BIOSYNTHESIS OF SULFIDE NANOPARTICLES BY *FUSARIUM OXYSPORUM*, PURIFICATION AND CHARACTERIZATION OF ENZYMES AND PROTEINS RESPONSIBLE FOR THE EXTRACELLULAR SYNTHESIS

A THESIS

SUBMITTED TO THE

UNIVERSITY OF PUNE

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY
IN
BIOTECHNOLOGY

BY

S. ANIL KUMAR

DIVISION OF BIOCHEMICAL SCIENCES
NATIONAL CHEMICAL LABORATORY
PUNE-411 008
INDIA

April 2007

Dedicated to my Beloved Parents and Wife...

"Where nature finishes producing its own species, man begins, using natural things and with the help of this nature, to create an infinity of species."

Nobel laureate:

Jean Marie Lehn.

TABLE OF CONTENTS

ACKNOWLEDGEMENT

CERTIFICATE

Certified that the work incorporated in the thesis entitled: **"Biosynthesis of sulfide nanoparticles by *Fusarium oxysporum*, Purification and characterization of enzymes and proteins responsible for the extracellular synthesis"**, submitted by **Mr. S. Anil Kumar**, to Pune University for the Degree of ***Doctor of Philosophy in Biotechnology***, was carried out by the candidate under my supervision at Biochemical Sciences Division, National Chemical Laboratory, Pune-411 008, India. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

Dr. M. I. Khan

(Research Guide)

DECLARATION BY THE CANDIDATE

I hereby declare that the thesis entitled **"Biosynthesis of sulfide nanoparticles by *Fusarium oxysporum*, Purification and characterization of enzymes and proteins responsible for the extracellular synthesis"**, submitted to Pune University for the Degree of *Doctor of Philosophy in Biotechnology*, was carried out by me during the period from **3rd Dec, 2001** to **15th Dec, 2006** under the guidance of **Dr. M. I. Khan** and has not formed the basis for the award of any degree, diploma, associateship, fellowship, titles in this or any other University or other institute of higher learning**.**

I further declare that the material obtained from other sources has been duly acknowledged in the thesis.

Date: Signature of the Candidate

                                                                           S. Anil Kumar

ABBREVIATIONS

β-ME	2, 4 -Mercapto ethanol
α-NADPH	Nicotinamide hypoxanthine dinucleotide phosphate reduced tetra sodium salt
AAS	Atomic absorption spectrometry
BSA	Bovine serum albumin
CdS	Cadmium sulfide
CdSe	Cadmium selenide
CM	Carboxymethyl
CPL	Chickpea lectin
CTAB	Hexadecyltrimethylammonium bromide
DEAE	Diethylaminoethyl
EDC	1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide
EDTA	Ethylene diamine tetra acetic acid
FTIR	Fourier transform infrared spectroscopy
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HTAC	Hexadecyl trimethylammonium chloride
kDa	Kilo dalton
K_m	Michaelis-Menten constant
MES	2 (N-Morpholino) ethanesulfonic acid
NIR	Near-infrared region
NTEE	3-nitro-L-tyrosine ethylester
QDs	Quantum dots
SAED	Single area electron diffraction
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
SMAD	Spray pyrolysis and solvated metal atom dispersion
TEM	Transmission electron microscope
UV/Vis	Ultraviolet/ visible spectroscopy
V_{max}	Maximum velocity
XPS	X-ray photoelectron spectroscopy
XRD	X-ray diffraction

ABSTRACT

An important area of research in nanotechnology is the synthesis of nanoparticles of different chemical compositions, sizes and controlled monodispersity. Currently, there is an ever-increasing need to develop environmentally benign nanoparticles synthesis processes. As a result, researchers in the field of nanoparticles synthesis and assembly have turned to biological systems for inspiration. This is not surprising given that many organisms, both unicellular and multicellular, are known to produce inorganic materials either intracellularly or extracellularly. Microorganisms, particularly prokaryotic bacteria, are often exposed to extreme environmental conditions, forcing them to resort to specific defense mechanisms to quell such stresses, including the toxicity of foreign metal ions or metals. Both prokaryotic (bacteria) and eukaryotic organisms such as yeast have been found to produce nanoparticles within the cell wall of the microorganisms. While enzymatic processes in sulfate reducing bacteria are relatively well understood and identified in the formation of biofilms of sphalerite ZnS and CdS and the intracellular synthesis of CdS within the yeast cells. The toxicity of metal ions is reduced or eliminated by changing the redox state of the metal ions and/or precipitation of the metals intracellularly, thus forming the basis of many important applications of microorganisms such as bioleaching, bioremediation, microbial corrosion, as well as the synthesis of nanoparticles. In a radical departure from the predominantly bacteria-based methods for the synthesis of inorganic nanoparticles, we have investigated the use of eukaryotic organisms such as fungi in nanomaterials synthesis and have demonstrated that the reaction of metal ions with *Verticillium* results in the intracellular synthesis of metal nanoparticles such as gold and silver. Recognizing that such biotransformation based protocols would better serve potential applications if nanoparticles synthesis could be accomplished extracellularly, we have recently identified the fungus *Fusarium oxysporum* for the synthesis of gold, silver and also semiconductor cadmium sulfide (CdS) nanoparticles extracellularly.

The present investigation details the extracellular biosynthesis of CdSe quantum dots by the fungus *Fusarium oxysporum* and also the mechanism by which it is able to reduce the metal salts and subsequently synthesize nanoparticles extracellularly. We have purified and characterized the enzymes and capping proteins

involved in the extracullular synthesis of nanoparticles. *In vitro* synthesis of different metal nanoparticles such as gold, silver and technologically important semiconductor CdS nanoparticles by using the purified enzymes and the capping peptide has been carried out. Conjugation of CdS quantum dots to different biologically significant proteins for diagnostic applications has been carried out. The present work has been organized under the following headings.

Chapter 1: General Introduction.

Chapter 2: Biosynthesis of extracellular CdSe quantum dots by the fungus, *Fusarium oxysporum.*

Chapter 3: Purification and characterization of α-NADPH dependent sulphite and nitrate reductases from the extracellular broth of *Fusarium oxysporum.*

Chapter 4: Sulphite reductase mediated synthesis of CdS nanoparticles capped by phytochelatin.

Chapter 5: Sulphite reductase mediated synthesis of gold nanoparticles.

Chapter 6: Nitrate reductase mediated synthesis of silver nanoparticles.

Chapter 7: Conjugation of CdS quantum dots to jacalin and chickpea lectin for diagnostic applications.

Chapter 8: General Discussion and Conclusion.

CHAPTER-I

General Introduction

This chapter presents a general introduction about various physicochemical aspects of nanomaterials. The different characteristic properties of nanomaterials, their chemically synthesized protocols, characterization techniques, and their applications are discussed in brief. Based on these reviews, the scope and objective of the present work have been outlined.

CHAPTER-II

Biosynthesis of extracellular CdSe quantum dots by the fungus, *Fusarium oxysporum*

This chapter focuses on the extracellular biosynthesis of CdSe quantum dots using *Fusarium oxysporum*. When the fungus was challenged with aqueous solution of $CdCl_2$, Na_2SO_3 and $SeCl_4$ solution, it resulted in the formation of extracellular CdSe quantum dots. The reaction mixture was monitored for the production of CdSe nanoparticles at regular intervals by subjecting to UV-Vis spectrophotometric measurements. The strong surface plasmon band centered at 370 nm clearly indicated the formation of CdSe nanoparticles.

CdSe nanoparticles were further characterized by using X-Ray Diffraction, Transmission Electron Microscopy and X-Ray Photo Electron Spectroscopy. The presence of intense peaks corresponding to (100), (111), (220), (311) and (222). Bragg reflections of CdSe agree with those reported for CdSe nanocrystals. The morphology of the nanoparticles is highly variable, with spherical and irregular shapes and in the size of range 9-15 nm. The photoelectron spectroscopy revealed the Cd $3d_{5/2}$ and $3d_{3/2}$ peaks at a binding energy of 406 eV and 412 eV respectively and is characteristic of metallic Cd and the Se 3d peak at a binding energy of 54.8 eV which is a characteristic of metallic Se.

CHAPTER-III

Purification and characterization of α-NADPH dependent sulphite and nitrate reductases from the extracellular broth of *Fusarium oxysporum*

This chapter focuses on the purification and characterization of enzymes (sulphite and nitrate reductases) responsible for the biosynthesis of nanoparticles by the fungus, *Fusarium oxysporum.*

Culture broth of *Fusarium oxysporum* grown for 48 h, was used for the purification of enzymes sulphite and nitrate reductases. The sulphite reductase purification was a two step process involving ion exchange chromatography on DEAE-Sephadex and gel filtration on Sephacryl S-300. The enzyme was homogenous as judged from the SDS-PAGE with a molecular mass of 35.6 kDa. The pI of the enzyme as determined to be 4.5. The enzyme exhibited an optimum pH of 6.0 and was stable in the pH range of 4

to 6. The optimum temperature for the enzyme activity was 45°C and it was stable in the temperature range of 35 to 50 °C and lost the total activity at 60 °C and above. Kinetic parameters were as follows V_{max} of 1.167 μ mol/sec. and K_m of 8.71 μ mol/sec. The amino acid analysis of the enzyme was performed and the enzyme was found to contain high amount of Gly and Lys, and one each of Cys and Trp.

The nitrate reductase purification was a three step process involving ion exchange chromatography on DEAE-Sephadex followed by ion exchange chromatography on CM-Cellulose and gel filtration on Sephacryl S-300. The enzyme was homogenous as judged from the SDS-PAGE with a molecular mass of 44.1 kDa. The pI of the enzyme was found to be 9.2. The enzyme exhibited an optimum pH of 7.0 and was stable in the pH range of 5 to 8. The optimum temperature for the enzyme activity was 55°C and it was stable in the temperature range of 40 to 60 °C and lost the total activity at 65 °C and above. Kinetic parameters were as follows: V_{max} = 7.69 μ mol/sec and K_m = 24.46 μ mol/sec. The amino acid analysis of the enzyme shows it contains high amount of Gly and Ser.

CHAPTER-IV

Sulphite reductase mediated synthesis of CdS nanoparticles capped by phytochelatin

This chapter focuses on a novel method for the synthesis of technologically important semiconductor metal sulfide nanoparticles using sulphite reductase purified from the extracellular broth of *Fusarium oxysporum* and the capping peptide. When the enzyme sulphite reductase was incubated in an aqueous solution of metal $CdCl_2$ and Na_2SO_3, highly stable quantum dots of cadmium sulfide nanocrystals are formed *in vitro*. The metal sulfide quantum dots are formed by reaction of Cd^{2+} ions with sulfide ions that are formed by the reduction of sulfate ions by the enzyme sulphite reductase in the presence of a co-factor NADPH. The surface plasmon band centered at 340 nm clearly indicated the formation of CdS nanoparticles.

CdS nanoparticles were further characterized by using X-Ray Diffraction, Transmission Electron Microscopy and X-Ray Photo Electron Spectroscopy. The presence of intense peaks corresponding to (111), (101), (002) and (220). Bragg reflections of CdS agree with those reported for CdS nanocrystals. The morphology of the nanoparticles is essentially spherical and in the size of range 5-20 nm. The

photoelectron spectroscopy revealed the Cd $3d_{5/2}$ and $3d_{3/2}$ peaks at a binding energy of 405.5 eV and 412.5 eV respectively and are characteristic of metallic Cd and the S 2p peak at a binding energy of 168.8 eV which is a characteristic of metallic S and these values corresponding well with the expected values for Cd bound to sulfur.

CHAPTER-V

Sulphite reductase mediated synthesis of gold nanoparticles

This chapter deals with the synthesis of metallic gold nanoparticles *in vitro* using the purified sulphite reductase and capping peptide in the presence α-NADPH. The exposure of sulphite reductase, to $HAuCl_4^-$ solution resulted in the reduction of metal ions and subsequent formation of gold nanoparticles *in vitro*. The strong surface plasmon band centered at 520 nm clearly indicated the formation of gold nanoparticles.

Gold nanoparticles were further characterized by using X-Ray Diffraction, Transmission Electron Microscopy and X-Ray Photo Electron Spectroscopy. The presence of intense peaks corresponding to (111), (200), (220) and (311). Bragg reflections of gold agree with those reported for gold nanocrystals. The morphology of the nanoparticles is varied with rhomboidal and spherical and in the size of range 7-20 nm. The photoelectron spectroscopy carried over a wide range of binding energies, i.e., 0 to 1400 e V, revealed the presence of Au, C, O, N and Na as the only prominent elements. The Au $4f_{7/2}$ and $4f_{5/2}$ peaks occurred at a binding energy of 83.9 eV and 87.7 eV respectively are characteristic of metallic Au.

CHAPTER-VI

Nitrate reductase mediated synthesis of silver nanoparticles

This chapter deals with the synthesis of metallic silver nanoparticles *in vitro* using the purified nitrate reductases and capping peptide in the presence 4-hydroxyquinoline and α-NADPH. The exposure of nitrate reductase, to $AgNO_3^-$ solution resulted in the reduction of metal ions and subsequent formation of silver nanoparticles *in vitro*. The strong surface plasmon band centered at 413 nm clearly indicated the formation of silver nanoparticles. Silver nanoparticles were further characterized by using X-Ray Diffraction, Transmission Electron Microscopy and X-Ray Photo Electron Spectroscopy.

The presence of intense peaks corresponding to (111), (200), (220) and (311). Bragg reflections of silver agree with those reported for silver nanocrystals. The morphology of the nanoparticles is spherical and in the size of range 10-25 nm. The photoelectron spectroscopy carried over a wide range of binding energies, i.e., 0 to 1400 e V, revealed the presence of Ag, C, O, N and Na as the only prominent elements. The Ag $3d_{5/2}$ and $3d_{3/2}$ peaks observed at a binding energy of 368.1 eV and 374.8 eV respectively are characteristic of metallic Ag

CHAPTER-VII

Conjugation of CdS quantum dots to jacalin and chickpea lectin for diagnostic applications

This chapter focuses on the conjugation of the *in vitro* CdS quantum dots to different proteins for diagnostic applications.

Conjugation of *in vitro* synthesized CdS quantum dots

CdS nanoparticles were synthesized as described in chapter III and conjugated to jacalin and chickpea lectin by EDC coupling reaction, an effective concentration of 5 mM, in 3 aliquots for a period of 1 hour at 30°C. The mixture was then passed through Sephacryl S-200 column. The fractions exhibiting fluorescence as well as lectin activity were pooled, dialyzed against water, concentrated and stored at 4°C until further use.

CHAPTER-VIII

General Discussion and Conclusion

The summary of the results obtained and the conclusions drawn are presented in this chapter. The scope for future work is also discussed.

CHAPTER 1

GENERAL INTRODUCTION

Nanotechnology

"Nanotechnology" deals with the controlled miniaturization of matter (< 100 nm) at the molecular level where its properties are significantly different from that of bulk materials (Feynman, 1991). It is also referred for designing, characterization, production and application of structures, devices and systems by controlling the shape and size on a nanometer scale (Bamford, 2000; Cao, 2004). In other words, nanoscience and nanotechnology is an area that focuses on the development of synthetic methods and surface analytical tools for building structures and materials to understand the change in physical and chemical properties due to miniaturization and use of these properties for the development of functional materials and devices (Rao et al., 2004; Mirkin, 2005).

It is well known that living cells are the best examples of machines that operate at a nano level performing numerous functions, ranging from generation of energy to extraction of targeted materials with very high efficiency (Godsell, 2004). Hence, the scientific interest in this interface of biology and technology is based on the perception that nanotechnology can lead to the development of potentially interesting and useful functional nanosystems.

Research in nanotechnology is as diverse as physics, chemistry, material science, microbiology, biochemistry and molecular biology. Hence, nanoscience in combination with biotechnology and biomedical engineering is an emerging area related to the development and applications of nano-structured materials in diagnosis, gene sequencing, and drug delivery. Thus, nanotechnology can enable one to learn more about the detailed functioning of individual cells and neurons, which inturn will be useful in the re-engineering of living systems (Lowe, 2000; Tsapis et al., 2002; Whitesides, 2003).

A number of factors influence the physical, chemical, optical, magnetic and electronic properties of nanomaterials. These factors inturn strongly modulate their properties viz. size (Buffat and Borel, 1976; McHale et al., 1997; Borel, 1981; Brus, 1983; Zhang and Banfield, 1998; Link and Sayed, 1999a; Glinka et al., 2001; Brust and Kiely, 2002; Dick et al., 2002; Skomski, 2003) shape (Link and Sayed, 1999b; EI-Sayed, 2001; Burda et al., 2005), surface composition (Chen et al., 1998, Chen and Huang, 2000; Chen and Pei 2001; Zhang and Sham 2002; Li and Li, 2003; Quinn et al., 2003), dielectric environment of the particle (Link et al., 1999; Templeton et al.,

2000; Itoh et al., 2001; Rechberger et al., 2003; Yan et al., 2003; Swanson and Billard, 2003) and interparticle interactions (AI-Rawasheh and Foss, 1997; Jensen et al., 1999; Xu et al., 1999; Kottmann and Martin Oliver, 2001; Su et al., 2003; Kelly et al., 2003; Schmid and Simon, 2005). The factors responsible for such remarkable variations in their properties are their dimensions comparable to that of De Broglie wavelength of the charge carriers and high surface to volume ratio (Zhang, 1997; Henry, 1998; Raimondi et al., 2005) In case of metal nanoparticles their color originates from surface plasmons i.e., the coherent charge density oscillations (Kreibeig and Vollmer, 1995). The excitation of the surface plasmons by an electromagnetic field at an incident wavelength, where the resonance occurs, results in the appearance of intense surface plasmon resonance (SPR) bands and an enhancement of local electromagnetic field (Hutter and Fendler, 2004). Such quantum size effects are best studied with semiconductor nanoparticles where the energy level spacing for a spherical particle is predicted to be inversely proportional to R^2 (Brus, 1986; Heath, 1995; Alivisators, 1996a). Thus, with decreasing size the effective bandgap increases, leading to relevant absorption and emission spectra blue shifts.

Apart from the optical properties, another beneficial aspect of miniaturization is that they result in a large increase in the surface to volume ratio of the materials compared to their bulk counter parts. Greater availability of the surface area facilitates better catalytic efficiency per unit volume of the catalyst (Lewis, 1993; Roucoux et al., 2002). Moreover, an interesting consequence of miniaturization is that, below a certain size limit, some of them start exhibiting catalytic activity, not shown by their bulk counterparts (Lewis, 1993; Roucoux et al., 2002). In fact, catalytic activities of some of the nanoparticles have also been shown to be dependent on their shape. In addition size also influences their melting point, magnetic properties and lattice structure.

The applications of nanoparticles range from drug and gene delivery (Mah et al., 2000; Panatarotto et al., 2003), to fluorescent labeling of biological samples (Bruchez et al., 1998; Chan and Nie, 1998; Wang et al., 2002), detection of pathogens (Edelstein et al., 2000), tumor destruction (Yoshida and Kobayashi, 1999), tissue engineering (Ma et al., 2003), catalysis (Lewis, 1993; Roucoux et al., 2002), solar cells (Hagfeldt and Graetzel, 2000; Bueno et al., 2004) and optical devices (Wang, 1991; Yoffe, 1993; Maier et al., 2001; Maier et al., 2002). These applications are due to their large surface area and small size. Electron transport phenomena like Coloumb

blockade (Feldheim and Keating, 1998) as well as the catalytic and thermodynamic properties of structures can be tailored where one can design materials on this scale. Hence, novel synthetic methods will permit one to design and control the composition on a nanometer scale leading to newer applications.

Preparation of nanomaterials

The widespread applications of nanoparticles have led to different approaches for the preparation of nanostructured materials and devices. Though the synthetic protocols are largely dominated by physical and chemical methods, attempts are also being made to evaluate the role of biological systems viz. microorganisms and plant extracts to develop ecofriendly processes for the synthesis of nanomaterials.

Physical methods

Some of the commonly used methods are: photoirradiation (Kurihara et al., 1983; Marignier et al., 1986; Yonezawa et al., 1987; Yonezawa et al., 1991; Henglein et al., 1992; Ershov and Henglein, 1993; Gutierrez and Henglein, 1993; Torigoe and Esumi, 1993; Itakura et al., 1995; Chen et al., 2002), radiolysis (Henglein, 1999, 2000), ultrasonication (Salkar et al., 2000), physical vaporization, spray pyrolysis and solvated metal atom dispersion (SMAD) (Klabunde et al., 1978, 1979; Davis and Klabunde, 1982; Wegner et al., 2002; Vitulli et al., 2002; Kim et al., 2002). Haes et al. (2005) described the synthesis of anisotropic shaped nanoparticles by nanosphere lithography where, a monolayer of polystyrene spheres was drop-coated on a substrate followed by vacuum deposition of the metal. Removal of the close packed sphere template, yielded highly ordered triangular shaped nanoparticles deposited in the void spaces. Interestingly, by removing the metal layer deposited on the spheres by dissolving the polystyrene spheres led to formation of nanocups (Love et al., 2002; Charnay et al., 2003). Crescent shaped metal particles were obtained by using a well-separated layer of polystyrene spheres and carrying out the deposition with a slight change in the angle (Liu et al., 2005; Shumaker-Parry et al., 2005). Liu and Bando, (2003) synthesized copper nanorods and nanowires by vacuum vapor deposition of copper on carbon films. In the opinion of the authors, the rough surface of the carbon

film was responsible for re-nucleation of the copper vapor leading to the linear growth of materials with uniform thickness. Zhou et al. (1999) obtained silver nanowires by solid-liquid phase arc discharge method where the arcs discharge of the silver filaments in $NaNO_3$ solution resulted in the formation of linear structures.

Chemical methods

Nanoparticles of metals, metal oxides and semiconductors are synthesized either by reduction or oxidation and precursor mediated precipitation in solution. The control of size, shape, stability and the assembly of nanoparticles can be achieved by incorporating different capping agents, solvents and templates. These capping agents range from simple ions to organic- and biopolymers (Toshima et al., 1991; Bradley et al., 1991; Bradley et al., 1992; Amiens et al., 1993; Toshima and Wang, 1994, Poulin et al., 1995; Naka et al., 1999; Warner et al., 2000; Jiang et al., 2001; Tan et al., 2003). Though water is most commonly used as solvent, reports exist on the use of organic solvents (Cardenas-Trivino et al., 1987; Brust et al., 1994; Brust et al., 1995). Apart from ionic liquids (Kim et al., 2004a) and supercritical fluids (Ohde et al., 2001; Chattopadhyay and Gupta, 2003; Viswanathan et al., 2003) soft and rigid templates like, micelles (Meyer et al., 1984; Lianos and Thomas, 1986; Petit et al., 1990; Pileni et al., 1992; Petit et al., 1994; Antonietti et al., 1995; Forster and Antonietti, 1998; Krieger et al., 2000; Capek, 2004) and organic polymers (Crooks et al., 2001; Minko et al., 2002; Zhang et al., 2004) are also employed for the synthesis of nanomaterials. Some of the biopolymers used are DNA (Braun et al., 1998; Richter et al., 2000), and Tobacco Mosaic Virus (Shenton et al., 1999; Fowler et al., 2001; Dujardin et al., 2003; Ongaro et al., 2005). Furthermore, mesoporous materials and preformed nanoparticles have been utilized for the controlled formation and assembly of nanoparticles.

Control of shape

Currently, increased attention is being directed towards controlling the shape of the nanoparticles rather than the size for which a number of methods already exist. Nanoparticles of various anisotropic shapes viz. rods and wires (Jana et al., 2001), tubes (Ou et al., 2004), dumbbells (Huang et al., 2004), cubes (Xiong et al., 2005), hexagons (Kuo et al., 2004), tetrahedrals (Ahmadi et al., 1996), decahedrons (Chen et

al., 2005), multipods (Teng and Yang, 2005), starshaped (Sau and Murphy, 2004a), discs (Maillard et al., 2002), triangles (Jin et al., 2001) and dendritic shaped (Zhou et al., 1999) have been synthesized. Various strategies applied for the controlled synthesis of anisotropic metal nanoparticles include: synthesis in micellar/surfactant solutions, use of soft and rigid templates, controlling the growth using physical confinements, use of nanosphere lithography, vacuum vapor deposition, direct synthesis in solution in presence/absence of additives and morphological transformations of the preformed nanoparticles by thermal, photo/ion irradiation processes.

Synthesis in micellar solutions: This is the most widely employed method for the synthesis of metal nanorods. They have been synthesized in presence of surfactants by electrochemical (Yu et al., 1997; Wang et al., 1999), photochemical (Esumi et al., 1995; Kim et al., 2002) and seed-mediated methods (Jana et al., 2001, 2001a, b, c; Murphy and Jana, 2002; Johnson et al., 2002; Busbee et al., 2003; Sau and Murphy 2004b; Gole and Murphy, 2004; Murphy et al., 2005a, b). Wang et al. (1999) synthesized gold nanorods by an electrochemical method in a micellar solution of hexadecyltrimethylammonium bromide (CTAB) using gold metal plate as anode and platinum plate as cathode. By this method, the bulk gold metal from anode was converted into gold nanoparticles at the interfacial region between cathodic surface and the electrolyte solution. Esumi et al. (1995) showed the synthesis of gold nanorods by photochemical irradiation in a micellar solution of hexadecyl trimethylammonium chloride (HTAC). The authors noted that the formation of elongated gold nanorods instead of spherical ones occur only when the concentration of HTAC is sufficient for the formation of rod like micelles in solution. Kim et al. (2002) using CTAB for the controlled synthesis of gold nanorods could also improve the yield by adding different amounts of $AgNO_3$. Though, similar observations were also made by Murphy et al. (2005), their synthetic method was based on carrying out the reduction of $AuCl_4^-$ ions in presence of CTAB and preformed spherical nanoparticles as seeds. Moreover, ascorbic acid was used for reduction because it reduces $AuCl_4^-$ ions to Au^0 (metallic gold) in presence of CTAB only after the addition of gold seeds, thus preventing fresh nucleation. According to the authors, slight changes in the experimental conditions could lead to the formation of nanomaterials of various morphologies like, blocks, cubes and tetrapods. The

formation of rod shaped nanoparticles was explained on the basis of either templating action of the micelles or preferential binding of CTAB molecules on the faces of fcc {110} gold (Gole and Murphy, 2004). Busbee et al. (2003) noted that gold nanorods of larger size are formed only when small gold seeds are used. Murphy et al. (2005) observed that the use of Br^- ions as counter ion is essential for the formation of nanorods whereas Cl^- and I^- ions were not effective. Pileni, (2003) synthesized copper nanoparticles of different shapes viz. cubes, rods and triangles using copper (II) bis (2-ethylhexyl) sulfosuccinate $Cu\text{-}(AOT)_2$-isooctane-water system and salts of different anions as the reaction medium. The results showed that, though the AOT micelle structure did not change much in presence of different salts, a drastic change in the morphology was observed. A method based on seeded growth in an aqueous solution containing the capping agent CTAB, gold ions ($HAuCl_4 \cdot 3H_2O$), ascorbic acid and NaOH was demonstrated for the synthesis of gold nanotriangles (Mirkin et al., 2005). Zheng et al. (2003) synthesized silver nanowires and dendrites in a mixture of CTAB and sodium dodecyl sulfate (SDS) by reduction of $AgNO_3$ with ascorbic acid. According to the authors by changing the experimental conditions viz. concentration of ascorbic acid and presence/absence of NaCl led to the formation of one-dimensional nanowires and dendrites.

Synthesis in presence of templates: Anisotropic metallic structures have been synthesized using both soft and rigid templates. DNA is an interesting template because of its linear structure and negatively charged phosphate backbone. Fabrication of one-dimensional parallel and two-dimensional crosses of paladium nanowire arrays, copper nanowires and silver nanorods were carried out on a solid substrate surface templated by DNA followed by reduction of metal ions (Deng and Mao 2003; Becerril et al., 2004). Wei et al. (2005) synthesized silver nanoparticles, nanorods and nanowires by initial complexation of silver with DNA and then reducing the complex with sodium borohydride. By this method, the diameter and the ratio of the silver nanorods and nanowires could be controlled by adjusting the DNA concentration and reduction time. The author's opined that the formation of uniform crystalline nanorods and nanowires arise due to template-directed aggregation of small particles and its subsequent recrystallization, rather than simple aggregation. Block polymers/copolymers are other versatile templates for generating anisotropic nanostructures (Zhang et al., 2001; Cornelissen et al., 2002; Djalali et al., 2002; Kim

et al., 2004b). Under appropriate conditions, different segments of the block copolymer formed regular arrays of cylinders with structures similar to self-assembled surfactants. Different regions of arrayed structure could be designed by selective interaction of functional groups with the precursor metal ions through physical adsorption or chemical bonding and its subsequent reduction resulting in the formation of one-dimensional nanostructures. By, using a wide range of copolymers this templating procedure has been exploited for the synthesis of silver nanowires (Zhang et al., 2001; Cornelissen et al., 2002) and gold nanowires and nanosheets (Djalali et al., 2002; Kim et al., 2004). Capillaries of single walled carbon nanotubes have also been used for the synthesis of nanowires of gold, silver, platinum and palladium (Kyotani et al., 1997; Sloan et al., 1999; Govindraj et al., 2000). Hao et al. (2002) used polystyrene mesospheres as templates for the synthesis of silver nanodiscs. Silver nanocubes and nanotriangles themselves have also been utilized for the synthesis of nanoboxes and triangular rings of gold (Sun et al., 2003; Sun and Xia, 2004). In this approach, the synthesis is facilitated by the transmetallation reduction of gold ions by metallic silver.

Synthesis under physical confinements: Nanorods of various metals have been synthesized within the pores of rigid materials. For example, polycarbonate and porous alumina membranes have been used for the synthesis of metal nanotubes and nanowires either by chemical reduction or by electrochemical deposition (Penner and Martin, 1987; Nishizawa et al., 1995; Menon and Martin, 1995; Hornyak et al., 1997; Jirage et al., 1997; Hulteen et al., 1998; Jirage et al., 1999; Kang and Martin, 2001; Lee and Martin, 2001). Chemical reduction yielded better anisotropic structures since the reduction was initiated in the walls of the pores. Similarly, metal nanowires were synthesized using mesoporous silica (Wang et al., 2003). The physical constraints of liquid-liquid and air-water interface have also been exploited for the formation of gold tapes, plates and fractal structures (Sanyal and Sastry, 2003; Swami et al., 2003, 2004a, b; Sastry et al., 2005).

Direct synthesis in presence and absence of additives: Pastoriza-Santos and Liz-Marzan, (2002) synthesized silver nanoprisms by boiling $AgNO_3$ in N, N-dimethylformamide in presence of poly-(vinylpyrrolidone). A similar method was used for the synthesis of nanowires of silver and platinum and nanocubes and

nanotriangles of silver (Sun and Xia, 2002; Sun and Xia, 2003; Chen et al., 2004). Caswell et al. (2003) demonstrated the synthesis of silver nanowires in templateless and seedless medium where the reduction of silver ions was carried out at 100 ^{o}C by sodium citrate in NaOH. A similar method was employed by Turkevich et al. (1951) for the synthesis of triangular nanoparticles of gold. Suito and Udeya, (1954) showed that plate like structures of gold nanoparticles could be obtained by reducing gold ions with salicylic acid. In this method, the formation of plate like structures was correlated to spiral growth mechanism apart from aggregation and recrystallization of the metal ion (Stranski, 1928; Frank, 1949; Fran, 1951; Suito and Udeya, 1960; Chiang and Turkevich, 1963).

Synthesis by induced shape transformation: Jin et al. (2003) described the transformation from spherical to triangular shaped nanoparticles of silver by photoirradiation whereas, a thermally induced transformation was employed for the conversion of spherical gold clusters to nanocubes (Jin et al., 2004). Li et al. (2003) used chemically induced transformation to convert gold spheres into planar forms. In an interesting approach, Roorda et al. (2004) synthesized gold nanorods by ion beam irradiation of spherical gold-silica core-shell nanoparticles. The ion beam irradiation is shown to deform the silica shell leading to the deformation of the core spherical gold nanoparticle. Similarly shape transformation by laser irradiation was shown to be responsible for the formation of Φ shaped nanoparticles from nanorods (Link et al., 2000).

Biological methods

The ability of biological systems to fabricate structural and functional inorganic materials of precise dimensions and controlled morphology in an ecofriendly manner has led nanotechnologists to explore the use of biological systems for the fabrication of nanomaterials. While there are several reports on the natural occurrence of biominerals, attempts are also been made to induce microorganisms to synthesize biominerals and semiconducting/metal nanoparticles. This route for the synthesis of nanoparticles is attractive due to the ability of microorganisms to withstand high metal ion concentration(s).

Bacteria and algae are capable of synthesizing inorganic materials, both intra- and extracellularly (Simkiss and Wilbur, 1989) by efflux systems, alteration of solubility and toxicity by changes in the redox state of the metal ions, extracellular complexation or precipitation of metals and lack of specific metal transport system (Rouch et al., 1995; Silver, 1996; Beveridge et al., 1997). The ability of microorganisms to interact with heavy metal ions in the above manner has been utilized in bioremediation (Stephen and Macnaughtom, 1999). Hence, material scientists studying detoxification of metal ions either by reduction (Temple and LeRoux, 1964; Zumberg et al., 1978; Blakemore et al. 1979, Beveridge and Murray, 1980; Hosea et al., 1986; Southam and Beveridge, 1996; Klaus et al., 1999; Joerger et al., 2000; Fortin and Beveridge, 2000; Joerger et al., 2001; Mukherjee et al., 2001a, b; Mukherjee et al., 2002; Ahmad et al., 2003; Senapati et al., 2005) or by sulphide formation (Aiking et al., 1982; Reese and Winge, 1988; Dameron et al., 1989; Smith et al., 1998; Cunningham and Lundie, 1993; Holmes et al., 1995; Labrenz et al., 2000; Kowshik et al., 2002a, b; Ahmad et al., 2002) are looking at the use of microorganisms as ecofriendly nanofactories. In an interesting development, Shankar et al. (2004) demonstrated the use of lemongrass extract for the synthesis of gold nanotriangles. According to the authors (Shankar et al., 2005) by simple variation in the experimental conditions it is possible to synthesize gold nanotriangles of various sizes which absorb near-infrared (NIR) region of the electromagnetic spectrum.

Role of enzymes

Enzymes, nature's catalysts, carry out reactions under mild conditions in a highly specific manner. The oxidation/reduction mechanism involved in the formation nanomaterials coupled with the ability of enzymes to carryout such reactions have prompted investigators to examine the role of reductases in the biotransformation of metals (Duran et al., 2005).

Characterization of nanomaterials

X-ray diffraction (XRD): X-ray diffraction is used for the determination the crystal structure of solids, including lattice constants and geometry, orientation of single

crystals, defects, etc. (Wang, 2000). Bragg's equation obtained on measurement of the diffraction pattern of the crystals relates to the distance between two *hkl* planes (*d*) and the angle of diffraction (2θ) as: $n\lambda = 2d\sin\theta$, where, λ = wavelength of X-rays, *n* = an integer is known as the order of reflection (*h*, *k* and *l* which represents Miller indices of the respective planes (Bragg and Bragg, 1949). Moreover, the average size of the nanoparticles can be estimated using the Debye–Scherrer equation: $D = k\lambda / \beta\cos\theta$, where *D* = thickness of the nanocrystal, *k* is a constant, λ = wavelength of X-rays, β = width at half maxima of (111) reflection at Bragg's angle 2θ (Birks and Friedman, 1946; Rau, 1962).

