



***An in vitro* assessment of the potential toxicity of Cadmium Selenide nanoparticles**

A thesis presented by

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Submitted in fulfilment for the award of the degree of

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Abstract

Nanotechnology is steadily finding its application in all aspects of the consumer industry, science and engineering. At a relative pace Cadmium Selenide (CdSe) nanoparticles are gaining increased attention for their potential use in biomedical applications such as bio-imaging of tissues, disease diagnosis and biological labelling due to its unique optical and electronic properties. Exposure of these particles to humans and other biological systems raise huge concerns with regards to their safety. In this study, water soluble cysteine capped CdSe nanocrystals, were prepared through a one pot green route method. The prepared CdSe nanocrystals were characterized using Transmission electron microscopy (TEM), High resolution transmission electron microscopy (HRTEM), Ultra violet spectroscopy (UV) and Photoluminescence (PL) analysis to establish the size, shape, dispersion, aggregation state, crystalline nature and optical properties of CdSe nanoparticles. The *in vitro* effects of CdSe nanoparticles on DNA stability, red blood cells (RBC's) and blood platelets were evaluated. DNA was exposed to CdSe nanoparticles and its assessment on DNA stability was confirmed by agarose gel electrophoresis and spectrophotometry. Damage to DNA structure was observed at 200 µg/ml of CdSe. *In vitro* assays carried out on RBC damage included reducing power and chelating activity of iron. The results showed that the CdSe nanocrystals exhibited high reducing power and sufficient chelating activity, which would be able to impair the function of haemoglobin. CdSe nanoparticles promoted platelet aggregation in a dose dependent manner. Based on the findings of this study the biosafety of CdSe nanoparticles is not guaranteed and further studies need to be conducted to ascertain the safety of CdSe nanoparticles for possible use in biological systems.

Dedication

This study is dedicated to my late Gurudev, Sri Saathiya Sai Baba. I am blessed everyday just knowing that you live forever in my heart. Thank you for always guiding me.

Declaration

I declare that this dissertation is my own, unaided work. It has been submitted for the degree of Master's in Science at the University of Zululand. It has not been submitted before for any degree or examination at any other University. I also state that all the sources that I have used have been duly acknowledged.

This _____ day of _____ 2012.

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List of Abbreviations and symbols

CdSe	Cadmium selenide
RBC's	Red blood cells
NaBH₄	Sodium borohydride
TEM	Transmission electron microscope
HRTEM	High resolution transmission electron microscope
UV	Ultra- violet
PL	Photoluminescence
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetra-acetic acid
TiO₂	Titanium dioxide
TAE	Tris-acetic EDTA
TCA	Trichloro acetic acid
ADA	Acid dextrose anti-coagulant

List of symbols

°C	Degrees Celsius
µg	microgram
ng	nano gram
bp	base pairs
nm	nanometers
ml	microliters
A₂₆₀	Absorbance at 260 nm
A₄₁₅	Absorbance at 415 nm
A₇₀₀	Absorbance at 700 nm

List of research outputs

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Chapter 1- Introduction and literature Review

This chapter provides an introduction to nanotechnology and its emerging counterpart nanotoxicology, providing the link to biomedical applications and highlighting the concern and importance of toxicity screenings, aim, hypothesis, specific objectives and approach for this project.

1.1 Introduction

Nanotechnology is the creation of new materials, devices, and systems through the control of matter on the nanometer-length scale, at the level of atoms and molecules (Sershen *et al.*, 2009). The essence of nanotechnology is the ability to work at these levels to generate nanostructures with fundamentally new molecular organization (Kelly *et al.*, 2003). Nanotechnology has a variety of applications in fields such as optics, electronics, bio-medicine, magnetics, mechanics, catalysis, energy science, physics and engineering amongst others (Pissuwan *et al.*, 2006).

In 1959, Professor Richard P. Feynman, unveiled the revolutionary concept of nanotechnology in his lecture titled, “*There’s Plenty of Room at the Bottom*”, (Kelly *et al.*, 2003), which focused on the idea of fabricating matter of roughly one to hundred nanometers. Scientists have since been able to design nanoparticles that exhibit unique physical and chemical properties. Today, medical and engineering researchers have designed nanomaterials with countless applications in consumer products, image technology, automated and aerospace mechanics, and medical devices (Jain *et al.*, 2006).

By definition nanoparticles are units of matter with at least one dimension between 1-100 nm. Nanostructured materials include carbon nanotubes, metal nanowires, semiconductor quantum dots and other nanoparticles produced from a huge variety of materials (Rao *et al.*,

2002). Nanoparticles are of great scientific interest as they are effectively a bridge between bulk materials and atomic or molecular structures. A bulk material should have constant physical properties regardless of its size. However, nanoparticles exhibit different characteristics as compared to their bulk material (Oberdoster *et al.*, 2005). Two principal factors cause the properties of nano materials to differ significantly from bulk materials: increased relative surface area to volume ratio and quantum effects (Rao *et al.*, 2002). These factors can change or enhance properties such as reactivity, strength and electrical characteristics. As a particle decreases in size, a greater proportion of atoms are found at the surface, as compared to those inside. For example, a particle of size 30 nm has 5% of its atoms on its surface, compared to a particle of size 10 nm, which will have 20% of its atoms on its surface, and even further compared to a particle of size 3 nm, which will have 50% of its atoms at its surface (Oberdoster *et al.*, 2005).

The principle is, by producing the material into smaller particle sizes, the total surface area increases. Thus nanoparticles have a much greater surface area per unit mass compared with larger bulk particles. As growth and catalytic chemical reactions occur at surfaces, this means that a given mass of material in nanoparticulate form will be much more reactive than the same mass of material made up of larger particles.

1.2 The use of nanoparticles in biomedical applications

Nanoparticles offer some attractive possibilities in biomedicine and bioanalysis (Lewinski *et al.*, 2008). They have controllable sizes ranging from a few nanometers up to tens of nanometers, which place them at dimensions that are smaller than or comparable to those of a cell (10 – 100 μm), a virus (20 – 450 nm), a protein (5 – 50 nm) or a gene (2 nm wide and 10 – 100 nm long). This means that they can get access to biological entities of interest. In addition to this, nanoparticles can be synthesized to have tunable sizes.

Of particular interest to this study is CdSe quantum dots or nanoparticles, which are made up of a colloidal nucleus consisting of cadmium and selenium atoms surrounded by one or more surface coatings (Oluwafemi *et al.*, 2008). CdSe nanoparticles exhibit a property known as quantum confinement that results when the electrons in a material are confined to a very small volume. Quantum confinement is size dependent, meaning the properties of CdSe nanoparticles are tunable based on their size (Peng *et al.*, 1997; Chen *et al.*, 2004). These features together with their unique optical and electrical properties facilitate their role in biomedical applications. Since CdSe nanoparticles exhibit size dependent fluorescence, they have the potential, for use in applications such as laser diodes. Using these particles, engineers are able to manufacture laser diodes that cover a large part of the electromagnetic spectrum (Talpin *et al.*, 2003).

Along similar lines, biomedical engineers are developing these materials for use in biomedical imaging applications. Human tissue is permeable to far infra-red light. By injecting appropriately prepared CdSe nanoparticles into injured tissue, it may be possible to image the tissue in those injured areas (Oberdorster *et al.*, 2005; Lewinski *et al.*, 2008).

According to Oberdorster *et al.* (2005), the biological activity of these nanoparticles will depend on physicochemical parameters such as, size distribution, agglomeration state, shape, crystal structure, chemical composition, surface area, surface chemistry, surface charge and porosity. It is these physicochemical characteristics that contribute to the toxicity of nanoparticles. However very limited information is available on exactly how each of these characteristics influence the toxicity of nanoparticles. The use of various nanoparticles in bioapplications has led towards research that seeks to understand their toxicity and confirm their biosafety.

1.3. Nanotoxicology

Toxicology is defined as "the study of the adverse effects of chemical, physical or biological agents on living organisms and the ecosystem" and is based on the 16th century principle that any substance can be toxic if consumed in sufficient quantity (Hoet *et al.*, 2004; Maynard, 2006). Hence, nanotoxicology refers to the toxicological effects of nanoparticles. Today, most developed countries have enacted laws and regulations to control the marketing of drugs, vaccines, food additives, pesticides, industrial chemicals, and other substances of potential toxicological risk. Such regulations often prescribe a specific regime of toxicity testing to generate information that will enable government regulators to determine whether the benefits of a particular substance outweighs its risks to human health and the environment. This process of regulatory risk assessment can be broken down into the following three main phases: hazard identification, exposure assessment and risk characterization. Hazard identification involves the determination of a substance's intrinsic toxicity through the use of various cytotoxicity and genotoxicity tests. These test results are analysed to determine what, if any, adverse effects occur at different exposure levels. Exposure assessment involves the determination of the extent of human and environmental exposure to a toxic substance including the identification of specific populations exposed and durations of exposure. Risk characterization, involves a composite analysis of the hazard and exposure assessment data to arrive at a "real world" estimate of health and ecological risk (Adams *et al.*, 2006).

A toxicity study can be further broken down into a dose metric analysis. Dose metrics are important components of a toxicity assessment which is based on the measurement of dose against a physical metric of particle mass, total surface area of particles or particle numbers for a well characterized material to enable quantitative interpretation of data.

These measurement points are used in establishing the lowest possible concentration of a substance that would induce toxicity and is an essential reference point in toxicity evaluations (Figure 1.1). Dose metrics of nanoparticles are essential components for evaluating toxicity because nanoparticles are large in particle number and total surface area and relatively small in mass (Oberdoster *et al.*, 1995).

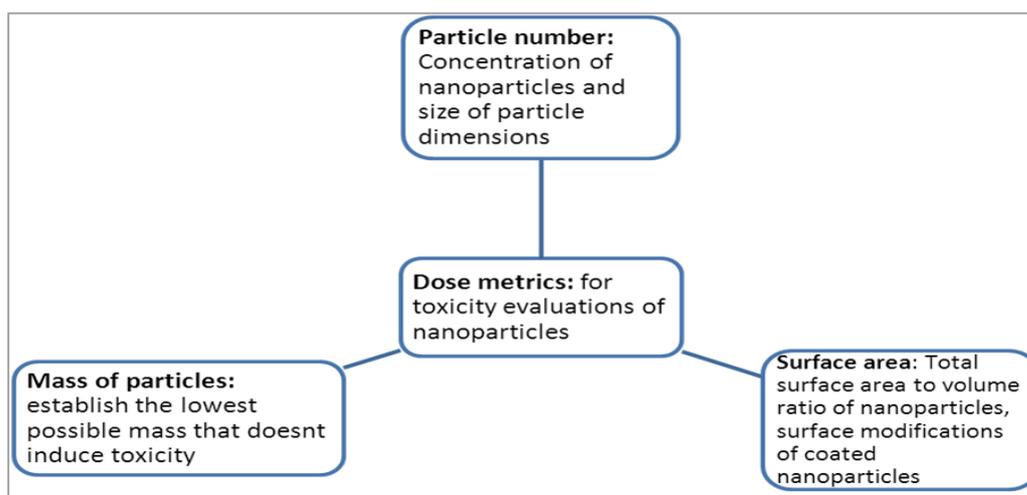


Figure 1.1 Dose metrics involved in toxicological characterization of nano materials

Particle number, surface area and mass of particles are the main features of dose metrics that are used to evaluate toxicity of nanoparticles.

Engineered nanomaterials possess unusual physical and chemical properties and are used in many products available to the public off the shelf. For example, titanium dioxide nanoparticles possess photocatalytic activity and are used as antibacterial coatings and in sunscreens (Oberdoster *et al.*, 2005; Lanone *et al.*, 2009). However to date, there are many contradictory studies with regards to the toxicity of titanium dioxide (Warheit *et al.*, 2003; Lanone *et al.*, 2009). Direct comparisons between these studies are very difficult as the physiochemical properties differ for each particle and the use of different experimental design and conditions (Lewinski *et al.*, 2008, Galloway *et al.*, 2010; Pujalte *et al.*, 2011).

Even though nanotoxicology is a fairly new field of research, there have been many studies where some nanomaterials have already being shown to be toxic. For instance carbon black

printex 90 is a well-known ingredient in rubber, plastics, inks, and paints with an annual production about 10 million tonnes (Ostiguy *et al.*, 2007; Tiede *et al.*, 2009). The toxic effects of Carbon Black have been well described *in vitro* and *in vivo*, making it an excellent benchmark material for nanotoxicity (Waalkes, 2003; Oberdoster *et al.*, 2005; Ostiguy *et al.*, 2007).

Gold nanoparticles are used in different biomedical applications, such as intracellular gene regulation, and drug delivery (Pissuwan *et al.*, 2006), as well as in optical and electronic applications (Kelly *et al.*, 2003). Gold nanoparticles also show great promise in the field of cancer research and diagnosis (Mroz *et al.*, 2008; Uboldi *et al.*, 2009). Findings on the *in vitro* cytotoxicity of gold nanoparticles have showed contradictory results, due to the use of different experimental conditions (Pissuwan *et al.*, 2006; Ostiguy *et al.*, 2007; Uboldi *et al.*, 2009). It is still unknown whether the properties arising from smaller particle sizes or the surface coating of many nanoparticles induces cellular damage and cytotoxicity *in vitro* (Oberdoster *et al.*, 1995; Oberdoster *et al.*, 2005).

The list of actual applications and uses for nanomaterials is already substantial, and will certainly become exponential in the future. It is important to have knowledge about the short term and long term effects of these nanoparticles, especially their effect on humans and the environment. Responsible development of any new materials requires that risks to health and the general environment associated with the development, production, use and disposal of these materials are addressed. This is necessary to protect workers involved in production and use of these materials, the public and the ecosystems within our environment (Kuhlbusch *et al.*, 2011). However, it also helps inform the public debate about the development of these new, potentially beneficial materials.

Assessment of health risks arising from exposure to chemicals or other substances requires understanding of the intrinsic toxicity of the substance, the levels and routes of exposure (by inhalation, by ingestion or through the skin) that may occur and any relationship between exposure and health effects (Oberdoster *et al.*, 2005; Tiede *et al.*, 2009). Concerns about the lack of knowledge and possible risks arising from exposure to nanoparticles exist (Oberdoster *et al.*, 2005; Ostiguy *et al.*, 2007). The fact that studies have shown that some nanoparticles are toxic when others report that they may be safe or less toxic (Oberdoster *et al.*, 2005; Jain *et al.*, 2006; Lewinski *et al.*, 2008; Lanone *et al.*, 2009) is testimony to the need for the development of standardized assays for assessment of nanoparticle safety.

