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How to cite this thesis


by

Leigh-Anne Koekemoer

DISSEYATION

Submitted in fulfilment of the requirements for the degree

MAGISTER SCIENTIAE

in

BIOCHEMISTRY

in the

FACULTY OF SCIENCE

at the

UNIVERSITY OF JOHANNESBURG
Auckland Park
South Africa

SUPERVISOR: Prof Liza Bornman
Co-Supervisor: Prof Mary Gulumian

October 2013
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Acknowledgements

I would like to thank:

God Almighty, “I can do all things through him who strengthens me” Philippians 4:13

My parents, for the support and motivational talks as well as the genuine interest you show in everything I do. Thank you for allowing me to pursue my dreams. I could not have achieved what I have without you.

Prof Mary Gulumian, for your mentorship and all the opportunities afforded to me while under your supervision. I have benefited greatly having you as my supervisor.

Kailen Boodhia, my best friend. What a long road this has been, but I am glad we got to travel it together. Thank you for laughing with me during the good times and being a shoulder to lean on in the stressful times. Thank you for being there.

Prof Michael Aschner, for the training received in your laboratory regarding astrocyte culturing, and for providing valuable advice and guidance with regards to western blots. Thank you for your hospitality.

Mary-Ann Costello and Patrick Selahle, for supplying the rat pups. Thank for you for your help in obtaining the dissection equipment and your valuable advice.

Tanyn James, for assisting with access to the University of Johannesburg Zoology department in order for me to perform the dissections. Sorry for the short notice.

Natasha Sanabria, for proof reading and editing my dissertation.

Charlene Andraos, for your help with the characterization results.

Melissa Vetten, for all the talks and help when needed.
Articles that have been prepared for publication in peer reviewed journals:


Awards received:

- IUTOX travel award to attend the 8th Congress of Toxicology in developing Countries (8CTDC) held in Bangkok (2012)

Parts of this study have been presented at the following conferences, congresses, meetings and symposia:

- Koekemoer L, Gulumian M. Characterization of the size distribution of MnO$_2$ dust particles collected from SAMANCOR and in vitro cytotoxicity and genotoxicity, 5th International Symposium on Nanotechnology, Occupational and Environmental Health, Boston (USA), August (2011) (Poster presentation)

- Koekemoer L, Gulumian M. Characterization of the size distribution of incidental MnO$_2$ dust nanoparticles collected from SAMANCOR and in vitro cytotoxicity and genotoxicity. ISO TC 229, Nanotechnologies meeting, Johannesburg (RSA), November (2011) (Poster presentation)

- Koekemoer L, Boodhia K, Aschner M, Gulumian M. *In vitro* cytotoxicity of MnO$_2$ dust particles collected from a ferromanganese smelter. The 46th Annual Congress of the SASBCP in association with the Dept. of Family Medicine (UP) and TOXSA, Pretoria, South Africa, 29 September – 2 October 2012. *(Oral presentation).*

- Koekemoer L, Gulumian M. Evaluating the effects of MnO$_2$ dust particles obtained from a South African smelter works on primary rat astrocytes. 10th International Particle Toxicology Conference, Dusseldorf, Germany, 4-7 June 2013 *(Oral presentation)*
Abstract

Manganese (Mn) is an essential trace element. Although it is vital for the normal development of mammals, too much Mn can be harmful. Most reported cases of toxicity have been found in occupational settings, such as welding, mining and ferro-manganese (FeMn) production plants. Long-term overexposure to Mn can result in lung epithelial necrosis and the development of a neurological disease, manganism. Even though evidence of Mn-associated diseases exists, some epidemiological studies have found no association between occupational exposure levels and possible indicators of neurotoxic effects. It is, therefore, important to establish Mn toxicity and the mechanisms involved in this toxicity, for a possible identification of biomarkers of exposure and effect.

The hypothesis formulated states that, FeMn particulate matter consists of nano and micro sized particles that, upon inhalation, may cause injury to the lungs and translocate to the brain. Since Mn-induced injury to the brain and lungs is a possibility, this study aimed to investigate the effects of FeMn dust, which was collected from a FeMn smelter works, on primary rat astrocytes and human bronchial epithelial (BEAS-2B) cells.

This was achieved by first characterizing the physicochemical properties of the particles by using a scanning mobility particle sizer (SMPS) and aerodynamic particle sizer (APS) for size distribution, Brunauer-Emmett-Teller (BET) for surface area determination and inductively coupled plasma atomic emission spectroscopy (ICP-AES) for elemental composition analysis. Cells were treated with 5, 10, 25 µg/cm² FeMn, and particle uptake, by astrocytes and BEAS-2B cells, was confirmed using dark field microscopy e.g. Cytoviva® hyperspectral imaging system. The viability and toxicity of FeMn was studied using the conventional toxicity assay systems, including 3-bis [2-Methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide salt (XTT), adenosine triphosphate (ATP) and lactate dehydrogenase (LDH) assays. It was, however, established that FeMn particles interfere with the final read-out produced by some of these assay systems. Therefore, a rare application of the xCELLigence real time cell analysis (RTCA) system was implemented, as a better option, in the assessment of the toxicity and viability of cells in the presence of FeMn particles. The ability of
FeMn particles to cause deoxyribonucleic acid (DNA) damage in both cell types was also
determined using the alkaline comet assay. Finally, the nuclear translocation of the antioxidant
transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) and inflammatory
transcription factor nuclear factor kappa B (Nf-κB), was studied using Western blotting.

The results showed that FeMn, in a dose dependent manner, could enter the cell, decrease the
viability, induce DNA damage, and initiate nuclear transport of the studied transcription factors.
The same methodologies were implemented to determine the physicochemical properties of Min-
U-Sil 5 crystalline silica, used as a positive control, to assess its toxicity and effect on cellular
viability. As well as its ability to induce DNA damage and initiate nuclear translocation of the two
transcription factors, in astrocytes and BEAS-2B cells. Similar to FeMn particles, crystalline silica
also enters the cells with subsequent reduction in cellular viability. It results in increased DNA
damage and increased nuclear translocation of the studied transcription factors. The effects of
crystalline silica on these cellular effects were, however, always higher than those produced by
FeMn particles.

To conclude, these results indicate that depending on the size distribution of particles in the work
environment, they may enter different regions of the lungs. However, for those particles in the
nano size region, direct access to the brain is a possibility. These results also indicate that after
deposition in the target organ, these particles will produce cellular changes through oxidative
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<table>
<thead>
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<th>Description</th>
</tr>
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<tbody>
<tr>
<td>% tail DNA</td>
<td>Percentage tail DNA</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>Cl</td>
<td>Cell index</td>
</tr>
<tr>
<td>cm</td>
<td>Centimetre</td>
</tr>
<tr>
<td>g</td>
<td>Acceleration due to gravity</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo dalton</td>
</tr>
<tr>
<td>kg</td>
<td>Kilogram</td>
</tr>
<tr>
<td>M</td>
<td>Molar/mole per litre</td>
</tr>
<tr>
<td>m</td>
<td>Meter</td>
</tr>
<tr>
<td>mA</td>
<td>Milliampere</td>
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</tr>
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<td>MW</td>
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</tr>
<tr>
<td>NCI</td>
<td>Normalized cell index</td>
</tr>
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<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometre</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µl</td>
<td>Microlitre</td>
</tr>
<tr>
<td>µm</td>
<td>Micrometre</td>
</tr>
<tr>
<td>µM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>V</td>
<td>Volt</td>
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#### A

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<thead>
<tr>
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<tbody>
<tr>
<td>Al</td>
<td>Aluminium</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APS</td>
<td>Aerodynamic particle sizer</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulphate</td>
</tr>
<tr>
<td>ARE</td>
<td>Antioxidant responsive element</td>
</tr>
<tr>
<td>As</td>
<td>Arsenic</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>Au</td>
<td>Gold</td>
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#### B

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<thead>
<tr>
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<tbody>
<tr>
<td>β-APP</td>
<td>Beta amyloid precursor protein</td>
</tr>
<tr>
<td>B</td>
<td>Boron</td>
</tr>
<tr>
<td>Ba</td>
<td>Barium</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma protein 2</td>
</tr>
<tr>
<td>Be</td>
<td>Beryllium</td>
</tr>
<tr>
<td>BEAS-2B</td>
<td>Bronchial epithelial cells</td>
</tr>
<tr>
<td>BET</td>
<td>Brunauer-Emmett-Teller</td>
</tr>
<tr>
<td>BHT</td>
<td>Butylated hydroxytoluene</td>
</tr>
<tr>
<td>Bi</td>
<td>Bismuth</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<table>
<thead>
<tr>
<th>Acronym</th>
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<tbody>
<tr>
<td>Ca</td>
<td>Calcium</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CAT</td>
<td>Catalase</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>Cd</td>
<td>Cadmium</td>
</tr>
<tr>
<td>Ce</td>
<td>Cerium</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>Co</td>
<td>Cobalt</td>
</tr>
<tr>
<td>COX 2</td>
<td>Cyclooxygenase 2</td>
</tr>
<tr>
<td>Cr</td>
<td>Chromium</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element binding protein</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebral spinal fluid</td>
</tr>
<tr>
<td>Cs</td>
<td>Caesium</td>
</tr>
<tr>
<td>Cu</td>
<td>Copper</td>
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<table>
<thead>
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<tbody>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DMT</td>
<td>Divalent metal transporter</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase</td>
<td>Deoxyribonuclease</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco’s phosphate buffered saline</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>Dy</td>
<td>Dysprosium</td>
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<table>
<thead>
<tr>
<th>Acronym</th>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF-R</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>Er</td>
<td>Erbium</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ESR</td>
<td>Electron spin resonance</td>
</tr>
<tr>
<td>EtBr</td>
<td>Ethidium bromide</td>
</tr>
<tr>
<td>Eu</td>
<td>Europium</td>
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<td>Description</td>
</tr>
<tr>
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<td>--------------------------------------------------</td>
</tr>
<tr>
<td>F1-ATPase</td>
<td>F1 domain-adenosine triphosphatase</td>
</tr>
<tr>
<td>Fas</td>
<td>Fatty acid synthase</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<td>Fe</td>
<td>Iron</td>
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<td>FeMn</td>
<td>Ferromanganese</td>
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<tr>
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<td>Gallium</td>
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<tr>
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<td>Gamma-aminobutyric acid</td>
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<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
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<tr>
<td>Gd</td>
<td>Gadolium</td>
</tr>
<tr>
<td>Ge</td>
<td>Germanium</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>GS</td>
<td>Glutamine synthetase</td>
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<tr>
<td>GSSG</td>
<td>Glutathione disulphide glutathione</td>
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<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
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<td>Glutathione-S transferase A2</td>
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<tr>
<td>H^+</td>
<td>Hydrogen ions</td>
</tr>
<tr>
<td>H_2O_2</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
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<tr>
<td>HF</td>
<td>Hydrofluoric acid</td>
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<td>Hf</td>
<td>Hafnium</td>
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<tr>
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<td>Mercury</td>
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<td>HMOX1</td>
<td>Heme oxygenase-1</td>
</tr>
<tr>
<td>HNO_3</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>HO-1</td>
<td>Heme oxygenase-1</td>
</tr>
<tr>
<td>Ho</td>
<td>Holmium</td>
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<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
</tr>
<tr>
<td>HS</td>
<td>Horse serum</td>
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<tr>
<td>HSI</td>
<td>Hyperspectral imaging</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intracellular adhesion molecule</td>
</tr>
<tr>
<td>ICP-AES</td>
<td>Inductively coupled plasma atomic emission spectroscopy</td>
</tr>
<tr>
<td>ICP-MS</td>
<td>Inductively coupled plasma mass spectroscopy</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
</tr>
<tr>
<td>IGEPAL CA-630-</td>
<td>Octylphenoxypolyethoxyethanol</td>
</tr>
<tr>
<td>IkB</td>
<td>NF-κB inhibitor</td>
</tr>
<tr>
<td>IKK</td>
<td>IkB kinase</td>
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</table>
IL    Interleukin
IL-1β    Interleukin 1 beta
In    Indium
Ir    Iridium

J
JNK    c-Jun N-terminal kinase

K
K    Potassium
K⁺    Potassium ions
Keap1    Kelch ECH Associating Protein 1

L
La    Lanthanum
LDH    Lactate dehydrogenase
Li    Lithium
LMPA    Low melting point agarose
LPS    Lipopolysaccharide
Lu    Lutetium

M
Maf    Musculoaponeurotic fibrosarcoma
MAPK    Mitogen activated protein kinase
MEM    Minimum essential medium
Mg    Magnesium
MHC    Major histocompatibility complex
MIP    Macrophage inflammatory protein
MIR    Manganese/iron ratio
Mn    Manganese
MnB    Blood manganese
MnO₂    Manganese dioxide
Mo    Molybdenum
MnS    Serum manganese
Mn-SOD    Manganese superoxide dismutase
MTT    (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) salt
MnU    Urine manganese
MnV    Saliva manganese
mRNA    Messenger RNA
MWCN    Multi-walled carbon nanotube
**N**

<table>
<thead>
<tr>
<th>Symbol</th>
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<td>Nitrogen</td>
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<tr>
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<td>Sodium</td>
</tr>
<tr>
<td>Na⁺</td>
<td>Sodium ions</td>
</tr>
<tr>
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<td>Niobium</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>Nd</td>
<td>Neodymium</td>
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<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide (reduced form)</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
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<td>NES</td>
<td>Nuclear export signal</td>
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<tr>
<td>Ni</td>
<td>Nickel</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localization signal</td>
</tr>
<tr>
<td>NMPA</td>
<td>Normal melting point agrose</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>NP</td>
<td>Nanoparticle</td>
</tr>
<tr>
<td>NQO1</td>
<td>NAD(P)H quinone oxidoreductase</td>
</tr>
<tr>
<td>Nrf2</td>
<td>Nuclear factor erythroid 2-related factor 2</td>
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**O**

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<thead>
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<tr>
<td>O₂</td>
<td>Oxygen</td>
</tr>
<tr>
<td>O₂⁻⁻</td>
<td>Superoxide</td>
</tr>
<tr>
<td>OH⁻⁻</td>
<td>Hydroxyl radical</td>
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**P**

<table>
<thead>
<tr>
<th>Symbol</th>
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<tr>
<td>p38</td>
<td>p38 MAPK</td>
</tr>
<tr>
<td>P</td>
<td>Phosphorus</td>
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<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Pb</td>
<td>Lead</td>
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<td>Pd</td>
<td>Palladium</td>
</tr>
<tr>
<td>pH</td>
<td>Power of hydrogen</td>
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<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3 kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PM</td>
<td>Particulate matter</td>
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<tr>
<td>PMS</td>
<td>Phenazine methosulfate</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
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<td>Pr</td>
<td>Praseodymium</td>
</tr>
<tr>
<td>Pt</td>
<td>Platinum</td>
</tr>
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<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>Rb</td>
<td>Rubidium</td>
</tr>
<tr>
<td>Rh</td>
<td>Rhodium</td>
</tr>
<tr>
<td>RHD</td>
<td>Rel homology domain</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>RTCA</td>
<td>Real time cell analysis</td>
</tr>
<tr>
<td>Sb</td>
<td>Antimony</td>
</tr>
<tr>
<td>Sc</td>
<td>Scandium</td>
</tr>
<tr>
<td>Se</td>
<td>Selenium</td>
</tr>
<tr>
<td>SAF</td>
<td>Submerged arc furnace</td>
</tr>
<tr>
<td>SAM</td>
<td>Spectral angle mapper</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
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<tr>
<td>SiO₂</td>
<td>Silicon dioxide</td>
</tr>
<tr>
<td>Sm</td>
<td>Samarium</td>
</tr>
<tr>
<td>SMPS</td>
<td>Scanning mobility particle sizer</td>
</tr>
<tr>
<td>Sn</td>
<td>Tin</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
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<tr>
<td>Sr</td>
<td>Strontium</td>
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<tr>
<td>Ta</td>
<td>Tantalum</td>
</tr>
<tr>
<td>Tb</td>
<td>Terbium</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
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<tr>
<td>TBST</td>
<td>TBS + Tween 20</td>
</tr>
<tr>
<td>Te</td>
<td>Tellerium</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylenediamine</td>
</tr>
<tr>
<td>Tfr</td>
<td>Transferrin receptor</td>
</tr>
<tr>
<td>Th</td>
<td>Thorium</td>
</tr>
<tr>
<td>Ti</td>
<td>Titanium</td>
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<tr>
<td>TiO₂</td>
<td>Titanium dioxide</td>
</tr>
<tr>
<td>Tl</td>
<td>Thallium</td>
</tr>
<tr>
<td>Tm</td>
<td>Thulium</td>
</tr>
<tr>
<td>TNF⁻α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>----------------------</td>
</tr>
<tr>
<td>U</td>
<td>Uranium</td>
</tr>
<tr>
<td>UFP</td>
<td>Ultrafine particle</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>V</td>
<td>Vanadium</td>
</tr>
<tr>
<td>Vis</td>
<td>Visible</td>
</tr>
<tr>
<td>W</td>
<td>Tungsten</td>
</tr>
<tr>
<td>X</td>
<td>3-bis[2-Methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide salt</td>
</tr>
<tr>
<td>Y</td>
<td>Yttrium</td>
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<td>Ytterbium</td>
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<tr>
<td>Zn</td>
<td>Zinc</td>
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<tr>
<td>Zr</td>
<td>Zirconium</td>
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</table>
Aim of Study

FeMn is produced as an unintentional by-product in ferroalloy smelter works and overexposure to manganese results in lung disease as well as neurotoxicity. However, some studies have found no significant relationship between neurotoxicity endpoints and measurements of exposure. The inconsistency in the relationship between “exposure levels” and “outcome effects” means that it is important to define mechanisms of toxicity for manganese in target organs. Since FeMn induced injury to the lungs and the brain is a possibility, this study aims to investigate the effects of FeMn dust, (collected from a FeMn works), on primary rat astrocytes and human bronchial epithelial cells (BEAS-2B), as representatives of target organs for manganese toxicity.

The hypothesis formulated states that, FeMn particulate matter consists of nano and micro sized particles and, therefore, upon inhalation, may translocate to the brain. This translocation occurs by two means. Firstly, the micro sized particles are inhaled and cause injury to the lungs, thus weakening the epithelial cells and allowing the particles to move into the blood stream facilitating their transfer to other organs (brain). Secondly, the nano sized particles can reach the brain directly via the olfactory pathway, which has already been shown in animal studies.

In order to test the hypothesis, the objectives of the study were:

- To characterize the FeMn dust and crystalline silica (bench mark particle) by determining:
  - Size distribution.
  - Surface area.
  - Elemental composition.
- To determine cellular uptake of the FeMn dust and crystalline silica using:
  - Dark field microscopy.
- To determine the effect of treatments, with FeMn or silica, on cell viability by investigating:
  - Adenosine triphosphate (ATP) levels.
  - Lactate dehydrogenase (LDH) release.
  - Mitochondrial activity.
  - Cellular adherence.
- To determine the effect of treatments with FeMn or silica, on NF-κB p65 and Nrf2 translocation to the nucleus.
- To determine the genotoxic effects of treatments with FeMn or silica by investigating:
  - DNA damage.
Chapter 1: Introduction

1.1. Airborne Particulate Matter

The following section is an introduction to particulate matter, its composition and characteristics. The term “airborne particulate matter” (PM) refers to a diverse mixture, differing in size and chemical composition. PM consists of both micro- and nano-sized particles. Micro particles can be classified as follows: (1) course particulate matter (PM 10-2.5), comprised of dust from demolitions, wind erosion and pollen, (2) fine particulate matter (PM 2.5), consisting of primary particles produced during combustion processes. As well as secondary particles produced by primary ultra fine particles which have grown by condensation and coagulation (refer to Figure 1; Politis et al., 2008; Hayens et al., 2010; Kelly and Fussell, 2012). Nanoparticles (NPs; PM 0.1) are smaller than 100 nm, with one or more dimensions within the nanoscale range. They include engineered NPs, natural biological NPs and ambient ultrafine particles (UFP; see Figure 1). UFPs are often described as incidental NPs, which are created as unintentional by-products of combustion processes, such as welding, smelting and other industrial processes (Oberdörster et al., 2005). Coarse PM is responsible for a large percentage of the total ambient PM mass. However, UFPs have the largest particle number and make up most of the surface area of the total PM mixture (Knol et al., 2009). Course particles, due to their large mass, do not stay airborne for long. Therefore, they do not travel the same distances and have the same deposition as UFPs. For this reason, UFPs have been perceived as the source of adverse health effects observed due to inhalation of PM (Chow and Watson, 2007; Weissenberg et al., 2010).
UFPs have a high surface area to volume ratio. Consequently, due to their small size, they have an extremely large surface area compared to that of fine and course particles. The increased surface area of UFP is known to be important for catalytic reactions, and could be responsible for inflammation caused by reactive oxygen species (ROS) that are produced on the surface of these particles. Other particle characteristics which may be responsible for UFP toxicity include chemistry, size, shape, dissolution, deposition, agglomeration state and dose (Beck-Speier et al., 2005; Oberdörster et al., 2005; Kelly and Fussell, 2012). Particulate matter is a complex heterogeneous mixture, containing organic materials and transition metals, such as manganese (Mn) amongst other things (Haynes et al., 2012). The following section will highlight the vital role Mn plays in normal development of mammals, and its use in industry.

1.2. Manganese

Manganese is the 12th most abundant naturally occurring element in the earth’s crust. It however, does not occur as a free metal. It is isolated using carbon to reduce the dioxide form and is found in several minerals, the most common of which being oxides, carbonates and silicates (Gerber et al., 2002; Au et al., 2008). Mn is not only found in rocks and soil, but also in food and water and is
classified as an essential trace element. The recommended daily intake of Mn is 1.8 and 2.3 mg/kg for women and men, respectively (Rivera-Manci´ et al., 2011). Since Mn functions as a co-factor for numerous enzymes, such as lyases, transferases and hydrolases, it is vital for normal development of mammals. Arginase, a Mn containing polypeptide, is present in mitochondria and is responsible for ammonia elimination in the liver; superoxide dismutase (SOD) is found in the mitochondria and protects against cellular oxidative stress; and pyruvate carboxylase is an important enzyme in gluconeogenesis (Crossgrove and Zheng, 2004; Schneider et al., 2006; Au et al., 2008). Other functions of Mn include formation of connective tissue and bone, reproduction regulation, carbohydrate and lipid metabolism, and normal brain function (Roth and Garrick, 2003). A substantial portion of Mn in the brain is used in the metabolism of glutamate to glutamine, a process linked to the astrocyte-specific enzyme glutamine synthetase (Crossgrove and Zheng, 2004; Au et al., 2008). In addition, Mn has also been found to interact with the cell surface integrin receptor, vitronectin and extracellular matrix (ECM), proteins which are important for neuronal development. This interaction can regulate attachment, differentiation, proliferation, and migration of cells. Via the interaction of Mn with the vitronectin receptor, several different signal transduction pathways, including the mitogen activated protein kinases (MAPK), extracellular signal-regulated kinase (ERK1 and 2), and the stress activated kinase, p38 (a MAPK), are stimulated (Roth and Garrick, 2003).

Other than being an essential nutrient, Mn is also used extensively in industry where Mn containing products include batteries, animal feed, welding metals, ceramics, and pigments. A substantial portion of the world’s Mn is mined in South Africa (Roth and Garrick, 2003; Rovetta et al., 2007). More than 90% of the excavated Mn ore is used in the production of steel, where it functions to (1) remove sulphur – making it easier to forge, (2) deoxidize the steel, and (3) act as an alloying agent – increasing the toughness and elasticity of steel (Aschner et al., 2006). It has been estimated that over 3700 tons of Mn is released into the atmosphere every year, during the mining and processing of Mn ore (Roth and Garrick, 2003). Therefore, the increased exposure of large populations to Mn-containing products substantiates the need for an accurate assessment of the possible health risk due to this metal (Gerber et al., 2002). The following section will discuss occupational exposure to Mn, as well as the resultant effects of Mn overexposure.
1.3. Manganese Overexposure

Manganese toxicity is not very common in humans, and if found, is usually dose dependent. For this reason most reported cases of toxicity have been found in the work place, with the most prominent sources being welding, ore-crushing, mining and ferromanganese production. In an occupational setting, there is an increased potential for chronic exposure to high levels of Mn, where the main exposure route has been found to be through inhalation (Weiss, 2006; Santamaria, 2008). Long term exposure to Mn levels (above the permissible exposure limit of 5 mg/m$^3$ set by the Occupational Safety and Health Administration), can result in lung epithelial necrosis, a characteristic of chemical pneumonitis (Lison et al., 1997). Inhaled Mn is also known to cause a variety of neurological effects similar to those observed in Parkinson Disease, where this form of the disease is called manganism (Lison et al., 1997; Jankovic, 2005; Winder et al., 2010). Symptoms include mood alterations, insomnia, increased tremor, as well as, decreased motor functions and memory (Bouchard et al., 2008).

