can be defined as either a top down approach or a bottom up approach [80–82]. The top down approach starts with a material of interest, which then undergoes size reduction via physical and chemical processes to produce nanoparticles. Importantly, nanoparticles are highly dependent on their size, shape, and surface structure and processing tends to introduce surface imperfections. These surface imperfections can significantly impact on the overall nanoparticle surface physicochemical properties [83]. In the bottom up approach, nanoparticles are built from atoms, molecules and small particles/monomers [84–86]. In either approach, the resulting nanoparticles are characterized using various techniques to determine properties such as particle size, size distribution, shape, and surface area. This is of particular importance if the properties of nanoparticles need to be homogeneous for a particular application.

In the case of chemical and biological synthesis of nanoparticles, the aqueous metal ion precursor from metal salts are reduced and as a result a colour change occurs in the reaction mixture. This is the first qualitative indication that nanoparticles are being formed. One interesting property of colloidal particles in solution, due to their size and shape, is their ability to be seen when a laser beam passes through the colloidal solution. This effect is known as the Tyndall effect and is a simple and straightforward technique that can be used to detect the presence of nanoparticles in solution [87]. After the reaction, nanoparticles can be separated from the colloid by high speed centrifugation and then examined using advanced nanocharacterization techniques.

Some of the spectroscopy and microscopy techniques routinely used include UV-visible spectroscopy (UV-vis), dynamic light scattering (DLS), atomic force microscopy (AFM), transmission electron microscopy (TEM), scanning electron microscopy (SEM), energy dispersive spectroscopy (EDS), powder X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FT-IR), and Raman spectroscopy. Microscopy based techniques such as AFM, SEM and TEM are considered direct methods of obtaining data from images taken of the nanoparticles. In particular, both SEM and TEM have been extensively used to determine size and morphological features of nanoparticles [87–90]. Spectroscopy based techniques such as UV-vis, DLS, XRD, EDS, FT-IR, and Raman are considered indirect methods of determining data related to composition, structure, crystal phase, and properties of nanoparticles. The UV-visible spectroscopy covers the UV range between 190 and 380 nm and the visible range between 380 and 800 nm. Both types of radiation interact with matter and promote electronic transitions from the ground state to higher energy states. Wavelengths between 300 and 800 nm are generally used for characterizing metallic nanoparticles ranging in size from 2 nm up to around 100 nm [87]. For example, absorption measurements for silver (Ag) nanoparticles are usually between 400 and 450 nm [91,92], while gold (Au) nanoparticles are generally detected by the presence of peaks between 500 and 550 nm [93,94]. DLS spectroscopy can be used to determine size distribution and quantify the surface charge of nanoparticles suspended in a liquid [87,95]. The elemental composition of nanoparticles can be determined via EDS mapping. Whereas XRD examination produces a diffraction pattern that is subsequently compared with data contained in a standard crystallographic database to determine structure information. Analysis of the XRD data identifies crystallite size, structure, preferred crystal orientation, and phases present.
3. Biological Synthesis of Nanoparticles

Recent studies have shown that green biologically based methods using microorganisms and plants to synthesize nanoparticles are safe, inexpensive, and an environment-friendly alternative [99,100]. Both microorganisms and plants have long demonstrated the ability to absorb and accumulate inorganic metallic ions from their surrounding environment. These attractive properties make many biological entities efficient biological factories capable of significantly reducing environmental pollution and reclaiming metals from industrial waste. Importantly, the ability of a biological entity to use its inherent biochemical processes to transform inorganic metallic ions into metal nanoparticles has led to a relatively new and largely unexplored field of research [101]. To date, the ability of microorganisms to interact, extract, and accumulate metallic materials from their surroundings has been capitalized on in a number of biotechnology applications such as bioremediation and bioleaching [102,103]. The capability of microorganisms to actively interact with their surrounding environment stems from the composition of their lipid-based amphipathic membranes enabling a variety of oxidation-reduction mechanisms to take place and promote biochemical conversions [104–106]. Studies have shown that both unicellular and multicellular organisms achieve both extracellular and intracellular synthesis of inorganic micron and nano-sized materials as presented in Table 1, and in the case of nanoparticle synthesis, culturing microorganisms in particular environments can also assist them in promoting coupled oxidation and reduction phenomenon [104,107]. The specific oxidation-reduction mechanisms, nucleation, and subsequent nanoparticle growth kinetics and the interaction of these processes with the microorganism metabolic processes have yet to be fully explained [108–111]. Hence, there is still a considerable level of research that needs to be undertaken to fully investigate and elucidate differences in nanoparticle size and morphology between different metals when synthesized using the same microorganism [65,105]. This is also true when considering the use of plants for synthesizing nanoparticles. The advantage of using plants over other eco-friendly biologically based systems such as bacteria and fungi, is that it avoids the use of specific, well-conditioned culture preparation and isolation techniques that tend to be expensive and elaborate. Conversely, biosynthesis of nanoparticles using plants or plant based extracts tends to be safe, have relatively short production times, and have a lower cultivation cost compared to other biological systems [112]. Furthermore, plant based biosynthesis is a relatively straightforward process that can be easily scaled up for large-scale production of nanoparticles.

As mentioned above, nanoparticles can be synthesised from a wide variety of biological entities such as actinomycetes, algae, bacteria, fungus, plants, viruses, and yeast. Each biological entity has varying degrees of biochemical processing capabilities that can be effectively used to synthesize particular metallic or metallic oxide nanoparticles. Not all biological entities can synthesize nanoparticles due to their enzyme activities and intrinsic metabolic processes. Therefore, careful selection of the appropriate biological entity is necessary to produce nanoparticles with well-defined properties such as size and morphology. Generally, biological entities with a potential to accumulate heavy metals have the best chance of synthesizing metallic nanoparticles [113–116]. In the case of a microorganism, culturing methods are very important. Hence optimisation of culturing parameters such as nutrients, light, medium pH, temperature, mixing speed, and buffer strength can significantly increase enzyme activity [74,117]. Recently, the biological synthesis of nanoparticles using plants and plant extracts appears be to an attractive alternative to conventional chemical synthesis and
the more complex culturing and isolation techniques needed for many microorganisms. Moreover, combinations of molecules found in plant extracts perform as both reducing and stabilizing (capping) agents during nanoparticle synthesis [118–120]. These biological molecules are chemically complex, but have the advantage of being environment-friendly.

**Table 1.** A selection of microorganisms used to synthesize nanoparticles.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Nanoparticle</th>
<th>Size (nm)</th>
<th>Extracellular/Intracellular</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinomycetes</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Rhodococcus sp.</td>
<td>Au</td>
<td>5 to 15, Spherical</td>
<td>I</td>
<td>[93]</td>
</tr>
<tr>
<td>Thermomonospora sp.</td>
<td>Ag</td>
<td>8, Spherical</td>
<td>E</td>
<td>[47,48]</td>
</tr>
<tr>
<td>Algae</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Chlorella vulgaris</td>
<td>Au</td>
<td>40 to 60, Spheroid, polyhedral</td>
<td>I</td>
<td>[121]</td>
</tr>
<tr>
<td>Sargassum wightii</td>
<td>Au, Ag</td>
<td>Spheroid</td>
<td>E</td>
<td>[122]</td>
</tr>
<tr>
<td>Bacteria</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>CdS</td>
<td>2 to 5, Spherical</td>
<td>I</td>
<td>[123]</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>Ag</td>
<td>8 to 10 Spherical</td>
<td>I</td>
<td>[124]</td>
</tr>
<tr>
<td>Pseudomonas stutzeri</td>
<td>Au</td>
<td>20 to 40 Spherical</td>
<td>E</td>
<td>[125]</td>
</tr>
<tr>
<td>Fusarium oxysporum</td>
<td>Au</td>
<td>20 to 40, Spherical, triangular</td>
<td>E</td>
<td>[126]</td>
</tr>
<tr>
<td>Volvariella volvacea</td>
<td>Ag &amp; Au</td>
<td>20 to 150, Spherical, hexagonal</td>
<td>E</td>
<td>[127]</td>
</tr>
<tr>
<td>Viral</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M13 bacteriophage</td>
<td>CdS, ZnS</td>
<td>Quantum dots, nanowires</td>
<td>E</td>
<td>[128]</td>
</tr>
<tr>
<td>M13 bacteriophage</td>
<td>HAP</td>
<td>Hydroxyapatite fibrils</td>
<td>E</td>
<td>[129,130]</td>
</tr>
<tr>
<td>Bacteriophage</td>
<td>Ca</td>
<td>Fibrils</td>
<td></td>
<td>[131,132]</td>
</tr>
<tr>
<td>Tobacco mosaic virus (TMV)</td>
<td>SiO$_2$, CdS, PbS, FeZnO$_3$</td>
<td>Various shapes</td>
<td>E</td>
<td>[133,134]</td>
</tr>
<tr>
<td></td>
<td>CdS</td>
<td>-</td>
<td>E</td>
<td>[60,135]</td>
</tr>
<tr>
<td>Tobacco mosaic virus (TMV)</td>
<td>Sb$_2$O$_3$, CdS</td>
<td>Nanotubes on surface</td>
<td>E</td>
<td>[60,135]</td>
</tr>
<tr>
<td>Yeast</td>
<td>Ag</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Candida glabrata</td>
<td>CdS</td>
<td>2, Spherical</td>
<td>I</td>
<td>[62]</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>PbS</td>
<td>3 to 10, Spherical</td>
<td>I</td>
<td>[77]</td>
</tr>
<tr>
<td>Candida glabrata (Yeast)</td>
<td>Ag</td>
<td>3 to 100, Spherical</td>
<td>I</td>
<td>[136]</td>
</tr>
<tr>
<td>Yeast strain MKY9</td>
<td>Ag</td>
<td>2 to 5, Hexagonal</td>
<td>E</td>
<td>[63]</td>
</tr>
<tr>
<td>Schizosaccharomyces pombe</td>
<td>PbS</td>
<td>1 to 2, Hexagonal</td>
<td>I, I</td>
<td>[62,110]</td>
</tr>
<tr>
<td>Torulopsis sp.</td>
<td>Ag</td>
<td>2 to 5, Spherical</td>
<td>I</td>
<td>[137]</td>
</tr>
</tbody>
</table>

The importance of developing environment-friendly sustainable metal nanoparticle producing technologies using the principles of green chemistry is discussed. The first part of this review briefly surveys the use of microorganisms and the second, more extensive part, examines the role of plants in synthesizing metal nanoparticles.

4. Microbial Routes for Nanoparticle Synthesis

Many studies have shown that microorganisms, both unicellular and multicellular have the ability to synthesize inorganic materials. The biological synthesis can be considered a bottom-up approach where nanoparticle formation occurs due to the reduction/oxidation of metallic ions via biomolecules such as enzymes, sugars, and proteins secreted by the microorganism [138]. However, a complete understanding of nanoparticle synthesis mechanism occurring in microorganisms is yet to be fully developed. This is because each type of microorganism tends to behave and interact differently with particular metallic ions. The interaction and biochemical processing activities of a specific microorganism and the influence of environmental factors such as pH and temperature ultimately determines the formation of nanoparticles with a particular size and morphology [50,100]. Nanoparticle formation can be either extracellular or intracellular depending on the microorganism as seen in Table 1 [139–143]. The following six sections briefly discuss some of the main microbial routes used to synthesise nanoparticles.
4.1. Actinomycetes

The literature reports extensively on the extracellular or intracellular synthesis of metallic nanoparticles via actinomycetes [144–146], with extracellular synthesis being the more common pathway. Intracellular reduction of metallic Au ions by the Rhodococcus sp. has revealed that Au nanoparticles were predominantly reduced on the cell membrane and cell wall, but not in the cytosol. Reduction of Au ions is believed to be the result of interacting enzymes being released from the cell membrane and cell wall while capping proteins stabilizes the formed nanoparticles. The biosynthesis process produced mono-dispersed Au nanoparticles ranging from 5 to 15 nm in size; the nanoparticles were non-toxic to the cell [144]. Similar studies with actinomycete cells have confirmed the intracellular reduction of Au and Ag ions by cell wall enzymes to form metallic Ag seeds/monomers that consequently initiate the growth of nanoparticles [147–150].

In an effort to explain the mechanism and conditions that favoured extracellular synthesis of nanoparticles in 2014, Karthik et al. undertook the reduction of silver nitrate (AgNO₃) ions by using Streptomyces sp. LK-3. This resulted in the efficient formation of Ag nanoparticles [145]. It is known that the nitrate reductase enzyme is generally involved in the cellular nitrogen cycle and is responsible for the reduction of nitrate to nitrite [151]. Their study indicated that Nicotinamide adenine dinucleotide (NADH-) dependent nitrate reductase enzyme, was indeed responsible for the reduction of Ag ions to metallic Ag via an electron transfer mechanism, and the subsequent formation of stabilized Ag nanoparticles. A similar nitrate reductase enzyme mechanism is seen in the reduction of Au ions from aqueous solutions containing gold chloride (AuCl₄⁻) ions [152]. During the electron transfer from NADH by NADH-dependent reductase, each Au ion receives an electron and it reduces to Au⁰ and subsequently forms stabilized Au nanoparticles [153,154]. Importantly, effective stabilization is necessary to prevent agglomeration due to the high-surface energy and protect the properties of the synthesized nanoparticle. Interestingly, biologically synthesized nanoparticles tend to have higher antimicrobial activity when compared with traditionally synthesized nanoparticles. The higher antimicrobial activity is believed to be the result of the action of synergistic proteins involved in capping and stabilizing the nanoparticles [155].

4.2. Algae

Algae are aquatic microorganisms and recent studies have shown that some of them not only accumulate heavy metals, but they can also be used to biologically synthesize metallic nanoparticles. For example, the dried unicellular alga Chlorella vulgaris was used to synthesize tetra-chloroaurate ions to form algal-bound gold that was subsequently reduced to form Au nanoparticles. The tetrahedral, decahedral and icosahedral shaped nanoparticles were found to accumulate near the cell surfaces [121]. A similar study using an extract from C. vulgaris was found to produce Ag nanometre scale plates at room temperature. The study indicated that proteins contained within the extract acted as reducing agent, shape-control modifier and stabilizing agent [156]. And a study by Govindaraju et al. revealed that a marine alga Sargassum wightii was capable of extracellular synthesis of Au, Ag and Au/Ag bimetallic nanoparticles [157]. Recently, Singaravelu et al. showed that S. wightii could rapidly synthesize Au nanoparticles. The extracellular synthesis produced nanoparticles ranging in size from 8 to 12 nm [122]. Rajasulochana et al. have also reported the synthesis of extracellular Au nanoparticles using Kappaphycus alvarezii [158]. While Mata et al. has reported on the biological reduction of Au using biomass derived from brown alga Fucus vesiculosus [159]. Additionally, Senapati et al. reported the intracellular synthesis of Au nanoparticles via Tetraselmis kochinensis [160]. And recently, Castro et al. reported using red Chondrus crispus and green alga Spirogyra insignis for synthesizing Au and Ag nanoparticles [161].
4.3. Bacteria

In nature, bacteria are frequently exposed to diverse and sometimes extreme environmental situations. Survival in these harsh conditions ultimately depends on their ability to resist the effects of environmental stresses. Natural defence mechanisms exist in bacteria to deal with a variety of stresses such as toxicity arising from high concentrations of metallic ions in the environment. Biological strategies for dealing with high concentrations of metallic ions include changes in metal ion concentration via redox state changes, efflux systems, intracellular precipitation, and accumulation of metals, and extracellular formation of complexes [162]. The major bacterial species used for the synthesis of metallic nanoparticles include Actinobacter sp., Escherichia coli, Klebsiella pneumonia, Lactobacillus spp., Bacillus cereus, Corynebacterium sp., and Pseudomonas sp. [65,163–165]. Bacteria are known to synthesise metallic nanoparticles by either intracellular or extracellular mechanisms. For example, Ag nanoparticles have been synthesized using Pseudomonas stutzeri AG259 bacterium via a mechanism involving the NADH-dependent reductase enzyme that donates an electron and oxidises to NAD⁺. The electron transfer results in the biological reduction of Ag ions to Ag nanoparticles [55]. In a similar study, Hussein et al. were able to reduce Au ions using Pseudomonas aeruginosa that resulted in the extracellular synthesis of Au nanoparticles [53]. However, some other researchers have also shown the non-involvement of biological enzymes. For example, Liu et al. were able to produce Au nanoparticles from dried cells of Bacillus megaterium [166]. A similar study by Sneha et al. using a Corynebacterium sp also revealed that a non-enzymatic reduction mechanism was involved in nanoparticle formation [167]. The reduction of nanoparticles is believed to be the result of a combination of several factors. The first factor is the presence of some organic functional groups at the cell wall that induce reduction, and the second depends on the appropriate environmental parameters such as pH and temperature being present [168]. For example, the dried biomass of Lactobacillus sp. A09 and Bacillus megaterium D01 can reduce Ag ions via the interaction of functional groups present on the cell wall to produce silver nanoparticles [169].

Size, shape, and composition of a nanoparticle can be significantly influenced by pH and temperature [170]. For example, particle size is an important factor since novel and unique physicochemical properties are more pronounced at smaller sizes. Therefore, there is a need to optimize synthesis parameters during nanoparticle formation to enhance the overall particle properties. In particular, selecting the appropriate culture media for a specific bacteria and the particular metallic salt is important since these two parameters form the basis of nanoparticle synthesis and can influence particle yield [49,51,171]. Studies by He et al. using bacterium Rhodopseudomonas capsulata have shown that particle size and morphology can be influenced by both metallic salt concentration and medium pH. At pH 6, dilute concentrations of AuCl₄⁻ tended to produce spherical Au nanoparticles ranging in size from 10 to 20 nm. Upon increasing the salt concentration, this reaction tended to produce Au nanowires at pH 6 [172]. Also, when the pH was changed to 4, dilute salt concentrations tended to produce both spheres and triangular nanometre scale plates [153]. The studies clearly indicated that controlling medium pH directly influenced nanoparticle morphology during formation. Table 1 summarizes the major bacterial species that have been used to synthesize a variety of nanoparticles along with composition, particle size range, and morphology.

4.4. Fungi

Biosynthesis of nanoparticles utilising fungi is widespread among many research groups globally and the synthesis occurs at both extracellular and intracellular locations. For example, fungi such as Aspergillus sp., Fusarium sp., and Penicillium sp. have been frequently reported for their biosynthetic ability to create both Ag and Au nanoparticles [124,125,127,173,174]. Moreover, studies have shown that fungi are capable of producing mono dispersed nanoparticles and particle sizes over a wide range of different chemical compositions as seen in Table 1. Fungi possess some additional attributes when compared to their bacterial counterparts for the synthesis of metallic
nanoparticles. For instance, fungi secrete large amounts of proteins and enzymes per unit of biomass, which results in larger amounts of nanoparticles being manufactured [175]. Studies have shown that some fungi possess high intracellular metal uptake volumes and the synthesised particles tend to be smaller in size [126,176]. However, the culture conditions can have a significant influence during the biosynthesis of metallic nanoparticles. For example, the biological reduction of Au ions was carried out using Trichothecium sp. biomass under stationary conditions synthesized extracellular nanoparticles. In contrast, agitation of the biomass tended to produce intracellular nanoparticles. One possible explanation suggested by this result was that non-agitation promoted the release of enzymes and proteins while agitation prevented their release [56]. Fluorescence spectra studies have indicated that extracellular synthesis of nanoparticles by the fungi results from the action of bioactive reducing agents secreted from the cell wall and it produces protein-stabilized nanoparticles. The study was able to show that the same proteins released by the fungal biomass were present in the solution and also bound to the surfaces of nanoparticles [5,177,178]. Both extracellular and intracellular synthesis of nanoparticles using fungi has been investigated. In the case of intracellular synthesis, extraction procedures in downstream processing suffer from the drawback of low yields. In contrast, extracellular synthesis produces nanoparticles at the cell surface or at the periphery of the cell, which means they can be readily recovered in downstream processing [162,173]. A very notable feature of some fungi is their ability to synthesize nanoparticles of different chemical compositions. For example, studies by Bansal et al. have shown that Fusarium oxysporum can biosynthesize silica and titania nanoparticles from aqueous solutions of Si$^2^-$ and TiF$^2^-$ respectively [179]. Furthermore, the synthesis of nanometre scale materials such as luminescent CdSe quantum dots [180], magnetite [181], zirconia [182], and oxide nanoparticles [183] have been reported in the literature.

4.5. Viruses

The use of viruses in the synthesis of nanomaterials is a novel technique that has been able to deliver inorganic materials such as silicon dioxide (SiO$_2$), cadmium sulphide (CdS), iron oxide (Fe$_3$O$_4$), and zinc sulphide (ZnS). Semiconductor materials such as CdS and ZnS are of particular interest to the electronics industry and green chemistry based methods for their synthesis have been extensively investigated. The use of viruses to synthesize quantum dots has been investigated over the last decade [60,62,128]. An attractive feature of viruses is their dense surface covering of capsid proteins that form a highly reactive surface capable of interacting with metallic ions [100]. For example, a typical plant virus such as the Tobacco mosaic virus (TMV) particle can have as many as 2130 capsid protein molecules covering its surface. The array of proteins can act as attachment points for the deposition of materials [184–187] or can be used to create three-dimensional vessels for pharmaceuticals [188–190]. In a recent study, low concentrations of TMV’s were added to Ag or Au salts before adding plant extracts of Nicotiana benthamiana (Round-leaved native tobacco) or Hordeum vulgare (Barley). The presence of the virus not only decreased the size of the synthesized nanoparticles, but also dramatically increased their numbers compared to the non-virus solutions [191]. The study also revealed that at higher TMV concentrations fewer free nanoparticles were formed and at the same time the TMV acted as a bio-template that underwent metallization to form nanowires. Similar studies have also shown the potential of viruses to be used as a template for the manufacture of nanometre scale structures such as nanowires and nanotubes [61,135].

4.6. Yeasts

Yeasts, like many other microorganisms, have the ability to absorb and accumulate significant amounts of toxic metals from their surrounding environment [105,107]. Adaptation to metal toxicity has resulted in yeast cells using a variety of detoxification mechanisms that cause activities such as chelation, bio-precipitation bio-sorption and extracellular sequestration. The variation in particle size, location, and particle properties is due to the different mechanisms used by yeast organisms to form and stabilise the nanoparticles during synthesis [170]. Studies by Dameron et al. have shown that
when Candida glabrata was exposed to cadmium salts the resulting intracellular synthesis produced CdS quantum dots [62]. Similar studies using Schizosaccharomyces pombe cells have also been able to find a link between the formation of CdS quantum dots and the growth phase of the yeast [110,192]. Moreover, intracellular synthesis of PbS quantum dots was possible when Torulopsis sp. were exposed to Pb2+ ions [137]. Also, Au nanoparticles ranging in size from a few nanometres up to around 100 nm have been intracellularly synthesized using Pichia jadinii. Importantly, it was found that during the synthesis step, the nanoparticle size and shape was easily regulated by controlling both the growth and cellular activities of P. jadinii [136]. In similar studies, the influence of biomass and Au salt concentration during extracellular synthesis [193] and intracellular synthesis [194] were studied using the marine yeast Yarrowia lipolytica, the studies revealed that both biomass and Au salt influenced the size and morphology of particles formed. For example, increasing Au salt concentration not only continued to produce nanometre scale spheres, but it also tended nanometre scale plates. Furthermore, the silver-tolerant yeast strain MKY3 has been used to synthesize extracellular Ag spherical nanoparticles ranging in size from 2 to 5 nm [63].

5. Biological Synthesis of Metal Nanoparticles via Plants
It has long been known that plants have the potential to hyper-accumulate and biologically reduce metallic ions [44,69]. Because of these interesting properties, plants have been considered a more environment-friendly route for biologically synthesizing metallic nanoparticles and for detoxification applications [66,69]. Plant extracts containing bioactive alkaloids, phenolic acids, polyphenols, proteins, sugars, and terpenoids are believed to have an important role in first reducing the metallic ions and then stabilizing them as seen in Figure 1 [195,196]. The variation in composition and concentration of these active biomolecules between different plants and their subsequent interaction with aqueous metal ions is believed to be the main contributing factors to the diversity of nanoparticle sizes and shapes produced as seen in Table 2 [197]. Importantly, the synthesis of nanoparticles from reducing metal salts via plants is a relatively straightforward room temperature process. The process begins by mixing a sample of plant extract with a metal salt solution. Biochemical reduction of the salts starts immediately and the formation of nanoparticles is indicated by a change in the colour of the reaction mixture. During synthesis, there is an initial activation period when process metal ions are converted from their monovalent or divalent oxidation states to zero-valent states and nucleation of the reduced metal atoms takes place [198]. This is immediately followed by a period of growth when smaller neighbouring particles amalgamate to form larger nanoparticles that are thermodynamically more stable while further biological reduction of metal ions takes place. As growth progresses nanoparticles aggregate to form a variety of morphologies such as cubes, spheres, triangles, hexagons, pentagons, rods, and wires [23]. In the final stage of synthesis, the plant extracts ability to stabilize the nanoparticle ultimately determines it’s most energetically favourable and stable morphology. Properties of the plants extract such as its concentration, metal salt concentration, reaction time, reaction solution pH, and temperature significantly influence the quality, size, and morphology of the synthesized nanoparticles [112,199].

Figure 1. Biological synthesis of nanoparticles using plant extracts.
Table 2. A selection of nanoparticles synthesized by various plants.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Nanoparticle Size (nm)</th>
<th>Shape</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aloe vera</td>
<td>Au &amp; Ag 50 to 350</td>
<td>Spherical, triangular</td>
<td>[200]</td>
</tr>
<tr>
<td>Aloe vera</td>
<td>InO3 5 to 50</td>
<td>Spherical</td>
<td>[201]</td>
</tr>
<tr>
<td>Camellia sinensis</td>
<td>Ag, Au 30 to 40</td>
<td>Spherical, triangular, irregular</td>
<td>[202]</td>
</tr>
<tr>
<td>Citrullus colocynthis</td>
<td>Ag 31</td>
<td>Spherical</td>
<td>[203]</td>
</tr>
<tr>
<td>Curcuma longa</td>
<td>Pt 10 to 15</td>
<td>Crystalline</td>
<td>[120]</td>
</tr>
<tr>
<td>Diopyros kaki</td>
<td>Pt 15 to 19</td>
<td>Spherical, triangular, hexagonal</td>
<td>[204]</td>
</tr>
<tr>
<td>Eucalyptus macrocarpa</td>
<td>Au 20 to 100</td>
<td>Spherical, cubes</td>
<td>[84]</td>
</tr>
<tr>
<td>Mangifera indica</td>
<td>Ag 10 to 100</td>
<td>Spherical, triangular, hexagonal</td>
<td>[92]</td>
</tr>
<tr>
<td>Rhododendron dauricum</td>
<td>Ag 25 to 40</td>
<td>Triangular, hexagonal</td>
<td>[206]</td>
</tr>
<tr>
<td>Psidium guajava</td>
<td>Au 25 to 30</td>
<td>Spherical</td>
<td>[207]</td>
</tr>
<tr>
<td>Pyrus sp. (Pear fruit extract)</td>
<td>Au 200 to 500</td>
<td>Spherical</td>
<td>[208]</td>
</tr>
<tr>
<td>Terminalia catappa</td>
<td>Au 10 to 35</td>
<td>Triangular, hexagonal</td>
<td>[209]</td>
</tr>
</tbody>
</table>

5.1. Factors Affecting Biological Synthesis of Metal Nanoparticles

During biological synthesis of metallic nanoparticles, a number of controlling factors are involved in the nucleation and subsequent formation of stabilised nanoparticles. These factors include pH, reactant concentrations, reaction time, and temperature. The following sections briefly discuss each of these factors in succession.

5.1.1. Influence of pH

The pH value of the reaction medium plays a significant role during the formation of nanoparticles [210]. Studies have shown that varying the pH of the reaction medium tends to produce variability in shape and size of nanoparticles synthesized. In particular, larger particles tend to be produced at a lower acidic pH values compared to high pH values [211,212]. For example, rod-shaped Au nanoparticles synthesized using Avena sativa (Oat) biomass were larger (25 to 85 nm) when formed at pH 2 and relatively smaller (5 to 20 nm) at pH 3 and 4 [213]. The study suggested that between pH 3 and 4 more accessible functional groups contained within the extract were available for particle nucleation. Conversely, at pH 2 fewer functional groups were available and resulted in particle aggregation to form larger Au nanoparticles. In a similar study, Ag nanoparticles were synthesized using Cinnamomum camphora (camphor) leaf extract and the number of particle synthesized increased with increasing concentrations of bark extract and at higher pH values (pH 5 and above) the shape of the nanoparticles tended to become spherical [214]. On the other hand, when Cinnamon zeylanicum bark extract was used to synthesize palladium (Pd) nanoparticles there was a slight increase in particle size with increasing pH. When the pH was less than 5 the particle ranged from 15 to 20 nm and when the pH was greater than 5 particles ranged in size from 20 to 25 nm [215].

