CHAPTER 1

INTRODUCTION

1.1. Statement of problems

Exposure to inhaled substances, including toxic gases, volatile organic compounds (VOCs), particulate matter (PM) and mixtures of these, causes both acute and chronic adverse health effects, especially in the respiratory system [(1)]. The routine toxicity testing of inhaled substances is done *in vitro* or *in vivo* (using an animal model). Conventional *in vitro* testing has been done by suspension of the substances in the culture medium in which the cultures are grown [(2)]. This method does not represent the *in vivo* exposure pattern to inhaled substances, because cell culture media can function as a chemical barrier. Beside the obvious ethical concerns, extrapolation from toxicity data from animal studies is still controversial [(3)]. Moreover, most animal studies have been done by oral and dermal exposure rather than inhalation exposure [(4, 5)]. Although studying the effects of inhaled substances is more challenging than other routes of exposure, promising in vitro exposure techniques have been recently developed [(6-9)]. However, these studies have been tested on generic compounds. Environmental pollutants are a complex mixture of gases, PM, VOC, and polycyclic aromatic hydrocarbons (PAHs). Humans are continuously exposed to this mixture, yet there are as yet no data comparable to that from *in vivo* exposure studies.

Our goal was to establish an *in vitro* exposure chamber in which airborne substances are delivered directly to the cells. In order to develop an *in vitro* technique

comparable to *in vivo* inhalation exposure, cells were cultured by the using air/liquid interface culture technique. Using this technique, target cells could be continuously exposed to airborne substances on their apical side, while being nourished from their basolateral side [10].

The aim of this study was to develop an exposure chamber that could mimic the human lung. Human alveolar epithelial cells (A549) were cultured in the chamber via an air/liquid interface technique. The study was divided into two parts; the comparison of the air/liquid interface to conventional cell culture and the establishment of an exposure chamber.

1.2. Literature review

1.2.1. Air pollution

Air pollution is the introduction of chemicals, particulate matter, or biological materials that cause harm or discomfort to humans or other living organisms, or cause damage to the natural environment or built environment, into the atmosphere.

1.2.2. Type of Air pollution

Air pollutants can be classified as primary or secondary. Primary pollutants are directly emitted from a process, such as ash from a volcanic eruption, the carbon monoxide gas from a motor vehicle exhaust or sulfur dioxide released from factories. Secondary pollutants are not emitted directly. They form in the air when primary pollutants react or interact. Some pollutants may be both primary and secondary that is, they are both emitted directly and formed from other primary pollutants.

1.2.3. Sources of Air pollution

Sources of air pollution refer to the various locations, activities or factors which are responsible for the releasing of pollutants into the atmosphere. These sources can be classified into two major categories:

1) Natural sources from natural processes that affect air quality include volcanoes, which produce sulfur, chlorine, and ash particulates. Wildfires produce smoke and carbon monoxide. Cattle and other animals emit methane as part of their digestive process.
2) Anthropogenic sources (human activity) mostly related to burning different kinds of fuel
    - Stationary Sources include smoke stacks of power plants, manufacturing facilities (factories) and waste incinerators, as well as furnaces and other types of fuel-burning heating devices.
    - Mobile Sources include motor vehicles, marine vessels, aircraft and the effects of sound.
    - Chemicals, dust and controlled burning practices in agriculture and forestry management. Controlled or prescribed burning is a technique sometimes used in forest management, farming, prairie restoration or greenhouse gas abatement. Fire is a natural part of both forest and grassland ecology and controlled fire can be a tool for foresters. Controlled burning stimulates the germination of some desirable forest trees, thus renewing the forest.
    - Fumes from paint, hair spray, varnish, aerosol sprays and other solvents

- Waste deposition in landfills, which generates methane. Methane is not toxic; however, it is highly flammable and may form explosive mixtures with air. Methane is also an asphyxiant and may displace oxygen in an enclosed space. Asphyxia or suffocation may result if the oxygen concentration is reduced to below 19.5% by displacement

1.2.4. Common Air pollution

1.2.4.1. Ozone

Ozone is found in two regions of the Earth's atmosphere, at ground level and in the upper regions of the atmosphere. Both types of ozone have the same chemical composition (O_3). While upper atmospheric ozone protects the earth from the sun's harmful rays, ground level ozone is the main component of smog.

Ozone in the air we breathe can harm our health typically on hot, sunny days when ozone can reach unhealthy levels. Even relatively low levels of ozone can cause health effects. People with lung disease, children, older adults, and people who are active outdoors may be particularly sensitive to ozone. [(11)]

Ozone is a highly reactive gas and a major constituent of photochemical smog. Breathing only slightly increased concentrations of ozone (60–120 ppb) results in a range of respiratory symptoms in a small proportion (10–20%) of the healthy population.[(11)] Symptoms include decreased lung function, increased airway hyper reactivity, and pulmonary inflammation. Those individuals with pre-existing conditions such as asthma and chronic obstructive pulmonary disease generally experience an exacerbation of their symptoms. Ozone is a relatively insoluble gas. It is very reactive, and uptake is directly related to reactions with substrates present in the lung lining fluid, the first compartment it encounters on entering the lung.

