Light-Dependent Biosynthesis of Silver Nanoparticles Mediated by Microalgal Cell Extract

by

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Abstract

Silver nanoparticles (AgNPs) are a promising nanomaterial with numerous applications and high level of commercialization. Biomass-mediated AgNP synthesis has emerged as a novel approach for producing AgNPs and microalgal biomasses have been found particularly advantageous. However, few studies have so far focused on microalgae-mediated biosynthesis and the mechanism of AgNP biosynthesis is still elusive.

The purpose of this study was twofold: 1) to investigate effects of different parameters on the biosynthesis of AgNPs; 2) to investigate the mechanisms involved in such a bioprocess. It was found that the cell extract of *Neochloris oleoabundans* prepared by whole cell aqueous extraction (WCAE) in boiling water bath was able to reduce Ag^+ to AgNPs. It was further discovered that sonication of algal cells before extraction could enhance the efficiency of cell extraction and enable AgNP biosynthesis using cell extract obtained by disrupted cell aqueous extraction (DCAE) at room temperature. Light was required for AgNP biosynthesis and rainbow tests showed that purple and blue lights were particularly necessary. Based on experimental results, we hypothesize the mechanism of microalgae-mediated AgNP synthesis to be a chlorophyll-mediated reaction, in which chlorophylls are excited upon absorbing photons in the purple and blue spectra and donate electrons to reduce Ag^+, the lost electrons are replenished by water-splitting reaction.

Résumé

Les nanoparticules d'argents (AgNPs) sont un nanomatériel prometteur ayant plusieurs applications et un excellent potentiel commercial. La synthèse d'AgNPs à partir de biomasse a émergé comme approche innovatrice pour la production d'AgNPs et les biomasses de micro-algues s'avèrent particulièrement avantageux. Par contre, peu d'études, à ce jour, se sont concentrées sur l'utilisation des micro-algues pour la biosynthèse d'AgNPs et le mécanisme derrière leur production est encore mal compris.

Cette étude avait pour but d'analyser les impacts de différents paramètres sur la biosynthèse d'AgNPs ainsi que les mécanismes responsables de leur production. Nous avons découvert que l'extrait de cellules *Neochloris oleoabundans,* préparé par extraction aqueuse de cellules entières (EACE) dans un bain d'eau bouillante, permet de réduire des ions Ag^+ en AgNPs. Nous avons également découvert que la sonification de cellules d'algues avant l'extraction améliore l'efficacité de l'extraction et permet la biosynthèse d'AgNPs en utilisant l'extrait obtenu par extraction aqueuse de cellules perturbée (EACP) à température ambiante. Dans le cas de l'EACE ainsi que de l'EACP, la lumière était requise pour la biosynthèse des AgNPs et la chlorophylle était un élément essentiel dans la réduction d'Ag^+. En effet, l'analyse arc-en-ciel a démontré que la lumière violette et la lumière bleue étaient particulièrement importants pour la biosynthèse d'AgNPs. Basé sur nos résultats expérimentaux, nous avons formulé l'hypothèse qu'une réaction dirigée par les chlorophylles excitées par l'absorption de photons provenant du spectre violet et bleu, est en fait le mécanisme responsable pour la synthèse d'AgNPs par les micro-algues. L'absorption permets aux chlorophylles de fournir des électrons pour réduire les ions Ag^+; c'est électrons perdus sont ensuite récupéré par une réaction de séparation d'eau.

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Nomenclature list

AgNP	Silver nanoparticle
WCAE	Whole cell aqueous extraction
DCAE	Disrupted cell aqueous extraction
λ_{max}	Absorption maxima
SPR	Surface plasmon resonance
SEM	Scanning electron microscopy
EDS	Energy dispersive X-ray spectrometry
TEM	Transmission electron microscopy
UV-Vis	Ultraviolet-visible spectroscopy
FTIR	Fourier transform infrared spectroscopy
FAAS	Flame atomic absorption spectroscopy
MBM	Modified Bristol medium
LB	Luria broth
DW	Deionized water
IZD	Inhibition zone diameter
E_{max}	Light extinction at absorption maxima

Chapter 1:

Introduction

1.1 Introduction

As a highly commercialized nanoparticle, silver nanoparticle (AgNP) accounted for over 50% of total nanomaterial based products [1]. The reason behind the high level of commercialization is AgNPs' various applications in different fields [2]. For example, AgNPs have already been used in public (e.g., railway stations) as antimicrobial agents [3] and they also showed cytotoxic effects against human breast cancer cells (MCF-7) [4]. The versatile applications of AgNPs led to extensive studies on AgNP synthesis, including physical, chemical, and biological methods [5]. Compared to conventional chemical or physical approaches where hazards chemicals and/or expensive apparatus were usually involved [5], [6], biosynthesis, including microalgae-mediated biosynthesis, of AgNPs has received more and more attentions from researchers.

Both biomasses [7] and aqueous cell extracts [8][9] of a variety of plants and microorganisms are able to mediate AgNPs biosynthesis when incubated with Ag^{+} under appropriate conditions. When biomasses are applied, cells either assimilate metallic ions and reduce them to form nanoparticles intracellularly [10] or cause metallic ions to reducing extracellularly on cell surfaces or in broth. In most cases, biomass-mediated biosynthesis produces AgNPs that are either stored intracellularly or bound to cell surface [12], which complicates the downstream process (i.e., product recovery). Aqueous cell extracts, obtained by whole cell aqueous extraction (WCAE) and disrupted cell aqueous extraction (DCAE), were also reported to enable AgNP synthesis. [8], [9], [11].

The objective of this study is threefold, 1) verify the feasibility of utilizing aqueous cell extracts of green algae *Neochloris oleoabundans* for AgNPs biosynthesis; 2) investigate the

effects of different conditions on the biosynthesis; and 3) study the mechanisms involved in the AgNP biosynthesis.

This thesis is composed of five chapters. Chapter 1 is a brief introduction for the thesis; Chapter 2 is a literature review focusing on different types of microalga cells used for mediating AgNP synthesis, techniques used for characterizing nanoparticles, and hypotheses proposed to explain the mechanisms of AgNP biosynthesis; Chapter 3 is an original research article focusing on the effects of different parameters, including extraction time, mixture pH, and Ag^{+} concentration on WCAE-mediated AgNPs biosynthesis; Chapter 4 is another original research paper discussing DCAE-mediated AgNP biosynthesis and the possible mechanism of it; and Chapter 5 is a summary of conclusions derived from this study and some recommendations for future studies.

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Chapter 2:

Literature review on microalgae-mediated biosynthesis of silver nanoparticles

Abstract

Silver nanoparticle (AgNP) has been extensively studied due to its practical applications in various fields and high degree of commercialization. Besides the conventional chemical or physical methods for the synthesis of AgNPs, biomass-mediated biosynthesis, especially microalgae-mediated biosynthesis, of AgNPs has attracted great interests among researchers because it is potentially more environmental friendly and cost effective. Ag^+ could be reduced to AgNPs by either algal biomass or cell extract. The former could be categorized into, on the basis of where AgNPs are produced, intracellular and extracellular (or on the surface) synthesis, while the latter includes AgNP synthesis mediated by whole cell aqueous extraction and that by disrupted cell aqueous extraction. This review aims at evaluating recent developments in microalgae-mediated AgNP biosynthesis to help identify knowledge gaps and facilitate further studies on this topic.

2.1 Introduction

Silver nanoparticles (AgNPs) have become a promising emerging nanomaterial due to its wide applications in diverse fields, including antibacterial, skin drug delivery, catalysis, cancer therapy, etc [1]–[4]. The various applications also lead to extensive studies on AgNP synthesis. While the conventional chemical and physical methods for synthesizing AgNPs have been established many years ago [5], [6], biological methods have emerged as a more environmental-friendly and cost-effective strategy for AgNP synthesis [7], [8]. Many plants or microorganisms were reported being used for AgNP biosynthesis, including leaves of plants (e.g., *Acalypha indica*) [9], fungus (e.g., *Fusarium oxysporum*) [10], bacteria (e.g., *Bacillus subtilis*) [11], micrioalgae (e.g., *Chlorella vulgaris*) [12], etc.

Among the possible bioproducers, more and more researchers have started focusing on microalgae-mediated AgNP synthesis for a few unique advantages. First, the production of AgNPs could be conjugated with the production of biofuels such as biodiesel. For instance, de-oiled microalgae were able to mediate AgNP biosynthesis [13]. Secondly, microalgae grow much faster compared to plants and large-scale cultivation of microalgae is well-developed [14].

The objective of this review is to provide a comprehensive and systematic overview of the recent developments in the field of microalgae-mediated biosynthesis of AgNPs. A criterion of classification based on the nature of biomass used for AgNP biosynthesis is proposed to organize the seemingly scattered information in this field to help identify knowledge gaps and facilitate studies in the field. According to this criteria, microalgae-mediated AgNP biosynthesis is categorized into two groups, i.e., whole cell and cell extract-mediated synthesis. Whole cell-mediated AgNP biosynthesis is further classified into intracellular and extracellular synthesis

while aqueous cell extract-mediated AgNP biosynthesis is classified into whole cell aqueous extraction (WCAE) and disrupted cell aqueous extraction (DCAE) synthesis. Commonly used characteristic techniques and some proposed hypotheses on the mechanisms of AgNP biosynthesis are also presented and analyzed in this review.

2.2 Microalgae-mediated AgNP biosynthesis

2.2.1 Cell-mediated biosynthesis

Cell-mediated biosynthesis refers to the biosynthesis of silver nanoparticles, or nanoparticles of other metals such as gold for this matter, that is mediated by whole cells of microorganisms including bacteria, fungi, and microalgae, although our focus is on microalgae. Cell mediated biosynthesis could be further classified into two groups according to the location of biosynthesis, i.e., extracellular and intracellular biosynthesis. Table 1 summarizes reports on cell-mediated biosynthesis of AgNPs involving microalgae.

Table 2- 1 Recent studies on microalgal whole cell-mediated AgNP synthesis.

Strains	Incubation Parameters			Separation and purification	Morphology		SPR peak (nm)	References
	pH	Light condition	$AgNO_3$ (mM)		Shape	Size (nm)		
Spyrogira insignis	6-10	Dark	0.5	Filtration	nonuniform	30	460	[17]
Chlorococcum humicola	-	-	5	-	Spherical	2 to 4	-	[15]
Chlamydomonas strain CC-124	-	-	1	Sonication and Filtration	Rounded/rectangular	5±1 to 35±5	470	[19]
Microcoleus sp.	6	-	0.95	-	-	44 to 79	370/440	[30]
Scenedesmus sp.	-	-	5	-	Spherical	15 to 20	420	[17]
Euglena gracilis	-	Light	1	-	Spherical	6 to 24	422	[14]
Euglena intermedia	-	Light	1	-	Spherical	6 to 24	413	[14]
Nannochloropsis oculata	-	-	1 to 5	-	-	-	-	[20]
Chlorella vulgaris	-	-	1 to 5	-	-	-	-	[20]
Chlorella vulgaris	-	Light and dark	1	Filtration	-	8 to 20	400	[22]

2.2.1.1 Intracellular AgNP synthesis

Some living microbial cells, including some microalga species, could assimilate Ag^+ and other metal ions into cells, where they are reduced by intracellular reducing agents to form nanoparticles. There have been a few studies reporting successful biomass-mediated AgNP synthesis using microalgae since 2011 [12], [15]–[18].

General protocol of intracellular biosynthesis of AgNPs follows the following steps: 1) cells are cultivated under specific conditions depending on strains to produce biomass; 2) biomass is then harvested to remove the trace of medium ions, which may cause side reactions when challenged by Ag^+; 3) the mixtures of biomass and Ag^+ are incubated under specific conditions (e.g., Ag^+ concentration, light, etc) for certain period of time to produce AgNPs; 4) AgNP recovery from cells.

Chlamydomonas reinharditii was reported to be able to reduce Ag^+ (1 mM) forming rounded/rectangular AgNPs of the size ranging from 5±1 to 35± 5 nm at room temperature. The location of produced AgNPs was further probed using SEM and AgNPs were found to be produced intracellularly, localized in periplasm and cytoplasm to be more exactly [16]. The authors considered the absorption maxima (λ_{max}) at 470 nm, caused by AgNP surface plasmon resonance (SPR) which will be discussed in details later, as the sign of AgNPs' presence and also suggested an approach to harvest AgNPs from cell that was to sonicate cells before using filtration to separate AgNPs from cell debris [16]. Cells of another microalga *Scenedesmus sp*. also showed ability of intracellularly synthesizing AgNPs by being incubated with 5 mM Ag^+ under 28 ^oC for 72 hours, and the produced AgNPs were found to be spherical with the average size of 15 to 20 nm [17]. This study also reported that AgNPs were densely synthesized at

cytoplasm and confirmed that no extracellular synthesis of AgNPs occurred by scanning the cell free reaction solutions from 190 to 1100 nm but no detectable AgNP characteristic peaks (λ_{max}=420 nm in the report) were found [17]. Intracellular synthesis of AgNPs by microalgae was also reported by a study using *Euglena gracilis* and *Euglena intermedia*, which indicated that AgNPs were synthesized in some vesicle-like structures instead of on the cytoplasmic membrane [18].

2.2.1.2 Extracellular AgNP synthesis

Although a majority of studies are concerned with intracellularly biossynthesis, AgNPs were found to be synthesized extracellular as well [12]. A 15 L continuous pilot-scaled system of microalgal cell-mediated AgNP synthesis was reported which mainly included: 1) ammonium chloride reservoir, 2) culture tank with *Chlorella vulgaris* cells suspended in deionized water, 3) filter paper of 0.45 μm pore size, and 4) collection tank for storage [12]. In this process, the ammonium chloride was maintained at 1 mM in the culture tank to maintain cell growth and the Ag^+ concentration was kept at 1 mM to supply Ag^+ while the filter paper was applied at the outlet of culture tank to withdraw cell free AgNP-containing effluent from the culture tank [12]. The results showed that AgNPs were detected both inside and outside of cells and the extracellular AgNPs were most likely synthesized by released biomolecules though unknown mechanism [12]. It is worth to note that *Chlorella vulgaris* reported in this study was found to be strongly tolerant to Ag^+ and AgNPs as almost 90% of cell's viability was maintained after 36 hours of operation [12]. The authors further suggested that extracellular synthesis rarely happened because limited microalgae were able to secrete reducing biomolecules [12]. However, more reports related to AgNP extracellular synthesis are found when the scope of search is broadened

to bacterium and fungus-mediated synthesis. For example, *Oscillatoria willei* was found to enable AgNP extracellular synthesis and the authors suggested that the released proteins/enzymes functioned as reducers of Ag^+ [19]. Similarly, AgNPs were reported to be produced using culture supernatant of *Enterobacteriacae* [20] and *Bacillus subtilis* [21].

Extracellular biosynthesis of metallic nanoparticles would be due to the reduction of metal ions by reducing agents secreted by cells as extracellular products. In one particular case, the authors proposed that nitrate reductase was responsible for Ag^+ reduction since nitrate reductase activity was detected in the culture [21]. However, the enzyme nitrate reductase, which was induced or repressed by using nitrate or ammonium as nitrogen source [22], was found to locate in the pyrenoid [23] but no other reports on extracellular nitrate reductase secretion was found after a quite extensive literature survey. Besides being mediated by released molecules, it was also suggested that extracellular AgNP synthesis could also be mediated by reducing groups on cell surface since microalgae had a large surface bound with numerous reducing functional groups [24], which had potentials of reducing Ag^+. For example, spherical AgNPs with the diameter of 25 ± 12 nm were found to be synthesized on the surface of *Verticillium* cells and the mechanism was speculated as the joint contribution of negatively charged carboxylate groups and enzymes since no AgNP production was found in solution [25].