1.4 Potential toxicity of CdSe nanoparticles

Cadmium is a toxic heavy metal, whereas selenium is only toxic at high levels. While it is well-known that bulk CdSe is cytotoxic, it has been suggested that CdSe nanoparticles are cytocompatible and safe for use in whole animal studies (Derfus *et al.*, 2003). This postulate is based in part on the use of protecting groups or surface modifications around the CdSe core of the quantum dot. These coatings have been shown to be protective, but their long-term stability has not been evaluated thoroughly (Derfus *et al.*, 2003). CdSe nanoparticles holds great promise in many fields of science, especially in bioapplications such as fluorescent dyes for cellular imaging (Brus, 1986; Peng *et al.*, 1997; Oluwafemi, 2009), however very little is known about its actual safety and potential toxicity. The possible uptake of CdSe nanoparticles during production through dermal and inhalation exposure raises many safety concerns, especially since cadmium containing compounds were reported to contribute to the carcinogenicity in human cell lines that were exposed, including epidemiological and mechanistic information that indicated a causal relationship between exposure to cadmium and cadmium compounds and human cancer (Waalkes, 2003; Joseph, 2009).

Other recent studies exploring the cytotoxicity of CdSe-core quantum dots using liver cell lines found that these quantum dots were acutely toxic under certain conditions (Derfus *et al.*, 2003). The cytotoxicity correlates with the liberation of free Cd²⁺ ions due to deterioration of the CdSe lattice. These data suggest that quantum dots can be rendered nontoxic initially for *in vivo* use when appropriately coated. However, the research also highlights the need to further explore the long-term stability of the coatings used, both *in vivo* and exposed to environmental conditions (Derfus *et al.*, 2003; Oluwafemi, 2009). Most of the cytotoxicity and genotoxicity research conducted, between 2002 and 2008, using CdSe quantum dots have being well summarized in a review by (Lewinski *et al.*, 2008).

1.5. Chemical synthesis of CdSe nanoparticles

CdSe is a semi-conducting material which has unique optical and electrical properties due to its large surface area to volume ratio and quantum confinement. Due to these features CdSe nanoparticles have gained considerable attention in many biological applications such as bio-imaging, biological labelling and even drug delivery systems. It is essential that CdSe nanoparticles are rendered water soluble if intended for use in biological applications. For the biological applications of nanoparticles, the control of size, shape, size distribution, crystallinity, and composition of the nanoparticles are of great importance because these characteristics determine their chemical and physical features and even affect the compatibility to biomaterials. The morphologic and structural characteristics are mostly controllable during the synthetic processes and strongly dependent on the synthetic methodology employed, strategic synthesis of nanoparticles with desired characters are required for the practical bioapplications of nanoparticles.

A variety of synthetic methodologies for the preparation of nanoparticles within a narrow size distribution are available. There are two distinct approaches for the synthesis and

functionalization of nanoparticles; bottom-up and top-down approaches (Brus, 1986; Chan *et al.*, 1998; Rao *et al.*, 2002) approaches. The bottom-up approach involves conversion of a precursor through a specific reaction such as reduction, decomposition or hydrolysis into nuclei that subsequently grow into mono-dispersed nanoparticles and the top-up approach is based on placing the bulk material into a hot solvent, which is then melted and sheared to produce colloidal spheres. Many very successful preparation methods have been established for the synthesis of high quality CdSe nanocrystals, including the colloidal route, the organometallic precursor route and the single molecule precursor route. This study however, focuses on the more recent one-pot solution based synthesis of CdSe nanoparticles.

1.5.1 Colloidal route to CdSe nanoparticles

The main method of preparation of semiconductor nanoparticles was, until recently, classical colloid chemistry, involving controlled arrested precipitation from colloidal solutions. The colloidal access to nanoparticles is achieved by carrying out a precipitation reaction in a homogenous solution in the presence of stabilizers, which functions in the role of agglomeration prevention and further growth (Jonson *et al.*, 1947; Peng *et al.*, 1997; Mekis *et al.*, 2003; Chen *et al.*, 2004). If the nucleation and growth processes were properly controlled, particles with nanosize dimensions could be reproducibly synthesized. The colloidal growth stability of the crystals can be improved by using solvents with low dielectric constants or by using stabilizers such as styrene/maleic acid copolymer. Work reported by Spenhel *et al.* (1987) and Brus *et al.* (1986) has made significant contributions to this method of preparation especially in the early work on cadmium sulfur nanoparticles. Although this method of synthesis is efficient, some important semi-conductor materials such as cadmium selenide, Gallium Arsenide, and Indium Phosphide cannot be easily synthesized using this method (Green and O'Brien, 1996).

1.5.2 Organometallic route to CdSe nanoparticles

This route, pioneered by Murray *et al.*, (2003), uses a volatile metal alkyl (dimethylcadmium) and a chalcogen source tri-*n*-octylphosphine selenide (TOPSe), dispersed in tri-*n*-octylphosphine (TOP) and injected into hot TOPO (tri-*n*-octylphosphine oxide). Nucleation of CdSe nanoparticles was achieved by the super saturation of the concentrated reagents resulting in the formation of nuclei, followed by slower growth and annealing, consistent with an Ostwald ripening process. The particles produced by this method were very monodispersed ($\pm 5\%$) and crystalline. The capping group or surfactant plays an important role in the nanocrystal growth (Scher *et al.*, 2003). At high temperatures (200–400 °C), the surfactant or capping molecules are dynamically adsorbed to the surface of the growing crystal, thereby stabilizing the particles in solution and mediating their growth. The surfactants can also be exchanged with other organic molecules with different functional groups and polarity to further modify its features.

1.5.3 Single-molecule precursor route to CdSe nanoparticles

Molecular precursor methods have developed in recent years as the most promising chemical routes to fabricate quantum dots for the semiconductor industry. The use of single-molecule precursors in which the metal-chalcogenide bond is available has proven to be a very efficient route to high-quality nanoparticles. Work reported by Trindade and O'Brien, (1997) investigated cadmium dithio- and diselenocarbamate complexes as precursors for the preparation of TOPO-capped II/VI semiconductor nanoparticles. The molecular precursors are decomposed in a coordinating solvent at relatively high temperatures, hence initiating the crystallinity and encapsulating or coating the dots surfaces. The formation of the nanoparticles is consistent with the mechanisms reported for colloids by Jonson *et al.* (1947). During this route of synthesis, the decomposition of the precursor drives the formation of the nanoparticles.

1.5.4 Synthesis of CdSe nanoparticles using a one-pot solution based route

Over the past decades research scientists have turned their focus onto developing “green” routes of synthesizing nanoparticles. These routes are time, cost and energy preserving by incorporating the use of reagents that are more stable. Many of the previously mentioned methods of synthesizing quantum dots require high temperatures, complex toxic reagents and expensive resources to proceed (Mekis *et al.*, 2003). The concern was to develop methods that are safe and environmentally friendly. Different kinds of safe, common and inexpensive compounds were proven to be ideal precursors for the synthesis of high quality CdSe nanoparticles (Chen *et al.*, 2004). In 2008, Oluwafemi *et al.*, reported a novel one-pot route for the synthesis of water soluble and biocompatible CdSe nanoparticles. This route does not require special reaction conditions and additional stabilizers and is both inexpensive and time conserving. Work done by Oluwafemi, (2009) highlights the need for creating “green”, reproducible, low cost techniques to create CdSe nanoparticles and surface coating agents. This idea was demonstrated by using starch, which is common, easily available and biodegradable, as a capping agent for the synthesis of CdSe quantum dots (Oluwafemi, 2008).

1.6 Interaction of nanoparticles with biological material

1.6.1 Interaction of nanoparticles with DNA molecules

It seems probable that life itself began its evolution with nucleic acids, as they are the only biological molecules that carry the remarkable potential for self-duplication. Nucleic acids are found within a nucleus, they serve as the repositories and transmitters of genetic information for every cell. The blueprint for an organism is encoded in deoxyribonucleic acid (DNA).

During the production and applications of various nanoparticles, different exposure routes, such as, dermal, oral, inhalation and injection, inevitably lead the nanoparticles into the blood circulatory system where they will be transported to the organs, tissues and cells. Once the nanoparticles enter the cell, they are capable of interacting with the cell membrane, cytoplasmic environment, mitochondria, cellular organelles and even the nucleus and DNA molecules. DNA plays a key role in the cell cycle, and in the development of organisms, hence its interaction with nanoparticles must be thoroughly researched. Many studies have shown that titanium oxide nanoparticles have adverse effects on DNA, gene expression and protein synthesis (Galloway *et al.*, 2009). A study by Mroz *et al.* (2008) observed a link between carcinogenesis pathways and nanoparticle driven DNA damage. It was reported that carbon black nanoparticles and urban dust particles caused both single and double stranded DNA breaks and significantly altered the cell cycle kinetics (Mroz *et al.*, 2008). Some studies place focus on nanoparticles that cause reactive oxygen species (ROS) that cause oxidative damage to DNA molecules. Genotoxicity studies on the effect of CdSe nanoparticles on DNA, using various cell lines and experimental conditions have been reported. The results are well documented in reviews by Ostiguy *et al.* (2007) and Lewinski *et al.* (2008).

1.6.2 Interaction of nanoparticles with Red blood cells

The main function of RBCs is to carry oxygen to all cells, tissues and organs within the body and then simultaneously remove carbon dioxide gaseous waste products from all cells within the body. This role of gaseous transport within the body must maintain its homeostatic balance. Within the RBC is a protein called hemoglobin. This protein is made up of four subunits, each containing an iron molecule which is responsible for binding to the gases during oxygen delivery and carbon dioxide excretion. Once the blood circulatory system is exposed to CdSe nanoparticles, interaction of RBCs and nanoparticles become inevitable. Understanding the interaction of nanoparticles with RBCs is very important, due to the fact

that there is a potential risk of the nanoparticles disrupting the integrity and function of the RBCs (Barbara *et al.*, 2008). Many studies on the interaction of various nanoparticles with RBCs have been reported. A study reported by Barbara *et al.* (2006), focused on how nanoparticles penetrate the RBC membranes. It was found that the surface charge and material of the nanoparticle did not influence their entry into the cell. These results showed that nanoparticles gained access to the RBC membrane by absorption mechanisms (Barbara *et al.*, 2006).

Silver nanoparticles interacted with bovine haemoglobin (BHb) causing changes in the absorption spectrum of the haemoglobin. Circular dichroism reports demonstrated conformational changes of BHb in the presence of silver nanoparticles. This effect was reported to increase as the thermodynamic parameters increased (Zolghadri *et al.*, 2009). The adverse effects of silver, gold and platinum nanoparticles on RBCs was reported by Asharani *et al.* (2009), gold and platinum nanoparticles were found to be haemocompatible, whilst silver nanoparticle resulted in various adverse effects to the RBC shape, causing cell lysis and agglutination. The products from the RBC destruction such reactive oxygen species were also able to induce DNA damage to other nucleated cells (Asharani *et al.*, 2009).

The effect of various sizes of mesoporous silica nanoparticles (MSNs) on human RBCs was investigated to understand what affect their size and surface properties would have on the RBC membrane. The study reported that small nanoparticles of MSNs of 100 nm and less were absorbed into the cell membrane without disturbing the membrane morphology, whilst larger particles of MSNs of 600 nm had caused membrane deformation, internalization of the particles within the RBCs and eventual hemolysis. Thus there is evidence that the interaction of CdSe nanoparticles with RBCs may affect haemoglobin function (Yannan *et al.*, 2011).

1.6.3 Interaction of nanoparticles with blood platelets

Blood is composed of many components, amongst them, platelets or thrombocytes. Platelets play an important role during blood clotting. Clotting is a natural process that allows the blood to thicken and solidify thereby preventing excessive bleeding. If the body did not have this unique ability to clot, then people would bleed to death after even a minor cut. Blood coagulation is highly conserved throughout biology. Figure 1.2 illustrates the potential pathways during nanoparticle exposure which lead to the blood circulatory system, providing an increased potential for blood platelet and nanoparticle interaction.

Nanoparticle induced platelet aggregation is an important area of research in nanotoxicology. A study reported by Radomski *et al*, (2005) examined the effects of both engineered and combustion derived carbon nanoparticles on human platelet aggregation *in vitro* and rat vascular thrombosis *in vivo*. The effects of multiple wall nanotubes (MWNT), single wall nanotubes (SWNT), C60 fullerenes (C60CS) and mixed carbon nanotubes (MCN) on blood clotting were investigated. All the nanoparticles except C60CS stimulated platelet aggregation and accelerated the rate of vascular thrombosis in rat ceratoid arteries. These observations are important for the pharmacological use of carbon nanoparticles. A clear understanding of nanoparticle interaction with all biological entities and systems are of key importance in generating and optimizing the related biomedical applications of some nano materials. Evidence from previous studies indicates the need for assessing the interaction of CdSe nanoparticles on blood platelet function (Radomski *et al*, 2005).