5.1.2. Influence of Reactant Concentration

The concentration of biomolecules found in plants extracts can significantly influence the formation of metallic nanoparticles. A study by Huang et al. found that by varying the amount of sundried Cinnamomum camphora (camphor) leaf extract in the reaction medium could significantly influence the shape of the synthesised Au and Ag nanoparticles [216]. For example, when the precursor chloroauric acid was subjected to increasing concentrations of extract, the resulting nanoparticle shape changed from triangular to spherical. Similarly, varying the amount of Aloe vera leaf extract in the reaction medium containing chloroaurate ions, Chandran et al. were able to influence the ratio of gold triangular plates to spherical nanoparticles [200]. The study also found that the carbonyl compounds present in the extract assisted in shaping particle growth. While changing
the extract concentration modulated particle size between 50 and 350 nm. Furthermore, decahedral, hexagonal, triangular, and spherical Ag nanoparticle shapes have been produced by varying the concentration of Plectranthus amboinicus leaf extract in the reaction medium [217].

5.1.3. Influence of Reaction Time

A recent study by Ahmad et al. revealed that the reaction time to synthesize spherical Ag nanoparticles using Ananas comosus (Pineapple) extract is an important factor indeed. In this particular case it produced a rapid colour change within 2 min [218]. Aqueous Ag(NO)₃ in the reaction medium was rapidly reduced and nanoparticles appeared within 2 min. The reaction continued up to 5 min, but after that only a slight variation in colour could be observed. The nanoparticles produced were spherical and had a mean size of 12 nm. In a similar study by Dwivedi and Gopal, Chenopodium album leaf extract was used to produce Ag and Au nanoparticles. During synthesize nanoparticles appeared within 15 min and continued to form over a 2-h period. Beyond the 2-h period very few nanoparticles were produced [199]. Moreover, a study by Prathna et al. revealed that when Azadirachta indica leaf extract and Ag(NO)₃ were combined, increasing the reaction time tended to produce particles with increasing size. The reaction time was varied between 30 min and 4 h to produce a change in particle size ranging from 10 to 35 nm [219].

5.1.4. Influence of Reaction Temperature

While it is generally known that reaction temperature is a crucial factor in any synthesis it has been found that temperature is also an important factor in determining the size, shape, and yield of nanoparticles synthesized via plant extracts [211,220]. For example, synthesis of Ag nanoparticles at a reaction temperature of 25 °C via Citrus sinensis (sweet orange) peel extract produced particles with an average size of around 35 nm. However, when the reaction temperature was increased to 60 °C the average particle size decreased to 10 nm [221]. Likewise, Song et al. using Diospyros kaki (persimmon) leaf extract was able to synthesize stable Ag nanoparticles over a reaction temperature range from 25 to 95 °C. It was also found by Armendariz et al., that thermal variation in the reaction conditions for Avena sativa (oat) biomass resulted in changes in the size and shape of Au nanoparticles formed [213]. Additionally, Gerbic and Pinches have shown that higher temperatures promote the higher formation rate for Au nanoparticles. At lower temperatures spherical-shaped Au nanoparticles were predominantly formed while at higher temperatures rod-like and plate-like nanoparticles were formed [64]. Reaction rate and particle formation rate appears to become faster when reaction temperature increases, however, the average particle size decreases and particle conversion rate steadily increases with increasing temperature.

5.2. Major Nanoparticles Synthesized by Plant Extracts

5.2.1. Gold and Silver Nanoparticles

Although Au nanoparticles have attracted significant interest due to their size, shape, and surface properties [13,222], because of these unique properties, Au nanoparticles have been investigated for potential applications in fields such as biosensors [223,224], hyperthermia therapy [225], delivery platforms for therapeutic drugs and genetic substances [226], and as antibacterial drugs [227,228]. Employing plants as biological factories has the potential to deliver an environmentally friendly source of Au nanoparticles via green chemistry based techniques. For example, Das et al. have been able to synthesize spherical shaped Au nanoparticles (~20 nm) using Nyctanthes arboristris (night jasmine) flower extract [229]. While Narayanan and Sakthivel were able to use Coriandrum sativum (coriander) leaf extracts to produce Au nanoparticles ranging in size from 7 to 58 nm. The synthesised particles also had diverse shapes such as decahedral, spherical, and triangular [119]. Moreover, several studies have independently reported the synthesis of Au nanoparticles using a variety of plants sources such as the leaves and bark of Ficus carica (fig) [230], Sphaeranthus amaranthoides [231].
and Putranjiva roxburghii [232]. Likewise, studies by Armendariz et al. have revealed that Avena sativa biomass produced Au nanoparticles ranging in size from 5 to 85 nm depending on reaction medium pH. The study also revealed a variety of shapes such as decahedral, hexagonal, isosahedral, irregular, and rod-shaped could be produced depending on reaction medium pH [213]. Also, in a recent study by Poinern et al. Eucalyptus macrocarpa leaf extract could be utilised to synthesize Au nanoparticles. The results of this study revealed that spherical particles ranging in size from 20 to 80 nm were the main product. However, coexisting with the spheres were a variety of shapes such as hexagonal pentagon and truncated triangles all ranging in size from 50 to 100 nm as seen in Figure 2 [84]. Historically, Ag is well known for its antimicrobial activity and as a result it is commonly used in a variety of medical preparations against pathogens [233–235]. For antimicrobial preparations, the size and high surface area to volume ratio of Ag nanoparticles enables them to closely interact with the bacterial cell membranes [236]. Recent antimicrobial studies have revealed that significant membrane damage and DNA toxicity can result from the interaction between Ag nanoparticles via bio-sorption and cellular uptake [31,237]. Among biological synthesis processes, plants are found to be more conducive and provide a faster pathway for manufacturing Ag nanoparticles compared to conventional microbial processes. For example, Edison and Sethuraman have used Terminalia chebula (harad) fruit extract to rapidly produce Ag nanoparticles [238]. Likewise, Poinern et al. have also used Eucalyptus macrocarpa leaf extract to synthesize cubic Ag nanoparticles ranging in size from 50 to 200 nm as seen in Figure 3 [92]. Studies by Geetha et al. have shown high antibacterial efficacy of Ag nanoparticles synthesized using Cymbopogon citratus (lemon grass) leaf extract. The Ag nanoparticles ranged in size from 15 to 65 nm with an average size of 34 nm. The shape of the particles was predominately cuboidal and rectangular. The antibacterial effect was found to be effective against Pseudomonas aeruginosa, Proteus mirabilis, Escherichia coli, Shigella flexaneri, Shigella sonnei, and Klebsiella pneumonia [239,240]. In addition to pure metal nanoparticles being synthesized by plants, several authors have also reported alloying Au and Ag to investigate the properties of the resulting bimetallic nanoparticle. Bimetallic nanoparticle synthesis involves the competitive reduction between two aqueous solutions each containing a different metallic ion precursor that is mixed with a plant extract. In the case of an Au-Ag bimetallic nanoparticle, Au having the larger reduction potential will form first to create the core of a resulting core-shell structure. Subsequent reduction of Ag ions results in Ag coalescing on the core to form the shell. Plants that have been successfully used to synthesize Au-Ag bimetallic nanoparticles include Azadirachta indica (neem) [79], Anacardium occidentale (cashew nut) [241], Swietenia mahagony (West Indies mahogany) [242], and cruciferous vegetable extracts [243].

Figure 2. Au nanoparticles synthesised using Eucalyptus macrocarpa leaf extract. (a) Plant and (b) typical transmission electron microscopy image (84)

Figure 3. Ag nanoparticles synthesised using Eucalyptus macrocarpa leaf extract (a) overview of agglomerated Ag nanoparticles and (b) enlarged view of Ag nanocubes [92].
5.2.2. Copper and Copper Oxide Nanoparticles

Copper (Cu) and copper oxide (CuO) nanoparticles have been synthesized by a variety of plant extracts. Cu nanoparticles have been biologically synthesized using magnolia leaf extract to produce stable nanoparticles ranging in size from 40 to 100 nm. Antimicrobial studies revealed that the Cu nanoparticles have potential antibacterial activity against Escherichia coli cells, a common pathogen [30]. Syzygium aromaticum (Clove) extracts can produce Cu nanoparticles with a mean particle size of 40 nm and a spherical to granular morphology [244]. Cu nanoparticles can be synthesised using stem latex of Euphorbia nivulia (Common milk hedge). These nanoparticles are coated and stabilized by peptides and terpenoids present in the latex; these nanoparticles are reported to be toxic to human adenocarcinomic alveolar basal epithelial cells (A549 cells) [245,246]. Furthermore, a study by Padil et al. using Sterculia urens (Karaya gum) extract was able to synthesize highly stable spherical Cuprous Oxide (CuO) nanoparticles with a mean particle size of 4.8 nm. The particles were found to have significant antimicrobial activity against common pathogens such as Escherichia coli and Staphylococcus aureus [247]. Similar studies have also shown that CuO nanoparticles exhibit both antioxidant and antibacterial behaviour [248,249].

5.2.3. Palladium and Platinium Nanoparticles

Palladium nanoparticles were synthesised by Satishkumar et al. in 2009, using an extract of taken from C. zeylanicum (cinnamon) bark [215]. Changing the bark extract concentration, reaction pH and temperature during synthesis was found not to influence particle size (15 to 20 nm) and morphology. Palladium nanoparticles ranging in size from 75 to 85 nm have also been synthesized using Annona squamosa ( Custard apple) peel extract [250], while the leaf extract of soybean (Glycine max) have been able to synthesise nanoparticles with a mean size of 15 nm [251]. And even common commercial products like Coffea arabica (Coffee) and Camellia sinensis (Tea) extracts have been utilised to synthesise palladium nanoparticles varying in size from 20 to 60 nm with faced centred cubic crystal symmetry [252]. Moreover, when an extract taken from Gardenia jasminoides (Cape jasmine)was used to synthesise palladium nanoparticles, antioxidants such as geniposide, chlorogenic acid, crocins, and crocetin were found to act as both reducing and stabilizing agents [253]. Subsequent analysis revealed particle sizes ranged from 3 to 5 nm and the study also found particle size was dependent on reaction temperature.

The first synthesis of platinium nanoparticles was reported by Song et al., in 2010 using a leaf extract taken from Diospyros kaki (Persimmon). The resultant nanoparticles ranged in size from 2 to 12 nm and showed that 90% of the platinum ions in solution were converted using a 10% concentration of leaf biomass at 95 °C [204]. A leaf extract taken from Ocimum sanctum (Holy basil) has also been used to synthesise platinum nanoparticles with a mean particle size of 23 nm from aqueous chloroplatinic acid at a reaction temperature of 100 °C [254]. And recently, the biological synthesis of platinum nanoparticles with particle size and shape control has also been reported by using plant wood nanometre scale materials [255]. For example, Coccia et al. have recently reported a one-pot synthesis technique for producing platinum and palladium nanoparticles using lignin isolated from red pine (Pinus resinosa) [25].

5.2.4. Titanium Dioxide and Zinc Oxide Nanoparticles

A number of plant extracts have been also been found to synthesize important metal oxide nanomaterials such as titanium dioxide (TiO₂) and zinc oxide (ZnO) nanoparticles. For example, Roopan et al. have found that Annona squamosa peel could be used to effectively synthesize TiO₂ nanoparticles [256], while Nyctanthes arbor-tristis leaf extracts have been found to produce spherical particles ranging in size from 100 to 150 nm [257] and Eclipta prostrata leaf extracts can produce particles ranging in size from 36 to 68 nm [258,259]. Velayutham et al. have used a Catharanthus roseus leaf extract to biologically synthesize TiO₂ nanoparticles. The resultant
nanoparticles were irregular in shape and ranged in size from 25 up to 110 nm. Assessment of the resulting TiO2 suspensions revealed that they were both adulticidal and larvicidal against Hippobosca maculata (hematophagous fly) and Bovicola ovis (sheep louse) [260]. The antibacterial and antioxidant properties of TiO2 nanoparticles synthesized via an extract from Psidium guajava were evaluated against Aeromonas hydrophila, Proteus mirabilis, Escherichia coli, Staphylococcus aureus, and Pseudomonas aeruginosa pathogens [261]. The nanoparticles were found to be most effective against Staphylococcus aureus and Escherichia coli. Furthermore, the antibacterial and antioxidant properties of nanometre scale and bulk TiO2 towards bacteria have also been examined and found to be deleterious towards a number of bacterial strains [249].

Zinc oxide nanoformulations is an important biomedical and cosmetic product. The latex from Calotropis procera has been used as both reducing and stabilizing agent for the synthesis of spherical shaped zinc oxide (ZnO) nanoparticles [262]. While stable and spherical ZnO nanoparticles have been synthesized using Aloe vera extract [263]. In addition, crystalline poly-dispersed ZnO nanoparticles with a mean particle size of 72.5 nm were synthesized via Physalis alkekengi extract [264] and nanoparticles synthesized from Sedum alfredii were pseudo-spherical in shape with a mean particle size of 53.7 nm. [265] A recent study by Vimala et al. has shown the ability of green synthesized ZnO nanoparticles to be used as drug delivery platforms for doxorubicin, which highlights the importance of developing novel green chemistry based techniques for developing new sources of nanoparticles [266].

5.2.5. Indium Oxide, Iron Oxide, Lead, and Selenium Nanoparticles

A number of other types of metal and metal oxide nanoparticles have been biologically synthesized using a variety of plants. Leaf extracts from Aloe vera (Aloe barbadensis Miller) have been used to synthesize Indium oxide (In2O3) nanoparticles. After initial synthesis, the precipitate was thermally treated between 400 and 600 °C to produce the nanoparticles. The resultant spherical nanoparticle size was dependent on treatment temperature and ranged from 5 to 50 nm [201]. Because of the importance of Iron (Fe) nanoparticles in a number of environmental remediation technologies, recent research has focused on green chemistry based methods to synthesize these Fe nanoparticles. For example, aqueous sorghum bran extracts have been used to biologically synthesize Fe nanoparticles at room temperature [29]. Recently Pattanayak et al. were able to synthesize Fe nanoparticle via extracts taken from plants such as Euphorbia milii, Tridax procumbens, Tinospora cordifolia, Datura innoxia, Calotropis procera, and Cymbopogon citratus (lemon grass tea). The smallest spherical nanoparticles size range (13 to 21 nm) were synthesized from the stem extract taken from Euphorbia milii and the widest size range (43-342 nm) occurred for particles synthesized using leaf extracts taken from Cymbopogon citratus [268]. Other significant metallic nanoparticles that have been biologically synthesized include lead (Pb) and selenium (Se). In the case of Pb nanoparticles, Joglekar et al. were able to use the latex from Jatropha curcas to synthesise spherical shaped particles ranging in size from 10 to 12.5 nm [269]. Recently, Sasidharan et al. were able to synthesise Selenium (Se) nanoparticles using the extracts taken from the peel of citrus reticulata to produce spherically shaped particles with a mean particle size of 70 nm [270].

6. Applications of Nanoparticles & Biologically Inspired Templates

The continually developing field of nanotechnology is expected to require a significant amount of optimised and functional nanomaterials. A wide range of conventional physicochemical processes has been used in the recent past to synthesise a wide variety of metal nanoparticles. These nanoparticles have been used in a diverse range of applications such as biosensors [271], targeted drug delivery platforms [10,14,272], diagnostics and therapeutics [273], cancer treatments [9,274], pesticides [275], and antimicrobials [276]. However, nanoparticles produced by environment-friendly
biological entities have only been exploited in relatively few practical applications. Ag nanoparticles have attracted considerable research interest due to its inherent antimicrobial activity and as a result it is already used as an antimicrobial agent in a wide range of commercially available medical and consumer products [18,20,277]. Another emerging application of nanoparticles and Ag nanoparticles in particular is in crop protection and the management of agricultural plant diseases [278,279]. Recent studies by Vivek et al. have demonstrated the antilungal effects of Ag nanoparticles [280]. Furthermore, Ag nanoparticles can be used to control a number of plant pathogens in a safer way compared to conventional fungicides [281] and these metallic Ag nanoparticles have also been found to be active against cancer cells and plasmoidal pathogens [282–285].

Traditionally, Au has been used in several medical applications. Au nanoparticles have attracted significant interest over the last decade as a medicinal material in treatment of tumours. For example, Au nanoparticles have the ability to passively accumulate in tumours due to their size and because of their unique optical and chemical properties can be used in thermal treatment procedures [286,287]. Moreover, studies have shown that biocompatible Au nanoparticles can be successfully used as carrier platforms for the targeted delivery of anticancer drugs thus improving delivery and minimizing treatment durations and side effects [13,226,288]. Studies have also shown that Au nanoparticles are effective antibacterial agents against a number of bacterial strains [84,289]. While Cu and CuO nanoparticles have also been found to be strong antimicrobial agents and their disinfecting properties against a number of infectious organisms means they can be used as an effective bactericide material to coat hospital equipment [244,290–292], Pt nanoparticles have the potential to be used in water electrolysis applications [254]. TiO2 nanoparticles, because of their antibacterial activity, have been used in antibacterial coatings and wastewater disinfection processes [293–295]. While ZnO nanoparticles display good antibacterial activity and have been used in food packaging and wastewater treatments [296,297]. Moreover, template assisted fabrication using biological entities permits the creation of more complex self-assembled structures at both the nanometre and micrometre scales. Bacteria, bacteriophages and viruses are attractive assemblers for manufacturing one dimensional structure into ordered arrays. For example, the tobacco mosaic virus has been used to assemble Au, Ag and Pt nanoparticles [298] and filamentous bacteriophages have been used to form silica fibres and nanotubes [299–302]. These nanometre scale entities are very effective templates for forming well-ordered 1D assemblies [303,304]. While entities such as silk sericin have been used to form nano-fibrous networks that direct the formation of needle like hydroxyapatite particles [305] and promote osteogenic properties of human bone marrow cells [306]. While magnetically controlled guidance of biomolecules via iron oxide nanoparticles has been able to produce high ordered 3D arrays used to support stem cell growth [307]. Furthermore, films incorporating Au nanoparticles have been assembled from genetically engineered bacteria and filamentous viruses to produce CdS quantum dots [308] and colourimetric sensors [309,310]. Recent studies by Wang et al. have demonstrated that viral nanofibres decorated with magnetic iron oxide nanoparticles can be used for the detection of human serum antibody biomarkers [311]. Nanoparticles and nanoparticle constructed structures have the potential to be used in a wide variety of applications as discussed above, especially if they can be synthesised using biological entities that can ensure clean, nontoxic, and eco-friendly methods of production.

The synthesis of metallic nanoparticles using a wide variety of biological entities, as discussed above, has been actively pursued in recent years as an alternative bottom up approach to self-assemble atoms to form nuclei and subsequently grow into nanometre scale particles. However, several factors have been identified that can significantly influence the viability of this eco-friendly process for synthesising nanoparticles. The most readily identified factors being particle size control, shape, and size distribution. These factors are all directly influenced by reaction medium pH, reactant moieties, reactant concentrations, reaction time, and temperature. As explained above, even small variations in these factors can significantly influence particle size, shape, and size distribution. For example, in the case of plant extracts, there can be noticeable variations in the chemical composition of
extracts taken at different times of the year and at different locations around the world for the same species. This compositional variation can often lead to different laboratories producing dissimilar results from the same plant extract and metal salt. This can be a serious drawback in using plant extracts to produce nanoparticles with consistent physical and chemical properties. Understandably, even with the current limitations, biosynthesis offers numerous advantages and has the potential to deliver nanoparticles with predetermined properties. For example, Shankar et al., using effective quality control and closely regulating the reaction medium pH, reactant concentrations, reaction time, and temperature during synthesis were able to reduce large quantities of triangular Au nanoprisms using Cymbopogon flexuosus (Lemongrass) extract [312]. Nearly 45% of the total nanoparticles reduced from the aqueous chloroaurate ion and extract solution was composed of Au nanotriangles. The triangles displayed truncated vertices similar to those seen for triangular Ag [313] and Au nanoprisms [314] synthesised by chemical and photochemical methods. Furthermore, repeated centrifugation (3400 g), washing, and re-dispersion of the reaction medium significantly improved the throughput of nanotriangle numbers (up to 90%). Interestingly, despite recent developments in conventional physical and chemical methods, many physical methods still require relatively expensive equipment and have operational requirements such as vacuum, pressurized gases, and high temperatures. While most chemical methods tend to use toxic materials such as organic solvents, reducing agents, and stabilizers. These economic and toxicity related emphasizes the importance and need for further research into eco-friendly biosynthesis methods factors further over the more traditional nanoparticle production processes.

7. Conclusions

Nanoparticles, in particular metallic nanoparticles have attracted considerable interest in many and diverse fields such as electronics, photonics, medicine, and agriculture. This review has summarized recent research into the synthesis of metallic nanoparticles using biological entities. However, owing to the diversity of biological entities ranging from microorganisms to plants, much of this field remains largely unknown and still remains to be discovered. The production of nanoparticles using biological entities has the potential to deliver new sources of novel materials that are stable, nontoxic, cost effective, environment-friendly, and synthesized using green chemistry approach. This green chemistry approach of using biological entities is in complete contrast with conventional chemical and physical processes that often use toxic materials that have the potential to cause environmental toxicity, cytotoxicity, and carcinogenicity. Whilst biological entities have been extensively used to produce nanoparticles, the use of plants offers a straightforward, clean, non-toxic, and robust procedure that does not need any special culture preparation or isolation techniques that are normally required for bacteria and fungi based techniques. In particular, the use of plant extracts for synthesizing nanoparticles is inexpensive, easily scaled up, and environment-friendly. Plant extracts have the potential to produce nanoparticles with a specific size, shape and composition. Plant synthesized nanoparticles have the potential to be widely used in current medical procedures involving nanoparticles such as fluorescent labelling in immunoassays, targeted delivery of therapeutic drugs, tumour destruction via heating (hyperthermia), and as antibacterial agents in bandages. On another front, plant synthesized nanoparticles have the potential to be used for the delivery of anti-microbial biological compounds for use as pesticides for agricultural crops. Moreover, agricultural crop wastes and food industry wastes are also excellent candidates for supplying sources of plant-based bio-chemicals with the potential to synthesize metallic nanoparticles and similar products. Despite the environmental advantages of using green chemistry based biological synthesis over traditional methods as discussed in this article there are some unresolved issues such as particle size and shape consistency, reproducibility of the synthesis process, and understanding of the mechanisms involved in producing metallic nanoparticles via biological entities. In the case of plant extracts, nanoparticle formation mechanisms vary between different plant species. Therefore, there is a need for more studies to evaluate and understand the actual plant dependent
mechanisms. This is a grossly unexplored field and requires much more research investment to fully utilize the green synthesis of metallic nanoparticles via biological entities.

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Biogenic Synthesis of Metal and Metal Oxide Nanoparticles via Marine Algae and Sea Grasses

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Abstract
Today there is a growing need to develop reliable, sustainable and eco-friendly protocols for manufacturing a wide range of metal and metal oxide nanoparticles. The biogenic synthesis via nanobiotechnology methods has the potential to produce clean technologies that can significantly reduce environmental and human health risks resulting from toxic chemicals and solvents generally used in conventional chemical and physical industrial fabrication processes. The largely unexplored marine environment that covers approximately 70% of the earth’s surface is home to many naturally occurring and renewable marine plants. The present review summarizes the current state of research into using two groups of plants, namely marine algae (commonly known as seaweeds) and sea grasses. Both groups of plants are a rich and abundant source of biologically active compounds and secondary metabolites that have the potential to act as biological factories in the manufacture of metal and metal oxide nanoparticles.

Keywords: biogenic synthesis, metal & metal oxide nanoparticles, marine algae

1. Introduction
In recent years there has been a convergence between biological based technologies, green chemistry and nanotechnology to create new materials and processes that reduce
or eliminate the use of hazardous substances [1]. An important field of research within nanotechnology is the synthesis of nanometre scale materials of different compositions and the direct control of particle morphology and dimensions. Nanometre scale materials have at least one dimension less than 100 nm and geometric shapes such as plates, sheets, tubes, wires and particles. Studies have shown that nanometre scale materials exhibit unique chemical, physical, electronic, optical, thermal, mechanical and biological properties that significantly differ from their bulk counterparts [2, 3]. It is the extremely small size, shape and size distribution that governs nanomaterial properties and because of their size they can act as bridge between bulk materials and molecular structures [4]. In terms of composition, nanomaterials can be broadly classified into two types, namely organic and inorganic. Organic generally defines carbon nanomaterials, while inorganic describes noble metal (gold, platinum and silver), magnetic (iron oxide Fe$_3$O$_4$) and semiconductor nanomaterials such as titanium dioxide and zinc oxide.

The physiochemical properties of most bulk materials are largely understood and as a result recent discoveries in material science have generally focused in the region between the atomic scale and bulk scale. The region between the two is the nanometre scale range and in recent years has attracted considerable interest. The interest stems from the fact that when bulk materials are reduced into much smaller amounts there is a significant increase in the surface area to volume ratio that results in changes in surface chemistry and a significant increase in chemical reactivity [5]. The large surface area to volume ratio is the major contributing factor to differences in the physiochemical properties of nanomaterials when compared to materials with same chemical composition at the much larger bulk scale [6]. These unique and novel size-dependent physicochemical properties have led to nanoparticles being promoted for a wide range of applications, including biosensors [7, 8], catalysts [9, 10, 11], environmental remediation [12-14], labelling for immunoassays [15, 16], hyperthermia treatment of tumours [17-19], antibacterial drugs [20, 21] and vector delivery of therapeutic drugs for cancer treatments [22-24]. However, studies have also shown that material properties of manufactured nanoparticles are heavily dependent on specific parameters such as particle size, morphology and size distribution [25]. Therefore, it very important that the synthesis process effectively controls particle size and shape during manufacture and enables customized production of nanoparticles for targeted application and purpose.

Nanoparticle manufacture can be broadly defined into two approaches. The first is the top down approach and involves a specific material undergoing significant size reduction via physical or chemical processes [26, 27]. During size reduction, the resulting particle size, shape and surface structure are heavily dependent on the technique used and tends to introduce surface imperfections that can significantly impact on the overall physicochemical properties of the fabricated nanoparticle. The second approach is a bottom up route that builds nanoparticles via the assembly of atoms, molecules and smaller particles or monomers [28, 29]. Unfortunately, many of the conventional chemical and physical processes suffer from several drawbacks such as low material conversion rates, technically difficult, high energy requirements and are generally expensive. Furthermore, many of these processes employ environmentally
hazardous chemicals such as reducing agents, organic solvents and non-biodegradable stabilising agents. For example, processes such as chemical precipitation and pyrolysis often result in toxic chemical species being deposited on the surface of newly formed nanoparticles, thus making them unsuitable for clinical and biomedical applications [30, 31]. Because of these drawbacks there has been a growing need to develop new environmental benign materials and methods for the manufacture of nanoparticles founded on the principles of green chemistry [32]. Accordingly, research in recent years has focused on manufacturing nanomaterials via nanotechnology-based processes that promote green chemistry principles and reduce or totally eliminate the use of hazardous chemicals. Thus environment-friendly green nanotechnology based processes have attracted considerable interest worldwide. Since they have the potential to deliver eco-friendly procedures that can be used to manufacture nanoparticles with a wide variety of compositions, sizes and morphologies [33, 34]. To emphasis this methodology, recent research has focused on using biological entities as an alternative to conventional chemical and physical processes for producing nanoparticles. Biosynthesis via unicellular and multicellular biological entities such as actinomycetes [35], bacteria [36], fungus [37], marine algae [38], plants [39], viruses [40] and yeast [41] have the potential to deliver an environment-friendly green chemistry approach. Each of these biological entities to varying degrees can perform as natural factories, with their inherent biologically active molecules and compounds acting as effective reducing agents and stabilising agents to produce nanoparticles with diverse sizes, shapes, compositions and physicochemical properties [42].