Studies conducted on non-human primates reported findings which strongly agree with those documented for human subjects (Santamaria, 2008). One of the studies tested the neurological effects of Mn at high doses and resulted in the primates developing similar symptoms to those seen in human cases, which included increased tremor and unsteady gait (Eriksson et al., 1987). These kinds of studies do aid in understanding Mn neurotoxicity, but it is still difficult to extrapolate the results to human cases due to the high doses of Mn used in these studies (Santamaria, 2008).

1.4. Studies Conducted on Manganese Overexposure in an Occupational Environment

This section will provide a summary of important studies performed to assess Mn overexposure in workers. Many epidemiological studies have been conducted on workers exposed to Mn, where the first central nervous system (CNS) symptoms of Mn exposure were described in the early 1800’s (Couper, 1837). Since then, conflicting reports have been published regarding the association between occupational exposure levels and the possible indicators of neurotoxic effects
caused by chronic Mn inhalation. Many studies identified differences between exposed workers and the reference groups used (Rodier, 1955; Young et al., 2005; Bowler et al., 2007; Bouchard et al., 2008). A study by Lucchini and colleagues used 58 male, Italian ferroalloy plant workers that were divided into three groups: high exposure (furnace workers), medium exposure (maintenance) and low exposure (clerks, foreman and laboratory technicians). In this study, they found that there were slight impairments of CNS function in workers of the high exposure group, even though no clinical symptoms were observed (Lucchini et al., 1995). In another study performed using 17 baggers from a manganese ore milling plant in Singapore and 17 referents, the investigators found a significant decline in motor speed, visual scanning, response speed and steadiness in the exposed group compared to the reference group (Chia et al., 1993). This study was, however, limited by the small sample size.

Other studies on the occupational exposure of Mn showed no effects. For example, Myers and colleagues used biological exposure and biological effect measures that included blood Mn (MnB), urine manganese (MnU) and prolactin. In addition a comprehensive range of endpoints in the form of specially designed questionnaires, to determine differences between an exposed group of 509 South African smelter workers and 67 referents were also used. Little evidence of any Mn effects was found (Myers et al., 2003a). Similar results were found in a study conducted on South African Mn mineworkers (Myers et al., 2003b). The difference in outcomes between the studies conducted on the smelter workers (Myers et al., 2003a) and the mine workers (Myers et al., 2003b), and other research groups, may be explained by studies showing that parkinsonism is rarely found in populations of African descent (Myers et al., 2009).

Meta-analyses, which describe a method of statistics focused on comparing and combining information from different studies, have been performed on published literature currently available (Lees-Haley et al., 2006; Greiffenstein and Lees-Haley, 2007). The authors of these meta-analyses concluded that the levels of Mn in the air (to which workers are exposed), may cause minor adverse effects on a cognitive and sensory level. These minor effects are detectable in population studies. However, they may be too small to be detected in individuals through clinical assessments currently used. For this reason, many studies find no noteworthy associations between neurotoxic effects and MnB, MnU, levels of Mn in the air and years of exposure. Other
reasons for the vast amount of conflicting literature available, is that all the studies differ in neuropsychological tasks used, number of measures analyzed, sample size, demographics of the subjects, cultural context and the amount of exposure (Santamaria, 2008). The following section will discuss MnB and MnU as biomarkers of Mn overexposure, and why they may not be an effective measure of Mn exposure.

1.5. Biomarkers of Manganese Overexposure

Any materials within the body that can be used to quantify exposure levels and predict likely adverse health effects are classified as “biomarkers”. There are three types of biomarkers: biomarkers of exposure, effect and susceptibility (Santamaria, 2008). Even though much research has been performed to establish whether biological tissues and fluids can serve as reliable indicators of Mn exposure, no established biomarkers have been found as yet (Crossgrove and Zheng, 2004). Biomarkers of exposure are actual substances or chemicals that can be measured within the body, or, once excreted from the body. Many potential biomarkers of Mn exposure have been studied over the years. Since Mn easily enters the blood circulation system after inhalation, the research originally centred on MnB concentration. However, it has been found that Mn readily collects in tissue (brain) and intracellular compartments, making MnB a poor measure of Mn exposure. Also, when using MnB it is also not possible to differentiate between current and historical exposure, as well as exposure due to inhalation or ingestion (Crossgrove and Zheng, 2004; Zheng et al., 2011). The MnU concentration has also been suggested as a marker of Mn exposure. However, the Mn found in urine is only a small portion of what may be present, due to the fact that most Mn within the body is excreted through the faecal route (Myers et al., 2003c; Zheng et al., 2011).

Iron (Fe) homeostasis is known to be affected by Mn and, therefore, the Mn/Fe ratio (MIR) was tested in either plasma or erythrocytes. This biomarker measures both the concentrations of the two elements, as well as changes in iron homeostasis. However, cut-off values for this ratio in the general population must still be established (Zheng et al., 2011). Serum prolactin has also been included in many assessments of Mn toxicity. This is because increased exposure to Mn causes changes in the levels of dopamine in the brain, which inhibits the release of prolactin. Even though
some studies have found positive correlations between MnB and prolactin, it should be noted that MnB is not an accurate assessment of Mn exposure (Montes et al., 2008). The Mn concentration in saliva (MnV) has also been suggested as a biomarker of Mn exposure. Nevertheless, research has shown that this marker is not more sensitive than serum Mn concentrations (MnS) used for determining Mn exposure levels (Zheng et al., 2011).

Overexposure to Mn can also result in oxidative stress, which is as a result of the overproduction of free radicals or ROS. Many enzymes and products involved in the production of ROS, such as SOD, glutamine synthetase (GS) or glutathione (GSH), could act as markers of Mn induced oxidative stress (Zheng et al., 2011).

“Biomarkers of effect” refers to changes that take place after an organism has been exposed to a particular substance and “biomarkers of susceptibility” refers to factors that make an organism more susceptible to the effects of a particular substance. Biomarkers of effect for Mn, such as neurological differences, must still to be validated and some studies have found that certain genes could act as susceptibility biomarkers for neurological diseases caused by Mn. Mn is an essential element for development therefore, baseline levels must be taken into consideration when analysing the use of various biomarkers (Santamaria, 2008).

1.6. Accumulation and Transport of Manganese in the Body

The inconsistency in the relationship between “exposure levels” and “outcome effects”, means that it may be important to define the possible exposure routes of Mn into a biological system. This section will highlight these exposure routes, as well as, the transport and accumulation of Mn following entry into the body. Ingestion is one route, where dietary Mn intake is closely regulated by the gut, and excess Mn readily excreted from the liver in bile (Au et al., 2008; Winder et al., 2010). Inhalation is another means by which Mn enters the body. One of the most noteworthy factors that influence pulmonary deposition is particle size. Many of the inhaled particles are within the size range of 0.1 µm to 2 µm, which are easily cleared by exhalation and larger inhaled particles are cleared by swallowing (Jankovic, 2005). Any particles in the size range of 20 nm to 100 nm, which are deposited in the lower airways, are either absorbed or translocated to other sites.
within the lungs. Some forms of Mn have a high bioavailability (soluble sulphates >> less soluble phosphates > tetraoxides), and are therefore, able to move from the lungs into the blood stream to secondary target organs (Dorman et al., 2006).

Accumulation of Mn tends to occur in pigment and mitochondrial rich tissues (Dorman et al., 2006). The entry of Mn into these tissues is dependent on its solubility, aggregation state, integrity of the epithelial lining and the site of deposition (Elder et al., 2006). Brain tissue has the capacity to retain Mn for long periods of time and Mn can enter the brain through three routes. These include the capillary endothelial cells of the blood-brain barrier, the blood-cerebrospinal fluid (CSF) barrier and lastly, the most important is the olfactory pathway (see Figure 2; Dorman et al., 2006). Transport of Mn into the brain was first thought to occur by active transport or facilitated diffusion. However, more recent studies have shown that Mn can enter the brain through a variety of transport proteins including: divalent metal transporter (DMT1), transferrin receptor (TfR), voltage-gated calcium (Ca) channels, sodium (Na)/ Ca exchanger, Na/magnesium (Mg) antiporter or mitochondrial active Ca uniporter (Crossgrove and Zheng, 2004; Au et al., 2008).

![Figure 2: Uptake and translocation of Mn along axons of the olfactory nerve has been shown in non-human primates and rodents (Oberdörster et al., 2005).](image-url)

There are conflicting reports regarding whether Mn transported via the olfactory pathway, leads to any toxicological effects. One group found changes in glial fibrillary acidic protein (GFAP) expression in the olfactory bulb of rats. This protein is a known marker of astrocytic damage
Dorman and colleagues, however, did not detect the same kind of changes, and this could have been due to the use of different doses and solubilities between the two studies, as well as, the presence of olfactory epithelial injury (Dorman et al., 2006). The uptake of Mn is also influenced by iron stores within the body and intake of iron through diet, where these metals compete for binding to transferrin and other transport systems (Roth and Garrick, 2003). Solubility and size are seen as important factors for transport of Mn through the olfactory pathway. Translocation of Mn phosphate, which is not very soluble, was measured by Dorman and colleagues and an increase in Mn concentration was only seen in the olfactory bulb (Dorman et al., 2004a). Elder et al., 2006 performed a similar study using Mn oxide and showed translocation of Mn to distal portions of the brain. The difference between the studies was that the phosphate particles had an aerodynamic diameter of 1.6 µm, whereas the aerodynamic diameter of the Mn oxide particles was 31 nm. They concluded that the particles must be ≤ 200 nm in size, since the axons of the olfactory neurons only have a diameter of 200 nm. Most studies regarding entry of Mn into the body, through the olfactory pathway, have been conducted on rats. Therefore, the results obtained could be misleading, as rats have larger olfactory mucosa than humans (Roth and Garrick, 2003; Santamaria, 2008; Bailey et al., 2009). However, using a predictive model Oberdörster and colleagues showed that the concentration of translocated particles can be up to 10X greater in the human olfactory bulb, than that found in rats (Oberdörster et al., 2005). Recent studies have also shown that Mn could accumulate in the striatum and not the olfactory tract or bulb (Bailey et al., 2009). Since translocation of Mn to the brain, via the olfactory pathway, is a possibility the following section provides a brief introduction to the CNS.

1.7. Central Nervous System

The nervous system is responsible for signal transmission and information storage within the body, as well as co-ordination of practically all physiological processes. The nervous system consists of two main parts: the CNS comprising of the brain and spinal cord, and the peripheral nervous system (PNS), consisting mainly of nerves. The CNS is made up of a complex network of cells called neurons and glial cells. Three types of glial cells have been identified, which include astrocytes, oligodendrocytes and microglia. These cells perform important functions, e.g. phagocytosis, neuronal support and maintenance, as well as, the regulation of blood brain barrier (BBB).
permeability. The presence of the BBB helps to insure the precise regulation of the extracellular fluid composition and the brain’s internal environment, by restricting the uptake of substances based on size and lipophilicity. The CNS has been found to be particularly vulnerable to neurotoxicants because of its relatively high lipid levels compared to other areas of the body. This predisposes the CNS to oxidant-induced lipid peroxidation, as well as, assists with the circulation of lipophilic chemicals throughout the nervous system. The CNS is also extremely complex and has a high metabolic rate, making it sensitive to disruption of mitochondrial function and energy metabolism. Due to the essential role played by glial cells in the normal functioning of the CNS, it is important to determine glial cell integrity when defining a substance as a neurotoxicant (Dorman, 2000; Au et al., 2007). The role of astrocytes in the CNS is further explained in the following section.

1.8. Functions of Astrocytes in the CNS

Astrocytes constitute the largest percentage of human brain cells and are present in all regions of the brain, in close contact with neurons. For many years it was thought that the only function of astrocytes was that of structural support for neurons. However, it has become increasingly clear that these cells are also vital for maintaining the physiologic and pathological states of neurons, as well as, sustaining brain homeostasis. Astrocytes secrete a number of factors e.g. growth factors, cytokines, extracellular matrix proteins, proteoglycans and cholesterol. These are essential for the survival, proliferation and differentiation of neurons (Rappold and Tieu, 2010; Giordano et al., 2009). The supportive functions of astrocytes include buffering the increase in extracellular potassium ions (K⁺) during neuronal activity. This is called K⁺ spatial buffering, which involves the redistribution of locally elevated K⁺ to distant sites, either passively or with the help of a Na⁺/K⁺ pump. Astrocytes are also involved in pH control of the brain via a lactate plus H⁺ transporter. This transporter controls the efflux of lactate and co-transported H⁺ from astrocytes, as well as, uptake of lactate and H⁺ into neurons thereby, affecting pH levels (Chen and Liao, 2002; Gee and Keller, 2005; Erikson and Aschner, 2006; Gunter et al., 2006; Tjalkens et al., 2006; Gonzalez et al., 2008; Giordano et al., 2009; Bitto et al., 2010; Kimelberg and Nedergaard, 2010).
Astrocytes were initially thought to respond to changes in neuronal activity only through membrane potential depolarization, due to these cells apparently not possessing any receptors. Later, this was found to be false as studies reported the presence of glutamate and GABA ionotropic receptors. It is, however, difficult to target astrocyte specific receptors, since astrocytes have been found to express receptors for the same ligands as neurons. Uptake of glutamate is one of the most important functions of astrocytes. Astrocytes contain a specific enzyme, namely glutamine synthetase, which inactivates glutamate by converting it to glutamine. This process is dependent on the consumption of ATP and ammonia, which is then taken up by the surrounding neurons and converted back to glutamate (Chen and Liao, 2002; Gee and Keller, 2005; Erikson and Aschner, 2006; Gunter et al., 2006; Tjalkens et al., 2006; Gonzalez et al., 2008; Giordano et al., 2009; Bitto et al., 2010; Kimelberg and Nedergaard, 2010).

Astrocytes also surround all the blood vessels in the brain, thereby controlling cerebral blood flow. Their interaction with vascular endothelial cells forms the tight junctions responsible for the development of the BBB. Recently it has been discovered that astrocytes contain water channels or aquaporins, which among other functions, help facilitate movement of oxygen (O$_2$), carbon dioxide (CO$_2$) and nitric oxide (NO). Astrocytes have also been found to have a number of antioxidant systems such as the glutathione disulfide–glutathione (GSSG–GSH) system, as well as, the enzymes catalase, glutathione peroxidase and SOD that help to neutralize reactive oxygen species and prevent the breakdown of essential lipids and proteins. Astrocytes are also responsible for immune functions, within the brain, and have been found to produce major histocompatibility complex (MHC) class I and II molecules in addition to intracellular adhesion molecules (ICAMs) (refer to Figure 3; Chen and Liao, 2002; Gee and Keller, 2005; Erikson and Aschner, 2006; Gunter et al., 2006; Tjalkens et al., 2006; Gonzalez et al., 2008; Giordano et al., 2009; Bitto et al., 2010; Kimelberg and Nedergaard, 2010).
1.9. Lung exposure to Manganese

The focus of Mn toxicity has mainly been on neurological effects due to the severity of the symptoms. However, pulmonary toxicity is also important to consider as the lungs are the first and main site of deposition. The following section therefore highlights Mn exposure in the lungs. Exposed individuals often develop bronchitis, pneumonia and various other pulmonary diseases, in addition to diseases associated with the brain (Roth and Garrick, 2003). These diseases usually develop as a result of inflammation, resulting in acute responses or exacerbating existing respiratory diseases (Hetland et al., 2004). Chronic inflammation can lead to lung fibrosis and mutations that ultimately result in tumour formation. Deposition of UFP in the lungs depends on its size and composition and occurs in one of four ways. Firstly, it occurs via interception, which is when the particle touches the airway surface. This is the main means of deposition for fibres. Secondly, it occurs via impaction, when particles collide with the airway surface due to a change in the initial trajectory of the particle. Thirdly via sedimentation, a particle’s mass overcomes its buoyancy and the particle settles on the airway surface (particles > 0.5 µm in size). Lastly via diffusion, particles deposit on the airway surface due to Brownian motion (particles < 0.5 µm in size), this being the main means of deposition for UFPs (Politis et al., 2008).
Using a predictive mathematical model, Oberdörster and colleagues illustrated the deposition, which is dependent on size, of inhaled particles within the respiratory system (Oberdörster et al., 2005). This model showed that 1 nm particles preferentially accumulate in the nasopharyngeal compartment, with no accumulation in the alveolar compartment, whereas 20 nm particles were found to accumulate with the highest efficiency in the alveolar region. Inhaled particles of 5 nm deposited equally in the nasopharyngeal, tracheobronchial and alveolar compartments (refer to Figure 4). These predicted differences in deposition could influence potential effects of the inhaled particles. Many particles deposited in the respiratory tract can, however, be cleared in two ways; (a) translocation across the lung epithelial cells into the blood (where the particles are circulated to different parts of the body including the brain) and (b) chemical mechanisms such as phagocytosis by alveolar macrophages (Oberdörster et al., 2005; Csavina et al., 2012).

Figure 4: Diagram presenting predicted fractional deposition of inhaled particles in the nasopharyngeal, tracheobronchial and alveolar region of the human respiratory tract during nose breathing (Oberdörster et al., 2005).
1.10. Manganese Toxicity

Now that Mn exposure routes have been established, this section briefly discusses Mn toxicity in general, followed by a brief summary of Mn toxicity in astrocytes and the lungs. The toxicity of Mn is not well understood, but it has become clear that it is a combination of factors that ultimately lead to Mn toxicity. Studies have found that the effects of Mn can be linked to iron concentrations within the blood as well as the speciation of Mn. The valance state of Mn can affect uptake behaviour, due to differences in solubility and toxicity, owing to its ability to participate in redox reactions (Crossgrove and Zheng, 2004; Jankovic, 2005; Charlet et al., 2012). Once Mn enters the cell, it has been found to influence the function of a number of organelles and pathways, one of these being the signalling pathway for apoptosis (Roth and Garrick, 2003). However, when specific apoptotic markers are inhibited, cytotoxicity was not prevented, thus, there are other mechanisms of cytotoxicity present. When Mn accumulates in the mitochondria, it impedes oxidative phosphorylation by affecting the function of F1 domain-adenosine triphosphatase (F1-ATPase), calcium efflux and inhibition of tricarboxylic acid cycle enzymes. It also causes chromosomal aberrations resulting in deoxyribonucleic acid (DNA) damage (Gerber et al., 2002; Roth and Garrick, 2003; Rovetta et al., 2007; Rivera-Manci´ et al., 2011). Although Mn accumulates mainly in the mitochondria, it has also been found to accumulate in the lysosomes and is involved in the fragmentation of the Golgi apparatus, as well as, the collapse of the endoplasmic reticulum (ER) (Towler et al., 2000; Villalobos et al., 2009).

1.10.1. Manganese Toxicity in Astrocytes

Due to the significant role astrocytes play in the maintenance of the CNS, it has been proposed that these cells could be neurotoxic targets for many compounds (Giordano et al., 2009). Manganese is one such compound and its levels within the CNS are tightly controlled by astrocytes using a high affinity, high capacity, specific transport system, which allows these cells to accumulate Mn at concentrations of up to 60 times that found in neurons (Hazell et al., 2006). Once Mn enters the cell, it is found at its highest concentration in the mitochondria. Some studies have shown that Mn can interact with nucleotides of DNA, ribonucleic acid (RNA) and ribosomes, as well as Pirin, a nuclear protein found within the nucleoplasma (Gonzalez et al., 2008; Kalia et al.,
Glutamine synthase, an astrocyte specific manganoprotein responsible for glutamate metabolism, accounts for a large portion of Mn found in the brain (Taylor et al., 2006).

If there is an overexposure to Mn, this could lead to production of ROS. This would inhibit glutamate transporters interfering with the removal of extracellular glutamate and subsequent detoxification of ammonia therefore, seriously impairing neuron function (Erikson et al., 2004; Taylor et al., 2006). Chen and colleagues found that astrocytes treated with Mn showed stellation, astrocytic swelling and decreases in antioxidant activity, DNA synthesis and energy production. It also enhanced interleukin-6 (IL-6) gene expression. These effects would decrease the ability of astrocytes to protect neurons from injury (Chen and Liao, 2002). Studies using microarray gene expression profiling and computational data analysis algorithms found similar results, where Mn was found to up-regulate 28 genes encoding various cytokines and pro-inflammatory chemokines, and, down regulate 15 genes responsible for functions involved in cell cycle progression (Sengupta et al., 2007; Park and Park, 2010). The Mn treatment of astrocytes has been found to influence the activity of nuclear factor κB (NF-κB), which is the transcription factor responsible for the inducible expression of nitric oxide synthase 2, (NOS2). This pathway can lead to over expression of NO resulting in apoptosis (Liu et al., 2004). Yin and colleagues also found that Mn, via the activation of caspase 3 and the ERK signalling pathway, can trigger apoptosis by causing the collapse of the mitochondrial inner membrane potential (see Figure 5; Yin et al., 2008).
Figure 5: The effects of particulate matter on the brain are complicated by the complex nature of these airborne particles but it is likely that CNS pathology is due to the interaction of multiple pathways as is shown here (Block and Calderon-Garciduenas, 2009).

1.10.2. Manganese Toxicity in the Lung

The adverse effects of UFP on lung tissue are dependent on deposition, surface area and the presence or absence of transition metals (Knol et al., 2009). Transition metals, which make up a large percentage of the total mass of airborne particles, have been found to be the greatest contributors to pulmonary inflammation, where copper (Cu) caused the most inflammation followed by Mn and nickel (Ni) respectively (Roth and Garrick, 2003). Particles are inhaled and deposit in the lungs, leading to activation of alveolar macrophages. In an attempt to attack and remove these particles from the lungs, the activated alveolar macrophages release ROS, which results in inflammation, triggering the emission of cytokines (Beck-Speier et al., 2005; Politis et al., 2008; Knol et al., 2009).

An increase in IL-8 and IL-6, as well as macrophage inflammatory protein-2 (MIP-2), have been shown in both human and animal studies (Hetland et al., 2004). The release of ROS can also increase the permeability of the lung epithelium allowing translocation of the particles, as well as the induced cytokines, to other areas of the body (Knol et al., 2009). Alveolar macrophages are also
responsible for phagocytosis, which is one of the key clearance methods in the lungs. Donaldson and colleagues used a macrophage cell line to determine the effects of ultrafine titanium dioxide (TiO$_2$) and carbon black on phagocytosis (Donaldson et al., 2001). They found that the UFP were able to inhibit phagocytosis, thereby hampering clearance of these particles from the lungs and allowing their accumulation (refer to Figure 6). This accumulation can then lead to the induction of both apoptosis and proliferation. Apoptosis is induced by the activation of c-Jun N-terminal kinase 1/2 (JNK1/2), whereas proliferation is stimulated by the activation of ERK1/2, both of which are dependent on the activation of epidermal growth factor receptor (EGF-R) (Weissenberg et al., 2010). Particle accumulation in the lungs can also lead to changes in the calcium concentration, which is another proinflammatory effect (Donaldson et al., 2001). From studies conducted on rats, it has become clear that all these adverse effects are dependent on the particle dose, expressed as surface area rather than to mass or volume (Wottrich et al., 2010).

Figure 6: A diagram showing of particulate matter induced toxicological effects in the lung (Michael et al., 2013).
1.11. Particulate Matter induced Oxidative stress

1.11.1. Transcription Factors Nrf2 and NF-κB

It would seem that the effects of oxidative stress and inflammation play a major role in particle induced toxicity. The following section will therefore briefly discuss the Nrf2 and Nf-κB pathways, their mechanisms of activation and the influence of Mn on each of these pathways. The PM-induced oxidative stress is as a result of ROS, such as hydroxyl radicals (OH·), hydrogen peroxide (H₂O₂) and superoxide (O₂⁻), as well as, redox-active metals (which can be found on the particle surface or produced as a result of a chemical reaction within the cell). The inflammation that results from oxidative stress leads to the increased expression of cytokines, chemokines and adhesion molecules, which are also known as signalling molecules (Michael et al., 2013). These signalling molecules have been found to activate the nuclear factor erythroid 2-related factor 2-Kelch ECH Associating Protein 1 (Nrf2-Keap1) pathway, which results in the induced expression of genes encoding for antioxidant phase II enzymes e.g. catalase (Rovetta et al., 2007) and SOD, and the antioxidant GSH (Giudice et al., 2010; Michael et al., 2013).

In normal, unstressed circumstances the cytoskeleton protein, Keap1, associates with the transcription factor Nrf2 via a ‘hinge and latch’ interaction. Thus, it keeps Nrf2 anchored in the cytoplasm and targets it for ubiquitination and subsequent 26S proteasomal degradation (latched). However, under stressed conditions, Keap1 senses the resultant change in the intracellular redox environment and undergoes oxidation of its cysteine residues and/or phosphorylation. This releases Nrf2 (the latch is opened, and Nrf2 hangs on the hinge), which is now no longer targeted for degradation (Figure 7). Keap1 then becomes saturated with Nrf2 and the concentration of free Nrf2 increases, which is now able to translocate into the nucleus using specific nuclear export (NES) and nuclear localization (NLS) signals. Once inside the nucleus, Nrf2 binds to another transcription factor called musculoaponeurotic fibrosarcoma (Maf) and triggers the cis-acting antioxidant responsive element (ARE)-dependent gene expression. Once conditions return to normal, Keap1 travels to the nucleus and dissociates Nrf2 from the ARE, subsequently, the Keap1-Nrf2 complex leaves the nucleus and Nrf2 is once again targeted for proteasomal degradation in the cytoplasm.
(refer to Figure 8; Kobayashi et al., 2006; Zhang, 2006; Copple et al., 2008; Bellezza et al., 2010; Giudice et al., 2010; Menshikova et al., 2010).

