DIFFERENTIAL EFFECT OF RESIDUAL OIL FLY ASH (ROFA), AMBIENT PARTICULATE MATTER AND DIESEL EXHAUST PARTICLE (DEP) ON THE HUMAN PULMONARY ALVEOLAR EPITHELIUM

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DECLARATION

I certify that this thesis is my original work and that all material included which is not my own work has been referenced accordingly.

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ABSTRACT

Epidemiological studies have shown that exposure to particulate matter (PM) contributes to cardiopulmonary morbidity and mortality. Evidence suggests that the diesel exhaust in ambient air in major cities mediates a significant proportion of the adverse health effects. However, it has been speculated that these effects might be increased in cities within the oil and gas producing countries as a result of a combined effect of inhaling both diesel exhaust particles (DEP) and residual oil fly ash (ROFA)

It is hypothesised that the nature and degree of alveolar epithelial reactivity will depend on the physico-chemistry of the particles, and that DEP/ROFA mixture will result in greater bio-reactivity compared to the same amount of DEP and ROFA alone. The specific aim is to determine the physico-chemical characteristics of ambient PM, DEP, ROFA and DEP/ROFA mixture and to compare their cellular reactivity/effects on human alveolar epithelium *in vitro*.

The physico-chemical composition of the particles was determined using transmission electron microscope and energy dispersive x-ray spectrometer (TEM/EDX) analysis. Primary human alveolar type 2 epithelial cells (AT2) and a transformed human alveolar type 1 (TT1) epithelial cell line were exposed to

ambient particles, ROFA, DEP and a ROFA/ DEP (1:1) mixture. Reactive oxygen species (ROS) production and cell viability were evaluated using the dichlorofluorescin (DCFDA) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium (MTT) assay respectively. Enzyme-linked immunosorbent assay (ELISA) was used to quantify release of CXCL8, IL-6, and CCL2.

TEM/EDX results revealed that transition metals and other reactive elements were present in the samples. The result also showed that ambient PM has an amorphous structure which made particle size difficult to determine. The estimated average diameters of ROFA and DEP were 74 ±17nm and 65 ± 2nm respectively. When mixed, it was 76 ± 24nm. Morphologically, these particles have large spherical, agglomerate and crystalline structures. Cellular exposures to PM were associated with significant increases in cytokine and chemokine release compared to non-treated controls ($P< 0.002$). At high concentrations (50 and 100μg/ml) the ROFA/DEP mixtures caused a significant increase in IL-6, CXCL8, and CCL2 release by TT-1 cells compared to exposure to an equivalent amount of DEP or ROFA alone ($P<0.05$). Ambient PM induced a higher level of mediator release compared to those observed with DEP while the DEP/ROFA mixture reduced cell viability and also triggerd rapid intracellular ROS release greater than that observed with ambient PM, DEP or ROFA alone.

All these changes are concentration- and exposure time-dependent. However, the results are not statistically significant.

In conclusion, DEP/ROFA mixture resulted in significant changes in cytokines/chemokines induction, ROS release and reduction in cell viability compared to DEP and ROFA alone (as well as PM). There was very little difference in particle size and shape, suggesting that differences between the reactivity of the test materials could be related to surface chemistry, for example elemental composition, although this could not be deduced in the current study. Other organic components might also be important, although they were not analysed in this investigation. Consequently, future work is needed to unravel the specific role of transition metals, as well as the organic and other inorganic components of the particles, in their cellular reactivity. It is also possible that mixing of ROFA and DEP causes reactions between volatile chemical components that enhances their cellular reactivity. These unique findings are of significance to those who live or work in cities where there are likely to be high levels of both types of emissions, which might have significant health effects.

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TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER ONE

CHAPTER TWO

LIST OF TABLES

ACRONYMS AND ABREVIATIONS

ASTM	American Society for Testing and Materials
AP-1	Activator protein 1
AQM	Air Quality Strategy
CAFE	Clean Air for Europe
CB	Carbon black
CDNP	Combustion–Derived Nanoparticle
DC	Dendritic Cell
DEP	Diesel Exhaust Particle
ECVAM	European Centre for the Validation of Alternative Method
EDX	Energy Dispersive X-ray Spectrometer
EFTEM	Energy Fitting Transmission Electron Microscopy
FA	Fly Ash
GSH	Glutathione
GSSG	Glutathione disulphide (oxidized glutathione)
ICAM	Intercellular Adhesion Molecule
IPL	Isolated Perfused Lung

LDH	Lactate Dehydrogenase
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
NF_kB	Nuclear Factor-Kappa B
NPCB	Nanoparticle Carbon Black
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
PAH	Polycyclic Aromatic Hydrocarbon
PCR	Polymerase Chain Reaction
PM	Particulate Matter
RAGE	Receptor for Advanced Glycation End Products
ROFA	Residual Oil Flash Ash
ROS	Reactive Oxygen Species
SEM	Scanning Electron Microscopy
SMMT	Society of Motor Manufacturers and Traders
TEM	Transmission Electron Microscope
TEER	Transepithelial Electrical Resistance
TSP	Total Suspended particle
VOC	Volatile Organic Carbon

CHAPTER ONE

1.0 INTRODUCTION

Anthropogenic air pollution is one of several major problems currently confronting humanity globally. About 2 million premature deaths were reported to have occurred annually due to urban outdoor air pollution and indoor air pollution (WHO European Region, 2005). It is now common knowledge that the health of more than 1.8 billion people may be at risk from poor air quality (WHO, 2009). With the world population now forecast to reach 7.5 billion by 2020, stabilising at approximately 9 billion by 2050; with 90% of the growth occurring in developing countries, most of which concentrating in urban areas, the current level of risk would increase 3-fold (Satterthwaite, 1993). Dincer (2001) ranks ambient air pollution alongside global issues such as acid rain, stratospheric ozone depletion and global warming. The quality of anthropogenic air quality has been under the constant influence of mankind's evolution, both in terms of increase in population, industrialization and in recent times globalisation (Anthony, 2000). These three broad concepts have contributed to ambient air pollution but it is important to note that their relative contributions are rarely quantified.

Historically, episodes of air pollution during the 17th century reported in Europe, and indeed, North America provided good evidence that high level ambient air pollution could result in morbidity and mortality (WHO, 2000). A fundamental pattern appeared to have emerged at the onset of industrial revolution in the 19th

century which became a pointer to the surge in the level of ambient air pollutants (Klumpp, Ansel & Klumpp, 2004). Among others, industrialization, especially in the developed world, set in motion the widespread use of fossil fuels (oil, gas and coal) which are now the main source of atmospheric air pollutants.

Today, the poor state of our ambient air is compensated in the variety of available consumer goods, the efficiency of transport systems, for successes recorded in today's technology and increased foreign exchange earnings for oil-rich countries, especially in developing economies (Qurashi & Hussain Tajammul, 2005).

Population growth is the second fundamental contributor to ambient air pollutant overload. With population numbers literally exploding around the world, the demand for food and other goods has continued to increase exponentially (Qurashi & Hussain Tajammul, 2005). This, according to industrial analysts, leads to expanded production and use of natural resources, which in its turn leads to higher utilisation of fossil fuel and consequent emission of particle pollutants in the ambient air (Anthony, 2000)

Globalization is also a major driver towards anthropogenic air pollution. In many cases, globalization has become an effective facilitator of poor air quality. In developing countries, manufacturers take advantage of lax air quality regulations and cause air pollution or what is now known as air pollutant dumping (Brennan, 1999). With this "benefit" of profit-maximization, made possible by the availability of raw materials, low tax, large work force as well as cheap labour, big industries

prefer to move their facilities to such "pollution havens" rather than work in more regulated markets.

Anthropogenic pollution is not limited to human-driven activities; however, the contributions of natural, also known as biogenic, sources have existed even before the advent of industrial revolution (Rothman & De Bruyn, 1998). Among these are volcanic eruptions which have for millions of years spewed particles and gases into the atmosphere. Forest fires have caused significant release of gases and particulate material during lightening strikes. Decomposition of organic matter, mainly plants and animals, increase the pollutant loads in the atmosphere during wind storms. Trees and other vegetative cover also contribute large amounts of pollen and spores to our atmosphere. These natural pollutants can be a source of risks at times, generally, they are not as much of a problem compared to anthropogenic sources (U.S. EPA, 2011).

Until the mid 1970s, it was generally thought that ambient pollution levels in Europe and America did not threaten human health (Holland et al., 1979). However, reviews from epidemiological studies during the last two decades have consistently shown that moderate and low concentrations of traditional pollutants, such as ambient particles, black smoke, sulphur dioxide and nitrogen oxides, can have both short- and long-term effects on health. Review of evidence from anthropogenic air pollution sources has also revealed that ambient air pollution is source-specific, for instance a change in emission sources influences the total

ambient load, which gives rise to corresponding changes to the morbidity pattern at any given time (Katsouyanni, 2003).

In the United Kingdom, with the number of vehicles increasingly becoming the most important source of ambient air pollution burden, especially in London, this has resulted in changes in the ambient air pollution mixture, now characterized by high concentrations of ambient particulate matter, nitrogen oxides and photochemical oxidants (Anderson et al., 1996). Comparatively, in developing countries, severe air pollution episodes have increased the risk of respiratory morbidity and mortality (Seaton et al., 1995). Today, many developing countries, especially oil-rich nations, are experiencing a unique industrial revolution of their own, with ever-increasing industrial growth, resulting in an ambient air pollution crisis (Hardoy & Satterthwaite, 1991)

Ambient air quality legislation has targeted anthropogenic sources in line with the level of existing knowledge. These sources are categorized into two broad categories, viz: mobile and stationary sources. Mobile sources of ambient air pollution include most forms of transportation covering: trains, trucks, passenger cars, aircrafts, marine vessels, motorcycles, tricycles, and small off-road equipment that can be moved from one location to another (e.g. recreation equipment, construction, agricultural). The contribution of mobile sources to ambient air emissions will continue to increase as long as there are increased numbers of mobile vehicles (Smith, 1993).

Stationary sources of ambient air pollution consist of non-moving sources such as power generating plants chemical plants, oil refineries, steel factories, glass and cement factories and many other industrial facilities. Area sources are stationary sources that are not yet subjected to a discharge permit from the government or local authority, but they emit significant pollutants into the ambient air, for instance, prescribed burning, residential wood use or communities of homes using wood stoves for heating, and dry cleaners. In contrast, a point source is a single stationery source of pollution, such as an industrial facility, that typically operates under some kind of government authorization (e.g. a permit, approval or regulation), such as a smokestack or storage tank that emits air pollutants.

Ambient air pollutants routinely measured in organised monitoring systems include various indicators of ambient air pollutant gases and particulates. These are referred to as "criteria pollutants" and are regulated under the clean air act (U.S. EPA, 2011) due to their historical importance, concentration, and overall effects on human plants and animals. These include sulphur dioxide (SO_2), oxides of nitrogen (NO_x: NO, NO_2), carbon dioxide (CO_2), ozone (O_3), lead (Pb) and particulate matter (PM).

1.1 Ambient particulate matter (PM)

A typical ambient air pollutant is a mixture of several components, mainly PM, CO, SO_2, NO_2, Pb and volatile organic compounds (VOCs) that have previously been described as "criteria pollutants". The independent effect of these pollutants and

the possibility of combined effects, led to the development of a European Union policy on Clean Air for Europe (CAFE) which commissioned a WHO review of the health effects of transport-related air pollution in Europe, which found evidence that exposure to air pollutants can affect human health in a variety of ways, the result of which was the establishment of air quality guidelines (U.S. EPA, 2004).

Much interest has been devoted to PM compared to other pollutants because of their increased health effects (Mills et al., 2009). The composition and quality of ambient PM is directly affected by the day-to-day activities of humans and this has a direct effect on both public health and ecosystems balance. PM is a complex mixture of various organic and inorganic substances comprising of soot, endotoxin, dust, pollen and liquid droplets distributed among the gaseous and particulate phases (Mills et al., 2009). The degree of PM complexity varies depending on sources and may exhibit much heterogeneity in size, morphology, elemental composition, biological activity, physical behaviour and chemical speciation (Mazzarella et al., 2007). Conventionally, PM is classified according to size, composition and origin. Thus, a given mass of PM may contain dusts, pollen, micro-organisms and many other substances, including inorganic material derived from combustion processes at very high temperature. The focus of this thesis is on combustion-derived inorganics capable of releasing particles within the coarse, fine and ultrafine range (Langley Data Centre, 2007). Combustion–derived particle

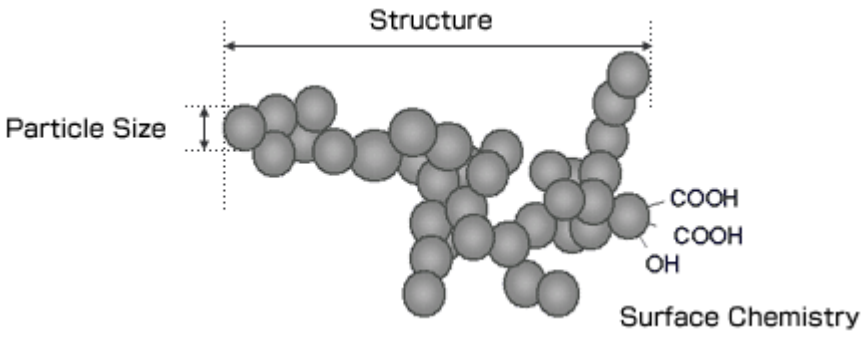
emissions (carbon black, diesel exhaust particles, residual oil fly ash and welding particles) are briefly described below.

1.1.1 Carbon black particles

Chemical analyses have shown that carbon black (CB) is a low-solubility particle produced industrially from the incomplete thermal decomposition of hydrocarbons (Donaldson et al., 2005). In some instances, the process of its generation is controlled to achieve a predefined and reproducible size, while, on the other hand, it is emitted from chimneys and exhausts pipes in a random manner. All these methods and processes produce furnace black, gas black, lamp black, and others. The basic properties of CB are defined in terms of their spherical particle nature denoted as "particle size," incorporating the surface chemistry (soluble polar end) structure as shown in figure 1.0

The surface of CB can be oxidized, once released into the atmosphere or *in situ*, by a variety of oxidants forming carbonyl, carboxyl, phenolic and quinone group (Studebaker et al., 1957, BeruBe et al., 2007) referred to as "surface chemistry". Research evidence (Donnet & Henrich, 1960) also revealed that the surface enrichment, particle size and internal structure, determines the blackness, solubility, dispensability and affinity of CB with other particles, thereby exerting toxic effects when acting alone or in mixtures with other particles or elements.

Figure 1.0 The physicochemical structure of carbon black



(Tsubokawa, 1992)

1.1.2 Diesel exhaust particles

Diesel exhaust particles (DEP) are a major product of internal combustion engines which represent about 20-40% of the fossil fuel utilization (BeruBe et al., 2007) and up to 80% of the mass of PM_{10} collected in urban areas mostly in developed countries (BeruBe et al., 2007). Diesel emissions consist of soot particles in the form of carbon black, surrounded by condensed organic compounds, and trace metals from lube oil and fuel additives (BeruBe et al., 2007). Earlier research by Matti Maricq (2007) revealed good evidence that DEP are normally agglomerates of hundreds of volatile/semi-volatile species adsorbed onto their refractory carbonaceous cores.

X-ray diffraction and TEM data revealed that the core of DEP spheres are composed of concentrically piled thermodynamically unstable carbon network. This unique organizational structure determines, to a large extent, the potential to hold material such as VOC, transition metals and sulphur, in large intra-network

spaces (Bayona, Markides & Lee, 1988). The outer skeleton is better ordered and more stable and is characterised by graphitic micro-crystallites of orientated carbon sheets. The structure of DEP and their potential to adsorb metals and other reactive substances contributes to their ability to elicit cellular effects. Some of these constituents are known carcinogens (Bérubé et al., 2006, BeruBe et al., 2007). Many toxicological studies have shown that CB has significantly less bio-reactivity than DEP. Evidence suggests that DEP are capable of inducing systemic inflammation by imparting oxidative stress in human cell systems (Stayner, Dankovic & Steenland, 1998, Solomon & Balmes, 2003).

1.1.3 Residual oil fly ash (ROFA)

ROFA is emitted as a by-product of fossil fuel oil combustion most commonly found in crude oil refineries, fossil fuel steam generators, and other large-scale oil-fired stationary sources and petrochemical plants. All these sources have organic liquid fuel as a primary source (Koike et al., 2002, Shima et al., 2006). ROFA can remain airborne for extended periods due to its smaller aerodynamic diameter, and also can deposit in the distal lung on inhalation (Gilmour et al., 2004).

Neutron activation and various characterisation techniques have revealed that ROFA contains transition metals and acids incorporated into a particulate carbonaceous core. The presence of toxic elements, such as arsenics, cobalt, Lithium, Zinc, and several PAHs such as naphthalene, acenaphthylene, fluorene, acenaphthene, anthracene, flouranthene and phyrene were also detected (Dreher et

al., 1997). A result of analysis of ROFA from a steam generator revealed that the predominant elements of ROFA from precipitated samples were soluble fractions of Ca, Mg, and Zn, while Fe , V, Ni, and Al were insoluble, and V was found to be mostly insoluble (Arantes-Costa et al., 2008).

1.1.4 Welding particles

The principal by-products of welding processes are welding fumes, radiation and gaseous particles. Different energy sources employed for welding include gas flame, electric arc, laser, electron beam, friction, and ultrasound. All these sources determine the welding type. Fumes generated during welding consist of particles whose chemical composition and size distribution depend on the type of welding process. Particles generated in stainless steel welding, for instance, are known to contain high concentrations of chromium and nickel (Chen & Lippmann, 2009)

Research evidence has shown that the particle fumes from stainless steel welding are particularly toxic (Chen & Lippmann, 2009). The surface of an individual particle may be enriched with more volatile elements, like manganese or the alkali metals, forming core-shell particle morphologies (Hooftman, Arkesteyn & Roza, 1988). Vaporized metal produced by the heat of the welding process oxidises to produce a fume containing metal oxides such as aluminium, cadmium, chromium, and copper and many of which are water soluble (Karlsen et al., 1992). The effects of welding particles have also been studied *in vitro* in both human and animal cell cultures, and both produced marked pro-inflammatory effects (Antonini, 2003).

These effects are driven largely by the metals (Naslund et al., 1990, Taylor et al., 2003, McNeilly et al., 2004) which undergo redox-cycling resulting in oxidative stress.

1.2 Particulate matter size distribution

Particle size covers a wide range, from tens of micrometers and a few nanometres. The degree of variation depends mostly on their aerodynamic diameter. The aerodynamic diameter (that is the size of a unit-density sphere with the same aerodynamic characteristics) is described as the particle size as seen in figure 1.0. Traditionally, the size of ambient particles varies over four orders of magnitude as shown in figure 1.1. The particle aerodynamic diameter also influences their transport, and removal in the air and their deposition within the respiratory system (Donaldson et al., 2005).

The distribution of particulate materials with respect to size is an important physical criteria governing PM behaviour (Witby & Sverdrup, 1980). For many mobile and stationary sources emitting particulate materials, the observed PM distribution in the gas stream approximates to what is known as a lognormal distribution. This generates a normal bell-shaped curve (figure 1.1) for each particle sub-group. Using the statistical notations, PM size distribution in terms of the logarithm of the particle diameter represented along the X-axis ($\log D_p$) and the measured differential concentration on the Y- axis expressed as the volume of particles per cubic meter of air having diameters in the size range from $\log D_p$ to log

($D_p+\Delta D_p$). Statistically, if $\Delta V/\Delta(\log D_p)$ is plotted on a linear scale, the volume of particles between D_p and $D_p+\Delta Dp$ is proportional to the area under the curve of $\Delta V/\Delta(\log D_p)$ versus $\log D_p$ as seen in figure 1.1. Similar considerations apply to distributions of number, surface area, and mass of PM

In PM research, four main conventions have been in use in the classification of particulate materials by size (Whitby, 1978). These include; (a) The modes, based on the observed size distributions such as the coarse, fine, nuclei and the accumulation modes (b) Occupational sizes, based on the entrance into various compartments of the respiratory system (c) Legally specified, regulatory sizes for air quality standards and (d) Cutpoint, usually based on the 50% cutpoint of the specific sampling device.

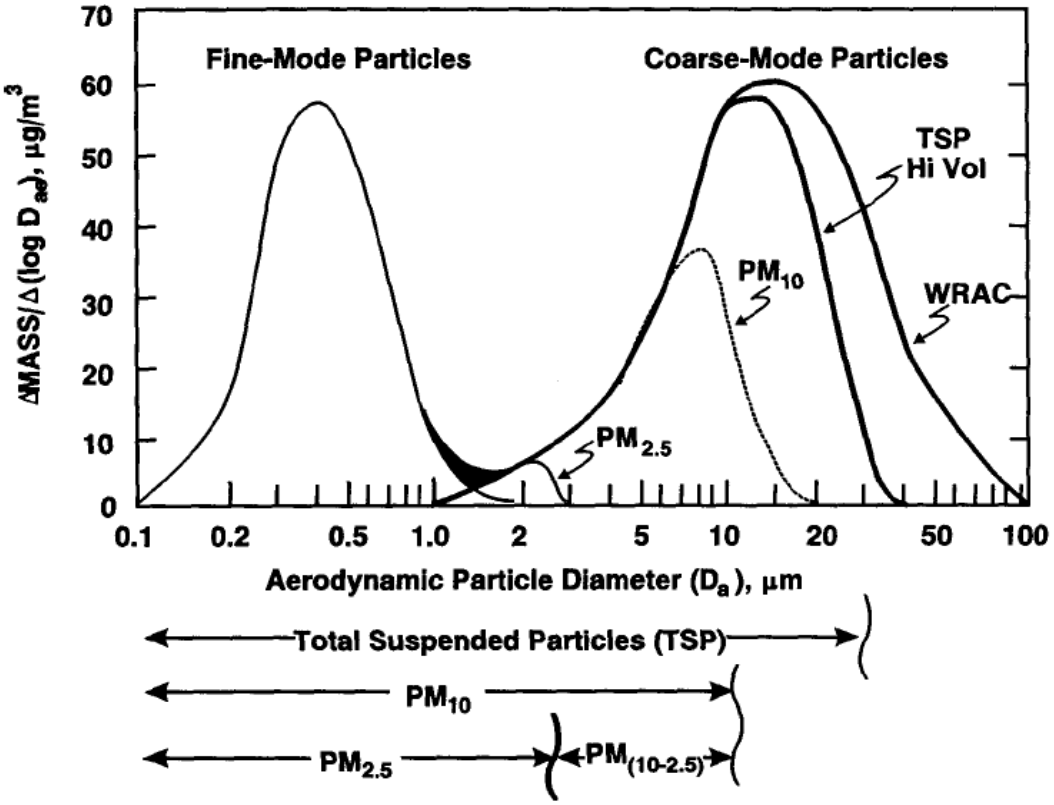
The consideration of size-selective sampling takes into account the collection of particulate materials within or below a specified aerodynamic size range. This is usually defined by the upper 50% cutpoint size, and has arisen in an effort to measure PM size fractions with some special significance including health and source apportionment.

Dichotomous samplers (figure 1.1) split a polydispersed PM population into two size fractions (larger and smaller) during sampling. For instance, total suspended particle (TSP) has always been defined by the design of the High Volume Sampler (hivol) which collects all of the fine particles but only part of the coarse particles. It should be noted that the upper cut off size of the hivol depends on the wind speed

and direction, and may vary from 25 - 40µm. In comparison, Cascade Impactors utilizes multiple size cuts to obtain a distribution of size cuts relevant for mass or chemical composition measurements. For instance, the Wide Range Aerosol Classifier (WRAC), for which a particle distribution is depicted in figure 1.1, was designed specifically to collect the entire coarse mode (Environmen & Burton, 1995)

Two rough size-based categories of ambient PM are commonly in use as a result of the ease to sample them in the ambient air. These groupings are generally referred to as fine and coarse particles (figure 1.1).

Figure 1.1 Schematic representation of the size distribution of fine- and coarse-mode particulate matter in ambient air



(Wilson & Suh 1997, Kreyling, Semmler-Behnke & Moller, 2006)

The largest particle type is called the coarse fraction, designated as $PM_{2.5-10}$. The particles have an aerodynamic diameter of between 2.5μm-10μm. The notation PM_{10} is also used to describe particles of 10 micrometers or less. These are released in many mechanical processes causing disintegration of large solid particles. The processes include: road construction, demolition, mining, quarrying and agricultural processes. Wind–blown dust is a product of these processes; other particles in this range include: sea sprays, mould spores, pollen, micro and macroscopic flora and fauna.

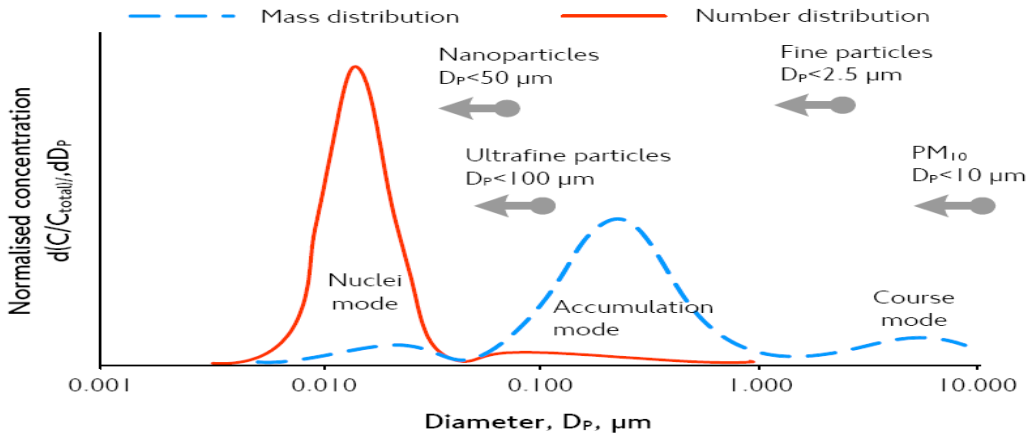
Research evidence has shown that the amount of energy required to break these particles increases as the resultant size decreases. This establishes a lower limit for coarse particle formation.

