DEVELOPING AN IN VITRO TECHNOLOGY TO STUDY THE INFLAMMATION POTENTIAL OF AMBIENT PARTICLE TYPES

by

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ABSTRACT

Elevated levels of suspended particles in the troposphere, termed particulate matter, elicit a myriad of adverse health effects in humans, ranging from shortness of breath and wheezing to myocardial infarction and death. It is currently believed that the adverse health effects associated with particulate matter are mediated by the inflammatory response initiated by the lung following particulate matter inhalation. What remains an area of much interest is elucidating the specific properties of particulate matter, physical or chemical, that cause the upregulation of proinflammatory mediators.

The basic premise of this thesis was to identify the specific chemical components of particulate matter responsible for its adverse health effects. To address this issue, instrumentation and methodology were developed wherein one could design, create, levitate and deposit particles of both known chemical composition and size onto lung cells, in vitro, followed by the monitoring of the downstream biological response.

An initial study focused on the role of the endotoxin component in particulate matter toxicity. Through a series of blocking studies we found that endotoxin acted synergistically with the particle core to elicit upregulation of proinflammatory mediators, including IL-1β, TNF-α and ICAM-1; all of which are associated with the NF-κB pathway. Through characterizing this relatively simple system, one observation became apparent: the presence of the insoluble particle core had a profound effect on the cellular response; that is to say, the particle core was not simply a delivery vector, but a determinant factor in the final intracellular location of the toxic chemical.

The latter observation held true as other particle types were studied and in addition, it was found that the nature of the actual chemical species itself plays a dual role in particle toxicity; first by retaining its toxic properties and second by altering the physical properties of the particle.
It stems from these findings that the toxicity of the chemical components must be studied in concert and not as individual entities.
DEDICATION

For mom and dad
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AC</td>
<td>alternating current</td>
</tr>
<tr>
<td>C</td>
<td>Celsius</td>
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<tr>
<td>CFCAS</td>
<td>Canadian Foundation for Climate and Atmospheric Science</td>
</tr>
<tr>
<td>cm</td>
<td>centimetre</td>
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<tr>
<td>DC</td>
<td>direct current</td>
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<tr>
<td>EDB</td>
<td>electrodynamic balance</td>
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<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbant assay</td>
</tr>
<tr>
<td>EPA</td>
<td>Environmental Protection Agency</td>
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<tr>
<td>g</td>
<td>gram</td>
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<tr>
<td>GC-MS</td>
<td>gas chromatography-mass spectrometry</td>
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<tr>
<td>h</td>
<td>hour</td>
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<tr>
<td>Hz</td>
<td>hertz</td>
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<tr>
<td>iCAPTURE</td>
<td>Imaging, Cell Analysis, and Phenotyping Toward Understanding Responsive, Reparative, Remodelling, and Recombinant Events</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IR</td>
<td>infrared</td>
</tr>
<tr>
<td>K</td>
<td>Kelvin</td>
</tr>
<tr>
<td>k</td>
<td>Boltzmann constant</td>
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</tbody>
</table>
kHz  kilohertz
L      litre
m/z    mass to charge ratio
M      matrix
MALDI  matrix assisted laser desorption/ionization
MHz    megahertz
MS     mass spectrometry
mL     millilitre
mm     millimeter
mmol   millimole
mol    mole
NF-κB  nuclear Factor-κB
ng     nanogram
nm     nanometre
NO₃    nitrate radical
NSERC  Natural Sciences and Engineering Research Council of Canada
OH     hydroxide radical
pg     picogram
PIP    proinflammatory potential
QIT    quadrupole ion trap
RF     radio frequency
ROS  reactive oxygen species
s  second
SFU  Simon Fraser University
T  temperature
TLF  time lag focusing
TNF-α  Tumour Necrosis Factor- α
TOF  time of flight
UBC  University of British Columbia
°  degree
μL  microlitre
μm  micrometer
CHAPTER 1

INTRODUCTION

From Air Pollution to Human Health

When one is concerned with the mysterious and wonderful functioning of the human body, cause and effect are seldom simple and easily demonstrated relationships. They may be widely separated both in space and time. To discover the agent of disease and death depends on a patient piecing together of many seemingly distinct and unrelated facts developed through a vast amount of research in widely separated fields.

Rachel Carson, Silent Spring (41)

It is said that Silent Spring launched the modern environmental movement in the west and, as a result, is arguably one of the most influential documents of the 20th century. When Rachel Carson wrote those words in 1962, she was discussing the hazard that rampant overuse of pesticides was having on the environment. She just as easily could have been talking about any anthropogenic species with the potential for causing adverse health effects to both man and nature.

The fundamental point of the above quote is that in the natural experiment that is the industrialized age, the identification of potentially harmful species is not trivial. However, whenever an association is observed, a wealth of research follows (figure 1.1). One such association, and the subject of this thesis, can be summed up in four words:

Air pollution is injurious
1.1 A Brief History of Air Pollution

Human health has been affected by air pollution ever since *Homo sapiens* lit their first fire. Caves that were inhabited thousands of years ago remain covered in a thick layer of soot, while mummified lungs from the Palaeolithic have a black tone (197).

The association of air pollution with human health has been hypothesized for over two thousand years. The first documented case of legislated air quality control was found over 2,000 years ago. This law was based on the commandment to "love your fellow as yourself," from the Mishnah Laws, which were based on the Torah in the form of the Halakhah. In Israel, during the first and second centuries A.D., it was required to regulate four nuisances: threshing floors, processing of carcasses, tanneries, and furnaces; tanneries being the nuisance most commonly referred to (190).

1,200 years later, the Hebrew philosopher and scientist Moses Maimonides drew parallels between air quality in the city relative to that in the desert and "waters that are befouled and turbid to waters that are fine and pure".

Some 500 years later in 1661, John Evelyn published *Fumifugium*, also called *The Inconveniencie of the Aer and Smoak of London*, the first book discussing the problem of air pollution in London and its effect on the survival rate of children less than 2 years of age (75). Again, industry was identified as a culprit, with the suggestion being made of moving some of London’s more polluting industry sectors out of town.

In the twentieth century, there were four definitive incidences wherein elevated levels of air pollution in the form of smog were shown to cause excess deaths within a population. In the Meuse Valley of Belgium in 1930, 63 people were killed by an air pollution episode (90, 248). In 1948, an inversion in Donora, Pennsylvania, killed 20 people and made 40 percent of the town's 14,000 inhabitants ill (203, 204, 275). During a five-day period in December of 1952 in London, elevated levels of smog promoted by heavy fogs and low altitude inversions, which ended up concentrating the pollutants in the boundary layer, resulted in the excess death of 4,000 people.
(69, 70, 187, 262). In response to this tragedy, Britain passed the Clean Air Act to reduce emissions. In 1962, when meteorological conditions of London were similar to those of December 1952 (205), a dramatic decline in the number of excess deaths was reported (down to 700 from 4,000), demonstrating the benefit of clean air legislation.

1.2 Identifying the Problem

Since the 1950s, the search to identify the causative agent of air pollution toxicity lead researchers to consider particulate air pollution. In 1979, a major report by a group of prominent British scientists suggested that elevated levels of particulate air pollution resulted in adverse health effects in humans (140). However, it wasn’t until the early 1990s that researchers from Harvard released a series of epidemiological studies that conclusively identified suspended particles in the troposphere as the primary component in air pollution responsible for an estimated 60,000 excess deaths per year in the United States alone (65). The implications of these findings resulted in the Environmental Protection Agency (EPA) producing legislation to regulate for fine soot in 1996. Controversy ensued, wherein critics claimed that such regulations, based on what they called “shaky science”, would result in unnecessary costs, and reduced profits (154). The basis of these claims was that the original studies, though they took into consideration other risk factors such as cigarette smoking, failed to consider that other pollutants produced alongside particulate matter, including nitrogen oxide, could be responsible for the observed change in mortality rates. These complaints were successful as they resulted in an 8-year delay in the implementation of the EPA proposed regulations with the call for direct evidence that the inhalation of particulate matter resulted in adverse health effects, such as asthma, emphysema, chronic obstructive pulmonary disease (COPD) and bronchitis. Since then, it has been shown that particulate air pollution is involved in the pathogenesis of cardiovascular diseases via the lung tissue inflammation, and systemic inflammation, that it causes.
The number of citations carrying the words “Particulate Matter” in the title or as a keyword per year over the past 50 years, as tabulated using Web of Science. In 1993, particulate matter was identified as the primary component of air pollution responsible for the observed adverse health affects in humans, resulting in a sharp increase in the number of citations produced per year. Note that the Web of Science keyword searches can only be done from 1991 to present.

The call for direct evidence resulted in a considerable increase in the number of publications looking at particulate matter toxicity (figure 1.1) where it was conclusively shown that, in fact, particulate matter was the primary component of air pollution responsible for the adverse health effects associated with air pollution.

1.3 The Big Picture, or Why Are We Here?

Research over the past decade on particulate matter toxicity has become increasingly focused on two major issues. First, the identification of the specific properties, either physical or chemical, that are responsible for the adverse health effects of particulate matter (19, 74), and second, the understanding of the mechanism in which inhalation of particulate matter within the lung results in adverse health effects (96, 213).
The goal of this thesis was to develop instrumentation and methodologies that can be employed to simultaneously consider the complexity of particulate matter chemistry (composition and reactions) while monitoring downstream biological responses of lung cells following incubation with those particles, all in the context of a single experiment. Early efforts during my thesis concentrated on developing an off-line mass spectrometry method to study organic/organic and organic/inorganic heterogeneous and multiphase reactions on particulate matter (118, 120). Once the potential for these reactions to occur in a laboratory environment in atmospherically relevant conditions was demonstrated, the project focus was expanded to incorporate the study of the potential adverse health effects associated with various ambient particle mimics (121, 122). To accomplish this, methodology was developed using similar instrumentation to generate a specific number of particles of known and designed chemical composition, deliver these particles directly onto a cell culture *in vitro*, and monitor numerous downstream biological responses. An illustration of the multi-faceted experimentation surrounding the development and application of an *in vitro* dose-response technology to address scientific issues regarding particulate air pollution is presented in figure 1.2.
Figure 1.2 Areas of particulate air pollution research that were studied over the course of this thesis. Early work focused on the tropospheric processing of suspended particles in the atmosphere (left), while later work looked at the effect such processing had on the subsequent downstream biological response (right). Different coloured arrows on the right side of the figure are indicative of different chemical fractions of the particle, such as the organic, aqueous or core. Different coloured arrows on the right are indicative of different particle/cell interactions such as endocytosis. Not depicted is the response of the cells to the particle insult.

1.4 Thesis Structure

The structure of this thesis is for the most part chronological. Additionally, it is what is known as a manuscript thesis; though documents of this nature are typically repetitive in nature, the methodologies employed throughout my studies were constantly evolving, making the document somewhat less repetitive.

Following a brief introduction to an instrument employed throughout, the electrodynamic balance, and a brief overview of the nature of particulate matter chemistry, studies undertaken by myself on the tropospheric processing of particulate matter are presented. In chapter 6, this document takes a turn into the realm of immunology, reflecting an expansion of the nature of my research from an environmental/analytical perspective to include a lung cell biology component.
After a concise description of particulate air pollution's role in causing adverse effects on human health, the remainder of the document describes the development and application of a methodology to study the role of particulate air pollution in the pathogenesis of cardiovascular and pulmonary related diseases.
Chapter 2

ELECTRODYNAMIC LEVITATION

A Summary of Levitation Technologies

*Using the Force, Yoda effortlessly levitates the X-Wing from the bog*

*Luke: “I don't, I don't believe it.”*

*Yoda: “That is why you fail.”*

*The Empire Strikes Back (29)*

The act of levitation, the phenomenon by which an object is suspended in air in defiance of gravity, has been employed for hundreds of years by magicians and Jedi alike for a multitude of purposes, ranging from lifting a volunteer off a table to retrieving one’s X-wing fighter from a swamp.

In actuality, instruments capable of levitation have been employed in science and engineering laboratories for the past hundred years to study numerous fundamental processes (202). Upon entering the Agnes group, I was introduced to one such device: the electrodynamic balance (EDB). The Agnes group has employed this apparatus since the late 1990’s in studies ranging from purely analytical chemistry development to reactions that probe the internal environment of a medium having net excess charge (82). All studies carried out through the course of this thesis revolve around the utility of the EDB to create, react and then deliver particles to a target. The EDB is an electrodynamic levitation device that can be used to suspend charged species. Presented here is a brief introduction to the history of, and theory behind, electrodynamic levitation, and how this tool made possible the research I undertook.
2.1 The Quadrupole Ion Trap

The first device employed for electrodynamic levitation was termed a three-dimensional quadrupole ion trap (QIT), commonly referred to as the Paul trap. Although the QIT was invented in 1953 by Paul and Steinwedel (232), it wasn’t until the mid 1980’s, when QITs began to be incorporated into commercially available gas chromatography-mass spectrometry (GC-MS) benchtop equipment, that the analytical potential of QIT was developed. Paul and Dehmelt, who first used the QIT to study levitated atoms, were awarded the Nobel Prize in Physics in 1989, for the development of the ion trap technology.

Physically, a QIT appears like an O-ring positioned between two halves of a ping pong ball, where the curved (parabolic) surfaces of the ball face each other (figure 2.1). The “two halves of a ping pong ball” are termed end-cap electrodes while the “O-ring” is aptly termed the ring electrode. The QIT operates under vacuum, with typical operating pressures between ~0.01 and 1 mTorr. Ions are either injected into the trap through an orifice in an end-cap, or created within the trap. Once inside, the ions are stored there by the electric field created by applying a radiofrequency (RF) waveform, at typically 1 MHz, to the ring electrode and holding the end-cap electrodes at ground potential. Depending on that electric field, ions having a mass to charge ratio (m/z) within a certain range will have stable trajectories, with the upper limit being around 5,000 m/z. Ions outside of that m/z range will not be stable, and will collide with the QIT electrodes and be neutralized.
The QIT is described as a dynamic instrument, which is to say that the motion of the ions within the trap is dictated by time dependant forces. The trajectory of the ions within the trap is a function of the quadrupole field within the trap itself. The quadrupole field is characterized as focusing, which is to say that as the ion deviates linearly from the centre of the trap, a quadratic restoring force pushes the ion back to the centre. The Mathieu equation, a second order differential equation originally derived to model vibrating stretched skins, has been employed successfully to model the motion of ions within the ion trap. The canonical form of the Mathieu’s differential equation is shown in equation 2.1.

\[
\frac{d^2y}{dx^2} + [a - 2q \cos(2x)]y = 0
\]  

An analytic solution to the Mathieu equation allows the stability of an ion within a QIT to be predicted as a function of two dimensionless parameters, \(a\) and \(q\).

\[
a_t = -2a_t = -\frac{16eU}{m_r^2 \omega^2}
\]

\[
qu_t = -2q_e = -\frac{8eV_{AC}}{m_r^2 \omega^2}
\]
Where $U$ is the DC voltage applied to the end-cap electrodes, $V_{AC}$ is the amplitude of the time-dependent waveform applied to the ring electrode, $\omega = 2\pi f$, $f$ is the RF frequency of the sinusoidal waveform applied to the ring electrode of the trap, $m$ is the mass of the ion, and $r_0$ is half the distance between the end-cap electrodes at their closest point. The stability region of an ion can also be viewed graphically, in the axial direction through plotting $a_r$ by $q_z$, and in the radial direction by plotting $a_r$ by $q_r$ (figure 2.2). By overlaying these two plots, areas of overlap are regions in which the ion is stable in both the radial and axial directions. Stability parameters $\beta_r$ and $\beta_z$, which are both functions of $a$ and $q$, are used to determine if an ion is stable within the QIT. Within these regions, the values of $\beta_r$ and $\beta_z$ range from 0 and 1. Ions must be stable in both the radial (r) and axial (z) directions to be levitated. The relationship between the ion stability in both the axial and lateral directions is evident in equations 2.4 and 2.5.

(Eq. 2.4) $$\beta_r \equiv \sqrt{a_r + \frac{q_r^2}{2}}$$

(Eq. 2.5) $$\beta_z \equiv \sqrt{a_z + \frac{q_z^2}{2}}$$
In retaining ions stored within the trap, the amplitude of the RF and DC potentials applied may be altered; however, the working range of the potential that can be physically applied to the ring and end-cap electrodes is relatively small. For instance, if a minimal AC potential ($V_{AC}$) required to stabilize a population of low molecular weight ions in the trap is around 1 kV and the maximum potential that can be applied to the electrodes before electric discharge ~10 kV, depending on the pressure, the working m/z range of the instrument is only one order of magnitude. If a proportionally larger ion is to be levitated, simply altering the amplitude of the potential on the electrodes will not suffice. In calculating both $a$ and $q$, the mass of the ion is inversely proportional to the subsequent $a$ and $q$ values calculated, as is the frequency of the sinusoidal waveform applied to the ring electrode squared. In other words, a lower frequency of the waveform applied to the ring electrode can be used to stabilize a larger ion. In a QIT, though the frequency of the waveform of the sine wave applied to the ring electrode can range from ~100 to 1,000 kHz, the circuit is tuned (eg. resonant) and adjusting the frequency in real time is unpracticed. Conceptually, if the frequency of the waveform of a QIT is lowered from 1 MHz to 30 Hz, a change of more than 5 orders of magnitude, an ion with a mass to charge ten orders of magnitude.
magnitude larger (ie. with an m/z < 3 x 10^{13}) could be levitated. Ions having such high mass to charge ratio could either be a massive single molecule with a single elemental charge, or the ions can be micrometer size particles (eg. total mass of tens of picograms) having a charge of typically thousands of elementary charges. Note that in this frequency range, from ~10 to 100 Hz, a tuned circuit is not a requirement. With the objective to levitate and study particles, several devices having different electrode geometry have been developed based on the figures of merit of the QIT, including the device employed throughout this thesis: the dihyperboloidal electrodynamic balance (EDB) (26).

2.2 The Electrodynamic Balance

Originally developed in 1959 by Wuerker and Langmuir, the EDB is an electrodynamic levitation apparatus capable of operating at atmospheric pressure (314). Since its inception, numerous forms of the device have been demonstrated, the most notable being the double-ring EDB that was characterized by Davis et al. in 1990 (59). Though there are differences between the EDB and the QIT, namely the ability to operate at higher pressures and the physical set up of the device, the trajectory of the charged particles within the EDB is governed by the Matheiu equation and as such is similar to that of the ions within the QIT. However, in terms of ion mobility, considerations for factors such as charged particle motion dampening due to collisions with background gases and Stokesian drag must be made. Consequently, the motion of ions within the EDB follow a modified Matheiu equation, but the relationships between the frequency of the waveform applied to the ring electrode and particle size remain the same, and the stability region of the ions as a function of \( a \) and \( q \) remain analogous to those described for the Paul trap.

The physical setup of the electrodes that form the double-ring EDB is similar to that of the QIT (figure 2.3). In replacement of two hyperbolic spheres, two flat conductive plates serve as end-cap electrodes and, in replacement of a single ring electrode, the EDB has two parallel ring electrodes (59). The parallel ring electrodes can be in the form of flat plates with holes of
appropriate size drilled into them or, as in the case in the Agnes lab, 1 mm diameter single conductor wire shaped in the form of a ring having a diameter of \( \sim 2.5 \text{ cm} \). Although the EDB is capable of operating without any walls shielding the ring electrodes from the ambient air, it is typically operated within a chamber of some sort to reduce the loss of levitated droplets due to air currents. A major difference between the two instruments is the source of the charged matter; charged matter being ions in a QIT and liquid and/or solid particles with net charge in an EDB. As such, the source of the charged species for each instrument is quite different (section 2.3.2).

![Figure 2.3](image)

**Figure 2.3** Comparison between the electrode geometry of an electrode dynamic balance (left) and a quadrupole ion trap (right).

With regards to atmospheric studies, the chamber encasing the EDB can play an active role in the experiment where the partial pressure of gases, ranging from reactive species such as ozone to simply water vapour, within the chamber can be controlled, thus allowing for reactions of those gases with the levitated droplets or particles to be monitored. The simplest of such studies are those where the mass of a droplet, containing organic species or electrolyte mixtures, is monitored as a function of the relative humidity (51, 52). These studies are made possible as the levitation of a droplet of a given mass in a single location within the EDB is a function of the settings of the electric field necessary to balance the force of gravity on the droplet.

For the most part, experiments wherein an EDB was employed studied the chemical and physical properties of the droplet or particle while it was levitated via such techniques as Raman
spectroscopy (34, 35). As a result, much effort was made to characterize the motion of a single levitated particle or droplet within the EDB, with the goal being to levitate and focus that single particle or droplet in a single location. However, this all changed in 2002 when the concept of using the EDB as a sample preparation technique was demonstrated.

2.3 Wall-Less Sample Preparation

On joining the Agnes group, a previous graduate student, Mike Bogan, had been working on a project that he termed wall-less sample preparation (WaSP) (25). WaSP employs the EDB as a sample preparation technique that when coupled with a matrix assisted laser desorption/ionization mass spectrometer (MALDI-MS), allows for detailed off-line analysis of a droplet or particle having complex chemical composition that had been prepared and reacted while levitated within the EDB. Any number of levitated droplets or particles levitated within the EDB, but not necessarily the entire population of levitated particles, can be extracted onto the stainless steel target plate in a controlled and reproducible fashion. Once on the stainless steel target plate, the chemical composition of individual particles or droplets can be characterized using MALDI-MS (119).

The primary goal of developing WaSP was to improve the sensitivity of MALDI-MS to the sub-attomole level for picoliter quantities of starting solution. WaSP was found to be successful in improving sensitivity through two distinctly different processes; first, the analyte was concentrated between 2 and 3 orders of magnitude through solvent removal during droplet levitation and second, by reducing the sample spot size, which itself has been shown to improve MALDI-MS sensitivity (72, 226). Originally developed as a purely analytical sample preparation strategy, the ability to use WaSP to deposit prepared droplets or particles of any given, designed chemical composition onto any target was recognized (figure 2.4).
Figure 2.4 Summary of potential applications of wall-less sample preparation (WaSP). These include, but are not limited to, optical microscopy, fluorescence microscopy, confocal microscopy, mass spectrometry and atomic force microscopy. The components of an EDB are indicated. A population of levitated droplets (A) and at a time during their extraction from the EDB (B) are shown in the inset photographs, including optical microscopy, confocal microscopy and mass spectrometry.

2.3.1 Electrode Configuration for an EDB Used for WaSP

The setup of an EDB used for WaSP is similar to a conventional EDB with one significant difference; one of the end-caps is replaced by a stainless-steel target plate, termed the deposition plate. Note that this difference is actually a conceptual difference because the position and shape of the deposition electrode need not be different from the end-cap electrode. Note that the relative positions of the deposition and induction electrodes are interchangeable. This enables
droplets to be injected into the trap from the bottom and the top, and consequently particles can be extracted from the top and bottom of the EDB as well.

2.3.2 Dispensing a Droplet with Net Charge

With this modified setup, levitation and deposition of a designed droplet within an EDB, or WaSP, is accomplished as follows. A starting solution from which droplets are dispensed containing both volatile and non-volatile components is selected. Approximately 10 µL of the starting solution is used to load the reservoir of a droplet-on-demand droplet dispenser (MJ-AB-01-60, MicroFab Technologies Inc., Plano, TX, USA). The nozzle of the droplet dispenser is then positioned 2 mm above the induction electrode and centred over the hole, 5 mm in diameter, cut into it. The induction electrode doubles as the top end-cap of the EDB, and is thus situated directly above the parallel ring electrodes of the EDB.

A single droplet is created by applying a voltage pulse to the piezoceramic fixed to the walls of the reservoir of the droplet dispenser. The shape of a typical waveform applied to the piezoceramic is shown in figure 2.5. The pressure wave thus created causes a jet of liquid to be expelled from the droplet dispenser nozzle.
Figure 2.5 Shape of the waveform applied to the piezoceramic within the MicroFab droplet dispenser employed throughout this thesis to dispense a single droplet.

The induction electrode is biased with a DC potential, positive or negative, of typically 100 V. The electric field between it and the nozzle of the dispenser induces a net charge on the jet of liquid such that, when it separates from the nozzle and collapses into a droplet, that droplet carries a net charge (figure 2.6). Repetition of this process, typically at 120 Hz, is used to dispense a population of droplets, some of which are captured and levitated in the EDB. The droplet capture efficiency ranges between 0 % to 100 %, depending on droplet dispensing and EDB parameters. Typical droplet capture efficiency in the EDB is around 20 %.
Figure 2.6 Illustration of selected moments in the process of dispensing a droplet with net charge.

(A) The positive potential applied to the induction electrode establishes an electric field that caused a net excess of anions to migrate to the tip of the dispenser and a net excess of positive ions to migrate away from the tip of the dispenser. (B) The separation of charge is maintained in the jet of fluid being ejected from the droplet dispenser and the resultant droplet thus acquires a net charge. (C) Basic electrostatics suggests that charge repulsion amongst the anions that compromise the net charge causes those ions to reside in a diffuse layer of the droplet-air interface. The red colour indicates a separation of charge. In this example, the droplet acquires a net negative charge.

The quantity of net charge on each droplet is a function of the potential applied to the induction electrode and the distance from the nozzle of the droplet dispenser to the induction electrode, where the following relationship is observed:
Net Charge on a Droplet = \( f(D_{\text{ind}+\text{DD}}^2, P_{\text{ind}}, C_{\text{sol}}, D_{\text{Disp}}, D_{\text{Size}}) \)

Where \( D_{\text{ind}+\text{DD}} \) is the distance between the induction electrode and the nozzle of the droplet dispenser, \( P_{\text{ind}} \) is the potential applied to the induction electrode, \( C_{\text{sol}} \) is the conductivity of the solution, \( D_{\text{Disp}} \) is the dispensing rate and \( D_{\text{Size}} \) is the diameter of the nozzle on the droplet dispenser. A typical setup for droplet dispensing has the nozzle of the droplet dispenser positioned 2 mm from the induction electrode to which a 100 V DC potential is applied. By way of example, the quantity of charge per initial droplet volume, 300 pL, originating from a starting solution consisting of 28 mM \( \alpha \)-cyano-4-hydroxycinnamic acid (CHCA) in 1:1 0.1% trifluoroacetic acid (TFA) in ddH\(_2\)O/acetonitrile (ACN), was 125 ± 25 fC (27).

The droplet net charge is a result of an excess population of cations or anions, depending on the induction potential. For example, if the ion was sodium, a charge of 200 fC equates to an excess of \( 1.25 \times 10^6 \) sodium ions relative to the total counter ion population. In terms of an overall shift in chemical composition of the droplet as a function of this charge, consider a droplet with a radius of 30 \( \mu \)m (27), a typical initial starting droplet radius formed in the laboratory, dispensed from a starting solution of 1 M NaCl would consist of \( 6.80 \times 10^{13} \) sodium chloride pairs. Thus the net excess charge is \( 1 \) Na\(^+\) or Cl\(^-\) per \( 5.45 \times 10^7 \) ion pairs of electrolyte in the solution.

### 2.3.3 Levitation of a Droplet with Net Charge

In each experiment, between 1 and 150 droplets with net charge were dispensed and a fraction of these droplets were captured and levitated by the electric field within the EDB (figure 2.7). Space charge repulsion between the like-charged droplets ensured none of the droplets would levitate at the null point of the EDB during the levitation period resulting in the droplets having amplitudes of motion of < 5 mm while levitated. The AC frequency applied to the ring electrodes was typically set to 60 Hz during droplet introduction to the EDB. Under these conditions, the trapped droplets move in stable trajectories at velocities of \( \sim 1 \) m/s.
Figure 2.7 A photograph of laser light scatter from a population of approximately 50 droplets levitated within an EDB. Note that the induction electrode is positioned below the ring electrodes in this set up. Droplets were dispensed upwards and deposited onto the target plate, positioned at the top of this photograph.

The volatile components of the droplets evaporate over a period of < 30 seconds, dependant on the relative humidity of the chamber and chemical composition of the original starting solution, after which time essentially all that remained in the levitated droplet were the compounds of low volatility present in the original starting solution. The resultant residue was either a liquid, a solid or a mixture of compounds in solid and liquid states. The relative abundances of all the components within the resultant residue were estimated based on the initial volume of the dispensed droplets and the known concentration of each compound in the starting solution.

The mass of the levitated droplet decreases dramatically during the evaporation of its volatile components. During this time, the overall volume of the droplet can decrease, typically between 1 to 3 orders of magnitude. To account for the change in mass, the levitation trajectories of the levitated droplets can be stabilized by, as indicated in equations 2.1 and 2.2, altering the electric field in the EDB. In the experiments conducted in chapters 4 and 5, the levitated particle
was stabilized by lowering the amplitude of the waveform from 4.5 kV\textsubscript{p-p} to 2.5 kV\textsubscript{p-p}, while the frequency remained at 60 Hz. In all subsequent experiments, a different waveform amplifier was employed which allowed for variation of both the frequency and amplitude. Using a variable frequency high voltage amplifier, the trajectories of the levitated particles were readily minimized by ramping the frequency of the waveform from 30 Hz to upwards of 1,200 Hz over a period of 5 seconds following the droplet dispensing event, with the amplitude held constant at 4.5 kV\textsubscript{p-p}. This ability to manipulate the waveform frequency by two orders of magnitude allowed for the levitation of particles with an overall smaller mass; with the variable frequency high voltage amplifier, a population of over 100 particles each having a diameter of less than 2 \( \mu \text{m} \) was readily levitated.

2.3.4 Particle Deposition

Droplets levitated in the EDB are removed from it by applying an attractive potential, typically \( \pm 500 \text{ V} \), to the deposition plate while at the same time reducing the trapping potential well depth by lowering the frequency of the AC field to below 30 Hz or by lowering the amplitude of the applied waveform, dependant on the waveform amplifier employed. The residues can be deposited directly onto the stainless steel deposition plate or onto any other substrate, which can also be a non-conductive material, positioned between the ring electrodes and the deposition plate. For instance, in the work described in chapters 7 through 11, the target was a human lung tissue culture grown on a glass coverslip.

2.4 Summary

Prior to the development of the WaSP technique, the EDB was seldom used to study a population of levitated particles. With the advent of WaSP by the Agnes group, developing the EDB as simply a tool to prepare and deliver particles to another substrate for further experimentation, the potential to use the EDB as a particle source was realized. These features of the WaSP technique were employed successfully throughout my studies.
Chapter 3

PARTICULATE MATTER AND ITS ANALYSIS

Chemical and Physical Properties of Suspended Particles in the Troposphere

The City is of Night; perchance of Death
But certainly of Night; for never there
Can come the lucid morning's fragrant breath
After the dewy dawning's cold grey air:
The moon and stars may shine with scorn or pity
The sun has never visited that city,
For it dissolveth in the daylight fair.

James Thomson, The City of Dreadful Night (292)

An unhealthy populace. Limited visibility. Foul odour. All characteristics of a polluted urban atmosphere, recognized and described for millennia, with London at the dawn of the industrial revolution being the most commonly described. Today, other cities in varied states of development all over the planet, such as Beijing, Tehran, Athens, Los Angeles and Mexico City can be similarly described. In most cases, anthropogenic sources like industry and vehicular exhaust are viewed as the source of the pollution. What all of these features share is that they are not merely a product of toxic gaseous species within the air, but rather they are the product of elevated levels of suspended particles within the air itself.

Described in this chapter is an introduction of the physical and chemical characteristics of suspended particles in the air, an overview of the chemistries occurring on these particles, and how recent advances in instrumental techniques have enabled their characterization.
3.1 Particulate Matter: Overview

Ambient particulate matter is defined as an aerosol, which may be either solid or liquid, with a diameter between ~0.002 and 100 μm that is suspended within the atmosphere. The lower end of this scale remains undefined as it is undetermined at which point a cluster of molecules becomes a particle whereas particles on the upper end of the scale fall out (e.g., gravitational settling) of the atmosphere and hence remain suspended in the air for only brief periods of time.

Particulate matter has a profound effect on a variety of processes, ranging from climate change (wherein elevated levels of particulate matter scatter incoming light (233), altering the atmosphere albedo), to cloud formation (wherein particulate matter acts as a nucleation site onto which water vapours condense) (106, 172), to the aforementioned health effects (65, 237). Given that the theme of this thesis is to study how the chemical composition of particulate matter elicits adverse health effects, no detailed discussion of the role of particulate matter on other issues, such as climate change or cloud formation, will be made.

3.2 Particulate Matter Characteristics

The chemical composition and size of suspended particles in the troposphere is a product of both its source and its atmospheric processing. In regards to source, this includes not only the direct emission of existing particles from sources such as fossil fuel consuming high temperature combustion engines, but also particles that are created within the atmosphere via nucleation (resulting in crystal formation) and coagulation (resulting in non-crystal solids), termed primary and secondary particles respectively. As a result, the size and chemical composition of suspended particles in the troposphere are related wherein different size fractions of these particles are associated with particular chemical compositions.

3.2.1 Physical Characteristics

Suspended particles in the troposphere are routinely described by three physical characteristics: size, surface area, and volume. The most important and easiest to characterize
through the use of inexpensive devices is size as it is related to both the particle’s source and where in the human respiratory tract such particles tend to deposit.

As mentioned above, particulate matter is described as having an average diameter, which suggests that each particle is spherical in shape. In reality, particles in the troposphere are irregular in shape. However, the diameter that particulate matter is described by is the aerodynamic diameter. Aerodynamic diameter, \( D_o \), is the diameter of a spherical water droplet that has the same terminal falling speed in air as the particle in question.

\[
(D_a) = D_o k \sqrt{\frac{\rho_p}{\rho_0}}
\]

Where \( D_o \) is the geometric diameter, \( k \) is the shape factor (which is 1.0 for a sphere), \( \rho_p \) is the density of the particle and \( \rho_0 \) is the density of water (1 g cm\(^{-3} \)). The aerodynamic diameter is found to be informative as it can be used to predict many things about the particle, most notably the location within the respiratory system where the inhaled particle are most likely to settle. For instance, the velocity in which a particle must travel for it settle out of air, termed the settling velocity \( (v) \), is a function of the aerodynamic diameter of the particle:

\[
v = \frac{D_o^2 \rho g C}{18\eta}
\]

where \( g \) is the gravitational constant, \( \rho \) is the density of the particle, \( \eta \) is the gas viscosity, and \( C \) is the Cunningham correction factor, which itself is approximated by:

\[
C = 1 + \frac{2.154l}{D}
\]

Where \( l \) is the mean free path between air molecule collisions and \( D \) is the particle diameter. From these equations, it is apparent that the smaller the particle, the slower the air velocity must be for a particle to settle; hence the deeper into the lung a smaller particle is able to travel before settling. This is believed to be one reason why smaller particles are more commonly associated
with adverse health effects than the larger particle fraction; the smaller particles are able to penetrate deeper into the lung (260).

The size distribution of particulate matter suspended in the troposphere, when graphed as the particle number as a function of size, cannot be fitted to a simple Gaussian distribution. Rather the distribution is best fitted by a log-normal distribution. Another characteristic of the size distribution of particulate matter is that it has three distinct populations, with three distinctly different modes. This is a result of the mechanisms by which the particulate matter was generated. The largest particles, those with a mean diameter of ~5 \( \mu m \), are typically generated by mechanical means, such as wind and sea salt spray (312). Smaller particles, those with a mean diameter of < 1 \( \mu m \) encompassing particles, are either directly emitted into the atmosphere by primary sources, such as diesel exhaust engines, or produced within the atmosphere through nucleation and coagulation. Of the latter two size modes, the smaller is nucleation (usually < 200 nm), and the other is the accumulation mode (usually 200 – 1,000 nm).

![Figure 3.1](image)

**Figure 3.1** A breakdown of the sources of suspended particles in the troposphere by mass. Adapted from values previously reported (295).
With regards to the relative abundances of these particles within the troposphere, based solely on source, particulate matter can be categorized as either fugitive or non-fugitive with the distinction being that non-fugitive particles are those that can potentially be controlled in that they are emitted from such devices as a smoke stack or a tail pipe, and thus can be filtered rather than released into the atmosphere (figure 3.1). Not only are the physical characteristics of these particle modes significantly different from one another, the chemical composition is as well.

### 3.2.2 Chemical Composition

The chemical composition of particulate matter is exceedingly complex and ever changing. As a geographical function, it varies on location, meteorological conditions and time of year. Additionally, the composition is a function of its source and the chemical processing of the particle during its lifetime. As mentioned in the previous section, the size of a specific particle tends to be associated with its final chemical composition; simply because both variables, size and composition, are a function of the original source of the particle.

Course particles are typically generated via mechanical means and as a result, have a chemical composition that is high in relatively chemically inert species such as silicates and other oxides of crustal elements. Additionally, since many particles of the course fraction originate as resuspended dust, this fraction tends to have higher abundances of biological material such as pollen, mould spores (98) and fragments of bacteria such as lipopolysaccharide (212, 229). The tropospheric lifetime of particles in the course fraction ranges from minutes to hours, resulting in both a limited travelling distance, typically between < 1 and 10 km, and little tropospheric processing, dependant on meteorological conditions (91, 231).

Fine particles, those with an aerodynamic diameter less than 2.5 μm, are formed typically through processes such as condensation of vapours in a combustion engine or in coal burning, nucleation of gases or through gas-phase reactions to name a few. These particle types are typically higher in nitrates and sulphates, polyaromatic hydrocarbons (PAHs) (37, 231) and
organic compounds. Since a major source of these particles is the combustion of fossil fuels and other forms of industry such as the smelting and processing of metals, they are high in metals such as lead, cadmium, zinc, iron, nickel and copper. The small size of these particles gives them an atmospheric lifetime of days to weeks, allowing them to travel distances in the thousands of kilometres range, again, dependant on the meteorological conditions.

The chemical composition of particulate matter is vast; therefore, it is exceedingly difficult to develop assays to study the entire composition of a particle within a single experiment. As a result, there tends to be a significant fraction of the chemical composition of particulate matter that remains unidentified (figure 3.2) (9).

![Figure 3.2](image)

**Figure 3.2** Results of a typical study on the total chemical composition of suspended particles in the troposphere.
Percent values used to construct this figure originated from work by Andrews et al. (9)

### 3.3 Particulate Matter: Chemistry

The chemistry of atmospheric particulate matter can be approached from three fronts: gas phase reactions that lead to the formation of particulate matter, reactions on the surface of the particle (also known as heterogeneous chemistry) and reactions within the particle following the uptake of the volatile species (also known as multiphase) (figure 3.3) (240). With regards to the scope of this thesis, an appreciation of all three of these processes is relevant. Additionally, to understand how the tropospheric processing results in the production and alteration of particulate matter, an overview of well established atmospheric chemistries is necessary.
3.3.1 Atmospheric Production of Oxidative Species

There are three main oxidative species present in the troposphere: ozone ($O_3$), nitrate radical ($NO_3$) and the hydroxyl radical (OH). These species are constantly produced and broken down by a variety of means. Given that these species are involved in the processing of numerous chemicals in the atmosphere, a brief overview as to the chemistry by which these species are synthesized in the atmosphere follows.