Figure 7: A diagram showing the ‘hinge and latch’ interaction between Keap1 and Nrf2. (A) In normal conditions Nrf2 is attached to Keap1 in the ‘latched’ position and is directed for proteasomal degradation. (B) Under stressed conditions Keap1 undergoes oxidation of its cysteine residues as well as phosphorylation thereby releasing Nrf2, which now hangs on a ‘hinge’ and is no longer targeted for degradation (Copple et al., 2008).

Figure 8: A diagram showing Nrf2 regulation. In unstressed or basal conditions Nrf2 is targeted for proteasomal degradation by its association with Keap 1 which acts as a substrate adaptor for a Cullin-dependent E3 ubiquitin ligase complex. However when induced by various stimuli protein kinases react by phosphorylating Keap-1 thereby allowing the accumulation of free Nrf2 which now translocates to the nucleus where it binds to ARE resulting in the transcription of genes encoding antioxidant enzymes (Zhang, 2006).
Nrf2 pathway activation is dependent on the amount of ROS present. Low levels of ROS result in the induction of the transcription factor Nrf2, whereas higher levels of ROS induce other transcription factors, e.g. NF-κB (Bellezza et al., 2010). NF-κB is a transcription factor activated by many different cytokines i.e. tumor necrosis factor alpha (TNFα), chemokines (IL-1β) and adhesion molecules, and it induces the expression of various pro-inflammatory genes that are important for cellular responses to stress and inflammation (May and Ghosh, 1998; Mercurio and Manning, 1999). In the majority of mammalian cell types, the NF-κB family of proteins comprises of an N-terminal region called the Rel-homology domain (RHD) that contains the dimerization, DNA-binding domains and the NLS. Five proteins belonging to the NF-κB/Rel family have been identified, namely: p65 (RelA), c-Rel, RelB, p50/p105, and p52/p100. In unstressed conditions, the p50 and p65 subunits of NF-κB form inactive homo- or hetero-dimers that are held in the cytoplasm by non covalent binding to inhibitory molecules. These are called NF-κB inhibitors (IkBs), usually IkBα, and cover the NF-κB DNA binding domains and NLS’s. Under stressed conditions, the IkBα molecule is phosphorylated by the IkB kinase complex (IKK) leading to its ubiquitination and degradation through the proteasome pathway. Freed NF-κB dimers then translocate to the nucleus, where NF-κB interacts with κB-like DNA sequences. It regulates the genes encoding IkBs, providing a feedback mechanism whereby resynthesized IkBs bind to DNA-bound NF-κB dimers and remove them from the nucleus (refer to Figure 9; Schmitz and Baueuerle, 1991; Beg et al., 1992; May and Ghosh, 1998; Me´met, 2006; Bellezza et al., 2010).
Figure 9: A diagram showing NF-κB regulation. Protein kinases react to a stimulus and phosphorylate the inhibitory molecule IκBα thereby inactivating it. The p50/p65 subunits of NF-κB are now free to translocate to the nucleus where the transcription of pro-inflammatory genes takes place (Verma et al., 1995).

1.11.2. Nrf2: Antioxidant Mediator

Many studies have shown that metal containing PM cause ROS mediated cell damage, resulting in adverse conditions, which include Parkinson Disease, aging, pulmonary fibrosis and acute pulmonary injury as well as inflammation (Zhang, 2006). To ensure their survival, mammalian cells have developed antioxidant mechanisms that neutralize the effect of oxidative stress. The transcription factor, Nrf2, is responsible for one such mechanism. Keap1 and Nrf2 are phosphorylated by several transducers including: protein kinase C (PKC), MAPK and ERK, as well as, phosphatidylinositol 3 kinase (PI3K), in response to oxidative stress. This in turn activates Nrf2, resulting in the transcriptional induction of genes encoding the expression of antioxidant enzymes heme oxygenase-1 (HMOX1), Nicotinamide adenine dinucleotide phosphate (NAD(P)H) quinone oxidoreductase (NQO1), and glutathione-S transferase A2 (GSTA2). Overexposure to metals can result in: 1) phosphorylation of Nrf2 due to activation of MAPK, 2) Sulphydryl group reduction in
Keap1 and 3) proteasomal pathway inhibition. These all result in the stabilization and activation of Nrf2 and increased production of antioxidant enzymes (Copple et al., 2008; Lee and Johnson, 2004; Simmons et al., 2011). In a study performed by Simmons and colleagues using a novel cell based transcriptional reporter assay to monitor Nrf2 dependent transcriptional activation in five different cell lines, they found that Mn, while being cytotoxic, did not activate Nrf2 (Simmons et al., 2011). This is, however, contradictory to a study performed by Casalino and colleagues, where they found Mn induced nuclear translocation of Nrf2 in the liver cells of rats (Casalino et al., 2007). The differences seen may be explained by the differences in the endpoints of the studies, being transcriptional activity and nuclear translocation respectively (Simmons et al., 2011).

The essential role of Nrf2 in pulmonary toxicity has also been established by use of mice lacking Nrf2. Exposure to diesel exhaust particles resulted in apoptosis and tissue inflammation, found to a greater extent in the Nrf2−/− mice than in the wild type (Aoki et al., 2001). In a different study, Nrf2−/− mice treated with the antioxidant butylated hydroxytoluene (BHT) developed acute respiratory disease syndrome (Chan and Kan, 1999). Nrf2 has also been found to play a major role in the protection of neurons. This protection seems to be due to the activation of Nrf2 in astrocytes (Satoh et al., 2006; Nguyen et al., 2009). Co-cultures of neurons and astrocytes were treated with an Nrf2 activator, sulforaphane and the researchers found that the Nrf2 activated astrocytes provided neuronal protection against glutamate and hydrogen peroxide (Johnson et al., 2007). It has also been discovered that microglial cultures, treated with astrocyte culture-conditioned media, showed an increase in ARE-dependent HO-1 promoter activity and suppression of ROS production induced by Interferon-gamma (IFN-γ), as well as, a reduction in inducible NOS expression and NO release (Tufekci et al., 2011). ARE-driven genes, such as NQO1, have also been found to protect IMR-32 human neuroblastoma cells from H$_2$O$_2$-induced apoptosis, and, Nrf2 over expression defends cells against fatty acid synthase (Fas)-induced apoptosis (Lee and Johnson, 2004).

1.11.3. NF-κB: Proinflammatory Mediator

Cells also have a number of proinflammatory pathways that are activated in response to the inflammation and stress caused by PM-induced oxidative stress. The primary oxidative stress-
response pathway is that of the transcription factor NF-κB. Phosphorylation of IκB leads to the activation of NF-κB, resulting in the transcription of genes encoding numerous stress response cytokines, chemokines and enzymes, some of which are also responsible for the activation of NF-κB (Mercurio and Manning, 1999; Li et al., 2010). NF-κB is activated by inducers such as TNFα, IL-1, ROS including H₂O₂ and lipopolysaccarides (LPS), and the JNK pathway also plays an important role in its activation (Li et al., 2010). Nf-κB can be found in virtually all cell types within the brain as well as the lungs. Activation of Nf-κB in the nervous system is through a number of ROS mediated pathways, which include diseases involving ROS, glutamatergic signal transduction and cytokine- and neurotrophin-mediated activation (Kaltschmidt et al., 1999). This activation results in the transcriptional regulation of the genes for TNF-α, IL-6, β-amyloid precursor protein (β-APP), manganese superoxide dismutase (Mn-SOD), B-cell lymphoma protein 2 (Bcl-2), inducible NOS and cyclooxygenase 2 (Cox 2). The brain is particularly vulnerable to oxidative stress and TNF is reported to control neuronal response to oxidative stress by the activation or repression of Nf-κB (Mattson and Camandola, 2001).

NF-κB response is both cell and stimulus specific (Kaltschmidt et al., 1999; Kaltschmidt et al., 2005). Bradykinin, a neural inflammatory molecule, activates NF-κB in astrocytes in response to injury resulting in the production of IL-6 and several other inflammatory cytokines (Mattson and Camandola, 2001). In astrocytes, the expression of NOS is regulated by NF-κB. Mn has been found to disrupt mitochondrial calcium buffering and decrease the uptake of glutamate, which in turn activates NF-κB in astrocytes in a cyclic guanosine monophosphate (cGMP)-dependent manner, through the MAPK/ERK pathway. This results in increased production of prostaglandins, TNF-α, NO and IL-1, thereby, increasing the expression of NOS. This astrocytic activation has been linked to various neurological diseases including manganism (Liu et al., 2005; Moreno et al., 2008; Moreno et al., 2011; Spilsbury et al., 2012). NF-κB is also able to induce the transcription of genes for antioxidant proteins and those involved in cellular calcium homeostasis (Mattson and Camandola, 2001).

TNF-α has also been reported as a strong activator of NF-κB in lung epithelial cells upon exposure to diesel exhaust particles (Li et al., 2010). Injuries seen in the lung, after exposure to diesel exhaust particles, have also been attributed to the activation of NF-κB. This led to the subsequent
expression of the c-fos proto-oncogene, after the release of IL-8 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Baeza-Squiban et al., 1999). Berlo and colleagues studied the effects of quartz particles on rat alveolar type II epithelial cells and found that the macrophages generated products that induced iNOS and NF-κB, whereas the quartz particles themselves induced HO-1 and COX-2 messenger RNA (mRNA) expression possibly due to the activation of Nrf2 (Figure 10; Berlo et al., 2010).

Figure 10: A diagram showing the production of reactive oxygen species within a cell and the subsequent activation of NF-κB (Mercurio and Manning, 1999).

There have been reports of cross-talk between the NF-κB and Nrf2 pathways, due to their respective suppression and activation by anti-inflammatory phytochemicals (Bellezza et al., 2010). Nair and colleagues analysed the promoter regions of human and murine Nrf2 and NF-κB using bioinformatics, and they found that several members of the MAPK family are involved in the regulation of these two transcription factors (Nair et al., 2008). It has also been shown that NF-κB negatively regulates Nrf2, by competing with it for binding to the cAMP (cyclic adenosine monophosphate) response element binding (CREB) protein, and promotes the binding of
corepressor histone deacetylase 3 to the Maf proteins, resulting in local histone hypoacetylation (Nair et al., 2008; Liu et al., 2008). However, Nrf2 stabilizes IκB through the inhibition of IκK, thereby suppressing the activity of NF-κB (see Figure 11). The anti-inflammatory effects of Nrf2 are also preserved by increased transcription of γ-glutamylcysteine synthase and glutathione reductase genes. The increased GSH/GSSG ratio then suppresses NF-κB activity, due to changes in the redox balance of the cell (Menshikova et al., 2010). When it comes to inflammation, NF-κB and Nrf2 play opposing roles, and a large number of stimuli including ROS activate both NF-κB and Nrf2-ARE. The activation or suppression of either pathway is largely dependent on the amount of stimulus present, but can also be influenced by the type of stimulus and the cell type in which these processes are taking place (Liu et al., 2008).

Figure 11: Effects of the Nrf2/ARE signalling system on NF-κB (Menshikova et al., 2010).
Chapter 2: Materials and Methods

Refer to Appendix 1 for all the manufacturers of reagents, apparatus and software mentioned below.

2.1. FeMn Dust Sampling

The Mn dust was sampled from the west plant filter area at the Meyerton works (Samancor), an industry that produces FeMn (see Figure 12). FeMn is produced from the reduction of manganese dioxides (MnO$_2$), found in Mn ores, in an electric submerged arc furnace (SAF) (Fahim et al., 2013).

![Figure 12: Schematic diagram of the Samancor (Meyerton works) showing where the manganese sample was collected.](image-url)
Min-U-Sil 5 is the crystalline silica sample that was used as the benchmark against which the toxicity of the Mn sample was measured in all experiments. Min-U-Sil 5 was obtained from US Silica (Berkely, West Virginia, USA). Silica was chosen as the reference particle due to its extensive characterization and well known toxicity (Fubini and Hubbard, 2003; Castranova, 2004).

2.1.1. Sample Preparation

The Mn sample was first sieved on an AS 200 Sieve Shaker in order to obtain the inhalable dust fraction. To separate the dust, the following sieves were used: 20, 40, 50, 63, 80 µm. Each sieve, as well as the collection pan, was weighed. The sieves were then assembled, in ascending order and 10 g of dust placed on the top sieve. The sieve stack was placed on the sieve shaker. The dust was then separated at 80 hertz for 8 hours. The resulting < 20 µm fraction of dust was used in all subsequent experiments.

Stock solutions of the samples were freshly prepared before each experiment, by weighing out 1 mg and diluting it in the appropriate culture medium, to a final concentration of 1 mg/ml. These stocks were then used in experiments at concentrations of 0, 5, 10 and 25 µg/cm² (0, 16, 32, 80 µg/ml).

2.2. Dust Particle Characterization

The physiochemical properties of particles play an important role in particle induced toxicity. Most notably the size, surface area and chemical composition of atmospheric particles have been found to be responsible for the toxicity induced by many inhaled particles.

2.2.1. Scanning Mobility Particle Sizer (SMPS) and Aerodynamic Particle Sizer (APS)

The size distributions of the FeMn and silica dust samples, in terms of number and mass, were analysed at the Toxicology and Biochemistry Department, NIOH (RSA) using a SMPS and APS. The SMPS and the APS use different measurement principles. The SMPS sort’s particles into different sizes based on their mobility in an electric field. This size depends on the cross section of the particle, which is regarded to be the same as its physical size. The APS determines aerodynamic
size, where particle time-of-flight is related to its aerodynamic size and the aerodynamic size is proportional to the physical size of the particle (Khlystov et al., 2004).

The samples were introduced into the particle sizer using the Small Scale Particle Dispenser (Model 3433) which is designed to disperse small quantities of dry powder in the range 1 to 50 µm. The Aerosol Instrument Manager Software (version 8.1.0) was used to collect sample data from the SMPS and APS instruments and the Data Merge Software (version 1.0.1) was then used to merge SMPS and APS data files to produce single particle size distributions.

### 2.2.2. Brunauer-Emmett-Teller (BET) Analysis

BET analysis was performed at the Department of Chemistry, University of the Witwatersrand (RSA) in order to determine the surface area and the porosity of the dust samples. The specific surface area of a powder is determined by physical adsorption of a gas (normally nitrogen or krypton) on the surface of the solid, at liquid nitrogen temperatures. By calculating the amount of adsorbed gas (measured by a volumetric or continuous flow technique), corresponding to a monomolecular layer on the surface, the surface area of the powder can be determined. Van der Waals forces are responsible for the physical adsorption between the adsorbed gas molecules and the adsorbent surface area of the powder (Dubois et al., 2010).

Samples weighing 1 g were degassed in N₂, at 150°C for 4 hours prior to analysis, using a Micromeritics Flow Prep 060 sample degas system. The surface areas and pore size distributions were then obtained at -196°C. The pore size distribution with specific surface areas of the samples, were determined via N₂ adsorption/desorption according to the BET method, using a Micromeritics Tristar surface area and porosity analyzer (continuous flow technique).

### 2.2.3. Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES)

ICP-AES was performed at Elliot Lake Research Field Station, Laurentian University (Canada), and was used to establish the trace elemental composition of the dust samples. A plasma source is used to dissociate the sample into its component atoms or ions, by exciting them to a higher energy level. When the atoms or ions return to their ground state, they emit photons of a
characteristic wavelength depending on the element present. This light is recorded by an optical spectrometer (Kocyigit et al., 2004).

Samples weighing 0.2 g were digested with an acid mixture of 9 ml hydrofluoric acid (HF) and 1 ml hydrochloric acid (HCl) in a Vulcan Digestion system, at 100°C with a ramp time of 30 minutes. This temperature was held for 280 minutes and the block cooled to 50°C. The above step was repeated at 110°C with a ramp time of 30 minutes. This temperature was held for 210 minutes and the block cooled to 50°C. The first step was repeated. However, this time, the samples were digested with an acid mixture of 7.5 ml nitric acid (HNO₃) and 7.5 ml HCl, heated to 110°C with a ramp time of 30 minutes. This temperature was held for 240 minutes and the block cooled to 50°C. The first step was repeated, again, with an acid mixture of 0.5 ml HF, 2ml HCl and 10 ml HNO₃ heated to 110°C with a ramp time of 30 minutes. This temperature held for 60 minutes. The total volume was reduced to 7 ml. The samples were then diluted with deionised water to 50 ml, stirred and left to stand overnight before analysis. Samples were analyzed by ICP-AES for elements of interest.

Elemental composition in mg/kg was calculated as follows:

\[ x \, \mu g/l \times \frac{50 \, ml}{1000} = y \, \mu g/50 \, ml = y \, \mu g/0.2 \, g \]

\[ y \, \mu g/0.2 \, g \times 0.2 = z \, \mu g/g = z \, mg/kg \]

where \( x \) is equal to the measured amount of element as determined by ICP-AES, 50 ml is the final volume of the sample prior to analysis, \( y \) is the measured amount of element in the originally weighed out 0.2 g of dust and \( z \) is the measured amount of element in mg/kg.

2.2.4. pH Measurements

The pH of RPMI 1640 medium was measured before and after addition of the dust samples, in order to determine the effect of the dust samples on the pH of culture media. Samples were dispersed in RPMI 1640 media, supplemented with 10% fetal bovine serum and 1%
penicillin/streptomycin to final concentrations of 25, 50 and 100 $\mu$g/cm$^2$, in a final volume of 200 µl. The samples were then analyzed using a Cyberscan pH6500 pH/ion meter at 37°C.

2.3. **Cell Culturing**

Primary rat astrocytes were isolated from rat pups obtained from University of the Witwatersrand Central Animal Services. Immortalized human bronchial epithelial cells (BEAS-2B) were obtained from Sigma Aldrich (St.Louis, Missouri, USA). Both cell lines are adherent and were used for the duration of this study.

2.3.1. **Primary Rat Astrocytes**

2.3.1.1. **Isolation**

Training in the isolation of astrocytes was obtained at Vanderbilt University (USA). Postnatal day 1 neonatal Sprague-Dawley rats were euthanized, with sodium pentobarbitone, by the University of the Witwatersrand Central Animal Services. The pups were then placed on ice and transported to the Zoology department at the University of Johannesburg, where the dissections took place. The head and neck of the pups were wiped down with 70% ethanol before the head was decapitated, using sterile dissection scissors. The head was then secured and using sterile fine angled dissecting scissors, the skin was cut from the base of the skull to the eyes along the midline in order to expose the brain. Using sterile curved forceps, the skull bone was bent back. Thereafter, the brain was gently removed to a petri dish kept on ice, and filled with cold minimum essential medium (MEM) supplemented with 10% horse serum (HS) and 1% Penicillin/Streptomycin (culture medium). Once all the dissections had been completed, the intact brains were placed under a dissection microscope. Two sterile forceps (one to hold the brain and the other to dissect) were used to remove the lower piece of the brain, and separate the cortices. Any extra tissue was also removed. Sterile Dumont forceps were used to gently remove the meningenial membranes on the cortical surface, and darker hippocampal crescents were also removed. The cortices were then moved back to the original petri dish on ice, until all the brains had been separated and cleaned.
2.3.1.2. **Dissociation**

The following steps were all performed under sterile conditions. The cortices were placed in a 50 ml glass beaker using forceps. A sterile glass pipette was used to break down the cortices by pipetting up and down slowly. Thereafter, 20 ml culture medium containing 40 mg Dispase was added to the beaker. Dispase is a protease which cleaves fibronectin and collagen, resulting in gentle dissociation of tissues. The tissue suspension was stirred for 30 minutes on a magnetic stirrer at low speed. After 10 minutes of stirring 100 µl deoxyribonuclease 1 (DNase I) was added. DNase is a nuclease which preferentially cleaves DNA. In this case, it was used to cleave DNA released by the cells during dissociation, preventing unwanted cell clumping. After 30 minutes, another 20 ml culture medium was added and the tissue suspension centrifuged at 200 g for 5 minutes. The cells were then counted using the trypan blue dye exclusion assay (refer to Section 2.3.3) and resuspended at 10 000 cells/cm$^2$, in the appropriate culture dishes using culture medium.

2.3.1.3. **Culturing**

After 24 hours, the astrocytes had attached to the culture flask. At this point, the media was changed to minimize neuronal, microglial and oligodendrocyte contamination of cultures. Following this, the cells were left to mature for 2 weeks, with the media being changed every three days. The astrocyte culture was maintained in MEM, supplemented with 10% HS and 1% Penicillin/Streptomycin. Cells were incubated at 37°C, with 5% CO$_2$ in a humidified atmosphere. Once the cells had matured they could be used for one month starting from the culture date.

2.3.2. **Human Bronchial Epithelial Cells (BEAS-2B)**

2.3.2.1. **Culturing**

The BEAS-2B cells were cultured in Roswell Park Memorial Institute medium (RPMI) 1640, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (culture medium). Cells were incubated at 37°C, with 5% CO$_2$ in a humidified atmosphere. The cells were subcultured twice a week as follows: the cells were washed with Dulbecco’s phosphate buffered saline (DPBS), followed by the addition of 3 ml 1X trypsin/ ethylenediaminetetraacetic acid (EDTA).
Trypsin/EDTA is used to detach adherent cells from culture vessels. Trypsin is inhibited by Mg\(^{2+}\) and Ca\(^{2+}\) ions, as well as some proteins found in culture medium. EDTA acts as an ion chelator, reducing the concentration of ions and proteins that inhibit trypsin. The flask was then incubated for 5 minutes at 37\(^{\circ}\)C. The trypsin/EDTA was inactivated with 5 ml culture medium and the cells were removed from the 75 cm\(^2\) culture flasks. The cells were then centrifuged for 5 minutes at 200 \(g\) and resuspended in 5 ml culture medium. Following this, cells were counted using the trypan blue dye exclusion assay (refer to Section 2.3.3) and seeded at \(2 \times 10^5\) cells/cm\(^2\).

2.3.2.2. Cell Stocks

Cell stocks were routinely generated by subculturing as described in section 2.3.2.1. The cells were then resuspended, at a concentration of \(1 \times 10^7\) cells/cm\(^2\), in cryogenic vials containing a cold solution of 90% FBS and 10% dimethyl sulfoxide (DMSO). The cells were placed in liquid nitrogen for storage until required. Cells were taken out of stock by thawing rapidly at 37\(^{\circ}\)C. Thereafter, they were transferred slowly to a tube containing 20 ml RPMI 1640, supplemented with 20% FBS and 1% penicillin/streptomycin (thaw medium). The cells were then centrifuged at 200 \(g\) for 5 minutes and resuspended using 5 ml thaw medium in a 25 cm\(^2\) flask. The following day, the cells were counted using the trypan blue dye exclusion method (refer to Section 2.3.3) and seeded at \(2 \times 10^5\) cells/cm\(^2\), using culture medium, in a 75 cm\(^2\) flask.

2.3.3. Trypan Blue Dye Exclusion Assay

The trypan blue dye exclusion assay was used to determine cell viability and concentrations at which the cells should be seeded. This assay is able to distinguish between viable unstained cells and stained dead cells. The cells were subcultured as previously described in sections 2.3.1 and 2.3.2.1 for astrocytes and BEAS-2B cells, respectively, and resuspended in 5 ml culture medium. Thereafter, 10 \(\mu l\) of the cell suspension was added to 10 \(\mu l\) trypan blue and mixed well. Of this mixture 10 \(\mu l\) was pipetted into one of the chambers of a plastic Countess™ slide, which was then inserted into the Countess™. The Countess™ determined the cell viability and concentration of the cell suspension.
2.4. Cellular Uptake Studies

2.4.1. CytoViva® Hyperspectral Imaging System

The CytoViva® hyperspectral imaging system is a dark field imaging system and was used to visualize the uptake and interaction of particles with the cells. Dark field microscopy was used in this study, due to its ability to detect structures too small to detect with conventional light microscopy (< 200nm). Dark field imaging optimizes differences in contrast, by collecting light scattered by the sample. This allows the detection of smaller structures, as well as, particles with no intrinsic fluorescence, as is the case in this study (Gibbs-Flournoy et al., 2011).

The slides were prepared as follows: The cells were subcultured as previously described in sections 2.3.1 and 2.3.2.1 for astrocytes and BEAS-2B cells, respectively, and seeded on cover slips, in 12 well plates, at a concentration of 2x10^4 cells/cm^2 in 1 ml culture medium. This was followed by a 24 hour incubation to allow the cells to adhere to the cover slips. The cell medium was replaced with fresh culture medium. The cells were treated with FeMn or silica at concentrations of 0, 5, 10 and 25 µg/cm^2 for treatment periods of 6 and 24 hours. Thereafter, the cover slips were washed thrice with culture medium and a further three washes with DPBS. The cells were then fixed in a solution of 4% formalin, diluted with 0.1 M Tris(hydroxymethyl)aminomethane (Tris)/HCl (pH 7.4) for 15 minutes at 4°C. The cover slips were then washed twice with DPBS and air dried. Following this, they were glued upside-down onto microscope slides, using Kaiser’s gelatine and left to dry over night.

