From the National Institute of Environmental Medicine, Division of Physiology,
The Unit for Experimental Asthma and Allergy Research

A novel technology for studying the disposition of drugs and toxicants in the lung; short inhalation exposures of the isolated and perfused rat lung to respirable dry particle aerosols

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Cover illustration: A macrophage (blue) that dwells in the alveoli of the lung is stretching a pseudopodia towards a diesel soot aggregate (yellow). The field of view is approximately 20 µm. Colours are for clarifying purpose. Photo kindly provided by Lennart Nilsson

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”Det var då Hunahären gick. Då var det en sme’ i Hånger socken som het Hunne. Så gick han in i sin smedja och gjorde sig en stor träklubba som han lät beslå med stora spikar.
Så gingo han ut och begynte Klubbekriget”

Ur ”Värend och Virdarna”

Gunnar-Olof Hyltén-Cavallius
ABSTRACT

The disposition of solutes following the deposition of inhaled substances in the lung is a complex process that proceeds along parallel paths; passive diffusion of solutes through the air/blood barrier and local metabolism followed by blood-borne clearance to the circulation. Using the isolated and perfused lung (IPL) the pulmonary disposition of inhaled particles can be studied in great detail without interference from distal organs and systemic exposure.

In this thesis the DustGun apparatus, a novel inhalation technology, was used for suspending dry powder particles to respirable aerosols for exposing the rat IPL and to fulfil the following aims; 1) to establish a method for performing short inhalation exposures to respirable particle aerosols in high concentrations 2) to study the dosimetry of increasing doses of an inhaled tobacco associated carcinogen 3) to describe the pharmacokinetics of three asthma drugs after inhalation exposure and 4) to measure the pulmonary perfusion flow rates after inhalation exposures to a corticosteroid.

1. The DustGun/IPL exposure technology was used to generate respirable aerosols from dry particles of diesel-soot, silica particles and some micronised pharmaceutical powders. The deposition of particles in the rat lung was in agreement with literature data.

2. With increasing exposures of the lung to the carcinogen benzo(a)pyrene (BaP), both the dissolution and metabolism were gradually saturated. The local concentration of the carcinogen in the lung was ten thousand-fold higher at the site entry than the average lung concentration and with increasing exposures the relative bioactivation of BaP in the rat lung was decreased. Consequently, this may have implications for the risk assessment of carcinogens using high- to low-dose extrapolation.

3. The exposure technology was used to measure the pharmacokinetics of three asthma drugs; budesonide, formoterol and terbutaline, representing different physico-chemical properties. After short inhalation exposures of the lungs to dry particle aerosols, the solutes appeared rapidly in the perfusate. The detailed absorption was followed for 85 minutes post exposure. At the end of the experiments the amount of solute remaining in the lungs was quantified and a mass balance of the inhaled substances was determined. Using only 1-3 mg powder per exposure, it was shown that terbutaline had a much more rapid penetration of the air/blood barrier than budesonide and formoterol. Consequently, budesonide and formoterol were markedly more retained in the lung tissue than terbutaline. These characteristics are in good agreement with clinical experience of the same drugs.

4. It was also demonstrated that budesonide had a significant vasoconstrictive effect on the pulmonary circulation and that this effect was partly α-adrenoceptor mediated. These findings are supported by clinical observations of vasoconstriction reducing the blood flow in the airway mucosa after inhalation of corticosteroids.

In the treatment of lung disorders, dry powder inhalation will remain an important route for drug delivery. Thus, the exposure technology presented in this thesis is suitable for studying the disposition of drug candidates (CDs) after short inhalation exposures of the lung. The low powder consumption for the generation of respirable aerosols warrants the use of the technology in early drug discovery/development.

Keywords; aerosol, inhalation, rat, lung, absorption, drugs, carcinogen, particles
LIST OF PUBLICATION

This thesis is based upon the following publications, which will be referred to in the text by their roman numeral


II Ewing P, Blomgren B, Ryrfeldt Å, and Gerde P.

Short inhalation exposures of the isolated and perfused rat lung to respirable dry particle aerosols; the detailed pharmacokinetics of budesonide, formoterol and terbutaline. 

Vasoconstriction after inhalation of budesonide: a study in the isolated and perfused rat lung. *Manuscript*
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INTRODUCTION

Aerosols

Effects of aerosols on human health

It has been known since long that the inhalation of particle aerosols can damage, but also improve pulmonary health. The evolution of the mammalian lung is an adaptation to terrestrial life and the human respiratory tract is well adapted to cope with exposures of e.g. dust and wood-smoke at reasonable levels.

As people learned new trades, people in occupations such as miners, chimney-sweepers, rail-road workers, waitresses and bakers etc. became exposed to dusts in much higher concentration. Thus, the respiratory system has never been adapted to cope with exposures to e.g. tobacco smoke and asbestos fibres of such high exposures. In high-income countries these occupational exposures have been considerably decreased due to the use of personal protection, improved ventilation and cessation of tobacco smoking.

When it comes to respiratory health the impact of smoking is still the number one hazard. During the past 20 years smoking rates have fallen in most high-income countries. While in developing countries smoking habit is increasing. Tobacco smoking, which both causes chronic obstructive pulmonary disease (COPD) and lung cancer, will for many decades to come continue to be the number cause of aerosol-associated deaths.

One often overlooked cause to aerosol-associated morbidity and mortality is the lack of proper stoves in developing countries. Since acute lower respiratory infection is the major cause of death in children in less developed countries, even small additional risks due to chronic indoor smoke exposure have important public health implications.

Another problem of most urban areas of the world, is the risks associated with chronic inhalation exposure to low levels of air pollution gases and particulate matter such
as of ozone, diesel particles and metals. One important task of epidemiology is to verify and quantitate the association between the sources of exposure to environmental particle aerosols and the health outcome in people – expressed as an increased risk in the population. For example, epidemiologists use exposure variables such as air pollution constituents, wind direction, temperature and distance to a road with high traffic intensity to test the association with diseases in the respiratory tract. From these studies, it has been verified that increased concentrations of particulate matter in an urban area is associated with an increased risk for hospitalisation of in particular the elderly. Episodes of high particle concentrations make it more difficult to fight respiratory tract infections in patients suffering from asthma and COPD, and these episodes also increase the risk for hospital admission of patients with cardiovascular disease.

While epidemiological studies may verify or not whether there is an association between an increased incidence of respiratory conditions and elevated levels of air pollution, epidemiology cannot prove the link between the disease and its cause. A coherence between epidemiology and mechanistic research is necessary to strengthen the causation between the condition and the source of exposure.

To find plausible mechanisms of disease in humans, studies in animals have to be conducted. In inhalation toxicology animals are exposed to a single substance or mixtures of substances at pre-defined aerosol concentrations. Complex mixtures of substances are used to mimic the situation of people being exposed to ambient particle aerosols in the street or tobacco smoke.

The deposition of particles and solutes in the lung, their subsequent absorption, distribution, metabolism and excretion is referred to as inhalation toxicokinetics. A primary objective of inhalation toxicokinetics is to verify whether the substance or its toxic
metabolites are available at the anatomical sites of interest in concentrations that could damage tissue.

Of primary importance is to assess the severity and distribution of pathological alterations of the respiratory tract. In the final analysis, toxicokinetic data, distribution of pathological findings and other markers of disease are evaluated to establish the risk assessment of a particular substance. However, the fact that rodents are not small human beings must always be taken into account. As an example, chronic inhalation studies have had difficulties in showing malignancies in animals within typical human exposure levels. It is most often assumed that there is linear dose-response between exposure and effect in target tissue. This controversial method in risk assessment is called high to low dose extrapolation.

Aerosols have also been used in the treatment of various lung conditions such as asthma and COPD as well as to achieve systemic exposure with remedies of low oral bioavailability. Before the 1950-ies, in patients suffering from bronchial asthma, few remedies were available to control the condition. Mainly, a high oral dose of corticosteroids was given to suppress the inflammation of the airways. However, these high oral doses were often associated with severe side-effects such as osteoporosis, diabetes and Cushing syndrome. The introduction of β2-adrenoceptor agonists for inhalation, with their short onset of action, resulted in a rapid relief for patients suffering from acute attacks of asthma. Inhaled corticosteroids with high local efficacy and low potential to induce systemic side effects were also introduced. More recently, the combination of long acting β2-adrenoceptor agonists and corticosteroids have provided an increased efficacy for patients with bronchial asthma.

Historically, mist producing apparatuses or nebulisers have been used for inhalation of various remedies to provide soothing of a soar throat or a congested nose. Also, nebulisation of β2-adrenoceptor agonists worked very nicely in the emergency room in patients with severe airway obstruction. However, in the daily lives of asthmatics, the dreadful sensation of...
shortness of breath comes rapidly and unexpectedly and the patient is in need of quick relief of symptoms. Thus, remedies that can be carried in the pocket were needed. Because of their size nebulisers do not work very nicely in the field, and as a result pressurised canisters containing drugs have become state of the art technology for inhalation therapies in the treatment of bronchial asthma.

Aerosol physics

Solid particles or liquid droplets dispersed in a gaseous phase are called an aerosol. An aerosol is heterogeneous in the micro scale but homogenous in the macro scale; so to some extent, an aerosol can be regarded as something in between a solution and a heterogeneous mixture. Combustion processes in nature such as wood fires is a good example of aerosol generation.