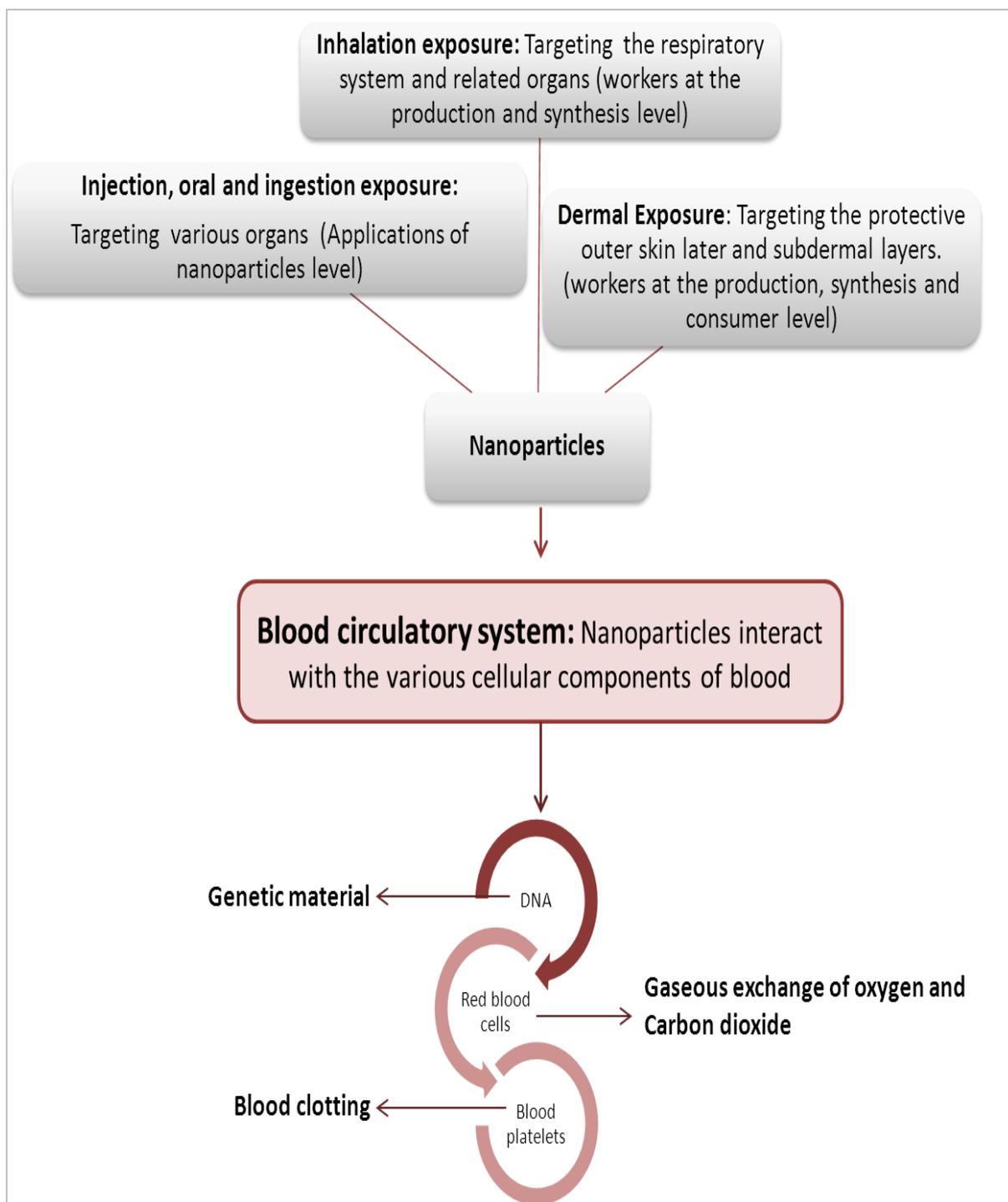


Figure 1.2 Nanoparticles exposure routes

Exposure routes lead nanoparticles into the blood circulatory system, resulting in the potential interaction of nanoparticles with blood cellular components.

1.7 Problem statement

Nanotechnology is steadily finding its place in all aspects of the consumer industry, science and engineering. Everything from cosmetics, appliances, hardware, medical treatment and pharmaceuticals are all being developed into a better performance product simply by incorporating the use of specific nanoparticles. The increased production, use and decomposition of nanoparticles raise huge concern in the area of nanotoxicology especially focusing on human and environmental exposure. It is, therefore, extremely necessary that all nanomaterials undergo a standardized toxicology assessment before been incorporated into products and applications. Furthermore, the safety of nanoparticles must be established so that not only the consumers of the product but also the workers at the synthesis, production and applications level are protected.

Many laws governing the ethics, use and need for nanotechnology are yet to be passed, especially here in South Africa. It is, therefore, very difficult to assess these particles in terms of a defined toxicity protocol. It is very important to note the fact that nanoparticles are so diverse in toxicity that tests for their safety need to be standardized. According to the research gathered thus far, a broad overview to define their properties stems from a combination of the following factors; decreased particle size and increased surface area to volume ratio and quantum physics confinement properties resulting in their unpredictability also leading to high reactivity.

1.8 Hypothesis

The use and applications of various nanoparticles is growing exponentially, leading to great technological advancements. Previous research does reveal that many nanoparticles do in fact induce toxicity. CdSe quantum dots hold special promise in the medical, biochemical and chemical applications. The aim of this study is to assess the *in vitro* toxicity of CdSe quantum dots on the function of red blood cells, blood platelets and stability of DNA. The application of CdSe quantum dots in human systems has the potential to influence metabolic processes, including cell cycle events. Modifications to the concentration of capping agent was used to create a coating layer that would stabilize the CdSe core and thereby induce less toxicity as the molar ratios between the capping agent and cadmium salt increased.

1.9 Specific objectives and methodological scope of this study

Specific objectives	Approach
1. Synthesis of water soluble, biocompatible CdSe nanoparticles	Synthesis of water soluble organically capped CdSe nanoparticles was done using a one-pot solution based route. This was conducted in the Department of Chemistry, University of Zululand
2.Characterization of CdSe nanoparticles according to the physicochemical parameters	Physicochemical characterization of CdSe nanoparticles according to size distribution, agglomeration state, shape, crystal structure and chemical composition using TEM, HRTEM (was done at University of Kwa-Zulu Natal); UV and PL analysis (University of Zululand).
3. <i>In vitro</i> toxicity studies	
3.1 Evaluation of the <i>in vitro</i> effect of CdSe nanoparticles on DNA structure and stability	DNA damage was assessed in the presence of CdSe nanoparticles based on analysis of agarose gel electrophoresis and spectrophotometry techniques. Department of Biochemistry and Microbiology, University of Zululand.
3.2. To determine the <i>in vitro</i> effects of CdSe nanoparticles on the function of red blood cells	- Reducing power and chelating activity was done using <i>in vitro</i> assays according to prescribed methods by Oyaizu. (1986); Decker and Welch, (1990).
3.3. To characterize the <i>in vitro</i> effects of CdSe nanoparticles on blood platelet function	-Platelet aggregation assay, based on method by Mekhfi <i>et al.</i> (2004), was done in the Department of Biochemistry and Microbiology, University of Zululand.

Chapter 2- Methodology

This chapter provides a brief description of all methods, reagents and equipment used for the synthesis, characterization and in vitro biochemical analysis of water soluble, cysteine capped CdSe nanoparticles.

2.1 Equipment:

Rotary Evaporator, Scale, pH meter, Spectrophotometer, Autoclave, Dissecting Kit, GRR Centrifuge, Microcentrifuge, Perkin-Elmer Lambda 20 UV-vis spectrophotometer, Biotek ELx 808 UI plate reader, Perkin-Elmer LS 55 luminescence spectrometer, JEOL JEM 3010 URP high resolution transmission electron microscope, Philips CM120 BIOTWIW transmission electron microscope.

2.2 Laboratory Animals

Ethical clearance for the use of animals in this study was obtained from the Research Ethics Committee of University of Zululand. The blood platelets used in this study were obtained from Sprague-Dawley Rats that are kept in the animal house in the Department of Biochemistry and Microbiology, University of Zululand.

2.3 Reagents

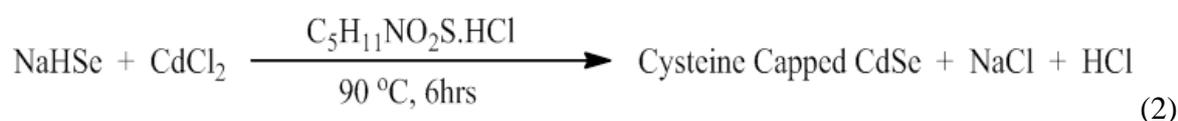
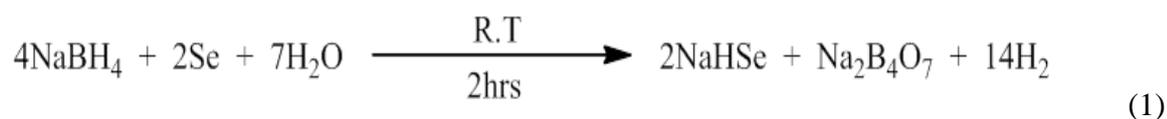
For a list of purchased reagents see appendix A, section A1. For a list of preparation of reagents see appendix A, section A2.

2.4 Methodology

2.4.1 Synthesis of cysteine capped CdSe nanoparticles

The synthesis of water soluble cysteine capped CdSe nanoparticles was prepared by the prescribed procedures as recorded by Oluwafemi *et al.* (2008). The synthesis process is broken down into three distinct phases; the reduction, capping and the purification phase.

Selenium powder (0.32 mmol) was mixed with 30 mL of deionized water in a three necked round bottom at room temperature. Sodium borohydride (0.81 mmol) was added to the reaction mixture under inert conditions. After 2 hours 20 mL of 3.2×10^{-4} M CdCl₂ (cadmium salt) and L-cysteine ethyl ester hydrochloride (capping agent) was added to the reaction mixture in molar ratios of 1:10, 1:20 and 1:30. The reaction mixture was then heated at 90 °C for 6 hours under nitrogen gas. The cysteine capped CdSe particles were separated from the mixture by filtration techniques and the sample was then concentrated by rotary evaporation. A brownish red to orange material was precipitated with acetone. This product was centrifuged to give the pellet that was then dried under vacuum and weighed out to give a material which is readily dispersible in water.



Scheme 1. Equations for the formation of Cysteine capped cadmium selenide nanoparticles (RT= Room temperature)

2.4.2 Characterization of CdSe nanoparticles using UV-vis absorption spectroscopy (UV)

A Perkin-Elmer Lambda 20 UV-vis spectrophotometer was used to carry out optical measurements in the 200-1100 nm wavelength range at room temperature. Samples were placed in quartz cuvettes (1cm path length) and the absorbance was recorded.

2.4.3 Characterization of CdSe nanoparticles using photoluminescence spectroscopy (PL)

At Room temperature photoluminescence (PL) spectra was recorded on a Perkin-Elmer LS 55 luminescence spectrometer with xenon lamp over range of 400-800 nm. The samples were

placed in quartz cuvettes (1cm path length) and the excitation peaks were analysed and recorded.

2.4.4 Characterization of CdSe nanoparticles using transmission electron microscopy (TEM)

Samples for analysis from all three batches (ratios of 1:10, 1:20 and 1:30) were prepared by putting an aliquot solution of water soluble CdSe nanocrystalline material onto an amorphous carbon substrate supported on a copper grid and then allowing the solvent to evaporate at room temperature. A Philips CM120 BIOTWIW transmission electroscope sample viewed at 80K was used for TEM analysis. Size distribution of the sample particles were calculated from the images obtained using TEM analysis.

2.4.5 Characterization of CdSe nanoparticles using high resolution transmission electron microscopy (HRTEM)

A JOEL JEM 3010 URP high resolution transmission electron microscope operated at 300 kV was used for high resolution analysis of a small aliquot of the CdSe samples. Images were recorded using a 1024 x 1024 CCD camera.

2.4.6 Purification of pGEMT Easy plasmid DNA

A 5 mL culture of *E. coli* XL1 Blue cells, transformed with the pGEMT Easy plasmid (supplied by Invitrogen) was centrifuged at 160 rpm. The resultant pellet fraction was used to purify the plasmid DNA as described below.

Qiagen purification kit was used in the following procedures:

1. Buffer P1 (0.3 mL) was added to the bacterial pellet.
2. Buffer P2 (0.3 mL) was added to reaction mixture from step 1, mixed thoroughly by vigorously inverting the sealed tube 4-6 times, and incubated at room temperature for 5 min.

3. Chilled Buffer P3 (0.3 mL) was added to reaction mixture from step 2, mixed immediately and thoroughly by vigorously inverting the sealed tube 4-6 times, and incubated on ice for 5 min.
4. The reaction mixture was centrifuged at maximum speed in a microcentrifuge for 10 minutes, followed by the removal of the supernatant containing Plasmid DNA promptly.
5. Equilibrate A QIAGEN-tip 20 was equilibrated by applying 1 mL Buffer QBT, and the column was allowed to empty by gravity flow.
6. The supernatant from step 4 was applied to the QIAGEN-tip 20 and allowed to enter the resin by gravity flow.
7. The QIAGEN-tip 20 by was washed with 2 x 2 mL Buffer QC.
8. DNA was eluted with 0.8 mL Buffer QF.
9. DNA was precipitated by adding 0.7 volumes (0.56 mL per 0.8 mL of elution volume) of room temperature isopropanol to the eluted DNA. Followed by immediate mixing and centrifugation at less than 10, 000 rpm for 30 min in a microcentrifuge. The supernatant was carefully decanted.
10. The DNA pellet was washed with 1 mL of 70 % ethanol and centrifuged at 10,000 rpm for 10 min. the supernatant was carefully decanted without disturbing the pellet.
11. The pellet was air dried for 5-10 min, and re-dissolved in sterile distilled water or suitable buffer.

2.4.7 Quantification of pGEMT Easy plasmid DNA

DNA (10 µL) was added to 990 µl of distilled water and placed into a 1mL quartz cuvette (Dilution factor 1:100). The spectrophotometer was switched on and left to warm up for 20 min, thereafter, the sample was read at 260 nm. 1 absorbance unit= 50 µg/µl of double stranded plasmid DNA.

2.4.8 Confirmation of the integrity of plasmid DNA using restriction analysis

The integrity of the transformed batch of DNA was confirmed using diagnostic analysis with restriction digest enzymes *Pvu* I and *Nco* I which cuts the plasmid at 2878 and 38 base pairs, respectively. Four test tubes were set up, each containing 4 µl of DNA, 2 µl of corresponding buffers, 16 µl of sterile distilled water. Test tube 1 containing an aliquot of DNA was not treated with restriction enzymes. Tube 2 was treated with 0.5 µl of *Pvu* I restriction digest enzyme and tube 3 was treated with 0.5 µl of *Nco* I restriction digest enzyme. Tube 4 was treated with 0.25 µl of *Pvu* I and 0.25 µl of *Nco* I. All four tubes were incubated at 37 °C for 3 hours. Thereafter the reaction was stopped by the addition of 4 µl of loading buffer. The products of this reaction were analyzed by agarose gel electrophoresis in order to estimate the sizes of the cut fragments of DNA.

2.4.9 Agarose gel electrophoresis and spectrophotometric analysis of DNA exposed to nanoparticles

DNA plasmid pGEMT Easy was exposed to cysteine capped CdSe nanoparticles from different batch ratios (1:10, 1:20 and 1:30) and of different concentrations (200, 600 and 1000 µg/mL). 6 µl of CdSe nanoparticles, 2 µl of pGEMT Easy DNA, 8 µl of sterile distilled water and 4 µl of phosphate buffer was added to each micro tube and incubated for 24 hours at 37 °C. After exposure, an aliquot of 5 µl of sample was taken and diluted up to 1 mL for spectrophotometric analysis at 260 nm. 5 µl of loading buffer was added to each remaining sample to stop the reaction, before running the samples on a 0.8 % agarose gel at 70 V for 1

hour 30 minutes (Refer to appendix A, section A2, for details on preparation of agarose gel)
All gels were viewed and imaged on the BIO- RAD gel doc system.

2.4.10 Assessment of the reducing power of CdSe nanoparticles on iron

Test tubes were set up in triplicate with 10 µg/mL of CdSe nanoparticles from different batch ratios (1:10, 1:20 and 1:30) and different concentrations (200, 400, 600, 800 and 1000 µg/mL), 2.5 mL of phosphate buffer and 2.5 mL of 1 % potassium ferricyanide were added in each corresponding test tube and incubated at 50°C for 20 minutes, followed by the addition of 2.5 mL of 10 % trichloroacetic acid and left standing for a further 5- 10 minutes before being centrifuged at 1000 rpm for 10 minutes. Thereafter 2.5 mL of supernatant was added to 2.5 mL of distilled water and 0.5 mL of 0.1 % ferric chloride. The absorbance was read at 700 nm.

