Active targeting of cancer cells using gemcitabine conjugated platinum nanoparticles

Submitted in fulfilment of the requirements of the Degree of Master of Applied Science in Biotechnology: in the Faculty of Applied Sciences at Durban University of Technology

Kriya Odayar

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PROMOTER: Professor B. Odhav

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REFERENCE DECLARATION

I, Ms. K Odayar - 20424285 and Professor Bharti Odhav (full name of promoter/ supervisor) do hereby declare that in respect of the following dissertation:

'**Active targeting of cancer cells using gemcitabine conjugated platinum nanoparticles**'

is my own work. It has not been submitted for any degree or examination in any other university or institute and that all the resources used or quoted have been indicated and acknowledged by means of complete references.

_____	_____
Signature of student	**Date**
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Signature of promoter/ supervisor	**Date**
_____	_____
Signature of co-promoter/ co-supervisor	**Date**

AUTHORS DECLARATION

This study presents original work by the author. It has not been submitted in any form to another academic institution. Where use was made of the work of others, it has been duly acknowledged in the text.

The research described in this dissertation was carried out in the Department of Biotechnology and Food Technology, Faculty of Applied Sciences, Durban University of Technology, South Africa, under the supervision of **Professor Bharti Odhav** and **Dr Viresh Mohanlall**

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Students' signature

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ABSTRACT

Nanotechnology is explained as the science of engineered materials and systems on a molecular scale. This innovation is currently used in a wide variety of applications which include using these nanoparticles as drug delivery vehicles. Such nanocarriers are relatively smaller than 100 nm in size with the ability to convey therapeutic drugs to a number of disease sites.

Platinum-based nanoparticles have been extensively used in a number of applications namely catalysts, gas sensors, glucose sensors and cancer therapy. The properties of platinum nanoparticles (PtNP's) typically depend on characteristics such as shape, particle size, elemental composition and structure, all of which can be manipulated and controlled in the fabrication process. Their unique size in the nanometer scale makes platinum nanoparticles an ideal candidate as targeted drug delivery vehicles.

To target an anticancer drug to a diseased site is a distinctive feature of most studies, which aim to transfer an adequate dosage of the drug to cancer cells. Transport systems used as carriers of anticancer drugs offer numerous advantages, which include improved efficacy and a decrease in toxicity towards healthy cells when compared to standard drugs.

The aim of this study was to determine the effect of platinum nanoparticles, gemcitabine and gemcitabine conjugated platinum nanoparticles (Hybrids) against cancer cells and healthy cells and to determine the mode of cell death and cell death pathways using flow cytometry.

Platinum nanoparticles were synthesized via the reduction of hexachloroplatinic acid using sodium borohydride in the presence of capping agents. Synthesized platinum nanoparticles and the hybrids were characterized by observing peaks at 301 nm and 379 nm respectively using UV-visible spectroscopy.

TEM images revealed that the PtNP's and the conjugate compounds were spherical in shape with core sizes of 1.14 nm - 1.65 nm and 1.53 - 2.66 nm respectively. The bioactivity platinum nanoparticles, gemcitabine and the hybrids were investigated using MCF7 and Melanoma cancer cells at different concentrations from 0.10 to 100 µg/ml.

Results indicated that conjugated nanoparticles induced the highest cell inhibition against both cell lines compared to gemcitabine and platinum nanoparticles. Bioactivity against PBMC (peripheral blood mononuclear) cells indicated that all three compounds show little or no effect towards the healthy cell line compared to the control.

Melanoma cell line was used to determine the mode of cell death. Apoptosis was detected using Annexin V-FITC to detect membrane changes, JC-1 to detect a loss in mitochondrial membrane potential and caspase-3 assay kits. Results indicated that a significant amount of cell death was caused by cleavage of caspase-3.

Nanoparticle drug delivery is an area that has shown significant promise in cancer treatment. Interaction of nanoparticles with human cells is an interesting topic for understanding toxicity and developing potential drug candidates.

Imagine, something that is atleast or more than 80,000 times smaller than the edge of the ridge on a fingertip and unlocks a new wilderness into cancer research. Nanotechnology, known as the science of minute, is changing the approach to cancer and especially future diagnosis and treatment.

Nanotechnology permits scientists to fabricate new apparatuses that are definitely smaller than cells, giving them the chance to attack tumor diseased cells. This innovation not just empowers practitioners to recognize malignancies prior but additionally holds the guarantee of halting cancer growth before it further develops. This progressive approach is so exact, specialists will in future be able to outline a unique treatment for an individual's own restorative and hereditary profile.

Researchers are designing nanoparticles that detect and destroy diseased cells and this optimistic innovation could be personalized for targeted drug delivery, enhanced imaging and ongoing affirmation of cancer cell death.

The National Cancer Institute remains hopeful that facilitated development, nanotechnology will drastically change cancer treatment.

CHAPTER 1: INTRODUCTION

1.1 Cancer

One of the largest and most common diseases in the world is cancer. Heart disease and cancer results in over 80% of deaths in many countries and incidences of cancer are dramatically increasing on a global scale. This phenomenally high death rate is because standardized medicine does not know what brings about cancer or how cancer cells spread. Resulting from this, there is no effective therapy for cancer available and the disease is expanding globally.

Each year 12 million individuals worldwide die due to atherosclerosis, coronary illness and strokes. These are by far the most widely recognized reasons for the death of our time. However, the cellular medicine field has officially discovered responses to these epidemics. Cancer is the second largest disease in the world. Cancer and coronary disease result in more than 80 % of all deaths in many industrialized nations. Conventional medicine does not know how cancer spreads nor its causes. Due to this, there is no effective cancer treatment accessible and the disease can continue developing on a worldwide scale (Rath 2001).

Some of the main causes of cancer include emotional stress, environmental factors, long term dietary problems and chronis stress which can be dircetly related to emotional stress.

Cancer is brought about when abnormal cells multiply without control and attack different tissues. These cancer cells can spread to different parts of the body through the blood and lymph framework. Most malignancies are named for the organ or kind of cell in which they begin. The primary classifications of cancer incorporate leukemia, carcinoma, sarcoma, lymphoma and central nervous system malignancies. To understand cancer, it is important to know how typical cells transform into tumor cells. Cells develop and separate in a controlled way to create more cells that are expected to keep the body healthy. At the point when cells get to be old, or harmed they pass on and are replaced by new cells. At the point when this procedure goes wrong, the genetic material of a cell can be harmed or damaged bringing on transformations that influence ordinary cell development. At the point when these changes occur, cells don't die and these additional cells might shape into a mass of tissue called a tumor. Tumors can be

malignant or benign and not all are necessarily harmful (Mathers *et al.* 2001; Rath 2001). In 2001, the World Health Organization (WHO) evaluated cancer as the main source of death overall bringing about 7.4 million deaths. The fundamental sorts of cancer diseases bringing on death where stomach, lung, liver, breast and colorectal cancer. The WHO assessed that by 2030 deaths from cancer worldwide are anticipated to keep ascending with an expected 11.5 million (Mathers *et al.* 2001).

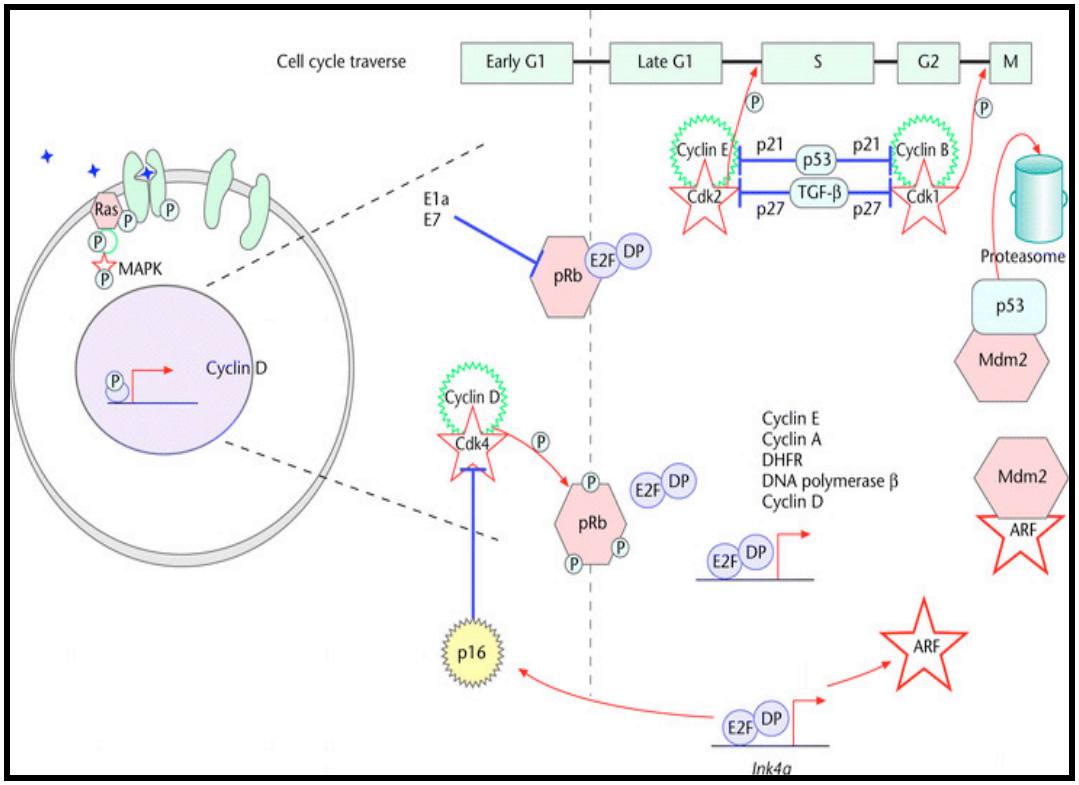

Figure 1: Overview of cell cycle regulation (Alison 2001)

Alison in 2001, discussed the regulation of the cell cycle and cancer development (Figure 1). Receptor actuation is brought about by ligand occupancy of the membrane-bound receptors, generally through phosphorylation of tyrosine deposits, activating downstream signal transduction pathways that deliver phosphorylated atoms to go about as translation components balancing gene expression. Mutational activation of any of the particles in these cascades can prompt constitutive signaling without the binding ligand, thus, adding to cancer advancement.

Cycles in eukaryotic cells are managed by intermittent activation with the use of various cyclin dependent kinases (Cdks), heterodimers of a protein kinase reactant subunit, the

Cdk, including a cyclin-initiating subunit. Diverse kinase complexes are needed to catalyze the addition of proteins that drive the cell cycle.

Cyclin D assumes a focal part; and is directed by development variables, and once the retinoblastoma protein (pRb) is phosphorylated by cyclin D–Cdk4, then E2F–DP interpretation components are allowed to intercede translation of various qualities encoding proteins that drive the cell cycle. In this way, once enacted, cyclin D goes about as a starter of the cell cycle; it refuels itself and affects cyclins for the progression of the cell cycle later on.

Cell cycle brakes are instructed by the Cdk inhibitors (CKIs), with as many as seven proteins having a place with either the Kip/Cip (kinase inhibitor protein/Cdk protein) family or the Ink4 (inhibitor of Cdk4) crew. Some Ink4 proteins, especially p16Ink4A, contend with cyclin D to tie Cdk4/6 thus square phosphorylation of pRb.

Accordingly, the Rb–cyclin D–Cdk4–p16 pathway is a noteworthy fuse box of development control. Brakes on the cell cycle are likewise given by the transcription component p53, upregulated by an assortment of cell stresses, inducing p21Cip1, a powerful inactivator of cyclin–Cdk edifices, and changing development variable B, inducing p27Kip1.

1.2 Nanotechnology

Nanotechnology manages the ability to picture, measure, model and control matter. It is principally concerned with the amalgamation of particles of various sizes, synthetic synthesis, shapes and their potential use for human advantages. Nanotechnology alludes the control of matter on a nuclear and atomic scale which brings about devices or materials that have no less than one measurement in the 1-100 nm range (Allhoff, Lin and Moore 2010). Nanoparticles of extremely small size offer unique physiochemical properties, for example, high reactivity and extensive surface area to mass proportion (Teow and Valiyaveettil 2010). Nanomaterials have a much bigger proportion of surface ratio to mass contrasted with normal materials. It is at the surface of materials that organic and substance responses happen and is in this manner expected that nanomaterials are more receptive than mass materials. These nanoscale materials regularly function uniquely in contrast to materials with a bigger structure do, even when the fundamental material (e.g., silver or carbon) is the same. They can have

distinctive fabrication procedures, physical, electrical and organic qualities. For instance, an aluminum can is safe for use, yet nano-sized aluminum is exceptionally toxic (Pelley and Saner 2009) and (Nair *et al.* 2010).

Currently, the primary purpose of exploration in nanotechnology concentrates on applications in areas of diagnostics, agriculture, drug delivery, medication and life sciences (Rathore 2014) and has incited materials for therapeutic applications with accentuation in diagnostics and treatment. Their interesting properties offer approaches to probe and control an assortment of biological and therapeutic procedures. By controlling their structures, one can control and alter their surface layer for improved solubility, biocompatibility and bioconjugation. Forming hybrid systems or conjugates makes way for bio-imaging, bio-detecting or biosensing and targeted drug delivery (Ajmal *et al.* 2015).

1.3 Nanoparticle action in targeting cancer/tumor cells

Nanotechnology has been discovered valuable to study drug targeting, stability and bioactivity. For instance, nanoparticles upgraded oral conveyance by enhancing the bioavailability of poorly consumed drugs by infiltrating cells to encourage cell internalization and connective tissue permeation. This empowered the drugs to be delivered to the objective tissue without clogging up vessels (Qin *et al.* 2016).

Figure 2 schematically displays a theoretical multifunctional nanoparticle that has been synthesized to incorporate many key components for drug delivery. These include the ability to target tumors, to avoid absorption by the reticuloendothelial system, to sense and report on molecular signatures and monitor disease sites and inhibit progression of the disease by delivering a therapeutic response to a biological or external trigger. Some of these components, for example, targeting, influence biological machinery. Others are derived artificially which empowers external probing or control that is generally not feasible in organic systems.

One of the long term goals in cancer therapy has been to provide the ability to physically target diseased cells to receive therapeutic drugs while avoiding particle uptake in normal healthy tissues (Ruoslahti 2002).

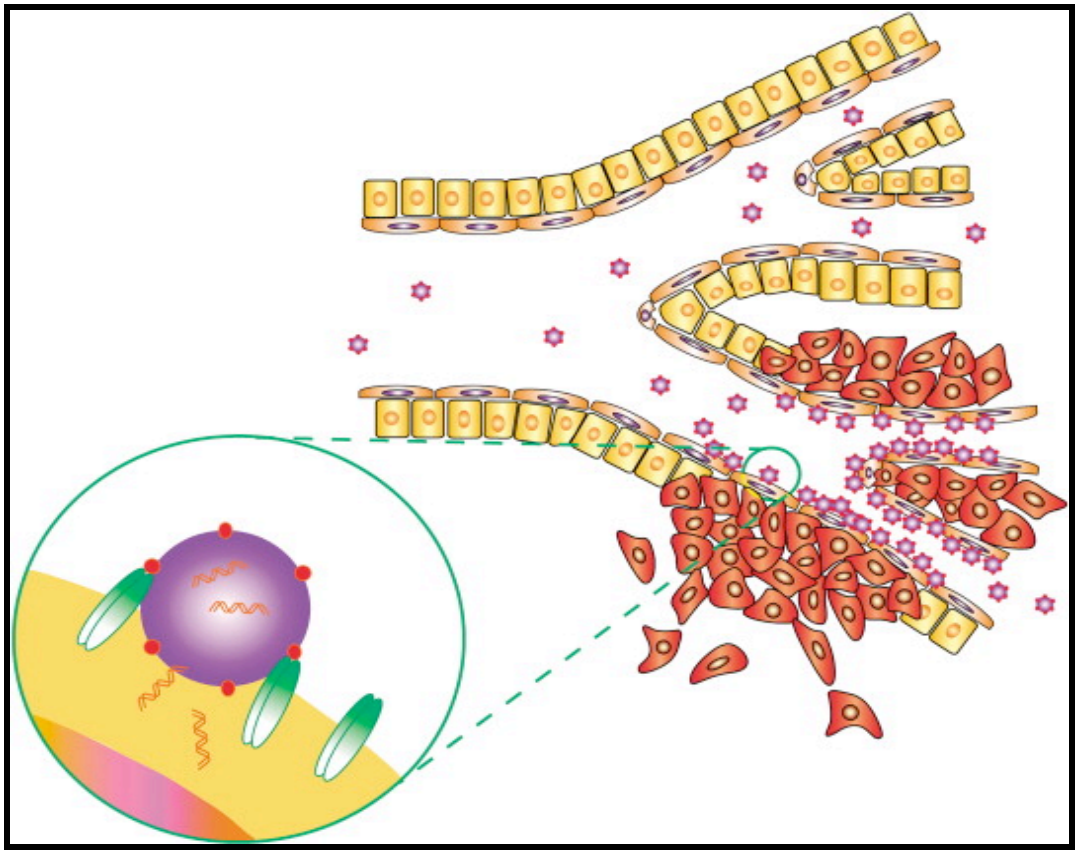

Figure 2: Schematic display of a multifunctional nanoparticle. A hypothetical nanoparticle targets the tumor, senses and reports molecular signatures resulting in delivery of a therapeutic in response to an external or biological trigger (Ruoslahti 2002).

Gold and silver nanoparticles have been extensively studied for the treatment of different cancers. Gold nanoshells have been used for photothermal ablation in superficial cancers that kills surrounding tumor tissue and silver nanoparticles for its incredible antimicrobial activity. More importantly, is the mechanism of action platinum nanoparticles to kill cancer cells. Platinum nanoparticles have been found to act as a reservoir that holds platinum ions which induce damage to the DNA of cancer cells (Yamada, Foote and Prow 2015).

Their extraordinary size in the nanometer scale makes platinum nanoparticles a perfect contender for targeted drug delivery vehicles. Platinum-based metals are known as D-block metals that can be found in mineral deposits. The greater part of the platinum group metals displays compound catalytic properties that can lead to the transformation of toxic chemicals (Kaushik, Snehit and Rasesh 2010).

A recent study has shown that platinum nanoparticles could hold a fundamental part in the medicinal field. Platinum nanoparticles have an activity closely related to mitochondrial complex 1, NADH. Reactive oxygen species are thought to be included in signal transduction pathways. Be that as it may, when they are overproduced, they participate in the onset and progression of numerous ailments. The platinum nanoparticles appeared to have an activity similar to NADH-ubiquinone oxidoreductase and it was expressed that platinum nanoparticles are a potentially therapeutic substance for oxidative diseases with suppressed mitochondrial complex 1 (Hikosaka *et al.* 2008).

Other nanoparticles used in cancer research and drug delivery is shown in table below.

Table 1: Nanoparticles used for cancer treatment

Type	**Result/ effect**	**Reference**
Gold NP	Reduces non-specific side effects and enables high dose delivery to target tissues	(Patra *et al.* 2010)
Silver NP	Demonstrates anticancer activity. These NP may be beneficial in lung cancer chemoprevention and chemotherapy, particularly at early stage	(He *et al.* 2016)
NP lasers	Method used to kill cancer cells and results show that cancer tumors are considerably damaged	(Jørgensen *et al.* 2016)
Quantum dots, nanoshells, nanowires and nanotubes	Proven to be promising applications for various cancer treatments	(Patel, Mistri and Prajapapti 2011)

1.4 Anticancer drugs and targeted drug delivery

Chemotherapy is one of the major therapeutic approaches for the treatment of cancer. It intends to extend life or mitigate side effects. Chemotherapy is frequently utilized in conjunction with other cancer treatments, for example, radiation or surgery. Tissue uptake of anticancer agents is of incredible importance for chemotherapy since anticancer drugs are not particular to cancer cells (Serpe 2006). This implies anticancer drugs annihilate cells that divide quickly under ordinary circumstances.

The greater part of drugs utilized for the treatment of cancer are cytotoxic drugs that meddle with the functioning of the cells' DNA. This can be brought about by the decimation of the DNA or restraint of DNA duplication (Alkylating agents or Mitomycin C), inhibition of the nucleic acid (DNA, RNA) amalgamation (Cytarabine) or interfering with the interpretation to hinder RNA synthesis (Doxorubicin). Cytotoxic drugs can possibly be harmful to the body unless they are particular to cancer cells. Lacking specificity, conventional approaches don't adequately differentiate between cancer cells and healthy cells. This regularly brings about systematic toxicity and antagonistic reactions with the loss of quality of life (Serpe *et al.* 2004a) (Serpe *et al.* 2004b).

Nanoparticles can promptly interact with particles at the surface and inside cells because of their minute size, yield better target specificity for therapeutics and diagnostics. Focusing on an anticancer drug to a diseased area is a particular component of most studies, which plan to transfer an adequate dose of the drug to the tumor. Drug delivery systems for use as vehicles for anticancer drugs offer various favorable advantages, which incorporate enhanced efficacy and reduced toxicity when contrasted with conventional dose forms (Rath 2001).

One of the significant challenges is to design new drugs that will be more specific for cancer cells and have lesser toxic impacts to healthy cells. One such drug could be gemcitabine, which is a pyrimidine analog and usually utilized for the treatment of different cancer types including lung, ovarian and breast cancer. This drug essentially kills cells undergoing DNA synthesis (Praetorius and Mandal 2007).

The aim of this study is to evaluate the effect of platinum nanoparticles, gemcitabine and platinum nanoparticle hybrids against cancer cells and normal cells and to determine the mode of cell death using flow cytometry.

1.5 Research Objectives

1. To synthesize platinum nanoparticles.

2. To characterize platinum nanoparticles using UV/Vis spectroscopy and transmission electron microscopy.

3. To conjugate gemcitabine and platinum nanoparticle and characterize the conjugate/hybrid using UV/Vis spectroscopy and transmission electron microscopy.

4. To perform cytotoxicity tests to investigate the effect of the gemcitabine, platinum nanoparticles and gemcitabine conjugated platinum nanoparticles (Hybrids) against cancer cells and healthy cells.

5. To determine the mode of cell death and cell death pathways using flow cytometry.

Interaction of nanoparticles with human cells is an interesting topic for understanding toxicity and developing potential drug candidates. This study focuses on the evolution of nanoparticles as carriers for anticancer drug delivery.

1.6 Dissertation Outline

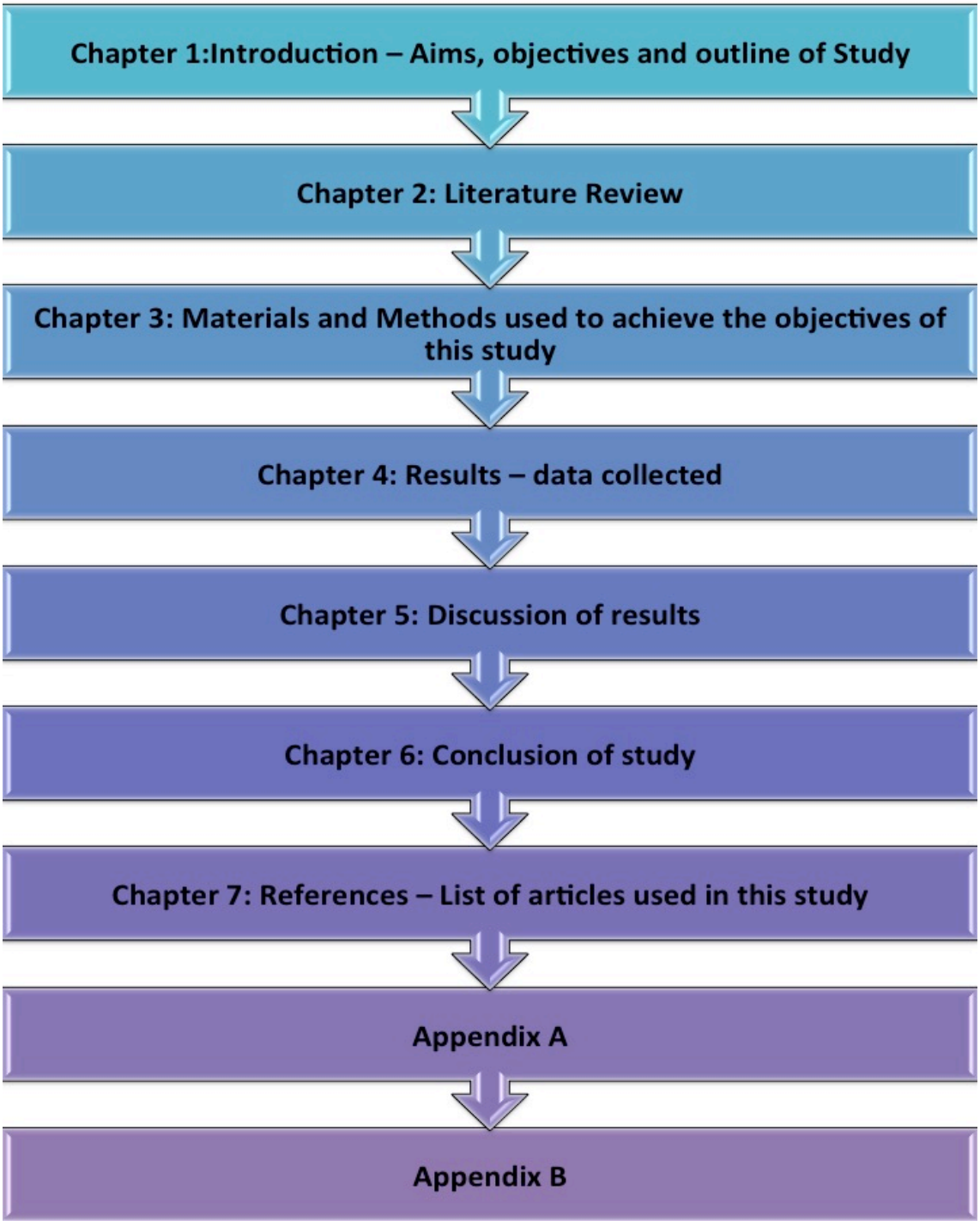

Figure 3: Dissertation Outline

CHAPTER 2: LITERATURE

2.1 Cancer

One of the largest and most common diseases in the world is cancer. Heart disease and cancer results in over 80% of deaths in many countries and incidences of cancer are dramatically increasing on a globally. This phenomenally high death rate is because standardized medicine does not know what brings about cancer or how cancer cells spread. Resulting from this, there is no effective therapy for cancer available and the disease will keep expanding globally.

