Toxicological effects of Nanoparticles: In vitro studies with Titanium Dioxide

Inaugural-Dissertation

zur

Erlangung des Doktorgrades der Mathematisch-Naturwissenschaftlichen Fakultät der Heinrich-Heine-Universität Düsseldorf

angefertigt am

Institut für Umweltmedizinische Forschung (IUF)

an der Heinrich-Heine-Universität Düsseldorf

vorgelegt von

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Düsseldorf 2005

Inaugural-Dissertation

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Erlangung des Doktorgrades der Mathematisch-Naturwissenschaftlichen Fakultät der Heinrich-Heine-Universität Düsseldorf

Gedruckt mit der Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Heinrich-Heine-Universität Düsseldorf

Gez.

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Tag der Prüfung.: *4. July.2005*

“Imagination is more important than knowledge”

Albert Einstein

For my parents and Lutz

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Abstract

Introduction – Epidemiological studies indicate an association between ambient concentrations of particulate matter (PM) and adverse health effects in susceptible populations. Although the fraction of ultrafine particles (<100nm) herein are considered to be the main causal factor, the exact mechanisms by which these adverse effects may occur are not yet clearly understood.

Hypothesis – Upon inhalation, ultrafine particles induce oxidative stress in lung epithelial cells which leads to the induction and release of pro-inflammatory cytokines. The surface area of the particles is more important than the surface chemistry for inducing these responses.

Aim and study design – The aim of this thesis was to scrutinise the role of size and surface chemistry of particles in initiating pro-inflammatory effects *in vitro*. The study was designed to characterise the particles for surface area, surface chemistry and to look at the particle induced cytotoxicity, oxidative stress, interleukin-8 (IL-8) release and cellular uptake of particles using A549 human lung epithelial cells on treatment with model TiO_2 particles. We compared five different sizes of model TiO_2 particles (10, 25, 50, 82 and 100 m^2/g) to look at the role of surface area in particle induced adverse effects. Two types of TiO_2 (10 and 50 m^2/g) were methylated to create the surface more hydrophobic and were evaluated to study the importance of the particle's surface chemistry.

Materials and Methods – Particles were characterised for surface area (BET-analysis according to Brunauer Emmett and Teller), for surface morphology (High-Resolution Transmission Electron Microscopy, HRTEM), surface chemistry (analysis of methylation-efficiency using Fourier-Transformed Infrared Spectromety, FTIR) and oxidative stress (using Electron Spin Resonance, ESR). To test the effects of particles in cells we chose human lung epithelial cells (A549) as a model and cytotoxicity in cells was assessed by measuring LDH release. The radical generating capacities of the particles were determined by using ESR and by measuring cellular glutathione depletion in the cells. The pro-inflammatory cytokine (IL-8) expression and release from the cells was determined by RT-PCR and ELISA respectively. TEM was used to determine endocytosis of the particles by the cells.

Results and discussion – All the particles of different BET-surface areas were tested at equal mass dose. The methylation was successful as revealed by FTIR spectroscopy and no significant formation of reactive oxygen species (ROS) was detected when the particles were measured in an acellular system. In A549 cells, none of the particle preparations caused significant toxicity in cells at lower doses; however at higher doses, some toxicity was observed. For ROS, glutathione depletion and IL-8 expression, clear surface area dependent responses were detected. We did not observe a significant effect of methylation on particle induced inflammatory effects.

Summary and General Discussion – Our findings suggest that for TiO_2 particles the surface area rather than mass is a better predictor for the pro-inflammatory effects *in vitro*. Our data is in concurrence with previous *in vivo* studies in animals which also verified a relation between inflammation and the surface area than mass of the particles. ROS and its impact on the redox imbalance in the cell could be a central mechanism dictating the induction of IL-8, and glutathione depletion might act herein as a fundamental unifying event. Supplementary experiments, e.g. using antioxidants and other inhibitors are needed to resolve and investigate the apparent missing link between ROS and IL-8.

Conclusions – We observed clear differences in the toxic and inflammogenic effects associated to the surface area of TiO_2 particles. Due to the emergence of so many unrequited questions, the contribution of ROS in IL-8 release remains an important area of exploration. Nevertheless, our current findings have implications for occupational exposure to the nanoparticles because the contemporary standards are set on the basis of mass. Adequate *in vivo* studies are mandatory to relate and authenticate these *in vitro* results.

Zusammenfassung

Einleitung – Epidemiologische Studien haben einen Zusammenhang zwischen Konzentrationen von Umweltpartikeln (englisch: particulate matter, PM) und gesundheitsschädigenden Effekten in besonders empfänglichen Bevölkerungsgruppen gezeigt.

Hypothese – Bei der Inhalation von ultrafeinen Partikeln wird in den betroffenen Lungenepithelzellen oxidativer Stress ausgelöst. Dies führt zu einer Induktion und Freisetzung von proinflammatorischen Zytokinen. Dabei ist die Größe der Partikeloberfläche wichtiger als deren chemische Zusammensetzung.

Forschungsziele – Ziel der vorliegenden Arbeit ist es, die Rolle von Partikelgröße und Oberflächenzusammensetzung auf die Auslösung entzündungsfördernder Prozesse in vitro zu untersuchen. Dabei wurden die Partikel hinsichtlich Oberflächengröße und -chemie charakterisiert. In humanen Lungenepithelzellen (A549) wurden Model-TiO_2-Partikel hinsichtlich Zytotoxizität, oxidativen Stress, Interleukin (IL)-8-Freisetzung und Partikelaufnahme untersucht. In einem Vergleich von fünf verschiedenen TiO_2-Partikelgrößen (10, 25, 50, 82 und 100 m^2/g) wurde die Bedeutung der Oberflächenbeschaffenheit auf die durch die Partikel induzierten Effekte analysiert. Außerdem wurde mittels Methylierung der Oberfläche die Hydrophobizität zweier TiO_2-Proben (10 und 50 m^2/g) geändert, um die Bedeutung der Partikelzusammensetzung zu evaluieren.

Materialien und Methoden – Zur Charakterisierung von Partikeln wurden die folgenden Partikeleigenschaften analysiert: Partikeloberfläche (BET-Analyse nach Brunauer, Emmett und Teller), Morphologie (hochauflösende Transmissionselektronenmikroskopie, HRTEM), Oberflächenchemie (Analyse der Methylierungs-Effizienz mittels Fourier-Transform-Infrarot-Spektrometrie, FTIR) und oxidativer Stress (Elektronen Spin Resonanz Spektroskopie, ESR). Zelluläre Partikeleffekte wurden in humanen Lungenepithelzellen (A549) getestet. Zytotoxizität wurde mittels LDH-Assay, die Radikalbildung der Partikel mittels ESR und Glutathion-Depletion bestimmt. Für die Untersuchung der Expression und Freisetzung des proinflammatorischen Zytokins IL-8 wurden RT-PCR und ELISA eingesetzt. Zur Analyse der Partikelaufnahme wurde TEM angewandt.

Resultate und Diskussion – Alle Partikeleffekte wurden bei gleicher Masse evaluiert. FTIR bestätigte die erfolgreiche Oberflächenmethylierung. Im azellulären System wurde keine signifikante Bildung reaktiver Sauerstoffspezies (ROS) durch die Partikel nachgewiesen. In A549 Zellen verursachten die Partikel bei niedriger Dosis keine und bei höherer Dosis eine geringe Toxizität. Zelluläre ROS-Bildung, Glutathion-Depletion und IL-8-Expression zeigten eine klare Korrelation zur Oberfläche. Partikelmethylierung hatte auf die untersuchten Parameter keinen signifikanten Effekt.

Zusammenfassung und allgemeine Diskussion – Unsere Ergebnisse weisen darauf hin, dass die Größe der TiO_2-Partikeloberfläche von entscheidenderer Bedeutung für die proinflammatorischen Effekte ist, als die Partikelmasse. Unsere Daten stimmen mit früheren Ergebnissen aus Tierversuchen überein, in denen eine Korrelation zwischen pulmonaler Entzündung und Partikeloberfläche nachgewiesen wurde. ROS und deren Einfluss auf den zellulären Redox-Status stehen möglicherweise in kausalem Verband zur Induktion von IL-8, und die Glutathion-Depletion nimmt in diesem Prozess wahrscheinlich eine zentrale Stellung ein. Weitere Experimente, z.B. unter Einsatz von Antioxidanzien und Inhibitoren, sind erforderlich, um den Verband zwischen Partikel-induzierter ROS-Bildung und IL-8-Expression aufzuklären.

Schlussfolgerung – Wir beobachteten klare Unterschiede in Toxizität und inflammatorischen Effekten von TiO_2 in direktem Verband zur Partikeloberfläche. Hinsichtlich der Vielzahl offener Fragen bleibt die spezifische Rolle von ROS in der IL-8-Freisetzung zu klären. Die Ergebnisse dieser Studie sind jedoch von wesentlicher Bedeutung für die berufsbedingte Belastung gegenüber Nanopartikeln, da diese bislang auf Massenbasis reguliert werden. Adäquate in-vivo-Studien sind zur Bestätigung der hier gezeigten in-vitro Ergebnisse erforderlich.

Abbreviations

$^{\cdot}OH$	Hydroxyl Radical
µl	Microlitre
A549	Human Caucasian Lung carcinoma
ADP	Adenosine diphosphate
AM	Alveolar Macrophages
ANOVA	Analysis of variance
AOE	Antioxidant enzyme
ARDS	Adult respiratory distress syndrome
ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage
BET	Brunauer-Emmett-Teller
CAT	Catalase
cDNA	Complementary deoxyribonucleic acid
CO_2	Carbon dioxide
COPD	Chronic Obstructive Pulmonary Disease
CuZnSOD	Copper zinc superoxide dismutase
DMPO	5, 5-dimethyl –1-pyrroline-N-oxide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
DPI	Diphenyleneiodonium
DQ12	Quartz
EPA	Environmental Protection Agency
F	Fine
FTIR	Fourier Transformed Infrared Spectroscopy
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSSG	Oxidized glutathione
H_2O_2	Hydrogen Peroxide
HBSS	Hank’s balanced salt solution
HRTEM	High Resolution Transmission Electron Microscopy
IL-8	Interleukin-8
kb	Kilobase
kD	Kilodalton
LDH	Lactate Dehydrogenase
LPS	Lipopolysaccharide
MF	Methylated Fine
ml	milliliter
MMD	Mass Median Diameter
MnSOD	Manganese superoxide dismutase
mRNA	Messenger ribonucleic acid
MUF	Methylated Ultrafine
NAC	N-acetylcysteine
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate

NF-?b	Nuclear factor-kappaB
NP	Nanoparticles
O_2	Molecular oxygen
O_2^-	Superoxide radical
O_3	Ozone
PBS	Phosphate buffered saline
PKC	Protein Kinase C
PM	Particulate Matter
PMN	Polymorphonucler nutrophils
QUARG	Quality of Urban Air Review Group
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RT	Room Temperature
RT-PCR	Reverse transcription polymerase chain reaction
SOD	Superoxide Dismutase
TEM	Transmission Electron Microscopy
TEMPOL	2, 2, 6, 6-tetramethylpiperidine-1-oxyl
TiO_2	Titanium Dioxide
TNF	Tumor Necrosis Factor
UF	Ultrafine
UFP	Ultrafine Particles
UV	Ultra Violet
XDH	Xanthine dehydrogenase
XO	Xanthine oxidase

1. Introduction

1.1 History

Once prehistoric man (70,000 BC Neanderthal man) had discovered fire, he could warm himself in front of the flames and cook the flesh of animals that he hunted. Fire brought light into the dark caves and as the time went by and men lived in houses, a fire in the hearth helped to create a comfortable homely atmosphere and a sense of security. With the aid of fire, men could build furnaces into which they put iron or a variety of other substances, mixed with charcoal. They used bellows to make the fires hotter and so were able to fashion tools for many purposes. Later they explored the remote and unpopulated areas of Europe, Asia, and finally the America by following a retreating glacier. Surviving the extreme cold climate was only possible because of fire. Fire could have made the difference between survival and extinction in the regions occupied by human beings and it was around campfires that the first seeds of civilisation and development were planted. The Prehistoric man probably first experienced harm from air pollution when they built fires in poorly ventilated caves and since then we have went on to pollute more and more of our environment.

The air pollution was common in large towns long before the industrial revolution. Early anthropogenic sources of air pollution probably included dust, wood smoke, tanneries, animal manure, smelting of metals, fireplaces and burning of land etc. Air pollution resulted from a variety of causes, not all of which were within human control. Dust storms in desert areas, volcanic eruptions and smoke from forest fires and grass fires, contribute to chemical and particulate pollution of the air. The next big step in particulate air pollution was probably when people started to burn fossil components such as coal instead of wood in cities that also had increasing population densities around the 12^{th} and 13^{th} century.

For many centuries man used fire to help him with the task of everyday life, although he was not skilled to control it completely until the invention of fuel ignition systems, without which the modern industry could not function. The invention of a more efficient (coal-powered) steam engine by James Watt in 1765 was one of the milestone factors that spurred on the Industrial Revolution. The Industrial Revolution in Europe in the 19^{th} century saw the

beginning of air pollution as we know it today, which has gradually become a matter of great global concern. This epoch saw a tremendous increase in factories and could definitely be viewed as the start of serious air pollution from industry. Modernisation and industrial progress have led to air getting more and more polluted over the years. Industries, vehicles, increase in the population, and urbanisation are some of the major factors liable for air pollution.

1.2 Air pollution and respiratory diseases

Air pollution as a trigger for the exacerbation of respiratory diseases has been recognised for more than 50 years due to large smog episodes in the Meuse Valley (1930s) and London (1950), and has led to the development of air quality standards in many countries world-wide that substantially decreased the levels of traditional air pollutants derived from the burning of fossil fuels, such as black smoke and sulphur dioxide. However, the recent dramatic increase in motor vehicle traffic has produced a tremendous increase in the levels of newer pollutants, such as particulate air pollution leading to a large number of premature deaths all over the world due to the respiratory and cardiovascular disorders (Samet et al. 2000; Dockery and Pope, 1996; Seaton et al. 1995).

1.3 Particle exposure and diseases

An extensive body of epidemiological studies has shown a correlation between exposure to elevated ambient particulate air pollution and mortality, exacerbation of respiratory disease like asthma, chronic bronchitis, ischemic heart diseases and strokes (Schwartz et al. 1994, 2002; Peters et al. 2000; Kaiser 1997; Dockey et al. 1993; Samet et al. 2000; Wichmann et al. 2000) especially in people with pre-existing lung disease (Schwartz 1994; Pope et al. 1995, 2000, 2002; Pope and Dockery 1999). Studies have shown that, amongst elderly, hospital admissions for cardiovascular disorders increased 1 % for each 10 μg increase in particulates, while there was 2 % increase in hospital admissions due to cardiopulmonary disorders (Samet et al. 2000).

Apart from the environmental exposure to the particulate matter, occupational exposures to mineral dusts have been already described since ancient cultures. Exposure to mineral dusts such as asbestos and silica in the occupational environment can lead to a variety of pulmonary disorders including lung cancers malignant mesotheliomas (Mossman and Churg 1998). Lung disorders due to the inhalation of several other mineral dusts such as granite, ceramic and pottery industries, mining and grinding coal, kaolin, gold, tin and sandblasting have been described in various occupational settings (McDonald 1994). Mineral dust induced lung disorders are regularly being reported in relation with other occupational exposures (Landrigan 1987, Cullen et al. 2000; Nemery et al. 1992).

1.4 Particulate Matter (PM_{10} , $PM_{2.5}$ and $PM_{0.1}$)

PM (Particulate Matter) is the term used to describe a heterogenous mixture of substances including carbon, metals, nitrates, sulphates, and volatile and particulate matter (Turnbull and Harrison 2000) in the atmosphere which may vary in size, mass, composition depending on season, source and weather conditions. Environmental particulate air pollution is measured by a global sampling convention called PM_{10} that measures mass of particles collected with 50 % efficiency for particles with aerodynamic diameter of 10 µm; a wide range of particle sizes are collected from coarse 10 µm down to the fine particulate ($PM_{2.5}$) (QUARG 1996).

The size distribution of particles in the urban air is conventionally characterised by three modes (Figure 1-1). The smallest of these, below 0.1 µm in diameter, is called the *nucleation mode* and is formed by condensation of hot vapour from combustion sources and from chemical conversion of gases to particles in the atmosphere. Particles of this size have a high chance of deposition in the gas-exchanging (alveolar) part of the lung; they are relatively short-lived and grow into larger particles between 0.1 and about 1 µm in diameter, known as the *accumulation mode*. These particles remain suspended for up to several weeks in the air, and are not readily removed by rain. The third, *coarse mode* comprises particles greater than about 2 µm in diameter. These are generally formed by break-up of larger matter, and include wind-blown dust and soil, particles from construction and sea spray. Their size means that they remain in the air for relatively short periods, but they make (in relation to their numbers) a disproportionate contribution to PM10 mass when this is measured close to a source.

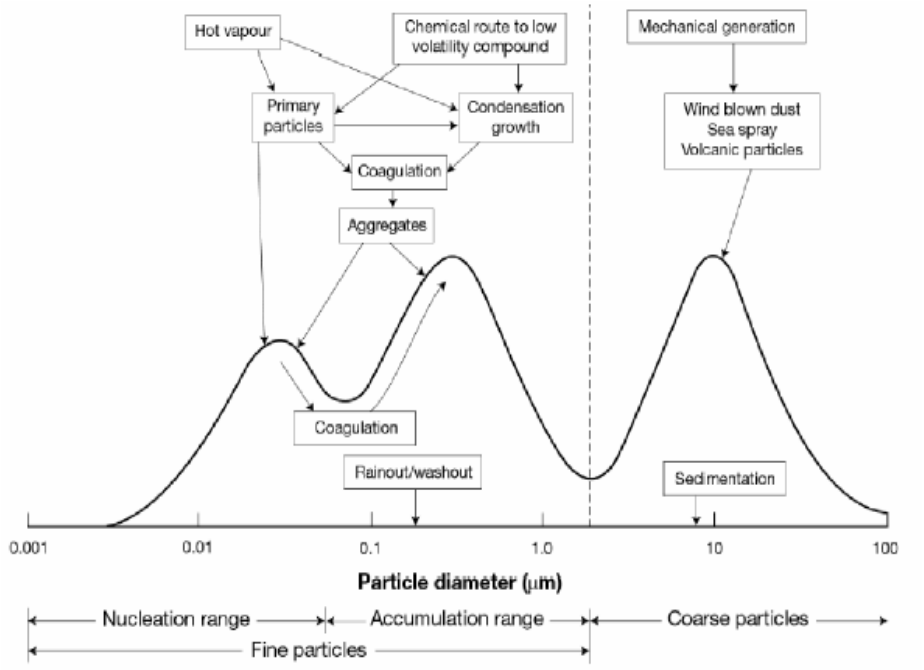

Figure 1-1 Typical size distribution of trimodal urban aerosol (From: EPA 1996)

The particle composition is critical in PM toxicity but all the components of PM_{10} are not on the whole very toxic, comprising in larger parts of sulphates, nitrates, chlorides, ammonium, carbonaceous materials, metals and wind blown crystal dusts (QUARG 1996). One of the major advances in PM research has been the recognition that the organic and metal components (Costa and Dreher 1997; Dreher et al. 1996) of PM can induce proinflammatory effects in the lung due to their ability to cause oxidative stress (Kumagai et al. 1997; Nel et al. 1998, 2001; Saldiva et al. 2002). Within the spectrum of particle sizes, ultrafine particles ($PM_{0.1}$), are suggested to have a central role in health effects of PM (Oberdörster and Utell 2002; Samet et al. 2000) and studies indicate that the ultrafine particles, which by count constitute the majority of urban particulate air pollution, may have an increased toxicity relative to larger particles under the same mass concentration (Ferin et al. 1992; Oberdörster et al. 1995, 2001; Stone et al. 1998; Li et al. 1999; Brown et al. 2001). Primary UFPs are formed during gas-to-particle conversion or during incomplete fuel combustion (HEI 2002). Due to their small size, high number concentration, and relatively large surface area per unit mass, UFPs have unique characteristics, including increased adsorption of organic molecules and enhanced ability to penetrate cellular targets in the lung and systemic circulation

(Frampton 2001; HEI 2002; Nemmar et al. 2002; Oberdörster 1996; Utell and Frampton 2000).

1.5 Ultrafine and Nanoparticles in historical context

So far the definition of ultrafines has not been consistent and defined differently by various disciplines. Some powder technology publications used the term ultrafine to denote particles smaller than 0.1 μm in diameter. Some defined ultrafine particles as particles with diameters below 45 μm. The term ultrafine particles was used by some scientists during WUFA (Workshop on Ultrafine Aerosols) in Vienna (Liu et al. 1982) and in 1996 EPA (Environmental Protection Agency) used the term ultrafine particles to characterize the particle size distribution with mass median diameter (MMD) below 0.1 μm (Pui and Chen 1997).

Physicist Richard P. Feynman (1918-1988) first talked about the concept of nanoscience in 1959 in his key lecture at the annual meeting of the American Physical Society, and the term nanotechnology was coined in 1974 by Japanese researcher Norio Taniguchi to mean "precision machining with tolerances of a micrometer or less". It refers to engineering on the molecular and atomic level. Twelve years later, Eric Drexler introduced the word nanotechnology and the concept into the public consciousness with his 1986 book Engines of Creation and a subsequent study on scholarly feasibility entitled "Nanosystems" where he predicted that nanotechnology could give rise to replicating assemblers, permitting an exponential growth of productivity and personal wealth.

However, the difference between the ultrafine and nanoparticles nomenclature seems not to be due to their size but because of the different disciplines of science. Particles which are less than 100 nm diameter are usually referred to as ultrafine particles by toxicologists (EPA 2004) and are called as nanoparticles by physicists (NNI 2004).

1.6 Ultrafine *versus* Nanoparticles

Ultrafine particle pollution was and is an unavoidable by-product of the industrial revolution and it received enormous attention due to its adverse effects on human health. However, it was a few decades ago when scientists discovered that the ultrafine particles are not only causing health risk but also possess lots of desirable properties (e.g. improved hardness, special optic and magnetic properties) which could be commercially exploited and thus leading to the large and uncontrolled commercial production of nanoparticles (Engineered nanomaterials).

Today, the engineered nanomaterials are used in a number of consumer and industrial products including sustainable energy, healthcare, automobiles, information and communication, cosmetics, food products (Table 1-1) and not only that, it is expected that in coming years there will be an immense increase in the number of consumer products relying on nanotechnology. Researchers forecast that manufactured nanoparticles may become suspended in the air during fabrication, distribution, use and disposal and can pose a threat to the environment (Oberdörster et al. 2005).

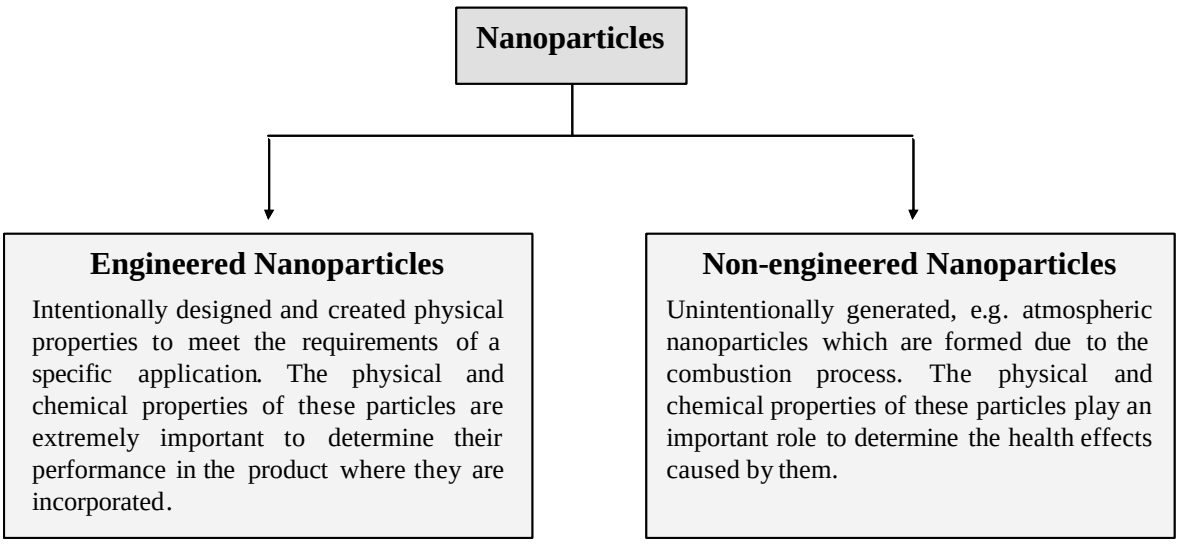

Figure 1-2 Engineered and Non-engineered Nanoparticles

The two main differences between intentionally designed engineered NP (like CB) and unintentionally generated UF (e.g. diesel exhaust particles) are surface chemistry and size, which can be controlled in case of synthetic NP while production.

The term “engineered nanomaterials” is used to describe inorganic materials of high uniformity, with at least one critical dimension below 100 nm, specifically engineered for commercial applications (Colvin 2003). Whereas, ultrafine particles are defined as particles with diameters under 100 nm, are chemically heterogeneous and polydisperse materials that bear little resemblance beyond their physical size to most engineered nanoparticles (Kleeman et al. 2000) compositions thus making comparisons with engineered nanomaterials problematic (Colvin 2003). Studies have shown that nanomaterials can enter the human body through several routes (inhaled, ingested or enter the body via the skin). However, accidental or involuntary contact during production or use is most likely to happen *via* the lungs from where a rapid translocation (Kreyling at al. 2002) through the blood stream to other vital organs is possible. In fact some of the nanoparticles produced for therapeutic purposes are fabricated to give them specific properties facilitating prolonged circulation in blood, reaching of specific organs or tissues, escape from phagocytosis, translocation through the blood-brain barrier and sustained release of drugs (Oberdörster and Utell 2002).

Table 1-1 Some commercially important nanomaterials

Nanoparticles	Commercial use
Carbon nanotubes	Field emission-based flat panel displays, novel semi- conducting devices, chemical sensors, and ultra-sensitive electromechanical sensors, energy storage, sport goods, automobiles
Titanium dioxide	Sunscreens, self cleaning glass and as photo-catalyst, cosmetics, textiles, coatings, electronics, military uses, decontaminants
Silicon/Germanium	Widely used in semiconductor manufacturing
Calcium oxide based materials	For bone replacements and reconstruction
Teflon	Textiles
Zinc Oxide	Sunscreen, cosmetics
Aluminium	Military uses, Decontamination
Silver	Medical uses
Iron Oxides	Pigments, drug delivery

Since there is only very limited information and evidence available to support the pros and cons of nanotechnology, the opponents and the pioneers of nanotechnology face extreme difficulties opposing or supporting this rather novel technology. Too little is known about the risks of nanoparticles and the scarcity of data gives rise to a host of fears and alarming scenarios (Colvin 2003). For example, larger titanium dioxide particles have been considered to be a safe physical, white, opaque sunscreen because it reflects and scatters UVB and UVA in sunlight (Judin 1993; Dunford et al. 1997). However, if the same TiO_2 crystals are reduced to nanoscale, it not only loses its characteristic white colour and becomes transparent but these TiO_2 nanoparticles from sunscreen are also found to cause free radicals in skin cells, damaging DNA (Dunford et al. 1997). While TiO_2 is generally considered to be inert in their larger size, nanosized TiO_2 can be highly photo reactive in presence of UV light (Serpone et al. 2001).

