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THE IMPACT OF NANOPARTICLES ON THE PROTEOME OF CULTURED HUMAN CELLS

by

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DECLARATION

I **Oladipupo Moyinoluwa David** declare that "**The impact of nanoparticles on the proteome of cultured human epithelial cells"** submitted for a PhD degree at the University of the Western Cape is my own work and has not been previously submitted for a degree at this or any other university, and that it is my own work, and that all sources used, cited, or quoted have been acknowledged and referenced accordingly.

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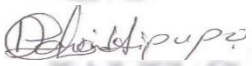
Signature:  **Date: September, 2021**

ABSTRACT

Living organisms are constantly being exposed to nanoparticles (NPs) in the environment via air, water, soil. Routes of exposure are usually in the form of industrial, occupational exposure, as well as therapeutic applications. This exposure could result in toxicity with potential harmful effects. The toxicity of nanoparticles depends on various factors such as surface interaction, shape, size, composition, aggregation and interaction with various cellular components.

Nanotoxicity refers to the possible harmful effects of environmentally generated and man-made nanoparticles on biological and environmental system. Assessing potential toxicity is vital for the probable use and safety of nanoparticles as well as understanding the routes of entry into organisms and their mechanism of action. Proteomics is a developing field of science that is being explored to understand protein composition, structure and interaction at the cellular level. This helps in detecting the presence, quantity, alteration and regulation of proteins within the biological system. The proteome analysis brings an additional information as it enables measurement of whole-protein (enzyme) expression levels, facilitating the construction of metabolic pathways and biomarker discovery for early disease diagnosis. Essentially, proteomic analysis reveals the consequence of stress on metabolic pathways necessary to maintain the energy homeostasis within the cells.

Interaction mechanisms between nanoparticles and biological systems in relation to proteomics are not yet fully understood. In addition, scientific knowledge about the mechanisms involved in nanoparticle–cell interaction has been accumulating in recent years and have shown that cells readily take up nanoparticles via either active or passive mechanism. Hence this research work seeks to investigate the effect of nanoparticles on the proteomic profile of human epithelial cells so as to improve the application and uses of nanoparticles in delivery, diagnostic, imaging, therapy

and environmental remediation. The nanoparticles selected for this study are silver (Ag) and titanium dioxide (TiO_2) nanoparticles with the aim of evaluating the possible harmful effects, inflammatory modulation, oxidative stress and uptake mechanism. The colon cancer epithelial cell line (Caco-2) was selected as a model for intestinal epithelium in this study for evaluation of the possible toxicity of the selected nanoparticles. The nanoparticles parameters evaluated included hydrodynamic size and zeta potentials. The exposure of Caco-2 cells to the respective nanoparticles assessed cell viability, proteome profile analysis, cell stress, inflammatory biomarkers, anti-angiogenic properties and uptake mechanisms.

The first set of objectives of this study was to elucidate the physiological effects that various media would have on AgNPs and TiO_2NPs hydrodynamic size and zeta potential. Thereafter the effects of these characterized nanoparticles on cell viability, cell stress, inflammatory biomarkers and anti-angiogenic properties on Caco-2 cells were evaluated. The results showed that the AgNPs were stable in physiological media, based on hydrodynamic size and zeta potential over the time period assessed 0 hour, 24 hours, 7 and 14 days. Whereas, the TiO_2NPs sizes increased over time, while zeta potentials were stable. The cytokine proteome profile membranes assessed for the AgNPs mostly revealed the same proteins, with the exception of activin A and dipeptidyl peptidase IV (DDPIV) which was only present in the control group. The intensity of certain proteins decreased in the presence of the IC_{50} value in comparison to the control. These proteins include: angiopoietin-2, endostatin and platelet-derived growth factor AA (PDGF-AA). Conversely, persephin was slightly upregulated in the presence of 100 μg/ml AgNPs in comparison to the control group. The AgNPs are cytotoxic to Caco-2 cells at high concentrations (IC_{50} = ± 100 μg/ml) and induced cell stress biomarkers at concentrations > 6.25 μg/ml AgNPs. The AgNPs modulated the inflammatory

cytokine IL-8 and reduced IL-6 production in a dose dependent manner. Also, possible anti-angiogenic markers of AgNPs were identified: angiopoietin-2 and PDGF-AA.

The TiO_2NPs proteome profile showed that the control (0 μg/ml TiO_2NPs) and NOAEL (100 μg/ml TiO_2NPs) supernatants assayed for potential angiogenic biomarkers revealed the same proteins, except persephin which was only evident in the control membrane. Based on the intensity of the dot, the protein dipeptidyl peptidase IV (DPPIV) and endostatin was more prominent on the NOAEL membrane. However, platelet derived growth factor AA (PDGF-AA) and angiopoietin-2 was more noticeable to cells exposed to control concentration. The level of inflammatory biomarkers was not affected. Subsequently, anti-angiogenic properties were exhibited when exposed to the no-observed-adverse-effect level (NOAEL) concentrations of the TiO_2NP. The TiO_2NPs induced cell stress biomarkers, which could be attributed to the NPs not being cytotoxic. The last objective of this research was to evaluate the uptake mechanism effects on the toxicity of nanoparticles through the cytotoxic and/or inflammatory pathways. Different pathway inhibitors were used such as Amiloride chloride for (micropinocytosis), ammonium chloride (phagocytosis), chlorpromazine (clathrin mediated endocytosis) and nystatin (caveolae mediated endocytosis). This is with the view to understand the pathway or mechanism whereby the nanoparticles induce cytotoxicity and inflammation. AgNPs were cytotoxic and modulated the inflammatory biomarkers (nitric oxide, interleukin-6, and macrophage migration inhibitory factor) at concentrations ≥ 12.5 μg/ml. However, the inhibitors did not significantly mitigate toxicity nor alter the inflammation caused by the AgNPs. Therefore, for NPs to be utilized to their fullest potential, it is important to evaluate their uptake with respect to toxicity and inflammation, which may also be used to predict any antagonistic cellular responses.

DEDICATION

This thesis is dedicated to God the Father, God the Son and God the Holy Spirit for perfecting this work through me.

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ACRONYMS AND ABBREVIATIONS

°C	degrees Celsius
2-D	two dimensional
AgNPs	silver nanoparticles
ANOVA	analysis of variance
ATCC	American Type Culture Collection interferon gamma
BSA	bovine serum albumin
Cells/ml	cells per millilitre
CO_2	carbon dioxide
DAS ELISA	double antibody sandwich enzyme-linked immunosorbent assay
DMEM	Dulbecco's modified Eagle's medium
DPBS	Dulbecco's phosphate buffered saline
ER	endoplasmic reticulum
FBS	fetal bovine serum
FBS/well	fetal bovine serum/well
FTIR	Fourier-transform infared spectroscopy
g/l	gram per liter
HRP	horseradish peroxidase
hrs	hours
HSA	human serum albumin
IC_{50}	half maximal inhibitory concentration
IFNα	Interferon alpha
IFNγ	interferon gamma

IL-6	interleukin 6
IL-8	interleukin 8
ILs	interleukins
K	potassium
K_2SO_4	potassium sulphate
KNO_3	potassium nitrate
LDH	lactate dehydrogenase
LPS	lipopolysaccharides
Mg	magnesium
Mg/l	milligram per litre
Mg/ml	milligram per millilitre
$MgCl_2$	magnesium chloride
MIF	macrophage migration inhibitory factor
mins	minutes
Mm	millilitre
mV	millivolts
n	sample size
$Nacl_2$	sodium chloride
NF-$_k\beta$	nuclear factor kappa beta
NK	natural killer cells
Nm	nanometer
NO	nitric oxide
NPs	nanoparticles

PAMPs	pathogen-associated molecular patterns
PEG	poly (ethylene glycol-amine)
pH	potential of hydrogen
PHA	phytohaemmagglutinin
ppb	parts per billion
ppm	parts per million
PRRs	pattern recognition receptors
ref	relative centrifugal force
ROS	reactive oxygen species
SD	standard deviation
Secs	seconds
SOD	superoxide dismutase
Tc	cytotoxic T-cells
TEM	transmission electron microscopy
TLRs	Toll-like receptors
TMB	3,3'.5,5'-tetramethylbenzidine
TNF-α	tumour necrosis factor alpha
Ti_2ONPs	titanium nanoparticles
UV-vis/UV	ultraviolet-visible spectrophotometry
v/v	volume per volume
w/v	weight per volume
WST-12-	(4-Iodophenyl) (-3-(4-nitrophenyl)-5-(2,4-dislfophenyl)-2H tetrazolium

Zn	Zinc
µg/g	microgram per gram
µg/l	microgram per liter
µg/ml	microgram per millilitre
µl	microliter
µm	micrometer
µM	micromolar

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CONTRIBUTION TO KNOWLEDGE DURING THE PERIOD

Drafted Manuscripts

- Immunomodulation and possible anti-angiogenic effects of AgNPs on human intestinal Caco-2 cells.
- Stability and anti-angiogenic properties of TiO_2NPs in Caco-2 cells.
- The uptake mechanism of AgNPs by human intestinal Caco-2 cells

Presentation

Edmund Pool, Oladipupo David, Okobi Ekpo, Maria Fildago (2019) Comparative toxicity of 20nm silver and titanium dioxide nanoparticles using CaCo-2 cells SETAC Europe 29th Annual Meeting Helsinki Finland

CHAPTER ONE: INTRODUCTION

1.1 Background

Nanotechnology is the scientific manipulation of matter and the process of controlling matter at the atomic and molecular level. Nanoparticles (NPs) on the other hand, are materials with dimensions that range between 1 and 100 nm (Bahadar *et al*., 2016). Nanotechnology now has applications in a wide range of fields and disciplines, including medicine, biology, chemistry, engineering, physics and environmental science, with immense benefits that are not limited to diagnostic, bioimaging, biosensing, drug delivery, drug therapy, pharmaceutical and home appliances (Oberdörster, 2010). The exponential growth in nanotechnology worldwide stems from various research discoveries which have promoted its use to upgrade and scale-up new nanomaterials (Breggin *et al*., 2016). Nanomaterials are referred to as "matter at the scale of 1 billionth of a meter" (i.e., 10^{9}m = 1 nm), usually studied at the atomic and molecular scale (Rai and Rai, 2017). With the advancement of nanotechnology, there seems to be a dearth of published toxicological data associated with the production, exposure, usage, interactions and disposal of nanoscale materials.

The demand for nanomaterials has increased over the years and many industries continue to place high demand on them due to their many product applications. End-user goods that contain nanomaterials were valued and estimated at $14,741.6 million in 2015, according to Allied Market Research, a global market research firm which also estimated a rise to the value of $55,016 million by 2022 (Mohajerani *et al*., 2019). Nanoparticles form a link between bulk materials and molecular structures as they have different physicochemical properties such as the small size, lower melting points, higher specific surface areas to volume ratio, specific optical properties, mechanical

strengths, and specific magnetizations (Horikoshi & Serpone, 2013). These properties can alter the solubility, dispersion state, aggregation, degree of agglomeration, chemical reactivity of the material and biological processes thereby causing toxicity (Azhdarzadeh *et al*., 2015). These parameters of NPs do not only differ in materials but also between different nanoforms of the same chemical substance and hence so do their effects and activities. The change in the physicochemical properties can lead to differences in the cellular uptake of these molecules, reactions within cellular components, levels of toxicity, and may also vary in concentration-response interactions describing toxicity (Schwirn *et al*., 2014).

Nanoparticles can be classified into different groups based on their physical and chemical characteristics, such as inorganic NPs (gold, silica, silver, superparamagnetic, paramagnetic iron oxides, copper, cobalt, zinc, titanium, and aluminum oxides) and organic (carbon, graphene, micelles, liposomes, dendrimers and polymers such as poly lactic-co-glycolic acid and polysacharrides chistosan) NPs (Khanna *et al*., 2015). Metallic NPs are preferred due to their low toxicities, effectiveness as well as useful industrial and medical applications. Silver and titanium dioxides are the most prospective constituents to be combined with other nanomaterials in consumer products (Vance *et al*., 2015) including drugs, food, cosmetics, electronics, home appliances etc., thus making them indispensable.

Silver nanoparticles (AgNPs) have become nanomaterials of choice over the years, due to their favourable properties especially their antimicrobial activities (Ahmed *et al*., 2017). Silver nanoparticles have gained economic importance as they are used in bioactive delivery systems, microbiological control, food processing, electronic appliances, clothing, cosmetics, infant

products, and flavour encapsulation (Zhang *et al*., 2016). In addition, AgNPs can also be used as active ingredients in the prevention of infection against microorganisms, leading to numerous medical inventions including nano-crystalline silver wound dressing and AgNP-coated ventricular catheters which are now commercially available (Naik & Kowshik, 2017). Environmental discharge of AgNPs occurs as a result of various processes such as production, usage, application and waste disposal (Du *et al*., 2018), all of which may pose a challenge to human health and the biota. Several studies have reported the *in vitro* and *in vivo* toxicity of AgNPs and their relative impact on the ecosystem (Tortella *et al*., 2019). The wide availability and growing usage of AgNPs may increase their entry into aquatic bodies, plants, animals and the biota. Therefore, it is imperative to elucidate the imminent danger that this valuable NP may portend to human populations and the biota.

Titanium dioxide (TiO_2) is one of the most common naturally occurring metal oxides (Armand *et al*., 2016). It has three different polymorphic forms, namely; brookite, anatase and rutile. Anatase and rutile forms of TiO_2 are commercially used compared to brookite form which is less produced and used industrially (Gea *et al*., 2019). Over the century, titanium dioxide nanoparticles (TiO_2NPs) have been used as pigments in cement and paint production due to their high refractive index, resistivity and brightness (Wang *et al*., 2019). TiO_2NPs have a wide range of application and usage, especially in food, cosmetics, paper, toothpaste, sunscreen and pharmaceutical products (Bettini *et al*., 2017; Louro *et al*., 2019). In addition, TiO_2NPs are used for water and air purification as well as in antimicrobial agents owing to their photocatalytic and catalytic properties (Gea *et al*., 2019). The mechanism of toxicity of TiO_2NPs is not yet fully understood as there are conflicting opinions from the studies done so far, possibly because of the physicochemical

properties, polymorphs, cell types and experimental setup involved (Botelho *et al*., 2014). TiO_2NPs being an inert NP, is considered to be harmless. However, recent studies showed that TiO_2NPs may be carcinogenic in humans (Dudefoi *et al*., 2017). Toxicity arising from TiO_2NPs may be attributed to cytotoxicity, inflammation, oxidative stress, genotoxicity, apoptosis and eventually cancer (Hanot-Roy *et al*., 2016; Kim *et al*., 2019; Skubalova *et al*., 2019). The indispensability and wide use in different products of TiO_2NPs has raised great concerns over their safety and harmful effects to living organisms and the environment.

Human activities have greatly contributed to different kinds of environmental pollutants such as air, water, and soil resulting from NP production. Hence, living organisms are constantly exposed to NPs, in their uninterrupted interactions with all forms of environmental activities emanating from industrial, occupational and daily applications. Nanoparticles enter their host through various routes such as lungs, gastrointestinal tracts and skin as a result of inhalation, injection, ingestion and implantation (Joris *et al*., 2013; Khanna *et al*., 2015). The entry of NPs into animals may pose danger by enabling penetration through the animal cells, tissues and organs systems leading to organ damage and possible adverse physiological conditions (Bakand & Hayes, 2016). Consequently, NPs may cause unwanted reactions with serious repercussions on humans and the biota. This may lead to an increased concentration of toxic substances within the organism causing profound complications linked to the alteration of essential biological processes (Oberdörster, 2010). Recent studies have shown that NPs may play a role in many chronic and degenerative diseases through increased reactive oxygen species (ROS), modulation of inflammatory cytokines, activation of signalling pathways, cytotoxicity, genotoxicity, oxidative stress, mitochondrial

dysfunction, and endoplasmic reticulum stress (Akhtar *et al*., 2012; Gu *et al*., 2016; Khanna *et al*., 2015).

Cellular uptake of NPs involves highly-controlled mechanisms with complex biological interactions to overcome the cell plasma membrane which acts as a barrier and separates intracellular components from the extracellular milieu. Generally, it is more difficult for large NPs to cross the capillary wall and distribute albeit more slowly, to the organs than small NPs (Zhao & Stenzel, 2018). Uptake and influx of NPs occurs through lysosomal or endosomal endocytosis (Behzadi *et al*., 2017). Macropinocytosis, scavenger receptor and clathrin-mediated pathways have been reported as mechanisms of NPs uptake in cells (Behzadi *et al*., 2017). Due to size and surface area, internalized NPs may enter such sub-cellular structures as the nucleus and mitochondria, where they interact with membrane proteins and cause complications of host cellular processes including alteration of cell morphology, inflammation, oxidative stress, DNA damage, genotoxicity, mitochondrial dysfunction, and consequently cell death by apoptosis or necrosis (Ahmed *et al*., 2017). Electron spin resonance research shows that dissolution of NPs can stimulate hydroxyl and other free radical formation in lysosomes, thus causing lysosomal rupture, the release of cathepsins into the cytoplasm, and ultimately lysosome-mediated apoptosis (Yang *et al*., 2014). NPs in the mitochondria cause mitochondrial membrane potential collapse, disruption of the respiratory chain, oxidative stress, inhibition of ATP synthesis and subsequent activation of the mitochondria-dependent intrinsic apoptotic pathway (Zhang *et al*., 2016). However, there is a paucity of information on cellular interaction mechanisms between NPs and the biological systems. Therefore, further studies are needed to understand the impact of NPs on human health and the environment.

Epithelial cells are a layer of cells present in all parts of an organism's body, especially the outer surfaces and inside inner organs (Parham, 2014). The diverse epithelial cells are derived from three of the primary embryonic germ layers namely, the ectoderm, mesoderm and endoderm (Parham, 2014). Epithelial cells are the body's first line of protection from physical, chemical and biological toxicity (Ahamed, 2011), by acting as gatekeepers of the body controlling permeability and allowing selective transfer of substances across barriers, as all substances that enter the body must cross an epithelium. Some epithelia often include structural features that allow the selective transport of molecules and ions across their cell membranes (Rabanel *et al*., 2012). These cells are capable of secretion and release of mucus and specific chemical compounds onto their apical surfaces against invasion by toxins. For example, the epithelium of the small intestine releases digestive enzymes, just as the cells lining the respiratory tract secrete mucus that traps incoming microorganisms and toxic particles (Parham, 2014). Furthermore, epithelial cells adjoin one another and lie upon a supporting extracellular stratum, the basement membrane. The adjoining cells that make the specialized intercellular connection between cell membranes are termed cell junctions. Some of these connections are adhering connections (junctions) (Capaldo *et al*., 2017) while the interconnecting junctions are zonula occludens (tight junctions) that seal the space between epithelial cells so that no ion or water can flow between them (Capaldo *et al.*, 2017). Zonula adherens physically bind the cells together in a zone near the apical pole while the macula adherens (desmosomes) physically bind cells together in smaller areas resembling spots. On the other hand, the gap junctions (nexus) connect the cells together via protein-lined channels that provide a means for crosstalk by allowing molecules to flow from cells to cells (Kierszenbaum & Tres, 2015). The open surface of the external membrane cells displays a little immobile cytoplasmic projection, called microvilli, and other structural specialization such as cilia. The

entire gratis of the tissue is frequently increased by means of folds, tubules, and in the intestine, large projections called villi. Alteration of epithelial cells by nanoparticles can possibly lead to calcium homeostasis imbalance, oxidative and endoplasmic reticulum stress, mitochondrial dysfunction and gene mutation (Chen *et al*., 2014).

The colon cancer epithelial cell line (Caco-2) is used extensively as a model for the intestinal epithelium in studies for drug, nutrient and xenobiotic absorptivity and transport as well as for the evaluation of the possible toxicity of nanoparticles (Georgantzopoulou *et al*., 2015; Lefebvre *et al*., 2015). The toxicology of inhaled and ingested nanoparticles has been scarcely studied compared to other routes of exposure, due to interaction rate and risk. It is most likely that when nanoparticles cross a cell's membrane barrier to enter the intracellular space, the immune system, which is seen as the defence machinery may be compromised as a result of its inability to detect and defend the host from microorganisms, toxic and foreign substances. This alteration of proteins of the immune system, especially chemokines and cytokines could possibly result in serious complications and diseases.

Proteomics is a developing field of science that is being explored in understanding protein composition, structure and interaction at the cellular level. This helps in detecting the presence, quantity, alteration and regulation of proteins within the biological system. In addition, the proteome is the entire set of proteins synthesized or altered by an organism. The level of alteration and modification of proteins is dependent on the cell type or organism that it is exposed to. Proteome analysis brings additional information as it enables the measurement of whole-protein (e.g. enzyme) expression levels, facilitating the metabolic pathways and biomarker discovery for

early disease diagnosis (Saptarshi *et al*., 2013). Essentially, proteomic analysis reveals the consequence of stress on metabolic pathways necessary to maintain the energy homeostasis within the cells. The toxicity of different NP cell interactions is dependent on the impact of the NPs on the plasma membrane, intracellular trafficking and the effect on basic biological functions (Panariti *et al*., 2012). Interaction mechanisms between NPs and biological systems in relation to the proteome remain elusive. However, scientific knowledge about nanoparticle–cell interaction mechanisms indicating that cells readily take up nanoparticles via either active or passive mechanisms, has been accumulating in recent years (Elsaesser & Howard, 2012). Nanoparticles toxicity can be detected by exploring the mechanisms and pathways whereby the cells take up the particles and cellular interaction. This mechanism of reactivity of NPs in cells and their relationship with proteins could be linked to their toxic effects, which is also associated with their physicochemical characteristics. e.g., smaller NPs tend to generally have higher toxic effects while other parameters such as surface area, protein corona formation, agglomeration, and cell type are often used to determine the cellular interaction of NPs (Ahmed *et al*., 2017). The production of reactive oxygen species, the dissolution and release of toxic ions, the disturbance of the electron/ion cell membrane transport activity, oxidative damage through catalysis, lipid peroxidation, etc. have been implicated to play an early and active role in the toxicity of nanoparticles (Zhang *et al*., 2016). This could lead to complicated processes in the cell that may elicit the malfunctioning of cellular processes in biological systems.

Organism exposure to toxic NPs can be minimized through the identification of biomarkers and specific pathways, which may be helpful in the early and successful diagnosis of possible risk exposure to NPs linked to such diseases as argyrosis, pleura damage, asthma, pulmonary fibrosis,

autoimmune diseases and cancer. In this research work, the interaction of silver and titanium NPs on the epithelial cells was studied, with a focus on the cytotoxicity, mechanisms of influx and effects on the proteome.

1.2 Research Problem

Concerns have been raised regarding the toxicity and possible adverse effects of NPs on some biological processes in humans. This is because living organisms can be exposed to nanoparticles through several applications such as diagnostics, bioimaging, biosensing, electronics, food processing and pharmaceuticals. Despite their unique and numerous applications and potential medicinal and economic benefits, NPs must be subjected to rigorous toxicity testing to ensure that they do not pose adverse risks to humans, the biota, and the environment. The exposure and possible influx mechanism of NPs into living organisms can potentially result in toxicity with potential harmful effects such as reactive oxygen species (ROS) generation. Although the cell has defence machinery to regulate the release of ROS such as the enzymes superoxide dismutase (SOD) and catalase, there is still some ROS leakage from the mitochondria which is potentially damaging to most cellular constituents including proteins, phospholipids and mitochondrial DNA.

The disruption of membranes, membrane activity, protein aggregation, protein conformation/folding, and transport processes also contribute to the possible effects of nanoparticles on the cells. Consequently, NPs may modulate the cellular system by their uptake and interactions with the proteome, intracellular organelles, immune system and biological fluids leading to pathological and physiological disorders. Due to insufficient data in literature, there is a need to investigate the immune modulatory effects and cytotoxicity of the selected nanoparticles. Furthermore, possible harmful effects of environmentally generated and self-mediated NPs on

biological and environmental systems need to be elucidated. The understanding of the cellular stress and immune response of an organism's interaction with NPs are very useful scientific evidence for the development of a harmless and environmentally-friendly NP in the nearest future.

1.3 Aims

This study is aimed at investigating the potential cytotoxicity and the different mechanisms by which silver and titanium dioxide nanoparticles affect the proteome of Caco-2 cells, a human colon epithelial cell line used as an *in vitro* model of the human intestinal epithelium.

1.4 Objectives

- To investigate the potential toxicity and different mechanisms by which AgNPs and TiO_2NPs affect the proteome of the human colon epithelial cell line (Caco-2).
- To analyse and identify potential biomarkers that can be used for early prognosis and diagnosis.
- To evaluate the different uptake mechanisms by which AgNPs and TiO_2NPs mediate cytotoxicity and inflammation.

1.5 Research Question

The possible research questions this research study seeks to address are:

- Do AgNPs and TiO_2NPs affect cell proliferation and viability of the Caco-2 epithelial cell line?
- What are the possible biomarkers which can be used to evaluate the potential toxicity effects of AgNPs and TiO_2NPs on the epithelial cells?

- What are the uptake mechanisms by which AgNPs and TiO_2NPs mediate cytotoxicity and inflammation in epithelial cells?

1.6 Hypothesis

H_0: AgNPs and TiO_2NPs are not cytotoxic and do not modulate the proteome of human Caco-2 colon epithelial cells.

H_1: AgNPs and TiO_2NPs are cytotoxic and modulate the proteome of human Caco-2 colon epithelial cells.

1.7 Thesis Structure

This thesis consists of six chapters; Chapter 1 is a general Introduction while Chapter 2 is the literature review. Chapter 3 will cover the effects of physiological media on TiO_2NPs hydrodynamic size, zeta potential and the effects of these characterized TiO_2NPs on cell viability, cell stress, inflammatory biomarkers and anti-angiogenic properties as well as the interaction of nanoparticles with the proteome using the inflammatory cytokine proteome profile membrane. In Chapter 4, some inflammatory biomarkers selected and analysed using the enzyme-linked immunosorbent assay (ELISA) to determine possible modulation and identify possible biomarkers for prognosis and diagnosis of toxicity of nanoparticles will be reported in detail. Chapter 5 will cover the report on the uptake mechanism that was evaluated to determine the possible route and effect on cytotoxicity and inflammatory biomarkers. The last chapter (Chapter 6) is the conclusion and recommendation.

The overview of the chapters is given below:

Chapter one: Introduction, objectives and thesis structure

This chapter gives an introduction to the general knowledge on nanoparticles, and identifies possible gaps. It also covers the aims, objectives and structure of the thesis.

Chapter two: Literature review

This chapter reviews the available studies on the *in-vitro* toxicity of NPs, various uptake mechanisms and the impact of nanoparticles on epithelial cells *in-vitro*.

Chapter three: Immunomodulation and possible anti-angiogenic effects of AgNPs on human intestinal Caco-2 cells.

This chapter elucidates the effects of physiological media on AgNPs hydrodynamic size and zeta potential and the effects of these characterized AgNPs on cell viability, cell stress, inflammatory biomarkers and anti-angiogenic properties.

Chapter four: Stability and anti-angiogenic properties of TiO_2NPs in Caco-2 cells.

This Chapter evaluates the interaction of nanoparticles with the proteome using the Inflammatory cytokine proteome profile membrane. Some inflammatory biomarkers were selected and analysed using the enzyme-linked immunosorbent assay (ELISA) to determine possible modulation and identify possible biomarkers for prognosis and diagnosis of TiO_2NPs toxicity.

Chapter five: The uptake mechanism effects on cytotoxicity and inflammatory biomarkers.

This chapter will report on the effects of various cellular uptake mechanisms on the toxicity of nanoparticles using cytotoxicity and/or inflammatory pathways evaluated in previous chapters. Different pathway inhibitors were used such as amiloride chloride for (micropinocytosis),

ammonium chloride (phagocytosis), chlorpromazine (clathrin-mediated endocytosis) and nystatin (caveolae-mediated endocytosis), with a view to understanding the pathway/s or mechanism by which the selected NPs induce cytotoxicity and inflammation.

Chapter six: Conclusion and recommendation

This last chapter focuses on the major outcomes of this research and also gives recommendation for future work.

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CHAPTER TWO: LITERATURE REVIEW

2.1 Nanoparticles

The term "nano" is derived from the ancient Greek word "nanos" which means "dwarf" and is increasingly being used as prefix for many scientific terms such as nanotubes, nanotechnology, nanowires, nanochips, nanoscience, nanotoxicity, nanometals, nanoscale, nanodrugs to mention a few (Buzea *et al.*, 2007). The field of nanotechnology could be said to have evolved from Richard Feyman, a physicist, who stated that a compound of many chemical characteristics could be built from the bottom up (Singh, 2015). Nanotechnology is defined as the science and technology that deals with materials ranging between 1 – 100 nm. It is designed to form a unique and sizable substance to meet a specific target and purpose (Iavicoli *et al.*, 2016). One is apt to assume that nanoparticles (NPs) e.g. dust or ash particles, although comparable in range to viruses, would be less toxic, as these substances do not possess the pathogenic capacity to reproduce. However, even as non-replicating foreign substances, NPs act indirectly by modulating biological processes, and may impede biological processes, thus affecting fundamental cellular functions, such as proliferation, metabolism and cell death (Buzea *et al.*, 2007). The increased use of NPs in industrial, pharmaceutical and medical applications has led to their increased production and a greater chance of their exposure and accumulation in humans and the biota.

2.1.1 Production of Nanoparticles

Nanoparticles (NPs) can either be naturally-occurring or synthesized chemically or biologically and are abundant in nature, being produced in many natural processes, including photochemical reactions, volcanic eruptions, forest fires and simple erosion, by plants and animals, e.g. shed skin and hair (Buzea *et al.*, 2007). Air pollution is often associated with human activities such as driving

cars, industrial processes, and charcoal burning, as well as such natural events as dust storms, volcanic eruptions and forest fires. These processes can produce vast quantities of NPs that could substantially affect the ecosystem. The most used technique for the synthesis of man-made NPs is the wet-chemical procedure. Many side effects are associated with this process as a result of which some chemical substances are being absorbed on the surface (Griffin *et al.*, 2018). The chemical methods used for NPs synthesis are cheap for maximum yield, but the presence of impurities from precursor compounds, the usage of toxic solvents, and the production of harmful by-products are some of the disadvantages. There is therefore a growing necessity to develop high-yield, affordable, harmless and ecologically non-threatening processes for the synthesis of nanoparticles (Ahmed *et al.*, 2016). Hence, the biological method for the synthesis of nanoparticles becomes imperative.

The development of biological synthesis over chemical and physical methods is ecologically friendly, cheap and can easily be scaled-up for mass production of nanoparticles. In addition, it does not require the use of high energy, pressure, temperature and toxic chemicals. There are two different approaches for synthesis of nanoparticles: the “bottom-up” method and the “top-down” method. Bottom-up, or self-assembly, refers to the building of a structure atom-by-atom, molecule-by-molecule, or cluster-by-cluster (Jeevanandam *et al.*, 2018). A benefit of the bottom-up method is the improved leeway of obtaining nanoparticles with reduced flaws and more standardized chemical conformation(s). In the top-down method, an appropriate preparatory material is condensed in size by physical or chemical means (Ahmed *et al.*, 2016).

2.1.2 Characterization of Nanoparticles

Nanoparticles can be characterized into different groups based on their physical and chemical characteristics, such as inorganic nanoparticles (gold, silica, silver, superparamagnetic, paramagnetic iron oxides, copper, cobalt, zinc, titanium, and aluminium oxides) and organic nanoparticles (carbon, graphene, micelles, liposomes, dendrimers and polymers such as poly lactic-co-glycolic acid and polysaccharide chitosan). Organic nanoparticles have been preferred over their metallic counterparts due to their low toxicities and abilities to function more effectively (Khanna *et al.*, 2015). while the inorganic ones are mostly applied in the industrial sector and in medical imaging.

2.1.3 Properties of Nanoparticles

Nanoparticles have different unique properties such as size, surface charge, shape, chemical composition, surface area, and surface chemistry, pH, surface reactivity, solubility, dispersion state, degree of agglomeration and aggregation (Azhdarzadeh *et al.*, 2015). The size enables NPs to adhere and interact with biological systems and to transcend different barriers such as the cell membrane and the blood-brain barrier, thus increasing their entry into cellular systems (Oberdörster *et al.*, 2009). NP size could also influence the efficiency of nanoparticle-encapsulated therapeutic agents (Nath Roy *et al.*, 2017). It also has been shown that the small size of nanoparticles has higher transfection ability compared to larger nanoparticles *in vitro (Han et al., 2013).* Earlier reports have revealed that particle size is a significant property that affects the intracellular uptake of nanoparticles, with smaller-sized particles, in general, having higher uptake. The surface area-to-volume ratio increases their interaction with the biological system, which includes uptake, distribution, metabolism and elimination from the system (Oberdörster *et al.*, 2005). Most NPs are unstable in dispersion, as they are susceptible to agglomeration or aggregation

and sedimentation, which affects NP intracellular uptake and toxicity (Joris *et al.*, 2013). Nanoparticles also show a reversal in their zeta potential (from anionic to cationic) in acidic pH (e.g. polylactic-co-glycolic acid nanoparticles) and can break away from the degradative endo-lysosomal compartment into the cytosol, whereas NPs that remain anionic at all pH values (e.g. polystyrene nanoparticles) do not exhibit endo-lysosomal escape (Panyam and Labhasetwar, 2003). Thus, the above physical characteristics of NPs are important in determining their intracellular uptake and crosstalk of nanoparticles with biological activities. These parameters also play an important role in the toxicity of NPs, in the way they interact with the environment and organisms. These unique properties can be altered by the preparation techniques, production, storage and when introduced into a biological system.