Cancer is brought on by the uncontrolled development and spread of abnormal cells. Effective ways of treatment include surgery, chemotherapy, radiotherapy, hormone treatment and immunotherapy. Each of these treatments offers advantages and disadvantages and a combination of one or more of the treatments is normally expected to create the best results. Since most human cancers (> 85%) are related to solid tumors, current cancer treatment typically includes procedures including the use of catheters for chemotherapy, with introductory chemotherapy to minimize any cancer present, surgery to have the tumor removed if conceivable, trailed by chemotherapy and radiation to kill the tumor cells.

Cancer includes six biological compatabilities which is acquired during the development of human tumors. These are known as the hallmarks of cancer and include sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis and adjusting invasion and metastasis (Carels *et al.* 2016).

Another type of medical treatment for cancer includes targeted therapy, otherwise known as molecular targeted therapy. Other types include hormonal therapy and cytotoxic chemotherapy. Tartgeted therapy is known to inhibit the growth of cancer cells interfering with specific molecules required for tumor growth. This method is significantly different as it does not interfere with rapidly dividing cells as traditional chemotherapeutic treatments do.

Another form of therapy includes nanoengineered enzymes which bind to tumor cells so that natural cell degradation is able to digest the diseased cell, effectively eliminating it from the body. Such types of targeted cancer treatment is expected to be more effective compared to older treatments and more importnatly, less harmful to normal cells (Sing-hung 2007).

Research endeavors to enhance chemotherapy in the course of recent years and has prompted a change in patient survival. The adequacy of the treatment and the possible side effects fluctuate among various agents. A few drugs might have phenomenal efficacy, as well as serious side effects influencing the quality of life. Moreover, they might be in constrained supply and hence, exceptionally costly. More successful and less costly anticancer drugs have been underdeveloped. An example is dEpoB, which was based on the activity of paclitaxel and is reportedly 30 times more viable than paclitaxel (Christina and Danielhefsky 1999). Be that as it may, it regularly takes no less than 10 years and billions of dollars to find another drug. The improvement of new anticancer drug delivery systems offers less costly but more powerful treatment with little or no side effects.

Normally, pharmaceutical agents will disseminate evenly inside of the body. However, perfect chemotherapeutics require a high local concentration of the drug at the ailment site, while the fixation in other non-target organs and tissues ought to be beneath a specific negligible level to prevent any negative symptoms. The idea of drug targeting likewise called the "magic bullet", originates from the experience of the nineteenth-century German scientific expert, Paul Ehrlich, who specifically recolored microscopic organisms for histological examination. The "magic bullet" as an element includes two segments: to recognize and bind the target while the other ought to provide a therapeutic action. In the clinical setting, anticancer payloads, for example, toxins, radionucleotides and chemotherapeutic agents, have been conveyed to tumors through monoclonal antibodies (Kohler and Milstein 1975). Distinctive pharmaceutical carriers, including solvent polymers, microcapsules, microparticles, cells, lipoproteins, liposomes and micelles, have been as of late used to duplicate the quantity of drug atoms per single targeting. Every one of them can help targeting somehow. For example, encapsulating anticancer drugs in liposomes empowers targeted drug delivery to cancer tissues and avoids damage to normal surrounding tissues.

Furthermore, anticancer drugs bound with magnetic beads have been infused into the blood vessel flow and guided to the tumor by a magnetic field for targeted drug conveyance.

There has been concentrated research into the improvement of biodegradable and biocompatible nanoparticles (distance across <100 nm) as successful drug delivery systems, particularly for chemotherapy and gene delivery. In passive targeting, liposomes, or macromolecular and nanoparticle bearers abuse the enhanced permeability membrane (EPR) impact, which is an outcome of the expanded vasculature and diminished the lymphatic capacity of tumors, to target the drug to the tumor. Pharmaceutical transporters, in this way, provide methodology which is more time and cost effective than new drug improvement (Kreuter 2002). Progress in nanoparticle innovation, cell and atomic physiology and pathology have added to headways in chemotherapy and the gene therapy of cancer and different ailments, ideally staying away from the near toxic dosages of non-particular agents (Kreuter, 2002).

2.2 Anticancer drugs

Targeting an anticancer drug to an infected area is an unmistakable component in many studies and the point is to convey a sufficient dose of the therapeutic drug to the tumor. A portion of the new delivery systems that are used as vehicles for anticancer drugs offer various advantages including, enhanced efficacy and reduced cytotoxicity contrasted with conventional dosage forms. Doxorubicin, paclitaxel and gemcitabine are only a few of the drugs utilized as a part of past studies.

Paclitaxel, a diterpernoid subsidiary, has a broad anti-neoplastic action against colorectal cancer cells and an exceptional mechanism of activity advancing the stabilization and polymerization of tubulin to microtubules. However, one of the major clinical issues of paclitaxel is its low solubility in water, because of its greatly hydrophobic nature (Serpe *et al.* 2004a).

An anthracycline anti-toxin, doxorubicin, is a standout amongst the most valuable anti-neoplastic agents, showing a wide scope of clinical activities against a few tumors. This anticancer drug applies its cytotoxic movement by repressing the synthesis of nucleic acids inside of the cell walls and the non-specific activity of doxorubicin cause

symptoms in patients which has been a noteworthy issue to unravel (Yoo *et al.* 2000). Doxorubicin has additionally been observed to be not all that dynamic against colorectal disease (Nielsen, Maare and Skivsgaard 1996).

Also, numerous carriers of doxorubicin, for example, polymeric and liposome nanoparticles have been studied on with the point of decreasing cardiovascular toxicity and enhancing therapeutic efficacy (Batist *et al.* 2001).

In this study Gemcitabine (Figure 4) was used. This anticancer drug is a nucleoside analog that displays antitumor activity.

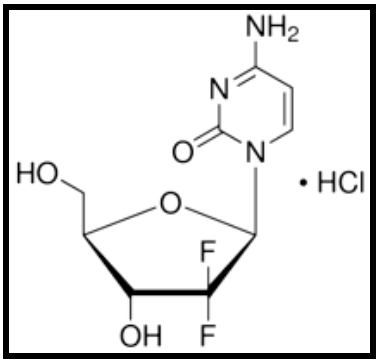

Figure 4: Chemical structure of Gemcitabine HCL (Gemzar 1996)

Gemcitabine displays cell phase specificity, basically executing cells experiencing DNA synthesis (S-phase) obstructing the movement of cells through the G1/S-phase boundary. Gemcitabine is metabolized intracellularly by nucleoside kinases to the dynamic diphosphate (dFdCDP) and triphosphate (dFdCTP) nucleosides (Gemzar 1996; O'Reilly and Abou-Alfa 2008; Fan *et al.* 2015).

The cytotoxic impact of gemcitabine is credited to a blend of two activities of the diphosphate and the triphosphate nucleosides, which prompts hindrance of DNA synthesis. To start with, gemcitabine diphosphate hinders ribonucleotide reductase, which is responsible for catalyzing the responses that create the deoxynucleoside triphosphates for DNA production. Restraint of this catalyst by the diphosphate nucleoside causes a reduction in the groupings of deoxynucleotides, including dCTP.

Second, gemcitabine triphosphate contends with dCTP for consolidation into DNA. The diminishment in the intracellular concentration of dCTP (by the activity of the diphosphate) enhances the incorporation of gemcitabine triphosphate into DNA. After

the gemcitabine nucleotide is fused into DNA, only one extra nucleotide is added to the developing DNA strands. After this expansion, there is restraint of further DNA synthesis. DNA polymerase epsilon is unable to evacuate the gemcitabine nucleotide and repair the developing DNA strands (conceal chain termination). In T-lymphoblastoid cells, gemcitabine instigates internucleosomal DNA fragmentation, one of the attributes of programmed cell death (Gemzar 1996; O'Reilly and Abou-Alfa 2008; Fan *et al.* 2015).

Another report stated that gemcitabine is viewed as the gold chemotherapeutic standard, yet just barely enhances lifespan because of its synthetic instability and low cell penetrance. Another worldview to enhance the therapeutic index of gemcitabine is to administer it in nanoparticles, which support its delivery to cells when under 500 nm in size (Papa *et al.* 2012).

2.3 Nanotechnology

Derived from the Greek word signifying 'Dwarf" and generally the length of three molecules next to each other, a nanometer is one billionth of a meter (Thakkar *et al.* 2010).

So why work with these nanoparticles? The answer lies in the novel properties controlled by these nanoparticles when contrasted with their naturally visible partners. Nanotechnology alludes to the control of matter on a molecular and atomic scale which brings about materials that have no less than one measurement in the 1-100 nm range (Allhoff, Lin and Moore 2010).

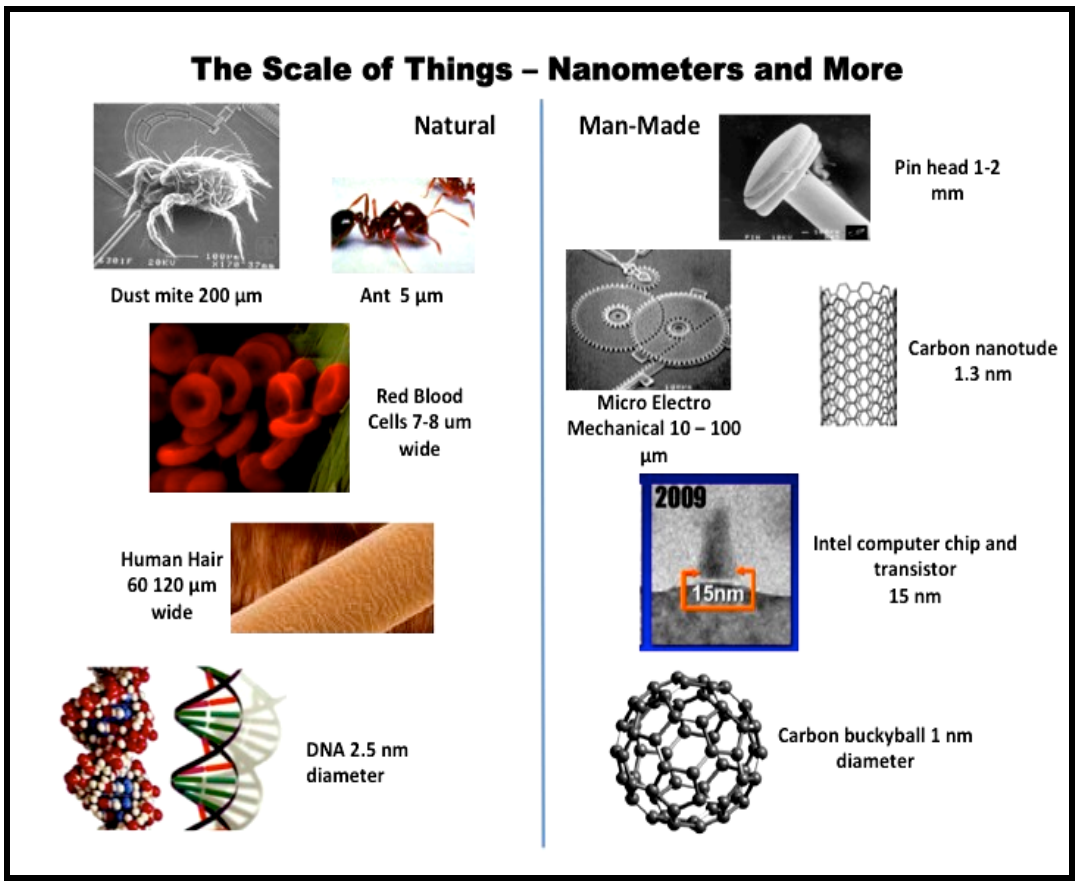

Figure 5: Scale of Things - Nanometers and more - image indicating sizes of objects natural and man-made (Usma 2015)

Crossing over any barrier between bulk materials and molecular or atomic structures, nanoparticles are of incredible scientific interest. A bulk material has steady physical properties no matter its size, however, at nanoscale this is frequently not the situation. Various well-characterized bulk materials have been found to have most fascinating properties when considered in the nanoscale. One truth adding to this is that nanoparticles have a very high aspect ratio. For instance, the high aspect ratio of silver nanoparticles permits them to effortlessly co-operate with different particles which increase their antibacterial productivity. As meager as 1 g of silver nanoparticles is known to exert anti-bacterial properties to large areas of the substrate material (Thakkar *et al.* 2010).

Metallic nanoparticles have diverse physical and synthetic properties from mass metals. These include higher surface areas, lower melting points, particularly optical properties, mechanical qualities and magnetizations, characteristics that may demonstrate appealing

in different modern applications. In any case, how a nanoparticle is seen and is characterized depends all that much on the particular application.
In this regard, Table 1 summarizes the definition of nanoparticles and nanomaterial's by various organizations.

Table 2: Definition of nanoparticles and nanomaterial's by various organizations (Horikoshi and Serpone 2013)

	Nanoparticles	Nanomaterials
ISO	A particle spanning 1–100 nm (diameter)	-
ASTM	An ultrafine particle whose length in 2 or 3 places is 1–100 nm	-
NIOSH	A particle with diameter between 1 and 100nm, or a fiber spanning the range 1-100nm	-
SCCP	At least one side is in the nanoscale range	Material for which at least one side or internal structure is in the nanoscale
BSI	All the fields or diameters are in the nanoscale range	Material for which at least one side or internal structure is in the nanoscale
BAuA	All the fields or diameters are in the nanoscale range	Material consisting of a nanostructure or a nano substance

Of specific significance, the optical property may be a key attraction and a trademark which is normal for a nanoparticle. For example, a silver nanoparticle is a yellowish grey shading and a gold nanoparticle (20 nm in size) has a trademark wine red shading. Palladium and Platinum based nanoparticles are shades of brown in color.

In 1818, Jeremias Benjamin Richters proposed a clarification for the distinctions in the different color arrangements of drinkable pink or purple gold solutions. It was further explained that these arrangements contained gold nanoparticles in the finest level of sub-division, however, yellow solutions were only found by the accumulation of fine particles

In 1857, in a surely understood production, Michael Faraday reported the development of dark red solution of colloidal gold by the reduction synthesis combining chloro-

aurate ($AuCl4^-$) and phosphorus in a two-stage system. Additionally research was done and he found that the optical properties of thin films was a result of the dried colloidal solutions. Here he noticed shading changes upon mechanical pressure (from blue-purple to green) (Horikoshi and Serpone 2013).

Nanotechnology is effortlessly evident in a number of old churches. A surely understood utilization of nanotechnology is the ruby red shading utilized for recolored glass windows amid medieval times. Excellent samples of these applications are seen in various glass stained/colored windows of numerous European churches.

An example of the recolored glass from medieval times is shown in Figure 6. Thereafter, science elucidated explanations for the formation of the different colors. The unmistakable clear hues were formed by controlling the state and more importantly, the size of the nanoparticles, namely, gold and silver. This relationship between particles and their related colors have been examined by Rafia Usma in 2015.

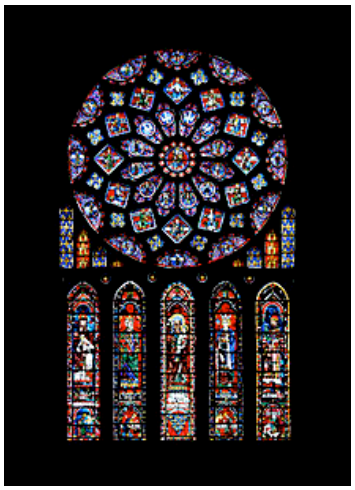

Figure 6: Rosacea stained glass in a Cathedral in France. Color changes depend on the size and shape of gold and silver nanoparticles (Usma, 2015). Red: Ag - 100 nm, triangle. Yellow: Au - 100 nm, spheres. Green: Au – 50 nm, spheres. Light Blue: Ag – 90 nm, spheres. Blue: Ag – 40 nm, spheres.

An outline of the foundation regarding nanoparticles is compressed in Table 2 (Horikoshi and Serpone 2013). The present innovation dealing with nanoparticles, basically nanotechnology, started from the uncommon optical wonder and the

foundation of a hypothesis describing the different physical phenomena that were followed resulting in the advancement of scientific instruments.

Table 3: Chronological table of the evolution of nanotechnology

Year	Remarks	Country/people
1676	Book published on drinkable gold that contains metallic gold in neutral media	J.von Lowenstern-Knuckel (Germany)
1718	Publication of a complete treatise on colloidal gold	Hans Heinrich Helcher
1857	Synthesis of colloidal gold	M. Faraday (The Royal Institute of Great Britain)
1908	Scattering and absorption of electromagnetic fields by a nanosphere	G. Mie (Germany)
1931	Transmission Electron Microscope (Oral *et al.*)	M. Knoll and E. Ruska (Technical University of Berlin)
1937	Scanning Electron Microscope (SEM)	M. von Ardenne (Forschungslaboratorium fur Elektronen-physik, Germany)
1960	Microelectromechanical systems (MEMS)	Igarashi (Toyota Central R&D Labs, Japan)
	Successful oscillation of a laser	T.H. Maiman (Hughes Research Laboratories, USA)
1974	Concept of nanotechnology proposed	N. Tangiguchi (Tokyo University of Science, Japan)
1976	Carbon nanofiber	M. Endo (Shinshu University, Japan)
1976	Amorphous silicon solar cells	D.E. Carlson and C.R. Wronski (RCA, USA)
1980	Quantum hall effect (Nobel Prize)	K. von Klitzing (University of Wurzburg, Germany)
1982	Scanning tunneling microscope (STM) (Nobel Prize)	G. Binnig and H. Rohrer (IBM Zurich Research Lab,., Switzerland)
1986	Atomic force microscope (AFM)	G. Binnig and H. Rohrer (IBM Zurich Research Lab, Switzerland)
1986	Three-dimensional space manipulation of atoms demonstrated (Nobel Prize)	S. Chu (Bell Lab, USA)
1987	Gold nanoparticle catalysis	M. Haruta (Industrial Research Institute of Osaka, Japan)
1991	Carbon nanotubes discovered	S. Iijima (NEC Co., Japan)

Materials at the small scale change the mechanics of atomic collaborations and this permits a great deal more sensitivity and permeability and empowers a more select way of interaction. Nanoparticles, which are the devices of nanotechnology, are produced chemically and research has moved towards producing nanoparticles using biological

preparations. Diverse biological systems including bacteria, fungi or plants, produce distinctive nanoparticles with varying behavior patterns. These particles are delivered extracellularly and are very steady and crystalline in solution (Gericke and Pinches 2006). Shape and size of these nanoparticles can be controlled by changing the parameters, for example, temperature and pH of the reactions. These qualities determine the properties of nanoparticles (Mohanpuria, Rana and Yadav 2008).

Nanoparticles likewise have a wavelength below the basic wavelength of light which renders them transparent and is a property that makes them exceptionally valuable for application in coatings, pressings and cosmetics products (Thakkar *et al.* 2010). Amid the previous decade, research in areas of nano science and nanomaterials have expanded in view of the acknowledgment that these small materials can be utilized for a huge number of mechanical and biochemical procedures (Oberdorster 2001).

Research in biology, material science and engineering drives investigation of the nanotechnology world. The design and synthesis of nanoparticles have filled the development of nanotechnology. There is enthusiasm for exploiting nanoparticles in different biochemical applications since their size is like that of biological particles (proteins and DNA) and structures (bacteria and viruses). Properties can be embedded into their configuration for control or detection of biological structures or systems (Chitrani, Ghazani and Chan 2010).

Nanoparticles have been found to increase lifespan and expand the therapeutic capability of drugs in the pharmaceutical industry. Be that as it may, some nanoparticles, for example, silica, titanium dioxide and zinc dioxide give rise to public concern because of safety and risk related issues. Toxicity of nanoparticles on the human body is one of these concerns (Oberdorster 2001; Oberdorster, Oberdorster and Oberdorster 2005).

Nanoparticles have the same similar size as proteins, thus, they are suitable for use in bio-labeling and tagging. Here, the nanomaterials are joined to bio-inorganic interfaces like antibodies or collagen that will render the nanoparticles biocompatible for use. As optical discovery strategies, nanoparticles will either change their optical properties or fluoresce. Their utilization has likewise been powerful in cancer treatment where the nanoparticles anticipate reactions of photodynamic cancer treatment. The dye that

causes the symptoms gets to be caught/ encased inside porous nanoparticles (Roy *et al.* 2003).

Progress has been made in the amalgamation of metallic nanomaterials, especially aiming to achieve materials that have the craved qualities as far as molecule size, elemental composition, shape and chemical stability, making them priceless for some applications (Peng and Yang 2009). Various systems have been defined before to incorporate noble metal nanoparticles, for example, gold, silver and platinum, with various sizes, compositions and controlled mono-dispersity.

Another conceived application for nanotechnology to medicine is in nanosurgery, an advancement of microsurgery. The advancement of semi self-sufficient robots that can be discharged into the blood, through which will travel to the wanted site and complete the required repair work. Cameras are embedded so they can travel through the digestive tract taking directions from specialists to take pictures of destinations of interest (Ramsden 2005). Metallic nanoparticles can be appended to single strands of DNA which open up avenues for medical applications. They can likewise transverse through veins and confine any objective organ which can possibly prompt novel therapeutic imaging and biomedical applications (Thakkar *et al.* 2010).

All the more essentially, nanomaterial has been utilized as a part of the universe of biology or medicine and these have included drug and gene delivery, bio-detection of pathogens (Edelstein *et al.* 2000), tissue building (Yoshida and Kobayashi 1999) and for early discovery and treatment of cancer. Some of which incorporate gold nanoparticles for biomedicinal application (Cai *et al.* 2008), silver nanoparticles for their cytotoxic impact against tumor cells (Devi and Bhimba 2012) and because of their extraordinary and adaptable properties, carbon nanotubes have indicated promise to enhance the properties of tissue designing platforms, drug delivery and imaging of engineering tissues (Paratala and Sitharaman 2011).

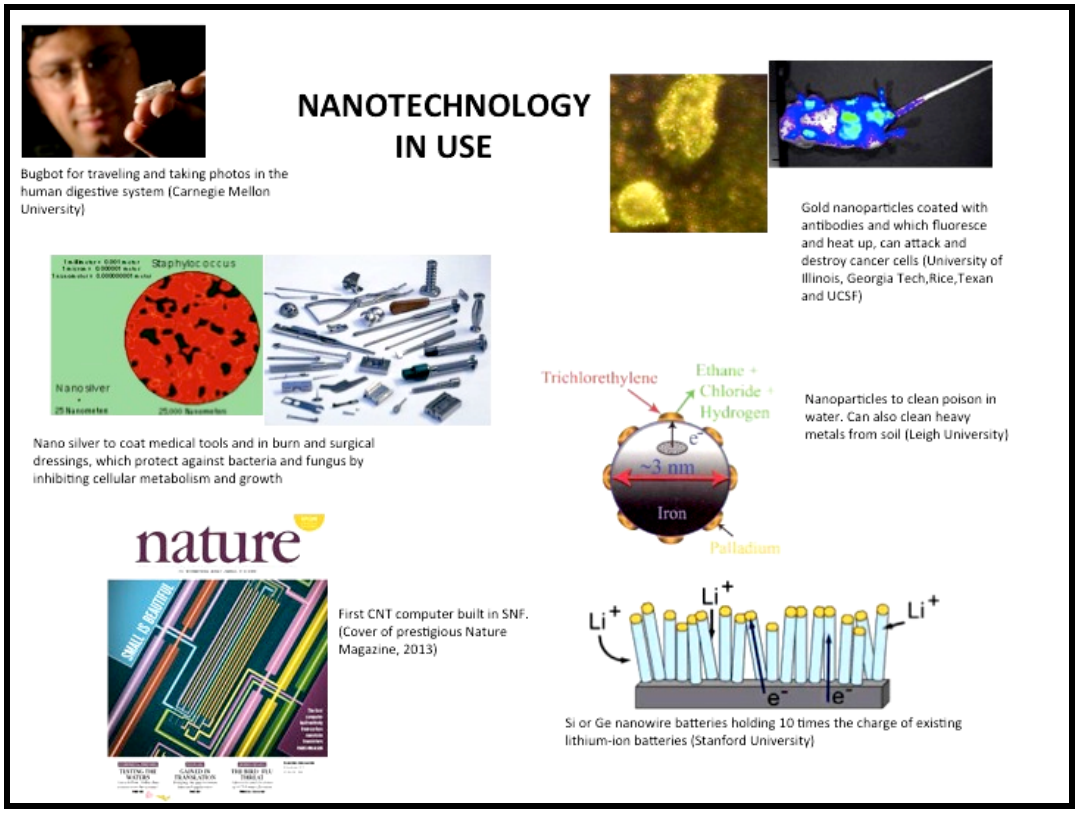

Figure 7: Nanotechnology in use. Examples of nanotechnology in various types of industry (Usma 2015; Dash 2005)

2.4 Size Control of Nanoparticles

The chemical and physical properties of nanomaterials depend on their composition as well as on the molecule size and shape (Heinglein 1989). A high-quality synthesis should first give control over molecule size and shape. For instance, if bigger Au (Gold) nanoparticles are synthesized, the surface plasmon resonance will be steadily moved from 530 nm to the more drawn out wavelength side. Hence, if nanoparticles contrast in size, their optical attributes will likewise change essentially (Marzan 2006).

