

Development and characterisation of air-liquid interface exposure systems for cell and tissue cultures

A thesis submitted in partial fulfilment of the requirements of London Metropolitan University for the degree
of Doctor of Philosophy

Shoaib Majeed

Supervisors:

Dr. Eirini Meimaridou

Prof. Kenneth White

DECLARATIONS

As part of my application for a PhD by prior output, I am pleased to submit a series of peer-reviewed journal articles I have contributed to. Each chapter in this thesis represents a different research article and provides a detailed summary of my work in that area. In submitting this thesis, I aim to demonstrate that I have met the requirements for a PhD in terms of both volume and academic content. I am proud to say that this collection of publications is not just a series of disconnected articles but represents a cohesive and comprehensive body of work. Throughout these articles, I have made significant and original contributions to the field of study, and I am confident that they demonstrate my ability to conduct rigorous and impactful research. I believe that this submission represents the culmination of years of hard work and dedication to my field, and I am excited to have the opportunity to share it with the academic community. I am deeply grateful for your consideration and support.

ABSTRACT

INTRODUCTION

Monitoring the delivery of inhaled materials to the respiratory tract requires consideration of particle deposition and gas dissolution within the complex structure of the respiratory system. Until the last decade or so, methodology relied heavily on animal models or limited, submerged cell culture models that did not capture air-liquid interactions, identifying the need for more comprehensive in vitro aerosol exposure systems to examine how aerosolised substances impact respiratory tissues, cellular responses, toxicity, and overall health. Aerosols possess opposing qualities, serving both as hazards and therapeutic tools, which underscores the critical necessity for rigorous safety testing. This thesis describes the development and characterisation of in vitro aerosol and smoke exposure systems for future testing, the Vitrocell® 24/48 and the InHALES systems.

RESULTS

The Vitrocell® 24/48 system was developed to simulate physiological conditions at the air-liquid interface using a stagnation flow setup with controlled temperature (37°C). Comprehensive characterisation revealed excellent reproducibility with a coefficient of variation of 12.2% across inserts and 15.6% overall between experimental runs. Quartz crystal microbalance measurements confirmed linear particle deposition ($R^2 > 0.95$) across dilution ranges from 7% to 69% cigarette smoke concentration. Chemical analysis revealed a consistent delivery of key smoke constituents, with eight carbonyls and nicotine exhibiting a strong linear correlation ($R^2 > 0.96$) with smoke concentration. Biological testing using A549 and BEAS-2B cells also showed concentration-dependent toxicity.

Additionally, the InHALES system is described, created to offer a more precise exposure platform in which the inhalation-exhalation cycle can be modelled and controlled. The research extends to practical applications, including the effects of cigarette smoke and aerosols on dental resin composites, where cigarette smoke caused 10-fold greater surface discolouration on tooth enamel compared to heated tobacco product aerosols after 14 days of exposure. Studies on 3D bronchial and nasal tissue cultures have revealed significant reductions in ciliary beating frequency following exposure to cigarette smoke, providing quantitative evidence of respiratory epithelial dysfunction. The research concludes with the application of the developed methodologies to address contemporary challenges, including the aerosol filtration testing of over 300 fabrics for the development of reusable masks, and the aerosol administration of chloroquine, which contributes valuable insights for public health improvements, especially during the COVID-19 pandemic.

CONCLUSION

The Vitrocell and InHALES platforms discussed in this thesis also bypass ethical concerns associated with traditional animal testing and propel biomedical research by offering a more relevant and compassionate approach. While much attention has been focused on cigarette smoke and tobacco-related aerosols, these in vitro systems have broader implications in inhalation science, extending to examining the effects of wood combustion and evaluating the impact of occupational and environmental aerosols on human health through inhalation. By bridging laboratory research with real-world applications during the COVID-19 pandemic, this research demonstrates how advanced exposure technologies can rapidly pivot to address urgent public health challenges, ultimately providing a more humane, accurate, and versatile approach to understanding the complex interactions between inhaled substances and human respiratory health.

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LIST OF ABBREVIATIONS

2D- Two-dimensional

3D - Three-dimensional

3R4F - Reference cigarette from University of Kentucky

3Rs - Replacement, Reduction, and Refinement

ALI - Air-liquid interface

ANOVA - Analysis of variance

API- Active pharmaceutical ingredient

APS - Aerodynamic particle sizer

AS - Artificial saliva

ASTM - American Society for Testing and Materials

CBF - Ciliary beating frequency

CDC - Centers for Disease Control and Prevention

CFP - Cambridge filter pad

CMAG - Condensation monodisperse aerosol generator

COVID-19 - Coronavirus disease 2019

CQ - Chloroquine

CS - Cigarette smoke

CV - Coefficient of variation

CYP - Cytochrome P450

DART-MS - Direct analysis in real-time mass spectrometry

DMSO - Dimethyl sulfoxide

DNPH - Dinitrophenylhydrazine

DSF - Disodium fluorescein

ENDS - Electronic nicotine delivery systems

FE - Filtration efficiency

FMPS - Fast mobility particle sizer

FTIR - Fourier-transform infrared spectroscopy

GC-MS - Gas chromatography-mass spectrometry

GVP - Gas-vapour phase

HBEC - Human bronchial epithelial cells

HCQ - Hydroxychloroquine

HCS - High-content screening

HEPA - High-efficiency particulate air

HnB - Heat-not-burn

HPLC - High-performance liquid chromatography

HRMS - High-resolution mass spectrometry

InHALES - Independent Holistic Air-Liquid Interface Exposure System

LC-MS - Liquid chromatography-mass spectrometry

MOC - Multi-organ-on-a-chip

MPPS - Most penetrating particle size

MS - Mainstream smoke

NAMs - New Approach Methodologies

NaCl - Sodium chloride

NHBE - Normal human bronchial epithelial

NPA - Network perturbation amplitude
OECD - Organisation for Economic Co-operation and Development
OD - Optical density
PBS - Phosphate-buffered saline
PDSP - Programmable dual syringe pump
PFE - Particle filtration efficiency
PG - Propylene glycol
PM - Particulate matter
PPE - Personal protective equipment
PSD - Particle size distribution
QCM - Quartz crystal microbalance
RH - Relative humidity
RNS - Reactive nitrogen species
ROS - Reactive oxygen species
SARS-CoV-2 - Severe acute respiratory syndrome coronavirus 2
SD - Standard deviation
SESI - Secondary electrospray ionisation
SMPS - Scanning mobility particle sizer
SPI-MS - Single-photon ionisation mass spectrometry
SS - Sidestream smoke
TEC - Tetric EvoCeram BulkFill
TEER - Transepithelial electrical resistance
TFE - Total filtration efficiency
THS - Tobacco Heating System
TiO₂ - Titanium dioxide
TPM - Total particulate matter
WHO - World Health Organization
WST-1 - Water-soluble tetrazolium salt

1.0 Introduction

1.1 Introduction:

The lungs are vital organs that directly interact with the outside environment, making them an ideal pathway for delivering local therapeutics via inhalation [1]. Delivering drugs directly to the lungs reduces the risk of side effects, and the drugs take effect immediately due to the extensive pulmonary capillary network and thin air-blood barrier (0.1-0.2 μm thickness). The lungs have an extensive surface area of approximately 70 square meters, along with a highly permeable epithelial lining. This unique structure makes them an ideal pathway for administering systemic drugs, especially those that exhibit lower bioavailability when taken orally. Such drugs often include large molecules like proteins, as well as other compounds that are vulnerable to breakdown during first-pass metabolism in the liver and intestines. By using the pulmonary route, these medications can be delivered more effectively into the bloodstream, potentially improving therapeutic outcomes for patients. Aerosols are widely used for therapeutic, diagnostic, and experimental purposes. Aerosol therapy has been recommended for centuries in treating asthma, but oral delivery of therapeutics has become more popular in the past decade [2]. Inhalation of cigarette smoke is a common practice and falls under one of the forms of inhalation. According to the World Health Organization (WHO), there are approximately 1.3 billion tobacco users globally (3).

In vitro testing methods are scientific experiments performed outside of a living organism, usually in a controlled laboratory setting. These methods involve the use of cell cultures, tissues, or biological molecules to explore different biological processes, examine drug effects, or study diseases mechanisms. By isolating specific components from their natural context, researchers can conduct detailed observations and analyses that aid in understanding cellular functions, performing toxicity tests, and evaluating the therapeutic efficacy of compounds. This approach is essential for developing new treatments and ensuring safety before progressing to in vivo studies, which involve whole organisms (3). These methods are crucial for ~~to~~ examining the effects of aerosols on human health, particularly given the increasing recognition of aerosol-mediated disease transmission and the growing concern about air pollution-related respiratory disorders (2).

Aerosols are minuscule particles suspended in the atmosphere that can be breathed into the respiratory system, causing various respiratory issues. However, they can also be utilised for therapeutic purposes when they are therapeutic aerosols. In vitro testing is a vital component of evaluating new products that may contain aerosols and ensuring their safety before they are introduced to the market. To achieve this, developing a more realistic exposure system for 2D cells/3D culture that utilises an air-liquid interface exposure technique is essential. It is crucial to acknowledge

the significant role that cell culture techniques play in important fields such as drug discovery, cancer research, and studies involving air-liquid interface exposures (4). By mimicking the physiological conditions of living organisms, these techniques offer valuable insights into cellular behaviours, enabling researchers to develop and test new therapies more effectively [5]. By increasing our focus on these techniques, we have the potential to make significant strides in advancing these fields (5).

Traditional cell culture methods use monolayer (2D) systems, where cells grow as a single layer on flat surfaces like tissue culture plates or flasks. Although these 2D techniques have offered useful insights into cellular processes, they do not truly mimic the structural complexity and physiological conditions of in vivo environments. [10]. In 2D cultures, cells exhibit unnatural polarity constraints, limited cell-cell contacts, and altered morphology that significantly differ from their in vivo counterparts [11].

The cellular microenvironment in living tissues comprises a complex network of biochemical and mechanical signals from the extracellular matrix (ECM), neighbouring cells, and soluble factors. These essential spatial and physical cues are largely absent from conventional 2D culture systems, where cells grow in a geometrically and mechanically homogeneous environment that lacks the three-dimensional context critical for physiologically relevant responses [12]. Consequently, cells cultured in 2D systems often display altered gene expression profiles, protein synthesis patterns, and metabolic activities compared to cells in native tissues [13]

1.1.1 The Evolution Toward 3D Culture Systems

3D cell culture refers broadly to any culture method where cells are grown in an environment that permits cell growth and interactions in all three spatial dimensions (x, y, and z axes), as opposed to the two-dimensional constraint (x and y axes only) of conventional cell culture plates. These 3D culture systems enable cells to form more physiologically relevant structures and interactions compared to 2D monolayers.

Basic 3D Culture Systems

Tissue insert-based 3D cultures: Cells are grown on semipermeable membrane inserts (e.g., Transwell®, Millicell®) that can support the formation of three-dimensional structures while permitting independent access to both apical and basolateral compartments. These insert systems are particularly important as they can be adapted for:

- **Submerged conditions:** Where both the apical and basolateral compartments contain culture medium, allowing for initial cell growth and establishment of 3D structures.
- **Air-liquid interface (ALI):** Where culture medium is removed from the apical compartment while maintained in the basolateral compartment, creating an air

exposure on the apical surface while providing nutrients from below. This configuration is especially valuable for respiratory and dermal applications.

Evidence from comparative studies clearly demonstrates that even basic 3D culture systems enable more physiologically relevant analysis of cell-to-cell interactions, metabolic profiling, stem cell differentiation, and disease modelling by better emulating the in vivo cellular environment. For instance, drug response studies in 3D tumour spheroids have shown significantly different results compared to the same cells in 2D cultures, with 3D models more accurately predicting clinical outcomes [13]. A review by Nayak et al. further supports this [14], suggesting that 3D tumor spheroid models "can correctly replicate some features of solid tumors (such as the secretion of soluble mediators, drug resistance mechanisms, gene expression patterns, and physiological responses) better than 2D cell cultures or animal models," providing concrete evidence of differential drug responses and emphasizing that these models are "essential to elucidate the complex biology of cancer and test new diagnostic and therapeutic options (14).

From Basic 3D Cultures to Organotypic Models

It is critical to distinguish between basic 3D cultures and true organotypic models. Simply culturing cells in a three-dimensional environment does not constitute an organotypic model. True organotypic cultures must recapitulate key aspects of organ-specific architecture, cellular composition, and functionality (14).

Basic 3D Cultures vs. Organotypic Models

Characteristic	Basic 3D Cultures	Organotypic Models
Growth Environment	Provide a three-dimensional growth environment	Replicate tissue-specific architecture and organisation
Cell Population	Often consist of homogeneous cell populations	Incorporate multiple cell types in physiologically relevant ratios and arrangements
Tissue Architecture	Lack organized tissue-specific architecture	Establish functional tissue-specific characteristics
Cellular Diversity	Limited cellular diversity and functional specialisation	Demonstrate specialised tissue functions

Characteristic	Basic 3D Cultures	Organotypic Models
Microenvironment	Minimal recapitulation of tissue-specific microenvironments	Recapitulate key aspects of the native extracellular matrix composition and organisation

For example, a simple ALI culture of bronchial epithelial cells may form a pseudostratified layer with some mucus production, but a true organotypic bronchial model would incorporate basal cells, ciliated cells, goblet cells, and club cells in appropriate proportions and spatial organisation, with functional cilia, mucus production, and barrier properties that reflect the native tissue [15].

