Effect of Titanium Dioxide Nanoparticle Aggregation on Mouse Myoblast Cellular Cytotoxicity and Nitric Oxide Synthesis

By

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PREFACE

The study described in this dissertation was carried out by Ms Wendy Nokhwezi Phoswa and has not been submitted in any other form to another University. This study was carried out in the School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa from March 2016 to March 2017 under the supervision of Prof Irene Mackraj and Mr Preenan Pillay.

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(211511344)

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Supervisor _____ Date 09/06/2017

Preenan Pillay

Co-supervisor _____ Date 09/06/2017

DECLARATION

I, Ms Wendy Nokhwezi Phoswa declare as follows:

1. That the work described in this thesis has not been submitted to UKZN or other tertiary institution for purposes of obtaining an academic qualification, whether by myself or any other party.

2. This thesis does not contain other person's writing, data, pictures, or other information unless specifically acknowledged as being sourced from other persons or researchers. Where other written sources have been quoted then:

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DEDICATION

This research project is dedicated to:

- God, my provider.
- My grandmother, Antonia Phoswa.
- My Mother, Daphney Nana Phoswa.

FUNDING

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- National Research Foundation (NRF)
- College of Health Sciences (UKZN)

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- Preenan Pillay, Dr K Moodley, Dr K Maduray and Mr Sashin Bejai for their help with planning and running experiments and for their constructive criticism and guidance throughout this work.
- My mum and grandmother for their spiritual support.
- Members of the placenta research group for their contributions to the success of this research project. My colleagues and friends

Contents

LIST OF FIGURES

Chapter 1: Literature review

Chapter 2: Manuscript

LIST OF ABBREVIATIONS

BH4	Tetrahydrobiopterin
BSA	Bovine serum albumin
CO_2	Carbon dioxide
eNOS	Endothelial nitric oxide synthase
Mg/ml	Milligram per mil
MTT	Methyl tetrazolium cytotoxicity
Nm	Nanometre
NPs	Nanoparticles
NO	Nitric oxide
NTA	Nanoparticle tracking analysis
OS	Oxidative stress
TEM	Transmission electron microscopy
TiO_2	Titanium dioxide
µL	Microliter
°C	Degrees Celsius
%	Percent

ABSTRACT

Introduction: The emerging interest of engineered titanium dioxide nanoparticles (TiO_2 NPs) in medical, agricultural, industrial and manufacturing sectors have raised health questions worldwide. Therefore, the objective was to assess the effect of physiochemical properties of titanium dioxide nanoparticles (TiO_2 NPs) on the cellular cytotoxicity, proliferation and physiological properties.

Methods: TiO_2 NPs were suspended in varying concentrations of bovine serum albumin (BSA γ-globulin) and characterised using nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM) for the determination of particle size, aggregation state, and zeta potential. The effect of TiO_2 physiochemical properties on cellular cytotoxicity and proliferation was assessed *in vitro* on mouse myoblast (C2C12) cells using the MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] and BrdU assay respectively. Nitric oxide (NO), a major signalling molecule was measured using a biochemical test. *in vitro*.

Results: There was an increase in size, distribution, surface charge and reduced aggregation in BSA stabilised TiO_2 NPs in comparison to non-stabilised TiO_2 NPs. Increased cytotoxicity of cells treated with monodispersed TiO_2 NPs compared to cells treated with aggregated TiO_2 NPs ($p<0.001$) was observed. A significant decrease in cell viability in cells treated with BSA (0.5, 0.8 and 1.0 mg/ml) stabilised TiO_2 NPs (40, 120, 240, 320 and 400 mg/ml) in a dose-dependent manner in contrast to cells treated with BSA (0.3 and 1.5 mg/ml) stabilised TiO_2 NPs (40, 120, 240, 320 and 400 mg/ml) dose dependent manner was observed ($p<0.05$). However, there was a greater decrease in cell viability in BSA (0.8 mg/ml) stabilised TiO_2 NPs (40, 120, 240, 320 and 400 mg/ml) compared to other BSA concentration ($p<0.05$). In addition, there was a significant difference in DNA proliferation of the control and treated cells. A significant difference in DNA damage was observed in cells treated with BSA compared to non-treated cells, especially at BSA concentrations of 0.8 and 1.5 mg/ml. A significant difference in DNA damage in cells treated with TiO_2 NPs in combination with BSA (0.8 and 1.5 mg/ml) was obtained. There was greater difference in DNA damage of cells exposed to TiO_2 NPs in combination with 0.8 mg/ml compared to TiO_2 NPs in combination with 1.5 mg/ml. More interestingly there was a significant difference between the levels of nitric oxide (NO) in 40 and 400 mg/ml TiO_2 NPs treated cells in comparison to cells treated with BSA (0.3-1.5 mg/ml) stabilised TiO_2 NPs (40 and 400 mg/ml) ($p<0.05$). There was a significance difference in the levels of NO between cells treated with 40 mg/ml TiO_2 NPs *vs* (0.3, 0.5, 0.8 and 1.0 mg/ml) BSA stabilised TiO_2 NPs (40 mg/ml) ($p<0.05$). However, there was greater significant difference between 400 mg/ml TiO_2 treated cells *vs* BSA (0.5, 0.8 and 1.0 mg/ml) stabilised 400 mg/ml TiO_2 NPs (400 mg/ml) ($p<0.05$).

Discussion/Conclusion: The use of BSA as a nanoparticle stabiliser impacted upon physiochemical properties for the determination of *in vitro* cytotoxicity. These findings indicate that particle size needs to be taken into consideration when assessing nanoparticle toxicity. The results also indicate that less aggregated TiO_2 NPs are more toxic than more aggregated TiO_2 NPs and have a potential to inhibit cellular signalling mechanisms such as NO signalling and cellular proliferation.

CHAPTER 1: BACKGROUND AND LITERATURE REVIEW

1.1 Background

The application of nanotechnology has revolutionised almost every technological and industrial sector of the world today. This technology has the ability to convert bulk materials to nanoscale and allows for specific properties (i.e. strength, durability, reactivity, conductance etc.) to be tailored towards each project of interest. The industries that have advantageously manipulated materials into nanoscale include medicine, food safety, environmental science, and many others. Numerous commercial and everyday products depend on the presence of nano-engineered materials in order to deliver the best possible outcomes following their use. In addition, the application of nanotechnology is rapidly advancing in various scientific sectors with researchers globally discovering remarkable properties and uses of nanoparticles (Gould, 1982). However, stringent control of nanoparticle usage, as well as a greater understanding of the toxicity mechanisms of nanoparticles, is necessary to minimise the harmful effects to humans and the environment. One of the most widely manufactured nanoparticles is titanium dioxide (TiO_2) which has numerous industrial applications (Fisher and Egerton, 2001, Kaida *et al.*, 2003, Wolf *et al.*, 2003, Weir *et al.*, 2012). Recently, TiO_2 nanoparticle application in nanomedicine includes drug delivery systems for the treatment of diseases (i.e. cancer), cell imaging, biosensors for biological assays, and genetic engineering (Nune *et al.*, 2009).

1.2 Titanium dioxide nanoparticles (TiO_2 NPs)

Titanium is the naturally occurring oxide of titanium with several crystalline structures. It is a white non-combustible and odourless powder with a molecular weight of 79.9 g/mol and size (<100 nm) (Shi *et al.*, 2013, Chen *et al.*, 2014). This low-solubility powder possesses high activity in the interaction with other chemical substances and has been previously considered to exhibit relatively low toxicity (ILSI, 2000, Safety and Health., 2005, Sager *et al.*, 2008). Recently TiO_2 have been widely used in industrial and consumer products due to its stronger catalytic activity in comparison to TiO_2 fine particles. TiO_2 NPs also exhibit different physiochemical properties compared to fine particles and this may alter their bioactivity and negatively impact human health and the environment (Shi *et al.*, 2013).

1.3 Applications of Titanium dioxide nanoparticles (TiO_2 NPs)

TiO_2 NPs are produced abundantly and used widely because of their high stability, anticorrosive and photocatalytic properties (Riu *et al.*, 2006). These NPs account for 70% of the total production volume of pigments worldwide (Baan *et al.*, 2006) and approximately four million tons of this pigment is consumed worldwide. This ranks it amongst the top five nanoparticles used in consumer products (Shukla *et al.*, 2011). Titanium dioxide can be used in various fields including medicine, industry, personal care products, food and environmental application (Kaida *et al.*, 2003, Wolf *et al.*, 2003, Wang *et al.*, 2007) (Table 1).

Table 1: Nanotechnological applications of TiO_2 NPs

Sector	Application	References
Environmental	• TiO_2 is also used in water treatment, air purification, remediation of organically contaminated soil and sludge.	(Pelaez *et al.*, 2012)
Industrial	• TiO_2 NPs provides whiteness and opacity to products such as paints, plastics, papers, and printing inks. • They are also used in self-cleaning ceramics and glass.	(Baan *et al.*, 2006, Kasanen *et al.*, 2009)
Medical	• TiO_2 NPs can be used as filters in polymeric materials used improve bone prostheses or as a component for articulating prosthetic implants, especially for hip and knee. • TiO_2 NPs can also be used for dental restoration. • Can also be used for cancer cells apoptosis under UV irradiations for cancer therapeutic treatment.	(Jovanović and Palić, 2012, Pelaez *et al.*, 2012, Yoo *et al.*, 2012, Dan *et al.*, 2015, Fujiwara *et al.*, 2015)
Food products	• Sometimes TiO_2 NPs can also be used as a colourant in food, e.g. skim milk. • Can also be used as a food additive and for food packaging.	(Reeves *et al.*, 2008, Trouiller *et al.*, 2009, Honarvar *et al.*, 2016)
Personal Care Products	• TiO_2 is also used in cosmetics as a pigment and in sunscreens due to its high refractive index.	(Weir *et al.*, 2012, Peters *et al.*, 2014, Dan *et al.*, 2015)

Abbreviations: TiO2, NP, UV

1. TiO2 Titanium dioxide
2. NPs Nanoparticles
3. UV Ultra violet

1.4 Physiochemical properties of Titanium dioxide nanoparticles

There are three crystal structures of TiO_2 namely anatase, rutile and brookite, with anatase being more chemically reactive and mostly used (Sayes *et al.*, 2006, Warheit *et al.*, 2007, Wu *et al.*, 2010, Markowska-Szczupak *et al.*, 2011). Anatase and rutile forms (Figure 1) have natural and industrial importance, while brookite is rarely used. Generally, anatase is more toxic than rutile and unfortunately being used abundantly (Wu *et al.*, 2010, Iswarya *et al.*, 2015). Recently, TiO_2 (NPs) have been widely used in industrial and consumer products due to their stronger catalytic activity when compared to fine particles (FPs) (Shi *et al.*, 2013). The increased catalytic activity of NPs has been attributed to their smaller sizes, which allow for larger surface area per unit mass (Shi *et al.*, 2013). Traditionally, FPs have been considered poorly soluble, low-toxicity particles (Hygienists, 1986, ILSI, 2000), whereas researchers have expressed concerns about the harmful effects of TiO_2 NPs on human health associated with decreased size (Andersson *et al.*, 2011, Wang and Li, 2012). TiO_2 NPs possess different physiochemical properties compared to FPs (Shi *et al.*, 2013). These properties include size, shape, surface charge (zeta potential) and aggregation state, which possibly influence bioactivity. Therefore based on this fact, adverse health effects of TiO_2 NPs should be carefully evaluated. It is recommended that researchers carefully characterise the physiochemical properties of TiO_2 NPs.