Scanning Electron Microscopy (SEM): This is one of the most widely used techniques for the characterization of nanostructures because of its high resolution. Unlike optical microscopy, this technique not only provides topographical information but also the chemical composition near the surface. The interaction between the electron beam and the sample gives different types of signals providing detailed information about the surface structure and morphology of the sample. When an electron from the beam encounters a nucleus in the sample, the resultant columbic attraction will lead to deflection in the electron's path, known as Rutherford elastic scattering. A fraction of these electrons will be backscattered resulting in reemergence from the incident surface. Since the scattering angle depends on the atomic number of the nucleus, the primary electrons arriving at a given detector position produces images yielding topological and compositional data (Lawes, 1987). The high-energy incident electrons can also interact with loosely bound conduction band electrons in the sample. However, the amount of energy given to these secondary electrons as a result of such interactions is small with a very limited range. Those secondary electrons produced within a very short distance from the surface escape from the sample giving high-resolution topographical images (Newbury et al., 1986).

Transmission Electron Microscopy (TEM): Transmission electron microscopy is typically used for high resolution imaging of thin films of solid samples for structural and compositional analysis. The technique involves: (i) irradiation of a very thin film by a high-energy electron beam, which is then diffracted by lattices of the crystalline or semicrystalline material and propagated in different directions, (ii) imaging and angular distribution analysis of the forward-scattered electrons (unlike SEM where

backscattered electrons are detected) and (iii) energy analysis of the emitted X-rays (Fryer, 1979). The topographic information obtained by TEM, in the vicinity of atomic resolution, can be utilized for structural characterization and identification of various forms of nanomaterials, *viz.*, hexagonal, cubic or lamellar (Wang, 2000) One of the limitations of TEM is that the electron scattering information on TEM originates from a three-dimensional image which is then projected onto a two-dimensional detector. Therefore, structural information along the electron beam direction is superimposed at the image plane.

TEM has also been used for determining the melting points of nanocrystals (Goldstein et al., 1992) and measurement of mechanical and electrical properties nanowires and nanotubes (Poncharal et al., 1999; Wang, 2000; Wang et al., 2000).

Selected area diffraction (SAD) offers a unique method to determine the crystal structure of nanomaterials such as nanocrystals and nanorods. In this method, the condenser lens is defocused to produce parallel illumination on the specimen and a selected-area aperture is used to limit the diffracting volume. SAD patterns are often used to determine the Bravais lattices and lattice parameters of crystalline materials by a method similar to the one used in XRD (Cullity and Stock, 2001).

*UV-VIS Spectroscopy***:** This method deals with the study of electronic transitions between orbitals or bands of atoms, ions or molecules in gaseous, liquid and solid state (Jorgensen, 1962). Metallic nanoparticles are known to exhibit characteristic colors. Mie, (1908), for the first time, explained the origin of color by solving Maxwell's equation for the absorption and scattering of electromagnetic radiation by small metallic particles. The absorption of electromagnetic radiation by metallic nanoparticles originates from the coherent oscillation of the valence band electrons induced by an interaction with the electromagnetic field (Farady, 1857). These resonances are known as surface plasmons occur only with nanoparticles (Papavassiliou, 1980). Hence, UV-Vis can be utilized to study the unique optical properties of nanoparticles (Link and Sayed, 1999; Burda et al., 2000).

Fluorescence Spectroscopy: In this technique, an incident light of a fixed wavelength is directed onto the specimen prompting the transition of electron from the ground to excited state. The excited state of the molecule then undergoes a non-radiative internal relaxation and the excited electron moves to a more stable excited

level. After a characteristic lifetime in the excited state, the electron returns to the ground state by emitting photons in the process with a characteristic wavelength in the form of light. This emitted energy can be used to obtain qualitative and quantitative information like, chemical composition, structure, impurities, kinetic process and energy transfer.

Fourier Transform Infrared Spectroscopy (FTIR): Fourier transform infrared spectroscopy involves the vibration of chemical bonds of a molecule at various frequencies depending on the elements and types of bonds. After absorbing electromagnetic radiation, the bond frequency increases leading to transition between ground and excited states. These absorption frequencies represent excitations of vibrations of the chemical bonds and are specific to the type of bond and the group of atoms involved in the vibration. The energy of these frequencies is in the infrared region (4000–400 cm^{-1}) of the electromagnetic spectrum. The term Fourier transform (FT) refers to a recent development wherein the data obtained is converted from an interference pattern to an infrared absorption spectrum similar to that of a molecular "fingerprint" (Griffiths and Haseth, 1986) The FTIR can be evaluate the presence of proteins in the solution, as the spectra, in 1400–1700 cm^{-1} region, provides information regarding the presence of –CO- and –NH- groups (Banwell and McCash, 1996).

X-Ray Photoelectron Spectroscopy (XPS): X-ray photoelectron spectroscopy is widely used for probing the electronic structure of atoms, molecules and condensed matter. When an X-ray photon of energy ($h\nu$) is incident on a solid matter, the kinetic energy (E_k) and the binding energy (E_b) of the ejected photoelectrons can be related as follows: $E_k = h\nu - E_b$. This kinetic energy distribution of the photoelectrons forms a series of discrete bands, which symbolizes the electronic structure of the sample (Fadley, 1978). The core level binding energies of all the elements (other than H and He) in different oxidation states are unique which provides information about the chemical composition of the sample. However, to account for the multiplet splitting and satellites accompanying the photoemission peaks, the photoelectron spectra should be interpreted in terms of many-electron states of the final ionized state of the

sample, rather than the occupied one-electron states of the neutral species (Egelhoff, 1987).

***Atomic absorption spectrometry (AAS)*:** The principle of atomic absorption is based on energy absorbed during transitions between electronic energy levels of an atom. When energy is provided to an atom in ground state at a high temperature (2100–2800°C), outer-shell electrons are promoted to a higher energy excited state. The absorbed radiation as a result of this transition between electronic levels can be used for quantitative analysis of metals and metalloids present on solid matrices in solution. The basis for the quantitative analysis depends on the measurement of radiation intensity and the assumption that radiation absorbed is proportional to atomic concentration. Analogy of relative intensity values for reference standards is also used to determine elemental concentrations (Robinson, 1975).

Properties of nanomaterials

Miniaturization of materials has pronounced effect on their physical properties, which are different from that of the corresponding bulk materials. Some of the physical properties exhibited by nanomaterials are due to (i) large surface area /surface energy (ii) spatial confinement, and (iii) reduced imperfections:

Optical Properties: The visible property of metallic nanoparticles is their colored colloidal solutions. Mie (1908) explained the red color of gold nanoparticles on the basis of Maxwell's equation for an electromagnetic light wave interacting with small metallic spheres. The color exhibited by metallic nanoparticles was due to the coherent excitation of all the "free" electrons within the conduction band, leading to an in-phase oscillation known as surface plasmon resonance. Hence, the difference in the color of metallic nanoparticles was correlated to their size and surface plasmon resonance.

Unique optical property of nanomaterials can also be due to quantum size effect, which arises primarily due to the confinement of electrons within particles of dimension smaller than the bulk electron delocalization length. This effect is more pronounced for semiconductor nanoparticles where, the band gap increases with a

decreasing size. The same quantum size effect is also shown by metal nanoparticles, when the particle size is >2 nm.

Magnetic properties: Magnetic properties of nanostructured materials are distinctly different from that of bulk materials. Ferromagnetic particles become unstable when the particle size reduces below a certain size and increase in surface energy provides sufficient energy for domains to spontaneously switch polarization directions to become paramagnetic. This transformed paramagnetism behaves differently from that of conventional paramagnetism and is referred to as super paramagnetism (Frankel and Dorfman, 1930; Bucher et al., 1991) In short; ferromagnetism of bulk material disappears and gets transferred to superparamagnetism in nanoscale due to high surface energy.

Mechanical properties: The mechanical properties of nanomaterials increase with the decrease in size. The enhanced mechanical strength is due to its high internal perfection. Generally, imperfections such as dislocations, micro-twins, impurities, etc. in crystals are highly energetic and needs to be eliminated from perfect crystal structures. Smaller the cross-section of nanowires less is the probability of any imperfections as nanoscale dimension permits the elimination of such imperfections.

Thermal properties: Metal and semiconductor nanoparticles have lower melting point or phase transition temperature compared to their bulk counterparts. The lowering of the melting point is observed when the particle size is >100 nm and is attributed to increase in the surface energy size reduction. The decrease in the phase transition temperature can be correlated to the changes in the ratio of surface to volume energy as a function of size.

Applications of nanomaterials

Nanotechnology has applications in electronics, optical communications, biological systems and new smart materials. The wide range of applications shown by nanostructures and nanomaterials are due to their small size and large surface area.

Molecular- and nanoelectronics: Past few years have witnessed a significant progress in molecular- and nanoelectronics (Klein et al., 1997; Chen et al., 1999; Service, 2001; Tseng and Ellenbogen, 2001; Schon et al., 2001a, b; Bachtold e al., 2001; Huang et al., 2001). In molecular electronics, single molecules are designed to control electron transport, for exploring the wide variety of molecular functions required for designing electronic devices. The control of electronic energy levels at the surface of conventional semiconductors and metals is achieved by assembling them on the solid surfaces followed by incorporating these molecules into a circuit. Use of biologically active molecules can be exploited for the development of bioelectronic devices (Klein et al., 1997; Vilan and Cahen, 2002).

Nanoelectronic devices like, tunneling junctions (Reed et al., 1997; Cui et al., 2001; Compano, 2001) electrically configurated switches (Chen et al., 1999), carbon nanotube transistors (Tans et al., 1998; Wind et al., 2002) and single molecular transistors (Kim et al., 1999; Liang et al., 2002; Park et al., 2002; Bell et al., 2003) have been developed.

The surface property and uniform size of gold nanoparticles, has led to their extensive use as a carrier for the attachment of various functional organic and bio molecules (Feldheim and Keating, 1998), in the construction of nanoscale   appliances like sensors and detectors (Persson et al., 1999; Brust and Kiely, 2002).

Nanorobots: Application of nanotechnology in medicine is referred to as nanomedicine. One of the attractive applications of nanotechnology in medicine is the creation of nanorobots for diagnosis and therapy (Haberzettl, 2002).

Biological applications: One of the most important biological applications of colloidal nanocrystals is in molecular recognition (Spinke et al., 1993; Stryer, 1995; Ludwig et al., 1997; Fritz et al., 2000; Liu et al., 2000). Molecular recognition is based on the ability of biological molecules to recognize and bind other molecules with high selectivity and specificity. For example, antigens to antibodies, lectins to glycoproteins and cofactors to enzymes.

Antibodies and oligonucleotides attached to the surface of nanocrystals have been used for labeling biomolecules (Alivisatos et al., 1996; Elghanian et al., 1997; Bruchez et al., 1998; Chan and Nie, 1998; Shaiu et al., 1993; Florin et al., 1994; Niemeyer, 2001; Parak et al., 2002; Parak et al., 2003; Mirkin, 2000; Taton, 2002).

Also, the change in color of gold nanoparticles from ruby-red to blue due to aggregation has been exploited for the development of very sensitive colorimetric methods for DNA analysis (Strhoff et al., 1998). Nanomaterials have also found application as fluorescent probes to label cells (Bruchez et al., 1998; Taton, 2001; Pathak et al., 2001; Klarreich, 2001; Weiss, 2001; Rosenthal et al., 2002; Dubertret et al., 2002; Akerman et al., 2002; Jaiswal et al., 2003), creation of chemical libraries (Han et al., 2001; Rosenthal, 2001) and artificial bones (Taton, 2001).

In catalysis: The large surface area of nanomaterials have been used in hydrogenolysis (Corrolleur et al., 1972a, b, c; Gault, 1981), oxidation (Liu et al., 2005), heterogeneous catalysis (Bond and Sermon, 1973a, b; Haruta et al., 1989; Haruta et al., 1993; Haruta, 1997; Bond and Thompson, 1999; Okumura and Haruta, 2000; Haruta and Date, 2001) hydrogen transfer reactions (Cha and Parravano, 1970; Galvano and Parravano, 1978) hydrogenation of cyclohexane (Mukherjee et al., 2002b) asymmetric dihydroxylation reactions (Li et al., 1999) carboxylic ester cleavage (Pasquato et al., 2000), electrocatalytic reductions by anthraquinone functionalized gold particles (Pietron and Murray, 1999) and particle-bound ring opening metathesis polymerization (Bartz et al., 1998). There have been quite a few interesting examples of nanostructured metal oxides and sulfides exhibiting unusual catalytic properties (Sun and Klabunde, 1999a, b; Thurston and Wilcoxon, 1999). Very recently, Liu et al. 2005 developed a highly efficient catalyst system for CO oxidation with Au-Ag alloy nanoparticles supported on the mesoporous MCM-41 and observed that the size effect is no longer a critical factor and silver is believed to play a key role in the activation of oxygen.

PRESENT INVESTIGATION

The unique properties observed on miniaturization of bulk materials have led to their extensive investigation both from basic as well as applied aspects. One of the important components of nanotechnology lies in the development of reliable ecofriendly processes for the synthesis of nanomaterials of various sizes, shapes and chemical composition. Majority of protocols for the synthesis of nanomaterials by chemical and physical processes require either hazardous chemicals or expensive machinary. Hence there is a need to develop ecofriendly methods using biological systems. The ability of bacteria and algae and even plant extracts to synthesize nanoparticles under mild conditions has led to extensive search for biological systems capable of synthesizing nanoparticles. As observed with both physical and chemical methods, the mechanism for the formation of nanoparticles involves oxidation/reduction reactions. Since enzymes are also capable of carrying out these reactions, attempts were made to study the role of oxidoreductases in the biosynthesis of nanomaterials.

REFERENCES

Ahmad, A., Mukherjee, P., Mandal, D., Senapati, S., Khan, M.I., Kumar, R., Sastry, M., (2002) *J. Am. Chem. Soc.* **124,** 12108-12109.

Ahmad, A., Mukherjee, P., Senapati, S., Mandal, D., Khan, M.I., Kumar, R., Sastry, M. (2003a) *Colloids Surf. B* **28,** 313-318.

Ahmad, A., Senapati, S., Khan, M. I., Kumar, R., Sastry, M. (2003b) *Langmuir* **19***, 3550-3553.

Ahmadi, T. S., Wang, Z. L., Green, T. C., Henglein, A., El-Sayed, M. A. (1996) *Science* **272**, 1924-1925.

Aiking, H., Kok, K., Van Heerikhuizen, H., Riet, J. V. (1982) *Appl. Environ. Microbiol.* **44**, 938-944.

Akerman, M. E., Chan, W. C. W., Laakkonen, P., Bhatia, S. N., Ruoslahti, E. (2002) *Proc. Natl Acad. Sci. USA*. **99**, 12617-12621.

Alivisatos, A. P. (1996a) *Science* **271**, 933-937.

Alivisatos, A. P., Johnsson, K. P., Peng, X., Wilson, T. E., Loweth, C. J., Bruchez, M. P., Schultz, P. G. (1996b) *Nature* **382**, 609-611.

Al-Rawashdeh, N., Foss, C. A., (1997) *Nanostruct. Mater.* **9**, 383-386.

Amiens, C., De Caro, D., Chaudret, B., Bradley, J. S., Mazel, R., Roucau, C. (1993) *J. Am. Chem. Soc.* **115**, 11638-11639.

Antonietti, M., Wenz, E., Bronstein, L., Seregina, M. (1995) *Adv. Mater.* **7,** 1000-1005.

Bachtold, A., Hadley, P., Nakanishi, T., Dekker, C. (2001) *Science* **294**, 1317-1320.

Bamford, D. H. (2000) *Curr. Biol.* **10**, R558-561.

Banwell, C. N., McCash, E. M. Fundamentals of Molecular Spectroscopy, McGraw-Hill, United Kingdom, (1996) Ch. 3, p. 55.

Bartz, M., Kuther, J., Seshadri, R., Tremel, W. (1998) *Angew. Chem. Int. Ed. Engl.* **37,** 2466-2468.

Becerril, H. A., Stoltenberg, R. M., Monson, C. F., Woolley, A. T. (2004) *J. Mater. Chem.* **14**, 611-616.

Bell, C., Burnell, G., Kang, D. J., Hadfield, R. H., Kappers, M. J., Blamire, M. G. (2003) *Nanotechnology* **14,** 630-632.

Beveridge, J. T., Hughes, M. N., Lee, H., Leung, K. T., Poole, R. K., Savvaides, I., Silver, S., Trevors, J. T. (1997) *Adv. Microb. Physiol.* **38**, 178-243.

Beveridge, T. J.,Murray, R. G. E. (1980) *J. Bacteriol.* 141, 876-887.

Birks, L. S., Friedman, H. (1946) *J. Appl. Phys.* **17**, 687-692.

Blakemore, R. P., Maratea, D., Wolfe, R. S. (1979) *J. Bacteriol.* **140,** 720-729.

Bond, G. C., Sermon, P. A. (1973a) *Gold Bull.* **6,** 102-109.

Bond, G. C., Sermon, P. A. (1973b) *J. Chem. Soc. Chem. Commun.* 444-445.

Bond, G. C., Thompson, D. T. (1999) *Catal. Rev. Sci. Eng*. **41,** 319-388.

Borel, J. P. (1981) *Surf. Sci.* **106,** 1-9.

Bradley, J. S., Millar, J. M., Hill, E. W., Behal, S., Chaudret, B., Duteil, A. *(*1991) *Faraday Discuss.* **92,** 255-268.

Bradley, J. S., Hill, E. W., Behal, S., Klein, C. (1992) *Chem. Mater.* **4,** 1234-1239.

Bragg, W. H., Bragg, W.L. The Crystalline State, McMillan, (1949) New York, Vol. **1**.

Braun, E., Eichen, Y., Sivan, U., Ben-Yoseph, G. (1998) *Nature* **391,** 775-778.

Bruchez, M., Moronne, M., Gin, P., Weiss, S., Alivisatos, (1998) A. P. *Science* **281,** 2013-2016.

Brus, L. E. (1983) *J. Chem. Phys.* **79,** 5566-5571.

Brus, L. E. (1986) *J. Phys. Chem.* **90,** 2555-2560.

Brust, M., Kiely, C. J. (2002) *Colloids Surf. A Physicochem. Eng. Asp.* **202,** 175-186.

Brust, M., Walker, M., Bethell, D., Schiffrin, D. J., Whyman, R. J. *J. (*1994) *Chem. Soc., Chem. Commun.* 801-802.

Brust, M., Fink, J., Bethell, D., Schiffrin, D. J., Kiely, C. J. (1995) *J. Chem. Soc. Chem. Commun*. 1655-1656.

Bucher, J. P., Douglass, D. C., Bloomfield, L. A. (1991) *Phys. Rev. Lett.* **70,** 2283-2290.

Bueno, J. T., Shchukina, N., Ramos, A. A. (2004) *Nature*. **430,** 326-329.

Buffat, P., Borel, J. P. (1976) *Phys. Rev. A* **13,** 2287-2292.

Burda, C., Chen, X., Narayanan, R., El-Sayed, M. A. (2005) *Chem. Rev.* **105,** 1025-1102.

Burda, C., Green, T., Landes, C., Link, S., Little, R., Petroski, J., El-Sayed, M. A. Characterization of Nanophase Materials, Ed, Z. L. Wang, Wiley-VCH, (2000) Weinheim, , Chapter 7, pp. 197.

Busbee, B. D., Obare, S. O., Murphy, C. J. (2003) *Adv. Mater*. **15,** 414-416.

Cao, G. (2004) Nanotructures and Nanomaterials, Synthesis, Properties and Applications, Imperial College Press, London.

Capek, I. (2004) *Advances in Colloid and Interface Science* **110,** 49-74.

Cardenas-Trivino, G., Klabunde, K. J., Dale, E. B. (1987) *Langmuir* **3,** 986-992.

Caswell, K. K., Bender, C. M., Murphy, C. J. (2003) *Nano Lett.* **3,** 667-669.

Cha, D. Y., Parravano, G. (1970) *J. Catal.* **18,** 200-211.

Chan, W. C. W., Nie, S. (1998) *Science* **281,** 2016-2018.

Charnay, C., Lee, A., Man, S.-Q., Moran, C. E., Radloff, C., Bradley, R. K., Halas, N. J. (2003) *J.Phys. Chem. B.* **107,** 7327-7333.

Chattopadhyay, P., Gupta, R. B. (2003) *Ind. Eng. Chem. Res.*, **42,** 465-472.

Chen, J., Reed, M. A., Rawlett, A. M., Tour, J. M. (1999) *Science* **286,** 1550-1552.

Chen, J., Herricks, T., Geissler, M., Xia.Y. (2004) *J. Am. Chem. Soc.* **126,** 10854-10855.

Chen, S., Huang, K. (2000) *Langmuir* **16,** 2014-2018.

Chen, S., Ingram, R. S., Hostetler, M. J., Pietron, J. J., Murray, R. W., Schaaff, T. G., Khoury, J. T., Alvarez, M. M., Whetten, R. L. (1998) *Science* **280,** 2098-2101.

Chen, S., Pei, R. *(*2000) *J. Am. Chem. Soc.* **123,** 10607-10615.

Chen, T., Chen, S., Sheu, H., Yeh, C. (2002) *J.Phys. Chem. B*, **106,** 9717-9722.

Chen, Y., Gu, X., Nie, C.-G., Jiang, Z.-Y., Xie, Z.-X., Lin, C. (2005) *J.Chem. Commun.*, 4181-4193.

Chiang, Y. S., Turkevich.J. (1963) *J.Colloid.Sci.*,**18,** 772-783.

Compano, R. (2001) *Nanotechnology* **12,** 85-88.

Cornelissen, J. J. L. M., Heerbeek, R. V., Kamer, P. C. J., Reek, J. N. H., Sommerdijk, N. A. J. M., Nolte, R. J. M. (2002) *Adv. Mater.* **14,** 489-492.

Corrolleur, C., Corrolleur, S., Gault, F. G. (1972) *J. Catal.* **24,** 385-401.

Corrolleur, C., Gault, F. G., Juttard, D., Maire, G., Muller, M. J. *(*1972a) *Catal.*, **27,** 466-.

Corrolleur, C., Tomanova, D., Gault, F. G. *(*1972b) *J. Catal.* **24,** 201-209.

Crooks, R. M., Lemon, B. I., III, Sun, L., Yeung, L. K., Zhao, M. (2001) *Top. Curr. Chem.* **212***,* 81-135.

Cui, X. D., Primak, A., Zarate, X., Tomfohr, J., Sankey, O. F., Moore, A. L., Moore, T. A., Gust, D., Harris, G., Landsay, S. M. (2001) *Science* **294,** 571-574.

Cullity, B. D., Stock, S. R. (2001) *Elements of X-ray Diffraction*, 3rd edition, Prentice Hall, Upper Saddle River, New York,.

Cunningham, D. P., Lundie, L. L. (1993) *Appl. Environ. Microbiol.* **59,** 7-14.

Dameron, C. T., Reese, R. N., Mehra, R. K., Kortan, A. R., Carroll, P. J., Steigerwald, M. L., Brus, L. E., Winge, D. R. (1989) *Nature* **338,** 596-597.

Davis, S. C., Klabunde, K. J. (1982) *Chem. Rev.* **82,** 153-208.

Deng, Z., Mao, C. (2003) *Nano Lett.* **3,** 545-576.

Dick, K., Dhanasekaran, T., Zhang, Z., Meisel, D. (2002) *J.Am. Chem. Soc.* **124,** 2312-2317.

Djalali, R., Li, S.Y., Schmidt, M. (2002) *Macromolecules* **35,** 4282-4288.

Dubertret, B., Skourides, P., Norris, D. J., Noireaux, V., Brivanlou, A. H., Libchaber, A. (2002) *Science* **298,** 1759-1762.

Dujardin, E., Peet, C., Stubbs, G., Culver, J. N., Mann, S. (2003) *Nano Lett.* **3,** 413-417.

Duran, N., Marcato, P. D., Alves, O., De Souza G. I. H., Esposito, E. (2005) *J. of Nanobiotech.* **3,** 8-14.

Edelstein, R. L., Tamanaha, C. R., Sheehan, P. E., Miller, M. M., Baselt, D. R., Whitman, L. J., Colton, R. J. (2000) *Biosensors Bioelectron* **14,** 805-813.

Egelhoff Jr., W. F. (1987) *Surf. Sci. Rep.* **6,** 253-415.

Elghanian, R., Storhoff, J. J., Mucic, R. C., Letsinger, R. L., Mirkin, C. A. (1997) *Science* **277,** 1078-1081.

El-Sayed, M. A. (2001) *Acc. Chem. Res.* **34,** 257-264.

Ershov, B. G., Henglein, A. (1993) *J. Phys. Chem.*, **97,** 3434-3436.

Esumi, K., Matsuhisa, K., Torigoe, K. (1995) *Langmuir* **11,** 3285-.

Fadley, C. S. Electron Spectroscopy, Theory, Techniques and Applications, Eds, C. R. Brundle, A. D. Baker, (1978) Academic Press, New York, Vol. 2, pp. 1–156.

Faraday, M. (1857) *Philos. Trans.* **147,** 145-181.

Feldheim, D. L., Keating, C. D. (1998) *Chem. Soc. Rev.* **27,** 1-12.

Feynman, R. P. (1991) *Science* **254,** 1300-1301.

Florin, E. L., Moy, V. T. Gaub, H. E. (1994) *Science* **264,** 415-417.

Forster, S., Antonietti, M. (1998) *Adv. Mater.* **10,** 195-217.

Fortin, D. and Beveridge, T. J., (2000) in Biomineralization. From Biology to Biotechnology and Medical Applications (ed. Baeuerien, E.), Wiley-VCH, Weinheim, p. 7.

Fowler, C. E., Shenton, W., Stubbs, G., Mann, S. (2001) *Adv. Mater.* **13,** 1266-1269.

Fran, F. C. (1951) *Acta.Cryst.* **4,** 497-507.

Frank, F. C. (1949) *Discussions Faraday Soc.* **5,** 48-54.

Frankel, J., Dorfman, J. (1930) *Nature* **126,** 274-275.

Fritz, J., Baller, M. K., Lang, H. P., Rothuizen, H., Vettiger, P., Meyer, E., Guntherodt, H. J., Gerber, C., Gimzewski, J. K. (2000) *Science* **288,** 316-318.

Fryer, J. R. (1979) Chemical Applications of Transmission Electron Microscopy, Academic Press, San Diego.

Gault, F. G. (1981) *Adv. Catal.* **30,** 1-95.

Glinka, Y. D., Lin, S. –H., Hwang, L. –P., Chen, Y. –T., Tolk, N. H. (2001) *Phys. Rev. B*, **64,** 085421-1-11.

Goldstein, A. N., Echer, C. M., Alivisatos, A. P. (1992) *Science* **256,** 1425-1427.

Gole, A., Murphy, C. J. *(*2004*) Chem. Mater.* **16***,* 3633-3640.

Goodsell, (2004) *Bionanotechnology, Lessons from Nature*, Wiley-Liss, Hoboken, New York.

Govindraj, A., Satishkumar, B. C., Nath, M., Rao, C. N. R. (2000) *Chem. Mater.*, **12,** 202-205.

Griffiths, P. R., De Haseth, J. A. (1986) Fourier Transform Infrared Spectrometry, John Wiley and Sons Inc., New York.

Gutierrez, M., Henglein, A. *J.* (1993) *Phys. Chem.* **97,** 11368-11370.

Haberzettl, C. A. (2002) *Nanotechnology* **R** 9**-**13.

Haes, A. J., Zhao, J., Zou, S., Own, C. S., Marks, L. D., Schatz, G. C., Van Duyne, R. P. (2005) *J. Phys. Chem. B*, **109,** 11158-11162.

Hagfeldt, A., Graetzel, M. (2000) *Acc. Chem. Res.* **33,** 269-277.

Hao, E., Kelly, K. L., Hupp, J. T., Schatz, G. C. (2002) *J. Am. Chem. Soc.* **124,** 15182-15183.

Haruta, M., Tsubota, S., Kobayashi, T., Kageyama, H., Genet, M. J., Delmon, B. (1993) *J.Catal.* **144,** 175-192.

Haruta, M., Yamada, N., Kobayashi, T., Iijima, S. *J. (*1989*) Catal.* **115,** 301-309.

Heath, J. R. (1995) *Science* **270,** 1315-1316.

Henglein, A. (2000) *J. Phys. Chem. B* **104,** 1206-1211.

Henglein, A. (1999) *Langmuir* **15,** 6738-6744.

Henglein, A., Mulvaney, P., Holtzworth, A., Sosebee, T. E., Fojitik, A. (1992) *Ber. Bunsenges. Phys. Chem.* **96,** 754-759.

Henry, C. R. (1998) *Surf. Sci. Rep.* **31,** 231-325.

Holmes, J. D., Smith, P. R., Evans-Gowing, R., Richardson, D. J., Russell, D. A., Sodeau, J. R. (1995) *Arch. Microbiol*. **163,** 143-147.

Hornyak, G. L., Patrissi, C. J., Martin, C. R. (1997) *J. Phys. Chem. B*, **101,** 1548-1555.

Hosea, M., Greene, B., McPherson, R., Heinzl, M., Alexander, M. D., Darnall, D. W. (1986) *Inorg. Chim. Acta.* **123,** 161-165.

Huang, C. –C., Yang, Z., Chang, H. –T. (2004) *Langmuir* **20,** 6089-6092.

Huang, Y., Duan, X. F., Wei, Q., Lieber, C. M. (2001) *Science* **291,** 630-633.

Hulteen, J. C., Jirage, K. B., Martin, C. R. *(*1998) *J. Am. Chem. Soc.* **120,** 6603-6604.

Hutter, E., Fendler, J. H. (2004) *Adv. Mater.* **16,** 1685-1706.

Itakura, T., Torigoe, K., Esumi, K. (1995) Langmuir **11,** 4129-4134.

Itoh, T., Asahi, T., Masuhara, H. (2001) *Appl. Phys. Lett.* **79,** 1667-1669.

Jaiswal, J. K., Mattoussi, H., Mauro, J. M., Simon, S. M. (2003) *Nature Biotechnol.* **21,** 47-51.

Jana, N. R., Gearheart, L. A., Murphy, C. J. (2001a) *Chem. Commun.* 617-618.

Jana, N. R., Gearheart, L., Murphy, C. J. (2001b) *Adv. Mater.* **13,** 1389-1393.

Jana, N. R., Gearheart, L., Murphy, C. J. (2001c) *J. Phys. Chem. B* **105,** 4065-4067.

Jana, N., Gearheart, L., Murphy, C. J. (2001d) *Langmuir* **17,** 6782-6786.

Jensen, T., Kelly, L., Lazarides, A., Schatz, G. (1999) *Cluster Sci.* **10,** 295-317.

Jiang, X., Xie, Y., Lu, J., Zhu, L., He, W., Qian, Y. (2001) *Langmuir* **17,** 3795-3799.

Jin, R., Egusa. S., Scherer. N. F. (2004) *J. Am. Chem. Soc.* **126***,* 9900-9901.

Jin, R., Cao, C., Hao, E., Metraux, G. S., Schatz, G. C., Mirkin, C. A. (2003) *Nature* **425,** 487-490.

Jin, R., Cao, Y. W., Mirkin, C. A., Kelly, K. L., Schatz, G. C.,. Zheng, J. G *(*2001) *Science* **294,** 1901-1903.

Jirage, K. B., Hulteen, J. C., Martin, C. R. (1997) *Science* **278,** 655-658.

Jirage, K. B., Hulteen, J. C., Martin, C. R. (1999) *Anal. Chem.* **71,** 4913-4918.

Joerger, R., Klaus, T., Granqvist, C. G., (2000) *Adv. Mater.* **12,** 407-409.

Joerger, T., Joerger, R., Olsson, E., Granqvist, C. G. (2001) *Trends Biotechnol.* **19,** 15-20.

Johnson, C. J., Dujardin, E., Davis, S. A., Murphy, C. J., Mann, S. *J. (*2002) *Mater. Chem.* **12,** 1765-1770.

Jorgensen, K. (1962) Absorption Spectra and Chemical Bonding in Complexes, Pergamon, New York,.

Kang, M. S., Martin, C. R. (2001) *Langmuir* **17,** 2753-2759.

Kelly, K. L., Coronado, E., Zhao, L. L., Schatz, G. C. *(*2003) *J .Phys. Chem. B* **107,** 668-677.

Kim, F., Song, J. H., Yang, P. (2002) *J. Am. Chem. Soc.* **124,** 14316-14317.

Kim, J. H., Germer, T. A., Mulholland, G. W., Ehrman, S. H. (2002) *Adv. Mater.* **14,** 518-521.

Kim, J., Yu,, Latyshev, I., Yamashita, T. (1999) *Appl. Phys. Lett.* **74,** 1156-1158.

Kim, K.-S., Demberelnyamba, D., Lee, H. (2004a) *Langmuir* **20,** 556-560.

Kim, U. K., Cha, S. H., Shin, K., Jho, J. Y., Lee, J. C. (2004b) *Adv. Mater.* **16,** 459-464.

Klabunde, K. J., Groshens, T., Brezinski, M., Kennelly, W. (1978) *J. Am. Chem. Soc.*, **19,** 59-86.

Klabunde, K. J., Timms, P. S., Skell, P. S., Ittel, S. (1979) *Inorg. Synth.* **19,** 59-86.

Klarreich, E. (2001) *Nature* **413,** 450-452.

Klaus, T., Joerger, R., Olsson, E., Granqvist, C. G., (1999) *Proc. Natl. Acad. Sci. USA* **96,** 13611-13614.

Klein, D. L., Roth, R., Lim, A. K. L., Alivisatos, A. P., McEuen, P.L. (1997) *Nature* **389,** 699-701.

Kottmann, J. P., Martin Oliver, J. F. (2001) *Opt. Lett.* **26,** 1096-1908.

Kowshik, M., Deshmukh, N., Vogel, W., Urban, J., Kulkarni, S. K., Paknikar, K. M. (2002a) *Biotechnol. Bioengineer.* **78,** 583-590.

Kowshik, M., Vogel, W., Urban, J., Kulkarni, S. K., Paknikar, K. M. (2002b) *Adv. Mater.* **14,** 815-818.

Kreibieg, U., Vollmer, M. (1995) *Optical properties of metal clusters*, , Springer, Berlin and New York.

Krieger, T., Boyen, M., Ziemann, G. H., Kabius, P. (2000) *Langmuir* **16,** 407-415.

Kuo, C.-H., Chiang, T.-F., Chen, L.-J., Huang, M. H. (2004) *Langmuir* **20,** 7820-7824.

Kurihara, K., Kizing, J., Stenius, P., Fender, J. H. (1983) *J. Am. Chem.Soc.* **105,** 2574-2579.

