

**Biological synthesis of gold nanoparticles by
Thermus scotoductus SA-01**

by

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Submitted in fulfilment of the requirements for the degree

PHILOSOPHIAE DOCTOR

In the

Department of Microbial, Biochemical and Food Biotechnology

Faculty of Natural Sciences

University of the Free State

Bloemfontein

Republic of South Africa

January 2010

Promoter: Prof. E. van Heerden

This thesis is dedicated to my family and the memory of my father.

ACKNOWLEDGEMENTS

I sincerely wish to express my gratitude to the following persons without who this dissertation would not be possible:

Prof. E. van Heerden for her invaluable assistance with the final preparation of this manuscript, as well as for her guidance and endless patience throughout the project.

Prof. D. Litthauer, Prof J. Albertyn, Dr. L.A. Piater and Dr. D.J. Opperman for their helpful advice during my studies and the preparation of this manuscript.

All the members of the Department of Microbial, Biochemical and Food Biotechnology for interest shown and support given. Special thanks to the members of the Extreme Biochemistry group for all that they have done, academically as well as socially.

The microscope units at the University of the Free State, University of Ghent, Nelson Mandela Metropolitan University and the University of Western Ontario for their assistance with data analysis.

Mintek, Project AuTEK, The National Research Foundation (NRF), The Ernst and Ethel Eriksen Trust, The World Gold Council, The Oppenheimer Memorial Trust and the Vice-Dean of the Faculty of Natural and Agricultural Sciences who supported this research.

And last but not least my family for their sacrifices, unfailing love and support throughout all my years of studying.

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LIST OF ABBREVIATIONS

A	Absorbance
ABC	ATP-binding cassette
ATP	Adenosine triphosphate
BBInternational	British Biocell International
BCA	Bicinchoninic acid
BIM	Biological induced biomineralization
BLAST	Basic Local Alignment Search Tool
BOB	Biological organised biomineralization
BSA	Bovine serum albumin
CD	Compact Disc
CID	Combat identification
cm	Centimeter
CM	Carboxymethyl
CNT	Carbon nanotubes
CVD	Chemical vapour deposition
DDT	Dichlorodiphenyltrichloroethane
DEAE	Diethylaminoethyl
DNA	Deoxyribonucleic acid
DVD	Digital Versatile Disc
EDS	Energy dispersive spectroscopy
EDTA	Ethylene diaminetetraacetic acid
EM	Electron microscopy
EPH	Ethopropazine hydrochloride
FAD	Flavin adenine dinucleotide

FADH	Reduced flavin adenine dinucleotide
FMN	Flavin mononucleotide
FSO	Physiological salt water
g	Gram
g.l ⁻¹	Gram per liter
gDNA	Genomic DNA
h	Hour
HIC	Hydrophobic interaction chromatography
HNAR	5-(2-hydroxy-5-nitrophenylazo)rhodanine
IPTG	Isopropyl β -D-1-thiogalactopyranoside
kb	kilobasepair
kDa	kilo Dalton
kV	kilo Volt
LB	Luria-Bertani
μ g	Microgram
μ g.ml ⁻¹	Microgram per milliliter
μ l	Microliter
μ m	Micrometer
μ M	Micromolars
μ M.ml ⁻¹	Micromolar per milliliter
M	Molar
MBP	Maltose binding protein
mg.ml ⁻¹	Milligram per milliliter
min	Minute
ml	Milliliter
ml.min ⁻¹	Milliliter per minute
mm	Millimeter

mM	Millimolar
MOPS	3-(N-Morpholino)propanesulfonic acid hemisodium salt
Mr	Relative molecular mass
MSD	Membrane spanning domain
MWCO	Molecular weight cut-off
MWNT	Multiwalled nanotubes
NAD ⁺	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NADP ⁺	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NBD	Nucleotide binding domain
ng	Nanogram
ng.μl ⁻¹	Nanogram per microliter
nm	Nanometer
NMWC	Nominal molecular weight limit
OD	Optical density
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PEM	Proton Exchange Membrane
ppb	Parts per billion
RFLP	Restriction fragment length polymorphisms
RNA	Ribonucleic acid
rpm	Revolutions per minute
SBP	Substrate-binding protein
SDS	Sodium Dodecyl Sulphate
SWNT	Single walled nanotubes

TE-buffer	Tris-EDTA buffer
TEM	Transmission Electron Microscopy
TMD	Transmembrane domain
Tris	2-Amino-2-(hydroxymethyl)-1,3-propandiol
TYG	Tryptone, yeast extract, glucose
U	Units
UF	Ultra filtration
US DOE	United States Department of Energy
UV	Ultraviolet
UV-Vis	Ultraviolet –visible
V/cm	Volts per centimetre
v/v	Volume per volume
VLS	Vapor-Liquid-Solid
W	Watt
w/v	Weight per volume
x g	Times gravity
x	Times
X-Gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranosidehosphate

CHAPTER 1

1. LITERATURE REVIEW

1.1. Introduction

Nanoscience and nanotechnology are fields that open up the possibility of understanding new and unique phenomena which may lead to novel applications that can be utilized at both the micro- and macro-scale. Nanoscience involves research to discover new behaviours and properties of materials with dimensions at the nanoscale (1 – 100nm), while nanotechnology is the field in which the discoveries made at the nanoscale are put to work. The applications of nanotechnology will not only boost the competitiveness of industry but could also create new products that will make positive changes in the lives of citizens (Bhat, 2003; Booker and Boysen, 2005; European commission 2004a; NNI, 2006; Ortego, 2008). On the other hand nanobiotechnology involves bridging the gap between inanimate and animate nature, where researchers learn from biology to create new micro-nanoscale devices to better understand life processes at the nanoscale. It thus aims at understanding the basic principles of biological functional units as well as creating extremely small elements at nanoscale in a controlled way with technical materials and interfaces (Harper, 2003; Hunt, 2004).

The focus on nanoscale sized particles has peaked in the last decade as the properties of materials at nanoscale can have some unexpected differences from their bulk form. These differences can be due to an increase in relative surface area and an increase in the dominance of quantum effects. These changes can cause the materials to become harder or lighter or more durable (Booker and Boysen, 2005; NNI, 2006; Sun and Xia, 2002). In some cases, simply making things smaller changes their optical, electronic or magnetic properties. Nanotechnology is not about only shrinking, but also fundamentally changing the internal structure of compounds.

1.2. Nanofabrication methods

The most commonly used approaches to create nanoscale materials can be divided into two categories: top-down and bottom-up construction (Dolye, 2006; Johans, 2003; Mijatovic *et al.*, 2005). The bottom-up approach is used to create things by manipulating atoms or molecules, while the top-down approaches involve taking larger objects and making them smaller and smaller until the required size is obtained by using machining and etching techniques.

The most common method for top-down nanofabrication is lithography. This technology is used to manufacture computer chips and virtually all other microelectronic systems. Materials can be reduced to about ten nanometers in scale and can be mass produced. These processes are very expensive, difficult and they require defect-free, flat surfaces (Whitesides and Love, 2001; Williams and Adams, 2007).

During bottom-up manufacturing, atoms and molecules are used to build up nanostructures by carefully controlled chemical reactions. Using these manufacturing methods nanostructures with dimensions between two and ten nanometers can be made inexpensively. Two of the most common bottom-up methods are those used to make nanotubes and quantum dots (Mijatovic *et al.*, 2005; Whitesides and Love, 2001). Although these processes are less expensive than the top-down processes, resulting nanostructures are randomly positioned unless combined with a top-down method and is, as yet, not suitable for nanofluidic devices as their function is more reliant on patterning than on dimensions. Longer, better controlled and interconnected structures could be achieved by combining top-down and bottom-up technologies.

1.3. Nanoparticle categorising

Single molecules and endless bulk systems are bridged to make up nanostructure systems. Individual nanostructures entail clusters, nanoparticles, nanocrystals, quantum dots, nanowires and nanotubes, while compilations of nanostructures involve arrays, assemblies, and superlattices of individual nanostructures. Entirely new and novel means to investigate structures and systems form part of the emerging field of nanotechnology. In addition to

well-established techniques of crystallography and transmission electron microscopy and spectroscopy, the modern methods of scanning probe and tunneling spectroscopies and of extended X-ray adsorption fine structure synchrotron radiation spectroscopy have provided powerful tools for the characterization of nanostructures (Aitken *et al.*, 2004; Jortner and Rao, 2002). A useful basis for characterizing nanoparticles is based on particle morphology and composition.

1.3.1. Nanotubes

Nanotubes are a particularly novel form of nanoparticles that are generating considerable interest. Carbon nanotubes (CNT) were first isolated and characterized by Iijima in 1991 when he was studying the material deposited on the cathode during arc-evaporation synthesis of the fullerenes (Teo *et al.*, 2004). CNT are essentially graphite sheets rolled up to form hollow tubes with diameters ranging from 0.4 to 500nm and lengths in the range of several micrometers to millimeters. Their structure is similar to fullerene; but while the fullerene's molecules form a spherical shape, nanotubes are cylindrical structures with the ends covered by half a fullerene molecule. There are two types of carbon nanotubes, namely single- and multiwalled nanotubes (Figure 1.1). Single walled nanotubes (SWNT) consist of a single layer of carbon atoms that wraps around to form a seamless tube which may be open at both ends or capped at one or both ends. Multiwalled nanotubes (MWNT) consist of multiple graphene layers telescoped about one another. The single-walled variety has been most studied in terms of its physical and electrical properties, because its behaviour is more easily predicted in this form (Theodore and Kunz, 2005; Wolf, 2004).

Nanotubes are molecularly perfect, which leads to their extraordinary properties, especially strength and flexibility. They also exhibit high conductivity, high surface area and potentially high molecular adsorption capacity. Their electrical properties are also very different and they can be excellent conductors, semiconductors, as well as insulators depending on their structure. CNT can be applied in several probable areas such as energy storage, molecular electronics, fibres and fabrics, catalysts as well as in the biomedical field (Silbergliitt, 2004; Theodore and Kunz, 2005).

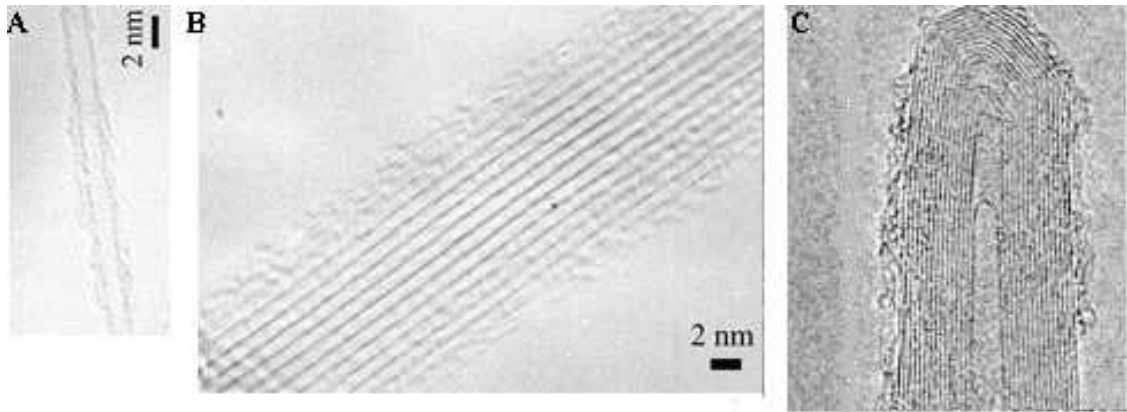


Figure 1.1 TEM images of (A) SWNT (B) bundle of SWNTs and (C) MWNT. (Taken from GTIR Nanotechnology Lab).

Fabrication of nanotubes are not difficult as they are found in common environments such as the flame of a candle, but the difficulty lies in controlling the size, orientation and structure, so they can be used for technological tasks. The synthesis of CNT can be performed by different kinds of techniques, which include arc-evaporation, chemical vapour deposition, laser ablation and sputtering (Köhler and Fritzsche, 2004; Wolf, 2004).

1.3.2. Nanowires

Nanowires are less than 100 nanometers in diameter and can be as small as three nanometers. Typically nanowires are more than 1000 times longer than their diameter and composed of either metals or semiconductors. Semiconductor nanowires are one-dimensional structures, with unique electrical and optical properties and show promise for use in applications such as memory devices, transistors, quantum devices, optoelectronics, field-emitters, photon ballistic waveguides and biomolecular nanosensors (Booker and Boysen, 2005; Takayanagi *et al.*, 2001).

Nanowires are not observed spontaneously in nature and must be produced in a laboratory through either suspension or deposition. A suspended nanowire is created in a vacuum chamber, made by chemically etching a bigger wire, by blasting a larger wire with high energy particles, or by pushing a nanoprobe's tip into the soft surface of a partly melted

metal and pulling it back to get a nanowire. On the other hand deposited nanowires are created using the Vapor-Liquid-Solid (VLS) synthesis method. This technique uses either ablated particles or a gas as source material. The source is exposed to a catalyst and deposited onto a substrate. This process produces crystalline nanowires for semiconductor components (Williams and Adams, 2007).

1.3.3. Nanocrystals, clusters and particles

Nanomaterials with at least one dimension $\leq 100\text{nm}$ and which are single-crystalline are often referred to as nanocrystals. They are bigger than molecules, but not as big as bulk matter. Although nanocrystals' physical and chemical characteristics change, one of their big advantages over larger materials is that their size and surface can be precisely controlled and properties such as melting point and charge conductivity can be modified (Williams and Adams, 2007).

Nanoclusters have at least one dimension between one and ten nanometers and a narrow size distribution. They are of particular interest in materials science, catalysis and other fields of applied sciences such as nanoelectronics. Nanoclusters can be distinguished from nanoparticles based on size. Nanoclusters consists of up to several hundred atoms, but when the number increases to 10^3 or more atoms they are called nanoparticles. Another way to differentiate between the two is to look at their properties. For nanoclusters each constituting atom can affect the properties in a significant and unique fashion, while for nanoparticles their properties gradually approach those of bulk materials or extended surfaces, i.e. are scalable with size (Bromely, 2009).

1.3.4. Quantum dots

Quantum dots are tiny semiconductor nanocrystals that glow when stimulated by an external source such as ultraviolet light. The number of atoms included in the quantum dot determines the size, which in turn, determines the colour of light emitted. Quantum dots are formed of various materials, currently the most commonly used materials include zinc sulphide, lead sulphide, cadmium selenide and indium phosphide. When energy is applied to

a quantum dot, electrons are energised and move to a higher level. When the electron returns to its lower and stable state, this additional energy is emitted as light corresponding to a particular frequency. The frequency or colour of light given off is not related to the material used in the quantum dot, but by the size of the quantum dot. Large quantum dots produce light with a long wavelength and small quantum dots produce light with small wavelengths (Figure 1.2) [Booker and Boysen, 2005; Williams and Adams, 2007].

Quantum dots can be manufactured by a number of processes from colloidal synthesis to chemical vapour deposition (CVD). The cheapest and simplest method is benchtop colloidal synthesis. Electrochemical techniques and CVD can be used to create ordered arrays of quantum dots on a substrate material. Quantum dots show promise for use in a wide range of applications from the quantum computers of the future to medical applications, high resolution television screens and household lighting (Booker and Boysen, 2005).

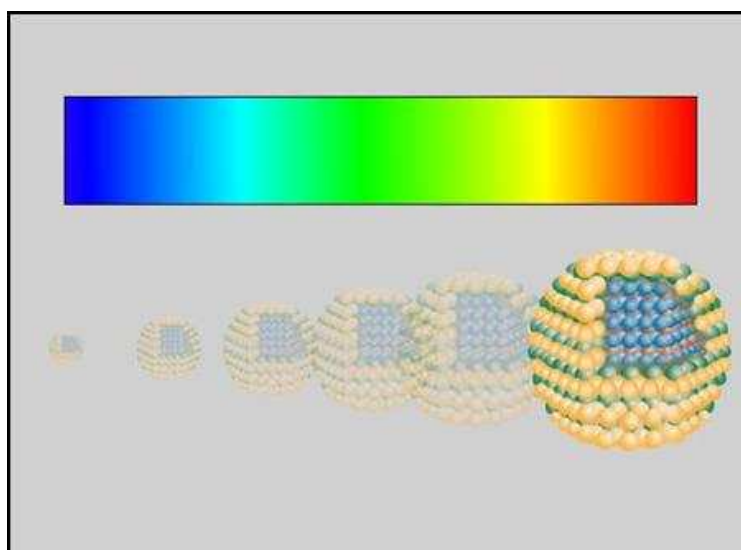


Figure 1.2 Quantum dots showing a change in colour with an increase in size. (Taken from Aerospace concepts Pty Ltd).

1.4. Focus areas and applications

Nanoscience can infuse virtually all technological sectors. It often merges different areas of science and benefits from an interdisciplinary approach and is expected to lead to new innovations. Nanotechnologies are gaining in commercial application and some sectors that have been receiving a great deal of attention and money to develop and use new applications for nanostructured materials are the electronic, magnetic, optoelectronic, medical, energy, catalytic and food and environmental sectors (Bhat, 2003; European Commission, 2004b)

1.4.1. Security

Nanotechnology has great importance in the field of security as it arms the war fighter with advanced technology to facilitate better force protection. One of the elements is the ability to identify friendly combat entities by means of Combat Identification (CID). Warfare results in unforeseen and unintentional death or injury to friendly personnel which can be eliminated by the use of CID. CID technologies can be divided into several groups which include: (a) Passive signalling devices, such as infrared paint and tape, identification panels and smoke markers. Infrared paint and tape can be visualized by using both image intensification and thermal imaging. The nature of the tape and paint provides flexibility of application and it is relatively inexpensive; (b) Active signalling devices which emit electromagnetic energy such as infrared beacons and chemical lights. Chemical lights come in a variety of visible colours and infrared spectrums, which are visible through night vision equipment (Nightmarker, 2008); (c) Interrogation / response systems that enable positive identification through the process of query and response (Boyd *et al.*, 2005; Evident Technologies, Inc, 2008).

1.4.2. Medical applications

The intersection of nanotechnology with life sciences has the potential to revolutionize medicine and health care. The present era of nanotechnology has reached a stage where scientists can create programmable and externally controllable materials and devices designed to interact with the body at sub-cellular levels with a high degree of specificity. This could be potentially translated into targeted cellular and tissue-specific clinical

applications aimed at maximal therapeutic effects with very limited adverse-effects. Some examples can be pharmacytes, microbivores, chromalloyocytes and respirocytes.

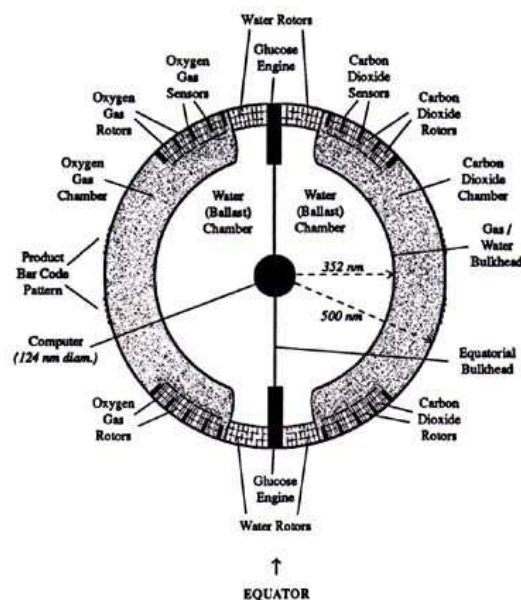


Figure 1.3 Equatorial cutaway view of a respirocyte. (Taken from Freitas, 1998).

For example: Respirocytes are artificial oxygen carriers, with a diameter of one micron, which floats along in the blood stream. These microscopic pressure tanks are made mostly of carbon atoms arranged as a diamond in a porous lattice structure inside a spherical shell. The tanks can safely be charged to 1000 atmospheres and be pumped full of oxygen and carbon dioxide (Figure 1.3). Respirocytes have molecular pumps on their surfaces that load and unload gasses from the pressurized tanks which are able to deliver 236 times more oxygen to tissues per unit volume than natural red blood cells (Freitas, 1998; Shanthi and Musunuri, 2007).

Primary applications will include transfusable blood substitution; partial treatment for anaemia, lung disorders, enhancement of cardiovascular or neurovascular procedures, tumour therapies and diagnostics; prevention of asphyxia; artificial breathing; and a variety of uses in sports, veterinary, battlefield as well as other uses.

1.4.3. Information technologies

Informatics includes hardware, design and modelling relating to large-scale integrated electronics and, information and communications technology. Technologies in this area are at the forefront of nanotechnology's commercialization. Progressive miniaturization of materials continues to be the trait of the electronics and microelectronics industry. Techniques such as soft lithography and bottom-up approaches to produce nanoscale components by self-assembly could produce cheap and effective microscale circuits (Taylor, 2002).

In the long term, the realization of molecular or biomolecular nanoelectronics, spintronics and quantum computing could open up new avenues beyond current computer technology. A number of applications which illustrate the potential for nanotechnology in informatics are photonic crystals and photonic integrated circuits, quantum information processing and quantum structure electronic devices. In microelectronics applications, the use of nanoscaled powders of precious metals provides significant advantages during the production of smaller and smaller electronics circuits with decreasing layer thickness, and such an approach can also reduce the overall consumption of precious metals (Theodore and Kunz, 2005).

A novel way to store information is by using colour lithography. Customarily the binary code (1 and 0) is used for information storage, where a transition between 1 and 0 or 0 and 1, define the amount of information that can be stored and retrieved. This technology is limited by the fact that a transition cannot represent two bits or more and has led to the search for alternative methods to store and retrieve information. Talla and co-workers (2008) introduced a new scheme for storage of information using lithography in colour, which is based on the hypothesis that each imprinted feature in colour can represent two or more bits as opposed to the single bit of conventional methods. There are four colours (red, green, blue and black) that can be used in a coding scheme, which can be made more efficient when the colour coding of bit pairs are incorporated. This can be done by analysing a sequence of 1000 bits for the frequency of occurrence of different bit pairs, and depending on how the colours are allotted there can be 24 different mappings (Figure 1.4). They also showed that this technology have superior performance in terms of information storage capacity when compared to CD and DVD technologies.

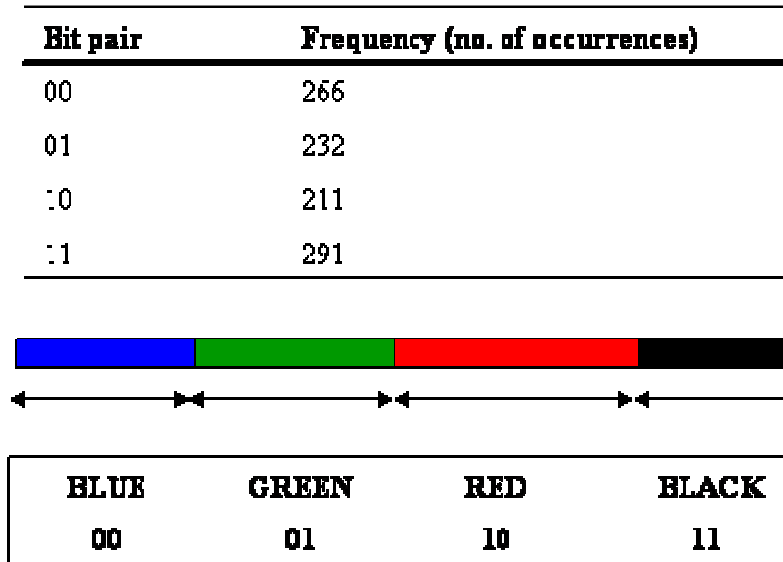


Figure 1.4 Schematic representation of the assignment of colour bit pairs based on their frequency of occurrence. (Taken from Talla et al., 2008).

1.4.4. Energy production and storage

As the world's fossil fuel resources dwindle, practical implementation of alternative energy systems is becoming increasingly important and the concerns with climate change have lead many to look towards the field of nanotechnology for the potential to deliver new, sustainable and environmentally friendly methods of providing energy solutions. Energy saving are anticipated *via* nanotechnological developments that lead to improved insulation, transport and efficient lighting. Energy production and storage can benefit from novel fuel cells or lightweight nanostructured solids that have the potential for efficient hydrogen storage (US DOE, 2002; Weston and Matcham, 2002).

Fuel cells will most likely be the energy source of the future, for vehicles, local off-grid power supply, and power sources for small and handheld electronic devices. Fuel cells are at the interface between energy and materials because of their advanced materials requirements and nanomaterials will be important as catalysts and key components of hydrogen storage systems. In addition, fuel cells are a clean technology, and the stack design makes it possible to add extra cells for more power, if necessary (Blum *et al.*, 2005; US DOE, 2000; Weston and Matcham, 2002).

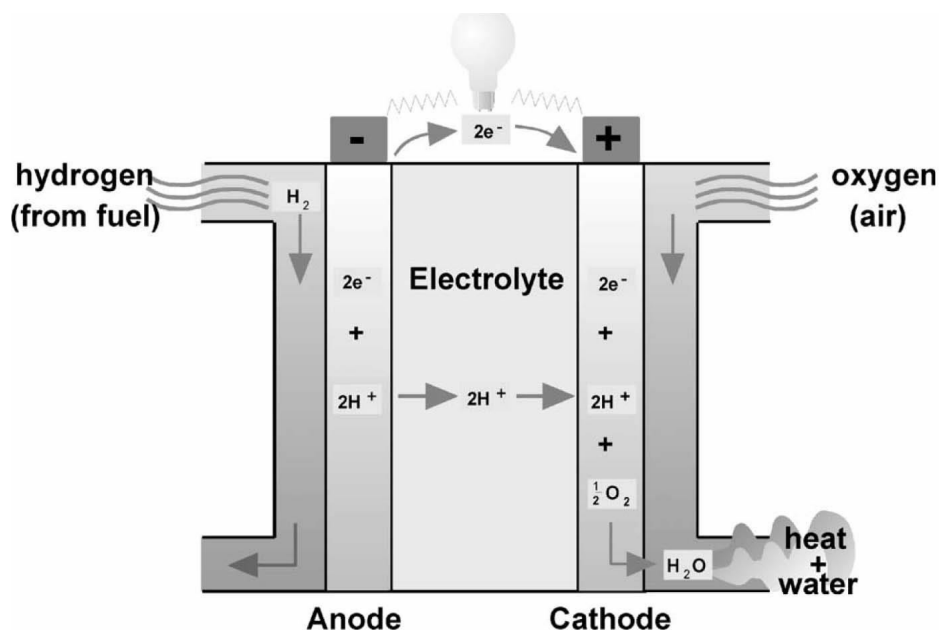


Figure 1.5 Schematic presentation of a PEM fuel cell operation. (Taken from Furman's Energy Options).

A fuel cell is an electrochemical conversion device. It produces electricity from various external quantities of fuel and an oxidant. It can utilize the hydrogen from any hydrocarbon fuel - from natural gas to methanol, and even gasoline. It will produce electricity and heat as long as hydrogen is supplied. A fuel cell consists of two electrodes, an anode and a cathode electrode sandwiched around an electrolyte. A catalyst causes the hydrogen atoms to split into protons and electrons (Figure 1.5). The electrons go through an external circuit, creating a flow of electricity, while the protons migrate through the electrolyte to the cathode where it reacts with oxygen, which is fed to the cathode to produce water and heat (Shukla *et al.*, 2004; Srinivasan, 2006; US DOE, 2000).

1.4.5. Food industry

Nanotechnology in the food industry can take a number of forms. These include the use of nanotechnology in packaging materials, farming practices, food processing and also in the foods themselves. Nanofood can be described as food in which nanotechnological techniques or tools are used during cultivation, production, processing or packaging. One vision of

nanotechnology is the manipulation of the molecules and atoms of food to design food with much more capability, lower costs and greater sustainability than at present (Traver, 2006).

Applications of nanotechnology to food manufacturing include antibacterial work surfaces, filters that can extract toxins and packaging that provides a better barrier against contamination, or can signal when its contents are spoiling, by changing colour. The use of nanotechnology in food packaging is already a commonplace reality and can be separated into two types: active packaging and smart packaging. Active packaging includes materials that constantly provide a certain feature like stopping oxygen from spoiling food, while smart packaging reacts to changes in the environment such as to indicate food spoilage. This recognition can be through nanosensors which selectively attaches to any number of food pathogens. These nanosensors can be detected by using either infrared light or magnetic particles (Doyle, 2006; Covered, 2004). Packaging that uses nanotechnology include plastic beer bottles made with nanocomposite materials, plastic films that increase shelf life and antimicrobial or antifungal packaging.

1.4.6. Commercial applications

Companies such as British Biocell International (BBInternational), NanoArmor and PlasmaChem are already selling nano-products with a variety of uses in all fields. BBInternational manufactures and sells gold and silver colloids which are widely regarded within the Diagnostic and Research industries as the "Gold Standard", due to their spherical nature, monodispersity and stringent size specifications. These properties make the colloids ideal for use in applications where consistent shape and scale is vital. They also offer assistance on several applications such as, advice on the optimum size of gold colloid to use in the application of your choice. They help with the selection of antibodies for conjugation and capture of the material, as well as the selection of an appropriate final storage buffer for the conjugates (BBI website).

1.5. Production of nanoparticles

Nanoparticles are very important materials for implementing nanotechnology in diverse areas and are abundant in nature as living organisms operate at a nanoscale. As nanoparticles exhibit interesting size- and shape-dependent physical and chemical properties, the synthesis of uniform nanoparticles with controlled sizes and shapes is of great importance. Nanoparticles are the end products of a wide variety of physical, chemical and biological processes, some of which are novel and radically different and others of which are quite commonplace (Aitken, 2004; Doyle, 2006; Johans, 2003). Particle production can occur either as part of the top-down or bottom-up processes.

1.5.1. Chemical processes

The most common approaches to synthesizing nanoparticles are chemical processes based on transformations in solution. Sol-gel processing, CVD, plasma or flame spraying synthesis, laser pyrolysis and atomic or molecular condensation are a few methods used for producing nanoscaled particles from various materials. These chemical processes rely on the availability of appropriate metal-organic molecules as precursors (Theodore and Kunz, 2005). The primary disadvantages of these techniques are high operating temperatures and pressures, use of organic solvents, potential lack of scalability and the limited control over crystalline dispersion (Mukherjee *et al.*, 2001).

Sol-gel processing is a wet-chemical method and differs from other chemical processes as nanoscale materials can be synthesized at low temperatures in direct contrast to the competing high temperature methods (Luther, 2004; Theodore and Kunz, 2005). Precursors used for sol-gel processing can follow one of two main routes, namely the inorganic and metal-organic routes. The inorganic route uses metal salts in aqueous solutions as raw materials, while the metal-organic route occurs in organic solvents and uses metal alkoxides as starting materials. The process consists of sol formation followed by gelling, shape forming, drying and densification.

In addition to sol-gel processing, CVD has also been used to produce nanoparticles from a variety of materials. During CVD the starting material is vaporized in a reaction chamber by activating a chemical reaction. The carrier gasses, containing the desired

compound flow over the heated surface onto which the solid is to be deposited. The heated surface allows for decomposition of the carrier gasses and for the mobility of the deposited atoms on the surface. This mobility helps to produce a highly ordered crystalline deposit (Wolf, 2004; Köhler and Fritzsche, 2004).

1.5.2. Biological processes

A variety of starting material has been employed for the biological production of nanoparticles using biomass obtained from plants and several microbial species (Gardea-Torresdey, 2002 and 2003; Sastry *et al.*, 2003; Shankar *et al.*, 2004; Williams *et al.*, 1996a and 1996b). Although microorganisms have been used in bioremediation technologies because of their ability to detoxify an environment by reduction of the metal, it has only been of late that they were considered useful as eco-friendly nanofactories. Many single-celled organisms can produce mineral structures from inorganic materials either intracellularly or extracellularly. Some examples include the magnetotactic bacteria which synthesizes magnetite or greigite, diatoms which synthesizes siliceous materials and the S-layer bacteria which have gypsum and calcium carbonate surface layers (Klaus-Joerger *et al.*, 2001; Sarikaya, 1999).

Magnetotactic bacteria synthesize nano-sized magnetite crystals that are highly consistent in size and morphology within bacterial species, each particle is surrounded by a thin organic membrane and is known as a magnetosome. The formation of magnetosomes is achieved by a biological mechanism that controls the accumulation of iron and the biomineralization of magnetic crystals (Bazylinski, 1999; Matsunaga *et al.*, 2007; Schüler, 1999). Magnetosomes are arranged in one or more chains, which confers a magnetic dipole moment to the organism causing it to align and swim along geomagnetic field lines. Examples of the morphologies of these particles are shown in Figure 1.6. These bacteria use megnetotaxis to migrate to environments more favourable to their survival. The synthesis of magnetic nanoparticles by a biological process promises advantages in terms of controlling crystal growth and structural properties. These particles could have applications in immunoassay and receptor binding assays, cell separation treatments, and could also be used as magnetic probes and as drug delivery systems.

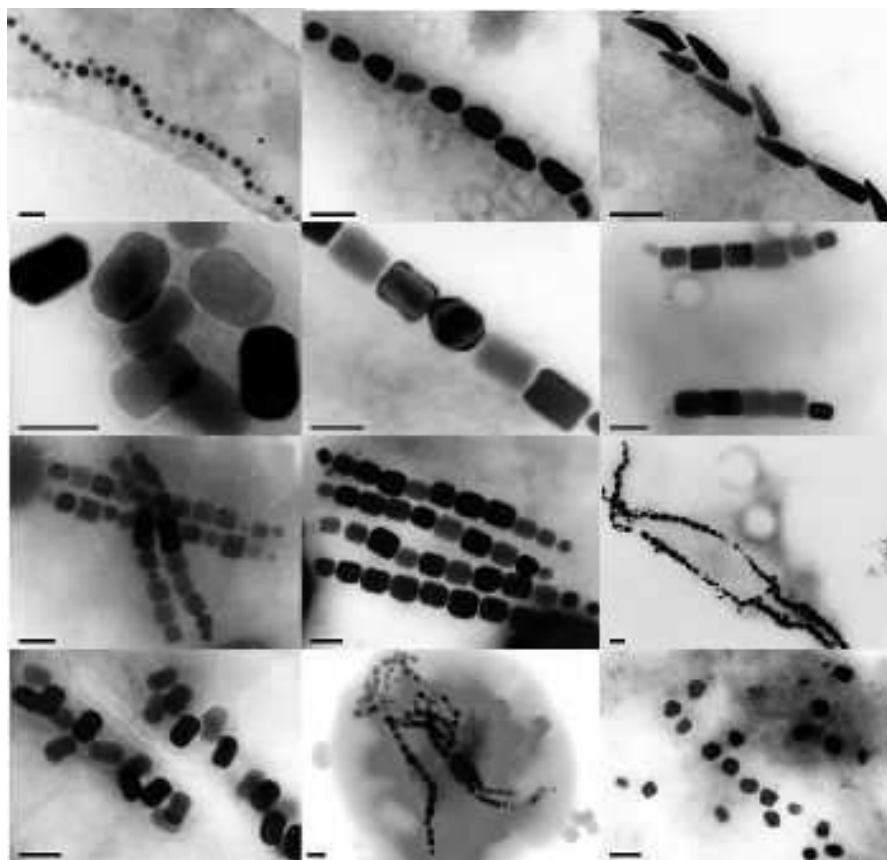


Figure 1.6 Electron micrographs of various magnetosomes. (Taken from Schüler, 1999).

In the last decade more microorganisms have been identified with the ability to produce inorganic particles. Most of these microorganisms were screened for the ability to produce metal particles with the noble metals gold (Ahmad *et al.*, 2003b and 2003c; Reyes *et al.*, 2009), silver (Ahmad *et al.*, 2003a; Maliszewska *et al.*, 2009; Vigneshwaran *et al.*, 2007) and platinum (Konishi *et al.*, 2007) having received the most attention. Another metal which has received attention is cadmium (Ahmad *et al.*, 2002; Bai *et al.*, 2009).

From the information supplied in this chapter it is clear that nanoparticles are of great scientific interest as they bridge the gap between bulk materials and atomic or molecular structures. A bulk material has constant physical properties regardless of its size, but at the nanoscale this is often not the case. A variety of chemical and physical procedures is available for synthesis of metallic nanoparticles. However, these methods are filled with many problems including use of toxic solvents, generation of hazardous by-products, and

high energy consumption. Accordingly, there is an essential need to develop environmentally friendly procedures for synthesis of metallic nanoparticles and a promising approach to achieve this objective is to exploit the range of biological resources available in nature.

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CHAPTER 2

2. INTRODUCTION TO PRESENT STUDY

2.1. Introduction

Nanotechnologists seek to produce and explore the applications of both novel and some natural nanomaterials in larger quantities and within a more consistent size range. The nanomaterial is then used to develop materials for use in new devices, components and products which will make positive changes in the lives of citizens (Bhat, 2003; Silberglitt, 2004). Numerous techniques are used to fabricate different nanomaterials. Nanoparticles can be produced from larger structures (top-down) by use of ultrafine grinders, lasers, and vaporization followed by cooling. For complex particles, nanotechnologists generally prefer to synthesize nanostructures by a bottom-up approach by arranging molecules to form complex structures with new and useful properties (Dolye, 2006).

Gold particles at nanoscale have a wide variety of uses since the material properties thereof differ from that of the bulk form. These differences can be due to an increase in relative surface area or in the dominance of quantum effects (Hunt, 2004; Sun and Xia, 2002). The unique properties of gold at nanoscale results in the application thereof in diverse areas such as electronics, catalysis, colours and coatings, as well as the biomedical sector (Corti *et al.*, 2004). The usual strategy to prepare nanoparticles involves the reduction of a gold salt in solution by various reducing agents in the presence of a stabilizer. Nanoparticles produced by chemical, biological and physical methods include nanocubes, nanorods / nanowires, nanodisks and nanotriangles / nanoprisms.

There are several chemical and physical methods for the production of nanoparticles (Aitken, 2004; Doyle, 2006; Johans, 2003), but there are evidence of the harmfulness of these methods on the environment. There is thus a growing need to develop clean, non-toxic and eco-friendly (“green chemistry”) procedures for the synthesis and assembly of nanoparticles. The potential of bio-organisms ranges from simple prokaryotic bacterial cells to eukaryotic fungi and even live plants. It has been shown that nanoparticles can be synthesized utilizing

these biological agents (Ahmad *et al.*, 2003; Armendariz *et al.*, 2004; Bhattacharya and Gupta, 2005; Sharma *et al.*, 2007).

Microorganisms such as fungi have been found to produce nanoparticles of different shapes and sizes, and the microbial interaction with metals also supply eco-friendly methods for nanoparticle production (Ahmad *et al.*, 2002; Maliszewska *et al.*, 2009; Mukherjee *et al.*, 2002; Sastry *et al.*, 2003; Vigneshwaran *et al.*, 2007). For tailor-made nanoparticle synthesis, the possibility of optimizing specific size and shape thereof has been demonstrated. Temperature, pH or other conditions were varied and specific shapes of gold nanoparticles obtained (Armendariz *et al.*, 2004; Gericke and Pinches, 2006; Kashefi *et al.*, 2001). No reaction has as yet resulted in uniform particles but processes hint towards a degree of control.

Many microorganisms that inhabit the earth's crust and soils bring about several biochemical and geochemical reactions. They encounter various metals and metalloids in their environments which can be enzymatically or non-enzymatically concentrated or dispersed (Ehrlich, 1997). Many of these metals can be used by microbes as an energy source by converting the ions from one electrical state to another (Spain, 2003); however some microbes can reduce metal ions without conserving energy from the reactions (Summers and Sugarman, 1974). These reduction reactions can then serve as a means to concentrate metals into what ultimately become ore deposits.

Morphological evidence that bacteria are involved in the formation of placer gold was obtained by Watterson (1991). Upon the examination of placer gold by means of scanning electron microscopy mineralized spheres the size of bacteria were observed. Additional experimentation by Southam and Beveridge (1994; 1996) led them to hypothesize that bacterial organics, mediate the formation of crystalline octahedral gold, as it was found that sulphur and phosphate were major components of the gold minerals examined. In 2006, Reith and co-workers extracted DNA from gold grains. Composition of the bacterial communities located on gold grains indicated a species common to all extracts. This bacterium was identified as *Ralstonia metallidurans* and it was shown that this bacterium could actively precipitate gold and survive in gold concentrations that is toxic to most other microorganisms. These findings suggest that microbes are involved in the geomicrobial cycling of gold. Recently more evidence was collected which indicated that microbiota is actively involved in the cycling of gold in the environment (Reith *et al.*, 2009). Gold grains collected from Queensland, Australia, showed that microbial biomass consisting of carbon,

nitrogen and oxygen were associated with the gold grains. Associated with the biofilms was nanoparticulate gold which resembled gold nanoparticles produced in the laboratory when *Cupriavidus metallidurans* was exposed to gold.

In the past decade several research groups have been actively studying the interaction of biological biomass with gold, silver and other metals. Most of the data was obtained by using intact or cell-free extracts to facilitate metal reduction and nanoparticle synthesis. Very few studies have been conducted on purified proteins and thus little is known about the biological mechanisms involved in gold(III) reduction and metal nanoparticle production, but speculation about possible mechanisms have been made (Duran *et al.*, 2005; He *et al.*, 2007; Nangia *et al.*, 2009). Theory centres around the oxido-reductase reactions which can either reduce or oxidize metals depending on whether the metal acts as electron acceptor or donor. Kumar and co-workers (2007) showed that a nitrate reductase purified from *Fusarium oxysporum* mediated the production of silver nanoparticles when NADPH was added to the protein mixture. A possible mechanism for gold(III) reduction was proposed (He *et al.*, 2007) which involves the electron shuttling during enzymatic metal reduction processes where NADH donates an electron which is used by the NADH-dependent reductase to reduce gold(III).