In order to deagglomerate and suspend solid particles in air to generate an aerosol, the repulsive forces on the aggregated particles must be greater than the attractive forces. An input of mechanical energy such as pressure is required to deagglomerate the particles. The most important attractive forces between particles are; Van der Waals forces, electrostatic forces, acid-base forces and solubilisation forces. As an aerosol is aging or cooling down the attraction between the surfaces of the individual particles forces particles to come together or agglomerate.

Aerosol generation

Aerosols are generated by many different mechanisms and can be divided into the following main categories: i) Combustion aerosols, these anthropogenic combustion emissions account for a major fraction of the fine particle air pollution and is a primary source of particulate organic matter. ii) Atomisation of liquid or aggregates of particles that are
exposed to mechanical energy in an orifice of a spray nozzle. This mechanism is used in inhalers. iii) Abrasion aerosols that could be caused by the tires of a vehicle by the friction between rubber and asphalt. iv) Cloud, fog or mist that is condensed water vapour in air.

**Medical aerosol generation technology**

The objective with inhalation of drugs is to deliver a local therapeutic dose to the target tissue. By inhalation of drugs a high local concentration at the target tissue could be obtained without causing a high systemic exposure. For the purpose of delivering pharmaceuticals to the deep lung particle atomisation is today the only available mechanism for aerosol generation. Particle atomisation could further be divided into two sub-categories; a) liquid atomisation and b) dry powder deagglomeration.

a) For the purpose of drug administration to the deep lung, liquids containing drugs are atomised in a device called a nebuliser. The nebuliser produces a mist that could be inhaled. Because the main advantage with nebulisers, since that they are active is that they require minimal training of patients prior to administration of drugs. Therefore nebulisers are suitable for children and patients with severe respiratory conditions. The recently introduced vibrating mesh technology seems to be very promising. A drawback with nebulisation is that the time of administration of drugs is significantly increased in comparison with dry powder inhalation. For example, the nebulisation of prostacyclin analogues to treat severe pulmonary hypertension, require the patient to inhale the drug for 12-15 minutes at 6-12 times every day.

b) Dry powder deagglomeration uses a driving gas to pressurise and suspend aggregates of dry powder particles into the gaseous phase. One main advantage with inhalation of dry powders in comparison to nebulisation is that a high deposited dose in the deep lung can be achieved in a shorter time span. This category could be further divided in two sub-categories, dry powder inhalers (DPI) that are passive and pressurised metered dose.
inhalers (pMDI) that are active. A shortcoming with pMDIs is that the hydrocarbon driving gas used to pressurise the canister, boils off fast and the rapidly expanding aerosol cloud of drug particles is likely to impact largely in the upper airways. A potential shortcoming for DPIs are that they are passive and thus, utilises the patients own inspiratory work to deagglomerate formulated powders. Therefore, this category of device requires more training of patients prior to administration of drug. However, it was recently shown that DPI’s were successfully used to dose the lungs of patients with varying degrees of COPD. Because dry powder inhalers from the clinic require industrial formulation and filling, dry powder inhalation of new chemical entities has not yet been commonly used in preclinical science.

**Preclinical assessment of drugs and toxicants via inhalation**

Generally, the rat is the first choice of species for the assessment of drugs and toxicants via inhalation. Other species that more resemble the human anatomy such as non-human primates and dogs are also used. For the purpose of exposing laboratory animals to an aerosol, an exposure atmosphere has to be produced. The exposure atmospheres can be produced with a variety of methods including combustion, vapour or dry powder aerosol generation. Many different technologies have been used for the generation of these exposure atmospheres such as nebulisation, fume collection and dry powder generators. Perhaps the most frequently used technology is the Wright Dust-feeder. This device generates aerosols by using a scraper to disperse a compacted cake of powder and feed the particles into a high-velocity airstream. The particles of the produced aerosol are often heterogeneous in size and impactors are needed to remove larger particles. Moreover, the powder consumption is very high. However, the system is very robust and the generation of aerosols can be maintained for days. Tracheal instillation is often used as an easier substitute for inhalation. One major problem with intratracheal instillation is the unevenness at which the test material is deposited in the lungs compared with inhalation exposures.
Concepts of lung physiology

Lung function
The lungs primary role is to provide a large area for gas exchange. The architecture of the airways and the gas-exchange/alveolar region of mammals is adapted to terrestrial life. As air passes through the dividing airways into the gas exchange area, the airflow rate is gradually slowed down and air flowing deeper into the lung is heated and moisturised. The number of divisions in the lung is different in different mammals. In humans the gas exchange area begins after 16-18 generations of airway divisions. Thus the inspired air reaches the alveolar area with optimal velocity, humidity and temperature for gas diffusion.

The diaphragm is the breathing muscle. The diaphragm is under control of the phrenic nerve, which is part of the peripheral nervous system. During inspiration the diaphragm is constricted and it relaxes during expiration. Bronchial smooth muscle produces a physiological muscular tone that is balancing two conflicting factors of airway physiology; too large airway calibres would require increased ventilation of dead space and may potentially lead to hypercapnia. Too small calibres may constrict the airways too much, which could cause asthma, hypoxia in peripheral tissue and increased breathing expenditure.

The surface area of the gas exchange region is approximately 100 $m^2$ and consists of an extremely thin biological barrier between air and blood. There is only a few hundred nanometres of tissue that separates the air lumen of an alveolar sac from the circulating blood. There are approximately 0.5 billion alveolar sacs in a human lung that are responsible for the bi-directional diffusion of oxygen and carbon-dioxide. The walls of the alveolar sacs are coated with surfactant. Surfactant is a detergent that lowers the surface tension and thus, prevents the lung from collapsing completely during expiration. During inspiration surfactant...
is thought to be relocated to the corners of hexagon-shaped alveolar sacs and thus, is believed to reinforce the alveolar wall.

The lungs are receiving the entire cardiac output. In contrast to the systemic circulation the pulmonary circulation is a low-pressure circuit. At rest the blood pressure of the pulmonary artery is approximately 20 mm hg and as the flow resistance increases in the alveolar capillaries, the pressure drops to roughly 8 mm hg. Normally, the vascular flow resistance of the peripheral lung is balanced so that the hydrostatic and osmotic pressures of the blood slightly exceeds the hydrostatic and osmotic pressure of the interstitial tissue, so that the interstitium absorbs a few mL/min of blood plasma. This filtrate is draining to lymphoid tissue, which is draining to venous blood vessels at the hilus. Inflammatory processes are associated with increased vascular permeability, vasodilatation and infiltration of inflammatory cells into the interstitium and epithelium. This will increase the absorption of fluids into the interstitium. When the maximum capacity of lymphoid drainage is reached, fluids start to accumulate in the interstitium. This process is called oedema and is reversible in the beginning. Eventually the increasing hydrostatic pressure of the interstitium can cause flooding of the alveolar region, which may become a fatal condition.

**Particle deposition in the respiratory tract**

The geometry of the nasal upper airways and peripheral airways in mammals plays an important protective role in filtering the inspired air, thereby minimising the damage of inhaled particles in the vulnerable gas exchange region. In a healthy adult mammal, the particle deposition within the different anatomical regions of the respiratory tract is mainly a function of the particle size distribution, the particle density and the electrostatic charge of the particles. There are three major mechanisms of particle deposition.
in the respiratory tract; impaction, sedimentation and diffusion. Particles that are not deposited in the airway tree are exhaled.

The airway calibre and the airflow rate decrease along the length of the dividing airway tree. The inertia of suspended particles in air increases with particle size and airflow rate. Thus, larger particles are more likely to deposit by impaction in the major branches of the airway tree. Particles with sizes larger than 10 µm are likely to deposit by impaction already in the head airways and in the major branches of bronchial tree, and will hardly reach the lower respiratory tract. This has implications for delivery of drugs to the deep lung, because particles deposited in the upper airways are likely to be swallowed and absorbed through the gastrointestinal tract.

Sedimentation; as both air velocity and airway calibre decreases in the peripheral airway tree, the free falling speed becomes significant and the particles tend to sediment to the airway walls. Sedimentation is therefore of greatest importance in the small airways and alveoli and is most pronounced for particles with larger diameters.

Diffusion; particles with smaller diameters are not affected by gravity at ambient pressures. Instead, with sizes smaller than 0.1 µm the Brownian motion of particles starts to overcome gravitational forces. So ultrafine particles with sizes smaller than 0.1 µm are increasingly deposited in the upper airways.

Most models for particle deposition in the rodent lung are based on morphometric data published by Raabe. In this paper the deposition after inhalation of monodisperse aerosols of different sizes in the different regions of the lung was examined by histopathology. Morphometric data and mathematical models have been successfully combined to predict particle deposition at different locations within the respiratory tract of several species. The relationship between particle size and deposition in the lower part of the airway tree has been demonstrated to be similar among different species.
**Dissolution of soluble particles in lung fluids**

Particles deposited in the lung have to be dissolved in the lung lining fluid prior to their absorption into the lung tissues. The kinetics of dissolution of particles are primarily dependent on the volume\textsuperscript{100} and composition of the lining fluid\textsuperscript{101} and physico-chemical properties of the inhaled substance. Such properties are the density and radius of the particle, the morphology of the particle surface (amorphous or crystalline), the lipophilicity of the solutes expressed as partition between water and octanol\textsuperscript{102}. Only a few studies have tried to measure particle dissolution \textit{ex vivo} /\textit{in vivo}\textsuperscript{103}. However, poor solubility of particles in the lung has profound effects on the pharmacokinetics of an inhaled particulate substance (PAPER II). A slow release and slow pulmonary absorption can be a potential positive effect of such a substance. On the other hand, slow release particles are more exposed to lung macrophages and to mucociliary clearance.