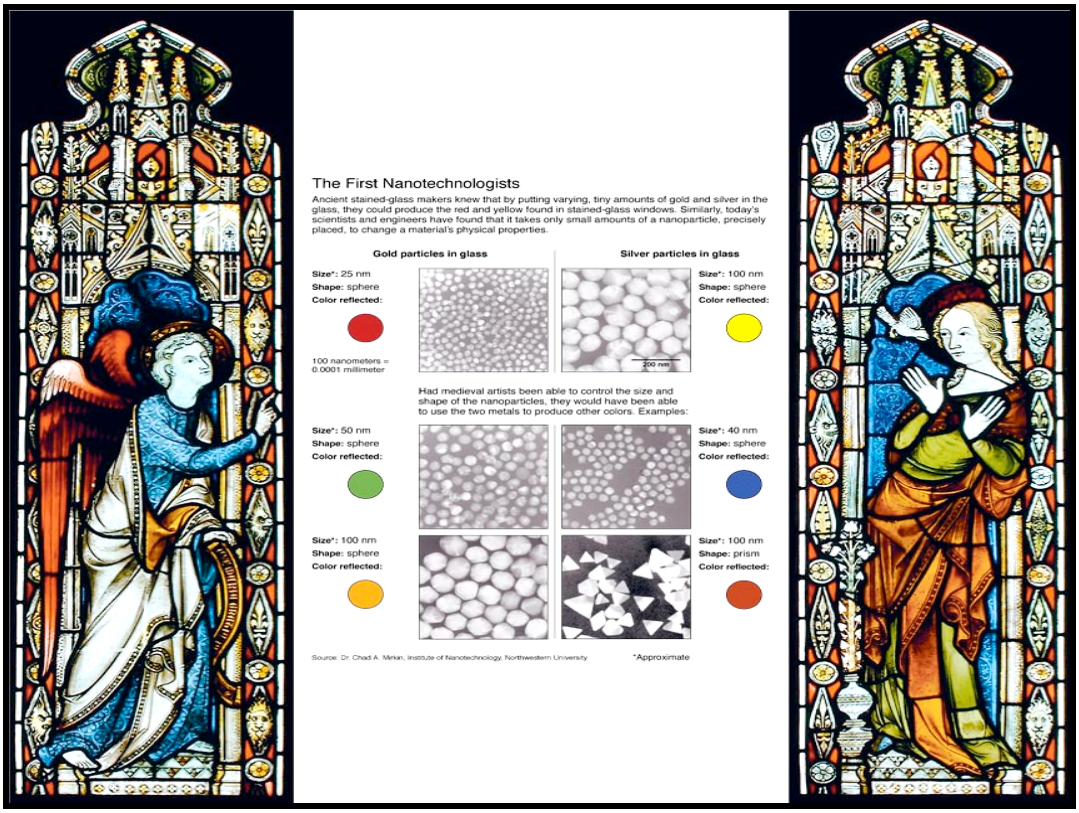

Figure 8: The First Nanotechnologists (Mirkin 2005)

Figure 8 represents how stained glass creators knew they would deliver the red and yellow in glass windows by including differing little measures of gold and silver to glass. Also, in late revelations, researchers have observed that only a small measure of nanoparticles are needed to change the physical properties of a material.

In optical uses of nanoparticles, simplification of the size dissemination of the particles turns into an imperative element. Thus, it is important to manufacture nanoparticles in light of a solitary target size. For the most part, with a specific end goal to plan mono-scattered nanoparticles, it is important that the nanoparticles grow slowly after the fast generation of the seed particles (Sugimoto 2000). In the event that the size of the nanoparticles diminishes (i.e., increase in specific surface area), then the expansion in the surface energy of such nanoparticles will encourage their aggregation. Thus, after their development of the ideal size, it will be important to stabilize the particulate surface by adding a dispersing agent (Mohanpuria, Rana and Yadav 2008).

Nanoparticles have been broadly contemplated as potential multifunctional carriers for imaging and drug delivery applications (Farokhzad and Langer 2006). To achieve the

objective diseased site, nanoparticles need to marginate toward the vascular wall, communicate with the receptors on the vascular surface, thereafter binding to the target area. The concentration of the drug at the objective site ought to be sufficiently high to kill the diseased cells with negligible side effects, hence making nanoparticle size and distribution critical in assessing therapeutic efficacy (Mathiowitz *et al.* 1997).

Nanoparticle distribution has been thought to be an important scientific factor in nanoparticle drug delivery. Assessment of nanoparticle distribution is extremely unpredictable on the grounds that it can be affected by different factors including molecule shape, size, surface area and flow conditions (Sanhai *et al.* 2008) and (Li *et al.* 2014).

As a major impact factor, size effect on distribution has been broadly studied. For instance, smaller NPs with sizes in the range of around 1nm and 200 nm are perfect for tumor delivery because of the enhanced permeability and retention (EPR) impact (Stolnik, Illum and Davis 1995; Mitragotri and Lahann 2009). A recent study likewise discovered that long worm-molded filo micelles might improve the circulation time in rodents (Geng *et al.* 2007) and are more effective in tumor stroma inhibition (Christian *et al.* 2009).

2.5 Platinum Nanoparticles

Platinum is one of the rarest and most costly metals. Known as 'Pt' on the periodic table, it has high erosion resistance and various catalytic applications including automotive exhaust systems and petrochemical cracking catalysts.

Platinum nanoparticles are typically utilized as a type of colloid or suspension in a fluid and they are extensively studied because of their antioxidant properties (Azonano 2013).

Platinum and platinum-based molecules as nanoparticles have been widely utilized in numerous applications, for example, coatings and plastics, polymer membranes, magentic nanopowders, catalysts in energy components, glucose sensors, gas sensors and cancer therapy. The properties of platinum nanoparticles rely upon molecule size, composition, shape and structure, which can be controlled during synthesis. Some of

which incorporate chemical reduction, polyol technique, green synthesis, gamma illumination and the photoreduction process (Gharibshahi and Saion 2012).

Figure 9: Platinum Nanoparticles in ready to use (Particular 2015)

According to Islam, Bhuiya and Islam (2014), platinum nanoparticles have been synthesized using a number of different techniques which include: chemical synthesis of platinum nanoparticles using hexacholoroplatinic acid and a stabilizing agent, sodium borohydride. This method results in size controlled naoparticles. Another method is growth controlled synthesis where the nanoparticles aggregate and form a nucleus with critical size and stability. This growth control process can be broken up into three methods:

Firstly, impregnation method which is characterized by depositing platinum precursors and thereafter a reduction step. This is a common method used for electrocatalyst preparation.

Secondly, the most common, colloidal method, involves the synthesis of metal nanparticles followed by the reduction step and a stabilizing agent with the use of capping agents that prevent aggregation. This method involves coating the nanoparticles with organic molecules/capping agents that can provide stabilzation. These include PVP and PVA.

Lastly, synthesis by microemulsion method, where controlling particle size, shape and distribution is possible.

Studies have demonstrated that receptive oxygen species (Grosse *et al.* 2009) are thought to be included in sign transduction pathways (Finkel 1998). However, they join

in the onset and progression of numerous diseases when they are overproduced. The primary tissue to produce ROS, particularly O_2^-, is the Mitochondria. O_2^- produced in mitochondria is a key ROS in controlling the redox state in the body. Oxidative stress, dysfunction of mitochondrial and the suppression of mitonchondrial electron transport complexes are included in the pathogenesis of human diseases, for example, atherosclerosis, inflammation, neurodegenrative illnesses and hepatitis. Treatment using antioxidants is one effective therapeutic treatment for these neurodegenerative illnesses (Beal 2005).

Another study has demonstrated that platinum nanoparticles have an activity like that of oxidizing NADH and decreasing CoQ. It was additionally recommended that platinum nanoparticles can copy part of the enzymatic elements of the Complex 1 and this study showed that if platinum nanoparticles could be conveyed to proper sites in the mitochondria, they could be valuable in the therapeutic treatment of oxidative anxiety illnesses (Turrens 2003).

In a similar study, it was demonstrated that platinum nanoparticles adequately protects against UV-induced inflammation by diminishing ROS generation and repressing apoptosis in keratinocytes (Yoshihisa *et al.* 2010).

Teow and Valiyaveeti, 2010, reported that platinum nanoparticles capped with polyvinyl pyrrolidone (PVP) were observed to be less lethal to MCF 7 breast cancer cells than platinum nanoparticles capped with folic acid. It was additionally reported that the cell viability was only influenced after exposure to a high dosage of 400 $\mu g\ m\ell^{-1}$. Also, exposure to Pt-PVP did not decrease cell viability following 72 hours. To enhance solubility in water, soluble molecules like PVP to cap the surface of nanoparticles can be used.

Using these reasons and its small scale ratio platinum nanoparticles were chosen for this study.

The hydrophilic polymer, PVP, can intensify stability and water distribution in cell culture medium and keeps singular particles from aggregating. It was noted that the stability of platinum nanoparticles determines the capping agents to be used and nanoparticles capped with PVP demonstrate high distribution and solubility especially

at increased concentrations in studies based on cell viability (Teow and Valiyaveeti, 2010).

A portion of the nanoparticles utilized as delivery vehicles for anticancer drugs incorporates paclitaxel, cisplatin, and oxaliplatin, which are platinum based drugs. Platinum-based chemotherapeutic drugs have a place with the most capable and broadly utilized agents in anticancer treatment (Jelinkova *et al.* 2014). Cisplatin, carboplatin, and oxaliplatin are effective against a scope of tumors, however, these compounds have shown toxicity toward healthy cells and have shown tumor resistance (Conde, Doria and Baptista 2012).

Cisplatin and its analogs are a standout amongst most classes of chemotherapeutic agents accessible for the treatment of tumors. However, they showcase extreme toxic reactions and cross-resistance, which are the significant downsides of the platinum-based drugs. It was specified that sulfur-containing biomolecules, for example, glutathione and metallothionein have a high affinity for platinum complexes and that these molecules bring about side effects and cross resistances of platinum complexes (Zhang *et al.* 2015b).

In addition, oxaliplatin as one of the leading platinum based antitumor drugs had been found to not be influenced in some cisplatin and carboplatin resistance tumors. They're in vitro cytotoxicity study demonstrated that platinum conjugated compounds exhibited selective toxicity towards some cancer cell lines.

Their application is regularly constrained by the undesired reactions and poor activity in some types of cancers because of their acquired resistance. These impediments bring out a quest for new platinum subsidiaries and better effectiveness with novel mechanisms of activity (Jelinkova *et al.* 2014).

2.6 Nanoparticles in cancer therapy

Extraordinary exploration has prompted a superior comprehension of cancer at the molecular, genetic and cellular levels. This has brought about techniques for expanding the anti-tumor capacity of drugs while diminishing unwanted secondary effects.

In cancer treatments, nanoparticulate delivery systems give better penetration of therapeutic drugs inside of the body at a lower risk in contrast with common/standard

cancer treatments. On account of their moderately little size, nanoparticle distribution results in extended movement times and its capacity to exploit tumor attributes. For instance, nanoparticulates under the size of 20 nm travel through cellular walls and these tiny particles allow for intravenous medication delivery and also subcutaneous injections (Praetorius and Mandal 2007). Moreover, nanoparticle size considers interactions with biomolecules within and on cell surfaces in a way that doesn't adjust the behavior and properties of those atoms (McNeil 2005).

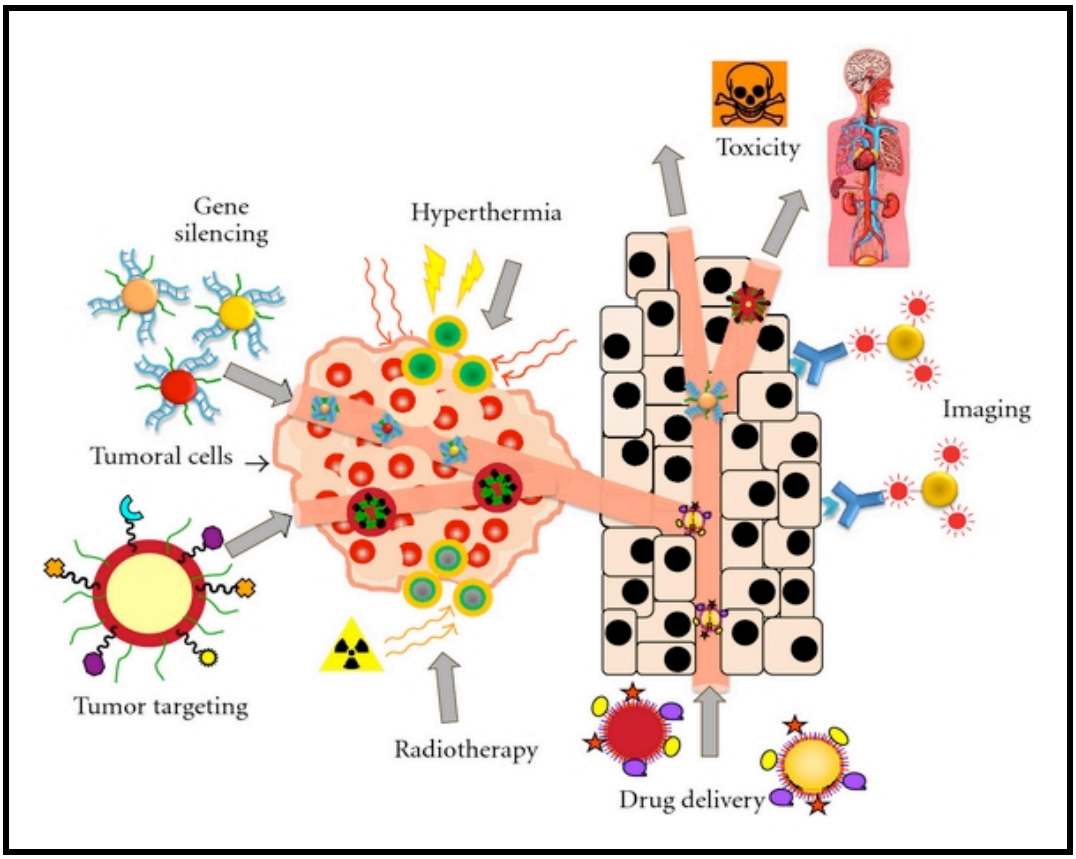

Figure 10: Metal nanoparticles for cancer therapy (Conde, Doria and Baptista 2012)

Figure 10 describes how metal nanoparticles play a role in cancer therapy (Conde, Doria and Baptista 2012). In their paper, it is discussed that once the tumor is associated with the principle blood circulation system, nanoparticles can exploit a few characteristics of the recently formed vasculature and productively target tumors.

Tumor cells are supplied by blood vessels that perfuse the cells of the tissue where nanoparticles would (i) passively accumulate or (ii) anchor through targeting on moieties to biomarkers overexpressed by tumor cells. It was also noted that

nanoparticles can act as therapeutic agents while simultaneously, inducing hyperthermia, silencing genes, improving radiotherapy and/or convey drugs to induce tumor cell death, and as imaging enhancers to help following the restorative impacts progressively.

Formulated from an assortment of materials, nanoparticles are designed to carry a variety of substances in a controlled and targeted way. They are built and designed to exploit cancer morphology and methods of advancement, for example, fast multiplication of cells, leaky tumor vasculature, and antigen expression. Nanoparticulate surface alterations incorporate linking or coating with proteins, folate, antigens, antibodies, enzymes, ligands, pH delicate agents and other substances. They are utilized as vehicles and synthesized to eliminate degradation of the load to be carried and shield substances being transported from coming into contact with any healthy cells. This decreases peripheral effects and increasing the relative measure of the load that reaches the diseased tissue (Praetorius and Mandal 2007).

Chemotherapeutic drugs are cytotoxic agents that are used to treat all types of cancers. They work by blocking elements of fast growing cells by disabling mitosis and additionally advancing apoptosis. Delivery of adequate concentrations of an effective drug to tumor cells without creating intolerable toxicity to the patient results in fruitful chemotherapy of cancer (Au and Wientjies 2006).

These tiny particles have turned out to be an effective delivery system for the delivery of anticancer drugs (Hosseinzadeh *et al.* 2012). In their study gemcitabine combined with nanoparticles were synthesized by a technique utilizing chitosan and a delivery vehicle. The cytotoxicity brought about by the nanoparticles was measured using a colon cancer cell line. A cytotoxicity test of gemcitabine conjugated nanoparticles demonstrated an increase in the cytotoxicity of gemcitabine implanted in the nanoparticles in correlation with drug alone. Their results recommend that nanoparticles could be considered as a proficient oral definition for colon cancer treatment.

Nanoparticles and their utilization in drug delivery is a more successful antitumor technique than routine chemotherapy, which is constrained by the toxicity of drugs to typical tissues, restricted solubility, and nonselectivity confining therapeutic efficacy (Li 2002).

2.7 Selective targeting by nanoparticles

The utilization of nanotechnology in pharmaceutical and all the more particularly drug delivery is set to increase rapidly. A huge number of substances are presently undergoing investigation for the synthesis of nanoparticles used as drug delivery systems. These include organic substances, phospholipids and gelatin, and more substances of a compound nature such as different polymers and metals that contain nanoparticles. Clearly, the potential interaction and toxicity with tissues and cells, extraordinarily relies on upon the actual composition of the nanoparticle definition (Zhu *et al.* 2016).

One of the profound difficulties in drug delivery is to transport the drug at the target site in the body in this manner avoiding potential toxicity to healthy organs or cells. This is particularly challenging in cancer therapy whereby the tumor might be confined as unmistakable metastases in different organs. It is the non-confined cytotoxicity of chemotherapeutics in this manner that limits the full utilization of the drug treatment potential. Drug conveyance or drug targeting results in expanded drug concentrations and gives strategies to more specific therapy. Interestingly, nanoparticles consist of particles as apparatuses to empower these strategies. Some of these advantages are for example, their small size, that permits infiltration of cell membranes, stabilization and binding of proteins, including escape of the lysosomal after endocytosis (Jong and Borm 2008).

Targeted drug delivery, which can convey drugs to particular organs or tissues, has been highlighted in cancer nanotechnology. These nanoparticles can target cells through the enhanced permeability and retention (EPR) impact resulting in the enhancement of anticancer efficacy These delivery systems use particular targeting agents for cancer cells minimizing the uptake of the anticancer agents by ordinary cells and improves their entry and retention in cancer cells (Di-Wen *et al.* 2015).

Either by size dependent passive targeting or either by dynamic targeting, nanoparticles can be conveyed to specific sites. Passive targeting is dependent on both tumor structure and the structure of encompassing inflamed tissues. Active targeting has achieved a high level of selectivity to specific tissues and improves the uptake of nanoparticles into target regions, for example, cancer cells. Nanoparticles are changed to target intrinsic

qualities of tumor cells, for example, quick proliferation and specific antigen presentation (Moghimi, Hunter and Morray 2001).

The change of nanocomposites through their therapeutic preparations using safe, cheap and aqueous synthesis strategies is key in diminishing their toxic quality. Specifically, gold nanoparticles can be orchestrated with exact size, surface chemistry and shape at the nanoscale level, promising enhanced safety in therapeutics (Ghodake *et al.* 2016).

Nanoparticle-based drug delivery systems have demonstrated huge focal points in the treatment of cancer in late decades in light of the fact that these systems might extend life, enhance the pharmacokinetics of therapeutic agents by expanding their uptake by tumor cells (Zhu *et al.* 2016).

2.8 The safety and quality of nanomedicines

In spite of its guarantee, there are still many concerns surrounding the quality and safety of these nanomedicines. The remarkable properties of nanoparticles, for example, high surface to volume ratio, reactivity, and minute size, that demonstrate valuable in targeting biological diseases, are likewise of concern when understanding the safety of these products. On the off chance that these particles are microscopic to the point that they can cross barriers of 100 nm in size, what is preventing them from voyaging past their proposed site(s) of treatment?

What are the toxicokinetic properties of nanoparticles in the human body, and in what capacity would we be able to guarantee that they display exact and safe absorption, circulation, metabolism and excretion?

Does bioaccumulation of nanoparticles force a greater risk to human wellbeing than the disease nanomedicines mean to treat?

Without performing preliminary examinations that are important to guarantee the safety of a product, nanomedicine headways will keep on sparking debate. While the U.S. Sustenance and Drug Administration has not distributed particular rules or prerequisites for the characterization of nanomedicines or other nanotechnology products, certain organizations have taken it upon themselves to comprehend the complexity of this field (Gerber 2004).

2.9 An introduction to apoptosis

Programmed cell death is of greek inception, carries the signifying "falling off or dropping off", similarly relates to the tumbling of leaves from trees or petals falling off flowers. Using this phrase, the similarity underlines that the death of cells is an indispensable and fundamental part of the cycle of life forms. This method of cell death is a dynamic and characterized process that assumes a critical part in the advancement of multicellular creatures and in the regulation as well as the upkeep of populations of cells in the tissue upon pathological and physiological conditions. More importantly, apoptosis is a well-defined and potentially a successive type of programmed cell death, but, non-apoptotic sorts of cell death likewise may hold biological significance (Leist and Jaatela 2001).

Programmed cell death was presented, suggesting that cell demise amid development is not accidental but rather takes after a succession of controlled strides prompting locally and transient obliteration (Lockshin and Williams 1964).

Apoptosis, otherwise called programmed cell death, typically plays a part in the improvement as well as the health of most multicellular life forms. Generally, cells die because of an assortment of activity they do in such in a regulated and controlled style. Amid programmed cell death, diminish in cell volume, nuclear changes with chromatin build-up, margination and fragmentation take place followed by cells blebbing and the breakdown of intact cells and nuclear membranes. This results in the formation of little fragmented apoptotic bodies containing cytoplasmic substance encompassed by the cell membrane, which is evacuated by the procedure of phagocytosis in the extracellular environment, keeping away from the inflammatory response. It is this process that differentiates apoptosis from necrosis. Necrosis is uncontrolled cell death prompts the loss of or breakage of the cell membrane, inflamatory reactions and conceivably, to serious health issues. By difference, programmed cell death is a procedure by which cells assume a dynamic part in their own death which is the reason programmed cell death is frequently alluded to as cell suicide (Trump *et al.* 1997).

Necrosis is an unintended procedure created by external cell damage by various stimuli. It is portrayed by the expansion in cell volume took after by amplification of cell organelles including the nucleus, loss of membrane integrity and the release of cellular

substance, which comprises of specific enzymes, for example, hydrolases that impact the adjoining cells prompting an inflammatory response in adjacent tissues (Lockshin and Zakeri 2001).

2.9.1 The significance of apoptosis

The advancement and upkeep of multicellular biological systems rely upon a sophisticated interaction between the cells forming the organism, it sometimes even appears to include an altruistic behavior of individual cells for the life form in general. Amid development, numerous cells are created in abundance which ultimately undergoes programmed cell death and in this manner add to sculpturing organs and tissues. These include the improvement of the conceptive organs (Meier, Finch and Evan 2000), development of the brain, amid which half of the neutrons initially created will die in later stages when the adult cerebrum is shaped (Hutchins and Barger 1998).

An especially informational sample for the ramifications of programmed cell death in animal advancement is the development of free and autonomous digits by massive cell death in the interdigital mesenchymal tissue (Zuzarte-Luis and Hurle 2002).

Apoptotic procedures are of biological significance. These include being involved in development, multiplication/homeostasis, differentiation, regulating and functioning of the immune system and in the evacuation of defect cells which are toxic cells. Accordingly, malfunction of the apoptotic system is embroiled in an assortment of pathological conditions. Imperfections in apoptosis could bring about cancer and spreading of viral infections as well as autoimmune diseases, while neurodegenerative disorders such as AIDS and ischaemic ailments are brought about by or enhanced by excessive apoptosis (Fadeel *et al.* 1999).

Because of its significance in such biological procedures, programmed cell death is a phenomenon, occurring in a wide range of metazoans (Tittel and Stellar 2000), for example, in mammals, creepy crawlies (Richardson and Kumar 2002), nematodes and cnidaria (Cikala *et al.* 1999). In addition, programmed cell death likewise may play a part in plant science (Solomon *et al.* 1999), and apoptosis-like cell demise mechanisms even have been detected and utilized as a model system in yeast (Skulachev 2002). Intriguing bits of knowledge into the origin and advancement of programmed cell death may possibly be given by the actuality, that programmed cell death is additionally a

vital part of the life cycle of eukaryotes and that even prokaryotes experience regulated cell demise (Ameisen 2002).