The biologically diverse marine environment covers around 70% of the earth’s surface is largely unexplored. However, recent studies have shown that naturally occurring marine plants have the potential to act as bio-factories in the manufacture of nanoparticles [43, 44]. During the last two decades significant interest into producing new and effective medicines from natural plant sources has revealed that marine algae [45] and seagrasses have the potential to produce a range of nanoparticles that can be used as antimicrobial agents [46, 47]. Marine algae encompass a diverse range of different species, which are generally classified into two groups, namely microalgae and macroalgae. Microalgae species such as phytoplankton survive suspended in the water column, while macroalgae or seaweed are plant-like organisms that can range in size from a few centimetres up to tens of metres in length. For example, the giant kelp grows up from the seafloor to form vast underwater forests. Seaweeds have adapted to living in a variety of habitat, ranging from small tidal rock pools close to shore or living several kilometres offshore in seawater depths capable of receiving sufficient light to promote photosynthesis. Algae are broadly classified into three groups based on the algal body or thallus pigmentation. The colour groups are brown algae (phaeophytes), green algae (chlorophytes), and red algae (rhodophytes) [48]. Brown and red algae are predominantly marine based, with some species of red algae being found in water depths where light levels are very low. While green algae is found in both marine and freshwater environments. Figure 1 presents a selection of marine algae and seagrasses found in coastal waters along the West Australian coastline.
Since the dawn of time, the combined effects of tides and wave action has resulted in large quantities of seaweed being washed up beaches and shorelines in many parts across the world. Consequently, for many centuries coastal communities have consumed seaweed since it was a readily available food source and a source for medicinal remedies. Even today seaweed is a staple food source and a medicinal remedy in daily use throughout South-East Asia and Japan [49, 50]. Studies have revealed that seaweeds are a rich source of biologically active compounds such as carbohydrates, carotenoids, polysaccharides, proteins, vitamins and numerous secondary metabolites [51-53]. To date, several studies have reported on the potential medicinal properties derived from biologically active compounds present in seaweeds and their complimentary use in conventional treatments and in alternative therapies [51, 54-56]. Several studies have also shown both anti-inflammatory and inhibitory properties associated with seaweed extracts and have been used to treat various medical conditions and suppress some forms of cancer [57-60]. Marine algae are capable of accumulating heavy metals [61] and secondary metabolites of several seaweeds have anti-biological fouling properties [62]. In addition, some studies have reported the biological reduction of metal ions in aqueous based seaweed solutions [36, 63-65]. Thus, indicating the viability of biologically active compounds present in seaweeds to act as both metal-reducing agents and capping agents during the formation of stable nanoparticles. Current studies by the authors have shown that some species of seagrass contain biologically active compounds that have the potential to synthesise stable metal nanoparticles. This review summarizes the current state of research into biological synthesis of metal and metal oxide nanoparticles via marine algae and seagrass. The review also discusses factors influencing the synthesis process and potential applications for nanoparticles produced via marine plants.
2. Toxicology and biosynthesis of nanoparticles

2.1. Toxicology of nanoparticles

Nanoparticles with their unique size-dependent properties are at the leading edge of the rapidly developing field of nanotechnology. However, the toxicological impact of nanoparticles, their potential threats to human health and the environment are of great concern too. The large surface area-to-volume ratio of a nanoparticle equates to more parameters that need to be considered when compared to working with bulk materials. For example, parameters such as particle size, morphology, composition, concentration, chemical reactivity, dispersion and aggregation can all effect the behaviour and interactions of the nanoparticle in a particular environment. In the case of nanoparticle size, studies have shown that particles around 10 nm can induce greater human cell death rates than larger particles ranging from 50 to 100 nm [66, 67]. Nanoparticles of the same composition can display behavioural differences in their ability to interact with different surrounding environments. These differences can result from slight differences in particle size, morphology, surface reactivity and surface coatings. Another source of behavioural differences seen in nanoparticles of the same material arises from the manufacturing process itself. Traditional chemical and physical manufacturing processes use hazardous chemicals such as surfactants that act as templating agents to direct particle growth and capping agents to stabilise the formed nanoparticles. The problem arises because it is extremely difficult to remove all the chemicals and solvents from the nanoparticle surface [68]. Furthermore, studies have shown that naked nanoparticles do not exist very long alone, (high surface energy effects) in biological or environmental surroundings due to the strong attraction and attachment of atoms and molecules surrounding it. In the environmental context, the surface properties of the nanoparticles rapidly change when they come into contact with air, water and soils [69]. In the biological setting, biomolecules such as enzymes and proteins rapidly attach to the nanoparticle to form a surface layer or corona as seen in Figure 2 [70].
Figure 2. Biosynthesis and subsequent formation of nanoparticle corona: a) biosynthesis of naked particle, b) stabilisation of nanoparticle by capping agents in algal extract solution c) naked nanoparticle in biological environment and d) proteins and enzymes firmly attach to the surface of nanoparticle to form an equilibrium layer with surrounding environment.

The difficulty in determining toxicity arises from how nanoparticles bind and interact with biological materials and multicellular living organisms. How these interaction mechanisms change the surface properties of nanoparticles and their influence during interactions in particular environments is not fully understood [71]. For example, silver (Ag) has been used as a successful antimicrobial agent for centuries and the use of Ag nanoparticles in recent years has significantly increased the effectiveness of Ag as a medicinal preparation [72, 73]. However, the disadvantage of using Ag nanoparticles in higher concentrations is that they can induce toxicity and produce a variety of health problems [74]. Furthermore, release of Ag nanoparticles into the environment can produce ecological problems [66, 75]. Therefore, toxicity could not only result from the nanoparticles size, morphology, composition and surface reactivity, but also from the presence of surface contaminants. This complication makes it not only necessary to understand nanoparticle composition, but also the interaction of formation chemicals during synthesis and ultimately the environment in which the nanoparticle will end up in when investigating potential toxicity issues [76, 77].

To reduce the toxicity complications normally associated with conventional chemical and physical synthesis processes, there needs to be greater attention paid to finding alternative clean, nontoxic and environment-friendly green chemistry approaches for the manufacture of nanoparticles. This is particularly important since the interactions between a nanoparticle and its surrounding biological environment is a surface phenomenon [78]. Thus, the nanoparticle surface and surface contaminants attached during synthesis are actively involved in the nanoparticle/biological interface rather than its core material. Because of the importance of nanoparticle/biological interactions, the presence of harmful toxic residues covering the surface will preclude the nanoparticle
being used in therapeutic applications. Thus, the medical use of nanoparticle-based pharmaceuticals will ultimately depend on their therapeutic benefit weighed against any toxicity risks. Therefore, in an attempt to achieve no toxicity risks, recent research has focused on biologically based synthesis techniques. Biosynthesis of nanoparticles via biological entities offers an eco-friendly route. In particular, the biomolecules present in marine algae and seagrasses have the potential to synthesis a variety of nanoparticles with very low to no toxicity risks.

2.2. Biosynthesis of nanoparticles

The biogenic synthesis of nanoparticles via marine algae and seagrasses is considered to be a clean, nontoxic and environment-friendly green chemistry approach that has the potential to deliver a wide range of particle sizes, morphologies, compositions and physicochemical properties. The use of natural renewable marine resources like seaweed-derived polysaccharides has been exploited for many years in the food industry [49, 79]. The cell walls of seaweed are composed of polysaccharides that mainly consist of small sugar units linked via glycosidic bonds that have hydrophilic surface groups, such as carboxyl, hydroxyl and sulfate groups [80]. Typical polysaccharides found in seaweeds include agar, alginate, carrageenan, fucoidan and laminarin [81]. Other biomolecules found in the cell walls are proteins and enzymes. The presence of these bioactive materials has resulted in significant research into developing food products, bioenergy and biomedical applications [82-84].

Currently, there is no exact understanding of the detailed mechanisms behind the formation of nanoparticles in the presence of seaweeds and seagrasses. However, studies have shown that the biomolecules present in the cell walls of various seaweeds can act as biocatalysts to assist in the reduction of precursor metal salts to nucleate metal and metal oxide nanoparticles [65, 85-87]. While other, much larger amphiphilic biomolecules present in the cell walls can act as surfactants, which direct and control nanoparticle growth [88]. Biosynthesis of nanoparticles is a relatively straightforward room temperature process that begins by mixing a metal salt solution with an aqueous solution containing seaweed or seagrass extract. Reduction immediately starts and is signalled by a colour change in the reaction mixture that indicates nanoparticle formation. For example, in a recent study by Arockiya et al the formation of gold (Au) nanoparticles via brown alga (Stoechospermum marginatum) occurred within 10 min and was clearly seen by the reaction mixture changing in colour from a pale brown to a ruby red colour [38]. Subsequent characterisation revealed the nanoparticles ranged in size from 18.7 to 93.7 nm and were predominantly spherical with smaller numbers of hexagonal and triangular shapes.

It is believed the growth of nanoparticles in solution begins with metal ions being converted from their mono or divalent oxidation states to zero-valent states. This is followed by metal ion reduction and subsequent nucleation [89]. Following initial nucleation, a kinetically controlled process takes place in which smaller neighbouring particles attach to low energy faces of the forming crystal to form larger nanoparticles that are thermodynamically stable. As growth progresses, biomolecules contained within the seaweed or seagrass extracts act as a natural surfactant that behaves as a
capping agent on specific facets of the forming crystal [90]. The adsorption of capping agents and their subsequent interactions on the crystal facets reduces interfacial energy and lowers surface tension [91]. The modified surface’s properties of the crystal facets tend to influence the orientation and assembly of subsequent growth [92]. Thus, growth occurs in preferential planes and explains the typical morphologies seen in biosynthesised nanoparticles such as cubes, hexagons, pentagons, rods, spheres, triangles, and wires [34, 93].

![Figure 3](image)

**Figure 3.** Bottom up assembly of atoms via biosynthesis using marine algae and metallic ionic sources to form metal or metal oxide nanoparticles

Naturally occurring biomolecules present in seaweed and seagrass extracts have the ability to influence particle size, morphology, composition and physicochemical properties that ultimately define the quality of the synthesised nanoparticles. However, significant research is needed to identify and determine the role of specific biomolecules involved in the synthesis process and the influence of individual biomolecules in dictating nanoparticle growth. Currently, physicochemical experimental parameters such as concentration of seaweed or seagrass extract, metal salt concentration, solution mixture pH, reaction time and temperature are being investigated by several researchers, since these parameters can have a significant effect on the quality and properties of the synthesised nanoparticles [31, 94].

3. Types of nanoparticles produced by marine algae and marine plants
At present, there is only a minor level of literature reports into the use of marine algae and other marine plants for the biosynthesis of nanoparticles. Recent studies have revealed that various forms of marine algae are capable of being used for the bioremediation of toxic metals [85]. The biosynthesis of nanoparticles *via* marine algae and marine plants is an emerging field of research since these biological entities have the potential to produce a wide range of metal and metal oxide nanoparticles using eco-
friendly methods [95, 96]. The following sections summarize and discuss the currently available literature reports on the biosynthesis of nanoparticles via marine algae. Table 1 presents a selection of various metal and metal oxide nanoparticles biosynthesised via marine algae.

3.1. Metal nanoparticles
The biosynthesis of metal nanoparticles via marine algae from metal salts is a facile room temperature process. Biogenic synthesis begins by mixing a metal salt solution together with marine algae extract solution. During an initial reduction period nanoparticle formation is revealed by a colour change in the reaction mixture. With the passage of time, the smaller neighbouring particles in the reaction mixture start agglomerating to form larger and more thermodynamically stable nanoparticles. The initial nucleonic particles tend to aggregate and self-assemble in the presence of templating biomolecules contained in the algae extract. During self-assembly, the most energetically favourable and stable shapes are formed. These include a variety of shapes such as cubes, hexagons, pentagons, rods, spheres, triangles and wires [97]. Studies have also shown algae concentration; metal salt concentration, reaction time, reaction solution pH, and temperature are important parameters in determining the quality, size, and shape of biosynthesized nanoparticles [34]. The majority of the studies to date have examined the biosynthesis of Ag and Au nanoparticles and the following sections summarise the results of this research.

3.1.1. Silver (Ag) nanoparticles
Historically, Ag has attracted a great deal of interest due to its antimicrobial properties [98]. Advances in nanotechnology based manufacturing of nanomaterials have enabled the production of nanometre scale Ag with unique physiochemical and antimicrobial properties. The enhanced antimicrobial activity and efficacy of Ag nanoparticles towards a wide range of microbial entities has resulted in its incorporation into a wide range of pharmaceuticals and medical protocols [98, 99]. The mechanisms associated with the antimicrobial properties are not fully understood, but the interaction between the Ag nanoparticles and microbial cell membranes is believed to cause significant membrane damage and bio-sorption. The increased cellular uptake of nanometre scale Ag results in significant toxicological damage to cellular DNA [100, 101]. Seaweeds are considered a valuable source of bioactive materials and are rich in polysaccharides. Because of the presence of these biomaterials, seaweeds have been identified as having significant medicinal and pharmaceutical properties [49]. Thus, the biosynthesis of Ag nanoparticles via seaweeds could deliver an additional synergic effect and potentially deliver particles with enhanced medicinal properties.

The biosynthesis of Ag nanoparticles via various species of seaweeds has been reported by a small number of researchers in recent years. For example, Rajeshkumar et al have reported the biosynthesis of Ag nanoparticles using brown seaweed Padina tetrastromatica. Their study revealed the nanoparticles were spherical in shape, had a mean particle size of 14 nm and displayed antibacterial activity [102]. Similarly, crystalline Ag nanoparticles were biosynthesised from Codium capitatum to produce spherical and cubic nanoparticles ranging in size from 3 to 44 nm, with a mean particle
size of 30 nm [103]. A comparable result was also seen for the green alga *Spyrogyra insignis*, which also produced spherical nanoparticles with a mean size of 30 nm [104]. While the macroalga *Padina tetrastromatica* also tended to produce crystalline spherical nanoparticles ranging in size from 5 to 35 nm [105]. The seaweed biosynthesised Ag nanoparticles have also been demonstrated to have antifungal [86, 106], antibacterial [107-111] and anticancer activities [112] as presented in Table 2. On another front, Ramkumar Vijayan *et al* have used an aqueous extract of the seaweed *Turbinaria conoides* to biosynthesis both Ag and Au nanoparticles to investigate their anti-biological film formation activity against marine biofilm forming bacteria [113]. The study found that Ag nanoparticles were promising agents against the formation of bacterial marine biofilms and were also found to be toxic to brine shrimp *Artemia salina*. While studies into antimicrobial agents have also revealed that AgCl nanoparticles biosynthesised via an aqueous extract of *Sargassum plagiothyllum* were effective antibacterial agents against bacterial pathogens such as *Escherichia coli* [114].

3.1.2. Gold (Au) nanoparticles
Au nanoparticles with their unique size dependent properties have been used in a variety of applications including: catalytic, biomedical, biosensors, pharmaceuticals, optical & imaging and electronics [115-120]. There is growing interest in using biological entities for the biosynthesis of Au nanoparticles. The biologically diverse marine environment is attracting considerable interest from many researchers worldwide and a number of articles describing the biosynthesis of Au nanoparticles by both marine and fresh water alga have appeared in the literature.

A study by Romero-Congalez *et al* has shown that de-alginated seaweed waste could be used to reduce Au ions in solution to form Au particles ranging from the nanometre scale range up to around 6 µm in size. The study found functional groups in the seaweed were able to produce stable particles with shapes such as irregular, decahedral, hexagonal, rods and tetrahedral platelets [85]. In a similar study, Mata *et al* were able to demonstrate an eco-friendly process that could recover Au from dilute hydrometallurgical solutions. The process involved the biosorption and bioreduction of Au by the brown seaweed *Fucus vesiculosus*. The Au nanoparticles produced varied in size and morphology [63]. Singaravelu *et al* have also reported the biosynthesis of Au nanoparticles using a marine alga *Sargassum wightii* Greville. The study revealed that the alga rapidly produced stable nanoparticles ranging in size from 8 to 15 nm and were spherical in shape [121]. In a similar study by Luangpipat *et al* (*Chlorella vulgaris*) [122], Rajasulochana *et al* (*Kappaphycus alvarezii*) [123] and Stalin Dhas *et al* (*Sargassum myriocystum*) [88] were able to produce a range of well-defined stable Au nanoparticles. While Senapati *et al* have reported the biosynthesis of Au nanoparticles using marine alga *Tetraselmis kochinensis* [124] and Rajeshkumar *et al* have also reported using *Turbinaria conoides* to produce Au nanoparticles ranging in size from 6 to 10 nm and shapes including spherical, pseudo-spherical and triangular [125]. In addition, Castro *et al*. have reported biosynthesising Au nanoparticles using green alga *Spirogyra insignis* and red alga *Chondrus crispus* [104]. And recently, Arockiya Aarthi Rajathi *et al*. have reported the biosynthesis of Au nanoparticles using a brown alga *Stoechospermum marginatum*. The nanoparticles were crystalline and ranged in size
from 18.7 to 93.7 nm and were predominantly spherical in shape with small numbers of hexagonal and triangular platelets. The study also found that hydroxyl groups present in the diterpenoids in the brown seaweed were directly involved in metal reduction. The Au nanoparticles were also found to display significant antibacterial activity against a range of selected bacterial pathogens [126]. Crystalline Au nanoparticles ranging in size from 7 to 11 nm have also been biosynthesised using brown seaweed (Turbinaria ornate) [127] and another brown seaweed (Padina pavonica) was also found to produce spherical nanoparticles ranging from 30 to 70 nm [128]. Additionally, some studies have also shown that species of freshwater algae such as green alga (Prasiola crispa) and red alga (Lemanea fluviatilis) are capable of biosynthesising Au nanoparticles [129, 130].

Furthermore, studies by the authors have shown the feasibility of biosynthesising Au nanoparticles at room temperature using a sea grass rhizome extract. The sea grass (Posidonia australis Hook. f.) is a marine flowering plant that has adapted to living in the near shore environment along the southern waters of temperate Australia. Figure 4 presents some preliminary results indicating metal ion concentration is an important factor. During biosynthesis all reactive mixtures changed from light yellow colour to brown indicating the formation of Au nanoparticles. Inspection of Figure 4 (b) reveals the mixture initially containing the 1 mL solution of 250 ppm gold chloride has turned the darkest shade of brown compared to the other concentrations. Thus indicating the influence of gold chloride concentration during the reaction. Electron microscopy analysis revealed particle sizes ranged from 30 nm up 80 nm, with a small number of larger hexagonal plates (400 nm to 1 µm) present in the agglomerate as seen in Figures 4 (c) and (d). Other shapes present in the images were spherical, cubic, and triangular.

3.1.3. Other metallic nanoparticles
There is a very little literature reporting the use of marine and freshwater algae in biosynthesising metal nanoparticles other than Ag and Au. However, Scarano and Morelli have reported using the marine phytoplanktonic alga (Phaeodactylum tricornutum Bohlin) to biosynthesis cadmium sulphide (CdS) nanocrystallites when the alga was exposed to aqueous solutions containing Cd ions [131]. Recently, Eroglu et al have reported the formation of crystalline spherical palladium (Pd) nanoparticles derived from aqueous Na₂[PdCl₄] via photosynthetic reactions within green microalgae (Chlorella vulgaris). The synthesised nanoparticles ranged in size from 2 to 15 nm, with a mean diameter of 7 nm [132]. Moreover, in a recent study by Parker et al, Pd nanoparticles were synthesized using alginic acid and the brown seaweed (Laminaria digitata). The study also reported the majority of nanoparticles produced by the seaweed ranged in size from 4 to 6 nm [133].
Figure 4. (a) Marine flowering plant (*Posidonia australis* Hook. f.), (b) influence of gold chloride concentration (250 to 1000 ppm) in the reactive mixture, (c) electron microscopy image of a representative sample, and (d) enlarged image of a Au nanohexagonal plate in the sample.

3.2. Metal oxide nanoparticles

Metal oxides are an interesting class of inorganic solids that have been extensively explored and studied due to their wide range of structures and properties. The character of metal oxides is more complex than pure metals, with metal-oxygen bonding varying from nearly ionic to highly covalent and even metallic. Metal oxides come in a variety of different forms, each possessing unique compositions, morphologies, structures, and physiochemical properties [7, 134]. In particular, metal oxide nanoparticles are of significant interest due to their unique and phenomenal optical, electronic and magnetic properties [135]. These unique properties give metal oxide nanoparticles significant industrial importance in applications such as catalytic processes, electronics, sensors, magnetic storage media and solar energy conversion [136-138]. Literature, describing the biosynthesis of metal oxide nanoparticles via marine algae is currently limited, mainly focused on three material types - copper oxide, ferric oxide and zinc oxide.

Abboud et al have reported the biosynthesis of copper oxide nanoparticles using brown alga extract (*Bifurcaria bifurcata*). The facile process produced both cuprous oxide nanoparticles (Cu$_2$O) and cupric oxide nanoparticles (CuO). The majority of the nanoparticles were spherical with a small percentage being elongated. The nanoparticles ranged in size from 5 nm to 45 nm and were found to have a mean particle size of 22.6 nm. Subsequent antibacterial studies using *Enterobacter aerogenes* and *Staphylococcus aureus* revealed the copper oxide nanoparticles displayed good antibacterial properties.
against both bacterial species [139]. A recent study by Khanehzaei et al reports the biosynthesis of copper cored copper oxide nanoparticles via red seaweed (*Kappaphycus alvarezii*). The seaweed acted as stabilising agent and the resulting copper cored-cuprous oxide coated nanoparticles were spherical in shape with a mean particle size of 53 nm. Their study also found the surface of the nanoparticles were capped by paired oxygen atoms, some hydroxyl and sulphates groups from the water-soluble sulphated polysaccharides derived from cell walls of the seaweed [140].

In a recent study by Mahdavi et al, ferric oxide (Fe$_3$O$_4$) nanoparticles were synthesized via a one-step green biogenic method using brown seaweed (*Sargassum muticum*). The aqueous seaweed extract when mixed with a ferric chloride solution produce Fe$_3$O$_4$ nanoparticles. The amino, carboxyl and hydroxyl functional groups derived from the water-soluble polysaccharide cell walls were found to act as both reducing agent and capping agent. The mean particle size was found to be 18 ± 4 nm, crystalline in nature and having cubic morphology [65]. Subsequent *in vitro* studies by Namvar et al using the derived Fe$_3$O$_4$ nanoparticles evaluated the cytotoxicity, cellular responses and anticancer activity towards human cell lines for leukemia, breast cancer, cervical cancer and liver cancer. The *in vitro* studies found the accumulation of Fe$_3$O$_4$ nanoparticles in treated cells tended to promote cell apoptosis and confirmed their potential use in the treatment of cancer [141].

Current biosynthesis research has also examined more versatile, renewable materials and innovative procedures for producing zinc oxide (ZnO) nanoparticles. ZnO nanoparticles have exceptional electrical and optical properties suitable for a wide range of applications such as biomedical, photo-catalysts and solar cells [142-144]. Recently Nagarajan et al examined three types of seaweed including green (*Caulerpa peltata*), red (*Hypnea Valencia*) and brown (*Sargassum myriocystum*) for the biosynthesis of ZnO nanoparticles. After investigating experimental parameters such as metal ion concentration, seaweed extract concentration, temperature, pH and reaction time, *Sargassum myriocystum* was found to have the natural capability to effectively biosynthesise ZnO nanoparticles [87]. Their study also revealed that soluble photo-chemicals present in *Sargassum myriocystum* such as alginic acid, ascorbic acid, protein, carbohydrates, flavanoids, tannins, mannitols and lipids acted as both reduction and stabilizing agents. The morphology of nanoparticles produced during biosynthesis includes spherical, triangular, hexagonal, rod and rectangular. Particle sizes ranged from 76 nm up 186 nm, and additionally both particle size and morphology were found to strongly influenced by the experimental parameters. For instance, both particle size and morphology were influenced by temperature, while biosynthesis carried out at pH 8 tended to predominantly produce nanoparticles with a mean size of 36 nm.

In a similar study by Azizi et al using the brown seaweed (*Sargassum muticum*) found the bioactive materials such as amino, sulfate, carboxyl and hydroxyl groups all played a significant role during the biosynthesis. The resulting ZnO nanoparticles were found to have a hexagonal crystal morphology, ranged in size from 3 to 57 nm and had a mean size of 42 nm [145]. Subsequent *in vitro* cytotoxicity studies using the biosynthesised ZnO nanoparticles on murine cancer cell lines indicated various levels of cytotoxicity
over the 72 h trial. However, suppression of cell growth and proliferation in the WEHI-3 cell line suggested the ZnO nanoparticles could offer an alternative chemotherapeutic treatment in the future [146]. An alternative ZnO biosynthesis method has also been examined by Francavilla et al using agar extracted from the red seaweed (*Gracilaria gracilis*) as a soft template material during milling. The reactive milling process used agar as a sacrificial template during which a zinc precursor Zn(NO$_3$)$_2$ was transformed into a highly crystalline ZnO. The hexagonal wurtzite structured ZnO nanoparticles ranged in size from 18 to 50 nm. After calcination at 600 °C, the porous ZnO nanoparticles were investigated and found to have excellent photo-catalytic properties towards the degradation of aqueous phenol [147].

Table 1. A selection of Metal and Metal Oxide Nanoparticles biosynthesised via marine alga

<table>
<thead>
<tr>
<th>Material</th>
<th>NP</th>
<th>Size (nm) &amp; Shape</th>
<th>Marine Alga</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metals</td>
<td>Ag</td>
<td>3 to 44, Spherical and Cubic</td>
<td><em>Codium capitatum</em></td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>Ag</td>
<td>30, Spherical</td>
<td><em>Spyrogyra insignis</em></td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>Ag</td>
<td>5 to 35, Spherical</td>
<td><em>Padina Tetrastromatica</em></td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>Au</td>
<td>8 to 15, Spherical</td>
<td><em>Sargassum wightii Greville</em></td>
<td>121</td>
</tr>
<tr>
<td></td>
<td>Au</td>
<td>6 to 10, Spherical &amp; Triangular</td>
<td><em>Turbinaria conoides</em></td>
<td>125</td>
</tr>
<tr>
<td></td>
<td>Au</td>
<td>18.7 to 93.7, Spherical</td>
<td><em>Stoechospermum marginatum</em></td>
<td>126</td>
</tr>
<tr>
<td></td>
<td>Pd</td>
<td>2 to 15, Spherical</td>
<td><em>Chlorella vulgaris</em></td>
<td>132</td>
</tr>
<tr>
<td></td>
<td>Pd</td>
<td>4 to 6, Spherical</td>
<td><em>Laminaria digitata</em></td>
<td>133</td>
</tr>
<tr>
<td>Metal oxides</td>
<td>Cu$_2$O, CuO</td>
<td>5 to 45, Spherical</td>
<td><em>Bifurcaria bifurcata</em></td>
<td>139</td>
</tr>
<tr>
<td></td>
<td>Cu/Cu$_2$O</td>
<td>53, Spherical</td>
<td><em>Kappaphycus alvarezi</em></td>
<td>140</td>
</tr>
<tr>
<td></td>
<td>Fe$_3$O$_4$</td>
<td>18 ± 4, Cubic</td>
<td><em>Sargassum muticum</em></td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>ZnO</td>
<td>3 to 57, Hexagonal</td>
<td><em>Sargassum muticum</em></td>
<td>145</td>
</tr>
<tr>
<td></td>
<td>ZnO</td>
<td>18 to 50, Hexagonal</td>
<td><em>Gracilaria gracilis</em></td>
<td>147</td>
</tr>
</tbody>
</table>

4. Applications and future perspectives

Nanoparticles have attracted considerable interest and accordingly have been extensively reported in the literature. The unique size and shape dependent physicochemical surface properties make nanoparticles more interactive and reactive to certain chemical species compared to bulk materials. The novel properties of nanoparticles have been extensively investigated and evaluated for a wide range of applications in a number of fields. Among noble metal nanoparticles, Ag nanoparticles have been found to be the most useful across a broad spectrum of antimicrobial applications in the biomedical field. Table 2 presents an overview of biosynthesised Ag nanoparticles via algae and their respective antimicrobial activity against various pathogens. Au nanoparticles have also displayed antimicrobial properties towards a variety of pathogens as seen in Table 2. To date, Ag and Au nanoparticles biosynthesised via marine algae have primarily focused on their antimicrobial properties. Therefore, there is a need for future studies to confirm Ag and Au
nanoparticles produced via marine algae will have similar physiochemical properties to those produce by more conventional manufacturing processes.