**Tissue diffusion of solutes**

The disposition of solutes following the deposition of inhaled substances in the lung is a complex process that proceeds along parallel paths; passive diffusion of solutes in membranes and in the aqueous domains of the air/blood barrier followed by blood-borne clearance to the circulation. Diffusion of solutes is the spontaneous net movement of molecules from an area of high concentration to an area of lower concentration. The area of the highest solute concentration is adjacent to a deposited particle. At the particle surface the concentration is close to saturation as long as the particle persists. Then, in a liquid film surrounding the particles, the solute will diffuse along a concentration gradient through tissues\textsuperscript{104, 105} or paracellular pores\textsuperscript{78} or junctions into to the underlying sub-mucosa. The persistence of such a liquid film and the steepness of the concentration gradient through the tissue is dependent upon the physico-chemical properties of the inhaled substance, the
thickness of the air/blood barrier and the rate of perfusion in the underlying sub-mucosa (PAPER II).

**Metabolism**

Clara cells are dome shaped and have short microvilli. The main function of the highly specialized Clara cells are to protect the bronchiolar epithelium by secreting a variety of products into the airway lining fluid, among them Clara cell secretory protein, which is a component of lung surfactant. They are also responsible for detoxifying harmful substances inhaled by the lungs. Metabolic detoxification is accomplished with Cytochrome p450 enzymes found in the their smooth endoplasmatic reticulum. Through a series of enzymatic oxidations of the substrate, the p450 system detoxifies lipophilic substances that otherwise could cause an imbalance in the lipid membranes. The final step often involves conjugation with glutathione and thus, a water-soluble conjugate is produced, which then can be excreted. However, for some substances, the metabolic intermediates of the p450 pathway can be very reactive, cytotoxic, and genotoxic. This is called bioactivation. Unfortunately, many very genotoxic substances are formed in the combustion of organic materials as in tobacco smoking. Primary lung cancer is predominantly a smokers disease.

**Mucociliary clearance**

Although the humidity in the lung is near 100%, the volume of the epithelial lining fluid is small. The thickness of the lining fluid in the airways is estimated to be 5-10 μm and decreases gradually along the airway tree into the alveoli, where the thickness is estimated to be about 0.05-0.08 μm. Under normal physiological conditions the volume and composition of the epithelial lining fluid is determined by active ion transport and passive water permeability of the tight respiratory epithelium. The surface liquids of the ciliated airways are composed of two phases: one aqueous epithelial lining fluid close to the cell...
surface in which cilia beat, and one gel phase of mucus on top of the aqueous phase. The mucus is produced by Goblet cells. The thickness of the mucus layer varies along the conducting airways, being about 8 µm in the trachea and about 2 µm in the bronchioles. The mucus layer is continuous in the larger human bronchial airways, but consists of discontinuous rafts in the smaller bronchi and bronchioles. A phospholipid layer between the phases lowers the surface tension between them.

The mucociliary clearance is an important mechanical host defense mechanism of the lung. By the coordinated movements of cilia, the mucus is swept out of the nasal cavity and lungs, respectively, towards the pharynx where it is swallowed. Slowly dissolving particles in lining fluid are thus, likely to swept out of the respiratory tract and swallowed (PAPER II).

**Phagocytosis**

The lung macrophages are cells that play an important role as the first line of defence against inhaled bacteria and particulates that have reached the peripheral lung. Particles deposited in the lungs of rabbits and rats have been demonstrated to be phagocytosed within a few hours. The macrophage is cleared from the alveoli to the bronchiole via the lining fluid and from the airways with the mucociliary escalator.
AIMS OF THIS THESIS

The aims of this thesis are to;

I) Establish a technology for short inhalation exposure of the IPL to respirable particle aerosols in high concentrations.

II) Study the bioactivation of the carcinogen benzo(a)pyrene after increasing exposures of the IPL to particle aerosols.

III) Demonstrate the detailed pharmacokinetics of the IPL after short inhalation exposures to drug aerosols.

IV) Study the acute nongenomic effect on the pulmonary circulation of the IPL after short inhalation exposures to corticosteroids

THE DUSTGUN EXPOSURE TECHNOLOGY

DustGun technology (PAPER I)

Fifty years after the invention of the Wright-dust feeder\textsuperscript{68} the DustGun aerosol generator was invented by Per Gerde. The technology was first assembled in 1999\textsuperscript{52}. The purpose of this technology is to efficiently suspend small amounts of collected dry powders into respirable aerosols. (PAPER I) The exposure system consists of an aerosol generator, an exposure manifold to direct the exposure, and a computerized control system. The generator unit of the system consists of three major parts, a fixed volume powder chamber, a variable-volume pressure chamber and a fast releasing valve (Figure 1). For the aerosol generation, a 1 mL portion of highly compressed air (100 bar) is loaded in the pressure chamber. Typically, a 0.5-3 mg portion of powder to be aerosolized was deposited in the powder chamber of the apparatus. By the release of a fast-acting valve, decompression of air in the pressure chamber causes an instant equilibration between the pressure chamber and the powder chamber, where the deposited powder is instantaneously pressurised and suspended.
Figure 1) Schematic drawing of the DustGun aerosol generator with exposure valves and tubings. In the exposure mode, V1 and V3 are opened, while V2 and V4 are closed. Valve V5 is only briefly opened to fill the pressure chamber.

The pressure was measured in the pressure chamber during one work cycle of the generator system. It was demonstrated in PAPER I that the rate of depressurisation in the powder chamber decreased in relation to the amount of powder loaded in the powder chamber.

Figure 2. Detail of the narrow exit conduit of the powder chamber.

Deagglomeration of particles is caused by expanding airflow that enters a hair-thin conduit in the dome-shaped top of the powder chamber and discharges into the holding chamber (Figure 2). Hence, in the DustGun dry powders are deagglomerated by gas
expansion controlled by the narrow conduit rather than by impaction or shearing more commonly used in most aerosol generator designs.

The greater the energy input during the generation of aerosols the larger the fraction generated aerosol that either consists of separated primary particle or small particle agglomerates. The filling pressure was typically 80-100 bars and the reset pressure was 10-50 bars. Closing the main valve at an elevated remaining pressure during the reset reduces the dilution of the aerosols with driving gas, allowing better control of the exposures. The produced aerosol is ejected into the holding chamber, and the pressure is instantly decreased to the ambient pressure. An exposure line was connected to the holding chamber of the apparatus. The exposure line ends in 3-way junction, also attaching to the exposure subject and to a total filter for collecting excess aerosol (Figure 3b).

For the purpose of this thesis the DustGun apparatus has been used to suspend an array of different particles into well characterised aerosols (Table 1). The aerosol generating system cannot fractionate particles, so in order to generate suitable aerosols with the DustGun apparatus the mass median diameter of the powder primary particles should be less than 10 \( \mu \text{m} \).

For studying the efficiency of the system, the deposition of particles within the system was measured using by spectrophotometric analysis and radiochemistry (PAPER I). Interaction between interior surfaces and the aerosol particles may increase deposition within the apparatus, which was assessed in detail for diesel soot. Of the loaded amount of diesel soot in the apparatus 82 \% exited into the holding chamber. For the diesel soot, the cumulative deposition of particles in the apparatus was found to be less than 50 \% of the loaded amount. Still, the majority of mass was available for inhalation. Since then, the powder
Figure 3a) Photograph showing experimental setup in the laboratory b) A schematic drawing of the exposure system. The total airflow ($Q_{\text{total}}$), produced by the vacuum pump ($V$), is drawn through, the DustGun (DG), the 3-way junction and the total filter (MF). The IPL is connected to the 3-way junction, without any valves; and the ventilation flow ($Q_{\text{vent}}$) is superimposed over the filter flow ($Q_{\text{filter}}$).
Figure 4a) Silica particles (arrowhead) 3.5 micron in MMAD deposited on the airway epithelium of a peripheral airway. Staining: hematoxylin and eosin. Scale bar=20µm. Photo kindly provided by MD Bo Blomgren.

b) A diesel soot aggregate (yellow) deposited in an alveoli of the rat lung. The field of view is approximately 50 µm. Colours are arbitrary. Photo kindly provided by Lennart Nilsson.
chamber has been redesigned. With the present design more than 95% of the loaded mass is injected into the holding chamber. The measured amount on the total filter was typically one fourth of the loaded amount, and for the majority of dry powders used in this thesis the cumulative deposition of particles within the apparatus was found to be more than 50% of the loaded amount. The main site of unwanted deposition was the walls of the holding chamber.

**Dry powder particles**

The dry powders used in this thesis display a wide array of characteristics. Synthetic amorphous silica particles were used both for deposition studies in the lung and as a carrier for chemicals. The carcinogen benzo(a)pyrene (BaP) was adsorbed onto diesel soot and silica particles, and can thus, be regarded as complex mixtures. By adsorbing a chemical to a carrier particle the carrier acts as a dilutant for the coating chemical. The dilution approach allows the exposure of subjects to a very wide range of exposure levels. The terbutaline sulphate powder represents a clinical formulation of a spheronized dry powder. The drugs tested in this thesis; budesonide, formoterol fumarate and terbutaline sulphate, represents an array of sparingly to increasingly water soluble substances.