2.4.11 Assessment of the chelation activity of CdSe nanoparticles on iron

Test tubes were set up in triplicate. Deionised water (3.75 mL), FeCl₂ (0.1 mL) and ferrozine (0.2 L) was added to 10 µg/mL of CdSe nanoparticles from different batch ratios (1:10, 1:20 and 1:30) and different concentrations (200, 400, 600, 800 and 1000 µg/mL) and left to stand for 10 minutes. Absorbance was read at 562 nm and the % chelating activity in relation to nanoparticle concentration was calculated using the following formula.

$$\% \text{ Chelating activity} = 1 - \frac{\text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

2.4.12 Assessment of the interaction of CdSe nanoparticles with blood platelets

Diethyl ether was used to anaesthetize the rats, immediately after the rats were dissected and blood was collected from the abdominal aorta. The collected blood was mixed with acid dextrose anti-coagulant (5:1 v/v). The platelets were obtained by a series of centrifugation and washing of the blood. The blood was centrifuged at 1200 rpm for 15 min, thereafter, at 2200 rpm for 2min before the supernatant was collected and centrifuged at 3200 rpm for 15

min. The sediment containing the platelets was resuspended in 5 mL of washing buffer and centrifuged at 300 rpm for 15 min. The supernatant was discarded and platelets were suspended in 10 mL resuspending buffer. Calcium chloride was added to the prepared platelets in the following ratio (0.4 mL platelet solution: 10 μ l of CaCl₂). A platelet activating enzyme, Thrombin was used as a positive control and Heparin was used as a negative control in this experiment. 150 μ l of platelets and 150 μ l of CdSe nanoparticles of different concentrations and different batch ratios (ranging from 200, 400, 600, 800 and 1000 μ l/mL) were added in each well of the micro plate. These treated samples were pre-incubated at 37°C for 5 minutes. The reaction was monitored at 415 nm for 20 min at 30 second intervals with the Biotek plate reader using Gen5 software. All methods were repeated in triplicate and the results were represented as mean \pm SEM (standard error of the mean). Students t-test was used to analyze statistical difference between controls and CdSe nanoparticle treated samples.

Chapter 3- Results

This chapter highlights the results and findings observed in this study.

3.1 The synthesis of cysteine capped Cadmium selenide nanoparticles

Water soluble cysteine capped CdSe nanoparticles were successfully prepared using the one-pot solution based route (Oluwafemi *et al.*, 2009). The addition of increasing molar ratios of cadmium salt to capping agent was used in this method, with the idea of creating a more stable or saturated capping agent layer that surrounds the quantum dot core. Molar ratios of 1:10, 1:20 and 1:30 were used. A colour change was observed as the molar ratios between cadmium chloride and cysteine increased. The batch ratio of 1:10 showed a light golden yellow colour, batch ratio 1:20 showed a darker orange colour and batch ratio 1:30 showed a dark red colour. The results for the synthesis of CdSe nanoparticles using different ratios are shown in Figure 3.1

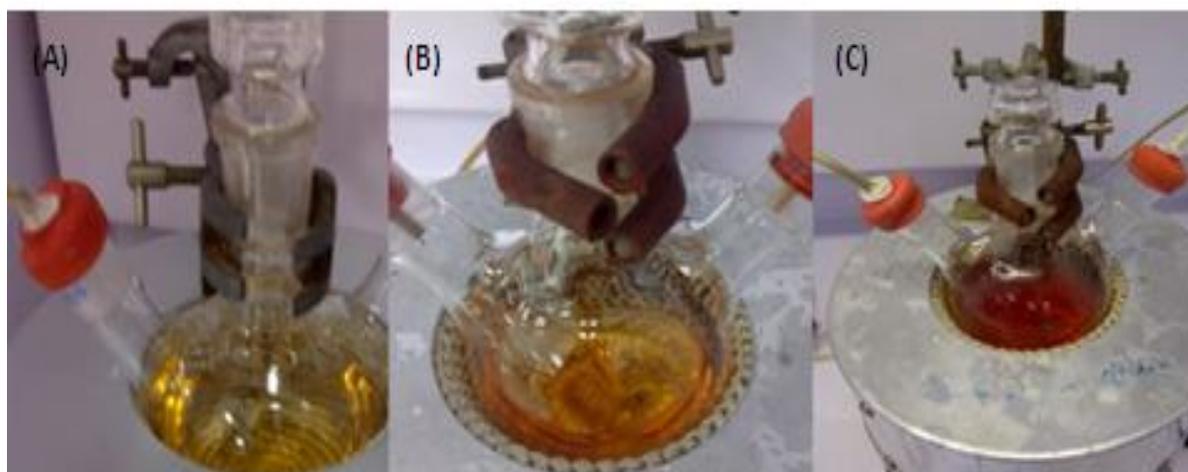


Figure 3.1 CdSe nanoparticles generated using various molar ratios of cadmium salt and coating agent. (A) Represents CdSe nanoparticles of sample batch ratio 1:10, note golden yellow colour. (B) Represents CdSe nanoparticles of sample batch ratio 1:20, note orange colour. (C) Represents CdSe nanoparticles of sample batch ratio 1:30, note dark red colour.

3.2 Characterization of CdSe nanoparticles

The prepared CdSe nanoparticles were characterized using Transmission Electron Microscopy (TEM) and High Resolution Transmission Electron Microscopy (HRTEM) techniques. Ultra-violet spectroscopy (UV) and Photoluminescence (PL) spectrophotometry

techniques were used to confirm the optical properties of the CdSe nanoparticles. UV-vis absorption spectroscopy and fluorescence spectroscopy techniques were performed simultaneously. PL spectroscopy studies transitions from the excited state to the ground state whereas UV absorption measures transitions from the ground state to the excited state. All the batches of CdSe samples had similar optical properties. Upon excitation, CdSe nanoparticles emit in the blue region with an emission maximum of 410 nm for each sample. The UV-vis absorption spectrum of aqueous solutions of CdSe nanoparticles showed an excitonic peak at 290 nm (Table 3.1). The increase in ratios of the cadmium salt and capping agent, during the synthesis of CdSe nanoparticles did not affect their optical properties; furthermore, these optical characteristics were consistent with the results reported by Oluwafemi *et al.* (2009) Table 3.1 shows the optical data for each batch ratio of cysteine capped CdSe nanoparticles emitting at the same range.

The band gap is defined as the energy gap between the valency and conduction band of a nanoparticle. The band gap is calculated using analysis of the UV- absorption peaks. As the nanoparticles get smaller, the band gap increases. This increase in band gap requires more energy to excite these nanoparticles, thereby releasing higher energies in fluorescence once the particle returns to its ground state. The band gap contributes to the optical characteristics of the nanoparticles. For graphical representations of the UV and PL characterizations (refer to Appendix B, section B1 and B2). The results shown in Table 3.1 demonstrate the concept described by Oluwafemi *et al.* (2009) which reports that cysteine is able to coat the CdSe surface without altering the optical features of CdSe nanoparticles.

Table 3.1 Optical characterization of CdSe nanoparticles

Batch ratio of CdSe nanoparticles	UV-vis absorption peak	Band gap	Photoluminescence emission maxima
1:10	290 nm	300 nm	410 nm
1:20	290 nm	300 nm	410 nm
1:30	290 nm	303, 3 nm	410 nm

The morphology of CdSe nanoparticles was studied using TEM and HRTEM techniques. These techniques allow a particle to be characterized according to the various physicochemical properties as described by Oberdoster *et al.* (2005). Figure 3.2 shows the TEM images of the cysteine capped CdSe nanoparticles. The TEM results showed that these nanoparticles appeared to be spherically shaped and monodispersed, with an average particle size of $15 \pm 2,12$ nm. The nanoparticles produced showed similar characteristics across each batch ratio. HRTEM sample analysis was used to confirm the crystalline nature of the particles (Figure 3.3). Well defined lattice fringes were observed. The interplanar distance, could be calculated from these images to provide detailed analysis of the lattice plane features observed in CdSe nanocrystals. After the synthesis and characterization of cysteine capped CdSe nanoparticles, the particles were purified using 0.2 μ m micro-filtration techniques and the concentration of the yields on average was calculated to ± 3000 ug. All methods were repeated in triplicate and the results were represented as mean \pm SEM.

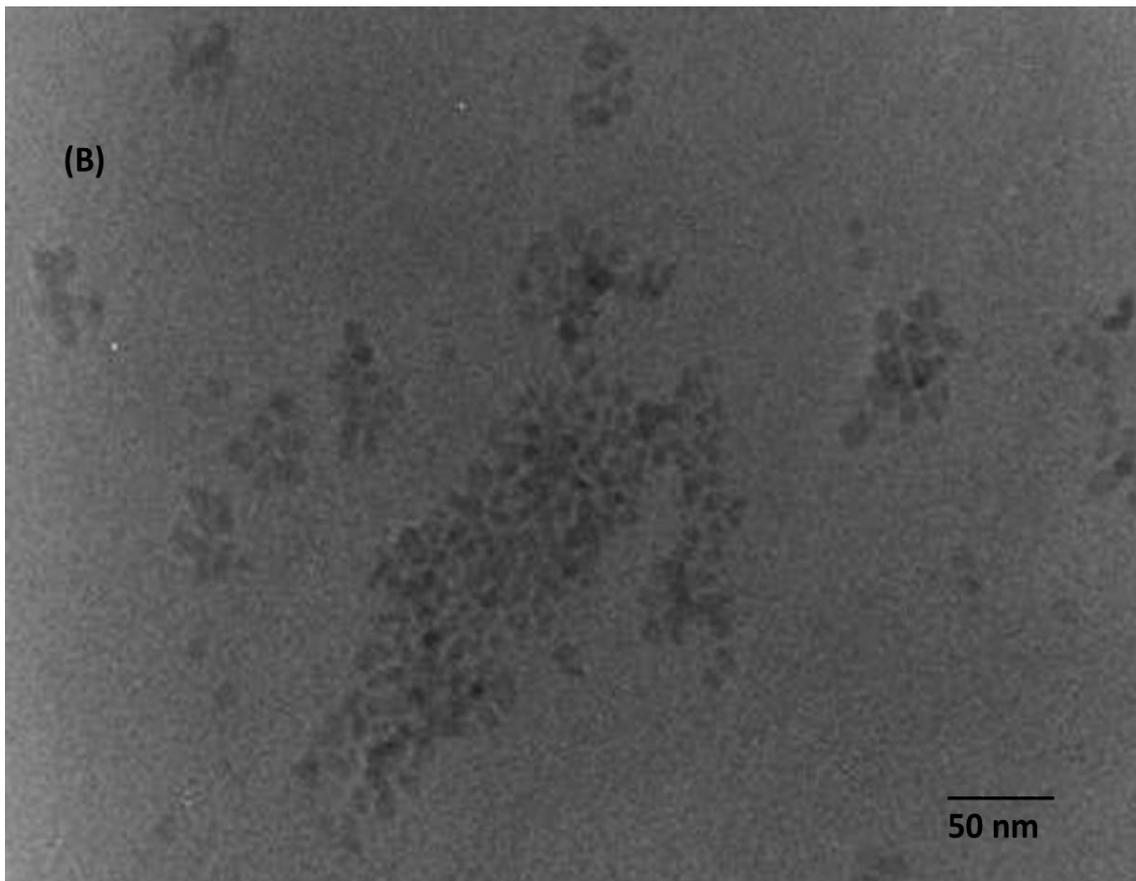
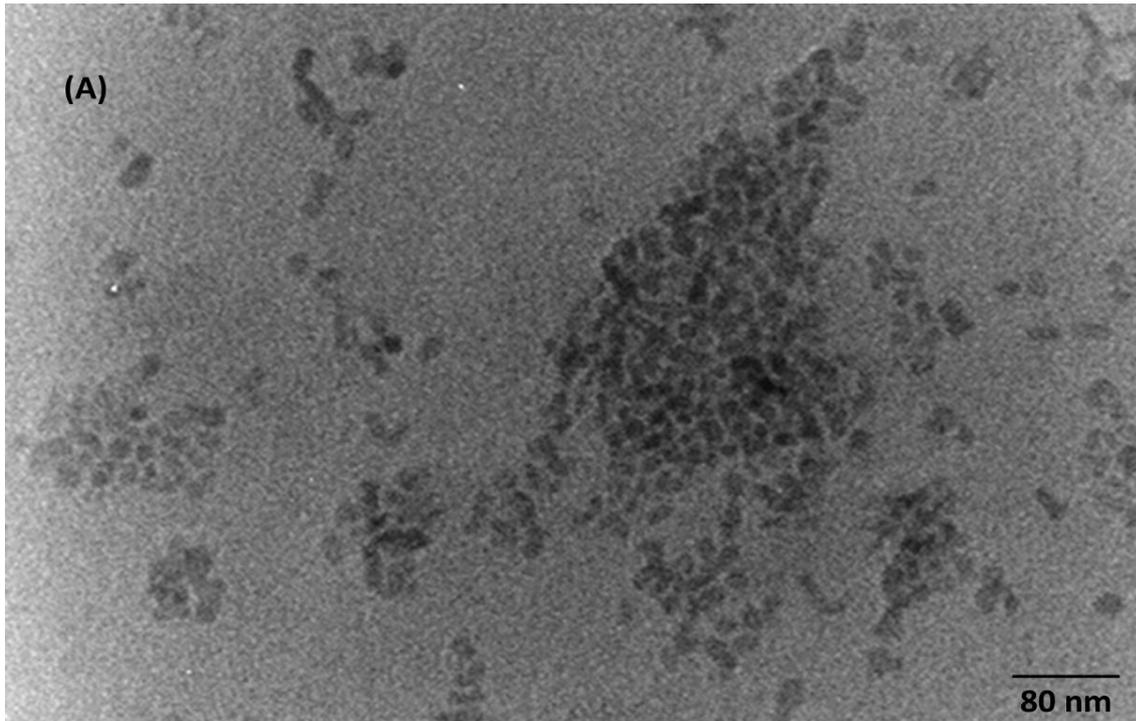


Figure 3.2 TEM image analysis of CdSe nanoparticles. (A) Shows mono dispersed, spherically shaped CdSe nanoparticles. Scale bar used was 80 nm (B) Shows CdSe particles that clump together to result in agglomeration of particles. Scale bar used was 50 nm.