2.2.2 Cell extract-mediated biosynthesis

Aqueous cell extract, which contains soluble intracellular materials, was also reported to enable AgNP synthesis, which was defined as (aqueous) cell extract-mediated biosynthesis. Cell extraction could be further classified into two groups: 1) whole cell aqueous extraction (WCAE), where cells are extracted by water of temperature nearly boiling point (i.e., in boiling water bath)

[13], [15], [17]; and 2) disrupted cell aqueous extraction (DCAE), where cell disruption is applied for the enhancement of cell extraction at room temperature [16], [18], [26], [27]. Table 2 summarizes reports on cell extract-mediated biosynthesis of AgNPs involving microalgae.

Table 2- 2 Recent studies on microalgal cell extract-mediated AgNP synthesis.

Strains	Aqueous Extraction		Incubation Parameters		Morphology		SPR peak (nm)	References
	Cell Disruption	Temperature °C	Light	$AgNO_3$ (mM)	Shape	Size (nm)		
Chlorococcum humicola	-	100	-	5	-	-	430-440	[15]
Chlamydomonas strain CC-124	Vortexing/sonication	4	Dark	1	Rounded/rectangular	5±1 to 15±2	470	[16]
Scenedesmus sp.	-	Room temperature	-	5	Spherical	50 to 60	430	[17]
Scenedesmus sp.	-	100	-	5	Spherical	5 to 10	420	[17]
Euglena gracilis	Vortexing/sonication	-	Dark	1	Spherical	15 to 60	425	[18]
Euglena intermedia	Vortexing/sonication	-	Dark	1	Spherical	15 to 60	418	[18]
Chlorella vulgaris	Mortaring	-	-	0.9	Spherical	50 to 70	436	[26]
Chaetoceros calcitrans	Mortaring	-	-	0.9	Spherical	30 to 35	420	[26]
Amphora sp.	Homogenization	-	Light	2	Spherical	20 to 25	413	[27]
Acutodesmus dimorphus	-	100	-	0.9	Spherical	5 to 20	420	[13]

2.2.2.1 Whole cell aqueous extraction (WCAE)

In WCAE, plant leaves, roots, or cells of bacteria, fungi, and microalgae are suspended in water, which is submerged in boiling water bath for extraction of cellular materials. The resulting aqueous cell extract is then used to mediate the biosynthesis of AgNPs. For instance, whole cell aqueous extract of a unicellular green alga *Chlorococcum humicola* was obtained by WCAE under 100 ^{o}C for 30 min, and the extract was further used for Ag^{+} reduction to produce AgNPs and the wavelengths of AgNP characteristic peaks of the solution were found to range from 430 to 440 nm [15]. In this study, the produced AgNPs were confirmed to have significant antimicrobial activity against human pathogen *Escherichia coli* and the FTIR test indicated that the AgNP synthesis, though not directly evident, was a redox reaction having protein participating [15]. In another study, *Scenedesmus sp.* was extracted by WCAE under room temperature for 5 days and 100 ^{o}C for 20 min, and the two cell extracts were identically incubated with 5 mM Ag^{+} under room temperature [17]. By comparing the AgNPs produced by

two cell extracts, it was found that boiled cell extract produced AgNPs in a much more rapid way and the produced AgNP suspensions were extraordinarily stable for over 30 days compared to AgNPs produced by room temperature extract agglomerating within a short period [17]. The authors explained their results by pointing out that higher temperature (e.g., 100 ^{o}C) increased reducing agent concentration in cell extract, which was of great important to AgNP synthesis in terms of particle size and stability [17]. The authors further indicated that, compared to the room temperature extract, there was a 33 times of increase in released protein concentration found in cell extract obtained under 100 ^{o}C, and those proteins were thereafter supposed to produce as well as stabilize AgNPs [17]. In another study, cells were dried under 60 ^{o}C and biofuel was extracted from dried cells using a group of organic solvents (e.g., chloroform, acetic acid, acetone, etc) [28]. The de-oiled cells were then extracted by WCAE under 100 ^{o}C for 5 min before being incubated with 1 mM Ag^{+}, and the results showed that the produced AgNPs were spherical with a size ranging from 2-20 nm [13]. The authors further indicated that –NH in proteins may be responsible for AgNP formation and those proteins coated with AgNPs functioned as stabilizers of AgNPs [13], which was compatible to the study just mentioned.

2.2.2.2 Disrupted cell aqueous extraction (DCAE)

Cell disruption prior to extraction increases the reducing agent concentration in cell extract, which was important to AgNP production and stability. It is thus reasonable that more researchers studied AgNP biosynthesis using cell extract obtained by DCAE [13], [16]–[18], [26], [27]. *Chlamydomonas reinhardtii* cells were disrupted by vortexing with glass beads followed by sonication, to avoid the effects of generated heat, 30 s of cooling was employed between every 1 min of agitation and the sonication was operated as 2 s on and 10 s off [16]. The

size of AgNPs was found to be 5 ± 1 nm to 15 ± 2 nm and it was also worth to note that the AgNP synthesis mediated by cell extract was slower than that mediated by cells, which was explained as the results of the lack of active biomolecules in living cells [16]. Another study which also employed vortexing and sonication for DCAE reported successful synthesis of AgNPs using *Euglena* microalgae, and a similar intermittent sonication (5 s on and 10 s off) was also applied for sample cooling [18]. Other than vortexing and sonication, mortaring was used for the DCAE of *Chlorella vulgaris* and *Chaetoceros clacitrans* and spherical AgNPs of size 50 to 70 nm and 30 to 35 nm, respectively, were found after the cell extract incubated with Ag^+ at 60 ^oC for one hour [26]. *Amphora sp.* cells were also homogenized, though the homogenization was not clearly explained, before the extraction, and the cell extract successfully reduced Ag^+ to spherical AgNPs (20 to 25 nm) with the surface plasmon resonance peak centered at 413 nm [27].

2.2.3 Comparison between whole cell and cell extract -mediated AgNP synthesis

AgNP synthesis mediated by cell extract, no matter WCAE or DCAE, was more popular than that mediated by whole cells (mostly intracellular synthesis) for the following reasons. When AgNPs were synthesized using cell extract, easy product recovery is the first advantage. As aforementioned, in cell extract-mediated biosynthesis, AgNPs are supposed to be produced in cell free solutions because cells and cell debris have already been removed. The separation of AgNPs from remaining Ag^+ and cell extract could be also be achieved by centrifugation [29]. In contrast, to recover the AgNPs synthesized intracellularly, typical strategy is to disrupt cells (e.g., sonication) before employing filtration [16]. Even for the AgNPs synthesized

extracellularly are difficult to isolate since the majority of them were found to be firmly bound with the cell surface [12].

Easy characterization of AgNPs is another advantage of using cell extract to produce AgNPs or study on the mechanism. For example, spectrophotometer is a frequently used apparatus for detecting AgNPs (which will be specified later). Compared to the spectra of cell extract-mediated biosynthesis mixtures, spectra of whole cell-mediated biosynthesis mixtures usually had no clear peaks because the presence of cells significantly increased the noise [13], [30].

Another advantage is the easy storage, components in cell extract can be stored at low temperature for long period of time and remain active. For example, chlorophyll, as a typical and common pigments in microalgae, was reported to be well stored and protected at -20 °C, which is not an extremely low temperature and allows quick recovery for use [31]. Proteins/enzymes (which are also potential reducers) were proved to be capable of being stored at low temperature. When microalgae are stored, they may be contaminated by zooplankton, bacteria, harmful algae, and virus, and the above biological pollutants can either graze algae or secrete extracellular compounds [32]. Cell lysis also results in the loss of stability of the culture, and cell lysis is hard to control since it was determined by various factors [33]. Freezing seems like a solution to this problem; however, freezing-thawing process also causes injury and the use of protective agent introduces other unwanted chemicals [34].

Cell extract-mediated AgNP synthesis also has its disadvantage. Firstly, cell disruption may be required in DCAE for AgNP production. Different from animal cells, microalgae cells have cell wall, which protects themselves from being disrupted or extracted [35]. Although

microalgae disruption has already been extensively studied [36], [37], methods can be used for cell extract-mediated biosynthesis are still limited because the mechanism and the functional components in cell extract are not clear and the cell disruption has to allow those unknown molecules remain active. The extraction process may have the same problem, it was reported that the synthesis of AgNPs using cell extract was much slower than that mediated by whole cells [16].

2.3 Characterization techniques

Characterization is essential to study on AgNP biosynthesis because the data collected using characterizing techniques may provide researchers with valuable information for understanding the reaction. Commonly used techniques of characterizing AgNPs are electron microscopy and spectroscopy.

2.3.1 Electron microscopy

Though electron microscopy techniques are relatively expensive and usually require experienced operators, they are still very important since electron microscopy can visually confirm the presence of AgNPs and examine particle morphology. Currently used electron microscopy techniques are scanning electron microscopy (SEM) and transmission electron microscopy (TEM). SEM is a useful surface imaging technique, which can be used for characterizing the shape and size of AgNPs; it can also determine the composition of samples once conjugated with Energy Dispersive X-Ray Spectroscopy (EDX) [38]. Compared to SEM, TEM is a much more powerful tool, which can be used for examining ultra-small particles. Besides AgNPs, cells also need to be examined to determine the morphology of cells and where AgNPs located are. The morphology of cells before and after reaction could be shown using

SEM [12], and the intracellular biosynthesis of AgNPs was also confirmed using detailed TEM images of cells [15].

2.3.2 Spectroscopy

2.3.2.1 Ultraviolet-Visible spectroscopy (UV-Vis)

Compared to the electron microscopy techniques introduced above, UV-Vis seems to be more popular and is being extensively used because it is a more portable apparatus without the need of experienced operators' help. Another reason is that spectrophotometers are typically priced at 5,000 to 9,000 CAD (by Fisher Scientific) while TEM costs 150,000 to 875,000 CAD (by TSS Microsocopy).

Surface plasmon resonance (SPR) is the resonant oscillation of free electrons on the surface of nanoparticles. The light absorption caused by SPR of nanoparticles can be detected using UV-Vis spectrophotometer to generate spectra [39], [40]. Technically speaking, in most cases, what spectrophotometer detects is not light absorption but actually light extinction, which consists of light absorption and scattering. It was reported that light extinction can be considered roughly equal to absorption, but this conclusion is not accurate when particle sizes range from 44 to 61 nm [41]. However, the spectra are still widely applied since absorption and extinction usually peak at the same wavelengths [41], and the absorption maxima is used for characterizing metal species [42].

Besides applied to AgNP characterization, UV-Vis has been widely used for characterizing nanoparticles of other metals like ZnO [43], Au [44], Cu [45], etc. However, UV-Vis also has its own disadvantage. One of the limit of this method is that the morphology of nanoparticles also affects suspension color [46] as well as the absorption maxima in the spectrum

[47]. Therefore, agglomeration of AgNPs or other impurities (e.g., cell debris) may shift the absorption maxima or cause noises, which disorders the results. In a study reporting a size-controlled synthesis of AgNPs, the results showed that the absorption maxima redshifts with the increasing size of particles [48]. However, according to the published data presented in Table 1 and Table 2, there is no convincing relationship between the absorption maxima and nanoparticle size probably because the absorption maxima is affected by multi-factors and the measurement of particle size is not accurate enough. Another limit is that the spectra of different components in samples may overlap. For example, chlorophyll and AgNPs were reported have characteristic peaks located at 420 nm [49], [50]. In this case, in the whole cell-mediated biosynthesis, absorption peak at 420 nm may be contributed by chlorophyll and/or AgNPs.

2.3.2.2 Fourier transform infrared spectroscopy (FTIR)

Besides AgNPs, bioagents also need to be defined and FTIR is such a frequently used technique for characterizing biomaterials [51]. FTIR generates spectrum by measuring the stretching vibrations of chemicals [52]. By comparing the results before and after reaction, the changes can help researchers deduce the contents of samples as well as the mechanism behind the reaction.

On the other hand, though many researchers did FTIR analysis, there is no convincing evidence showing the mechanism of biosynthesis of AgNPs. The reason is the components inside cells are much more complex than the information FTIR analysis can provide. We can only know the changes of functional groups by FTIR spectroscopy; however, the data is still too limited to hypothesize the structure of biomolecules and probably several biomolecules take participate in this reaction together.

2.3.2.3 Flame atomic absorption spectroscopy (FAAS)

Biosynthesis of AgNPs as a biochemical reaction, researchers also concern its kinetics. Currently, since there is no portable way of quantifying AgNPs concentration, one strategy is to determine the concentration of Ag^+ remaining in samples and the amount of Ag^+ consumed is corresponding to the amount of AgNPs produced [47]. FAAS is a widely used method of determining concentration of specific metal ions because it is easy to use, reliable, and inexpensive [53]. The mechanism of FAAS is easy to understand. Firstly, metal ions are atomized by heat (flame), and the atoms are capable of absorbing light of particular wavelength to be excited; the light absorption can be measured by FAAS to determine ion concentration in tested samples [54].

The performance of FAAS is not very satisfied when the concentration of ions to be determined is low [55], [56]. Another limit is that the AgNPs concentration was considered as equal to the Ag^+, but this hypothesis is based on no AgNPs degradation and Ag^+ can only be reduced to form AgNPs.

2.4 Mechanisms

As the biosynthesis of AgNPs is a 'bottom-up' approach as well as a redox reaction [57], Ag^+ and reducing agents are necessary to the synthesis. Based on Marcus theory, the electrons are transferred from electron donors (reducing agents) to electron acceptors (Ag^+), and the reaction preferably occurs in polar solvents like water [58]. However, even the scope was narrowed down to microalgae-mediated AgNP biosynthesis, researchers have not reached an agreement on the mechanism behind this bioprocess. Three representative functional bioagents

which are hypothesized as providers of reducing power: protein/enzyme [16], polysaccharide [47], and pigment [27].

As aforementioned, many researchers speculated or confirmed the involvement of proteins/enzymes in AgNP synthesis, either as reducing agents or as stabilizers. Some proteins, including histone and ATP synthase, were found to be of great importance to AgNP synthesis because the reaction rate was significantly decreased and the produced AgNPs were larger after proteins were depleted using CM-sepharose and DEAE-sepharose ion exchange chromatographic column [16]. While the mechanism was not clear, proteins were found to be bound with synthesized AgNPs [16]. The same research group also reported that in *Microcoleus sp.*-mediated AgNP synthesis, Ag^+ was reduced by a protein, i.e., nitrate reductase [30].

Polysaccharides were also considered to be a potential reducing agent for metallic ion reduction. For example, in a study using ground dry biomass of the green alga *Spyrogira insignis* to mediate AgNPs [47], the authors suggested that pectins (a polysaccharides rich in galacturonic acid) were the reducing agents because the they have reducing hydroxyl groups [47]. Besides polysaccharides on cell wall or inside cells, a green alga *Neochloris oleoabundans* was found to produce extracellular biopolymers (polysaccharides) [59], which may potentially reduce suitable ions. However, no experimental evidences (e.g., oxidization of these groups) were provided to support this hypothesis.

Pigments have become a new candidate since some researchers, including our group, proved that the AgNP biosynthesis was a light dependent reaction [12], [27] while other reports showed that the biosynthesis of AgNPs using microalgae did not require light [16], [47]. It was further discovered that fucoxanthin could produce AgNPs with illumination [27]. This result was

confirmed by the consistent UV-Vis spectra of cell extract and fucoxanthin. We did not find any direct evidence showing how does light function in synthesis of AgNPs, but there are reports studying on such light-induced reactions [60], [61]. Inspired by the photosynthesis, light may be in charge of exciting electron transfer in the presence of particular pigments [62]. However, the concern is that, though the role of light in the reaction is not clear, it has been widely admitted that light is harmful to the stability of nanoparticles. It was reported that AgNPs can be well protected in dark but significantly changed when exposed to light [63], [64]. In addition, as aforementioned, some of reactions occurred in darkness.

Due to the conflicts among the existing mechanisms reported by different groups, we realized that there was probably more than one mechanism behind the reaction and AgNP biosynthesis was actually the results of several bioagents functioning either jointly or individually.