2.1.4 Application of Nanoparticles

This field of science and technology research has progressed considerably and finds application and uses in many other fields including medicine, biology, chemistry, engineering, physics and environmental science. Specific applications of nanoparticles include usage as diagnosing tools, bioimaging, biosensing, drug delivery, drug therapies and pharmaceuticals (Arvizo *et al.*, 2012). Nanoparticles drug delivery is to enhance the process of targeting specific organ/cell/tissue due to the ability of NPs to facilitate both protein and drug conjugation by enhancing protein stability, reducing immunogenicity and the ability to cross different barriers due to their sizes (Giri *et al.*, 2014). Nanoparticles are also used for the therapeutic management due to their porous behaviour, advanced oral bioavailability, maintenance of drug/gene effect in target tissue, increased solubility of drugs for intravascular delivery, and improved stability of therapeutic agents against enzymatic degradation (Fisher *et al.*, 2012). Special attention is especially paid to a therapeutic agents of interest such as protein, peptide, and nucleic acids drugs. Industrially, NPs are used for a vast array

of products ranging from sensors, electronics, chemical synthesis, catalyst, pollutant removal, food additives and textile production (Yohan and Chithrani, 2014). However, in environmental remediation, NPs are mostly used in wastewater treatment, waste control, pollution control and renewable energy (Becker *et al.*, 2014). Fullerenes, liposomes and carbon nanotubes have been widely used in polymer nanocomposites, biomedical applications and electronics due to their physical, chemical, thermal and optical properties and also for drug delivery purposes due to their multiple attachment points responsible for binding, and also offers high electrical conductivity and strength. A number of NPs are used for different applications, which will be briefly discussed in the sections that follow.

Gold NPs are used for cancer drug carrier systems and molecular imaging particularly in magnetic resonance imaging. Silver NPs are being used as antibacterial agents for the treatment of infections (Rinna *et al.*, 2015). Silica NPs have found application in chemical mechanical polishing, as additives to pharmaceutical drugs, cosmetics, printer toners, food and in biomedical and biotechnological fields such as biosensors, biomarkers and cancer therapy. Thus, the contact of humans with silica NPs cannot be avoided. Titanium oxide (TiO_2NPs) is a biologically inert and poorly soluble particle that has been widely used as a white pigment in the production of paint, paper and several kinds of plastics, and as a food additive or food colorant with recent rapid advances in nanotechnology (Song *et al.*, 2015). However, smaller TiO_2NPs particles have been used in cosmetics and pharmaceuticals, resulting in potential widespread exposure during both manufacturing and use (Zhao *et al.*, 2013). Copper oxide NPs are being used in various applications such as antimicrobial preparations, heat transfer fluids, semiconductors, and intrauterine contraceptive devices (Boyles *et al.*, 2016). Extensive research on carbon nanotubes

(CNT) has confirmed their unique physical, chemical, and electrical properties generating considerable interest in their potential biomedical applications, e.g. drug delivery, tumour hyperthermic ablation, and tissue engineering (Chen *et al.*, 2012). Apart from their conventional utilizations in electronics, composite materials and optics, more recent applications of multi-walled carbon nanotubes (MWCNT) include biomedical engineering, biosensors, drug delivery and gene therapy (Hussain *et al.*, 2014). Recently, nanoparticles are being explored as modems for biomarkers for early disease diagnosis and detection thus creating a new approach in omics development (Gioria *et al.*, 2016). Although NPs have numerous increasingly growing benefits, they can potentially pose health risks. Therefore, benefits versus risks need to be carefully assessed before using and releasing NPs into the environment.

2.2 Nanotoxicity

Nanotoxicity refers to the possible harmful effects of environmentally generated and man-made NPs on biological and environmental systems. The potential usage and safety of NPs can be achieved by introducing precautionary measures upon reviewing the uptake and interactions ofNPs with cellular processes through nanotoxicology. Increased exposure of NPs to the biota (air, water, soil) usually occur during production, occupational exposure and applications (Wongrakpanich *et al.*, 2016). This could result in nanotoxicity with potential harmful effects. Most studies have shown that NP toxicity involves oxidative stress, endoplasmic reticulum stress, inflammation, cytotoxicity, genotoxicity and membrane disruption (Khanna *et al.*, 2015). The toxicity of nanoparticles depends on various factors such as surface interaction, shape, size, composition, aggregation and interaction with various cellular components and very importantly the specific organism and cells that are exposed to the NP. Based on these factors, there is need for further

studies on the possible effects of NPs on the proteome. The immune responses of the host organism could be of valuable importance to understanding nanotoxicity (Farrera and Fadeel, 2015).

2.2.1 Cytotoxicity

Cytotoxicity is the process by which toxic agents or foreign substances alters or disrupts cellular activities, thereby hindering the proliferation of cells (Domey *et al.*, 2013). Chemical, biological and physical substances may cause cytotoxicity or decrease cell viability. Exposure of cells to cytotoxic compounds may result in different effects intracellularly and extracellularly. These substances may cause cytotoxicity by weakening the membrane integrity, inhibiting the biological processes, through interactions with the proteins and interfering with the organelles and cellular metabolic pathways (Istifli *et al.*, 2019). Consequently, the disruption of cell homeostasis may result in programmed cell death (apoptosis) or uncontrolled cell death (necrosis) due to loss of membrane integrity resulting in cell lysis (Donaldson *et al.*, 2009, Oberdörster *et al.*, 2009). Apoptosis is known to result in a cascade of cellular injuries such as nuclear condensation, cytoplasmic shrinkage and DNA damage (Foldbjerg *et al.*, 2015). The cells undergoing apoptosis also eventually lead to secondary necrosis in which the membrane integrity and metabolic activities of the cells are impaired. Cells undergoing necrosis release their intracellular content to extracellular space due to disruption of the membrane and impairment of metabolic processes (Riss and Moravec, 2004).

When cytotoxicity is measured, the most commonly studied parameter is cell viability, usually determined by the number of viable/living cells. There are several *in vitro* methods used for determining cell viability; these include tetrazolium reduction, resazurin reduction, protease markers, and ATP detection. The most available ones are the tetrazolium compounds such as MTT,

MTS, XTT, WST-1 and CCK-8. These salts are in two categories, MTT which is positively charged and the others which are negatively charged (Riss *et al.*, 2016). These compounds are reduced from their tetrazolium salts to formazans by metabolically active cells, followed by a change in colour that can be detected by ultraviolet/visible (UV/V) spectroscopy. The more intense the colour change, the higher number of viable cells. Other viability assays include but are not limited to lactate dehydrogenase (LDH), which measures necrosis, a process in which the cell membrane is damaged resulting in intracellular proteins leaking into the culture media (Chan *et al.*, 2013). Membrane integrity can also be determined through the usage of dyes such as neutral red and trypan blue, in which the dyes penetrate the dead cells and stain intracellular components (Khalili Fard *et al.*, 2015). Also the annexin V/propidium iodide (PI) assay helps to distinguish the mechanism of cell death via either apoptosis or necrosis (Rieger *et al.*, 2011).

2.2.2 Oxidative Stress

Toxicological effects of NPs have been reported by various studies to be oxidative-stress related (Avalos *et al.*, 2014, Khanna *et al.*, 2015). Oxidative stress is the state in which free radicals or reactive oxygen species (ROS) and reactive nitrogen species (RNS) exceed the antioxidants production in living organisms. The ROS include the hydroxyl radicals (·OH), superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) (Abdal Dayem *et al.*, 2017, Ganguly *et al.*, 2019), while the RNS mostly include nitric oxide (NO·), a potent oxidant, harmful to biological systems. ROS are intrinsically produced through inflammatory responses, microsomes, peroxisomes and mitochondria respiration, while nanoparticle generated ROS extrinsically in response to overproduction of free radicals (Di Meo *et al.*, 2016, Yu *et al.*, 2015). This increase leads to the failure of the antioxidant system to detoxify the formed reactive intermediates or repair the cellular damage (Ganguly *et al.*, 2019). The free radicals generated play a dual function which can either

be beneficial or detrimental to a biological system (Beer *et al.*, 2012). They play an important role in various signalling pathways, maintenance of homeostasis and machinery of the immune system but if generated in excess due to interaction with nanoparticles, it results in a deleterious effect such as mitochondrial membrane damage, alteration of cellular activities, and modification of genetic processes (Sun *et al.*, 2016). Metallic NPs such as iron, copper, chromium, vanadium and silver, titanium, and gold are involved in ROS generation through the Haber-Weiss and Fenton reaction mechanism (Abdal Dayem *et al.*, 2017). Metal NPs intrinsically generate harmful radicals, which interfere with redox reactions, catalyze Fenton reactions and modulate cellular uptake of nanoparticles as shown in Fig. 1 (Tee *et al.*, 2016). ROS generated during various normal physiological processes are controlled by specific antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), as well as antioxidants not limited to vitamin E, glutathione, ascorbic acids and flavonoids (Abdal Dayem *et al.*, 2017) and to combat the effects of metallic NP exposure cells must upregulate the antioxidant systems. Amongst some commonly used assays to determine oxidative stress include the dichloro-dichloro-fluorescein diacetate (DCFH-DA) assay and the use of cell stress markers such as SOD, CAT, GSH, heat shock proteins (HSPs) as well as measuring the levels of non-enzymatic antioxidants such as vitamin C and E (Tee *et al.*, 2016). The levels of most of the cell stress markers can be measured in biological samples through immunohistochemistry, Western blotting, enzyme linked immunosorbent assays (ELISAs), and gel electrophoresis. However, with the range of possible targets and varying reactivity of cellular antioxidants, it is uncertain how activities of ROS generation can be regulated. Therefore, other contributing factors may include reactive species generated, duration of oxidant production, localization of their source, target organelles, cell type, and interaction with the proteome (Di Meo *et al.*, 2016, Ganguly *et al.*, 2019, Sun *et al.*, 2017).

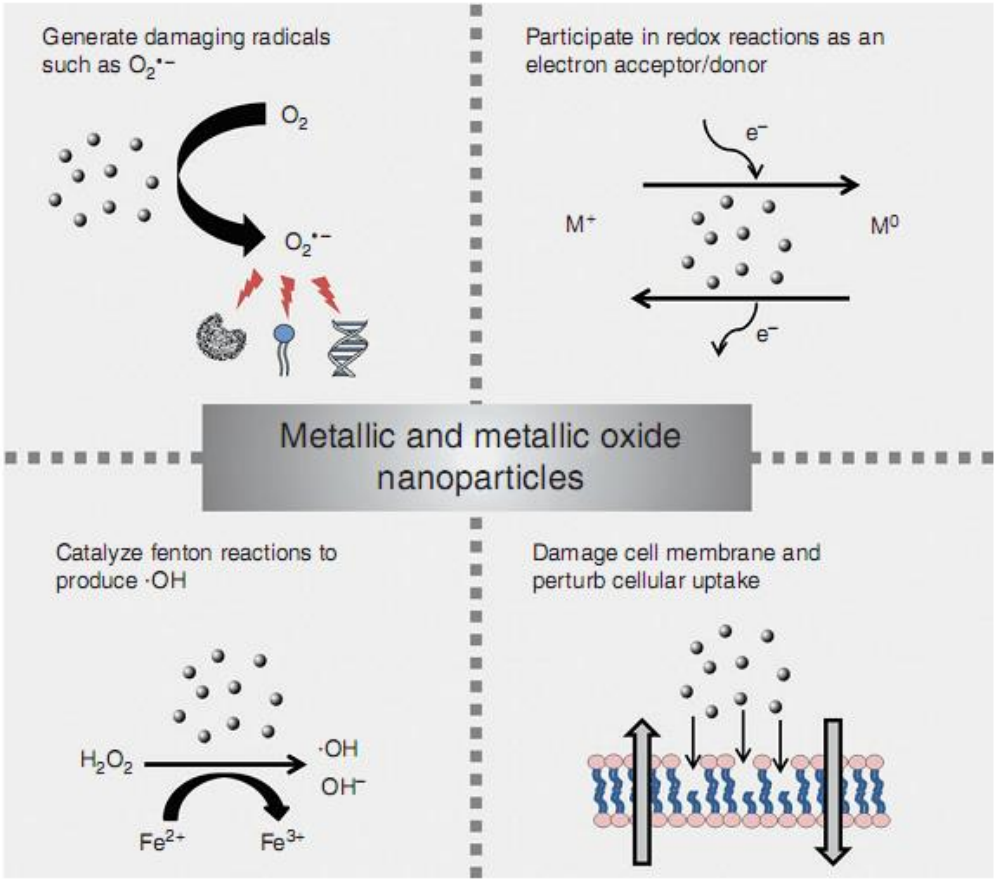

Figure 2.1: Nanoparticles mechanism for intrinsically mediated cellular oxidative stress (Tee *et al.*, 2016).

2.2.3 Genotoxicity

Genotoxicity is an important parameter in assessing the toxicity of NPs for their safety applications. It is essential to evaluate the potential mutagens and possible human carcinogens through the detection of primary DNA lesions, chromosomal damage and gene mutation (Rodriguez-Garraus *et al.*, 2020). NPs produce unpredictable genotoxicity effects related to DNA damage which lead to carcinogenesis and mutagenesis (Heshmati *et al.*, 2019). Owing to the physicochemical parameters of NPs, they can cross the cellular membranes and interact with the proteome and the DNA directly. Subsequently, as shown in Fig. 2 the interaction of NPs with the

DNA may cause double and single strand breaks, deletion, genomic instability, altered cell cycle kinetics, chromosomal aberrations and modulation of such protein as such p53 which is involved in the control of various cell stresses that cause DNA damage and repair (Shi *et al.*, 2013). Among the assays used to determine genotoxicity induced by NPs include the comet assay (neutral, alkaline or enzyme-modified assay), the micronucleus (MN) test, the hypoxanthine-guanine phosphoribosyl transferase (HPRT) and the thymine kinase (TK) gene for mammalian cell gene mutation tests *in vitro* (Rodriguez-Garraus *et al*., 2020). The comet assay known as single gel electrophoresis, is a highly sensitive, quick and easy technique for detecting DNA damage for single cells (Luque-Garcia *et al.*, 2013, Rodriguez-Garraus *et al.*, 2020).

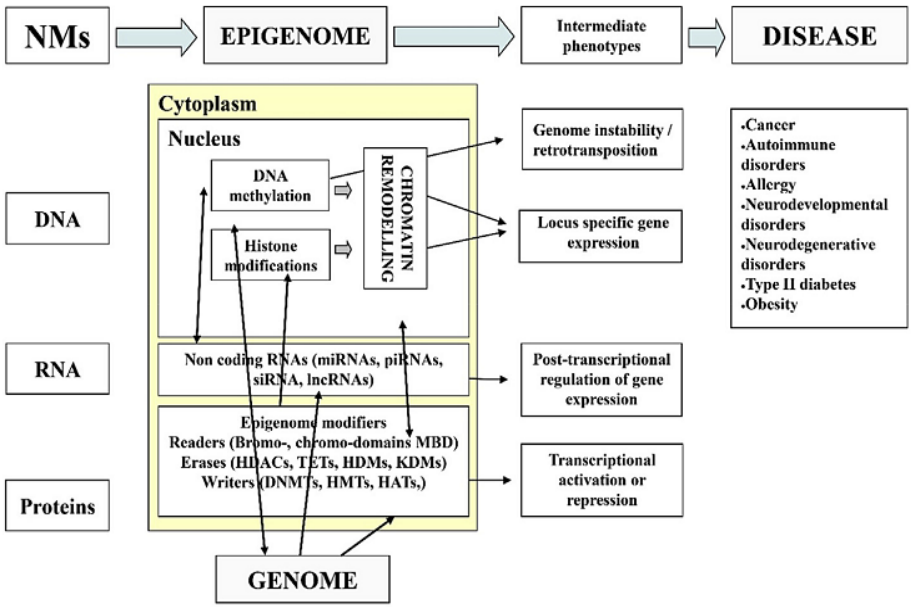

Figure 2.2: Schematic diagram of Genotoxicity of Nanoparticles (Dusinska *et al.*, 2017)

2.2.4 Immunotoxicity

The physical and chemical properties of NPs can lead to adverse effects on biological systems. Assessing the potential immunotoxicity of NPs is key to toxicological studies with respect to

production, application, and disposal of NPs. Nanoparticle applications may alter cellular processes and negatively affect the immune system. The fundamental function of the immune system is to prevent or reduce the risk of infection by microbes and toxic substances. The immune system is made up of several cells, organelles and tissues, which together form an effective, collaborative and complex network that protects the body from harmful substances (Moser and Leo, 2010). The immune system can be categorised into two types, known as natural (innate) immunity inherited from birth and adaptive (acquired) immunity developed after exposure to pathogenic agents (Murphy and Weaver, 2016). The essential structures of the immune system are: antibodies, bone marrow, complement systems, lymphatic systems, white blood cells, spleen, and thymus.

Innate immunity is the first line of defense against noxious substances (Moser and Leo, 2010). However, this type of immunity only recognizes organisms with certain conserved regions and is memory deficient. Prior to the invasion of the host by foreign substances and microorganisms, these harmful substances encounter various barriers, including anatomical barriers such as skin, hairs, mucous membranes etc. and physiological barriers like stomach acids, lysozyme, interferon, complement etc. (Moser and Leo, 2010). This serves as the first line of protection for the host from foreign and harmful substances. The innate immune response is activated by pattern-recognition receptors (PRPs), pathogen-associated molecular patterns (PAMPs), toll-like receptors (TLRs), phagocytosis and complement system (Najafi-Hajivar *et al.*, 2016). The recognition of microorganisms and harmful substances is the initiation process of defense in a host by the immune system. The innate immunity responds rapidly to attacks by foreign noxious agents by initiating inflammation

Inflammation serves as a defensive mechanism against tissue damage. In the inflammatory systems various cells go into impaired tissue and produce inflammatory mediators that triggers sensitive reactions resulting into fever, pain, redness and swellings (Ryu et al.2013). The impaired tissues result in production of bioactive substances like interleukins (ILs), nitric oxide (NO), tumour necrosis factor-α (TNF-α) and prostanoids. The initiation of the complement system arises from the activation of three complement pathways, viz.; the alternative pathway, the classical pathway, and the lectin pathway. The activation of the different pathways is specific for the respective pathway. However, all three pathways eventually results in the formation of the membrane attack complex (MAC) leading to the eradication of noxious agents (Noris and Remuzzi, 2013). The complement system also coordinates the acquired /adaptive immune responses, and also directs non-inflammatory removal of dead cells from the body (Makene and Pool, 2019).

Acquired immunity is adapted from repeated infectious attacks by pathogens, thus developing a robust defense mechanism overtime that is able to recognize and protect the host against future attacks by the same pathogen (Newton and Dixit, 2012). It is facilitated and sustained by B and T lymphocytes. B-cells, the precursor of antibody secreting cells, detect antigens through their B-cell receptor (Brewer, 2013). T lymphocytes and B-lymphocytes (for type 3 antigens) require antigen presenting cells (APCs) to process and present the antigen to them.

Both innate and adaptive immunity as shown in fig. 3, elicit functional responses in a coordinated way by initiating the activation of some important immune modulators such as cytokines (Wang *et al.*, 2013). Cytokines are small soluble protein molecules or peptides that are secreted by specialized cells of the immune system that controls a variety of processes, such as apoptosis,

hematopoiesis, immunity and inflammation (House, 2001). The cytokines that mediate and regulate the innate, humoral and cell mediated immune responses include IL-6, IL-10 and IFNγ respectively (Harvey *et al.*, 2015). The intentional and unintentional exposure of NPs through their usage and application may lead to immunotoxicity, resulting in the alteration and modulation of the immune system and its functions (Alsaleh and Brown, 2018). Consequently, the toxicological effect of NPs on immune function may cause hypersensitivity, immunosuppression, autoimmunity, immunogenicity, immunostimulation and possibly exacerbate the different physiological and pathological disease conditions (Najafi-Hajivar *et al.*, 2016). Hypersensitivity is an immunological sensitization to a substance, which may elicit critical immune responses. Immunosuppression arises from the alteration of the component of the immune system, affecting the optimum function of the immune system (Zufferey *et al.*, 2017). Autoimmunity is an immune response to self-antigen. Immunogenicity occurs as a result of an allergic response to a specific substance and/or its metabolites or multiple exposure to the substance (DeWitt *et al.*, 2012, Corsini *et al.*, 2013). Immunostimulation refers to the uncontrolled activation of some components of the immune system. The interaction of NPs with the immune system may result in the imbalance of the innate and acquired immune response. Therefore, it is important to elucidate these interactions to ensure the immune system is not compromised. Accordingly, to ensure safety and application of NPs, there is a need to understand the immune systems interaction with NPs. These interactions could enhance our understanding with regards to proteolytic degradation of extracellular molecules, growth factors stimulation of endothelial cells, capillary tubule production and proliferation of endothelial cells which results in angiogenesis.

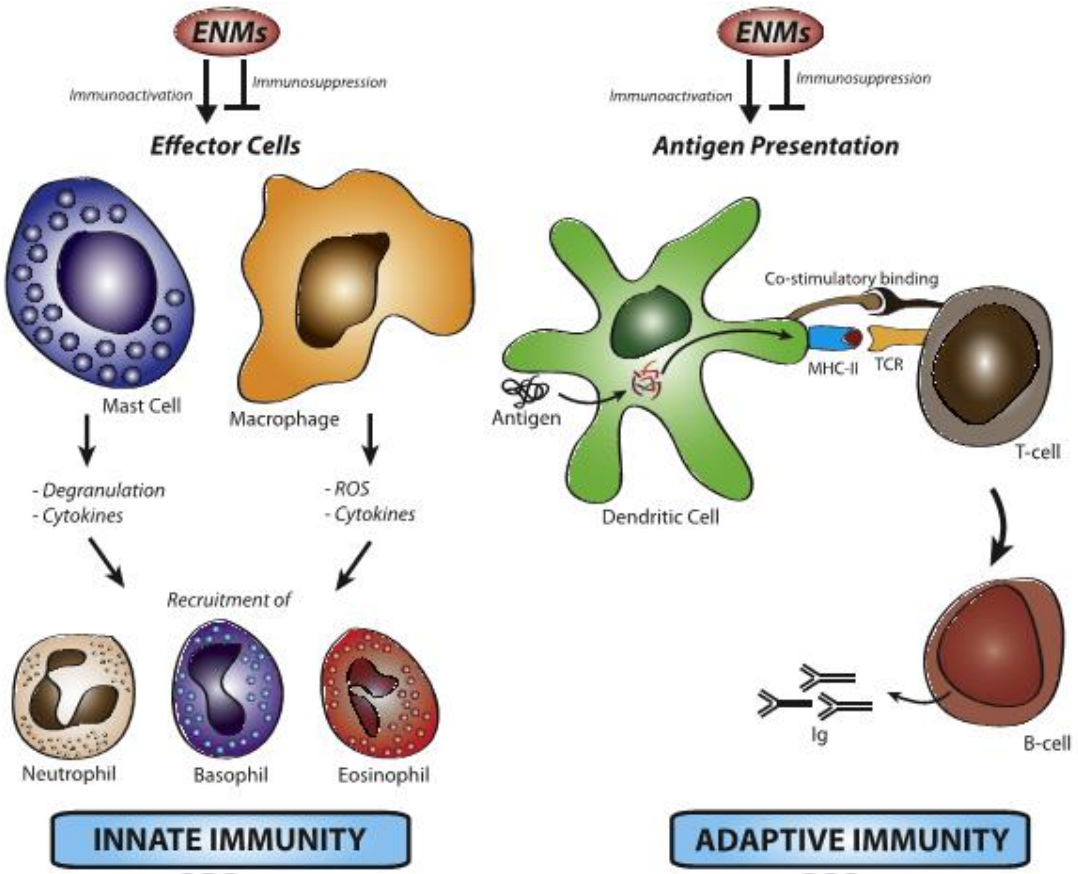

Figure 2.3: Schematic diagram of possible NPs mechanism of action on immune system (Alsaleh and Brown, 2018).

2.2.5 Angiogenesis

Angiogenesis is the formation of new blood vessels from previously existing blood vessels and necessary for growth and development, especially physiological processes such as ovulation, embryo growth and wound healing (Hashemi Goradel *et al.*, 2018, Tiwari, 2012). Impaired angiogenesis may also be involved in the progression of many illnesses such as arthritis, metastasis and diabetic retinopathy (Saeed *et al.*, 2019). Angiogenesis is an intricate process involving proliferation and migration of endothelial cells, formation of capillary tubes, stimulation of endothelial cells by growth factors, and proteolytic degradation of the extracellular matrix (Hashemi Goradel *et al.*, 2018). Furthermore, the balance of pro-angiogenic and anti-angiogenic

substances in the tissue helps maintain the vasculature in an inactive state, in most normal adult tissues (Tiwari, 2012). Vascular Endothelial Growth factor (VEGF), is a renowned angiogenesis activator that is over-secreted during tumour growth and metastasis. It is an important factor in the progression of angiogenesis, that functions through interactions with tyrosine kinase receptors (TKRs), which results in the supply of nutrients and oxygen to tumour cells (Saeed *et al.*, 2019). The up regulation of the expression of VEGF is triggered by NF-kB, with VEGF initiating the anti-apoptotic proteins expression A1 and Bcl-2 (Hashemi Goradel *et al.*, 2018). Accordingly, studies available revealed that NP activities in angiogenesis may lead to deprivation of oxygen and nutrients to tumour cells. Thereby inhibiting angiogenesis and directly decreasing cancer progressions (Hashemi Goradel *et al.*, 2018, Saeed *et al.*, 2019). However, there is need for further studies to ascertain the limitations and risk that this may pose to human health and underlying conditions.

2.2.6 Inflammation

Inflammation is the immediate response of the body to foreign assaults arising from the damage of cells and tissues (Newton and Dixit, 2012). The role of an inflammatory response is to protect the body against harmful biological, physical and chemical substances such as NPs, microorganisms and toxins (Stevenson *et al.*, 2011). The affected area increases blood vessel permeability resulting in the increase of white blood cells, with the migration of monocytes and neutrophils to sites of inflammation (Luo *et al.*, 2015). Signs of inflammation include pain, swelling, redness and alteration of cellular functions (Newton and Dixit, 2012). Recent studies showed that interaction and exposure of NPs to cells can result in the secretion of various chemokines and cytokines by epithelial cells (Mortezaee *et al.*, 2019). The resulting inflammatory response involves immune cells such as macrophages derived from circulating monocytes, which

destroys invading toxic substances, pathogens and dead cells (Makene and Pool, 2019). In addition, as shown in Fig. 4, macrophages trigger the secretion of inflammatory cytokines, which results in the upregulation of inflammatory mediators such as NF-kB (Mortezaee *et al.*, 2019). The interaction of NPs with the cells may lead to acute or chronic inflammation resulting in continuous secretion of inflammatory cytokines and ROS, which further impair biological processes and cellular functions. Inflammatory responses result in the secretion of several inflammatory mediators such as nitric oxide (NO) and interleukins (ILs) for example interleukin 6 (IL-6) and interleukin 8 (IL-8). IL-6 is pleiotropic cytokine, i.e. it can act as both pro-inflammatory and anti-inflammatory cytokine with diverse functions involved in cellular processes, induction of acute phase reactions and supporting chronic inflammatory reactions. IL-8, also known as neutrophil chemotatic factor, is a cytokine produced by macrophages, epithelial cells, endothelial cells and airway smooth muscle cells, and induce chemotaxis in target cells at the site of attack (Alessandrini *et al.*, 2017). Nitric oxide is a key mediator of the immune response that is involved in DNA damage and repairs, autoimmune disease, cancer development, hypotension, etc. (Mortezaee *et al.*, 2019). Several assays are used to determine inflammatory responses, with ELISAs being the most common.

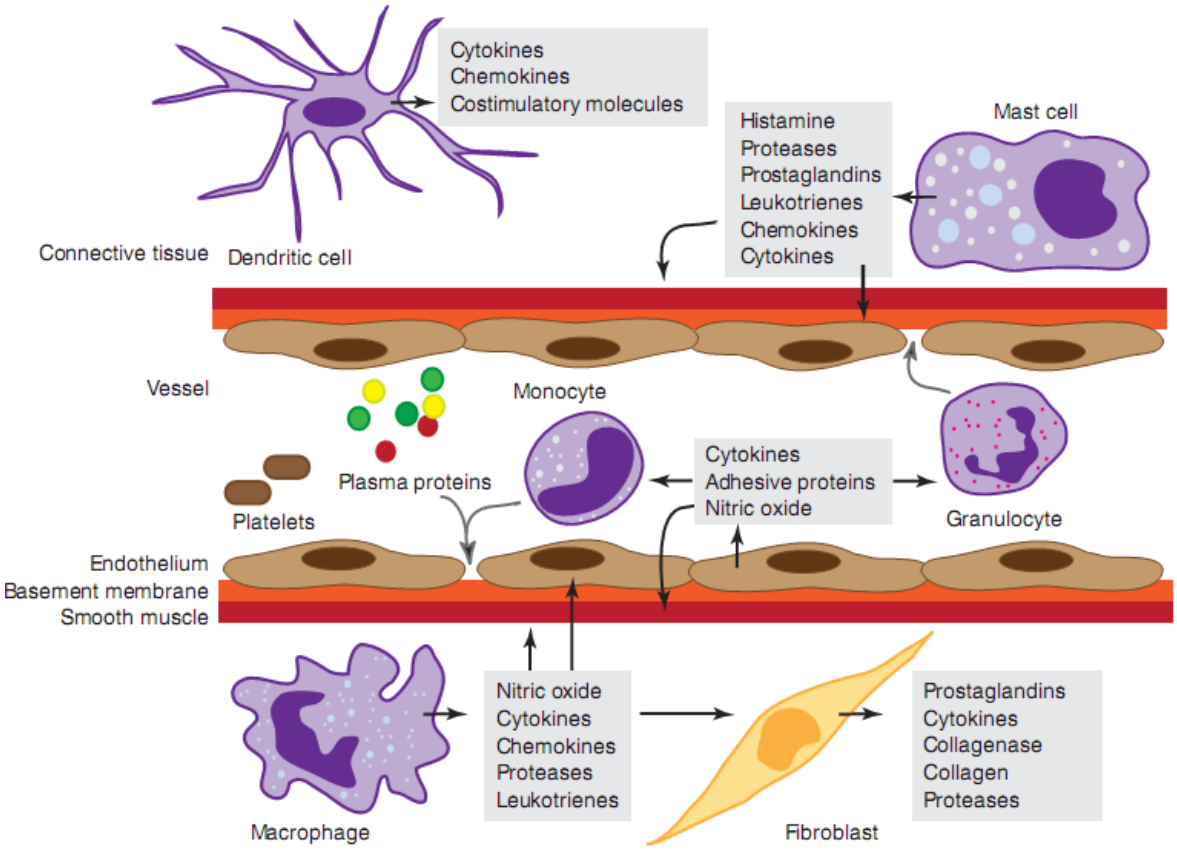

Figure 2.4: Cells and mediators of the inflammatory response. Molecules derived from plasma proteins and cells in response to tissue damage or pathogens mediate inflammation by stimulating vascular changes, plus leukocyte migration and activation (Newton & Dixit, 2012).

2.3 Silver Nanoparticles

Silver (Ag) is one of the fundamental elements that makes up the earth. It is a naturally-occurring metal, that is malleable and ductile. It has high thermal and electrical conductivities compared to other metals and very low contact resistance (Nordberg and Gerhardsson, 1988). Natural processes and human activities like burning of fossil fuels, weathering of rocks, ores processing and manufacturing of compounds leads to the release of silver into the atmosphere (Akter *et al.*, 2018). It can also enter rivers and groundwater through rain and industrial water treatment plants. Silver

occurs in different oxidation states: Ag^0, $Ag^{+,}$ Ag^{2+}, Ag^{3+} with the latter two being the most abundant (Wijnhoven *et al.*, 2009). Ag has been used in ancient times in daily life and for medicinal purposes, for example it is used as storage containers to keep and transport fresh water. The antimicrobial usage of Ag was unknown until it was scientifically described in the nineteenth century (Russell and Hugo, 1994). Successively, Ag has been used in a wide range of medical devices such as surgical instruments and needles, catheters, bone prostheses, dental devices, cardiac implants, wound therapy and textiles equipment. Industrially the largest users were the photographic, silverware and the jewellery industries (Foldbjerg *et al.*, 2015).

