SYNTHESIS OF SILVER NANOPARTICLES FOR ANTIBACTERIAL AND LARVICIDAL APPLICATIONS

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FACULTY OF SCIENCE
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ABSTRACT

The present study deals with the green synthesis of silver nanoparticles (AgNPs) using the extract of plant. AgNPs were synthesized by reacting aqueous $AgNO_3$ solution with apple extract (reducing agent). Synthesis conditions such as: ratio of silver salts/apple extract, reaction time, comparison of heating and non-heating method, surfactant as well as pH were investigated and optimized. The results of this investigation showed that AgNPs could be synthesized and optimized by controlling the synthesis conditions. The X-ray diffraction pattern confirmed the presence of only face centered cubic (FCC) Ag crystallites, and the dynamic light scattering estimates the average sizes of the AgNPs to be 5-150 nm. Fourier Transform Infrared (FTIR) as well as UV-vis spectroscopy identified ethylene groups as the reducing agent and capping agent for the formation of the AgNPs. The absorption maxima of the synthesized AgNPs showed characteristic Ag surface plasmon resonance (SPR) peak. The usage of surfactant has been observed to be an important parameter in controlling the shape and also play an important role in dispersing the AgNPs. Solution with pH 4 was found to be suitable in producing spherical shape of AgNPs. The AgNPs in suspension showed bactericidal activity of the AgNPs against Gram-negative and Gram-positive bacteria. Minimum bactericidal concentrations (MBCs) were found to be in the range of 15 μg/mL to 1000 μg/mL. Larvicidal activity of AgNPs against L4 *Aedes aegypti* was also determined. The AgNPs showed moderate larvicidal effects against fourth larval instar (LC_{50} = 15.76 ppm and LC_{90} = 27.70 ppm). In addition, comparison of larvicidal activity performance of AgNPs at high concentration prepared using two different methods showed that Ae. aegypti larvae was fully eliminated within 2.5 hours. This study has proven that green synthesis could provide an economic, eco-friendly, and clean synthesis route of the AgNPs.

ABSTRAK

Kajian ini berkaitan dengan sintesis hijau nanopartikel perak menggunakan ekstrak tumbuhan. Nanopartikel perak telah disintesis melalui tindakbalas akueus $AgNO_3$ dengan ekstrak epal (agen penurunan). Keadaan sintesis seperti: nisbah garam perak/ekstrak epal, masa tindak balas, perbandingan pemanasan dan kaedah bukan pemanasan, ejen pemuka serta pH telah dikaji dan dioptimumkan. Hasil kajian menunjukkan bahawa nanopartikel perak boleh disintesis dan dioptimumkan dengan mengawal parameter tersebut. Corak pembelauan sinar-X mengesahkan kehadiran hanya wajah berpusat padu (FCC) kristal perak, dan cahaya berselerak dinamik menganggarkan saiz purata nanopartikel perak sekitar 5-150 nm. Fourier Transform Infrared (FTIR) dan juga UV-vis spektroskopi mengenal pasti kumpulan etilena sebagai ejen penurunan dan ejen pengehad untuk pembentukan nanopartikel perak tersebut. Penyerapan maksimum oleh nanopartikel perak yang disintesis ditemui mempunyai ciri yang sepadan dengan puncak plasmon resonan permukaan (SPR) perak. Kesan penggunaan ejen pemuka dalam penyediaan sampel menunjukkan ianya satu parameter penting dalam mengawal bentuk dan juga memainkan peranan penting dalam melarutkan nanopartikel perak. Larutan dengan pH 4 didapati menghasilkan bentuk sfera nanopartikel perak yang baik. Larutan nanopartikel perak telah menunjukkan aktiviti terhadap bakteria Gram-negatif dan Gram-positif. Kepekatan bakteria minimum (MBCs) didapati dalam julat 15µg/mL hingga 1000 µg/mL. Aktiviti larvicidal nanopartikel perak terhadap L4 *Aedes aegypti* juga ditentukan. Nanopartikel perak menunjukkan kesan larvicidal sederhana terhadap instar larva keempat (LC_{50} = 15.76 ppm dan LC_{90} = 27.7 ppm). Di samping itu, perbandingan prestasi aktiviti nanopartikel perak pada kepekatan yang tinggi yang disintesis dengan menggunakan dua kaedah yang berbeza menunjukkan bahawa larva *Ae. aegypti* telah dihapuskan sepenuhnya dalam masa 2.5 jam. Kajian ini telah membuktikan bahawa sintesis hijau boleh merintis

kepada perkembangan teknik sintesis nanopartikel perak yang ekonomik, mesra alam, dan bersih.

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LIST OF ABBREVIATIONS

DNA	Deoxyribonucleic acid
EDX	Energy dispersive X-ray analysis
FESEM	Field emission scanning electron microscope
HRTEM	High-resolution transmission electron microscopy
ICP-MS	Inductively coupled plasma mass spectrometry
PSD	Particle size distribution
ROS	Reactive oxygen species
SPR	Surface plasmon resonance
TEM	Transmission electron microscopy
UV-Vis	UV-Visible spectroscopy
XRD	X-ray diffraction

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CHAPTER 1

INTRODUCTION

1.1. Introduction

The potential of nanotechnology was first put forth by physicist Richard Feynman in his lecture to the American Physical Society in 1959 entitled “there’s plenty of room at the bottom”(Feynman, 1960). Feynman envisioned the idea of manipulating the very small, even down to the level of individual atoms for technological purposes. Half a century later, the global nanotechnology industry has an estimated industrial market value of almost $500 billion (US), and predicted to rise to 17.5 % by 2022.

While the initial inspiration was to use nanotechnology for technological purpose, other researches extended into biotechnology as it has been discovered that nanomaterials possess properties that could contribute in biological applications. For example, various nanomaterials have shown antimicrobial properties that may be used to control microbial populations including those that have evolved resistance to antibiotics. For instance, silver nanoparticles have proven to display antibacterial properties against these drug-resistant bacteria such as *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Klebsiella pneumonia* ATCC 700603, *Staphylococcus aureus* ATCC 29213, and *Staphylococcus epidermidis* ATCC 12228 (Ion-Nedelcu et al., 2009; Saeb et al., 2014; Sunenshine et al., 2007). These drug resistant bacteria are also known to be responsible for nosocomial infection, a hospital-acquired infection that could be fatal. Apart from that, the growing number of dengue cases has also opened the possibility to use nanotechnology as larvicidal agents (Angajala et al., 2014; Roopan et al., 2013), with AgNPs, ZnO and few other nanomaterials exhibiting the larvicidal properties.

The role of technology in improving human life has been undeniable. Life is getting better and more comfortable. The demand for a better technological devices is rising. The advent of nanotechnology has been the answer. Machine is getting smaller and more efficient. Inventions and inovations are blossoming. Newer technological devices are produced at a faster rate. Industries are booming.

1.2 Problem Statement

In order to cater these needs, more factories have been set up. While the large-scale production has led to profitable business and gives positive impact towards development of the technoloy, it also leaves some adverse effects. Toxic effluents have been reported to be released into the water stream. According to a report by United Nations Environment Program (UNEP), every second 310 kg of toxic chemicals are released into our air, land, and water by industrial facilities around the world (Dadax, 2010). This amounts to approximately 10 million tons of toxic chemicals each year. Of these, over 2 million tons per year are recognized carcinogens.

While strict regulation has been imposed on the industries by the government, the problem continues to exist. This is probably due to the high cost involved to set up the wastewater treatment facility which sometimes the operator might feel not wanting to obligate and therefore neglect the recommended procedure.

To overcome such problems, green technology has emerged and been proposed hoping to solve the problems. Numerous efforts have been done and this can be observed by massive works of scientists all over the world. Reseaches on battery, solar cell, nanomaterials are all now slowly shifting towards using natural organic materials.

For green technology to be good and competitive the product must have the quality as good as the product produced by conventional method. For example, natural organic reducing agent has been known for its capability to be used in the synthesis of silver nanomaterial. The question is; does the AgNPs have the same properties and quality as the one produced by conventional method (eg; chemically reduced, electrolysis etc).

Works on green synthesized AgNPs have been massive (Ibrahim, 2015; Murugan et al., 2015; Oluwafemi et al., 2013; Reddy et al., 2014; Soni et al., 2012; Umoren et al., 2014). Most of these works have focussed on searching newer plant as the novelty of the work. With the number of plant species reaching up to thousands, this could be a never-ending work and effort. However, works on improving and enhancing the green synthesized AgNPs properties are very scarce.

Recently, Lokina et al. reported synthesis of AgNPs using apple as reducing agent (Lokina et al., 2014). The AgNPs were nearly spherical in shape and has size in the range of 10-40 nm. Antibacterial tests carried out on Staphylococcus aureus, Pseudomonas aeruginosa, *Escherichia coli*, *Citrobacter koseri* and *Bacillus cereus*, and against the fungus *Candida albicans* showed that the best minimum inhibitory concentration (MIC) result was found on *Staphylococcus aureus* (0.0781 mg/ml). While their results was to prove that apple can be used in synthesizing AgNPs, their report is limited to only analyze the antibacterial properties through minimum MIC and therefore can be expanded further.

In this study, the topic will be approached from different aspects. It will show what can be done to reduce the size and improve dispersibility of the AgNPs. Synthesis of AgNPs will be carried out with a control over several experimental parameters to understand the structural effects on the synthesized AgNPs.

In their study, Lokina et al. carried out the test using only one type of AgNPs by MIC method. In this study the antibacterial performance of three different AgNPs with different sizes and stability/dispersibility will be compared using minimum bactericidal concentration (MBC) technique. Moreover, Lokina et al. works was to show that the synthesized AgNPs possessed antibacterial properties whereas in this study, it is to show that antibacterial properties can differ if the size and dispersibility of the AgNPS differ. By carrying the test with several AgNPs, relationship of antibacterial performance and physical properties of the AgNPs can be established and understood. Apple was chosen because it is available everywhere although in some parts of the world it is not cheap.

By definition, the terminology "green" is still vague and obscure. There is no definite and conclusive definition of it. Although most of the publications or articles related to green synthesis used no inorganic materials, there is also no rule stating that a green synthesis or products cannot combine process with any inorganic chemicals.

Several definitions of 'green' have been discussed in literatures. One of them by (Ottman et al., 2006) suggest that " *although no consumer product has a zero impact on the environment, in business, the terms 'green products' or 'environmental product' are used commonly to describe those that strive to protect or enhance the natural environment by conserving energy and/or resources and reducing or eliminating use of toxic agents, pollution, and waste*". Based on this definition, it is understood that any effort to reduce reliance towards anything that is unfriendly towards environment can be the subset of green definition and moreover it does not in any way state that it must be 100% free of chemical use.

Apart from that, previous works (in literature review) show that there is limitation in the green synthesis of AgNPs. Agglomeration/aggregation problem is dominant. To overcome such problem, certain aspect has to be compromised. Chemicals will be used in order to reduce the agglomeration and increase the dispersibility. The need to be ‘green’ must be realistically balanced with the fact that producing green products without quality will only severe and jeopardize its potential to be used in real life.

Therefore, works focussing on this aspect are deemed to be very important and much needed in order to evaluate the full potential of the green approach especially in the field of biotechnology applications.

1.3 Objectives

The main aim of this work is to obtain green synthesized AgNPs with an improved antibacterial and larvicidal properties.

There are four main objectives:

(i) To synthesize AgNPs using apple extract and control its morphological properties by varying the operating temperature condition, surfactant, time, pH as well as ratio of extract to the Ag salts.

(ii) To characterize the green synthesized AgNPs using physical methods.

(iii) To determine the antibacterial efficacy of the green synthesized AgNPs.

(iv) To determine the larvacidal effect of the green synthesized AgNPs.

CHAPTER 2

LITERATURE REVIEW

2.1 Nanotechnology

Nanotechnology refers to a technology that utilizes any material with a dimension lower than 100 nm. A physicist by the name of Richard Feynman has been dubbed as the father of nanotechnology owing to his visionary ideas of a process where individual atoms and molecules could be manipulated and controlled. He suggested that nanosized devices, machines and robot could possibly be used to develop a wide range of atomically precise microscopic instrumentation and manufacturing tools, which could be applied to produce various nanoscale and microscale robots and a vast quantities of small computers. Feynman's idea remained largely undiscussed until the mid-1980s, when the Massachusetts Institute of Technology (MIT) educated engineer K Eric Drexler published a book “Engines of Creation”, to popularize the potential of molecular nanotechnology (Drexler, 1986). Prior to that, it was in 1981 that the first Atomic-force microscopy (AFM) machine was invented (Binnig et al., 1986).

Nanotechnology has been used by medieval people as early as in the 4th century. For example, a dichroic glass known as *Lycurgus Cup* was invented by the Romans using colloidal gold and silver making it to appear opaque green when lit from outside but translucent red when light shines through the inside. Recently a study by Reibold et al. (2006) on the sword of Damascus has observed the presence of carbon nanotubes, nanowires and other extremely small, intricate structures that might explain their unique features. Damascus swords, first made in the 8th century A.D., are renowned for their complex surface patterns, strength and sharpness. It is said that the blades could cut a piece of silk in half as it falls to the ground and maintain their edge after cleaving through metal, stone or even after striking other swords. However, it has been suggested that these effects were achieved unintentationally and the craftmen did not really understood the mechanism or the presence of nanomaterials in their product.

In this modern age, nanotechnology is a usual thing and present in everything. For example, light-emitting diode (LED) light using nanomolecules are designed to use less energy, producing brighter and sharper display as a substitute to liquid crystal display (LCD) technology. This has helped to revolutionize the electronic displays for car stereos, cell phones, digital cameras and computer screens. While the development in medical field is still on-going, the most significant impact of nanotechnology is expected to be realized in drug delivery and development of smart drug. Nanoparticles would enable physicians to target drugs at the source of the disease, which will increase the efficiency and minimize side effects.

Nanotechnology has been adding new functionalities, thus increasing efficiencies and capability in many fields. Reducing the particle size of materials is found to be an efficient and reliable approach to achieve this objective. It is because, nanosized material behaves differently compared to its bulky counterpart. Therefore numerous sizes and shapes of nanomaterials have been developed to cater the industrial needs.

In general, nanomaterials can be clasiffied into zero dimension, 1-dimension, 2-dimension and 3-dimension (Figure 2.1). Zero dimensional material has nano-dimensions in all the three directions. Quantam dots are the perfect example of this kind of nanoparticles. They are tiny particles or nanocrystals of a semiconducting material with diameters in the range of 2-10 nanometers (10-50 atoms). For 1-dimensional nanostructures, they are usually long but with a diameter of few nanometers. Nanowires, nanorods and nanotubes of metals, oxides and other materials are few examples of this kind of materials. Two dimensional (2-D) nanomaterials has two dimensions outside the nanometer range. These include different kinds of nano films such as thin-film-multilayers, nano sheets and coatings. The area and width of the nano films can be large, but the thickness is always in nano scale range. Three dimensional (3-D) nanomaterials have all dimensions outside the nano meter range. These include bulk materials composed of the individual blocks which are in the nanometer scale (10-100 nm).

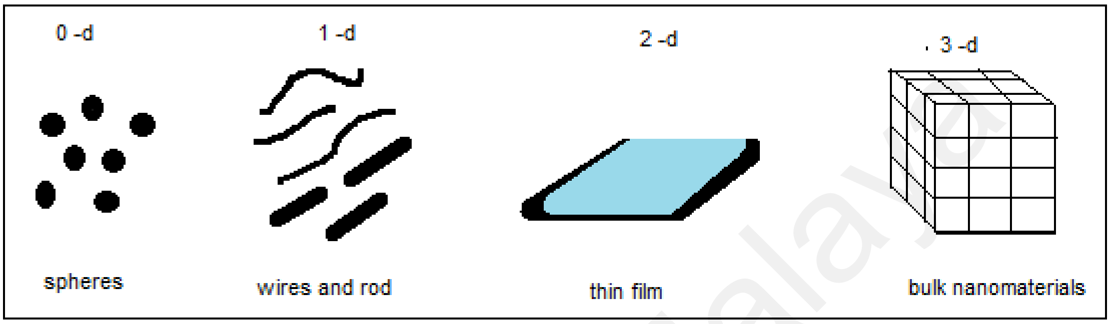


Figure 2.1: Illustrations of nanomaterials classification.

2.2 Importance of Silver

Silver (Ag) is the metallic element with a molar mass of 107.8682 g/mol. It has a face-centered crystal structure. It possesses the highest electrical conductivity, thermal conductivity, and reflectivity compared to other metals. Historically the usage of Ag has been traced back as early as 4 BC by inhabitants of modern day Turkey. Ag was first mined in Anatolia and used as coins. The usage of Ag continued to flourish through several world civilizations in Europe and Egypt as currencies and decorations.

Silver nanoparticles (AgNPs) have attracted extensive research interests because of their excellent optical, electronic, and chemical properties which depend on their size, shape, composition, crystallinity, and structure (Balan et al., 2007; Chen et al., 2009; Jiang et al., 2005). They have been widely exploited for use as antibacterial materials (Martinez-Castanon et al., 2008; Morones et al., 2005), catalytic materials (Bastús et al., 2014; Venkatesham et al., 2014) sensor materials (Velichko et al., 2013; Yi et al., 2013; Zhang et al., 2016) and microelectronic materials (Siow, 2012, 2014) owing to these unique properties. For example, the melting point of AgNPs can be drastically reduced because the surface energy of the nanoparticles tremendously increases because of the extremely small size of the particles (Qiao et al., 2014).

With the emergence and increase of microbial organisms resistant to multidrug antibiotics (Magiorakos et al., 2012), as well as the continuing demand on cheaper health-care costs, many researchers have tried to develop new, low-cost but effective antimicrobial drug. Such problems and needs have led to the revival in the possible use of Ag-based drug that have already been known for its ability to eliminate broad spectrum of bacteria such as *E. Coli, P. Aeruginosa, MRSA* and *S. Aereus* (Ali et al., 2016; Hernández-Sierra et al., 2008; Ismail et al., 2013).

The antibacterial effects of Ag and Ag salts have been noticed since a long time ago. Indian Ayurvedic System of Medicine has been using Ag as early 5000 years B.C (Barve et al., 2011). The Ayurvedic therapy proposed using water boiled with Ag ingot as a healing drink. This was also practices of many ancient Egyptian Kings for a hygenic water supply. However, the first clear record of Ag nitrate being used as a medical agent was reported by Gabor in 702–705 A.D, and in 980 A.D. Avicenna used Ag filings as a blood purifier and also to prevent palpitations of the heart and to treat offensive breath. In1520, Paracelsus used Ag internally and also applied Ag nitrate as a caustic for the treatment of wounds, a practice that continues until today (Alexander, 2009).

Another growing interest in the usage of Ag is in the larvicidal application. Numerous studies (Murugan et al., 2015; Muthukumaran et al., 2015; Patil et al., 2012; Subarani et al., 2013) recently have reported on its ability to eliminate broad species of larva such as *Anopheles subpictus Grassi, Culex quinquefasciatus Say, Culex quinquefasciatus, Aedes agyptae* and many others. It is a known fact that mosquito can be a vector to transmit disease and causing endemic problem. Taking dengue for instance, it is spread by female *Aedes agyptae* mosquito. The transmission occurs when a mosquito bites an infected person and then bites someone else.

As of now, there is no effective vaccine or specific treatment for dengue fever while current control methods (eg; space spraying with insecticides or fogging, public education, larviciding, legally enforced breeding site reduction, etc.) are deemed not enough to stop the spread of the disease. Therefore it is an urgent need to evaluate any promising new technologies like the usage of AgNPs in eliminating the larva.

2.2.1 Mechanism of Ag in antibacterial and larvicidal properties

Exact mechanism for antibacterial and larvicidal properties of Ag is still not known. While some studies (Kumar et al., 2005; Morones-Ramirez et al., 2013) have proposed the mechanism in which Ag particles eliminate bacteria, it is believed that the same mechanism may also be used to explain the larvicidal properties of the Ag (Sundaravadivelan et al., 2014). The Ag could disrupt the cell of larva by interupting enzyme cycles and production (Yamanaka et al., 2005) as well as anchoring to the cell wall, penetrate it and cause structural changes that lead to the cell's death (Palza, 2015; Prabhu et al., 2012). Typically antibacterial properties are based on (i) dissolved Ag ions interaction and (ii) reactive oxygen species (ROS). In the former, dissolved ions interrupt the bacteria cell's ability to form bonds essential for its survival. These bonds produce the cell's physical structure and therefore when bacteria meet Ag ions they would literally fall apart. Ag also disrupts multiple bacterial cellular processes, including disulfide bond formation, metabolism, and iron homeostasis resulting in increase of permeability of the cell wall (Morones-Ramirez et al., 2013). For the latter, a recent study suggested that ROS generation by Ag nanoparticles or Ag^+ ions could also be responsible for the strong antibacterial agents (Kim et al., 2007; Kumar et al., 2005). Figure 2.2 illustrates the summary of the possible Ag reactions towards bacterial cell.

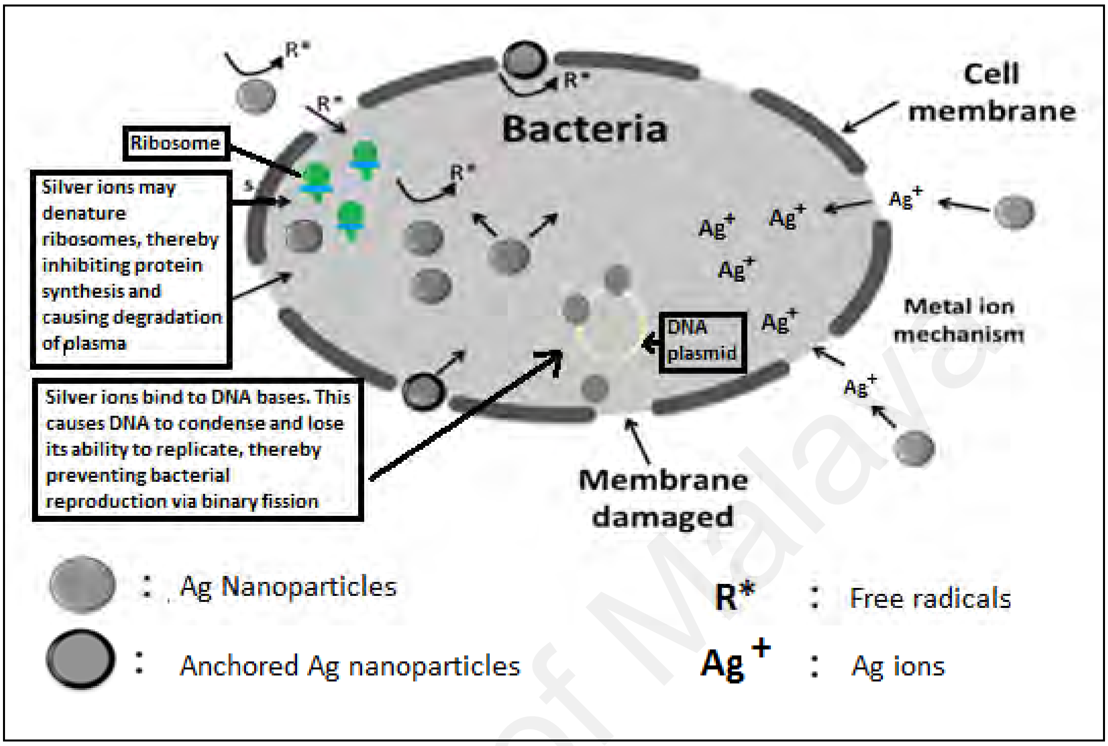


Figure 2.2: A summary of the mechanisms associated with the antibacterial behaviour of AgNPs: anchor/attachment to the cell surface, radical formation and release of Ag^+ ions. Modified from Palza (2015) and Chaloupka et al. (2010). Reproduced with permission (Appendix B1).

There are three types of ROS commonly associated with AgNPs: singlet oxygen $^1O_2$, hydroxyl radical ·OH, and peroxide radical. Among these, $^1O_2$ is said to be the most detrimental to cells. Although Ag particles are used instead of its ionic form (Ag^+), it has been reported that metallic silver can be oxidized to silver ion (Kumar et al., 2005) especially if they are in nanosize. On the other hand, study on ROS generation by Xu et al. (2012) showed that the usage of AgNP does produce ROS when tested against *Escherichia coli*.

2.2.1.1 Factors affecting toxicity of Ag

Reducing the particle size of materials is an efficient and reliable tool to improve Ag toxicity in the biomedical application. The larger surface area-to-volume ratio will increase the Ag contact with microbes and its ability to permeate cells (Morones et al., 2005; Nel et al., 2006). Particles sufficiently small would be able to pass through transmembrane porins (typical internal pore size in nm) for transport across cell membranes and therefore could cause the damage of cellular constituents and metabolism (Choi et al., 2008).

Shrinkage in size may also create discontinuous crystal planes that will increase the number of structural defects which could establish specific surface groups that could function as reactive sites (Nel et al., 2006). This could contribute to possible increase in the free-radical production by the nanosized material. Those surface properties can lead to toxicity when electron donor or acceptor active sites (chemically or physically activated) are in interaction with molecular dioxygen (O_2). Electron capture can lead to the formation of the superoxide radical ($O_2.^{-}$), which through dismutation or Fenton chemistry can generate additional ROS (Nel et al., 2006).

2.2.2 Other nanomaterials

Different types of nanomaterials like titanium, zinc, copper, gold and silver have come up but AgNPs have proven to be most effective as it has good antimicrobial efficacy against bacteria, viruses and other eukaryotic micro-organisms (Gong et al., 2007). For example, comparison of performance between Ag, ZnO, Cu_2O, CuO and Cu against a range of bacterial pathogen by Ren et al. (2009) also showed a consistent and lowest concentration needed by Ag to eliminate all the bacteria (Table 2.1). *Streptococcus mutans*, a bacteria associated to the teeth problem showed higher sensitivity towards Ag compared to Au and ZnO. It is reasonable to believe that chemical composition possibly plays a primary role in the toxicological effects of different nanomaterials (Yang et al., 2009).

Table 2.1: Minimum bactericidal concentration (MBC) performance of different nanoparticles conducted by Ren et al. (2009).