Kyotani, T., Tsai, L. F., Tomita, A. *J. (*1997) *Chem. Soc., Chem. Commun.* 701-702.

Labrenz, M., Druschel, G. K., Thomsen-Ebert, T., Gilbert, B., Welch, S. A., Kemner, K. M., Logan, G. A., Summons, R. E., De Stasio, G., Bond, P. L., Lai, B., Kelly, S. D., Banfield, J. F. (2000) *Science* **290,** 1744-1747.

Lawes, G. (1987) Scanning Electron Microscopy and X-ray Microanalysis, John Wiley and Sons Ltd. Chichester.

Lee, S. B., Martin, C. R. (2001) *Anal. Chem.* **73,** 768-775.

Lewis, L. N. (1993) *Chem. Rev.* **93,** 2693-2730.

Li, H., Luk, Y. Y., Mrksich, M. (1999) *Langmuir* **15,** 4957-4959.

Li, D., Li, J. (2003) *Chem. Phys. Lett.* **372,** 668-673.

Li, G., Lauer, M., Schulz, A., Boettcher, C., Li. F., Fuhrhop, J.-H. (2003) *Langmuir***19,** 6483-6491.

Liang, W., Shores, M. P., Bockrath, M., Long, J. R., Park, H. (2002) *Nature* **417,** 725-729.

Lianos, P., Thomas, J. K. (1986) *Chem. Phys. Lett.* **125,** 299-302.

Link, S., Burda. C., Nikoobakht El-Sayed. M. A. *J. (*2000) *Phys. Chem. B* **104,** 6152-6163.

Link, S., El-Sayed, M. A. (1999a) *J. Phys. Chem. B* **103,** 4212-4217.

Link**,** S., El-Sayed, M. A. (1999 b) *J. Phys. Chem. B*, **103,** 8410-8426.

Link, S., Mohamed, M. B., El-Sayed, M. A. (1999) *J. Phys. Chem. B* **103,** 3073-3077.

Liu, J. H., Wang, A. Q., Chi, Y. S., Lin, H. P., Mou, C. Y. (2005) *J. Phys. Chem. B* **109,** 40-43.

Liu, J., Alvarez, J., Kaifer, A. E. (2000) *Adv. Mater.* **12,** 1381-1383.

Liu, Z., Bando, Y. (2003) *Adv. Mater.* **15**, 303-305.

Love, J. C., Gates, B. D., Wolfe, D. B., Paul, K. E., Whitesides, G. M., (2002) *Nano Lett.* **2,** 891-894.

Lowe, C. R. (2000) *Curr. Opin. Struc. Biol.* **10,** 428-434.

Ludwig, M., Dettmann,W., Gaub, H. E. *(*1997) *J. Biophys.* **72,** 445-448.

Ma, J., Wong, H., Kong, L. B., Peng, K. W. (2003) *Nanotechnology.* **14,** 619-623.

Mah, C., Zolotukhin, I., Fraites, T. J., Dobson, J., Batich, C., Byrne, B. (2000) *J. Mol Therapy*, **1,** 214-239.

Maier, S. A., Brongersma, M. L., Kik, P. G., Atwater, H. A. (2002) *Phys. Rev. B* **65,** 193408-183411.

Maier, S. A., Brongersma, M. L., Kik, P. G., Meltzer, S., Requicha, A. G., Atwater, H. A. (2001) *Adv. Mater.* **13,** 1501-1505.

Maillard, M., Giorgio, S., Pileni, M.-P. (2002) *Adv. Mater.* **14,** 1084-1086.

Maisels, A., Kruis F. E., Fissan, H., Rellinghaus, B., Zahres, H., (2000) *Appl. Phys. Lett*. **77,** 4431-4433.

Marignier, J. L., Belloni, J., Delcourt, M. 0., Chevalier, J. P. (1986) *Nature* **317,** 344-345.

McHale, J. M., Auroux, A., Perotta, A. J., Navrotsky, A. (1997) *Science* **277,** 788-791.

Menon, V. P., Martin, C. R. (1995) *Anal. Chem.* **67,** 1920-1928.

Meyer, M., Wallberg, C., Kurihara, K., Fendler, J. H. (1984) *Chem. Commun.* 90-91.

Mie, G. (1908) *Ann. Physik.* **25,** 377-445.

Minko, S., Kiriy, A., Gorodyska, G., Stamm, M. (2002) *J. Am. Chem. Soc.* **124,** 10192-10197.

Mirikin, C. A. (2000) *J. Nanoparticle Res.* **2,** 121-122.

Mirkin, C. A. (2005) *Small* **1,** 14-16.

Mukherjee, P., Ahmad, A., Mandal, D., Senapati, S., Sainkar, S. R., Khan, M. I., Parischa, R., Ajayakumar, P. V., Alam, M., Kumar, R., Sastry, M., (2001a) *Nano Lett.* **1,** 515-519.

Mukherjee, P., Patra, C. R., Ghosh, A., Kumar, R., Sastry, M. (2002a) *Chem. Mater.* **14,** 1678-1684.

Mukherjee, P., Senapati, S., Mandal, D., Ahmad, A., Khan, M.I., Kumar, R., Sastry, M. (2002b) *Chem. BioChem.* **3,** 461-463.

Mukherjee,P., Ahmad,A., Mandal,D., Senapati,S., Sainkar,S.R., Khan,M.I., Ramani,R., Parischa,R., Ajayakumar, P. V., Alam, M.,Sastry,M., Kumar.R (2001b) *Angew. Chem. Int. Ed.* **40,** 3585-3588.

Murphy, C. J., Jana, N. R. (2002) *Adv. Mater.* **14,** 80-82.

Murphy, C. J., Sau, T. K., Gole, A. M., Orendorff, C. J., Gao, J., Gou, L., Hunyadi, S. E., Li, T. *J. (*2005a) *Phys. Chem. B.* **109,** 13857-13870.

Murphy, C. J., Sau, T. K., Gole, A., Orendorff, C. J. (2005b) *MRS Bull.* **30,** 349-355.

Naka, K., Yaguchi, M., Chujo, Y. (1999) *Chem. Mater.* **11,** 849-851.

Natter, H., Hempelmann, R. (2003) *Electrochim. Acta.* **49,** 51-61.

Newbury, D. E., Joy, D. C., Echlin, P., Fiori, C. E., Goldstein, J.I. (1986) Advanced Scanning Electron Microscopy and X-ray Microanalysis, Plenum Press, New York.

Niemeyer, C. M. (2001) *Angew. Chem. Int. Ed. Engl.* **40,** 4128-4158.

Nishizawa, M., Menon, V. P., Martin, C. R. (1995) *Science* **268,** 700-702.

Ohde, H., Hunt, F., Wai, C. M. (2001) *Chem. Mater.* **13,** 4130-4135.

Okumura, M., Haruta, M. (2000) *Chem. Lett.* 396-397.

Ongaro, A., Griffin, F., Beecher, P., Nagle, L., Iacopino, D., Quinn, A., Redmond, G., Fitzmaurice, D. (2005) *Chem. Mater.* **17,** 1959-1964.

Panatarotto, D., Prtidos, C. D., Hoebeke, J., Brown F., Kramer E., Briadd, J. P., Muller, S., Prato, M., Bianco, A. (2003) *Chemistry & Biology* **10,** 961-966.

Papavassiliou, G. C. (1980) *Prog. Solid State Chem.* **12,** 185-193.

Parak, W. J., Gerion, D., Pellegrino, T., Zanchet, D., Michael, C., Williams, S. C., Boudreau, R., Le Gros, M. A., Larabell, C. A., Alivisatos, A. P. (2003) *Nanotechnology* **14,** R15-27.

Parak, W. J., Gerion, D., Zanchet, D., Woerz, A. S., Pellegrino, T., Michael, C., Williams, S. C., Seitz, M., Bruehl, R. E., Bryant, Z., Bustamante, C., Bertozzi, C. R., Alivisatos, A. P. (2002) *Chem. Mater.* **14,** 2113-2119.

Park, J., Pasupathy, A. N., Goldsmith, J. I., Chang, C., Yaish, Y., Petta, J. R., Rinkoski, M., Sethna, J. P., Abruna, H. D., McEuen, P. L., Ralph, D. C. (2002) *Nature* **417,** 722-725.

Pasquato, L., Rancan, F., Scrimin, P., Mincin, F., Frigeri, C. (2000) *Chem. Commun.* 2253-2254.

Pastoriza-Santos. I., Liz-Marzan. L. M. (2002) *Nano Lett.* **2,** 903-905.

Pathak, S., Choi, S. K., Arnheim, N., Thompson, M. E. (2001) *J. Am. Chem. Soc.* **123,** 4103-4104.

Penner, R. M., Martin, C. R. (1987) *Anal. Chem.* **59,** 2625-2630.

Persson, S. H. M., Olofsson, L., Hedberg, L. *(*1999) *Appl. Phys. Lett.* **74,** 2546-2551.

Petit, C., Lixon, P., Pileni, M. P. (1990) *J. Phys. Chem.* **94,** 1598-1603.

Petit, C., Jain, T. K., Billoudet, F., Pileni, M. P. (1994) *Langmuir* **10,** 4446-4450.

Pietron, J. J., Murray, R. W. (1999) *J. Phys. Chem. B* **103,** 4440-4446.

Pileni, M. P. (2003) *Nature Mater*. **2,** 145-150.

Pileni, M. P., Motte, L., Petit, C. (1992) *Chem. Mater.* **4,** 338-345.

Poncharal, P., Wang, Z. I., Ugarte, D., De Heer, W. A. (1999) *Science* **283,** 1513-1516.

Poulin, J. C., Kagan, H. B., Vargaftik, M. N., Stolarov, I. P., Moiseev, I. I. (1995) *J. Mol. Catal.* **95,** 109-113.

Qu, L., Shi, G., Wu, X., Fan, B. (2004) Adv. Mater. **16,** 1200-1203.

Quinn, B. M., Liljeroth, P., Ruiz, V., Laaksonen, T., Kontturi, K. (2003) *J. Am. Chem. Soc.* **125,** 6644-6645.

Raimondi, F., Scherer, G. G., Kötz, R., Wokaun, A. (2005) *Angew. Chem. Int. Ed.* **44,** 2190-2209.

Rao, C. N. R., Muller, A., Cheetham, A. K. (Eds.), Wiley-VCH, (2004) Weinheim, Vol. 1.

Rau, R. C. (1962) Advances in X-ray Analysis, Ed, W. M. Mueller, Sir Isaac Pitman and Sons Ltd., London, , Vol. **5**, pp. 104–116.

Rechberger, W., Hohenau, A., Leitner, A., Krenn, J. R., Lamprecht, B., Aussenegg, F. R. (2003) *Opt. Commun.* **220,** 137-141.

Reed, M. A., Zhou, C., Muller, C. J., Burgin, T.P., Tour, J. M. (1997) *Science* **278,** 252-254.

Reese, R. N., Winge, D. R. (1988) *J. Biol. Chem.* **263,** 12832-12835.

Reetz, M. T., Helbig, W. (1994) *J. Am Chem. Soc.* **116,** 7401-7402.

Reetz, M. T., Helbig, W., Quaiser, S. A., Stimming, U., Breuer, N., Vogel, R. (1995) *Science* **267,** 367-369.

Reetz, M. T., Winter, M., Breinbauer, R., Thurn-Albrecht, T., Vogel, W. (2001) *Chem. Eur. J.* **7,** 1084-1094.

Richter, J., Seidel, R., Kirsch, R., Mertig, M., Pompe, W., Plaschke, J., Schackert, H. K. (2000) *Adv. Mater.* **12,** 507-510.

Robinson, J. W. *Atomic Absorption Spectroscopy*, Marcel Dekker, (1975) New York.

Rodriguez-Sanchez, L., Blanco, M. C., Lopez-Quintela, M. A. (2000) *J. Phys. Chem. B* **104,** 9683-9688.

Rodriguez-Sanchez, M. L., Rodrigues, M. J., Blanco, M. C., Rivas, J., Lopez-Quintela, M. A. (2005) *J. Phys. Chem. B* **109,** 1183-1191.

Roorda, S., van Dillen, T., Polman, A., Graf, C., van Blaaferen, A., Kooi, B. (2004) *J. Adv. Mater.* **16,** 235-237.

Rosenthal, S. J. (2001) *Nature Biotechnol.* **19,** 621-622.

Rosenthal, S. J., Tomlinson, I., Adkins, E. M., Schroeter, S., Adams, S., Swafford, L., McBride, J., Wang, Y., DeFelice, L. J., Blakely, R. D. (2002) *J. Am. Chem. Soc.* **124,** 4586-4594.

Rouch, D.A. Lee, B. T., Morby, A. T. *(*1995) *J. Ind. Micro.* **14,** 132-141.

Roucoux, A., Schulz, J., Patin, H. (2002) *Chem. Rev.* **102,** 3757-3778.

Salkar, R. A., Jeevanandam, P., Kataby, G., Aruna, S. T., Koltypin, Y., Palchik, O., Gedanken, A. (2000) *J. Phys. Chem. B* **104**, 893-897.

Sanyal, A., Sastry, M., (2003) *Chem.Commun.* 1236-1237.

Sastry, M., Swami, A., Mandal, S., Selvakannan, PR. *(2005) J. Mater. Chem.* **15,** 3161-3174.

Sau, T. K. , Murphy, C. J. (2004a) *J. Am. Chem. Soc.* **126,** 8648-8649.

Sau, T. K., Murphy, C. J. *(2004b) Langmuir* **20,** 6416-6422.

Schmid G., Simon U. (2005) *Chem. Commun.* **6,** 697-710.

Schon, J. H., Meng, H., Bao, N. (2001a) *Nature* **413,** 713-7`6.

Schon, J. H., Meng, H., Bao, N. (2001b) *Science* **294,** 2138-2140.

Senapati, S., Ahmad, A., Khan, M.I., Sastry, M., Kumar., R (2005) *Small* **1,** 517-520.

Service, R. F. (2001) *Science* **293,** 782-785.

Shaiu, W. L., Larson, D. D., Vesenka, J., Henderson, E. (1993) *Nucl. Acids. Res.*, **21,** 99-103.

Shankar, S. S., Rai, A,, Ankamwar, B., Singh, A., Ahmad, A., Sastry, M. (2004) *Nat. Mater.* **3,** 482-488.

Shankar, S. S., Rai, A., Ahmad, A., Sastry, M. (2005) *Chem. Mater.* **17,** 566-572.

Shenton, W., Douglas, T., Young, M., Stubbs, G., Mann, S. (1999) *Adv. Mater.* **11,** 253-256.

Shumaker-Parry, J. S., Rochholz, H., Kreiter, M. (2005) *Adv. Mater.* **17,** 2131-2134.

Silver, S. (1996) *Gene* **179,** 9-19.

Simkiss, K., Wilbur, K. M. (1989) Biomineralization, Academic Press, New York.

Skomski, R (2003) *J. Phys., Condens. Matter* **15,** R841-896.

Sloan, J., Wright, D. M., Woo, H.-G., Bailey, S., Brown, G., York, A. P. E., Coleman, K. S., Huchison, J. L., Green, M. L. H. (1999) *Chem. Commun.* 699-700.

Smith, P. R., Holmes, J. D., Richardson, D. J., Russell, D. A., Sodeau, J. R., (1998) *J. Chem. Soc., Faraday Trans.* **94,** 1235-1241.

Southam, G., Beveridge, T. J. (1996) *Geochimica et Cosmochimica Acta* **60,** 4369 4376.

Spinke, J., Liley, M., Guder, H. J., Angermaier, L., Knoll, W. (1993) *Langmuir* **9,** 1821-1825.

Stephen, J. R., Macnaughton, S. J. (1999) *Curr. Opin. Biotechnol.* **10,** 230-233.

Storhoff, J. J., Elghanian, R., Mucic, R. C., Mirkin, C. A., Letsinger, R. L. (1998) *J. Am. Chem. Soc.* **120,** 1959-1964.

Stranski, I. N. (1928) *Z.Phys.Chem.* **136,** 259-278.

Stryer, L. (1995) *Biochemistry* 4th edition, Freeman, New York.

Su, K.-H., Wei, Q.-H., Zhang, X., Mock, J. J., Smith, D. R., Schultz, S. (2003) *Nano Lett.* **3,** 1087-1090.

Suito, E., Uyeda, N. (1954) *Proc. Intern. Conf. Elect. Micro.* London, 224.

Suito, E., Uyeda. (1960) N. *Nature* **185,** 453-454.

Sun, N., Klabunde, K. J. (1999a) *J. Catal.* **185,** 506-512.

Sun, N., Klabunde, K. J. (1999b) *J. Am. Chem. Soc.* **121,** 5587-5588.

Sun, Y., Mayers, B., Xia, Y. (2003) *Adv. Mater.* **15,** 641-646.

Sun, Y., Xia, Y. (2002) *Science* **298,** 2176-2179.

Sun, Y., Xia, Y. (2004) *J. Am. Chem. Soc.* **126,** 3892-3901.

Sun, Y., Xia. Y. (2003) *Adv. Mater.* **15,** 695-699.

Swanson, N. L., Billard, B. D. (2003) *Nanotechnology* **14,** 353-357.

Swami. A., Kumar, A., Selvakannan, P. R., Mandal, S., Pasricha, R., Sastry, M (2003) *Chem. Mater.* **15,** 17-19.

Swami.A, Kasture, M.,   Pasricha, R., Sastry, M. (2004a) *J.Mater. Chem.* **14,** 709-714.

Swami. A., Kumar, A., D`Costa, M., Sastry, M. (2004b) *J. Mater.Chem.* **14,** 2696-2702.

Swihart M. T. (2003) *Curr. Opin. Colloid Interface Sci.* **8,** 127-133.

Tan, Y., Dai, X.,Y. Li, , Zhu, D. (2003) *J. Mater. Chem.* **13,** 1069-1075.

Tans, S. J., Verschueren, A. R. M., Dekker, C. (1998) *Nature* **393,** 49-51.

Taton, A. A. (2002) *Trends Biotechnol.* **20,** 277-279.

Taton, A. A. (2001) *Nature* **412,** 491-492.

Templeton, A. C., Pietron, J. J., Murray, R. W., Mulvaney, P. (2000) *J. Phys. Chem. B* **104,** 564-570.

Teng, X., Yang, H. (2005) *Nano Lett.* **5**, 885-891.

Thurston, T. R., Wilcoxon, J. P. (1999) *J. Phys. Chem.* **103**, 11-17.

Torigoe, K., Esumi, K. (1993) *Langmuir* **9,** 1664-1667.

Toshima, N., Harada, M., Yonezawa, T., Kushihashi, K., Asakura, K. (1991) *J. Phys. Chem.* **95**, 7448-7453.

Toshima, N., Wang, Y. (1994) *Adv. Mater.* **6,** 245-252.

Tsapis, N., Bennett, N. D., Jackson, B., Weitz, D. A., Edwards, D. A. (2002) *PNAS*, **19**, 12001-12005.

Tseng, G. Y., Ellenbogen. (2001) J. *Science* **294,** 1293-1294.

Turkevich, J., Hiller, J., Stevenson, P. C. (1951) *Discussions Faraday Soc***. 11,** 55-75.

Vilan, A., Cahen, D. (2002) *Trends Biotech.* **20,** 22**-**29.

Viswanathan, R., Lilly, G. D., Gale, W. F., Gupta, R. B. (2003) *Ind. Eng. Chem. Res***.** **42,** 5535-5540.

Vitulli, G., Bernini, M., Bertozzi, S., Pitzalis, E., Salvadori, P., Coluccia, S., Martra, G. (2002)*Chem. Mater.* 14, 1183-1186.

Wang, D., Zhou, W. L., McCaughy, B. F., Hampsey, J. E., Ji, X., Jiang, Y.-B., Xu, H., Tang, J., Schmehl, R. H., O'Connor, C., Brinker , C. J., Lu, Y. (2003) *Adv. Mater.* **15**, 130-133.

Wang, S., Mamedova, N., Kotov, N. A,, Chen, W., Studer, J. (2002) *Nano Letters* **2,** 817-822.

Wang, Y. (1991) *Acc. Chem. Res.* **24,** 133-139.

Wang, Z. I., Poncharal, P., De Heer, W. A. (2000) *J. Phys. Chem. Solids* **61,** 1025-1030.

Wang, Z. L. (2000) *Adv. Mater.* **12,** 1295-1298.

Wang, Z. L. Characterization of Nanophase Materials, Ed, Z. L. Wang, (2000) Wiley-VCH, Weinheim, Ch 3, pp. 37.

Wang, Z. L., Mohamed, M. B., Link, S., El-Sayed, M. A. (1999) *Surf. Sci.* **440,** L809.

Warner, M. G., Reed, S. M., Hutchison, J. E. (2000) *Chem. Mater.* **12,** 3316-3320.

Wegner, K., Walker, B., Tsantilis, S., Pratsinis, S. E. (2002) *Chem. Eng. Sci* **57,** 1753-1762.

Wei, G., Zhou, H., Liu, Z., Song, Y., Wang, L., Sun, L., Li, Z. (2005) *J. Phys. Chem. B.* **109,** 8738-8743.

Weiss, P. S. (2001) *Nature* **413,** 585-586.

Whitesides, G. M. (2003) *Nature Biotechnol.* **21,** 1161-1165.

Wind, S. J., Appenzeller, J., Martel, R., Derycke, V., Avouris, P. (2002) *Appl. Phys. Lett.* **80,** 3817-3819.

Xiong, Y., Chen, J., Wiley, B., Xia, Y., Yin, Y., Li, Z.-Y. (2005) *Nano Lett.* **5,** 1237-1242.

Xu, H., Bjerneld, E. J., Kall, M., Borjesson, L. (1999) *Phys. Rev. Lett*. **83,** 4357-4360.

Yan, B., Yang, Y., Wang, Y. (2003) *J. Phys. Chem. B* **107,** 9159-9164.

Yoffe, A. D. (1993) *Adv. Phys.* **42,** 173-266.

Yonezawa, Y., Sato, T., Ohno, M., Hada, H. (1987) *J. Chem. Soc., Faraday Trans.* **183,** 1559-1567.

Yonezawa, Y., Sato, T., Kuroda, *S.* (1991) *J. Chem. Soc.Faraday Trans.* **87,** 1905-1910.

Yoshida, J., Kobayashi, T. (1999) *J Magn Magn Mater* 194**,** 176-184.

Yu, Y.-Y., Chang, S.-S., Lee, C.-L., Wang, C. R. C. (1997) *J. Phys. Chem. B. 101*, 6661-6664.

Zhang, D., Qi, L., Ma, J., Cheng, H. (2001) *Chem. Mater.* **13**, 2753-2755.

Zhang, H., Banfield, J. F. (1998) *J. Mater. Chem* **8**, 2073-2076.

Zhang, J. H. (1997) *Acc. Chem. Res.* **30**, 423-429.

Zhang, M., Drechsler, M., Muller, A. H. E. (2004) *Chem. Mater.* **16,** 537-543.

Zhang, P., Sham, T. K. (2002) *Appl. Phys. Lett*. **81,** 736-738.

Zheng, X., Zhu, L., Yan, A., Wang, X., Xie, Y. (2003) *J. Colloid Interface Sci.* **268,** 357-361.

Zhou, Y., Yu, S. H., Cui, X. P., Wang, C. Y., Chen, Z. Y. (1999) *Chem. Mater.* **11**, 545-546.

CHAPTER 2

BIOSYNTHESIS OF EXTRACELLULAR CdSe QUANTUM DOTS BY THE FUNGUS, *FUSARIUM OXYSPORUM*

SUMMARY

Fusarium oxysporum, when incubated with a mixture of selenium tetrachloride and cadmium chloride in distilled water could synthesize semiconductor cadmium selenide (CdSe) nanoparticles extracelluarly at room temperature. The nanoparticles were characterized by X-ray diffraction, transmission electron microscopy, X-ray photoelectron spectroscopy, UV-Vis optical absorption, and Fluorescence spectroscopy. Characterization of the nanoparticles showed them to be spherical and polydisperse with sizes ranging from 9-15 nm.

INTRODUCTION

Semiconductor quantum dots (QDs) have attracted considerable attention due to their application as luminescent probes (Alivisators, 1996a; Nirmal and Brus, 1999). These crystalline clusters of semiconductors, also known as quantum dots, are in the range of 1-10 nm and exhibit unique optical properties due to their quantum confinement effects (Efros and Efros, 1982; Efros and Rosen, 2000). Their high photostability, controllable and narrow emission spectra along with high quantum yield offer significant advantages over conventional fluorescent dyes for imaging biological samples (Michalet et al., 2001, Chan et al., 2002). Although, many physical and chemical methods exist for the synthesis of CdSe nanoparticles, most of them involve the use of high temperature, toxic chemicals and poor reproducibility. Since, microorganisms (Kowshik et al., 2002a, b; Ahmad et al., 2002; Bai et al., 2006) and plant materials (Shankar et al., 2004) can also synthesize different metal nanoparticles; they offer a viable alternative for the development of ecofriendly process for their synthesis. This Chapter describes the use of an endophytic fungus viz. *Fusarium oxysporum* for the extracellular synthesis of CdSe quantum dots.

MATERIALS

Cadmium chloride (CdCl$_2$), selenium tetrachloride (SeCl$_4$), nicotinamide adenine dinucleotide phosphate, reduced form (α-NADPH) and sodium nitrate, (Sigma chemicals Co., St Louis, MO, U.S.A) were used. All other chemicals used were of analytical grade.

METHODS

Microorganism and growth

Fusarium oxysporum was maintained on PDA slants (potato 20% w/v, dextrose 2% w/v and agar 2% w/v) at 25 ^{o}C. The fermentation was carried out by inoculating a 1 cm diameter colony, from a 7 day old PDA slant, into a 100 ml liquid MGYP medium (0.3% w/v malt extract, 1.0% w/v glucose, 0.3% w/v, yeast extract and 0.5% w/v peptone), in 500 ml Erlenmeyer flasks, followed by incubation at 26±1°C on a rotary shaker (200 rpm) for 96 h**.** After the fermentation period the mycelia was collected by centrifugation (4500 g, 20 min at 10°C) washed extensively with distilled water under aseptic conditions and used for further studies.

Extracellular synthesis of CdSe quantum dots

Twenty grams (wet weight) of the mycelia was incubated with 100 ml of sterile distilled water containing 1 mM $CdCl_2$, 5 mM $NaNO_2$ and 1 mM $SeCl_4$, in 500 ml Erlenmeyer flasks, for 96 h under shaking (200 rpm) at room temperature. Samples were removed at fixed time intervals and subjected to UV-Vis spectroscopy to check for the formation of nanoparticles. After the fermentation period, the unbound proteins were removed by precipitation with 2 volumes of absolute ethanol and the nanoparticles were subjected to characterization.

UV-Vis and fluorescence measurements

UV-Vis spectrophotometric measurements were performed on a Shimadzu dual-beam spectrophotometer (model UV-1601 PC) operated at a resolution of 1 nm.

Fluorescence measurements were carried out on a Perkin-Elmer LS 50B luminescence spectrofluorimeter, with slit width of 2 nm for both the monochromators and scan speed 300 nm/min. Samples were excited at 370 nm and the emission spectra were recorded in wavelength ranging from 380 nm to 700 nm.

Life time measurements were performed on a time correlated single photon counting FLS920 spectrofluorimeter (Edinburgh Instruments, Livingston, UK). A hydrogen flash lamp of pulse width 1 ns was used for excitation and an nF-900 nanosecond flash lamp was used to detect the fluorescence. Sample was exited at 370 nm and emission was recorded at 440 nm. Slit widths of 15 and 15 nm were used on the excitation and emission monochromators, respectively. The resultant decay curves

were analyzed by a multiexponential iterative fitting program supplied by Edinburgh Instruments.

Transmission electron microscopy (TEM)

Samples were prepared by drying a drop of CdSe nanoparticles solution on carbon coated TEM copper grids followed by measurements on a JEOL Model 1200 EX Transmission electron microscope operated at an accelerating voltage of 80 kV.

X-ray diffraction measurements (XRD)

Thin films of CdSe nanoparticles were prepared on Si (111) substrates by drop-coating with nanoparticles solution. The films on Si wafers were then subjected to X-ray diffraction (XRD) on a Philips PW 1830 instrument operated at 40 kV and at 30 mA with Cu Kα radiation.

X-ray photoelectron spectroscopy (XPS)

Chemical analysis of a drop-coated film of CdSe nanoparticles on a conductive substrate was carried out on a ESCALAB MK II X-ray photoelectron spectrometer (V. G. Scientific, UK) with A1 k_α as the exiting source (hγ = 1486.6 e V) operating at an accelerating voltage of 10 kV and 20 m A at a pressure of about 10^{-8} Pa.

RESULTS AND DISCUSSION

The fungus, *Fusarium oxysporum* when incubated with a mixture of $CdCl_2$/$SeCl_4$ showed a gradual change in the color from colorless to reddish brown (Fig. 2.1) suggesting the formation of CdSe nanoparticles. Although the mechanism for the formation of nanoparticles in biological media is still not clear, in case of chemical methods it has been attributed to oxidation/reduction reactions which can be followed by observing the change in the color of reaction mixture due to excitation of surface plasmon resonance in the nanoparticles (Henglein, 1993; Sastry et al., 1997; Sastry et al., 1998). In the present studies the change in color of the incubation medium suggested the reduction of the metal salts leading to the formation of CdSe nanoparticles.

Visual inspection

Figure 2.1 shows test tubes of the $CdCl_2$, $SeCl_4$ reaction solution at the beginning (time $t=0$ (A)) and after 96 h of reaction (B) with the *Fusarium oxysporum* biomass. The appearance of reddish brown color clearly indicated the formation of CdSe nanoparticles.

A   **B**

Fig. 2. 1: Formation of CdSe nanoparticles in presence of fungal biomass.
A: At 0 h and B**:** At 96 h.

UV/Vis spectroscopy

The UV-Vis spectra (Fig.2.2) showed a well-defined surface plasmon band centered at around 370 nm, characteristic of CdSe nanoparticles (Henglein, 1993; Sastry et al., 1997; Sastry et al., 1998). Absorption between 270-280 nm suggests the presence of proteins in the extracellular broth (Eflink and Ghiron, 1981).

The nanoparticles were extremely stable, with no evidence of flocculation of the particles even after a month. The long-term stability of the CdSe nanoparticles in solution can be correlated to the presence of the capping peptide/protein that binds to the surface of the nanoparticles preventing its flocculation.
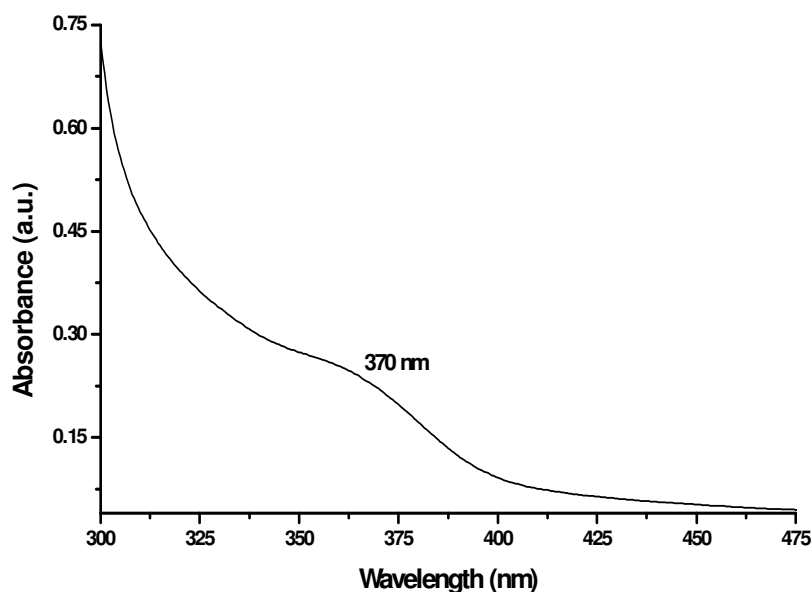
Fig. 2. 2: UV-Vis spectra of the CdSe nanoparticles.

Fluorescence spectroscopy

Fluorescent measurements showed a red shift in the emission spectra characteristic of CdSe nanoparticles. A red shift in the emission peak at 440 nm (Fig. 2.3) was observed that can be correlated to the band gap or near band gap emission resulting from the recombination of the electron-hole pairs in the CdSe nanoparticles
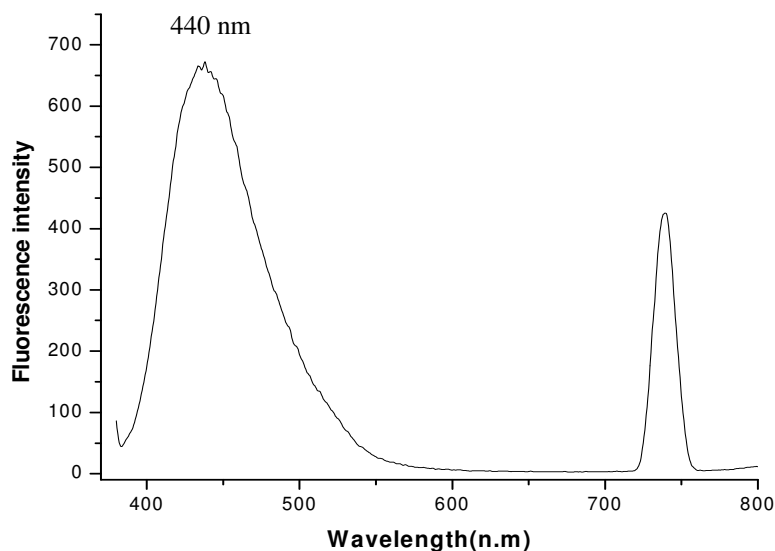
Fig. 2. 3: Fluorescence emission spectra of the CdSe nanoparticles.

The small stokes` shift in the band indicates that the nanocrystals possess a continuous surface with most surface atom exhibiting the coordination and oxidation states of their bulk counterparts (Brus, 1986; Gallardo et al., 1989; Machol et al., 1994) Although the intensity of the peak gradually increases with reaction time, the

emission spectra shows the same general features. In the present case, the absence of any red shifted emission band with respect to time suggested the presence of uniformly distributed cadmium selenide nanoparticles.

The luminescence characteristics shown by semiconductor nanoparticles have potential implications in the field of optoelectronic devices (Steigerwald and Brus, 1990; Nirmal and Brus, 1999; Empedocles and Bawendi, 1999) and biosensors (Alivisators, 1996b; Peng et al., 1998; Brucher et al., 1998; Chan and Nie, 1998). This property is dependent on the nature, physical dimensions and the chemical environments of the nanoparticle (Spanhel et al., 1987; Henglein, 1989; Steigerwald and Brus, 1990; Nirmal and Brus, 1999; Empedocles and Bawendi, 1999).