2.2. The aims of this study were to:

1. Screen bacterial isolates for the ability to reduce gold(III) with subsequent nanoparticle formation.
2. Evaluate the effect of varying physico-chemical parameters on nanoparticle formation using intact biomass.
3. Purify and identify the protein(s) involved in gold(III) reduction and nanoparticle formation.
4. Characterize the purified protein(s) directly responsible for gold(III) reduction and nanoparticle formation by varying the physico-chemical parameters (pH; temperature; gold salt concentrations; reaction time).
5. Heterologously express the purified protein in the mesophile *Escherichia coli*.

6. Characterize the recombinant proteins with respect to gold reducing activity and nanoparticle formation as well as evaluating the effect of environmental parameters (pH; temperature; gold salt concentrations; reaction time; sodium dithionite concentrations) on particle formation.

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CHAPTER 3

3. GOLD REDUCTION AND NANOPARTICLE FORMATION BY BACTERIA ISOLATED FROM SOUTH AFRICAN MINES

3.1. Introduction

Generally speaking, a metal is a material with high reflectivity and conductivity that can usually be deformed plastically. A metal reflects light like a mirror unless the surface has been corroded. The term metal also refers to the metallic elements even when these are combined with other elements to form non-metallic compounds such as salts and oxides. Metals will display different characteristics depending on various physico-chemical parameters. Metals can combine in almost any proportion offering a vast range of alloys which generally show all the characteristics of a metal and are therefore regarded as metals (Hillert, 1997).

Microorganisms require certain metallic elements for growth and function. Essentially, they function as catalysts for biochemical reactions; they stabilize proteins and bacterial cell walls, and serve in maintaining osmotic balance (Hughes and Poole, 1991). These metallic elements include the bulk elements potassium and magnesium, as well as the trace elements such as manganese, iron, copper, zinc and molybdenum. Even though some metals are essential trace elements, at high concentrations most can be toxic to all branches of life, including microbes, by forming complex compounds within the cell (Nies, 1999). Heavy-metal cations, such as Hg^{2+} , Cd^{2+} and Ag^+ are such toxic complex-formers that they are too dangerous for any biological function.

3.1.1. Metal-microbe interactions

Since some environments (volcanic soils, deep-sea vents) naturally contain high concentrations of toxic metals, microbes have been exposed to such materials long before mankind began increasing local concentrations through industrial activity. Microbes have evolved several mechanisms to tolerate the presence of metals, i.e. efflux, complexation or

reduction of metal ions both assimilatory and dissimilatory. Because the intake and subsequent efflux of metal ions by microbes usually includes a redox reaction involving the metal, bacteria that are resistant to, and grow on metals also play an important role in the biogeochemical cycling of those metal ions (Ahmann *et al.*, 1994; Ehrlich, 1997; Gadd, 2001; Spain, 2003).

Microorganisms can reduce a variety of metals in metabolic processes that are not related to metal assimilation by using a variety of proteins. Some microorganisms can conserve energy to support growth by coupling the oxidation of simple organic acids and alcohols or hydrogen to the reduction of certain metals (Lovley, 1993; Williams and Silver, 1984). Microorganisms that use metals as terminal electron acceptors, or reduce metals as a detoxification mechanism play an important role in the cycling of both organic and inorganic species in a variety of environments, including aquatic sediments, submerged soils and terrestrial subsurface.

Besides metal reduction reactions microorganisms can interact with elements in various other ways which could lead to particle formation. These particles can either be associated with the biomass or separate from the biomass. Formation of these products can be described by biomineralization processes. The next section will discuss these processes in more detail.

3.2. Biomineralization processes

Organisms are capable of forming a diverse array of minerals. When organisms convert a substance from an organic to an inorganic substance the process is known as biomineralization. It is a widespread phenomenon and the minerals formed are deposited at many different locations both inside and outside of the cell (Frankel and Mann, 1994; Weiner and Lowenstam, 1989). Biomineralization can be divided into two fundamentally different processes, namely biological induced mineralization (BIM) and biological organized biomineralization (BOB) [Lowenstam, 1981].

BIM is induced by the organisms as a result of interaction between biologically produced metabolites and cations in the environment. An organism thus modifies its local microenvironment in such a way as to favour mineralization. Mineral particles without

unique morphology and with a broad particle size distribution are formed as the organism has no control over the crystallization process as this is an unintentional and uncontrolled sequences of mineral deposition (Frankel and Bazylinski, 2003; Lowenstam, 1981; Weiner and Lowenstam, 1989). During BOB, minerals are grown in a pre-formed organic structural framework which allows for greater control over the nucleation and growth of minerals as opposed to the mineral formed by the BIM pathway. These mineral particles are structurally well-ordered with a narrow size distribution. It is thought that this process is under metabolic and genetic control as the intra-vesicular conditions are controlled by the organism which make mineral formation less sensitive to external environmental parameters. Minerals formed at a particular site may have several different fates. The mineral may be retained in the same location or transported to other sites, excreted, dissolved and replaced continuously, periodically or occasionally (Bazylinski and Frankel, 2003; Frankel and Mann, 1994; Lowenstam, 1981; Veis, 2003).

As BOB processes require the cell structure to mineralize the substances, it can be described as occurring extra-, intra- or intercellularly. BOB is characterized as such based on the location of the mineralization site within the cell structure. In extracellular mineralization a framework is produced by the cell which consist of different macromolecules, such as proteins, polysaccharides or glycoproteins. This matrix becomes the site of mineralization (Weiner and Dove, 2003). Intracellular mineralization occurs within sealed membrane-enclosed compartments (vesicles). These vesicles have selected permeability to certain metals ions and molecules and direct the nucleation of biominerals within the cell. After mineralization the vesicles containing the mineralized products may remain inside the cell or be transported out of the cell or elsewhere in the cell (Mann, 2006; Weiner and Dove, 2003). The intercellular mineralization process is not widespread and occurs between cells in a community. During mineralization the cell envelope of the cells are used as the organic substrate for nucleation and at first glance this might appear to be a type of BIM but it has been shown that the individual organism directs the polymorph and shape of the biomineral formed (Weiner and Dove, 2003).

Biologically induced and biologically controlled deposition of magnetic iron minerals by bacteria has been recognized for some time now. Magnetotactic bacteria contain intracellular chains of single magnetite crystals which are bound to the membrane. The magnetite crystals are held in place by an elongated sheath of organic material and help the

organism to migrate in a magnetic field (Frankel and Bazylinski, 1994; Schüler, 1999). Non-magnetotactic dissimilatory iron-reducing and sulphate-reducing bacteria produce magnetite, siderite, vivianite, and iron-sulfides by BIM processes. These bacteria export ferrous ions and sulphide ions into the environment inducing the formation of these minerals (Coker *et al.*, 2006; Frankel and Bazylinski, 2000).

3.2.1. Geochemistry of gold

Gold is one of the rarest elements in the earth's crust, ranging at about 3-6 ppb and with concentrations between 5 and 50 ppt in seawater (McNulty, 1994). It is widely distributed and occurs as nuggets or grains in rocks, in veins and alluvial deposits. It can be found in nature either as a free metal or in tellurides. Gold is one of the least reactive metals; it is stable in air under normal conditions, but can be attacked by and dissolved in alkaline solutions of sodium cyanide. It can also be dissolved in a mixture of acids to form chloroauric acid (Kerrick, 1999). Chemically gold is a transition metal and can form cations and in its pure state it is a soft, ductile metal. Improved strength and durability is conveyed by forming an alloy and it is a good conductor of heat and electricity (Butterman and Amey, 2005).

Gold has a variety of applications in various fields, including the biomedical (Del Campo and Bruce, 2005), electronic (World Gold Council, 2009a) and catalytic (World Gold Council, 2009b) fields. It might be asked why gold is an ideal material for such a variety of applications. One important material characteristic is its resistance to surface oxidation. Another factor contributing to the wide array of applications is the change in optical properties from the bulk form to the nanoscale. This results in a colour varying from red to purple depending on the size of the particles (Corti *et al.*, 2004).

3.2.2. Microbial interaction with gold

Microbial processes influence the mobilization, distribution and speciation of many metals and microorganisms which are exposed to pollutants in the environment, such as metal ions, thus may have the ability to fight that metal stress (Reith *et al.*, 2006b). Recent evidence

suggests that some dissimilatory metal-reducing microorganisms can reductively precipitate gold from solution (Kashefi *et al.*, 2001) and it is believed that biological material was involved in the formation of the Precambrian Witwatersrand deposits in South Africa (Dyer *et al.*, 1988; Hallbauer *et al.*, 1977; Schildowski, 1965). More investigations indicated that bacteria were also involved in the formation of Alaskan placer gold. Microscopic analysis of Alaskan placer gold showed the presence of micrometer sized spheroids and these did not consist of solid gold (Watterson, 1991). Reith and co-workers (2006a) found that genetic material on gold deposits which confirms the involvement of microorganisms in gold deposition processes (placer gold, grains or nuggets). The DNA of 30 different species was associated with the gold of which *Ralstonia metallidurance* was the only species not observed in the surrounding soil. *R. metallidurance* can actively precipitate gold and survive in concentrations that is toxic to most other microorganisms. Very little experimentation has been done on using microorganisms *in situ* for gold immobilization, but several studies on *in vitro* immobilization have been published (Southam and Beveridge, 1994 and 1996; Southam *et al.*, 2000).

3.2.2.1. Bacteria, Actinomycetes and Archaea

Gold recovery from waste products is essential as gold is a very scarce metal. Recovery is possible by different leaching methods which includes treatment with cyanide. If bacteria with the ability to solubilise gold or with the ability to produce low concentrations of cyanide can be used *in situ*, it would lead to gold recovery without pollution of the environment with excess cyanide. One such cyanide-producing bacterium is *Chromobacterium violaceum*. Faramarzi and co-workers (2004) demonstrated the ability of *C. violaceum* to solubilise gold used in printed circuit boards, while Campbell and co-workers (2001) showed that *C. violaceum* were able to colonize on gold-covered surfaces and was able to solubilise gold from gold ore concentrates, indicating the possibility to use this bacterium for *in situ* gold recovery.

Other than solubilising gold, bacteria and fungi can also selectively accumulate gold via absorption onto their cell walls or via absorption into the cell itself. These processes may play an important role in the precipitation of gold by microorganisms (Reith, 2003). Some of the bacteria, actinomycetes and archaea examined which had the ability to accumulate gold

include *Bacillus subtilis* (Nakajima, 2003; Southam and Beveridge, 1994), *Pseudomonas maltophilia* (Nakajima, 2003), *P. aeruginosa* (Karthikeyan and Beveridge, 2002), *Rhodococcus* sp. (Ahmad *et al.*, 2003b), *Thermomonospora* sp. (Ahmad *et al.*, 2003a) and *Shewanella algae* (Konishi *et al.*, 2006).

There is no clear understanding of the mechanism by which bacteria are able to precipitate gold. Studies by Lengke and co-workers (2006) showed that the cyanobacterium, *Plactonema boryanum*, reduces gold(III) stepwise by first forming a gold(I) species intermediate on the cell wall, which is then deposited as metallic gold near the cell surfaces or as a colloidal suspension. Earlier studies by Ulberg and co-worker (1992) suggested that bioaccumulation of gold in *Bacillus cereus* is dependent on the presence of a proton gradient. Gold accumulation was only possible in metabolic active cells, and was dependent on fully functioning ATPases located in the plasma membrane.

Microbial gold precipitation can be either associated with the cell or with the external environment. Prokaryotic gold precipitation usually occurs as cell associated processes and even though extracellular gold precipitation using prokaryotes is a rare occurrence, some have been identified with this ability. *Thermomonospora* sp. are actinomycetes which have the ability to produce gold nanoparticles extracellularly. When *Thermomonospora* sp. was incubated in the presence of aqueous chloroaurate ions, gold nanoparticles with good monodispersity were formed in the external media while the cells remained colourless (Ahmad *et al.*, 2003a). A bacterium which can produce external gold nanoparticles is *Pseudomonas aeruginosa*. Bacterial cell supernatant was exposed to hydrogen tetrachloroaurate and well-dispersed particles with a size range of 15-30nm were produced (Husseiny *et al.*, 2007). The effect of pH on the extracellular formation of gold nanoparticles has been illustrated by using the bacteria *Rhodopseudomonas capsulate*. A decrease in pH resulted in the formation of nanoplates in addition to the spherical particles observed at a higher pH. The nanoplates were predominantly triangular in shape (He *et al.*, 2007). An example of bacteria which have the ability to precipitate gold intracellularly is *Shewanella algae*. Gold was precipitated in the periplasmic space only when the bacteria were incubated in the presence of hydrogen, which leads to the assumption that a hydrogenase is involved in the reduction and subsequent precipitation of gold ions (Konishi *et al.*, 2006). After exposure of the actinomycete, *Rhodococcus* sp., to gold ions, gold nanoparticles were observed on the

cell wall and cytoplasmic membrane. These particles were essentially spherical and fairly monodisperse and had an average size of about 12nm (Ahmad *et al.*, 2003b).

Metal interaction with microorganisms are not restricted to microorganisms isolated from metal rich environments as shown by Nair and Pradeep (2002) when they used *Lactobacillus* strains isolated from buttermilk to form gold, silver and gold-silver alloy particles. These metal particles were synthesized inside the cells after exposure to the corresponding metal ions. Another means by which microorganisms can be used to precipitate gold is to use cell-free extracts of the microorganisms. When cell-free extracts from *Rhodopseudomonas capsulata* was incubated with varying concentrations of gold ions different morphologies of gold nanoparticles were formed. Lower concentrations resulted in the formation of mainly spherical particles, while higher concentrations resulted in the formation of nanowires with a network structure (He *et al.*, 2008).

3.2.2.2. Yeast and Fungi

As opposed to prokaryotic gold reduction eukaryotes can precipitate gold both intracellularly and extracellularly. Fungi secrete many compounds which may influence the mobility of metals (Gadd, 1999; Gadd and Sayer, 2000). Yeasts with the ability to interact with gold include *Candida utilis* (Savvaidis *et al.*, 1998; Tsuhuta, 2004), *Debaryomyces hansenii* (Savvaidis *et al.*, 1998; Tsuhuta, 2004) and *Saccharomyces cerevisiae* (Karamushka and Gadd, 1999; Lin *et al.*, 2005). Fungi with the ability to interact with gold include *Aspergillus niger* (Gomes *et al.*, 1998; Xie *et al.*, 2007), *Fusarium oxysporum* (Mukherjee *et al.*, 2002) and *Verticillium sp.* (Mukherjee *et al.*, 2001).

After exposure of *Verticillium sp.* to gold ions, particles were observed within the fungal cells, specifically on the cytoplasmic membrane and mycelia surfaces. No reduction occurred in the external environment indicating intracellular reduction and precipitation of the gold ions. The particles had an average diameter of about 20nm with good monodispersity. The particles were mostly spherical but a few triangular and hexagonal particles were produced (Mukherjee *et al.*, 2001). A mycelia-free spent medium of *Aspergillus niger* was exposed to gold ions and it was found that nanoparticles were formed ranging from polydisperse small spheres to large triangles and hexagons. It was suggested that the triangles and hexagons were flat and they made-up 50% of the total particles

produced. The triangles ranged from 50 to 200nm in size and were approximately 10 to 20nm thick (Xie *et al.*, 2007). Gomes and co-workers (1998) indicated that *A. niger* might be used for the recovery of gold and silver from cyanide-containing solutions as it was possible for the fungus to accumulate these metals from solutions containing high cyanide concentrations. Fungi such as *Fusarium oxysporum* have the ability to produce extracellular gold nanoparticles. When the *F. oxysporum* biomass was exposed to gold ions the external solution turned purple while the biomass remained unchanged. The secreted proteins were purified from solution and it was observed that an NADH dependent reductase was involved in the reduction of gold ions and the subsequent formation of nanoparticles, as in the absence of NADH no nanoparticles were formed (Mukherjee *et al.*, 2002). This fits with the proposed mechanism by He and co-workers (2007) where they speculate that a NADH dependent reductase is involved in the reduction of gold(III). When the effect of gold on *Saccharomyces cerevisiae* growth was evaluated it was found that an increase in gold concentration causes a strong inhibitory effect. When gold concentrations increased above 0.2mM no growth occurred (Karamushka and Gadd, 1999). Gold particles accumulated in the cell wall when waste biomass was used (Lin *et al.*, 2005) indicating that microorganisms need not always be metabolically active to reduce gold and produce nanoparticles.

These metal-microbe interactions have already found an important role in biotechnological applications. It is only in the last decade that microorganisms have been explored as potential biofactories for synthesis of metallic nanoparticles (McNulty, 1994).

The aims of this chapter were:

1. Screen bacterial isolates for the ability to reduce gold(III) with subsequent nanoparticle formation.
2. Evaluate the effect of varying physico-chemical parameters on nanoparticle formation.

3.3. Materials and methods

3.3.1. Bacterial isolation and enrichment

Most of the bacteria used in this study were isolated as part of the Life in Extreme Environment (NSF LEXEN site) and Research Education for Undergraduates (REU site) projects. These projects have been yielding microbial isolates since 1999. The bacteria were isolated and enriched from soil, biofilm or water samples collected from South African mines (Table 3.1). The bacteria were cultured in 5ml nutrient broth (Meat extract 1g.l⁻¹; Yeast extract 2g.l⁻¹; Peptone 5g.l⁻¹; NaCl 8g.l⁻¹) tubes at their respective isolation temperatures ranging from 30 to 65°C. Cultures were incubated for 24 to 48h where after the biomass suspensions were plated on nutrient agar plates. Pure cultures were obtained through sub-culturing on nutrient agar plates.

Pure isolates were stored at -80°C using 15% glycerol as cryoprotectant. The isolates were revived by inoculating 0.5ml culture into 5ml nutrient broth and incubating at the isolation temperature for 24h.

Table 3.1 Microbial isolates screened for the ability to reduce gold(III) and produce nanoparticles.

Culture	Isolation temperature	Origin	Culture	Isolation temperature	Origin
<i>Acinetobacter</i>	30	UFS culture collection	UV 33	37	BHP [§]
<i>Chryseomonas luteola</i>	30	UFS culture collection	UV 35	37	Merriespruit*
<i>Flavobacterium</i>	30	UFS culture collection	UV 36	37	BHP [§]
<i>Micrococcus kristinae</i>	30	UFS culture collection	UV 37	37	Merriespruit*
<i>Pseudomonas maltophilia</i>	30	UFS culture collection	UV 23	45	BHP [§]
<i>Pseudomonas alcaligenes</i>	30	UFS culture collection	UV 25	45	BHP [§]
<i>Bacillus brevis</i>	30	UFS culture collection	UV 29	45	BHP [§]
<i>Erwinia sp.</i>	30	UFS culture collection	UV 31	45	BHP [§]
<i>Staphylococcus epidermidis</i>	37	UFS culture collection	UV 32	45	BHP [§]
<i>Staphylococcus chromogenes</i>	37	UFS culture collection	UV 34	45	BHP [§]
<i>Bacillus badius</i>	37	UFS culture collection	UV 38	45	BHP [§]
UV 20	37	Evander *	UV 39	45	BHP [§]
UV 26	37	Merriespruit*	UV 41	45	BHP [§]
UV 27	37	Merriespruit*	UV 42	45	BHP [§]
UV 28	37	Merriespruit*	UV 43	45	BHP [§]
UV 21	37	Kloof [†]	UV 44	45	BHP [§]
UV 9	37	Evander *	UV 45	45	BHP [§]
UV 10	37	Evander *	UV 46	45	BHP [§]
UV 11	37	Evander *	UV 47	45	BHP [§]
UV 13	37	Gravelotte [‡]	<i>Bacillus stercorophilus</i>	45	UFS culture collection
UV 14	37	Evander*	UV 22	45	BHP [§]
UV 15	37	Gravelotte [‡]	UV 2	55	Gravelotte [‡]
UV 16	37	Evander *	UV 6	55	Gravelotte [‡]
UV 17	37	Evander *	UV 8	55	Gravelotte [‡]
UV 18	37	Evander *	<i>Thermus scotoductus</i> SA-01	65	UFS culture collection
UV 19	37	Gravelotte [‡]			

* Merriespruit and Evander are Harmony Gold Mining Company LTD mines

[†] Kloof is a Goldfields Limited mine

[‡] Gravelotte is a Gravelotte Mines Limited mine

[§] BHP is a BHP Bilton mine

3.3.2. Screening of cultures for gold(III) accumulation and nanoparticle synthesis

Screening was conducted at the respective isolation temperatures of the individual cultures. Pure cultures were grown for 24 to 48h in test tubes containing 5ml nutrient broth in shake incubators (200rpm). The biomass was separated from the medium by centrifugation (5000x g; 10min) and washed three times with sterile physiological salt water (FSO; 0.8%). The biomass was standardized by resuspending the recovered biomass to a final OD₆₀₀ of 1. Gold(III) in the form of HAuCl₄ was added to a final concentration of 0.5mM. The mixtures were then incubated at the respective temperatures for 1 to 7 days, monitoring gold nanoparticle accumulation daily. The accumulation and reduction of gold was followed by visual observation of the mixture turning pink to purple, indicating the formation of gold nanoparticles and indicate their relative sizes (Figure 3.1) [Webexhibits website; Lazarides and Schatz, 2000]. Viability of the cells was not evaluated but a control with autoclaved cells was included. The control sample showed some gold(III) reduction and particle formation but significantly less than for the active cells and no specific particles were observed by TEM analysis.

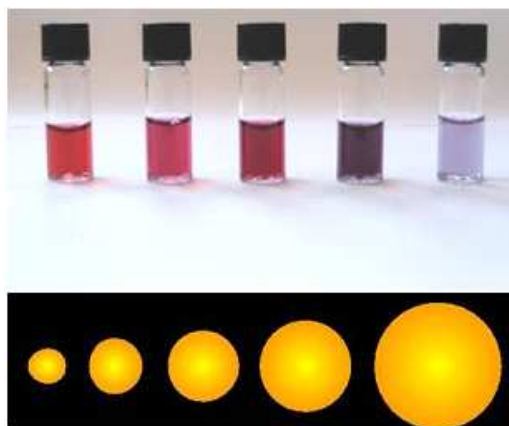


Figure 3.1 The diameter of gold nanoparticles determines the wavelengths of light absorbed. The colours in this diagram illustrate this effect. (Taken from Webexhibits website).

Cultures that displayed positive nanoparticle formation were subjected to a second round of screening. Increased gold(III) concentration was used to differentiate between efficiency of the cultures to reduce gold(III). Cultures were grown as described above and exposed to 0.5

and 1mM gold(III), these concentrations were chosen as most of the experiments conducted by other groups incubated the biomass in gold(III) concentrations ranging from 0.1 to 1mM (Ahmad *et al.*, 2003a; Hussein *et al.*, 2007; He *et al.*, 2008). Colour formation was followed visually over time.

3.3.3. Assay development

Three colorimetric assays were evaluated for use in determining gold(III) concentrations.

3.3.3.1. 5-(2-hydroxy-5-nitrophenylazo)rhodanine

Zaijun and co-workers (2003) designed and synthesized a reagent that could selectively bind to gold(III) forming a stable yellow complex with maximum absorbance at 480nm. The 5-(2-hydroxy-5-nitrophenylazo)rhodanine (HNAR) was synthesized in our laboratory according to the methods described by Zaijun and co-workers (2003). The reagent (HNAR) however was not stable and deteriorated rapidly. Several attempts to stabilize the complex were unsuccessful and this assay was discarded.

3.3.3.2. Association complex formation between Gold-Thiamine and Phloxine

Fujita and co-workers (1999) studied the colorimetric reaction of gold(III) by utilizing an association complex with an acidic dye and a ligand. The gold(III) containing sample was added to a solution containing 0.5% methylcellulose, 0.01M thiamine, 0.001M phloxine B and water. The mixture was incubated at 40°C for 20 minutes. After incubation, the solution was cooled for 5 minutes in a water bath and the absorbance read at 570nm against a reagent blank. Triplicate values were used to construct a standard curve (Figure 3.2). When constructing the standard curve with varying gold(III) concentrations (0.005 to 0.05mM) it became increasingly clear that the assay was not reproducible as colour formation was dependent on various factors, including the addition order of the reagents and the degree of mixing after each addition. Also observed was the break down of the colour-complex soon

after formation. The colour-complex was stable for about 5 minutes and even the reagent blanks gave significant differences. This assay was abandoned.

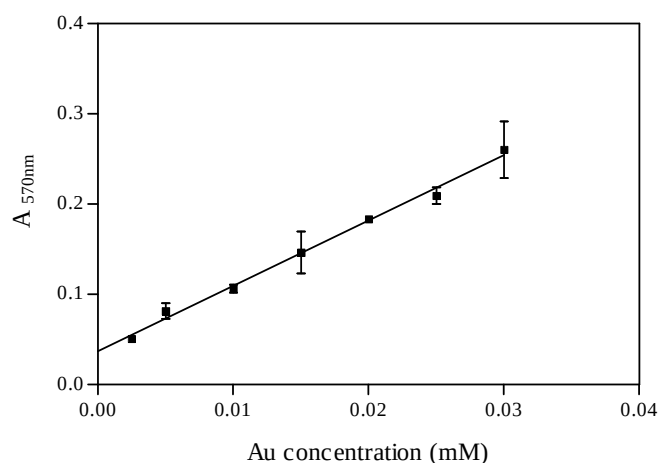


Figure 3.2 Standard curve determination of the Phloxine B assay for gold(III). Error bars indicate standard deviations.

3.3.3.3. Ethopropazine hydrochloride method (EPH)

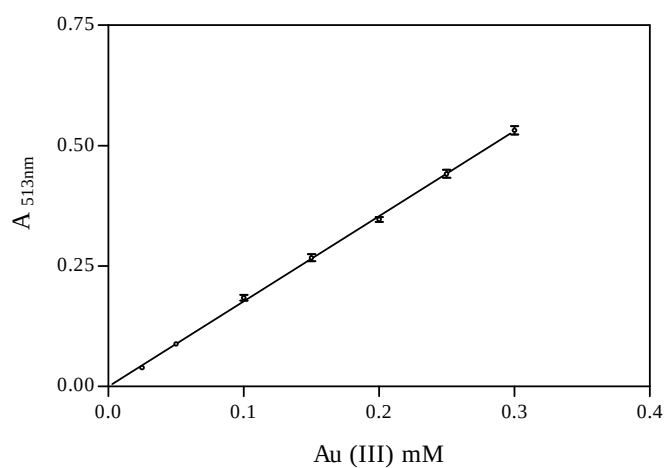


Figure 3.3 Standard curve determination of the Ethopropazine hydrochloride assay for gold(III). Error bars indicate standard deviations.

A third assay method was explored (Melwanki *et al.*, 2002). This assay is based on the oxidation of phenothiazines by gold(III) to give red radical cations absorbing maximally at 513nm. Two hundred microliter sample was added to 800µl 10M phosphoric acid and 800µl distilled water, followed by the addition of 200µl 0.2% ethopropazine hydrochloride. The solution was mixed well and the absorbance values were read immediately at 513nm against a reagent blank as the formed complex is only stable for 5 minutes. Triplicate values were used to construct a standard curve (Figure 3.3). This assay was used for further characterization as it was reproducible, exhibited low deviation and could assess gold(III) over a wider range than the previously mentioned assay methods.

3.3.4. Electron microscopy techniques

Samples were prepared for EM analysis by separating the biomass from the liquid by centrifugation (5000x *g*; 10min; 4°C). The biomass was washed twice in 0.1M cacodylate buffer, pH 7.2 and fixed for 1h in 3% glutaraldehyde solution, prepared in cacodylate buffer, pH 7.2. Recovering and washing of the biomass was done by centrifugation. For TEM analysis, the biomass was enrobed in 1% agar and dehydrated in a graded acetone-water series. Infiltration and embedding were accomplished by spurr-epoxy resin, followed by two changes of spurr. Blocks were polymerised at 70°C for 8h and the embedded material sectioned with an ultra-microtome, yielding sections of approximately 0.08µm. Sections were mounted on copper grids and electron micrographs taken with either a Philips CM 100 or 200 kV Philips CM 20 TEM (van Wyk and Wingfield, 1991). TEM analysis of the cell-free extracts and protein samples were carried out on carbon coated formvar grids. A drop of the sample was placed on the grid and removed after a minute using blotting paper and the grid air-dried before analysis.

3.3.5. Culturing conditions of the chosen isolate

Thermus scotoductus SA-01, a thermophilic bacterium, isolated from an AngloGold Ashanti mine near Carletonville, Republic of South Africa, was chosen for characterization of gold reduction and nanoparticle formation using the intact cells under resting conditions.

3.3.5.1. Monitoring growth and gold(III) reduction

Thermus scotoductus SA-01 was grown from a glycerol stock, plated out onto TYG-agar plates (5g.l⁻¹ tryptone, 3g.l⁻¹ yeast extract, 1g.l⁻¹ glucose and 18g.l⁻¹ bacteriological agar) and grown for 24h. A 20-24h old culture was used to prepare the pre-inoculum (a loop full of culture in 50ml TYG in a 250ml Erlen-meyer flask) and grown until mid-exponential growth phase (~8h). The inoculum was also grown to mid-exponential growth phase (~8h) and was prepared by adding 10ml of the pre-inoculum to 90ml TYG (in a 500ml Erlen-meyer flask). Five millilitres of the inoculum was inoculated into 95ml TYG (in a 500ml Erlen-meyer flask) for the growth experiments. One millilitre samples were withdrawn over time and the OD was measured at 600nm. Growth studies were done in triplicate.

The gold(III) reducing capabilities of the cells were correlated to growth phase. A flask was sacrificed at each time interval and the cells washed three times and resuspended in a 1:20 w/v ratio in 50mM sodium acetate buffer, pH 5.0 (Kaufmann and Lovley, 2001). Gold(III) reducing activity of the cells at each time interval was evaluated by incubating the washed, resuspended cells at 65°C in the presence of 2mM. The gold(III) concentrations were determined at times 0 and 60 minutes using the EPH assay method as described in section 3.3.3.3.

3.3.5.2. Whole cell nanoparticle characterization

Cells were grown as described in section 3.3.5.1 and resuspended in a 1:20 (w/v) ratio after washing. A 10x dilution of this suspension was used to evaluate the effect of environmental parameters on nanoparticle formation under resting conditions. Parameters evaluated included varying the pH, temperature, gold(III) concentration and the exposure time. Standard incubation conditions were 2mM gold(III), pH 5.0 and incubated at 65°C for 24 hours.

3.3.5.2.1. Effect of growth phase on particle production

Biomass recovered at specific time intervals through centrifugation was used for determining the effect of growth phase on particle formation. Gold(III) to a final concentration of 2mM

was added to the cell suspensions (section 3.3.5) and incubated at 65°C for 24h. The samples were prepared for TEM analysis as described in section 3.3.4. Visual observations were conducted over time to follow the colour change from yellow to blue / pink.

3.3.5.2.2. The effect of pH on particle production

The effect of pH on particle formation was evaluated by incubating the 10x diluted cell suspension (section 3.3.5.2) in pH specific buffers ranging from pH 3.6 to 9.5. Usually pH characterization is conducted using a buffer cocktail containing equimolar concentrations of the components, but the cocktail had a high chemical reduction rate. Sodium acetate buffer (50mM) was used for the range below pH 6, 50mM sodium phosphate buffer for the neutral pH range and 50mM borax buffer for the alkaline pH range. Incubation was done at the isolation temperature of 65°C in the presence of 2mM gold(III). Samples were withdrawn over time and a UV-Vis spectrum (400 – 700nm) was obtained using a Beckman DU-800 spectrophotometer, to evaluate the plasmon band associated with gold nanoparticles. The 24h sample was prepared for TEM analysis as described in section 3.3.4.

3.3.5.2.3. The effect of temperature on particle production

A temperature range between 30 and 65°C was chosen to evaluate the effect of temperature on particle formation. The cell suspension was incubated for 24h at the desired temperature in 50mM sodium acetate buffer, pH 5.0, containing 2mM gold(III). The samples were prepared for TEM analysis as described in section 3.3.4. Visual observations were conducted over time to follow the colour change from yellow to blue / pink.

3.3.5.2.4. The effect of gold(III) concentration on particle production

Gold(III) concentrations varying from 0 – 10mM were used to evaluate the effect thereof on particle formation. The cell suspension was incubated for 24h in the des

ired gold(III) concentration in 50mM sodium acetate buffer, pH 5.0. Samples were prepared for TEM analysis as described in section 3.3.4. A UV-Vis spectrum (400 – 700nm) was

obtained using a Beckman DU-800 spectrophotometer to evaluate the plasmon band associated with gold nanoparticles.

3.3.5.2.5. The effect of exposure time on particle production

The cell suspension was incubated at 65°C in 50mM sodium acetate buffer, pH 5.0, containing a final gold(III) concentration of 2mM. Samples were withdrawn in hourly intervals. The cells were prepared for TEM analysis as described in section 3.3.4. A UV-Vis spectrum (400 – 700nm) was obtained using a Beckman DU-800 spectrophotometer to evaluate the plasmon band associated with gold nanoparticles.

3.4. Results and discussion

3.4.1. Screening for gold(III) reducing and nanoparticle producing bacteria

Table 3.2 Mesophilic bacteria screened for gold(III) reduction and nanoparticle formation.

Cultures	Temperature	Colour change	Cultures	Temperature	Colour change
<i>Acinetobacter</i>	30	no	UV 21	37	no
<i>Chryseomonas</i>	30	no	UV 9	37	no
<i>luteola</i>					
<i>Flavobacterium</i>	30	no	UV 10	37	yes
<i>Micrococcus</i>	30	no	UV 11	37	no
<i>kristinae</i>					
<i>Pseudomonas</i>	30	no	UV 13	37	no
<i>maltophilia</i>					
<i>Pseudomonas</i>	30	no	UV 14	37	no
<i>alcaligenes</i>					
<i>Bacillus brevis</i>	30	no	UV 15	37	no
<i>Erwinia sp.</i>	30	no	UV 16	37	no
<i>Staphylococcus</i>	37	no	UV 17	37	no
<i>epidermidis</i>					
<i>Staphylococcus</i>	37	no	UV 18	37	no
<i>chromogenes</i>					
<i>Bacillus badius</i>	37	no	UV 19	37	yes
UV 20	37	no	UV 33	37	no
UV 26	37	no	UV 35	37	no
UV 27	37	no	UV 36	37	no
UV 28	37	no	UV 37	37	no

Bacteria spanning both the mesophilic and thermophilic ranges were screened for the potential to reduce gold(III), with subsequent nanoparticle formation as described in section 3.3.2. Few of the mesophilic bacteria were capable of reducing gold(III) and producing nanoparticles (Table 3.2), while all but one of the thermophilic bacteria were able to produce nanoparticles (Table 3.3). Particle production was dependent on the organism as the control reactions showed no colour change.

Table 3.3 Thermophilic bacteria screened for gold(III) reduction and nanoparticle formation.

Cultures	Temperature	Colour change	Cultures	Temperature	Colour change
UV 23	45	yes	UV 44	45	yes
UV 25	45	yes	UV 45	45	yes
UV 29	45	yes	UV 46	45	yes
UV 31	45	yes	UV 47	45	yes
UV 32	45	yes	<i>Bacillus stereothermophilus</i>	45	yes
UV 34	45	yes	UV 22	45	yes
UV 38	45	yes	UV 2	55	yes
UV 39	45	yes	UV 6	55	yes
UV 41	45	yes	UV 8	55	no
UV 42	45	yes	<i>Thermus scotoductus</i> SA-01	65	yes
UV 43	45	yes			

All the isolates that were able to reduce gold(III) and form nanoparticle were subjected to another round of screening, where the gold(III) concentration was varied. Table 3.4 represents the highest concentration gold(III) at which the bacteria could produce nanoparticles under resting conditions and the incubation time required for particle formation (colour change from yellow to blue / pink). Once again the thermophiles out-performed the mesophiles, by being able to produce nanoparticles at higher gold(III) concentrations and over a shorter incubation time. Identification of the isolates by 16S RNA sequencing indicated that most of the isolates belonged to the *Bacillus* genus. *Thermus scotoductus* SA-01 was chosen for further experimentation on the grounds that it is unique to South Africa and can grow at high temperatures and reduce gold(III) to produce nanoparticles at a satisfactory rate. This bacterium can reduce a number of compounds as electron acceptors

through the dissimilatory pathway under anaerobic conditions and these compounds includes Fe(III), Mn(IV), Co(III)-EDTA, Cr(VI) and U(VI) (Kieft *et al.*, 1999).

Table 3.4 Comparison between the capability to produce nanoparticles by mesophilic and thermophilic bacteria.

Culture	Temperature	Colour change	[Au] (mM)	Time (h)
UV 10	37	+	0.5	168
UV 19	37	+++	0.5	120
UV 22	45	++	0.5	22
UV 23	45	+	0.5	42
UV 25	45	+++	1	8
UV 29	45	+++	1	8
UV 31	45	+	1	42
UV 32	45	++	0.5	22
UV 34	45	++	1	22
UV 38	45	+	0.5	22
UV 39	45	+++	0.5	22
UV 41	45	+	0.5	22
UV 42	45	+++	0.5	22
UV 43	45	+	0.5	42
UV 44	45	+	0.5	42
UV 45	45	+	0.5	42
UV 46	45	+	1	22
UV 47	45	+	0.5	42
UV 2	55	++	1	21
UV 6	55	+	0.5	50
<i>Thermus scotoductus</i> SA-01	65	+++	0.5	24

+ Poor reduction



++ Moderate reduction



+++ Good reduction



3.4.2. Growth and reduction

The pre-inoculum, inoculum and growth of *Thermus scotoductus* SA-01 was monitored to see when the cells reached the mid-exponential phase of growth and is necessary to standardize the growth procedure. The gold(III) reducing capability of cells in different growth phases was determined by harvesting cells at different stages of growth and assessing their ability to reduce gold(III) under non-growth conditions as described in sections 3.3.5.2.1 and 3.3.3.3. It was found that maximum gold(III) removal or reduction was reached after 8h

of growth, which corresponds to the late exponential - early stationary growth phase (Figure 3.4). This is not surprising as the cells are metabolically active and protein synthesis as a maximum. After 8h the reducing abilities of the cells gradually tapers off, with about 60 to 70% of the gold(III) remaining.

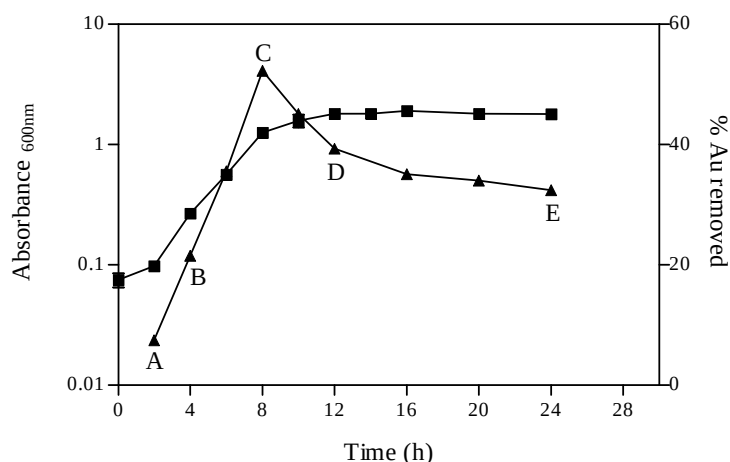


Figure 3.4 Growth curve for *Thermus scotoductus* SA-01, illustrating correlation between growth phase (■) and activity (▲). A-E corresponds to the TEM micrographs in Figure 3.5. Error bars indicate standard deviations but were smaller than the symbols used.

Shankar and co-workers (2004) indicated that metal accumulation may be dependent on the growth phase of cells. To evaluate the effect of growth phase on the ability of cells to accumulate and reduce gold(III), the biomass was harvested over time as described in section 3.3.5.2.1 and exposed to 2mM gold(III) for 24 hours at 65°C. TEM analysis (section 3.3.4) indicated that the nanoparticles produced were associated with the biomass (Figure 3.5), it may be concluded that the cells might have a higher degree of control over the particle morphology as the BOB pathway of mineralization is observed (Weiner and Dove, 2003; Bazylinski and Frankel, 2003; Veis, 2003).

The TEM micrographs (Figure 3.5) illustrates the difference between the particles produced at each time interval. The growth phase had no effect on the particle shape, but a decrease in the number of particles produced was observed with cells harvested in the stationary phase of growth (Figure 3.5 D and E). The late-exponential growth phase cells

formed a high number of particles and were able to remove the majority of the gold(III) from solution as illustrated in Figure 3.4. A possible explanation for this might be that cells in the exponential phase of growth produces higher amounts of proteins than the stationary phase cells, which may be involved in the accumulation and reduction of gold(III).