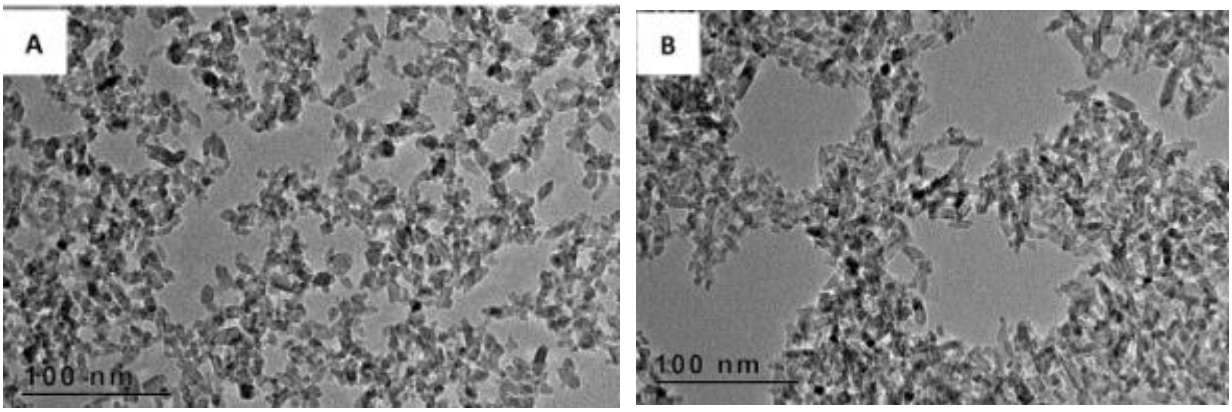

Figure 1: TEM micrographs of the two crystal structures of TiO_2 NPs namely anatase **(A)** and rutile **(B)**, **Anatase (A)** is composed of egg-shaped particles of 11–18 nm, while **Rutile (B)** is more elongated and the particles have a mean size diameter of 10–35 nm. Images adapted from (Uboldi *et al.*, 2016).

1.5 Nanoparticle mode of entry

The main routes of entry of nanoparticles into the human body include inhalation, ingestion, medical (capsule coatings) and dermal application (cosmetic products). Inhalation is the most common form of entry for a wide variety of airborne nanoparticles found in industrial and urban environments. This inhalation of NPs can harshly affect cardiovascular or extrapulmonary organs (Adamson *et al.*, 1999, Oberdörster *et al.*, 2005b, Maynard and Kuempel, 2005, Valavanidis *et al.*, 2008). In the case of biomedical applications, nanoparticles are often introduced into the human body through the intravenous, subcutaneous, intramuscular or intraocular pathways. Another most common route of exposure is dermal application and ingestion, which includes approximately 88% of foods and personal care products in concentrations ranging from 0.02 to 9.0 mg TiO_2/g (Powers, 2006, Long *et al.*, 2007). Therefore, the widespread use of titanium dioxide nanoparticles and its potential entry into the body poses a considerable health risk to humans (Iavicoli *et al.*, 2012, Shi *et al.*, 2013, Shah *et al.*, 2017). Numerous studies have attempted to determine the toxicity of nanoparticles on cell types and organs ranging from endothelium, blood, spleen, liver, nervous system, heart and kidney (Nemmar *et al.*, 2002, Oberdörster *et al.*, 2002, Vickers *et al.*, 2004, Oberdörster *et al.*, 2005b). However, more recently there has been interesting in the physiochemical properties of NPs such as size, surface charge (zeta potential) and aggregation state of nanoparticles and the effect it has on cellular toxicity.

1.6 The effect of titanium dioxide nanoparticle size and aggregation on toxicity

Several investigations have suggested that the agglomerative state of nanoparticles may affect the particle mechanism of action and thus may be the most significant factor in inducing *in vitro* cytotoxicity (Powers *et al.*, 2006, Grassian *et al.*, 2007). Whilst it has been suggested that the disruption of cellular physiology by nanoparticles is induced by the internalisation of particles of varying sizes (Geiser *et al.*, 2005, Blank *et al.*, 2006, Chithrani and Chan, 2007), the mechanism of action is not clearly understood. It has been established that nanoparticle cytotoxicity, is dependent on the agglomeration status and concentration, and may result in either cellular death or physiological imbalance as the uptake mechanism differs between monodispersed and aggregated nanoparticles (Albanese and Chan, 2011, Panariti *et al.*, 2012). Additionally, studies have reported that nanoparticle toxicity not only depends on the type of nanoparticle being tested but it also depends on the physiological cellular response and the metabolic pathways affected when exposed to nanoparticles (Lankoff *et al.*, 2012). Titanium dioxide NPs are mostly found in aggregates (agglomerates) form rather than the monodispersed form (Baveye and Laba, 2008).

Aggregation of nanoparticles have also been associated with functional alterations in cellular physiology, such as inhibition of replication, transcription, and cell proliferation (Yildirimer *et al.*, 2011). This may be due to a decreased surface area which in turn may affect the cellular responses (Park *et al.*, 2011). In a study done by Okuda- Shimazaki *et al.*, 2010, they have reported that sub-micro large

titanium aggregates showed a larger effect on cell viability and gene expression when compared with the small aggregates. They also speculated that the cells incorporate too much large titanium nanoparticles *via* phagocytosis into the cytoplasm and that large titanium nanoparticles (~25 and ~300 nm) induce cytotoxic effects. In contrast, some researchers have shown that toxicity is not dependent upon particle size, but rather on surface characteristics (Warheit *et al.*, 2006, Warheit *et al.*, 2007, Karlsson *et al.*, 2009). Therefore, the precise characterization of the concentrations and properties of the nanoparticles in their respective micro-environment is essential. The present study therefore focused on the characterization of the size, surface charge and aggregation state of TiO_2 nanoparticles prior to *in vitro* testing. Characterising the state of NPs (such as size, surface charge and degree of aggregation) and understanding the parameters that affect this state are crucial for toxicity investigations as these parameters may have a significant impact on observed toxicological responses (Jiang *et al.*, 2009).

1.7 Experimental consideration in assessing nanoparticle toxicity: Characterization of titanium dioxide nanoparticles (TiO_2 NPs)

1.7.1 Effect of bovine serum albumin (BSA) on size, surface charge and aggregation state of TiO_2 NPs

Interestingly, previous studies have focused on improving experimental conditions involving NPs by achieving less aggregated nanoparticles through the addition of surfactants or additives such as bovine serum albumin (BSA) (Skebo *et al.*, 2007, Tantra *et al.*, 2010). Albumin is one of the most abundant plasma proteins and has a role in maintaining colloid osmotic pressure needed for proper distribution of body fluids between intravascular compartments and body tissues (Zhang *et al.*, 1998). BSA is of great importance in pharmacology as the conjugation of drugs to albumin decreases their toxicity (Kragh-Hansen, 1981). It has also been reported that BSA conjugated nanoparticles have improved stability against flocculation, increased quantum yield and low toxicity (Derfus *et al.*, 2004). Bovine serum albumin has been shown to improve particle characterization by generating small agglomerates of primary particles and improved the stability of dispersions and thus also been shown to be relevance in *in vitro* toxicity tests (Vippola *et al.*, 2009). Additionally, nanoparticle toxicological studies have shown BSA to have an impact on improving cellular uptake of nanoparticles (Johnston *et al.*, 2010).

Bovine serum albumin does not only affect the state of aggregation of NPs, but also the particles zeta potential (Dominguez-Medina *et al.*, 2012). Zeta potential is extremely important in determining nanoparticle reactivity, stability and interaction with cells (Honary and Zahir, 2013). It is known that zeta potential is dependent on the aggregation state of a nanoparticle and both the factors affect the response of organisms upon exposure. The state of aggregation and zeta potential are known to be inversely proportional, which indicates that an increase in aggregation state causes a decrease in zeta potential vice versa (Vandsburger, 2009). Therefore the accurate characterization of nanoparticle dispersion is essential for in order to obtain accurate data pertaining nanotoxicology investigation

(Holmberg *et al.*, 2013). Figure 2 illustrates the relationship between zeta potential and aggregation state of NPs and their cytotoxic effect *in vitro*.

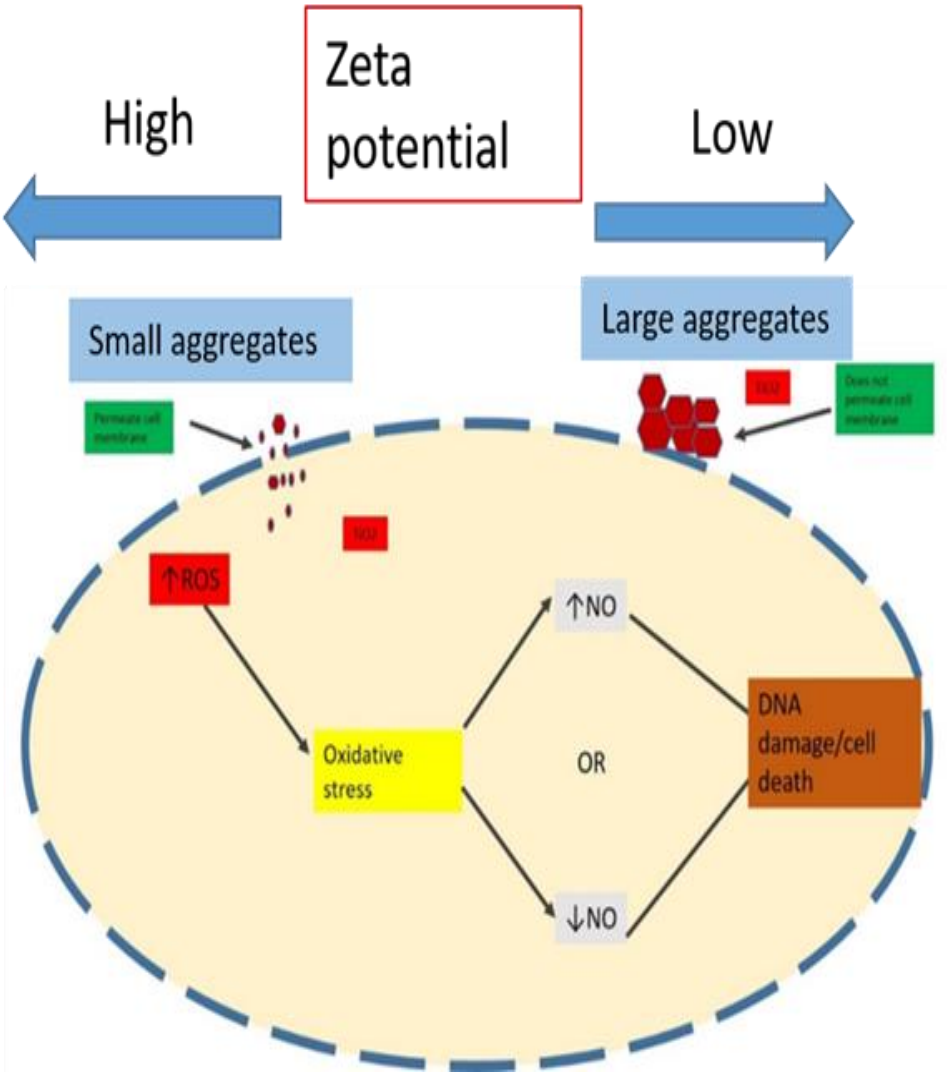

Figure 2: Schematic showing the effects of smaller and larger titanium dioxide nanoparticles aggregates on cellular physiology. Smaller aggregated titanium dioxide nanoparticles permeate easier through cell membrane in comparison to larger particles. This causes an increase in ROS which results in cellular oxidative stress that either reduces or increase NO bioavailability. This results in inadequate cell proliferation, DNA damage and cell death (Adapted from Fairhurst, 2013).