Langford et al. referred to this mechanism as “reactive absorption”.[12] The uptake of ozone is thus related not only to its concentration but also availability of substrates within the lung lining fluid compartment. Following reaction with a target substrate, ozone is consumed, disabling it from transiting the lung lining fluid compartment. Cellular responses to ozone are therefore not the result of direct reaction of ozone with cell surface components, but are mediated through a cascade of secondary, free radical derived, ozonation products. Children are at greatest risk from exposure to ozone because their lungs are still developing and they are more likely to be active outdoors when ozone levels are high, which increases their exposure. Children are also more likely than adults to have asthma. Breathing ozone can trigger a variety of health problems including chest pain, coughing, throat irritation, and congestion. It can worsen bronchitis, emphysema, and asthma. Ground level ozone also can reduce lung function and inflame the linings of the lungs. Repeated exposure may permanently scar lung tissue.

1.2.4.2. Particulate Matter

Particulate air pollution refers to an air suspended mixture of solid and liquid particles that vary in size, composition, origin, and effects. The term 'aerosols' refers to a stable mixture of suspended particles and gases and therefore implies smaller sized particles. Particulate air pollution is formed by condensation of gases or vapors, or by direct generation through mechanical processes. The different processes of formation lead to characteristic differences in size and composition of particles.

Particle size is expressed in terms of its aerodynamic diameter, defined as the diameter of a unit density sphere that has the same settling velocity as the particle in

question. The size distribution of suspended particles in the atmosphere is bimodal. Large particles are 2.5 to 30 µm aerodynamic diameter and most often have a basic pH. These large particles are derived from uncontrolled combustion and mechanical breakup of soil and other crustal materials. Biological particles such as pollen and spores are also found in this large particle range. Smaller particles are < 2.5 µm aerodynamic diameter and are often acidic. These fine particles include soot and acid condensates derived from vehicle emissions, manufacturing, power generation, and agricultural burning. Sulfate and nitrate aerosols generally make up the largest fraction of small particles by mass. Deposition, Clearance, Toxicity, and Particle Size are the most important characteristics influencing deposition in the respiratory system. Models of inhaled particle deposition relate aerodynamic particle diameter to the site of deposition. Most inhaled particles of greater than 5µm aerodynamic diameter deposit in the upper airways or larger lower airways. Particles smaller than 5µm aerodynamic diameter are more likely to deposit in the smaller airways, e.g. the bronchioles and the alveoli. Particle clearance is achieved by several mechanisms. Particles deposited in the trachea and bronchioles rise on the mucociliary ladder to be expelled by coughing or to be swallowed. Particles deposited beyond the terminal bronchioles are cleared largely by lung macrophages that, in tum, transport the ingested particles onto the mucociliary ladder or into the lymphatic system. A small fraction of these distally deposited particles migrate through alveolar tissue directly into the lymphatic circulation. Biologic effects of a particle are determined by the physical and chemical nature of the particle itself (particularly its solubility), the physics of deposition and distribution in the respiratory tract, and the physiologic events that occur in response to the particle's presence. Controlled human studies have

focused on airway effects of single agents or simple mixtures. Toxicological studies have generally focused on chronic effects and single agents.

Epidemiological studies have consistently reported associations between particulate air pollution, especially PM10, and adverse health effects, increasing morbidity and mortality.[13] It is by no means clear how exposure to PM, typically as low as 30 $\mu g/m^3$, can produce these health effects and which components of PM mediate them. Although epidemiological evidence suggests that it is the fine (PM2.5) or ultrafine (PM0.1) fraction that contains the toxic components, there is no general agreement. The wide spectrum of disease endpoints (from cardiovascular death to asthma attack) suggests that more than one component may be driving the health effects. Ambient particles contain a large number of soluble metals including transition metals that are capable of redox cycling. The idea has therefore been developed that oxidative stress underlies much of the toxicity of ambient particles.[14] Studies involving residual oil fly ash (ROFA) have helped develop and refine this theory. ROFA contains about 10% by weight of water soluble Fe, Ni, and V, and its intra tracheal instillation in rats leads to aldehyde generation. Moreover, oxidative stress has been shown by electron spin resonance (ESR) measurements in animals instilled with ROFA,[15] and exposure to aqueous extract of ROFA produces effects similar to those of unfractionated ROFA. Pulmonary inflammation induced by ROFA is reproducible by installation of mixtures of V, Ni, and Fe in proportions similar to those seen in the water soluble fraction of ROFA. Likewise, exposure of phagocytic cells to ambient particles collected from different urban settings causes oxidative stress which correlates with the iron content of the particles. This and other work has led to the suggestion that the dose of bioavailable transition metal, rather than

particulate mass, may be the primary determinant of the acute inflammatory response.[16] Evidence is also accumulating to suggest that organic components carried on the particle surface play an important role in mediating the toxic effect. For example, polycyclic aromatic hydrocarbons (PAHs) can induce oxidative stress indirectly, through biotransformation by cytochrome P450 and dihydrodiol dehydrogenase to generate redox active quinones that act as catalysts for free radical production. Other work has highlighted the potential role of particle bound endotoxin in driving the toxicity associated with particulate exposure.