Most recently, AgNPs have been considered a valuable material due to their promising properties, which differs from their bulk materials (Ferdous and Nemmar, 2020). The physico-chemical characteristics of AgNPs have increased their exploration in several applications such as electronics, biotechnology, medicine, agriculture, bioremediation, pharmaceuticals and food industries (Mukherjee *et al.*, 2012). Since new applications for AgNPs are discovered every day, rapid growth is anticipated in global AgNPs production. Among the tons of NPs manufactured daily, AgNPs represent one fifth of it, with total yearly production between 320 to 420 tons (Pulit-Prociak and Banach, 2016, Vance *et al.*, 2015). The synthesis of AgNPs is multifaceted involving green and biological techniques using algae, plants and microorganisms, and also chemical methods. Due to the quantities of AgNPs needed to meet production demands, different techniques have been used to synthesize it on an industrial scale, such as electrochemical methods, chemical reduction and gamma ray radiation (Tortella *et al.*, 2019). Industrial production and application of AgNPs may lead to the environmental pollution of the air, water and soil, causing potential toxicity to the ecosystem. Besides ecological toxicity, AgNPs can harm useful organisms in the biota by

altering significant processes in the ecosystem. AgNPs from industrial processes pass through the sewage treatment plants and are partly released via water effluents into the water bodies. Also, some AgNPs that precipitate as sludge will be deposited into the soil if the sludge from the sewage facilities is used for fertilization processes (Guo *et al.*, 2018). Consequently, living organisms and humans are at the receiving end of this pollution and toxicity may result due to exposure either through occupational processes or application and usage. Therefore, the potential harmful effects of AgNPs needs to be constantly elucidated to prevent the threat posed due to rapid application and usage.

2.3.1 Toxicological Effects of AgNPs

The possible toxicological impacts of AgNPs may arise from their synthesis and production due to several factors and physicochemical parameters such as size, shape, charge, agglomeration and stability in the processes involved in large scale production (Akter *et al.*, 2018). AgNPs are unstable and their oxidation results in the release of ions arising from their reactions with oxygen and protons or other biomolecules. The oxidation processes are influenced by the temperature, pH, ionic strength, ligands and coating. Therefore, altering the particles properties and interaction with other biological processes thus making them potentially toxic to living organisms and hazardous to human health and the environment (Cameron *et al.*, 2018). The toxicity of AgNPs as shown in Fig. 5, is quite sensitive to prediction because of their properties, characteristics, interactions with biological processes and behaviour in biological systems. Likewise, the available published data of the toxicological effects of AgNPs on biological systems *in vitro* and *in vivo* shows different and even conflicting results. Nevertheless, the toxicity of AgNPs include cytotoxicity, oxidative stress, endoplasmic reticulum stress, membrane damage, mitochondrial dysfunction, apoptosis,

inflammation, immunotoxicity, autophagy, lethality and genotoxicity (Du *et al.*, 2018, Gioria *et al.*, 2018).

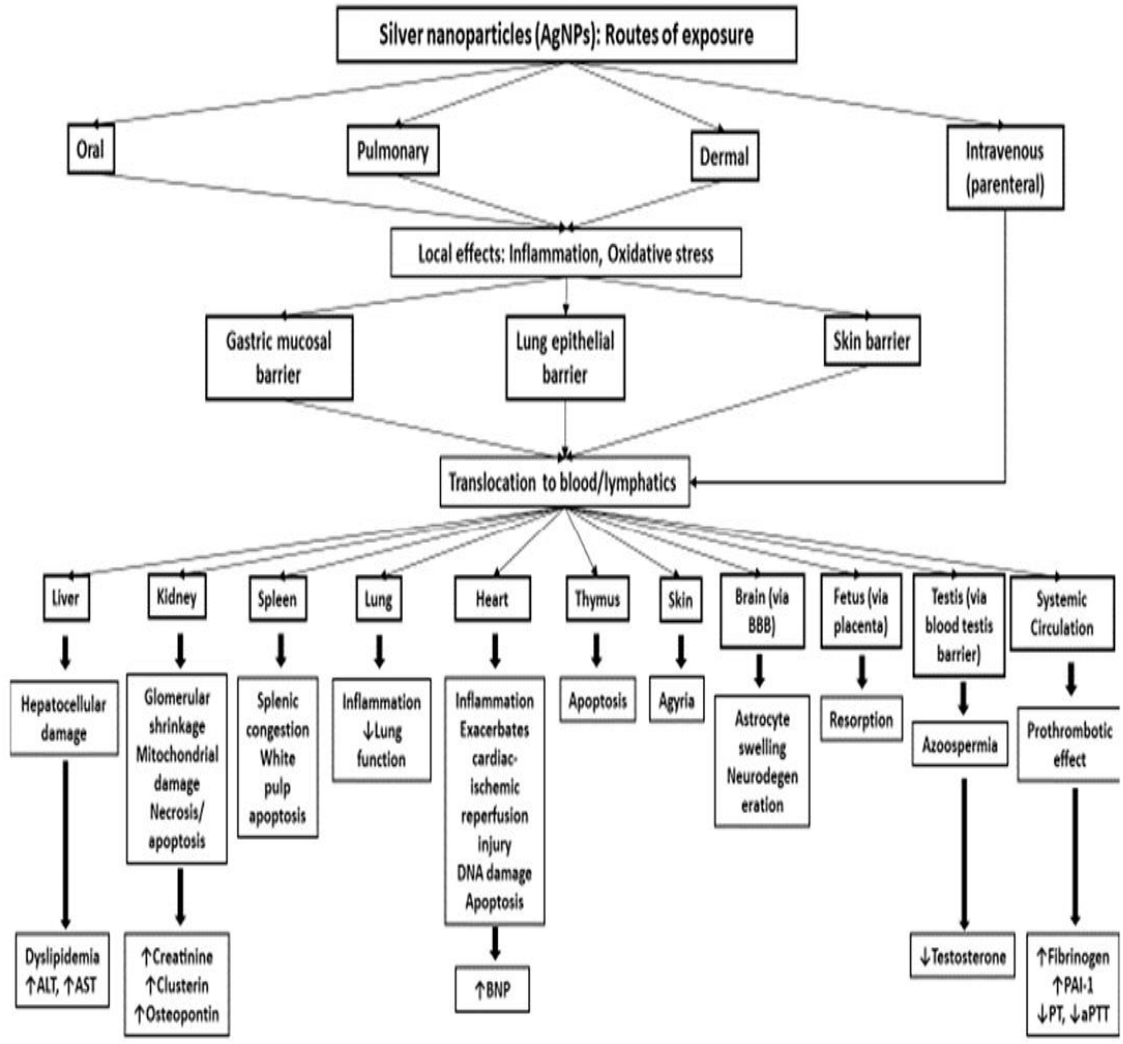

Figure 2.5: A Model for AgNps bio-distribution toxicological action adapted from Ferdous and Nemmar, 2020.

2.3.1.1 In vitro Toxicity of AgNPs

Recent *in vitro* studies available from various cell line models show that AgNPs lead to several forms of toxicity resulting from interaction with different cell lines (Table 1). Reduced cell

viability, cytotoxicity, membrane damage, mitochondria dysfunction, ROS generation and oxidative stress appears to be mostly reported in various cell lines for different sizes of AgNPs (Asharani *et al.*, 2012, Avalos *et al.*, 2014, Chen *et al.*, 2015, Foldbjerg *et al.*, 2011, Liu *et al.*, 2010b). For example, investigation of cellular and molecular toxicity of AgNPs in normal human lung cells IMR-90 and human brain cancer cells U251 revealed that protein expression and gene function were altered resulting in inflammation, DNA damage and proliferation arrest (Asharani *et al.*, 2012). AgNPs were cytotoxic and genotoxic to A549 and also caused oxidative stress (Foldbjerg *et al.*, 2011). RAW 264.7 and human whole blood culture WBCs exposed to AgNPs concentrations (0 – 250 ug/ml) modulated inflammatory cytokines (Lategan *et al.*, 2019). AgNPs induced apoptosis, cell cycle arrest, decreased viability and metabolic rate in A549 (Lee *et al.*, 2014). Cell proliferation, DNA damage, cell stress and apoptosis were revealed when A431, A459 and RAW 264.7 were exposed to AgNPs (50 and 100 nm) by Kaur and Tikoo, 2013. AgNPs inhibited cell proliferation, induced morphological changes, inflammation and oxidative stress in human colon epithelial cells Caco-2 (Bohmert *et al.*, 2014, Bohmert *et al.*, 2012, Bohmert *et al.*, 2015). Consequently, a search on the published reviewed articles for a period of 20 years from (2000 -2020) revealed that there is still dearth of information available on the immunotoxicity of AgNPs compared to other studies such as cytotoxicity, membrane damage, ROS generation, oxidative stress, inflammation and genotoxicity (Fig. 6).

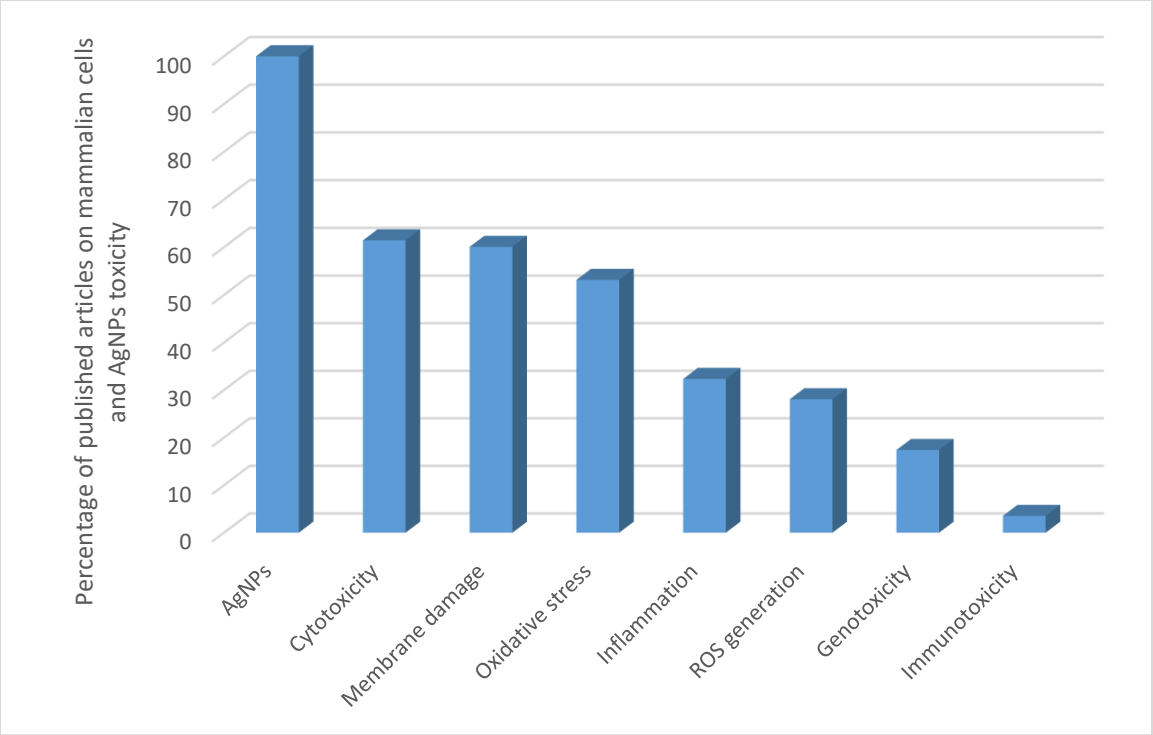

Figure 2.6: Graphical representation of a Science Direct search of the percentage of peer reviewed articles for a period of 20 years (2000-2020) using the term 'mammalian cells, silver nanoparticles' or 'mammalian cells, silver nanoparticles, Cytotoxicity' and then replacing the cytotoxicity with membrane damage, oxidative stress, inflammation, ROS generation, genotoxicity and immunotoxicity for each search as of 01 August, 2020.

Table 2.1: A non-exhaustive summary of in vitro toxicity effects of silver nanoparticles on various cell lines

Cell Lines	Cell types	Concentrations	Time	Size	Major Findings	References
IMR-90 & U251	Norman human lung cells, human brain cancer cells	0, 25, 50, 100, 200, 400 µg/ml	24, 48, 72 hrs	6-20 nm	Cytotoxicity, Genotoxicity, Inflammation, DNA damage, chromosomal aberrations and cell cycle arrest.	(Asharani *et al.*, 2012)
HepG2, HL-60	Human hepatoma cells, tumoral human leukemia cells,	0-13-45 µg/ml	24,48,72 hrs	4.7 & 42 nm	Cytotoxicity, ROS production, Glutathione depletion, SOD inactivation.	(Avalos *et al.*, 2014, Avalos *et al.*, 2016)
CHO	Chinese hamster ovary cells	0, 25, 50, 100 µg/ml	24 hrs	10 nm	Cytotoxicity, Oxidative stress, Apoptosis and DNA damage.	(Awasthi *et al.*, 2015)
HepG2	Human hepatoma cells	0, 5, 11 µg/ml	24 hrs	30 nm	Cytotoxicity, Genotoxicity and Apoptosis.	(Bastos *et al.*, 2017)
Caco-2	Human colon carcinoma epithelial cells	5-100 µg/ml	24, 30, 48 hrs	20 & 40 nm	Morphological changes, cytotoxicity, cell death, Inflammation and Oxidative stress.	(Bohmert *et al.*, 2015)
RBCs	Fish red blood cells	0-20 µg/ml	2 hrs	15, 50 & 100 nm	Cytotoxicity, membrane injury, Oxidative stress and Hemolysis.	(Chen *et al.*, 2015)
A549	Human alveolar carcinoma epithelial cells	0-20 µg/ml	24 hrs	30-50 nm	Cytotoxicity, Genotoxicity and Oxidative stress.	(Foldbjerg *et al.*, 2011)
TK6 & V79-4	Human B-lymphoblastoid cells,	0.31, 0.63, 1.25, 2.5 µg/ml	2 & 24 hrs	20 nm	Cytotoxicity, membrane damage, Cell cycle arrest, cell	(Huk *et al.*, 2015)

	chinese hamster lung fibroblast cells				death, DNA damage and Gene mutation.	
A431, A549, RAW 264.7	Human skin epithelium carcinoma cell, human lung carcinoma cell, murine macrophage cell	0, 5,10, 25, 50, 75, 100 µg/ml	24 hrs	50 & 100 nm	Cytotoxicity, DNA damage, Apoptosis and Cell stress.	(Kaur and Tikoo, 2013)
RAW 264.7, WBCs	Murine macrophage cells, whole blood cells	0-250 µg/ml	24 hrs	20-40 nm	Modulation of inflammatory markers.	(Lategan *et al.*, 2019)
A549	Human lung carcinoma cell	5, 10, 50, 200 µg/ml	12, 24, 48, 72 hrs	100 nm	Decreased cell viability, Cell cycle arrest, decreased metabolic activity and Apoptosis.	(Lee *et al.*, 2011)
NIH 3T3	Mouse embryonic fibroblast	0, 2, 5, 10, 15, 30, 40 µg/ml	12, 24, 48 hrs	20 nm	Cell damage, ROS generation, Autophagy and Apoptosis.	(Lee *et al.*, 2014)
THP-1	Human monocytic cell	0.-100 µg/ml	24 hrs	24 nm	Cytotoxicity.	(Martinez-Gutierrez *et al.*, 2012)
LoVo	Human colon carcinoma cell	0-10 µg/ml	24, 48 hrs	10, 20, 40, 60, 100 nm	Cytotoxicity, Inflammation, ROS generation, Mitochondrial dysfunction and Apoptosis.	(Miethling-Graff *et al.*, 2014)

J774A.1, HT29	Murine Macrophage cell, human colon cancer epithelial cells	1-250 µg/ml	24 hrs	24-26 nm	Cytotoxicity, ROS generation, Oxidative stress, Inflammation, DNA damage and Apoptosis.	(Nguyen *et al.*, 2016)
HCT 116	Human colon carcinoma cells	0, 50, 100, 150, 200 µg/ml	24, 48, 72 hrs	20 nm	Apoptosis, Decreased cell viability and DNA damage.	(Satapathy *et al.*, 2013)
RAW 264.7, J774.1, A549, A498, HepG2, Nuro 2A	Murine macrophage cells, Human lung adenocarcinoma cells, human renal epithelial cells, human hepatic cells, human neuronal cell.	1, 3, 10, 50, 100 µg/ml	72 hrs	43.9 nm	Mitochondrial damage, Apoptosis and Cell death.	(Singh and Ramarao, 2012)
CHO-K1, CHO-XRS5	Chinese hamster ovary fibroblast cells,	0.025-5.0 µg/ml	24-72 hrs	10, 100 nm	Cytotoxicity and Genotoxicity.	(Souza *et al.*, 2016)
A549	Human lung adenocarcinoma cells	10, 25, 50, 100 µg/ml	24, 48 hrs	<100 nm	Cytotoxicity, Oxidative stress, DNA damage, Inflammation and ROS generation.	(Suliman *et al.*, 2015)

A549, HepG2	Human lung adenocarcinoma cells	0-200 µg/ml	24 hrs	<100 nm	Cytotoxicity, Oxidative stress and cell damage.	(Xin *et al.*, 2015)
HepG2, L02	Human hepatic cells, Normal human hepatic cells	20-160 µg/ml	24, 48 hrs	23.4 nm	Reduced cell viability, Membrane leakage, Mitochondrial injury, ROS generation, Oxidative stress and Apoptosis.	(Xue *et al.*, 2018)

Furthermore, toxicological effects of AgNPs on microorganisms and *in vivo* studies including invertebrates, vertebrates, aquatic life and higher plants has been comprehensively reviewed (Antony *et al.*, 2015, de Lima *et al.*, 2012, Du *et al.*, 2018, Akter *et al.*, 2018, Ahamed *et al.*, 2010, Kim and Ryu, 2013, Kruszewski *et al.*, 2011, Marambio-Jones and Hoek, 2010, Prasath and Palaniappan, 2019, Reidy *et al.*, 2013, Rodriguez-Garraus *et al.*, 2020, Volker *et al.*, 2013, Wijnhoven *et al.*, 2009). Human toxicological effects of long term exposure to silver have been reported to cause skin discolouration (argyria) and eye discolouration (argyrosis) as well as affecting reproductive health (Olugbodi *et al.*, 2020). In addition, studies have also shown that different mammalian organs exposed to AgNPs may lead to different physiological and pathological conditions (Gaillet and Rouanet, 2015). More specifically, concentration, physicochemical properties, cell types, environment, experimental setup and media may lead to different outcomes of toxicity by AgNPs.

2.4 Titanium Oxide Nanoparticles

Titanium dioxide (TiO_2) is the only naturally occurring form of titanium in nature. Like other metals, it is malleable, thermally stable and chemically resilient with great photocatalytic activities (Baranowska-Wojcik *et al.*, 2020). It is known to have three different crystalline polymorphs; rutile, anatase and brookite as shown in fig 7, with rutile being the most available compared to the other two and mostly used industrially due to its abundance and stability in nature (Baranowska-Wojcik *et al.*, 2020, Kaur and Singh, 2019). TiO_2NPs possess considerably different physicochemical characteristics compared to the bulk materials and have been known to be used in the past for commercial production of whiteners and pigments in paints and cements respectively (Armand *et al.*, 2016). In recent times these unique properties have made titanium oxide nanoparticles indispensable. It has been explored in a wide range of applications, which

includes production of cosmetics, paints, sunscreens, plastics, paper, inks, toothpaste, food, pharmaceuticals, air and water treatment (Lappas, 2015, Mu *et al.*, 2019, Skocaj *et al.*, 2011, Sukwong *et al.*, 2017, Wang *et al.*, 2019). However, rapid development, wide usage and application of these particles poses intentional and unintentional risk of exposure. This could possibly pose danger to useful microorganisms, aquatic life, human health and the environment. Researchers, workers and consumers are at high risk of being adversely affected (Song *et al.*, 2016). Nevertheless, owing to the several explications in daily life, it is important to elucidate their potential threat to the biota and possible physiological and pathological challenges on human health. Likewise, studies have reported that TiO_2NPs leads to cytotoxicity, inflammation, fibrosis, lung tumours, pulmonary damage, oxidative stress, apoptosis and genotoxicity (Gea *et al.*, 2019, Kranc *et al.*, 2015, Kumar *et al.*, 2018, Song *et al.*, 2016). Although there are conflicting studies on the toxicity of TiO_2NPs with data showing low or absence of toxicity, this may be due to experimental procedures, cell types, concentration and biological media (Botelho *et al.*, 2014, Dudefoi *et al.*, 2017).

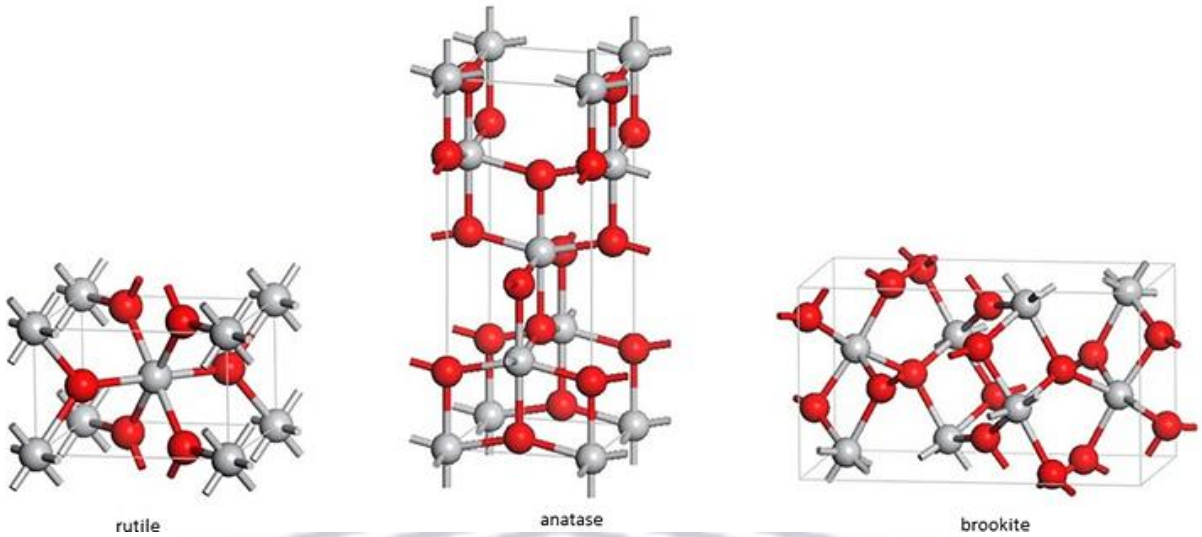

Figure 2.7: Tetragonal structure of crystalline form of rutile, anatase and brookite TiO2NP sphere: red O_2, grey- Ti adapted from Baranowska-Wojcik *et al.*, 2020.

2.4.1 Toxicity of Titanium Oxide Nanoparticles

In recent years, the harmful effects of TiO_2NPs on human health have resulted in different perspectives due to the available data. The polymorphic structure and properties of TiO_2NPs may alter the toxicity of TiO_2NPs. The potential properties, inertness and wide applications has raised concerns regarding the toxicity and safety of TiO_2NPs (Chen *et al.*, 2014b). It has been recently classified as a potential carcinogenic factor from group 2B by the international agency for research on cancer (IARC) due to the experimental tests done on animals concerning exposure by inhalation (Baranowska-Wojcik *et al*., 2020; Chen *et al*., 2014). Toxicological *in vitro* studies revealed a number of harmful effects on mammalian cells not limited to decreased cell viability, DNA damage, increased ROS generation, inflammation and genotoxicity (Table 2). The effect of TiO_2NPs on a wide range of microorganisms was reviewed and reported by (Hou *et al.*, 2019) with insight to understanding the mechanism of action and toxicity of TiO_2NPs in living organisms. The study reported that TiO_2NPs was harmful to several species of microorganisms such as

Escherichia coli, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Enterococcus faecalis*, etc. with special indications to the biotoxic diversity of TiO_2NPs on growth inhibition of microorganisms. Likewise, many *in vivo* studies revealed in Fig. 7 (Shakeel *et al.*, 2016) show that TiO_2NPs exposure result in possible entry into the bloodstream leading to systemic circulation and potential accumulation in major organs causing damage to organs and resulting into various physiological and pathological conditions (Czajka *et al.*, 2015). Subsequently, these harmful processes may result in neuroinflammation and further brain damage (Baranowska-Wojcik *et al.*, 2020, Czajka *et al.*, 2015). In addition, the ecological toxicity of TiO_2NPs in aquatic animals is usually studied in terms of acute, sub-acute and sub-chronic conditions. Studies showed that the toxicity of TiO_2NPs may cause ROS generation, damage of cellular components and alteration of metabolic activities (Raja *et al.*, 2020). TiO_2NPs affects human health through the alteration of cell cycle, apoptosis, constriction of nuclear membranes, genotoxicity, interruption of absorption of nutrients, disturbances of lipid and glucose homeostasis, liver swelling and cardiac injuries (Baranowska-Wojcik *et al.*, 2020, Raja *et al.*, 2020). Non-allergic activation of mast cells and no toxicity were observed in some adult mammals, implying age played a key role in the adverse effects caused by TiO_2NPs (Baranowska-Wojcik *et al.*, 2020). A search on the published reviewed articles for a period of 20 years from (2000 -2020) revealed that there is still dearth of information available on the immunotoxicity of TiO_2NPs compared to other studies such as cytotoxicity, membrane damage, ROS generation, oxidative stress, inflammation and genotoxicity (Fig. 9).

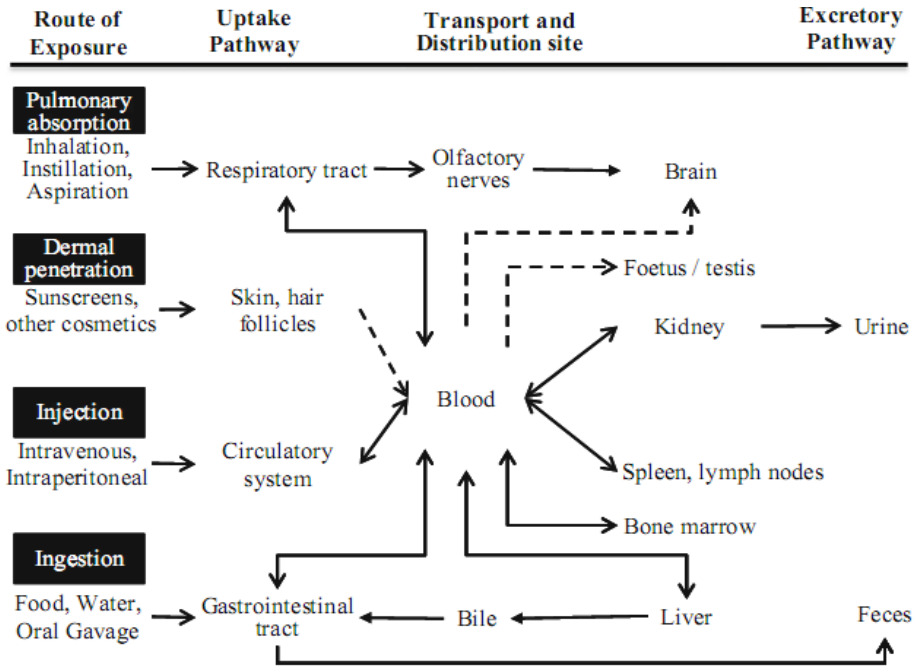

Figure 2.8: Schematic diagram of TiO2NPs exposure, distribution, excretion and toxicity adapted from Shakeel *et al.*, 2016.

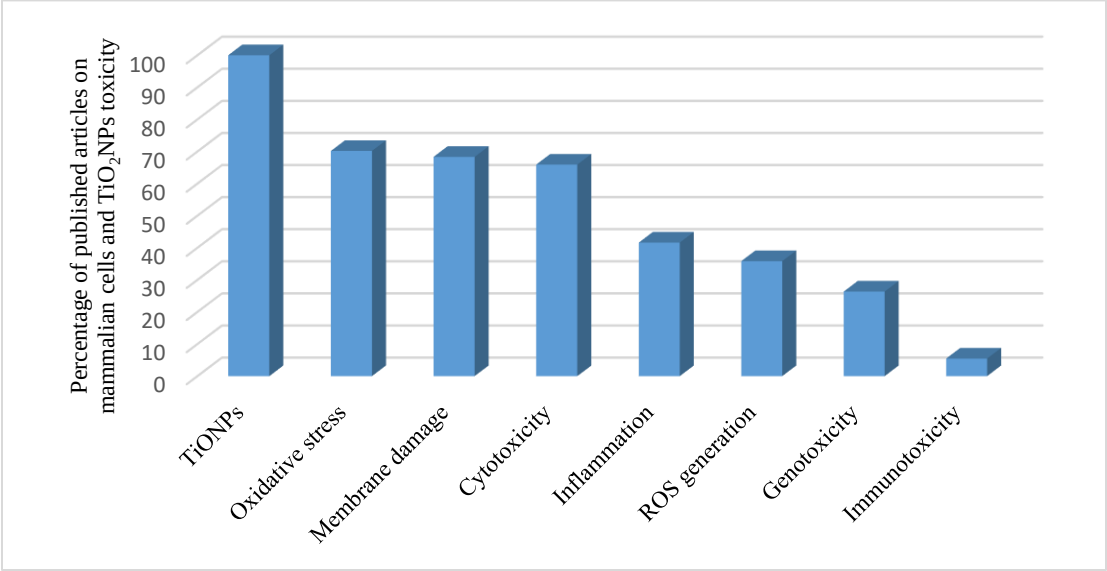

Figure 2.9: Graphical representation of a ScienceDirect search of the percentage of peer reviewed articles for a period of 20 years (2000-2020) using the term 'mammalian cells, titanium oxide nanoparticles' or 'mammalian cells, titanium oxide nanoparticles, Cytotoxicity' and then replacing the cytotoxicity with Membrane damage, oxidative stress, inflammation, ROS generation, genotoxicity and immunotoxicity for each search as of 01 August, 2020.

Table 2.2: A non-exhaustive summary of in vitro toxicity effects of titanium oxide nanoparticles on various cell lines

Cell Lines	Cell types	Concentrations	Time	Size	Major Findings	References
A549	Human lung carcinoma cells	0, 1,2.5,5,10,50 µg/ml	Long term	21 nm	Alterations of biological pathways, mitochondrial activity, glucose metabolism, DNA damage and cell stress.	(Armand *et al.*, 2016)
A549	Human lung carcinoma cells	25-500 µg/ml	24 hrs	25 nm	Cytotoxicity, cell death, ROS generation and No effects on HSP 70 and GRP 78.	(Aueviriyavit *et al.*, 2012)
AGS	Human gastric epithelial cancer cells	20, 40, 60, 80, 100, 120, 150 µg/ml	3, 6, 24 hrs	21 nm	Increase in cell proliferation, increased oxidative stress and increased genotoxicity.	(Botelho *et al.*, 2014)
Chago-K1	Human bronchial epithelial cells	0.1-2 µg/ml	24 hrs	75 nm	Cytotoxicity through apoptotic pathway.	(Chen *et al.*, 2008)
NHBE, A549, BEAS-2B	Normal human bronchial epithelial cells, Human alveolar epithelial cells, human bronchial epithelial cells	0.1-400 µg/ml	24 hrs	9, 5, 14, 25, 60 nm	Inflammation and ROS generation.	(Ekstrand-Hammarström *et al.*, 2012)

A549, Caco-2	Human lung carcinoma cells, human colon epithelial cells	0.1-100 µg/ml	48 hrs	18, 30, 87 nm	Dose and size dependent cytotoxicity through decreased cell viability, increased LDH and ROS generation.	(Gandamalla *et al.*, 2019)
RAW 264.7	Murine macrophage cells	10^{-1}-10^{-9} µg/ml	48 hrs	21, 35 nm	Cytotoxicity, Mitochondrial damage, Inflammation and Autophagy.	(Hu *et al.*, 2019)
PC12	Rat pheochromocytosoma cells	1,10,50, 100 µg/ml	6, 12, 24, 48 hrs	20-50 nm	Dose and time dependent decrease in cell viability, Induced intracellular ROS generation and Apoptosis.	(Liu *et al.*, 2010a)
THI-1, HMC-1	Human monocytic leukemia, human mast cells	1,10,20,50 µg/ml	24 hrs	20-40 nm	Reduced cell viability, Concentration dependent.	(Madhubala *et al.*, 2019)
BEAS-2B	human bronchial epithelial cells	5,10,20,40 µg/ml	24, 48, 72, 96 hrs	21 nm	Cytotoxicity, Cell death, ROS increase, Glutathione decrease, Induction of oxidative stress related gene, Apoptosis and Inflammation.	(Park *et al.*, 2008a)
A_1	Human hamster hybrid cells	25, 50, 100, 200 µg/ml	72 hrs	5,15, <100 nm	Cytotoxicity, genotoxicity and Mitochodria dysfunction in time and size dependent manner.	(Wang *et al.*, 2019)

2.5 Uptake of Nanoparticles

The uptake of NPs by cells is a critical process involving the biological activities of molecules, and is dependent on the interaction between NPs and the components of the cell membrane (Mosquera *et al.*, 2018). The cell membrane, found in all cells, is a critical part of the cell that forms the outer layer of the cells, by separating and protecting the intracellular organelles from the extracellular environment (Kettler *et al.*, 2014). Most importantly it gives structural support to the cells, regulates the transport of molecules and nutrients in and out of the cells, and maintains cell homeostasis and ion concentration gradients (Behzadi *et al.*, 2017). Subsequently, most cell membranes have a similar structural component. They are made up of two important components namely lipids and proteins to form an amphiphilic bilayer, having a hydrophilic head and hydrophobic tail. The proteins help to provide cellular signalling and regulate the interchange of molecules between the intracellular and the extracellular space, while the lipids form a morphological bilayer that shields the intracellular organelles from the extracellular environment (Mosquera *et al.*, 2018). The uptake of NPs by cells occurs via various transport mechanisms such as active transport, simple and facilitated diffusion. The diffusion mechanisms require the movement of molecules by a concentration, or charged gradient supported by membrane proteins, while active transport requires energy from the cells (Mosquera *et al.*, 2018).