The major mechanism in which ozone is produced in a clean stratospheric atmosphere is through a series of photochemical reactions known as the Chapman cycle, named after Sir Sydney Chapman who first hypothesized them in 1930 (45, 46):

(Eq. 3.4) \[ O_2 + \text{hv} \rightarrow 2O \]

(Eq. 3.5) \[ O + O_2 \rightarrow M \rightarrow O_3 \]

(Eq. 3.6) \[ O + O_3 \rightarrow 2O_2 \]

(Eq. 3.7) \[ O_3 + \text{hv} \rightarrow O + O_2 \]
Where M is another molecule, such as O₂ or N₂. Since ozone is both produced and broken down within this cycle, a steady state occurs wherein the concentration of ozone remains relatively constant. Stratospheric ozone is beneficial as it protects life on the planet’s surface from the ultraviolet (UV) radiation of the sun. However, ozone is also found during both diurnal and nocturnal time periods within the troposphere. Tropospheric ozone is identified as a pollutant as it is both an irritant and a reactive species, which itself is involved in numerous chemical reactions such as secondary aerosol formation. Ozone is formed in the troposphere by the following sequence of reactions:

(Eq. 3.8) \[ \text{NO} + \frac{1}{2} \text{O}_2 \rightarrow \text{NO}_2 \]  
(slow reaction)

(Eq. 3.9) \[ \text{NO}_2 + \text{hv} \rightarrow \text{NO} + \text{O} \]

(Eq. 3.10) \[ \text{O} + \text{O}_2 + \text{M}_p \rightarrow \text{O}_3 \]

Where M_p is a third body such as N₂. NO is produced both from natural sources such as forest fires and lightning, and from anthropogenic sources such as fossil fuel burning.

The hydroxyl radical (OH) is produced during daytime hours, as it is produced photochemically, and is extremely reactive making it relatively short lived. A major mechanism of OH production in both a clean and polluted atmosphere is through the photodissociation of O₃:

(Eq. 3.11) \[ \text{O}_3 + \text{hv} (\lambda < 340 \text{ nm}) \rightarrow \text{O}^1\text{D}) + \text{O}_2 \]

This leads to the production of O(¹D), a form of electronically excited oxygen that in turn reacts with water to form OH radicals:

(Eq. 3.12) \[ \text{O}^1\text{D}) + \text{H}_2\text{O} \rightarrow 2\text{OH} \]

In the absence of water vapour, O(¹D) will collisionally deactivate to its ground state O(^3P). Unlike OH, NO₃ is detected within the troposphere only at night, as it is rapidly photolyzed by sunlight. NO₃ is formed through the oxidation of NO₂ by ozone:

(Eq. 3.13) \[ \text{NO}_2 + \text{O}_3 \rightarrow \text{NO}_3 + \text{O}_2 \]
In the absence of a species to oxidize, such as volatile organic compounds (VOC), NO$_3$ will typically react with NO$_2$ to form the more stable N$_2$O$_5$.

(Eq. 3.14) \[ \text{NO}_3 + \text{NO}_2 + M_p \rightarrow \text{N}_2\text{O}_5 + M_p \]

N$_2$O$_5$ is not only a daytime sink for NO$_3$, but it may also be broken down into nitric acid, a major component of acid rain.

(Eq. 3.15) \[ \text{N}_2\text{O}_5 + \text{H}_2\text{O} \rightarrow 2\text{HNO}_3 \]

These oxidative species, O$_3$, OH and NO$_x$ (NO$_x$ includes both NO and NO$_2$), are involved in nearly all facets of tropospheric chemistry, including the chemical processing of particulate matter. Once an aerosol is in the troposphere, whether it is a primary aerosol, such as those emitted by forests fires, or a secondary aerosol, oxidative processing of these aerosols continues throughout the particle’s lifetime. As a result, the rate of a chemical species being produced in the troposphere must be compared to the rate in which that species reacts with these oxidative species (eg. removal rate).

### 3.3.2 Secondary Aerosol Formation

The combustion of fossil fuels generates numerous intermediates that can react further under appropriate meteorological conditions to yield many products, as suggested by the equation (88):

(Eq. 3.16) \[ \text{VOC} + \text{NO}_x + \text{hv} \rightarrow \text{O}_3 + \text{PAN} + \text{HNO}_3 + \text{Particles} \]

Where PAN is peroxyacetyl nitrate.

Secondary aerosols found in the troposphere tend to be dominated by NH$_4$SO$_4$, NH$_4$NO$_3$ and organic compounds, where the organic compounds originate from a volatile organic species that has condensed onto an existing particle or has nucleated within the troposphere. The compounds that are involved in the formation and growth (accumulation mode) of secondary organic aerosols are those formed within the troposphere when VOCs are oxidized, forming compounds of lower volatility and higher boiling points (146). This results from their reactions in the gas phase with oxidative species, namely ozone (O$_3$), nitrate radical (NO$_3$) and the hydroxyl
radical (OH). In addition to the production of secondary aerosols, these oxidative species also play a role in the reactivity of chemical species on a tropospheric particle.

### 3.3.3 Heterogeneous Chemistry

Heterogeneous chemical reactions are defined as reactions that occur between species at the interface between two phases of matter (240). Though common in solid-state chemistry, designing experiments to study tropospherically relevant reactions occurring on the air-solid or air-liquid interface of particulate matter remains difficult. As a result, the measured number of reaction rates of heterogeneous reactions occurring under tropospherically relevant conditions remains relatively low. The limited number of heterogeneous reaction rates included in atmospheric models makes understanding their role in the atmosphere unclear. Although the global impact of atmospheric heterogeneous chemistry remains to be determined, the impact of these reactions within a localized environment is appreciated, as evidenced by the Antarctic stratospheric ozone depletion event every spring (269, 294).

Atmospheric heterogeneous reactions can be divided into two groups with some overlap between them; organic and inorganic. Of the two, inorganic heterogeneous reactions have been studied more heavily as the technology to study inorganic species was developed earlier due to the important role of inorganic heterogeneous chemistry in the Antarctic stratospheric ozone depletion event (269, 294).

Other inorganic heterogeneous reactions that have been studied include direct substitution style reactions, such as those between sodium chloride with nitric acid or with sulphuric acid.

(Eq. 3.17) \[ \text{NaCl}(s) + \text{HNO}_3(g) \rightarrow \text{NaNO}_3(s) + \text{HCl}(g) \]

(Eq. 3.18) \[ 2\text{NaCl}(s) + \text{H}_2\text{SO}_4(g) \rightarrow \text{Na}_2\text{SO}_4(s) + 2\text{HCl}(g) \]

These reactions result in a net depletion of the halide in the particle and in some instances result in release of halogen gaseous atoms into the atmosphere. In other cases, inorganic heterogeneous reactions of sea salt spray may result in the release of halogen gases.

(Eq. 3.19) \[ \text{NaCl}(s) + \text{ClONO}_2(g) \rightarrow \text{NaNO}_3(s) + \text{Cl}_2(g) \]
These reactions, and others, result in an elevation of gas phase halogenated species in the marine boundary layer. These halogenated species can then, in turn, affect the chemistry of hydrocarbons in these air parcels. How such substitution reactions alter the overall toxicity of suspended particles remains unclear.

Organic heterogeneous chemistry reactions that are typically studied are those made between an organic molecule, in a solid or liquid state, with oxidative species such as ozone or nitrate radical. Inherently, the product of these reactions will have an increased polarity, which results in a reduced hydrophobicity. Additionally, if the organic molecule of interest is unsaturated, such as oleic acid, the product will have an increased hygroscopicity (76, 253). Hygroscopicity is defined as the propensity for an object, in this case a chemical species, to extract water from the atmosphere through absorption. These fundamental changes in the physical characteristics of organic compounds on a particle following heterogeneous oxidation reactions have a profound effect on numerous atmospheric processes, such as cloud and fog formation (172). How such addition reactions alter the overall toxicity of suspended particles remains unclear.

3.3.4 Multiphase Reactions

Multiphase reactions are defined as reactions that occur between species once one species in one phase, such as a vapour, is taken up into another phase, such as a droplet. Relative to heterogeneous reactions, the homogeneous reaction portion of multiphase reactions is more readily studied and the chemical reactions monitored are simpler to interpret, as the chemistry occurring within these liquid aerosols is akin to the chemistry occurring within a beaker. With regards to inorganic chemistry, the production of acid rain through the oxidation of SO₂ to sulphuric acid by oxidants such as H₂O₂ and ozone and its uptake into particles, has made it the most studied multiphase reaction occurring in the troposphere (44, 104); however, many mechanistic details remain to be elucidated. This category of chemistry concerning atmospheric
particles involving hydrophobic functional groups on oxidized organic compounds is not well described in the atmospheric literature.

### 3.3.5 Points to Consider

In the undertaking of a laboratory study, with the set goal of mimicking a reaction occurring within the environment, one must strive to incorporate as many of the natural components and factors of the actual system as possible; with regards to this thesis, this meant that the particles studied must be of a tropospherically relevant composition and size. The role of oxidative species in the chemical processing of these particles must also be considered as it is of little importance to the atmospheric community if the product of any reaction is rapidly oxidized and broken down at a rate faster than it is produced in, or emitted into, the troposphere. However, from the perspective of the products of such compounds accumulating on particulate matter, such short-lived compounds could influence the potential of the particle to cause tissue injury.

With an idea as to the type of chemistry that will be studied, a discussion of how one measures the concentration of these compounds on a particle, whether it be performed *in situ* in the troposphere or in the laboratory, follows.

### 3.4 Technologies for Particulate Matter Analysis

Numerous analytical techniques have been developed and employed over the past 20 years to study in great detail the chemical composition of, and reactions occurring on or within, particulate matter. These techniques can be categorized into two groups, those that study particles as they exist within the troposphere, which may be off-line or in real-time, and those that are used to study designed particles within a laboratory setting, which also may be off-line or in real-time. The methodology for the use of an electrodynamic balance (EDB) introduced in chapter 2 pertains to the latter group.
3.4.1 Off-Line Analysis of Ambient Particles

Early methods developed for the study of tropospheric particulate matter were typically two-step processes, beginning with the collection and removal of a population of particles from a given volume of sampled tropospheric air through some form of filtration device, followed by the off-line analysis of the chemical composition of the sampled particle population using analytical instrumental methods. These methods include, but are by no means limited to, inductively coupled plasma (ICP), atomic emission spectroscopy (AES) and atomic absorption spectroscopy (AAS), used to identify and quantify components of the inorganic fraction, to gas chromatography-mass spectrometry (GC-MS) (112) and liquid chromatography-mass spectrometry (LC-MS) (183), used for the identification and quantification of the components of the organic fraction.

While this approach contributed much knowledge regarding bulk assays of ambient particles, the ability of these methods to accurately determine the composition of an ambient particle as it exists in air remained unaddressed. Specifically there were sampling issues regarding the alteration of the chemical composition during the particle collection process that were not readily addressed using the bulk analytical methods. Once collected on the filter, the particles were extracted from it through either sonication, or physically by simply shaking or scraping the filter (24). Ultrasonication, which is a common form of particle removal, uses gas bubbles in solution whose interior, when they collapse, can reach both high temperatures and pressure (284). Under these conditions, chemical processing of the particle itself (283), likely mediated through the hydroxyl radical, can occur. The act of particle collection and filtration likely resulted in the removal of soluble components from each particle and hence, the actual chemical composition of the particle was altered.

Moreover, the collected particles are not analyzed individually, resulting in an average composition being reported. As a result, the exact chemical speciation of individual particles was
not known. Given that particulate matter is a complex mixture of many particles, each with a different size and each speculated to have a different chemical composition, the reporting of an average meant that associations between individual particle sizes with chemical compositions or between specific compounds, or species, remained uncertain. Furthermore, with regards to this thesis, this problem was paramount as the primary hypothesis of this work was that the biological response to a particle is dependant on both the size and chemical composition of that particle. Thus, dosing a population of cells with a mixture of particle types collected from the troposphere would result in a mixture of biological responses, with no discernable trend observed.

3.4.2 Real-Time Analysis of Ambient Particles

The ability to analyze ambient particles in the air in real-time, as they exist in the troposphere allows for the creation of a database of most probable individual particle chemical compositions. The true power of the database stems from the fact that there is comparatively less alteration of the sample during its analysis. During the 1990’s, aerosol mass spectrometers capable of analyzing individual particles sampled directly from the troposphere in real-time were developed (39, 135, 219). Over the following decade, numerous versions of these devices were developed (267) and employed across the globe to produce enormous data sets with regard to individual particulate composition and size in numerous locations of the boundary layer, all of which afforded detailed real-time characterization of individual particles within a given volume of air (186, 214, 215, 219).

Aerosol mass spectrometers typically consist of three chambers; the aerosol sampling chamber, the particle sizing chamber and the particle chemical composition analysis chamber (219). Each chamber is operated at a different pressure, with the pressure of each getting significantly lower from one chamber to the next. Particles are first channelled from the atmosphere into a vacuum chamber through an aerodynamic aerosol sampling inlet. Though the system is open to the atmosphere, the pressure within the chamber remains low through the
constant running of a vacuum pump. The particles are then sized, one at a time, as they pass through an aerosol sizing chamber using Mie scattering in conjunction with particle time of flight measurement (56). In the last chamber, the particle is ionized by either electron impact (EI), laser desorption/ionization (LDI) (39) or vaporization by impact onto a hot plate followed by EI, and the mass to charge ratio of the ions produced are measured using a mass spectrometer. Both quadrupole and time of flight (TOF) mass spectrometers (267) have been used for this purpose. The quadrupole mass spectrometer has a mass to charge limit of ~5,000, and as such aerosol mass spectrometers fitted with quadrupoles are incapable of identifying larger chemical species, such as polymers, or biological species, such as proteins. In contrast, aerosol mass spectrometers that use a TOF mass analyzer have a much higher theoretical mass range. However, relative to MS/MS using quadrupole analyzers, MS/MS using TOF mass analyzers is not a mature technology and requires extensive and expensive instrumentation modification. Thus, depending on the mass of the analyte of interest, the appropriate mass spectrometer should be selected.

These devices are capable of collecting enormous quantities of data regarding the composition of ambient particles. To date, only a limited number of studies have been published where they have been employed to monitor specific chemical reactions involving particles (100) and to obtain the rates of those reactions.

3.4.3 Analysis of Particles Created in a Laboratory Setting

To fully characterize a reaction, that is to determine all the reactants and products as well as all of the relevant reaction rates, it is prudent to perform such experimental work initially in a controlled laboratory environment. A key feature of this strategy is that the chemistry occurring on aerosols or surfaces in a laboratory environment can be probed using a suite of instrumental methods, assuming they possess sufficient sensitivity to address many different species. Though many strategies have been successfully employed to look at the rates of reactions occurring on the
surface of aerosols, such as measuring reaction rates on films, this section will focus on those methods where the reaction rates were measured on the surface of individual aerosols.

The chemistry occurring on unlevitated aerosols is typically studied by first nebulizes an aqueous solution of known chemical composition onto a hydrophobic film. The presence of the film limits the aqueous droplets from spreading and thus a convex shape is retained. The film covered with aerosol droplets is then placed into a controlled environment where the experimental factors such as humidity, temperature, or gaseous reactive species are introduced. Numerous common analytical techniques can then be employed to monitor the reactions occurring on the aerosol droplets including attenuated reflectance infrared spectroscopy (ATR-IR) and UV-Vis absorption spectroscopy.

As mentioned in chapter 2, levitation techniques have been developed to study chemical reactions occurring on levitated particles in real-time. The technology employed for the levitation of a single micrometer sized aerosol droplet has been described in great detail in chapter 2. Common instrumental techniques used to monitor the chemical composition of a single levitated droplet or particle in real-time include Raman spectroscopy and Fourier transform infrared microscopy (FT-IR).

3.4.4 Selecting a Detection Strategy for the Compounds Used in These Studies

One of the goals of this thesis was to develop an analytical method for monitoring organic chemical reactions occurring on levitated particles within a laboratory setting to ascertain the rates of such reactions and hence predict the likelihood of such reactions occurring within the troposphere, thus enabling subsequent investigation of the effect these compounds on a particle’s ability to cause injury to lung tissue. Given the nature of the research group, coupled with the availability of the appropriate technologies, it was decided to use off-line mass spectrometry, specifically laser desorption/ionization mass spectrometry (LDI-MS), to monitor the reactions occurring on particles levitated within the electrodynamic balance. The advantage of using LDI-
MS is that it provides a simultaneous measurement of numerous chemical species within a single particle at the same time.

Given the extent to which LDI-MS and matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS) was used throughout the thesis, an introduction to these techniques is provided here.

3.5 Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

A common instrument found in a modern analytical laboratory is the mass spectrometer. Though now typically coupled with a form of separation science (e.g., gas or liquid chromatography), recent technological advances have led to mass spectrometers being found more often as a stand-alone instrument. Over the past two decades, the field of mass spectrometry has enjoyed a massive surge in popularity and overall use.

The three main parts of the mass spectrometer are the ion source, the mass analyser and the detector. The function of the ion source is to generate ions from inorganic and organic compounds in a sample; the mass analyser then separates these ions based on their mass to charge ratio (m/z) and their relative abundance of the ions is measured by the detector.

3.5.1 Ionization

As mentioned, ionization of a molecule is the required first step in the measurement of its mass by mass spectrometry. Over the past fifty years, numerous mechanisms have been developed to ionize a molecule in the gas phase, including chemical and electron impact ionization; however, the recent growth in popularity of mass spectrometry can be attributed to the advent of soft ionization techniques for thermally fragile compounds having low vapour pressure. These advances in ion sources have lead to the emergence of a new field of study termed proteomics. The two soft ionization techniques largely responsible for these developments are electrospray (309, 316, 317) and MALDI (157-159). The importance of these techniques has been recognized, with the individuals responsible for the founding development of these two
techniques being co-recipients of the Nobel Prize in chemistry in 2002. In the context of this thesis, only the process of MALDI will be described as electrospray was not used.

MALDI itself is a derivative of the technique laser desorption/ionization (LDI). Since the ionization technique of LDI is employed extensively in both chapters 4 and 5, a description of it is provided below, followed by a description of MALDI.

First described in the late 1960s soon after the development of pulsed lasers, LDI was found to be an effective ionization technique for low mass (< 1,000 Da), light absorbing species. In LDI, a sample is placed onto a conductive plate, which itself is positioned into the ion source region of a mass spectrometer. The sample is irradiated by pulses from the laser, which in the case of the mass spectrometer located in the Agnes laboratory is a nitrogen laser (λ = 337 nm, with a pulse duration of 3 ns). The laser output is focused using a long focal length lens resulting in an oval spot size of ~130 µm by ~200 µm in dimension. If the absorption cross-section of a molecule in the sample is non-zero for the laser output, the energy absorbed from a laser shot can cause the molecule to vaporize and ionize. The molecule can be detected as a molecular ion or extensively fragmented depending on the compound and the laser fluence. In LDI, ionization of the sample occurs when an electron is captured or removed from the molecule, resulting in an ion with a +1 or −1 charge. The process by which this occurs is either via photoionization, charge exchange or electron capture in the gas phase.

In MALDI, the process by which ionization of analyte species occurs is somewhat different than in LDI. First, it is not necessary, but rather desirable, that the analyte does not absorb energy from the output of the laser. The first requirement of sample preparation for MALDI is the analyte cocrystallization within a host compound that is termed the matrix. Matrix molecules are typically weak organic acids that have strong absorption coefficients for the laser output. The matrix molecule is mixed with the analyte molecule at an approximate mole ratio of 10,000 to 1 respectively. As the energy of the laser is not absorbed by the analyte, the subsequent benefit of MALDI over LDI is that it enables the ionization of molecules that are thermally
fragile, namely proteins and polymers that have molecular weights in the hundreds of thousands of Daltons, with little to no fragmentation of the analyte. The mechanism by which analyte ionization in MALDI occurs is the subject of much research, as numerous processes are speculated to be simultaneously competing during the desorption/ionization step in MALDI.

Ionization of the analyte can take place on the stainless steel plate via simple acid-base chemistry or by gas-phase proton transfer in the expanding plume of desorbed material immediately following sample irradiation. It remains unclear whether ionization on the plate or in the plume is the most important mechanism of analyte ionization. Upon irradiation of the laser, much like LDI, the molecules capable of absorbing the energy of the laser, in this case the matrix, are desorbed from the surface, physically carrying with them the analyte molecules. Ionization of the analyte can then occur in the plasma plume up to a few hundred nanometers above the stainless steel plate through analyte and matrix collisions that result in proton or alkali metal transfer. Interferences arise when the matrix molecules themselves are ionized, as well as when clusters, groups of numerous molecules bound together non-covalently that appear as a single ion in a mass spectra and that likely result from incomplete disruption of the host crystal, are desorbed from the sample material. This creates a significant problem, as it greatly increases the number of observed peaks in the mass spectrum. It is for these reasons that MALDI is not well suited for the analysis of low mass (<1,000 Da) analyte compounds.

A list of the types of ions that are formed by LDI and MALDI are shown in table 3.1.
**Table 3.1** Ions formed by LDI or MALDI

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Positive ions</th>
<th>Negative ions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Single:</strong></td>
<td><strong>Single:</strong></td>
</tr>
<tr>
<td>Non-Polar</td>
<td>$M^{\ddagger}$</td>
<td>$M^\dagger$</td>
</tr>
<tr>
<td>Medium Polarity</td>
<td>$M^{\ddagger}$</td>
<td>$M^\dagger$</td>
</tr>
<tr>
<td></td>
<td>[M + H]$^+$</td>
<td>[M + H]$^+$</td>
</tr>
<tr>
<td></td>
<td>[M + alkali]$^+$</td>
<td>[2M]$^+$</td>
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<tr>
<td></td>
<td></td>
<td>[2M + H]$^+$</td>
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<tr>
<td></td>
<td></td>
<td>[2M + alkali]$^+$</td>
</tr>
<tr>
<td></td>
<td><strong>Clusters:</strong></td>
<td><strong>Clusters:</strong></td>
</tr>
<tr>
<td></td>
<td>[2M]$^{\ddagger}$</td>
<td>[2M]$^\dagger$</td>
</tr>
<tr>
<td></td>
<td>[2M + H]$^{\ddagger}$</td>
<td>[2M + H]$^\dagger$</td>
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<td></td>
<td>[2M + alkali]$^{\ddagger}$</td>
<td>[2M + alkali]$^\dagger$</td>
</tr>
<tr>
<td></td>
<td><strong>Adducts:</strong></td>
<td><strong>Adducts:</strong></td>
</tr>
<tr>
<td></td>
<td>[M + Ma + H]$^+$</td>
<td>[M + Ma]$^+$</td>
</tr>
<tr>
<td></td>
<td>[M + Ma + alkali]$^+$</td>
<td>[M + Ma - H]$^+$</td>
</tr>
<tr>
<td>Polar</td>
<td><strong>Single:</strong></td>
<td><strong>Single:</strong></td>
</tr>
<tr>
<td></td>
<td>[M + H]$^+$</td>
<td>[M - H]$^-^\dagger$</td>
</tr>
<tr>
<td></td>
<td>[M + alkali]$^+$</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Exchange:</strong></td>
<td><strong>Exchange:</strong></td>
</tr>
<tr>
<td></td>
<td>[M - H$<em>n$ + alkali$</em>{n+1}$]$^+$</td>
<td>[M - H$<em>n$ + alkali$</em>{n+1}$]$^-^\dagger$</td>
</tr>
<tr>
<td></td>
<td><strong>Clusters:</strong></td>
<td><strong>Clusters:</strong></td>
</tr>
<tr>
<td></td>
<td>[nM + H]$^+$</td>
<td>[nM - H]$^-^\dagger$</td>
</tr>
<tr>
<td></td>
<td>[nM + alkali]$^+$</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Adducts:</strong></td>
<td><strong>Adducts:</strong></td>
</tr>
<tr>
<td></td>
<td>[M + Ma + H]$^+$</td>
<td>[M + Ma - H]$^-^\dagger$</td>
</tr>
<tr>
<td></td>
<td>[M + Ma + alkali]$^+$</td>
<td></td>
</tr>
<tr>
<td>Ionic</td>
<td>$C^+$</td>
<td>$A^-$</td>
</tr>
<tr>
<td></td>
<td>[C$<em>n$ + A$</em>{n+1}$]$^+$</td>
<td>[C$_n$ + A$_n$]$^-^\dagger$</td>
</tr>
</tbody>
</table>

Where C is a Cation, A is an Anion, M is the Matrix and n is a positive integer
Modified from “Mass Spectrometry”, Gross.(114)

Ion formation via LDI primarily results in only $M^+$ and $M^{\ddagger}$ ions being formed whereas MALDI can generate all of the ions shown in Table 3.1. As a result of this, LDI mass spectra typically have fewer peaks in the low mass range than MALDI mass spectra (figure 3.4).
Figure 3.4 MALDI-TOF-MS (top) spectrum and LDI-TOF-MS (bottom) spectrum of 1,8-diaminonaphthalene.

The settings of the mass spectrometer used for the generation of each spectrum are the same. Note the number of peaks in the MALDI spectrum is much greater than in the LDI spectrum. The matrix was α-cyano-4-hydroxycinnamic acid (CHCA).

It is this ability to produce a relatively clean mass spectrum in the low mass region that the technique of LDI-MS was selected for the experiments on heterogeneous and multiphase chemistry on molecules that have non-zero absorption coefficients for the N₂ laser output, the results of which studies are presented in Chapters 4 and 5. The ability to detect molecules having high mass, such as the proteins studied in chapters 9 and 11, necessitated that the technique of MALDI-MS be employed.

3.5.2 Mass Analyzer

Throughout the years, numerous mass analyzers have been developed, including the quadrupole ion trap (QIT) discussed in Chapter 2. One of the more robust mass analyzers, with respect to its ability to measure ions of an exceedingly large range of m/z, is the time-of-flight (TOF) analyzer. Though the TOF analyzer was originally introduced in 1946, it wasn’t until the
1990’s that its commercial availability became more prevalent. This was a direct result of the advent of MALDI and its ability to ionize biomolecules, since a TOF is well suited to be coupled with a “batch” ion source.

Like most mass analyzers, the TOF operates under high vacuum (pressure typically below \( \sim 10^{-6} \) Torr, depending on the drift tube length). The basic premise behind the TOF predicts that the rate at which an ion of a given kinetic energy travels across a field-free drift region of known length is a function of its m/z. An ion has an electric charge equal to the number \( z \) of eliminating electronic charges \( e \) and a mass of \( m \). Upon ionization, the ion is imparted with energy \( E \) by the applied voltage \( U \):

\[
E = ezU
\]

(Eq. 3.20)

Equation 3.20 represents the potential energy that accelerates the ions prior to entering the field-free drift region. At the entrance to the drift region, the ions have a kinetic energy equal to its potential energy:

\[
ezU = \frac{1}{2}mv^2
\]

(Eq. 3.21)

Thus, the velocity \( v \) of the ion passing across through the drift tube is inversely proportional to the m/z of the ion:

\[
v = \sqrt{\frac{2ezU}{m}}
\]

(Eq. 3.22)

As such, the time \( t \) it takes for an ion of a given m/z to pass through the field free drift tube of a given length \( s \), also termed the time of flight, is expressed as follows:

\[
t = \frac{s}{v} = \frac{s}{\sqrt{\frac{2ezU}{m}}}
\]

(Eq. 3.23)

Further rearrangement of equation 3.23 reveals that the flight time of an ion is a function of the square root of the m/z of the ion.
\[ t = \frac{s}{\sqrt{2eU}} \sqrt{\frac{m}{z}} \]

Thus by measuring the flight time of an ion, the m/z of the ion can be determined.

Figure 3.5  A pictorial summary of a MALDI-TOF-MS experiment and instrumentation. Ions are first generated by (A) MALDI prior to being injected into (B) the flight tube of the time-of-flight mass analyzer. Larger ions travel at a slower velocity through the flight tube. Shown on the right is the Micromass MALDI-TOF-MS situated in the Agnes laboratory.

3.5.3 So, Why Mass Spectrometry?

The ability for LDI-MS to identify numerous chemical species within a single sample made it an attractive readout device for off-line monitoring of heterogeneous and multiphase reactions occurring on levitated aerosols. In addition, the same instrument can be used in
MALDI mode for the detection of proteins harvested from lung cell culture supernatants, as described in Chapters 9 and 11.

3.6 Summary

Each suspended particle in the troposphere is a miniature chemical reactor, in and upon itself. Every particle, like a snowflake, is unique, having its own size, shape and composition. For this reason, the real-time study of particulate chemistry, as it pertains to the troposphere, is exceedingly difficult. Thus, to gain useful kinetic data, it is advantageous to undertake experiments in a controlled laboratory setting.

To try to address this issue, we set out to develop controlled laboratory experiments wherein we could study one or two chemical reactions occurring at a time on a particle using LDI-MS as the analytical tool to provide chemical readout. We felt that the use of a mass spectrometry readout would potentially yield more detailed chemical characterization of the particles before, during and after the reaction periods than the more conventional spectroscopy readouts such as Raman or IR. As a result, the potential to learn more about the fundamental reactions occurring on suspended particles in the troposphere could be explored.
Chapter 4

**Organic Heterogeneous Chemistry**

A Class of Heterogeneous/Multiphase Organic Reactions Studied on Droplets/Particles Levitated in a Laboratory Environment: Aldehyde + 1,8-Diaminonaphthalene = Imine

Never before has urban man known so much about a vital resource and yet so little in relation to what he must know. Air, which is the collection of gas and materials that make up the atmosphere, is a flow resource and is renewable. By precipitation, oxidation, and absorption into the oceans, the atmosphere can cleanse itself of all known pollutants, given sufficient time.

*Spencer W. Havlick, The Urban Organism (127)*

The composition of the atmosphere is constantly changing, with chemical species constantly being produced while others are being broken down. The extent to which a chemical species will remain in the air long enough to be measured is dependant on the comparison between the rate at which the chemical species is being produced with respect to the rate at which the species is being broken down. Prior to 2004, the general assumption in the atmospheric chemistry community was that organic species in the troposphere underwent oxidation, becoming structurally smaller and more hydrophilic, prior to their inevitable removal through precipitation. The working hypothesis of the work presented in this chapter was that under suitable conditions, more hydrophobic organic compounds with a larger molecular weight could be produced from the same organic species in the troposphere at an appreciable rate.
4.1 Context and Contributions

Sections 4.2 to 4.7 of this chapter have been previously published in their entirety in the journal Atmospheric Environment (Atmospheric Environment, Volume 38, Number 4, February 2004, pp. 545-556). The authors of the manuscript were myself and George R. Agnes. Experimental design along with all data collection and processing was undertaken by myself. This was the first manuscript from the Agnes group to focus solely on atmospheric chemistry and it includes many features conclusive to that, such as modelling and reaction rate estimation. By publishing this work in a journal such as Atmospheric Environment, our intent was to raise the awareness of the atmospheric community to our technology and to the observations we were making. Our goal of raising awareness was fruitful when, in 2005, Dr. Kimberly Prather, an atmospheric chemist known internationally for her role in the development of real-time individual particle chemical analysis, cited our work in a review article concerning environmental analysis published in the journal Analytical Chemistry (281).

4.2 Abstract

A condensation reaction between aldehydes and a primary amine on the surface of particulate matter created and suspended in a laboratory environment has been studied. The methodology developed for these studies made use of a non-volatile chromophore, 1,8-diaminonaphthalene, as the starting amine which facilitated detection of the starting and product compounds. This reactive chromophore was located on glycerol droplets, saturated salt water droplets, or salt particles that were levitated in an electrodynamic balance. Following an exposure of the levitated droplets/particles to the vapour of different aldehyde compounds, the droplets/particles were deposited onto a stainless steel plate and the reaction products, imines, were characterized using laser desorption ionization mass spectrometry. The starting compound, 1,8-diaminonaphthalene and the imine reaction product compounds were detected as molecular radical cations. The rate of the heterogeneous/multiphase reaction between the amine and
benzaldehyde was measured to be $9 \times 10^{-19}$ cm$^3$ molecule$^{-1}$ s$^{-1}$ on glycerol droplets, which makes the rate of imine formation comparable with amine consumption via ozone or hydroxyl radical under conditions of slightly elevated aldehyde concentrations. As such, imine formation via a heterogeneous/multiphase reaction could occur under certain conditions in the troposphere. This reaction on particulate matter reduces the hydrophilic character of the product compound relative to the starting compounds, and the implications of this class of reactions with respect to the toxic properties of suspended particles in the troposphere are briefly speculated upon.

4.3 Introduction

A substantial amount of data linking adverse effects on human health to particles suspended in the atmosphere has been accumulated (33, 144, 164, 209). Particles suspended in the troposphere are less than 10 μm in diameter (PM$_{10}$) (89). These particles, and particularly those that are smaller than 2.5 μm in diameter (PM$_{2.5}$) penetrate deep into the lungs (251). There, the exchange of air is less efficient and particles settle onto epithelial cells in the alveoli of an individual's lungs. Individuals exposed to high concentrations of particles experience a systemic response that peaks in severity 24–48 hours later (213, 234, 289). The symptoms experienced range from shortness of breath to death, depending on the exposure and health of the individual (90, 252, 259, 263).

The composition of suspended particles that are responsible for the adverse effects on human health remains a subject of much attention (88, 195, 201, 257, 274). The type of particle core and the compounds in the films that coat the particles have been implicated. For example, copper ions from a mineral particle, or organic compounds such as polycyclic aromatic hydrocarbons and polychlorinated biphenyls that are found in the films are known to have toxic and carcinogenic properties (6, 84, 200, 293).

Many other compounds on suspended particles are likely to contribute to this particle air pollution problem. For example, of the estimated $7.5 \times 10^8$ tons of volatile organic compounds
emitted into the atmosphere every year (89), gas-phase chemical reactions initiated by attack from tropospheric oxidants such as OH, NO₃, and O₃ produce numerous product compounds that readily condense onto particles (8, 89, 175, 235). There are in addition, gas–particle heterogeneous chemistries that increase the diversity of compounds on particulate matter (100, 110, 143, 240). That line of research can be traced to the realization that catalytic gas-particle heterogeneous chemistry was occurring in the stratosphere, and its effect on stratospheric O₃ concentrations was significant (79, 206). In the organic compound-rich troposphere, the most abundant species after sulfates on particles are organic compounds (146), and recent studies under conditions relevant to the troposphere have shown evidence for acid catalyzed heterogeneous reactions of aldehydes on organic aerosols (148, 149).

Given the vast variety of the organic compounds found on suspended particles, heterogeneous and multiphase chemistries involving only organic compounds on particles and droplets, respectively, in the troposphere could be occurring. We speculate that those reactions could be of significant relevance to the toxicity of suspended particles. We report here the results of our first studies of gas–particle heterogeneous and gas-droplet multiphase chemistry that involved a condensation reaction between an aldehyde and a primary amine on particles and droplets levitated in a laboratory environment. These species were studied because aldehydes can be found in urban and indoor environments (Table 4.1), amines readily adsorb/absorb onto suspended particles, and the bulk phase reactions between aldehydes and amines have been well characterized.
Table 4.1 Common sources\(^a\), concentrations and octanol-water partition coefficients for selected atmospheric particulate amines and aldehydes

<table>
<thead>
<tr>
<th>Species</th>
<th>Industrial</th>
<th>Plastics</th>
<th>Tobacco Smoke</th>
<th>Automobile</th>
<th>Diesel</th>
<th>Sawdust Treatment</th>
<th>Atm ospheric Concentrations (ppb)(^b)</th>
<th>Log (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Urban</td>
<td>Remote</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Species</td>
<td>Imine(^e)</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>▲ ▲ ▲ ▲ ▲</td>
<td>▲ ▲ ▲ ▲</td>
<td>▲ ▲ ▲ ▲ ▲ ▲</td>
<td>▲ ▲ ▲ ▲ ▲ ▲</td>
<td>1 to 60</td>
<td>0.3 to 2</td>
<td>0.35</td>
<td>2.7</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>▲ ▲ ▲ ▲ ▲</td>
<td>▲ ▲ ▲ ▲</td>
<td>▲ ▲ ▲ ▲ ▲ ▲</td>
<td>▲ ▲ ▲ ▲ ▲ ▲</td>
<td>1 to 18</td>
<td>0.1 to 1</td>
<td>0.156</td>
<td>1.92</td>
</tr>
<tr>
<td>Crotonaldehyde</td>
<td>▲ ▲ ▲ ▲ ▲</td>
<td>▲ ▲ ▲ ▲</td>
<td>▲ ▲ ▲ ▲ ▲ ▲</td>
<td>▲ ▲ ▲ ▲ ▲ ▲</td>
<td>0.1 to 0.5</td>
<td>0.09</td>
<td>0.51</td>
<td>3.05</td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>▲ ▲ ▲ ▲ ▲</td>
<td>▲ ▲ ▲ ▲</td>
<td>▲ ▲ ▲ ▲ ▲ ▲</td>
<td>▲ ▲ ▲ ▲ ▲ ▲</td>
<td>0.1 to 1</td>
<td>0.01 to 0.5</td>
<td>1.64</td>
<td>2.9</td>
</tr>
</tbody>
</table>

Aniline ▲ ▲ ▲ ▲ ▲ | ▲ ▲ ▲ ▲ | ▲ ▲ ▲ ▲ ▲ ▲ | ▲ ▲ ▲ ▲ ▲ ▲ | 1 | 0 | 0.94 |
Methylamine ▲ ▲ ▲ ▲ | ▲ ▲ ▲ ▲ | ▲ ▲ ▲ ▲ ▲ ▲ | ▲ ▲ ▲ ▲ ▲ ▲ | \(\sim 1^{cd}\) | 0\(^c\) | -0.57 |

PM\(_{10}\) ▲ ▲ ▲ ▲ ▲ ▲ | ▲ ▲ ▲ ▲ ▲ | ▲ ▲ ▲ ▲ ▲ ▲ | ▲ ▲ ▲ ▲ ▲ ▲ | 20,000 to 100,000 | 11,000 |
PM\(_{2.5}\) ▲ ▲ ▲ ▲ ▲ ▲ | ▲ ▲ ▲ ▲ ▲ | ▲ ▲ ▲ ▲ ▲ ▲ | ▲ ▲ ▲ ▲ ▲ ▲ | 50,000 to 80,000 | 5,000 to 25,000 |

\(^b\) Unless otherwise noted, values from: Finlayson-Pitts, B.J.; Pitts, J.N. Chemistry of the Upper and Lower Atmosphere: Theory, Experiments and Applications, 2000, Academic Press.
\(^c\) Concentration on particulate matter
\(^e\) The Log P for the imine is for the product of the aldehyde and aniline

In each of our experiments, imine product compounds formed by this dehydration reaction (Eq. 4.1) were detected.

\[(\text{Eq. } 4.1)\] \[\text{R}_1\text{CHO} + \text{H}_2\text{N-R}_2 \rightarrow \text{R}_1\text{-CH=N-R}_2 + \text{H}_2\text{O}\]

### 4.4 Experimental

To facilitate studies of gas-particle heterogeneous and gas-droplet multiphase chemistry within a laboratory environment, we developed methodology that enables us to characterize reactions that have taken place on particles levitated in an electrodynamic balance (EDB) (figure 4.1). A typical experiment involves the following steps. Creation of droplets that have net charge from a starting solution, and levitation of those droplets in an EDB. The volatile solvent within them evaporates within seconds (266), resulting in levitated droplet residues that were either droplets or particles depending on humidity and the composition of the starting solution. These residues, still levitated, were then exposed to the vapour of one or more compounds, and following that, deposited onto a stainless steel plate and characterized using laser desorption
ionization (LDI) time of flight (TOF) mass spectrometry (MS) (25). Details of this experimental procedure are described below.