Dark-field images were captured at 60X magnification, using the CytoViva 150 Unit, integrated onto the Olympus BX43 microscope. Images were acquired using a Dagexcel X16 camera and the DAGE Exponent software. Hyperspectral imaging (HSI) was performed at 60X magnification, using the HSI System 1.1 and ENVI software. In addition to the scans of the cells treated with FeMn or silica as described, scans were also performed on the FeMn or silica particles alone. For this analysis, particles were suspended in culture medium and placed on a microscope slide, spread out and allowed to dry. A cover slip was placed on the slide prior to the acquisition of an HSI scan at 60X magnification. Spectral libraries were collected by selecting ten spectra of randomly chosen
particles. The image classification algorithm SAM (spectral angle mapper) was performed, using the ENVI software to map the spectral libraries onto the scans of the cells incubated with particles. These spectral libraries were visually compared to those of the FeMn or silica particles found within the cells. Scale bars were inserted using Image J software.

2.5. Cell Viability and Cytotoxicity Assays

2.5.1. XTT Assay

The 2,3-bis[2-Methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide salt (XTT) based _In Vitro_ Toxicology Assay Kit was used to evaluate the mitochondrial activity of viable cells. Mitochondrial activity in living cells was analysed by measuring the ability of mitochondrial dehydrogenase to reduce the tetrazolium ring of XTT to a water soluble formazan derivative. This increased production of formazan product was measured spectrophotometrically in order to determine the number of viable cells present. Research shows, that upon entry into the cell, Mn accumulates in the mitochondria (Dorman _et al._, 2006). Since, the XTT assay uses mitochondrial activity to evaluate cell viability, it was chosen as one of the assays performed in this study.

The cells were subcultured as previously described in sections 2.3.1 and 2.3.2.1 for astrocytes and BEAS-2B cells, respectively. The cells were then seeded in a sterile 96-well plate at 1x10^4 cells/cm^2, in 100 µl cell culture media, followed by a 24 hour incubation, in order to allow the cells to adhere to the plate before treatment. Wells containing culture medium only, were also pipetted and used as blanks. The cell media was then replaced with fresh culture medium, supplemented with 10% FBS and 1% penicillin/streptomycin. The cells were treated with FeMn or silica at concentrations of 5, 10 and 25 µg/cm^2, for a treatment period of 1 hour. Following this, 20 µl XTT stock solution (XTT with 1% phenazine methosulfate (PMS) in RPMI 1640 without phenol red and without serum), was added to each well. The plate was incubated at 37°C, with 5% CO2 in a humidified atmosphere for 2 hours. Thereafter, the absorbance was measured at 450 nm, using an automated microplate reader (ELx800) and KC 4 (version 3.4) software.
2.5.2. ATP Assay

The CellTiter-Glo® Luminescent Cell Viability Assay was used to determine the amount of ATP produced by viable cells. This ATP quantification involves the conversion of luciferin to oxyluciferin and light, in a reaction catalysed by luciferase in the presence of ATP, Mg$^{2+}$ and O$_2$. The light produced is then measured as luminescence, and is directly proportional to the number of viable cells present. The ATP assay was chosen as a comparison assay in the current study, because the mitochondria are involved in energy production. Therefore, any disturbances in the mitochondria would affect the levels of ATP (Ulukaya et al., 2008).

The cells were subcultured as described in sections 2.3.1 and 2.3.2.1 for astrocytes and BEAS-2B cells, respectively. The cells were then seeded in a sterile, opaque 96-well plate at 1x10$^4$ cells/cm$^2$, in 100 µl cell culture medium, followed by a 24 hour incubation, in order to allow the cells to adhere to the plates before treatment. Wells containing culture medium only, were used as blanks. The cell medium was then replaced with fresh culture medium. The cells were treated with FeMn or silica at concentrations of 0, 5, 10 and 25 µg/cm$^2$, for a treatment period of 1 hour. Following this, 100 µl CellTiter-Glo® reagent (CellTiter-Glo® substrate and CellTiter-Glo® buffer) was added to each well, to lyse the cells and release any ATP present. The plate was then incubated at room temperature for 10 minutes to stabilize the luminescent signal, which was recorded using a Flx 800 Fluorescence Microplate Reader and KC4 (version 3.4) software.

2.5.3. LDH Assay

The CytoTox-ONE™ Homogeneous Membrane Integrity Assay was used to assess membrane integrity. LDH, which is released through the membrane of damaged cells, is quantified by the conversion of lactate to pyruvate (catalysed by LDH). This reaction drives the secondary conversion of substrate, resazurin, by diaphorase to fluorescent product, resorufin. The fluorescence generated is directly proportional to the number of non-viable cells present. Research shows that particles interact and attach to cell membranes (Mu et al., 2012). This interaction of particles with the cell membrane may have adverse effects and this was tested using the LDH assay.
The cells were subcultured as previously described in sections 2.3.1 and 2.3.2.1 for astrocytes and BEAS-2B cells respectively. The cells were then seeded in a sterile, opaque 96-well plate at 1x10^4 cells/cm^2, in 100 µl cell culture media, followed by a 24 hour incubation, in order to allow the cells to adhere to the plates before treatment. Wells containing culture medium only, were used as blanks. The cell medium was replaced with fresh culture medium. The cells were treated with FeMn or silica at concentrations of 0, 5, 10 and 25 µg/cm^2, for a treatment period of 1 hour. Following this, 100 µl CytoTox-ONE™ reagent (substrate mix and assay buffer) was added to each well and the plate incubated at room temperature for 10 minutes. Thereafter, 50 µl stop solution was added and the fluorescence measured at an excitation wavelength of 560 nm, and an emission wavelength of 590 nm, using a Flx800 Fluorescence Microplate Reader and KC4 (version 3.4) software.

2.5.4. Particle Interference Studies (in the absence of cells)

2.5.4.1. XTT Assay

Reaction of particles in dispersion medium only: Particles were suspended in dispersion medium only, and the absorbance was recorded at 450 nm, in order to determine if particles absorb light at the same wavelength as the formazan chromophore product.

Reaction of particles with formazan chromophore product: Formazan was produced from XTT substrate, in the presence of 0.1 mM NADH, in a sterile 96 well plate. The plate was allowed to incubate at 37°C, with 5% CO_2 in a humidified atmosphere for 4 hours, to ensure that product formation reached saturation (plateau). FeMn or silica dust particles were added to the wells containing formazan at concentrations of 0, 25, 50 and 100 µg/cm^2, in a final volume of 100 µl culture medium (RPMI 1640 without phenol red, supplemented with 1% FBS and 1% Penicillin/Streptomycin). The absorbance was recorded at 450 nm, using an automated microplate reader (ELx800) and KC4 (version 3.4) software. Control wells received dispersion medium only.
2.5.4.2. ATP Assay

Reaction of particles in dispersion medium only: Particles were suspended in dispersion medium only, and the luminescence was recorded in order to determine if particles emit light at the same wavelength as the oxyluciferin product.

Reaction of particles with oxyluciferin product: Oxyluciferin was produced from luciferin substrate, in the presence of 1.5 µM ATP, in a sterile opaque 96 well plate. The plate was mixed for 2 minutes and allowed to incubate at room temperature for 10 minutes to allow saturated product formation. FeMn or silica dust particles were added to the wells containing oxyluciferin luminophore at concentrations of 0, 25, 50 and 100 µg/cm², in a final volume of 100 µl culture medium (RPMI 1640 without phenol red supplemented with 1% FBS and 1% Penicillin/Streptomycin). The luminescence was recorded using a Flx800 Fluorescence Microplate Reader and KC4 (version 3.4) software. Control wells received dispersion medium only.

2.5.4.3. LDH Assay

Reaction of particles in dispersion medium only: Particles were suspended in dispersion medium only, and the fluorescence was recorded at 560/590 nm in order to determine if particles fluoresce at the same wavelength as the resorufin fluorophore product.

Reaction of particles with resorufin product. Resorufin was produced from resazurin substrate, in the presence of 0.1 mM NADH, in a sterile opaque 96 well plate. The plate was allowed to incubate at room temperature for 10 min to allow saturated product formation. FeMn or silica dust particles were added to wells containing resorufin at concentrations of 0, 25, 50 and 100 µg/cm², in a final volume of 100 µl culture medium (RPMI 1640 without phenol red supplemented with 1% FBS and 1% Penicillin/Streptomycin). The fluorescence was recorded at an excitation wavelength of 560 nm and an emission wavelength of 590 nm, using a Flx800 Fluorescence Microplate Reader and KC4 (version 3.4) software. Control wells received dispersion medium only.
2.5.5. Real Time Cell Viability and Cytotoxicity Studies

Real time measurements of cell viability and cytotoxicity, based on cellular adherence were obtained using the xCELLigence RTCA system and RTCA (version 1.2.1) software. This system makes use of sensor electrodes on the bottom of special 96 well plates. Cells act as insulators on the electrode surface leading to an increase in impedance. Viable cells will spread and attach strongly, resulting in greater impedance of the electrodes. This electrode impedance is defined by a unitless parameter known as the cell index (CI). Verification was done using the resistor plate provided, with the RTCA station outside the incubator and then, again, when the RTCA station is placed inside the incubator.

The CI value is calculated as follows:

\[ CI = \max_{i=1}^N \frac{R_{\text{cell}}(f_i)}{R_b(f_i)} - 1 \]

where \( R_{\text{cell}}(f_i) \) is frequency-dependent electrode impedance at any time, \( R_b(f_i) \) is background impedance measured at the initial time without cells, and \( N \) is the number of the frequency points at which the impedance is measured.

The normalized CI (NCI) value is calculated as follows:

\[ NCI_i = \frac{CI_i(t)}{CI_i(t \text{ of dose})} \]

where \( CI_i(t) \) is CI at any time and \( CI_i(t \text{ of dose}) \) is CI at the time of particle dosing.

2.5.5.1. Proliferation Curves

Prior to running experiments, proliferation curves were established for both cell lines in order to determine the optimum cell seeding densities. Firstly, 50 µl cell culture medium was added to each well of an E-Plate 96 and the plate left to equilibrate for 30 minutes, at room temperature, in a sterile environment. The E-Plate 96 was then placed in the RTCA station and read to establish a baseline. Following this, the cells were subcultured as previously described in sections 2.3.1 and
2.3.2.1 for astrocytes and BEAS-2B cells, respectively, and 100 µl of cell suspension at various concentrations was added to each well of the E-Plate 96. It was left to equilibrate for 30 minutes, at room temperature, in a sterile environment. The E-Plate 96 was then placed in the RTCA station at 37°C, with 5% CO₂ in a humidified atmosphere. The proliferation of BEAS-2B cells was monitored over a period of 3 days and the proliferation of astrocytes was monitored over 2 weeks.

2.5.5.2. **Cytotoxicity Experiments**

Once the growth curves had been established, cytotoxicity experiments were performed, with both cell lines seeded at a concentration of 1x10⁴ cells/cm². The BEAS-2B cells were left to proliferate for 24 hours, prior to treatment. The astrocytes were allowed to mature for 2 weeks, with the culture medium being changed every three days, prior to treatment. During this time, readings were taken every 15 minutes. The medium was then replaced with fresh culture medium and cells treated with FeMn or silica at concentrations of 0, 5, 10 and 25 µg/cm². Readings were taken at 5 minutes intervals for the first 2 hours and then at 15 minutes intervals for the remainder of the experiment.

2.5.6. **Nuclear Translocation of Nrf2 and Nf-κB**

Western blots were used to determine the effects of FeMn or silica dust particles on the translocation of transcription factors, Nf-κB and Nrf2, from the cytoplasm to the nucleus. Western blots were used to detect and quantify a specific protein of interest by using gel electrophoresis to separate native proteins based on their size. The separated proteins were then transferred to a membrane (nitrocellulose in this case), where the proteins were detected using antibodies specific to the target protein. The target proteins for this study were the translocating proteins (A) Nrf2, responsible for the transcription of genes encoding numerous antioxidant enzymes and one of the first mechanisms activated in order to overcome oxidative stress, and (B) Nf-κB, responsible for the transcription of the genes for numerous pro-inflammatory markers, is activated as the final step before apoptosis and mitochondrial damage occur.
2.5.6.1. **Cell Fractionation**

The cytoplasmic and nuclear fractions were obtained using the Cellytic™ NuCLEAR™ Extraction Kit. The cells were subcultured as described in 2.3.1 and 2.3.2.1 for astrocytes and BEAS-2B cells, respectively, and were seeded in 12 well plates at $1 \times 10^4$ cells/cm$^2$ in 1ml culture medium. This was followed by a 24 hour incubation to allow the cells to adhere to the plates. The cell medium was then replaced with fresh culture medium. The cells treated with FeMn or silica at concentrations of 0, 5, 10 and 25 µg/cm$^2$ for treatment periods of 6 and 24 hours. Cells treated with 100 µM H$_2$O$_2$ for 15 minutes on ice, were included as a positive control. The cells were washed once with 500 µl cold DPBS, and following the second addition of DPBS, the cells were removed from the plates by scraping on ice using a cell scraper. The scraped cells were placed in eppendorf tubes and centrifuged at 450 g for 5 minutes at 4°C. The supernatant was discarded. The pellet was gently resuspended in 125 µl cold 1X hypotonic lysis buffer (10X hypotonic lysis buffer, 0.01 M dithiothreitol (DTT), protease inhibitor) and the tubes were incubated on ice for 15 minutes. The protease inhibitors were added in order to prevent degradation, of the protein of interest, by proteases and phosphatases. DTT was added in order to reduce the disulfide bonds present (MacPhee, 2010). Thereafter, 1.5 µl 10% octylphenoxypolyethoxyethanol (IGEPAL), a non-ionic non-denaturing detergent, was added and the tubes were vortexed vigorously for 10 seconds. The tubes were centrifuged at 10 000 g for 1 minute at 4°C. The supernatant was transferred to a fresh eppendorf tube on ice (cytoplasmic fraction). The pellet was resuspended in 35 µl extraction buffer (extraction buffer, 0.01 M DTT, protease inhibitor) and vortexed for 15 minutes. The tubes were centrifuged at 20 000 g for 5 minutes at 4°C. The supernatant was transferred to a fresh eppendorf tube on ice (nuclear fraction). The cytoplasmic and nuclear fractions were snap frozen in liquid nitrogen and stored at -70°C until needed.

2.5.6.2. **Bicinchoninic Acid (BCA) Protein Determination Assay**

The BCA assay relies on two reactions to determine the total protein concentration in a solution. The peptide bonds in proteins reduce copper (Cu$^{2+}$) ions from the cupric sulfate to Cu+. The amount of Cu$^{2+}$ reduced is proportional to the amount of protein present in the solution. The BCA chelates with the Cu$^+$ ions. This reaction forms a purple-coloured product that strongly absorbs light at 562 nm. However, this reaction can also be measured at 540 nm.
The samples were thawed on ice, during which 24.5 ml of Pierce® BCA Protein Assay Reagent A and 500 µl BCA™ Protein Assay Reagent B was added together (reagent mix). Thereafter, 1 ml of the reagent mix was added to small glass test tubes, one for each sample, as well as eight standards. The bovine serum albumin (BSA) standards were prepared at concentrations of 0, 50, 100, 200, 400, 600, 800, 1000 µg/ml, where 20 µl of each standard was pipetted into the standard tubes. Following this, 2 µl of each sample was pipetted into each sample tube, as well as 18 µl of distilled water. The tubes were then vortexed briefly, and placed in a waterbath at 37°C for 30 minutes. The samples were allowed to cool, and 200 µl of each sample was pipette, in duplicate, into a 96 well plate. The plate was read spectrophotometrically at 540 nm, on an automated microplate reader (ELx800) using KC4 (version 3.4) software.

2.5.6.3. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Casting the Gels

Tris-glycine SDS-PAGE gels are most commonly used, and were used in the current study. SDS is a strong anionic detergent that binds to the hydrophobic regions of denatured proteins. The amount of bound SDS is proportional to the molecular mass of the polypeptide chains. The now, negatively charged SDS-protein complexes undergo directional migration and are separated based on polypeptide size (MacPhee, 2010).

The Mini PROTEAN Tetra Cell Casting module was assembled. A 12% separating gel consisting of 30% Acrylamide, 0.8% bisacrylamide, water, 1 M Tris pH 8.8, 10% sodium dodecyl sulphate (SDS), 10% ammonium persulphate (APS) and N,N,N′,N′- tetramethylenediamine (TEMED), was prepared, cast and allowed to polymerize for 1 hour. A 5% stacking gel (30% Acrylamide, 0.8% bisacrylamide, water, 1 M Tris pH 6.8, 10% SDS, 10% APS and TEMED) was then prepared, cast, the combs inserted and allowed to polymerize for an hour. The Mini-PROTEAN® II electrophoresis cell was assembled and 1X running buffer (10X Tris/Glycine/SDS Buffer) added. The wells were cleaned and the gels were pre-run for 20 minutes at 70 V.
**Sample Preparation and Electrophoresis**

The samples were diluted with laemmli sample buffer consisting of 475 µl laemmli sample buffer and 25 µl mercaptoethanol. The laemmli sample buffer consists of glycerol, which increases the density of the sample, facilitating sample loading. As well as, bromophenol blue, this serves as a dye front to monitor band migration. The mercaptoethanol is added to reduce disulphide bonds. The samples were then heated at 100°C for 5 minutes and centrifuged at 11,000 g for 1 minute. The samples (15 µg) and 5 µl of Precision Plus Protein™ Standards Kaleidoscope™ were loaded into the wells and the gels were run for 90 minutes at 120 V and 40 mA.

**2.5.6.4. Preparation of the Nitrocellulose Membrane and Transfer of Proteins**

Nitrocellulose was used in the current study due to its sensitivity and ability to bind proteins better than PVDF. Nitrocellulose membranes bind proteins non-covalently by means of hydrophobic interactions (Alegria-Schaffer *et al.*, 2009). The nitrocellulose membrane was marked for orientation and cut in half, then soaked in 1X transfer buffer (10X Tris-Glycine, 20% methanol), along with extra thick filter paper and sponges. Methanol was added to the transfer buffer in order to improve adsorption between the polypeptides and the membrane, and to prevent protein distortion, as well as, gel swelling (MacPhee, 2010). The gels were removed from the Mini-PROTEAN® II electrophoresis cell and the transfer cassettes assembled with the pre-soaked sponges, extra thick filter paper, gels and the nitrocellulose membrane. The transfer cassettes were then placed in the transfer tank filled with 1X transfer buffer. The gel was orientated to the cathode (negative electrode - black) and an ice brick added. The entire apparatus was then covered in ice and the proteins left to transfer for 90 minutes at 200 V and 400 mA.

**2.5.6.5. Blocking and Antibody Treatment**

The blot is blocked using a non-relevant protein, like non-fat milk powder or BSA, both of which were used in this study, dissolved in TBST. The optimal blocking conditions are sometimes stated on the antibody data sheet, as was the case with the Nf-κB antibody used in this study. Blocking serves two purposes. The first is to prevent non-specific binding of the antibody to hydrophobic
sites on the membrane. The second is to support renaturation of antigenic sites (Alegria-Schaffer et al., 2009; MacPhee, 2010).

2.5.6.5.1. **Nrf2**

After protein transfer to the nitrocellulose membrane, the membrane was removed, cut to size and washed thrice with 1X TBST buffer (10X Tris buffered saline (TBS) and 0.1% Tween 20), for 5 minutes. The membrane was then placed in 10 ml blocking solution (5% blocking grade milk, 1X TBST), for 1 hour. This was followed by incubation with rabbit primary antibody, directed against human Nrf2 (1:200 in blocking solution) overnight at 4°C. The membrane was washed thrice with 1X TBST buffer for 5 minutes. Goat anti-rabbit secondary antibody, with a horse radish peroxidase (HRP) conjugate, was added to the membrane (1:5000 in blocking solution) and incubated for 2 hours at room temperature. The membrane was washed thrice with 1X TBST buffer, for 5 minutes, followed by incubation in PIERCE SuperSignal® West Dura Chemiluminescent substrate, for 30 seconds in order to allow for the detection of the HRP.

2.5.6.5.2. **NF-κB p65**

After protein transfer to the nitrocellulose membrane, the membrane was removed, cut to size and washed thrice with 1X TBST buffer (10X TBS, 0.1% Tween 20), for 5 minutes. The membrane was then placed in 10 ml blocking solution (5% BSA, 1X TBST) for 1 hour. This was followed by incubation with rabbit primary antibody, directed against human Nf-κB p65 (1:2000 in blocking solution), overnight at 4°C. The membrane was washed thrice with 1X TBST buffer, for 5 minutes. Goat anti-rabbit secondary antibody, with a HRP conjugate, was added to the membrane (1:5000 in blocking solution) and incubated for 2 hours at room temperature. The membrane was washed thrice with 1X TBST buffer for 5 minutes, followed by incubation in PIERCE SuperSignal® West PICO Chemiluminescent substrate, for 1 minute to allow for the detection of the HRP.

2.5.6.6. **Detection**

The indirect method of detection, which was used in this study, makes use of a labeled secondary antibody bound to an unlabeled primary antibody. The secondary antibody is conjugated to an enzyme which is most often HRP. Luminol (5-amino-2,3-dihydro 1,4- phthalazinedione)–based
substrates are most often used to generate the chemiluminescent signal, during the oxidation of luminol by H$_2$O$_2$ (Alegria-Schaffer et al., 2009). Once the exposed blots have been captured under optimized conditions, the accompanying analytical software, of imaging systems, can be used to quantify the relative abundance of a specific protein using densitometry (MacPhee, 2010).

The membranes were analysed using the auto exposure feature of the G:Box Chemi XR5 gel documentation system and GeneSys software (version 1.3.3.0). The density of the bands was analysed using the GeneTools software (version 4.03b).

### 2.5.6.7. Loading Control

The relative densities measured, are an estimation of the protein expression levels and are based on the assumption that equal amounts of protein were loaded when the gels were run. In order to correct for any loading errors, it is important to use a loading control. The loading control is often a house keeping protein such as, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β-actin, and β-tubulin (Li and Shen, 2013). These proteins are used, because their expression remains constant from sample to sample, cell to cell or treatment to treatment. β-actin is one of the most abundant proteins found in eukaryotic cells, where it assists with motility and cytoskeleton maintenance (Ruan and Lai, 2007). β-actin was used as the loading control in this study.

After visualizing the protein of interest, the membrane was stripped in 10 ml stripping buffer for 30 minutes. Thereafter, the membrane was washed thrice with 1X TBST for 5 minutes. The membrane was then placed in 25 ml blocking solution, for 1 hour. This was followed by incubation with mouse primary antibody directed against β-actin (1:50 000 in blocking solution), overnight at 4°C. The membrane was washed thrice with 1X TBST buffer for 5 minutes. Goat anti-mouse secondary antibody, with HRP conjugate, was added to the membrane (1:5000 in blocking buffer) and incubated for 2 hours at room temperature. The membrane was washed thrice with 1X TBST buffer for 5 minutes, followed by incubation in PIERCE SuperSignal® West PICO Chemiluminescent substrate for 1 minute to allow for the detection of the HRP. Detection was performed as described in section 2.5.6.6.
2.6. Genotoxicity Study

2.6.1. Alkaline Comet Assay

The alkaline comet assay was performed, in order to determine the genotoxicity of the FeMn and silica particles, by studying the DNA damage that these particles induce. The comet assay is a sensitive technique, which can detect different types of DNA damage. It involves the encapsulation of cells in agarose gel, after which the cells are lysed and the DNA allowed to unwind under alkaline conditions (pH > 13). Supercoiled DNA is attached to a nuclear scaffold by a series of loops. During DNA denaturation or unwinding, if there are any breaks in these loops, the loops will be able to relax and, under the influence of an electrophoretic field, migrate towards the anode. The basic principle of the comet assay is that if a substance is highly genotoxic it will result in numerous strand breaks. The higher the number of strand breaks, the more frequently the relaxed DNA loops occur, resulting in a greater percentage of DNA in the observed comet tail (Bowden et al., 2003; Stone et al., 2009; Karlsson, 2010; Azqueta and Collins, 2013).