Various other studies (Table 1-2) have shown that the toxicity of nanoparticles appears to be more related to their size than to the material from which they are made which means that the toxicity of a material in larger form does not tell what its toxicity will be when it is nanosized. Therefore, while appreciating the promising properties of nanoparticles one must be aware of the gigantic difference (Figure 1-2) between inevitable ultrafines generated as a combustion by-product and deliberate large scale commercial fabrication of nanoparticles. As the risks arising from exposure to many types of nanoparticles are not yet entirely understood, control strategies should be based on a principle of reducing exposure as much as possible, especially for the protection of the workers involved in the industrial production of NPs.

Table 1-2 Crucial findings on the biological effects of Nanoparticles (NP) (Modified from Borm and Kreyling, 2004)

Key studies on toxicological effects of NP	References
NP have a higher deposition probability particularly in the small airways and the alveolar region of the lungs	Bair et al. 1994
NP have a high specific surface area, which can catalyse reactions and which can adsorb high amounts of toxic substances (like PAH), making them a carrier into the deep lung during inhalation	Seaton et al. 1995
NP are taken up by other cells of the respiratory epithelia such epithelial cells, dendritic cells	Ferin et al. 1992; Geiser et al. 2000
NP are less well phagocytised by alveolar macrophages than larger particles and inhibit their phagocytic ability, inhibit macrophage motility	Lundborg et al. 2001; Möller et al. 2002
NP adversely affect cardiac functions and vascular homeostasis	Stone and Godleski 1999
NP may form complexes with similar sized proteins and bio molecules which may result in functional changes of the latter	Borm and Kreyling 2004
NP have greater access to interstitial spaces than larger particles	Stearns et al. 1994; Oberdörster et al. 2000
NP affect immunity	Behrendt and Becker 2001
NP can cause more inflammatory effects than larger particles	Donaldson et al.2001
NP have access to systemic circulation	Nemmar et al. 2002; Oberdörster et al. 2002; Kreyling et al. 2002
NP induce more oxidative stress than fine particles	Stone et al. 1998, 2001
The large surface area of ultrafine particles and its composition and structure play a pivotal role in the above mentioned interactions with biological target cells, body fluids and tissues	Donaldson et al. 2002

1.7 Hypothesis for the factors influencing the effects of UF particles

Various studies (Table 1-3) done in the last decade suggest that the ultrafine fraction of PM_{10} is responsible for causing the adverse health effects. Ultrafine particles, i.e. particles with diameter 0.1 µm and less, represent a substantial component, in terms of particle numbers, in PM_{10}, although they represent a relatively small fraction of the total mass (Peters et al. 1997) and have a much larger surface area, hence, more toxic potential (Oberdörster et al. 2001; Donaldson et al. 2001). Discussing all the points mentioned in the Table 1-2 is beyond the scope of this thesis. Therefore two of the most important factors influencing ultrafine effects, and which we are also going to test in this thesis work, are discussed in following sections *1.7.1* and *1.7.2*.

1.7.1 Particle surface area

In vivo studies have shown that ultrafine particles elicit more lung injury and pathology than those exposed to the same deposited mass of fine respirable particles of the same material (Oberdörster et al. 1994; Donaldson et al. 1998; Höhr et al.2001) which is due to the large surface area available on small sized particles. It has been suggested that the particle number and size is more important than the particle mass in inducing a biological response (Peters et al. 1997; Maynard and Maynard 2002). It is estimated that a 10 % increase in PM_{10} would represent a very large change in both number and surface area of the particles (COMEAP 1995). In addition, recent studies suggest that inflammation observed in rats exposed to ultrafine carbon black and polystyrene particles is due to either the surface area or to particle number effects, in the absence of transition metals (Brown et al. 2000, 2001; Duffin et al. 2002). The large surface area of UFPs can catalyse reactions and adsorb high amounts of toxic substances, thus enabling the particles to carry these substances deep into the lung during inhalation (Seaton et al. 1995).

Due to the increasing demand for tailored nanomaterials, a deeper understanding of the governing process parameters from detailed kinetic studies are necessary to meet the requirements of the respective application. Currently there is a lack of data on the potential toxicity of manufactured nanoscale materials. Most of the information about nanoparticles

toxicity is derived from studies performed on ultrafine particle inhalation toxicology (Oberdörster et al. 2005). It suggests that particle size can impact toxicity equally if not more so than chemical composition and hints at the intricacy of the topic. The distinctive and diverse physicochemical properties of nanoscale materials suggest that toxicological properties may differ from materials of similar composition but dissimilar size. There are indications in the literature that manufactured nanoscale materials may be distributed in the body in unpredictable ways and certain nanoscale materials have been observed to preferentially accumulate in particular organelles. Surface properties can be changed by coating nanoscale particles with different materials, but surface chemistry is also influenced by the size of the particle. This interaction of surface area and particle composition in eliciting biological responses adds an extra dimension of complexity to evaluating potential adverse events that might result from exposure to these materials.

1.7.2 Particle surface chemistry

The degree of hydrophilicity/hydrophobicity of a surface is an important property to evaluate, since it regulates cell-surface adhesion, protein denaturation at the interface, and the selective adsorption of components from the liquid phase (Van Oss 1994). Variations in the hydrophobicity of the surface can result in different translocation routes in various biological compartments, different coatings of the surface by endogenous materials, and differences in the interfacing of the solid with cells (Fubini 1997). For example, coating poly (methyl methacrylate) nanoparticles with different types and concentrations of surfactants significantly changes their body distribution (Araujo et al. 1999). Water soluble fullerenes, for example, associate strongly with cell membranes due to their hydrophobic nature (Foley et al. 2002). Furthermore, surface properties are implicated in the ability of particles to generate free radicals and reactive oxygen species (ROS) which are believed to play a major role in the inflammatory effects induced by them (Schins and Donaldson 2000; Schins 2002).

Respirable quartz which has been classified as a human lung carcinogen (IARC 1997) is the best example for explaining the importance of surface reactivity. The surface reactivity is one of the key factors contributing to the pathogenicity associated with quartz inhalation (Donaldson and Borm 1998; Fubini1998; Fubini et al. 1990) and studies have shown that quartz-induced silicosis could be inhibited by the administration of compounds such as aluminium salts and polyvinylpyridine-*N*-oxide (PVNO), which are known to modify the

surface reactivity of the quartz particles (Brown et al.1989; Bouffant et al.1975; Begin et al. 1987; Schlipköter et al.1961; Goldstein and Rendall 1987). As a model for studying the protective effects of aluminium silicate clays on the quartz surface, various investigators (Brown et al. 1990; Begin et al. 1987; Duffin et al. 2001; Albrecht et al. 2004) have utilized aluminum lactate and demonstrated that brief incubation of a highly inflammatory quartz sample in a solution of this compound dramatically ameliorates the pro-inflammatory effects. Various agents such as lipid and proteinaceous surfactant materials, the polymer polyvinyl-pyridine-n-oxide (PVPNO), and organosilane, have been shown to coat the surface of quartz and to decrease its toxicity (Nolan et al. 1981; Wallace et al. 1985; Vallyathan et al. 1991; Antonini and Reasor 1994; Castranova et al.1996a; Albrecht et al. 2004).

Pott et al. (1998) showed that TiO_2 which was coated with a silane compound to change the hydrophilic surface to a hydrophobic one appeared highly toxic and lethal for rats when the dusts were instilled at doses exceeding 2 mg. However, later studies showed that the intratracheal doses of 250 and 500 µg of the same hydrophobic, silanised ultrafine TiO_2 did not show toxicity and induced a much lower pulmonary inflammation in comparison to the hydrophilic, uncoated dusts (Oberdörster 2001). Furthermore, the studies from Warheit et al. 2002 showed that the OTES coating on the pigment grade TiO_2 particle does not cause significant pulmonary toxicity. Rehn at al. (2002) also showed that the hydrophobic coating did not affect the sub-chronic pulmonary toxicity of pigment grade TiO_2 (Table 1-3).

Surface coating or chemically modifying a variety of engineered nanoparticles is an important aspect of nanomaterials synthesis and is currently an active area of research. Such coating allows modification and tailoring of physical and chemical properties of the nanomaterial. It is well known that there is a relationship between the surface chemistry (e.g. hydrophobicity) and uptake of particles by the cells and this effect is exploited in nanomedicine to develop stealth particles (Gupta and Gupta 2005). Stealth particles are the carrier systems which can avoid phagocytosis and thus can circulate longer in the body.

All information regarding coating that we have till now comes from respiratory toxicology. One important variable characteristic appears to be the surface reactivity of quartz. In the industrial production of various nanoparticles, the adjustment of the nanoparticle diameters by coating the nanoparticles with thin layers or shells is done to introduce novel optical absorption behaviour which is exploited to fabricate very precise, high performance, optical

sensors from the IR to the UV range. These surface modified particles can effectively deliver active ingredients that exert local and systemic physiological effects. In particular, these NP are assumed to be effective for targeted and controlled delivery of biologically active ingredients into the periodontal pocket for oral care treatment. The NP used for drug delivery consists of solid hydrophobic nanospheres, having an average particle size of 0.01 to 1 micron. The nanospheres have a high ionic surface charge density to enhance their adsorption onto cell surfaces causing a higher local concentration. The NP used for drug delivery has properties like enhanced stability of ingredients and prolonged product shelf life. If these particles are released in the environment, it would pose a threat due to their poor biodegradability.

Table 1-3 Overview of intratracheal instillation studies that have used hydrophobic TiO_2

Studies	Animal species	TiO_2 and exposure	Dose exposure	Findings
Pott et al. (1998)	Rat Wistar-female	T805, 45 m^2/g, 21 nm versus P25. Instillation in 1% Tween 80 with CO_2 anesthesia	0.1- 120 mg, 24 hrs	LD50 T805, 1 mg. Lungs contained large amounts of erythrocytes
Rehn et al. (2002)	Rat Wistar-female	T805, P25 and DQ12 in lecithin (0.25 %) PBS	0.15, 0.3, 0.6 and 1.2 mg	Acute (3 days) inflammation mild and not different between T805 and P25. At 90 days also no difference in proliferation and PL
Oberdörster (2001)	Rat	T805 versus P25(material obtained from Pott)	50 and 500 µg	Less acute inflammation with T805, i.e. hydrophobic TiO_2, at both doses
Höhr et al. (2002)	Rat Wistar-female	Methylated fine (180 nm, 10 m^2/g) and ultrafine (P25, 20 nm) TiO_2 versus untreated TiO_2. No Tween	1 and 6 mg, 18 hrs	Less acute inflammation with hydrophobic TiO_2 at 1 mg dose. Effect seen with fine and ultrafine TiO_2
Warheit et al. (2002)	Rat SD-male	Fine TiO_2 with OTES coating, Tween 80 suspension	10 mg each, 24 hrs, 1wk, 3 months	Largest acute inflammation with uncoated TiO_2.No effect of coating or combination with Tween 80

1.8 Hypothesis for mechanisms and mediators involved in toxicity induced by ultrafine particles

Inhalation of particles is possible when they remain suspended in the air for a longer time and the likelihood of a particle remaining suspended in the air depends upon its size, shape and density. After generation, particles may be removed from the atmosphere by depositing on the floor, walls and other surfaces (in workplace exposures) or settle as a result of gravity. The gravitational settling velocity of the particle is proportional to the diameter. In contrast to smaller particles created by combustion processes (0.001-0.1 μm), coarse particles generated by processes like mining or grinding (3-20 μm) are relatively large and will therefore settle and will be removed from the stable aerosol.

Airborne UFP will settle down much more slowly than the larger particles and gravitational settling is not an effective removal process. This might lead to higher, longer exposures and hence UFP will be more likely to be inhaled and deposited on the inner surface of the lung. The efficiency of deposition of the particles in various anatomical compartments of the lungs depends on the MMAD (mass median aerodynamic diameter) of the particle. MMAD is the aerodynamic diameter around which the mass is centred and it is considered to be the most important parameter governing the deposition of particles in the respiratory tract (Clarke and Yates 1994).

Since the last decade, studies have shown an association between particulate matter and adverse health effects but the underlying biological mechanisms of particle induced toxicity are not yet clear. In a number of animal studies the inflammatory responses induced by particles have been linked to particle overload (Mauderly et al.1996; Morrow 1998; Bellmann 1992). It has been shown that upon inhalation, particles can reach the deep alveolar regions. These particles can activate and also impair the alveolar macrophage-mediated lung clearance, which occurs when the deposition rate is greater than the alveolar clearance rate. Due to this, accumulation and interstitialisation of particles (Ferin et al. 1991, 1992; Oberdörster et al. 1992a, 1994), excessive release of inflammatory mediators from activated phagocytic cells (Becker et al. 1996; Vanhee et al. 1995; Driscoll and Maurer, 1991; Driscoll

et al. 1990a; Borm et al. 1988), damage to epithelium, and the activation of fibroblasts (Driscoll et al. 1990b, 1995, 1997; Rom 1991) occur in the alveolar region.

As mentioned above, a number of mechanisms and mediators have been suggested to be involved in particle induced inflammation. Among those, mechanisms of particle uptake, causation of oxidative stress and the subsequent induction of inflammatory mediators play a crucial role and will be elaborated in the following paragraphs.

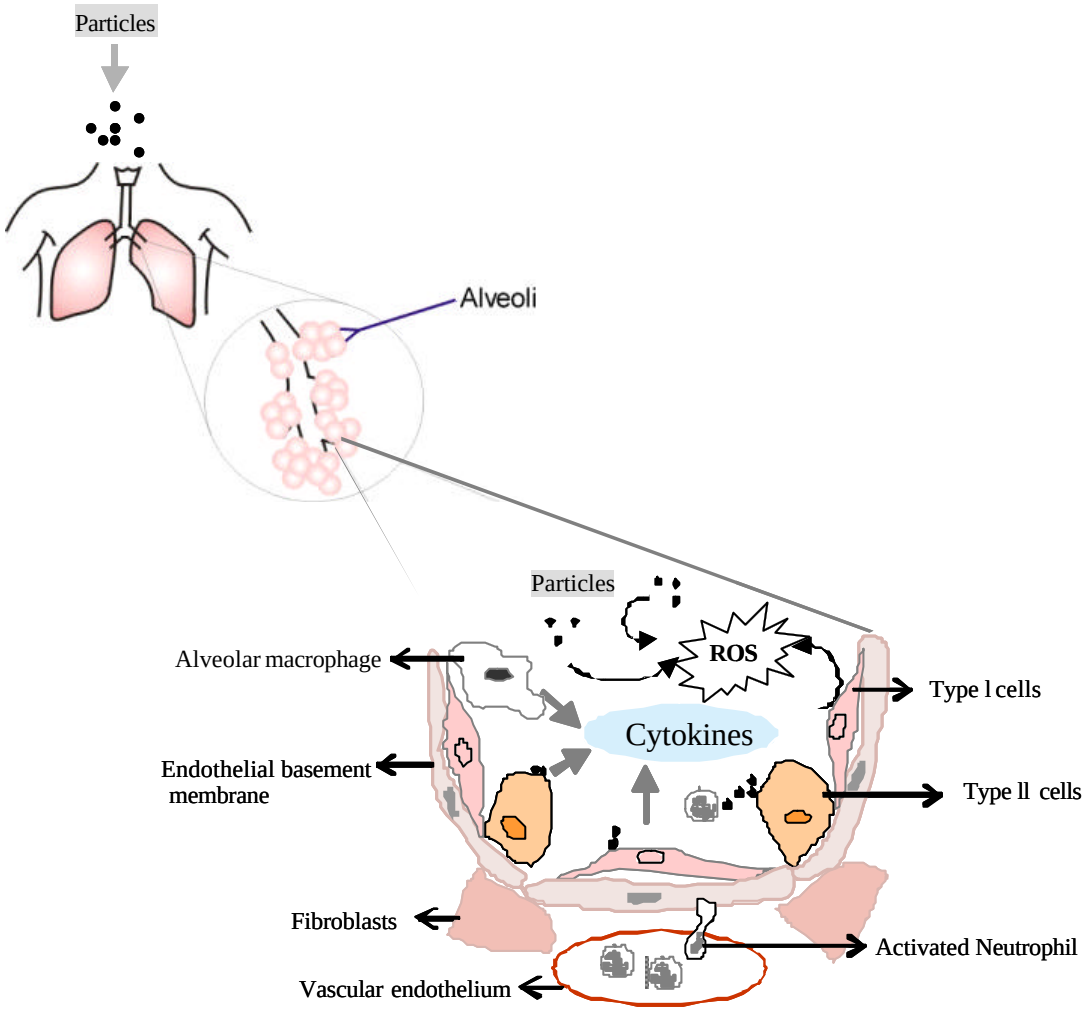

Figure 1-3 Mechanisms of particle induced lung injury

The alveolar macrophages usually the first cell encountered by inhaled particles, and become activated. The activated macrophage produces not only IL-8, but also early - response cytokines, IL-1 and TNF which further activates epithelial cells, fibroblasts and endothelial cells. The particle itself can escape the macrophage surveillance and interact directly with epithelium, activating them and thereby establishing a significant IL-8 chemotactic gradient, culminating in the recruitment of neutrophils in lung parenchyma.

1.9 Particle Uptake

The entry of particles into epithelial and sub-epithelial tissues is known to be associated with a variety of deleterious effects including release of inflammatory and fibrogenic cytokines, genotoxicity, oxidative cell injury, cell death and interstitial fibrosis. It has been proposed that the adverse effects caused by ultrafine particles can be explained by their high tissue access due to their smaller size, leading to high levels of mediator release (Oberdörster et al. 1995; Seaton et al. 1995; Schins 2001).

Ultrafine particles are phagocytised less efficiently by alveolar macrophages and therefore are more likely to be internalised by epithelial cells and translocated to interstitial sites (Ferin et al. 1991; Stearns et al. 1994). Because of impaired phagocytosis, the epithelial lining of the respiratory tract likely represents the dominant target of the toxic effects of inhaled ultrafine particles. To understand the mechanism of particle induced inflammatory effects, it becomes necessary to clarify the interaction between particles and lung epithelial cells. The alveolar epithelium is composed of two different cell types, namely type I cells (which constitute more than 90 % of the alveolar surface and account for more than 7 % of the parenchymal lung cells) and type II cells (which cover about 10 % of the alveolar surface and account for 15 % of all parenchymal cells). Although type II cells represent a minor part of the alveolar surface, they play an important regulatory role in modulating the intra-alveolar inflammatory response. However, the mechanisms by which the particles are taken up by the pulmonary epithelial cells are not yet very clear (Churg 1996). Its speculated that there may be specific uptake mechanisms for particles in epithelial cells and these may differ with particle properties. Such differences may in turn lead to different cytokine responses.

1.10 Oxidative stress in Lung

The lung is a vulnerable organ to oxidant damage because of its location, anatomy and function (Crystal 1991). The lung epithelium is not only exposed to the free radicals which are constantly generated internally as a part of normal metabolism but also to numerous

exogenous chemicals and physical agents, which includes particulate matter (as previously described) as well as ozone (Graham and Koren 1990; Johnston et al. 2002), nitrogen dioxide (Speizer et al. 1980. Samet et al. 1987), sulphur (Harrison et al. 1995), ionising radiation (Janssen et al. 1993), and tobacco smoke (Church and Prior 1985; Howard et al. 1998).

Free Radical is any chemical species is capable of independent existence possessing one or more unpaired electron in the outer (valence) shell of the molecule (Halliwell and Gutteridge 1989). This is the key factor in the structure of this species (Greenwald 1991; Halliwell 1995) and is the reason why they are highly reactive. Reactive Oxygen Species (ROS) is a collective term often used to describe oxygen radicals and certain non-radicals that are either oxidising species or that can easily converted into radicals (Table 1-4).

Table 1-4 Free Radical vs. Non radicals

Reactive Oxygen Species - ROS have often been loosely categorised as free radicals, but this is incorrect because not all ROS are free radicals.

Free Radicals	Non radicals
$O_2^{\cdot -}$ Super oxide	H_2O_2 Hydrogen Peroxide
$^{\cdot}OH$ Hydroxyl	HOCl Hypochlorous acid
$RO_2^{\cdot}$ Peroxyl	O_3 Ozone
$RO^{\cdot}$ Alkoxyl	$^1O_2$ Singlet Oxygen
$HO^{\cdot}_2$ Hydroperoxyl	ROOH Hydroperoxide

The lung has a well-developed antioxidant system which detoxifies ROS efficiently but sometimes the cellular production of ROS overwhelms its antioxidant capacity leading to oxidative stress. The term oxidative stress is defined as a disturbance in the prooxidant-antioxidant balance in favour of the prooxidant, leading to potential damage (Sies 1985, 2000). Oxidative stress is thought to contribute to the pathogenesis of a number of human diseases (Cross et al. 1987; Halliwell et al. 1992) including those of the lung. Inflammatory lung diseases, such as asthma and chronic obstructive pulmonary disease (COPD), are characterised by systemic and local chronic inflammation and oxidative stress (Repine et al. 1997; Kaminsky et al. 1999). There is now overwhelming evidence of the importance of

oxidative stress in a number of pathological situations in the lungs, where the epithelium represents a major target of oxidant injury (Shackelford et al. 2000).

Particles can directly or indirectly generate ROS through the interaction of the surface area of ultrafine particles with the target cells, such as macrophages and epithelial cells. Both processes may elicit transcription of proinflammatory cytokines and result in a cascade of inflammatory events (Stone et al. 1998). The generated oxidative stress is suggested to be a central mediator of ultrafine particle toxicity. This is due to the fact that ultrafine particles have a smaller size and hence a greater surface area than the fine particles at the same mass. This also increases the capacity of ultrafine particles to carry free radicals (Donaldson et al. 1998; Zhang et al. 1998). The difference in the inflammatory potential of the particles may therefore be explained on the basis of their free radical generating activity.

Emerging evidence suggests that reactive oxygen species (ROS) at low concentration may function as signalling intermediates of cellular responses (Schreck et al. 1991). However, the mechanisms through which ROS act are still poorly understood. Recently, ROS have been shown to stimulate signalling pathways (Finkel 1998) implicated in growth factor and cytokine effects through activation of their important components, such as MAPK, extra cellular-regulated protein kinase (Sundaresan et al. 1995; Guyton et al. 1996), and c-Jun NH_2-terminal kinase (Lo et al. 1996), and transcription factors such as NF-κB (Manna et al. 1998; Schreck et al 1991) and AP-1 (Lo et al. 1995). Although a large number of signalling pathways appear to be regulated by ROS, the signalling molecules targeted by ROS are less clear. NF-kappa-B, a transcription factor that regulates the expression of a number of genes involved in immune and inflammatory responses, has long been considered oxidant responsive (Meyer et al. 1993; Schreck et al. 1992).

Some of these ROS, especially hydrogen peroxide (Rhee 1999), are key signalling molecules, while others appear to be extremely detrimental to biological systems. To be considered as a potential signalling molecule, ROS must be produced by a cell, should have an action within the cell which produces the ROS, or a nearby cell producing them, and it should be removed in order to turn off, or reverse, the signal. Hydrogen peroxide readily permeates membranes and it is therefore not compartmentalized in the cell. It is now clear that some ROS, in particular hydrogen peroxide and superoxide, fulfil these criteria.

1.10.1 General Antioxidants Defence in Lung

As previously mentioned, the lung has a well-built antioxidant system (Figure 1-4) which is a defence mechanism to combat the damaging effects of ROS. It includes superoxide dismutase (SOD), catalase, and glutathione-dependent enzymes (Seidman et al 1999) which are described in following section. Epithelial cells (which are the primary target of ROS) of the lower respiratory tract likely consume intracellular antioxidants such as superoxide dismutases, catalases and the glutathione system to shield themselves against the toxic effects of oxidants generated (Crapo et al. 1980; McCord et al. 1969). However, in some circumstances, the production of free radicals can be so massive that the cell defence system is not able to detoxify them efficiently and thus leading to oxidative stress in the cell. Previous studies suggest that antioxidant activity is impaired in lung cancers (Coursin et al.1996; Guner et al. 1996; Melloni et al. 1996).

1.10.2 Superoxide dismutase

Superoxide dismutase (SOD) is an enzyme of the oxido-reductase class that catalyses the reduction of superoxide anions to hydrogen peroxide, protecting cells against dangerous levels of superoxide. The hydrogen peroxide is further broken down to oxygen and water. SOD enzymes include the intracellular Mn SOD and CuZn SOD and an extra-cellular SOD that is present in epithelial lining fluid and blood vessels (Erzurum et al, 1993). The mechanism of reaction catalysed by SOD is as follows:

$2O_2^{\cdot -} + 2H^+ \longrightarrow H_2O_2 + O_2$

a) Manganese superoxide dismutase, (MnSOD) - is considered to be one of the most important intracellular antioxidant enzymes. MnSOD is a homotetramer with manganese at its active sites, and has a molecular weight of 88 kDa (Fridovich and Freeman 1986) and is localized to mitochondria. Its promoter area contains binding sites for several transcription factors one of the most important being NF-? B (Das et al. 1995). MnSOD has been localised to type II pneumocytes, alveolar macrophages, and bronchial epithelium of rat (Clyde et al. 1993; Coursin et al. 1992; Chang et al. 1995) and at least bronchial epithelial cells of human lung (Kinnula et al. 1994, Coursin et al. 1996). MnSOD is induced by changes in the cellular redox state, inflammatory cytokines such as tumor necrosis factor alpha (TNF-a) (Wong and

Goeddel 1988; Tsan et al. 1990; Visner et al. 1990), interleukin-6 (IL-6) (Tsan et al. 1992;Warner et al. 1996), lipopolysaccharide (LPS) (Clerch et al. 1996), cigarette smoke (Gilks et al. 1998), chronic ozone exposure (Weller et al. 1998), asbestos fibers (Mossman et al. 1986) and oxidants such as H_2O_2 (Das et al. 1997, Warner et al. 1996, Jackson et al. 1998). MnSOD constitutes approximately 10-15 % of the total SOD activity in most tissues (Tsan 2001).

b) Copper zinc superoxide dismutase, (CuZnSOD) - is an intracellular enzyme mainly localised in cytosol (Crapo et al. 1992), and although more abundant than MnSOD, it is not inducible to the same extent (Shull et al. 1991; Kinnula et al. 1995). CuZnSOD is a homodimer with molecular weight of 32,5 kDa and contains both copper and zinc at its active sites (Fridovich and Freeman 1986). Copper is essential for the enzyme's catalytic activity, and zinc imparts stability to the protein structure (Fridovich 1975). SOD is an endogenously produced intracellular enzyme present in essentially every cell in the body. In contrast to MnSOD, CuZnSOD can also act as a superoxide reductase and a superoxide oxidase (Liochev and Fridovich 2000).

c) Extracellular superoxide dismutase, (ECSOD) - is a secretory tetrameric gycoprotein with a molecular weight of 135 kDa (Marklund 1984). It contains Cu and Zn in its active site and is found in interstitial spaces of tissues and also in extracellular fluids, accounting for the majority of the SOD activity in plasma, lymph, and synovial fluid (Marklund 1980; Sun et al. 1995). EC-SOD is the only isoform of SOD that is released from cells into the extra-cellular space (Marklund 1982). EC-SOD is the primary extra-cellular antioxidant enzyme and is highly expressed in blood vessels, (Stralin et al. 1995; Oury et al. 1996) uterus, (Sandström et al. 1993) and airways (Oury et al. 1996). Lung is the major tissue that expresses ECSOD which is very similar for both murine and human lung (Folz et al. 1997; Ookawara et al. 1998). In human lung ECSOD mRNA expression is found in alveolar type II cells, bronchial epithelial cells, alveolar macrophages, endothelial cells and chondrocytes (Folz et al. 1997; Su et al. 1997).