Endocytosis is the main entry point for NPs, via membrane invagination and endocytic vesicles, after which the NPs are conveyed to other intracellular compartments (Zhao *et al.*, 2011). The mechanism of endocytosis involves two pathways namely pinocytosis and phagocytosis (Kuhn *et al.*, 2014). Phagocytosis is the process whereby large molecules are taken up and this process is normally performed by the specialist phagocytes such as monocytes, neutrophils, dendritic cells, and macrophages (Iversen *et al.*, 2011). Pinocytosis is the process whereby fluids and smaller

molecules are taken up by the cells. It is present in all cell types with multiple forms depending on the cell type, function, and origin (Behzadi *et al.*, 2017, Kou *et al.*, 2013). Pinocytosis can be classified into different types based on the protein involved in the uptake, such as micropinocytosis, caveolae-mediated endocytosis, clathrin mediated endocytosis, and clathrin- and caveolae-independent endocytosis as shown in Fig. 10 (Kou *et al.*, 2013). Studies have also reported that NPs explore more than one pathway to gain access into the cell (Saeed *et al.*, 2010).

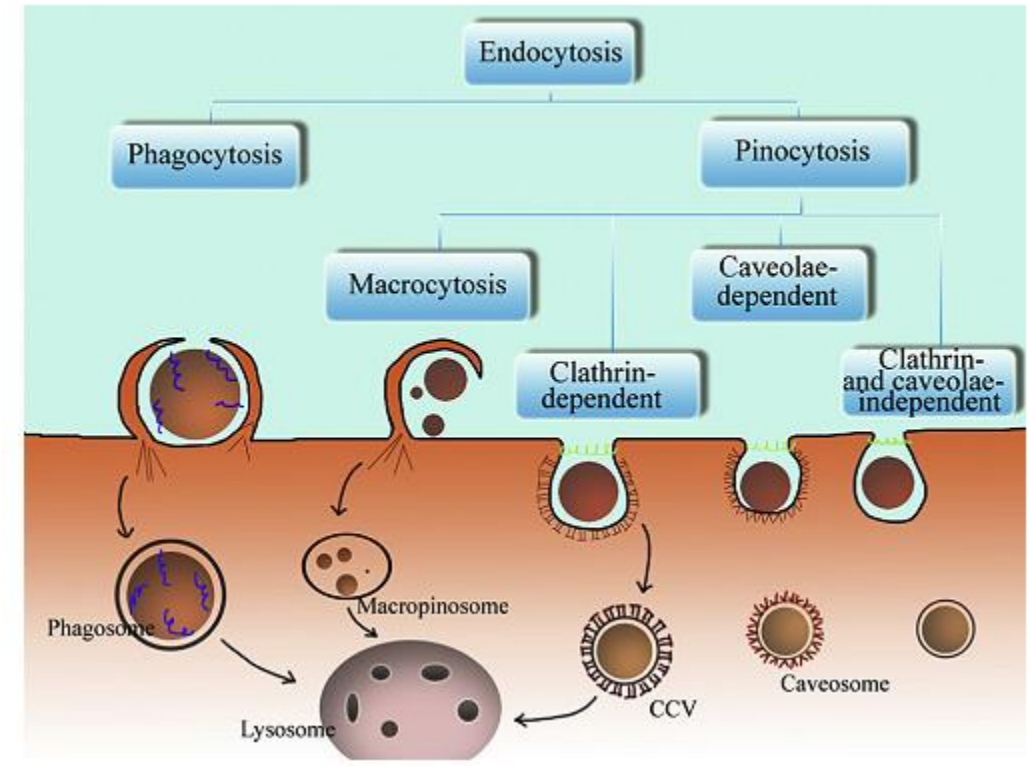

Figure 2.10: The pathway mechanism of nanoparticles in mammalian cells adapted from Kou *et al.*, 2013.

2.5.1 Phagocytosis

Phagocytosis is a process by which large particles (> 500 nm) are taken up by cells, predominantly phagocytes, such as macrophages, dendritic cells, neutrophils and monocytes (Kou *et al.*, 2013).

Phagocytic activities occur to a lesser extent in other cell types such as epithelial, endothelial and fibroblast. The NPs that enters the cells through this pathway are firstly recognized by opsonins such the blood proteins (fibronectin, laminin, etc.), immunoglobulins (IgG and IgM), complement components (C3, C4, and C5) and others (Sahay *et al.*, 2010). Therefore, the opsonized NPs bind to the surface and interact with the protein receptor on the membrane, forming a cup-shaped protrusion. The NPs are then enveloped by protrusion and then internalized forming a phagosome. Subsequently, the phagosomes fuse with the lysosomes forming a phagolysosome, and the cargo enveloped by the phagosomes is then destroyed by enzymolysis and acidification in the lysosomes (Sahay *et al.*, 2010). Physicochemical parameters of NPs such as surface charge, size and shape are critical in the phagocytic process. The mechanism of phagocytosis and other cascades of events are also dependent on the receptors involved (Behzadi *et al.*, 2017). Investigation of this mechanism can be elucidated by some inhibitors such as dynasore and cytochalasin D (Iversen *et al.*, 2011).

2.5.2 Pinocytosis

Pinocytosis is an important uptake mechanism through which fluids, solutes and small molecules enter the cells. It is categorized into different pathways based on the proteins used in the mechanism such as micropinocytosis, clathrin- and caveolae independent endocytosis, clathrin mediated endocytosis, and caveolae mediated endocytosis (Kuhn *et al.*, 2014, Zhao and Stenzel, 2018).

2.5.2.1 Clathrin-mediated/dependent endocytosis (CME)

Clathrin-mediated endocytosis (CME) is the uptake mechanism by which cells internalize nutrients. This uptake route enables the cells to obtain plasma membrane components such as

cholesterol through low-density lipoproteins, (LDLs), iron via the transferrin carrier and nutrients (Foroozandeh and Aziz, 2018). The CME mechanism takes place through the non-specific adsorptive uptake also known as receptor independent clathrin-mediated endocytosis or receptor specific uptake. In receptor-independent CME, uptake occurs through non-specific electrostatics or hydrophobic forces, which initiates the uptake without directly binding with the components of the membrane (Kou *et al.*, 2013). The receptor-specific CME occurs in an area of the cell membrane that is rich in clathrin. The clathrin assembly is made up of a three-legged structure comprising three light and heavy chains known as triskelion. This specific protein with others is important in the formation of a co-assembly into a complex structure that produces and stabilizes a budding vesicle and then the curvature (Behzadi *et al.*, 2017). Adaptor proteins are recognition sites for various cargos and sorting signals that are employed as docking sites on the cytoplasmic face of the cell membrane. Nucleation facilitates an assembly of the clathrin triskeleton into curved lattices comprising of a pentagon and hexagon, which induces invagination of the membrane into clathrin coated pits and subsequently stabilizes the distortion points at the membrane (Foroozandeh and Aziz, 2018, Sahay *et al.*, 2010). This route can be studied through the use of inhibitors or other factors such as potassium depletion, cytosol acidification, chlorpromazine hydrochloride and a hypertonic solution (Zhao and Stenzel, 2018).

2.5.2.2 Caveolae-mediated endocytosis

Caveolae-mediated endocytosis is key to various biological processes, such as transcytosis, cell signalling, regulation of membrane proteins, fatty acids and lipids (Behzadi *et al.*, 2017). This uptake mechanism involves flask shaped membrane invaginations known as the little caves (caveolae). Caveolae are composed of a membrane protein called caveolin-1,2, and 3 which gives them the flask shaped form, and are present in different cells such as fibroblasts, adipocytes, and

endothelial, epithelial and muscles cells (Kou *et al.*, 2013, Yameen *et al.*, 2014). Many organisms such as bacteria and viruses employ this uptake mechanism because of their ability to bypass the lysosomes and avoid hydrolytic enzymes and degradation (Foroozandeh and Aziz, 2018). When this protein binds to the receptor on the membrane, NPs or pathogens then interact with the receptor to form flask shaped vesicles, which are then sliced off from the membrane by dynamin. They also require actin like the CME to move and the interaction of microtubules to crosstalk within the cell. The caveolae moves to combine with multivesicular bodies or caveosomes which have a neutral pH (Behzadi *et al.*, 2017). The pharmaceutical inhibitors used for the study of this mechanism include cholera toxin, Filipin, genistein, and nystatin (Kuhn *et al.*, 2014, Zhao and Stenzel, 2018).

2.5.2.3 Clathrin-and Caveolae-independent endocytosis

Clathrin-and Caveolae-independent endocytosis uptake mechanisms do not make use of caveolae and clathrin. They make use of specific lipid composition and cholesterol in which various substances such as growth hormones, and cellular fluid are taken up by cells in the absence of clathrin and caveolae (Behzadi *et al.*, 2017). This pathway is categorized into Rhoa-dependent, Arf6-dependent and Cdc42-dependent. Dynamins play an active role in this pathway, but the exact mechanism requires further elucidation (Kou *et al.*, 2013). Moreover, folic acid is reported to be taken up by the cell through this route, for example folate-functionalized NPs in which the folate binds to its receptor leading to a non-disruptive entry of folate-modified NPs into the cytoplasm (Behzadi *et al.*, 2017, Kou *et al.*, 2013).

2.5.3 Macropinocytosis

Macropinocytosis is a specialized form of uptake mechanism that is caveolae-and-clathrin independent, transient, actin-driven and growth factor-induced endocytosis (Kuhn *et al.*, 2014). It

utilizes a lipid raft or pit forming proteins in which ruffles are formed due to the rearrangement of the cytoskeleton, which then fuse back into the membrane, creating a large vacuole that internalizes surrounding fluid (Behzadi *et al.*, 2017, Kou *et al.*, 2013). Macropinocytosis can be found in all cell types with the exception of brain microvessel endothelial cells (Oh and Park, 2014). The mechanism is initiated by an external stimulation in which tyrosine kinases are activated and then triggers a signalling matrix that results in the formation of membrane ruffles (Kuhn *et al.*, 2014). In the macrophages, macropinosomes enter the cytosol and fuse with lysosomes after separating from the membrane. The fate of macropinosomes is dependent on the cell type (Kou *et al.*, 2013). Finally, macropinocytosis is important in many physiological functions such as an entry point for various microorganisms and antigen presentation (Maruyama *et al.*, 2015). As a result of this mechanism, a large vacuole is created, this is key to the uptake of larger NPs that may not enter the cell through the clathrin- or caveolae-mediated pathway (Behzadi *et al.*, 2017). This mechanism is studied through different inhibitors such as sodium proton exchange, amiloride chloride, wortmannin, jasplakinolide, dynasore and cytochalasin D (Iversen *et al.*, 2011, Zhao and Stenzel, 2018).

2.6 Epithelial Cells

Epithelium (plural epithelial) is a layer of cells in all parts of the entire human body, mostly the outer surfaces of the body to the least open facets inside the inner organs (Parham, 2014). They are adjoining cells and lie upon a supporting extracellular stratum of the basement membrane. The open surface of the external cell membrane displays a little immobile cytoplasmic projection, called microvilli, and other structural specializations such as cilia (Giepmans and van Ijzendoorn, 2009). The entire gratis of the tissue is frequently increased by means of fold, tubules, and, in the intestine, large projections called villi. The diverse epithelial cells are derived from three of the

primary embryonic germ layers, namely the ectoderm, mesoderm and endoderm (von Knebel Doeberitz and Wentzensen, 2008). Their roles are connected in that they line free surfaces, underscoring their protective ability in covering the exterior and body aperture. Other roles such as movement of mucus and other molecules are carried out by ciliated epithelium located in the respiratory and genital ducts (Hammad and Lambrecht, 2015). The epithelia of the kidney, intestine and certain organs are highly involved in absorption and secretion of substances into and from the lumen (Parham, 2014). The sole function of the exocrine gland is to provide secretory products that will reach an outer surface. Due to their exposed position on free surfaces, epithelial cells are also important in sensory reception as in the case of taste buds and olfactory mucosa. Highly modified epithelial cells nurture and maintain the reproductive cells of the ovary and testis.

The epithelial cells can be classified based on their cell layers as simple, stratified and pseudo stratified and into squamous, cuboidal and columnar based on their shape (Hay, 2013). The simple epithelial cells consist of a single layer of cells which could be (squamous, cuboidal or columnar), lining the body cavities and tracts, majorly the kidney, blood vessels, lungs and skin, which aids in osmosis and diffusion processes in the body (Kierszenbaum and Tres, 2015). The stratified epithelial cells are packed together in multiple layers linked with tiny spaces between them, and mostly located in the skin, digestive tract and reproductive tracts and helps in the regulation of water loss from the body and defense against microbes and invading toxins (Hay, 2013). The pseudo stratified epithelial cells is false stratified epithelium possessing hair like extension called cilia and unicellular glands called goblet cells that produce mucus commonly found in the upper respiratory tract and the non-ciliated located in the glandular ducts and urethra and helps in absorption and protection by removing toxins and unwanted particles from the respiratory tract

(Greenburg and Hay, 1982). The epithelial cells show some polarity with differences in function and structure between the apical facing surface of the cell and the basal surface close to the underlying body structures. The basal lamina, a mixture of glycoproteins and collagen, provides an attachment site for the epithelium, separating it from underlying connective tissue. Sloughing off of damaged or dead cells is a characteristic of surface epithelium and allows our airways and digestive tracts to rapidly replace damaged cells with new cells (Mokhtar, 2017). The various routes of exposure to nanoparticles are dermal, lungs, and gastrointestinal tracts as a result of inhalation, injection, ingestion and implantation. The epithelial cell lining the skin, parts of the mouth and nose, and the anus develop from the ectoderm. Cells lining the airways and most of the digestive system originate in the endoderm. The epithelium that lines vessels in the lymphatic and cardiovascular system derives from the mesoderm and is called an endothelium. The skin is not the only part of the body exposed to the outside; other parts include the digestive tract, the urinary system, the reproductive systems and the airways all of which are lined by the epithelial cells (Sherwood, 2015). Other parts of the body that are not connected to the external part of the body such as the cardiovascular system are also lined by the epithelial cells. Most epithelial tissues are basically bulky sheets of cells covering all the surfaces of the body opened to the outer world and lining the outside of organs. Epithelium also forms the greater part of the glandular tissue of the body.

The adjoining cells that make the specialized intercellular connection between cell membranes is termed cell junction. The epithelial cells have some connections allowing different kinds of interactions between the cells. Three are adhering connections (junctions) and one provides a means of communication between cells (Parham, 2014). The four junctions are the zonula

occludens (tight junctions) that seal the space between epithelial cells so that no ion or water can flow between them, the zonula adherens that physically bind the cells together in a zone near the apical pole, the macula adherens (desmosome) that physically bind cells together in smaller areas resembling spots, and the gap junctions (nexus) that connect the cells together via protein lined channels that provide a means for crosstalk by allowing molecules to flow from cells to cells as shown in fig 11(Chang *et al.*, 2019, Kierszenbaum and Tres, 2015). Epithelial cells provide the body with its first line of protection from physical, chemical, and biological toxicity (Li *et al.*, 2012, Nel *et al.*, 2009). The epithelial cells act as gatekeepers of the body controlling permeability and allowing selective transfer of substances across barriers. All substances that enter the body must cross an epithelium. Some epithelia often include structural features that allow the selective transport of molecules and ions across their cell membranes (M Rabanel *et al.*, 2012). The epithelial cells are capable of secretion and release mucous and specific chemical compounds onto their apical surfaces against invasion by toxins. The epithelium of the small intestine releases digestive enzymes, while the cells lining the respiratory tract which secretes mucus that traps incoming microorganisms and toxic particles (Parham, 2014).

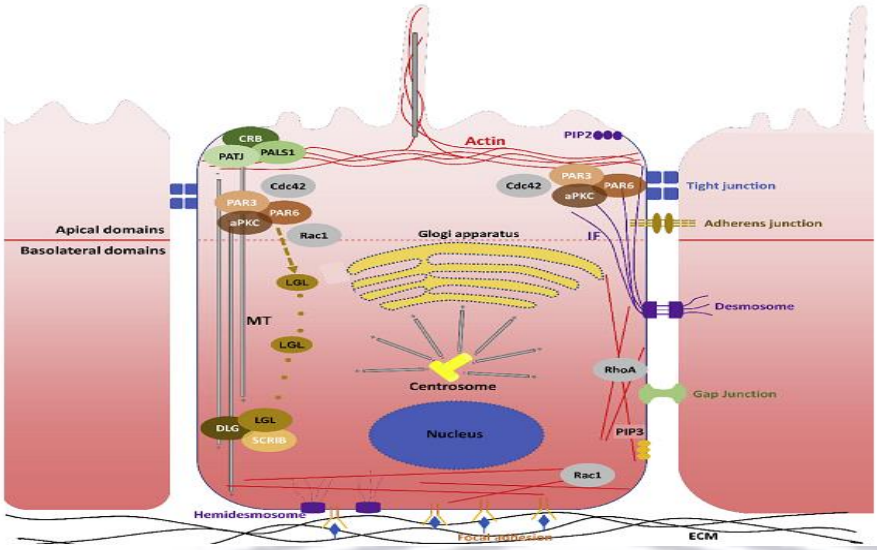

Figure 2.11: Epithelial cells with illustration of intracellular junctions, cytoskeleton, organelles, molecules and cell polarity. Adapted from Chang *et al.*, 2019.

2.6.1 Effect of Nanoparticles on Epithelial Cells

Alteration of epithelial cells by nanoparticles can possibly lead to calcium imbalance, oxidative stress and endoplasmic reticulum stress, mitochondrial dysfunction and gene mutation (Akhtar *et al.*, 2016, Chen *et al.*, 2014a, Iavicoli *et al.*, 2016). While studies are still ongoing to ascertain the level of toxicity and interaction of these particles with the components of the cells (endoplasmic reticulum, nucleus, cytoplasm, mitochondria and golgi body) that can cause or lead to degenerating disease conditions. It is therefore important to understand whether the numerous application possibilities of NPs in medicine and other fields outweigh their toxicity and possible adverse effects (Table 3).

Table 2.3: A None exhaustive summary of the in vitro effect of nanoparticles on epithelial cells

Nanoparticles	Cell lines	Cell types	Concentrations	Time	Effect	Reference
Cerium oxide NPs	BEAS-2B	Human lung epithelial cells	5, 10, 20, 40 µg/ml	24, 48, 72 & 96 hrs	Cell death, ROS increase, GSH decrease, and the inductions of oxidative stress-related genes such as hemeoxygenase-1 (HO-1), catalase, glutathione, S-transferase and thioredoxin reductase .	(Park *et al.*, 2008b)
Cerium oxide NPs	BEAS-2B	Human lung epithelial cells	15, 30 & 45 µg/ml	24 hrs	Significant increases in the cellular ROS concentrations, subsequently leading to the strong induction of HO-1 via the p38-Nrf-2 signalling pathway.	(Eom and Choi, 2009)
Copper Oxide NPs	A549 & HBEC	Human lung adenocarcinoma cells, human bronchial epithelial cells	9.2 µg/ml	2 & 4 hrs	Cell viability, increased lactate dehydrogenase (LDH) release and elevated levels of ROS and IL-8 in a dose-dependent manner.	(Jing *et al.*, 2015)
Copper oxide NPs	A549	Human lung adenocarcinoma cells	5, 10 & 15 µg/ml	24 hrs	Concentration dependent induction of DNA damage, micronuclei (MN), oxidative stress, which was indicated by the induction of ROS and lipid peroxidation along with gluthathione (GSH) depletion.	(Akhtar *et al.*, 2016)

Dolomite NPs	A549	Human lung adenocarcinoma cells	100, 200 & 400 µg/ml	48 hrs	Oxidative stress, genotoxicity and inflammatory responses, as seen by significant induction of ROS, LPO, MN, TNF-□, IL-1 and IL-6.	(Patil *et al.*, 2012)
Gold NPs	SAECs	Small airway epithelial cells	1 µg/ml	72 hrs	DNA damage, increase in lipid peroxide, oxidative stress-related cytotoxicity and genotoxicity.	(Ng *et al.*, 2013)
Iron oxide Magnetite Fe_3O_4 NPs	A549	Human lung adenocarcinoma cells	20-60 µg/ml	24 hrs	Increased production of ROS, activation of c-Jun N-terminal kinases (JNK) without increased nuclear factor kappa-B (NF-kB)-binding activity but delayed IkB-degradation was observed and genotoxicity.	(Könczöl *et al.*, 2011)
Latex polystyrene NPs	TT1	Human alveolar epithelial type1-like cells	50 & 100 µg/ml	4 & 24 hrs	Induction of cell detachment, cytotoxicity, Apoptosis and increased release of IL-8.	(Ruenraroengsak *et al.*, 2012)
MWCNTs	A549	Human lung adenocarcinoma cells	12.5, 25, 50, 100 & 200 µg/ml	6 hrs	Significant ROS generation and GSH depletion which reduced the cellular antioxidant level could be the major factor of cytotoxicity, the activation of cell autophagy with the intracellular	(Wang *et al.*, 2014)

					ATG16L1 level increase as a defense mechanism.	
MWCNT	HBE	Human bronchial epithelial cells	1.5-24 µg/ml	4,18,24,48 hrs	Induced NLRP3 inflammasome dependent pyroptosis in HBE cells in a time- and dose-dependent manner. HBE cells induced significant increase in mRNA expression of pro-fibrotic markers (TIMP-1, Tenascin-C, Procollagen 1, and Osteopontin).	(Hussain *et al.*, 2014)
MWCNT	A549	Human lung adenocarcinoma cells	5,10 & 50 µg/ml	6,12,24 hrs	Induced the production of ROS and malondialdehyde along with significant decrease in the activity of catalase and GSH.	(Srivastava *et al.*, 2011)
MWCNT	A549	Human lung adenocarcinoma cells	30 µg/ml	24 hrs	Cell cycle changes, apoptosis, or DNA damage, significant changes in protein expression. Proteins involved in several cellular processes including proliferation, stress, and cellular skeleton organization. In particular, MWCNT treatment causes increases in actin expression. This increase has the potential to contribute to increased migration capacity and may be mediated by ROS.	(Ju *et al.*, 2014)

Nickel NPs	HepG2		2–100 µg/ml	24 hrs	Induced cytotoxicity (cell death) and ROS generation in cells in a dose-dependent manner, Micronuclei induction, chromatin condensation and DNA damage in cells, suggested that induced cell death is via the apoptotic pathway, the expression level of mRNA of apoptotic genes (bax and caspase-3) were up-regulated whereas the expression level of anti-apoptotic gene Bcl-2 was down-regulated.	(Ahamed *et al.*, 2013)
Nickel NPs	A549	Human lung adenocarcinoma cells	0, 1, 2, 5, 10 & 25 µg/ml	24 & 48 hrs	Reduced mitochondrial function and induced leakage of LDH in a dose and time-dependent manner, induce oxidative stress in a dose and time-dependent manner indicated by depletion of GSH and induction of ROS and LPO. Further, activity of caspase-3 enzyme, marker of apoptosis was significantly higher in treated cells with time and dosage.	(Ahamed, 2011)
Quantum dots	HSF-42	Human skin epithelial cells	440 – 680 µg/ml	2, 8 & 24 hrs	Significant DNA damage in a time- and dose-dependent manner, induced	(Ju *et al.*, 2013)

					cytotoxic effects, including the inhibition of cell growth.	
Silica NPs	BEAS-2B	Human lung epithelial cells	50 µg/ml	24 hrs	Exerted toxicity via oxidative stress, which lead to the induction of HO-1 via the Nrf-2–ERK MAP kinase signalling pathway.	(Eom and Choi, 2009)
Silica NPs	Caco - 2	Human colorectal adenocarcinoma cells	10 – 200 µg/ml	24,48 & 72 hrs	At a higher concentration and longer exposure period, silica NPs do not induce the apoptosis/necrosis of cells, but arrest cell cycle and inhibit the cell growth.	(Yang *et al.*, 2014)
Silica NPs	A549	Human lung adenocarcinoma cells	100, 200 & 400 µg/ml	48 hrs	Induced dose dependent cytotoxicity and oxidative stress, had little effect on intracellular GSH level and the activities of glutathione metabolizing enzymes; glutathione reductase (GR) and glutathione peroxidase (GPx).	(Akhtar *et al.*, 2010)
Silica NPs	TR146	Human oral mucosa epithelial cells	12.5-125 µg/ml	2,4,6,8 & 12 hrs	Dock and cross the cellular membrane barrier in a dose–time-dependent manner, exert significant oxidative stress with concomitant upregulation of inflammatory genes IL-6 and TNF-□□ and apoptosis.	(Tay *et al.*, 2013)

Silver NPs	C2BBe1	Human intestinal epithelial cell	1.25 µg/ml	24 hrs	Induced 15 % necrotic cell death and an 80 % reduction in metabolic activity and decreased the GSH/GSSG ratio, indicating oxidative stress. G2/M phase cell cycle arrest and complete inhibition of cell proliferation.	(McCracken *et al.*, 2015)
Silver and Gold NPs	MCF- 7	Human breast carcinoma	50, 100, 150, 200 & 250 µg/ml	24 & 48 hrs	Provocation of intracellular ROS that cause damage to various cellular components, upregulation of Bax, Bcl2, caspases-6 and -9, PARP, p53 and downregulation of Bcl-2 depicts the induction of apoptosis and mitochondrial dysfunction.	(Jeyaraj *et al.*, 2015)
SWCNTs	A549	Human lung adenocarcinoma cells	4 and 8 µg/ml	1, 3, 6, 24 hrs	It induces changes in IL-8 expression under dynamic cell growth.	(Baktur *et al.*, 2011)
Titanium oxide	A549	Human lung adenocarcinoma cells	40 – 300 µg/ml	2 & 4 hrs	Elicited oxidant generation, IL-8 release and triggers inflammatory responses that appear to be driven by their large surface area.	(Singh *et al.*, 2007)
TiO_2NPs	(WISH) cells	Human amnion epithelial cell	0.625–10 µg/ml.	24 hrs	Revealed the concentration dependent cytotoxic effects, exhibited significant reduction in catalase activity and GSH level, increase in intracellular ROS	(Saquib *et al.*, 2012)

					generation, increase in G2/M cell cycle arrest and the formation of DNA double strand breaks.	
TiO_2NPs	A549	Human lung adenocarcinoma cells	25, 50, 75 & 100 µg/ml	4, 8, 16 ,24 & 48 hrs	Induce single-strand breaks and oxidative lesions to DNA, together with a general oxidative stress and impair cell ability to repair DNA.	(Jugan *et al.*, 2012)
TiO_2NPs	NHB, A549 & BEAS-2B	Norman human bronchial epithelial cells, human lung adenocarcinoma cells & human lung epithelial cells	5, 50, 100, 200 & 250 µg/ml	24 hrs	Induced ROS and secretion of the neutrophil chemoattractant IL-8 in all the cells, provoked release of the inflammatory mediators: IL-6, G-CSF and VEGF, in NHBE cells but not in the other two cell lines, provoked release of the inflammatory mediators: IL-6, G-CSF and VEGF, in NHBE cells but not in the other two cell lines.	(Ekstrand-Hammarström *et al.*, 2012)

Due to insufficient literature data, as seen in the above tables with respect to interaction of NPs with the proteome of human epithelial cells, there is a need to investigate the immune modulatory effects and cytotoxicity of nanoparticles. Also elucidating the interaction of NPs with cellular processes are vital as it tends to be valuable for the improvement of nanoparticles applications in the closest future.

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CHAPTER THREE: IMMUNOMODULATION AND POSSIBLE ANTI-ANGIOGENIC EFFECTS OF SILVER NANOPARTICLES ON HUMAN INTESTINAL CACO-2 CELLS

ABSTRACT

Silver nanoparticles (AgNPs) are present in numerous products used for various applications. Due to the large volumes AgNP products produced, environmental and human exposure to this NP become inevitable. Nanomaterials are being investigated for therapeutic applications such as angiogenesis. This study aimed to elucidate the effects of physiological media on the AgNPs. The study also explored the effects of AgNPs on Caco-2 cell viability, inflammatory activity and cell stress biomarkers, as well as angiogenesis using proteome profiling. This study shows that the NPs are stable in physiological media, based on hydrodynamic size and zeta potential over the time period assessed. The AgNPs are cytotoxic at high concentrations (IC_{50} = ± 100 μg/ml) and induced cell stress biomarkers at concentrations > 6.25 μg/ml AgNPs. The NPs modulated the inflammatory cytokine IL-8 and reduced IL-6 production in a dose dependent manner. Possible anti-angiogenic markers of AgNPs have been identified namely angiopoietin-2 and PDGF-AA, and these markers need to be investigated in more detail.

3.1 Introduction

In 2020, it was expected that the production and use of nanomaterials would increase to 58 000 tons due to the rapid increase in various applications and the anticipated use of silver nanoparticles (AgNPs) is estimated at 55 tons (Gaillet and Rouanet, 2015b, Sharma, 2009). This is due to the rapid research resulting in the generation of new nanomaterials and nanodevices, which have a range of applications, including electronics, medicine and energy production (Aueviriyavit *et al.*, 2014). Silver nanoparticles, in particular is attracting lots of attention due to their antibacterial properties and due to their various applications in consumer products (Park *et al.*, 2011). These consumer products include water purification systems, preservation of food products, pharmaceuticals, pesticides, electronics and paints (Böhmert *et al.*, 2012).

Since AgNPs are used in pharmaceutical and food products for their antimicrobial properties, there is an increased risk of human and environmental exposure (Vila *et al.*, 2018). Despite AgNPs having various applications, there is an absence of information concerning the impact these nanomaterials have on human health, especially the gastrointestinal system. To our knowledge, limited data is also available on how physiological medias would impact the NPs. Nanoparticles encounter the gastrointestinal system due to the direct ingestion of the materials and this can have toxicological implications.

A review conducted by Hadrup and Lam (2014) reported that *in vivo* AgNP exposure, resulted in the nanomaterial being distributed to all of the organs, including the intestine and stomach (Hadrup and Lam, 2014). The authors also noted that the animals exhibited dose dependent toxicity, and stated that weight loss, impaired liver activity, cardiac enlargement, immunological effects and death were evident. *In vitro* exposures to AgNPs have reported the induction of reactive oxygen

species (ROS), DNA damage and apoptosis in a number of cell lines (Costa *et al.*, 2010, Foldbjerg *et al.*, 2009, Ahamed *et al.*, 2008, Piao *et al.*, 2011). It is postulated that these effects are due to the release of silver ions (Ag^{+}) from the AgNPs causing the induction of ROS and this results in oxidative stress in cells, cell membranes, organelles and nucleus, directly causing apoptosis or necrosis (Vila *et al.*, 2018, Gaillet and Rouanet, 2015a, Park *et al.*, 2011).

Therapeutic applications to induce/supress angiogenesis is another avenue for which nanomaterials are being investigated. Angiogenesis is the process of neovascularization from pre-existing vessels (Walia *et al.*, 2015). A study explored the anti-angiogenic effects of AgNPs on bovine retinal endothelial cells. It was found that AgNPs could inhibit vascular endothelial growth factor (VEGF), which is involved in neovascularization (Gurunathan *et al.*, 2009). This could spark further interest with regards to AgNPs.

The human colon adenocarcinoma, Caco-2 cell line has been used extensively in toxicology research due to its capacity to undergo spontaneous differentiation, which leads to the formation of a cell monolayer. In addition to it mimicking small intestine enterocytes, both morphologically and functionally, it also simulate with *in vivo* absorption in humans (Carr *et al.*, 2012, Vila *et al.*, 2018). This study aims to elucidate how various physiological medias would impact AgNPs physicochemical characteristics, as well as the effects of AgNPs on Caco-2 cell viability, innate immune system biomarkers, cell stress biomarkers and potential angiogenesis biomarkers using proteome profiling.

3.2 Materials and Methods

3.2.1 Nanoparticle Characterization

Silver nanopowder, with ploy(vinylpyrrolidone) (PVP) as a dispersant (Sigma-aldrich Cat No. 576832) were previously characterized by Walters et al. (2013)(Walters *et al.*, 2013). Briefly, the authors determined that the NPs were spherical in shape and formed loosely packed aggregates via scanning and transmission electron microscopy (SEM and TEM), and that the NPs were crystalline in nature via X-ray diffraction (XRD). It was also found that when the NPs were in water, approximately 35 % were between 20- 40 nm and 15 % were between 70-1000 nm. It was also determined that in water, the zeta potential was -18.8 mV.