2.6.1.1. Cell Treatment

The cells were subcultured as previously described in section 2.3.1 and 2.3.2.1 for astrocytes and BEAS-2B cells, respectively, and were seeded in 12 well plates at 1x10^6 cells/cm^2, in 1ml culture medium. This was followed by a 24 hour incubation to allow the cells to adhere to the plates. The cell medium was then replaced with fresh culture medium. The cells were treated with FeMn or silica at concentrations of 0, 5, 10 and 25 µg/cm^2 for treatment periods of 1, 6 and 24 hours. Cells treated with 100 µM H_2O_2 for 15 minutes on ice, were included as a positive control for DNA damage. Cells were then washed once with 500 µl cold DPBS. Following the second addition of DPBS, the cells were removed from the plates by scraping on ice using a cell scraper. The scraped cells were placed in eppendorf tubes and centrifuged at 200 g for 5 minutes at 4°C, after which the supernatant was discarded. The pellet was resuspended in 500 µl cold DPBS, centrifuged at 200 g for 5 minutes at 4°C and the supernatant was discarded. The tubes were then stored on ice.
2.6.1.2. Embedding the Cells in Agarose Gel

Microscope slides had been pre-coated with 1% normal melting point agarose (NMPA) gel, with the excess gel removed from the underside of the slides. The slides were left to dry over night. Cells were embedded by mixing the cell pellet with 150 µl 0.8% low melting point agarose (LMPA) gel. Of this gel mixture, 70 µl was pipetted onto the pre coated slides, in duplicate. Each gel was covered with a cover slip and allowed to solidify for 15 minutes at 4°C.

2.6.1.3. Cell Lysis

The lysis solution usually contains a high salt concentration, as well as, a detergent like Triton-X. The salt is responsible from removing the cellular and nuclear membranes, and the detergent dissolves the histones found within the supercoiled DNA (Azqueta and Collins, 2013). The cover slips were removed and the slides were incubated in coplin jars, filled with cold lysing solution (2.5 M sodium chloride (NaCl), 100 mM EDTA, 10 mM Tris and 1% Triton-X pH 10), for 1 hour at 4°C.

2.6.1.4. DNA Unwinding and Electrophoresis

The slides were then washed twice with rinsing solution (1X PBS pH 7.4). The slides were placed in the centre of a horizontal electrophoresis tank and orientated so that the DNA migrated from the cathode (black) to the anode (red). The remainder of the tank was filled with blank slides. Sufficient cold electrophoresis buffer, consisting of 300 mM NaOH and 1 mM EDTA at a pH >13 (made from 10 M sodium hydroxide (NaOH) and 200 mM EDTA stock solutions), was added to the tank in order to cover the slides. The slides were then incubated for 40 minutes at 4°C. Electrophoresis was performed across the slides at 25 V and 300 mA, at 4°C for 1 hour. The slides were then neutralized for 10 minutes in coplin jars filled with neutralizing buffer (0.4 M Tris, pH 7.5). They were removed and left to dry overnight.

2.6.1.5. Visualization and Scoring

When reporting DNA damage, a variety of parameters are utilized. The most commonly used include, tail length, percentage tail DNA (% tail DNA), and tail moment. Tail length is only useful at low concentrations of DNA damage, and plateaus once the tail is established. Tail length is also
sensitive to the background settings of the analysis software used. Tail moment, which is the product of tail length and tail intensity, does not linearly represent the dose and gives no information regarding the comets appearance. For these reasons, relative tail intensity or % tail DNA was chosen as the parameter to represent DNA damage in this study. Percentage tail DNA is the recommended parameter for use, because it is not affected by background settings, it is able to discriminate damage over a wide range and provides an indication of comet appearance (Bowden et al., 2003; Collins, 2004; Azqueta and Collins, 2013).

The slides were stained with a 10 mg/ml ethidium bromide (EtBr) solution. The visualization and scoring was accomplished using an Olympus BX41 Microscope, with fluorescent burner and Comet Assay IV software.

When performing these experiments, it was considered important to include a number of controls. In addition to the bench mark particle (silica), H₂O₂ was used as a positive control in this study. Lazarova and colleagues tested the known genotoxicity of H₂O₂, on various primary rat cells (lymphocytes, testicular cells, type II pneumocytes and hepatocytes), by treating them with 50 - 200 µM H₂O₂. The results indicated a dose dependent increase in DNA damage in all cell types tested. H₂O₂ genotoxicity resulted from the catalase dependent decomposition into O₂ and the production of OH• through the Fenton or Haber–Weiss reactions (Lazarova et al., 2006).

In most genotoxicity experiments, viability studies are run concurrently, usually in the form of the trypan blue exclusion assay. This assay however, indicates damage to the cell membrane only, which could be caused during harvesting of the cells. Cells are able to recover from this kind of membrane damage and, therefore, cells that stain positive may in fact be viable. The best indication of viability, when using the comet assay, is represented by untreated cells that have a % tail DNA of approximately 10% (Collins, 2004).
2.7. Statistical Analysis

All results requiring statistical analysis were expressed as the standard error of the means (SEM). Differences between the treatment groups were analyzed using one-way analysis of variance (ANOVA), followed by Bonferroni’s post hoc test. Statistical significance was set at \( P < 0.05 \). The number of replicates for each test is stated in the figure legends. Data analysis was carried out using Graph-Pad prism. These statistical tests were chosen as they have been used in combination for similar studies (Yin, et al., 2007; Ni, et al., 2011). ANOVA is used to establish changes in the group means of more than two groups, and assists in eliminating type 1 errors that may result from performing multiple t-tests. Bonferroni’s post hoc test is used as a multiple comparison test to eliminate false results and ensures that no results obtained occurred by chance.
Chapter 3: Results

3.1 Characterization of FeMn and Silica Particles

In order to elaborate on the toxicity results obtained for the FeMn and silica particles, some of the physicochemical characteristics of these particles were analyzed. The characterization included particle size distribution, surface area analysis, pH measurements and determination of trace elemental composition. Prior to any analysis, the dust samples were first sieved using a < 20 µm sieve in order to obtain the inhalable fraction of dust.

3.1.1. Size Distribution

It was important to analyze the size distribution of the particles, because their fate and transport within the organism, as well as, possible target organs could then be ascertained. The size distributions were analyzed using a SMPS and an APS. The aerodynamic diameter refers to a physical property of a particle in air. Particles, in general, are asymmetrically shaped, hence, making it difficult to measure their actual diameter. The aerodynamic diameter is, therefore, used to describe particles behaviour in air, as if it were a perfect sphere. The SMPS measures particles with aerodynamic diameters ranging between 7 and 300 nm. The APS measures particles with aerodynamic diameters ranging between 0.5 and 20 µm.

The size distribution of FeMn obtained using the SMPS and APS, is presented in Figure 13. Specifically, Figure 13A represents the number of particles per cm$^3$ (#/cm$^3$) relative to aerodynamic diameter (nm), indicating the number of particles per cm$^3$ that have the same aerodynamic size. Figure 13B is the size distribution as mass of particles per m$^3$ (µg/m$^3$), relative to aerodynamic diameter (nm), indicating the mass of particles per m$^3$ with the same aerodynamic size. According to the APS and SMPS analysis, FeMn has a median aerodynamic diameter of 1.518 µm (in the micron range) and of 114 nm (in the nano range) (refer to Figure 13 and Table 1). The combined size distributions of the APS and SMPS, based on mass of particles per m$^3$ air, show a large mass value in the micron-range. This is due to the presence of larger particles, which weigh more than
nano particles (refer to Figure 13B). Conversely, when the size distributions based on number of particles per cm$^3$ air are used, a large number of particles are observed in the nano-range (refer to Figure 13A). Hence, for a specific mass of FeMn, the percentage of nanoparticles with lower mass is higher than that of micron particles with higher mass.

![Figure 13: The size distribution of FeMn particles, represented as number of particles per cm$^3$ (#/cm$^3$) (A) and as mass of particles per m$^3$ (µg/m$^3$) (B), relative to aerodynamic diameter (nm). Data in the figures are single particle size distributions of merged results obtained from the SMPS (indicated by the green vertical lines) and the APS (indicated by the pink vertical lines).](image)

The combined size distributions of the APS and SMPS, based on mass of particles per m$^3$ air for silica, also show a large mass value in the micron-range (refer to Figure 14B), similar to what was found for FeMn. When the size distributions based on number of particles per cm$^3$ air are used, a large number of particles are observed in the nano-range (refer to Figure 14A). This, therefore, means that regarding a specific mass of silica, there is a higher percentage of particles within the nano region with low mass compared to particles within the micron region that have a higher mass. According to the APS and SMPS analysis, silica has a median aerodynamic diameter of 1.861 µm in the micron range and of 168 nm in the nano range, and is shown to have a bimodal size distribution (refer to Figure 14 and Table 1). The median aerodynamic diameter of 1.861 µm in the micron range, for commercially available silica, was found to be similar to aerodynamic diameter of 1.6 µm provided by the supplier (Min-U-Sil 5 data sheet, supplied in Appendix 3).
Figure 14: The size distribution of silica particles, represented as number of particles per cm$^3$ (#/cm$^3$) (A) and as mass of particles per m$^3$ (µg/m$^3$) (B), relative to aerodynamic diameter (nm). Data in the figures are single particle size distributions of merged results obtained from the SMPS (indicated by the green vertical lines) and the APS (indicated by the pink vertical lines).

3.1.2. Surface Area

It was also considered important to measure the specific surface area, as the surface area of a particle is inversely proportional to its size. In addition, the larger the surface area, the more reactive the particle is thought to be. Specific surface area is defined as the surface area per unit weight of particulate material. The surface area of both particles was determined using BET analysis, which uses the adsorption of nitrogen onto the surface of the particles in order to measure specific surface area expressed as m$^2$/g. The FeMn particles were found to have a surface area of 3.7 m$^2$/g and the silica particles had a surface area of 5.1 m$^2$/g, which correlates well to the expected surface area of 5 m$^2$/g specified by the supplier. The pH of culture media, following the suspension of FeMn and silica, was also measured and was found to be similar to the pH under normal physiological conditions (refer to Table 1).
Table 1: Characterization results for FeMn and silica particles.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>FeMn</th>
<th>Silica</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size</td>
<td>114 nm&lt;sup&gt;a&lt;/sup&gt;</td>
<td>168 nm&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>1.518 µm&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.861 µm&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Specific surface area</td>
<td>3.7</td>
<td>5.1</td>
</tr>
<tr>
<td>(m²/g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH at 37°C</td>
<td>7.77</td>
<td>7.69</td>
</tr>
<tr>
<td>Colour</td>
<td>Dark brown</td>
<td>White</td>
</tr>
</tbody>
</table>

<sup>a</sup> Median aerodynamic diameter determined with the SMPS Model 3080

<sup>b</sup> Median aerodynamic diameter determined with the APS Model 3321

3.1.3. Elemental Composition

The elemental compositions of both the FeMn and silica particles, as determined using ICP-AES, are listed in Table 2. The elements making up the bulk of the FeMn sample are highlighted in blue. Those found at high concentrations in the silica sample are highlighted in purple. FeMn is an incidental particle and as such, the sample was found to contain high concentrations of aluminium, calcium, iron, magnesium, sodium, lead and zinc with the largest portion of the sample being made up of Mn. The Mn was present in the sample at a concentration of 352 000 mg/kg. Since the silica sample is manufactured, it is almost exclusively made up of silica in the form of silicon dioxide (SiO₂), where most other elements were below the limit of detection. However, the sample did contain small concentrations of aluminium, calcium and titanium, where aluminium was present at the highest concentration of all the elements tested (1520 mg/kg).
Table 2: The elemental composition, expressed as mg/kg, of the FeMn and silica particles analysed using ICP-AES. Elements present at high concentrations in the FeMn sample were highlighted in blue. Those present in high concentrations in the silica sample were highlighted in purple.

<table>
<thead>
<tr>
<th>Element (mg/kg)</th>
<th>FeMn</th>
<th>Silica</th>
<th>Element (mg/kg)</th>
<th>FeMn</th>
<th>Silica</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aluminium (Al)</td>
<td>12900</td>
<td>1520</td>
<td>Molybdenum (Mo)</td>
<td>4.46</td>
<td>&lt;DL</td>
</tr>
<tr>
<td>Arsenic (As)</td>
<td>16.5</td>
<td>0.209</td>
<td>Sodium (Na)</td>
<td>3230</td>
<td>&lt;DL</td>
</tr>
<tr>
<td>Gold (Au)</td>
<td>&lt;DL</td>
<td>&lt;DL</td>
<td>Niobium (Nb)</td>
<td>3.98</td>
<td>&lt;DL</td>
</tr>
<tr>
<td>Boron (B)</td>
<td>256</td>
<td>25.8</td>
<td>Neodymium (Nd)</td>
<td>3.9</td>
<td>0.992</td>
</tr>
<tr>
<td>Barium (Ba)</td>
<td>223</td>
<td>7.61</td>
<td>Nickel (Ni)</td>
<td>48.4</td>
<td>0.564</td>
</tr>
<tr>
<td>Beryllium (Be)</td>
<td>0.203</td>
<td>&lt;DL</td>
<td>Phosphorus (P)</td>
<td>353</td>
<td>36.9</td>
</tr>
<tr>
<td>Bismuth (Bi)</td>
<td>15.3</td>
<td>&lt;DL</td>
<td>Lead (Pb)</td>
<td>2520</td>
<td>0.486</td>
</tr>
<tr>
<td>Calcium (Ca)</td>
<td>23600</td>
<td>1030</td>
<td>Palladium (Pd)</td>
<td>&lt;DL</td>
<td>&lt;DL</td>
</tr>
<tr>
<td>Cadmium (Cd)</td>
<td>1.06</td>
<td>&lt;DL</td>
<td>Praseodymium (Pr)</td>
<td>1.1</td>
<td>0.271</td>
</tr>
<tr>
<td>Cerium (Ce)</td>
<td>10.6</td>
<td>2.58</td>
<td>Platinum (Pt)</td>
<td>&lt;DL</td>
<td>&lt;DL</td>
</tr>
<tr>
<td>Colbalt (Co)</td>
<td>35.6</td>
<td>0.0509</td>
<td>Rubidium (Rb)</td>
<td>118</td>
<td>0.123</td>
</tr>
<tr>
<td>Chromium (Cr)</td>
<td>104</td>
<td>1.02</td>
<td>Rhodium (Rh)</td>
<td>&lt;DL</td>
<td>&lt;DL</td>
</tr>
<tr>
<td>Caesium (Cs)</td>
<td>17.8</td>
<td>&lt;DL</td>
<td>Antimony (Sb)</td>
<td>2.03</td>
<td>&lt;DL</td>
</tr>
<tr>
<td>Copper (Cu)</td>
<td>33.3</td>
<td>1.21</td>
<td>Scandium (Sc)</td>
<td>4.03</td>
<td>1.22</td>
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<tr>
<td>Dysprosium (Dy)</td>
<td>0.689</td>
<td>0.182</td>
<td>Selenium (Se)</td>
<td>19.4</td>
<td>&lt;DL</td>
</tr>
<tr>
<td>Erbium (Er)</td>
<td>0.402</td>
<td>0.182</td>
<td>Samarium (Sm)</td>
<td>0.776</td>
<td>0.187</td>
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<tr>
<td>Europium (Eu)</td>
<td>0.158</td>
<td>&lt;DL</td>
<td>Tin (Sn)</td>
<td>3.17</td>
<td>&lt;DL</td>
</tr>
<tr>
<td>Iron (Fe)</td>
<td>71800</td>
<td>222</td>
<td>Strontium (Sr)</td>
<td>88.3</td>
<td>4.78</td>
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<tr>
<td>Gallium (Ga)</td>
<td>12.9</td>
<td>0.515</td>
<td>Tantalum (Ta)</td>
<td>&lt;DL</td>
<td>&lt;DL</td>
</tr>
<tr>
<td>Gadolinium (Gd)</td>
<td>0.728</td>
<td>0.187</td>
<td>Terbium (Tb)</td>
<td>0.101</td>
<td>0.0118</td>
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<tr>
<td>Germanium (Ge)</td>
<td>10.8</td>
<td>&lt;DL</td>
<td>Tellurium (Te)</td>
<td>4.34</td>
<td>&lt;DL</td>
</tr>
<tr>
<td>Hafnium (Hf)</td>
<td>0.869</td>
<td>&lt;DL</td>
<td>Thorium (Th)</td>
<td>2.91</td>
<td>&lt;DL</td>
</tr>
<tr>
<td>Mercury (Hg)</td>
<td>&lt;DL</td>
<td>&lt;DL</td>
<td>Titanium (Ti)</td>
<td>593</td>
<td>90.2</td>
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<tr>
<td>Holmium (Ho)</td>
<td>0.12</td>
<td>&lt;DL</td>
<td>Thallium (Tl)</td>
<td>1.82</td>
<td>&lt;DL</td>
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<tr>
<td>Indium (In)</td>
<td>&lt;DL</td>
<td>&lt;DL</td>
<td>Thulium (Tm)</td>
<td>0.0433</td>
<td>&lt;DL</td>
</tr>
<tr>
<td>Iridium (Ir)</td>
<td>&lt;DL</td>
<td>&lt;DL</td>
<td>Uranium (U)</td>
<td>0.605</td>
<td>0.144</td>
</tr>
<tr>
<td>Potassium (K)</td>
<td>15700</td>
<td>30.8</td>
<td>Vanadium (V)</td>
<td>12.3</td>
<td>0.271</td>
</tr>
<tr>
<td>Lanthanum (La)</td>
<td>5.11</td>
<td>1.53</td>
<td>Tungsten (W)</td>
<td>1.4</td>
<td>&lt;DL</td>
</tr>
<tr>
<td>Lithium (Li)</td>
<td>7.79</td>
<td>&lt;DL</td>
<td>Yttrium (Y)</td>
<td>4.02</td>
<td>1.33</td>
</tr>
<tr>
<td>Lutetium (Lu)</td>
<td>0.0441</td>
<td>&lt;DL</td>
<td>Ytterbium (Yb)</td>
<td>0.395</td>
<td>0.128</td>
</tr>
<tr>
<td>Magnesium (Mg)</td>
<td>11800</td>
<td>&lt;DL</td>
<td>Zinc (Zn)</td>
<td>5730</td>
<td>0.531</td>
</tr>
<tr>
<td>Manganese (Mn)</td>
<td>352000</td>
<td>2.07</td>
<td>Zirconium (Zr)</td>
<td>35.9</td>
<td>18.9</td>
</tr>
</tbody>
</table>

< DL Below the limits of detection
3.2. Cell Based Studies

All the cell based investigations were performed on primary astrocytes, isolated from 1 day old Sprague-Dawley rat pups, as well as on BEAS-2B. LDH, ATP and XTT assays were performed on the BEAS-2B cells, only.

3.2.1. Cellular Uptake of FeMn and Silica Particles

In order to establish the interaction of the FeMn and silica particles, with astrocytes and BEAS-2B cells, the cellular uptake of the particles was visualized using the CytoViva® Hyperspectral imaging system and DAGE exponent software. The two cell lines were treated with FeMn or silica, at concentrations of 5, 10 and 25 µg/cm², for periods of 6 and 24 hours. Untreated cells were also included as a negative control. The cells were then fixed with formalin and viewed under an oil lens at 60X magnification.

Images of the astrocytes were taken 6 hours after treatment with different concentrations of FeMn or silica (refer to Figure 15). Most of the FeMn and silica particles appeared to form large aggregates once suspended in culture medium. A large portion of these aggregates were seen adsorbed to the surface of the cells. However, some particle uptake can also be observed. The aggregates increased in size as the particle concentration increased. Adherence of particles to the cell surface, as well as, and particle uptake, increased from the 6 hour (refer to Figure 15) to the 24 hour (refer to Figure 16) treatment periods. From the uptake studies, it was observed that the particles were not uniformly distributed throughout the cell, but rather accumulated within specific intracellular regions. This intracellular accumulation increased from the 6 hour (Figure 15) to the 24 hour (see Figure 16) treatment periods.
Figure 15: Representative images of FeMn or silica uptake, and interaction with astrocytes treated for 6 hours. The images were taken using the CytoViva® hyperspectral imaging system. Astrocytes were treated with 5, 10 and 25 µg/cm² FeMn (on the left) or silica (on the right). The cells were then fixed and visualized using an oil lens at 60X magnification.
Figure 16: Representative images of FeMn or silica particle uptake, and interaction with astrocytes treated for 24 hours. The images were taken using the CytoViva® hyperspectral imaging system. Astrocytes were treated with 5, 10 and 25 µg/cm² FeMn (on the left) or silica (on the right). The cells were then fixed and visualized using an oil lens at 60X magnification.
Images of BEAS-2B cells were also taken 6 hours after treatment with different concentrations of FeMn or silica (refer to Figure 17). Again, particle aggregates could be seen in these images, adsorbing to the surface of the BEAS-2B cells. Similar observations could be made after incubation of the cells with different concentrations of particles for 24 hours (refer to Figure 18). Again, it was observed that the particles were not uniformly distributed throughout the cell, but rather accumulated within specific intracellular regions. This intracellular accumulation increased from the 6 hour (Figure 17) to the 24 hour (Figure 18) treatment periods.

In all the images analysed, it could be noted that for the same dose of particles, the silica samples contained a larger number of particles compared to that of FeMn.
Figure 17: Representative images of FeMn or silica uptake, and interaction with BEAS-2B cells treated for 6 hours. The images were taken using the CytoViva® hyperspectral imaging system. BEAS-2B cells were treated with 5, 10 and 25 µg/cm² FeMn (on the left) or silica (on the right). The cells were then fixed and visualized using an oil lens at 60X magnification.
Figure 18: Representative images of FeMn or silica uptake, and interaction with BEAS-2B cells treated for 24 hours. The images were taken using the CytoViva® hyperspectral imaging system. BEAS-2B cells were treated with 5, 10 and 25 µg/cm² FeMn (on the left) or silica (on the right). The cells were then fixed and visualized using an oil lens at 60X magnification.

In order to confirm the presence or absence of FeMn or silica particles in the cell or adsorbed to the surface of the cell, the first step was to collect spectral profiles of these particles. Figure 19A and 19B represent the spectral profiles of FeMn and silica particles, respectively. Particles suspended in medium were spread on a slide and allowed to dry prior to hyperspectral imaging.
Ten randomly selected spectra were obtained from particles. In the figures, each coloured line represents the spectrum from a single pixel, and these collections of representative spectra form a known spectral library of the particles. In Figure 19, it can be seen that FeMn and silica have homogenous curves within the sample. In addition, both particles have a unique spectral profile, which may be used to confirm the presence of either particle inside the cell, or adsorbed to the surface of the cell.

![Figure 19: Spectral profiles of FeMn (A) and silica (B) particles. Ten spectral profiles of randomly selected FeMn and silica particles were collected. Each coloured line represents the spectrum from a single pixel.](image)

The subsequent analyses used SAM to verify the presence or absence of FeMn or silica in the two cell types. SAM is an analysis tool, part of the ENVI software, used for the identification of a known material. It uses a unique spectral profile in order to assess the presence of this known material, in an image acquired, using hyperspectral imaging. The process is performed by comparing the known spectra for a material, to unknown spectra. It identifies pixels in the unknown image that map to the known spectra, irrespective of the light intensity. Using this process on scans of the untreated astrocytes, a representative output was obtained (refer to Figure 20). In this figure, the HSI scan of untreated astrocytes represents an “unknown” spectral image (see Figure 20A). The known spectral library of the FeMn and silica particles was mapped against this image (refer to Figure 20B and 20C). Both the FeMn and silica spectral libraries did not map onto the image of the cells, thereby verifying the absence of FeMn or silica particles in the untreated cells.
Figure 20: Representative images of the SAM analyses, performed on untreated astrocytes. (A) HSI scan of untreated astrocytes; (B) SAM image indicating pixels of (A) that matched the spectral library of FeMn particles; (C) SAM image indicating pixels of (A) that matched the spectral library of silica particles.

Since the absence of particles was verified in the untreated cells, it was important to verify the presence of particles in treated cells. SAM was again used on scans of astrocytes incubated with FeMn or silica particles in order to obtain a representative output (see Figure 21). In this figure, the HSI scan of astrocytes incubated with 5 µg/cm² FeMn or silica for 6 and 24 hours represent “unknown” spectral images (refer to Figure 21 Ai, Bi, Ci and Di). The known spectral library of the FeMn and silica particles was mapped against this image indicated in blue (see Figure 21 Aii, Bii, Cii and Dii). Both the FeMn and silica spectral libraries mapped onto the images of the cells, thereby verifying the presence of FeMn or silica in the astrocytes.
Figure 21: Representative images of SAM analyses, performed on astrocytes treated with FeMn or silica, for 6 and 24 hours. (A, i) HSI scan of astrocytes treated for 6 hours with 5 µg/cm² FeMn; (A, ii) SAM image indicating pixels of (A, i) that matched the spectral library of FeMn particles shown in blue; (B, i) HSI scan of astrocytes treated for 24 hours with 5 µg/cm² FeMn; (B, ii) SAM image indicating pixels of (B, i) that matched the spectral library of FeMn particles shown in blue; (C, i) HSI scan of astrocytes treated for 6 hours with 5 µg/cm² silica; (C, ii) SAM image indicating pixels of (C, i) that matched the spectral library of silica particles shown in blue; (D, i) HSI scan of astrocytes treated for 24 hours with 5 µg/cm² silica; (D, ii) SAM image indicating pixels of (D, ii) that matched the spectral library of silica particles shown in blue.