![Diagram](image)

**Figure 4.1** A simple schematic of the droplet/particle generation and levitation apparatus used throughout the course of the study. The MALDI plate was fabricated from stainless steel.

To detect the molecular ion of an organic compound on these residues via LDI-TOF-MS, each compound must absorb at the output of the laser used, in this case a nitrogen laser ($\lambda = 337$ nm). To account for this, the aromatic amine 1,8-diaminonaphthalene was selected. The low vapour pressure of 1,8-diaminonaphthalene ($2.93 \times 10^{-5}$ mmHg) coupled with its ability to absorb at 337 nm made it a suitable amine for the study. Additionally, several properties of 1,8-diaminonaphthalene suggest that it can be used as a surrogate to the common tropospheric aromatic amine aniline in an environmental model. The similarities between 1,8-diaminonaphthalene and aniline are as follows: they have similar basicity/acidity (both have a pKa of 4.6 (247), they are structurally similar in that they are both-NH$_2$ substituted aromatic rings, and in house studies in acetonitrile found that the reaction rate of aniline or 1,8-diaminonaphthalene with benzaldehyde were similar (1,8-diaminonaphthalene was found to be 25% lower, likely due to steric hindrance). The reaction of aniline itself was not monitored in
these studies as a molecular ion of it was not able to be detected on the levitated droplet residues by LDI-TOF-MS.

Starting solutions containing either 8% glycerol in methanol with 0.01 M 1,8-diaminonaphthalene, or 1.5 M NaCl in water with 0.005 M 1,8-diaminonaphthalene were prepared. The solubility of 1,8-diaminonaphthalene in water (850 mg/L) lead to two different concentrations to be used. A syringe was filled with one of these solutions, and that solution was used to fill the reservoir of an ink-jet style droplet dispenser. Adhered to opposite sides of the rectangular reservoir were two piezoelectric strips that were used to create a pressure wave that, with suitable backing pressure, caused the ejection of a jet of liquid from a 40 μm diameter nozzle fixed to the other end of the reservoir. Suitable backing pressure was obtained by placing the syringe containing the starting solution 10 – 15 cm above the nozzle of the droplet dispenser. An induction electrode mounted 0 – 2 mm above the nozzle induced an image charge on this jet of liquid, such that when that jet collapsed to form a droplet, that droplet carried a net elementary charge. The initial droplet size was ~40 μm in radius. For a starting solution that had 0.01 M 1,8-diaminonaphthalene, each of the droplets contained ~1.6 pmol of this reactive chromophore.

Each droplet created flew upward from the nozzle, through a hole in the induction electrode, and into the centre of a two-ring EDB (1, 59). The droplet was trapped there by the electric field created from a sinusoidal waveform (frequency = 60 Hz, amplitude = 1000 – 2500 V_0-p) applied to the ring electrodes, and a DC field applied between the two plate electrodes. These two plates, referred to as the induction electrode and the deposition plate were mounted, respectively, below and above the ring electrodes. The DC field was used to offset the force of gravity on the droplet/particle.

During any one experiment, 10 – 20 droplets/particles were levitated inside the EDB, which was enclosed in a Plexiglas chamber (23 cm x 14 cm x 14 cm) to eliminate loss of levitated droplets/particles due to air currents. Within seconds of being levitated, the majority of the volatile solvent had evaporated (266), leaving behind a residue consisting of any solvents of low
volatility plus non-volatile solutes. These levitated droplet residues were then used as the template to study the heterogeneous and multiphase reactions between primary amines and aldehydes that produced imines. The reaction was initiated with the introduction of the vapour of a common tropospheric aldehyde into the levitation chamber in which there was a population of levitated droplets/particles. During any one experiment, the air inside the levitation chamber was isolated from the laboratory air, but the air that was used to fill the levitation chamber at the start of an experiment was from the laboratory. The humidity inside the chamber was monitored using a Traceable Digital Hygrometer/Thermometer (Control Company, Friendswood, TX).

Following the exposure of the levitated droplets/particles to the vapour of an aldehyde, the droplets/particles were deposited onto the deposition plate in a manner so as to prevent co-deposition of the droplets. Details of the procedures to deposit levitated droplets/particles on top of one another (25) or without co-deposition (83) have been reported elsewhere. The latter procedure was used here, and basically that procedure involved a single, large step in the potential applied to the deposition plate to electrostatically extract all levitated droplets/particles simultaneously from the EDB. The area on the deposition plate over which droplets/particles deposited was dictated by the droplet–droplet (or particle–particle) space charge repulsion (25). In this work, the typical levitated glycerol droplet residue was ~15 μm in diameter, but its deposition onto a plate caused it to smear to a diameter of ~40 μm, whereas the salt particle did not deform or shatter upon deposition. The deposited droplets or particles were examined with an optical microscope (model B5 Professional Series, Moticam 1300, Richmond, BC). The separation between any two deposited droplets was typically 125 μm.

In all of our experiments, the deposition plate was mounted inside the levitation chamber at the start of each experiment, and thus it was also exposed to the aldehyde vapour. To account for the reaction that occurs during deposition between the amine on the particle/droplet with the aldehyde on the plate, the following control experiment was developed. The solution that was present in the tip of the droplet generator during the levitation period was removed to ensure that
the solution used subsequently had no imine present. The reservoir of the droplet dispenser was reloaded and 10 droplets were produced by the droplet dispenser and directed onto different regions of the plate without being levitated. By not allowing for the methanol solvent to evaporate, the low viscosity of the droplet-solution allowed the 1,8-diaminonaphthalene to spread as a thin layer over a larger region of the deposition plate as compared to the glycerol droplets that had been deposited previously. This enabled all of the 1,8-diaminonaphthalene available to react with any aldehyde adsorbed onto the plate. Immediately following deposition, the plate was removed from the levitation chamber, and analyzed by LDI-TOF-MS.

The plate was inserted into a commercial laser desorption/ionization (LDI) time-of-flight (TOF) mass spectrometry (MS) instrument (model Voyager DE, Perceptive Biosystems, Framingham, MA) and the output of the N₂ pulsed laser was centered over one droplet or one particle at a time. The laser output was focused to a spot size of 173 µm × 90 µm (25), thus the entire droplet/particle was irradiated by each laser shot, and only one droplet/particle was likely to have been targeted at any one time. Each mass spectrum, or data point presented is the sum of the ions detected from firing the laser eight times at a single droplet or particle. The pulse energy of the N₂ laser was measured using a power meter (model 840, Newport Optical) set at 337 nm and detector (model 818-UV, Newport optical).

The ability of matrix assisted LDI-TOF-MS to provide semi-quantitative results has been previously shown (111, 302). In that work, the relative concentration of the analyte was determined through self-normalization of its signal intensity divided by the major ion peak in the spectrum. We applied the same methodology to our LDI-TOF-MS results to estimate the relative concentration of imine formed as a function of time, but in our study, the signal intensity was self-normalized by the sum of all the ion peaks in the spectrum. Because both the starting compound and the imine product were detected as molecular radical cations, using their ion signal intensities, the second-order rate constant of the heterogeneous/multiphase reaction between benzaldehyde and 1,8-diaminonaphthalene on a levitated glycerol droplet was evaluated.
4.5 Results

4.5.1 Reactions of 1,8-Diaminonaphthalene with Aldehydes on Levitated Glycerol Droplets

A starting solution containing 8% glycerol and 0.01 M 1,8-diaminonaphthalene in methanol was used to create a population of levitated droplets that, after the evaporation of the majority of the methanol, consisted of a residue that contained glycerol and 1,8-diaminonaphthalene. Optical microscopic analysis indicated that 1,8-diaminonaphthalene was insoluble in glycerol, resulting in 1,8-diaminonaphthalene precipitates being formed within, and distributed evenly throughout, the droplet. Plotted in figure 4.2A is a spectrum representative of the blank for this experiment, which was the levitation of these glycerol droplets for 20 min prior to depositing them onto a plate. The ion peak at $m/z = 158$ is the molecular radical cation of the starting amine, 1,8-diaminonaphthalene. The ion signals in the range of $m/z$ from 270 – 350 are believed to be from solidified 1,8-diaminonaphthalene within the glycerol droplet. The ion signal at $m/z = 161$ was only observed in the LDI-TOF-MS analysis of glycerol droplets that contained unreacted 1,8-diaminonaphthalene. The source of this ion is unclear at this time.
Figure 4.2  LDI-TOF-MS spectra of glycerol droplets that contained 1,8-diaminonaphthalene that had been levitated in the presence of the vapour from four different aldehydes. (A) no aldehyde was introduced (396 µl/pulse), (B) formaldehyde (413 µl/pulse), (C) acetaldehyde (378 µl/pulse), (D) crotonaldehyde (378 µl/pulse), and (E) benzaldehyde (440 µl/pulse).

The laser energy per shot used to generate the spectra are indicated in parentheses. The structures shown are the molecular radical cations of the imine product compounds on the glycerol droplets.
We proceeded to expose populations of levitated glycerol droplets to an aldehyde vapour that was deliberately introduced into the chamber. 5 mL of either pure formaldehyde, acetaldehyde, crotonaldehyde, or benzaldehyde was placed into a beaker positioned inside the levitation chamber. These aldehydes were selected as they are among the most common aldehydes in the troposphere (89). Following a 20 minute levitation period in which the 1,8-diaminonaphthalene containing glycerol droplets were levitated in the presence of the vapour from one of these aldehydes, the droplets were deposited and analyzed by LDI-TOF-MS. In the mass spectra obtained from these experiments, the imine product compounds were readily detected (figure 4.2B - E). The starting amine, 1,8-diaminonaphthalene, was not detectable following the levitation period during which an aldehyde vapour was present, suggesting that the reaction equilibria were not reversible on the glycerol droplet under the condition of 60% humidity in the levitation chamber.

In comparing the spectra plotted in figure 4.2A to that of figure 4.2B, where, respectively, formaldehyde vapour was not and was deliberately added to the levitation chamber, the product compound at m/z = 170 was detected in both experiments. At that point in our studies, we had not introduced any formaldehyde into the laboratory. The source of this aldehyde was the background concentration of formaldehyde in the air in our laboratory. At the elevated formaldehyde vapour concentration (figure 4.2B), the imine product compound from the addition of two formaldehyde equivalents (m/z = 182) was detected, as were clusters consisting of these two imine compounds plus one molecule of formaldehyde (m/z = 200 and 212).

The concentrations of the aldehydes that had been deliberately added to the levitation chamber to this point in our studies were several orders of magnitude higher than those experienced in an urban environment. The next results presented were from experiments that used aldehyde vapours at much lower concentrations. As previously described, we created a population of 10 – 20 glycerol droplets from a starting solution containing 8% glycerol and 0.01 M 1,8-diaminonaphthalene. A 1.0 mL aliquot of a $4.7 \times 10^{-5}$ M solution of benzaldehyde in methanol was
placed in a beaker inside the levitation chamber. The addition of this aliquot raised the concentration of gaseous benzaldehyde inside the chamber to an estimated 1 ppb. The levitated glycerol droplets were exposed to the benzaldehyde vapour from this solution for a period of 180 min, then the droplets were deposited and analyzed for imine production using LDI-TOF-MS. A mass spectrum of the compounds detected from one of these droplets is shown in figure 4.3A. The 1,8-diaminonaphthalene reacted with benzaldehyde to form an imine, the molecular radical cation of which was detected at m/z = 246. In addition, the 1,8-diaminonaphthalene also reacted with formaldehyde and acetic acid, and the compounds formed by these two reactions were also detected as molecular radical cations, at m/z = 170 and 200, respectively. Their sources were, contamination from the cleaning of the walls of the levitation chamber with acetic acid between experiments, and formaldehyde from the ambient air.
Immediately after the glycerol droplets that were levitated for 180 minutes had been deposited, a control experiment as described in the experimental section was performed to obtain a measure of the maximum amount of imine that could be produced as a result of the droplet deposition procedure. The ion signal peak intensities for the 1,8-diaminonaphthalene starting compound ($m/z = 158$) relative to the imine product formed by its reaction with benzaldehyde ($m/z = 246$) in the residues of the non-levitated droplets yielded a ratio of $41 \pm 26$. In comparison, the ratio for the same ion peaks in the levitated droplets was $3 \pm 2$. This result implies that at least 10 times more imine was produced while the glycerol droplet was levitated relative to that which could have formed during the deposition procedure.
Next, a blank for this experiment was performed after wiping clean the chamber using neat methanol solvent and then heating the chamber to 50°C for 2 h. Upon cooling the chamber to the ambient laboratory temperature, a fresh volume of the same starting solution was used to create another population of glycerol droplets that were levitated for 180 minutes, during which time there was no benzaldehyde vapour deliberately introduced to the levitation chamber. An LDI-TOF-MS spectrum from one of those droplets is shown in figure 4.3B. There was no signal intensity for the imine product for the reaction between the 1,8-diaminonaphthalene and benzaldehyde. The ion peaks for 1,8-diaminonaphthalene, and clusters of it that had been detected in previous blank experiments were detected. Similarly, an ion signal for the imine produced as a result of formaldehyde reacting with 1,8-diaminonaphthalene was detected, as would be expected if the source of the formaldehyde was from ambient levels in the air in our laboratory.

In a second experiment, 0.5 mL of a 3.6×10⁻⁷ M solution of acetaldehyde in methanol was placed in a beaker that was then positioned inside the levitation chamber. This concentration and volume of acetaldehyde solution created an atmosphere inside of the levitation chamber that had an estimated acetaldehyde concentration < 1 ppb. After a 165 minute levitation period, the glycerol droplets were deposited and analyzed by LDI-TOF-MS. As described previously, a control experiment to determine the maximum possible imine formed as a result of the deposition procedure was performed. Based on the LDI-TOF-MS characterization of the 10 non-levitated droplet residues, the ion signal intensity ratio of the starting material to product imine measured was 2.4 ± 1.4. In comparison, the ratio of the ion signal intensity of the starting material to product imine in the levitated droplets was 0.4 ± 0.4. The difference between these ratios suggests that at least six times more imine formed on the levitated glycerol droplets as compared to the maximum quantity of imine that could have formed upon deposition of the glycerol droplet onto the plate. This implies that there is not enough aldehyde on the plate to form a significant quantity of the imine.
4.5.2 Reaction of Benzaldehyde with 1,8-Diaminonaphthalene on a Super-Saturated Salt Water Droplet and a Salt Particle

A starting solution containing 1.5 M NaCl and 5.0 mM 1,8-diaminonaphthalene was used to create a population of levitated droplets. Microscopic analysis (model Hyperion 1000, Bruker, Milton, Ont.) of the levitated salt droplets showed that sodium chloride does not precipitate out of solution until the relative humidity was lowered to 35%, in agreement with that reported by Choi and Chan (49). Because the humidity was ~ 60% over the course of this experiment, the levitated residues were supersaturated salt droplets. The droplets were levitated for 160 minutes prior to introducing a beaker that contained 5 mL of pure benzaldehyde inside the levitation chamber. The salt droplets were levitated in the presence of benzaldehyde vapour for 60 minutes before being deposited and analyzed by LDI-TOF-MS. A mass spectrum obtained from one of the salt droplets in this experiment is shown in figure 4.4. The imine product compound, detected as the molecular radical cation at m/z = 246, that formed on the droplet by the reaction of 1,8-diaminonaphthalene with benzaldehyde was readily detected. As previously observed, the imine generated at m/z = 170 from the reaction between the 1,8-diaminonaphthalene and ambient levels of formaldehyde vapour was again detected. The large ion signal at m/z = 170 can be attributed to the increased exposure time, of an additional 160 minutes, that ambient formaldehyde had to react with the 1,8-diaminonaphthalene prior to the introduction of the benzaldehyde.
Figure 4.4 LDI-TOF-MS spectrum from a saturated saltwater droplet containing 1,8-diaminonaphthalene that had been levitated in the presence of benzaldehyde (estimated concentration 167 ppm). The imine product compound formed as a result was detected as the molecular radical cation at m/z = 246. The N₂ laser energy was set to 331 µJ/pulse.

In the control experiment, no ion signals at m/z = 246 were detected with the laser energy set at 331 µJ/pulse. That energy per pulse was equal to the setting used to acquire the spectrum plotted in figure 4.4. Only by raising the laser energy to 556 µJ/pulse was a small ion signal for the imine product detected (S/B ~ 3). Based on these data, we conclude that the imine product detected at m/z = 246 in the spectrum plotted in figure 4.4 was generated on the levitated droplet and that the quantity of imine formed when those droplets were deposited onto the plate was by comparison, insignificant.

The above experiment was repeated under the conditions of lower relative humidity (30%). Under these conditions, the starting solution formed a solid salt particle (efflorescence point of 35%) spiked with solid 1,8-diaminonaphthalene. A population of salt particles were levitated prior to introducing a beaker containing 2 mL of pure benzaldehyde into the levitation
chamber. The levitation reaction period was 40 minutes, after which time the particles were deposited and analyzed via LDI-TOF-MS. The presence of the benzaldehyde + 1,8-diaminonaphthalene imine product (m/z = 246) verified that the reaction was able to occur on a solid substrate (figure 4.5A).

![Figure 4.5](image.png)

**Figure 4.5** LDI-TOF-MS spectrum from a salt particle coated with 1,8-diaminonaphthalene that had been levitated in, (A) the presence and, (B) the absence of benzaldehyde. For (A) the estimated concentration of benzaldehyde was 167 ppm. The imine product compound formed as a result was detected as the molecular radical cation at m/z = 246. The \( \text{N}_2 \) laser energy was set to 493 \( \mu \text{J/pulse} \).

### 4.5.3 First estimate of the reaction rate

The pseudo-first-order rate constant of the reaction between benzaldehyde in the gas phase with a glycerol droplet spiked with 1,8-diaminonaphthalene was determined as described below. A starting solution containing 8% glycerol and 0.01 M 1,8-diaminonaphthalene was used to generate a population of 5 – 10 droplets. After 10 minutes, 1 mL of pure benzaldehyde was introduced to the reservoir within the levitation chamber. After a given period of time, as indicated by the data points in figure 4.6, the residues were deposited onto the stainless steel plate and the relative imine concentration within each residue was determined via LDI-TOF-MS. The
imine signal intensity detected from each droplet was measured once by firing the N\textsubscript{2} laser eight times, meaning the sum of the ions detected over the range of m/z = 0 – 2000 from each droplet was monitored. Two populations of droplets were levitated in the presence of benzaldehyde for each of the five different time periods for which data points were obtained. As such, for each time period, between 12 and 20 mass spectra from separate droplets were collected. After each levitation period, the chamber was wiped down with methanol and heated with a hot air gun for 5 min to ensure that at t = 0, the benzaldehyde concentration within the chamber was not detectable in the blank experiments.
Figure 4.6 Mass spectra (A) used to generate the data (B) that were used to evaluate the reaction rate between benzaldehyde in the gas phase with 1,8-diaminonaphthalene on a glycerol droplet. Reaction times in (A) were: (i) 30 min, (ii) 7 min, (iii) 5 min and (iv) 3 min. The laser energy setting was 440 µJ/pulse. In (B), the error bars represent 95% confidence intervals.
The results of the rate study are shown in figure 4.6. A sample mass spectrum for each of the time periods has been plotted in figure 4.6A. The signal to noise ratio for the m/z = 246 peak increased with longer reaction periods for the levitated droplets. The area of the peak at m/z = 246 was self-normalized to the sum of all the ion signals in the entire spectrum, and those data points were used to generate the graph in figure 4.6B. The 95% confidence intervals for each of the data points suggest that the LDI-TOF-MS technique was capable of quantitative analysis of the droplet residues provided that variables such as residue size, composition, environment within the levitation chamber, and laser intensity were held constant. By plotting the data in figure 4.6B as $\ln (c/c_0)$ vs. time (s) (figure 4.7), the pseudo-first-order rate constant was estimated to be $3.6 \times 10^{-3}$ s$^{-1}$.

![Graph](image_url)

**Figure 4.7** The concentration of imine present on the levitated glycerol droplet as a function of time. The slope of the least squares fit through the data is (-0.00361) with an $R^2$ of 0.9594.
Using the estimated concentration of gas phase benzaldehyde within the chamber (4.1×10^{15} \text{ molecules cm}^{-3}), the second-order rate constant between the heterogeneous reaction of 1,8-diaminonaphthalene and benzaldehyde was evaluated to be 9×10^{-19} \text{ cm}^3 \text{ molecules}^{-1} \text{ s}^{-1}.

Tropospheric aerosols containing elevated concentrations of amines tend to be positively correlated with elevated levels of organic and inorganic acid (47, 55, 271). As such, the imine formation reaction is likely to proceed under mildly acidic conditions, at low- and high-humidity in the troposphere. It has been well established that the highest rate of imine formation in solution occurs at pH between 4 and 5 (150, 188) due to acid catalysis (290). As such, lowering the pH of the levitated droplets to between 4 and 5 could increase the rate of the reaction over that which was measured in this work. Recent results from our group have shown that the uptake of the nitric acid, whether introduced prior to benzaldehyde introduction, post benzaldehyde introduction or simultaneously with the benzaldehyde, does not impede imine formation (120). Given that 1,8-diaminonaphthalene is a diamine, an aldehyde may react differently with a protonated monosubstituted primary amine, as such, it can be only concluded that the presence of acid is not likely to stop imine formation from occurring.

4.5.4 Estimated Environmental Lifetimes and Concentrations of Benzalaniline as Predicted with Environmental Modelling Software

Using the rate constants and reactant concentrations presented in Table 4.2, a model was generated with the STELLA modeling program (High Performance Systems, Lebanon, New Hampshire) to predict the likelihood of benzalaniline formation occurring within an urban environment. In the model, the rate constant for the reaction of benzaldehyde and 1,8-diaminonaphthalene determined in this study was used as an estimate of the heterogeneous/multiphase reaction rate constant for benzaldehyde and aniline.
Table 4.2 Concentrations and rate constants used to generate the atmospheric model

<table>
<thead>
<tr>
<th>Species</th>
<th>Concentrations (molecules/cm³)</th>
<th>Reaction Rates With: (cm³/molecules x sec)</th>
<th>Kp (m³/μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low</td>
<td>High</td>
<td>Ozone</td>
</tr>
<tr>
<td>Aniline</td>
<td>2.46 x 10^10</td>
<td>2.21 x 10^12</td>
<td>1.12 x 10^{-13}b</td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>1.23 x 10^10</td>
<td>2.46 x 10^10</td>
<td>---</td>
</tr>
<tr>
<td>Ozone</td>
<td>1.97 x 10^12</td>
<td>3.69 x 10^12</td>
<td>---</td>
</tr>
<tr>
<td>Hydroxyl Radical</td>
<td>5.00 x 10^5</td>
<td>1.00 x 10^7</td>
<td>---</td>
</tr>
<tr>
<td>Benzaniline</td>
<td>0</td>
<td>0</td>
<td>1.71 x 10^{-11}d</td>
</tr>
<tr>
<td>TSP</td>
<td>0 μg/m³</td>
<td>100 μg/m³</td>
<td>---</td>
</tr>
</tbody>
</table>

a = Unless otherwise stated, values in this table are from: Finlayson-Pitts, B.J.; Pitts, J.N. Chemistry of the Upper and Lower Atmosphere; Theory, Experiments and Applications, 2000, Academic Press
e = Experimentally determined in this work

Briefly, the model included the following processes. The uptake of aniline onto the particulate matter was assumed necessary for the reaction to take place. The rate of aniline uptake onto the particulate matter competed with the breakdown of aniline by O₃ and OH. The rate of benzalaniine ozonolysis is an estimation based on the ozonolysis rate of styrene. With these assumptions, it was found that altering the concentration of total suspended particulates [TSP] or the Kp (of aniline) had little to no effect on imine production. As such, for the all of the models [TSP] and Kp were held constant at 50 μg m⁻³ and 1 m⁻³ μg⁻¹, respectively. The reservoir volume was set as 64 m³. The concentrations of O₃ and OH were adjusted to estimate feasible maximum and minimum imine concentrations (Table 4.2), and the concentration of the imine formed over a 20,000 second time period was evaluated. The results of the environmental model generated are shown in figure 4.8. The starting mass of aniline was 1 g in the gas phase and the starting mass of benzaldehyde was either held constant at 1 ppb (figure 4.8A), or 1 g only was added once to the gas phase (figure 4.8B). In figure 4.8A, at high [O₃] (3.69 X 10^{12} molecules/cm³) and [OH] (1.00 X 10^{7} molecules/cm³), the peak imine concentration was found to be 0.004 ppb, while at low [O₃] (1.97 X 10^{12} molecules/cm³) and [OH] (5.00 X 10^{5} molecules/cm³), the peak imine concentration was found to be 0.06 ppb. In figure 4.8B, at high [O₃] and [OH], the peak imine concentration was
found to be 21 ppb, while at low [O$_3$] and [OH], the peak imine concentration was found to be 1 ppm.

Figure 4.8 Graphical presentation of results generated from an environmental model created using STELLA software. Rate constants used in the model can be found in Table 2. (A) The benzaldehyde concentration was held constant at 1 ppb while 1 g of aniline was added to the system at t = 0. (B) 1 g of aniline and 1 g of benzaldehyde were introduced into the system simultaneously at t = 0.

4.6 Discussion

Throughout the experimental work, the ambient concentration of formaldehyde in the air in our laboratory was sufficient to react with 1,8-diaminonaphthalene to form readily detectable quantities of the imine product. Results obtained in this study suggest that imine formation can occur on suspended particulate matter within the troposphere independent of humidity, either by multiphase or heterogeneous reaction pathways. Combined, these data suggest that imines are
likely to be present on particles suspended in the troposphere, as supported by the model that was
developed to estimate imine production and lifetime. There are many more aldehydes in the
troposphere than were studied here that could also form imines, and those reactions would
compete with amine consumption by reaction with hydroxyl radicals and ozone, thus it is
reasonable to speculate that the total mass of imines being formed could be higher than the values
predicted by the model.

For imine production to occur, a specific environment wherein elevated levels of amines,
aldehydes and particulate matter are required. One such environment is a plume of cigarette
smoke, where aldehydes, amines and particulate matter are all found in abundance (4, 11). At this
time, estimation of the formation and lifetime of imines in specific environments, either locally or
globally, are beyond the current understanding of this reaction. We are pursuing further studies of
imine formation on levitated droplets/particles that more closely mimic the composition of
particles found in the troposphere, at conditions pertinent to the remote and urban troposphere.
Developments in the methodology used herein are ongoing, with emphasis on enabling
quantitative measurement of compounds on droplets/particles that had been suspended in
controlled environments using purified air, and in air sampled from the troposphere.

Imine production on the surface of particles suspended in the troposphere is interesting
from the following perspective. Gas-phase reactions of organic compounds in the troposphere are
dominated by attack from radicals or ozone, and those reactions lead to increased oxidation of the
organic compounds, and thus an increase in the hydrophilic character of those compounds. In
contrast, imine product compounds produced by heterogeneous and multiphase particle/droplet
chemistry have decreased hydrophilic character relative to the starting compounds. Upon delivery
of imine compounds to a lung cell, following inhalation, we speculate that they would interact
and possibly react with protein receptors expressed on the lung cell surface, and because they
have octanol/water partition coefficients in the range of 2 – 3 (185, 211), they would also diffuse
across the cellular membrane where they could be hydrolyzed inside the lung cell. Either or both
of these processes could lead to inflammation of the lung cell. Intra-cellular hydrolysis of an imine compound would liberate an aldehyde, and aldehydes are known to have toxic and clastogenic properties (129). Formaldehyde is also known to react in forming covalent cross-links between proteins (94, 145, 227, 228). The possibility that there could be a transport mechanism for aldehydes into cells via imine-carriers needs further investigation. These considerations need to become well understood before it can be concluded whether or not imines are factors in the toxicity of suspended particle air pollution.

4.7 Conclusions

We have developed methodology that was used to study imine product compound formation by a reaction between aldehydes and 1,8-diaminonaphthalene on levitated droplets/particles. The key features of the methodology were the use of an electrodynamic balance to suspend droplets and particles of known composition in the presence of aldehyde vapours, followed by controlled spatial deposition of those particles onto a target, and the extensive use of a non-volatile chromophore, 1,8-diaminonaphthalene, as a reactant compound. The reaction rate between benzaldehyde and 1,8-diaminonaphthalene on levitated droplets was estimated to be $9 \times 10^{-19}$ cm$^3$ molecules$^{-1}$ s$^{-1}$. Using this rate, an environmental model was developed and used as an estimate of imine formation within the troposphere. Results from that model suggest the imine production under specific, and tropospherically relevant, conditions is possible.
Chapter 5

INORGANIC + ORGANIC
HETEROGENEOUS CHEMISTRY

Off-line LDI-TOF-MS monitoring of simultaneous inorganic and organic reactions on particles levitated in a laboratory environment

Calvin: Curiosity is the essence of the scientific mind. For example, you know how milk comes out your nose if you laugh while drinking?
(Calvin then inserts two straws into his nostrils)
Calvin: Well, I’m going to see what happens when I inhale milk into my nose and laugh!
Susie: Idiocy is the essence of the male mind.
Calvin: I’m guessing it will shoot out my ears.

Bill Watterson, Homicidal Psycho Jungle Cat (305)

Having demonstrated the ability to monitor heterogeneous organic reactions that occur on the levitated aerosols created in the laboratory, the next step was to add another degree of complexity. This would serve as a way to exhibit the utility of our methodology and to further characterise the rate of imine formation in a polluted atmosphere. To do this, we monitored the inorganic heterogenous processing of the aerosol alongside the organic reactions. Of particular interest was how these reactions, which would be occurring in concert with one another, affected the reactions of one another. Specifically, we studied how the presence of nitric acid vapour in the system affected the rate of imine formation.
5.1 Context and Contributions

Sections 5.2 to 5.8 of the following chapter have been previously published in their entirety in the Journal of Aerosol Science (Journal of Aerosol Science, Volume 36, Number 4, 2005, pp. 521-533). The authors of the manuscript were myself, Xiao Feng, Ray Nassar, Michael Bogan and George Agnes. All experiments, data collection and processing was undertaken by myself excluding the purely inorganic reaction of nitric acid with sodium chloride (Section 5.5.1).

5.2 Abstract

Simultaneous inorganic and organic reactions occur on tropospheric particles. The outcomes of these parallel reaction pathways, and particularly the minor reaction products that collectively sum to an appreciable fraction of the particle's mass, are the focus of this work. To characterize condensed phase organic, inorganic, and metal-organic reaction products, we are developing methodology that involves, firstly, the creation of picoliter droplets that carry net charge that are trapped and levitated by an electrodynamic balance in a laboratory environment for the duration of the experiment. Following the rapid evaporation of volatile solvents from the droplets (i.e. seconds), either a residual droplet or a particle remains levitated depending on the atmospheric conditions inside the levitation chamber and the composition of the starting solution from which the initial droplets were created. These droplet residues, whose initial composition is known, are then exposed to gaseous compounds and oxidants that cause transformation of the composition of the levitated droplets/particles by multiphase and heterogeneous reactions that proceed dependent on the species condensing and reacting on the particles. The droplets/particles are then deposited onto a substrate that allows subsequent, and detailed characterization by instrumental methods. In the studies reported here, laser desorption ionization time of flight mass spectrometry was used to characterize reaction products that had formed on the levitated droplets/particles. This apparatus and methodology were used to study the effect of the inorganic reaction of nitric acid vapour with NaCl$_{(aq)}$ and NaCl$_{(s)}$ on the formation of imines by dehydration.
reaction between the functional groups of a primary amine and an aldehyde. The product yield of imines was found to be dependent on the sequence of introduction of the gaseous reactants to the levitation chamber.

5.3 Introduction

Collectively, particles suspended in the troposphere are involved in atmospheric processes that influence climate, such as cloud formation, light absorption and scattering, and visibility reduction (87, 88). In addition, the composition of suspended particles is believed to determine health outcomes following inhalation exposure (2, 65, 237, 251, 252, 270, 289, 296, 301, 310). Improved characterization of individual particle composition, and the reactions involved in their transformations could lead to effective strategies that mitigate the adverse effects on climate and on human health from inhalation exposure to suspended particles (32).

The chemical composition of the particulate matter suspended in the troposphere is representative of its formation processes as well as its chemical alteration within the troposphere through homogeneous, heterogeneous and multiphase reactions during its lifetime (123, 240). Particle cores range from inorganic wind blown dusts and sea spray to purely organic particle cores in secondary organic aerosols, or both, as found in carbonaceous particles derived from high temperature combustion sources. These particles also contain varied quantities of water, inorganic (nitrates and sulfates) and organic compounds depending on the airmass and the local environmental conditions. Particle phase reactions that proceed by heterogeneous and multiphase pathways have been implicated between the reactive functional groups of oxidized organic compounds, in the formation of polymeric compounds (148, 155) and on the involvement of radicals initiated by attack from tropospheric oxidants in the further transformation of a particle's composition. In addition to those reactions whose product yields are the subject of current investigation, many other compounds generated at low absolute yields that could be toxic to human lung cells are expected. Metal organic compounds, for instance, could be produced within
the films that coat suspended particles in the troposphere and the toxicity of such compounds are poorly characterized.

Here we report a laboratory apparatus and associated methodology (figure 5.1) that will be applied to the study of parallel organic, inorganic, and metal-organic reactions on suspended particles. We utilize particle levitation technology (1, 58, 61, 297, 314) with methodology developed by our group to extract a synthesized particle from a controlled atmosphere in a levitation chamber and cause it to impact onto a stainless steel plate (25). That last step is key to our methodology because the deposition of individual particles onto separate locations on a deposition plate allows a number of instrumental techniques to be utilized in the identification of the compounds on each particle, the distribution of these compounds within each particle, and their relative abundance. Here we demonstrate the potential of this apparatus by introducing high concentrations of nitric acid and aldehyde vapours into a chamber in which NaCl containing droplets and NaCl particles were levitated, and characterize using LDI-TOF-MS the outcomes of the parallel condensation and reaction of these vapours on the particle.
Figure 5.1 A pictorial summary of the methodology for off-line LDI-MS characterization of reactions that occurred on a levitated particle.

(A) The levitation apparatus. (B) (i) Droplet generation and injection into the EDB, (ii) heterogeneous/multiphase reactions on the resultant levitated particle, (iii) extraction of the particle from the EDB and impact onto the deposition plate to enable subsequent LDI-TOF-MS analysis. (C) A brief summary of the heterogeneous/multiphase particle phase reactions. The (i) starting compounds and (ii) product compounds of the reactions. Note that only a small portion of the top-most region of the particle is depicted in panels (i) and (ii).
5.4 Experimental

5.4.1 Chemicals

Reagent grade methanol, glycerol, sodium chloride, and nitric acid were purchased from BDH Inc. (Toronto, Ont., Can.). The sodium chloride used contained 0.01% bromide, 0.003% nitrate, 0.005% potassium. Benzaldehyde and 1,8-diaminonaphthalene were purchased from Aldrich (Oakville, Ont., Can.).

5.4.2 Droplet Dispensing and the Electrodynamic Balance

The droplet dispenser, the ring electrodes of the electrodynamic balance (EDB), and the substrate were enclosed in a Plexiglas chamber (figure 5.1A) (25). This chamber eliminated air convection in the laboratory from disturbing the trajectories of levitated objects.

The droplet dispenser used in this work was purchased from Uni-photon Systems (Brooklyn, NY, USA). Starting solutions were loaded into the internal reservoir using a micropipette. The droplet dispenser was equipped with a 40 μm diameter nozzle from which discrete $37 \pm 3$ μm radius droplets were produced as outlined here. A time variant waveform was applied to the piezoceramic element of the droplet dispenser that caused it to constrict, with the resultant pressure wave causing a volume of liquid to be ejected as a jet from the nozzle. During separation from the nozzle, the jet of liquid collapsed to form a single droplet. The nozzle of the droplet dispenser was positioned 1 mm from a plate electrode, and centred over a 5 mm diameter hole that had been cut in this electrode. A DC bias potential (0 – 250 V) was applied to this electrode. The electric field created by applying the DC potential on the plate induced charge separation in the jet, which resulted in each droplet having net charge. Hereafter, this electrode will be referred to as the induction electrode.

The EDB consisted of two 2 cm diameter ring electrodes that were fabricated from 1 mm diameter wire and mounted parallel at a separation distance of 8 mm. A 60 Hz sine wave ranging in amplitude from 500 – 3000 $V_{o.p}$ was applied to the electrodes in phase. The two end cap
electrodes consisted of the induction electrode and another flat plate as the top electrode. The top electrode was a stainless steel matrix assisted laser desorption/ionization (MALDI) sample platform, and it will be referred to hereafter as the deposition plate. During the droplet generation process, a sheath placed between the ring electrodes of the EDB and the deposition plate prevented unlevitated droplets from contaminating the deposition plate. The sheath was a piece of weighing paper. A DC potential was also applied to the stainless steel deposition plate. The operating characteristics of the EDB and the details of extracting a single levitated particle from the EDB and depositing it onto a deposition plate were reported elsewhere (25).

5.4.3 Levitated Particles of User-Defined Composition

The volume of the initial droplet formed from this dispenser can be controlled through variation of the DC amplitude applied to the piezoelectric strips of the droplet dispenser, use of a different sized nozzle, or changing the composition of the starting solution. We have found it most practical to vary the concentration of dissolved solids or the amount of non-volatile solvent in the starting solution to vary the mass of non-volatile material in the levitated particle. The majority of the volatile solvent in the starting solution evaporated within a few seconds following the droplet formation event (266), which left behind a residual droplet or particle.

Two starting solutions were used. For the studies involving the dispensing of droplets containing sodium chloride to create sodium chloride particles, the starting solution consisted of 3.0 M NaCl in distilled deionized water. The studies that involved the simultaneous organic and inorganic reaction monitoring were performed using a single starting solution that contained 0.25 M NaCl and 0.01 M 1,8-diaminonaphthalene in 80:20 water:methanol. The droplets/particles created from the latter starting solution each contained 1.6 pmol of 1,8-diaminonaphthalene, and the diameter of the resultant particles were typically 20 μm. A population of 20–30 particles/droplets was levitated in any one trial of an experiment.
5.4.4 Reactions on the Levitated Particles

Throughout, the relative humidity was monitored using a Traceable Digital Hygrometer/Thermometer (Control Company, Friendswood, TX). The environment in the levitation chamber was not controlled differentially from that in the laboratory. The relative humidity within the laboratory, and therefore also within the levitation chamber ranged from 15% to 35% dependent on the day in which the experiment was performed. Experiments were performed at relative humidity < 35% as to ensure particle formation (49). Given that imine formation occurs through a dehydration reaction, the presence of water on the particle may play a role in the reaction. However, Haddrell and Agnes have shown that imine formation can occur under conditions of elevated relative humidity (> 60%) through a multiphase reaction (118). As such, the presence of water on the particle is believed to have a negligible effect on the rate of imine formation.