1.2.4.3. Carbon Monoxide

Carbon monoxide (CO) is one of many ubiquitous contaminants of our environment that requires prevention and control measures to insure adequate protection of public health. A primary focus of air pollution control by industrialized societies has been the regulation of CO in ambient air and occupational settings. The term ambient air is interpreted to mean outdoor air available to the general public. The recommended multi hour ambient air quality standard of 9 ppm (10 mg: m^3) CO for 8 h is intended to protect susceptible population groups from adverse effects resulting from CO exposures in the outdoor environment [17].

Carbon monoxide is impossible to detect by an exposed person because it is colorless, tasteless, odorless, and nonirritating. When inhaled, CO is readily absorbed from the lungs into the bloodstream, where it forms a tight but slowly reversible complex with hemoglobin (Hb) known as carboxyhemoglobin (COHb). The presence of COHb in the blood decreases the oxygen carrying capacity, reducing the availability of oxygen to body tissues and resulting in tissue hypoxia. A reduction in

oxygen delivery because of the elevated COHb level, exacerbated by impaired perfusion resulting from hypoxic cardiac dysfunction, potentially will impair cellular oxidative metabolism. This occurs because hypoxia and reductions in blood flow may allow CO to bind to cytochrome C oxidase, which inhibits aerobic adenosine triphosphate synthesis. The disturbance in mitochondrial electron transport also causes generation of oxidative stress, measured as an increase in the hydroxyl like radical fraction, and causes generation of hydroxyl like radicals [18, 19]. Energy production and mitochondrial function are restored slowly after COHb levels decrease because of continued inhibition of respiration A proposed pathological mechanism of CO, which may be independent of hypoxic stress, is related to an elevation in the steady state concentration of the free radical, nitric oxide (*NO). This phenomenon has been documented *in vitro* with human and rat platelets, and bovine lung endothelial cells, and *in vivo* in both lung and brain of experimental animals [20]. The elevation of *NO can occur with exposures as little as 22 nM CO, which is the concentration expected with an interstitial CO partial pressure of about 20 ppm and a COHb level of 7% [21]. The mechanism for enhanced *NO release appears to be based on competition between CO and *NO for intracellular hemoprotein binding sites, rather than on a net increase in enzymatic production of *NO [20, 21] Vascular oxidative stress from *NO can cause leakage of high molecular weight substances into organ parenchyma and trigger leukocyte adherence: activation [22]. The health risks associated with CO vary with its concentration and duration of exposure. Effects range from subtle cardiovascular and neurobehavioral effects at low concentrations to unconsciousness and death after prolonged exposures or after acute exposures to high concentrations of CO. Risks associated with the relatively low

ambient concentrations found in the environment and in contaminated work places have been reviewed in several excellent [23]. Carbon monoxide poisoning is a major public health problem for a significant percentage of all poisoning deaths. In fact, CO may be responsible for more than one half of all fatal poisonings that are reported worldwide each year. The frequency of health problems associated with sub lethal levels of CO is difficult to quantify. Certain indoor and outdoor environments exist where the risk of exposure to dangerous levels of CO can be anticipated. Outdoors, concentrations of CO are highest near traffic intersections, in congested traffic, near exhaust gases from internal combustion engines and from industrial combustion sources, and in poorly ventilated areas such as parking garages and tunnels. Indoors, CO concentrations in the workplace and in homes with faulty or unvented combustion appliances or downdrafts and backdrafts, have been measured in excess of 100 ppm, which is estimated to result in COHb levels of greater than 10% after 8 h of exposure, the COHb levels of victims are sufficiently high to suspect that deaths in more than one half were caused by CO poisoning [24]. Because of the risk of occult poisoning, some communities now require the installation of CO detectors in residences, along with smoke detectors and fire alarms. Despite efforts in prevention, and in public and medical education, CO intoxication remains frequent, severe, and too often overlooked [25]. The incidence of mortality and morbidity from CO exposure is similar worldwide [26]. In some places, continuous surveillance is performed through the recording of all cases hospitalized in the region, yielding annual epidemiological reports at the local level. Often, individuals suffering from CO poisoning are unaware of their exposure because symptoms are similar to those associated with viral illness

or clinical depression. A recent study estimated over 40,000 emergency department visits annually for recognized acute CO poisoning in the United States. [27]

1.2.4.4. Nitrogen Oxides

There are seven oxides of nitrogen that may be found in the ambient air. Nitrous oxide (N_2O) is a greenhouse gas with significant anthropogenic sources contributing to its worldwide abundance (~ 0.3 ppm). However, nitric oxide (NO) and nitrogen dioxide (NO_2) are the two principal nitrogen oxides associated with combustion sources. Ambient concentrations of these two gases vary widely according to local sources and sinks, but can exceed a total concentration ($NO + NO_2$) of 500 $\mu g/m^3$ in dense urban areas. Nitrous acid is a common pollutant in ambient and indoor environments, produced by the reaction of nitrogen dioxide with water. Nitric oxide is oxidized in air to form nitrogen dioxide. In its liquid form, nitrogen dioxide is colourless to brown. While the boiling point of nitrogen dioxide is 21.15 °C, in normal ambient conditions its low partial pressure in the atmosphere prevents condensation so that it exists in the air in its gaseous form. In that form, nitrogen dioxide is volatile, reddish-brown in colour and heavier than air, and has a characteristic pungent odour perceptible from a concentration of 188 $\mu g/m^3$ (0.1 ppm). It is a strong oxidant, corrosive and poorly soluble in water. Its molecular weight is 46.01 g/mol, melting point –11.2 °C, boiling point 21.15 °C and density 1.59 (air = 1). It reacts with water and is soluble in sulfuric and nitric acids.