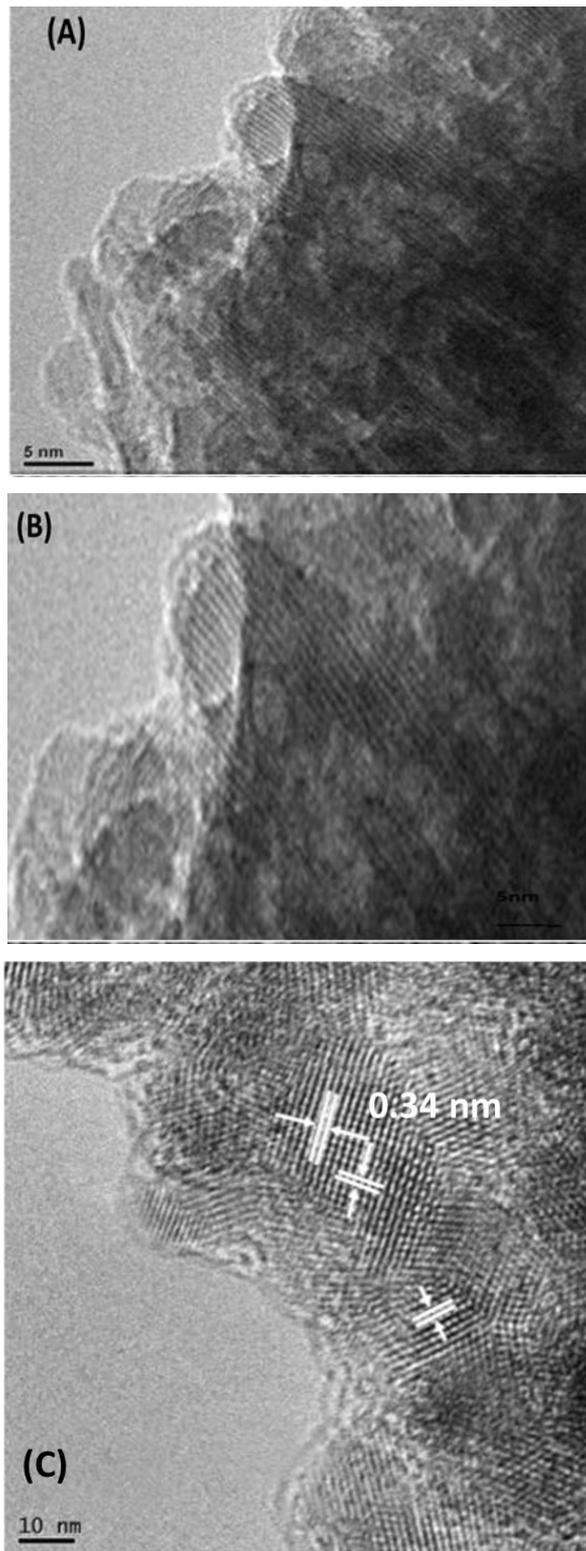


Figure 3.3 HRTEM characterizations of CdSe nanoparticles. (A) HRTEM images from batch ratio 1:10 note the overlapping crystal lattices from particles that are overlapping each other. (B) The presence of clearly defined lattice fringes to confirm the crystallinity of the CdSe nanoparticles. (C) Shows the interplanar distance of 0.34 nm which is indexed to the (111) plane of cubic CdSe.

3.3 CdSe nanoparticles cause DNA damage

This study sought to investigate the effect of CdSe nanoparticles on DNA stability using *in vitro* techniques of agarose gel electrophoresis and spectrophotometry. Plasmid DNA, pGEM T Easy was transformed using the Qiagen midi kit. The DNA was then quantified according to standard protocols and the concentration was calculated at 2200 ng/mL. Restriction digest enzymes were used to confirm the integrity of the transformed DNA plasmid. pGEM T Easy plasmid is 3016 base pairs long and the sites at which these restriction enzymes cut the plasmid is highly specific. Restriction digest enzyme *Pvu* I cuts the plasmid DNA at 2878 base pairs or at 1782 base pairs. *Nco* I cuts the plasmid at 38 base pairs (Figure 3.4).

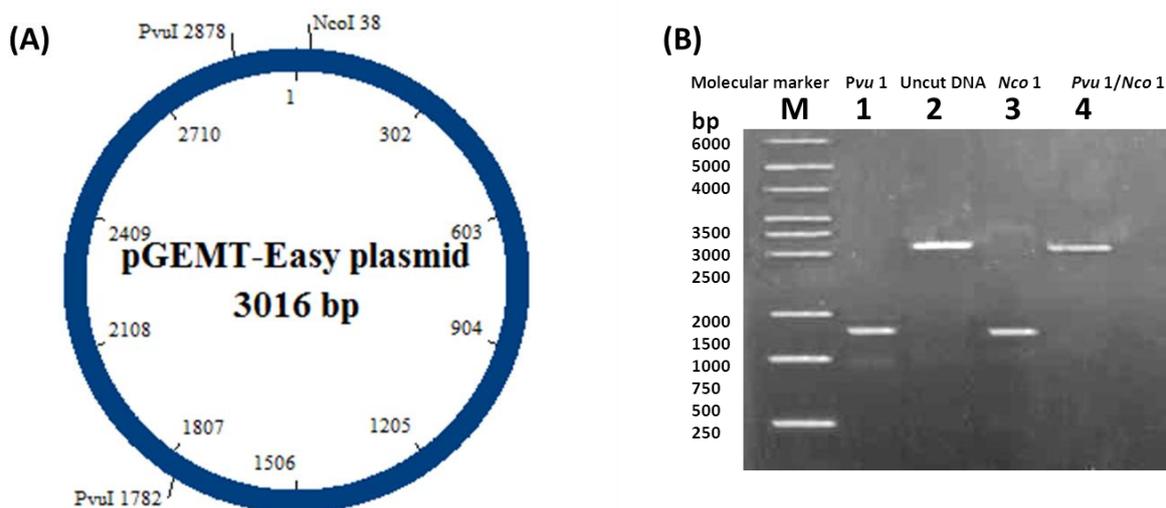


Figure 3.4 Restriction digest analysis of DNA plasmid pGEMT Easy.

(A) Restriction map of pGEMT Easy (B) Restriction digest results obtained from Ethidium bromide stain of 0.8 % agarose gel. Lane M: the molecular marker which is used to estimate fragment sizes. Lane 1: pGEMT Easy plasmid digested with *Pvu* I. Lane 2: Uncut plasmid estimated at above 3000 base pairs. Lane 3: pGEMT Easy plasmid digested with *Nco* I. Lane 4: pGEMT Easy digested with equal amounts of *Pvu* I and *Nco* I

The transformed plasmid DNA, pGEMT Easy was exposed to different concentrations of CdSe nanoparticles from different batch ratios to assess the *in vitro* interaction and effect of these nanoparticles on DNA structure and stability. The concentrations of CdSe nanoparticles

used in this experiment were 200, 600 and 1000 $\mu\text{g}/\text{mL}$, for each batch ratio. The study was conducted at 37 $^{\circ}\text{C}$ and at pH 6.6 .After 24 hours, agarose gel electrophoresis was conducted on the DNA samples. The concentration of agarose in the gel was 0.8 % and the voltage used was 70 V. Figure 3.5 shows the results observed for the *in vitro* interaction of DNA and CdSe nanoparticles. The single band of DNA observed in the negative control, marked as supercoiled form, lane N, shows that under physiological conditions the DNA plasmid that was untreated remained in a supercoiled form. Lane P, the positive control (titanium dioxide) shows two bands of DNA, the first band marked as supercoiled form, indicates the supercoiled covalently closed circular plasmid estimated at just above 3000 bp, the second DNA band, marked as linearized form indicates a larger size of linearized form of DNA, this conformational change from supercoiled to relaxed linear form observed in the positive control represents DNA damage.

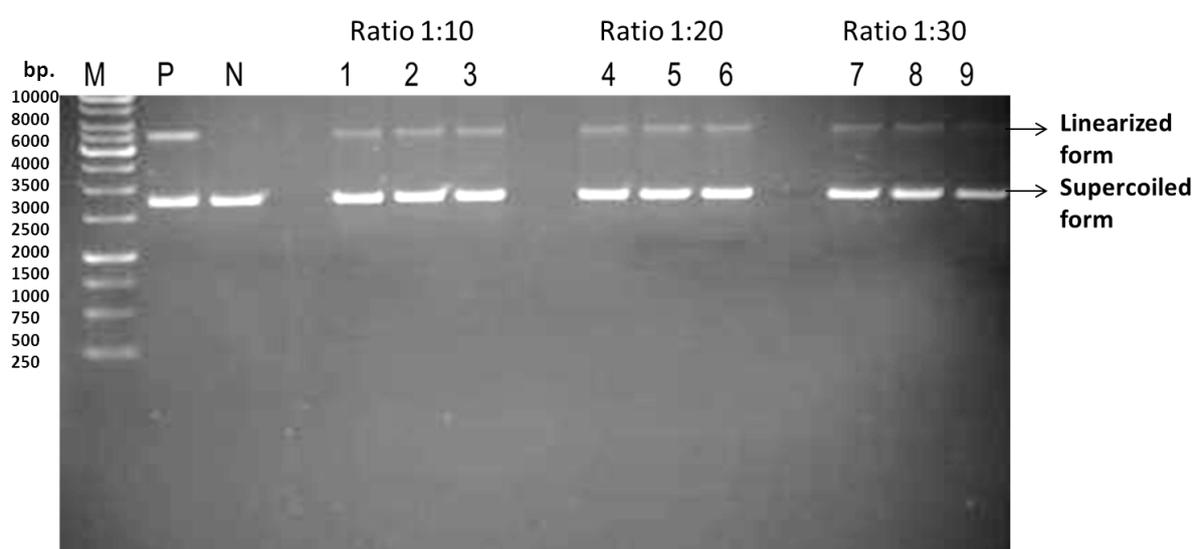


Figure 3.5 The *in vitro* effect of CdSe nanoparticles on plasmid DNA pGEMT Easy.

Ethidium bromide stain of 0.8 % agarose gel. **Lane M:** Molecular marker, **Lane P:** Positive control- titanium dioxide, **Lane N:** Negative control- untreated DNA, **Lane 1:** DNA treated with 200 $\mu\text{g}/\text{mL}$ of CdSe nanoparticles from batch ratio 1:10. **Lane 2:** DNA treated with 600 $\mu\text{g}/\text{mL}$ of CdSe nanoparticles from batch ratio 1:10. **Lane 3:** DNA treated with 1000 $\mu\text{g}/\text{mL}$ of CdSe nanoparticles from batch ratio 1:10. **Lane 4:** DNA treated with 200 $\mu\text{g}/\text{mL}$ of CdSe nanoparticles from batch ratio 1:20. **Lane 5:** DNA treated with 600 $\mu\text{g}/\text{mL}$ of CdSe nanoparticles from batch ratio 1:20. **Lane 6:** DNA treated with 1000 $\mu\text{g}/\text{mL}$ of CdSe nanoparticles from batch ratio 1:20. **Lane 7:** DNA treated with 200 $\mu\text{g}/\text{mL}$ of CdSe nanoparticles from batch ratio 1:30. **Lane 8:** DNA treated with 600 $\mu\text{g}/\text{mL}$ of CdSe nanoparticles from batch ratio 1:30. **Lane 9:** DNA treated with 1000 $\mu\text{g}/\text{mL}$ of CdSe nanoparticles from batch ratio 1:30.

In lanes 1 to 9, the plasmid DNA was treated with various concentrations of CdSe nanoparticles. The bands of DNA observed in those lanes were compared to the negative and positive controls in this experiment. It is observed that across lane 1 to 9, there were two bands of DNA present, marked as supercoiled and linearized form. The presence of the second linearized band of DNA, in lanes 1 to 9, show that the DNA plasmid had undergone conformational changes from the supercoiled to relaxed linear form. These conformational changes confirm that CdSe nanoparticles induced DNA damage to plasmid pGEMT Easy. To confirm these findings, a complementary experiment, using Spectrophotometry was done. Aliquots of nanoparticle treated DNA samples were analyzed at 260 nm. Figure 3.6 shows the graphical results for this experiment, indicating the absorbance reading at 260 nm for each sample. An absorbance reading of 0.038 nm was observed in the negative control of untreated DNA. A higher absorbance reading of 0.26 nm was observed in the positive control of Titanium Dioxide treated DNA. These absorbance readings indicate, that when DNA is damaged, the conformation of the plasmid changes, thereby increasing the total surface area and size of the DNA fragment, hence the absorbance reading increases. The absorbance readings of DNA treated with CdSe nanoparticles increased in a dose dependent manner from lanes 1 to 8 (Figure 3.6; Appendix B3). These readings further confirm that the DNA was damaged upon exposure to CdSe nanoparticles.

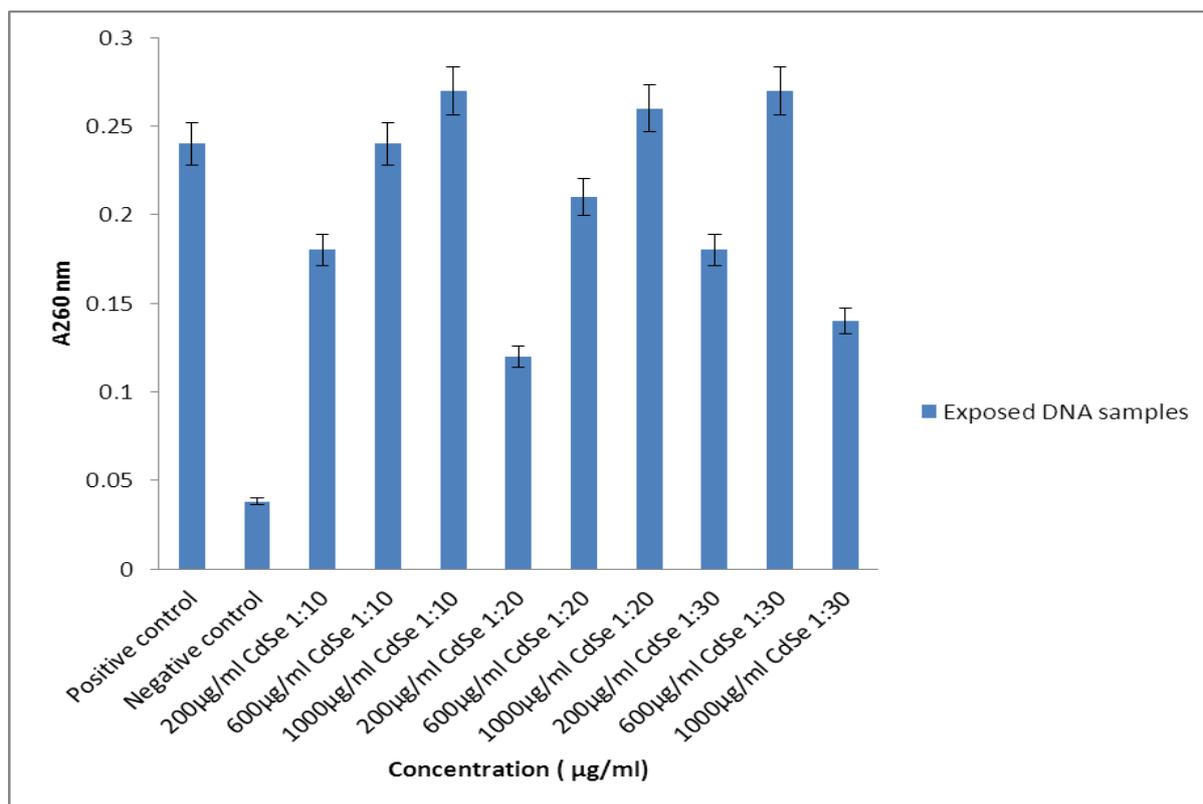


Figure 3.6 Spectrophotometric analysis of exposed DNA (Data is expressed as mean \pm SEM, n=3) These results show that as DNA is damaged the absorbance reading increases in a dose dependent manner.