In all experiments the particles were levitated for 10 minutes prior to introducing reactive vapours. Nitric acid vapour was introduced to the chamber in which particles were levitated by simply placing into the chamber a 10 mL beaker containing 1 mL of concentrated nitric acid, resulting in a final concentration of nitric acid vapour of ~79,000 ppm. Haddrell and Agnes have shown previously that imine formation occurs under conditions of both low and high aldehyde concentrations (118). In experiments in which organic and inorganic reactions were allowed to proceed, two 10 mL beakers were placed inside the levitation chamber, one beaker contained 1 mL of concentrated nitric acid and the other 1 mL of benzaldehyde (gas phase concentration of benzaldehyde in the levitation chamber was ~170 ppm). The beakers were removed from the levitation chamber prior to depositing the droplets/particles onto the deposition plate. Each particle was deposited onto a separate location on the deposition plate, approximately 125 μm apart (118).
5.4.5 Offline Particle Laser Desorption Ionization Time-of-Flight Mass Spectrometry

All mass spectra were collected after removing the deposition plate from the levitation chamber and inserting it into a Perseptive Biosystems Voyager-DE matrix assisted laser desorption ionization TOF-MS (Framingham, MA, USA). The delay time between the particle deposition event and LDI-MS analysis ranged from 3 to 5 minutes. The output of the N₂ laser (337 nm) was attenuated using a variable density filter in the range from 331 to 763 µJ/pulse. The detection of the starting material, 1,8-diaminonaphthalene and its reaction products absorbed the 337 nm output of the N₂ laser, and hence their detection as molecular radical cations required relatively low laser pulse irradiance. Conversely, the detection of ionized salt clusters, by desorption ionization of the NaCl particles, required pulse irradiance energies > 550 µJ/pulse.

Haddrell and Agnes have shown that under similar conditions, with respect to time, humidity and temperature, the reactions between NaCl or 1,8-diaminonaphthalene with nitric acid or benzaldehyde, adsorbed onto the deposition plate during the period of droplet/particle levitation, were negligible relative to the quantity of product compounds that had formed on the levitated particle (118). To verify this in this work, unlevitated droplets were dispensed directly onto a remote location on the deposition plate immediately after the particle deposition. Upon mass spectral analysis, it was shown that imine production was not detectable on the deposition plate during the delay time (3 – 5 min).

5.5 Results

5.5.1 An Inorganic Multiphase/Heterogeneous Reaction

The results described in this section characterize the production of an inorganic compound on suspended particles, which later in this study will be built upon by introducing organic compounds to the system. NOₓ emissions from high temperature combustion sources undergo oxidation to form HNO₃, which undergoes further reactions on NaCl particles (57, 62, 86, 87, 93, 165, 307).
The anion exchange reaction that results in NaNO₃ production is indicated as Eq. 5.1 (100, 303).

\[
\text{HNO}_3(\text{g}) + \text{NaCl}_{\text{particle}} \rightarrow \text{HCl}(\text{g}) + \text{NaNO}_3(\text{particle})
\]

We proceeded to generate isolated NaCl particles that were then exposed to high concentrations of HNO₃(g) for 1 h. In figure 5.2, four sequential LDI-TOF-MS spectra from one such particle are plotted. In acquiring that data, the irradiation energy of the laser and the position of the laser focus were not adjusted. Figure 5.2A shows the ions detected from the first four laser shots fired at the particle. The expected ions of Na⁺, [Na⁺(NaCl⁺)], and [Na⁺(NaCl⁺)] were detected, as was an ion signal indicative of a heterogeneous reaction product, [Na⁺(NaNO₃)]. The surface specificity of the reaction (40) and the analysis by LDI-TOF-MS, can be appreciated by comparison of the ion signal intensities in figure 5.2A to those in figure 5.2B–D. As the number of laser shots increased, the original surface material on the particle was removed, as indicated by the decrease in signal intensity of the cluster [Na⁺(NaNO₃)]. Coinciding with this observation was the appearance of three new cluster ion peaks in figure 5.2B at m/z = 139, 141 and 143, corresponding to the isotopic distribution expected for chlorine in [Na⁺(NaCl)]₂. The signal intensity of these ions increased with subsequent laser shots, suggesting that the ions observed in figure 5.2D originated from material representative of the NaCl particle core.
The LDI-TOF mass spectra collected from a single NaCl particle that had been exposed to HNO$_3$ for 1 h. The ion signal intensities in each mass spectrum were normalized to the [Na$^+$ (Na$^{35}$Cl)] peak. Each mass spectrum is the average of four consecutive laser pulses (A) 1–4, (B) 5–8, (C) 9–12, (D) 13–16. The laser irradiation energy was set to 664 $\mu$J/pulse.

The ion peaks labelled as Na$^+$, K$^+$, $^{52}$Cr$^+$, $^{56}$Fe$^+$, and $^{58}$Ni$^+$ ions were background ions. These ions were also observed from the clean stainless steel plate using laser intensities > 495 $\mu$J/pulse on the Perceptive Biosystems Voyager-DE. This grouping of background ions from a stainless steel plate has also been reported by Cody et al. who also irradiated a stainless steel plate with the output of a nitrogen laser (50). The ions at m/z = 87 and 89 are assigned as [Cr$^+$ $^{35}$Cl] and [Cr$^+$ $^{37}$Cl]. The broad spectral width of these ion peaks suggests that these ions were formed by a different desorption/ionization mechanism than other ions in the spectrum.

Currently, the presence of potassium and sodium ions in the background hinders our ability to monitor their distribution within the particle. The ability to monitor this would be of
interest as multicomponent aerosol crystallization creates particles that do not have homogeneous chemical morphology, and they often exhibit a surface layer enriched by minor components (101-103). Zhaozhu et al. have shown that the K+/Na+ peak area ratio observed in droplets created with identical NaCl and KCl mole fractions increased as the relative humidity increased, indicating that the surface properties of the aerosol particles could be quite different from the composition of the droplet's core (101, 102). The effect of the Na+/K+ signal intensity is but one aspect of the problem of matrix effects in quantitative analysis, and thus is proving a tremendous challenge in single particle analysis (17, 54, 101-103, 113, 156, 224, 255, 313, 315). The capability to obtain multiple samplings from a single particle may allow a profile of a particle's cross sectional composition (figure 5.1C), and possibly a better understanding of the matrix effects.

5.5.2 Simultaneous Inorganic and Organic Reactions

Having characterized the production of NaNO₃, the complexity of the system was incremented by the introduction of organic compounds. A population of particles created using the starting solution containing 0.01 M 1,8-diaminonaphthalene and 0.25 M NaCl in distilled deionized water were dispensed, and levitated. Benzaldehyde and nitric acid were then introduced, in separate reservoirs, to the levitation chamber. The 1,8-diaminonaphthalene reacts with aldehydes by way of a dehydration reaction to form an imine (118). After a 40 minute levitation period, the nitric acid and benzaldehyde were removed from the chamber and the levitated particles were then deposited onto the deposition plate.

One of these particles was characterized by LDI-TOF-MS using the following procedure. First, the laser output was centred over the deposited salt particle. The laser irradiation energy was set to 467 μJ/pulse and the ions detected from firing the laser 8 times were summed and then the average ion signal plotted as the mass spectrum identified as figure 5.3A. The laser was then fired at the salt particle at that laser irradiation energy until no further ion signals were detected (typically 100 – 150 laser shots), at which point, the laser irradiation energy was incremented by
27 μJ/pulse, and another mass spectrum from the first 8 laser shots was acquired. This cycle was repeated until the laser irradiation energy reached 556 μJ/pulse, at which point, ionized clusters of the inorganic compounds in the particle were detected, including a peak indicative of the inorganic heterogeneous reaction product, [Na⁺(NaNO₃)]. Figures 5.3D and 5.3E contain the ion signals of both the starting materials and products of the inorganic and organic heterogeneous/multiphase reactions. In figure 5.3F there were no ion signals indicative of the reaction between 1,8-diaminonaphthalene and benzaldehyde, suggesting that the organic species had been desorbed from the particle surface by the preceding laser shots.
Figure 5.3 LDI-TOF mass spectra collected from a single NaCl particle dosed with 1.6 pmol of 1,8-diaminonaphthalene that had been exposed to both nitric acid vapour and benzaldehyde vapour for 40 minutes. The laser irradiation energies used were (A) 467 (B) 493 (C) 537 (D) 556 (E) 585 (F) 614 μJ/pulse. Each mass spectrum is the average of first eight consecutive laser shots at each laser irradiation energy setting.
5.5.3 **Effect of the Sequence of Reactant Addition on Imine Formation**

Having established that the simultaneous introduction of nitric acid and benzaldehyde vapor does not prevent the imine reaction from proceeding, the order in which the two reactants were added to the chamber on the outcome of imine formation was investigated.

A blank, in which a population of particles was levitated for 30 min without the addition of beakers containing nitric acid or benzaldehyde was performed. A mass spectrum from the LDI-TOF-MS characterization of one of the particles at a laser irradiation energy of 440 μJ/pulse allowed detection of the starting material, 1,8-diaminonaphthalene as its radical cation, and an imine resultant from its reaction with ambient formaldehyde that was present in the air in the laboratory (figure 5.4A).
Figure 5.4  LDI-TOF collected from four different salt particles, each dosed with 1.6 pmol of 1,8-diaminonaphthalene, that were levitated in a chamber under four different conditions. (A) Pure starting material that had been passed through the droplet dispenser and levitated in a laboratory environment for 30 minutes. (B) 1 mL of pure benzaldehyde and nitric acid were introduced into the chamber simultaneously. (C) 1 mL of pure benzaldehyde was introduced into the chamber for a period of 45 minutes, then the benzaldehyde was removed and 1 mL of nitric acid was placed into the chamber for 55 minutes. (D) 1 mL of nitric acid was placed into the chamber for 2 h, after which time, 1 mL of benzaldehyde was introduced into the chamber for 1 h (note that the nitric acid remained in the chamber for 3 h). The laser irradiation energies used for the four trials were as follows: (A) 440 μJ/pulse, (B) 413 μJ/pulse, (C) 440 μJ/pulse and (D) 614 μJ/pulse. Each spectrum is an average of eight consecutive laser pulses.

In the first iteration, both nitric acid and benzaldehyde were simultaneously placed into separate reservoirs within the levitation chamber containing a population of levitated particles. Following a 30 minute levitation/reaction exposure period, the particles were deposited and analyzed via LDI-TOF-MS. As shown in the mass spectrum identified as figure 5.4B, an ion signal indicative of the imine product at m/z = 246 was detected. Equilibrium vapour pressures for benzaldehyde and nitric acid were 1.7 x 10^{-4}, and 7.9 x 10^{-2} atm, respectively. As such, the
nitric acid concentration in the gas phase was higher than that of benzaldehyde over the course of the reaction, yet there appeared little inhibition of the imine formation in this experiment.

In the second iteration, a population of droplets dosed with 1,8-diaminonaphthalene was injected into the EDB and levitated prior to introducing 1 mL of benzaldehyde into the levitation chamber. After 45 minutes, the beaker containing the benzaldehyde was removed and 1 mL of nitric acid in a different beaker was introduced to the chamber. After an additional period of 55 minutes, the particles were deposited and analyzed via LDI-TOF-MS (figure 5.4C). From a relatively low laser pulse energy of 440 μJ/pulse, no ion signal for the starting material was detected, yet a large ion signal of the product (m/z = 246) was observed. This indicates that the nitric acid did not break down the imine once it had formed suggesting that the imine reaction was not reversible at these conditions. As such, the imines formed by such reactions could have lifetimes longer than an initial estimate of ~ 6 h (118).

In the final run, a population of salt particles plus 1,8-diaminonaphthalene was levitated, with 1 mL of pure nitric acid in a reservoir placed within the chamber. After 2 h, the nitric acid was removed and 1 mL benzaldehyde in a separate reservoir was placed within the chamber. After 1 h, the particles were deposited and analyzed via LDI-TOF-MS (figure 5.4D). The ion signal of the product imine at m/z = 246 was detected, but note that a very high laser irradiation energy (614 μJ/pulse) was necessary in order to observe this ion (no ion signal indicative of the imine product was observed at a laser irradiation energy of 440 μJ/pulse). This indicates that the amount of the imine formed during the course of this levitation period was greatly reduced. To understand this result, the reaction mechanism for the formation of an imine must be considered (figure 5.5). The highest rate of imine formation occurs in a pH range between 4 and 5 (150, 188). The rate-determining step of the reaction is the initial attack of the lone pair electrons of the amine on the carbonyl carbon of the aldehyde, followed by stabilization of that intermediate by the introduction of an electron acceptor. However, the exposure of the amine to nitric acid was found to significantly decrease the rate of imine formation. The reduction in imine formation can
be attributed to the fact that the nitric acid vapour was given sufficient time to interact with the levitated particles containing 1,8-diaminonaphthalene prior to the introduction of the benzaldehyde vapour. The results presented also indicate that the protonation of the reactant 1,8-diaminonaphthalene by the uptake of HNO₃ under the conditions studied was irreversible. As indicated by the reaction scheme in figure 5.5B, the nitric acid protonates the primary amine, effectively preventing the lone pair on the N atom of the amine group from attacking the carbonyl carbon. Although 1,8-diaminonaphthalene has two amine groups, protonation of both groups may not be required to significantly decrease the rate of imine formation as the two amine groups are conjugated, and as such a positive charge on one of the amines will alter the basicity of the other.

![Reaction scheme](image)

**Figure 5.5** The reaction mechanism of imine formation, dependent on whether acid was added to the system (A) after or (B) before the introduction of the aldehyde.

### 5.6 Discussion

The laser irradiation energy that was used to acquire each mass spectrum needs consideration when interpreting the LDI-TOF-MS data. For instance, both spectra identified as
figures 5.3A and 5.4B were obtained from similarly produced particles. Differences in the
spectra, such as the detection of the starting compound, 1,8-diaminonaphthalene (molecular
radical cation at m/z = 158) was due to differences in the laser irradiation energies used during
sample characterization. Another example of this is evident in the decreased imine abundance,
relative to the ion signals of the starting reactants, detected from a particle that had been first
exposed to nitric acid (compare ion signal intensity at m/z = 246 in figure 5.4A versus figure
5.4D). The relatively low ion signal intensity of the imine's radical cation (figure 5.4D), coupled
with the high laser irradiation energy required to generate those ion signals, indicates that its
production was relatively slow during the levitation period.

A feature of the methodology used to acquire the mass spectra reported herein is that each
particle levitated in the EDB can be viewed as an individual reaction vessel. Here, LDI mass
spectral analysis was carried out on each individual particle, but other instrumental methods, such
as force microscopy (179) and optical microscopy could each be invoked to provide
complementary, or new data. We anticipate that this strategy can be used as a platform with
which standard analytical methodologies can be implemented for improved quantitative
compound characterization of individual particles. The use of femtosecond laser pulses (268)
could allow for increased specificity for the characterization of a particle's surface composition.
Such a platform can also be used to develop models to account for chemical composition
evolution of individual particles in a tropospheric airmass. The potential for this was
demonstrated through the differential production of the imine by variation of the order of
introduction of nitric acid versus aldehyde vapour to the levitation chamber. This could be the
most significant finding in this study and it best exemplifies why methodology capable of
monitoring simultaneously occurring organic and inorganic particle-phase reactions is necessary.
Thus, not only do the relative rates of a reaction need to be considered, but the particle's history
and local environment also needs to be considered.
5.7 Conclusions

Currently, the simultaneous monitoring of organic and inorganic homogeneous/heterogeneous/multiphase reactions described here is qualitative, though standard quantitation strategies can be incorporated into the method to enable semi-quantitative results (118). Nevertheless, the methodology described herein is a step toward understanding, and possibly monitoring of the rates of inorganic and organic reactions occurring simultaneously on suspended particles in the troposphere.

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Chapter 6

PARTICULATE AIR POLLUTION AND HUMAN HEALTH

From Inhalation to Expiration

Air is a physical substance; it embraces us so intimately that it is hard to say where we leave off and air begins. Inside as well as outside we are minutely designed for the central activity of our existence – drawing the atmosphere into the centre of our being, deep into the moist, delicate membranous labyrinth within our chests, and putting it to use.

David Suzuki, The Sacred Balance (287)

In humans, the inhalation of air is necessary for life to be sustained. It is our constant requirement of oxygen that demands a continuous cycling of air from the atmosphere into the lungs and from there to every cell in our body. Unfortunately, along with oxygen, numerous other toxic species are also inhaled, most notably particulate matter. Though there are filtration systems in place, such as nasal hair, to remove as much of these species as possible, it is inevitable that some will reach the surface of the lung. The question thus becomes how the inhalation of micrometer sized particles into the lung result in acute respiratory and cardiovascular events, such as myocardial infarction and death.

The role of this chapter is to overview the complex interactions occurring between the human lungs and inhaled particulate matter, as well as to overview current opinions regarding how those interactions are involved with the pathogenesis of numerous diseases.
6.1 The Respiratory System and Particulate Matter

The respiratory system is a 40 to 120 m⁻² conducting system employed by the body for the exchange of gases between the atmosphere and the bloodstream. While oxygen is transported from the atmosphere into the bloodstream, carbon dioxide is transported from the bloodstream back into the atmosphere. The volume of air inhaled by an average adult per day ranges, based on activity, from 10,000 to 20,000 L, making lungs the most challenged organ in the body (251).

The respiratory system is comprised of an upper respiratory tract and a lower respiratory tract. The primary function of the upper respiratory tract, which consists of the nasal cavity, paranasal sinuses and the nasopharynx, is to filter, humidify and adjust the temperature of inspired air. The primary function of the lower respiratory tract, which consists of everything below the larynx, is the transfer of gaseous species into and out of the blood stream. Since the upper respiratory system is well evolved to remove particles using cilia and mucous, the remaining discussion will focus on the lower respiratory tract.

Beginning at the larynx, the lower respiratory tract continues into the thorax as a complex series of branching tubes, each having a smaller diameter than the previous one with further penetration into the lung. At this point, the larynx becomes the trachea, which then divides into left and right primary bronchi, which themselves further divide into secondary and then into tertiary bronchi, each division resulting in progressively smaller airways. The tertiary bronchi gives rise to airways orders of magnitude smaller, the smallest of which are known as the terminal bronchioles. The terminal bronchioles further divide into a series of transitional airways involved in gaseous exchange called respiratory bronchioles and alveolar ducts; which finally terminate into the alveolar sacs. At each branching point, there is the potential for particles suspended in the inhaled air to settle onto tissue either by straight collision with the airway wall or through the formation of pockets of relatively stagnant air, much like the eddy in a river, where the velocity of the air is low enough for the particles to settle out. Recall from chapter 3 that the velocity in which a particle will settle out of the air is a function of the aerodynamic diameter of
the particle (equation 3.2); therefore, as the air currents slow down, larger particles settle out first, while the smallest particles settle out of the most stagnant air. For this reason, smaller particles travel deeper into the alveolar sac before their deposition onto lung tissue. The mechanism by which the structure of these branches affects the deposition of particles onto various locations in the lung is an area of active research.

Comprehensive computer models of an ideal mouth and lung have been used extensively to try to determine the locations within the lung in which particles preferentially settle. In constructing these models, factors considered include breathing rate, size and shape of airways, as well as the size of the particle being inhaled. Generally speaking, it has been shown that as the breathing rate and particle size decreases, particles deposit deeper in the lung. Studies using radioactive particles have demonstrated that while particles with an aerodynamic diameter greater than 10 μm are either filtered from the air in the upper airway or land in a site further up in the airway where they are removed by ciliary activity; particles of a size fraction less than 2.5 μm settle deeper within the lung, even as deep as the alveoli surface. Numerous epidemiological studies have shown that particles with an aerodynamic diameter less than 2.5 μm show a greater association with adverse health effects in humans than PM$_{10}$. Particles with an aerodynamic diameter less than a micrometer have even been hypothesized to penetrate through the respiratory interstitium and into the blood, where they may potentially cause even more adverse health effects. However, it is unclear whether it is simply the site of particle deposition that causes the observed increase in overall toxicity, or whether it is the chemical composition of these particles that makes them so toxic. This is not simple to elucidate for, as mentioned in chapter 3, the size and chemical composition of a particle is a function of its source and thus its not trivial to differentiate between the two properties using primary particle extracts in laboratory studies. Addressing this issue became the focus of the remainder of my thesis; by injuring cells with designed particles of known chemical composition and size created using electrodynamic
levitation technology, an association between particle composition with an overall injury to the lung cells was observed.

Cells exhibit injury in numerous means, ranging from oxidative stress or the expression of proinflammatory mediators to apoptosis. It has been demonstrated that exposure to particulate matter results in both local and systemic inflammation. Even though, inflammation is essential line of defense, when chronic it can lead to numerous diseases due to the constant injury inflicted to the tissue which leads to constant remodelling that may result in structural changes due to disordered coagulation and extravascular fibrin deposition. It is currently believed that a sustained systemic inflammatory response due to particulate matter exposure results in a myriad of adverse health effects to both the pulmonary system, such as chronic and deficient lung tissue remodelling resulting in impaired oxygen exchange due to increased vascularization, subepithelial fibrosis, loss of epithelial organization and smooth muscle hyperplasia, which can lead to asthma, and to the cardiovascular system, causing both atherosclerotic plaque proliferation and destabilization. It was deemed necessary to monitor the expression of multiple proinflammatory mediators in order to best assess the extent of cellular injury.

6.2 Inflammation

The body is constantly being challenged by infectious pathogens such as virus, fungi and parasites. To protect itself, the body has evolved three main lines of defence: protective surface mechanisms (anatomical barriers such as skin), non-specific tissue defence (also known as inflammation) and specific immune defence. Broadly, specific immunity, also known as acquired immunity, is divided into cellular and humoral (antibody base) immunity. The former is mediated by Th1 cytokines and it targets intracellular pathogens such as viruses and intracellular bacteria, while the second is mediated by Th2 cytokines and targets extracellular pathogens such as bacteria and fungi. Interestingly, while in most individuals there is an equilibrium between Th1
and Th2 immunity, in asthmatics this equilibrium is shifted towards Th2 immunity, which may result in a chronic ‘allergic’ state.

Tissue damage initiates inflammation, which is a complex non-specific biological process wherein the end result is the removal of dead tissue and foreign matter, followed by regeneration of normal tissue (7). The general process of inflammation is summarized as follows (figure 6.1). With injury, affected cells express and secrete cytokines, which include intercellular signalling proteins that alert neighbouring cells and, if the damage is severe enough, neighbouring tissue and organs. These cytokines serve three primary functions. First, a specific subset of cytokines termed chemokines is secreted from intracellular vesicles to direct neutrophils and monocytes to the area of injury. Second, if the damage is large enough, or sustained long enough, some of these cytokines will trigger the release of either other granulocytes (non-specific immunity) or lymphocytes (specific immunity) from the bone marrow in a systemic inflammatory response. Third, other cytokines will activate leukocytes upon arrival to the site of injury; the most notable example of this is the transformation of monocytes into macrophages.
van Eeden et al. have shown that systemic inflammation can be triggered by the inhalation of particulate air pollution (213). Upon inhalation, particles settle onto epithelial cells in the lung and cause injury to it through mechanisms that remain the subject of much research. A prominent hypothesis is that the particles induce oxidative stress onto the exposed cells, resulting in tissue damage. Following injury to the lung tissue, proinflammatory pathways are activated, resulting in the expression and secretion of numerous proinflammatory mediators; cytokines and chemokines, including TNF-α, GM-CSF, IL-1β, IL-6, and IL-8; TNF-α and IL-1β themselves cause further activation of the above cytokines, thus propagating the inflammatory response. The presence of these mediators in the blood stream stimulates the bone marrow to reduce the migration time of leukocytes from the bone marrow, resulting in an elevation of the number of leukocytes, both mature and immature, in the blood stream. Neutrophils, a type of leukocyte, migrate from the bone marrow to the site of injury through chemotaxis, following the concentration gradient of chemokines secreted by the injured cells.
Inflammation can be either acute or chronic. In terms of particulate air pollution, acute exposure can lead to moderate responses such as the exacerbation of asthma while constant exposure can result in chronic inflammation within the lung. The near constant state of inflammation, as well as the physical particle itself, leads to the destruction of lung tissue. Wound repair under inflammatory conditions, or simply ineffective wound repair, leads to remodelling of the airways within the lung. Over time, significant airway remodelling will lead to an overall reduction in lung function, and hence the progression of numerous lung related illnesses including emphysema and chronic obstructive pulmonary disorder (COPD).

The cardiovascular system, the series of arteries and blood vessels involved in the transport of blood throughout the body, is intrinsically related to the pulmonary system; thus the systemic inflammatory response initiated by particulate air pollution exposure does not only have a profound effect on the lungs, but on the arteries as well. One of the most dramatic effects observed following such exposure is the growth and destabilization of atherosclerotic plaques, which are fat deposits ensembled on the inner lining of arterial walls. The growth and rupturing of these plaques leads to thrombus formation, commonly referred to as a blood clot, and the subsequent restriction of blood flow, which in turn can lead to organ damage in the form of heart attack or stroke. The location and degree of this restriction will lead to an acute cardiovascular event, ranging from chest pain to stroke or heart attack. It is believed that the association between particulate air pollution exposure and atherosclerotic plaque formation is mediated by the proinflammatory response (99).

In fact, the adverse health effects initiated by particulate matter exposure are a by-product of the chronic systemic inflammation that it causes. It is believed that exposure to different chemicals might induce different cellular responses; thus it is necessary to characterize how the chemical composition of particulate matter affects the proinflammatory response of lung tissue following exposure (125, 254).
6.2.1 The Nuclear Factor (NF)-κB Pathway

Inflammation is the product of the expression of numerous proinflammatory proteins. Cytokines, a class of proinflammatory mediators, are expressed following the activation of biological signal transduction cascades. The NF-κB pathway, which has been shown to be activated following exposure to particulate air pollution (265), is a major proinflammatory pathway found in numerous cell types that, when activated, results in the transcription of mRNA of numerous proinflammatory mediators, including TNF-α, IL-1β, IL-6 and intercellular adhesion molecule (ICAM)-1 (figure 6.2) (14, 116, 198, 318).

NF-κB amplifies and extends the length of the immune response by promoting the transcription of certain pro-inflammatory mediators, such as TNF-α and IL-1β, which are involved in the positive feedback activation of the pathway (225, 299, 304).

NF-κB is also involved in cell proliferation. This role, in addition to it’s association with inflammation, has lead to linking defects in several of the NF-κB pathway components to numerous diseases, including cancer (160), septic shock, inflammatory and autoimmune diseases, viral infection and abnormal immune development (80, 81, 107, 220).
There are three triggers to the NF-κB pathway, oxidative stress (116, 117, 177), toll-like receptor (TLR) activation or through TNF-α via the TNF-α receptor (figure 6.2). Given the relatively short lifetimes of reactive oxidative species within a biological system, the exact mechanism by which oxidative stress activates NF-κB translocation remains unclear (116).
However, the pathways by which the activation of the TNF-α receptor or the TLR results in NF-κB translocation have been well studied (13, 14).

NF-κB is a protein complex that consists of either homo or heterodimers resulting from the combination of any of the five different NF-κB proteins (21, 191). Unactivated NF-κB proteins are noncovalently bound to inhibitor proteins termed inhibitor-κB (I-κB) (105); seven of which have been identified. Following cell stimulation, activation of an I-κB kinase (IKK) results in the phosphorylation of two serine residues of the I-κB protein, which targets its ubiquitination and degradation (191, 299) (291). The NF-κB dimer, thus freed, translocates then to the nucleus (figure 6.2) (191) where it facilitates the binding of RNA polymerase to DNA by binding to a specific decameric DNA sequence (GGG ACT TTC C) (116) and, as such, facilitating transcription (264).

The NF-κB pathway can be monitored indirectly by following the expression of the pro-inflammatory mediators transcribed following its activation. An understanding as to which environmental stimulants cause the activation of this pathway may shed some insight into their toxicity. Thus, the focus of this thesis became the study of the relationships between the chemical composition of a particle and its effect on this major proinflammatory pathway.

### 6.2.2 Intercellular Adhesion Molecule (ICAM)-1

A critical step of the inflammatory process is the extraction of leukocytes, also known as white blood cells, from the bloodstream and into the tissue (78). Neutrophils, a type of leukocyte, are removed from the bloodstream by first adhering to the endothelium. This process is mediated by membrane bound proteins such as intercellular adhesion molecules and selectins (38, 178, 273, 277). Through the use of animal models, it has been shown that the expression of ICAM-1 in the lung is critical for inflammation (124, 218, 272, 273, 306). In fact, administration of anti-ICAM-1 antibodies decreases both neutrophil adhesion and injury following animal LPS
exposure, suggesting that antibodies directed towards ICAM-1 may serve as an appropriate therapeutic for patients suffering from excess lung inflammation (124, 173).

A brief summary of the leukocyte extraction process is as follows. First, the activated epithelium will express both E and P selectins that bind to sulfated-sialyl-Lewis moieties of certain leukocyte glycoproteins. At the same time, the ICAM-1 from the endothelium will bind β2-integrins on the leukocyte and, as chemokines, cause conformational changes in the latter so that the leukocyte will stop rolling akin to a tennis ball rolling down a Velcro mat. Once bound, the leukocyte will then begin to migrate into the tissue, between the endothelial cells, following a gradient of chemokines. While some leukocytes will mature only upon migration into the tissues (monocytes in the blood become macrophages in the tissues), others (neutrophils) undergo no such transformation.
ICAM-1 is a cell surface glycoprotein expressed in numerous cell types. In the lung, it is expressed by epithelial cells (208). Throughout the rest of the body, it is constantly expressed in the membranes of leukocytes and endothelial cells in low numbers, but upon stimulation by cytokines, such as interleukin-1β and tumour necrosis factor (TNF)-α, its expression is upregulated.

Since ICAM-1 is critical for the intercellular adhesion necessary for inflammation to take place (figure 6.3), and the amount of its extracellular expression is a direct result of the regulation of the NF-κB pathway, it is an ideal target for immunological studies.
6.3 Common Biological Assays and Supplies Used

Throughout the remainder of this thesis, a series of common biological assays and equipment are employed. Over the course of the rest of this chapter, a brief overview of these samples and techniques will be presented.

6.3.1 The A549 Cell Line

Primary cells are those taken directly from a biopsy, and then grown in culture. These cells are the most useful, sought after and precious cells available given that they are considered the best representation of cells within a living tissue. The problem is that primary cells do not divide indefinitely. Over time they will either become replicative senescent, quit dividing, or mutate to the point where they are no longer phenotypically representative of the tissue from which they have been extracted. To address these issues, primary cell cultures have been immortalized into cell lines.

Cells can be immortalized in a number of ways. The two more common methods are through inactivation of tumor suppressor genes, which confer replicative senescence, and through the maintainance of telomere lengths, which normally shrink with each cell division. The former method is achieved through viral transformation, the process by which viral genes from viruses such as Simian virus 40 (SV40) or Epstein-Barr virus (EBV) are transfected into the host cell; the latter method is achieved through expression of the telomerase reverse transcriptase protein (TERT), which reforms shortened telomers.

The A549 cell line is a human transformed cell line whose genotype and phenotype resemble that of type II alveolar epithelial cells. 96% of the surface area of the pulmonary epithelium is covered by Type I cells while the remaining is covered by Type II. Though Type II cells cover far less surface area, they have more functionality than Type I, including the ability to divide and produce surfactants (92).
The A549 cell line was initiated by D.J. Giard in 1972 from an explant of a lung carcinomatous tissue from a 58 year old male (184). Over the past 30 years, numerous studies have been undertaken using this cell line, and it has become somewhat of a cell standard for the alveolar type II epithelial cell type (244). Thus a major benefit of using this cell line is that it is well characterized within the literature and is relatively easy to culture.

The inherent problem with using A549 cells is that they are a cell line. Thus any results obtained from using it, such as “'X' particle type causes ‘Y’ response from lung cells”, may in reality be simply “'X' particle type causes ‘Y’ response from A549 cells”. Thus, for far reaching, high impact results, studies employing multiple cell lines, or a primary cell culture, should be used.

6.3.2 Immunocytochemistry

The location of a protein on or within a cell can tell much about its function and role within that cell. The location of a protein can be found by labelling it with an antibody, which itself is labelled with a dye or fluorescent tag. This labelling process is termed immunocytochemistry where “immuno” refers to the antibodies and thus the immune system, “cyto” to the cells grown in culture and “chemistry” to the chemicals used to label the molecules of interest.

Immunocytochemistry is a multistep process, as depicted in figure 6.4. Once the cell culture has been perturbed, and undergone the appropriate incubation period, the cell culture is fixed with a 10% acetone solution, which kills the cells and fixes the relative locations of all the proteins within the cell. After fixation, a blocking agent is added to the cell culture. This agent, typically serum, ensures that no non-specific binding between the antibody and molecules other than its antigen occurs. The primary antibody, which is specific to the protein of interest, is added next.
After incubation, the secondary antibody, which is specific for the Fc fragment of the primary antibody, is added. In order to ensure that no cross-reactivity will take place, the secondary antibody, which typically is an immunoglobulin G (IgG) protein, is raised in a different animal than the primary antibody. The primary antibody used in my studies was produced in mice (mouse anti-human ab), while the secondary antibody was raised in a goat (goat anti-mouse ab), hence it was termed a goat anti-mouse IgG. In most cases, the secondary antibody itself is labelled (either by fluoresce or by conjugating it to an enzyme); however, in instances when signal amplification is desired, a labelled tertiary antibody may be used.

Each time an antibody is added in the labelling process, the likelihood of observing the protein of interest is increased. This is simply a product of the tree-like structure of antibodies formed during the labelling process. Multiple antibodies bind to the previously bound antibody, which itself is bound to the protein of interest. In this way, the ability to detect a single protein molecule is increased as that single molecule becomes bound to numerous labelled molecules. Thus, the amount of labels observed is directly proportional to the number of proteins expressed in the sample, making immunocytochemistry a very sensitive quantitative technique for membrane bound proteins.
Tag

*Tertiary Antibody*

*Secondary Antibody*

*Primary Antibody*

*Blocking Agent*

*Antigen*

**Figure 6.4** Graphical representation in which antibodies bind to the protein of interest in an immunocytochemistry assay. The blocking agent ensures that only specific binding occurs. The primary antibody binds the antigen. Secondary antibodies then bind the primary antibody. Lastly, tertiary antibodies, which are labelled with a fluorescent of chromatic dye, bind to the secondary antibodies.

### 6.3.3 Fluorescence Microscopy

Fluorescence is the multistep phenomenon in which a molecule absorbs, and thus is excited by, the energy of a photon and then, in non-resonance fluorescence, loses some of the absorbed energy through vibration or rotation, to finally release the remaining energy through the emission of a photon of less energy.

Fluorescence microscopy is simply a tool to view fluorescent species at high magnification. The microscope used in the Agnes laboratory is fitted with a mercury lamp. Next, it must be coupled with two filters; the first to select a specific wavelength to be used to illuminate the sample and the second one to allow only the fluorescent light to reach the detector. The detector can be a digital camera, thus allowing for the image collected to be converted into a digital format, which enables the eventual quantification of the overall fluorescing signal. The physical setup of a fluorescent microscope is shown in figure 6.5.
6.3.4 Confocal Microscopy

Confocal microscopy shares many similarities with fluorescence microscopy, both in instrumental setup (compare figures 6.5 and 6.6) and in sample analysis. However, confocal microscopy has the added advantage of being able to focus only a small slice along the Z-axis of (or into) the sample at a time. This is accomplished through placing a pinhole between the light source and the sample, and another between the sample and the detector. The former pinhole ensures that only a small three dimensional portion of the sample is ever illuminated at a single time, while the latter ensures that only the emitted light from the sample at the focal point of the laser reaches the detector. Therefore, the size of that three dimensional portion is a function of the diameter of the two pinholes.
Figure 6.6 Physical setup of a confocal microscope.
The presence of the two pinhole filters limits the amount of laser light that reaches the sample, and the amount of fluorescent light that reaches the detector.

Since only the small portion of emitted light from the focal point ever reaches the detector at any given time, analysis of the entire sample requires the sample to raster through the focal point of the laser in the X, Y and Z directions, making it necessary to install a precise computer controlled translation stage into the confocal microscope. As each point passes through the focal point, the relative fluorescence of the sample in the focal point is measured. Therefore, based on the size of the sample, and the desired resolution of the image collected, more than $10^7$ individual measurements are required for any given sample, demanding high processing power. Imaging software is then used to convert the fluorescent signal intensity array data into a series of images. Processing of these images allows for one to create three dimensional images of the sample. Additionally, by employing multiple filters and fluorescent labels, the relative location, and in some instances the colocalization of, numerous proteins can be measured.

This ability to determine the relative location of a molecular species, such as a protein, within a sample, such as a cell, has made the confocal microscope a valuable tool in many cellular biology laboratories.
6.3.5 Enzyme-Linked ImmunoSorbent Assay

Many assays have been developed to quantify the concentration of secreted proteins within a sample, many of which make use of labelled antibodies. One of the most sensitive and more commonly used assays that employ labelled antibodies is the Enzyme-Linked ImmunoSorbent Assay (ELISA). Due to its high sensitivity and reliability, several modifications of this assay have been developed. A summary of the protein labelling process for ELISA is shown in figure 6.7.

![Diagram of ELISA process](image)

**Figure 6.7** Graphical representation in which antibodies bind to the protein of interest in the Enzyme-Linked ImmunoSorbent Assay (ELISA). Unlike immunocytochemistry, a capture antibody coats the sample well and binds the free floating antigen, thus concentrating it on the surface. The primary antibody then binds a different epitope of the antigen, followed by the enzyme-linked secondary antibody binding to the Fc chain of the primary. The final step is the activation of the substrate by the enzyme, causing the substrate to either change colour or fluoresce.

In this ELISA variety, an antigen-specific capture-antibody is bound to the plastic plate as to further concentrate the desired molecule on the surface. Once fixed to the surface and all unfixed antibody removed, the sample containing the analyte, which may range in composition from an aqueous solution to serum, is introduced. After an incubation period in which the capture-antibody binds the antigen, the primary antibody, raised in a different animal than the first one and which recognizes a different epitope of the antigen, is added. Finally, after removing any unbound antibody, the secondary antibody, which is specific for the Fc region (note that Fc regions are animal specific, thus the need for having the capture and the primary antibodies raised
in different animals) of the primary antibody, is added. This last antibody, which is raised in yet a
third animal, is coupled to an enzyme such as horseradish peroxidase (HRP). Once all unbound
enzyme-linked secondary antibody is removed through rinsing, a solution containing the substrate
is added. The substrate is then converted by the enzyme to a product that releases a chromogenic
or fluorescent signal. The amount of colour produced by, or the amount of fluorescence emitted
from, the sample is directly proportional to the amount of antigen in the sample. Hence the
measurement of either of these signals through the use of a spectrometer allows for the
quantification of the amount of antigen in the original sample.