In ambient air, the oxides of nitrogen are formed by various combinations of oxygen and nitrogen at high temperatures during the combustion process. The higher the combustion temperature, the more nitric oxide is generated. Indeed, 90–95% of

the nitrogen oxides are usually emitted as nitric oxide and only 5–10% as nitrogen dioxide, although substantial variations from one source type to another have been observed. In ambient conditions, nitric oxide is rapidly oxidized in air to form nitrogen dioxide by available oxidants (such as oxygen, ozone and VOCs) and this rapid oxidation velocity is such that it is nitrogen dioxide that is usually considered as a primary pollutant. In indoor air, this oxidation process is generally much slower [(28)]. Road traffic is the principal outdoor source of nitrogen dioxide. The most important indoor sources include tobacco smoke and gas, wood, oil, kerosene and coal burning appliances such as stoves, ovens, space and water heaters and fireplaces, particularly unflued or poorly maintained appliances. Outdoor nitrogen dioxide from natural and anthropogenic sources also influences indoor levels. Occupational exposures can be elevated in indoor spaces, including accidents with silage and in ice arenas with diesel- or propane-fuelled ice resurfacing machines and underground parking garages [(29)]. In ambient conditions, both outdoors and indoors, nitrogen dioxide exists in its gaseous form, and inhalation is therefore the major route of exposure at room temperature. Exceptionally, direct contact with the eyes and associated membranes may lead to eye irritation, although this is more likely to occur in industrial settings after accidental contact with relatively high gaseous nitrogen dioxide concentrations [(28)]. The relationship between indoor and outdoor levels has been studied in various countries [(30)]. The study also found that the presence of a gas fired heating furnace was the major factor in the elevated nitrogen dioxide concentrations [(31)]. The absence of an extractor fan when cooking, the absence of central heating, and cigarette smoking [(32)]. Combustion sources influence indoor levels. Annual mean concentrations in urban areas throughout the world are generally in the range of 20–90 μg/m^3[(30)]. The air rate

exchange between indoors and outdoors affects nitrogen dioxide levels in buildings. Indoor levels vary widely depending on the presence of indoor sources, air mixing within and between rooms, the characteristics and furnishing of buildings, and reactive decay on interior surfaces. Further, it has been shown that car exhausts containing nitrogen dioxide may enter a house from an attached garage.

In the absence of indoor nitrogen dioxide sources, indoor levels will be lower than outdoor levels. Under normal ventilation conditions, the indoor: outdoor ratio has been found to vary from 0.88 to 1 [(33)]. This is attributable to the removal of nitrogen dioxide by the building envelope and its reactions with interior surfaces and furnishing [(34)]. However, in the presence of indoor sources, especially unvented combustion appliances, indoor levels may exceed those found outdoors [(35)] with an increase in the indoor: outdoor ratio from 0.7 without an indoor source to 1.2 in the presence of an indoor source. These ratios reflect average levels over several days of measurement and do not reflect the more extreme indoor/outdoor differences that one would expect to see over shorter periods of time. The average nitrogen dioxide concentration over a period of several days may exceed 150 $\mu g/m^3$ when unvented gas stoves are used [(36)]. While most studies of indoor air pollutant exposures from biomass burning in developing countries have focused on airborne particulate matter, nitrogen dioxide levels can also be elevated [(22)], it has been shown that, in homes, peak concentrations are typically related to the use of combustion appliances for cooking and heating [(37-40)]. In particular, occurrences of peaks are strongly associated with the use of gas and solid fuel stoves, the highest nitrogen dioxide concentrations coinciding with the time of meal preparation [(41)].

In addition to the direct release of nitrogen oxides, indoor combustion sources emit various co-pollutants including ultrafine particles, which are also produced during cooking [41]. Secondary reactions, such as the production of nitrous acid from surface chemistry involving nitrogen dioxide, can contribute to indoor pollutant concentrations that directly affect health [42]. The role of these co-pollutants in the health effects attributed to nitrogen dioxide in field studies is unknown, but abatement measures for nitrogen dioxide, such as improved ventilation, will be beneficial in reducing co-exposures also. High nitrogen dioxide concentrations are also associated with the use of candles and mosquito coils. In chamber (18 m^3) tests, maximum nitrogen dioxide concentrations up to 92 $\mu g/m^3$ were observed during incense burning [43]. The link between direct source exposure and high nitrogen dioxide levels was noted in a small study of unvented natural gas fireplaces [33]. Seasonal variability can be significant, owing to variations in source use (e.g. heaters and stoves) and seasonal fluctuations in air exchange rates. This variability results typically in higher indoor concentrations during winter months [44]. A nitrogen dioxide is a free radical, it has the potential to deplete tissue antioxidant defenses and, as a consequence, cause injury and inflammation as shown in a variety of in vitro test systems. Exposure of human blood plasma to 26,230 $\mu g/m^3$ (13.95 ppm) nitrogen dioxide resulted in a rapid loss of ascorbic acid, uric acid and protein thiol groups, in addition to lipid peroxidation and a depletion of alpha-tocopherol (vitamin E) [45].