2.9.2 Morphological features of apoptosis and necrosis

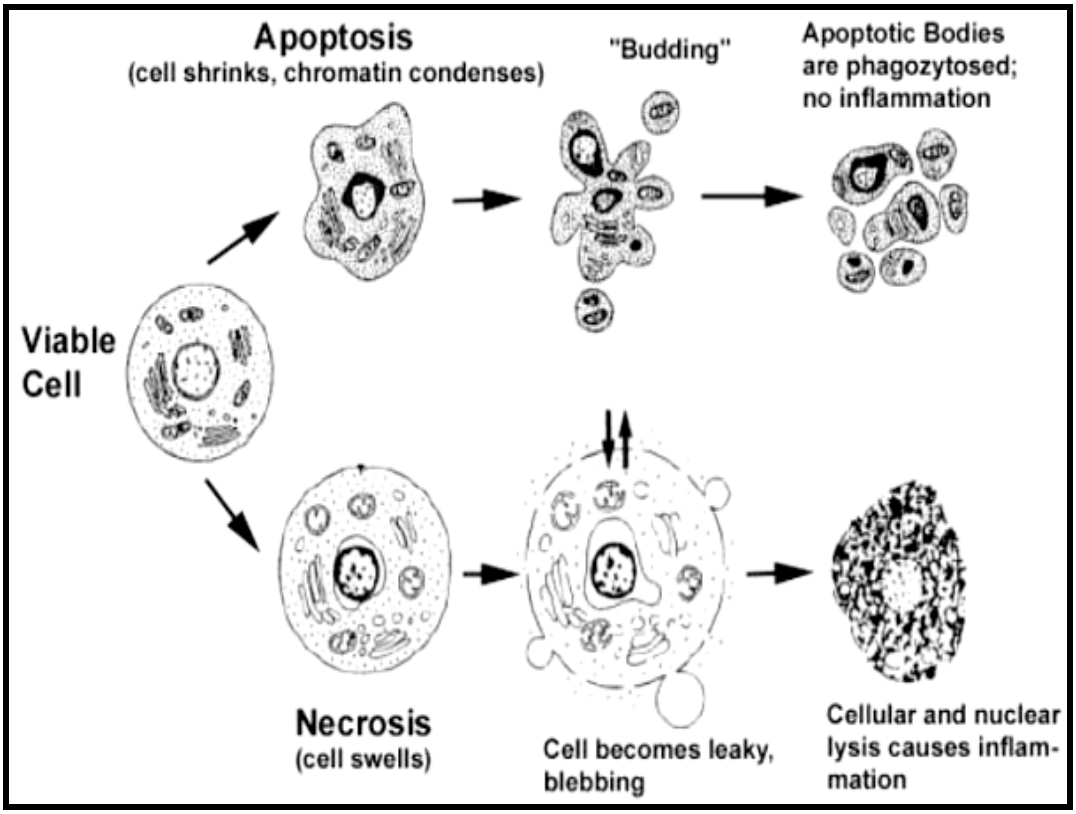

Figure 11: Apoptotic and necrotic cell death process (Cruchten and Broek 2002)

Apoptotic cells are readily be perceived by cliché morphological changes which include: shrinkage of the cell which demonstrates deformation and loss of contact with neighboring cells. The chromatin consolidates, accumulates near the nuclear membrane, plasma membrane blebbing and lastly fragmentation of the cell into small membrane-enclosed structures. These are called 'apoptotic bodies' and contain the condensed chromatin, organelles and cytosol (Figure 11). The bodies are immersed by macrophages resulting being expelled from the tissue and not resulting in inflammation. These morphological changes are a result of characteristic reactions taking place in an apoptotic cell, remarkably the initiation of proteolytic enzymes which intercede the cleavage of DNA into fragments and the cleavage of a huge number of protein substrates that generally decide shapes and integrity of the cytoplasm and/or the organelles (Saraste and Pulkki 2000).

Apoptosis, as opposed to the necrosis, brings about the loss of membrane integrity and the cell disruptures and swells. Amid necrosis, the cell contents are discharged uncontrollably into the cell's surroundings which bring about damage to cells resulting in inflammation in the tissue (Figure 11) (Leist and Jaatela 2001; Cruchten and Broek 2002).

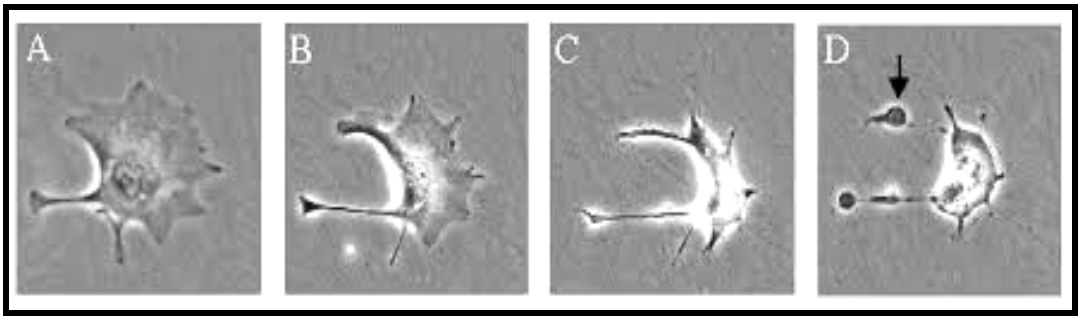

Figure 12: Morphology of an apoptotic trophoblastic cell captured by time-lapse microscopy taken over a 6 hour period (Dash 2005).

Apoptotic cells show particular morphology amid the apoptotic process, and this can be seen in Figure 12. Ordinarily, the cell starts to shrink taking after the cleavage of lamins and actin filaments in the cytoskeleton (A). The breakdown of chromatin in the nucleus frequently prompts nuclear condensation and the apoptotic cells take on a "horse shoe" like appearance (B). Cells keep on shrinking (C), packing themselves into a structure that takes into account their evacuation by macrophages. These phagocytic cells are in charge of clearing the apoptotic cells from tissues in a clean and tidy form that stays away from a number of the issues associated with necrotic cell death.

Apoptotic cells frequently undergo plasma membrane changes that trigger the macrophage reaction. One such change is the translocation of phosphatidylserine from within the cell to the external surface. The end phases of apoptosis are frequently described by the presence of membrane blebs (D) or blisters process. Little vesicles called apoptotic bodies are likewise observed (D, arrow) (Dash 2005).

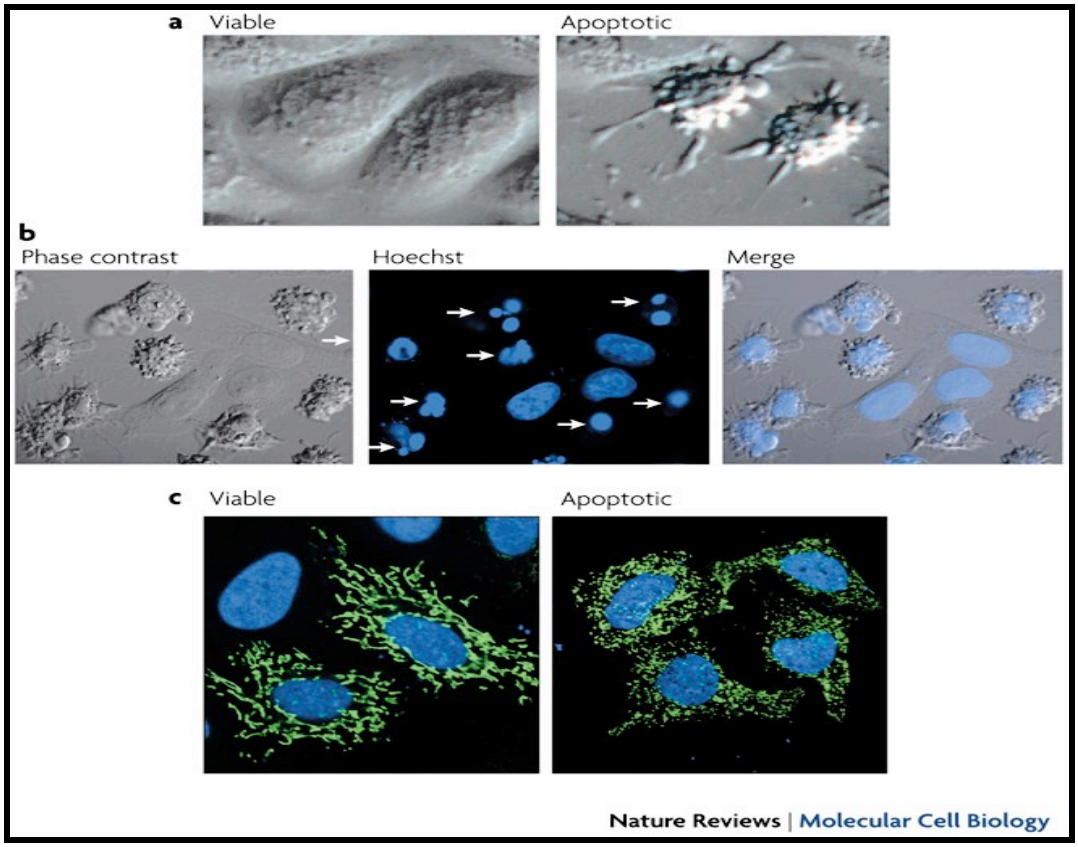

Figure 13: Morphology of apoptosis by Hela cells exposed to anticancer drug, daunrubicin. (a) Left panel show cells after 2h of exposure where cells appear healthy. Right panel show cells after 4,5 h of exposure with typical apoptotic morphology. (b) Features of apoptosis displaying condensation and fragmentation of the nucleus. Apoptotic cells bring about plasma membrane blebbing and nuclei are condensed or fragmented. (c) Fragmentation of mitochondria. Untreated cells shown on the left panel and cells treated with antinomycin D to induce apoptosis (right panel) (Delivani *et al.* 2006).

Taylor, Cullen and Martin in 2008 studied apoptosis using Hela cells exposed to daunrubicin, an anticancer drug. Figure 13 displays what happens during apoptosis which is similar to what happens during mitosis. They explained that cells initially become rounded following separation the from neighboring cells. This process usually brings about plasma membrane blebbing which form small vesicles called apoptotic bodies (Taylor, Cullen and Martin 2008).

2.9.3 Apoptotic signaling network

Apoptosis is firmly regulated and a very effective cell death program which requires the interchange of a large number of components. The segments of the apoptotic signaling system are genetically encoded and are thought to be for the most part set up in a nucleated cell prepared to be activated by a death prompting stimulus (Ishizaki *et al.* 1995; Weil *et al.* 1996).

Apoptosis can be activated by different stimuli from outside or inside the cell, e.g. by ligation of cell surface receptors, by DNA damage as a reason for imperfections in DNA repair mechanisms, treatment with irradiation or cytotoxic drugs, by an absence of survival signals, conflicting cell cycle signaling or by formative death signals. Death signs of such an assortment of origins appear to activate typical cell death machinery prompting the trademark features of apoptotic cell death (Weil *et al.* 1996).

2.9.4 The role of mitochondria in apoptosis

Mitochondria play an imperative part in the regulation of cell death. They contain pro-apoptotic proteins, for example, Apoptosis Inducing Factor (AIF), Smac/DIABLO and cytochrome C. These components are discharged from the mitochondria taking after the development of a pore in the mitochondrial membrane called the permeability transition pore, or PT pore. These pores are formed through the activity of the pro-apoptotic individuals from the bcl-2 group of proteins, which thusly are initiated by apoptotic signals, for example, cell stress, free radical damage or growth deprivation. Mitochondria additionally assume a critical part in intensifying the apoptotic signaling from the demise receptors, with receptor enrolled caspase-8 actuating the pro-apoptotic bcl-2 protein (Bauer *et al.* 1999).

2.9.5 The role of caspases in apoptosis

The term caspases come from cysteine-dependant aspartate proteases: their catalytical action relies on upon a basic cysteine residue inside a profound dynamic site pentapeptide QACRG, and the caspases particularly cleave their substrates after Asp deposits (Richardson and Kumar 2002).

Caspases are a group of proteins that are one of the principle agents of the apoptotic process. They are catalysts or enzymes known as cysteine proteases and exist inside the

cell as inactive pro-structures or zymogens. These zymogens can be cleaved to form dynamic enzymes after the induction of apoptosis.

Apoptosis is induced by means of death receptors and normally brings about the actuation of an initiator caspase, for example, caspase-8 or caspase-10. These caspases can then initiate different other caspases in a cascade. This cascade, in the end, prompts the enactment of the effector caspases, for example, caspase-3 and caspase-6. These caspases are in charge of the cleavage of the key cell proteins, for example, cytoskeletal proteins, that results in morphological changes in cells experiencing apoptosis (Denault and Salvesen 2002).

In the cell, caspases are incorporated as inactive zymogens, the alleged procaspases, which at their N-terminal convey a prodomain followed by a two subunits which are isolated by a linker peptide. Upon development, the procaspases are proteolytically developed between the two subunits, bringing about a small and a large subunit. The prodomain is evacuated amid the reactivation process frequently but not necessarily. A heterotetramer comprising of two small and two expansive subunits then forms an active caspase.

The pro-apoptotic caspases can be separated into initiator caspases including procaspases-2, 8, 9 and 10, and into executioner caspases which include procaspases-3, 6, and 7. While the executor caspases have just short pro-domains, the initiator caspases have long pro-domains, containing death effector domains on account of procaspases-8 and 10 or caspase recruitment domains (CARD) as on account of procaspase-9 and procaspase-2. By means of their pro-domains, the initiator caspases are selected to and actuated at death instigating signaling complexes either in light of the ligation of cell surface demise receptors (extrinsic apoptosis pathways) or because of signals starting from inside the cell (intrinsic apoptosis pathways) (Earnshaw, Martins and Kaufmann 1999).

2.9.6 Extrinsic and intrinsic apoptotic pathways

To date, research demonstrates that there are two principle apoptotic pathways: the extrinsic also known as the death receptor pathway and the mitochondrial or intrinsic pathway. Research shows that these two pathways are connected and that particles in either pathway can impact the other (Igney and Krammer 2002).

An additonal pathway exists which includes T-cell mediated cytotoxicity and perforin granzyme dependant execution of the cell. This perforin/granzyme pathway can affect apoptosis by means of granzyme B.

The extrinsic and granzyme B pathways merge on the same terminal or execution pathway. This pathway is started by the cleavage of caspase-3 and results in DNA discontinuity, degradation of nuclear proteins and the cytoskeletal, cross linkage of proteins, the arrangement of apoptotic bodies, articulation of ligands for phagocytic cell receptors and lastly the uptake by phagocytic cells.

The granzyme A pathway enacts a parallel, cell death pathway which is caspase-independent by means of damaging single stranded DNA (Martinvalet, Zhu and Lieberman 2005).

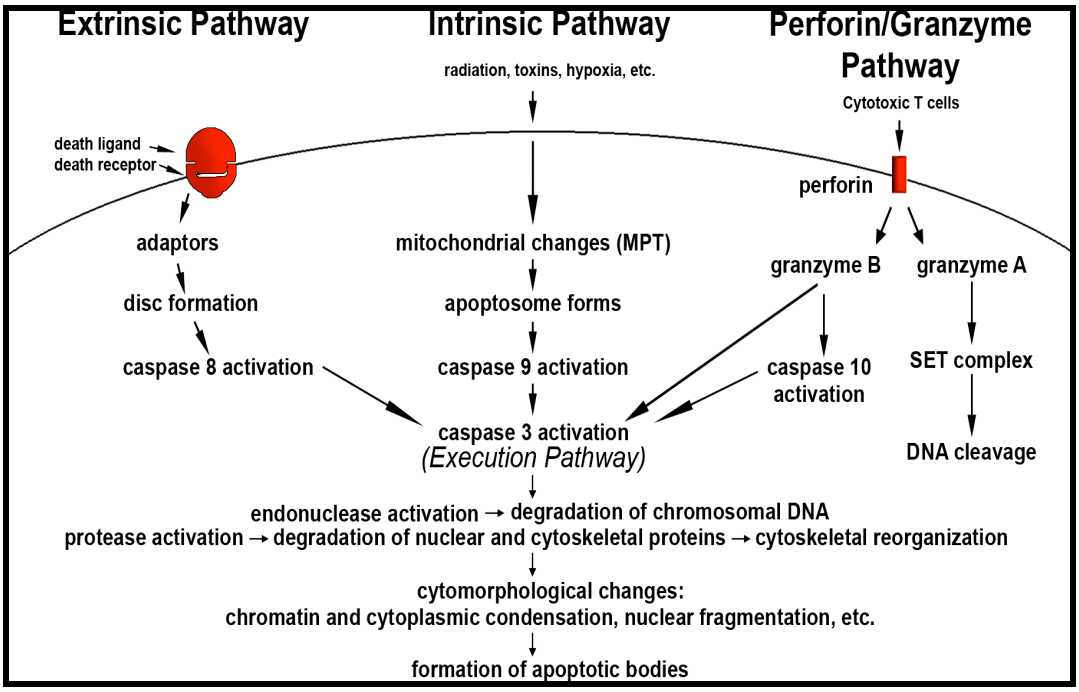

Figure 14: Schematic representation of apoptotic events. The two main pathways of apoptosis are extrinsic and intrinsic as well as a perforin/ granzyme pathway (Elmore 2007)

In the above scheme (Figure 14), each pathway requires particular activating signals to start an energy dependent course of molecular events. Each pathway actuates its own particular initiator caspase (8, 9, 10), which thus will enact the executor caspase-3. Be that as it may, granzyme A works in a caspase free form.

The execution pathway results in trademark cyto-morphological features which include cell shrinkage, chromatin condensation, the formation of blebs, apoptotic bodies and lastly phagocytosis of the apoptotic bodies by nearby parenchymal cells, neoplastic cells or macrophages (Elmore 2007).

2.9.7 Caspases and chromatin breakdown

One of the signs of apoptosis is the cleavage of chromosomal DNA into nucleosomal units. The caspases assume an imperative part in this procedure by initiating DNases, repressing DNA repair enzymes and separating basic proteins in the nucleus. The role of the caspases in the breakdown of chromatin is represented in Figure 15.

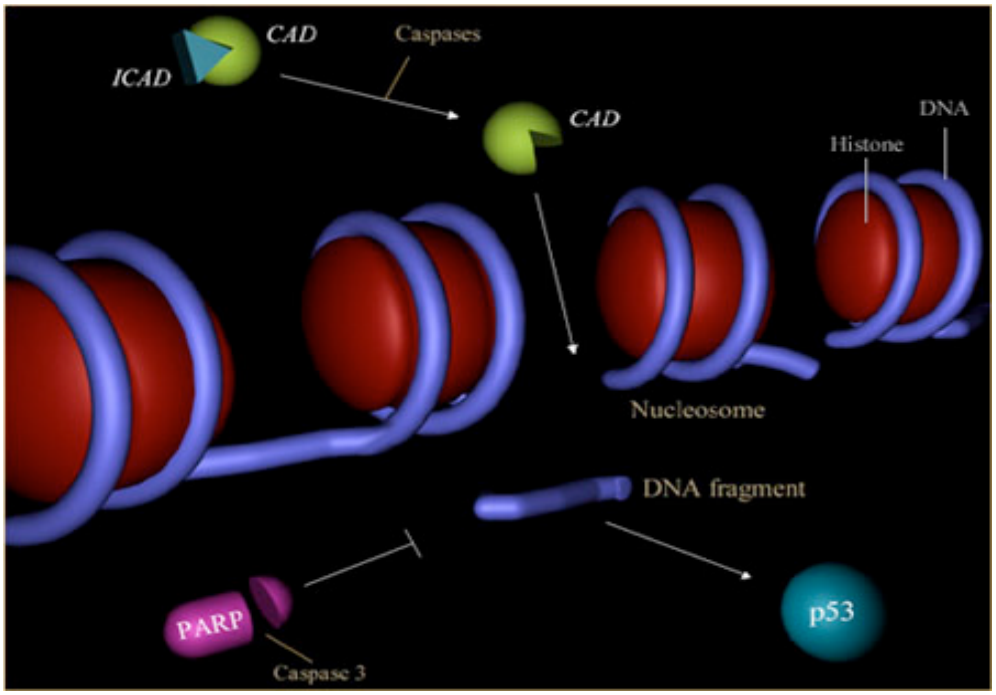

Figure 15: Breakdown of chromatin during apoptosis (Dash 2005)

1) Inactivation of catalysts included in DNA repair. The compound poly (ADP-ribose) polymerase, or PARP, is an imperative DNA repair enzyme and was one of the principal proteins recognized as a substrate for caspases. The capacity of PARP to repair DNA damage is averted taking after cleavage of PARP by caspase-3.

2) Breakdown of structural nuclear proteins. Lamins are intra-nuclear proteins that maintain the nucleus shape and intercede interactions between the chromatin and the nuclear membrane. Defragmentation of lamins by caspase 6 results in the chromatin

3) Fragmentation of DNA. The fragmentation of DNA into nucleosomal units is brought on by an enzyme known as CAD, or caspase activated DNase. Ordinarily, CAD exists as an inert complex with ICAD (inhibitor of CAD). Amid apoptosis, ICAD is cleaved by caspases, for example, caspase 3, to discharge CAD. Rapid fragmentation of the nuclear DNA takes after condensation and nuclear discontinuity (Dash 2005).

A number of studies recommend that defects along apoptotic pathways assume a critical part in carcinogenesis and that numerous new treatment strategies focusing on apoptosis are achievable and might be utilized as a part of the treatment of different sorts of cancers. Some of these revelations are preclinical while others have as of now entered clinical trials. A large number of these new operators or treatment strategies have likewise been incorporated into combined therapy including traditional anticancer drugs in a few clinical trials, which might enhance presently accessible treatment modalities.

In any case, some puzzling and troubling inquiries, for example, whether these treatment systems affect resistance in tumors and whether they will bring about ordinary cells to die in large numbers still stay unanswered. This is a genuine concern if lessons somehow happened to be learned from the routine anticancer drugs, which wipe out both healthy cells and tumor cells and cause ruthless side effects and tumor resistance (Wong 2011).

2.9.8 Analysis of cell death pathways using flow cytometry

Recent advances in both cellular and molecular biology aid in the investigation of cells. This includes their capacity, the structures, cooperations within their environment, division, life cycle and death. These characteristics helped scientists pick up a more profound comprehension of diseases. Propelling comprehension of the functioning of cells, cell populations and the contrasts between different types of cells lies crucial in investigating cell disease and biology.

Flow cytometry, plays a critical part in facilitating such understanding and is an advanced innovation for counting, sorting and examining cells. Just as imperative to this development was the parallel revelation of antibodies which permitted scientists to recognize and label particular populations of cells (Biosciences 2012).

CHAPTER 3: METHODOLOGY

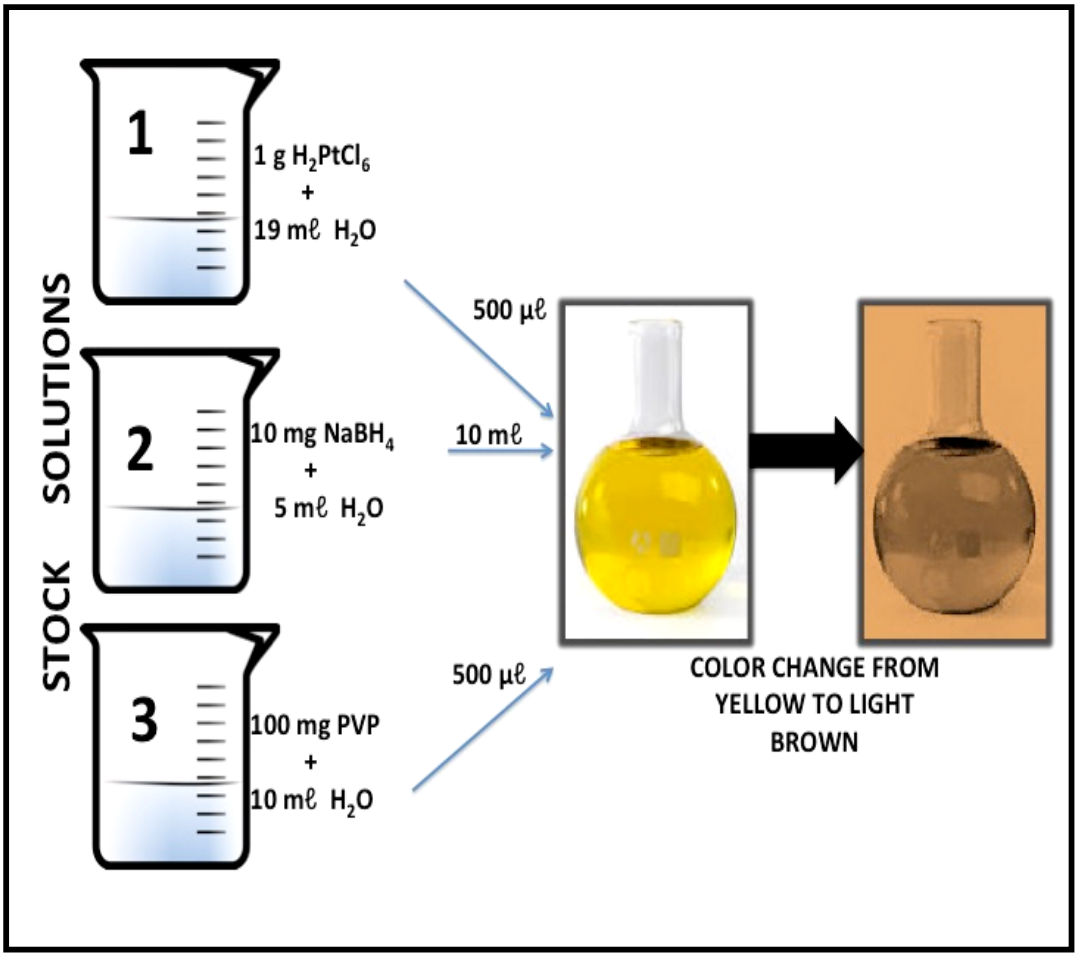

Figure 16: Synthesis of platinum nanoparticles

3.1 Synthesis of platinum nanoparticles

Platinum nanoparticles were synthesized as per Teow and Valiyaveeti (2010) with some modifications. Hexachloroplatinic acid hexahydrate (37.50 % as Pt), $H_2PtCl_6.6H_20$, sodium borohydride, $NaBH_4$ (98 %) and PVP with an average molecular weight 10 000, were purchased from Sigma-Aldrich.

Platinum nanoparticles were synthesized via the reduction of Hexachloroplatinic acid (Figure 16). Three stock solution were made from Hexachloroplatinic acid (stock solution 1), sodium borohydride (stock solution 2) and PVP (stock solution 3). 1 g of $H_2PtCl_6.6H_20$ was added to 19 mℓ of distilled water to make 100 mM stock solution 1. Stock solution 2 was made by adding 10 mg sodium borohydride to 5 mℓ distilled

water. 100 mg PVP was added to 10 mℓ distilled water to make stock solution 3. 500 µℓ of stock solution 1 was reduced under constant stirring with the addition of 10 mℓ PVP stock solution 3. Lastly, 500 µℓ sodium borohydride was added to the colloidal solution. The color of the solution changed from yellow to light brown during the reduction process, resulting in the formation of platinum nanoparticles. Stirring continued for a further 2 hours at room temperature for the reaction to complete and stabilize. Samples were then transferred into Eppendorf tubes and refrigerated at 4 ^{0}C.

PVP, a hydrophilic polymer, enhances stability and water dispersion in cell culture medium. Teow and Valiveeti reported that using PVP as a capping agent reduced the need for adding a capping agent or organic solvents which can be toxic to the cells.