Strain	**Minimum bactericidal concentration (MBC) (μg/mL)**				
	Ag	**ZnO**	**Cu_2O**	**CuO**	**Cu**
EMRSA-16	100	5000	2500	1000	250
EMRSA-15	100	5000	2500	250	250
MRSA	100	> 5000	2500	1000	1000
Staphylococcus aureus (golden)	100	2500	2500	2500	1000
Staphylococcus aureus (golden)	100	5000	2500	100	250
Staphylococcus epidermis SE-51	100	2500	2500	2500	500
Staphylococcus epidermis SE-4	100	2500	2500	2500	1000
Escherichia coli NCTC	9001	>5000	500	250	250
Proteus spp.	100	>5000	5000	5000	2500
Pseudomonas aeruginosa PAOI	100	>5000	2500	5000	2500

TiO_2 has the ability to kill Gram-positive and Gram-negative bacteria owing to its toxicity that can be attributable to reactive oxygen species, (ROS). ROS is induced by visible light, near-UV or UV. It can cause damage to membrane, DNA, and many other macromolecules and functions of the bacterial cell (Beyth et al., 2015). The dependency of TiO_2 on UV light to activate its antibacterial activity can be modified by doping with Ag. Doping TiO_2 with Ag allows the synergistic bactericidal activity of the Ag-

TiO_2 nanoparticles in both light and dark conditions (Li et al., 2011). They reported that under UV light, all hybrid Ag-TiO_2 nanoparticles exhibited stronger bactericidal activity compares to the use of UV alone, Ag/UV, or UV/TiO_2. The Ag-TiO_2 has also exhibited an improved antibacterial performance for experiments conducted in the dark. Bactericidal activity of Ag-TiO_2 nanoparticles was reported to be greater than either bare TiO_2 (inert) or pure AgNPs. Guin et al. showed that 5% of Ag in TiO_2 nanoparticles showed the highest inhibitory activity on E. coli and is more effective in eliminating bacteria compared to pure TiO_2 (Guin et al., 2007). Owing to its natural white color, it has also been used in dental applications. Addition of TiO_2 in an orthodontic composite has exhibited antibacterial effects against *Streptococcus mutans,* a commonly found bacteria in human oral cavity and a significant contributor to tooth decay (Poosti et al., 2013).

ZnO nanoparticles have shown to have a wide range of antimicrobial activity against various bacteria such as *Salmonella enteritidis* (Jin et al., 2009), *Streptococcus mutans* (Hernández-Sierra et al., 2008), *Klebsiella pneumonia* (Reddy et al., 2014) and *E. coli* (Dasari et al., 2013). ZnO nanoparticles have also proven to be effective as bactericidal agents against the drug-resistants bacteria such as *methicillin-sensitive S. aureus* (MSSA) (Musarrat et al., 2015), *methicillin-resistant S. aureus* (MRSA) (Vijayakumar et al., 2015), and *methicillin-resistant S. epidermidis* (MRSE) strains. The ZnO depends on the ROS production as one of the route to eliminate the bacteria. Just like the TiO_2, ZnO are photo-reactive and produce ROS only under irradiation of light. Therefore, under dark condition, no ROS is produced (Dasari et al., 2013). In dark environment, they proposed that the toxicity towards bacteria comes from the attachment of the particles to the cell wall, although there is claim that ion leaching could as well contribute. The white color and UV-blocking of the ZnO enables it to be used in fabric and glass industries as coating materials designated for medical related devices. ZnO also depend on ROS production to eliminate the bacteria.

Generally, Au can be considered inert and lack antimicrobial properties except for the nanosized particles (Beyth et al., 2015). In comparison to Ag, AuNPs are less potent and have almost no antibacterial effect by themselves (Majdalawieh et al., 2014). The mechanism of Au in the elimination of bacteria occurs in two steps. First, the Au causes the membrane potential to change and adenosine triphosphate (ATP) synthase activities to reduce, causing reduction in the metabolism process. Secondly, they impede the subunit of the ribosome for tRNA binding, thus collapsing its biological mechanism. Smaller size Au has higher bactericidal activities because the interaction and contact with the bacteria is improved. Small sized Au can also easily bind to the outer membrane of the cell, hence penetrate or cause structural changes to the cell. Moreover, AuNPs with a small size and enhanced surface area produce some electronic effects which are beneficial for enhancing the surface reactivity of NPs (Shamaila et al., 2016). Nanocomposite of chitosan-gold has also been developed in an effort to create a less toxic material to human but efficient in its bactericidal activities. Regiel-Futyra et al. reported that the composites did not show any cytotoxicity against mammalian somatic and tumoral cells but did cause damages to the bacterial cell wall of *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Regiel-Futyra et al., 2015). Using Ag to create alloy with the Au nanoparticles can improve the antibacterial performance. The Ag–Au alloy nanoparticle solution exhibited excellent antibacterial activity against the bacteria such as *Escherichia coli*, *Bacillus cereus*, *Pseudomonas aeruginosa and Bacillus subtilis* the performance was better than the use of Au nanoparticles alone (Bankura et al., 2014).

CuO antibacterial efficacy is inferior to that of Ag or ZnO (Beyth et al., 2015). CuO eliminate bacteria by ROS production and causing membrane disruption. ROS production is related to the size of the CuO particle, with smaller sizes produce a greater amount of ROS (Applerot et al., 2012). They proposed that combined actions of the strong bind of the CuO nanoparticles to the cell membrane, as well as ROS generation on the particles' surface, cause an increase in cell permeability, leading to an uncontrolled transport of CuO particles through the cytoplasmic membrane and finally leads to cells death. This event increases in the case of smaller CuO particles is used, owing to their higher surface-to-volume ratio, resulting in the formation of more ROS per unit weight, and to their higher probability to pass the cell membrane. Cu can be utilized for efficacy enhancement in the form of nanocomposites. A firm name Cupron incorporates micron-sized CuO particles into polymers which are further made into wound dressings. These dressings have effectively prevented infection and increased the rate of wound healing, compared to standard treatments (Gabbay et al., 2006).

2.3 Method to Synthesize Nanomaterials

Over the last decades, many synthesis methods have been developed and reported for the preparation of nanomaterials. They can be classified as: i) top-down and ii) bottom-up approach. In top-down process, bigger particle is 'sliced' to nanomater scale whereas in bottom up process, individual atoms are assembled to form bigger size particles. Moreover, the synthesis method can also be classified into (i) chemical and (ii) physical approach.

It is a common practise to use additional chemicals known as surfactants to stabilize dispersive nanoparticles during the metal nanoparticle preparation and avoiding their agglomeration. Surfactants can stabilize particle growth, and protect particles from sedimentation and agglomeration.

As the concern over the climate change and pollution are hugely growing, there is consistent calls for a 'cleaner' and eco-friendly method to be developed. Green chemistry has emerged as a possible answer. It uses biological approach. Owing to the availability of numerous organisms and plants, this approach has since gained a lot of attention worldwide.

2.3.1 Chemical approach

The most common approach for synthesis of AgNPs is chemical reduction by inorganic and organic reducing agents. Generally, different reducing agents such as sodium citrate (Guzman et al., 2012), sodium borohydride ($NaBH_4$) (Dong et al., 2010), polyol process (Silvert et al., 1997), Tollens reagent (Yin et al., 2002) and poly (vinyl pyrrolidone) (PVP) (Washio et al., 2006) are used for reduction of Ag ions (Ag^+) in aqueous solutions. These reducing agents reduce Ag^+ and lead to the formation of metallic Ag (Ag^0).

While this simplistic approach has successfully produced Ag particles, overtime it has been modified to further improve the nature of the Ag such as the sizes and stability. Introduction of additional parameters such as heating, irradiation and electroplating has expanded the simple technique into becoming more complex and expensive yet produce smaller size and more stabilized AgNPs. The subtopic 2.3.1.1-2.3.1.5 elucidates the concept and insights of the techniques in the chemical synthesis method.

2.3.1.1 Chemical reduction

Chemical reduction is widely used to synthesize AgNPs owing to its simple and wide-availability of the materials required to carry out the process. Sodium borohydride ($NaBH_4$) has been widely used as the reducing agent (Ahmad et al., 2011; Shameli et al., 2011; Dong et al., 2012). $NaBH_4$ which is a strong reducing agent has been reported to be able to produce ultra small and well-controlled size of silver nanoparticles with an improved size distribution (Dong et al., 2012). $NaBH_4$ reduction of $AgNO_3$ is:

$$AgNO_3 + NaBH_4 \rightarrow Ag + \frac{1}{2} H_2 + 1/2B_2H_6 + NaNO_3$$

Polymeric materials such as gelatin and chitosan have been used to control size and act as stabilizers. Ahmad et al. compared the average diameters of the AgNPs produced by Ag/chitosan, Ag/chitosan/gelatin and Ag/gelatin systems were 10.5, 3.37 and 4.38 nm, respectively (Ahmad et al., 2011). The stability of the AgNPs in solution was also remained up to 4 months.

Shameli et al. (2011) discussed some insight into how polymer works as stabilizer. Based on their work using PVP as stabilizer, they proposed that after the formation of Ag, the Ag will either aggregate each other or interact with PVP. The later will result in PVP to bind to the surface of Ag particles. The PVP-bounded-Ag will inhibit the aggregation and keep each of the Ag isolated.

The advantage of the technique is its simplicity, low-energy consumption as well as ability to control particle size and morphology very effectively. Environmental pollution is a disadvantage of the chemical method since it involves the use of reducing and stabilizing agents.

2.3.1.2 Electrochemical method

Reetz et al. have described an electrochemical procedure as to obtain particles in which a metal sheet is anodically dissolved and the intermediate metal salt formed is reduced at the cathode, giving rise to stabilized metallic particles (Reetz et al., 1994).

In this method, the choice of the right solvent, supporting electrolyte, type of electrode, and the current density are several important parameters to consider (Rodriguez-Sanchez et al., 2000). For example, they observed that increasing current density would result in smaller size distribution.

It is possible to control the particle size by adjusting electrolysis parameters and to improve homogeneity of Ag particles by changing the composition of electrolytic solutions (Yin et al., 2003). The usage of PVP as surfactant was found to reduce the silver deposition rate and accelerates the rate of silver particle formation and also prevents the particles formed from being agglomerated.

Khaydarov et al. reported the synthesis of AgNPs suspended in water solution that were nearly spherical and size distribution lies in the range of 2 to 20 nm, with the average size being about 7 nm. However, one obvious problem in this technique is the deposition of Ag in the electrode. This impedes the reduction of Ag ions in the solution. To overcome the problem, they periodically changed the polarity every 4 mins. AgNPs synthesized by the proposed method were sufficiently stable for more than 7 years even under ambient conditions (Khaydarov et al., 2009).

Some of the advantages of this method are the high purity of the particles and the possibility to precisely control particle size by simply adjusting the current density. This method is also eco-friendly because it avoids the use of reducer agents that usually are toxic. However, electrochemistry has not been employed as a means of preparing large numbers of metal nanoparticles (Zoval et al., 1996) because the consumption of electric would be very high.

2.3.1.3 High energy radiation

In this technique, no reducing agent is required. The electron will be produced by the water radiolysis as a result of irradiating the precursor solution with gamma ray. The hydrated electrons generated hence can reduce the Ag^+ to form Ag.

Long et al. (2007) used gamma ray with oligochitosan [(GlcN)x] as stabilizer to produce AgNPs with average particle size to in the range of 5 and 15 nm. Varying the irradiation dose between 5 and 20 kGy was reported to affect the size of the AgNPs. No AgNPs was formed at a dose lower than 5 kGy whereas large particles will be formed and black precipitation was observed at a dose higher than 20 kGy.

Naghavi et al. described the technique as a dose-dependent process (Naghavi et al., 2010). At high doses, where the nucleation event is more than the total ions, the radiation synthesis produced smaller sizes of nanoparticles following aggregation. On the other hand, at low doses where the nucleation event is less than the total ions, the radiation synthesis produced larger sizes of nanoparticles following aggregation.

The gamma-irradiation-induced reduction synthesis offers some advantages over the conventional methods because of its simplicity and ability to prepare AgNPs of high-purity and highly-stable state. Moreover, the absence of reducing agents or undesired oxidation by-product impurities makes it a harmless or environmentally friendly process. The disadvantage of the method is that the gamma source is very expensive and requires proper facilities to hold the gamma source.

2.3.1.4 Microwave assisted

Reaction to chemically produce AgNPs is exothermic hence heat supply is necessary. Conventional heating method used heating bath to achieve the purpose. Recently, microwave heating has been widely used as it shortens reaction and crystallization time (Nadagouda et al., 2011; Pastoriza-Santos et al., 2002). Microwave heating consistently yields nanostructures with smaller sizes, narrower size distributions, and a higher degree of crystallization (Nadagouda et al., 2011). It also consumes less energy compared to conventional heating (Raghavendra et al., 2016).

The use of microwave irradiation provides an extra degree of control of the reduction process (Pastoriza-Santos et al., 2002). Its ability to heat the solution uniformly results in a more homogeneous nucleation therefore encourage the formation of mono-dispersed colloids.

While the stability of AgNPs can be contributed by concentration of reducing agent, Ag precursor as well as the usage of stabilizers, the power of the microwave used does also play role. Aswathy et al reported that AgNPs formed using 180 watt has the highest stability compared to 300, 450, 600 and 800 watt (Aswathy et al., 2011). They suggested that at higher microwave powers, excess concentration of Ag_2O layers were formed as a result of surface oxidation on the surface of the AgNPs. These Ag_2O layers favored agglomeration. Although the use of microwave is slightly expensive than the conventional heating, the advantages of the technique have outweighed this factor.

2.3.1.5 Tollen method

It is based on using Tollen's reagent as a way to prove presence of aldehyde in solution. Tollen reagent is an alkaline solution containing a silver–amine complex ion, $[Ag(NH_3)_2]^+$. Typically, Tollen reagent is prepared by ammonium solution and silver nitrate solution. The complex ion is stable (Madan, 2005) and formed as a result of strong affinity of ammonia towards Ag+.

Studies have shown that cellulose is among the common reducing agent to obtain the Ag from the ion complex. Montazer et al. used cellulose to reduce the silver-amine complex ion to synthesis nanosilver on cotton (Montazer et al., 2012).

It has also been suggested that ammonia concentration and the nature of the reducing agent plays a major role in controlling the Ag nanoparticle size (Sharma et al., 2009). The average particle size increased with increasing NH_3 concentration. The difference in structure of monosaccharides and disaccharides as the reducing agents could give influence to the particle size with disaccharides giving on average smaller particles than monosaccharides. Moreover, the usage of HTAB (n-hexadecyltrimethylammonium bromide) as reducing agent also gave AgNPs of different morphologies: cubes, triangles, wires, and aligned wires.

The ability to reduce size, improve the stability and produce different shapes of the AgNPs is the advantage of this technique. However, its reliance to the chemical solutions which are mostly toxic makes it not environmentally friendly.

2.3.2 Physical approach

Evaporation-condensation and laser ablation are two key strategies in physical approach. This condition is created using heating or laser ablating process. Typically tube furnace or ceramic heater is used to evaporate the source materials. The evaporated vapor can be condensated at the upstream of a tube furnace owing to the existence of temperature gradient. Inert gas like argon is sometimes employed as a carrier to transport the heat vapor from hot to a cooler region in the tube furnace. This process has been widely used to synthesize nanoparticles in thin film form. Zinc oxide (ZnO) and titanium dioxide (TiO_2) of different morphologies such as nanowires, nanorods and nanotubes have also been reported (Pradhan et al., 2003; Ratanatawanate et al., 2008; Roy et al., 2003; Yang et al., 2002). Among factors affecting the size and shape of the nanostructures are type of carrier gas, gas flow-rate, type of substrate, catalyst and operating temperature. Lin reported that the usage of lower flow-rate resulted in smaller SiO_2 nanowires (Lin et al., 2005).

Nanoparticles can also be created using laser ablating process. The high power of the laser beam induces large light absorption on the surface of target, which makes temperature of the absorbing material increases rapidly. As a result, the material on the surface of target vaporizes into laser plume. The vaporized materials can be made to condense into cluster and particle with or without any chemical reaction. Introducing external reactants to react with the vaporized material can form new materials. The condensed particle will be either deposited on a substrate or collected through a filter system consisting of a glass fiber mesh. The laser ablating technique can be carried out in solution or air environment. The ablation efficiency and the characteristics of produced nanosilver particles depend upon many parameters (Amendola et al., 2013; Elsayed et al., 2013; Fong et al., 2013; Moradi et al., 2013; Swarnkar et al., 2016), including the wavelength of the laser impinging the metallic target, the duration of the laser pulses (in the femto, picoand nanosecond regime), the laser fluence, the ablation time duration and the effective liquid medium, with or without the presence of surfactants. One significant advantage in the laser ablation technique is the ability to produce pure and stable nanoparticles (Elsayed et al., 2013).

2.3.3 Biological approach

In recent years, the development of efficient green chemistry methods for synthesis of metal nanoparticles has become a major focus. The green chemistry has been investigated in order to find an eco-friendly technique for production of well-characterized nanoparticles. One of the most considered methods is production of metal nanoparticles using organisms. Among these organisms plants seem to be the best candidate and are suitable for large-scale biosynthesis of nanoparticles. Nanoparticles produced by plants are more stable and the rate of synthesis is faster than in the case of microorganisms. The nanoparticles are more various in shape and size in comparison with those produced by other organisms.

2.3.3.1 Plants extract as reducing agents

A number of biomolecules in plant extracts have been shown to successfully act as reducing agents in the green synthesis of AgNPs. For example, leafs extract of black tea (Uddin et al., 2012) and *Mangifera indica* (Philip, 2011) have been used for the biosynthesis of AgNPs with sizes averaging 20 nm. Extracts from fruits such as the red fruits of the piquin pepper (*Capsicum annuum var. aviculare*) have also been shown to produce AgNPs in the range of 3–10 nm (Mendoza-Reséndez et al., 2013). The aqueous extract of Hovenia dulcis fruit produces AgNPs with sizes of 45 nm (Basavegowda et al., 2014).

The –OH groups present in the plant extracts could be acting as the reducing agent in the green synthesis approach. Source of this –OH groups could be attributed to flavonoids/limonoids/carotenoid (Balch, 2003; Kumar & Pandey, 2013; Tringali, 2003). A study (Yuan et al., 2017) reported that quercetin, an element belongs to the class of flavonoids, which are well known polyphenolic compounds in fruits, and have antioxidant properties have the properties of electron donation or hydrogen atom donation from the free hydroxyl groups. It can also act as capping agents.

2.3.3.2 Microorganism as reducing agents

An alternative way of synthesising metallic nanoparticles is by using living organisms such as bacteria, fungi and plants. Reports showed that the protein in the extract plays role as the reducing agent. Fayaz et al. in their study suggested that the release of extracellular protein molecules could possibly perform the function of the formation and stabilization of AgNPs in aqueous medium (Fayaz et al., 2010).

There are two possible routes that microorganism such as bacteria can be utilized to synthesize AgNPs: (i) bacteria releases biomolecules into the precursor solution to reduce Ag^+ to AgNPs and/or (ii) nanoparticles are produced inside the bacteria cell and later are discharged outside. Several hypotheses have been proposed to explain the role of bacterial proteins, genes and enzymes in both events.

Liu et al. (2012) proposed that Gluconacetobacter xylinum discharges Cl^- from the cytoplasm and generates reductases to reduce the Ag+ to form Ag/AgCl nanoparticles as by-product. Moreover, nucleation of clusters of Ag^+ during the initial phase of AgNP synthesis could induce an electrostatic interaction between the ions and negatively charged carboxylate groups of the cell wall (Wang et al., 2012). Any Ag^+ adsorbed on the bacterial surface can thereby be reduced by cellular reductases and other redox proteins released by the cells to AgNPs.

In the case of AgNPs produced inside the cell, cysteine-rich metalloprotein inside the bacteria cell has been suggested as binding sites to accumulate AgNPs (Shakibaie et al., 2003). The process starts by uptaking the Ag^+ and binds the ions to the histidine sites. Ions are presented to bacterial Ag reduction machinery where biomolecules, generated by reduction machinery, bind to the ions and reduce them to metallic Ag nuclei or seed nanoparticles. These particles undergo growth and assembly to form AgNPs. Three major gene homologues, namely silE, silP and silS of resistance machinery have been suggested to be responsible in production of AgNPs (Parikh et al., 2008).

2.3.3.3 Summary of biological approach

Organic materials released by bacteria and plant act as natural capping and stabilizing agents for AgNPs (Nagaich et al., 2016), thereby preventing their aggregation and providing stability for a longer time. In the case of the AgNPs remain attached to the bacterial cell wall (Parikh et al. 2011), mild sonication could be utilized for recovery.

Although $AgNO_3$ salt has been widely used in the production of AgNPs, other Ag precursor, like silver sulfide (Ag_2S) and silver oxide (Ag_2O) nanoparticles, have also been reported (Debabov et al. 2013; Dhoondia and Chakraborty 2012).

Moreover the protein could probably act as stabilizing agent. Ethylene groups detected by FTIR has been reported to have capability to act as reducing or capping agent (Li et al., 2014). Mohan et al. proposed that the capping properties exist through electrostatic cross-linking between the protein and the synthesized AgNPs (Mohan et al., 2014).

In contrast to other works on ZnO or other metal oxide materials where numerous shapes have been synthesized, work on green synthesized AgNPs have been mostly spherically shaped. While there are few works manage to synthesize AgNPs with different shapes, the shape is usually non-uniform in which we can assume it to exist as a coincidence. Choi et al. reported AgNPs of spherical, triangular, pentagonal, hexagonal with nonequilateral edges, irregular, and urchin-like shapes (Choi et al., 2014). These AgNPs are sometimes referred as anisotropic.

In general, although biological or green approach can be regarded as safe and biocompatible, it is difficult to control the morphology of the nanoparticles (Wadhwani et al. 2014). Contrary to chemical methods in which only a single reducing agent is responsible for the reduction of metal ions to nanoparticles, and hence, easing the process to produce monodispersed nanoparticles (Li et al. 2012), biological methods involve numerous enzymes, proteins, amino acids, genes, etc. This results in formation of polydispersed nanoparticles in bacteriagenic synthesis. To overcome the problem, Shedbalkar et al. suggested that regulation over reaction conditions has could possibly be applied to control the dispersion, morphology and yield of AgNPs (Singh et al., 2015).

2.4 Growth Control Agent

Nanoparticles are prone to clustering either via agglomeration (reversible) or aggregation (irreversible) processes. Agglomerates are an assembly of particles joined together at the corners or edges by a weak adhesion to form larger particles units. On the other hand, aggregates are an assembly of particles that have grown together and are aligned side by side to form also larger units of particles. Agglomeration and aggregation have been a major problem in the synthesis of nanoparticles. It results in structural and morphological non-uniformity.

Particle on nucleation growth will grow on all directions. In the presence of capping agent or surfactant, the nucleated particle will be surrounded and restricts the particle from growing. In general, capping agent is a chemical that prevents nanoparticles from growth whereas surfactant is an agent to prevent agglomeration. Capping agents are also used to prepare nanoparticles in certain desired configuration (size, shape, distributions or dispersion) so that optimum performance can be derived for their applications. On the other hand, surfactant is known to act as stabilizing and dispersing agent. In the growth of nanostructures, the steric forces as a result of the surfactant will keep the nucleated particles separated from each other thus prevents from agglomeration. While the function and role of capping agent and surfactant may seem different, they might actually be the same molecule. For example, PVP (Ahmad et al., 2017; Koczkur et al., 2015; Luo et al., 2015) and CTAB (Naresh et al., 2013; Singla et al., 2009) are used for both purposes.

Different type of capping agent has different growth-preventing strength. For example, comparison between polyethylene glycol (PEG), ethylenediaminetetraacetic acid (EDTA), polyvinyl pyrrolidone (PVP) and polyvinyl alcohol (PVA) in the synthesis of AgNPs showed that the average size was in the order of PEG-AgNPs > EDTA-AgNPs

> PVP-AgNPs > PVA-AgNPs (Ajitha et al., 2016). Capping agents may also act as reducing agent. PVP for instance donates lone pair electrons of oxygen and nitrogen atoms to Ag^+ and thus forms the Ag ions and PVP complex structure (Jovanović et al., 2012). PVA molecules with activated OH-groups free from H-bonding can also adsorb Ag^+ cations to produce Ag^o (Gautam et al., 2006).

Apart from tuning particle size, a low degree of agglomeration of the nanoparticles is highly desirable. Surfactant has been widely used to prevent agglomeration. It is an organic molecule with a structure containing tail (hydrophobic group) and head (hydrophilic group). There are 4 types of surfactants: nonionic, cationic, anionic and amphoteric. These classifications are based upon the composition of the polarity of the head group (Figure 2.3).

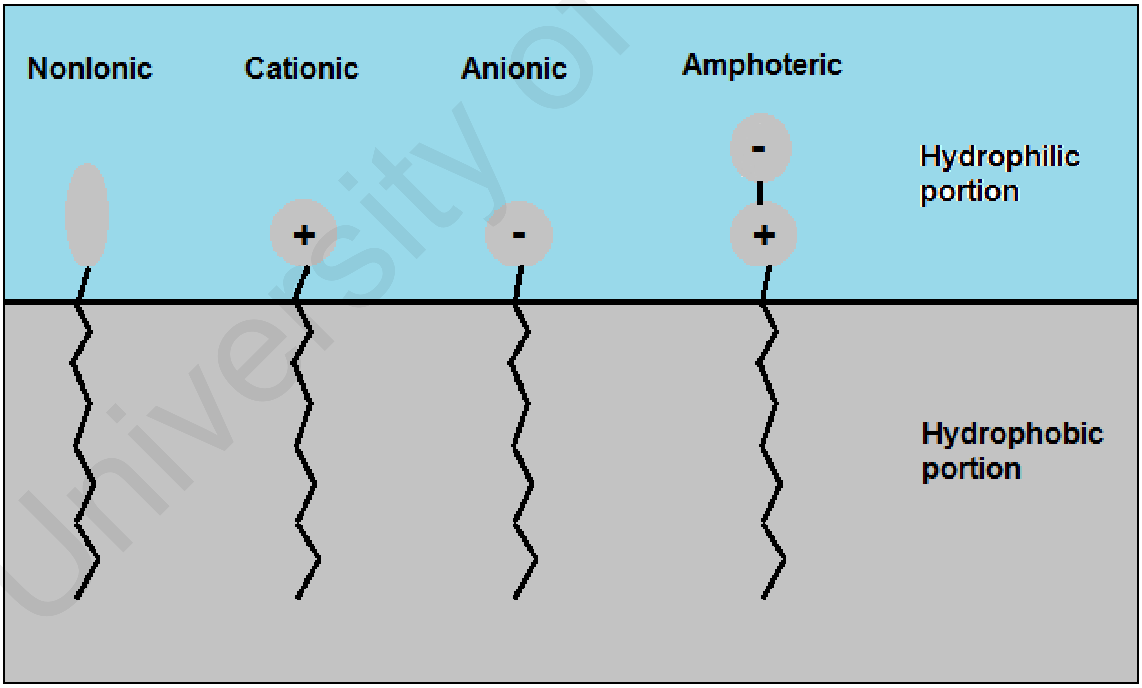


Figure 2.3: Different type of surfactants.