The fine fraction or mode are typically produced from gases and combustion processes or are formed within the atmosphere by chemical processes (Whitby & Sverdrup, 1980). Unlike coarse particles, major sources of fine particles include all types of combustion activities (motor vehicles, power plants, wood burning, etc.) and certain industrial processes. They have an aerodynamic diameter of 2.5 μm or less. Fine particle ($PM_{2.5}$) fractions have substantially longer residence times and have greater potential to affect PM concentrations further away from emissions sources, compared to coarse fractions with aerodynamic diameters exceeding 3μm. Therefore, fine particles behave more like gases (U.S. EPA, 2004). An earlier study by Oberdorster (1996) revealed that particles congregate in different sub-ranges

according to their formation process. The smallest particles, less than 0.1 µm are formed by nucleation. Hence, particles in nucleation range are also known as ultrafine particles. This consists of particles with diameters less than approximately 0.1 µm that are emitted directly from combustion sources or that condense from cooled gases soon after emission (Whitby, Husar & Liu, 1972).

It has been revealed that the efficiency of both coagulation and condensation decreases as particle size increases, which effectively produces an upper limit such that particles do not grow by these processes beyond 1µm. Thus particles tend to "accumulate" between 0.1 and 1µm, giving rise to the so-called accumulation range. Most combustion-derived particles described above may produce particles not only in the nucleation range but also in the accumulation range. Oberdorster, (1996) revealed that relative numbers of particles produced in the nucleation range, compared to the accumulation range, depends not only on combustion process, but also on dilution and cooling conditions.

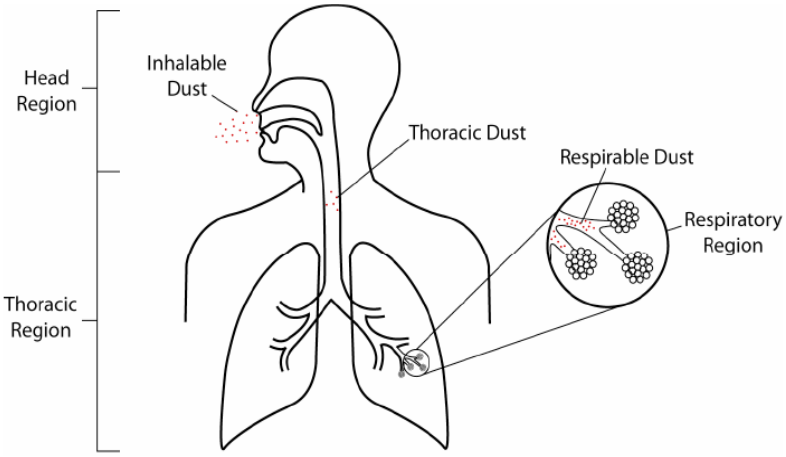
Figure 1.2 Diesel particle size distributions



(Zellner,1986)

Research evidence from systematic reviews show that particles less than 10μm in diameter (PM_{10}) pose a significant health concern (Zellner, 1986, Oberdorster, Oberdorster & Oberdorster, 2005). This is because they can be inhaled into and accumulates in the respiratory system. However, particles less than 2.5μm in diameter ($PM_{2.5}$) which are also present in PM_{10} are believe to pose the greatest health risks (Kreyling, Semmler-Behnke & Moller, 2006). Many jurisdictions and the U.S. EPA defined PM_{10} as the ambient particle indicator to be used for regulatory purposes and in the last decade, $PM_{2.5}$ and $PM_{0.1}$ has been added (U.S EPA, 2011). It is important to note that the old particle-size convention is still being used by some authors. This convention classifies particle size according to their deposition site within the respiratory system. Knowledge of this old particle-size convention use makes PM research progress more interesting. The human respiratory system and specific PM deposition sites are shown in the figure below:

Figure 1.3 The particulate matter size fraction and their size deposition in the lung

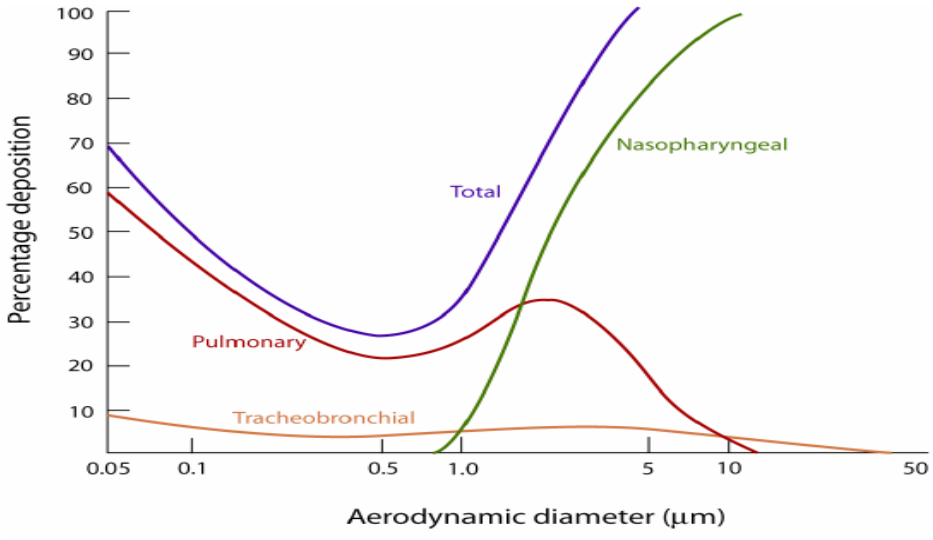


(Oberdorster, Oberdorster & Oberdorster, 2005)

A review of evidence from the workplace and environmental exposure, using a range of personal samplers, led to changes in the "total suspended particle-size" convention to be replaced based on inhalability (Oberdorster, 2001). These regulations specify that health-related sampling should be based on one or more of the three progressively finer, particle size-selective fractions. These include;

i. The inhalable fraction (<100 µm aerodynamic diameter). Particles in this fraction can reach the nose or mouth
ii. Thoracic fraction (<25 µm aerodynamic diameter). This is the sub-fraction of inhalable particles which can penetrate into the respiratory tract below the larynx.
iii. Respirable fraction (<10 µm aerodynamic diameter).This includes the sub-fraction of the particle that can penetrate beyond the terminal bronchioles to the gas exchanging alveolar region of the lungs.

Figure 1.4 Particulate matter site deposition



(James et al., 1994)

1.3 Health effects of ambient particulate matter

The percentage of diesel powered vehicles in many cities in Europe and America has increased over the years. This has continued to drive the emission of PM to up to six times more than their gasoline-powered counterparts (James et al., 1994). Apart from studies in Europe and America, which reflected mostly vehicular emissions as the major source of PM, elsewhere in oil-rich countries like Canada, Venezuela and many oil host communities in the Middle East and Africa, ROFA emissions have equally been very dominant in ambient air pollution reduction strategy.

The World Health Organization, in their 2002 global update, revealed that PM_{10} inhalation is responsible for 1,000,000 premature deaths annually (Jiménez et al., 2009). It pointed out that the range of health effects due to inhalation of PM_{10} is broad, but predominantly affecting the respiratory and cardiovascular systems. Of much concern is that the whole population is affected, though susceptibility may vary with age or current health status (WHO, 2002). Equally of great significance is a marked increase in risk with exposure level, and there is nothing to suggest a threshold below which no adverse health effects would be anticipated, since research in Western Europe and United States has demonstrated adverse health effects for fine and ultrafine particulate matter across diverse groups (Ware, 2000).

PM can act as a prime vehicle for the transport of toxic chemicals into the human respiratory and cardiovascular system on a daily basis. There is substantial evidence

that the acute cardiovascular effect of air pollution has significantly increased in recent years with experts agreeing that PM plays a leading role in triggering cardiovascular events among those at risk of cardiovascular pathologies (WHO European Region, 2005, Brook et al., 2010).

Recent reviews and meta-analysis of air quality studies provide evidence that exposure to PM, especially $PM_{2.5}$, is positively associated with cardiopulmonary diseases including exacerbation of asthma cases and lung function deterioration, frequent hospitalization and complications arising from respiratory disorders (Künzli et al., 2010, Mills et al., 2009). Specifically, exposure to diesel particles in early childhood alters lung development and also reduces its optimal function in later life (Miller et al., 2007).

A review of evidence on air pollution and children's health development, and a meta analysis of time-series studies conducted by Anderson and colleagues (WHO working group, 2004), and within the WHO Systematic Review Projects (WHO working group, 2004), noted that an increase of $10\mu g/m^3$ of PM_{10} led to a significant increase in all cause mortality, including respiratory and cardiovascular diseases. This report revealed that the increase also caused an estimated 0.8% of premature deaths world-wide, including respiratory and cardiovascular diseases, cancer of the lungs, bronchus, trachea and acute infections. The report also observed that the pattern of pollution has been paralleled by a progressive increase in the proportion of sensitive populations especially children, the elderly aged 65

and over, suffering from asthma, allergic diseases and cardiovascular conditions who are more susceptible to changes in ambient air pollution (WHO Working Group, 2004, Tibboel & Jobe, 2010). Although the specific characteristics of these subgroups have not been well understood, the existence of an association between PM and chronic disease deterioration and general health status is easy to find, according to researchers, it is precisely what makes the members of this group so particularly vulnerable to the impact of PM concentration dynamics.

1.4 Statement of the issue

The potential health effects of inhaling PM emitted as a result of human activities mentioned above have been a cause of much concern. The recurring issue is that monitoring of ambient PM simply in terms of size/mass is insufficient to provide measures that protect human health. In recent times, regulation has considered the inclusion of general PM physico-chemical composition and specific cellular changes as being adequate to provide adequate warning measures (Dockery et al., 1993). However, research evidence has not been adequate on the exact physico-chemistry and toxicological properties of ambient PM that might trigger adverse health effects**.** Thus the exact risk factors and the mechanisms involved in the effects of air pollution health cannot be fully documented. To ease these difficulties, researchers have focused mostly on individual PM types, for example, PM_{10} on every occasion and the reason is that each of the particle types contributes a more than higher proportion of the ambient PM components, for instance, DEP

in London ambient air. Secondly, increases in vehicle number in our urban areas have continued to attract research focusing on the health and environmental impact of vehicle emissions, without regard to other important sources, for example, oil refinery operations and gas flaring.

A review of air pollution in urban environment like London, revealed an important results associated with health morbidities and mortalities traceable to DEP alone (Pope et al., 2004, Ostro et al., 2007), however, urban areas of Niger-Delta in Nigeria present a different ambient PM scenario, in which the major sources of ambient PM are vehicular emissions and oil production activities. Therefore, evaluation of health effects in any urban environment should take into account the major sources of ambient PM, in the case of Nigeria, it depends on DEP and ROFA mixtures.

The absence of research covering all scenarios like the one described above creates an important gap. This obvious vacuum has made ambient PM guidelines from WHO (2000) inadequate to protect life. Thus, the question is whether there are significant differences in public health risks from exposure to PM in oil-rich areas, especially refinery areas, and those living around urban locations, which also have a high dominance of vehicular emissions, in which environment ROFA and DEP would be the principal polluting particles. This thesis has focused on prospective scenarios where ambient PM consisting of such mixtures might exist: are any toxic effects of ROFA and DEP synergistic, antagonistic or simply additive?

1.5 Why Nigeria and the United Kingdom?

Nigeria was chosen for this study for a number of reasons. It is the most populous country in Africa and 7th in the whole world population. It also ranks as the largest oil producer in Africa, 11th in the world and the 8th largest exporter in the whole world, averaging 2.28 million barrels per day, with 42% of its export to the United States. As a developing country, it has very weak air pollution regulations and has been reported to be a "pollution haven" with the highest emission record in the oil and gas industry. Recent estimates from the United Nations reported that 64.4 % of the population live below one USA dollar ($1) per day while about 54.7% live below the national poverty line (UNEP, 20011). It is important to note that about 63.8% of the national averages are in the rural areas (Vigotti, 1999). Specifically, about 800,000 Nigerian children die every year before their fifth birthday, the highest in Africa, and one of the worst in the world, with about 94 deaths in every 1000 live births. It has one of the highest percentage mortality rates related to air pollution (John, Vogel & Minshall, 2001, Katsouyanni et al., 2001, National Bureau of Statistics 2006, National Population Commission, 2009). Consistent with results from cities around the world, air pollution in Nigeria is attributed to the emissions from transport, energy and domestic sources and, most importantly, the oil and gas industry.

Therefore, the study focuses on PM in the ambient air of the Niger-Delta of Nigeria made up of six states, Rivers, Bayelsa, Cross River, Edo, Delta and Akwa

Ibom states in the South-South geographical zone of the country, where the majority of the oil production and fossil power generation plants have continued to overload the ambient air. The Niger-Delta population, both in local and urban areas, is significantly exposed to high amounts of ambient air pollution concentrations. This region harbours the major thermal power stations and the bulk of the petrochemical activity, including oil refining and gas flaring. High traffic densities are also prevalent in the Niger-Delta due to an influx of activities related to oil exploration. This makes this region an ideal study scenario for adverse health effects related to DEP and ROFA mixtures.

On the other hand, air quality in the UK has improved significantly compared to Nigeria since the great London smogs of the 1950s. With great success already recorded because coal combustion is no longer the main cause of particulate emissions and therefore no longer poses a threat to the population (Asindi, Ibia & Udo, 1991). The United Kingdom is among the developed countries where air pollution has become an important focus for local, regional and nation policy makers. Legislation has continued to focus on reducing the adverse health effect of air pollution as recent findings place the cardiovascular and pulmonary diseases as top killers; however, acid rain and ecosystem damage has continued to receive increased attention (LAQN, 2011). The report of Diddy, (2011) which concluded that about 24,000 deaths were due to the short-term effects of air pollution remains fresh to researchers in Britain. The Clean Air Acts, which focused on the successful reduction of vehicle emissions contributed in no small measure in reducing the

trend (POST, 2002). Other policy directives, which included the establishment of Air Quality Strategies and a system of local and air quality management, remain in force. The dramatic impacts of a series of unusually hot summers throughout many parts of Europe, especially in the UK provide evidence of elevated levels of air pollution.

This study has focused on London city because, among the cities in the United Kingdom, London is one of the most polluted places in Europe, having recorded dangerous levels of PM for the 36th time this year (LAQN, 2011).This places a higher number of vulnerable people in a population of about 7.8 million people at the risk of cardiopulmonary morbidity and mortality. According to research estimates, there has been a six–fold increase in road traffic between 1955 and 2001 (POST, 2002). This has since increased three fold, to date (LAQN,2011). According to the figures released from Clean Air in London, it found up to 1,148 London schools are within 150m and 2, 270 schools are within 400m, of roads carrying about 10,000 vehicles a day (Darren, 2011, LAQN, 2011). In related research, there is evidence that exposure to a high level of ambient air pollution could lead to 15% to 30% of new cases of asthma in children. The report by the London Air Quality Network (2011) provided evidence that London contributes more to air pollution than any other UK city and thus has continued to drive the amount of PM over and above other air pollutants and as such is the main target for reductions (POST, 2002). Specifically, London and the Niger-Delta present a unique ambient PM scenarios, with a higher concentration of DEP and a mixture

of DEP and ROFA in their urban environment respectively. This calls for physico-chemistry and toxicological evaluation.

1.6 Hypothesis and study objectives

The alveolar epithelium encounters about 50% of the respirable fraction of PM reaching the lung parenchyma that cannot be removed by mucocilliary mechanism (Ayres, et al., 2008). The post-deposition events in the alveolar space that might be characteristic of exposure to these particles include, an increased respiratory burst and redox activity of bound and soluble transition metals, the bioavailability of these metals for reaction, the interaction between the different metals in the reaction, the redox cycling by complex organic contaminants and oxidative stress delivered by increased particle surface area (Ayres et al., 2008). The response of the inflammatory cells to these extracellular stimuli initiates the signalling cascades that are mediated by various protein kinases that target the nuclear transcription factors.

It is important to note that alveolar epithelial cells exposures to DEP and its organic extracts activate JNK, P38 MAPK, and NF-kB pathways and associated downstream signalling and inducing cytokine production. The exposure of ROFA or vanadium-rich chemical compound instillation produced a marked increase in the activation of P-ERK1/2 and the transcription factors c-Jun and ATF-2. Substrates of JNK and P38 were markedly phosphorylated in cells treated with Cr, Cu, V, and Zn (Samet et al., 1998). The redox potential of DEP is dependent on size, PAH, quinone and redox inactive metals that deplete cellular antioxidants

particularly thiols, however, ROFA reactivity relies solely on size and soluble transition metals (V, Cr and Ni) all triggering redox sensitive signalling pathways (Pourazar, et al., 2005). In all, it was reasoned that there could be an additive or synergistic effect when these particles are mixed in an ideal cellular micro-environment.

It was hypothesized that the nature and degree of alveolar epithelial reactivity will depend on the physico-chemistry of the particles and that DEP/ROFA mixture will result in increased (additive) oxidative stress and inflammatory cytokines and chemokines production compared to DEP or ROFA alone.

The specific objectives of the current work are:

a. To characterize the ambient PM, ROFA, DEP and DEP/ROFA mixture with respect to particle size, morphology and elemental composition.
b. To identify which particles are most biologically reactive with human alveolar epithelial cells *in vitro*.
c. To determine which attribute(s) of the particles are likely to confer toxicity to the alveolar epithelial cells *in vitro*.

CHAPTER TWO

2.0 BACKGROUND TO STUDY COUNTRIES

2.1 Introduction

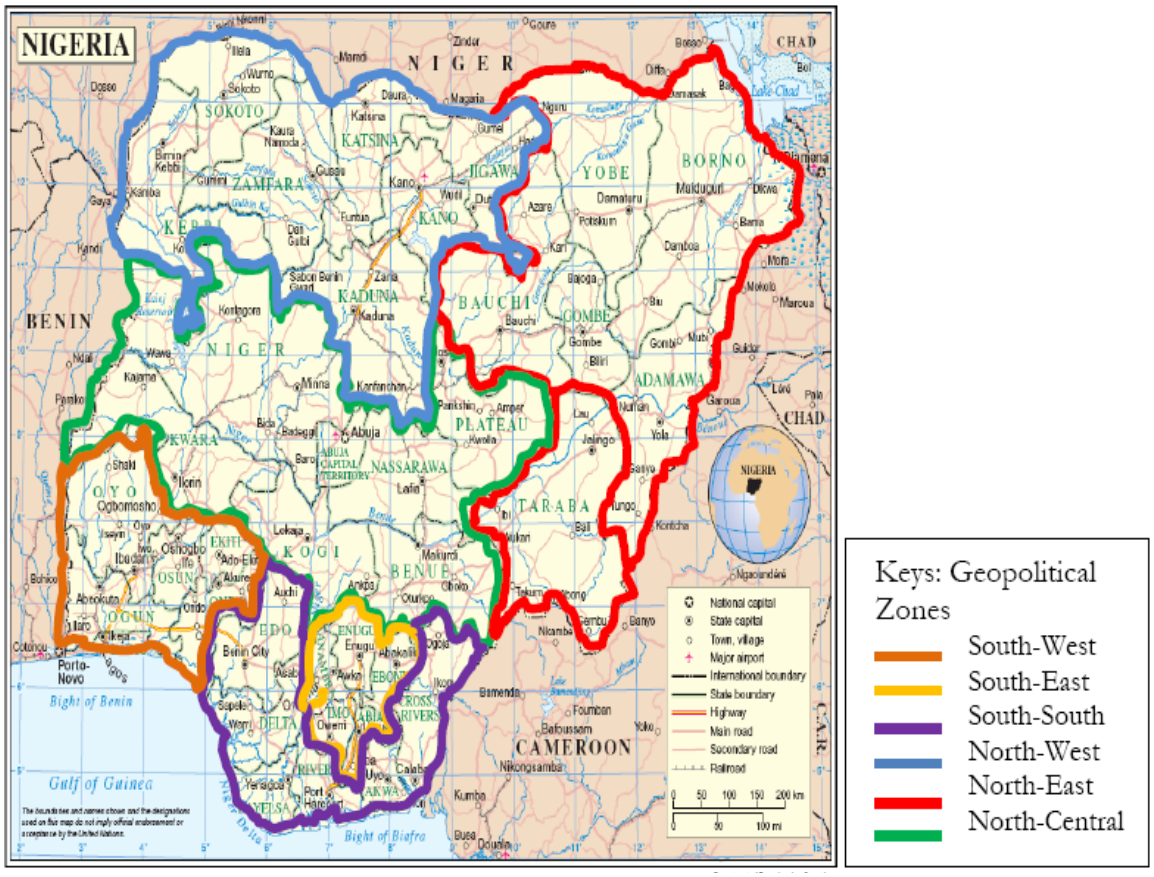
Nigeria is a federal constitutional republic made up of 36 states, 774 local government areas (LGA) and the Federal Capital Territory (FCT), Abuja. The country is divided into six geopolitical zones as shown in Figure 2.1. The 2006 census estimated the population in the country to be 140 million (NPC, 2009).

The onset of oil and gas exploration and industrial development in Nigeria brought about a drastic change in the composition of the atmospheric air pollutants signature including particulate matter (PM) and other criteria pollutants (that is, the six most common components of air pollution). Absolute dependence on crude oil for export and local consumption led to the neglect of the country's strong but young manufacturing and dominant agricultural bases before 1970. The anthropogenic causes leading to the emission of these pollutants are oil production activities (crude oil refinery and gas flaring) including transportation and petrochemical industrial activities in this region (DPR, 2002).

During the last two decades, the Federal Ministry of Health and Ministry of the Environment have increasingly recognized the high risk of PM exposure to public health and the natural environment and have since established the Federal Environmental Protection Agency (FEPA) whose statutory responsibility is for

overall protection of the environment, and setting and enforcing ambient emission standards for air, water and noise pollution (FEPA, 1991). The ambient air quality

Figure 2.1 Map of Nigeria showing the six geopolitical zones



Source: The National Space Research and Development Agency (NASRDA)[1]

limit for suspended particulate matter (PM_{10}) was set by FEPA in its effort to protect public health (National Population Commission, 2009). This limit as at 1989, when the legislation was passed, placed the daily limits for SPM (PM_{10}) at 150μg/m^3. However, enforcement and monitoring, including annual reviews of this

[1] The National Space Research and Development Agency (NASRDA) is a research institutions under the Federal Ministry of Science and Technology, Nigeria (http://www.nasrda.gov.ng)

target, could not be achieved due to poor governance and weak democratic structure that pervaded the agency. Not minding the ambitious steps taken by many countries, especially the developed world, in achieving the WHO targets, Nigeria is still struggling to transfer from total suspended particles (TSP) monitoring to using PM_{10} and $PM_{2.5}$ standard concentrations reporting. It is important to note that most of the ambient air studies carried out in Nigeria, especially in the Niger-Delta, still use TSP nomenclature in reporting as surrogate for PM_{10} and $PM_{2.5}$ concentrations.

2.1.1 Niger-Delta: Geography

The Niger-Delta is located in the southern part of Nigeria and is geopolitically described as the South-South geopolitical zone, as shown in figure 2.2. It is a coastal lowland. The rich ecology of the zone is very significant to its people. The zone has a vast mangrove forest and fresh water swamps with an area cover estimated to be $600Km^2$ and $11,700Km^2$ respectively (Moffat & Linden, 1995). Ecologically and geopolitically, the region is made up of nine states which include Akwa Ibom, Bayelsa, Edo, Cross River, Delta and Rivers, Imo, Abia and Ondo States. These nine states have an estimated area of $110,446Km^2$ which is about 7.5% of the Nigerian landmass (FEPA, 1991). The region is rich in petroleum and natural gas reserves. The Niger-Delta is the location in Nigeria that harbours one of the world's richest crude oil and natural gas deposits. This makes it the eleventh

richest oil producer controlling about 2.5% of the world oil reserves (Moffat & Linden, 1995; Klett et al., 1997).

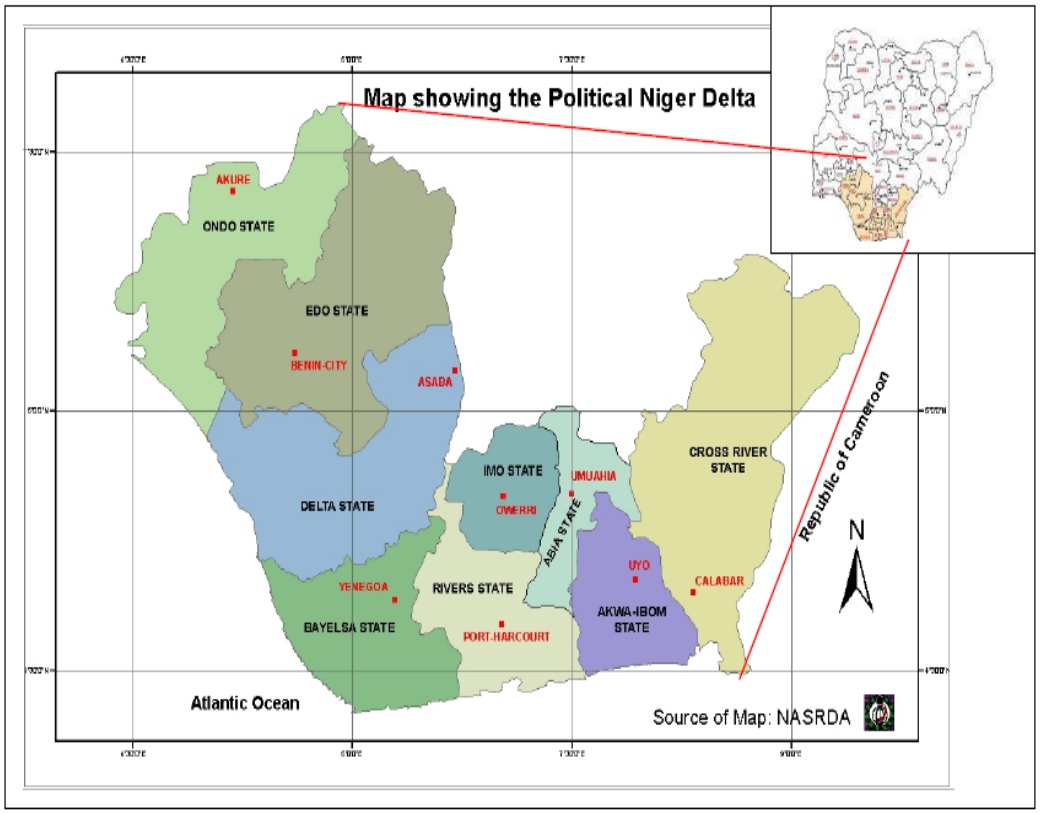
2.1.2 Niger-Delta: Climate

All regions in Nigeria, including the Niger-Delta, observe two seasons, the rainy and dry seasons. The dry season is characterised by dust-laden air mass (Harmattan) from the Sahara desert into West Africa, and across northern Nigeria before being transported to southern Nigeria by winds (FEPA, 1991). From available evidence and daily experiences, this air mass has been shown to dominate Nigeria's climate during the dry season from December to March. The dusty air mass has less potential of forming rain due to low humidity within it, thus creating a hazy atmosphere within West Africa and Nigeria when it predominates. The Niger-Delta, like most southern parts of Nigeria, experiences a prevailing tropical maritime air mass in most periods of the year. The rainy season is characterized by rain and humid air from the tropical maritime air mass originating from the South Atlantic Ocean (Dajab, 2006).