SAM was again used on scans of BEAS-2B cells, incubated with FeMn or silica particles, in order to obtain a representative output (refer to Figure 21). In this figure, the HSI scan of BEAS-2B cells incubated with 5 µg/cm² FeMn or silica for 6 and 24 hours, represent “unknown” spectral images (see Figures 21 Ai, Bi, Ci and Di). The known spectral library of the FeMn or silica particles was mapped against this image indicated in blue (refer to Figures 21 Aii, Bii, Cii and Dii). Both the FeMn and silica spectral libraries mapped onto the images of the cells, thereby verifying the presence of FeMn or silica in the BEAS-2B cells.
The particle uptake results indicated that both FeMn and silica particles tend to aggregate when suspended in media. In addition, these aggregates are then either taken up by the cells, or adsorbed to the surface of the cells. SAM analysis was then able to verify the absence of FeMn or silica in the untreated cells, as well as, the presence of FeMn or silica in the treated cells.

3.2.2. **Cell Viability and Cytotoxicity Assays**

3.2.2.1. **Kit Based Assays**

3.2.2.1.1. **Viability Studies**

Once the ability of both cell types to internalize FeMn and silica particles had been confirmed, the effects of these particles on cell viability and cytotoxicity were investigated. There are a number of kits commercially available for testing both cell viability and cytotoxicity. Numerous studies in
literature have reported that particles may interfere with assays implemented to assess cell viability and cytotoxicity, producing false-positive or -negative results (Kroll et al., 2009; Stone et al., 2009; Oostingh et al., 2011). To ensure reliability of results, it was recommended to first perform more than one type of assay, as well as to investigate the influence of the particles on these assay systems. Cell viability was measured using the In Vitro Toxicology Assay Kit (XTT based) and the CellTiter-Glo® Luminescent Cell Viability Assay. Cytotoxicity was measured using CytoTox-ONE™ Homogeneous Membrane Integrity Assay.

Using these assay systems, the BEAS-2B cells were treated with FeMn or silica at concentrations of 5, 10 and 25 µg/cm² for a period of 1 hour (refer to Figure 23). The absorbance and luminescence generated by the XTT (see Figure 23A) and ATP (see Figure 23B) assays, respectively, were expressed as a percentage of the control, in order to normalize the data for comparison between biological repeats. The fluorescence generated by the LDH assay (refer to Figure 23C) was expressed as a percentage of the LDH max, which is the maximum LDH released by the cells. As seen in Figure 23C, there was no significant change ($P > 0.05$) in the viability or cytotoxicity in the BEAS-2B cells after treatment with FeMn or silica. This was observed in all three assays used. The XTT assay did, however, show a significant increase in viability for cells treated with 10 µg/cm² silica ($P < 0.01$) and 25 µg/cm² silica ($P < 0.0001$).

From these results, it could be concluded that FeMn particles are not toxic to BEAS-2B cells at all the concentrations tested, using the XTT, ATP and LDH assay systems. Silica particles were not toxic to BEAS-2B cells, when assessed using the ATP and LDH tests, but were able to increase the cell viability when using the XTT assay.
Figure 23: XTT, ATP and LDH analysis of FeMn or silica treated BEAS-2B cells. The toxic effects of FeMn or silica particles was measured using the XTT toxicity assay (A), CellTiter Glo assay (ATP) (B) and CytoTox One assay (LDH) (C). In each assay BEAS-2B cells were treated with FeMn or silica particles at concentrations of 5, 10 and 25 µg/cm² for an incubation period of 1 hour. A negative control (untreated cells) was included. For the XTT toxicity assay and CellTiter Glo assay (ATP) viability is represented as a percentage of the control and for the CytoTox One assay (LDH), cytotoxicity is represented as a percentage of the maximum LDH release (LDH max). Values were expressed as the mean ± SEM (standard error of the mean). **P < 0.01, and ****P < 0.0001 denote significance compared to the control (n = 3).

3.2.2.1.2. Particle Interference Studies (in the absence of cells)

In order to explain the increase in viability of the BEAS-2B cells when treated with silica particles, observed from the XTT assay, as well as, to investigate the interference of FeMn and silica particles with the XTT, ATP and LDH assays, further experiments were conducted. These included the investigation of, (a) particle interference at the wavelengths of the optical read-out (absorbance, luminescence, fluorescence) and (b) particle interference with the assay components i.e. product. In order to test for interference of the particles with the optical read-out, NPs were suspended in dispersion medium only. The absorbance/luminescence/fluorescence was recorded at the wavelength used for each assay (to determine whether the particles interfere with the signal). In
order to test for interference of the particles with the assay components, particles were incubated with the assay product. The absorbance/luminescence/fluorescence was recorded to determine whether particles interfere with these components.

3.2.2.1.2.1. XTT Assay

FeMn or silica particles were first suspended in dispersion medium only. The absorbance was recorded at 450 nm to determine if these particles interfere with the absorbance at the wavelength at which the final product, formazan chromophore, is measured (refer to Figure 24A). The introduction of FeMn resulted in a significant increase in the absorbance measured for both 10 µg/cm$^2$ ($P < 0.001$) and 25 µg/cm$^2$ ($P < 0.0001$) FeMn. Silica at all concentrations also resulted in a significant ($P < 0.0001$) increase in the absorbance measured at 450 nm. In the next step, FeMn or silica particles were incubated with formazan, representing the converted product (see Figure 24B). The addition of FeMn at all concentrations resulted in a significant ($P < 0.0001$) decrease in absorbance measured. The addition of 25 µg/cm$^2$ silica resulted in a significant ($P < 0.01$) increase in the absorbance of the formazan product. Although non-plasmonic in nature (i.e. does not exhibit characteristic absorbance maximum), FeMn and silica are optically dense, i.e. it absorbs light at all wavelengths in the UV-vis spectrum. Note that Figure 24C shows the results after subtraction of the absorbance values obtained for FeMn or silica particles alone in dispersion medium. A significant ($P < 0.0001$) decrease in absorbance at 450 nm was seen for all concentrations of FeMn and silica. Therefore, it can be concluded that such interference by FeMn or silica particles with XTT components was significant enough to alter the results of a cell-based study shown in Figure 23, which may be falsely interpreted as an overestimation of cell viability.
3.2.2.1.2.2.

ATP Assay

The interference of particles with the ATP assay system was investigated next. FeMn or silica particles were first suspended in dispersion medium only. The luminescence was recorded to determine if these particles interfere with the luminescence at the wavelength at which the final product, oxyluciferin, is measured (refer to Figure 25A). Although luminescence values were detected for all particles, the relative luminescence units were approximately 1% of what is generally obtained in the presence of ATP assay components. Subtracting the optical interference, from the data, did not cause any significant change in the luminescence recorded (refer to Figure 25C). Thereafter, FeMn or silica particles were incubated with oxyluciferin, representing the...
converted product (see Figure 25B). A slight increase in luminescence was observed for the silica treatment. The measurements reached significance ($P < 0.05$) at 100 $\mu g/cm^2$, when incubated with luciferin and ATP. This increase in luminescence may falsely be interpreted as an overestimation of cell viability.

Figure 25: Reaction of FeMn or silica with the CellTiter Glo assay (ATP), in the absence of cells. (A) The absorbance of FeMn or silica at 25, 50 and 100 $\mu g/cm^2$ in dispersion medium only, was recorded to determine whether FeMn or silica particles interfere with the read-out signal. (B) Reaction of FeMn or silica in the conversion of luciferin substrate to oxyluciferin product in the presence of 1.5 $\mu M$ ATP cofactor. Control represents luciferin and 1.5 $\mu M$ ATP. (C) Subtraction of the absorbance of FeMn or silica particles in dispersion medium only (shown in A) from the absorbance of FeMn or silica in oxyluciferin product and ATP cofactor (shown in B). Values were expressed as the mean $\pm$ SEM. *$P < 0.05$ denotes significance compared to the control ($n = 3$).

3.2.2.1.2.3. LDH Assay

The interference of FeMn or silica with the LDH assay system was investigated next. FeMn or silica particles were first suspended in dispersion medium only. The fluorescence was recorded to determine if these particles interfere with the fluorescence at the wavelength at which the final product, resorufin, is measured (refer to Figure 26A). None of the particles showed any significant
(P > 0.05) fluorescence values at 560 nm excitation, 590 nm emission, as well as, the wavelengths used to detect the resorufin product in the LDH assay. In the next step, FeMn or silica particles were incubated with resorufin, which represents the already converted product (see Figure 26B). None of the particles showed interference with the LDH assay components in the presence of NADH co-factor. The lack of interference, therefore, shows that the results obtained with the LDH assay represent the true toxicity (or lack thereof) of the FeMn or silica particles.

Figure 26: Reaction of FeMn or silica with the CytoTox One assay (LDH), in the absence of cells. (A) The absorbance of FeMn or silica at 25, 50 and 100 µg/cm² in dispersion medium only was recorded to determine whether FeMn or silica particles interfere with the read-out signal. (B) Reaction of FeMn or silica in the conversion of resazurin substrate to resorufin product in the presence 0.1 mM NADH co-factor. Control represents resazurin and 0.1 mM NADH. (C) Subtraction of the absorbance of FeMn or silica particles in dispersion medium only shown in A from the absorbance of FeMn or silica in resorufin product and NADH co-factor shown in B. Values were expressed as the mean ± SEM (n = 3).
3.2.2.2. Real Time Monitoring of Cell Viability and Cytotoxicity

The toxicity of FeMn and silica were also investigated, using the xCELLigence RTCA. This system is known to be unaffected by particle interference since that it does not rely on an optical read-out system or dyes to generate results.

Initially, growth curves or characteristic RTCA profiles, for both cell lines were established. This was done in order choose the optimal cell density at which to carry out the cytotoxicity studies. The cells were seeded at different cell concentrations and left to proliferate. The astrocytes (refer to Figure 27), were monitored for a period of a month (30 days) and the BEAS-2B cells (see Figure 28) for a period of 24 hours. The manufacturer recommends that the cell index (CI) be above 1 before starting any experiments. In addition, the chosen cell density should be in the CI/cell number linear range. For both cell types, high cell densities resulted in a loss in proportionality. However, low cell densities lead to impractical CI values. A seeding density of 10 000 cells/cm$^2$ (refer to Figure 27 purple growth curve and Figure 28 pink growth curve) was chosen for use in all subsequent experiments for both cell lines, this seeding density best fulfilled the manufacturers recommendations.

![Figure 27: Representative data showing the characteristic RTCA profile/growth curve of astrocytes, obtained using the xCELLigence RTCA system. The astrocytes were seeded at different cell concentrations and monitored over a period of a month. A seeding density of 10 000 cells/cm$^2$ was chosen for use in all subsequent experiments (shown in purple). A slope represents the mean of three wells.](image-url)
Figure 28: Representative data showing the characteristic RTCA profile/growth curve of BEAS-2B cells, obtained using the xCELLigence RTCA system. The BEAS-2B were seeded at different cell concentrations and monitored over a period of three days. A seeding density of 10 000 cells/cm$^2$ was chosen for use in all subsequent experiments (shown in pink). A slope represents the mean of three wells.

Subsequently, cytotoxicity experiments were then performed using the xCELLigence RTCA system. The CI was normalized at the point of treatment. The normalized CI (NCI) indicates the level of adhesion and is, therefore, associated with the viability of the cells. Significant differences of treated cells from the control were determined using the slope of the curves. The slope describes the changing rate of the CI within the time period, i.e. from the point of treatment to the point of confluence, which was determined from the curve of the untreated cells.

Figure 29 illustrates the NCI over time of astrocytes, when treated with 5, 10 and 25 µg/cm$^2$ FeMn (see Figure 29A) and silica (refer to Figure 29B). In both cases, astrocytes were treated for 48 hours after being allowed to proliferate for two weeks, where the CI was normalised at the point of treatment. Treatment was carried out whilst the cells were in the early stages of log growth phase, as determined by the unique RTCA growth profile in Figure 27. Astrocytes treated with FeMn showed a significant ($P < 0.001$) decrease in CI values compared to the untreated controls (see Figure 29A). Even though the decrease in CI for cells treated with 5 µg/cm$^2$ FeMn was not found to be significant ($P > 0.05$), it still showed that there was a concentration dependent decrease in the viability of the astrocytes, after treatment with FeMn. Astrocytes treated with silica also showed a concentration dependent decrease in CI. However, only those cells treated with 25 µg/cm$^2$ silica showed a significant ($P < 0.01$) decrease in CI (refer to Figure 29B).
Figure 29: Representative data of normalized cell index values, obtained after treatment of astrocytes with FeMn or silica, as analyzed by the xCELLigence RTCA system. Astrocytes were seeded and left to grow for two weeks after which they treated with FeMn (A) or silica (B) particles at concentrations of 5, 10 and 25 µg/cm². The treated cells were then monitored for changes in cell index compared to the untreated cells. A slope represents the mean of three wells. **P < 0.01, and ***P < 0.001 denotes significance of each slope compared to the control slope (n = 3).
Figure 30 illustrates the NCI over time of BEAS-2B cells, when treated with 5, 10 and 25 µg/cm² FeMn (refer to Figure 30A) and silica (refer to Figure 30B). In both cases, BEAS-2B cells were treated for 24 hours after seeding and the CI was normalised at the point of treatment. Treatment was carried out whilst the cells were in the early stages of log growth phase, as determined by the unique RTCA growth profile in Figure 28. BEAS-2B cells treated with 10 µg/cm² FeMn, showed a significant ($P < 0.05$) decrease in CI value, compared to the untreated controls. BEAS-2B cells treated with 25 µg/cm² FeMn also showed significant ($P < 0.0001$) decreases in CI values (see Figure 30A). Even though it was not significant ($P > 0.05$), the decrease in CI for BEAS-2B cells treated with 5 µg/cm² FeMn showed that there was a concentration dependant decrease in the viability of cells when treated with FeMn. BEAS-2B cells treated with silica showed a significant ($P < 0.0001$) concentration dependent decrease in CI values, compared to the untreated controls (see Figure 30B).

The results obtained from the xCELLigence system indicated that the viability of astrocytes and BEAS-2B cells decreased after treatment in relation to increasing concentrations of FeMn or silica particles. The CI values obtained for both cell types, after treatment with FeMn or silica, also indicate that astrocytes may be more sensitive to treatment with these particles than BEAS-2B cells, i.e. BEAS-2B cells showed some recovery after treatment with low concentrations of FeMn.

The lack of interference by FeMn and silica, with the xCELLigence RTCA system is also illustrated in Figures 29 and 30 by the purple and green growth curves respectively. Wells containing 25 µg/cm² FeMn or 25 µg/cm² silica, suspended in medium without cells, did not result in an increase in CI values and remained zero over time. These results confirm that the xCELLigence can be used as a better alternative to conventional kit based assays.
Figure 30: Representative data of normalized cell index values, obtained after treatment of BEAS-2B cells with FeMn or silica, as analyzed by the xCELLigence RTCA system. BEAS-2B cells were seeded and left to grow for 24 hours after which they treated with FeMn (A) or silica (B) particles at concentrations of 5, 10 and 25 µg/cm². The treated cells were then monitored for changes in cell index compared to the untreated cells. A slope represents the mean of three wells. *P < 0.05, and ****P < 0.0001 denotes significance of each slope compared to the control slope (n = 3).
3.2.3. Nuclear Translocation of Nrf2 and Nf-κB

In the previous section, the xCELLigence RTCA system was used to analyze any changes in viability of astrocytes and BEAS-2B cells when treated with different concentrations of FeMn or silica. It was found that treatment with these particles resulted in a decrease in viability, in both cell types. These findings highlighted the need to conduct experiments in order to elucidate pathways that may be activated by treatment with these particles. This prompted an investigation into the effects of FeMn or silica treatment, of astrocytes and BEAS-2B cells, on the nuclear translocation of the transcription proteins Nrf2 and Nf-κB, using western blot analysis. The transcription factor Nrf2 is activated as the primary defence against low levels of ROS and other harmful substances, since it forms part of the antioxidant defence system. Nf-κB is a pro-inflammatory transcription factor, activated once the primary defence systems (anti-oxidants) have failed in order to neutralise harmful substances that may be present in the cell.

Astrocytes and BEAS-2B cells were treated with FeMn or silica at concentrations of 5, 10 and 25 µg/cm², for incubation periods of 6 and 24 hours. The cytoplasmic and nuclear protein fractions were isolated using the CelLytic™ NuCLEAR Extraction kit. Western blot analysis was then performed on both the cytoplasmic and nuclear protein fractions, to detect the presence of Nrf2 and Nf-κB, as well as, the degree of nuclear translocation for both these proteins, following treatment with FeMn or silica. The density of the Nrf2 and Nf-κB specific bands were normalized to β-actin, because it was present in the cell at constant concentrations and was not affected by most treatments to which the cells were subjected. The results were then expressed as a fold of the control, for comparison between biological repeats.

3.2.3.1. Nrf2

Figure 31 shows the Nrf2 levels in the nuclear and cytoplasmic fractions of astrocytes treated with FeMn or silica. As indicated, the cytosolic Nrf2 increased in a concentration-dependent manner, after 6 hour FeMn or silica treatment. However, the cytosolic levels of Nrf2 were still lower than that of the control value during this treatment period. Under the same conditions, the nuclear Nrf2 protein level showed an increase after treatment with 25 µg/cm² FeMn and 100 µM H₂O₂, compared to the levels of nuclear Nrf2 in the control. After treatment with 5 µg/cm² FeMn for 24
hours, the cytosolic Nrf2 increased significantly ($P < 0.01$). It then decreased, in a concentration dependent manner, after treatment with higher concentrations of FeMn. Treatment with silica resulted in a concentration dependent increase in the levels of cytosolic Nrf2, after treatment for 24 hours. Only the 25 $\mu$g/cm$^2$ silica induced an increase in Nrf2 levels greater than that of the control. Under the same conditions, treatment with FeMn or silica for 24 hours resulted in a decrease in the levels of nuclear Nrf2, compared to that of the control value.

The nuclear fractions were compared to their corresponding cytoplasmic fractions, where treatment with 25 $\mu$g/cm$^2$ FeMn for 6 hours, showed a significant ($P < 0.05$) increase in Nrf2 protein levels, in the nuclear fraction. Astrocytes treated with 5 $\mu$g/cm$^2$ silica also showed a significant ($P < 0.05$) increase in Nrf2 protein levels in the nuclear fraction. Conversely, astrocytes treated with FeMn or silica for 24 hours, showed significant decreases in Nrf2 levels between the nuclear and cytoplasmic fractions.

![Figure 31: Nrf2 protein levels in isolated cytoplasmic and nuclear cellular fractions, of astrocytes treated with FeMn or silica, for 6 and 24 hours. The Nrf2 protein level in cytoplasmic and nuclear cellular fractions was analyzed by western blot analysis. Astrocytes were treated with FeMn or silica particles at concentrations of 5, 10 and 25 $\mu$g/cm$^2$. Negative (untreated cells) and positive ($H_2O_2$ treated cells) controls were included. Values were expressed as the mean ± SEM. *$P < 0.05$, **$P < 0.01$ denotes significance compared to the control (n = 2). Each cytoplasmic fraction was also compared to its corresponding nuclear fraction where $†P < 0.05$, $‡‡P < 0.001$, and $‡‡‡‡P < 0.0001$ denotes a significant difference between the nuclear fraction and its corresponding cytoplasmic fraction.]}
Figure 32 shows the Nrf2 levels in the nuclear, as well as, cytoplasmic fractions of BEAS-2B cells treated with FeMn or silica. As shown in Figure 32, levels of cytosolic Nrf2 remained unchanged after a 6 hour FeMn or silica treatment, compared to that of the control value. Under the same conditions, the nuclear Nrf2 protein level showed an increase after treatment with 5 and 10 µg/cm² FeMn, as well as 100 µM H₂O₂, compared to the levels of nuclear Nrf2 in the control. Treatment with 10 µg/cm² FeMn for 24 hours resulted in a significant ($P < 0.01$) increase in the cytosolic Nrf2 levels. A significant ($P > 0.05$) increase in the cytosolic Nrf2 was also observed in BEAS-2B cells treated with 25 µg/cm² silica. Under the same conditions, treatment with FeMn or silica for 24 hours resulted in a decrease in the levels of nuclear Nrf2 compared to that of the control value. An exception was observed for BEAS-2B cells treated with 10 µg/cm² silica. This treatment condition was found to induce a significant increase in nuclear Nrf2 levels.

The nuclear fractions were compared to their corresponding cytoplasmic fraction, where treatment with 25 µg/cm² silica for 6 hours resulted in a significant ($P < 0.05$) increase in nuclear Nrf2 protein levels. Treatment with FeMn at concentrations of 5 and 10 µg/cm², also showed an increase in the levels of Nrf2 in the nuclear fraction, even though it was not found to be significant ($P > 0.05$). Conversely, after treatment of BEAS-2B cells for 24 hours with FeMn or silica, significant decreases in nuclear Nrf2 levels were seen for all FeMn treatment conditions, as well as after treatment with 25 µg/cm² silica.
Figure 32: Nrf2 protein levels in isolated cytoplasmic and nuclear cellular fractions, of BEAS-2B cells treated with FeMn or silica, for 6 and 24 hours. The Nrf2 protein level in cytoplasmic and nuclear cellular fractions was analyzed by western blot analysis. BEAS-2B cells were treated with FeMn or silica particles at concentrations of 5, 10 and 25 µg/cm². Negative (untreated cells) and positive (H₂O₂ treated cells) controls were included. Values were expressed as the mean ± SEM. *P < 0.05, **P < 0.01 denotes significance compared to the control (n = 2). Each cytoplasmic fraction was also compared to its corresponding nuclear fraction where ††P < 0.01, ††††P < 0.0001 denotes a significant difference between the nuclear fraction and its corresponding cytoplasmic fraction.

3.2.3.2. Nf-κB

Figure 33 shows the Nf-κB levels in the nuclear and cytoplasmic fractions of astrocytes treated with FeMn or silica. As indicated, cytosolic Nf-κB decreased significantly after a 6 hour FeMn or silica treatment. Under the same conditions, the nuclear Nf-κB protein level showed a significant (P < 0.01) increase after treatment with 25 µg/cm² FeMn, when compared to the levels of nuclear Nf-κB in the control. After treatment with FeMn and silica for 24 hours, the cytosolic levels of Nf-κB remained unchanged, compared to the control value. Under the same conditions, treatment with FeMn or silica for 24 hours resulted in an increase in the levels of nuclear Nf-κB, compared to that of the control value.

When comparing the nuclear fractions with their corresponding cytoplasmic fractions of astrocytes, after a 6 hour incubation with FeMn, significant increases in Nf-κB levels were observed
after treatment with 5 µg/cm² FeMn (P < 0.001), 10 and 25 µg/cm² FeMn (P < 0.0001). The same increase in Nf-κB levels was also seen for astrocytes treated with 5 µg/cm² silica (P < 0.001). A slight, but not significant (P > 0.05), increase was also seen after treatment with 10 µg/cm² silica. Astrocytes treated for 24 hours, with FeMn or silica, showed significant increases in Nf-κB levels. An exception was observed for those cells treated with 10 µg/cm² FeMn and 5 µg/cm² silica which still showed an increase in Nf-κB levels. However, this increase was not found to be significant (P > 0.05) (refer to Figure 33).

Figure 33: Nf-κB protein levels in isolated cytoplasmic and nuclear cellular fractions, of astrocytes treated with FeMn or silica, for 6 and 24 hours. The Nf-κB protein level in cytoplasmic and nuclear cellular fractions was analyzed by western blot analysis. Astrocytes were treated with FeMn or silica particles at concentrations of 5, 10 and 25 µg/cm². Negative (untreated cells) and positive (H₂O₂ treated cells) controls were included. Values were expressed as the mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 denotes significance compared to the control (n = 2). Each cytoplasmic fraction was also compared to its corresponding nuclear fraction, where ††P < 0.01, †††P < 0.001, and ††††P < 0.0001 denotes a significant difference between the nuclear fraction and its corresponding cytoplasmic fraction.

Figure 34 shows the Nf-κB levels in the nuclear and cytoplasmic fractions of BEAS-2B cells treated with FeMn or silica. As indicated, cytosolic Nf-κB remained unchanged after a 6 hour FeMn or silica treatment. Similarly under the same conditions, the nuclear Nf-κB protein level remained unchanged, when compared to the levels of nuclear Nf-κB in the control. After treatment with FeMn and silica for 24 hours, the cytosolic levels of Nf-κB remained unchanged, compared to the
control value. However, under the same conditions, treatment with FeMn or silica for 24 hours resulted in an increase in the levels of nuclear Nf-κB, compared to that of the control value.

When comparing the nuclear fractions with their corresponding cytoplasmic fractions of BEAS-2B cells, the only significant ($P < 0.05$) increase in nuclear fraction protein levels was seen in BEAS-2B cells treated with $H_2O_2$ for 24 hours.