2.5 Conclusions

Existing protocol of producing AgNPs using microalgae can be categorized into synthesis mediated by whole cells or cell extract. Whole cell-mediated synthesis (including intracellular and extracellular synthesis) requires no cell disruption but cell extract biosynthesis was more popular because the produced AgNPs do not need to be separated or recovered from cells. Cells were extracted using water (i.e., WCAE) and cell disruption may apply prior to extraction (i.e., DCAE). Cell extracts obtained by WCAE and DCAE were both found to be able to produce AgNPs. Several techniques were applied to AgNP characterization, including SEM, TEM, UV-Vis, FTIR, and FAAS. Researchers have been trying to find the reaction mechanism and the potential reducing agents were found to be protein/enzyme, polysaccharide, and pigment.

However, the scarce of convincing evidence makes the mechanism still to be prevailed and the underlying conflicts among different theories probably suggest various pathways of such a bioprocess.

2.6 References

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Chapter 3:

Light-dependent biosynthesis of silver nanoparticles using dilute aqueous cell extract of green alga *Neochloris oleoabundans**

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Abstract

Spherical silver nanoparticles (AgNPs) were synthesized by incubating the mixture of $AgNO_3$ solution and aqueous cell extract of *Neochloris oleoabundans* prepared by boiling water bath with illumination. The AgNPs thus synthesized are spherical with a mean particle diameter of 15.8 nm and exhibited decent infirmity and exceptional antibacterial activities. Results suggest that the reactive components are stable at a temperature of 100 ^{o}C, soluble or at least slightly soluble in water, and at least one of them requires light for the reaction that led to the reduction of Ag^{+} to Ag. It was hypothesized that electrons of chlorophylls, which were excited by absorbing photons, were delivered to Ag^{+} to reduce it to Ag and then water splitting catalyzed by the photosystem II of the photosynthetic electron transport chain of *N. oleoabundans* was utilized to replenish the lost electrons in chlorophyll molecules through reactions similar to the light-dependent reactions of photosynthesis.

Keywords: biosynthesis; silver nanoparticles; microalgae; chlorophyll; light-dependent reaction.

3.1 Introduction

Particles smaller than 100 nm are regarded as nanoparticles (NPs), which are characterized by large surface to volume ratio [1]. The extraordinarily small size and large surface to volume ratio of NPs allow their unique and improved performance in numerous applications [2], making production of NPs a topic of extensive interests of both the academic and industrial communities [3]. Among various metallic nanoparticles, silver nanoparticles (AgNPs) are one of the most promising and versatile and have found numerous applications such as in disinfection as antimicrobials [4], [5] and as catalysts [6].

Methods of NPs production can be categorized into three groups: physical, chemical, and biological [7]. Physical and chemical approaches are in general costly, involving complex facilities and/or involving physical and chemical hazards [8]. On the other hand, synthesis of NPs utilizing different forms of bio-derived materials including bacteria, fungi, microalgae, and plants have shown potentials to produce NPs in a cost-effective, environmental friendly, and sustainable manner [9].

Microalgae may prove to be particularly advantageous because of their fast reproduction [10], low or non-toxicity [11], rich contents of various bioactive materials [12], and the fact that they are easy to disrupt using commercial equipment [13]. Furthermore, they are one of the major primary producers that could be produced in a sustainable manner in combination with dynamic carbon dioxide recycling and are therefore excellent candidates for green NP synthesis [14].

A few pioneers have investigated alga-mediated NP synthesis. For instance, it was reported that the synthesis of AgNPs mediated by cell extracts of *Euglena gracilis* and *Euglena*

intermedia obtained using ultrasonscation [15]. It was also reported that cell extract of *Amphora sp.* (IMMTCC-46) was successfully used for producing AgNPs [16]. Approximately 20 research articles were published in 2014 and 2015 on algae-mediated synthesis of metallic NPs [3] and the information in this field is still scarce. Extensive research efforts are therefore warranted to better our understanding to the mechanisms involved in alga-mediated (or in a more general sense biomass-mediated) nanoparticle synthesis and to eventually bring it to a commercially viable level.

In this study, we investigated for the first time the synthesis of AgNPs using extract of green algae *Neochloris oleoabundans* under different conditions including $AgNO_3$ concentration, pH, extraction time, and light/dark conditions. It was demonstrated that the extremely dilute aqueous cell extracts of *N. oleoabundans* under boiling water batch could effectively mediate the bio-reduction of Ag^+ to Ag with illumination. This finding may prove to have significant theoretical and practical significance.

3.2 Materials and methods

3.2.1 Silver Source

$AgNO_3$ powders (99.85%) were purchased from Acros Organics and a 10 mM $AgNO_3$ stock solution was prepared with distilled water. The solution was kept in a brown bottle and stored at 4 °C.

3.2.2 Microbial strains

Green alga *Neochloris oleoabundans* (*N.oleoabundans*, UTEX 1185) was purchased from Culture Collection of Algae at the University of Texas in Austin. *Escherichia coli* (*E. coli*) K-12, which was used for testing the antibacterial activities of AgNPs, was obtained from ATCC.

3.2.3 Media

Media for algal cultivation: The Modified Bristol Medium (MBM), which was adopted in our previous studies [17] was used in this study for the cultivation of *N. oleoabundans*. It was composed of (per litre): 0.35 g $NaNO_3$, 0.138 g K_2HPO_4, 0.0823 g $MgSO_4$, 0.025 g $CaCl_2$, 0.322 g KH_2PO_4, 0.025 g NaCl, 0.0068 g $FeCl_3$, and 1 mL A5 solution. The A5 solution was composed of the following components (per litre): 1.6423 g EDTA-Fe, 2.86 g H_3BO_3, 1.81 g $MnCl_2{\cdot}4H2O$, 0.22 g $ZnSO_4{\cdot}7H_2O$, 0.079 g $CuSO_4{\cdot}5H_2O$, and 0.039 g $(NH_4)_6Mo_7O_2{\cdot}4H_2O$. All chemicals used in the medium were of analytical grade. The media were sterilized using autoclave at 121°C for 20 min.

Media for antibacterial activity tests: Luria Broth (LB), which was composed of (per liter) 10 g tryptone, 5 g yeast extract, and 10 g NaCl, was used to grow *E. coli* inoculum. LB agar, which was composed of LB plus 15 g agar per litre, was used to make agar plates. Both LB and LB agar were autoclaved at 121°C for 20 min. To make agar plates, the autoclaved LB agar was cooled to approximately 55 °C and then about 10 mL medium was transferred into each sterile petri dish under sterile conditions. Agar plates were then put on surface of lab bench further cooled down to room temperature to form solid agar plates.

3.2.4 Algal culture

The microalgae were cultivated in 500mL Erlenmeyer Flasks containing 100 mL sterile MBM for 8 to 10 days at 27 °C in an illuminated incubator until the microalgal cell density reached approximately 1.6×10^7 cells/mL.

3.2.5 Algal cell extract

To avoid the influence of ions in the medium, 100 mL microalgal culture was centrifuged at 7750 g and rinsed with deionized water (DW) three times to produce salt-free biomass, which was re-suspended in 100 mL DW. The biomass suspension was incubated in boiling water bath for a certain period of time as specified in the text and then centrifuged at 7750 g to remove cells and cell debris. The supernatant was defined as (aqueous) cell extract in this study. The contents of chlorophyll a and b in the cell extracts were estimated by measuring the light extinction at different wavelength and then calculated according the following equations as reported as follows [18].

$$\text{Chlorophyll a (nmol/mL)} = 13.43 \times E663.8 - 3.47 \times E646.8 \quad (1)$$

$$\text{Chlorophyll b (nmol/mL)} = 22.90 \times E646.8 - 5.38 \times E663.8 \quad (2)$$

Where E663.8 and E646.8 are the light extinction at 663.8 nm and 646.8 nm, respectively.

It should be noted that the above equations were originally proposed for the calculation of chlorophyll contents in Dimethylformamide (DMF) solutions, which has the largest polarity among three given solvents, i.e., DMF, methanol, and acetone.

3.2.6 Synthesis of AgNPs

5 mL of cell extract was added with different volumes (as specified in text) of $AgNO_3$ stock solution (10 mM), sterile deionized water was then added to make the total volume of the mixture to 10 mL. The mixture was mixed by hand shaking before being incubated at 27 °C for

12 hours for synthesis of AgNPs with illumination at 5000 lux. For AgNP synthesis in darkness, the test tubes were wrapped completely with aluminum foil.

3.2.7 Antibacterial activity of AgNPs

E. coli was transferred sterilely from a work stock slant stored in a refrigerator at 4 °C to a 100 ml flask containing 25 ml liquid LB and incubated at 37 °C overnight. The culture was then transferred to pre-prepared LB agar plates, 20 uL each, and spread evenly onto the agar surface using a sterile glass triangle. Small pieces of sterile filter papers, which had been submerged in the AgNP suspensions of appropriate dilutions beforehand, were transferred using a pair of sterile tweezers to appropriate locations on the surface of agar plates. Labelling the locations of filter papers on the bottom of the plates, which were then incubated at 37 °C for 12 hours. The transparent circles surrounding the filter paper coupons and their sizes were used to estimate the antibacterial activities of the AgNP solutions of different dilutions.

3.2.8 Others

Cell number was counted using a hemocytometer (Improved neubauer, Phase Counting Chamber w/2 cover glass, USA) under a phase-contrast microscope (Infinity II BX40, Olympus, Canada) at a magnification of 200 times.

Spectrometric measurements were carried out by scanning the cell extract or AgNP suspensions from 300 nm to 800 nm using a GENESYS 10S UV-VIS Spectrophotometer. An absorbance peak located at 420 was used to confirm the presence of AgNPs [15].

The morphology of AgNPs was observed by subjecting the AgNP suspension samples to a TEM (FEI Tecnai G2 Spirit Twin TEM) at the Centre for Catalysis Research and Innovation

(CCRI) in the University of Ottawa. Pictures containing scale bars were used for measuring particle size using ImageJ.

3.3 Results

3.3.1 Essential components for AgNPs synthesis

As presented in Table 3-1, all three components, i.e., $AgNO_3$ solution, aqueous cell extract of *N. oleoabundans*, and light (illumination) were required for the synthesis AgNPs. The aqueous cell extracts were obtained by incubating flasks containing *N. oleoabundans* biomass in boiling water bath for varied times ranging from 0.5 to 9 hours. These results indicate that one or more water soluble components that are stable at 100 °C served as the electron donor for the reduction of Ag^+ in $AgNO_3$ to Ag in AgNPs and the reduction reaction is light-dependent.

Table 3- 1 Results of AgNP synthesis with or without Ag^{+}, light, and cell extract.

$AgNO_3$	Light	Cell extract	AgNPs
-	-	+	-
-	+	-	-
-	+	+	-
+	-	-	-
+	-	+	-
+	+	-	-
+	+	+	+

3.3.2 Effects of $AgNO_3$ concentration, pH value, and extraction time on the synthesis of AgNPs

3.3.2.1 Effects of $AgNO_3$ concentration

Figure 3-1 shows the effect of $AgNO_3$ concentration on the AgNPs synthesis at pH 5 with 30-min cell extract. Light extinction at 420 nm (E420) increased significantly from 0.319 to 0.463 when $AgNO_3$ increased from 0.2 to 0.4 mM, however, dropped to 0.177 when $AgNO_3$ concentration further increased to 0.6 mM. The E420 decreased slowly but steadily with minor fluctuations when $AgNO_3$ concentration further increased from 0.6 to 5.0 mM. These results indicate that the optimal $AgNO_3$ for AgNPs synthesis is around 0.4 mM under the specific conditions.

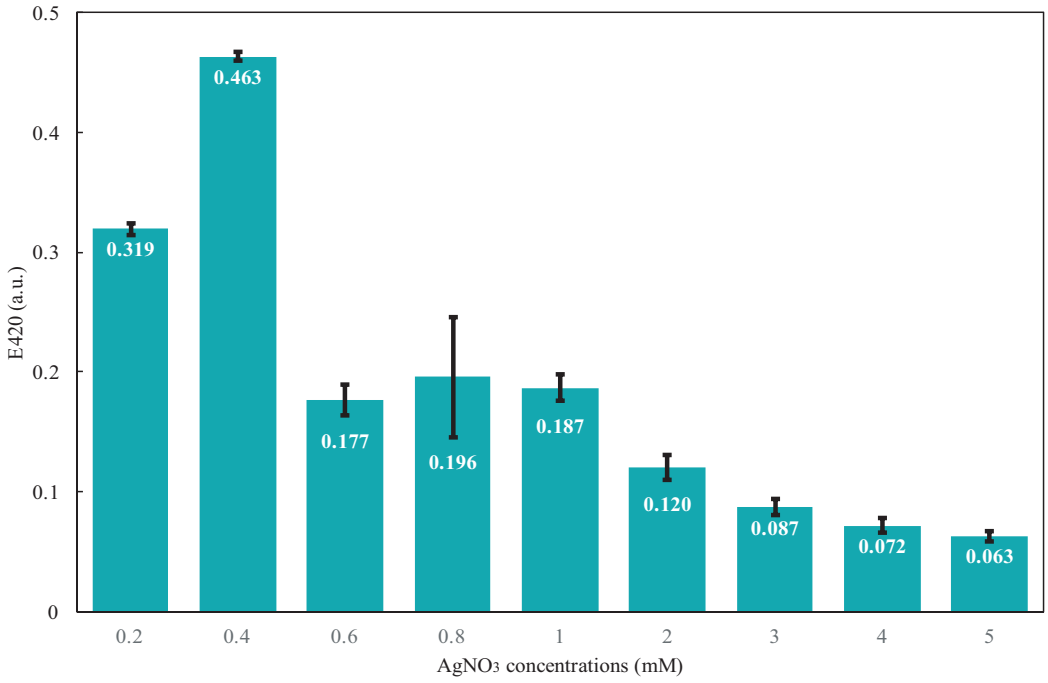

Figure 3- 1 E420 of AgNPs suspensions at different concentrations of $AgNO_3$ at 30 mins extraction, pH 5, and 12 hours incubation with illumination (reported are mean values of triplets).

3.3.2.2 Effects of pH

Figure 3-2 shows the UV-Visible spectra of AgNP suspensions produced at different pH in the range of pH 3-7 with 30-min cell extract and 0.4 mM $AgNO_3$. It is clear that pH 5 gave the highest AgNPs production, at which the E420 was 0.826. Under pH 6 and 7, the production of AgNPs was similar with E420 being 0.16 and 0.17 for pH 6 and 7, respectively. However, the synthesis of AgNPs under pH 3 and 4 seemed to be significantly inhibited since there were no distinguishable peaks at 420 nm under these pH conditions.

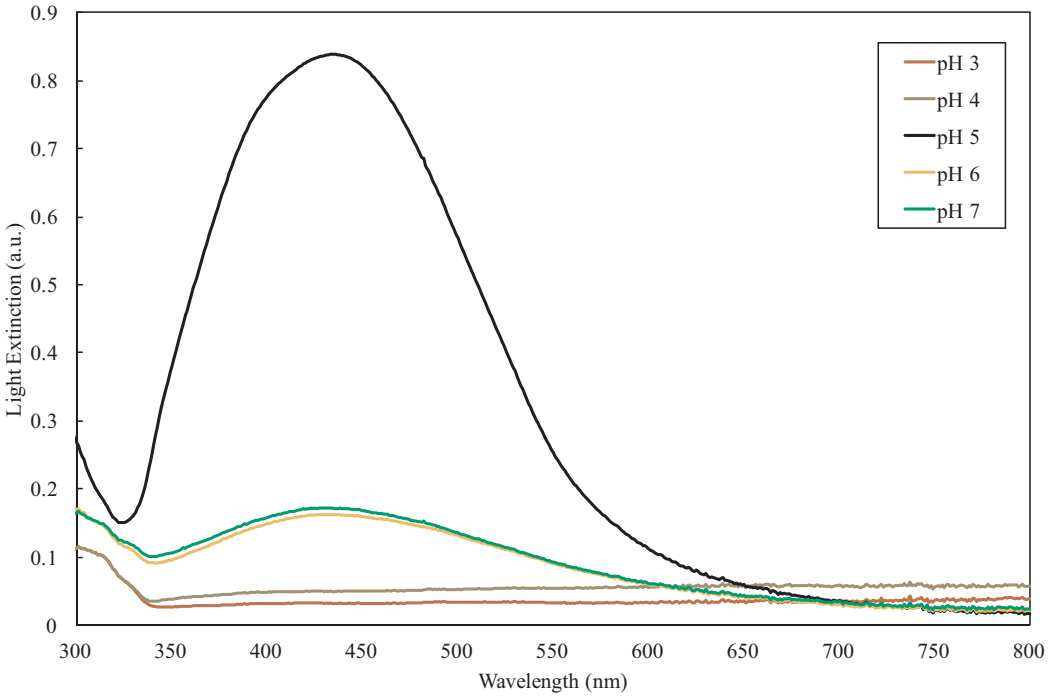

Figure 3- 2 UV-Visible spectra of AgNP suspensions produced at different pH values at 30 mins extraction time, 0.4mM $AgNO_3$, and 12 hours incubation with illumination.