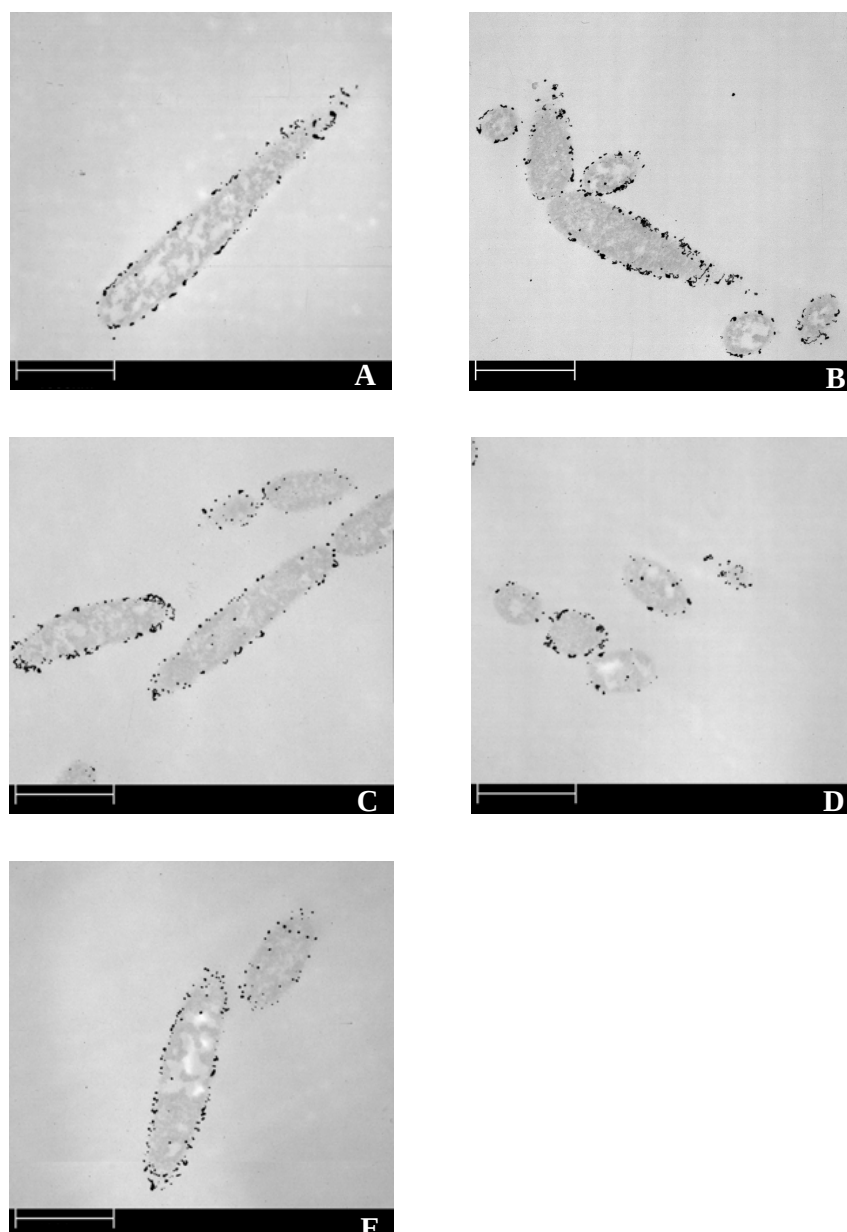


Figure 3.5 TEM micrographs illustrating the effect of growth phase (A) 2 (B) 4 (C) 8 (D) 12 and (E) 24h on particle formation using cells under resting conditions. Scale bars equal to 1000nm.

3.4.3. Physico-chemical parameters

3.4.3.1. The effect of pH on particle production

This experiment was conducted as described in section 3.3.5.2.2. Specific care was taken when choosing the individual buffers, depending on the rate of chemical reduction. This type of characterization is usually done with a cocktail buffer but individual buffer components gave very little to no reduction, while the cocktail buffer gave a higher chemical reduction rate.

From literature it is known that gold nanoparticles exhibit pink-ruby red colours, which arises due to excitation of surface plasmon vibrations in the gold nanoparticles. UV-Vis spectroscopy can be used to record the surface plasmon band of the gold nanoparticles as described in section 3.3.5.2.2. Wavelength scans on the samples showed that pH 5.5 was the first to show changes in the ~540nm range and it occurred at 8h of incubation (Figure 3.6.A). The surface plasmon band at ~540nm for pH 5.5 steadily increased in its intensity as a function of reaction time (Figure 3.6.B). Hussein and co-workers (2007) found that an increase in particle size results in a colour shift from pink to blue due to the surface plasmon of gold nanoparticles, thus looking at Figure 3.7 it can be concluded that the particles produced at lower pH values are larger than particles produced at higher pH values as they appear to be more blue than pink in colour. This was confirmed with TEM analysis as described in section 3.3.4. Figure 3.8 A-D illustrates the increase in particle size corresponding to the decrease in pH, but contrary to suggestions by He and co-workers (2007) that varying the pH, particle sized and distribution can be controlled, it was found that although pH has an effect on particle size, distribution and shape could not be controlled at this level. Also observed in Figure 3.8 A is the formation of octahedral gold which points to mineral diagenesis, while Figure 3.8 C shows intracellular gold reduction which leads to cell death *versus* Figure 3.8 D where immobilization is focused in the cell envelope.

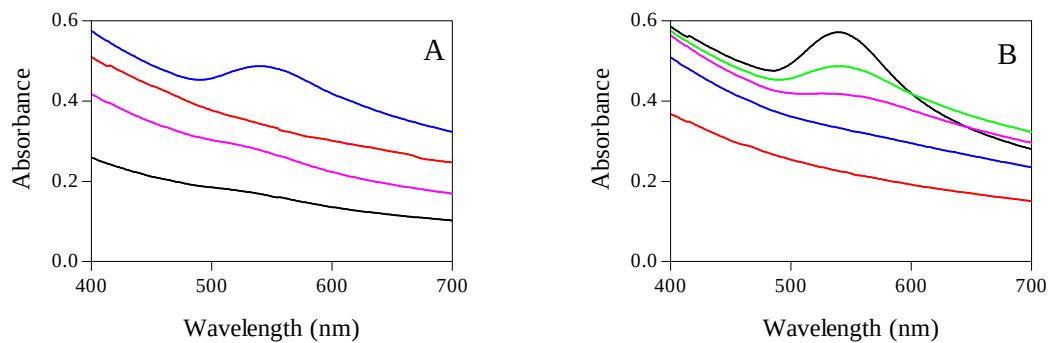


Figure 3.6 (A) Wavelength scans conducted at 8h of incubation in the presence of 2mM gold(III) at 65°C with varying pH. (—) pH 3.6, (—) pH 5.5, (—) pH 7.5 and (—) pH 9.5. (B) Wavelength scans conducted over time on the pH 5.5 sample. (—) time 0h, (—) time 4h, (—) time 8h, (—) time 12h and (—) time 24h.



Figure 3.7 Colour change observed after 8h of incubation at 65°C in the presence of 2mM gold(III). F.l.t.r. pH 3.6; pH 5.5; pH 7.5 and pH 9.5.

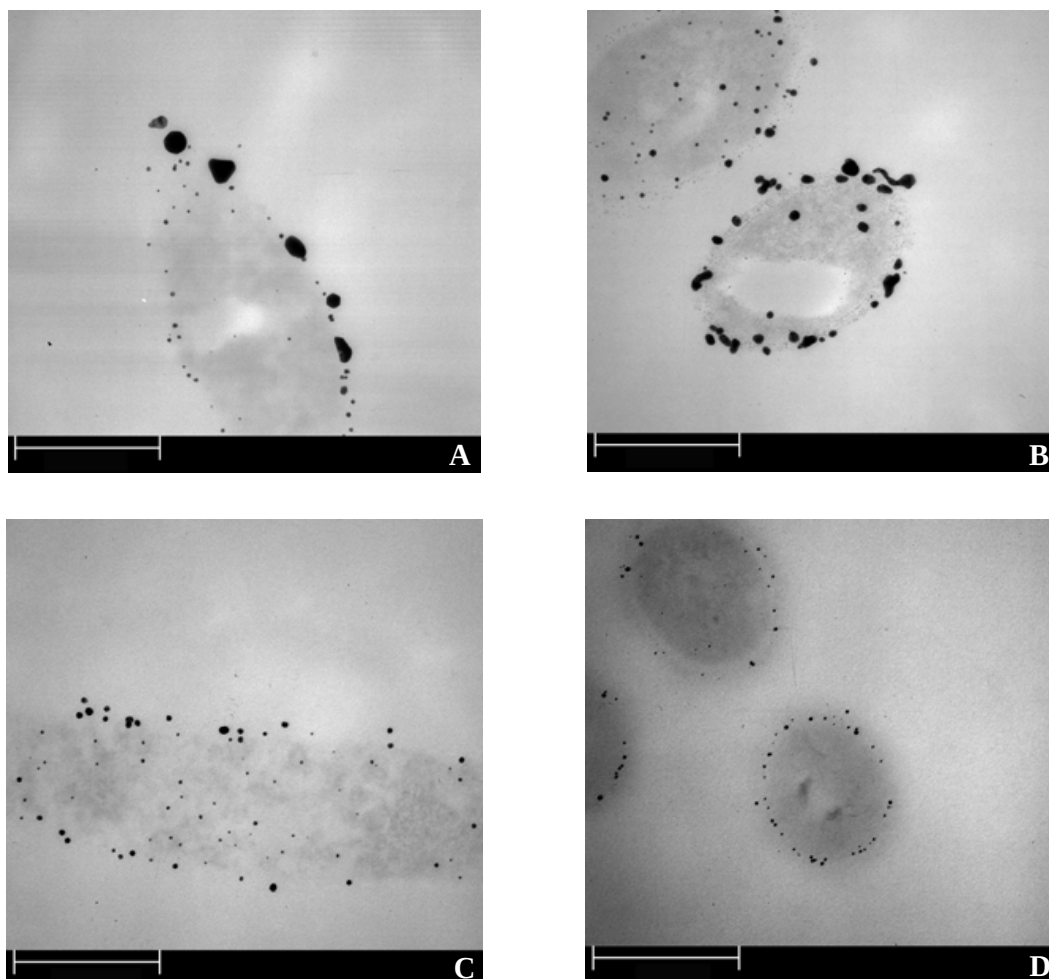


Figure 3.8 TEM micrographs illustrating the effect of pH (A) 3.6 (B) 5.5 (C) 7.5 and (D) 9.5 on particle formation using cells under resting conditions. Scale bars equal to 400nm.

3.4.3.2. The effect of temperature on particle production

The percentage gold(III) removed by *Thermus scotoductus* SA-01 cells were evaluated by determining the remaining amount of gold(III) after 2 and 8h incubation at the desired temperature with an initial gold(III) concentration of 2mM using the method described in section 3.3.5.2.3.

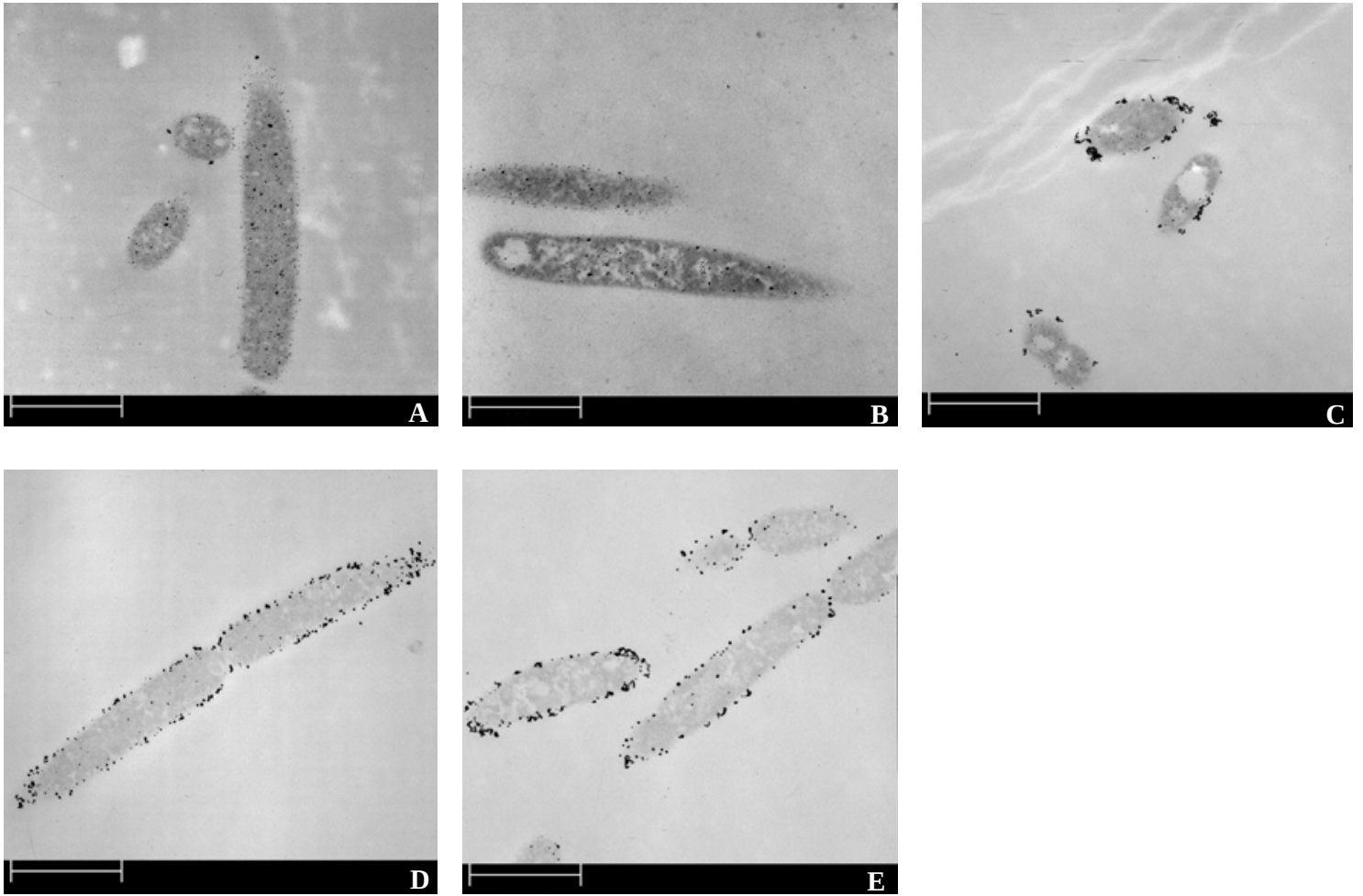


Figure 3.9 TEM micrographs illustrating the effect of temperature (A) 30 (B) 37 (C) 45 (D) 55 and (E) 65°C on particle formation using cells under resting conditions. Scale bars equal to 1000nm.

A longer incubation time resulted in an increase in the amount of gold(III) removed from solution. An increase in temperature also resulted in an increase of gold(III) removal from solution, with the cells at 65°C removing about 80% of the gold(III), while cells at 30°C only removed about 30% of the gold(III). TEM micrographs (section 3.3.4) show that temperature has an effect on particle formation as shown in Figure 3.9 A-E. Fewer and smaller particles are produced at lower temperatures, while the rate of particle formation increased with an increase in temperature. No specific shapes are visible, mostly the particles are amorphous.

3.4.3.3. The effect of gold(III) concentration on particle production

Although initial studies followed literature, adaptations were made in this study to facilitate a higher gold(III) concentration. Incubation of whole cell suspensions (section 3.3.5.2.4), under resting conditions with varying gold(III) concentrations resulted in the formation of particles both in and out of the nano-range, as can be observed in Figure 3.10. A plasmon band is only associated with particles which falls into the nanometer range. Concentrations above 5mM gold(III) gave no characteristic peak in the ~540nm range (Husseiny *et al.*, 2007) suggesting that the particles produced do not fall in the nanometer range. This was confirmed by both the colour formation (Figure 3.11) and the TEM micrographs (Figure 3.12. A-E). At concentrations exceeding 5mM particles appear to rupture the cell walls and are released into the external environment. These particles do not seem to be associated with a specific cell component. From this experiment it is clear that the optimum gold(III) concentration for whole cell reduction experiments are in the range between 1 and 5mM.

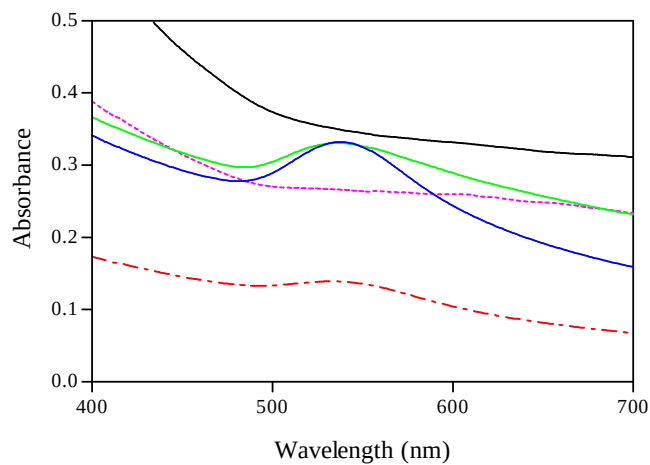


Figure 3.10 Wavelength scans conducted with varying gold(III) concentrations at 65°C after 24h incubation (—■—■—) 0.5; (—■—) 1; (—■—) 1.5; (—■—) 5 and (—■—) 7.5mM.



Figure 3.11 Colour change observed after 24h of incubation at 65°C in the presence of varying gold(III) concentrations. F.l.t.r. 0.1; 0.5; 1; 1.5; 5; 7.5 and 10mM.

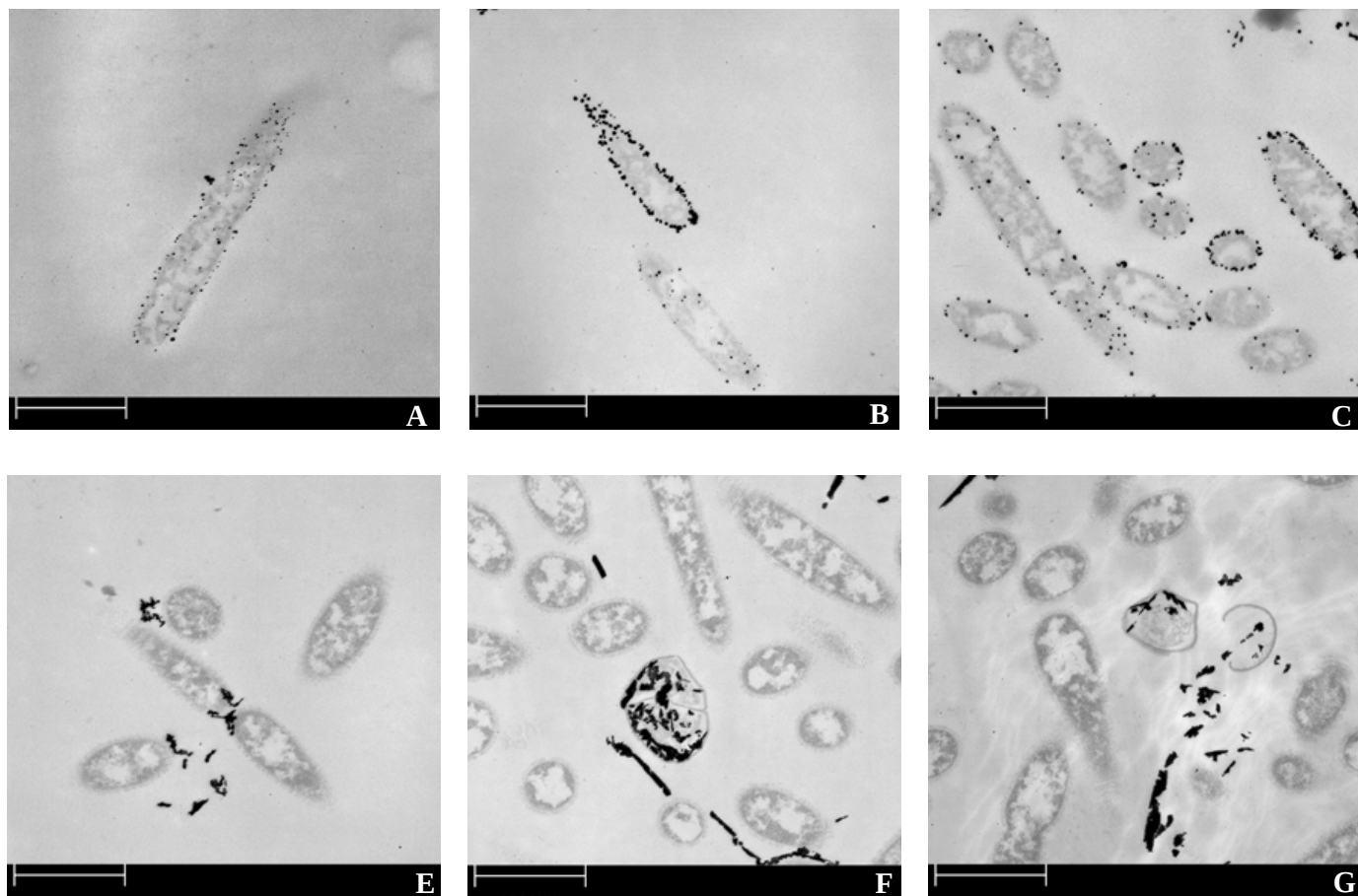


Figure 3.12 TEM micrographs illustrating the effect of gold(III) concentration (A) 0.5 (B) 1 (C) 1.5 (D) 2 (E) 5 (F) 7.5 and (G) 10mM on particle formation using cells under resting conditions. Scale bars equal to 1000nm.

3.4.3.4. The effect of exposure time on particle production

The effect of exposure time on particle synthesis was evaluated as described in section 3.3.5.2.5. Smaller particles are produced when exposure time is limited and it seems as if particle formation occurs both on the cell wall and in the cytoplasmic regions (Figure 3.14. A and B). Particles produced on the cell walls might be due to chemical reduction as the cell walls contains sugars which has the ability to reduce gold(III) with subsequent nanoparticle formation (Gole *et al.*, 2001; Panigrahi *et al.*, 2004 and 2005). Particles produced inside the cell might be due to enzymatic reduction of the gold(III) but further experimentation is needed to elucidate the specific protein/s involved in gold(III) reduction and nanoparticle formation. An increase in exposure time led to an increase in particle size, suggesting that initial particles formed acts as nucleation site for particle growth (Mikheenko *et al.*, 2008; Tang and Hamley, 2009). Plasmon resonance of the particles can only be noted after prolonged exposure to the gold ions (Figure 3.13).

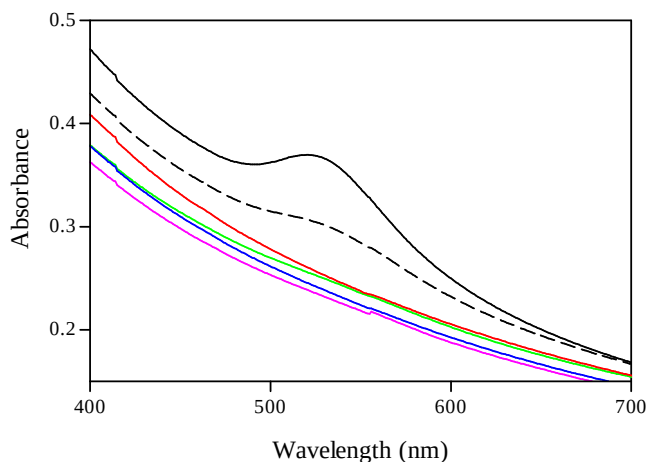


Figure 3.13 Wavelength scans conducted over time in the presence of 2mM gold(III) at 65°C. (—) 0; (—) 1; (—) 2; (—) 4; (---) 12 and (—) 24 hours.

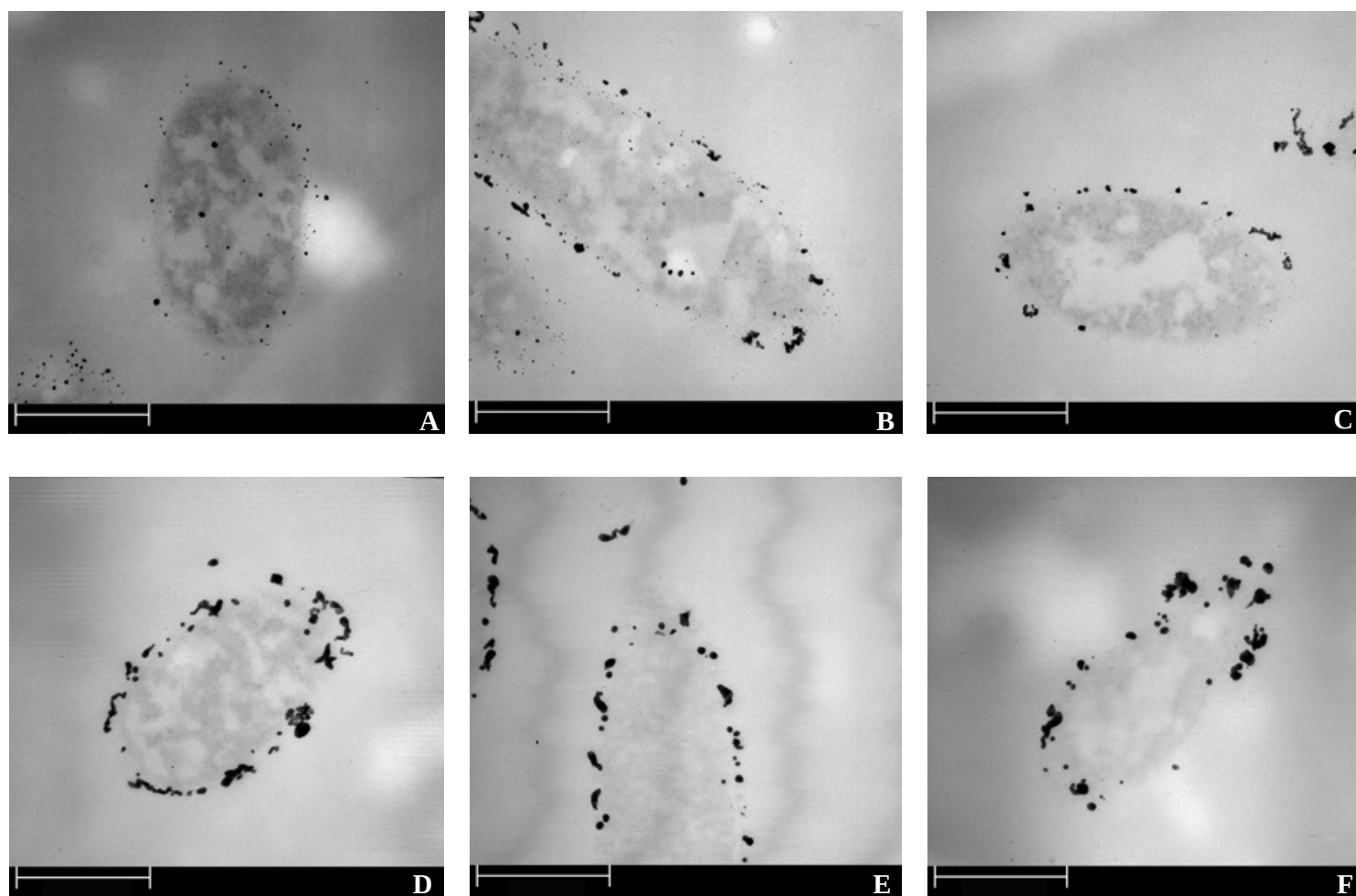


Figure 3.14 TEM micrographs illustrating the effect of exposure time (A) 1 (B) 2 (C) 4 (D) 12 (E) 24 and (F) 48h on particle formation using cells under resting conditions. Scale bars equal to 400nm.

3.5. Conclusions

Comparison of the mesophilic and thermophilic bacteria's abilities to reduce gold(III) and produced nanoparticles it can be concluded that the thermophilic bacteria are better equipped to deal with gold(III) reduction and nanoparticle production. This makes them better candidates for the industrial production of nanoparticles as they are heat stable, which will lower production costs as it eliminates the need for cooling processes. The thermophilic bacteria could also tolerate higher gold concentrations and were able to produce nanoparticles at a rate exceeding that of the mesophilic bacteria.

The ability of the chosen bacterium, *Thermus scotoductus* SA-01, to produce gold nanoparticles was affected by physico-chemical parameters. A variance in pH affected not only the size and shape of particles produced but also the amount (concentration) of particles formed per cell. This indicates that changes in this parameter could play an important role during optimization of processes controlling particle morphology, while incubation time might be used to manipulate the size of the particles. Gold reduction primarily occurred in the cell envelope which is strong evidence for a gold 'specific' reduction process.

Microorganisms are very complex structures with a variety of metabolic functions which all contribute towards the proper working of the cell. Many of these activities could be involved in the reduction of gold(III) and the formation of nanoparticles, but a better understanding of the mechanism might be obtained if the protein/s involved in these reactions are elucidated. The next chapter will discuss the purification and characterisation of a gold(III) reducing and nanoparticle producing protein from *Thermus scotoductus* SA-01.

3.6. References

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CHAPTER 4

4. PURIFICATION AND IDENTIFICATION OF THE NANOPARTICLE SYNTHESIZING PROTEIN

4.1. Introduction

The ability to produce gold nanoparticles with specific shapes and controlled sizes could result in interesting new applications that can potentially be utilised in areas such as optics, electronics and the biomedical field (Corti *et al.*, 2004). The benefits of using a biological system include the predictable production of uniform metal nanoparticles due to the highly structured activities of microbial cells, environmentally friendly production methods and the ability to produce nanoparticles with unique shapes and composition (Ahmad *et al.*, 2002; Mukherjee *et al.*, 2002; Sastry *et al.*, 2003). It is important to gain an understanding of the mechanism of gold ion accumulation and reduction on a molecular and cellular level. Identification and analysis of the biopolymers involved in the process could potentially allow for a process in a cell-free environment, where the size and shape of particles can be controlled by specific proteins/enzymes (He *et al.*, 2008). It would also be advantageous from a process point of view, since it would eliminate the need to harvest the nanoparticles formed within the cells, simplifying the process and lowering operation costs.

It is important to establish a reliable controlled protocol for the synthesis of nanoparticles, as size and shape play a vital role in their potential applications. Several biological systems have been researched to facilitate in producing these protocols (Ahmad *et al.*, 2002; Kumar *et al.*, 2007). Several hypotheses have been formulated about the mechanisms involved in nanoparticle synthesis. These are all based on enzymes with metal reductase (oxidoreduction) abilities (Duran *et al.*, 2005; He *et al.*, 2007).

4.1.1. Metal reductases

Many organisms require metallic elements at low concentrations for growth and function but at high concentrations, these metals become toxic. These organisms have developed several

mechanisms to detoxify their environments when these metals exceed the required concentrations, as well as means to solubilise insoluble metal compounds occurring in the natural environment prior to utilization. Utilization of iron, copper and manganese by microorganisms are dependent on a change in the valence state (Crane *et al.*, 1985; Wakatsuki *et al.*, 1988) and valencies of mercury and chromium are changed by reducing systems of cells in the process of detoxifying themselves (Horitsu *et al.*, 1983; Tonomura *et al.*, 1968). Reduction of heavy metals by microorganisms requires appropriate electron donors produced by specific energy metabolism systems. Microorganisms have electron transport systems in the plasma membrane and reduce heavy metals in the cell surface layer. Metal ion reductases are also found in the cytoplasm. These metal ion-specific reducing enzymes require NADH or NADPH as an electron donor and FMN or FAD as an electron carrier component. Oxidoreduction of metals are important for biological systems for metal-autoregulation and metal-mediated regulation. Microorganism can thus regulate movement of metals helping them to detoxify the environment as well as utilizing essential elements (Wakatsuki, 1995). The prokaryotes include organisms with the ability to oxidize manganese(II), iron(II), copper(I) or reduce manganese(IV), iron(III), cobalt(III) on a large scale and conserve energy from these reactions. Some microbes have the ability to reduce metal ions such as mercury and silver to their elemental counterparts, but does so without conserving energy from these reactions (Ehrlich, 1997).

4.1.2. Hypothesis of metal nanoparticle synthesis

The ability of microorganisms to reduce metals and produce nanoparticles have led to several hypotheses about the probable mechanisms involved in metal reduction and particle formation.

Rhospseudomonas capsulate was able to produce gold nanoparticles of varying shapes and sizes (He *et al.*, 2007). This bacteria is known to produce co-factor NADH- and NADPH-dependent enzymes that may be responsible for the bio-reduction of gold(III) to elemental gold with the subsequent production of nanoparticles. He and co-workers (2007) proposed a possible mechanism which is involved in the electron shuttling during enzymatic metal reduction processes (Figure 4.1) where NADH donates an electron which is used by the NADH-dependent reductase to reduce gold(III), however, the exact mechanism of electron

shuttling and the reduction of gold ions have not been discussed. Other studies also indicated that NADH- and NADPH-dependent enzymes are important factors during metal reduction and nanoparticle formation (Mukherjee *et al.*, 2002; Ahmad *et al.*, 2002; Senapati *et al.*, 2005). The same hypothesis was made about the production of silver nanoparticles using *Fusarium oxysporum* strains (Duran *et al.*, 2005). A nitrate-dependent reductase, specific to *Fusarium oxysporum*, was involved in the reduction of silver ions and the subsequent production of nanoparticles. This hypothesis was proven when Kumar and co-workers (2007) illustrated the involvement of an α -NADPH-dependent nitrate reductase purified from *Fusarium oxysporum*. Silver ions were reduced in the presence of nitrate reductase leading to the formation of a stable silver hydrosol.

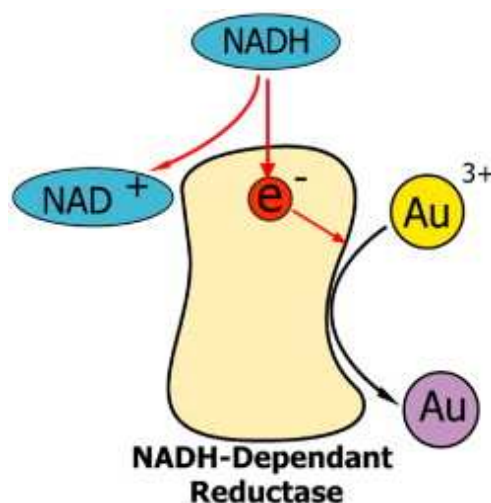


Figure 4.1 Possible mechanisms for bioreduction of gold ions. Taken from He *et al.*, 2007.

4.1.3. Other enzymatic production of metal nanoparticles

4.1.3.1. Hydrogenase

Sulphate-reducing bacteria have the ability to reduce metallic ions and the unspecified periplasmic hydrogenases of *Desulfovibrio desulfuricans* facilitate the reduction of technetium, palladium and uranium (Mikheenko *et al.*, 2008). It has been suggested that metal reductase activity seems to be a common feature of hydrogenases. Using wild-type and

hydrogenase-deficient *Desulfovibrio fructosivorans* strains, it was shown that palladium nanoparticles were only found in the regions containing hydrogenases, suggesting that hydrogenases act as the nucleation site and assist with initial palladium nanoparticle growth probably by supplying the electrons for palladium reduction. More proof that hydrogenases are involved in metal reduction was obtained when a hydrogenase from a consortium of sulphate-reducing bacteria was purified. This hydrogenase was able to reduce platinum in the presence of its electron carrier, cytochrome c_3 (Rashamuse *et al.*, 2008).

4.1.3.2. Sulphate reductase

Fusarium oxysporum can be used to produce CdS nanoparticles extracellularly by an enzymatic process. The partial purification of the excreted proteins from *Fusarium oxysporum* indicated a possible sulphate-reducing enzyme-based process for the synthesis of CdS starting from Cd^{2+} and SO_4^{-2} ions, a process which required NADH (Ahmad *et al.*, 2002). Extracellular proteins from *Fusarium oxysporum* has also been used to produce silver (Ahmad *et al.*, 2003) and gold nanoparticles (Mukherjee *et al.*, 2002).

4.1.3.3. Glutathione reductase

Scott and co-workers (2008) demonstrated that the mechanism of glutathione reductase can be harnessed to produce metallic nanoparticles. Glutathione reductase from *Escherichia coli* is a flavoenzyme of known structure. It is a homodimer with 450 amino acid residues (Mr 49560) and one FAD per subunit. It belongs to the family of FAD-dependent disulphide oxido-reductases (Mittl and Schultz, 1994). This enzyme is important in maintaining a reducing environment within the cell (Akerboom *et al.*, 1982) and catalyzes the NADPH-dependent reduction of oxidized glutathione *via* a disulphide exchange reaction involving two active site cysteine residues. Glutathione reductase accepts electrons from reduced NADPH into the noncovalently bound FAD to give the FADH_2 enzyme form. The electrons from FADH_2 are then transferred to an internal disulphide bond formed between the two cysteine residues in the active site. The reduced cysteines then catalyze the reduction of the oxidized glutathione *via* a disulphide exchange reaction.

Glutathione reductase catalyzes the NADPH-dependent reduction of gold(III) to form gold nanoparticles at the active site that are tightly bound through the catalytic cysteines (Figure 4.2). Scott and co-workers (2008) concluded, after several independent experiments, that gold(III) is reduced at the glutathione binding site of the glutathione reductase and that the catalytic cysteines are directly involved in the reduction. Nanoparticles produced in this manner had a relatively narrow size distribution (mean diameter of 2.13nm) [Scot *et al.*, 2008]. This could be due to a decrease in the rate of gold(III) reduction with an increase in particle size. The enzymes with fewer atoms of reduced gold will preferentially reduce the remaining gold ions resulting in a rather even distribution of nanoparticle size. These particles separate from the enzyme upon the addition of a thiol.

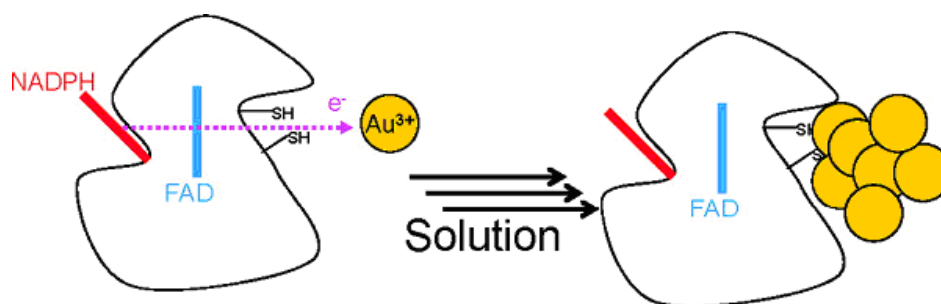


Figure 4.2 Synthesis of gold nanoparticle at the glutathione reductase active site. Taken from Scott *et al.*, 2008.

From the previous chapter it can be concluded that *Thermus scotoductus* SA-01 has the ability to reduce gold ions, with the subsequent production of nanoparticles. It was also shown that particle morphology was dependent on physico-chemical parameters such as pH, temperature and gold ion concentration. According to literature an oxido-reductase enzyme is expected to be involved in the reduction of gold(III) leading to particle formation. This part of the study was done to:

1. Purify and identify the protein(s) involved in gold(III) reduction and nanoparticle formation.

2. Characterize the protein(s) directly responsible for gold(III) reduction and nanoparticle formation by varying the physico-chemical parameters.

4.2. Materials and methods

4.2.1. Microorganism

Thermus scotoductus SA-01 was cultured as described in section 3.3.5.1.

4.2.2. Preparation of subcellular fractions

The supporting skeleton of the bacterial cell wall consists of a regular polymer, the peptidoglycan, murein. This macromolecule is a heteropolymer made up of chains of alternating molecules of N-acetylglucosamine (GlcNAc) and its lactic acid ester, N-acetyl muramic acid (MurNAc) linked by 1,4- β -glycosidic bonds. These heteropolymers are straight chains without branching and form the backbone of murein. In gram-negative bacteria the murein network consists as a single layer. Attached to the outer surface of the murein skeleton are large quantities of lipoproteins, lipopolysaccharides and other lipids. This lipopolysaccharide layer appears to require calcium ions to maintain its stability; removal of the calcium ions by treatments with EDTA liberates the lipopolysaccharides which make the murein layer accessible to degradation by the enzyme lysozyme. Lysozyme is an acetyl muramidase that attacks the glycosidic bonds between the C-1 atom of N-acetylmuramate and the C-4 atom of N-acetylglucosamine. Lysozyme thus degrades murein to GlcNAc-MurNAc (Schlegel, 1997). The first step, in most fractionation methods, is the removal of the cell wall by treatment with lysozyme in the presence of EDTA.

Subcellular fractions were prepared as described by Kaufmann and Lovley (2001). *Thermus scotoductus* SA-01 was grown as described in section 3.3.5.1. The cells were resuspended in a 1:20 (w/v) ratio in 50mM sodium acetate buffer, pH 5.0, containing 25% (w/v) sucrose. To accomplish cell wall lysis, lysozyme (1mg.ml⁻¹) was added to the cell suspension and stirred for 20 minutes. Na₂-EDTA was added to a final concentration of 5mM and stirred for another 20 minutes. Finally MgCl₂ was added to a final concentration of 13mM and the suspension was stirred for another 20 minutes. Separation of the spheroplasts

from the periplasmic fractions was obtained by centrifugation (20000x *g*; 30min; 4°C). Spheroplasts were resuspended in 50mM sodium acetate buffer, pH 5.0.