1.1.2 New Approach Methodologies (NAMs) for Respiratory Research

The emergence of New Approach Methodologies (NAMs) represents a significant advancement in respiratory disease research, as "NAMs are increasingly being used for regulatory decision making by agencies worldwide because of their potential to reliably and efficiently produce information that is fit for purpose while reducing animal use" [16]. These innovative approaches address the limitations of traditional testing methods while providing more human-relevant data, as "NAMs have the potential to fundamentally transform today's regulatory work by allowing more human-relevant decision-making" (17).

1.1.3 The Regulatory Context: From Animal Testing to NAMs

The regulatory landscape for respiratory toxicology has undergone substantial transformation, with NAMs gaining formal acceptance through OECD guidelines and regulatory frameworks. Recent developments include "Defined Approaches (DAs) - specific combinations of data sources (e.g. in silico, in chemico, and/or in vitro data) with fixed data interpretation procedures" that have been formally adopted by OECD, with multiple new guidelines published in 2022-2023 [17]. A landmark achievement occurred when "3D organotypic lung cultures used to assess the hazard of a fungicide (chlorothalonil) ultimately replaced a 90-day inhalation toxicology study in rats", as documented in an official OECD case study [18]. This regulatory integration demonstrates that NAMs now provide "more protective and/or relevant models that have a reduced reliance on animals" while meeting stringent regulatory requirements for respiratory safety assessment (18).

1.1.4 Addressing Critical Gaps in Aerosol and Inhalation Toxicology

1.1.4.1 The Challenge of Physiologically Relevant Exposure

While the regulatory acceptance of NAMs represents a significant milestone, a fundamental challenge remains in accurately replicating human inhalation exposure scenarios. Current challenges include ensuring that "exposure methods and cell/tissue-based test systems" appropriately represent physiological conditions, as "direct liquid applications to an in vitro differentiated primary respiratory cell model altered the transcriptome, biological pathways, activation of several cellular signalling pathways" compared to air-liquid interface exposures [19]. This methodological limitation undermines the physiological relevance of toxicological assessments, as it fails to account for the complex aerodynamics, particle deposition patterns, and localised dosimetry that characterise human inhalation exposure.

To fully comprehend the critical significance of aerosol deposition within the respiratory tract, it is imperative to have a comprehensive understanding of the intricate anatomy and physiology of the airways and lungs. This knowledge is essential for developing exposure systems that accurately represent how aerosols interact with the respiratory system and their subsequent health impacts (18).

1.1.4.2 Advancing Aerosol Exposure Assessment Through 3D NAMs

The evolution of NAMs has coincided with the development of sophisticated in vitro aerosol exposure systems specifically designed to evaluate diverse aerosol types, including cigarette smoke, electronic cigarettes, heated tobacco product vapour, inhaled pharmaceuticals, and environmental exposures. These systems mark a significant progress, as many reviews on in vitro inhalation toxicity testing models have been published, showing that it is possible to replicate specific regions of the human respiratory tract and their responses in vitro [19].

The regulatory urgency for these advanced methods has been intensified by the U.S. Food and Drug Administration's recent final deeming rule for inhalable products, which has created an immediate need to evaluate numerous products and ingredients across various industries. Traditional approaches demonstrate limitations as "NAMs are increasingly being used for regulatory decision making by agencies worldwide because of their potential to reliably and efficiently produce information that is fit for purpose while reducing animal use" [17].

1.1.4.3 The Scientific Imperative

Developing robust in vitro testing approaches that incorporate physiologically relevant aerosol exposure is, therefore, essential for providing critical insights into how inhaled substances affect lung health and contribute to pulmonary diseases. This understanding addresses a fundamental global health need, as respiratory illnesses linked to inhalation exposure account for a substantial number of deaths worldwide. The integration of advanced 3D respiratory models with sophisticated aerosol delivery systems represents a convergence of technological innovation and regulatory necessity (39).

These adapted in vitro aerosol exposure systems are continuously being refined to accommodate new product categories and now serve instrumental roles in supporting product development, regulatory submissions, and validation of safety claims [17]. The data generated from these systems bridges the critical gap between mechanistic understanding and regulatory compliance, enabling more accurate risk assessment while reducing reliance on animal testing (39).

1.2 Respiratory System

To understand how smoke, aerosols, or air pollution affect our health, we must first examine the respiratory system. The lungs are the primary site where airborne pollutants interact with the body, featuring a vast internal surface area of approximately **70 m²** (11). As an organ that exchanges gases, the lung separates inhaled air from the bloodstream through a barrier that's only 0.1 µm thick. Despite its size, the respiratory system is fragile, and it has evolved over time to protect itself from harmful substances, whether they are natural or man-made (cigarette smoke or diesel exhaust). Thus, the respiratory system has developed mechanisms to avoid inhaling potentially dangerous substances and to clear itself of anything that manages to enter. The human respiratory system is divided into three primary regions (see Figure 1.1). The first region is the nasopharyngeal region, which includes the nose and pharynx. The second region is the tracheobronchial region, which consists of the trachea, bronchi, and bronchioles. The bronchi, can be further divided into the main, lobar, and segmental bronchi. The third region is the alveolar region, which includes the alveolar bronchioles and alveoli. The exchange of gases between the lungs and the blood occurs during the process of respiration. This exchange happens in the alveoli, which are tiny air sacs found in the lungs. Oxygen diffuses through the alveoli walls and binds to red blood cells in the surrounding capillaries. Simultaneously, carbon dioxide, produced by the body's cells, is released into the alveoli, from where it can be exhaled. This exchange of gases is vital for the body to function correctly, as oxygen is needed to produce energy, while carbon dioxide is a waste product that must be removed. These three regions collaborate to facilitate the process of respiration, which is essential for human survival. The process of exchanging gases between

inhaled aerosol or smoke and the bloodstream occurs exclusively in the alveolar bronchioles and the alveoli. As we move from the nasopharyngeal to the innermost region (the alveolar region), the processes that prevent airborne pollutants from entering or remove deposited particles gradually change transformation. Coughing and sneezing serve as the primary means of defence against inhaled particles. These actions occur in the nasopharyngeal and upper tracheobronchial regions. When particles reach the lower tracheobronchial region, they can get trapped in the mucus that lines the airway epithelium. Along with any deposited material, the mucus is then moved by ciliary activity toward the pharynx, where it is swallowed and eliminated. However, even after the mucociliary escalator filters out a large portion of particles, some may still reach the bronchiolar and alveolar regions. In these areas, the mucociliary activity is less efficient, and the alveolar macrophages become the primary mechanism for epithelial clearance. Lung-resident phagocytes and pulmonary dendritic cells act as guards against fine particles in the lungs. The phagocytes remove any deposited material they come across, but their clearance process is slower than the mucociliary escalator [22].

The movement of deposited particles from the epithelium into the connective tissue, bloodstream, or lymphatic circulation and their uptake by various cell types remains incompletely understood. However, it appears that this process involves both active and passive mechanisms. The protection and clearance mechanisms play a crucial role in understanding how air pollution or smoke/aerosol affects the respiratory system. It should be noted that these mechanisms solely provide protection against particulate air pollution or smoke and do not effectively counter gaseous compounds. The respiratory epithelium, a thin layer of cells lining the airways, is essential for gas exchange between inhaled air and the underlying tissues bloodstream. This process requires the free movement of gases into the respiratory system. The distribution of airborne particles within the respiratory system is primarily determined by their size, with smaller particles able to penetrate more deeply into the lungs and reach the more peripheral lung regions. In contrast, larger particles tend to remain in the upper airways. Optimal deposition efficiency is observed for particles ranging in size from 1-100 nm in the tracheobronchial and alveolar regions. As particles penetrate deeper into the respiratory system, they have more opportunities to interact with or cross the epithelium. Small particles are anticipated to have a more significant interaction due to their ability to enter deeper into the respiratory system.

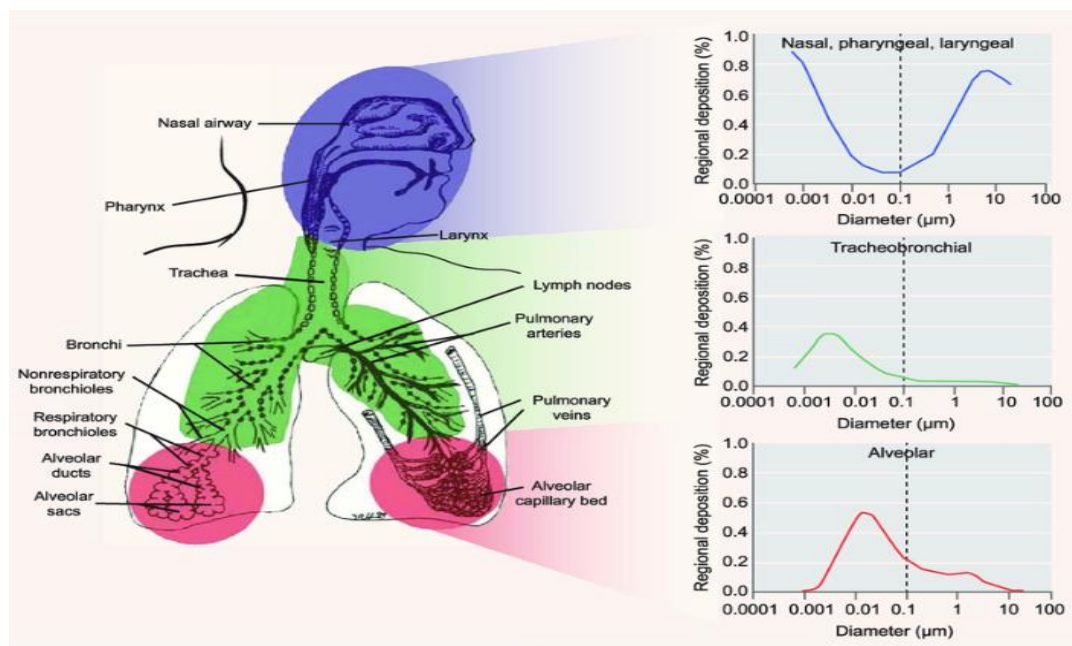


Figure 1.1 Respiratory system: This figure illustrates the different respiratory tract regions and how inhaled particles' deposition varies depending on their size. The estimated particle deposition in various parts of the human respiratory tract—namely, the nasopharyngeal, tracheobronchial, and alveolar regions—differs significantly based on particle size during nose breathing. Nano-sized particles, in particular, exhibit distinct behaviours compared to larger particles. Those in the 10–100 nm range tend to deposit efficiently in the alveolar region, primarily through diffusion. This occurs because airflow in the alveoli is minimal, allowing smaller agglomerates to settle more effectively than larger ones. Density is essential in the deposition of larger agglomerates within the respiratory system. Denser particles tend to deposit more readily in the lungs due to their weight and aerodynamic properties. It is important to note that the overall density of agglomerated particles is typically lower than that of the original material, which affects how these particles behave when inhaled. Additionally, the shape of the particles significantly influences their deposition characteristics. Irregularly shaped particles may experience turbulence and exhibit different settling patterns compared to spherical particles, which can further impact where they deposit within the respiratory tract. Understanding these factors is essential for evaluating the health implications of inhaling various particulate substances [24].

1.3 Aerosol/Tobacco Smoke

The composition of the air we breathe extends beyond gases, encompassing suspended solid and liquid particles referred to as aerosols. Aerosols can manifest in the form of fog or dark emissions emitted by factories or from diesel exhaust produced by vehicles. In essence, aerosols are a stable amalgamation of solid or liquid particles within a gas medium, such as air. Individuals continuously

inhale these aerosols. The classification of aerosols is not universally standardised. Smoke, for instance, serves as a perceptible aerosol resulting from the incomplete combustion of tobacco (Figure 1.2). The particles inherent in aerosols, whether solid or liquid, generally have a diameter of less than 1 μm and may aggregate, similar to fume particles [25] (26).