1.10.3 Catalase

Catalase is a homotetrameric enzyme of four identical subunits (220,000 to 350,000 kD), each with a heme prosthetic group at the catalytic centre. It has a molecular weight of 240 kDa,

mainly localised to peroxisomes of nearly all aerobic cells and serves to protect the cell from the toxic effects of hydrogen peroxide by catalysing the decomposition of H_2O_2 to water and oxygen (Fridovich and Freeman 1986; Deisseroth and Dounce 1970; Zamocky and Koller 1999), and is detectable especially in alveolar type II pneumocytes and macrophages (Kinnula et al. 1995). In humans, the highest levels of catalase are found in the liver, kidney, and erythrocytes, where it is believed to account for the majority of H_2O_2 decomposition. Catalase has one of the highest catalytic activities reported, near the diffusion-controlled limit. The mechanism of catalysis is not entirely elucidated, but the overall reaction is as follows:

$2H_2O_2 \longrightarrow 2H_2O + O_2$

1.10.4 ***Glutathione***

A typical feature in the antioxidant defence of human lung is the high glutathione content in the epithelial lining fluid (approximately 140 times higher than in the circulating blood) (Cantin et al. 1987) and based on this, glutathione (a low molecular weight thiol) and enzymes associated with its maintenance have been suggested to constitute one of the fundamental antioxidant defence mechanisms of human lung. It is abundant (3 to 10 mM) in cytoplasm, nuclei, and mitochondria and is the major soluble anti-oxidant in these cell compartments. It is a tetrameric protein 85,000 D. It has 4 atoms of selenium (Se) bound as seleno-cysteine moieties that confer the catalytic activity. Reduced glutathione (GSH), a tripeptide (g-glutamylcysteinylglycine) with a free thiol group, is a major antioxidant in human tissues that provides reducing equivalents for the glutathione peroxidase catalysed reduction of hydrogen peroxide and lipid hydroperoxides to water and the respective alcohol. During this process GSH becomes oxidized glutathione (GSSG). The GSSG is then recycled to GSH by using the nicotinamide adenine dinucleotide phosphate (NADPH) dependent enzyme glutathione reductase (GR).

$H_2O_2 + 2GSH \longrightarrow 2H_2O + GSSG$

$GSSG + 2NADPH \longrightarrow 2GSH + 2NADP$

Under normal conditions the balance of the equation is far in the direction of maintaining cellular glutathione in its reduced state (GSH> 99 %). The oxidative stress have been shown to result in GSSG formation (decrease in GSH/GSSG ratio) leading to GSSG accumulation

and a short-run depletion of GSH in the cell. In some cases a rebound effect occur resulting net increases in organ GSH levels between 12 and 48 h after initial stress. Studies have shown that lung GSH can be significantly elevated above control levels after 24 h of exposure to oxygen concentrations >98 %. Analysis of the GSSG level, or the GSH/GSSG ratio, is a useful measure of oxidative stress.

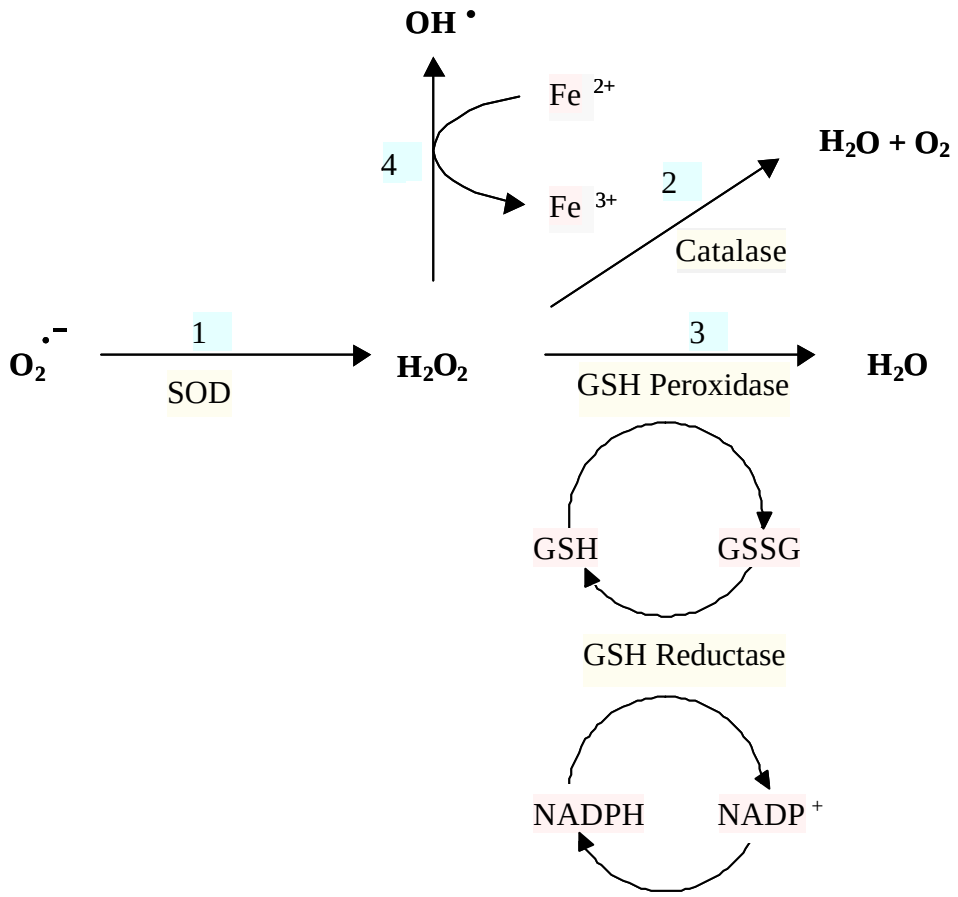

Figure 1-4 Fate of ROS in the cell

1) The function of SOD enzymes is to convert superoxide anion to H_2O_2. The univalent reduction of superoxide produces hydrogen peroxide which is not a free radical because all of its electrons are paired. 2) Catalase converts H_2O_2 to water and oxygen. 3) GSH reduces H_2O_2 via glutathione peroxidase reaction. NADPH is used to reduce the oxidized glutathione (GSSG) via glutathione reductase reaction. 4) The well-known reactivity of H_2O_2 is not due to its reactivity per se, but requires the presence of a metal reductant to form the highly reactive hydroxyl radical which is the strongest oxidising agent known and reacts with organic molecules at diffusion-limited rates. The respective enzymes that interact with superoxide and H_2O_2 are tightly regulated through a feedback system. An increase in the production of SOD without a subsequent elevation of catalase or glutathione peroxidase leads to the accumulation of hydrogen peroxide, which gets converted into the hydroxyl radical in presence of iron (Fenton reaction).

1.11 Inflammatory mediator: Interleukin-8

It is known that these biological responses to particles are driven by the production of cytokines (Kelley 1990), chemokines and other inflammatory mediators locally produced in the airways. The correlation between particle inhalation and the initiation of inflammation has been demonstrated in several studies already mentioned in the previous sections which indicate that the inhaled particles can cause the production of ROS that leads to the antioxidant depletion causing oxidative stress in the lung epithelial cells and this is assumed to be the most important mechanism for the commencement of inflammation. Due to the oxidative stress, epithelial cells produce inflammatory mediators that cause the infiltration of inflammatory cells (neutrophils) in the lung parenchyma. These inflammatory cells also contribute further to the oxidative stress at the site of particle deposition by generation ROS and releasing cytokines (Figure 1-3). This oxidative stress may initiate the damage to the cell membranes and also may cause the depletion in GSH and hence leading to the redox imbalance in the cells. The redox imbalance within the cells is an important factor leading to the induction of redox sensitive transcription factors like NF-kB (Schreck et al. 1992) and AP-1 (Meyer et al. 1994) that control the transcription of pro-inflammatory and proliferative genes such as IL-8 (Rahman and MacNee 1998).

IL-8 (Albelda et al. 1994; Strieter et al. 1996; Kunkel 1991) is a member of CXC chemokine family which is one of the most stable and potent chemotactic and activating factors known for polymorphonuclear leukocytes (PMNs). It plays an important role in the pathogenesis of airway inflammation and is an important biomarker which has been shown to be increased in sputum and bronchial lavage fluid of patients with chronic bronchitis, asthma and plays an important role in the development of clinical respiratory diseases (Keatings et al. 1996; Borm and Schins 2001; Barnes 2001). Although alveolar macrophages (AM) are known to be major producers of IL-8, mesothelial cells and alveolar epithelial cells (A549 cells) have also been shown to synthesise IL-8 in response to various stimuli (Standiford et al. 1990), including asbestos fibers (Boylan et al. 1992; Rosenthal et al. 1994).

Elevated IL-8 levels, detected in the lungs of COPD patients, are thought to play a major role in airway inflammation in this disease, particularly in the recruitment of neutrophils to the

lungs. Furthermore, IL-8 has been shown to be upregulated in response to components of PM_{10}, such as diesel exhaust and residual oil fly ash (ROFA) through mechanisms involving transition metals. Elevated IL-8 concentrations and neutrophil influx were demonstrated in BAL fluid of healthy volunteers on 24h post-instillation of the PM extracts (Ghio and Devlin 2001). IL-8 is CXC chemokine which is shown to be induced by oxidative stress (De forge et al. 1993; Massion et al. 1996, Stringer and Kobzik 1998) and is produced by various types of cells in the lung (Bagglioni et al. 1989),including epithelial cells(Nakamura et al. 1989, Strieter et al. 1989). The alveolar type II cells are known to be one of the major sources of this cytokine (Becker et al 1993; Marini et al, 1992). The potential for lung epithelial cells to participate in inflammation and disease is increasingly recognised (Oberdörster 1995; Vincet 1990; Churg 1996).

Previous studies indicated that environmental particulates can interact with lung epithelial cells (Berry et al. 1978; Watson and Brain 1979), and can also mediate production of proinflammatory cytokines from this cell type. Because of their capacity for ROS production, epithelial cells are likely to be an important target for oxidative stress. Studies have shown that A549 cells are capable of expressing IL-8 upon exposure to particles (Stringer et al. 1996). In our studies we used IL-8 as a biomarker of inflammation.

1.11.1 The human IL8 gene

Interleukin-8 (IL-8), a member of the CXC (Baggionlini et al. 1989) chemokine family, is an important activator and chemoattractant for neutrophils and has been implicated in a variety of inflammatory diseases. IL-8 is secreted in a stimulus specific manner by a wide variety of cell types and is regulated primarily at the level of gene transcription. Chemokines are a group of small (approximately 8 to 14 kD), mostly basic, structurally related molecules that regulate cell trafficking of various types of leukocytes through interactions with a subset of 7-transmembrane, G protein-coupled receptors. Chemokines also play fundamental roles in the development, homeostasis, and function of the immune system, and they have effects on cells of the central nervous system as well as on endothelial cells involved in angiogenesis or angiostasis. Chemokines are divided into 2 major subfamilies, CXC and CC, based on the arrangement of the first 2 of the 4 conserved cysteine residues; the 2 cysteines are separated by a single amino acid in CXC chemokines and are adjacent in CC chemokines. CXC

chemokines are further subdivided into ELR and non-ELR types based on the presence or absence of a glu-leu-arg sequence adjacent and N terminal to the CXC motif.

The human IL8 gene (SCYB8) has a length of 5.1 kb and contains four exons. It maps to human chromosome 4q12-q21. The mRNA consists of a 101 base 5' untranslated region, an open reading frame of 297 bases, and a long 3' untranslated region of 1.2 kb. The 5' flanking region of the IL8 gene contains the usual CAT and TATA boxlike structures and in addition, contains a number of potential binding sites for several nuclear factors including activation factor-1, activation factor-2, IFN regulatory factor-1, hepatocyte nuclear factor-1, a glucocorticoid responsive element, and a heat shock element. Transcription factors in these families bind the IL-8 promoter as dimers, and several distinct subunit combinations have been identified as important for IL-8 transcription. In addition, these factors can act in concert to synergistically activate the IL-8 promoter. IL8 transcription appears to be activated by a promoter recruitment mechanism where inducible transcription factor binding to the IL-8 promoter is required for binding of constitutively active TATA box-binding proteins and formation of a stable preinitiation complex (Kenneth A Roebuck 1999).

IL8 is a non-glycosylated protein of 8 kDa (72 amino acids). It is produced by processing of a precursor protein of 99 amino acids. Processing of this precursor by specific proteases yields N-terminal variants of IL8. The IL8 protein contains four cysteine residues participating in disulphide bridges (Cys-7/Cys-34; Cys-9/Cys-50).

2. Aim and Design

As mentioned in the previous chapter, particles reach the lung alveoli (Figure 1-3) where they are recognised and phagocytosed by the alveolar macrophages (AM). At high exposure, AM clearance may get overwhelmed with recognition and picking up all the ultrafine particles in the alveolus. Although AM do phagocytose particles in their direct vicinity, the probability that AM ingest the particles more distant decreases rapidly with distance. Therefore, the particles are retained on the epithelium longer where they are subject to endocytosis by the epithelial cells and their transport through the epithelium (Kreyling 2003). It is well known that epithelial cells lining the alveolus play a key role in the initiation and progression of particle-induced inflammation and injury in lung (Vincent 1990; Oberdörster 1995; Churg 1996).

As already discussed, particles can generate ROS on their surfaces (direct) or upon interaction with cells (indirect), and both processes are considered important for ultrafine particles due to their large and active surface area. We assume that the interactions between particles and epithelial cells initiate a cascade of events that underlie the adverse effects associated with inhaled particles. We also assume that this cascade begins with the induction of cellular oxidative stress, e.g. as reflected by the changes in the cellular glutathione status (GSH and GSSG) and culminating in transcription of the inflammatory genes (Rahman and MacNee, 2000) including IL-8. Thus there is a clear sequence of events leading from oxidative stress and GSH depletion to the activation of NF-?B and, subsequently, the transcription of genes for chemokines such as IL-8, that attract neutrophils (PMN) and alveolar macrophages (AM) to the site of particle deposition to produce inflammation. The present study will examine the mechanisms of particle induced toxicity, oxidative stress and inflammatory effects in the lungs using in vitro systems. Specifically, we will examine the effects that directly impact on human alveolar type II epithelial cells, as represented by A549 cells. As mentioned before, epithelial cells are among the first cells in the respiratory tract to come into contact with inhaled particles and therefore we suggest that damage to these cells can serve as a direct and highly sensitive measure of adverse effects induced by inhaled nanoparticles.

2.1 Hypothesis and Study Questions

We hypothesise that the interaction between TiO_2 particles and epithelial cells can initiate a cascade of events that induces the adverse effects associated with the particle inhalation. First we suggest that the surface area of the particles drives the oxidative stress in epithelial cells leading to the oxidation of cellular glutathione and subsequent induction of IL-8.

This thesis is intended to understand the mechanisms involved in particle induced inflammatory responses in the airways of humans, based on the *in vitro* studies. More specifically this study anticipates answering the following questions:

What is the effect of particle surface on the particle induced inflammatory responses?

What is the role of particles surface chemistry in inducing inflammatory responses?

In order to answer these questions, the following set of subsequent experimental clusters is carried out:

- Characterisation of physio-chemical properties (surface area, surface chemistry, surface morphology and contamination with endotoxin) of TiO_2 particles which may play a role in their activity (BET, Methylation, FTIR, HRTEM, LAL assay).
- Investigation of the ROS generation by the particle surface area itself (acellular ESR) as well as due to the interaction of particles with the cells (cellular ESR).
- Investigation of the cytotoxicity of the different TiO_2 particles used in this thesis (LDH).
- Investigation of the role of particle size and surface properties on inducing intra-cellular oxidative stress (GSH depletion).
- Investigation of the role of particle surface area and surface chemistry in their uptake by A549 cells and their subcellular localisation (TEM).
- Investigation of the effect of dose, surface area and surface properties of TiO_2 particles to elicit activation of IL-8 mRNA and protein expression by airway epithelial cells (RT-PCR and ELISA).

2.2 Schematic presentation of the thesis

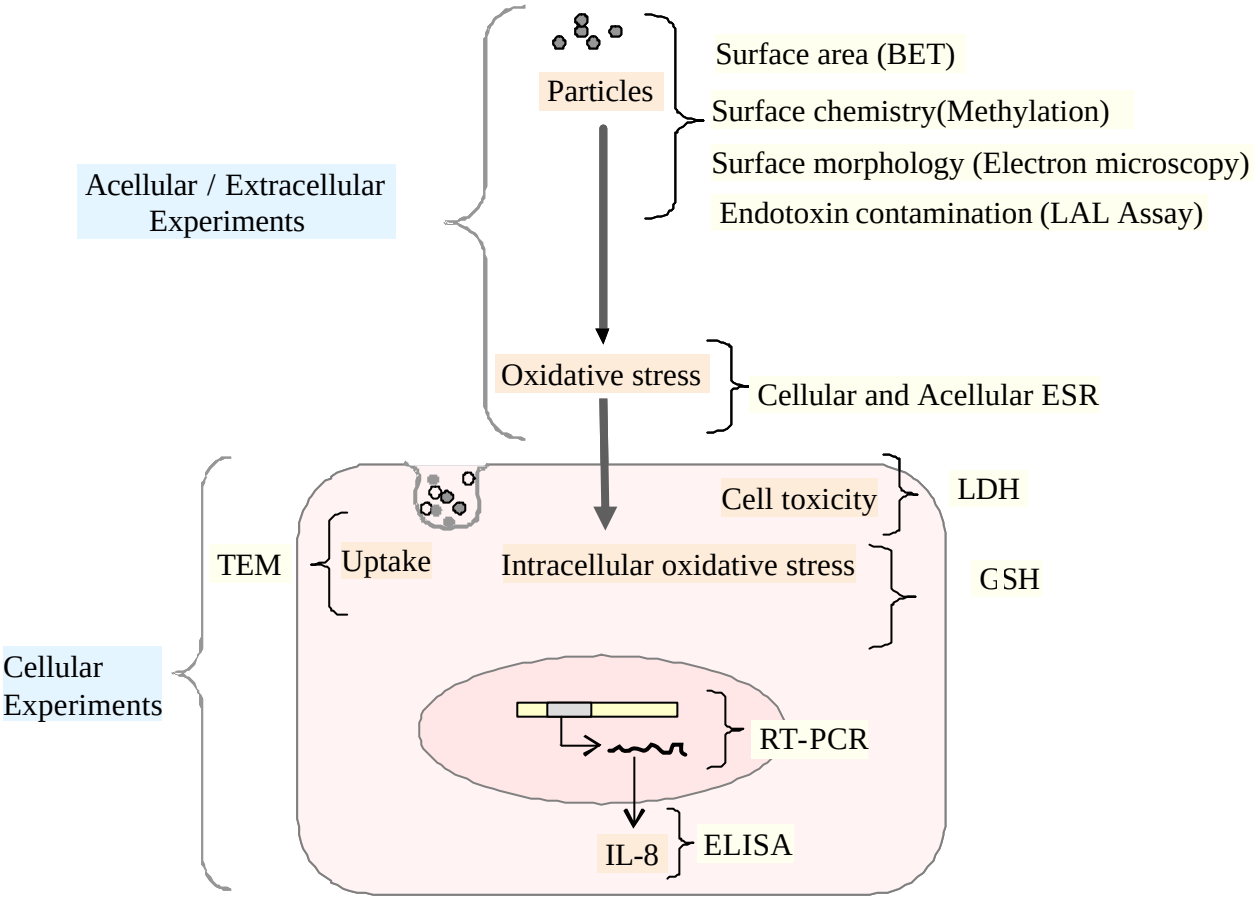

Figure 2-1 Diagram illustrating the study hypothesis, the strategy and accordingly applied methods

To study these mechanisms TiO_2 model particles of different size, surface area and surface chemistry were used. The purpose, principle and the description of these methods are elaborated in chapter 3, while chapter 4 reports the outcome of this experiment clusters and discuss the details of specific experiments. In chapter 5, general findings are discussed and put into perspective and overall conclusions are covered in chapter 6.

3. Materials and methods

3.1 Particles

Five types of microcrystalline TiO_2 powders (Table 3-1) were studied in this thesis, i.e. TiO_2 Merck (pure anatase phase, B.E.T. specific surface area 10 $m^2 \cdot g^{-1}$), TiO_2-SSA (B.E.T. 25 $m^2 \cdot g^{-1}$), TiO_2 Degussa-P25 (80 % anatase 20 % rutile, B.E.T. 50 $m^2 \cdot g^{-1}$), TiO_2-HSA Degussa (80 % anatase 20 % rutile, B.E.T. 82 $m^2 \cdot g^{-1}$) and TiO_2-SAC Sachtleben Chemie(pure anatase, B.E.T. 100 $m^2 \cdot g^{-1}$). DQ12 quartz (Batch 6, IUF), as well as Tumour Necrosis Factor alpha (TNF-α) were used as particulate and non-particulate positive controls respectively (Fiedler et al. 1998; Schins et al. 2002b) at the indicated concentrations. BET is a measure of specific surface area which is defined as the ratio A/m (m^2/g) between the absolute surface area of a solid and its mass. The surface area includes all parts of accessible inner surfaces (mainly pore wall surfaces). Precise measurement of the specific surface area of solids by gas adsorption (nitrogen, krypton) was performed according to Brunauer, Emmett and Teller (BET method). The entire surface area of a material is formed of external geometric contours of its constituents and the internal surface area as well. The latter is composed of the entirety of pore walls' surfaces and such of primary particles. These can only be determined if they are accessible for sorption of a gas. For this the low-temperature nitrogen adsorption according to BET method was applied, which has been proved as a standard procedure. The dependence of adsorbed gas quantity on equilibrium gas pressure p at the temperature of liquid nitrogen is analyzed. In a defined pressure range, one can represent adsorption isotherm as a straight line from whose slope and the axis section a monolayer capacity is calculated, where under the number of gram moles of gas is to be understood which is required to form a monomolecular layer on the surface area of 1g of substance.

Table 3-1 Specific surface area (m^2/g) of different TiO_2 used for this thesis measured by Brunauer-Emmett-Teller (BET) Method

The specific surface of a solid material (e.g. powder, dust) is the surface related to the mass which is freely accessible for gases. The BET method is based on the adsorption of nitrogen on a sample surface related to the mass that is freely accessible for gases (including the "inner" surface) at the temperature of liquid nitrogen.

 # Antase rutile ratio not known.

TiO_2	BET value in m^2/g	Anatase Rutile ratio
Fine	10	100% Anatase
SSA	25	#
P25	50	100:20
HSA	82	100:20
SAC	100	100% Anatase

3.2 Particle morphology HRTEM

Size and morphology of the TiO_2 microcrystals (T10 and T50) were detected by high resolution transmission electron microscopy (HRTEM) measurements carried out with a Jeol 2000 EX microscope equipped with polar piece and top entry stage. Before the introduction in the instrument, the samples were ultrasonically dispersed in isopropyl alcohol, and a drop of the suspension was deposited on a copper grid covered with a lacy carbon film. The samples were prepared and analysed in the Department of Inorganic Chemistry, Torino, Italy, by our collaborators Prof. Bice Fubini and Prof. Gianmario Martra

3.3 Modulation of the Particle surface by Methylation

As discussed in Chapter 1, the degree of hydrophilicity/hydrophobicity of the particle surface is an important property to evaluate since it determines the particle behaviour, uptake,

induction of cytokine expression, oxidative stress and toxicity in the cells. The body distribution of particles is strongly dependent on their surface characteristics. For example, coating poly (methyl methacrylate) nanoparticles with different types and concentrations of surfactants significantly changes their body distribution (Araujo et al. 1999).

In our studies, we chose the TiO_2 Merck and TiO_2 Degussa-P25 for methylation procedure. For methylation, the samples were prepared and analysed (by FTIR) in the Department of Inorganic Chemistry, Italy Torino, Italy, by our collaborators Prof. Bice Fubini and Prof. Gianmario Martra.

The purpose to methylate TiO_2 Merck (B.E.T. 10 $m^2 \cdot g^{-1}$), and TiO_2 Degussa-P25 (B.E.T. 50 $m^2 \cdot g^{-1}$), was to modify the particle surface i.e. making the particles more hydrophobic and compare them with their less hydrophobic counterparts. Both TiO_2 dusts were out gassed at room temperature for 1 h using a residual pressure of $1x\ 10^{-6}$ torr. After heating the samples to 200°C for 1 h, CH_3OH was admitted (150 torr) and the samples were kept in contact with methanol for 1 h and out gassed at 200°C (1 h). Using the above methylation procedure, a total of four different TiO_2 samples were obtained (i.e. the native forms of TiO_2 Merck (B.E.T. 10 $m^2 \cdot g^{-1}$), and TiO_2 Degussa-P25 (B.E.T. 50 $m^2 \cdot g^{-1}$), as well as the methylated forms.

The efficiency of methylation of the TiO_2 dusts was investigated by Fourier Transformed Infrared Spectroscopy (FTIR). Infrared spectroscopy is an important technique in organic chemistry and it is an easy way to identify the presence of certain functional groups in a molecule. Also, one can use the unique collection of absorption bands to confirm the identity of a pure compound or to detect the presence of specific impurities. Therefore, the TiO_2 powders were pressed in the form of self-supporting pellets (*ca.* 20 $mg \cdot cm^{-2}$) and were placed in a conventional IR quartz cell equipped with KBr windows, permanently connected to a vacuum line (residual pressure: 1.0×10^{-6} Torr; 1 Torr= 133.33 Pa) allowing all thermal adsorption-desorption experiments to be carried out *in situ.*

3.4 Evaluation of endotoxin contamination by Limulus Amebocyte Lysate (LAL) Assay

Endotoxins are part of the outer membrane of the cell wall of Gram-negative bacteria and are invariably associated with Gram-negative bacteria whether the organisms are pathogens or not. Although the term "endotoxin" is occasionally used to refer to any cell-associated bacterial toxin, it is properly reserved to refer to the lipopolysaccharide complex associated with the outer membrane of Gram-negative bacteria such as *E. coli, Salmonella, Shigella, Pseudomonas, Neisseria, Haemophilus,* and other leading pathogens. The biological activity of endotoxin is associated with the lipopolysaccharide (LPS). The use of Limulus Amebocyte Lysate (LAL) for the detection of endotoxin evolved from the observation by Bang in year 1956 that Gram-negative infection of *Limulus polyphemus* resulted in fatal intravascular coagulation. Later in 1964, Levin and Bang demonstrated that this clotting was a result of the action between endotoxin and a clottable protein in the circulating amebocytes of *Limulus* blood. Following the development of a suitable anti-coagulant for *Limulus* blood, Levin and Bang in 1968, prepared a lysate from washed amebocytes which was an extremely sensitive indicator of the presence of endotoxin. Solum (1970, 1973) and Young, Levin, and Prendergast (1972) have purified and characterised the clottable protein from LAL and have shown the reaction with endotoxin to be enzymatic. Gram-negative bacterial endotoxin catalyses the activation of a proenzyme in the Limulus Amebocyte Lysate (Young et al 1972). The initial rate of activation is determined by the concentration of endotoxin present. The activated enzyme (coagulase) hydrolyzes specific bonds within a clotting protein (coagulogen) also present in Limulus Amebocyte Lysate. Once hydrolysed, the resultant coagulin self-associates and forms a gelatinous clot.