**Preparation of test particles**

Diesel soot was collected from a tractor engine. The diesel soot was pre-extracted with toluene in a Soxhlet apparatus to denude the native content of organics adsorbed on the carbon core of the particles. The diesel soot was then radiolabeled with tritiated benzo(a)pyrene (3H-BaP). Next, the radiolabeled soot was diluted with unlabeled, organic-denuded soot to obtain a suitable specific radioactivity. The amount of radiolabeled soot in solution or tissues was measured by liquid scintillation counting (LSC) following total combustion of the samples.
Synthetic particles of amorphous silica were used as a model for a micron-size carrier particle of inhaled drugs. Silica particles were chosen as carriers for the $^3$H-BaP into the lungs due to their inert properties and weak adsorptive binding of the hydrocarbon. Silica particles were found particularly suitable with a rather high internal surface area provided by pores of 80 Å diameters. $^3$H-BaP in toluene solution and silica powder were transferred and mixed in a glass ampoule. After evaporation to dryness with argon gas, the ampoule was sealed under a reduced argon atmosphere. The ampoule was heated to allow the $^3$H-BaP to distribute evenly over the silica surface.

**Exposure configurations**

Within the scope of this thesis two different exposure configurations have been developed; the IPL exposure configuration, and the aerosol measurement configuration. During the work of this thesis the DustGun technology$^{52, 120}$ have been continuously developed, and has focused upon;

i) Reduction of dead-space in the system

ii) Sampling of the test atmospheres

iii) Characterisation of aerosols

iv) Scanning electron microscopy

v) Bench testing of the deposited dose

vi) Automation of exposures

i) The dead-space in the exposure line have been reduced from 35 mL in PAPER I to 15 mL in PAPER III. The dead-space in the tracheal catheter have been reduced from 0.5 mL in PAPER I to 0.2 mL in PAPER IV. ii) The sampling of the test atmosphere was changed from a U-tube immersed in liquid nitrogen, suitable for radio-labelled substances to a total filter system (MF), both located downstream of the exposure catheter. The total filter system was
introduced in PAPER II and the geometry of the elements were revised and finalised in PAPER III. iii+iv) a system for aerosol characterisation, aerosol sampling for scanning electron microscopy and bench testing was developed v) Online acquisition of ventilation flows and tidal volume during the exposures was finalised

**Aerosol measurements**

The most important parameter of aerosol characterisation is the particle size distribution of aerosols generated by the DustGun apparatus. During the work of this thesis a protocol for the assessment of particle morphology and particle size distribution was standardised. The general objective of the standardised aerosol measurements was to produce and characterise aerosols that are representative of the aerosols inhaled by the IPL. Scanning electron microscopy was used to visualise the morphology of particles on filters and in the rat lung (Figure 4b).

For measuring particle size distribution, the DustGun apparatus was used to expose cascade impactors to aerosols in high concentration at well controlled airflows. The cascade impactor was coupled to the exposure line of the DustGun apparatus. The airflow through the DustGun apparatus and the cascade impactor was created by a downstream vacuum source. The loaded amount of dry powder, the exposure time and the internal dead spaces of the exposure of the aerosol measurements setup were kept close to the exposure parameters used during the IPL exposures. However, the airflow was increased to 2 L/min for the 7-stage Marple (MSP corporation, Shoreview MN, U.S.A) with the size interval of 0.5 – 12 µm, and to 30L/min for the 10-stage MOUDI (MSP corporation, Shoreview MN, U.S.A) with the size interval of 0.1 – 12 µm. The airflow of the IPL exposure configuration was set to 0.4 L/min.
After the test exposure the mass deposition on each impactor stage was gravimetrically measured. The mass deposited on each of the 7-10 stages was plotted vs. the impactor stage. This was repeated three times for each substance. The particle size distribution expressed as mass median aerodynamic diameter (MMAD) and the related geometric standard deviation (GSD) were calculated. The MMAD corresponds to the X-axis value that divides the mass distribution in half. The GSD is the MMAD divided by the X-axis value corresponding for the lower 16th percentile of the Y-axis distribution. The fractional deposition on each stage could also be plotted vs. the log-normal distribution of the stages (PAPER I and III)\(^{121}\) (Figure 5).

![Figure 5](image)

*Figure 5) The size distribution of pharmaceutical aerosols suspended by the DustGun aerosol generator. The MMAD and GSD for the aerosols are given in table 1. A) budesonide B) formoterol fumarate C) terbutaline sulphate*
**Isolated perfused rat lungs**

**Perfusion strategy**

After inhalation of soluble drugs or toxicants in people, the deposited substance may exert its action on the lung either first-pass while being absorbed through the epithelium to the circulating blood or, in a second exposure following the blood returning to the lung\textsuperscript{28}. The pharmacokinetics in people after inhalation of soluble substances is contributed either from first-pass absorption in the lung or from the GI-tract via systemic clearance and elimination processes (Figure 6). While the systemic exposure level can be easily assessed through a blood sample, the pulmonary first-pass component is much more difficult to measure.

![Perfusion strategy](image)

*Figure 6) Perfusion strategy of this thesis a) single pass perfusion b) recirculating perfusion c) in vivo. Absorption (Aa), Perfusion flow rate (Q), Volume of distribution (Vss), Renal and Hepatic Clearance (CLR, CLH)*

The *ex vivo* isolated and perfused and ventilated rat lung model (IPL) has been extensively used in pharmacological\textsuperscript{122-124}, toxicological\textsuperscript{125, 126} and pharmacokinetic\textsuperscript{127-129} studies with drugs and toxicants administered to the lung via inhalation. For pharmacokinetic studies of
inhaled drugs and toxicants the IPL is especially advantageous, because the IPL does not involve recirculation of blood from distal compartments. Because the perfusate is passed only once through the lung (single-pass perfusate) the measurements reflect the maximum capacity of the air/blood barrier to absorb solutes\textsuperscript{58, 127}. In contrast, by allowing perfusate to return to the lung via the pulmonary artery\textsuperscript{128} (recirculating perfusate), the rate of absorption can be reduced when recirculating solute return to the lung via blood.

However, critical to the use of the DustGun IPL-system in the assessment of disposition of drugs and toxicants is how well data from this experimental set up correlates with \textit{in vivo} data. The major differences between inhalation exposures of the IPL-system and the corresponding exposure of a live animal are:

i) No systemic compartments. With single-pass perfusion the incoming perfusate have a zero concentration of solute. The perfusate, thus, have a maximum extraction capacity towards the solute throughout the experiment.

ii) Much fewer components of the typical perfusate compared with blood may give a reduced extraction capacity, particularly for lipophilic substances.

iii) The rate of perfusion of the IPL can be maintained only at about 15% of the cardiac output in the rat. The rate of perfusion of the IPL in this thesis was 15-20 mL/min compared to a typical cardiac output of 120 mL/min \textit{in vivo}\textsuperscript{130}.

iv) The isolation of the organ does not allow deposition of particles in upper airways.

\textbf{Preparation method}

The rat was anesthetized with an overdose of barbiturates, and heparin was injected into the heart of the unconscious animal. The pulmonary artery was cannulated and the pulmonary vein entering the left atrium was cannulated through the left ventricle of the heart. Silicone tubing was attached to both catheters. The lung circulation was perfused with a Krebs-Ringer buffer containing 2% serum albumin. A stainless steel tracheal catheter was
placed in an incision in the trachea and firmly sutured. The heart and lung was carefully dissected *en bloc* from the thorax and placed in the artificial thorax. The negative thorax pressure of -0.4 kPa inside the container promotes the reconstitution of the collapsed lung and the lung was ventilated at tidal volume of 1 mL. The negative pressure in the thorax was produced by a vacuum source and monitored on a vertical U-tube. The perfusate was passed through the pulmonary circulation at a constant hydrostatic pressure of 10 cm H$_2$O. A pH level of 7.35-7.45 was maintained. The lung was allowed to stabilize for 10 minutes before any intervention. Lung function was monitored mainly as pulmonary resistance (mL/min/mm H$_2$O) and dynamic compliance (mL/mL/min).