3.3.2.3 Effects of extraction time

Figure 3-3 shows the change of E420 of AgNPs suspensions with extraction time in the range of 0.5-10 hours with 0.4 mM $AgNO_3$ at pH 5. The E420 of the AgNPs suspension increased with extraction time continuously until 6 hours, after which the increase leveled off gradually.

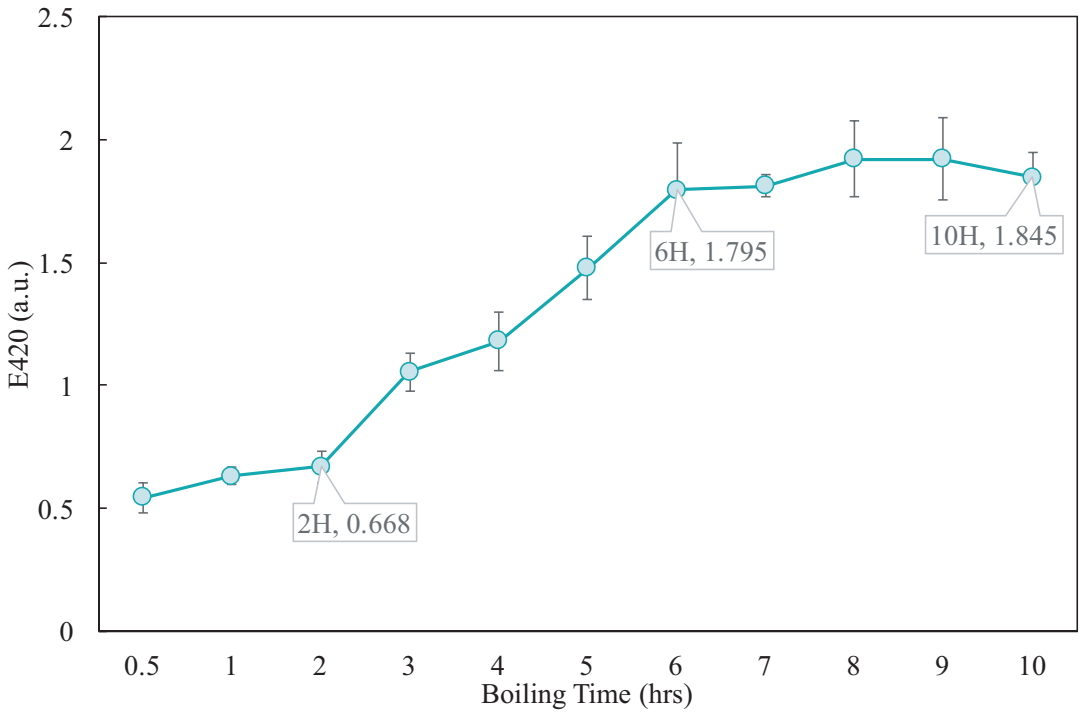

Figure 3- 3 E420 of AgNP suspensions produced with cell extracts of different extraction time at pH 5, 0.4 mM $AgNO_3$, and 12 hours incubation with illumination (reported are means of triplets).

3.3.2.4 Joint effects of different conditions on the synthesis of AgNPs

An orthogonal experiment (Table 3-2) was designed to analyze the significance of each factor [19] and the results are summarized in Table 3-3.

Table 3- 2 Orthogonal experimental plan.

Runs	Factors		
	A	B	C
1	A1	B1	C1
2	A1	B2	C2
3	A1	B3	C3
4	A2	B2	C2
5	A2	B3	C3
6	A2	B1	C1
7	A3	B3	C3
8	A3	B1	C1
9	A3	B2	C2

A: $AgNO_3$ Concentration	B: pH Value	C: Aqueous Extraction Time
A1: 0.4 mM	B1: pH 3	C1: 3 H
A2: 0.8 mM	B2: pH5	C2: 6 H
A3: 1.2 mM	B3: pH7	C3: 9 H

Table 3- 3 Between-subjects effects.

Source	SSE	MSE	P
$AgNO_3$ Concentration	0.565	0.283	0.051
pH Value	0.790	0.395	0.037
Aqueous Extraction Time	1.775	0.888	0.017
Error	0.030	0.015	

The *P* values of pH and aqueous extraction time were less than 0.05 while that of $AgNO_3$ concentration (0.051) was approximately 0.05. As shown in the Figure 3-4, the $AgNO_3$ concentration showed no visible effect on E420 when it was increased from 0.4 mM to 0.8 mM but significant inhibitive effect was demonstrated when it further increased to 1.2 mM. When the extract time increased from 3 to 9 hours the marginal mean value of E420 increased continuously. Finally, the optimal pH for the AgNP synthesis was found to be pH 5. These results show the same trends as when these parameters were individually tested although at different scales. The estimated results of all the 27 combinations were further calculated based

on 95% confidence interval and were summarized in Table 3-4, which show the optimal combination of conditions to be 0.8 mM $AgNO_3$, pH 5, and 9-hour extraction time with a predicted E420 value of 1.84. While some negative values are shown in Table 3-4 as the predicted concentrations of AgNPs, they are not physically realistic and should be treated as zeros.

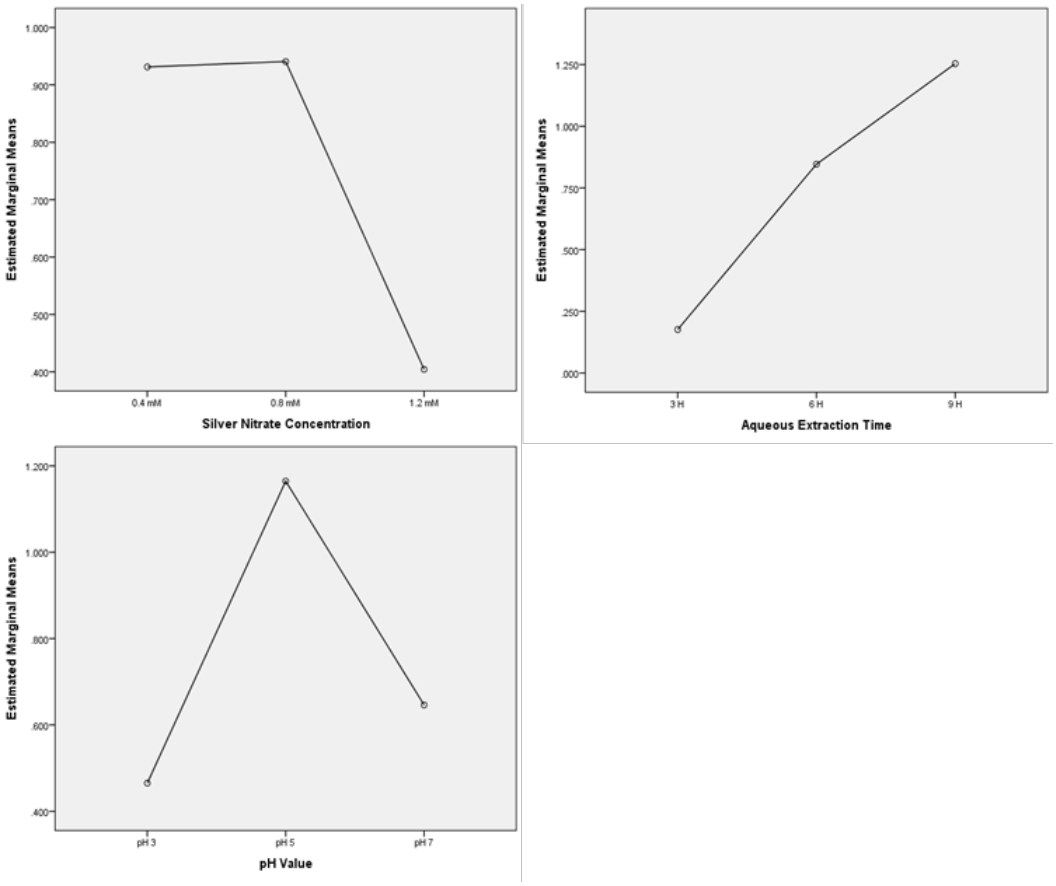

Figure 3- 4 Estimated marginal means of E420 versus $AgNO_3$ concentration, pH value, and extraction time.

Table 3- 4 Estimates of AgNPs synthesis using all the combinations of three parameters.

Parameters			Mean	Std.Error	95% Confidence Interval	
$AgNO_3$ Concentration	pH Value	Aqueous Extraction Time			Lower Bound	Upper Bound
0.4 mM	pH 3	3 H	0.055	0.108	-0.410	0.521
		6 H	0.725	0.108	0.260	1.191
		9 H	1.133	0.108	0.667	1.598
	pH 5	3 H	0.755	0.108	0.289	1.220
		6 H	1.425	0.108	0.959	1.890
		9 H	1.832	0.108	1.367	2.298
	pH 7	3 H	0.236	0.108	-0.229	0.702
		6 H	0.906	0.108	0.441	1.372
		9 H	1.314	0.108	0.848	1.779
0.8 mM	pH 3	3 H	0.065	0.108	-0.401	0.530
		6 H	0.735	0.108	0.269	1.200
		9 H	1.142	0.108	0.677	1.608
	pH 5	3 H	0.764	0.108	0.299	1.230
		6 H	1.434	0.108	0.969	1.900
		9 H	1.841	0.108	1.376	2.307
	pH 7	3 H	0.246	0.108	-0.220	0.711
		6 H	0.916	0.108	0.450	1.381
		9 H	1.323	0.108	0.858	1.789
1.2 mM	pH 3	3 H	-0.472	0.108	-0.937	-0.006
		6 H	0.198	0.108	-0.267	0.664
		9 H	0.606	0.108	0.140	1.071
	pH 5	3 H	0.228	0.108	-0.238	0.693
		6 H	0.898	0.108	0.432	1.363
		9 H	1.305	0.108	0.840	1.771
	pH 7	3 H	-0.291	0.108	-0.756	0.175
		6 H	0.379	0.108	-0.086	0.845
		9 H	0.787	0.108	0.321	1.252

3.3.3 Characterization of AgNPs

Synthesis of AgNPs under the optimal conditions as concluded from the afore discussed experiments, i.e., 9-h cell extract, 0.8 mM $AgNO_3$, pH 5, and 12-h incubation with illumination, were subjected to characterization in terms of UV-visible absorbance spectrum, particular morphology, and antibacterial activities. As shown in Figure 3-5, the suspension of AgNPs obtained under the specified conditions exhibited an E420 of 1.2, which was 27.9 times of that of the cell extract (E420 = 0.026). Visual inspection of the mixtures of cell extract and $AgNO_3$ under illuminated conditions indicated that they changed gradually from the original light greenish color to yellow. This color change is caused by surface plasmon resonance (SPR), which is an indicator showing the presence of AgNPs [20].

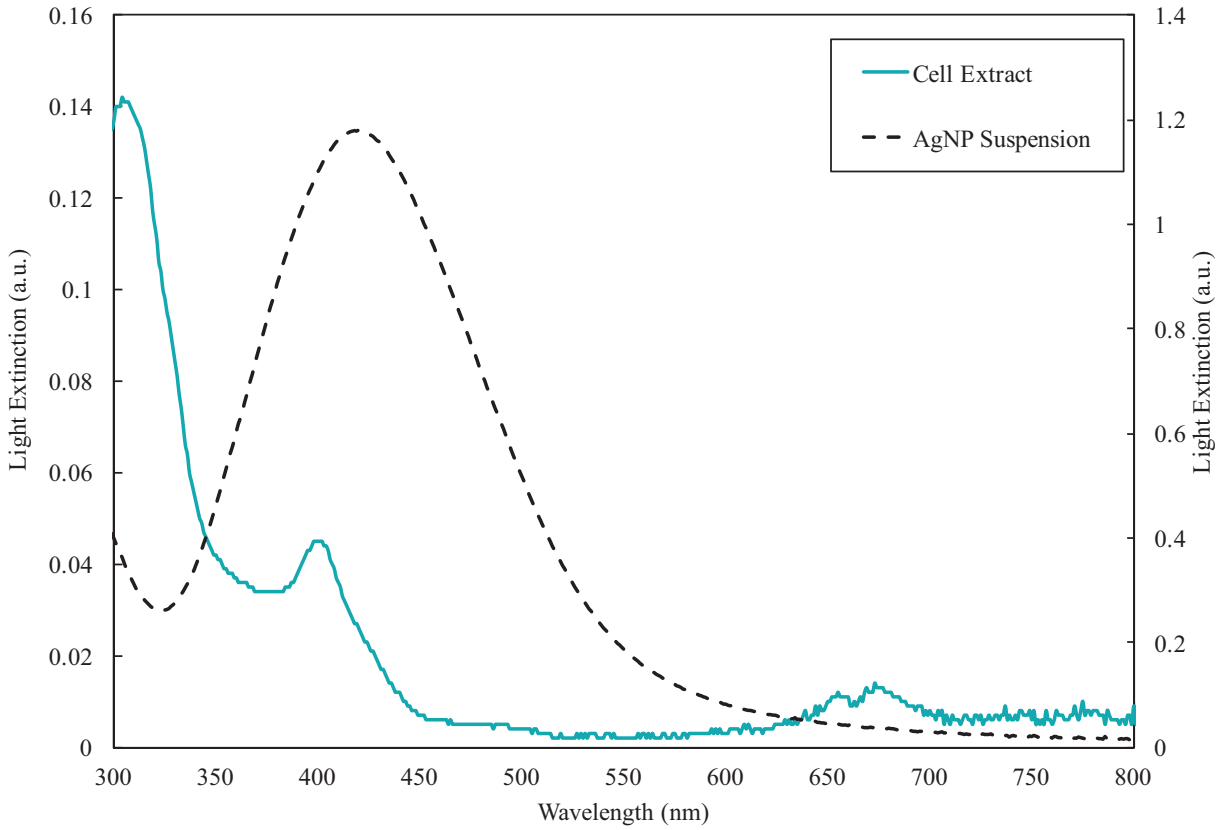

Figure 3- 5 UV-Vis spectra of cell free extract obtained at 9 hours extraction time and the suspension obtained by incubating the mixture of this cell extract and 0.8 mM $AgNO_3$ at pH 5 with illumination at room temperature for 12 hours.

As shown in Figure 3-6, the AgNPs synthesized in this process were nearly spherical and more than 50% of them have a diameter in the range of 12 to 18 nm, showing excellent uniformity. The mean diameter of AgNPs was 15.8 nm, which was relatively small comparing to the bio-mediated AgNPs reported in literature [21].