The AgNPs were subsequently characterized in physiological media over a 2-week period to determine whether their characteristics would alter. A 10 mg/ml AgNPs stock suspension in water was prepared by sonication on ice for short bursts for a total of 5 minutes. Thereafter, the AgNPs were placed in various physiological medias (pH 7): 150 mM sodium chloride (NaCl) (Sigma-aldrich); 1 x phosphate buffered saline (PBS) (Sigma-aldrich); Incomplete Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-aldrich) containing 0.1 % v/v glutamax (Sigma-aldrich), 0.1 % antibiotic/antimycotic solution (Sigma-aldrich) and 0.05 % v/v gentamicin (Sigma-aldrich) ; and complete DMEM media, containing the same constituents as incomplete media with 10 % v/v heat inactivated foetal bovine serum (FBS) (Sigma-aldrich), to yield a final concentration of 10 μg/ml AgNPs. The various medias were then incubated at 37 °C for 0 hr, 24 hrs, 7 and 14 days. After the various incubation periods, the media was centrifuged at 10 000 rpms (Eppendorf Centrifuge 5810 R), washed and resuspended in dH_20. Thereafter, disposable folded capillary cells (Malvern Pananlytical) were used to determine the hydrodynamic size and zeta potential of the nanoparticles with a ZetaSizer Nano ZS (Malvern Instruments).

3.2.2 Nanoparticle Preparation for Cell Culture

A 10 mg/ml stock concentration of AgNPs were prepared in distilled water. The NPs were sonicated (QSonica, LLC. Misonixsonicators, XL-200 Series) on ice for short burst for approximately 5 minutes. Nanoparticles were freshly prepared prior to each experiment.

3.2.3 Cell Culture

The human colorectal adenocarcinoma (Caco-2) epithelial cell line was obtained from the American Type Culture Collection (ATCC HTB-37). Standard tissue culture conditions were used to maintain the cells in complete DMEM medium. Cells were sub-cultured approximately every 3-4 days using 0.05 % trypsin-ethylenediaminetetraacetic acid (EDTA) (Gibco).

Caco-2 cells were seeded at a density of 4 x 10^3 cells/ml in 24 well tissue culture treated plates (Nunc) and were approximately 60 % confluent before nanoparticle treatment. Cells were exposed for 48 hrs to 0-200 μg/ml AgNPs. Thereafter, supernatants were removed and cell viability assessed. Supernatants were centrifuged at 12.100 rcfs for 1 min (MiniStar Plus Super Mini Centrifuge) before evaluating innate inflammatory biomarkers and angiogenesis biomarkers. Experiments were repeated in triplicate. The cells were harvested after AgNP exposure using lysis buffer solution (1 X PBS, 0.1% tween and 200μl protease inhibitor). The cells were then scraped with a scrapper after which cells were then sonicated using (QSonica, LLC. Misonixsonicators, XL-200 Series) on ice for short burst for 20secs and centrifuged at 12 100rcf for 1min. Protein concentration of the cell homogenate was quantified using Bradford reagent. The cell homogenates at 300 μg/ml protein were used to evaluate cell stress biomarkers potentially induced by the nanoparticle. The cell homogenates 300 μg/ml were used to evaluate cell stress biomarkers induced by the nanoparticles.

3.2.4 Cell viability Assay

Cell viability was monitored using the sodium 3'[1-[(phenylamino)-carbony]-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene-sulfonic acid hydrate) (XTT) assay (Sigma-Aldrich). The assay monitors the conversion of the XTT tetrazolium salt to a soluble formazan salt by metabolically active cells (Aslantürk, 2018). Therefore, formazan formation is directly proportional to cell viability. Supernatants were removed and cells washed with PBS to remove any excess NP that may interfere with the assay. A 1:50 ratio of XTT coupling reagent to XTT labelling reagent was prepared. This mixture was further diluted in complete medium to yield a final ratio of 1:3. Plates were immediately read at 450 nm (FLUOstar Omega, BMG Labtech) after the addition of XTT. Plates were then incubated at 37 °C for 1 hr, thereafter, an additional reading was made. The change in absorbance at 450 nm over time was calculated and the percentage viability analysed.

3.2.5 NO Assay

The amount of nitrite produced by the Caco-2 cells exposed to the respective nanoparticle concentrations was assessed in the cell culture supernatant as an indication of NO production. This assay is based on the Griess reaction (Granger *et al.*, 1996). The amount of nitrite produced by the cells was measured against the nitrite standard range (Sigma-Aldrich) (0-100 μM). A 1:1 of culture supernatant or standard was mixed with the Griess reagent (1:1 of 1% sulphanilamide and 0.1% naphtylethlemidimine-dihydrochloride in 2.5 % sulphuric acid (all obtained from Sigma-Aldrich). The plate was subsequently read at 540 nm (FLUOstar Omega, BMG Labtech) and the amount of nitrite produced by the cells quantified.

3.2.6 Innate Inflammatory biomarkers IL-6 and IL-8

The double antibody sandwich enzyme linked immuno-sorbent assays (DAS-ELISA) were run according to the manufacturer's instructions. Both the IL-6 (Invitrogen) and IL-8 (R & D Systems) assays were run using undiluted supernatants.

3.2.7 Cellular Protein Quantification

Cell protein extracts were quantified using the Bradford assay (Bio-Rad). The assay was performed as per the manufacturer's instructions.

3.2.8 Cell Stress Biomarkers

The production of cell stress biomarkers potentially induced by exposing the cells to the nanoparticles were assessed by performing SOD, Phospho-HSP27 and HSP70 ELISAs (all purchased from R & D Systems). The experiments were all performed as per the manufacturer's instructions. All samples were run using the cell lysates at 300 μg/ml.

3.2.9 Angiogenesis Proteome Profile

The angiogenesis proteome profiler (R & D Systems) contained 4 membranes, each spotted in duplicate with 55 different angiogenesis antibodies. The assay was performed as per the manufacturer's instructions. The concentrations selected were 0 and 100 μg/ml AgNPs. The concentration selected represented the IC_{50} (100 μg/ml AgNPs). The membranes were subjected to an ultra-sensitive chromogenic 3,3',5,5'-Tetramethylbenzidine (TMB) substrate (Thermofisher) to show sample-antibody complexes labelled with streptavidin-horse radish peroxidase (HRP). Pictures were taken of the membranes after substrate exposure.

3.2.9.1 Statistical analysis

All experiments were performed in triplicate and the data calculated using Microsoft Excel. The data is represented as mean ± standard deviation (SD). A one-way analysis of variance (ANOVA) using SigmaPlot 12.0 (Systat Software Inc.) was used to determine statistical differences, with $p < 0.01$ deemed significant.

3.3 Results

3.3.1 Nanoparticle Characterization

The AgNPs were exposed to various physiological media over a 2-week period. The temperature (37 °C) and pH (pH7) were kept constant. The hydrodynamic size and zeta potentials were assessed at various time points (i.e. 0 hr, 24 hrs, 7 days and 14 days) of exposure. The hydrodynamic size of the NPs after being exposed to 150 mM NaCl for 14 days remained stable. However, the size of the NP only notably increased ($p<0.001$) at day7 compared to 0 hr, from an initial size of 416.57 ± 68.64 to 830.93 ± 121.61 nm (Table 1). At 14 days, the NPs had the highest zeta potential ($p<0.001$) across the time frame of -31.73 ± 1.5 mV compared to the initial time point of -22.4 ± 0.44 mV (Table 2). Silver nanoparticles in the presence of 1x PBS showed a gradual but significant increase ($p<0.001$) in size across the time periods assessed, increasing from an initial 398.37 ± 28.47 nm (0 hr) to 801.3 ± 115.64 nm (14 days). The zeta potential reflected this noteworthy increase ($p<0.002$) only at day 14, where it increased to 28.37 ± 2.27 mV after an initial -15.2 ± 1.48 mV (0 hr). When the NPs were observed in DMEM alone, the size was constant across the 14-day period, with no significant changes noted. This was echoed with the zeta potential data as no changes were seen across all time periods assessed. The size of the NPs that were evaluated in DMEM supplemented with 10 % FBS (culture medium) displayed the same trend as NPs assessed in DMEM alone. No change in hydrodynamic size across all time points

were observed. However, the zeta potential was constant after 24 hrs, and at day 14, only showing a significant decrease ($p<0.009$) in zeta potential at day 7 (-17.57 ± 3.64 mV).

Table 3.1: Hydrodynamic size of AgNPs after being exposed to various physiological medias over a 2-weeks period

MEDIA	Number of Days			
	0	**1**	**7**	**14**
150 Mm NaCl	416.57±68.64	632.7±30.48	830.93±121.61*	548.35±89.74
1 X PBS	398.37±28.47	647.53±56.17***	717.50±105.04**	801.3±115.64*
DMEM	629.9±13.19	619.57±90.68	733±212.85	647.53±56.18
DMEM (10% FBS)	619.33±90.58	647.53±56.18	924.60±198.60	812.17±119.21

Data expressed as mean ± SD. Significance demarcated by (*) indicating significant difference of $p<0.001$, (**) $p<0.003$ and (***) $p<0.007$ compared to the relative 0 hr control.

Table 3.2: Zeta Potential of AgNPs after being exposed to various physiological medias over a 2-weeks period

MEDIA	Number of Days			
	0	**1**	**7**	**14**
150 Mm NaCl	-22.4±0.44	-25.65±2.1	-23.15±2.96	-31.73±1.5*
1 X PBS	-15.2±1.48	-20.13±3.48	-15.24±3.75	-28.37±2.27**
DMEM	-25.27±0.93	-18.83±0.97	-21.51±0.84	-21.55±4.13
DMEM (10% FBS)	-26.13±0.74	-23.12±2.5	-17.57±3.64***	-28.77±2.13

Data expressed as mean ± SD. Significance demarcated by (*) indicating significant difference of $p<0.001$, (**) $p<0.002$ and (***) $p<0.009$ compared to the relative 0 hr control.

3.3.2 Cell Viability

Exposure of Caco-2 cells to AgNPs at concentrations ≤ 50 µg/ml had no effect on cell viability (Fig 1). However, cell exposures to concentrations ≥ 100 µg/ml AgNPs exhibited a significant reduction ($p<0.001$) in cell viability. The percentage reduction in viability at 100 and 200 µg/ml AgNPs were approximately 60 and 90 % respectively in comparison to the control.

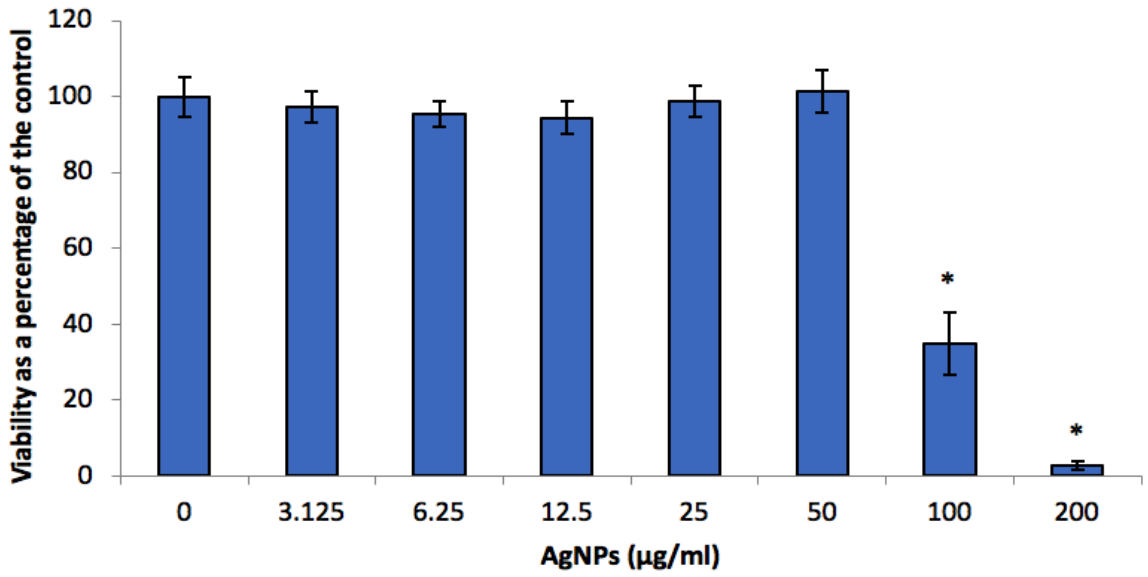

Figure 3.1: Caco-2 cell viability after 48 hr exposure to a range of AgNP concentrations. Data represented as mean ± SD. Significance demarcated by *, indicating a significant difference of $p<0.001$.

3.3.3 The Effects of AgNP Exposure on Caco-2 Cell Secretion of the Inflammatory Biomarker, NO

After the 48 hr exposure period of the Caco-2 cells to varying AgNPs concentrations, a number of innate immune system inflammatory biomarkers were assessed. It was noted that the NPs significantly upregulated ($p<0.006$) NO production from the cells at 50 µg/ml AgNPs and a further

increase ($p<0.001$) in its production at concentrations 100 and 200 µg/ml AgNPs respectively (Fig 2). However, this increase would not be deemed majorly significant in a system.

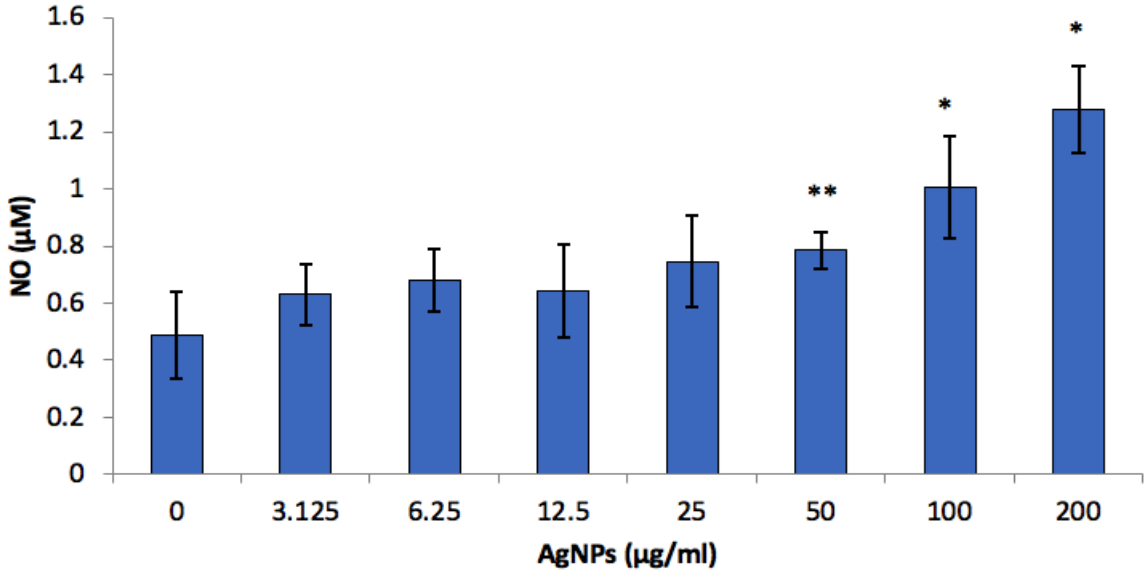

Figure 3.2: Caco-2 Nitric oxide (NO) after 48 hr exposure to a range of AgNP concentrations. Data represented as mean ± SD. Significance demarcated by *, indicating a significant difference of $p<0.001$ and (**) $p<0.006$.

3.3.4 The Effects of AgNP Exposure on Caco-2 Cell Secretion of the Inflammatory Biomarker, IL-6

There was a notable dose dependent decrease ($p<0.001$) in the release of the cytokine IL-6 when the cells were exposed to AgNP concentrations ≥ 6.25 µg/ml (Fig 3). It is also noted that there is an approximate 50 and 30 % reduction in IL-6 production at 100 and 200 µg/ml AgNP respectively.

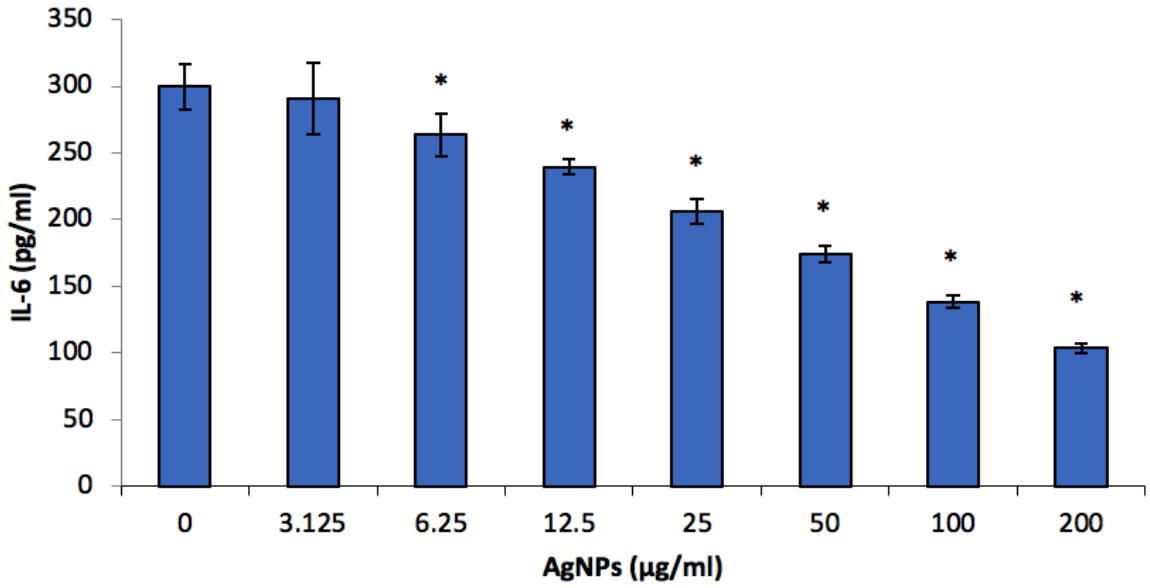

Figure 3.3: Caco-2 Interleukin 6 (IL-6) after 48 hr exposure to a range of AgNP concentrations. Data represented as mean ± SD. Significance demarcated by *, indicating a significant difference of p<0.001.

3.3.5 The Effects of AgNP Exposure on Caco-2 Cell Secretion of the Inflammatory Biomarker, IL-8

The IL-8 initially showed an upregulation ($p<0.006$) at 3.125 µg/ml and a further significant increase ($p<0.001$) at 6.25 µg/ml AgNP in comparison to the control (Fig 4). Thereafter, the production of IL-8 notably decreased ($p<0.006$) after the cells were exposed to the AgNP range 25-100 µg/ml. However, the biggest reduction ($p<0.001$) of IL-8 occurred at 200µg/ml AgNP.

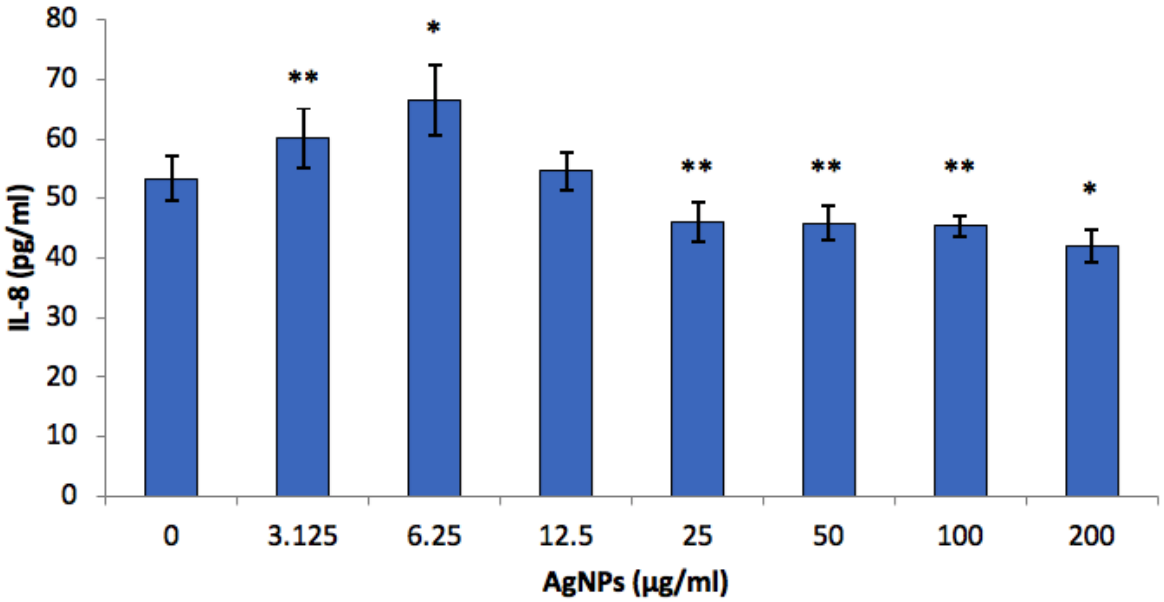

Figure 3.4: Caco-2 Interleukin 8 (IL-8) after 48 hr exposure to a range of AgNP concentrations. Data represented as mean ± SD. Significance demarcated by *, indicating a significant difference of p<0.001 and (**) p<0.006.

3.4 Cell stress Biomarkers

Cell lysates of the cells exposed to AgNPs for 48 hrs were examined for a number of cell stress biomarkers. All three biomarkers (i.e. Phospho-HSP-27, HSP-70 and SOD-2) evaluated in this study exhibited the same trend. The study showed that there was a steady, significant dose dependent increase in the respective biomarker synthesized by the cells after AgNP exposure (Fig 5,6,7). Moreover, HSP-70 synthesis was the most sensitive to AgNP exposure as the amount of biomarker produced was significantly upregulated ($p<0.001$) at all the concentrations assessed (0-50 µg/ml AgNPs) in the study (Fig 5). Phospho-HSP-27 and SOD-2 followed the same trend, but proved to be less sensitive than HSP-70. Both phospho-HSP-27 and SOD-2 showed a significant increase ($p<0.003$) in the respective biomarker at 12.5 µg/ml AgNPs and a further substantial increase ($p<0.001$) at 25 and 50 µg/ml AgNPs respectively.

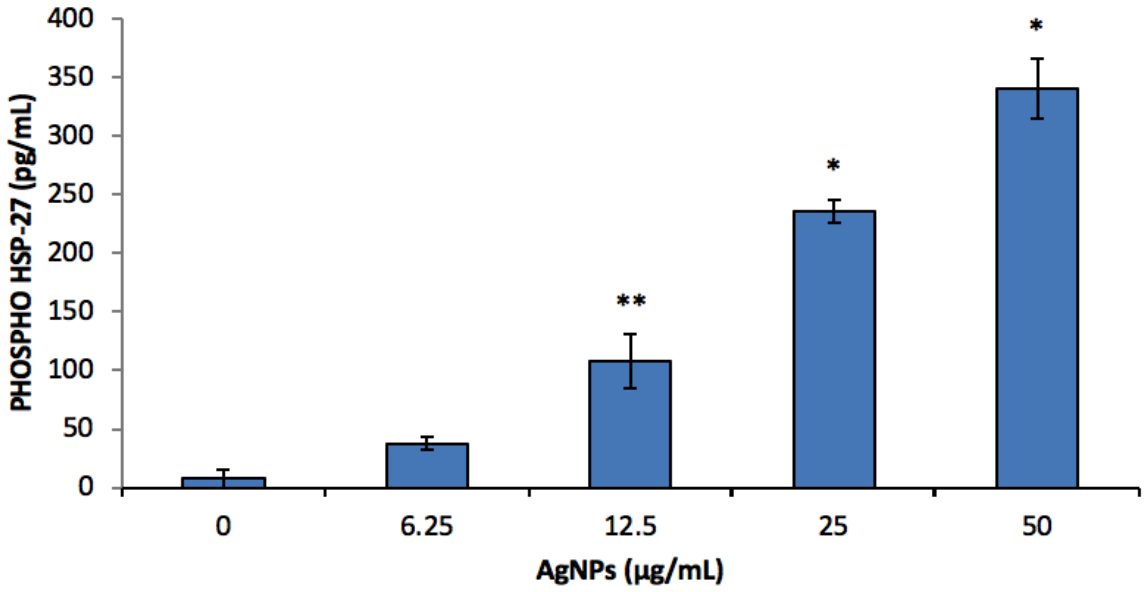

Figure 3.5: Phospho-HSP-27 cell stress biomarkers after exposing Caco-2 cells to various AgNP concentrations for 48 hrs. Data represented as mean ± SD. Significance demarcated by (*), indicating a significant difference of p<0.001 and (**) p<0.003.

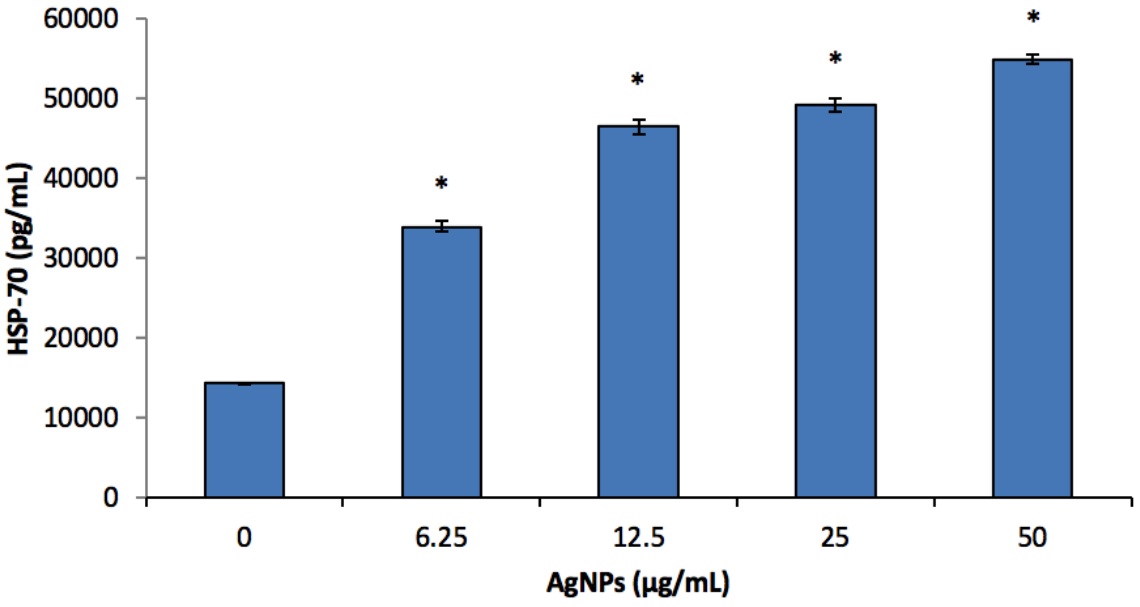

Figure 3.6: HSP-70 cell stress biomarkers after exposing Caco-2 cells to various AgNP concentrations for 48 hrs. Data represented as mean ± SD. Significance demarcated by (*), indicating a significant difference of p<0.001.

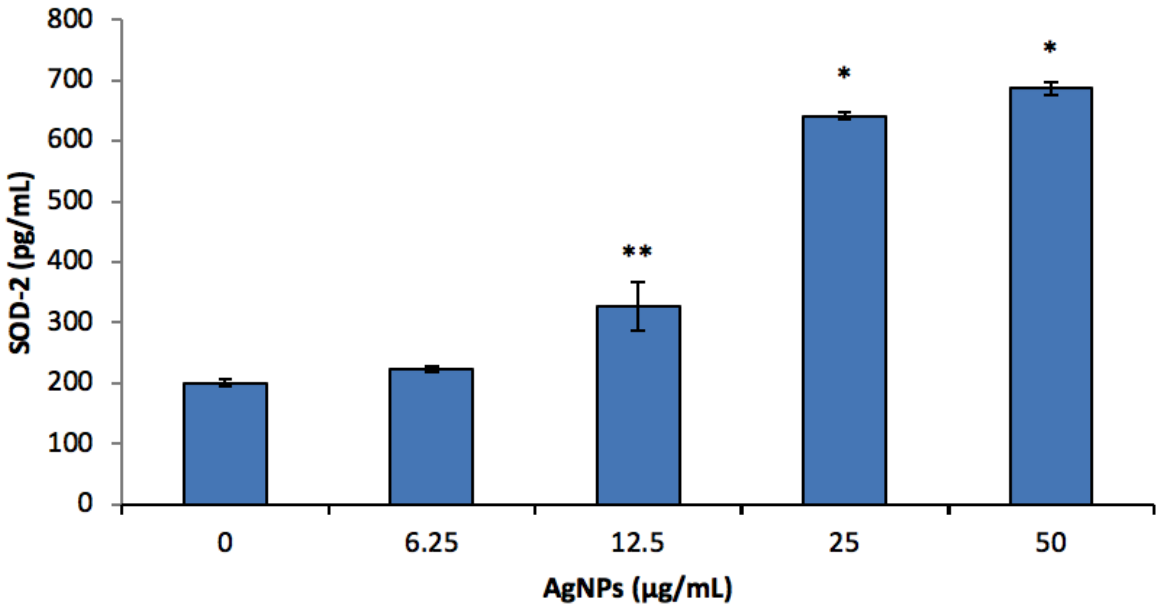

Figure 3.7: SOD-2 cell stress biomarkers after exposing Caco-2 cells to various AgNP concentrations for 48 hrs. Data represented as mean ± SD. Significance demarcated by (*), indicating a significant difference of p<0.001 and (**) p<0.003.

3.5 Angiogenesis Proteome Profile

Angiogenesis proteome profiles, using cell culture supernatants for cells exposed to the control (0 µg/ml AgNPs) and IC_{50} value (100 µg/ml AgNPs) were analysed and revealed differences in markers between the exposure groups (Fig 8). The membranes assessed mostly revealed the same proteins, with the exception of activin A and dipeptidyl peptidase IV (DDPIV) which was only present in the control group (Fig 8a). The intensity of certain proteins decreased in the presence of the IC_{50} value in comparison to the control. These proteins include: angiopoietin-2, endostatin and platelet-derived growth factor AA (PDGF-AA) (Fig 8b). Conversely, persephin was slightly upregulated in the presence of 100 µg/ml AgNPs in comparison to the control group.

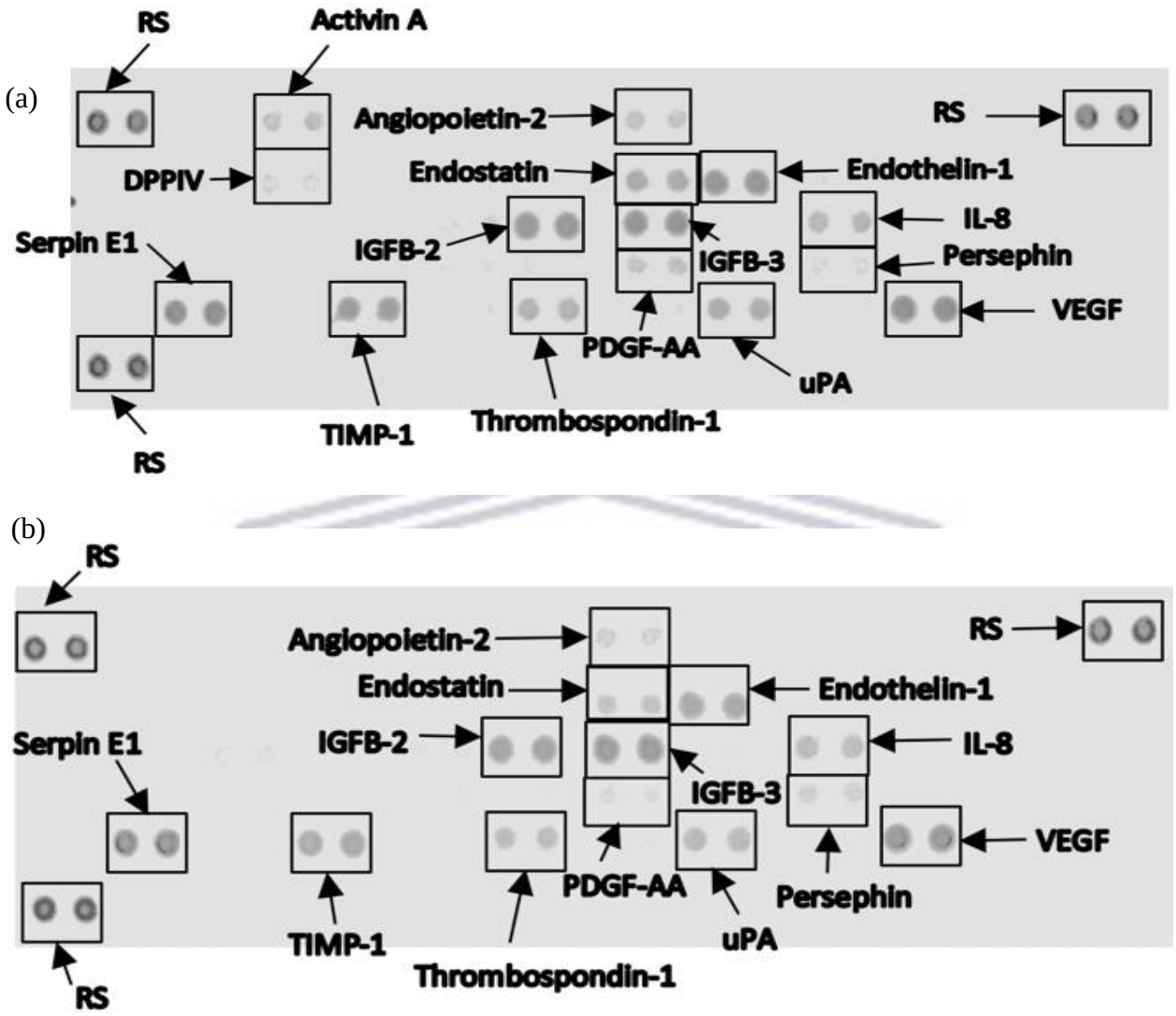

Figure 3.8: Angiogenesis proteome profile using culture supernatants of Caco-2 cells exposed to (a) control (0 µg/ml AgNPs) and (b) IC50 value (100 µg/ml AgNPs) for 48 hrs. (b) control (0 µg/ml AgNPs) and (b) IC50 value (100 µg/ml AgNPs) for 48 hrs.