3.4 Possible interaction between CdSe nanoparticles and the iron in RBCs

This experiment aimed to investigate the *in vitro* reducing power and chelating activity of CdSe nanoparticles on iron. The results from these *in vitro* assessments were used to hypothesize what may happen if CdSe nanoparticles entered the RBC and haemoglobin. There would be a possible interaction of CdSe nanoparticles with the iron molecules present within the haemoglobin protein subunits, with emphasis placed on the role of iron in gaseous exchange within the RBCs. During the reducing power experiment CdSe nanoparticles interact with iron. Reduction is defined as a gain of electrons, in this case Fe^{3+} (ferric ion) is reduced to Fe^{2+} (ferrous ion). The presence of the component ions (cadmium and selenium ions) reduces iron by donating an electron. During the chelation activity experiment CdSe nanoparticles interact with iron. Chelating agents are defined as organic compounds which complex or chemically bind metal ions. Ferrozine forms a complex with Fe^{2+} . In the presence of a chelating agent, the complex formation between ferrozine and Fe^{2+} is disrupted. The

colour change of this complex is a measure of the chelation activity of CdSe nanoparticles on iron.

Citric acid was used as a positive control for the reducing power experiments and showed 76% reducing power of iron at 1000 $\mu\text{g/mL}$. CdSe nanoparticles of the following concentrations 200, 400, 600, 800 and 1000 $\mu\text{g/}$ exhibited reducing power of iron in a dose-dependent manner across all batch ratios (Figure 3.7) The batch ratio of 1:10 of CdSe nanoparticle samples showed 90 % reducing power at 1000 $\mu\text{g/mL}$. Batch ratio 1:20 of CdSe nanoparticle samples showed 33 % reducing power at 800 $\mu\text{g/mL}$. Batch ratio 1:30 of CdSe nanoparticle samples showed a 20 % reducing power at 800 $\mu\text{g/mL}$. The results shown in Figure 3.7 further indicate that as the molar ratios of each batch of CdSe nanoparticles increased, their reducing power of iron decreased. Batch ratio 1:20 and 1; 30 showed poor reducing power of iron. These findings indicate that the increase in concentration of cysteine in the higher batch ratios of CdSe nanoparticles played a role in inducing less reducing power of iron.

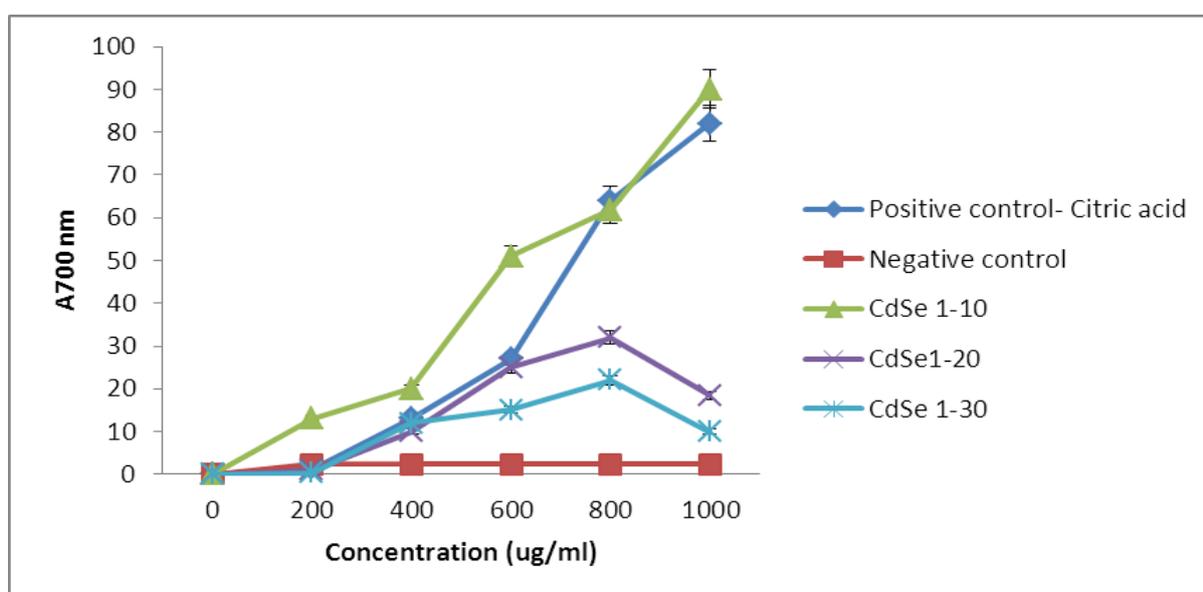


Figure 3.7 Reducing power activity of CdSe nanoparticles on iron

Iron is reduced by CdSe nanoparticles in a dose dependent manner. The lowest Batch ratio (1:10) exhibited the highest reducing power. All experiments were done in triplicates. The data is expressed as a mean \pm SEM, n=3

The *in vitro* chelating activity of CdSe nanoparticles on iron aimed to assess the chelation effects of CdSe nanoparticles on iron. EDTA was used as a negative control, as it has well-known chelating properties and its activity was observed at 80 % chelation at 1000 $\mu\text{g/mL}$. CdSe nanoparticles were used in the following concentrations 200, 400, 600, 800 and 1000 $\mu\text{g/mL}$ to evaluate their chelating activity on iron. The chelation activity of the batch ratios indicated that as the molar ratios (1:10, 1:20 and 1:30) of the CdSe nanoparticles decreased the chelation activity increased respectively (Figure 3.8). Batch ratio 1:10 showed the highest chelation activity of 65 % at 1000 $\mu\text{g/mL}$ and batch ratios 1:20 and 1:30 showed poor chelation activity of less than 20 % at 1000 $\mu\text{g/mL}$. The results show that CdSe nanoparticles from Batch ratio 1:10 exhibited the highest reducing power and chelating activity of iron.

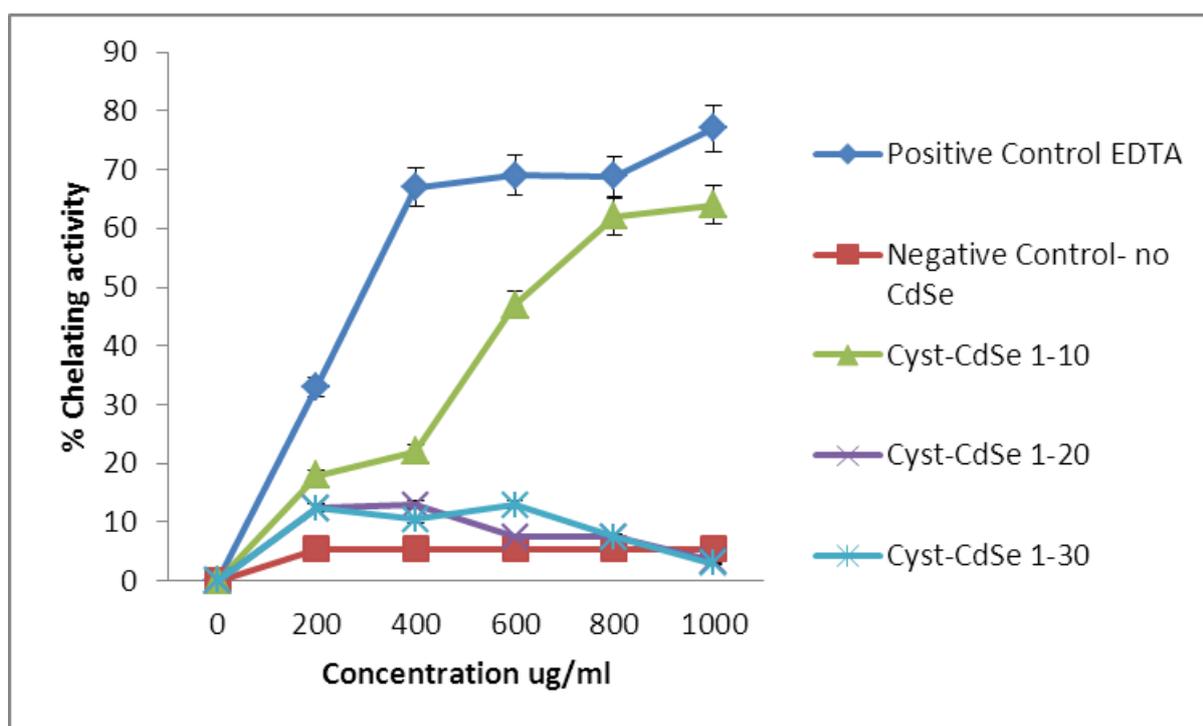


Figure 3.8 Chelation activity of CdSe nanoparticles on iron

Iron is chelated by CdSe nanoparticles in a dose dependent manner for batch ratio 1:10. Batch ratio (1:20 and 1:30) exhibited poor chelation activity across all concentrations. All experiments were done in triplicates. The data is expressed as a mean \pm SEM, n=3

3.5 CdSe nanoparticles promote platelet aggregation

This experiment sought to investigate the *in vitro* effects of CdSe nanoparticles on blood platelets obtained from rat blood. Thrombin was used as a positive control to promote platelet aggregation and heparin was used as a negative control to suppress platelet aggregation. Different batch ratios (1:10, 1:20 and 1:30) and concentrations of CdSe nanoparticles ranging from 200, 400, 600, 800 and 1000 $\mu\text{g}/\text{mL}$ were used in the study. The platelets were treated with thrombin, heparin and CdSe nanoparticles (Figure 3.9) and the interaction was observed using a Biotek plate reader. Large complexes were formed as platelets began to aggregate causing an increase in the absorbance readings of those samples. Figure 3.9 shows that platelet aggregation was observed at the higher concentrations of CdSe nanoparticles (1000 and 800 $\mu\text{l}/\text{mL}$) for batch ratios 1:10 and 1:20. The batch ratio 1:30 of CdSe nanoparticles exhibited increased platelet aggregation activity in a dose dependent manner (Figure 3.10; Appendix B, section B4).

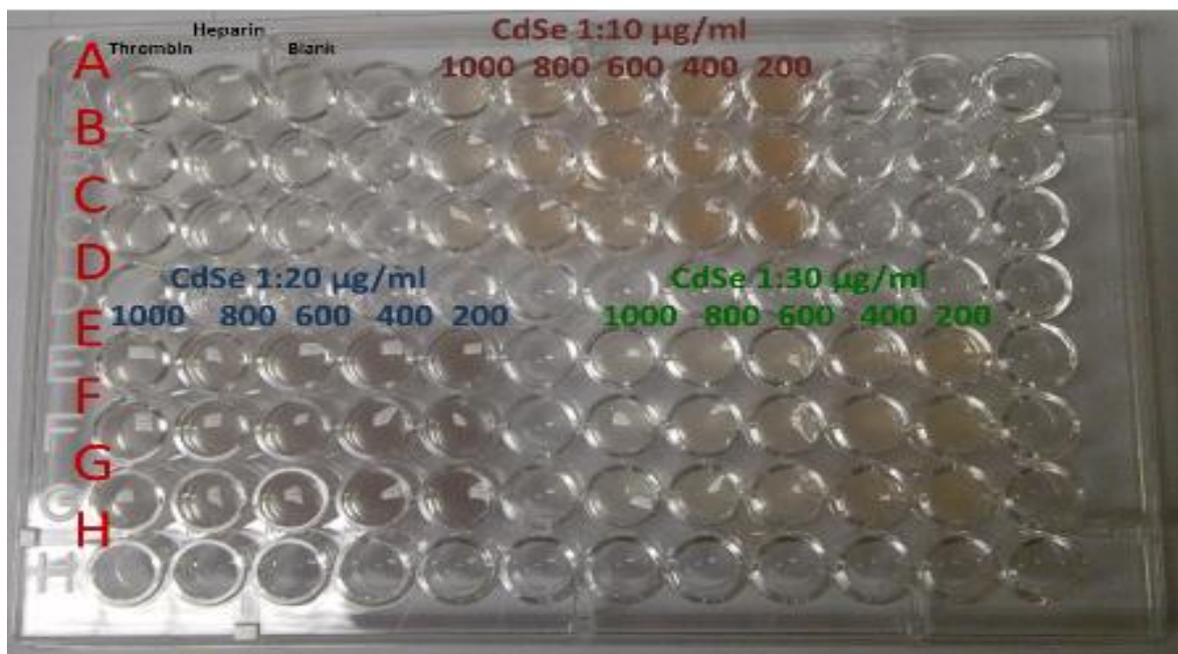


Figure 3.9 Illustration of CdSe nanoparticles of various concentrations, interacting with blood platelets Thrombin was used as the positive control to initiate platelet aggregation and heparin was used as the negative control to suppress platelet aggregation. The CdSe nanoparticles effect on platelet function was compared to both controls. All experiments were repeated in triplicates. The data is expressed as a mean \pm SEM, n=3

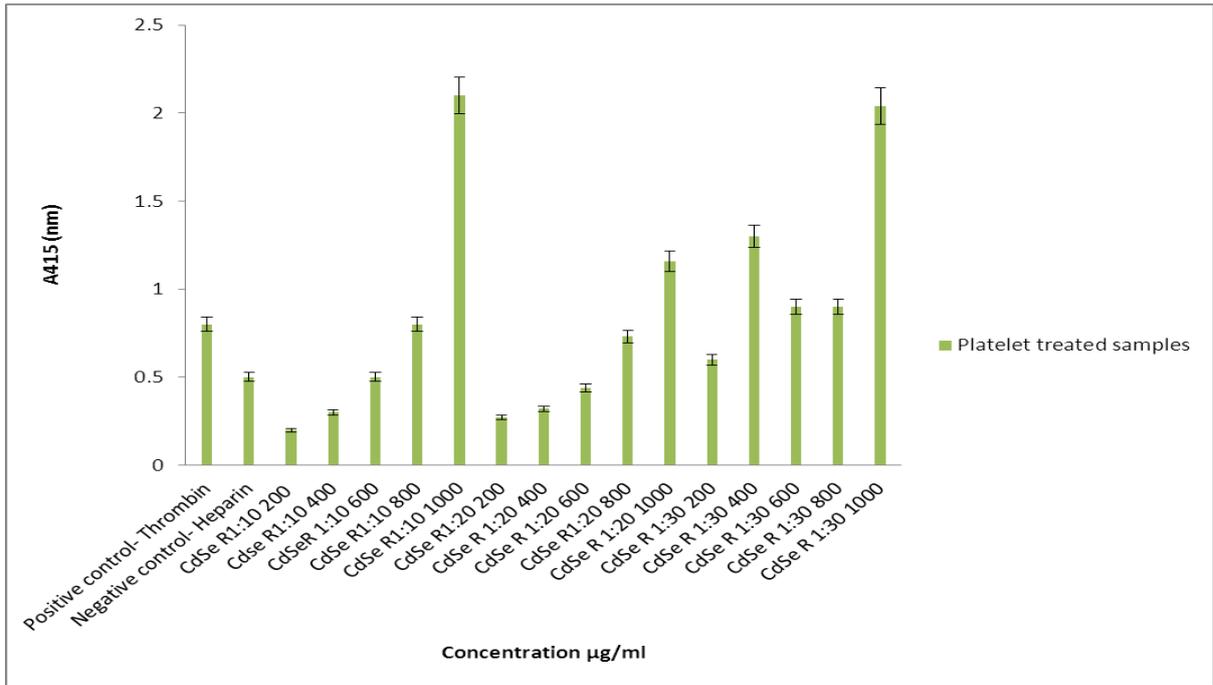


Figure 3.10 effects of CdSe nanoparticles on platelet function

Thrombin has a higher absorbance reading than Heparin. Note batch ratio 1:30 exhibits the highest platelet aggregation activity in a dose dependent manner.