1.7.2 Effect of titanium dioxide nanoparticles on cell biology and toxicity

The utilisation of TiO_2 raised health related concerns which had led to several *in vivo* and *in-vitro* studies which have investigated the toxicological properties of TiO_2 in, animal and cellular models. Numerous studies have reported the cytotoxic effects of TiO_2 NPs to be associated with its physiochemical properties such as size and aggregation state (51, 52). Titanium dioxide NPs have a size range of ± 100 nm (Shi *et al.*, 2013). Prolonged exposure to titanium dioxide has been shown to have negative effects on cellular physiology such as increased lipid peroxidation, DNA damage (Wang *et al.*, 2007, Wang *et al.*, 2009, Hackenberg *et al.*, 2010, Guichard *et al.*, 2012), caspase activation which leads to micronuclei formation (Rahman *et al.*, 2002), chromatin condensation (Chen *et al.*, 2014) and eventual cell death via apoptosis (52). In addition, the presence of titanium dioxide nanoparticles, have been reported to induce oxidative stress (Donaldson *et al.*, 2003, Long *et al.*, 2007, Park *et al.*, 2008, Guichard *et al.*, 2012) and inflammation and affects degradation pathways leading to appearance of autophagosomes and lysosomal dysfunction, in autophagy and apoptosis (Gurr *et al.*, 2005). The oxidative stress has been reported to be due to upregulation of heme oxygenase 1 gene expression, catalase activities and malondialdehyde (Liu *et al.*, 2011). Cells treated with titanium dioxide nanoparticles can also exhibit the features of non-apoptotic (e.g., necrotic) cell death, such as cytoplasmic membrane rupture (Hussain *et al.*, 2005, Sayes *et al.*, 2006, Thevenot *et al.*, 2008).

1.7.3 Effects of nanoparticles physiochemical properties on cellular uptake

Physiochemical properties, such as particle size, shape and surface charge, play a key role in the cellular uptake of nanoparticles. The uptake of nanoparticles by cells involves a two-step process: first, a binding step on the cell membrane and second, the internalisation step (Ciani *et al.*, 2007). The attachment of nanoparticles to cell membrane seems to be most affected by the surface charge (zeta potential) of the particles (Patil *et al.*, 2007, Chen *et al.*, 2010). Variation of the particle surface charge could potentially control binding to the tissue and direct NPs to cellular compartments both *in vitro* and in *vivo*. It is known that cellular surfaces are dominated by negatively charged sulphated proteoglycans molecules that play pivotal roles in cellular proliferation, migration and motility (Mislick and Baldeschwieler, 1996, Bernfield *et al.*, 1999). Cell surface proteoglycans consist of a core protein anchored to the membrane and linked to one or more glycosaminoglycan side chains (hepiran, keratin or chondroitin sulphates) to produce a structure that extends away from the cell surface. The internalisation of NPs inside the cell leads to degradation of glycosaminoglycans polymers and disruption of intracellular organelles (Hartig *et al.*, 2007). Nanoparticles with higher surface charge (zeta potential) are reported to binding strongly to the cell membrane and result in higher cellular uptake. After the internalisation of the nanoparticles on the cellular membrane, the uptake occurs via several possible mechanisms such as pinocytosis, nonspecific or receptor-mediated endocytosis or phagocytosis (Wilhelm *et al.*, 2003, Sahay *et al.*, 2010).

1.8 Mechanism of Cellular Uptake of TiO_2 NPs

Nanoparticles exert their actions on cellular cytotoxicity through two mechanisms i.e. internalisation and signal transduction. It has been established that the main way cells internalise aggregates (agglomerates) is cellular endocytosis (Conner and Schmid, 2003). Different types of endocytotic mechanisms are known, such as phagocytosis, pinocytosis, and macropinocytosis (Muhlfeld *et al.*, 2008). Endocytosis of nanoparticles depends on the size, shape, charge of nanoparticles and cell type (Harush-Frenkel *et al.*, 2007, Lai *et al.*, 2007, Harush-Frenkel *et al.*, 2008). There are three main types of endocytotic pathways in non-phagocytic cells, namely, clathrin-mediated endocytosis (CME), caveolin-mediated endocytosis (Cav-Me) and macropinocytosis (Thurn *et al.*, 2011). Therefore, taking into account the dimension of titanium dioxide nanoparticles and their aggregates; phagocytosis and macropinocytosis have been identified as the most probable uptake mechanisms leading to induction of cytotoxicity (Churg *et al.*, 1998, Singh *et al.*, 2007, Simon-Deckers *et al.*, 2008, Hussain *et al.*, 2009, Andersson *et al.*, 2011).

1.9 Induction of cell death by titanium dioxide nanoparticles (TiO_2 NPs)

A recent study provided molecular evidence that TiO_2 NPs preferentially cause cell death through the lysosomal pathway through conversion of these particles to ionic titanium in lysosomes. In Figure 3, ionic titanium, also known as a Fenton-type reagent, leads to the generation of hydroxyl radicals (Tengvall *et al.*, 1989b, Tengvall *et al.*, 1989a) enhanced generation of reactive oxygen species and subsequent cellular damage due to severe oxidative stress (Zhu *et al.*, 2012). In addition, severe oxidative stress is associated with lysosomal membrane permeabilization and subsequent necrosis, which is controlled by complex signalling pathways (Vandenabeele *et al.*, 2010, Tang *et al.*, 2011). Moreover, there are signalling cascades that have been associated with TiO_2 NPs cytotoxicity. They involve mechanisms such as P53 mediated pathway (Tucci *et al.*, 2013) and reactive oxygen species (ROS)-mediated fas upregulation and bax activation (Yoo *et al.*, 2012).

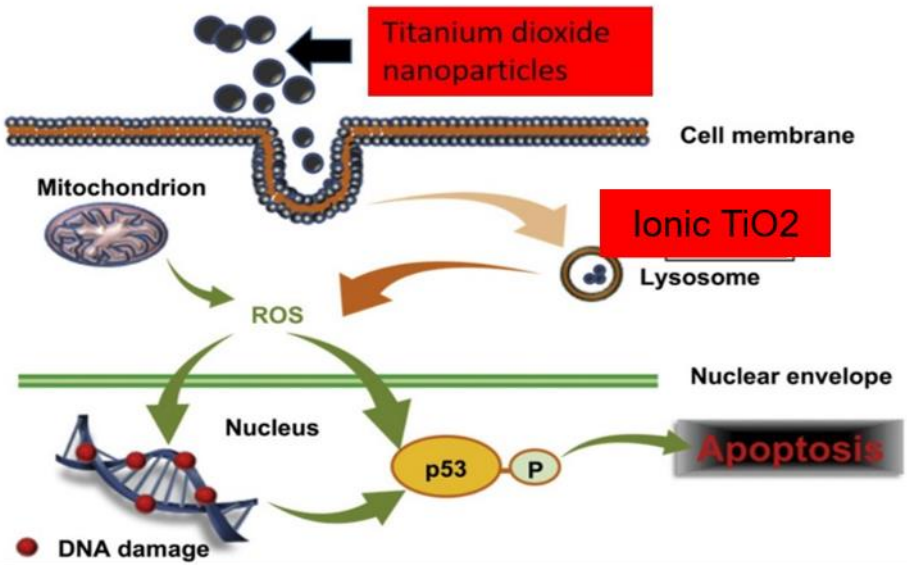

Figure 3: **Proposed cellular response mechanism involving p53 pathway**. TiO_2 NPs underwent extracellular and intracellular dissolution to form ionic titanium dioxide. Ionic Titanium dioxide raises reactive oxygen species (ROS) levels by an unknown mechanism. Increased ROS levels trigger p53 pathway directly or indirectly through DNA damage and the apoptotic machinery is activated (Ng *et al.*, 2011).

1.10 The effect of titanium dioxide nanoparticles (TiO_2 NPs) on nitric oxide (NO) induced cytotoxicity

Nitric oxide (NO) is the body's most ubiquitous second messenger which plays a pivotal role in vascular functions such as endothelium-dependent relaxation (Persson *et al.*, 1990), angiogenesis (Matsunaga *et al.*, 2002), and leukocyte adhesion (Nabah *et al.*, 2005). Alterations in NO bioavailability has been associated with exposure to particulate matter *in vivo* (Knuckles *et al.*, 2008) and *in vitro* (Nurkiewicz *et al.*, 2004). This alteration is thought to be due to the uncoupling of endothelial nitric oxide synthase (eNOS). eNOS is an enzyme responsible for the regulation of vascular endothelial cell function through production of NO. However, its bioavailability is affected by multiple factors, including increased oxidative stress which results in uncoupling of eNOS with subsequently less NO and more superoxide generation (Figure 2). This occurs due to the enhanced oxidation of BH4 (tetrahydrobiopterin) which in turn leads to a decrease in BH4 bioavailability. As a pivotal factor, BH4 is necessary for optimal eNOS activity which facilitates NADPH-derived electron transfer from eNOS reductase to the oxygenase domain to convert L-arginine to NO and L-citrulline. Therefore, when BH4 levels are inadequate, eNOS becomes unstable and uncoupled, leading to subsequently less NO production and more superoxide generation followed by oxidative stress and cell death (Kuzkaya *et al.*, 2003, Kietadisorn *et al.*, 2012). In this state, eNOS produces reactive oxygen species (ROS) rather than NO.

It has been shown that microvascular nitric oxide production is reduced after titanium dioxide particles exposure due to the fact that ROS is capable of consuming NO (Förstermann and Münzel, 2006). In contrast, Gurr *et al* 2005, reported that titanium dioxide nanoparticles are capable of causing or inducing the generation of hydrogen peroxide and nitric oxide, leading to lipid peroxidation and oxidative DNA damage *in vitro* (Gurr *et al.*, 2005).

There have been other reports on increased nitric oxide production in lung epithelial cells treated with titanium dioxide nanoparticles (Fu *et al.*, 2014). This was attributed to ROS formation in the cells which enhances cytosolic calcium concentration and activation of transcription factors, triggering the up-regulation of several proinflammatory genes, including the gene for inducible nitric oxide synthase (Blackford Jr *et al.*, 1997). Although studies have observed both decreases and increase in nitric oxide production to be associated with cell death when cells are treated with titanium dioxide nanoparticles; the mechanism driving nitric oxide cytotoxicity remain unelucidated.

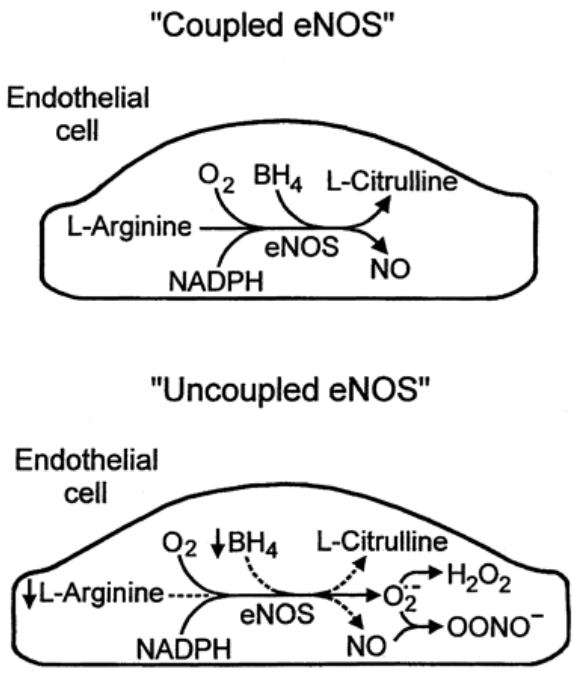

Figure 4: Schematic representation of "uncoupling" of nitric oxide (NO) synthesis. Suboptimal concentrations (↓) tetrahydrobiopterin (BH4) are required for "uncoupling." Superoxide anion (O_2^-); hydrogen peroxide (H2O2); peroxynitrite anion (OONO−); endothelial NO synthase (eNOS) adapted from (Katusic, 2001).