3.6 Discussion

Due to the varying applications of AgNPs, environmental and human exposure is inevitable. However, there is a lack of knowledge regarding the toxicity of NPs, especially in the gastrointestinal system. This study aimed to elucidate how various physiological medias would

impact AgNPs as well as the effects of AgNPs on Caco-2 cell viability, innate immune system inflammatory biomarkers, cell stress biomarkers and the angiogenesis proteome profile.

The size and zeta potential of AgNPs in the culture medium were stable across the 24 hr exposure period, with sizeable changes only occurring after 7 days (Table 1 and 2). Therefore, it can de deduced that the NPs were stable in the 48 hrs experimental procedure used in this study. As Tejamaya et al. (2012) characterized PVP and polyethylene glycol (PEG) coated AgNPs in standard Organisation for Economic Co-operation and Development (OECD) media for *Daphnia* sp. over a 21 day period (Tejamaya *et al.*, 2012, OECD, 2004). The authors found that the PVP coated NPs were the most stable, with no aggregation or changes in dissolution found.

In the present study, an approximate 50 and 30 % decrease in Caco-2 cell viability was exhibited at 100 and 200 μg/ml AgNPs respectively (Fig 1). A study exposing AgNPs to Caco-2 cells for 24 hrs exhibited a dose dependent decrease in viability using the MTT assay, with the authors reporting an IC_{50} value of 16.7 μg/ml AgNPs (Aueviriyavit *et al.*, 2014). This is remarkably lower compared to our results. However, different time points were assessed and the authors did not report which dispersant for the NP was used and could account for the discrepancy in the IC_{50} values. In another study, 20 and 40 nm peptide coated AgNPs reduced toxicity of Caco-2 cells after 48 hrs at concentrations ≥ 5 and ≥ 50 μg/ml respectively (Böhmert *et al.*, 2012). The coating and size of the NP, as well as the resuspension of the dried NP in 0.015 N hydrochloric acid (HCl) could account for the effects seen. The cell stress biomarkers assessed (SOD-2, phospho-HSP-27 and HSP-70) after AgNP exposure all increased at concentrations > 6.25 μg/ml (Fig 5,6,7). SOD to the contrary, was found to be reduced after 24 hr exposure to Caco-2 cells at concentrations >

50 μg/ml AgNPs, in conjunction with no ROS activity (Kang *et al.*, 2015). Similarly, Song et al. (2014) found that AgNPs did not induce SOD or ROS at the concentrations evaluated in the study (0-200 μg/ml) after 24hr exposure (Song *et al.*, 2014). This would imply that the cell was not undergoing oxidative stress after 24 hrs. Though the type of NP dispersant was not stated in both studies and similar concentrations were used in comparison to the current study. Limited data is available regarding the heat shock response of intestinal cells against AgNPs. However, a proteomic study of Caco-2 cells exposed to AgNPs for 72 hrs found that mitochondrial heat shock (HSP-60) and HSP-70 were deregulated to counteract the oxidative damage to the cells (Gioria *et al.*, 2018). When human liver HepG2 cells were exposed to AgNPs for 24 hrs, the authors found that the cells responded to the imposed threat by upregulating stress response genes such as HSPs and metallothioneins (Sahu *et al.*, 2015). SOD is one of the antioxidant species which protects cells from damage by catalyzing the dismutation of superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2) which is consequently detoxified to oxygen (O_2) and water by catalase and glutathione peroxidases (Asakura and Kitahora, 2018, Younus, 2018). The induction of HSPs are rapid and intense, which aids in shielding the cell from insults such as hypoxia and cytotoxic exposure (Miller and Fort, 2018, Lindquist and Craig, 1988). This could explain why toxicity is only evident at very high concentrations of the NP as the SOD and the HSPs assist to protect the cell from damage. Similar to the discrepancies found in viability, the exposure period, size and dispersant of NP could influence the results seen with regards to the cell stress biomarkers. The viability data could be attributed to the upregulation of the cell stress biomarkers as one of the accepted modes of action resulting in these effects is the induction of ROS, which results in oxidative stress in cells, cell membranes, organelles and nucleus, directly causing apoptosis or necrosis, although the

release of Ag^+ from the NPs were not assessed in this study (Vila *et al.*, 2018, Gaillet and Rouanet, 2015a).

Pro-inflammatory chemokines, IL-6 and IL-8 are known to have pleiotropic effects, and are involved in the recruitment of neutrophils and other granulocytes to sites of infection during acute and chronic infection (Tarantini *et al.*, 2015, Martirosyan *et al.*, 2016). To our knowledge the *in vitro* inflammatory response of intestinal systems to AgNPs has not been well established, especially the effect these NPs have on IL-6 production. This inflammatory cytokine was found to be inhibited in a dose dependent manner at concentrations ≥ 6.25 μg/ml AgNPs (Fig 3). The effects of other nanoparticles on IL-8 production, such as chitosan, silica and titanium dioxide have been investigated (Krüger *et al.*, 2014, Chalew and Schwab, 2013, Tu *et al.*, 2016, Tarantini *et al.*, 2015). Chitosan NPs were found to inhibit IL-8 production from Caco-2 cells stimulated by LPS (Tu *et al.*, 2016). Chalew and Schwabb (2013) established that the exposure of commercially available NPs, silver and titanium, to human intestinal Caco-2 and SW480 cells increased IL-8 cytokine generation (Chalew and Schwab, 2013). Another study found that AgNPs at 30 and 45 μg/ml reduced IL-8 synthesis in a monoculture of Caco-2 cells (Martirosyan *et al.*, 2016). In the current study IL-8 production was modulated as an increase was evident between 3.125-6.25 μg/ml AgNPs (Fig 4). The concentration of IL-8 decreased at AgNP concentrations ≥ 25 μg/ml. With the results obtained in this article and previously published work, one can see the varying reports with regards to IL-8 production in response to AgNP exposure. The differences in viability and cell stress markers found in various studies could be attributed to exposure time, size and dispersant of the NP.

Nanomaterials are currently being investigated for therapeutic applications such as angiogenesis. Angiogenesis is the process of neovascularization from pre-existing vessels (Walia *et al.*, 2015). In 2009, Gurunathan *et al*. explored the anti-angiogenic effects of AgNPs on bovine retinal endothelial cells. The authors found that AgNPs could inhibit vascular endothelial growth factor (VEGF), which is involved in neovascularization (Gurunathan *et al.*, 2009). In this study, an angiogenesis proteome profile of Caco-2 cells exposed to the control (0 μg/ml AgNPs) and IC_{50} value (100 μg/ml AgNPs) revealed the modulation of a few proteins (Fig 8). The proteins activin A and DDPIV were only present on the control membrane (Fig 8a). Activin A, a member of the transforming growth factor beta (TGF- β) superfamily, regulates cell proliferation and differentiation in various organs and DDPIV is constitutively expressed on endothelial cells (Kitlinska *et al.*, 2004, Kaneda *et al.*, 2011). The inhibition of activin A at the IC_{50} value could aid in what was seen with the viability data (Fig 1 and 8b). The production of other proteins exposed to 100 μg/ml AgNPs was reduced, namely: angiopoietin-2, endostatin and PDGF-AA. Angiopoietin-2 initiates the onset of angiogenesis and is needed for normal formation of lymph vessels (Linares *et al.*, 2014, Gurunathan *et al.*, 2009). Endostatin is an inhibitor of angiogenesis, endogenously produced by cells but the mechanism is not fully understood (Walia *et al.*, 2015). PDGF is expressed in microvascular endothelium *in vivo* when the endothelial cells are activated and can cause angiogenesis, this suggests that PDGF may have a direct effect on endothelial cells (Risau *et al.*, 1992). These markers need to be further investigated as angiopoietin-2 and PDGF-AA are suggesting anti-angiogenic effects of AgNPs. The inhibition of endostatin may induce angiogenesis, although the loss in cell viability needs to be considered.

3.7 Conclusions

The AgNPs are cytotoxic to intestinal cells *in vitro* at high concentrations after 48 hrs and this could be the result of cell stress markers being induced at low concentrations. Ag^{+} release from the NPs were not explored in this study. The NPs also modulate and reduce the chemokines, IL-8 and IL-6 respectively. The anti-angiogenic markers identified, angiopoietin-2 and PDGF-AA needs to be further investigated. These proteins could illuminate the potential anti-angiogenic potential of AgNPs. Although not consistently reported in the literature, variations in results for AgNPs exposure by publications necessitate that the size, dispersant and exposure time to the NP be reported to make comparisons.

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CHAPTER FOUR: STABILITY AND ANTI-ANGIOGENIC PROPERTIES OF TITANIUM DIOXIDE NANOPARTICLES (TIO_2NPS) USING CACO-2 CELLS

ABSTRACT

Nanoparticles, such as titanium dioxide nanoparticles (TiO_2NPs) are found in a large range of products such as sunscreen, paints, toothpaste and cosmetics due to their white pigment and high refractive index. This could result in the direct or indirect exposure of these NPs to humans and the environment. Conflicting levels of toxicity has been associated with these NPs and should be investigated for these and other applications. This study aimed to investigate the effects of exposure media on TiO_2NPs properties (hydrodynamic size and zeta potential), and the effects of TiO_2NPs exposure on human colorectal adenocarcinoma (Caco-2) epithelial cell viability, inflammatory and cell stress biomarkers and angiogenesis proteome profile. The NPs increased in size over time in the various media, while zeta potentials were stable. TiO_2NPs induced cell stress biomarkers, which could attribute to the NPs not being cytotoxic. TiO_2NPs exposure had no effects on the level of inflammatory biomarkers produced Caco-2. Anti-angiogenic properties were exhibited when exposed to the NOAEL of TiO_2NP and requires further in-depth investigation.

4.1 Introduction

Titanium dioxide nanoparticles (TiO_2NPs) are known to exist in various forms, of which anatase, brookite and rutile are the most abundant. The rutile form has been studied extensively due to its electrical, optical and thermal properties (Reddy *et al.*, 2003). These NPs have a white pigment, and due to the brightness and high refractive index these NPs have a variety of applications (Shi *et al.*, 2013). These applications include paints, food products, cosmetics, toothpastes, plastics, industrial photocatalytic processes, and very commonly in sunscreens, as it helps protect the skin from UV light (Shi *et al.*, 2013, Trouiller *et al.*, 2009, Iavicoli *et al.*, 2011). Based on these characteristics, TiO_2NPs were one of the earliest industrially produced nanoparticles and are reportedly the most manufactured globally (Iavicoli *et al.*, 2011). Some recent reports have indicated that TiO_2NPs may potentially be detrimental to the environment and humans due to the increased presence of these NPs in consumer products, and could potentially be consumed directly (Chen *et al.*, 2009).

The *in vivo* effects of TiO_2NPs exposure have indicated acute toxicity in several organs such as lung, kidney and liver, and as well as immune toxicity (Chen *et al.*, 2009, Liu *et al.*, 2010, Czajka *et al.*, 2015, Liu *et al.*, 2013, Chen *et al.*, 2014). However, there are conflicting reports where some indicated that TiO_2NPs did not significantly absorb in the liver, kidneys, and small intestine (Cho *et al.*, 2013, Janer *et al.*, 2014). The main route of excretion was found to be via the renal pathway (Xie *et al.*, 2011). Similarly, there are also contradictory reports regarding *in vitro* toxicity and TiO_2NPs exposure. Some report genotoxicity and cytotoxicity in some cell lines, where others do not (Trouiller *et al.*, 2009, Chen *et al.*, 2016, Saquib *et al.*, 2012, Alinovi *et al.*, 2015, Huerta-García *et al.*, 2014, Magdolenova *et al.*, 2012, Shukla *et al.*, 2011, Chen *et al.*, 2014). The rationale for these inconsistent reports is the cell types, animal models, dose and size of the nanoparticle

used. An extensive review conducted on the *in vitro* toxicology of TiO_2NPs on mammalian cells indicated that there were limited studies on the effects of these NPs on intestinal cells. The review also noted that the NPs could cross the intestinal epithelium layer via transcytosis, without damaging the cells integrity but only resulting in subtle effects (Iavicoli *et al.*, 2011).

To our knowledge, limited studies have assessed how these NPs react when in physiological media containing serum constituents. It is thought to affect aggregation and stability of the NP. A study conducted by Fatisson *et al*. (2012) noted that engineered NPs (ENPs) were only moderately affected by cell culture media, however, in the presence of serum, the NPs were destabilized after 24 hrs (Fatisson *et al.*, 2012). Another study noted an increase in NP size when in cell culture media and these factors can attribute to the conflicting reports of TiO_2NP toxicity *in vivo* and *in vitro* (Dinesh *et al.*, 2017). This study aimed to evaluate the effects of physiological media on TiO_2NP hydrodynamic size and zeta potential. In addition, elucidate the effects of these characterized TiO_2NPs on cell viability, cell stress and inflammatory biomarkers. Due to the many applications of TiO_2NPs, they could potentially have anti-angiogenic properties. Thus, an angiogenesis proteome profile analysis was conducted to identify potential biomarkers.

4.2 Materials and Methods

4.2.1 Nanoparticle Characterization

The aeroxide P25 TiO_2NPs were provided by the manufacturer (Evonik Degussa Corporation) and the manufacturers reported a spherical shape and an average primary particle size of 21 nm. This was confirmed by further testing (Romanello and de Cortalezzi, 2013). It is also noted that it is a hydrophilic fumed TiO_2 mixture of rutile and anatase forms. These NPs were subsequently further characterized and were noted to form aggregates of 200-300 nm in aqueous solutions, at pH values

where agglomeration is favourable (Romanello and de Cortalezzi, 2013). The TiO_2NPs were subsequently characterized in physiological media over a 2-week period to determine whether their characteristics would alter. A 10 mg/ml TiO_2NPs stock solution in water was prepared and suspended in solution by short sonic bursts using (QSonica, LLC. Misonixsonicators, XL-200 Series) for a total of 5 minutes on ice. Thereafter, the TiO_2NPs were placed in various physiological medias (pH 7): 150 mM sodium chloride (NaCl) (Sigma-aldrich); 1 x phosphate buffered saline (PBS) (Sigma-aldrich); Incomplete Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-aldrich) containing 0.1 % glutamax (Sigma-aldrich), 0.1 % antibiotic/antimycotic solution (Sigma-aldrich) and 0.05 % gentamicin (Sigma-aldrich) ; and complete DMEM media, containing the same constituents as incomplete media but containing additional 10 % heat inactivated foetal bovine serum (FBS) (Sigma-aldrich), to yield a final concentration of 10 μg/ml TiO_2NPs. The TiO_2NPs in the various medias were then incubated at 37 °C for 0 hr, 24 hrs, 7 and 14 days respectively. After the various incubation periods, the media was centrifuged at 10 000 rpms (Eppendorf Centrifuge 5810 R), and the NP pellets were then washed and resuspended in dH_20. Thereafter, disposable folded capillary cells (Malvern Pananlytical) were used to determine the hydrodynamic size and zeta potential of the nanoparticles with a ZetaSizer Nano ZS (Malvern Instruments).

4.2.2 Nanoparticle Preparation for Cell Culture

A 10 mg/ml stock of TiO_2NPs was prepared in distilled water. The NPs were sonicated (QSonica, LLC. Misonixsonicators, XL-200 Series) on ice for short burst for approximately 5 minutes. Nanoparticles were freshly prepared prior to each experiment.

4.2.3 Cell Culture

The human colorectal adenocarcinoma (Caco-2) epithelial cell line was obtained from the American Type Culture Collection (ATCC HTB-37). Standard tissue culture conditions were used to maintain the cells in complete medium. Cells were sub-cultured approximately every 3-4 days using 0.05 % trypsin ethylenediaminetetraacetic acid (EDTA) (Gibco). Caco-2 cells were seeded at a density of 4 x 10^3 cells/ml in 24 well tissue culture treated plates (Nunc) and were approximately 60 % confluent before nanoparticle treatment. Cells were exposed for 48 hrs to 0-500 μg/ml TiO_2NPs, respectively. Thereafter, supernatants removed and cell viability assessed. Supernatants were centrifuged at 12.1 rcfs for 1 min (MiniStar Plus Super Mini Centrifuge) before evaluating innate inflammatory biomarkers and performing an angiogenesis proteome profile. Experiments were repeated in triplicate. The cells were harvested after NP exposure using lysis buffer solution (1 X PBS, 0.1% tween and 200μl protease inhibitor). The cells were then scraped with a scrapper after which cells were then sonicated using (QSonica, LLC. Misonixsonicators, XL-200 Series) on ice for short burst for 20secs and centrifuged at 12 100rcf for 1min. Protein concentration of the cell homogenate was quantified using Bradford reagent. The cell homogenates at 300 μg/ml protein were used to evaluate cell stress biomarkers potentially induced by the nanoparticle.

4.2.3.1 Cell viability assay

Cell viability was monitored using the sodium 3'[1-[(phenylamino)-carbony]-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene-sulfonic acid hydrate) (XTT) assay (Sigma-Aldrich). The assay monitors the conversion of the XTT tetrazolium salt to a soluble formazan salt in metabolically active cells (Aslantürk, 2018). Therefore, an increase in the conversion to formazan is directly proportional to cell viability. Supernatants were removed and cells washed with PBS to remove

any excess NP that may interfere with the assay. A 1:50 ratio of XTT coupling reagent to XTT labelling reagent was prepared. This mixture was further diluted in complete medium to yield a final ration of 1:3. Plates were immediately read at 450 nm (FLUOstar Omega, BMG Labtech) after the addition of XTT. Plates were then incubated at 37 °C for 1 hr after which an additional reading was made. The change of absorbance over time was calculated and the percentage viability analysed.

4.2.4 NO Assay

The amount of nitrite produced by the Caco-2 cells exposed to the respective nanoparticle concentrations was assessed in the cell culture supernatant as an indication of NO production. This assay is based on the Griess reaction (Granger *et al.*, 1996). The amount of nitrite produced by the cells was measured against the nitrite standard range (Sigma-Aldrich) (0-100 μM). A 1:1 of culture supernatant or standard was mixed with the Griess reagent (1:1 of 1% sulphanilamide and 0.1% naphtylethlemidimine-dihydrochloride in 2.5 % sulphuric acid (all obtained from Sigma-Aldrich). The plate was subsequently read at 540 nm (FLUOstar Omega, BMG Labtech) and the amount of nitrite produced by the cells quantified.

4.2.5 Innate Inflammatory Biomarkers IL-6 And IL-8

The double antibody sandwich enzyme linked immuno-sorbent assays (DAS-ELISA) were run according to the manufacturer's instructions. Both the IL-6 (Invitrogen) and IL-8 (R & D Systems) assays were run using undiluted supernatants.

4.2.6 Protein Quantification

Protein samples were quantified using the Bradford assay (Bio-Rad). The assay was performed as per the manufacturer's instructions.

4.2.7 Cell Stress Biomarkers

The production of cell stress biomarkers induced by exposing the cells to the nanoparticles were assessed by performing SOD, Phospho-HSP27 and HSP70 ELISAs (all purchased from R & D Systems). The experiments were all performed as per the manufacturer's instructions. All samples were run at a cell homogenate protein concentration of 300 μg/ml. The exposure concentration range selected was 0 and 31.25-500 μg/ml TiO_2NPs.

4.2.8 Angiogenesis Proteome Profile

The angiogenesis proteome profiler (R & D Systems) contained 4 membranes, each spotted in duplicate with 55 different angiogenesis antibodies. The assay was performed as per the manufacturer's instructions. The concentrations selected were 0 and 100 μg/ml TiO_2NPs. The concentration selected represented the control and the no observed adverse effect level (NOAEL) (100 μg/ml TiO_2NPs). The membranes were subjected to an ultra-sensitive chromogenic 3,3',5,5'-Tetramethylbenzidine (TMB) substrate (Thermofisher) to show sample-antibody complexes labelled with streptavidin-horse radish peroxidase (HRP). Pictures were taken of the membranes after substrate exposure.

4.2.9 Statistical Analysis

All experiments were performed in triplicate and the data calculated using Microsoft Excel. The data is represented as mean ± standard deviation (SD). A one-way analysis of variance (ANOVA)

using SigmaPlot 12.0 (Systat Software Inc.) was used to determine statistical differences, with $p < 0.01$ deemed significant.

4.3 Results

4.3.1 Nanoparticle Characterization in Physiological Media

The NPs in the presence of a 150 mM NaCl solution indicated a steady increase in size across the period assessed. The hydrodynamic size over the 2 week incubation period significantly increased ($p<0.001$) from 297 ± 43.55 to 647.53 ± 56.18 nm (14 days) (Table 1). However, the zeta potential for the same period exhibited a notable decrease in NP charge at 14 days. It decreased from an initial -23.3 ± 0.66 (0 hr) to -12.43 ± 0.45 mV (14 days) (Table 2). When the TiO_2NPs were incubated in the presence of PBS, it was noted that size did not change across the 24 hr period. It was also notable that the size of the NPs at 0 hrs were the same for PBS and DMEM, in the absence and presence of serum (787.33 ± 65.24 nm). Subsequently, TiO_2NPs in PBS for 14 days exhibited a significant increase ($p<0.014$) in size from 787.33 ± 65.24 (0 hr) to 1055.8 ± 86.95 nm (14 days) (Table 1). Nonetheless, the zeta potential did not reflect this. The surface charge notably increased ($p<0.001$) from -26.03 ± 2.29 to -16.05 ± 2.77 mV over the 24 hrs period. It then decreased to -27.87 ± 1.97 mV at day 7 and then another significant increase ($P<0.001$) at day 14 with a charge of -16.48 ± 1.29 mV. Thus, 0 hrs and 7 days had similar surface charges and 24 hrs and day 14 have comparable zeta potentials (Table 2). The TiO_2NPs in the presence of DMEM, without serum displayed a notable increase ($p<0.014$) in size from 0 hr to 7 days, with sizes ranging from 787.33 ± 65.24 to 1442.35 ± 491.99 nm respectively (Table 1). The surface charge of the NPs was stable after 24 hrs. The zeta potential increased ($p<0.014$) from -14.1 ± 0.87 to -8.29 ± 1.9 mV between 0 hr and day 7 respectively (Table 2). TiO_2NPs exposed to DMEM in the presence of serum showed a similar trend to the NPs in 150 mM NaCl, where there was an increase in particle size

over time. The size increased ($p<0.003$) from 787.33 ± 65.24 to 1121.26 ± 216.6 nm at day 7 and another subsequent increase ($p<0.003$) to 1821.8 ± 450.9 nm after 14 days (Table 1). Surface charge remained consistent and stable after exposing the NPs to complete DMEM for 7 days even though particle size increased. However, zeta potential of the TiO_2NPs notably increased ($p<0.014$) at day 14 (-16.45 ± 1.1 mV) compared to incubation period 0 hr to 7 days (Table 2).

Table 4. 1: Hydrodynamic size (nm) of TiO2NPs after being exposed to various physiological medias over a 2-weeks period.

MEDIA	Number of days			
	0	**1**	**7**	**14**
150 Mm NaCl	297.33±43.55	431.87±59.81	347.94±72.48	647.53±56.18*
1 X PBS	787.33±65.24	787.33±65.24	915.3±113.18	1055.8±86.95****
DMEM	787.33±65.24	962±140.63	1442.35±491.99****	685.04±121.19
DMEM (10% FBS)	787.33±65.24	911.67±75.06	1121.26±216.26	1821.8±450.9**

Data expressed as mean ± SD. Significance demarcated by (*) indicating significant difference of $p<0.001$, (**) $p<0.003$, and (****) $p<0.014$ compared to the relative 0 hr control.

Table 4. 2: Zeta Potential (mV) of TiO2NPs after being exposed to various physiological medias over a 2-weeks period

MEDIA	Number of days			
	0	**1**	**7**	**14**
150 Mm NaCl	-23.3±0.66	-23.82±1.49	-20.06±5.84	-12.43±0.45***
1 X PBS	-26.03±2.29	-16.05±2.77	-27.87±1.97	-16.48±1.29*
DMEM	-14.1±0.87	-13.76±1.66	-8.29±1.9****	-12.62±2.41

DMEM (10% FBS)	13.03±0.85	-13.4±1.35	-13.7±1.18	-16.45±1.1****

Data expressed as mean ± SD. Significance demarcated by (*) indicating significant difference of $p<0.001$, (**) $p<0.003$, (***) $p<0.007$ and (****) $p<0.014$ compared to the relative 0 hr control.

4.3.2 Cell Viability

The XTT assay used to evaluate potential cytotoxicity of TiO_2NPs, indicated no effect on viability after the cells were exposed to the NP range assessed in this study (Fig. 1).

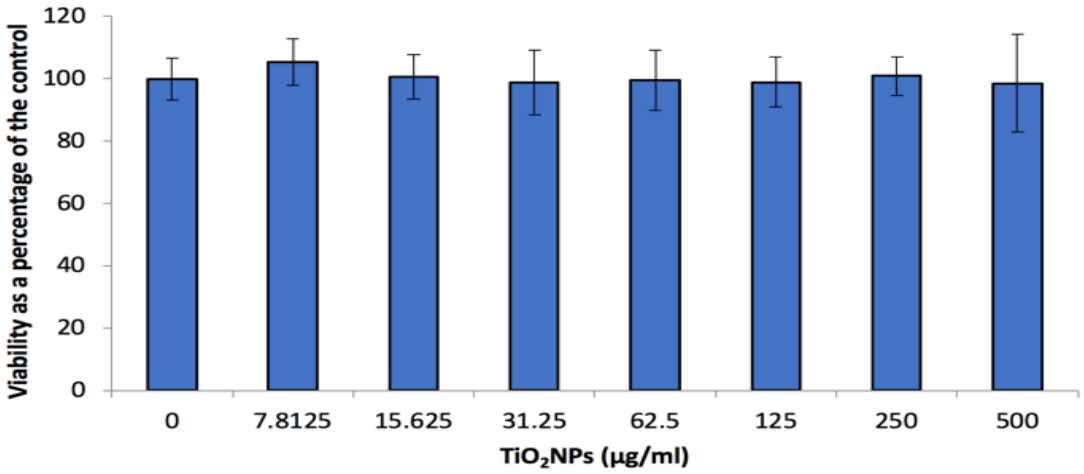

Figure 4.1: Caco-2 cell viability after 48 hr exposure to a range of TiO2NP concentrations. Data represented as mean ± SD.

4.3.3 Inflammatory Biomarkers

The exposure of Caco-2 cells to TiO_2NPs for 48 hrs resulted in a significant increase ($p < 0.001$) in NO released from the cells at concentrations ≥ 62.5 µg/ml TiO_2NPs (Fig. 2). However, the increase would not be deemed significant in a physiological system.

Contrary to the NO data, the other inflammatory markers (i.e. IL-6 and IL-8) secretion levels from the cells remained unaffected after a 48 hr exposure period (Fig. 3 and 4). IL-6 levels secreted by the cells were approximately 3 times higher compared to IL-8 secretion.

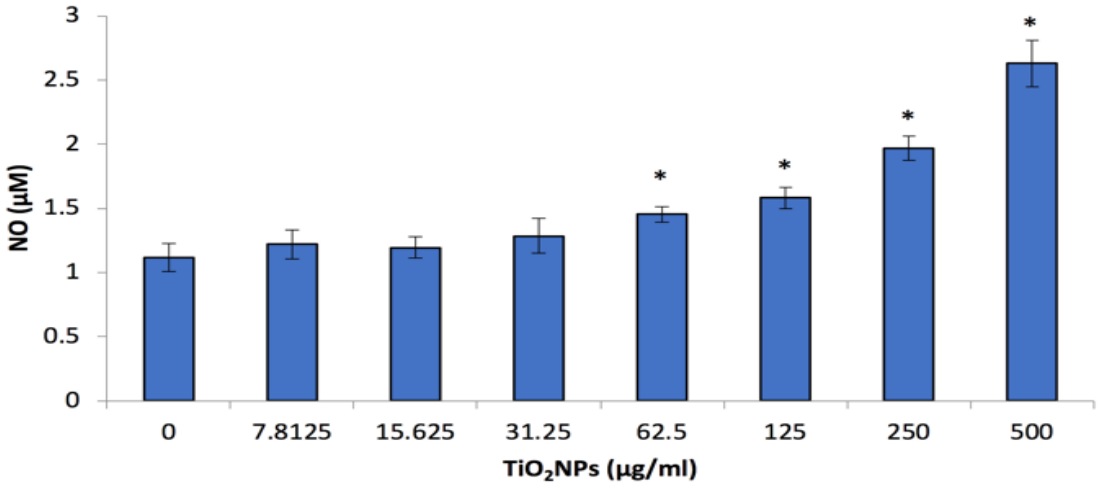

Figure 4.2: Caco-2 Nitric oxide (NO) after 48 hr exposure to a range of TiO2NP concentrations. Data represented as mean ± SD. Significance demarcated by *, indicating a significant difference of p<0.001.

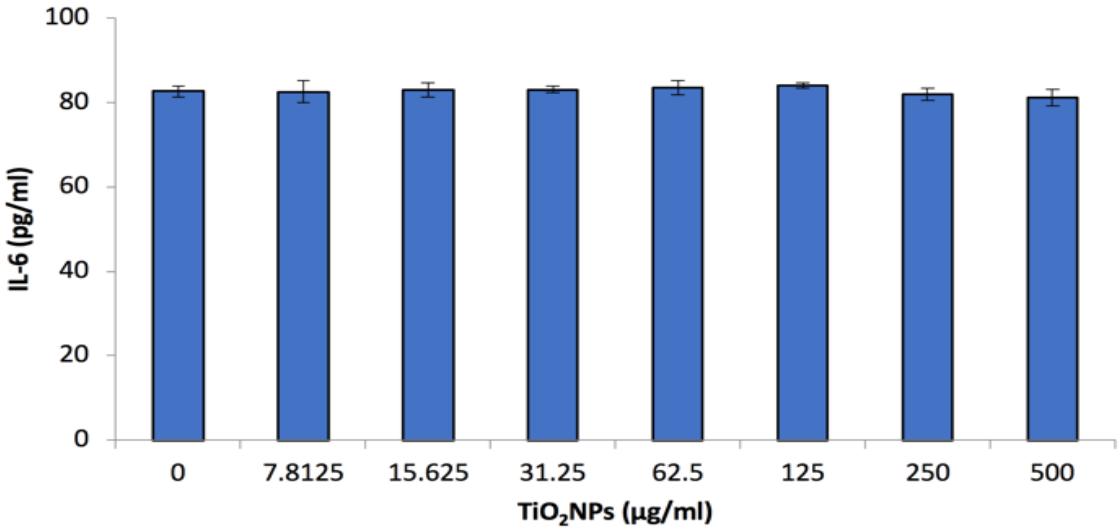

Figure 4.3: Caco-2 Interleukin 6 (IL-6) after 48 hr exposure to a range of TiO2NP concentrations. Data represented as mean ± SD. Significance demarcated by *, indicating a significant difference of p<0.001.

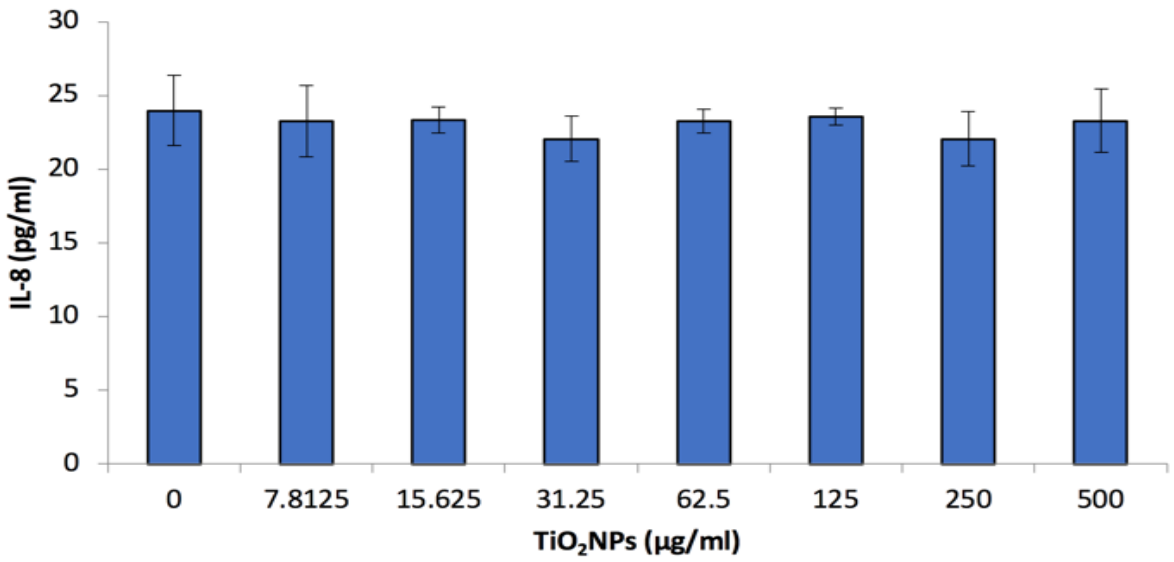

Figure 4.4: Caco-2 Interleukin 8 (IL-8) after 48 hr exposure to a range of TiO2NP concentrations. Data represented as mean ± SD. Significance demarcated by *, indicating a significant difference of p<0.001 and (**) p<0.006.