Surfactant will provide nanoparticles with specific superficial hydrophobic or hydrophilic properties, enabling the dispersion in a targeted medium such polar or nonpolar. Cationic surfactants such as cetyltrimethylammonium chloride (CTAC) and cetyltrimethylpyridinium chloride (CPyC) have been reported to stabilize nanoparticles in water (polar solvent) (Mehta et al., 2009). On the other hand, nonionic monomeric and polymeric thiols surfactants were reported in stabilizing AgNPs in paraffin oil (non-polar solvent) (Azzam et al., 2011). Apart from that, report (Mehta et al., 2009) also showed that addition of surfactants during the synthesis process will restrict the growth hence provide the ability to control the size of nanoparticles which previously discussed as the role of capping agent. Moreover, the ability of the adsorbed surfactant molecules to remain intercalated between the particles during post-production of nanoparticles (drying process) has prevented the permanent fusion of the nanoparticles to form bigger particles and therefore keep the stability when redispersed in water.

Stabilizing nanoparticles can be achieved through electrostatic or steric stabilizers. Electrostatic stabilization is the repulsive electrostatic force, which nanoparticles experience, when they are surrounded by a double layer of electric charges. On the other hand, steric stabilization is the encapsulation of nanoparticles by long chain molecules which have hydrophilic and hydrophobic end. In general, steric stabilizer is also the surfactant. Some authors showed that steric stabilizers results in more stable nanoparticles than electrostatic stabilizers (Tejamaya et al., 2012).

The past two decades have seen the introduction of a new class of surface active substance, known as polymeric surfactants. Unlike conventional surfactant which has hydrophobic and hydrophilic group to its tail and head, respectively, polymeric surfactants are macromolecules which contain both hydrophilic and hydrophobic parts in their structure (Raffa et al., 2016). By using polymeric surfactant to establish the steric stabilization, shape and size of nanoparticles can be controlled by changing the polymer chain length (Luo et al., 2005; Shimmin et al., 2004). The use of PVP with different polymer chain lengths could also result in particles to have a different degree of stability although the size is the same (Pastoriza-Santos et al., 2002). Moreover, polymeric surfactant was also used as capping agent to synthesize Ag nanowires (Zeng et al., 2014).

In the case of using polymers to control the stability of nanoparticles during the synthesis stage, formation of nanoparticles can take place either before or after the interaction between the metalic ions (eg; Ag^+, Zn^{2+}) and the polymers. In the former case where reduction process precedes the interaction, no complex formation takes place between metal ions and polymer. Therefore, the growth of the metal particles cannot be properly controlled by the polymers and the protection effect is only obtained after the particle is formed. However, in later case, metal ions and polymer can form a complex before the reduction of the metal ions takes place. In this case, the obtained metal atoms retain their interaction with the polymer (Pastoriza-Santos et al., 2002).

Although a rarity, stabilizer is also used in green synthesis. Shameli et al. reported the use of PEG to stabilize AgNPs synthesized using β-D-glucose as its reducing agent (Shameli et al., 2011). Ahmad et al. used PEG as stabilizer in the green synthesis of AgNPs using chitosan (Ahmad et al., 2011). The former and the latter reported the smallest size of the AgNPs to be 10.6 ± 3.75 and 5.50 ± 1.33 nm, respectively.

On the other hand, PEG, a short chain polymer has also been used as reducing agent in the synthesis by chemical approach. Its reducing strength has been reported to be dependent on its polymer chain length can (Luo et al., 2005). Some work even suggested that PEG can act as both a reducing and stabilizing agent (Nam et al., 2011).

Solvent has also been known to control the growth and nucleation of nanoparticles. Crystallinity and crystallite size of nanoparticles can be tailored by changing solvent (Kumar et al., 2016). It has been demonstrated that concentration of the solvent could also be the factor in affecting the shape and the surface structure (Liao et al., 2008).

This is because the polar characteristic of the solvent could affect both nucleation and growth process (Khoza et al., 2012). For example, short chain length alcohol such as ethanol has exhibited the ability to slow down the nucleation and growth process (Hu et al., 2003).

2.5 Factors Affecting sizes and shapes of the AgNPs in Biosynthesis

Biosynthesis technique provides valuable advantages over the chemical and physical nanoparticles synthesis methods such as cost-effective, environmentally compatible and simple. It requires no manipulation over the pressure, temperature, toxic chemicals and energy of the system. Nevertheless, since the properties of the nanoparticles closely depend on their formation kinetics therefore the composition of the reaction solution condition could play an important role in the kinetics of AgNPs formation.

2.5.1 Effect of pH

The pH of the medium plays important role in the synthesis of nanomaterial by affecting the size and monodispersity of the nanoparticles. Higher or basic pH has been reported to produce highly monodispersed nanoparticles. Roopan et al. (Roopan et al., 2013) reported that at pH 11 highly monodispersed AgNPswere obtained with an average size of 23 ± 2 nm. A difference in pH has been shown to affect the amount of nanostructures formed, whereby higher production of AgNPs was obtained at pH 9 compared to pH 5 (Chitra et al., 2014). Since a variety of biomolecule proteins are postulated to be involved in nanoparticle biosynthesis, there is also a possibility that these biomolecules are likely to be inactivated under the extremely acidic conditions. Roopan et al. (2013) and Ibrahim (2015) reported that no formation of AgNPs was observed at pH 2.The pH also affects the dispersibility and stability of the synthesized AgNPs. Sathishkumar et al. (2009) reported AgNPs were highly dispersed at basic condition (pH > 9) probably due to the electrostatic repulsion at higher pH (Sadowski et al., 2008). In her work, Sathishkumar reported that low pH resulted in formation of big nanoparticles and low dispersing level whereas the effect of high pH is vice-versa. Another work reported on the instability of AgNPs at acidic pH (Sadowski et al., 2008). Table 2.2 summarizes the pH effect on the dispersibility and stability of the AgNPs.

Table 2.2: The pH effect on size and dispersion level of the AgNPs.

pH	**Nanoparticles**	**Dispersing level/Stability**	**Explanation (Sathishkumar et al., 2009)**
Acidic	• Size is big • Low yield	Low	At low pH, the aggregation of Ag nanoparticles to form larger nanoparticles was believed to be favored over the nucleation to form new nanoparticles.
Alkaline	• Size is small • Higher yield	High	At higher pH, however, the large number of functional groups available for Ag binding facilitated a higher number of Ag(I) and subsequently form a large number of nanoparticles with smaller diameters.

2.5.2 Effect of temperature

Temperature can affect the process of ionic Ag reduction. It is a well-known fact that when the temperature is increased, the reactants are consumed rapidly leading to the formation of smaller nanoparticles (Park et al., 2007). The size of silver nanoparticles was reported to decrease with an increase in incubation temperature when the fungus *Trichoderma viride* was employed (Fayaz et al., 2009). This is also in agreement with Ibrahim (2015) who reported that relation by means of surface plasmon resonance (SPR) peak. The AgNPs produced at higher temperature was observed to show peak at lower wavelength region (~412 nm) indicating smaller nanoparticle size whereas those produced at lower temperature showed peak at higher region indicating bigger nanoparticles. In contrast to these reports, Pourmortazavi reported uncertainty or no specific trend in the sizes of AgNPs with respect to the temperature as reactions at 25°C, 50°C, 100°C produce AgNPs of 55, 165, 21 nm, respectively (Pourmortazavi et al., 2015).

2.5.3 Effect of silver salt concentration

The use of silver nitrate ($AgNO_3$) as Ag^+ source is often the choice in the green synthesis method. The Ag^+ is the source for Ag formation. This has been observed in many reports (Banu et al., 2014; Fayaz et al., 2010; Gnanadesigan et al., 2011; Ibrahim, 2015; Panáček et al., 2009; Pasupuleti et al., 2013; Sundaravadivelan et al., 2014). The $AgNO_3$ is soluble in water at room condition. $AgNO_3$ is a strong oxidizing agent. Typically a very low concentration 0.001 M is used for the AgNPs synthesis. Several studies have gone into investigating the effect of Ag^+ concentration to the size of the AgNPs. However, no significant relationship has been observed as reported by Pourmortazavi (2015). The use of low concentration does not always result in the bigger or smaller AgNPs sizes. As opposed to that, another study suggests that when the initial Ag^+ concentrations are increased, AgNPs with smaller sizes are produced (Darroudi et al., 2012).

Apart from that, basic chemistry dictates that higher concentration of Ag^+ will result in more Ag formation. This is typically observed and reported by means of SPR peaks intensity, whereby higher intensity equates to higher yields (Darroudi et al., 2012).

2.5.4 Effect of reaction time

In most studies, UV-vis spectrum has been used to observe and discuss the effect of reaction time. The spectrum could reveal several things: (i) size (ii) yield amount and (iii) level of aggregation/agglomeration. Studies revealed that there will be a gradual increase in the UV-vis absorption intensity when duration of reaction is increased. This corresponds to bigger size and higer yield. Absorption band will also shift. For example a shift from 440 nm to 446 nm, indicates higher aggregation of silver nanoparticles AgNPs (Lu et al., 2013).

2.5.5 Effect of plant extract

Eversince the work of green synthesis started, numerous plant extracts have been used as reducing agent. Reports (Eslami et al., 2016; Khan et al., 2013; Yadav et al., 2015) have gone from very low cost plant like wild grass to the most expensive herbs such as saffron. While the type of the plant could be different, there is not much studies reporting on the significant differences in the morphology of the AgNPs produced.The shape is mostly non-spherical. Sizes are different and not homogenous. Agglomeration and aggregation still dominant. It is inferred that these extracts are only different in terms of their reducing strength which give effect to how fast the extract can reduce the $AgNO_3$ solution.

Table 2.3: AgNPs synthesized by different plant extracts.

No	**Extracts**	**AgNPs (size)**	**Shape**	**Reference**
1	*Crataegus douglasii* fruit	29.28 nm	Nearly spherical shape	(Ghaffari-Moghaddam et al., 2014)
2	*Ganoderma neo-japonicum*	range of 5 nm	spherical	(Gurunathan et al., 2013)
3	*Alternanthera dentata* leaf extract	-	-	(Kumar et al., 2014)
4	leaf extract of *Mimusops elengi*, L.	55-83 nm	spherical shape	(Prakash et al., 2013)
5	*Artocarpus heterophyllus Lam.* seed	average size 10.78 nm	irregular in shapes	(Jagtap et al., 2013)
6	carob leaf	5-40 nm	spherical particles	(Awwad et al., 2013

Table 2.3: AgNPs synthesized by different plant extracts (continued).

No	**Extracts**	**AgNPs (size)**	**Shape**	**Reference**
7	flower extract of *Nyctanthes arbortristis*	5-20 nm		(Gogoi et al., 2015)
8	seeds of *Nyctanthes arbor-tristis*	50-80 nm	mainly uniform spherical shaped	(Basu et al., 2016)
9	leaves extract of *Skimmia laureola*	46 nm	irregular spherical, hexagonal	(Ahmed et al., 2015)
10	*Boerhaavia diffusa*	25 nm	spherical	(Kumar et al., 2014)
11	*Hydrangea paniculata* flower	36-75 nm	spherical and ellipsoidal	(Karunakaran et al., 2017)
12	*Peumus boldus*	18 nm	spherical	(Sánchez et al., 2016)
13	*Capuli cherry*	40-100 nm (white light) 20-80 nm (blue LED light)	nanospheres	(Kumar et al., 2016)
14	*Lantana camara* leaf	3-27 nm	nearly spherical	(Ajitha et al., 2015)

2.6 Agglomeration and Aggregation in Green Synthesis

As can be found in numerous reports (Chen et al., 2008; Jagtap et al., 2013; Murugan et al., 2015; Suganya et al., 2014), most of the green synthesis works opted to use a very diluted system or low concentration of the silver salts (eg; silver nitrate) such as 0.1 mM, 1 mM, 10 mM presumably to reduce the agglomeration and aggregation problem. However, this approach does not seem to prevail. Figure 2.3 shows the field emission scanning electron microscopy (FESEM) images of the AgNPs. While transmission electron microscope (TEM) has always been used to avoid showing poor dispersion and morphological results (Figure 2.4a-f), FESEM can reveal the actual level of both of agglomeration and aggregation. Hence it is a good preference to use FESEM instead of TEM to assess the agglomeration and aggregation level of the synthesized AgNPs.

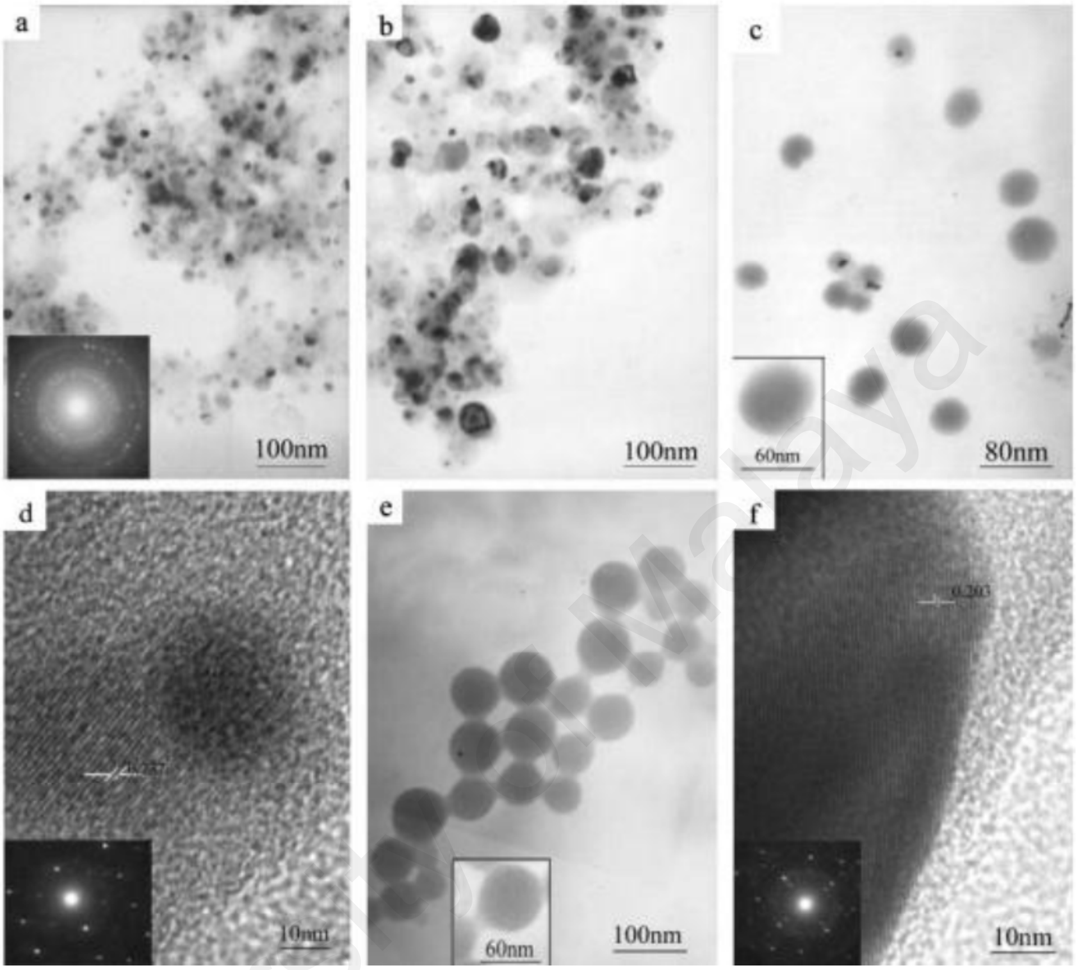


Figure 2.4: (a-f) TEM and HRTEM images of silver NPs green synthesized using Capsicum annuum L. Extract (Li et al., 2007). Reproduced with permission (Appendix B2).

As the agglomeration and aggregation problem is severe and difficult to control, several works have incorporated capping agent to solve the problems (Nadagouda et al., 2009; Sharma et al., 2009). This in some ways resulted in a more uniform sizes and lesser agglomeration as well as aggregation. One work (Raveendran et al., 2003) has shown that it is possible to achieve uniformity in size and stability of the AgNPs without using any capping agent but only starch as the reducing agent.

2.7 Apple and Green Synthesis

Organic materials have been used in green synthesis as a reducing agent owing to their ability to donate electron. The ability to donate electron is directly related to the antioxidant properties of the organic materials. From a medical point of view, an antioxidant is a stable molecule that donates an electron to a rampaging free radical and neutralize it, thus reducing its capacity to damage (Lobo et al., 2010).

Apple is known for its health benefits. It is rich with phytonutrients and phytochemicals. Certain antioxidants in apples have health promoting and disease prevention properties. These properties can be attributed to the content of phenolics and ascorbic acid. Phenolics, are a class of chemical compounds consisting of a hydroxyl group (-OH) bonded directly to an aromatic hydrocarbon group. Phenolic compounds include phenolic acids, flavonoids, tannins, stilbenes, curcuminoids, coumarins, lignans, quinones, and others. The polyphenolic antioxidants contained in apples are responsible for most of the antioxidant activities of the fruit (Huang et al., 2009; Lee et al., 2003) since the antioxidant activity by ascorbic acid is less than 0.4% (Eberhardt et al., 2000).

According to Boyer et al., phytochemicals contents of apple include quercetin, catechin, phloridzin and chlorogenic acid, all of which are strong antioxidants (Boyer et al., 2004). Quercetin is a type of flavonoid antioxidant, catechin is a type of natural phenol, phloridzin is a family of bicyclic flavonoids and chlorogenic acid is a polyphenolic compound.

Apple has always been considered a fruit with low chlorophyll and carotenoid contents (Delgado-Pelayo et al., 2014). On the other hand, using reflectance spectral measurement, Solovchenko et al. showed that apple has no carotenoid contents at all (Merzlyak et al., 2003). Apple also contains protein (Zheng et al., 2013). In general for an apple weighing approximately 182 g, the protein is around 0.5 g.

A study by Yuan et al. reported that quercetin has antioxidant properties and ability for electron donation or hydrogen atom donation owing to its free hydroxyl groups (Yuan et al., 2017). As other reports has suggested, quercetin can reduce Ag^+ and also act as stabilizing agent to prevent agglomeration (Osonga et al., 2016). On the other hand, Lokina et al. proposed that it was the role of polyphenols present in the apple extract that act as a reducing and stabilizing agent to produce the AgNPs (Lokina et al., 2014).

2.8 Green Synthesized AgNPs in Biological Applications

In recent years, AgNPs play a major role in antibacterial and mosquito control programs. Numerous green methods have been proposed as a means to synthesize AgNPs for these purposes. Of the AgNPs synthesized by fungus and bacteria, AgNPs synthesized using plant extract gain more interest in antibacterial and mosquito control owing to its availability, simplicity and low-cost (Ali et al., 2016).

2.8.1 Antibacterial application of biosynthesized AgNPs

The usage of biosynthesized or green synthesized AgNPs in antibacterial application has been growing owing to its ability of eliminating broad range of bacteria(Awwad et al., 2013; Jagtap et al., 2013; Khalil et al., 2014). *Escherichia coli (E. coli), Pseudomonas aeruginosa (P. aeruginosa)*and *Klebsiella pneumoniae (K. pneumonia)* are the Gram-negative bacteria, whereas *Methicillin-Resistant Staphylococcus aureus (MRSA)* and *Staphylococcus aureus (S. aureus)* are the Gram-positive bacteria that are responsible for majority of hospital-acquired infections, namely, nosocomial infections. Urinary tract infections are the most common type of nosocomial infections (Krein et al., 2012). Surgical site infections, bloodstream infections, and *pneumonia* are the other most common types (Inweregbu et al., 2005).

Toxicity of AgNPs against bacteria can bedirectly assessed by minimum bactericidal concentration (MBC), i.e., the lowest concentration of nanoparticles that kills 99.9% of the bacteria (Ruparelia et al., 2008). MBC can be regarded as the most basic laboratory measurement, yet effective and reliable to assess antibacterial properties.

2.8.2 Larvicidal application of biosynthesized AgNPs

Aedes aegypti is a vector responsible in spreading several arbovirus diseases including dengue, chikungunya, and dengue hemorrhagic fever (Yang et al., 2009). Cases involving this vector are globally increasing. The statistical numbers of dengue cases have exceeded 1.2 million in 2008 while > 2.2 million were reported in 2010 with an estimated 500,000 individuals infected with dengue (mainly severe dengue) being hospitalized annually. Of these, almost 2.5% of the affected population succumbed to death (Organization, 2012). In many countries, such as in Malaysia, dengue has became endemic (Halstead, 2008). Since the first reported case in Malaysia has been documented in 1902 (Skae, 1902), several dengue outbreaks have been reported in 1974, 1978, 1982 and 1990, with the total number of dengue cases has increased (Lam, 1994). There is currently no effective specific treatment for dengue. However, with an effective and practical control strategies such as frequent mosquito growth eradication, the transmission and spread of the dengue virus carrier can be reduced and inhibited.

Green synthesis of AgNPs have been reported to possess anti-larvae application (Santhoshkumar et al., 2011). Soni and Prakash (2013) had reported the potential of AgNPs synthesized by a fungus *F. oxysporum* and found LC_{50} and LC_{90} values of 4 and 11.48 against *Ae. aegypti*, respectively. Owing to its simplicity and cheap procedures, the green approach in the anti-larvae application has been gaining interest worldwide.

Synthesis of AgNPs by filamentous fungus *Cochliobolus lunatus* has been reported to have its larvicidal activity against the third instar larvae of *Ae. aegypti* (LC_{50} = 1.48; LC_{90} = 3.33) (Suganya et al., 2014). The value of LC_{50} = 0.79 ppm and LC_{90} = 1.09 ppm have been observed for *Ae. aegypti* treated with *B. bassiana* (Bb) silver nanoparticles (AgNPs) (Banu et al., 2014). Banu et al. had reported the activity of synthesized AgNPs using Bacillus thuringiensis extract against the third larvae of *Ae. aegypti* and found that LC_{50} = 0.10 ppm and LC_{90} = 0.39 ppm (Banu et al., 2014). Synthesized AgNPs using fungus *C. tropicum* extract tested against the third-instar larvae of *Ae. aegypti* resulted in the values of LC_{50} and LC_{90} to be 4 and 8.91 ppm, respectively (Soni et al., 2012). LC_{50} and LC_{90} against *Ae. aegypti* using AgNPs synthesized from mangroove plant extract were found to be 0.585 and 2.615 mg/L (Gnanadesigan et al., 2011).

2.9 Summary and Research Scope

Numerous physical and chemical methods have been developed with an aim to facilitate and cater demands of nanotechnology. Each method has an edge over the other. As nanotechnology advances, the concern over environmental issue arise from this sector comes into play. Pollution due to the release of chemical wastes into rivers and oceans is gaining attentions worldwide. To address such concern, synthesis of nanomaterials using high energy radiation has been proposed and researched. The technique do not requires any reducing chemicals, hence reduce the dependencies towards toxic chemicals. While it can produce nanomaterials with properties comparable to the other methods, its low-productivity and high-cost owing to its reliance towards the usage of radiation source remains a hurdle.

This has opened up to a new branch of research which is low cost, simple and reliable. Green approach has been proposed as it has shown some good results in developing nanomaterials. Considering the availability of numerous natural plants and microorganism to be exploited for the purpose, this highly motivates the efforts to improve the research.

As much as the conventional methods (chemical and physical methods) facing the problems in producing nanomaterials with high stability and less agglomeration, the green synthesis also face the same problems. It is highly anticipated because this technique deals with organic compounds to act as substitution to those toxic chemicals. Organic compound contains numerous other elements that need to be studied. One direct argument in the ability of natural extracts and microorganism to produce nanomaterials lies in their antioxidant properties, which is the ability to donate electrons. Moreover, it is noteworthy to mention that these compounds are also degradable due to its organic nature.

In conventional methods, every chemical used is known to its details on how it will impact the process because the content of the chemical is known. This is in contrast with organic compound. Although the green approach is advantageous in term of cost and simplicity, the stability and agglomeration of the products remain a problem. Therefore, while the ability to produce nanomaterials by green approach has been proven, the trend of research which seems to abandon these problems must be addressed.

As reviewed, some works have suggested the use of PEG in their green synthesis method as a stabilizer. However, the stability achieved and reduction in the degree of agglomeration have not been discussed and comparison was not made with respect to the AgNPs synthesized without the PEG.

Therefore, this study will replicate those works in order to study the contribution of the PEG to the stability/dispersion, agglomeration and aggregation of AgNPs. As the use of stabilizing agent could be seen as 'not-so green' approach and in order to avoid dependency on the PEG, the use of ethanol in the system will be proposed as a possible substitute for the PEG. This is because solvent is also known to affect the growth of nanoparticles. PEG and ethanol will act as the growth control agent in this synthesis process. As heat, duration, concentration and pH have been shown (in literatures) to affect the synthesis, these parameters will also be studied on their effect towards the morphological and stability of the AgNPs. Antibacterial and larvicidal properties of the synthesized AgNPs will be compared. Observations on how the level stability, agglomeration, aggregation and sizes affect the antibacterial and larvicidal properties of the AgNPs will be discussed and their relationship will be derived.

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Introduction

In this chapter, the experimental set up for the synthsis of AgNPs nanostructures and its antibacterial and larvicidal applications are presented. In section 3.2, the preparation and procedures of different synthesis parameters such as heating time, heating vs non-heating method, different ratio of Ag salt/extract ratio, growth control agent (solvent/stabilizing agent) and pH are discussed. In section 3.3, procedures for sample characterisation are presented. In section 3.4, antibacterial and larvicidal tests setup are presented. Working principle of the characterization instruments are discussed in Appendix A.