The Niger-Delta rainfall has always remained at a minimum around December through January at an estimated 20-75mm, reaching a 300-700mm maximum in June. The region has an annual average rainfall of between 1,400 and 3,000mm with slight variation in some coastal towns (Fagbeja et al., 2008). It has the tropical monsoon climate at the coasts, with rainfall peaks in June and September/October. As a result, the region experiences partial or full cloud coverage almost all year

round. The significant changes in the season leads to a corresponding variation in atmospheric temperature in Nigeria, which can go as high as 44 °C (111.2 °F) in some parts of the northern states and as low as 21°C to 25°C during the raining season. However, the maximum temperature of 33°C is recorded in the Niger-Delta around January to March; this drops to 21°C as the minimum in July and December. While during the rainy and dry seasons, a mean monthly record of 24°C to 25°C and 27°C to 29°C has continued to be observed.

Figure 2.2 Map of the nine states of the Niger-Delta



Source: National Space Research and Development Agency (NASRDA)[1]

2.2 Sources of air pollution in the Niger-Delta

The anthropogenic sources of air pollution in the Niger-Delta are wide and varied. Poverty remains one of the main barriers to the adoption of cleaner fuels in the Niger-Delta and Nigeria as a whole (United Nation, 2011). In recent times, both the urban and rural households rely heavily on unprocessed biomass fuels in the form of fire wood, sawdust, impure coal, animal dung, crop residues and kerosene for domestic cooking and lighting (Klett et al., 1997). These are burnt indoors and in most cases in open fires or poorly fabricated functioning stoves resulting in indoor, and a significant contribution to outdoor air pollution to which women, especially those responsible for cooking, and their young children are most heavily exposed (Olaniran, 1986). According to the Nigerian National Bureau of Statistics (2006), about 70.0% of Nigerians rely on firewood for cooking. 26.6% depend on the use of kerosene, 1.1% depends on liquefied natural gas while only 0.5% use electricity.

In most urban areas generally, major sources of human-generated air pollution sources may easily be divided into mobile and stationary sources. Mobile sources of air pollution predominantly include: motor vehicles (buses, cars and heavy duty trucks), trains, motorcycles, tricycles, airplanes, generators and other earth moving or construction equipment. Most vehicles affordable to the majority of Nigerians, according to Anozie et al., (2007) are prone to emitting high quantities of PM and carbon monoxide and nitrogen oxide. There are more heavy duty vehicles that

serve the refineries and oil service companies in the Niger-Delta than anywhere else in Nigeria. The emissions from these vehicles include ambient PM (DEP) and other criteria pollutants. These are over and above the regulatory threshold (UNEP, 2011). All these differ remarkably from stationary sources of air pollution (petrochemical industries, incinerators, refineries and gas flaring) which emit a much greater percentage of ambient PM in the form of residual oil fly ash and other criteria pollutants (Ogunsola et al., 1993)

2.2.1 Oil production activities

Oil and gas deposits in the Niger-Delta accounted for more than 98% of export earnings and about 83% of federal government revenue in Nigeria (Federal Ministry of finance, 2002). Since Nigeria's discovery of oil and gas, its estimated reserve has continued to increase as more discoveries are made within the region (DPR, 2010). The abundant oil and gas deposits in the Niger-Delta are responsible for the strategic importance of the region and the country in the current global economic realities. This is also responsible for the current level of urbanisation, industrialisation and the large influx of people from other parts of the globe, making the region the fastest growing region in Africa (Obioh et al., 2005). As stated previously, the Niger-Delta regions play host to the major Nigeria energy generation and supply companies, oil and gas multinational companies, three among the four refinery companies, and oil and gas service companies, petro-chemical and allied companies and many other industries that leverage on the

population and the sea ports for imports and exports of manufactured goods and raw materials in Nigeria.

Figure 2.3 Agip gas flares at Ebocha, Rivers State, Niger-Delta

(Adeyinka, Bankole & Olaye, 2005)

Apart from crude oil, the Niger-Delta is also endowed with the tenth largest proven natural gas reserves (DPR, 2010). The World Bank, in its recent estimate, reported that almost 2.5 billion cubic feet of the 3.5 billion cubic feet (100,000,000 m^3) of associated gas (AG) produced annually is burnt during crude oil production, more than is flared anywhere else in the world (Ahmad, 1994). This estimate equals about 25% of the UK's total natural gas consumption, and was the equivalent to 40% of the entire African continent's gas consumption in 2001 and equivalent to

the total annual power generation in sub-Saharan Africa (DPR, 2010). A report from FEPA estimated that there are about 100 gas flaring sites in Niger-Delta some of which have been burning ceaselessly for 40 years as shown in figure 2.3 (Adeyinka, Bankole & Olaye, 2005). Gas flaring, according to experts, not only wastes valuable resources but is also a major contributor to ambient air PM in the Niger-Delta and Nigeria as a whole.

2.3 Ambient PM in the Niger-Delta

The high concentration of PM and other pollutants and their increased risk to public health, especially to the exposed population in the Niger-Delta, has led to increased hostilities and targeted sabotage of oil and gas installations in the Niger-Delta, aimed at stopping the major oil companies who engage in gas flaring and other unwholesome practices that emit significant amounts of pollutants into the ambient air (FEPA, 1991). Evidence suggests that, Lagos city, reputed to be one of the most polluted cities in the world, may not rival the Niger-Delta (FEPA, 1991). This is because the major source of air pollution in Lagos is limited to vehicular emissions, domestic combustion, indoors and few industrial emissions and electricity generating sets. However, the Niger-Delta combines these with huge emissions from oil and gas activities.

While DEP account for the majority of ambient air pollutants in Lagos, ROFA and DEP dominate the ambient air in the Niger-Delta due to the presence of crude oil refineries, off-shore and on-shore flaring sites and petrochemical industrial

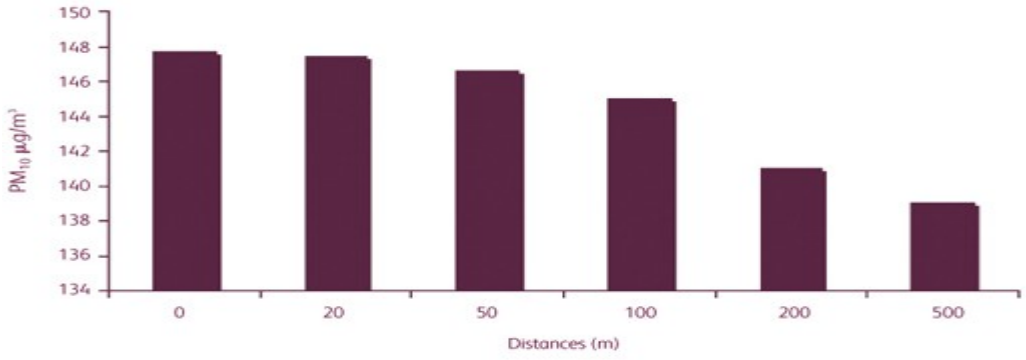
emissions. These emission sources are located close to public offices and dwelling places, with emission concentrations increasing as the distances decrease. This is shown in figure 2.4. The evidence of human exposure and its relationship to point sources has been reported by many researchers (Abdulkareem,Odigure & Abenege, 2009, Obioh et al., 2005).

2.4 Health effects of ambient PM in the Niger-Delta

There is little current epidemiological research into the epidemiological impacts of PM pollution on human health in Niger-Delta. Nevertheless, current practices dating back to the discovery of oil and gas in commercial quantities offer significant evidence linking ambient PM pollution to respiratory and cardiovascular morbidity and mortality (Zou et al., 2009). Specifically, in Niger-Delta, oil and gas host communities have had the highest share of this burden, especially for children and the elderly (Efe, 2008). Current assessment of the impact of PM reveals that most urban dwellers and road users are constantly exposed to ambient PM hazards. Road users with increased risk of exposure are cyclists, passengers, street traders, hawkers and motor vehicle drivers. Oftentimes, these groups cover their nostrils with handkerchieves, while car owners wind up their windows, especially at traffic junctions to reduce exposure to PM and other pollutants (Obioh et al., 2005). Some researchers observed that those who lived close to traffic-clogged areas and road junctions, linking oil refinery and flaring sites, experience increased risk (Ana, Sridhar & Bamgboye, 2009). Ogunsola et al., (1994) specifically noted that residents

of the crude oil refinery industrial areas in Port Harcourt and Warri, and those living along the refinery road corridor (including other high-density residential and commercial districts in the metropolis) were the most affected. The common symptoms include eye irritations and respiratory problems, increased asthma exacerbation and frequent hospital and primary health centre visits (Obioh et al., 2005).

Figure 2.4 Relationship between ambient PM and distance to the emission source



(Efe, 2008)

Recent evidence of the effect of ambient PM on human health in the Niger-Delta has been reported (Efe, 2008). Efe (2008) asserted that high rates of respiratory diseases and hospital admissions, including infant mortality, occasioned by increased PM_{10} concentrations were experienced by residents of most urban areas in the Niger-Delta compared to other cities. Efe (2008) noted that over 15% of increased cases recorded among infants and schoolchildren were associated with high PM concentrations and maintained that the relationship is causal.

2.5 Air Pollution in the United Kingdom

Air quality in the UK has improved significantly following the infamous smogs of the 50s and the 60s (POST, 2002). Since then there has been a concerted effort to reduce air pollutants. Early accounts of air pollution showed an association with rapid increases in population growth, changes in fuel use and urbanization (POST, 2002). Historically, the early transition from wood to coal for domestic fireplaces was very significant. Much of the indoor air pollution in the 17th and 18th century resulted in coal combustion for domestic heating and cooking. Industrial boilers and furnaces burning coal were also reported to be accelerated in the early part of 19th the century (POST, 2002).

Smoke and sulphur dioxide emitted from cooking and heating practices according to research evidence were major air pollutants (Chauhan & Johnston, 2003). As mentioned earlier, a high reliance on coal for heating during cold winters resulted in increased concentrations of smoke and SO_2 which led to frequent pollution episodes owing to the formation of smogs (combination of smoke and fog). The health impact of these pollution episodes were well documented in the later part of the 17th century (POST, 2002). These became more frequent, increasing in severity at the conclusion of the19th century. All efforts to reduce air pollution and the risk to public health were increased in 1953, following the disastrous smog in December, 1952 which led to the premature death of about 4000 people and a higher morbidity cases in London alone (POST, 2002, Chauhan & Johnston, 2003).

Most of the recommendations from the committee on air pollution, that investigated the causes, nature, effects and preventive measures of the 1952 episodes, formed the basis of the Clean Air Act, 1956 that has undergone several modifications to date.

Main sources of air pollution in the United Kingdom in the 20th century include, railway engines and ships, industrial complexes, domestic premises, road traffic, coal mines and electricity generating stations (Ministry of Health, 1954)

To date, indoor air pollution has become less of a public health risk because of the improved reduction in smoke and SO_2 and with advancement in domestic heating, cooking technologies and strong regulation in building standards (Black, 2003). Other European countries, for instance, the Republic of Ireland, recorded a significant decline in air pollution and reduced number of attributable deaths in the year following the ban in the sale of coal in Dublin (Colvile et al., 2001). However, much of the gain recorded in reducing smoke and SO_2 as the main pollutant has been eclipsed due to an approximate six–fold increase in vehicle traffic between 1955 and 2001 (Colville et al., 2001). Vehicular traffic on the roads and streets of Britain today is the main source of criteria pollutants. Results from epidemiological studies have shown consistent evidence that low and moderate concentrations of the traditional pollutants, especially ambient PM, can have both long and short-term effects on health (Clancy et al., 2002). Evidence from a review of some of

these studies led to the establishment of ambient air quality guidelines by WHO and DEFRA (Colvile et al., 2001).

2.5.1 Ambient PM guidelines

Over the past forty years, the ambient air composition of traditional air pollutants especially PM, have declined significantly as a result of clean air legislation (Katsouyanni, 2003). Importantly, the current WHO guidelines on air quality for Europe do not have any (Atkinson et al., 2009) evidence for a PM concentration threshold below which there is no effect of PM on health. Hence, only concentration-response data for acute health effects are used for guidance, based on studies mostly using PM_{10} and a few using $PM_{2.5,}$ as an indicator of particulate air pollution and a relative risk of long–term effects. The initial effort to reduce ambient particle concentrations was first addressed, using air quality limit values for $PM_{10,}$ by the European Commission (1999). This is contained in the first daughter directive (99/30/EC) under the air quality framework directive (96/62/EC). The World Health Organization has, equally, set out an ambitious target to reduce the concentration of ambient PM drastically (Katsouyanni, 2003). The current WHO guideline for particulate matter (WHO, 2000) is shown below:

- $PM_{2.5}$: 10μg/m^3 annual mean
- 25 μg/m^3 24-hour mean
- PM_{10}: 20 μg/m^3 annual mean
- 50 μg/m^3 24- hour mean

Consequently, the United Kingdom, through DEFRA, has revised their existing guidelines in the context of local constraints, economic capabilities and public health priorities (DEFRA, 2008). This gave rise to the PM air quality strategy (AQS). With ambient air quality directives incorporating measures of fine and coarse particulate material, these measures have been used to establish new national ambient PM levels to be achieved by 2020. Nevertheless, recent short term epidemiological evidence of particulate matter emission in London city, and other UK hot spots, suggests that cardiovascular and respiratory morbidity and mortality persists (Samoli et al., 2008) even at the present revised levels of the United Kingdom revised Air Quality Strategy, as shown below:

- For the 24 h levels, 50 μg/m^3 of PM_{10} should not be exceeded more than 35 times per year by 2005 and more than seven times per year by 2010;
- For the annual levels, 40 μg/m^3 of PM_{10} should not be exceeded by the year 2005
- $PM_{2.5:}$ 25 μg/m^3 annual mean with a target of 15% reduction at urban background to be achieved by 2020

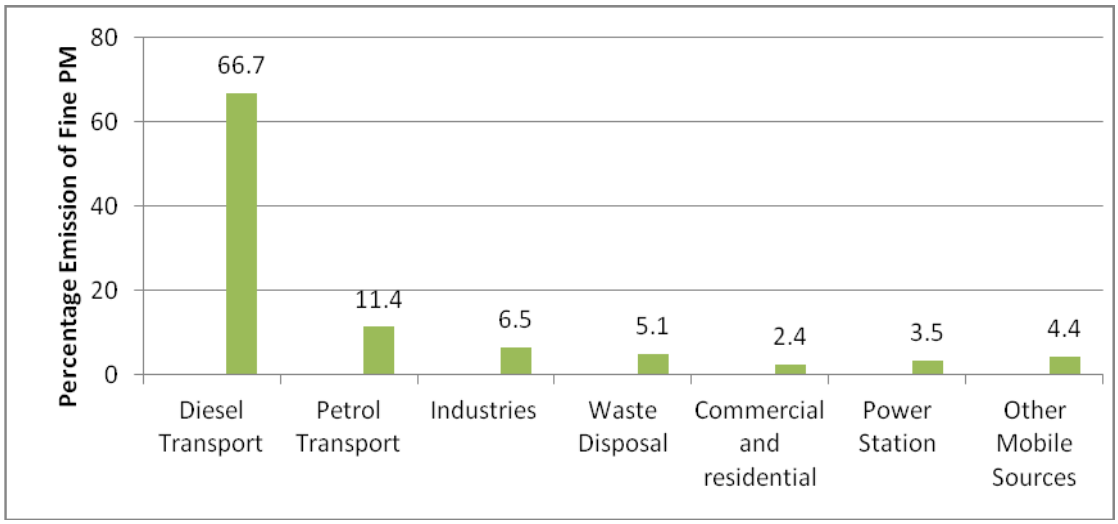
2.6 Vehicular transport in London and ambient PM

As reported previously, vehicular transport is the major sources of ambient air PM in the United Kingdom, especially in London city (Figure 2.5). Air quality monitoring in London shows evidence of increased levels of criteria pollutants. This has continued to drive the amount of PM over and above other air pollutants

monitored by the London air quality network (Krzyzanowski, 2008). It has been estimated that 20%–80% of the primary PM_{10} emissions can be attributed to DEP and in some parts of London, DEPs can account for as much as 87% of the mass of PM_{10} emissions. Vehicles operating on fossil fuel emit PM, but diesel engines, according to the evidence, emit more PM per vehicle kilometre compared to gasoline engines (Hubbard, 2006).

Study of the PM sources in the heavily trafficked London roads has revealed that the annual increase in mean PM_{10} concentration is attributable to an increase in the primary PM_{10} emission sources between 1999 and 2004 (Fuller & Green, 2006, de Kok et al., 2006).

Figure 2.5 Sources of ambient PM in London



(Charron & Harrison, 2005)

This increase is in direct contrast with the emissions inventory predictions, based on ambitious and progressive PM_{10} abatement strategies in London, including introduction of a congestion charging scheme and fitting of particle filters to

Transport for London (TFL) buses that are mostly diesel–powered (Harrison, Jones & Lawrence, 2004)

Documented evidence revealed that the benefits of PM_{10} abatement may have been offset by the increasing proportion of diesel-powered engines in the UK vehicle fleet and in London in particular. This likely reflects that diesel vehicle fuel use rose from 16% in 1997 to 38% in 2006 (Fuller & Green, 2006).

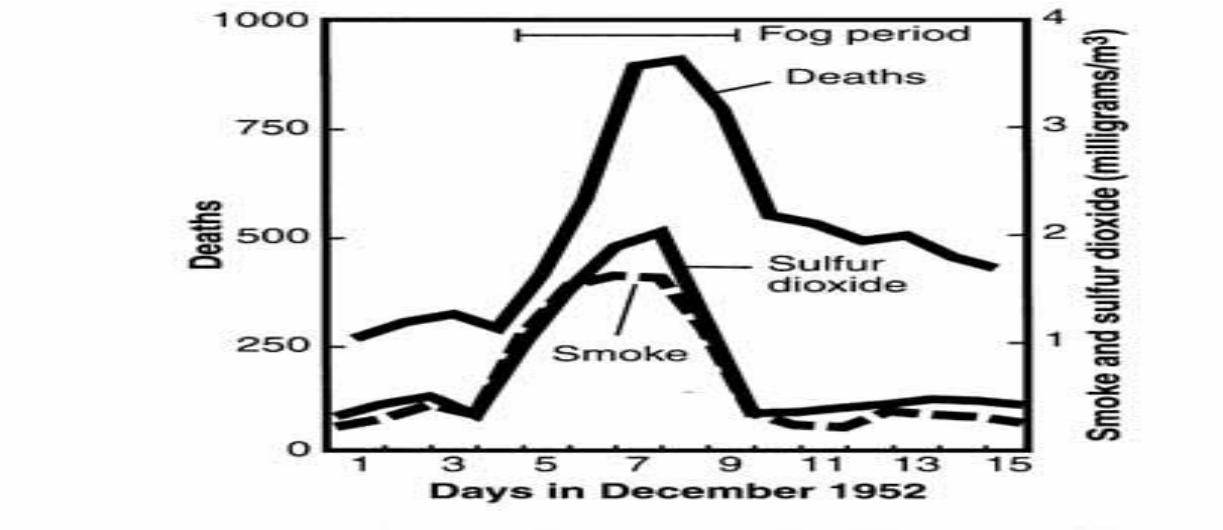
Additionally, new commercial vehicles rose by 41% between 1997 and 2006 and most interestingly, over 97% of commercial vehicles sold in 2006 were diesel engines (SMMT, 2007). It is important to note that new vehicle sales provide an insight for change in the total vehicle fleet, but it is the total fleet that must be considered for the purpose of predicting PM emissions. The Society of Motor Manufacturers and Traders (2007) in its report inferred that between 1997 and 2005 the total diesel vehicles in the UK increased by 127% and overall sales of diesel fuel in the UK increased by 30%. Therefore, unless technologies continue to improve, increase in diesel powered vehicles could limit the rate at which ambient PM, and most precisely DEP, are forecast to fall in London.

2.6.1 Health effects of ambient PM in London

It is suggested that the gain recorded in the reduction of black smoke and SO_2 in the last three decades (Figure 2.6), is reflected in recent improvement in health (Figure 2.7). However, there is evidence that thousands of people may die prematurely every year owing to exposure to ambient PM. There is evidence that

the process leading to death can be accelerated during extreme weather (Colville et al., 2001).

Figure 2.6 Death rate, smoke and SO_2 concentration during the Great Smog in December, 1952



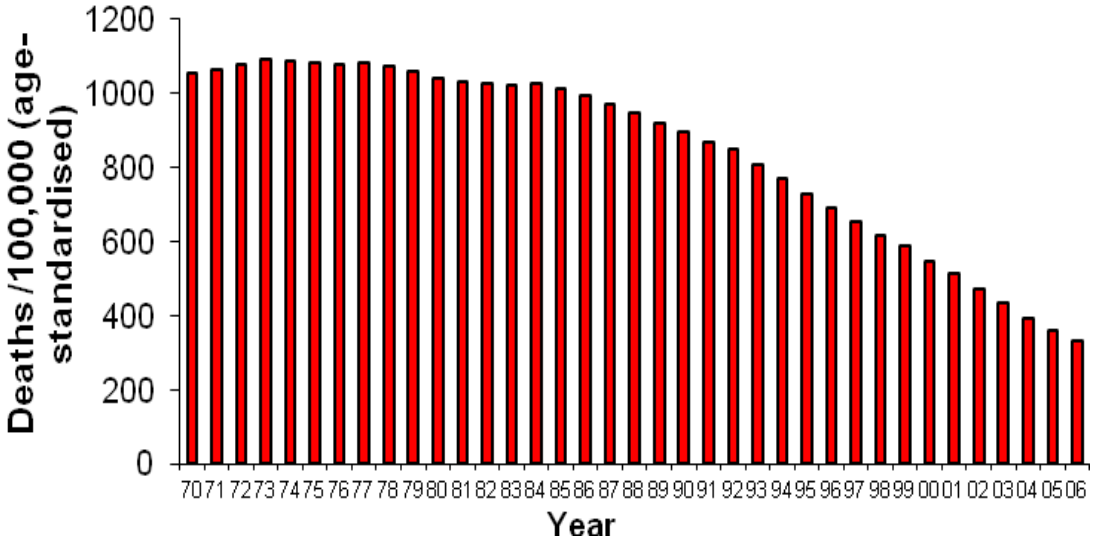
(POST, 2002)

In related studies, significant increases have been reported in adverse health effects at the current ambient PM air pollution levels in most urban areas in United Kingdom, resulting in a range of endpoints including cardiovascular and respiratory admissions, premature mortalities, asthma exacerbation, loss of lung function and other respiratory symptoms. According to a recent report from the British Heart Foundation (Figure 2.7), the mortality and morbidity related to air pollution is still high compared to other killers like pneumonia, cancer, diabetes and bronchitis.

There is also a worrying trend in that the young, the sick and the elderly are mostly affected. Evidence from reviews and meta-analysis revealed that these effects are strongest for ambient PM and the corresponding association is causal

(Katsouyanni et al., 1997).Transport is one of the four underpinning priorities in London health strategy. A transport-related, health impact assessment report, commissioned by the NHS (Watkiss et al., 2000) provided evidence on the effect of ambient PM on London health; it revealed that acute changes in PM_{10} levels cause premature deaths, which arise primarily from respiratory and cardiovascular causes, including early and extra hospital admissions.

Figure 2.7 Death rates from Coronary Heart Disease (CHD), for people aged 65 to 74 in England



(British Heart Foundation, 2009)

The report also contained some evidence that chronic exposure to increased concentrations of PM have a marked effect on mortality, with increased risk of developing respiratory diseases. It also validated a linear relationship on studies reporting acute effects with no PM threshold, down to less than 20μg/m^3 of PM_{10}

The studies on which the report was based include those that examined the effect of ambient PM related to vehicular emissions in London.

These include epidemiological evidence of positive short-term associations. For instance, Katsouyanni et al., (2001) reported that a 31μg/m^3 increase in PM_{10} was strongly associated with increased accident and emergency visits for asthma and other respiratory symptoms for all age groups. Watkiss et al., (2000) have also reported that increases in most pollutants were significantly associated with respiratory mortality between 1992 and 1994. The study also observed the largest effect for PM_{10} with 4% increase in respiratory mortality compared to other pollutants (nitrogen dioxide, ozone, sulphur dioxide, and carbon monoxide) considered in the study. This evidence is consistent with the relationship between PM_{10} increase and daily GP consultations for asthma and other lower respiratory tract conditions also reported in the study and elsewhere (Atkinson et al., 1999). Similarly, Bremner et al., (1999), reported that the effect estimate of most pollutants, particularly PM_{10} was much larger in both children and adult populations during the cold and warm seasons.

Other studies that showed air pollution due to PM is associated with increased daily mortality and hospital admissions for respiratory and cardiovascular problems, and asthma, in London have also been published (Ponce de Leon, 1996, Poloniecki,1997, Atkinson et al., 1999) as well as the APHEA project report (Hajat et al., 1999).