Time resolved fluorescence spectroscopy

The fluorescence decay curves of the CdSe nanoparticles obtained from the time resolved measurements are given in Figure 2.4. The decay curve profile could be best fitted to bi-exponential function ($\chi^2 \leq 1.1$). The life time values obtained for CdSe nanoparticles were 6.397 and 1.424 ns ($\chi^2 = 1.125$) which is comparable to the one reported by chemical methods 6 and 1 ns (Firth et al., 2004).
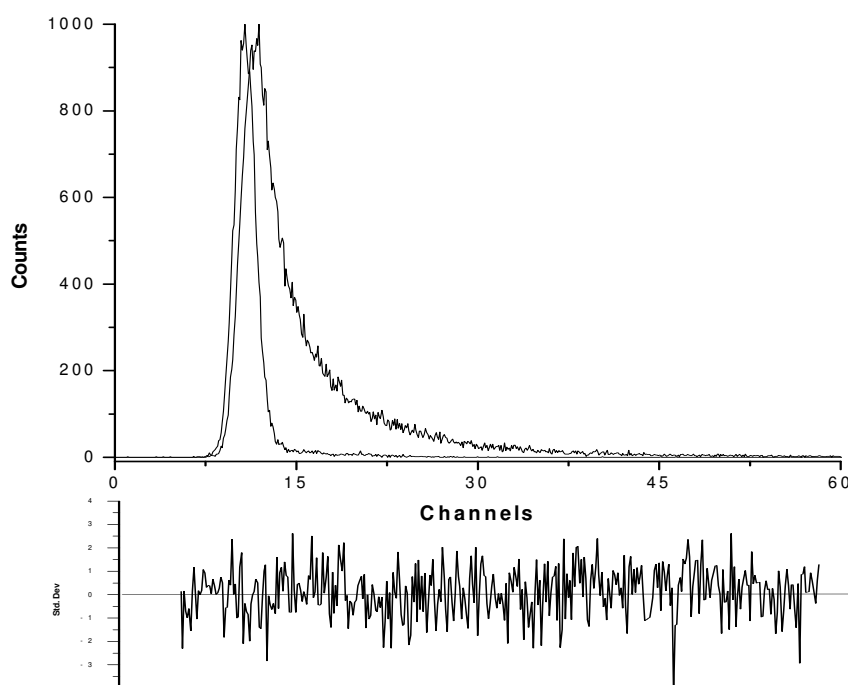
Fig.2.4: Time-resolved fluorescence profile of the CdSe nanoparticles. The solid line corresponds to the nonlinear least square fit of the experimental data to a bi-exponential function. The lower panels represent the residuals.

Analysis of the decay profile also yielded the relative contributions of each component of the total fluorescence intensity. For CdSe nanoparticles, α_1 was 0.020, corresponding to 55.85% of the total fluorescence intensity while that of α_2 was 0.070 amounting to 44.15% contribution to the total fluorescence intensity.

X-Ray diffraction

X-ray diffraction (XRD) pattern of CdSe nanoparticles, on Si biofilm is given in Figure 2.5.
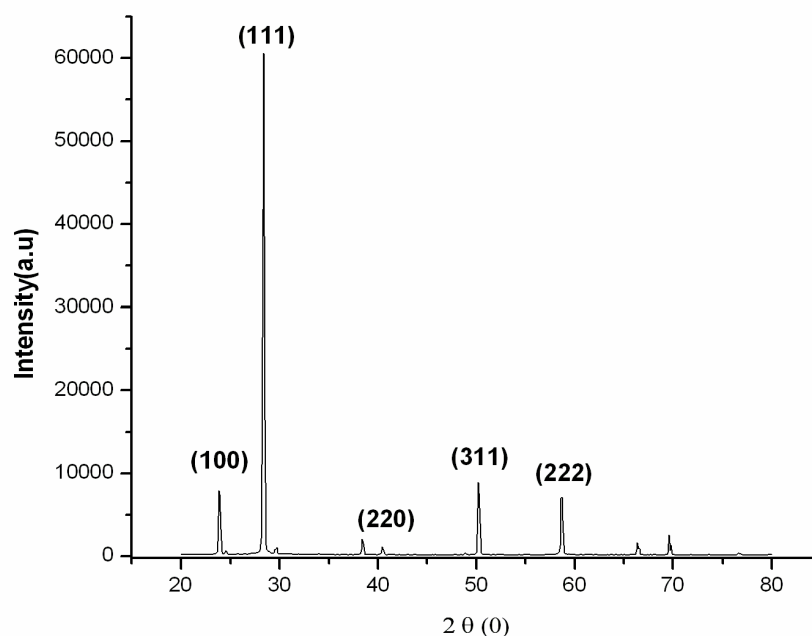
Fig. 2.5: XRD pattern of the CdSe nanoparticles.

Intense peaks corresponding to (100), (111), (220), (311) and (222) in the 2θ range 20°-70° was observed which are in agreement with those reported for the CdSe nanocrystals (Colvin et al., 1992; Kundu et al., 1997). The broadening of Bragg peaks indicates that the CdSe particles formed are of nanoscale dimensions.

Transmission electron microscopy

TEM analyses of the CdSe nanoparticles showed that they are polydisperse and are in size ranging from 9-15 nm with an average of 11 $\pm$ 2 nm (Fig. 2.6). The morphology of the nanoparticles was essentially spherical.
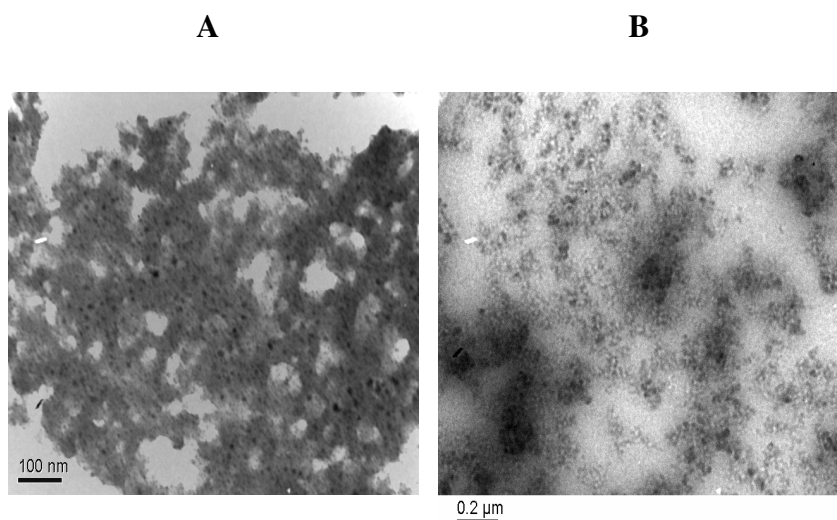
Fig.2.6: TEM micrograph image of the CdSe nanoparticles at different magnifications. A: At 100 nm resolution and **B:** At 200 nm resolution.

X-Ray photoelectron spectroscopy

The presence of CdSe nanoparticles was also confirmed by analyzing the samples by X-ray photoelectron spectroscopy is shown in Figure 2.7. The results showed the presence of Cd, Se, C, O, N and Na as the prominent elements.
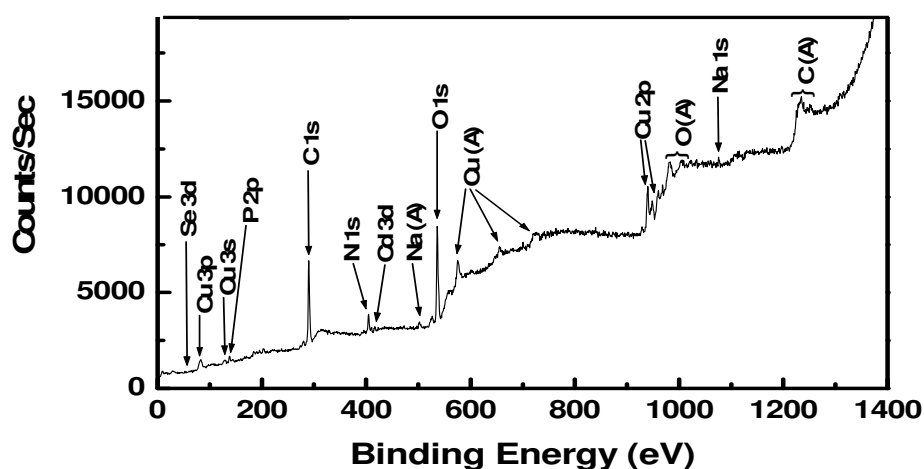
Fig.2.7: X-ray photoelectron spectroscopy of the CdSe nanoparticles.

The core level binding energies and atomic percentages of various elements detected in the reaction mixture is given in Table 1.

Table 2.1*:* **Core level binding energies and atomic percentages of various elements detected in the reaction mixture.**

Element	C1s	O1s	N1s	Cd 3d$_{5/2}$	Se 2P
Atomic %	70.4	22.5	4.6	1.3	1.2
Binding energy [e V]	285.0	531.1	399.7	405.5	64.8

The Cd 4f spectrum could be decomposed into two spin-orbit components 1 and 2 (spin-orbit splitting ~ 6 eV). The Cd 3d$_{5/2}$ and 3d$_{3/2}$ peaks occurred at a binding energy of 406 eV and 412 eV respectively, which agrees with the core level binding energies and are characteristic of metallic Cd (Fig. 2.8.A).
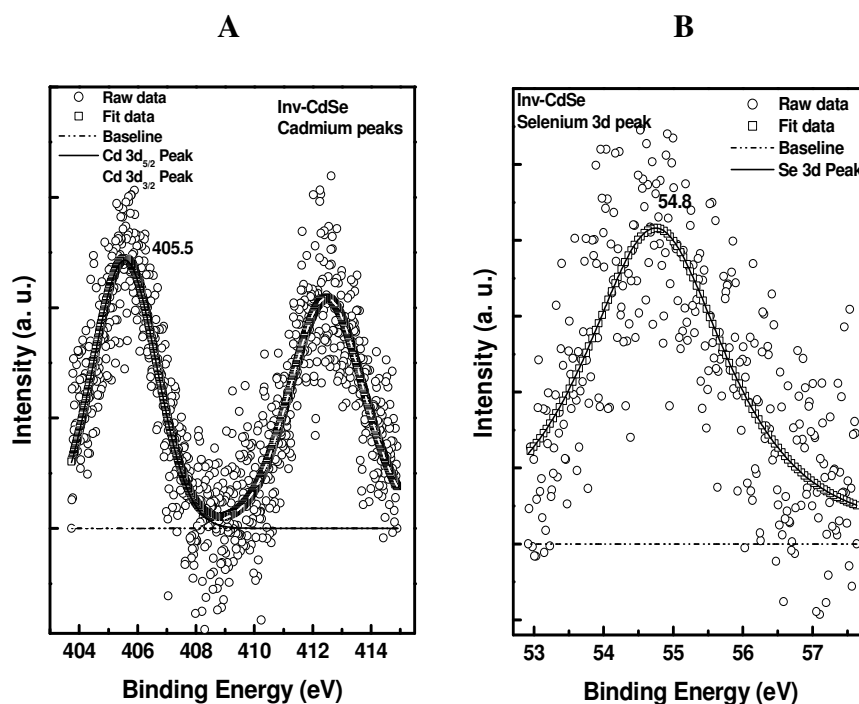
Fig. 2.8: Core level spectra of CdSe nanoparticles. A: Cd 3d and **B:** Se 3d*.*

Moreover, the binding energy of 54.8eV for Se 3d peak is comparable to the core level binding energies and is typical for metallic Se (Fig. 2.8.B). The absence of a higher binding energy for both Cd $3d_{5/2}$ and Se 3d clearly shows that all the metal ions are fully reduced by the fungus *Fusarium oxysporum* and are in metallic form. Our results are in agreement with that of Chastain (1992).

CONCLUSION

The present studies show that *Fusarium oxysporum* can be used for the biosynthesis of CdSe nanoparticles. Our observations are of importance since it demonstrates that this fungus can be used for the development of a reliable ecofriendly process for the synthesis of CdSe nanoparticles at ambient temperature and pressure.

REFERENCES

Ahmad, A., Mukherjee, P., Mandal, D., Senapati, S., Khan, M.I., Kumar, R., Sastry, M., (2002) *J. Am. Chem. Soc.* **124,** 12108-10129.

Alivisatos, A. P. (1996a) *Science* **271,** 933-937.

Alivisatos, A. P. (1996b) *J. Phys. Chem.* **100,** 13226-13239.

Bai. Hong-Juan., Zhao-Ming, Z., Jungong, (2006) *Biotechnol. Lett.* **28,** 1135-1139.

Bruchez, Jr. M., Moronne, M., Gin, P., Weiss, S., Alivisatos, A. P. (1998) *Science* **281,** 2013-2016.

Brus, L. (1986) *J. Phys. Chem.* **90,** 2555-2560.

Chan, W. C. W., Nie, S. (1998) *Science* **281,** 2016-2018.

Chan, W. C., Maxwell, D., Gao, X., Vailer, R., Han, M., Nie, S. (2002) *Curr. Opin. Biotechnol.* **13**, 40-46.

Chastain, J. (1992) Handbook of X-ray Photoelectron Spectroscopy, Perkin-Elmer, Norwalk.

Colvin, V. L., Goldstein, A. N., Alivisators, A. P. (1992) *J. Am. Chem. Soc.* **114,** 5221-5230.

Eflink, M. R., Ghiron, C. A. (1981) *Anal. Biochem.* **114,** 199-277.

Efros, A. L., Efros, A. L. (1982) *Sov. Phys. Semicond.* **16,** 772-775.

Efros, A. L., Rosen, M. (2000) *Annu. Rev. Mater. Sci.* **30,** 475-521.

Empedocles, S., Bawendi, M. G. (1999) *Acc. Chem. Res.* **32,** 389-396.

Firth, A. V., Haggata, S. W., Khanna, P. K., Williams, S. J., Allen, J. W., Magennis, S. W., Irof Samuel D. W., Cole-Hamilton, D. J. (2004) *J. Liminesc.* **109**, 163-172.

Gallardo, S., Gutierrez, M., Henglein, A., Janata, E. (1989) *Ber. Bunsen-Ges. Phys. Chem.* **93,** 1080-1090.

Henglein, A. (1989) *Chem. Rev.* **89,** 1861-1873.

Henglein, A. (1993) *J. Phys. Chem.* **97,** 5457-5471.

Kowshik, M., Vogel, W., Urban, J., Kulkarni, S. K., Paknikar, K. M. (2002a) *Adv. Mater.* **14,** 815-818.

Kowshik, M., Deshmukh, N., Vogel, W., Urban, J., Kulkarni, S. K., Paknikar, K. M. (2002b) *Biotechnol. Bioengineer.* **78,** 583-590.

Kundu, M., Khosravi, A. A., Kulkarni, S. K. (1997) *J. Mater. Sci.* **32,** 245-248.

Machol, J. L., Wise, F. W., Patel, R., Tanner, D. B. (1994) *Phys. A* **207,** 427-434.

Michalet, X., Pinaud, F., Lacoste, T. D., Dahan, M., Bruchez, M. P., Alivisatos, A. P., Weiss, S. (2001) *Single. Mol.* **2,** 261-276.

Nirmal, M., Brus, L. (1999) *Acc. Chem. Res.* **32,** 407-414.

Peng, X., Wickham, J., Alivisatos, A. P. (1998) *J. Am. Chem. Soc.* **120,** 5343-5344.

Sastry, M., Bandyopadhyay, K., Mayya, K. S. (1997) *Colloid. Surf. A* **127,** 221-228.

Sastry, M., Patil, V., Sainkar, S. R. (1998) *J. Phys. Chem. B* **102,** 1404.

Shankar, S. S., Rai, A,, Ankamwar, B., Singh, A., Ahmad, A., Sastry, M. (2004) *Nat. Mater.* **3,** 482-488.

Spanhel, L., Haase, M., Weller, H., Henglein, A. (1987) *J. Am. Chem. Soc.* **109,** 5649-5655.

Steigerwald, M. L., Brus, L. E. (1990) *Acc. Chem. Res.* **23,** 183-188.

CHAPTER 3

PURIFICATION AND CHARACTERIZATION OF
α-NADPH DEPENDENT SULPHITE AND
NITRATE REDUCTASES FROM THE
EXTRACELLULAR BROTH OF *FUSARIUM
OXYSPORUM*

SUMMARY

Fusarium oxysporum when incubated with a solution containing cadmium chloride and selenium tetrachloride synthesized cadmium/selenide nanoparticles extracellularly. The formation of nanoparticles was due to the presence of oxidoreductases in the extracellular medium. The sulphite reductase was purified to homogeneity by DEAE-Sephadex chromatography followed by gel filtration on Sephacryl S-300, with an over all yield of 20%. The purified enzyme is a 35.6 kDa protein with a pI of 4.5 and contains 5.3% neutral sugar. The enzyme required α-NADPH as a cofactor. Its optimum pH and optimum temperature was 6.0 and 45°C respectively. Nitrate reductase was purified to homogeneity by negative adsorption on DEAE- Sephadex and chromatography on CM-Sephadex followed by gel filtration on Sephacryl S-300. It is a 44.1 kDa protein with pI of 9.2 and contains 8.4% neutral sugar. The optimum pH and optimum temperature of the purified enzyme was 7.0 and 55°C respectively.

INTRODUCTION

In the previous chapter, we have demonstrated the use of fungus, *Fusarium oxysporum* for the extracellular synthesis of CdSe nanoparticles. Exposure of microorganisms to high concentration of metal ions is known to result in the secretion of enzymes and proteins (Martino et al., 2002). Since nanoparticles formation involves oxidation/reduction reactions attempts were made to look for oxidoreductases in the extracellular broth of *Fusarium oxysporum*, purify and characterize them to evaluate their role in the synthesis of nanoparticles.

MATERIALS

Sephacryl S-300 (Pharmacia Fine Chemicals, Uppsala, Sweden), acrylamide, SDS molecular mass markers, 2 (N-Morpholino) ethanesulfonic acid (MES), nicotinamide adenine dinucleotide phosphate, reduced form (α-NADPH), Coomassie Brilliant Blue G-250, DEAE-Sephadex, CM-Sephadex, SDS, sodium sulfite, sodium nitrate, N-(1-napthyl)-ethylenediamine dihydrochloride and sulfanilamide (Sigma Chemicals Co., St Louis, MO, U.S.A) were used. All other chemicals used were of analytical grade.

METHODS

Detection of sulphite and nitrate reductases

This was carried out by mixing 100 µl of the extracellular broth (Chapter 2, page 34) with 1 ml of 1 mM $AuCl_4$ or $AgNO_3$ and following the color development. Formation ruby-red and brown color indicted the presence of sulphite and nitrate reductases, respectively.

Enzyme assays

Determination of sulphite reductase activity: This was carried out according to Yashimoto et al. (1968). The total reaction mixture 1.4 ml contained 1.0 mM of freshly prepared sodium sulfite, in 200 mM MES buffer pH 6.0, containing 1.0 mM EDTA, 0.15 mM NADPH and appropriately diluted enzyme. The reaction was initiated by the addition of NADPH followed by incubation at 45°C. The oxidation of NADPH was monitored spectrophotometrically at 340 nm. Enzyme samples incubated in the absence of sodium sulfite served as blank. One unit of sulphite reductase activity is defined as the amount of enzyme required to oxidize 1 mole of NADPH/min under the assay conditions.

Determination of nitrate reductase activity: This was performed as described by Snell et al. (1949). The total reaction mixture of 1.5 ml, in one side arm of a Thunberg tube, contained 10 µ mole of sodium nitrate in 200 µM of phosphate buffer pH 7.0 and appropriately diluted enzyme. The other arm of the Thunberg tube contained 1mg of freshly prepared sodium dithionate and 1 mM α-NADPH, in 1ml of 200 µM phosphate buffer pH 7.0. Subsequently the tubes were evacuated and the reaction was initiated by the addition of α-NADPH and sodium dithionate followed by incubation at 55°C for 10 min. The reaction was terminated by opening the Thunberg tube to oxidize excess α-NADPH. To 1 ml of the above reaction mixture, 1ml of sulfanilamide reagent (1% w/v in 1 M HCl) and 1 ml of N-(1-napthyl)-ethylenediamine dihydrochloride (0.02% w/v in water) was added and left at room temperature for 10 min. The red color developed was measured at 540 nm after making up the volume to 9.5 ml with water. One unit of the enzyme is defined as the amount of enzyme required to produce 100 n mole of nitrite under the assay conditions. Reaction mixture incubated in the absence of enzyme served as blank.

Protein determination

Protein concentration was determined according to Lowry et al. (1951) using BSA as standard. However, during enzyme purification steps protein concentrations were determined using the formula $1.55 \, A_{280} - 0.76 \, A_{260} = $ protein (mg/ml) (Stoscheck, 1990).

Microorganism and growth

Fusarium oxysporum was maintained on PDA slants (potato 20% w/v, dextrose 2% w/v and agar 2% w/v) at 25°C. The fermentation was carried out by inoculating a 1 cm diameter mycelium from 7 day old PDA slant, into a 100 ml liquid MGYP medium (0.3% w/v *m*alt extract, 1.0% w/v *g*lucose, 0.3% w/v, yeast extract and 0.5% w/v *p*eptone), in 500 ml Erlenmeyer flasks, followed by incubation at 26±1°C on a rotary shaker (200 rpm) for 96 h. After the fermentation period, the mycelia was collected by centrifugation (4500 *g*, 10°C, 20 min) washed with distilled water under sterile conditions and then suspended in 100 ml sterile distilled water, in 500 ml Erlenmeyer flask for 48 h under shaking (200 rpm). The supernatant was collected by centrifugation (4500 *g,* 20 min), concentrated by lyophilization, dialyzed extensively against Milli Q water followed by dialysis against 20 mM phosphate buffer, pH 7.2 and used as the source of enzymes.

Mr determination

SDS-polyacrylamide gel electrophoresis (pH 7.0) was carried out according to Weber and Osborn (1969) with phosphorylase b (Mr 97.4 kDa), bovine serum albumin (Mr 66kDa), ovalbumin (Mr 43 kDa), carbonic anhydrase (Mr 29.1 kDa) soyabean trypsin inhibitor (Mr 20.1 kDa) and lysozyme (Mr 14.3kDa) as reference proteins. After electrophoresis the gels were stained with Coomassie Brilliant Blue R-250.

Isoelectric focusing (IEF)

This was carried out on a Rotofor system (Bio-Rad), using ampholytes over the pH range of 3-10.

Carbohydrate content

Purified enzyme (400 µg in 400 µl water) was incubated with 400 µl of 5% (w/v) phenol at for 10 min at room temperature. Two ml of sulphuric acid was then added and the

mixture was allowed to cool for 20 min at room temperature. The color developed was then measured spectrophotometrically, at 490 nm by using mannose as standard (Duboin et al., 1956).

Amino acid analysis

The amino acid composition was determined on an AccQ-Fluo (Waters Corporation, USA) equipped with a fluorescent detector. Salt free protein sample (50 µg) was hydrolyzed in 6 N constant boiling HCl, in a vacuum sealed hydrolyzing tube, for 24 h at 110°C. After hydrolysis, the sample was again lyophilized, dissolved in 100 µl of borate buffer (0.5 M, pH 9.0) and derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC). One µl of the hydrolysate was subjected to analysis on an AccQ-Tag column equipped with a fluorescent detector.

Enzyme purification

A. Purification of sulphite reductase

DEAE-Sephadex chromatography: The concentrated and dialyzed sample obtained from the above step was loaded on to a DEAE-Sephadex column (2.5x25 cm) pre-equilibrated with 20 mM phosphate buffer, pH 7.2, at a flow rate of 30 ml/h. The column was then washed with the above buffer till A_{280} was < 0.1. The bound protein was then eluted with a step wise gradient of NaCl (100-500 mM) in the same buffer at a flow rate of 30 ml/h. Fractions of 3 ml were collected and assayed for sulphite reductase activity and protein. The 100 mM eluates containing the sulphite reductase activity were pooled, lyophilized, dissolved in minimum amount of Milli Q water and dialyzed extensively against Milli Q water followed by 20 mM phosphate buffer, pH 7.2 containing 150 mM NaCl and used for the next step.

Gel filtration on Sephacryl S-300: The fractions containing sulphite reductase obtained from the above step was loaded on to a Sephacryl S-300 column (2x100 cm) pre-equilibrated with 20 mM phosphate buffer, pH 7.2 containing 150 mM NaCl at a flow rate of 12 ml/h. Fractions of 2 ml were collected and checked for enzyme activity and

protein. Active fractions were pooled, concentrated by lyophilization, dialyzed against Milli Q water and stored at 4°C till further use.

RESULTS AND DISCUSSION

Fusarium oxysporum produces sulphite and nitrate reductase in a ratio of approximately 1:2. In the present case, DEAE-Sepadex chromatography at pH 7.0 completely separated sulphite reductase from nitrate reductase. Subsequently, chromatography on Sephacryl S-300 yielded a homogenous enzyme. The results of a typical procedure for the purification of sulphite reductase are summarized in Table 3.1.

Table 3.1: Purification of sulphite reductase.

Step	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Fold Purification	Recovery (%)
Crude	6889.00	415.0	16.60	1.0	100.0
DEAE-Sephadex (pH 7.2)	88.27	12.0	7.35	15.3	43.9
Sephacryl S-300 (pH 7.2)	13.20	4.0	3.30	20.6	20.0

The enzyme was purified approximately 21-fold, with an overall recovery of 20% and a specific activity of 0.825 U/mg protein. The purified enzyme is a glycoprotein and contains 5.3% neutral sugar. The purified enzyme could be stored in Milli Q water at -20 °C for a year without any apparent loss of activity.

The Mr of the purified enzyme determined by SDS-PAGE was 35.6 kDa (Fig.3.1).
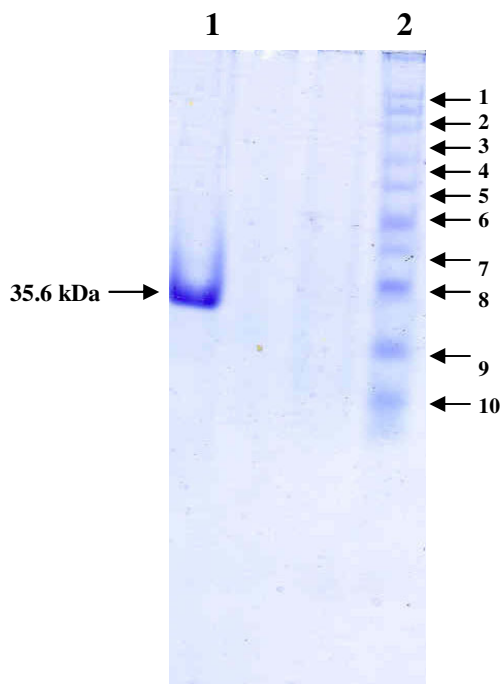
Fig. 3.1*:* **Mr determination of sulphite reductase by SDS-PAGE (Weber and Osborn).**
Lane 1: purified sulphite reductase. Lane 2: molecular mass markers (1) Myosin rabbit
muscle (205 kDa) (2) β-galactosidase (116 kDa) (3) Phosphyrelase rabbit muscle (97 kDa)
(4) Fructose 6 -phosphokinase (84 kDa) (5) Bovine serum albumin (66 kDa) (6) Glutamic
dehydrogenase (55 kDa), (7) Ovalbumin (45 kDa (8) Glyceraldehyde 3-phosphate
dehydrogenase (36 kDa), (9) Carbonic anhydrase (29 kDa) and (10) Trypsinogen (24 kDa).

The Mr based on amino acid composition was 30.91 kDa as shown in Table 3.2.
The Mr of *Fusarium* sulphite reductase is lower than those from *Salmonella typhimurium*
(66 kDa) (Ostrowski et al., 1989) and Spinach (69 kDa) (Kruger and Siegel, 1982).
However, *E. Coli* (Siegel et al., 1973), *Sacharomyces cerevisiae* (Kobayashi and
Yashimoto, 1982) and *Thiobacillus denitrificans* (Schedel and Truper, 1979) are very
high Mr proteins with Mr. of 607 kDa, 604 kDa and 160 kDa respectively. It is an acidic
protein with a pI of 4.5 (Fig. 3.2).

Table 3.2. Amino acid composition of sulphite reductase.

Amino acid	No. of residues/mol
AsX	25
Thr	24
Ser	14
GlX	16
Pro	15
Gly	42
Ala	14
Cys[a]	2
Val	10
Met	6
Ile	9
Leu	14
Tyr	18
Phe	10
His	14
Lys	30
Trp[b]	4
Arg	14
Total	**281**

Determined spectrophotometrically according to: **a.** (Cavallini et al., 1966) and **b**. (Spande and Witkop, 1967) respectively.
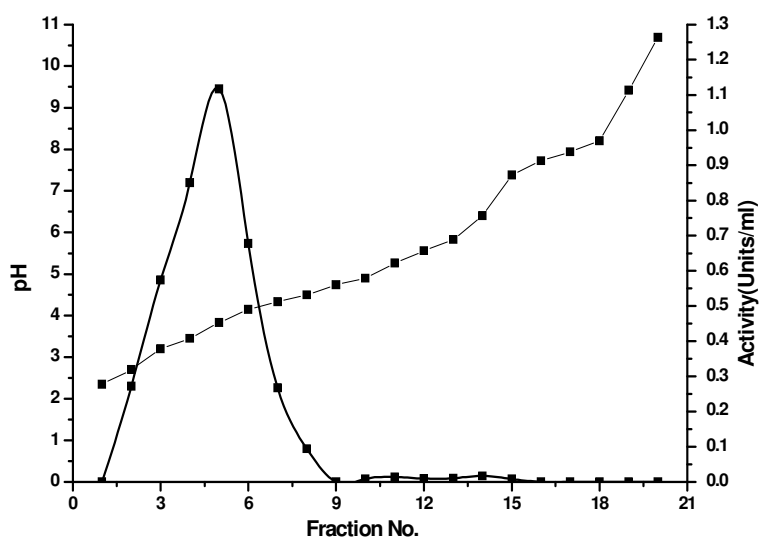
Fig. 3.2: **Isoelectric focussing of sulphite reductase.** pH (■), Activity (●).

Effect of pH and temperature

Fusarium oxysporum sulphite reductase exhibited an optimum pH of 6.0 (Fig. 3.3A), similar to that reported for *Thiobacillus denitrificans* sulphite reductase (Schedel and Truper, 1979). However, enzymes from *Sacharomyces cerevisiae* (Kobayashi and Yashimoto, 1982), *Salmonella typhimurium* (Ostrowski et al., 1989), *Spinach* (Kruger and Siegel, 1982) and *E. coli* (Siegel et al., 1973) showed an optimum pH of 7.3, 7.7, 7.75 and 7.7, respectively. The optimum temperature of the purified enzyme was 45°C (Fig. 3.3B).
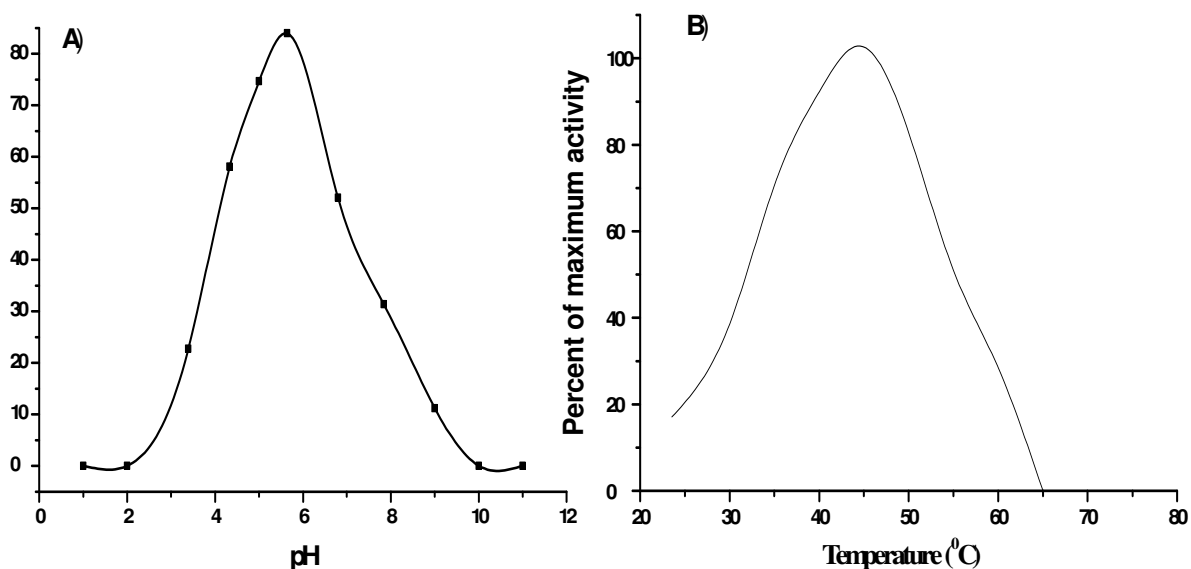
Fig. 3.3: Effect of pH and temperature on the activity of sulphite reductase. A: pH and **B:** temperature.

Effect of substrate concentration

The K$_m$ and V_{max} of the purified sulphite reductase were 9 μmole and 1.167 μ mole/sec, respectively (Fig. 3.4). The K$_m$ of *Fusarium oxysporum* sulphite reductase is lower than those reported from *Sacharomyces cerevisiae* (17 μ mole) (Kobayashi and Yashimoto, 1982) and *Spinach* (600 μ mole) (Kruger and Siegel, 1982).

Fig. 3.4: **Lineweaver-Burk plot for sulphite reductase.** The purified enzyme was assayed in a series of substrate concentrations (2- 12 µmole) at pH 6.0 and 45°C.

B. Purification of nitrate reductase

*DEAE-Sephadex chromatography***:** The concentrated and dialyzed extracellular extract (Page 47) was loaded on to a DEAE-Sephadex column (2.5x25 cm) pre-equilibrated with 20 mM phosphate buffer, pH 7.2, at a flow rate of 30 ml/h. The unadsorbed fractions containing nitrate reductase activity were pooled, concentrated by lyophilization and dialyzed extensively against Milli Q water followed by 20 mM sodium acetate buffer, pH 4.5 and used for the next step.

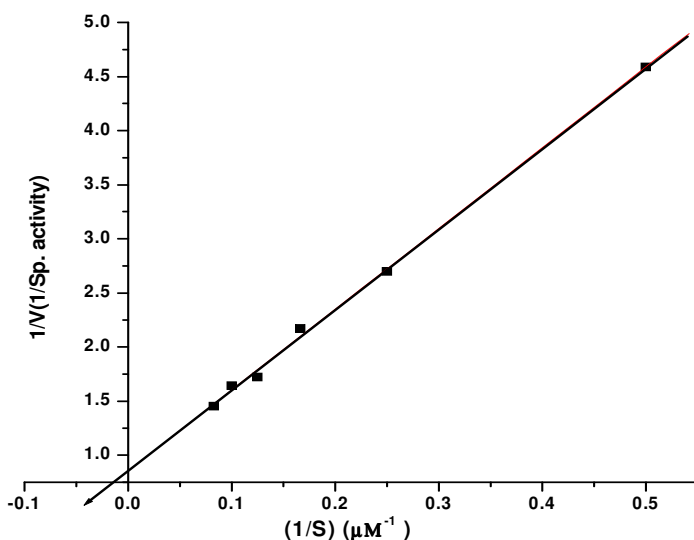*CM-Sephadex column chromatography* The enzyme from the above step was chromatograped on a CM-Sephadex column (25x2.5 cm) pre-equilibrated with 20 mM sodium acetate buffer, pH 4.5. The column was then washed with the same buffer till A_{280} was < 0.1.The bound protein was then eluted with a step wise gradient of NaCl (100-500 mM) in the above buffer at a flow rate of 30 ml/h. Fractions of 2 ml were collected and assayed for nitrate reductase activity and protein . The 200 mM eluate containing nitrate reductase activity was lyophilized, dissolved in minimum amount of Milli Q water and then dialyzed extensively against Milli Q water followed by   20 mM phosphate buffer containing 150 mM NaCl, pH 7.2 and used for gel filtration.