Chapter 4- Discussion

This chapter includes a discussion on the findings from this study and highlights the importance of the data obtained.

Water soluble cysteine capped Cadmium Selenide nanoparticles were prepared, using the one-pot solution based route. The cadmium salt (cadmium chloride) and the capping agent (L-cysteine ethyl ester hydrochloride) were added to the reduced selenium in molar ratios of 1:10, 1:20 and 1:30. It was anticipated that increasing the concentration of cysteine in the reaction, would result in a more saturated and structurally stable capping layer which surrounds the surface CdSe quantum dot core. This would ensure the generation of a product that is stable, especially under physiological conditions. Oxidation can occur between pairs of cysteine side chains to form disulfide bonds. The presence of such bonds plays important structural roles in stabilizing the surface coating layer around the CdSe core. As CdSe nanoparticles become smaller in size and exhibit larger band gap energies, which results in the component ions (cadmium and selenium ions) having extraordinary optical, electrical and redox abilities. Therefore, it is important that the core and the component ions remain stable, to ensure that CdSe nanoparticles maintain their integrity during interaction with biological entities.

Capping these quantum dots with cysteine serves many additional functions. Previous studies show that cysteine is the most suitable thiol that is able to encapsulate the CdSe nanoparticle without disturbing its optical and electronic features (Oluwafemi *et al.*, 2008). Cysteine functions as an agent for solubilisation in water, stabilization of the core complex within the quantum dot and finally for the possible conjugation with biomolecules. The CdSe core is attached to cysteine through the mercapto group. This means that the mercapto group binds to the cadmium atom and the polar amino side groups (COO^- and NH_3^+) of cysteine, making CdSe hydrophilic nanoparticles. These features give rise to great potential in terms of

bioapplications such as drug, molecular or gene delivery as the free ammonium and carboxylate groups are available for covalent binding to many biomolecules such as amino acids, proteins, hormones and nucleic acids. All batch ratio samples of Cysteine capped CdSe nanoparticles showed similar characteristics during analysis with TEM, HRTEM, UV and PL (Section 3.2: Figure 3.2; Figure 3.3 and Table 3.1). TEM images revealed spherically shaped mono dispersed nanoparticles of sizes less than 100 nm (Figure 3.2). These features are important as they all accumulatively contribute to the potential toxicity of CdSe nanoparticles (Oberdoster *et al.*, 2005) HRTEM images confirmed the crystalline nature of the semiconducting nanoparticles. In most samples the lattice fringes was clearly observed. UV and PL readings also revealed similar excitation peaks and emitting ranges for each batch ratio, confirming that irrespective of the increase in capping agent concentration, CdSe nanoparticles still exhibited previously reported optical properties (Oluwafemi *et al.*, 2008).

The purified CdSe nanoparticles were exposed to DNA, RBC components and blood platelets to examine the *in vitro* interactions of CdSe nanoparticles on these important biological components. The blood circulatory system is considered one of the most important biological components, since it is the first point of contact for intravenously administered substances. It could also be assumed that all exposure routes inevitably lead to the blood system (Oberdoster *et al.*, 2005; Kuhlbusch *et al.*, 2011), where CdSe nanoparticles are bound to interact with the above mentioned biological components. The importance of assessing these interactions would provide useful information for the further use of CdSe nanoparticles bioapplications such as drug delivery or bio-optic systems. The interaction of CdSe nanoparticles with DNA was assessed using agarose gel electrophoresis and spectrophotometry techniques.

The plasmid exists as a double stranded covalently closed circular DNA. This supercoiled conformation makes the plasmid smaller in size, hence these DNA fragments migrate faster and further through the gel pore. In order for the plasmid to remain in the supercoiled conformation it is important that the strands remain intact. However, once a strand of the double helix is nicked or cut, either by restriction enzymes, free radicals and even nanoparticles, they assume the uncoiled relaxed state, resulting in a plasmid supporting an open-circular or relaxed conformation. This change to an open circular relaxed conformation makes the DNA fragment move slower during agarose gel electrophoresis. If both strands of the double helix are nicked or cut, the circular double helix becomes linear. This linear conformation allows DNA fragments to migrate along the gel at a relative speed, according to its size. The changes in conformation of DNA from supercoiled to the relaxed linearized form indicates DNA damage upon exposure to CdSe nanoparticles (Section 3.3, Figure 3.5)

Spectrophotometry analysis of plasmid pGEMT Easy DNA, provides further evidence that CdSe nanoparticles induced DNA conformational changes, hence DNA damage (Section 3.3; Figure 3.6).

Supercoiled DNA is compact and small in size, hence has a small surface area, less light is absorbed and therefore the reading of DNA of this conformation is much lower. When the DNA plasmid conformation changes to open circular or relaxed, the size of the DNA fragment becomes larger, hence an increase in the total surface area of the DNA molecule. This requires more light to be absorbed into the DNA sample and, therefore, the reading of damaged DNA fragments is much higher. Agarose concentration used in this experiment was 0.8 %. Higher concentrations cause the gel matrix to become denser, creating small pores which create higher frictional resistances against the DNA fragments, resulting in slower migration of DNA along the gel. A lower concentration of agarose make the gel matrix less

dense and cause the larger pores which exert less frictional resistance and allows easier migration of DNA along the gel. The applied voltage was used in this experiment was 70 V because decreasing the voltage allows the DNA to migrate along the gel matrix for a longer period of time and hence gives a better resolution and less diffusion of the DNA fragments in the gel. The DNA damage observed (Section 3.3, Figure 3.5) raises safety concerns with regards to the bioapplications of CdSe nanoparticles. If DNA is damaged by CdSe nanoparticles, there is an increased risk of gene mutations, generation of reactive oxidative species formation and carcinogenesis.

The reducing power and chelating activity experiments were based on *in vitro* assays to determine the direct interaction of CdSe nanoparticles with iron. The results confirm that CdSe nanoparticles reduce iron by donating an electron to Fe^{3+} (Ferric ion), resulting in Fe^{2+} (ferrous ion). CdSe nanoparticles interacted with iron, batch ratio 1:10 exhibited high chelation activity on iron, whilst batch ratio 1:20 and 1:30 exhibited very poor chelation activity. The explanations for these findings are related to the oxidative and reductive abilities of the cadmium and selenium ions released from the quantum dot core. As the particle size decreases, the band gap increases, allowing the component ions of the CdSe core to exhibit high oxidative and reductive properties. When the capping agent or coating layer is degraded or compromised the component ions within the quantum dot core are released, these ions are responsible for reducing and chelating the iron. The batch ratio 1:20 and 1:30 showed that by increasing the concentration of cysteine which surrounds the CdSe surface, the nanoparticles were stabilized in these experiments. This experiment can be related to an apparent interaction that CdSe nanoparticles could have with RBCs and haemoglobin if they had to interact, hence making iron both chemically and structurally unable to fulfill its role in gaseous exchange of oxygen and carbon dioxide within the haemoglobin of the RBC.

These observations are based on a qualitative *in vitro* assessment. To thoroughly understand the chemical mechanisms behind the reducing and chelating abilities of CdSe nanoparticles, further analysis of the molecular features of CdSe nanoparticles must be investigated.

During platelet aggregation a complex of platelets are formed and this complex requires more light to pass through it in order for the absorbance to be read. Therefore, the readings in samples with blood platelet aggregation are much higher than in samples with no platelet aggregation. Upon further observations, the results show that the sample batch having the highest cysteine concentration (1:30) had promoted platelet aggregation in a dose dependent manner. This could be due to the platelets interacting with the higher concentrations of the cysteine on the surface of the CdSe nanoparticle. These results raise issues surrounding biological malfunctions of cellular components and biological systems, which could include unnecessary clotting of blood platelets, DNA damage and impairment of the gaseous exchange processes during from exposure to CdSe nanoparticles through various bioapplications.

Chapter 5- Conclusion

This chapter provides possible conclusions and future work that may arise from this study.

5.1 Conclusion

The cysteine capped Cadmium selenide nanoparticles was synthesized in three batch ratios to produce a more stable nanoparticle that could be used in bioapplications whilst inducing less toxicity. This is theoretically expected. However, in practice, it was observed that even the highest batch ratio did induce nanoparticle toxicity and showed some adverse effects to the biological entities used in this study. From the results obtained one can conclude that all batches regardless of the different batch ratios, exhibit comparable optical and morphological properties. It is only upon investigating the effects of the CdSe nanoparticles on biological material that one can establish what effect, if any does increasing the molar ratios between the cadmium salt and capping agent have on the toxicity of the CdSe nanoparticles.

Damage done to DNA conformation indicates potential mutations of genes especially since cadmium is a known human carcinogen. The reducing and chelating activities exhibited by CdSe nanoparticles suggests that further tests need to be carried out in order to investigate the mechanism behind these abilities. In the presence of CdSe nanoparticles blood platelets aggregated in a dose dependent manner further highlighting the potential toxic risks these nanoparticles can induce to important biological entities within the system. These results raise issues concerning the safety of CdSe nanoparticles, the integrity of the capping agent and the intended use of CdSe nanoparticles in bioapplications.

5.2 Future work from this project

Future work from this project would include a more detailed characterization of the nanoparticles and the development of methods to synthesize more biosafe nanoparticles in order to expand its applications in biomedicine and biochemistry technologies. Cell culture

cytotoxicity assays and aspects of molecular biology could also be applied to expand the toxicity testing of these nanoparticles in future research projects.

Appendix A

A1. Special chemical Reagents

Name of reagent	Name of supplier
Acetic anhydride	Merck
Acetone	Sigma-Aldrich
Agarose	Merck
Ammonium persulphate	Merck
Ampicillin	Calbiochem
Broth	Merck
Cadmium chloride	Sigma-Aldrich
Calcium chloride	Merck
Citric acid	Merck
Chloroform	Sigma-Aldrich
Dextrose	Merck
Diethyl ether	Sigma-Aldrich
Ecoli XL Blue cells	Fermantas Life Sciences
EDTA	Sigma-Aldrich
Ethanol	Sigma-Aldrich
Ethidium Bromide	Merck
Ferric chloride	Sigma-Aldrich
Ferrous chloride	Sigma-Aldrich
Ferrozine	Sigma-Aldrich
Gene ladder 1 kb	Fermantas Life Sciences
Glacial acetic acid	Sigma-Aldrich
Glucose	Merck
Glycerol	Merck
Hydrochloric acid	Sigma-Aldrich
L-cysteine ethyl ester hydrochloride	Sigma-Aldrich
Nutrient agar	Merck
pGEMT Easy plasmid DNA	Fermantas Life Sciences
Potassium Ferricyanide	Merck
Qiagen kit	Fermantas Life Sciences
Red blood cells	Sigma-Aldrich
Restriction enzymes	Merck
Selenium	Sigma-Aldrich
Sodium borohydride	Sigma-Aldrich
Sodium chloride	Merck
Sodium hydroxide	Merck
Tris	Merck
Trisodium citrate	Sigma-Aldrich
Thrombin	Sigma-Aldrich
Tryptone	Oxoid, England
Yeast	Merck

Appendix A2 - Preparation of reagents

A1. Tris buffer: 2.79g of EDTA, 7.88g of Tris HCL and 10.22g of NaCl was dissolved in distilled water, and then the solution was brought up to 1L by further addition of sufficient distilled water.

A2. Resuspending buffer: 8.18 g of 0.14 M NaCl, 2.36 g of 15 mM Tris HCL and 0.9 g of 0.005 mM of glucose was dissolved in distilled water and then the solution was brought up to 1L by further addition of sufficient distilled water.

A3. Phosphate buffer: 18 mL of 0.2M KOH and 50 mL of 0.2M KH_2PO_4 were mixed and dissolved in distilled water and then made up to 1L by further addition of sufficient distilled water.