2.7 Ambient PM sampling and monitoring

Since the early smog of the 1950's the government and devolved assemblies have introduced statutory air quality strategies and a system of local air quality management. Air Quality Strategy and Air Quality Framework Directives are carried out through regular monitoring and sampling, where necessary, in places where ambient PM and other air pollution standards are not being met; the local authority under the law must declare an air quality management area (AQMA) with specific reference to the zone affected, and then prepare and implement a remedial action plan to improve the ambient air quality situation for that area.

Sampling and monitoring for PM originally relied on common techniques for the measurement of particulate mass concentration and particle size in gas streams. Local air quality measurement relied on the use of a national and European reference sampler, which is a gravimetric device (Anderson et al., 1996). PM measurement using this device is based on a non-continuous (discrete) measurement process, where PM mass is collected on a filter over a specified period of time and subsequently weighed to determine the increase in weight. However, there are significant disadvantages to this method. These include the ability of the sampler to measure only 24-hour mean concentration of particles, and the inability to disseminate data to the public in real time. The labour involved in its operation and handling of the filter for measurement and analysis is also a cause for concern. This problem has been offset by the current use of continuous (real-

time) monitors. In continuous monitoring, PM is constantly measured, with improved advantage of reduced labour, and the data are processed, stored or automatically transmitted to a central database.

The Tapered Element Oscillating Microbalance (TEOM) analyser has predominantly been used for real-time measurement in combination with a gravimetric sampler in the United Kingdom PM sampling and monitoring campaigns. However, the TEOM has been reported to under-read exact values in comparison with the gravimetric method (Katsouyanni et al., 2001). A major concern with the TEOM is that the filter is held at an elevated temperature (50°C) in order to minimise errors that may be associated with the evaporation and condensation of water vapour. It is suggested that this has led to the evaporation of the more volatile species, including volatile organic compounds (VOC) and nitrates. In this respect, Soutar et al., (1999) discovered that volatile PM, which makes up a considerable portion of $PM_{2.5,}$ will therefore not be measured if the sample stream is heated in such a way that these materials volatilize and subsequently are not deposited on the sampler filter media. Recently, a default correction factor of 1.3 has been applied to TEOM data, in order to generate a nominal "gravimetric-equivalent result" (EC Working Group on Particulate Matter, 2002).

In contrast to the United Kingdom, there has been no government strategy to monitor air pollution in Nigeria. Current data on air quality has been through the efforts of individual researchers funded by international agencies or the oil

companies. However, most studies that have been funded by oil companies and it has been suggested that the outcome has been modified to satisfy their interest. Some of these studies have focused on urban centres, where industrial processes, oil production activities and traffic congestion constitute the major sources of ambient PM pollutants. However, these studies are not controlled by government recommendations as yet, thus there are currently no established standards or systematic measurements, nor the equipment required for ambient air quality sampling. The newly established National Environmental Standard Regulatory Enforcement Agency (Eatough et al., 2003) is currently addressing this issue.

2.7.1 The London air quality network (LAQN)

The London Air Quality Network (LAQN) was set up in 1993 to coordinate and improve air pollution monitoring campaigns in London. It provides extended air pollution measurements that are appropriate to underpin air quality management and health risk. LAQN is managed by the air quality management group of the Environmental Research Group (ERG) at Kings College, London which also oversees and operates the Marylebone Road sites and maintains many of the LAQN affiliate's sites to the United Kingdom Automatic Urban and Rural Network (AURN). 31 of the 33 London boroughs currently supply measurements to the network, in addition to the data being supplemented by local authorities surrounding London, thereby providing an overall perspective of air quality in London and surrounding counties.

Much of the monitoring has relied on TEOM instruments for PM_{10} measurement, while few long-term measurements of $PM_{2.5}$ are available in London. Following application of the DEFRA recommended 1.3 gravimetric correction factor to compensate for losses of volatile particulates, the data emanating from these TEOMs are not only reliable but also comparable to the gravimetric measurements. Ambient PM in London remains one of the major pollutants as determined by the LAQN. The LAQN database revealed that mean concentrations for PM_{10} in London decreased in the late 90's but showed a steady rise since then, at a mean annual rate of 0.4% (Green, Fuller & Barratt, 2001). Thus, although, AQS and EU limit values for PM_{10} and $PM_{2.5}$ have been attained in background sites, these values are regularly breached along the busiest part of London trunk road networks and within the city's areas marked as hot spots, due in part to operation of diesel powered engine buses.

2.8 Ambient PM characterisation

Characterisation of ambient PM has become a very important part of the strategies aimed at protecting the public health and natural environment. Knowledge of the physical and chemical properties of the particulate fraction is significant from the standpoint of understanding the fundamental relationship or correlation between particle properties and adverse health effects (Grobéty et al., 2010). Rigorous characterisation process is also important in establishing a health-based ambient air quality standard to take account of the varying toxicity potential of different

constituents of the particulate fractions. In addition to using these analyses in source apportionments, most studies have also been concerned with measuring specific constituents which could be useful in evaluating time trends of specific components. Identification of the source of particles and relative contribution to the PM sample concentration at any monitoring station would help in implementing adequate abatement strategies.

The principal method of collection of PM for physico-chemical characterisation is based on the gravimetric approach, which involves measuring the weight of a given particulate size fraction collected on a filter per unit volume of air sampled (EC Working Group on Particulate Matter, 2002). As specified in the European Union manual reference method for particles measured, a typical mass loading on filters for the low to high volume and super-high volume samplers (Wide range aerosol classifier) should be less than 5 mg, yet many chemical species are present at levels of less than 1 µg for a sampling period of 24 hours for PM_{10} and $PM_{2.5}$ fractions (Grobéty et al., 2010).

The next step in the characterisation procedure is the extraction of particles from the filter. The composition of the extracts is subsequently analysed using different techniques depending on the nature of the particle, the operator and subsequent analysis of the material. Common techniques include: ion chromatography, atomic absorption spectroscopy, gas chromatography mass spectrometry, high performance liquid chromatography.

The Clean Air for Europe (CAFE) and the National Ambient Air Standard (NAAS) organization have relied on measurement of PM_{10} and $PM_{2.5}$ fractions to establish the air quality limit measures for ambient particulate material. However, since the particles in PM_{10} samples may have volumes as small as 1 μm^3 and trace elements present in as little as 1 nanogram per particle, electron beam techniques such as SEM, TEM and X-ray microanalysis, with wavelength or energy dispersive X-ray analyzers, are ideally suited for the analysis of such samples (Geller et al., 2006). The use of transmission electron microscopy (TEM) has been employed by many research institutes in the UK and elsewhere because of its higher resolution compared to SEM. The analytical information of SEM/EDXA or TEM/EDXA can be combined to provide statistical output and description of particles with respect to their chemical and/or elemental composition and morphology.

CHAPTER THREE

3.0 REVIEW OF CELL CULTURE MODELS TO DETERMINE THE TOXICITY OF PM

3.1 Introduction

A variety of *in vitro* models have been used to determine the mechanism of toxicity and bio-reactivity of air-borne particles, and there are many cell culture models providing possible alternatives to animal exposures for analyzing particle toxicity (Card et al.,2008, Rothen-Rutishauser et al., 2008, Simkó & Mattsson, 2010). However, several methodological criticisms have been expressed regarding the suitability or credibility of some experimental procedures due mainly to the relevance of the *in vitro* models that have been employed to mimic the human system (Rothen-Rutishauser et al., 2008). This possible unsuitability has likely led to a remarkable difference between studies and has raised the need to review these experimental models through proper evaluation of their biochemical, morphological and bio-kinetic characteristics more appropriate to the *in vivo* situation and where a more relevant result might be obtained.

The aim of this chapter therefore, is to identify an appropriate *in vitro* model for particle toxicity, generating results that are reproducible, more controllable and which offers a reliable precursor to refining subsequent *in vivo* procedures.

The objectives of this review are:

- To conduct a thorough literature search and identify which *in vitro* models have been developed to study particle toxicity.
- To evaluate these models based on their phenotypic and bio-kinetic properties
- To identify any limitations, in order to improve *in vitro* research which might be usefully translated into man to modify environmental and public health, as well as clinical practice.

3.2 The physiology of the respiratory tract

The internal surface area of the respiratory tract is large. For a normal human at rest, it is estimated that the volume of air entering the nostrils every 24 hours is between 10,000-15,000 litres. The anatomical design and sequential passage of the respiratory tract is made up of the nasal cavity, the pharynx, the larynx, the trachea, the bronchi, the bronchioles and the pulmonary alveoli within the parenchyma (Gruber & Hartung, 2004). These structures make it feasible for efficient delivery of oxygen to the circulating red blood cells passing through the lungs and the removal of carbon dioxide from the blood into the air on expiration. The respiratory system is divided into an upper and lower respiratory tract. While the nasal passages, pharynx and the larynx make up the upper tract, the lower

respiratory tract is composed of the trachea, the primary bronchi and the parenchymal respiratory zone (Steimer, Haltner & Lehr, 2005).

Conventionally, the larynx is considered to be the boundary between the upper and lower respiratory tract. The upper region extends from the external nares to the larynx and the lower region from the larynx to the visceral pleura (Rothen-Rutishauser et al., 2008). Functionally and physiologically, the respiratory system can also be divided into conducting, transitional and respiratory zones. The major function of the conducting airway zone includes air conditioning, warming, humidification and cleansing of the inhaled air mainly for potentially harmful dust particles, gases and microorganisms (Hong et al., 2004). This biological process in the lungs involves the mucocilliary clearance, nervous reflexes causing bronchoconstriction, mucus release and cough. The transitional zone is the proximal region of the interstitial alveolar region, consisting of the respiratory bronchioles. The major function of this zone is air conduction and gas exchange.

The respiratory zone is the second peripheral area of the interstitial alveolar region. It is composed of the peripheral airway, the alveolar ducts with their walls opening into numerous alveoli and terminal alveolar sacs. Adult human lungs have about 300 million alveoli, each measuring about 250µm in diameter when expanded. Unlike other regions, the main function of this region is gas exchange. The clearance of particles in this zone is not very efficient and depends largely on the presence of alveolar macrophages. Evidence suggests that a high proportion of

particles within the nano range reach the alveolar epithelium (Rothen-Rutishauser et al., 2008).

3.3 The large airway epithelium

Airway epithelium is a major air conduction channel. It is the first line of defence against inhaled toxicants and other biological agents and plays a vital role in the maintenance of physicochemical homoeostasis (Phalen & Oldham, 1983). While preventing entry of noxious agents into the underlying tissues, it also isolates the host from the external environment (Bair, 1995). Recent evidence, however, suggests that this barrier plays a critical role, not only in the disease pathogenesis, including asthma, but also in maintaining maximum interaction with the surrounding cells both directly, and indirectly, releasing and metabolizing mediators (Rothen-Rutishauser et al., 2008).

The predominant epithelial cell types in the airways are columnar and basal cells which form a pseudo-stratified cell lining (Davies & Devalia, 1992). Another major function of airway epithelial cells is the production of complex secretions or mucous. The columnar cells are composed of both ciliated and non-ciliated secretory cells, of which the latter comprise the goblet, serous and clara cells. The ciliated cells propel the secretion and all the inhaled trapped particles proximally, forming the mucociliary escalator. Ciliated cells are joined to each other at the luminal surface by tight junctions, which act as a permeability barrier and maintain the integrity of the epithelium (Takizawa, 1990). Recent study has revealed that

basal cells not only play a role in the attachment of columnar cells of the airway basement membranes, but are also multipotent progenitors with the potential to renew the epithelium (Gruenert, Finkbeiner & Widdicombe, 1995). Epithelial cells can also modulate inflammation by expressing adhesion molecules for inflammatory cells, presenting antigen, preventing apoptosis, and producing chemokines, antiproteases and antioxidants.

3.4 The alveolar epithelium

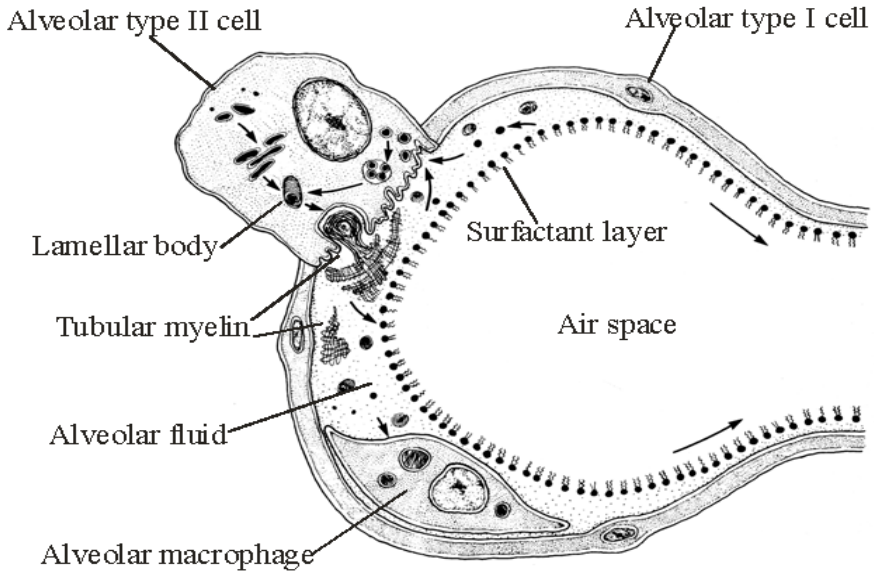
Inhaled PM that reaches the distal lung is likely to injure, interact with and might be transported across the alveolar epithelium due to various biological processes, including phagocytosis, endocytosis, sedimentation and diffusion (Elia et al., 1988, Hong et al., 2004). This is because of their small size and surface chemistry (Nemmar et al., 2001). Electron microscopy shows that the alveolar epithelium is largely a simple squamous lining that is in continuity with the columnar epithelium of the conductive airways and this is separated from the underlying connective tissue and capillaries of the interstitium by a shared basement membrane that makes up the air-blood barrier (Lighty, Veranth & Sarofim, 2000, Kim & Malik, 2003)

3.5 The Structure of alveolar unit

The alveolar unit is made up of unique interlinked sac-like structures consisting of the alveolar cells and the surfactant system (Weibel, 2011). The major function of this unit is the production of lung surfactant, gaseous exchange and host defence.

Oxygen from the inhaled air diffuses through the walls of the alveoli and adjacent capillaries into the red blood cells.

Figure 3.0 Alveolar structures and its defence system



(Jeffery, 2004)

Surfactant is a lipoprotein complex containing phospholipids (fats) and four different types of surfactant proteins: hydrophilic (water-attracting) proteins SP-A and SP-D and the hydrophobic (water-repelling) proteins SP-B and SP-C**.** Some studies have limited the relevance of surfactants to the reduction of surface tension at the air–liquid interface of the lung (Wright, 2005). However, recent studies show that surfactant also functions in pulmonary host defence. As an innate component of the lung's immune system, it helps to maintain sterility and balance immune reactions in the distal airways (Hawgood & Clements, 1990, Wright, 2005). Three principal cells make up the alveolar unit. They are the type 1 and type 2 epithelial

cells or squamous and cuboidal cells respectively. The third one is the alveolar macrophage.

3.5.1 Type 1 alveolar epithelia cell (AT1)

ATI cells are also called type 1 pneumocytes, and squamous epithelial cells. These squamous epithelial cells make up 97% of the alveolar surface due to their large diameter (50-80μm) but account for less than 8% of distal lung cells. AT1 cells are specialized to serve as very thin (about 0.1μm in thickness) gas-permeable cells at the blood–air barrier. They provide a structural lining with a very large surface area and short diffusion distance for gas exchange between alveolar air and blood in the capillaries (Chroneos, Sever-Chroneos & Shepherd, 2010). Electron micrographs reveal that AT1 prominent organelles (endoplasmic reticulum, Golgi complex and mitochondria) cluster around the nucleus, which forms the deepest part of the AT1 cell. Recent studies illustrate that AT1 cells also play a prominent role in preventing fluid loss, facilitating protein transport–transcytosis (Phelps, 2001). AT1 cells are attached to the neighbouring epithelial cells by occludin junctions and desmosomes and can be distinguished from the adjacent capillary endothelial cells by their position bordering the alveolar lumen and their cytokeratin-positivity.

3.5.2 Type 2 alveolar epithelial cells (AT2)

AT2 cells are also referred to as Type 2 pneumocytes, great alveolar cells, and alveolar septal cells. They are cuboidal in shape and cover about 3-7% of the total

alveolar surface, contributing 60% of alveolar epithelial cells and form 16% of the total cells in the distal lungs (Crapo, Barry & Gehr, 1982). AT2 cells occur most often in small groups at the angles where alveolar septal walls converge. They are interspersed among the AT1 cells, to which they attach by desmosomes and occludin junctions. Electron micrographs reveal that AT2 cells contain many mitochondria and a well-developed golgi complex, and are mainly characterized by the presence of large membrane-limited lamellar bodies. The lamellar bodies exhibit many closely apposed concentric and/or parallel membranes that contain glycosaminoglycans and phospholipids.

It has been noted that the major function of AT2 cells is secretory. As mentioned previously, they synthesize and release the pulmonary surfactant (Kim & Malik, 2003). Surfactant is assembled and stored in the lamellar bodies, which also transport the surfactant to the apical cytoplasm. There, the lamellar bodies fuse with the apical plasma membrane and release surfactant onto the alveolar surface. Other metabolic functions of AT2 cells include the production of antioxidants, antiproteases, defensins, surfactant proteins, cytokines/chemokines and many other molecules that play a significant role in lung defence and in the maintenance of pulmonary homeostasis (Stone et al., 1992). AT2 cells serve as a precursor for type 1 epithelial cells during lung growth and repair, leading to the maintenance of alveolar integrity through proliferation and differentiation into type 1 cells.

3.5.3 Alveolar macrophages

Alveolar macrophages are also found both on the surface of alveolar septa and in the interstitium (Figure 3.0). They are the predominant cell type within the alveolar space, and undoubtedly serve as the first line of host defence against inhaled organisms and soluble and particulate matter (Wright, 2005). Macrophages are important in removing any debris that escapes the mucus and cilia in the conducting portion of the respiratory system (Kemp et al., 2008). The complex immunoregulatory role of the macrophage has also been documented. They have been shown to produce a wide variety of pro- and anti-inflammatory agents including cytokines (e.g. IL-1, IL-6, and tumour necrosis factor-α), chemokines (including interleukin-8), which modulate leukocyte function, and growth factors which promote fibroblast migration and replication.

3.6 Migratory cells and barrier components

The epithelial cells also maintain a reservoir of migratory cells. For instance, polymorphonuclear leucocytes are always recruited under acute inflammatory circumstances. Others include: macrophages, lymphocytic and dendritic cells (Fels & Cohn, 1986). Most common in healthy lungs are alveolar macrophages and together with lung dendritic cells, they play a very important role in the immune defence system of the lungs against inhaled organic and inorganic particles. Recent work by (Rubins, 2003) has revealed a synergism between dendritic cells and macrophages to form a transepithelial interdigitating network which counteracts

foreign nanoparticlutes. It has been noted that most of the clearing processes are major barrier components. These structures include the airway route (Reid et al., 2005), the aqueous surface lining layer which makes up the mucociliary escalator (Blank, Rothen-Rutishauser & Gehr, 2007), the alveolar surfactant film (Brain, 1988), the tight junctions and adherens junctions between the epithelial cells (Kilburn, 1968). Other structural barriers that facilitate solute transport and filter unwanted materials include capillary endothelium (Schurch et al., 1990), basement membrane (Godfrey, 1997) and the interstitial connective tissue. It is important to note also that these barriers have to withstand changing hemodynamic pressures from the blood capillary side, to tolerate surface tension forces from the air side, and will encounter many different physical, chemical, and biological insults (Schneeberger, 1977)

3.7 Combustion-derived particle interactions in the lungs

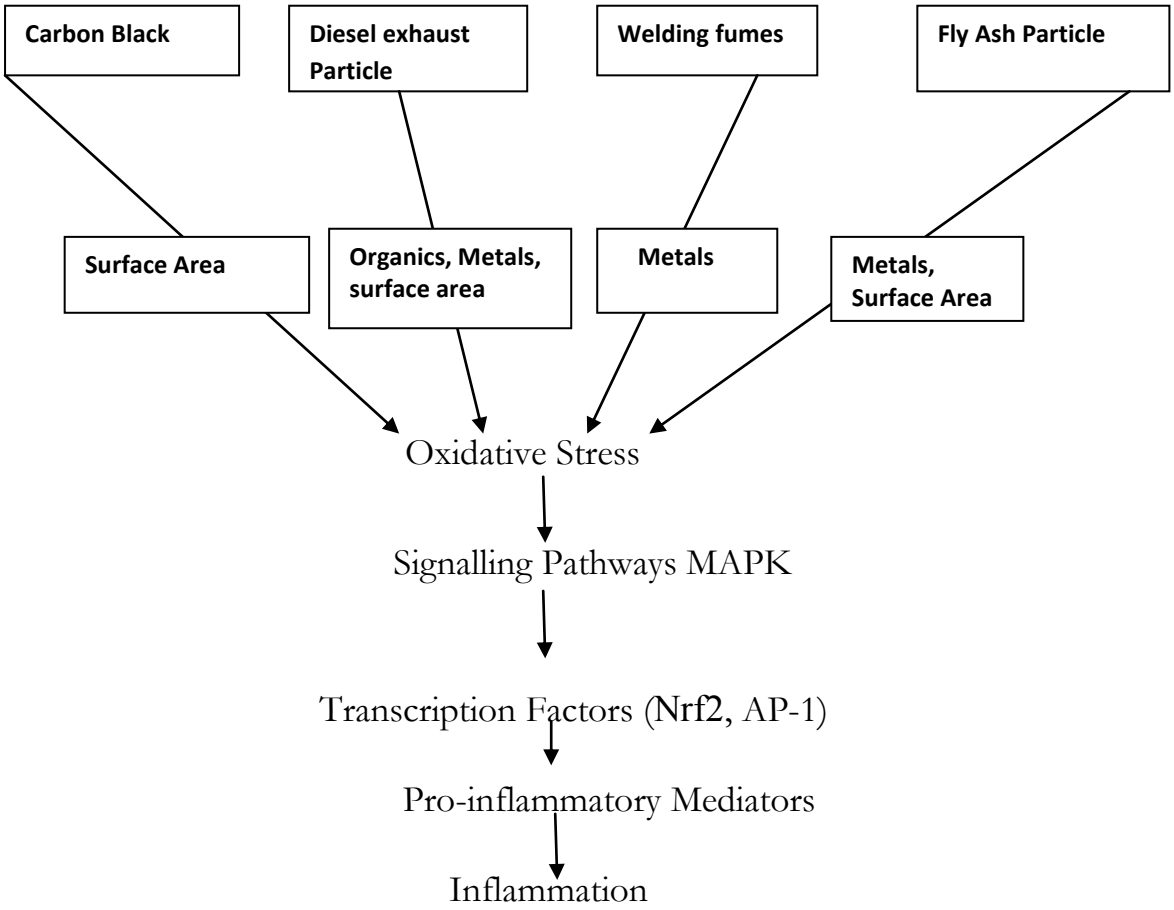
Combustion-derived nanoparticles (CDNP) deposit with high efficiency in the lungs and have significant potential to trigger inflammation due to high particle number and increased surface area per unit mass (Maina & West, 2005). A variety of mechanisms have been linked to CDNP interaction in the lungs. These include the direct effect of the particle components on the intracellular sources of ROS and indirect effects due to pro-inflammatory mediators released from particle-stimulated macrophages within the ultrastructures (Maina & West, 2005).

Inflammation plays a vital role in tissue regeneration, very important to lung injury and repair, but can be harmful in excessive amounts and under inappropriate settings. However, oxidative stress, in addition to being a consequence of normal cell respiration and metabolism, is a common accompaniment to cellular injury. Researchers have identified three important arms of oxidative stress (Rahman &MacNee, 2000, Donaldson el at., 2005). These are as follows:

- *A sensory arm:* This responds to the oxidative stress as indicated by an increase in the production of molecules such as oxidised glutathione (GSSG) and 4 hydroxynonenal within the cells (Donaldson et al., 2005)
- *An effector arm:* This involves the increase in GSSG and 4-hydroxynonenal that stimulates the activation of oxidative-stress-response transcription factors such as Nrf2 and AP-1 that bind to DNA leading to transcription of key pro-inflammatory genes (DNA gene expression)
- *A response arm:* This occurs in the form of translated proteins such as the pro-inflammatory mediators, cytokines, and proteins that act to remove oxidative stress through restoration of redox balance; examples are superoxide dismutase and catalase.

Figure 3.1 summarizes these mechanisms and show how different components of different CDNP might cause oxidative stress through redox sensitive pathways that cause inflammation. It can also be seen that CDNP, produced in a range of situations, could share similar toxicity mechanisms.

Figure 3.1: Possible mechanisms of toxicity of a variety of CDNP



(Donaldson et al., 2005)

DEP has been reported to induce apoptosis in macrophages through ROS generation, with subsequent activation of caspase cascades, loss of membrane integrity, and DNA damage (Donaldson et al., 2005). The components of DEP involved include the organic fraction (Donaldson et al., 2005) and transition metals (Hiura et al., 1999). Organics, such as PAH, have been shown to induce both apoptotic and anti-apoptotic signals (Bonvallot et al., 2001). For carbon black, particle surface area burden has been reported as important and is much greater for a given mass of nanoparticle carbon black (NPCB) than the same mass of

respirable, non-nanoparticle carbon black (Ball et al., 2000). Exposure to welding fumes has been associated with inflammatory cytokine increase and systemic oxidation triggered by the generation of free radicals and soluble metal components (Solhaug et al., 2004). For coal fly ash and residual oil fly ash particles, the bioavailability of iron, vanadium and nickel can lead to redox cycling which liberates oxidants (Gilmour et al., 2004).