**Lung integrity and measurements**

It is well known that the IPL preparation and the related physiology is gradually deteriorating with time. Therefore the integrity of the IPL preparation has to be assessed. For the purpose of this thesis four different tests were applied i) Histopathological assessment ii) Lung weight post 10 and 100 minutes of perfusion iii) Dry weight to wet weight ratio iv) Transfer rate over the air/blood barrier of inhaled dry powder aerosol of 4KDa FITC-Dextran.

i. **Perivascular oedema is best characterised by histopathology.** Histology was performed on a few control lungs of this thesis. Histopathological examination of control lungs after 60 minutes of perfusion, showed only very limited if any signs of perivascular oedema.

ii. **“In methods in pulmonary research”**$^{130}$, the editors suggest that a lung weight increase of 2-10 % is expected per hour of perfusion. For PAPER III the lung weight was 1.55±0.2 (mean±SD N=17). In PAPER I lungs were collected and individual lung lobes was weighed. The weight of these lungs was 1.4±0.09 (mean±SD N=3). This was an increase with 10 % for 90 minutes of perfusion.
iii. Wet to dry weight ratios is a sensitive test of oedema formation. The wet to dry weight ratio in PAPER III was 8.7±0.9 (mean±SD N=17) after 90 minutes of perfusion and in PAPER II the wet to dry weight ratio was 8.4±1.2 (mean±SD N=9) after 90 minutes of perfusion, (unpublished data PAPER II). For baseline comparison from data of lungs perfused for 30 minutes, the wet to dry weight ratio was 7.4±0.6 (mean±SD N=46). It was concluded, that an increase of wet to dry weight ratio per hour of 12% in PAPER II and 15% PAPER III was acceptable.

iv. A pivotal increase in interstitial hydrostatic pressure could alter the filtration coefficient of macromolecules. The airway and alveolar epithelium may become permeable to macromolecules such as albumin. With macromolecules water will follow and flooding of the alveolar lumen is a reality. Alveolar flooding leads rapidly to devastating wet to dry weight ratios >20 in a few minutes. Inhaled FITC-Dextran has been used as a marker for the integrity of the airway epithelium. Preliminary data from a dry powder aerosol inhalation of 4-kDa FITC-Dextran (4.85 MMAD, 1.85 GSD) in the IPL resulted in a transfer rate into single pass perfusate less than 10% of 50 µg deposited mass. This data was in agreement with data from Tronde, where, the transfer rate of 10-kDa FITC-Dextran in to perfusate was approximately 10% of the deposited mass during 120 minutes of perfusate recirculation. Thus, it was concluded supported by this preliminary data, for the conditions of ventilation and perfusion used for this thesis, a low transfer rate of FITC-Dextran (4kDa) was an indication of maintained airway epithelium and an unchanged filtration coefficient (Figure 7). However by decreasing the thoracic pressure down to –0.8 kPa there was a rapid infiltration of inhaled 4-kDa FITC-Dextran into the circulation, indicating a deteriorated epithelial integrity (Figure 7).
Perfusate sampling

To measure the concentration of solutes in the perfusate, discrete samples of perfusate were taken at predetermined time intervals. The interval between samples was determined by practical reasons, such as workload and costs for the bio-analysis. Generally, fewer samples or longer intervals between samples should be used in the end of the perfusate sampling period. An automated fraction collector was used to sample the perfusate. The collector consists of a computer controlled motorised XY-axis positioner and an automatic 3-way valve so that perfusate could either be sampled at any permitted XY position or directed to drain.

Short inhalation exposures of the IPL

The generation of aerosols with the DustGun technology produces high aerosol concentrations, and thus, only short exposures of the lung are needed in order to reach the objective of particle deposition in the lung. For the purpose of this thesis the length of the
exposures was 10-120 seconds. The exposure line of the DustGun ends in the 3-way junction. The stainless tracheal catheter of the IPL was connected to the 3-way junction (Figure 3). The exposure air containing the aerosols was entering and exiting the 3-way junction with a 45° degree angle to the horizontal plane. Using a downstream precision controlled vacuum source, rebreathing of depleted aerosol was prevented by maintaining a unidirectional flow ($Q_{\text{filter}}$) that was at least three times the average ventilation flow ($Q_{\text{vent}}$). $Q_{\text{vent}}$ was 75-100 mL/min and was produced by the rodent ventilator. The sum of $Q_{\text{filter}}$ and $Q_{\text{vent}}$ was $Q_{\text{total}}$ (Eqn.1) and the resulting $Q_{\text{total}}$ was typically 430 mL/min. Downstream of the 3-way junction a total filter (MF) was positioned. The MF collects aerosol that was either bypassing the lung or exhaled from the lung. When the exposure cycle of the DustGun was finished the MF filter was collected and gravimetrically assessed. The concentration of aerosol was calculated for each exposure (Eqn. 2 and Table 2). The theoretical deposited mass was calculated; the flow corrected mass on the filter was multiplied with the total lung deposition factor (Hoffmann) for a monodisperse aerosol of a specific diameter in the rat lower respiratory tract$^{90}$ (Eqn. 3).

Eqn. 1 $Q_{\text{total}} = Q_{\text{filter}} + Q_{\text{vent}}$

Eqn. 2 $\dot{C}_{\text{aerosol}} = \frac{\text{MF}}{Q_{\text{total}}} \times \frac{1}{t}$

Eqn. 3 $M_{\text{dep}} = \text{MF} \times \frac{Q_{\text{vent}}}{Q_{\text{total}}} \times F_{\text{dep}}$

$C_{\text{aerosol}}$ = aerosol concentration (mg/mL)  
MF = total aerosol mass on end filter (mg)  
$Q_{\text{vent}}$ = minute ventilation (mL/min).
$Q_{\text{total}} =$ total average flow (mL/min).

t = time of exposure (min)

$M_{\text{dep}} =$ Mass deposited (µg) (theoretical dose estimate)

$F_{\text{dep}} =$ fractional deposition of anatomical model prediction of an aerosol with the measured MMAD.

Figure 8) The distribution of diesel soot deposited in the lung lobes of individual rat IPLs. The total deposition of 19.8±1.1 µg in the lungs represented 0.9% of the amount loaded to the aerosol generator or 9.5% of the amount inhaled by the lungs. The rest was exhaled.
**Table 1a**  Administration of toxicants to the rat IPL.

<table>
<thead>
<tr>
<th>Dry powder</th>
<th>TEXP</th>
<th>Metered</th>
<th>Average</th>
<th>Inhalable</th>
<th>Lung dose</th>
<th>Deposition</th>
<th>Theoretical</th>
<th>Deposition factor</th>
<th>Ratio</th>
<th>Dose estimate</th>
<th>Theroretical</th>
<th>Mass deposited of the carrier particle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diesel soot</td>
<td>2.2±0.1</td>
<td>120</td>
<td>1.3</td>
<td>990±50</td>
<td>19.8±1.1</td>
<td>2.0</td>
<td>0.55±2.2</td>
<td>0.09</td>
<td>20±1.0</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Silica</td>
<td>10</td>
<td>120</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>3.5±1.7</td>
<td>N/A</td>
<td>0.3</td>
<td>12</td>
<td>0.6</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Silica+BaP</td>
<td>3.0±0.1</td>
<td>60</td>
<td>0.5</td>
<td>200</td>
<td>23±4</td>
<td>11.0</td>
<td>3.5±1.7</td>
<td>0.3</td>
<td>22</td>
<td>0.8</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Silica+BaP</td>
<td>3.0±0.1</td>
<td>60</td>
<td>0.9</td>
<td>380</td>
<td>27±4</td>
<td>7.1</td>
<td>3.5±1.7</td>
<td>0.3</td>
<td>53</td>
<td>1.0</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Silica+BaP</td>
<td>3.0±0.1</td>
<td>60</td>
<td>2.1</td>
<td>910</td>
<td>53±2</td>
<td>5.8</td>
<td>3.5±1.7</td>
<td>0.3</td>
<td>53</td>
<td>1.0</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

This aerosol was representative for an aerosol with a methylated silica surface,
\(^{b}\) this aerosol was representative for an aerosol with particle surface of crystalline BaP,
\(^{c}\) time of exposure in seconds,
\(^{d}\) mass deposited in coldtrap,
\(^{e}\) the determined dose in lung tissue,
\(^{f}\) deposition factors taken from (Hofmann 2000),
\(^{g}\) calculated according to Eqn. 3.
\(^{1}\) calculations of aerosol concentration have been revised according to Eqn. 2.
Table 1b) Administration of drugs to the IPL.

<table>
<thead>
<tr>
<th>Dry powder</th>
<th>Metered dose (mg)</th>
<th>TEXP (s)</th>
<th>Average Aerosol Conc (µg/mL)</th>
<th>Inhalable dose (µg)</th>
<th>Lung dose (µg)</th>
<th>Lung/Inhalable Dose (%)</th>
<th>MMAD/GSD (µm)</th>
<th>Deposition factor</th>
<th>Theoretical dose estimate (µg)</th>
<th>Ratio: estimated/deposited</th>
<th>PAPER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Budesonide</td>
<td>1.5</td>
<td>12</td>
<td>1.2</td>
<td>100±18</td>
<td>N/A</td>
<td>N/A</td>
<td>2.3/1.9</td>
<td>0.26</td>
<td>3.3±0.1</td>
<td>N/A</td>
<td>III</td>
</tr>
<tr>
<td>Budesonide</td>
<td>3.0</td>
<td>120</td>
<td>0.9</td>
<td>740±83</td>
<td>N/A</td>
<td>3.1</td>
<td>2.3/1.9</td>
<td>0.26</td>
<td>27±5.5</td>
<td>1.2</td>
<td>III</td>
</tr>
<tr>
<td>Formoterol</td>
<td>1.5</td>
<td>12</td>
<td>1.0</td>
<td>84±23</td>
<td>N/A</td>
<td>N/A</td>
<td>4.7/1.7</td>
<td>0.42</td>
<td>5.2±1.9</td>
<td>N/A</td>
<td>III</td>
</tr>
<tr>
<td>Formoterol</td>
<td>3.0</td>
<td>120</td>
<td>0.8</td>
<td>710±47</td>
<td>N/A</td>
<td>5.2</td>
<td>4.7/1.7</td>
<td>0.42</td>
<td>37±2.3</td>
<td>1.0</td>
<td>III</td>
</tr>
<tr>
<td>Terbutaline</td>
<td>1.5</td>
<td>12</td>
<td>1.4</td>
<td>120±16</td>
<td>N/A</td>
<td>N/A</td>
<td>5.3/2.2</td>
<td>0.5</td>
<td>9.7±1.4</td>
<td>N/A</td>
<td>III</td>
</tr>
<tr>
<td>Terbutaline</td>
<td>3.0</td>
<td>120</td>
<td>1.0</td>
<td>880±140</td>
<td>61±6.1</td>
<td>6.9</td>
<td>5.3/2.2</td>
<td>0.5</td>
<td>73±14</td>
<td>1.2</td>
<td>III</td>
</tr>
</tbody>
</table>

Time of exposure in seconds, mass deposited on the total filter, the determined dose in lungs, deposition factors taken from (Hofmann 2000), calculated according to Eqn. 3. N/A (not available).
Assessment of biological effects in the lung after short inhalation exposures to the IPL

Particle deposition in the IPL PAPER I, II and III

Particle deposition in the IPL occurs along the airways to the pulmonary acinus. The dominant mechanisms of particle deposition in the IPL for the aerosols used in this thesis are sedimentation and impaction\textsuperscript{95}.