3.2 Synthesis of AgNPs

Red apples were bought from a local grocery shop, and $AgNO_3$ was purchased from Sigma-Aldrich (M) Sdn BhD. The apple extract was prepared by cutting the apples into small pieces, which were then thoroughly washed with running tap water. Next, 100 g of the small cut apples was put in 200 mL of deionized water, which was heated for 1 h at 80°C. The extract was filtered using filter paper, and the filtrate was later used as the reducing agent for AgNP preparation. The synthesis of AgNPs was carried out by using 50 mL of the apple extract in 50 mL of 0.1 M aqueous $AgNO_3$ solution. The mixture was stirred and heated at 80°C.

3.2.1 Effect of heating time

The effect of heating time was assesed by analyzing the absorption spectra of the AgNPs. Preparation of AgNPs was carried out at 80°C and 1.5 mL of samples from the reaction medium were withdrawn at different time intervals, which were 0, 5, 10, 15, 20, 30, 60 and 90 min to record the UV-vis absorption spectra of the formed AgNPs at these time intervals.

3.2.2 Effect of heating vs non-heating method

The experiment with heating approach was carried out at 80 °C for 1-h whereas the non-heating sample was prepared at room temperature (27 °C) for also 1-h. The other parameters were set constant: concentration of $AgNO_3$ solution (0.1 M) and volume ratio of $AgNO_3$ solution : apple extract (1:1).

3.2.3 Effect of Ag salt/extract ratio

Volume ratio of $AgNO_3$ solution to the apple extract was varied to observe its effect towards the shape of the nanoparticles. The ratios of 1:4, 1:9, 4:1 and 9:1 were used. The other parameters were set constant: no heat, concentration of $AgNO_3$ solution (0.1 M) and duration of reaction was set for 24 h.

3.2.4 Effect of growth-control agent

Solvent and polymers can be used to stabilize nanoparticles. In this study, ethanol as solvent and polyethlene glycol (PEG) as stabilizing agent will be used to control the growth. For each sample preparation, the $AgNO_3$ solution, apple extract and ethanol/PEG were mixed with 1:1:1 ratio. The experiment was carried out using non-heating approach and at room temperature (27 °C) for 24 h. The concentration of the solvent and stabilizing agent was varied. Table 3.1 lists the concentrations and setup used.

Table 3.1: Concentration of the PEG and ethanol.

No	Growth control agent	Concentration (vol. %)		
1	Polyethlene glycol (PEG)	10	30	50
2	Ethanol	10	30	50

3.2.5 Effect of pH

To investigate the effect of pH towards the AgNPs formation, the experiment was carried out at both acidic and alkaline conditions. The pH of the precursor was adjusted to be 1, 4, 7 and 11 by using nitric acid (HNO_3) and sodium hydroxide (NaOH).

3.3 Characterisation of AgNPs

The AgNPs were characterized by FESEM equipped with energy dispersive x-ray spectroscopy (EDX), x-ray diffraction (XRD), high resolution transmission electron microscopy (HRTEM), Uv-Vis spectroscopy, Fourier transform infrared (FTIR), Zeta Potential and particle size distribution (PSD).

The procedures for sample preparation for characterisation are as follows:

a) Field Emission Scanning Electron Microscope (FESEM)

For FESEM characterisation, the samples were first cleaned by rinsing the sample in deionized water and centrifuged in 1.5 mL vials for 2 min. The centrifugal speed was set at 6500 rpm. The process of rinsing and centrifuging was repeated three times. The precipitate from the vials was dried on an aluminium foil in a furnace at 300 °C for 2 h. Observation of the nanoparticles was performed by a SU9000 HITACHI (SE, 2.00 kV).

b) UV-Vis Spectroscopy

1.5 mL of the AgNPs solution was withdrawn and transfered into vials. Measurement of wavelength was set and taken from 300 to 700 nm.

c) Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

The measurement required the solution to be very dilute. Therefore, a series of sample dilutions was prepared. Dilution factor of 1:10, 1:50 and 1:100 were used. 2 mL of samples were then filled into plastic vials before inserted into the ICP-MS instrument (Agilent's HMI technology).

d) X-ray diffraction (XRD)

A 1 g of sample was loaded onto a silicone plate, inserted into the measuring chamber and automatically run by the machine. The 2θ-wavelength for the measurement was set from 20 to 90 degrees with a scan rate of 0.02 s^{-1}.

e) Zeta Potential & Particle Size Distribution

The measurement of the zeta potential and particles size distribution was outsourced to an outside company due to inavailability of the equipment in the lab. For the measurement, 10 mL solution of each sample was used.

f) Fourier Transform Infrared (FTIR)

By using a micropipette, approximately 100 ul of AgNPs solution was dropped onto the KBr plate. Second plate was placed on top of it, sandwiching the liquid samples. The plates were subsequently put into a sample holder and measurement was recorded from 4000 to 400 cm^{-1} with resolution of 4 cm^{-1}.

g) HRTEM

AgNPs were dissolved in ethanol. The mixture was ultrasonicated to mix and produce a uniform suspension of the AgNPs in the ethanol. The suspension was later centrifuged. Supernatant of the suspension was taken and dropped onto the TEM grid mesh. The grid mesh was allowed to dry before the viewing process was proceeded. Observation of nanoparticles was performed by a TEM (FEI Tecnai TF20) operated at 200 kV.

3.4 Antibiorganism Applications

3.4.1 Antibacterial test

Minimum bactericidal concentrations (MBCs) by broth dilution method were used to test the antibacterial properties of the synthesized AgNPs against five bacterial strains, namely, *Escherichia coli, Pseudomonas aeruginosa, S.Pneumonia, methicillin-resistant Staphylococcus aureus (MRSA)* and *Staphylococcus aureus*. AgNPs at 1000, 500, 250, 125, 62.50, 31.25, 15.60 and 7.80 μg/mL in suspensions were used to determine the lowest bactericidal concentration needed to prevent the growth of bacteria. Live cells of the bacteria at concentrations of 5×10^5 CFU/mL were inoculated with different concentrations of AgNPs for 24 h. For each bacterial strain, three replications were done.

3.4.2 Larvicidal test

The larvicidal activity was conducted using the established protocols provided by World Health Organization (WHO) (LARVICIDES, 2005) and as per the method of Gnanadesigan et al. (2011) with some modifications introduced. Screening test to determine the LC_{50} and LC_{90} was carried out. The LC_{50} and LC_{90} are the general parameter used to measure the lethal concentration required to eliminate 50% and 90% of the larva, respectively. For each bioassay test, 20 fourth instar larvae of *Ae. aegypti* were placed in 3 batches containing a mixture of 100 mL of distilled water and 1.0 mL of the Ag solution. The control containing only 100 mL of distilled water and 20 fourth instar larvae of *Ae. aegypti* was also set up in the present study. To assess the toxicity of the AgNPs at high concentration, 500 ppm of the AgNPs were used. The numbers of dead larvae were monitored and counted each 10 min.

CHAPTER 4

RESULTS

4.1 Synthesis of Ag Nanoparticles

4.1.1 Heating time

To observe the effect of heating time towards sizes and morphology of the AgNPs, different heating times were used: 15, 30 and 60 min. Heating temperature and ratio of silver nitrate/extract were set constant to 80 °C and 1:1, respectively. FESEM images in Figure 4.1 show that the AgNPs formed are in the range of 30-70 nm and agglomeration

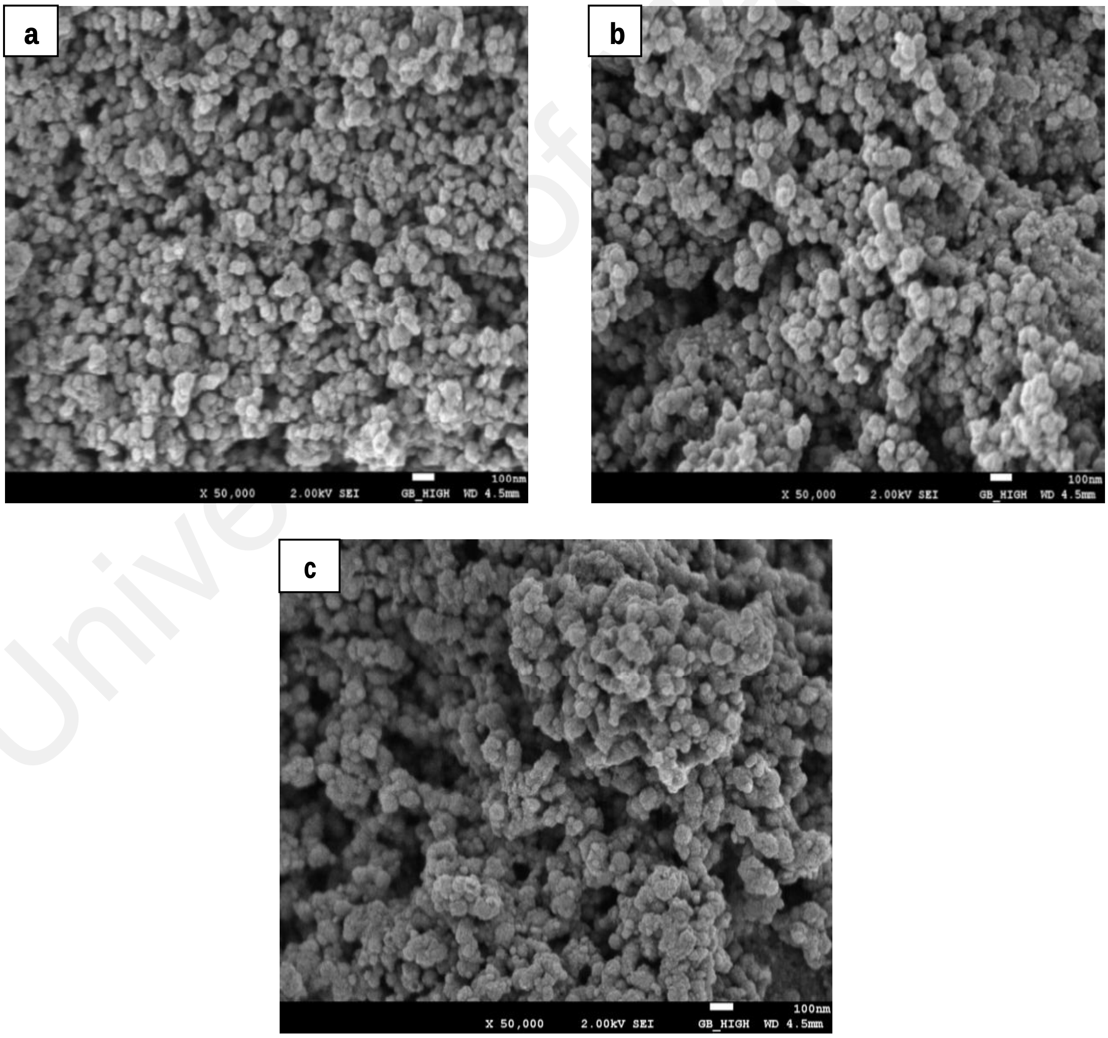


Figure 4.1: AgNPs with different heating times of a) 15 b) 30 and c) 60 min.

as well as aggregation seems to be dominant. No signifant morphological different is observed between the three samples. The samples were agglomerated and aggregated.

UV-vis spectroscopy has been widely used to detect the presence of AgNPs during green synthesis (Karuppiah et al., 2013; Philip, 2010). In particular, absorbance in the range of 420-450 nm has been used as an indicator to confirm the reduction of Ag^+ ions to metallic Ag (Dong et al., 2014; Karuppiah et al., 2013). In this study, the formation of AgNPs was monitored by measuring UV-vis spectra at different time intervals (Figure 4.2). As the time increased, the intensity of this absorbance increased, indicating increase in the amount of AgNPs produced from the mixture. Further prolonging reaction duration to 90 min, absorption intensity decreased. This is because particles start to coalesce, forming big agglomerates which are heavier and not well dispersed in the solution. These agglomerates increase further in size and finally will precipitate and therefore they will not contribute to the measurement anymore (Ziegler et al., 2011). Absorption band shifts from 440 nm to 446 nm, which indicates higher aggregation or formation of bigger size silver nanoparticles (Lu et al., 2013).The broad spectrum could probably be attributable to several reasons: (i) encapsulation of AgNPs by organic elements which originated from apple extract (ii) aggregation (Lu et al., 2013) and (iii) extra fine nature (Ahmad et al., 2011) or high homogeneity (Ahmad et al., 2012; Zargar et al., 2011) of the AgNPs.

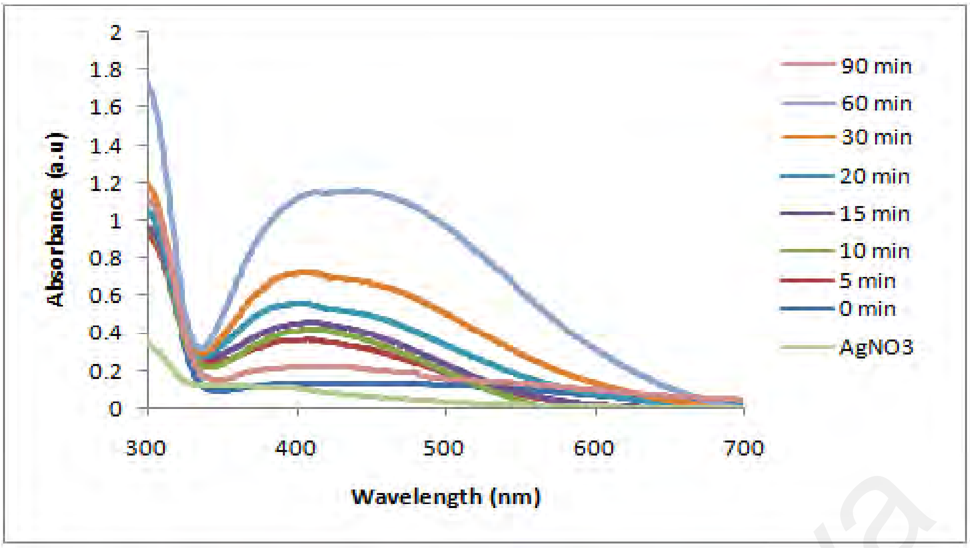


Figure 4.2: UV-Vis spectra of AgNPs measured at different time intervals.

AgNPs are well-known for their surface plasmon resonance (SPR) properties, which originate from collective oscillation of their conduction electrons in response to optical excitation (Yong et al., 2006). The SPR frequency has been shown to depend on sizes, shape and aggregate. Due to its size-dependent nature, typically, the peak plasmon resonance will shift to longer wavelengths and broaden as diameter and aggregation increases. When aggregate, the particles become electronically coupled and this coupled system has a different SPR than the individual particles (Oldenburg, 2012). Therefore, the plasmon resonance will also be red-shifted to a longer wavelength than the resonance of an individual nanoparticle. In this study, however, as the duration of heating increased, the peaks of the AgNPs did not demonstrate any significant degree of shifting towards longer wavelength. Only broadening of spectrum was observed. The shift was negligible. This indicates that the size and level of aggregation did not much differ among the samples. As can be seen through FESEM images (Figure 4.1), the sizes, shape and aggregation were indeed almost the same in all the samples.

The XRD spectrum confirmed the crystalline structure of the precipitate as Ag (Figure 4.3). The peak values at 2θ = 38.15°, 44.35°, 64.59°, 77.47°, and 81.60° correspond to the (111), (200), (220), (311), and (222) lattice planes of the face-centered cubic crystal structure of AgNPs, respectively. Pure Ag crystallite was obtained, while some other studies reported the presence of peaks corresponding to unassigned peaks (Karuppiah et al., 2013), weak peaks (Oluwafemi et al., 2013), oxide of Ag (Mendoza-Reséndez et al., 2013), or incomplete peaks (Saxena et al., 2012). Philip et al. proposed that unassigned peaks were probably observed due to the fact that the crystallization of bioorganic phase occurs on the surface of the nanoparticles (Philip et al., 2011), which in our case does not occur.

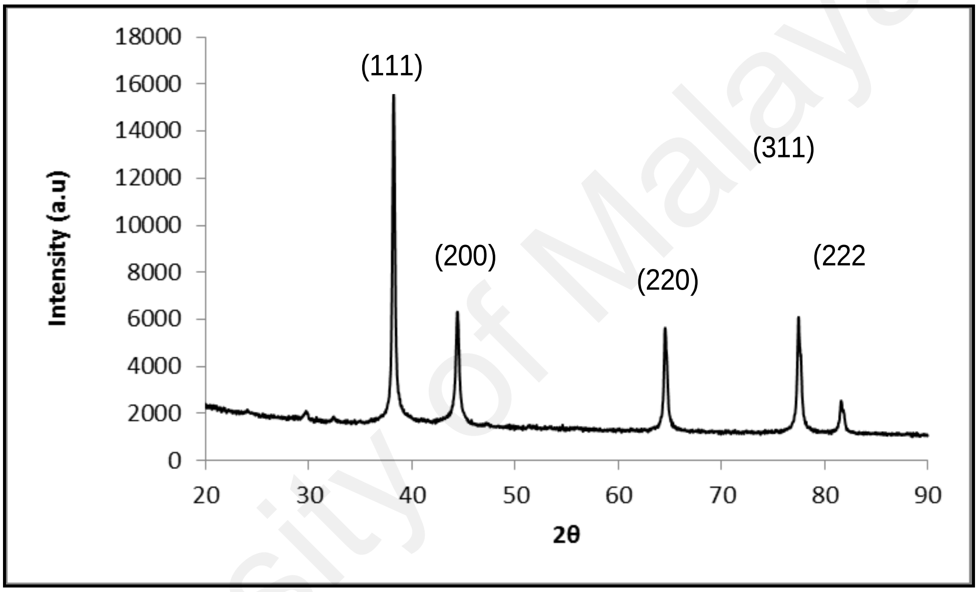


Figure 4.3: XRD spectrum of AgNPs green-synthesized using apple extract.

The FTIR spectra of AgNPs and apple extract are shown in Figure 4.4. The AgNPs demonstrated peaks at 3366 cm^{-1} for O-H stretching of alcohols (Basavegowda et al., 2014) and 1658 cm^{-1} for C=O strecthing of amide (Subashini et al., 2014). The C-O stretching of esters peaks at 1142 cm^{-1} and 1052 cm^{-1} were also recorded, which is in the same range as Edison who reported the C-O stretching of esters at 1032 cm^{-1} (Edison et al., 2012). Apple extract revealed peaks at 3412 cm^{-1} attributable to O-H stretching of alcohols, 2981 cm^{-1} to C-H stretching, 1727 cm^{-1} to C=O stretching of carbonyl or aldehyde, 1663 cm^{-1} to C=O stretching of amide, 1037 cm^{-1} to C-O stretching of ester, 836 cm^{-1} to =C-H bending of alkene. The shift in the wavenumber of alcohol, amide and ester observed at the AgNPs with respect to the apple extract indicates that these functional groups could probably involved in the synthesis of the AgNPs.

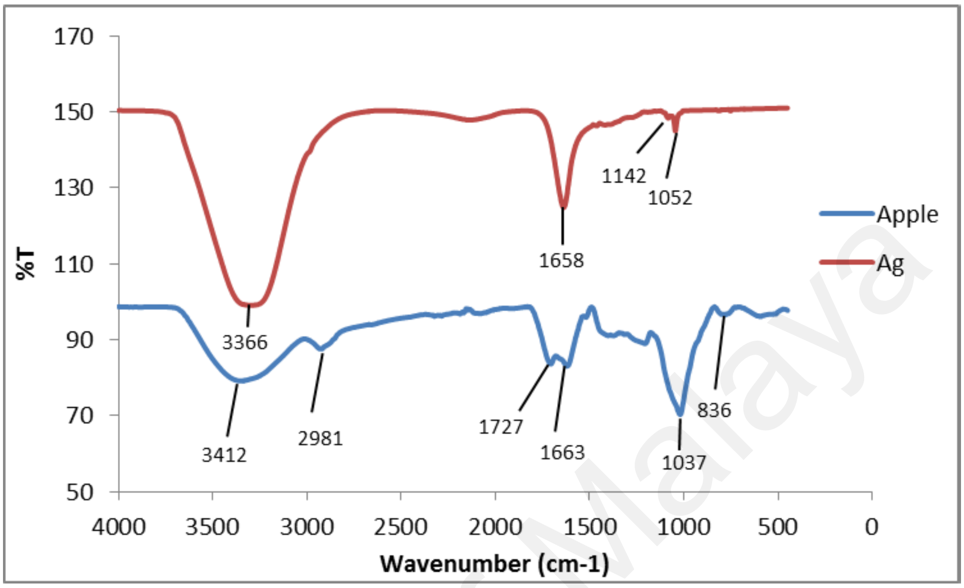


Figure 4.4: FTIR spectrum of AgNPs green-synthesized using apple extract.

4.1.2 Heating condition: Heating and without heating

In this section comparison between heating and non-heating approach is carried out. Quantifiable data is neccessary to analyse the quality of the particle. Zeta value and size distribution have been used as the main indicators for this purpose. Apart from that, other characterizations were also carried out to complement the observation. Figure 4.5 shows the visual observation of both samples obtained by FESEM. However, no significant morphological differences can be observed. The AgNPs was observed to be agglomerated, aggregated and non-uniformed spherical shapes.

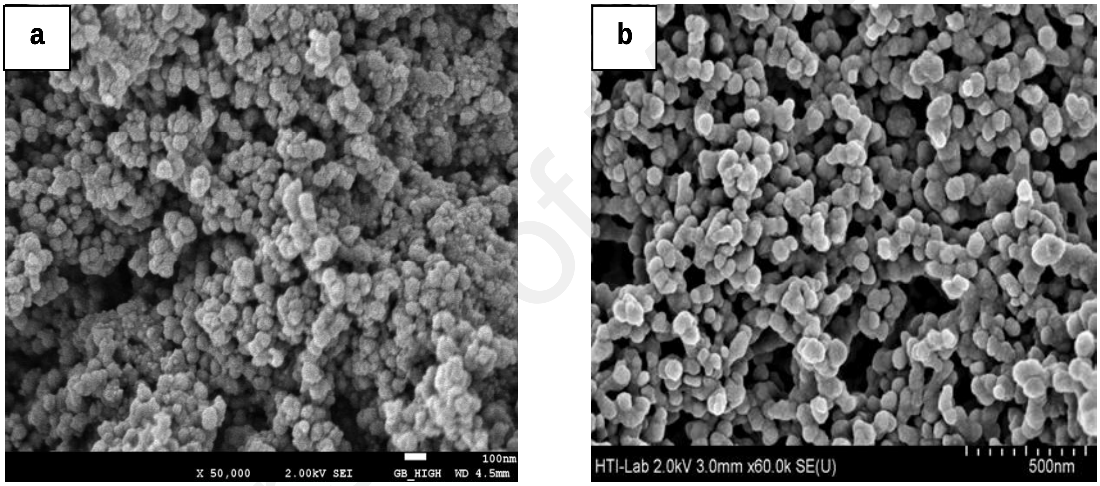


Figure 4.5: FESEM images of (a) AgNPs-T (heat 30 min) and (b) AgNPs-RT.

Zeta potential is an essential characteristic parameter for nanosuspensions (Müller et al., 2001). It gives information on the stability and dispersibility of a material in a certain solution. High dispersibility of the AgNPs in the solution would allow better interaction between the AgNPs and the bacteria or larvae, therefore increasing the effectiveness of the solution. The zeta potential of AgNPs was observed to be 10.1 ± 1.11 mV and 4.57 ± 0.50 mV for AgNPs-RT and AgNPs-T, respectively (Table 4.1). High zeta potential value (AgNPs-RT) indicates electrically stabilized particles while particles with low zeta value (AgNPs-T) tend to coagulate or aggregate. Particle size distribution (PSD) tests showed that the average size of AgNPs-RT and AgNPs-T to be 79.3 ± 2.64 nm and 93.2 ± 3.20 nm, respectively (Table 4.1 & Figure 4.6).

Table 4.1: Summary of the zeta value and size of the AgNPs synthesized by both method.

Sample	Zeta value (mV)	Size (nm) by PSD	Size (nm) by XRD
AgNPs (heat)	10.7 ± 1.25	93.2 ± 3.20	29.20
AgNPs (room temperature)	40.1 ± 5.44	79.3 ± 2.64	13.32

Studies (Hyung et al., 2007; Martí et al., 2007; Roy et al., 1997) have suggested that there are possibilities that the organic compound (eg; microbes, plant) may as well act as stabilizing agent. Stabilizing agent promotes better stability and dispersibility. The organic stabilizing agent adhere to the surface in the form of thin layer encapsulating the AgNPs. However, the organic compound is typically temperature-sensitive. It can be decomposed or degraded by heat. If the degradation or decomposition of the organic compound takes place, it would affect the dispersibility of the AgNPs. This is probably the reason why the AgNPs prepared by heat has lower zeta value as compared to the AgNPs prepared at room temperature. The heat has decomposed the organic compound encapsulating the AgNPs therefore reduced its dispersibility.