Therefore, the four different TiO_2 samples used (Figure 3-1), were also analysed for possible endotoxin contamination, in order to avoid uncontrolled effects of this potent IL-8 stimulating agent during the particle handling which was necessary for the methylation procedure. The four different particle preparations, were suspended in endotoxin free water, and then subjected to a quantitative kinetic chromogenin Limulus Amoebocyte Lysate (LAL) method (Bio Witthaker), using *Escheria coli* 055:B5 endotoxin (Bio Witthaker) as standard.

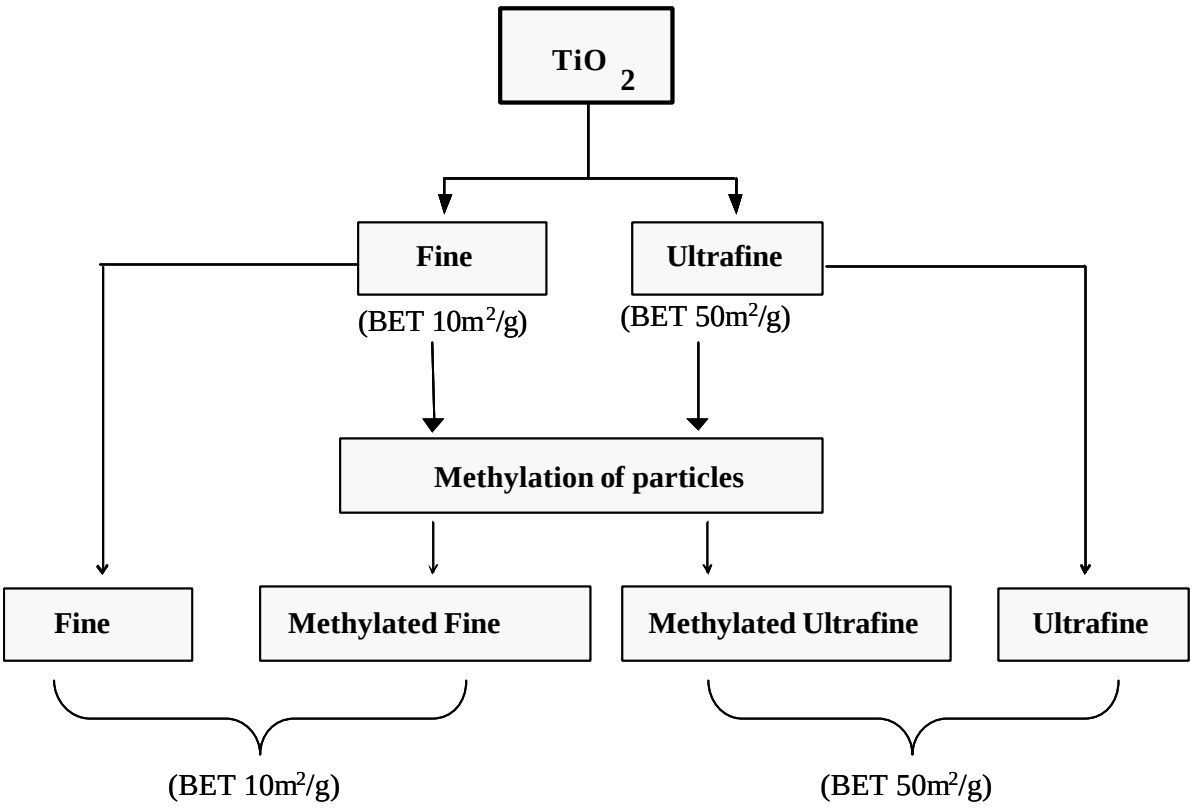

Figure 3-1 Model TiO_2 particles for investigating the role of size and surface chemistry.

3.5 Detection of reactive oxygen species

Since many free radicals occur at low concentration in biology and have very short half-lives due to their high reactivity with other reactive species and metabolites, one requires extremely sensitive measuring techniques. Superoxide anions and hydroxyl radicals are two reactive oxygen species that are usually present at very low concentrations in biological systems. Among the different reactive oxygen species, •OH is probably the most unstable with a half-life of 10^{-9} sec (Pryor, 1986). This compound is so reactive that it reacts with any reactive species in the neighbourhood of approximately 30 Å. H_2O_2 is more stable, but it is highly metabolised either enzymatically by catalase and GSH peroxidase, or non enzymatically by low concentrations of transitional metals such as iron. Thus, the lifetime of H_2O_2 in the cell is depending on the concentration of protective enzymes in the close environment. Meanwhile, due to its high diffusibility, H_2O_2 is able to cross the membranes. The lifetime of $O_2^{\bullet-}$ cannot

be calculated without a precise knowledge of local concentration of SOD and other substrates. However, the lifetime of $O_2^{\bullet -}$ in a cellular environment is expected to be very short.

A number of direct or indirect analytical methods have been proposed for the detection and/or the quantification of ROS generation and only the most common methodologies used to assess the ROS is shown in Table 3-2. In this thesis work, particle induced acellular and cellular generation of ROS was determined directly by Electron Spin Trapping and its consequences for cellular oxidative stress was investigated using glutathione depletion in A549 cells.

Table 3-2 Various methods available to detect ROS in vitro and in vivo

Assay	ROS detected	Advantages	Disadvantages
Chemiluminescene	Oxygen radicals	Quantitative	Specificity
Salicylate	•OH and $ONOO^-$	Quantitative	Limited to •OH and $ONOO^-$ detection only
Cytochrome C	ROS and RNS	Quantitative and Simple	Only *in vitro*, no information about the nature of ROS
DCDHF	ROS	Both intra and extra cellular ROS can be detected, Visualisation	Autocatalytic degradation , no information about the nature of ROS
Product analysis	ROS	Employs well- established analytical techniques	Does not provide unequivocal evidence
Total GSH depletion	ROS	Simple	Does not provide GSH:GSSG ratio
ESR	Free radicals	Used both *in vitro* and *in vivo*, Quantitative, structural information	Not possible to calibrate *in vivo*
Inhibition by SOD	Superoxide	Simple, Highly specific	Only applicable to superoxide
Inhibition by antioxidants	ROS	Simple	Little information about structure of radical

Electron spin (paramagnetic) resonance (ESR or EPR) is a spectroscopic technique that detects the unpaired electron present in a free radical. As such, it is the only approach (other than inhibition of superoxide dismutase) that can provide direct evidence for the presence of a free radical. In addition, the analysis of the ESR spectrum generally enables the determination of the identity of the free radical. Electron spin resonance or electron paramagnetic resonance (EPR) spectroscopy in combination with spin-trapping agents is a technique used extensively to detect and identify many short-lived free radical compounds. Spin trapping is a technique in which a short-lived, reactive free radical combines with a diamagnetic spin trap to form a more stable radical adducts which can be detected by electron spin resonance. Even if the free radical is diatomic such as the superoxide or hydroxyl radical and therefore undetectable in solution, its radical adduct will be polyatomic and, in principle, detectable. With this approach we determined the formation of reactive oxygen species by the different preparations of TiO_2 in acellular and cellular conditions.

3.6 ESR in acellular system

The formation of hydroxyl-radicals was investigated using the spin trapping agent DMPO (5, 5-dimethyl –1-pyrroline-N-oxide). DMPO (Figure 3-2 DMPO reaction) is a widely used nitrone spin trap for trapping oxygen-derived radicals due to the relative stability and diverse spectral characteristics of the spin adducts. It is more likely that that DMPO is more efficient at trapping extracellular radicals that might arise from, e.g. inflammatory cell activation or radicals produced at the cell membrane surface. Although intracellular trapping of radicals is also believed to occur with DMPO (Pietri et al 1992; Cova et al. 1992), this is more difficult to characterise in vivo. In additions, DMPO has low cytotoxicity, accessibility to the cell and high rate constant with •OH radicals (Figure 3-2). DMPO was purified prior to use by activated charcoal and the concentration of DMPO in aqueous solution was measured spectroscopically as described (Shi et al. 2003). Particles were suspended in double distilled deionized water (0.8 mg/ml) and sonicated for 5 minutes. Then, 450 µl of this particle suspension was mixed with 50 µl 1M DMPO (prepared in PBS). The mixture was incubated in dark at indicated time points at 37°C in shaking water bath. The suspension then vortexed and was transferred into a 50 µl glass capillary and measured with a Miniscope MS100 EPR spectrometer (Magnettech, Berlin, Germany). The ESR-spectra were recorded at room

temperature using the following instrumental conditions: Microwave frequency: 9.39 GHz, Magnetic field: 3360 G, sweep width: 100 G, scan time: 30 sec, number of scans: 3, modulation amplitude: 1.8 G, receiver gain: 1000. To verify that the obtained spectrum was specific to •OH, samples were also subjected to ESR with DMPO in the presence of ethanol (10 % of volume).

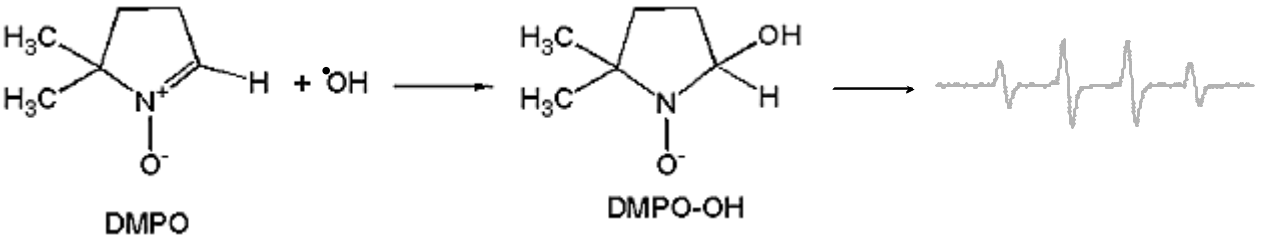

Figure 3-2 DMPO reaction

DMPO (5, 5-dimethyl –1-pyrroline-N-oxide) reaction with hydroxyl radical to give DMPO-OH adducts which can be detected as a typical 1:2:2:1 spectrum by ESR.

To determine the formation of superoxide ($O_2^{\cdot-}$) by the different particle preparations, we used 4-hydroxy-2, 2, 6, 6-tetramethylpiperidine-*N*-oxyl (TEMPOL) as the spin trapping agent. TEMPOL is a stable piperidine nitroxide that has a relatively low molecular weight and permeates biological membranes. This nitroxide compound has been reported to act as a genuine "SOD mimetic", producing anti-oxidative activity in various biological systems at molecular, cellular, and laboratory animal levels. It is oxidised by protonated superoxide to oxoammonium cation which in turn oxidise another superoxide to molecular oxygen (Figure 3-3). 450 µl of 0.8 mg/ml particle suspension were sonicated for 5 mins and incubated with 50 µl of 0.05 mM TEMPOL (prepared in PBS). The mixture was then incubated in dark at indicated time points at 37°C in shaking water bath. The suspension then vortexed and was transferred into a 50 µl glass capillary and measured with a Miniscope MS100 ESR spectrometer (Magnettech, Berlin, Germany) using these settings.

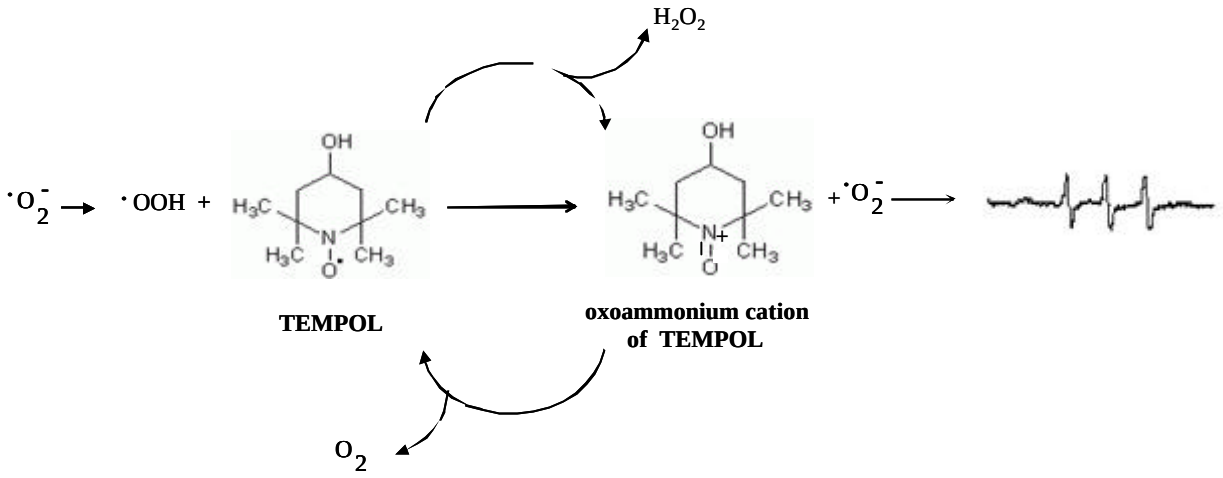

Figure 3-3 Tempol reaction with superoxide radical

Tempol reacts with superoxide radical and gives a three-line spectrum which can be detected by ESR. TEMPOL, one of the nitroxide spin probes, is considered to act as a SOD-mimic in the redox mechanism, that is, TEMPOL gets readily oxidised by protonated superoxide to oxoammonium cation, which in turn oxidise another superoxide to molecular oxygen. Studies have demonstarted that tempol is capable of dismuting two $O_2^{\cdot-}$ molecules by a direct reaction with $O_2^{\cdot-}$ or its $O_2^-\cdot OOH$ form. When its concentration is sufficiently high, tempol reacts with $\cdot OOH$, a form of $O_2^{\cdot-}$, to produce H_2O_2 and oxoammonium. Then this cation form of tempol directly reacts with $O_2^{\cdot-}$ to produce O_2 and regenerate tempol. In this reaction, tempol produces H_2O_2 with a rate constant of 107 M^{-1} s^{-1}. As a catalyst, however, tempol concentrations remain constant; therefore, it will more efficiently remove $O_2^{\cdot-}$ than will the stoichiometric scavengers.

3.7 Culturing and treatment of A549 cells

Since epithelial cells are the first cells in the respiratory tract to come in contact with the inhaled particles, we examined the effects of particles on human type II cells, represented by A549 cells. A549, a human lung cancer cell line, has been widely used in various kinds of studies. It was initiated in 1972 by D.J. Giard et al. through explant culture of lung carcinomatous tissue from a 58 year-old Caucasian male, and the culture in passage 68 was deposited by M. Lieber into American Type Culture Collection (ATCC) in 1976. These cells have a human karyotype and appear to have been derived from a single parent cell. All A549 cells at both early and late passage levels contain multilamellar cytoplasmic inclusion bodies

typical of those found in type II alveolar epithelial cells of the lung. At early and late passage levels, the cells synthesise lecithin with a high percentage of disaturated fatty acids utilising the cytidine diphosphocholine pathway; such a pattern of phospholipid synthesis is expected for cells believed to be responsible for pulmonary surfactant synthesis (Imanishi et al. 1989; Lieber et al. 1976; Honma et al. 1996).

The A549 cells (American Type Culture Collection/ATTC), were grown as described previously (Schins et al. 2000b) in Dulbecco's Modified Eagle's Medium (DMEM; Life Sciences), supplemented with 10 % heat inactivated fetal calf serum (FCS; Life Sciences), L-glutamine (Life Sciences), and 30 IU/ml penicillin-streptomycin (Life Sciences) at 37°C and 5 % CO_2. For experiments, cells were grown until near confluency (90-95%) in complete culture medium. Cells were then starved for 24 hours in serum free medium. Immediately before the start of the incubations, particle suspensions were prepared in DMEM/HBSS and sonicated for 5 minutes. The A549 cells were then rinsed with PBS and then treated with the particle suspensions at the indicated concentrations and incubation time intervals. DQ12 quartz (Batch 6, IUF), as well as Tumour Necrosis Factor alpha (TNFa) were used as well-known positive controls (Fiedler et al. 1998; Schins et al. 2002b) at the indicated concentrations.

3.8 Culturing and treatment of human primary cells

In some specific investigations we determined the ability of particles to elicit chemokine release from human primary epithelial cells. Therefore, human nasal polyps or turbinates were obtained from patients who underwent polypectomy or turbinectomy. They were washed and incubated with 2 mg/ml Pronase (Protease XIV, Sigma) in DMEM/F12 (Gibco) supplemented with 50 U penicillin and 50mg/ml streptomycin at 4°C for 16-20 hours under gentle rotary agitation (80 rpm). 10 % foetal calf serum (FCS) was then added in order to neutralise the enzyme. After washing, the cell suspension was filtered on a 30 µm diameter filter and centrifuged at 400 g for 5 minutes. The supernatant was eliminated and the cells were resuspended in DMEM/F12. Aggregates were discarded and dissociated cells were preplated for 2 h at 37°C on plastic dishes (Falcon Merck-Eurolab, Strasbourg, France) to eliminate most contaminating fibroblasts, and epithelial cells were counted. Cells were cultured on 6

well plates at 500 000 cells/well for 6 days. The particles were prepared in DPL and were sonicated for 4mins and the cells were treated with 10 µg/cm^2 particle dose. The supernatant were collected at 4 h and 24 h, centrifuged at maximum speed for 5 minutes and the cell free supernatant was frozen at –20°C and IL-8 and GM-CSF were measured later by ELISA. These experiments were performed in the lab of our collaborator Prof. Dr. F. Marano (Laboratorie de Cytophysiology Et de Toxicologie Cellulare Paris).

3.9 ESR in A549 cells

The generation of free radicals within the living cell is of increasing interest. However, only indirect detection methods are widely applied such as the use of fluorescent dyes or measuring cytochrome c-oxidase activity. Since electron spin resonance spectroscopy is the only method to study directly and specifically radicals, it provides a promising and valuable complementary method to detect ROS generation in cell culture.

Briefly, the A549 cells were grown as mentioned before, starved for 24 hours, and were then rinsed with PBS and the treated with particle suspensions at the indicated concentrations prepared in HBSS. The cells were incubated for 1h and then either 50µl of 0.05 mM TEMPOL or 1M DMPO was added. The supernatant was taken out at indicated time points and transferred to a 50 µl glass capillary for analyses by ESR spectrometer. The ESR-spectra were recorded at room temperature using the following instrumental conditions: Magnetic field: 3360 G, sweep width: 100 G, scan time: 30 sec, number of scans: 3, modulation amplitude: 1.8 G, receiver gain: 1000.

3.10 LDH-assay

To select the particle concentrations that were non-toxic for the assays to assess pro-inflammatory marker (IL-8) and oxidative stress (GSH depletion), toxicity was evaluated using the LDH assay in A549 cells following treatment with a range of particle concentrations. Lactate dehydrogenase (LDH) is a cytosolic enzyme present within all mammalian cells, which is based on the measurement of cytoplasmatic enzyme activity

released by damaged plasma membranes. Lactate dehydrogenase, also called lactic dehydrogenase, is an enzyme found in the cells of many body tissues, including the heart, liver, kidneys, skeletal muscle, brain, red blood cells, and lungs. LDH is responsible for converting lactic acid into pyruvic acid, an essential step in producing cellular energy and is rapidly released into the cell culture supernatant upon damage of the plasma membrane. The normal plasma membrane is impermeable to LDH, but damage to the cell membrane results in a change in the membrane permeability and subsequent leakage of LDH into the extracellular fluid (Rae, 1977). *In vitro* release of LDH from cells provides an accurate measure of cell membrane integrity and cell viability. As a result, the release of lactate dehydrogenase has proved to be a popular and reliable test for cytotoxicity.

The LDH-assay was measured as follows: Following treatment of the cells, supernatants were collected and immediately centrifuged (5 minutes, 15000 rpm) to get rid of cells and particles. The resulting supernatants were stored at 4°C for a maximum of 24 hrs. LDH was analysed is an assay coupled to the reaction of residual pyruvate with 2, 4- dinitrophenylhydrazine, as described previously (Schins et al. 2002). Briefly, 20 µl of supernatant was added to 50µl of pre-warmed NADH (1 mg/ml) in 0.75 mM sodium pyruvate solution. After thorough mixing, the samples were incubated for 30 minutes at 37°C. The reagent 2, 4-dinitrophenyl-hydrazine (50 µl, 0.2 mg/ml 1 N HCl) was added to each sample and incubated for further 20 min at room temperature. NaOH (50 µl, 4N) was added for 5 minutes before reading the absorbance of the samples by a microplate reader at 540 nm.

Cytotoxicity was expressed as the percentage of LDH activity in the medium as a percentage of the control incubations. Lactate dehydrogenase (LDH) activity in the supernatant provides a means of determining cell membrane damage induced by particles as indicated by leakage of the enzyme, LDH, out of the cells into the medium.

3.11 Measurement of total Glutathione Depletion

Glutathione (GSH), the major intracellular non protein thiol, is mainly known as an important protector against free radical damage by providing reducing equivalents for several key antioxidant enzymes and also by scavenging hydroxyl radicals and singlet oxygen. Depletion of GSH has been used as indicator of oxidative stress following particle exposure (MacNee et

al. 1997; Li et al. 1997). According to the method described previously (Schins et al. 2004), the total glutathione depletion from the A549 cells was determined as an indicator of oxidative stress.

Total glutathione (GSH + GSSG) was measured by the kinetic, microtitre plate method of Baker et al. (1990), with some modifications: Briefly, after the removal of the medium, cells were rinsed with PBS twice. Then 1ml ice-cold extraction buffer (100 mM phosphate buffer (pH 7.5) containing 1 mM EDTA, 0.1 % Triton and 0.6 % sulfosalicylic acid) was added into each well, and cells were homogenised using a pipette. The cell suspensions were centrifuged at 5000 rpm for 5 min at 4 °C, and 20 µl of supernatant was then used for the determination of total glutathione, as follows: 120 µl of enzyme reaction mix containing 300 mM β -NADH, 225 mM DTNB, and 20 units glutathione reductase in phosphate buffer was added immediately before analysis of the kinetic absorbance development at 412 nm over 3 min. The total glutathione concentration in the samples was then determined by regression analysis using the values obtained from the standard curve. GSH was expressed as µM per mg of protein, as determined using the BCA protein assay kit (Sigma).

3.12 Determination of particle uptake in A549 cells by electron microscopy

The Transmission Electron Microscope (TEM) was the first type of Electron Microscope to be developed and is patterned exactly on the Light Transmission Microscope except that a focused beam of electrons is used instead of light to "see through" the specimen. It was developed by Max Knoll and Ernst Ruska in Germany in 1931.

Currently, there is major interest in the uptake of nanosized particles by mammalian cells, since this is believed to be of major impact for the toxicological properties of these materials (Oberdörster et al. 2005). Among the characteristics which are considered to be of major importance for uptake of particles by cells is their size as well as their surface properties. Here we have characterised the ability of A549 cell to internalise fine and ultrafine TiO_2 and their methylated counterparts. Therefore, monolayer cultures were grown in 35 mm culture dishes until 90 % confluence, and then starved in serum free medium for 24 h. The cells were then

washed twice with PBS and treated with 16 or 80 µg/cm^2 native or methylated UF-TiO_2 for 4h in complete medium. At the end of the incubation the monolayers were rapidly washed three times with serum free medium and fixed with 2 % glutaraldehyde in 0.1 M sodium cacodylate buffer for 1 h at 4°C. After post fixation in 2 % OsO_4 in 0.1 M sodium cacodylate buffer for 1 h at 4°C the samples were en bloc stained with 1.5 % uranylacetate dihydrate and phosphotungstic acid, dehydrated in ethanol series and embedded in epoxy resin, Epon (Serva, Heidelberg, Germany) (Luft 1961). Morphologic characteristics of the cells and the distribution of particles within the cells were investigated using ultra thin sections (50 nm) placed on 150 mesh grids and examined by transmission electron microscopy (STEM CM12, Philips) in combination with a digital imaging system (SIS, Münster, Germany) and an elemental dispersive X-ray analyser for chemical analysis.

3.13 Total RNA extraction

Total RNA was extracted from the treated cells with TRIZOL reagent (Invitrogen) using the following protocol. The cells were seeded in 60 mm culture dishes. Subsequent to the particle treatment, the culture medium was removed and the cells washed twice with cold calcium/magnesium free phosphate buffered saline (Sigma). 600 µl of Cold TRIzol (Invitrogen) was added to each well; the cells were scraped and were transferred into sterile tubes and kept at RT for 5 mins. 200 µl of chloroform was added to each tube, vortexed and incubated on RT for 5-10 min. The samples were then centrifuged at 12,000 g for 15 min at 4°C. The top colourless aqueous phase from each tube was transferred to a new tube and 450 µl of isopropanol was added and samples were kept at RT for 5-10 min, vortexed briefly and again centrifuged at 12,000 g for 10 min. The supernatants were then discarded and pellets washed in 1 ml of cold 75 % ethanol, vortexed and centrifuged for 5 min at 7500 g at 4 °C. The supernatants were discarded and pellets dissolved in 50 µl of diethylpyrocarbonate (DEPC) treated water. Samples were stored at -70°C until used. For spectrophotometrical analysis, the RNA sample was diluted 250 fold, so the calculation included a dilution factor (D=250) and RNA concentration was determined spectrophotomically (Beckman DU 640 Spectrophotometer) by reading the optical densities at 260 and 280 nm. RNA yield was quantitated by analysis using the standard, 1 absorbance unit at 260 nm equals to 40 µg RNA

per ml and purity checked by the 260 and 280 nm absorbance readings. RNA samples having an absorbance 260/280 ratio of > 1.6 were used.

3.14 Reverse transcriptase-polymerase chain reaction

Numerous techniques have been developed to measure gene expression in cells and tissues which include Northern blots, coupled reverse transcription and PCR amplification (RT-PCR), RNase protection assays, *in situ* hybridisation, dot blots and S1 nuclease assay. Out of these methods, RT-PCR is the most sensitive and versatile technique and can be used to determine the presence and absence of a transcript, to estimate expression levels and to clone cDNA products without the necessity of constructing and screening a cDNA library.

In our experiments, human IL-8 messenger RNA (mRNA) expression was detected by reverse transcriptase-polymerase chain reaction (RT-PCR), as described previously (Schins et al. 2000 TAP). The Promega Access RT-PCR System kit was used for the amplification of human IL-8 mRNA and the human housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Sequences of the primers were respectively *5'- CGA TGT CAG TGC ATA AAG ACA -3'* (sense) and *5'- TGA ATT CTC AGC CCT CTT CAA AAA -3'* (anti-sense) for IL-8, and *5'- CCA CCC ATG GCA AAT TCC ATG GCA -3'* (sense), and *5'- TCT AGA CGG CAG GTC AGG TCC ACC -3'* (anti-sense) for GAPDH. All primers were synthesised by Gibco Life Sciences and diluted to 100*p*mol/µl with DEPC water. RT-PCR was performed according to manufacturer's instructions. Briefly, each reaction mixture contained 0.3µg sample RNA, AMV reverse transcriptase (5 units), *Tfl* DNA polymerase (5 units), 1x AMV/*Tfl* reaction buffer, dNTP mix (0.2 mM), $MgSO_4$ (1 mM) and 1 µM each of the appropriate sense and anti-sense primers to give a final volume of 50µl. RT-PCR of IL-8 and GAPDH mRNA was carried out in separate tubes. RNA was reverse-transcribed for 45 minutes at 48°C and resulting cDNA was denatured for 2 minutes at 94°C followed by a 25-cycle, three-step amplification stage (94°C for 30 seconds, 60°C for 1 minute and 68°C for 2 minutes) and a final extension step (68°C for 7 minutes). Linearity of RT-PCR was assessed for the amplification of cDNA's and was found to be optimal for GAPDH at 25 cycles and for IL-8 cDNA at 30 cycles (results not shown). Negative controls containing water as a substitute for either RNA or the primer pairs were also included.