1.11 The effects of NO in cellular signalling

Nitric oxide is a diatomic free radical and one of the most essential signalling molecules in mammalian physiology. Nitric oxide mediates various physiological events such as smooth muscle relaxation,

vasodilation, neurotransmission, inhibition of platelet aggregation, and immunomodulation (Beckman and Koppenol, 1996, Murad, 2006). Additionally, at the cellular level, NO regulates cell growth, survival, apoptosis, proliferation and differentiation (Murad, 2006) via NO-cGMP dependent or independent pathway; however, manipulation of the NO-cGMP pathway by employing activators and inhibitors has been shown to have vast effects on cellular differentiation. The cGMP-dependent mechanism is mediated through its receptor soluble guanylyl cyclase (sGC) and cGMP-independent effects of NO are mediated through its interaction with metal complexes, oxygen (O_2) and superoxide (O_2^-) that mediates various downstream events (Beckman and Koppenol, 1996).

1.11.1 Nitric oxide (NO) induced cell cycle effect

Some studies have reported on other mechanisms involving NO that include the formation of NO-induced, post-translational modifications (Bian *et al.*, 2006, Villalobo, 2006, Martínez-Ruiz *et al.*, 2011). These modifications activate pathways that are cGMP-independent such as apoptotic proteins as well as mitochondria-related mechanisms (Figure 5). The involvement of cGMP in growth inhibition has been described in vascular smooth muscle cells (VSMCs), in which NO activates GC (guanylate cyclase) with a subsequent increase in cGMP leading to the phosphorylation of a vasodilator-stimulated phosphoprotein and subsequent inhibition of the epidermal growth factor (EGFR) signalling pathway (Yu *et al.*, 1997, Chen *et al.*, 2004).

Nitric oxide has been suggested to be a pathophysiological modulator of cell proliferation, cell cycle arrest and apoptosis (Napoli *et al.*, 2013). Anti-proliferative effects induced by endogenous NO has been found in various cell types, including vascular smooth muscle cells (VSMCs) (Garg and Hassid, 1989, Nakaki *et al.*, 1990, Hogan *et al.*, 1992, Nunokawa and Tanaka, 1992, Albrecht *et al.*, 2003, Villalobo, 2006). In contrast, studies have reported that NO can also promote cell proliferation under certain conditions, although the mechanisms responsible for this effect have remained poorly understood (Bian and Murad, 2003, Thomas *et al.*, 2008).

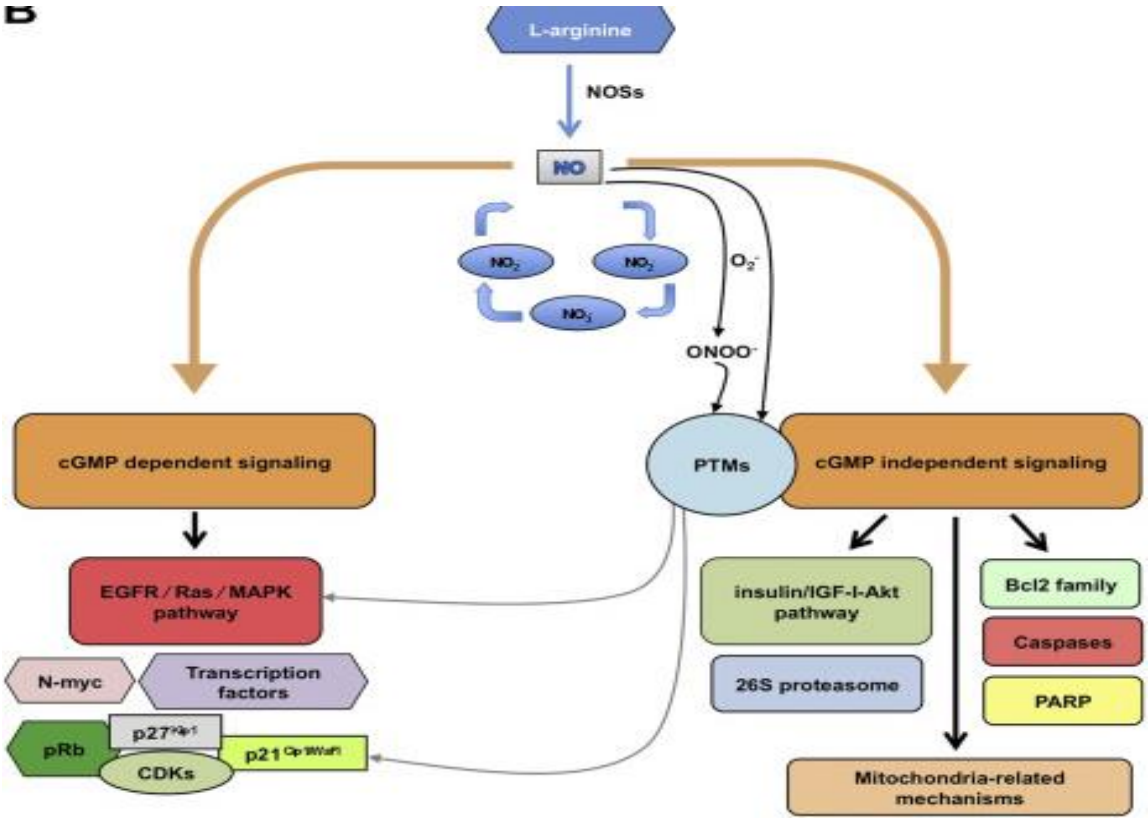

Figure 4: Molecular Mechanisms Involved in the Effects of NO on the Cell Cycle. cGMP-dependent signalling pathway activates subsequent kinases responsible for cell grow. cGMP-independent signalling pathway activates (Beckman and Koppenol, 1996) proliferation kinases and apoptotic proteins as well as mitochondria-related mechanisms adapted from (Napoli *et al.*, 2013).

1.12 Significance of the Study

Nanoparticle characterisation with respect to the size, zeta potential and aggregation is an important factor to consider when improving experimental procedures. However, before attempting nanoparticle characterisation, standardising procedures and methods are critical. Nanoparticle stabilisation is one of the essential factors during nanoparticle characterisation as it influences nanoparticle physiochemical properties such as size, zeta potential and aggregation.

These properties have been shown to alter extracellular signalling mechanisms and negatively affects cellular physiology, leading to cellular toxicity. Therefore, there is a need to investigate the stabilisation of TiO_2 NPs to obtain a true reflection of the cytotoxic effects of TiO_2 NPs.

The present study focused on the stabilisation of TiO_2 NPs with various concentrations of BSA Using BSA will be advantageous in nanotechnological application as it could result in the production of less toxic commercial products coated with TO_2 NPs.

1.13 Hypothesis

In this study, we investigated the effects of BSA-stabilized TiO_2 NP on physiochemical properties such as size, zeta potential and aggregation state, and their effects on cell viability, cellular proliferation and NO production in a mouse myoblast cell line. We hypothesised that smaller titanium dioxide NPs aggregation will enhance cytotoxicity when compared to larger particle aggregates due to the fact that they induce cellular oxidative stress, resulting in the dysregulation of NO synthesis thereby contributing to cellular DNA damage and death.

1.14 Aim

The main aim of the study is to investigate the *in vitro* cytotoxic effects of stabilised titanium dioxide nanoparticles aggregation using mouse myoblast cells.

1.15 Objective

I. To characterize TiO_2 nanoparticle aggregation using nanoparticle tracking analysis and transmission electron microscopy.
II. To determine the anti-proliferative and cytotoxic effect of titanium dioxide (TiO_2) aggregation on mouse myoblast cell lines (C2C12) using a BrdU DNA and MTT assay respectively.
III. To determine the effect of titanium dioxide nanoparticles on the synthesis of nitric oxide *in vitro* using a nitric oxide colorimetric biochemical assay.

Chapter 2: Manuscript Submitted to the Journal of Nanotoxicology

Investigating the *in vitro* Cytotoxic Effects of Bovine Serum Albumin Stabilised Titanium Dioxide Nanoparticles using Mouse Myoblast Cells

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Abstract

Titanium dioxide nanoparticles NPs (TiO_2 NPs) is one of the most widely used nanoparticles in many industrial sectors worldwide. However, extensive research, as well as stringent regulations, are necessary in order to ensure their safe usage. Recently, concerns about the harmful effects of TiO_2 NPs on human health have been associated with its physiochemical properties namely size, surface charge and aggregation state. Therefore, this study investigated the efficacy of bovine serum albumin (BSA) as a stabilising agent to reduce TiO_2 NPs aggregation for *in-vitro* cytotoxic testing using mouse myoblast cells (C2C12). Titanium dioxide NPs were stabilised with varying concentrations of BSA (0.3, 0.5, 0.8, 1.0 and 1.5 mg/ml) before size, surface charge and state of aggregation were characterised using nanoparticle tracking analysis and transmission electron microscopy. The *in-vitro* cytotoxic effect of BSA-TiO_2 and TiO_2 (excluding BSA) NPs on C2C12 cells were assessed using the MTT assay, BrdU DNA and functional effects using the NO biochemical colorimetric assay. Results showed an increase in size distribution, surface charge and reduced aggregation in BSA-TiO_2 NPs in comparison to TiO_2 NPs. There was a significant decrease in cell viability percentage and proliferation in cells treated with BSA (0.8 mg/ml) stabilised TiO_2 NPs in comparison to cells treated with TiO_2 NPs excluding BSA ($p<0.05$). A significant difference in NO concentration was also observed in cells treated with BSA-TiO_2 NPs ($p<0.05$) in comparison to cells treated with TiO_2 NPs excluding BSA ($p<0.05$). Therefore, the findings of this study indicated that BSA at a concentration of 0.8 mg/ml reduced aggregation of TiO_2 NPs resulting in increased cytotoxicity that decreased cell proliferation and NO production.

KEYWORDS: Nanoparticles, Titanium dioxide, Bovine serum albumin, Aggregation, Cytotoxicity

Introduction

Nanotechnology, the manipulation of matter on a near-atomic scale to produce new structures, materials and devices has revolutionised almost every technological and industrial sector of the world today. The key focus of nanotechnology is the synthesis of engineered nanoparticles (NPs) that exhibit characteristics such as small size, large surface area to mass ratio, shape, surface charge (zeta potential), reactive surface groups, dissolution rate, the state of aggregation or dispersal. These characteristics confer on NPs properties that are substantially different from their bulk counterparts. (Handy and Shaw, 2007, Ling *et al.*, 2011, Li and Nel, 2011). These properties offer immense opportunities for the development of new NPs for industrial, environmental and biomedical applications. Extensive research has been conducted to advance nanotechnology and its applications. However, the occupational health and safety, nanotoxicology and nano-risk aspects of NPs are still in its formative phase (Oberdörster *et al.*, 2005a, Yokel and MacPhail, 2011, Clift *et al.*, 2011). Therefore, there is a significant need to elucidate the interactions of NPs with other biomolecules, cells, and tissues since these studies could provide a foundation for engineering the next generation of nontoxic nanomaterials, that can effectively target and accumulate in diseased cells (Nel *et al.*, 2009) as advanced drug delivery, diagnostic and therapeutic agents (McGowan, 2012).

Titanium dioxide (TiO_2) is a common example of one of the most widely manufactured and commercially used NP world-wide. These NPs are found primarily in the form of the mineral rutile, anatase and brookite (Hedenborg, 1988, Duan *et al.*, 2010). They have been reported to exhibit thermal stability, relatively low toxicity, and excellent physiochemical properties which makes them biocompatible and are widely used in paints, printing ink, rubber, paper, cosmetics, sunscreens, food products, car materials, cleaning air products, decomposing organic matter, orthopaedic and dental prostheses coatings (Jacobs *et al.*, 1991, Wolf *et al.*, 2003, Warheit *et al.*, 2007, Iavicoli *et al.*, 2012). Although this NP is widely used commercially and medically, its toxicological profile is not fully profiled with several concerns pertaining to potential toxic effects of TiO_2 NPs with regards to the harmful interaction with biological systems and the environment (Nel *et al.*, 2006).