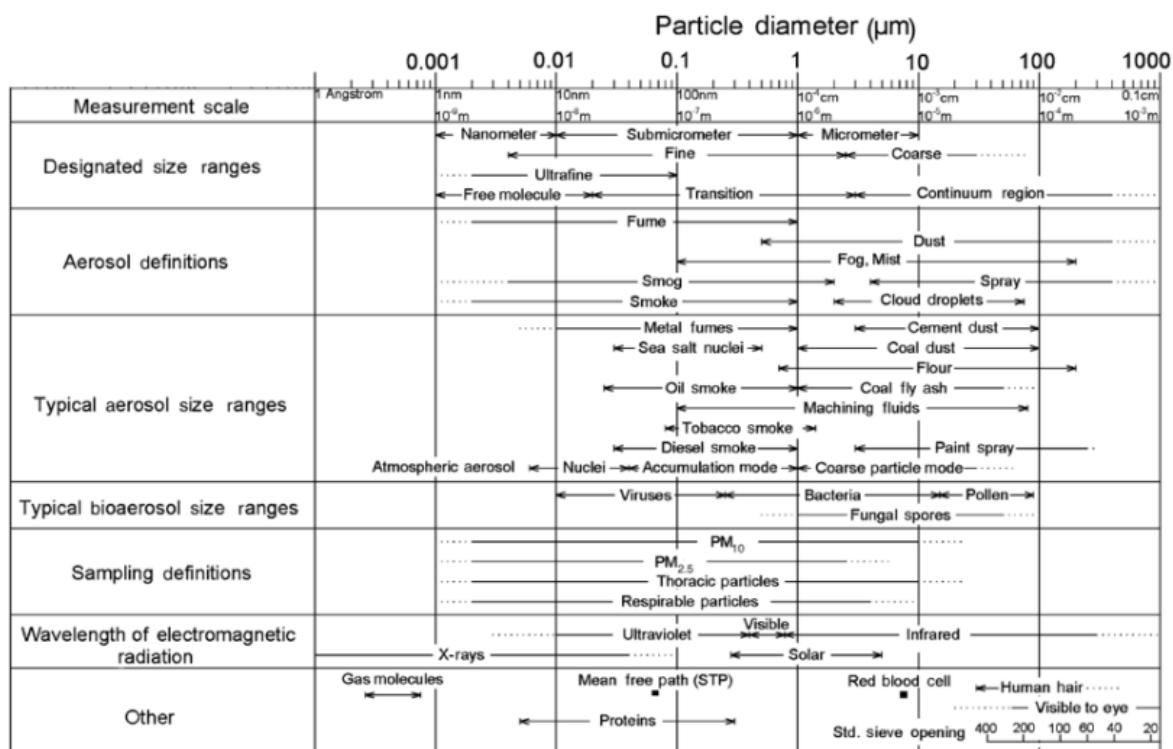


Figure 1.2 Size ranges of common types of aerosol and air-borne particles: A distinction is made at 1 μm , which defines the upper limit for sub-micrometre particles and the lower limit for micrometre particles (1-10 μm). The range illustrated in the figure extends from gas molecules to millimetre-sized particles, covering a size range of 10^7 . The aerosols shown in the figure have particle sizes ranging from 0.01 to 100 μm . Generally, dust, ground materials, and pollen fall within the micrometre range or larger, while fumes and smoke are considered submicrometer particles. The smallest aerosol particles are comparable in size to large gas molecules and share many of their properties (26).

1.3.1 Tobacco Smoke

Tobacco smoke comprises a complex mixture of gases and particles, containing numerous chemical substances. It can be divided into two main phases: the gas-vapour phase (GVP) and the total particulate matter (TPM) phase, as shown in Figure 1.3. The GVP is a gas containing a high concentration of aldehydes (carbonyls), chemicals that can irritate the respiratory system. The TPM consists of small particles that contain polycyclic aromatic hydrocarbons and tobacco-specific

compounds nitrosamines. These particles are small enough to enter the lungs and can cause damage to the respiratory system [26].

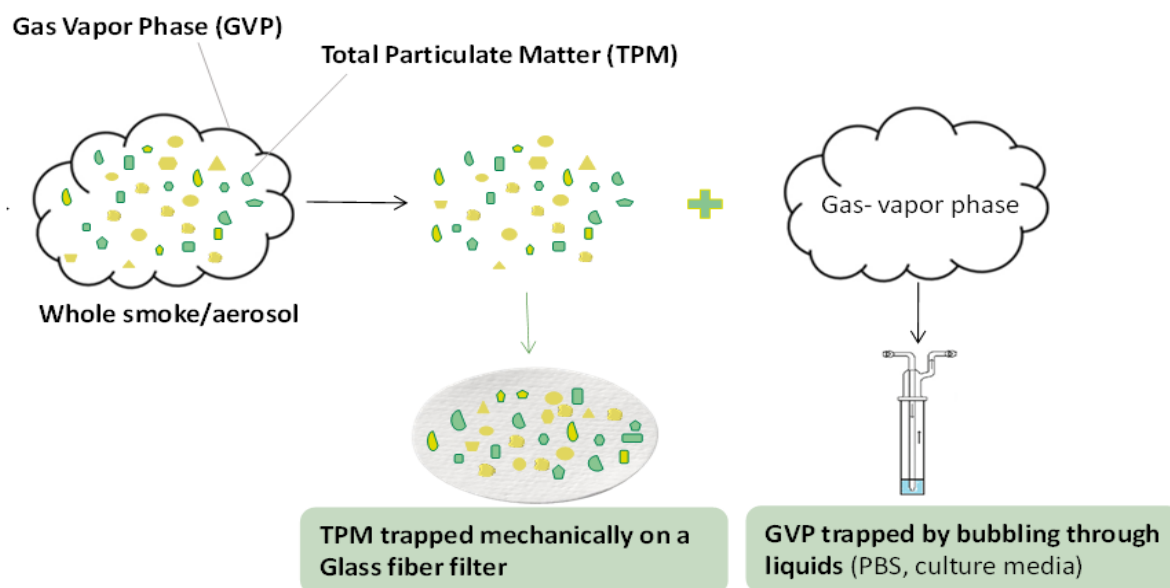


Figure 1.3 Aerosol/smoke Phases: Aerosol/smoke can be divided into two distinct phases. The particulate phase, also known as Total Particulate Matter (TPM), can be captured on a glass fibre filter. The Gas-Vapour Phase (GVP) can be captured by bubbling through wash bottles.

1.3.2 Particle Size

Particle sizes play a crucial role in understanding aerosol behaviour. All properties of aerosols are contingent upon particle size, with specific properties significantly impacted by it. Additionally, particle size offers important insights into how aerosols interact within various regions of the respiratory tract during inhalation. Five mechanisms facilitate particle deposition in the lungs: interception, impaction, sedimentation, diffusion, and electrostatic precipitation [26].

Interception: When a particle in the aerosol approaches and contacts the surface of the airway passages, it becomes trapped or lodged. This process is essential for fibrous particles such as asbestos—the location where the particle deposits in the respiratory tract depends on the fibre's length. For example, a fibre measuring 1 μm in diameter and 200 μm in length is expected to settle in the bronchial tree, as shown in Figure 1.4. [27].

Impaction: Particles in the air usually follow the airflow, but when the airway bends, many particles can't keep up with the change in direction and instead impact and adhere to the surface along their initial path. This behavior is influenced by the airflow velocity and the particles' mass. Typically, particles with an aerodynamic diameter greater than 10 μm are deposited in the nose or throat and cannot reach the deeper regions of the respiratory tract (see Figure 1.4) [26, 28].

Sedimentation: Airborne particles lose their ability to float due to gravity and air resistance as they move through the air, eventually settling on surfaces in the lungs, particularly in the bronchi and bronchioles. When particles are less than $0.5\ \mu\text{m}$ in diameter, they are less likely to sediment (Figure 1.4) [26, 28].

Diffusion: Particles smaller than $0.5\ \mu\text{m}$ display random movement, known as "Brownian motion," similar to gas molecules air. Particles tend to deposit on lung walls mainly through random Brownian motion. As particle size decreases, their movement becomes more vigorous. Deposition in small airways and alveoli mainly occurs through diffusion. Additionally, very fine particles, $0.01\ \mu\text{m}$ or smaller, can also be trapped in the upper airway (Figure 1.4) [26, 28].

Electrostatic precipitation: Although less prominent than other mechanisms, electrostatic forces can influence the deposition of charged particles that are near the surfaces of the airways, which have the ability to induce charges of the opposite polarity. These charged particles tend to be attracted to the walls of the airways, resulting in a higher level of deposition in comparison to neutral particles (Figure 1.4) [26, 28].

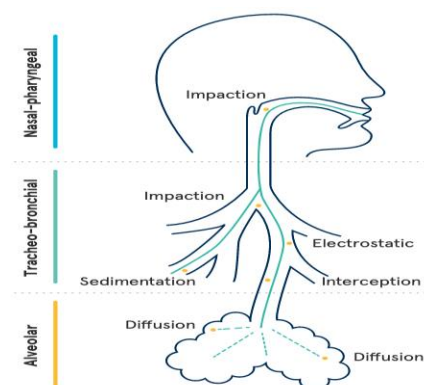


Figure 1.4 Particles deposition in the lungs: Demonstrates how aerosol particles deposit in different regions of the respiratory system, depending on their size and the speed of the aerosol flow [26].

1.3 Mechanisms underlying adverse health effects of exposure to smoke/aerosol
Cigarette smoke is a complex and diverse blend of over 4,000 different toxic chemical compounds, each with its unique properties and effects on the body. Among these compounds, at least 250 have been identified as being toxic and contributing to various smoking-related illnesses [26]. These illnesses can include respiratory diseases, like chronic bronchitis and emphysema, as well as cardiovascular problems, such as heart disease and stroke. Smoking has also been linked to several types of cancer, including lung, bladder, and throat cancer. In the 1990s, the harmful effects of particulate air pollution were believed to be caused by pulmonary inflammation. However, the mechanism by which inhaled particles induce inflammation was not yet understood. The aerosol

technology industry has recently made significant advances by introducing new Heat-not-burn (HnB) tobacco products, or Electronic Nicotine Delivery Systems (ENDS) in global markets. These products are designed to help people quit smoking, which is the most effective way to reduce the risks associated with smoking-related diseases. These products work by heating tobacco or nebulising liquids, or by vaping while maintaining nicotine levels instead of burning tobacco. [29].

1.4 Mechanisms that underlie the beneficial health impacts of exposure to therapeutic aerosols

In the medical field, intensive research has been conducted to deliver various types of drugs via aerosolisation. This technique involves converting a liquid drug into an aerosol that can be inhaled into the lungs. This method is particularly beneficial for medications that require direct delivery to the lungs, such as treatments for asthma and other respiratory diseases. Oberdörster and his colleagues conducted a study in 1994 that demonstrated that mice that were intratracheally instilled with ultrafine titanium dioxide (TiO₂) particles, with an average diameter of approximately 20 nm, showed more robust inflammatory responses than those that received the same mass of fine TiO₂ particles with an average diameter of about 250 nm [30]. Due to its low reactivity, titanium dioxide has been frequently used as a negative control particle. This finding suggested that the surface area and chemical composition of the particles were crucial in particle toxicology. Further research indicated that particle toxicity was induced by oxidative stress, specifically the generation of reactive oxygen and nitrogen species (ROS/RNS). It is also important to note that this includes inhaling other aerosols, such as cigarette smoke and aerosols from tobacco products. To understand how different aerosols and smoke affect our bodies and cause illnesses, we first need to comprehend the complex behaviour of smoke or any aerosol. To do this, we must develop exposure techniques both in vitro (in a laboratory setting) and in vivo (in a living organism). By doing so, we can identify the key elements and processes that contribute to the harmful health effects of exposure to different aerosols and smoke [31].

1.5 In-vitro Exposure Studies

The development of in vitro exposure systems has primarily been motivated by the shortcomings of traditional smoke exposure methods, which mainly address only the particulate phase of cigarette smoke and neglect the vapour phase and semi-volatile compounds during analysis. Conversely, whole smoke exposure systems provide the flexibility to examine all phases of smoke—either combined or separately—based on the experimental needs. This enables researchers to customise their studies to include both particulate and vapour phases, leading to more complete and informative data. Several whole smoke systems are available, and most of them can also be used to deliver individual aerosols or other complex aerosol mixtures to cell cultures. Researchers evaluated the effects of whole smoke

by exposing cells at the air-liquid interface (ALI). In this setup, cells were cultured on a porous membrane, receiving liquid nourishment from one side and exposure to air on the other. This approach more effectively assesses the impact of whole smoke [31].

This thesis describes the development of technology for testing exposures to smoke and aerosols, as well as understanding the effects of smoking or exposure to various environmental aerosols on human lungs. Developing exposure systems that use whole smoke can provide more accurate results than using only parts of the smoke. These systems can expose cells to both the gas and particle phases of smoke, which is more closely representative of what occurs in real-life situations. These systems are also being used to study other aerosols, including those from ENDS and inhaled drugs. The improvements made in the studies presented in this thesis can lead to better drug screening and reduce the number of animal tests required. This work also helps to increase confidence in in vitro sciences [32].

1.6 In-vitro exposure systems

A wide range of whole smoke exposure systems is available on the market, designed to deliver either individual aerosol compounds or complex mixtures to cultured cells. While these systems are widely used in the tobacco sector, they also have potential applications in other fields. However, there is still a lack of comprehensive guidelines and robust data for evaluating airborne chemical substances using in vitro approaches. Consequently, establishing standardised protocols and procedures for in-vitro exposure systems is essential to guarantee consistent, reproducible, and reliable results. As a result, the importance of standardising and validating in vitro aerosol methodologies has become more critical than ever [33].