Table 2. A selection of Nanoparticles and their antimicrobial against various pathogens

<table>
<thead>
<tr>
<th>NP</th>
<th>Size (nm) &amp; Shape</th>
<th>Alga</th>
<th>Antimicrobial Activity</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag</td>
<td>14, Spherical</td>
<td>Padina tetrastromatica. Gracilaria Corticata</td>
<td>Pseudomonas sp., Escherichia coli, Bacillus subtilis, Klebsiella planticola</td>
<td>102</td>
</tr>
<tr>
<td>Ag</td>
<td>18 to 44 Spherical</td>
<td>Gelidiella acerosa</td>
<td>Huminola insolens, Fusarium dimerum, Mucor indicus, Trichoderma reesei</td>
<td>106</td>
</tr>
<tr>
<td>Ag</td>
<td>22 Spherical 8-27</td>
<td>Sargassum wightii</td>
<td>Staphylococcus aureus, Bacillus rhizoidis, Escherichia coli, Pseudomonas aeruginosa</td>
<td>107</td>
</tr>
<tr>
<td>Ag</td>
<td>20-30 Spherical</td>
<td>Urospora sp.</td>
<td>Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia, Bacillus subtilis</td>
<td>108</td>
</tr>
<tr>
<td>Ag</td>
<td>20 Spherical 2-32</td>
<td>Ulva lactuca</td>
<td>Bacillus subtilis, Bacillus pumalis, Enterococcus faecalis, Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa, Aspergillus niger, Candida albicans, Saccharomyces cerevisiae</td>
<td>109</td>
</tr>
<tr>
<td>Ag</td>
<td>5-22 Spherical</td>
<td>Sargassum wightii</td>
<td>Pseudomonas aeruginosa, Vibrio cholera, Klebsiella pneumonia, Staphylococcus aureus, Escherichia coli, Staphylococcus pneumoniae Salmonella typhi</td>
<td>111</td>
</tr>
<tr>
<td>Ag</td>
<td>50 to 100 Spherical</td>
<td>Saragassum polycystum C. Agardh</td>
<td>Pseudomonas aeruginosa, Klebsiella pneumonia, Escherichia coli, Staphylococcus aureus, MCF Brest Cancer</td>
<td>112</td>
</tr>
<tr>
<td>Ag &amp; Au</td>
<td>&lt; 60 Spherical &amp; Triangular</td>
<td>Turbinaria conoides</td>
<td>Salmonella sp., Escherichia coli, Serratia liquefaciens, Aeromonas hydrophila</td>
<td>113</td>
</tr>
<tr>
<td>AgCl</td>
<td>21-48 Spherical</td>
<td>Sargassum plagiophyllum</td>
<td>Escherichia coli</td>
<td>114</td>
</tr>
<tr>
<td>Au</td>
<td>18.7-93.7 Spherical Hexagonal Triangle</td>
<td>Stoechospermum marginatum (kützing)</td>
<td>Pseudomonas aeruginosa, Klebsiella oxytoca, Enterobacter faecalis, Klebsiella pneumoniae, Vibrio parahaemolyticus, Vibrio cholerae, Escherichia coli, Salmonella typhi, Salmonella paratyphi, Proteus vulgaris</td>
<td>126</td>
</tr>
<tr>
<td>Au</td>
<td>30-70 Spherical</td>
<td>Padina pavonica</td>
<td>Bacillus subtilis, Escherichia coli</td>
<td>128</td>
</tr>
<tr>
<td>Cu2O</td>
<td>5-45 Spherical</td>
<td>Bifurcaria bifurcata</td>
<td>Enterobacter aerogenes, Staphylococcus aureus</td>
<td>139</td>
</tr>
<tr>
<td>CuO</td>
<td>96-110 Spherical</td>
<td>Sargassum myriocystum</td>
<td>Staphylococcus mutans, Micrococcus luteus. Vibrio cholera, Klebsiella pneumonia, Neisseria gonorrhoea</td>
<td>87</td>
</tr>
</tbody>
</table>
This is of particular importance since conventionally manufactured Au nanoparticles have been used in a variety of biomedical applications such as carriers for anticancer drugs [16, 19, 119, 148], biosensors [6] and targeted platforms in tumours for thermal treatment therapies [149, 150]. Another interesting type of nanometre scale material with unique super paramagnetic properties suitable for a variety of biomedical applications are Fe$_3$O$_4$ nanoparticles. The magnetic properties of the magnetite nanoparticles makes them ideal for magnetic resonance imaging (MRI) and targeted drug delivery [16, 151]. However, very few studies have studied using marine algae for producing super paramagnetic nanoparticles as seen in Table 1. Similarly, there are very limited studies reporting the biosynthesis of zinc oxide and copper oxides via marine algae. Oxides are of particular interest, since copper oxides have displayed antibacterial properties [139] and ZnO has displayed both therapeutic properties [146] and photocatalytic properties [147]. Surveying the literature reveals biosynthesising metal and metal oxide nanoparticles via marine algae and marine plants is a relatively new and largely unknown research field. This review has summarized the current state of research in the field and highlighted that marine algae and plants are a rich source of biomolecules that have the potential to act as both reducing agents and stabilizing agents. However, further studies are needed to fully explore the prospective properties of marine algae and marine plants for the biosynthesis of metal and metal oxide nanoparticles and their potential to contribute to new pharmaceuticals and medical treatments.

Conclusions
The biogenic synthesis of nanostructured materials, especially metal and metal oxide nanoparticles, has attracted considerable interest in recent years due to their unique properties that make them highly desirable for a wide range of pharmaceutical and biomedical applications. Biosynthesis via marine algae and marine plants has the potential to deliver facile, green and eco-friendly technologies for producing nanoparticles. Clean green chemistry based technologies have the potential to significantly reduce the toxic effects of chemicals and solvents generally used in conventional chemical and physical manufacturing processes. The polysaccharides, proteins, and other active bioactive chemicals found in the cell membranes of marine alga discussed in this review have proven to be effective reducing and capping agents. However, as highlighted in this review, only a relatively small number of marine alga and marine plants have been studied to date and it is not expected that all algal and plant species will be suitable for the biogenic synthesis of nanoparticles.

The biogenic synthesis of metal and metal oxide nanoparticles via marine algae and marine plants is still a largely unknown research field and offers some unique opportunities for exploration and developing new green chemistry based biogenic processes. This review will hopefully inspire much needed research in this relatively unexplored and sustainable source of bioactive molecules and materials.

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Author Contributions
All authors contributed equally to this work.

Conflicts of Interest
The authors declare no conflict of interest.

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Chapter 3 - Biosynthesis studies with indigenous Australian plant species

3.1. Overview and author contributions

Research carried out as part of this chapter significantly advances knowledge in the field. The chapter addresses the first aim of the research project, namely, manufacture metallic nanometre scale particles using a green environmentally friendly biological synthesis process that uses Australian indigenous plants. The three indigenous plants involved in the studies were Eucalyptus macrocarpa (rose of the west), Xanthorrhoea glauca (grass tree), and Anigozanthos manglesii (red and green kangaroo paw). And the two types of noble metal nanoparticles produced via the indigenous plants were Au and Ag. Both Au and Ag were selected because they have been extensively studied elsewhere and the results of the present studies could be easily compared to those reported in the literature. The chapter is composed of three cases studies, each one exploring the biogenic potential of an individual indigenous Australian plant to produce such noble metal nanoparticles. Case Study 1 examines the ability of Eucalyptus macrocarpa to synthesis Ag nanoparticles at low energy input. The study found that the leaf extract, at room temperature was capable of producing Ag nanocubes ranging in size from 10 nm up to 50 nm. Case Study 2 studied the Eucalyptus macrocarpa leaf extract potential and its proficiency to synthesis Au nanoparticles. During the study a parallel path method was used, the first path synthesised Ag using a similar technique used in Case Study 1 and the second path synthesised Au nanoparticles. The study compares and contrasts the results of the biogenic synthesis techniques used. Case Study 3 presents a microscopy study of Xanthorrhoea glauca leaves and the ability of the leaves and leaf extracts to biologically synthesis Ag nanoparticles.

Overall, the main author contributions in all three Case Studies consisted of G.E.J. Poinern acting as principal supervisor who designed the overall concept of the experimental studies with M. Shah. In the first Case Study, M. Shah carried out the bulk of the experimental work and subsequent nanoparticle characterisation and analysis. During characterisation M. Shah was assisted by P. Chapman. M. Shah significantly
contribution to the paper by writing text, and preparing tables and images with the technical assistance of D. Fawcett. All authors were all involved in addressing various technical difficulties encountered during the preparation of the manuscript and with the editorial changes to the manuscript as recommended by reviewers. In the second Case Study, M. Shah conducted all the experimental work and subsequent characterisation of the synthesised Ag and Au nanoparticles. D. Fawcett provided technical assistance, while M. Shah was actively involved with data analysis and manuscript preparation. All authors assisted in the preparation of the manuscript and with editorial changes recommended by reviewers before article were accepted for publication.

In the third Case Study all experimental work and characterisation was carried by M. Shah. G. Thomson assisted M. Shah with the preparation of optical slides and optical microscopy studies. M. Shah conducted data analysis and prepared the manuscript with the assistance of Derek Fawcett. All authors actively participated in the preparation of the manuscript and with the editorial changes to the manuscript as recommended by reviewers of the peer reviewed journal. Throughout all three case studies, all authors contributed to the peer reviewed research articles that formed the content of chapter 3.

3.2. Published Research Articles

**Case Study 1**

**Case Study 2**
**Case Study 3**

**Monaliben Shah, Gordon Thomson, Gérard Eddy Jai Poinern, Derek Fawcett.**


### 3.3. Chapter Summary

The primary focus of the chapter was to undertake biosynthesis studies to determine the feasibility of using Australian indigenous plants to biologically synthesise noble metal nanoparticles. The present work was composed of three Case Studies that investigated various aspects of biogenic synthesis using Australian indigenous plants. Each of the Case Studies selected have clearly demonstrated that each plant’s cellular content was easily capable as acting as a biological factory in the manufacture of noble metal nanoparticles from their respective metal salts. In each case, the process was found to be straightforward, clean, and eco-friendly and sustainable. The room temperature process (typically around 24 °C) used various leaf extracts to produce nanoparticles with a variety of particle sizes, shapes and size distributions.

Studies of Eucalyptus *macrocarpa* have revealed that the biogenic process had effectively reduced and subsequently stabilised a variety of Ag and Au nanoparticle sizes and morphologies. Thus, confirming the leaf extract was capable of acting as both reducing agent and stabilising agent. XRD and EDS analysis confirmed the presence of metallic Ag and Au, and both TEM and FESEM images revealed spherical and non-spherical particle morphologies for both Au and Ag nanoparticles. In particular, TEM images of Au taken after 1 h showed spherical particles ranging in size from 20 nm up to around 100 nm. The images also revealed a smaller number of triangular, pentagon and hexagonal shapes ranging in size from 50nm to 100 nm. However, longer periods revealed the smaller particles continued to grow and end up as micrometre scale platelets typically 2 µm in size. Similarly, spherical Ag nanoparticles ranging in size from 10 to 100 nm, cubes ranging from 10 to 50 nm, and some triangular particles were seen after 1 h of reduction. But longer periods tended to produce micrometre scale cubes (50 nm up to 1 µm) with the occasional triangular and hexagonal plate.
Another important aspect of this research was to investigate regions within a representative leaf to determine potential synthesising sites for noble metal nanoparticles. Case Study 3 investigated this property by carrying out an extensive microscopy study of Ag nanoparticle synthesis sites within *Xanthorrhoea glauca* leaves. In the first part of the microscopy study, leaf structure was investigated via a series of transverse and longitudinal sections. The optical microscopy and scanning electron microscopy studies revealed that the long reed-like leaves had a complex structure. Importantly, the results indicated that important phytochemicals present in the cell membranes of the plant were effective in reducing AgNO₃ solutions to form Ag nanoparticles. After 20 minutes exposure to the AgNO₃ solution nanoparticles were formed near the cell membranes. Microscopy examination revealed the morphology of the nanoparticles cubes included cubes, truncated triangular and hexagonal plates and particle sizes ranging from 50 nm and 200 nm. Notably, the nanoparticles formed *in situ* were similar to those produced by leaf extract and AgNO₃. Thus, indicating the phytochemicals present in the leaf extract had come initially from the cell membranes. However, the phytochemicals present in the leaf extract and actively involved in Ag nanoparticle formation have yet to be individually identified. This unresolved issue forms the basis for future work and will be discussed in Chapter 5.
Green biosynthesis of silver nanocubes using the leaf extracts from 
Eucalyptus macrocarpa

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ABSTRACT: In this preliminary study, we present for the first time a facile and environmentally friendly process for the green synthesis of silver nanoparticles using the leaf extract from an indigenous Australia plant Eucalyptus macrocarpa. The synthesis process is performed at room temperature and the leaf extract acts as both reducing agent and stabilising agent. The synthesis process is clean, non-toxic and straightforward and does not need complex processing equipment. The formation of the silver nanoparticles was confirmed by UV-visible spectroscopy, X-ray diffraction, transmission electron microscopy and field emission scanning electron microscopy.

KEYWORDS: silver nanoparticles, green synthesis, Eucalyptus macrocarpa

1. Introduction

Metallc nanoparticles prepared from metals, such as copper (Cu), gold (Au) and silver (Ag) have attracted considerable interest in many fields such as medicine, biotechnology, materials science, photonics and electronics [1-4]. The size, shape and surface morphology of the nanoparticles can have a profound influence on its chemical, physical, optical and electronic properties [5, 6]. This is indeed the case for medical preparations that use Ag nanoparticles as their active antimicrobial ingredient, since the antimicrobial effect of the nanoparticles is attributed to their size and high surface area to volume ratio, which enables them to closely interact with the bacterial cell membrane [7]. Recent antimicrobial studies have shown that membrane damage and toxicity can result from the bio-sorption and cellular uptake of nanoparticles by bacteria [8]. However, the processes behind nanoparticle inhibition of bacterial growth are not fully resolved, but some studies have suggested that the size, shape and surface modifications could influence the antibacterial properties of the nanoparticles [8, 9]. Conventional methods of producing nanomaterials involve the use of expensive chemical and physical processes that often use toxic materials with potential hazards such as environmental toxicity, cytotoxicity and carcinogenicity [10]. Furthermore, during these processes there is a potential for toxic chemical species to be absorbed onto the surface of the nanoparticles which ultimately leads to adverse effects occurring during their application. Therefore, it is imperative to develop alternative chemical synthesis processes that can produce metal nanoparticles without the problems of environmental toxicity, cytotoxicity and carcinogenicity. In recent years, the challenge has been to optimise the usage of environmentally friendly, naturally occurring extracts for the synthesis of metal nanoparticles.

An attractive alternative to the traditional manufacturing techniques used for the production of nanoparticles involves using a green, environmentally friendly technology based on biological systems such as plants [11, 12], bacteria [13, 14], fungus [15, 16] and similar organisms [17, 18]. Synthesising nano-particles via biological systems offers a clean, nontoxic and environmentally friendly method with the potential to deliver a wide variety of nanoparticle types, sizes, shapes and morphologies. To be an effective alternative, the green biosynthesis process needs to address three important parameters, namely: 1) an environmentally friendly solvent medium; 2) an effective and benign reducing agent; and 3) a nontoxic capping agent to stabilize the particles and prevent particle agglomeration [4]. Out of the several biological systems mentioned above, the biosynthesis of nanoparticles at ambient temperature via leaf extracts from plants is a relatively straightforward technique. A leaf extract is an abundant source of phytochemicals that can potentially function as both reducing agent and stabilizing agent for the synthesis of nanoparticles [19]. The use of leaf extracts also has the advantage of not needing any special culture preparation or isolation techniques that are normally required for bacteria and fungi.

For centuries, silver and silver compounds have been successfully used as an effective antimicrobial agent for the treatment of infections. Silver nanoparticles (Ag NPs), like its bulk counterpart have also been found to be an
efficient antimicrobial agent capable of interacting with the cell membrane, interfering and damaging cellular nucleic acids [20]. Recent studies have shown that Ag NPs possess both anti-bacterial and anti-inflammatory properties that can promote faster wound healing and as a result have been incorporated into wound dressings, pharmaceutical preparations and implant coatings [21-23]. Because of the biomedical importance of Ag NPs and the need for new environmental friendly processes, it is necessary to investigate plant flora for potential candidates that can act as both reducing agent and stabilising agent.

The South West corner of the Australian continent is a global biodiversity hot spot and is also the indigenous region of the exquisite Eucalyptus macrocarpa, which is also known as the Rose of the West or the Mottlecah [24, 25]. The Mottlecah is easily recognized by its beautiful silvery foliage, which consists of distinctive ovate shaped leaves that can grow up to 12 cm in length and has prominent red flowers (see Fig. 1a). The silvery grey appearance of the plants leaves are the result of nano-structured features formed by the epicuticular waxes that give the leaves their remarkable wetting and self-cleaning properties which enhances the plants survival in its arid climate [26]. Because of its attractive floral arrangement, this particular eucalyptus is not an endangered species and has been successfully propagated across the west coast of Australia.

In this paper, we report for the first time the green synthesis of stable Ag NPs by the direct reduction of silver nitrate, (AgNO₃) via Mottlecah leaf extracts without using conventional stabilising agents. The advantages of using this approach include: 1) the leaf extract acts as both reducing agent and stabilising agent during the synthesis process; 2) the aqueous synthesis process is environmental friendly and produces no toxic waste products; and 3) the technique is simple, straight forward and does not require specialised equipment.

2. Materials and methods

2.1. Chemicals

The source of Ag⁺ ions used throughout all nanoparticles synthesis procedures was silver nitrate [AgNO₃, (99.99%)] and was supplied by Sigma-Aldrich (Castle Hill, NSW, Australia). The Ag nanoparticles used as a control were synthesised by using sodium borohydride (NaBH₄) reduction of AgNO₃ in the presence of sodium citrate (C₆H₅Na₃O₇) as the stabilizing and capping agent. Both NaBH₄ and C₆H₅Na₃O₇ were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia) and used without further purification. Milli-Q® water was used throughout all synthesis procedures involving aqueous solutions and was produced by a Barnstead Ultrapure Water System D11931 (Thermo Scientific Dubuque IA 18.3 MΩ cm)

2.2. Leaf material and preparation of leaf extract

Eucalyptus macrocarpa or Mottlecah leaves were collected from several locations around the Murdoch University campus in Perth, Western Australia. A wide selection of healthy Mottlecah leaves, ranging from young to mature leaves was harvested from various locations on each plant. Generally, 5 locations were selected (top, north, south, east and west) and on average 10 leaves were taken from each of the locations. Both the adaxial and abaxial sides of the leaf were examined, with only healthy leaves free from damage being harvested. The leaves were washed several times with Milli-Q® water to
remove any dust or debris. After cleaning, 10 g of Mottlecab leaves were finely cut into small strips and then added to a 100 mL solution of Milli-Q® water. The aqueous mixture was then added into the blending bowl of an IKA® T25 Digital Ultra-Turrax® Homogenizer. The mixture was homogenized at 5000 rpm for 10 min at a room temperature of 24 °C. At the end of this time the solution was first filtered using a Hirsch funnel to remove leaf debris. This was followed by two further filtrations using a 0.22 µm Millipore® (33 mm Dia.) syringe filter unit. At the end of the resulting filtration procedure, the leaf extract was placed in a glass vial ready for the synthesis of Ag nanoparticles.

2.3. Synthesis of Ag nanoparticles

The Ag nanoparticles used as the control were synthesised by first adding a 1.0 mL solution of 1 mM AgNO₃ to a 10 mL solution of Milli-Q® water while the solution was stirred vigorously. This was followed by adding a 1.0 mL solution of 1 mM sodium citrate (stabilizing and capping agent) to the aqueous solution at room temperature (24 °C) and stirred for 10 minutes. The reduction of the Ag nanoparticles was initiated by the addition of a 1.0 mL solution of 0.01 M sodium borohydride to the aqueous solution. The reduction process was allowed to proceed at room temperature (24 °C).

Biological reduction of a 1.0 mL solution of 1 mM AgNO₃ was investigated using 3 solutions with varying amounts of leaf extract. The quantities of leaf extract used to make up the solutions consisted of 1 mL for s₁, 2 mL for s₂ and 3 mL for s₃. Once the AgNO₃ was added to each quantity of leaf extracts, the solutions were then vigorously stirred for 1 minute. The reduction process was allowed to proceed at room temperature (24 °C).

2.4. Leaf droplets

A clean Mottlecab leaf was selected, and laid flat in the horizontal plane to prevent the various droplet types from rolling off the leaf surface. Then using a fluid specific clean glass pipette fitted with a rubber bulb a series of 4 droplets were placed onto the leaf surface. The droplets consisting of: (i) raw leaf extract; (ii) solution of Ag nanoparticles; (iii) pure AgNO₃ solution; and (iv), solution s₁ consisting of AgNO₃ and leaf extract solution (1:1). The leaf and droplets were photographed over a period of time using a Canon EDS 600 D digital camera (Canon Inc, Tokyo Japan fitted with macro lens EF 100 mm 1:2.8 USM).

2.5. Characterisation of biologically reduced Ag NPs

All samples were examined and characterised using five advanced analysis techniques. These included: UV-visible spectrum analysis, X-ray diffraction spectroscopy (XRD), energy dispersive X-ray spectroscopy (EDAX), transmission electron microscopy and (TEM) field emission scanning electron microscopy (FESEM).

2.5.1. UV-visible spectrum analysis.

A series of samples were prepared. The first set consisted of three controls: 1) Milli-Q® water; 2) pure AgNO₃ solution; and 3) pre-filtered pure leaf extract (filtered twice, each time using a new Whatman 0.22µm syringe filter). The test solutions consisted of the 3 Ag colloids s₁, s₂ and s₃. The UV-visible spectra of each of the samples was then measured using a Varian Cary 50 series UV-Visible spectrophotometer version 3, over a spectral range from 200 to 1100 nm, with a 1 nm resolution over the first hr at room temperature of 24 °C.

2.5.2. XRD spectroscopy

After the end of each reduction procedure, samples for XRD examination were extracted from each glass vial using a clean glass pipette fitted with a rubber bulb. Then two to three drops of each sample were dispersed over the surface of a specific glass microscope slide. Then each glass slide was then dried under vacuum for a period of 4 hours. At the end of this time, the dried samples were then characterised using XRD spectroscopy. The XRD spectra were recorded at room temperature (22 °C), using a Bruker D8 series diffractometer [Cu Kα = 1.5406 Å radiation source] operating at 40 kV and 30 mA. The diffraction patterns were collected over a 2θ range from 15° to 80° with an incremental step size of 0.04° using flat plane geometry. The acquisition time was 2 seconds. The powder XRD spectrum was used to identify the size of the Ag particles and their crystalline structure. The particle size was calculated using the Debye-Scherrer equation [Equation 1] from the respective XRD patterns and estimated from both TEM and FESEM images.

2.5.3. EDAX spectroscopy

Each sample for EDAX examination was initially deposited onto a thin mica strip using a glass pipette, the mica strip was attached to a SEM stub using carbon tape. The samples were then dried under vacuum overnight. The following day, all samples were sputter coated with a 3 nm layer of Platinum. The samples were then examined using an Oxford Instruments EDS X-ray detector (EDAX) and Oxford Instruments energy dispersive X-ray detectors (EDS). The electron backscatter diffraction analysis (EBSD) used during the analysis was set with an EDS aperture of 60 µm and operated at 20kV.

2.5.4. TEM

The size and morphology of the Ag NPs was investigated using TEM. Sample preparation consisted of filtering the suspensions 2 times, each time using a new Whatman 0.22µm syringe filter. After filtration a single drop from each sample was deposited onto its respective carbon-coated copper TEM grid using a micropipette and then allowed to slowly dry over a 24 hour period. After sample preparation a bright field TEM study was carried out us-
ing a Phillips CM-100 electron microscope (Phillips Corporation Eindhoven, The Netherlands) operating at 80kV.

2.5.5. FESEM

Each sample for FESEM examination was initially deposited onto a thin mica strip using a glass pipette, the mica strip was attached to a SEM stub using carbon tape. The samples were then dried under vacuum overnight. The following day, all samples were sputter coated with a 3 nm layer of Platinum. The particle size and morphological features of the samples were investigated using a high resolution FESEM (Zeiss Neon 40ESB FIBSEM) at 5 kV with a 30 µm aperture operating under a pressure of 10⁻¹⁰ Torr.

3. Results and discussion

The present study reports the green synthesis of cubic Ag NPs by the biological reduction of aqueous silver ions using the leaf extract from Eucalyptus macrocarpa (Mottlecah). The water soluble ingredients present in the leaf extract were not only found to be a highly effective reducing agent, it also turned out to be an efficient stabilising agent. The formation of the Ag NPs could be easily monitored by the change in colour of the reactive mixture. The reactive mixture changes to a brown colour due to the excitation of surface plasmon vibrations of the formed Ag NPs. In the case of solution s1 (1:1 ratio of AgNO₃ and leaf extract) the change in colour of the reaction mixture occurs within 10 minutes, see droplet (iv) in Fig. 1(b). There was no colour change in the control droplet, (iii), which only contains the pure aqueous AgNO₃ solution.

A qualitative evaluation of the reducing agents in the Mottlecah leaf extract could be made by comparing the colour change (mixture turning brown), the time of complete colour change and the concentration of AgNO₃ to leaf extract in the mixture. The reduction of the silver ions was quite rapid, with colloids s1 (1:1) only taking 10 minutes to react. While in the case of colloids s2 (1:2) it took 60 min to achieve the same colour change as s1 and solution s1 (1:3) took 90 minutes. This simple qualitative analysis reveals that the efficiency of the nanoparticle synthesis decreases with increasing amounts of leaf extract in the AgNO₃ to leaf extract colloid. The UV-Vis spectrum for solution s1 (1:1) is presented in Fig. 2(a). The maximum absorbance occurs at 430 nm, which is similar to the maximum absorbance reported by Singh et al using ginger rhizome [27] and by Masurkar et al using Cymbopogon citrates (lemon grass) leaf extract [28]. The results of an EDAX spectroscopic examination of each colloid confirmed the presence of metallic Ag in each of the leaf extracts. A typical EDAX spectrum is presented in Fig. 2(b) for the s1 solution and clearly indicates a significant metallic Ag peak.

To investigate the crystalline nature of the Ag NPs, dried Ag NP powder samples were investigated using XRD spectroscopy. The Ag crystalline phases present were found to be consistent with phases incorporated in the ICDD (International Centre for Diffraction Data) databases. The results of the XRD studies are presented in Fig. 3. Four diffraction patterns are presented for comparative purposes; the first is pattern (a), which represents the spectra of the leaf extract. Inspection of pattern (a) reveals that there are no diffraction peaks present. The second pattern (b) is the spectra of metallic Ag deposited on glass by Tollen’s reaction, while pattern (c) is the spectra of Ag NPs synthesised via the reduction of AgNO₃ by sodium borohydride, with sodium citrate being used as the stabilizing and capping agent. Comparison of patterns (b), (c) and (d) reveals that the Bragg reflection peaks are present in all three. Examination of the dominant three peaks located at 38.5°, 44.5° and 64.5°, which corresponds to the (111), (200), and (220) lattice plane sets. The spectra of Ag NPs synthesised using the leaf extract, pattern (d) were then indexed to reveal a face centred cubic structure for the Ag powder sample. The crystalline size, \(d_{(hkl)}\), of Ag NPs was calculated from the XRD pattern using the Debye-Scherrer equation presented in eq. (1) below:

\[
I( hkl) = \frac{0.9\lambda}{B \cos \theta( hkl)}
\]
The typical side dimension of the larger micrometre scale Ag cubes was found to be around 1 µm. There are also many nanometre structures still present along with the organic matrix of the leaf extract. At this stage there are relatively few spherical NPs, but there are small numbers of triangular structures, these are circled in red for easier identification in Fig. 4(c).

where, \( \lambda \) is the wavelength of the monochromatic X-ray beam, \( B \) is the Full Width at Half Maximum (FWHM) of the peak at the maximum intensity, \( \theta_{(hkl)} \) is the peak diffraction angle that satisfies Bragg’s law for the \((h k l)\) plane and \( t_{(hkl)} \) is the crystallite size. An estimate of the mean crystallite size using the FWHM of the \((111)\) was calculated to be 38 ± 2 nm for the Ag NPs synthesised using the leaf extract.