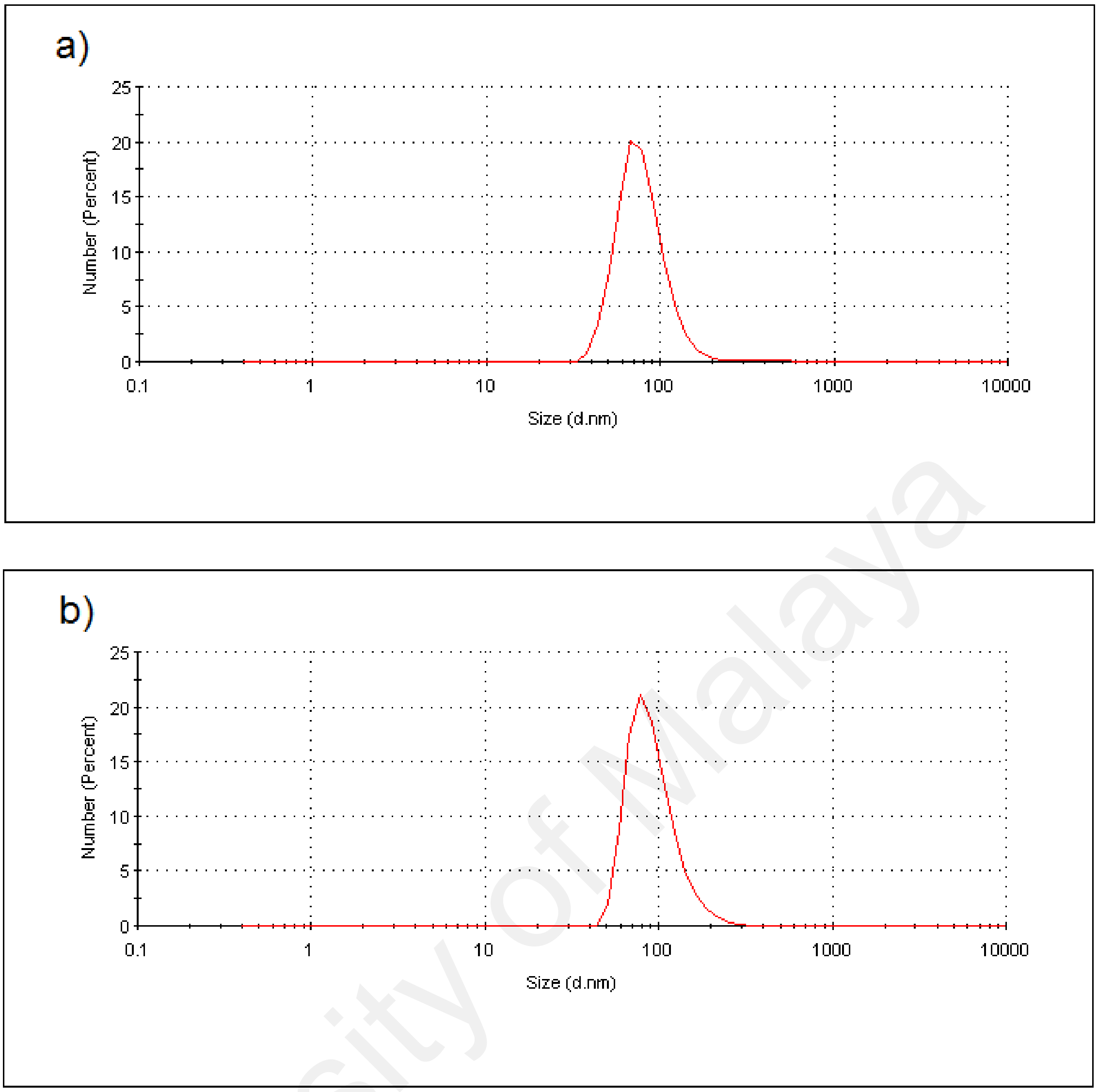


Figure 4.6: Particle size distibution by number of (a) AgNPs-RT and (b) AgNPs-T.

The X-ray diffraction pattern intense peaks were observed at 2θ values of 38.24°, 44.42°, 64.55° and 77.50° for AgNPs- RT and 38.21°, 44.35° and 64.55° and 77.50° for AgNPs-T. These peaks correspond to (111), (200), (220) and (311) Bragg's reflections based on the fcc structure of AgNPs, respectively. The average particle size of silver nanoparticles can be estimated using Debye-Scherrer equation

$$n = \frac{K\lambda}{\beta cos\theta}$$

where K is the Scherrer constant, λ is the X-ray wavelength (1.5418 Å), β is the width of the XRD peak at half height and θ is the Bragg angle. The strongest peak from both samples was identified and from the Scherrer equation, the average crystallite sizes are found to be around 29.20 nm and 13.32 nm for AgNPs-T and AgNPs-RT, respectively. The difference in average crystallite size obtained from PSD measurement and Debye-Scherrer equation (based on XRD spectrum) probably arises because of the different working component and precision of the instrument; PSD uses laser whereas XRD uses X-ray to measure angular diffraction when beam is scattered through. Scattering is dependent on the difference in the electron density of particles (Merkus, 2009). Therefore, a difference in wavelength used can result in different scattering and diffracting amount, hence affecting the data used to approximate the size of the AgNPs.

The AgNPs were also evaluated through UV-Vis spectrophotometer in a range of wavelength from 300 to 700 nm (Figure 4.7). The maximum peak at 420 nm indicates the production of AgNPs. This is similar to the surface plasmon vibrations with characteristic peaks of AgNPs prepared by chemical reduction (Banu & Balasubramanian, 2014; Santhoshkumar et al., 2011). As the intensity of UV-Vis spectrum proportionates to the amount of Ag, therefore higher intensity of the AgNPs-T spectra compared to the AgNPs-RT's suggests that the usage of heat has resulted in the production of more AgNPs.

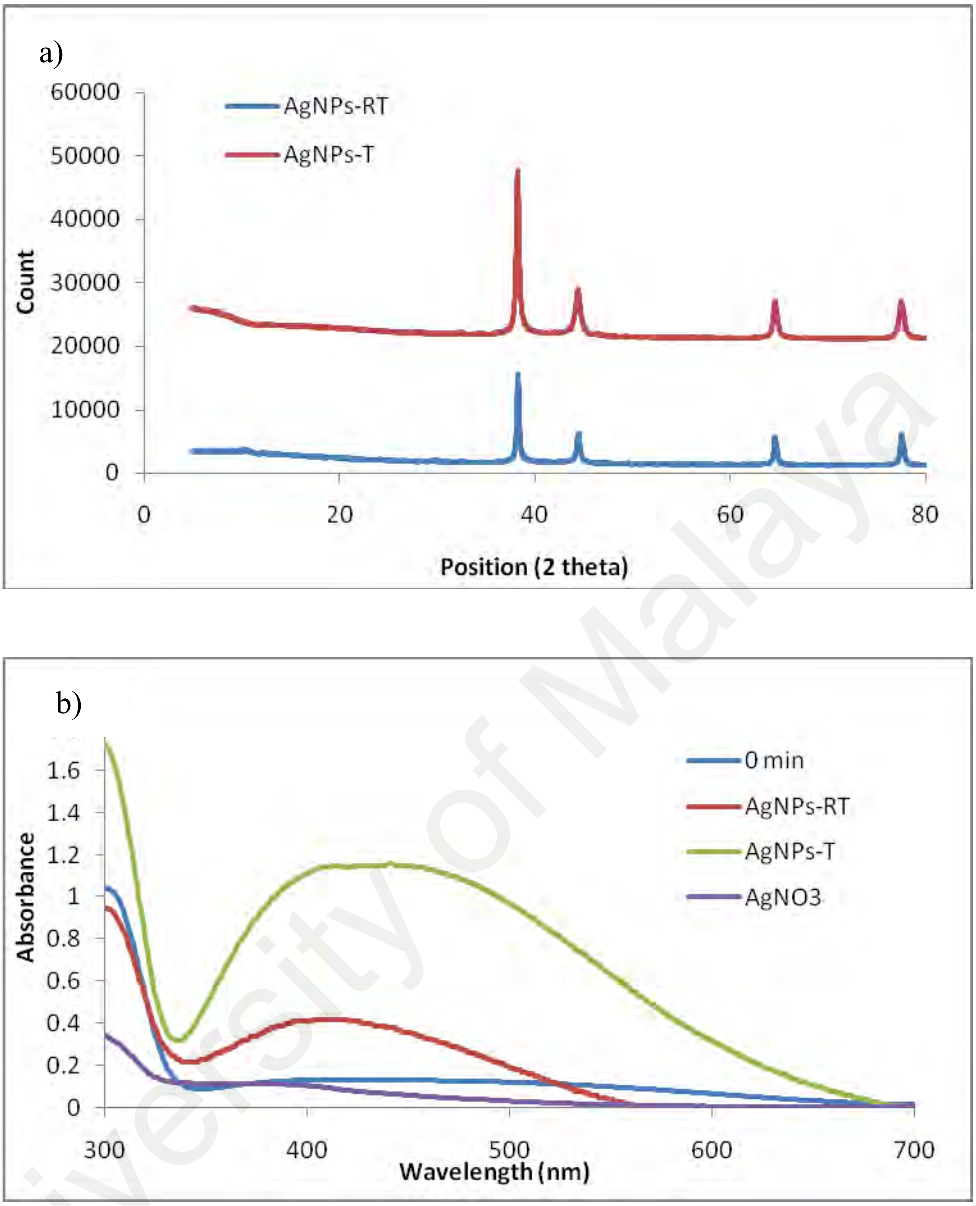


Figure 4.7: a) XRD and b) UV-Visible spectra of the AgNPs.

4.1.3 Ratio of silver nitrate and extract

In section 4.1.2, it has been shown that the preparation of AgNPs without heat yield better results in terms of particle size and agglomeration level. Therefore the non-heating method has been employed in this section onwards to investigate the effect of different ratios of the silver nitrate and the extract. Four different ratios were used to investigate their effect towards particle aggregation. The ratios of silver nitrate to extract used were 1:4, 1:9, 4:1 and 9:1. It can clearly be observed using FESEM that aggregations are still obvious in all of the samples (Figure 4.8). The shape, morphology and size were also observed to be almost the same. In general, the AgNPs were spherical in shape but aggregated and attached together. The size of the spherical structures was in the range of 50-150 nm. The formation of nanoparticles is evidently still in favor of aggregation.

Khalil et al. reported that modifying leaf extract ratio by increasing its concentration from 1 to 5 ml in the reaction mixture decreases the particle size (Khalil et al., 2014). Moreover, apart from being the reducing agent, it was also found that at higher concentration the leaf extracts act as capping agent to successfully prevent aggregation, whereas at lower concentration aggregation prevails. This is because at lower concentration, the quantity of the extracts is not sufficient to act as capping agent. Another study suggested that increasing concentration of Ag^+ in the system increases the mean size of AgNPs (Zain et al., 2014). According to the study, on contrary, increasing concentration of extracts decreases the size. This is because the extracts may prevent the growth of the nanoparticles by adsorbing onto their surfaces. As a comparison, the findings of this study do not conform to what is obtained by these studies probably due to the higher concentration of $AgNO_3$ was used (0.1 M), whereas both of the studies used very diluted $AgNO_3$ concentration ($1x10^{-3}$ M).

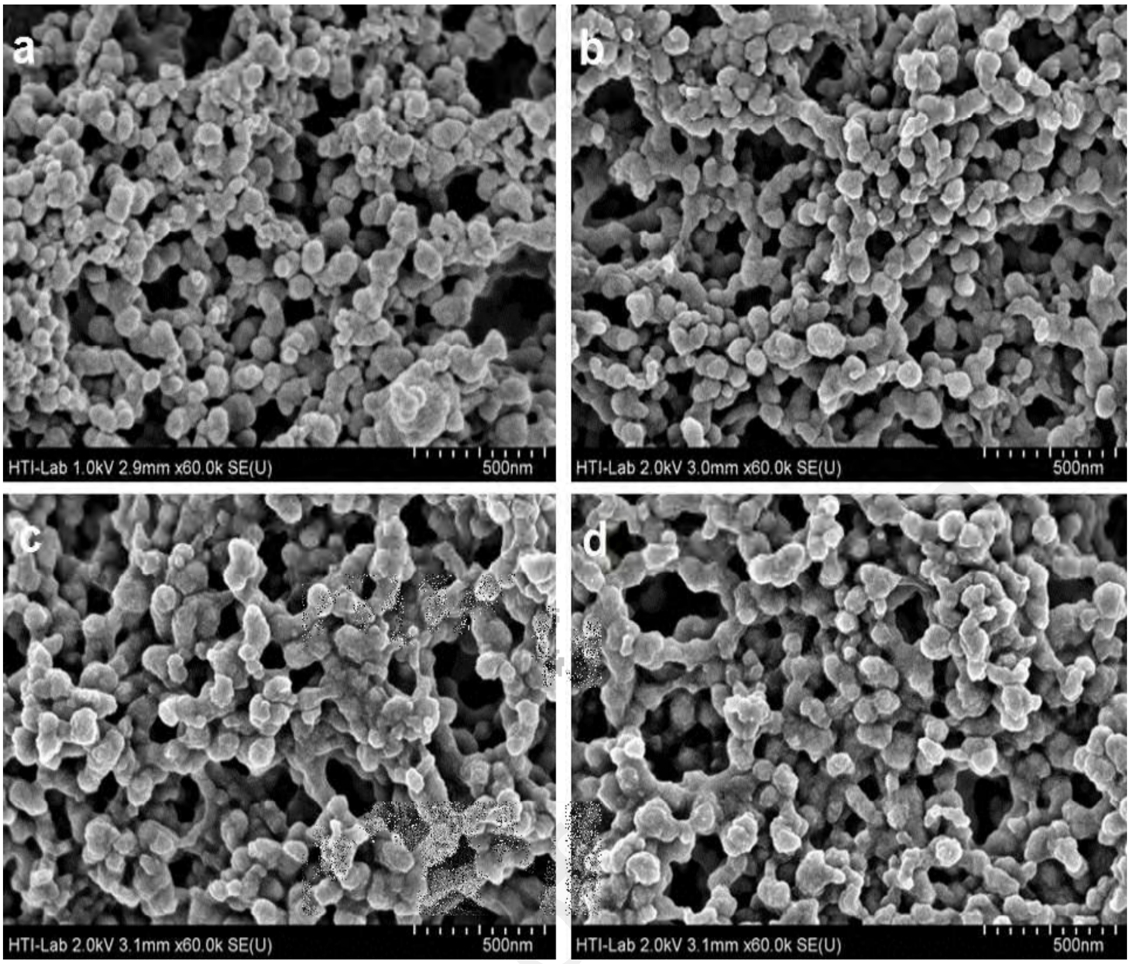


Figure 4.8: AgNPs with different ratio of silver nitrate and extract: a) 1:4, b) 1:9, c) 4:1 and d) 9:1.

4.1.4 Growth control agent

To further modify the shape, solvent and stabilizing agent were used, namely ethanol and polyethylene glycol (PEG). Apart from FESEM and TEM to view the nanoparticles, PSD and Zeta potential were also used to assess the size and stability of the AgNPs in deionized water suspension.

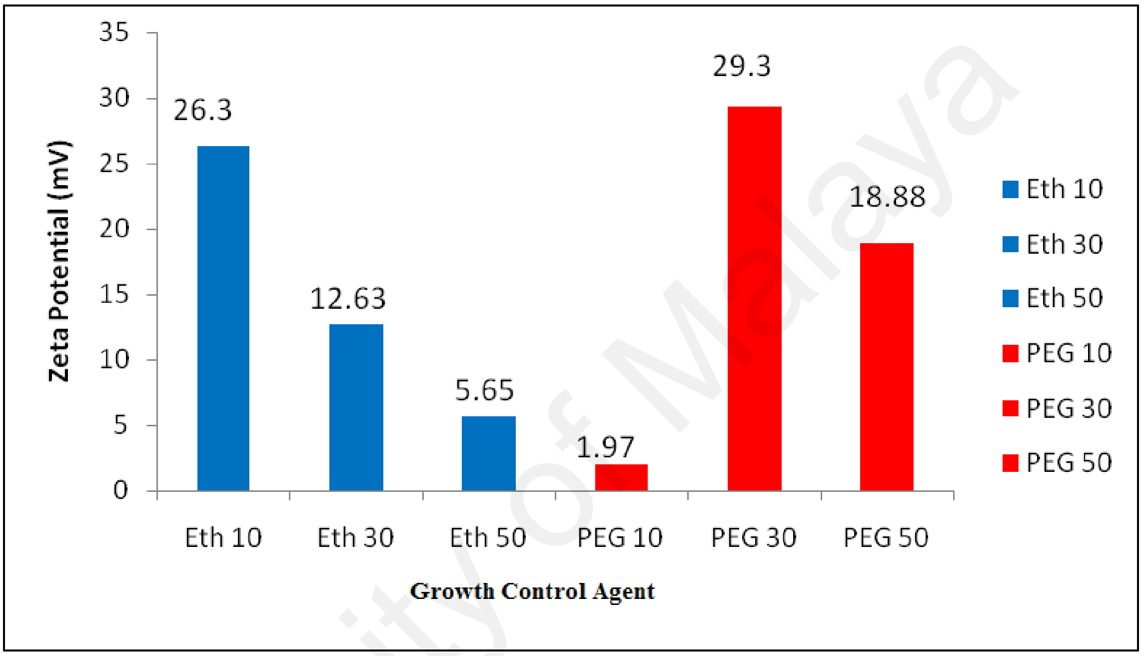


Figure 4.9: Zeta potential of the AgNPs with the amount of ethanol and PEG used in the synthesis.

The AgNPs with PEG 30% and ethanol 10% give comparable zeta potential values of 29.3 mV and 26.3 mV, respectively (Figure 4.9). Figure 4.10 shows FESEM of both samples. The shape of both samples is observed to be spherical. The agglomeration and aggregation seem to be significantly reduced as compared to Figure 4.8 in section 4.1.3. The AgNPs synthesized with PEG 30% has size distribution between 10-70 nm whereas with ethanol 10% between 10-800 nm (Figure 4.11).

Relationship between zeta potential value and concentration of PEG is not directly proportional. The zeta potential value increased until it reached certain concentration of PEG value, and later decreased as higher concentration of PEG was used. On the other hand, the effect of ethanol concentration is inversely proportional; increase in ethanol concentration led to decreasing zeta potential value which indicates instability. This is probably due to the charge cancellation mechanism (Jódar-Reyes et al., 2006). The ethanol could probably differs in sign of charge, therefore induce reduction of the surface charge of the bare AgNPs. In this study, the highest concentration of growth control agent (GCA) used was limited to only 50% because higher amounts will defeat the objective to promote green approach and reduce the dependency towards the use of chemicals.

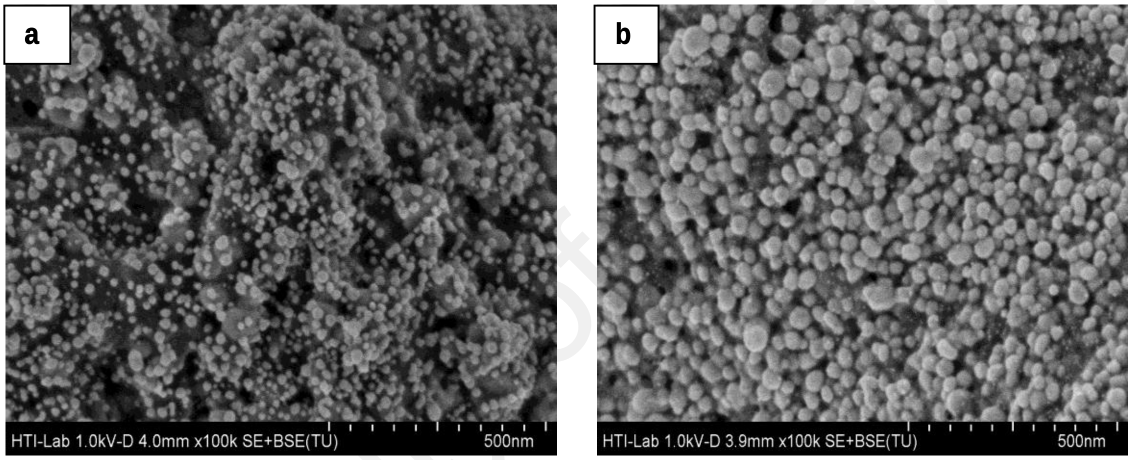


Figure 4.10: FESEM images of AgNPs by using a) PEG 30% and b) ethanol 10%. Both samples have comparable zeta potential values of 29.3 mV and 26.3 mV, respectively.

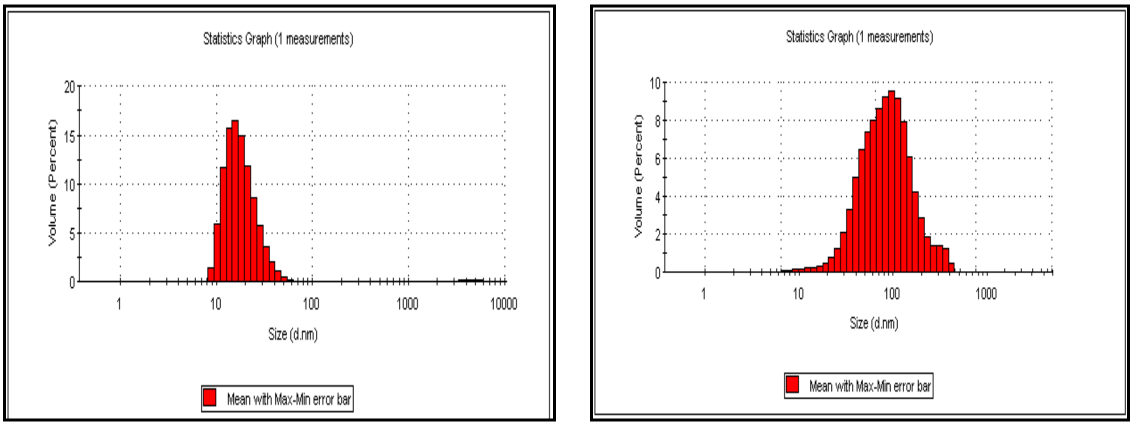


Figure 4.11: Size distribution of the AgNPs synthesized with a) PEG 30% and b) ethanol 10%.

However AgNPs synthesized with 30% of ethanol (Figure 4.12) was also observed for comparison with PEG 30% (Figure 4.10). The PSD estimated the highest distribution size of the AgNPs was in the range 5-150 nm (34.6 ± 5.32) which is much smaller than the AgNPs synthesized with ethanol 10% (10-800 nm). The sizes estimated through FESEM were 10-25 nm and 5-25 nm for AgNPs synthesized with 30% of ethanol and PEG 30%, respectively. The zeta value was 12.63 mV, which was twice lower than AgNPs synthesized with PEG 30%, 29.3 mV. The characteristics of the AgNPs synthesized with 30% ethanol is given in Figure 4.12 and 4.13. TEM images reveal that the shape of the AgNPs to be spherical, trigonal, rectangular and hexagonal shapes (Figure 4.13a). The sizes were estimated to be in the range of 5-60 nm. The lattice spacing for the planes was measured to be 2.30 Å (Figure 4.13b). This is in agreement with interplanar spacing calculated for plane (111) from XRD diffractogram of the AgNPs which is approximately 2.36 Å. It is also in agreement with other studies that synthesized AgNPs by green approach and chemical reduction, with the plane distance reported to be 2.30 Å (Hebeish et al., 2013) and 2.32 Å (Ariyanta et al., 2016), repectively.

It is noteworthy to mention that the difference in working principle has probably given rise to the different measurement of the AgNPs sizes by PSD and the FESEM observation. The PSD requires the AgNPs to be in solution whereas for FESEM the AgNPs have to be dry. The PSD technique utilizes light scattering concept to measure the induced speed of particles undergoing Brownian motion. Estimation of the size is subsequently aproximated from the speed. Moreover, the speed of the Brownian motion is also influenced by shape, sample viscosity, sedimentation level and temperature of the solution. On the other hand, FESEM uses a beam of electrons to bombard sample to generate secondary electrons which are used to approximate the surface structure.

As can be seen, when ethanol and PEG were respectively used as solvent and stabilizing agent, the stabilities of the AgNPs were observed to decrease in comparison to AgNPs synthesized under bare system (Table 4.1). Without the use of ethanol or PEG, the stability is higher; zeta potential reading was 40.1 ± 5.44 mV. Nevertheless, although it gives good stability, the AgNPs were highly aggregated and the sizes were bigger. Therefore, while the use of solvent or stabilizing agent seems to slightly sacrifice the stability, it has successfully alter the degree of aggregation and reduce the sizes of the AgNPs.

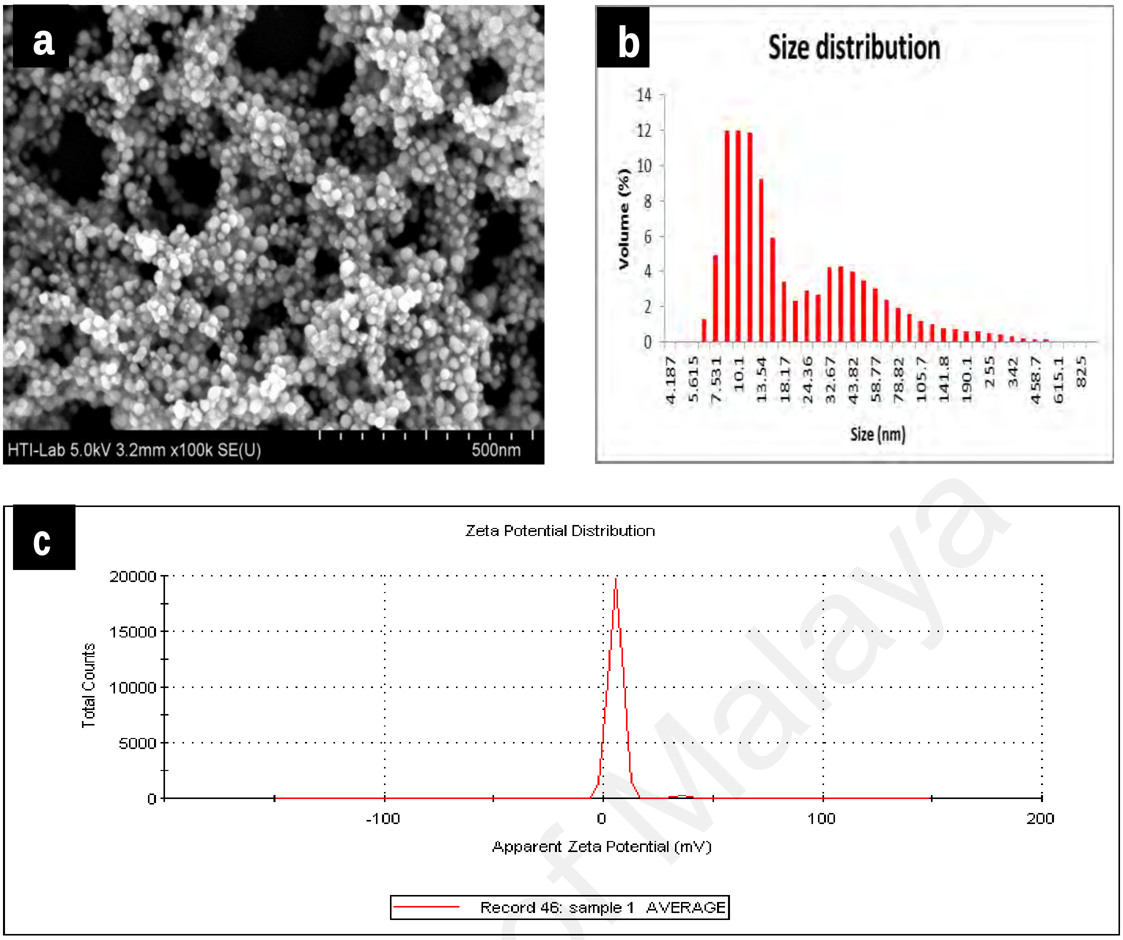


Figure 4.12: (a) FESEM images (b) particle size distribution and (c) zeta potential spectra of 30% ethanol AgNPs.