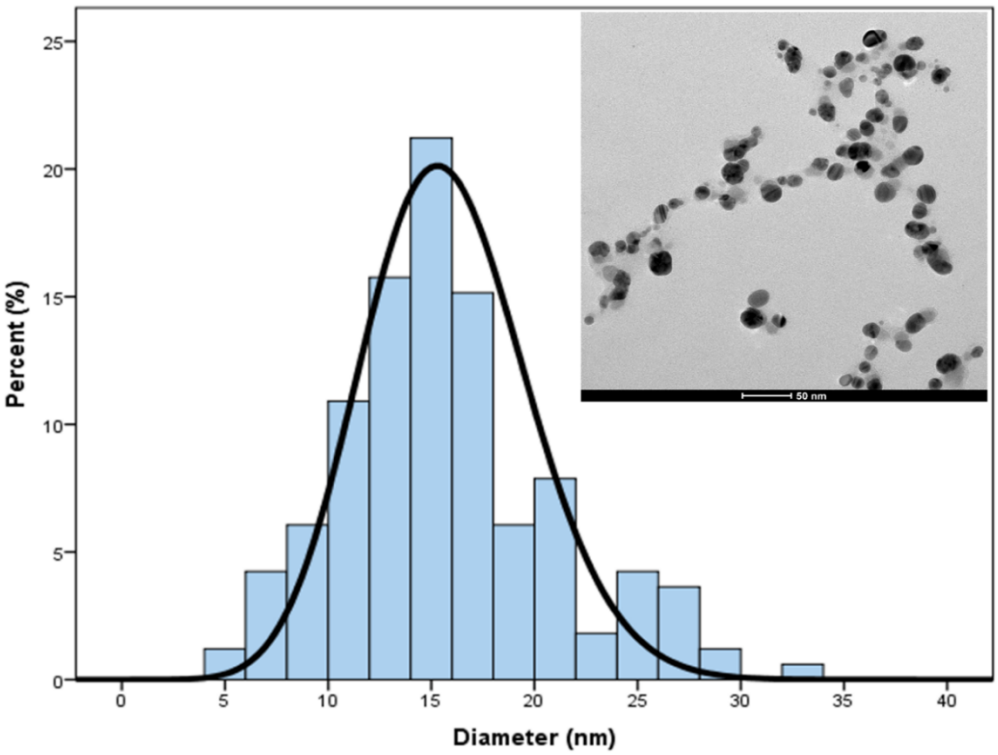

Figure 3- 6 Size distribution of AgNPs produced at 0.8 mM $AgNO_3$, pH 5, and 9-hour extraction time.

The original AgNPs suspension showed excellent antibacterial activities. While the inhibition zone diameter (IZD) to *E. coli*, a gram positive bacterium that has been frequently used as the indictor strain for tests of antibacterial activities of different metal NPs [22]–[24], decreased with the increase of dilutions, the inhibition zone was still visible when the AgNPs suspension was diluted 20 times.

The AgNPs had a mean diameter of 15.8 nm and a SPR at 420 nm. It was reported that the wavelength of SPR peak was related to the size of particles [25]. Smaller particle size caused a blueshift of peak and possessed better antibacterial activity. It was also reported that peak at 420 nm corresponded to the particle dimeter of 50 nm [25]. However, it was not in agreement with another research focusing on linking the SPR peak to particle sizes [26]. For example, a peak at

411 nm corresponded to particle size of 30 nm in Agnihotri et al.'s report but around 4.4 nm in Baset et al.'s. Therefore, SPR peak might be affected not only by mean particle size but also by other factors such as particle size distribution and shape as well. As for the antibacterial tests, a report specifically studying the effects of AgNPs on *E. coli* showed that low concentration of AgNPs prolonged the lag phase and concentration above a certain level (10 ug/mL in the study) caused no bacterial growth within 7 days [27]. Our results also confirmed that our homemade AgNPs were able to inhibit the growth of *E. coli*.

3.4 Discussion

3.4.1 Effects of operating conditions

In the single factor experiments with varied $AgNO_3$ concentration with 0.5-h aqueous algal extract and pH 5, it was shown (Figure 3-1) that 0.4 mM was the optimal concentration of $AgNO_3$ for synthesizing AgNPs. One possible explanation was that, from 0.2 mM to 0.4 mM, reducing power in the extract was excessive and more silver ions were available to be reduced to form more AgNPs when $AgNO_3$ increased in this range. On the other hand, beyond 0.4 mM, excessive silver ions became inhibitive to the reaction. It has been well established that, as a heavy metal ion, high concentration of Ag^+ had inhibiting effects on bioactive agents [28], which are important carriers of reducing power in this reaction.

The optimal $AgNO_3$ concentration was found to be 0.8 mM in the optimal set of conditions obtained in the orthogonal experiments, where the joint effects of different factors were investigated with boiling time for production of aqueous algal extract increased to 9 hours. These observations could be explained by the hypothesis that more reductive components were

extracted from *N. oleoabundans* cells when the boiling time was increased from 0.5 hours in the single factor experiments to 9 hours in the orthogonal experiments.

It was demonstrated that the optimal pH of the reaction mixture was pH 5 for AgNP biosynthesis while nearly no AgNPs were formed at the pH range 2-4. The effects of pH to AgNP synthesis could be twofold: 1) affecting the activities of the bioactive components that were extracted from *N. oleoabundans* biomass; and 2) the increased H^+ concentration at low pH could lead to formation of strong oxidant nitric acid [29], which could consume the reducing power (electron donors) in the cell extract.

As shown in Figure 3-3, prolonging extraction time increased the production of AgNPs, and the rate of increase slowed down after 6 hours. Many researchers reported the successful synthesis of AgNPs using boiled plants [30]. Of particular relevance, it was reported the successful synthesis of AgNPs using aqueous banana peel extract, which was obtained by stewing banana peel at 90 °C for 30 min [31]. Those results indicated that the active agents were thermally stable and can be easily extracted from cells.

Microalgal cells were not disrupted before or during the preparation of aqueous cell extracts. As a result, the concentration of reactive components in the extracts was extremely low. Indeed, the concentrations of chlorophyll a and b, the two most likely pigments that were responsible for mediating the AgNPs synthesis, were only 0.0334 and 0.0298 nmol/mL, respectively. Such low concentration of active components in cell extracts may prove to have practical significance. First of all, it implies that these components are extremely effective in mediating AgNPs formation and points to a potentially high efficiency and low cost process when the extraction of these components is improved in the future. Secondly, the extremely low

concentration of these components in the reaction mixture makes the separation of AgNPs from the mixture straightforward and cost-effective.

3.4.2 Mechanisms of AgNPs synthesis

The synthesis of AgNPs from Ag^+ is a reductive reaction that requires reducing power (i.e., electron donors).

$$Ag^+ + e^- \rightarrow Ag$$

As shown in Table 3-1, three components were essential for the above reaction to take place, 1) $AgNO_3$ aqueous solution, 2) aqueous extract of algal biomass, and 3) light. While $AgNO_3$ is the source of Ag^+ and aqueous extract of algal biomass the source of electron donors and other reactive agents (e.g., enzymes if applicable), the light requirement of the reaction suggests that this is probably a pigment-mediated reaction. It should be noted that the synthesis of silver and other metal nanoparticles mediated by algae [21], [32] or other biomasses [31] were reported not to need light. Nevertheless, there are reports demonstrating pigment-mediated AgNPs with extracts of microalgal biomass. For instance, it was reported that AgNP synthesis mediated by cell extract of *Amphora* sp. obtained by cell homogenization was light-dependent [16], which is similar to our findings. The pigments responsible for mediating AgNPs biosynthesis seemed to be chlorophyll a and b, which are abundant in *N. oleoabundans*.

As discussed above, the concentration of active components in aqueous cell extracts was extremely low. The fact that such small amounts of active components were able to effectively provide the electrons required for the reduction of a significant amount of Ag^+ to Ag suggests that they were not the ultimate electron donors. A likely scenario is that, similar to the light-dependent reactions of biosynthesis, two electrons of a chlorophyll pigment molecule were

excited by absorbance of a photon and were passed on to two Ag^+ ions to reduce them to two Ag atoms. Then the lost electrons of the chlorophyll molecule were replenished by acquiring electrons through water splitting. This serious of reactions are the first steps of the light-dependent reactions of the photosynthesis pathway commonly found in microalgae, cyanobacteria, and plants, which are catalyzed by enzymatic complexes in the respiratory chain such as Photosystem II (PS II, alias water-plastoquinone oxidoreductase).

The cell extracts were obtained by incubating biomass samples in boiling bath for up to 9 hours, indicating that the reactive components must be soluble to water and stable under such conditions. It was reported that, although chlorophyll a is not thermally stable, chlorophyll b has an impressive half-life time $t_{1/2}$=177.73 and 277.26 min at 100 and 90 °C, respectively [33]. The high thermal stability makes it possible for chlorophyll b to remain active for Ag^+ reduction after the aqueous extraction. The difference in the structures of chlorophyll a and b [34] is on the C7 group, where on chlorophyll a there is a $–CH_3$ group while on chlorophyll b a –CHO. The more polar –CHO group seems to be responsible for the higher thermal stability of chlorophyll b in comparison to chlorophyll a and may also render it higher water solubility than chlorophyll a.

There are however two difficulties to this hypothesis. First of all, the thermal stability of the PSII and/or other enzymatic complexes at a temperature close to 100 ^oC is questionable. Secondly, it was reported that the light was necessary the AgNPs synthesis mediated by the supernatant *Klebsiella pneumoniae* [35], which is a heterotrophic Gram-negative bacterium. These seemingly contradictory results need to be examined in a more systematic manner.

3.5 Conclusions

In conclusion, cell extract obtained by aqueous extraction showed ability of reducing Ag^+ to fabricate AgNPs, which are spherical nanoparticles with excellent antibacterial activity on *E. coli* at large dilutions. Extraction time of 9 hours, reaction mixture at pH 5 and 0.8 mM $AgNO_3$ were determined to be the optimal combination under the investigated conditions. It is hypothesized that the biosynthesis of AgNPs was mediated by chlorophylls with the possible participation of PSII, which are required to replenish chlorophyll molecules the lost electrons via water-splitting. The function of light is likely to excite the electrons from chlorophylls.

3.6 Compliance with Ethical Standards

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Conflict of Interest: Zeqing Bao declares that he has no conflict of interest. Jiahui Cao declares that he has no conflict of interest. Guangbo Kang declares that he has no conflict of interest. Christopher Q. Lan declares that he has no conflict of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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Chapter 4:

Light-dependent biosynthesis of silver nanoparticles mediated by disputed cell extract of *Neochloris oleoabundans**

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*Submitted to **Colloids And Surface B**

Abstract

This study investigated the role of chlorophyll and light in the biosynthesis of silver nanoparticles (AgNPs) using disrupted cell aqueous extract of *Neochloris oleoabundans*. While increasing sonication time increased the percentage of disrupted cells and efficiency of aqueous cell extraction, prolonged sonication reduced AgNP production, indicating reactive cellular materials sensitive to sonication were involved in AgNP biosynthesis. AgNP biosynthesis required illumination of white, blue, or purple light while AgNP formation was undetectable under dark condition or illumination of orange or red light, indicating that only photons of high energy levels among photosynthetic active radiations are capable of exciting the electrons of chlorophylls to a state that is sufficient for Ag^+ reduction. Chlorophylls were demonstrated to be an essential component mediating the reduction of Ag^+ and results of mass balance suggests oxidized chlorophylls after donating their electrons need to be recycled, which were hypothetically achieved by water splitting catalyzed by photosynthetic enzyme complexes such as photosystem II to supply electrons.

Keywords: biosynthesis; silver nanoparticles; chlorophyll; mechanism; light-dependent reaction.

4.1 Introduction

Silver nanoparticles (AgNPs) are drawing extensive attention from researchers all over the world mainly for two reasons: 1) they have been widely applied to various fields, including anti-bacteria [1], tumor therapy [2], high-conductivity elements fabrication [3], etc; and 2) they have high level of commercialization as they were reported to account for over half of the total nanomaterial-based products in 2014 [4].

Biosynthesis of AgNPs was reported to be mediated by cells or aqueous cell extracts of plants [5], bacteria[6], fungi [7], and microalgae [8]. Of particular relevance, our previous studies have demonstrated that aqueous cell extract of green alga *Neochloris oleoabundans* enabled AgNP synthesis. Aqueous cell extraction mainly consists of whole cell aqueous extraction (WCAE) and disrupted cell aqueous extraction (DCAE), the former employs water of different temperature to extract cells while the latter applies cell disruption prior to cell extraction at room temperature.

While studies on biosynthesis of AgNPs have increased remarkably in the last ten years [9], the mechanism of AgNP biosynthesis has not been well established [10]. A few hypotheses have been proposed to explain the mechanism involved in the reduction of Ag^+ with three representative functional bio-reagents as electron donors, i.e., proteins and enzymes [11], polysaccharides [12], and pigments [8]. However, these hypotheses are in general very vague and are characterized by lack of quantitative verification. For instance, it is well known that proteins and enzymes are typically thermally sensitive while relatively high temperature (e.g., 100 ^{o}C) usually apply in the process and may cause protein denaturation [13].

To this end, the main purpose of this work was to investigate the AgNP biosynthesis mediated by disrupted cell aqueous extract and the roles of chlorophyll and lights aiming at facilitating further studies in this area. Based on experimental results, a hypothetic mechanism of this light-dependent microalgae-mediate biosynthesis of AgNPs was also proposed for the first time. The reaction was hypothesized to be achieved by photoinduced electron transfer where Ag^+ received electrons from chlorophylls excited by lights.

4.2 Materials and methods

4.2.1 Culture and medium free culture

Neochloris oleoabundans UTEX 1185 was purchased from Culture Collection of Algae at the University of Texas in Austin.

The Modified Bristol Medium (MBM), which was adopted in our previous studies [14] was used in this study for the cultivation of *N. oleoabundans*. It was composed of (per litre) 0.35 g $NaNO_3$, 0.138 g K_2HPO_4, 0.0823 g $MgSO_4$, 0.025 g $CaCl_2$, 0.322 g KH_2PO_4, 0.025 g NaCl, 0.0068 g $FeCl_3$, and 1 mL A5 solution. The A5 solution was composed of the following components (per litre): 1.6423 g EDTA-Fe, 2.86 g H_3BO_3, 1.81 g $MnCl_2{\cdot}4H2O$, 0.22 g $ZnSO_4{\cdot}7H_2O$, 0.079 g $CuSO_4{\cdot}5H_2O$, and 0.039 g $(NH_4)_6Mo_7O_2{\cdot}4H_2O$. All chemicals used in the medium were of analytical grade. The media were sterilized using autoclave at 121 °C for 20 min.

The microalgae were cultivated in 500 mL Erlenmeyer Flasks containing 400 mL sterile MBM for 8-10 days at 27 °C in an illuminated incubator (model: LI15, manufactured in USA by Sheldon Manufacturing INC) until the microalgal cell density reached approximately 1.6×10^7 cells/mL. The culture was then centrifuged at 7750 g and rinsed with deionized water three times

to produce medium free cell culture, which was stored at -80 °C in a ULT freezer (model:706, manufactured in USA by Thermo Electron Corporation) for future use.

4.2.2 AgNP synthesis

$AgNO_3$ was used in this study as the silver source for AgNP production. $AgNO_3$ powders (99.85%) were purchased from Acros Organics and a 10mM $AgNO_3$ stock solution was prepared with distilled water. The solution was kept in a brown bottle and stored at 4 °C.

20 mL microalgal culture thawed at room temperature before being sonicated (40% amplitude, programmed as 3s on and 3s off) for certain period of time (as specified in the text). An ice-water bath was applied to cool the sample to avoid the effects of heat generated by sonication. The sonicated cell suspensions were then centrifuged at 7750 g to separate cells and cell debris, and the supernatant was defined as disrupted cell aqueous extract in this study for AgNP synthesis.

9 mL of disrupted cell aqueous extract was added with 1 mL of $AgNO_3$ stock solution (10 mM). The mixture was homogenized by hand shaking before being incubated at 27 °C for 6 hours with illumination of 5000 lux, an identical set of experiments was carried out in dark.

Spectrometric measurements were carried out by scanning the cell extract or AgNP suspensions from 300 nm to 800 nm using a GENESYS 10S UV-VIS Spectrophotometer.

4.2.3 Cell number

Cell number was counted using a hemacytometer (Improved neubauer, Phase Counting Chamber w/2 cover glass, USA) under a phase-contrast microscope (Infinity II BX40, Olympus, Canada) at a magnification of 200 times. Disrupted cells suspensions were centrifuged at 7750 g

to remove cells and cell debris, and the supernatant was defined as disrupted cell aqueous extract in this study.