This ingenious use of antibodies confers ELISA high specificity and low detection limits.
Thus, ELISA has been used in numerous studies to measure the upregulation and secretion of
proinflammatory mediators in both tissue culture (176) and whole blood (223). However, given
that the technique is an antibody assay, its inherent limitation is that it can only be used to look
specifically for analytes for which an antibody is added. Thus, in order to screen for other
potential proinflammatory mediators, MALDI-MS was used in conjunction with ELISA in the
studies undertaken in chapters 10 and 11.

6.3.6 Endotoxin

One of the most toxic elements of Gram-negative bacteria is the lipopolysaccharide
(LPS) component of their outer wall: endotoxin (figure 6.8) (16). The response to LPS exposure
ranges from the activation of the NF-κB pathway by way of its receptor toll-like receptor (TLR)-
4 (12, 115, 128, 189, 288), resulting in the initiation of inflammation, to septic shock and Gram-
negative bacteria-induced pneumonia (199). Gram negative bacteria are affiliated with animal
waste, and as a result are found in essentially all locales across the planet.
Recent studies have measured significant quantities of endotoxin on cigarette smoke (126) and particulate matter (212, 229, 241). Given the size and source of endotoxin, the mechanism by which endotoxin ends up on ambient particles is likely to be mechanical; that is to say that endotoxin is first located on the ground, bound to dirt and dust, and eventually blown into the air by either traffic or wind (28, 241). With regards to particles in cigarette smoke, 1% of the total endotoxin originally in the tobacco leaves survive combustion to remain within the smoke (126). Therefore, the question becomes, is the quantity of endotoxin on particulate matter enough to illicit a proinflammatory response (16)?

6.4 The Take Home Message

The concepts discussed in this chapter are crucial for the context in which the rest of this thesis was undertaken. Some key points to be taken away from this chapter are:

1) Particulate air pollution damages the lung by inducing inflammation, both local and systemic.
2) Chronic inflammation in the lung leads to airway remodelling, which in turn leads to the progression of numerous cardiovascular diseases, including lung cancer and chronic obstructive pulmonary disease (COPD).
3) A major proinflammatory pathway in the lung epithelial cells is the NF-κB pathway, which among many other proinflammatory mediators, regulates the expression of ICAM-1 protein.
4) By monitoring the expression of ICAM-1 through immunocytochemistry, one may be able to predict the potential of a specific particle type to induce a proinflammatory response, and hence be involved in the pathogenesis of diseases such as asthma and COPD.
5) Develop new methods to screen cytokine secretion using soft-ionization MS to complement immunocytochemistry and ELISA results and possibly add new information regarding the downstream biological response.

With this knowledge in hand, we set out to try to develop methods to enable measurement of the extent to which exposure to a particle of a given chemical composition will result in A549 cells exhibiting an inflammatory response. The idea, illustrated in figure 6.9, was to first dose lung cells grown in vitro with a known number of particles with designed chemical composition, generated within an AC trap. Then monitor the downstream biological response by the cells exposed, such as the expression of the proinflammatory mediator ICAM-1 or by measuring the secretion of proinflammatory mediators such as TNF-α or IL-1β.

Figure 6.9 The biological response to particulate air pollution processed in the troposphere as it occurs in vivo as compared to the in vitro methodology designed and developed during the course of this thesis.

Through measuring the relative expression of these proinflammatory mediators across numerous particle types, we hoped to gain insight into what are the potentially most harmful chemical components of particulate matter, as well as potentially learn some fundamentals about particle cell interactions.
Chapter 7

**DOSING ONE CELL WITH A SINGLE PARTICLE**

Apparatus for Preparing Mimics of Suspended Particles in the Troposphere and Their Controlled Deposition onto Individual Lung Cells in Culture with Measurement of Downstream Biological Response

*There's so much pollution in the air now that if it weren't for our lungs there'd be no place to put it all.*

*Robert Orben*

With regards to any toxicological study, many factors need to be considered, but not all can necessarily be addressed. In this chapter, the vector, or route, by which a potentially toxic species is introduced to the human body is studied. The primary toxicological question being addressed throughout this thesis is: “What role does each of the chemical species found on particulate matter play in initiating the proinflammatory response?” To properly address this question, methodologies must be used wherein the vector, in this case the particle, is considered. Observing the response that follows to the act of simply pouring a chemical species found on particulate matter onto a cell culture is not enough, for it does not consider the physical role of a particle core and the possibility of it acting in cooperation with other compounds (synergy or antagonism). The goal of this chapter was to further develop the methodology used in chapters 4 and 5 to study particulate matter toxicity by dosing a cell culture with particles of complex...
chemical composition generated in an electrodynamic balance (EDB) and observing the downstream biological response.

### 7.1 Context and Contributions

Sections 7.2 to 7.8 of this chapter have been previously published in their entirety in the journal Analytical Chemistry (Analytical Chemistry, Volume 77, Number 11, 2005, pp. 3623-3628). This was the first publication resulting from the collaboration between the Agnes group from SFU and the van Eeden group from James Hogg iCAPTURE Centre at St. Paul’s Hospital. The authors of the manuscript were myself, Hiroshi Iishi, Stephan van Eeden and George Agnes; however, all experiments, data collection and processing was undertaken by myself. Hiroshi Iishi was a post-doctoral fellow who was working under the supervision of Dr. van Eeden at the time in which these experiments were undertaken. Though he was not directly involved in any of the experimentation, he was responsible for training me in the field of cellular biology; for that reason, I selected him as a co-author for the manuscript, as without him the results in the manuscript may have not come to be. Hiroshi trained me in numerous areas, including cell culture techniques, primary cell extraction (collecting viable cells from a recently harvested organ) and immunocytochemistry, to enumerate just a few.

The following chapter can be viewed as simply the reporting of methodology developed by us to enable relevant, quantitative dose-response methodology for \textit{in vitro} particulate air pollution research. The goal of publishing this work was to report the ability to not only dose a cell culture with designed particles made within an EDB, but also to measure a response from the cell culture following particle deposition. This may appear trivial, but the challenge lies in the fact that only 1 to 120 particles are deposited onto the cell culture, which consists of $10^5$ to $10^6$ cells in any one trial. Once the capability of measuring a cellular response was demonstrated in the literature, the technique would then be able to be developed further to include more biological
readouts, thus allowing for the potential for more substantive dose-response relationships to be explored.

7.2 Abstract

Inhalation exposure to particles < 10 μm in size that are suspended in the troposphere (PM$_{10}$) is a factor in respiratory and cardiovascular diseases. The extent of the injury, local to systemic inflammation, is dependent on the number, size, and composition of the particles to which an individual is exposed. The physical properties of and compounds on PM$_{10}$ that are responsible for these adverse effects on human health are the subject of intense investigation. Here, we report a laboratory method that involved the creation of 1-120 particles per trial that were of known size and composition, followed by deposition of them directly onto individual human lung cells within a cell culture, and after an incubation period, a downstream biological response was measured. To illustrate this methodology, particles that each contained 50 pg of lipopolysaccharide were created and deposited onto individual cells over a region < 0.36 mm$^2$ within a genetically modified A549 cell culture. The biological readout was the relative expression of intercellular cell adhesion molecule (ICAM)-1 after 24 h of incubation using an immunocytochemistry assay. The apparatus and methodology introduced here enables studies at the interface between the relevant but diverse areas of atmospheric particle chemistry and lung cell biology to identify the chemical and physical factors of PM$_{10}$ that cause/exacerbate respiratory and cardiovascular diseases by triggering various biological pathways.

7.3 Introduction

The biological response following inhalation exposure to elevated concentrations of PM$_{10}$ ranges from local and systemic inflammation to increases in mortality and morbidity rates from respiratory and cardiovascular diseases (32, 65, 90, 252, 263, 282, 285, 310, 311). Through in vitro experimentation, components of the inflammatory response, such as tumor necrosis factor (TNF)-α, granulocyte macrophage colony stimulating factor (GM-CSF), interleukin (IL)-1β, IL-6,
IL-8, leukemia inhibitory factor (LIF), and oncostatin M (OSM), in addition to other pathways that include upregulation of matrix metalloproteinases, have been found (95-97, 132, 192, 278, 296). Specific components within the particulate matter that are responsible for causing these effects are beginning to be elucidated (207). The challenges in identifying the species responsible can be appreciated with consideration of the complexity of the individual particles in PM$_{10}$ with respect to size and composition as a result of their formation and reactivity in the troposphere, including homogeneous and heterogeneous pathways (85, 89, 148, 149, 155, 240). There is evidence that the organic and inorganic compounds that together comprise the soluble and insoluble fractions of PM$_{10}$ contribute to its toxicological properties (2, 3, 15, 18, 23, 68, 96, 132, 146, 169, 182, 221, 242, 250, 300).

A strategy to study particulate matter toxicity in vitro involves the collection of PM$_{10}$ on filters, with subsequent fractionation of the soluble versus insoluble PM$_{10}$ components as a result of extracting those materials from the filters for later use in toxicity studies (2, 3, 24, 68, 300). Another strategy involves incubation of lung cells with compounds that have been identified on PM$_{10}$, such as transition metals. Typically, an individual compound is used so as to characterize the lung cell injury caused by that compound alone (18, 43, 71, 217, 239, 242). In another strategy, particles in the exhaust of an internal combustion engine were deposited directly onto cells cultured on transwell plates (48).

Here, we introduce an apparatus that provides in vitro mimicry of the direct delivery of all compounds on the individual particles that comprise PM$_{10}$ as it occurs in vivo. The apparatus was developed around an electrodynamic balance (EDB) which has been used extensively to facilitate the characterization of individual particles levitated in air (1, 34, 216, 238). In this work, levitated carbon particles that contained lipopolysaccharide (LPS), an endotoxin that has been identified on PM$_{10}$ (212, 254, 276), were generated and then deposited directly onto cells in a culture, and a downstream biological response was monitored using immunocytochemistry. The results presented herein were acquired during the development of this methodology, and as such,
they provide an indication as to how this apparatus and methodology could be applied generically in dose-response studies in addition to PM$_{10}$ toxicity studies.

7.4 Experimental

The apparatus that was used can be seen in the photograph identified as figure 7.1A. Particles were generated by the evaporation of volatile solvents from individual droplets dispensed and levitated in an EDB (59) prior to causing those droplets to deposit onto a target plate using previously described WaSP methodology (25). The target plate was either a bare glass coverslip or a glass coverslip on which was a culture of cells. The levitation chamber containing the EDB and droplet dispenser were situated in a biological safety cabinet (Nuaire Inc., NU-425-600, Plymouth, MN). Details of the experimental procedure were as follows.
Figure 7.1 Apparatus for particle formation and depiction of the steps to deposit them onto a cell culture.

(A) Photograph and schematic of the levitation apparatus. (B) With a population of droplets levitated, a cell culture was prepared for receipt of the particles by draining the growth medium and placing the coverslip supporting the culture on the mounting platform for the bottom electrode of the EDB. (C) Without introducing convection into the levitation chamber that would otherwise cause some of the particles to be lost from the EDB, the culture was positioned below the ring electrodes of the EDB. A population of levitated particles illuminated by forward laser light scattering can be seen in the photograph, and scattered laser light inside the levitation chamber illuminated the ring electrodes. (D) Delivery of the levitated particles to the culture by adjustment of the electric fields in the EDB. The photograph illustrates a population of particles whose trajectories were displaced downward, toward a target plate by the adjustment of the attractive DC potential applied to the bottom electrode. (E) Having received the particles, the cell culture is removed from the levitation apparatus and placed into a sterile Petri dish that is, in turn, placed in an incubator. The scale bars in the photograph and the schematic of the levitation apparatus in panel A is 10 cm, and the scale bar for panel E is 100 μm.

An ~3 μL aliquot of a starting solution was used to load the internal reservoir of the droplet dispenser. The diameter of the nozzle on the droplet dispenser was 40 μm (model MJ-AB-01-40, MicroFab Technologies Inc., Plano, TX). The droplet dispensing rate was 120 Hz. The
initial volume of droplets dispensed was determined to have been 26 ± 3 pL (initial droplet radius 18.4 ± 0.7 μm) on the basis of optical microscopic measurement of the size of the spherical particles generated using methodology described in this section.

To enable electrodynamic levitation of a droplet, each droplet necessarily had to have a net charge. A net charge was induced onto the droplet during its formation by positioning the nozzle of the droplet dispenser 2 mm from a DC biased electrode. The magnitude of the induced net charge was 100 ± 20 fC, measured by dispensing droplets directly onto a plate to which an electrometer (model 6517A, Keithley Instruments, Cleveland, OH) was connected (27). A 5 mm diameter hole was cut in the induction electrode to allow the droplets to fly through that aperture into the EDB. The nozzle of the droplet dispenser was centred over the 5 mm diameter hole. Within the EDB, the combination of a DC field between the two endcap electrodes and an AC field between the 2-ring electrodes and the endcap electrodes (59) created forces that allowed droplets to be levitated in air. Note that the top endcap electrode of the EDB also functioned as the induction electrode during droplet dispensing.

Each trial commenced with continuous dispensing of droplets to load the EDB. This was visually ascertained by observation of forward-scattered laser light from the droplets levitated inside the EDB. This procedure to load the EDB was completed in 2 s. The volatile solvent, distilled deionized water, in the starting solutions evaporated from the droplets within ~10 s (266). As a consequence, upon visual observation of a population of droplets levitated, the DC potential applied to the induction electrode was switched to 0 V, and the frequency of the sine wave (4.5 kV₀,₉) applied to the ring electrodes of the EDB was ramped within a period of 1 - 5 s from 50 Hz to a frequency in the range from 150 to 950 Hz. The different AC frequencies were necessary to track the evaporative mass loss of water from the droplet, and the final frequency of the sine wave required for retention of the resultant particles in the EDB was inversely related to the concentration of low-volatility solutes in the starting solutions. The EDB used in these studies
is best described as an AC trap, but the equations of motion for particles in a Paul trap can be used to describe the limits of the levitation stability for evaporating droplets (193).

The percent water remaining in the residual levitated particle was dependent on the relative humidity inside the chamber in which the particles were levitated. The relative humidity in the levitation chamber was 30 ± 5%. Therefore, the concentrations of the low-volatility components in the starting solutions from which the droplets were dispensed determined to a first approximation the size and composition of the residual levitated particle, because the same droplet dispenser was used throughout, and the initial droplet volume was invariant within the range of starting solution compositions used. Starting solutions that contained 0.1 - 25% percent India ink (Speedball, Product no. 3338, Statesville, NC) were used to create carbon particles. India ink itself is 7 vol % dispersed carbon nanometer-sized particles (nanoparticles) in water. Those nanoparticles are considered inert, and they have been used as carbon controls in a previous toxicology study (96). Within each of the droplets dispensed, the carbon nanoparticles aggregated as the droplet's solvent evaporated, creating a single large spherically shaped residue, referred to as the particle. The sizes of the particles were measured using a calibrated optical microscope after depositing them onto a coverslip (model B5, Professional, Motic, Richmond, BC, Canada) (25). Several additional starting solutions contained India ink and 2 mg mL⁻¹ LPS. The LPS used was derived from *Escherichia coli* serotype 0111:B4, L-2630 (Lot no. 76H4099, Sigma Chemical Co., St. Louis, MO) (10, 63, 152, 163). Though we did not further process the composition of the levitated particles in this study, there exists the capability to alter the particle composition by changing the dissolved/dispersed solids in the starting solutions and also by introducing vapours of other compounds or tropospheric oxidants, such as ozone, hydroxyl radical, and nitrate radical, into the levitation chamber to "age" the particle's composition (118).

With a population of between 1 and 120 particles levitated in the EDB and ready to be deposited (figure 7.1B), a cell culture was prepared for receipt of the particles as follows. A culture of genetically modified A549 cells (American Type Culture Collection, Manassas, VA)
grown to 95 - 100% confluence on an 18-mm glass coverslip in a 6-well plate (Corning) in minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) in 5% CO2 at 37 °C was removed from the incubator. This cell line consists of human type II alveolar-like cells originally derived from a patient with bronchioalveolar carcinoma that were stably transfected with adenovirus 5 E1A (138, 163, 194). This modification causes the transfected A549 cells to express intercellular cell adhesion molecule-1 (ICAM-1) upon interaction with LPS. The growth medium in which the cell culture had been bathed was drained from the cell culture, and the coverslip supporting the culture was placed on top of the mounting platform for the bottom electrode. The cell culture was inserted into the levitation chamber and positioned below the ring electrodes of the EDB (figure 7.1C). The DC potential applied to the bottom electrode was adjusted, changing the electric field to effect their instability in the EDB, causing their ejection and deposition onto cells in the culture (figure 7.1D). The location of particle deposition on the culture can be as small as 0.03 mm², or as large as the culture on a glass coverslip (25). In this work, a rapid particle deposition mode was used to minimize the time that the cell culture was outside the incubator. The rapid particle deposition mode causes the particles to deposit over a region defined by the charge-charge repulsion between the particles that each had net charge of the same polarity. For this study, the area over which a population of levitated particles were deposited spanned from 0.03 to 0.36 mm² for 1 and 43 particles deposited, respectively. Following the delivery of the particles to the cell culture, the coverslip supporting the culture was removed from the levitation chamber (figure 7.1E) and placed back in a dry Petri dish inside the incubator (37 °C and 100% humidity). The total time elapsed between removing the cell culture from the EDB, draining the growth medium, depositing the particles, and then replacing the culture back into the incubator was < 60 s. Growth medium was not added to the cell culture following the particle deposition.

Cells were fixed after incubating with particles for a 24 h period using a 1% acetone solution for 10 minutes. During fixation, the number of deposited particles was counted. Cell
surface ICAM-1 expression was demonstrated by the alkaline phosphatase anti-alkaline phosphatase (APAAP) method using mouse anti-human ICAM-1 monoclonal antibody (Immunotech, Marseille, France) (97, 163). The positive control for these studies was a cell culture exposed to a 1μL aliquot of 10 mg mL⁻¹ LPS. The negative control was an untouched cell culture. The control used in the staining was mouse immunoglobin G (IgG) as the primary antibody.

7.5 Results and Discussion

Droplets of initial volume 26 ± 3 pL were dispensed from one of five different starting solutions that contained India ink at a concentration that spanned from 0.1 to 25 vol %. The droplets were injected into and captured in an EDB and levitated for 2 minutes prior to causing their deposition onto a clean glass cover slip. Images of representative particles viewed using an optical microscope are presented in figure 7.3Ai-Di and 7.E. Next, carbon particle delivery onto cells in culture was visually ascertained using optical microscopy. After 40 minutes of incubation, > 95% of the deposited particles were unable to be rinsed from the cell culture, indicating significant cell-particle interaction. The deposition of a population of 23 carbon particles onto a cell culture did not affect cell viability, as verified using a Trypan Blue staining assay after 6 h of incubation (figure 7.2).
Figure 7.2 The effect of particle deposition on cell viability as shown by a Trypan Blue assay in which this dye is taken up by passive diffusion into the cells, and from which viable cells actively transport the dye back out to the extracellular medium. Image of cells in cultures following a Trypan Blue assay that indicate (A) cells that had been dead and (B) viable cells that had been dosed with $8.8 \pm 0.5 \mu m$ carbon particles prior to the time of the assay. The scale bar length is $100 \mu m$.

Using the same five starting solutions, five new populations of particles were individually generated and deposited onto different cultures. Following a 24 h incubation period, the particles were viewed using an optical microscope (figure 7.3Aii-Dii). Observation of particle surface alteration and changes in the relative transparency were together used as relative measures of particle breakdown. Particle alteration by the cells ranged from partial breakdown of the larger particles deposited (i.e., figure 7.3Aii) to complete breakdown of the smallest particles (i.e., figure 7.3Dii). For instance, the particles in figure 7.3Ai and 7.3Aii show little to no changes in these criteria for particle breakdown, whereas the changes between the particles observable in figure 7.3Di versus 7.3Dii indicate extensive breakdown. The products of the breakdown were suspensions of carbon nanoparticles that originated from the India ink added to the starting solutions. Possible cellular uptake of the nanoparticles was not measured. The carbon particles that had been $1.7 \mu m$ in diameter when initially deposited onto the culture were broken down to
the extent that there were no observable residues of those particles following the 24 h incubation period. The mean and 95% confidence interval for each of the particle size distributions produced from the five different starting solutions at the time of their deposition onto a glass coverslip (3 populations per starting solution, 30 particles per population) are plotted in the graph identified as figure 7.3F. This range of particle size, from 1.7 to 10 µm in diameter, encompasses the larger particles in PM$_{2.5}$ plus the coarse fraction of PM$_{10}$.
Figure 7.3 Populations of carbon particles of different sizes generated by dispensing droplets from starting solutions containing different percentages of India ink by volume. (Ai-Di, E) Particles viewed on a glass coverslip after having been deposited from the EDB. (Aii-Dii) Particles deposited from an EDB directly onto a cell culture and viewed following a 24-h incubation period. The scale bar shown in the image identified as E was 10 μm in length, and it is accurate for all images depicted. (F) The mean and 95% confidence interval for the size of carbon particles deposited onto glass coverslips.

Variation of the percentage of India ink in starting solutions used to generate particles having different sizes was a single demonstration of a generic strategy to vary particle size and composition by dispensing droplets from starting solutions having different composition. For instance, other compounds measured or speculated to exist on PM₁₀ can be added to starting solutions to form particles of known size and composition for the purpose of replicating the in vivo delivery of those particles, intact, rather than as separate fractions for the purpose of delineating the factors of PM₁₀ that are responsible for its toxic properties.
To illustrate this aspect of the methodology, two starting solutions containing 10% India ink by volume in distilled deionized water and 0 or 2 mg mL\(^{-1}\) LPS were prepared. Depending on the starting solution used, carbon particles 8.8 ± 0.5 µm in diameter that contained either 0 or 50 pg LPS were generated. This abundance of LPS per particle had been liberally estimated on the basis of a prior study by Fujii et al., who introduced 10 mL of medium containing 1 µg mL\(^{-1}\) of LPS into a medium containing ~2.5 to 3 x 10^6 cells per dish, which induced significant expression of ICAM-1 from the same cell line as used in this work (96). Had all of the LPS added to the cell culture in the experiments performed by Fujii et al. been taken up equally by all of the cells, ~3 pg of LPS would have interacted with each cell in the culture, but note that the total LPS introduced to the cultures was 10 µg in that study.

Thirteen particles containing 50 pg LPS were deposited onto a cell culture, and 15 particles containing no LPS were deposited onto a different culture. The trajectories of the particles in the balance, due to charge-charge repulsion, made accurate accounting of the levitated particles very difficult. As such, determination of the number of particles deposited was performed after the 24 h incubation period. The differential expression of ICAM-1 following immunocytochemistry was observable between the two cultures that were each dosed with one of the two particle types (figure 7.4). The images identified as figure 7.4A and 7.4B were acquired by taking a series of images along a line across each of the two cultures that were then cropped and assembled into two 3 X 20 mm images. The insets in figure 7.4Ai and 7.4Bi include the portion of the culture that encompassed the region within which the 13 and 15 particles had been deposited, respectively. In comparing the staining observable in these images, significant expression of ICAM-1 was induced over an area of ~16 mm\(^2\) in the culture that was dosed with LPS-containing particles. Cell staining was not uniform due to the cells' being at different stages in their cell cycle. Assuming a diffusion coefficient for LPS of 4.5 X 10\(^{-10}\) m\(^2\) s\(^{-1}\), the maximum area over which LPS could have diffused from intact particles during the 24 h incubation period would have been ~16 mm\(^2\). On the basis of the area stained in figure 7.4A, the differential
expression of ICAM-1 was localized to within an area of size comparable to the area over which the LPS was expected to have diffused.

![Image of ICAM-1 expression](image)

**Figure 7.4** Differential ICAM-1 expression after a 24 h incubation period made apparent by immunocytochemistry (red staining) on different cultures that occurred as a result of depositing (A) 13 particles of elemental carbon that each contained 50 pg of LPS, and (B) 15 particles of elemental carbon that each contained 0 pg of LPS. These particles remained visible on the culture following the incubation period. The scale bar in panels Ai-Bi indicate a distance of 300 μm, and the area of particle deposition was < 0.36 mm².

However, a marked difference in the area over which ICAM-1 was expressed in a culture was repeatedly observed when a larger number of particles, each containing 50 pg of LPS, were deposited onto a culture. On the basis of the results of 34 trials, 17 of which involved deposition of less than 30 particles and 17 involved deposition of more than 30 particles onto different cultures, the extent of ICAM-1 upregulation in the latter set of 17 trials could not be accounted for on the basis of diffusion of LPS alone. To illustrate this result, images from two cell cultures that are representative of the results obtained within this set of 34 trials are presented in figure 7.5. With only 5 particles deposited over a region < 0.36 mm² (figure 7.5A), the extent of ICAM-1 expression was observed to be localized to a region of ~16 mm² centred at the site of particle deposition. When 43 particles were deposited onto a cell culture, also over a region < 0.36 mm², the extent of ICAM-1 expression was observable in cells that were 7 mm removed from the site.
of particle deposition (figure 7.5B). Significant ICAM-1 expression, relative to the controls, was consistently observed over an area > 320 mm² in each culture that received a dose of more than 30 particles, suggesting that the cells near the site of particle deposition reported their injury to other cells in the culture. We speculate intercellular communication occurred, likely by upregulation of proinflammatory cytokine secretory pathways that were triggered when greater quantities of LPS-containing particles were deposited onto a culture. Work is in progress to add quantitative measurement of the differential ICAM-1 expression, cytokine secretion, and other biological responses. When that has been demonstrated, an in vitro methodology would be available for toxicological studies of PM₁₀ wherein the dose of synthesized particles deposited onto a small region of a cell culture would be known quantitatively, and downstream biological responses of cells as a function of their spatial location in the culture would also be quantitatively measured.

Figure 7.5 ICAM-1 expression indicated by immunocytochemistry (red staining) at (i) the site of particle deposition and (ii) 7 mm distal from the site of particle deposition. (A) 5 and (B) 43 particles deposited onto different cultures. Each particle contained 50 pg of LPS.
7.6 Conclusions

Methodology has been developed that offers synthesis of particles suspended in air whose composition and size are known, followed by their direct delivery to cells within a culture. This methodology mimics the deposition of the larger particles in PM$_{10}$ onto tissue in the pulmonary system. A feature of this technology is that it allows quantitative dose-response study of the injury caused to a cell by the direct delivery of particles whose compositions were engineered to mimic that of ambient particles, and it also enables measurement of the effect of such interactions on cells distal to the site of particle deposition. As such, it represents a new tool with which factors in PM$_{10}$ that are toxic can be elucidated.

7.7 Acknowledgments

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Chapter 8

**DOSE RESPONSE RELATIONSHIPS AT THE SINGLE PARTICLE LEVEL**

Dose–response studies involving controlled deposition of less than 100 particles generated and levitated in an AC trap onto lung cells, *in vitro*, and quantitation of ICAM-1 differential expression

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*The human lung, with all its cleverness of design and tolerance to insult, is ill equipped to deal with more subtle dangers of air pollution. The larger particles are quite easily trapped and ejected by the vigilant cilia. But the smaller the particle, the more chance it has to slip past these defenders. Highly toxic or even cancer-causing particles can invade deep into the lung and remain in direct contact with sensitive tissue for long periods; gases or other chemicals cling to these particles. There is virtually no defence from the tiniest particles and gases that invade the lung.*

*William Longgood, The Darkening Land*

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The above words were written in 1970 to remind us of the hazard of particulate air pollution to human health. Concern in those days focused on either the toxic nature or the cancer causing potential of these particles. Since those days, much has been learned about the effects of particulate air pollution human health, most notably its subtle ability to induce inflammation and how inflammation is a factor in the pathogenesis of many diseases. The goal of the work described in this chapter was to further improve the methodology developed in chapter 7 as to quantify the proinflammatory response observed.
8.1 Context

Sections 8.2 to 8.7 of this chapter have been previously published in their entirety in the journal Toxicology in Vitro (Toxicology in Vitro, Volume 20, Number 6, 2006, pp. 1030-1039). This pivotal publication was the second one resulting from the collaboration between the Agnes group from SFU and the van Eeden group from James Hogg iCAPTURE Centre at St. Paul’s Hospital. This was the first manuscript to appear in a purely biology oriented journal, which allowed for it to be showcased to an audience whom, until that point, may have been unaware of our efforts to contribute new technology to the in vitro study of particulate air pollution. Additionally, through the reviewing process, much was learned of the data requirements for a publication in this kind of journal. From this, future experiments were modified to incorporate additional controls and the like. The authors of the manuscript were myself, Stephan van Eeden and George Agnes. All experiments, data collection and processing was undertaken by myself.

Like the previous one, this chapter can be described as a method development chapter. The goal of publishing this work was two fold; first, to showcase this technology to a new audience in our target demographic and second, to demonstrate the ability to quantify dose-response relationships.

8.2 Abstract

A developing area of interest regarding the relationship between the adverse health effects associated with particles suspended in the troposphere is an understanding of how particle chemical composition influences different biological outcomes. Described is the development and application of an apparatus and methodology wherein a known number of particles of tropospherically relevant chemical composition can be designed and levitated in an alternating current (AC) trap followed by their controlled deposition directly from the ac trap onto air-liquid interface cultured lung cells. A downstream biological response, differential upregulation of intercellular adhesion molecule (ICAM)-1, was measurable using fluorescence microscopy in the
air-liquid interface human lung cell cultures even though the dose per culture was 0-100 lipopolysaccharide (LPS)-containing elemental carbon particles (52 pg LPS per 6.3 μm diameter particle). Fluorescence emission intensity data measured from a 1 mm² area centred over the site of particle deposition were fitted using a least squares linear regression line. Because the total mass of each different compound comprising each of the particles delivered to the culture was known, the data generated with this methodology can be expressed as a pro-inflammation potential (in this case ICAM-1 expression) per particle number and composition. Also described is how this methodology affords opportunities to quantitatively study pro-inflammatory intercellular signalling, leading to ICAM-1 expression at sites distal to the site of particle deposition.

8.3 Introduction

Chronic and acute inhalation exposure to particulate matter suspended in the troposphere (PM₁₀) have been correlated to an increase in the frequency of respiratory and cardiovascular diseases (30, 32, 65, 66, 74, 258, 260, 282). Recent epidemiological studies suggest that proximity and residence time near roadways having major vehicular traffic are significant factors in cardiopulmonary morbidity and mortality (36, 137, 174, 261). Observed world-wide, this correlation is linked through the inflammatory response, where the extent of the injury following exposure to PM₁₀ ranges from being localized within lung tissue to systemic inflammation (20, 22, 95, 96, 109, 213, 223, 285, 286).

Lung epithelial and macrophage cells, and possibly endothelial cells, that interact directly with particles initiate a response by reporting their injury to other cells and tissues through the upregulation and secretion of pro-inflammatory cytokines (95, 96). Nuclear factor (NF)-κB is activated when lung cells are exposed to particulate matter air pollutants (136, 160, 265). NF-κB is a transcription factor that when activated, results in the production of numerous pro-inflammatory proteins including interleukin (IL)-8, tumour necrosis factor (TNF)-α, interferon...
(IFN)-γ, granulocyte colony-stimulating factor (G-CSF) and intercellular adhesion molecule (ICAM)-1 (21, 296). Although other pathways have been proposed to cause arrhythmia (64, 243, 245, 280), and the acute coronary syndrome (261, 279, 308) following inhalation exposure to ambient particles, most studies implicate the systemic inflammatory response resulting from local lung inflammation in causing the adverse downstream cardiovascular effects.

Elucidation of how the size, number density, and chemical composition of ambient particles initiates and effects various biological responses would improve our understanding of how atmospheric particles trigger and exacerbate respiratory and cardiovascular diseases (figure 8.1A) (32, 125). While the relative toxicity of specific single component particles have been studied, such as titanium dioxide (67), the chemical composition of the individual particles comprising PM$_{10}$ is complex and the diversity of particle types in PM$_{10}$ is large (53, 89, 196, 246). In consideration of this, many investigators have collected ambient particles on filters for subsequent use in animal and in vitro toxicology studies, but the sampling step itself and any steps subsequent to remove the particles from the filter can cause their chemical composition to be altered from that of the original particle (figure 8.1B)(147). For instance, the relative toxicity of soluble fractions (96) and/or the insoluble fraction (95, 96, 109, 301) of particles collected from the troposphere have been measured to be different. In consideration of the overall toxicity of PM$_{10}$ that has been learned from these studies, there is potential for multiple chemical components on a particle to behave synergistically. However, a technological challenge for existing in vitro dose–response strategies is to identify instances of synergy.
Herein, we describe a levitation apparatus and a methodology to generate particles of known size whose composition is of relevance in populated regions, and then deliver those particles directly onto cells \textit{in vitro}. The particles used in this study were comprised of elemental
carbon plus bacterial lipopolysaccharide (LPS) or endotoxin. Endotoxin from *Escherichia coli*, was selected because recent studies have found significant concentrations of endotoxins in the coarse fraction of PM$_{10}$ (size range from 2.5 to 10 µm in diameter), with daytime average concentrations as high as 100 endotoxin units (EU)/mg ambient particles (42, 130, 212). Furthermore, many investigators have suggested that endotoxins play a substantial role in determining the overall toxicity of PM$_{10}$ (5, 126, 254). Following an incubation period, quantitation of a downstream biological response, the differential upregulation of ICAM-1 from cells at different locations across the cell culture was measured.

### 8.4 Experimental

#### 8.4.1 Materials

As described in the following sub-sections, the particles were themselves derived from droplets that were dispensed from starting solutions containing 5% India ink (Speedball, product #3338, Statesville, NC, USA) by volume in distilled deionized water with either 0 or 2 mg/mL of LPS from *E. coli* (Serotype 0111:B4, L-2630, lot; #76H4099, Sigma Chemical Co., St. Louis, MO, USA). India ink is an aqueous solution containing carbon nanoparticles and surfactants, the latter compounds prevent the nanoparticles from aggregating until the ink is used at which time the water solvent is lost due to evaporation. Individual droplets of known volume were dispensed, and upon evaporation of the volatile solvents, the resultant aggregate of elemental carbon nanoparticles from each droplet was spherical in appearance when viewed using an optical microscope and had a diameter of 6.3 ± 0.4 µm (mean and SD), and they contained either 0 or 52 pg of LPS depending on the starting solution used. Each of the latter aggregates had ~280 EU of LPS/mg particle (121). The range of particle size follows a Gaussian distribution because of the random error in initial droplet volume dispensed and its net charge, and also on the injection and trapping of the dispersed droplets by the AC trap operated under static conditions.
To characterize the extent to which the soluble components of the deposited particles (i.e., LPS) diffused across the cell culture, a population of rhodamine 6G (R6G) containing residues were deposited onto the cell culture. These residues were generated from starting solutions that contained R6G chloride at $4.18 \times 10^{-3}$ M and either 0 or 5% India ink by volume in distilled deionized water. The extent of R6G diffusion following a 24-h incubation period was then measured using fluorescence microscopy.

8.4.2 Cell Culture

A549 cells (American Type Culture Collection, Manassas, VA, USA), a human type II alveolar-like cell line originally derived from a patient with bronchioalveolar carcinoma was transfected with the adenovirus 5 E1A gene. Regular type A549 cells do not upregulate ICAM-1 upon exposure to E. coli derived LPS. However, following transfection with the adenovirus 5 E1A gene, previous studies have shown that the transfected A549 cells show significant ICAM-1 upregulation following exposure to an aqueous solution containing LPS (97, 133, 134, 161, 162, 222). As such, this transfected cell line was used in the development of this methodology since a straightforward dose–response relationship using an aqueous LPS solution had been established.

All cell cultures were grown to > 95% confluence over a region no smaller than $18 \times 18$ mm$^2$ on a glass coverslip in a 6-well plate (Corning) in minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum in 5% CO$_2$ at 37 °C. The negative control used in this study was a cell culture grown to > 95% confluence and then had all but 15.9 ± 2.5 μL (mean ± SD) of its medium removed prior to being placed into an incubator. The volume of growth medium remaining on the cultures was determined gravimetrically using a 20 minute drying period to achieve constant mass. The positive control used in this study was bathed in 2 mL of growth medium, to which 1 μL of a solution consisting of 10 mg/mL of LPS in water was added, resulting in a final LPS concentration of 5 μg/mL.
8.4.3 Particle Generation and Deposition onto Lung Cells in vitro

10 µL of a starting solution was placed into the reservoir of a droplet-on-demand dispenser (MJ-AB-01-60, MicroFab Technologies Inc., Plano, TX, USA). The droplet dispenser was then positioned above an induction electrode for downward dispensing (figure 8.2A). Activation of the piezoceramic element of the droplet dispenser caused the ejection of a jet of liquid from the nozzle of the droplet dispenser. A 150 V DC potential applied to the induction electrode affected ion mobility in the jet that, upon the jet separating from the nozzle and collapsing to form a droplet, each droplet carried an induced net charge of 200 ± 25 fC (figure 8.2B) (27). Droplets were generated at a rate of 120 Hz with an initial diameter of 60 µm. The net charge on the droplets allowed them to be levitated by an electric field produced by applying potentials to the ring electrodes of the AC trap (figure 8.2C) (59). A droplet dispensing period of 5 s or less was used, and some of the dispensed droplets were captured in the AC trap and levitated. Typical populations of levitated droplets ranged from 10 to 120. Tallying of the number of particles levitated and actually delivered to each cell culture was determined post deposition. The dimensions of the electrodes comprising the AC trap and the sinusoidal waveforms applied to those electrodes were based on the two-ring electrodynamical balance (59, 120). The water within the droplets quickly evaporated (i.e., within seconds) leaving behind an aggregate, or residue, comprised of the solutes of low volatility that were in the dispensed droplets. Hereafter, these residues are referred to as particles (figure 8.2D).
Figure 8.2 A summary of the methodology for direct deposition of designed particles onto lung cells, in vitro.

(A) A schematic of the AC trap used. (B) Dispensing of a droplet having net charge. (C) A droplet levitated by the electric field inside the AC trap. The inset is a photograph of a population of particles levitated in an AC trap. The scale bar length represents 11 mm. (D) Following a brief period of levitation (<10 s), the volatile solvents have evaporated leaving behind a residue (i.e., a particle). (E) An air–liquid interface cell culture grown on a coverslip is positioned below the ring electrodes of the AC trap. (F) A DC potential applied to the bottom electrode of the AC trap extracts the levitated particles onto the culture, which is then placed into an incubator for 24-h. The inset shows particles on an air–liquid interface culture as viewed through an optical microscope. The deposition of the particles is such that no one cell interacts with more than one particle. The scale bar length represents 100 μm. (G) Following an incubation period, the cells are fixed and then (i) a protein is fluorescently labelled using an antibody and (ii) its differential expression relative to controls is determined using microscopy to measure fluorescence emission signals. Depicted are the two area scans acquired per culture of fluorescence emission relative to the site of particle deposition.