Gel filtration on Sephacryl S-300: The fractions containing nitrate reductase obtained from the above step was loaded on to a Sephacryl S-300 column (2x100 cm) pre-equilibrated with 20 mM phosphate buffer containing 150 mM NaCl, pH 7.2, at a flow rate was 12 ml/h. Fractions of 2 ml were collected and those with nitrate reductase were pooled, concentrated, dialyzed against Milli Q water and stored at 4°C till further use.

RESULTS AND DISCUSSION

Purification

As mentioned earlier, *Fusarium oxysporum* produces sulphite and nitrate reductase in a ratio of 1.2. The flow-through fractions obtained after DEAE-Sephadex chromatography, when subjected to CM-Cellulose chromatography and gel filtration on Sephacryl S-300 yielded a homogenous enzyme preparation. The results of typical procedure for the purification of nitrate reductase are summarized in Table 3.3.

Table 3.3: Purification of nitrate reductase

Step	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Fold Purification	Recovery (%)
Culture filtrate	13778.00	415.0	33.2	100.0	1.0
DEAE-Sephadex (pH 7.2)	204.48	12.0	17.04	5.6	18.0
CM-Sephadex (pH 4.5)	140.13	9.0	15.57	5.1	21.6
Sephacryl S-300 (pH 7.2)	41.6	4.0	10.4	3.45	32.5

 The enzyme was purified with 32.5-fold, with an overall recovery of 3.45% and a specific activity of 2.6 U/mg protein. It is a glycoprotein and contains 8.4% neutral sugar.

The purified enzyme could be stored in Milli Q water at -20 ^{o}C for a year without any apparent loss of activity.

The Mr of the purified enzyme determined by SDS-PAGE was 44.1 kDa (Fig. 3.5).
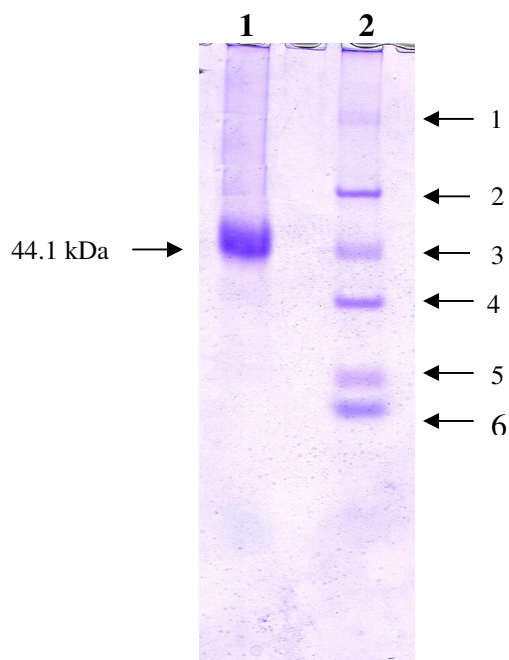
Fig. 3.5*:* **Mr determination of the purified nitrate reductase by SDS-PAGE (Weber and Osborn).** Lane 1: purified nitrate reductase. Lane 2: molecular mass markers (1) Phosphorylase b (97.4 kDa) (2) Bovine Serum Albumin (66 kDa), (3) Ovalbumin (43 kDa), (4) Carbonic anhydrase (29.1 kDa) (5) Soyabean trypsin inhibitor (20.1 kDa) and (6) Lysozyme (14.3 kDa).

The Mr based on amino acid composition (Table 3.4) was 39.4, comparable that obtained by SDS-PAGE. The Mr of *Fusarium oxysporum* nitrate reductase is very small compared to nitrate reductases from *Porphyra yezoensis* - 220 kDa (Nakumura and Ikawa, 1998), *Skeletonema costatum* - 110 kDa (Gao et al., 1993), *Emiliania huxleyi* - 514 kDa (Iwamoto and Shiraiwa, 2003) and *Rhodotorula glutinis* - 130 kDa (Morozkina et al., 2005). Moreover, it is a highly basic protein with a pI of 9.2 (Fig. 3.6).

Table 3.4. Amino acid composition of nitrate reductase.

Amino acid	No. of residues/ mol
AsX	28
Thr	18
Ser	45
GlX	36
Pro	12
Gly	61
Ala	38
Cys[a]	8
Val	6
Met	5
Ile	9
Leu	18
Tyr	23
Phe	3
His	9
Lys	11
Trp[b]	2
Arg	26
Total	**358**

Determined spectrophotometrically according to: **a**. (Cavallini et al., 1966) and **b**. (Spande and Witkop, 1967) respectively.
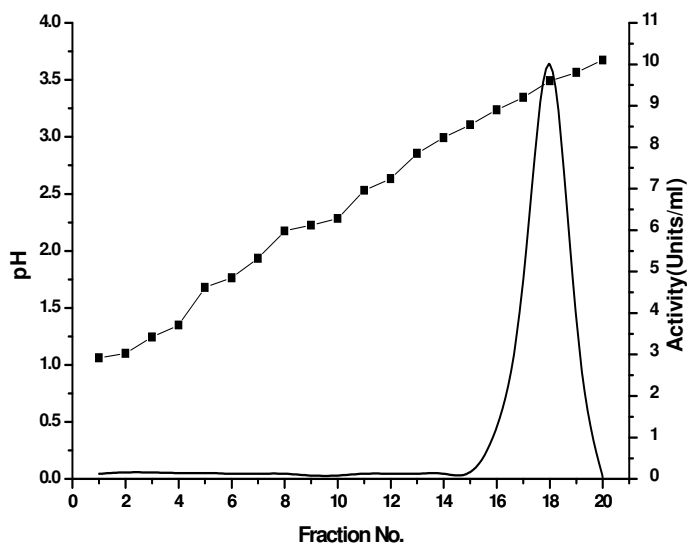
Fig. 3.6: **Isoelectric focussing of nitrate reductase.** pH (■), Activity

Effect of pH and temperature

The nitrate reductase exhibited an optimum pH of 7.0 (Fig. 3.7A), similar to that reported for *Rhodotorula glutinis* (Morozkina et al., 2005). However enzymes from *Skeletonema costatum* (Gao et al., 1993) *Porphyra* (Nakumura and Ikawa, 1998), *Haloferax mediterranei* (Martinez-Espinosa et al., 2001) *Emiliania huxleyi* (Iwamoto and Shiraiwa, (2003) showed an optimum pH of 7.4, 8.3, 9.0 and 8.0 respectively. The optimum temperature of the purified enzyme was 55°C (Fig. 3.7B).
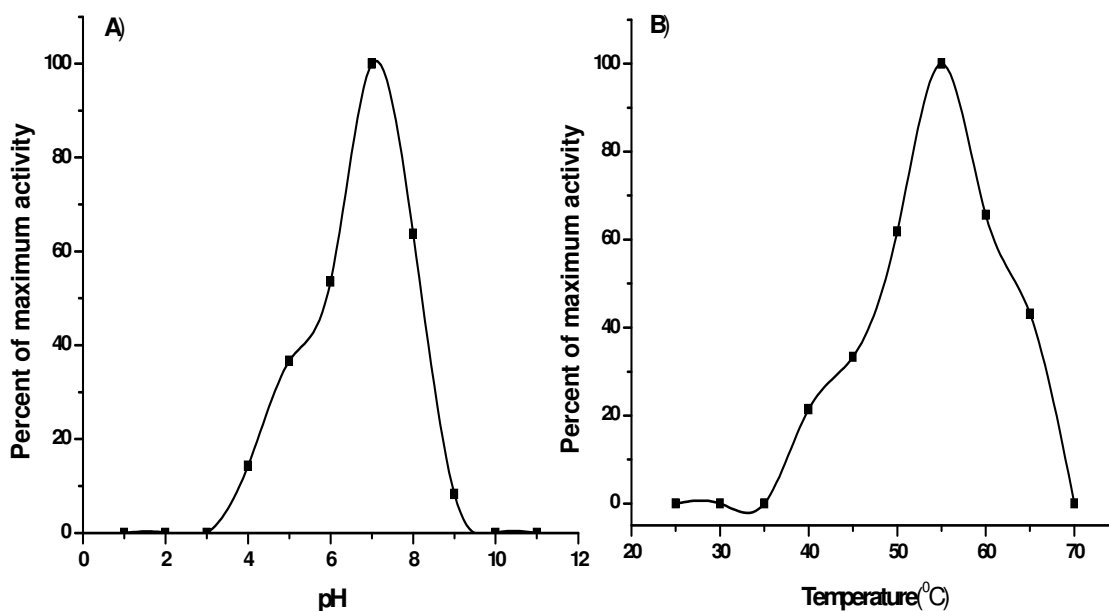

Fig. 3.7: Effect of pH and temperature on the activity of nitrate reductase. A: pH and **B:** temperature.

Effect of substrate concentration

The K_m and V_{max} of the purified nitrate reductase were 25 μ mole and 7.69 μ mole/sec respectively (Fig. 3.8).
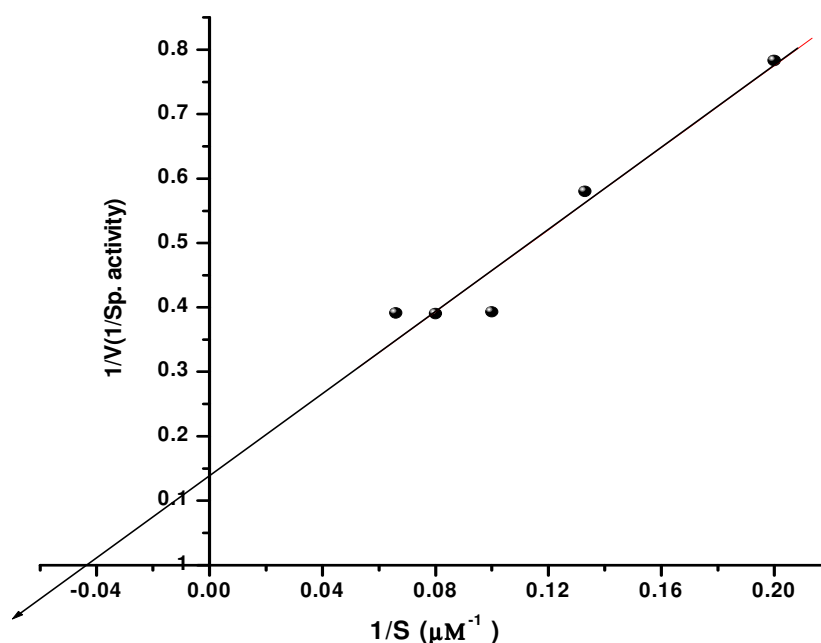
Fig. 3.8: **Lineweaver-Burk plots of nitrate reductase.** The purified enzyme was assayed in a series of substrate concentrations (2- 12 μmole) at pH 7.2 and 55°C.

The K_m of the *Fusarium oxysporum* nitrate reductase was lower than reported for nitrate reductases from *Skeletonema costatum* (296 μ mole) (Gao et al., 1993), *Porphyra* (64 μ mole) (Nakumura and Ikawa, 1998), *Emiliania huxleyi* (104 μ mole) (Iwamoto and Shiraiwa, 2003) and *Rhodotorula glutinis* (740 μ mole) (Morozkina et al., 2005).

REFERENCES

Cavalini, D., Graziani, M. T. and Dupre, S. (1996) *Nature* **212**, 294-295

Gao, Y., Smith, G. J., Alberte, R. S. (1993) *J. Phycol*. **7**, 1437-1445.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., Smith, F. (1956) *Anal. Chem.* **28,** 360-356.

Iwamoto, K., Shiraiwa, Y. (2003) *Mar. Biotechnol*. **5**, 20-26.

Kobayashi, K., Yashimoto, A. (1982) *Biochemica. Biophysica. Acta*. **705**, 348-356.

Krueger, R. J., Siegel, L. M. (1982) *J. Biol. Chem*. **21**, 2892-2904.

Lowry, O. H., Rosenbrough, N. H., Farr, A., Randall, R. J. (1951) *J. Biol. Chem*. **193**, 265-275.

Martinez-Espinosa, R. M., Marhuenda-Egea, F. C., Bonete, M. J. (2001) *FEMS Microbiology Letters* **204**, 381-385.

Martino, E., Franco, B., Piccoli, G., Stoccli, V., Perotto, S. (2002) *Mol. And Cell. Biochem.* **231**, 179-185.

Morozkina, E. V., Nosikov, A. N., Zvyagilskaya, R. A., L'vov, N. P. (2005) *Biochemistry (Moscow)*, **70**, 980-986.

Nakamura, Y., Ikawa, T. (1998) *Plant Cell Physol*. **34**, 1239-1249.

Ostrowski, J., Barber, M. J., Rueger, D. C., Miller, B. E., Siegel, L. E., Kredich, N. M. (1989) *J. Biol. Chem*. **264**, 15796-15808.

Schedel, M., Truper, H. G. (1979) *Biochemica. Biophysica. Acta*. **568**, 454-467.

Siegel, L. M., Murphy, M. J., Kamin, H. (1973) *J. Biol. Chem.* **248***,* 251-264.

Snell, F. P., Snell, C. T. (1949) *Colorimetric methods of analysis* **3**, 804-805.

Spande, T. F., Witkop, B. (1967) *Methods Enzymol*. **11**, 498-506.

Stoscheck, C. M. (1990) *Methods Enzymol.* **182**, 50-68.

Weber, K., Osborn, M. (1969) *J. Biol. Chem.* **244,** 4406-4412.

Yoshimoto, A., Sato, R. (1968) *Biochim. Biophys. Acta*. **153**, 555-575.

CHAPTER 4

SULPHITE REDUCTASE MEDIATED SYNTHESIS OF CdS NANOPARTICLES CAPPED BY PHYTOCHELATIN

SUMMARY

Cadmium chloride, sodium sulphite when incubated with NADPH-dependent sulphite reductase in presence of phytochelatin could synthesize semiconductor cadmium sulfide (CdS) nanoparticles *in vitro*. The nanoparticles formed were polydisperse, predominantly spherical with sizes ranging from 5-20 nm.

INTRODUCTION

Colloidal semiconductor nanoparticles have attracted wide attention due to their quantum confinement effects and size dependent photoemission characteristics (Bawendi et al., 1990; Weller, 1993; Alivisators, 1996a; Fendler, 1998; Sun et al., 2000; Michler et al., 2000; Murray et al., 2000; Klabunde, 2001; Hyeon, 2003). The semiconductor nanoparticles have found application in light-emitting diodes, electroluminescent devices, photovoltaic devices, lasers, and single-electron transistors (Chan and Nie, 1998; Hu et al., 2001; Sundar et al., 2002). The ability to tailor the optical properties of semiconductor nanoparticles by changing their size upon UV irradiation is particularly attractive in diverse areas such as cell labeling (Wu et al., 2003), cell tracking (Parak et al., 2002), *in vivo* imaging (Dubertret et al., 2002), diagnostics and DNA detection (Taylor et al., 2000; Xu et al., 2003).

Steigerwald et al. (1988) demonstrated the synthesis of sulfide nanoparticles by reacting of an alkaline solution of the metal salt with H_2S in the presence of a stabilizing agent. Recently, several groups reported the synthesis of sulfide nanoparticles by thermolysis of single-source precursors (Lee et al., 2002; Jun et al., 2002; Pradhan and Efrima, 2003). However, the above methods suffer from agglomeration and non-uniform in size distribution. Biomaterials and biological structures of higher complexity can act as active units for the synthesis of nanoparticles. Mukherjee et al. (2001a, b) reported the synthesis of different metal nanoparticles using the fungus *Verticillium* and *Fusarium oxysporum* (Mukherjee et al., 2002; Ahmad et al., 2002). The intracellular formation of CdS nanoparticles was demonstrated on exposure using the bacterium, *Klebsiella aerogenes* (Holmes et al., 1995; Smith et al., 1998), *Schizosaccharomyces pombe* and *Candida glabrata* (Dameron et al. (1989). On the other hand, Kowshik et al. (2002a, b)

used *Turolopsis* sp. for the synthesis of intracellular PbS nanoparticle However; few reports exist on the use of enzymes for the synthesis of nanomaterials. Duran et al. (2005) suggested the involvement of nitrate-dependent reductase in the extracellular synthesis of silver nanoparticles by several *Fusarium oxysporum* strains. In contrast, Willner et al. (2006) noted the synthesis of gold nanoparticles during the oxidation of glucose to gluconic acid by glucose oxidase. Similar observations were also made by Yasui and Kimizuka (2005) during the conversion of glucose to gluconolactone in presence of glucose oxidase.

In the preceding chapter we described the detection, purification and characteristics of NADPH dependent sulphite and nitrate reductases. This chapter describes the use of NADPH dependent sulphite reductase and a capping peptide for the *in vitro* synthesis of CdS nanoparticles.

MATERIALS

Cadmium chloride, sodium sulfite and NADPH (Sigma chemicals Co., St Louis, MO, U.S.A) and phytochelatin (Geno Mechanix, Florida, U.S.A) were used. All other chemicals used were of analytical grade. Sulphite reductase was purified as described in chapter 3 (Page 48).

METHODS

Enzyme mediated synthesis of CdS nanoparticles *in vitro*

The total reaction mixture of 3 ml contained 1.0 mM each of freshly prepared $CdCl_2$ and Na_2SO_3, 100 μg of phytochelatin, 1.0 mM α-NADPH and 1.66 U (100 μg protein) of sulphite reductase was incubated, under anaerobic conditions, at 25^0C. Reactions performed in the absence of the enzyme, NADPH, phytochelatin as well as the inactivated enzyme served as control. Samples were removed at regular intervals and subjected to UV-Vis spectroscopy to check for the formation of nanoparticles. On completion of the reaction, the unbound proteins were removed by extraction with 50% (v/v) 1, 4-dioxane and the nanoparticles formed were characterized.

UV-Vis and fluorescence measurements

UV-Vis spectrophotometric measurements were performed on a Shimadzu dual-beam spectrophotometer (model UV-1601 PC) operated at a resolution of 1 nm.

Fluorescence measurements were carried out on a Perkin-Elmer LS 50B luminescence spectrofluorimeter, with slit width of 2 nm for both the monochromators and scan speed 300 nm/min. Samples were excited at 340 nm and the emission spectra were recorded in wavelength ranging from 350 nm to 700 nm.

Life time measurements were performed on a time correlated single photon counting FLS920 spectrofluorimeter (Edinburgh Instruments, Livingston, UK). A hydrogen flash lamp of pulse width 1 ns was used for excitation and an nF-900 nanosecond flash lamp was used to detect the fluorescence. Sample was exited at 340 nm and emission was recorded at 440 nm. Slit widths of 15 and 15 nm were used on the excitation and emission monochromators, respectively. The resultant decay curves were analyzed by a multiexponential iterative fitting program supplied by Edinburgh Instruments.

Transmission electron microscopy (TEM)

Samples were prepared by drying a drop of CdS nanoparticles solution on carbon coated TEM copper grids followed by measurements on a JEOL Model 1200 EX Transmission electron microscope operated at an accelerating voltage of 80 kV.

X-ray diffraction measurements (XRD)

Thin films of CdS nanoparticles were prepared on Si (111) substrates by drop-coating with nanoparticles solution. The films on Si wafers were then subjected to X-ray diffraction (XRD) on a Philips PW 1830 instrument operated at 40 kV and at 30 mA with Cu Kα radiation.

X-ray photoelectron spectroscopy (XPS)

Chemical analysis of a drop-coated film of CdS nanoparticles on a conductive substrate was carried out on a ESCALAB MK II X-ray photoelectron spectrometer (V. G.

Scientific, UK) with A1 k_α as the exiting source ($h\gamma$ = 1486.6 e V) operating at an accelerating voltage of 10 kV and 20 m A at a pressure of approximately 10^{-8} Pa.

RESULTS AND DISCUSSION

When $CdCl_2$, Na_2SO_3, phytochelatin were incubated with NADPH-dependent sulphite reductase, resulted in the formation of CdS nanoparticles. No nanoparticle formation was observed when the reactions were carried out in the absence of any one of the aforementioned components and the inactivated enzyme. These results suggest apart from active enzyme, phytochelatin also has role in the formation of CdS nanoparticles.

UV/Vis spectroscopy

The UV-Vis spectra for the reaction mixture, as a function of time, showed a well-defined surface plasmon at 340 nm, characteristic of CdS nanoparticles (Fig. 4.1).
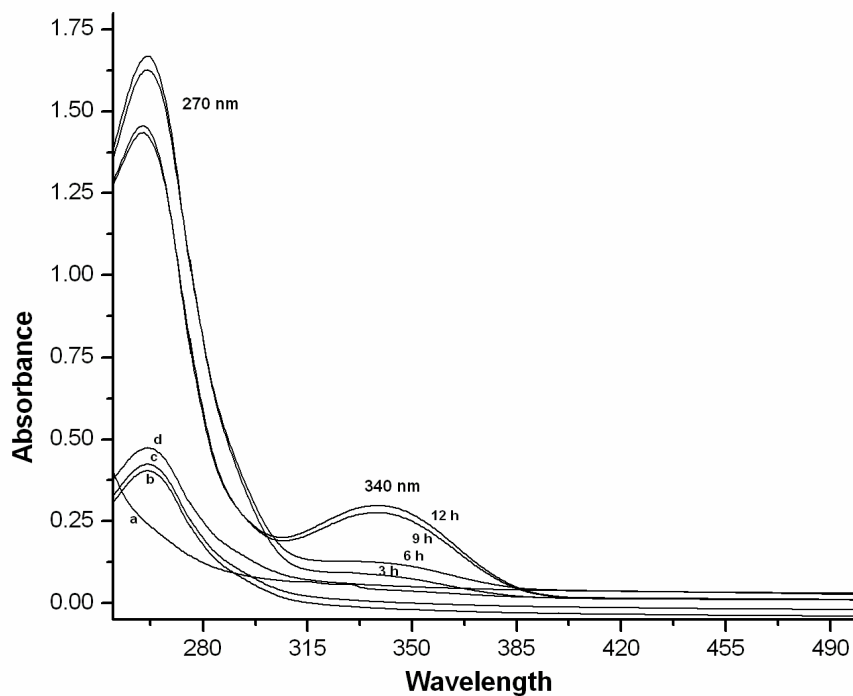

Fig. 4.1: UV-Vis spectra of CdS nanoparticles as a function of time. Curves a, b, c and d represent the reactions performed in the absence enzyme, phytochelatin, NADPH and denatured enzyme respectively.

Similar observations were made by Henglein, (1993) and Sastry et al. (1997, 1998) while studying the CdS nanoparticle formation using chemical methods. Despite the removal of

proteins by extraction with 1, 4-dioxane, the peak at 270 nm (Fig.4.1) can be correlated to the continued presence of phytochelatin on the nanoparticles. The CdS nanoparticles were extremely stable with no evidence of flocculation even after one month. The absence of a peak at 340 nm, for the reaction carried out in the absence of active and denatured enzyme, NADPH, and phytochelatin (Fig.4.1) showed that all the above ingredients and an active enzyme are essential for the formation of the nanoparticles.

Fluorescence spectroscopy

Fluorescence measurement of the CdS nanoparticles, after excitation of the reaction mixture at 340 nm, showed an emission band at 450 nm with a red shift (Fig.4.2). The intensity of the peak, at 450 nm, gradually increased with the reaction time but the emission spectra showed the same features. The absence of any red shifted emission band with respect to time indicated the formation of uniformly distributed CdS nanoparticles The small stroke's shift in the peak suggests that the nanocrystals possess a continuous surface with most surface atoms exhibiting the coordination and oxidation states of their bulk counterparts. Our results are in agreement with those reported by Brus (1986), Gallardo et al. (1989) and Machol et al. (1994).
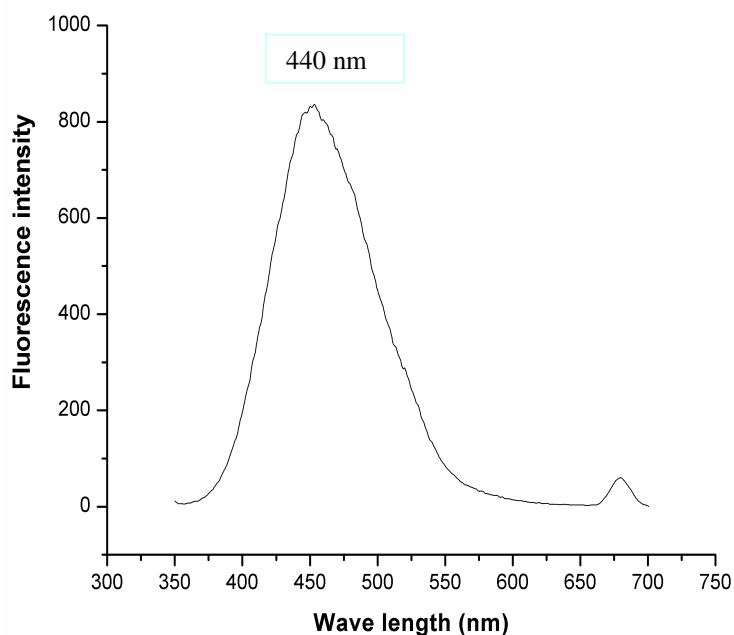

Fig.4.2: Fluorescence emission spectrum of CdS nanoparticles. The excitation was carried out at 340nm.

Time Resolved Fluorescence Spectroscopy

The fluorescence decay curves of CdS nanoparticles obtained from the time resolved measurements are given in Figure 4.3.
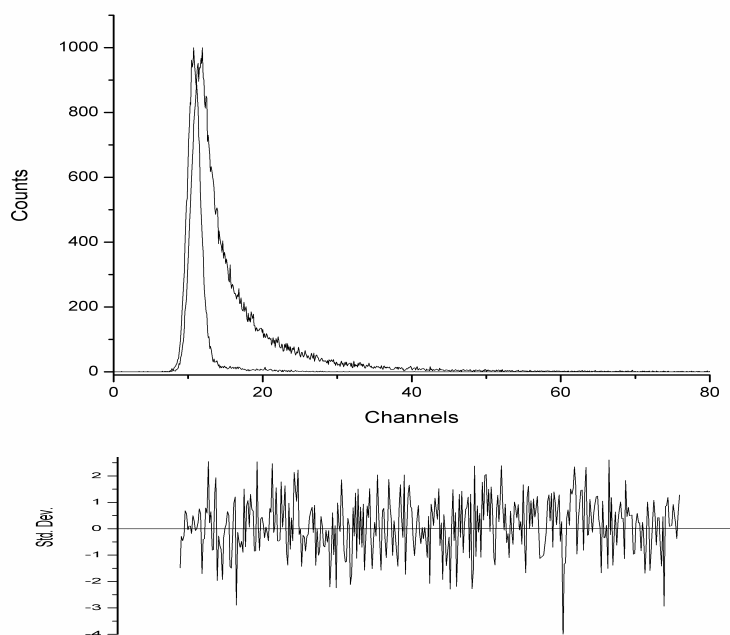
Fig. 4.3: Time-resolved fluorescence decay profile of the CdS nanoparticles. The solid line corresponds to the nonlinear least square fit of the experimental data to a bi-exponential function. The lower panels represent the residuals.

The decay profile CdS nanoparticles could be best fitted to be bi-exponential function ($\chi^2 \leq 1.1$). The life time values obtained CdS nanoparticles were 6.445 and 1.476 ns ($\chi^2 = 1.121$) and were comparable to the one reported by Wu et al. (7.9 and 2.5 ns), Paramita et al. (7.52 and 2.46 ns) synthesized using chemical methods. The relative contribution obtained from the decay profile of the CdS nanoparticles showed α_1 and α_2 to be 0.020 and 0.069 contributing to 55.28% and 44.72% respectively, of the total fluorescence intensity.

X-Ray diffraction

The X-ray diffraction pattern of CdS nanoparticles showed intense peaks at (111), (101), (002) and (220) in the 2θ range 20°-70° (Fig. 4.4).
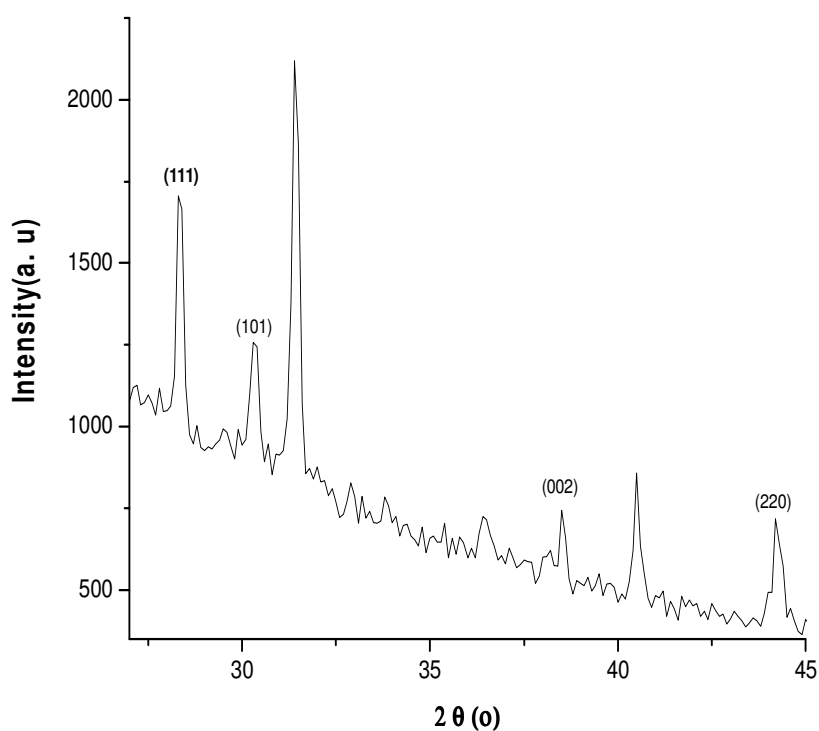
Fig. 4.4: X-ray diffraction pattern of the CdS nanoparticles.

The broadening of Bragg peaks indicated that the CdS particles formed are of nanoscale dimensions. Similar observations were made by Colvin et al. (1992) and Kundu et al. (1997) while characterizing CdS nanocrystals synthesized by chemical methods.

Transmission electron microscopy

TEM analyses of the CdS nanoparticles showed that they are polydisperse and essentially spherical (Fig. 4.5A). The nanoparticles were crystalline (Fig. 4.5B) and their size ranged from 5-20 nm with an average of 9 ± 2 nm (Fig. 4.5C).
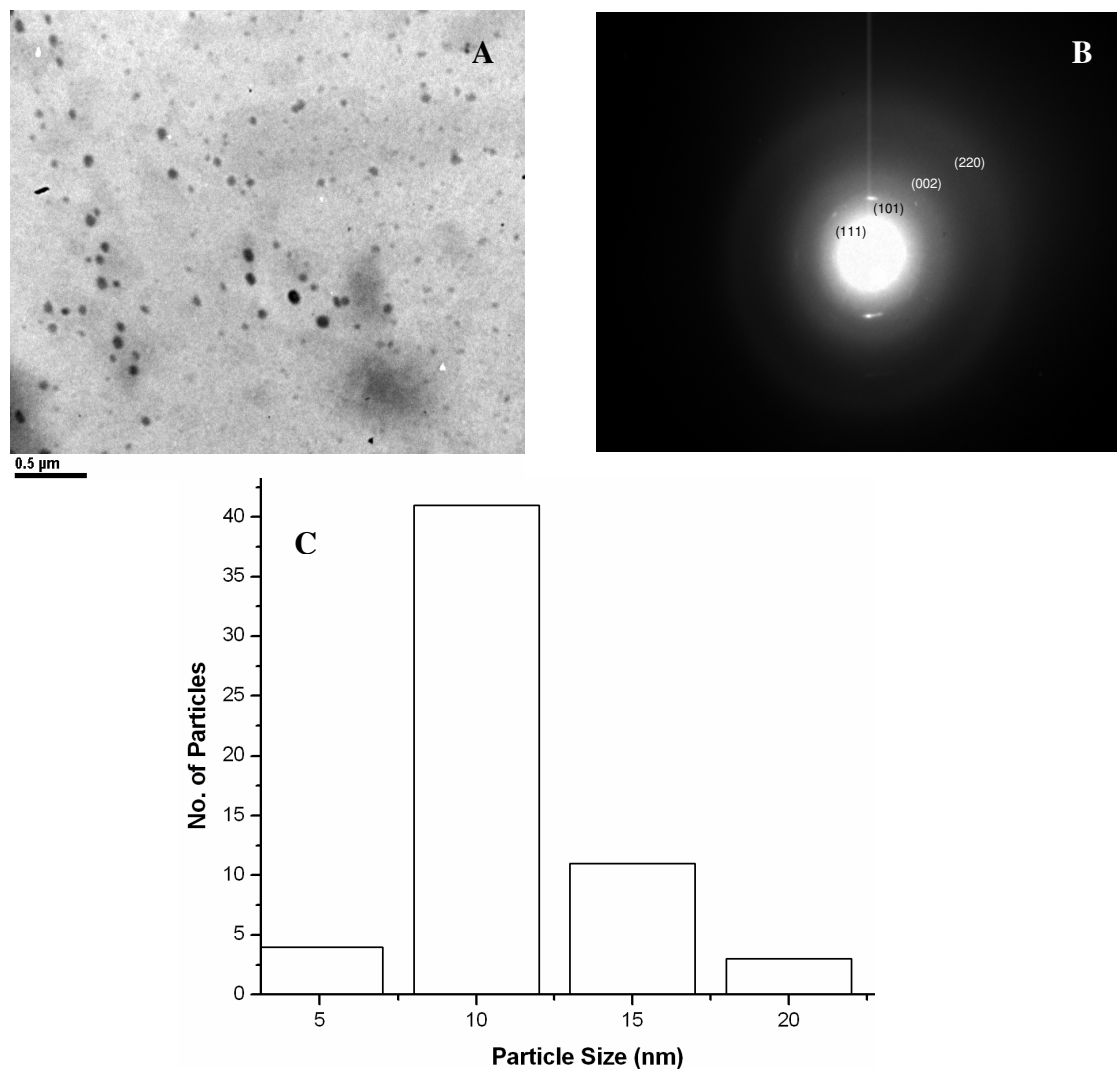
Fig. 4.5: A: TEM micrograph of CdS nanoparticles B: Selected area of electron diffraction pattern recorded from one of the CdS nanoparticles shown in Fig. A. C: Histogram of size distribution of the CdS nanoparticles.