The final XRD pattern in Fig. 3 is (d), which also reveals the presence of a number of other peaks in the leaf extract being treated with AgNO₃. The presence of metallic Ag in the leaf extract/AgNO₃ mixture is indicated by the two diffraction peaks at 38.8° and 44.5°, which correspond to the \((111)\) and \((200)\) lattice planes respectively. These peaks are identified by filled circles in pattern (d). Inspection of pattern (d) also indicates the presence of two significant, but unidentifiable peaks and one extremely weak unidentifiable peak. All three peaks are indicated in pattern (d) by the presence of a filled triangle over the peak. Similar unidentified peaks resulting from the interaction between AgNO₃ and a fungus (Aspergillus flavus) have also been reported by Vigneshwaran et al [29]. The three unidentified peaks formed by the interaction between leaf extract and AgNO₃ are currently being investigated by the authors.

Figure 4(a) presents a typical TEM image of Ag NPs taken within 1 h after the reduction process. The image reveals spherical and non-spherical morphology, with a number of nanometre sized cubes clearly present these are circled in blue for easier identification. The spherical Ag NPs range in size from 10 to 100 nm, while the nanometre sized cubes range in size from 10 to 50 nm. At this point in time there are also a few nanometre size triangles present. Figure 4(b) presents an FESEM image of Ag nanocubes taken 90 minutes after the initial reduction process, at this point in time the cubes range from 50 to 200 nm. If the colloidal solution is allowed to age for several hours, many of the Ag NPs grow into micrometre size particles, which are predominantly cubic in structure as shown in Fig. 4(c).
Further investigations are needed in order to determine the functional groups present in the *Eucalyptus macrocarpa* leaf extract that are involved in the reducing, capping and stabilizing the Ag NPs. In addition, the antimicrobial properties of the synthesised Ag NPs need to be investigated along with any potential synergistic effects resulting from the leaf extract. Earlier studies by Murata *et al* isolated a novel antibacterial compound (macrocopal A), while subsequent studies by Yamakoshi *et al* isolated a further six antibacterial compounds (macrocopal B to G) from *Eucalyptus macrocarpa* leaf extracts [30, 31]. In their studies the structure of the macrocarpal compounds were determined using both XRD and NMR analysis, which revealed that the compounds were composed of phloroglucinol dialdehyde diterpene derivatives. At this stage it is not known if these compounds are involved in the synthesis of the Ag NPs, or whether these compounds could have a potential synergistic effect when used in conjunction with the Ag NPs.

### 4. Conclusion

In this study, a natural, clean and environmentally friendly plant based agent was used to synthesis Ag NPs. The study for the first time demonstrated the ability of an indigenous Australian plant leaf extract derived from *Eucalyptus macrocarpa* to rapidly synthesis stable Ag nanocubes and other morphologies. The synthesis process was performed at room temperature and the leaf extract performed as both reducing agent and stabilising agent. The Ag NPs synthesised from AgNO3 treated leaf extract were found to have an UV-visible absorption peak at 430nm, while the mean crystallite size was calculated from XRD data and found to be 38 ± 2 nm. TEM micrographs of the resultant Ag NPs indicate the presence of both spherical and cubic morphologies. The spherical Ag NPs ranged in size from 10 to 100 nm, while the nanometre sized cubes ranged in size from 10 to 50 nm. Subsequent FESEM images of AgNO3/leaf extract mixtures taken after a sufficient aging period, usually several hours, the predominant morphology is cubic and is in the range from 50 nm up to 1 µm.

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### Disclosure

The authors report no conflict of interest in this work.

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Biogenic Synthesis of Gold and Silver Nanoparticles using the Leaf Extract from Eucalyptus Macrocarpa

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Abstract: The present work presents the results of a straightforward, no-toxic and eco-friendly process for the green synthesis of gold and silver nanoparticles using the leaf extract from Eucalyptus macrocarpa. The process was conducted at room temperature (24 °C) with the leaf extract acting as both reducing agent and stabilising agent. Particle formation was monitored during synthesis using UV-visible spectroscopy, X-ray diffraction analysis and Energy Dispersive Spectroscopy (EDS) confirmed the presence of metal nanoparticles. While transmission electron microscopy (TEM) and field emission scanning electron microscopy (FESEM) were used to determine and confirm particle size and morphology.

Keywords: gold, silver, nanoparticles, green biosynthesis

1. Introduction

In recent years, Gold (Au) and Silver (Ag) nanoparticles with well-defined size, shape and surface morphology have attracted a considerable amount of interest in fields such as medicine, materials science and optoelectronics [1-4]. The chemical, physical, optical and electronic properties of nanoparticles are directly influenced by their size, shape and surface morphology [5]. A wide range of physicochemical procedures has been used to nanoparticles with different compositions, sizes and logies [6]. However, their manufacture is generally ve and often employs the use of hazardous solvents another complication arises from the use of potentially face capping agents, (e.g. tri-n-octylphosphine oxide) nt nanoparticle aggregation [7].

Currently there is a need to significantly reduce or completely eliminate the use of toxic and environmentally damaging materials in the synthesis of nanoparticles. Developing green chemistry based techniques via biological systems such plants, bacteria, fungus and similar organisms offer a reliable and eco-friendly processes for the green synthesis of nanoparticles [8-11].

Compared with other biogenic sources the use of plant extracts for the synthesis of nanoparticles is relatively straightforward and as a result research into plant-mediated synthesis is being vigorously pursued worldwide. Studies have shown that source of the plant extract can significantly influence the formation of metal nanoparticles [12, 13]. This arises from different extracts having different concentrations and combinations of biomolecules. The biomolecules present in plant extracts can act as both reducing agents and stabilizing agents during the synthesis of nanoparticles. Because of the number of different biomolecules involved, the biological reduction, formation and growth of nanoparticles is quite a complex process [14]. Plant extract-mediated synthesis involves mixing the aqueous extract with an aqueous solution containing the metallic ion source at room temperature. During synthesis a number of controlling parameters such as pH, reactant concentrations, reaction time and temperature are actively involved during nanoparticle formation. These parameters and their subsequent variation can significantly influence the quality, size and morphology of the synthesized nanoparticles [15].
The present study investigates the plant-mediated synthesis of Au and Ag nanoparticles at room temperature via leaf extracts taken from Eucalyptus macrocarpa as seen in Figure 1 (b). The indigenous Australian plant is also known as the Rose of the West or the Mottlecah. The silvery coloured leaves produce an extract that was found to act as both reducing agent and stabilising agent during the synthesis process. The formation of Au and Ag nanoparticles was monitored during synthesis using UV-visible spectroscopy. X-ray diffraction analysis confirmed the presence of Au and Ag nanoparticles. While transmission electron microscopy (TEM) and field emission scanning electron microscopy (FESEM) were used to determine particle size and morphology.

2. Materials and methods

2.1. Materials

In this study, the source of Au ions was derived from gold chloride [HAuCl₄ (99.999%)] and the Ag ions were derived from silver nitrate solution [AgNO₃ (99.99%)] All chemicals were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia) and used as supplied without any further purification. Milli-Q water was used in all aqueous solutions used in the synthesis of nanoparticles and was produced by a Barnstead Ultrapure Water System D11931 (Thermo Scientific Dubuque IA 18.3 MΩ cm⁻¹).

2.2. Harvesting Eucalyptus leaf extract

A wide selection of healthy leaves free from damage were harvested from various locations on each plant from the local campus. Generally, 5 locations were selected (top, north, south, east and west) and on average 10 leaves were taken from each location. Leaves were washed several times with Milli-Q water to remove surface contamination. After, cleaning, 10 g of Mottlecah leaves were finely cut into small strips and placed into a blending bowl containing 100 mL of Milli-Q water. The mixture was homogenized at 5000 rpm for 10 min at a room temperature (24 °C) using an IKA® T25 Digital Ultra-Turrax® Homogenizer. The homogenized solution was then filtered using a Hirsch funnel to remove leaf debris. Two further filtrations using a 0.22 μm MilliEX® (33 mm Dia.) syringe filter unit were carried out before the leaf extract was transferred to clean glass vials ready for nanoparticle synthesis.

2.3. Biosynthesis of nanoparticles

In general, biological reduction of a 1.0 mL solution of metal ion source solution was investigated using 3 solutions with varying amounts of leaf extract. In the case of Au, a 1.0 mL solution of 10 mM aqueous AuCl₄⁻ ion solution was added to three leaf extracts solutions in the quantities of 1 mL for s1, 2 mL for s2 and 3 mL for s3. While in the case of Ag, a 1.0 mL solution of 1 mM AgNO₃ ion solution was added to three leaf extracts solutions in the quantities of 1 mL for s1, 2 mL for s2 and 3 mL for s3. Once each respective metal ion source was added to each respective leaf extract, the solutions were then vigorously stirred for 1 minute. The reduction process was allowed to proceed at room temperature (24 °C).

2.4. Characterisation of nanoparticles

All samples were examined and analysed using the following five advanced characterisation techniques. UV-visible spectrum analysis typically consisted of three controls: 1) Milli-Q water; 2) pure metal ion source solution; and 3) pre-filtered pure leaf extract (filtered twice, each time using a new Whatman 0.22μm syringe filter). The two sets of test solutions consisted of the three Au colloids [g1, g2 and g3] and three Ag colloids s1, s2 and s3. The respective UV-visible spectra of each sample was then measured using a Varian Cary 50 series UV-Visible spectrophotometer V3, over a spectral range from 200 to 800 nm, with a 1 nm resolution at room temperature of 24 °C. XRD samples were extracted from each glass vial using a clean glass pipette fitted with a rubber bulb. Two to three drops of each sample were dispersed on a glass microscope slide and dried under vacuum for a period of 4 hours. The sample was then characterised using XRD spectroscopy. The XRD spectra were recorded at room temperature (22 °C), using a Bruker D8 series diffractometer [Cu Kα = 1.5406 Å radiation source] operating at 40 kV and 30 mA. The diffraction patterns were collected over a 2θ range from 15° to 80° with an incremental step size of 0.04° using flat plane geometry. The overall acquisition time was 2 seconds. A single drop from each sample was deposited onto its respective carbon-coated copper TEM grid using a micropipette and then allowed to slowly dry over a 24 hour period. After sample preparation a bright field TEM study was carried out to determine size and morphology using a Philips CM-100 electron microscope (Philips Corporation Eindhoven, The Netherlands) operating at 80kV. Samples for FESEM were prepared by pipetting two drops onto a thin mica strip attached to a SEM stub using carbon tape. After drying, samples were sputter coated with a 3 nm layer of Platinum. The particle size and morphological features were investigated using a high resolution FESEM (Zeiss Neon 40EsB FIBSEM) at 5 kV with a 30 μm aperture operating under a pressure of 10⁻¹⁰ mbar. While sample elemental composition was analysed using an Oxford Instruments energy dispersive X-ray detectors (EDS).

3. Results and discussions

The biological reduction of aqueous metal ions using the leaf extract derived from Eucalyptus macrocarpa (Mottlecah) was investigated in this study. The pH value of the leaf extract was found to range from 5 to 5.5 and reduction took place at room temperature (24 °C). Two metal ion sources were investigated, the
first being Au and the second being Ag. In both cases the biomolecules present in the leaf extract were found to be an effective reducing agent in the formation of Au and Ag nanoparticles. Furthermore, after synthesis the biomolecules also acted as an effective stabilising agent preventing nanoparticle agglomeration. Formation of the respective nanoparticles could easily be seen via the colour change of the reactive ionic mixture. For example, the reactive solution s2 (1:2 ratio of AgNO₃ to leaf extract) changed from light green to dark brown in 10 minutes, see Figure 1 (d). The brown colour results from the excitation of surface plasmon vibrations of the Ag nanoparticles formed in the mixture. A representative UV-Vis spectrum for Au nanoparticle synthesis via solution g2 (1:2) is presented in Figure 1 (a) and representative spectrum for Ag nanoparticle synthesis via solution s2 (1:2) is presented in Figure 1 (c). Inspection of spectrum presented in Figure 1 (a) reveals that maximum absorbance occurred at 570 nm for solution g2. This value is similar to the maximum absorbance’s reported by Philip (573 nm) for Hibiscus rosa sinensis [16] and Singh et al (560 nm) for ginger (Zingiber officinale) [17]. In the case of Ag, the spectrum presented in Figure 1 (c) reveals a maximum absorbance of 430 nm for solution s2.

Figure 1.UV-visible spectrophotometer analysis of Au nanoparticles (a) and Ag nanoparticles (c); Eucalyptus macrocarpa (Mottlecah) (b) and colour change induced by the reduction and stabilisation of Ag nanoparticles.

XRD spectroscopy was used to confirm the formation of crystalline Au and Ag in the respective samples. Representative XRD patterns of dried samples are presented in Figure 2 and all Au and Ag crystalline phases present in the respective samples were consistent with phases listed in the ICDD (International Centre for Diffraction Data) databases. Inspection of the XRD pattern in Figure 2 (a) reveals the presence of intense peaks that correspond to the main (hkl) indices for Au: (111), (200), (220) and (311). The existence and location of these peaks confirms the presence of pure crystalline metallic Au consisting of a face centred cubic (fcc) lattice structure. A similar result for Ag could be seen in the representative XRD pattern presented in Figure 2 (c) that revealed the presence of peaks associated with pure crystalline (fcc) metallic Ag. TEM images were taken within 1 h after reduction and revealed both spherical and non-spherical morphology. Figure 2 (b) presents a typical TEM image of the various Au nanoparticles present in the samples. The main product found in the samples are spherical particles ranging in size from 20 nm up to around 100 nm. Also present in smaller numbers are equilateral or truncated triangular (yellow circles), pentagon and hexagonal shapes (green circles) ranging in size from 50nm to 100 nm. Figure 2(d) presents a typical image of Ag nanoparticles formed after 1 h of reduction and also reveals spherical and non-spherical morphology. Spherical Ag nanoparticles range in size from 10 to
100 nm. Also present are nanocubes ranging in size from 10 to 50 nm (red circles) and in smaller numbers are truncated and un-truncated triangular shapes that are around 100 nm (blue circle).

Figure 2. XRD spectroscopy analysis of samples containing Au (a) and Ag (c) nanoparticles and TEM images of reduced Au (b) and (d) nanoparticles respectively.

FESEM was not only used to examine the reduced nanoparticles, but it was also used to study the subsequent growth into the micrometre range. The study also revealed atomically flat surfaces which were a characteristic feature of the plate-like particles. Also carried out as part of the FESEM study was an EDS elemental analysis of the samples. Figure 3 presents the results of the FESEM and EDS studies of Au, while Figure 4 presents the results of the Ag studies. Figure 3 (a) presents a representative FESEM micrograph in backscatter mode showing an array of various sized Au particles. Figure 3 (b) presents the corresponding EDS elemental analysis of the sample and clearly shows the strong presence of Au. The EDS result confirms the results of the XRD analysis and confirms Au was biologically reduced by the leaf extract. Figure 3 (c) presents an enlarged micrograph showing the presence of spherical and plate-like particles in the sample. Morphologies seen include hexagonal, triangular and truncated triangular shaped plates. Typically, the length of the truncated triangular sides was found to be 3 µm and the sides of the hexagonal plates was around 2 µm. The thickness of the plates tended to range from 200 to 500 nm. Also present in the sample aggregate were spherical and semi-spherical particles with diameters varying between 200 nm up to 1.5 µm. Figure 3 (d) presents an enlarged FESEM image to highlight the morphology of the truncated triangular and hexagonal Au plates.
Biogenic Synthesis of Gold and Silver Nanoparticles using the Leaf Extract from Eucalyptus Macrocarpa

Figure 3 (a) Representative FESEM micrograph (backscatter mode) showing an array of various sized Au particles; (b) EDS elemental analysis showing the strong presence of Au; (c) Enlarged micrograph showing the variety of particle morphologies, and (d) Enlarged micrograph highlighting particle morphology.

Also present are a few spherical to rounded non-spherical particles, but there are also a small number of triangular structures. Figure 4 (d) highlights a typical triangle with a red circle and a representative cube with a brown circle for easier identification.

The present work has shown that the leaf extract taken from *Eucalyptus macrocarpa* (Mottlecah) has the capability of biologically reducing both Au and Ag from respective ion sources. Analysis of the results from the various characterisation techniques has confirmed the formation of Au and Ag nanoparticles with a morphology of spherical, semi-spherical, cubic, hexagonal, triangular, and truncated triangular. The synthesis process can be summarized by the following equation:

\[
\text{Metal ions + Mottlecah leaf extract} \rightarrow \text{Stabilised metal nanoparticles}
\]
Biogenic Synthesis of Gold and Silver Nanoparticles using the Leaf Extract from Eucalyptus Macrocarpa

Figure 4  (a) Representative FESEM micrograph showing an array of various sized Ag particles; (b) EDS elemental analysis showing the strong presence of Ag; (c) Micrograph of various particle morphologies, and (d) highlighted particles.

4. Conclusion
The present work has demonstrated a straightforward, clean, and eco-friendly process for biologically synthesising Au and Ag nanoparticles via leaf extracts from an indigenous Australian plant Eucalyptus macrocarpa. The studies revealed that the leaf extract at room temperature (24 °C) was able to act as both reducing agent and stabilising agent. XRD and EDS analysis confirmed that biological reduction produced metallic Au and Ag from their respective ion sources. UV-visible spectroscopy analysis found that the respective absorption peaks (Au: 570 nm and Ag: 430 nm) were consistent with Au and Ag peaks reported in the literature for similar plant based synthesis methods. Furthermore, analysis of TEM and FESEM images confirmed the reduction and subsequent stabilisation process had produced a variety of nanoparticle sizes and morphologies. Both TEM and FESEM images revealed spherical and non-spherical particle morphologies for both Au and Ag nanoparticles. TEM images of Au taken after 1 h revealed spherical particles ranging in size from 20 nm up to around 100 nm. Also present in smaller numbers were triangular, pentagon and hexagonal shapes ranging in size from 50nm to 100 nm. Longer reduction times permitted particles to grow into micrometre scale platelets typically 2 µm in size. Spherical Ag nanoparticles ranging in size from 10 to 100 nm, cubes ranging in size from 10 to 50 nm and the occasional triangle were seen in TEM images after 1 h of reduction. Subsequent FESEM images taken after longer periods revealed the predominant morphology was micrometre scale cubic (50 nm up to 1 µm) with smaller numbers of triangles and hexagonal plates.

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Disclosure
The authors claim no conflict of interest in this work.

References
Biogenic Synthesis of Gold and Silver Nanoparticles using the Leaf Extract from Eucalyptus Macrocarpa

Microscopy Study of Xanthorrhoea glauca Leaves and Preliminary Investigation into Biogenic Synthesis of Silver Nanoparticles

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Abstract: The present study examines an eco-friendly room temperature method for synthesising Ag nanoparticles using the phytochemicals present in indigenous Xanthorrhoea glauca leaves. The study investigated the transverse and longitudinal sections of the long reed-like leaves via optical microscopy and scanning electron microscopy to determine its structure. Microscopy was also used to determine the sites of nanoparticle synthesis within the leaf structure. While the leaf extract itself was also used to produce Ag nanoparticles from AgNO₃. Particle shapes produced during the 20 minute incubation period consisted of cubes, truncated triangular and hexagonal plates ranging in size from 50 nm and 200 nm. Both energy dispersive spectroscopy (EDS) and transmission electron microscopy (TEM) were used to confirm Ag nanoparticle formation.

Keywords: silver nanoparticles, biogenic synthesis, green chemistry

1. Introduction

Nanoparticles exhibit new and unique chemical, physical, optical and electronic properties compared to their macro scale equivalents [1]. These distinctive properties are derived from specific characteristics such as particle size, distribution and shape. Noble metal nanoparticles such as gold (Au), silver (Ag) and platinum (Pt) have been widely used in a variety of fields such as biotechnology, medicine, optoelectronics, pharmaceuticals and materials engineering [2, 3]. Furthermore, nanoparticles have been widely used in commercial products such as detergents, cosmetics, shampoos and even toothpastes [4]. Historically, the disinfecting properties of Ag have been well known and have been used in past traditional medicinal practices. In recent times Ag nanoparticles have shown themselves to be non-toxic towards humans and effective antimicrobial agents against bacteria, fungi and viruses [5]. The exact antimicrobial mechanism involved is not fully understood, but it is believed the Ag nanoparticles size, shape and surface chemistry interacts and damages both the membrane and nucleic acids of the microbe [6]. Because of the attractive antimicrobial and its anti-inflammatory properties, Ag nanoparticles are currently being used in a variety of commercial pharmaceutical products especially with the advent of antimicrobial resistance to common antibiotics [7, 8]. Advancement of this field not only requires the control of predetermined physiochemical properties, but also on the use of eco-friendly methods to synthesise nanoparticles.

Traditional chemical and physical approaches used to produce nanoparticles tend to be complex, use toxic chemicals and have low material conversions. Furthermore, these approaches are generally not eco-friendly and present a wide range of hazards [9]. The use of biological entities and methods employing microorganisms or plant extracts offers a green chemistry based approach that is a viable eco-friendly alternative to traditional chemical and physical approaches [10-12]. Phytochemicals present in plant extracts have the potential to act as both bio-reducing and bio-capping agents. The presence of these chemical agents make plant extracts an acceptable eco-friendly method for synthesising Ag nanoparticles.
nanoparticles. Reviewing the literature reveals an extensive selection of plant extracts have been investigated and employed to synthesise a variety of nanoparticles [13-15]. These studies have revealed that nanoparticle formation is dependent on parameters such as phytochemicals present in the plant species and their concentration, pH, reaction time and temperature. The interplay between these various parameters have been found to directly influence nanoparticle properties such as size, size distribution, surface chemistry and morphology [16].

In the present study, microscopy techniques were used to investigate the indigenous leaf structure of Xanthorrhoea glauca, location of particle synthesis and resulting various nanoparticle properties such as size and shape. The aqueous based technique was found to be straightforward, eco-friendly and produced no toxic waste products. During synthesis silver nitrate was reduced at room temperature to form stable Ag nanoparticles. The nanoparticles were subsequently characterized using UV-visible spectroscopy, transmission electron microscopy (TEM), scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDS) to determine particle size, morphology and composition.

2. Materials and Methods

2.1. Materials
Silver nitrate solution [AgNO₃, (99.99%)] analytical grade was used as received from Sigma-Aldrich (Castle Hill, NSW, Australia) and all aqueous solutions were prepared from Milli-Q® water produced from a Barnstead Ultrapure Water System (D11931 - Thermo Scientific Dubuque IA 18.3 MΩ cm⁻¹).

2.2. Nanoparticle Synthesis
Prior to biosynthesis at room temperature (24 °C), several 1 mL solutions of 0.1M AgNO₃ were prepared using Milli-Q® water. The individual AgNO₃ solutions were added to three leaf extracts consisting of 1 mL for s1, 2 mL for s2 and 3 mL for s3. The various mixtures were agitated for 1 minute before being permitted to stand, during which time the colour change of each respective mixture was monitored. In the dipping tests, leafs were vertically placed into 1 mL solutions of 0.1M AgNO₃ and progress of AgNO₃ permeation was monitored using microscopy.

2.3. Optical Microscopy, Scanning Electron Microscopy and Energy Dispersive Spectroscopy
Prior to optical microscopy, Johansen’s Safranin O and Fast Green stains were used to stain the respective sectioned Xanthorrhoea glauca leaf material. While Formalin-Acetic-Alcohol (FAA) was used as a fixing agent to preserve the leaf sections. Leaf material was sectioned in two planes (transverse and longitudinal) as shown in Figure 1 (b). The leaf sections were sectioned using a Olympus BX51 compound microscope (Olympus Optical Co. Ltd., Tokyo, Japan) and were photographed with the integrated DP 70 camera attachment. Leaf sections were also studied using characterisation techniques such as SEM, TEM and EDS to determine particle elemental analysis, particle size and morphology. TEM particle analysis consisted of placing a single drop of reaction medium onto a carbon-coated copper TEM grid. The sample was then allowed to dry over a 24 hours period before undergoing examination.

Figure 1 (a) photographic image of Xanthorrhoea glauca Plant (b) Leaf sectioning orientations used in microscopy studies.

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Microscopy Study of Xanthorrhoea Glauca Leaves and Preliminary Investigation into Biogenic Synthesis of Silver Nanoparticles

The bright field study was carried out using a Phillips CM-100 electron microscope operating at 80kV (Phillips Corporation Eindhoven, The Netherlands). Leaf sections were examined using a NeoScope™ (JCM-6000) SEM fitted with a secondary energy dispersive spectrometer. Samples were mounted on SEM stubs using carbon tape and then coated with a 2 nm layer of gold using a Cressington 208HR sputter coater to improve electrical conductivity.

Figure 2 (a) typical optical image of transverse section of leaf clearly showing vascular system; (b) and (c) optical and SEM images of a representative vascular bundle (phloem & xylem), and (d) and e) optical and SEM longitudinal sections of surface and inner leaf structure.

3. Results and Discussions
Figure 1 presents optical and SEM microscopy images of sectioned leaf material in transverse and longitudinal planes. Figure 2 (a) presents a representative optical image of a transverse leaf section showing its internal structure and overall square shape. The image reveals a waxy cuticle and epidermis layers covering a relative thin layer of palisade mesophyll cells that contain a large interior composed of spongy mesophyll cells surrounding an array of vascular bundles. Figures 2 (b) and 2 (c) present optical and SEM images of a typical vascular bundle that is used to transport xylem (water and minerals) and phloem (food energy) throughout the leaf. Figures 2 (d) and 2 (e) present optical and SEM images of a representative longitudinal section showing the internal structure of a leaf. In the optical images Safranin stain appears as a brilliant red in chromosomes, nuclei and cutinized cell walls. While the Fast green stains colours the cytoplasm and cellulosic cell walls. During the dipping procedure AgNO₃ could be visually seen moving up the leaf (yellow arrow) via a very noticeable colour change as seen in Figure 3 (a). Initially the AgNO₃ solution was clear but within 20 minutes it had become black, indicating bio-reduction had taken place. Optical microscopy revealed that AgNO₃ enters the leaf via stomata and vascular bundles (red arrows) as seen in Figure 3 (b) and (c). However, much larger quantities of AgNO₃ end up in the region of the cells than the vascular bundles (red arrows) as seen in Figures (b) and (d). Enlarged images presented in Figures 4 (a) and (b) also indicates the biosynthesis of Ag nanoparticles (red arrows) via the reduction of AgNO₃ occurs predominantly at the cell walls.
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Figure 3 (a) leaf after dipping in AgNO$_3$; (b) and (c) transverse sectional images showing principle regions of AgNO$_3$ reduction to form Ag nanoparticles, and (d) longitudinal section showing Ag NPs along the cells.

TEM analysis of the samples after 20 minutes revealed the biosynthesis process had produced particles that had an anisotropic character. Particle shapes found in representative samples include bars (blue circle), truncated triangular (yellow circle) and hexagonal plates (red circle) as seen in Figure 4 (c). Also present in smaller numbers were cubes and spherical shapes. The nanoparticles ranged in size between 50 nm and 200 nm, with mean size around 100 nm. Nanoparticles biosynthesised using the pure leaf extract were also found to produce particles with similar shapes and size range. EDS elemental compositional analysis conducted after biosynthesis indicated the presence of metallic Ag in the samples. The very strong signal for metallic Ag, as seen in Figure 4 (d) confirmed the results of the TEM study that clearly indicated the presence of Ag nanoparticles.

Figure 4 Enlarged views of leaf showing actual sites of Ag nanoparticle biosynthesis (a) transverse section and (b) longitudinal section; (c) representative TEM image of Ag nanoparticles produced, and (d) EDS analysis confirming the presence of metallic Ag nanoparticles.

This preliminary study has revealed that indigenous Xanthorrhoea glauca leaves contains the necessary phytochemicals needed to act as both reducing agent and stabilising agent during the synthesis of Ag nanoparticles. Initial results indicate that phytochemicals present in the cell membranes were effective in reducing AgNO$_3$ solutions to form Ag nanoparticles. The size and shape of nanoparticles formed in the leaf were found to be similar to those formed when an aqueous solution of AgNO$_3$ was mixed with a leaf extract solution. The resultant reaction mixture after 20 minutes produced nanoparticles of similar shape and size range as those produced in the leaf as seen in Figure 5 (a). Clearly seen in Figure 5 (a) are the hexagonal plates and a truncated triangular plate highlighted by a yellow circle. In addition, the elemental compositional analysis after a 20 minute incubation period revealed a the strong Ag peak indicating Ag nanoparticle formation had taken place as seen in Figure 5 (b).