With a population of particles levitated, a cell culture grown on a coverslip was then drained of all but 15.9 ± 2.5 μL of the growth medium that bathed the culture. A culture so prepared is hereafter referred to as an air–liquid interface culture. The cover slip supporting the culture was then placed on top of the bottom electrode of the AC trap, and the centre of the culture was manually aligned directly below mid-point of the ring electrodes of the AC trap, which is the location in the AC trap where the particles were levitated (figure 8.2E). The particles were then extracted from the AC trap through the application of a 500 V (DC) potential to the bottom electrode and they impacted onto the air–liquid interface culture (figure 8.2F) (25).
Previous studies have shown that the applied potential does not measurably affect cell viability (120). The particles deposited over an area < 0.4 mm$^2$ of the culture using this procedure (25).

Immediately following particle deposition, the culture was placed into a sterile (35 mm × 10 mm) tissue culture dish that was then placed into an incubator set at 100% humidity, 37 °C and 5% CO$_2$ for 24 h. No additional medium was added to the culture following particle deposition, and visual analysis of the cell culture following the 24-h incubation period showed no signs of the cell culture drying out. To ensure maximum possible ICAM-1 upregulation, the cell culture was incubated for 24-hour (171).

8.4.4 Antibody Assay

Following the 24-h incubation period, the cells were fixed with a 1% acetone solution for 10 min. During the fixation period, the number of particles that had been deposited were counted using optical microscopy. The primary antibody was mouse anti-human ICAM-1 monoclonal antibody (Immunotech, Marseille, France) and the secondary antibody was a fluorescently labelled goat anti-mouse IgG (Alexa Fluor 546, Molecular Probes, Eugene, OR, USA) (figure 8.2Gi).

8.4.5 Fluorescence Microscopy and Image Analysis

A Zeiss Axioplan 2 (North York, Ont., Canada) fitted with an excitation filter (BP-546/12) and emission filter (LP-590) was used to collect all images of fluorescence emission from fluorescently tagged antibodies bound to ICAM-1 (figure 8.2Gii). For each sample, fluorescence emission collected from a 1.00 mm × 1.25 mm area centred over the site of particle deposition was acquired, and the image generated (1.00 mm$^2$) is hereafter referred to as a deposition site scan. This image viewed an area of the culture that was well in excess of the area of particle deposition, which was < 0.4 mm$^2$. The < 0.4 mm$^2$ area that was the site of particle deposition was first positioned to be in the centre of the field-of-view of the microscope’s objective using optical microscopy prior to acquiring the fluorescence image that is termed the deposition site scan. A second fluorescence image used for quantitative analysis of ICAM-1 expression was acquired.
from each culture as follows. Including the deposition site scan, a series of $1 \times 1.25 \text{ mm}^2$ images were collected in an arbitrary direction from the site of particle deposition to generate the data needed to post re-construct a single image. Each of these images had 33% overlap with the previous image to ensure suitable reconstruction of a single combined image using an image processing software package (Photoshop, Adobe). The combined image, cropped to an area of $1.00 \text{ mm} \times 6.75 \text{ mm}$, is hereafter referred to as the $7 \text{ mm}^2$ scan. For each culture, the signal intensity of fluorescence emission, indicative of ICAM-1 expression, within these two scanned areas of the culture, the deposition site scan and the $7 \text{ mm}^2$ scan, was determined using Image J software (Research Services Branch, National Institute of Mental Health, Bethesda, MD, USA) and integrated using Excel. The extent of ICAM-1 upregulation was reported as a percentage of the total signal relative to the positive control.

8.4.6 Statistical Analysis

For each of the scans, the $1 \text{ mm}^2$ deposition site and $7 \text{ mm}^2$ scans, the fluorescence data was compiled and ranked based on the number of deposited particles. The data were binned into five groups based on the number of particles deposited. The bin sizes were 0–20, 21–40, 41–60, 61–80, or 81–100 deposited particles. The average and 95% confidence interval of both the number of deposited particles and the relative signal intensity for each group was then calculated.

8.5 Results

The airway surface liquid (ASL) is a complex aqueous solution containing ions, glycoproteins and numerous other proteins including lysozyme, antimicrobial surfactant proteins, and human salivary histatin. The ASL is thought to play an important role in airway hydration, innate immunity, and antimicrobial defence (298). The typical depth of the ASL in a normal healthy human lung ranges from 5 $\mu\text{m}$ to 20 $\mu\text{m}$. For patients suffering from cystic fibrosis, the typical ASL depth is 50 $\mu\text{m}$. In this study, the air–liquid interface culture composition consists of MEM growth medium containing FBS, glutamine, and vitamins. The average depth of the air–
liquid interface culture in this study was $49 \pm 8 \mu m$. As such, the air–liquid interface culture used in this study was comparable to that of the ASL in human lungs in terms of depth, but different in terms of the composition.

Deposition site scans of fluorescence emission images indicating ICAM-1 upregulation in air–liquid interface cultures resulting from the deposition of 26 and 74 LPS-containing carbon particles are shown in figure 8.3A. The extent of, or more specifically, the distance from the site of particle deposition that ICAM-1 upregulation was observed was confined to an area not far removed from the site of deposition of the 26 particles. In contrast, much more extensive ICAM-1 upregulation was observed across the culture to which 74 particles were delivered. This observation was reproduced in numerous dose–response experiments (figure 8.3B).
Figure 8.3 (A) Representative fluorescence emission profiles of fluorescently labelled antibodies bound to ICAM-1 from air-liquid interface cell cultures following the deposition of (i) 26 and (ii) 74 LPS-containing carbon particles. The quantity of LPS per particle was 50 pg. The circle denotes the site of particle deposition. (B) Representative fluorescence emission in 7 mm x 1 mm² scans of fluorescently labelled antibodies bound to ICAM-1 from air-liquid interface cell cultures: (i) A positive control, (ii) 91, (iii) 66, (iv) 47, (v) 34, (vi) 23 LPS-containing carbon particles deposited, where the quantity of LPS per particle was 50 pg, and (vii) 80 carbon particles deposited. (C) Representative fluorescence emission of rhodamine 6G in a 7 mm x 1 mm² scan 24 h following particle deposition onto a cell culture. For all images in B and C, the scale bars represent a distance of 2 mm, and the site of particle deposition was at the left-hand side of these images.

To address this distinct change in area of the culture from which ICAM-1 was differentially expressed, the extent of ICAM-1 upregulation as a function of particle dosage in the particle deposition scan (figure 8.4A) and also in the 7 mm² scan (figure 8.4B) was determined. The measured dose-response relationship for the upregulation of ICAM-1 in the site of particle deposition scan as a function of particle dosage was fit with a least squares regression line. In
contrast, the extent of ICAM-1 upregulation as a function of the particle dosage in the 7 mm² scans was fitted to a sigmoidal curve. Particles comprised of elemental carbon from India ink alone did not cause differential upregulation of ICAM-1.

![Graph A and B showing differential expression of ICAM-1 as a function of particle dosage](image)

**Figure 8.4** Differential expression of ICAM-1 as a function of particle dosage in (A) the particle deposition site scan (1 mm²) and in (B) the 7 mm² scan. Each 6.3 µm diameter elemental carbon particle contained either 0 (•) [number of assays = 37, R²(A) = 0.0908, R²(B) = 0.3314] or 50 (■) pg of LPS [number of assays = 149, R²(A) = 0.9863, R²(B) = 0.0021]. All fluorescence emission signals measured from the air-liquid interface cultures incubated with particles were scaled relative to the positive control. Error bars represent the 95% confidence interval.

To estimate the fraction of LPS that remained bound within the matrix of the carbon particle over a 24-h incubation period, droplets containing LPS but no carbon were delivered directly (i.e., levitation period = 0 s) to air–liquid interface cultures. The size of the generated droplets was a function of the settings on the droplet dispenser and the AC trap, but the droplets containing LPS with no carbon contained the same amount of LPS as the LPS-containing carbon particles. Following an incubation period of 24-h, fixing of the cells, and immunochemical staining for ICAM-1, the extent of ICAM-1 differential expression was determined based on the signal intensity of fluorescence emission from the site of droplet deposition scan (figure 8.5A)
and in the 7 mm² scan (figure 8.5B). As few as 25 LPS droplets effected widespread differential expression of ICAM-1. Conversely, cells exposed to < 40 LPS-containing carbon particles did not affect widespread ICAM-1 upregulation. ICAM-1 upregulation is the result of the cell culture responding to the presence of soluble LPS. The absolute quantity of LPS in each droplet and each particle was the same within experimental error, thus any difference in measured ICAM-1 upregulation between 25 droplets and 25 particles being deposited onto the cell culture can be attributed to the LPS being entrapped to the carbon particle. A comparison of the data plotted in figure 8.5A versus figure 8.4A suggests that a majority of the LPS within the LPS-containing carbon particle remained entrapped in the carbon particle core.

![Graph](image)

**Figure 8.5** Differential upregulation of ICAM-1 as a function of droplet dosage in (A) the deposition site scan (1 mm²) and in (B) the 7 mm² scan (n = 21 for both scans). Each droplet contained 50 pg LPS. Error bars represent the 95% confidence interval.

Based on the data plotted in figure 8.4B, a dosage of 45–116 carbon particles containing 52 pg of LPS per particle, with all of those particles deposited within an area < 0.4 mm² of the
culture, was sufficient to cause differential upregulation of ICAM-1 expression across the entire cell culture (18 × 18 mm²). This observation suggested that culture-wide pro-inflammatory signalling between cells occurred at a threshold delivery of LPS-containing carbon particles to an air–liquid interface culture. Mediators, such as TNF-α and IL-1β are known to be produced by these cells on activation, and we suspect that these mediators initiated intercellular communication that resulted in widespread upregulation of ICAM-1. Alternatively, this observation could have been the result of simple diffusion of some of the LPS from the carbon particles across cultures dosed with 45–116 particles. To obtain a measure of the maximum extent to which LPS could have diffused from the site of particle deposition, R6G-containing elemental carbon particles, generated with the same methodology used to create the LPS-containing elemental carbon particles, were prepared and delivered onto air–liquid interface cell cultures. In addition, an unlevitated population of 100 R6G-containing droplets having no elemental carbon was delivered to another cell culture. In bypassing the AC trap to deliver droplets directly to the cells from the droplet dispenser, a known number of unlevitated droplets were deposited onto the cell culture. Following a 24-h incubation period, the extent to which the R6G diffused from the site of particle (figure 8.3C) and droplet deposition was measured using fluorescence microscopy and analyzed using Image J software (figure 8.6). The integrated fluorescence signal intensity under each curve was the same indicating the constant total R6G deposited onto the cell cultures in these experiments. However, the areas from which fluorescence emission was detected were different between particles versus droplets. R6G diffused approximately 2 and 3 mm from the site of particle and droplet deposition, respectively. The larger area of diffusion of R6G from the droplets is attributed to it being dissolved in a larger droplet (droplet diameter = 18 μm) versus being entrapped within a particle (particle diameter = 6.3 μm) upon deposition, thus resulting in a larger initial deposition site, < 0.4 mm² versus < 5 mm², because the droplets wetted the cell culture. The extent of R6G diffusion in these cultures as evaluated from theory is also plotted in figure 8.6.
Figure 8.6 The extent to which R6G diffused in air–liquid interface cell cultures from the site of deposition of 92 particles or 100 droplets over a 24 h period in an incubation chamber. The lines indicate the extent of R6G diffusion from particles (thin line), from droplets (dashed line), and theory (thick line).

Based on the relative difference in molecular weights, the extent of diffusion of R6G across the culture was assumed to overestimate the extent to which LPS would diffuse under similar conditions. The diffusion coefficient of R6G is $2.80 \times 10^{-10}$ m$^2$/s (170). The profiles for R6G diffusion in figure 8.6 indicate that the culture-wide differential upregulation of ICAM-1 was not due to simple LPS diffusion, but rather they support the hypothesis that pro-inflammatory mediators secreted by the cells affected the observed widespread upregulation of ICAM-1 on the air–liquid interface cell cultures. Visual comparison between the upregulation of ICAM-1 across
the coverslip following LPS-containing carbon particle deposition (i.e., figures 8.3Bi and 8.3Biii) versus that of the diffusion of R6G from R6G-containing carbon particles following deposition, provides support for a conclusion that the extent of ICAM-I upregulation across the coverslip was not simply a result of LPS diffusing across the coverslip. This hypothesis is the subject of current investigations using routine proteomic technologies to identify biomacromolecules differentially secreted from lung cells exposed to particles.

8.6 Discussion

The utility of the technology and methodology described here was demonstrated with its ability to provide results that were fitted using a linear dose–response relationship regarding ICAM-I upregulation at the site of particle deposition, where the mass of a given compound on each particle, and the number of particles delivered directly onto an air–liquid interface culture was known (figure 8.4A). As such, this methodology can generate data that can be used to quantitatively rank the pro-inflammatory potential of designed particles. The AC levitation technology allows ready formation of particle types with various chemical cores that range from elemental carbon to secondary organic aerosols and mineral dusts (58, 60, 89). The known chemical composition of these designed particles can be made increasingly more complex through the addition of numerous non-volatile chemical species to the original starting solution from which droplets are dispensed, as demonstrated simplistically by the two component LPS-containing elemental carbon particles used in this study. Furthermore, the chemical composition of the particles can be altered through heterogeneous and homogeneous chemical processing while being levitated in the AC trap (118, 120). For instance, introducing unsaturated volatile organic compounds and an oxidant, such as ozone, into the levitation chamber would result in the condensation of some of the compounds that had been oxidized by homogeneous gas phase chemistry onto the levitated particles. As such, the methodology introduced here enables the preparation of reasonable mimics of ambient particle types in terms of the chemical composition.
to incorporate water soluble and insoluble compounds, and the capability to vary the distribution of those compounds on the particles. The direct delivery of such levitated particles onto an air-liquid interface cell culture has also been coupled with the quantitation of a downstream biological response. Work is underway to incorporate quantitative measurement of other biological responses into this methodology. For instance, it is known that LPS alone may induce the secretion of numerous other cytokines, such as TNF-α, that may affect ICAM-1 upregulation through the NF-κB pathway.

Another characteristic of this particle levitation/deposition technology is that it enables investigation of the biological responses of cells that did not interact directly with the particles, or any of the soluble compounds that diffused from the particles during the incubation period. It is for this reason that the standard technique such as flow cytometry was not used, as removing the cells from the coverslip would result in loss of spatial information regarding the biological response of cells distal to the site of particle deposition. We conclude that this methodology affords opportunities for detailed investigation of the influence of particle composition (plus particle size and number) on downstream biological responses of cells. Such information is anticipated to improve the understanding as to how inhalation exposure to ambient particulate matter affects various biological outcomes.

8.7 Acknowledgments

This research was funded by the British Columbia Lung Association, the Canadian Foundation for Climate and Atmospheric Sciences (CFCAS), and the Natural Sciences and Engineering Council of Canada (NSERC). Dr. Stephan van Eeden is an American Lung Association Career Investigator and William Thurlbeck Distinguished Researcher.
Chapter 9

**ADDING MASS SPECTROMETRY READOUT**

Development of a methodology to measure changes in the secreted proteome of a cell culture exposed to < 200 ambient particle mimics

*Real knowledge is to know the extent of one’s ignorance.*

Confucius

Quantitative analysis of samples in numerous bioanalytical experiments is often restricted to the scanning and measurement of only the species believed to be present. For instance, the major limitation of an antibody assay is that the only species that can be detected are not only those that are scanned for, but also those for which antibodies are available. However, with the advent of genome and proteome methodologies, such as DNA microarray and mass spectrometry respectively, the ability to observe differential changes in numerous biomacromolecules, many of which may not have been initially hypothesized to be relevant, has become possible. Until this point in our studies, we had only monitored the differential expression of a single proinflammatory mediator, ICAM-1. The monitoring of only a single mediator severely confined the amount of information we could obtain thus limiting our understanding of the system on the whole. For this reason, we set out to add a mass spectrometry readout of the downstream biological response to the methodology we had developed thus far.
9.1 Context
The contents of this chapter are unpublished. All method development, experimentation and data analysis were undertaken by myself.

9.2 Abstract
Particulate air pollution initiates a myriad of adverse health effects on the human body, through prolonged inflammation. Detailed understanding of how the chemical composition of particulate air pollution affects upregulation of proinflammatory mediators is of considerable interest. We have previously described a methodology to create a small population (< 200) of particles with known chemical composition, dose them onto lung cells in vitro, and monitor the upregulation of a single proinflammatory response. Here we report the ability to monitor changes in the secreted proteome of a cell culture dosed with < 200 designed particles using a mass spectrometry readout. Through dosing a modified A549 cell line with LPS, carbon particles, or LPS containing carbon particles, we demonstrate that the particle core serves as a mechanism by which to deliver potentially toxic species to a cell, and it acts synergistically with those chemical species to initiate additional changes in the secreted proteome. We hypothesize that an insoluble particle core and the other components of the particle exhibit synergy with respect to effecting changes in the secreted proteome, which may be the reason it has proven challenging to determine the specific chemical species on particulate air pollution responsible for the observed adverse health effects.

9.3 Introduction
Inhalation of particulate air pollution results in local and systemic inflammatory response (213) that in turn can lead to a range of acute responses, such as wheezing and tightness of chest to myocardial infarction (31, 32), as well as to a range of chronic responses, such as airway remodelling and atherosclerotic plaque growth and destabilization (285, 310). The first step in the inflammatory response is the secretion of proinflammatory mediators, known as cytokines
A number of studies have correlated the presence of elevated levels of these proinflammatory mediators in the blood and in tissues with the pathogenesis of the majority of the adverse effects on human health that result from particulate air pollution.

Previously, we have demonstrated the ability to measure the downstream differential expression of a membrane bound proinflammatory mediator, intercellular adhesion molecule (ICAM)-1, that results from dosing a cell culture with up to 150 designed particles that had been formed in an AC levitation trap (73, 121, 122). ICAM-1 is a product of the major proinflammatory pathway nuclear factor (NF)-κB’s activation and as a result the measurement of the ICAM-1 protein has yielded valuable data with regards to the association of the chemical composition of particulate matter with its overall toxicity (73, 121, 122). However, there are numerous other proinflammatory pathways, as well as hundreds of other intercellular signalling molecules. The ability to monitor changes in all molecules, a subset of which is the secreted proteome, as a function of the particle chemical composition would provide more information regarding the cellular response, from which an improved understanding of particulate air pollution can be obtained.

Numerous methods have been developed to quantify the secreted proteins in a sample. The majority of these techniques are antibody based, such as the ELISA technique that is both selective and sensitive (96, 213); it is recognized that such assays are hypothesis driven, meaning that information only on those proteins that are specifically selected can be obtained. However, this is not the case when the readout device is capable of detecting molecules for which no previous information is necessary, such as soft ionization mass spectrometry, where the ionization sources such as matrix assisted laser desorption/ionization (MALDI) or electrospray (ES) are used. These ion sources are not highly-selective with respect to what compounds are ionized. Therefore, a technique such as MALDI-MS can in theory be used to detect many of the biomacromolecules present in a sample. The goal of this study is to develop sample processing methodology in which a commercially available MALDI-TOF-MS can be used to monitor the
differential expression in the secreted proteome of a human lung cell culture of $10^5$ to $10^6$ cells dosed with up to 150 particles of known and designed chemical composition. Thus, the challenge of this study was to differentiate between the relative abundances of secreted biomacromolecules from a small population of cells dosed with an even smaller population of particles. Though both the cell count and particle dose is low, the potential to detect differential changes is feasible. For instance, as measured by an ELISA, a cell culture of $3 \times 10^6$ cells grown in which all cells were stimulated has been measured to generate a secreted mass of $\sim 20$ pg of interleukin (IL)-8. Assuming all cells to be equivalent, each cell secreted $\sim 6.7$ attograms. If every cell on an 18 mm$^2$ coverslip, with a population of $\sim 500,000$ cells, were to secrete this mass of IL-8, this would equate to 0.79 femtomoles of IL-8, which may potentially be detectable using a mass spectrometry readout. In our methodology however, the number of particles dosed onto the cell culture from the AC levitation trap (< 200) will be insufficient to elicit a response from every cell in the culture. This makes the core challenges of this study the extraction and concentration of enough of the secreted biomacromolecules in the medium in order to observe biomolecule ion signals of sufficient S/N and reproducibility that differences in the secreted proteome are measurable. Because the nature of this study was method development, and numerous intercellular mediators involved in the inflammatory response have a molecular weight between 10,000 and 20,000, the mass range of the mass spectrometer was set between 10,000 and 20,000. It is recognized however, that a broader mass range would lead to a more complete picture as to what is going on in the system on the whole.

9.4 Experimental

9.4.1 Materials

The particles generated throughout this study consist of a solid elemental carbon core, where the source of the carbon is dispersed carbon nanoparticles (India ink, Speedball, product
and/or lipopolysaccharide (LPS), an endotoxin, from *Escherichia coli* (Serotype 0111:B4, L-2630, lot; #76H4099, Sigma Chemical Co., St. Louis, MO, USA).

Starting solutions used to levitated particles consisted of 5% India Ink or 5% India Ink with 2 mg/mL LPS. These solutions resulted in the formation of an aggregate of elemental carbon nanoparticles, that had a spherical appearance (with a diameter that follows a Gaussian distribution with a mean and SD of 6.3 ± 0.4 μm (121)) when viewed using an optical microscope, containing either 0 or 52 pg of LPS depending on the starting solution used. Each of the latter aggregates had ~280 EU of LPS/mg particle (121).

### 9.4.2 Cell Culture

A549 cells (American Type Culture Collection, Manassas, VA, USA), a human type II alveolar-like cell line originally derived from a patient with bronchioloalveolar carcinoma was transfected with the adenovirus 5 E1A gene. The transfection increased the likelihood that a response to the LPS would occur given that regular type A549 cells exposed to LPS derived from *E. coli* does not activate the NF-κB pathway, whereas the transfected cell line does (97, 133, 134, 161, 162, 222).

All cell cultures were grown to >95% confluence on an 18 × 18 mm² glass coverslip in a 6-well plate (Corning) in minimum essential medium supplemented with 5% heat-inactivated fetal bovine serum in 5% CO₂ at 37 °C.

### 9.4.3 Particle Generation and Deposition onto Lung Cells *in vitro*

10 μL of a starting solution were placed into the reservoir of a droplet-on-demand dispenser, equipped with a nozzle that had a diameter of 60 μm (MJ-AB-01-60, MicroFab Technologies Inc., Plano, TX, USA). The dispenser was then positioned 1 mm above the 2 mm hole in the induction electrode of the AC trap (figure 9.1). The piezoceramic element of the droplet dispenser was activated, at a rate of 120 Hz, causing a jet of liquid to be ejected from the nozzle of the droplet dispenser at 120 Hz. Each jet of liquid collapsed to form a single droplet.
Each droplet had a net charge resulting from the elevated number of anions that was a result of a DC potential applied to the induction electrode that created an electric field which effected ion mobility in the liquid at the nozzle and consequently charge separation in the jet. With a $+100\text{ V}$ DC potential, the electric field induced a net charge of $-200 \pm 25\text{ fC}$ onto each droplet (27). The presence of the net charge allowed the droplets to be levitated by the electric fields within the AC trap (figure 9.1A) (59). In any given experiment, a population of 50 to 100 droplets were levitated (121, 122). Within seconds of each droplet being formed, the water in it had evaporated, leaving behind an aggregate that consisted of the solutes of low volatility and nanoparticles that were in the dispensed droplets (figure 9.1B). Hereafter, these residues are referred to as particles.

With a population of particles levitated, a cell culture grown to 95 % confluence on an 18 X 18 mm$^2$ coverslip was then prepared for particle deposition through first draining of all but $15.9 \pm 2.5 \text{ µL}$ of the growth medium (122), and manually positioning the coverslip atop the deposition electrode of the AC trap. Application of a 500 V DC potential to the deposition electrode extracted the levitated particles from the AC trap and they impacted on the cell culture over an area $< 0.4 \text{ mm}^2$ (figure 9.1C) (25). The coverslip was then removed from the apparatus and placed into a sterile (35 mm X 10 mm) tissue culture dish, which itself was placed into an incubator set at 100% humidity, 37 °C and 5% CO$_2$ (figure 9.1D). The negative control was a cell culture grown to > 95 % confluence that had all but $15.9 \pm 2.5 \text{ µL}$ (mean $\pm$ SD) of its medium removed prior to being placed into an incubator set to 5 % CO$_2$, 100 % humidity and 37 °C (122).

The abundance of species having low volatility in the starting solution consisting of only LPS was insufficient to form a particle large enough to be levitated. For this starting solution, it was loaded into the droplet dispenser and a population of 100 droplets were delivered directly onto the centre of prepared cell cultures.
9.4.4 Collection of the Supernatant

The supernatant was collected 0.5, 6, 12 or 18 hours following particle deposition through five washes of a 100 μL aliquot of PBS over the entire coverslip (figure 9.1 E), then transferred to a micro-centrifuge tube and stored in a freezer at -20 C, where it was kept until further analysis. Through microscopic analysis, little to no cell lysates was observed as a result of the washing process.

9.4.5 MALDI-TOF-MS Preparation and Analysis of the Supernatant

A C18 ZipTip (Millipore, Bedford MA) was used in accordance with the method described in its accompanying manual to purify and concentrate the biomacromolecules in the supernatant. Briefly, the pH of the supernatant washing was adjusted by adding 4 μL of 2.5 % trifluoroacetic acid (TFA), then loaded onto the C18 ZipTip column, rinsed with a 0.1 % TFA solution, and finally eluted using 4 μL of 0.1 % TFA/50 % ACN solution onto a stainless steel MALDI plate that had been pre-coated with a layer of sinapic acid (Sigma, St. Louis MO). The layer was prepared by first dissolving sinapic acid in acetone past the point of saturation, then
aliquoting ~20 µL of the solution onto the stainless steel MALDI plate and then letting the droplet dry.

The mass spectrometer (MS) used was a MALDI-TOF-MS (model MALDI-LR, Waters Corp., Manchester, UK). The MS was operated in linear mode with a pulse voltage = 1400 V, source voltage = 15000 V, a multichannel plate detector potential difference of 1800 V and a TLF delay of 1500 ns (figure 9.1F).

9.4.6 Analysis of MALDI-TOF-MS Data

All mass spectra were smoothed five times with a Savitzky-Golay algorithm using a window size of 3, and then background subtracted. The m/z and peak intensity of every peak in the range from m/z = 10,000 to 20,000 were tabulated. For each spectrum, the ion with a m/z of 12,426 was selected as a ion signal reference because the S/B for it had a RSD = 16 % across all samples. For comparative data analysis, the relative abundance of every other ion in a given spectrum was normalized to the abundance of the ion at m/z = 12,426. Differences in the relative abundance of a single ion resulting from the exposure of different particle types were deemed significant if the difference between the averages of each ion’s normalized signal was greater than the cumulated standard deviation of each.

9.5 Results

9.5.1 Reproducibility Between Sequential Mass Spectra from a Single Sample

Given that MALDI-TOF-MS analysis is accomplished through the rastering of a laser over a heterogeneous solid sample, reproducibility is an issue as evidenced by “hotspots”. These are localized areas within a sample being analyzed via MALDI-MS where a larger abundance of analyte ions are detected. It is this large variation in ion signal intensity between laser shots that has lead to the notion that MALDI-MS is not an appropriate means by which to collect quantitative data. However under the conditions employed in this study, the absolute abundances of the ions in the mass spectra were found to be fairly consistent, as demonstrated by the 27.4 %
RSD for the m/z = 15,446. Given that the relative signal intensity within a single sample was important for this work, the overall effect resulting from the differences observed between sequential laser shots was of interest. Shown in figure 9.2 are sequential mass spectra collected from a single sample that had been dosed with a population of < 100 LPS containing carbon particles.

![MalDI-TOF mass spectra](image)

**Figure 9.2** Sequential MALDI-TOF mass spectra of a single supernatant of an A549 cell culture dosed with a population of LPS containing carbon particles. Each mass spectrum is the accumulated average of 10 individual spectra. Shown in the order in which the spectra were collected, beginning from (A) and finishing at (I).

The relative abundance, once normalized to the reference ion m/z = 12,426, of all ions present in each spectrum were similar, as demonstrated by the 9.5 % RSD for the m/z = 15,446. This data shows that even though there is some variation of the absolute abundance of an ion of interest (27.4 % RSD), the variation can be significantly reduced (down to 9.5 % RSD) once normalized to a reference ion. Therefore, quantitative data regarding relative abundance of molecules can be collected via MALDI.
9.5.2 MALDI-TOF-MS of Collected Supernatants

Four representative mass spectra collected for each particle type at each of the incubation periods are plotted as figures 9.3 to 9.6. Each mass spectrum shown is the cumulative average of > 50 individual mass spectra collected from an individual sample.

![MALDI-TOF mass spectra](image)

**Figure 9.3** MALDI-TOF mass spectra of the supernatants of A549 cell cultures dosed with a population of LPS containing carbon particles, LPS droplets, carbon particles or negative following a 30 minute incubation period.
Figure 9.4 MALDI-TOF mass spectra of the supernatants of A549 cell cultures dosed with a population of LPS containing carbon particles, LPS droplets, carbon particles or negative following a 6 hour incubation period.
Figure 9.5 MALDI-TOF mass spectra of the supernatants of A549 cell cultures dosed with a population of LPS containing carbon particles, LPS droplets, carbon particles or negative following a 12 hour incubation period.
9.5.3 Processing of the MALDI-TOF-MS Spectra of the Collected Supernatant

The relative intensity of every ion peak in each mass spectrum was calculated using equation 9.1.

(Eq. 9.1) \[ \text{Relative Intensity (Ion)} = \frac{\text{Signal(Ion)}}{\text{Signal(12426)}} \]

Where Signal(Ion) is the intensity of the ion of interest and Signal(12426) is the intensity of the ion with a m/z = 12,426. The relative intensity, as calculated from Eq. 9.1, for each ion collected from the negative was subtracted from the relative intensity of each ion of interest for each particle type and each time point, and then plotted as a function of time (figure 9.7).
Figure 9.7 Relative abundance of ions observed in the MALDI-TOF mass spectra of the supernatant collected from an A549 cell culture dosed with a population of LPS containing carbon particles, LPS droplets, carbon particles or no particle, at various lengths of incubation.

Error bars indicate one standard deviation of the cumulated mass spectra as calculated based on four different samples. The mean relative intensity for a given ion obtained for the negative is set to zero for each time point, for clarity of illustrating instances of both up and down regulation. Also, at each time point, the data points for different particle types are offset for viewing clarity.
Of the 14 ions observed in the m/z range from 10,000 to 20,000, 13 were observed to experience differential expression at one or more time points, relative to the negative control.

9.6 Discussion

9.6.1 Demonstrating the Use of MALDI-TOF-MS to Collect Quantitative Data

To address the issues associated with the ability of using MALDI-MS to collect quantitative data and to attempt to ascertain reproducible data, each sample was prepared in the same fashion and multiple spectra for each sample were collected. That, coupled with the use of the apparent homeostatic ion with an m/z of 12,426 as a reference resulted in sufficient reproducibility between samples. Reproducibility was observed for each ion in both the sequential laser shots within a single sample (figure 9.2, RSD = 9 % for m/z = 15,446) and between multiple samples dosed with the same particle type and incubated a given period of time (figures 9.3 to 9.6, an average of all the RSD collected for every ion in each group of 42 %). Note that the RSD of the relative ion signal intensity is much larger between samples than within a single sample; it is speculated that this variation results from the efficiency of biomacromolecule capture during supernatant collection, rather than from the MALDI-MS.

With regards to extrapolating relative concentration through comparing the peak heights of multiple ions within a single spectrum, of major concern are the ionization efficiencies of the analyte molecules. For this reason, it is a daunting task to ascertain the relative abundance of any species through simply looking at a single mass spectrum. However, the relative abundance of a single ion in one sample versus the same ion in another sample can be deduced through comparing the relative abundance of the ion in each spectrum; the ionization efficiencies of compounds detected should be consistent within each laser shot event, provided the sample preparation for all samples were performed in a reproducible manner, and if the settings on the mass spectrometer were the same during the collection of the mass spectra for both samples, makes direct comparison permissible.
However absolute quantitation, in the form of a concentration measurement, of secreted biomacromolecules present in the sample can not be readily obtained with this methodology. For this reason, only shifts in the form of significant up or down regulation, relative to that of a negative control, are reported. Further characterization of these shifts, in the form a specific dose (number of particles) – response (picograms of cytokine secreted) relationship would require additional analysis and the method of standard addition, as well as independent confirmation by an alternative, well established technique such as an ELISA.

9.6.2 Preliminary Observations of Mass Spectra Collected

At the 30 minute time point, differences relative to the negative control were observed in the form of both up and down regulation of various biomacromolecules. The biomolecules that were observed to be upregulated in this short of a time frame are believed to be premade and stored in vesicles within the cell, ready to be released on a moments notice. The species that are down regulated are possibly those mediators that are taken up by the cell once the culture is stimulated; significant down regulation in this short of a period of time suggests that numerous receptors for these molecules must be premade by the cell and readily expressed. Additionally, a relatively high reproducibility between the mass spectra from similar samples was observed, in spite of the fact that each cell culture was dosed with a low particle count of below 150.

Following a 6 hour incubation period, relative to the negative control, no differential upregulation of any of the signals was observed; however, some ion signals decreased, indicating that down regulation occurred. Following both the 12 and 18 hour incubation periods, there is the potential for proteins produced through transcription to be secreted and hence detected. Significant up and down regulation of numerous biomacromolecules were observed at these longer incubation time points.
9.6.3 Tentatively Identifying the Ion Peaks of Interest

Based solely on their mass, a preliminary identification of the observed ions is presented in Table 9.1. The search was limited to the secreted biomolecules of human lung cells, including cytokines, chemokines and β-defensins. This resulted in the tentative identification of eight of the fifteen biomacromolecules of interest, those that were significantly upregulated or downregulated with respect to the negative, based solely on m/z.

Table 9.1 Preliminary identification of ions observed in the mass spectra collected from the supernatant of A549 cells dosed with LPS, carbon particles or LPS containing carbon particles and incubated for varying lengths of time. The triangle symbol indicates that the respective ion was observed to be differentially expressed relative to the control.

<table>
<thead>
<tr>
<th>m/z of Peak</th>
<th>Preliminary Identification</th>
<th>LPS</th>
<th>Carbon</th>
<th>LPS + Carbon</th>
</tr>
</thead>
<tbody>
<tr>
<td>10368</td>
<td>XCL2</td>
<td>▲</td>
<td>▲</td>
<td>▲</td>
</tr>
<tr>
<td>10637</td>
<td>Unknown</td>
<td>▲</td>
<td>▲</td>
<td>▲</td>
</tr>
<tr>
<td>10938</td>
<td>Unknown</td>
<td>▲</td>
<td>▲</td>
<td>▲</td>
</tr>
<tr>
<td>11425</td>
<td>CCL23</td>
<td>▲</td>
<td>▲</td>
<td>▲</td>
</tr>
<tr>
<td>11741</td>
<td>CXCL9</td>
<td>▲</td>
<td>▲</td>
<td>▲</td>
</tr>
<tr>
<td>13649</td>
<td>Unknown</td>
<td>▲</td>
<td>▲</td>
<td>▲</td>
</tr>
<tr>
<td>14289</td>
<td>CCL25</td>
<td>▲</td>
<td>▲</td>
<td>▲</td>
</tr>
<tr>
<td>15055</td>
<td>IL-3</td>
<td>▲</td>
<td>▲</td>
<td>▲</td>
</tr>
<tr>
<td>15446</td>
<td>IL-21</td>
<td>▲</td>
<td>▲</td>
<td>▲</td>
</tr>
<tr>
<td>15980</td>
<td>Unknown</td>
<td>▲</td>
<td>▲</td>
<td>▲</td>
</tr>
<tr>
<td>16900</td>
<td>IL-22</td>
<td>▲</td>
<td>▲</td>
<td>▲</td>
</tr>
<tr>
<td>17335</td>
<td>TNF-alpha</td>
<td>▲</td>
<td>▲</td>
<td>▲</td>
</tr>
<tr>
<td>18089</td>
<td>BD-29</td>
<td>▲</td>
<td>▲</td>
<td>▲</td>
</tr>
<tr>
<td>18547</td>
<td>IL-10</td>
<td>▲</td>
<td>▲</td>
<td>▲</td>
</tr>
</tbody>
</table>

The inherent limitation of one dimensional mass spectrometry readout is that positive identification of the ion of interest is tenuous without the use of methods such as standard addition, which could improve the likelihood of a correct assignment and an initial estimate of that molecule's concentration. In order to positively identify these peaks, additional experimentation such as an antibody assay like an ELISA or bottom-up or top-down MS/MS would be required. However, the true benefit of the mass spectrometry readout is that it can be used to screen for potential biomacromolecules, many of which may not be initially considered as molecules of interest.

Of the potentially identified peaks, we speculate that the ion peak with an m/z = 17,335 is TNF-α given the incubation time at which it was observed in the mass spectra and the stimulus to
which its upregulation was effected (163). TNF-α is both rapidly secreted by stimulated cells and again many hours later following transcription via the NF-κB pathway, which is itself initiated by TNF-α. However, to verify this further experimentation must be undertaken.

### 9.6.4 Differential Expression as a Result of Exposure to LPS

A series of biomacromolecules are secreted following exposure to LPS; a response was observed whether the LPS was delivered onto the cell culture on an elemental carbon particle or simply directly delivered as soluble LPS (table 9.1).

Of the molecules tentatively identified as a byproduct of LPS exposure, both IL-3 and TNF-α, are known to be secreted by alveolar epithelial cells following LPS exposure. In addition, β-defensins are produced by the body to fight bacterial infection. It was not surprising then, to find that exposure of the cell culture to LPS, an indicator of bacterial infection, would cause the differential expression of a β-defensin, in this case BD-29.

### 9.6.5 Evidence of Synergy

A biomacromolecule of particular interest is m/z = 10,637. Like the ions with an m/z of 13,649 and 18,089, m/z = 10,637 is secreted both 30 minutes and then 18 hours following exposure, suggesting that the molecule is both stored in vesicles for immediate secretion and transcribed later to sustain the initial response. However, unlike these other two molecules, m/z = 10,637 is secreted following a 30 minute incubation period upon the exposure to only the LPS containing carbon particle. Unfortunately, screening based on the measured m/z thus far has failed to tentatively identify this ion. We view this discrepancy of secretion pattern as a potential sign of synergy, wherein the LPS containing carbon particle behaves as both a mechanism by which LPS is delivered to the cell culture and as an entity in and upon itself wherein the cell culture responds to the LPS containing carbon particle as its own unique species. We speculate that such an observation illustrates the overall challenge in the analysis of particulate matter toxicity; the particle itself changes the nature by which the compounds on it interact with the cell and thus alters the responses the cell then has towards these compounds. This change may both
enhance the response, as observed with m/z = 10,637, or limit the response, as observed in m/z = 13,649, m/z = 10,938, m/z = 11,425, m/z = 15,446, m/z = 15,980, and m/z = 18,547.