X-ray photoelectron spectroscopy

X-ray photoelectron spectroscopy (XPS) carried out over a wide range of binding energies, i.e. 0 to 1400 e V (Fig. 4.6) showed the presence of Cd, S, C, O, N and Na as the prominent elements.
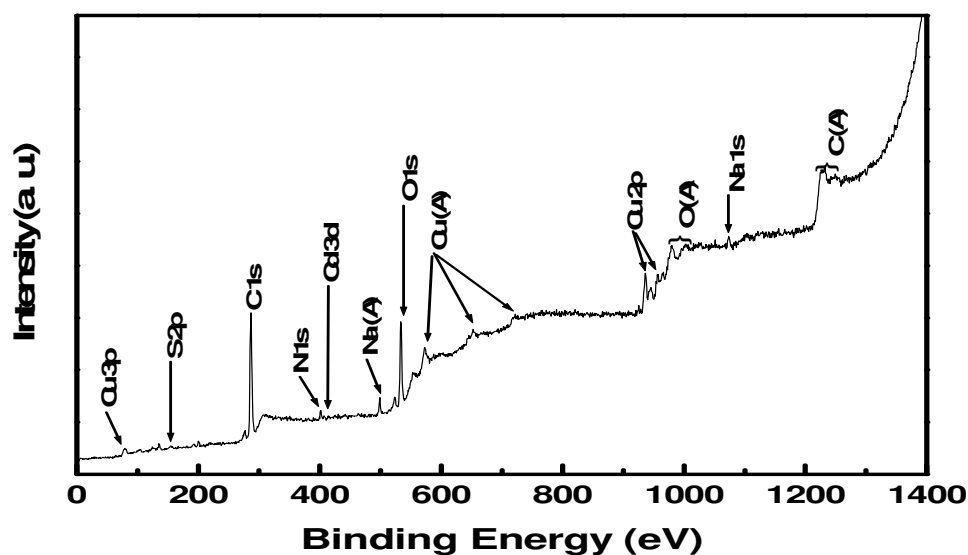
Fig. 4.6: X-ray Photoelectron spectroscopy of the CdS nanoparticles.

The core level binding energies and atomic percentages of various elements detected in the reaction mixture are given in Table 4.1.

Table 4.1. Core level binding energies and atomic percentages of various elements detected in the reaction mixture.

Element	C1s	O1s	N1s	Cd 3d$_{5/2}$	S 2P
Atomic %	71.4	22.1	4.9	0.3	1.2
Binding energy [e V]	285.0	531.1	399.7	405.5	168.8

The Cd and S peaks in the X-ray photoelectron spectra are observed at 405.5 e V and 168.8 e V respectively, corresponding well with the expected value for Cd bound to sulfur. The Cd 3d spectrum could be resolved into two spin-orbit components viz. Cd 3d $_{5/2}$ and Cd 3d $_{3/2}$ (spin orbit splitting ~ 7.0 eV).
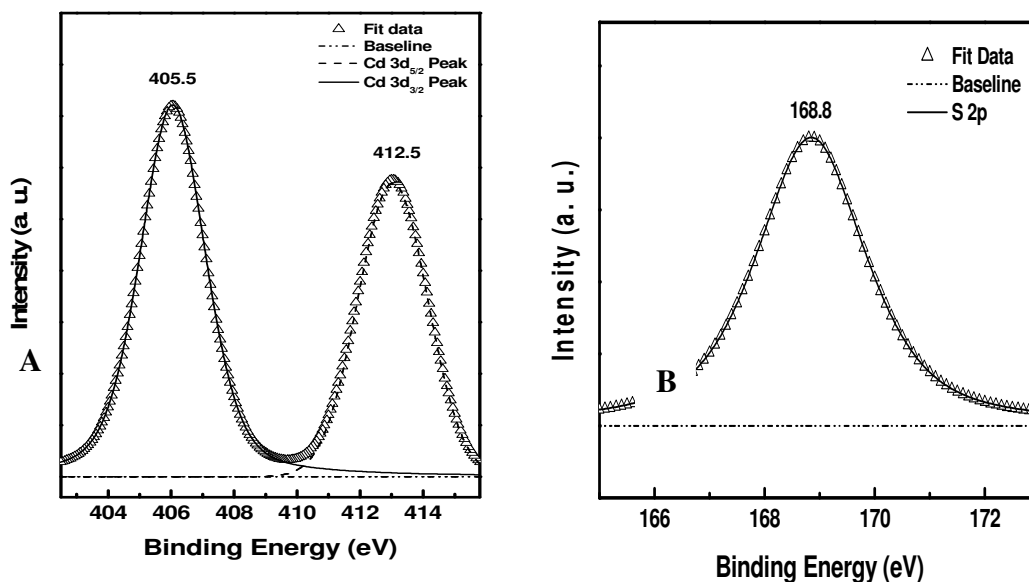
Fig. 4.7: Core level spectra of CdS nanoparticles. A:. Cd 3d and **B:** S 2p.

The peaks corresponding to a binding energy of 405.5 eV and 412.5 eV, respectively are characteristic of metallic Cd (Fig. 4.7A) and agree with the core level binding energies reported earlier (Chastain, 1992) Moreover, the S 2p peak corresponding to binding energy of 168.8 eV is characteristic of metallic S (Fig. 4.7B) is similar to the core level binding energies reported earlier (Chastain, 1992). The absence of a higher binding energy for both Cd 3d and S 2p components clearly reveals that all the Cd^{2+} and SO_3^{2-} ions are fully reduced and are in metallic form.

In conclusion, the present studies demonstrate the application of NADPH-dependent sulphite reductase for the *in vitro* synthesis of CdS nanoparticles in presence of phytochelatin. The role phytochelatin lies in the prevention of flocculation of the particles.

REFERENCES

Ahmad, A., Mukherjee, P., Mandal, D., Senapati, S., Khan, M.I., Kumar, R., Sastry, M., (2002) *J. Am. Chem. Soc.* **124**, 12108-12109.

Alivisatos, A. P. (1996a) *Science* **271,** 933-937.

Bawendi, M. G., Steigerwald, M. L., Brus, L. E. (1990) *Annu. Rev. Phys. Chem.* **41,** 477-496.

Brus, L. (1986) *J. Phys. Chem.* **90,** 2555-2560.

Chan, W. C. W., Nie, S. (1998) *Science* **281,** 2016-2018.

Chastain, J. (1992) Handbook of X-ray Photoelectron Spectroscopy, Perkin-Elmer, Norwalk

Colvin, V. L., Goldstein, A. N., Alivisators, A. P. (1992) *J. Am. Chem. Soc.* **114,** 5221-5230.

Dameron, C. T., Reese, R. N., Mehra, R. K., Kortan, A. R., Carroll, P. J., Steigerwald, M. L., Brus, L. E., Winge, D. R. (1989) *Nature* **338,** 596-597.

Dubertret, B., Skourides, P., Norris, D. J., Noireaux, V., Brivanlou, A. H., Libchaber, A. (2002) *Science* **298,** 1759-1762.

Duran, N., Marcato, P. D., Alves, O., De Souza G. I. H., Esposito, E. (2005) *Journ. of Nanobiotech.* 3, 8-14.

Fendler, J. H. (1998) Nanoparticles and Nanostructured Films, Wiley-VCH, Weinheim: Germany.

Gallardo, S., Gutierrez, M., Henglein, A., Janata, E. (1989) *Ber. Bunsen-Ges. Phys. Chem.* **93,** 1080-1090.

Henglein, A. (1993) *J. Phys. Chem.* **97,** 5457-5471.

Holmes, J. D., Smith, P. R., Evans-Gowing, R., Richardson, D. J., Russell, D. A., Sodeau, J. R. (1995) *Arch. Microbiol*. **163,** 143-147.

Hu, J. Li, L. S., Yang, W., Manna, L., Wang, L. W., Alivisatos, A. P. (2001) *Science* **292,** 2060-2063.

Hyeon, T. (2003) *Chem. Commun*. 927-934.

Jun, Y. W., Jung, Y. Y., Cheon, J. (2002) *J. Am. Chem. Soc*. **124,** 615-619.

Klabunde, K. J. (2001) Nanoscale Materials in Chemistry, Wiley-Interscience, New York.

Kowshik, M., Deshmukh, N., Vogel, W., Urban, J., Kulkarni, S. K., Paknikar, K. M. (2002a) *Biotechnol. Bioengineer.* **78,** 583-590.

Kowshik, M., Vogel, W., Urban, J., Kulkarni, S. K., Paknikar, K. M. **(**2002b) *Adv. Mater.* **14,** 815-818.

Kundu, M., Khosravi, A. A., Kulkarni, S. K. (1997) *J. Mater. Sci.* **32,** 245-248.

Lee, S. M., Jun, Y. W., Cho, S. N., Cheon, J. (2002) *J. Am. Chem. Soc.* **124,** 11244-11245.

Machol, J. L., Wise, F. W., Patel, R., Tanner, D. B. (1994) *Phys A* **207,** 427-434.

Michler, P., Imamoglu, A., Mason, M. D., Carson, P. J., Strouse, G. F., Buratto, S. K. (2000) *Nature* **406,** 968-970.

Mukherjee, P., Senapati, S., Mandal, D., Ahmad, A., Khan, M.I., Kumar, R., Sastry, M. (2002) *ChemBioChem* **3,** 461-463.

Mukherjee,P., Ahmad,A., Mandal,D., Senapati,S., Sainkar,S.R., Khan,M.I., Ramani,R., Parischa,R., Ajayakumar, P. V., Alam, M.,Sastry,M., Kumar.R (2001a) *Angew. Chem. Int. Ed.* **40,** 3585-3588.

Mukherjee, P., Ahmad, A.,. Mandal, D., Senapati, S., Sainkar, S. R., Khan, M. I., Parischa, R., Ajayakumar, P. V., Alam, M., Kumar, R., Sastry, M., (2001b) *Nano Lett.*, **1,** 515-519.

Murray, C. B., Kagan, C. R., Bawendi, M. G. (2000) *Annu. Rev. Mater. Sci.* **30,** 545-610.

Parak, W. J., Boudreau, R., Gros, M. L., Gerion, D., Zanchet, D., Micheel, C. M., Williams, S. C., Alivisatos, A. P., Larabell, C. (2002) *Adv. Mater.* **14,** 882-885.

Paramita, S. C., Pushpal, G., Amitava, P. (2007) *J. of Luminescence* **124,** 327-332.

Pradhan, N., Efrima, S. (2003) *J. Am. Chem. Soc*. **125,** 2050-2051.

Sastry, M., Bandyopadhyay, K., Mayya, K. S. (1997) *Colloid. Surf. A* **127,** 221-228.

Sastry, M., Patil, V., Sainkar, S. R. (1998) *J. Phys. Chem. B* **102,** 1404-1410.

Smith, P. R., Holmes, J. D., Richardson, D. J., Russell, D. A., Sodeau, J. R., (1998) *J. Chem. Soc., Faraday Trans.* **94,** 1235-1241.

Steigerwald, M. L., Alivisatos, A. P., Gibson, J. M., Harris, T. D., Kortan, R., Muller, A. J., Thayer, A. M., Duncan, T. M., Douglass, D. C., Brus, L. E. (1988) *J. Am. Chem. Soc.* **110,** 3046-3050.

Sun, S., Murray, C. B., Weller, D., Folks, L., Moser, A. (2000) *Science* **287,** 1989-1992.

Sundar, V. C., Eisler, H. J., Bawendi, M. G. (2002) *Adv. Mater*. **14,** 739-743.

Taylor, J. R.**,** Fang, M. M.**,** Nie, S. (2000) *Anal. Chem.* **72,** 1979-1986.

Willner, I., Baron, R., Willner, B. (2006) *Adv. Mater*. **18,** 1109-1120.

Wu, X.**,** Liu, H.**,** Liu, J.**,** Haley, K. N.**,** Treadway, J. A.**,** Larson, J. P.**,** Ge, N.**,** Peale, F.**,** Bruchez, M. P. (2003) *Nature Biotechnol.* **21,** 41-46.

Wu, F., Zhang, J. Z., Kho, R., Mehra, R. K. (2000) *Chemical Physics Letters* **330**, 237-242.

Xu, H., Sha, M. Y., Wong, E. Y., Uphoff, J., Xu, Y., Treadway, J. A., Truong, A., O'Brien, E., Asquith, S., Stubbins, M., Spurr, N. K., Lai, E. H., Mahoney, W. (2003) *Nucleic Acids Res.* **31,** e 43.

Yasui, K., Kimizuka, N., (2005) *Chem. Lett.* **34,** 416-417.

CHAPTER 5

SULPHITE REDUCTASE MEDIATED SYNTHESIS OF GOLD NANOPARTICLES

SUMMARY

A solution of AuCl$_4$, when incubated in presence of NADPH dependent sulphite reductase and phytochelatin could synthesize gold nanoparticles. The nanoparticles were stable with dimensions in the range of 7-20 nm. These studies will help in designing a rational enzymatic strategy for the synthesis of nanomaterials of different chemical composition, shapes and sizes.

INTRODUCTION

The area of nanotechnology, which spans the synthesis of nanoscale matter, understanding/utilizing their exotic physiochemical and optoelectronic properties, and organization of nanoscale structures into pre defined super structure, promises to play an increasingly important role in many key technologies (Schmid, 1994, 1996).

The common method used for the preparation gold colloids is the chemical reduction of salts. Other techniques include ultraviolet irradiation, aerosol technologies, lithography, laser ablation, ultrasonic fields, and photochemical reduction. While these methods produce pure and well-defined Au nanoparticles, they are expensive and involve the use of hazardous chemicals. In this respect, bioreduction of metals using microorganisms, offer viable alternative for the formation of nanoparticles (Duncan et al., 1997; Stephen and Maenaughton, 1999), yeast (Mehra and Winge, 1991), algae (Greene et al., 1986; Hosea et al., 1986; Kuyucak and Volesky, 1989) and fungi (Mukherjee et al., 2001, 2002). In the last chapter, we described the enzyme mediated synthesis of CdS nanoparticles. This chapter describes the application of NADPH-dependent sulphite reductase for the *in vitro* synthesis of gold nanoparticles.

MATERIALS

Auric Chloride (HAuCl$_4$), Na$_2$SO$_3$ and NADPH, (Sigma chemicals Co., St Louis, MO, U.S.A) and phytochelatin (Geno Mechanix, Florida, U.S.A) were used. All other chemicals used were analytical grade. Sulphite reductase was purified as described in chapter 3 (Page 48).

METHODS

In vitro synthesis of gold nanoparticles

The total reaction mixture of 3 ml contained 1.0 mM each of freshly prepared $AuCl_4$ and Na_2SO_3, 100 µg of phytochelatin, 1.0 mM α-NADPH and 1.66 U (100 µg protein) of sulphite reductase. The reaction mixture was incubated, under anaerobic conditions, at 25^0C. Reactions performed in the absence of the enzyme, NADPH, phytochelatin as well as the inactivated enzyme served as control. Samples were removed at regular intervals and subjected to UV-Vis spectroscopy to check for the formation of nanoparticles. On completion of the reaction, gold nanoparticles were collected by centrifugation (30000 *g*, 30 min), washed twice with Milli Q water and the unbound proteins were removed by treating with 80% (v/v) ethanol.

UV/Vis spectroscopy

UV-Vis spectrophotometric measurements were performed on a Shimadzu dual-beam spectrophotometer (model UV-1601 PC) operated at a resolution of 1 nm.

Transmission electron microscopy (TEM)

Samples were prepared by drying a drop of gold nanoparticles solution on carbon coated TEM copper grids followed by measurements on a JEOL Model 1200 EX Transmission electron microscope operated at an accelerating voltage of 80 kV.

X-ray diffraction measurements (XRD)

Thin films of gold nanoparticles were prepared on Si (111) substrates by drop-coating with nanoparticles solution. The films on Si wafers were then subjected to X-ray diffraction (XRD) on a Philips PW 1830 instrument operated at 40 kV and at 30 mA with Cu Kα radiation.

X-ray photoelectron spectroscopy (XPS)

Chemical analysis of a drop-coated film of gold nanoparticles on a conductive substrate was carried out on a ESCALAB MK II X-ray photoelectron spectrometer (V. G.

Scientific, UK) with A1 k_α as the exiting source ($h\gamma$ = 1486.6 e V) operating at an accelerating voltage of 10 kV and 20 m A at a pressure of about 10^{-8} Pa.

RESULTS AND DISCUSSION

Freshly prepared $HAuCl_4$ when incubated with Na_2SO_3, phytochelatin and NADPH-dependent sulphite reductase, under anaerobic conditions, at 25^0C for 4 h resulted in the formation of gold nanoparticles as evidenced by the change in the color of reaction mixture from colorless to ruby-red (Fig.5.1).
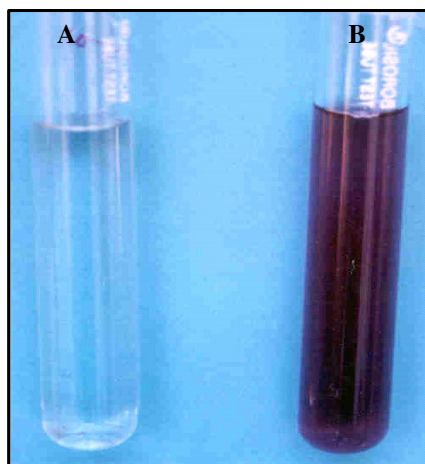

Fig. 5.1: Formation of gold nanoparticles by sulphite reductase A: At 0 h and **B:** At 4 h.

The change in color is due to surface plasmon resonance (SPR) of the metal nanoparticles (Mulvaney, 1996).

UV/Vis spectroscopy

The UV-Vis spectra for the reaction mixture, as a function of time, showed a well-defined surface plasmon at 520 nm, characteristic of gold nanoparticles (Fig.5.2).
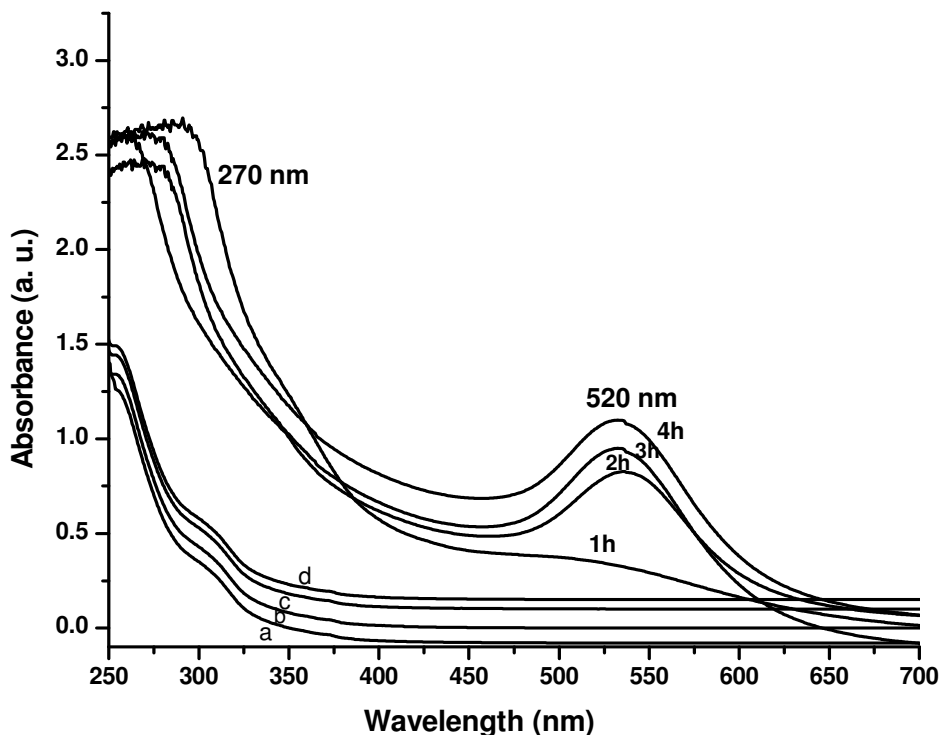
Fig. 5.2: UV-Vis spectra of gold nanoparticles as a function of time. Curves a, b, c and d represent the reactions performed in the absence enzyme, phytochelatin, NADPH and denatured enzyme respectively.

The solution was extremely stable, with no evidence of flocculation of the particles even a month after reaction. The absence of surface plasmon resonance at 520 nm of gold nanoparticles, in the absence of active and inactivated enzyme (Fig. 5.2), clearly indicates involvement of NADPH-dependent sulphite reductase in the reduction of gold.

Moreover, the obligate requirement of phytochelatin (Fig. 5.2), for the formation of nanoparticles suggests the role of a capping peptide in the prevention of their aggregation since its removal by treatment with 2% SDS led to the reaggregation of the particles.

X-Ray diffraction

The X-ray diffraction pattern of gold nanoparticles showed intense peaks at (111), (200), (220) and (311) in the 2θ range 20°-70° (Fig. 5.3) and agree with those reported for the gold nanocrystals.

.
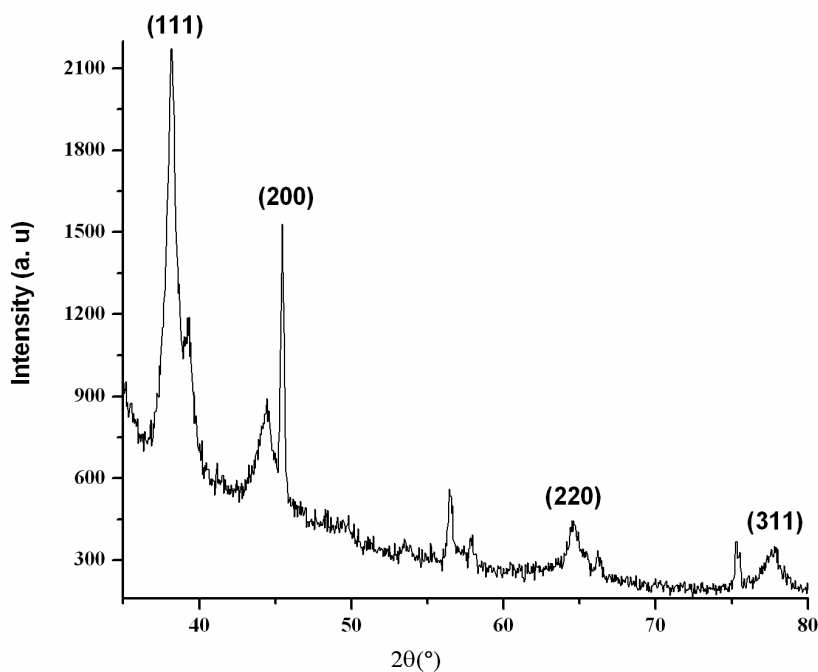
Fig. 5.3: XRD pattern of the gold nanoparticles.

Transmission electron microscopy

TEM analyses of the gold nanoparticles showed that they are polydisperse and are essentially spherical (Fig. 5.4A). The nanoparticles were crystalline in nature (Fig. 5.4B) and their size were in the range of 7-20 nm with an average of 15 ± 2 nm (Fig. 5.4C).
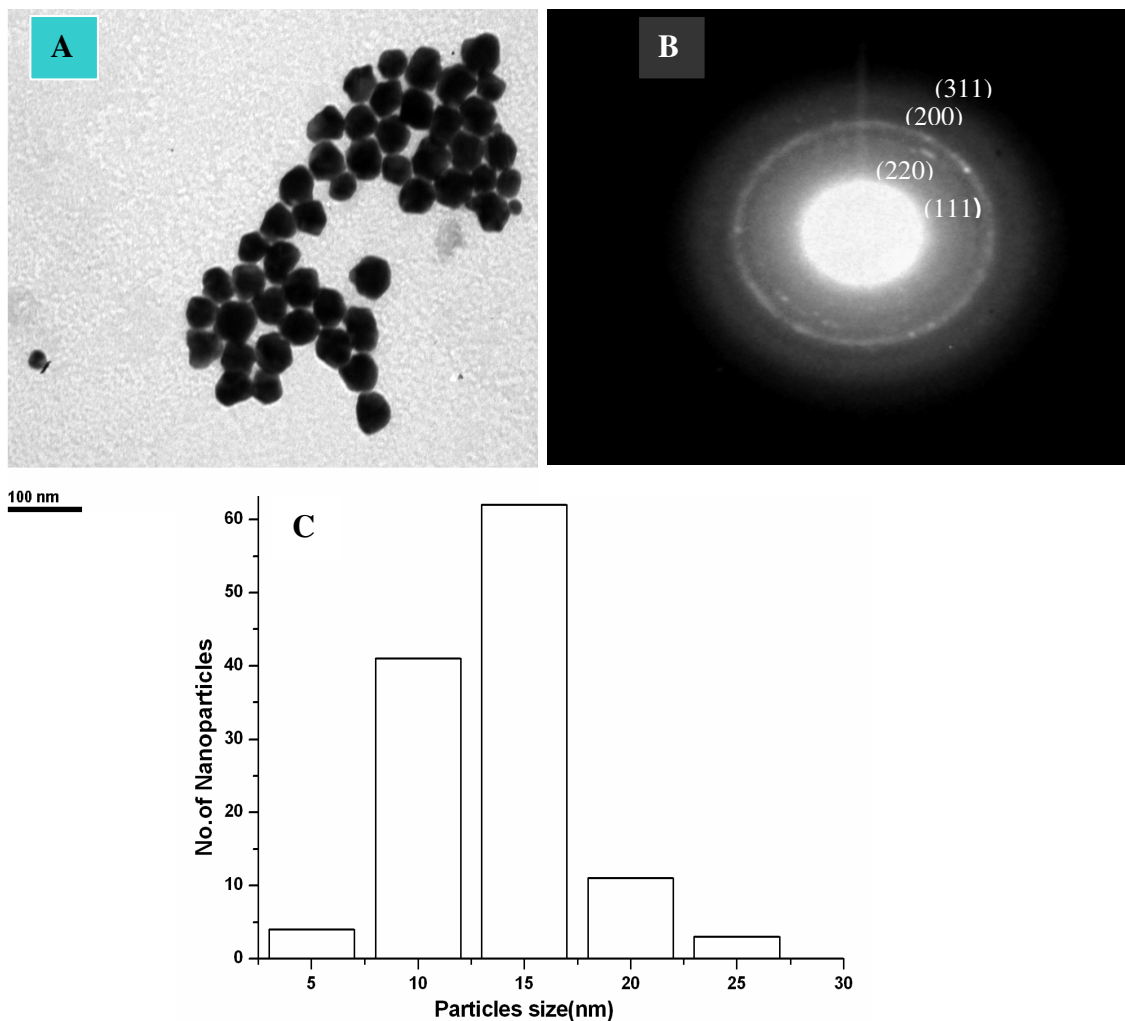
Fig. 5.4: A: TEM micrograph of gold nanoparticles B: Selected area of electron diffraction pattern recorded from one of the gold nanoparticles shown in Fig. A. C: Histogram of size distribution of the gold nanoparticles.

X-ray photoelectron spectroscopy

X-ray photoelectron spectroscopy (XPS) carried out over a wide range of binding energies, i.e. 0 to 1400 e V (Fig. 5.5) showed the presence of Au, C, O, N and Na as the prominent elements.
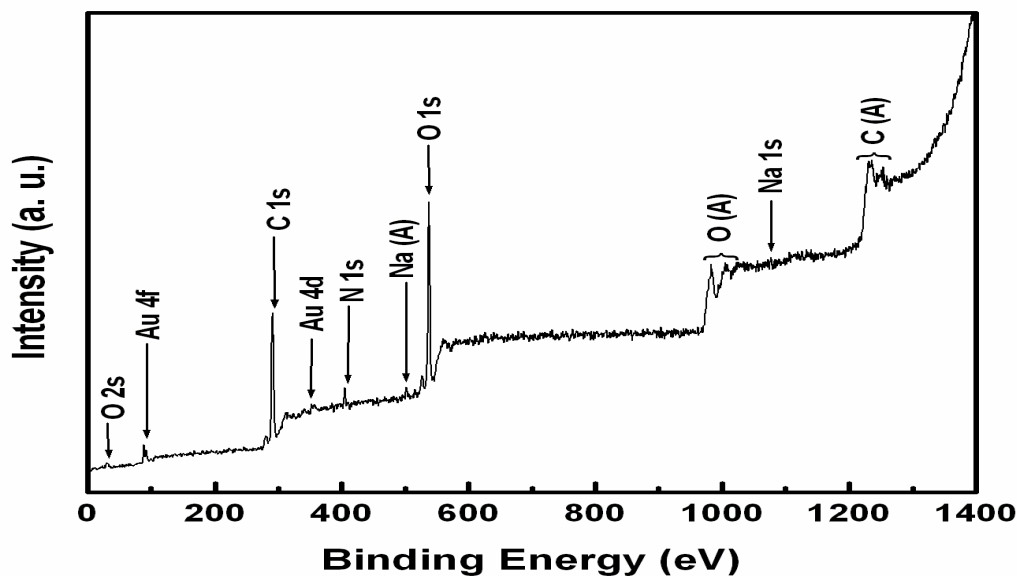
Fig. 5. 5: X-ray Photoelectron spectroscopy of the gold nanoparticles.

The core level binding energies and atomic percentages of various elements detected in the reaction mixture is given in Table 5.1.

Table 5.1. Core level binding energies and atomic percentages of various elements detected in the reaction mixture.

Element	C1s	O1s	N1s	Au 4f$_{7/2}$
Atomic %	63	28.5	6.3	2.2
Binding energy [e V]	285.0	531.1	399.7	83.9

The Au 4f spectrum could be resolved into two spin-orbit components viz.1 and 2 (spin-orbit splitting ~ 3.8 eV).
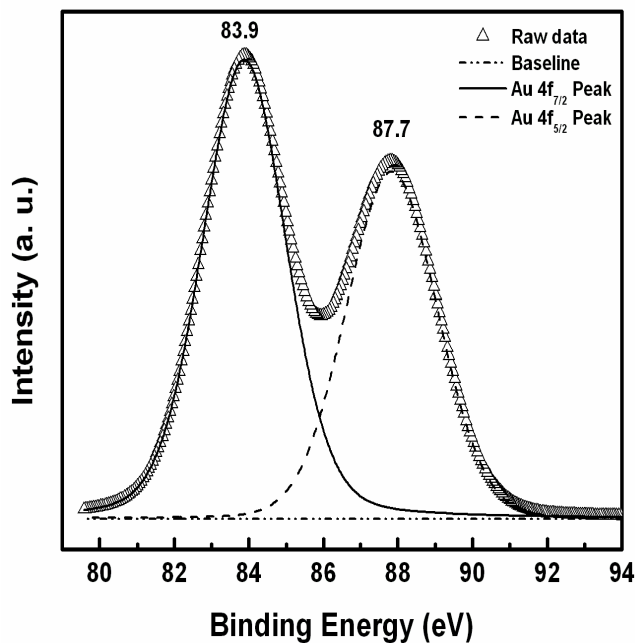
Fig. 5. 6: Au 4f core level spectra of the gold nanoparticles.

The Au $4f_{7/2}$ and $4f_{5/2}$ peaks occurred at a binding energy of 83.9 eV and 87.7 eV respectively which are characteristic of metallic Au (Fig. 5.6) and agree with the core level binding energies reported by Chastain (1992). The absence of higher binding energy for Au 4f component shows that all the chloroaurate ions are fully reduced and are in metallic form.

In conclusion, the present studies, for the first time, demonstrate the application of an enzymatic route for synthesis of gold nanoparticles.

REFERENCES

Chastain, J. (1992) Handbook of X-ray Photoelectron Spectroscopy (Perkin-Elmer: Norwalk)

Duncan, J. R.; Brady, D.; Wilhelmi, B. (1997) *Bioremediation protocols*, **2,** 91-97.

Greene, B.; Hosea, M.; Mc Pherson, R.; Henzi, M.; Alexander, M. D.; Darnall, D. W. (1986) *Environ. Sci. Technol*. **20,** 627-632.

Hosea, M.; Greene, B.; Mc Pherson, R.; Henzi, M.; Alexander, M. D.; Darnall, D. W. (1986) *Inorg. Chem. Acta*. **123,** 161-165.

Kuyucak, N. and Volesky, B. (1989) *Biorecovery* **1,** 189-204.

Mehra, R. K. and Winge, D. R. 91991) *J. Cell. Biochem.* **45,** 30-40.

Mukherjee, P.; Ahmad, A.; Mandal, D.; Senapati, S.; Sainkar, S. R.; Khan, M. I.; Ramani, R.; Parischa, R.; Ajaykumar, P. V.; Alam, S.; Sastry, M. (2001) *Angew. Chem. Int. Ed.* **40,** 3585-3588.

Mukherjee, P.; Senapati, S.; Mandal, D.; Ahmad, A.; Khan, M. I.; Kumar, R.; Sastry, M. (2002) *ChemBio-Chem* **3,** 461-463.

Mulvaney, P. (1996) *Langmuir* **12,** 788-800.

Schmid, G. (1996) (Eds. Edelstein, A. S. and Cammarata, R. C.), IOP, Bristol.

Schmid, G. (1994) Wiley-VCH, New York.

Stephen, J. R. and Maenaughton, S. J. (1999) *Curr. Opin. Biotechnol***. 10,** 230-233.

CHAPTER 6

NITRATE REDUCTASE MEDIATED
SYNTHESIS OF SILVER NANOPARTICLES

SUMMARY

NADPH-dependent nitrate reductase, in presence of phytochelatin and 4-hydroxyquinoline, could reduce AgNO₃ leading to the formation of stable silver nanoparticles of dimensions in the range of 10-25 nm. The nanoparticles formed were predominantly spherical, interspersed with rhomboids and triangles.

INTRODUCTION

Nanotechnology (Feynman, 1991) which deals with the formation of nano-meter sized objects of dimensions less than 100 nanometers has generated considerable interest because of its perceived impact on the energy, chemical, electronics and space industries and drug/gene delivery (Rao et al. 2001). Hence the development of experimental procedures for the synthesis of nanoparticles of different chemical compositions, sizes, shapes and controlled polydispersity is important. Currently, there is an ever-increasing need to develop ecofriendly processes to replace synthetic protocols involving toxic ingredients. On this background, increasing attention is being paid on the use of microorganisms for the synthesis of nanomaterials (Klaus et al. 1999, 2001; Mukherjee et al. 2001a, b, 2002; Ahmad et al. 2002, 2003 a, b,). The preceding chapters we described the *in vitro* synthesis of CdS and gold nanoparticles using NADPH-dependent sulphite reductase. This chapter describes the application of NADPH-dependent nitrate reductase for the synthesis of silver nanoparticles.

MATERIALS

Silver nitrate (AgNO₃), NADPH and 4-hydroxyquinoline (Sigma Chemicals Co., St Louis, MO, U.S.A) and phytochelatin (Geno Mechanix, Florida, U.S.A) were used. All other chemicals used were of analytical grade. Nitrate reductase was purified as described in chapter 3 (Page 53).