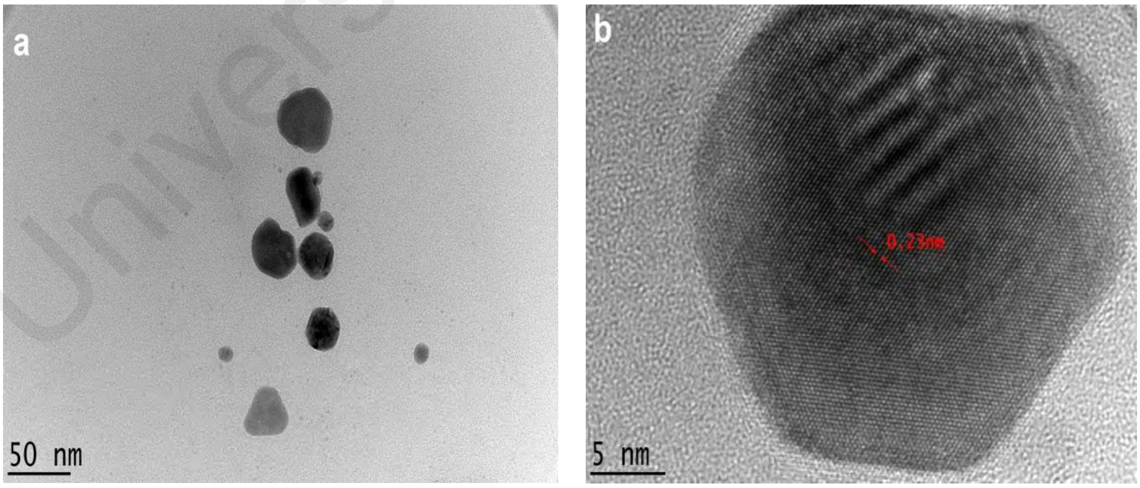


Figure 4.13: TEM images of AgNPs synthesized with 30 % ethanol at a) 50 nm and b) 5 nm scale.

Figure 4.14 shows the diluted sample of AgNPs synthesized with and without the use of stabilizing agent. As can be observed, AgNPs with PEG show a very light yellowish color, the lightest color amongst all; in comparison to AgNPs synthesized with ethanol and non-GCA. On the other hand, the AgNPs synthesized without any GCA was observed to be brownish in colour and was the darkest sample. It is a known fact that darker colour is equivalent to more AgNPs formation. As the experiment was conducted over the same period of time (24 h), therefore, rate at which AgNPs were formed can be presumably suggested and arranged as follows:

AgNPs without GCA > AgNPs with ethanol > AgNPs with PEG

From here it can be proposed that more AgNPs were formed when no growth control agent is used and that the sample has the highest formation rate. To confirm the observation, inductively coupled plasma mass spectrometry (ICP-MS) was used to quantify the amount of AgNPs in the samples.The amount of AgNPs was 3184000, 30000 and 22710 ppb for non-GCA , ethanol and PEG system, respectively. This measurement agrees with the color comparison (darker colour equates to more AgNPs formation) which indicates that the system with no GCA produced more AgNPs than the systems with ethanol and PEG as the solvent and stabilizing agents.The presence of both ethanol and PEG seems to suppress and slow down the formation of AgNPs.

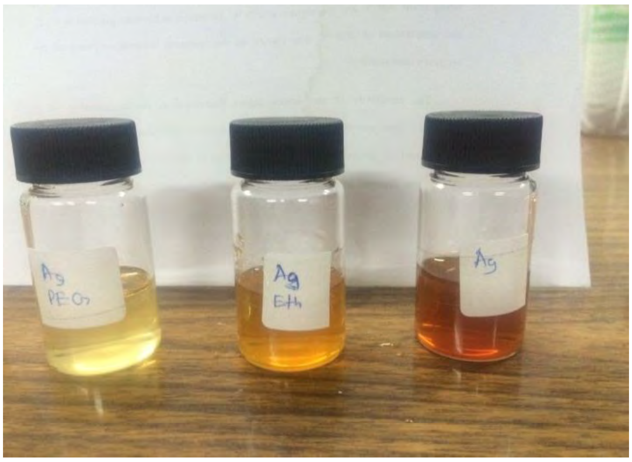


Figure 4.14: The diluted sample of the AgNPs synthesized using PEG (Ag PEG), ethanol (Ag Eth) and comparison with the bare AgNPs (Ag) sample.

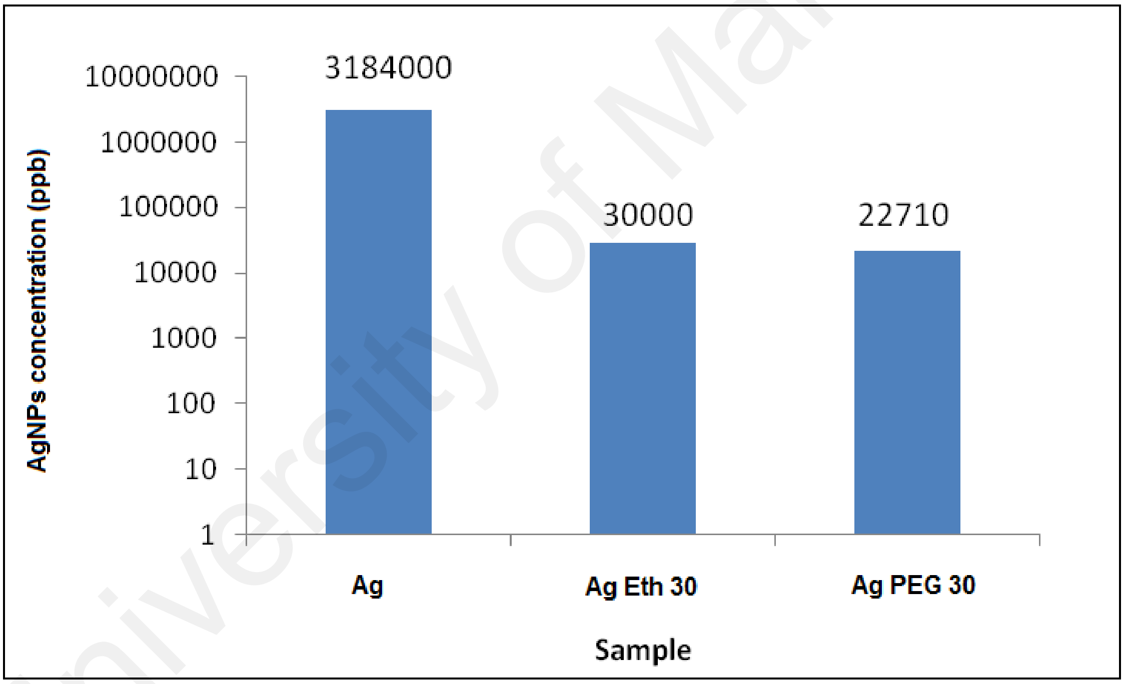


Figure 4.15: The amount of AgNPs quantified by ICP-MS measured in parts per billion (ppb).

Based on the result, it was observed that the use of PEG and ethanol as the solvent and stabilizing agent seemed to slow down, suppress and limit the undergoing reaction. This was reflected by the color of the solution and the ICP-MS test where smaller amount of AgNPs was detected when they were used. Therefore, the slowed down reaction as a result of the use of GCA has probably hindered the formation of Ag from Ag^+.

As the morphology of the AgNPs produced by using PEG and ethanol were much smaller and uniform in shape, it is proven that introducing solvent and stabilizing agent could improve the quality of the AgNPs. Detailed discussion on how stabilizing agents works in green synthesis is scarce because in general, the works in green synthesis try to avoid the use of any additional chemicals. However there are few works that reported the use of stabilizing agents in their works (Ahmad et al., 2011; Shameli et al., 2012). Nevertheless, their studies investigated the effect of polymer chain length of the PEG on the stability of the green synthesized AgNPs, and not on the concentration-stability relationship.

According to the zeta potential readings, higher concentration of stabilizing agents does not always result in higher zeta potential value. The relationship between them is not directly proportional. This was the case of PEG where the concentration of 50% resulted in lower zeta potential value compared to concentration of 30%. The zeta readings were increased and later decreased as the concentration was increased. According to Jodar-Reyes et al. (Jódar-Reyes et al., 2006), the declining stability of the nanoparticles when concentration of PEG is increased in the system can still be explained using the phenomenon of micelle formation, although PEG is a nonadsorbing polymer (Liu et al., 2013) and not a surfactant.

As nanoparticle was formed from the nucleation process, it will be encapsulated by the PEG. The presence of the stabilizing agent will lower surface tension of the solution therefore dissolving or creating a soluble-like condition for the AgNPs. At optimum concentration, PEG adsorb on the particles and PEG in the solution will be in equilibrium. The dispersion at this level is also optimum. After this point, any increase in the concentration of the PEG to the system will result in the equilibrium to shift. The PEG will leave the surfaces of the AgNPs to start formation of micelles. Micelles form only when the concentration of PEG is greater than the CMC. Particle surfaces are no longer repulsed, and energy of the system favors reagglomeration. Figure 4.16 illustrates the possible explanation for the nanoparticles-PEG relationship.

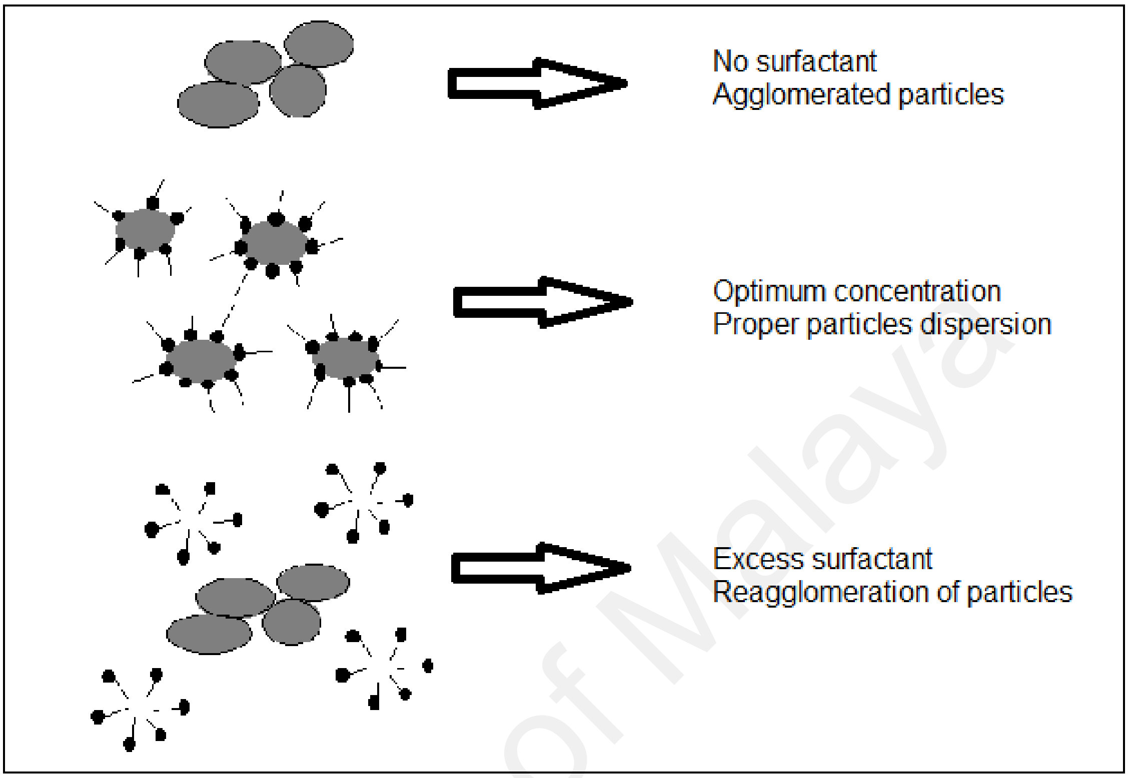


Figure 4.16: Ilustration of the surfactant and micelle formation.

4.1.5 pH of solution

The pH of the solution is known to affect the formation, size and shape of nanoparticles (Amaladhas et al., 2012; Chowdhury et al., 2015; Khalil et al., 2014; Sathishkumar et al., 2009). In this part, the pH of the solution was varied from acidic to basic; 1, 4, 7, 11 (Figure 4.17). In this experiment, pH 4 was the natural condition when the silver nitrate, apple extract and ethanol were mixed. It can be observed that under acidic conditions (pH 1 & 4), the shape of AgNPs was spherical. However, the AgNPs synthesized at pH 4 showed a smaller size as compared to pH 1. The sizes of the AgNPs estimated by PSD at pH 1 and 4 were 10-50 nm and 5-25 nm, respectively. The shape was also spherically uniform. As the pH was set to 7, the spherical shape of the AgNPs gradually changed. Signs of clustering, irregular-sized particles and aggregation started to appear. As the pH was set to 11 these signs became highly evident. The AgNPs synthesized at pH 11 showed a formation of aggregation.

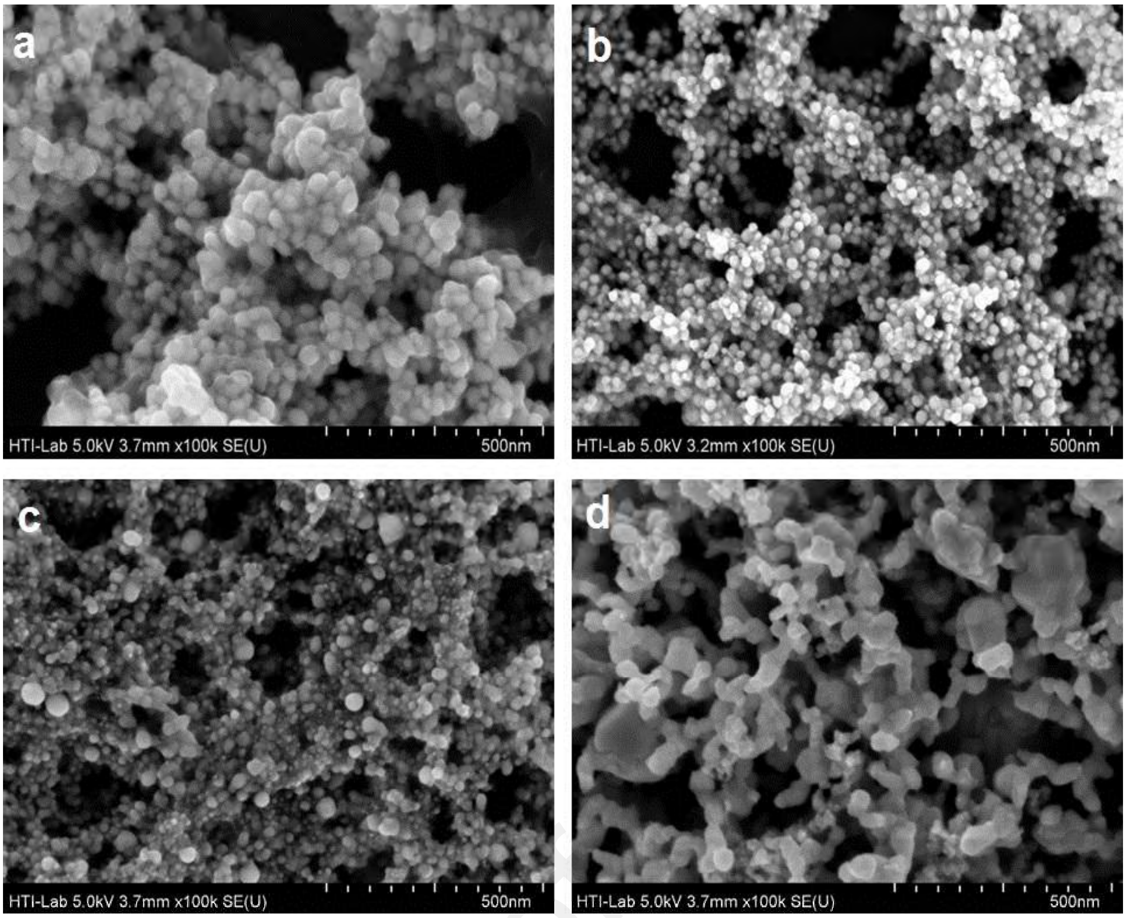


Figure 4.17: AgNPs at different pH of a) 1, b) 4 c) 7 and d) 11.

The synthesis of the AgNPs was tested over a wider pH range of 1–11. Less aggregated nanoparticles were formed at lower pHs (1 & 4), whereas a highly aggregated nanoparticles were formed at high pHs (7 & 11). At high pH, the aggregation of AgNPs to form larger nanoparticles was believed to be favored over the nucleation to form new nanoparticles. At lower pH, smaller diameters of AgNPs were formed. In addition, at higher pH the shape of the nanoparticles formed were more distorted compared to a more spherically structured at lower pH. This result confirms the very important role played by pH in controlling the shape and size of the AgNPs synthesis. Earlier works have shown and derived the pH effect to the shape of AgNPs only based on the UV-Vis spectrum instead of FESEM images (Dubey et al., 2010; Mochochoko et al., 2013). For example, Dubey et al. concluded from the blue shift observation that bigger particle size are produced at acidic pH as compared to the basic pH (Dubey et al., 2010). Ahila et al.

suggested that increment in pH has resulted in the blue shift which correspond to small and homogeneous distribution of the AgNPs (Ahila et al., 2016).

The results obtained in this work are in contradiction to those reports by Dubey et al. (2010) and Ahila et al. (2016). For instance, smaller sizes of the AgNPs were obtained at lower pH instead of at higher pH. The sizes of the AgNPs also do not become smaller at higher pH. Higher pH environment seemed to only promote aggregation to the AgNPs.

Through FESEM, this work has revealed the actual pH effect to the morphologies of the AgNPs as well as their agglomeration and aggregation level. Previous works that use UV-Vis spectroscopy lacks precision because it cannot identify the shape and aggregated level of the particles accurately as compared to the FESEM.

4.2 Antibacterial Results

Minimum bactericidal concentration (MBC) is defined as the lowest concentration of antimicrobial agent that kills 100% of the initial bacterial population (Ansari et al., 2015). In order to derive and understand relationship between the stability, aggregation and sizes of the AgNPs to the antibacterial performances, three AgNPs (Table 4.2 & Figure 4.18) prepared using three methods were evaluated in terms of their effectiveness in inhibiting and eliminating five types of bacteria namely *Escherichia coli, Pseudomonas aeruginosa, S.Pneumonia, methicillin-resistant Staphylococcus aureus (MRSA)* and *Staphylococcus aureus.* Each of the AgNPs has different degree of stability, aggregation and sizes. Table 4.2 summarizes the information of the all the AgNP samples. Figure 4.18 showed the FESEM image of the corresponding samples.

Table 4.2: Information of the AgNPs used in the antibacterial tests.

AgNPs	**Preparation method/system**	**Zeta (mV)**	**Sizes (nm)**
Sample 1	• Silver nitrate : Apple extract = 1:1 • The mixture is stirred and heated for 1 hour at 70 C	10.7 ± 1.25	93.2 ± 3.20
Sample 2	• Silver nitrate : Apple extract = 1:1 • The mixture is allowed to react at room temperature (~ 27 C) for 24 hours. • No heat is used	40.1 ± 5.44	79.3 ± 2.64
Sample 3	• Silver nitrate : Apple extract : Ethanol (30%) = 1:1:1 • The mixture is allowed to react at room temperature (~27 C) for 24 hours • No heat is used	12.6 ± 1.46	34.6 ± 5.32

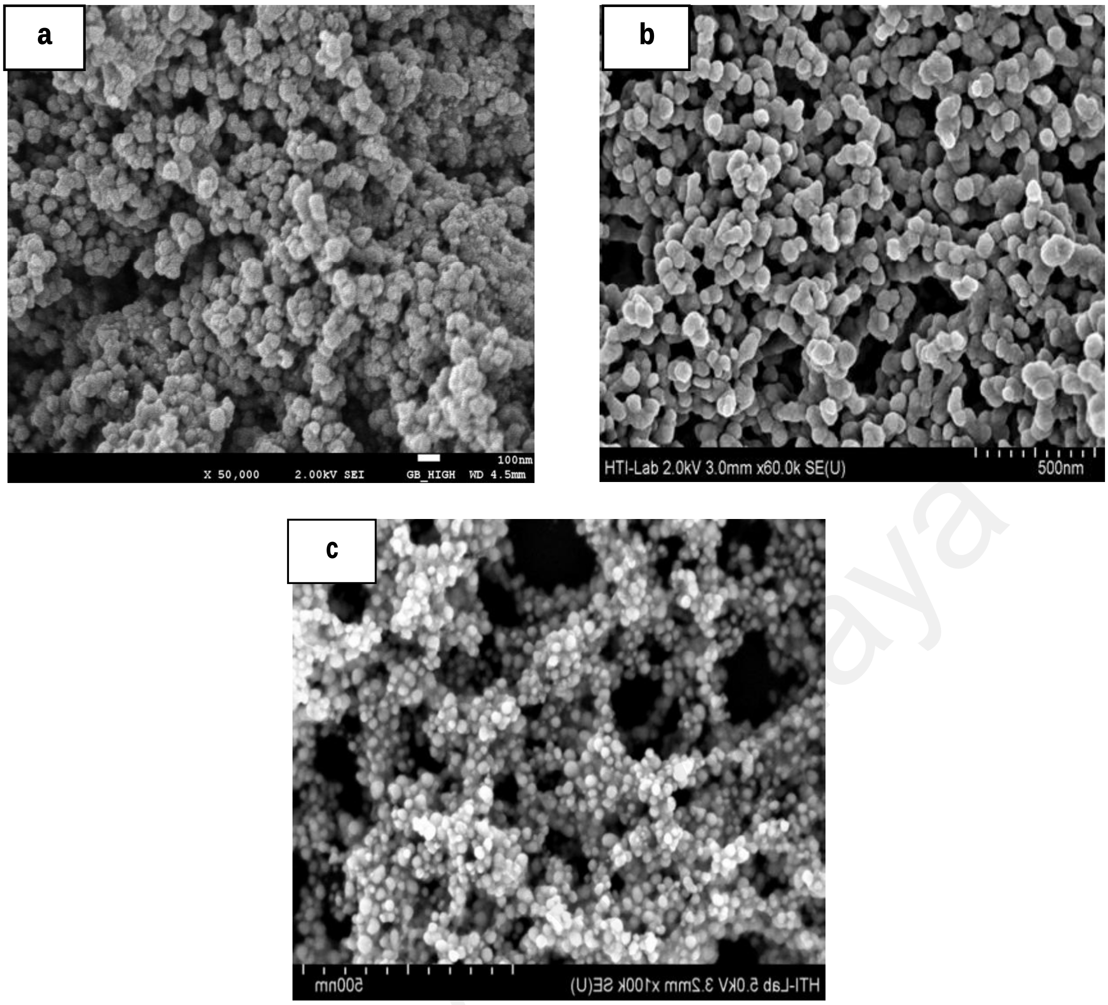


Figure 4.18: FESEM images of (a) Sample 1 (b) Sample 2 and (c) Sample 3.

The observed MBC values for the AgNPs were summarized and graphically compared as shown in Figure 4.19. Sample 3 showed the lowest MBC, and the best results for all the bacteria tests. The lowest MBC of 15.62 µg/mL was recorded by Sample 3 for *E. coli*, *P. aeruginosa* and *S. pneumonia* whereas *S. aureus* and *MRSA* were 62.5 and 125 µg/ mL, respectively. Except in the case of *S. pneumonia*, the efficiency of the AgNPs can thus be arranged as: Sample 3 > Sample 2 > Sample 1.