4.2.4 Chlorophyll extract

30 mL of medium free cell culture was centrifuged (7750 g) to harvest biomass, which was then resuspended in 5 mL anhydrous methanol in dark at room temperature for 4 hours with gentle mixing by periodical hand shaking [15]. The cell suspension was centrifuged again (7750 g) to remove cells and the supernatant was the chlorophyll methanol solution, which was kept in dark at 4 °C for future use.

4.2.5 Calculations

1) The efficiency of cell disruption was determined by:

$$\varphi = \frac{\text{Cell number before sonication} - \text{Cell number after sonication}}{\text{Cell number before sonication}} \times 100\%$$

2) The concentration of chlorophyll was considered as the total of chlorophyll a and b in disrupted cell aqueous extract:

Chlorophyll a (nmol/mL) = $13.43 \times E_{663.8} - 3.47 \times E_{646.8}$

Chlorophyll b (nmol/mL) = $22.90 \times E_{646.8} - 5.38 \times E_{663.8}$

Where $E_{663.8}$ and $E_{646.8}$ are the light extinction at 663.8 nm and 646.8 nm, respectively. It should be noted that the above equations were originally proposed for the calculation of chlorophyll contents in Dimethylformamide (DMF) solutions, which has the largest polarity among three given solvents, i.e., DMF, methanol, and acetone [16].

3) The concentration of AgNPs was estimated by:

AgNP concentration (mg/mL) = $0.0082 \times E_{max}$

Where E_{max} is the light extinction at absorption maxima (λ_{max}) and the number 0.0082 is the conversion factor, which was obtained as the slope of an experimentally determined AgNPs concentration vs E_{max} calibration curve (R^2=0.9998) using commercial AgNPs (sigma-aldrich; provided by MilliporeSigma Canada Co. at 2149 Winston Park Dr. Oakville, Ontario L6H 6J8), which had a mean particle size of 40 nm.

4) Reaction rate of AgNPs synthesis was determined by:

Reaction rate at t_2 (mg/(mL×hr)) = (Concentration(t_2)-Concentration(t_1))/(t_2-t_1)

4.3 Results and discussion

4.3.1 Effects of sonication on AgNP synthesis

As shown in Figure 4-1, increasing sonication time increased the percentage of disrupted cells. While 30 min of sonication disrupted over 80% cells, over 95% cells were disrupted by 50 min of sonication.

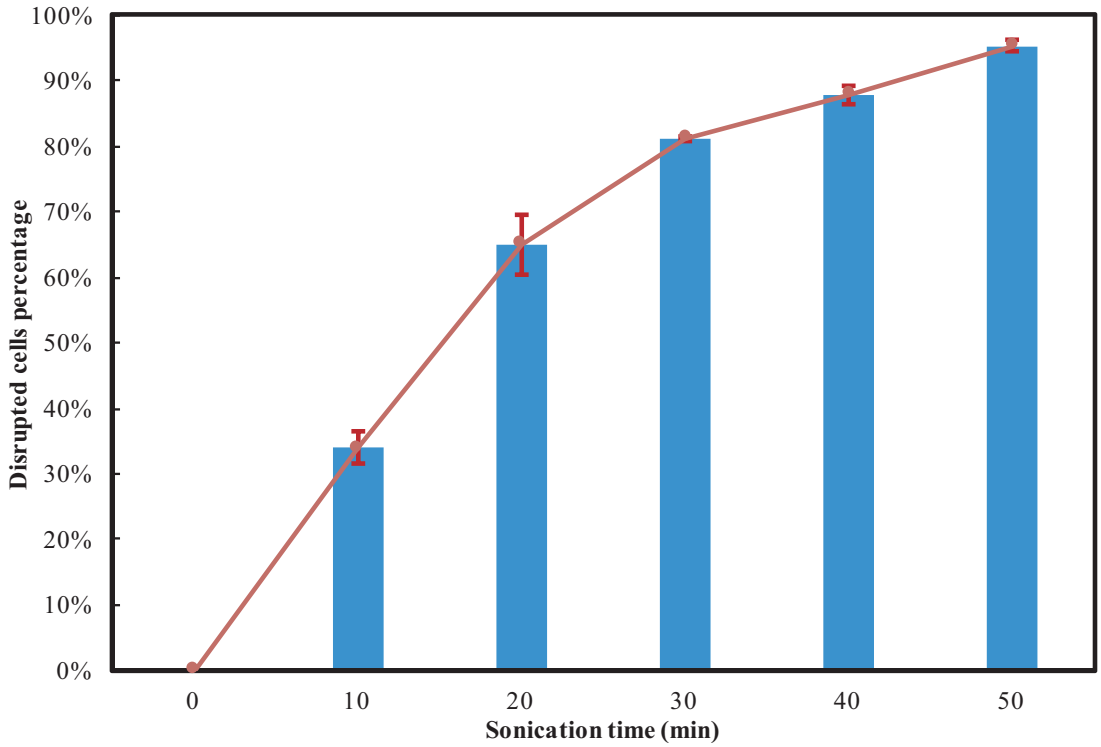


Figure 4- 1 Percentage of cells disrupted by 0 to 50 min of sonication.

Sonication is an efficient way of disrupting cells, which has been widely applied to lipid extraction enhancement [17]–[19] and pigment extraction [20], [21]. DACE with the help of sonication was reported was also successfully employed for microalgae-mediated AgNP synthesis [22].

Disrupted cell aqueous extract obtained by 12.5, 25, 37.5, and 50 minutes of sonication was defined as 12.5, 25, 37.5, and 50 min-DACE, respectively. As shown in Figure 4-2a, longer

time of sonication gave the disrupted cell aqueous extract higher concentration of aqueous soluble materials that led to larger light extinction. Furthermore, the spectra shown in Figure 4-2a were compatible with the spectra of chlorophylls in plants [23]. The peaks located between 650 to 700 nm were considered as the evidence of the presence of chlorophyll and have been used for quantifying the content of chlorophylls [12], [20]. In addition, DCAE (with the help of sonication) was also reported as an efficient way of extracting chlorophyll from green algae [24]. Since sonication of longer time disrupted more cells, the increase of light extinction was caused by more cell extract released from disrupted cells and chlorophyll showed a good stability under the sonication.

Figure 4-2b shows the spectra of AgNP suspensions produced by 12.5, 25, 37.5, and 50 min- DCAEs. Based on the values of E_{max} of the produced AgNP suspensions, 37.5 min-DCAE, not the 50 min-DCAE, gave the highest AgNP production. These results suggest some reactive components, which were likely proteins, in disrupted cell aqueous extracts were sensitive to prolonged sonication under the investigated conditions. 37.5 min-DCAE was thus applied to the following tests.

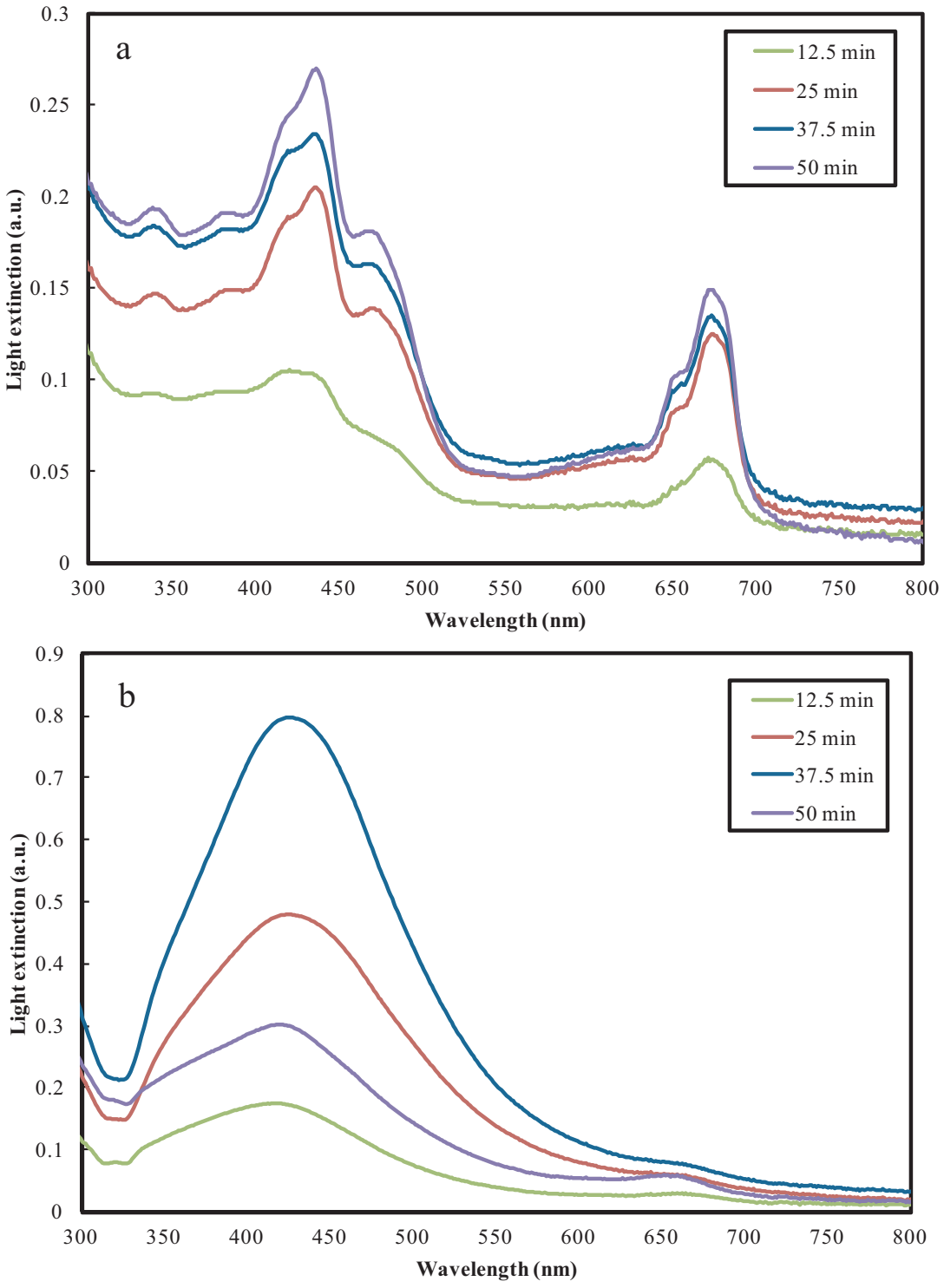


Figure 4- 2 UV-Vis spectra of cell extract obtained by disrupting cells by 12.5-50 min of sonication a) before incubated with $AgNO_3$ and b) after incubated with 1mM $AgNO_3$ for 6 hours under light conditions.

While it was reported that sonication was able to cause protein denaturation and to affect proteins' functionalities [25], the involvement of proteins in AgNP biosynthesis was also demonstrated by a study conducted with *Chlamydomona reinhardtii*, a unicellular green alga [11]. Also reported by that study, *Chlamydomona reinhardtii* was disrupted using vortexing and sonication before extraction and the disrupted cell aqueous extract was reported to enable AgNP synthesis but the reaction rate was significantly decreased and bigger AgNPs were produced after proteins were depleted by employing CM-sepharose and DEAE-sepharose ion exchange chromatographic column [11].

4.3.2 Light dependency of AgNP synthesis

As shown in Figure 4-3a, λ_{max} increased from 426 to 431 nm when the E_{max} kept increasing with reaction time in the range of 1 to 10 hours. Figures 4-3b and 3c show the time vs reaction rate and λ_{max}, respectively. The reaction rate was slow at the beginning, and then increased rapidly but started decreasing after 3 hours. Meanwhile, the λ_{max} continuously shifted to longer wavelength (i.e., redshift).

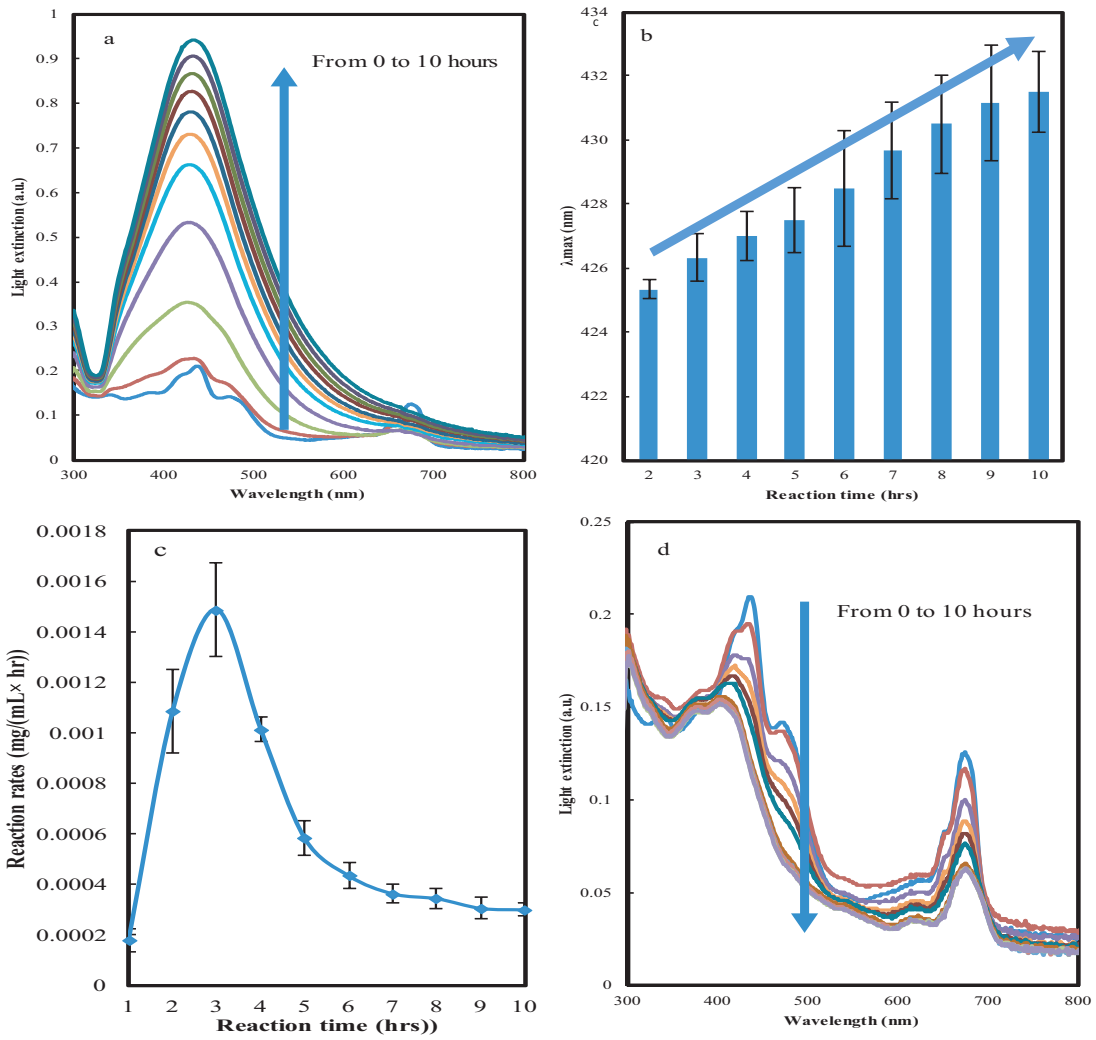


Figure 4- 3 a) UV-Vis spectra, b) absorption maximum, and c) reaction rates of the mixture of 37.5 min-DCAE and 1mM $AgNO_3$ incubated with illumination from 0 to 10 hours and d) UV-Vis spectra of an identical set of experiment conducted in dark.

The AgNP characteristic peak shown in Figure 4-3a increased as time passed, indicating that AgNPs were being produced as the reaction proceeded [26]. The color change of the suspension occurred fast during AgNP biosynthesis and the greenish mixture of 37.5 min-DCAE and 1 mM $AgNO_3$ solution turned to bright yellow within 2 hours, which then gradually became brownish.