The contemporary landscape of in vitro aerosol exposure systems is presently exhibiting unprecedented diversity, which presents a challenge in selecting the ideal option [34]. The main goal of this thesis is to evaluate the effectiveness of the newly developed Vitrocell® 24/48 in vitro aerosol exposure system, using mainstream cigarette smoke and liquid aerosols for characterisation. This research also aims to verify the system's efficacy in exposing various aerosols to in vitro settings. Additionally, the secondary objective is to develop a new system using insights from the current system's strengths and limitations. The proposed system is engineered to replicate the entire human respiratory tract mechanically and can expose cell cultures at the air-liquid interface within a model representing each key respiratory compartment. Additionally, it is designed to independently sample and generate test atmospheres, eliminating the need for additional aerosol sources such as syringe pumps or aerosol supplies chambers [35].

Several novel techniques have also been developed for the characterisation of the in-vitro exposure system and are presented in this thesis. These include a new fluorescence-based method and the trapping of aerosols within metallic cell culture inserts containing PBS for downstream analysis of various target compounds. The innovative approaches undertaken in this particular area of research have yielded significant advances, allowing researchers to gain a deeper and more comprehensive understanding of the effects of aerosols and particulate matter on human health. These sophisticated methods have enabled researchers to conduct more detailed and thorough studies, resulting in valuable insights that aid in developing effective strategies to mitigate the harmful impacts of airborne pollutants on human health. By utilising advanced technologies and research techniques, scientists can now better grasp the complex interactions among aerosols, particulate matter, the environment, and human biology—establishing a basis for future targeted, evidence-based policies and interventions.

Comparison of Currently Used ALI In Vitro Exposure Systems

Given the critical importance of physiologically relevant in vitro models for respiratory research, several commercial systems have been developed to facilitate air-liquid interface exposures. Each system employs different operating principles and offers distinct advantages for specific research applications. The following comparison evaluates the major commercially available ALI exposure systems currently used in respiratory toxicology and inhalation research.

Table 1.1. Comparison of Currently Used ALI In Vitro Exposure Systems

System	Operating Principle	Key Features	Advantages	Disadvantages
VITROCELL® Systems[35]	Stagnation flow with individual exposure modules	• Modular design • Direct exposure to the test atmosphere • Temperature-controlled exposure • Integrated dose measurement options • Available for gases, aerosols, and nanoparticles	• Precise control of flow rates • Quantifiable dose monitoring (e.g., QCM microbalance) • Multiple concentrations in parallel • Adaptable to different cell culture systems	• Relatively high cost • Complex setup requiring technical expertise • Potential for variability between intra and inter runs • Labour-intensive cleaning process

System	Operating Principle	Key Features	Advantages	Disadvantages
VITROCELL® Cloud[25]	Single droplet nebulisation with sedimentation	• Direct nebulisation of liquid substances • Dose-controlled exposure • Smaller and more affordable than full systems	• Simpler setup than flow-through systems • Uniform particle deposition • Suitable for pharmaceuticals/liquid aerosols • Less expensive than continuous flow systems	• Limited to liquid/soluble substances • Less suitable for gases/vapours
CULTEX® RFS [36]	Radial flow technology with electrostatic precipitation	• Radial flow distribution • Electrostatic particle deposition • Compatible with particles, gases, and complex mixtures	• Enhanced particle deposition efficiency • Uniform radial distribution • Validated for regulatory purposes • Good reproducibility between labs	• System complexity • Limited number of parallel doses • Chamber design may impact flow patterns
Borgwaldt RM20S [37]	Syringe-based dilution with BAT exposure chamber	• Multiple independent syringes • Wide range of dilutions (1:2-1:4,000) • Specific for smoke/aerosol testing • Paired with BAT exposure chamber	• Independent control of each exposure • Wide dilution range capability • Well-characterised for tobacco research	• Primary focus on tobacco smoke testing • Less versatile for other applications • Syringe system requires calibration • Particle loss in delivery system (~47%)
InHALES (Independent Holistic Air-Liquid Exposure System)[38]	Active breathing simulation with human respiratory tract structure	• Simulates active breathing patterns • Replicates human respiratory tract structure	• Delivers human-relevant doses • Mimics physiological breathing mechanics • Higher translational relevance	• Relatively new system with fewer validation studies • More complex setup • Higher technical requirements

System	Operating Principle	Key Features	Advantages	Disadvantages
		• Compatible with medical inhalers and tobacco products • Accounts for particle deposition kinetics	• Can operate medical inhalers for direct testing	• Limited commercial availability
PreciseInhale [39]	Precision-controlled aerosol generation with multiple exposure modules	• Aerosol generation from minimal amounts of test substance • Multiple exposure modules (in vitro, ex vivo, in vivo) • Compatible with both dry powders and nebulised solutions • Interchangeable modules for translational research	• Precise dose control • Efficient substance use (as little as 50mg for a full study) • The same aerosol can be used for multiple exposure types	• Higher cost • Specialised training required • Primarily focused on research applications • Limited regulatory validation compared to established systems

1.7 Aims and Objectives & Thesis Overview

This thesis thoroughly examines the development, characterization, and use of in vitro air-liquid interface (ALI) exposure systems in inhalation toxicology. Recognising the limitations of traditional animal models and submerged cell culture methods, it emphasises creating more physiologically accurate models to improve the assessment of inhaled substances' impact on human respiratory health.

The specific aims and objectives of this thesis are to:

- **Develop and Characterise Novel ALI Exposure Systems:** Design, construct, and validate advanced *in vitro* exposure systems, specifically the Vitrocell® 24/48 and InHALES platforms, capable of simulating realistic aerosol deposition patterns and exposure conditions.
- **Establish Methods for Aerosol Characterisation and Dosimetry:** Develop and implement innovative techniques for quantifying aerosol properties, including particle size distribution, chemical composition, and deposition kinetics, within the developed exposure systems.
- **Investigate the Biological Effects of Inhaled Substances:** Utilise the characterised exposure systems to evaluate the impact of various inhaled substances, including cigarette smoke, ENDS aerosols, and pharmaceutical formulations, on human respiratory cells and tissues.
- **Promote Non-Animal Testing Methodologies:** Contribute to the advancement of *in vitro* methodologies as reliable and ethical alternatives to animal testing in inhalation toxicology, aligning with the principles of the 3Rs (Replacement, Reduction, and Refinement).

Thesis Overview:

This thesis is structured as a collection of interconnected research articles, each building upon the previous to present a cohesive narrative. The main argument is that accurately characterised *in vitro* ALI exposure systems, along with reliable analytical methods, can offer important insights into inhalation toxicity mechanisms and guide the creation of safer inhaled products therapies.

- **Chapter 2** focuses on the comprehensive characterisation of the Vitrocell® 24/48 system, using mainstream cigarette smoke as a model aerosol. This chapter establishes the system's ability to deliver consistent and quantifiable exposures to cell cultures.
- **Chapter 3** shifts the focus to e-cigarette aerosols, presenting a framework for *in vitro* systems toxicology assessment of e-liquids and their aerosols. This chapter addresses the challenges associated with the diverse range of ENDS products and proposes a tiered testing approach.
- **Chapter 4** introduces a novel fluorescence-based method for characterising *in vitro* aerosol exposure systems, providing a cost-effective and accessible technique for assessing aerosol deposition and distribution.

- **Chapter 5** builds upon the previous chapters by applying the fluorescence-based method to characterise the Vitrocell® 24/48 system for exposures to liquid aerosols, demonstrating its versatility and applicability to different types of inhaled substances.
- **Chapter 6** examines how cigarette smoke and aerosols impact the colour stability of dental resin composites, emphasising the wider uses of* *in vitro** exposure systems beyond respiratory health.
- **Chapter 7** examines how repeated exposure to cigarette smoke affects 3D bronchial and nasal tissue cultures, offering insights into the biological responses triggered by cigarette smoke at the tissue level.
- **Chapter 8** details the development and testing of Inhales, a new aerosol exposure system aimed at more accurately mimicking the human respiratory tract and enhancing the physiological relevance of *in vitro* inhalation studies.
- **Chapter 9** focuses on the application of secondary electrospray ionisation coupled with high-resolution mass spectrometry for chemical characterisation of thermally generated aerosols, providing a powerful analytical tool for assessing the composition of inhaled substances.
- **Chapter 10** assesses single-photon ionisation mass spectrometry for online monitoring of *in vitro* aerosol exposure experiments.
- **Chapter 11** provides aerosol filtration testing of fabrics for the development of reusable face masks.
- **Chapter 12** describes the pulmonary delivery of aerosolised chloroquine and hydroxychloroquine to treat COVID-19: *in vitro* experimentation to human dosing predictions.

By integrating these studies, this thesis provides a holistic understanding of *in vitro* inhalation toxicology, contributing to the development of more effective and ethical methods for assessing the impact of inhaled substances on human health.

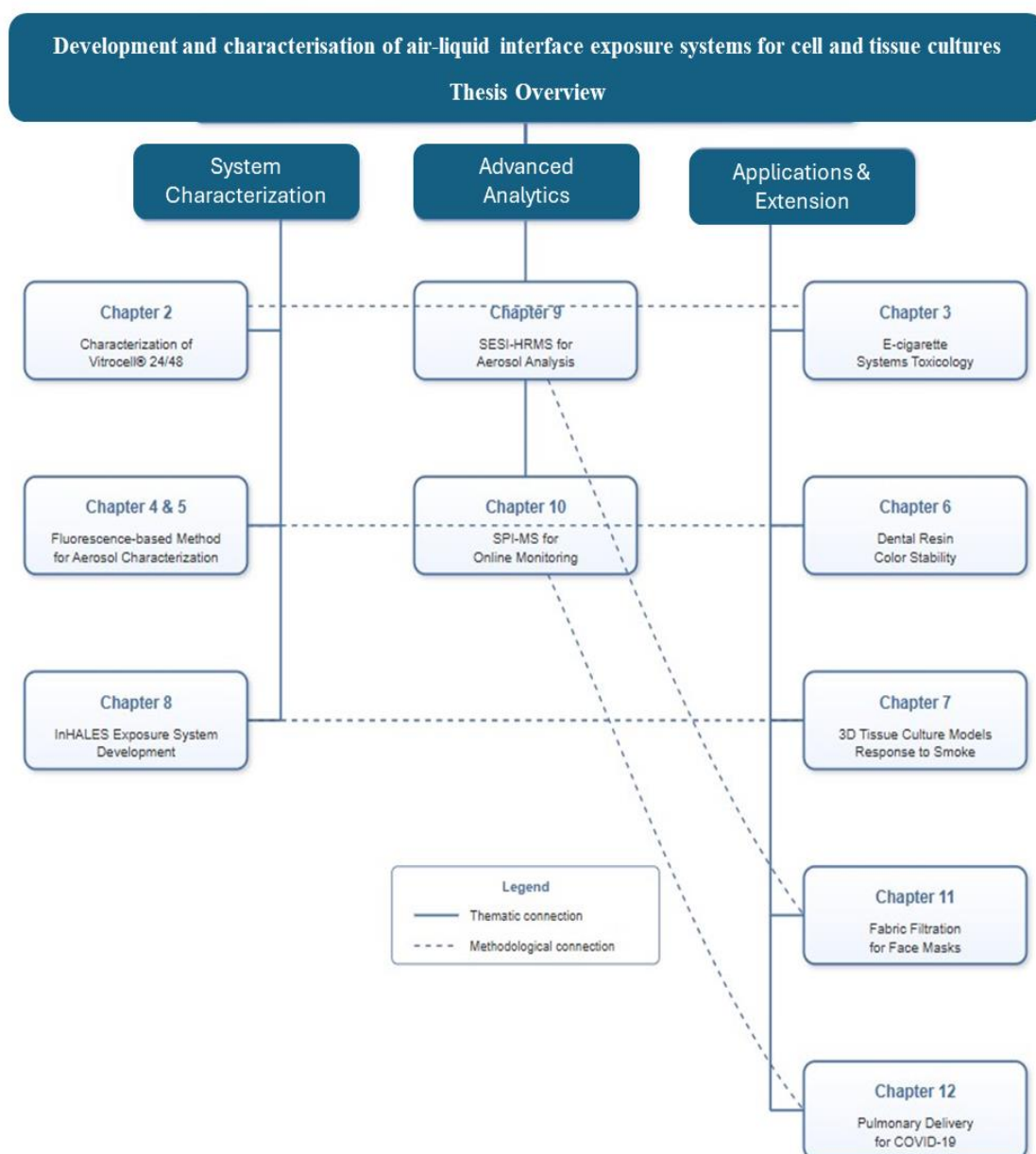


Figure 2.5: Flowchart illustrating the organisation and connections among the chapters of the thesis.

The flowchart presents the organisation and interconnections of the thesis, which is structured around three primary research themes:

System Characterisation: This theme includes chapters that focus on the development and validation of in vitro exposure systems.

- Chapter 2: Characterisation of Vitrocell® 24/48
- Chapters 4 & 5: Fluorescence-based Method for Liquid Aerosol Characterisation

- Chapter 8: InHALES Exposure System Development (Development and testing of a new-generation aerosol exposure system: The independent holistic air-liquid exposure system (InHALES))

Advanced Analytics: Chapters under this theme cover innovative measurement and analysis methods.