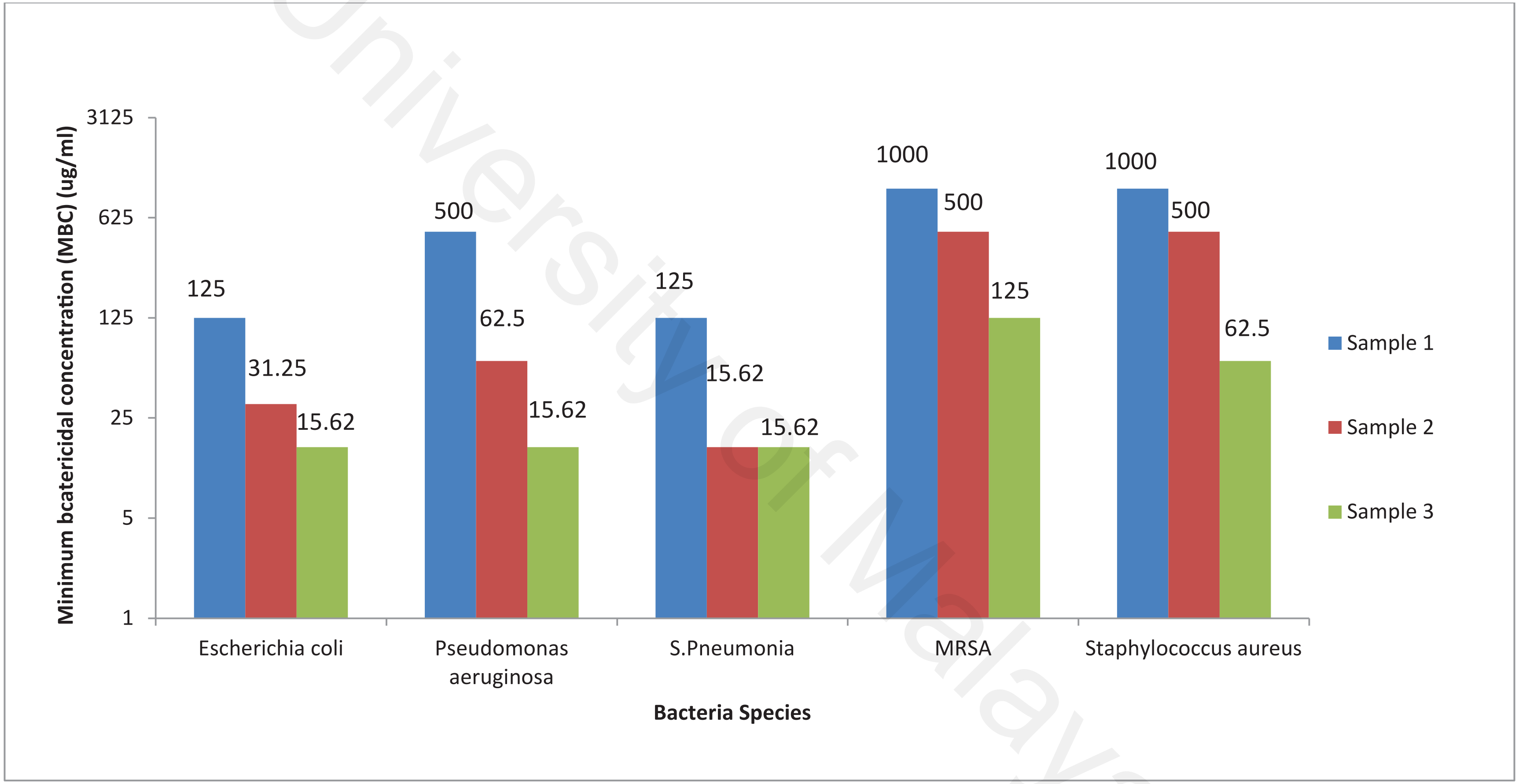


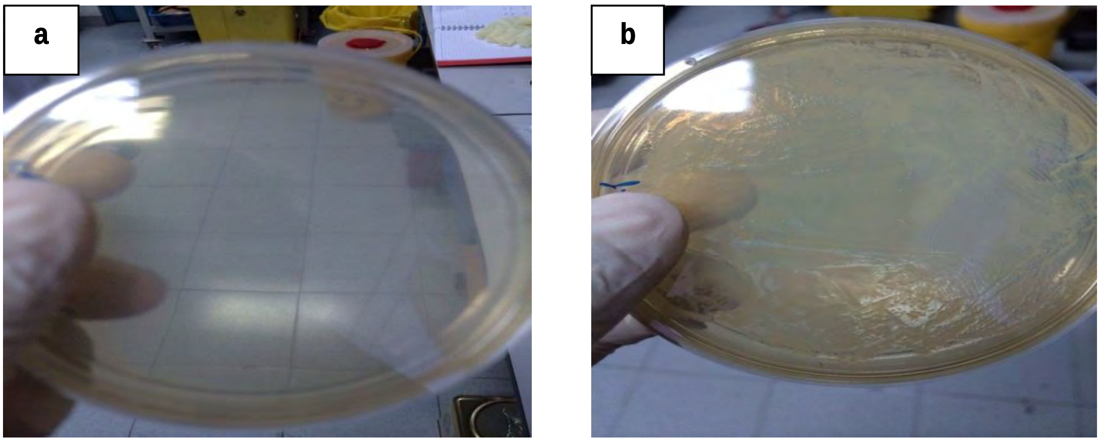
Figure 4.19: Minimum Bactericidal Concentration (MBC) against five bacteria for AgNPs prepared using three different condition.

Table 4.3 compares the performance of Sample 3 with AgNPs produced by others reports. Sample 3 is observed to show the best performance only to *E. coli* and *P. aeruginosa* compared to other reports. Therefore, the antibacterial properties of the AgNPs synthesized in this work are comparable to others.

Table 4.3: Comparison performance of AgNPs from other studies.

Extract	**Size (nm)**	**MBC of AgNPs for bacteria (µg/ml)**				**Reference**
		MRSA	***S. Aureus***	***E coli***	***P. aeruginosa***	
Eucalyptus globulus leaf	1.9-25	30	-	42	36	(Ali et al., 2015)
Ficus benghalensis and *Azadirachta indica*	40-50	-	-	100	100	(Nayak et al., 2016)
Rosmarinus officinalis leaf	10-33	-	193.31	193.31	96.65	(Ghaedi et al., 2015)
Grape Seed	25-50	-	51.0	-	-	(Xu et al., 2015)
Rhus chinensis	150	-	400	400	600	(Patil et al., 2016)
Apple	5-25	125.0	62.50	15.62	15.62	Sample 3

The inhibition effect of the AgNPs can be observed in Figure 4.20 with Sample 1 containing Ag with concentration of 125 µg/mL and the one without Ag (control) for *E. coli.* Figure 4.20(a) shows clear and transparent petri dish, indicating that the presence of AgNPs has prevented survival of the bacteria, whereas in Figure 4.20(b) an obscured and blurry dish was obtained, indicating the survivability of the *E. coli* when no AgNPs were employed.



(a) (b)

Figure 4.20: Petri dish of *E. coli* plates (a) containing 125 μg/mL AgNPs of sample 1 and (b) control.

4.3 Larvicidal Against *Aedes Agyptae*

Two AgNPs samples were used in larvicidal studies against *Aedes agyptae*. The study was carried out to verify the inference that elimination of larva by AgNPs is dependent on the concentration and the quality of the AgNPs used. Two factors used to determine the quality of the AgNPs were i) size and ii) zeta potential value.

Two samples of AgNPs were prepared using i) heating and ii) non-heating method and labelled as AgNPs-T and AgNPs-RT, respectively (Figure 4.21). The average sizes of AgNPs-T and AgNPs-RT as estimated by PSD were 93.2 ± 3.20 nm and 79.3 ± 2.64 nm, respectively. The size distibution of the particles is given in Figure 4.22. The zeta potential values of the AgNPs were 10.7 ± 0.12 mV and 40.1 ± 0.54 mV, respectively. The sample prepared without heating showed smaller average size and higher zeta potential value compared to AgNPs prepared using heating method. Higher zeta potential value indicates its better stability.

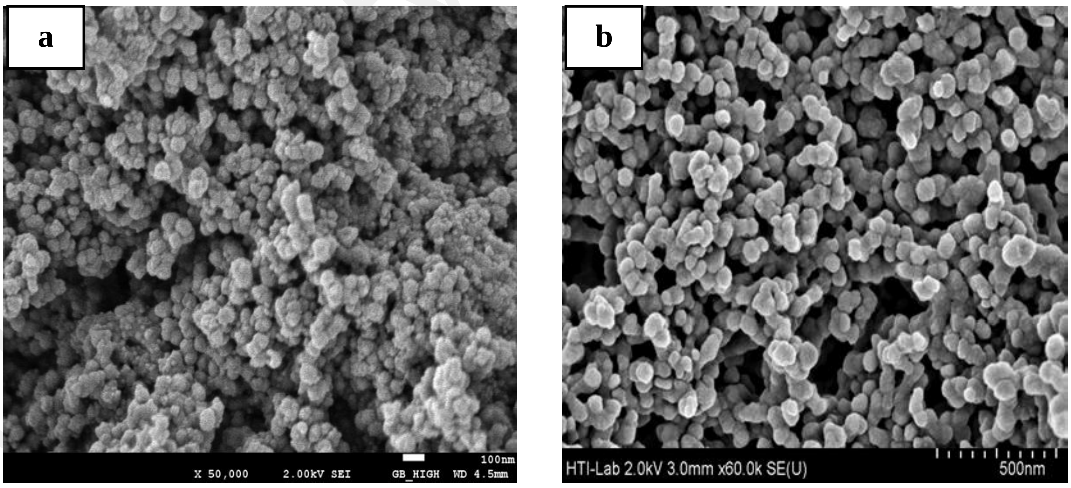


Figure 4.21: FESEM images of (a) AgNPs-T and (b) AgNPs-RT.

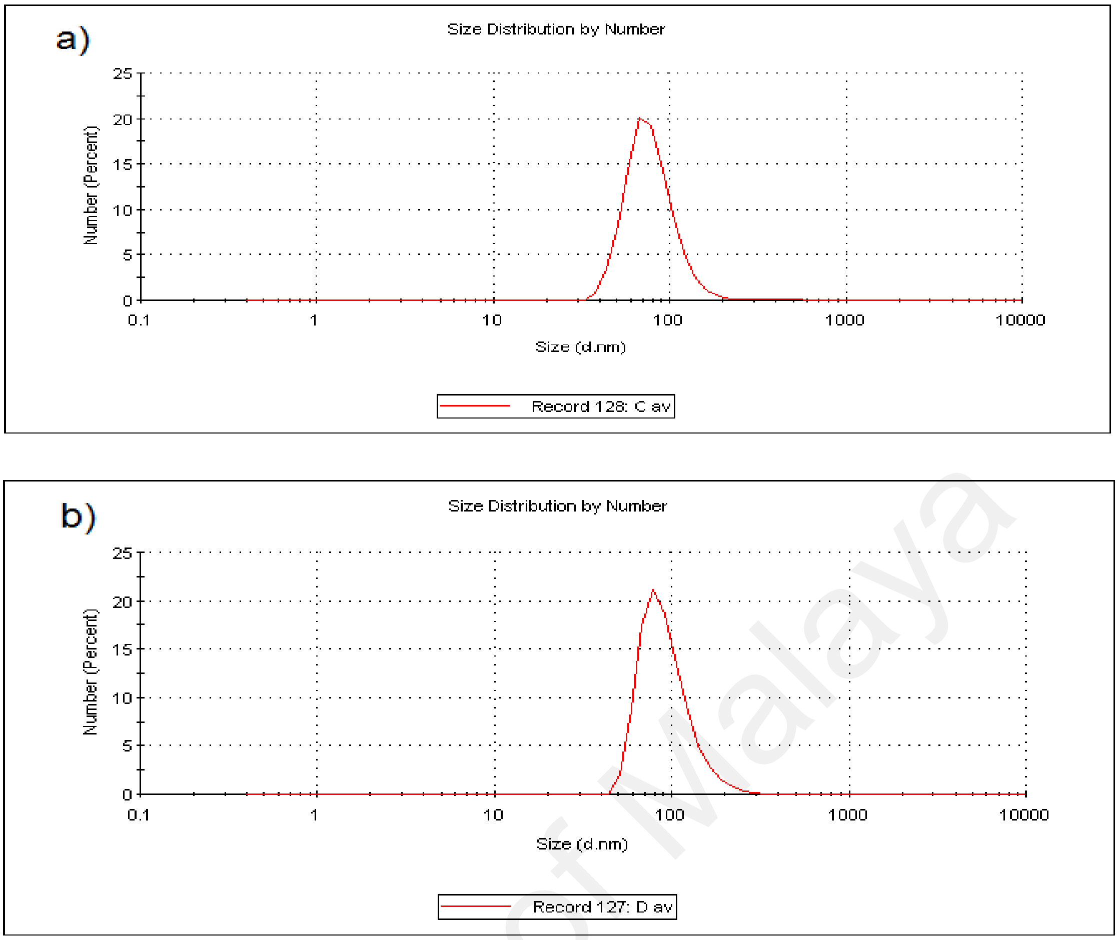


Figure 4.22: Particle size distibutions of (a) AgNPs-RT and (b) AgNPs-T.

The larvicidal activity of AgNPs, as presented in Table 4.4, showed moderate toxic effect on the larvae after 24 h of exposure. The LC_{50} = 29.81 ppm, LC_{90} = 42..3 ppm for AgNPs-T and LC_{50} = 15.76 ppm, LC_{90} = 27.7 ppm for AgNPs-RT were recorded, respectively. For both cases, the mortality increased as the concentration of the AgNPs increased (Table 3). Control samples were observed to show 0% of mortality. Based on this preliminary screening results, we carried out the test of high concentration on the larvae was tested. The pattern of elimination is shown in Figure 4.23. Comparison of performance between AgNPs-T and AgNPs-RT was also conducted. AgNPs-RT showed a slightly better performance. The AgNPs-RT successfully eliminated all the larvae after 100 min whereas the AgNPs-T after 160 min. The difference in the toxicity could be attributed to the smaller size and better dispersibility of the AgNPs-RT.

Table 4.4: Larvicidal activity of AgNPs-RT and AgNPs-T against fourth instar larvae of *Ae. aegypti*.

Sample	Concentration (ppm)	% Mortality MEAN	LC_{50}(ppm)	LC_{90}(ppm)
AgNPs-RT	500	100	15.76	27.70
	250	100		
	50	100		
	25	93.3 ± 4.41		
	12.5	16.7 ± 1.67		
	5	3.33 ± 1.67		
AgNPs-T	500	100	29.81	42.30
	250	100		
	50	100		
	25	30.0 ± 12.5		
	12.5	13.3 ± 1.67		
	5	10.0 ± 2.89		

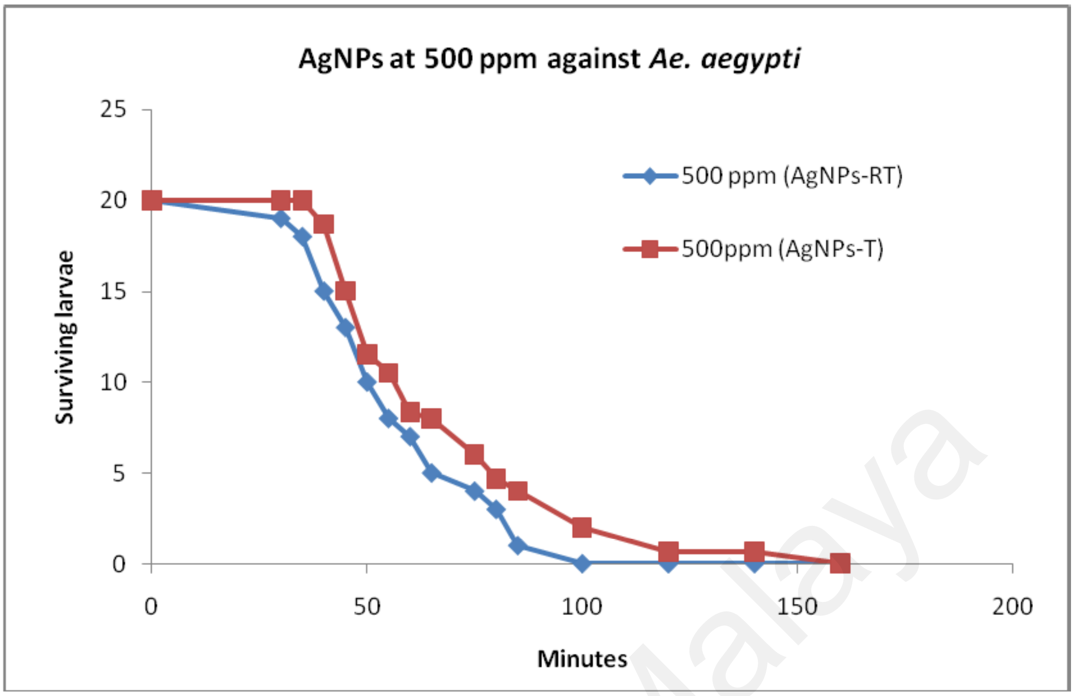


Figure 4.23: Pattern of the elimination of *Ae. aegypti*.

CHAPTER 5

DISCUSSION

5.1 Synthesis of AgNPs

The nanoparticles synthesized AgNPs have been confirmed with XRD to be pure Ag crystallite. This makes the apple extract a good reducing agent in producing AgNPs. Based on the FTIR results, it is proposed that the organic compounds from the apple extract could have been acting as the reducing agent in the formation of Ag. Ethylene groups detected by FTIR have also been reported by (Li et al., 2014) to be capable of acting as reducing or capping agent. Lokina et al. (2014) reported that the -OH groups present in the apple extract could involve the reduction of Ag^+ to Ag^0 through oxidation of alcohol to aldehyde group. Quercetin from the groups can donate electron (Osonga et al., 2016; Yuan et al., 2017). Sources of this –OH groups are flavonoids/limonoids and their reaction with the $AgNO_3$ salts can form silver flavonoids/limonoids complex (Balch, 2003; Kumar et al., 2013; Tringali, 2003). This complex mechanism has also been put forth by Kumar et al. (2014) for their ZnO green synthesis work. Figure 5.1 illustrates the possible reaction of the -OH groups and Ag^+ in forming AgNPs.

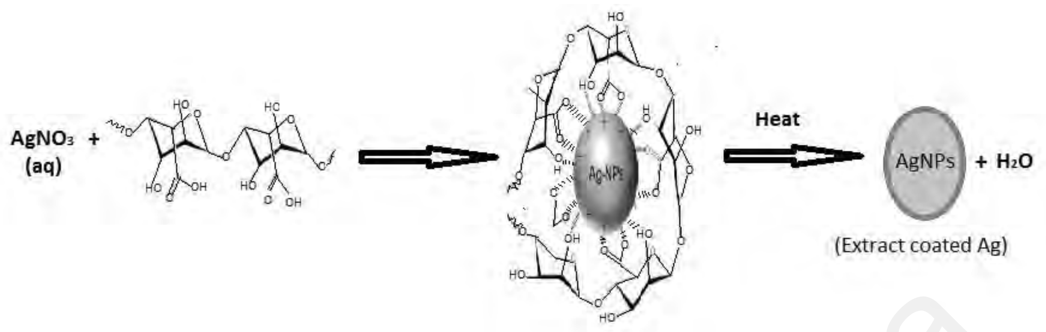


Figure 5.1: Schematic illustration of the mechanism for the formation of AgNPs. Modified from Balavandy et al. (2015) and Kumar et al. (2014). Reproduced with permission (Appendix B3).

In the case of using PEG as the stabilizing agent, the reaction could be slightly different. Polyethylene Glycol (PEG) is a water-soluble polymer with a general formula $H(OCH_2CH_2)_nOH$. Several studies have suggested that PEG is a good stabilizer for AgNPs (Chen et al., 2007; Popa et al., 2007; Shkilnyy et al., 2009). In one of these research works, Popa et al. (2007) reduced $AgNO_3$ in the presence of PEG and suggested that stabilization can be achieved owing to the presence of monomer chains, which establish steric forces that impedes aggregation and agglomeration.

It can be suggested that after addition of Ag^+ in the PEG aqueous solution, PEG reacted with the Ag+ to form a PEG complex $[Ag(PEG)]^+$ (Shameli et al., 2012), which then reacted with apple extracts to form [Ag(PEG)]. The schematic illustration of the synthesis of AgNPs capped with PEG is depicted schematically in Figure 5.2.

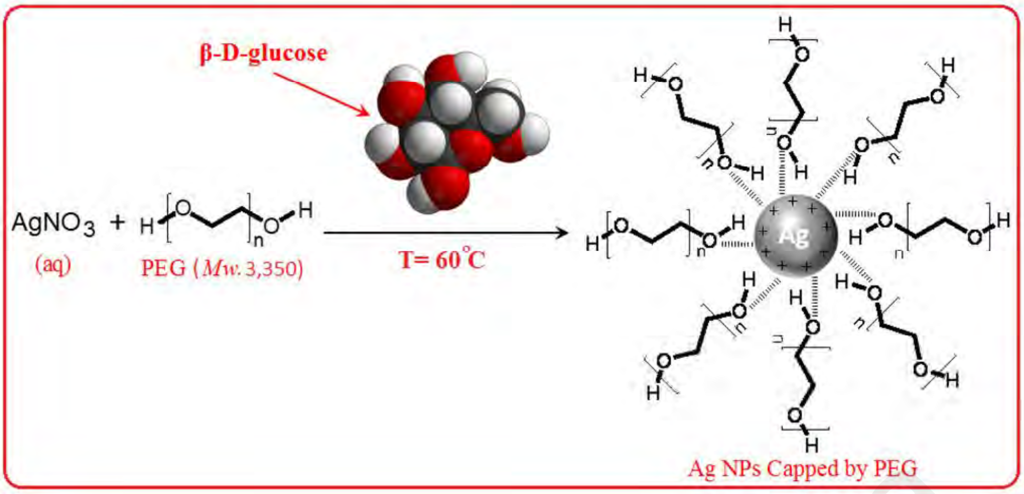


Figure 5.2: Formation of AgNPs by apple extracts in the presence of PEG. Reproduced under fair used open access publication (Appendix B4).

Hydroxyl group of PEG can cover the surface of AgNPs and acts as capping agent because of the positively-charged surface of the AgNPs (Shameli et al., 2012). The positively-charged surface and the negatively charged oxygen groups present in the molecular structure of the PEG result in the presence of attractive van der waals forces between them, hence creating a stabilized Ag(PEG) compound (Dallas et al., 2011).

As an alternative to the use of PEG, AgNPs was also synthesized in ethanolic environment. Solvent is known to be able to tune crystallite size of nanoparticles (Kumar et al., 2016) and surface structure (Liao et al., 2008). Ethanol is a very polar molecule due to its hydroxyl (OH) group, with the high electronegativity of oxygen allowing hydrogen bonding to take place with other molecules, hence give the ability to attract polar and ionic molecules. The ethyl (C_2H_5) group in ethanol is non-polar and become attractive to non-polar molecules. Therefore, ethanol can dissolve both polar and non-polar substances. The polar characteristic of solvent was proposed to be the main factor that affects both nucleation and growth (Khoza et al., 2012). The polarity or dissolving nature of the active ingredients of the plant extract in the ethanolic system is known to be better than aquous solution (Nagappan, 2012). On the other hand, Pace et al. reported that proteins will be unstable in most polar solvents such as ethanol (Pace et al., 2004). A medium-sized apple (app. 182 g) contains approximately 0.5 g of protein. Considering all these facts, it can be proposed that while the reducing reaction might follow the usual route of plant extracts reducing the Ag+, the presence of ethanol in the system could have somehow interacted with the extracts hence affecting the reducing and growth process of the AgNPs, as ethanol has been reported to slow down the nucleation and growth process (Rath et al., 2016).

It is known that most of the work on the synthesis of AgNPs cannot escape from the agglomeration and aggregation problems. While nanoparticles often have superior performance owing to its high-surface to volume ratio, agglomeration and aggregation cause these nanoparticles to clog together and reduce the area. Therefore, while it is known that AgNPs can be produced using the green approach, there is still more work needed to be done in order to address this problem. It is noteworthy to mention that although the agglomeration and aggregation prevail, the sizes of the AgNPs produced in this study are still comparable to other reports; 5-50 nm (Basavegowda et al., 2014) and 40-50 nm (Velmurugan et al., 2013).

As observed in Chapter 4, presence of heating, use of stabilizing agent and pH variation have led to changes in agglomeration and aggregation of the AgNPs. Based on the results, agglomeration can be reduced when no heat is used but aggregation still prevails and can only be reduced when stabilizing agent is used. Although it is known that an organic compound that is involved in the reduction process may as well act as the surfactant (Klem et al., 2005), it is still not suffiecient to reduce the aggregation of the process.

Unlike agglomeration, aggregation often involves a strong bond between two or more particulates together forming a big particle. According to definition by IUPAC, agglomerate corresponds to clusterings of dispersed particles held together by weak physical interactions forming large size precipitates. Attractive short-range van der Waals forces will keep the particles attached. The agglomerating particulates can be separated using physical dispersion technique such as ultrasonic dispersion (Figure 5.3a). On the other hand, an aggregate is defined as comprising strongly bonded colloidal particles, and the bonding formed is very strong that it may prevent separation once the particles have attached (Rodriguez-Navarro et al., 2005). Unlike agglomeration, the aggregated particles can only be separated using heavy physical force such as ball-milling. Figure 5.3b illustrates the difference between agglomeration and aggregation.

Figure 5.3: Illustration of (a) reversible process on agglomerating particles and b) structural difference of agglomerated and aggregated particles.

Using stabilizing agent or surfactant is a common approach to treat both agglomeration and aggregation problem (Kumar et al., 2014; Leo et al., 2013; Salem et al., 2016). They work by attaching to the surface of nanoparticles, altering the surface charge of the nanoparticles and creating repulsive forces that inhibit the interaction between the nanoparticles, thereby reducing agglomeration and aggregation.

In an attempt to understand how solvent and stabilizing agent succesfully reduces aggregation, it is better to understand how particles are formed and started to aggregate.

It is generally accepted that nucleation and growth are the key steps in the nanoparticle formation process. Nuclei may initially follow diffusional growth, with growth spread outwards forming spherical shape particle known as primary small particles (Figure 5.4a). In the case of agglomeration, these primary particles would coalesce, agglomerate into bigger particles owing to an attractive charge present in the surface of the particles. Aggregate on the other hand could take place because of dislocations or defects exist on the surface of the particles. The dislocations or defects provide growth points thus providing structural transformation (Frank, 1949). A new growth will develop from the new points forming an inseparable particle structure. Figure 5.4b shows the illustration of the dislocations or defects on the particle surface.