4.3.4 Cell stress Biomarkers

The cell stress biomarkers, phospho-HSP-27 and HSP-70, notably increased ($p<0.001$) at concentrations ≥ 31.25 µg/ml TiO_2NPs. Phospho-HSP-27 levels doubled at 31.25 µg/ml TiO_2NPs and tripled at 125 µg/ml TiO_2NPs, compared to the 0 µg/ml TiO_2NP control, and then increased to 7-fold at 250 and 500 µg/ml TiO_2NPs (Fig. 5). However, with regards to the HSP-70 levels, there was a notable dose dependent increase in the secretion of this biomarker (Fig. 6). HSP-70 levels were also noted to be a 1000 x higher in comparison to the phospho-HSP-27 levels.

Dissimilar to the other cell stress biomarkers, the production of SOD-2 was only considerably upregulated ($p<0.001$) at 250 µg/ml TiO_2NPs, with an approximate 1.8-fold increase in its production in comparison to the control (Fig. 7).

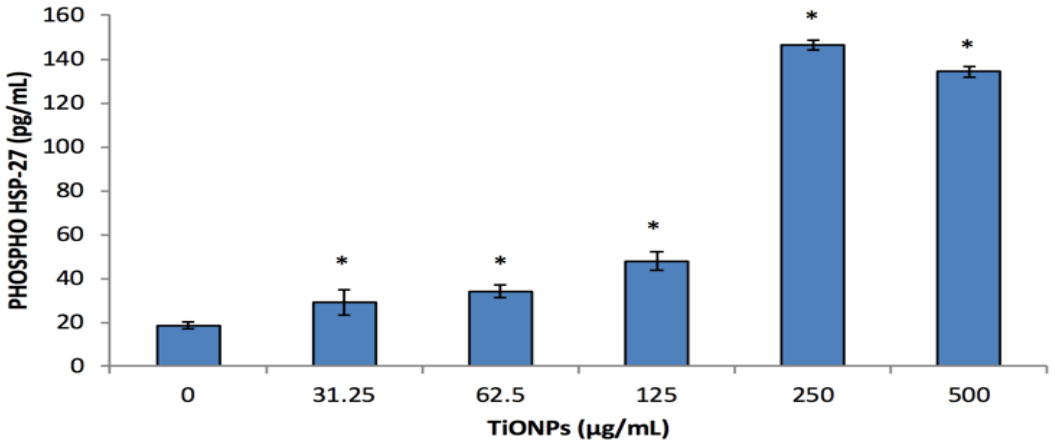

Figure 4.5: Phospho-HSP-27 cell stress biomarkers after exposing Caco-2 cells to various TiO2NPs, concentrations for 48 hrs. Data represented as mean ± SD. Significance demarcated by (*), indicating a significant difference of p<0.001.

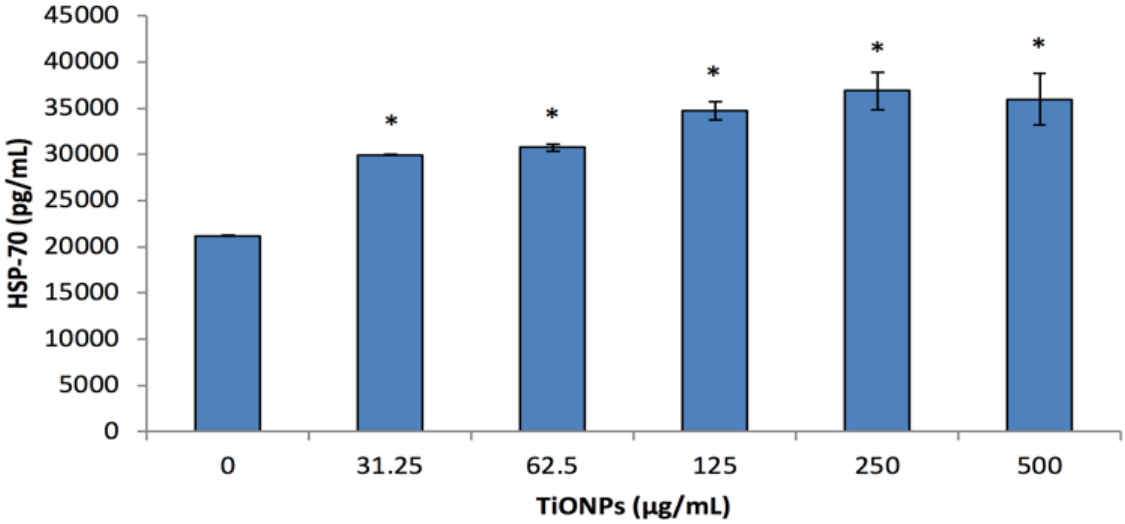

Figure 4.6: HSP-70 cell stress biomarkers after exposing Caco-2 cells to various TiO2NPs, concentrations for 48 hrs. Data represented as mean ± SD. Significance demarcated by (*), indicating a significant difference of p<0.001.

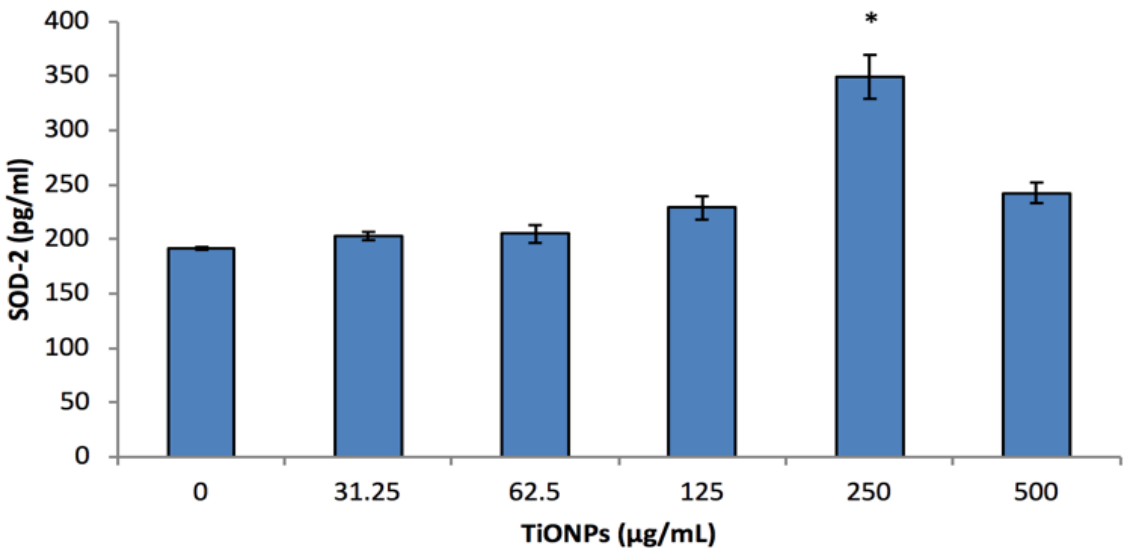

Figure 4.7: SOD-2 cell stress biomarkers after exposing Caco-2 cells to various TiO2NP concentrations for 48 hrs. Data represented as mean ± SD. Significance demarcated by (*), indicating a significant difference of p<0.001.

4.3.5 Angiogenesis Proteome Profile

The control (0 µg/ml TiO_2NPs) and NOAEL (100 µg/ml TiO_2NPs) supernatants assayed for potential angiogenic biomarkers revealed the same proteins, except persephin which was only evident on the control membrane (Fig. 8). Based on the intensity of the dot, the proteins dipeptidyl peptidase IV (DPPIV) and endostatin was more prominent on the NOAEL membrane (Fig. 8b). However, platelet derived growth factor AA (PDGF-AA) and angiopoietin-2 was more noticeable to cells exposed to the control concentration (Fig. 8a).

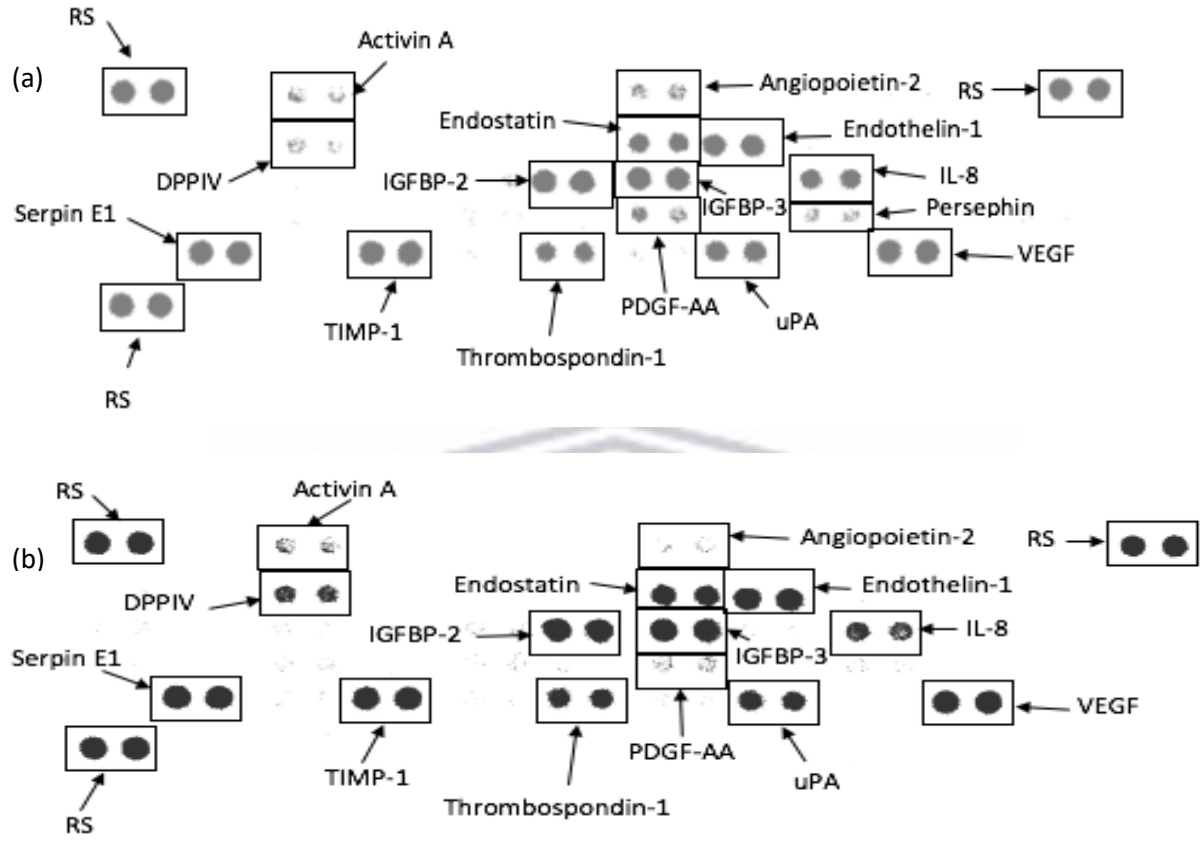

Figure 4.8: Angiogenesis proteome profile using culture supernatants of Caco-2 cells exposed to (a) control (0 □g/ml TiO2NPs) and (b) NOAEL concentration (100 □g/ml TiO2NPs) for 48 hrs. *RS-Reference spot

4.4 Discussion

The initial size of TiO_2NPs in complete DMEM was 787.33 ± 65.24 nm (Table 1) which was similar to what was found by another study where the initial hydrodynamic size of TiO_2NP in complete DMEM was 843 ± 69 nm (Ji *et al.*, 2010). However, their zeta potential was -7.4 ± 2.5 mV which is much higher compared to the data generated in this study (-13.03 ± 0.85 mV) (Table 2). It is also proposed by other studies that probe ultrasonication does not easily break down agglomerates but in fact endorses agglomeration due to the enhanced particle-particle interaction

(Ji *et al.*, 2010, Jiang *et al.*, 2009). In conjunction with probe sonication the high ionic strength present in the culture media can also promote agglomeration (Ji *et al.*, 2010). This was evident as after 24 hrs the particle size was 911.67 ± 75.06 nm and 1121.26 ± 216.26 nm after a 7-days incubation period, respectively. This trend was consistent with all the medias in which TiO_2NPs was evaluated and proposes that the NPs were stable after 48 hrs, the time frame used in this study. The zeta potential remained mostly stable after size increase in all medias used (Table 2). A study where different reducing agents, namely glycine (gly-TiO_2NP) and L-alanine (ala-TiO_2NP), was used evaluated how complete DMEM impacted the size and surface charge of TiO_2NP. The authors noted an increase in size when placed into cell culture media for 24 hrs, with hydrodynamic size being 1842.6 ± 263 nm and 1296 ± 662 nm for gly-TiO_2NP and ala-TiO_2NP, after an initial size of 85.5 and 72.8 nm respectively (Dinesh *et al.*, 2017). The surface charge after being exposed to complete DMEM with gly-TiO_2NP and ala-TiO_2NP was -7.9 ± 0.4 and -8.2 ±. 0.2 mV respectively. These results imply that the reducing agent does not impact size once in culture medium as can be seen when comparing the data found in this study.

The TiO_2NPs were not cytotoxic to the cells at the assessed concentrations (Fig.1). This could be attributed to the increase in NP size (Table 1), as this could impact cellular uptake. This has been investigated by numerous studies, looking at various types of nanoparticles and cells. The authors have ascertained that an increase in particle size would impact cellular uptake and in turn decrease the level of toxicity (Park *et al.*, 2011, Feng *et al.*, 2018, Saquib *et al.*, 2012, Truong *et al.*, 2019, Tarantini *et al.*, 2015). Several questions remain as a limited number of nanoparticles have been tested, as well as difficulty comparing data reported due to disparities in surface chemistry, purity and size uniformity of the nanoparticles used (Truong *et al.*, 2019, Khan *et al.*, 2019, Alkilany and

Murphy, 2010). Uptake of these NPs require further investigation to corroborate the proposed mechanism for the lack of cytotoxicity. However, other studies have found similar results with regards to TiO_2NPs not being cytotoxic to human colon carcinoma cells (Zhang and Sun, 2004, Koeneman *et al.*, 2010, Shi *et al.*, 2013, Peters *et al.*, 2004). The lack of cytotoxicity could also be attributed to the induction of cell stress biomarkers (Fig. 5,6 and 7). As phospho-HSP-27, HSP-70 and SOD-2 are produced to aid in protecting the cells from damage such as hypoxia and cytotoxic exposure (Asakura and Kitahora, 2018, Younus, 2018, Miller and Fort, 2018, Lindquist and Craig, 1988). These systems regulate reactive oxygen species (ROS) formation and protect biological systems from ROS induced oxidative damage (Bigagli and Lodovici, 2019). In contrast to what we have found, other studies have found that TiO_2NPs induce cyto-and genotoxicity (Trouiller *et al.*, 2009, Chen *et al.*, 2014, Saquib *et al.*, 2012). This disparity can be attributed to NP size, cell type and dosage.

The TiO_2NPs were found not to affect the production of inflammatory cytokines, IL-6 and IL-8 (Fig. 3 and 4). The IL-6 data generated agrees with other studies that found that Caco-2 cells exposed to TiO_2NPs did not affect the expression levels of this cytokine (Krüger *et al.*, 2014, De Angelis *et al.*, 2013). The IL-8 data is different from other studies that investigated the effected TiO_2NPs on Caco-2 cells IL-8 expression levels. Kruger et al. (2014) elucidated that TiO_2NPs activates IL-8 and IL-8-related pathways (Krüger *et al.*, 2014). This was confounded by other studies, which indicated that exposure of human endothelial cells to TiO_2NPs resulted in an increase of IL-8 levels after a 24 hr exposure period (Sharma, 2009, De Angelis *et al.*, 2013). This trend of increasing IL-8 levels was also evident when Caco-2 cells were exposed to nanosilica (Tarantini *et al.*, 2015). An *in vivo* study looking at inflammatory cytokines such as IL-6 and IL-8

secretion from the small intestine upon TiO_2NPs exposure found no change in their expression levels, which is in agreeance with our data. However, they did find an increase in other inflammatory cytokines such as IL-4, IL-12, TNF-α and IFNγ (Nogueira *et al.*, 2012).

The angiogenesis proteome profile revealed very little differences between the control and 100 μg/ml TiO_2NPs (Fig. 8). There was an inhibition of the protein and pro-angiogenic molecule, persephin upon exposure to 100 μg/ml TiO_2NPs. However, the protein, DPPIV was more prominent in the NOAEL exposure compared to the anti-angiogenic molecule, endostatin which was also upregulated at the same exposure concentration. The other pro-angiogenic proteins, PDGF-AA and angiopoietin-2 were higher in the control compared to NOAEL. The inhibition of persephin, and suppression PDGF-AA and angiopoietin-2 in the NOAEL exposure indicates the potential anti-angiogenic effects of TiO_2NPs. As the activation of persephin, angiopoetin-2 and PDGF-AA triggers the angiogenic process (Gurunathan *et al.*, 2009, Linares *et al.*, 2014, Risau *et al.*, 1992, Jo *et al.*, 2014). This is further supported by the upregulation of endostatin in the 100 μg/ml TiO_2NPs exposure as this is produced endogenously and inhibits angiogenesis (Walia *et al.*, 2015). The upregulation of DPPIV in the NOAEL exposure is not significant as this protein is constitutively expressed on endothelial cells (Kaneda *et al.*, 2011, Kitlinska *et al.*, 2004). These results are supported by Jo et al. (2014) who found TiO_2NPs to be anti-angiogenic *in vitro* as there was a reduction in vascular endothelial growth factor (VEGF) (Jo *et al.*, 2014). Identifying these potential biomarkers are important as angiogenesis promotes the development of new blood vessels from existing ones (Walia *et al.*, 2015). This has a direct relation to cancer as tumour growth and metastasis relies on the initiation of angiogenesis and lymphangiogenesis which can

be triggered by chemical signals (Nishida *et al.*, 2006). However, this requires further in-depth investigation as the size and exposure period of the NP might impact these factors.

4.5 Conclusions

Caco-2 cells express anti-angiogenic markers (i.e. persephin, angiopoetin-2, PDGF-AA and endostatin) upon exposure to 100 μg/ml TiO_2NPs and could be a potential candidate for use in cancer therapy. This, with the lack of inflammatory cytokine production and cytotoxicity makes this NP a good candidate. However, further comprehensive investigations are required as the exposure period, dosage and size of the NP could greatly impact the above-mentioned factors.

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CHAPTER FIVE: UPTAKE MECHANISMS OF SILVER NANOPARTICLES BY HUMAN INTESTINAL CACO-2 CELLS ON TOXICITY AND INFLAMMATION.

ABSTRACT

Living organisms are exposed to nanoparticles intentionally and unintentionally. Therefore, concerns have been raised regarding the possible adverse effects of nanoparticles on some cellular processes in humans. Despite their numerous applications and potential biomedical benefits, especially drug delivery, nanoparticles must be subjected to rigorous toxicology testing to prevent potential adverse risk to the environment and humans. Uptake mechanisms may also influence sensitive biological processes, thus elucidating the interactions of nanoparticles with biological processes as to whether such interactions might cause toxicity or modulate inflammation is imperative. Different uptake mechanisms that could possibly mediate toxicity and modulate inflammatory immune biomarkers were elucidated using specific pharmaceutical inhibitors (amiloride hydrochloride. ammonium chloride, chlorpromazine, and nystatin) for specific pathways such as clathrin, caveolae, macropinocytosis and pH acidification using Caco-2 epithelial cell lines. Cells pretreated for 1 hour with the previously mentioned uptake inhibitors, after which the cells were exposed to AgNPs for 48 hours. Cell viability and inflammatory biomarkers were then assayed. AgNPs were cytotoxic and modulated the inflammatory biomarkers (nitric oxide, interleukin-6, and macrophage migration inhibitory factor) at concentrations ≥ 12.5 µg/ml. However, the inhibitors did not significantly mitigate toxicity nor alter the inflammation caused by the AgNPs. Therefore, for AgNPs to be utilized to their fullest potential, it is important to evaluate their uptake with respect to toxicity and inflammation, which may also be used to predict any antagonistic cellular responses.

5.1 Introduction

The current rapid increase in production, usage and disposal of silver nanoparticles (AgNPs) is of great concern (Tortella *et al.*, 2019). This is because of various reported health risks and possible hazardous effects posed by AgNPs to the biota, especially human life. However, the benefits of AgNPs are well recognized in various fields, due to their antimicrobial and metallic properties. AgNPs have become an indispensable material employed in everyday usage in home appliances, kitchen utensils, food preservatives, pharmaceuticals, water and air remediation, microbial growth control, catheters, surgical instruments, biomedical applications, such as biosensors, biomarkers for cellular imaging, cancer therapy, DNA and drug delivery systems (Zivic *et al.*, 2018, Miethling-Graff *et al.*, 2014, Tortella *et al.*, 2019).

The growing increase in AgNPs demand and inevitable exposure may alter biological system by their interaction with cellular processes, intracellular organelles and biological fluids leading to possible pathological and physiological disorders (Wang *et al.*, 2012, Pourmand and Abdollahi, 2012). Recent studies have reported various toxicity mechanisms underlying the harmful effects of AgNPs such as mitochondrial dysfunction, cell cycle arrest, necrosis, apoptosis, inflammation, oxidative stress, membrane damage, cytotoxicity, genotoxicity and carcinogenicity (Akter *et al.*, 2018, Rodriguez-Garraus *et al.*, 2020, Antony *et al.*, 2015). Prolonged exposure and application may also result in adverse conditions such as argyria and argyrosis, a permanent bluish-grey discolouration of the skin and eyes respectively (Al-Doaiss *et al.*, 2020). The toxicity mechanisms may be dependent on the dosage, exposure time, and physicochemical parameters of NPs as well as the cell line used (Bannunah *et al.*, 2014, Zhang *et al.*, 2016).

The uptake mechanisms from various studies revealed that NPs enters the cells through both passive and active mechanisms (Gitrowski *et al.*, 2014, Rothen-Rutishauser *et al.*, 2014). Passive uptake occurs in cells that lacks endocytotic properties and is studied using red blood cells (Kuhn *et al.*, 2014b). While active mechanisms occur primarily in other cell types and are known as receptor mediated endocytosis. Endocytosis may be categorized into two main mechanisms, phagocytosis which are involved in the uptake of larger particles (> 500 nm) and pinocytosis which is responsible for the uptake of smaller molecules, and further subdivided into clathrin-dependent endocytosis, caveolae-dependent endocytosis, micropinocytosis and clathrin/caveolae independent endocytosis (Kuhn *et al.*, 2014b, Vetten and Gulumian, 2019). Scientific evidence revealed that functional physico-chemical characteristics of nanoparticles such as shape, size, surface area, charge and agglomeration are involved in their uptake and toxicity (Zhao and Stenzel, 2018). Gitrowski *et al* (2014) investigated the effects of different crystal structures of TiO_2NPs in colon cancer intestinal epithelial cell line Caco-2, the result showed that energy mediated vesicular processes is involved in the uptake of NPs with possible disturbances in electrolyte homeostasis of the cell (Gitrowski *et al.*, 2014). Furthermore, Vetten and Gulumian (2019) established that size, surface coating and functional properties of gold nanoparticles (AuNPs) are involved in clathrin and caveolae–mediated pathway dependent intracellular uptake in human bronchial epithelial cell line BEAS-2B (Vetten and Gulumian, 2019). Similarly, Wu et al, (2019) varied the diameter of AgNPs as it is an important factor in cellular uptake, transportation and its accumulation within B 16 mouse melanoma cell line. These findings indicate that size and coating are important in the clathrin-mediated endocytosis pathway efficiency of AgNPs cellular uptake (Wu *et al.*, 2019).

The elucidation of uptake mechanisms is important in understanding safety and the toxicological effects of NPs in general. There is a lack of published information on the role of different pathways

responsible for AgNPs uptake and their possible role in toxicity, modulation of immune biomarkers and inflammation. The pathway mechanisms responsible for AgNPs uptake can be studied using specific pharmaceutical inhibitors (amiloride hydrochloride. ammonium chloride, chlorpromazine, and nystatin) for pathways such as clathrin, caveolae, micropinocytosis and pH acidification and as such this research was undertaken.

5.2 Methodology

5.2.1 Characterization of AgNPs

Commercially available AgNPs (Sigma-aldrich Cat No. 576832) were supplied as a black powder with a purity of 99 % and a specific surface area of 5.0 m^2/g. The AgNPs nanoparticle size distribution and shape was characterized by transmission electron microscopy (TEM) to determine the morphology and size of the AgNPs. Samples were imaged with Gatan Microscopic software. Scanning electron microscope (SEM) with energy dispersive x-ray was used to confirm elemental silver (Ag) shape and size. X-ray diffraction (XRD) was done by Philip expert pro MPD X-ray diffractometer using Cu-k radiation at 40 kv and 40 mA to determine the structure of the AgNPs as previously characterized by Walters et al. (2014) (Walters *et al.,* 2014). The AgNPs were shown to be spherical in shape and formed loosely packed aggregates via scanning and SEM and TEM, and that the NPs were crystalline in nature via XRD. It was also found that when the NPs were in water, approximately 35 % were between 20- 40 nm and 15 % were between 70-1000 nm. It was also determined that in water, the zeta potential was -18.8 mV.

5.2.2 Nanoparticle Preparation for Cell Culture

A 5 mg/ml stock concentration of AgNPs were suspended in distilled water. The NPs were sonicated (QSonica, LLC. Misonixsonicators, XL-200 Series) on ice in short bursts for a total of 5 minutes. Stocks were freshly prepared prior to each cell culture assay.

5.2.3 Cell Culture

The human colorectal adenocarcinoma (Caco-2) epithelial cell line was obtained from the American Type Culture Collection (ATCC HTB-37). Standard tissue culture conditions were used to maintain the cells in complete Dulbecco's Modified Eagle's Medium (DMEM)(Lonza). The medium was supplemented with 10 % heat inactivated fetal bovine serum (FBS)(Biowest), 1 % glutamax (Thermofisher), 0.5 % gentamicin (Sigma-Aldrich), and 1 % antibiotic and antimycotic mixture (Sigma-adrich). Cells were sub-cultured approximately every 3-4 days using 0.05 % trypsin ethylenediaminetetraacetic acid (EDTA) from Gibco. Caco-2 cells were seeded at a density of 4 x 10^3 cells/ml in 96 well tissue culture treated plates (Nunc) and were approximately 60 % confluent before nanoparticle treatment.

5.2.4 Inhibitor Assay

Preliminary studies were done to ascertain efficiency and optimum concentration and exposure time of the NPs and inhibitors. Cell viability was assessed due to previous reports on toxicity of these inhibitors (Vetten and Gulumian, 2019), data not shown. Cells were then pretreated with the different endocytic pathway inhibitors; chlorpromazine hydrochloride (CPZ, Sigma Aldrich), nystatin (Nystatin, Sigma Aldrich), amiloride hydrochloride (AMH, Sigma Aldrich) and ammonium hydrochloride (NH_4Cl, May and Baker, limited Dagenam, England) for 1 hour at 37°C

as shown in Table 1 below. Followed by exposure to AgNPs at different concentrations (0, 12.5, 25 μg/ml) for 48 hours.

Table 5.1: Inhibitors of endocytosis. The final concentration and the targeted endocytosis pathways of the endocytosis inhibitors used in this study

INHIBITORS	ENDOCYTOSIS PATHWAY	CONCENTRATION
CHLOPROMAZINE	Clathrin mediated endocytosis	5 μg/ml
NYSTATIN	Caveolae mediated endocytosis	20 μM
AMILORIDE	Macropinocytosis	10 μg/ml
NH_4Cl	pH acidification	10 mM

5.2.5 Cell Viability Assay

Cell viability was measured using the sodium 3'[1-[(phenylamino)-carbony]-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene-sulfonic acid hydrate) (XTT) assay (Sigma-Aldrich). The assay monitors the conversion in the XTT tetrazolium salt to a soluble formazan salt in metabolically live cells. Consequently, an increase in the formation of formazan is directly proportional to viable cells (Aslantürk, 2018). Culture supernatants were removed at the end of the inhibitor/NP exposure period after which the cells were washed with PBS to remove any excess NP that may interfere with the viability assay. XTT coupling reagent and XTT labelling reagent were prepared according to the manufacturer's instructions. Plates were read at 450 nm (FLUOstar Omega, BMG Labtech) immediately after the addition of the prepared XTT reagent at time 0. Plates were then incubated at 37 °C for 1 hour, thereafter, another reading was taken. The change in OD was used to calculate cell viability as a percentage of the control.

5.2.6 NO Assay

The culture supernatant was used to determine the amount of nitrite produced by the Caco-2 cells exposed to the different inhibitors and NP concentrations. This experiment is based on the Griess reaction (Granger *et al.*, 1996). A 1:1 of culture supernatant or standard was mixed with the Griess reagent (1:1 of 1 % sulphanilamide and 0.1 % naphtylethlemidimine-dihydrochloride in 2.5 % phosphoric acid all purchased from Sigma-Aldrich). The plate was then read at 540 nm (FLUOstar Omega, BMG Labtech) and the amount of nitrite produced by the cells quantified. The concentration of nitrite released by the cells was quantified against nitrite standards (Sigma-aldrich) (0-100 μM).

5.2.7 Modulation of the Inflammatory Biomarkers, Interleukin 6 (IL-6) and Microphage Migratory Inhibitory Factor (MIF), Produced by Cells.

The double antibody sandwich enzyme linked immuno-sorbent assay (DAS-ELISA) were used to assess inflammatory markers released by the Caco-2 cells upon inhibitor and NP exposure. The IL-6 (Invitrogen) and Microphage Migratory Inhibitory factor (MIF) (R & D Systems) assays were performed according to the manufacturer's instructions. Cell culture supernatants were assayed neat.

5.2.8 Statistical Analysis

The assays were performed in triplicate and results calculated using Microsoft Excel. Data is presented as mean ± SD. Statistical differences between groups were compared against the control using SigmaPlot 12.0, by one-way analysis of variance (ANOVA). A p-value of < 0.001 was considered as significantly different.

5.3 Results

5.3.1 The Effect of AgNPs and Inhibitors on the Viability of Caco-2 Cells

In the preliminary study to ascertain the concentration range of the AgNPs to be used in subsequent experiments, it was noted that cytotoxicity was induced by AgNPs in a concentration dependent manner ($p < 0.001$) at concentrations ≥ 12,5 μg/ml AgNPs. Where the loss of cell viability ranged from approximately 40 – 90 % between the concentration range 12,5 – 100 μg/ml AgNPs (Figure 1). Therefore, the AgNP concentrations selected was 0, 12.5, 25 μg/ml AgNPs for the subsequent experiments.

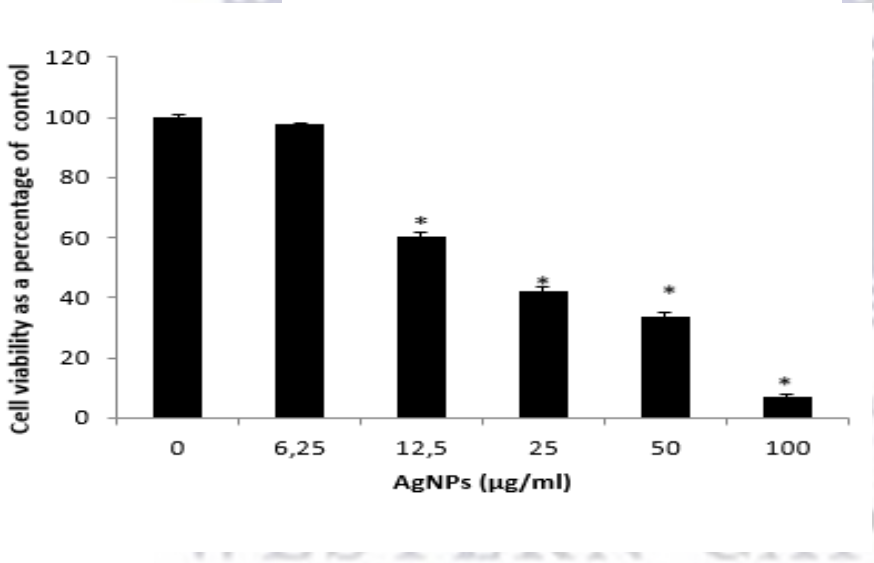

Figure 5.1: Cell viability of Caco-2 cells exposed to AgNPs. Data represents mean percentage ± SD. Error bars marked with (*) indicate significant difference (P < 0.001) compared to control.