The results of the preliminary study indicate leaf material of Xanthorrhoea glauca has the ability to reduce and produce stable Ag nanoparticles in situ directly from AgNO$_3$ solutions, (even without having to make an extract). However, further studies are needed to identify the exact phytochemicals involved in nanoparticle reduction and those involved in stabilizing the nanoparticles. Also, further studies are needed to investigate potential antimicrobial
properties of the synthesised Ag nanoparticles by
indigenous Xanthorrhoea glauca leaves.

Figure 5 (a) Ag nanoparticles formed via AgNO$_3$/leaf extract reaction solutions and (b) EDS elemental analysis confirming metallic Ag nanoparticle formation via reaction mixture.

4. Conclusion
The present study has examined an eco-friendly room temperature method for producing Ag nanoparticles via the phytochemicals present in Xanthorrhoea glauca leaves. The phytochemicals present in the cell membranes of the leaf were able to reduce Ag nanoparticles from AgNO$_3$ solutions, then coat and stabilise the nanoparticles. The formed nanoparticles were anisotropic in nature and were similar to those synthesised in AgNO$_3$/leaf extract mixtures after a 20 minute incubation time. Nanoparticle shapes included cubes, truncated triangular and hexagonal plates ranging in size from 50 nm and 200 nm. However, further research is needed to identify individual phytochemicals involved in Ag nanoparticle formation, physiochemical properties of the nanoparticles and potential applications such as antimicrobial pharmaceuticals.

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Disclosure
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Chapter 4 – Antimicrobial Studies of Gram positive and Gram negative bacteria using Au & Ag nanoparticles

4.1. Overview and author contributions

Chapter four addresses the third aim of the research project; namely, identify antimicrobial properties of the synthesised Ag and Au nanoparticles produced by indigenous Australian plant species. Both Case Study 4 and Case Study 5 have advanced knowledge in the field of nanoparticle synthesis using indigenous Australian plant species. Research in this field is currently limited, with relatively few publications in the field. Both Case Studies provide a much needed insight into the biogenic synthesis of noble metal nanoparticles and the subsequent investigation into their antimicrobial properties. This is of particular importance since antimicrobial resistance to current antibiotic medications has been identified as the most serious health threat to humanity today [1, 2]. In recent years several bacterial pathogens have become resistant and continue to proliferate in the presence of therapeutic levels of many commonly used antibiotics [3]. Ominously, the growth in new antibiotic medications in recent years has declined and during this same period numbers of antibiotic resistant bacterial pathogens have increased [4]. On the other hand, during the same period there has been a major effort into developing new and effective medicinal preparations directly from a variety of natural plant sources. A large proportion of this interest has focused on biologically synthesising metal nanoparticles for medical and pharmaceutical applications [5]. In particular, studies have shown that noble metal nanoparticles such as Ag and Au have antimicrobial properties that makes them suitable as antimicrobial agents in new pharmaceuticals products [6].

In an earlier study by the team of Murdoch Applied Nanotechnology Research Group, Eucalyptus macrocarpa leaf extracts were found to be effective reducing and stabilising agents in the formation of Au nanoparticles. A variety of sizes and shapes were formed, including spherical particles (20 to 80 nm) and crystalline shapes (equilateral or truncated triangular, pentagon and hexagonal) ranging in size from 50 to 100 nm. The
study found a synergistic effect between the Au NPs and leaf waxes in the leaf extracts and a Combination of Au nanoparticles and leaf extracts (with wax) created an effective antibacterial agent against both *Bacillus subtilis* and *Escherichia coli* [7]. Building on this earlier work, Case Study 3 established that Eucalyptus *macrocarpa* leaf extracts were also capable of biologically synthesising Ag nanoparticles. This result was important since Ag and in particular Ag nanoparticles have been incorporated into a wide range of pharmaceuticals and medical protocols [8, 9]. For this reason Ag nanoparticle synthesis via other indigenous Australian plants offered another pathway of producing Ag nanoparticles with unique properties via a green eco-friendly route. Therefore, the two Cases Studies forming Chapter 4 focused on two indigenous Australian plants, namely, *Xanthorrhoea glauca* and *Anigozanthos manglesii*. Both plants had been identified by the Murdoch Applied Nanotechnology Research Group as plants of interest. Both Case Studies investigated the ability of each respective plant to biologically synthesise Ag nanoparticle and then to examine the antimicrobial properties of the respective nanoparticles.

The overall concept for both case studies was developed by M. Shah and G.E.J. Poinern (Principal supervisor). In both Case Studies, M. Shah carried out the bulk of the experimental work and subsequent nanoparticle characterisation and analysis. During the antimicrobial studies M. Shah was assisted by G. Thomson from the School of Veterinary and Life Sciences, Murdoch University. D. Fawcett provided technical assistance, while M. Shah was actively involved with data analysis and manuscript preparation for both Case Study four and five. All authors actively participated in the preparation of both manuscripts and with the editorial changes to each manuscript as recommended by the respective reviewers of the peer reviewed journals.
4.2. Published Research Articles

Case Study 4

Monaliben Shah, Gérrard Eddy Jai Poinern, Derek Fawcett. Biosynthesis of silver nanoparticles using *Xanthorrhoea glauca* leaf extract and their antibacterial activity against Escherichia coli and Staphylococcus Epidermis.

Case Study 5

Monaliben Shah, Gérrard Eddy Jai Poinern, Derek Fawcett. Biogenic synthesis of silver nanoparticles via an *Anigozanthos manglesii* (red and green kangaroo paw) leaf extract and its potential antibacterial activity.

4.3. Chapter Summary

Chapter four was composed around two Case Studies, both of which examined the biological synthesis of Ag nanoparticles via indigenous Australian plants and the subsequent examination of the antimicrobial properties of the nanoparticles produced by each respective plant leaf extract. For the first time *Xanthorrhoea glauca* and *Anigozanthos manglesii* were studied from the perspective of eco-friendly nanoparticle synthesis. In both Case Studies, the cellular leaf extract from each respective plant was found to act as both reducing agent and capping agent. The green chemistry based method was non-toxic, eco-friendly and offered a viable alternative to conventional physical and chemical processing techniques. Advanced characterisation studies revealed that *Xanthorrhoea glauca* produced crystalline, anisotropic Ag nanoparticles ranging in size from 50 nm up to 200 nm with range of shapes including bars, cubes, triangular and hexagonal plates. *Anigozanthos manglesii* produced crystalline and anisotropic Ag nanoparticles ranging in size from 50 nm up to around 150 nm. While shapes produced consisted of cubic, triangular and hexagonal plates.
Antimicrobial studies of nanoparticles produced by *Xanthorrhoea glauca* leaf extracts displayed varying degrees of antibacterial activity against *Escherichia coli* and *Staphylococcus Epidermis*. The leaf extract showed no antibacterial properties so there was no synergistic effect seen in any of the samples. However, antimicrobial studies using *Staphylococcus Epidermis* revealed a larger zone of inhibition of around 11 mm. Antibacterial studies of Ag nanoparticles produced by *Anigozanthos manglesii* revealed that *Deinococcus* was sensitive, while both *Escherichia coli* and *Staphylococcus Epidermis* were found to be resistant. The studies also revealed that there was no synergistic effect produced by the leaf extract. Both Case Studies have addressed the third aim of the research project, namely, identify antimicrobial properties of the synthesised Ag nanoparticles produced by the indigenous Australian plant species under examination. The Case Studies have added to the knowledge in this field. However, further studies are needed to confirm the medicinal properties of the leaf extract mediated Ag nanoparticles, the influence of biomolecules in forming the nanoparticles and their subsequent interaction with various microorganisms. Also, the current studies have only focused on Ag and there is a need to examine the synthesis of other metal nanoparticles. This aspect of future research is discussed in Chapter

**References**


Research Article

Biosynthesis of silver nanoparticles using indigenous Xanthorrhoea glauca leaf extract and their antibacterial activity against Escherichia coli and Staphylococcus epidermis

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ABSTRACT

Background: This study for the first time presents an environmentally friendly, room temperature procedure for synthesizing silver (Ag) nanoparticles via the leaf extract taken from Xanthorrhoea glauca.

Methods: The simple and straightforward green chemistry based technique uses the leaf extract that acts as both reducing agent and capping agent to produce Ag nanoparticles which are subsequently quantified using advanced characterisation techniques. In addition, antibacterial studies were conducted using the Kirby-Bauer sensitivity method.

Results: Advanced characterisation revealed the synthesised particles had a variety of shapes including cubes, truncated triangular and hexagonal plates, and ranged in size from 50 nm up to 200 nm. The Gram-positive bacteria Staphylococcus epidermis showed the maximum zone of inhibition at 11 mm.

Conclusions: The study has shown that the leaf extract was able to synthesis Ag nanoparticles with antibacterial activity against Escherichia coli and Staphylococcus epidermis.

Keywords: Silver nanoparticles, Antimicrobial activity, Green bio synthesis, Xanthorrhoea glauca

INTRODUCTION

Noble metal nanoparticles such as gold (Au), silver (Ag) and platinum (Pt) have attracted considerable interest in many fields such as medicine, biotechnology, materials science, photonics and electronics.¹⁻⁴ The interest in nanoparticles arises from their size, shape and surface morphology that ultimately determines their chemical, physical, optical and electronic properties. ⁵ Conventional chemical and physical methods used to prepare nanoparticles often use toxic materials and processes that are not environmentally friendly and present many hazards.⁶⁻⁷ Biological methods for synthesizing nanoparticles offers a green and environmentally friendly technology that can overcome many of the harmful effects produced by conventional chemical and physical methods. Green chemistry methods using biological systems such plants, bacteria, fungus and similar organisms are an attractive and environmentally friendly alternative to chemical and physical methods.⁸⁻¹² Plant based synthesis of nanoparticles is normally carried out at moderate temperatures and pH values at atmospheric pressure. The green chemistry based approach also has the advantages of being straightforward processing and eco-friendly. Surveying the literature reveals a wide range of plant extracts have been used to synthesize metallic nanoparticles, in particular Au and Ag have been extensively reported.¹³⁻¹⁷ In the case of plants, phytochemicals present in the extracts act as both reducing agent and stabilising agent. Nucleation and
subsequent formation of nanoparticles is dependent on plant species, pH, phytochemical concentration, reaction time and temperature. These factors significantly influence size, morphology and properties of nanoparticles produced over reaction times ranging from a few minutes up to many hours depending on plant species.18,19

Historically, the antimicrobial properties of Ag compounds have been used in the medical treatment of infections. The strong antimicrobial activity of Ag against bacteria, fungi and viruses has led to this metal and in particular Ag nanoparticles being extensively studied in recent years.20 Although the precise mechanisms involved in producing the antimicrobial properties are not fully understood, it is believed the nanoparticles size, well-developed shape and large surface area provides maximum contact with bacteria, fungi and viruses. The particle size, morphological and surface chemistry are believed to be factors influencing the interaction and damage to the cell membrane, and inhibit and damage cellular nucleic acids.21 Current research has confirmed both antimicrobial and anti-inflammatory properties of Ag nanoparticles. And because of these results Ag nanoparticles have been incorporated into a number of pharmaceutical preparations and wound dressings.22–23 Future development and incorporation of Ag nanoparticles as antimicrobial agents in pharmaceutical products will require close control of particle size, morphology and particle stability. Furthermore, bio-inspired synthesis of Ag nanoparticles via plants avoids contaminating particles with harmful chemicals and solvents traditionally used in conventional chemical and physical methods.

In the present study, we report for the first time a novel green method for the synthesis of Ag nanoparticles from silver nitrate at room temperature via leaf extracts from an Australian indigenous plant Xanthorrhoea glauca also known as “Grass Tree” leaves as seen in Figure 1c. Biological synthesis was adopted since previous studies by the authors on other Australian indigenous plants have shown that the leaf extracts can act as both reducing agent and stabilizing agent during the synthesis process.24 Also, the aequous based technique is straightforward, eco-friendly and produces no toxic waste products.

The Ag nanoparticles synthesized using the leaf extract have been characterized by UV-visible spectroscopy, X-ray diffraction (XRD), energy dispersive X-ray spectroscopy (EDS), transmission electron microscopy (TEM), field emission scanning electron microscopy (FESEM) and Fourier transform infrared spectroscopy (FT-IR) to determine particle size, morphology, composition and role of the different functional groups in the synthetic process. The antimicrobial properties of the synthesized Ag nanoparticles were evaluated against gram-positive bacteria (Staphylococcus epidermidis) and Gram-negative bacteria (Escherichia coli) using the sensitivity method of Kirby-Bauer.

METHODS

Chemicals

Silver nitrate (AgNO₃, purity: 99.99%) and sodium borohydride (NaBH₄, purity: 99%) were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia) and used without further purification. Milli-Q® water was used to make all aqueous solutions used in processing the leaf extracts and chemical synthesis procedures. Milli-Q® water was produced using a Barnstead Ultrapure Water System D11931 (Thermo Scientific Dubuque IA 18.3 MΩ cm⁻¹).

Leaf material and preparation of leaf extract:

Xanthorrhoea glauca also known as “Grass Tree” leaves were collected from several sites around the Murdoch University campus in Perth, Western Australia. A range of healthy leaves free from damage and ranging from young to mature leaves were harvested from various locations on each plant. The leaves were first washed several times using Milli-Q® water to remove surface contaminants. After washing, 10 g of Xanthorrhoea leaves were finely diced and mixed using a standard domestic blender. The leaf mixture was then stirred into a 50 mL solution of Milli-Q® water before being poured into a blending bowl of an IKA® T25 Digital Ultra-Turrax® Homogenizer. The aqueous leaf mixture was homogenized at 5000 rpm for 10 min at room temperature (24°C). After homogenization the mixture was initially filtered using a Hirsch funnel to remove leaf debris. Two further filtrations were carried out using a 0.22 µm Milllex® (33 mm Dia.) syringe filter unit. After filtration, the leaf extract was placed into a glass vial ready for nanoparticle synthesis.

Biologically synthesized Ag nanoparticles

Biological synthesis of Ag nanoparticles was examined using varying amounts of leaf extract. Leaf extract solutions consisted of 1 mL for s1, 2 mL for s2 and 3 mL for s3. A 1.0 mL solution of 0.1M AgNO₃ was added to each of the 3 leaf extract solutions. Each mixture was vigorously stirred for 1 minute and then allowed to stand at room temperature (24°C). During which time the colour change of each respective mixture could be easily seen.

Biologically synthesized Ag nanoparticles were characterized using visible spectroscopy. X-ray diffraction (XRD), energy dispersive X-ray spectroscopy (EDS), transmission electron microscopy (TEM), field emission scanning electron microscopy (FESEM) and Fourier transform infrared spectroscopy (FT-IR) to determine particle size, morphology, composition and role of the different functional groups in the synthetic process. The antimicrobial properties of the synthesized Ag nanoparticles were evaluated against gram-positive bacteria (Staphylococcus epidermidis) and Gram-negative bacteria (Escherichia coli) using the sensitivity method of Kirby-Bauer.

Characterization techniques included;

UV-visible spectrum analysis

Analysis was carried out on controls and test samples. Control solutions consisting of Milli-Q® water, AgNO₃ solution and filtered pure leaf extracts, (filtered twice, each time using a new Whatman 0.22µm syringe filter). Test solutions consisted of the 3 Ag/leaf colloids s1, s2 and s3. All controls and samples were examined using a Varian Cary 50 series UV-Visible spectrophotometer.
Transmission electron microscopy (TEM)

Ag nanoparticle size and morphology was studied using TEM. Sample preparation started with filtering the respective Ag/leaf extract suspensions (filtered twice, each time using a new Whatman 0.22 μm syringe filter). A glass pipette fitted with a rubber bulb was then used to transfer a single drop from each respective sample to its respective carbon-coated copper TEM grid. The grids were then allowed to slowly dry over a 24 hour period. After preparation a bright field TEM study was carried out using a Philips CM-100 electron microscope (Phillips Corporation Eindhoven, The Netherlands) operating at 80 kV.

X-ray diffraction (XRD) spectroscopy

Crystalline phases present in the samples were identified using XRD spectroscopy. Sample preparation consisted of depositing two to three drops of colloid onto a glass slide via a clean glass pipette fitted with a rubber bulb. The droplets were dispersed over the slide and then dried under vacuum for a period of 4 h. Spectroscopy was performed on each sample in turn at room temperature using a GBC® eMMA X-ray Powder Diffractometer (Cu Kα = 1.5406 Å radiation source). XRD operated at 35 kV and 28 mA in flat plane geometry mode with each scan taking 2 seconds. The respective diffraction patterns were collected over a 20° range of 20° to 90°.

Fourier transform infrared spectroscopy (FT-IR)

FT-IR spectroscopy was used to identify the major functional groups and vibration modes present in the pure leaf extract and the respective colloids. A Perkin–Elmer Frontier FT-IR spectrometer with Universal Single bounce Diamond ATR attachment was used to analyse samples over a range starting at 525 up to a maximum of 4000 cm⁻¹ in steps of 4 cm⁻¹.

Scanning electron microscopy (SEM) and energy dispersive spectroscopy (EDS): Micrographs showing particle size and morphology were taken using a JCM-6000 (NeoscopeTM) fitted with an energy dispersive X-ray spectroscopy attachment to determine elemental composition of the samples. Individual samples were mounted on substrate holders using carbon adhesive tape before being sputter coated with a 2 nm layer of gold to prevent charge build up using a Cressington 208HR High Resolution Sputter coater.

Antibacterial activity of synthesized Ag nanoparticles: Ag nanoparticle antibacterial activity was studied using two bacterial strains (Escherichiacoli and Staphylococcus epidermis) via the Kirby-Bauer sensitivity method. Bacteria cultures were sub-cultured in Petri dishes (90 mm Dia.) containing a nutrient agar medium. Each strain was uniformly spread over the agar surface in each individual Petri dish using a sterile cotton swab. Then using a micropipette, 15 μL of nanoparticle solution was deposited onto a 6 mm diameter sterile disk (Whatman® AA 2017-006) and then allowed to air dry for 20 minutes. Once dried, the respective disks were placed on the agar media using sterile forceps before the being incubated overnight at 37°C. After incubation, the diameters of the different zones of inhibition were measured. Sample testing was carried out in duplicate and the mean inhibition zone diameters were used in the subsequent data analysis.

RESULTS

Figure 1a presents a representative UV-Visible spectra for a colloid containing 2.0 mL leaf extract and 1.0 mL of 0.1M AgNO₃ (s2). The spectra displays an optical Surface Plasmon Resonance (SPR) peak at 440 nm, which is typical for metallic Ag nanoparticles. Addition of AgNO₃ to the leaf extract changed the colour from clear to a reddish dark brown in 20 minutes. Previous studies report the brown colour indicates the formation of Ag nanoparticles in the reaction mixture due to excitation of surface plasmon vibrations as seen in Figure 1b. Mie’s theory predicts a single intense SPR peak in the absorption spectra for spherical metal nanoparticles. However, the broad peak centred at 440 nm in this study was due to the anisotropic nature of the nanoparticles. Subsequent TEM analysis revealed particles ranging in size from 50 nm up to 200 nm and a variety of shapes including bars, cubes, truncated triangular and hexagonal plates as seen in the representative image presented in Figure 1d.

A representative XRD pattern of a dried sample is presented in Figure 2 (a) and reveals three intense peaks corresponding to (111), (200) and (311) Bragg reflections. These peaks indicate an FCC structure present in the synthesized Ag nanoparticles. Also present in the XRD pattern is some degree of peak broadening that indicates the formation of nanoparticles and is
consistent with the results seen in the TEM images. Also present in the XRD patterns were a number of intense peaks (indicated by green stars) associated with unidentified materials present in the plant extract. These materials are currently being investigated and will be reported in a future article. Analysis of the respective XRD patterns obtained in this study reveal that they are consistent with similar reports for the biosynthesis of Ag nanoparticles (Figure 2).27,28

XRD study. Figure 3 also contains images of Ag particle growth and presents typical sizes and morphologies present in the samples after 1 h. The dominant shape present at this stage of growth is cubic and the maximum size seen is typically around 1 µm. Hexagonal plates are also seen in smaller numbers and there also a number of truncated triangular plates present. Both triangular and hexagonal plates are also typically around 1 µm in size (Figure 3).

Figure 3: (a) Representative EDS elemental analysis of synthesised Ag NPs; (b)-(d) SEM images of Ag nanometre and micrometre particles.

Antibacterial studies were conducted using the Kirby-Bauer sensitivity method. The method revealed the biologically synthesized Ag nanoparticles displayed significant antibacterial activity against both Escherichia coli and Staphylococcus Epidermis.

Representative results of Ag nanoparticles synthesised from an s2 reaction mixture are presented in Figure 4. The mean diameter of the inhibitory zones were measured and graphically displayed in Figure 4d. The Gram-positive bacteria Staphylococcus Epidermis showed the maximum zone of inhibition at 11 mm (Figure 4).

Figure 4: Microbial studies of AG antibacterial effects of (a) pure leaf extract; (b) leaf synthesised AG nanoparticles against e coli, (c) leaf synthesised AG nanoparticles against s epidermis, and (d) zones of inhibition.

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DISCUSSION

In the present study Ag nanoparticles were biological synthesized using *Xanthorrhoea glauca* leaf extract. The presence of nanoparticles in the reaction medium was visualised via a colour change (clear to dark brown) as seen in Figure 1b insert. UV-Visible spectroscopy revealed a broad SPR peak located at 440 nm, which is similar to peaks reported by Ahmad, N, Sharma using *Ananas comosus* and by Masurkar et al using *cymbopogan citratus* (lemon grass) leaf extract.\(^{33,34}\) In this study the broad peak was due to the anisotropic nature of the nanoparticles that ranged in size from 50 nm up to 200 nm. But unlike many studies that report spherical morphology, the particles produced by *Xanthorrhoea glauca* leaf extract have a variety of shapes such as bars, cubes, truncated triangular and hexagonal plates as seen in Figure 1d.\(^{33}\) The anisotropic nature of the nanoparticles synthesised in this study are of particular interest, since studies have shown Ag nanoparticles undergo shape-dependent interactions with cell membranes of micro-organisms.\(^{35}\)

FTIR studies have revealed that carboxy and amide groups appearing in the AgNO\(_3\) treated leaf extract suggest the involvement of phytochemicals in the synthesis of Ag nanoparticles as highlighted by the appearance of IR peaks at 1642 cm\(^{-1}\), 1556 cm\(^{-1}\), 1536 cm\(^{-1}\) and 1320 cm\(^{-1}\).\(^{1,36}\)

The Bragg reflections seen in the XRD patterns were indexed to the (111), (200) and (311) planes of face centred cubic (FCC) orientation and revealed the nanoparticles were pure crystalline Ag. Elemental analysis confirmed the formation of pure crystalline Ag in the samples. The peak associated with the (111) plane was found to be more intense than the other planes and indicated this plane was the predominant orientation. Also present in the XRD patterns were a number of unknown peaks associated with the leaf extract and are currently being investigated. The presence of these peaks highlights the complex array of phytochemicals present in the leaf extract.

The Ag nanoparticles demonstrated varying degrees of antibacterial activity against *Escherichia coli* and *Staphylococcus Epidermis* as indicated by the inhibition zone diameters. While the leaf extract, like the disk controls didn’t display any antibacterial activity as seen in Figure 4.

The Gram-positive bacteria *Staphylococcus epidermis* showed the larger of zone of inhibition (11 mm), compared to Gram negative *Escherichia coli* (8 mm). The variation in inhibition zone size suggests the physical and chemical properties of the cell membranes are disturbed by the attaching Ag nanoparticles. This interaction results in important cellular functions such as permeability and respiration being disrupted. Further damaged is inflicted on bacterial cells by invading Ag nanoparticles interacting with constituents such as DNA and proteins.\(^{35,37}\)

CONCLUSION

Ag nanoparticles were produced by the interaction of AgNO\(_3\) with an aqueous solution containing *Xanthorrhoea glauca* leaf extract. The green chemistry based method is simple and straightforward and was conducted at room temperature. The leaf extract was found to act as both reducing agent and capping agent. Characterisation studies found the synthesised Ag nanoparticles were crystalline, anisotropic, ranged in size from 50 nm up to 200 nm and had a wide range of shapes such as bars, cubes, truncated triangular and hexagonal plates.

The Ag nanoparticles demonstrated varying degrees of antibacterial activity against *Escherichia coli* and *Staphylococcus Epidermis*, with *Staphylococcus epidermis* showing a larger zone of inhibition (11 mm). The leaf extract itself showed no antibacterial properties so no synergistic effect was seen in the samples. This green chemistry based procedure is cost-effective, non-toxic and an eco-friendly alternative to conventional physical and chemical processing methods. The procedure used in this study has the potential for large- scale room temperature production of Ag nanoparticles that could be incorporated into antimicrobial based pharmaceutical products.

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Research Article

Biogenic synthesis of silver nanoparticles via indigenous Anigozanthos manglesii, (red and green kangaroo paw) leaf extract and its potential antibacterial activity

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ABSTRACT

Background: Metallic silver nanoparticles with antibacterial properties were biosynthesised for the first time using an indigenous Australian plant Anigozanthos manglesii.

Methods: A practical, straight-forward and eco-friendly technique used the Anigozanthos manglesii leaf extract, which acted as both reducing and capping agents to create stable silver nanoparticles. The antibacterial activities of the nanoparticles were investigated using the Kirby-Bauer sensitivity method.

Results: Characterisation revealed the nanoparticles ranged in size from 50 nm up to 150 nm, and their morphologies included cubes, triangular plates and hexagonal plates. Antibacterial studies revealed Deinococcus was sensitive and susceptible to the biosynthesised nanoparticles. Escherichia coli and Staphylococcus Epidermis strains were also found to be less susceptible to the silver nanoparticles.

Conclusions: The present study has shown that silver nanoparticles biosynthesised using Anigozanthos manglesii leaf extracts have antibacterial activity against Deinococcus, Escherichia coli and Staphylococcus Epidermis bacterial strains

Keywords: Antibacterial, Anigozanthos manglesii, Green chemistry, Silver nanoparticles

INTRODUCTION

Over the last decade research in nanotechnology and biological sciences has converged to create the new field of nano-biotechnology. This new multi-disciplined research field combines the principles of green chemistry, biology and nanotechnology to deliver sustainable, eco-friendly and state-of-the-art solutions for a wide range of biomedical, environmental remediation and optoelectronic applications.

Much of the research in this field has focused on synthesising metal nanoparticles. Since metals, typically gold (Au) and silver (Ag), at the nanometre scale have unique and distinctive physiochemical properties that are directly related to their size, size distribution and morphology. Conventional physical and chemical methods of producing metal nanoparticles have a number of inherent limitations such as cytotoxicity, carcinogenicity and environmental toxicity that result from the use of toxic chemicals and solvents during manufacture. Understandably, because of toxicity concerns research has focused on developing alternative manufacturing protocols that are clean, non-toxic, and eco-friendly to implement.

However, developing effective eco-friendly methods for producing nanoparticles with a specific size, shape,
composition, desirable surface chemistry and yield still remains a challenge. However, many biological entities offer a viable alternative green chemistry based procedure for producing non-toxic and eco-friendly nanoparticles.

Literature articles have reported the synthesis of a variety of nanoparticles via biological entities such as actinomycetes, bacteria, fungus, plants, viruses and yeasts. These articles have shown that biological entities can act as factories capable of producing nanoparticles in a nontoxic and eco-friendly manner. Among biological entities, plant-derived extracts offer an eco-friendly source of active biomolecules. Studies have shown biomolecules present in plant extracts can produce stable metal nanoparticles with a wide range of sizes, shapes, compositions and physicochemical properties. In particular, nanoparticles synthesised via plant extracts are formed much quicker and are generally more stable than those produced by microorganisms.

Ag nanoparticle synthesis

The influence of varying concentrations of leaf extract on Ag nanoparticle synthesis at room temperature (24°C) was investigated by adjusting the amount of leaf extract (1 mL for s1, 2 mL for s2 and 3 mL for s3). The procedure involved a fixed amount of processed leaf extract being mixed via stirring with a 1.0 mL solution of 0.1M AgNO3 for 1 minute. After combining the mixture was allowed to stand while silver reduction took place. Control nanoparticles were formed by first stirring a 1.0 mL solution of 1 mM AgNO3 to a 10 mL solution of MQ water. This was followed by adding a 1.0 mL solution of 1mM sodium citrate (stabilizing and capping agent) while stirring. Reduction of Ag nanoparticles was initiated by stirring a 1.0 mL solution of 0.01 M sodium borohydride to the mixture at room temperature (24°C). After homogenization initial filtration was carried out using a Hirsch funnel to remove leaf debris. This was followed by two further filtrations using two separate 0.22 µm Millex® (33 mm Dia.) syringe filters. The processed leaf extract was then stored in glass vials ready for use.