- Chapter 9: SESI-HRMS for Aerosol Analysis (Application of Secondary Electrospray Ionization Coupled with High-Resolution Mass Spectrometry in Chemical Characterization of Thermally Generated Aerosols)
- Chapter 10: SPI-MS for Online Monitoring (Assessment of Single-Photon Ionization Mass Spectrometry for Online Monitoring of *in Vitro* Aerosol Exposure Experiments)

Applications & Extensions: This section showcases the practical applications of the research across various fields.

- Chapter 3: A framework for *in vitro* systems toxicology assessment of e-liquids
- Chapter 6: Dental Resin Colour Stability (Effects of cigarette smoking and aerosols on colour stability of dental resin composites)
- Chapter 7: Impact Assessment of Repeated Exposure of Organotypic 3D Bronchial and Nasal Tissue Culture Models to Whole Cigarette Smoke
- Chapter 11: Fabric Filtration for Face Mask (Aerosol Filtration Testing of Fabrics for Development of Reusable Face Masks)
- Chapter 12: Pulmonary Delivery for COVID-19 (Pulmonary Delivery of Aerosolized Chloroquine and Hydroxychloroquine to Treat COVID-19: *In Vitro* Experimentation to Human Dosing Predictions)

The flowchart illustrates both direct connections between chapters, represented by solid lines, and methodological connections using dashed lines to indicate how techniques developed in one area can inform applications in another.

This visual representation underscores the thesis's logical progression, beginning with the development of foundational systems, advancing through innovative analytical methods, and culminating in diverse practical applications. It also highlights how the core methodological work lays the groundwork for the applications and extensions explored in the later chapters.

1.8 Achievements and contributions to knowledge

To ensure that animal testing adheres to the ethical principles of "Replace, Reduce, Refine", developing relevant *in vitro* models is essential. Rather than merely observing molecular changes within a cell, the latest *in vitro* toxicology methods focus on understanding the toxic mechanisms in systems that can simulate the *in vivo* environment. These methods utilise three-dimensional (3D) organotypic culture models to more robustly investigate exposure effects. Using an *in vitro* exposure system allows researchers to adhere to the 3R principle. Having a well-characterised *in vitro* exposure system readily accessible is crucial for minimising animal testing and ensuring that testing procedures are properly conducted ethically.

This thesis focuses on characterising in vitro exposure systems, aiming to develop reliable methods to accurately evaluate and pre-validate both the systems and the tested aerosol types. This helps ensure the systems are suitable for their intended use. The research has led to new opportunities in inhalation and systems toxicology, resulting in over 30 publications in reputable international journals.

In-vitro exposure systems have proven to be very useful in exposing 3D tissues at an air-liquid interface (ALI), and in exposing various dental materials and resins to better understand the discolouration induced by cigarette smoke. Research on the pharmacological effects of existing drugs via in vitro screening suggests that chloroquine (CQ) and hydroxychloroquine (HCQ) could be effective against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Inhalable formulations of CQ and HCQ were created for thermal aerosolisation, a method that helps lower the dose-limiting toxicity linked to oral use. This method enables direct and rapid delivery to target tissues, leading to a patent for these formulations. However, hydroxychloroquine is not recommended for the treatment or prevention of COVID-19. In March 2020, the FDA granted emergency use authorisation for these drugs for COVID-19 based on non-human trials and began monitoring health outcomes. By April 2020, due to concerns regarding heart risks, the FDA recommended against using these medications outside of hospitals or clinical trials. Our research emphasises the potential for an alternative route of administration for drugs typically administered orally, and suggests that other drugs, like hydroxychloroquine and chloroquine, could also be tested through aerosolisation. However, we do not endorse the use of CQ or HCQ to treat COVID-19.

The development of a novel and state-of-the-art aerosol exposure system, termed InHALES (Independent Holistic Air-Liquid Interface Aerosol Exposure System), represents a significant advancement in respiratory research. This system is structurally and functionally modelled on the human respiratory tract, enabling realistic simulation of aerosol delivery and test atmosphere exposure within a living respiratory system. It facilitates precise alignment between in vitro and in vivo dosimetry, thereby improving the translational relevance of experimental data. In addition, the integration of emerging artificial intelligence (AI) tools—such as machine learning algorithms for image analysis, exposure modelling, and real-time data interpretation—can further enhance the system's capabilities. These tools offer the potential to optimise experimental design, automate complex analyses, and improve predictive accuracy, making InHALES a powerful platform for studying respiratory toxicology and aerosol behaviour. Recent research highlights the usefulness of AI and machine learning in aerosol science, such as predicting aerosol levels and dispersion patterns with convolutional autoencoders and neural networks [40]. The preliminary version of the InHALES system has undergone successful testing, demonstrating its technical proficiency in replicating the structure and function of the respiratory tract and delivering test atmospheres to cell cultures with

reproducibility. Additionally, the system is compatible with 3D organotypic cell cultures, which are a complex and physiologically relevant model system that can better mimic the human respiratory system's response to inhaled substances [41].

The development of the InHALES system (Figure 1.6) represents a significant advancement in respiratory research, providing a more precise and dependable simulation of real-world inhalation exposure scenarios. This technology could revolutionise the study of respiratory diseases and improve the assessment of the safety and efficacy of inhaled drugs, environmental pollutants, and other airborne substances [39]. It represents the cutting-edge solution for a range of applications within the field of inhalation sciences, including Inhalation toxicology, Product assessment, Aerosol dosimetry, and Aerosol engineering [43].

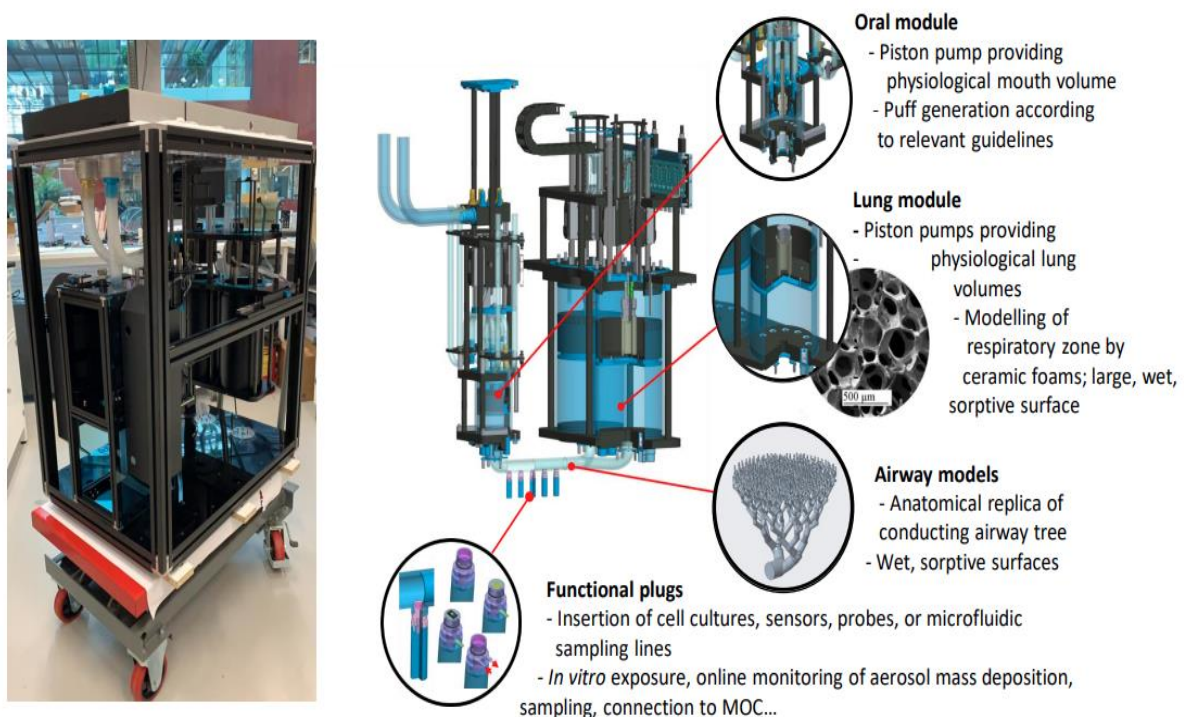


Figure 1.6 InHALES system: InHALES is a groundbreaking, innovative system designed to mimic the human respiratory tract with the ability to simulate human breathing and aerosol delivery to the lungs closely. It provides an air-liquid interface environment and operates independently. The system is comprehensive and takes a holistic approach to aerosol exposure research [39].

2.0 Characterization of the Vitrocell® 24/48 *in vitro* aerosol exposure system using mainstream cigarette smoke

2.1 Introduction and Contribution

In this chapter, I will discuss my research paper, "Characterization of the Vitrocell® 24/48 *in vitro* aerosol exposure system using mainstream cigarette smoke"(Majeed S, Frentzel S, Wagner S, Kuehn D, Leroy P, Guy PA, Knorr A, Hoeng J, Peitsch MC. Chem Cent J, 2014. 8(1): p. 62)(15). As the primary and first author of this article, I was responsible for designing the experiments, compiling the results, and preparing the manuscript for publication.

2.2 Background

In Chapter 1, we briefly discussed that cigarette smoke contains over 4,000 compounds, with at least 250 of them known to be toxic. Various diseases related to smoking have been linked to the toxic compounds found in cigarettes, including respiratory and cardiovascular issues, as well as cancer. Cigarette smoke comprises two distinct phases: a gas-vapour phase and a particulate phase, each containing unique elements. *In vitro* studies have examined the impact of individual components, smoke fractions, or whole smoke exposure on cells to understand the harmful effects of cigarette smoke. Typically, researchers use submerged cell culture systems to perform these studies, exposing cells to specific fractions of cigarette smoke. An alternative method involves utilising either filter-trapped or solution-trapped particulate and gas-vapour phase fractions for *in vitro* investigations using submerged cell culture systems. Nonetheless, conducting *in vitro* experiments with whole smoke aerosols presents technical difficulties and can only be accomplished if the cells are positioned at the air-liquid interface (ALI). In the ALI cell culture system, cells are cultured on permeable membranes inside culture inserts, where they receive nutrients from the basolateral side. The apical side is exposed to air (see Figure 2.1). This configuration permits direct contact of cells with whole smoke, allowing interaction with both gaseous and particulate components simultaneously.[39, 42, 43].

Mainstream smoke (MS) refers to the smoke inhaled by a smoker from a cigarette, cigar, or pipe. On the other hand, sidestream smoke (SS) is the smoke released into the air from a smoking tobacco product between puffs. SS is a significant contributor to environmental tobacco smoke (ETS), which is caused by the burning of tobacco products. It is important to note that only MS was tested in this research study [43].

In vitro studies examining the effects of cigarette smoke often use lung cell lines such as A549, BEAS-2B, and 16HBE. The A549 cell line is derived from human alveolar epithelial cells and exhibits features similar to type II alveolar epithelial cells. They produce a type of lecithin rich in saturated fats, similar

to the substances that help the lungs expand and contract. Ke et al. have developed a novel human bronchial epithelial cell line with a significantly prolonged lifespan for culture, achieved through the introduction of SV40 virus T antigen genes [21]. Whilst cell lines can proliferate quickly when grown as monolayers, models of three-dimensional (3D) organotypic cell culture more accurately reflect the inherent architecture of the bronchial epithelium, emulating a multi-layered arrangement that features both ciliated and non-ciliated cell types. Nonetheless, the creation of 3D organotypic cell cultures presents a more complex and lengthy process. Consequently, current research utilises epithelial cell lines cultivated in an air-liquid interface (ALI), specifically concentrating on A549 or BEAS-2B cells, to examine how smoke exposure influences cellular health [44].

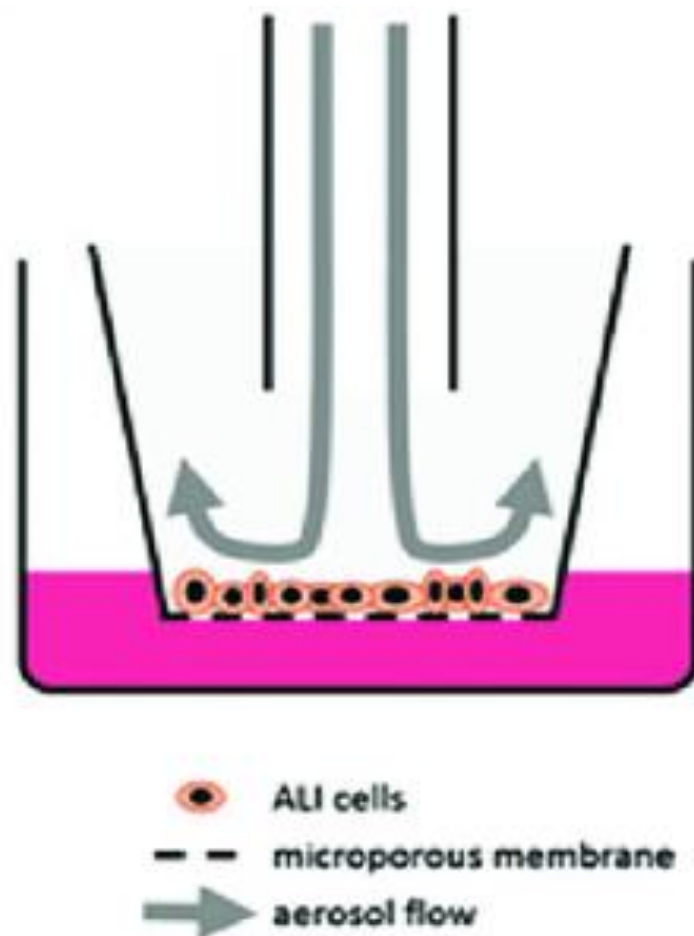


Figure 2.1 ALI culture: In the ALI cell culture system, cells are grown on porous membranes placed on culture inserts. The cells receive medium from the bottom while the upper side is exposed to air.