9.6.6 Further Characterization of the E1A-A549/LPS Model

We have previously reported a preliminary study of the model system used in this study, the dosing of LPS containing carbon particles on A549 cells transfected with adenovirus E1A (121, 122), through measuring the upregulation of ICAM-1 as a function of particle dosage. In those studies, cell culture wide expression of ICAM-1 was observed 24 hours following exposure to both LPS and LPS containing carbon particles. It is our current hypothesis that in order for culture wide ICAM-1 expression to occur, intercellular signalling between cells must occur rapidly, as a time period of 18 to 24 hours is required for ICAM-1 expression through translation to take place. For this reason, there was an interest in the thirty minute time point, where differentially secreted molecules relative to the control following exposure to both LPS and LPS containing carbon particles would be of significance. Note that this particular differentially secreted molecule must not be secreted following exposure to carbon particles. The biomacromolecule with an m/z = 13,649 is the only one to fit such a criteria and thus we hypothesize that it is a mediator of significance for the observed culture wide response. Unfortunately, at this stage we have failed to tentatively or positively identify this molecule.

9.7 Conclusions

The ability to dose a cell culture with up to 150 discrete particles and monitor changes in the secreted proteome has been demonstrated.

9.8 Acknowledgments

Discussions with Shizu Hayashi. Funding provided by the Canadian Foundation for Climate and Atmospheric Sciences (CFCAS), the Natural Sciences and Engineering Council of Canada (NSERC), the British Columbia Lung Association and the Heart and Stroke Foundation of Canada.
Chapter 10

SYNERGISTIC TOXICITY OF ENDOTOXIN AND ELEMENTAL CARBON

Endotoxin on elemental carbon particles synergistically induce ICAM-1 expression in alveolar epithelial via TLR-4

It is this problem – global air pollution – that presents the true strategic threat to which we must now respond. The political battles against local air pollution are the easiest to organize because the direct effect on human health can be seen vividly in the hazy, smog-choked skies and heard loudly in the hacking and coughing of the affected citizenry. The battles to control regional air pollution are more complex because the people who are most affected often live in a different, downwind region from the people most responsible for causing it. Still, this problem is finally being addressed, even as heated arguments continue over cause and effect.

Al Gore, Earth in the Balance (108)

In drafting clean air legislation, politicians attempt to balance public health with economic growth. The focus is typically on minimizing the economic impact of the legislation, and thus there is an emphasis on reducing the emissions of only the most toxic species; the challenge is in elucidating the identity of these species. Described in this chapter is the first study that used the methodology developed thus far in this thesis where we studied the proinflammatory effect of a chemical species commonly associated with particulate matter, endotoxin.
10.1 Context

Sections 10.2 to 10.7 of this chapter have been submitted and reviewed positively in their entirety in the journal American Journal of Physiology: Lung, Cell and Cellular Biology. This was the third publication resulting from the collaboration between the Agnes group from SFU and the van Eeden group from James Hogg iCAPTURE Centre at St. Paul's Hospital. Though the impact factor of AJP is similar to that of JACS, the overall impact of this publication will in all likelihood prove to be greater than the work published in Chapter 7 as this was the first publication that used our particle delivering methodology for hypothesis driven studies; the findings of which may have large implications in the particulate matter toxicity field.

The authors of the manuscript were myself, Danielle Balik, Teresita Mariana Cruz-Sanchez, Tillie-Louise Hackett, Gurpreet Singhera, Stephan van Eeden and George Agnes. Danielle Balik, an undergraduate student, assisted with the confocal microscopy, and Teresita Mariana Cruz-Sanchez, Tillie-Louise Hackett, Gurpreet Singhera ran the ELISA samples, while all other experiments, data collection and all processing was undertaken by myself.

10.2 Abstract

It has been hypothesized that endotoxins, such as lipopolysaccharide (LPS), in coarse particulate matter is responsible for differential expression of proinflammatory mediators by lung tissue. Soluble LPS introduced directly into the lung is known to induce a large proinflammatory response through initiation of the nuclear factor (NF)-κB pathway via toll-like receptor (TLR)-4. However, it remains unclear if the quantity of LPS delivered to the lung via particulate matter is sufficient to elicit a significant proinflammatory response. To address this issue, we developed an apparatus with an associated methodology wherein particles having known multi-component compositions are generated while levitated in air and deposited directly onto a cell culture to study downstream biological responses of human lung tissues. A549 cells, a type II alveolar epithelial cell line known not to have the CD14 cofactor, were used. Less than 100 synthesized
particle mimics of endotoxin containing carbon particles, each containing 2.5 ± 0.2 pg LPS per particle corresponding to ~14 endotoxin units per milligram (EU/mg), which is similar to that typically measured on coarse ambient particle fractions, induced significant expression of intercellular adhesion molecule (ICAM)-1. In contrast, no ICAM-1 upregulation was measured for either endotoxin or carbon particles alone. A549 cells dosed with as few as 50 endotoxin containing carbon particles, each having 52 ± 3 pg LPS per particle corresponding to ~280 EU/mg, onto an area of the cell culture < 0.5 mm² produce IL-1β that contributes to the propagation of ICAM-1 expression in cell cultures. LPS containing carbon particles enhance particle-cell interaction and recruit intracellular TLR-4 to the site of particle deposition within 30 minutes following their delivery to A549 cultures. TNF-α augmented the intercellular signalling of the IL-1β. These results illustrate that the presentation of LPS to a cell on a carbon particle effected synergy with respect to the outcome of ICAM-1 upregulation. By analogy, these in vitro results suggest that ambient particles can present biological compounds such as endotoxin to lung cells in vivo in a manner such that minute concentrations elicit a significant downstream pro-inflammatory response.

10.3 Introduction

Acute inflammation within the lung, initiated by exposure to elevated levels of ambient particles in the troposphere (PM₁₀) (89), has been implicated in the pathogenesis of numerous pulmonary and cardiovascular diseases (32, 65, 66, 166, 258). Though the fine and ultrafine fractions of PM₁₀ are strongly associated with cardiovascular induced morbidity and mortality (260, 279, 280, 308), by mass, the coarse fraction of PM₁₀ has been shown to induce a larger proinflammatory response in the lung than the fine fraction (131, 229, 254). From these findings it has been hypothesized that the lipopolysaccharide (LPS) component of the outer-wall of Gram-negative bacteria, commonly referred to as endotoxin (figure 10.1A), plays a significant role in PM₁₀ induced inflammation in the lung (254). Concentrations of endotoxin are typically elevated
in the coarse fraction of PM$_{10}$, and coarse fraction PM$_{10}$ tends to be generated by mechanical means, such as wind or traffic (130, 212). What remains unclear is the quantity of endotoxin on PM$_{10}$ required to (i) induce local injury and/or a systemic response, and (ii) how the presence of the particle affect the LPS/cell interactions.

The study of the pulmonary injury from PM$_{10}$ bound endotoxin is challenging for a number of reasons. First is the issue of dose. Assays employed to quantify the LPS bound to PM$_{10}$, the Limulus Ambeocyte Lysate (LAL) assay and lipid A by gas chromatography-mass spectrometry (GC-MS) (figure 10.1B), were designed to measure soluble LPS and as such yield inconsistent results, as matrix effects attributed to LPS-particle interactions influence accuracy. It is not uncommon for multiple laboratories to measure the same sample of PM$_{10}$ and report significantly different endotoxin concentrations (241). The inability to accurately and consistently quantify endotoxin on PM$_{10}$ leads to erroneous interpretation of dose-response results. Secondly, endotoxin is not a compound, but a class of compounds. The source of the endotoxin greatly affects the extent to which cells secrete proinflammatory mediators (142, 168). Hence conclusions in manuscripts range from endotoxin being the major inflammatory agent in PM$_{10}$ to endotoxin being considered an insignificant contributor to the overall response to PM$_{10}$. 

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To address these issues, we developed a methodology wherein we can design and create particles of known size and chemical composition. In this study the particles consist of elemental carbon plus a known, tropospherically relevant quantity of LPS (Table 10.1). The endotoxin selected in this study, *Escherichia coli* serotype 0111:B4, is a relatively innocuous form; this endotoxin serotype alone elicits little to no response from A549 cells (167, 168). The A549 cell line is derived from a bronchoalveolar carcinoma with phenotypic type II pneumocyte characteristics (184, 244). Alveolar epithelium type II pneumocytes secrete the surfactants that prevent the alveoli from collapsing onto themselves and constitute only ~5% of the alveolar surface area, even though type I pneumocytes comprise ~40% of the alveolar epithelial cells (92). A549 cells lack both the receptor for LPS (115) and its co-receptor (168, 212), suggesting that this cell line is an appropriate model for the study of potential synergy between LPS and a carbon particle with respect to causing injury that elicits measurable differential downstream biological response.
Figure 10.1 A summary of two assays used to determine endotoxin concentration on PM$_{10}$.
(A) The source and chemical structure of endotoxin. (B) Conventional assays for endotoxin. Particles are first collected on and extracted from a filter. Endotoxin extracted from particles is then analyzed by one of two assays, the Limulus Amoebocyte Lysate assay, or chemical hydrolysis to liberate 3-hydroxy fatty acid that is measured using GC-MS. (C) The particle creation, levitation and deposition methodology employed in this study to dose A549 cells with particles of a designed chemical composition.
The hypothesis of this study was that the quantity of endotoxin present on coarse particles in the troposphere causes injury to lung tissue that is communicated by the upregulation of proinflammatory mediators. To address this, LPS-containing carbon particle types were prepared and deposited, *in vitro*, onto alveolar type II cells prior to monitoring the downstream biological response (figure 10.1C) (121, 122). The downstream biological response selected for this study was the expression of intercellular adhesion molecule (ICAM)-1, interleukin (IL)-6, IL-8, IL-13 and tumor necrosis factor (TNF)-α. ICAM-1 was the primary biomarker of interest for four reasons: it is a crucial mediator in the inflammatory response as it is involved in the critical step of leukocyte recruitment (78); its upregulation can be stimulated in type II pneumocytes, as opposed to in type I pneumocytes where it is expressed constitutively, along with IL-6 and IL-8 (167, 256); its expression is triggered by the NF-κB pathway (77, 160); it is membrane bound and as such its differential expression as a function of separation distance from the site of particle deposition on the cell culture afforded opportunities to investigate intercellular signaling (121, 122). Results are presented that demonstrate the particle core acts synergistically with LPS to induce ICAM-1 expression and IL-1β secretion by A549 cells. Relative to a carbon particle, LPS containing particles were being internalized by the cell at a higher rate, thus enabling LPS to permeate the cell membrane where it interacted with intracellular toll-like receptor (TLR)-4. The partial internalization of LPS containing carbon particles facilitated ICAM-1 differential expression through TLR-4 in the absence of CD14, suggesting that the presentation of LPS on a particle itself was mediating TLR-4 activation. Additionally, this injury to the cells by the LPS containing carbon particles effected IL-1β secretion that promoted widespread ICAM-1 upregulation across the entire cell culture. The IL-1β induced upregulation of ICAM-1 was augmented by the secretion of TNF-α (139). Taken together, these results show that LPS containing carbon particles cause a pronounced proinflammatory response locally through TLR-4 and distally through the secretion of IL-1β.
10.4 Experimental

10.4.1 Cell Culture

The A549 cell line (American Type Culture Collection, Manassas, VA, USA) was grown to > 95% confluence on an 18 mm X 18 mm glass coverslip in a 6-well plate (Corning) in minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum in 5% CO₂ at 37 °C and 100% humidity. No antibiotics were added to the growth medium.

The negative control used in this study were cell cultures grown to > 95% confluence on an 18 mm X 18 mm glass coverslip that had all but 15.9 ± 2.5 μL of their medium removed prior to being placed into an incubator (122). The positive control used in this study were cell cultures grown to > 95% confluence on an 18 mm X 18 mm glass coverslip, each bathed in 2 mL of growth medium, to which 10 μL of a solution consisting of 10 μg/mL of TNF-α (Sigma-Aldrich, T6674-10UG, Oakville, ON, Canada) in water had been added.

10.4.2 Particle Generation and Deposition onto Lung Cells in vitro

Starting solutions consisted of 5% India ink (Speedball, product #3338, Statesville, N.C., USA) by volume in distilled deionized water with either 0, 0.1 or 2 mg/mL of LPS from Escherichia coli (Serotype 0111:B4, L-2630, lot; #76H4099, Sigma Chemical Co., St. Louis, MO, USA) in distilled water; concentrations were prepared through serial dilution of the stock solution. Prior studies have used India ink as a source of elemental carbon (109). India ink is an emulsion of ~20 nm diameter elemental carbon particles.

A 10 μL aliquot of a starting solution was placed into the reservoir of a droplet-on-demand dispenser (MJ-AB-01-60, MicroFab Technologies Inc., Plano, TX, USA), which itself was positioned above the induction and ring electrodes of the AC trap (figure 10.1C). Upon activation of the piezoceramic element of the droplet dispenser, a jet of liquid was ejected from the nozzle of the droplet dispenser. The momentum of the jet caused it to separate from the liquid within the dispenser and collapse into a single droplet. Within the jet, while it was still attached to
the dispenser, a 200 V DC potential applied to the induction electrode positioned 3 mm from the
dispenser nozzle affected ion mobility to the extent that each resultant droplet had an induced net
charge of 200 ± 25 fC. The presence of this net charge enabled the droplets to be levitated by the
electric field created in an AC trap by application of a sinusoidal waveform (4.5 kV<sub>o-p</sub>, 60 Hz) to
the ring electrodes of the trap (121).

The volatile compounds within each droplet, in this case water, evaporated within 20
seconds under ambient conditions to leave behind a residue that consisted of the solutes of low
volatility, in this case carbon and LPS. The rapid aggregation and precipitation of the dispersed
nanoparticles and dissolved solids, respectively, in these residues resulted in LPS molecules being
encapsulated throughout, and on, elemental carbon nanoparticle aggregates. These residues,
which are large micrometer sized particles were thus each comprised of nanoparticles bound by
noncovalent forces. The residues are hereafter referred to as particles.

The final diameters of the particles are a function of numerous factors, including the
droplet dispenser nozzle dimension, the waveform used to actuate the piezoceramic element of
the dispenser, the percentage by volume of the nonvolatile components that make up the initial
droplet volume, and the electric field in the AC trap. Due to small fluctuations in dispensed
droplet volume and induced net charge, the size of the final particles generated followed a
Gaussian distribution with an average diameter of 6.3 ± 0.4 μm (mean and SD), which places
these particles into the size range of coarse particulate matter. Depending on the starting solution
used, each particle contained 0, 2.5 ± 0.2 or 52 ± 3 pg of LPS, 0, ~14 or ~280 EU/mg,
respectively. The amount of LPS per particle was calculated given that a known quantity of
standardized LPS was added to the starting solution, and the initial volume of the dispensed
droplet was known. With a population of between 1 and 100 particles levitated, a cell culture
grown on a coverslip was drained of all but 15.9 ± 2.5 μL of its growth medium. The coverslip
supporting the culture was immediately placed on top of the bottom electrode of the AC trap,
directly below the ring electrodes. The particles were then extracted from the AC trap onto the cell culture through the application of a 500 V DC potential to the bottom electrode. The voltage applied to the bottom electrode during particle deposition was found to cause no differential ICAM-1 expression (figure 10.2). The particles were deposited over an area < 0.5 mm² of the culture using this procedure.

![Diagram](image)

**Figure 10.2** The effect of the voltage applied to the bottom electrode during particle deposition on the ICAM-1 expression by an A549 culture. Cultures were exposed to an elevated concentration of LPS (5 µg/mL) for 60 minutes, prior to placing the coverslip supporting the culture onto the bottom electrode of the EDB and applying the indicated potential. Note that a potential of 500 V was used to extract a population of particles from the EDB. Following a 24-hour incubation period, the relative upregulation of ICAM-1 was measured.

Within 10 seconds of particle deposition, the culture was placed into a sterile (35 mm X 10 mm) tissue culture dish that in turn was placed into an incubator set at 100 % humidity, 37 °C and 5 % CO₂. Dependant on the analysis, the incubation period ranged from 30 minutes to 24 hours. No additional medium was added to the culture following particle deposition.
10.4.3 Blocking Studies

0.5 µg of either goat anti-human TNF-α affinity isolated antibody (Sigma-Aldrich, T6674-10UG, Oakville, ON, Canada), rabbit anti-TLR-4 polyclonal antibody (Invitrogen, San Francisco, California), mouse anti-human IL-1β monoclonal antibody (R&D Systems, Minneapolis, Minnesota), or 10 µL of a 10 mM N-acetylcysteine (NAC, Aldrich) solution was added to 2 mL of growth medium that contained the cell culture grown in a six well plate 60 minutes prior to particle deposition.

10.4.4 Antibody Assay

The cells were fixed with a 1 % acetone solution for 10 minutes following a 24-hour incubation period. The primary antibody was mouse anti-human ICAM-1 monoclonal antibody (Immunotech, Marseille, France) and the secondary antibody was a fluorescently labelled goat anti-mouse IgG (Alexa Fluor 546, Molecular Probes, Eugene, OR, USA).

10.4.5 Fluorescence Microscopy

Analysis of fluorescently labelled cell cultures was undertaken using a Motic AE31 inverted fluorescence microscope (Motic, Ontario, Canada) fitted with an Epi-FI filter block (Green MG-1, Motic, Ontario, Canada) (180). Images were collected at the site of particle deposition (1.00 mm X 1.25 mm), hereafter referred to as the deposition site scan.

In addition, a series of images were collected along a vector of arbitrary direction from the site of particle deposition toward an edge of the coverslip. These sequential images contained at least 33 % overlap to ensure suitable reconstruction of a single image when they were combined using imaging software (Photoshop, Adobe) to construct a 6.75 mm X 1.00 mm scan wherein the site of particle deposition was at one end of the scan. This scan is hereafter referred to as the 7 mm² scan.
10.4.6 Image and Statistical Analysis

The intensity of the fluorescence emission in the scans was calculated using Image J software (Research Services Branch, National Institute of Mental Health, Bethesda, MD, USA) and integrated using Excel (Microsoft, Seattle, Washington). The extent of ICAM-1 upregulation per scan is reported as a percentage of the total fluorescence signal relative to positive controls, and plotted as a function of the number of deposited particles. Using the Excel Data Analysis package, a least squares linear regression fit was applied to the data. A significance test of the dose-response relationship was performed, and a p value less than 0.05 (90 %) was deemed significant (210).

The ability to deposit a known number of particles having a known composition and size onto a cell culture is a feature of this methodology. To compare the relative injury caused by various particle types across numerous studies, a data presentation concept termed proinflammatory potential (PIP) was developed. PIP is the relative ability of a specific particle type to induce differential expression of pro-inflammatory mediators. The numerical value of the PIP is the slope of a least squares linear fit to the dose-response data, which is a plot of the number of deposited particles to the relative expression of a proinflammatory mediator. For example, ICAM-1 upregulation was measured relative as the relative fluorescence emission at the site of particle deposition (73), termed PIP(ICAM-1). The error of the PIP(ICAM-1) value is the standard deviation of the slope of the linear regression fit.

10.4.7 NF-κB Translocation

Following particle deposition and a 2.5 hour incubation period, cells were fixed with a water solution containing 4.5% formaldehyde, then permeabilized with 0.2 % Triton X-100. Following a 30 minute pretreatment with a Protein Block (DakoCytomation, Carpinteria, California), cells were labelled with rabbit anti-human NF-κB p65 antibody (Invitrogen, San Francisco, California) as the primary antibody while the secondary antibody was a fluorescently
labelled goat anti-rabbit IgG (Alexa Fluor 546, Molecular Probes, Eugene, OR, USA). Images of
the site of particle deposition were then collected using a Motic AE31 inverted fluorescence
microscope (Motic, Ontario, Canada) fitted with an Epi-FL filter block (Green MG-I, Motic,
Ontario, Canada). The positive control was an A549 cell line that was transfected with the
adenovirus 5 E1A gene which causes the cell line to activate NF-κB following exposure to 1 µg
mL⁻¹ of LPS in medium. The negative control was the transfected cell line incubated with regular
medium.

10.4.8 Supernatant Analysis Via Enzyme-Linked Immunosorbent Assay

Following particle deposition and a 30 minute or a 12 hour incubation period, the
supernatant of the cell culture was collected. A 100 µL solution of PBS was washed over the cell
culture, taking care to ensure little to no cell lyses occurred; this was verified via optical
microscopy.

The levels of each cytokine in the supernatant were measured by enzyme-linked
immunosorbent assay (ELISA) and the concentration corrected for total protein. Human TNF-α
ELISA (limit of detection of 0.3 pg/mL) was purchased from R&D Systems Europe Ltd,
Abingdon, UK. Human IL-6 and IL-8 were measured using commercially available ELISA
Duosets from Biosource Europe, SA (limits of detection 0.5 pg/mL, 0.5 pg/mL, respectively).
The manufacturer's protocol was followed for each ELISA. The concentration of IL-13 was
determined with an in house protocol using human-IL-13 as the standard (R&D Systems.
Cat#213-IL-005), an anti-IL-13 mouse-anti-human monoclonal antibody (R&D Systems. Cat#
MAB2131) as the primary and anti-mouse horse radish peroxidase as the secondary.

10.4.9 Confocal Fluorescence Microscopy

30 minutes following particle deposition, the culture was fixed with a 1 % acetone
solution for 10 minutes, then permeabilized with 0.2 % Triton X-100 in PBS for 5 minutes.
Following a 30 minute pretreatment with a Protein Block (DakoCytomation, Carpinteria,
California, cells were then labelled with rabbit anti-human TLR-4 polyclonal antibody (Invitrogen, San Francisco, California) as a primary antibody and the secondary antibody was a fluorescently labelled goat anti-rabbit IgG (Alexa Fluor 546, Molecular Probes, Eugene, OR, USA).

A series of images were collected with a confocal fluorometer (Zeiss, Laser Scan Microscope 410 Invert). The vertical distance between each scan was set to 0.5 μm. The vertical resolution of the microscope was 1 μm.

10.5 Results

10.5.1 LPS Containing Carbon Particles Behave Synergistically to Induce ICAM-1 Expression on A549 Cells

The relative expression of ICAM-1 was measured at the site of particle deposition using fluorescence microscopy following the deposition of varying populations of LPS droplets, carbon particles, or LPS containing carbon particles onto a cell culture (figure 10.3). Neither LPS or carbon alone was found to cause significant ICAM-1 upregulation in the form of a dose-response relationship (p = 0.251 and p = 0.091 respectively), whereas the LPS containing carbon particles were found to cause significant ICAM-1 expression in a dose dependant manner (p = 2 x 10^{-10}). This was evidence of synergy as each component delivered individually elicited an insignificant response.
Figure 10.3 Differential expression of ICAM-1 as a function of dosage in particle deposition site scans.

A549 cells were dosed with populations of residues consisting of (A) 52 pg of LPS, (B) 6.3 μm diameter elemental carbon particles or (C) 6.3 μm diameter LPS containing elemental carbon particles, with 52 pg LPS per particle. All fluorescence emission signals measured from the air–liquid interface cultures incubated with particles were scaled relative to the positive control (TNF-α). Error bars represent the 95% confidence interval. Each of the data sets were fitted to a least squares linear regression line. The raw data used for panel C of figure 2 (n = 34) yielded an $R^2$ of 0.6639.

The PIP(ICAM-1) values were measured for an LPS droplet and LPS containing carbon particles having 0, 14 or 280 LPS EU per mg of particle (figure 10.4). The PIP(ICAM-1) was found to increase in a dose dependant manner as a function of the quantity of LPS on each carbon particle. These results indicate that exposure to < 100 coarse ambient particles, having tropospherically relevant levels of LPS, are able to induce the expression of pro-inflammatory mediators.
Figure 10.4 The proinflammatory potential (PIP) for ICAM-1 of A549 cells dosed with LPS, carbon particles, or LPS containing carbon particles. Error bars shown indicate the standard deviation of the slope of the fitted least square straight line. (*) indicates a significant (p < 0.05) dose-response relationship was observed.

10.5.2 The Observed ICAM-1 Upregulation is a Result of NF-κB Activation

As shown in figure 10.5, the translocation of the NF-κB p65 protein following LPS exposure was observed to be dependant on the presence of the carbon particle core. This result is also suggestive of the synergistic behaviour of LPS with the elemental carbon core to induce ICAM-1 expression is mediated by the NF-κB pathway.
Figure 10.5 Translocation of the NF-κB p65 protein in A549 cells.
(A) (i) Negative and (ii) positive control. Representative cell cultures dosed with (B) (i) no particles, (ii) LPS, or (iii) LPS containing carbon particles are shown.

10.5.3 LPS Containing Carbon Particles Initiate the Secretion of IL-1β and TNF-α Resulting in Culture Wide ICAM-1 Upregulation

The methodology employed in this study allowed for a small population of cells within an area, < 0.5 mm², termed the particle deposition site, to interact with the population of particles delivered. Typically only one cell interacted with any one particle. Thus, the effect of the particle dose on cells well removed from the site of particle deposition could be observed. The relative expression of ICAM-1 across the 7 mm² scans following the deposition of LPS droplets, carbon particles, or LPS containing carbon particles onto a cell culture is shown in figure 10.6. Like the deposition site scans, the 7 mm² scans indicated that the LPS droplets and carbon particles each elicited minimal ICAM-1 upregulation, whereas a dose of > 50 LPS containing carbon particles effected culture-wide ICAM-1 upregulation.
Figure 10.6 Differential expression of ICAM-1 as a function of particle dosage in the 7 mm² scans. A549 cells were dosed with populations of residues consisting of (A) 52 pg of LPS, (B) 6.3 μm diameter elemental carbon particles or (C) 6.3 μm diameter LPS containing elemental carbon particles, with 52 pg of LPS per particle. All fluorescence emission signals measured from the air–liquid interface cultures incubated with particles were scaled relative to the positive control (TNF-α). Error bars represent the 95% confidence interval.

The extent to which LPS can diffuse from the site of particle deposition in a 24 hour period is < 3 mm (122), which implies that the widespread upregulation of ICAM-1 was a result of secretion of mediators of intercellular communication. Two common proinflammatory mediators of intercellular communication are IL-1β and TNF-α, both of which are known to elicit differential expression of ICAM-1 in alveolar type I and II cells through the activation of NF-κB (151, 236).

Cells were incubated with a neutralizing antibody towards either TNF-α or IL-1β, or the antioxidant NAC for 60 minutes prior to LPS containing elemental carbon particle deposition. The subsequently determined PIP was calculated using the deposition site scans (figure 10.7).
NAC was used as a control because ICAM-1 differential expression is inhibited post-translationally by the presence of NAC, as evidenced by the low PIP (p = 0.817). In the presence of the TNF-α antibody, the cell culture responded to the LPS containing carbon particles in a dose dependant manner (p = 3.92 x 10^{-10}), though a slight reduction of the PIP(ICAM-1) was observed. In contrast, presence of the IL-1β antibody reduced the PIP(ICAM-1) of the LPS containing carbon particles such that no significant dose-response relationship was observed (p = 0.115). These results indicate that the widespread ICAM-1 upregulation was primarily driven by the initial secretion of IL-1β.

**Figure 10.7** The proinflammatory potential (PIP) for ICAM-1 of A549 cells dosed with LPS containing carbon particles in the absence or presence of antibodies or an antioxidant. Error bars shown indicate the standard deviation about the slope of the fitted least square straight line. * indicates a significant (p < 0.05) dose-response relationship was observed.
10.5.4 The Amount of TNF-α Secreted From the Cell Culture Following Exposure to LPS or LPS Containing Carbon Particles is Not Enough to Elicit Differential Expression of ICAM-1

ELISA analysis of the supernatant 0.5 and 12 hours following particle deposition for TNF-α was undertaken. It was found that the quantity of LPS dosed onto the cell culture was enough to elicit the secretion of TNF-α (figure 10.8A). However, the quantity of TNF-α secreted was too low to cause differential expression of ICAM-1 (figure 10.8B).

**Figure 10.8** (A) The quantity of TNF-α in the supernatant of an A549 cell culture 30 minutes and 12 hours following the deposition of LPS, carbon particles or LPS containing carbon particles, as determined by an ELISA. (B) The relative upregulation of ICAM-1 as a function of the quantity of TNF-α added to the cell culture and incubated for 18 hours. For all the studies that monitor the upregulation of ICAM-1, 5000 pg/mL of TNF-α was used as the positive control.

10.5.5 The amount of IL-13, but not of IL-8 and IL-6, is a product of the dosage of LPS and Elemental Carbon

Following the deposition of a population of LPS droplets, or carbon or LPS containing carbon particles and a 30 minute incubation period, the amount of secreted IL-6, IL-8 and IL-13
was measured via an ELISA (figure 10.9A). It was found that the amount of secreted IL-13 was a product of the particle type in which the cell culture was exposed to, but unlike the ICAM-1 expression the response was observed to be simply additive rather than synergistic. A similar result was not observed when looking at cytokines more commonly associated with the NF-κB pathway, namely IL-6 and IL-8, where no significant differential expression as a function of particle type was observed.

Given that 30 minutes is too short a time period for the secretion of proteins not stored in the cell to occur, a second set of experiments were undertaken wherein a 12 hour incubation period was given as to allow for transcription and translation of IL-6 and IL-8 to occur (figure 10.9B). However, like in the 30 minute incubation period, no differential expression was observed.
Figure 10.9 Concentration of IL-6, IL-8 and IL-13 in the supernatant of A549 cell cultures (A) 30 minutes and (B) 12 hours following the deposition of LPS, carbon particles, or LPS containing carbon particles, as determined by an ELISA. Note that the concentration of IL-13 is plotted on ng/mL scale, whereas the concentration of IL-6 and IL-8 are plotted on a pg/mL scale.

10.5.6 LPS Containing Carbon Particles Induce ICAM-1 Expression via TLR-4

The addition of TLR-4 antibody in the growth medium resulted in no significant change in the PIP(ICAM-1) value (figure 10.7), as a linear dose-response relationship was still observed (p = 0.0041), indicating that interaction between membrane bound TLR-4 with the particle bound LPS was not responsible for the observed expression of ICAM-1.

Confocal fluorescence microscopy (CFM) was then employed to make observations of intracellular TLR-4 distributions following incubation with particles. The distributions of TLR-4 30 minutes following the deposition of either carbon particles or LPS containing carbon particles were compared (figure 10.10). Starting at the bottom of the stack of the images identified as
figure 10.10Ai, ii, which was closest to the bottom of the cell culture, and scanning towards the surface, it is apparent that the intracellular TLR-4 was recruited to the vicinity of the site of LPS containing carbon particle deposition. Similar recruitment of TLR-4 following deposition of a carbon particle was not observed.
Figure 10.10 Confocal fluorescence microscopy of A549 cells that had been dosed with (A) LPS containing carbon particles or (B) carbon particles, and then fluorescently labeled for intracellular TLR-4.

(i) and (ii) are a series of images taken from below the cell culture (bottom image) to above the cell surface (top image). The vertical separation between each image was 0.5 μm. (iii) and (iv) are images taken from four different cell cultures. The absence of fluorescence at the site of particle deposition was interpreted as the presence of a particle and hence is indicative of significant particle-cell interaction; the number of images that contained these areas devoid of fluorescence was indicative of the depth of cell membrane penetration by the particle into the cell. The black and white images were taken using light microscopy to illustrate the site of particle deposition. Scale bars are equal to 10 μm.
The size of the impression on the cell surface, taken as the circular region devoid of fluorescence, and therefore TLR-4, across numerous scans indicating penetration into the cell membrane, caused by LPS-containing carbon particles was considerably greater than the impression depth left by the carbon particles (figure 10.11). Moreover, the spatial distribution of intracellular TLR-4 is not correlated to the site of carbon particle deposition. Taken together these images show a significant change in which the cells physically interact with the cell culture, and that the interaction is affecting the relative location of TLR-4 within the cell.
Figure 10.11  Epi and confocal fluorescence microscopy images of A549 cells that had been dosed with (A) LPS containing carbon particles or (B) carbon particles. These representative images show the extent of particle-cell interaction by the lack of fluorescence in the location corresponding to the sites of particle deposition. Cells were incubated for 30 minutes, fixed and permabilized prior to labelling for intracellular TLR-4. Scale bar equal to 10 μm.

10.5.7 Cells Incubated With Soluble LPS Prior to Deposition of < 40 Carbon Particles Express ICAM-1

Cell cultures were bathed in growth medium containing 0.1 mg/mL of LPS for 60 minutes, after which carbon particles were deposited directly onto the cell cultures as per the
method described; the LPS remained in the medium throughout the incubation period. Following a 24-hour incubation period, the relative expression of ICAM-1 was measured using fluorescence microscopy (figure 10.12A) at the site of particle deposition. The differential ICAM-1 expression observed following the deposition of < 40 carbon particles was significantly greater (t = 10.65, df = 22, p < 0.0005) than the response of a cell culture not bathed in LPS, but dosed with a similar population of LPS containing carbon particles. Like in figure 10.3, there appears to be a slight response of the cell culture to the LPS in the medium; this response appears to be higher than that observed in figure 10.12A as the overall quantity of LPS in this system was much greater.
Figure 10.12 The relative expression of ICAM-1 by an A549 cell culture grown in medium containing 0.1 mg/mL LPS and dosed with a population of elemental carbon particles, as (A) determined by the relative fluorescence of a fluorescently labeled cell culture at the site of particle deposition. (B) the relative distribution of TLR-4, as measured using confocal fluorescence microscopy, following the deposition of carbon particles onto an A549 cell culture incubated with soluble LPS.

The series of images taken from below the cell culture (bottom image) to above the cell surface (top image), with a vertical separation between each image of 0.5 μm. Scale bar is equal to 10 μm. \(*t = 6.434, df = 14, p < 0.0005\) and \(**t = 10.65, df = 22, p < 0.0005\).
CFM of the distribution of TLR-4 30 minutes measured following the deposition of carbon particles onto a LPS primed cell culture is shown in figure 10.12B. The association of the TLR-4 to the site of particle deposition is analogous to the TLR-4 distribution observed following the deposition of LPS containing carbon particles, as well as similar observations of increased impression on the cell membrane by these particles. Such favorable particle-cell uptake was however observed for ~60% of these carbon particles (figure 10.12B).

10.6 Discussion

Through the use of particulate matter mimics, we were able to independently study the in vitro injury caused by the LPS component of a carbon particle and by the carbon particle itself. The downstream biological response of the A549 cell culture following the interaction with these two particle types was quite different. Exposure to carbon particles having sizes from 2.0 to 6.3 \( \mu \text{m} \) in diameter resulted in an observably small contact area between the particle and cell with no measurable evidence of membrane penetration (figure 10.10) and no significant expression of surface ICAM-1 (figure 10.4). In contrast, LPS containing carbon particles were observed to have comparably larger contact area with the cell (figure 10.10) and cause significant dose dependent increase in ICAM-1 expression across the cell culture (figure 10.6) following both NF-\( \kappa \text{B} \) activation (figure 10.5) and secretion of IL-1\( \beta \) from the perturbed cells (figure 10.7). The presence of the LPS on the carbon particle increased the rate of cell membrane permeation by the particle relative to a carbon particle, suggesting that the carbon particle core acted as a vector by which the LPS gained access into the cell interior. Once inside the cell, the LPS lead to the recruitment of intracellular TLR-4 (figure 10.10). Prior studies have reported that A549 cells lack membrane bound TLR-4 and have provided further evidence to suggest that it is intracellular, suggesting that LPS must first permeate the cell membrane prior to initiating a response (11). It has also been previously shown that A549 cells do not express CD14 (22, 27), and that the soluble form of CD14 (sCD14) is required for these cells to respond to soluble LPS (23, 30). We
suspect that the sCD14 independent increase in ICAM-1 expression by A549 cells (figure 10.4) is caused by the elemental carbon particle either presenting the LPS to the cell much like CD14, or the elemental carbon particle simply enabling LPS to gain entrance to the cell allowing interaction with the intracellular TLR-4. Interaction of LPS with intracellular TLR-4 causes activation of intracellular pathways, including the NF-κB (figure 10.5) (34-37), resulting in the downstream production of cytokines and adhesion molecules such as ICAM-1 (figures 10.6 and 10.7). It remains unclear if the secretion of the IL-1β resulted from, or was independent of, the interaction of LPS with intracellular TLR-4.

Secretion of TNF-α following exposure to LPS was also observed (figure 10.8A). However, the quantity of secreted TNF-α was not enough to elicit ICAM-1 expression (figure 10.8B). Thus we hypothesize that the role of TNF-α in this model is to amplify the cellular response that is primarily driven by IL-1β. The dominant role of IL-1β in causing widespread ICAM-1 expression was further demonstrated by the secretion pattern of IL-13, IL-6 and IL-8 (figure 10.9). It has been previously shown that IL-1β causes the secretion of IL-6, IL-8 and IL-13. However, in lung cells, it has also been shown that IL-1β causes more IL-13 to be secreted than either IL-6 or IL-8 and a similar trend was observed in figure 10.9A. Thus, this is further evidence that the widespread ICAM-1 upregulation was driven by the initial secretion of IL-1β. Of note was the inability to detect significant changes in the amount of secreted IL-6 or IL-8 12 hours following particle deposition, meaning that any changes in the quantity of these secreted cytokines was below the sensitivity of the experiment. Significant changes in the quantity of secreted IL-13 were possible as there was ~10 X more IL-13 present in the supernatant than the IL-6 or IL-8.

The extent to which the cell culture responds to the LPS containing elemental carbon particles was speculated to be a direct function of the amount of bioactive LPS present on the particle’s surface, where the particle contacted the cell. To address this, another experiment was
performed in which LPS would only be present on the surface of dosed carbon particles; cells were primed with soluble LPS in the growth medium prior to being dosed with < 40 carbon particles containing no LPS (figure 10.12). For comparison, cells grown in LPS free medium were dosed with < 40 LPS-containing carbon particles (figure 10.4). Significant ICAM-1 upregulation was observed when elemental carbon particles were delivered to an A549 culture bathed in LPS-containing growth medium, whereas no significant upregulation was observed following a dose of < 40 carbon particles, each containing 52 pg of LPS, where the LPS is distributed throughout the particle. The modification of the carbon particles post deposition (figure 10.12A), and not the priming of the cells by the LPS is speculated to have caused the measured proinflammatory response, as evidenced by the observation that only 60% of the carbon particles deposited onto the LPS primed cell culture resulted in recruitment of intracellular TLR-4 (figure 10.12A); the resulting increased number of contacts between intracellular TLR-4 with the LPS component of the particle drives the response. Additionally, this observation suggests that the quantity of LPS present on the surface of each carbon particle was a factor in these outcomes. Based on these results, a maximum quantity of LPS sorbed onto the surface of each carbon particle was estimated as 150 fg, corresponding to an overall dosage of ~6 pg of carbon particle-surface bound LPS necessary to effect culture-wide upregulation of ICAM-1 (40 carbon particles deposited, each having 150 fg LPS, figure 10.12). This value was calculated by assuming unity LPS uptake coefficient onto a carbon particle and homogeneous endotoxin distribution in the growth media. With these assumptions, all LPS in a volume of growth medium, approximated as a cylinder of diameter equal to the diameter of the particle and height equal to the depth of the growth medium, became sorbed onto the carbon particle surface as it sank down to the A549 cells. Given the nature of surface properties of primary particle types, such as soil and mineral dusts, and the chemical properties of LPS (figure 10.1A), it is likely that LPS would bind more strongly to these particle types relative to carbon.
It is unclear whether the uptake of the LPS containing carbon particles was a result of active endocytosis or passive diffusion resulting from the changes at the cell-LPS containing carbon particle interface. What is clear is that there is a physical change in the interaction between the particle and the cell based on the presence of LPS on the particle, as observed by different distributions of intracellular TLR-4 within the cell cultures. Detailed study of particle surfaces, the physical interaction between the particles and the individual cells, and downstream changes to the surfaces of these participants is warranted. Additionally, the quantity of surface bound, and thus bioavailable, LPS on PM$_{10}$ necessary to cause injury to lung tissue, and potentially initiate a systemic response, is likely in the low picogram per particle range; in vitro only 40 such particles were required. Implicit in these results is that delivery of LPS together with carbon particles represents a path for transport of LPS to the interior of A549 cells that is otherwise not accessible. We speculate that assessing the potential for an ambient particle type to cause injury must be based on assays that mimic the in vivo PM$_{10}$-lung tissue interactions as closely as possible; conversely, assessment of any given compound known to be present on PM$_{10}$ on its own is likely to yield a significantly underestimated pro-inflammatory potential.