PVP, also a water-soluble polymer and has a large number of consumer uses mainly due to its biological compatibility, adhesive characteristics, film forming, low toxicity and its resistance to thermal degradation when in solution (Giri *et al.* 2011).

3.2 Synthesis of gemcitabine conjugated platinum nanoparticles

Gemcitabine was purchased from Sigma-Aldrich. The gemcitabine conjugated platinum nanoparticles was synthesized following the procedure as per (Tom *et al.* 2004). A 0.0025 M aqueous stock solution was prepared by dissolving 0.0083 g of gemcitabine into 10 mℓ of distilled water. After filter sterilizing, the stock solution was then refrigerated overnight at 4 ^{0}C. To prepare the conjugate, 5 mℓ of 0.0025 M gemcitabine stock solution was added to the nanoparticle solution (as prepared in 3.1) in a 250 mℓ glass beaker. The solution was stirred for 6 hours with a color change from light to dark brown.

3.3 Characterization of platinum nanoparticles and gemcitabine conjugated platinum nanoparticles

3.3.1 UV-Visible spectrophotometric analysis

Platinum nanoparticles were characterized using UV/Visible spectroscopy at the Durban University of Technology. Samples were observed at a range between 100 nm and 800 nm using a Varian-Carey UV-Vis spectrophotometer. The wavelength at the maximum of the absorption band will give information about the structure of the molecule or ion and the extent of the absorption is proportional to the amount of the species absorbing

the light (Skoog, West and Holler 1992; Kenkel 1994). The best way of following the conversion to platinum nanoparticles is by monitoring the UV-Vis absorption spectra of the solution.

3.3.2 Transmission electron microscopy

Platinum nanoparticles and the gemcitabine conjugated platinum nanoparticles structure, morphology, size, distribution and arrangement was observed using a JEOL model JEM-2100 transmission electron microscope at CSIR (Pretoria), The National Center for Nanostructured Materials. All samples were transported on ice and refrigerated at 4 ^{0}C at the facility. For observation, a drop of each sample was added to a carbon-coated copper grid and allowed to air dry. The samples were then viewed using the microscope at the highest magnification. Measurements of particles observed were taken from different areas on the copper-coated grid for both samples.

3.4 The effect of platinum, gemcitabine and gemcitabine conjugated platinum nanoparticles on cancer cells and healthy cells

Procedures including cell maintenance and sub-culturing, storage, regeneration, enumeration and MTT assay were carried out as per Mossman (1983), with some modifications.

3.4.1 Cell Lines

Three cell lines were used In this study. MCF-7, a breast cancer cell line, UCAA-62, a melanoma cell line and a healthy cell line, PBMC (human peripheral blood mononuclear) (Figure 17). MCF-7 and UCAA-62 were supplied by Natasha Kolesnikova from the Council for Scientific and Industrial Research (Bioscience, CSIR). When cells reached 80 % confluency they were transferred into 75 cm^2 flasks and incubated at 37 ^{0}C in a humidified incubator containing a 5 % CO_2 atmosphere.

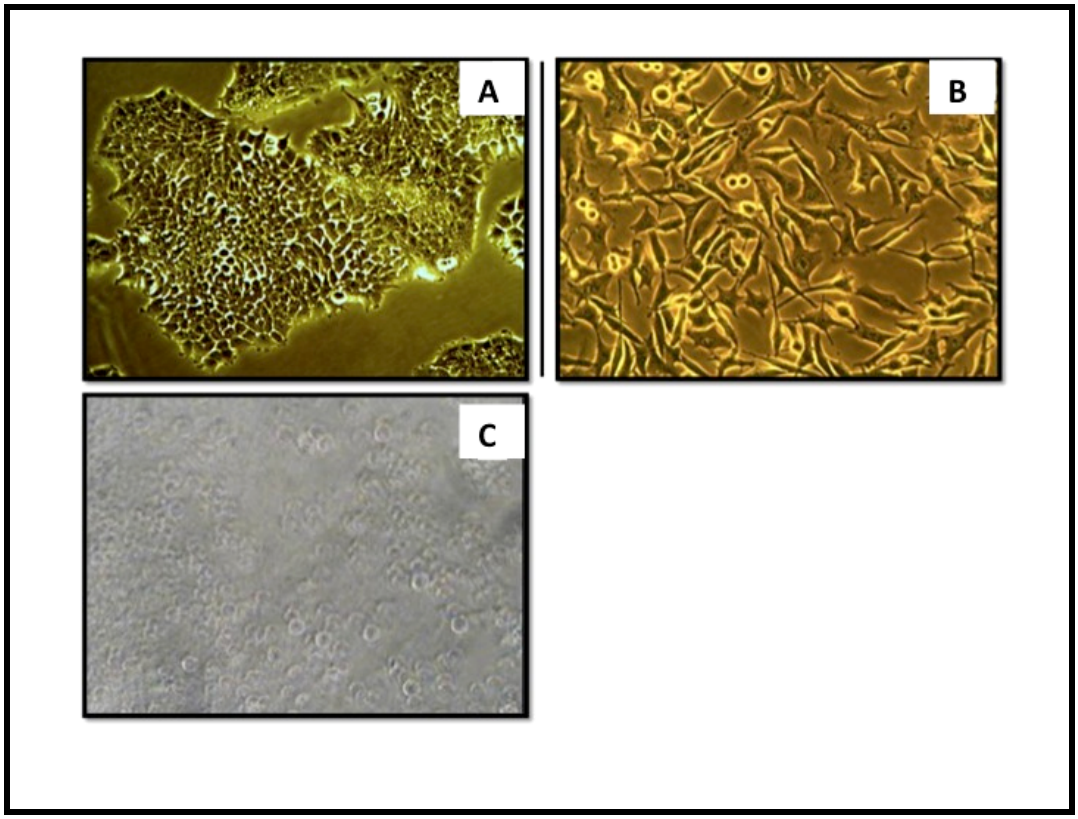

Figure 17: Microscopic view of cell morphology MCF-7 (A), Melanoma UCAA-62 (B), and Human Peripheral Blood Mononuclear cells (PMBC) (C) respectively. Morphology observation shows spindle-shaped cells at 10X magnification.

3.4.2 Cell Maintenance and Sub-culturing

In order to obtain an aseptic environment, all cell culture experiments were carried out in a laminar flow cabinet (Scientific Engineering, INC). Prior to work done, sterilization was done by exposing the laminar flow to UV light for approximately 30 minutes and frequently using 70 % ethanol (Merck, South Africa) during experiments. Dulbecco's Modified Eagle Medium (DMEM) was used to grow cells in two monolayers. DMEM was purchased from Sigma-Aldrich and contains 4.5 $g\ell^{-1}$ of glucose, 1 mM sodium pyruvate, and 1 mM L-glutamine. Additionally, the medium was supplemented with 1 % penicillin/streptomycin antibiotic and 10 % heat inactivated fetal calf serum. Cells were maintained every two to three days when confluency was reached to atleast 80 %.

To subculture cells, the medium was removed from the flask followed by washing the monolayer of cells with phosphate saline buffer (PBS). A 1 $m\ell$ aliquot of trypsin was added to flasks followed by incubation at 37 ^{0}C for approximately three minutes in a

humidified incubator with 5 % CO^2. To detach the cells, the flask was gently tapped on the side for 30 seconds. 10 mℓ of fresh supplemented media was then added to the flask and 1 mℓ of cell culture was sub-cultured to each flask. Finally, 20 mℓ of fresh supplemented medium was added to each flask followed by incubation at 37 ^{0}C in a humidified incubator containing 5 % CO_2. Monitoring of cells was done daily and medium color and turbidity changes were noted during the incubation period. The cell growth was examined and monitored using an inverted Nikon microscope.

3.4.3 Storage of cells

Only cell culture flasks that were atleast 80 % confluent were used. Cells were washed with 5 mℓ PBS following trypsinization as described in 3.4.2. Thereafter, 10 mℓ of DMEM was added and the cells were then centrifuged at 1500 rpm for 10 minutes. The pellet formed during centrifugation was then resuspended in 2 mℓ of Cryoprotective medium (10 % DMSO, 80 % DMEM and 10 % FCS). From the 2 mℓ solution, 1 mℓ aliquots were transferred to cryotubes and were placed on ice to allow slow cooling. A thermos flask was used to store the cryotubes overnight at -20 ^{o}C. Following 24 hrs the cells were then stored in an ultra freezer at -85 ^{o}C.

3.4.4 Regeneration of cells

Cells were quickly thawed out from freezer storage of -80 ^{0}C and transferred to pre-warmed supplemented DMEM medium (1 % penicillin/streptomycin antibiotic and 10 % heat inactivated fetal calf serum) at a volume of 20 mℓ in 75 cm^2 tissue culture flasks. The flasks containing the cells were then incubated at 37 ^{0}C in a humidified incubator containing a 5 % CO_2 atmosphere.

3.4.5 Enumeration of cells

An exclusion dye, Trypan Blue, was used to count viable cells.

Intact (viable) cells do not take up the trypan blue dye whereas cells with altered membranes (non-viable) do. Visualization of cell morphology is also facilitated from staining with trypan blue resulting in blue non-viable cells and transparent viable cells. For cell enumeration, 100 µℓ trypan blue was mixed with 100 µℓ cell suspension cultures, using centrifuge tubes to hold the sample. The sample was then incubated at room temperature for 1 minute. A Naubauer hemocytometer was used to count cells. 10 µℓ aliquots were added to the two chambers of the hemocytometer. Counting of cells

included the center square and the four corner squares (1 mm) of the two hemocytometer chambers.

The equation used to determine the number of cells in suspension is as follows:

Total cell count	=	16 squares x 4
	=	cell counts in 4 sets of 16 squares
16 squares	=	2×10^4/ mℓ
Cells/ mℓ	=	$\frac{\text{total cell count}}{4} \times 2 \times 10^4$ per mℓ

3.4.6 Isolation of monocytes from peripheral blood mononuclear cells (PMBC)

Peripheral blood mononuclear cells (PBMC) was collected, separated and isolated as described by (Boyum 1967), with some modifications. All materials used during this step was stored in a Class II Biological Safety cabinet and aseptic techniques were practiced throughout the isolation procedure of the monocytes from PBMCs. All reagents used were brought to room temperature and sprayed with 70 % ethanol before being placed into the laminar flow working area.

For the isolation of monocytes, 50 mℓ was supplied by the South African National Blood Services (SANBS). 50 mℓ of pre-warmed Hanks' Balanced Salt Solution (HBSS) was added to the 50 mℓ Buffy coat sample. Using a pre-warmed Histopaque 1077 (Sigma), the mixture was carefully layered in a 5:4 ration using sterile 15 mℓ centrifuge tubes (Greiner). The sample was then centrifuged at 2000 rpm for 30 minutes at room temperature. Centrifugation separated the sample into four layers as seen in figure 9. The monocytes present between the HBSS and Histopaque layers were carefully aspirated using a sterile pasteur pipette and transferred to a sterile centrifuge tube. The cells were washed with HBSS, followed by centrifugation for 15 minutes at 1200 rpm and washed again to remove any residual histopaque. The cells formed pellets which were resuspended in 1 mℓ RPMI 1640 (supplemented with 10 % unheated fetal calf serum, 2 mM glutamine, 100 IU/mℓ penicillin and 100 Ig/mℓ streptomycin) (Bioscience) (Figure 18).

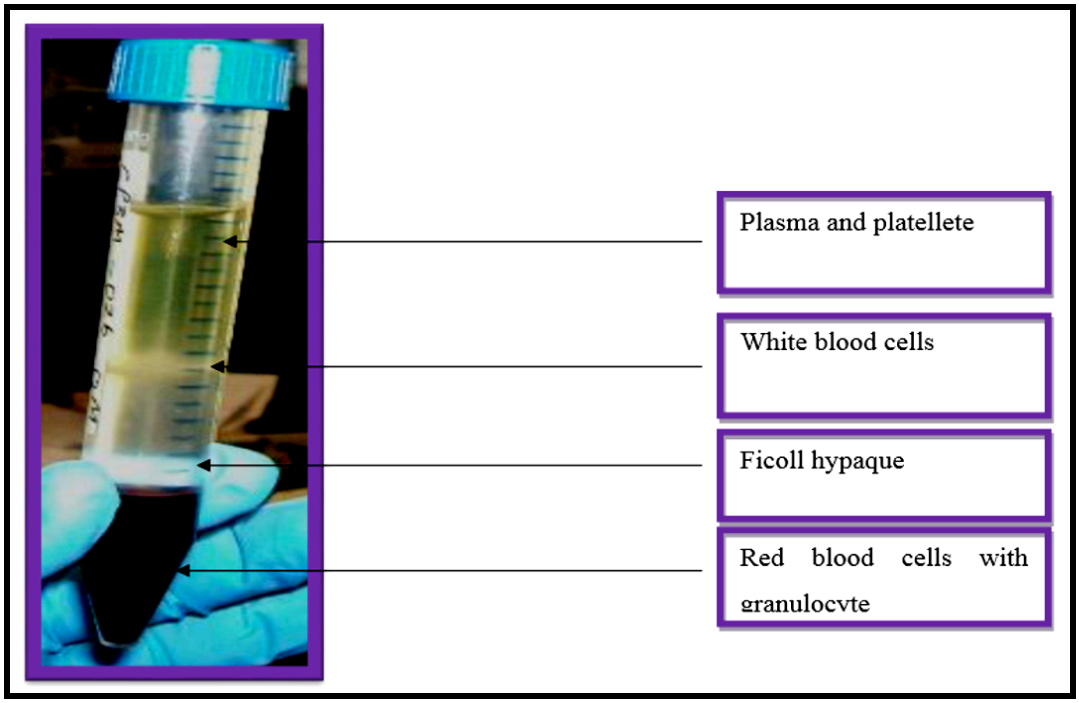

Figure 18: Isolation of Human Peripheral Blood Mononuclear Cells.

3.5 Cytotoxicity assay of platinum nanoparticles, gemcitabine and gemcitabine conjugated platinum nanoparticles

The effect of platinum nanoparticles, gemcitabine and gemcitabine conjugated platinum nanoparticles on the two cancer cell lines and one healthy cell line was evaluated using the MTT (3-4,5 dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) assay method as described by Mossman (1983), with some modifications.

Yielding reproducible and accurate results, the MTT method is simple and is based on mitochondrial dehydrogenases. In this case, succinate dehydrogenase, also a mitochondrial dehydrogenase, is present in metabolically active cells. The succinate dehydrogenase cleaves the tetrazolium ring resulting in a color change from yellow (MTT) to purple formazan crystals. These crystals are dissolved by isopropanol or 100 % DMSO. The resulting purple solution can then be measured spectrophotometrically. Mossman noted that the amount of formazan formed is related to the degree of cytotoxicity caused by the test compound.

Using a 96 well microtitre plate, 90 µℓ of cell culture – 1 x 10^5 cells/ mℓ were pipetted into each well and the outer wells were filled with phosphate saline buffer (PBS). PBS was used to prevent the medium from evaporating during the incubation period. The

plates were then incubated at 37 ^{0}C for 24 hours during which the cells attach to the wells of the plate. A stock solution of 50 µg/ mℓ was diluted using growth medium to ensure consistency of samples. Each test well was then treated with 10 µℓ of varying concentrations of platinum, gemcitabine and gemcitabine conjugated platinum nanoparticles. DMSO (Dimethyl sulfoxide) was used as a negative control in the study. Test compounds and the negative control were each tested in triplicate. The concentrations used for this assay were 0.10, 0.20, 0.78, 1.56, 3.13, 12.25, 50 and 100 µg/ mℓ respectively. All values calculated were mean ± SD. Following incubation of the cell containing microtiter plates at 37 ^{0}C for 48 hours (in a humidified incubator containing 5 % CO_2), 5 µg / mℓ MTT was added to each test well and incubated for a further 4 hours at 37 ^{0}C (5 % CO_2 atmosphere). The medium was removed and 100 µℓ DMSO was added to each test well. The DMSO dissolves the formazan crystals that form in metabolically active cells. Plates were then incubated for an additional hour at 37 ^{0}C (5 % CO_2 atmosphere). An ELIZA plate reader was then used to evaluate the absorbance of formazan at 590 – 630 nm.

Cell viability and cytotoxicity were calculated using the following equations:

% Cell Viability	=	$\frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100$
% Cytotoxicity	=	100 - (Cell Viability)

Thereafter, comparison of cell viability was done for the three compounds and Graph Pad Prism6 software was used to determine inhibitory concentrations of the three compounds that caused cell death by 50 % by plotting the percentage of inhibition versus the concentration.

3.6 Apoptosis determination using flow cytometry

3.6.1 How does flow cytometry work?

Flow cytometry involves the study of cells in a fluid system. Flow cytometers contain three main systems—the fluidics, the optics, and the electronics. The fluidics system funnels a sample of cells (for example, a sample of human blood) into a single stream so that the cells pass one at a time through a laser beam. As each cell passes through the

beam, it scatters light and may emit fluorescent light. These light signals are collected by the optics system and routed to various detectors. The signals received by the detectors then are converted into numerical values by the electronics system. Results can be displayed on the screen or saved for future analysis using specially designed software (FlowJo 10.0.6 software was used to analyze results of this study).

As each cell moves through the beam, its parameters (characteristics) are measured and recorded, along with the time that it passed through the beam. Typically data is collected for at least 10,000 cells per sample.

Flow cytometry analyzes physical and chemical characteristics of cells at an increased rate (over a thousand cells/second).

It is a widely used to technique to study many aspects of cell biology and is routinely used in the diagnosis of disease and in basic and clinical research and has applications in a number of fields including pathology, molecular biology, medicine, microbiology, plant biology, marine biology, and nanotechnology (Biosciences).

For the flow cytometry experiments, Melanoma (UCAA-62) cells were grown in 25 cm^2 flasks at a concentration of 5 x 10^5. Cells were incubated for 24 hours in order for cells to attach before the various drugs were added. 1 mℓ of each of the three compounds (gemcitabine, platinum nanoparticles, and the conjugated molecule) was then added to separate flasks before incubating for a further 24 hours. Following incubation, the cells were then removed and washed with PBS. Adherent cells were harvested by trypsinization using the cell dissociation buffer. Floating cells were then collected by centrifugation.

3.6.2 Using the Annexin V Kit to detect membrane changes

The Annexin V apoptosis kit procedure was followed as per manufacturer's instructions BD Biosciences.

Cell pellets were suspended in 100 µℓ of the staining solution that was supplied with the Annexin V kit. The cell pellet (stained) was then incubated for 15 minutes at room temperature. Thereafter, 400 µℓ of the binding buffer was added to the stained cells and the cells were analyzed using flow cytometry. Using a dot plot graph, early apoptotic cells were localized in the lower right quadrant using Annexin V against PI (propium

iodide). Controls used for this experiment were Doxorubicin (6 µg/ mℓ), DMSO (50 µg/ mℓ), cells stained with FITC, unstained cells, cells stained with Annexin V only (no PI), cells stained with PI only (no FITC Annexin V) and DMEM.

Cell fluorescence was measured by flow cytometry using an FL2 channel at 565 – 605 nm with a minimum of 10 000 events needed per sample tested.

3.6.3 Mitochondrial Membrane Potential using JC-1 Kit

The JC-1 apoptosis kit procedure was followed as per manufacturer's instructions (BD Biosciences).

To start, 0.5 mℓ of the JC-1 working solution was added to each pellet of cells. Thereafter, using JC-1 working solution cells were suspended and vortexed at low speed to gently break the clumps followed by incubation at 37^0C in a CO_2 incubator for 10 -15 minutes. The cells were then centrifuged and the supernatant was removed and discarded. After washing the cells twice using the 1 X Assay buffer, cells were re-suspended in 0.5 mℓ 1 X Assay buffer and analyzed by flow cytometry.

Controls used in this experiment were Untreated unstained cells, untreated stained cells, DMSO (50 µg/ mℓ) and Doxorubicin (6 µg/ mℓ).

The flow cytometer was used to measure cell fluorescence by comparing the ratio of fluorescence between FL1 and FL2 channels. A minimum of 10 000 events was needed per test sample.

3.6.4 Apoptosis detection using Caspase-3 Kit

The Caspase-3 Kit procedure was followed as per manufacturer's instructions (BD Pharmagen).

0.5 mℓ of BDcytofix/Cytoperm was added to 1 x 10^6 cells followed by incubation on ice for 20 minutes. Cells were then pelleted by centrifugation and the cytofix/cytoperm solution was discarded. The pelleted cells were then washed twice using 0.5 mℓ BD Perm/Wash^{TM} 1 X Buffer at room temperature. The cells were then resuspended in BD Perm/Wash^{TM} 1 X Buffer plus the antibody. The resuspended cells were then incubated at room temperature for 30 minutes. Following 30 minutes, the cells were pelleted via centrifugation and the BD Perm/Wash^{TM} plus antibody solution was removed and

discarded. The cells were thereafter washed once using 1 mℓ of BD PermWash™ 1 X Buffer. Cells were resuspended in 0.5 mℓ of BD Perm/Wash™ 1 X Buffer and analyzed using flow cytometry.

Controls used in this test was Doxorubicin (6 µg/ mℓ) and DMSO (50 µg/ mℓ).

The flow cytometer was used to measure cell fluorescence by comparing fluorescence between Comp-FL2-A and Comp-FL1-A channels. A minimum of 10 000 events was needed per test sample.

CHAPTER 4: RESULTS

4.1 Synthesis of platinum nanoparticles

The synthesis of platinum nanoparticles was confirmed when the reaction mixture turned from light yellow to dark brown, indicating hexachloroplatinic acid hexahydrate reduction as reported by Teow and Valiyaveeti (2010) as well as by Rakap (2015). Inset in figure 19 visually confirmed the brown platinum nanoparticle solution formed. Further characterization via UV-visible spectroscopy and transmission electron microscopy was performed to confirm the presence of platinum nanoparticles in solution.

4.2 Synthesis of gemcitabine conjugated platinum nanoparticles

Following the addition of 5 mℓ of 0.0025 M gemcitabine stock solution and continuous stirring for 6 hours, gemcitabine conjugated platinum nanoparticles were synthesized. A slight color change from light to dark brown was observed. Further characterization, as stated above, was performed to confirm the presence of the conjugated molecule in solution.

4.3 Characterization of platinum nanoparticles and gemcitabine conjugated platinum nanoparticles

4.3.1 UV-Visible Spectrophotometric Analysis

The UV-visible spectrophotometric analysis was done as part of the confirmation of platinum nanoparticles, gemcitabine and gemcitabine conjugated platinum nanoparticles in solution. Platinum nanoparticles were characterized by the formation of an absorbance peak at a wavelength of 301 nm (Figure 19).

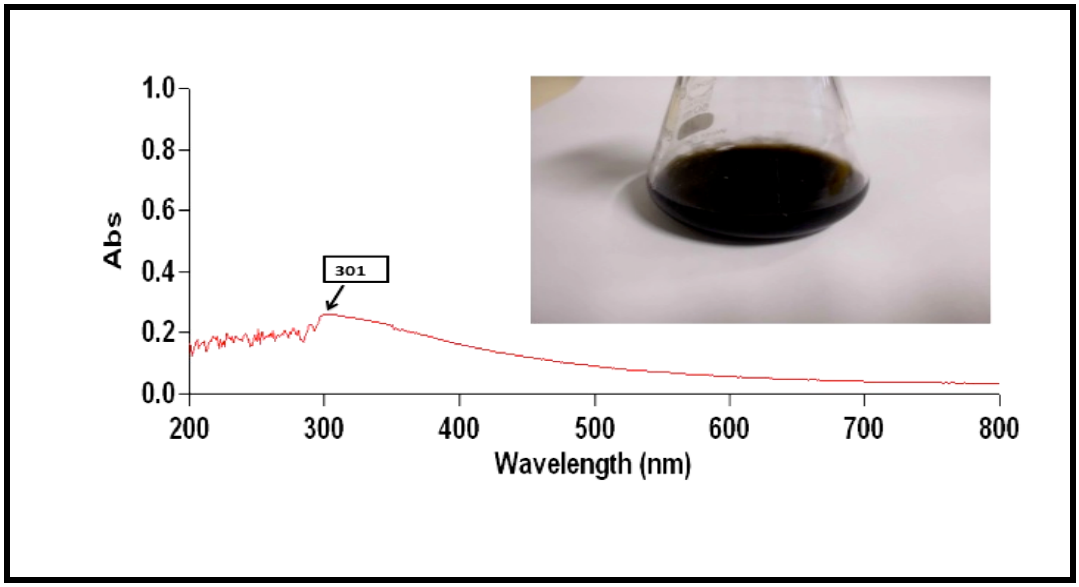

Figure 19: UV scan of platinum nanoparticles detected at 301 nm. Insert indicating synthesized platinum nanoparticles with characteristic brown color.

Figure 20 indicates the UV scan of gemcitabine. Although gemcitabine was a purchased drug, a UV scan was done in order to detect changes in absorbance peaks between platinum nanoparticles and the gemcitabine conjugated platinum nanoparticles. An absorbance peak at a wavelength of 283 nm was detected.