Advanced characterisation

UV–visible spectroscopy (Varian Cary 50 series UV-Visible spectrophotometer V3) was used to monitor nanoparticle formation and record the maximum surface plasma resonance over the 200 to 800 nm spectral range. The crystalline structure of the synthesised Ag nanoparticles were examined using a Bruker D8 series diffractometer with flat plane geometry operating at 40 kV and 30 mA [Cu Kα = 1.5406 Å radiation source]. While the diffraction pattern was collected over a 20 range starting at 15° and finishing at 80°.

A JEOL JCM-6000, NeoScopeTM scanning electron microscope (SEM) was used to ascertain particle size and morphology. The energy dispersive spectroscopy (EDS) attachment was used to identify the presence of Ag and other elements in the samples. All samples were mounted on holders using carbon adhesive tape before being coated with a 2 nm layer of gold via a sputter coater (Cressington 208HR) to prevent charge build up.

For transmission electron microscopy (TEM), a single sample drop was pipetted onto a carbon-coated grid and then allowed to dry at room temperature. Micrographs were taken using a Phillips CM-100 electron microscope.

Anigozanthos manglesii is an indigenous plant found in Western Australia and is more commonly known as the Red and Green Kangaroo Paw. The plant grows annually from an underground rhizome to produce a red stalk that reaches a height of around 1 m as seen in Figure 1 (b). During spring and summer, the stalk is crowned with an array of red and green tubular flowers that open at the apex to form a claw structure that resembles the paw of a kangaroo, hence its name.

This article reports for the first time the synthesis of Ag nanoparticles using a leaf extract derived from the Red and Green Kangaroo Paw plant. The nanoparticles were characterised using UV–visible spectroscopy, transmission electron microscopy (TEM), X-ray diffraction (XRD), scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR) and energy dispersive spectroscopy (EDS). The report also presents a potential medicinal application via an antibacterial study using the Kirby-Bauer sensitivity method. The results of the antibacterial study revealed that Deinococcus was sensitive and susceptible, while both Escherichia coli and Staphylococcus Epidermis were resistant to the synthesised Ag nanoparticles.

METHODS

The source of Ag+ ions came from analytic grade silver nitrate (AgNO3, 99.99%) supplied by Sigma-Aldrich (Castle Hill, NSW, Australia) and was used as supplied. The Milli-Qa water (MQ water) used to make all aqueous solutions was produced by a Ultrapure Water System (D11931 Barnstead, 18.3 MΩ cm-1) supplied by Thermo Scientific. Healthy Anigozanthos manglesii leaves were randomly selected from several plants (Figure 1) located at various sites around the Murdoch University campus. The leaves were thoroughly washed in MQ water before being processed in a standard domestic blender. After blending 50 mL’s of MQ water was slowly added to the blend under the influence of slow stirring. The blend was then homogenised at 5000 rpm for 10 min at room temperature (24°C) using a IKA® T25 Digital Ultra-Turrax® Homogeniser.

After combining the mixture was allowed to stand while silver reduction took place. Control nanoparticles were formed by first stirring a 1.0 mL solution of 1 mM AgNO3 to a 10 mL solution of MQ water. This was followed by adding a 1.0 mL solution of 1mM sodium citrate (stabilizing and capping agent) while stirring. Reduction of Ag nanoparticles was initiated by stirring a 1.0 mL solution of 0.01 M sodium borohydride to the mixture at room temperature (24°C).
operating at 80kV. The major functional groups and vibration modes of the samples were determined by a Perkin-Elmer Frontier FT-IR spectrometer fitted with a universal single bounce diamond (ATR) attachment.

**Antibacterial assay**

The antibacterial activity of the synthesised Ag nanoparticles was investigated using the sensitivity method of Kirby-Bauer. Bacteria used in the antibacterial challenge were Deinococcus, Escherichia coli and Staphylococcus Epidermis. The sub-cultured bacteria were swabbed uniformly over nutrient agar medium coated Petri dishes (Dia. 90) using a sterile cotton swab. Then 50 µL nanoparticle sample solutions were deposited on sterile disks (6 mm Whatman AA 2017-006) using a micropipette. The disks were then allowed to air dry for 20 minutes before being placed on the respective bacteria treated agar plates using sterile forceps. The plates were then incubated at 37 °C for 48 h. Following incubation, the zone of inhibition of the various bacterial strains was measured, compared and the antibacterial performance of the Ag nanoparticles appraised.

**RESULTS**

A Biogenic synthesis of Ag nanoparticles occurred within 20 minutes, with the results of the reaction clearly visible. Initially, the pale green coloured mixture containing leaf extract and AgNO₃ progressively turned dark brown as seen in a representative sample s2 in Figure 1 (a) insert. Figure 1 (a) also presents a representative UV-Visible spectra for an s2 sample showing an optical Surface Plasmon Resonance (SPR) peak at 435 nm. Studies reported in the literature indicate the resulting brown colour is typical and shows the formation of metallic Ag nanoparticles (Figure 1).

Figure 2 (a) and (b) representative high-resolution TEM images of reduced Ag nanoparticles and (c) XRD analysis of a typical sample containing Ag nanoparticles showing the crystalline phases present.

Figure 3 (a) Representative SEM micrograph showing an array of various sized Ag nanoparticles, insert (b) showing higher magnification image highlighting the various polygonal nanoparticle shapes present and (c) FT-IR spectroscopy analysis of representative samples of a pure leaf extract sample and a leaf extract/AgNO₃ reaction mixture.

The FT-IR spectra of pure leaf extract and bio-reduced Ag nanoparticles from leaf extract and AgNO₃ are presented in Figure 3 (c). The pure leaf extract consists of only two adsorption bands located at 1635 and 3328.
cm⁻¹, while the leaf broth mixture contained a further seven adsorption bands located at 1031, 1082, 1231, 1318, 1374, 1533 and 1639 cm⁻¹ (Figure 3).

Potential antibacterial properties of Ag nanoparticles were studied using the sensitivity method of Kirby-Bauer. The results of the antimicrobial study revealed a null result for the leaf extract. But, the biologically synthesised Ag nanoparticles when challenged by the three bacterial strains produced zones of inhibition, which were measured. Deinococcus recorded a mean inhibition zone of 14 mm, Escherichia coli a mean value of 9 mm and Staphylococcus Epidermis a mean value of 8 mm. A typical antibacterial test petri dish is presented in Figure 4 (b) and representative results of the antibacterial challenge are summarised and presented graphically in Figure 4 (c) (Figure 4).

The XRD results were found to be consistent with nanoparticle seen in TEM micrographs presented in Figure 2 and are consistent with similar reports for biologically synthesised Ag nanoparticles.26-27 Furthermore, a much larger landscape SEM image of synthesised Ag nanoparticles presented in Figure 3 (a) and corroborates the presence cubic, triangular and hexagonal shaped particles seen in the TEM micrographs. The enlarged SEM image presented in Figure 3 (b) confirms that the biologically synthesised nanoparticles are smaller than 200 nm, with many of them smaller than 100 nm.

Analysis of the FT-IR spectra revealed that the 1031, 1318, and 1639 cm⁻¹ bands were characteristically associated with the biological reduction of Ag ions. The strong and broad band located at 3328 cm⁻¹ in the samples results from bounded hydroxyl (-OH) or amine (-NH) groups present in the bio-macromolecules in the leaf extract. The weak band located at 1639 cm⁻¹ and the much stronger band located at 1533 cm⁻¹ indicates the presence of amide I and amide II, and arise from carbonyl stretching and -N-H stretching vibrations respectively. While the adsorption band located at 1318 cm⁻¹ corresponds to C≡C stretching of aromatic amines. As a final characterisation, EDS analysis confirmed the formation of Ag nanoparticles in the leaf broth mixture as seen in Figure 4 (a) by the presence of a strong elemental signal for Ag.

**DISCUSSION**

Biogenic synthesis of Ag nanoparticles turned the reaction fluid dark brown in colour and produced a (SPR) peak at 435 nm. Studies in the literature have reported modelling based on Mie’s theory of the absorption spectra tends to produce a single intense SPR peak for spherical metal nanoparticles. The broad peak located at 435 nm seen in absorption spectra presented in Figure 1 (a) suggests the synthesised Ag nanoparticles have an anisotropic nature.

The anisotropic nature was confirmed by TEM images that revealed the presence of nanoparticles with different sizes and shapes. Figures 2 (a) and 2 (b) presented representative micrographs taken after a 20 minute synthesis period. Examination of the images revealed the nanoparticles ranged in size from 50 nm up to around 150 nm. The shapes seen in the micrographs included cubes, triangular plates and hexagonal plates.

Figure 4 (a) EDS elemental analysis showing the strong presence of metallic Ag, (b) A representative petri dish sample of *Staphylococcus epidermis* being challenged by leaf extract mediated Ag nanoparticles and (c) Graphical Summary of antibacterial challenge test results for the three bacterial strains.

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The anisotropic nature was confirmed by TEM images that revealed the presence of nanoparticles with different sizes and shapes. Figures 2 (a) and 2 (b) presented representative micrographs taken after a 20 minute synthesis period. Examination of the images revealed the nanoparticles ranged in size from 50 nm up to around 150 nm. The shapes seen in the micrographs included cubes, triangular plates and hexagonal plates.

Figure 4 (a) EDS elemental analysis showing the strong presence of metallic Ag, (b) A representative petri dish sample of *Staphylococcus epidermis* being challenged by leaf extract mediated Ag nanoparticles and (c) Graphical Summary of antibacterial challenge test results for the three bacterial strains.
sterile pad treated with chemically synthesised Ag nanoparticles (control) were challenged by each respective bacterial strain and each zone of inhibition was recorded.

The challenge recorded a zone of inhibition of 16 mm for *Deinococcus*, 9 mm for *Escherichia coli* and 10 mm for *Staphylococcus Epidermis*. Finally, biologically synthesised Ag nanoparticles were challenged by the respective bacterial strains and zones of inhibition were measured. *Deinococcus* recorded a mean inhibition zone of 14 mm, *Escherichia coli* a mean value of 9 mm and *Staphylococcus Epidermis* a mean value of 8 mm. A representative antibacterial test petri dish is presented in Figure 4 (b) and the results of the antibacterial challenge are summarised and presented graphically in Figure 4 (c).

Inspection of Figure 4 (c) reveals the zones of inhibition for all disks treated with Ag nanoparticles produced via leaf extract tend to be slightly smaller or equal to the control treatment. *Deinococcus* was found to be sensitive and susceptible to Ag nanoparticles produced by both synthesis routes. While both *Escherichia coli* and *Staphylococcus Epidermis* were found to be resistant to Ag nanoparticles produced by both synthesis routes.

Differences in the size of the zones of inhibition between the two Ag nanoparticle treatments is believed to result from surface attachment of biomolecules present in the leaf extract. During biological synthesis, biomolecules present in the leaf extract act initially to reduce the AgNO₃ to form metallic Ag nanoparticles. Following formation, other biomolecules present in the leaf extract act as a capping agent to stabilise the nanoparticles and prevent particle agglomeration. The presence of biomolecules coating the surface of Ag nanoparticles appears to moderate their antibacterial properties and reduce their effectiveness against the bacterial strains studied as seen in Figure 4 (c).

The types of biomolecules involved in first reducing and then capping the formed Ag nanoparticles is currently under investigation. Also currently under investigation is the influence of capping biomolecules, coating structure on Ag nanoparticles and their interaction with microorganisms. The results of these studies will be published in a future article.

**CONCLUSION**

The present work has shown that a leaf extract taken from *Anigozanthos manglesii* (Red and Green Kangaroo Paw) was capable of synthesising stable Ag nanoparticles. The room temperature and eco-friendly process followed the principles of green chemistry. Nanoparticles ranged in size from 50 nm up to around 150 nm and produced cubic, triangular and hexagonal plate morphologies.

While UV-Visible spectroscopy revealed a SPR peak at 435 nm and EDS analysis confirmed the presence of a strong elemental signal for Ag in the samples. Subsequent XRD pattern analysis indicated a face centred cubic structure was present in the formed Ag nanoparticles and peak broadening confirmed nanoparticle formation.

The room temperature synthesis process confirmed that biomolecules present in the leaf extract acted as both reducing agent and stabilising agent. Antibacterial studies using the sensitivity method of Kirby-Bauer revealed that *Deinococcus* was sensitive, while both *Escherichia coli* and *Staphylococcus Epidermis* were found to be resistant to the synthesised Ag nanoparticles.

However, further studies are needed to confirm the medicinal properties of the leaf extract mediated Ag nanoparticles, the influence of biomolecules in forming the nanoparticles and their subsequent interaction with various microorganisms.

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**REFERENCES**


Chapter 5 - General Discussions, Conclusions and Future Work

In recent years there has been a worldwide search into alternative efficient, eco-friendly green chemistry based methods for producing metallic nanoparticles. These unique nanoparticles and nanostructures offer a variety of materials that can be exploited for a wide variety of unique applications. Conventional physical and chemical manufacturing processes have a number of inherent limitations such as cytotoxicity, carcinogenicity and environmental toxicity [1]. These limitations result from the use of toxic chemicals and solvents being used during the manufacturing process [2]. The toxicity arises from the inability to completely remove all chemicals and solvents from the nanoparticle surface after manufacture [3]. Toxicity issues have been the main driver for the search for new alternative manufacturing protocols. These new protocols must be straightforward, clean, non-toxic, and eco-friendly. Implementing these new alternative protocols will ultimately lead to manufacturing processes that can produce metal nanoparticles suitable for both biomedical and pharmaceutical applications. Recent studies have shown that many biological entities actinomycetes, bacteria, fungus, plants, viruses and yeasts offer a viable alternative green chemistry based procedure for producing non-toxic and eco-friendly nanoparticles [2, 4, 5]. The advantages of using plants for nanoparticle synthesis, besides being eco-friendly, include relatively short production times and much have lower cultivation costs compared to other biological entities such as bacteria and yeasts [5, 6]. However, finding suitable plants and developing effective protocols to produce metal nanoparticles with predetermined physiochemical properties still remains a challenge. Hence, the need for the present research work that has clearly demonstrated an effective alternative eco-friendly process for manufacturing noble metal nanoparticles using three indigenous Australian plants

5.1. Research Results and Achievements

Research work embarked on during this thesis project was designed to develop alternative eco-friendly green chemistry based methods for producing Ag and Au nanoparticles via indigenous Australian plant species. The research undertaken in this
thesis is at the forefront of the rapidly developing field of biogenic synthesis of nanoparticles via plant extracts. For the first time extracts taken from three indigenous Australian plant species were found to have the necessary chemical properties to synthesis Ag and Au nanoparticles. During the discovery stage, the three indigenous Australian plant species were identified and examined. The plant species Eucalyptus macrocarpa (rose of the west), Xanthorrhoea glauca (grass tree) and Anigozanthos manglesii (red and green kangaroo paw). The thrust of the research was centred on three aims: 1) discovery; 2) synthesis optimisation & particle characterisation, and 3) identification of particle/plant antimicrobial properties. Each aim was addressed by a number of individual case studies that permitted a detailed investigation into the various aspects of nanoparticle synthesis, nanoparticle physiochemical properties and nanoparticle/plant antimicrobial properties.

Chapter two’s focus was to review the literature in the field and accordingly contains two review articles. The first review article entitled “Green Synthesis of Metallic Nanoparticles via Biological Entities” was a comprehensive literature review of the current state of research in the field. The review discusses the convergence between nanotechnology and biology to create the new field of nanobiotechnology. With the main thrust of the review presenting recent trends in synthesizing nanoparticles via biological entities and their potential applications. Biological entities discussed were actinomycetes algae, bacteria, fungi, viruses, yeasts, and plants. The experimental parameters that governed the performance of the biochemical and biophysical processes used during nanoparticle synthesis were discussed at length. The advantages of using plant extracts was particularly emphasised since it had a direct bearing on the present studies. Furthermore, during the literature searches it was found that there were relatively few research articles reporting the biogenic synthesis of nanoparticles using marine plants. Importantly, there was a distinct absence of any reviews in the literature summarising the results of the reported nanoparticle/marine plant studies. To rectify this gap in knowledge a second review article was prepared from currently available studies reported in the literature. The second review focuses on two groups of marine plants, namely marine algae (commonly known as seaweeds) and sea grasses. In particular, seaweeds are a rich source of biologically active compounds such as carbohydrates, carotenoids, polysaccharides, proteins, vitamins and numerous secondary metabolites [7]. Currently, seaweed is a staple food source and a medicinal remedy commonly used
throughout South-East Asia [8]. Interestingly, contemporary studies have revealed some types of seaweed extracts have anti-inflammatory and inhibitory properties that can assist in the treatment of several medical conditions including the suppression of some cancers [9-11]. Combined with the ability of some seaweeds to biologically synthesis metal nanoparticles and the medicinal properties of seaweeds there is the potential to produce biocompatible nanoparticles with enhanced medicinal properties derived from seaweed extracts. It is believed that the importance of the second review article will come from informing the scientific community of this largely unexplored area of study and promote further research in this very promising field.

Chapter Three is made up of three cases studies, namely, Case Study One, Two and Three. Each Case Study explores the biogenic potential of a small selection of indigenous Australian plants to produce two noble metal nanoparticles. Research carried out in Chapter Three addresses the first two aims of the thesis, namely, 1) discovery and 2) synthesis optimisation & particle characterisation. The three indigenous plants selected for these studies were *Eucalyptus macrocarpa* (rose of the west), *Xanthorrhoea glauca* (grass tree), and *Anigozanthos manglesii* (red and green kangaroo paw). The two types of noble metal nanoparticles synthesised in these exploratory studies were Au and Ag. Importantly, both noble metal nanoparticles have been synthesised by various techniques have also been extensively reported in the literature. Thus, allowing comparisons to be made between biologically synthesised nanoparticles and nanoparticles synthesised by more conventional physiochemical methods.

Case Study one extends the research initially started by the Murdoch Applied Nanotechnology Research Group into plant mediated synthesis of Au nanoparticles using *Eucalyptus macrocarpa*. The earlier work had established that *Eucalyptus macrocarpa* leaf extracts could synthesis Au nanoparticles with a variety of shapes such as hexagonal, triangular and truncated triangular shaped plates. The bulk of the current work carried out in the first Case Study was to expand on the earlier work, improving the earlier synthesis techniques and optimising the experimental parameters. After optimisation it was possible for the first time to synthesise Ag nanometre scale cubes using leaf extracts taken from *Eucalyptus macrocarpa*. The refined optimisation procedures developed in Case Study One were applied in Case Study Two to confirm and further explore the Au forming capabilities of *Eucalyptus macrocarpa*. The results
of the second case study established the viability of the techniques used and confirmed the effectiveness of the optimised experimental procedures. The suitability of the optimised experimental procedure was also found to be applicable for the synthesis of other metal and metal oxide nanoparticles such as copper (Cu), copper oxide (CuO, Cu₂O) and zinc oxide (ZnO). This aspect of the research is discussed in more detail in the following future work section.

Case Study Three was an in depth microscopy study of the structure of the *Xanthorrhoea glauca* structure and the identification of possible synthesis sites within the leaf structure for producing Ag nanoparticles. A typical optical image of a transverse leaf section reveals an overall square shaped leaf with waxy cuticle and epidermis layers covering a relative thin layer of palisade mesophyll cells. This outer layer contains a large interior composed of spongy mesophyll cells surrounding an array of vascular bundles. To study the *in-situ* synthesis of Ag nanoparticles, leaf samples were dipped into AgNO₃ solutions and with time the solutions were drawn into the vascular bundles. Traveling up through the leaf and spreading out from the vascular bundles AgNO₃ came in contact with surrounding cell membranes. During this AgNO₃ solution and cell membrane interaction, the AgNO₃ was reduced to form Ag nanoparticles. The importance of this study is that it clearly identifies the cell membranes as the main instigator in synthesising Ag nanoparticles. Bioactive compounds such as phospholipids, sterols, proteins and carbohydrates interspersed within the cell membranes are believed to be involved in first reducing and then stabilising the Ag nanoparticles. Reduction of AgNO₃ resulted in nanoparticles with shapes such as cubes, truncated triangular and hexagonal plates. While the nanoparticle size ranged from 50 nm to 200 nm. The study also highlighted the need for further research into determining to the identity of individual bioactive compounds directly involved with reducing AgNO₃ and those involved with forming stable Ag nanoparticles. This further work is discussed at length in the following section.

Chapter four focused on studying the antimicrobial properties of Ag nanoparticles synthesised by *Xanthorrhoea glauca* and *Anigozanthos manglesii* leaf extracts. Two detailed Case Studies were carried out and each study focused specifically on one particular plant species. Case Study Four examined the biosynthesis of Ag nanoparticles using *Xanthorrhoea glauca* leaf extracts and the antibacterial activity of the
nanoparticles against Escherichia coli and Staphylococcus Epidermis. While Case Study
Five investigated the biogenic synthesis of Ag nanoparticles using *Anigozanthos manglesii* (red and green kangaroo paw) leaf extracts and then examined the
antibacterial properties of the nanoparticles against *Deinococcus, Escherichia coli and Staphylococcus Epidermis*.

Ag nanoparticles synthesised by *Xanthorrhoea glauca* were found to have varying
degrees of antibacterial activity against Escherichia coli and Staphylococcus Epidermis. The leaf extracts themselves were found to have no antibacterial activity towards the
two bacterial pathogens. In the case of gram-positive bacteria *Staphylococcus Epidermis*
the presence of Ag nanoparticles produced an 11 mm zone of inhibition and in the case
of gram-negative Escherichia coli an 8 mm inhibition zone was produced. The
interaction between the Ag nanoparticles and both pathogens indicates an antibacterial
property. Similarly, Ag nanoparticles synthesised by *Anigozanthos manglesii* leaf
extracts also displayed varying degrees of antibacterial activity against Escherichia coli
and Staphylococcus Epidermis, and also Deinococcus. The results of this particular
antibacterial study revealed *Deinococcus* was sensitive to Ag nanoparticles displaying a
16 mm inhibition zone, while both Escherichia coli (9 mm) and Staphylococcus
Epidermis (10 mm) were found to be resistant to the Ag nanoparticles. The results of
both Case Studies have clearly shown that Ag nanoparticles synthesised from leaf
extracts of both plant species have antibacterial properties to varying degrees. The
variation in inhibition zones suggests the physical and chemical properties of the cell
membranes are disturbed to varying degrees by the attaching Ag nanoparticles. Like
previous studies looking at the antimicrobial properties of Ag nanoparticles synthesised
by various routes, the present Case Studies have also shown that interactions between
the particles and the membranes results in the disruption of important cellular functions
such as permeability and respiration. The present work also supports the invasive nature
of the Ag nanoparticles entering the bacteria cells and damaging cell components such
as DNA and proteins [12-14].
5.2 Recommendations for Future Work

The research undertaken as part of this thesis was highly successful, (with six published peer-reviewed research papers) and has demonstrated that the leaf extracts from the three-selected indigenous Australian plant species could be used to biologically synthesise both Ag and Au nanoparticles with unique and desirable properties. This particular field is new and largely unexplored and as a result holds many opportunities for exploration and discovery. In particular, the second review article testifies to how little research has been done in the field of nanoparticle synthesis via marine plants. Similarly, research into using indigenous Australian plant species is relatively new and this thesis is believed to be the first of its kind in this new and emerging field. Accordingly, the scope for future work is large since very few indigenous Australian plant species have been studied for their biosynthesis capabilities in terms of producing nanoparticles. For example, the present work has only examined three plant species and only two noble metal nanoparticles.

Focusing on the present work, only three indigenous Australian plant species have been investigated for their biogenic synthesis capabilities. In each case, the respective leaf extracts taken from each plant species have proven to be capable of first reducing the respective metal salt and then acting as a capping agent to prevent nanoparticle aggregation in the aqueous mixtures. Generally, FTIR analysis performed on the respective leaf extract samples before the reduction of AgNO$_3$ reveals the presence of a number of O-H functional groups. After reduction, the presence of amide I and amide II resulting from carbonyl stretching and –N-H stretching in the amide linkages in the kproteins appear in the FTIR spectrum. Also present in the spectrum was C=C stretching of aromatic amines and O-H functional groups. All these groups are from phytochemicals present in the leaf extracts and are involved in the biogenic synthesis of Ag nanoparticles [32]. However, what needs to be done in the near future is to identify the various individual phytochemicals present in the leaf extracts. To date, no phytochemical studies of the three indigenous Australian plant species have been reported in the literature. This study would be of particular importance since it would make possible the identification phytochemical and active biomolecules involved in
first reducing the metal salt, templating the growth of the nanoparticle and those involved in capping the newly formed nanoparticles.

Further studies are needed to explore the wide range of indigenous Australian plant species both terrestrial and marine. Both published review articles have highlighted the need for much more exploration, since there are numerous candidates there have not been investigated. On another front, Ag and Au nanoparticle synthesis by other various plant based routes have been extensively reported. However, there are relatively few articles in the literature reporting the biosynthesis of other noble metals, non-noble metals and metal oxides. In this regard, there is plenty of scope to investigate the synthesis of other types of nanoparticles. In the case of terrestrial plants the literature reports the synthesis of: 1) Copper and Copper Oxide Nanoparticles; 2) Palladium and Platinum Nanoparticles; 3) Titanium Dioxide and Zinc Oxide Nanoparticles, and 5) Indium Oxide, Iron Oxide, Lead, and Selenium Nanoparticles by a number of plant species [2]. And in the case of marine plants, the number of species studied and types of nanoparticles biosynthesised is relatively small. This is also the case for indigenous Australian plant species, with the present work being the first of its kind. Thus, further work is planned that will use the plant species identified in this thesis and other species of interest to biosynthesise other noble metals, non-noble metals and metal oxides. Preliminary investigations towards this future work has already revealed that Eucalyptus *macrocarpa* has the potential to biosynthesise metal and metal oxide nanoparticles. These exploratory studies have revealed that copper (Cu), copper oxide (CuO, Cu$_2$O) and zinc oxide (ZnO) nanoparticles can be produced. Thus, directing the future pathway for a more comprehensive study that explores a wide range metals and metal oxides. This future work could also examine the potential of both *Xanthorrhoea glauca* and *Anigozanthos manglesii* towards producing metal and metal oxide nanoparticles. The future work could then be broadened to encompass a wider range of terrestrial and marine indigenous Australian plant species.

Future work will also need to take into account that only a relatively few practical applications have used nanoparticles synthesised from plants. So future work will need to find an effective translational approach that can produce nanoparticles for specific practical applications for this alternative environment-friendly process to be widely
accepted. In recent years Ag nanoparticles have attracted considerable interest due to their antimicrobial properties towards a wide range of pathogens and as a result they have been incorporated into several commercially available medical and consumer products [15, 16]. While Cu and CuO nanoparticles have also been identified as antimicrobial agents and can be used as bactericide coatings on hospital equipment [17, 18]. Moreover, Au nanoparticles can be used as antibacterial agents against a number of bacterial strains [19] and can be used as carrier platforms for the targeted delivery of anticancer drugs [20]. However, before eco-friendly biogenic synthesis can become a viable alternative to traditional nanoparticle manufacturing processes and produce nanoparticles suitable for the above mentioned applications a number of factors need to be tackled. Typically, the readily identifiable factors include particle size control, shape, and size distribution. Since even small variations in these factors can significantly influence the properties of the nanoparticles. Importantly, all these factors are directly influenced by reaction medium pH, reactant moieties, reactant concentrations, reaction time, and temperature. Moreover, there are also noticeable variations in the chemical composition of extracts taken from particular plant species at different times of the year and at different geographical locations. Accordingly, future research is needed to resolve these variations and inconsistencies currently associated with the biogenic synthesis of nanoparticles via plant extracts. Once these factors have been addressed then scale-up of the process can take place to produce economically sustainable amounts of tailored nanoparticles suitable for pharmaceutical and medical applications.

References


Appendix

Overview and author contributions

Apart from my research work of synthesis of nanoparticles using Australian indigenous plants, as a part of my initial training at Murdoch applied nanotechnology research group I have helped with the carbon nanospheres project. I have worked as one of the team members in this project. Nanoparticles of functionalized carbon nanospheres (CNS) have the potential to improve the photothermal properties of the working fluid. CNS was produced by the pyrolysis of acetylene gas in a tube-based electric furnace/chemical vapor deposition apparatus. The reaction takes place at 1000°C in the presence of nitrogen gas without the use of a catalyst. The synthesized CNS were examined and characterized using field-emission scanning electron microscopy, transmission electron microscopy, X-ray diffraction spectroscopy, Raman spectroscopy, thermal gravimetric analysis, and ultraviolet-visible analysis.