METHODS

In vitro synthesis of silver nanoparticles

The total reaction mixture of 3 ml contained 1.0 mM of freshly prepared $AgNO_3$, 100 μg of phytochelatin, 1 mM 4-hydroxyquinoline, 1.0 mM α-NADPH and 3.32 U (100 μg protein) of nitrate reductase. The reaction mixture was incubated, under anaerobic conditions, at 25^0C. Reactions performed in the absence of the enzyme, NADPH, 4-hydroxyquinoline, phytochelatin as well as the inactivated enzyme served as control. Samples were removed at regular intervals and subjected to UV-Vis spectroscopy to check for the formation of nanoparticles. On completion of the reaction, silver nanoparticles were collected by centrifugation (75000 *g*, 30 min), washed twice with Milli Q water and the unbound proteins were removed by treating with 80% (v/v) ethanol.

UV/Vis spectroscopy

UV-Vis spectrophotometric measurements were performed on a Shimadzu dual-beam spectrophotometer (model UV-1601 PC) operated at a resolution of 1 nm.

Transmission electron microscopy (TEM)

Samples were prepared by drying a drop of silver nanoparticles solution on carbon coated TEM copper grids and subjected to Transmission electron microscopy (JEOL Model 1200 EX) operated at an accelerating voltage of 80 kV.

X-ray diffraction measurements (XRD)

Thin films of silver nanoparticles were prepared on Si (111) substrates by drop-coating with nanoparticles solution. The films on Si wafers were then subjected to X-ray diffraction (XRD) on a Philips PW 1830 instrument operated at 40 kV and 30 mA with Cu Kα radiation.

X-ray photoelectron spectroscopy (XPS)

Chemical analysis of a drop-coated film of silver nanoparticles on a conductive substrate was carried out on a ESCALAB MK II X-ray photoelectron spectrometer (V. G. Scientific, UK) with A1 k_α as the exiting source ($h\gamma$ = 1486.6 e V) operating at an accelerating voltage of 10 kV and 20 m A at a pressure of about 10^{-8} Pa.

RESULTS AND DISCUSSION

Freshly prepared AgNO3, phytochelatin, 4- hydroxyquinoline and NADPH when incubated with nitrate reductase at 25°C for 5 h, under anaerobic conditions, resulted in the formation of silver nanoparticles as evidenced by the change in the color of reaction mixture from colorless to brown (Fig.6.1).
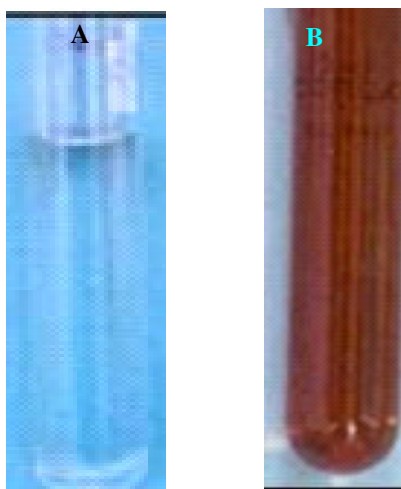
Fig. 6. 1: Formation of silver nanoparticles by nitrate reductase. A: At 0 h and **B:** At 5 h.

The coloration was due to the excitation of surface plasmon resonance of the metal nanoparticles (Mulvaney 1996).

UV/Vis spectroscopy

The UV-Vis spectra for the reaction mixture, as a function of time, showed a well-defined surface plasmon at 413 nm, characteristic of silver nanoparticles (Fig.6.2).
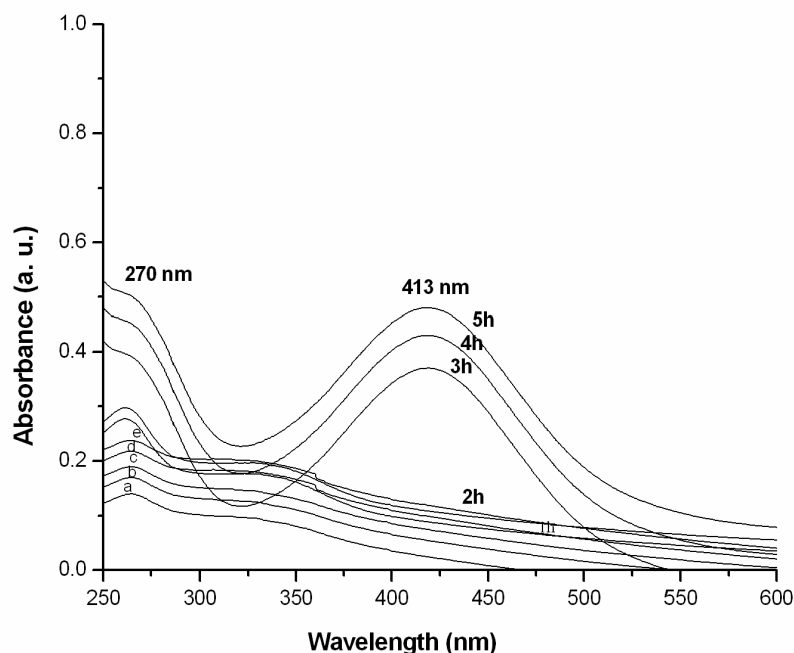
Fig. 6. 2: UV-Vis spectra of silver nanoparticles as a function of time. Curves a, b, c, d and e represent the reactions performed in the absence enzyme, phytochelatin, hydroxyquinoline, NADPH and denatured enzyme respectively.

The solution was extremely stable, with no evidence of flocculation of the particles after a month's storage at room temperature. The absence of surface plasmon resonance at 413 nm of silver nanoparticles, in the absence of active and inactivated enzyme (Fig. 6.2), clearly indicates involvement of NADPH-dependent nitrate reductase in the reduction of silver. The obligate requirement of phytochelatin (Fig. 6.2), in the formation of silver nanoparticles suggests the role of capping peptide in the prevention of their aggregation since its removal by treatment with 2% SDS resulted in the reaggregation of the particles.

Moreover, hydroxyquinoline might be acting as an electron shuttle transferring the electrons, generated during the reduction of nitrate, to Ag^{2+} ions and its subsequent conversion to metallic Ag.

X-Ray diffraction

The X-ray diffraction pattern of silver nanoparticles showed intense peaks at (111), (200), (220) and (311) in the 2θ range 20°-70° (Fig. 6.3) and agree with those reported for the silver nanocrystals.
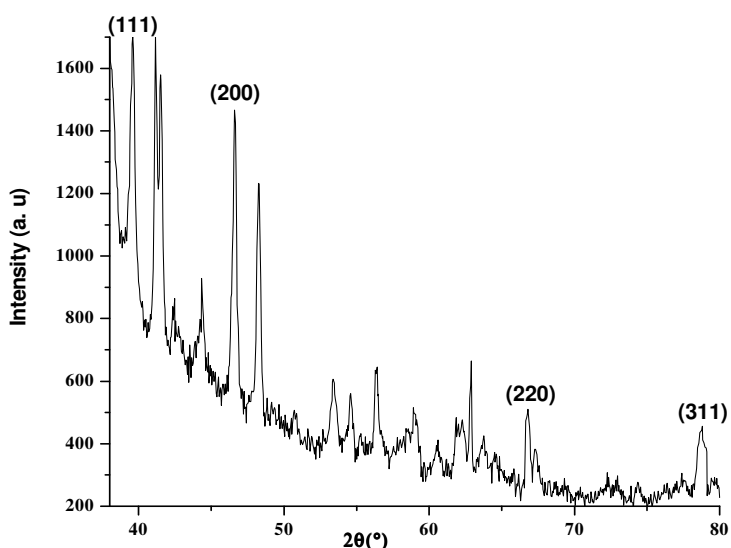
Fig. 6.3: XRD pattern recorded from the silver.

The Bragg reflection of silver, along with a Lorentzian fit (111) is given in Fig. 6.3. Determination of the size of the nanoparticles using Debye Scherrer formula (Jeffrey 1971) gave a value of 15 nm comparable to that obtained by TEM (Fig. 6.4A).

Transmission electron microscopy

TEM analyses of the gold nanoparticles showed them to be polydisperse and spherical (Fig. 6.4A). The nanoparticles were crystalline (Fig. 6.4B) and their size were in the range of 10-25 nm with an average of 15 ± 2 nm (Fig. 6.4C).
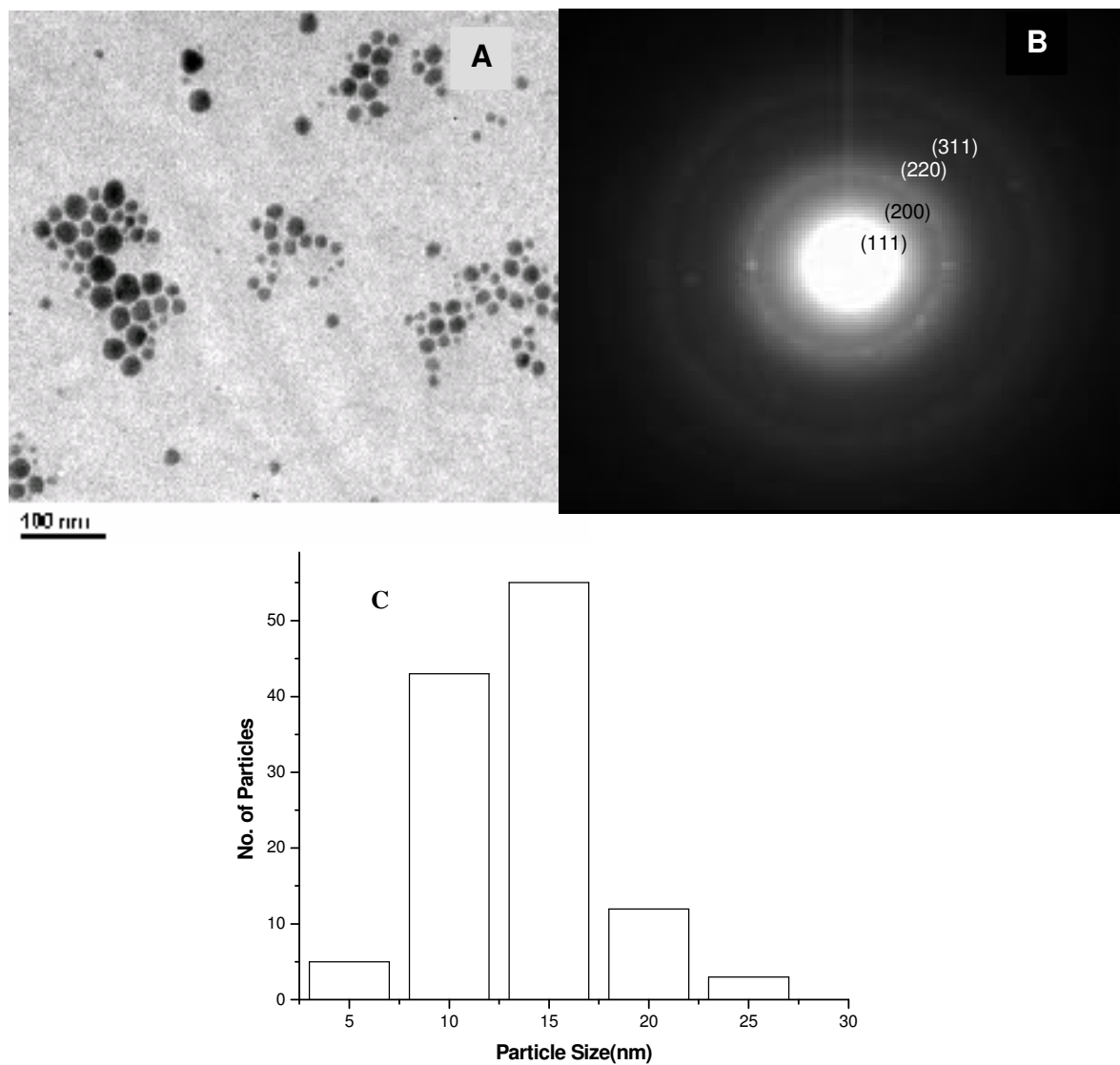
Fig. 6.4: A: TEM micrograph of silver nanoparticles B: Selected area of electron diffraction pattern recorded from one of the silver nanoparticles shown in Fig. A. C: Histogram of size distribution of the silver nanoparticles.

X-ray photoelectron spectroscopy

X-ray photoelectron spectroscopy (XPS) carried out over a wide range of binding energies, i.e. 0 to 1400 e V (Fig. 6.5) showed the presence of Ag, C, O, N and Na as the prominent elements.
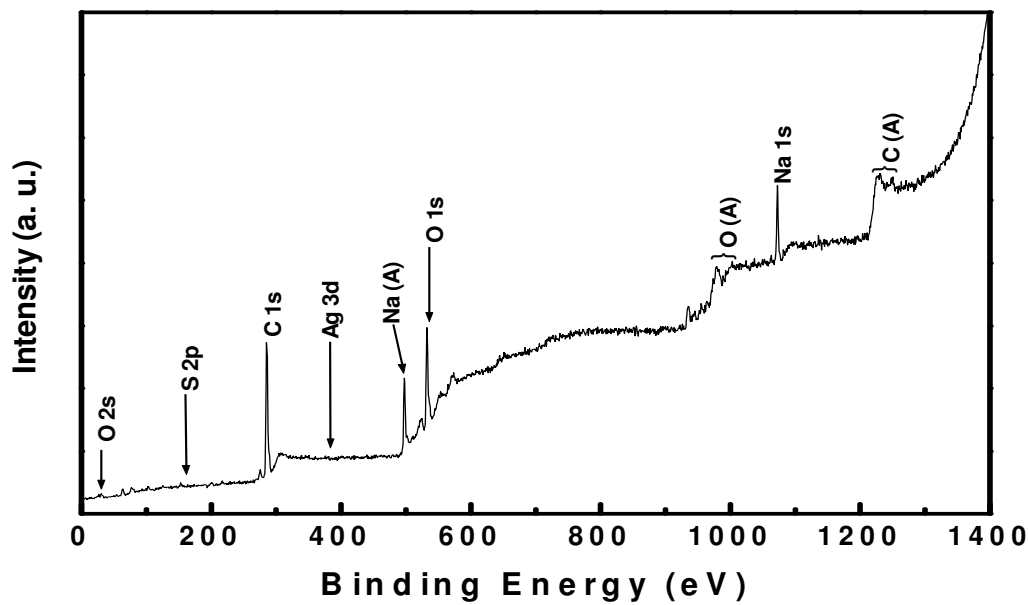
Fig. 6.5: X-ray Photoelectron spectroscopy of the silver nanoparticles.

Table 6.1 shows the core level binding energies along with atomic percentages of the detected elements from the reaction mixture.

Element	C1s	O1s	N1s	Ag 3d$_{5/2}$
Atomic %	75	22.5	1.8	0.7
Binding energy [e V]	285.0	532	399.7	368.1

The Ag 3d spectrum could be resolved into a single spin-orbit pair (spin-orbit splitting ~6 eV). The Ag 3d5/2 and 3d3/2 peaks were observed at binding energies of 368.1 eV and 374.8 eV, respectively which are characteristic of metallic Ag (Fig. 6.6) and agree with the core level binding energies reported by Chastain (1992).
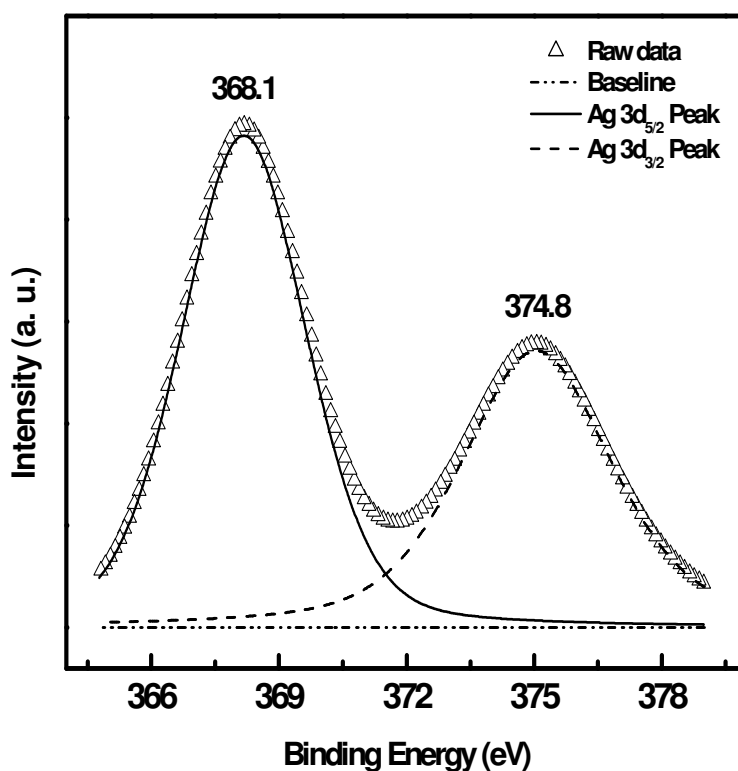
Fig. 6.6: Ag 3d core level spectra of the silver nanoparticle.

The absence of a higher binding energy Ag 3d clearly indicates that all the silver ions are fully reduced and are in metallic form.

In conclusion, the present studies demonstrate the application of NADPH-dependent nitrate reductase for synthesis of silver nanoparticles. Our observations are of importance since it can be used for the development of a gentle process for the synthesis of nanomaterials.

REFERENCES

Ahmad, A., Mukherjee, P., Mandal, D., Senapati, S., Khan, M. I., Kumar, R., Sastry, M. (2002) *J. Am. Chem. Soc.* **124**, 12108-12109.

Ahmad, A., Senapati, S., Khan, M. I., Kumar, R., Sastry, M. (2003) *Langmuir.* 19, 3550-3553.

Ahmad, A., Mukherjee, P., Senapati, S., Mandal, D., Khan, M. I, Kumar, R., Sastry, M. (2003) *Colloids Surf.* B **8**, 313-318.

Chastain J (1992) Handbook of X-ray Photoelectron Spectroscopy (Perkin-Elmer, Norwalk).

Feynman, R. P. (1991) *Science* **254,** 1300-1301.

Jeffrey, J. W. (1971) Acedemic press, New york

Klaus, T., Joerger, R., Olsson, E., Granqvist, C. G. (1999) *Proc. Natl. Acad. Sci.* U.S.A. **96,** 13611-13614.

Klaus-Joerger, T., Joerger, R., Olsson, E., Granqvist, C. G. (2001) *Trends Biotechnol*. **19**, 15-20.

Mukherjee, P., Ahmad, A., Mandal, D., Senapati, S., Sainkar, S. R., Khan, M. I., Ramani, R., Parischa, R., Ajaykumar, P. V., Alam, M., Sastry, M. (2001a) *Angew. Chem. Int. Ed.* **40**, 3585-3588.

Mukherjee, P., Senapati , S., Mandal, D., Ahmad, A., Khan, M. I., Kumar, R., Sastry, M. (2002) *Chem Bio-Chem.* **3**,461-463.

Mukherjee, P., Ahmad, A., Mandal, D., Senapati, S., Sainkar, S. R., Khan, M. I., Parischa, R., Ajayakumar, P. V., Alam, M., Kumar, R., Sastry, M. (2001b) *Nano. Lett.* **1**, 515-519.

Mulvaney, P. (1996) *Langmuir.* **12**, 788-800.

Rao, C. N. R., Cheetham, A. K. (2001) *J. Mater. Chem*. **11,** 2887-2894.

CHAPTER 7

CONJUGATION OF CdS QUANTUM DOTS TO
JACALIN AND CHICKPEA LECTIN FOR
DIAGNOSTIC APPLICATIONS

SUMMARY

Conjugation of the i*n vitro* synthesized CdS nanoparticles to jacalin and chickpea lectin was carried out using 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC). Conjugation to jacalin was carried out at pH 6.0 during which the free carboxyl groups of the capping peptide associated with CdS nanoparticles, are cross-linked with the reactive amine groups of jacalin. For the conjugation with chickpea lectin (CPL), CdS nanoparticles were first derivatized with ethylene diamine. The underivatized CdS peptide contains a free carboxyl, and cross links the CdS-peptide acid groups with the active site amino group present in the CPL. In order to prevent this cross linking amine derivatized CdS nanoparticles were used for conjugation. During the derivatization reaction, all the four CdS-peptide acid groups were derivatized to free amino groups using ethylene diamine. In both the above reactions, the concentration of lectin was maintained at 50 μg/ml in order to prevent the formation of inter-molecular lectin conjugates.

INTRODUCTION

Biotechnology is witnessing the impressive advances in the synthesis of biocompatible surfaces for the immobilization of a range of biomolecules (Fang et al., 1999; Loidl-Stahlhofen et al., 2001; Lei et al., 2002) with applications in biosensing and medicine (Rembaum and Dreyer, 1980; Albers, 2001; Gilardi and Fantuzzi, 2001; Park et al., 2002). The availability of colloidal semiconductor nanoparticles with controlled optical and electrical properties has sparked widespread interest in their biologically relevant applications such as luminescent tagging and imaging, medical diagnostics, drug delivery and implantable nanoelectronics (Niemeyer, 2001, 2003). Examples of colloidal particle-biomolecule conjugates include immunomicrospheres, which can be used to react in a specific way with antibodies, target cells, DNA, peptides and proteins. A number of templates have been used for enzyme immobilizations such as silica nanotubes (Mitchell et al.; 2002), phospholipid bilayers (Hamachi et al., 1994; Chen et al., 1999), self-assembled monolayers (Mrksich et al., 1995; Fang et al., 1996), Langmuir-Blodgett films (Nicolini et al., 1993; Boussand et al., 1998), polymer matrices (Yang et al., 1995; Franchina et al., 1999), galleries of α-zirconium phosphate (Kumar and McLendon,

1997), mesoporous silicates such as MCM-41 (He et al., 2000), silica nanoparticles (Qhoboshean et al., 2001) and thermally evaporated lipid films (Sastry et al., 2002).

The adsorption of proteins on both polymers (Elgersma et al., 1990; Schmitt et al., 1997; Basinka et al., 1999; Caruso et al., 1999; Molina-Bolivar et al., 1999) and inorganic colloidal particles of oxides/metals has also been shown. Functionalized γ-Fe$_2$O$_3$ magnetic nanoparticles (Dyal et al., 2003) have been used as a support for the enzyme lipase. In the area of metal nanoparticle-enzyme conjugate materials, the formation and enzymatic activity of nanoparticles complexed with horseradish peroxidase (Stonehuerner et al., 1992), xanthine oxidase (Zhao et al., 1996) as well as glucose oxidase and carbonic anhydrase molecules (Crumbliss et al., 1992) has been demonstrated. Conjugation of pepsin (Gole et al., 2001a) and fungal protease (Gole et al., 2001b) with nanoparticles has also been reported. This chapter discusses the conjugation of *in vitro* synthesized CdS quantum dots to jacalin and chickpea lectin for diagnostic applications.

MATERIALS

2-(N-Morpholino) ethanesulfonic acid (MES), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), 3-nitro-L-tyrosine ethylester (NTEE) (Sigma chemicals Co., St Louis, MO, U.S.A) were used. All other chemicals used were of analytical grade. CdS nanoparticles were synthesized as described in chapter 3.

METHODS

Estimation of free carboxyl group

The total reaction mixture 3 ml containing 100 µg of CdS nanoparticles in 50 mM MES/HEPES buffer (75:25 v/v) pH 6.0, 50 mM EDC and 30 mM NTEE was incubated at 30°C for 45 minutes. Subsequently the reaction was arrested with the addition of 1 ml of 10% TCA and the precipitated CdS-peptide complex was collected by centrifugation, washed extensively with chilled acetone, air-dried and redissolved in 1 ml of 100 mM

NaOH. The number of nitrotyrosyl group incorporated was determined spectrophotometrically at 430 nm using a molar absorption coefficient of 4600 $M^{-1}cm^{-1}$.

Estimation of free amino group

Total reaction volume of 1 ml contained 100 µg of CdS nanoparticles, 500 µl of 4% sodium carbonate buffer pH 8.4, 50 µl of 0.1 % TNBS was incubated in dark at 30°C for 2 hours. The number of amino groups modified was determined spectrophotometrically at 335 nm using a molar absorption coefficient of 9950 $M^{-1}cm^{-1}$.

In vitro **conjugation of CdS nanoparticles**

(a) Jacalin

The volume of the reaction mixture 3 ml, containing CdS nanoparticles-peptide complex (1 mg) in 50 mM MES/HEPES buffer (75:25 v/v) pH 6.0, 500 µg of jacalin, was adjusted such that the effective concentration of jacalin is 50 µg/ml. To the above mixture, EDC was added, to an effective concentration of 5 mM, in 3 aliquots over a period of 1 hour at 30°C. Then the mixture was allowed to equilibrate for 15 minutes, concentrated to 1 ml by lyophilization and loaded onto a Sephacryl S-200 column (2x100) pre-equilibrated with 20 mM phosphate buffer containing 150 mM NaCl. The flow rate of elution was 10 ml/h and fractions of 1.5 ml were collected. The fractions exhibiting fluorescence as well as jacalin activity were pooled, dialyzed against water, concentrated and stored at 4°C for further use.

(b) Chickpea lectin

Derivatization of CdS nanoparticles: The total reaction mixture 3 ml containing 100 µg of CdS nanoparticles in 50 mM MES/HEPES buffer (75:25 v/v) pH 6.0, 100 times molar excess of ethylene diamine was incubated in the presence of 50 mM EDC at 30°C for 3 h. Subsequently the reaction mixture was passed through Sephadex G-25 gel filtration column to remove excess ethylene diamine and used for coupling. The column was pre-equilibrated with 20 mM phosphate buffer containing 150 mM NaCl. The flow rate of elution was 10 ml/h and fractions of 1.5 ml were collected. The fractions exhibiting

fluorescence were pooled, dialyzed against water, concentrated and used for the next step.

In vitro conjugation: The volume of the reaction mixture 3 ml containing amine derivatized CdS nanoparticles (1 mg) in 50 mM MES/HEPES buffer (75:25 v/v) pH 6.0, 500 µg of lectin, was adjusted such that the effective concentration of lectin is 50 µg/ml. To the above mixture, EDC was added, to an effective concentration of 5 mM, in 3 aliquots for a period of 1 hour at 30°C. Then the mixture was allowed to equilibrate for 15 minutes, concentrated to 1 ml and loaded onto Sephacryl S-200 column (2x100) pre-equilibrated with 20 mM phosphate buffer containing 150 mM NaCl. The flow rate of elution was adjusted to 10 ml/h and 1.5 ml fractions were collected. The fractions exhibiting fluorescence as well as lectin activity were pooled, dialyzed against water, concentrated and stored at 4°C for further use.

Hemagglutination test

Hemagglutination tests were performed in standard microtitre plates by the two-fold serial dilution method. A 50 µl aliquot of the erythrocytes (native as well as pronase treated) suspension was mixed with 50 µl of serially diluted CdS coupled lectin and agglutination was examined visually after incubation for one hour. The unit of hemagglutination activity (U) was expressed as the reciprocal of the highest dilution (titre) of the lectin that showed complete agglutination. The specific activity of the lectin is defined as the number of hemagglutination units per milligram of the protein (U/mg).

Fluorescence spectroscopy

Fluorescence measurements were carried out on a Perkin-Elmer LS 50B luminescence spectrofluorimeter, with slit width of 2 nm for both the monochromators and scan speed 300 nm/min. Samples were excited at 340 nm and the emission spectra were recorded in wavelength ranging from 350 nm to 700 nm.

Life time measurements were performed on a time correlated single photon counting FLS920 spectrofluorimeter (Edinburgh Instruments, Livingston, UK). A hydrogen flash lamp of pulse width 1 ns was used for excitation and an nF-900 nanosecond flash lamp was used to detect the fluorescence. Sample was exited at 340 nm

and emission was recorded at 440 nm. Slit widths of 15 and 15 nm were used on the excitation and emission monochromators, respectively. The resultant decay curves were analyzed by a multiexponential iterative fitting program supplied by Edinburgh Instruments.

RESULTS AND DISCUSSION

Bioconjugation preparation techniques typically involve incubating biomolecules with nanoparticles that are densely passivated by protective molecule or by linker molecules that form a bridge between nanoparticles and biomolecules. In this work, we have chosen to study the conjugation of lectins from chickpea and jack fruit to colloidal CdS nanoparticles. Lectins are a well known class of multivalent carbohydrate binding proteins of non-immune origin which recognize diverse sugar structures in a non-catalytic manner (Sharon and Lis, 1989). They have been implicated in cellular signaling, malignancy, host pathogen interactions, scavenging of glycoprotein's from the circulatory system, cell-cell interactions in the immune system, differentiation, protein targeting etc. (Sharon and Lis, 1989; Ashwell and Harford, 1982; Springer and Lasky, 1991).

The CdS nanoparticles synthesized *in vitro*, were precipitated with 1, 4 dioxane to remove the excess proteins and peptides that were used for their synthesis. The number of free carboxyl groups and free amino groups present in the capping peptide associated with the CdS nanoparticles were determined to be 4 and 0, per molecule respectively.

The conjugation of CdS nanoparticles was carried out at pH 6.0 by using the free carboxyl group of the capping peptide associated with CdS nanoparticles, for cross-linking with reactive amine groups of jacalin. The reaction utilizes the water-soluble 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) to catalyze reactions between CdS-peptide acid groups and jacalin amine groups In the above reaction, the concentration of jacalin was maintained at 50 μg/ml in order to prevent the formation of inter-lectin conjugates. De La Fuente et al. (2005) has used similar method for the conjugation of non-natural tiopronin directly to CdS nanoparticles.

CdS nanoparticles derivatized with ethylene diamine were used for conjugation with chickpea lectin. The CdS-chickpea lectin conjugates were prepared as described

above. Underivatized CdS peptide contains a free carboxyl that forms cross link between the CdS-peptide acid group with the catalytic amino group present in the CPL. In order to prevent this cross linking amine derivatized CdS nanoparticles were used for conjugation. During the derivatization reaction, all the four CdS-peptide acid groups were derivatized to free amino groups using ethylene diamine.

The conjugated CdS-jacalin and CdS-chickpea showed hemagglutination activity (Fig. 7.1) as well as fluorescence intensity indicating successful conjugation. The CdS-jacalin conjugate showed hemagglutination with normal as well as pronase treated erythrocytes but the CdS-chickpea conjugate showed activity only with pronase treated erythrocytes, similar to that reported for native jacalin (Sastry et al., 1986) and chickpea lectin (Katre et al., 2004).
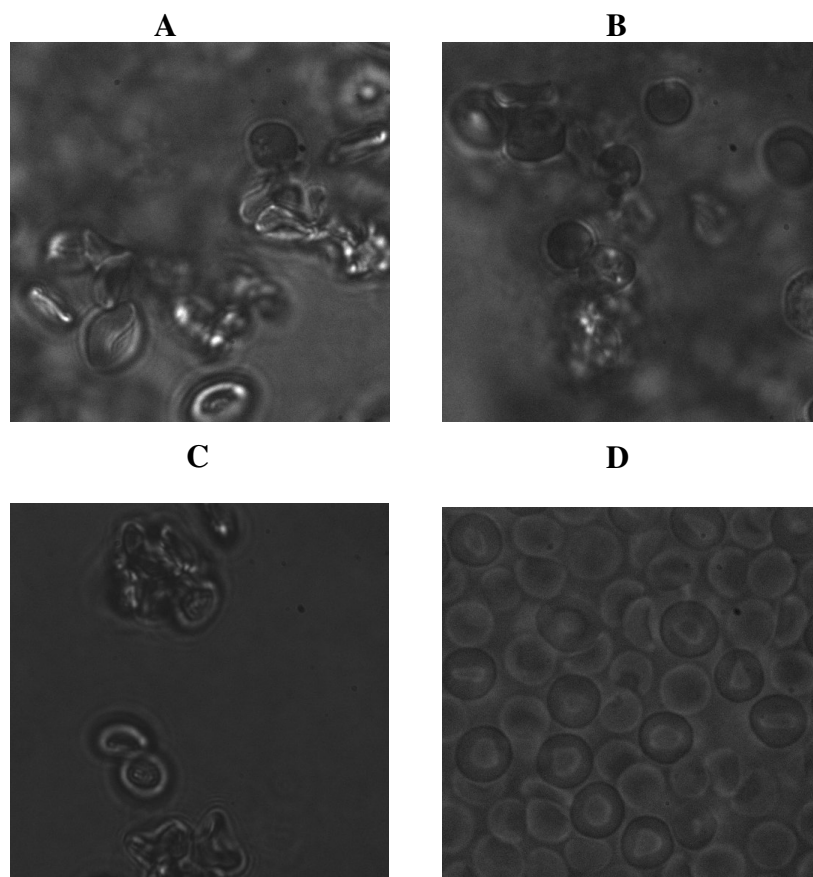
Fig.7.1: Phase contrast images of hemmagglutination activity for CdS-lectin conjugates. For CdS-jacalin conjugate A: With pronase treated erythrocytes **B:** With pronase untreated erythrocytes, **for CdS-chickpea conjugate C:** With pronase treated erythrocytes **D:** With pronase untreated erythrocytes.

Fluorescence spectroscopy

Fluorescence measurements of CdS nanoparticles, after excitation of the reaction mixture at 340 nm, showed an emission peak at 440 nm (Fig 7.2), where as CdS-lectin conjugate showed two emission peaks one sharp peak at 400 nm (Fig.7.3) that is not
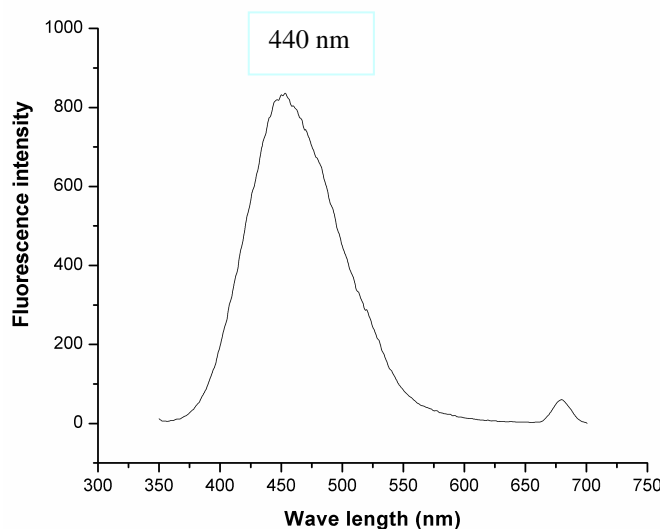
Fig.7.2: Fluorescence emission spectrum of CdS nanoparticles. The excitation was carried out at 340nm.

present in unconjugated CdS nanoparticles and another broad peak similar to that of CdS nanoparticles centered at 440 nm (Fig.7.2), for both CdS-jacalin and CdS-chickpea lectin.
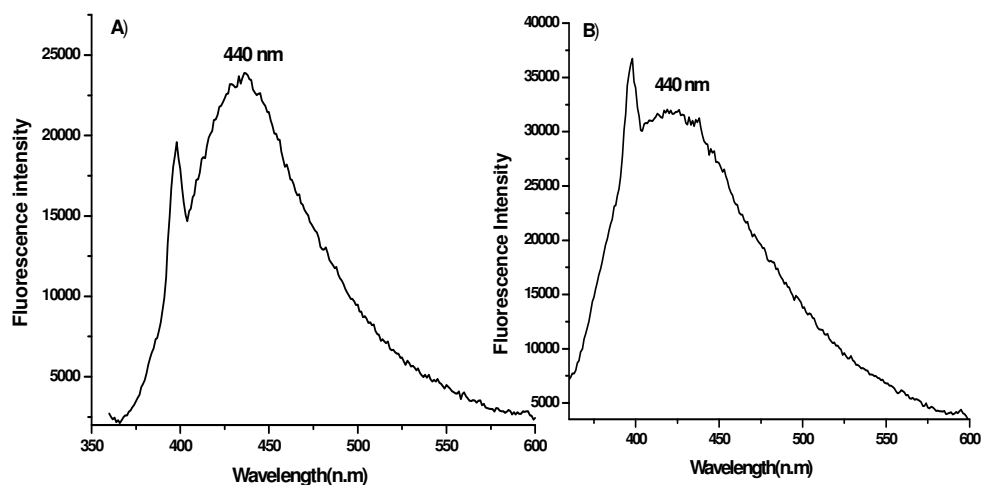
Fig.7.3: Fluorescence emission spectrum. A: CdS-jacalin conjugate and **B:** CdS-chickpea conjugate. The excitation was carried out at 340 nm.