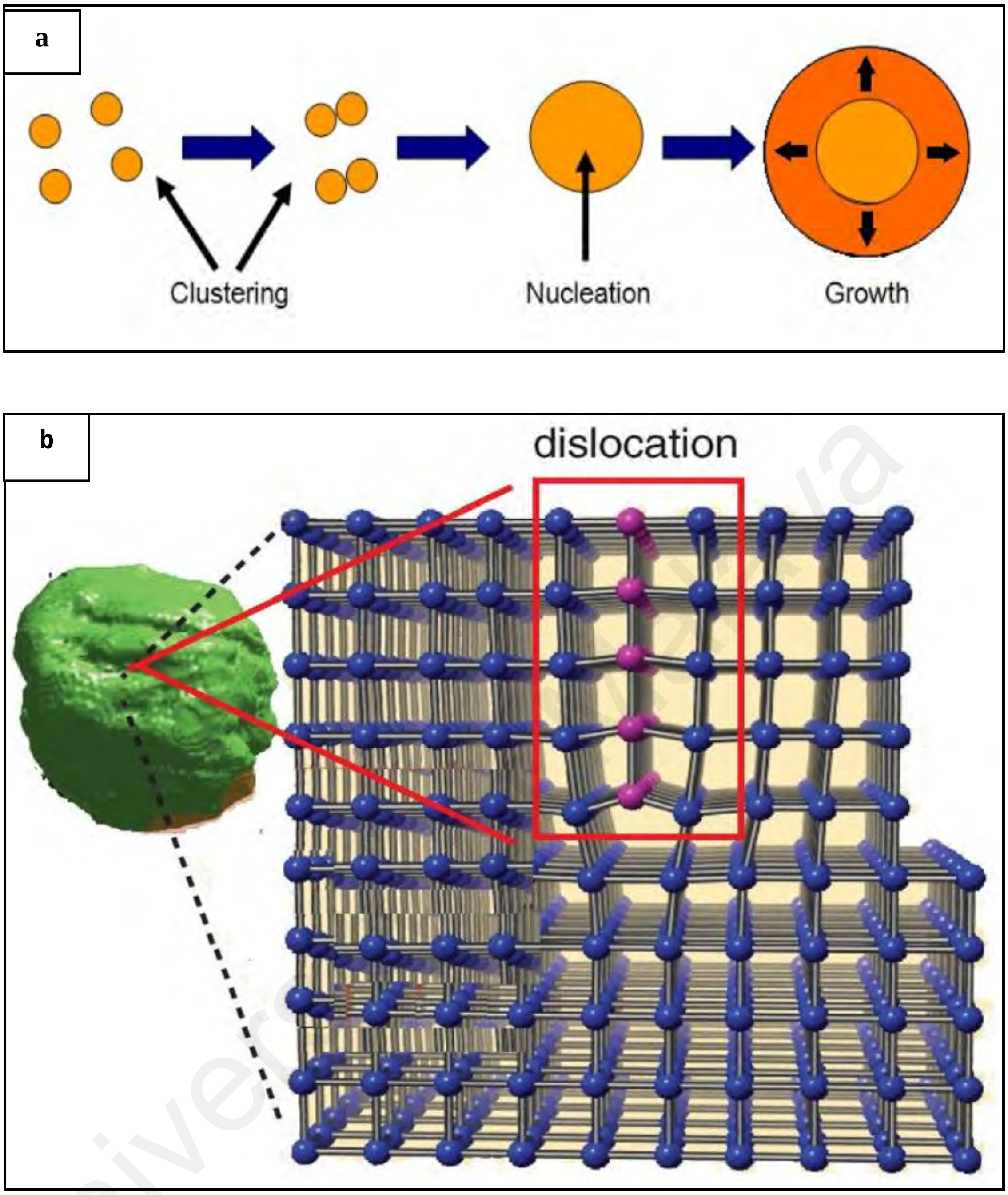


Figure 5.4: a) Illustration of particle growth and b) dislocation of unit cell structure on the surface of particles. Figure reproduced and modified from Ulvestad et al. (2015) with permission (Appendix B5).

Dislocations or defects may occur due to atomic collission during rapid formation of nanoparticles. Therefore, preventing the rapid formation could probably be the answer to reduce aggregation. It is understood from the data in Chapter 4 that the presence of solvent and stabilizing agent in the system does slow down the reaction. The morphology and shape of the AgNPs produced with solvent and stabilizing agents (ethanol and PEG) have significantly improved in term of homogeneity and sizes.

Based on this, it is proposed that the use of PEG and ethanol has probably prevented the rigourous reaction that might cause defects and dislocations to the particles therefore impeding formation of growth point which could result in the aggregated particles. The ability of ethanol to scavenge and quench the OH radical species (Dang et al., 2009; Rath et al., 2016) that responsible for electron donation could probably be the reason the reaction slowed down. Moreover, the significant reduction level of agglomeration may also be attributed to the surface charge alteration by the stabilizing agents.

The pH also significantly affects the morphology, agglomeration and aggregation of the AgNPs. In this work, the best morphology and smallest size of AgNPs were obtained at pH 4. Increasing to higher pH resulted in increasing the aggregation of the AgNPs. As known, variation in pH does affect the agglomeration level of a dispersed solution (Edison et al., 2012; Singh et al., 2009). Variation in pH on the other hand could probably play significant role in the aggregation of the AgNPs by influencing the nucleation and growth process. The use of unsuitable pH may promote uncontrolled growth or create defects or dislocations that act as growth points, thereby reversing and opposing the good effect brought in by the surfactant. Hence, searching for optimized level of pH and stabilizing agent is very important.

5.2 Antibiorganism Applications

The AgNPs synthesized using apple extract have shown toxicity to both bacteria and larva. As the toxicity tests were carried out using different sample of AgNPs, these samples showed different level of toxicity. Toxicity of Sample 3 in bacterial test was the highest whereas AgNPs-T in larvicidal test was the highest, respectively. Based on the synthesis parameter results, Sample 3 has the smallest sizes, moderate dispersibility and spherically structured compared to Sample 1 and Sample 2, which have bigger sizes, highly aggregated and agglomerated structures. This undoubtedly shows that these properties could boost the toxicity of AgNPs towards bacteria. The effect was the same for the larvicidal tests. Smaller sizes and higher dispersibility were key factors to high toxicity. The agglomeration effect may have affected the bactericidal efficiency and MIC/MBC values as also suggested by Gan et al. (2004).

There have been several proposed mechanisms on how AgNPs work as antibacterial although the exact mechanism is still unknown. Several reports (Kumar et al., 2005; McDonnell et al., 2001; Pal et al., 2007; Sondi et al., 2004) suggested that the AgNPs could produce Ag^+ ions which will damage the cell membrane, interrupt the metabolic activity, and subsequently lead to denaturation of protein and finally cell death. AgNPs could also produce reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), hydroxyl radical, (•OH) and hydrogen peroxide (H_2O_2) which are toxic to the bacteria (Carlson et al., 2008). Figure 5.5 illustrates the possible antibacterial mechanism of the AgNPs.

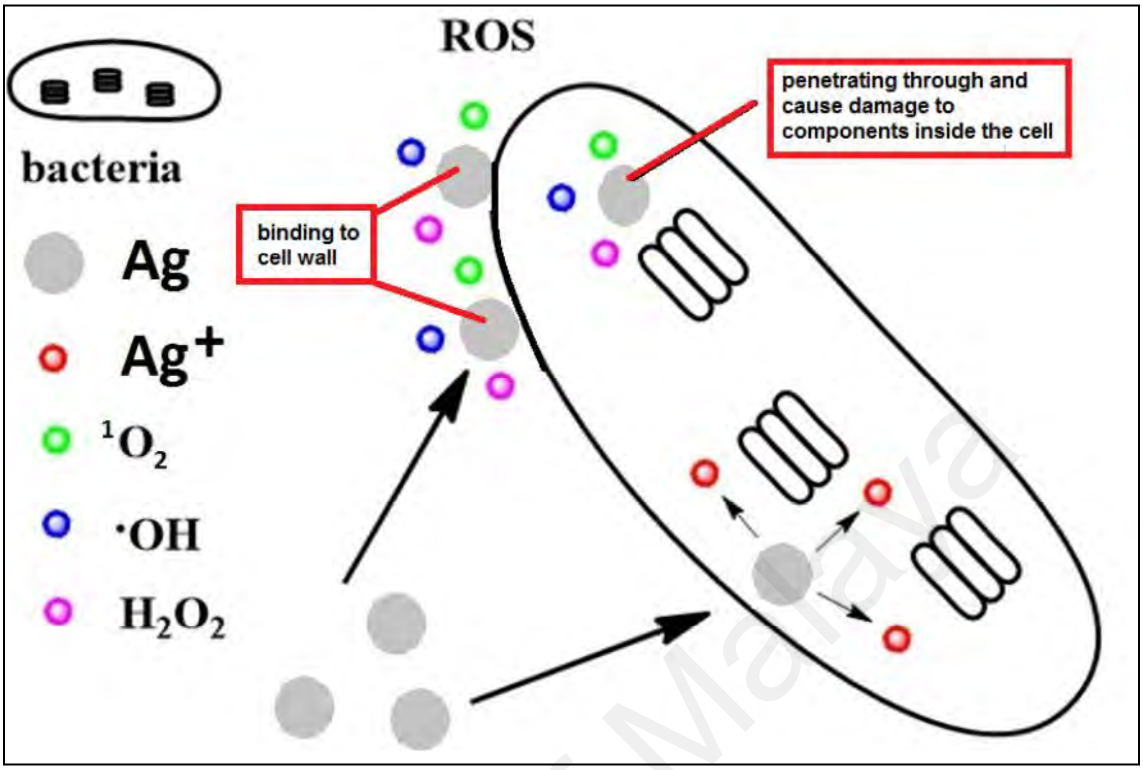


Figure 5.5: Possible reaction mechanism of Ag, Ag^+ and ROS towards bacteria. Reproduced under open access (Appendix B6).

As for the mechanism of larvicidal activity, it is also still unclear. However, it is believed that the AgNPs has to be in contact with the larvae in order to eliminate it. Sundaravadivelan et al. (2014) believed the idea put forth by Yamanaka et al. (2005) on the mechanism of antibacterial study could also be applicable in the case of larvae. Yamanaka proposed that interaction of the biologically synthesized Ag with cytoplasm in the interior of the cell could denature the ribosome, resulting in the suppression of enzymes and proteins essential for ATP production and eventually leads to cell disruption.

CHAPTER 6

CONCLUSION

6.1 Conclusion

AgNPs have been succesfully synthesized using apple extract and $AgNO_3$ aqueous solution. The face-centered cubic crystalline nature of the AgNPs is evident from sharp peaks in the XRD diffractogram. The size of the AgNPs estimated from FESEM was around 5-25 nm, which is slightly bigger than the average size estimated by particle size distribution (PSD DLS Analyser); 8-15 nm. The zeta potential value of 12.63 mV indicated moderate stability in a solution. FTIR analysis suggested that ethylene and aldehyde groups from the apple extract could be the reducing agent responsible for the reduction of Ag^+ into Ag. FESEM analysis showed that size and degree of agglomeration as well as aggregation can all be improved and reduced by controlling the presence of heat, pH and the usage of surfactant. Controlling rate of formation (reaction) by means of heat and using diluted system did not fully improve the agglomeration and aggregation problems. Surfactant was necessary to address these problem. Therefore in order to produce AgNPs with a uniformity in sizes and shape as well as low agglomeration and aggregation, parameters such as pH, heat, duration of reaction, ratio of extract/salts and surfactant must be carefully optimized and controlled.

The present investigation also highlights the potential of the synthesized AgNPs in the antibacterial and larvicidal applications. Regardless of size, all of the synthesized AgNPs exhibited antibacterial and larvicidal properties. The differences found were only in the strength of elimination. The AgNPs with the smallest size, low agglomeration and aggregation have proven to be the most effective in both applications. A range of 15 to 1000 µg/mL was required to eliminate the Gram-positive and Gram-negative bacteria with Sample 3, the AgNPs with the smallest size and low agglomeration and aggregation were found to be the most effective. Sample 3 required the lowest MBC of 15.6 µg/mL to eliminate *E. coli, P. aeruginosa* and *S.pneumonia* and 125 µg/mL and 62.5 µg/mL for *MRSA* and *S. aureus*, respectively. On the other hand, the best LC_{50} & LC_{90} against the dengue vector *Ae. aegypti* for the AgNPs were 15.76 ppm and 27.70 ppm, respectively.

This method of producing AgNPs is environmentally friendly, low cost and simple. It can promote and boost the application of green technology. Although AgNPs is known to have good antibacterial properties, its high cost makes its usage scarce. Therefore, by using the green approach, it is expected that more antibacterial products based on the AgNPs can be developed. The toxicity of the AgNPs must be carefully studied and analyzed prior to clinical testing. Addressing this issue is required if a truly industrial sized market for AgNPs is ever to become a reality.

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Ali, Z. A., Roslan, M. A., Yahya, R., Sulaiman, W. Y. W., & Puteh, R. (2016). Eco-friendly synthesis of silver nanoparticles and its larvicidal property against fourth instar larvae of Aedes aegypti. *IET Nanobiotechnology*, *11*(2), 152-156.

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APPENDIX A

FUNCTION AND WORKING PRINCIPLE OF INSTRUMENTS

A1 - X-ray Powder Diffraction

X-ray powder diffraction (XRD) is a technique used for phase identification of a crystalline material. It provides information on unit cell dimensions where atomic spacing and crystallite size can be derived.

X-ray diffraction is based on constructive interference of monochromatic X-rays and a crystalline sample. These X-rays are generated by a cathode ray tube, filtered to produce monochromatic radiation, collimated to concentrate, and directed toward the sample. The interaction of the incident rays with the sample produces constructive interference (and a diffracted ray) when conditions satisfy Bragg's Law ($n\lambda=2d \sin \theta$). This law relates the wavelength to the diffraction angle and the lattice spacing in a crystalline sample. These diffracted X-rays are then detected, processed and counted.

By scanning the sample through a range of 2θ angles, all possible diffraction directions of the lattice could be obtained due to the random orientation of the material. Conversion of the diffraction peaks to d-spacings allows identification of the mineral because each mineral has a set of unique d-spacings. Comparison of d-spacings with standard reference patterns is then carried out for identification process.

A2 - Inductively Coupled Plasma Mass Spectrometry

Inductively Coupled Plasma Mass Spectrometry (ICP-MS) is an analytical technique used for elemental identification and quantification. It is capable of detecting metals and several non-metals at concentrations as low as one part in 10^{15} (part per quadrillion, ppq). The ICP-MS consists of two main parts which are (i) high-temperature ICP (Inductively Coupled Plasma) source and (ii) a mass spectrometer. The ICP source converts the atoms of the elements in the sample to ions. These ions are then separated and detected by the mass spectrometer.

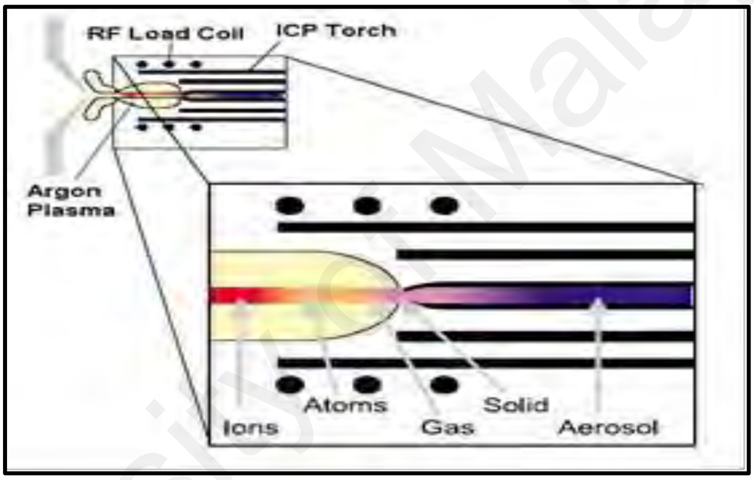

Figure 1: The ICP Torch showing flow of the sample

Figure 3.1 shows a schematic representation of an ICP source in an ICP-MS. Argon gas flows inside the concentric channels of the ICP torch. The radio-frequency (RF) load coil is connected to a RF generator. As power is supplied to the load coil from the generator, oscillating electric and magnetic fields are established at the end of the torch. When a spark is applied to the argon flowing through the ICP torch, electrons are stripped off of the argon atoms, forming argon ions. These ions are caught in the oscillating fields and collide with other argon atoms, forming an argon discharge or plasma.

The sample is typically introduced into the ICP plasma as an aerosol, either by aspirating a liquid or dissolved solid sample into a nebulizer or using a laser to directly convert solid samples into an aerosol. Once the sample aerosol is introduced into the ICP torch, it is completely desolvated and the elements in the aerosol are converted first into gaseous atoms and then ionized towards the end of the plasma. Once the elements in the sample are converted into ions, they are then brought into the mass spectrometer via the interface cones. Once the ions enter the mass spectrometer, they are separated and identified by their mass-to-charge ratio.

A3 - Zeta Potential

Zeta potential is a measure of magnitude of repulsive or attractive charges between particles. It can provide information on the stability of a particular solid or suspension in a solution which could be useful in determining its level of dispersion or aggregation.

Many nanoparticles or colloidal particles have a surface charge when they are in suspension. When an electric field is applied, the particles move due to the interaction between the charged particles and the applied field. The direction and velocity of the motion is a function of the particle charge, the suspending medium, and the electric field strength. Particle velocity is then measured by observing the Doppler shift in the scattered light. The particle velocity is proportional to the electrical potential of the particle at the shear plane which is the zeta potential. Thus, this optical measurement of the particle motion under an applied field can be used to the determine zeta potential.

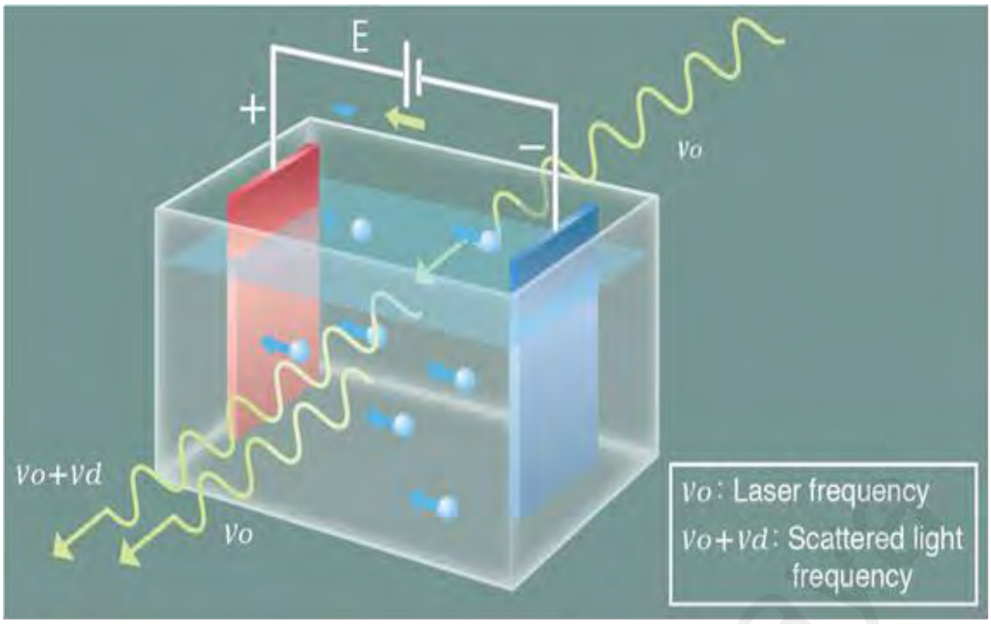

Figure 2 : Zeta potential working principle

Particle motion under an applied electric field is known as electrophoresis. Sample particles are suspended in a solvent af known refractive index n, velocity η and dielectric constant ε. The sample is irradiated with laser light of wavelength λ. An electric field with strength E is applied. Due to the electric field, the particles are moving. Since the particles are moving, the scattered light at angle θ is measured and the particle velocity V is determined from the frequency shift. Mobility is then readily obtained as the radio of velocity to electric field strength V/E. Zeta potential is then found mobility using a model, the most common of which is the Smulochowski model. The following equation is used for the relationship between the calculated electrical mobility and zeta potential. Typically, the calculation will be automatically carried out by the instrument.

$$U = \frac{\lambda \cdot Vd}{2 \cdot E \cdot n \cdot \sin(\theta/2)}$$

A4 - Field Emission Scanning Electron Microscope and Energy-dispersive X-ray

FESEM has been an important instrument used to characterize and visualize morphology of nanostructures. It achieves its high resolution because it uses electron instead of light. It produces images of a sample by scanning it with a focused beam of electrons namely primary electrons. The electrons interact with atoms in the sample, causing the atom to excite and emit electrons, namely secondary electrons. The angle and velocity of these secondary electrons relates to the surface structure of the object. A detector collects the secondary electrons and produces an electronic signal which later translate into an image displaying the topography of the surface.

Electrons can be generated by heating a tungsten filament using a current to a temperature of about 2800°C. Electrons are also produced using crystal of lantanumhexaboride (LaB6) for better resolution. Owing to the advancement of technology and the needs to enhance the resolution, electron is produced using a cold approach; known as cathode-anode configuration. Cathode made of an extremely thin and sharp tungsten needle can produce electron when gradient of potential between 0.5 to 30 KV is created between the cathode and the anode. This kind of electron production is called field emission.

In addition to low energy secondary electrons that are vital to visualize the morphology of the sample, backscattered electrons and X-rays are also generated as a result of the primary electron bombardment. The intensity of backscattered electrons can be correlated to the atomic number of the element within the sampling volume. Hence, some qualitative elemental information can be obtained. The analysis of characteristic X-rays (EDX or EDS analysis) emitted from the sample could provide a quantitative elemental information.

A5 - UV-Vis Spectrometer

UV-VIS Spectrophotometer is an instrument ideal for measuring the absorbance spectra of various chemical and biochemical compounds. Different molecules absorb radiation of different energy or wavelengths. When an atom or molecule absorbs energy, electrons are promoted from their ground state to an excited state or antibonding orbitals. Potentially, three types of ground state orbitals may be involved; i) σ (bonding) molecular orbital, ii) π (bonding) molecular orbital and iii) n (non-bonding) atomic orbital. In addition, two types of antibonding orbitals may be involved in the transition; i) σ* (sigma star) orbital and ii) π* (pi star) orbital.

A transition in which a bonding *s* electron is excited to an antibonding σ orbital is referred to as σ to σ* transition. In the same way π to π* represents the transition of one electron of a lone pair (non-bonding electron pair) to an antibonding π orbital. Thus the following electronic transitions can occur by the absorption of ultraviolet and visible light: σ to σ*, n to σ*, n to π*, π to π.

A6-Fourier Transform Infrared Spectrometer

Fourier Transform Infrared (FTIR) spectrometer is an instrument to identify organic functional group present in a chemical system. The identification is based on the vibration modes at different infrared wave numbers. The presence or absence of functional groups, their protonation states, or any changes due to new interactions can be monitored by analysing the position and intensity of the different infrared absorption bands[1].

When infrared (IR) radiation is passed through a sample, some radiation is absorbed by the sample and some passes through (is transmitted). The resulting signal at the detector is a spectrum representing a molecular ‘fingerprint’ of the sample. Michelson interferometer is used to provide light of different wavelength and spectrum. The light will be shone on the sample. The molecules will absorb specific frequencies that are characteristic of their structure. The frequency of the absorbed radiation matches the transition energy of the bond or group that vibrates. These energies are determined by the shape of the molecular potential energy surfaces, the masses of the atoms and the associated vibronic coupling. The transmitted output of the light will then be compared with the reference. The spectra at which the IR is absorbed can be matched with the corresponding enegry of the functional group.

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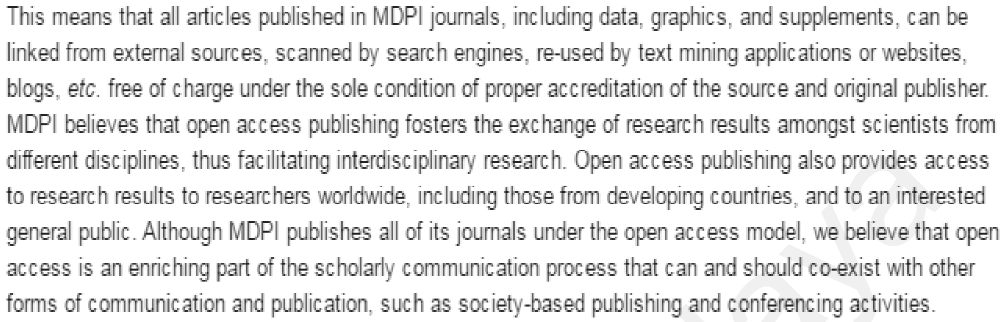

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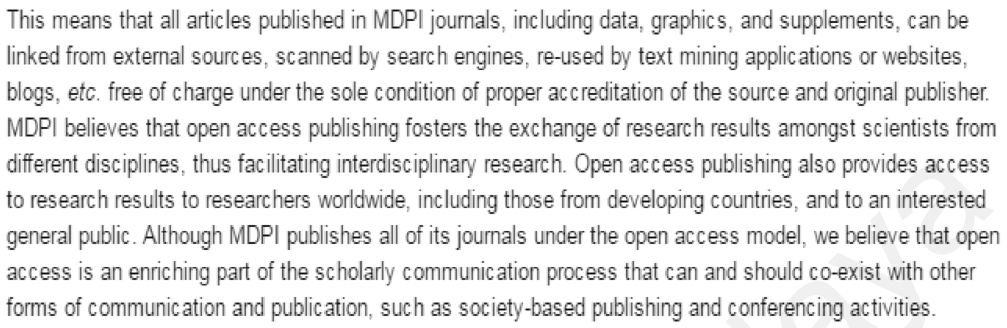
B6 - Materials

Author: Zheng-Yong Zhang and Huan-Ming Xiong
Article: Photoluminescent ZnO Nanoparticles and Their Biological Applications
Volume: *8*(6), 3101-3127
Publisher: MDPI



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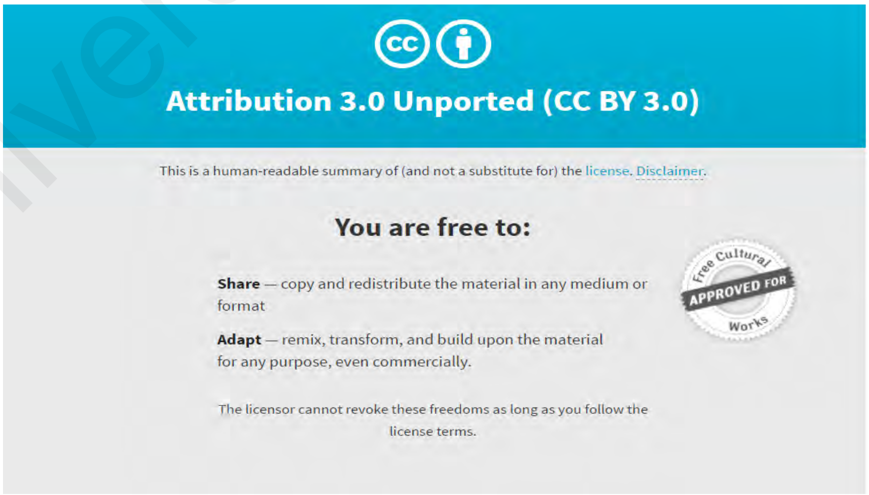
B7 - Bioinorganic Chemistry and Applications

Author**:** Brajesh Kumar, Kumari Smita, Luis Cumbal, and Alexis Debut
Publisher: Hindawi
Article: Green Approach for Fabrication and Applications of Zinc Oxide Nanoparticles



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