The author contributions consisted of G.E.J. Poinern acting as principal supervisor who designed the overall concept for the paper with S. Brundavanam being the major contributor to the papers. All text and images were carried out by S. Brundavanam under the supervision of D. Fawcett. S. Brundavanam was assisted by G.E.J. Poinern, D. Fawcett, M. Shah, L. Laava in overcoming some of the various technical difficulties encountered during the paper preparation and with the editorial changes to the manuscript as recommended by reviewers. All authors provided feedback during the preparation of the paper which was coordinated by S. Brundavanam.
Published Research Articles

Photothermal response of CVD synthesized carbon (nano)spheres/aqueous nanofluids for potential application in direct solar absorption collectors: a preliminary investigation

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Abstract: Direct-absorption solar collectors have the potential to offer an unlimited source of renewable energy with minimal environmental impact. Unfortunately, their performance is limited by the absorption efficiency of the working fluid. Nanoparticles of functionalized carbon nanospheres (CNS) have the potential to improve the photothermal properties of the working fluid. CNS are produced by the pyrolysis of acetylene gas in a tube-based electric furnace/chemical vapor deposition apparatus. The reaction takes place at 1000°C in the presence of nitrogen gas without the use of a catalyst. The synthesized CNS were examined and characterized using field-emission scanning electron microscopy, transmission electron microscopy, X-ray diffraction spectroscopy, Raman spectroscopy, thermal gravimetric analysis, and ultraviolet-visible analysis. The CNS powders with a mean particle size of 210 nm were then functionalized using tetraethylammonium hydroxide ([C2H5]4NH[OH]) and used to produce a series of aqueous nanofluids with varying mass content. The photothermal response of both the nanofluids and films composed of CNS were investigated under 1000 W/m² solar irradiation.

Keywords: solar absorption, carbon nanospheres, nanofluids, photothermal

Introduction

The development of new and efficient energy technologies that can deliver environmentally friendly, practical, and economically sustainable sources of energy is an important factor in tackling global warming and reducing carbon dioxide emissions. Sunlight falling on Earth offers a solution, since the hourly solar flux incident on Earth’s surface is greater than the annual human consumption of energy in a year. It is also the largest source of renewable energy, and has been collected, concentrated, and converted into usable forms of energy. However, the major obstacles in optimizing the use of this renewable energy lie in efficiently collecting and converting it into other useful forms of energy. One of the most common methods of collection is through solar thermal collectors, which vary in design for collecting solar radiation and efficiently converting the energy. A typical solar thermal collector consists of a black absorber surface, usually plates or tubes that are coated with a spectrally selective material that enhances the absorption of solar energy.

The absorber then transfers heat to a working fluid or heat-transfer fluid flowing in tubes encased or attached to the absorber. There are clearly significant advantages to using this type of solar energy collection system: it is potentially 100% renewable, produces no emissions, and greatly reduces the cost of heating the working fluid.
However, the efficiency of conventional solar thermal collectors is not only dependent on how effectively the absorber can capture solar energy but also on how effectively heat can be transferred to the working fluid and heat losses from the collector. It is therefore not surprising that there is considerable interest in improving solar energy collection efficiency, which will in turn produce greater heating efficiencies. To significantly reduce the shortcomings of the conventional solar thermal collector, an alternative collector design was proposed in the 1970s. The so-called direct-absorption solar collector has the potential to directly absorb solar energy within the working fluid volume and significantly enhance the heating efficiency of the collector. For high-flux applications, the collector consists of a closed-loop heat-exchange circuit that separates the heat-transfer fluid from the potable water circuit. Initially, the solar energy is directly absorbed by working fluid in the absorber panels; the heated working fluid then flows through the piping circuit to the heat exchanger, where it transfers its heat to the potable water circuit. Typical examples of traditional working or heat-transfer fluids such as water, ethylene glycol, water/ethylene glycol mixtures, and oils have been shown to have extremely low absorptive properties over the solar spectrum (0.25 to 2.5 μm). Therefore, for the direct solar absorption collector to operate at its optimal efficiency, its working fluid must have superior absorption properties over the solar spectrum. Hence the need for seeding the working fluid with suitable particles that can enhance the absorption properties of the base fluid. Research in the late twentieth century focused on developing black liquids that contained small particle sizes ranging from the millimeter scale down to the micrometer scale that could be used to enhance the absorptive properties of the working fluid. Despite having good absorption properties, many of these particle-seeded fluids or microfluids were abrasive and suffered from particle precipitation and sedimentation, which tended to block tubes, valves, and pumps in the collector piping system. Recent developments in nano-technology have seen the discovery of many new materials with novel properties that are significantly different from their bulk form. The inclusion of nanoparticles in liquids to form stable suspensions has also been investigated, since nanofluids have the potential to significantly improve a number of fluid properties, such as thermal conductivity, heat transfer and transport properties, optical properties, and viscosity. In addition, several researchers have reported that nanofluids have the potential to become an effective working fluid in direct solar thermal collectors. For example, a study by Tyagi et al revealed that a nanofluid composed of aluminum nanoparticles and pure water had an increased absorption capacity of 10% compared to a pure-water working fluid in a convention flat-plate solar collector. In a similar study, Han et al were able to demonstrate that nanofluids composed of carbon black particles displayed significant temperature- increase enhancements compared to pure water. As early as 1985, the discovery of buckminsterfullerenes, or buckyballs, heralded a renewed interest in new forms of carbonaceous materials. These new carbon nanostructured materials from the C family consist of nanotubes (CNTs), nanofilaments, nanocapsules, and the recently discovered carbon nanospheres (CNS). CNTs are the blackest materials discovered to date, with excellent thermal and electrical conductivities, which makes them highly desirable for a number of applications, such as particle additives to the working fluids of direct solar absorption collectors. The nano-sphere form of carbon has been synthesized and studied by a number of research groups worldwide due to its novel properties and potential applications. For example, the luminescent and fluorescent properties of carbon quantum dots are being explored as a low-toxicity alternative to metal-based quantum dots. Serp et al have classified carbon spheres into three categories: the first contains the C family and the well graphitized onion-like structures that have diameters in the range of 2–20 nm; the second contains the less graphitized nanosized spheres that range in diameter from 50 nm to 1 μm; and the final category contains the carbon beads that range in diameter from 1 to several μm. The properties of CNS are similar to those of graphite or fullerene. Some of these properties include high temperature stability, a large packing density, and excellent electrical conductivity. These properties make CNS an attractive material for a variety of potential applications, such as lubricating materials, energy-storage devices, supercapacitors, catalyst supports, superconductivity, and special rubber additives. Recent studies by Wang et al have demonstrated the possible application of carbon-based graphene nanostructures in computer hardware and nanoelectronic devices. The advantage of replacing copper and other metallic interconnections in micro- and nanoelectronic devices with graphene sheets and carbonaceous nanomaterials is that it will increase performance and overcome the large failure rate in metallic interconnections that has resulted from the trend in miniaturization. This paper investigates the potential use of CNS in an aqueous-based nanofluid that has the potential to be used as the working fluid in a direct solar absorption collector. Many techniques have been used to produce CNS to date: the carbonization of pitch, arc discharge, the pyrolysis
of hydrocarbons such as styrene, toluene, benzene, hexane, and ethene,\(^\text{36}\) chemical vapor deposition (CVD),\(^\text{38,37}\) the use of micelles,\(^\text{38}\) and ultrasonic processing.\(^\text{39}\) The CVD technique is considered to be one of the most effective processes available to produce CNS. In 1996, using a CVD equipped with a catalyst bed composed of mixed- valent oxides and rare earth metals, with methane gas as the carbon source and operating with a reaction temperature of 1100°C, Wang and Kang were able to produce monodispersed CNS.\(^\text{40}\) The composite oxide catalyst played a key role in releasing oxygen, which in turn reacted with hydrogen atoms of the methane molecule. The net result of this reaction process was the production of carbon nanostructures. Using a similar technique to Wang and Kang,\(^\text{40}\) but using an iron catalyst, Serp et al\(^\text{38}\) were able to produce CNS with a size range starting from 10 nm up to a maximum of 300 nm in diameter. Also using a CVD experimental setup operating with reaction temperature range of 650°C–850°C, with a catalyst bed of transitional metal salts supported by kaolin, Miao et al were able to produce CNS and carbon beads ranging in size from 400 nm to 2 \(\mu\)m in diameter.\(^\text{41}\) CVD techniques that produce CNS without the use of catalysts have also been investigated by several researchers. The pyrolysis of both methane and benzene in a hydrogen atmosphere was investigated by Govindaraj et al for the production of CNS.\(^\text{42}\) In addition, Qian et al devised a noncatalyzed CVD process that used toluene as the carbon source for producing CNS that ranged in size from 60 nm to 1 \(\mu\)m in diameter.\(^\text{37}\) Furthermore, Jin et al have reported the use of a noncatalyzed process for producing CNS that used the direct pyrolysis of a hydrocarbon.\(^\text{36}\) The range of hydrocarbons investigated included benzene, ethane, hexane, styrene, and toluene. The size of the CNS produced ranged from 50 nm to 1 \(\mu\)m in diameter.\(^\text{36}\)

This study is composed of two parts. In the first part, CNS were synthesized using a single-step CVD process that was capable of producing high yields of CNS without the use of a catalyst bed. In the second part, a two-step method of producing CNS-based nanofluids was adopted. In the first part, the process involved the direct pyrolysis of acetylene gas at a reaction temperature of 1000°C. The use of acetylene as the carbon feedstock made the process more economical than using conventional carbon sources such as hexane and toluene. After pyrolysis, the CNS powder was collected from the deposition sites in the CVD chamber or over water in the collecting chamber, and then examined and characterized using six advanced analysis techniques. These included field-emission scanning electron microscopy (FESEM), transmission electron microscopy (TEM), Raman spectroscopy, X-ray diffraction spectroscopy (XRD), thermogravimetric analysis (TGA), and ultraviolet (UV)-visible analysis. After characterization, the CNS powders were then functionalized using tetraethylammonium hydroxide (TEAH). This was necessary because untreated CNS are extremely hydrophobic, and a reactant was needed to attain suspension stability of the nanofluid.

The second part investigates the photothermal response of five CNS-based nanofluids and a CNS base film when exposed to solar irradiation. The CNS film was also examined for its electrical conductivity.

**Materials and methods**

**Chemicals**

All chemicals used were of chemical-grade purity, purchased from Sigma-Aldrich (Castle Hill, NSW, Australia) and used without further purification. Milli-Q water (Barnstead Ultrapure Water System D11931; Thermo Scientific, Dubuque, IA) (18.3 MΩ cm) was used throughout all synthesis procedures involving aqueous solutions. The reactant/surfactant used in the preparation of the CNS was TEAH ([C\(_2\)H\(_5\)]\(_4\)N[OH]).

**Synthesis of carbon nanospheres**

The pyrolysis of instrument-grade acetylene (C\(_2\)H\(_2\)) gas in the presence of high-purity nitrogen (N\(_2\)) was carried out using a CVD apparatus to synthesize carbon CNS. The CVD setup consisted of a simple electrical furnace equipped with a horizontal quartz tube that was 3.8 cm in diameter and 90 cm in length (Figure 1A). Once the furnace temperature was stabilized at 1000°C, the gases were introduced into the quartz tube of the CVD. The flow rates of both gases were monitored using flow meters (Dwyer Instruments, Michigan City, IN); the acetylene flow rate was 400 standard cubic centimetres per minute (sccm), while the nitrogen flow rate was 1200 sccm. During the pyrolysis process, no metallic catalysts were used, and nitrogen was used as a buffer gas. When both gases entered the quartz tube, the pyrolysis of acetylene immediately took place, and the formation of carbon soot could easily be seen. The soot formed on the inside wall of the quartz tube and also collected on the surface of Milli-Q water in the carbon-collecting chamber.

The difference in weight of the CVD collecting components resulting from carbon deposition over a set period of time enabled the determination of the mass flow rate during the pyrolysis process. Over a series of 5-minute time intervals, a mean carbon deposition rate was found to be 1.990 g
per 5 minutes or 0.398 g per minute. The mass balance indicated that all of the carbon originally contained in the acetylene was deposited onto the collecting components within the CVD. The hydrogen from the acetylene combined with nitrogen to form ammonia (NH₃). Thus, by mass, 92.3% of the acetylene was deposited as carbon in the form of soot during the pyrolysis process. The collected carbon soot was then investigated and characterized using FESEM, TEM, XRD, Raman spectroscopy, TGA, and UV-visible analysis.

Characterization of carbon nanospheres

The particle size and structural and morphological features of the carbon soot were investigated using both FESEM and TEM. The morphological features of the soot were investigated using a high resolution FESEM (1555 VP-FESEM; Zeiss, Oberkochen, Germany) at 3 kV with a 30-µm aperture operating under a pressure of 1.0–10⁻¹⁰ Torr, (Figure 2). TEM scans were taken using a Philips (Amsterdam, Netherlands) CM100 Twin TEM operating at 1.0–10⁻¹⁰ Torr pressure and a voltage of 80 kV. Images from both techniques were also used to graphically determine the mean CNS diameter.

The XRD spectra were recorded using a Siemens (Berlin, Germany) D500 series diffractometer (Cu Kα = 1.5406 Å radiation source) operating at 40 kV and 30 mA. The diffraction patterns were collected at room temperature over the 2θ range from 20°C to 60°C with an incremental step size of 0.04°. The acquisition time was 2.0 seconds. The Raman spectra of the samples were recorded using a LabRAM model 28 dispersive Raman spectrophotometer (Horiba, Kyoto, Japan) that was equipped with a 2-mW helium–neon (λ = 632 nm) laser. All spectra were collected between 4000 cm⁻¹ and 125 cm⁻¹ using a diffraction grating consisting of 600 lines per millimeter.

TGA was used to investigate the thermal stability of the CNS using an SDT 2960 (TA Instruments, New Castle, DE). Initially, two 8-mg carbon soot samples were prepared; the first sample was used in a high-purity argon atmosphere, and the second sample was used in normal atmospheric air. The TGA procedure determined the start and completion of the oxidation process of each sample and the respective atmosphere in turn as the temperature was increased, (heating rate of 10°C/minute) from room temperature (24°C) to a maximum temperature of 900°C, (see Figure 3). The UV-visible response of the carbon soot was examined by first dissolving a small amount of CNS in ethanol. The mixture was then passed through a 0.22 µm Whatman Millipore syringe filter, and the resulting filtrate was examined using a Varian (Palo Alto, CA) Cary 50 series UV-visible spectrophotometer version 3, over a spectral range of 200–800 nm, with a 1-nm resolution over 1 hour using a scan rate of 400 nm per sec at a room temperature of 24°C.

Functionalization of carbon nanospheres and synthesis of nanofluids

The synthesized CNS powders were found to be extremely hydrophobic and needed to be functionalized before being dispersed in Milli-Q water (see Figure 4B insert). Functionalization consisted of adding 1–2 mL of 20% TEAH to a specific mass of CNS powder in a mortar. The range of CNS sample masses functionalized was 0.005, 0.010, 0.020, 0.030, and 0.040 g. Each individual sample was then ground for 15 minutes to produce a smooth paste using a pestle and mortar. The paste was then added to a small vial containing a solution of 10 mL of 20% TEAH and 90 mL of Milli-Q water. The mixture was then sonicated for 1 hour using an ultrasonic processor (UP50H, 100% amplitude, 50 W, 30 kHz, MS7 Sonotrode [7 mm diameter, 80 mm length]; Hielscher Ultrasonics, Teltow, Germany) to disperse the CNS.

Photothermal response of a CNS-based film in air

The effectiveness of using a CNS film for temperature enhancement of a substrate surface exposed to solar irradiation was investigated. A functionalized CNS paste was made by mixing 1–2 mL of 20% TEAH with 0.05 g CNS powder in a mortar. The mixture was then ground for 15 minutes to produce a smooth paste using a pestle and mortar. Two 15-mm squares of Parafilm M (SPI Supplies, West Chester, PA) sealing film were cut. The first was untreated and used as the control. The second was coated with a thin layer of CNS paste that was evenly spread over the upper surface. The film was then allowed to naturally air dry. Both film samples were then laid on a standard laboratory glass slide for support and handling. The slide was then placed into a specifically designed light box fitted with a solar light source (1000 W/m²). Both films were initially photographed using a thermal imager (Ti25; Fluke, Everett, WA) and then irradiated for 160 seconds. A thermal image was taken using the thermal imager (Ti25; Fluke, Everett, WA) after thermal images (see Figure 5A).

Photothermal response of nanofluids

The photothermal response of the nanofluids was carried out in a specifically designed light box fitted with a solar light source (1000 W/m²). The nanofluids were sealed in...
glass vials, all filled to the same level to ensure the same thermal transfer area. The temperatures of each nanofluid sample were monitored using a QM-1600 meter (Digittech, Hounslo, UK) with the thermocouple inserted into the fluid. Three sets of temperature measurements were carried out and recorded in real time, with the average value being used. The average atmospheric temperature was 28°C.

Electrical conductivity measurements

Thin films with a mean thickness of 0.3 mm composed of functionalized CNS were examined for their electrical conductivity. The functionalized CNS paste was made by mixing 1–2 mL of 20% TEA-H with 0.05 g CNS powder in a mortar. The mixture was then ground for 15 minutes using a pestle and mortar to produce a smooth paste. Then three 15-mm squares of Parafilm M sealing film were cut; these were used as backing support for the CNS film. The Parafilm M films were then coated with a thin layer of CNS paste that was evenly spread over the upper surface. The film was then allowed to naturally air-dry. The electrical conductivity characteristics of all three CNS films were carried out using a power supply (Escort dual tracking, model 3030TD; Microtek Instruments, Chennai, India) as the voltage source. The current and voltage measurements were taken using two digital multimeters (True RMS Digital Multimeter model 506; Protek, Morwood, NJ). The voltmeter probes were positioned 1 cm apart on the surface of the CNS film. Three sets of measurements were carried out on each film, and average values from all three sets of film measurements were used to produce a representative current–voltage curve for the film.

Results and discussion

The structural and morphological features of the CNS produced from the pyrolysis of acetylene were examined using both TEM (Figure 2B) and FESEM (Figure 2A). Figure 1 presents TEM images of the carbon soot particles that were collected directly from the collecting components within the CVD. The FESEM images (Figure 2) reveal that the untreated soot, in bulk, appears black and is composed of nanosized carbon spheres. The sphere diameters range in size from 100 nm to 400 nm. This spherical particle morphology was confirmed by the TEM analysis. The images reveal that the CNS are consistently spherical in shape and range in size from 100 nm to 500 nm in diameter. Graphical analysis of both FESEM and TEM images revealed that the mean particle size of the CNS was 210 nm. The images of both FESEM and TEM also clearly demonstrate that a metallic catalyst was not needed during the pyrolysis of acetylene to form CNS.

The bonding and thermal stability of the produced CNS was investigated using both Raman spectroscopy and XRD. The Raman spectrum presented in Figure 3A reveals two prominent peaks, the first located at 1325 cm⁻¹ and the second at 1583 cm⁻¹. The first peak represents the D band, while the second peak results from the graphitization of the carbon soot (stretching mode of the carbon–carbon bonds). The peak intensity ratio of the D and G bands (I_D/I_G) is commonly used to describe the degree of graphitization of a carbon material.43,44 A recent study involving the noncatalytic pyrolysis of toluene to synthesize CNS by Qian et al produced a peak intensity ratio of 0.84,37 while Xu et al.45 reported a value of 1.15 for pyrolyzing a mixture of CrCl3 and Fe(OH)3.46 This investigation found a peak intensity ratio value of 1.14; this value indicates that the CNS produced in this work have a lower degree of graphitization and the presence of disordered carbon.

To confirm the degree of graphitization that has taken place in producing the CNS, an XRD study was undertaken. The results of the XRD study are presented in Figure 3B.
rapid onset of oxidation. Just above 500°C, the oxidation process began and rapidly increased until a sharp declining slope developed at 562.12°C, indicating the onset of a rapid oxidation rate. The steepness of this slope indicates that there are a large number of lattice defects in the structure of the CNS that enable oxygen to travel into the structure and facilitate a high oxidation rate. The oxidation process continued until all the carbon soot was completely oxidized, and the reaction abruptly ended at 658.19°C.

UV-visible analysis was used to investigate the fluorescent properties of the CNS produced in this work. The ultraviolet response of oxidized carbon nanoparticles formed in candle soot was first demonstrated by Liu et al. In their study, they found that the fluorescent properties of the candle soot were dependent upon the size of the carbon nanoparticles formed.

A similar technique was used in this work to investigate the UV-visible response of the CNS. In general, carbon soot is highly hydrophobic; however, the CNS produced in this study were able to be dissolved in alcoholic solutions. A small quantity of CNS were dissolved in ethanol, filtered using a 0.22-µm syringe filter and then submitted to UV-visible analysis. The absorbance peaks in the UV-visible spectrum are presented in Figure 3B. The preliminary optical results indicate that the CNS produced by the pyrolysis of acetylene show absorbance peaks, which could relate to the different CNS sizes. Furthermore, under UV irradiation, the CNS in ethanol are fluorescent, as seen in the Figure 3B insert. This preliminary study clearly indicates that further investigation is needed.

Nanofluid stability is influenced by particle properties such as morphology and surface chemistry and the chemistry of the base fluid. The CNS synthesized in this work have a surface chemistry that makes them extremely hydrophobic (see Figure 4B insert, i). Therefore, it was necessary to functionalize the surface of the CNS using TEAH, which effectively modified the surface chemistry of the CNS. The functionalization process effectively increased the wettability of the CNS surface, which allowed easier dispersion of the CNS into the Milli-Q water, hence forming the CNS-based aqueous nanofluid suspension (see Figure 4B insert, ii). The CNS-based aqueous nanofluid suspensions were stable for periods ranging from 2 to 3 weeks. After 2 weeks of standing in a glass vial under standard laboratory conditions, CNS particle precipitation could be observed. And by the end of the third week, sedimentation could be easily seen in the bottom of the vial. Further investigation is needed to improve the long-term stability of the CNS nanofluids for periods greater than 2 weeks, when the nanofluid is left standing for extended periods.

Figure 2 (A–F) Field-emission scanning electron microscopy images of carbon nanospheres produced by the pyrolysis of acetylene gas taken at various magnifications ranging from 100 µm down to 100 nm.
When a thin CNS layer film deposited on a Parafilm M film was exposed to 1000 W/m² of solar irradiation, there was a significant photothermal response. After 30 seconds, the temperature of CNS-coated film was 4.5°C higher than the uncoated Parafilm M film, and after 160 seconds the temperature difference was 6.2°C. Thermal images of the CNS-coated and uncoated films are presented in Figure 5A. The complete thermal data for the 160-second test period are presented; the graphical plots reveal that there was a significant temperature difference throughout the test period. The data confirm that the CNS coating was able to provide a significant temperature enhancement to the Parafilm M film. In addition, the results of electrical conductivity measurements revealed that the current–voltage characteristics of the TEAH-functionalized CNS films were linear (ohmic behavior) over the experimental range examined (see Figure 6B). The slope of the line fit indicates that the resistance of the CNS films was relatively high, with a mean value of 920 ± 30 kΩ between the test probes (1 cm spacing).

A similar temperature-enhancement trend was also seen with the inclusion of CNS in an aqueous-based nanofluid. The base solution was composed of a solution of 10 mL 20% TEAH and 90 mL Milli-Q water. The presence of TEAH in the base solution had no significant photothermal response when compared to the pure Milli-Q water control solution; therefore, the photothermal response seen in all
CNS nanofluids was the result of the CNS present. This can be seen in Figure 5B, in which increasing amounts of CNS in the base solution produce a greater photothermal response. In the case of the nanofluid with the smallest mass of CNS present (0.005 g); the temperature enhancement after 60 minutes was 3.5°C, while the nanofluid with the largest mass of CNS (0.04 g) had a temperature enhancement of 8.1°C for the same period of time (see Figure 6A). This result clearly indicates increasing CNS mass content in the nanofluids significantly improves the photothermal properties in the experimental range.

However, a similar study by Han et al using carbon black nanofluids found that the temperature enhancement of a 7.7-vol% carbon black nanofluid was similar to a 6.6-vol% sample thus indicating that the photothermal properties did not increase significantly above the 6.6-vol% sample. The 7.7-vol% carbon black nanofluid was found to produce a 7.2°C temperature enhancement compared to pure water. This is similar to the 8.1°C temperature enhancement of the 0.04-g CNS nanofluid, the only major difference being in the type of carbon and its content. In terms of volume fraction, the 0.04-vol% (0.04 g) used in this study was significantly less than the values used.

Figure 4 (A) Raman spectrum and (B) X-ray diffraction spectroscopy pattern of carbon nanospheres (CNS) produced by the pyrolysis of acetylene gas; insert (b) untreated CNS in Milli-Q water and functionalized CNS in tetraethylammonium hydroxide (TEAH)/Milli-Q water-based nanofluid.
by Han et al. In addition, the CNS functionalized with TEAH appear to have enhanced photothermal properties compared to carbon black when we consider the low volume fractions being used in this work. Han et al also pointed out that the temperature enhancements of their carbon black nanofluids were higher than those of Mu et al’s TiO$_2$/water, SiO$_2$/water, and ZrC/water nanofluids (0.1 wt%). The superior photothermal response of the carbon black nanofluids is believed to be due to the high concentration and good solar absorption of carbon black present in the water based fluid compared to the metallic nanoparticles used by Mu et al. The CNS used in this work also display the superior solar absorption properties of carbon black, but with much lower volume fractions. The lower fractions of CNS used this work make them highly suitable for incorporation into water based working fluids within a conventional pumping system, since unlike larger micrometer-sized particles, the nanosized CNS will have few detrimental effects, such as increased fluid friction, sedimentation, and blockages.

**Conclusion**

CNS were produced via the noncatalyzed pyrolysis of acetylene gas at 1000°C in the presence of nitrogen gas in a simple

![Graph showing the photothermal response of carbon nanosphere (CNS) coated film and five CNS-based nanofluids of varying CNS mass content.](image)

**Figure 5** (A) Photothermal response of a carbon nanosphere (CNS)-coated film; (B) photothermal response of five CNS-based nanofluids of varying CNS mass content. Abbreviation: TEAH, tetraethylammonium hydroxide.
electric furnace–based CVD apparatus. The resultant carbon soot contained CNS that ranged in size from 100 nm to 500 nm in diameter. Raman spectroscopy, XRD, and TGA analysis indicated a lower degree of graphitization in the CNS, which produced stable spherical structures with a mean particle size of 210 nm. The preliminary UV-visible analysis of filtered CNS dispersed in ethanol revealed some interesting features that will need further investigation. In addition, a detailed study is needed to investigate the effect of different reaction conditions on the size, structure, and morphology of CNS being formed during pyrolysis. The results of this further study would provide information needed to effectively control the formation mechanisms operating during the pyrolysis process.

Refinement of the formation mechanism would allow fine tuning of the CNS size, structure, and morphology.

The CNS were then functionalized using TEAH before being dispersed in Milli-Q water to form nanofluids of varying CNS mass content. All of the CNS nanofluids had favourable photothermal responses to solar irradiation over the exposure period. The largest CNS mass content (0.04 g) nanofluid had the largest temperature enhancement of 8.1°C, which clearly demonstrates efficient absorption capabilities of the CNS nanofluids towards solar irradiation compared to Milli-Q water.

The results indicate that functionalized CNS nanofluids have the potential to effectively improve the solar absorption capabilities of direct- absorption solar collectors.

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Disclosure

The authors report no conflict of interest in this work.

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28. Sun YP, Zhou B, Lin Y, et al. Quantum photothermal response of carbon nanosphere based aqueous nanofluids. Nanotechnology. Science and Applications is an international, peer-reviewed, open access journal that focuses on the science of nanotechnology in a wide range of industrial and academic applications. It is characterized by the rapid reporting across all sectors, including engineering, optics, bio-medicine, cosmetics, textiles, resource sustainability and sciences. Applied research into nano-materials, particles, nano-structures and fabrication, diagnostics and analytics, drug delivery and toxicology constitute the primary direction of the journal. The manuscript management system is completely online and includes a very quick and fair peer-review system, which is all easy to use.

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