Since TiO_2 NPs are used in various fields, they have gained increasing interest in research (Oberdörster *et al.*, 2005b, Nel *et al.*, 2006, Lewinski *et al.*, 2008) and they are the main target for toxicological studies due to the differences in their physicochemical properties. Several studies have investigated the toxicity of nanoparticles based on various characteristics, such as shape, size, surface chemistry, chemical composition, surface activity, solubility and aggregation. Although studies have been published showing experimental evidence of the toxic effects of TiO_2 NPs, the cellular physiological and biochemical effects of TiO_2 NPs *in vitro* still need elucidation. It is, therefore, of importance to understand the toxicological properties of TiO_2 NPs relating to direct and indirect environmental exposure *in vitro,* and the mechanisms leading to toxicity of these nanoparticles. Regarding TiO_2 NP physiochemical properties, aggregation per se, has been shown to be the primary factor in determining cytotoxicity as this factor has a direct impact on cellular effects (Baveye and Laba, 2008, Waters *et al.*, 2009, Okuda-Shimazaki *et al.*, 2010). There is a paucity of reports on the effects of TiO_2 NP aggregation and cellular activity. Furthermore, despite the growing interest in NPs and their effect on the body, standardised procedures have not yet been outlined for the evaluation of nanoparticle toxicity (Teeguarden *et al.*, 2007, Vippola *et al.*, 2009, Dhawan and Sharma, 2010). Optimising experimental procedures for nanoparticle toxicity assessment in order to gather reliable data is a priority as there are major gaps in knowledge related to risk assessment for the use of TiO_2 NPs (Williams *et al.*, 2005, Kong *et al.*, 2011).

The present study aims to investigate the efficacy of bovine serum albumin (BSA) as a stabilising agent to reduce TiO_2 NPs aggregation for *in vitro* cytotoxic and functional testing using mouse myoblast cells. The stabilisation of TiO_2 NPs would be beneficial in toxicological studies as the variations in aggregation state would affect the magnitude of the cellular toxicity and cellular function.

Methods

Nanoparticles

Titanium (IV) oxide nanopowder, (Aeroxide® TiO2 P-25; 21 nm primary particle size; 80% anatase/ 20% rutile; TEM; ≥ 99.5 trace metals basis) was purchased from Sigma-Aldrich, 71.

Cell Culture

C2C12 mouse myoblast cells (ATCC® CRL-1772™) were cultured in Dulbecco's Modified Eagle Medium (Lonza, Verviers, Belgium), 10% fetal bovine serum and 1% penicillin. Cells were cultured to a confluence of 60-80%. The cells were maintained at 37°C with 5% CO_2.

Characterisation of the Size, Surface Charge and Aggregation State of Titanium Dioxide Nanoparticles (TiO_2 NPs)

Titanium dioxide nanoparticles were prepared by dissolving in dH_2O to achieve a concentration of 1.0 mg/ml and thereafter sonicated for 10 minutes. Bovine serum albumin lyophilized powder (Bio-Rad Laboratories, Hercules, CA, USA), with fatty acid and globulin content smaller than 1% was prepared by dissolving in dH_2O, followed by sonication at 10 min and sterile filtration through a 0.2 mm low-protein-binding syringe filter (Cellulose acetate, GVS™, Europe). Bovine serum albumin (BSA) was used as a stabilising agent to influence TiO_2 nanoparticle aggregation. Various concentrations of BSA (0.3, 0.5, 0.8, 1.0, and 1.5 mg/ml) were used to suspend the TiO_2 NPs The nanoparticle suspension and respective BSA solution was mixed (1:1 ratio) prior to characterisation. The characterization of TiO_2 in BSA was determined using Nanoparticle Tracking Analysis (NTA) and Transmission Electron Microscopy (TEM).

Nanoparticle Tracking Analysis (NTA)

Quantification and size distribution of titanium dioxide nanoparticles (TiO_2 NPs) was determined using the NS500 equipped with a 405 nm laser and sCMOS camera (NanoSight NTA 3.0 Nanoparticle Tracking and Analysis Release, Version Build 0069). Samples were diluted with PBS prior to analysis to obtain particle distribution of 10 and 100 particles per image (optimal, 50 particles per image) before analysis with NTA system. Samples were introduced into the sample chamber using the following script: PUMPLOAD, REPEATSTART, PRIME, DELAY 10, CAPTURE 60, REPEAT 5. Videos were recorded at a camera level of 10, camera shutter speed of 20 ms and camera gain of 600, these settings

were kept constant between samples. Each video was then analysed to give the mean particle size together with the concentration of particles. The size titanium dioxide nanoparticles were represented as the mean particle size ± SD.

Transmission Electron Microscopy

Samples were dropped cast onto formvar-coated 200 mesh copper grids. The overall volume of the drop cast was chosen to be V=5 µL (Michen *et al.*, 2015). The drop cast was dried overnight under a fume hood before TEM imaging. Electron micrographs of the TiO2 NPs were taken with JEOL 1010 transmission electron microscope (JEOL, Peabody, MA, USA) at the Electron Microscopy Unit, University of KwaZulu-Natal. Both 10 kV and 20 kV electron micrographs were captured.

Cytotoxicity

Cell viability was assessed using an MTT Cell Proliferation Assay Kit (Roche), after 12 hrs of exposure. This assay quantitatively assesses viable cells which have functional mitochondrial dehydrogenase enzymes which can reduce MTT to formazan. C2C12 myoblast cells were seeded onto 96-well plates at a cell density of 1.69×105 cells/well, and the plates were incubated overnight at 37°C, 5% CO_2. Thereafter, cells were exposed to TiO_2 nanoparticle concentrations ranging from 40 to 400 mg/ml for 24h at 37 °C, 5% CO_2. Post exposure, the cell culture media was carefully aspirated and analysed using light microscopy to determine cellular morphology. MTT labelling reagent (final concentration of 0.5 mg/ml) was added to each well and incubated at 37°C, 5% CO_2 overnight. After incubation, the samples were removed from the incubator and mixed with 100 µL solubilizing reagent and incubated at 37 °C, 5% CO_2 overnight. The absorbance of the samples was measured using a microplate reader. The wavelength to measure the absorbance of the formazan product was between 550 nm and 600 nm. An absorbance of 555 nm was selected. The reference wavelength was less than 640 nm. Data was expressed as a percentage cell viability. Untreated controls were also run under identical conditions.

Cell Proliferation

For cell proliferation assays, ethynyl-2′-deoxyuridine (EdU) incorporation assays were performed. C2C12 cells were seeded in 24-well plates and thereafter incubated with TiO_2 nanoparticles in their respective BSA concentrations. After 24-h incubation, cells were assayed using a Click-iT EdU Alexa Fluor® 488 cell proliferation assay kit (Invitrogen). Cell fixation,

permeabilization, and EdU detection were performed according to the manufacturer's instructions. 4′,6-Diamidino-2-phenylindole (DAPI) staining was used to identify nuclei for determination of cell number. EdU and DAPI signals were captured with the EVOS FLoid fluorescence microscope. The EdU and DAPI signals for each sample were captured and analysed.

Nitric Oxide Assay

Treated cells (1 $X10^5$ cells/treatment) (50 µl) was added in triplicate to the wells of a 96 well microplate. Sodium nitrite standards were also prepared (0 µM- 200 µM) and 50 µl of each was added in triplicate to the wells. This was followed by addition of 50 µl Vanadium "cocktail" to the standards, then 50 µl of Vanadium Chloride (VCl3), 25 µl 2% sulphanilamide (SULF) and 50 µl 0.1% N-1-napthylethylenediamine dihydrochloride (NEDD). The plate was then incubated (37 ºC) for 45 min in the dark, and the optical density read at 540/690 nm using a spectrophotometer (Bio-Tek MQx200, South Africa). A standard curve was constructed using the results obtained from the nitrite standards, and the resultant NO concentration for each sample was determined by extrapolation.

Statistical Analysis

Data was presented by arithmetic means of at least three independent experiments ± standard error measurements. One-way ANOVA (Tukey post hoc test) was used to determine intergroup variances for the level of significance between treatment methods. A p-value less than 0.05 was considered statistically significant. Statistical calculation was done using GrapPad Prism 5.

Results

Characterisation of the Aggregation state of Titanium Dioxide Nanoparticles

Figure 1 shows that the aggregation state of TiO_2 is affected by altered concentrations of BSA. TEM micrographs and NTA profiles indicate that particle aggregation is induced at a concentration greater than and less than 0.8 mg/ml BSA. It is evident that the NTA profiles are consistent with the TEM micrograph size distribution. There was a significant decrease in the modal particle size distribution of TiO_2 particles suspended in BSA at a concentration of 0.5-1.5 mg/ml in comparison to the control (Fig. 2, $p<0.05$). A significant decrease in the modal size distribution of TiO_2 suspended in 0.8 mg/ml in comparison to 0.3, 0.5, 1.0 and 1.5 mg/ml BSA (Fig. 2A, $p<0.05$). The cumulative percentage point of diameter distribution of TiO_2 in BSA is represented as 10, 50 and 90 % (Fig. 2B, C & D). A significant decrease in the D10, D50 and D90 of TiO_2 suspended in 0.8 mg/ml was obtained in comparison to the control, 0.3 and 0.5 mg/ml BSA.

The Cytotoxic Effect of Titanium Dioxide Nanoparticles (TiO_2 NPs) Aggregation on Cellular Proliferation

Bovine serum albumin (0.3, 0.5, 0.8, 1.0 and1.5 $mg.ml^{-1}$) was used as a stabiliser to reduce particle aggregation. A significant decrease in percentage cell viability was obtained using 0.8 $mg.ml^{-1}$ BSA in comparison to 0.3, 0.5, 1.0 & 1.5 $mg.ml^{-1}$ BSA (51.44 ± 14.74 vs. 61 ± 8.21, 68 ± 9.21, 62 ± 8.21, 64 ± 9.21% Fig 3a, $p<0.05$). These results indicate that reduced TiO_2 aggregation results in enhanced cellular cytotoxicity which was further validated by assessing cellular proliferation by the qualitative measure of DNA synthesis (Fig 3b). These results show that that TiO_2 stabilised with 0.8 $mg.ml^{-1}$ BSA has enhanced inhibitory effects on cellular proliferation in comparison to the controls and 1.5 $mg.ml^{-1}$ BSA.