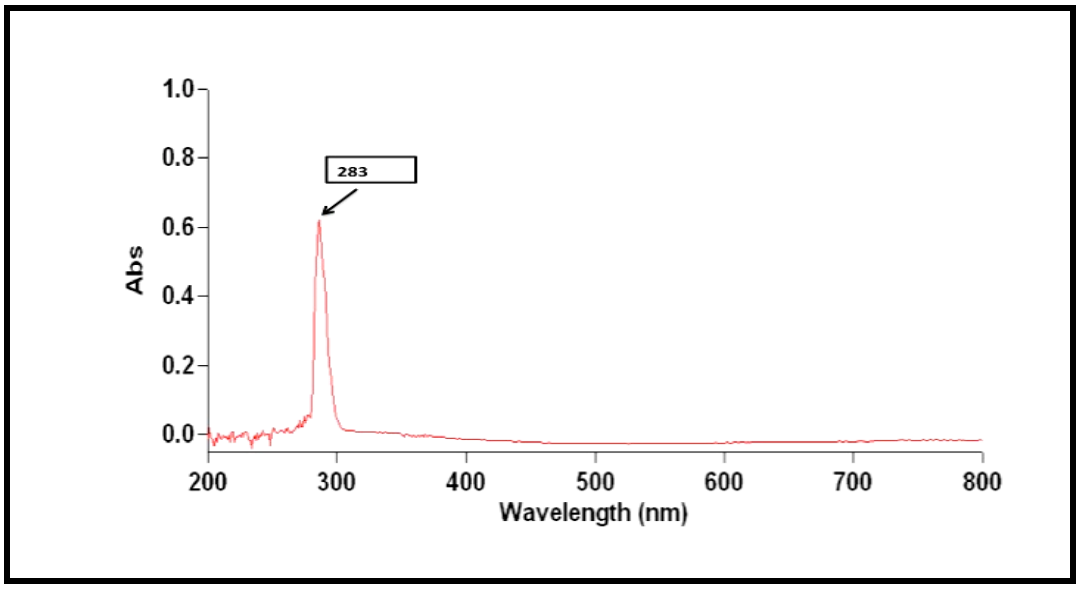

Figure 20: UV scan of gemcitabine detected at a wavelength of 283 nm.

Figure 21 indicates the UV scan of the conjugated molecule. An absorption peak at a wavelength of 379 nm was detected. Unbound platinum nanoparticles and gemcitabine were detected at wavelengths of 302 nm (peak 2) and 283 nm (peak 1).

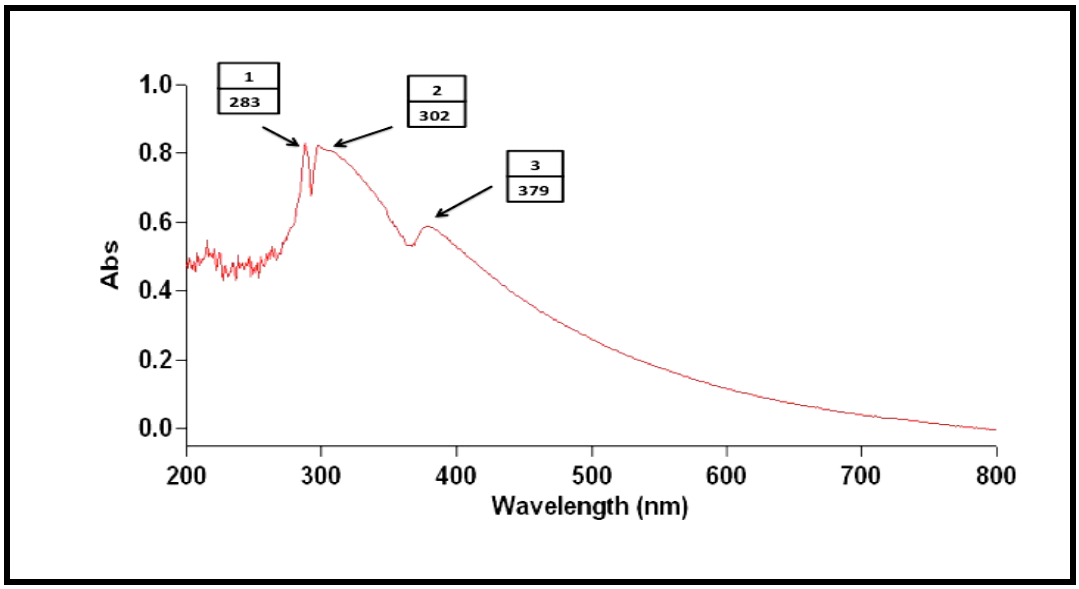

Figure 21: UV scan of gemcitabine conjugated platinum nanoparticles. The conjugated molecules are detected at 379 nm (3). Unbound platinum nanoparticles (2) and unbound gemcitabine (1) are detected at 302 nm and 283 nm respectively.

4.3.2 Ultrastructural analysis of platinum nanoparticles and gemcitabine conjugated platinum nanoparticles by transmission electron microscopy

The size and morphology of platinum nanoparticles and gemcitabine conjugated platinum nanoparticles were analyzed by TEM.

Both compounds were detected using a scale of 5 nm and 10 nm. Measurement of the platinum nanoparticles and the hybrid are indicated on selected areas on the copper coated grid used. The core size of platinum nanoparticles detected ranged in size from 1.14 nm to 1.65 nm (Figure 22). The hybrid molecule ranged from 1.53 nm to 2.66 nm in diameter (Figure 23). A slight increase in sizes between platinum nanoparticles and the hybrid was noted.

TEM images also revealed that the platinum nanoparticles and the hybrid molecule were predominantly spherical in shape with good particle dispersion in the sample. No

significant agglomeration was observed amongst particles for platinum nanoparticles and gemcitabine conjugated platinum nanoparticles.

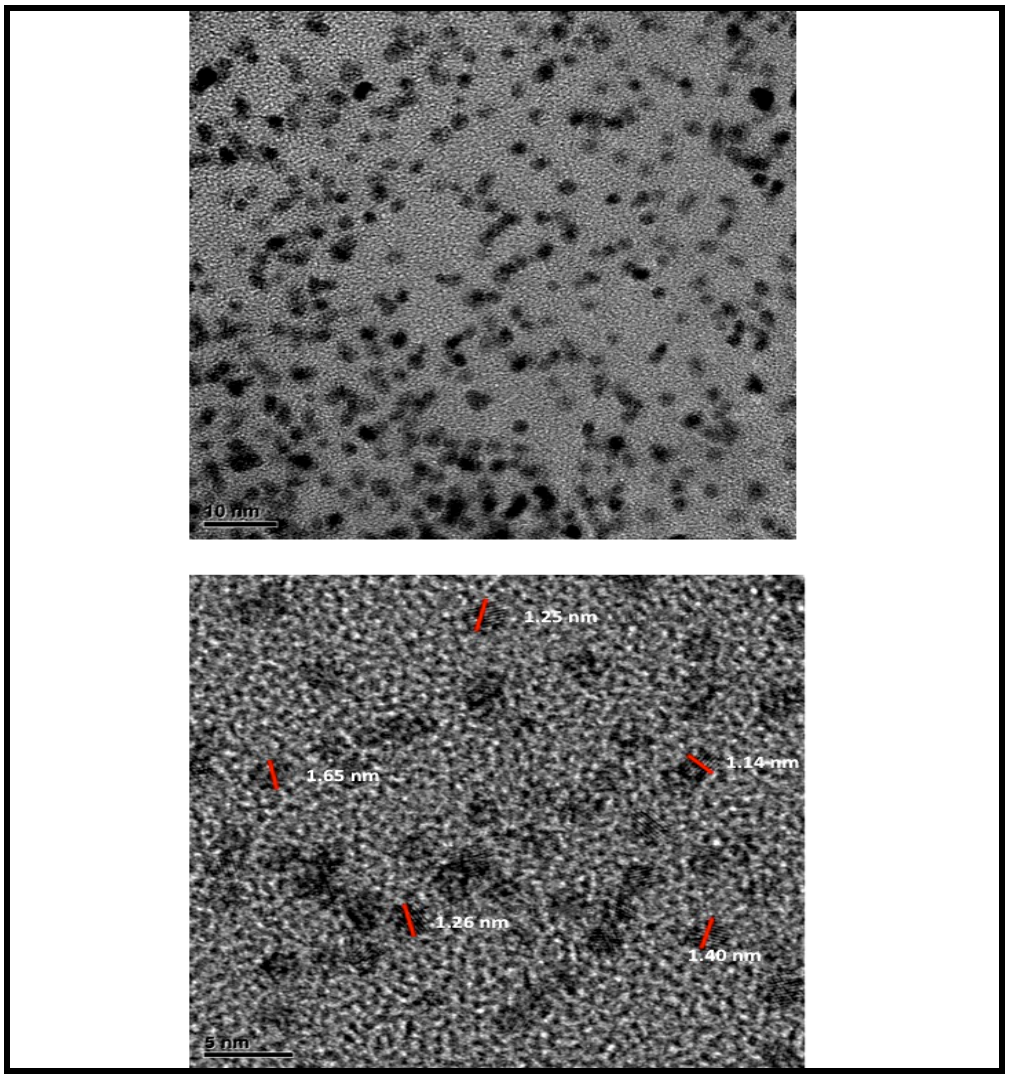

Figure 22: TEM image of platinum nanoparticles prepared by the reduction of H_2PtCl_6. The sample was deposited on a copper-coated grid and analyzed using TEM. Platinum nanoparticles were detected measuring between 1.14 and 1.65 nm in diameter.

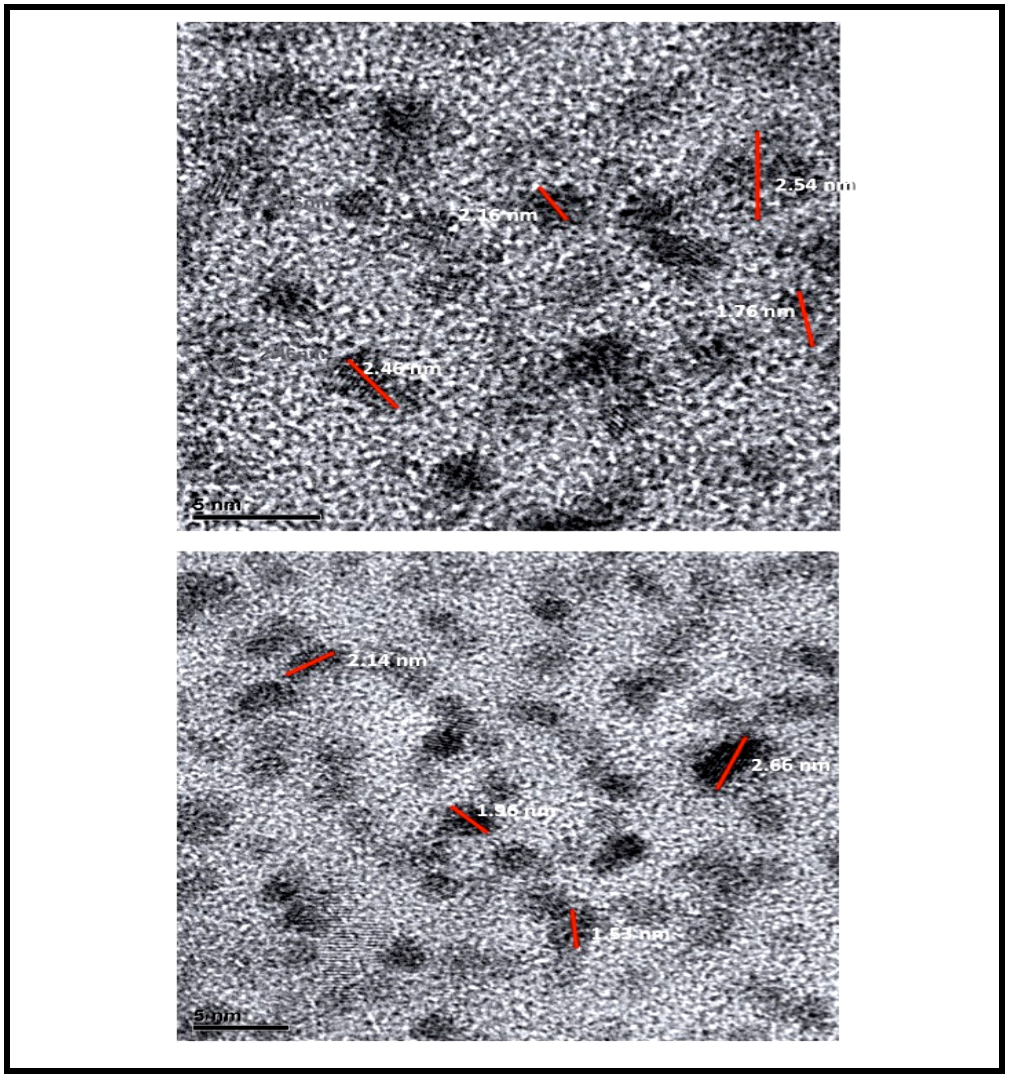

Figure 23: TEM image of gemcitabine conjugated platinum nanoparticles. Conjugated nanoparticles were detected measuring between 1.53 nm and 2.66 nm in diameter.

4.4 Cytotoxicity assay of platinum nanoparticles, gemcitabine and gemcitabine conjugated platinum nanoparticles on cancer cells and healthy cells

Using the MTT assay, platinum nanoparticles, gemcitabine and the gemcitabine conjugated platinum nanoparticle compounds were screened for toxicity against Breast cancer (MCF-7), Melanoma (UACC-62), and Peripheral blood mononuclear (PBMC) cell lines. The concentrations used for this assay were 0.10, 0.20, 0.78, 1.56, 6.25, 3.13, 12.25, 25, 50 and 100 µg/ mℓ respectively. All values are mean ± SD (n=3).

4.4.1 Cytoxicity results against MCF-7 cell line

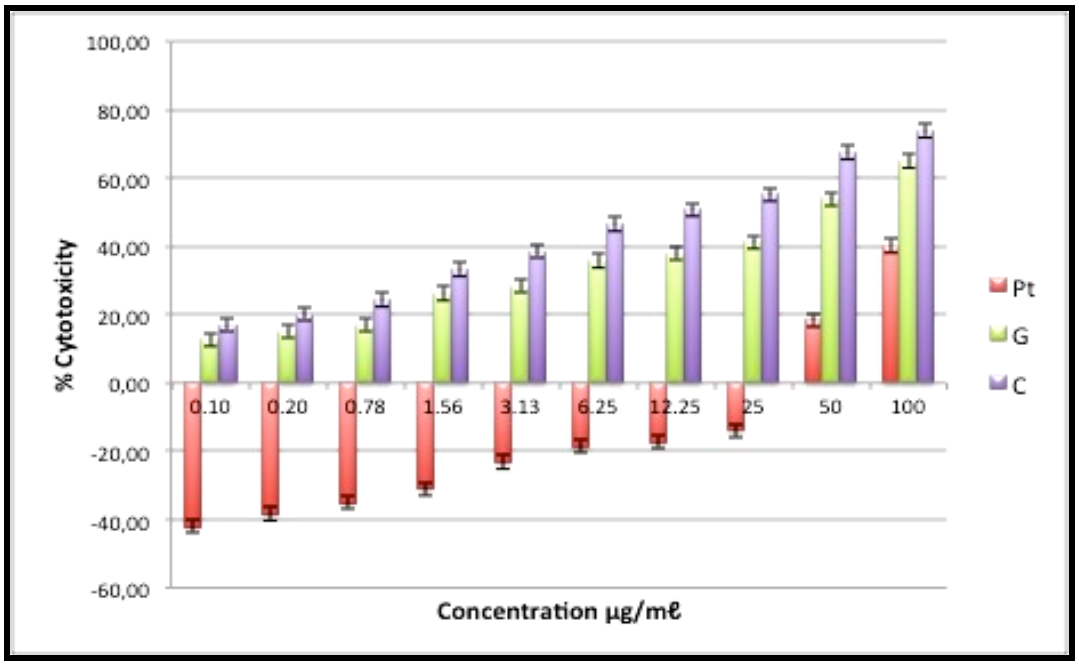

Figure 24: Cytotoxicity comparison of platinum nanoparticles, gemcitabine and gemcitabine conjugated platinum nanoparticles against MCF-7 cell line

Table 4: Concentration vs. % Cytotoxicity of platinum NP, gemcitabine and the conjugate against MCF-7 cell line

	0.10	0.20	0.78	1.56	3.13	6.25	12.25	25	50	100
Platinum	-42.03	-38.30	-34.99	-30.95	-23.19	-18.63	-17.63	-13.98	18.43	40.06
Gemcitabine	12.76	15.03	17.01	26.12	28.32	35.69	37.81	41.31	53.83	64.92
Conjugate	16.93	20.05	24.30	33.18	38.42	46.39	59.80	55.20	67.43	73.80

Conjugated platinum nanoparticles showed the highest toxicity against breast cancer cells (Figure 24 and Table 4). The cytotoxicity effect from lowest to highest concentration was 16.93, 20.05, 24.30, 33.18, 38.42, 46.39, 59.80, 55.20, 67.43 and 73.80 %.

Cytotoxicity of gemcitabine was lower than that of the conjugated nanoparticles. These results included 12.76, 15.03, 17.01, 26.12, 28.32, 35.69, 37.81, 41.31, 53.83 and 64.92 respectively.

Platinum nanoparticles showed toxicity to MCF-7 cells at 50 µg/ mℓ (18.43 %) and 100 µg/ml (40.06 %) with other toxicity values ranging from -42.03, -38.30, -34.99, -30.95, -23.19, -18.63, -17.63 and -13.98 from concentration 0.10 to 25 µg/ mℓ respectively (Table 4).

4.4.2 Cytoxocity results against Melanoma cell line

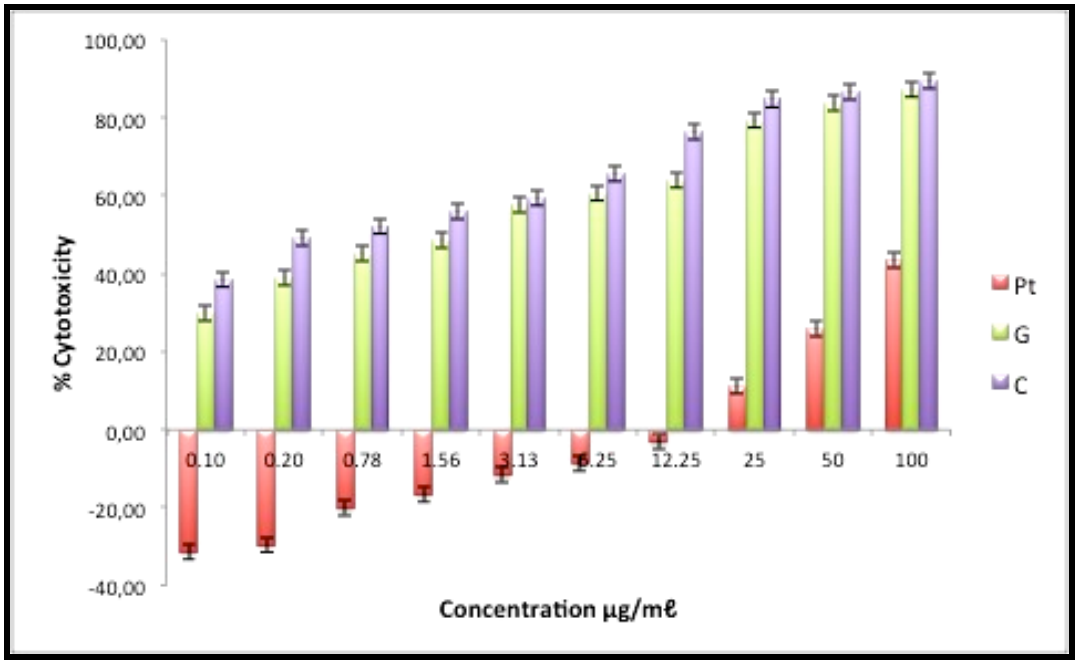

Figure 25: Cytotoxicity comparison of platinum nanoparticles, gemcitabine and gemcitabine conjugated platinum nanoparticles against Melanoma cell line

Conjugated nanoparticles showed the highest toxicity against Melanoma cells (Figure 25 and Table 5).

Table 5: Concentration vs. % Cytotoxicity of platinum NP, gemcitabine and the conjugate against Melanoma cell line

	0.10	**0.20**	**0.78**	**1.56**	**3.13**	**6.25**	**12.25**	**25**	**50**	**100**
Platinum	-31.28	-29.46	-20.20	-16.55	-11.77	-8.63	-3.26	11.24	25.74	43.28
Gemcitabine	29.92	39.03	45.24	48.55	57.66	60.77	64.08	79.50	83.60	87.37
Conjugate	38.61	48.96	52.28	55.90	59.52	65.73	76.29	84.68	86.54	89.54

Percentage cytotoxicity concentrations for the conjugated compound was 38.61, 48.96, 52.28, 55.90, 59.52, 65.73, 76.29, 84.68, 86.54 and 89,54 respectively.

Cytotoxicity of gemcitabine was lower than the conjugated nanoparticles. Percentage cytotoxicity was 29.92, 39.03, 45.24, 48.55, 57.66, 60.77, 64.08, 79.50, 83.64 and 87.37.

Platinum nanoparticles displayed toxicity at 25, 50 and 100 µg/ mℓ. These results were 11.24, 25.74 and 43.28 percent respectively (Table 5). Other cytotoxicity values included -31.28, -29.46, -20.20, -16.55, -11.77, -8.63 and -3.26 from concentrations 0.10 to 12.25 µg/mℓ respectively.

4.4.3 Cytoxicity against PBMC cell line

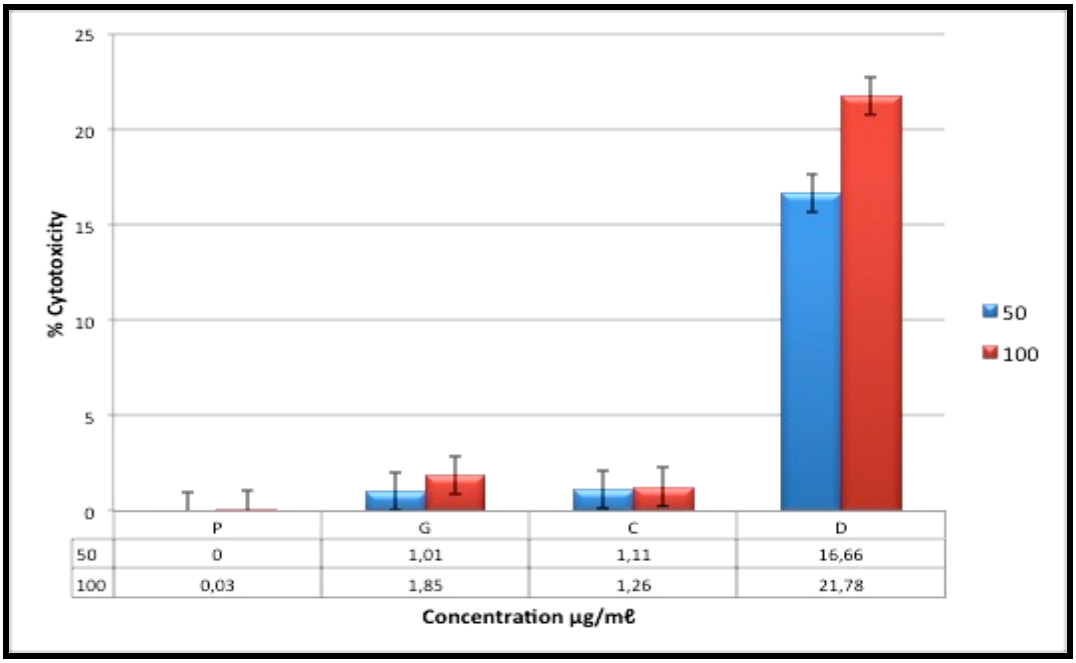

Figure 26: Cytotoxicity comparison of platinum nanoparticles, gemcitabine and gemcitabine conjugated platinum nanoparticles against healthy cell line (PBMC)

To test the cytotoxicity effect on normal cells, peripheral blood mononuclear cells were used as a reference. Figure 26 illustrates results showing doxorubicin (+ control) having the highest cytotoxicity against PBMC. Gemcitabine conjugated platinum nanoparticles showed 1.11 and 1.26 % cytotoxicity at 50 and 100 µg/ mℓ respectively. Gemcitabine displayed 1.01 and 1.85 % toxicity and platinum nanoparticles inhibited 0 % of cells at 50 µg/ mℓ and 0,03 % at 100 µg/ mℓ.

4.5 IC_{50} determination of platinum nanoparticles, gemcitabine and gemcitabine conjugated platinum nanoparticles

The inhibitory concentration required for 50% cytotoxicity (IC_{50}) value was determined using the Prism dose-response curve (Prism GraphPad, Prism version 6 for Mac, GraphPad Software) and plotting the percentage of inhibition versus the concentration.

The IC_{50} values of platinum nanoparticles, gemcitabine, and gemcitabine conjugated platinum nanoparticles were determined using UACC-62 (Melanoma) and MCF-7 (Breast cancer) cell lines. Results in table 6 indicate the minimum inhibitory concentration required to kill cells by 50 % for both cancer cell lines.

Table 6: IC_{50} (µg/ mℓ) of compounds against UACC-62 (Melanoma) and MCF-7 (Breast cancer) cells

Compounds	UACC-62 (Melanoma)	MCF-7 (Breast Cancer)
Platinum Np	50.06 ± 0.01	58.43 ± 0.01
Gemcitabine	14.03 ± 0.05	16.07 ± 0.01
Conjugate Np	8.03 ± 0.03	9.20 ± 0.04
Doxorubicin (+ Control)	0.09 ± 0.01	1.61 ± 0.01

Values are mean ± SD (n=3)

Both the conjugated nanoparticles, gemcitabine, and doxorubicin showed significant cytotoxicity against both cell lines.

Platinum nanoparticles had an IC_{50} of 58.43 for MCF-7 and 50.06 for Melanoma cell lines.

Gemcitabine displayed a low IC_{50} value of 14.04 for UACC-62 and 16.07 for MCF-7.

The conjugate compound exhibited the lowest IC_{50} values compare to platinum nanoparticles and gemcitabine with 8.03 for UACC-62 and 9.20 for MCF-7 cell lines.

Lower IC_{50} values give the indication that the drug is potentially stronger. These results express similar results as the cytotoxicity assay. IC_{50} values for platinum NP,

gemcitabine and the hybrid was observed to be lower for Melanoma cells than MCF-7 (Table 6).

4.6. Apoptosis determination using flow cytometry

To evaluate whether platinum nanoparticles, gemcitabine or the gemcitabine conjugated platinum nanoparticles induced apoptosis, apoptotic cells were identified by the use of four methods. These methods involved (a) observation of morphological changes associated with apoptosis (b) using the Annexin V assay to detect plasma membrane changes (c) using the JC-1 kit to determine changes in mitochondrial membrane potential and (d) using the caspase-3 assay to determine caspase activation.