To obtain the membrane and soluble fractions, a protocol adapted from Gaspard and co-workers (1998) was used. DNase (10µg.ml⁻¹) was added to the EDTA-lysozyme-treated cell suspension, and the cells broken by ultrasonic treatment. Sonification consisted of cycles of 60 seconds burst with 30 seconds resting time. This was done at 75% output and done six times using a sonifier (Bandelinn Sonopuls) in an ice-water bath. The suspension was centrifuged (4000x *g*; 10min; 4°C) to remove the cellular debris (pellet). The supernatant was subjected to ultra-centrifugation, using an Optima™ L-100 Ultracentrifuge (Beckman Coulter) (100000x *g*; 90min; 4°C) to separate the soluble proteins (supernatant) and the membranes (pellet). The pellet was resuspended in a 1:5 (w/v) ratio in 50mM sodium acetate buffer, pH 5.0. All the fractions obtained were assayed for gold reducing activity as described in section 4.2.6.1.

The soluble fraction of *Thermus scotoductus* SA-01 was separated into different fractions using an Amicon stirred cell (model 8050) fitted with a membrane (Osmonics Ultra Sep Disc. UF 10 and 30kDa NMWC). The soluble fraction was first filter through a 30kDa cut-off membrane, and the filtrate, containing components smaller than 30kDa, was kept for further filtrations, while the retentate, containing components larger than 30kDa was washed four times with 50ml 50mM sodium acetate buffer, pH 5.0. The filtrate was then filtered through a 10kDa cut-off membrane, and the new filtrate, containing components smaller than 10kDa, was kept for further analysis, while the retentate, containing components sized between 10 and 30kDa, was washed four times with 50ml 50mM sodium acetate buffer, pH 5.0. All the fractions obtained were assayed for gold(III) reducing activity as described in section 4.2.6.1 and subjected to SDS-PAGE and TEM analysis as described in sections 4.2.4 and 4.2.7.1.

4.2.3. Protein determination

Protein concentrations were determined using the bicinchoninic acid (BCA) method (Smith *et al.*, 1985). The Pierce BCA protein assay reagent is a highly sensitive reagent for the spectrophotometric determination of protein concentration. The commercially available kit from Pierce was used according to the manufacturer's instructions. Standard curves relating

protein concentration to absorbance at 562nm were constructed (Figure 4.3). A set of protein standards was prepared with bovine serum albumin (BSA). Fifty microliters of each standard / unknown sample was pipetted into an eppendorf (50 μ l of diluent was used for blanks); 1ml of working reagent was added to each tube and vortexed. Tubes were incubated at either 37°C (Figure 4.3 A) or 60°C for 30 minutes (Figure 4.3 B) and the absorbance read at 562nm using a GENESYS 5 (Spectronic) spectrophotometer. Protein concentrations between 125 and 1000 μ g.ml⁻¹ could be detected using the incubation at 37°C, while protein concentrations between 0 and 250 μ g.ml⁻¹ could be detected using the 60°C incubation.

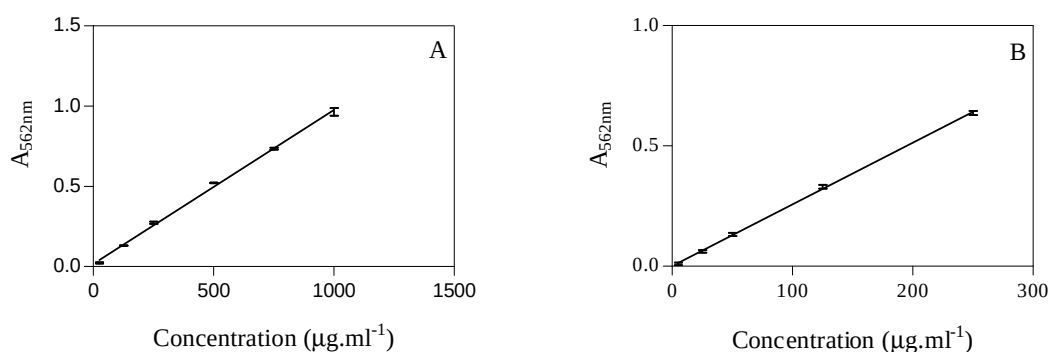


Figure 4.3 Standard curve for the BCA-protein assay with BSA as the protein standard for the eppendorf protocol at (A) 37 and (B) 60°C. Error bars indicate standard deviations, but are smaller than the symbols used.

4.2.4. SDS-PAGE analysis

The homogeneity of the proteins present in each fraction was determined by electrophoresis under denaturing conditions or sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed using the “Mighty Small” miniature slab gel electrophoresis unit, SE 200, from Hoefer Scientific Instruments. The protocol used was that described by Laemmli (1970) using a 10% resolving gel and 4% stacking gel. Precision Plus Protein Standards (BIO-RAD) was used as molecular weight markers in SDS-PAGE, and proteins were visualized by staining polyacrylamide gels with either Coomassie- (Fairbanks *et al.*, 1971) or silver nitrate (Rabilloud, 1988).

4.2.5. Liquid chromatography

Purification of the protein(s) involved in nanoparticle formation is important to understand the synthesis mechanism, which could supply a means to manipulate particle characteristics during synthesis. As co-factor dependency may influence the ability of the enzyme to function, an alternative activity was chosen to assist purification of the gold(III) reducing and nanoparticle producing protein. Nitrate reduction was chosen based on the results obtained by Kumar co-workers (2007) where it was shown that a purified nitrate reductase from *Fusarium oxysporum* had the ability to produce silver nanoparticles. See supplementary notes (section A.1).

All chromatography steps were carried out at room temperature and enzyme preparations were stored at -20°C without loss of activity. Purification to homogeneity entailed six chromatography steps: ion-exchange, dye-affinity, hydrophobic interaction and size-exclusion chromatography, using the Akta Prime Purification System (GE Health).

4.2.5.1. Ion exchange chromatography

Different strong and weak ion exchange resins were tested for the ability to separate the gold(III) reducing proteins from the non-reducing proteins. CM-Toyopearl 650M (TosoHaas) and SP-Toyopearl 650M (TosoHaas) were chosen for cation exchange, while DEAE-Toyopearl 650M (TosoHaas) and Super Q-Toyopearl 650S (TosoHaas) were chosen for anion exchange. The resins were washed and equilibrated according to the manufacturer's instructions. The column was loaded with protein (1ml) from fractions >30kDa. The non-interacting compounds were removed by washing the column with 50mM sodium acetate buffer, pH 5, for the cation exchangers and 50mM sodium phosphate buffer, pH 7.4, for the anion exchangers. The columns were then washed with 0.5M NaCl in the appropriate buffer followed by washing with 1M NaCl in the same buffer. All the resulting fractions were assayed for gold(III) reducing abilities as described in section 4.2.6.1.

4.2.5.1.1. Anion exchange

Super Q-Toyopearl exchange resin column (2.5 x 7cm) was used as the first purification step. The resin was washed and equilibrated according to the manufacturer's instructions with a flow rate of $5\text{ml}\cdot\text{min}^{-1}$. The $>30\text{kDa}$ fraction in sodium phosphate buffer (50mM; pH 7.4) was loaded onto the column and the proteins that had no interaction with the column were eluted with 50mM sodium phosphate buffer, pH 7.4. Binding proteins were eluted with a linear gradient of 0 – 0.7M NaCl in the same buffer. Fractions were assayed for nanoparticle formation as described in section 4.2.6.1 and for TEM analysis at 24h as described in section 4.2.7.1, and SDS-PAGE analysis (section 4.2.4).

4.2.5.1.2. Cation exchange

SP-Toyopearl column (2.5 x 7cm) was used as the second purification step. The resin was washed and equilibrated according to the manufacturer's instructions with a flow rate of $5\text{ml}\cdot\text{min}^{-1}$. The chosen fractions (Figure 4.10 peak A-A), obtained from the Super Q column, were pooled and dialysed against 50mM sodium acetate buffer, pH 5.0. The sample was loaded onto the column and the proteins that had no interaction with the column were eluted with 50mM sodium acetate buffer, pH 5.0. The interactive proteins were eluted with a linear gradient of 0 – 1M NaCl in the same buffer. Fractions were assayed for nanoparticle formation as described in section 4.2.6.1. The nitrate reducing ability was also evaluated as described in section 4.2.6.2. Samples were prepared for TEM analysis at 24h as described in section 4.2.7.1 and subjected to SDS-PAGE analysis (section 4.2.4).

The active fractions were pooled, dialysed against 50mM sodium acetate buffer pH 5.0 and again loaded onto the SP-Toyopearl ion-exchange resin. The proteins that had no interaction with the column was eluted with 50mM sodium acetate buffer, pH 5.0. The interactive proteins were eluted with a linear gradient of 0 – 0.3M NaCl in the same buffer. Selected fractions were assayed for gold- (section 4.2.6.1) and nitrate reducing (section 4.2.6.2) activity and subjected to SDS-PAGE analysis (section 4.2.4).

4.2.5.2. Dye-affinity chromatography

The active fractions, obtained from the SP-Toyopearl column, were pooled and dialysed against 50mM sodium phosphate buffer, pH 7.4 and loaded onto a regenerated Blue Sepharose CL-6B (Sigma-Aldrich) column (1.5 x 13cm) at a flow rate of 2ml.min⁻¹. The non-interacting proteins were eluted with 50mM sodium phosphate buffer, pH 7.4, and the interacting proteins were eluted with a linear gradient of 0 – 0.6M NaCl in the same buffer. Selected fractions were assayed for gold- (section 4.2.6.3) and nitrate reducing (section 4.2.6.2) activity as well as being subjected to SDS-PAGE analysis (section 4.2.4).

4.2.5.3. Hydrophobic interaction chromatography (HIC)

The active fractions, obtained from the dye-affinity column, were pooled and dialysed against 50mM sodium phosphate buffer, pH 7.4. Ammonium sulphate, to a concentration of 1M, was added to the protein solution and loaded onto the Phenyl-Toyopearl (TosoHaas) column (2.5 x 7cm) at a flow rate of 5ml.min⁻¹. The interacting proteins were eluted from the resin using a 1 – 0M (NH₄)₂SO₄ linear gradient in 50mM sodium phosphate buffer, pH 7.4. Selected fractions were assayed for both nitrate- (section 4.2.6.2) and gold(III) [section 4.2.6.3] reduction as well as being subjected to SDS-PAGE analysis (section 4.2.4).

4.2.5.4. Size-exclusion chromatography

The final purification step was size-exclusion chromatography. The active fractions, obtained from the HIC column were pooled, dialysed against 50mM sodium phosphate buffer, pH 7.4 and concentrated to 3ml. The sample was loaded onto the Sephacryl S200HR (Sigma-Aldrich) column (2.5 x 65cm) after the addition of ~5% glycerol at a flow rate of 1ml.min⁻¹. The proteins were eluted with 50mM sodium phosphate buffer, pH 7.4, containing 50mM NaCl. The resulting peak was assayed for nitrate- (section 4.2.6.2) and gold reducing activity (section 4.2.6.1) and subjected to SDS-PAGE analysis (section 4.2.4).

4.2.6. Activity determination

The plasmon resonance band associated with the gold nanoparticles was obtained for each reaction by collecting a UV-Vis spectrum using a Beckman DU-800 spectrophotometer. This was done to verify the presence of gold nanoparticles.

4.2.6.1. Gold assay

The gold(III) reducing ability of the protein solutions was evaluated by adding 2 μ l 100mM gold(III) to 100 μ l protein solution and incubating at 65°C for 24h.

4.2.6.2. Nitrate reductase assay

For the assay of nitrate reductase, 100 μ l sample was added to the following reaction mixture: 100 μ l 50mM sodium phosphate buffer, pH 7.4, 100 μ l 200 μ M sodium nitrate, 100 μ l 1 μ M methyl viologen and 500 μ l distilled water. The reaction was started by adding 100 μ l 46 μ M sodium dithionite, dissolved in 100 μ M sodium hydrogen carbonate, to the reaction mixture. After slowly inverting the mixture it was incubated at 65°C for 10 minutes, the reaction was stopped by shaking the tube to oxidize the remaining dithionite and reduced methyl viologen. To determine the nitrite concentration, two parts of 4% sulfanilamide in 25% HCl solution was mixed with one part of 0.08% N-1-naphthylethylene-diamine dihydrochloride; 300 μ l of this mixture was added to the nitrite containing sample. After 10 minutes, the absorbance was read at 540nm against a reagent blank using a GENESYS 5 (Spectronic) spectrophotometer (Showe and DeMoss, 1968). See supplementary notes (section A.1).

4.2.6.3. Adapted gold-nitrate reductase assay

To determine if the protein solutions had gold(III) reducing and nanoparticles producing capabilities, the nitrate reductase assay described in section 4.2.6.2 was modified. Hundred microliters sample was added to the following reaction mixture: 100 μ l 50mM sodium phosphate buffer, pH 7.4, 100 μ l 20 μ M gold(III), 100 μ l 1 μ M methyl viologen and 500 μ l distilled water. The reaction was started by adding 100 μ l 46 μ M sodium dithionite, dissolved

in 100 μ M sodium hydrogen carbonate, to the reaction mixture. After slowly inverting the mixture it was incubated at 65°C for 24h, the reaction was stopped by shaking the tube to oxidize the remaining dithionite and reduced methyl viologen. The formation of a pink to purple colour indicated gold nanoparticle production (Showe and DeMoss, 1968; Lazarides and Schatz, 2000). No significant colour change was observed for the control reactions.

4.2.7. Nanoparticle characterization using either cell-free extracts or the purified protein

4.2.7.1. Electron microscopy

TEM analysis of the cell-free extracts and protein samples were carried out on carbon coated formvar grids. The samples were washed twice with and resuspended in distilled water. A drop of the sample was placed on the grid and removed after a minute using blotting paper, and the grid air-dried before analysis (van Wyk and Wingfield, 1991). Electron micrographs were taken with either a Philips CM 100 or 200kV Philips CM 20 TEM operating at 60kV. Elemental analysis on the particles was carried out using energy dispersive spectroscopy (EDS) coupled to the 200kV Philips CM 20 TEM. Care was taken to remove all salts but some crystals might have been formed thus care has to be taken not to over interpret results without EDS results.

4.2.7.2. The effect of Gold(III) concentration on particle production

The effect of gold(III) concentration on particle formation using cell-free extracts were evaluated by incubating the extracts in gold(III) concentrations varying from 0 – 20mM. The extracts were incubated for 24h in the desired gold(III) concentration in 50mM sodium acetate buffer, pH 5.0. The samples were prepared for TEM analysis as described in section 4.2.7.1.

For the purified protein, the adapted nitrate reductase assay (Section 4.2.6.3) was used to determine the effect of gold(III) concentration on nanoparticle production. The reactions containing 100 μ g.ml⁻¹ protein were incubated at 65°C in 50mM sodium phosphate buffer, pH 7.4 with gold(III) concentrations ranging from 0 – 10mM for 24 hours. The samples were prepared for TEM analysis as described in section 4.2.7.1. A hypothesis of the mechanism of

gold(III) reduction was slowly forming by calculating the stoichiometry by converting the protein, as well as, the gold(III) concentrations to μM values.

4.2.7.3. The effect of temperature on particle production

The effect of temperature on the ability of the purified protein to produce nanoparticles was evaluated by using the adapted nitrate reductase assay (Section 4.2.6.3). The reactions containing $100\mu\text{g.ml}^{-1}$ protein were incubated at temperatures ranging from 30 – 75°C in 50mM sodium phosphate buffer, pH 7.4 with 2mM gold(III) for 24 hours. The samples were prepared for TEM analysis as described in section 4.2.7.1.

4.2.7.4. The effect of pH on particle production

The effect of pH on the ability of the purified protein to produce nanoparticles was evaluated by using the adapted nitrate reductase assay (Section 4.2.6.3). The reactions containing $100\mu\text{g.ml}^{-1}$ protein were incubated at 65°C with 2mM gold(III) in pH buffers ranging from 3.6 – 9.5 for 24 hours. The samples were prepared for TEM analysis as described in section 4.2.7.1. Usually pH characterization is conducted using a buffer cocktail containing equimolar concentrations of the components, but the cocktail had a high chemical reduction rate, thus individual buffers were used. Sodium acetate buffer (50mM) was used for the range below pH 6, 50mM sodium phosphate buffer for the neutral pH range and 50mM borax buffer for the alkaline pH range.

4.2.7.5. The effect of protein concentration on particle production

The effect of protein concentration on the ability of the purified protein to produce nanoparticles was evaluated by using the adapted nitrate reductase assay (Section 4.2.6.3). The reactions were incubated at 65°C in 50mM sodium phosphate buffer, pH 7.4 with 2mM gold(III) containing protein concentrations ranging from 10 - $100\mu\text{g.ml}^{-1}$ for 24 hours. The samples were prepared for TEM analysis as described in section 4.2.7.1. A hypothesis of the mechanism of gold(III) reduction was slowly forming by calculating the stoichiometry by converting the protein, as well as, the gold(III) concentrations to μM values.

4.2.7.6. The effect of exposure time on particle production

The effect of exposure time on the ability of the purified protein to produce nanoparticles was evaluated by using the adapted nitrate reductase assay (Section 4.2.6.3). The reactions containing $100\mu\text{g.mL}^{-1}$ protein were incubated for 2 – 48 hours at 65°C in 50mM sodium phosphate buffer, pH 7.4 with 2mM gold(III). The samples were prepared for TEM analysis as described in section 4.2.7.1.

4.2.7.7. The effect of sodium dithionite concentration on particle production

The effect of sodium dithionite concentration on the ability of the purified protein to produce nanoparticles was evaluated by using the adapted nitrate reductase assay (Section 4.2.6.3). The reactions containing $100\mu\text{g.mL}^{-1}$ protein were incubated at 65°C in sodium phosphate buffer, pH 7.4 with 2mM gold(III) for 24 hours. Sodium dithionite concentrations varied from 0 – 100 μM . The samples were prepared for TEM analysis as described in section 4.2.7.1. Understanding the importance of stoichiometry relating the protein concentration to the sodium dithionite concentration was important (Supplementary notes A.2).

4.2.8. N-terminal sequencing

The 4.4mg of the purified protein was freeze dried and sent to the Protein Chemistry Facility of the Centro de Investigaciones Biológicas (CSIC; Madrid, Spain). N-terminal sequencing was performed by automated Edman degradation with an Applied Biosystems 477A gas-phase sequencer (Foster City, California).

4.3. Results and discussion

4.3.1. Cellular fractionation

Through fractionation (section 4.2.2) the cells were separated into the soluble components and the membrane fraction. All the resulting fractions were incubated in the presence of gold(III) as described in section 4.2.6.1. The soluble fraction turned purple within 24h,

which corresponded to the disappearance of the gold(III) from solution while it took the membrane fraction 48h to remove the gold(III). Protein analysis (section 4.2.4) of these fractions indicated a difference in protein composition between the soluble fraction and the membrane fraction (Figure 4.4), which could explain the difference in the ability of these fractions to produce nanoparticles and that there might be more than one protein present with the ability to reduce gold(III).

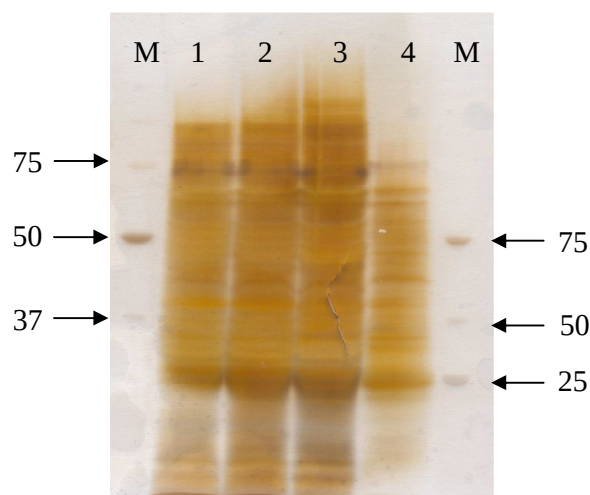


Figure 4.4 SDS-PAGE of Thermus scotoductus SA-01 extracted and separated protein fractions. (M) marker, (1) spheroplasts (2) cell-free extract, (3) soluble fraction and (4) membrane fraction.

TEM micrographs taken of the cell-free extract (section 4.2.7.1) after exposure to 2mM gold(III) for 4h illustrated the formation of various amorphous particles which vary in both size and shape. Some of the shapes observed are shown in Figure 4.5 (A to C), with no specific shape being prominent. Micrographs (Figure 4.6 A to C) of the soluble fraction illustrated an improvement of particle shape. Although these particles are still not perfectly shaped, they are much better developed than the particles obtained for the cell-free extract. This could be due to the removal of interfering substances. Initially 4h was chosen as incubation time for the reduction reactions as a colour change was already visible at this stage; however further reductions experiments were incubated for 24h as better colour development was observed after 24h than after 4h. Also visible are nanoparticles with sheet-

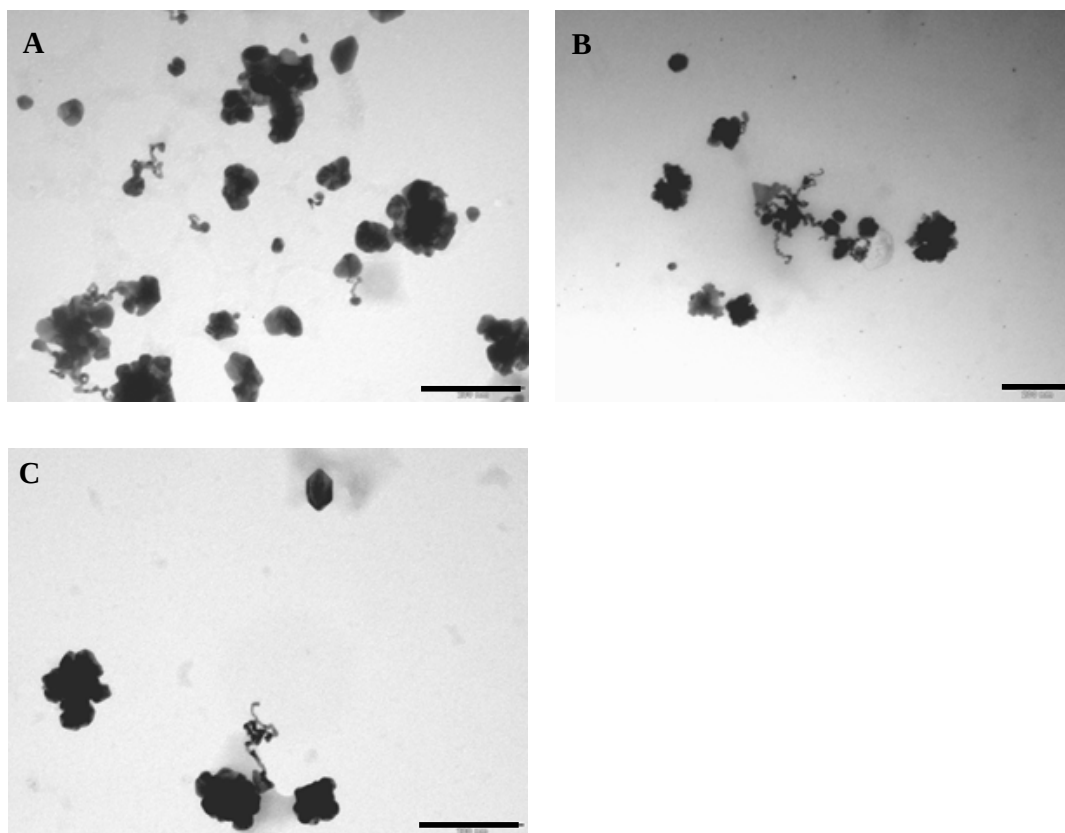


Figure 4.5 TEM micrographs of the cell-free extract after 4h exposure to 2mM gold(III) at 65°C, showing nanoparticles. Scale bars are equal to 200nm.

like morphologies. Gold nanosheets have attracted much attention because of their unique optical properties and their potential in gas sensors, in the fabrication of nanodevices and substrate materials (Li *et al.*, 2006; Kim *et al.*, 2004; Shankar *et al.*, 2005). The exact mechanism of nanosheet formation is not fully understood. When Wei and co-worker (2007) used chitosan to reduce gold ions they found that when the gold ions reached supersaturation they started to nucleate and grow and when the chitosan concentration was favourable the polar groups adsorbed preferentially on the sites of the {111} planes of the gold nuclei which leads to preferential growth along the $\langle 110 \rangle$ direction (Sun *et al.*, 2005). This leads to the formation of gold nanosheets with triangular, hexagonal or truncated shapes. When the chitosan concentrations were too high the growth of different crystal faces were induced and they suggested that the formation of large nanoplates were dependent on the chitosan to gold ion ratio. Similar conclusions were made by Li and co-workers (2006) when gold nanosheets

have been synthesized in large quantities through a solution phase process and by Shankar and co-worker (2005) when gold nanotriangles were synthesized using lemongrass extract.

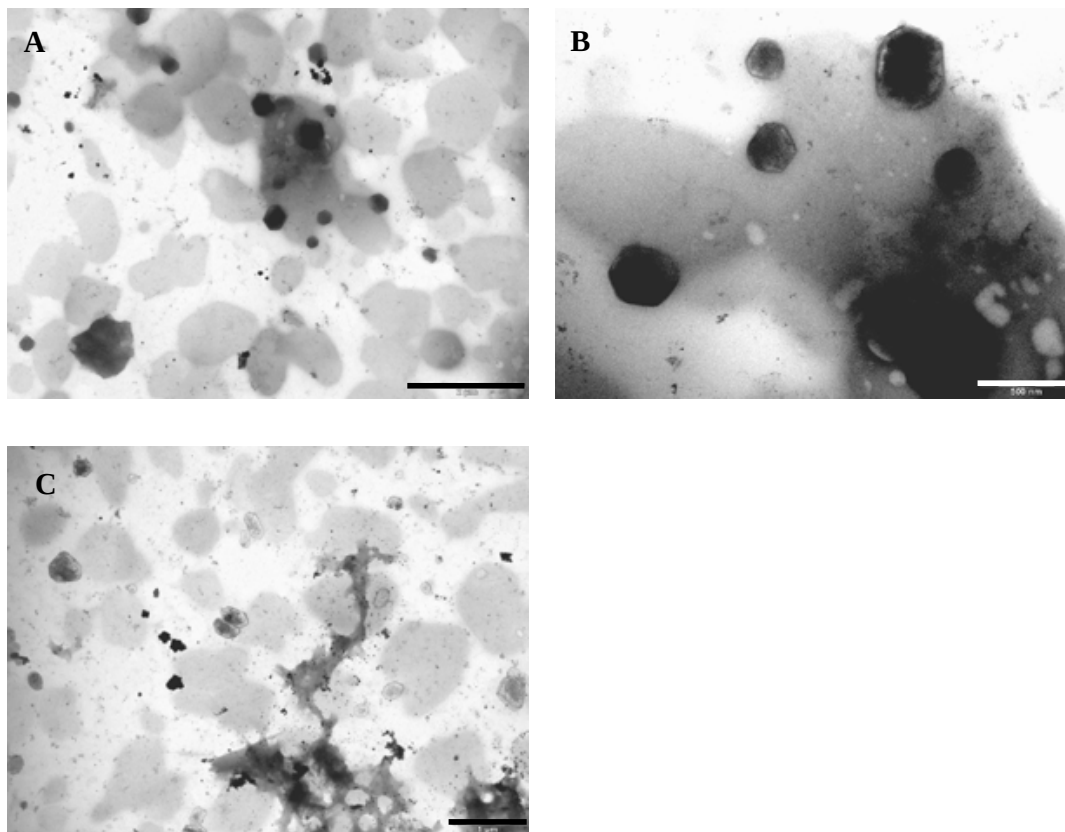


Figure 4.6 TEM micrographs of the soluble fraction after 4h exposure to 2mM gold(III) at 65°C, showing nanoparticles. Scale bars are equal to (A) 2μm (B) 500nm and (C) 1μm.

Size fractionation of the soluble fraction resulted in a fraction containing components larger than 30kDa, a fraction containing components sized between 10 and 30kDa and a fraction containing components smaller than 10kDa. The visual assay (section 4.2.6.1) for nanoparticles displayed different colours for these fractions (Figure 4.7) and it may be concluded that there is a difference in the size, and possibly the shape, of the nanoparticles formed (Underwood and Mulvaney, 1994). The fraction containing components sized between 10 and 30kDa showed no colour change, indicating no reduction and nanoparticle formation, while the fractions containing components smaller than 10kDa and larger than

30kDa had different colours ranging from pink to purple. The fraction containing components larger than 30kDa was concentrated 10 times to increase the reducing potential, however to ensure the removal of contaminating components, for example remaining organic acids and carbohydrates which have the ability to act as reducing agents and produce nanoparticles (Handley, 1989), the fraction was dialysed (SnakeSkin 7000 MWCO; Pierce) with a buffer change overnight against 50mM sodium acetate buffer, pH 5.0. This fraction was used to determine the effect of gold(III) concentration on gold nanoparticle formation as described in section 4.2.7.2.

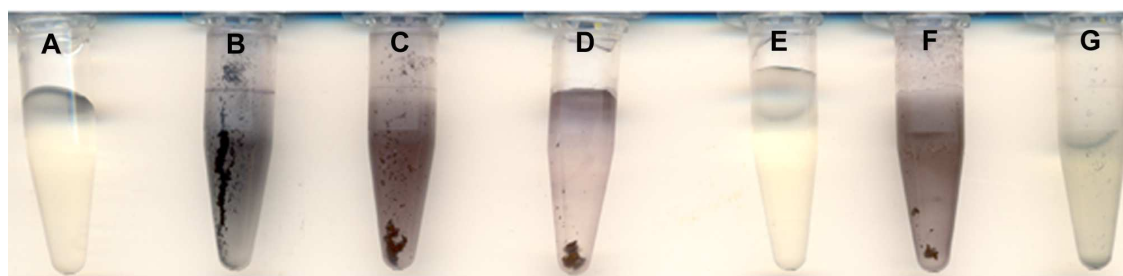


Figure 4.7 The cell-free extract and the ultrafiltered soluble fractions were exposed to 2mM gold(III) for 24h at 65°C. (A) Blank (B) cell-free extract (C) soluble fraction (D) fraction containing components <10kDa (E) fraction containing components >10<30kDa (F) fraction containing components <30kDa and (G) fraction containing components >30kDa. All the fractions were diluted to the original volume to maintain the protein ratios.

Representative micrographs (Figure 4.8) taken after 24h of exposure to varying gold(III), as described in section 4.2.7.2, illustrated that the particles synthesized varied in shape and size. A mixture of small amorphous particles and nebula-like formations were observed for the 2 and 5mM concentrations (Figure 4.8 A). Addition of 10 and 20mM gold(III) resulted in the presence of very large particles, with definite crystalline surfaces (Figure 4.8 B and C). These particles had better shape definition than the lower concentrations, morphologies were nanosheet-like, with shapes being mostly triangular and hexagonal. A colour difference between the different concentrations was also visible after incubation as illustrated in Figure 4.9, confirming that colour is indicative of particle size, with the pinkish colour representing smaller particles than the purplish colour. These results give an indication that gold(III)

concentration might be a physico-chemical parameter with the ability to manipulate the size of the particles produced (Wei *et al.*, 2007). Analysis done on the nanosheets formed during extract incubation with 20mM gold(III) indicated that the nanosheets were crystalline with no twinning present, and that the herring motif was most prominent. The triangular nanosheets showing three-fold symmetry were in the $\{111\}$ orientation (Figure 4.8 C) and dark lines visible through the crystals are bend contours (Rodriguez-Gonzalez, 2006).

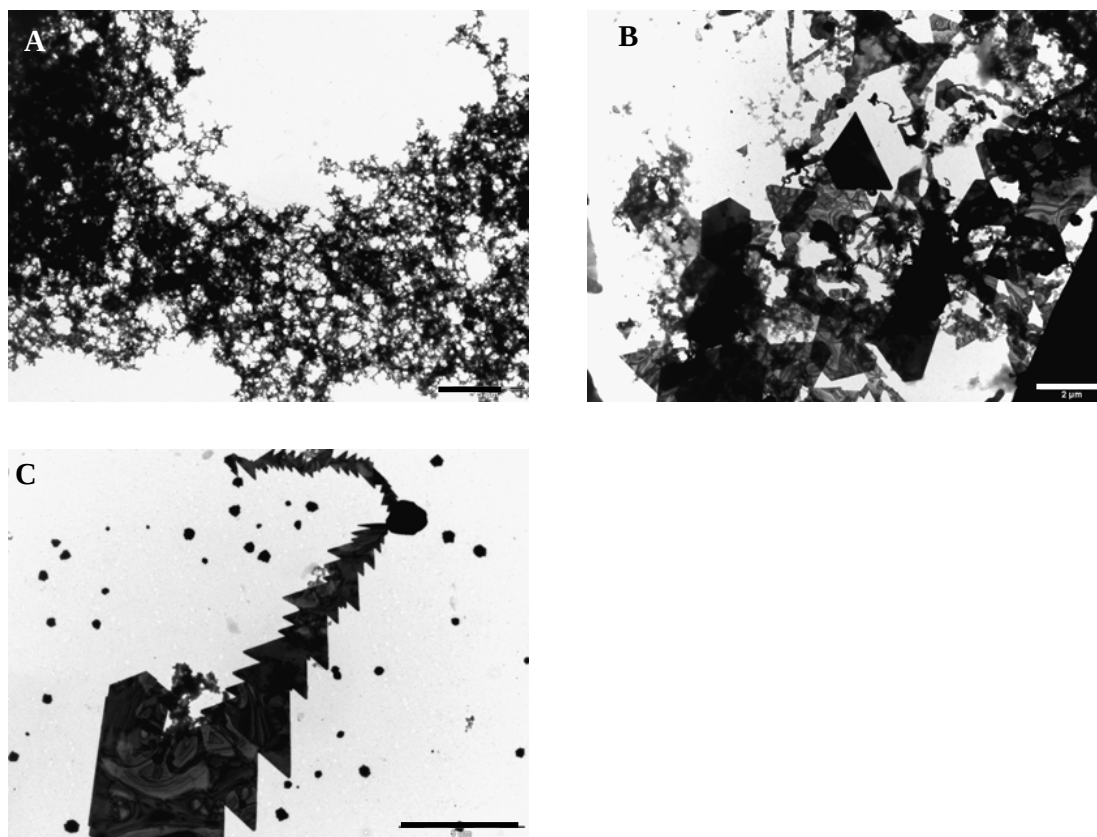


Figure 4.8 TEM micrographs showing the effect of gold(III) concentration (A) 2 (B) 5 (C) 10 and (D) 20mM, on nanoparticle formation in the presence of cell components >30kDa. Scale bars in A and D is equal to 5 μ m, and in B and C equal to 0.2 μ m and 2 μ m, respectively.

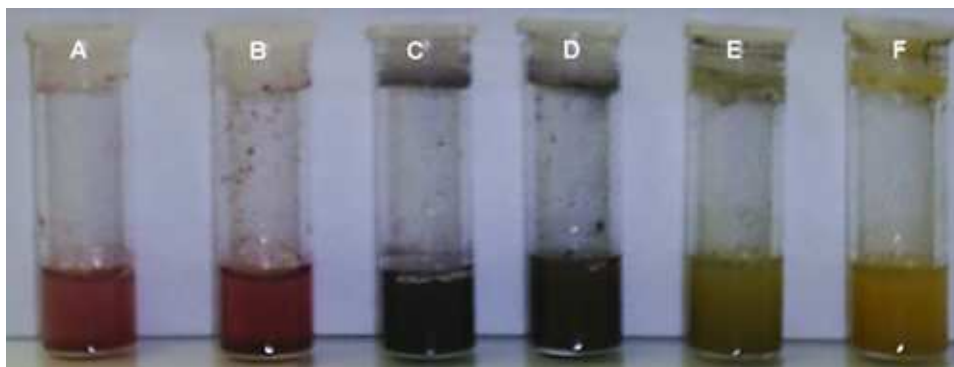


Figure 4.9 Exposure of the >30kDa fraction to various gold(III) concentrations after 24h at 65°C. (A) 0.5 (B) 1 (C) 2 (D) 5 (E) 10 and (F) 20mM.

4.3.2. Purification strategy

Organic acids and carbohydrates have the ability to act as reducing agents and produce gold nanoparticles (Handley, 1989). For this reason the fraction containing components sized larger than 30kDa, obtained from the fractionation experiments (section 4.2.2), was chosen for further purification and identification of the gold(III) reducing and nanoparticle producing protein.

4.3.2.1. Ion exchange

4.3.2.1.1. Anion exchange (Super Q-Toyopearl)

The elution profile obtained for the first step of purification using Super Q-Toyopearl (section 4.2.5.1.1) showed several protein peaks (Figure 4.10), the A_{280} readings can be correlated to protein concentration. There were many proteins not interacting with this resin (Figure 4.10 peak A-A) and especially since these fractions could also produce nanoparticles, these fractions were dialyzed against 50mM sodium acetate buffer, pH 5.0, concentrated and separated using the cation exchanger SP-Toyopearl (section 4.2.5.1.2). The peak eluting with the salt gradient (Figure 4.10 peak B-B) displayed activity as illustrated by the colour change in Figure 4.11 when the fractions were incubated in the presence of gold(III) [section 4.2.6.1], indicating that it is possible to separate the active proteins from each other. The colour between the different protein peaks also differed and might indicate a difference in the

size of the particles produced (Figure 4.11 blocks A and B). Peak C-C (Figure 4.10) displayed no gold reducing activity and was not subjected to further purification.

Subsequent purification of the pooled interacting fractions (Figure 4.10 peak B-B) using the HIC column Phenyl-Toyopearl resulted in the loss of the reducing potential of the proteins and was not subjected to further purification.

TEM micrographs of the various pooled fractions after each column step showed a variety in shape and size of the particles formed (Figure 4.12 A and B). Micrographs indicates there is a differences in particle quality and EDS analysis (Figure 4.12 C) showed that the particles in Figure 4.12 A consisted of gold and sodium. Other elements present in the EDS spectrum can be contributed to the grid, which consisted of copper, carbon and oxygen.

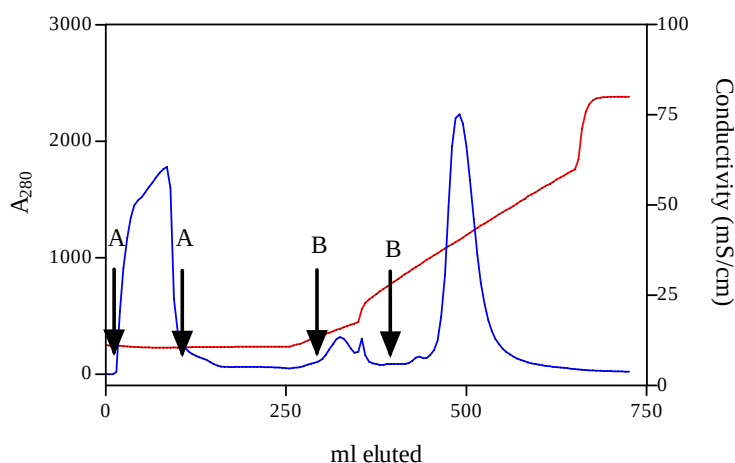


Figure 4.10 Elution profile obtained for the purification step on Super Q-Toyopearl anion exchange resin. The regions between the arrows (A-A) and (B-B) were pooled separately for subsequent purification. (—) A_{280} and (—) conductivity.

A typical gold nanostructure synthesis process can be summarized in four steps: (1) Shortly after the addition of the reducing agent the gold(III) is gradually reduced to gold(I). The yellow colour disappears and a translucent solution remains. (2) When the reduction of gold(III) to gold(I) is almost complete, the reduction of gold(I) to gold(0) begins. This causes

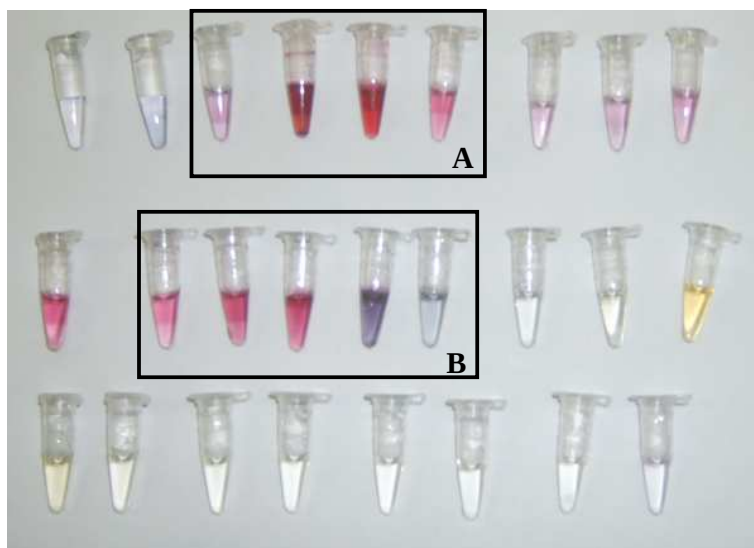


Figure 4.11 Visual assay of every third fraction obtained from the first step of purification, which had been incubated at 65°C in the presence of 2mM gold(III), showing a difference in colour between the fractions. Block A corresponds to peak A (Figure 4.10) and block B corresponds to peak B (Figure 4.10).

a nucleation process in the solution. (3) Nuclei grow gradually into plates when sufficient gold(I) ions are present. The stabilising agent attaches to the formed plates and directs crystallization, the solution turns a light orange colour. (4) In the final stages when almost all of the gold(I) ions have been consumed, the gold nanoplates are formed (Tang and Hamley, 2009). Variations to this process (using different reducing agents and combination of reducing agents and stabilisers) indicated that the reducing agents and the stabilisers can control the crystallization of gold and consequently yield special forms of gold crystals. Using different reducing agents and combination of reducing agents and stabilisers, different sized and shaped nanoplates were observed. Most of the shapes observed were derivatives of pseudotrigonal, octahedral gold (diamonds, pentagonal shields, isosceles trapezoids and rods). Correlating these findings to the particle shapes observed for the reduction experiments using the protein peak B (Figure 4.10) the most dominant shapes are diamonds and the rods (Figure 4.12 A and B) and that the proteins acts as reducing / stabilising agents. Experiments conducted by Kawamura and Nogami (2009) showed that different particles are produced depending on the initial type seed. When single crystalline and multiple twinned particles were used as seed particles, nanorods and nanobipyramids were produced.