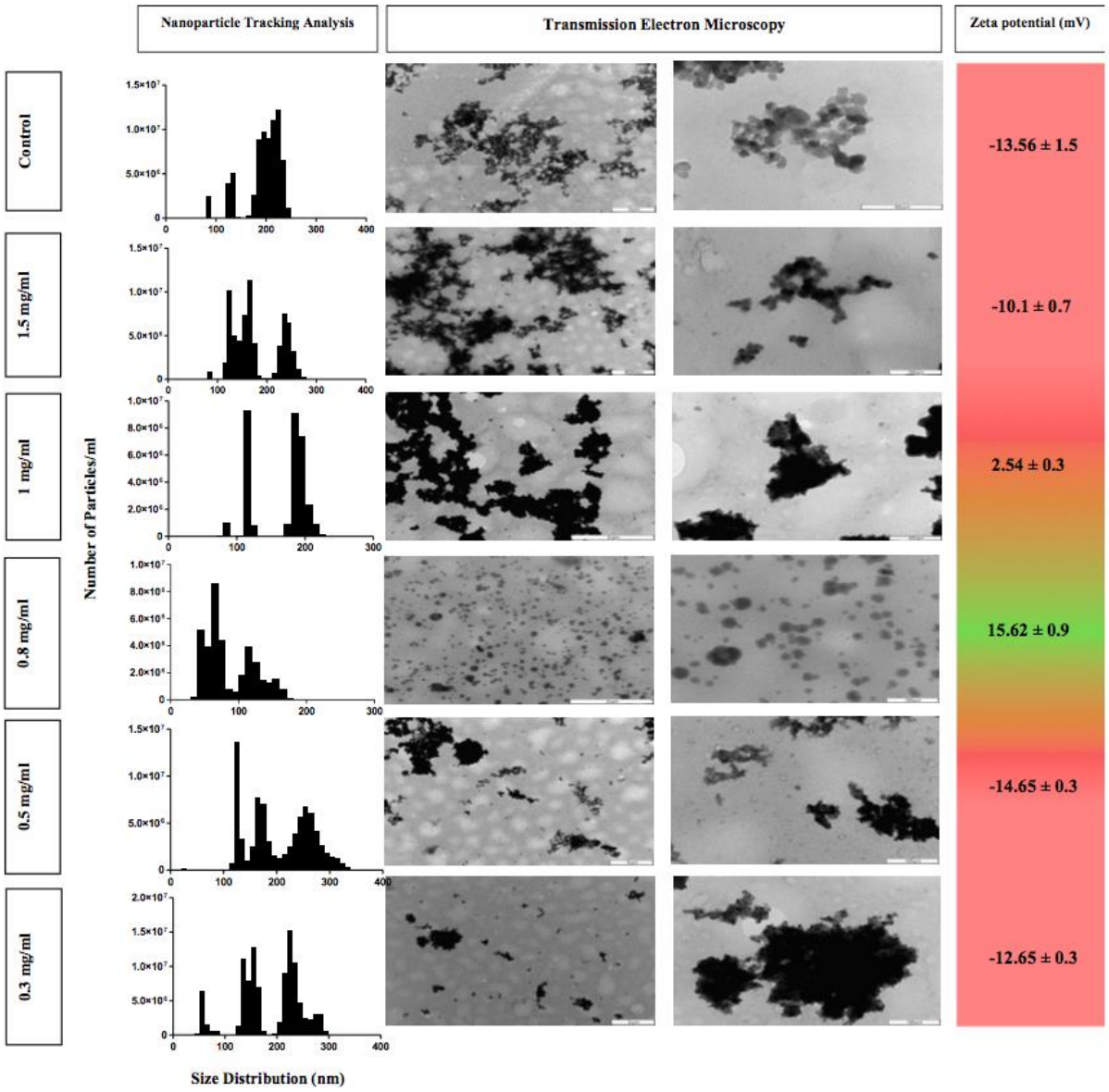

Figure 1: Effect of varying BSA concentrations on the surface charge and aggregation state of titanium dioxide nanoparticles. Nanoparticle tracking analysis shows the concentration and size profile of TiO_2 using the NanoSight500. Transmission electron micrographs show representative size distribution (10kV) and TiO_2 aggregates (50kV).

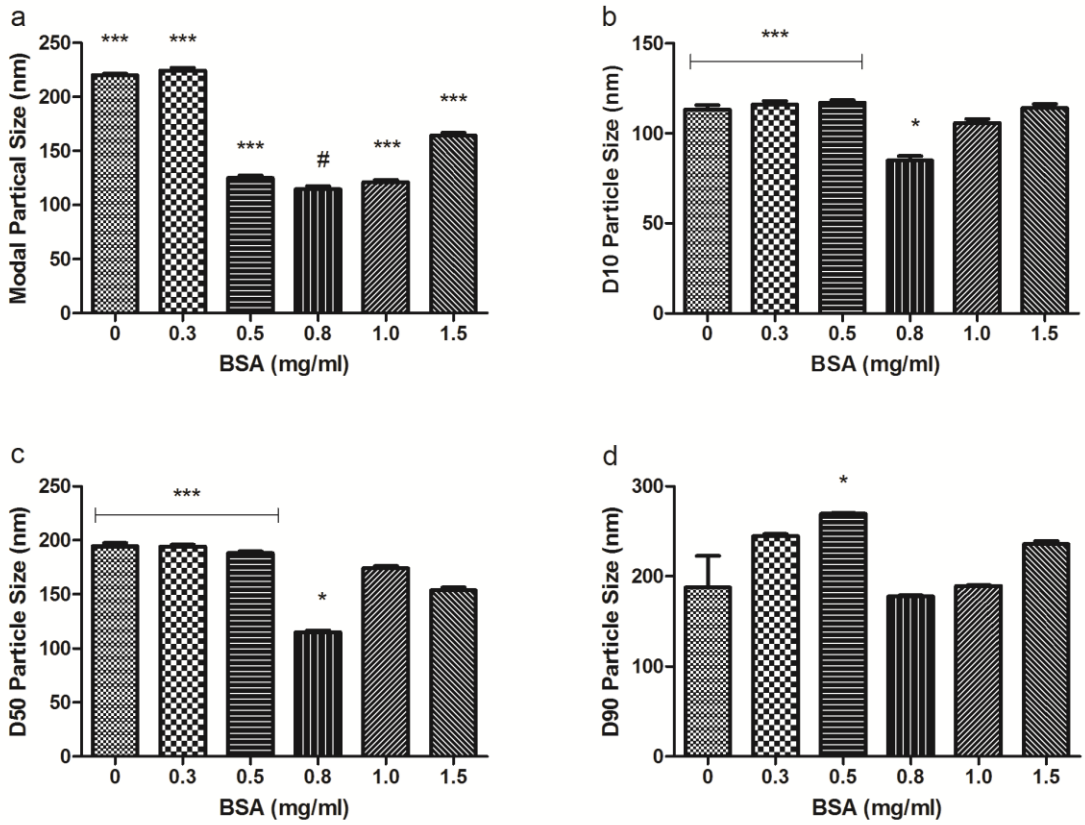

Figure 2: Particle Size Distribution of TiO_2 in BSA. Data is represented as the modal size (a) and the cumulative 10 (b), 50 (c) and 90 (d) % point of diameter. In A ***$p<0.001$ 0, 0.3, 0.5, 1.0, 1.5 vs 0.8 $mg.ml^{-1}$ BSA; #$p<0.05$ 0.8 vs 1.5 $mg.ml^{-1}$ BSA. In B & C ***$p<0.001$ 0, 0.3, 0.5 vs 0.8 $mg.ml^{-1}$ BSA; #$p<0.05$ 0.8 vs 1.0, 1.5 $mg.ml^{-1}$ BSA. In D *$p<0.05$ 0.5 vs 0.8 $mg.ml^{-1}$ BSA.

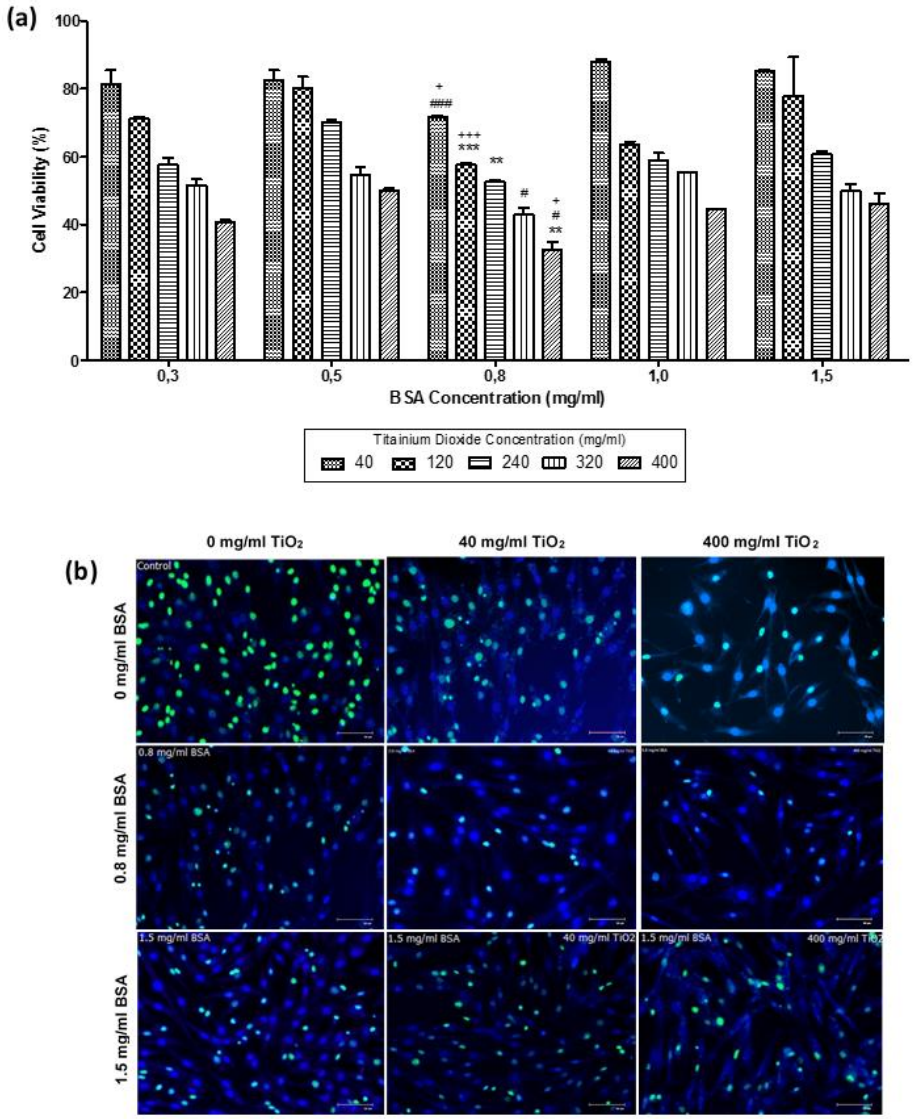

Figure 3: Effect of TiO_2 aggregation on cellular toxicity and proliferation. (a) Cytotoxicity (% Cell Viability) of TiO_2 aggregation stabilised in BSA (0.3 -1.5mg.ml^{-1}). (b) The effect of TiO_2 aggregation on cell proliferation using the Edu DNA incorporation assay. In A **p<0.01 & ***p<0.001 0.8 vs 0.5 mg.ml^{-1} BSA; #p<0.05 & ##p<0.01 0.8 vs 1.0 mg.ml^{-1} BSA; +p<0.05 & +++p<0.001 0.8 vs 1.0 mg.ml^{-1} BSA.

The effect of titanium dioxide nanoparticles (TiO_2 NPs) aggregation on Nitric Oxide (NO) Synthesis

We determined the effect of TiO_2 NP aggregation on nitric oxide synthesis using the NO colorimetric assay. Bovine serum albumin (0.3-1.5mg.ml-1) was used as a stabiliser to reduce particle aggregation. A significant decrease in cell nitrates was obtained using 0.8 mg.ml-1 BSA in comparison to 0.3, 0.5, 1.0 & 1.5 $mg.ml^{-1}$ BSA (0.94 ± 0.03 vs. 1.495 ± 0.05, 1.56 ± 0.06, 1.39 ± 0.04, 1.33 ± 0.06 $mg.ml^{-1}$ Fig 4, $p<0.05$). These results indicate that reduced TiO_2 aggregation results in NO synthesis (Fig 4). These results show that that TiO_2 stabilised with 0.8 mg.ml-1 BSA has enhanced inhibitory effect on NO synthesis in comparison to the controls and 1.5$mg.ml^{-1}$ BSA.