![Figure 34: Nf-κB protein levels in isolated cytoplasmic and nuclear cellular fractions, of BEAS-2B cells treated with FeMn or silica, for 6 and 24 hours. The Nf-κB protein level in cytoplasmic and nuclear cellular fractions was analyzed by western blot analysis. BEAS-2B cells were treated with FeMn or silica particles at concentrations of 5, 10 and 25 µg/cm². Negative (untreated cells) and positive ($H_2O_2$ treated cells) controls were included. Values were expressed as the mean ± SEM. (n = 2). Each cytoplasmic fraction was also compared to its corresponding nuclear fraction, where †$P < 0.05$ denotes a significant difference between the nuclear fraction and its corresponding cytoplasmic fraction.](image)

These results indicated that treatment of astrocytes with FeMn and silica for 6 hours, resulted in the activation of Nrf2 and its subsequent translocation to the nucleus. However, after 24 hours the cytosolic Nrf2 levels were still high, but there was much less nuclear translocation taking place. This may suggest that the anti-oxidant activities of Nrf2 were overcome after 24 hours treatment with FeMn or silica and that the cells had now activated other pathways, e.g. the Nf-κB pathway. This suggestion was supported by the increased levels of activation and nuclear translocation of Nf-κB that were observed after treatment of astrocytes with FeMn or silica for 6 and 24 hours. Treatment of BEAS-2B cells with FeMn or silica for 6 hours, resulted in higher levels
of Nrf2 activation, compared to astrocytes. However, once again, after 24 hours the cytosolic Nrf2 remained high and there was an observed decrease in nuclear translocation of Nrf2. This could possibly again be explained by the activation of other pathways. The increased levels of Nrf2 seen in BEAS-2B cells, indicated their increased ability to overcome the effects of harmful substances. This may explain their decreased sensitivity to treatment with FeMn or silica, when compared to astrocytes.

### 3.2.4. Genotoxicity Study

The decrease in cell viability observed in both cell types upon treatment with FeMn or silica, may indicate an inability of the cells to repair DNA damage. Consequently, further studies were performed on both astrocytes and BEAS-2B cells, in order to determine the levels of DNA damage induced by treatment with different concentrations of FeMn or silica. These studies were carried out using the alkaline comet assay.

Both astrocytes and BEAS-2B cells were treated with FeMn or silica at concentrations of 5, 10 and 25 µg/cm², for incubation periods of 1, 6 and 24 hours. The resulting DNA damage was then quantified using the alkaline comet assay. Images of 50 randomly captured comets, of 25 cells per slide were examined, in duplicate, using Comet IV software. The computerized image analysis system acquired images and estimates the comet cell components. Undamaged cells had an intact compact nucleus without a tail, and damaged cells had a tail that resembled a comet (see Figure 35). The parameter % tail DNA, was used to represent the amount of DNA damage.
Figure 35: Representative images of (A) undamaged cell with compact nucleus and (B) damaged cell with DNA tail. These images were taken of BEAS-2B cells after performing the alkaline comet assay and stained with ethidium bromide.

Figure 36 illustrates the exposure of astrocytes to 5, 10 or 25 µg/cm² FeMn or silica, for 1, 6 and 24 hours. This resulted in a significant ($P < 0.0001$) dose-dependent increase in % tail DNA, compared to the % tail DNA produced in the control cells. An exception was observed for astrocytes treated with 5 µg/cm² silica for 24 hours, which had a similar % tail DNA to that of the control. The % tail DNA was seen to peak after treatment with FeMn or silica, for 1 hour, after which the % tail DNA remained constant at all treatment concentrations for the remaining treatment periods. Figure 36 also showed an increase in % tail DNA for silica treated astrocytes, compared to those cells treated with different concentrations of FeMn.
Figure 36: Percentage tail DNA obtained following treatment of astrocytes with FeMn or silica, for 1, 6 and 24 hours, as analyzed using the alkaline comet assay. Astrocytes were treated with FeMn or silica particles at concentrations of 5, 10 and 25 µg/cm². Negative (untreated cells) and positive (H₂O₂ treated cells) controls were included. Values were expressed as the mean ± SEM. ****P < 0.0001 denotes significance compared to the control (n = 4).

As illustrated in Figure 37, exposure of BEAS-2B cells to 5, 10 or 25 µg/cm² FeMn or silica for 1, 6 and 24 hours, resulted in a significant (P < 0.0001) dose-dependent increase in % tail DNA. This was in relation to the % tail DNA produced in the control cells for all particle concentrations and time periods used. An increase in % tail DNA was also noted between the 1, 6 and 24 treatment periods. Figure 37 showed an increase in % tail DNA for silica treated BEAS-2B cells, compared to those cells treated with different concentrations of FeMn.
These results indicated that both FeMn and silica particles were able to induce DNA damage, in astrocytes as well as BEAS-2B cells, in a concentration dependent manner. Silica, however, induced the highest % tail DNA between the two particles, indicating its increased ability to cause DNA damage.
Chapter 4: Discussion

Human overexposure to Mn is not common. However, most reported cases have been found in an occupational setting, with the most prominent sources being welding, ore-crushing, mining and FeMn production (Santamaria, 2008). Overexposure to Mn can be attributed to inhalation of high levels of FeMn particulate matter over long periods of time, and results in numerous pulmonary diseases and neurological effects (Winder et al., 2010). However, conflicting literature exists regarding the relationship between Mn exposure levels and outcome effects. It is hypothesized that FeMn particulate matter consists of nano and micro sized particles and therefore, upon inhalation may translocate to the brain. Since FeMn induced injury in the lung and the brain is a possibility, this study aimed to investigate the effects of FeMn dust, collected from a FeMn smelter works, on primary rat astrocytes and also BEAS-2B cells.

4.1. Characterization of FeMn and Silica Particles

It is known that inhalation of PM is responsible for the development of numerous diseases, and aggravation of existing conditions such as asthma. However, the mechanisms of toxicity responsible for the development of these diseases still require clarification. Before the toxicity of a particle can be determined, it is important to perform characterization studies. Many studies have shown that particle properties such as size, surface area and composition play an important role in toxicity (Gillespie et al., 2013; Jankovic, 2005; Oberdörster et al., 2005).

When a particle is inhaled, its fate within the body is determined by its size (Dieme et al., 2012). Since the size range of airborne PM is very wide, a combination of instruments must be used to determine their size distribution. In this study the SMPS and the APS were employed, since the combination of these two instruments has reportedly been used successfully in many aerosol-based studies (Shi et al., 2001; Hand and Kreidenweis, 2002; Shen et al., 2002; Khlystov et al., 2004).
The combined size distributions of the SMPS and APS, based on mass of particles per m³ air, showed that for a fixed mass of FeMn, particles existing in the micron-range constitute the bulk of the sample mass, while particles in the nano range largely make up the particle number (refer to Figure 13). Similar observations were also made for silica particles (see Figure 14). In other words, the dust samples contain larger particles, which weigh more than nano particles. According to the SMPS and APS results, FeMn has a median aerodynamic diameter of 1.518 µm in the micron range and 114 nm in the nano range (refer to Figure 13). However, silica has a median aerodynamic diameter of 1.861 µm in the micron range and 168 nm in the nano range (see Figure 14). This means that the particles in the silica sample are slightly larger than those of the FeMn dust sample. However, due to the presence of nano particles in both dust samples, it can be concluded that translocation to the brain is a possibility.

The characteristic small size of UFPs will translate into a large surface area to volume ratio. This is thought to be responsible for the increased catalytic activity of UFPs and production of ROS on their surface (Kelly and Fussell, 2012). The present study, has implemented the commonly used BET methodology in order to define the surface area of particles (Dubois et al., 2010; Eisazadeh et al., 2013). The FeMn particles were found to have a surface area of 3.7 m²/g and silica 5.1 m²/g (see Table 1). This would indicate that due to the increased surface area for silica, these particles would be more reactive, and hence may induce more inflammation than the FeMn particles. Substantial evidence indicates that particle-induced inflammation, is correlated to the increased surface area of the particles (Sager et al., 2008; Sager and Castranova, 2009).

It is known that in addition to their chemical composition, the high surface area of UFPs will enable their surfaces to adsorb molecules readily, e.g. carbonaceous species and different trace metal ions (Kocyigit et al., 2004). The FeMn sample under study herein was found to contain high concentrations of Al, Ca, Fe, Mg, Na, Pb and Zn. Where the largest portion of the sample was made up of Mn. Mn was present in the sample at a concentration of 352 000 mg/kg. The silica sample contained small concentrations of Fe, Ca and Ti with Al being present at the highest concentration of all the elements tested (1520 mg/kg) (see Table 2). Trace metals are required as co-factors for numerous enzymes but some; e.g. Fe and Cu, are involved in oxidative reactions (Fenton and Haber-Weiss reactions). At high concentrations, these transition metals may account for the
overproduction of free radicals, resulting in oxidative stress (Kocyigit et al., 2004). Consequently, these elements Al, Fe, Pb, Zn and Mn have all be found to cause disease at high exposure levels (Kabadayi and Cesur, 2010). This implies that in addition to the particle effects, the transition metal ions adsorbed to the surface, will also contribute to the particle toxicity.

4.2. Cell Based Studies

Despite the fact that in vitro systems may not represent the complexity seen in the body, there are many advantages to using in vitro testing systems including: reduced variability between experiments, cost effectiveness, the possibility of standardization, small quantities of test sample required, and the possibility to use human cells (Kroll et al., 2009; Kandarova and Letasiova, 2011). In vitro testing systems have been designed as an alternative to animal experimentation, and are used as a rapid method to screen toxic materials. In vitro systems can be used to study particular mechanistic pathways under controlled conditions, as well as to establish starting doses for in vivo work (Oberdörster et al., 2005a; Park et al., 2009). It has also been shown that responses observed in vitro regarding particle uptake, cell morphology and protein production, are often similar to those seen in vivo (Stone et al., 2009).

When designing an experiment, it is important to choose cell lines based on the particle exposure route and target organs. Primary cells are ideal because they respond to stimulus in a manner that is similar to that of normal cells, in a normal individual. However, this is not always possible due to the difficulty in obtaining new cells, since they have a finite lifespan. As an alternative, transformed cells can be used. These cells have an infinite lifespan, thus making them easier to use and the results obtained more reproducible (Oostingh et al., 2011).

Specific for airborne PM, the primary exposure route is through inhalation (Kong et al., 2011). The inhaled particles will come into contact with epithelial cells once deposited in the lungs, therefore BEAS-2B cells were used in this study to represent this exposure route. There is also substantial evidence that once particles have deposited in the lung, they are able to move into the blood stream where they are transported to various target organs (brain). Studies have already shown that UFPs can enter the brain directly through the olfactory pathway (Oberdörster et al., 2005).
The information provided, along with evidence that the Mn concentration in the brain is regulated by astrocytes, prompted the use of primary rat astrocytes in this study, as a representation of the target organ, i.e. the brain.

Numerous studies have shown that particle toxicity may be concentration dependent. Therefore, the particle dose is an important factor to consider when determining toxicity. In particle toxicology, dose is expressed in particle mass, number or surface area per volume (Sager et al., 2008; Park et al., 2009). Suggestions were also made that for cell culture studies, doses could be expressed as the mass of particles per cell number (μg/10^6 cells) (Stone et al., 2009; Kong et al., 2011). It has also been shown that particles often settle due to gravity and agglomeration (Stone et al., 2009). Therefore, due to the fact that both cell lines used were adherent, the particle dose in the current study was expressed as mass per unit surface area of the culture dish (µg/cm^2). This metric takes into account gravitational settling and can be compared to in vivo studies, where dose is often expressed as mass per unit surface area of the lung (mg/cm^2 of alveolar epithelium). Using this dose metric, concentrations of 5, 10 and 25 µg/cm^2 were used. As for exposure periods, most studies in the literature recommend exposure periods ranging between 4 and 24 hours (Park et al., 2009). Exposure periods of 6 and 24 hours were chosen for the cytotoxicity studies and 1, 6 and 24 hours were selected for the genotoxicity studies, in order to observe the toxic effect of FeMn over short and longer exposure periods.

Finally, when investigating the toxicity of an unknown particle, it was recommended in the literature, to include particles of known toxicity (Oberdörster et al., 2005a; Park et al., 2009). Therefore, it was decided to use Min-U-Sil, crystalline silica, of known toxicity with well established membranolytic and surface activities (Park et al., 2009; Stone et al., 2009). In addition, silica has the ability to activate transcription factors, such as Nf-κB and to initiate apoptosis (Fubini and Hubbard, 2003; Castranova, 2004).

4.3. **Cellular Uptake and Particle Interaction with Cells Leading to Toxicity**

The study of cellular uptake, as well as the interaction of particles with cells, is an important aspect of particle toxicology. This interaction is influenced by the physicochemical properties of NPs
In addition, the toxicity they produce is dependent on whether they are adsorbed on the cellular surfaces, or internalized by these cells (Brandenberger et al., 2010).

In order to study the uptake as well as the interaction of FeMn or silica with astrocytes (see Figures 15 and 16) and BEAS-2B cells (see Figures 17 and 18), a dark field imaging system known as the CytoViva® Hyperspectral imaging system was used. This imaging system has been used previously to determine the localization of multi-walled carbon nanotubes (MWCNT) in lung tissue, as well as, the uptake and cellular toxicity of silver nanoparticles in U937 cells (Vetten et al., 2013). Using this methodology, it was possible to show uptake and adsorption of particles, in both astrocytes and BEAS-2B cells visually, using the high signal-to-noise dark-field images. It was also possible to confirm the identity of the internalised particles as FeMn or silica, through SAM analyses (refer to Figures 20, 21 and 22). SAM analyses have previously been used to confirm the identity of internalised gold nanoparticles in BEAS-2B cells (Vetten et al., 2013).

The cellular uptake images that were obtained for FeMn and silica, showed that for the same particle dose used, the number of silica particles was much higher than that of the FeMn particles. This could possibly be due to the fact that the FeMn contained a higher number of particles in the nano-size region (refer to Figure 13). Therefore, it tended to agglomerate, forming larger particles in solution and a decrease in particle number. However, the silica sample contained a higher number of particles in the micron-size region (see Figure 14), with less agglomeration and, therefore, they seemed to have an increased particle number when compared to FeMn samples.

The images taken in the current uptake studies reveal that both FeMn and silica tend to agglomerate in cell culture media, with the size of the agglomerates increasing with an increase in particle concentration. FeMn and silica in both astrocytes and BEAS-2B cells showed some uptake, with particles not uniformly distributed throughout the cell, but rather accumulating in specific intracellular regions. The intracellular accumulation increased between the 6 hour (refer to Figures 15 and 17) and 24 hour (see Figures 16 and 18) treatment periods. However, the majority of the particles tended to be adsorbed onto the surface of the cells. Wittmaack, 2011 suggested that gravitational settling of particles, during treatment of cell cultures, would ‘coat’ the cells resulting
in the disruption of normal membrane functioning (Wittmaack, 2011). Other studies have found that aggregated particles are not able to pass through the membrane by passive diffusion (Vranic et al., 2013). Thus, it may be possible to eliminate this pathway as a means for the FeMn or silica particles to enter the cell in the current study. Further investigations are still needed to elucidate the mechanisms involved in the internalisation of FeMn and silica into different cells.

Once internalised, the mechanisms of the toxicity observed for FeMn and silica could be explained via the different mechanisms of uptake. It is shown in literature, that engineered Mn NPs enter the cell through a “Trojan horse” type of mechanism. This results in the transfer of large amounts of Mn into the cell, significantly increasing its capacity to cause damage. Upon entering cells, these NPs could release Mn ions through dissolution, thereby contributing to the production of oxidative free radicals (Limbach et al., 2007; Studer et al., 2010; Frick et al., 2011). On the other hand, it is shown that silica has an affinity for lipid bilayers, which may result in the interaction of these particles with cellular membranes, leading to cell membrane disruption (Mu et al., 2012). This may account for the increased toxicity of the silica particles, compared to the FeMn particles.

4.4. Cell Viability and Cytotoxicity using Conventional Kit-based Assays

*In vitro* screening methods have been used to determine particle potency as well as details regarding mechanisms of effect (Nadeau et al., 1996). Numerous methods have been developed to facilitate the measurement of cell proliferation, viability and cytotoxicity *in vitro*. For increased convenience, these assays have been designed for use in 96 well plates, allowing for the simultaneous analysis of many samples (Weyermann et al., 2005). Three of these assays were chosen for this study, in order to determine the effects of FeMn and silica on cell viability and cytotoxicity, namely the XTT, ATP and LDH assay.

In this study, no release of LDH or changes in ATP levels were observed in BEAS-2B cells after treatment with FeMn or silica for 1 hour (see Figure 23). However, a concentration dependent increase in mitochondrial activity was seen for both FeMn and silica when using the XTT assay (refer to Figure 23). The XTT results are slightly different from those found in literature. It was previously reported that PC12 cells treated with Mn for 24 hours, indicated inhibition of
mitochondrial respiration, without simultaneous depletion of ATP levels or release of LDH (Hirata, 2002). It was suggested that depletion of ATP may not result from inhibition of oxidative phosphorylation alone, but that the inhibition of both glycolysis and oxidative phosphorylation are required for ATP depletion (Hirata, 2002). However, Roth and colleagues found that Mn-induced necrosis in PC12 cells was due to depletion in ATP levels (Roth et al., 2000). Hirata, further explained that this difference in outcomes may have been due to differences in experimental conditions, taking into consideration that the production of ATP and mitochondrial activity depend on the glucose concentration in culture medium (Hirata, 2002).

The performance of these cell viability and cytotoxicity assays can also be influenced by a number of other conditions, including pH and the presence of phenol red in the culture medium (Vistica et al., 1991; Johno et al., 2010; Kupcsik, 2011). In the current study, precautionary measures were applied in order to minimize the influence of some of these conditions. Studies in literature have previously reported on the pH dependence of all the assays used in this study (Fischer et al., 2010; Kroll et al., 2009). Hence, the pH of the culture media after the addition of FeMn or silica was measured in this study, and no difference in pH was observed over a period of 24 hours (see Table 1). Additionally, to overcome changes in pH and glucose levels, the culture medium was changed prior to addition of the assay reagents. Culture medium without phenol red was also used, as suggested by Johno and colleagues (Johno et al., 2010). The presence of serum in the cell culture medium has also been found to affect the outcome of these assays (Funk et al., 2007). For this reason the serum concentration was reduced to 1%, prior to performing the assays.

When performing the LDH assay, it has been found that treatment with particles may influence cell proliferation and, in turn, the cell number. The decrease in cells would therefore result in a decrease of released LDH and a subsequent underestimation of cytotoxicity. This effect was taken into consideration in the current study by including a control of total released LDH (max LDH), compared to the control cells. These cells are treated with Triton X-100, a detergent known to permeabilize cell membranes (Stone et al., 2009).
4.5. Influence of Particle Interference on Kit-Based Assays

It is difficult to compare particle toxicology studies, due to lack in standardization of reagents and methods used by different laboratories. Traditional toxicological testing involves optical measurements such as absorbance, fluorescence and luminescence. These assay systems, however, were designed for testing chemicals and drugs, not particles (Kroll et al., 2009; Shapiro et al., 2009; Oostingh et al., 2011). Particles, particularly UFPs, have unique features including increased surface area, resulting in increased adsorption properties, as well as increased catalytic activity and optical properties. All of these result in interference of the particles with assay components and commonly used detection systems (Kroll et al., 2009; Dhawan and Sharma, 2010). Without proper validation, these interferences can lead to the production of unreliable results and conflicting data.

In the current study with XTT, a concentration dependent light absorption due to the effects of light scattering was observed following the addition of FeMn particles. This resulted in a decrease in absorbance values measured (refer to Figure 24). This would lead to an underestimation of cell viability as observed in Figure 23. These results were similar to the light scattering effects seen for TiO$_2$ (Kroll et al., 2012). However, when silica was tested, the absorbance increased with increasing particle concentration (see Figure 24). This increase in absorbance, after addition of particles, may have resulted in the increased absorbance values seen during the viability studies (refer to Figure 23). In literature, this increase in absorbance is explained by the decreased translucence of the solution after addition of particles (Kroll et al., 2012). Based on the observed interferences by particles in this assay system, the recommendation will be that the XTT assay should not be used in the assessment of the toxicity of these particles.

There are few studies on the interference of compounds with the ATP assay. However, Shapiro and colleagues state, that when detection of the end product is indirect, as in the case of the ATP assay, the test compound can interact with the product or detection reagent in order to produce a false increase or decrease in the luminescent signal detected (Shapiro et al., 2009). In the current study, the addition of FeMn or silica particles to the ATP product did not alter the luminescent signal produced. The only exception occurred after the addition of 25 µg/cm$^2$ silica (see Figure 25).
These results indicate that the ATP assay may only be suitable for use with low concentrations of particles, i.e. ≤ 10 µg/cm². Finally, in this study, the addition of FeMn or silica particles to the LDH product, did not alter the fluorescent signal produced (refer to Figure 26). This indicates that the opaque nature of these particles did not interfere with this assay system. Therefore, in future studies, this assay may possibly be used to determine membrane integrity of cells treated with the FeMn or silica particles.

Particle interference of assay systems exists. However, there are methods available that may help to overcome the worst of these effects. Many authors suggest the use of two or more independent assay systems to validate data obtained (Dhawan and Sharma, 2010). Therefore, three assay systems were used in this study. Removal of particles from the solution by means of washing and centrifugation has also been suggested, but this does not remove all particles. It may therefore be useful to reduce the concentration of particles used (Kroll et al., 2012). This was illustrated in the current ATP interference study. In addition, the use of appropriate controls remains the most important factor. The three controls that should be included when using conventional assay systems are A) nanoparticles only, B) nanoparticles plus assay reagents and C) nanoparticles in the test matrix, spiked with a reference standard for the assay. It is also important to note the each assay needs to be validated on a particle by particle basis (Dobrovolskaia et al., 2009).

4.6. Real Time Monitoring of Cell Viability and Cytotoxicity: An Alternative to Kit-based Assays

The validations mentioned in the previous section can become time-consuming and expensive, and may not overcome all interference produced by certain particles (Vetten et al., 2013). Additionally, these kit-based methods only provide endpoint measurements which could result in loss of data, if the incorrect exposure periods are chosen. Real time cellular impedance has therefore been suggested as a means to determine viability (Huang et al., 2008; Vetten et al., 2013). The xCELLigence RTCA technology uses cell impedance as an alternative non-invasive, real time, label-free measure of cell viability, proliferation and migration (Limame et al., 2012; Leme et al., 2011). Impedance based technology has already been used in particle toxicology with quantum dots, gold nanoparticles and nanorods, as well as quartz (Huang et al., 2008; Vetten et al., 2013).
Using this technology, the viability of primary rat astrocytes and BEAS-2B cells, following treatment with different concentrations of FeMn or silica, was assessed. The influence of FeMn or silica, alone, was tested in parallel with exposure of the cells to the particles. This was done by including wells that only contained FeMn or silica particles. The curves obtained for these wells were identical to wells containing media only, indicating no interference by the particles (refer to Figures 29 and 30). The observation of non-interference by FeMn or silica particles whilst using this system, was similar to that obtained for quartz particles (Huang et al., 2008). 

Prior to treatment of cells with the particles investigated, it was essential to establish a growth curve for each cell type in order to optimize cell density. The cell density may strongly impact cell adhesion, spreading, lag and exponential growth phases, as well as confluence. A cell index of 1 or greater is recommended by the manufacturer, and the cell density chosen should be within the cell index/cell number linear range (Atienzar et al., 2011). Following this recommendation, the growth curves obtained for both cell lines showed loss in proportionality at high seeding densities and inadequate cell growth at low seeding densities (refer to Figures 27 and 28). A cell density of 10 000 cells/cm$^2$ was chosen because this growth curve showed the best linearity for both astrocytes and BEAS-2B cells. Most studies use seeding densities ranging between 1000 and 20 000 cells/well, this value is however cell type specific (Atienzar et al., 2011).

According to the results obtained by the xCELLigence RTCA system in the present study, treatment with either FeMn or silica resulted in a dose dependent decrease in the viability of both astrocytes (see Figure 29) and BEAS-2B cells (see Figure 30). The effects of silica seemed to be more toxic than that of FeMn. The xCELLigence results also indicated an increased sensitivity of astrocytes to both particles, compared to the BEAS-2B cells, where BEAS-2B cells showed some recovery after treatment with low concentrations of FeMn. In literature, most studies observe increased cytotoxicity with an increase in treatment period (Park et al., 2009). This is similar to the trend observed for astrocytes treated with FeMn or silica. However, there were some studies that reported decreased cytotoxicity with an increase in treatment period (Park et al., 2009). Those reports are similar to the trend observed for BEAS-2B cells treated with FeMn or silica. The reported decrease in cytotoxicity was attributed to the detoxification of particles in cellular compartments or the adaptive antioxidant response of the cell (Park et al., 2009).
Atienzar and colleagues have found that a decrease in the CI value does not always indicate cell death. In some cases, it may just indicate morphological changes (Atienzar et al., 2011). For this reason it was important to use the xCELLigence RTCA system in combination with other techniques, such as microscopy, in order to confirm and accurately explain the results obtained using this system. The darkfield images obtained using the Cytoviva® HSI (see Figures 15, 16, 17 and 18), have therefore confirmed that no morphological changes were produced in the cells after treatment. By a process of elimination, the decrease in CI values was reasoned to be due to a decrease in cell viability.