In another study, exposure to nitrogen dioxide over a lower concentration range (94–1,880 $\mu g/m^3$; 0.05–1.0 ppm) resulted in the antioxidant defenses, uric acid and ascorbic acid being depleted in human bronchoalveolar lavage (BAL) fluid [46]. This was explained by decreased production as a result of an inhibition of NADPH

oxidase and complex III of the respiratory chain, and to a lesser extent increased scavenging brought about by enhanced glutathione peroxidase and CuZn-superoxide dismutase mRNA expression and enzyme activities. Evidence for the role of oxidative stress in the effects of nitrogen dioxide on respiratory virus-induced injury comes from a study that found that pretreatment of cultured primary human nasal epithelia cells and cells of the BEAS-2B line with the antioxidant N-acetylcysteine inhibited the production of IL-8 following exposure to 3,160 $\mu g/m^3$ (2 ppm) nitrogen dioxide for three hours in combination with human rhinovirus type 16 (RV16)[47].

Cell culture systems have also been used to describe nitrogen dioxide mediated cell injury and inflammation. One system exposed cultured human bronchial epithelial cells to 7,520 and 15,040 $\mu g/m^3$ (4.0 and 8.0 ppm) nitrogen dioxide and elicited cell membrane damage and increased membrane permeability [48]. It should be remembered that confluent airway epithelial cell monolayers *in vitro* are not fully differentiated and posses a markedly decreased level of resistance to pollutants when compared to the epithelium in the intact human. However, in a more physiologically relevant system, nitrogen dioxide (200 and 800 $\mu g/m^3$;0.1 and 0.43 ppm) has also been shown to trigger inflammation in cultured human nasal mucosa explants, using histamine release into the culture medium as a marker of the inflammatory response [49]. The early pro-inflammatory responses following exposure to a brief high concentration of nitrogen dioxide up to a maximum of 84,600 $\mu g/m^3$ (45 ppm) over 50 minutes have also been assessed using normal human bronchial epithelial (NHBE) cells as an *in vitro* model of inhalation injury [50]. While immunofluorescence studies confirmed oxidant induced formation of 3-nitrotyrosine, the nitrogen dioxide exposed cells exhibited marked increases in the levels of nitrite, IL-8, IL-1β and TNF-α.

Further, to simulate a preexisting "inflammatory" condition of the bronchial epithelium, such as would exist in asthma and other hyper reactive airway diseases, cells were pre-treated with various proinflammatory cytokines (IFN-γ, TNF-α, IL-1β and IL-8) for 24 hours prior to exposing them to nitrogen dioxide. The combination of cytokine treatment and nitrogen dioxide exposure consistently enhanced the generation of nitric oxide and IL-8. More recently, further findings have been published on the early changes in NHBE cells on exposure to a brief high dose (84,600 μg/m^3; 45 ppm) of nitrogen dioxide, focusing on the nature and time-course of nitrogen-dioxide-mediated cell death and, more generally, on the cellular mechanisms by which the various pro-inflammatory mediators affect the target cells [(51)]. Cells were found to undergo apoptotic cell death during the early post nitrogen dioxide period, independent of any significant increase in caspase-3 activity, while necrotic cell death was more prevalent at later time intervals. Exposed cells also exhibited increased expression of hemeoxygenase-1 (HO-1), a redox-sensitive stress protein, at 24hours and increased adhesion to neutrophils, which in turn resulted in an increased NHBE cell death. Earlier reports of an involvement of nitric oxide [(50)] were supported by the significant decrease in cell death and neutrophil adhesion in the presence of nitric oxide synthase inhibitors (L-NAME and 3-aminoguanidine)[(51)].

Nitrogen dioxide has been shown to exert a range of biological effects on experimental animals, including changes in lung metabolism, structure, function, inflammation and host defense against infectious pulmonary disease. Such effects vary widely, however, depending on the species and strain exposed, the concentration and duration applied, and the age and sex of the animals [(52)]. It is the anatomical and physiological differences between animals and humans that represent a particular

challenge. For example, we have known for some time from mathematical modeling that the distribution of nitrogen dioxide deposition within the respiratory tracts of rats, guinea-pigs, rabbits and humans appears to be similar [53].

More recently, Tsujino et al. [54], using mathematical airway models of rats, dogs and humans, demonstrated that interspecies variations in anatomy and respiratory patterns do cause significant differences in the concentration of nitrogen dioxide in the airways and alveoli. Despite some limitations, owing to many simplifications and assumptions necessary to construct the airway model and carry out calculations, intra-airway nitrogen dioxide concentrations were higher in the upper and lower airways of humans compared with rats and dogs, while those in the alveolar regions were lowest in humans.