3.15 Agarose gel electrophoresis of PCR product

Gel electrophoresis is the technique by which mixture of charged macromolecules, especially nucleic acids and proteins are separated in an electrical field according to their mobility which is directly proportional to macromolecule's charge to mass ratio.

The expected product sizes for IL-8 and GAPDH were 200 bp and 600 bp, respectively. An aliquot (12 µl) of the PCR product was run on 1.2 % agarose gel that contained ethedium bromide. For preparing 1.2 % agarose gel, 1.25 gm of agarose was added in 100 ml of 1x TBE Buffer and dissolved by heating in microwave. After cooling it to about 50°C, ethedium bromide was added, stirred and poured into a horizontal gel chamber. 10 µl of loading buffer was added to 50 µl PCR product, centrifuged briefly at maximum speed and 12 µl sample was loaded into the gel. The gel was run at 40 V at RT for about 2-3 hrs. The PCR products were visualised and photographed under UV light and the bands were quantified by computed assisted densitometric scanning (GS-700 Imaging Densitimeter; Bio-Rad Laboratories). IL-8 mRNA was analysed relative to the intensity of corresponding housekeeping gene GAPDH cDNA and subsequently expressed as a percentage of the relative expression as calculated for the control incubations.

3.16 Interleukin-8 ELISA

Interleukin-8 (IL-8) is an important biomarker which has been shown to be increased in sputum and bronchial lavage fluid of patients with chronic bronchitis, asthma and plays an important role in the development of clinical respiratory diseases (Keatings et al. 1996; Borm and Schins 2001; Barnes 2001). Although alveolar macrophages (AM) are known to be major producers of IL-8, mesothelial cells and alveolar epithelial cells (A549 cells) have also been shown to synthesise IL-8 in response to various stimuli (Standiford et al. 1990), including asbestos fibers (Boylan et al. 1992; Rosenthal et al. 1994).

Briefly, cell supernatants were collected after incubation with particle preparations, and immediately microfuged (5 min, 15000 rpm) to get rid of cells and particles. The resulting supernatants were stored at –20°C until measurement of IL-8 concentrations with an enzyme-linked immunosorbent assay (ELISA) from PeliKine compact ^{TM} human IL-8 ELISA kit. The PeliKine compact ^{TM} human IL-8 ELISA kit is a sandwich-type of enzyme immunoassay in which a monoclonal anti-hulL antibody is bound on the polystyrene microtiter wells. Human IL-8 present in a measured volume of sample or standard is captured by the antibody on the microtiter plate, and non-bound material is removed by washing. Subsequently, a biotinylated sheep antibody to huIL-8 is added. This antibody binds to the huIL-8 antibody complex present in the microtiter well. Excess biotinylated antibody is removed by washing, followed by addition of horseradish peroxidase (HRP) conjugated streptavidin, which binds onto the biotinylated side of the huIL-8 sandwich. After removal of non-bound HRP conjugate by washing, a substrate solution is added to the wells. A coloured product is formed in proportion to the amount of huIL-8 present in the sample or standard. After the reaction has been terminated by the addition of a stop solution, absorbance is measured in a microtiter plate reader at 450 nm. From the absorbance of samples and those of a standard curve, the concentration of huIL-8 is determined by interpolation with the standard curve.

3.17 Statistical Analysis

Data are expressed as mean ± SEM unless stated otherwise. Treatment-related differences were evaluated using one-way analysis of variances (ANOVA), with Dunnet post hoc testing. A difference was considered significant at $P<0.01$ and $P<0.05$ as indicated. Pearson's correlation procedure was used to investigate the relation between particle BET surface area and the various biological readouts.

4. Results and Discussion

4.1 High resolution Transmission electron micrographs

The HRTEM images (Figure 4-1) of Fine (T10) and Ultrafine (T50) titanium dioxides reveal that the samples are polydispersed in nature. The particles are observed as clusters or aggregates of smaller singlet particles for both fine and ultrafine particles. In the figure, the obvious size difference fine and ultrafine particles can be clearly seen and also the small pores on the particle surface are clearly visible. It is not only the outer surface but also the internal accessible pore on the particles on the surface adds to the surface the total area which is measured by the BET method. The surface area (measured by BET method) of ultrafine particles is five times higher than the fines when tested at equal mass (See *Table 3-1*).

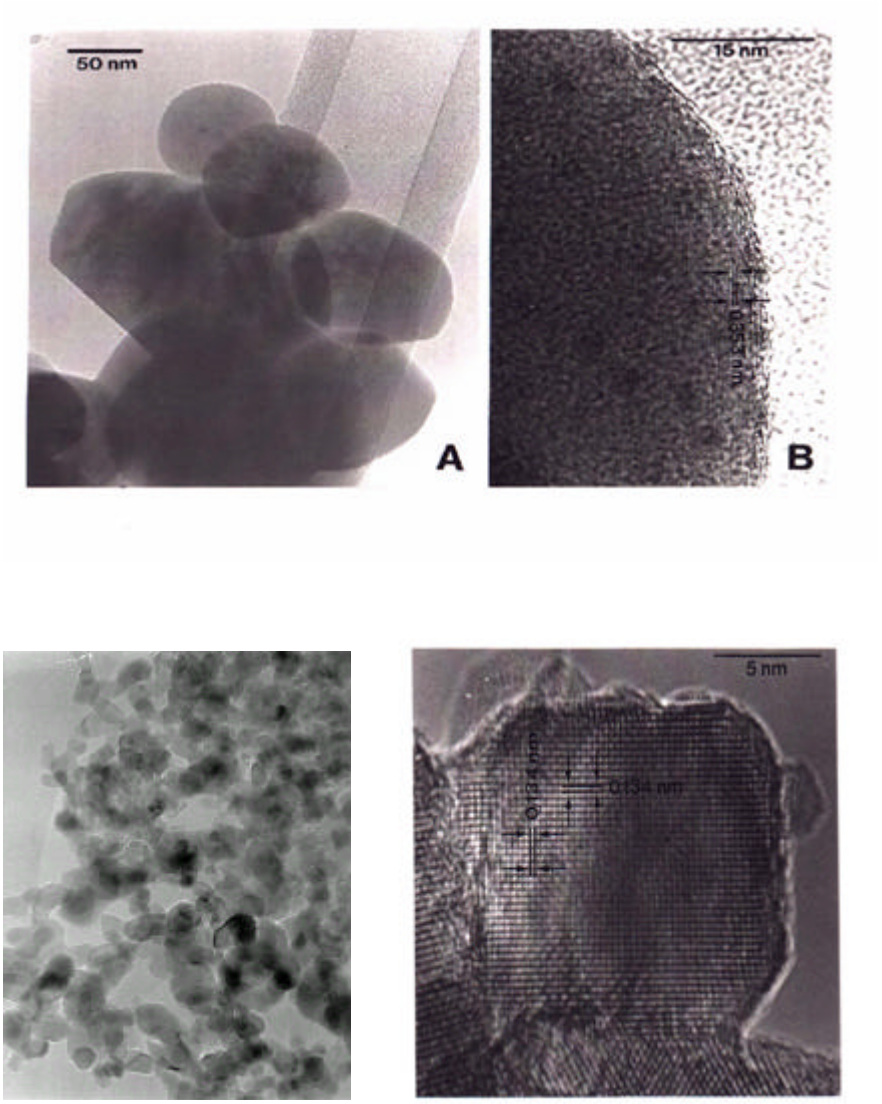

Figure 4-1 HRTEM images of Fine and Ultrafine TiO_2 used in this study

Fine TiO_2 Upper graph and Ultrafine TiO_2 Lower graph, at lower and higher magnifications.

4.2 Analysis of the methylation efficiency

The efficiency of the methylation procedure of the particles was determined by Fourier transform infrared (FTIR) spectroscopy. The representative IR spectrum of TiO_2 in Figure 4-2 depicts the absorbance bands which are attributed to the presence of individual chemical groups in the particles before and after methylation. Spectrum (a) shows TiO_2 outgassed at room temperature at 45 minutes. In this condition a full monolayer of hydroxyl groups, both vibrationally "free" (i.e. not interacting through hydrogen bonding with neighbour OH and hydrogen bonded, and water molecules coordinated to surface Ti^{4+} cations coordinatively unsaturated are observed. Spectrum (b) shows TiO_2 after treatment at 250°C in CH_3OH (40 torr) for 45 minutes and subsequent outgassing at the room temperature. It can be noticed that the bands due to water molecules disappeared because of water desorption. The same occurred for the signals of hydroxyl groups which are substituted by $–CH_3$ groups. Spectrum (c) shows TiO_2 after readmission of water at room temperature on the methylated sample. The bands due to absorbed water molecules reappeared, whilst this does not occur for the bands due to hydroxyl groups. This indicates $–CH_3$ are not removed from the surface and they should replace surface of the hydroxyl groups even when the methylated particles are dispersed in water.

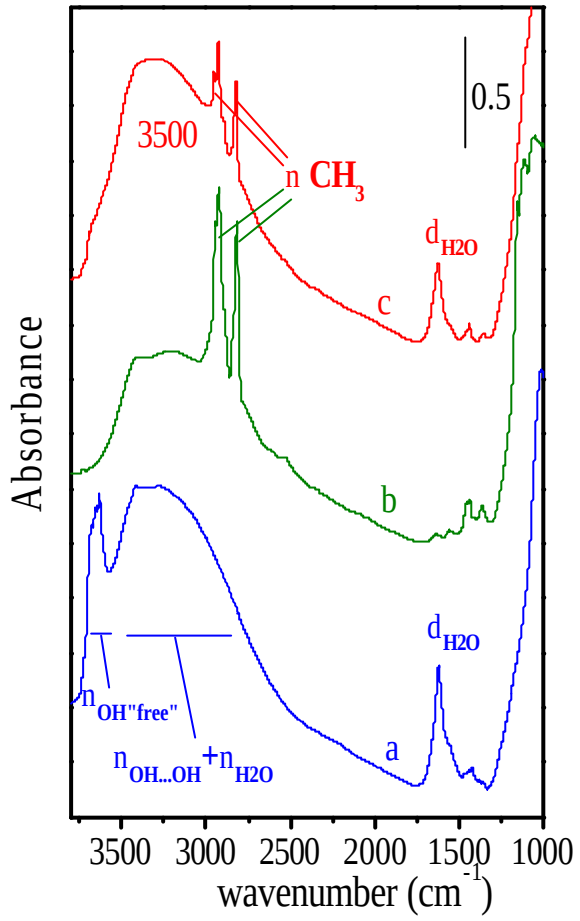

Figure 4-2 Infrared spectra of the native and methylated TiO_2 dusts

This FTIR spectrum shows absorbance bands which are attributed to the presence of distinctive CH_3 (a methyl-group is a hydrophobic Alkyl functional group) and H_2O groups on the particles. UF-TiO_2 (a) After treatment at 250°C in CH_3OH(40 torr) for 45 minutes and subsequent outgassing (b) and after readmission of H_2O at room temperature (c). Approximately 50 % of the surfaces OH-groups of the particles were substituted by OCH_3 groups after being dissolved in water.

4.3 Endotoxin contamination of the TiO_2 particles (LAL Assay)

In order to evaluate whether the sampling handling would lead to contamination of the samples, we determined endotoxin levels of the different TiO_2 samples by using LAL Assay. Endotoxin determinations of the different dusts suspended in endotoxin free water showed values of 0.006, 0.007, 0.005, and 0.009 EU/mg dust, respectively for F-TiO_2, UF-TiO_2, MF-TiO_2, and MUF-TiO_2. These data showed that the endotoxin contamination was negligible for all samples used (i.e. <0.003 EU/ml during cell treatment), and would not affect the outcome of cell experiments.

PM- mediated lung injury is suggested due to the bacterial derived endotoxin bound to the surface of the particles (Becker et al. 1996, 2003; Dong et al. 1996; Long et al. 2001). Endotoxins are well recognised to cause airway inflammation (O'Grady et al. 2001) and have been shown to act as priming agent for particle induced inflammation (Oberdörster et al. 2000). It is also known that LPS can induce IL-8 in A549 cells (Standiford et al. 1990; Hansen et al. 1997).

Therefore, we did a dose dependent LPS treatment of A549 cells to look at the IL-8 response. As can be seen in the following graph, LPS induces IL-8 in A549 cells but only at much higher concentrations than those detected on our test particles.

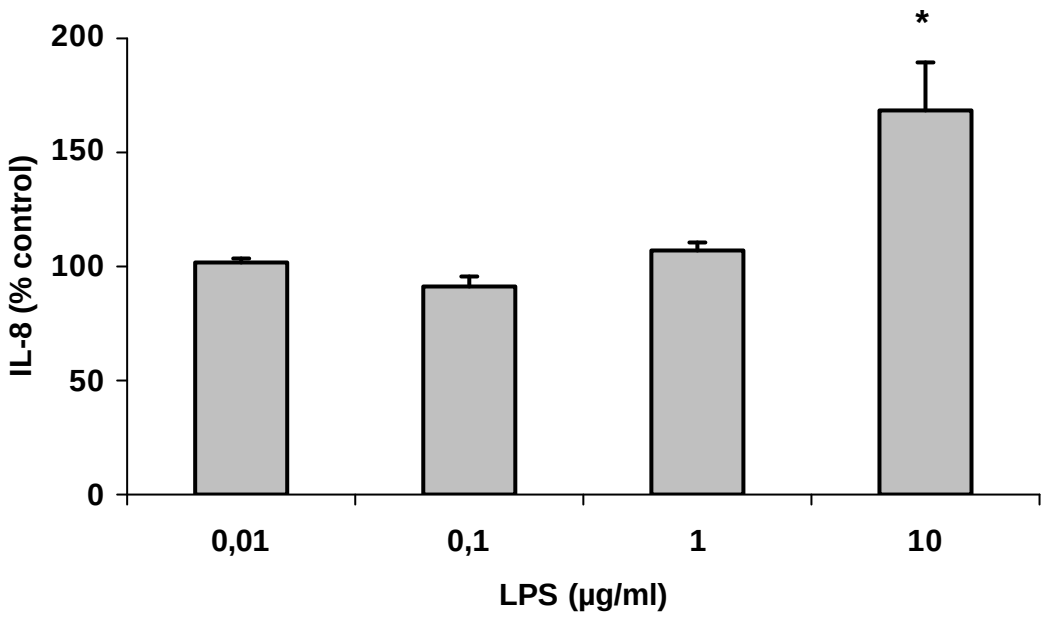

Figure 4-3 IL-8 release in A549 cells after treating with different doses of LPS

Measurement of IL-8 in A549 cells after treatment with different concentrations of LPS. The results are expressed as IL-8 release as a percent of control untreated cells. Cells treated with 10 µg/ml LPS showed a significant increase in IL-8 release (P-value<0.05).*

4.4 ROS formation by the different particle preparations in acellular system

The formation of hydroxyl-radicals and superoxide-radicals by the different TiO_2 samples was measured by ESR with the spin trapping agent DMPO and TEMPOL respectively. A characteristic spectrum as observed with different TiO_2 is shown in Figure 4-4 and Figure 4-5.

In case of DMPO, the DMPO-OH adducts can arise from either trapping the hydroxyl radical or the decomposition of DMPO-OOH, e.g. as resulting from superoxide anion. To confirm that the obtained 1:2:2:1 quartet pattern (Figure 4-4 A) was specific to ·OH, ESR was also performed in the presence of the ·OH scavenger ethanol (10 % v/v). As can be seen in the Figure 4-4 B, addition of ethanol yielded a distinctive six–line ESR spectrum, due to the involvement of the DMPO-C_2H_4OH radical adduct. The comparative evaluation at equal mass

the four different TiO_2 samples are shown in (Upper bar graph). As can be seen in the graph, all samples caused considerable formation of •OH, which however, was not different for the different particle preparations on equal mass basis.that is despite their contrasting surface areas. Without particle suspension (negative control), no signal was observed.

In order to determine the ability of the different samples to elicit formation of superoxide (O_2•-), we used TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl) as a spin trapping agent which is a free radical scavenger and superoxide dismutase mimic. The decay of the TEMPOL signal intensity as compared to the control was considered to reflect the production of superoxide by the particle preparations. As mentioned in chapter 3, in case of TEMPOL, radical induced by the particles will neutralise the spin label and thus decrease its ESR signal by pairing electrons. TEMPOL was added to particle suspensions (0.8 mg/ml), mixed by vortexing and incubated in dark at 37°C for in a shaking water bath. The incubation of TEMPOL to the particle preparation did not lead to the reduction in the intensity of the triplet ESR signal (Figure 4-5 A) which indicates the absence of significant O_2•$^-$ generation by the particles. Quantification of the signals was carried out as the average of the sum of the amplitudes of the ESR signal, and outcomes are expressed in arbitrary units (Figure 4-6).is quantification and the average of three independent experiments. Interestingly, none of the particle preparations showed a change in the ESR signal.

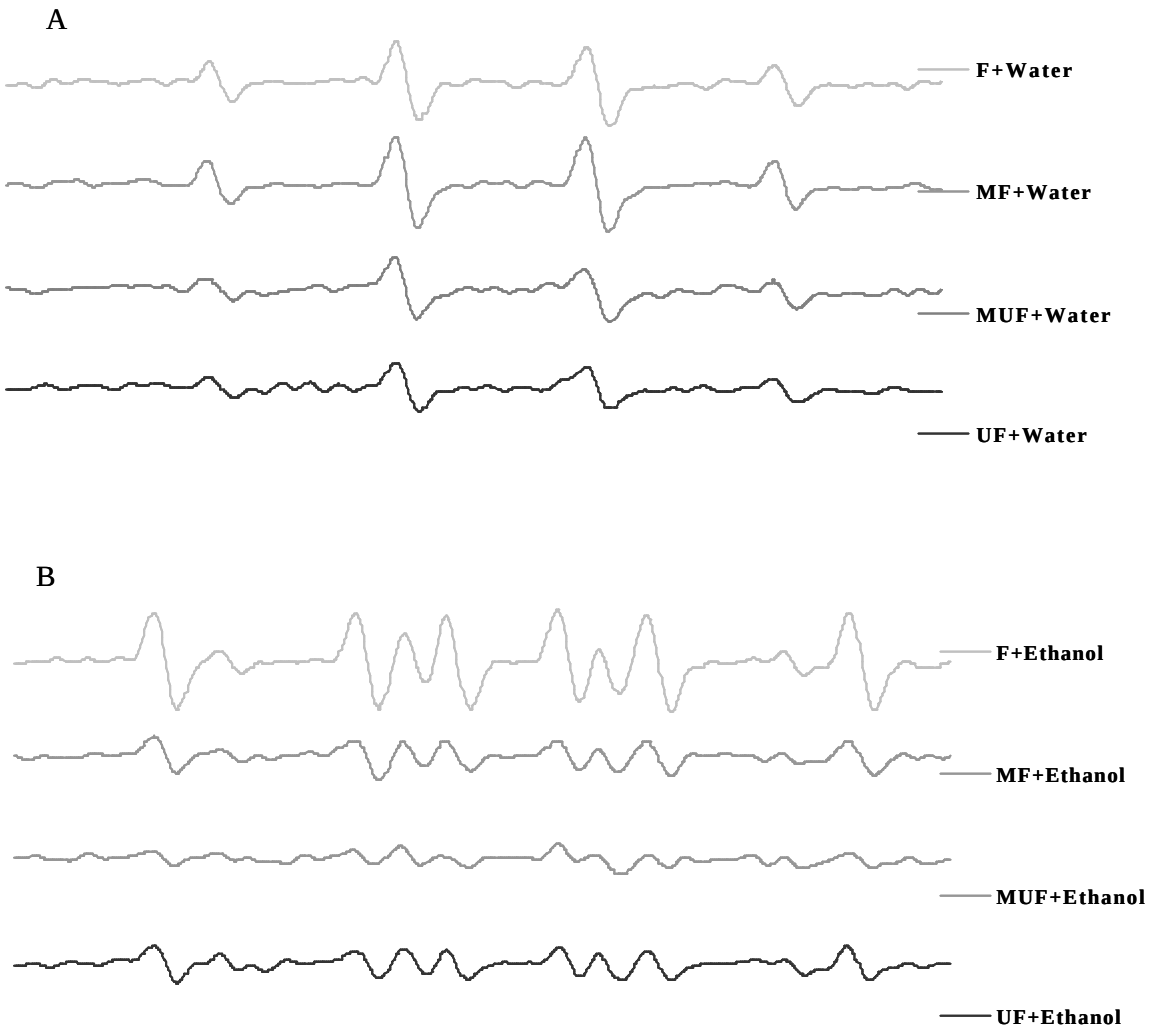

Figure 4-4 ESR spectra of DMPO adduct trapping ·OH radical

Graph A represents ESR spectra of DMPO adduct trapping ·OH radical generated by different preparations of TiO_2 in acellular system and Graph B is ESR spectra of DMPO spin trapping adducts of methylated and nonmethylated fine and ultrafine TiO_2 particle suspensions in presence and absence of ethanol (10 % of volume). The spectrum shows the ESR spectra of DMPO spin trapping adducts of different preparations of Titanium dioxide 0.8 mg/ml of particles suspension was mixed with 50 µl of 1M DMPO and was incubated in dark for 4 hrs at room temp. The spectrum was recorded at room temperature. The resulting spectra in Figure A show the characteristic 1:2:2:1 DMPO-OH quartet. Figure B show the change in spectrum of ethanol on radical generation. The splitting caused by the DMPO-C_2H_4OH adducts in panel B confirms the hydroxyl radical formation by exhibiting a distinctive six-line ESR spectrum by the particles.

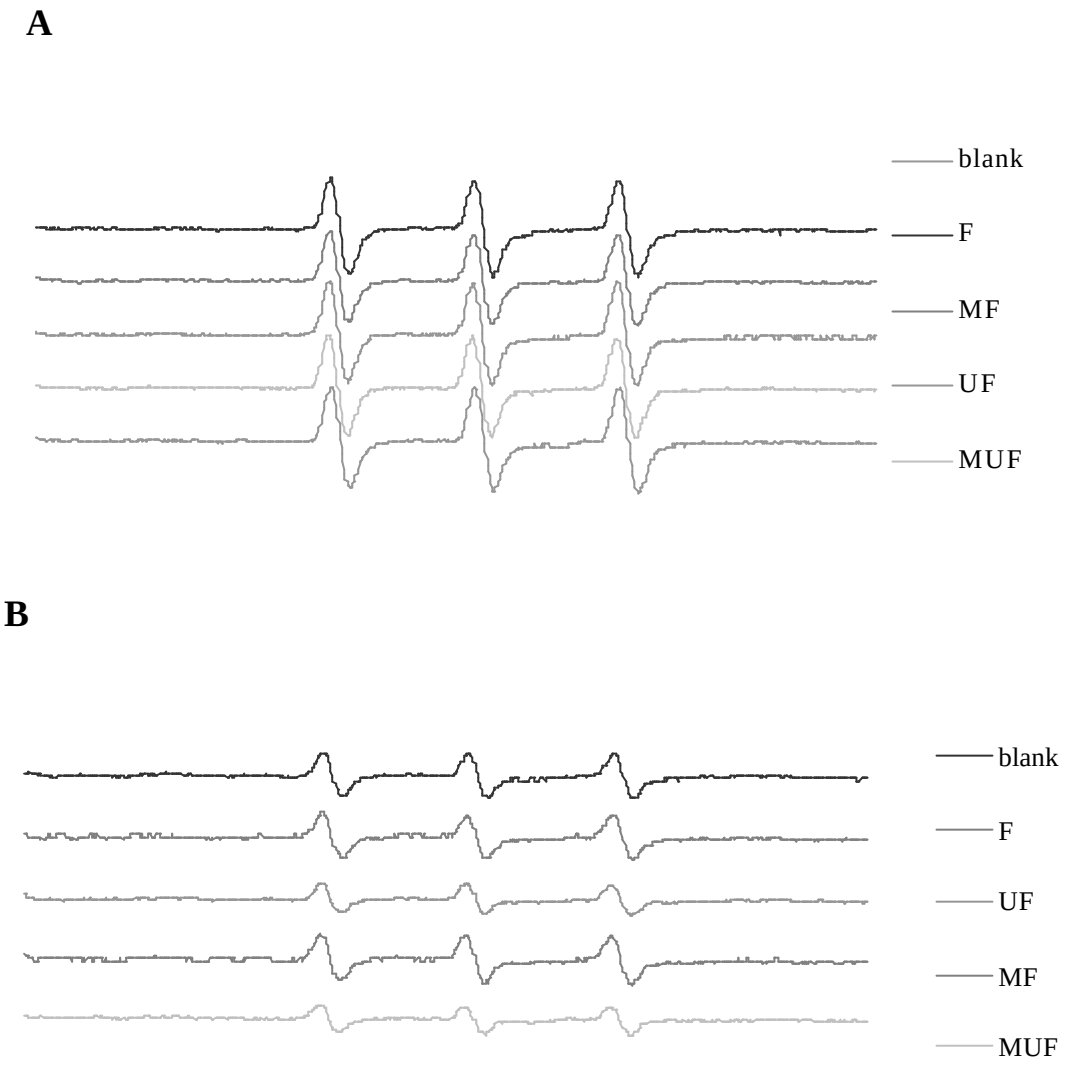

Figure 4-5 ESR spectra of TEMPOL adduct trapping $O_2^{\cdot-}$ radical generated by different preparations of TiO_2

ESR spectra of TEMPOL adduct trapping $O_2^{\cdot-}$ radical generated by different preparations of TiO_2 in acellular (Graph A) and cellular (Graph B) system. The spectrum shows the three line ESR spectra of TEMPOL spin trapping adducts of different preparations of TiO_2. 0.8 mg/ml of particles suspension was mixed with 50µl of 0.05 mM TEMPOL and was incubated in dark for 4 hrs at 37°C in shaking water bath. The spectrum was recorded at room temperature.

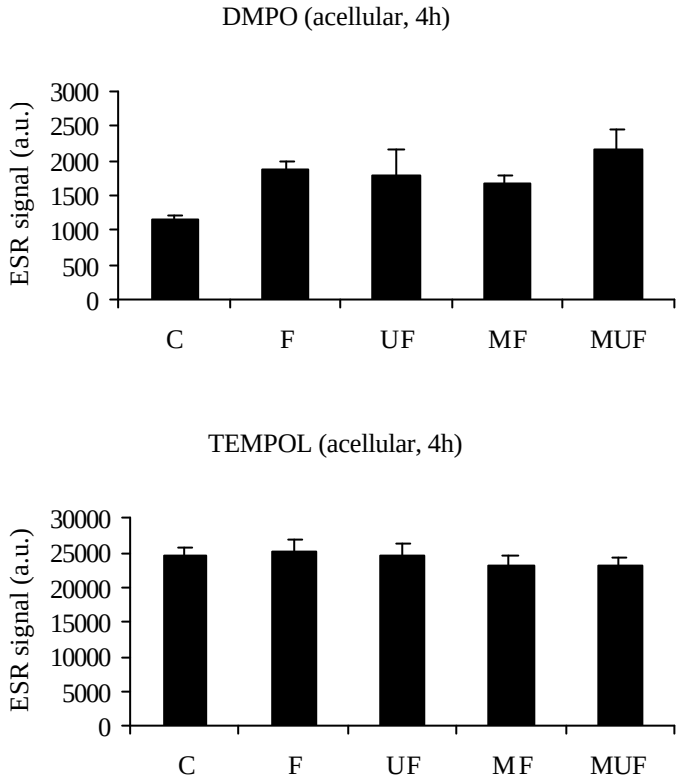

Figure 4-6 Acellular Hydroxyl and Superoxide radical generation by particles

(Upper graph) and superoxide (lower graph) radical generation by different TiO_2 particle preparation (0.8 mg/ml) at 4 h in cell free system. The incubation of the samples was performed in dark at 37°C in shaking water bath and the spectrum was recorded at room temperature. Data are expressed as mean and SD of the resulting DMPO and TEMPOL signal in arbitrary units (a.u). No statistically significant differences were observed between the test samples (P>0.05). C= Control, F= Fine, UF= Ultrafine, MF= Methylated Fine, MUF= Methylated Ultrafine.