In all, a variety of endpoints have been used to determine the toxic potential of the CDNP, these include cell death, cell viability, necrosis/apoptosis, release of LDH, intracellular ROS production, pro-inflammatory mediator release, including IL-1, IL-6, CXCL8 and elevated levels of oxidised GSH (Smith et al., 2000)

3.8 Models to study particle-cell interactions

Assessing the effect of inhaled nanoparticles after inhalation has been a challenging task. The lungs are normally equipped with an efficient defence mechanism that eliminates exogenous substances that enter through the airways. These mechanisms include a number of biological pathways. But the uncertain behaviour of these nanoparticles, that can evade the anatomical complexity of the lungs, has continued to be implicated in alveolar toxicity. Therefore, to better understand the functional and pathological disorders triggered by these particles, direct investigations of their effects are required. Several *in vivo*, *in vitro* and ex-vivo models have been used to understand the mechanisms of particle toxicity (Gilmour et al., 2004).

3.8.1 *In vivo* models

There is abundant literature on *in vivo* inhalation models to examine the deposition and effects of particles in the lung. As reviewed in Particle and Fibre Toxicology, inhalation is the preferred method of exposure of the respiratory tract for hazard identification and to obtain dose response data (Donaldson & Stone, 2003). While it is important to generate physico-chemical information regarding the particle properties and behaviour, effort should also be made for the particle preparations to be well controlled for size and concentration during exposure. This is important because it is possible to generate chamber aerosols of high concentrations which do not occur in life, and which consequently generates agglomerated particles that are so large that very few will gain access to the pulmonary system during the test procedures. Thus, PM of interest should be small enough that the inhaled particles can reach the deeper portions of the lung and should reflect those that exist in life. Reproducing human exposure or environmental conditions will equally guarantee a more reliable result.

Inhalation exposure research include: the whole body, head/nose/mouth–only or lung-only models (Aufderheide, 2005). Driscoll, (1996) reported that intratracheal instillation, pharyngeal or laryngeal aspiration are acceptable methods for pulmonary exposure to evaluate the relative toxicity of PM; however, it was argued that these methods were not adequate substitutes for inhalation studies because they do not represent the actual event of exposure. Secondly, they use less material

than whole body or nose-only inhalation exposure; the latter uses more efficient and controllable concentrations and allows testing of parameters (e.g. blood sample) during exposures.

The problem is that these *in vivo* models are expensive utilising significant numbers of animals, and it is difficult to set up particle preparations representative of those generated in ambient air. Thus preliminary and mechanistic studies *in vitro* provide alternative models in the first instance to help make decisions regarding *in vivo* exposure studies and to therefore address the 3Rs, to replace, reduce and refine animal experimentation

3.8.2 *In vitro* models

Cell cultures have gained considerable importance in basic research and research work employing the use of these models has rapidly expanded in recent years (Shvedova et al., 2003). This is because, compared to *in vivo* techniques, cultured human and animal cells are simpler to use, can be robust and offer a direct test system with the potential to be more focused, controllable and reproducible (Driscoll, 1996). Furthermore, the benefit of minimizing operational cost and research delays (especially in seeking ethical approval) make *in vitro* models clinically and economically desirable (Hartung et al., 2002). In addition, as mentioned above, the *in vitro* models support the European Centre for the Validation of Alternative Methods (ECVAM) efforts, which require that the commission and the member states actively endorse the development, validation and acceptance of methods that

could reduce, refine or replace the use of laboratory animals (Gruber & Hartung, 2004). *In vitro* models are therefore seen as an important adjunct. These models have relied on the cells or tissues freshly isolated from animals or human donors, in the case of primary cells, or at the other extreme, may utilise a laboratory–adapted strain or cell line that has been transformed and serially propagated and maintained in continuous culture (continuous/transformed cell lines). The major difference between these types of tissue culture systems is that primary cells differentiate rapidly in culture, and have a limited capacity to multiply and survive passage; in contrast, continuous cell lines have the ability to proliferate *in vitro* for long periods of time. Despite the fact that a continuous cell line is associated with genetic modification with a high probability of losing many of the phenotypic characteristic of the original cell type *in vivo*, it benefits from a long serial propagation of up to 50 doublings (Gruber & Hartung, 2004) and if used properly, observing the best quality control practices, it can help to a large extent to understand what happens *in vivo*. Another advantage of these models is that they allow specific biological pathways to be tested under controlled conditions, as well as isolation of relevant pathways of interest, that is not always feasible *in vivo* (Ball et al., 2000). Primary human cell cultures can, at times, represent a more heterogeneous population of cell phenotypes, where each isolate could be unique and impossible to reproduce exactly; furthermore, the limited number of cells that can be obtained at each isolation, together with the problem of donor variation, make total reliance on *in*

vitro studies of primary human cells less attractive for routine toxicity testing (Kemp et al., 2008).

3.9 Principles of alveolar epithelial cell evaluation

Many animal and human cell culture models have been adapted to obtain a simplified and more controllable and yet reproducible environment that can compare favourably to processes going on *in vivo*. Thus, in the past, while researchers have continued to improve the quality of their research in epithelial transport kinetics of test molecules, there are reported cases that some of the *in vitro* models did not reproduce the *in vivo* characteristics. Therefore, it has been necessary to focus on these defining characteristics. The most important of these properties which can be used to evaluate *in vitro* epithelial models includes:

- Ability of the cell to form tight junctions
- Presence of molecular markers
- Presence of tight junction proteins or adherens and gap junction proteins
- The viability and integrity of the cell culture model
- Morphology
- Function (e.g. surfactant synthesis)

3.9.1 Ability of the cell to form tight junctions

In the alveoli of the lungs, the tight junctions are seen as a series of focal close contacts where outer lipid leaflets of adjacent unit membranes appear to merge,

thereby eliminating the intracellular space and restricting the passage of electron dense tracers through the pathway (Oberdorster, Oberdorster & Oberdorster, 2005). The tight junction forms the primary solute selectively permeable barrier between the apical and basolateral compartments. Research has revealed that large and smaller solutes, including proteins and possibly nanoparticles, utilize the transcellular and paracellular pathways respectively.

3.9.2 Presence of molecular markers

Most cells have unique and clear signatures that make their characterization less tedious. More recently, Gstraunthaler and Hartung (2002) reported that R3/1 express some markers typical for type 1 pneumocytes including T1α, ICAM, connexin -43, receptor for advanced glycation end products (RAGE) and Caveolin -1 and -2 . This is also corroborated in a review of studies by McElroy & Kasper, (2004). Aquaporin- 5 (McElroy & Kasper, 2004) and aquaporin-4 (Liu & Li, 2005, Zhu, Li & He, 2008), ubiquitous channel proteins that facilitate the transport of water across cell membranes, are also markers of alveolar type 1 and type 2 cells respectively (Table 3.0). The discovery of other type 1 and 2 cell-specific markers have also enhanced their accurate characterisation and identification, and further markers have been identified, to include cell surface lectins, specific monoclonal antibodies and carboxypeptidase M, which appears to be differentially expressed between type 1 and type 2 cells (McDowell & Trump, 1983, Carlstedt & Sheehan, 1988). In a review and characterization studies of *in vitro* cell culture models for

pulmonary assessment of drugs, McElroy and Kapsper (2004), and Horálková et al., (2009) presented some common molecular markers and lectins that have been identified in alveolar type 1 and type 2 cells of human and animal origin, as shown in table 3.0

Table 3.0 Some molecular markers and lectins for alveolar type 1 and type 2 cells

Alveolar type 1 cell (AT 1)	Alveolar Type 2 Cells (AT 2)
Caveolin -1 T1α* P2 Purinergic Receptor 7 (P2X7)* Interferon-induced protein* BCl2-Associated Protein* Aquaporin-5(AQP-5) Reception for advanced glycation end products Soybean lectin FGFR AP-1* ICAM Connexin *Bauhinin Purpurea agglutinin* *Erythrina Cristagalli lectin* *Lycopersicon esculentum lectin* *Ricinus communis agglutinin*	Surfactant Protein (SP)–A, B,C, and D Aquaporin-4 γ- Aminobutyric acid receptor π–subunit (GABRP)* *Maclura promifera agglutinin* *Helix Pomatia lectin* *Sambucus nigra agglutinin*

Not yet confirmed in human pneumocytes

3.9.3 Presence of the tight, adherens and gap junction proteins

A standard *in vitro* epithelial cell culture model should have a full complement of proteins forming the tight junctions. These include: occludin, zonula occludinens (ZO-1,-2,-3), claudins, tricellium (Sporty, Horálková & Ehrhardt, 2008, Horálková et al., 2009) as well as alpha-beta catenin and alpha catenin, which have strong coupling properties, facilitating attachment to the cytoskeleton. Also of high significance is the integral membrane protein in adherens junctions, cadherin which

is responsible for calcium-dependent cell-cell adhesion (Furuse et al., 1993). This is a family of glycoproteins that span the plasma membrane. Other members of the cadherin family include E-Cadherin, N-Cadherin and P-Cadherin (Rogers et al., 1993). Also significant are the cell–cell associating proteins such as gap junction protein connexins, which connect two adjacent lateral plasma membranes, resulting in a space or gap of about 2-4nm (Halbleib & Nelson, 2006). These proteins might be well defined and evaluated, particularly if cell-cell interactions and membrane permeability are a focus of the research. A variety of methods have been used to screen for tight junction proteins or adherens junctions proteins. These include western blotting, immunofluorescence, gel electrophoresis and real-time PCR, (Furuse et al., 1993, Halbleib & Nelson, 2006).

3.9.4 The viability and integrity of the cell culture model

For any *in vitro* model used in particle toxicology and translocation research, it is important to determine the viability and integrity of the model as a matter of priority (Saetta et al., 2000). Determining the transepithelial electrical resistance (TEER) of the model is particularly important. This is performed by measuring the potential difference across the epithelium, i.e. the degree of tightness of the cell–cell contact within the epithelium. The unit of TEER is Ohms centimetre squared (Ωcm^2). Rothen-Rutishauser et al., (2008) reported about $1000\Omega cm^2$ for primary alveolar type 2 epithelial cell cultures, while a review of human bronchial cell lines by Kwang-Jin (2002) reported values between 300 and $400\Omega cm^2$. These values are

greater than that of the human pulmonary A549 adenocarcinoma cell line. An alternative procedure (Elbert, Schafer & Schafers, 1999) relies on the apparent permeation value of the epithelial barrier by a paracellular marker which includes some smaller solutes, like ^{14}C-Mannitol with a value of 1.41 ± 0.13 x 10^{-6} cm/sec in Madine-Darby canine kidney culture. Other techniques have also been evaluated which are very useful in determining the apparent permeation co-efficient values for *in vitro* epithelial models (Forbes, 2000).

3.10 Evaluation of selected *in vitro* alveolar epithelial culture models

The fundamental function of the alveolar epithelium is not only to provide a large surface area for gas exchange, but also to act as blood-gas barrier across which endogenous nano-sized particles, including proteins and solutes, are trafficked through extracellular and cellular compartments, via active and passive processes, from whence they can react with the vasculature (Kwang-Jin, 2002). Lung epithelial cell lines emanate from various regions of the airways, including trachea, bronchi for example Calu-3 and 16HBE14 (Audus et al., 1990, Imanidis et al., 1996) and alveolar epithelium and have been very useful for toxicological studies. However, the results of these researchers have sometimes been variable in diffusive transport kinetic studies due to their poor ability to discriminate various molecules, including CDNP (Kemp et al., 2008). In this study, a well characterised alveolar epithelial cell models with proving evidence of many universally consistent morphological and molecular properties described above as a gold standard was used.

It has been shown that alveolar epithelial type 2 cell cultures can provide a tight epithelial barrier similar to the pulmonary barrier *in vivo*. Availability of reliable and continuously growing cell lines that possess pulmonary epithelial cell morphology and phenotype is scanty, but a majority of the research for particle transport and drug absorption studies has relied on these cell lines. For such studies, the ability of the cells to form a confluent, highly polarised monolayer and tight junctions which give the cell a high transepithelial electrical resistance, is a prerequisite.

3.10.1 Pulmonary A549 adenocarcinoma cell line

The A549 cell line has many important biological characteristics of alveolar epithelial bodies, for example, the cytoplasmic inclusion bodies similar to lamellar bodies of type 2 cells (Rothen-Rutishauser et al., 2008). The phospholipid composition of the cell line according to the work by Sakagami (2006) is similar to the primary isolates of type 2 cells in total phosphatidylcholine, disaturated phosphatidylcholine, and palmitate and saturated fatty acid. Many other ultrastructural components of this cell line include tight junctions, distinct polarization, tight junctional proteins, carboxylic enzymes, and specialised phenotypic markers which have been likened to AT2 cells. However recent studies in this laboratory, and others, show that the A549 cell line is very different from primary human AT2 cells (Shapiro, Nardone & Rooney, 1978), and, in particular, does not necessarily express surfactant proteins A or C.

3.10.2 TT1 cell line

Another cell line that has increased relevance in nanoparticle research is the immortalised human alveolar type 1 epithelial cell (Nardone & Andrews, 1979). Primary human alveolar type 2 cells were immortalised by transduction with catalytic subunits of telomerase and simian virus 40 large-tumour antigens. Characterisation of this cell line revealed a positive result for caveolin-1 and RAGE and pan-cytokeratin, all phenotype markers associated with ATI cells suggesting that the AT2 cells had differentiated into type 1 cells (Swain et al., 2010). These proteins are constitutively expressed by the AT1 cells and have been suggested to mediate the endocytic event reported earlier (Kemp et al., 2008). These studies have also revealed the AT2 cell's potential to differentially internalise positively and negatively charged polystyrene latex microspheres (50nm and 100nm) modified with amine and carboxyl functions, respectively (Stearns, Paulauskis & Godleski, 2001). This means that the cells can discriminate particle size and surface chemistry on the basis of surface charge and size, properties that have been implicated in particle cytotoxicity studies. However, Ruenraroengsak, Thorley & Tetley, (2009) using the TT-1 model, has identified a number of toxicological endpoints which are most marked following exposure to 50nm cationic nanospheres (amine modified). The results showed that the particles stimulated cellular ROS in a time dependent manner. Also observed were significant cell detachment, death and decrease in cell viability. The toxic dose inducing 50% reduction in cell viability activity was 41μg/cm^2 in 24 hours indicating a necrotic and apoptotic effects.

Carboxyl-modified and neutral spheres did not induce such marked, toxic effects even at very high concentrations above 50μg/cm^2, where 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was above 80% of the control.

3.11 Mono- and co-culture models

The aim in particle toxicology is to choose a model that will help to clarify the mechanism of how particles that are inhaled and deposited on the lung surface might interact with the cells and induce a response. While feasible, there is the uphill task of reproducing an *in vitro* environment that will mimic the *in vivo* situation. Several *in vitro* lung cell culture models have been described (Teeguarden et al., 2007, Kemp et al., 2008, Ruenraroengsak, Thorley & Tetley, 2009). Studies comparing mono- and co-cultures have shown differences in the cellular reactions upon particle exposure, related mainly to the magnitude of the response, but also to mechanisms of particle interactions (Rothen-Rutishauser et al., 2008, Sayes, Reed & Warheit, 2007). Thus, the complex cellular reactions upon exposure to PM, and the interplay of different lung cell types in a co-culture, have been shown to substantially modulate the oxidative stress and the inflammatory responses. However, the result from the mono-culture studies offers a better insight into the exact involvement and response of individual cell components and metabolic pathways.

3.12 Limitation of *in vitro* models

There are many significant limitations to *in vitro* models which may hamper their use for risk assessment and determination of toxic potential of PM. It can be seen that, depending on which *in vitro* model is chosen, there can be variability with respect to the biochemical, morphological, functional and bio-kinetic features. There is also reported evidence of disruption of cellular structural integrity and intercellular relationships after dissociation of the tissue during primary cell isolation, which make the cells differ significantly and sometimes respond differently, compared to when they are *in situ*. Evidence suggests that the study of mechanisms regulating a response against inhaled nanoparticles is complex and diverse, and is influenced by the interaction of different cell types, cytokines and variety of other biological components of the cell. This complexity cannot be completely reproduced by an *in vitro* system. According to Rothen-Rutishauser et al., (2008), it is difficult to demonstrate the exact *in vivo* situation in a submerged or indirect exposure in an *in vitro* model system; they noted that the gaseous compounds cannot be included in these studies, and the particle characteristics may be changed during the process of collection and re-suspension. In addition, it may be difficult to determine the precise particle dose which will likely come into contact with the tissues. Finally, cytological differences may arise as a result of differences in tumour micro-environment, for instance, lack of vascularisation, and hypoxia may introduce active sub-cellular fractions that may lead to important phenotypic limitations. Therefore, to make the study more reliable, the model of

choice should be systematically characterised with respect to the requirements –i.e. aims and objectives needed for each investigation.

3.13 Conclusion

If chosen carefully, *in vitro* models of the lung can be used to predict the toxic potential of combustion-derived particles, and more importantly, to facilitate the design and interpretation of animal models of toxicity, and their extrapolation to humans for risk assessment purposes. Thus, such models have aided in the understanding of the mechanisms of how inhaled particles that deposit on the epithelial surface of the lungs can interact and trigger cellular responses. Even though these models exhibit some limitations, there exists a significant balance of benefits which makes their use in toxicological research highly desirable.

When compared to *in vivo* models, it can be seen that *in vitro* methods can be established, may be relatively inexpensive and simple to run and automate, hence, they can yield quick results and permit replication and good quantification (Rothen-Rutishauser et al., 2008). These important properties make them reliable models which can be adapted for high throughput screening of large numbers of newly developed particles (engineered nanoparticles) in a very short time; at the same time, some of these models can be used to estimate the ability of such particles to overcome the air–blood barrier.

TT1 cells have been extensively used for nanoparticle screening in this laboratory. This is because the cells have been well characterised, and offer a reliable yield of relevant phenotypic markers for toxicological studies. Thus they resemble AT1 cells that exist in, and cover 95% of, the alveolar space; they can internalise and discriminate different particles on the basis of surface charge, size and other properties. Therefore, the current work utilised a mono-culture of TT1 cell line to better understand the differential effects of ambient PM, DEP, ROFA and a DEP + ROFA mixture at the epithelial gas liquid interface of the lung.

CHAPTER FOUR

4.0 MATERIALS AND METHODS

4.1 Reagents

The reagents and solvents used for the research were sourced from a reputable company. Dimethyl sulphoxide (DMSO), sodium chloride, trypsin, Hank's balanced salt solution (HBSS), deoxyribonuclease 1 (DNase), phosphate buffered saline (PBS) tablets, bovine serum albumin (BSA), sulphuric acid (H_2SO_4) were obtained from Sigma-Aldrich, Co. Ltd (Poole, UK). Low protein hybridoma medium (LPHM), penicillin-streptomycin-glutamine (PSG) and newborn calf serum (NCS) were purchased from Invitrogen Life Science (Paisley, UK). High protein serum-free medium (DCCM-1) was obtained from React Scientific (Troone, UK) while monoclonal anti-human IL-6 and CXCL8 antibody and all the ELISA reagents were obtained from R&D systems. The MTT assay was used to determine cell viability. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) a yellow tetrazole was also obtained from Invitrogen Life Science (Paisley, UK).

4.2 Culture of the transformed alveolar epithelial cell line

Stocks of transformed (TT1) alveolar epithelial type 2 cells (AT2 cells) at 20-30 passages (Kemp et al 2008) were plated in DCCM-1 containing 0.05% (w/v) G418 (Geneticin), 1% (v/v) PSG (Penicillin/Streptomycin/L-Glutamine) 10% (v/v)

Newborn Calf Serum (NCS) at a density of 10^4cells/well in 96 well plates. The cells reached confluence within 24-48 hours at 37^0C, 5% CO_2. These cells show the same morphological and phonotypical characteristics as AT-1 cells *in vivo* (Teeguarden et al., 2007, Rothen-Rutishauser et al., 2008).

4.2.1 Isolation of primary human alveolar epithelial type-2 (AT2) cells

Primary AT2 cells were isolated from regions of lung tissue with normal appearance as previously described (Kemp et al., 2008) with ethical approval from the Royal Brompton and Harefield Ethical Committee. The AT2 cells were then seeded at a concentration of 100,000 cells/well in 96-well tissue culture plates that had previously been coated with 300µl of a 1% (v/v) solution of collagen (Purecol Solution, Leimuiden, Netherlands: type 1 collagen) and allowed to air dry. Cells reached confluence by 48 hours and had greater than 95% purity assessed by positive staining for alkaline phosphatase (Kemp et al., 2008)

4.3 Preparation of particulate matter stock solution

Industrial forklift DEP (Standard Reference material 2975) was obtained from the National Institute of Standard and Technology (NIST), Gaithersburg, USA. Residual Oil Fly Ash (ROFA) was obtained from the Southern Research Institute Birmingham, Alabama, USA. The ambient PM sample was a gift from Dr. Ian Mudway, while a mixture of DEP and ROFA in equal amounts was used as the fourth sample (50:50). The particle-DCCM-1 suspensions were prepared at a

concentration of 1mg/ml prior to dilution to the appropriate concentration for cell exposure. The solution was vortexed for 30 sec then sonicated for 5 min in a sonicating water bath.

4.4 Preparation and characterisation of particulate matter

Particle size validation, morphology and elemental compositions were determined. Particles were suspended in DCCM-1 medium at a concentration of 50µg/ml and sonicated in a sonicating water bath for 5 min. Sonication was used to disperse the PM further to prevent agglomeration. 10µl of the suspension was then applied to a copper grid and left to dry at room temperature for 10 min. Any remaining liquid was gently removed by adsorption using filter paper.

The physico-chemical properties (elemental or metal composition, size and morphology) of the particles were determined using Transmission Electron Microscopy (TEM) equipped with Energy Dispersive X-ray (INCAx-sight, Oxford Instruments). The TEM analysis was carried out with an accelerating voltage of 200 kV and a specimen tilt angle of 15° was used to optimize the signal for X-ray microanalysis. Copper grid studs with holey carbon film were used for mounting the PM samples for TEM/EDX analysis. The bright-field (BF) and dark-field (DF) features were used for imaging in the TEM. In the latter method, images were generated using the direct electron beam, which consists of forward scattered electrons. Automated imaging processing approaches were used to estimate the particle size.

4.5 Exposure of epithelial cells to particulate matter

Briefly, cells were exposed to DEP, ROFA, ambient particulate matter and 50% (w/v) DEP + 50% (w/v) ROFA mixture at a concentration of 5, 10, 25, 50 and 100µg/ml. Cells were exposed to each condition in triplicate for 24 hrs and the medium was then harvested, centrifuged to remove particles and the supernatant stored at -80°C until analysis (detailed below). Thereafter, the cells were washed with Dulbecco's PBS (DPBS) to remove the residual, non cell-associated particles prior to processing the cells for MTT (cell viability) analysis.

4.6 Measurement of cytokines and chemokines

CXCL8 is a pro-inflammatory chemokine that mediates a number of inflammatory events in the lung, including neutrophil recruitment, a well recognised phenomenon (Witherden et al., 2004, Kemp et al., 2008). Whilst IL-6 works in concert with other cytokines as an important mediator of the acute phase response, CCL2 is expressed by monocytes and other cells such as pulmonary type 2 epithelial cells in culture. CCL2 is chemotactic for monocytes and has been described as essential in monocyte trafficking across the endothelial and epithelial barrier both *in vitro* and *in vivo* (Teran et al., 1997). The study also revealed that expression of CCL2 in pulmonary type 2 alveolar epithelial cells provoked a massive accumulation of monocytes within the alveolar air spaces. Prior to analysis of cytokine release into the conditioned media, supernatants were transferred to microcentrifuge tubes (Eppendorf tubes) and centrifuged (290g, for 10 min; 20^0C)

so as to remove the residual particles which could otherwise interact with the cytokines and/or interfere with the assay. Thereafter, CXCL8, IL-6 and CCL2 concentrations were determined by ELISA (R&D Systems, Abingdon, UK). The assays were performed according to the manufacturer's protocol. The results were expressed as picogram per millilitre (pg/ml) of culture medium. The threshold limit of detection of the assays is 3.4pg /ml for CCL2, 0.039 pg /ml for IL-6, and 1.26pg/ml for CXCL8. The inter-assay coefficient of variance was <5% for all assays conducted.

4.7 Determination of cellular reactive oxygen species (ROS) production

Intracellular ROS were assessed using a fluorometric assay. This utilises 2',7'-dichlorofluorescin (H_2DCF-DA), an oxidation-sensitive fluorescent probe, to evaluate intracellular ROS. H_2DCF-DA can cross the intact cell membranes. Once inside, this probe is deacetylated by intracellular esterases forming H_2DCF, which in the presence of a variety of intracellular peroxides is oxidized to a highly fluorescent end-product, known as 2_,7_dichlorofluorescein (DCF). Stock H_2DCF-DA solution was made at 20 mM in DMSO and stored at -20°C. Before exposure to particles, cells were incubated with 100 mM DCFH-DA for 30 min and thereafter washed in HBSS, for microplate fluorescent analysis. The DCFH-DA-treated cells were exposed to one concentration of each of the test particles. The emitted fluorescence is directly proportional to the concentration of ROS produced. Therefore, the intracellular DCF fluorescence can be used as an index to quantify the overall ROS in cells using a fluorescent plate reader (Singer &

Sansonetti, 2004), and compared with baseline fluorescence in control, unexposed cells.