1.2.4.5. Sulfur Dioxide

Sulfur dioxide (SO_2) is one of a group of highly reactive gases. The largest sources of SO_2 emissions are from fossil fuel combustion at power plants (73%) and other industrial facilities (20%). Smaller sources of SO_2 emissions include industrial processes such as extracting metal from ore, and the burning of high sulfur containing fuels by locomotives, large ships, and non-road equipment. SO_2 is linked with a number of adverse effects on the respiratory system.[55]

Current scientific evidence links short term exposures to SO_2, ranging from 5 minutes to 24 h with an array of adverse respiratory effect including bronchoconstriction and increased asthma symptoms. These effects are particularly important for asthmatics at elevated ventilation. Studies also show a connection between short term exposure and increased visits to emergency departments and

hospital admissions for respiratory illnesses, particularly in at risk populations including children, the elderly, and asthmatics[56]. EPA's National Ambient Air Quality Standard for SO_2 is designed to protect against exposure to the entire group of sulfur oxides (SO_x). SO_2 is the component of greatest concern and is used as the indicator for the larger group of gaseous sulfur oxides (SO_x). Other gaseous sulfur oxides (e.g. SO_3) are found in the atmosphere at concentrations much lower than SO_2. Emissions that lead to high concentrations of SO_2 generally also lead to the formation of other SO_x. Control measures that reduce SO_2 can generally be expected to reduce people's exposures to all gaseous SO_x. This may have the important co-benefit of reducing the formation of fine sulfate particles, which pose significant public health threats. SO_x can react with other compounds in the atmosphere to form small particles. These particles penetrate deeply into sensitive parts of the lungs and can cause or worsen respiratory disease, such as emphysema and bronchitis, and can aggravate existing heart disease, leading to increased hospital admissions and premature death[57].

1.2.4.6. VOC

Volatile organic compounds (VOCs) are organic chemicals that have a high vapor pressure at ordinary, room-temperature conditions. Their high vapor pressure results from a low boiling point, which causes large numbers of molecules to evaporate or sublimate from the liquid or solid form of the compound and enter the surrounding air. An example is formaldehyde, with a boiling point of –19 °C, slowly exiting paint and getting into the air.

VOCs are numerous, varied, and ubiquitous. They include both human-made and naturally occurring chemical compounds. Most scents or odors are of VOCs. VOCs play an important role in communication between plants. Some VOCs are dangerous to human health or cause harm to the environment. Anthropogenic VOCs are regulated by law, especially indoors, where concentrations are the highest. Harmful VOCs are typically not acutely toxic, but instead have compounding long-term health effects. Because the concentrations are usually low and the symptoms slow to develop, research into VOCs and their effects is difficult.

1.2.5. Health effects and Toxicity mechanism of PM

1.2.5.1. Mechanisms of PM-induced acute pulmonary injury

Recent research has investigated the biological mechanisms for both the initial pulmonary injury and the consequent systemic effects following PM exposure. The initial pulmonary injury may be related to one or more properties of PM and its constituents including physical, chemical and biological characteristics [58]. Physical characteristics include gravimetric mass, particle number, particle size, and particle surface area. In the ultrafine hypothesis, particles smaller than 0.02 mm are suggested to elicit a strong and persistent pulmonary inflammation [59, 60]. Some epidemiological studies have found a stronger association with counts of the number of ultrafine particles [61], but clinical studies have suggested that surface properties [62] and chemical composition [63, 64] may be more important factors in the toxicity of ultrafine particles. Chemical characteristics involve the direct action of an agent in a process that consumes that agent. In the acid hypothesis, the acidity delivered to the airways and alveoli through the deposition of particles is responsible for the observed health effects. Other chemical agents include hydrogen peroxides, nitrates, sulfates, organic

carbon and acid aldehydes. Catalytic characteristics transition metal hypothesis, are a special category of chemical characteristics that involve processes by which reactive metal ions produce an oxidative lung injury due to the formation of reactive oxygen and/or reactive nitrogen species [65]. Various scientists have suggested that nickel, vanadium, and copper [66] might play an important role in PM-induced acute cardio-pulmonary toxicity. The endotoxin hypothesis states that the toxicity may involve an immunological reaction to a biological constituent of PM, such as bacterial endotoxin. Endotoxin exposures are known to cause respiratory problems in certain industries [67, 68]. Other bio aerosols include allergens such as fungal spore fragments, pollen grains and antigens associated with house dust mites and cockroaches. [69].

1.2.5.2. Mechanisms of PM-induced acute health events

Several hypotheses have been proposed to explain the pathophysiology of PM-induced health effects. The most obvious mechanism involves the reduction in pulmonary function in response to the pulmonary inflammation. In the hypoxia hypothesis, the susceptible population consists of individuals with severe respiratory disease whose pulmonary reserve capacity is already near the minimum compatible with life. Exposure to PM further lowers their pulmonary function levels and results in emergency hospitalization and death. Direct mechanisms involve the penetration of PM constituents through the lung into the circulation and their subsequent effects on distal organs such as the heart. In the ultrafine hypothesis, ultrafine particles penetrated into the blood, deposited in cardiac tissue, and caused cardiac arrhythmia and death [60]. Immunological mechanisms cytokine hypothesis, involve the release of secondary messengers cytokines into the circulation by various pulmonary cells [58]. Alveolar macrophages, lymphocytes, and neutrophils can be stimulated to release a