4.5 Formation of ROS in A549 cells upon treatment with the different particles

When A549 cells were incubated with different TiO_2 suspensions in the presence of DMPO, all the samples exhibited typical 1:2:2:1 ESR spectra confirming the formation of DMPO-OH adducts. Interestingly, ultrafine and methylated ultrafine TiO_2 suspensions significantly

increased the formation of DMPO-OH adducts as compared to the fine TiO_2 preparations. Significant differences between the ROS generation by different TiO_2 preparations were observed which was in contrast to the acellular hydroxyl radical generation where all the particle preparations produced more or less similar ROS. All four peaks were quantified by measuring the amplitudes and the average of the sum of the amplitudes of the ESR signals are expressed in arbitrary units (Figure 4-7).

For detecting the production of superoxide radicals by the particles, different TiO_2 suspensions were incubated with A549 cells in the presence of TEMPOL, all the samples exhibited typical three lined ESR spectra. Tempol is a low molecular weight, membrane permeable, SOD mimic, stable free radical that is electron paramagnetic resonance detectable. TEMPOL is considered to act as a SOD-mimic in the redox mechanism, that is, it was readily oxidised by protonated superoxide to oxoammonium cation (Figure 3-3) which in turn oxidised another superoxide to molecular oxygen. The decay rate of the TEMPOL signal intensity is considered to reflect the production of superoxide. Quantification of the signal was carried by accumulating three different spectra, each averaging three different scans. All three peaks were quantified by measuring the amplitudes and the average of the sum of the amplitudes of the ESR signals are expressed in arbitrary units (Figure 4-7). As can be seen in the figure, the decrease in Tempol signal clearly indicates the radical production by all the particle preparations. Interestingly, both UF and MUF TiO_2 preparations show a statistically significant ROS production after 2 h as well after 4 h.

The ESR results in acellular system indicated that radicals might not be generated by particles itself whereas the ESR results in cellular system shows a difference in the radical generation by different particle preparations indicating that the particle might induce the cells to produce radicals. These observations were made using two independent spin-trapping agents, i.e DMPO and TEMPOL. From the observations, we hypothesised that the cellular ROS generation could be due to activation of membrane bound NADPH oxidase complex by the ultrafine particles. To further evaluate this, subsequent experiments were also performed with the NADPH oxidase inhibitor Diphenyleneiodonium (DPI). In cells that were pretreated with DPI and subsequently with ultrafine TiO_2, indeed a reduced formation of ROS was observed indicated by ESR measurements using TEMPOL as the spin trapping agent. However, also in the absence of particle treatments, as well as upon treatment with quartz particles (which

failed to increase cellular ROS alone) DPI reduced ROS generation (Data not shown). As such, the effect of ultrafine TiO_2 on activation of NADPH oxidase could not be confirmed.

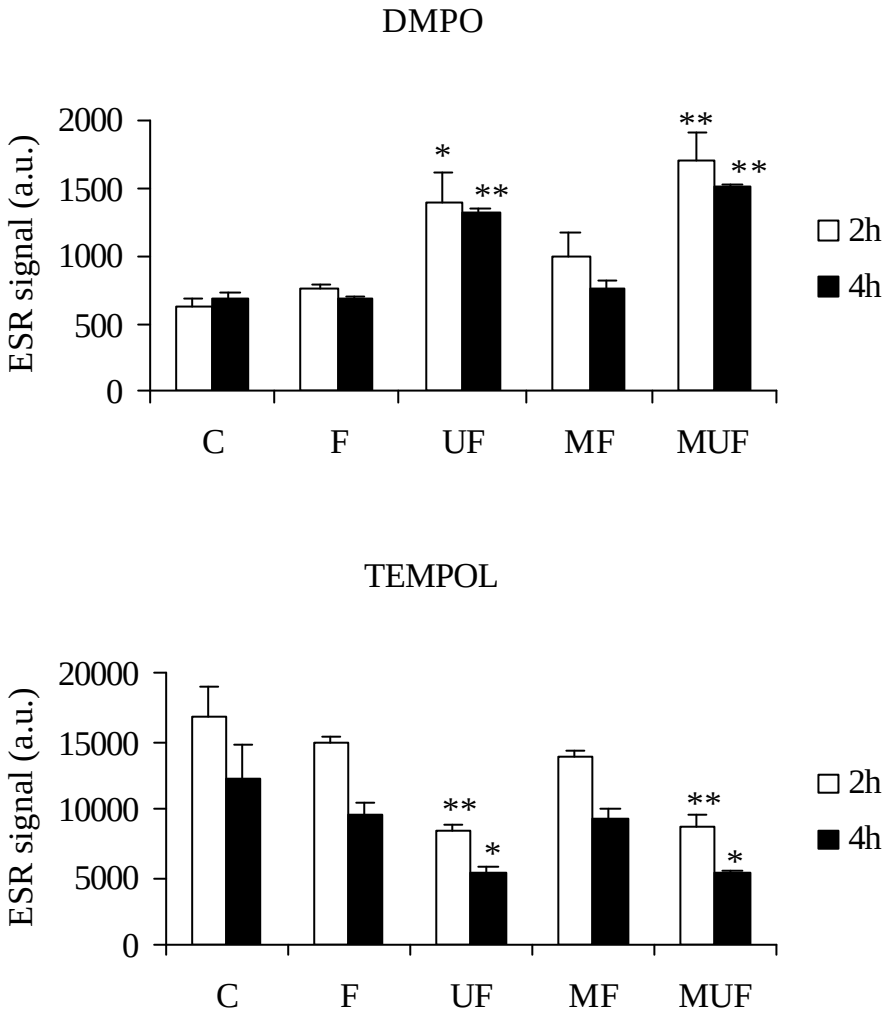

Figure 4-7 Radical generation in A549 cells

*Upper bar graph shows DMPO-OH formation in A549 cell. Increase in ESR signal indicates the increased hydroxyl radical in the cells. The lower bar graph showing hydroxyl and superoxide radical generation in A549 cells. Diminished TEMPOL signal indicates the increased superoxide radicals in the cells by the particles. The spectrum was recorded at room temperature. * and ** indicates $P<0.05$ and $P<0.01$ (significantly different than control) resepectively. Data represents the mean of three independent experiments (ANOVA with Dunnett post hoc comparison). 2 h (white bars) and 4 h (black bars).*

C= Control, F= Fine, UF= Ultrafine, MF= Methylated Fine, MUF= Methylated Ultrafine

4.6 LDH

A549 cells were treated with different particle concentrations to determine the cytotoxic concentrations using leakage of cytoplasmic lactate dehydrogenase (LDH assay) after the treatments as a marker of cytotoxicity. The purpose was to identify the particle concentrations that would give little or no LDH release after treatments. In serum-free or serum-reduced conditions, no significant release of LDH could be detected at 4 h in A549 cells after particle treatment. However LDH release was detected after 24 h at higher concentrations. The treatments used in this study did not produce any substantial toxicity.

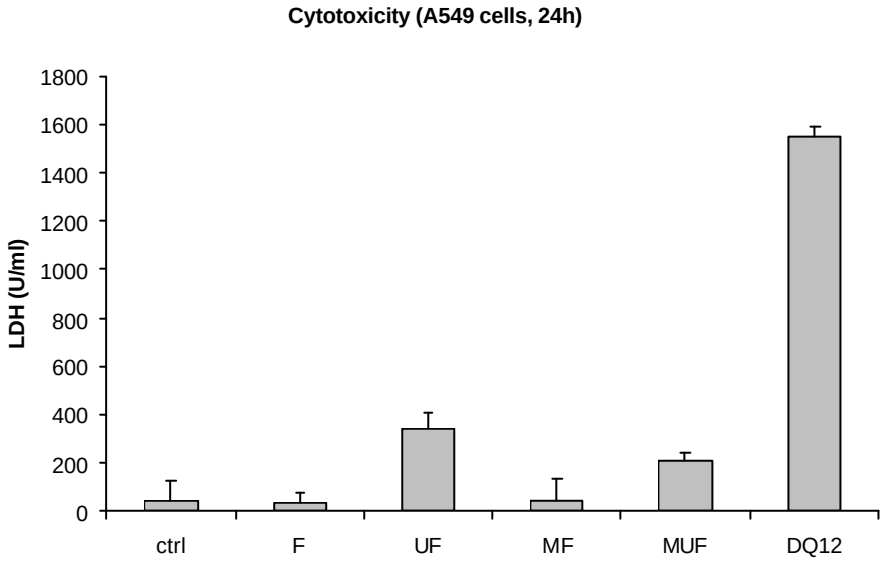

Figure 4-8 Cytotoxicity in A549 cells after treating with different particles preparations (400µg/cm^2)

Ctrl= Control, F= Fine, UF= Ultrafine, MF= Methylated Fine, MUF= Methylated Ultrafine, DQ12=Quatrz.

As seen in Figure 4-8, the methylation status does not affect the LDH leakage, but the UF (independent of methylation) tended to show stronger toxicity than their fine counterparts, in line with our ESR results in cells. DQ12, which is used as a positive control shows strongest

affect on LDH leakage. This indicates a possible role of cellular ROS generation in the membrane damaging effects of ultrafine particles.

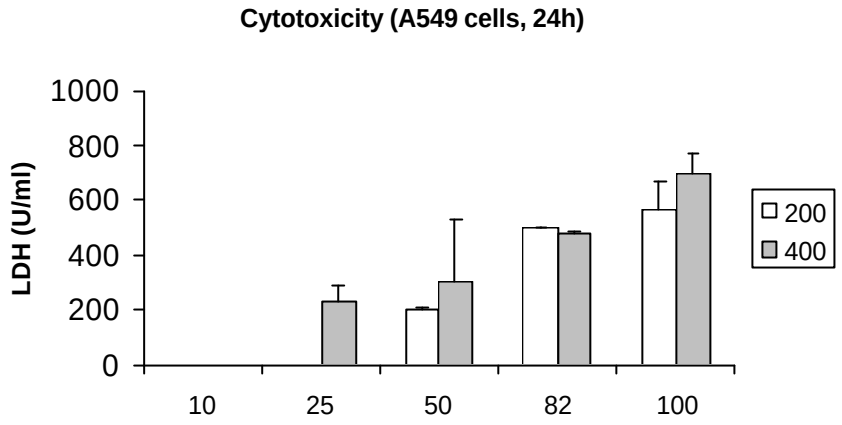

Figure 4-9 Cytotoxicity in A549 cells after treating with different TiO_2 preparations at 200 and 400 µg/cm²

The A549 cells were treated with 200 µg/cm² and 400 µg/cm² of T10, T25, T50, T82 and T100 (for particle details see table 3-1). The supernatants were collected after 24 hours and measured for LDH leakage.

The data tend to show increased toxicity with increased surface area, with the exception of T82 for which no clear dose-dependency was observed. One possible explaination could be that high surfaces of TiO_2 would interfere with the LDH assay, for instance by binding or inactivation of the LDH enzyme. However, such an affect was not observed for the T100 dust. To further evaluate this possibility of LDH inactivation, the subsequent experiment was performed. A549 cells were incubated with 0.1 % Triton X to allow the complete LDH release into the culture medium. This medium was then incubated with increasing concentrations of T50 or T100 for 4 hours. The supernatants were then centrifuged and the supernatants were then subjected o the LDH assay. The results are shown in Figure 4-10. As can be seen in this figure, the particles did not interfere with the resulting LDH measurements even at very high concentrations (up to 8 mg/ml of TiO_2).

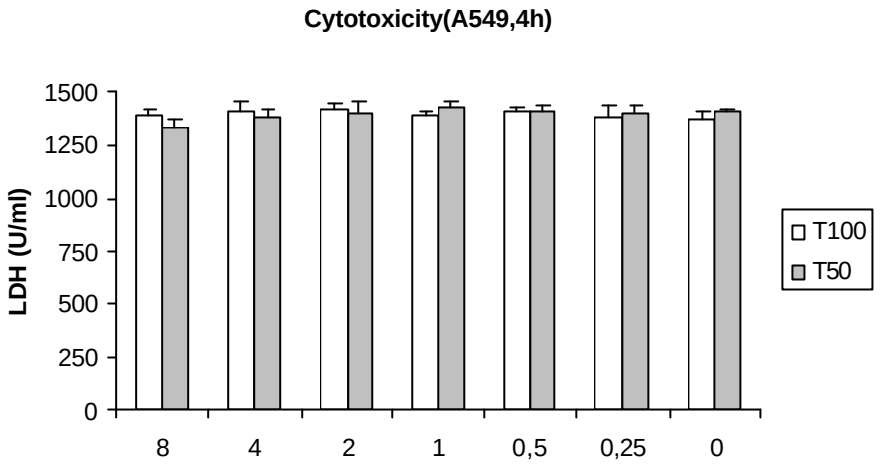

Figure 4-10 Spiking experiment in A549 cells using T100 and T50 particle preparations at different doses

4.7 Glutathione depletion

Depletion of total cellular glutathione was measured as an indicator of oxidative stress. Results are shown in Figure 4-11. At a dose of 100 µg/cm^2 none of the dusts was found to cause a significant GSH depletion both at 4 hours and 24 hours. At a dose of 200 µg/cm^2, a significant GSH depletion was found to occur at 4 hours for all TiO_2 dusts except for T10. The strongest depletion was observed with the T100 sample ($p<0.01$). After 24 hours, similar effect were observed, except for T50, which was borderline significant ($p = 0.051$), and the effect of T100 appeared to be less pronounced compared to 4 hours. Over all, our observations indicate a correlation between the BET value of the dusts and the related GSH depletion, indicative of a surface area driven effect on cellular oxidative stress. Upon treatment of 200 µg/cm^2 the correlation coefficient between BET value and cellular GSH status was found to be $r = -0.657$ ($p<0.005$) and $r = -0.665$ for 4 hours and 24 hours respectively.

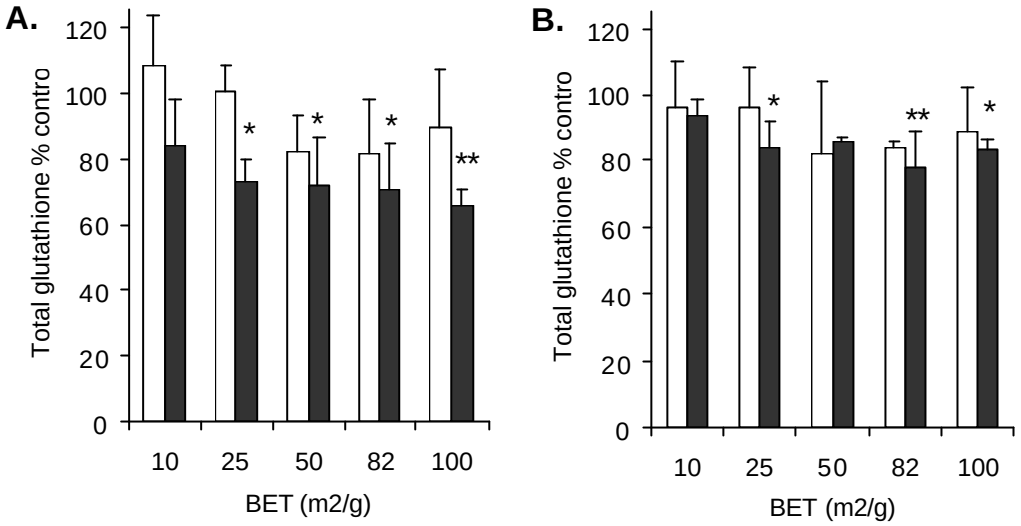

Figure 4-11 Total glutathione depletion by TiO_2 dusts of different BET surface area

*A549 cells were collected after 4h (graph A) and 24h (graph B) incubation with particles and analysed for glutathione depletion. (r^2= 0.431, P=0.003, P< 0.05 *, P<0.01** (significantly different from control). Cells treated with medium and DQ12 were taken as negative and positive control respectively. Data represents the mean of three independent experiments (n=3) ANOVA with Dunnett post hoc comparison). Open bars 100 µg/cm^2 and closed bars 200 µg/cm^2. DQ12 (100µg/cm^2) caused maximum GSH depletion.*

4.8 Release of interleukin-8 – The size effect

The release of IL-8 into the supernatant of A549 cells upon treatment with the different dusts was used as an indicator of the inflammogenic potency of the dusts. Results for this analysis are shown in Figure 4-12. At 4 hours treatment, no significant differences in IL-8 release were observed for any of the dusts (ANOVA), whereas at 24 hours the IL-8 release by the T82 sample was found to be significantly high compared to control at both treatment doses ($p<0.05$). DQ12 (100 µg/cm^2) caused a significant increase in IL-8 production compared to controls both after 4 hours (2782 ± 1446 pg/ml, $p<0.001$) and after 24 hours (17169 ± 7963 pg/ml, $p<0.001$).A clear trend for increased IL-8 release was observed with increasing particle surface area for the samples T10, T25, T50 and T82, although remarkably, the sample with the highest surface area (T100) failed to cause a strong IL-8 release. Upon exclusion of the T100 sample a significant correlation ($p<0.05$) was found between the BET surface area and IL-8 release, at both treatment concentrations (i.e. 100 µg/cm^2 and 200 µg/cm^2) as well as

treatment time intervals (4 h, 24 h). The best correlation was observed upon 24h treatment with 100 µg/cm^2 ($r = 0.767$, $p<0.001$), and from these data one could deduce that the IL-8 release from A549 cells would be doubled roughly for each doubling of the BET value. As such, the expected IL-8 release for the T100 dust would be anticipated to be at lease 2000 pg/ml at this dose. To further evaluate the apparent lack of IL-8 production by the TiO_2 dust with the largest surface area (T100), experiments were also carried out at a lower dose range and the T100 sample caused a rather strong IL-8 release at early time points (4 h), whereas no enhanced IL-8 release was observed after 24 hours (Data not shown) which indicated that apart from surface area some other effects play a modulating role in IL-8. Further studies are needed to explain this phenomenon. To some extent the apparent lack of IL-8 release after treatment with T100 at high dose, may be explained by adsorption of the IL-8 protein by the particles. To evaluate this, spiking experiments were performed with recombinant human-IL-8 using the T100 sample at different doses. These results showed that the IL-8 can be adsorbed onto high particle surfaces (Data not shown).

As mentioned in Chapter 3, in order to further evaluate the importance of surface chemistry, the native and methylated counterparts of T10 and T50 particles were used. IL-8 release from A549 cells by F/UF TiO_2 and their methylated counterparts were comparatively evaluated at different mass concentrations; these results are shown in the following section.

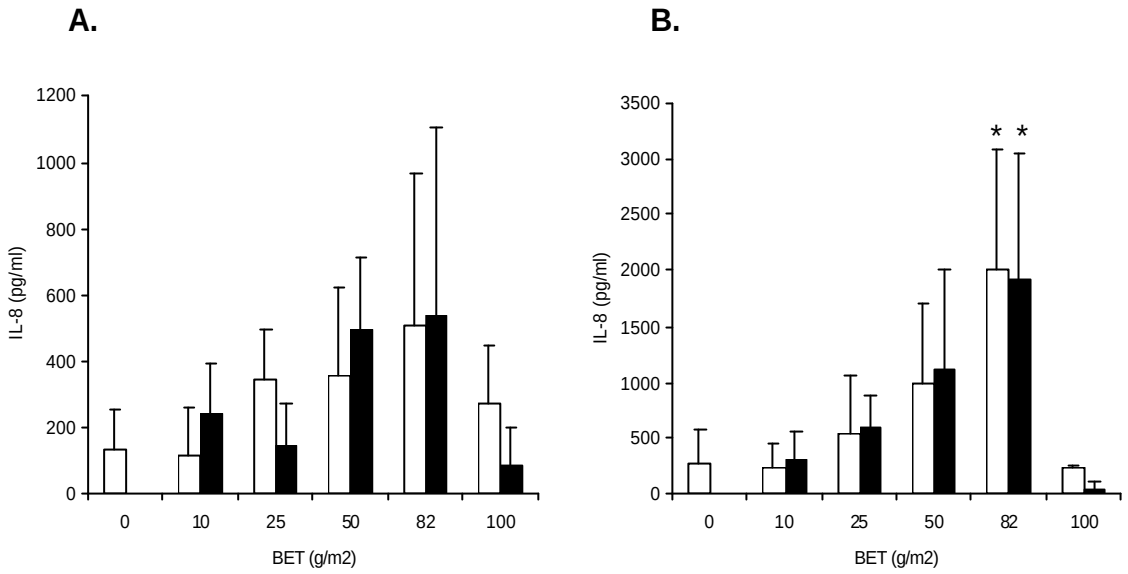

Figure 4-12 Interleukin-8 released in A549 cells exposed to 5 different TiO_2 at two different mass doses

Cells were incubated with particles suspensions for 4h (graph A) and 24h (graph B). The supernatants were collected and quantified for IL-8 release by ELISA assay. Each bar represents the mean SD of three independent experiments. (n=3) mean and standard deviation asterisk significance p<0.05 ANOVA with Dunnett post hoc comparison). Open bars and closed bars represent 100 µg/cm^2 and 200 µg/cm^2 particles treatments respectively.DQ12 (100 µg/cm^2) used as positive control caused a significant increase in IL-8 production compared to controls both after 4 hours (2782 ± 1446pg/ml, p<0.001) and after 24 hours (17169 ± 7963 pg/ml, p<0.001). At 4 h, 100 µg/cm^2 (r=0.537, p<0.05), 4h 200 µg/cm^2 (r=0.529, p<0.05) at 24 h, 100 µg/cm^2 (r=0.767, p<0.001), 24 h 200 µg/cm^2 (r=0.735, p<0.005).

4.9 Evaluation of the role of membrane damage in IL-8 release from A549 cells

Since our experiments showed increased toxicity with increased surface area of TiO_2 samples, as well as increased IL-8 release, we determined whether the observed IL-8 appearance in the cell supernatants could be due to membrane damaging effect. Therefore, A549 cells were treated with increasing concentrations of the membrane-damaging compound Triton X-100, after which the cell supernatants were analysed for LDH activity as well as IL-8 release. The results are shown in Figure 4-13 and Figure 4-14. As can be seen in the figure, at high concentrations a clear increase in LDH was found (Figure 4-13), but in contrast, no enhanced IL-8 levels were detected (Figure 4-14).These results confirm that the increased IL-8 levels are found upon treatment of A549 cells due to the particles with increasing BET surface area, did not result from leakage of the constitutive amounts of intracellular IL-8.

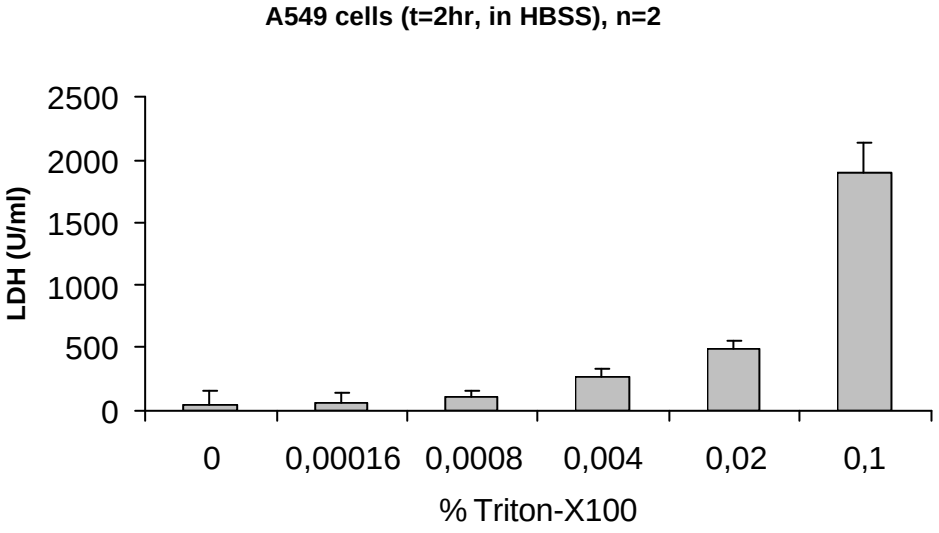

Figure 4-13 LDH activity in A549 cells on treatment with Triton X-100

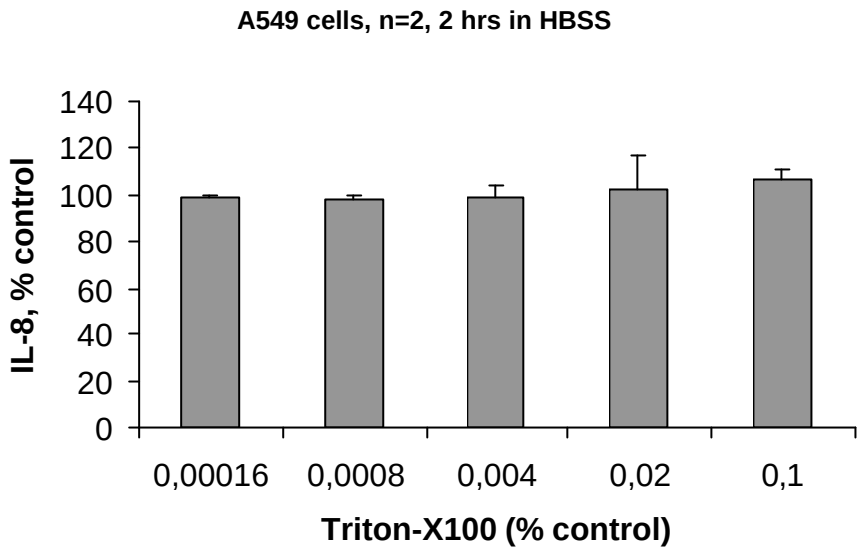

Figure 4-14 IL-8 release in A549 cells on treatment with Triton X-100

4.10 IL-8 mRNA expression and protein release - The methylation effect

RT-PCR was also used to determine the effects of the different particle samples (F-TiO_2 and UF-TiO_2 and their mathylated counterparts) on the ability to induce IL-8 mRNA expression in A549 cells. Glyseradehyde-3-phosphate dehydrogenase (GAPDH) is a catalytic enzyme involved in glycolysis. Because of its relative constitutive expression in all cells it was used as a house keeping gene to normalise mRNA quantitation in the RNA samples. A representative gel is shown in Figure 4-15.

These experiments clearly indicated that the UF-TiO_2 and its methylated counterpart (MUF-TiO_2) caused an enhanced IL-8 mRNA expression in comparison to the untreated cells. Enhanced IL-8 mRNA expression was also observed with TNF and DQ12 quartz, which served as non-particulate and particulate positive controls respectively. However, the IL-8 expression as seen with both fine types (i.e. F-TiO_2 and MF-TiO_2) did not differ from the negative control.