The regional distribution of particle deposition in individual lung lobes was measured for diesel soot exposed lungs in PAPER I (Figure 8). For these diesel soot exposed lungs, deposition in respectively extrapulmonary bronchi and lung lobes were in close agreement with the data of deposition from similar sized monodisperse particle aerosols by Raabe\textsuperscript{95}.

However, the purpose of most theoretical models and in vivo experiments on aerosol deposition is to relate deposition to the whole animal. So compared with the IPL, the basis for regional deposition fractions derived in such studies also includes inhalability and upper airway deposition\textsuperscript{95}. Yet, in two multi path models based on morphometric data from the rat lung\textsuperscript{90, 131}, the deposition fractions of the lung are normalised to the number of particles entering the trachea, not the nose. Therefore, the fractional deposition of particles in the lungs of the IPL model will be higher than typical deposition fractions based on data for the whole animal. The predicted lung deposition (µg) (the sum of tracheobronchial- and pulmonary deposition) of the different particles as based on theoretical deposition fractions, aerosol concentrations, and ventilation patterns was compared with the measured total mass balance (µg) (Eqn. 7). Despite a few exceptions, generally, the ratio of predicted to measured depositions for the aerosols spanning a fairly wide MMAD came out close to 1 (Table 1).
However, these theoretical estimates of regional particle deposition must be cautiously interpreted. To test the frequency and loci of the deposition of a monodisperse silica particle aerosol in the IPL, a stereological approach was developed. For the monodisperse silica particles an alveolar deposition of more than 30% would be expected. However, from stereological estimations of particle distribution within the lung it was found that more than 90% of the deposited particles were deposited in peripheral bronchi (Figure 4a) PAPER II. It has been suggested that this increased bronchial deposition was related to electrostatically charged particles that induced mirror charges on peripheral airways as particles passed through the most narrow passages of an airway.

**Methods of quantitation in perfusate and lung tissue** PAPER I, II and III

To measure the concentration of solutes in perfusate and tissues two different methods have been used for this thesis; a) Liquid scintillation counting LSC of tritiated benzo(a)pyrene (3\(^{11}\)HBaP) (PAPER I and II) b) Tandem mass spectrometry LC-MS/MS (PAPER III). To facilitate reliable measurement of solute retained in tissue two different methods have been used. a) for the quantification of 3\(^{11}\)HBaP in tissues a method with total combustion over a platinum catalyst followed by LSC was used to determine the amount of tritium present in the tissue (PAPER I and II) b) a microwave oven extraction of solutes followed by LC-MS/MS (PAPER III). The amount of 3\(^{11}\)HBaP-eq. in perfusate and tissues was calculated from the specific activity of each labelled/unlabelled BaP-eq. mixture used for the different exposure levels. The amount the 3\(^{11}\)HBaP-eq. in perfusate from the different exposure levels was measured by liquid scintillation counting (LSC) and the amount of 3\(^{11}\)HBaP-eq. in tissue for the different exposure levels was measured by liquid scintillation counting (LSC) following total combustion of tritium-labelled soot.
Pharmaco-/toxicokinetics PAPER II and III

After measuring the concentration of solutes in perfusate and tissues after short inhalation exposures of the IPL to drugs and toxicants, some pharmacokinetic parameters was established. However, the pharmacokinetic and toxicokinetic concepts of inhalation exposures are well established, as are the parameters to describe these concepts; absorption, distribution, metabolism and excretion of a given drug (Figure 6). However, in the IPL, the use of these well established concepts are not applicable. Therefore, the concepts described in the following section are explicitly interpreted for the single pass IPL.

Cmax was defined as the peak concentration in perfusate. Tmax was defined as the time in minutes to reach the maximum concentration in perfusate. The amount of substance appearing in the perfusate during the entire perfusion period was integrated by trapezoid integration, thereby obtaining the cumulative clearance (Mcc) of drugs and toxicants (Eqn.4).

Eqn. 4 \[ \text{Mcc} = \sum_{n=1}^{n} \left( \frac{C_n + C_{n+1}}{2} \right) \times Q \times t \]

Eqn. 5 \[ \text{Fraction}_{\text{lung}} = 1 - \frac{\sum_{n=1}^{n} \left( \frac{C_n + C_{n+1}}{2} \right) \times Q \times t}{M_{\text{cc}} + M_{\text{tissue}}} \]

Eqn. 6 \[ \text{Fraction}_{\text{perfusate}} = \frac{1}{V} \times \frac{\left( \frac{C_n + C_{n+1}}{2} \right) \times Q \times t}{M_{\text{cc}} + M_{\text{tissue}}} \]

Eqn. 7 \[ \text{Dose}_{\text{DEP}} = \text{Mcc} + M_{\text{tissue}} \]

\[ C_n = \text{Concentration of the nth sample (mole/mL)} \]
Δt = interval of period (min)

Fraction_{lung} = Fraction retained of the substance in lung tissue (mole retained/mole deposited)

Fraction_{perfusate} = Fraction of deposited dose transferred per mL perfusate (mole/mL perfusate/mole deposited) 1/mL

M_{ccalc} = Amount of substance cumulatively cleared (mole)

M_{TISSUE} = Amount of substance in tissue (mole)

Q_{PERF} = Perfusion flow (mL/min)

V_{n} = Volume of the nth sample (mL)

DOSE_{DEP} = total mass balance of the inhaled aerosol (mole)

A mass balance of the inhaled dose was calculated by the addition of cumulative clearance with mass retained in tissue at the end of the perfusion time (Eqn. 7). The fraction of drug retained in tissues as normalised to the deposited dose, was calculated after each perfusate sample (Eqn. 5). The fractional retention values were plotted versus time and the first and second half-lives of pulmonary absorption (t½) was calculated. Similarly, the amount of solute transferred per mL perfusate as normalised to the deposited dose was calculated after each perfusate sample (Eqn. 6). The maximum rate of solute penetrating the air/blood barrier was denoted as Fraction_{perfusate(maximum)} or F_{max}. The fraction of the deposited dose transferred per mL perfusate throughout the perfusion interval was plotted versus time. For the purpose of the discussion of this thesis the unit of Fraction_{perfusate} was changed to 1/100mL (Table 2).
High local concentration of benzo(a)pyrene in the lungs PAPER II

Increasing exposure levels of benzo(a)pyrene (BaP) administered as short inhalation exposures to the rat lung caused an abrupt change in the absorption kinetics of this hydrocarbon in the lung (Figure 9 and Table 2). For the low exposure level, the absorption followed a first-order absorption process. At the medium exposure level absorption to the perfusate reached a plateau that lasted for about 12 minutes and was then followed by a first-order absorption process. This plateau in the perfusate concentration is a strong indication that physicochemical saturation was briefly reached in the immediate vicinity of the deposited silica particles in the lung. For the high dose exposure level, physicochemical saturation totally dominated the absorption process changing it into a zero-order process for the last two-thirds of the experiment. With increasing exposure there was an increasing fraction of BaP-eq. that was retained in the lung tissues (Figure 10 and Table 2).

Figure 9) Left panel; first-pass pulmonary absorption of BaP for the three different exposure levels. Right panel; The Fraction_{perfusate} of the deposited dose of BaP appearing per mL perfusate. Please note! the opposite order between the curves in the left and right panel.
The rate of metabolism of BaP in the lungs increased with increasing exposure levels. However, when related to the amount of BaP deposited, metabolism in the lungs decreased drastically with increasing exposure levels. Therefore, the fractional concentration of BaP metabolites decreased with increasing exposure levels in the tissues as well as in the perfusate. The high level of parent compound retained in the mucosa was comprised of one fraction of BaP dissolved in the tissues and another fraction of BaP lingering as crystalline material on the carrier particles. At the 75 min time point the metabolic pattern in the perfusate could be compared with that of the tissues. It was evident that on average the metabolite mix leaving the lungs in the perfusate was much more polar than the fraction remaining in the lungs. This metabolite balance was in accordance with the physicochemical mechanism of higher mobility of less lipophilic solutes in tissues than of more lipophilic solutes.

Figure 10) The total mass balance and the distribution of inhaled BaP-eq. between perfusate and lung tissue at the end of the perfusion period, for the low, medium and high exposure levels, respectively.