variety of cytokines including platelet activating factor and tumor necrosis factor [70-72]. Airway epithelial cells have been shown to act directly as immune effector cells by releasing cytokines, such as IL-8 [73], granulocyte–macrophage colony stimulating factor [74, 75], and platelet activating factor. Cytokines, including tumor necrosis factor and interleukins, have been linked to cardiac arrhythmias [76] and platelet activating factor has been implicated in atherosclerosis and cardiac thrombosis [77]. The principal objection to the cytokine hypothesis is the short half-lives of cytokines in the blood. Neural mechanisms, for example the vagal nerve hypothesis, involve the response of the autonomic nervous system to pulmonary irritants. The C-fibers in the lung and other receptors detect the initial irritation of airways and alveoli by PM and the signal is transmitted by the afferent arm of the vagus nerve to the respiratory centers [78]. This signal stimulates the sympathetic nervous system and inhibits the parasympathetic nervous system. A decrease in the efferent arm of the vagus nerve is associated with a decrease in heart rate variability, especially high frequency variability, and an increase in heart rate. Measures of autonomic dysfunction, such as increased heart rate and decreased heart rate variability, have been associated with the incidence of adverse coronary events and death.

1.2.5.3. Mechanisms of PM-induced chronic disease

Some multi-stage process must be involved that increases the size of the susceptible population as well as causing illness and mortality within this susceptible population. One possible pathophysiological mechanism for PM-induced chronic disease involves hematological changes such as increased platelet activation and plasma viscosity [79]. These hematological changes may lead to the induction of atherosclerosis.

1.2.6. The structure of lung tissue membrane

A series of structural and functional barriers protects the respiratory system against harmful and innocuous particulate material. Most of the clearing structures are part of the barrier components of the lungs. They include the surfactant film, the aqueous surface lining layer including the mucociliary escalator, a thin, membrane like epithelium that allows gases diffusion while against the solutions leakage into the alveoli. This thin layer of epithelium is composed of alveolar type I cells and alveolar type II cells. Tight junctions form a gasket-like seal between adjoining cells and help maintain their structural and functional polarity.

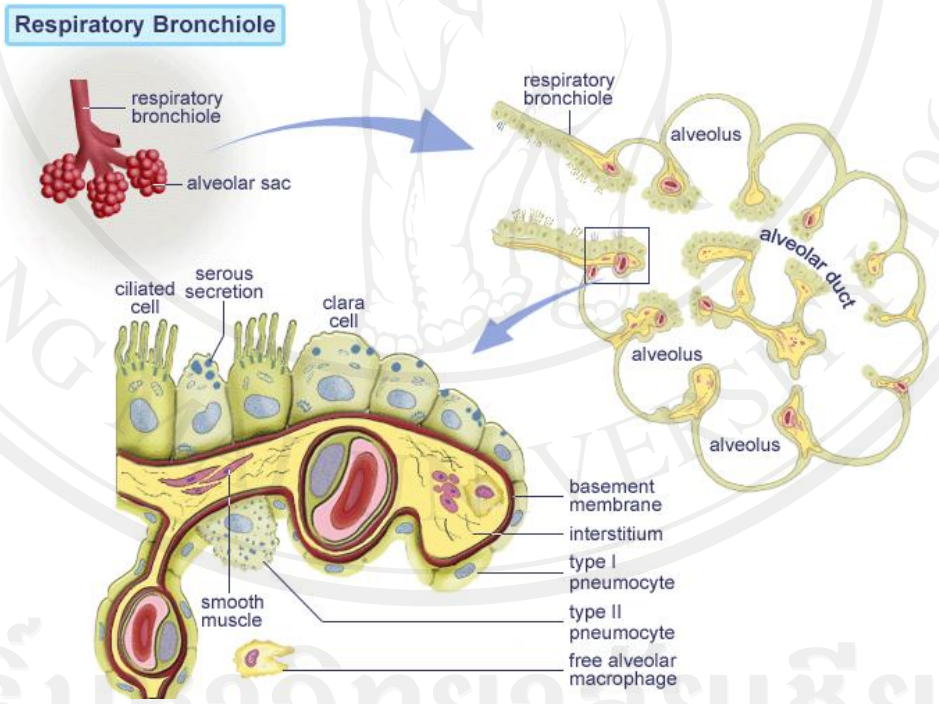

Figure 1.1 The structure of lung tissue membrane

Alveolar type I cells are large, flat squamous cells with a relatively simple structure that functions mostly as a thin, gas-permeable membrane. There are also

pinocytotic vesicles in the peripheral region of the cytoplasm and at both alveolar and interstitial surfaces of the cells. The vesicles are thought to be involved in protein transportation between cells and alveoli.[80]

Alveolar type II cells are small and cuboidal in shape. The cells are structurally and functionally polarized due to the existence of tight junctions at the lateral cell surface that divide the cell into apical and basolateral domains. The apical membrane contains molecules not found in basolateral membrane, such as glycolprotein 330.[81] The apical cell membrane also has numerous short microvilli. Secretion and endocytosis take place mostly in the apical domain.[82]

Macrophages or phagocytes exist in the airways and in the alveoli, the epithelial cellular layer, and the network of dendritic cells inside and underneath the epithelium. Besides the lung epithelium, the basement membrane, the connective tissue, and the capillary endothelium serve as structural barriers against inhaled particulate material.