However, it is very difficult to understand the relationship between the particle seed and the resulting nanostructures. During particle growth, the original seed structure may or may not be maintained, and the stabilising agents may influence the structural morphologies of the nanostructures (Seo *et al.*, 2008).

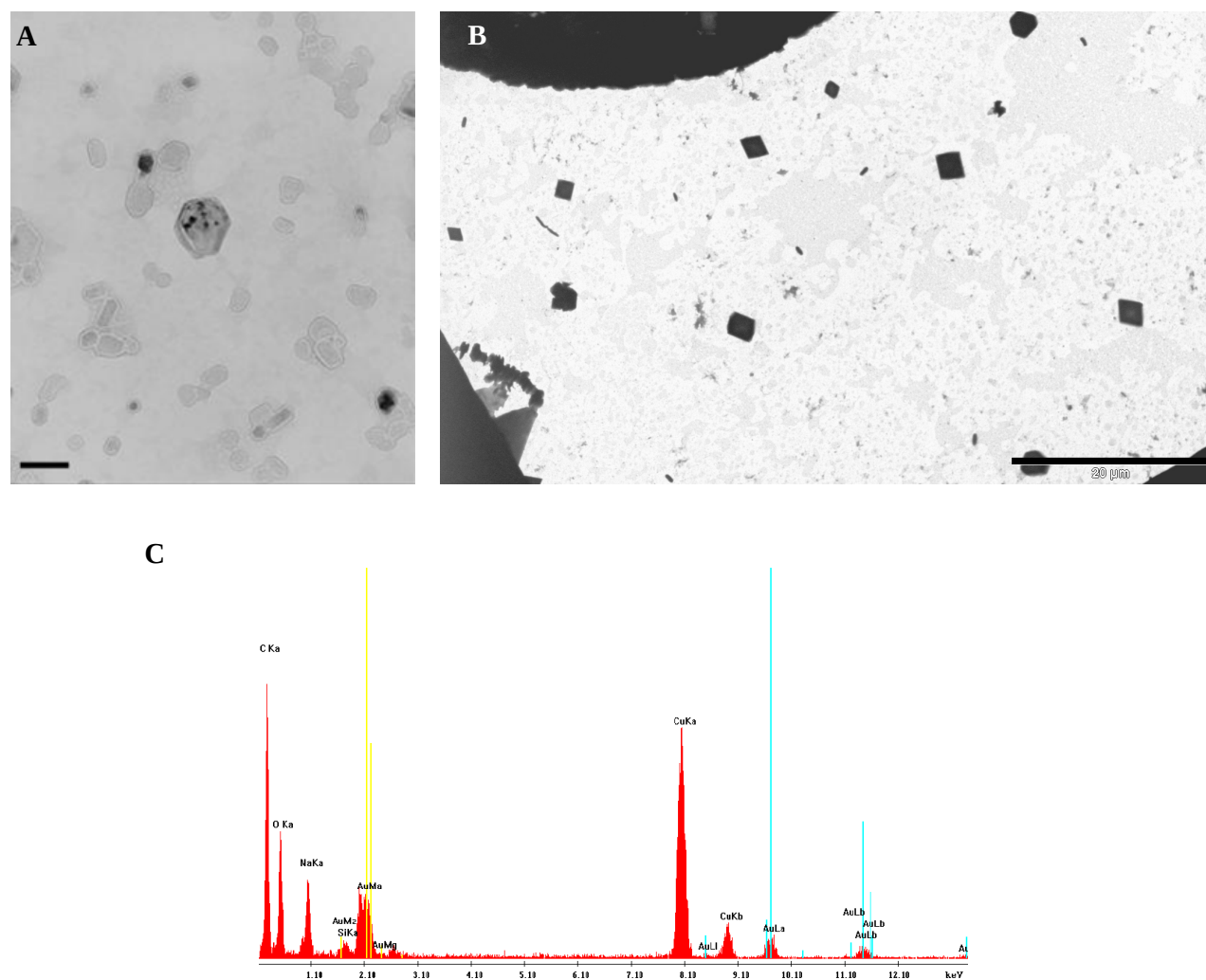


Figure 4.12 (A) and (B) TEM micrograph illustrating particle shape and (C) EDS spectrum for the particles produced by the interacting protein fraction obtained after the first step of purification using the anion exchanger Super Q. Scale bar equal (A) 100nm and (B) 20 μ m.

4.3.2.1.2. Cation exchange (SP-Toyopearl)

Second purification attempt: The non-interacting proteins collected during the anion purification attempt (Figure 4.10 peak A-A) was prepared for the next step of purification as described in section 4.2.5.1.2. The elution profile obtained for the cation purification step using SP-Toyopearl resin (Figure 4.13), showed several protein peaks eluted with the salt gradient. The peak indicated by the space between the arrows in Figure 4.13 displayed gold reducing activity but poor resolution. These fractions were pooled and subjected to another cation resin interaction.

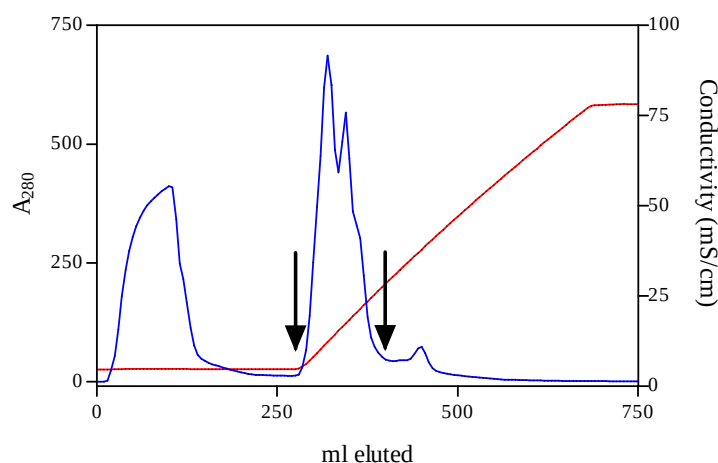


Figure 4.13 Elution profile obtained for the purification steps on SP-Toyopearl cation exchange resin. The region between the arrows were pooled for subsequent purification. (—) A_{280} and (—) conductivity.

The elution profile obtained for the second SP-Toyopearl column (Figure 4.14), illustrated several protein peaks eluting with the stretched salt gradient. The two protein peaks obtained between the arrows A (refer to as SP2P1) and B (refer to as SP2P2) in Figure 4.14 both had nitrate (section 4.2.6.2) and gold(III) reducing (section 4.2.6.1) capabilities. The activity peaks correlated very well with each of the protein peaks and were pooled separately for further purification attempts.

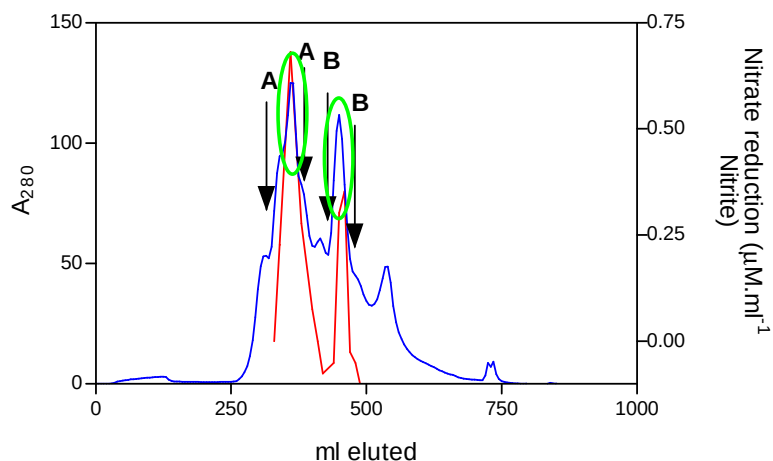


Figure 4.14 Elution profile obtained for the second purification step on SP-Toyopearl cation exchange resin. (—) A_{280} , (—) Nitrate reducing activity and (—) Au reduction activity. The region between the arrows were pooled for subsequent purification.

4.3.2.2. Dye-affinity chromatography (Blue Sepharose Cl-6B)

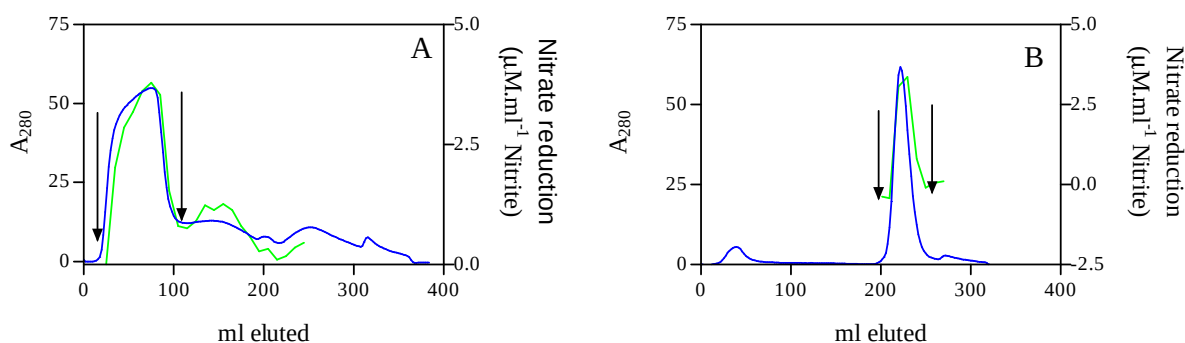


Figure 4.15 Elution profiles obtained from the Blue Sepharose CL-6B resin when loaded with (A) SP2P1 and (B) SP2P2. (—) A_{280} and (—) nitrate reducing activity. The region between the arrows were pooled for subsequent purification.

Subsequent purification of the pooled peaks SP2P1 and SP2P2 obtained from the second SP-Toyopearl step on Blue Sepharose CL-6B (section 4.2.5.2) showed pooled SP2P1 mainly did not interact (Figure 4.15 A), while the pooled SP2P2 did interact with the resin (Figure 4.15

B). Neither of these peaks showed any gold reducing ability (section 4.2.6.1) but both had nitrate reducing activity corresponding to the major peaks obtained.

4.3.2.3. Hydrophobic interaction and size exclusion chromatography

Separation on HIC of SP2P1 resulted in several protein peaks with low homogeneity and no gold(III) reducing abilities (section A.1). The interacting peak (SP2P2), on the other hand, obtained from the Blue Sepharose CL-6B column, was used for purification on the hydrophobic resin (section 4.2.5.3). The HIC purification profile showed one major peak and corresponded to the nitrate reducing activity (Figure 4.16 A) obtained as described in section 4.2.6.2). When this peak was loaded onto the gel filtration resin (section 4.2.5.4) only one peak was observed (Figure 4.15 B). Neither nitrate- (section 4.2.6.2) nor gold reducing abilities (section 4.2.6.1) could be observed, however when the adapted gold assay was used (section 4.2.6.3) a colour change was observed indicating gold(III) reducing and nanoparticle formation. This implies that the purified protein is dependent on the electron shuttling system supplied in the reaction mixture. See supplementary notes (section A.1).

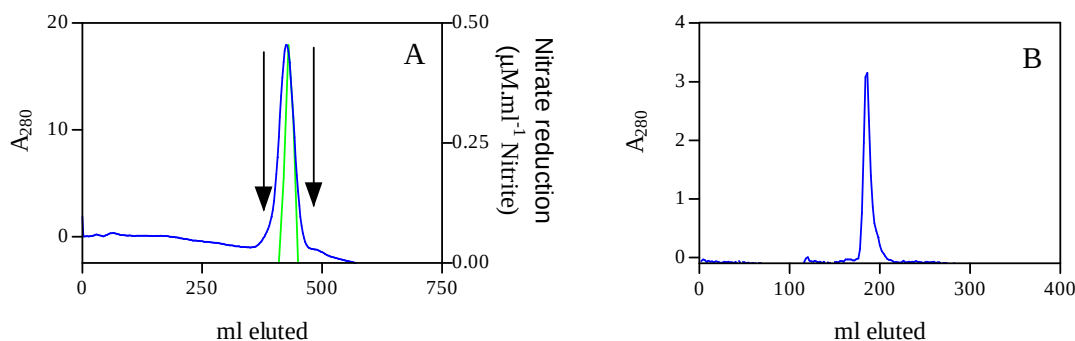


Figure 4.16 Elution profiles obtained from the (A) Phenyl and (B) Sephacryl S200HR resins. (—) A_{280} and (—) nitrate reducing activity. (A) The region between the arrows were pooled for subsequent purification.

The purified protein was denatured by autoclaving for 20 minutes and was assayed for gold(III) reducing and nanoparticle producing abilities. The denatured protein showed no

colour change indicating that the protein needs to be in its native conformation to facilitate gold(III) reduction and subsequent nanoparticle formation.

4.3.3. SDS-PAGE analysis

SDS-PAGE analysis (section 4.2.4) of the protein fractions obtained during the different steps of purification (sections 4.2.5.1.1 through 4.2.5.4) showed that there is a difference in the protein profiles and an increase in the homogeneity of the proteins (Figure 4.17 A and B). Figure 4.18 A shows the protein profile of the pooled active peak obtained from the Phenyl-Toyopearl resin and Figure 4.18 B shows the protein profile for the peak obtained from the Sephacryl S200HR resin. These results shows that a protein was purified and that the protein has gold(III) reducing and nanoparticle producing capabilities when the modified gold assay (section 4.2.6.3) was used for activity determination.

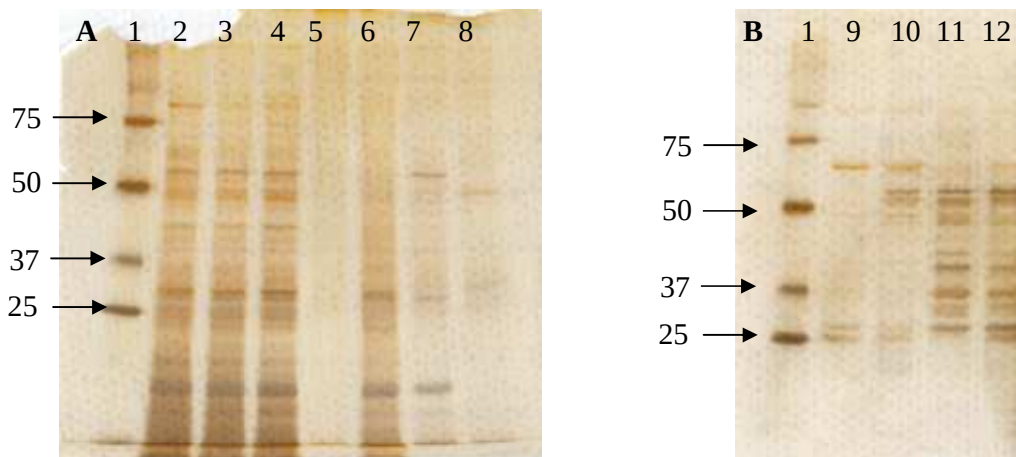


Figure 4.17 SDS-PAGE analysis of the purification steps. Each lane was loaded with 2 μ g protein. Lane (1) marker (2) cell-free extract (3) soluble proteins (4) >30kDa (5) >10<30kDa (6) <30kDa (7) Super Q-Toyopearl, non-interacting proteins (8) Super Q-Toyopearl, interacting proteins (9) Blue Sepharose CL-6B, SP2P2, interacting proteins (10) Blue Sepharose CL-6B, SP2P2, non-interacting proteins (11) Blue Sepharose CL-6B, SP2P1, interacting proteins (12) Blue Sepharose CL-6B, SP2P1, non-interacting proteins.

Purification fold and yield could not be calculated as the specific gold reducing activity could not be determined. The only means of testing activity included visual observations (colour change) and the recording of the plasmon resonance band.

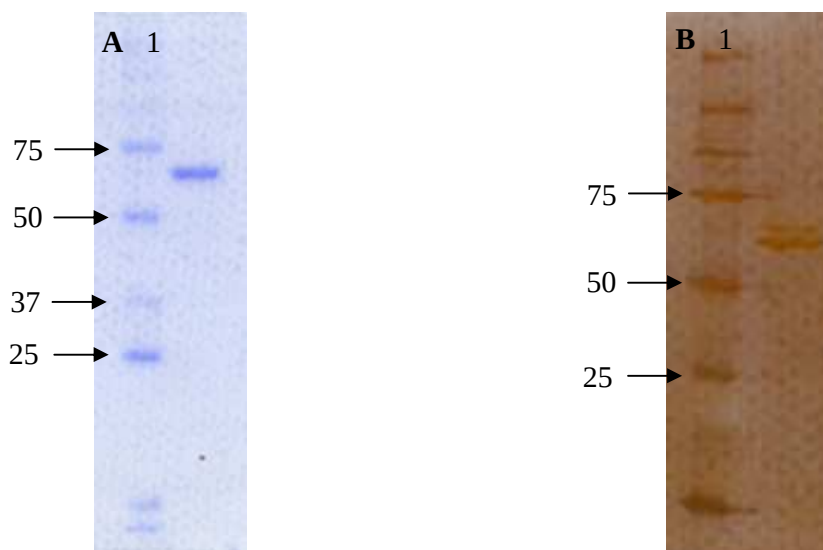


Figure 4.18 SDS-PAGE analysis of the pooled active protein peak on the (A) Phenyl-Toyopearl and (B) Sephacryl S200HR resin. Lane (1) represents the marker.

4.3.4. N-terminal sequencing

In order to identify the protein catalyst the N-terminal sequence of the soluble protein was determined using Edman degradation (section 4.2.8). N-terminal sequencing requires a homogenic protein, which was determined by using SDS-PAGE analysis in conjunction with coomassie staining. Two bands closely associated with each other were observed, which would interfere with the sequencing, thus attempts were made to separate these bands. Before loading the SDS-PAGE gel the sample is denatured by boiling the protein sample in the presence of DTT to facilitate the separation of proteins based on size. The sample was boiled over time and loaded onto a 10% SDS-PAGE gel to determine if the protein was degraded during the boiling process. It was found that the top band decreased in intensity over time while the bottom band increased in intensity (Figure 4.19), showing that the boiling process does influence the protein integrity. To determine the effect of boiling in the

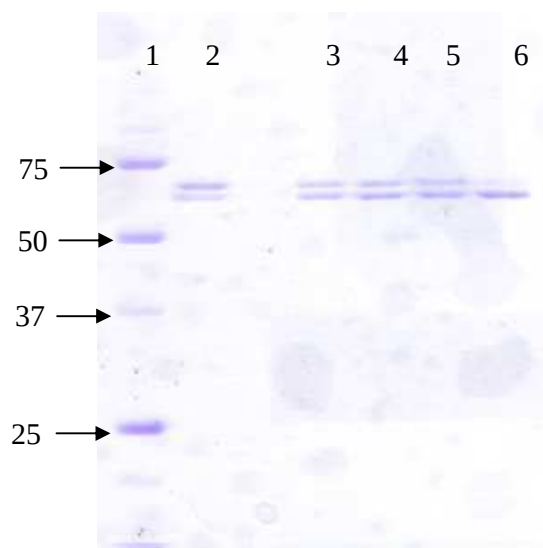


Figure 4.19 SDS-PAGE analysis of the protein purified on the Phenyl-Toyopearl resin subjected to boiling over time. Lane (1) marker (2) 10 (3) 15 (4) 20 (5) 30 and (6) 40 minutes.

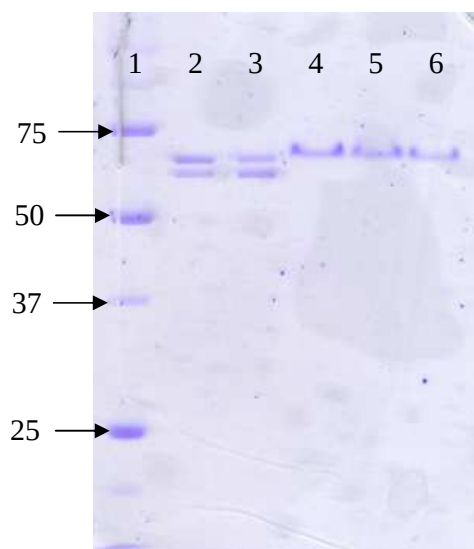


Figure 4.20 SDS-PAGE analysis of the protein purified on the Phenyl-Toyopearl resin subjected to boiling in the presence and absence of DTT. Lane (1) marker (2) +DTT boil for 5min (3) +DTT boil for 10min (4) +DTT no boiling (5) –DTT boil for 10min and (6) – DTT no boiling.

presence and absence of DTT, another gel was run with protein samples denatured under different conditions. It was found that in the absence of DTT, boiling had no effect on the protein integrity (Figure 4.20). The N-terminal sequence of the purified protein was determined as: GPQDNSLVIGAS. NCBI BLASTp results indicated that only one protein in the database shared the complete amino acids sequence. It was identified as an ABC transporter, peptide-binding protein from *Thermus thermophilus* (YP_144900).

4.3.5. Characterization of the purified protein

For each of the parameters tested one reaction was done at the standard incubation conditions [2mM gold(III), 24h, 100 μ g.ml⁻¹ protein, 4.6 μ M sodium dithionite and 65°C]. Particles produced under these conditions are depicted in Figure 4.21 D and E.

4.3.5.1. The effect of pH on particle production

The effect of pH on particle formation was evaluated as described in section 4.2.7.4. It seems as if lower pH values are more conducive to producing nanoplate-like structure with a larger aspect ratio, while a higher pH results in the formation of more spherical and smaller particles. This could also be concluded by looking at the colour change (Figure 4.22), as the higher pH value buffers had a pinkish colour corresponding to the smaller particles (Underwood and Mulvaney, 1994). Similar results were obtained by He and co-workers (2007), where they show that the starting pH could have an important effect on both the size and the shape of the gold nanoparticles produced. Different pH values would regulate the proton concentration of the buffer and this results in the control of nanoparticle morphology. The purified protein has the potential to produce particles with defined edges (Figure 4.21 E), but optimum conditions have not yet been established. More experimentation is required to narrow the range of physico-chemical parameters.

Comparing these results to the results obtained for the whole cell characterization (section 3.4.3.1), it was found that in both cases the lower pH buffers resulted in larger particles than the higher pH buffers. However, using the purified protein, nanosheets instead

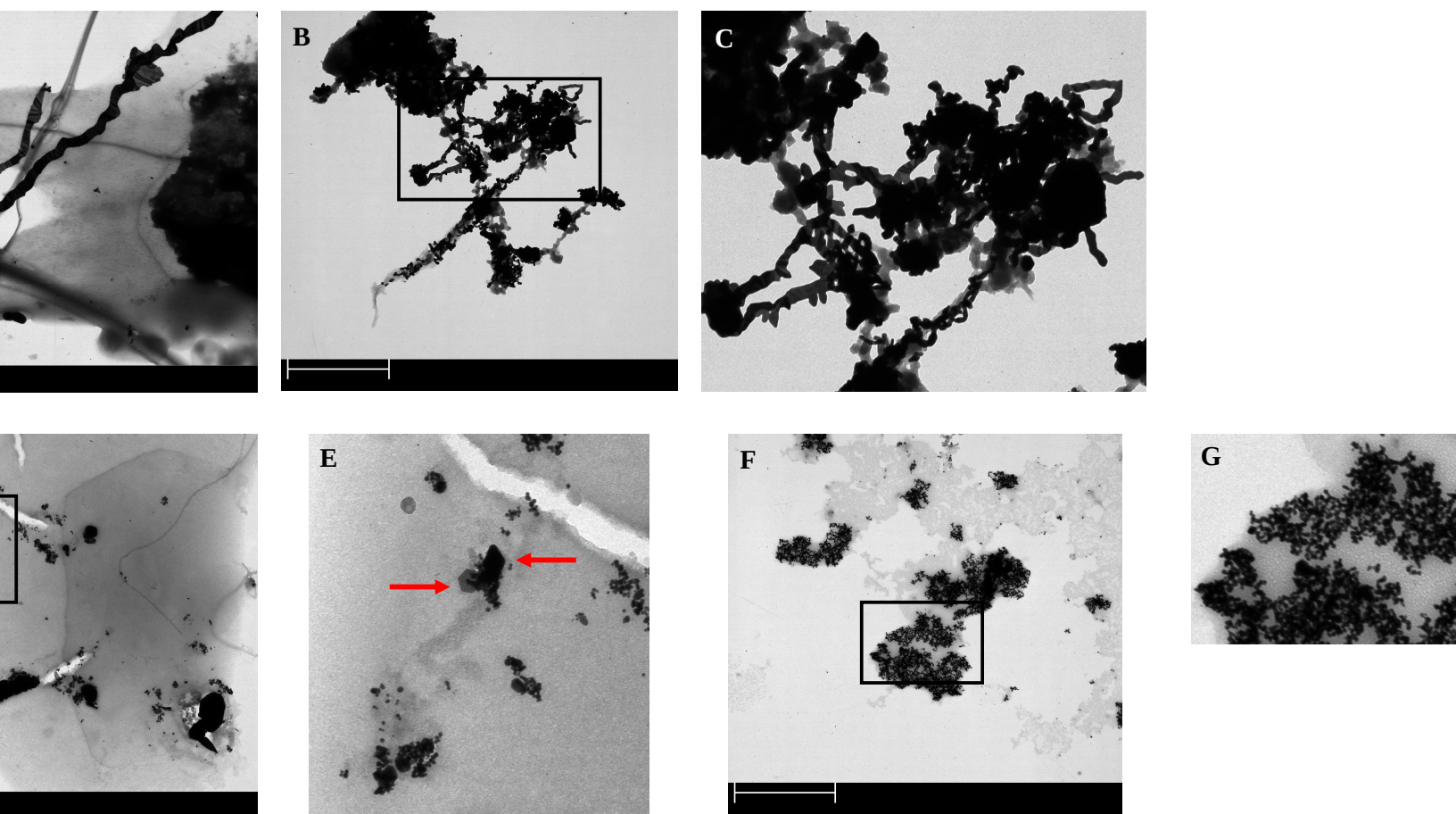


Figure 4.21 TEM micrographs of the purified protein exposed to different pH buffers. (A) pH3.6, scale bar equals 2 μ m, (B) pH 5.5, scale bar equals 1000nm, (C) enlargement of B, (D) pH 7.4, scale bar equals 1000nm, (E) enlargement of B (F) pH 9, scale bar equals 1000nm and (G) enlargement of F.

of nanoparticles were observed for the lower pH buffers. This indicates that separation of the proteins from the biomass results in different types of particles that can be produced.

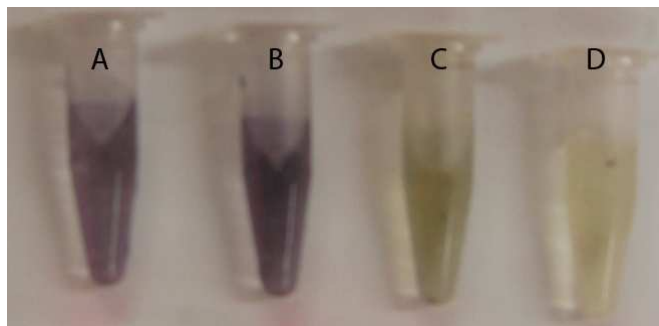


Figure 4.22 Photo representing the colour change observed when the purified protein was exposed to different pH buffers. (A) pH 3.6, (B) pH 5.5, (C) pH 7.4 and (D) pH 9.

4.3.5.2. The effect of temperature on particle production

The effect of temperature on particle formation was evaluated as described in section 4.2.7.3. From the micrographs (Figure 4.24 and Figure 4.21 D and E) it seems as if a lower temperature give better shape definition at this stage. The gold assay of the 42°C sample had a pinkish colour (Figure 4.23) while the higher temperatures had a more bluish colour. The gold assay of the 42°C sample (Figure 4.24 A) produced particles with defined edges, while the gold assay of the 65°C sample (Figure 4.21 D and E) produced particles which were less defined but some edges were still visible. It thus seems as if a lower temperature might be a contributing factor to produce particles with morphologies other than spherical. The particles produced at 75°C (Figure 4.24 B) were mostly spherical, roughly the same size as particles produced at lower temperatures. Thus temperature has no effect on the size of the particles but seems to influence nucleation of the particles as the nucleation seed plays a role in determining the end shape of the particle (Kawamura and Nogami, 2009). The data obtained from the micrographs (Figure 4.24) do not correlate to the colour production (Figure 4.23), suggesting that colour alone is not a good measurement for determining the size range of the particles produced, especially when determining optimum temperature. The colour formation suggests that the particles produced at high temperature should be larger than the particles produced at lower temperatures. The lack of increase in the size of the particles could be attributed to a depletion of gold(I) ions needed for particle growth (Tang and Hamley, 2009).

Comparing these results to the results obtained for the whole cell characterization (section 3.4.3.2), it was found that in both cases the temperature has no effect on the size of the particles produced. Purification of the protein from the cell indicates that a restriction is eliminated as the number of particles produced by the purified protein at lower temperatures is much greater in number than the amount of particles produced by the intact cells. Quantitative analysis on particle volumes was not conducted, thus a specific ratio could not be determined.

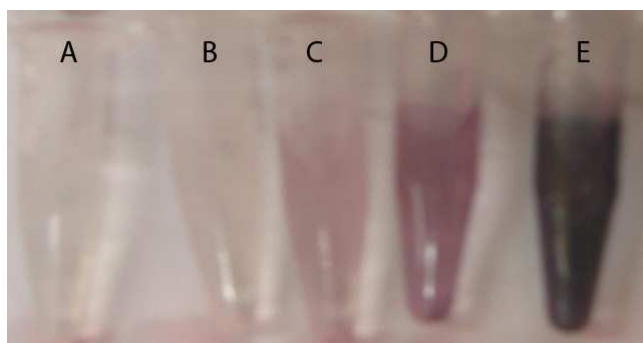


Figure 4.23 Photo representing the colour change observed when the purified protein was exposed to different temperatures. (A) 30°C, (B) 42°C, (C) 55°C, (D) 65°C and (E) 75°C.

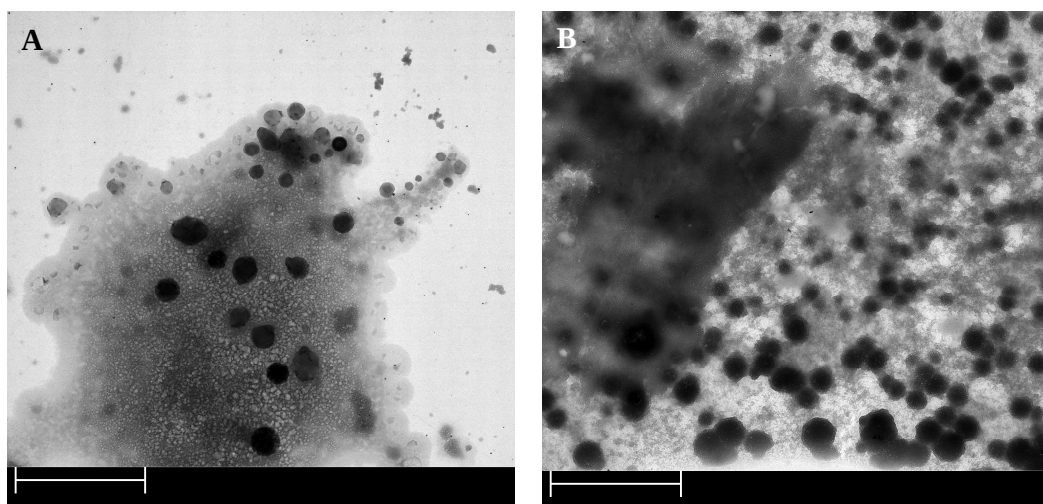


Figure 4.24 TEM micrographs of the purified protein exposed to different temperatures. (A) 42°C and (B) 75°C. Scale bars equal to 1000nm.

4.3.5.3. The effect of gold(III) concentration on particle production

The effect of gold(III) concentration on particle formation was evaluated as described in section 4.2.7.2. The 0.5mM sample does not have definitive shape and is very small compared to a higher gold(III) concentration. The 2mM sample had some shape definition with defined edges (Figure 4.21 D and E), but increasing the concentration to 5mM (Figure 4.25 C and D) resulted in the loss of the edges and the particles appear to be larger than those produced at lower gold(III) concentrations. Increasing the gold(III) concentration to 10mM (Figure 4.25 E and F) resulted in the formation of plate-like structures, the colour change by the 10mM gold(III) mixture did not turn the customary pink-purple colour but gold flake were observed with the naked eye (Figure 4.26), which could be related to the nanoplate structures formed. A few well defined shapes (Figure 4.25 F; triangles, indicated by the red arrows) could be observed. These results indicate that gold(III) concentration plays both a role in the shape and the size of the nanostructures produced. Similar observations were made by He and co-workers (2008) when they exposed cell-free extract of *Rhodopseudomonas capsulata* to varying gold(III) concentrations. The stoichiometry was calculated by converting the protein, as well as, the gold(III) concentrations to μM values. It was found that the gold ions were always in excess. The greater the ratio the bigger the particles indicating that the ratio of protein to gold ions play a role in the morphology of the nanoparticles (Kawamura and Nogami, 2009; Tang and Hamley, 2009). Elemental analysis was not done on these particles and care needs to be taken on interpreting particle composition.

Comparing these results to the results obtained for the whole cell characterization (section 3.4.3.3), it was found that in both cases an increase in gold(III) concentrations resulted in multiple nucleation events. Purification of the gold reducing and nanoparticle producing protein resulted in the formation of nanoplates when 10mM gold(III) was used, which differs from that of the particles produced by the intact cells. The particles produced in the cells, caused the cells to lyse when they became too large, thus the cell causes a restriction to the size of the particles that can be produced. The purified protein had no such limits and particles could grow into nanosheets.

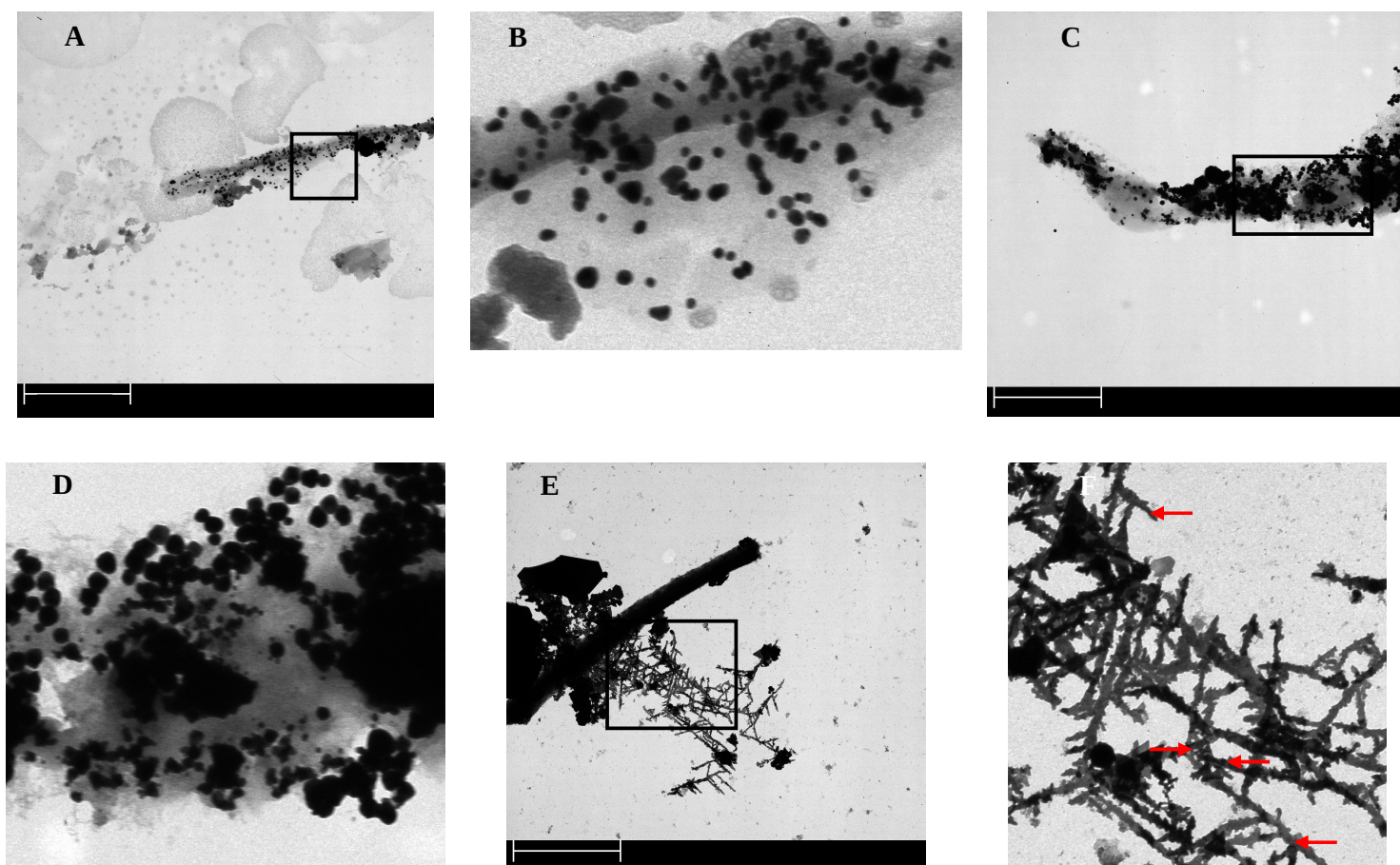


Figure 4.25 TEM micrographs of the purified protein exposed to varying gold(III) concentrations. (A) 0.5mM gold(III), scale bar equals 1000nm, (B) enlargement of A, (C) 5mM gold(III), scale bar equals 1000nm, (D) enlargement of C, (E) 10mM gold(III), scale bar equals 2 μ m and (F) enlargement of E.

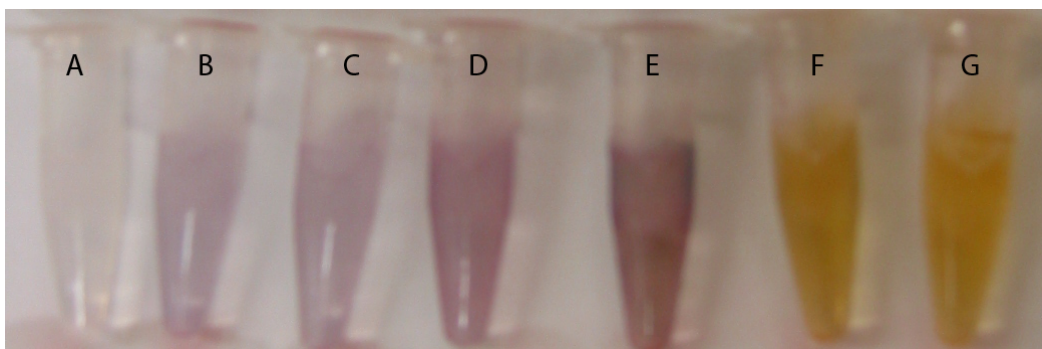


Figure 4.26 Photo representing the colour change observed when the purified protein was exposed to varying gold(III) concentrations. (A) 0.1mM, (B) 0.5mM, (C) 1mM, (D) 2mM, (E) 5mM, (F) 7.5mM and (G) 10mM.

4.3.5.4. The effect of protein concentration on particle production

The effect of protein concentration on particle formation was evaluated as described in section 4.2.7.5. From the micrographs (Figure 4.27) it seems as if a lower protein concentration is better suited to produced particles with shape definition as opposed to higher protein concentrations where the particles seem to have little to no definition. Also for the $20\mu\text{g.mL}^{-1}$ protein sample (Figure 4.27 A) the particles produced appear to be larger than the particles produced by the $100\mu\text{g.mL}^{-1}$ protein sample (Figure 4.21 D and E) which also gave a few particles with defined edges. The colour change observed (Figure 4.28) for these reactions showed that the lower protein concentrations had a slightly more blue tint than the higher protein concentrations, which corresponds to the micrographs (Figure 4.27). This could be because less proteins means fewer nucleation sites for gold nanoparticle initiation which allows for the particles to grow (Mikheenko *et al.*, 2008; Scott *et al.*, 2008).

Looking at the stoichiometry of protein molecules to gold(III) concentration, it was once again seen that the gold(III) was in excess no matter which protein concentration was used. This corresponds to the reasoning that a higher ratio of gold to protein (reductant) would result in larger particle as all the possible protein nucleation sites were occupied by a gold ion (Tang and Hamley, 2009). The excess gold ions can now be used for particle growth, thus the less nucleation site available the faster particles can grow as there will be

less competition for the available gold ions to act either as part of the nucleation process or as to help with the grow of the particle.