As demonstrated in PAPER II, the mucosa will reach physicochemical saturation at relatively low exposure rates to BaP. Very important to note is that the saturation process is driven primarily by a low mobility in tissues, not a low solubility. The distinction is
important because an erroneous conclusion of a limiting solubility would indicate that the substance is not bioavailable and is not likely to reach toxic quantities in the entrance epithelium, whereas the low mobility mechanism clearly indicates high local concentrations in the site-of-entry epithelium\textsuperscript{69}. The silica particles with the medium concentration of BaP were used to control whether physicochemical saturation is a likely explanation for the plateau signalled by the absorption curve. If the entire content of BaP on the silica particles of the medium exposure would dissolve in a cylindrical section of the air/blood barrier below the particles deposited in the bronchioles, this saturated cylinder would be about 4 µm in diameter and about 5 µm high above the basement membrane. This is clearly a reasonable dimension for a saturated section of the air/blood barrier surrounding the silica carrier particles in the bronchioles. Thus, it was reasonable believe that local saturation corresponding to a cellular concentration of 2.3 nM BaP-eq occurs, in a lung the size as that of the rat at acute cumulative inhalation exposures of only 36 ng BaP. Thirty-six ng is about the content of BaP in the smoke from a single cigarette\textsuperscript{134}. The detected limit of saturation gives a strong reminder of how uneven the cellular doses are in the body when highly lipophilic toxicants such as BaP are inhaled. The concentration of BaP in the highest exposed cells surrounding the carrier particle lingers at levels around 5 million times higher than the average initial body burden projected for the rat the lungs came from.

**Pharmacokinetics of budesonide, formoterol and terbutaline PAPER III**

After short inhalation exposures of the IPL of respirable aerosols of budesonide, formoterol and terbutaline (Figure 5 and Table 1), the three inhaled drugs appeared rapidly in the perfusate. The concentration of budesonide and terbutaline peaked at a significantly shorter Tmax than did formoterol, both in the low and high dose exposures (Table 2).

In the case where cumulative clearance measurements were combined with quantitation of substance remaining in the lungs after the perfusion period, an overall mass
balance over the lungs throughout the perfusion interval was calculated (Table 2). For this exposure level fractional retention at the end of the perfusion period was found to be respectively 0.19±0.05, 0.19±0.06, 0.04±0.01 (mean±SD, n=3) for budesonide, formoterol and terbutaline. The pulmonary retention of drugs was possible to quantitate throughout the perfusion period by subtracting the appearance of drug in the perfusate from the total amount of drug recovered from tissues and perfusate (Eqn. 5). The fraction retained in lung tissue

Figure 11) Comparable pharmacokinetics of a short acting β₂-adrenoceptor agonist (terbutaline) versus long acting β₂-adrenoceptor agonist (formoterol). Left panel: A comparison of the rate of penetration of the air/blood barrier of terbutaline (dotted line) and formoterol (solid line). Right pane: A comparison of the solute retention in tissues between terbutaline (dotted line) and formoterol (solid line). The low retention of terbutaline in tissues after the perfusion time is a strong indication of well perfused lungs.
### Table 2a) Toxicokinetics of the inhaled carcinogen benzo(a)pyrene b) Pharmacokinetics of budesonide, formoterol and terbutaline

<table>
<thead>
<tr>
<th>Dry powder</th>
<th>Tmax (min)</th>
<th>Fmax (1/100 mL)</th>
<th>Cmax (nM)</th>
<th>t½</th>
<th>Fraction Lung&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Total dose (ng)</th>
<th>Mcc&lt;sup&gt;b&lt;/sup&gt; (ng)</th>
<th>Tissue dose (ng)</th>
<th>PAPER</th>
</tr>
</thead>
<tbody>
<tr>
<td>BaP Low</td>
<td>3.7±0.4</td>
<td>0.14</td>
<td>0.01±0.002</td>
<td>25±1.5</td>
<td>0.3</td>
<td>2.2±0.4</td>
<td>1.7±0.3</td>
<td>0.5±0.2</td>
<td>II</td>
</tr>
<tr>
<td>BaP Medium</td>
<td>3.4±1.4</td>
<td>0.07</td>
<td>0.10±0.02</td>
<td>65±1.0</td>
<td>0.5</td>
<td>35±4</td>
<td>17±2.0</td>
<td>17±1.0</td>
<td>II</td>
</tr>
<tr>
<td>BaP High</td>
<td>40&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0.03</td>
<td>8.5±0.2</td>
<td>N/A</td>
<td>0.7</td>
<td>8400±1200</td>
<td>2400±550</td>
<td>5800±120</td>
<td>II</td>
</tr>
</tbody>
</table>

<sup>a</sup> Fraction of solute remaining in tissue at the end of experiment  
<sup>b</sup> cumulative clearance

<sup>1</sup> First-pass absorption crested at 40 minutes post exposure. N/A (not available)

<table>
<thead>
<tr>
<th>Dry powder</th>
<th>Tmax (min)</th>
<th>Fmax (1/100 mL)</th>
<th>Cmax (nM)</th>
<th>T½</th>
<th>Fraction Lung&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Total dose (µg)</th>
<th>Mcc&lt;sup&gt;b&lt;/sup&gt; (µg)</th>
<th>Tissue dose (µg)</th>
<th>PAPER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Budesonide</td>
<td>2.2 ±0.2</td>
<td>0.22</td>
<td>110± 44</td>
<td>40±12</td>
<td>0.19</td>
<td>54±41</td>
<td>43±34</td>
<td>11±7.4</td>
<td>III</td>
</tr>
<tr>
<td>Formoterol</td>
<td>6.7±4.0</td>
<td>0.15</td>
<td>180±20</td>
<td>32±11</td>
<td>0.19</td>
<td>87±20</td>
<td>70±11</td>
<td>17±8.6</td>
<td>III</td>
</tr>
<tr>
<td>Terbutaline</td>
<td>2.2 ±1.1</td>
<td>0.38</td>
<td>1030±260</td>
<td>14±4.5</td>
<td>0.04</td>
<td>220±12</td>
<td>210±8</td>
<td>10±3.1</td>
<td>III</td>
</tr>
</tbody>
</table>

<sup>a</sup> Fraction of solute remaining in tissue at the end of experiment  
<sup>b</sup> cumulative clearance.
(Fraction\textsubscript{lung}) was, thus, calculated and plotted versus time for the entire perfusion period (\textit{Figure 11}). From the fractional retention curves, a second half-time of absorption (t\textsubscript{1/2}\textsubscript{β}) was calculated to be respectively 40±12, 32±11, 14±4.5 min (mean±SD, n=3) for budesonide, formoterol and terbutaline. The amount of inhaled drug penetrating the air/blood barrier of the rat lung per 100 mL perfusate was normalised to the deposited dose and thus, the Fraction\textsubscript{perfusate} was calculated for all samples (Eqn. 6) and plotted versus time (\textit{Figure 11}). The Fraction\textsubscript{perfusate} peaked at respectively 0.22±0.1, 0.15±0.05 and 0.38±0.1 l/ 100 mL (mean±SD, n=3) for budesonide, formoterol and terbutaline, respectively.

The relatively long tissue retention of both budesonide and formoterol found in the present study, have been well documented in many preclinical and clinical studies\textsuperscript{44,135}. The longer acting β\textsubscript{2}-adrenoceptor agonist formoterol had a markedly longer tissue retention in the lungs than did the short acting β\textsubscript{2}-adrenoceptor agonist terbutaline. This may explain the increased clinical effect duration of formoterol in comparison with terbutaline, which is thought to be caused by a higher lipophilicity of the former substance\textsuperscript{136}. A possible mechanism of the delayed clearance of budesonide is intracellular fatty acid esterification coupled with the high lipophilicity, as previously documented after \textit{in vitro}, as well as \textit{in vivo} administration. This reversible esterification has the potential to prolong the anti-inflammatory effect of budesonide and improve its airway selectivity\textsuperscript{137}.

A high plasma concentration after the inhalation of a compound chosen to exert a local action in the lung, is often associated with systemic side effects\textsuperscript{138}. The critical contribution to this phenomenon from a rapid pulmonary absorption was documented in detail in the present model. In PAPER III it was shown that the more water-soluble adrenergic β\textsubscript{2}-adrenoceptor agonist terbutaline had a distinctively more rapid absorption and higher perfusate concentration than did the β\textsubscript{2}-adrenoceptor agonist formoterol and the corticosteroid budesonide. The amount of drug that penetrated the air/blood barrier for terbutaline per mL
perfusate peaked at a 2.5 times higher level than did formoterol (Figure 11). It was reasonable to assume that a rapid absorption into the perfusate of the IPL will in general correspond to a high peak concentration in arterial blood samples following in vivo deep lung inhalation exposures, thereby increasing the potential for systemic side effects.

This set of data on rates of absorption and tissue retention of budesonide, formoterol and terbutaline in the rat IPL was in reasonable agreement with published clinical findings. This study (PAPER III), shows the potential of the DustGun technology in combination with a short inhalation protocol as used in the clinic, to generate IPL data of inhaled dry powder aerosols of potential drugs in the discovery phase and early development.

Vasoconstriction after inhalation of budesonide: a study in the isolated and perfused rat lung PAPER IV

Recently it has been demonstrated that inhaled corticosteroids exert a vasoconstrictive effect on the bronchial circulation\textsuperscript{81,139}. This effect is considered to be a non-genomic effect of corticosteroids\textsuperscript{40,140}. During the experiments of PAPER III an anomaly was noticed. After short inhalation exposures of budesonide to the IPL a decrease in the pulmonary perfusion flow rate was seen. Thus, a method was established where a constant hydrostatic pressure was used to perfuse the IPL for measuring the relative pulmonary perfusate flow rate after short inhalation exposures of the IPL to lactose and budesonide. Perfusate was only allowed to pass once through the lung or single-pass perfusion.