In the present study, the particles were found to induce toxic effects in BEAS-2B cells when using the xCELLigence system. However, when the conventional viability assays were used to establish toxicity, the particles showed no toxicity. These conflicting results may be attributed to interference of particles in the conventional assay systems, as well as, to the period of incubation used in each assay system.

4.7. Nuclear Translocation of Nrf2 and Nf-κB

To ensure their survival, mammalian cells have developed antioxidant mechanisms, which neutralize the effects of oxidative stress. One such mechanism is Nrf2 (Zhang, 2006). If the antioxidant defence systems of an organism become overwhelmed, a number of pro-inflammatory pathways become activated in response to the inflammation and stress caused; namely Nf-κB (Li et al., 2010). Both of these transcription factors are capable of translocation. This implies that upon activation they are able to move from the cytoplasm to the nucleus, where transcription takes place. In the current study, the protein levels of Nrf2 and Nf-κB were analyzed in both the cytoplasmic and nuclear fractions, of astrocytes and BEAS-2B cells, treated with FeMn or silica.

In the case of astrocytes (refer to Figure 31), Nrf2 translocation was observed after a treatment period of 6 hours. The most significant (P < 0.05) translocation was seen in cells treated with 25 µg/cm² FeMn. This was indicative of the astrocytes’ attempt to overcome particle induced stress. After 24 hours, there was a decrease in nuclear translocation of Nrf2 for all concentrations and treatments. These results indicated that after 24 hours, the antioxidant defences were unable to
overcome the oxidative stress and as such, this pathway was no longer active. Pro-inflammatory pathways may have been activated instead. A similar increase after a 6 hour treatment and decrease after a 24 hour treatment was also observed for the BEAS-2B cells. The most significant ($P < 0.05$) increase was obtained with 25 µg/cm$^2$ silica (refer to Figure 33). The initial increase and subsequent decrease of Nrf2 nuclear translocation may be due to DJ-1, a protein responsible for Nrf2 stabilization. This stabilization results in the dissociation of Nrf2 from KEAP1, allowing Nrf2 to escape degradation, leading to its activation as suggested by Malhotra and colleagues (Malhotra et al., 2008). The decrease in Nrf2 levels observed after 24 hours may then be due to an increase in oxidative stress. Subsequent proteasomal degradation of DJ-1, implies it will no longer be available to stabilize Nrf2, which will then be targeted for degradation. These findings were similar to experiments conducted on BEAS-2B cells that reported increased oxidative stress induced by cigarette smoke, resulting in a decrease in Nrf2 levels, due to proteasomal degradation of DJ-1 protein (Malhotra et al., 2008).

The nuclear translocation of NF-κB in astrocytes treated with FeMn over a 6 hour period (refer to Figure 32), increased in a concentration dependent manner, with the same increase observed after 24 hours. These results indicated that the NF-κB pathway was activated 6 hours after treatment and remained activate for 24 hours. It should be noted that activation of this pathway may have occurred earlier, however, more time periods would need to be studied in order to confirm this. Signalling pathways that activate IKK and result in the phosphorylation of IkBα, the inhibitory subunit of NF-κB, are thought to be responsible for its activation (Liu et al., 2005; Lee et al., 2012). On the other hand, for astrocytes treated with different concentrations of silica, a concentration dependent decrease in nuclear NF-κB was seen after 6 hours. This may be due to the antioxidant activities of Nrf2. After 24 hours, the levels of silica induced nuclear NF-κB are similar to those induced by FeMn. Finally, the NF-κB results for BEAS-2B cells treated with FeMn or silica showed no significant nuclear translocation. This may be due to the large standard errors resulting from insufficient replicates. Even though it was not deemed to be significant, there was an increase in nuclear NF-κB levels after treatment with FeMn and silica for 24 hours, indicating activation of the pro-inflammatory pathway in BEAS-2B cells treated with both particles (see Figure 34). Some studies have found that cells treated with Mn, require pre-treatment with an inflammogen (LPS) in order to observe any NF-κB activation. Since LPS is a common component of environmental dusts
(Filipov et al., 2005), it may be present in the FeMn and silica samples. This may, in part, account for the increase in the measured Nf-κB levels.

When comparing the effects of FeMn or silica treatment, on Nrf2 and Nf-κB levels, between the two cell lines, it would seem that the Nrf2 pathway is more strongly activated in the BEAS-2B cells, compared to the astrocytes. Contrary to this observation, the Nf-κB pathway seemed to be more strongly activated in astrocytes, compared to BEAS-2B cells. This would indicate that BEAS-2B cells may be able to overcome oxidative stress and are, therefore, less sensitive to treatment with FeMn or silica, compared to astrocytes. This also would correlate well with the increased sensitivity observed in the astrocytes, compared to the BEAS-2B cells, when using the xCELLigence system.

4.8. Genotoxicity

The comet assay is an easy, fast and sensitive technique that can be used to detect various forms of DNA damage, including single and double stranded DNA breaks, oxidative DNA damage, DNA cross-links and alkali-labile sites (Lazarova et al., 2006). In this study, the alkaline version of the comet assay was used to determine DNA damage that was caused by FeMn or silica particles, in both astrocytes and BEAS-2B cells.

Astrocytes treated for 1, 6 and 24 hours showed a significant ($P < 0.0001$) dose dependent increase in % tail DNA, after treatment with both FeMn and silica (refer to Figure 36). The results obtained after 24 hours were similar to those obtained after 6 hours, indicating that the maximum DNA damage that these particles could exert on astrocytes was reached after 6 hours. Similarly, BEAS-2B cells treated with FeMn or silica (see Figure 37), showed significant ($P < 0.0001$) dose dependent increases in % DNA for all time periods analyzed. The only observed difference was that the % tail DNA damage continued to increase at 24 hours for all the concentrations used. Based on current literature, the possible mechanisms that may be responsible for the observed genotoxicity of FeMn and silica, may be through stimulation of the cells to produce oxidants through the disruption of mitochondrial electron transport. In addition, FeMn and silica may interact directly with DNA. This may occur during mitosis when the nuclear membrane is broken down (Knaapen et al., 2002). The sensitivity of astrocytes, in relation to the BEAS-2B cells, was once again apparent
and indicated by the increased % tail DNA in astrocytes when compared to that induced in BEAS-2B cells. Overall, the genotoxicity of silica was concluded to be slightly higher than that of FeMn in astrocytes, whereas the degree of DNA damage caused by the two particles was observed to be of a similar level in BEAS-2B cells.

The observed increases in % tail DNA after treatment of the astrocytes and BEAS-2B cells with FeMn or silica are supported by reports in current literature. Dutta and colleagues treated human peripheral lymphocytes with MnO₂, and established it as a genotoxicant, able to cause DNA strand breaks (Dutta et al., 2006). During cellular uptake, quartz particles have been found to disrupt the cell membrane, resulting in lipid peroxidation and subsequent DNA damage due to OH· (Knaapen et al., 2002). Zhong and colleagues used the alkaline comet assay in order to compare the DNA damage induced by Min-U-Sil 5, in Chinese hamster lung fibroblasts (V79 cells) and human embryonic lung fibroblasts (Hel 299 cells). They found significant increases in tail length, for all concentrations used, after a treatment period of 3 hours. It was suggested that silicic acid leached from the silica particles, causing damage to the secondary structure of DNA, resulting in DNA strand breaks (Zhong et al., 1997).
Chapter 5: Conclusion

Respirable particles in an occupational environment are composed of a heterogeneous mixture of organic and inorganic materials. Occupational settings, where human exposure to particles may often occur, include mining, welding and smelting. Depending on the composition and size of particles, the development of numerous respiratory diseases and, as was recently established, neurological diseases, may ensue. The aim of this study was to investigate the effects of FeMn dust collected from a ferromanganese smelter works, as well as Min-U-Sil 5 crystalline silica used as a positive control, on primary rat astrocytes and on immortalized human bronchial epithelial (BEAS-2B) cells.

By using SMPS, APS, BET and ICP-AES, it was possible to characterize the FeMn and crystalline silica dusts in terms of size distribution, surface area and trace elemental composition. The presence of both micro and nano sized particles was established in both the FeMn and silica samples. Therefore, upon inhalation, deposition of the micro-sized particles in the alveolar region, as well as the translocation of the nano-sized particles to the brain through the olfactory pathway, may occur. These studies have also revealed that the vast majority of the FeMn sample consisted of Mn, and to a lesser extent, Fe. However, they also contained trace amounts of other known toxic metals including Al, Fe, Pb and Zn. The presence of the identified transition metals (in a large concentration) and of known toxic heavy metals (albeit in lower concentrations) may be responsible for the observed FeMn toxicity.

Using the CytoViva® HSI it was possible to visually show the uptake of FeMn and crystalline silica, into both astrocytes and BEAS-2B cells. This was a novel application for an incidental UFP. The identity of the internalised particles was confirmed as FeMn or silica, through SAM analyses. Using the rare application of the xCELLigence RTCA system, a dose dependent decrease in cell viability was shown. In addition, by using the alkaline comet assay technique, a dose dependent increase in % tail DNA by FeMn and silica was shown.

Finally, by implementing western blot analysis, it was possible to study the nuclear translocation of an antioxidant transcription factor (Nrf2) and inflammatory transcription factor (Nf-κB), after
exposure of astrocytes and BEAS-2B cells to FeMn or crystalline silica. Short term exposure to FeMn and silica resulted in an increase, whereas long term exposure resulted in a decrease in translocation of both of these transcription factors, in both cell types tested. This indicated that after longer periods of exposure, the antioxidant defences were unable to overcome the oxidative stress induced by these particles.

In conclusion, in all the experiments conducted, the toxicity of crystalline silica was more pronounced than the toxicity of FeMn, despite the higher concentrations of transition metal ions present in the latter particles. In addition, the extent of the toxicity by FeMn and crystalline silica observed in astrocytes was more pronounced than that observed in BEAS-2B cells. This indicated that the differences in the ability of these cells to respond to oxidative stress, was central to the extent of the total cell toxicities observed.

Further experiments may need to be conducted in order to confirm the ability of FeMn and silica to generate ROS. This would be achieved by using the electron spin resonance (ESR) technique. Moreover, the role of ionic state of Mn (Mn$^{2+}$ vs. Mn$^{3+}$) in FeMn particles, with regards to the generation of free radicals, may also be assessed using ion chromatography coupled with ICP-MS, or pulse anodic stripping voltammetry (DPSV) technologies. In addition, the subcellular localisation of the particles after they are internalised should also be investigated using Electron Microscopy (EM) techniques. Finally, further experiments must be conducted to assess the involvement of various kinases, in order to further elucidate the mechanisms involved in particle induced activation of the transcription factors Nrf2 and Nf-κB.

The results of this study indicate that depending on the size distribution of particles in the work environment, they may enter different regions of the lungs. However, for those particles in the nano size region, direct access to the brain may occur. After deposition in the target organ, these particles will produce cellular changes through oxidative stress, leading to inflammation, decreased cellular viability and increased toxicity. Therefore, this study and others like it, provide a better understanding into mechanisms of Mn toxicity, and when these results are combined with in vivo studies, could assist in the establishment of reliable biomarkers of Mn exposure and effect.
References


human cell-based assays to determine nanoparticle-induced immunomodulatory effects. *Particle and Fibre Toxicology*, 8


Appendix 1: Reagents, Materials, Apparatus, Software

Reagents

10x Phosphate Buffered Saline, pH 7.2 (Diagnostics & Technical Services cc, Randburg, RSA)

2-Mercaptoethanol, (Sigma, Germany)

30% Acrylamide/Bis solution 37.5:1 (Biorad, China)

Adenosine 5’-triphosphate (ATP) disodium salt (Sigma, St. Louis, MO, USA)

Affinity Purified Antibody Peroxidase labelled, Goat Anti-Mouse IgG (KPL, MO, USA)

Agarose, low gelling temperature (Sigma, St. Louis, MO, USA)

Agarose, Type II (Sigma, St. Louis, MO, USA)

Albumin from bovine serum (Sigma, St. Louis, MO, USA)

Ammonium Persulfate (APS) (Biorad, Japan)

Bovine Serum Albumin 30% (Biowest, Nuaille, France)

Celllytic™ NuCLEAR™ Extraction Kit (Sigma, St. Louis, MO, USA)

CellTiter Glo® Luminescent Cell Viability Assay (Promega Corporation, Madison, WI, USA)

CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega Corporation, Madison, WI, USA)

Deoxyribonuclease (DNase I) (Sigma, St. Louis, MO, USA)

Dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO, USA)

Dispase® prepared from Bacillus Polmyxa (Life Technologies-Invitrogen, Grand Island, NY, USA)

Dulbecco’s Phosphate Buffered Saline (DPBS), without Ca²⁺ and Mg²⁺ (Lonza, Verviers, Belgium)

Ethidium Bromide Solution 10 mg/ml (Biorad, Hercules, CA, USA)

Ethylene diaminetetra-acetic acid (EDTA) disodium salt (Associated Chemical Enterprises (Pty) Ltd., Southdale, RSA)

Extra thick blot paper (filter paper) (Biorad, USA)

Fetal Bovine Serum (FBS) (Thermo Scientific, Northumberland, UK)

Formaldehyde Solution 37% (Merck, Darmstadt, Germany)

Glycerol, Molecular Biology Grade (Calbiochem, USA)

Horse Serum (Lonz, Verviers, Belgium)

Hydrochloric acid 37% (Associated Chemical Enterprises (Pty) Ltd., Southdale, RSA)
Hydrogen Peroxide 30% (*Merck, Darmstadt, Germany*)

In Vitro Toxicology Assay Kit, XTT based (*Sigma, St. Louis, MO, USA*)

Kaiser’s glycerol gelatine (*Merck, Darmstadt, Germany*)

Laemmli Sample Buffer (*Biorad, USA*)

Methanol (*Merck, Darmstadt, Germany*)

Minimum Essential Medium (MEM) Alpha Medium (1x) with phenol red (*Life Technologies-Invitrogen, Grand Island, NY, USA*)

Minimum Essential Medium (MEM) Alpha Medium (1x) without phenol red (*Life Technologies-Invitrogen, Grand Island, NY, USA*)

Monoclonal Anti-β-Actin, antibody produced in mouse (*Sigma, St. Louis, MO, USA*)

Nicotinamide adenine dinucleotide (NADH), disodium salt (*Roche Diagnostics, Mannheim, Germany*)

Nitrocellulose Membranes 0.45 µm (*Biorad, Germany*)

Nf-kappaB p65 Rabbit Antibody (*Cell Signalling Technology, Danvers, MA, USA*)

N,N,N’,N’-tetramethylethylenediamine (TEMED) (*Biorad, USA*)

Penicillin/Streptomycin (PEN-STREP) (*Lonza, Verviers, Belgium*)

Pierce® Antibody Goat Anti-Rabbit IgG: Horseradish Peroxidase (*Thermo Scientific, Rockford, IL, USA*)

Pierce® BCA Protein Assay Reagent A (*Thermo Scientific, Rockford, IL, USA*)

Pierce® BCA Protein Assay Reagent B (*Thermo Scientific, Rockford, IL, USA*)

Poly-L-lysine hydrobromide (*Sigma, St. Louis, MO, USA*)

Polyoxyethylene sorbitan monolaurate (Tween) 20 (*Biorad, USA*)

Precision Plus Protein™ Kaleidoscope™ (*Biorad, USA*)

Protease Inhibitor Cocktail (*Sigma, St. Louis, MO, USA*)

Radio Immunoprecipitation Assay (RIPA) Buffer (*Sigma, St. Louis, MO, USA*)

Restore™ Western Blot Stripping Buffer (*Thermo Scientific, Rockford, IL, USA*)

Roswell Park Memorial Institute medium (RPMI) 1640 with L-Glutamine (*Lonza, Verviers, Belgium*)

Roswell Park Memorial Institute medium (RPMI) 1640 without L-Glutamine and phenol red (*Lonza, Verviers, Belgium*)

Sodium Chloride (*Merck, Darmstadt, Germany*)

Sodium dodecyl sulphate (SDS) Solution 20% (w/v) (*Biorad, USA*)

Sodium Hydroxide pellets (*Merck, Darmstadt, Germany*)
Super Signal® West Dura Kit *(Thermo Scientific, Rockford, IL, USA)*

Super Signal® West Pico Kit *(Thermo Scientific, Rockford, IL, USA)*

Tris (hydroxymethyl)-aminomethane (Tris) *(Merck, Darmstadt, Germany)*

Tris Buffered Saline 10x (TBS) *(Biorad, USA)*

Tris/Glycine/SDS (TGS) 10x Buffer *(Biorad, München, Germany)*

  1x solution: 25 mMTris, 192 mM glycine, 0.1% (W/V) SDS, pH 8.3

Tris/Glycine (TG) Buffer 10x *(Biorad, München, Germany)*

  1x solution: 25 mMTris, 192 mM glycine, pH 8.3

Triton® X-100 *(Merck, Darmstadt, Germany)*

Trypan Blue Solution 0.4% *(Life Technologies-Invitrogen, USA)*

Trypsin-EDTA solution (10x) *(Sigma, St. Louis, MO, USA)*

**Consumables**

10 ml Sterile plastic pipettes *(LP Italiana Spa, Milano, Italy)*

25 ml Sterile disposable plastic pipettes *(Sterilin, Newport, UK)*

50 ml CELLSTAR® tubes *(Greiner bio-one, Frickenhausen, Germany)*

75 cm² nuclon™ treated flasks *(nunc™, Denmark)*

12 well plates *(nunc™, Denmark)*

Cell Scrapers *(Greiner bio-one, Frickenhausen, Germany)*

25 cm² Cell Culture Flasks *(Greiner bio-one, Frickenhausen, Germany)*

96 well Cell Culture Plates *(Corning Incorporated, NY, USA)*

1.8 ml CryoTube™ Vials *(nunc™, Denmark)*

15 ml CELLSTAR® Tubes *(Greiner bio-one, Frickenhausen, Germany)*

Countess™ Cell Counting Chamber Slides *(Invitrogen, OR, USA)*

Frosted Microscope Slides *(Menzel-Glaser®, Braunschweig, Germany)*

22x50 mm coverslips *(Menzel-Glaser®, Braunschweig, Germany)*

10 ml glass pipettes *(Corning Incorporated, NY, USA)*

Millicell® EZ slides *(Millipore™, Ireland)*

2 ml Graduated Microcentrifuge tubes *(Quality Scientific Plastic, USA)*
96 well Black walled clear bottom plates (*nunc™, Denmark*)

22x22 mm coverslips (*Marienfield, Lauda-Königshafen, Germany*)

E-Plate 96 (*ACEA Biosciences Inc, China*)

**Apparatus**

Aerodynamic Particle Sizer (*TSI Incorporated, Shoreview, MN, USA*)

Mini-PROTEAN® II. Electrophoresis Cell (*Biorad, Hercules, CA, USA*)

Allegra X-22R Centrifuge (*Beckman Coulter, Palo Alto, CA, USA*)

AS 200 Sieve Shaker (*Retsch, Haan, Germany*)

Automated microplate reader (ELx800) (*Biotek Instruments Inc, Winooski, VT, USA*)

CO₂ water-jacketed Incubator, (*Nuaire, Plymouth, MN, USA*)

Countess™ Automated Cell Counter (*Invitrogen, Carlsbad, CA, USA*)

CyberScan pH 6500 Meter (*Thermo Scientific, NY, USA*)

CytoViva® Hyperspectral Imaging system (*CytoViva® Inc, Auburn, AL, USA*)

Eppendorf 5415R Centrifuge (*Merck, Wadeville, RSA*)

Eppendorf 5804R Centrifuge (*Merck, Wadeville, RSA*)

Forma Series II CO₂ Incubator (*Thermo Scientific NY, USA*)

G:Box Chemi XR5 gel documentation system (*Syngene, Cambridge, UK*)

LabStir™ (*Gilson, Germany*)

Microplate Fluorescence reader (FLx800) (*Biotek Instruments Inc, Winooski, VT, USA*)

Mini-PROTEAN® tetra cell (*Biorad, Hercules, CA, USA*)

Olympus BX41 Microscope (*Olympus, Barcelona, Spain*)

Scanning Mobility Particle Sizer (*TSI Incorporated, Shoreview, MN, USA*)

Small Scale Particle Dispenser Model 3433 (*TSI Incorporated, Shoreview, MN, USA*)

Super Aurius 3000 series spectrophotometer (Cecil 3021) (*Cecil Instruments limited, Cambridge, UK*)

xCELLigence Real Time Cell Analysis System (*ACEA Biosciences Inc, San Diego, CA, USA*)
Analysis Software

Aerosol Instrument Manager software (AIM version 8.1.0) *(TSI Incorporated, Shoreview, MN, USA)*

Comet Assay IV software *(Perceptive Instruments Ltd, Bury St Edmunds, UK)*

Data Merge software (DMS version 1.0.1) *(TSI Incorporated, Shoreview, MN, USA)*

DAGE Exponent software (DageMTI XL16 400) *(DAGE-MTI, Michigan City, IN, USA)*

ENVI software (version 4.4) *(Exelis VIS, Boulder, CO, USA)*

GeneSys capturing software (version 1.3.3.0) *(Syngene, Cambridge, UK)*

GeneTools analysis software (version 4.03b) *(Syngene, Cambridge, UK)*

Graph-Pad prism *(GraphPad Software, San Diego, CA)*

Image J software *(National Institute of Health, Bethesda, MD, U.S.)*

KC 4 software (version 3.4) *(Biotek Instruments Inc, Winooski, VT, USA)*

RTCA software (version 1.2.1) *(ACEA Biosciences Inc, San Diego, CA, USA)*
Appendix 2: Ethics Approval

Faculty Ethics Committee
Faculty of Science
University of Johannesburg
Kingsway
Auckland Park Campus

Date: 27 February 2012

Researcher: Ms L-A Koekemoer
Supervisor: Prof L Bornman
Department: Biochemistry
Project title: Characterization of Manganese dioxide (MnO₂) dust particles collected from a ferromanganese smelter and in vitro cytotoxicity and genotoxicity studies
Programme: MSc Biochemistry

Protocol No. 8 February 2012
Name Koekemoer
Student No. 200513141

Dear Sir/Madam

APPLICATION FOR RESEARCH ETHICS CLEARANCE

Thank you for submitting your proposal for ethics clearance.

The Faculty Ethics Committee of the Faculty of Science, University of Johannesburg reviewed and approved the application to use rat pups as experimental animals in the project. The NSPCA approved the Standard Operating Procedure (SOP) for the keeping and handling of rats for the experiments.

Species: Sprague-Dawley rat pups (postnatal day 1 neonatal)
Number of individuals: 5 once a month
Period of study approval: January 2012 (duration of study)
Notes: Until the student has completed her master's degree

Yours sincerely

Chairperson/Faculty Ethics Committee
Faculty of Science
Appendix 3: Min-U-Sil 5 data sheet

MIN-U-SIL® 5
FINE GROUND SILICA

PLANT: BERKELEY SPRINGS, WEST VIRGINIA

PROJECT DATA

PARTICLE SIZE DISTRIBUTION
(CUM % FINER THAN)

PERCENT

100
50
0

EQUIVALENT SPHERICAL DIAMETER (MICRONS)

TYPICAL PHYSICAL PROPERTIES

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<tr>
<th>Property</th>
<th>Value</th>
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<td>BULK DENSITY-COMPACTED (lbs/ft³)</td>
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<tr>
<td>BULK DENSITY-UNCOMPACTED (lbs/ft³)</td>
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<tr>
<td>HARDNESS (Mohs)</td>
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<tr>
<td>HEMGAN</td>
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<tr>
<td>MEDIAN DIAMETER (Microns)</td>
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<tr>
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<tr>
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<tr>
<td>5 MICRON (%)</td>
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<tr>
<td>+325 MESH (%)</td>
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<td>REFLECTANCE (%)</td>
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<td>YELLOWNESS INDEX</td>
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<td>SPECIFIC GRAVITY</td>
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TYPICAL CHEMICAL ANALYSIS %

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<td>SiO₂ (Silicon Dioxide)</td>
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</tr>
<tr>
<td>Fe₂O₃ (Iron Oxide)</td>
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<tr>
<td>Al₂O₃ (Aluminum Oxide)</td>
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<tr>
<td>TiO₂ (Titanium Dioxide)</td>
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<tr>
<td>CaO (Calcium Oxide)</td>
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<tr>
<td>MgO (Magnesium Oxide)</td>
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<tr>
<td>Na₂O (Sodium Oxide)</td>
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<td>K₂O (Potassium Oxide)</td>
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<tr>
<td>LOI (Loss on Ignition)</td>
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</tbody>
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October 1, 2010

DISCLAIMER: The information set forth in this Product Data Sheet represents typical properties of the product described; the information and the typical values are not specifications. U.S. Silica Company makes no representation or warranty concerning the Products, expressed or implied, by this Product Data Sheet.

WARNING: The product contains crystalline silica - quartz, which can cause silicosis (an occupational lung disease) and lung cancer. For detailed information on the potential health effect of crystalline silica - quartz, see the U.S. Silica Company Material Safety Data Sheet.

U.S. Silica Company        P.O. Box 187, Berkeley Springs, WV 25411-0187       (800) 243-7500       (304) 258-2500