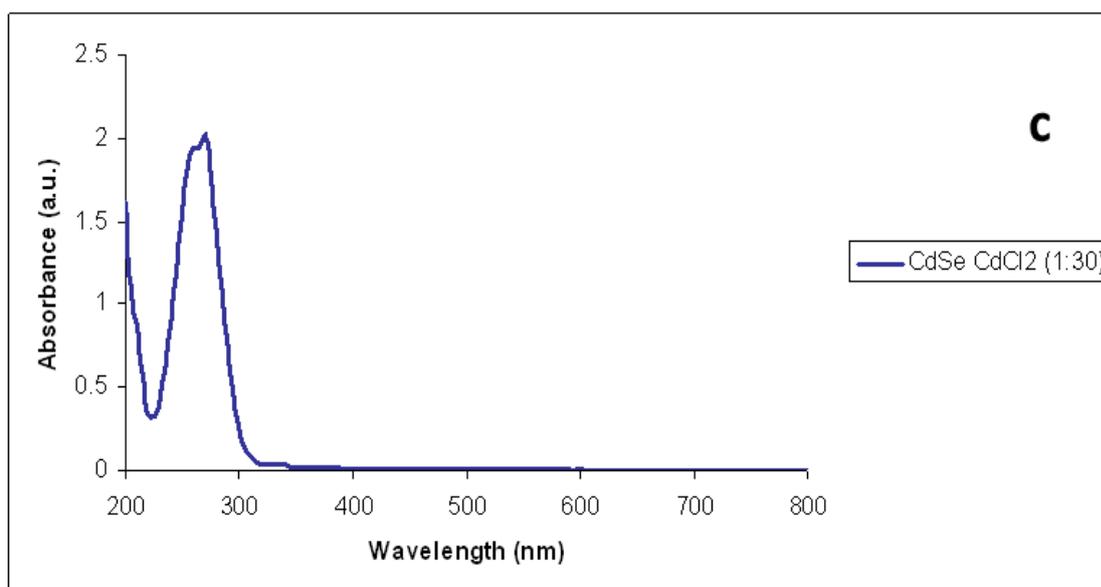
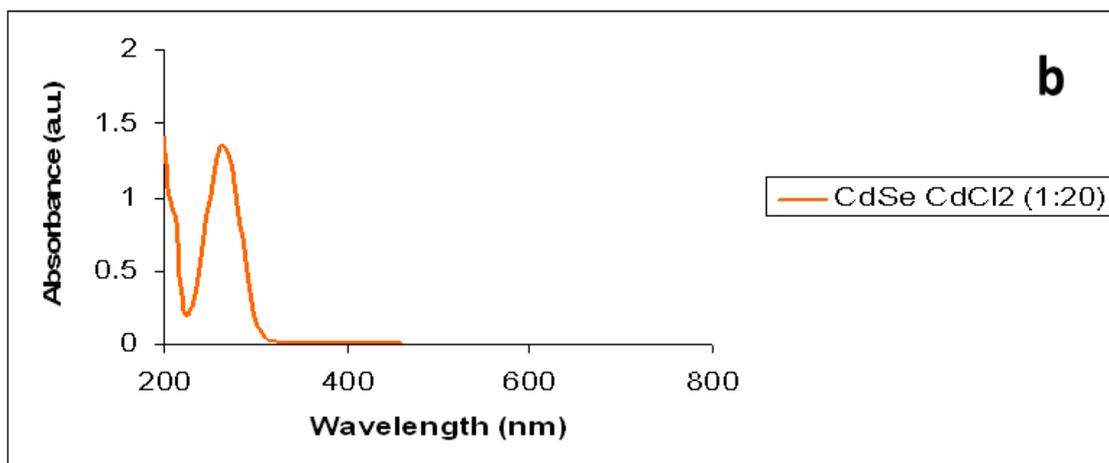
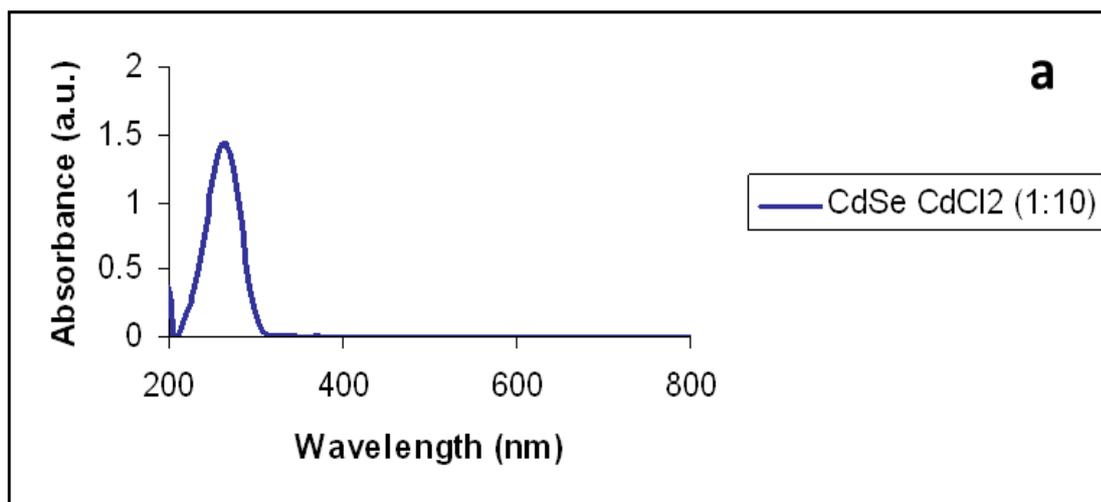
A4. ADA: Acid Dextrose anti-coagulant: 20g of Dextrose, 13,66g of 0,065 M Citric acid and 25g of 0.085M of Trisodium citrate mixed together and thereafter dissolved in 500 mL of distilled water.

A5. Washing buffer (pH 6.5): Washing buffer was prepared by the addition of 32.77g of 0.113 M of sodium chloride together with 3.053g of 4.3 mM Na_2HPO_4 , 3.741g of 4.3 mM of K_2HPO_4 , 14.64g of 24.4 mM of NaH_2PO_4 , 5.45g of 5.5 mM Glucose and 1.86g of 1 mM EDTA and dissolve in 5000 mL of distilled water.

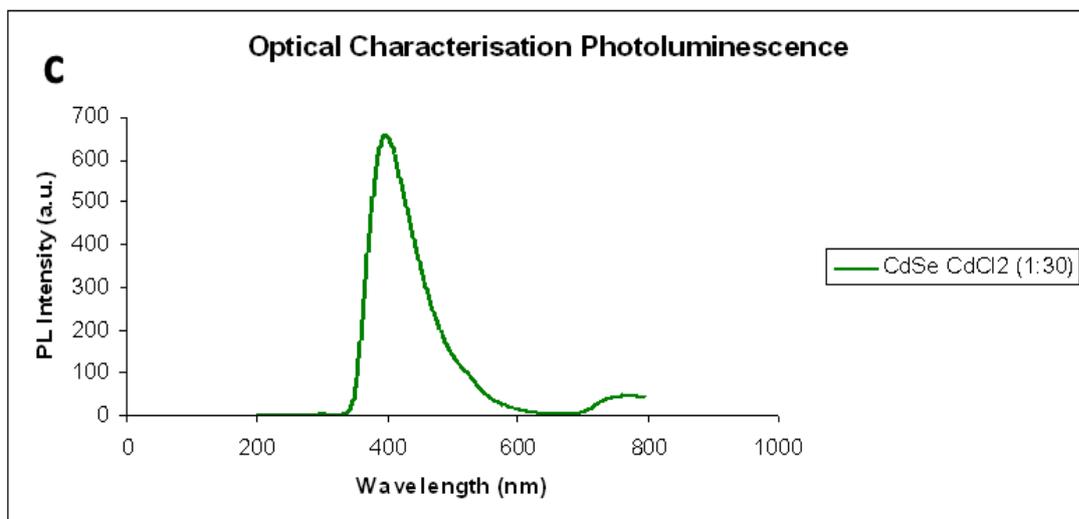
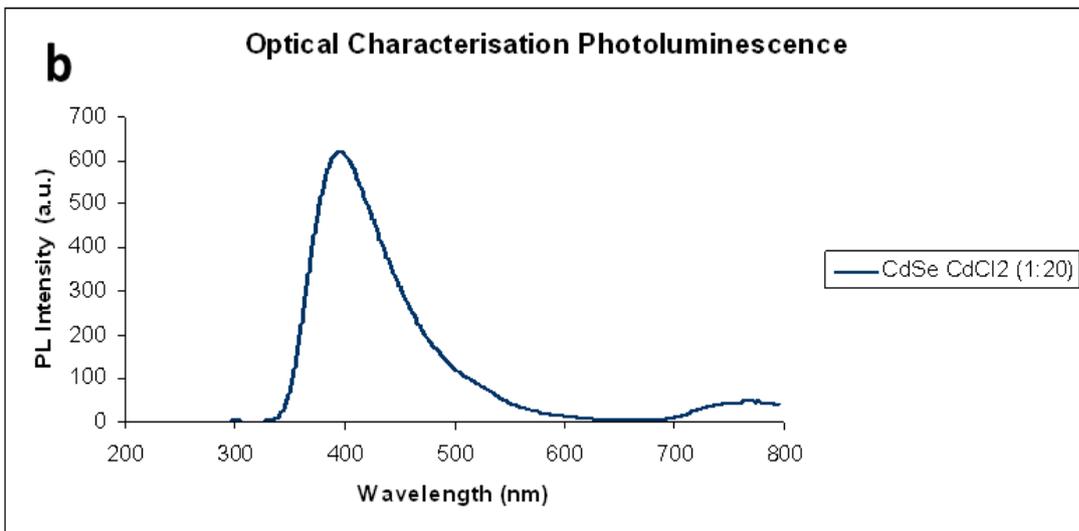
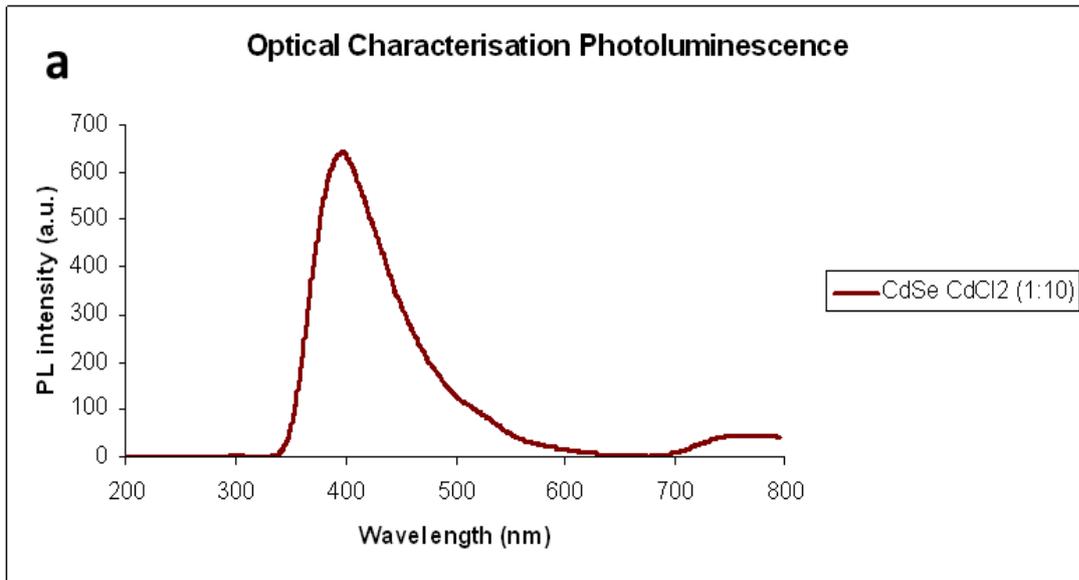
- A6. 10x TAE buffer:** 48.8 g of Tris + 10,9g of acetic acid + 3g EDTA- top up to 1000mL /1L.
- A7. 0.2M phosphate buffer:** 18mL of 0,2M KOH and 50mL of 0,2M KH_2PO_4 was mixed and dissolved in distilled water then brought up to 1L.
- A8. 0.8 % Agarose gel:** 0,8g agarose, 10mL of 10x TAE buffer and 90mL distilled water was mixed and boiled after cooling, 10 μl of ethidium bromide was added to the agarose mixture and poured into casting tray+comb and allowed to set. Load DNA and run gel using the appropriate voltage.
- A9.Ethidium Bromide:** Add 0.25 % Ethidium bromide to 30% glycerol.
- A10. 1 % potassium ferricyanide:** 1g of potassium ferricyanide and top up to 100mL.
- A11. 10 % Trichloroacetic acid:** 10g top up to 100mL.
- A12. 1% TBA:** 50% glacial acetic acid +1g TBA top up to 100mL.
- A13. 0.1 % ferric chloride:** 0,1g of ferric chloride and top up to 100mL.
- A14. 0.1M Calcium chloride:** 1, 47 grams of Calcium chloride was dissolved and topped up to 100 mL of distilled water.

Appendix B- Supplementary data

B1. UV Characterizations for each batch ratio of CdSe nanoparticles



B2. PL characterizations for each batch ratio of CdSe nanoparticles



B3. Absorbance reading from the spectrophotometric analysis

(Compare with graphical results in Chapter 3, section 3.3)

Sample	Absorbance reading (nm) mean value
Positive control (Titanium dioxide)	0.24
Negative control (Untreated DNA)	0.038
1-200 µg/mL Ratio 1:10 CdSe	0.18
2-600 µg/mL Ratio 1:10 CdSe	0.24
3-1000 µg/mL Ratio 1:10 CdSe	0.27
4-200 µg/mL Ratio 1:20 CdSe	0.12
5-600 µg/mL Ratio 1:20 CdSe	0.21
6-1000 µg/mL Ratio 1:20 CdSe	0.26
7-200 µg/mL Ratio 1:30 CdSe	0.18
8-600 µg/mL Ratio 1:30 CdSe	0.27
9-1000 µg/mL Ratio 1:30 CdSe	0.14

B4. The effect of CdSe nanoparticles on blood platelets.

Note that absorbance readings ≤ 0.5 nm shows no platelet aggregation and absorbance readings ≥ 0.8 nm show platelet aggregation. (Compare with graphical results in Chapter 3, section 3.5)

Samples exposed to platelets	Mean absorbance	Standard Deviations \pm SEM	Observed Results
Positive control- Thrombin	0.8	0.186	Platelet aggregation
Negative control- Heparin	0.5	0.269	No aggregation
CdSe Ratio 1:10			
200 µg/ml	0.2	0.200	No aggregation
400 µg/ml	0.3	0.258	No aggregation
600 µg/ml	0.5	0.693	No aggregation
800 µg/ml	0.8	0.304	Platelet aggregation
1000 µg/ml	2.1	0.156	Platelet aggregation
CdSe Ratio 1:20			
200 µg/ml	0.27	0.754	No aggregation
400 µg/ml	0.32	0.525	No aggregation
600 µg/ml	0.44	0.437	No aggregation
800 µg/ml	0.73	0.277	Platelet aggregation
1000 µg/ml	1.16	0.171	Platelet aggregation
CdSe Ratio 1:30			
200 µg/ml	0.6	0.287	No aggregation
400 µg/ml	1.3	0.198	Platelet aggregation
600 µg/ml	0.9	0.322	Platelet aggregation
800 µg/ml	0.9	0.034	Platelet aggregation
1000 µg/ml	2.04	0.230	Platelet aggregation

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