In vitro experiments often rely on the particulate and gas-vapour phases, commonly known as cigarette smoke (CS) condensate fraction. Nonetheless, these techniques are not without drawbacks. The trapping process can alter the chemical makeup of these phases. Some of the more volatile chemicals might not be trapped entirely, and the filtration and collection apparatus might introduce

extraneous substances into the sample. Additionally, the solvents used could interact with components within the smoke. There is also a chance that examining separate aerosol/smoke fractions does not fully account for the combined toxic risks associated with smoking [25].

Many researchers are adopting culture systems to address these challenges, particularly those that expose cells to tobacco smoke at the air-liquid interface (ALI). This technique involves the use of a smoking apparatus to channel tobacco smoke into an exposure chamber housing cell culture inserts maintained at the ALI. Various institutions and companies have devised sophisticated smoke exposure systems capable of conducting in vitro experiments on ALI and submerged cell cultures. These systems are composed of a smoking device and chambers for cell culture exposure. Although the systems' designs and specifications may vary, they allow researchers to manipulate the concentration of the complete smoke [25].

2.3 Study Introduction

The current study utilised the Vitrocell® 24/48 system developed by VITROCELL® Systems GmbH, which features a dual inlet for dilution and distribution, allowing for flow rates of dilution between 0.1 and 3 litres per minute. This system is also equipped with six identical wells on the cultivation base module, each of which can be subjected to variations in dilution airflow. Additionally, separate trumpets deliver the CS to each individual well. A vacuum pump is attached to the base module to generate negative pressure, enabling CS to be sampled through the trumpets. The airflow used for sampling can be adjusted between 2 to 5 mL/min, but only 2 mL/min was utilised for the experiments described below. An SM 2000 carousel smoking machine, equipped with 30 ports, is responsible for generating cigarette smoke (CS). This smoke was then channelled directly into the Vitrocell® system's 24/48 inlet for further dilution and distribution. Additionally, the system also incorporates a sensor-regulated heating plate and an environmental chamber that encases both the dilution/distribution mechanism and the cultivation base module [7].

While controlling the smoke dilution and smoking regimen is possible, measuring the deposition of CS on culture inserts is challenging. Various techniques have been used to assess the total smoke levels in in-vitro exposure systems, but each method is specifically designed to measure either gas-vapour components or particulate matter components. The Vitrocell® apparatus comes with an integrated tool within its exposure unit known as Quartz Crystal Microbalances (QCMs). Originating in the 1950s,

QCMs were initially invented to gauge contaminants in the environment. By analysing changes in the resonance frequency of a quartz crystal, researchers can detect particles that settle on its surface in real-time. The resonance frequency of a quartz crystal microbalance (QCM) directly correlates with the mass accumulated on its surface. The sensitivity depends on the crystal's intrinsic properties—10 nanograms per square centimetre as specified by Vitrocell. This setup allows QCMs to be integrated into specific channels of the Vitrocell 24/48 dilution and distribution module, facilitating the simultaneous exposure of multiple replicate cell cultures under controlled conditions. [25].

This study uses the chemical reactivity of cigarette smoke (CS) with the redox-sensitive indicator dye WST-1 to evaluate the uniformity of CS distribution within the Vitrocell® 24/48 system's. WST-1, a tetrazolium solution from Roche Applied Science commonly used for assessing cell viability, is applied here without cells. Upon exposure to CS, WST-1 undergoes reduction likely due to the high concentration of oxidants present in both the gas-vapour and particulate (tar) phases of CS [21]. This reduction process produces coloured formazan, which can be measured using a plate reader at an optical density (OD) of 430 nm. Thus, WST-1 reduction provides a simple way to assess CS distribution across the cultivation base module of the Vitrocell® 24/48 system. Additionally, we conducted an extensive analysis measuring the concentrations of specific components within CS, specifically nicotine and various carbonyls, using the Vitrocell dilution/distribution system. Finally, we assessed how cigarette smoke affects the viability of A549 and BEAS-2B cells placed within the Vitrocell® 24/48 system [25].

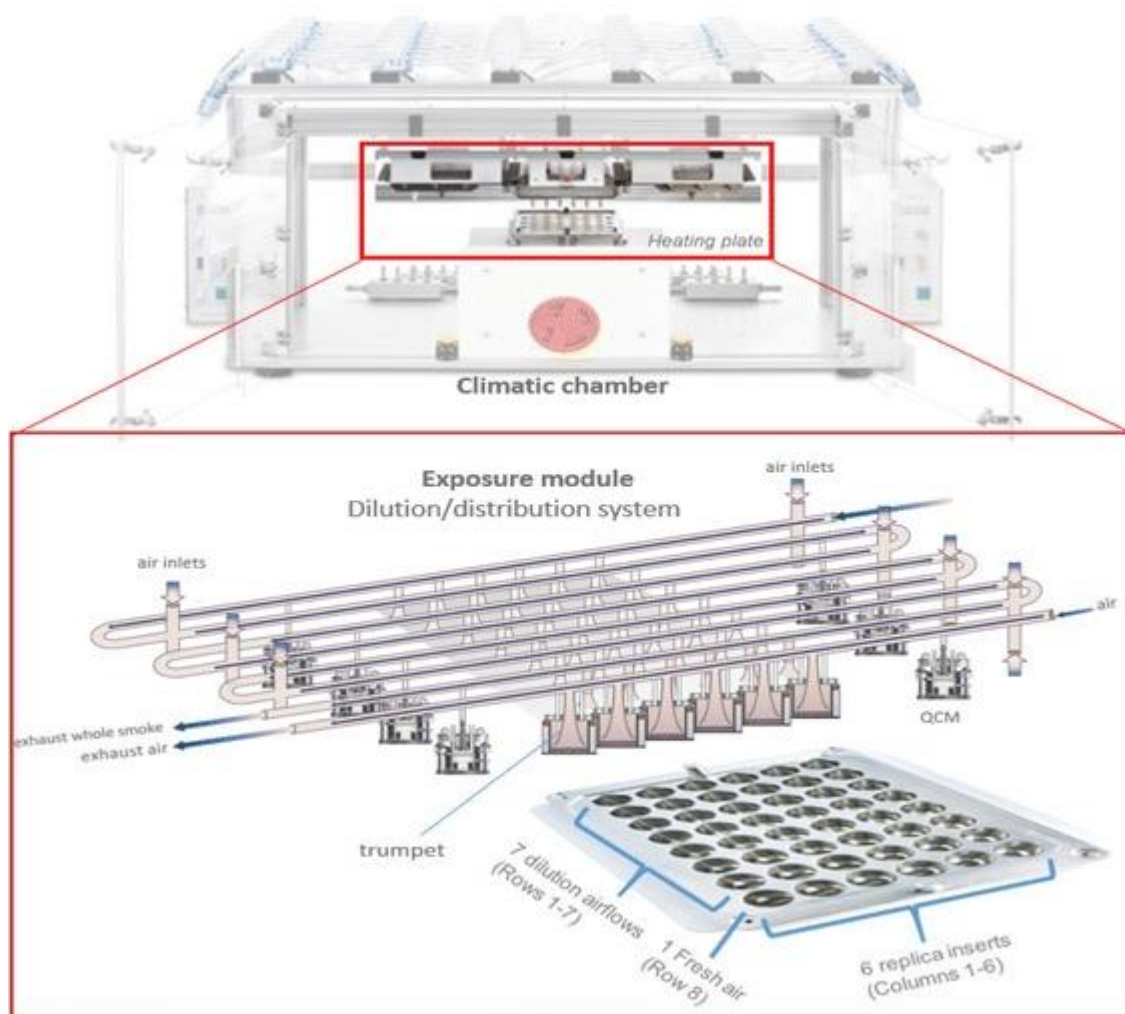


Figure 2.2 Vitrocell® 24/48 exposure system: A representation of the Vitrocell® 24/48 exposure system. The system comprises a climatic chamber housing an exposure module. The module includes a dilution/distribution system on top of a cultivation base module. The base module has the capability to simultaneously expose up to 48 wells in an 8-row, 6-column format. The cultivation base module has seven airflow rows for dilution and one row for exposing the negative control to fresh air. Individual trumpets deliver the whole smoke to the wells of the module through the dilution/distribution system at a 2 mL/min flow rate. Each of the seven rows in the dilution/distribution system is fitted with a QCM that tracks particle deposition [25].

2.4 Materials and Methods

The process of generating and delivering mainstream cigarette smoke to the Vitrocell® 24/48 exposure chamber is described below. The reference cigarettes used in the experiments were obtained from the University of Kentucky and conditioned according to ISO guidelines. To maintain a consistent aerosol output, the cigarettes were utilised in conjunction with an adapted Health Canada smoking protocol[25]. The smoke was delivered to the Vitrocell using a 30-port carousel smoking machine. Two

cigarettes in the carousel produced four puffs per minute. The experiments involved diluting the cigarette smoke at different airflow rates, creating a range of cigarette smoke concentrations from 7% to 69% in the Vitrocell. The concentration of Total Particulate Matter (TPM) in 100% cigarette smoke (CS) was measured by capturing undiluted mainstream smoke from 3R4F reference cigarettes using a Cambridge filter pad connected to a smoking apparatus machine. The filter pad was weighed both before and after smoke collection to measure the mass of particulate matter retained. This mass was then converted into a concentration (mg/L) by dividing it by the total volume of smoke that passed through the filter during the trapping process, resulting in a TPM concentration of 42.4 mg/L.

To assess the effects of different smoke concentrations within the exposure system, we conducted a series of dilutions using airflow rates of 0.1, 0.2, 0.5, 1.0, 1.5, 2.0, and 3.0 L/min. These dilution rates resulted in specific concentrations of CS and corresponding values of TPM:

0.1 L/min- 69% CS = 29.2 mg/L

0.2 L/min- 54% CS = 22.2 mg/L

0.5 L/min- 32% CS = 13.0 mg/L

1.0 L/min 19% CS = 7.7 mg/L

1.5 L/min- 13% CS = 5.4 mg/L

2.0 L/min- 10% CS = 4.2 mg/L

3.0 L/min- 7% CS = 2.9 mg/L

This systematic approach allowed for a thorough assessment of how different concentrations of cigarette smoke affected particulate matter levels [25].

In all experiments, exposure to fresh air served as the control. Dilution air was adjusted to a relative humidity of 60%, while the climate chamber and heating plate were set at a temperature of 37°C. The trumpet apparatus (Figure 2.3), delivering the cigarette smoke to the inserts, was positioned 2 mm above the membrane [25].

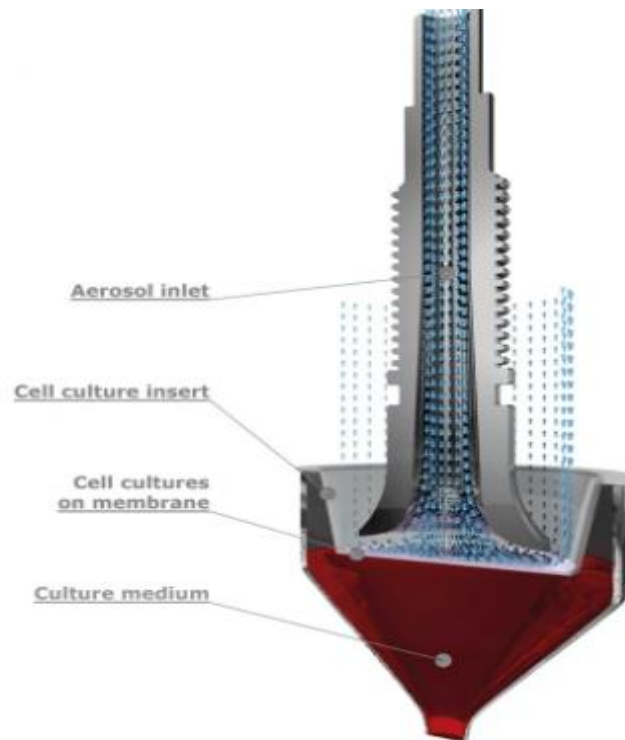


Figure 2.3 Stagnation flow setup: The geometry of the trumpet inside the Vitrocell® 24/48 exposure chamber, including ALI cultures on a microporous membrane combined with a stagnation flow setup, enables the flow of aerosol/smoke [25].