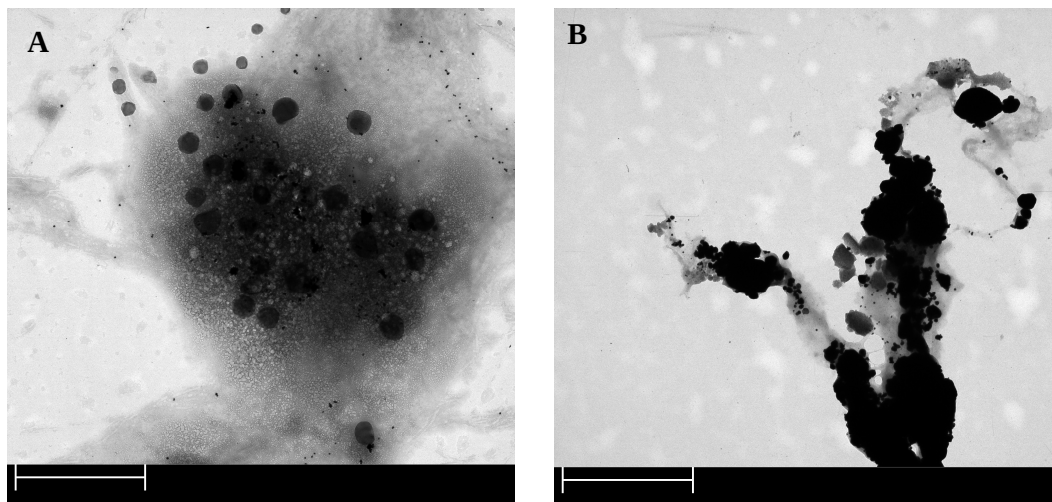


Figure 4.27 TEM micrographs of the purified protein exposed to varying protein concentrations. (A) $20\mu\text{g.mL}^{-1}$ and (B) $50\mu\text{g.mL}^{-1}$. Scale bars equal to 1000nm.

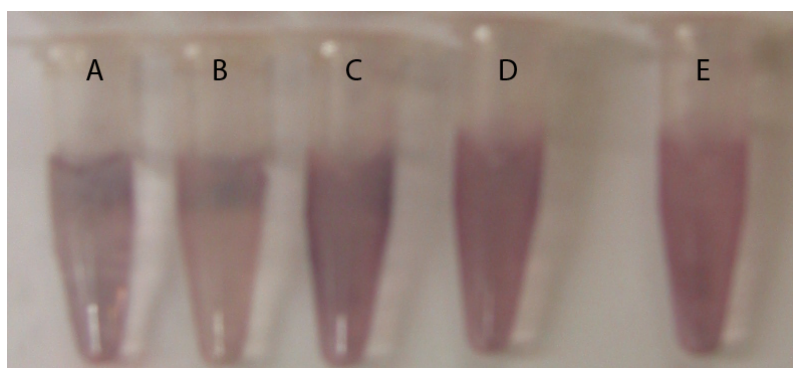


Figure 4.28 Photo representing the colour change observed when the purified protein was exposed to varying protein concentrations. (A) $10\mu\text{g.mL}^{-1}$, (B) $20\mu\text{g.mL}^{-1}$, (C) $50\mu\text{g.mL}^{-1}$, (D) $75\mu\text{g.mL}^{-1}$ and (E) $100\mu\text{g.mL}^{-1}$.

4.3.5.5. The effect of exposure time on particle production

The effect of exposure time on particle formation was evaluated as described in section 4.2.7.6. Figure 4.29 shows that particle production was already visible after 8 hours of incubation. These particles were roughly the same size as for the 24 (Figure 4.21 D and E) and 48 hour incubation periods (Figure 4.29 B), indicating that exposure time does not really effect particle size. Similar to the findings for temperature (section 4.3.5.2), these results however are not portrayed in the photos of the colour change (Figure 4.30). The photo would suggest that the particles produced after a longer incubation period should be larger than the particles produced at shorter time intervals. It seems as if the particles formed at the 48h interval were smaller and more spherical than the particles produced at shorter incubation time. An explanation for this is still unclear as it would be expected to produce larger particles as a longer growth period is supplied. This could mean that all the possible nucleation sites are not occupied and that an increase in incubation times allows for more of the sites to be accessed by the gold ions with causes the ions to be used for nucleation processes instead of for growth agents (Mikheenko *et al.*, 2008; Tang and Hamley, 2009).

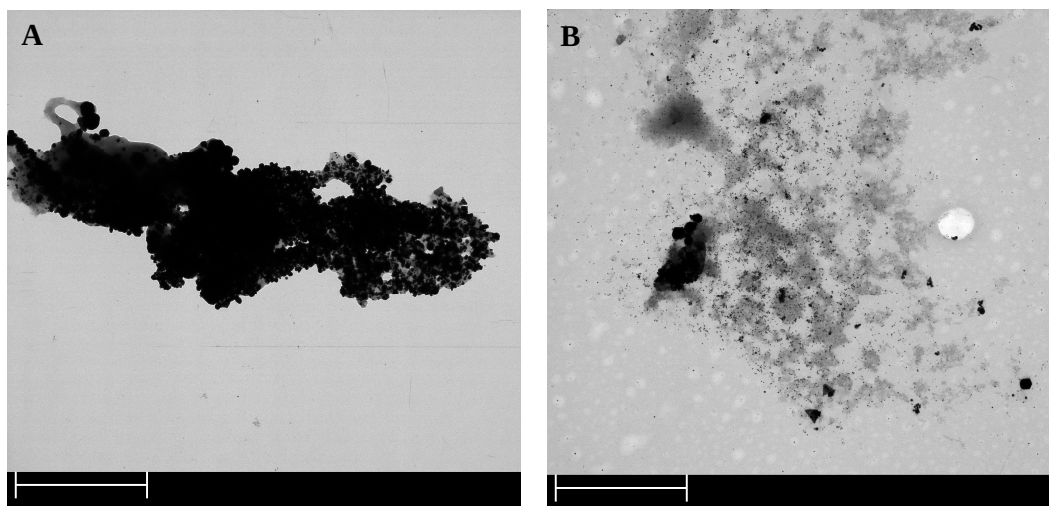


Figure 4.29 TEM micrographs of the purified protein exposed to varying incubation times. (A) 8h and (B) 48h. Scale bars equal to 1000nm.

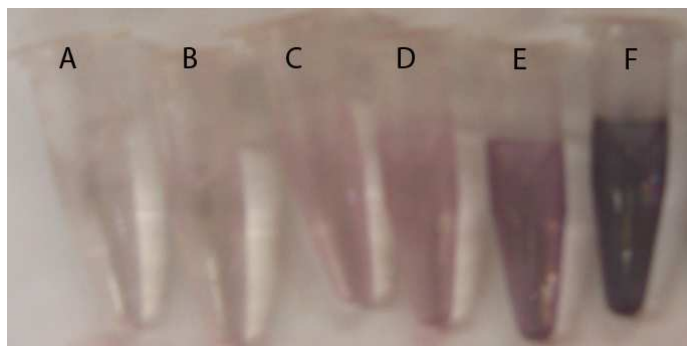


Figure 4.30 Photo representing the colour change observed when the purified protein was exposed to varying incubation times. (A) 2h, (B) 4h, (C) 8h, (D) 12h, (E) 24h and (F) 48h.

4.3.5.6. The effect of sodium dithionite concentration on particle production

The effect of sodium dithionite concentration on particle formation was evaluated as described in section 4.2.7.7. Significant reduction of gold(III) only occurred in the presence of the protein and then only when sodium dithionite was added to the reduction mixture. See supplementary notes (section A.2). If the sodium dithionite concentration is too high (Figure 4.31 B and C) few particles are produced and those which are formed have no definitive shape - just a big black mass. Sodium dithionite concentration equal to that used for the nitrate reductase assay described by Showe and DeMoss (1968) gave the best particle production implying that sodium dithionite need not be in excess for the disulphide bond to be reduced and act as nucleation site for gold(III) reduction and particle formation. Even when no dithionite (Figure 4.31 A) was added to the reaction mixture a small amount of reduction still occurred as the particle formed had no shape or clearly defined edges, while $4.6\mu\text{M}$ dithionite gave particles with defined edges (Figure 4.21 D and E). Stoichiometry calculations revealed that a ratio of protein to sodium dithionite of lower than 1:1 gives the better results in terms of particle shape. These parameters still need further investigation as optimum parameters have not been established. Particle morphology could also be dependent, not only on the protein to sodium dithionite ratio, but also on the ratio of protein, sodium dithionite and gold(III). Thus changing only one parameter at a time might not give optimum conditions. It is also suggested to test different reducing agents to determine the effect of reducing agent as well as concentration of the particular reducing agent on nanoparticle synthesis.

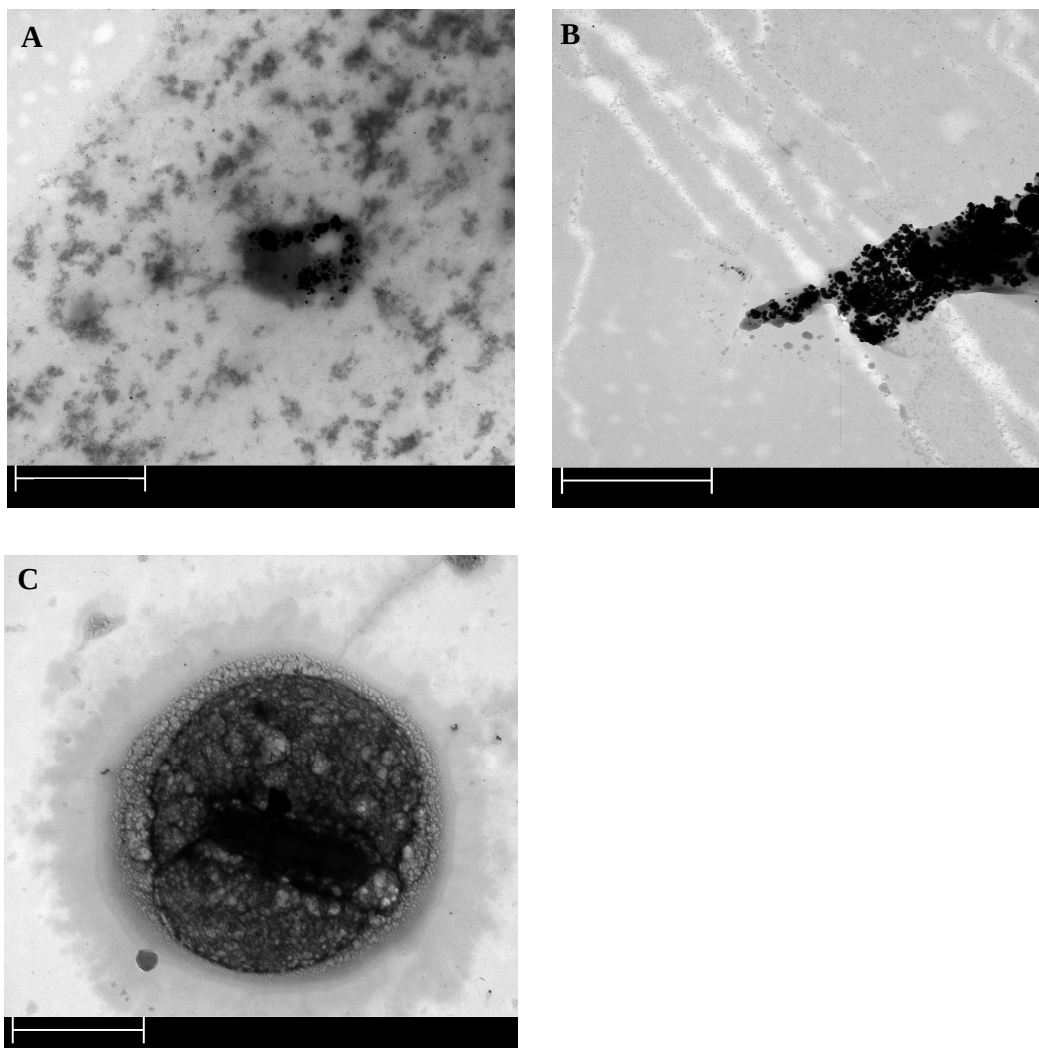


Figure 4.31 TEM micrographs of the purified protein exposed to varying sodium dithionite concentrations. (A) 0 μ M (B) 50 μ M and (C) 100 μ M. Scale bars equal to 1000nm.

4.4. Conclusions

The soluble fraction had better particle formation than the cell-free extract, which could lead to the conclusion that interfering substances are removed during the purification steps. This might be the first point of optimization. Initial data indicated the possible involvement of a nitrate reductase-like protein but the N-terminal sequencing showed that the protein was an ABC transporter, peptide-binding protein. This was not expected as transporters are not

oxido-reductases. The ABC transporter, peptide-binding protein has a disulphide bond which leads to a hypothesis involving the reduced state of the disulphide bridge, thus aiding in the shuttling of electrons. This is strengthened by the addition of sodium dithionite in the assay mixture which is needed to reduce the sulphide residues, which are then able to transfer the electrons to the gold ions, reducing it to elemental gold. The protein thus acts as a seeding point for particle formation. This hypothesis is somewhat confirmed by the data supplied by Scott and co-workers (2008) where they showed that the catalytic cysteine residues of the glutathione reductase are directly involved in the reduction of gold(III). Not unlike what is suggested in the present study.

Characterization of the pure protein show that the physico-chemical parameters play a role in particle formation. Temperature, protein concentration and exposure time has an effect on the size of the particles while pH, gold(III) and sodium dithionite concentrations have an effect on the shape as well as on the size of the particles. Despite these experiments particles lack monodispersity and no optimum parameters could be concluded. At this stage the discovery of the protein identity almost convinces the researcher that the physico-chemical parameters are not the key to control reduction and particle production. Further research is still needed to determine the optimal conditions for gold(III) reduction and nanoparticle formation.

4.5. References

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CHAPTER 5

5. EXPRESSION AND CHARACTERIZATION OF THE ABC TRANSPORTER, PEPTIDE-BINDING PROTEIN

5.1. Introduction

The membranes of cells are made-up of lipids which confer an inherent resistance to a great majority of hydrophilic solutes required for cell nutrition as well as resistance towards many of the waste products and / or toxins that must be excreted. The membranes contain highly selective gates that promote the passage of certain materials in certain directions and block others altogether (Mathews *et al.*, 2000). These gates are proteins, which sole function is to catalyze the translocation of substrates through the membrane (Figure 5.1).

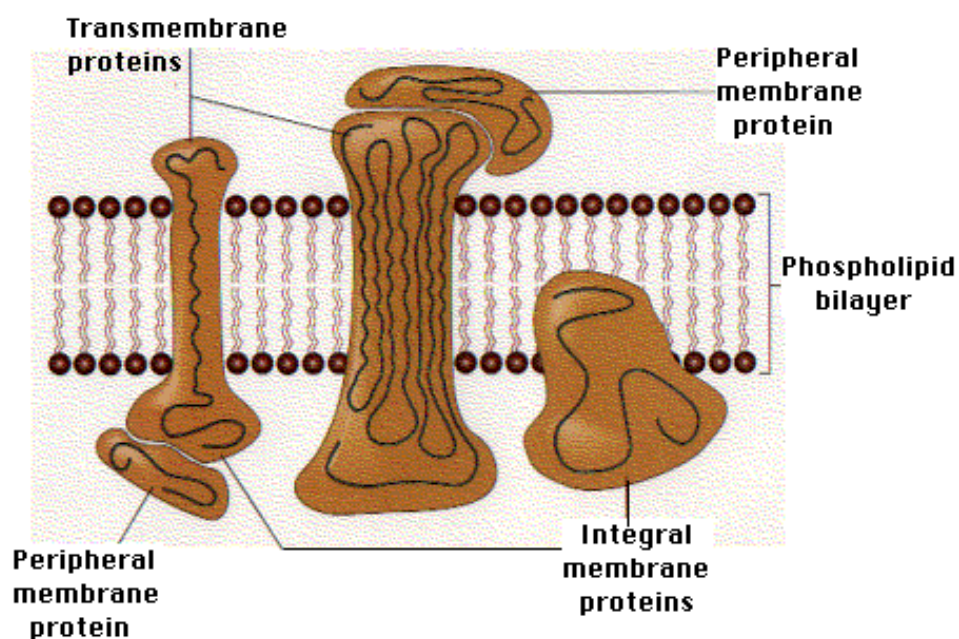


Figure 5.1 Cell membrane cross section. (Taken from *Cell Membranes*, 2003).

Free-living microorganisms often inhabit environments where nutrients are in short supply, and different species must compete with each other for the available metabolites. Accordingly they couple expenditure of metabolic energy to inward transport of essential nutrients to achieve intracellular concentrations sufficient for optimal growth. Furthermore, the process of eliminating metabolic wastes and / or toxins such as antibiotics is often coupled to the expenditure of metabolic energy, an indication of its importance for survival (Henderson, 1998; Mathews *et al.*, 2000). Solutes can cross the membrane either by passive diffusion, facilitated diffusion or active transport. Active transport processes embrace a variety of molecular mechanisms, in which energy may be derived from light, oxidoreduction, ATP hydrolysis or pre-existing solute gradients. ABC transporters are one such group of membrane spanning proteins which requires the hydrolysis of ATP to facilitate transport of a substrate across the membrane.

5.1.1. ABC transporters

ATP binding cassette (ABC) transporters are ubiquitous membrane proteins that facilitate unidirectional substrate translocation across the lipid bilayer by utilizing the energy obtained from the hydrolysis of ATP and are found in both eukaryotes and prokaryotes. They are classified as importers and exporters depending on the direction of translocation of their substrates, and are very diverse with respect to their physiological function and substrate (Braibant *et al.*, 2000; Locher and Borths, 2004; Saurin *et al.*, 1999). Importers are found exclusively in prokaryotes, while exporters are found in both prokaryotes and eukaryotes. As the main source of ATP is found in the cytosol of living organisms, import is defined as the transport of a molecule into the cytosol, while export is the transport of a molecule out of the cytosol, even if the final location is an intracellular organelle (Dassa and Bouige, 2001).

ABC transporters are from a large superfamily of multi-subunit permeases. A typical ABC transporter has four domains, consisting of two hydrophobic membrane spanning domains (MSDs) associated with two cytoplasmic nucleotide binding domains (NBDs). The NBDs of ABC transporters bind ATP and couple ATP hydrolysis to the transport process. They are the best conserved domains of the transporters, sharing about 30-40% sequence homology regardless of their substrate specificity or of their origin (Braibant *et al.*, 2000; Davidson, 2002; Davidson and Chen, 2004). Three of these highly conserved regions are: (a)

the Walker_A and Walker_B motifs which form an ATP binding pocket, (b) the ABC transporter family signature, a characteristic pattern located between the two Walker_A and Walker_B motifs (about 100 residues downstream of the Walker_A motif and 10 residues upstream of the Walker_B motif), (c) and a six amino acid motif located downstream of the Walker_B motif which contains a highly conserved histidine residue (Figure 5.2). The transmembran domains (TMDs) are made-up of two highly hydrophobic proteins which create a channel that allows the translocation of the substrate through the membrane and are less conserved than the NBDs. The MSDs are bundles of four to eight α -helices that cross the membrane multiple times in a zig-zag manner (Biemans-Oldehinkel, 2006; Braibant *et al.*, 2000).

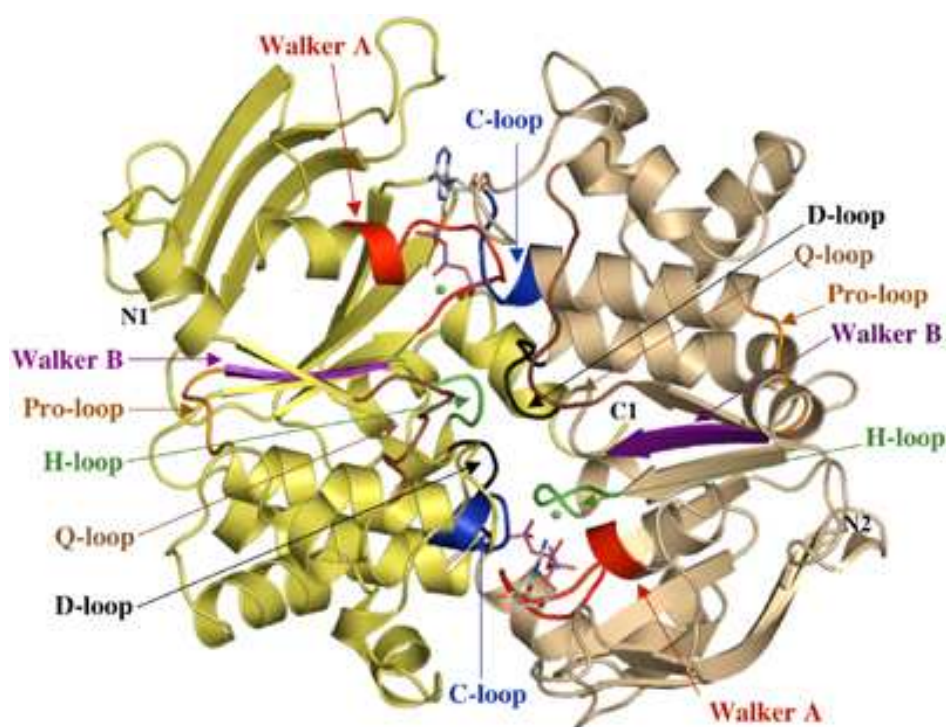


Figure 5.2 Crystal structure of the HlyB-NBD H662A dimer with bound ATP/Mg²⁺. ATP in stick representation and Mg²⁺ (green spheres) are sandwiched at the interface of the two HlyB-NBD monomers (shown in light tan and light yellow). N- and C-termini of the individual monomers are labelled. Conserved motifs are coloured in red, brown (Q-loop), blue (C-loop or ABC signature motif), magenta (Walker B), black (D-loop), and green (H-loop) and labeled accordingly. (Taken from Zaitseva *et al.*, 2005).

5.1.2. Classification of ABC transporters

Most ABC transporters are primary transporters which transport molecules in one direction over the membrane. Each is specific for a single substrate or group of related substrates that can range from inorganic ions to sugars and amino acids, complex polysaccharides and proteins (Locher and Borths, 2004; Saurin *et al.*, 1999). There are two main families, namely, the exporters (efflux) and the importers (uptake). Efflux ABC transporters consist of NDB and MSD components, often fused in one protein. Two of these half-transporters will interact to complete the four-domain structure of the ABC transporter. Exporters are involved in the export of molecules out of the cytoplasm. These molecules include peptides, polysaccharides, proteins and hydrophobic drugs. The uptake ABC transporters consist of three different proteins which function together to import metabolites into the cell. The three components are the NDB, the MSD and a periplasmic substrate-binding protein (SBP). The SBP is found in the periplasm in gram-negative organisms and is attached to the cytoplasmic membrane in gram-positive organisms. Importers are involved in the uptake of a wide variety of solutes (Braibant *et al.*, 2000; Saurin *et al.*, 1999). There are dozens of families within the ABC superfamily, and transporter proteins have been grouped by a number of functional criteria, including mechanism (e.g. sodium-solute symporters), topology (e.g. 12-transmembrane helix transporters), intracellular location (e.g. mitochondrial transporters) and possession of amino acid sequence (e.g. ATP binding cassette (ABC) transporters).

5.1.2.1. DRI family involved in drug resistance

This family is comprised of systems similar to that of the DRA family, which is characterized by strong sequence conservation of the ABC subunits. Typically organization consists of one ABC and one or two MSDs. The DRI family systems are found in prokaryotes and have been involved in antibiotic resistance and in bacteriocin and lantibiotic immunity (Dassa and Bouige, 2001). The DRI family contains various subfamilies, one of which is the DRB subfamily which is involved in peptide antibiotic resistance. The branched cyclic dodecyl peptide bacitracin resistance determinant BcrABC in *Bacillus licheniformis* is the best characterized system in this subfamily (Podlesek *et al.*, 1995). The BcrA protein shares a high degree of homology with the ATP-binding components of the ABC transporter family, while the BcrB and BcrC genes encode for hydrophobic proteins which may function as the

TMDs. These genes also confer resistance upon *Escherichia coli* and it is hypothesized that antibiotic resistance is due to the active efflux of the drug through the membrane.

5.1.2.2. ABC-2 nodulation protein and ABC-2 polysaccharide exporter families

Members of the ABC-2 nodulation family comprises of the NODJ nodulation protein which mediate the export of modified oligosaccharides, while members of the ABC-2 polysaccharide exporter family mediate polysaccharide export. These transporters are only found in gram-negative bacteria and the proteins are not homologous to any other ABC transporter families, although their transport mechanism is believed to involve bound ATP and to be similar to that of other ABC transporters (Griffith and Sansom, 1998; Reizer *et al.*, 1992).

5.1.2.3. The OPN family specific for di- and oligopeptides and nickel

Members of the OPN family have been found in all prokaryotic genera and are specific for the uptake of di- and oligopeptides and nickel. They are characterized by the fact that the two ABC subunits are encoded by different genes. Oligopeptide-like transporters have been implicated in the uptake of a class of opines such as agrocinosins, agropinic and mannopinic acids (Dassa and Bouige, 2001; Hayman *et al.*, 1993)

5.1.2.4. ATP binding protein-dependent transporters

Although binding protein-dependent transport systems are poorly characterized, the members of this superfamily are structurally, functionally, and evolutionary related to each other. These transporters are found in bacteria, yeasts, plants, and animals including man, and include both import and export systems. Although any single system is relatively specific, different systems handle very different substrates which can be inorganic ions, amino acids, sugars, large polysaccharides, or even proteins (Higgins, 1990).

The ABC-associated binding protein-dependent peptide transporter family mediate the uptake of oligopeptides and are associated with the cytoplasmic elements of the ABC

superfamily. Proteins in this superfamily use the energy derived from ATP hydrolysis to pump substrates across cell membranes. Two cytoplasmic chains containing the ATP binding domains associates with two transmembrane domains of the ABC-associated binding protein-dependent peptide transporter family to form the active complex. According to statistical analysis of multiple amino acid sequence comparisons, this family is most closely related to the ABC-associated binding protein-dependent maltose transporter family (Higgins, 1990; Griffith and Sansom, 1998).

5.1.3. Mechanism of ABC transporters

The architecture of the two MSDs and the two NBDs across the entire ABC transporter superfamily are conserved, and thus it could be expected to have a common translocation mechanism for both ABC uptake and efflux systems. The transport cycle is initiated by the interaction of substrate with the MSDs. Substrate binding causes the transport proteins to under go a conformational change involving the bending of a hinge (NBDs) that joins the two lobes. This initiates the hydrolysis of ATP. The transport proteins take on a more open conformation in absence of the transported substrate, and the substrate binding promotes bending at the hinge and closing the cleft around the substrate (Davidson and Chen, 2004; Higgins, 2001). The substrate is transferred from a high-affinity binding site on one side of the membrane to a low-affinity binding site on the other side of the membrane. Once the substrate is released into the environment the transport protein must be reset and the binding sites re-orientated.

The most uncomplicated model of substrate binding protein-dependent uptake entails four steps (Figure 5.3). The mechanism will be described using the maltose binding transporter system as it is the most closely related to the ABC-associated binding protein-dependent peptide transporter family (Griffith and Sansom, 1998). Firstly the maltose binds to the maltose binding protein (MBP) causing it to close. The maltose containing MBP docks onto the TMDs and the NBDs are signalled to bind ATP. This causes the TMDs to change conformation from an open to a closed state, exposing an internal sugar-binding site. The MBP opens and donates the maltose to the sugar-binding site. ATP is hydrolyzed and causes the dissociation of the NBD dimmers, resulting in the conformation change of the closed state

to the open state upon release of the substrate into the cytoplasm (Biemans-Oldehinkel *et al.*, 2006; Davidson and Chen, 2004).

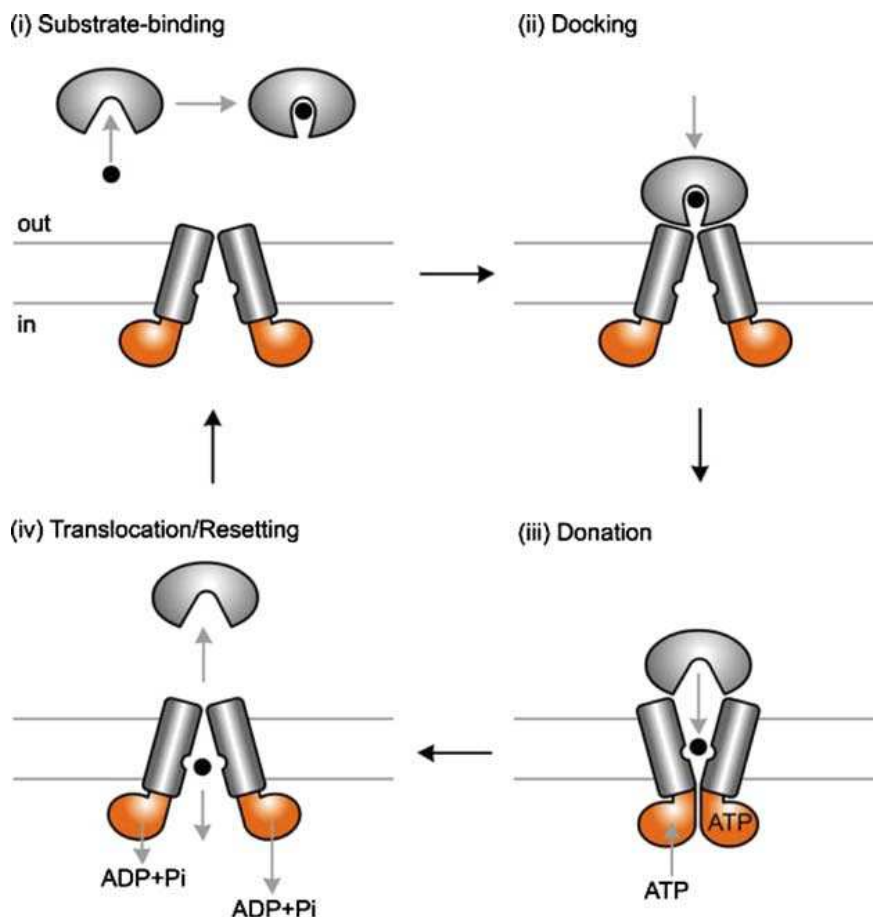


Figure 5.3 Model of the translocation mechanism of a SBP-dependent ABC transporter. (Taken from Biemans-Oldehinkel *et al.*, 2006).

In the previous chapter a native gold(III) reducing and nanoparticle producing protein from *Thermus scotoductus* SA-01 was purified and characterized. The aims of this chapter became:

1. The heterologous expression of the ABC transporter peptide-binding protein in the mesophile *Escherichia coli* to confirm gold(III) reducing and nanoparticle producing capabilities.

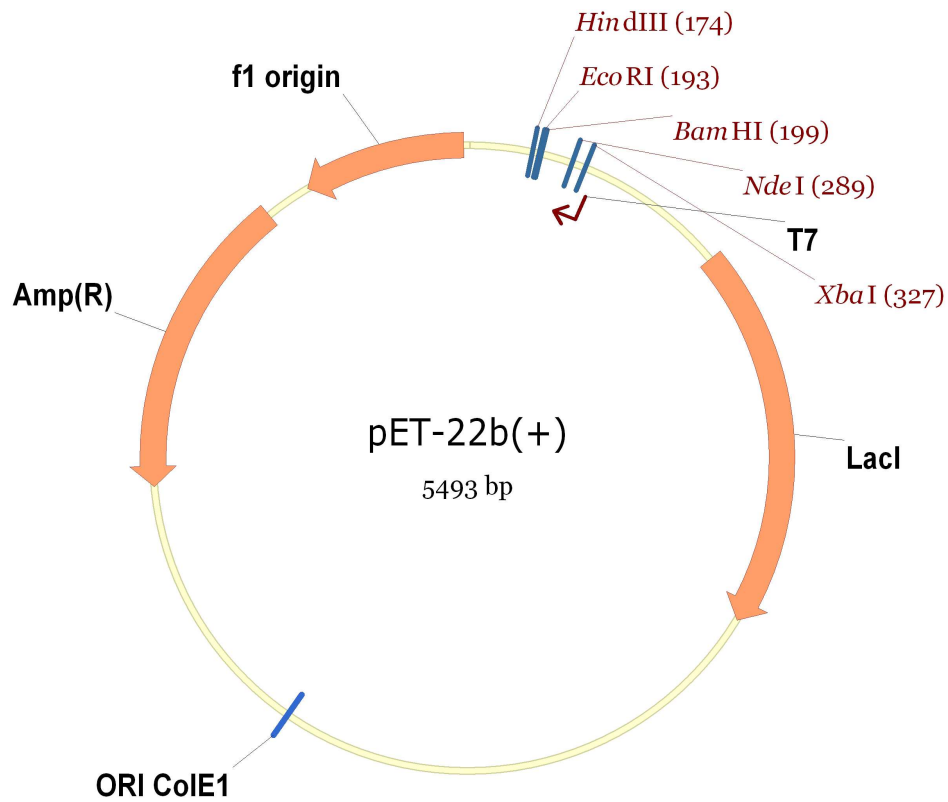
2. Characterization of the recombinant proteins with respect to the above mentioned under various changing parameters and to study the effects on nanoparticle dispersity.

5.2. Materials and methods

5.2.1. Bacterial strains, plasmids and culture conditions

Thermus scotoductus SA-01 was cultured in TYG medium as described in section 3.3.5.1. *Escherichia coli* strains TOP10 (Invitrogen) and BL21(DE3) [Lucigen] were used as hosts for genetic manipulation and expression of proteins respectively. *E. coli* strains were grown in Luria-Bertani (LB) medium (10g.l⁻¹ tryptone, 5g.l⁻¹ yeast extract and 5g.l⁻¹ NaCl, pH 7) at 37°C with aeration (200rpm). Kanamycin (30µg.ml⁻¹) or ampicillin (100µg.ml⁻¹) were used as selective pressure when required.