The intensity of the peak, at 440 nm, gradually increased with the reaction time but the emission spectra showed the same features. The small stroke's shift in the peak suggests that the protein conjugated nanocrystals possess a continuous surface with most surface atoms exhibiting the coordination and oxidation states of their unconjugated and bulk counterparts.

The fluorescence decay curves of the CdS nanoparticles (Fig. 7.4A), CdS-jacalin (Fig. 7.4B) and CdS-chickpea (Fig. 7.4C) were determined by time resolved measurements.
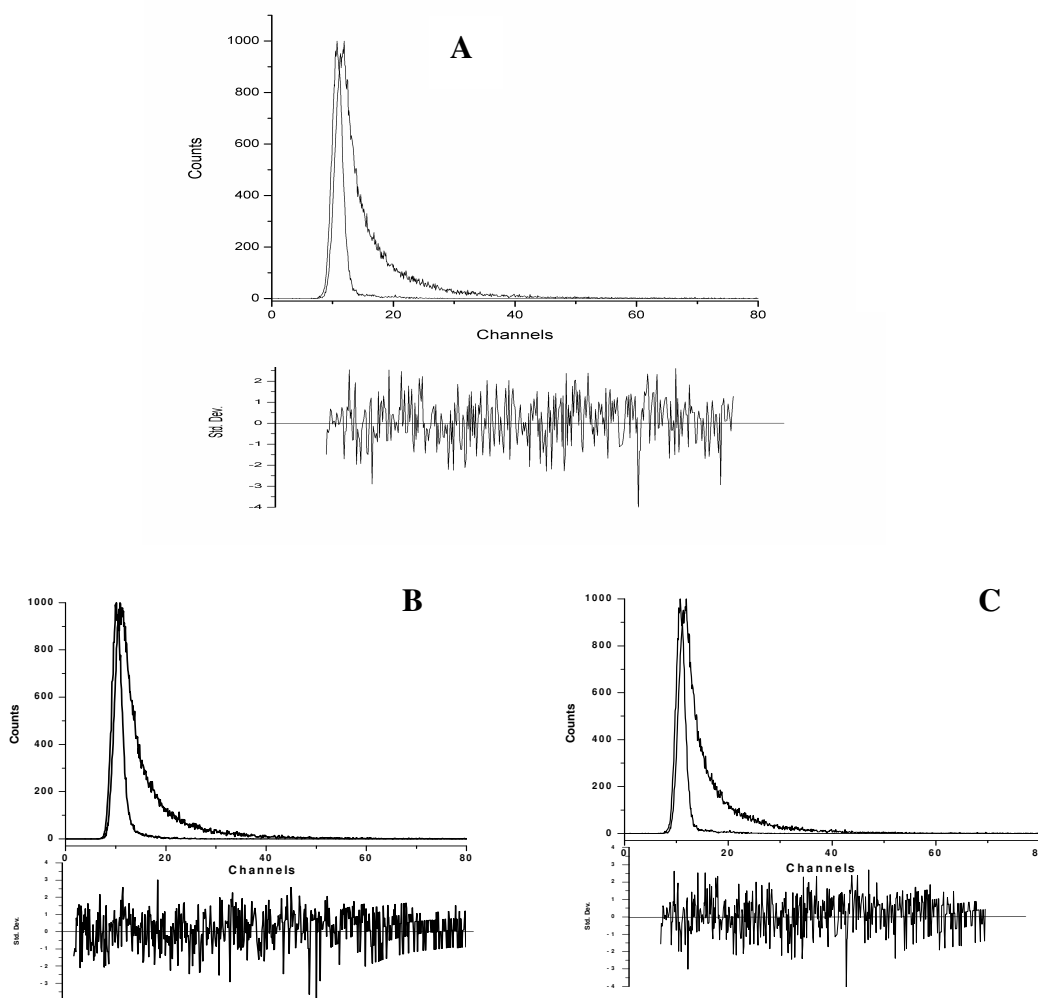
Fig.7.4: Time-resolved fluorescence profile of the conjugated CdS nanoparticles. **A:** CdS nanoparticles **B:** CdS-jacalin conjugate and **C:** CdS-chickpea conjugate. The solid line corresponds to the nonlinear least square fit of the experimental data to a bi-exponential function. The lower panels represent the residuals.

The decay curve profile could be best fitted to bi-exponential function ($\chi^2 \leq 1.1$). The life time values obtained are given in Table 7.1.

Source	τ1	τ2	α1	α2	χ^2
CdS-Jacalin	7.869 ns	1.869 ns	0.013	0.064	1.005
CdS-Chickpea	6.932 ns	1.741 ns	0.015	0.066	1.086
CdS	6.445 ns	1.476 ns	0.020	0.069	1.121

There was no significant difference in the lifetime values of the CdS nanoparticles, after conjugating to both jacalin and chickpea lectin. The life time values obtained for the CdS nanoparticles are 6.445 and 1.476 ns that are slightly lower than those observed for CdS-jacalin conjugate (7.869 and 1.869 ns) and CdS-chickpea conjugate (6.932 and 1.741 ns). The life time values obtained are in congruence with the CdS nanoparticles synthesized by physical and chemical methods reported earlier by Wu et al. (7.9 and 2.5 ns), Paramita et al. (7.52 and 2.46 ns).

In conclusion, the present studies demonstrate the conjugation of CdS nanoparticles for with jacalin and chickpea lectin to improve staining methodologies for cell-biology studies by a straight forward and economical EDC coupling methodology.

REFERENCES

Albers, W. M., Vikholm, I., Viitala, T., Peltonen, J. (2001) Handbook of surfaces and interfaces of materials. **Vol. 5**, Chapter 1, Academic Press, San Diego, USA.

Ashwell, G., Harford, J. (1982) *Annu. Rev. Biochem.* **51**, 531-554.

Basinska, T., Calwell, K. D. (1999) *ACS Symp. Ser.* **731,** 162-177.

Boussand, A., Dziri, L., Arechabaleta, N. J., Tao, N. J., Leblanc, R. M. (1998) *Langmuir* **14**, 6215-6219.

Caruso, F., Mohwald, H. (1999) *J. Am. Chem. Soc.* **121**, 6039-6046.

Chen, X., Hu, N., Zeng, Y., Rusling, J. F., Yang, J. (1999) *Langmuir* **15**, 7022-7030.

Crumbliss, A. L., Perine, S. C., Stonehuerner, J., Tubergen, K. R., Zhao, J., O'Daly, J. P. (1992) *Biotech. Bioeng.* **40**, 483-490.

De La Fuente, J. M., Fandel, M., Berry, C. C., Riehle, M., Cronin, L., Aitchison, G., Curtis, A. S. G. (2005) *Chem BioChem* **6**, 989-991.

Dyal, A., Loos, K., Noto, M., Chang, S. W., Spagnoli, C., Shafi, K. V. P. M., Ulman, A., Cowman, M., Gross, R. A. (2003) *J. Am. Chem. Soc.* **125**, 1684-1685.

Elgersma, A. V., Zsom, R. L. J., Norde, W., Lyklema, J. (1990) *J. Colloid and Interface Sci.* **138**, 145-156.

Fang, J., Knobler, C. M. (1996) *Langmuir* **12**, 1368-1374.

Fang, X., Liu, X., Schuster, S., Tan, W. (1999) *J. Am. Chem. Soc.* **121**, 2921-2922.

Franchina, J. G., Lackowski, W. M., Dermody, D. L., Crooks, R. M., Bergbreiter, D. E., Sirkar, K., Russell, R. J., Pishko, M. V. (1999) *Anal. Chem.* **71**, 3133-3139.

Gilardi, G., Fantuzzi, A. (2001) *Trends. Biotechnol.* **19**, 468-476.

Gole, A., Dash, C., Mandale, A. B., Rao, M., Sastry, M. (2000b) *Anal. Chem.* **72**, 4301-4309.

Gole, A., Dash, C., Ramakrishnan, V., Sainkar, S. R., Mandle, A. B., Rao, M., Sastry, M. (2001a) *Langmuir* **17**, 1674-1679.

Hamachi, I., Fujita, A., Kunitake, T. (1994) *J. Am. Chem. Soc.* **116**, 8811-8812.

He, J., Li, X., Evans, D. G., Duan, X., Li, C. (2000) *J. Molecular Catalysis. B: Enzymatic.* **11**, 45-53.

Katre, U. M., Gaikwad, S. M., Bhagyawant, S. S., Deshpande, U. D., Khan, M. I., Suresh, C. G. (2005) *Acta. Crystallograph.* **61,** 141-143.

Kumar, C. V., McLendon, G. L. (1997) *Chem. Mater.* **9**, 863-870.

Lei, C., Shin, Y., Lui, J., Ackerman, E. J. (2002) *J. Am. Chem. Soc.* **124**, 11242-11243.

Loidl-Stahlhofen, A., Schmitt, J., Noller, J., Hartmann, T., Brodowsky, H., Schmitt, W., Keldenich, J. (2001) *Adv. Mater.* **13**, 1829-1834.

Mitchell, D. T., Lee, S. B., Trofin, L., Li, N., Nevanen, T. K., Soderlund, H., Martin, C. R. (2002) *J. Am. Chem. Soc.* **124**, 11864-11865.

Molina-Bolivar, J. A., Ortega-Vinuesa, J. L. (1999) *Langmuir* **15**, 2644-2653.

Mrksich, M., Sigal, G. B., Whitesides, G. M. (1995) *Langmuir* **11**, 4383-4385.

Nicolini, C., Erokhin, V., Antolini, F., Catasti, P., Facci, P. (1993) *Biochim. Biophys. Acta.* **1158**, 273-278.

Niemeyer, C. M. (2001) *Angew. Chem., Int. Ed.* 113, 4128-4158.

Niemeyer, C. M. (2003) *Angew. Chem., Int. Ed.* 42, 5796-5800.

Qhoboshean, M., Santra, S., Zhang, P., Tan, W. (2001) *Analyst* **126**, 1274-1278.

Park, S. A., Taton, T. A., Mirkin, C. A. (2002) *Science* **295**, 503-1506.

Paramita, S. C., Pushpal, G., Amitava, P. (2007) *J. of Luminescence* **124,** 327-332.

Rembaum, A., Dreyer, W. J. (1980) *Science* **208**, 364-368.

Sastry, M., Rao, M., Ganesh, K. N. (2002) *Acc. Chem. Res.* **35**, 847-855.

Sastry, M. V. K., Banarjee, D., Sankhavaram, R., Patanjuli, M., Swamy, J., Swarnalatha, G. V., Surolia, A. *J. Biol. Chem.* (1986) 25, **261**, 11726-11733.

Schmitt, A., Fernandez-Barbero, A., Cabrerizo-Vilchez, M., Hidalgo-Alvarez, R. (1997) *Prog. Colloid Sci.* **104**, 144-147.

Sharon, N., Lis, H. (1989) *Science* **246**, 227-234.

Springer, T. A., Laskya, L. A. (1991) *Nature* **349**, 196-197.

Stonehuerner, J. G., Zhao, J., O'Daly, J. P., Crumbliss, A. L., Henkens, R. W. (1992) *Biosens. Bioelectron.* **7**, 421-428.

Wu, F., Zhang, J. Z., Kho, R., Mehra, R. K. (2000) *Chemical Physics Letters* **330**, 237-242.

Yang, Z., Mesiano, A. J., Venkatasubramanian, S., Gross, S. H., Russel, A. J. (1995) *J. Am. Chem. Soc.* **117**, 4843-4850.

Zhao, J., O'Daly, J. P., Henkens, R. W., Stonehuerner, J., Crumbliss, A. L. (1996) *Biosens. Bioelectron.* **11**, 493-502.

CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

SUMMARY

The main emphasis of the work presented in this thesis has been to demonstrate the synthesis of different metal and semiconductor metal nanoparticles using biological systems as an environmentally benign alternative to chemical and physical methods. The aspects that have been covered in this thesis are: 1) Biosynthesis of extracellular CdSe quantum dots using the fungus, *Fusarium oxysporum,* 2) purification and characterization of enzymes sulphite and nitrate reductases, 3) sulphite reductase mediated synthesis of semiconductor CdS nanoparticles, 4) sulphite reductase mediated synthesis of gold nanoparticles, 5) nitrate reductase mediated synthesis of silver nanoparticles, 6) conjugation of CdS nanoparticles to jacalin and chickpea lectin for diagnostic applications.

Chapter 1 presents a general introduction about nanoscience and nanotechnology and their consequences to different fields of science. Different physical, chemical and bio-based synthesis routes of nanomaterials and their characterization techniques are discussed. It also gives an account of different properties and applications of nanomaterials with particular emphasis to biological application. Based on literature survey, the scope of the present work has been summarized at the end of the chapter.

Chapter 2 deals with the extracellular synthesis of cadmium selenide (CdSe) quantum dots using eukaryotic fungi. The fungus, *Fusarium oxysporum*, when exposed to $CdCl_2/SeCl_4$ solutions resulted in the extracellular formation of highly stable CdSe nanoparticles. Characterization of the nanoparticles showed them to be spherical and polydisperse with sizes ranging from 9-15 nm. The high stability of the nanoparticles solution is due to the secretion of capping peptide by the fungus in the reaction mixture. The CdSe nanoparticles synthesized using biological method correlates with the one synthesized by physical and chemical methods (Lei et al., 2006; Firth et al., 2004; Yang et al., 2005). Fluorescence measurements of the CdSe nanoparticles when exited at 370 nm showed an emission band at 440 nm which is comparable to the emission peaks of CdSe nanoparticles synthesized by chemical methods (Lei et al., 2006). This has been attributed to the band gap or near band gap resulting from the recombination of the electron hole pairs in the CdSe nanoparticles (Donahue et al., 1998). The life time values obtained for the CdSe by our method was 6.397 and 1.424 ns which is also comparable to

the one reported by chemical methods 6 and 1 ns (Firth et al., 2004). Further more XRD analysis of the CdSe nanoparticles showed intense peaks at 110, 111, 220, 311 and 222 and agree with those reported for CdSe nanocrystals (Colvin et al., 1998). The chemical analysis by XPS revealed the presence of $Cd3d_{5/2}$ and $3d_{3/2}$ peaks at binding energies of 406 eV and 412 eV respectively and the Se3d peak at 54.8 eV that agree with the core level binding energies reported earlier (Chastain, 1992).

Chapter 3 deals with the purification and characterization of sulphite and nitrate reductases, enzymes responsible for the formation of extracellular nanoparticles. The sulphite reductase was purified to homogeneity by DEAE-Sephadex chromatography followed by gel filtration chromatography on Sephacryl S-300, with an over all yield of 20%. The purified enzyme is a 35.6 kDa protein and is lowest among the sulphite reductases reported so far from various sources *Salmonella typhimurium-* 66 kDa (Ostrowski et al., 1989), Spinach- 69 kDa (Kruger and Siegel, 1982), *E. Coli,* 607 kDa (Siegel et al., 1973) *Sacharomyces cerevisiae-* 604 kDa (Kobayashi and Yashimoto, 1982) and *Thiobacillus denitrificans-* 160 kDa (Schedel and Truper, 1979). The pI of the enzyme was 4.5 and requires α-NADPH as a cofactor. *Fusarium oxysporum* sulphite reductase exhibited an optimum pH of 6.0, which was similar to that reported for *Thiobacillus denitrificans* sulphite reductase (Schedel and Truper, 1979) and different with the enzymes from *Sacharomyces cerevisiae-* 7.3 (Kobayashi and Yashimoto, 1982), *Salmonella typhimurium,* 7.7 (Ostrowski et al., 1989), *Spinach-* 7.75 *(*Kruger and Siegel, 1982) and *E. coli-* 7.7, (Siegel et al., 1973). The optimum temperature of the purified enzyme was 45°C.

Nitrate reductase was purified to homogeneity by successive chromatography on DEAE- and CM-Sephadex followed by gel filtration on Sephacryl S-300. It is a 44.1 kDa protein with pI of 9.2. The Mr of *Fusarium oxysporum* nitrate reductase is also very small compared to nitrate reductases from various sources viz. *Porphyra yezoensis* - 220 kDa (Nakumura and Ikawa, 1998), *Skeletonema costatum* - 110 kDa (Gao et al., 1993), *Emiliania huxleyi* - 514 kDa (Iwamoto and Shiraiwa, 2003) and *Rhodotorula glutinis* - 130 kDa (Morozkina et al., 2005). The nitrate reductase exhibited an optimum pH of 7.0 that was similar to that reported for *Rhodotorula glutinis* (Morozkina et al., 2005). However enzymes from *Skeletonema costatum-* 7.4 (Gao et al., 1993) *Porphyra-* 8.3

(Nakumura and Ikawa, 1998), *Haloferax mediterranei*- 9.0 (Martinez-Espinosa et al., 2001) *Emiliania huxleyi*- 8.0 (Iwamoto and Shiraiwa, (2003) have a different optimum pH. The optimum temperature of the purified enzyme was 55°C.

Chapter 4 deals with the enzymatic synthesis of semiconductor cadmium sulfide (CdS) nanoparticles using sulphite reductase purified from the extracellular broth of *Fusarium oxysporum*. The *in vitro* synthesis of CdS is due to NADPH-dependent sulphite reductase, which results in the reduction of SO_4^{2-} ions to S^{2-} and subsequent formation of CdS nanoparticles which was easily followed by UV-Vis spectroscopy. Surface plasmon resonance of CdS nanoparticles exhibited an emission band at 340 nm. This is in congruence with that reported earlier (Dameron et al., 1989). The life time values obtained for the CdS by our method was 6.445 and 1.476 ns which were comparable to the one reported by Wu et al. (7.9 and 2.5 ns), Paramita et al. (7.52 and 2.46 ns) synthesized using chemical methods. The CdS nanoparticles formed were in the size range of 5-20 nm. Further more XRD analysis of the CdS nanoparticles showed intense peaks at (111), (101), (002) and (220) in the 2θ range 20°-70° and agree with those reported for the CdS nanocrystals (Colvin et al., 1992; Kundu et al., 1997). The chemical analysis by XPS revealed the presence of Cd3d$_{5/2}$ and 3d$_{3/2}$ peaks at binding energies of 405.5 eV and 412.5 eV respectively and the Se2P peak at 168.8 eV that agree with the core level binding energies reported earlier (Chastain, 1992).

Chapter 5 deals with the enzymatic synthesis of gold nanoparticles using sulphite reductase purified from *Fusarium oxysporum*. The *in vitro* synthesis of gold is due to α-NADPH dependent sulphite reductase, which results in the reduction of HAuCl^4 ions to Au and subsequent formation of gold nanoparticles which was easily followed by UV-Vis spectroscopy. It is well known that surface plasmon resonance of gold nanoparticles exhibit ruby red color and an emission band at 510-540 nm (Richard, 1978). The fact that gold nanoparticles peak remained close at 520 nm even after a month of reaction indicating the stabilization due to the capping peptide present in the reaction mixture. The gold nanoparticles formed were in the size range of 7-20 nm. Further more XRD analysis of the gold nanoparticles showed intense peaks at 111, 200, 220 and 311 that agree to the Bragg's reflection of the gold nanoparticles in reported literature (Jeffrey, 1971). The chemical analysis by XPS revealed the presence of Au 4f$_{7/2}$ and 4f$_{5/2}$ peaks at a binding

energy of 83.9 eV and 87.7 eV respectively and agree with the core level binding energies reported earlier (Chastain, 1992).

Chapter 6 deals with the enzymatic synthesis of silver nanoparticles using nitrate reductase purified from *Fusarium oxysporum*. The *in vitro* synthesis of silver is due to α-NADPH dependent nitrate reductase, which results in the reduction of $AgNO_3$ ions to Ag and subsequent formation of silver nanoparticles which was easily followed by UV-Vis spectroscopy. It is well known that surface plasmon resonance of silver nanoparticles exhibit ruby red color and an emission band at 400-430 nm (Richard, 1978). The fact that silver nanoparticles peak remained close at 413 nm even after a month of reaction indicating the stabilization due to the capping peptide present in the reaction mixture. The silver nanoparticles formed were in the size range of 10-25 nm. Further more XRD analysis of the silver nanoparticles showed intense peaks at 111, 200, 220 and 311 that agree to the Bragg's reflection of the silver nanoparticles reported literature (Jeffrey, 1971). The chemical analysis by XPS revealed the presence of Ag 3d5/2 and 3d3/2 peaks at binding energies of 368.1 eV and 374.8 eV, respectively and agree with the core level binding energies reported earlier (Chastain, 1992).

Chapter 7 focuses on the application of the *in vitro* synthesized CdS nanoparticles by conjugating to jack bean and chickpea lectin to improve staining methodologies for cell-biology studies by a straight forward and economical EDC coupling methodology. De La Fuente et al. (2005) had used the similar method for the conjugation of non-natural tiopronin directly to CdS nanoparticles. The CdS-lectin conjugate showed hemagglutination activity as well as fluorescence intensity at 440 nm when excited at 340 nm indicating the successful conjugation. There was no difference in the lifetime values of the CdS nanoparticles, after conjugating to both jacalin and chickpea lectin. The life time values obtained for the CdS nanoparticles are 6.445 and 1.476 ns that correspond to the life time values after CdS conjugation to lectin. The life times values of CdS-jacalin conjugate are 7.869 and 1.869 ns and CdS-chickpea conjugate are 6.932 and 1.741 ns. The life time values obtained are in congruence with the CdS nanoparticles synthesized by physical and chemical methods reported earlier by Wu et al. (7.9 and 2.5 ns), Paramita et al. (7.52 and 2.46 ns).

SCOPE AND IMPORTANCE

One of the important areas of nanotechnology is the development of reliable processes for the synthesis of nanomaterials over a range of sizes, shapes and chemical composition. Hence, the current research is directed towards the development of different experimental protocols for the synthesis of nanomaterials of variable sizes and shapes. Chemical and physical processes require either hazardous chemicals or expensive machinary. Thus, there is a need to develop environmentally benign processes that do not employ toxic chemicals and require expensive equipment in the synthesis protocols. This has fuelled the researchers to look at biological systems. Recently, the utilization of prokaryotic cells (such as bacteria, algae) has emerged as novel methods for the synthesis of nanomaterials. Although, several reports of the use of microbes has been addressed for nanoparticle synthesis but the exposure of different inorganic salts to these prokaryotic cells resulted in the synthesis of nanoparticles intracellularly. However, such biotransformation based nanoparticles synthesis strategies would have greater commercial viability if the nanoparticles could be synthesized extracellularly. Towards this objective, the thesis will focus on the extracellular synthesis of CdSe quantum dots using the fungus, *Fusarium oxysporum.* In these cellular systems, however many proteins, carbohydrates and biomembranes contribute to the overall biological reaction. Better control over nanoparticles size, shape, characteristics and the separation of biological materials are some of the inherent problems associated with biological systems. Therefore, it is desirable to develop more simple enzymatic routes for the synthesis nanomaterials, especially those catalyzed by a single oxidoreductase.

There are very few reports in the use of enzymes for the synthesis of nanomaterials. Very recently Nelson Duran et al. (2005) for the first time proposed a mechanism for the synthesis of silver nanoparticles by several *Fusarium oxysporum* strains. Willner et al. (2006) demonstrated the synthesis of gold nanoparticles using the enzyme glucose oxidase during the oxidation of glucose to gluconic acid. Yasui and Kimizuka (2005) reported the synthesis of gold nanoparticles using glucose oxidase during the conversion of glucose to gluconolactone.

Studies have been presented here demonstrating the synthesis of different metal and semiconductor metal nanoparticles using enzymes, sulphite and nitrate reductases

purified from the extracellular broth of *Fusarium oxysporum*. The expected benefits by following this method is that, analogous to the biological synthesis of metal nanoparticles using microorganisms, efficient synthesis can still be achieved in an environmentally benign process. It is likely to be more cost effective with a scope for large-scale production and the synthesis can be achieved *in vitro*.

FUTURE PROSPECTIVES

The ability of eukaryotic and prokaryotic microorganisms to reduce inorganic metals has opened up a new exciting green chemistry approach towards the development of natural 'nano-factories'. However, a number of issues have to be addressed from the nanotechnology and microbiology point of view, before such a biosynthesis approach can compete with the existing physical and chemical methods. The elucidation of biochemical pathways leading to metal ion reduction and formation of nanoparticles is essential in order to develop a rational bio-based nanoparticle synthesis procedure. Similarly, an understanding of the surface chemistry of the biogenic nanoparticles (i.e., the nature of reductases/capping peptides) is equally important. This would then lead to the possibility of genetically engineering fungi/actinomycetes to over express specific reducing molecules and capping agents, thereby controlling not only the size of the nanoparticles but also their shape. The rational use of enzymes to modulate nanoparticle size and shape is an exciting possibility. The range of chemical compositions of nanoparticles currently accessible by bio-based methods is currently extremely limited and confined to metals, metal sulfides and iron oxide. Extension of the protocols to enable reliable synthesis of nanomaterials of other oxides (TiO_2, ZrO_2, etc.) and nitrides, carbides etc. using enzymes could make this synthesis protocol a commercially viable proposition.

The enzyme mediated approach towards the synthesis of nanoparticles has many advantages such as ease with which the process can be scaled up, economic viability, control of shape, size, separation of nanomaterials etc. Compared to microbial fermentations, in which the process technology involves the use of sophisticated equipment for getting clear filtrates from the colloidal broths and also the synthesis of nanoparticles would be of importance in catalysis and other applications such as non-

linear optics. The nanoparticles especially quantum dots can be immobilized to different matrices, diagnostically important proteins and antibodies and can be used in various applications viz. cell and molecular labeling, fluorescence detection of pathogens, antibodies and proteins, diagnosis etc.

REFERENCES

Chastain J. (1992) Handbook of X-ray Photoelectron Spectroscopy, Perkin-Elmer, Norwalk.

Colvin, V. L., Goldstein A. N., Alivisators, A. P. (1992) *J. Am. Chem. Soc.* **114,** 5221-5230.

Dameron, C. T.; Reese, R. N.; Mehra, R. K.; Kortan, A. R.; Carroll, P. J.; Steigerwald, M. L.; Brus, L. E.; Winge, D. R. (1989) *Nature* **338,** 596-597.

De La Fuente, J. M., Fandel, M., Berry, C. C., Riehle, M., Cronin, L., Aitchison, G., Curtis, A. S. G. (2005) *Chem BioChem* **6**, 989-991.

Donahue, E. J., Roxburgh A., Yurchenko, M. (1998) *J. Mater. Res. Bull.* **33,** 323-329.

Duran, N., Marcato, P. D., Alves, O., De Souza G. I. H., Esposito, E. (2005) J. of Nanobiotech. **3,** 8-14.

Firth, A. V., Haggata, S. W., Khanna, P. K., Williams, S. J., Allen, J. W., Magennis, S. W., Irof Samuel D. W., Cole-Hamilton, D. J. (2004) *J. Liminesc.* **109**, 163-172.

Gao, Y., Smith, G. J., Alberte, R. S. (1993) *J. Phycol***. 7**, 1437-1445.

Iwamoto, K., Shiraiwa, Y. (2003) *Mar. Biotechnol*. **5**, 20-26.

Jeffrey, J. W. (1971) Methods in crystallography, Academic press, New York.

Kobayashi, K., Yashimoto, A. (1982) *Biochemica. Biophysica. Acta***. 705**, 348-356.

Krueger, R. J., Siegel, L. M. (1982) *J. Biol. Chem.* **21**, 2892-2904.

Kundu, M.; Khosravi, A. A.; Kulkarni, S. K. *J. Mater. Sci.* **1997,** 32, 245.

Lei, Z., Wei, X., Fan, Y., Liu Y., Bi, S. (2006) *J. Coll and Interf. Sci.* **304**, 402-407

Martinez-Espinosa, R. M., Marhuenda-Egea, F. C., Bonete, M. J. (2001) *FEMS Microbiology Letters* **204**, 381-385.

Morozkina, E. V., Nosikov, A. N., Zvyagilskaya, R. A., L'vov, N. P. (2005) *Biochemistry* (Moscow), **70**, 980-986.

Nakamura, Y., Ikawa, T. (1998) *Plant Cell Physol*. **34**, 1239-1249.

Ostrowski, J., Barber, M. J., Rueger, D. C., Miller, B. E., Siegel, L. E., Kredich, N. M. (1989) *J. Biol. Chem*. **264**, 15796-15808.

Paramita, S. C., Pushpal, G., Amitava, P. (2007) *J. of Luminescence* **124,** 327-332.

Richard, J. P. (1978) The chemistry of gold, Elsevier, Amsterdam.

Schedel, M., Truper, H. G. (1979) *Biochemica. Biophysica. Acta*. **568**, 454-467.

Siegel, L. M., Murphy, M. J., Kamin, H. (1973) *J. Biol. Chem.* **248***,* 251-264.

Willner, I., Baron, R., Willner, B. (2006) *Adv. Mater.* **18,** 1109-1120.

Wu, F., Zhang, J. Z., Kho, R., Mehra, R. K. (2000) *Chemical Physics Letters* **330**, 237-242.

Yang Y. J., Xiang, B. J. (2005) *J. Crys. Growt.* **284,** 453-458

Yasui, K.; Kimizuka, N.; (2005) *Chem. Lett.* **34,** 416-417.

PUBLICATIONS

1. **Anil Kumar**, **S.,** Abu Ayoobul Ansary, Absar Ahmad and Khan M. I. Biosynthesis of extracellular CdSe quantum dots by the fungus, *Fusarium oxysporum"* **Journal of Biomedical Nanotechnology** (**Accepted, In Press**).

2. **Anil Kumar**, **S.,** Majid Kazemian, Gosavi, S. W., Kulkarni S. K., Renu Parischa, Absar Ahmad and Khan M. I. "Nitrate reductase mediated synthesis of silver nanoparticles from $AgNO_3$." **Biotechnology Letters 29, 439-445, 2007**.

3. **Anil Kumar**, **S.,** Majid Kazemian, Gosavi, S. W., Kulkarni S., Absar Ahmad and Khan M. I. "Sulphite reductase mediated synthesis of gold nanoparticles capped by phytochelatin." **Biotechnology and Applied Biochemistry** (**Accepted, Online, DOI: 10.1042/BA20060205**).

4. **Anil Kumar**, **S.,** Abu Ayoobul Ansary, Absar Ahmad, Krishna Sastry and Khan. M. I. *"CdS Quantum dots: Sulphite reductase mediated synthesis, characterization and bioconjugation to jack bean and chickpea lectin"* (**Communicated to Chem BioChem**).

5. Singh. V. B., **Anil Kumar**, **S.,** Isaac Kirubakaran, S., Sunish Kumar, R. and Sakthivel N. "Comparison of exopolysaccharides produced by *Xanthomonas oryzae* pv. *oryzae* strains, BXO1 and BXO8 that show varying degrees of virulence in rice (*Oyrza sativa* L.)." **Journal of Phytopathology, 154, 410-413, 2006**.

6. **Anil Kumar**, **S.,** Abu Ayoobul Ansary, Absar Ahmad and Khan M. I. "CdSe-Zns core shell nanoparticles: Enzyme mediated synthesis and characterization." (Manuscript under preparation).

CONFERENCES/ABSTRACTS/POSTERS/ORAL PRESENTATIONS

1. **XVII International Symposium on Glycoconjugates**, IISc, Bangalore, India Jan 12-16, 2003.

2. **XXXIII National Seminar on Crystallography**, NCL, Pune, India, Jan 8-10, 2004.

3. **Prospecting of Fungal Diversity and Emerging Technologies**. Agharkar Research Institute, Pune, India, Feb 6-7, 2003.

4. **Eighth International Conference on Nanostructured Materials** (Nano 2006). Indian Institute of Science, Bangalore, India, 20 – 25 August, 2006.

PRESENTATIONS

ORAL

1. **32nd annual meeting of the Mycological Society of India**, JGI, India Feb 2-3, 2006, "*In vitro* synthesis of nanoparticles using the purified enzymes and proteins from *Fusarium oxysoporum*" **S. Anil Kumar**, Ahmad. A and Khan. M. I.

2. **Indo-French meeting held at Sophia-Antipolis**, France Sep 8-10, 2004, "Extracellular synthesis of nanoparticles by the fungus, *Fusarium oxysporum*" M. I. Khan, Absar Ahmad, Murali Sastry, Rajiv Kumar, **S. Anil Kumar** and S. Senapati.

3. **Indo-French meeting held at Goa**, India Feb 4-7, 2006 "*In vitro* synthesis of nanoparticles using enzymes" M. I. Khan, **S. Anil Kumar**, P. Nithya Priya, Shadab Ali Khan and Absar Ahmad.

4. **National Seminar on Nanobiotechnology** held at NBRI, Lucknow, India Nov 17-19, 2005," *In vitro* synthesis of inorganic nanoparticles using enzymes and proteins from microbes" Absar Ahmad, M. I. Khan and **S. Anil Kumar.**

5. **National seminar on Nanoscience and Nanobiotechnology**, Pondichery Central University, Pondichery, India Dec 5-6, 2005, "*In vitro* synthesis of nanomaterials using enzymes and proteins from fungi and actinomycetes" Absar Ahmad, M. I. Khan and **S. Anil Kumar**.

6. **Half day symposium on "Biomimetic Materials"**, MRSI, Pune, India Nov 10th, 2005, "*In vitro* synthesis of nanoparticles using enzymes" M. I. Khan, **S. Anil Kumar** and Absar Ahmad.

POSTER

1. **XVII International Symposium on Glycoconjugates**, Indian Institute of Science, Bangalore, India Jan 12-16, 2003. "Chickpea lectin, a lectin with complex sugar specificity" Sameer Bhagyawant, Atul Thakur, **S. Anil Kumar**, Feroz Khan, Absar Ahmad and M. I. Khan.

2. **Eighth International conference on nanostructured materials** (Nano 2006). Indian Institute of Science, Bangalore, India, 20-25 August, 2006. "*In vitro* synthesis of different metal nanoparticles using the purified enzymes and proteins from *Fusarium oxysporum*". **S. Anil Kumar**, Abu Ayoobul Ansary, Absar Ahmad and M. I. Khan.