The release of IL-8 protein into the supernatant of A549 cells upon 24 hour treatment with the different samples is shown in Figure 4-16. As can be seen in the figure, there was a

concentration-dependent trend for all samples. A significant increase in IL-8 production was observed at the highest particle mass dose with both UF-TiO_2 and MUF-TiO_2 as well as with the positive control DQ12 quartz. There was no significant increase in IL-8 production by F-TiO_2 or MF-TiO_2. Importantly, no differences were observed between the native UF-TiO_2 samples and its methylated counterparts (MUF-TiO_2). The data clearly indicate that the production of IL-8 was merely driven by surface area, and not affected by surface methylation.

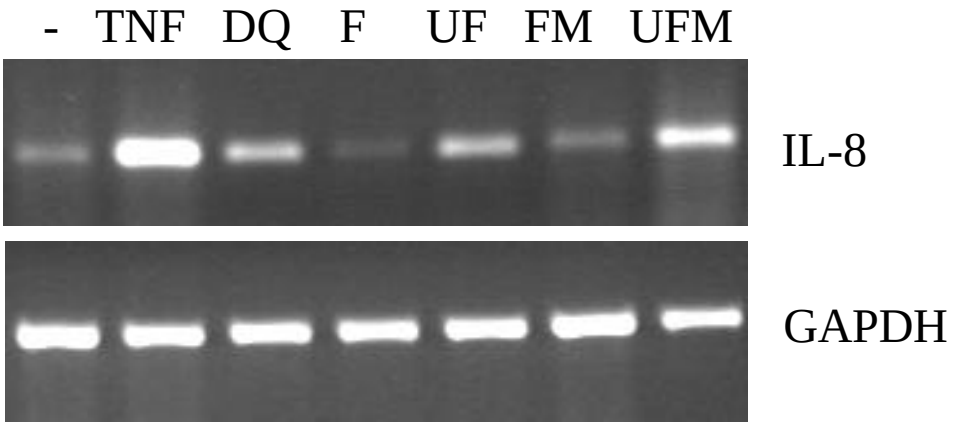

Figure 4-15 Induction of IL-8 mRNA by different preparations of TiO_2 in A549 cells

The cells were stimulated for 4 h with the particles (80 µg/cm^2).Total cellular RNA was extracted and RT-PCR was performed. The lower panel shows the housekeeping gene GAPDH to indicate the amount of RNA loaded into each lane. Negative control-not exposed cells. Positive control: TNF (10 ng/ml) and DQ12 (80 µg/cm^2).

C= Control, F= Fine, UF= Ultrafine, MF= Methylated Fine, MUF= Methylated Ultrafine, TNF= Tumor Necrosis Factor, DQ12= Quartz.

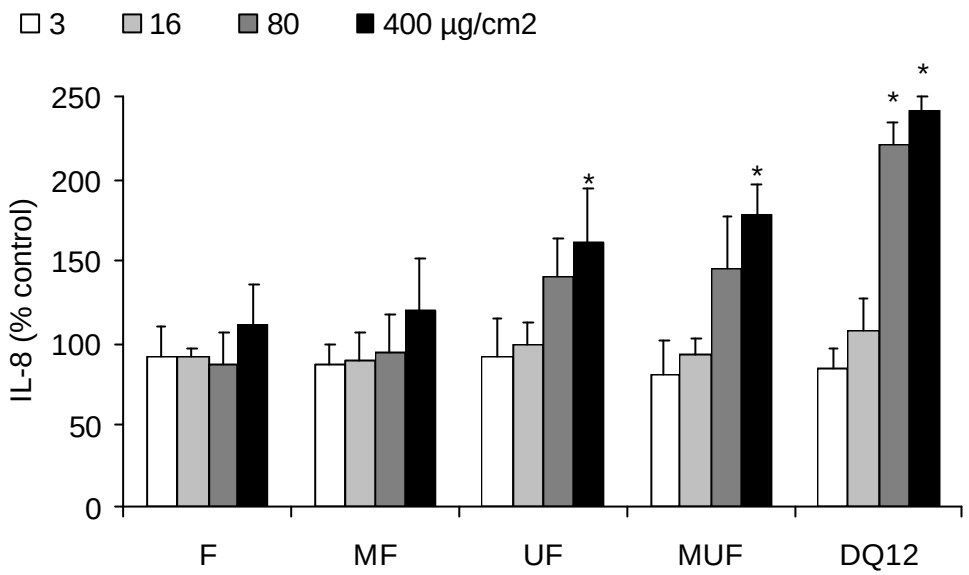

Figure 4-16 Effect of different doses of fine and ultrafine particles preparations on IL-8 protein release in A549 cells measured by ELISA

*Cells were overnight starved at confluence and then were incubated with increasing concentrations of different particle preparations (as indicated) in the histogram. Supernatants were collected 24 hours after treatment with particles and assessed for IL-8 release by ELISA. *P<0.05. The mean of three independent experiments and S.D. are indicated in the histogram. Negative control-not exposed cells. DQ12 is used as positive control.*

C= Control, F= Fine, UF= Ultrafine, MF= Methylated Fine, MUF= Methylated Ultrafine, DQ12- Quartz.

4.11 Particle uptake

The human epithelial cell line A549 has been proved to be a suitable *in vitro* model for studying endocytosis of particles (Stearns et al. 2001). In order to determine particle surface modifications on particle uptake of two different sizes TiO_2 and their methylated counterparts were analysed by particle exposed monolayer cultures of A549 cells using electron microscopy. The identity and composition of electron dense particles assessed to be TiO_2 was determined by EDX-analysis and elemental mapping. The particles which were first identified inside or outside of the epithelial cells by their specific ultra-structure at high microscopic magnification, showed the characteristic X-ray emission line of the element Ti which refers to an unequivocal identification of the particles (Data not shown).

The TEM analysis demonstrated a rapid internalisation of the TiO_2 particles in the A549 cells after 4 h. The majority of the TiO_2 particles were associated as aggregates and entered the cells by phagocytosis. Small aggregates containing 3-5 ultrafine primary TiO_2 particles (size 20-30 nm) were seldom endocytosed by clathrin coated vesicles (pinocytosis). The particle clustering limited the determination of the exact particle number that entered the cells and therefore it was impossible to correlate particle number to inflammatory effects. Therefore in our experiments we used the number of particle aggregates and their maximally mean diameter as parameters for quantifying particle uptake. The number of particle aggregates was about 2-fold higher in the A549 cells phagocytosing ultrafine TiO_2 particles than in the cells internalising the non-ultrafine particles (Figure 4-18). At the low dose of 16 µg TiO_2/cm^2 the methylation of both fine and ultrafine particles tended to cause a slight enhancement of their cellular uptake whereas at the higher dose of 80 µg/cm^2 the surface properties had no increasing effect on the number of ultrafine TiO_2 particle aggregates. Furthermore the methylation of the hydrophilic TiO_2 particles did not change the diameters of the primary particle aggregates (Data not shown). About half of the fine TiO_2 particle aggregates had a size range of 200-500 nm whereas most of the ultrafine particles showed the tendency to the lower size range of 0-200 nm. Neither the different size of the particles nor their surface properties had an influence upon the intracellular particle distribution. Fine and ultrafine particle aggregates were predominantly incorporated in membrane-bound vacuoles (Figure 4-17). Particles were also found associated with both loosely and highly packed lamellar bodies, the main compartment of surfactant production. Association of particles with lamellar bodies has been noticed in other studies (Corrin 1969; Sorokin et al. 1975). Multivesiculated bodies, which are regarded as acid phosphatase containing lysosomes mixed with residual membraneous material and other cell debris, were enriched with TiO_2 particles. Membrane-enclosed particle aggregates of different size were often observed next to the nucleus but never inside the nucleus. Golgi apparatus, rough ER and mitochondria were TiO_2 particle free cell compartments. TiO_2 particles in the space between the cells were never observed moving through the tight junctions into the cells.

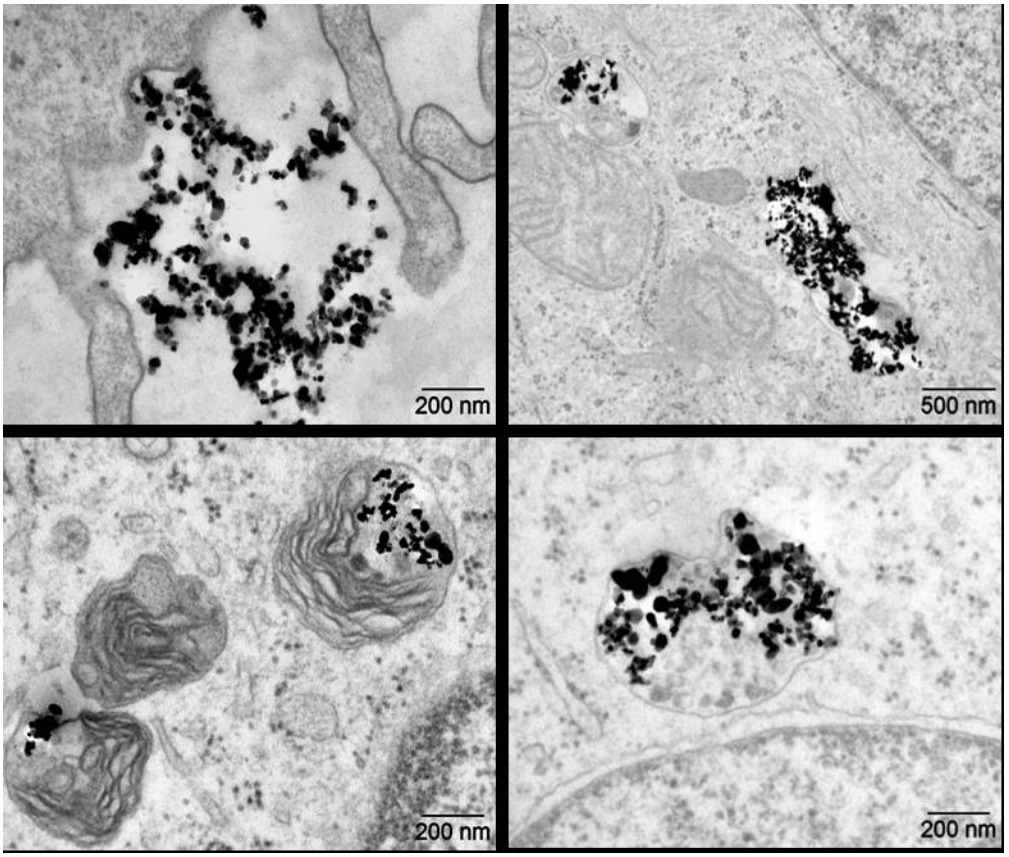

Figure 4-17 TEM images of A549 cells treated with ultrafine TiO_2 at low dose 16µg/cm^2

Upper left picture indicates the cell lamellipodia engulfing the particles aggregates. Lower left picture shows the TiO_2 particles inside the lamellar body. The upeer right and lower right picture indicate membrane bound aggregates of particles near mitochondria and perinuclear space respectively.

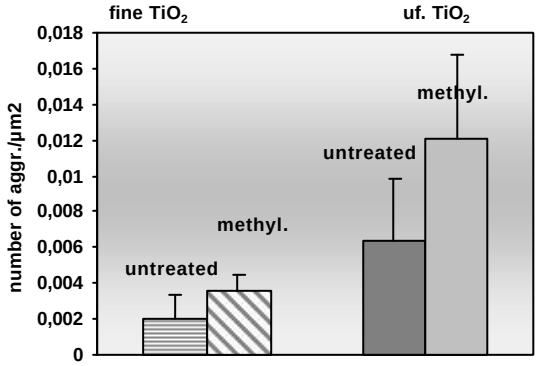

Figure 4-18 The number of particle aggregates at low dose 16/µg/cm^2

The number of particle aggregates was about 2-fold higher in the A549 cells phagocytosing ultrafine TiO_2 particles than in the cells internalising the non-ultrafine particles. At the low dose of 16 µg TiO_2/cm^2 the methylation of both fine and ultrafine particles tended to cause a slight enhancement of their cellular uptake.

5. Summary and General Discussion

5.1 Background

Titanium dioxide is a non-combustible, odourless white powder which is used for a number of industrial applications such as a food colorant, important additive in pharmaceuticals, cosmetic industry and for a wide range of other consumer products due to its photophysical properties (Hayashi and Kobayashi 1996; Ogawa et al. 1996; Bingham et al. 2001). TiO_2 has been used as a non-toxic control dust in many studies related to particle toxicology, at least under nonoverload conditions (Baggs et al. 1997; Warheit et al. 1997). TiO_2 was considered biologically inert (Ferin and Oberdörster 1985; Lee et al. 1985; Driscoll et al. 1990; Lindenschmidt et al. 1990; Muhle et al. 1991; Baggs et al. 1997) until studies done with ultrafine particles by some groups (Ferin et al.1992; Oberdörster et al. 1994), showed that ultrafine titanium dioxide particles (20 nm in diameter) elicited a higher inflammatory response in the rat lung than larger sized particles (250 nm in diameter). Later studies showed that when the instillation or inhalation doses were expressed in terms of particle surface area then the responses of the ultrafine and fine TiO_2 particles fell on the same dose-response curve indicating that surface area is an important parameter in toxicity of ultrafine particles (Osier and Oberdörster 1998; Tran et al. 2000). Similar observations, (Li et al. 1996) in rats that have been instilled intratracheally with ultrafine carbon black versus fine carbon black confirmed the role of surface area in particle induced inflammatory effects (Greim et al. 2001; Borm et al. 2004). There is a debate about the enhanced toxicity of ultrafine particles and it is suspected that independent of particle's chemistry (Brown et al. 2000, 2001; Oberdörster 2001) they are more likely to induce inflammatory responses compared to the same mass of fine particles (Ferin et al. 1990; Donaldson et al. 1998; Oberdörster 2001).

Our Hypothesis revisited

To investigate the inflammatory effects of the TiO_2 dusts, we hypothesised that the toxicity of the TiO_2 might be due to its surface area and to test this we compared five different TiO_2

dusts of different surface areas by doing toxicity and inflammatory assays. In addition, for all these dusts, we measured the glutathione depletion to determine the correlatelation between inflammatory effects and cellular oxidative stress. The intention behind it was to see if the particles surface area drives the oxidative stress in human lung epithelial cells (A549) leading to the oxidation of cellular glutathione and subsequent induction of IL-8.

The lung epithelium serves as a barrier and a direct stimulatory effect of particles on it may represent an alternative pathway by which inhaled particles may elicit inflammation in the lung (Driscoll et al. 1997). Therefore to clarify the mechanism behind particle induced adverse effects we used *in vitro* cultured lung epithelial cell lines. We hypothesised that the interaction between particles and epithelial cells can initiate a cascade of events that induce the adverse effects associated with the particle inhalation. More specifically, we hypothesised that the ingestion of particles by A549 cells is a crucial phenomenon associated with the IL-8 release. Therefore we sought to quantify the uptake of fine and ultrafine TiO_2 particles by the A549 cells (using TEM) and then measuring the IL-8 release by the cells.

In addition to the size or surface area related effects, we also wanted to elucidate the importance of hydrophobicity/hydrophilicity of the TiO_2 particles ROS generation, uptake and their subcellular localisation in order to understand the involvement of uptake for the IL-8 release in the A549 cells. We expected a directly proportional effect between uptake and IL-8 release.

In several studies, ROS are suggested to play an important role in the pathophysiological interaction of particles and lung epithelial cells. In this study, we also attempted to demonstrate whether the particles itself produce ROS or could induce ROS in A549 cells, and whether this would relate to subsequent IL-8 release.

5.2 TiO_2 - The surface area driven effects

Our results (Chapter 4) clearly demonstrate that the surface area of TiO_2 particles is an important factor for inducing oxidative stress and depleting the total glutathione levels in A549 cells after treating with different sized TiO_2 particles. We observed that the glutathione depletion in A549 cells was directly proportional to the particle surface area as well as particle

dose. In combination, these observations demonstrate the important role of particle surface area in causing cellular oxidative stress. Interestingly, less glutathione depletion was observed at 24 h as compared to the 4 h time point. This could be explained by the fact that oxidative stress reduces cellular GSH but at the same time it also activates signalling pathways that leads to the induction of GSH pathway enzymes and *de novo* synthesis of GSH. Depending on the amount of initial stress, complete restoration of the GSH pool or even an 'overshoot' of GSH might take place in the cells (Rahman and MacNee, 1999). Rahman and MacNee have discussed this concept in relation to contrasting observations on GSH levels in the lungs of acute *versus* chronic smoking conditions. The evaluation of this mechanism for the TiO_2 powders used in this study is beyond the scope of the current work, but remains an interesting issue for future research concerning acute versus chronic inhalation of ultrafine particles.

Previous studies (Stone et al. 1998) in A549 cells using fine and ultrafine carbon black have suggested a greater free radical activity based on the depletion of glutathione levels in case of ultrafines as compared to the fine particles. This also suggests a role of oxidative stress in toxicological effects of the ultrafine carbon black particles. This phenomenon was further confirmed in studies done *in vivo* by the same group in 1998 after instillation of rats with fine and ultrafine carbon black. This showed greater inflammation and GSH depletion in the lung lavage fluid on ultrafine CB treatment compared to fine particles (Li et al. 1999). From our studies and the studies done from the other groups, we can conclude that the particle surface area plays a significant role in depleting GSH and inducing IL-8 release in A549 cells which indicates the possible involvement of particle induced oxidative stress in inflammatory response.

Our IL-8 results are in line with the findings from Brown et al. (2001) who also showed an increase in IL-8 expression in A549 cells after treatment with ultrafine polystyrene particles. This also suggests that ultrafine particles composed of low toxicity materials such as polystyrene or TiO_2 have pro-inflammatory activity due to their large surface area. However, the polystyrene particles used in their studies were rather monodisperse (uniform in shape, size and composition) which contrasts with the TiO_2 particles used in our studies. The TiO_2 particles used in our studies were characterised according to their BET values and consisted of a mixed population of particles characterised by a distribution of sizes.

Hetland et al. (2001) have reported that the surface area is a critical determinant when the potency of different sized quartz dusts is compared. In their experiments, A549 cells were

exposed to different size fractions of quartz and all particles induced an increased release of the proinflammatory cytokine IL-8. When cells were exposed to equal masses of quartz, the smallest size fraction was the most potent inducer of IL-8. These differences however, disappeared when cytokine release was related to equal surface areas. Importantly however, the dusts used by Hetland et al. were generally in the non-ultrafine range, and quartz is well known for its high reactivity when compared to other dusts including TiO_2 at the non-ultrafine size range (Schins et al. 2000). Furthermore, other studies with commercial quartz showed a 10 fold variability of inflammatory response at equal surface dose (Bruch 2004).

5.3 TiO_2 - surface chemistry driven effects

As mentioned in chapter 1, the degree of hydrophilicity/hydrophobicity of a surface is also considered as an important property for its toxicity and biological activity. Therefore the surface chemistry of the particles may be one of the important factors explaining the particle induced adverse health effects. In this work, we compared the effect of surface chemistry by methylating the surface of Fine (BET of 10 m^2/g) and ultrafine (50 m^2/g) TiO_2. We evaluated and compared the effects of methylated and nonmethylated particles concerning uptake, ROS production, IL-8 expression and release.

The important parameter which determines particle uptake and transport is particle size and studies claim that smaller particles enter the epithelial cells and are transported to the interstitium to a much greater extent than the larger particles of the same material (Moosman et al. 1978; Adamson et al. 1981; Ferin et al. 1992; Churg et al. 1996). It is speculated that there may be specific uptake mechanisms for particles in epithelial cells and these may differ with particle properties. Such differences may in turn lead to different cytokine responses.

Ingestion of particles by epithelial cells has been postulated to be an important mechanism for particle deposition in the lung interstitium (Berry et al. 1978; Brody et al. 1981; Adamson 1981). The entry of the particles into the epithelial and sub-epithelial tissues is associated with inflammation, genotoxicity and cell injury. Several studies have described qualitatively the ability of epithelial cells to bind and ingest various types of particulates. Churg et al. (1998a) examined the relationship between *in vitro* particle uptake by pulmonary epithelial cells and particle size. They exposed rat tracheal explants to fine particles (0.12 µm) or ultrafine

particles (0.021 µm) of titanium dioxide for 3 or 7 days. Their results suggest that the behaviour of particles of different sizes is complex and ultrafine particles persist in the tissues as relatively large aggregates, whereas the size of fine particle aggregates becomes smaller over time. Ultrafine particles appear to enter the epithelium faster, and once in the epithelium, a greater proportion of them are translocated to the sub-epithelial space compared to fine particles. However, if it is assumed that the volume proportion is representative of particle number, the number of particles reaching the interstitial space is directly proportional to the number applied i.e., overall, there is no preferential transport from lumen to interstitium by size.

Another study done by Stearns et al. (2001) used an *in vitro* model of type II lung epithelium to evaluate the cell's ability to ingest ultrafine particles (titanium dioxide, 50 nm diameter). After treating the human epithelial cell line A549 with 40 µg/ml TiO_2 particles for 3, 6, and 24 h, aggregates of the ultrafine particles were observed in cytosolic, membrane-bound vacuoles after 3 h. There were considerably more intracellular aggregates of membrane-bound particles, and aggregated particles were found enmeshed in loosely and tightly packed lamellar bodies, after 24 h of exposure time.

The main aim of our uptake studies was to evaluate uptake and sub-cellular distribution of the particles in relation to their size and hydrophobicity. Our data indicates that surface methylation of ultrafine TiO_2 does not affect the particle uptake in A549 cells significantly. Interestingly at lower dose, there was a small difference in the uptake of methylated and nonmethylated ultrafine particles. However, we did not observe a drastic difference in the uptake of methylated and nonmethylated particles at higher doses. One possibility could be that in case of very small and inert particles like TiO_2, the surface chemistry effects are almost negligible. Another explanation can be the instability of the methyl groups on the surface of the particles. We tested the stability of the methylation procedure by FTIR spectrophometry after suspending the methylated particles in water. The FTIR results clearly show the success of the methylation procedure showing an extra peak for the $O\text{-}CH_3$ binding. It is also possible that the methylated particles lose their surface methylation to some extent in the cell medium and act like the native hydrophilic particles but we have no experimental method to detect this. Once in medium, particles will be coated by the proteins and perhaps that obscures the methylation difference.

Our TEM data shows that the particles were enmeshed in lamellar bodies and no particles were observed in nucleus, mitochondria or any other vital cells organelles. These findings are in line with the previous endocytosis observations (Stearns et al. 2001) which also reported the phagocytosis of UFTiO$_2$ by type II cells as aggregates enmashed in lamellar bodies. Lamellar bodies are members of a subclass of lysosome-related organelles referred to as secretory lysosomes. The principal constituents of the lamellar body are surfactant phospholipids. Surfactant is synthesised exclusively by alveolar type II epithelial cells where it is stored in inclusions (lamellar bodies). Interactions between particles and surfactants have been little studied and the uptake results showing particles enmeshed in the lamellar bodies highlights the importance of investing this aspect also.

The ESR data indicate that our TiO$_2$ particle preparations do not show any significant difference in radical generation in a-cellular experiments which initially made us think that radicals are not an important factor for inducing IL-8 expression. However we observed very different results when we measured ESR signal in the presence of lung epithelail cells (A549). In cells ultrafines produced more superoxide as well as hydroxyl radicals than the fine TiO$_2$. Methylation had no effect in enhancing or reducing the radical generating properties of both Fine and UF TiO$_2$ particles. Again, this confirms that surface properties after our methylation procedure do not influence acellular and cellular ROS generation. In addition, we found that ROS measurement by ESR provides a useful quantitative tool to study the induction of oxidative stess by the particles in the cells. Our data also establish the utility of A549 cell line for this purpose. ROS generation as observed may also have consequences for cellular effects which were currently not investigated, such as cytotoxicity, apoptosis, and genotoxicity, for the lungs but also on other organs.

Along the same line of arguments, if ROS play an important role, one would expect a similar profile in the IL-8 expression. In our IL-8 expression (RT-PCR) and IL-8 release (ELISA) data we observed IL-8 expression which could be correlated to the ESR data. The methylation of the particle did not play any role in modifying the particle effects. Indeed, our IL-8 data show that TiO$_2$ mediated IL-8 protein release and mRNA expression is associated with the surface area and less so with the surface properties in A549 cells. It clearly demonstrates that when tested at equal mass, ultrafine TiO$_2$ is more inflammogenic than the fine TiO$_2$. Furthermore, changing the particle surface properties by methylation did not affect the toxicity in a dramatic manner, and both preparations of ultrafine TiO$_2$ induced similar mRNA

expression and release of IL-8 from the A549 cells. Höhr et al. (2002) have compared the *in vivo* effects of fine and ultrafine TiO_2 in relation to the surface methylation. In agreement with observations by others, the ultrafine TiO_2 caused stronger inflammatory effects than the fine TiO_2 dust in rat lungs. However, in our current *in vitro* studies, no marked difference in lung inflammation was caused by the methylation of TiO_2 species. This also indicates that our *in vitro* model may be relevant, at least to some extent, to predict inflammation in vivo by ultrafine particles in the lung.

In previous studies, ROS have been shown to function as subcellular messengers in gene regulatory and signal transduction pathways (Allen and Tresini 2000). Our ESR results show a similar trend like IL-8 response in A549 cells indicating the possible role of particle induced oxidative stress in triggering the inflammatory response. Our *in vitro* observations with TiO_2 are in line with the *in vivo* data which suggest that the inflammation is predominantly caused by the particle number and the particle surface and this is relatively independent of the surface chemistry (Lehnert 1990; Oberdörster et al. 1992, 1994; Driscoll et al. 1996; Oberdörster 1996). In summary, we found no difference in radical generation by particles at the same mass in acellular system whereas in the presence of cells, we observed a significant difference in pattern of radical generation by fine and ultrafine particle preparations. This indicates the complexity of the particle induced inflammatory mechanism and also questions the relevance of acellular results for effects in cellular system. In interpreting this *in vitro* study one should remember that the health effects associated with particulate air pollution are very complex and are not a consequence of a single pathogenic mechanism.

Our uptake data shows that at higher dose (80 $\mu g/cm^2$) there is not significant difference in the uptake of Fine and Ultrafine particles by the cells. Whereas we do see a big difference in the IL-8 expression induced by Fine and Ultrafine particles at the very same dose of 80 $\mu g/cm^2$. The precise mechanisms of particle induced IL-8 has not yet been clarified but most likely it does not involve the uptake of particles by the target cells in our experiments. The generation of extra- and intra-cellular ROS and the subsequent release of inflammatory mediators seem correlated.

5.4 Extrapolation of findings

The aim of this *in vitro* study was to enhance understanding of the underlying biological mechanisms involved in particle induced inflammatory effects and toxicology. We intended to investigate the mechanisms of action of particles size and surface chemistry in order to gain insight into the particle induced inflammatory effects which could also help to meet regulatory requirements for ambient and occupational nanoparticle exposure. This study was aimed at studying the mechanism rather than studying the response to a realistic particle dose. A disadvantage of working with A549 cells is that they respond to relatively high doses of particles. In fact, almost all *in vitro* and *in vivo* models that examine the effects of PM particles use unrealistically high doses because low doses tend not to produce any effects (Tao et al. 2003).