1.2.7. *In vivo* cell exposure studies of air pollution

Inhalation toxicity tests are carried out on test animals to identify the median lethal concentration (LC50) of airborne chemicals causing death as a toxic endpoint in 50% of exposed animals. Some inhalation chambers may provide whole body exposure while many others accommodate oral–nasal exposure only. Both the concentration and fixed time period are critical parameters and approximately 30–50 animals are needed to carry out the LC50 test for a single chemical. Standard protocols have been recommended by regulatory agencies for both short-term and long-term inhalation tests. There are some difficulties unique to inhalation studies that have been identified. Estimating the dose received by animals is one of the

challenging issues of inhalation toxicology as several factors may influence the actual dose, such as: airborne concentration, exposure duration and pulmonary characteristics of the test animal which modulate the deposition/absorption pattern of the airborne chemical. Therefore, the selection of animal species for inhalation studies is a crucial consideration that may influence the outcome of in vivo studies and consequently the estimated human adverse health effect. Generation and characterization of high-volume test atmospheres and reproducible exposure conditions is a more complicated and expensive procedure than that required for oral and dermal exposures. This process requires specialized equipment and techniques to generate, maintain and measure test atmospheres. Inhalation exposure systems involve several efficient and precise subsystems including a conditioned air supply system, a suitable gas or aerosol generator for the test chemical, a dilution and delivery system, flow monitoring system, exposure chamber, real-time monitoring or sampling, an analytical system, and an exhaust/filter or scrubbing system.

1.2.8. *In vitro* cell exposure studies of air pollution

Exposure systems with cells cultured under submerged conditions *in vitro* have been widely used to assess the toxicity of particulate matter. These studies have typically been performed under submerged culture conditions, where the agent to be investigated is added to the culture medium, which completely covers the cells grown on the bottom of the cell culture dish. With this approach a wide variety of biological endpoints and PM have been investigated including ambient and occupational particles (e.g. soot and welding fume particles). While testing under submerged culture conditions is a simple and well-proven approach for soluble molecular toxins, this method has several deficiencies when applied to airborne particles. First, for

primary contact organs such as the lung (or the skin or retina), submerged exposure represents an unrealistic form of exposure for airborne particles, since the *in vivo* exposure to airborne particles occurs at the air liquid interface (ALI, epithelial cells represent the boundary between ambient air and inner tissue of the organisms) and not under fully immersed (submerged) conditions.

Furthermore, unlike dissolved molecular substances, the biological effect of PM may be altered by surface adsorption of molecules contained in the medium and agglomeration of individual particles may result in much larger and hence biologically different structures. Finally, it is difficult to infer the cell-deposited nanoparticle dose from the nanoparticle concentration in the cell medium. Consequently, the reliability of dose–response relationships obtained under submerged conditions is limited unless all parameters (particle/agglomerate size, height of the medium above the cells, etc.) are well controlled.

With the increasing awareness of the inherent problems of using submerged cell cultures for PM testing, several *in vitro* exposure systems have recently been developed, which allow for controlled PM exposure of cells cultured at the Air/Liquid Interface. The ALI exposure not only represents a more realistic exposure scenario for PM inhaled into the lungs, it also provides more control over the effective PM dose interacting with the cells, which facilitates more reliable dose-response measurements

ALI cell culture techniques were introduced in the 1970s by growing the cells on micro-porous membranes, where the cells can be nutrified from the basal side of the membrane and the apical part with the cultivated cells is in direct contact with the test atmosphere consisting of gaseous and/or particulate compounds. The CULTEX®

system is based on the use of a culture insert system, which offers the unique possibility of culturing cells on membranes permeable to culture media. The cultures on the insert are run as conventional submersed cultures in 6 well plates at 37°C in an incubator (5% CO2, 100% humidity) and used in the CULTEX® system. Here cells are only immersed in medium, the apical medium being removed; they are maintained and exposed to the air/liquid interface until the end of the experiment. The CULTEX® system allows exposing the cells maintained in the air/liquid interface to different mixtures of gases via negative pressure through the module. The newest generation of in-vitro cell exposure systems adopts two main technological improvements: enhancement of the nanoparticle deposition efficiency and real-time monitoring of the cell-deposited nanoparticle dose. Electrostatic precipitation is one of the main mechanisms to improve the deposition efficiency. For bipolarly charged 50-600 nm (airborne) particles, deposition efficiencies can be enhanced to 15-35% under the influence of an alternating electrostatic field. Larger deposition efficiencies are not possible with typically used diffusion chargers, since bipolar diffusion charging leaves the majority of submicron-sized particles uncharged. The so-called EPDExS system selects a monodisperse subfraction of nanoparticles from a bipolarly charged polydisperse sample aerosol with a differential mobility particle sizer (DMA) and deposits the selected unipolar (positive or negative) subfraction with an efficiency of near unity onto a cell covered substrate using a constant electrical field.

1.3 Objectives

1.3.1 To develop the direct alveolar epithelial cells-air exposure prototype.

1.3.2 To test toxicity of particulate matter collected from Chiang Mai by using the developed direct alveolar epithelial cells-air exposure prototype.