2.4.1 Cell culture and CS exposure or treatment

In this study, two human lung cell lines were used, the alveolar basal epithelial cell line A549 and the bronchial epithelial cell line BEAS-2B. Frozen cells from the stock pool were thawed and grown in 75 cm² flasks until they covered the surface of the flask. A549 cells were cultured in F12k medium (Life Technologies, Zug, Switzerland), and BEAS-2B cells were cultured in KGM-2 medium (LONZA, Basel, Switzerland). Before treating or exposing cells to CS, 24-well polyethylene terephthalate transparent membrane inserts (ThinCert™ by Greiner Bio-One International AG based in Kremsmünster, Austria), which feature a pore size of 0.4 µm, were prepared by soaking the inserts in the designated cell culture medium for a duration of four hours. After being washed twice with phosphate-buffered saline (PBS) without Ca²⁺ and Mg²⁺, the cells were trypsinised. The number of cells was counted using an electronic cell counter (CASY® Scharfe System, Roche Innovatis AG, Reutlingen, Germany)[25].

For the initial seeding, 50 x 10³ A549 cells, or 60 x 10³ BEAS-2B cells were placed per insert.. The cells were then incubated for 24 hours to allow for cell growth and attachment to the inserts.[25]

After 24 hours, the culture medium was removed from the apical side of each insert, and the cell monolayers were washed with PBS containing Ca^{2+} and Mg^{2+} , 18 hours before exposure. . This ensured that the cells were in optimal condition and ready for exposure [25].

2.4.2 Mainstream CS distribution in the Vitrocell® 24/48 system

The insert-to-insert variability to exposure of CS across the 24-well plate was measured using WST-1 (Roche Applied Science, Rotkreuz, Switzerland) in cell culture media only. WST-1 (1 mL) was mixed with 4 mL Dulbecco's modified Eagle's medium supplemented with 0.1% gentamycin. A 75- μL aliquot of this mixture was transferred to cell culture inserts without cells and exposed to six 3R4F reference cigarettes (66 total puffs) over a range of dilutions, or air only as a negative control. The flow rate was 0.5 L/min, and the total exposure time was 18 min. After exposure to CS, 50 μL aliquots of the WST-1 medium were transferred into a 96-well plate, and the optical density was measured at 430 nm. OD measurements taken from three independent smoke exposure runs were analysed to determine the effect of different runs on the overall variability.

During the same CS exposure runs where the WST-1 colourimetric assay was used, particle deposition was tracked by attaching individual Vitrocell quartz crystal microbalances (from Vitrocell Systems GmbH, Waldkirch, Germany) to each row of the dilution/distribution system. Particle deposition was monitored in real-time during exposure, and the final mass after the last cigarette was smoked was measured to compare particle distributions across different CS concentrations [25].

2.4.3 Viability assay

In the actual experiment, inserts with cells were exposed to a series of six 3R4F cigarettes producing 15% and 10% concentrations of mainstream smoke using the Vitrocell® system; each session lasted 18 minutes. The viability of the cells was evaluated post-exposure using a resazurin-based assay at 4, 24, and 48 hours after exposure. For this assessment, 150 μL of resazurin solution (120 $\mu\text{g}/\text{mL}$ in the culture medium) was added to each insert and incubated for 2 hours at 37°C, in an environment of 5% CO_2 and 95% humidity. The subsequent fluorescence reading was taken from 100 μL aliquots from each well, measured at wavelengths of 560 nm for excitation and 590 nm for emission. Cells exposed to a 1% Triton X-100 concentration in their basal media served as a positive control for maximum cell viability loss—Non-exposed inserts cultured at the air-liquid interface (ALI) and kept in the incubator served as negative controls to represent baseline conditions. Additionally, non-exposed cells seeded on inserts but maintained under submerged conditions in the same incubator acted as supplementary controls, enabling differentiation between effects caused by ALI exposure and those due to standard submerged culture [25].

2.4.4 Analysis of eight carbonyl compounds using liquid chromatography

To capture carbonyl compounds from cigarette smoke, PBS served as an effective surrogate for the mucus layer in the lungs. In the Vitrocell® 24/48 system, 18.5 mL of PBS was placed into each row of the cultivation base module (six positions in the row connected from below and shared the same media supply). The procedure consisted of three separate experimental runs, each with ten 3R4F cigarettes, and all were replicated technically three times. Multiple dilutions of CS were carried out at airflow rates varying from 0.1 to 3.0 L/min to estimate levels of carbonyl contents in cigarette smoke. PBS exposed to fresh air only was used as a negative control for comparison. The duration of exposure was set to 28 minutes [25].

Post-exposure, 1.2 mL aliquots of PBS from each position in the base module that had been exposed to smoke or air, were mixed with 1.8 mL of a dinitrophenylhydrazine (DNPH) mixture containing 15 mM DNPH in acetonitrile and 25 mM perchloric acid, to derivatise the carbonyl compounds by chemical reaction with DNPH for subsequent detection. The reaction was allowed to run for 30 min at ambient temperature and was quenched by adding 150 µL of pyridine. 500 µL of each derivatised sample was transferred into an LC-MS vial, containing 485 µL acetonitrile and 15 µL of an internal standard solution with specific concentrations of acetone-d₆-DNPH and methyl-ethyl-ketone-d₅-DNPH [25].

The quantitative assessment involved identifying various aldehydes, including formaldehyde, acetaldehyde, and crotonaldehyde, among others, as well as acetone, using liquid chromatography (model Agilent 1200) paired with mass spectrometry featuring electrospray ionisation (5500 QqQ model by AB Sciex). Separation of the aldehyde was performed in isocratic mode on a Chromolith Speedrod RP-18e HPLC column using water, acetonitrile, tetrahydrofuran, and isopropanol (59:30:10:1, v/v/v/v) at a flow rate of 2.5 mL/min (column set at 40°C equipped with a post-column splitter 1:6 before entrance into the MS). MS detection was performed in multiple reaction monitoring mode, and the concentration of the carbonyl compound (expressed in µg/cigarette) was calculated using an external calibration curve [25].

2.4.5 Nicotine Determination

To assess nicotine levels in diluted tobacco smoke, samples were taken using Extrelut 3NT columns saturated with 0.5M sulfuric acid, affixed to the outflow of the Vitrocell® 24/48 system's dilution mechanism. Smoke dilutions at the following concentrations: 69%, 54%, 32%, 19%, 13%, 10%, and 7%

were analysed in triplicate samples and the entire procedure was replicated three times. After collection, isoquinoline, used as an internal standard, was dispensed onto the column. Nicotine and the internal standard were eluted using 5% triethylamine in N-butyl acetate. For sample injection, an Agilent 7890A gas chromatograph was used on a split mode setting with an injector temperature maintained at 220°C and a split ratio of 1:20. The chromatographic separation took place in a constant temperature oven held at 140°C using a 15m long DB-5 fused silica capillary column with an internal diameter of 0.25 mm, and a film thickness of 0.25 µm. Helium was used as the carrier gas at a constant flow rate of 1.4 mL/min, and flame ionisation detection allowed for both compound identification and nicotine quantification via an internal standard calibration curve [25].

2.4.6 Statistical analysis

The decomposition of variance was performed using the MIXED procedure with the COVTEST option and the method of restricted maximum likelihood in SAS 9.2. The model incorporated random effects, including row, column, and exposure run. The Wald z-test was employed to assess the significance of random effects' variances, determining whether they differed significantly from zero. To identify significant differences in WST-1 reduction across different insert positions or amongst various QCMs, ANOM analysis was applied. Additionally, to account for multiple comparisons in our data, we adjusted the raw p-values using the Nelson-Hsu approach. Statistics for cell viability assays were derived from a mixed-design ANOVA model [25].

2.5 Results

2.5.1 Mainstream CS distribution inside the Vitrocell® 24/48 exposure chamber

The performance evaluation of the Vitrocell® 24/48 system included an analysis of the distribution pattern of CS from standard 3R4F reference cigarettes. This analysis employed a modified smoking protocol developed by Health Canada [25]. The process began with monitoring the reduction of WST-1 by CS, determined by measuring optical density (OD) at a wavelength of 430 nm post-exposure. Setting the airflow to a dilution rate of 0.5 L/min, which equates to a 32% concentration of CS, revealed that the system effectively and uniformly dispersed CS across the Vitrocell® 24/48 system without noticeable variation in distribution. OD measurements, adjusted for control values, were plotted for each separate insert within the cultivation base module, and statistical significance was noted by calculating p-values. It is essential to note that there was only one instance of a statistically significant deviation (p-value < 0.01) due to an outlier in one of the three exposure trials (Figure 2.4). Upon examining the average optical densities (ODs) together with their standard deviations (SD) for each of the six samples per row in the cultivation apparatus, apparent differences were observed between

the rows treated with cigarette smoke (CS; rows 1–7) and the row that was subjected to fresh air. Three separate exposure experiments were carried out to investigate the causes of this variance. Subsequent variance decomposition analysis revealed that a significant part of the variation, totalling 39.5%, could be linked to the different exposure experiments themselves. Additionally, when considering just the variability among the inserts exposed to CS—disregarding the exposure experiments—the coefficient of variation (CV) was found to be relatively small at 12.2%. The overall CV, which encompasses both within- and between-trial variability, reached 15.6%. When considering the differences attributed to specific locations within the rows or columns of the cultivation apparatus, these proved insignificant compared to random errors, with rows showing a CV of 4.8% and columns at 1.4% versus an unpredicted error percentage of 54.2% (Figure 2.4) [25].

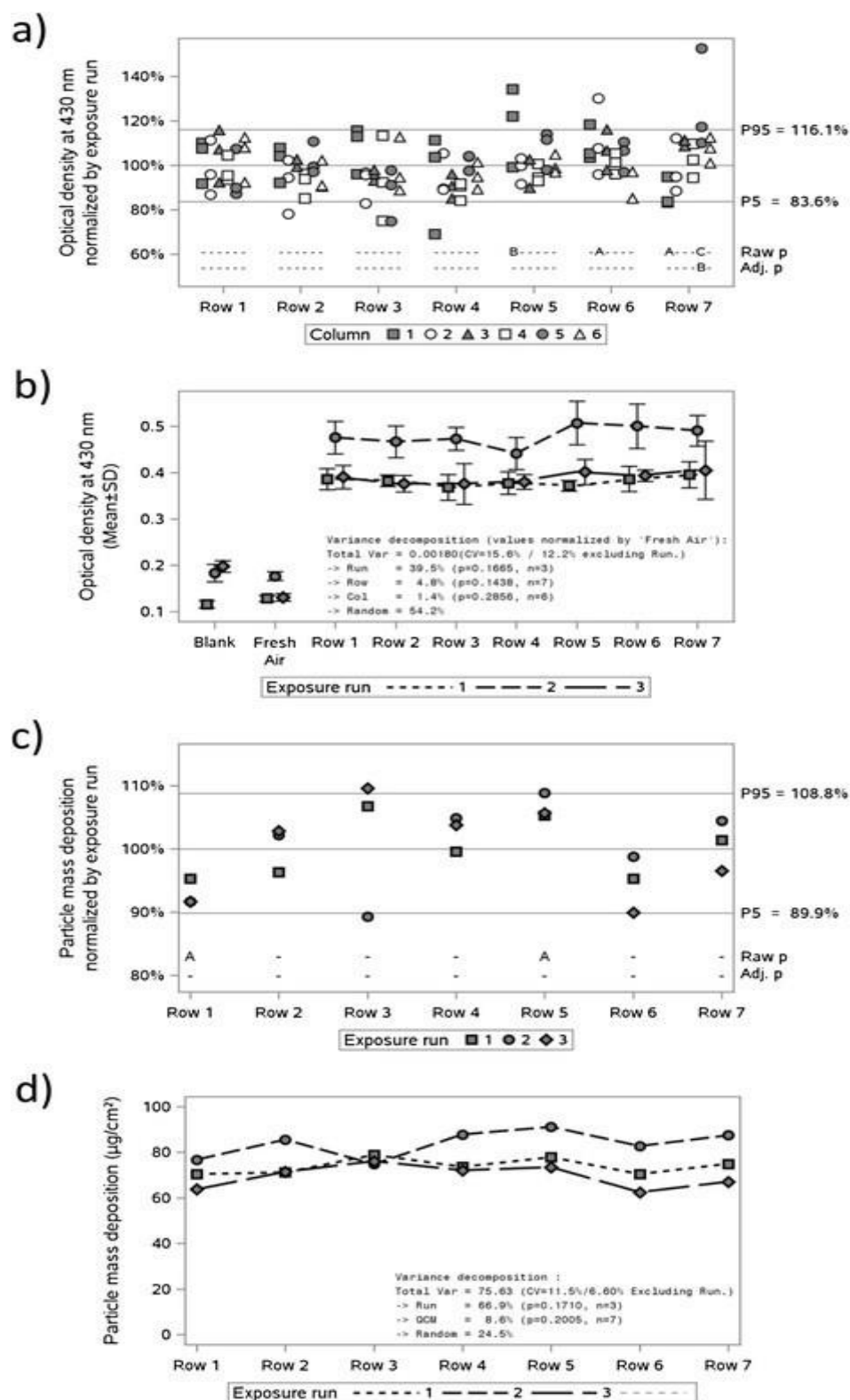


Figure 2.4 displays the uniform distribution of CS aerosols at a concentration of 32% (0.5 L/min):
The figure includes different parts:

(a) Scatter plot showing the blank corrected OD values for WST-1 reduction, normalised to 100%, for each insert of the cultivation base module, from three independent CS exposure runs. Different insert

positions in the respective rows of the cultivation base module (Rows 1–7) are marked with different symbols. Raw p-values according to ANOM (Raw p) and adjusted p-values for multiple testing (Adj. p) are indicated at the bottom of the graph for each insert position (A: $p < 0.05$; B: $p < 0.01$ and C: $p < 0.001$).