5.3.2 The Effect of AgNPs and Inhibitors on the Viability of Caco-2 Cells

Cytotoxicity was induced by AgNPs in a concentration dependent manner ($p < 0.001$) at concentrations ≥ 12,5 μg/ml AgNPs. However, the inhibitors did not significantly mediate cytotoxicity at the different concentrations assessed in this study (Figure 2). As cell viability was

reduced by 40 and 60 % at 12,5 and 25 µg/ml AgNPs, respectively in both the presence and absence of the inhibitors (Figure 2 A, C, D). However, no toxicity was exhibited when exposed to AgNPs and nystatin (Figure 2 B).

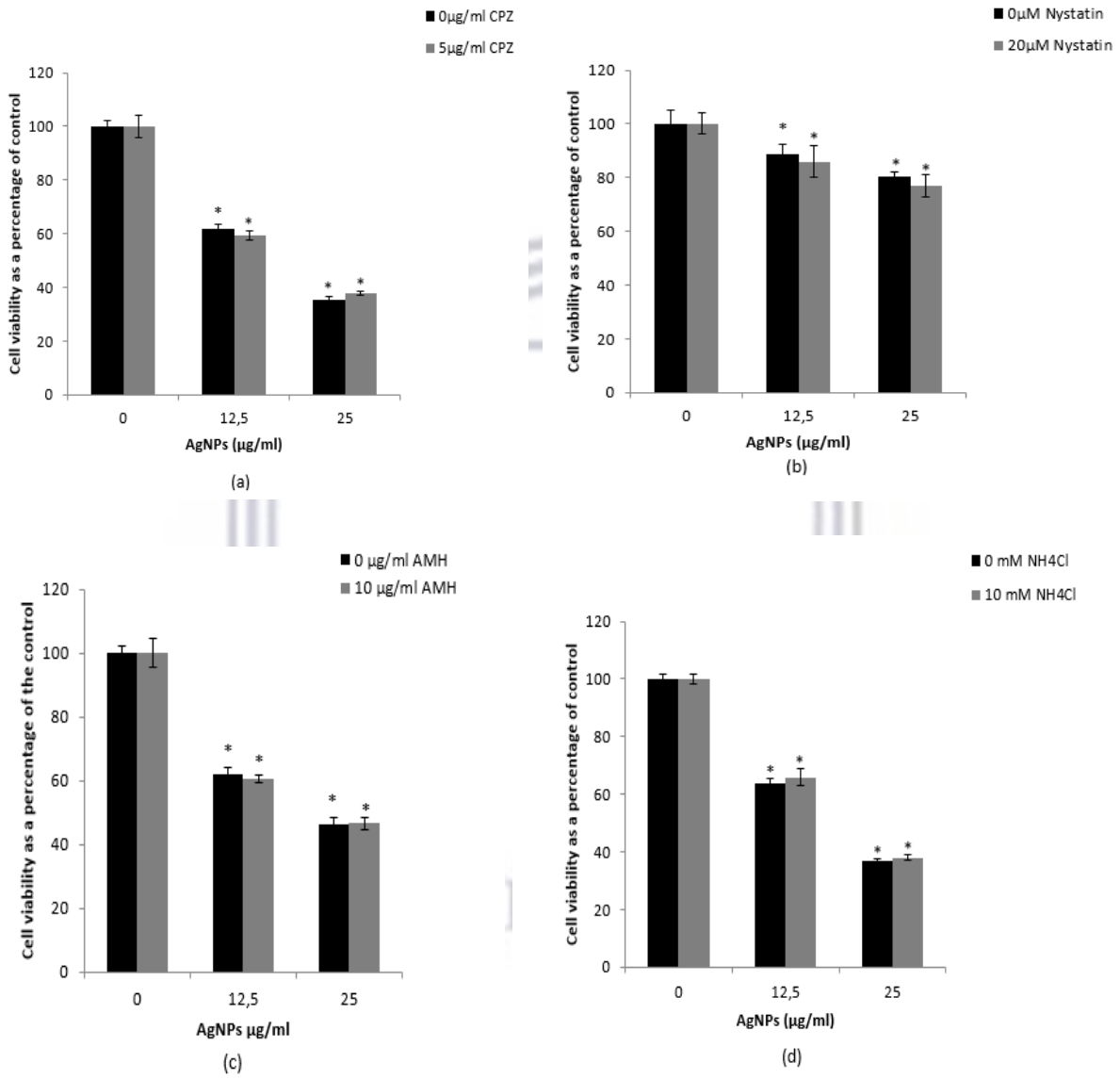

Figure 5.2: Cell viability of Caco-2 cells exposed to AgNPs after pretreatment with different inhibitors. (a) 10 µg/ml Amiloride chloride (AMH), (b) 5 µg/ml Chlopromazine (CPZ), (c) 10 mM Ammonium chloride (NH4Cl) and (d) 20 µM Nystatin. Data represents mean perce

5.3.3 The Effect of AgNPs and Inhibitors on Inflammatory Biomarkers NO Using Caco-2 Cells

Exposure of the cells to AgNPs in the presence of the various inhibitors did not influence the upregulation of NO produced by the Caco-2 cells (Figure 3). AgNPs along with the respective inhibitors (i.e. CPZ, AMH and NH_4Cl) upregulated NO production in the cells in a concentration dependent manner ($p < 0.001$) at concentrations ≥ 12,5 μg/ml AgNPs by approximately 3 fold (Figure 3 A, C, D). However, cells exposed to AgNPs and the inhibitor nystatin had no effect for the same concentration range compared to the control (Figure 3D).

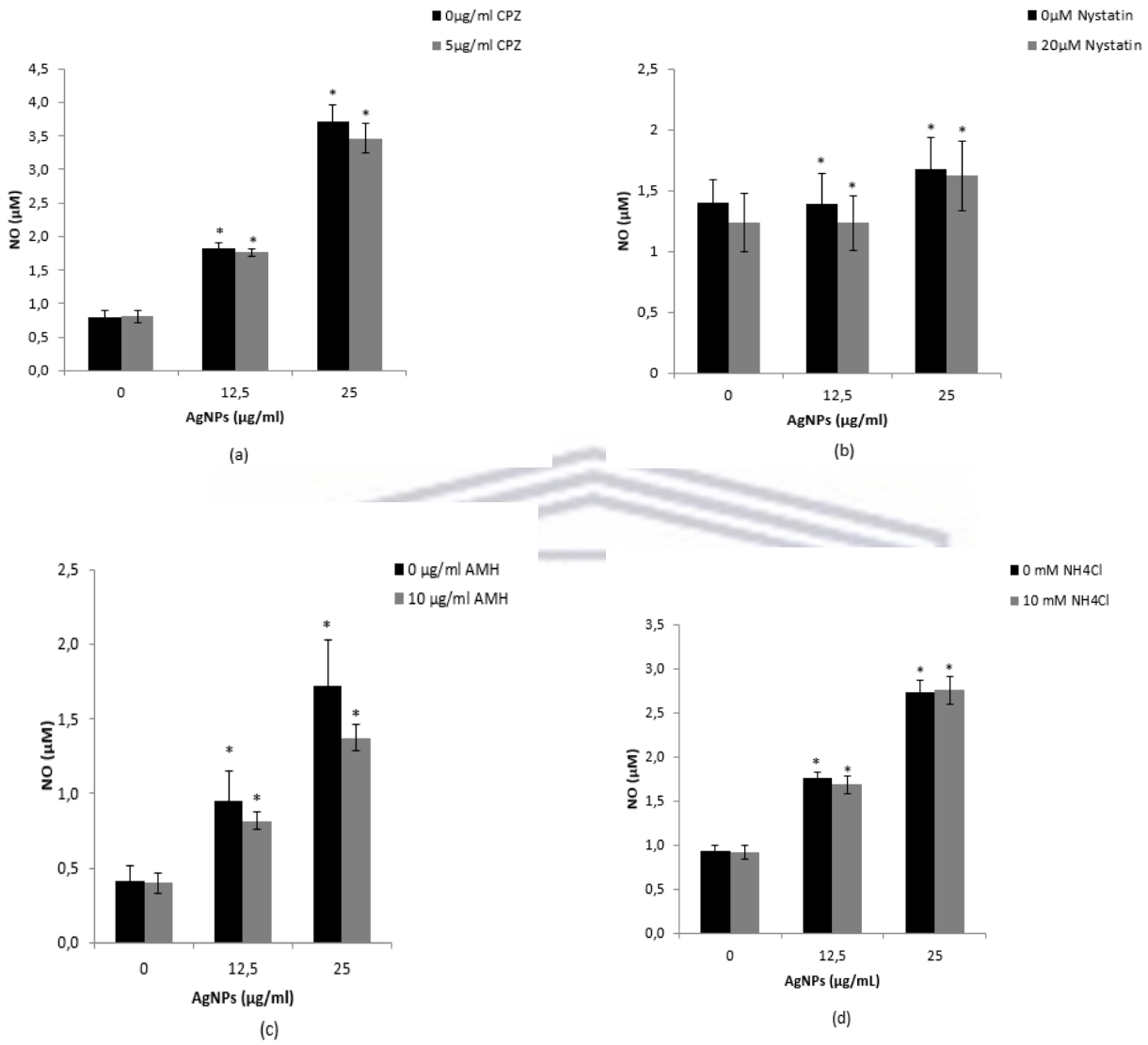

Figure 5.3: NO levels of Caco-2 cells exposed to AgNPs after pretreatment with different inhibitors. (a) 10 µg/ml Amiloride chloride (AMH), (b) 5 µg/ml Chlopromazine (CPZ), (c) 10 mM Ammonium chloride (NH4Cl) and (d) 20 µM Nystatin. Data represents mean ± SD. Error

5.4 The Effect of AgNPs and Inhibitors on Inflammatory Biomarkers IL-6 Using Caco-2 Cells

AgNPs at the concentrations tested decreased IL-6 production in a concentration dependent manner ($p < 0.001$) at concentrations ≥ 12,5 µg/ml AgNPs. The inhibitors did not notably alter the

inflammatory effect at the different concentrations screened compared to the control (Figure 4). The level of IL-6 produced by the cells decreased by ≥ 50 % when in the presence of AgNPs and the inhibitors; CPZ, AMH and NH_4Cl (Figure 4 A, C, D). Whereas, no effect was observed for cells exposed to AgNPs and nystatin for the same concentration range (Figure 4 B).

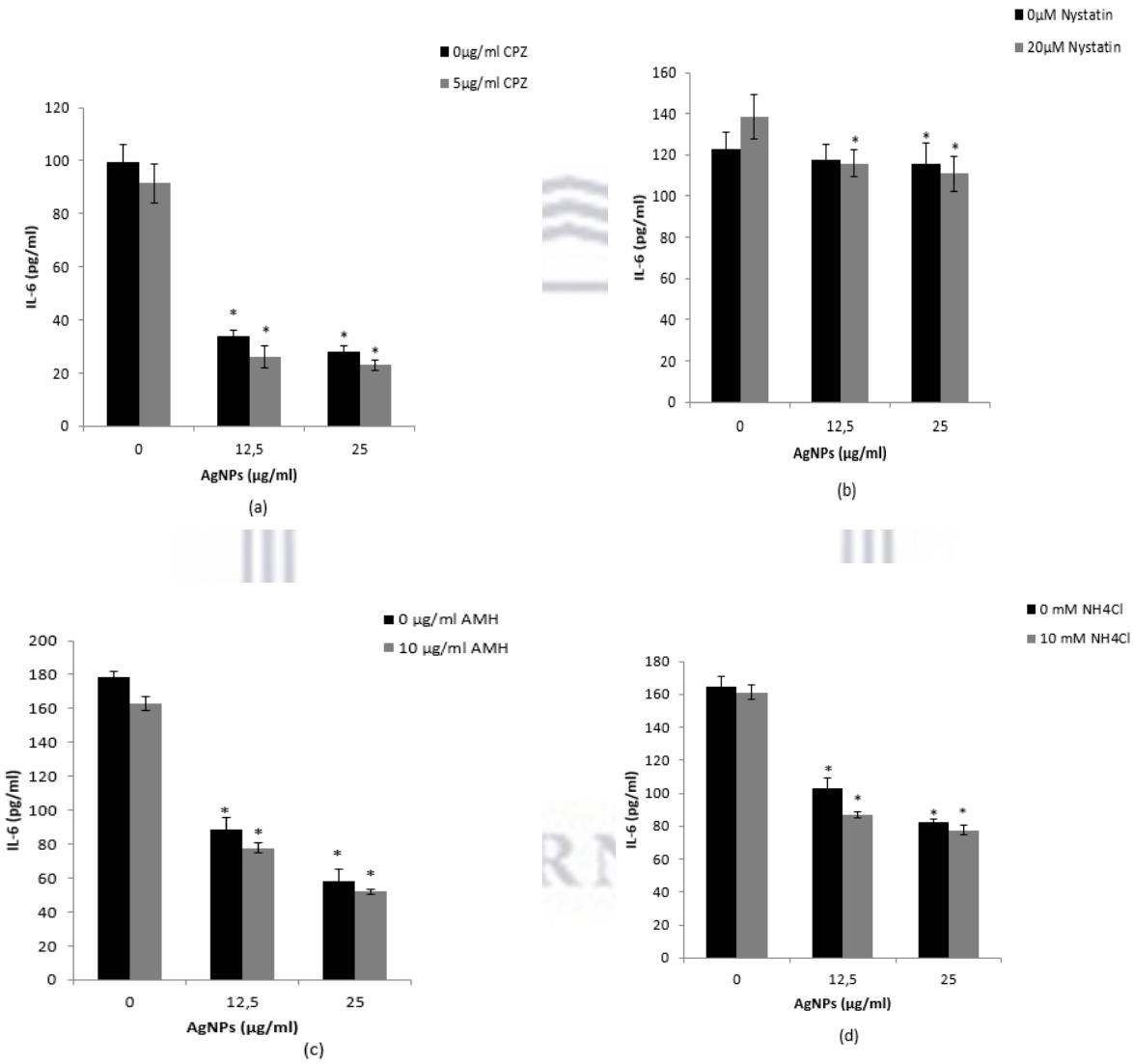

Figure 5.4: IL-6 levels of Caco-2 cells exposed to AgNPs after pretreatment with different inhibitors. (a) 10 µg/ml Amiloride chloride (AMH), (b) 5 µg/ml Chlopromazine (CPZ), (c) 10 mM Ammonium chloride (NH4Cl) and (d) 20 µM Nystatin. Data represents mean percentage ± SD. Error bars marked with (*) indicate significant difference (P < 0.001) compared to control.

5.5 The Effect of AgNPs and Inhibitors on Inflammatory Biomarkers MIF Using Caco-2 Cells

AgNPs at the concentrations tested increased the MIF secretion in a concentration dependent manner ($p < 0.001$) at concentrations ≥ 12,5 μg/ml AgNPs. However, the inhibitors did not alter the inflammatory effect at the different concentrations assayed compared to the control (Figure 5). The levels of MIF were consistently upregulated by approximately 4-fold or greater compared to the control, irrespective of the inhibitor used.

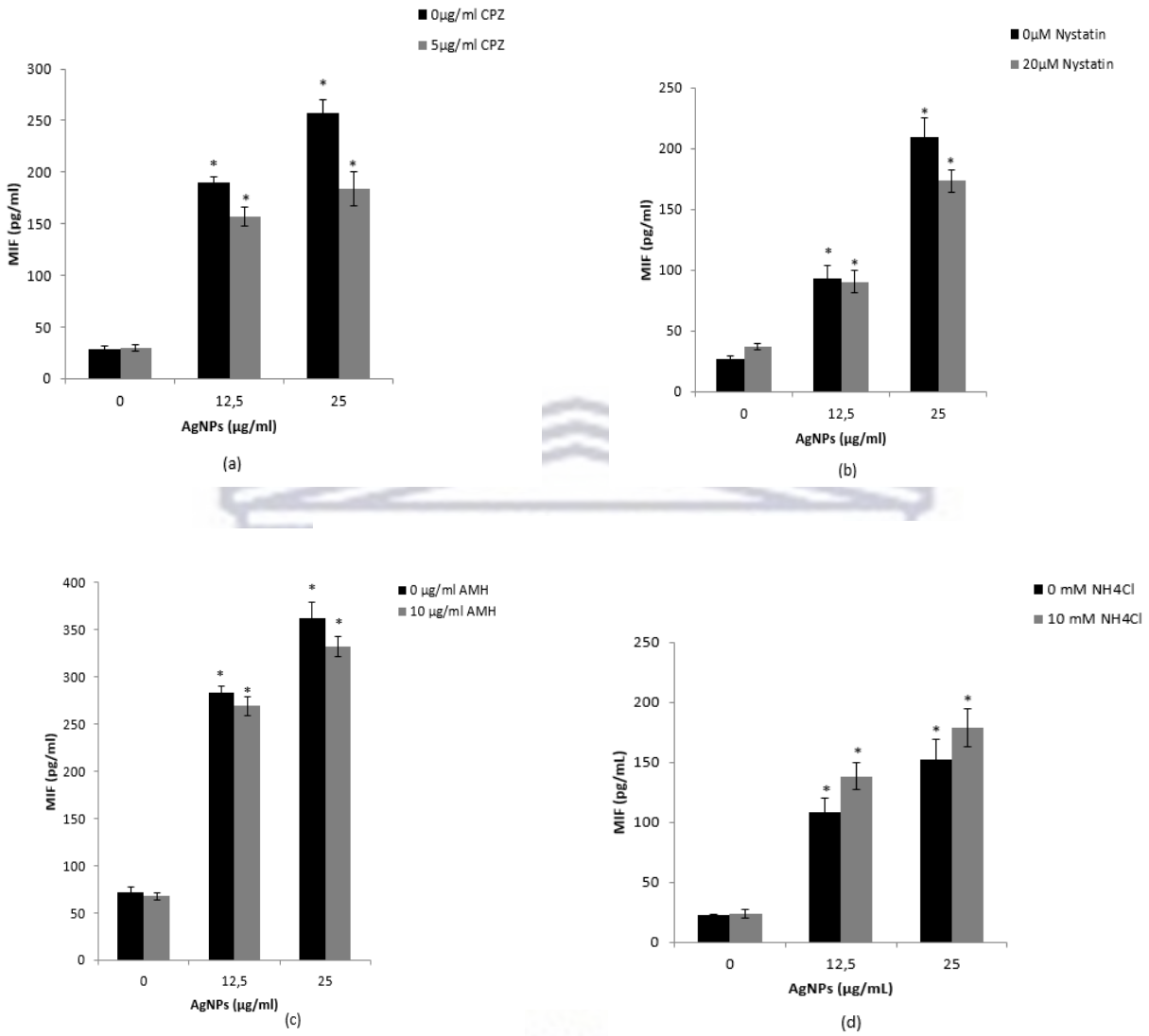

Figure 5.5: MIF levels of Caco-2 cells exposed to AgNPs after pretreatment with different inhibitors. (a) 10 µg/ml Amiloride chloride (AMH), (b) 5 µg/ml Chlopromazine (CPZ), (c) 10 mM Ammonium chloride (NH4Cl) and (d) 20 µM Nystatin. Data represents mean percentage ± SD. Error bars marked with (*) indicate significant difference (P < 0.001) compared to control.

5.6 Discussion and Conclusion

Understanding interactions of AgNPs with biological processes, particularly cells, is of importance due to their increasing application in medical applications and materials, and subsequent biological and environmental exposure. For AgNPs to be utilized to their full potential, it is important to evaluate their uptake with respect to toxicity and inflammation, which may also be used to predict any antagonistic cellular response. A fundamental mechanism for AgNPs to impart toxicity or modulate inflammation is to gain access into the cell. Many parameters affect the behaviour of AgNPs in a cellular environment particularly their interactions with cell membranes, such as the concentration, time of exposure, and physicochemical characteristics of the NPs. These physicochemical characteristics of the NP include size, charge, shape, and coatings, culture environment such as cell types and media composition and external factors such as pH, temperature, and humidity which are key parameters that could influence uptake mechanisms and could possibly impact toxicity and inflammation (Kuhn *et al.*, 2014a, Rothen-Rutishauser *et al.*, 2014, Wu *et al.*, 2019, Akter *et al.*, 2018).

Entry of NPs into the cell involves highly regulated mechanisms with complex biochemical interactions with the plasma membrane. This biological membrane acts as a protective barrier and separates a cell's intracellular components from the extracellular environment (Behzadi *et al.*, 2017). When NPs are suspended in biological solutions, different types of biomolecules (predominantly proteins) interact with the NPs, developing a protein corona, which may modify their biochemical characteristics, thus altering the uptake mechanism (Mosquera *et al.*, 2018). The NPs uptake mechanisms interacts with membrane characteristics such as the phospholipid-based bilayer, proteins and other biomolecules resulting in an alteration of membrane structure and

function such as receptors, channels and transporters (Donahue *et al.*, 2019, Behzadi *et al.*, 2017), which may lead to a cascade of reactions such as toxicity and inflammation.

Literature revealed that NPs enters the cell through various pathways described as simple diffusion, facilitated diffusion, and active transport (Mosquera *et al.*, 2018). Active transport is the most widely used mechanism and requires energy and biomolecules to allow entry of NPs into the cells, through a process known as endocytosis. Endocytosis can be further classified as phagocytosis and pinocytosis (clathrin-mediated endocytosis, caveolin-mediated endocytosis, clathrin/caveolae-independent endocytosis, and micropinocytosis) (Mosquera *et al.*, 2018). To understand the endocytotic mechanisms, pharmaceutical inhibitors are used to block specific pathways to elucidate whether the uptake is through the pathways or alternative mechanisms. Moreover, there is evidence from different studies that supports the entry of NPs into the cells by these various pathways (Kuhn *et al.*, 2014b, Bannunah *et al.*, 2014, Fan *et al.*, 2016). These studies suggest that elucidation of uptake mechanism is important to understanding safety and risk associated with exposure to AgNPs.

In this study, we investigated the different uptake pathways that could possibly mediate toxicity, and modulate inflammatory immune biomarkers using specific pharmaceutical inhibitors (amiloride hydrochloride. ammonium chloride, chlorpromazine, and nystatin) for definite pathways such as clathrin, caveolae, macropinocytosis and pH acidification in Caco-2 epithelial cell lines. The intestinal epithelium is made up mostly of differentiated epithelial cells. During differentiation, epithelial cells are biochemically and functionally modified resulting in discrete apical and basolateral membrane domains that are separated by tight junctions may affect the

uptake and interactions of nanoparticles (Fan *et al.*, 2016). NPs uptake have been previously reported to be inhibited by the clathrin mediated endocytosis (CME) (Rothen-Rutishauser *et al.*, 2014). Chlorpromazine hydrochloride, is a cationic amphiphilic drug, which blocks the clathrin pathway by transferring clathrin and its adaptor proteins from the extracellular matrix into the intracellular space. Caveolae mediated endocytosis (CavME) have also been proved by many studies to be involved in the entry of NPs into the cell (Felix *et al.*, 2017). Caveolae are flask-shaped invaginations found in the plasma cell membrane, interspersed among regions of dense bodies anchoring the cytoskeleton. It plays a critical role in many biological processes, such as regulation of membrane proteins, lipids, fatty acids and cell signaling (Behzadi *et al.*, 2017). Nystatin inhibits the caveolae pathway through the lipid raft internalization mechanism by depletion of cholesterol resulting in an invagination on the cell membrane (Kuhn *et al.*, 2014b). Macropinocytosis is a class of non-specific cellular uptake mechanisms that occurs by the internalization of surrounding fluid into large vacuoles by actin-dependent membrane protrusions, forming large intracellular vacuoles known as macropinosomes (Mosquera *et al.*, 2018). Amiloride hydrochloride inhibits macropinocytosis by the alteration of the membrane pH and the Na^+/H^+ pump through modulation of membrane signaling and cytoskeleton rearrangement (Koivusalo *et al.*, 2010). Ammonium chloride alters the endosomal acidification (Wang *et al.*, 2019). Therefore, an insightful understanding of the uptake and interactions of nanoparticles with biological processes is requisite for their exposure, safety and usage to mitigate possible toxicity and harmful effects.

In the present study, the cell viability assay (Fig 1) showed a significant reduction in viable cells in a concentration dependent manner (≥ 12,5 μg/ml AgNPs), as also observed by Bohmert et al,.

2012 and Chen et al 2016 in Caco-2 cells (Bohmert *et al.*, 2014, Bohmert *et al.*, 2012, Bohmert *et al.*, 2015, Oberemm *et al.*, 2016, Chen *et al.*, 2016). Most previous studies also corroborated AgNPs cytotoxicity in various cells types alluding to the effect of membrane disruption, mitochondrial activity disturbance and release of silver ions into solution (Akter *et al.*, 2018) (Chairuangkitti *et al.*, 2013). However, there was no significant effect by the inhibitors on cytotoxicity (Fig 2). These insignificant changes in CavME may be due to low detectable levels of caveolae in Caco-2 epithelial cells as a result of specialized tissue functions of caveolae as reported by studies from Kumari *et al.*, 2010, and Mirre *et al.*, 1996 (Kumari *et al.*, 2010, Mirre *et al.*, 1996). Rothen-Rutishauser *et al.*, 2013 reported that nystatin has no effects on the epithelial cell A549 in the quantification of gold nanoparticles uptake under controlled conditions (Rothen-Rutishauser *et al.*, 2014). The other pathways CME, macropinocytosis and endosomal acidification showed no effect on the cytotoxicity which may be due to their involvement in the uptake of nutrient from the media. The cell inability to access nutrients from the media may lead to reduction in proliferation and possible cell death.

The inflammatory mediators assayed in this study, NO, IL-6 and MIF are known for their pleiotropic expressions, and are involved in the mobilization of neutrophils and other granulocytes to infection sites during exposure of cells to biological and chemical substances (Tarantini *et al.*, 2015, Martirosyan *et al.*, 2016). To our understanding, the uptake mechanism of AgNPs in relation to their modulation of *in vitro* inflammatory cytokines of gastrointestinal systems has not been well established in literature, particularly the effect of these uptake mechanism on inflammatory biomarkers. NO is a key inflammatory mediator in response to cell and or tissue injury and its upregulation results in toxicity rather than cellular defence and persistent NO production may lead

to reduction of enzymatic substrates and cofactors (Asghari *et al.*, 2018). The results revealed that NO production was slightly upregulated by AgNPs (Fig 3) this corroborated our previous studies on RAW 264.7 macrophage cells (Lategan *et al.*, 2019). Subsequently, the inhibitors did not significantly alter the increased NO production, thus unable to mitigate the inflammatory responses caused by the AgNPs. Conversely, IL-6 is an important regulator of the innate immune function as it acts as a pro-inflammatory and anti-inflammatory cytokine. There was a decrease in the IL-6 level with cells treated with AgNPs only (Fig 4), suggesting the involvement of AgNPs in the alteration and/or suppression of inflammation due to regulation by other inflammatory mediators. The results correspond with that of Tang *et al* 2017 on rat kupper cells (Tang *et al.*, 2017). Conversely, there was no significant effect by the inhibitors on the alteration of the IL-6 reduction. Nonetheless, Tang *et al* 2017 studies on the effect of endocytosis inhibitors on folate conjugated NPs on kupper cells revealed a minor decrease in IL-6 production (Tang *et al.*, 2017). MIF is a pro-inflammatory cytokine that is critical to the regulation of the innate immune system. MIF plays a vital role in the pathogenesis of shock which may lead to vascular leakage induction (White *et al.*, 2013). The results revealed a marked increase in MIF at concentrations >12,5µg/ml AgNPs (Fig 5). The marked increase of MIF could be attributed to the oxidative stress caused by the AgNPs as recent studies revealed that MIF can induce autophagy via the generation of reactive oxygen species (Chen *et al.*, 2015). Contrariwise, there was no significant effect by the inhibitors on the modulation of the increased MIF production caused by AgNPs. Several clinical studies have also highlighted MIF as a potential biomarker for diverse infections relating to inflammatory component which include metabolic disorders such as type 2 diabetes, obesity systemic infections, sepsis, autoimmune diseases, and cancer (Grieb *et al.*, 2010). Therefore, this can possibly help in the monitoring of toxicity of nanoparticles, their applications and exposure. Current studies have

also corroborated the upregulation of inflammatory cytokines by AgNPs in Caco-2 cells (Gioria *et al.*, 2018).

The study needs to be further elucidated as several factors such as temperature, cell types, could impact on the insignificant effect of these inhibitors on cytotoxicity and inflammation. Subsequently, the inevitable exposure and release of AgNPs into the biota can possibly impact on human health as these nanoparticles are used in biomedical and drug delivery systems among other applications. Furthermore, the current study is crucial for development and safety of living organisms.

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CHAPTER 6: CONCLUSIONS AND RECOMMENDATION

6.1 General Conclusions

This study was done to investigate the potential cytotoxicity and the different mechanisms by which silver and titanium dioxide nanoparticles affects the proteome of human colon epithelial cells. This study also proposed to identify potential biomarkers that may be may be used for early prognosis and diagnosis as well as to evaluate the different uptake mechanisms by which these nanoparticles mediated cytotoxicity and inflammation. This was evaluated by exposing silver and titanium dioxide nanoparticles Caco-2 cells, a human colon epithelial cell line used as an *in vitro* model of the human intestinal epithelium. The cell line was exposed to the nanoparticles at various physiological medias and conditions. The physiological medias varied are (150 Mm NaCl, DMEM, 1 X PBS, DMEM +10% FBS), the conditions included various time points (i.e. 0 hr, 24 hrs, 7 days and 14 days) of exposure, while the temperature and pH were kept constant. The research was of importance because concerns have been raised regarding the toxicity and possible adverse effects of nanoparticles on some biological processes in humans. This is because living organisms can be exposed to nanoparticles through several applications such as diagnostics, bioimaging, biosensing, electronics, food processing and pharmaceuticals. In spite of their unique and numerous applications and potential economic and medicinal benefits, nanoparticles must be subjected to rigorous toxicology testing to ensure that they do not pose a potential adverse risk to the environment and humans.

Chapter 3 elucidated the effects physiological media on AgNPs hydrodynamic size and zeta potential and the effects of these characterized AgNPs on cell viability, cell stress, inflammatory biomarkers and anti-angiogenic properties. The study also explored the effects of AgNPs on Caco-

2 cell viability, inflammatory and cell stress biomarkers, as well as an angiogenesis proteome profile. This study shows that the AgNPs are stable in physiological media, based on hydrodynamic size and zeta potential over the time period assessed. The AgNPs are cytotoxic at high concentrations (IC_{50} = ± 100 μg/ml) and induced cell stress biomarkers at concentrations > 6.25 μg/ml AgNPs. The AgNPs modulated the inflammatory cytokine IL-8 and reduced IL-6 production in a dose dependent manner. Possible anti-angiogenic markers of AgNPs were identified, (angiopoietin-2 and PDGF-AA), as potential biomarker for nanoparticle exposure via proteome profiling.

Chapter 4 showed the effects physiological media had on TiO_2NPs properties (hydrodynamic size and zeta potential). As well as the effects of TiO_2NPs on human colorectal adenocarcinoma (Caco-2) epithelial cell viability, inflammatory and cell stress biomarkers, as well as an angiogenesis proteome profile. The NPs increased in size over time, while zeta potentials were stable. The TiO_2NPs induced cell stress biomarkers, which could attribute to the NPs not being cytotoxic. The level of inflammatory biomarkers was not affected. Anti-angiogenic properties were exhibited when exposed to the NOAEL TiO_2NP concentration and requires further in-depth investigation.

Chapter 5 established that cytotoxicity was induced by AgNPs in a concentration dependent manner at concentrations ≥ 12,5 μg/ml AgNPs. Where the loss of cell viability ranged from approximately 40 – 90 % between the concentration range 12,5 – 100 μg/ml AgNPs. The inhibitors did not notably alter the inflammatory effect (NO, IL-6 and MIF) at the different concentrations screened compared to the control. To this end definite pathways such as clathrin, caveolae, macropinocytosis and pH acidification in Caco-2 epithelial cell lines, did not significantly mitigate toxicity nor alter the inflammation caused by the AgNPs.

6.2 Future Perspectives and Recommendations

- In future proteome profile of other biological processes should be considered, such as chemokines, cytokines, adipokines, apoptosis, and receptor kinases at chronic and acute exposures so as to have a better understanding of the interactions of nanoparticles with cellular processes.
- The anti-angiogenic markers of AgNPs identified, (angiopoietin-2 and PDGF-AA), as potential biomarker for nanoparticle exposure via proteome profiling needs to be further elucidated so as to ascertain the possible usage for prognosis and diagnosis tools for effective production and safety applications of nanoparticles.
- The lack of inflammatory cytokine production and cytotoxicity makes TiO_2NPs a good candidate for usage and application. However, further comprehensive investigation is required such as the exposure period, dosage and size of the nanoparticles which may significantly influence these factors.
- Further investigation is also required for anti-angiogenic markers of TiO_2NPs (i.e. persephin, angiopoetin-2, PDGF-AA and endostatin) upon exposing the Caco-2 cells to 100 μg/ml TiO_2NPs and could be marked for use in cancer therapy.
- Few *in vivo* studies involving nanoparticles are available with regards to immune response, further investigation can be done with animal studies to ascertain the safety of these nanoparticles.