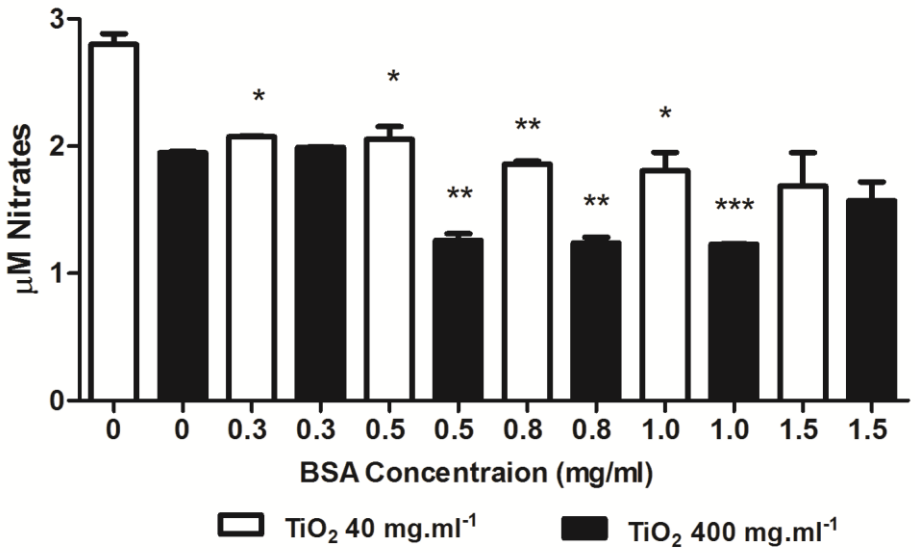

Figure 4: Effect of TiO_2 nanoparticle aggregation on cellular nitric oxide (NO) sythesis. (a) Cytotoxicity (nitrites µM) of TiO_2 aggregation stabilised in BSA (0.3 -1.5mg.ml-1). (b) The cytotoxic effect of TiO_2 aggregation on myoblast NO synthesis using NO biochemical assay. In TiO_2 40 mg/ml vs 0.3 mg/ml BSA, 0.5 mg/ml and 1.0 mg/ml *$p<0.05$ & **$p<0.05$ TiO_2 40 mg/ml vs 0.8 mg/ml BSA. **$p<0.05$ TiO_2 400 mg/ml vs 0.5 mg/ml BSA; **$p<0.05$ TiO_2 400 mg/ml vs 0.8 mg/ml; ***$p<0.05$ TiO_2 400 mg/ml vs 1.0 mg/ml BSA

Discussion

Titanium Dioxide Nanoparticles are a key constituent of various food and personal care products due to its photocatalytic properties; it is, therefore, necessary to understand the potential cytotoxic effects using standardised test methods. One of the potential factors influencing cytotoxicity is nanoparticle aggregation which is inversely proportional to cytotoxicity, an assertion, which is contested by some. Therefore this study investigated the cytotoxic effects of stabilised titanium dioxide nanoparticles in mouse myoblast cells (C2C12) at various degrees of aggregation using BSA as a stabilising agent. Bovine serum albumin has been explored as an effective dispersing agent for TiO_2 NPs due to synergistic effects of its multiple protein components (Bihari *et al.*, 2008, Mahl *et al.*, 2010, Graf *et al.*, 2012). This has also been supported by previous studies, which have shown that BSA can stabilise gold NPs via an electrostatic mechanism and prevent aggregation (Brewer *et al.*, 2005, Dominguez-Medina *et al.*, 2012). Whilst studies have demonstrated increased stabilisation with BSA, the effects of varying concentrations have not been explored and optimised, particularly in muscle cells. Therefore, in this study, varying concentrations BSA was used to study its effect on TiO_2 NP aggregation in mouse myoblast cell lines. Our findings indicate that an accurate measure of cellular toxicity can only be obtained from a particular state of aggregation. Importantly, the state of TiO_2 aggregation has also shown to have a direct relationship with optimum cellular function as indicated by nitric oxide synthesis. Titanium dioxide nanoparticles stabilised with 0.8 mg/ml of BSA had resulted in an optimum, monodisperse particle distribution which had a significant impact on cellular toxicity and nitric oxide synthesis in murine myoblast cells.

The effect of TiO_2 nanoparticle aggregation on cellular toxicity

Previous studies have not clearly elucidated the relationship between physiochemical properties of NPs and their toxicity (Shah *et al.*, 2017). These studies have however suggested that the cytotoxic effects of NPs are associated with its state of aggregation (Gatoo *et al.*, 2014, Shin *et al.*, 2015). It has also been suggested that NP aggregation is associated with functional alterations in cellular physiology, such as inhibition of replication, transcription, and cell proliferation (Yildirimer *et al.*, 2011). Therefore this study investigated the cytotoxic effects of TiO_2 NPs aggregation *in vitro*.

Several studies have shown that smaller/less aggregated particles are more toxic than larger particle aggregates (Pan *et al.*, 2007, Napierska *et al.*, 2009). Similarly, in our study, we observed increased cytotoxicity in less aggregated TiO_2 NPs **(Figure 3a, b and figure 4).**

However, we speculate that this is due to the ability of smaller aggregates to penetrate the cell membrane and locate itself to other cellular compartments such as DNA. In contrast, Sun *et al.,* 2017 found that larger TiO_2 NPs exhibited higher cellular uptake, suggesting that larger NPs strongly induced more cytotoxicity when compared to smaller size NPs. Interestingly, these findings further conclude that the cellular uptake of different sizes of NPs was energy dependent, suggesting that there are size-dependent uptake pathways. Albanes *et al.,* 2011 indicated that aggregation state of NPs does not elicit a unique toxic response, but it results in an increased uptake pattern of nanoparticles in monodispersed particles compared to aggregated particles. This suggested that the mechanism of interactions may play a significant role in understanding cellular uptake and cellular interactions with nanoparticles particles (Kong *et al.,* 2011).

Therefore the significant decrease in cellular viability and DNA replication observed with TiO_2 NPs (40, 120, 24, 320, 400 mg/ml) stabilised with 0.8 mg/ml of BSA was as a result of the reduced particle aggregation. In this study, we have shown that TiO_2 NP aggregation had an inverse relationship with myoblast cellular cytotoxicity and DNA replication. This finding is in keeping with other studies which have reported that prolonged exposure to titanium dioxide has negative effects on cellular physiology such as increased lipid peroxidation, DNA damage (Wang *et al.*, 2007, Wang *et al.*, 2009, Hackenberg *et al.*, 2010, Guichard *et al.*, 2012), caspase activation which leads to micronuclei formation (Rahman *et al.*, 2002), chromatin condensation (Chen *et al.,* 2014) and eventual cell death via apoptosis (52). The reduction in DNA replication was indicative of the reduction in cellular proliferation which could have occurred due to the enhanced oxidative stress which induced cellular cytotoxicity(Donaldson *et al.*, 2003, Long *et al.*, 2007, Park *et al.*, 2008, Guichard *et al.*, 2012). These findings suggest that the effects of BSA as a stabilising agent is effective in reducing TiO2 NP aggregation at an optimal concentration which is imperative for future NP toxicological studies.

The effect of TiO_2 nanoparticle aggregation on nitric oxide synthesis

A significant decrease in NO concentration was observed in TiO2 NPs (40, and 400 mg/ml) stabilised with 0.8 mg/ml BSA-stabilized. Nitric oxide is known to be involved in various physiological events such as smooth muscle relaxation, vasodilation, neurotransmission, inhibition of platelet aggregation, and immunomodulation (Beckman and Koppenol, 1996, Murad, 2006). Additionally, at the cellular level, NO regulates cell growth, survival, apoptosis, proliferation and differentiation (Murad, 2006) via NO-cGMP dependent or independent

pathway. NO-cGMP independent pathway has been reported to be mostly affected by cellular interaction with metal complexes, oxygen (O_2) and superoxide (O_2.-). We, therefore speculate that cellular interaction of monodisperse TiO_2 NPs induces ROS activation which in turn may affect the NO-cGMP independent pathway, resulting in inadequate NO thereby leading to a decrease in cellular proliferation (Beckman and Koppenol, 1996). This finding suggests that the translocation of monodisperse TiO_2 NPs from the dermis or gut to skeletal muscle could negatively impact skeletal muscle biology related to mitochondrial biogenesis and muscle contractility.

Conclusion

This study demonstrates a significant increase in cellular toxicity due to reduced TiO_2 NP aggregation with an optimal concentration of BSA. These findings suggest that stabilising nanoparticles using optimum concentrations of BSA prior to *in vitro* testing is required for future NP toxicological studies. However, additional studies are required to understand the toxicological effects of TiO2 NP physiochemical properties (such as size, shape, aggregation state) *in-vivo.*

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Disclosure

The authors of this study declare that there are no conflicts of interest present.

Authors' contribution

Professor I Mackraj and Mr P Pillay contributed to the concept and design of the study. Mr P Pillay, Dr K Moodley in the analysis of TiO_2 NPs cytotoxicity and statistical analysis. Dr K Maduray and Dr K Moodley worked on the NO assay. All of the authors contributed to compiling and editing the manuscript.

Ethics

Ethical approval for this study was obtained from the University of Kwa-Zulu Natal Biomedical Research Ethics Committee on 11/07/16. The BREC reference number allocated to this study is BE230/16.

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CHAPTER 3: SYNTHESIS, CONCLUSION AND RECOMMENDATION

3.1 Synthesis

The global demand for engineered nanoparticles (NPs) has increased worldwide with titanium dioxide (TiO_2) NPs emerging as one of the most extensively used NPs in food, drug and cosmetic products due to their physiochemical properties, such as strength, resistance to corrosion, machinability, biocompatibility and photocatalytic properties (Chen *et al.*, 2009, Morishige *et al.*, 2010). In recent years, the influence of TiO_2 nanoparticle physiochemical properties (size and aggregation) on cellular toxicity has become a health risk. This is mainly due to the ability of TiO_2 NPs to permeate cellular membranes and interact with components of cells, such as proteins and lipids which compromise cellular functions, leading to cytotoxicity (Wu *et al.*, 2014, Logan *et al.*, 2015, Catauro *et al.*, 2015). It is therefore necessary to determine the effect of physiochemical properties of stabilised TiO_2 NPs on cellular function in order to assess the true toxicological properties of TiO_2 NPs.

3.1.1 Stabilisation of Nanoparticles

The stabilisation of nanoparticles is normally carried out before their actual use in any technological application (Jadhav *et al.*, 2015). Various organic compounds are used to stabilise nanoparticles by altering their surface properties and preventing them from aggregation. Simple surfactants (Alexandridis, 2011), bi-functional organic compounds (Venugopal *et al.*, 2013, Li *et al.*, 2013, Mourdikoudis and Liz-Marzán, 2013, Xu *et al.*, 2013), natural polymers or biological materials (Jadhav, 2012, Jadhav and Bongiovanni, 2012); oligomers or polymers bearing different functional groups are used for this purpose (Gasilova *et al.*, 2010, Iravani, 2011, Nicolás *et al.*, 2013). The interacting groups from these stabilisers react with the complementary groups present on the surface of nanoparticles or directly with the surface atoms forming stable monolayers by chemisorption. The functional groups present in the stabiliser or ligand's structure greatly alter the properties of the nanoparticles (Jadhav *et al.*, 2015), which may affect the toxicological profiles of these molecules.

In this study, BSA was used as a stabilising agent to reduce TiO_2 NPs aggregation prior to *in vitro* cellular testing. Bovine serum albumin has been shown to generate small agglomerates of primary particles and improve the stability of dispersions (Vippola *et al.*, 2009). Nanoparticle characterization is critical for the production process of drugs and cosmetics. More importantly, it is a vital control point in the pharmaceutical industry in order to evaluate the efficacy of the drug delivery strategy and to ascertain potentially dangerous, fatal and immunogenic responses in patients treated with nanoparticle-based drugs (Cho *et al.*, 2013b).

Previous studies have used various techniques for the characterization of nanoparticle and stabiliser interactions. Some studies have used Dynamic Light Scattering (DLS), zeta-potential measurements,

and scanning electron microscopy, while others have used disc centrifugation for the size characterization of nanoparticles (Tantra *et al.,* 2010, Cho *et al.*, 2013a). However, these techniques have their limitations, in that they do not offer sufficient sensitivity and selectivity to probe into the mechanism of the interaction in great detail (Tantra *et al.*, 2010). The additional use of techniques such as nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM) plays a crucial role in determining the mechanism behind the nanoparticle–stabiliser interaction. Nanoparticle tracking analysis detects individual NPs and thus provides a high size resolution of nanoparticles in suspension. (Saveyn *et al.*, 2010). Furthermore, this technique is easily reproducible, accurate, less expensive and suitable for a low resource setting (White *et al.*, 2012). In addition, TEM is advantageous as it confirms characterization of NPs by deriving data on the particle shape, size and distribution as well as enables imaging of the degree of aggregation

In the present study, we assessed TiO_2 NP size distribution, zeta potential (surface charge) and aggregation using NTA, and TEM analysis. Our results showed that an optimal concentration of BSA improved particle size distribution, surface charge and degree of aggregation. These findings are in keeping with others who demonstrated that BSA coated nanoparticles resulted in improved nanoparticle colloidal state stability and reduced aggregation (Zuo *et al.*, 2015).