The results obtained in this study demonstrate that LPS has a role, possibly a significant role, in the overall injury caused by PM$_{10}$ exposure. In humans, initiation of IL-1β secretion by the lung epithelium is believed to be a key pro-inflammatory pathway in the pathogenesis of numerous diseases, including asthma and cystic fibrosis (2, 24). The ability of LPS containing carbon particles to induce IL-1β secretion, whose inflammatory potential is amplified by the secretion of TNF-α, suggests that the LPS component of PM$_{10}$ could also be involved in the pathogenesis of other respiratory, and possibly cardiovascular, diseases.

10.7 Acknowledgments

Discussions with Shizu Hayashi. Funding by the Canadian Foundation for Climate and Atmospheric Sciences (CFCAS), the Natural Sciences and Engineering Council of Canada
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Chapter 11

HETEROGENEOUS PROCESSING OF PARTICULATE MATTER AND ITS EFFECT ON THE DOWNSTREAM BIOLOGICAL RESPONSE

The role of the heterogeneous processing of oleic acid on its proinflammatory potential

We can't solve problems by using the same kind of thinking we used when we created them.

Albert Einstein

The effect that tropospheric processing of particulate air pollution has on adverse health outcomes in humans is complex and difficult to elucidate. Given the complexities of both health and atmospheric research, traditionally there has been an effort by researchers to focus on only one area. The result is a disconnect between the understanding of particulate matter chemistry by the health researchers and of the adverse health effects of particulate matter by the atmospheric chemists. In an attempt to design methodologies that could be used to bridge this gap, we undertook the following interdisciplinary study where the downstream biological response of a cell culture was monitored following exposure to organic aerosols that had undergone varying forms of heterogeneous processing.
11.1 Context
This chapter resulted from the first collaboration between the Agnes group from SFU, the van Eeden group from James Hogg iCAPTURE Centre at St. Paul’s Hospital, and the Bertram group from UBC. Chemical processing of oleic acid, and the characterization thereof, were undertaken by Simone Gross of the Bertram group. All other experiments, data collection and processing was undertaken by myself. This is the second chapter where the emphasis of the chapter moved beyond method development and into hypothesis driven studies.

11.2 Abstract
The effect of heterogeneous processing of the organic fraction of particulate matter on its ability to induce a proinflammatory response was studied using an in vitro methodology where particles consisting of elemental carbon, oleic acid, the products of the oxidation of oleic acid by ozone or NO₃, or a combination thereof were dosed onto a cell culture and the downstream biological response was monitored. It was found that the upregulation of ICAM-1 via exposure to particles containing oleic acid was lowered following the oxidation of oleic acid by either NO₃ or ozone. Through analysis of the secreted proteome, four distinct groups of intracellular mediators, where the secretion of one member coincided with the secretion of another, were observed. The role of these mediators in the proinflammatory response remains unclear. Taken together, the results of this study suggest that the oxidation of organic aerosols reduces the potential for the aerosol to induce inflammation through lowering the hydrophobicity of the organic particle, thus reducing the potential for passive cell membrane permeation.

11.3 Introduction
The chemical makeup of the troposphere is ever changing, with chemical species constantly being produced, emitted, and altered through homogeneous, heterogeneous and multiphase processing, before finally being removed from the air, through gravitational settling and precipitation. The processing of organic aerosols by trace oxidants in the atmosphere is of
particular interest as it has implications in both climate regulation and overall atmospheric chemistry. Organic aerosols are a fraction of a larger class of species known as particulate matter whose potential to induce adverse health effects in humans through initiating a systemic proinflammatory response (96) has been well documented (33, 65, 237). The question then becomes: What is the overall effect that heterogeneous processing of organic aerosols has on the biological response following exposure?

Oleic acid is a monosaturated fatty acid common to both animal and vegetable oils. The presence of elevated levels of oleic acid in the troposphere, along with the chemical nature of the species itself, has lead to it becoming a model system to study heterogeneous reactions. The products of the ozonolysis of oleic acid have been well characterised, with some of the more abundant low volatility product compounds being azelaic acid, nonanoic acid, 9-oxononanoic acid, and nonanal. Numerous other oxidized compounds are also produced. All of these species are of a higher hydrophilicity and lower volatility relative to oleic acid. With regards to atmospheric chemistry, this change in chemical properties results in the production of potential cloud condensation nuclei, which in turn has a direct effect on both weather patterns and the overall albedo of the planet. With regards to toxicology, the effect that this processing has on human health is considerably less studied.

Injection of oleic acid has become a well established standard method by which to experimentally induce symptoms characteristic of the early phase of acute respiratory distress syndrome (ARDS) in a range of laboratory animal species, including dogs, sheep and pigs (153, 230, 249). ARDS is characterized by inflammation in the lung and impaired gas exchange. Given that injection of oleic acid is known to induce inflammation, which plays a role in the pathogenesis of numerous diseases, and oleic acid particles are prevalent within the industrialized troposphere (from incomplete cooking and simple volitization from olive oil), the goal of this study was to monitor the effect by which inhalation of oleic acid induces inflammation in the lung, and how the heterogeneous processing of oleic acid affects its ability to induce a
proinflammatory response. We have developed a methodology wherein we can design and create micrometer sized particles of known chemical composition and size and dose these particles onto a lung cell culture in vitro in a way that mimics the cell-particle interaction observed in vivo (73, 121, 122). We use this methodology to dose lung cells grown in culture with particles consisting of elemental carbon and organic compounds, either oleic acid or the products of the oxidation of oleic acid by ozone or NO$_3$. Following a subsequent incubation period, the potential injury caused to the cells, as reported as the upregulation of proinflammatory mediators, was assessed through measurement of biomolecules in the supernatant by MALDI-MS.

11.4 Experimental

11.4.1 Materials

The source of the carbon is India ink (Speedball, product #3338, Statesville, NC, USA); which itself is an aqueous solution containing carbon nanoparticles and surfactants, from which, upon removal of the water due to evaporation, the carbon nanoparticles aggregate. The organic fraction of the particles were provided by adding to the starting solution either oleic acid (99% pure, Aldrich), or products of the oxidation of oleic acid by ozone or NO$_3$. The resultant aggregate, hereafter referred to as a particle, formed using the levitation methodology employed in this study were spherical in appearance and when viewed using an optical microscope had a diameter of 6.3 ± 0.4 μm (mean and SD).

Oleic acid was oxidized using either NO$_3$ or ozone. NO$_3$ was obtained by thermal decomposition of N$_2$O$_5$ in a Teflon coated glass oven at 150 - 160 °C. N$_2$O$_5$ was synthesized by reacting NO$_2$ with an excess amount of O$_3$. N$_2$O$_5$ was then trapped in a glass trap at -75 °C and used in He carrier gas.

11.4.2 Cell Culture

A549 cells (American Type Culture Collection, Manassas, VA, USA), a human type II alveolar-like cell line originally derived from a patient with bronchioloalveolar carcinoma was
used. All cell cultures were grown to > 95% confluence on an 18 X 18 mm² glass coverslip in a 6-well plate (Corning) in minimum essential medium supplemented with 5% heat-inactivated fetal bovine serum in 5% CO₂ at 37 °C. The negative control was a cell culture grown to > 95% confluence that had all but 15.9 ± 2.5 μL (mean ± SD) of its medium removed prior to being placed into an incubator (122). The positive control was a cell culture grown to > 95% confluence on an 18 X 18 mm² glass coverslip and bathed in growth medium with a TNF-α (Sigma-Aldrich, T6674-10UG, Oakville, ON, Canada) concentration of 0.5 μg/mL.

11.4.3 Heterogeneous Processing of Oleic Acid with Ozone

A 5 μL droplet of oleic acid was exposed for 4 h at room temperature to 6331 ppm of O₃ in an O₂ gas flow at atmospheric pressure. This resulted in an O₃ exposure concentration of approximately 1.6 x 10¹⁷ molecules cm⁻³, under which conditions all of the oleic acid reacted (reaction rate of oleic acid with ozone is on the order of 10⁶ s).

11.4.4 Heterogeneous Processing of Oleic Acid with Nitrate

1-2 μL droplet of oleic acid was exposed for 2 h under vacuum, pressure: 2 - 2.5 Torr, at room temperature to NO₃, with a concentration approximately 1 - 5 x 10¹¹ molecules cm⁻³.

11.4.5 Particle Generation and Deposition onto Lung Cells in vitro

10 μL of a starting solution was placed into the reservoir of a droplet-on-demand dispenser (MJ-AB-01-60, MicroFab Technologies Inc., Plano, TX, USA), which itself was positioned above the induction electrode of the AC trap. A 100 V DC potential was applied to the induction electrode, creating an electric field in which the relative abundance of anions and cations in a volume of starting solution at the tip of the droplet dispenser is perturbed due to ion mobility so that when the piezoceramic element of the droplet dispenser is activated, the jet of liquid ejected from the nozzle of the droplet dispenser had a net charge imparted onto it. Droplets were generated at a rate of 120 Hz with an initial diameter of 60 μm and had a net charge of 200 ± 25 fC (27). The presence of the net charge on the droplets allowed them to be levitated by the electric fields within the AC trap (59). In any given experiment, a population of 10 to 120
droplets were levitated, the total of which was verified post deposition (121, 122). Within seconds, the water contained in the droplets evaporated, leaving behind an aggregate, or residue, consisting of the solutes of low volatility that were in the dispensed droplets. Hereafter, these residues are referred to as particles.

Once a population of particles was levitated, a cell culture grown on an 18 X 18 mm$^2$ coverslip was then prepared for particle deposition by first draining all but 15.9 ± 2.5 μL of the growth medium that bathed the culture and manually positioning the coverslip below the centre of the ring electrodes and directly above the bottom end-cap of the AC trap. Application of a 500 V DC potential to the bottom end-cap then extracted the levitated particles from the AC trap and onto an area < 0.4 mm$^2$ on the cell culture (25). The coverslip was then removed from the system and placed into a sterile (35 mm X 10 mm) tissue culture dish, which itself was then placed into an incubator set at 100% humidity, 37 °C and 5% CO$_2$. The length of time of incubation was dependant on the technique used to assay or measure downstream biological response.

11.4.6 Antibody Assay

After a 24 h incubation period, cells were fixed with a 1% acetone solution for 10 minutes, during which time the number of particles that had been deposited were tabulated by optical microscopy. The primary antibody was mouse anti-human ICAM-1 monoclonal antibody (Immunotech, Marseille, France) and the secondary antibody was a fluorescently labelled goat anti-mouse IgG (Alexa Fluor 546, Molecular Probes, Eugene, OR, USA).

11.4.7 Fluorescence Microscopy and Image Analysis

A Zeiss Axioplan 2 (North York, Ont., Canada) fitted with an excitation filter (BP-546/12) and emission filter (LP-590) was used to collect all of the fluorescence emission images of the samples fluorescently tagged with antibodies for ICAM-1. For each fluorescently labelled sample, the fluorescence emission collected from a 1.00 mm X 1.25 mm area centred over the site of particle deposition was acquired; the image generated is hereafter referred to as a deposition site scan. The signal intensity of fluorescence emission for each sample, indicative of ICAM-1...
expression, was determined using Image J software (Research Services Branch, National Institute of Mental Health, Bethesda, MD, USA) and integrated using Excel (Microsoft, Seattle, Washington, USA). The degree of ICAM-1 upregulation was reported as a percentage of the total signal relative to the positive control.

11.4.8 Statistical Analysis of the PIP-ICAM-1 Data

For each of the 1 mm² deposition site scans, the fluorescence data was graphed as a function of the number of deposited particles. The proinflammatory potential (PIP) for a given particle type and proinflammatory mediators, in this case ICAM-1, measured is defined as the slope of least squares linear regression fit to the data, which was calculated using the Excel Data Analysis package (73, 122). The standard error about the slope is the error of the PIP.

11.4.9 Collection of the Supernatant

The supernatant was collected 30 minutes following particle deposition through washing a 100 μL aliquot of PBS over the entire coverslip five times. Care was taken to ensure that little to no cell lysis occurred during the washing process; cell lyses would release intracellular biomolecules into the supernatant. To increase the abundance of secreted biomacromolecules in the sample, the same 100 μL aliquot was used to collect the supernatant from two individual coverslips. Samples were then prepared for mass spectral analysis.

11.4.10 MALDI-TOF-MS Preparation and Analysis of the Supernatant

Biomacromolecule purification and concentration was carried out using a C18 ZipTip (Millipore, Bedford MA). The pH of the 100 μL collected supernatant was adjusted by adding 4 μL of 2.5 % trifluoroacetic acid (TFA), resulting in an overall 0.1 % TFA solution. Sinapic acid (Sigma, St. Louis MO) was used for the matrix. Mass spectra were collected using MALD-TOF-MS (MALDI-LR, Waters Corp., Manchester, UK) in linear mode. The MS was operated with a pulse voltage = 1,400 V, source voltage = 15,000 V, a multichannel plate detector potential difference of 1,800 V and a TLF delay of 500 ns. Prior to analysis, all mass spectra were
smoothed five times with a Savitzky-Golay algorithm with a window size of 3, and then background subtracted.

Scans were acquired in the mass range from an m/z of 3,000 to 10,000 and in the mass range from an m/z of 10,000 to 25,000.

11.4.11 Statistical Analysis of MALDI-TOF-MS Data

For each biomacromolecule of interest, the relative abundance was calculated using equation 11.1.

(Eq. 11.1) \[ \text{Signal} = \frac{\text{Signal(Ion)}}{\text{Signal(Rf)}} \]

Where the signal was the peak height of the ion and Rf was the reference ion. In the low mass range, the reference ion was at an m/z of 8,546; it was selected for the internal reference as it was present in each spectrum at a similar relative abundance between samples and it was present in mass spectral analysis of the pure, cell free, medium. In the high mass range, the reference ion was at an m/z of 10,076 was selected for the internal reference as it was present in each spectrum from all cultures including control cultures as a major ion peak and at a similar relative abundance (leading to a low standard deviation) between samples. The noise of the background was calculated by taking the standard deviation of the average of 50 data points taken from a mass range devoid of ion signals, then dividing the standard deviation by the average.

Unlike the PIP(ICAM-1), MALDI-TOF-MS was not used to generate dose-response relationships. Rather, the role of the MALDI-TOF-MS analysis was to screen for the differential expression of potential proinflammatory mediators in the supernatant as a function of the particle type in which the cell culture is exposed.

11.5 Results

11.5.1 The Effect of Heterogeneous Processing on PIP-ICAM-1

The addition of the insoluble carbon core in the organic aerosol dosed onto the cell culture was found to increase the PIP(ICAM-1) in every sample, except for oleic acid oxidized by
ozone (figure 11.1); this despite the fact that the carbon particles alone were found to cause no significant ICAM-1 upregulation. This has been viewed as a form of synergistic toxicity, and appears to correlate with previous results obtained (chapter 10).

![Graph showing proinflammatory potential (PIP) for different treatments](image)

Figure 11.1 The proinflammatory potential (PIP)-intercellular adhesion molecule (ICAM)-1 of an A549 cell culture dosed with lipopolysaccharide (LPS), oleic acid, or the oxidized products of oleic acid, all either with or without an insoluble elemental carbon core. The yellow colour indicates the value was previously reported in chapter 10, and added here simply for reference.

Whether the elemental carbon core is simply a means of maintaining a high concentration of, in this case, oleic acid in a small area for a prolonged period of time or whether the physical core plays a more active role remains unclear. What is clear however, is that the presence of the insoluble core has a profound effect on the potential toxicity of the chemicals adhered to the particle.

Oxidation of oleic acid by either ozone or nitrate radical was found to reduce the PIP(ICAM-1) (figure 11.1). Based on these results, it was hypothesized that the PIP was correlated with the physical properties of the chemicals coating the particle, namely their hydrophobicity. The oxidation of oleic acid results in the synthesis of lower molecular weight compounds that are more polar. As a result, the likelihood of these chemicals permeating the cell
membrane through simple passive diffusion is reduced. To further investigate this, the PIP as a function of the octanol-water coefficient (log P) was plotted (figure 11.2).

Figure 11.2 The proinflammatory potential (PIP)-intercellular adhesion molecule (ICAM)-1 as a function of the octanol-water coefficient of oleic acid or its oxidized derivatives constituting or coating the particle. The Log P values used in the construction of this graph were the weighted average of the products of the oxidation of oleic acid by ozone (log P = 2.8) and nitrate radical (log P = 4.1). The data was fit to a least squares linear regression, with the R² values equalling 0.9694 and 0.9361 for particles having no carbon, and carbon, respectively.

The presence of a linear correlation between the octanol-water coefficient and the PIP suggests that the physical nature of the chemical bound to the particle directly affects its ability to stimulate ICAM-1 expression.

11.5.2 The Effect of Heterogeneous Processing on the Secreted Proteome

The supernatant of cell cultures dosed with a single population of 50 to 100 particles consisting of elemental carbon (C), oleic acid (OA), oleic acid with elemental carbon (OAC), products of the complete reaction between oleic acid with ozone with (OACO3) or without
elemental carbon (OAO3) or products of the complete reaction between oleic acid with nitrate radical with (OACNO3) or without elemental carbon (OANO3) was collected and analyzed via MALDI-MS (summary shown in Table 11.1).

Table 11.1  Acronyms for the Particle Types Used in this Study. Triangles signify inclusion of the compounds indicated in the particle type.

<table>
<thead>
<tr>
<th>Carbon</th>
<th>Oleic Acid</th>
<th>Oleic Acid Oxidized by:</th>
<th>Label</th>
</tr>
</thead>
<tbody>
<tr>
<td>▲</td>
<td>▲</td>
<td>Ozone</td>
<td>C OAO3</td>
</tr>
<tr>
<td>▲</td>
<td>▲</td>
<td>Nitrate Radical</td>
<td>OANO3</td>
</tr>
<tr>
<td>▲</td>
<td>▲</td>
<td></td>
<td>OAC OACO3</td>
</tr>
<tr>
<td>▲</td>
<td>▲</td>
<td></td>
<td>OACNO3</td>
</tr>
</tbody>
</table>

Representative mass spectra of the supernatant collected 30 minutes following particle deposition is presented (figures 11.3 and 11.4).
Figure 11.3 Representative mass spectra (m/z range from 3,000 to 10,000) of the supernatant of a cell culture dosed with a population of particles consisting of elemental carbon, oleic acid or products of the oxidation of oleic acid by ozone or NO$_3$. 
Figure 11.4 Representative mass spectra (m/z range from 10,000 to 25,000) of the supernatant of a cell culture dosed with a population of particles consisting of elemental carbon, oleic acid or products of the oxidation of oleic acid by ozone or NO$_3$.

From the mass spectra presented in figures 11.3 and 11.4, changes in the relative abundances of numerous ions as a function of particle type were observed. To ascertain the
degree in which these changes occur, the relative abundances of these ions of interest were tabulated using equation 11.1 and then plotted (figures 11.5 and 11.6).

**Figure 11.5** The relative abundances of biomacromolecules preliminarily estimated to be differentially expressed measured using MALDI-MS in the m/z range of 3 to 10 kD. The number of samples equals 3 for each particle type (n = 1 for OAC). Error bars represent a single standard deviation of the relative signal intensity.
Figure 11.6 The relative abundances of biomacromolecules preliminarily estimated to be differentially expressed measured using MALDI-MS in the mass range of 10 to 25 kD. The number of samples equals 3 for each particle type (n = 1 for OAC). Error bars represent a single standard deviation of the relative signal intensity.
Incidences of differential biomacromolecule expression were observed for multiple particle types. However, no significant incidences of synergy, like that observed in the PIP(ICAM-1) scans, were observed; the pattern of the relative abundance of the ion m/z = 6082 as a function of particle type to which the cell culture was exposed appears to be similar to the pattern observed in the PIP(ICAM-1); however, these changes appear not to be significant. This was further demonstrated through plotting the relative abundance of the ion at m/z = 6082 as a function of PIP(ICAM-1) (data not shown), which yielded no significant linear relationship; furthermore, this observation was held true across all the ions monitored in this study.

The m/z of the biomacromolecules of interest was used for preliminarily identification. A list of a 119 potential candidates, consisting of chemokines, cytokines and β-defensins was used in the screening process. The potential candidates were selected based on fitting the criteria as a biomacromolecule that is potentially secreted from human lung cells. Based solely on their m/z, nine of the twenty biomacromolecules of interest were preliminarily identified (Table 11.2).
Table 11.2  Preliminary Identification of Biomacromolecules Differentially Expressed Following Exposure to Particles Containing Pure or the Oxidized Derivatives of Oleic Acid and Elemental Carbon

<table>
<thead>
<tr>
<th>m/z of Peak</th>
<th>Preliminary Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>3618</td>
<td>Unknown</td>
</tr>
<tr>
<td>4215</td>
<td>Unknown</td>
</tr>
<tr>
<td>6082</td>
<td>Unknown</td>
</tr>
<tr>
<td>6216</td>
<td>Unknown</td>
</tr>
<tr>
<td>8187</td>
<td>Unknown</td>
</tr>
<tr>
<td>8404</td>
<td>CCL26</td>
</tr>
<tr>
<td>10200</td>
<td>CCL15</td>
</tr>
<tr>
<td>10829</td>
<td>Unknown</td>
</tr>
<tr>
<td>11289</td>
<td>CCL23</td>
</tr>
<tr>
<td>12256</td>
<td>CCL21</td>
</tr>
<tr>
<td>12319</td>
<td>IL-13</td>
</tr>
<tr>
<td>13500</td>
<td>Unknown</td>
</tr>
<tr>
<td>13979</td>
<td>Unknown</td>
</tr>
<tr>
<td>14927</td>
<td>IL-17F</td>
</tr>
<tr>
<td>15100</td>
<td>IL-17A</td>
</tr>
<tr>
<td>16000</td>
<td>Unknown</td>
</tr>
<tr>
<td>17045</td>
<td>Unknown</td>
</tr>
<tr>
<td>17157</td>
<td>Unknown</td>
</tr>
<tr>
<td>18034</td>
<td>BD-29</td>
</tr>
<tr>
<td>20859</td>
<td>IL-6</td>
</tr>
</tbody>
</table>

Given that no single mediator monitored via MS was found to correlate with the upregulation of ICAM-1, we then hypothesized that potentially multiple mediators may be acting in concert with one another to drive the response. Thus the relative abundance of each ion in every spectrum was systematically plotted with one another as to elucidate whether groups of biomacromolecules were secreted in conjunction with one another. From the 231 individual comparisons, 17 significant linear relationships demonstrating a positive correlation were observed (figures 11.7 and 11.8), while no significant linear relationships demonstrating a negative correlation were observed. Significance was defined as an \( R^2 \) value greater than 0.5 of the linear least squares fit of the plot of the relative abundance of one ion with the other. The basis for being able to make these comparisons was that we hypothesized that the cellular response was driven by the carboxylic acid component of oleic acid and its products following oxidation by ozone or NO\(_3\). The presence of multiple linear relationships verified this.
Figure 11.7 The correlations between the relative abundances of biomacromolecules present in the MALDI mass spectra of the supernatant of a cell culture dosed with various particle types, group 1. The m/z of the biomacromolecules of interest is located on each of the axis. The correlation between the coexpression of the two molecules is reported as the $R^2$ value, and deemed significant if it was over 0.5.

The groups of species that were positively correlated with one another were further divided into three groups; the basis of this separation was the fact that the abundance of one species in each group was found to be correlated with the abundance of numerous other species within that group and none of the species outside of it, where a correlation with an $R^2$ of over 0.5 was deemed significant. The group containing the most members (totalling ten) was termed
group 1; the observed correlations between species within the group is shown in figure 11.7.

Groups 2 and 3 consist of five and one member respectively; the observed correlations between these species are shown in figure 11.8.

![Graph showing correlations between species](image)

**Figure 11.8** The correlations between the relative abundances of biomacromolecules present in the MALDI mass spectra of the supernatant of a cell culture dosed with various particle types, groups 2 (A) and 3 (B). The m/z of the biomacromolecules of interest is located on each of the axis. The correlation between the coexpression of the two molecules is reported as the $R^2$ value, and deemed significant if it was over 0.5.

Group 1 consisted of 9 different species; of the 45 different potential relationships between these species, only 11 significant correlations were observed. For group 2 which consisted of 4 species, only 5 significant relationships of potentially 10 were observed. This suggested that all the biomacromolecules of each group are not secreted en masse following a 30 minute incubation period; rather the degree in which each is secreted ranges, yet on the whole they all appear to respond together. It remains unclear which, if any, of these groups is driving the upregulation of ICAM-1.

The relative abundance of 15 of the 20 species of interest was found to be associated with the relative abundance of at least one other species. This leaves five species (termed group 4) that
were found to be up and down regulated independently of all other species monitored. A summary of ions, and which group they are associated with is presented in Table 11.3.

Table 11.3 Preliminary Identification of Biomacromolecules Differentially Expressed Following Exposure to Aerosols Containing Oleic Acid or the Oxidized Derivatives of Oleic Acid: Species Grouped Together Were Found to be Secreted to the Same Stimulus

<table>
<thead>
<tr>
<th>Group</th>
<th>m/z</th>
<th>Preliminary Identification</th>
<th>Brief Overview of Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>10829</td>
<td>Unknown</td>
<td>Chemoattractant for T cells, monocytes and neutrophils</td>
</tr>
<tr>
<td></td>
<td>11289</td>
<td>CCL23</td>
<td>Chemoattractant for naïve T cells, B cells and mesangial cells</td>
</tr>
<tr>
<td></td>
<td>12256</td>
<td>CCL21</td>
<td>B cell growth and differentiation. Inhibits macrophage inflammatory cytokine production</td>
</tr>
<tr>
<td></td>
<td>12319</td>
<td>IL-13</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>16000</td>
<td>Unknown</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>17045</td>
<td>Unknown</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>17157</td>
<td>Unknown</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>18034</td>
<td>BD-29</td>
<td>Antibacterial cationic peptide, destabilizes the plasma membrane</td>
</tr>
<tr>
<td></td>
<td>20859</td>
<td>IL-6</td>
<td>Chemoattractant for CD4+ T cells, monocytes and eosinophils</td>
</tr>
<tr>
<td>#2</td>
<td>13500</td>
<td>Unknown</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>13979</td>
<td>Unknown</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>14927</td>
<td>IL-17F</td>
<td>Inhibits angiogenesis. Activates T cells and PBMCs</td>
</tr>
<tr>
<td></td>
<td>15100</td>
<td>IL-17A</td>
<td>Related to neutrophil chemotaxis through induction of IL-8 release</td>
</tr>
<tr>
<td>#3</td>
<td>6082</td>
<td>Unknown</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>6216</td>
<td>Unknown</td>
<td>---</td>
</tr>
<tr>
<td>#4</td>
<td>3618</td>
<td>Unknown</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>4215</td>
<td>Unknown</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>8187</td>
<td>Unknown</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>8404</td>
<td>CCL36</td>
<td>Chemoattractant for eosinophils and basophils</td>
</tr>
<tr>
<td></td>
<td>10200</td>
<td>CCL15</td>
<td>Chemotaxis of T cells, monocytes, neutrophils and dendritic cells</td>
</tr>
</tbody>
</table>

11.6 Discussion

Oleic acid initiates a proinflammatory response in cells by causing an imbalance of reactive oxygen species (ROS) in a biological system that interferes with the cellular repairing mechanism; a phenomena collectively known as oxidative stress (249). ROS are produced in the mitochondria under various conditions and it is at this organelle where oleic acid has been shown to modulate oxidative stress. Oxidative stress is known to trigger the major proinflammatory pathway NF-κB, of which ICAM-1 is a product. Hence it was of little surprise that exposure to oleic acid would cause ICAM-1 expression in a dose-dependant manner.

There are no receptors on the cell for oleic acid, suggesting that the interaction of the cell with oleic acid is a chemical based interaction (ie. the production or reduction of ROS in the cell) rather than a biological one (ie. antigen-receptor interaction). The translocation of oleic acid into the cell is likely a critical step for it to induce oxidative stress. The octanol-water coefficient (log(P)) is a good indicator of the extent to which a chemical species will reside in the organic...
fraction of a given system. The correlation between the PIP(ICAM-1) and log(P) suggests that it is the physical properties of the chemicals dosed on the cell that affects the expression of ICAM-1; thus penetration of oleic acid into the cell is a major step in initiating a response. This also explains why signs of synergy were observed. The insoluble particle core acts as a vessel on which the oleic acid may potentially permeate the cell membrane.

The lack of a correlation between the relative abundance of any of the biomacromolecules monitored in the mass spectra detected 30 minutes after particle deposition with the PIP(ICAM-1) suggests that the observed upregulation of ICAM-1 in a dose dependant manner was solely a product of cellular response to the particle type dosed onto the cell culture and was not driven by a single intercellular mediator secreted by the cells dosed with the particle. ICAM-1 requires 18 to 24 hours to be expressed on the surface of the cell and thus for the widespread response to be driven by a single intracellular mediator, the mediator must be present in the supernatant shortly after particle deposition. This was different than the response observed in chapter 10, where the upregulation of ICAM-1 was primarily driven by the initial production of IL-1β. Another possibility is that multiple mediators are acting in conjunction with one another, wherein the presence of all mediators is required for a widespread ICAM-1 upregulation. This may be the case here, where 4 distinctly different groups of mediators respond to various particle types. At this point it is unclear whether this is the case, and to ascertain which of these groups may be involved in the widespread upregulation of ICAM-1, additional characterization of the system will be required.

Each member of a given group (of those listed in table 11.2) may be acting in concert with other species within the same group because they are either all part of the same pathway or they are all initiated by a similar molecule. Given that the identity of all the peaks in each group remains unknown, it is yet not possible to determine whether the species were in fact the byproduct of a single pathway. With regards to the correlated responses being initiated by some preliminary trigger expressed by the cells initially dosed with the particles, the identity of this
molecule remains unclear, as demonstrated by the lack of a correlation between the PIP and relative abundance of any of the ions of interest.

11.7 Conclusions

Dosing a cell culture with particles consisting of oleic acid, its oxidative derivatives or elemental carbon, or a combination thereof, was found to cause injury to cell culture; which was reported as the dose-dependant upregulation of ICAM-1.
Chapter 12

**FUTURE DIRECTIONS**

The perceived impact and current applications of the methodology developed during this thesis

*Prediction is very difficult, especially about the future.*  
*Niels Bohr*

For the most part, the nature of studies undertaken throughout the course of thesis was method development. As a result, much of the focus was on demonstrating the utility of the methodology, with less on the actual application of it. For instance, only three of the eight data chapters employed the method developed solely to address specific hypotheses. So, like many documents discussing a new technique, the questions then become, where does the technique developed here reside in the toolkit of the modern scientist, and can it be used to gain useful information that is unattainable by other means? Simply put, what does the future hold for this new technique?

In an attempt to address these questions, the following chapter will first discuss how the technique was used to gain insight in the area for which it was designed, at the interface of the atmospheric chemistry and toxicology fields. Then, the focus turns to the experiments currently being undertaken that employ this new technique. Lastly, some experiments that potentially can be carried out are mentioned.
12.1 How Does This Work Fit…

12.1.1 … into the Atmospheric Chemistry Field?

As mentioned before, when my studies in the atmospheric toxicology field began, the
conventional wisdom was that organic species were typically broken down into more polar
molecules by oxidizing species present in the atmosphere while on their way to being removed
from the atmosphere. The findings in this thesis demonstrated the production of larger organic
molecules in the atmosphere was also possible through multiphase and heterogeneous processing,
which compliment other publications produced shortly before (155) and after (141) the
publication of my work. It has even been demonstrated that polymers formed through the photo­
oxidation of aromatic hydrocarbons are the major component of organic aerosols (155), further
altering the fundamental view of particulate matter chemistry.

12.1.2 … into the Toxicology Field?

Over the past decade, an inordinate amount of research has been performed that
demonstrate that primary component of air pollution most significantly responsible for the
observed adverse health effects was the particulate fraction. Most of the work undertaken during
the course of this thesis related to the toxicology field revolved around the role of the endotoxin
component of particulate matter associated with the adverse health effects following exposure to
particulate air pollution. There were two reasons for looking at this. First, in designing the
system, endotoxin coupled with E1A transfected A549 cells was viewed as an appropriate model
to design a novel dose-response apparatus as the cellular response to the endotoxin had been well
characterized. Second, the role of endotoxin in particulate matter toxicity was the subject of
much debate, with conflicting evidence showing that endotoxin was both the primary component
of particulate matter responsible for initiating the adverse health effects and that endotoxin played
no role in initiating adverse health effects.
The studies presented here show that the amount of endotoxin typically found bound to particulate matter is sufficient to induce inflammation. However, given that historically the fine and ultrafine portion of particulate matter, typically deficient in endotoxin, illicit a larger proinflammatory response than the course fraction; it is likely that endotoxin is not the primary component of particulate matter responsible for the adverse health effects observed.

12.1.3 … into the Atmospheric Toxicology Field?

With the advent of dosing levitated designed particles onto an air-liquid interface (ALI; cell culture grown on a porous membrane, allowing for cell differentiation, which are much more intricate than the ALIs that I had termed my cell cultures in this work), we have constructed a unique methodology for mimicking lung exposure to particulate air pollution. Additionally, the capability to simultaneously alter these designed particles through heterogeneous processing enables this technology to address relevant hypotheses. As a result, there is potential to learn information about the fundamental processes involved in particulate air pollution toxicity.

12.2 Current Applications

The methodology developed through the course of this thesis enabled the detailed design of complex particles of known composition and the ability to administer a known number of these particles to a cell culture followed by the means in which to measure the cellular response. This capability has afforded us the opportunity to address hypotheses that had been challenging to directly test through experimentation.

For instance, the ability to design particles of complex chemical composition allows for the systematic study of complete particle types, with the end goal of ascertaining the most toxic species in such a particle. Michael Eleghasm is using this methodology to study the role of the inorganic fraction of particulate matter within regards to its ability to induce inflammation. In a similar fashion, Alice Kardiputri is using this methodology to look at the role of a selected fraction of the compounds on ambient particles, specifically the functional group of a long chain
hydrocarbon associated with an elemental carbon particle, on the secretion of proinflammatory mediators. Another study being undertaken by Teresita Mariana Cruz-Sanchez consists of generating elemental carbon particles containing viable Respiratory syncytial virus (RSV) that can be dosed simultaneously in the form of a discrete particle onto asthmatic and normal cell lines grown on an ALI. The goal of her project is to measure the effect of particulate air pollution and viral infection on the secreted mediators involved in the exacerbation of asthma.

12.3 Where to Go From Here

Other than the areas currently being addressed, there are a few others I feel that the methodology developed during this thesis can be utilized.

12.3.1 From Epidemiology to in vitro

Recently, a series of epidemiological studies have been released associating traffic generated air pollution with adverse health effects (36, 261). Specifically they have shown that people who live within 100 feet of a major roadway are more likely to suffer from both cardiovascular and pulmonary diseases, and that the reduction of the traffic on these roadways through the construction of a by-pass resulted in both a significant lowering of pollutant levels and an alleviation of respiratory systems for people who reside in the affected area (36). The significance of this is that it narrows the focus onto the specific elements of air pollution that are most problematic with regards to the overall health of the population. Results from epidemiological studies like these can be used in the selection of potential target particle types to study using the technology designed throughout this thesis.

12.3.2 From Pulmonary to Cardiovascular

Recent studies have begun to characterize that the association between particulate air pollution with cardiovascular events, such as stroke and myocardial infarction, is potentiated through numerous factors including the production of atherosclerotic plaques, destabilization of those plaques, and physiological events such as arrhythmia and increased blood pressure. Given
how intrinsically related the cardiovascular and pulmonary systems are, it is of little surprise that a species that harms one would be affecting another. The question to consider at this point is how the chemical composition of particulate air pollution affects the cardiovascular system indirectly, using the pulmonary system as a mediator.

Experiments to study the relationship between these two systems with regards to their response to particulate air pollution would involve the use of both epithelial (from the lung) and endothelial (from the artery or vein) cells. First, designed particles would be dosed onto epithelial cells. Following a subsequent incubation period, the supernatant of the culture would be transferred to the endothelial cell culture. The downstream biological response of the endothelial cell culture would then be monitored. The basic premise of these co-culture style experiments is that one cell culture is used to monitor changes in the secreted proteome of the other. This style of experiment is not new, with similar experiments being used to study the interactions between macrophages and epithelial cells following particulate matter exposure (95).

12.3.3 Fundamental Responses to Particulate Air Pollution at the Cellular Level

There remains much debate as to how, at the cellular level, particulate air pollution inhalation results in systemic inflammation. A prominent mechanism is the oxidative stress paradigm (181). The basis of this hypothesis is that the ultrafine particle fraction, which is more toxic than the course and fine fractions, is also able to generate chemical and biochemical reactive oxygen species (ROS). It would be beneficial to further develop this methodology to monitor changes in the occurrences of these ROS in a cell. Additionally, given that the exact moment of particle deposition is known, there lies the potential to do detailed kinetic studies, with the generation of these ROS as the biological readout.
12.4 Final Thoughts

The method by which the experiments were approached through the course of this thesis can be summarized by the following exchange:

Hobbes: “But you’re not painting in the lines and you’re not using the colors that correspond to the numbers.”
Dramatic Pause.
Calvin: “If I did THAT, I’d get the picture they show on the box.”
Hobbes: “Ah.”

Bill Watterson, Homicidal Psycho Jungle Cat (305)

By coloring outside the lines, and not sticking to the numbers, I believe we have developed a useful way in which future scientists may attempt to address fundamental questions about how the human lung interacts with particulate matter. It is through these experiments that trends observed in epidemiological studies can be further understood at the cellular level, leading to an improved understanding as to how air pollution is injurious.
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