Plasmids pET 22b(+) and pET 28b(+) (Novagen) were used for expression of the proteins in *E. coli* BL21(DE3). The pET 22b(+) vector are designed for fusion of target proteins to signal sequences that facilitates export into the periplasmic space. Unfused proteins can also be expressed by cloning into the *NdeI* site of the vector (Figure 5.4). The pET 28b(+) vector are designed for generating target proteins fused with cleavable N-terminal His-tag / T7-tag. Proteins having their native N-terminus can be produced by using the *NdeI* site (Figure 5.5). The vector encodes for a protease recognition site for the removal of the N-terminal tag (Novagen, 2002-2003).



```

Bg/II      T7 promoter      lac operator      Xba I      rbs
AGATCTCGATCCCGGAAATTAATACGACTCACTATAGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTGTTAACTTTAAGAAGGAGA
Nde I      BspM I      pelB leader      Msc I Nco I      BamH I EcoR I Sac I
TATACATATGAAATACCTGCTGCCGACCGCTGCTGCTGGTCTGCTGCTCCTCGCTGCCAGCGGCGATGGCCATGGATATCGGAATTAATTCGGATCCGAATTCGAGCTCC
MetLysTyrLeuLeuProThrAlaAlaAlaGlyLeuLeuLeuLeuAlaAlaGlnProAlaMetAlaMetAspIleGlyIleAsnSerAspProAsnSerSerSer
Sai I      Hind III      Eag I      Ava I*      His-Tag      signal peptidase      Bpu1102 I
GTCGACAAGCTTGCGGCGGCACTCGAGCACCACCACCACCACCTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAAC
ValAspLysLeuAlaAlaAlaLeuGluHisHisHisHisHisHisEnd
T7 terminator
TAGCATAACCCCTTGGGGCCTCTAAACGGGCTTGAAGGGTTTTTGT

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Figure 5.4 Vector map of pET 22b(+) indicating the ampicillin resistance gene, ColE1 origin of plasmid replication, lacI coding sequence and the multiple cloning site under the T7 promoter.

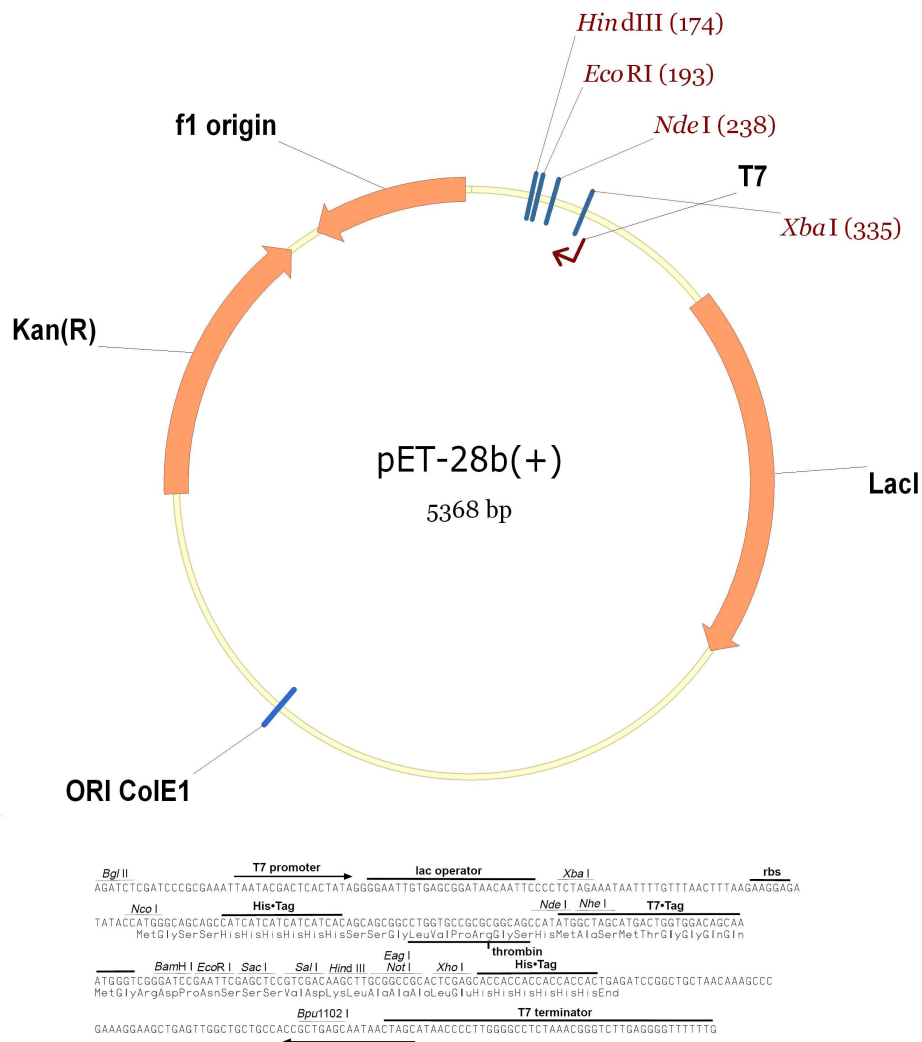


Figure 5.5 Vector map of pET 28b(+) indicating the kanamycin resistance gene, ColE1 origin of plasmid replication, lacI coding sequence and the multiple cloning site under the T7 promoter. Sequence of the pET 28b(+) cloning region showing the ribosome binding site and configuration for the N-terminal His-tag and thrombin cleavage site fusion.

5.2.2. Genomic DNA isolation

Genomic DNA was extracted from *Thermus scotoductus* SA-01 by the Proteinase K / Phenol method (Towner, 1991). Cells (0.5g) were washed in TE-buffer pH 8 (0.01M Tris [trishydroxymethylaminomethane] and 1mM EDTA) and centrifuged (3000x g; 5 min; 4°C) to pellet the cells. The pellet was resuspended in 3.2ml 50mM Tris-HCl pH 8 with 0.7mM

sucrose and 600 μ l lysozyme (20mg.ml⁻¹ in water), and the suspension was placed on ice for 5 minutes. Six hundred microliters EDTA (0.5M) and 500 μ l SDS (10%) was added to the sample after which it was mixed gently and placed on ice for 5 minutes. Ten milliliters digestion buffer (1% SDS, 50mM Tris-HCl pH 8, 0.1M EDTA, 0.2M NaCl and 0.5mg.ml⁻¹ Proteinase K) was added and the sample incubated overnight with mild shaking at 55°C. pH calibrated phenol was added (1x sample volume) to the sample and mixed gently for 3h at room temperature. Centrifugation (4000x g; at room temperature) was used to separate the aqueous and organic phases. The aqueous phase was transferred to a new tube and 1x sample volume chloroform:isoamyl alcohol (24:1) was added and mixed gently by inverting. The sample was centrifuged (4000x g; at room temperature) and the aqueous phase transferred to a new tube. This was placed on ice for 5 minutes and 1:10 (v/v) 5M NaCl added. Ten milliliters high purity 100% ice cold (-20°C) ethanol was added, the DNA spooled and placed in a 1.5ml tube. The spooled DNA was washed twice with 70% ethanol (1ml) and centrifuged (12 000rpm; 1min; 4°C). The resulting DNA pellet was air dried and dissolved in 500 μ l TE buffer, pH 8, by incubation for 1h at 50°C. The integrity of the genomic DNA was assessed by electrophoresis as described in section 5.2.3. Sterile reagents and aseptic techniques were used throughout the procedure.

5.2.3. Polymerase chain reaction (PCR)

The complete ABC gene was amplified by PCR from genomic DNA. Primers were designed using the N-terminal sequence and the gene sequence obtained from the *Thermus scotoductus* SA-01 genome database (Table 5.1). The genome sequence of *Thermus scotoductus* SA-01 was completed using a combination of GS20 and FLX-pyrosequencing (Gounder, 2009). PCR reactions were performed in a total reaction volume of 50 μ l using a Thermal Cycler (PxE 0.2, Thermo electron corporation). Reaction mixture contained 5 μ l of the 10x Super-Therm reaction buffer, MgCl₂ (2mM), dNTP's (0.8mM), Super-Therm polymerase (2.5U), 0.2 μ M of both the forward (ABC_F_Nde) and reverse (ABC_R_Eco) primers (Table 5.1) and 50ng of template, filled to the reaction volume with sterile redistilled water. Reaction conditions consisted of an initial denaturing step at 95°C for 5 minutes, followed by 30 cycles of denaturing at 95°C (30 seconds), annealing at 53.4°C (40 seconds) and elongation at 72°C (2.5 minutes). A final elongation step of 10 minutes at 72°C was added to ensure complete elongation of amplified product.

Table 5.1 Oligonucleotide primers used for PCR amplification and sequence PCRs.

Primer	Sequence	T _m (°C)
ABC_F_Nde	5' - <u>CAT ATG</u> AGA AAA GTA GGC AAG CTG GCT - 3'	59.5
ABC_R_Eco	5' - <u>GAA TTC</u> TTA CTT GAC GGA AAG AGC GTA - 3'	57.4
ABC_Int_F	5' - AGG ATG CGG AGA GGC TC - 3'	51.9
ABC_Int_R	5' - CGC TGG ATG TAG TCG TCG - 3'	51.9

Underlined sequences indicate introduced restriction sites for *Nde* I and *Eco* RI

All PCR and DNA products were analyzed with the use of agarose gel electrophoresis. All of the agarose gels consisted of 0.8% agarose in TAE buffer (0.04M Tris-HCl, 1mM EDTA pH 8.0 and 0.021mM glacial acetic acid) with 0.6µg.mL⁻¹ ethidium bromide. DNA was separated within the agarose gels at 5.6V.cm⁻¹ for 60 minutes. DNA was visualized under a high radiation UV source (ChemiDoc XRS Gel Documentation system; BioRad Laboratories), while DNA to be isolated from agarose gels for further studies was visualized using a low radiation UV source (Darkreader), and was isolated using the BioFlux BioSpin Gel Extraction Kit according to manufacturer's recommendation (Bioer Technology Co., Ltd). Nucleic acid concentrations were determined using a NanoDrop[®] ND-1000 spectrophotometer (NanoDrop Technologies, Inc).

DNA fragment size was estimated based on electrophoretic mobility relative to that of the MassRuler[™] DNA Ladder standards (Fermentas) during the same electrophoretic run.

5.2.4. Ligations

The ~1.9kb PCR purified product was ligated into the pGEM[®]-T Easy Vector system (Promega). Protocols for ligations were performed as per manufacturer's instructions. The ligation reaction was performed in a total reaction volume of 10µl and consisted of 2x Rapid Ligation Buffer (5µl), pGEM[®]-T Easy vector (2.5ng), PCR product (3µl) and T4 DNA Ligase (0.3 Weiss units) with a 1:1 molar ration of vector:insert DNA. The ligation reactions were incubated overnight at 4°C.

5.2.5. Plasmid propagation and cloning

Competent *Escherichia coli* TOP10 (Invitrogen) and BL21(DE3) cells were prepared according to the method described by Hanahan (1983) with modifications. Flasks containing Psi broth (5g.l⁻¹ yeast extract, 20g.l⁻¹ tryptone, 5g.l⁻¹ magnesium sulphate, pH 7.6 with KOH) were inoculated with 1ml of an overnight culture and grown at 37°C until an absorbance of 0.6 at 600nm was reached. Cells were placed on ice for 30 minutes and centrifuged (3000x g; 10min; 4°C) to collect cells. Cells were resuspended in 40ml TfbI buffer (30mM potassium acetate, 100mM rubidium chloride, 10mM calcium chloride, 15% glycerol, pH 5.8) and incubated on ice for 15 minutes. Cells were collected through centrifugation (3000x g; 10min; 4°C) and resuspended in 4ml TfbII buffer (10mM MOPS-NaOH, 75mM calcium chloride, 10mM rubidium chloride, 15% glycerol, pH 6.5). The cell suspensions were incubated on ice for 10 minutes, aliquoted and snap frozen in liquid nitrogen followed by storage at -80°C.

The vector of interest / ligated mixture was transformed into competent *E. coli* TOP10 cells for propagation. A stock (50µl) of the chemically competent *E. coli* strain was removed from -80°C and placed on ice to thaw. The ligation mixture was transferred to the competent cells and the transformation mixture kept on ice for 30 minutes after which it was heat shocked at 42°C for 40 seconds and placed back on ice for 2 minutes. Two hundred microliters pre-heated (37°C) SOC media (20g.l⁻¹ tryptone, 5g.l⁻¹ yeast extract, 0.01M NaCl, 2.5mM KCl, 0.01M MgCl₂, 0.01M MgSO₄ and 0.02M glucose) was added to the transformation mixture and incubated at 37°C for 1h with gentle shaking.

For the pGEM[®]-T Easy vector various aliquots of the transformed culture was plated onto LB plates containing ampicillin (0.1mg.ml⁻¹), isopropyl β-D-thiogalactoside (IPTG, 0.12mg.ml⁻¹) and 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal, 0.08mg.ml⁻¹) and incubated overnight at 37°C to verify DNA ligation using blue / white selection. Positive transformants were inoculated into 5ml LB media containing 0.1mg.ml⁻¹ ampicillin to maintain selective pressure and grown for 16h at 37°C. DNA mini-preparations followed the 16h growth of the positive transformants. This was performed using the BioFlux BioSpin Plasmid Extraction Kit as per manufacturer's recommendations (Bioer Technology Co., Ltd). Restriction fragment length polymorphism (RFLP) was used as screening method using *EcoRI* and *NdeI* in a double digestion reaction.

For the pET vectors selective pressure was maintained by using LB plates containing 0.1mg.ml⁻¹ ampicillin [pET 22b(+)] or 0.03mg.ml⁻¹ kanamycin [pET 28b(+)]. Positive transformants were inoculated into 5ml LB media containing the required antibiotic to maintain selective pressure and grown for 16h at 37°C. DNA mini-preparations followed the 16h growth of the positive transformants. This was performed using the BioFlux BioSpin Plasmid Extraction Kit as per manufacturer's recommendations (Bioer Technology Co., Ltd). RFLP was used as screening method using *EcoRI* and *NdeI* in a double digestion reaction.

5.2.6. Sequencing

In order to confirm the nucleotide composition of the relevant gene, purified positive template was used in sequencing reactions. Sequencing was performed using the ABI Prism[®] Big Dye[™] Terminator Cycle Sequencing Ready Reaction Kit V. 3.0 or 3.1 (Applied Biosystems, USA) according to the manufacturer's instructions along with the primers listed in Table 5.1. Approximately 30 – 50% of each sequencing reaction was loaded onto a 4% acrylamide gel, separated at 1.6kV and data collected on an ABI Prism[®] 377 DNA Sequencer (Perkin Elmer Biosystems, USA). The data was analyzed using Sequencing Analysis v. 3.3. Sequences were assembled using AutoAssembler v. 3.3. Reverse complementation and alignments were performed using DNAssist v. 2.0. Analyzed sequences were used to search the Genbank Database.

5.2.7. Construction of expression plasmids

The pGEM[®]-T Easy plasmid containing the desired insert was propagated and the plasmids double digested using *EcoRI* and *NdeI* for ligation into pET 22b(+) and pET 28b(+) similarly digested. The insert and the digested pET 22b(+) and pET 28b(+) vectors were cleaned from an agarose gel using the BioFlux BioSpin Gel Extraction Kit according to manufacturer's recommendation (Bioer Technology Co., Ltd). Ligations were performed in a 1:1 molar ratio using 50ng of vector and in a total reaction volume of 20µl overnight at 4°C with T4 DNA Ligase (1.5 Weiss units). The ligation mixtures were transformed into competent *E. coli* TOP10 cells and positive clones were identified through plasmid isolation and RFLP as described in section 5.2.5.

5.2.8. Expression of the ABC protein

The pET 22b(+) / 28b(+) ABC constructs were transformed into *E. coli* BL21(DE3) competent cells (Lucigen) for expression. Positive clones were identified through selection on LB plates containing 30 $\mu\text{g.ml}^{-1}$ kanamycin or 100 $\mu\text{g.ml}^{-1}$ ampicillin and inoculated into LB medium also containing the appropriate antibiotic pressure. Cells were incubated with shaking (200rpm) until an OD₆₀₀ of approximately 0.8 – 1 was reached. IPTG was added as inducer to a final concentration of 1mM and cells grown for an additional 4h. Cells were harvested through centrifugation (5000x *g*; 10min) and washed using 50mM sodium phosphate buffer, pH 7.4.

5.2.9. Purification of the recombinant ABC protein

Harvested cells containing the pET 28b(+) vector were resuspended in 20mM sodium phosphate buffer, pH 7.4, containing 20mM imidazole and 0.5M NaCl. Cells were broken by ultrasonic treatment for 5 minutes (100W) and unbroken cells and debris were removed through centrifugation (5000x *g*; 10min). The soluble fraction (cytoplasm) was separated from the insoluble fraction (membranes) through ultracentrifugation (100000x *g*; 90min).

The recombinant ABC protein was purified through immobilized metal-affinity chromatography (IMAC) and size exclusion chromatography.

The soluble fraction was loaded onto a HisTrap FF column (5ml, Amersham Biosciences) and unbound proteins eluted (5ml.min⁻¹) using 20mM sodium phosphate buffer, pH 7.4 containing 20mM imidazole and 0.5M NaCl. Bound proteins were then eluted in the same buffer using a linear gradient (100ml) of imidazole up to 0.5M. Active fractions were pooled and concentrated to 3ml using an Amicon stirrer cell through a 30kDa MWCO membrane (Osmonics Inc.). The 3ml concentrated sample was loaded onto a Sephacryl S200HR column (2.5 x 63cm, Sigma) equilibrated with 50mM sodium phosphate buffer, pH 7.4, containing 50mM NaCl. Proteins were eluted using the same buffer at a flow speed of 1ml.min⁻¹.

Harvested cells containing the pET 22b(+) vector were resuspended in 20mM sodium phosphate buffer, pH 7.4. Cells were broken by ultrasonic treatment for 5 minutes (100W)

and unbroken cells and debris were removed through centrifugation (5000x *g*; 10min). The soluble fraction (cytoplasm) was separated from the insoluble fraction (membranes) through ultracentrifugation (100000x *g*; 90min). The recombinant ABC protein was purified from the soluble fraction by using heat to denature the non-thermal stable proteins followed by size exclusion chromatography.

The soluble fraction was incubate at 70°C for 90 minutes. The denatured proteins were separated by centrifugation (12000x *g*; 30min; 4°C) from the thermal stable proteins (supernatant). The supernatant was concentrated to 3ml using an Amicon stirrer cell through a 30kDa MWCO membrane (Osmonics Inc.). The 3ml concentrated sample was loaded onto a Sephacryl S200HR column (2.5 x 63cm, Sigma) equilibrated with 50mM sodium phosphate buffer, pH 7.4, containing 50mM NaCl. Proteins were eluted using the same buffer at a flow speed of 1ml.min⁻¹.

5.2.10. Characterization of recombinant ABC proteins

5.2.10.1. Protein concentration

Protein concentrations were determined as described in section 4.2.3.

5.2.10.2. SDS-PAGE analysis

SDS-PAGE analysis was done as described in section 4.2.4.

5.2.10.3. The effect of temperature on particle production

The effect of temperature on the ability of the purified protein to produce nanoparticles was evaluated by using the adapted nitrate reductase assay (Section 4.2.6.3). The reactions containing 100µg.ml⁻¹ protein for the pET 28b(+) recombinant protein and 20µg.ml⁻¹ for the pET 22b(+) recombinant protein were incubated at temperatures ranging from 30 – 75°C in 50mM sodium phosphate buffer, pH 7.4 with 2mM gold(III) for 24 hours. The samples were prepared for TEM analysis as described in section 4.2.7.1

5.2.10.4. The effect of pH on particle production

The effect of pH on the ability of the purified protein to produce nanoparticles was evaluated by using the adapted nitrate reductase assay (Section 4.2.6.3). The reactions containing $100\mu\text{g.mL}^{-1}$ protein for the pET 28b(+) recombinant protein and $20\mu\text{g.mL}^{-1}$ for the pET 22b(+) recombinant protein were incubated at 65°C with 2mM gold(III) in pH buffers ranging from 3.6 – 9.5 for 24 hours. The samples were prepared for TEM analysis as described in section 4.2.7.1. Usually pH characterization is conducted using a buffer cocktail containing equimolar concentrations of the components, but the cocktail had a high chemical reduction rate, thus individual buffers were used. Sodium acetate buffer (50mM) was used for the range below pH 6, 50mM sodium phosphate buffer for the neutral pH range and 50mM borax buffer for the alkaline pH range.

5.2.10.5. The effect of gold(III) concentration on particle production

The effect of gold(III) concentration on the ability of the purified protein to produce nanoparticles was evaluated by using the adapted nitrate reductase assay (Section 4.2.6.3). The reactions containing $100\mu\text{g.mL}^{-1}$ protein for the pET 28b(+) recombinant protein and $20\mu\text{g.mL}^{-1}$ for the pET 22b(+) recombinant protein were incubated at 65°C in 50mM sodium phosphate buffer, pH 7.4 with gold(III) concentrations ranging from 0 – 10mM for 24 hours. The samples were prepared for TEM analysis as described in section 4.2.7.1. A hypothesis of the mechanism was slowly forming by calculating the stoichiometry by converting the protein, as well as, the gold(III) concentrations to μM values.

5.2.10.6. The effect of protein concentration on particle production

The effect of protein concentration on the ability of the purified protein to produce nanoparticles was evaluated by using the adapted nitrate reductase assay (Section 4.2.6.3). The reactions were incubated at 65°C in 50mM sodium phosphate buffer, pH 7.4 with 2mM gold(III) containing protein concentrations ranging from 10 - $100\mu\text{g.mL}^{-1}$ for 24 hours. The samples were prepared for TEM analysis as described in section 4.2.7.1. A hypothesis of the

mechanism was slowly forming by calculating the stoichiometry by converting the protein, as well as, the gold(III) concentrations to μM values.

5.2.10.7. The effect of exposure time on particle production

The effect of exposure time on the ability of the purified protein to produce nanoparticles was evaluated by using the adapted nitrate reductase assay (Section 4.2.6.3). The reactions containing $100\mu\text{g.ml}^{-1}$ protein for the pET 28b(+) recombinant protein and $20\mu\text{g.ml}^{-1}$ for the pET 22b(+) recombinant protein were incubated for 2 – 48 hours at 65°C in 50mM sodium phosphate buffer, pH 7.4 with 2mM gold(III). The samples were prepared for TEM analysis as described in section 4.2.7.1.

5.2.10.8. The effect of sodium dithionite concentration on particle production

The effect of sodium dithionite concentration on the ability of the purified protein to produce nanoparticles was evaluated by using the adapted nitrate reductase assay (Section 4.2.6.3). The reactions containing $100\mu\text{g.ml}^{-1}$ protein for the pET 28b(+) recombinant protein and $20\mu\text{g.ml}^{-1}$ for the pET 22b(+) recombinant protein were incubated at 65°C in 50mM sodium phosphate buffer, pH 7.4 with 2mM gold(III) for 24 hours. Sodium dithionite concentrations varied from 0 – $100\mu\text{M}$. The samples were prepared for TEM analysis as described in 4.2.7.1. It was important to understand the stoichiometry relating to the protein concentration to sodium dithionite concentration.

5.2.11. Modelling

A homology model of the ABC transporter peptide-binding protein of *Thermus scotoductus* SA-01 (Thsc-ABC) was built using Yasara Structure & Whatif. The template used was the oligopeptide-binding protein of *Thermus thermophilus* HB8 (2D5W-B) which has a 90% identity (95% similarity) with the ABC transporter, peptide-binding protein of *Thermus scotoductus* SA-01 .

5.3. Results and discussion

5.3.1. DNA isolation

The protein purified in chapter 4 that had the ability to reduce gold(III) and produce nanoparticles was identified as an ABC transporter, peptide-binding protein. Genomic DNA ($1075\text{ng.}\mu\text{l}^{-1}$) was extracted from *Thermus scotoductus* SA-01 as described in section 5.2.2. The gDNA isolated was of good quality ($A_{260}/A_{230} = 1.9$) and integrity as illustrated in Figure 5.6 A. The gene was amplified as described in section 5.2.3 for cloning and expression studies. A band of approximately 1.9 kb was obtained which confirmed amplification of the ABC protein Figure 5.6 B) [Gounder, 2009]. The band was purified from the gel and stored at -20°C until required for downstream experiments.

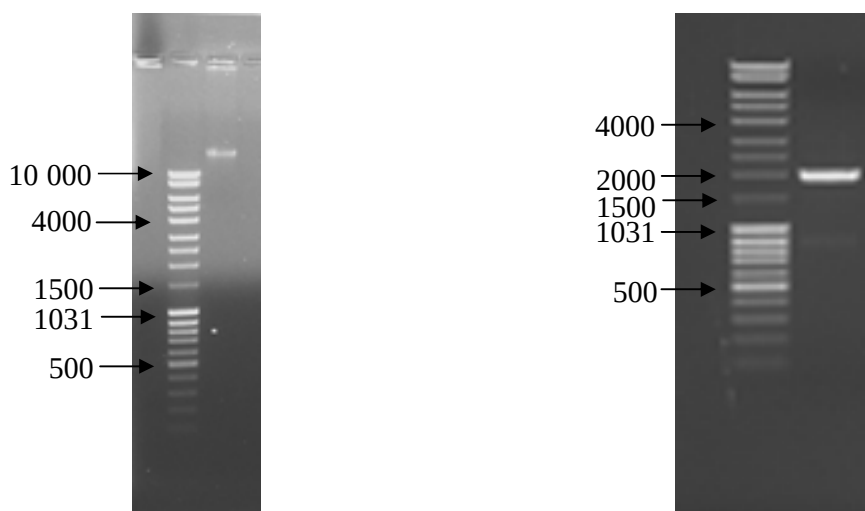


Figure 5.6 (A) Genomic DNA isolated from *Thermus scotoductus* SA-01 and (B) the amplified ABC transporter, peptide-binding protein.

5.3.2. Cloning and sequencing of the ABC gene from *Thermus scotoductus* SA-01

The amplified, gel purified ABC gene was ligated into the pGEM[®]-T Easy vector system (section 5.2.4). The ligation mixture was transformed into the *E. coli* TOP10 cells and five clones were selected for plasmid extraction (section 5.2.5). RFLP of a double digest with

EcoRI and *NdeI* confirmed insertion of the cloned gene (Figure 5.7). Sequencing of the insert (section 5.2.6) showed 100% identity to the reference gene (Figure 5.8)

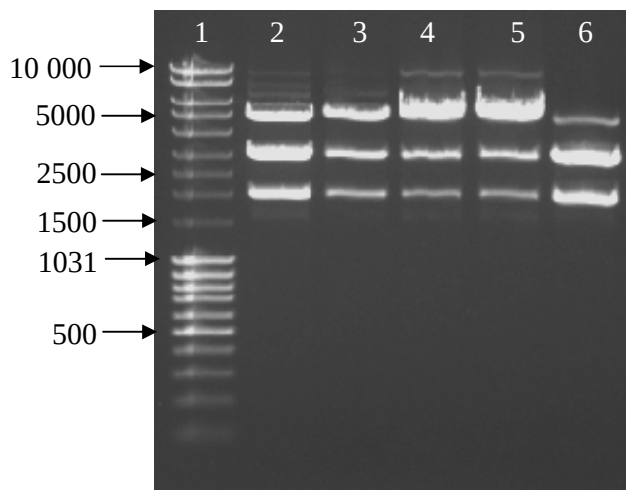


Figure 5.7 *pGEM[®]-T Easy* plasmid containing the *ABC* gene digested with *EcoRI* and *NdeI*. Lane 1 represents the marker and lanes 2 to 6 the clones screened for inserts.

The *pGEM[®]-T Easy* insert was removed from the plasmid backbone using a double digest with *EcoRI* and *NdeI*. The insert was purified from the gel and ligated into the double digested *pET* vectors and propagated using *E. coli* TOP10 cells (section 5.2.7). Five clones of each plasmid were selected for plasmid extraction (section 5.2.5). Clones were grown on either ampicillin [*pET* 22b(+)] or kanamycin [*pET* 28b(+)] containing LB plates overnight and plasmid extracted. The plasmids were screened for the insert of the correct size using a double digest with *EcoRI* and *NdeI* (Figure 5.9). One of each correct clone was selected for expression studies.

Reference gene: ATGAGAAAAGTAGGCAAGCTGGCTGTATTGCGTTTAGCCGCCCTGGGCTTGGCCCTGGCG
T.scotoductus SA-01: ATGAGAAAAGTAGGCAAGCTGGCTGTATTGCGTTTAGCCGCCCTGGGCTTGGCCCTGGCG
Reference gene: GGGCCCCAGGACAACAGCCTGGTCATAGGGGCTTCGACGAGAGCCCCGGGTTCTGGCGGGG
T.scotoductus SA-01: GGGCCCCAGGACAACAGCCTGGTCATAGGGGCTTCGACGAGAGCCCCGGGTTCTGGCGGGG
Reference gene: GACTTCCTAAGCATCATCTCCAACCAAGTCCATCAAGTTGGAGATCGAGCAGTACCTCTTC
T.scotoductus SA-01: GACTTCCTAAGCATCATCTCCAACCAAGTCCATCAAGTTGGAGATCGAGCAGTACCTCTTC
Reference gene: GCCCCCTCATCGGTTTCAACGCCAACAGCGAAAACCTTCCCGTGCTGGTCACCGAGGTG
T.scotoductus SA-01: GCCCCCTCATCGGTTTCAACGCCAACAGCGAAAACCTTCCCGTGCTGGTCACCGAGGTG
Reference gene: CCCACCCGGCAAAACGGGCGTTTGCGGGTGACGGACATCGGCGGGGGCAAGAAGCGCTTG
T.scotoductus SA-01: CCCACCCGGCAAAACGGGCGTTTGCGGGTGACGGACATCGGCGGGGGCAAGAAGCGCTTG
Reference gene: GAGATGGACCTCACCATCCGGCCCGATGCCGCTGGTCCGACGGCAAGCCCATCACCACC
T.scotoductus SA-01: GAGATGGACCTCACCATCCGGCCCGATGCCGCTGGTCCGACGGCAAGCCCATCACCACC
Reference gene: GAGGATGTGGCCTTCTACTACGAGGTGGGCAAGGCCAAGGGATGCCGCTGCTCAACCCG
T.scotoductus SA-01: GAGGATGTGGCCTTCTACTACGAGGTGGGCAAGGCCAAGGGATGCCGCTGCTCAACCCG
Reference gene: GACTACTGGGAGCGGGTGAACCTCCGGGTGAGGGACGCCGCAACTTACCCTGATCTTT
T.scotoductus SA-01: GACTACTGGGAGCGGGTGAACCTCCGGGTGAGGGACGCCGCAACTTACCCTGATCTTT
Reference gene: GAGCCCGCTACTACTACGACACCTACGGCGGCACCTACGGCTCCCCATCGGCTACGCT
T.scotoductus SA-01: GAGCCCGCTACTACTACGACACCTACGGCGGCACCTACGGCTCCCCATCGGCTACGCT
Reference gene: CCCAAGCACATCATGGCGCCGAGTGGGAGAAGGTGAAAGCGGCGGCCGGAACCTGGAT
T.scotoductus SA-01: CCCAAGCACATCATGGCGCCGAGTGGGAGAAGGTGAAAGCGGCGGCCGGAACCTGGAT
Reference gene: CCCGATAAGGATGCGGAGAGGCTCAACGAGCTCTACCGCAACTTCTTCTCAAGTTGCGC
T.scotoductus SA-01: CCCGATAAGGATGCGGAGAGGCTCAACGAGCTCTACCGCAACTTCTTCTCAAGTTGCGC
Reference gene: ACTCCCCAGGCCCTAAACCGGGGAGCCATGGTCTACTCGGGGCGCTTCAAGCTGCGGCG
T.scotoductus SA-01: ACTCCCCAGGCCCTAAACCGGGGAGCCATGGTCTACTCGGGGCGCTTCAAGCTGCGGCG
Reference gene: TGGGTGCCGGGAACCTCCATTGAGATGGAGCGGAACCCCAACTTCCCCATCAAGCCCGAG
T.scotoductus SA-01: TGGGTGCCGGGAACCTCCATTGAGATGGAGCGGAACCCCAACTTCCCCATCAAGCCCGAG
Reference gene: GGTGGGGAGAGCCGGTACGTGCAGAGGGTGGTCTACCGCTTCATCCAGAACAACCACTCC
T.scotoductus SA-01: GGTGGGGAGAGCCGGTACGTGCAGAGGGTGGTCTACCGCTTCATCCAGAACAACCACTCC
Reference gene: CTCCTGGTGGCGCTCCTGGGCGGGAGCATTGACGCCACCTCCAGCGTCTCCCTACCTTT
T.scotoductus SA-01: CTCCTGGTGGCGCTCCTGGGCGGGAGCATTGACGCCACCTCCAGCGTCTCCCTACCTTT
Reference gene: GACCAAGGCCGTAGCCGCCAGCTCACCTCCCGGGCCCTGGCGGCTTGACATCTGGTTG
T.scotoductus SA-01: GACCAAGGCCGTAGCCGCCAGCTCACCTCCCGGGCCCTGGCGGCTTGACATCTGGTTG
Reference gene: GTGCCCGGGCCATCTGGGAGCACATTGACGTCAACAAGTTTGAGAATGCCAGGCGGTG
T.scotoductus SA-01: GTGCCCGGGCCATCTGGGAGCACATTGACGTCAACAAGTTTGAGAATGCCAGGCGGTG
Reference gene: CGCGACTTGGGCTGAACGACGTCCGCACCCGTGCGGGCCCTCCTCCAGCTGTGAACCGG
T.scotoductus SA-01: CGCGACTTGGGCTGAACGACGTCCGCACCCGTGCGGGCCCTCCTCCAGCTGTGAACCGG
Reference gene: GAGGGGTTGGTCAAGGCTTCTTTGACGGCTCCAGCCGTGGCCACACCTGGATCGCC
T.scotoductus SA-01: GAGGGGTTGGTCAAGGCTTCTTTGACGGCTCCAGCCGTGGCCACACCTGGATCGCC
Reference gene: CCGGTCAACCCCTCTTCAACCCCAATGTGCGGAAGTACGAGTTTGACCTGAAGAAGGCG
T.scotoductus SA-01: CCGGTCAACCCCTCTTCAACCCCAATGTGCGGAAGTACGAGTTTGACCTGAAGAAGGCG
Reference gene: GAGGCGCTCTTGGCGGAGATGGGCTGGAGGAAGGGGCGGACGGCATCCTTCAGCGCACC
T.scotoductus SA-01: GAGGCGCTCTTGGCGGAGATGGGCTGGAGGAAGGGGCGGACGGCATCCTTCAGCGCACC
Reference gene: GTGGGTGGCCGACCGTGCCTTTGAGATTGAGTTGCTCACCACCGCGGCAACGCTATC
T.scotoductus SA-01: GTGGGTGGCCGACCGTGCCTTTGAGATTGAGTTGCTCACCACCGCGGCAACGCTATC
Reference gene: CGGGAGCGCACCAGCACTTCTTCGCCGAGGACCTGAAGAAGATCGGCATCGCGTCAAG
T.scotoductus SA-01: CGGGAGCGCACCAGCACTTCTTCGCCGAGGACCTGAAGAAGATCGGCATCGCGTCAAG
Reference gene: ATCAATAACGCCCCAGCGCCGTGGTCTTCGCCGACGACTACATCCAGCGGGCCAGCGAG
T.scotoductus SA-01: ATCAATAACGCCCCAGCGCCGTGGTCTTCGCCGACGACTACATCCAGCGGGCCAGCGAG
Reference gene: TGCAAGTGGACCGGGCTGTTGAGTTGCTTGGGTTTCCAACCTGGCCGAGGATGGCTCC
T.scotoductus SA-01: TGCAAGTGGACCGGGCTGTTGAGTTGCTTGGGTTTCCAACCTGGCCGAGGATGGCTCC
Reference gene: CTCTTCCAGTACAAGAACCTGAACACCGGGCCATCATGGTGGCCACCAAGGAGAACAAC
T.scotoductus SA-01: CTCTTCCAGTACAAGAACCTGAACACCGGGCCATCATGGTGGCCACCAAGGAGAACAAC
Reference gene: TACCAAGGGGAGAACATCGCGGCTGGCGCAACGACGAGTTTGACCTGTGACGAGCCAG
T.scotoductus SA-01: TACCAAGGGGAGAACATCGCGGCTGGCGCAACGACGAGTTTGACCTGTGACGAGCCAG
Reference gene: GGTGTCTTGGAGTTTGACGAGGCCAGGCGGAAGCAGCTCTTCTGGAGGGCCAGGAGATC
T.scotoductus SA-01: GGTGTCTTGGAGTTTGACGAGGCCAGGCGGAAGCAGCTCTTCTGGAGGGCCAGGAGATC
Reference gene: TGGGCCGAGGAGCTGCCTGCCCTTGCCTCTACTTCCGCGCTAACCCTTACGTGGTGCGG
T.scotoductus SA-01: TGGGCCGAGGAGCTGCCTGCCCTTGCCTCTACTTCCGCGCTAACCCTTACGTGGTGCGG
Reference gene: AAGGGCTTGGTCAACTACGTGGCCAGCGCTTACGCGGGCGGCTACGGTTACCCCGGCTGG
T.scotoductus SA-01: AAGGGCTTGGTCAACTACGTGGCCAGCGCTTACGCGGGCGGCTACGGTTACCCCGGCTGG
Reference gene: AACGCTTGGGAGATCGGCTGGGAGAGCCGCGCGCGGTGAAGAAGTGGGACCAGGCGAAG
T.scotoductus SA-01: AACGCTTGGGAGATCGGCTGGGAGAGCCGCGCGCGGTGAAGAAGTGGGACCAGGCGAAG
Reference gene: TACGCTCTTTCCGTCAAGTAA
T.scotoductus SA-01: TACGCTCTTTCCGTCAAGTAA

Figure 5.8 Sequence alignments of the reference ABC gene form the *Thermus scotoductus* SA-01 genome database and the cloned ABC gene form *Thermus scotoductus* SA-01.

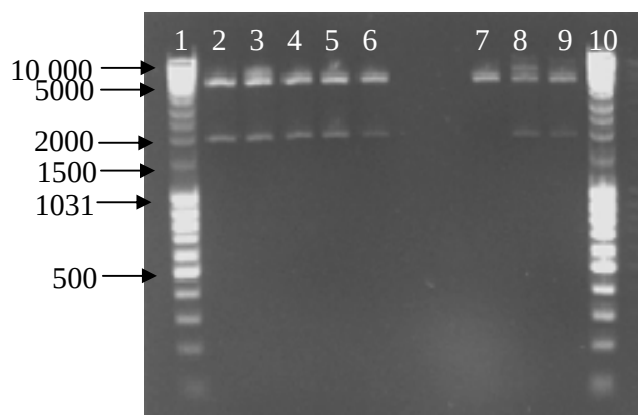


Figure 5.9 pET 22b(+) and 28b(+) plasmids containing the ABC gene digested with EcoRI and NdeI. Lanes 1 and 10 represents the marker, lanes 2 to 6 the pET 22b(+)- and lanes 7 to 9 the pET 28b(+) clones screened for inserts.

5.3.3. Expression and purification of the recombinant ABC proteins

The main aim of chapter 5 was to over-express the protein purified from *Thermus scotoductus* SA-01 in chapter 4 for upscale applications and to determine if the recombinant proteins have the same gold(III) reducing and nanoparticle producing capabilities.

The gene sequence was obtained from the *Thermus scotoductus* SA-01 genome database constructed through pyrosequencing (Gounder, 2009). Reverse and forward primers were designed using the ORF and restriction sites were incorporated to facilitate cloning of the gene into the desired plasmids (section 5.2.3). The pET 22b(+) and pET 28b(+) plasmids containing the ABC transporter, peptide-binding protein was used to over-express the ABC transporter, peptide-binding protein either with or without a N-terminal His-tag (sections 5.2.8 and 5.2.9). His-tag expression was done to aid the purification of the recombinant protein and to determine whether the His-tag influences the proteins ability to reduce gold(III) and produce nanoparticles.

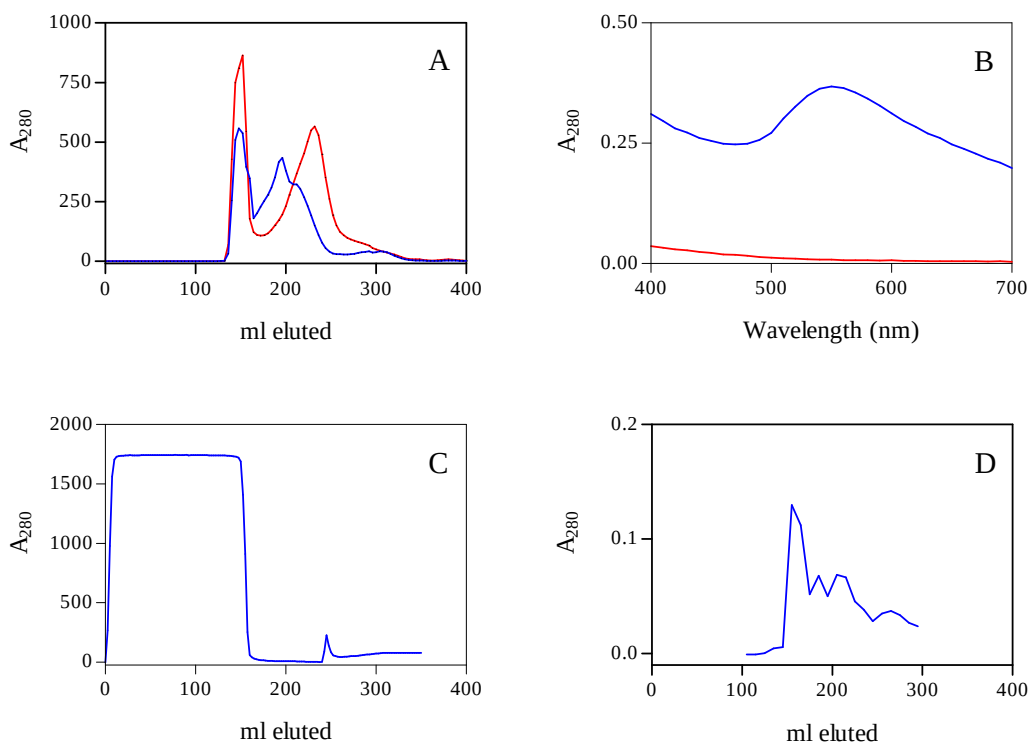


Figure 5.10 (A and D) Elution profile obtained from the Sephacryl S200HR resin. (B) Wavelength scan conducted at 24h of incubation in the presence of 2mM gold(III) at 65°C, pH 7.4. and (C) Elution profile obtained from the Metal-affinity resin. (—) Vectors containing the ABC gene and (—) the negative control.

During purification of the recombinant proteins (section 5.2.9), SDS-PAGE (section 4.2.4) was run to indicate which fractions contained the expressed protein as more than one peak was observed when the protein fraction was loaded onto the size-exclusion resin (Figure 5.10 A and D). The SDS-PAGE results also indicated the degree of homogeneity of the recombinant proteins after purification steps (Figure 5.11 A and B). The pET 22b(+) recombinant protein does not have a His-tag to help with purification, thus a heat shock step was performed before loading of the fraction onto the size-exclusion resin to remove the non thermostable proteins. The heat shock step gave good results as many proteins were removed from the solution, but several contaminating proteins were still present (Figure 5.11 A). After the size-exclusion run (Figure 5.10 A) more of the contaminating proteins were removed and the recombinant protein was close to being homogeneous (Figure 5.11 A). The negative control (empty plasmid) did not possess the ability to produce nanoparticles as the