4.6.1 Morphological observation of cells

Defined by characteristic changes in the nuclear morphology, apoptosis includes chromatin condensation, overall cell shrinkage, blebbing of the plasma membrane and fragmentation resulting in apoptotic bodies containing nuclear or cytoplasmic material. Apoptosis is also referred to as type 1 cell death or programmed cell death (Dash 2005; Zhang *et al.* 2015a).

Melanoma (UACC-62) cell line was used for morphological observation. Cells treated with Doxorubicin (positive control) at 6 µg/mℓ, DMSO (negative control), platinum nanoparticles, gemcitabine and gemcitabine conjugated platinum nanoparticles at 50 µg/mℓ respectively, were observed under light microscopy.

The results observed in figure 27 shows untreated melanoma (27A) and the negative control, treated with 50 µg/ mℓ DMSO (27B) appear normal. All other compounds including doxorubicin at 6 µg/ mℓ (27F) displayed detachment of cells, changes in cell shape and clumping which is observed in images C (gemcitabine conjugated platinum nanoparticles) and D (gemcitabine).

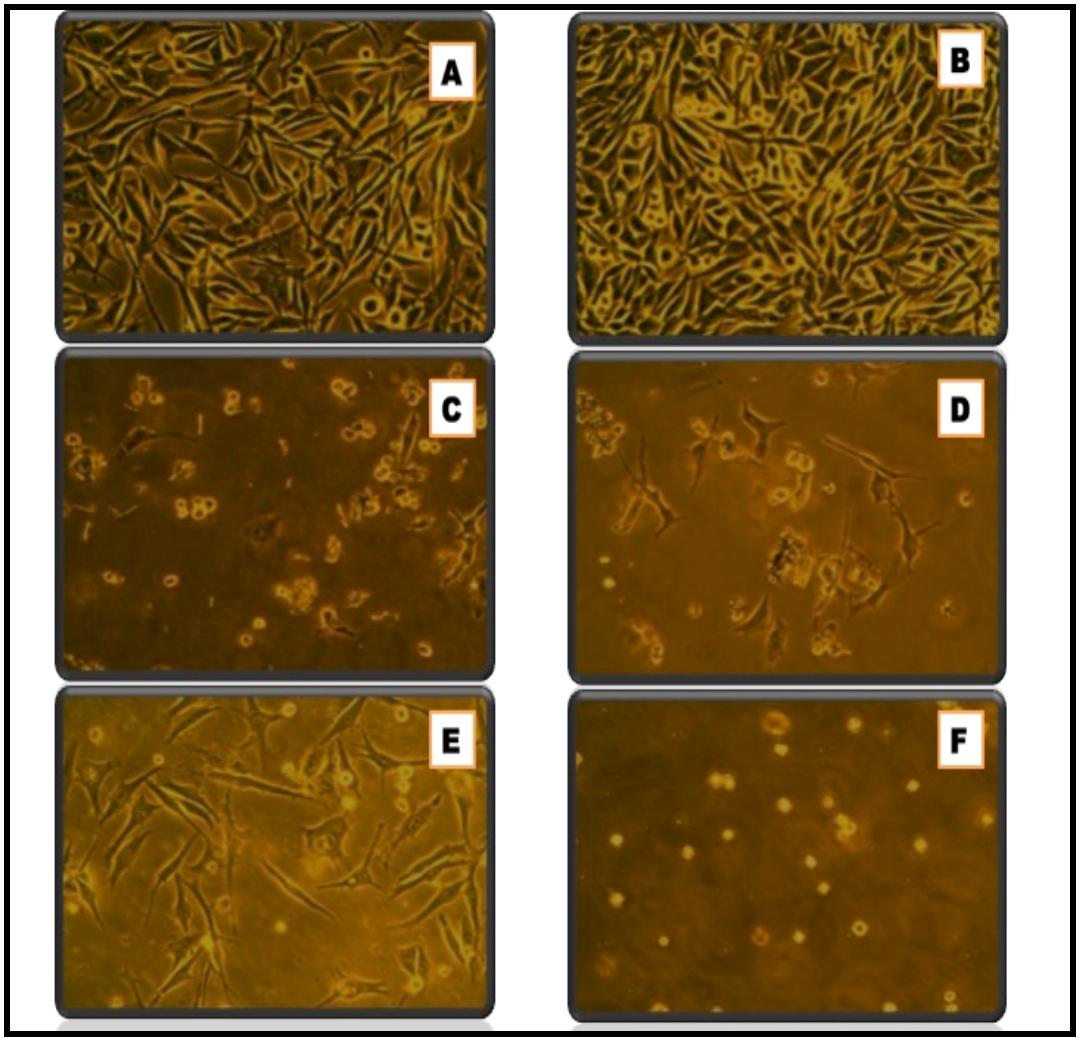

Figure 27: Morphological observation of Melanoma (UACC-62) cells under light microscopy. (A) Untreated Melanoma cells. Treated with 50 µg/ mℓ of (B) DMSO, (C) Gemcitabine conjugated platinum nanoparticles, (D) Gemcitabine, (E) Platinum nanoparticles respectively and (F) Doxorubicin (6 µg/ml).

4.6.2 Using Annexin V to detect changes cell membranes

The Annexin V apoptosis kit procedure was followed as per manufacturer's instructions BD Biosciences). This assay is used to quantitatively determine the percentage of cells undergoing apoptosis. Annexin V-FITC assay measures cell fluorescence using an FL2 channel (565 -605 nm). 10 000 events were acquired as a minimum per sample to perform the assay.

Table 7: UACC-62 (Melanoma) cells treated with platinum nanoparticles, gemcitabine, and gemcitabine conjugated platinum nanoparticles and stained with Annexin V-FITC/PI

Treatment	Viable (%)	Apoptosis (%)	Late Apoptosis (%)	Necrotic (%)
Untreated unstained (A)	100	0	0	0
PI Control (B)	15.4	0	9.21	84.6
Untreated stained(C)	80.6	9.22	6.56	3.62
Doxorubicin (D)	3.18	0.05	56.4	39.9
DMSO (E)	80.5	7.75	10.4	3.62
Annexin only (F)	75.3	4.58	5.65	14.5
Platinum (G)	82.1	0.056	0.12	17.7
Gemcitabine (H)	90.1	2.98	4.76	2.19
Conjugate (I)	68.6	3.85	9.15	18.4

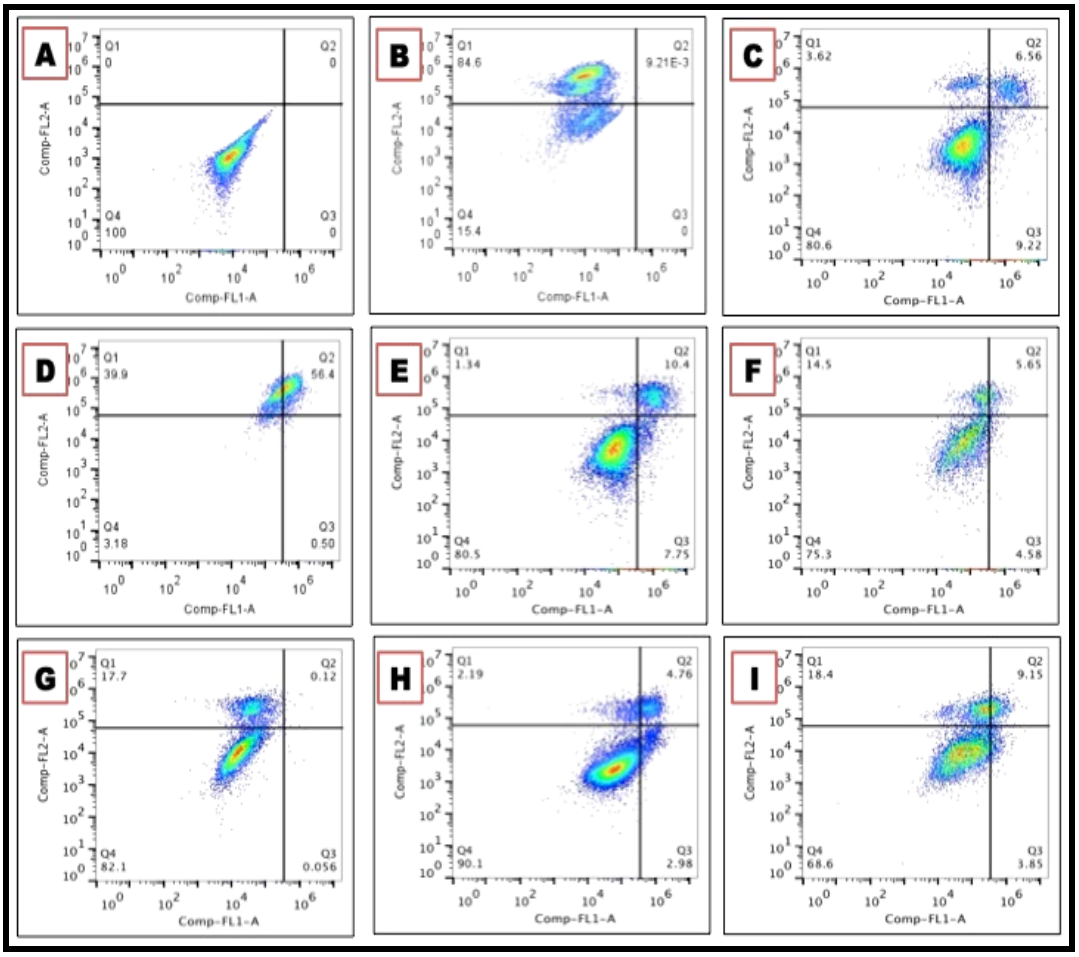

Figure 28: Apoptosis evaluation of compounds using Annexin V-FITC. The lower right quadrant (Q3) indicates early apoptotic cells and Q2 indicates late apoptotic cells in the top right quadrant. Necrotic cells are indicated in the upper left quadrant (Q1) with viable cells in the lower left quadrant (Q4). (A) Untreated unstained cells, (B) PI Control, (C) Untreated cells stained with Annexin/PI, (D) Doxorubicin 6 µg/ml, (E) DMSO 50 µg/ mℓ, (F) Annexin only, (G) Platinum, (H) Gemcitabine and (I) Conjugate at 50 µg/ml respectively

Table 7 and Figure 28 indicate results for cells that induced early apoptosis, late apoptosis and cells undergoing necrosis. Platinum nanoparticles induced apoptosis at very low percentages of 0.056 for early apoptosis and 0.12 for late apoptosis. Gemcitabine-induced apoptosis at 2.98 % for early stage and 4.76 % at the late stage. Conjugate nanoparticles showed the highest percentages of three compounds with 3.85 % early stage and 9.15 % late stage. Doxorubicin (positive control) was used to validate these results inducing late apoptosis at 56.4 %. These results indicate that

platinum nanoparticles, gemcitabine, and gemcitabine conjugated platinum nanoparticles have the potential to induce apoptosis.

4.6.3 Mitochondrial membrane potential using JC-1

The mitochondrial membrane potential assay procedure was followed as per manufacturer's instructions (BD Biosciences). One of the early markers for apoptosis is changes in the mitochondrial membrane. JC-1 assay uses monomers and aggregates that have different emission spectra to detect changes in mitochondrial membrane potential (BD Bioscience). FL1 and FL2 channels were used to measure cell fluorescence and 10 000 events were acquired as a minimum per sample to perform the assay.

Table 8: Table of JC-1 mitochondrial membrane potential

Treatment	Stained Viable Cells (%)	Apoptosis (%)
Untreated unstained cells (A)	100	0
Untreated stained cells (B)	93.6	4.58
DMSO (C)	92.3	6.50
Doxorubicin (D)	84.8	14.2
Platinum nanoparticles (E)	91.7	6.42
Gemctiabine (F)	85.6	12.0
Conjugate (G)	84.8	13.1

Compounds which induced apoptosis using the JC-1 assay are indicated in Figure 29 and Table 8. Platinum nanoparticles had an apoptotic activity of 6.42 % whilst gemcitabine showed apoptotic activity at 12.0 %. Gemcitabine conjugated platinum nanoparticles again showed the highest activity of the three compounds at 13.1 %. These results were verified by using doxorubicin (6 µg/ mℓ) as a positive control which displayed activity at 14.2 %. DMSO was used as a negative control.

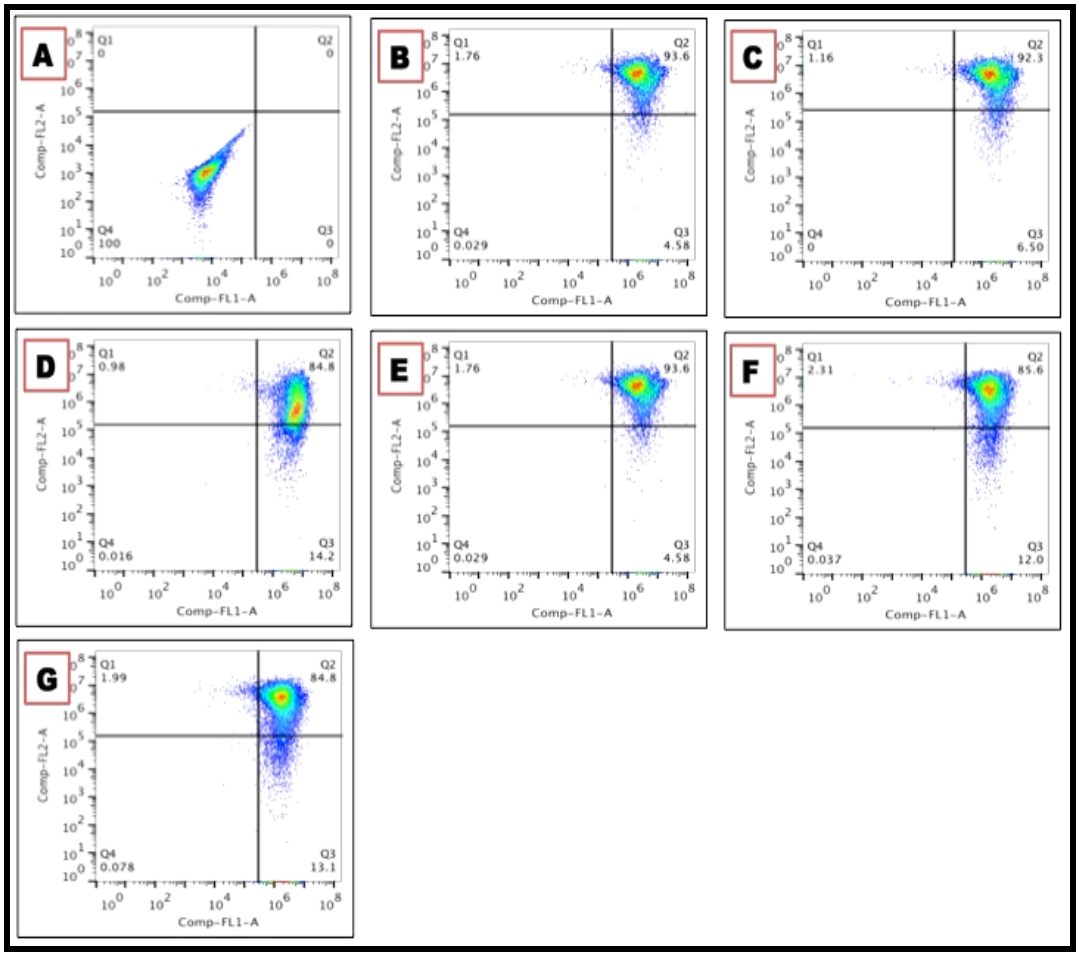

Figure 29: Mitochondrial membrane potential displaying apoptotic cells. Apoptotic cells are localized in the lower right quadrant and viable cells in the high right quadrant (Q2). (A) Untreated unstained cells, (B) Untreated stained cells, Cells treated with (C) 50 µg/ mℓ DMSO, (D) 6 µg/ mℓ Doxorubicin, (E) Platinum nanoparticles, (F) Gemcitabine and (G) Conjugate

4.6.4 Apoptosis detection using the caspase-3 assay

The Caspase-3 assay was followed as per manufacturer's instructions (BD Pharmagen). Caspases cleave proteins which result in cell death and this characteristic is used to identify apoptotic cells.

Cells are stained using the PE conjugate polyclonal active caspase-3 antibody. Cell fluorescence was measured by comparing fluorescence between channels Comp-FL2-A and Comp-FL1-A. A minimum of 10 000 events was acquired per test sample.

Table 9: Caspase-3 activity of platinum nanoparticles, gemcitabine and gemcitabine conjugated platinum nanoparticles

Treatment	Caspase negative PE-A-	Caspase positive PE-A+
Negative control (A)	99.7	0.29
Doxorubicin (B)	0.21	99.8
Platinum nanoparticles (C)	98.2	1.84
Gemcitabine (D)	53.9	46.1
Conjugate (E)	46.2	56.8

Significant caspase-3 activity was observed for gemcitabine conjugated platinum nanoparticles with 56.8 % PE-A^+ (Figure 30E). Gemcitabine-induced caspase-3 activity at 46.1 % (Figure 30D) and platinum nanoparticles at the lowest activity of 1.84 % (Figure 30C). Doxorubicin (positive control) administered at 6 µg/ mℓ induced the highest caspase-3 activity of 99.8 % (figure 30B). This was used as validation for caspase-3 results.

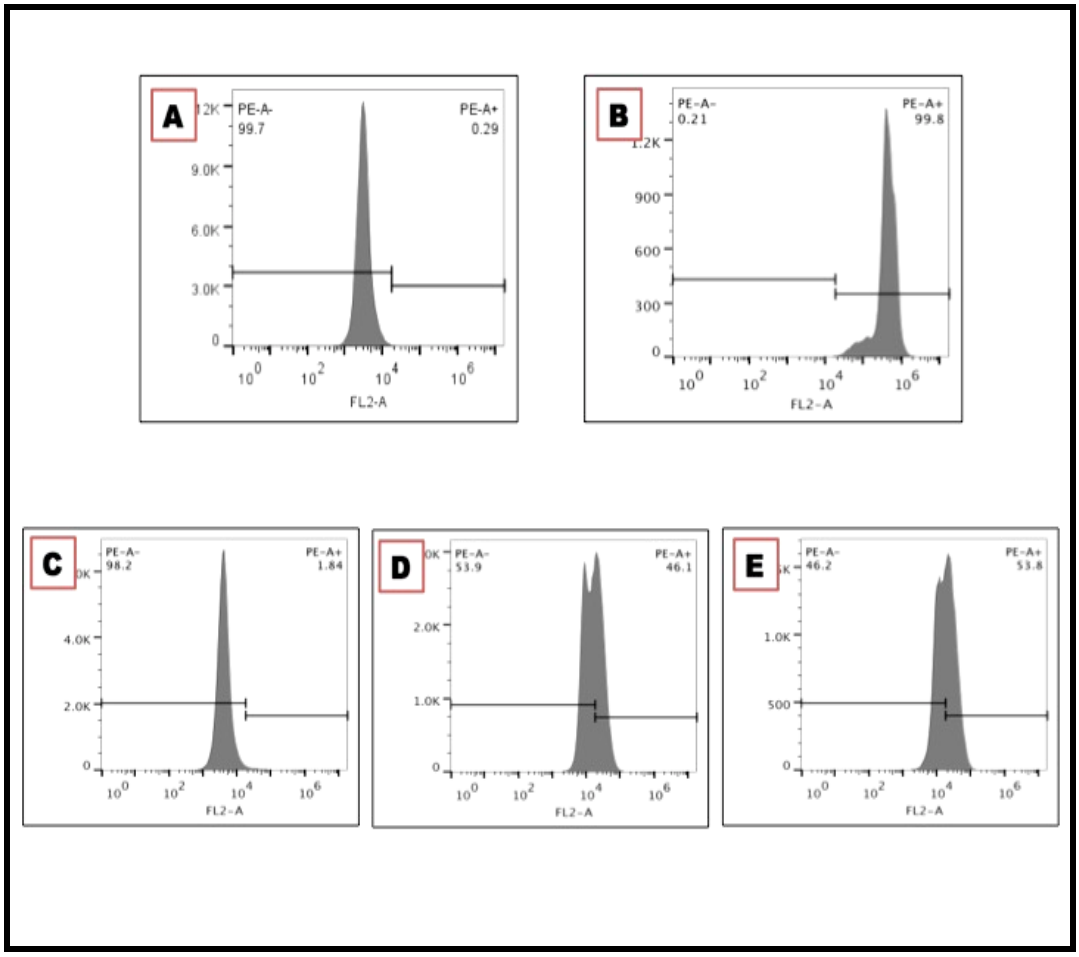

Figure 30: Histogram of caspase-3 activity. PE-A+ indicates apoptotic cells and viable cells are indicated by PE-A-. (A) Untreated cells, (B) treated with 6 µg/ mℓ Doxorubicin, and cells treated with 50 µg/ mℓ (C) Platinum nanoparticles, (D) Gemcitabine and (E) Conjugate

CHAPTER 5: DISCUSSION

Nanoparticle interaction with human cells has lead to an interesting topic for understanding toxicity and for the improvement of potential drug candidates. Recently, targeted cancer therapeutics has been one of the significant leaps forward in cancer treatment. Clinical results have escaped some routine conventional drugs for the treatment of leukemia and breast cancer. Interest in combining molecular targeting and nanoparticle delivery has increased and has originated from the achievement of these molecular targeting agents.

Platinum drugs fall in an alkylator's class where interest has been in creating nanoparticle therapeutics. Some of these platinum's have an extensive variety of applications in cancer including oxaliplatin, cisplatin and carboplatin. Liposomal formulations of cisplatin have exhibited incredible preclinical data without entering the trial stage (Wang, Langer and Farokhzad 2012).

5.1 Synthesis and characterization of platinum nanoparticles using Uv-Visible spectrophotometric analysis

PVP- capped platinum nanoparticles were prepared via the reduction of hexachloroplatinic acid hexahydrate resulting in the color change from a yellow to a light brown solution. It was also observed that the platinum nanoparticle solution displayed good dispersity in water and the solution remained stable for a long period of time at room temperature. The color change was used an indicator for the presence of platinum nanoparticles in solution as reported by Teow and Valiyaveeti (2010) as well as by Rakap (2015). Teow and Valiyaveeti (2010) also noted that both platinum nanoparticles capped with PVP and platinum conjugated with folic acid displayed good dispersity in water with light and dark brown solutions.

Poly vinyl pyrrolidone was used to cap nanoparticles which allowed for enhancement of stability and water dispersion in cell culture medium and it reduced the need for adding a capping agent or organic solvents which can be toxic to the cells.

UV-Vis spectrum analysis was performed to indicate the absorption of platinum nanoparticles. It was found that platinum nanoparticles had an absorption peak at 301 nm (figure 19). These results are near the absorption peak to that found by Borodko *et*

al. (2006). Transmission electron microscopy was done to further verify that platinum nanoparticles were, in fact, present in solution.

5.2 Synthesis and characterization of gemcitabine conjugated platinum nanoparticles using Uv-Visible spectrophotometric analysis

Following the addition of 5 mℓ of 0.0025 M gemcitabine stock solution and continuos stirring for 6 hours, gemcitabine conjugated platinum nanoparticles were synthesized. Similar to the platinum nanoparticle solution, it was also observed that the conjugated nanoparticle displayed good dispersity in water and the solution remained stable for a long period of time at room temperature.

To verify the presence of the conjugated nanoparticle in solution UV-Vis spectrum analysis was performed. An absorbance peak was detected at 379 nm with unbound platinum nanoparticles at 302 and unbound gemcitabine at 283 nm. These unbound particles could be due to excess particles in solution or where conjugation did not occur.

Upon conjugation with gemcitabine, a color change from the light brown to dark was observed. After the gemcitabine was added, a change in the absorption plasmon is observed with a shift to a larger wavelength. This phenomenon was reflected on the UV scan (figure 21) with an increase in wavelength from 301 nm (platinum nanoparticles) to 379 nm (conjugate). The shift in absorbance and aggregation of nanoparticles is described by Pissuwan *et al.* (2010) and Tom *et al.* (2004).

In 2010, in a report by Teow and Valiyaveeti, platinum nanoparticles were conjugated to folic acid using polyvinyl pyrrolidone (PVP) as a capping agent and the bioactivity was tested on commercially available cell lines. In their study, it was found that platinum nanoparticles capped with PVP were less toxic (with greater that 80 % viability) than folic acid capped platinum nanoparticles. It was also estimated in their elemental analysis that Pt-FA (conjugate) displayed a higher platinum content than the Pt-PVP.

5.3 Ultrastructural analysis of platinum nanoparticles and gemcitabine conjugated platinum nanoparticles

Nanoscale materials are those that have bigger surface area compared to larger scale materials of similar volumes. This implies these particles have more surface region accessible for interaction with different materials around them (Oberdorster, Oberdorster and Oberdorster 2005; Mohanpuria, Rana and Yadav 2008).

The utilization of nanoparticles has turned out to be always more vital in nanomedicine. Their size is like that of cellular components allowing nanoparticles to specifically interface with the cells and organelles (Noel, Simard and Girard 2016).

Transmission electron microscopy serves as a method for establishing platinum nanoparticles and the conjugated particle formation. This was helpful to show and observe size, morphology and distribution of the nanoparticles mentioned. This sort of microscopy is a path by which particles are directly observed and measured. Particles are expressed as a diameter of a sphere.

In this study nanoparticles analyzed by TEM at the highest magnification using a scale of 5 nm and 10 nm. Both platinum nanoparticles and the hybrid have been observed to fall in the nanoscale range (1–100 nm). The core size of platinum nanoparticles (Figure 22) detected had a diameter of between 1,14 nm and the largest of 2,66 nm for the conjugated molecule respectively (Figure 23).

Conjugated nanoparticles were noted to be slightly larger particles than platinum nanoparticles. The resulting bigger hybrid molecule could be due to the binding of gemcitabine to the platinum nanoparticle.