4.8 Cell viability MTT assay

MTT 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole, is reduced to purple formazan in living cells by mitochondrial reductase. This allows the cell viability and proliferation to be determined, depending on the project aims. It can also be used to evaluate the cytotoxic potential of PM. Mosmann's (1983) MTT-assay protocol was followed with some modifications to limit PM-interference. Briefly, confluent monolayers of TT1 cells were maintained in 96-well plates leaving 3 wells with medium, but without cells as control to measure background, minimum absorbance. The cells were incubated overnight in serum free medium. The particle suspensions were added to the wells in replicates at the concentrations described previously. The plate was incubated for 4 and 24 hours at 37°C in a humidified incubator under 5% CO_2. The medium was removed, and 10µl/100ml tissue culture medium containing MTT reagent (5mg of MTT reagent in 1ml of HBSS) was added and the cells incubated for 2 hours. 200µl of DMSO was then added and left for 3min to solubilise the cells and release formazan. Thereafter, each of the cell lysates was transferred, using an identical template, into a 96 well v-bottom microtiter plate and centrifuged (290g, 10 min, 20^0C) to remove the particles. 100µl of the supernatant was transferred to a 96 well plate, again using the same template, and optical absorbance was measured at

570nm. Cell viability was expressed as a percentage of control (100% viable cells) for all sample concentration exposures. This was calculated from the ratio of mean absorbance for the test sample concentration and control as shown below;

$$\text{Percentage (\%)Viability} = \frac{\text{Mean absorbance of test sample at 570nm}}{\text{Mean absorbance of control sample at 570nm}}$$

1.9 Rationale for the concentrations of ambient particulate matter, DEP and DEP/ROFA mixtures, used in the *in vitro* exposure studies

This thesis focuses on the physico-chemical composition and toxicological potential of ambient air PM and mixtures of particulate materials from different sources. The assessment of risks to public health will be limited to a number of *in vitro* toxicology assays which may be extrapolated to suggest mechanistic processes that may occur *in vivo*. Much of the expected benefits of this thesis are to provide an alternative entry point for a monitoring campaign that considers the source and composition of the PM, rather than just the PM mass and size. Specifically, to concentrate on ROFA, DEP and DEP/ROFA mixtures which can abound in different ambient climes, to provide insight for targeted reduction strategies in these countries and possibly useful in reducing public health risks.

Most studies have reported on the contribution of traffic emissions to the level of ambient air pollution in Europe (WHO, 2005). Thus, 80% of particulate pollutants (PM10) in urban cities have been shown to consist of DEP (WHO European region, 2005). Specifically, in the Greater London Area, approximately 87% of primary PM10 emissions were derived from diesel exhaust emissions (COMEAP,

1998). However, this proportion is significantly different in the Niger-Delta due to increased levels of ROFA emissions, as a result of oil production and refining operations. In this study, knowing that the concentration of DEP would be less than the 80% observed in Europe, an assumption was made that DEP and ROFA may be contributing an equivalent amount by weight to the PM. Hence, a ratio of 50:50 (by weight) for DEP and ROFA was used in this study as a pilot study.

To accurately evaluate the toxicological potential of the ambient PM and other particulate materials used in the present study, a wide range of working concentrations of 5, 10, 25, 50 and 100μg/ml were used (although it should be noted that in the older literature researchers used significantly greater, non-physiological quantities). A concentration of 50μg/ml would be considered to represent a "hot spot" of PM deposition within the lung (Heintz, 2009) whilst 100mg/ml would be considered unlikely but possible in some, highly polluted situations, not normally seen in the UK. Thus, the lower concentrations represent realistic exposure scenarios, albeit during an air pollution episode. Thus the chosen concentrations reflect those occurring within ambient environmental exposures of humans and can be correlated to varying densities (mass/volume) of airborne PM. These concentrations were expressed in microgram per millilitre (μg/ml) or microgram per cubic centimetre of the tissue culture well (μg/cm^3) and can be converted to microgram per cubic meter (μg/m^3) commonly used and derived from many ambient PM sampling equipment. While the lower ranges of these

concentrations may sometimes exist in many regulated ambient environments during periods of air pollution, the top ranges and much higher concentrations more closely relate to those recorded in areas of high ambient PM load such as in the Niger-Delta areas. These high concentrations may also occur as a result of PM accumulation and agglomeration, for example in occupational settings (e.g., oil refinery and coal mines).

4.10 Statistical analyses

The results are expressed as the median (range) values for three independent experiments. The data did not fit the assumptions of a normal distribution (parametric), therefore, a Kruskal-Wallis test was carried out instead of the analysis of variance (ANOVA). Subsequent to a significant finding, a post hoc test (Mann-Whitney test for non-normal data) was carried out to analyze specific PM pairs to determine significant differences following exposure to PM, in cell viability, ROS generation, and CCL2, IL-6 and CXCL8 release. A P value <0.05 was considered to be statistically significant.

CHAPTER FIVE

5.0 RESULTS AND DISCUSSION

5.1 Characterisation of particulate matter

TEM allows the direct (real space) visualization, measurement, analysis and processing of the PM information. TEM provides particle size analysis from individual particles observed in a transmission electron micrograph. The TEM-grid-sample was photographed by a TEM (INCAx-sight, Oxford Instruments). The micrograph was digitalised with high resolution closed-coupled device camera and an image processing data acquisition system to analyse the particle size and shape. This gives localised shape and size information from the areas of the sample where the images were obtained. TEM micrographs data (Figure 5.1, 5.2 and 5.3) revealed that the mean diameter of DEP and ROFA was 65± 2nm and 74 ±18nm respectively (Figures 5.1, 5.2). When ROFA and DEP were mixed, they had a mean diameter of 76 ± 24nm (Figure 5.3). The resultant increase in size may be attributed to potential binding/agglomeration of the mixed particle surfaces. The automated digital particle size data results shown were number-averaged instead of volume averaged, and the TEM analysis was also based on 'thresholding' the intensities from each pixel of an image, and exploiting the differences in intensity between particles and the background (Maus et al., 2002). The morphology (shape) of the particles differed which likely reflects the source of the particles, mode of collection as well as their chemical composition, which will influence the degree of

agglomeration and interaction between particles. Thus, ambient PM was amorphous in shape (Figure 5.4 A, B), while DEP, ROFA and DEP+ROFA mixture were of spherical, crystalline morphology (Figures 5.5 A, B, 5.6 A, B, 5.7 A, B).

Compared to DEP and ROFA, ambient PM has an irregular shape, showing more agglomeration and clusters of particles which consisted of tens and hundreds of tiny individual spherules of indeterminate size. The exact particle size diameters are difficult to establish using TEM due to their amorphous nature. If the ambient PM particles had been collected using cascade impaction, the various size fractions would have been collected separately and would have been easier to analyse using TEM. However, for these exposure studies we wanted to assess the toxicity of all the available particle fractions, so this sample included all particles below an aerodynamic diameter of 10μm (i.e. PM_{10}). In contrast, electron diffraction analysis showed crystalline structures for DEP, ROFA and DEP + ROFA, shown in dark field (Figures 5.4C, 5.5C 5.6C, 5.7C). The morphology of the DEP compares well with other studies that have used the same DEP standard (Wang & Joseph, 1999).

5.2 Chemical composition

There were also differences between the samples in elemental composition as shown in the EDX spectrums (Figure 5.4D, 5.5D, 5.6D, 5.7D). We used DCCM-1 medium to suspend the particles for analysis as this medium was used in the in-vitro exposure studies. Ethanol has also been used as the solvent of choice for

EDX analysis (Ozkaya, 2008). EDX showed the major elements of ambient PM to be Cr, Si, and Sn, of ROFA to be Cl, K, Na, Zn and S, of DEP to be Fe, Cl, Ta, Sn and Zr, and of DEP+ROFA to be Fe, C, O, C, S, Sn, Mo, Zn and Na. These profiles do not exactly match those in the literature. This may be due to differences in the exact source of the samples and "contamination" from a variety of environmental sources. However, it is possible that suspension of particles in tissue culture medium prior to analysis affected the outcome, introducing some elements present in DCCM-1 medium, or masking elements, possibly affecting their solubility, or the interactions of some organic and inorganic components of the particles. However, it was considered that possible effects of DCCM-1 on the elemental profile of the particles should be taken into account, and thus analysis was performed in DCCM-1 medium. Further studies should analyse particles in a number of media/solvents to establish whether this is an important issue that might influence *in vitro* studies, but which may also be important *in vivo*, for example in lung lining liquid.

Figure 5.1 Aerodynamic diameter of DEP. The middle arrow shows the centre of the particle while the red dot indicates the mean size

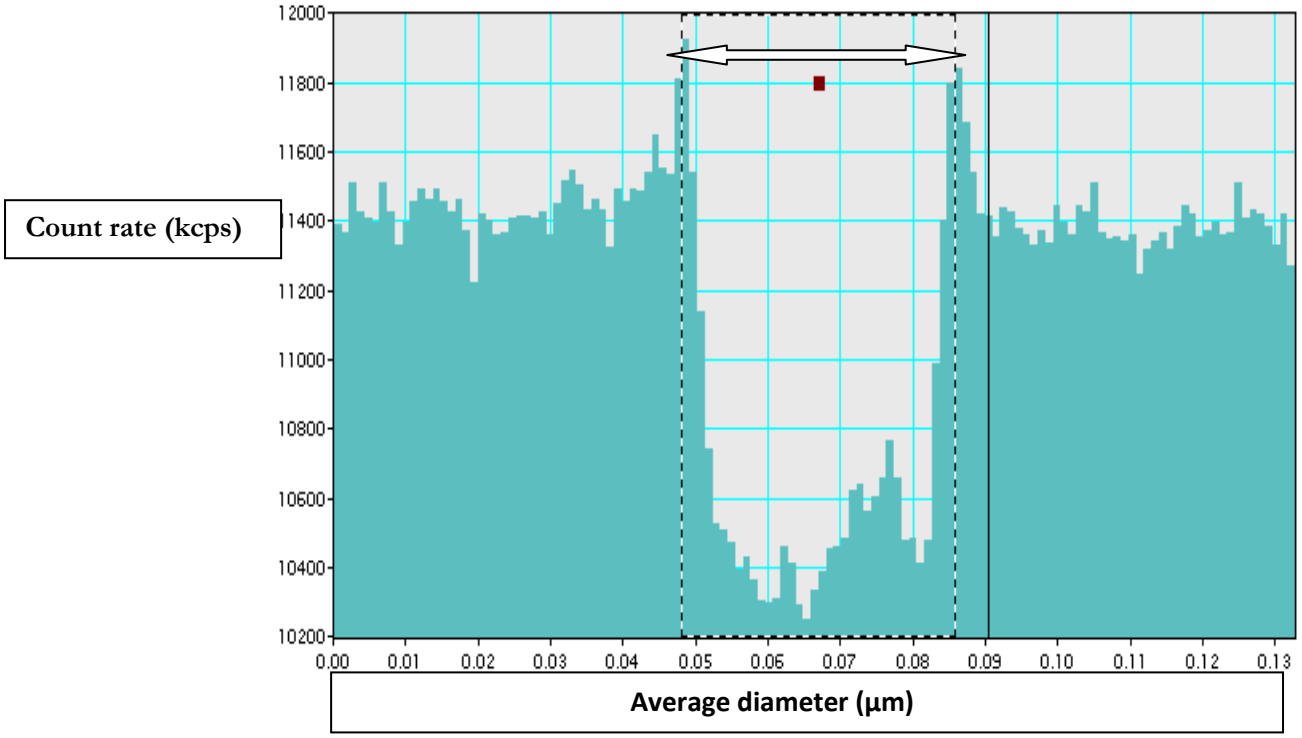


Figure 5.2 Aerodynamic diameter of ROFA mixture. The middle arrow shows the centre of the particle while the red dot indicates the mean size.

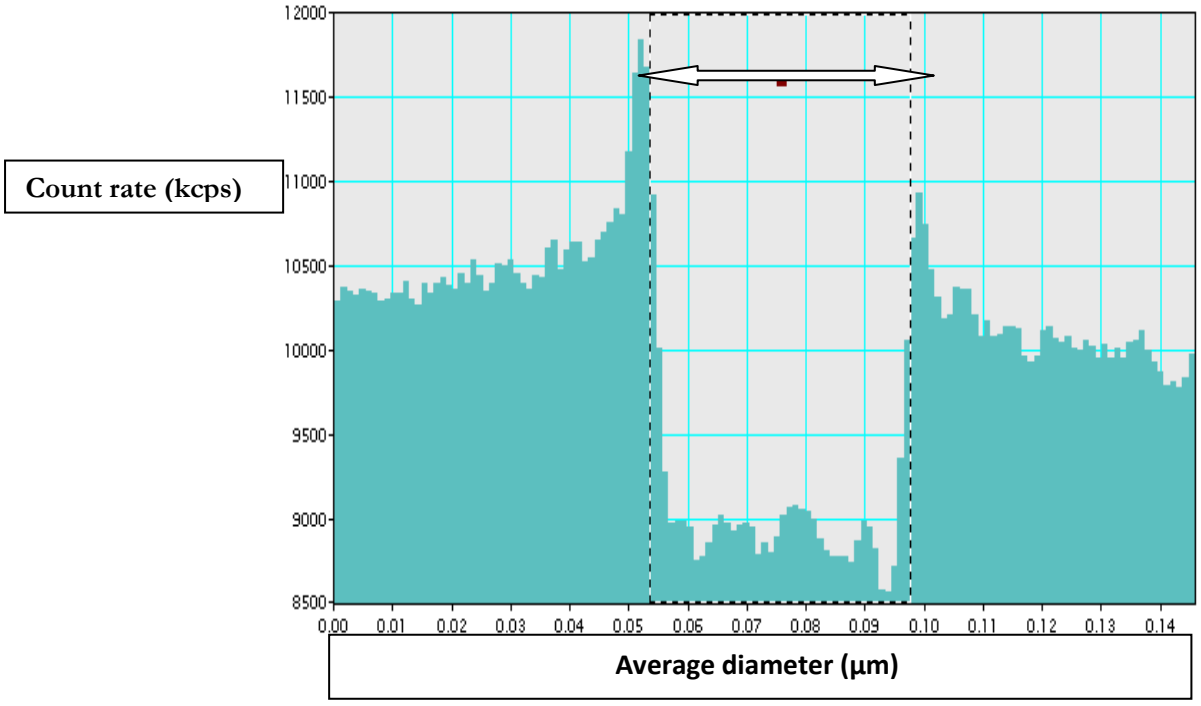


Figure 5.3: Aerodynamic diameter of DEP+ROFA. The middle arrow shows the centre of the particle while the red dot indicates the mean size

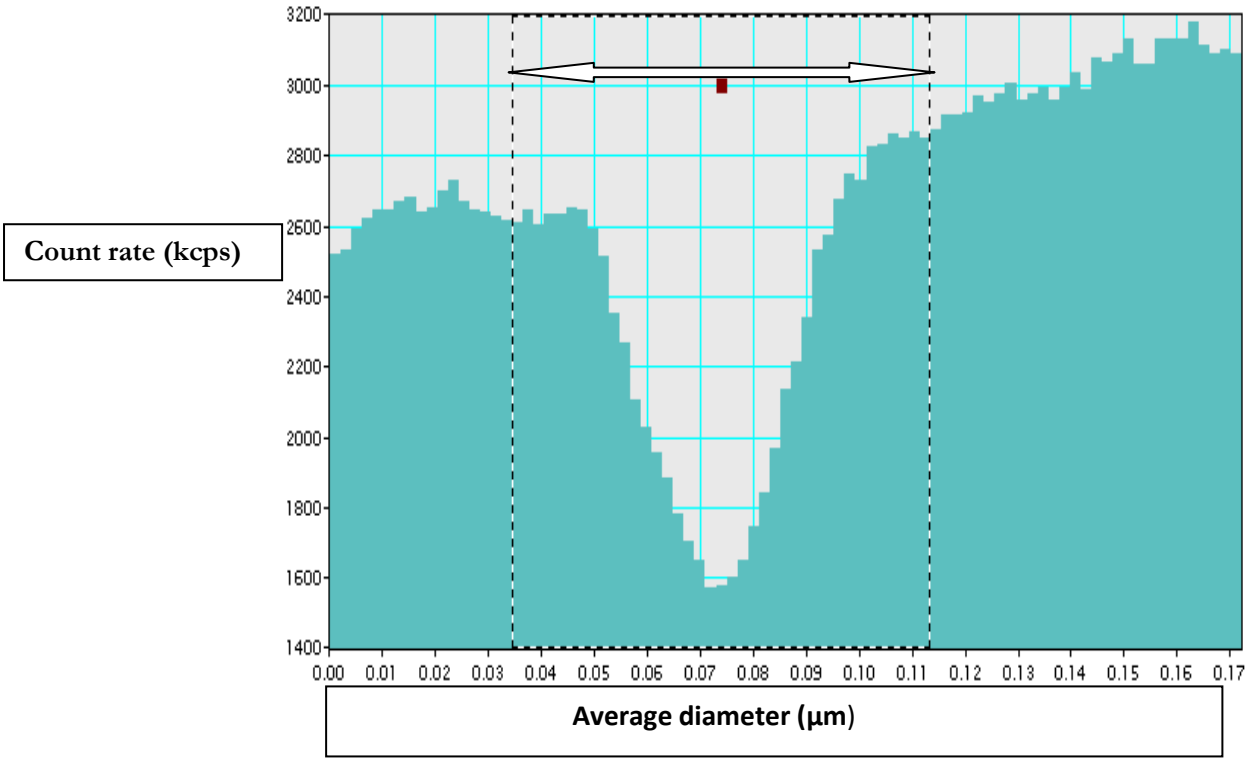
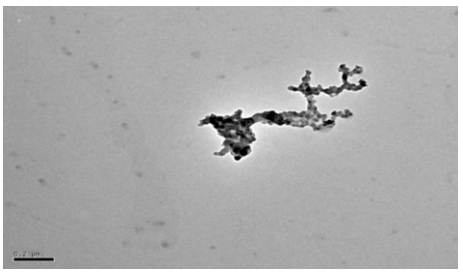
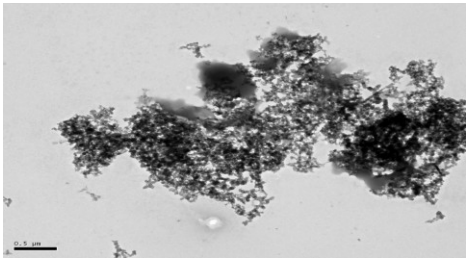


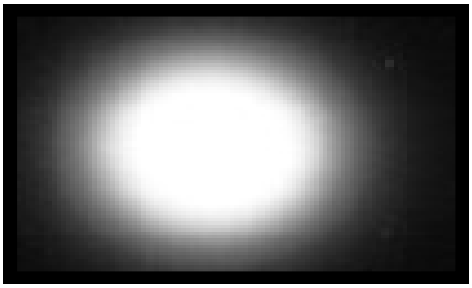
Figure 5.4: TEM micrograph of (A) Amorphous ambient PM, x2000 (B) x4000 of A (C) Electron diffraction (D) EDX spectrum



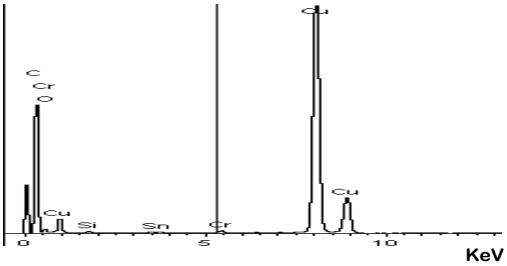
Ambient PM morphology X 2000 Mag.



Ambient PM Morphology x4000 Mag.

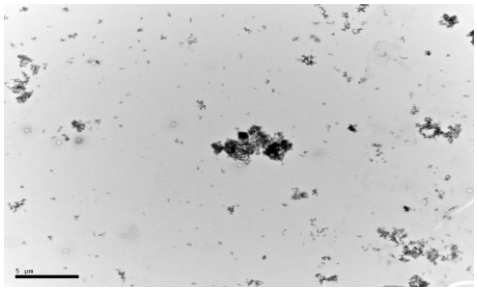


Ambient PM electron diffraction Showing amorphous structure

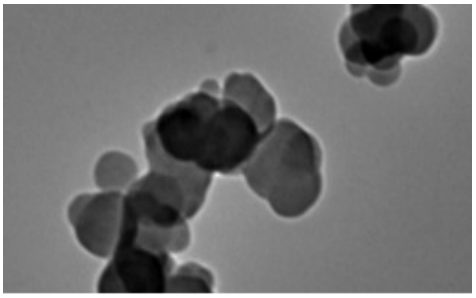


Ambient PM EDX spectrum showing elemental composition

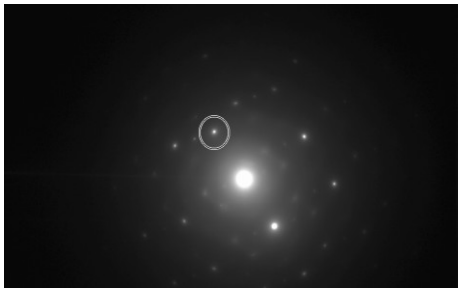
Figure 5.5 TEM micrograph of (A) ROFA morphology, x 4000 (B) ROFA morphology, x 25000(C) Electron diffraction (D) EDX spectrum



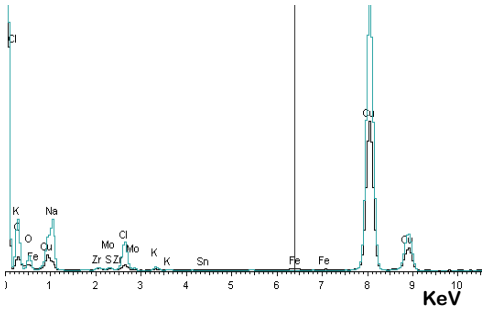
ROFA Morphology x4000 Mag.



ROFA Morphology x25,000

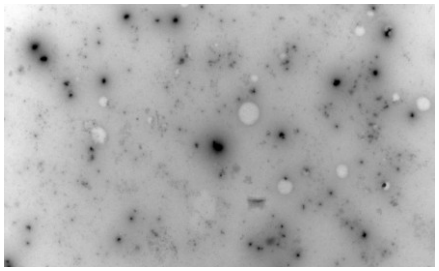


ROFA electron diffraction
Showing crystalline structure

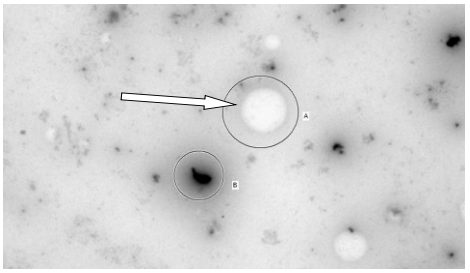


ROFA EDX spectrum
Showing elemental composition

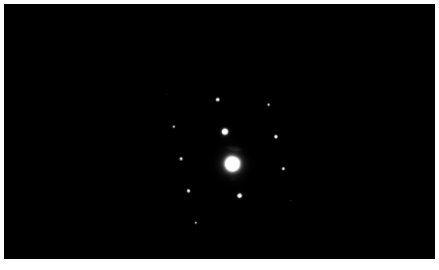
Figure 5.6 TEM micrograph of (A) Iron-rich DEP morphology, x4000 (B) DEP morphology, x8000. Circled area (arrow) showing where the EDX measurements were taken (C) Electron diffraction (D) EDX spectrum.



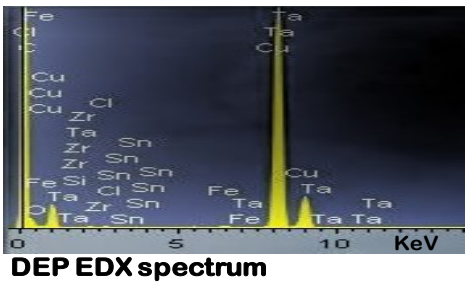
DEP morphology X 4000 Mag.



DEP Morphology x8000 Mag.

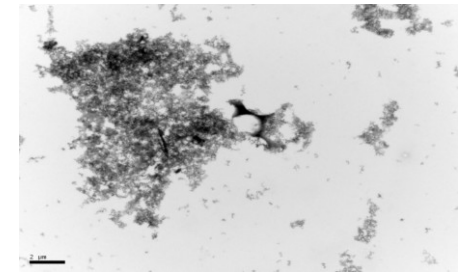


DEP Electron diffraction
Showing crystalline structure

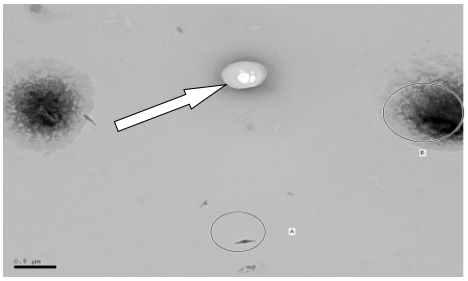


DEP EDX spectrum
showing elemental composition

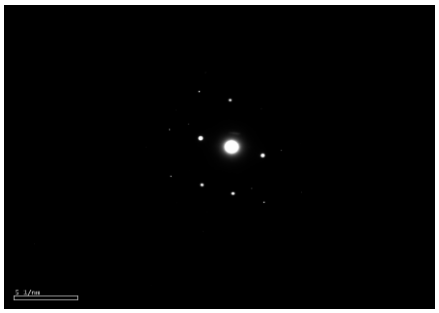
Figure 5.7 TEM micrograph of (A) DEP + ROFA morphology, x4000 (B) DEP+ROFA morphology x25000. *Circled area (arrow) showing where the EDS measurements were taken.* (C) Electron diffraction (D) EDX spectrum



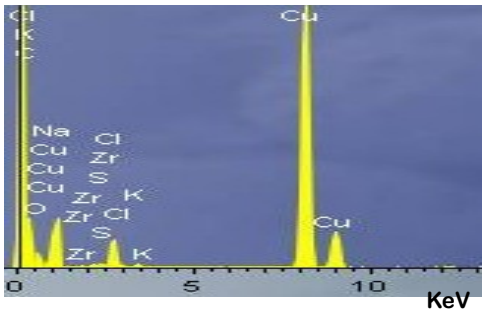
DEP+ROFA morphology X 4000 Mag.



DEP+ROFA morphology X 25000 Mag.



DEP+ROFA electron diffraction Showing crystalline structure



DEP+ROFA EDX spectrum showing elemental composition

5.3 Cell Viability

Changes in TT1 cell viability in response to particle exposure were determined using the MTT assay. After 4 hours, there was no sigificant reduction in cell viability for any of the test samples (Figure 5.8). However, after 24 hours, viabilities were reduced in a concentration related response where the lowest concentrations caused 10-20% cell death and the highest dose caused 40-50% cell death (Figure 5.9), which was most marked following exposure to DEP+ROFA. These changes are not statistically significant.