3.1.2 The effect of BSA on nanoparticle aggregation

We observed that TiO_2 NPs stabilized with 0.8 mg/ml BSA, in comparison to other BSA concentrations (0.3, 0.5, 1.0 and 1.5 mg/ml) resulted in significantly increased TiO_2 size distribution, surface charge and decreased aggregated NPs (p-value <0.05) (figure 1, 2, and 3).This could be due to the stabilising properties of BSA, as it forms a thin protective layer that masks the Van der Waals interactions between particles (Bihari *et al.*, 2008, Ji *et al.*, 2010, Mahl *et al.*, 2010, Wiogo *et al.*, 2010). The BSA protein coating maintains the colloidal stability, reduces particle-particle interaction, improves particle surface charge (zeta potential) and prevents aggregation. It has also been shown to improve dispersion of nanoparticles. Our findings with BSA-coated titanium dioxide are in agreement with these findings (Brewer *et al.*, 2005, Bihari *et al.*, 2008, Mahl *et al.*, 2010, Graf *et al.*, 2012, Dominguez-Medina *et al.*, 2012). Another important aspect of this study was to determine the effect of TiO_2 NP aggregation state on *in vitro* cellular toxicity.

3.1.3 Effect of BSA-stabilized NPs on cytotoxicity

Aggregation of nanoparticles have been shown to have an extensive impact on cytotoxicity and may result in either cellular death or physiological imbalances since the uptake mechanism differs between monodispersed and aggregated NPs (Albanese and Chan, 2011, Yildirimer *et al.*, 2011, Panariti *et al.*, 2012). In addition, studies have reported that reduced particle aggregation is more toxic in comparison

to larger particle aggregation due to its ability permeate the cell membrane and disrupt the cellular compartments (Yildirimer *et al.*, 2011). In the current study, we have shown that less aggregated TiO_2 NPs results in enhanced cytotoxicity, as evidenced by a significant decrease in cell viability. These findings are in accordance with other studies that found smaller TiO_2 NPs in comparison to larger ones had higher oxidative stress. Interestingly, the above study has also reported that smaller TiO_2 NPs can also induce genotoxic effects, chromosome aberrations, DNA double-strand breaks and cell death (Hussain and Kharisov, 2016). We also observed severe DNA damage in cells treated with smaller TiO_2 NPs aggregates (Figure 5). In contrast, a study done by Okuda- Shimazaki *et al.* 2010, has reported that large titanium aggregates showed enhanced cytotoxicity when compared with the small aggregates. Hence large TiO_2 NPs were more cytotoxic that small TiO_2. They speculated that the cells incorporate increased large titanium nanoparticles v*ia* phagocytosis into the cytoplasm, due to size-dependent uptake mechanisms inherent in different cell types (Okuda-Shimazaki *et al.*, 2010).

Whilst it has been suggested that the disruption of cellular physiology by nanoparticles is induced by the internalisation of particles of varying sizes (Geiser *et al.*, 2005, Blank *et al.*, 2006, Chithrani and Chan, 2007), the mechanism of action is not clearly understood. Additionally, studies have reported that nanoparticle toxicity is determined by the type of NP as well as the physiological cellular responses and metabolic processes that are affected (Lankoff *et al.*, 2012).

This study showed a significant increase in the cytotoxicity of TiO_2 NPs stabilised with BSA at concentration of TiO2 NPs with 0.5, 0.8 and 1 mg/ml BSA compared to the controls and TiO_2 NPs in combination with 0.3 and 1.5 mg/ml BSA. Our findings correspond with a similar study which indicated that reduced particle aggregation results in enhanced cytotoxicity (Naljayan and Karumanchi, 2013). Therefore, the stabilisation of NPs using varying concentrations of BSA demonstrated that the concentration of BSA that reduces TiO_2 NP aggregation enhances cellular cytotoxicity.

3.1.4 The impact of nanoparticle aggregation on cellular function

A reduction in particle aggregates enables the particles to easily permeate the cell membrane and affect cellular components by locating in the cellular DNA which results in anti-proliferation effects and DNA damage, therefore, inhibiting cell growth and altering cellular morphology. Furthermore, recent studies have reported that the physiochemical properties of TiO_2 NPs and type of cell model used impacts the cytotoxic activities of TiO_2 NPs.There is currently no literature on the effects of TiO_2 NP aggregation on mouse myoblast cell line. Therefore, the present study used mouse myoblast cells as a model for *in vitro* testing of the cytotoxic effects of TiO_2 NPs aggregates. The use of established myoblast C2C12 cells, has numerous advantages for cytotoxicity testing, as they provide repeatable and reproducible systems, which reduce the number of animal studies (Ikeda *et al.*, 2017). These cells also differentiate rapidly, forming contractile myotubes and producing characteristic muscle protein (Allen *et al.*, 2005) which make them a good model to study signalling molecules affected during cytotoxicity.

It is known that TiO_2 NPs induce cellular cytotoxicity via independent apoptotic signalling pathways or necrotic cell death, such as cytoplasmic membrane rupture (Hussain *et al.*, 2005, Sayes *et al.*, 2006, Thevenot *et al.*, 2008). Another common feature emerging from current studies is that exposure of TiO_2 NPs increases the generation of reactive oxygen species (ROS) and activates oxidative stress (OS) mediated pathway. This has been reported to be probably due to the conversion of TiO_2 NPs to ionic TiO_2 in the lysosome. The ionic TiO_2 raises reactive oxygen species (ROS) levels by an unknown mechanism and results to induced oxidative stress followed by DNA damage (Ng *et al.*, 2011). In the present study, increased cellular DNA damage in cells treated with TiO_2 NPs with less aggregation was observed. We speculate that this DNA damage was due to increased cellular ROS, which resulted in oxidative stress and cell DNA damage. A decrease in nitric oxide bioavailability on cells treated with TiO_2 NPs was also observed in this tudy (figure 4). Several studies have associated a decrease in NO to be strongly linked to the increase in ROS (Vaziri and Ding, 2001, Hsieh *et al.*, 2014). We, therefore, also speculate that TiO_2 NPs induces ROS activation which results in inadequate NO bioavailability. It has been reported that at the cellular level, NO regulates cell growth, survival, apoptosis, proliferation and differentiation (Murad, 2006) via NO-cGMP dependent or independent pathway. Interestingly, the NO-cGMP independent pathway has been reported to be mostly affected by cellular its interaction with metal complexes, oxygen (O_2) and superoxide (O_2.-). We, therefore speculate that cellular interaction with TiO_2 NPs induces ROS activation which in turn in the activates the OS-mediated pathway and affects NO-cGMP independent pathway and results in decreased NO bioavailability which in turn reduces cellular differentiation (Beckman and Koppenol, 1996). However, whether or not the decrease of NO bioavailability is truly responsible for the cytotoxic effect of NPs is still unknown.

In addition, given the evidence of lysosomal involvement in TiO_2 NPs cytotoxicity NPs cytotoxicity has been linked with severe oxidative stress which is associated with lysosomal membrane permeabilization and subsequent necrosis which is controlled by complex signalling pathways. Interestingly, Zhu *et al.*, 2012 have reported that necrosis might account for cell death in TiO_2 NPs treated cells. This corresponds with our results as we also found reduced cell viability and proliferation in cells treated TiO_2 NPs.

Conclusions

This study demonstrates the importance of stabilising TiO_2 nanoparticles prior to *in vitro* toxicity testing in order to study the possible toxicological properties of TiO_2 nanoparticles before their use in nanotechnological applications. This can be useful in producing less toxic products coated with TiO_2 NPs and may reduce health threats to humans exposed to TiO_2 NPs coated products. Our results indicate that consideration should be given to nanoparticle physiochemical properties such as size, and

aggregation state, as these properties may have the ability to compromise important cellular signalling molecules and lead to cell death.

Recommendations

Several studies have shown that preparation and characterization of nanoparticles are pivotal before attempting to use them in toxicological studies. These studies show variations in the toxic effects of stabilised nanoparticles, and they also show different types of cellular signalling mechanisms the induce TiO_2 NPs cytotoxicity. Amongst these mechanisms, the lysosome-mediated pathway has also been recently identified to be involved in inducing TiO_2 NPs cytotoxicity. Therefore, since exosomes are a product of the lysosomal pathway it is probable that TiO_2 alters the biogenesis of exosomes which in turn impacts cellular proliferation and toxicity. It is, therefore, necessary to further investigate the effects of TiO_2 NPs on exosome production *in vitro* as this will enable us to gain better understanding of the underlying mechanisms involved in the toxicity of TiO_2 NPs. Additionally, it would be imperative to test the cytotoxic properties of TiO_2 NPs in end organ derived cell lines in order to identify the physiological characteristics of TiO_2 NPs in relation to various cellular biochemical pathways. This will provide the foundation for understanding the cellular signalling mechanisms altered by TiO_2 NP-induced cytotoxicity.

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APENDIX A

Ethical Approval from the University of Kwazulu-Natal Biomedical Research Ethics Committee (BREC)

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

11 July 2016

Ms W Phoswa
Discipline of Physiology
School of Laboratory Medicine and Medical Sciences
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Protocol: The effects of titanium dioxide nanoparticles on the nitric oxide pathway in placental trophoblastic and endothelial cells.
Degree: MSc
BREC reference number: BE230/16

EXPEDITED APPLICATION

The Biomedical Research Ethics Committee has considered and noted your application received on 31 March 2016.

The study was provisionally approved pending appropriate responses to queries raised. Your response received on 31 May 2016 to queries raised on 23 May 2016 have been noted and approved by a sub-committee of the Biomedical Research Ethics Committee. The conditions have now been met and the study is given **full ethics approval.**

This approval is valid for one year from **11 July 2016.** To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be **RATIFIED** by a full Committee at its meeting taking place on **16 August 2016.**

We wish you well with this study. We would appreciate receiving copies of all publications arising out of this study.

Yours sincerely

[redacted]

Professor J Tsoka-Gwegweni
Chair: Biomedical Research Ethics Committee

cc supervisor: [redacted]
cc postgrad: [redacted]

Biomedical Research Ethics Committee
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Ethical Approval from the University of Kwazulu-Natal Biomedical Research Ethics Committee (BREC) Amendment Letter

UNIVERSITY OF KWAZULU-NATAL
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29 May 2017

Ms W Phoswa
Discipline of Physiology
School Of Laboratory Medicine and Medical Sciences
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Dear Ms Phoswa

Protocol: The effects of titanium dioxide nanoparticles on the nitric oxide pathway in placental trophoblastic and endothelial cells.
Degree: MSc
***BREC reference* number: BE230/16**

NEW TITILE: *Effect of Titanium Dioxide Nanoparticle Aggregation on Mouse Myoblast Cellular Cytotoxicity and Nitric Oxide Synthesis*

Your correspondence received on 29 May 2017 submitting an application for Amendments to change the title to the above has been **noted and approved** by a subcommittee of the Biomedical Research Ethics Committee.

NB: PI to submit protocol to/or seek further advise from Animal Research Ethics Committee (AREC) since animal cell lines are now being used.

This approval will be ratified at the next meeting to be held on 13 June 2017.

Yours sincerely

[redacted]
Senior Admin Officer: Biomedical Research Ethics Committee

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cc postgraduate office: tarinm@ukzn.ac.za