Because of their nanoscale size, nanoparticles pose great interest in many industrial applications. Their surface to volume ratio as well as their size has brought about many size dependent phenomena. These include chemical, electronic and mechanical properties. Furthermore, their melting points are decreased with nanoparticles as small as 1 and 2 nm in scale (Akbari *et al.* 2010).

Nanoparticles have been broadly contemplated as potential multifunctional carriers for drug delivery applications and one of their properties contributing to this is their size.

Their small size makes it easier for the nanoparticles to reach the objective diseased site, by marginating toward the vascular wall, communicating with the receptors on the vascular surface and thereafter binding to the target area. This contributes to the concentration of the drug at the objective site remaining sufficiently high to kill the diseased cells with negligible side effects, hence making nanoparticle size and distribution critical in assessing therapeutic efficacy.

Nanoparticle distribution has been thought to be an important scientific factor in nanoparticle drug delivery. Assessment of nanoparticle distribution is extremely unpredictable on the grounds that it can be affected by different factors including molecule shape, size, surface area, and flow conditions (Sanhai *et al.* 2008) and (Li *et al.* 2014).

In a report by Teow and Valiyavveti (2010), the core size of platinum nanoparticles was found to be in the range of 2-6 nm in diameter, whilst the conjugated Pt-FA nanoparticle was found to be 10-15 nm in diameter. It was also reported that platinum nanoparticles in water showed no signs of agglomeration and that this factor is imperative in making sure that individual molecules are transported across cell membranes.

TEM images, as in this study, also revealed that the platinum nanoparticles and the hybrid molecule were predominantly spherical in shape.

The chemical and bio-reactivities of NPs are frequently higher than bigger particles (Aitken, Creely and Tran 2004). Size is a key element for determining the toxic effect of a molecule, and nanoparticles are known to exhibit greater toxicity than their larger counterparts. The higher chemical reactivity of NP's prompts increased generation of ROS, including free radicals. ROS production has been seen in a few NPs, for example, carbon nanotubes, carbon fullerenes and metal oxide NPs. ROS and free radicals produced by NPs are the essential sources of NP toxicity, which might bring about inflammation, oxidative stress and DNA damage, proteins and membranes (Finkel 1998; Beal 2005; Grosse *et al.* 2009).

Shape, surface charge, chemical arrangement and structure, solubility and aggregation are factors that influence the toxicity of nanoparticles (Holsapple *et al.* 2004).

Nanoparticles promptly enter into the human body, crossing membranes and gaining access to cells, tissues and organs; that bigger particles ordinarily cannot. NPs are transported inside cells and taken up by the nucleus and mitochondria. In this manner, they can readily bring about major destruction to mitochondria, prompting damage to DNA and even cause cell death (Hett 2004).

As mentioned earlier, the chemical and physical properties of nanomaterials depend on their composition as well as on the molecule size and shape. A high-quality synthesis should first give control over molecule size and shape. For instance, if bigger nanoparticles are synthesized, the surface plasmon resonance will be steadily moved from out to the bigger wavelength side. Hence, if nanoparticles contrast in size, their optical attributes will likewise change essentially.

5.4 Cytotoxicity assay of platinum nanoparticles, gemcitabine and gemcitabine conjugated platinum nanoparticles

In this study, the procedure used to conduct the cytotoxicity assay was carried out as per instructions by Mosman (1983) with some modifications. The positive control used was doxorubicin for MFC-7 (breast cancer), UACC-62 (melanoma) and PBMC (peripheral blood mononuclear) cell line assays. Platinum nanoparticles, gemcitabine and gemcitabine conjugated platinum nanoparticles were screened against these cell lines.

PBMC was used to verify that platinum nanoparticles and the conjugated molecule do not cause cell inhibition to healthy cells.

From the MTT assay using the MCF-7 cell line (Figure 24), it was found that platinum nanoparticles did not inhibit cell growth at low concentrations. However, it must be noted that there was an increase in cell inhibition as the concentration increased. Platinum nanoparticles displayed toxicity to MCF-7 cells at 50 µg/ mℓ (18.43 %) and 100 µg/ mℓ (40.06 %) (Table 4). It was observed that at low concentrations platinum nanoparticles proliferated cell growth with cytotoxicity percentages as low as -42.03, -38.30, -34.99, -30.95, -23.19, -18.63, -17.63 and -13.98 % from concentrations 0.10 to 25 µg/ mℓ respectively. These values show that the percentage of viable cells exceeded dead cells. Platinum nanoparticles displayed the lowest toxicity towards MCF-7 cells compared to gemcitabine and gemcitabine conjugated platinum nanoparticles.

Conjugated platinum nanoparticles (Hybrids) displayed the highest toxicity against breast cancer cells. The cytotoxicity effect from lowest to highest concentration was 16.93, 20.05, 24.30, 33.18, 38.42, 46.39, 59.80, 55.20, 67.43 % and a high of 73.80 % at 100 µg/ mℓ (Table 4). Cytotoxicity of gemcitabine was lower than the conjugated molecule but higher than platinum nanoparticles.

From the MTT assay using UACC-62 (melanoma) cell line, platinum nanoparticles inhibited cell growth the least with percentage cytotoxicity values of -31.28, 29.46, -20.20, -16.55, -11.77, -8.63 and -3.26 % from concentrations 0.10 to 12.25 µg/ mℓ respectively (Table 5). These values show that the percentage of viable cells exceeded dead cells. Furthermore, platinum nanoparticles inhibited cell growth as the concentration increased with the highest toxicity at 25, 50 and 100 µg/ mℓ. These results were 11.24, 25.74 and 43.28 % respectively.

Gemcitabine conjugated platinum nanoparticles (hybrids) displayed the highest cytotoxicity against UACC-62 cell line with the highest cytotoxicity percentage of 89,54 at 100 µg/ mℓ (Table 5). Cytotoxicity of gemcitabine was lower than the conjugate but higher than platinum nanoparticles.

When comparing the cytotoxicity of platinum nanoparticles, gemcitabine and the hybrid to both cell lines, the following differences are noted. Platinum nanoparticles proliferated growth in both cell lines at low concentrations. As the concentration increases, cell inhibition occurs. Also to be noted that platinum nanoparticles displayed increased cytotoxicity towards against melanoma cells than breast cancer cells.

Gemcitabine conjugated platinum nanoparticles displayed the highest cytotoxicity against both cancer cell lines. However, cytotoxicity was greater towards melanoma cells (89,54 %) than breast cancer cells (73,80 %) at 100 µg/ mℓ.

Gemcitabine displayed cytotoxicity towards both cell lines with cell death of 64,92 % at 100 µg/ mℓ for breast cancer and 87,37 % at 100 µg/ mℓ for melanoma cells. Gemcitabine displayed higher cytotoxic effects against both cells lines compared to platinum but lower than the conjugated compound for both cell lines.

The results of this study prove that platinum nanoparticles, gemcitabine and the hybrid used displayed increased cytotoxic effects towards Melanoma cells than MCF-7 cells.

Platinum nanoparticles displayed toxicity to both cell lines as the concentration increased and this shows that platinum nanoparticles could potentially be used as a chemotherapeutic drug alone without the addition of a conventional drug.

These results also show that combining nanoparticles with current anticancer drugs can cause cancer cells to be targeted and that nanoparticles could allow for anticancer drugs to be more site specific. This would result in higher cytotoxicity towards cancer cell lines.

The cytotoxicity of platinum nanoparticles, gemcitabine and the hybrid was thereafter tested against peripheral blood mononuclear cells (PBMC) as a verification procedure that the conjugated nanoparticles do not inhibit the cell growth of healthy cells.

The results show that platinum nanoparticles do not have a cytotoxic effect on the healthy cell line at 50 µg/ mℓ (0 % cytotoxicity) but at 100 µg/ mℓ the cell inhibition was 0,03 %. Gemcitabine exhibited cytotoxicity of 1.01 % at 50 µg/ mℓ and 1.85 % at 100 µg/ mℓ. The conjugated compound displayed cytotoxicity of just 1.11 % at 50 µg/ mℓ and 1.26 at 100 µg/ mℓ. Doxorubicin was used as a positive control in this experiment and exhibited high cytotoxic effects at both 50 µg/ mℓ (16.66 %) and 21.76 % at 100 µg/ mℓ.

Gemcitabine displayed higher cytotoxicity to PBMC at 100 µg/ mℓ (1.85 %) than the conjugated compound (1.26 %).

These results indicated that platinum nanoparticles, gemcitabine and the hybrid compounds displayed cytotoxicity but in minute percentages towards PBMC. Cell death at such low values could be due to stress or other external unknown factors. Furthermore, platinum nanoparticles at low concentrations do not affect healthy cells. These results show that conjugated nanoparticles as drug carriers express lower cytotoxic effects to healthy cells compared to conventional drugs such as Doxorubicin. The cytotoxic effect of doxorubicin towards healthy cell line was 21.78 % at 100 µg/ mℓ.

Also, it should be noted that as the concentration increases cell inhibition to healthy cells could possibly increase.

Nanoparticles are said to be diverse systems created with a variety of shapes and sizes that empower them to be utilized for particular applications. Be that as it may, when planning drug delivery systems the primary consideration is to accomplish an increase in the quantity of effective treatments and reducing cytotoxic impacts to healthy cells (Alexis *et al.* 2010).

Wang, Liu and Ma (2014) reported that, mechanistic pathways have been established for a percentage of the functionalized platinum-based anticancer drugs, for instance, a platinum drug that had been conjugated with the Nrp-1 that targeted peptides particularly bound to Nrp-1 receptor, had lead to enhanced uptake by cells and cell toxicity. The pro-drug cisplatin was conjugated to LDH nanoparticles and enhanced the cellular uptake of platinum and additionally genomic DNA platination in cancer cells. Their study demonstrated that the platinum-based drug, cisplatin, induced apoptosis in cancer cells with no cytotoxicity to healthy cells (Wani *et al.* 2016).

Chopade *et al.* (2015) evaluated the cytotoxicity of platinum complexes combined with dimethyl pyrazoles based selenium ligands. Their cytotoxic action was tried against human colon, ovarian and bladder tumor cell lines and their activity was contrasted with cisplatin and adriamycin. The results demonstrated that the concentration of the complexes including cisplatin and adriamycin was more prominent than >100 μM to inhibit 50 % of cancer cells. It was noticed that the cytotoxic impacts of platinum-based complexes are dependent on the types of cell lines utilized and the dosage of test complex. Cytotoxicity of four of the platinum edifices towards human colon and bladder cancer cell lines showed comparable results to cisplatin, however, were poor for ovarian cell lines.

In a recent study, platinum complexes displayed potential antitumor impacts towards diverse cancer types. It was found that the platinum (II) complex (Oral *et al.* 2015) had a significant cytotoxic impact against MCF-7 and MDA-MB-231 (human breast cancers).

Yamada, Foote and Prow (2015), reported that platinum nanoparticles have the capacity to enter cells bringing about DNA damage. These nanoparticles also displayed antioxidant response changes when tested in vitro. Their study showed that smaller

nanoparticles brought about an increase in toxicity, proving that the size of nanoparticles readily plays a role in nanoparticle uptake across cell membranes.

Metal nanoparticles as delivery vehicles have been vastly studied (Wang *et al*, 2012). Gold or titanium nanoshells have been utilized for their controlled discharge as a part of chemotherapy. It must be noticed that a critical portion of the particles are retained in the body after it is administered, which can bring about an aggregation of particles following repeated administration and lead to toxicity. On account of this, most work on metal nanoparticles as drug delivery vehicles has stayed in the preclinical stage (Wang, Langer and Farokhzad 2012).

5.5 IC50 determination of platinum nanoparticles, gemcitabine and gemcitabine conjugated platinum nanoparticles

Both the conjugated nanoparticles, gemcitabine, and doxorubicin showed significant cytotoxicity against both cell lines. Platinum nanoparticles had an IC_{50} of 58.43 for MCF-7 and 50.06 for Melanoma cell lines. Gemcitabine displayed a low IC_{50} value of 14.04 for Melanoma and 16.07 for MCF-7. The conjugate compound exhibited the lowest IC_{50} values compare to platinum nanoparticles and gemcitabine with 8.03 for Melanoma and 9.20 for MCF-7 cell lines.

IC_{50} is defined as the drug concentration required to inhibit 50 % of cell growth by MTT assay following 48-hour drug exposure (Zhang *et al.* 2015b).

In a previous study by Bielawska *et al.* (2010), a platinum (II) complex was tested for activity against MCF-7 and MDA-MB-231 breast cancer cells. Reported results show that the IC_{50} values were 32 ± 2 μM and 20 ± 2 μM, respectively.

Lower IC_{50} values give the indication that the drug is potentially stronger, requiring a higher concentration to inhibit cell growth by 50 %. IC_{50} values for all three compounds was observed to be lower for Melanoma cells than MCF-7. From these results, it can be concluded that gemcitabine conjugated platinum nanoparticles exhibit the highest toxicity to both cell lines with the lowest IC_{50} values. These results express similar results as the cytotoxicity assay for both cell lines.

5.6 Apoptosis determination using flow cytometry

Apoptosis was first described by Kerr, Wylie and Currie (1972) is represented by a complex mechanism during which a number of molecules interact in an elaborate manner. Currently, there are several techniques as well as markers that help with the detection of apoptosis in vitro.

Apoptotic procedures are of biological significance to all life forms. Apoptosis is involved in the development, regulating and many other functions of the immune system. It also plays a role aim the evacuation of damaged or harmful cells.

Accordingly, dysfunction of the apoptotic system is embroiled in an assortment of pathological conditions. Imperfections in apoptosis can bring about cancer, spreading of viral infections and autoimmune diseases, while neurodegenerative disorders such as AIDS and other ailments are brought on or enhanced by excessive apoptosis.

5.6.1 Morphological observation

As discussed earlier, apoptosis includes chromatin condensation, overall cell shrinkage, blebbing of the plasma membrane and fragmentation resulting in apoptotic bodies containing nuclear or cytoplasmic material.

In the MTT assay, it was clear that gemcitabine and gemcitabine conjugated platinum nanoparticles significantly inhibited cell growth for the melanoma cell line. These results show that it is possible for these compounds to induce morphological changes that would be characteristic of cells undergoing the apoptosis process. The results in figure 25 show normal cell features for the untreated melanoma sample and the negative control, DMSO. Following treatment of the three compounds and the positive control (doxorubicin), cells appear to exhibit characteristic features of apoptosis (figure 27C, D, E and F). Clumping of cells can be seen in figures 27C and 27D with the detachment of cells in the latter four images. This is similar to an indication by Hacker (2000).

Apoptotic cells can be perceived by these stereotypical morphological change which includes the shrinkage of the cell which demonstrates deformation and looses contact with nearby cells. Its chromatin consolidates and marginates at the nuclear membrane, blebbing of the plasma membrane. The cells also fragment into small membrane

enclosed structures which are called 'apoptotic bodies' that contain the condensed chromatin, cytosol and organelles.

These apoptotic bodies are immersed by macrophages and in this way they are expelled from the tissue without bringing about an inflammatory reaction. Those morphological changes are a result of characteristic molecular and biochemical reactions taking place in an apoptotic cell, most remarkably the initiation of proteolytic enzymes which mediate the cleavage of DNA into oligonucleosomal fragments and the cleavage of a huge number of specific protein substrates which generally decide the shape and integrity of the cytoplasm or organelles (Saraste and Pulkki 2000).

5.6.2 Using Annexin V to detect changes in cell membranes

Annexin V was previously known as human placental anticoagulation protein. This protein contains a sequence comparing to a $Ca2^{+}$ dependent phospholipid binding site. Phospholipids are asymmetric and are circulated between the inner and external layers of the plasma membrane. Phosphatidylcholine and sphingomyelin are present on the outer layer and phosphatidylserine on the inner layer confronting the cytosol. Since PS is situated to the external membrane layer, cells which experience apoptosis lose the phospholipid asymmetry of their plasma membrane. Apoptotic cells can then be investigated by measuring the annexin V which ties to PS exposed in the external layer. Loss of membrane integrity and the intracellular binding of the protein can be brought about by necrosis. Necrotic cells likewise demonstrate a high annexin V binding capacity so cells can be stained with the non-vital dye propidium iodide (PI) (Grosse *et al*, 2009).

In this study, it was found that platinum nanoparticles, gemcitabine and the hybrid inhibited cell growth in melanoma cells by inducing apoptosis (Table 6). The percentage of apoptosis was quantified by flow cytometry. Results showed that the majority of untreated melanoma cells unstained with Annexin V were healthy indicating 100 % viability (Figure 26A). There was a decrease in viable melanoma cells tested against platinum nanoparticles, gemcitabine and the hybrid indicating cells undergoing apoptosis.

It was found that cell populations treated with platinum nanoparticles, gemcitabine and the hybrid and stained with Annexin V underwent early apoptosis (0.056 – 3.85 %) and

late apoptosis (0.12 – 9.15 %). Gemcitabine conjugated platinum nanoparticles exhibited the highest percent of cells displaying early apoptosis (3.85 %) and late apoptosis (9.15 %). These results suggest that a minimal amount of cells undergo apoptosis via changes in the cell membrane.

5.6.3 Mitochondrial membrane potential using JC-1

The mitochondrial membrane potential in this study was determined by flow cytometry. One of the earliest markers for apoptosis is changes in the mitochondrial membrane (BD Biosciences). For the JC-1 assay, monomers and aggregates that have different emission spectra were used to detect changes in the cells mitchondria.

JC-1 dye forms complexes known as J-aggregates which emit intense red fluorescence in healthy cells. These cells exhibit high mitochondrial membrane potential. In apoptotic or unhealthy cells, however, the mitochondrial membrane potential collapses. This means that JC-1 dye no longer accumulates within the mitochondria and yields only green fluorescence.

The mitochondrial membrane potential was quantitatively detected to assess the cell death mechanism when melanoma cells were exposed to platinum nanoparticles, gemcitabine and the hybrid (test compounds).

Results in Table 7 showed that untreated unstained cells exhibited the highest percentage of viable cells (100 %) and no apoptosis took place. Untreated cells stained with JC-1 exhibited 4.58 % of cells undergoing apoptosis. Doxorubicin was used as a positive control at 6 µg/mℓ and exhibited the highest percentage of cells undergoing apoptosis (14.2 %). Of the three test compounds, gemcitabine conjugated platinum nanoparticles showed the highest percentage of cells undergoing apoptosis (13.1 %) Gemcitabine displayed 12 % of cells undergoing changes in mitochondria whilst there was a decrease in membrane potential displayed by platinum nanoparticles with 4.58 % of cells undergoing apoptosis.

The results indicated a significant loss of mitochondrial membrane potential at a concentration of 50 µg/mℓ. These results show that apoptosis of melanoma cells may have been caused by the loss of mitochondrial membrane potential function.

It was described in a recent study how the loss of mitochondrial membrane potential is a mechanism of cell apoptosis. In the study it was mentioned that mitochondrial membrane potential loss is chiefly because of the opening of a huge cell channel known as the mitochondrial permeability transition pore. This pore changes the matrix exchange between the external and internal mitochondrial membrane. This membrane potential breakdown was the necessary condition of the cells experiencing apoptosis and this procedure is irreversible (Zhang *et al.* 2015a).

5.6.4 Apoptosis detection using the caspase-3 assay

There have been extensive studies done on apoptosis in the most recent two decades and it has been demonstrated that caspases (particularly caspase-3, 8, and 9) assume critical roles in the apoptotic process (Sawai and Domae 2011). One of the signs of apoptosis is the cleavage of chromosomal DNA into nucleosomal units.

Cell death through apoptosis incorporates two major pathway (extrinsic and intrinsic). Both of these pathways lead to the enactment of proteases which comprise of caspases 3, 6, and 7. Of these caspases, caspase-3 is primarily responsible for the cleavage events amid apoptosis (Huang *et al.* 2009).

Caspases cleave proteins which result in cell death and this characteristic is used to detect apoptotic cells. Cells in this assay were stained with a PE conjugate called polyclonal active caspase-3 antibody. Cells were detected using the flow cytometer by their fluorescence when stained.

Doxorubicin exhibited apoptosis with 99.8 % of cells as caspase positive PE-A^+. Gemcitabine conjugated platinum nanoparticles exhibited the highest percentage of cells undergoing apoptosis at 56.8 % for caspase PE-A^+. Gemcitabine exhibited 46.1 % of cells for caspase positive PE-A^+ activity, whereas, platinum nanoparticles displayed the lowest activity at 1.84 % of cells undergoing apoptosis (Table 8). As expected, the results for apoptosis correlate with MTT results showing gemcitabine conjugated platinum nanoparticles displaying the highest cytotoxicity against the melanoma cell line. Gemcitabine and platinum nanoparticles also fall in line with MTT results.

Apoptosis detection using the melanoma cell line indicates that the highest percentage of cells undergo cell death via cleavage of caspase-3 proteins.

CHAPTER 6: CONCLUSION

Current research is aimed at achieving specific targeted delivery of anticancer drugs utilizing new and innovative avenues of directing drugs to cancer cells. It is evident that the versatility of nanoparticles makes them very suitable as drug delivery vehicles. This is due to their characteristic small size, increased surface area and volume ratio. An increasing number of nanoparticles are being investigated as delivery vehicles of anticancer drugs due to their versatile physiochemical properties. Nanoparticles can be easily fuctionalized, are readily available and they possess good biocompatibility with low cytotoxicity.

In this study, platinum nanoparticles, gemcitabine and gemcitabine conjugated platinum nanoparticles were synthesized and characterized in order to evaluate their bioactivities towards MCF-7 (breast cancer) and UACC-62 (melanoma) cancer cell lines. Their bioactivity was also tested against PBMC (peripheral blood mononuclear) cells as a verification that platinum nanoparticles and the conjugate do not inhibit cell growth of healthy cells.

TEM images revealed the core size of platinum nanoparticles in the range of 1 - 2 nm and slightly larger particles (1.5 – 2.6 nm) for the conjugate. Studies have shown that this characteristic small size of nanoparticles could enhance the uptake of anticancer drugs in tumor cells by ease of transport through blood vessels and vascular cell wells.

Platinum nanoparticles, gemcitabine and the hybrid displayed cytotoxicity towards MCF-7 and UACC-62 cell lines with the hybrid resulting in the highest percentage of cell death at 100 μg/mℓ against both breast cancer and melanoma cell lines. Platinum nanoparticles, gemcitabine and the hybrid displayed little or no toxicity towards healthy cells and were observed to induce apoptosis by bringing about morphological and membrane changes, disruption of the mitochondrial membrane and most significantly by cleavage of caspase-3.

This process of apoptosis results in the cleavage and fragmentation of the cells DNA by directly attacking the cells nucleosomal structure.

Research shows that platinum nanoparticles readily cause DNA damage in cancer cells

and that smaller nanoparticles can pass through cell membranes more efficiently allowing a sufficient dose of anticancer drugs to be delivered to the treatment site (Yamada, Foote and Prow 2015).

In conclusion, such toxicity, combined with the possibility to incorporate functional organic molecules as capping agents, can be used for developing new drug candidates.

The utilization of nanoparticulate drug vehicles can address numerous difficulties in drug delivery including enhancing drug solvency and dependability; augmenting drug half-lives in the blood; diminishing negative impacts in non-target organs as well as the concentration of drugs to diseased sites.

This study shows that combining nanoparticles to anti-cancer drugs can enhance cytotoxicity against cancer cells with little or no effect towards healthy cells. The important factor is to have cancer detected and treated as early as possible. This would require advanced detection as well as targeting methods, which can be combined with nanotechnology.

It must be noted that further extensive research into the toxicity of nanoparticles need to be carried out which is why most studies on nanoparticles have remained the pre-clinical trial phase.

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APPENDIX A

Materials, Suppliers and Equipment

1. Synthesis of platinum nanoparticles and gemcitabine conjugated platinum nanoparticles

Reagent	**Empirical Formulae**	**Supplier**	**Catalogue Number**
Hexachloroplatinic acid hexahydrate	H_2PtCl_6	Sigma Aldrich	P5775
Sodium Borohydride	NaBH4	Sigma Aldrich	480886
Polyvinyl pyrollidone	PVP	Sigma Aldrich	P2307
Gemcitabine hydrochloride	$C_9H_{11}F_2N_3O_4.HCl$	Sigma Aldrich	G6423

2. MTT Assay

Media/ Consumables	**Supplier**	**Catalogue Number**
Ethanol	Sigma Aldrich	
Dulbecco's Modified Eagle Medium (DMEM)	Sigma Aldrich	D6429
10 % heat activated foetal calf serum	Sigma Aldrich	F0685
1 % antibiotic-penicillin streptomycin solution	Sigma Aldrich	A2213
Trypsin	Sigma Aldrich	T1763-25UN

Phosphate buffered saline (PBS)	Sigma Aldrich	P5493
100 % dimethyl sulfoxide (DMSO)	Sigma Aldrich	D8418
3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide (MTT reagent)	Sigma Aldrich	M2003
Cell culture flasks	Sigma Aldrich	CLS3920

3. Equiment

Experimental Use	**Equipment**
UV-sprectrum analysis	Cary 100 UV-Vis spectrophotometer
FT-IR analysis	PerkinElmer Spectrum 100 spectrophotometer
Transmission electron microscopy (TEM)	JEOL JEM-1010 transmission electron microscope
MTT assay - plate reader	BiohitPlc, e-Lisa XL
MTT assay – cell culture incubation	Snijders Scientific CO19IR CO_2 incubator
Flow cytometry	BD Bioscience

APPENDIX B

Equation and Formulae

1. MTT assay

MTT assay plates were scanned for absorbance readings using the e-Lisa plate reader and percentage cell viability was calculated using the following equation:

Percentage viability (%)	=	$\frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100$
Percentage cytoxicity	=	100 – (percentage viability)

MTT assay was perfomed in triplicate for all samples including controls. An average of the three absobance readings was used to calculate % viability for each concentration. Standard devaition and standard errors were determined for each concentration.