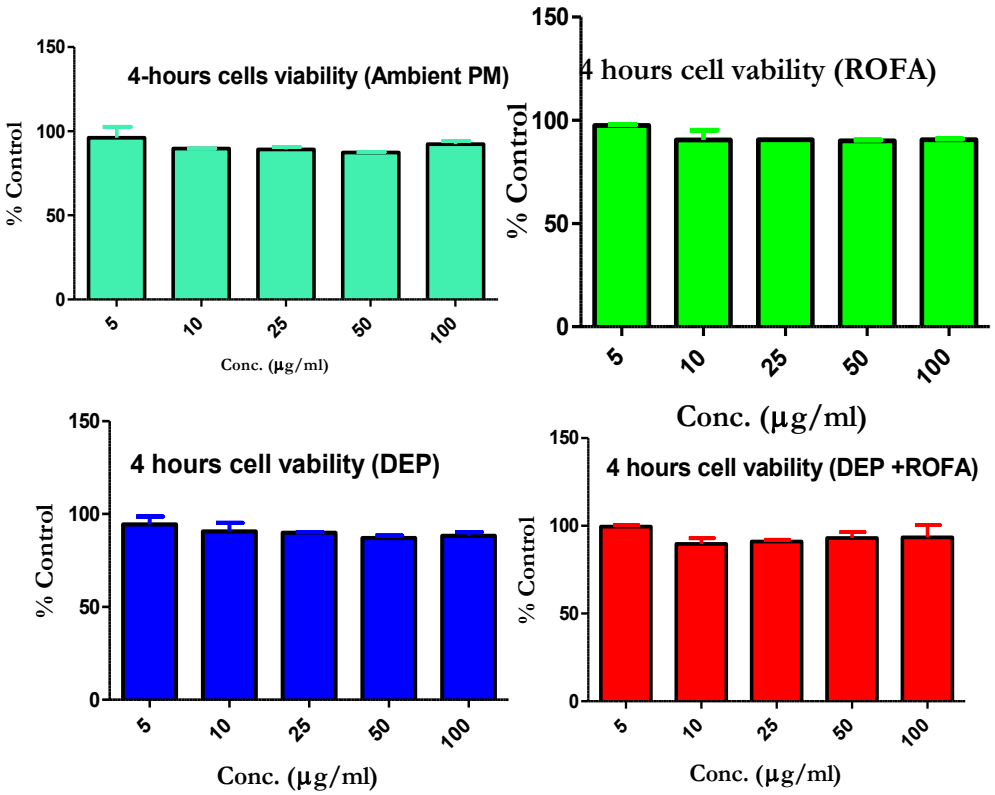


Figure 5.8: Cell viability of T'T1 cells after 4 hrs of exposure to ambient PM, ROFA, DEP and DEP+ROFA. Cell viability was expressed as a percentage of control. Data are shown as median (raange) for three independent experiements (n=3). *There was no significant differences in cell viability for DEP+ROFA mixture compared to DEP, ROFA and ambient PM.*

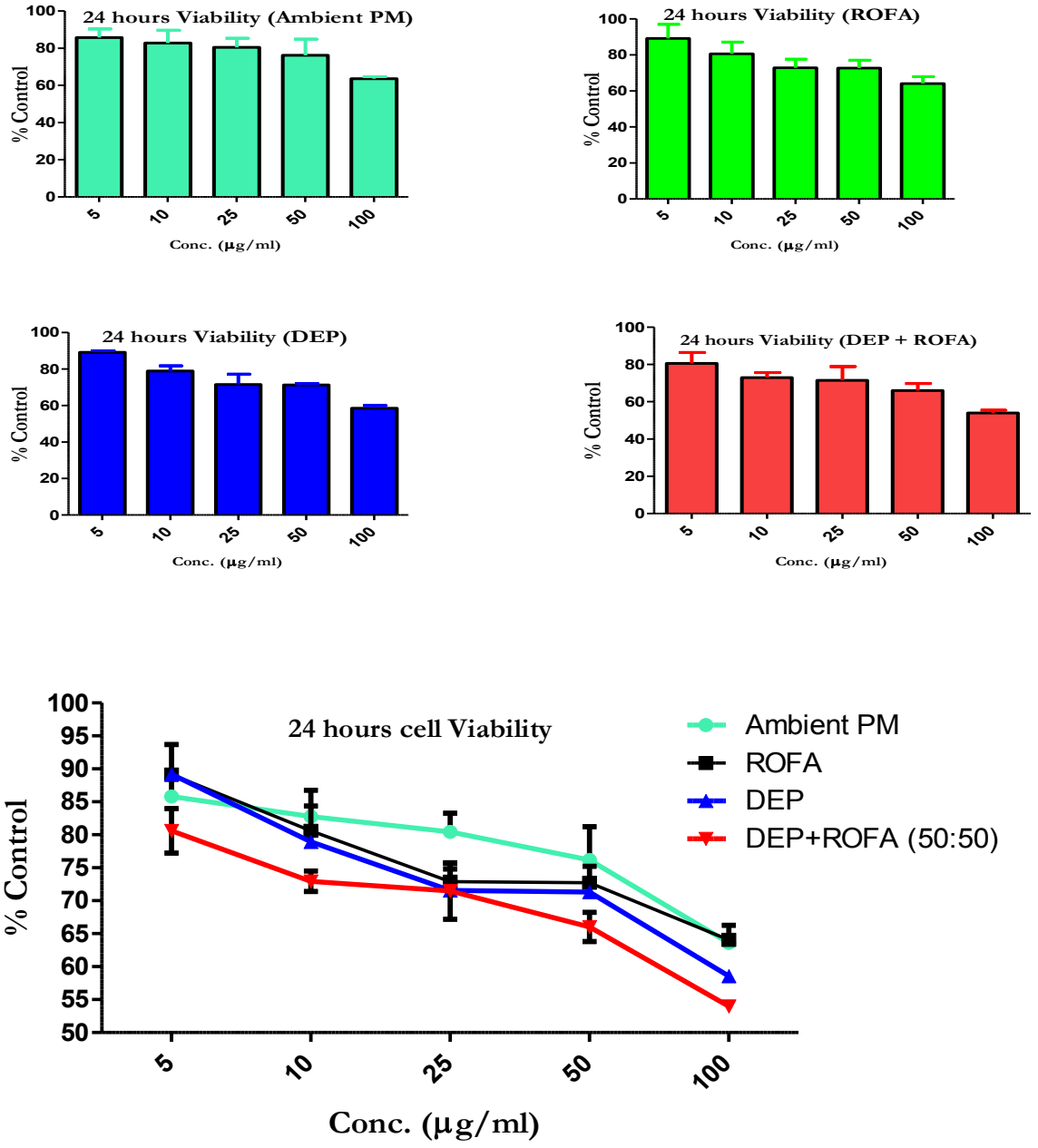


Figure 5.9: Cell viability of TT1 cells after 24 hrs of exposure to ambient PM, ROFA, DEP and DEP+ROFA. Cell viability was expressed as a percentage of control. Data are shown as median (range) for three independent experiements (n=3). *There was no significant differences in cell viability of DEP+ROFA mixture compared to DEP, ROFA and ambient PM.*

5.4 Reactive oxygen species (ROS) production

As expected, within 30 minuts of exposure, there was a strong response and ROS increased significantly above baseline control levels for all 4 types of particle exposure. Exposure to DEP + ROFA caused the most marked increase, compared to DEP or ROFA alone ($P<0.05$). However, ambient PM caused an increase in ROS that was above DEP and ROFA, but lower than DEP + ROFA, but this difference was not statistically significant. After 60 min, although ROS production fell compared to that at 30 min, it remained high, again higher for DEP + ROFA than for the other samples ($P<0.05$). From 120-210 min post-exposure, ROS continued to fall and the differences in ROS levels between particle types disappeared.

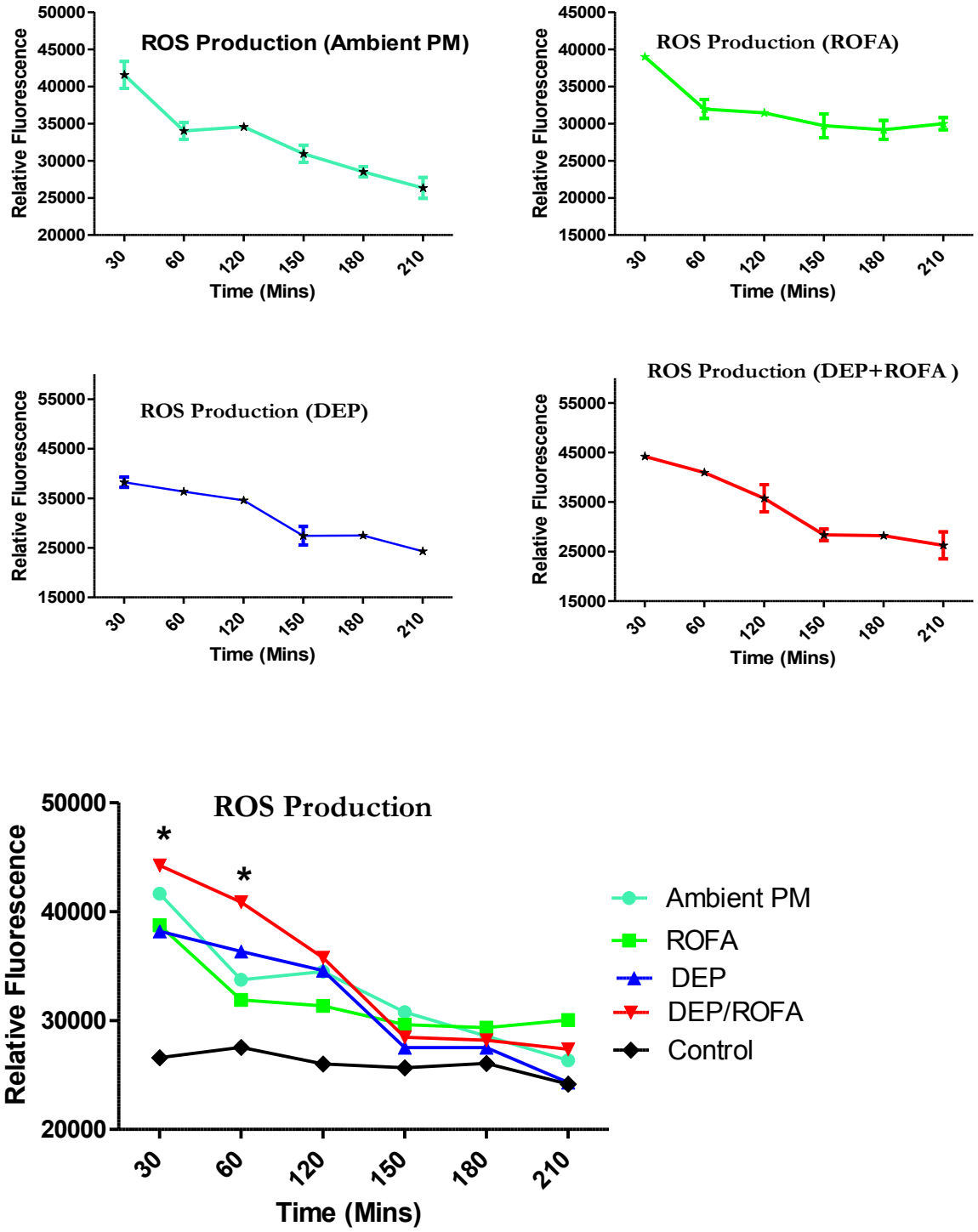


Figure 5.10: ROS production of TT1 cells at different times of exposure to 100µg/ml of ambient PM, ROFA, DEP and DEP+ROFA. The data are presented as median (range) values for three independent experiements (n=3). The asterisks represent significant differences in ROS production from DEP+ROFA exposed cells compared to DEP and ROFA alone, * P < 0.05

5.5 Cytokine and Chemokine release

The particles used in this study significantly potentiated the release of CXCL8, IL-6, and CCL2 by TT1 cells, above those of control, but by varying amounts (Figure 5.11A, 5.12A, 5.13A, 5.14A) over 24 hrs. For DEP+ROFA, these increases were all concentration-dependent, with >50µg/ml in each case triggering a significant increase compared to the non-treated control (**P< 0.002). The response to ROFA for CXCL8 and CCL2 release was also concentration-dependent. However, although DEP and ambient PM stimulated an increase in release in all these cytokines at the lowest concentration, the degree of increase remained the same at the higher concentrations, showing that the profile and degree of stimulation of cytokine release is highly dependent on the particle type (Figure 5.11A, 5.12A, 5.13A).

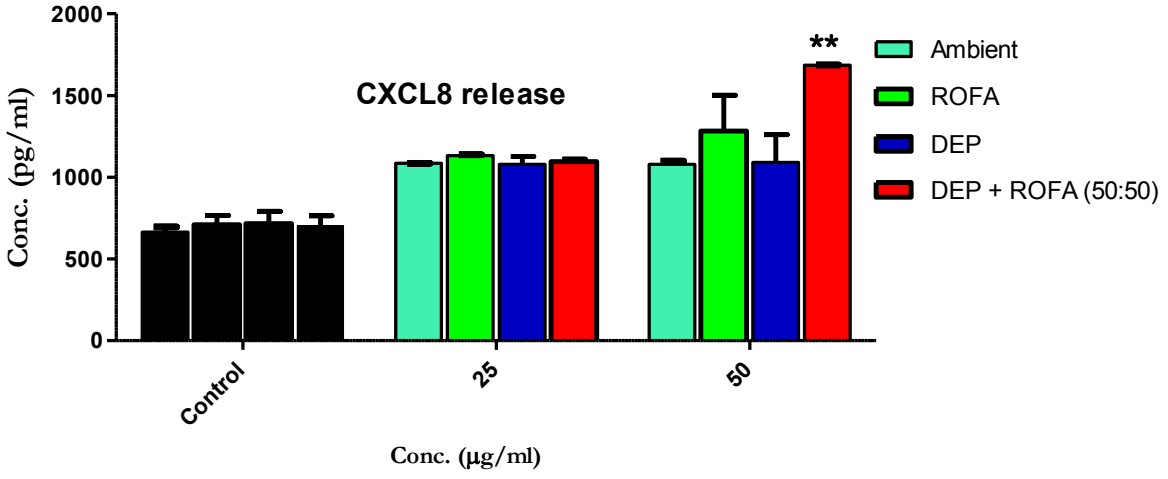


Figure 5.11A: The effect of increasing PM concentrations on the release of CXCL8 compared to control. The data are presented as median (range) values in picograms per millilitre of the supernatant for three independent experiments (n=3). The asterisk denotes significant difference *P<0.002

Notably, the DEP + ROFA mixture consistently induced higher cytokine and chemokine release when compared with ambient PM, DEP and ROFA alone, in CXCL8 (Figure 5.11 B), IL-6 (Figure 5.12B, 5.12C) and CCL2 outputs (Figure 5.13B).

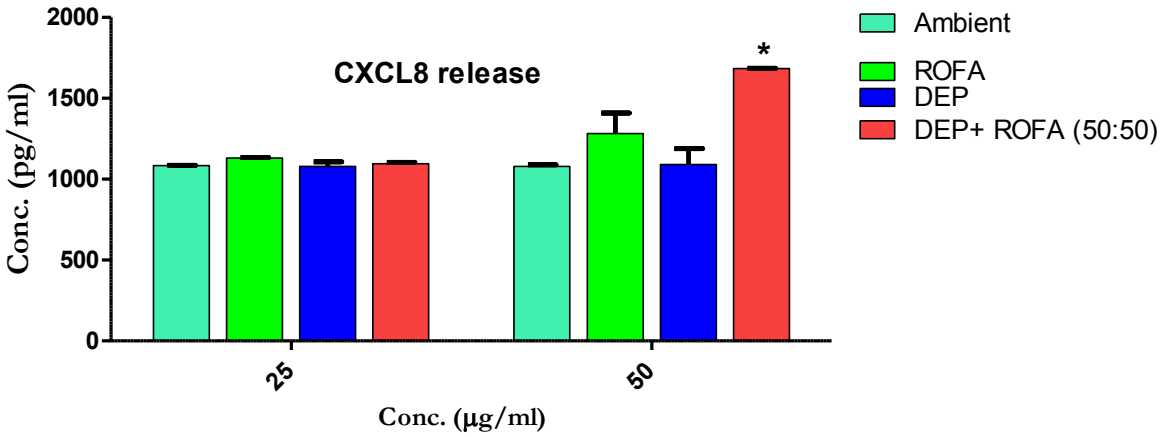


Figure 5.11B: The effect of increasing PM concentrations on the release of CXCL8. The data are presented as median (range) values in picograms per millilitre of the supernatant for three independent experiment (n=3). The asterisk denotes significant difference between DEP+ ROFA and ambient PM, DEP or ROFA alone, *P<0.05

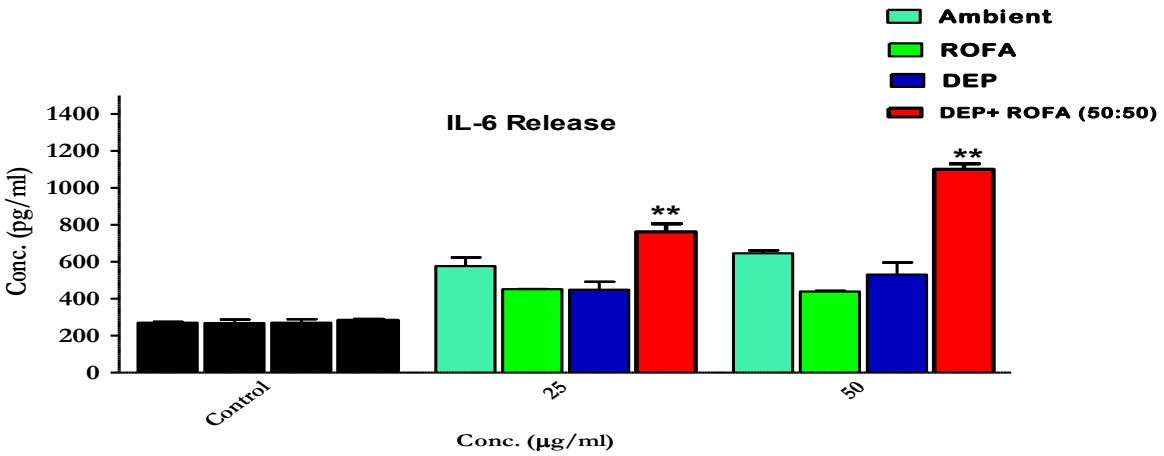


Figure 5.12A: The effect of increasing PM concentrations on the release of IL-6 compared to control. The data are presented as median (range) values in picograms per millilitre of the supernatant for three independent experiment (n=3). The asterisk denotes significant difference, **P<0.002

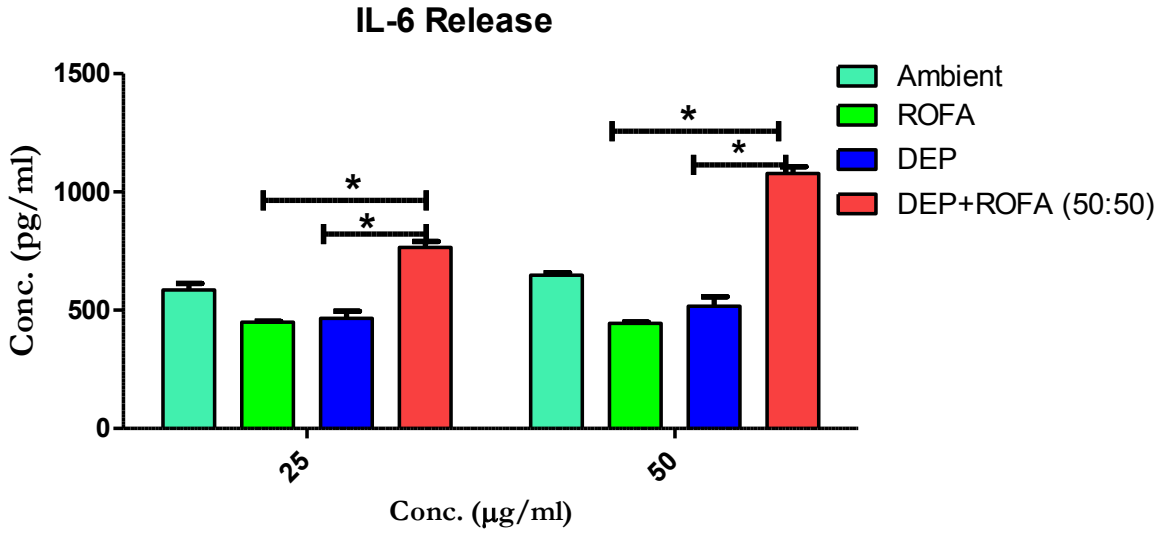


Figure 5.12B: The effect of increasing PM concentrations on the release of IL-6. The data are presented as median (range) in picograms per millilitre of the supernatant for three independent experiment (n=3). The asterisk denotes significant difference between DEP+ ROFA and DEP or ROFA alone, *P<0.05

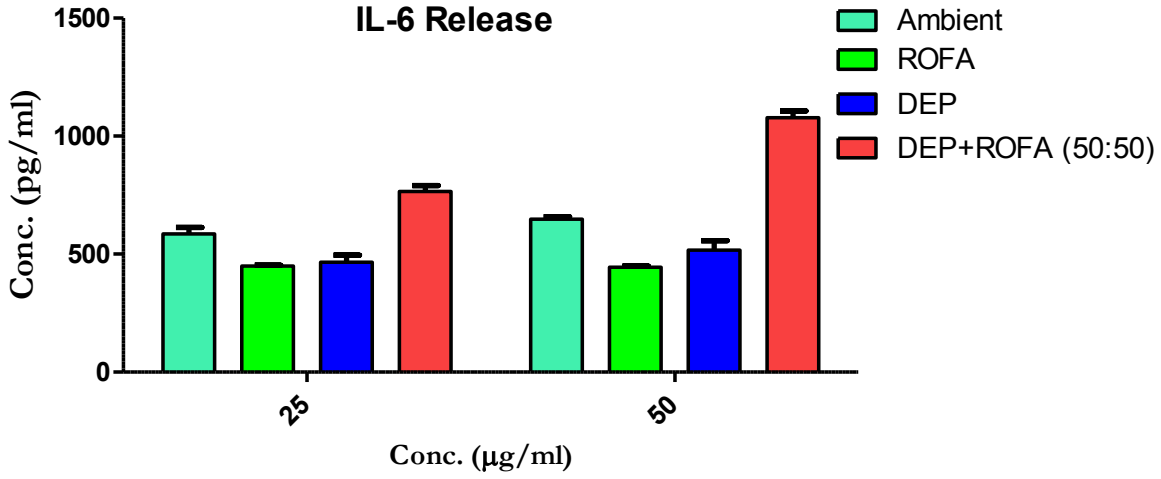


Figure 5.12C: The effect of increasing PM concentrations on the release of IL-6. The data are presented as median (range) values in picograms per millilitre of the supernatant for three independent experiment (n=3. *There was no significant difference between ambient PM and DEP or ROFA.*

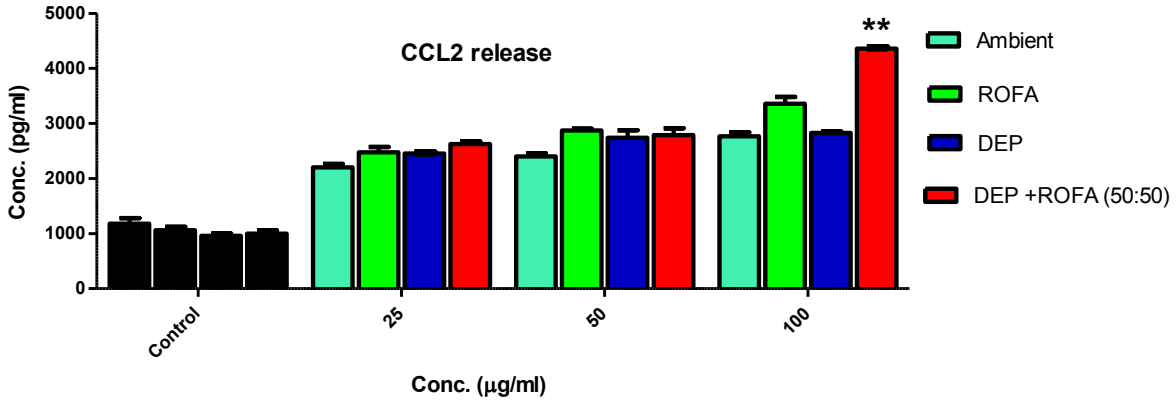


Figure 5.13A: The effect of increasing PM concentrations on the release of CCL2 compared to control. The data are presented as median (range) values in picograms per millilitre of the supernatant, (n=3) ± SEM. The asterisk denotes significant difference, **P<0.002

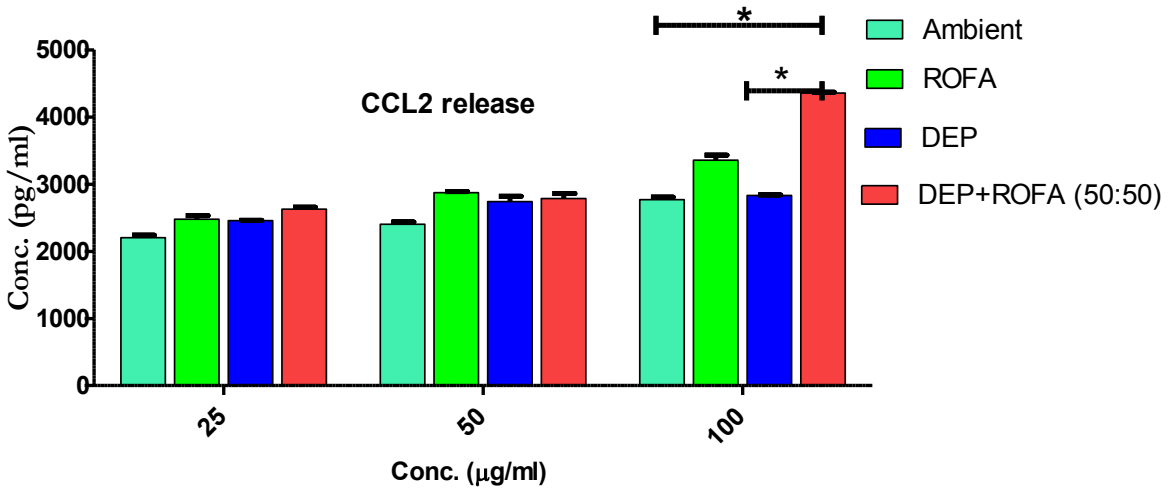


Figure 5.13B: The effect of increasing PM concentrations on the release of CCL2. The data are presented as median (range) values in picograms per millilitre of the supernatant for three independent experiments (n=3). The asterisk denotes significant difference between DEP+ROFA and ambient PM and DEP alone, *P<0.02