As shown in Figure 4-3b, at the beginning of the reaction, the reaction rate was slow but increased as time elapsed until 3 hours, at which the peak rate was achieved. This type of kinetics could be well explained by the model of the auto-catalyzed reaction, which involves two stages: nucleation and seeded-growth [27]. In nucleation, metal ions receive electrons to be reduced as individual particles while, in seeded-growth, metal ions are reduced on the surface of existing particles, which would increase particle size with time as the surface area of particles growing with time [27]. Therefore, what happened in AgNP synthesis could be described by three different stages: 1) nucleation was dominant at the start of the reaction due to the low concentration of AgNPs; 2) seeded-growth became dominant since more AgNPs were produced and silver ions started being reduced on the surface of existing nuclei, which caused the reaction to accelerate as the diameter of particles increased with time; and 3) As the reaction preceded, the reaction rate decreased and became slow when the concentration of the reactants, either silver ions or the reducing agents, became limiting upon the consumption by reaction.

Figure 4-3c shows that λ_{max} gradually increased as the reaction proceeded. The redshift of λ_{max} indicates that AgNPs were growing and the diameter became bigger [4]. As what we alluded to in the previous discussion, the increasing particle diameter could be explained by the seeded-growth model.

As afore mentioned, 37.5 min-DCAE could mediate AgNP production under light conditions but no detectable AgNPs were produced in dark conditions. To further demonstrate this point, Figure 4-3d shows the change of spectra of the mixture of 37.5 min-DCAE with 1 mM $AgNO_3$ aqueous solution conducted in darkness with time.

The light extinction of the mixtures kept decreasing from time 0 to 10 hours. There were no characteristic peaks of AgNPs at 420-430 nm and the mixture color did not turn to yellow, which was the typical color of AgNP suspensions. Furthermore, the typical λ_{max} of chlorophylls at both 430 nm and 670 nm decreased with time, which was explained by the structure of chlorophyll. Chlorophyll has Mg^{2+} located at the center of its tetrapyrrole ring and Mg^{2+} is the necessary ion for chlorophyll functioning. Heavy metals (mercury, cadmium, lead, etc) were reported to be capable of substituting the Mg^{2+}, causing the 'Mg-substitution' [28]. That study also reported that almost all Mg^{2+} was substituted by heavy metals at low light intensity but very few Mg-substitution (less than 2%) occurred when the light intensity was high [28]. It was also reported that the substituted chlorophyll (Cu- or Zn- chlorophyll) had lower level of light absorption [29]. Therefore, the gradual decrease of light absorbance by the 37.5 min-DCAE and $AgNO_3$ mixture at dark conditions may be hypothetically explained by substitution of Mg^{2+} with Ag^{+} in chlorophyll.

While light was demonstrated beyond doubt as a necessary component in this study and our previous studies, there are other studies (as shown in Table 4-1) showing AgNP biosynthesis achieved avoiding light. Besides our studies, at least two other studies on microalgae-mediated AgNP synthesis reported light as a necessity.

Table 4- 1 Recent studies on biosythensis of AgNPs.

Species		Reactive agents	Light conditions	Absorption Maxima	AgNP Morphology	References
Plants	*Banana peel*	90 C-aqueous extract	Dark	433 nm	Spherical (23.7 nm)	[37]
	Acalypha indica leaf	60 C-aqueous extract	Dark	420 nm	Spherical (20-30 nm)	[35]
	Morinda citrifolia root	100 C-aqueous extract	Dark	413 nm	Spherical (30-55 nm)	[38]
	Sesuvium portulacastrum L.	100 C-aqueous extract	Dark	420 nm	Spherical (5-20 nm)	[39]
Fungi	*Fusarium oxysporum*	Raw biomass	Dark	413 nm	Spherical (5-15 nm)	[40]
	Aspergillus fumigatus	Cell filtrate	Dark	420 nm	Spherical (5-25 nm)	[7]
	Aspergillus flavus	Mycelium	Dark	420 nm	Spherical (8.92±1.61 nm)	[41]
	Penicillium brevicompactum	25 C-aqueous extract	Dark	420 nm	Not mentioned (23-105 nm)	[42]
Microalgae	*Chlamydomonas strain CC-124*	Disrupted cells' extract	Dark	470 nm	rounded/rectangular (5±1-15±2)	[11]
	Spyrogira insignis	Raw biomass	Dark	460 nm	nonuniform (30 nm)	[7]
	Euglena gracilis	Raw biomass	Light	422 nm	Spherical (6-24 nm)	[22]
	Euglena gracilis	Disrupted cells' extract	Dark	425 nm	Spherical (15-60 nm)	[22]
	Amphora sp.	Disrupted cells' extract	Light	413 nm	Spherical (20-25 nm)	[8]
	Neochloris oleoabundans	100 C-aqueous extract	Light	420 nm	Spherical (15.8 nm)	Our studies
	Neochloris oleoabundans	Disrupted cells' extract	Light	425-431 nm	Not measured	Our studies

One of the light-dependent AgNP biosynthesis was conducted using disrupted cell aqueous extract of *Euglena* [22]; however, whether the synthesis would occur in darkness was not mentioned and that work also reported AgNPs produced using living *Euglena* cells in dark condition[22]. In another study, which clearly compared the effects of light and dark conditions, fucoxanthin was reported as the pigment mediating AgNPs biosynthesis [8].

4.3.3 The role of chlorophylls

As shown in Figure 4-4, the reaction rate of AgNP synthesis was increased by adding additional chlorophyll extracted from cells using methanol, which would not have included intracellular proteins. As the concentration of disrupted cell aqueous extract did not change, the increase of the reaction rate was contributed by the added chlorophyll. Furthermore, the mixture of extracted chlorophyll and $AgNO_3$ without disrupted cell aqueous extract did not show ability of producing AgNPs (not shown in the figure). These results indicate the necessity of the

combined functions of chlorophylls and intracellular cell materials, most likely proteins.

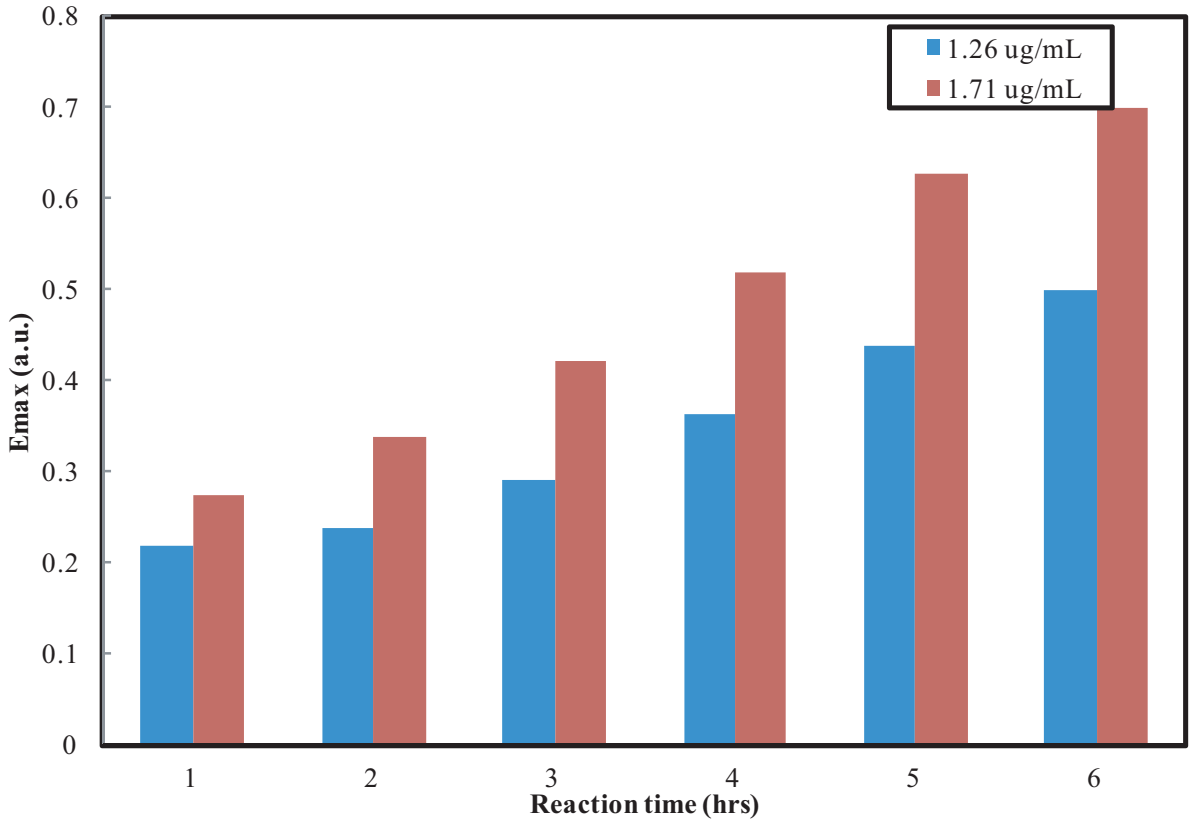


Figure 4- 4 Emax of mixtures of 37.5 min-DCAE and 1 mM $AgNO_3$ with (red bars) or without (blue bars) additional chlorophyll, which contained 1.26 or 1.71 ug/mL of chlorophyll, incubated with illumination from 1 to 6 hours.

Chlorophylls contain networks of alternating single and double bonds in their tetrapyrrol rings. They have very strong absorption bands in the visible region of the spectrum with peaks in the purple/blue light range and orange/red light range. Upon absorbance of a photon of the proper wavelength, the energy of the photon excites an electron from its ground energy level to an excited energy level. If there is a suitable electron acceptor nearby, the excited electron can move from the chlorophyll molecule to the acceptor [30]. More specifically, excited electrons of chlorophylls were observed to be able to be transferred to metal ions or metal nanoparticles. For instance, it was reported that gold nanoparticles were able to be negatively charged by excited

chlorophyll a and thus helped shuttle the electrons to another acceptor [31]. Excited electrons of chlorophylls were also observed to be transferable to Au^{3+} for its reduction under light conditions through photosynthetic electron transport system [32]. It is therefore reasonable to hypothesize that the AgNP biosynthesis observed in this study was mediated by chlorophyll, i.e., light was absorbed by chlorophylls and the excited electrons of chlorophyll molecules were transferred to Ag^{+}, which was consequently reduced to AgNPs.

4.3.4 The role of active components in disrupted cell aqueous extract other than chlorophylls

To further understand the role of chlorophyll in AgNP biosynthesis, the concentration of chlorophyll in 37.5 min-DCAE and the produced AgNPs was estimated and the results showed that the total molar concentration of chlorophyll a and b in 37.5 min-DCAE was 1.56 uM, which was only 2.6% of the 0.06 mM Ag^{+} that was reduced to Ag atom after 6 hours of reaction. Therefore, it would be necessary for the positively charged chlorophyll after electron donation to be reduced and therefore recycled to continuously provide reducing power (i.e., electrons). This hypothesis points to an ultimate electron donor in the reaction mixture that is not chlorophyll to supply electrons for Ag^{+}.

One possible scenario is that the lost electrons of the chlorophyll molecules were replenished by acquiring electrons through water splitting, which is what happens in the light-dependent reactions of the photosynthesis. As chlorophylls were of low concentration and in charge of donating electrons for Ag^{+} reduction, the chlorophyll donating electrons could be the rate-determining step. This explains the observation that adding chlorophyll extract to disrupted cell aqueous extract accelerated the AgNP synthesis. The requirement of reactive components beyond chlorophylls is supported by the aforementioned observations that methanol extracted

chlorophyll alone could not mediate AgNP biosynthesis and that overtime sonication, which may lead to protein denaturation, resulted in decrease of AgNP biosynthesis.

4.3.5 Rainbow tests - effects of light frequency

As shown in Figure 4-5, biosynthesis conducted under illumination of white light (i.e., the positive control) produced the most AgNPs while reactions conducted under blue and purple light illumination produced (after 24 hours) 76% and 62%, respectively, of the AgNPs produced in the positive control. While chlorophylls absorb red and orange light as well, no AgNPs were produced under red or orange light illumination. These results suggest that the energy of photons of orange (590-620 nm) and red light (620-750 nm) were not sufficient to excite the chlorophyll electrons to a state that could be accepted by Ag^+ for its reduction while the energy of photons of blue (450-595 nm) and purple light (380-450 nm) was sufficient.

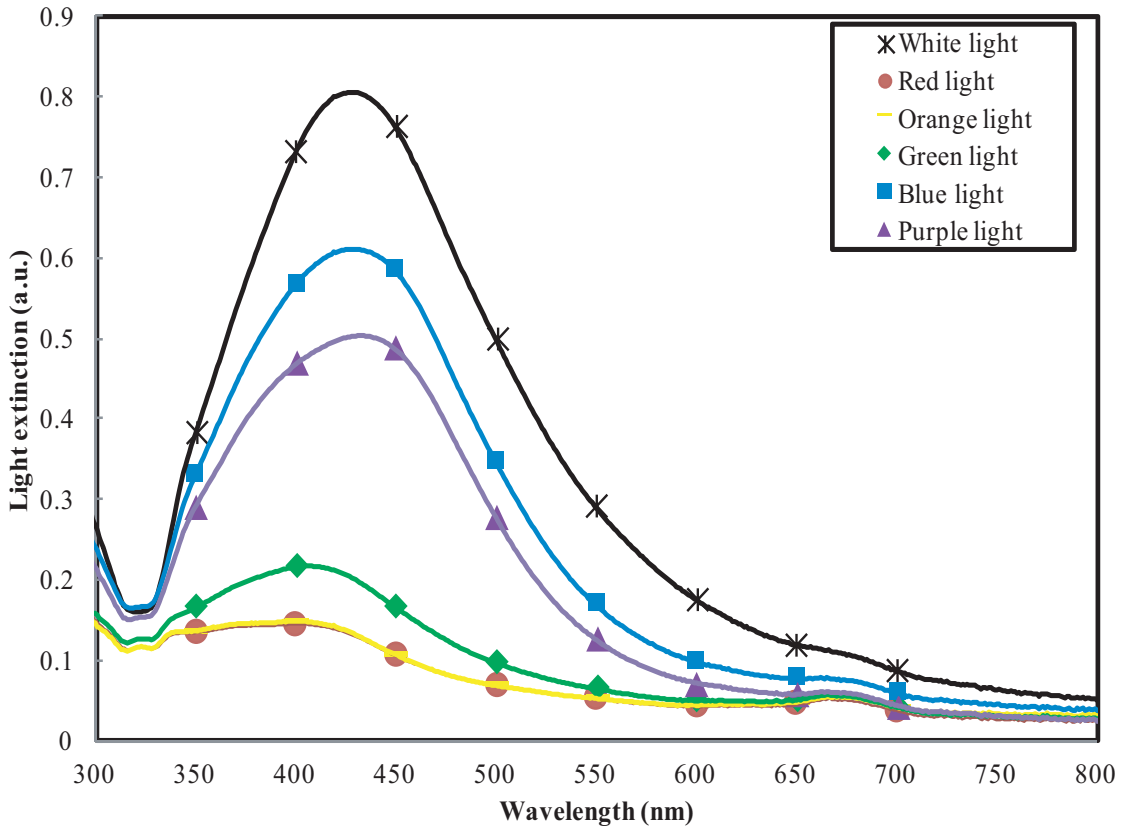


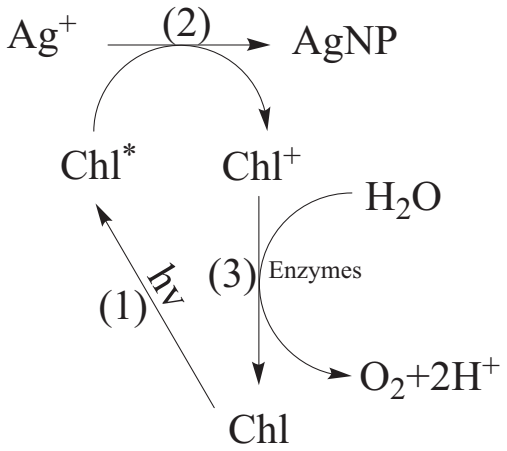
Figure 4- 5 UV-Vis spectra of AgNPs synthesized by 37.5 min-DCAE and 1 mM $AgNO_3$ under white, red, orange, green, blue, and purple lights.