UV-Vis spectra showed no peak in the 540nm range (Figure 5.10 B). No particles were produced through chemical reduction when the solutions were incubated in the presence of 2mM gold(III) at 65°C for 24 hours (section 4.2.6.3) as no colour change was observed. On the other hand the purification of the pET 28b(+) recombinant protein was done using the nickel-affinity resin because the His-tag has an affinity for nickel and can be displaced by imidazole as they have basically the same functional group, but imidazole has a higher affinity for the nickel than the His-tag. Only one binding peak (Figure 5.10 C) was observed and it was loaded onto the size-exclusion resin to remove the imidazole from the protein solution and to remove contaminating proteins. Four distinct peaks (Figure 5.10 D) were observed and SDS-PAGE analysis (section 4.2.4) showed that most of the contaminating proteins were removed and it indicated which of the peaks represented the recombinant protein (Figure 5.11 B).

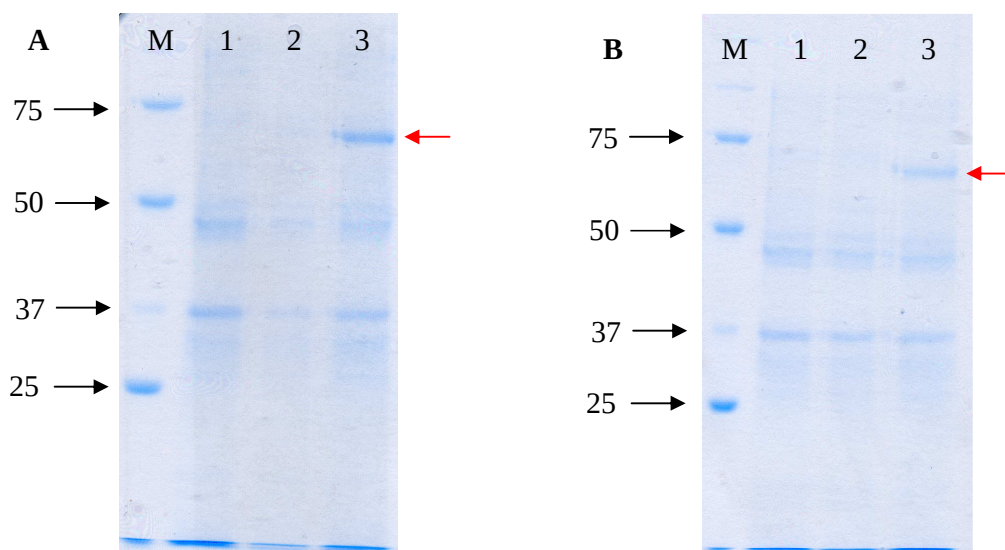


Figure 5.11 SDS-PAGE analysis of the recombinant ABC protein when using the (A) pET 22b(+) and (B) pET 28b(+) vector system. (M) marker, (1) negative control at 4h post-induction, (2) ABC insert at 0h post-induction, (3) ABC insert at 4h post-induction.

5.3.4. Characterization of the recombinant ABC proteins

The recombinant proteins were evaluated for the ability to reduce gold(III) and produce nanoparticles by visually following colour formation (yellow to pinkish-bluish) and by obtaining UV-vis spectrums to monitor the plasmon resonance band. The recombinant proteins were characterized using the same physico-chemical parameters used for characterizing the native protein. However, when the pET 22b(+) recombinant protein was used at $100\mu\text{g.mL}^{-1}$ no colour formation and no plasmon resonance band was observed for any of the reactions. A protein range ($0 - 100\mu\text{g.mL}^{-1}$) was incubated in the presence of 2mM gold(III), $4.6\mu\text{M}$ sodium dithionite for 24h at 65°C to determine if reduction of gold(III) was possible when using a lower protein concentration (section 5.2.10.6). Results showed that a colour change could be observed when protein concentrations were less than $20\mu\text{g.mL}^{-1}$. It was thus decided to characterize the pET 22b(+) recombinant protein using a protein concentration of $20\mu\text{g.mL}^{-1}$. The characterization of the pET 28b(+) recombinant protein was done using a protein concentration of $100\mu\text{g.mL}^{-1}$.

5.3.4.1. The effect of temperature on particle production

The effect of temperature on particle formation was evaluated as described in section 5.2.10.3. From the micrographs (Figure 5.13) a difference in nanoparticle production was observed between the His-tagged and untagged recombinant proteins when comparing the lower incubation temperatures. No difference was observed for the higher temperatures. Compared to the particles produced by the native protein (section 4.3.5.2) the recombinant proteins had less shape definition at the lower temperatures but at 65°C the untagged recombinant protein (Figure 5.13 A and B) had about the same degree of shape definition. Unlike the native protein which produced mainly spherical particles at 75°C the recombinant proteins produced no shape specific particles. From the TEM micrographs (Figure 5.13) and the visual observations (Figure 5.12) it seems as if the 65°C temperature range is optimum for the recombinant proteins to produced shape specific nanoparticles. There does not seem to be a significant difference in the ability of the recombinant proteins to produced nanoparticles when compared to the native protein when temperature is the physico-chemical parameter which is varied. Since the disulphide bond is surface exposed, reduction and particle formation is not a temperature induced binding or stabilization of a transition state.

Although an increase in temperature might cause more reduced activated species, which in turn would readily reduce gold(III) and / or serve as nucleation sites.

The His-tagged recombinant protein was not as effective as the native and untagged recombinant protein in producing shape specific nanoparticles. This could be the result of the presence of the N-terminal His-tag which may influence protein folding and thus the positioning of the cysteine residues. Also this protein is expressed in a mesophilic host which may also influence the folding of the protein (supplementary notes section A.2). However further analysis needs to be done before any concrete hypotheses can be drawn.

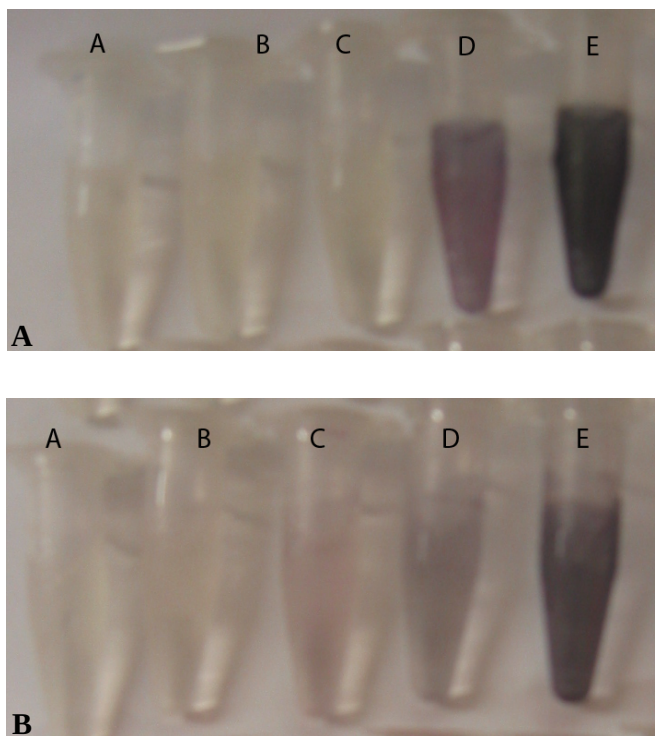


Figure 5.12 Photo representing the colour change observed when the purified recombinant (A) untagged and (B) His-tagged proteins were exposed to different temperatures during the gold assay. (A) 30°C, (B) 42°C, (C) 55°C, (D) 65°C and (E) 75°C.

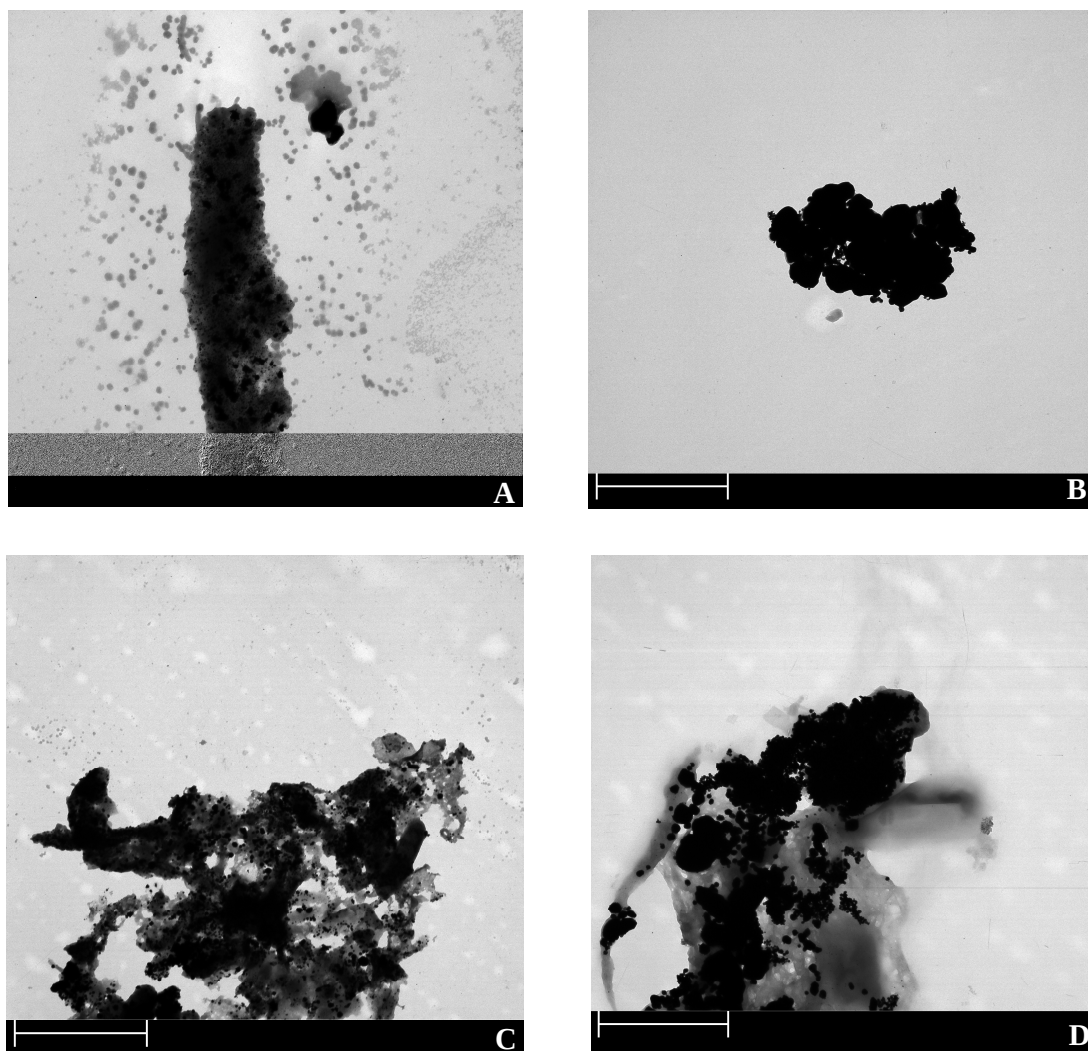


Figure 5.13 TEM micrographs of the purified recombinant (A and B) untagged and (C and D) His-tagged proteins exposed to different temperatures during the gold assay. (A and C) 42°C and (B and D) 75°. Scale bars equal to (A, B and D) 1000nm and (C) 2 μ m.

5.3.4.2. The effect of pH on particle production

The effect of pH on particle formation was evaluated as described in section 5.2.10.4. Visual observations (Figure 5.14 A and B) indicated no significant difference in gold(III) reduction and nanoparticle production ability between the recombinant proteins. The recombinant proteins did not possess the ability to produce particles with clear morphologies at the lower pH ranges as opposed to the native protein (4.3.5.1) which in the low pH ranges is conducive to the formation of nanoplates / nanowires. Once again it is seen that the initial pH has an

effect on the type of particles produced as was observed by He and co-workers (2007). The ability of the recombinant proteins to produce nanoparticles at a neutral pH was similar to that of the native protein as in both cases shape specific particles was observed, although the particles produced by the His-tagged recombinant protein were mostly spherical. An alkaline pH resulted in the loss of shape which was observed for the neutral pH range. At this stage of characterization it seems as if the presence of the N-terminal His-tag has a negative effect on the ability of the recombinant protein to reduce gold(III) and produce nanoparticles. So even though the His-tag facilitates the purification of the protein it is not conducive to shape specific particle production.

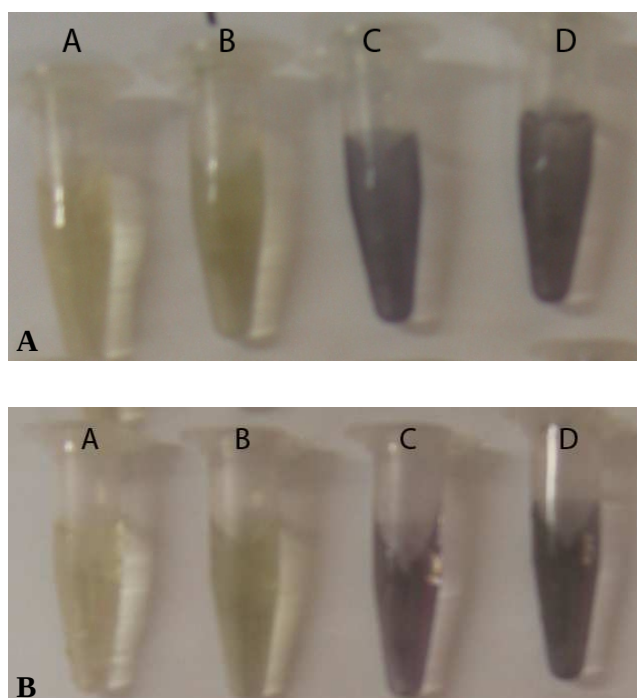


Figure 5.14 Photo representing the colour change observed when the purified recombinant (A) untagged and (B) His-tagged proteins were exposed to different pH buffers. (A) pH 9, (B) pH 7.4, (C) pH 5.5 and (D) pH 3.6.

5.3.4.3. The effect of gold(III) and sodium dithionite concentration on particle production

The effect of gold(III) and sodium dithionite concentration on particle formation was evaluated as described in sections 5.2.10.5 and 5.2.10.8. Visual observations (Figure 5.15) indicated a significant difference between the recombinant proteins ability to reduce gold(III) and produce nanoparticles when gold(III) concentration is varied. The untagged recombinant protein could produce particles in the nanometer range at gold(III) concentrations less than 5mM while at concentrations greater than 5mM the particles were outside the range. On the other hand the His-tagged recombinant protein could only produce particles which fall into the nanometer range at gold(III) concentrations between 2 and 5mM. The particles produced at gold(III) concentrations below 5mM were mostly spherical to amorphous, with a few shape specific particles. This differs greatly from the data collected for the native protein experiments (section 4.3.5.3). All three of the proteins gave nanoplate structures when incubated with 10mM gold(III) [Figure 5.16]. It seems as if the presence of the His-tag does not have any effect on particle production when the gold(III) concentration exceeds 10mM. This could imply that protein morphology and position of the disulphide bond may not play a significant role in the reduction and particle formation process when gold exceeds the optimum stoichiometric ratio of protein to gold(III) concentration. Ongoing studies where particles are produced using similar techniques showed no salts present in the samples when subjected to elemental analysis (M. Erasmus, M.Sc. study)

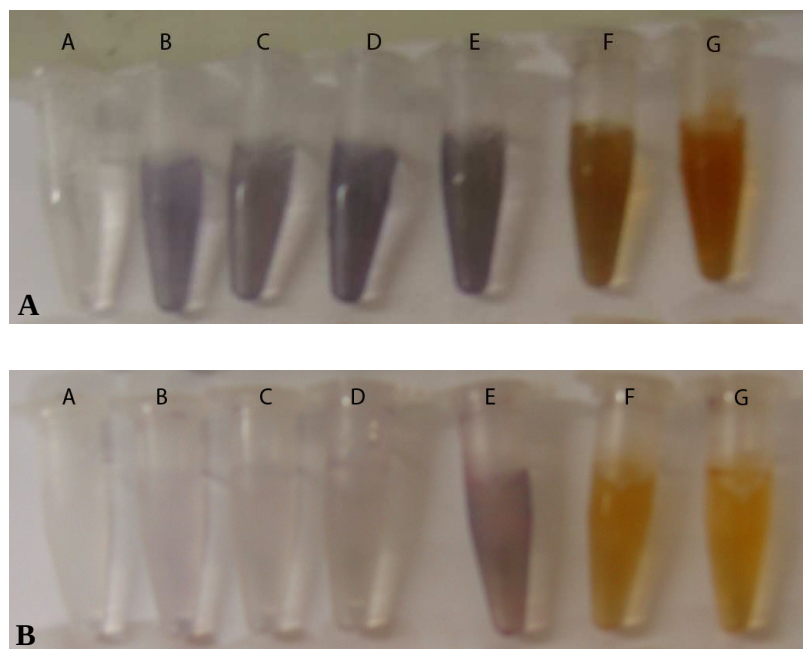


Figure 5.15 Photo representing the colour change observed when the purified recombinant (A) untagged and (B) His-tagged proteins were exposed to varying gold(III) concentrations. (A) 0.1mM, (B) 0.5mM, (C) 1mM, (D) 2mM, (E) 5mM, (F) 7.5mM and (G) 10mM.

Reduction of gold(III) only occurred in the presence of the protein and then only when sodium dithionite was added to the reduction mixture. Different concentrations of sodium dithionite had no effect on the shape and size of the particles produce. It can be concluded that sodium dithionite acts only to reduce the disulphide bridge in the protein, which then supplies an initiation site for gold reduction and subsequent nanoparticle formation. As the disulphide bond is located on the surface of the protein there is no real access restrictions for the sodium dithionite and gold(III) to reach the disulphide bond and this may not be the area to manipulate for control over nanoparticle production. The influence of temperature, pH and gold(III) concentration on particle shape and size might also be attributed to the particle growth rate, where slower growth rates yield defined shapes but a too-rapid growth only leads to amorphous particles – a phenomena often encountered in protein crystallisation, as well as metal crystal growth.

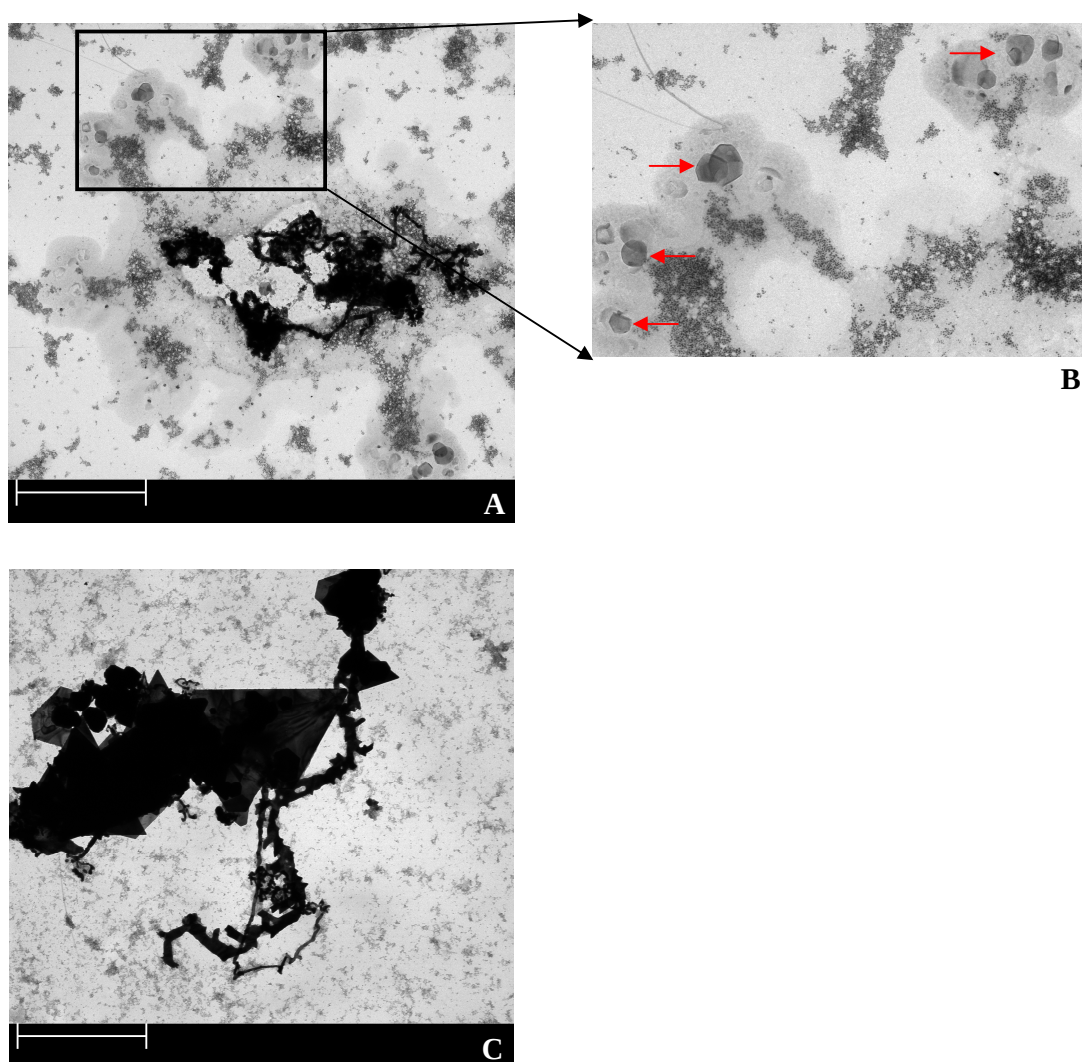


Figure 5.16 TEM micrographs of the purified recombinant (A and B) untagged and (C) His-tagged proteins when exposed 10mM gold(III). (B) enlargement of A. Scale bars equal (A) 20mm and (C) 1000nm.

5.3.4.4. The effect of exposure time on particle production

The effect of exposure time on particle formation was evaluated as described in section 5.2.10.7. As for the native protein (section 4.3.5.5) particles could be observed for the recombinant protein after 8 hours of incubation. These particles are extremely small and the visual observations (Figure 5.17 A and B) only indicated a slight change in colour. However, the results for the 48 hour incubation reaction was different than those obtained for the native protein. The particles were larger and less monodisperse. The optimum exposure time for

the synthesis of shape specific particles is 24 hours as no other period resulted in any defined shapes. None of the incubation times gave any shape specific particles and it seems as if incubation time can not be used to manipulate shape when using the recombinant proteins but it can be used to manipulate the size of the particles as an increase in incubation times allows for longer contact time between the reduced gold and the seed particles allowing the nanoparticles to grow (Kawamura and Nogami, 2009; Tang and Hamley, 2009).

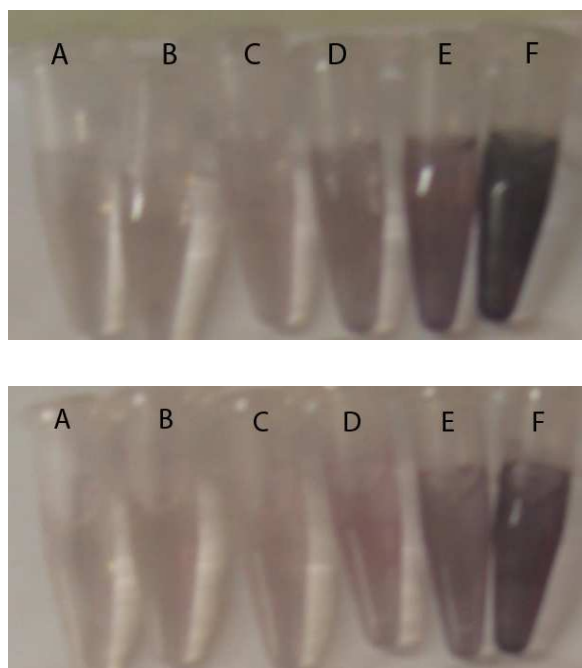


Figure 5.17 Photo representing the colour change observed when the purified pET 22b(+) recombinant protein was exposed to varying incubation times. (A) 2h, (B) 4h, (C) 8h, (D) 12h, (E) 24h and (F) 48h.

5.3.5. Modelling of the native protein

Modelling of the protein showed a disulphide bond (Figure 5.18) which is accessible from the exterior of the protein, supplying a possible nucleation site for gold(III) reduction and particle production once the bond has been broken by the addition of a reducing agent such as sodium dithionite. No other reducing agents were tested but varying the reducing agent might increase the efficiency of the protein to act as nucleation site.

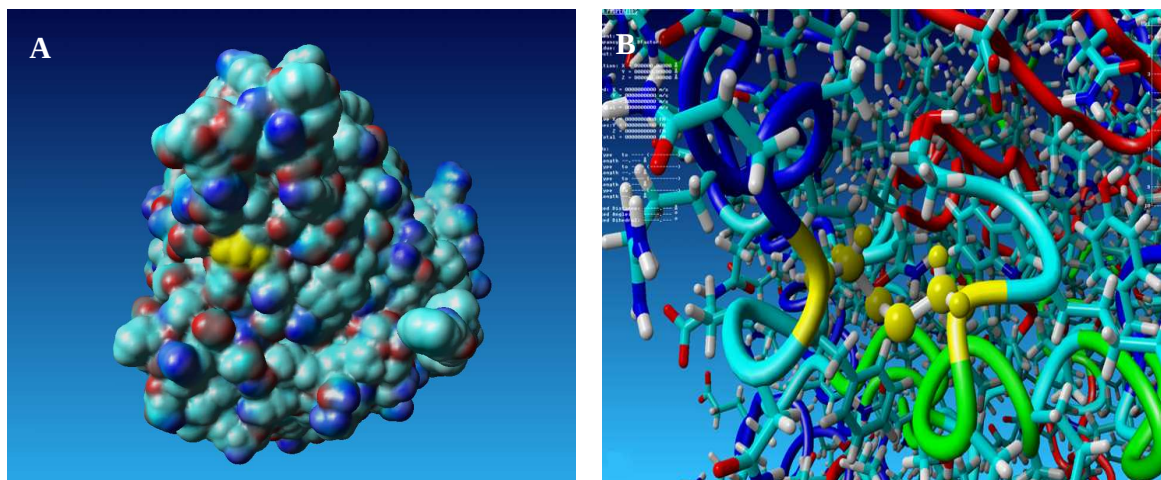


Figure 5.18 (A) Homology model of Thsc-ABC. The surface exposed disulphide bond which is hypothesized to be involved in the reduction of gold is shown in yellow. (B) Detail of the environment of the disulphide bond.

5.4. Conclusions

The ABC transporter, peptide-binding protein of *Thermus scotoductus* SA-01 can be readily over-expressed in *E. coli* as a soluble protein which retain its gold(III) reducing and nanoparticle producing capabilities even though the gold(III) reducing and nanoparticle producing activity of the recombinant proteins were not similar to that of the native protein's activity. The difference in the ability of these proteins to reduce gold(III) and produce nanoparticle might be contributed to miss-folding of the recombinant proteins as the purified ABC transporter, peptide-binding protein is a thermophilic protein which is expressed in a mesophilic host.

Studies indicated the involvement of the disulphide bond in gold(III) reduction and nanoparticle production similar to that observed from other literature but it was shown here that by manipulating the reaction conditions particle formation could not be controlled. From the purification strategies it was clear that *Thermus scotoductus* SA-01 contains others proteins with the capability to reduce gold(III) and produce nanoparticle. Future studies might include purification of the other proteins to determine whether they are better suited for

manipulation to produce monodisperse particles. Also viable is the use of site-directed mutagenesis to remove the cysteine residues of the disulphide bond thought to be the reducing and / or nucleation site for gold(III) reduction and nanoparticle production. This can be done to evaluate whether the protein will retain the gold(III) reducing and nanoparticle producing ability once there are no cysteine residues to form a disulphide bond.

The recombinant proteins could possibly be used in upscale production of nanoparticles once the optimum physico-chemical parameters are established. Expression of the recombinant proteins without the leader peptide and cleaving of the His-tag might influence the ability of the protein to produce nanoparticles as both the leader peptide and the His-tag may effect the protein folding and thus the conformation of the protein. Thus without these peptide sequences the conformation might be closer to that of the native protein and the gold(III) reducing and nanoparticle producing ability might be more similar than the results obtained in this chapter. As this is all speculation it would be a viable option to express the proteins without the leader peptide and to remove the N-terminal His-tag after purification and then compare the characterization results to the results obtained for the native protein.

5.5. References

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SUPPLEMENTARY NOTES A

A. EXPERIMENTAL METHODOLOGY

A.1. Purification strategies

Initial strategies to purify a gold(III) reducing and nanoparticle producing protein from *Thermus scotoductus* SA-01 were derived from the hypotheses made by Duran and co-workers (2005), He and co-workers (2007) as well as Nangia and co-workers (2009). Their hypotheses focuses on the involvement of NADH- and NADPH-dependent enzymes in metal reducing reactions. Some examples of NAD(P)H dependent reduction of metals include the reduction of Cr(VI) to Cr(III) by *Pseudomonas ambigua* G-1, where the reduction of one mol Cr(VI) to Cr(III) required the addition of three mol NADH. When NADPH instead of NADH was used as electron donor the activity of the reductase increased 63% (Suzuki *et al.*, 1992). Another example is the reduction of Fe(III) to Fe(II) by the hyperthermophilic archaeon *Archaeoglobus fulgidus*. This bacterium contains a ferric reductase which can use both NADH and NADPH as electron donors for iron reduction. Kinetic studies on this enzyme showed that ferric reductase had a slightly higher affinity towards NADH as electron donor (Vadas *et al.*, 1999).

Throughout all the purification steps the gold(III) reducing and nanoparticle producing capabilities were evaluated by visually following colour formation (yellow to pinkish-bluish) and by obtaining UV-vis spectrums either in the absence or presence of co-factors as described in sections 4.2.6.1 and 4.2.6.3.

An anion-exchange resin (Super Q-Toyopearl) was used as first step of the purification strategy. Here it was found that both the interacting and non-interacting protein fractions showed the ability to synthesize nanoparticles. The interacting fraction was further subjected to purification on the anion resin with a stretched gradient to increase the separation resolution. The protein peaks with reducing capabilities were further purified by using the HIC column Phenyl-Toyopearl. This step resulted in the loss of the ability to reduce gold(III) and to produce nanoparticles. This approach was abandoned.

The non-interacting protein fractions, obtained from Super Q-Toyopearl resin, with gold(III) reducing abilities were used for the next purification strategy. As they did not interact with the anion-exchanger, a cation exchanger was chosen as an alternative step of purification. Here the interacting protein fractions had the ability to reduce gold(III). Even though activity was still observed without the addition of a co-factor, the reaction time was longer than previously observed, suggesting that most of the native co-factors have been removed. Reactions were supplemented with NADH to increase the rate of gold(III) reduction and nanoparticle production as literature suggested NADH as electron donor (Mukherjee *et al.*, 2002; Senapati *et al.*, 2005). It was found that NADH did in fact increase the reduction rate as a colour change (Figure A.1) was visible after 30 minutes while the reactions incubated without the addition of NADH only changed colour after overnight incubation. These results enhanced the thought that a possible NADH-dependent protein might be involved in the production of gold nanoparticles. The hypotheses of Duran and co-workers (2005) and He and co-workers (2007) as well as this data indicated that the use of the dye-affinity resin, Blue Sepharose CL-6B, was an option as this resin has the ability to bind several enzymes including kinases, dehydrogenases and most enzymes requiring adenylcontaining co-factors (including NAD⁺ and NADP⁺) [Amersham Biosciences, 2002].

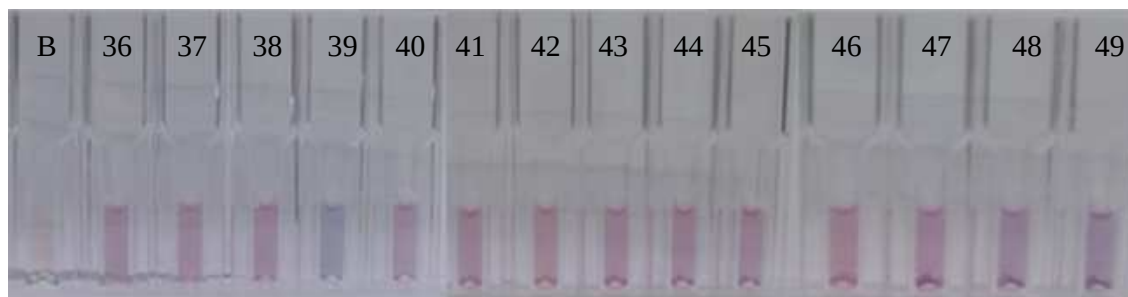


Figure A.1 Visual assay after 30 minutes incubation at 45°C in the presence of 0.1mM Au(III) and 0.3mM NADH. (B) is the blank and the numbers corresponds to the fractions obtained from the SP-Toyopearl column elution.

The active fractions obtained from the second SP-Toyopearl resin was pooled and loaded onto the Blue Sepharose CL-6B resin. Two distinct peaks were eluted, one associated with the non-interacting protein fractions and the other with the interacting protein fractions

(Figure A.2). SDS-PAGE analysis on the interacting protein peak showed that there is still a number of proteins present in these fractions (Figure A.3). Alternatively the active fractions obtained from the SP-Toyopearl resin were pooled into two separate fractions (SP2P1 and SP2P2). Each of these fractions was loaded onto the Blue Sepharose CL-6B resin. The SP2P1 eluted mostly in the non-interacting protein fraction with various small peaks in the interacting protein fractions. On the other hand the SP2P2 pooled fraction eluted with the interacting protein fraction indicating that conservative pooling of the fractions might enhance separation. Neither of these steps resulted in the protein fractions which had the ability to reduce gold(III) and produce nanoparticles. When these fractions were assayed for nitrate reducing activity it was found that nitrite production was associated with the interacting protein peak (SP2P2) and was widely detected for the SP2P1 fraction. It was decided to continue downstream purification using the interacting protein peak obtained from the Blue Sepharose CL-6B run when the pooled SP2P2 fraction was used. This was decided as the non-interacting protein peak obtained from the Blue Sepharose CL-6B run when the pooled SP2P1 fraction was used were loaded onto the HIC resin resulted in several protein peaks (Figure A.4). SDS analysis showed that all of the peaks had low homogeneity (Figure A.5).

The next resin used was the HIC resin Phenyl-Toyopearl. Only one peak was observed and was located in the interacting protein fraction. SDS analysis indicated a homogeneous protein but to confirm this a size exclusion chromatography step was included. The S200HR resin was chosen and SDS analysis now showed two closely associated protein bands. As a homogeneous protein is necessary for N-terminal sequencing, a re-evaluation of the SDS protocol indicated that this step affected the integrity of the protein. Now it was confirmed that the protein was indeed homogenous and it was sent for N-terminal sequencing. Although these fractions did not show any gold(III) reducing capabilities when using the standard assay method (section 4.2.6.1), activity was observed when the nitrate reductase assay was adapted (section 4.6.2.3). This assay requires sodium dithionite to work and implies that the protein needs to be in its reduced state to function as a gold reductase. This could indicate the presence of sulphur containing amino acids which might be involved in the reduction process, which was different from published literature but not too cumbersome as data published by Scott and co-workers (2008) indicated the involvement of disulphide moieties in the reduction of gold(III).

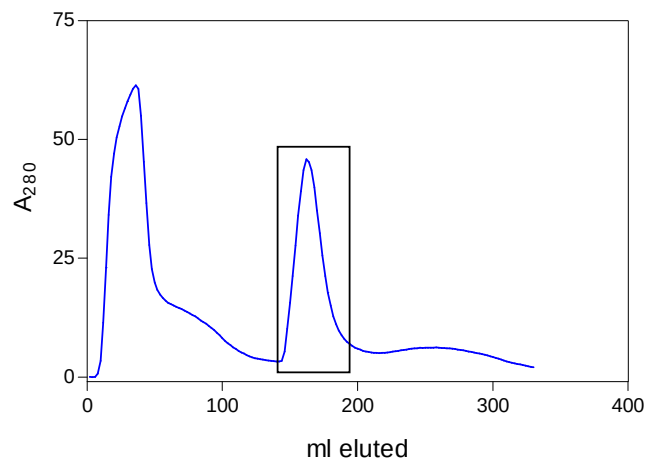


Figure A.2 Elution profile obtained from the Blue Sepharose CL-6B resin when loaded with the pooled fraction obtained from the SP-Toyopearl resin.

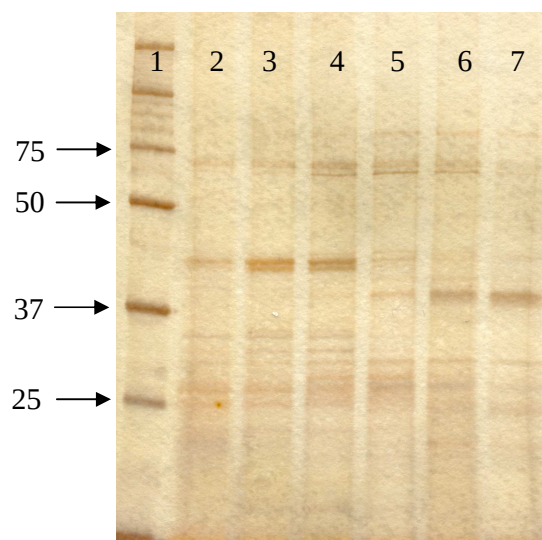


Figure A.3 SDS-PAGE analysis of the interacting protein fractions obtained from the Blue Sepharose CL-6B resin. Each lane was loaded with $2\mu\text{g}$ protein. Lane (1) marker and (2-7) corresponds to the protein fractions indicated by the block in Figure A.2.

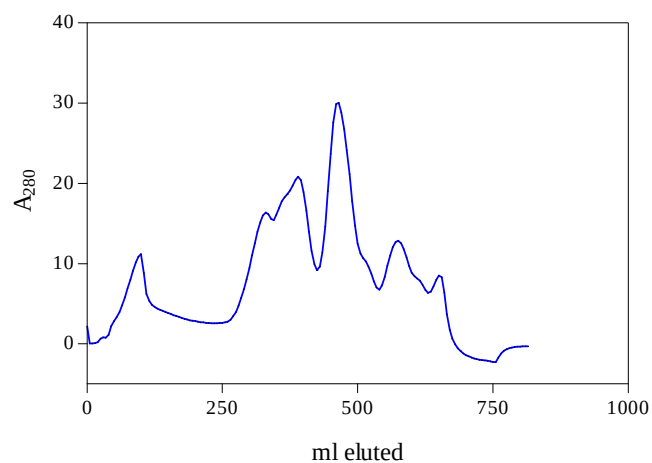


Figure A.4 Elution profile obtained for the Phenyl resin when loaded with the SP2P1 fraction.

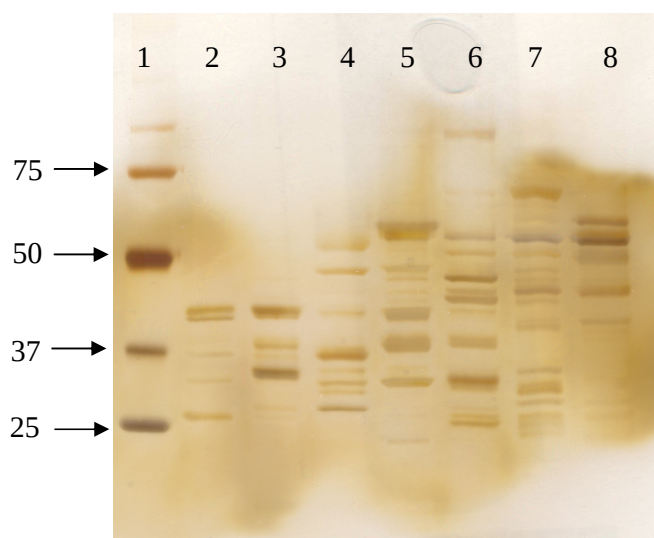


Figure A.5 SDS-PAGE analysis of the interacting protein fractions obtained from the Blue Sepharose CL-6B resin. Each lane was loaded with 2 μ g protein. Lane (1) marker and (2-8) corresponds to the protein fractions indicated by the block in Figure A.2.

A.2. Justification of the adapted assay

N-terminal sequencing showed that the purified protein was not the expected oxido-reductase. Instead it was identified as an ABC transporter, peptide-binding protein. This protein has no cavity for the binding of NAD(P)H, but it has two cysteine residues which are involved in the formation of a disulphide bridge. Scott and co-workers (2008) demonstrated that the mechanism of glutathione reductase can be harnessed to produce metallic nanoparticles. They concluded, after several independent experiments, that gold(III) is reduced at the glutathione binding site of the glutathione reductase and that the catalytic cysteines are directly involved in the reduction.

Our hypothesis is based on the presence of the cysteine residues. The cysteine residues are reduced by the addition of a reducing agent (sodium dithionite). These residues then acts as nucleation site for gold(III) reduction and subsequent particle formation. No visible reduction (colour change) could be observed in the absence of sodium dithionite.

A.3. References

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SUMMARY

The usual strategy to prepare gold nanoparticles involves the reduction of a gold salt in solution by various reducing agents in the presence of a stabilizer. These particles are mostly spherical with poor monodispersity. An alternative means is to use biological material to mediate particle synthesis. Microorganisms such as fungi have demonstrated the ability to produce nanoparticles of different shapes and sizes extending beyond the scope of chemical means, and the microbial interaction with metals also supply eco-friendly methods for nanoparticle production.

It has been hypothesized that the proteins involved in nanoparticle synthesis require a co-factor such as NADH / NADPH, as previous studies have indicated that NADH- and NADPH-dependent enzymes are important factors in the biosynthesis of metal nanoparticles.

Thermus scotoductus SA-01, a thermophilic bacterium, isolated from an AngloGold Ashanti mine near Carletonville, Republic of South Africa, was used for purification of a gold(III) reducing and nanoparticle synthesizing protein. This bacterium has the ability to produce gold nanoparticles, and more than one pathway can be followed to produce these particles. A protein was purified to homogeneity by using a combination of several liquid chromatography resins. The N-terminal sequence was obtained by using automated Edman degradation. The protein purified is not a classical oxido-reductase and was identified as an ABC transporter peptide-binding protein (~70kDa). This discovery shows that gold nanoparticles can be produced by proteins other than oxidoreductases. The interaction of the protein extracted and purified from *Thermus scotoductus* SA-01, as well as the recombinant proteins, with liquid gold under varying physico-chemical conditions have been studied using TEM, EDS, and by measuring the plasmon resonance band, to illustrate the effect on particle morphology and to elucidate the protein mechanism.

The size and the shape of particles could, to an extent, be manipulated by controlling the environmental parameters. The purified protein as well as the recombinant proteins was only able to produce nanoparticles in the presence of sodium dithionite and it is thus hypothesized that the donation of electrons *via* the disulphide bridge in the protein is involved in the reduction of the gold ions. Even though the recombinant proteins had the ability to

produce nanoparticles they were not as efficient as the native protein, but when the optimum parameters for the recombinant proteins are established they could be used in the upscale production of gold nanoparticles or gold nanosheets.

Key words: Metal nanoparticles; gold; biological reduction; ABC transporter; nanobiotechnology

OPSOMMING

Die algemeenste strategie vir die voorbereiding van nanopartikels behels die reduksie van 'n opgeloste goudsout deur verskeie reduceermiddels in die teenwoordigheid van 'n stabiliseerder. Hierdie partikels is meestal sferies met lae monodispersiteit. 'n Alternatiewe manier om partikels te vorm is om biologiese materiaal te gebruik. Mikroörganismes, soos fungi, beskik oor die vermoë om nanopartikels van verskillende vorme en groottes te produseer, wat 'n verbetering is op die gebruik van chemiese metodes. Hierdie mikrobiese interaksie met metale verskaf ook 'n omgewingsvriendelike metode vir die produksie van nanopartikels.

Vorige studies het getoon dat NADH- en NAD(P)H- afhanklike ensieme belangrike faktore is in die biosintese van metaal nanopartikels. Dit het gelei tot die hipotese dat proteïene wat betrokke is by goud nanopartikel sintese 'n kofaktor soos NADH / NAD(P)H benodig.

'n Proteïen wat in staat is om goud te reduceer en nanopartikels te vorm, is gesuiwer vanuit die termofiele bakterium, *Thermus scotoductus* SA-01. Hierdie bakterium was geïsoleer vanuit 'n AngloGold Ashanti myn naby Carletonville, Republiek van Suid-Afrika en het die vermoë om goud nanopartikels te produseer deur meer as een biologiese weg te volg. Die proteïen is gesuiwer deur gebruik te maak van 'n kombinasie van verskeie vloeistof-chromatografie harse. Die N-terminale proteïen volgorde is verkry deur gebruik te maak van Edman degradering. Die gesuiwerde proteïen is nie 'n klasieke oksido-reduktases nie, maar is geïdentifiseer as 'n ABC transporter-peptied-bindings proteïen (~70kDa). Hierdie ontdekking wys dat goud nanopartikels geproduseer kan word deur ander proteïene as oksido-reduktases. TEM, EDS en die meting van die plasmon resonans band is gebruik om die effek van variërende fisiese-chemiese kondisies op partikel morfologie te toon en om die proteïen meganisme op te klaar. Vir die doel is die gesuiwerde proteïen vanuit *Thermus scotoductus* SA-01 asook rekombinante proteïene gebruik.

Die grootte en vorm van die partikels kon tot 'n mate gemanipuleer word deur omgewingsfaktore te beheer. Die gesuiwerde proteïen asook die rekombinante proteïene kon slegs nanopartikels produseer in die teenwoordigheid van natriumdithionaat en 'n hipotese is

geformuleer dat die disulfied brug in die proteïen betrokke is by die reduksie van die goud ione. Alhoewel die rekombinante proteïene oor die vermoë beskik om nanopartikels te produseer is dié nie so effektief soos die gesuiwerde proteïen nie. Indien die optimale kondisies vir die rekombinante proteïene uitgeklaar word, sal dié waarskynlik in die grootskaalse produksie van goud nanopartikels of goud nanoplate gebruik kan word.

Sleutel woorde: Metaal-nanopartikels; biologiese reduksie; ABC transporter; goud; nanobiotegnologie