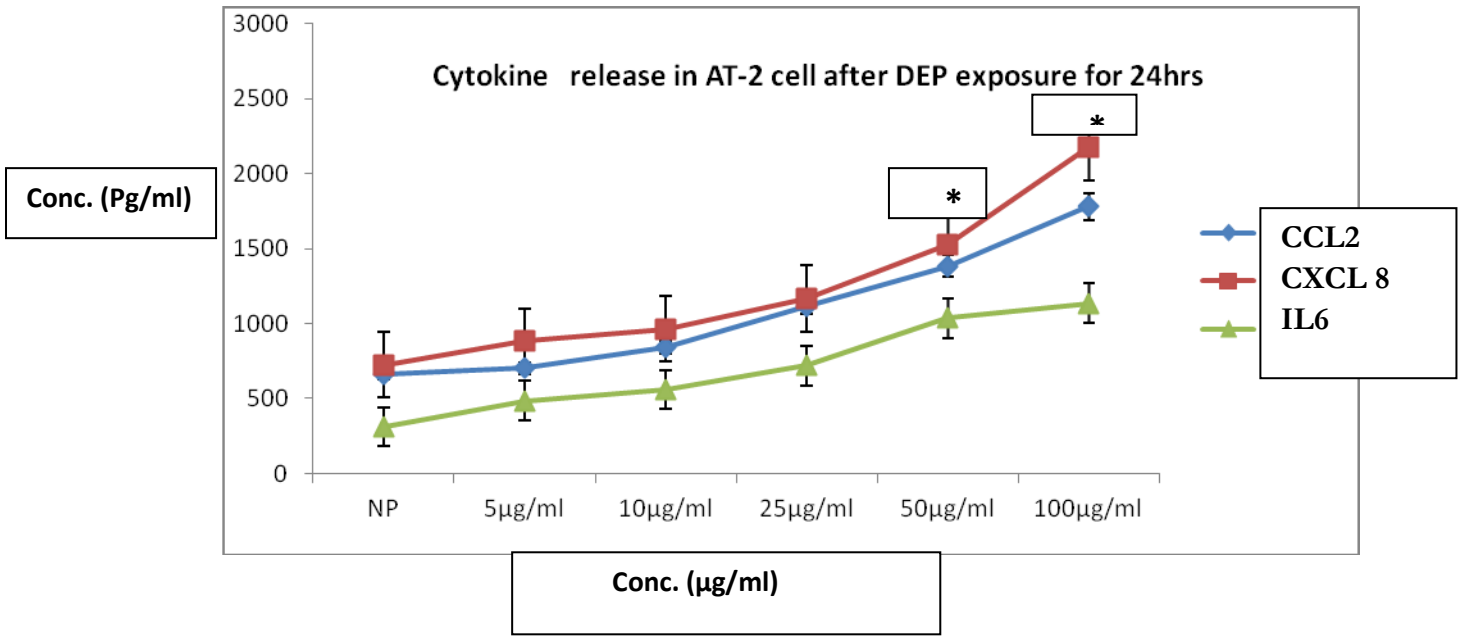


Figure 5.14: The effect of increasing DEP concentrations on the release of CXCL8, IL-6 and CCL2 from AT2 cells. The data are presented as median (range) values in picograms per millilitre of the supernatant (n=3). The asterisk denotes significant differences,*P<0.02

5.6 Discussion

Many studies evaluating the toxicity of particulate materials in human epithelial cells have provided a good basis for the evaluation of the toxic potential of PM (Singh et al., 2004, William & Douglas, 2008). Most of these studies have used cell lines or primary cell culture models (Donaldson et al., 2005, Mitschik et al., 2008). Even though these models yield significant toxicological endpoints, some of them might be limited in scope due to poor particle discrimination owing to lack of adequate characterization. However, the TT1 cell culture model used in this study is well characterised, and has offered a more physiologically relevant approach

being similar to the AT1 cells found in the human alveolar space. The phenotypic characteristics of this model are robust and very suitable for toxicological screening of particulate materials and their mixtures on the bases of surface charge, size and other physico-chemical properties (Amakawa et al., 2003, Kemp et al., 2008)

In the present study, ambient PM, DEP, ROFA and DEP + ROFA mixture differed significantly in terms of morphology, crystallinity and elemental composition but were somewhat similar in primary particle size, except for ambient PM whose size was difficult to estimate due to its amorphous nature. The TEM micrographs of ambient PM morphology showed that it has an irregular particle format which may have been a consequence of the collection procedures and gaseous phase coagulation, high temperature vaporization or condensation under low vapour pressure (Mundandhara, Becker & Madden, 2006). The observed results may also be due to gas-phase adsorption of other complex materials and incomplete combustion of carbonaceous matter resulting in an amorphous carbon black complex (Kemp et al., 2008).

Particle agglomeration was, in fact, observed in all the samples especially the DEP. This compares well with other studies where the same DEP sample has been used (Heinrich & Slama, 2007). The result also revealed that DEP, ROFA and their mixtures have crystalline structures as seen by electron diffraction (Figure. 5.5C, 5.6C and 5.7C). While differences may exist for volatile and semi-volatile organic and inorganic components (PAHs, nitro-PAH, organic carbon) not analysed here,

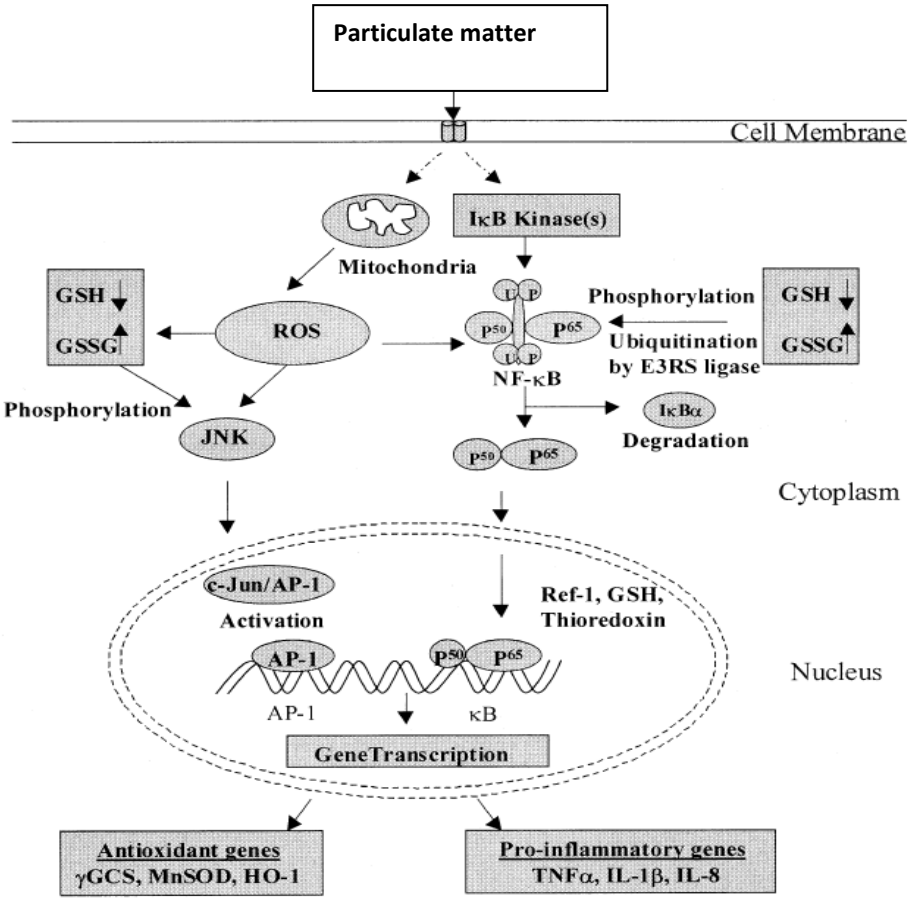
our study revealed a number of reactive elements and transition metals present in the particulate materials under investigation. These include: iron, sulphur, silicon, zinc, molybdenum, zirconium, vanadium, oxygen, chromium, sodium, copper, chlorine, potassium and tin (Figure 5.4D, 5.5D, 5.6D, 5.7D)

Since PM emissions from various sources constitute a major part of ambient air PM burden, knowledge of their *in vitro* toxicity may also provide insight into plausible causative agent(s) and biological mechanisms responsible for any adverse health effects (Risby & Sehnert, 1988). A number of studies (Singh et al., 2004, Dockery et al., 1993) have revealed the ability of transition metals present in PM to participate in Fenton-like chemical reactions (redox cycling). Their studies found that a transition metal's ability to produce reactive oxygen species could trigger tyrosine phosphorylation, nuclear factor kappa B (NF-kB) and Nrf2 activation, and other transcription factor activation, inflammatory lung injury, induction of inflammatory mediators and subsequent expression of genes encoding pro-inflammatory mediators such as cytokines and adhesion molecules regulated by these transcription factors. Possible biological mechanisms have been reviewed by (Donaldson et al., 2005), as illustrated in Figure 5.15.

Consistent with the current findings, previous *in vitro* and animal model studies have also demonstrated that exposure to ambient PM, DEP and ROFA alone have the potential to induce expression and release of CXCL8 and CCL2 by alveolar epithelial cells (Rahman & MacNee, 2000); furthermore, ROFA and DEP have

vanadium and iron as the major transition metals respectively (Aust et al., 2002 Smith et al., 2006).

Figure 5.15 The oxidative stress pathway of NF-$_k$B and AP-1 activation triggered by oxidants



(Rahman & MacNee, 2000)

These metals may have played a similar role in the observed cytotoxicity in the investigations described here.

However, there are no other studies considering the effect of respirable DEP + ROFA mixtures, even though this is a situation that is likely to dominate the properties of ambient air in most oil producing cities in countries such as Nigeria.

Dye et al., (1999) demonstrated that ROFA exposure resulted in acute pulmonary injury and inflammation, including increased airway hyperresponsiveness and pulmonary fibrosis in experimental animals. The relevance of the observed greater effects of DEP+ROFA shown in this study cannot be overemphasised in the light of the positive epidemiologic associations between PM air pollution exposure and decreased pulmonary function, including asthma exacerbations. We have demonstrated here that one or more components of such respirable particles could generate cellular, pulmonary epithelial ROS; the responsible components might be transition metals, organic and/or inorganic components (Dye et al., 1999, Ghio et al., 2003). Some studies have clearly distinguished the participation of different metals, predominantly transition metals including iron, nickel, vanadium, zinc and chromium, which appear to be responsible for their cellular reactivity (Smith et al., 2000, Rahman et al., 2001, BeruBe et al., 2007). DEP and Coal Fly Ash (CFA), were demonstrated to induce ferritin, the iron storage protein, which indicates that bio-available iron was released (Ball et al., 2000a, Nel & Diaz-Sanchez, 2001) from DEP and CFA.

Our studies showed that one or more components of ambient PM, ROFA, DEP or DEP+ ROFA mixture can generate ROS that potentiate the release of cytokines and chemokines, possibly via ROS production as proposed previously (Figure 5.10). Furthermore, DEP + ROFA mixtures stimulate what appears to be an additive increase in IL-6, CXCL8 and CCL2 compared to the equivalent weight of DEP and ROFA alone ($P<0.05$), which was also above that of ambient PM

particles; PM stimulated a greater IL-6 release compared to DEP or ROFA alone, however, this greater change was not statistically significant. There was no enough time to examine possible synergistic effects of the DEP+ROFA mixtures, for example, using isobologram analyses.

Interestingly, there was no significant difference in cytokine/chemokine release when DEP was compared to ROFA. It is possible that this greater effect of DEP + ROFA mixture reflects the combined effects of the metal components and other chemicals (not analysed) leading to oxidative stress, as speculated earlier (Figure 5.15)

In the study of ROS induction, the amount of ROS released in the first 30 min following exposure to DEP was the same when compared to ROFA; however, ROS release for DEP + ROFA mixtures was significantly greater when compared to either of the particles individually ($P<0.05$) at 30 and 60 min post-exposure.

This extended period suggests that transition metals present in the particles may not be the only possible trigger of ROS generation, and that the observed increase could be a combined effect of not only the transition metals, which would react rapidly, but also other slower reacting organic and inorganic components of the particle mixtures. Other studies have identified organic and inorganic compounds including polyaromatic hydrocarbons (PAH), quinones and toluene (Smith et al., 1998), which may be important in activating ROS. These organic components have also been shown to play a major role in the induction of cytotoxicity and

production of CXCL8 in a human epithelial cell line (Singh et al., 2004). The smaller less significant change or cytotoxic effect observed with lower concentrations of DEP + ROFA mixture could have been due to transition metal content, the particle size, composition (bioavailability) of organic compounds including the PAH content, and their interactions, as well as pH and surface properties all of which were found to influence the toxicity of DEP and ROFA (Vecchi et al., 2009).

Previous *in vitro* studies suggest the involvement of ROS in the signal transduction and cytokine release induced following exposure to a range of different particles (Marano et al., 2002). Thus ROS were thought to have originated from transition metal activity (Li et al., 2003), organic compounds (Gonzalez, 2005), and/or the carbonaceous core of the particles (Donaldson et al., 1997). In our samples, any or all these properties could account for cellular oxidative stress and subsequent stimulation of cytokines release, even though ROS decreased over the 24 hours of study, this may be due to corresponding decrease in cell viability

Previous analysis of ROFA has revealed a complex mixture containing nitrates sulphates and metals. The majority of these metals including Fe, V and Ni occur relatively in high concentrations and are water-soluble. This is consistent with studies by (Baulig et al., 2003), where physico-chemical analysis of ROFA revealed Na, Mg, Si, Ca, S, Fe, V, and Ni; Ni and V were the most abundant. Characterisation of the ROFA used in the present study did not reveal the presence

of some of these transition metals; we suspected that the tissue culture medium used for particle suspension may have affected their availability forming, for example ion complexes. This will be determined in our further work and such processes may be important in complex environments where serum and other body fluids are present (e.g. lung secretions, sweat, gastrointestinal secretions). There is evidence in this study of a more complex interaction between the constituents in the DEP + ROFA mixture that was responsible for inducing a significantly more ROS and mediator release. We have already suggested that the chemical composition is important, particularly the metallic and organic materials, but the differences in reactivity between the different particle sources may be due to differences in surface properties and size (Dick et al., 2003) as shown in figure 3.1. Although the aerodynamic diameters of ambient PM, DEP and ROFA were somewhat similar, the surface properties are different, which could be very important. Toxicology studies have demonstrated the importance of particle size on cellular effects, where nanosize is often (not but always) more toxic than bulk material of the same material. It seems possible that this may have played a significant role in our own study especially given that the source and composition of the particles were different (Dreher et al., 1997). The combined actions of the various metals in the mixture with the physical properties of the particles may have contributed to the observed differences between particles in their reactivity. This is consistent with other *in vitro* studies that revealed both synergistic and antagonistic

metal interactions in the cytotoxicity of heavy metals on the alveolar macrophage (Geertz et al., 1994).

Another possibility for the increased effects of the mixture of DEP + ROFA is the presence of varying quantities of sulphates and nitrates, which together might confer a higher acidic potential compared to ROFA or DEP alone. Such processes have been suggested to explain the finding that the kinetics and severity of injury induced by ROFA and transition metal sulphate mixtures, triggering a change in pH (lower) following exposure to a transition metal sulphate mixture containing $Fe_3^+ + V_2^+ + Ni_2^+$, leads to increases in BAL protein, albumin, LDH, neutrophil, and eosinophil levels (Donaldson et al., 2005)

Respirable particles have been implicated in the cytotoxicity and reduced viability of the A549 adenocarcinoma cell line *in vitro* (Donaldson et al., 2005). We have used a more appropriate cell line, which resembles human AT1 cells, and monitored cell viability using the MTT assay as a proxy for mitochondrial activity and injury after 4 and 24 hours of particle exposure (Dreher et al., 1997). Previous studies suggest that DEP exposure is not cytotoxic at the dose range (20 μg/cm^2; 24 h) studied in murine LA-4 alveolar type 2-like epithelial cells, as well as primary murine tracheal epithelial cells (Okeson, Riley & Riley-Saxton, 2004). This contrasts with the present study of human AT1 cells, where, although increased exposure dose, up to 100μg/ml, in TT1 cell line caused no large changes in cell viability after 4 hours, after 24 hours the cell viability was reduced by 40% at the top

concentration of DEP (Figure 5.9). Interestingly, DEP + ROFA induced the greatest degree of cell death possibly related to induction of higher levels of ROS. The combined cell viability data plot (Figure 5.10) shows that the DEP+ROFA mixture was more toxic that the equivalent amount of DEP or ROFA alone. The least toxic was ambient PM, although high concentrations of all the particulate materials induced marked cell death. It could be seen that the marked differences were not statistically significant. This was not surprising because the data generated were not sufficiently powered. Nevertheless, this will be corrected in our subsequent study.

These unique studies of relevant human AT1-like epithelial cells suggest that the alveolar epithelium is likely to be more sensitive to deposition of fine and nano-sized particles than other types of lung epithelial cells.

We speculated that the PM damage might have been mediated by hydroxyl ion. In a similar study (Pan et al., 2009), ambient PM did cause damage to the DNA that was mediated by hydroxyl radicals, as shown by inhibition of the injury with mannitol. The particle-associated hydroxyl radical activity was confirmed using a HPLC based assay to measure the hydroxyl radical. Possible involvement of hydroxyl radicals with DEP + ROFA mixtures could be tested in our future work.

Another factor in the reactivity of particulate material is surface reactivity. The amount of stable free radicals on the surface of different types of DEP particles was measured using electron paramagnetic resonance technology (Manzo et al.,

2010), and it was shown that DEP-SRM 2975 had significantly increased surface free radical concentrations compared with an automobile–derived DEP (A-DEP) used in the same study (Donaldson et al., 1997, Singh et al., 2004). It is possible that DEP + ROFA mixture and/or the ambient PM may have had higher surface free radical activity, something that could be determined in future studies.

DEP, ROFA and ambient PM had very similar reactivity, especially in the induction of CXCL8 (at concentration of 25µg/ml) and CCL2 (at 25µg/ml and 50µg/ml). What is interesting is that DEP+ROFA induced levels significantly higher than DEP, ROFA or ambient PM alone. Numerous studies have shown that DEP has increased pro-inflammatory potential compared to ambient PM (Miller et al., 2009, Singh et al., 2004). Particle characterisation has revealed that ambient air in busy roads or streets, which have a high volume of diesel-powered vehicles, contain 85-90% of DEP (Becker et al., 1996) and might be expected to be very reactive. The present findings showed that ambient PM resulted in increased induction of IL-6 over that of DEP or ROFA alone (not significant). Alternatively, since endotoxin has been shown to be associated with the insoluble fraction of PM, (Bleck et al., 2010), and is considered to explain some of the pro-inflammatory properties of PM, this may have been responsible for the observed effect. However, ambient PM had a very similar stimulatory effect to DEP and ROFA, on CXCL8 and CCL2 release. It is possible that the marked induction of IL-6 following ambient PM exposure reflected a specific toll-like receptor (TLRs) mediated cellular response. TLRs are pattern recognition receptors (PRR) and/or

evolutionarily conserved trans-membrane molecules broadly shared by pathogens . As soon as these molecules (alone or associated with pathogens) have breached the host physical barriers such as the skin, they are recognized. This helps the immune system to recognize pathogen associated molecular patterns (PAMP). It could be possible that the endotoxins (LPS) present in the ambient PM may have differentially stimulated some of the many intracellular signalling dependent and independent pathways, leading to the activation of transcription factor Nrf2, which in turn promotes pro-inflammatory cytokine production and release**.** The inherent limitation to this is that most of the TLR pathways could induce the pro-inflammatory mediators measured here due to Nrf2 activation, as could the ambient PM itself. Therefore, we cannot make a firm conclusion about the effect of ambient PM on the TLR pathway and thus the innate immune system as a whole. One way of determining the contribution of endotoxin to the responses measured here would be to add polymyxin, an inhibitor of endotoxin, to a parallel set of exposures, to determine whether it reduces the overall response.

There is evidence that the surface charge and lower pH of PM play a significant role in their interaction with epithelial cells (Veronesi et al., 1999, Weckwerth, 2001, Schaumann et al., 2004). We speculated that the surface charge and lower pH of the PM used in our study may have mediated the induction of cytotoxicity and release of cytokines (IL-6). Accordingly, a morphometric analysis of surface charge (i.e., zeta potential) and pH values of microscopically visible (≥2.0 μm) particulate materials, including synthetic ROFA (filtrate containing soluble acid and metals)

suspended in either a Hepes-buffered KCl solution or keratinocyte growth medium, revealed that that ROFA has a high zeta potential of −36 ± 2 compared to coal fly ash and ambient PM (Veronesi et al., 2002).The evidence further indicated that lower pH (acidity) and surface charge was correlated with cytokine (IL-6) release an increase in intracellular calcium (Ca^{2+}) in immortalised tracheal-bronchial epithelial cells (BEAS-2B). One of the possible explanation is that ROFA is composed largely of soluble metals and acidic sulphates (Antonini et al., 2004, Roberts et al, 2009), hence, the acidity of ROFA may have stimulated the cytokine release through activation of pH-sensitive irritant receptors that are differentially sensitive to acid pH (Veronesi et al., 1998), and that its chemistry not only contributes to high cytokine release relative to other PM but also predicts its low number of PM above 2μm. These characteristics are considered relevant predictors of oxidative stress and inflammation (Gavett et al., 1997, Kleeman et al., 2000; Neas, 2000)

Our data indicate that the lower pH and surface charge (i.e., zeta potential) carried on our particulate materials might have predicted their differential release of inflammatory cytokines and induction of ROS in TT1 cells.

5.7 Conclusion and future work

In conclusion, the DEP + ROFA mixture appeared to induce an additive cellular response, compared to ambient PM, DEP and ROFA alone, which themselves were bioreactive. Although evidence suggests that lower pH, surface charge,

particle size, transition metals and other organic and inorganic components of the particles may have been responsible in the observed cellular changes, this has not been resolved. Furthermore, this work can be considered inconclusive given that other components of the particle mixtures have not yet been factored in. Therefore, based on the present results, it cannot be said at this point which transition metals, if any, have the most significant cellular effects, nor can we say that the particle size and endotoxin, rather than PAH, surface charge and other organic components are likely to generate the largest oxidative and inflammatory burden at the epithelial air-liquid interface. Most significantly, we do not yet have complete understanding of how mixtures of DEP and ROFA can induce a greater cytotoxic and pro-inflammatory effect than DEP or ROFA alone.

Despite the fact that the data generated in this preliminary study were not sufficiently powered to detect even a slight increase cellular response, it was interesting to discover a significant difference in toxic potential of DEP +ROFA compared to DEP, ROFA and ambient PM at the present level. In addition to this statistical challenge, more work is required to obtain a consistent physico-chemical analysis of the composition of the particulate materials which may have contributed to PM toxicology *in vitro*. The medium (DCCM) used for PM dispersion prior to characterisation may have introduced some artefacts in the particle analysis. In future, to examine specific metal or elemental effects, appropriate metal chelating agents could be used to block the activity of specific metals and therefore precisely monitor the cellular effects brought about by the

transition metals. This would help to determine whether these transition metals act either individually or as soluble mixtures, or whether any of the interactions could be described as antagonist, synergistic or just additive in their effects.

Future planned work also includes further characterisation and investigation of the amount of these metals, organic and inorganic particle components, including their surface charge (zeta potential) and the influence of pH on their specific contribution to the observed effects and the exact bio-reactive mechanisms. Previous studies support the hypothesis that differences in the surface charge carried by PM and oxidative stress might relate to their differential inflammatory and cytotoxic effects on cultured respiratory epithelial cells (Veronesi et al., 1999, Veronesi et al., 2002, Moshammer & Neuberger, 2003). However, further work would address whether the differences in the zeta potential carried on particles from the various PM are due to PM-specific compounds and transition metals. This knowledge would help to establish a relationship between surface charge of not only PM_{10}, $PM_{2.5}$ and smaller (nano) size particles but also their possible inflammatory properties. Such information, in addition to the present study, may further elaborate which physicochemical properties of PM and their mixtures contribute most to their toxicity.

A number of sub-studies would involve an epidemiological evaluation of a possible association of DEP/ROFA and cardiovascular disease. This would provide new evidence through routine air sampling and evaluation of total exposure and further

evaluation of biological mechanisms, including laboratory procedures directed specifically at the role this unresearched PM mixture could play in the induction and exacerbation of respiratory diseases like asthma.

Irrespective of the above limitations, our findings clearly demonstrate that not only DEP+ROFA mixtures, but also exposure to individual PM, can significantly potentiate cellular responses. In the wider context of public health discussion, the evidence presented here could, for the first-time, implicate DEP+ROFA mixtures as stimulating cellular effects above those of each particle alone. A wider implication is increasing host susceptibility to infection in the presence of high levels of these PM's, adding to pre-existing knowledge, and *in vitro* data, implicating DEPs and other nano-sized particulate materials (Veronesi et al., 2002, Bhattacharjee et al., 2010) in worsening susceptibility to infection. The differences observed could be linked to the apparent difference in physico-chemical properties including size, surface charge, organic and inorganic compounds, and elemental compositions. Although further particle characterisation is needed, understanding the biological mechanism through which the particles' components act seems imperative. Nonetheless, with increasing levels of ambient PM in most cities combined with particles generated in oil producing areas, there is urgent need for improved policy action to control anthropogenic particulate material emissions.

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