4.3.6 Hypothetic mechanism of light-dependent AgNP biosynthesis

Based on the above results and discussion, we propose that the AgNP biosynthesis mediated by disrupted cell aqueous extract of *N. oleoabundans* is an auto-catalyzed reduction of Ag^+, which is carried out in three stages, 1) nucleation in which individual Ag^+ ions are reduced, 2) seeded-growing in which the surface of existing Ag nuclei surfaces accelerates Ag^+ reduction and deposition of Ag atoms on to the surfaces, and 3) stabilized phase when a reactant became rate-limiting due to the consumption of it in the reaction.

As shown in Figure 4-6, the reduction of Ag^+ to Ag is consisted of three reactions: chlorophyll excitation upon photon absorption (1), Ag^+ reduction (2), and water splitting for

chlorophyll recycling (3). These processes may involve one or more enzymes catalyzing light-dependent reactions of biosynthesis, e.g., photosystem II (alias water-plastoquinone oxidoreductase) for water splitting [33]. This hypothesis is supported by the following evidences: 1) the presence of chlorophyll in disrupted cell aqueous extract was confirmed by spectrophotometric method [34]; 2) the reaction rate was increased by adding extra chlorophyll which was extracted from whole cells using methanol and therefore contained no intracellular macromolecules such as proteins; 3) the biosynthesis specifically requires blue and purple lights, both of which could be absorbed by chlorophylls; 4) chlorophyll was reported to be excited by light to donate electrons to reduce suitable acceptors [30]-[32]; 5) chlorophyll did not produce AgNPs without disrupted cell aqueous extract.



(1) Chlorophyll excitiation
(2) Ag^+ reduction
(3) Chlorophyll recycling

Figure 4- 6 Graphic description of the mechanism of AgNP synthesis.

Above all, the disrupted cell aqueous extract contained chlorophyll as well as proteins (which would be decomposed by longtime sonication). When the synthesis was conducted with illumination, chlorophyll was excited by blue light, purple light, orange light, and red light to become excited chlorophyll. However, only the electrons excited by blue and purple lights had the adequate energy to reduce the Ag^+ to produce AgNPs. With the help of proteins, AgNPs were stabilized and the oxidized chlorophyll was reduced back to non-excited chlorophyll to be excited by lights again. In addition, AgNP synthesis was a three-stage synthesis, where at the beginning, nucleation was dominant and smaller AgNPs were synthesized. When more AgNPs were produced, Ag^+ started being reduced on the surface of existing particles, which accelerated reaction rates as well as caused the increase of particle size and redshift of AgNP characteristic peaks. The reaction rates then decreased when reactants were significantly consumed.

It was found that polysaccharides were speculated to be in charge of Ag^+ reduction [12]. However, polysaccharides are polymeric carbohydrate molecules composed of long chains of monosaccharide and the carboxyl groups are reducing groups, which means that each long chain of polysaccharide contains only one reducing carboxyl group, indicating that the reducing agents provided by polysaccharides are thus limited for AgNP production. In another study, nitrate reductase was considered as a possible reactant reducing Ag^+ to AgNPs [35]. We designed experiments to study this hypothesis by cultivating microalgae using NH_4^+ or NO_3^- as nitrogen source since it was reported that using only NH_4^+ or NO_3^- as nitrogen source for cell cultivation could reduce or induce the nitrate reductase produced in cells, respectively [36]. Our results showed that there were no significant effects of the change of nitrate reductase concentration on AgNP synthesis. In addition, neither polysaccharides nor enzymes were reported to require light

to provide reducing power, so they were less likely to mediate the light-dependent AgNP synthesis.

4.4 Conclusion

The synthesis was an auto-catalyzed reaction which involved two models of AgNP formation: nucleation and seeded-growth. Additional chlorophyll was found to accelerate reaction rate while chlorophyll without cell extract did not show ability of producing any AgNPs, indicating that besides chlorophyll, there were other reactive agents in cell extract jointly functioning with chlorophyll for AgNP synthesis. The unknown reactive agents were sensitive to longtime sonication. Only light of specific wavelengths, i.e., purple and blue lights, which coincidence with one of the absorbance peak of chlorophylls, could induce the reduction of Ag^{+}. A hypothesis was proposed to explain the mechanism of this light-dependent biosynthesis of AgNPs mediated by microalgal extract, i.e., Ag^{+} was reduced by excited electrons of chlorophyll molecules upon absorbance of photons of the appropriate energy level (i.e., blue and purple lights) and the positively charged chlorophyll was hypothetically reduced by accepting electrons from water splitting, which was catalyzed by photosystem II, an enzymatic protein found in the photosynthetic electron chain. The involvement and function of photosystem II have yet to be confirmed by direct experimental evidences.

4.5 Acknowledgements

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Chapter 5:

Conclusions and recommendations

5.1 Conclusions

5.1.1 In conclusion, this study proved that aqueous cell extract obtained by whole cell aqueous extraction (WCAE) and disrupted cell aqueous extraction (DCAE) of green alga *N. oleoabundans* could reduce Ag^+ and thus produce AgNPs, which potentially provides a green approach to produce AgNPs in a sustainable manner.

5.1.2 It is determined, according to experimental data, that the biosynthesis of AgNPs, mediated either by WCAE or by DCAE, is light-dependent, to which both chlorophylls and non-chlorophyll cellular materials which are most likely proteins, are essential.

5.1.3 The kinetics of the DACE-mediated biosynthesis of AgNPs indicates that is an auto-catalyzed reaction that could be separated into three phases, i.e., nucleation, seeded growth, and reactant-limiting reaction.

5.1.4 The whole cell aqueous extract obtained by boiling water-bath aqueous extraction of whole *N. oleoabundans* cells, which contained extremely dilute cellular material (e.g., chlorophyll concentration 0.0632 μM), was able to mediate the reduction of up to 0.09 mM of Ag^+ to AgNPs.

5.1.5 Results of DCAE-mediate biosynthesis with addition of external chlorophylls and the rainbow tests indicate the biosynthesis was chlorophyll-mediated.

5.1.6 Chlorophyll concentration in whole cell aqueous extract was only 0.069 % of AgNPs produced and that was 2.6 % in disrupted cell aqueous extract. In the sense of practical application, they suggest that a high-efficiency AgNP biosynthesis process could be established once a more effective aqueous extraction process is developed to drastically increase the

concentration of reactive agents such as chlorophylls in the cell extracts. In theoretical reasoning, these results indicate that reducing cellular materials alone, which existed at concentrations magnitude lower than the AgNPs produced, could not possibly provide all the electrons required for the reduction of Ag^+. In other words, the final electron donor(s) for Ag^+ reduction should be something abundant in the reaction mixture and water splitting was hypothesized to be the means of electron supply in the biosynthesis.

5.1.7 Three parameters, including Ag^+ concentration, pH value, and extraction time were found to have significant effects on AgNP production. The individual effects of the three parameters on AgNP production were tested and 0.4 mM Ag^+, pH 5, and 6 hours of boiling extraction were found to be optimal conditions of AgNP production. The joint effects of the mentioned parameters were further studied by orthogonal tests and it was found that 0.8 mM Ag^+, pH 5, and extraction time of 9 hours were the best combination for AgNP production. The AgNPs thus synthesized were spherical with a mean particle diameter of 15.8 nm with decent uniformity, an SPR peak at 420 nm, and exceptional antibacterial activities.

5.1.8 For DCAE-mediated biosynthesis of AgNPs, sonication was applied prior to extraction at room temperature to obtain disrupted cell aqueous extract, which was similarly proved to be able to produce AgNPs with illumination. The reaction rate was slow at the beginning since nucleation was dominant where AgNPs of small size were synthesized. As the reaction proceeded, seeded-growth became dominant where Ag^+ started being reduced on existing nuclei, which led to bigger particle size as well as redshift of absorption maxima. Chlorophyll was found to mediate AgNP synthesis since higher chlorophyll concentration led to higher reaction rate. In rainbow tests, blue and purple lights (380-595 nm) were found

particularly necessary to the reaction since only lights in this spectra were able to excite chlorophyll to give electrons with adequate energy for Ag^+ reduction. Based on experimental results, for the first time, Ag^+ reduction was hypothesized to be achieved by photo-induced electron transfer where electrons were donated by excited chlorophyll by absorbing blue and purple lights. Chlorophyll molar concentration was far less than that of reduced Ag^+, the excited chlorophyll thus needed to be reduced and the lost electrons were potentially replenished by water splitting.

Overall, this project was about studying on AgNP biosynthesis using microalga *N. oleoabundans*. A potentially industrialized way of sustainably produce AgNPs was proved and optimized, that was to used whole cell aqueous extract to reduce Ag^+ to AgNPs. In addition, a hypothetic theory was proposed to explain the reaction mechanism, which was a chlorophyll-mediated Ag^+ reduction.

5.2 Recommendations

This project demonstrated the great potential of biosynthesis of AgNPs mediated by aqueous extract of either whole cells or disrupted cells of *N. oleoabundans*. The following are some recommendations on future studies.

5.2.1 Studies on reaction mechanism

The mechanism proposed for the biosynthesis of AgNPs mediated by the aqueous cell extracts of *N. oleoabundans* according to the results of this study needs to be further verified with experimental evidences and the following studies are recommended.

1. Using extracted chlorophylls and pure photosynthetic respiratory chain components including photosystem II (PS II) to verify the involvement of PS II in AgNP biosynthesis;
2. Alternatively, selectively remove or denature PS II from aqueous cell extract and test the capacity of the residual cell extract for mediating AgNP biosynthesis;
3. Identify major components in the aqueous cell extracts capable of mediating AgNP biosynthesis.

5.2.2 Studies on process development

1. Systematically optimize DCAE conditions for more efficient cell disruption which minimizes denaturation of reactive components;

2. Systematically study the effects of biomass concentration on efficiency of extraction and AgNP biosynthesis;

3. Systematically study the effects of Ag^{+} concentration, temperature, pH, and light intensity on AgNP biosynthesis;

4. Develop continuous process for AgNP biosynthesis.

Appendix

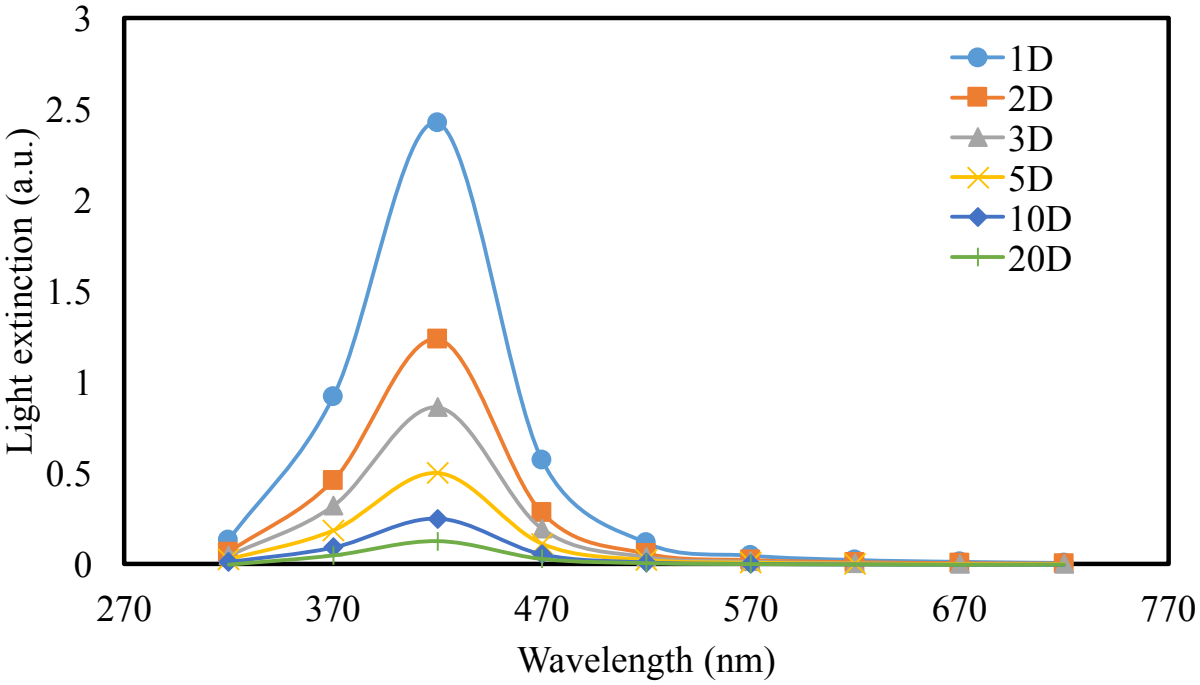

Figure A- 1 UV-Vis spectra of commercial AgNPs (mean size of 40 nm) of 1 to 20 dilution rates.

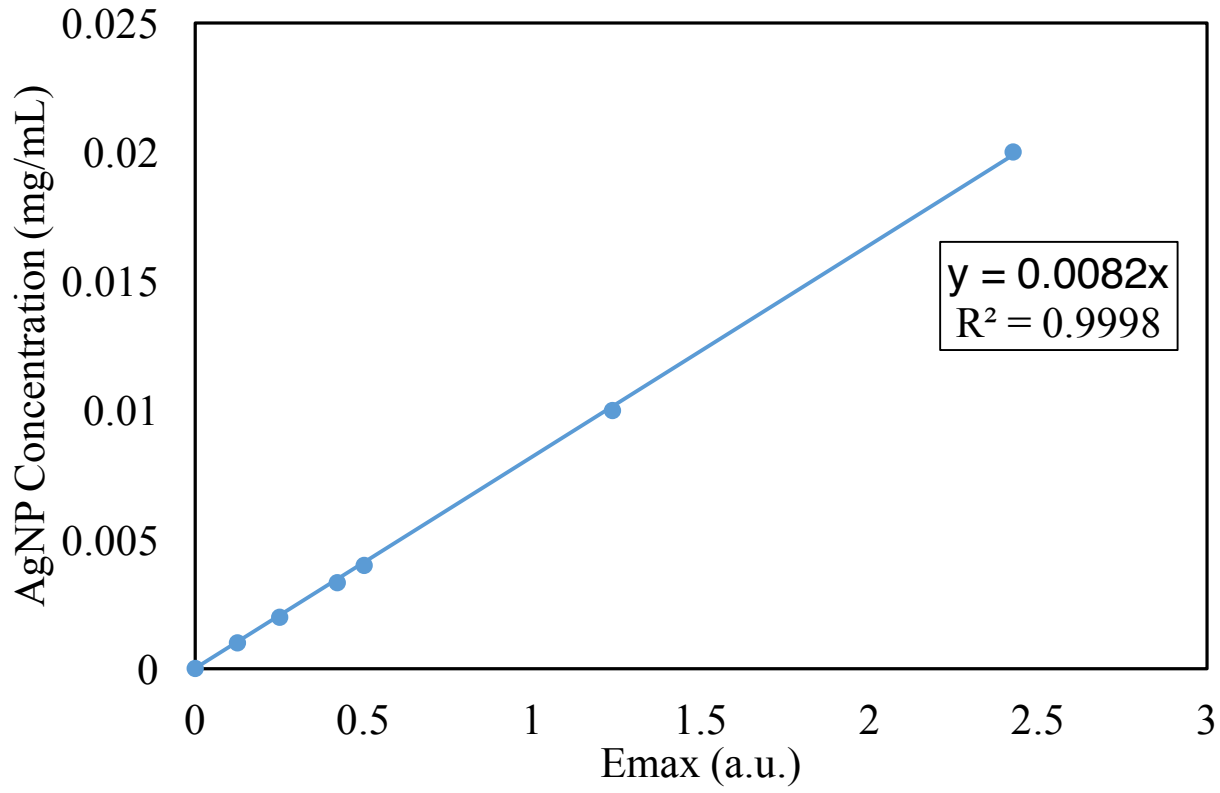

Figure A- 2 AgNP concentration (mg/mL) vs Emax calibration curve.