In order to look at the effects of particles using a sensitive model, we did some preliminary studies on human primary nasal epithelial cells (Data not shown). Primary cells isolated from human tissue, represent a model system much closer to the *in vivo* situation than long established and often highly transformed cell lines. The advantages of primary cultures are that the cells have not been modified in any way (other than enzymatic or physical dissociation). Therefore we also looked at IL-8 induced in primary nasal epithelial cells to simulate the real life situation using much lower doses. Our results (Data not shown) indicated that the primary cells are a sensitive system to conduct experiments, as the cells could be stimulated at very low doses which represent the realistic doses present in ambient air. The disadvantages of primary cultures are the mixed nature of each preparation, limited life span of the culture and the potential contamination problems. The other important factor is that the primary cells are normally isolated from the tissues of patients who already suffer with some disease. Such patients, on one hand represent a sensitive model simulating the real life situation but on the other hand the reproducibility of the data migh differ from patient to patient, as other factors like the age, sex (spitzer 1999) and health status of each patient differs.

6. General Conclusions

Based on the outcomes of our present study we can draw a set of conclusions which are illustrated in Figure 6-2. As it is already discussed in the previous chapters, our results clearly indicate that ultrafine particles are more inflammogenic than the fine particles and methylation does not play a significant role. Since one big part of the thesis was the characterisation of particles that were used in this study, a number of experiments in acellular system were performed. Therfore, before discussing the cellular outcomes, the potential factors which might lead to the biasing of our cellular responses are briefly discussed in the following lines.

In acellular system, only negligible endotoxin content was detected in the particles. Therefore the likelihood of detecting IL-8 due to the possible endotoxin content in particles is entirely ruled out. The methylation procedure was successful as proved by FTIR results but the stability of the methylation on the particles still remains an important discussion. Looking at the HRTEM pictures of T10 and T50 particles, we can clearly see that not only the particle diameter but also the porous surface adds to the total surface area of the particles. It is well known that a high surface area can be attained either by fabricating small particles where surface to volume ratio of each particle is high, or by creating materials of high porosity. It can be therefore speculated that for the particles with complex porous surface will give different results than the particle of same size but of negligible porosity. Since pores or cervices on the particle surface adds more to the surface area, we can predict that the surface area is much more important factor than the size. In our studies, the specific surface area of the particles was measured using gas absorption technique (BET method) using nitrogen gas which measures not only the external surface but also the internal pore surface. For particles of very small size and complex pore structure, the size of the gas molecule may affect the penetration of the gas molecule into the pores and therefore not being able to predict the actual surface area. In this case the surface area would depend on the precision and sensitivity of method used.

Another important factor to discuss here is the Anatase/Rutile ratio of the particles that we used in our study. Several studies (Dunford et al. 1997; Uchino et al. 2002), demonstrated that anatase is more active in generating ROS than rutile. By looking at the Anatase/Rutile ratio of the five different particles that we used, one might speculate that the differences in the

observed results are due to that. However the Anatase/Rutile ratios of our particles (Table 3-1) are the same for T10 and T100 or T25 and T82 but that did not influence the oxidative stress and IL-8 inducing potential which was shown to be totally driven by the particle surface area. Once again this proves the importance of surface area above surface chemistry.

All samples caused considerable formation of DMPO-OH adducts indicating the production of •OH radicals as compared to the negative control. This however, was not different for the different particle preparations on equal mass basis despite their contrasting surface areas. In addition, no $O_2^{\bullet -}$ was detected in any sample as indicated by the Tempol results. Studies have shown that TiO_2 produces •OH radical, H_2O_2 and $O_2^{\bullet -}$ under UV irradiation (Clechet et al. 1979; Harbour et al. 1985; Cai et al. 1992b; Lawless et al. 1997).

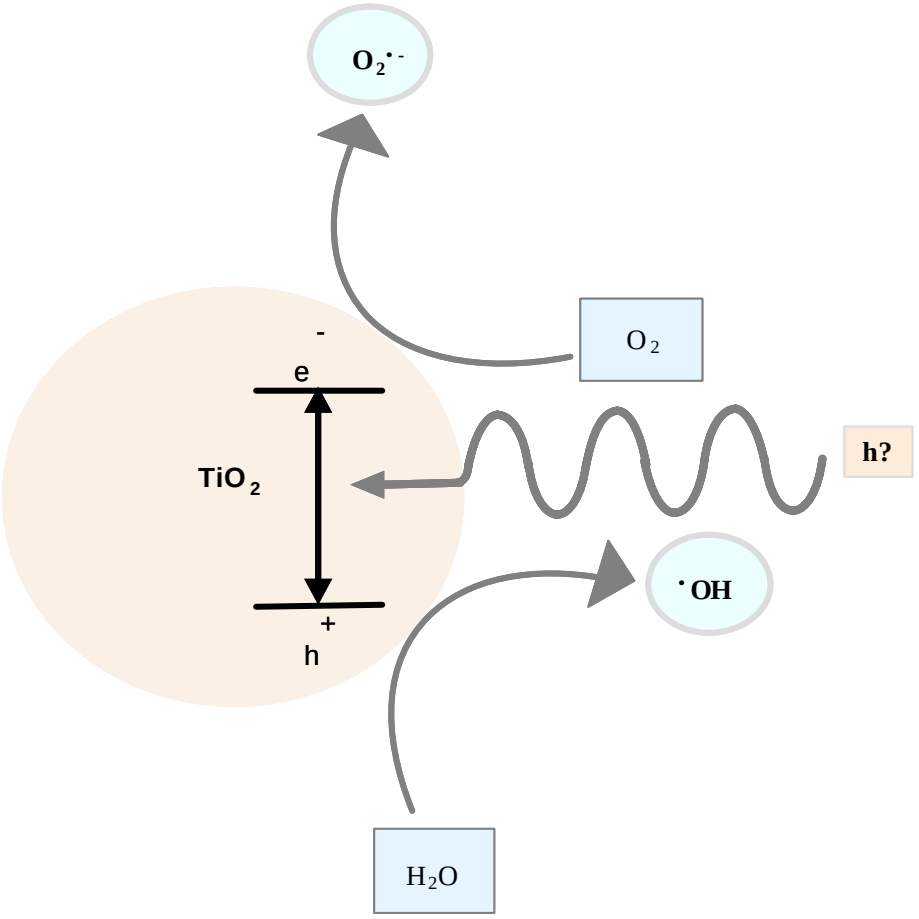

Figure 6-1 Schematic representation of the photochemical generation of superoxide and hydroxyl radicals at the surface of TiO_2 particles

Infact there are some studies showing that ROS by TiO_2 is detectable only after UV illumination (Serpone 1996; Uchino et al. 2002) and the radical generation is dependent on anatase or rutile content of TiO_2. Using spin trapping and ESR detection, Jaeger and Bard (1979) have observed ESR spectra consistent with formation of •OH and $O_2^{\bullet -}$ following absorption of UV radiation by TiO_2 (anatase). Sclafani and Herrmann (1996) reported that anatase form was more active in photocatalysis than the rutile form.

In our studies, we did not treat the particles with UV light but still observed •OH radicals in acellular ESR experiments. Therefore we thought that the sonication of the particles (which is a normal particle preparation procedure) might cause this increase in •OH radicals. To substantiate, we performed ESR experiments looking at the effects of sonication on ROS generation but we did not see any effect of 5-10 minutes sonication (Data not shown) on radicals generation by the particles. Additional studies are therefore needed to understand the mechanism by which •OH radicals are generated without UV radiation of the TiO_2 particles. After looking at our acellular results for ROS generation, we can conclude that ROS generation by particle itself is not very important. The low DMPO-OH signal as detected in our samples might also be due to some impurities of transition metals leading to Fenton – type reaction leading to the generation of •OH radicals.

We also studied the involvement of NADPH oxidase system in the induction of extracellular ROS. NADPH oxidase is the best characterised plasma membrane oxidase in phagocytic cells which serves as a specialised function in host defence against invading micro-organisms. Studies have suggested that the functional components of the phagocytic NADPH are also present in non-phagocytic cells (Meyer et al. 1999; Bauskin et al. 1998; Moulton et al. 1998; Fukui et al. 1995; Hiran et al. 1997; Jones et al. 1994). Since the experiments with DPI which is an NADPH oxidase inhibitor did not show any positive results, the role of NADPH oxidase remains to be investigated.

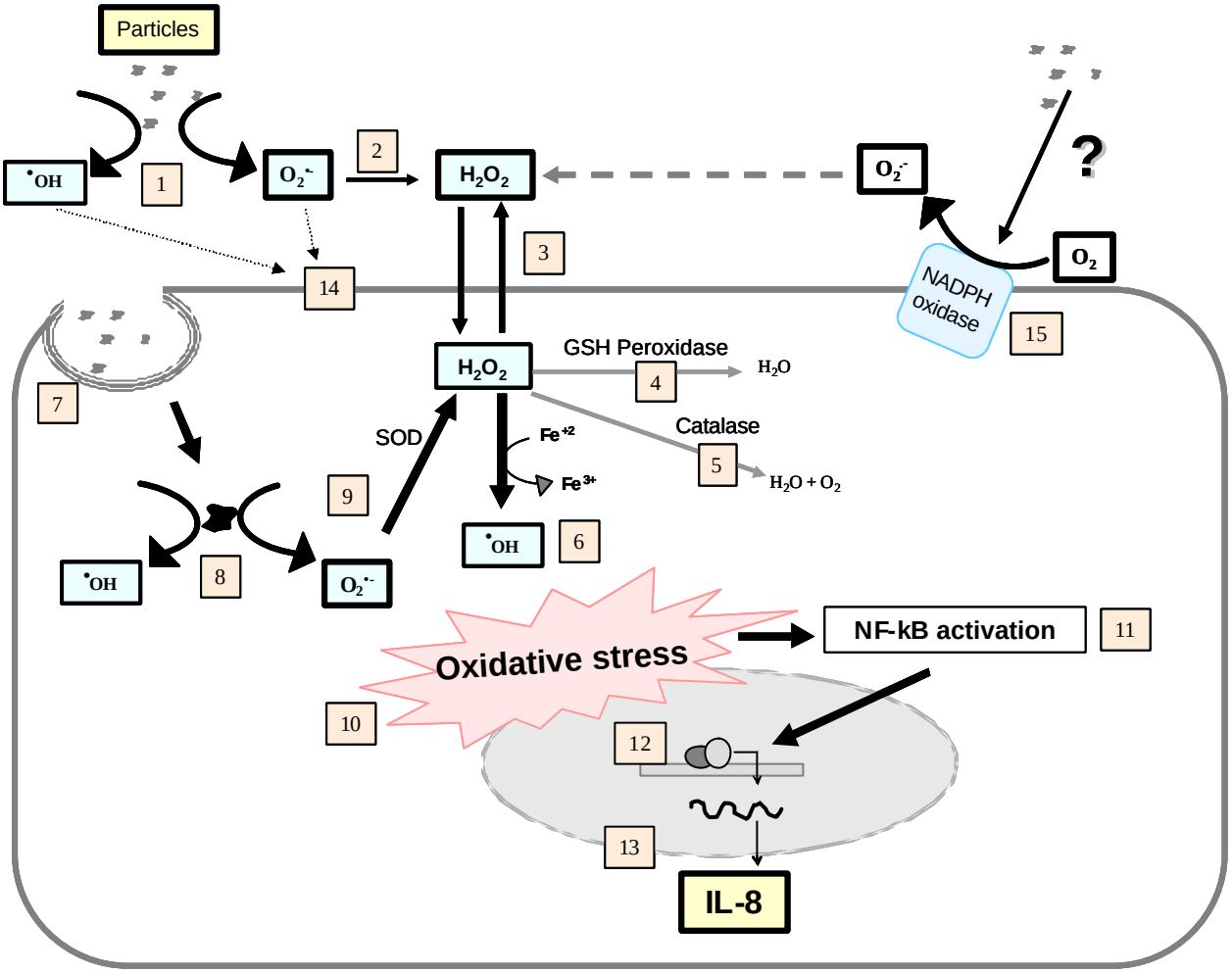

Figure 6-2 Scheme combining our hypothesis, study scheme and obtained results

The particles can induce $^{\cdot}OH$ and $O_2^{\cdot-}$ either outside (1) or inside (7, 8) the cell. $O_2^{\cdot-}$ can either spontaneously or in presence of extracellular (2) or intracellular (9) SOD, dismutates to H_2O_2 (2,9). Unlike $O_2^{\cdot-}$ and OH, H_2O_2 is not a free radical and is much more stable. H_2O_2 is able to diffuse across the biological membranes (3), whereas $O_2^{\cdot-}$ not. At lower concentartion, the H_2O_2 can be broken down into H_2O and O_2 by catalase and peroxidases (4, 5). But in the situation of oxidative stress, excess H_2O_2 can undergo fenton reaction to form OH radicals (6). We hypothesized that the activation of NADPH oxidase might be involved in extracellular ROS (15) which adds up to the pool of oxidative stress of the cells. This oxidative stress can lead to the nuclear translocation of NF-kB complex leading to IL-8 transcription and translation (11, 12, and 13). Oxidative stress causes membrane damage (14) leading to LDH leakage.

The minor fissure in our understanding the connecting link between ROS and IL-8 is because in our hands, unfortunately neither the addition of catalase nor SOD diminished the ROS (ESR) and IL-8 release (RT-PCR and ELISA) (Data not shown). In ESR experiments, one reason could be that either the concentration of antioxidant was too low as compared to the concentration of spin trap or the rate of reaction between the ROS and the spin trap was much faster than that of the ROS and antioxidant. No change in IL-8 expression (RT-PCR) on adding antioxidants might be due to the fact that the SOD and catalase are extracellular antioxidants and hence cannot enter the cells. Therefore we did not observe any reduction in the IL-8 signal. However, when we look at the mechanism of action of SOD, the missing change in the IL-8 expression on adding it to the cells is not a revelation. Adding SOD speeds up the dismutation process and hence leading to more H_2O_2 in the system which can induce IL-8. H_2O_2 is a potent inducer of IL-8 and we also proved it experimentally in our A549 cells which on H_2O_2 treatment gave a dose dependent response (Data not shown). The absence of the catalase effect on IL-8 can also be explained by the fact that the extracellular catalase breaks down the H_2O_2 into H_2O and O_2 outside the cell. Simultaneously, some of the extracellular H_2O_2 can also diffuse into the cell that can still induce the intracellular oxidative stress and cell signalling leading to IL-8 release. To further confirm this hypothesis, future experiments with cell permeable antioxidants in combination with extracellular antioxidants are suggested to provide some valuable clues in this regard.

Another possibility is that the •OH radicals might be responsible for IL-8 induction. It is well known that H_2O_2 generates hydroxyl radicals by means of Fenton reactions (Lloyd et al. 1997). This might also be a possible phenomenon in our experiments since almost all the biological system contains Iron. In addition, hydrogen peroxide and superoxide radicals in the presence of ferric ions can participate in iron-catalyzed Haber-Weiss reaction to yield more hydroxyl radicals. •OH radicals are known to upset the intracellular redox balance, leading to the activation of transcription factors such as NF-?B (Jimenez et al. 2000) or AP-1 (Timblin et al. 1998). However, additional work is required to elucidate the specie(s) contributing to the IL-8 induction in our experiments. All these factors are represented in a schematic diagram in Figure 6-3.

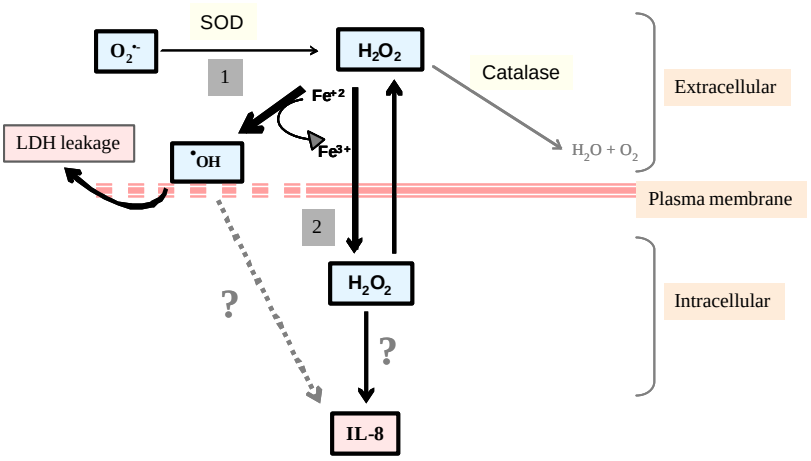

Figure 6-3 ROS and IL-8 – The speculation

Adding SOD at step 1 speeds up the dismutation process and hence leading to more H_2O_2 which can induce IL-8. However, no effect of catalase on IL-8 can also be explainable by the fact that the extracellular catalase breaks down the H_2O_2 into H_2O and O_2 but at the same time H_2O_2 can diffuse into the cell (where extracellular catalase canot reach) and can still induce IL-8 (step 2). The $^{\cdot}OH$ radicals (fenton reation) formed near to the cell membrane can lead to the membrane damage causing leakage of LDH. Apart from that, $^{\cdot}OH$ radicals can upset the redox balance within the cell, leading to the activation of transcription factors such as NF-kB and thus leading to IL-8 expression.

So, from our studies we have an adequate amount of evidence that UF particles induce more oxidative stress and IL-8 release than the fine particles but we could not prove it by using antioxidants. We can also speculate that the particle generate IL-8 which is absolutely independent of oxidative stress (Epiphenomenon*).

We have however incomplete evidence about the nature of reactive oxygen species involved in the IL-8 induction in A549 cells but regardless of the nature of oxidative species involved, there is substantial evidence that the cell membrane damage is the direct result of the oxidative damage. Cellular membranes and especially the plasma membrane are primary targets of ROS. The fatty acid side chains of membrane phospholipids undergo peroxidation under oxidative stress (Nachbar and Korting 1995). Once the cell membrane barrier is compromised, TiO_2 particles can exert oxidative actions directly and various cellular components can be subject to oxidative modifications including cytosolic and nuclear lipids and proteins leading to cell signalling.

*An epiphenomenon is a secondary phenomenon that occurs alongside a primary phenomenon.

Apart from several *in vitro* studies showing particle uptake by cells, there is also *in vivo* evidence of particle uptake of particles by the cells (Lee 1985). In our uptake results, there was no preferential uptake of ultrafine particles which we would expect if we consider uptake is important phenomenon for IL-8 response. However, methylation of particles did show some effect on the particle uptake at low dose, but apart from this, the methylation did not show any effect in any measured endpoints in this study.

In vivo studies done by Höhr et al. (2002) also used the same methylated and nonmethylated TiO_2 particles and they observed that the UF particles were more inflammogenic than fine particles and less acute inflammation with hydrophobic TiO_2 at 1 mg dose. The possible elucidation to this is that *in vivo* experiments, the number of inflammatory cells is measured in BAL fluid which denotes the inflammatory effects induced by the particles. In addition to epithelial cells, the inflammatory cells (PMNs) present in lungs also play an important role which might enhance the inflammation *in vivo*. In our studies, we measured IL-8 as a marker of oxidative stress in submerged cell culture containing epithelial cells (A549) only. Other critical factor to be considered is that the IL-8 is not produced in rats and MIP-2 is the rat equivalent of IL-8. In this case, the comparison of MIP-2 and IL-8 might provide a better justification to our *in vitro* results as compared to the *in vivo* oxidative stress represented by the number of PMNs. Our uptake data (TEM) shows the rapid internalisation of particles by A549 cells and uptake of fine particles is more or less similar to the uptake of the ultrafine. In contrast, our IL-8 data shows that the ultrafine induce more IL-8 than the fine particles. This indicates that in our experiments, the uptake is not a decisive factor for inducing IL-8 release.

From our studies and the studies done by other groups, we can conclude that the particle size, and more explicitly the surface area might play a significant role in the inflammatory potency of poorly soluble particles such as TiO_2 (Greim et al. 2001; Tran et al. 2001; Borm et al. 2004). Particle surface properties apparently play an important role in case of very reactive particles like quartz which is already very toxic and inflammogenic at fine particle size because of its extremely reactive surface (Schins 2002; Albrecht 2004; Fubini 2004). This can also be confirmed in the experiments where we used DQ12 quartz as a positive control. In addition, the likelihood that the effects we observed in our study are very specific only for TiO_2 particles can not be ruled out. Therefore, we emphasise that additional investigations are necessary using other materials in the nanoparticles size range in order to see to which extent our current results represent general features of nanoparticles toxicology. Moreover, it is

important to be cautious about interpretation of our current results in relation to the understanding of the health effects of particulate air pollution in general. TiO_2 particles are not a surrogate for ultrafine particles in ambient air, but these particles served as a model to attain valuable information about principles of ultrafine particle behaviour and mechanisms related to their toxicity and inflammatory effects in general. Dick et al. (2003) used four different kinds of same sized ultrafines and observed particle-material specific effects. This may indicate that our results might be relevant for TiO_2 particles only and not for all ultrafine particles in general. The implication of our *in vitro* data to occupational exposure is limited, but several test methods employed in this thesis may be used to screen hazards of nanomaterials based on fine or ultrafine TiO_2 with chemical surface modifications. As such this study may serve as a first step towards a conceptual understanding of nanoparticles toxicology.

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Acknowledgements

This study was carried out at the Institute of Environmental Medicine of Heinrich Heine University, Duesseldorf, during the years 2002-2004. Finishing this thesis work was a step by step progression, accompanied with hardships, resentment, disappointment, encouragement; faith and kind help of a number of people. It was indeed the teamwork which helped me to finish my work. Though it will not be enough to express my gratitude in words to all those people who helped me, I would still like to give my loads of thanks to all these people.

I wish to convey my sincere gratitude to Professor Paul J.A Borm, former Head of the Department of Particle Toxicology, and Professor Jean Krutman, Director of the Institute, for providing excellent research facilities.

My supervisor Dr. Peter Frans Roeland Schins, who introduced me to the mesmerizing world of ultrafine particles, deserves my innermost gratitude for all the help, guidance, encouragement and support he has given me during these years. With his enormous amounts of liveliness, integrity, fortitude, dedication and patience he has gained my greatest approbation and uppermost respect. Thank you Roel for your unfailing optimism and efforts during this project. I am indebted for your productive criticism and valuable annotations during the preparation of this manuscript. You are a perfect proof-reader and it is hard to imagine how you could manage to finish editing in such a short time. Thank you for keeping embarrassing mistakes in my thesis away from seeing the light of day. Dankewell !!

Professor Regine Kahl, who in the course of time eventually became my important collaborant and my erstgutacher, has greatly stunned me not only with her scientific achievements, but also with the effectiveness and serenity she makes them. I am also extremely thankful to her for giving me a golden opportunity to attend her lectures and PET courses.

I would like to express my deep sense of gratitude to Professor Paul J.A Borm, who created an extraordinary and stimulating research environment for the particle research group. He is always been inspiring, encouraging and had excellent opinions for arranging the thesis. I am indebted to him for editing my thesis, despite of having and extremely hectic schedule. My sincere thanks also to Prof Wunderlich, for revising my thesis.

Dr. Tingming Shi, our ESR specialist, has generously shared with me his gigantic knowledge of Oxidative Stress, has been a perpetual source of advice and encouragement, and also has an astonishing capacity to see a silver lining even in a darkest cloud, for all of which I am perpetually grateful. Shey Shey!!

I also want to thank our collaborators, Prof. Fubini, Prof. Martra (Department of Inorganic Chemistry, Italy) for providing particles and useful information related to them. I immensely enjoyed the working atmosphere at the Department of cell physiology and cell toxicology, University Paris and want to affectionately thank everyone for extremely useful discussions of various problems with laboratory practice, protocols etc. My very special thanks Professor Marano for kindly giving me the opportunity to visit her laboratory at Paris to learn novel techniques and to Armelle who introduced me to the Primary cell culture techniques and for her skilful and friendly collaboration. I also wish to express my gratitude to Chiara Schiolla and all those who participated in

this project before me. Professor Engel is gratefully acknowledged for kindly providing the first opportunity to do research in Germany.

I'd also like to thank all the members of our glorious particle research team, especially Dr Catrin Albrecht, Dr. Karim Bagate, Dr. Ad Knappen, James Justus Meiring, Damien van Berlo, Hui Li (sunshine in the morning), Petra Haberzettl, Mrs. Veena Suri and Mrs. Wick for sharing these years of scientific work with me. I would like to convey my special Thanks to Catrin and to Damien for their unselfish help that enabled me to present my thesis more smoothly and unmistakably in German and English. Dr. Doris Höhr deserves a very special gratitude for skillfully performing most of the Electron Microscopic work of the TiO_2 samples used in this study. A special thanks to Dr. Andrea Becker for her expertise with the RT-PCR method. Jemo and Demo thank you very much for your fabulous sense of humor which has enlightened countless otherwise grey days. It was not only for being such pleasure to work with, but also for treasured friendship of you all.

All through this study, I have been incredibly fortunate to enjoy skilful technical assistance of Ms. Astrid Winzer who has provided invaluable help with cell culture, Ms. Kirstin Lederman for Western blotting and Ms. Cristal Weishaupt our ELISA specialist.

Nicht zu vergessen möchte ich Frau Angelika Simons ganz herzlich für ihre Geduld und Mühe danken, die sie mir vor der Abgabe entgegengebracht hat.

I'd like to express my gratitude to all my friends and family members for being there for me all the time. There are far too many friends to be mentioned individually. However I owe my special thanks to Gonja, Semi, Anja, Suman, CP, Ruchika, Sushma, Jahid, Sonja for their valuable friendship. My sisters Neelam, Ute, Renu, Kirstin, Rekha and Birtie; brother-in-laws, Anil, Jan, Dharmedra, Heemendra, Jörn; nephews Anubhav, Johannes, Mark, Mallika, Utkarsh, Shivansh, Chinu and Maulik and Parents-in-law for providing priceless love and affection. I owe my very special thanks to my brother- in- law Jörn Meyer Ohlendorf for his never-failing patience with me during the plentiful hours he exhausted for preparing lay-outs for my thesis. Jörni, I would like to also thank you for always uncomplainingly and compassionately advising me.

I am greatly indebted to my mother, father, and parents in law for their love, encouragement, and persistent faith in me.

This work was financially supported by German Ministry of Environment, European Graduate School, which are all gratefully acknowledged for creating organizational and financial opportunities to accomplish this project.

At last, but far from least, I would like to convey my gratefulness to my husband Lutz who has contributed to the genesis of this thesis through his loving support in thousand and one wonderful ways than a page can hold.

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Düsseldorf, July 2005

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List of publications

6.1.1 Published abstracts

Singh, S.; Shi, T.; Höhr, D.; Martra, G.; Fubini, B.; Borm, P.; Schins,R. The Expression of Interleukin-8 in A549 Human Lung Epithelial Cells by Fine and Ultrafine Titanium Dioxide and its relation to particle surface properties *Archives of Pharmacology*, Volume 367.supplement 1. march 2003.

Singh, S.; Shi, T.; Höhr, D.; Martra, G.; Fubini, B.; Borm, P.; Schins,R. Interleukin-8 expression by titanium dioxide in A549 cells: effects of particle surface area and surface coating *Toxicology and Applied Pharmacology* Volume 197, number 3, June 15, 2004.

6.1.2 Full Papers

Roel P.F Schins, Seema Singh, Gabriele Wölke, Joachim Heinrich. Whole blood cytokine production by particulate matter in relation to its endotoxin content and toll-like receptor 4 gene variants *(in press)*.

Seema Singh, Rodger Duffin, Catrin Albrecht, Paul J.A. Borm, Gianmario Martra, Bice Fubini, Roel P.F. Schins. Cytotoxic and inflammatory effects of titanium dioxide dusts in relation to particle surface area *(to be published).*

Seema Singh, Tingming Shi, Rodger Duffin, Catrin Albrecht, Bice Fubini, Gianmario Martra, Paul J.A. Borm, Roel P.F. Schins. IL-8 release from human lung epithelial cells upon treatment with ultrafine titanium dioxide particles involves surface area-driven cellular oxidative stress *(to be published).*