After short inhalation exposures to lactose and budesonide, the theoretical dose estimate was calculated for each exposure group (Table 3). Lactose exposed lungs were chosen as an aerosol exposure control, for comparison with budesonide exposed lungs. There was a non-significant decrease in the mean perfusion flow rate of lactose in comparison to Sham (Figure 12). The three exposure levels of budesonide (BUD-2, BUD-10 and BUD-50) were chosen to obtain a five fold dose increase between successive dose levels (Table 3). The
perfusate flow rate after dry particle aerosol exposure was followed up to 100 minutes post intervention.

Table 3) Estimates of lung dose of budesonide and lactose

<table>
<thead>
<tr>
<th>Drug</th>
<th>n</th>
<th>Mass deposited (µg) a</th>
<th>Lung average conc. (µM) b</th>
</tr>
</thead>
<tbody>
<tr>
<td>BUD-2</td>
<td>5</td>
<td>2.5±0.3</td>
<td>10</td>
</tr>
<tr>
<td>BUD-10</td>
<td>5</td>
<td>11±0.8</td>
<td>40</td>
</tr>
<tr>
<td>BUD-50</td>
<td>5</td>
<td>48±9.3</td>
<td>200</td>
</tr>
<tr>
<td>BUD-10 +Prazosin</td>
<td>5</td>
<td>12±0.2</td>
<td>40</td>
</tr>
<tr>
<td>Lactose</td>
<td>5</td>
<td>40±11</td>
<td>N/A</td>
</tr>
</tbody>
</table>

aThe theoretical deposited mass (µg) after inhalation of budesonide and lactose in the IPL,
bthe calculated initial average lung concentration of budesonide in 1.5 g lung tissue(µM).

There was a rapid and significant decrease in mean perfusion flow rate in BUD-10 and BUD-50 in comparison to BUD-2 and lactose exposed lungs (Figure 12), the latter which did not differ significantly. The onset of decrease in the mean relative perfusion flow rate was significant for BUD-10 and BUD-50 in comparison to lactose at 40 minutes post exposure. Hence, in PAPER IV, a dose dependent onset of the decrease in the mean perfusion flow rate in the pulmonary circulation was measured. This flow reduction was most likely caused by vasoconstriction of pulmonary blood vessels.

One important aspect of comparing vasoconstriction in clinical and preclinical models is whether the applied doses to target tissues are approximately similar. The data in humans were derived from vapour disappearance into the bronchial circulation in the trachea and main stem bronchi81, whereas the preclinical data of the IPL model was derived from
vasoconstriction in the pulmonary circulation involving the entire peripheral lung. Significant vasoconstriction in the IPL model was found to require a higher initial dose than was observed in the clinic. Most likely, the difference in anatomy and site of particle deposition within the respiratory tract of the two species may explain the difference in potency of budesonide to induce vasoconstriction PAPER (IV). Thus, the current IPL model could serve as a valuable complement to more advanced clinical models for studying the influence of inhaled drugs on the circulation in the lungs.

Figure 12) Reduction of relative perfusate flow rate (% qlung) in the lungs averaged over different time intervals after inhalation exposures of the IPL to budesonide. For all treatments from left to right respectively and as indicated in figure; air control, lactose, BUD-2, BUD-10, BUD-10+praz., BUD-50 (mean±SD, N=5), * denotes significantly different from lactose, § denotes significantly different from BUD-10, # denotes significantly different from BUD-10
* = p<0.05, ** = p<0.01, §§ = p<0.01 and ## = p<0.01.
Budesonide exposures of the lungs had a pronounced influence on the measured perfusate flow rates. While the flow rate of the BUD-2 exposures showed no significant effect, the BUD-10 exposures substantially reduced the perfusion flow rates of the lungs. Then at the BUD-50 exposures there was again a smaller reduction in the perfusion flow rate, causing a bell-shaped dose-response. This smaller reduction for BUD-50 was significant than noted for BUD-10 during the last 60 minutes of perfusion but for the total period, the difference in perfusion flow rate was non-significant (Figure 12). Interestingly, in healthy subjects and asthmatics, inhaling fluticasone propionate a “bell-shaped” dose response curve was seen. The mechanism behind these observations is not known.

In an attempt to address the underlying mechanism behind the budesonide induced reduction of perfusate flow rates, the addition of a selective $\alpha_1$-adrenoceptor antagonist prazosin to the perfusate reduced the budesonide induced vasoconstriction by about 50% (Figure 12). Thus the following mechanism was suggested; most likely budesonide reduces the extraneuronal uptake of noradrenalin by inhibition of transporters of noradrenalin uptake\textsuperscript{141, 142}. This leads to an increased noradrenalin concentration at neuromuscular junctions causing vasoconstriction via stimulation of $\alpha_1$-adrenoceptors. The selective $\alpha_1$-adrenoceptor antagonist prazosin competes with noradrenalin at the binding site of the $\alpha_1$-adrenoceptor and in this way reduces the budesonide induced vasoconstriction. Thus, it was suggested in PAPER IV that the vasoconstriction seen in the IPL model after inhalation of corticosteroids (ICS) represents a nongenomic effect,\textsuperscript{81} that was mediated, at least partly, by corticoid interference with noradrenalin uptake\textsuperscript{141}.

Thus, the presented technology for short inhalation exposures of the IPL with detailed measurements of pulmonary perfusion flow gives the opportunity to study rapid effects on vasomotor tone in the IPL after interventions with various pharmacological tools.
The method can be particularly suitable for investigating effects of the rapid nongenomic response seen after exposures of the IPL to corticosteroid receptor agonists at therapeutic- and supratherapeutic doses of ICS.

GENERAL CONCLUSIONS

DustGun Technology

The DustGun exposure technology generated respirable aerosols from a great variety of dry powders such as silica particles and diesel soot and several pharmaceutical powders for the treatment of asthma. The common traits for these exposures were;

i) Low powder consumption per exposure ranging from 0.5-3 mg.

ii) Short exposure time spanning an interval from 9-120 seconds.

iii) High concentration of aerosols in the air, ranging from 0.5-2 µg/mL.

iv) High reproducibility in the generation of aerosols

The dose dependent and site specific dosimetry of inhaled carcinogens

Increasing exposures of airway epithelial cells to benzo(a)pyrene (BaP), greatly influence its toxicity. The disposition of BaP in the lung was abruptly altered by saturation of both dissolution and metabolism of BaP at the site of entry. With increasing exposures of the airway epithelium saturation of metabolism resulted in a relatively decreased bioactivation of parent BaP. This different rate of bioactivation in sub- and supra-saturation exposures may explain the well-known difficulties of inducing lung cancer in laboratory animals using very high concentrations of carcinogenic hydrocarbons, in inhalation exposures during limited life-spans.
**DustGun/IPL inhalation technology for early drug discovery/development**

Technical shortcomings with aerosol exposures have so far often delayed collection of data to predict the kinetics of absorption and drug retention in the lung in the drug discovery/development process. As a result, other procedures, rather than the short inhalation exposures to highly concentrated powder aerosols as later used in the clinic have been the only means available to produce data in the preclinical discovery/development phase.

Hence, by exposing the IPL to respirable particle aerosols in high concentrations, critical pharmacokinetic data from inhalation exposures can be collected. The rate of absorption of the drug into single-pass perfusate was measured after short inhalation exposures of the IPL and was combined with measuring of the fraction of drug retained in lung tissues after the experiment. The low powder consumption during generation of the aerosols warrants the use of the DustGun/IPL technology in early drug discovery/development.

**Vasoconstriction after inhalation of corticosteroids**

Inhalation of budesonide into the rat lung resulted in vasoconstriction of pulmonary vessels that was; a) partly $\alpha_1$-adrenergic mediated b) the dose response to induce vasoconstriction seemed to be bell-shaped. Interestingly, similar dose-response relationships have also been observed in clinical measurements of airway mucosal blood flow after the inhalation of corticosteroids.\(^{81}\)

Thus, the presented technology for short inhalation exposures of the IPL with detailed measurements of pulmonary perfusion flow gives the opportunity to study rapid effects on vasomotor tone in the IPL after interventions with various pharmacological tools. The method is particularly suitable for investigating effects of the rapid nongenomic response seen after exposures of the IPL to corticosteroid receptor agonists at therapeutic- and supratherapeutic dose regimen of ICS.
Final conclusion and outlook

a) Short inhalation exposures using well characterised particle aerosols in high concentration are made possible with the DustGun technology for preclinical assessment of substances with low powder consumption.

b) The resulting deposition of particles in the lung is in good agreement with the corresponding toxicokinetic data for the rat lung (PAPER I and II). Pharmacokinetic and pharmacodynamic data is in agreement with the clinical experience of the tested drugs (PAPER III and IV). Nevertheless, the predictive power of the technology will be considerably increased by adding \textit{in vivo} exposures to the IPL exposure scheme using the same exposure technology.

c) Finally, this thesis demonstrates the utility of the DustGun/IPL exposure technology for studying interactions between inhaled particle substances and the isolated and perfused lung (PAPER II and IV), which can be difficult/impossible to perform \textit{in vivo} (animals/man) due to practical/ethical considerations.
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REFERENCES