(b) Cellular Viability via WST-1 Assay: The average optical density at 430 nm, indicative of WST-1 reduction, was measured following exposure to mainstream cigarette smoke (CS). Data represent the mean $\pm$ standard deviation (SD) from six inserts located in rows 1–7 of the cultivation base module. Comparative measurements included inserts exposed to fresh air and blank controls. Each line in the graph corresponds to one of the three independent exposure runs.

(c) Particle Mass Deposition: A scatter plot illustrates the normalised particle mass deposition (set to 100%) across QCMs attached to rows 1–7. Statistical analysis was performed using ANOM (Analysis of Means), with both raw p-values and adjusted p-values for multiple comparisons reported beneath the graph. Inserts with statistically significant deviations ($p < 0.05$) are marked accordingly. Distinct symbols denote the three separate exposure runs.

(d) Particle Number Distribution: The number of particles deposited on QCMs connected to each row of the dilution system was quantified. As in previous panels, data from three independent exposure runs are shown.

In panels (a) and (c), horizontal lines indicate the 5th (P5) and 95th (P95) percentiles of all measured values, providing a visual reference for variability. Variance decomposition was applied to each dataset to assess the contribution of different sources of variability, with corresponding p-values and sample sizes (n) reported [25].

2.5.2 Smoke constituents measurement inside the Vitrocell® 24/48 system

The research objective was to identify and evaluate the variety of volatile and semi-volatile compounds that are emitted in the mainstream smoke (MS) from cigarettes when drawn in a Vitrocell environment. To achieve that, nicotine, along with eight distinctive carbonyl compounds, were captured within the Vitrocell® 24/48 system under different concentrations of diluting airflows. Subsequent liquid chromatography-mass spectrometry (LC-MS) analysis was conducted on these samples. The outcomes indicated a pronounced direct proportionality between the amount of nicotine captured and the concentration of cigarette smoke (with an R^2 value exceeding 0.95) spanning the entire observed cigarette smoke concentration spectrum, which ranged from 7 to 69 percent. Similarly, the levels of various carbonyl compounds—which include acetaldehyde, acetone,

acrolein, butyraldehyde, crotonaldehyde, formaldehyde, methyl ethyl ketone, and propionaldehyde—also showed a robust correlation to the levels of cigarette smoke exposure (with an R^2 value greater than 0.96). These results indicate the efficacy and potential utility of the methods employed in this study for accurately measuring both the composition and concentration levels of substances present in mainstream cigarette smoke (Figure 2.5) [25].

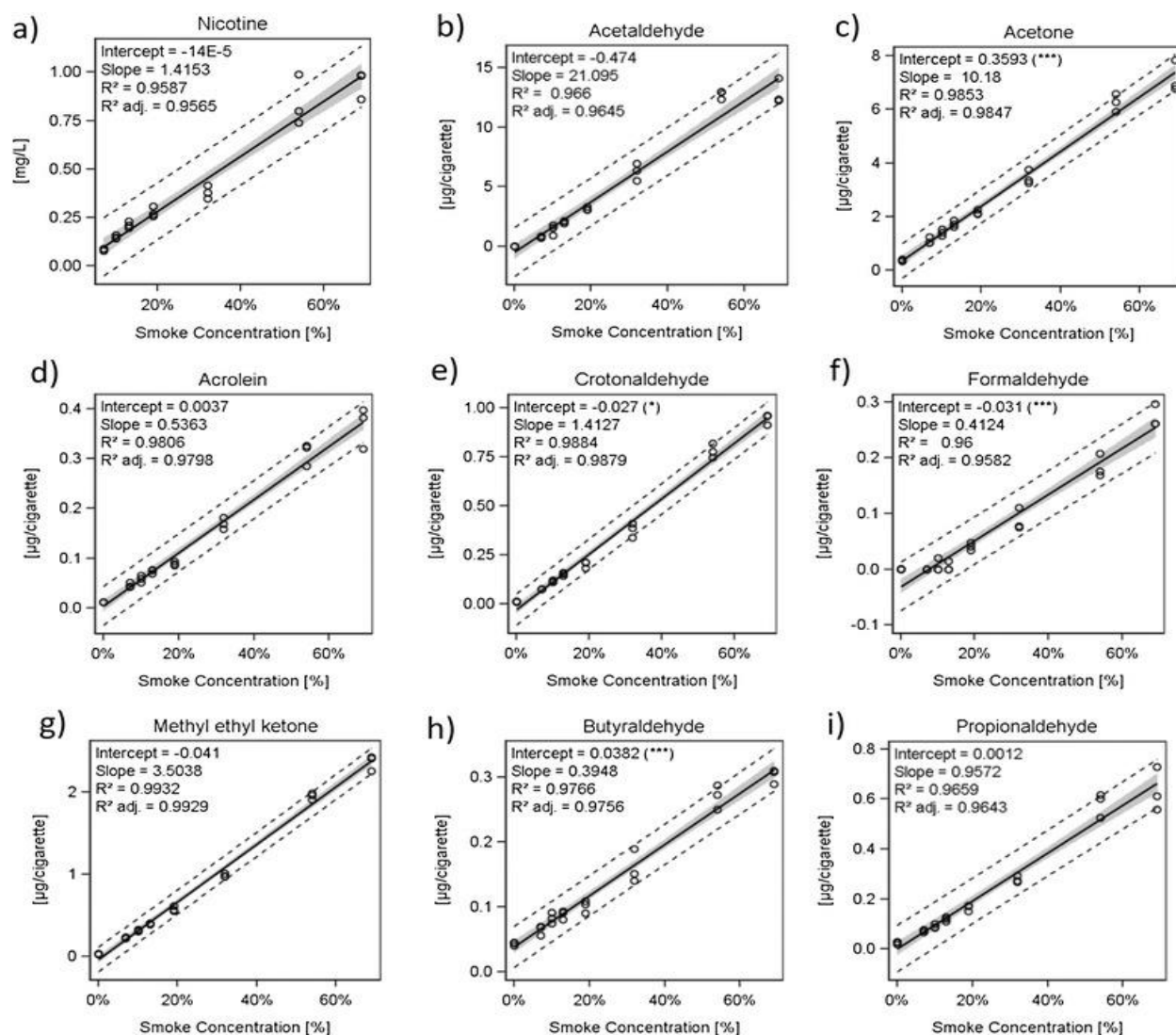


Figure 2.5 Quantitative Assessment of Key CS volatiles: The Vitrocell® 24/48 exposure chamber was used to quantitatively assess key cigarette smoke components in a range of dilutions of mainstream cigarette smoke.

2.5.3 Mainstream CS effects on the cell viability of different cells

In investigating the effects of cigarette smoke (CS) on cellular health using the Vitrocell 24/48 exposure system, we analysed two cell types, A549 and BEAS-2B, cultivated under an air-liquid interface (ALI). These cells were exposed to CS at concentrations of 10% and 15%, corresponding to dilution airflows

of 1.95 L/min and 1.25 L/min, respectively. Post-exposure times of 4, 24, and 72 h were selected to monitor both acute effects and the cells' potential recovery. Figures 2.6a and b show that, compared with fresh air, CS exposure was toxic to cells, which became less capable of reducing resazurin, a dye commonly used to determine cells' metabolic activity as a cell viability marker. BEAS-2B cells were more sensitive to CS than A549 cells, particularly after 24 or 48 hours post-exposure, by which time the A549 cells had shown signs of recuperation. Moreover, the higher concentration of CS inflicted more significant damage in both types of cells. We incorporated a positive control for toxicity into our study, using a 1% Triton X-100 solution to lyse cells to achieve maximum viability reduction for comparison. The data confirm that cells can maintain viability at an ALI for up to 48 hours, with viability comparable to that of cells grown under normal, submerged conditions. [46]

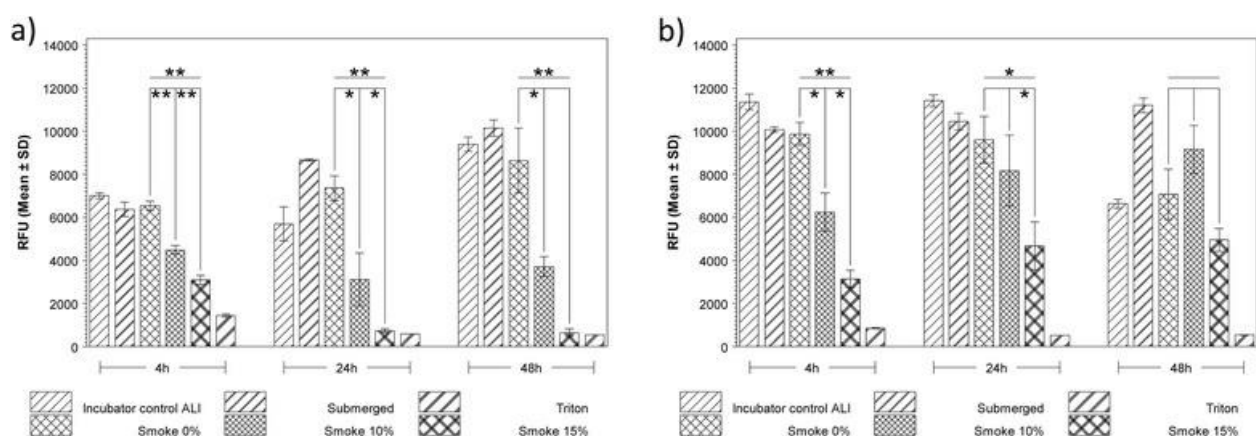


Figure 2.6 Mainstream CS exposure of cells: The diagram illustrates the effects of mainstream cigarette smoke (CS) exposure on the viability of BEAS-2B and A549 cells at 4, 24, and 48 hours post-exposure. For each time point, three independent sets of inserts were analysed. Treatment groups (n = 6), comprising two CS exposure runs and three technical replicates, were statistically evaluated using a mixed-design ANOVA model, with the exposure run treated as the error term. Cell viability was quantified via Relative Fluorescence Units (RFU), and statistical significance was denoted by asterisks: $p < 0.05$ (*) and $p < 0.01$ (**). To ensure robust interpretation, a positive control using 1% Triton X-100 was included. Additionally, two control conditions were assessed: incubator ALI controls (non-exposed inserts maintained at the air-liquid interface) and submerged controls (non-exposed inserts cultured under submerged conditions). The bar graph accompanying the data illustrates the percentage of CS applied across the different treatment groups [25].

2.6 Discussion

This study evaluated the effectiveness of the Vitrocell 24/48 system for exposing cells to CS. This highly adaptive system enables the concurrent exposure of six replica inserts and can analyse up to seven varying concentrations of smoke. It can assess 48 inserts with CS or a combination of 42 test inserts

and six controls with fresh air in a single cycle. Results from the study showed consistent CS distribution across the system, as evident from the uniform decrease in WST-1 levels across all examined culture inserts when subjected to a consistent level of whole CS (Figure 2.4). Additionally, particle mass accumulation, as determined by QCMs, was comparably homogeneous across the entire set of CS concentrations. When conducting statistical analysis, the coefficient of variation (CV) for diverse inserts loaded with WST-1 and QCMs was found to be stable across three separate runs, ranging from 69% to 7%, using the Vitrocell 24/48 system [25].

The variability noted in this study was mainly due to the run effect, which probably results from factors like how the smoking machine delivers smoke or how the assay technology performs. It's important to note that directly comparing these results with studies using different Vitrocell® exposure modules is not simple. Variations in module design, such as trumpet or insert diameters, along with differences in smoking regimes, sampling flow rates, and the types of smoking machines used, can substantially affect the results. Nevertheless, a consistent pattern has been observed across studies, particularly the even distribution of aerosol and concentration-dependent effects responses. For example, QCM measurements using the Vitrocell® 6/4 Stainless Steel module with the Vitrocell® VC10 Smoking Robot showed no notable positional variability within the exposure module. However, other studies have identified statistically significant differences between module positions, indicating that equipment maintenance—especially of the smoking machine—may influence inter-study variability. These findings support the conclusion that the most substantial sources of variability are external to the Vitrocell® exposure system itself, underscoring the importance of standardised operational procedures and rigorous equipment maintenance in aerosol exposure studies [25].

Studies demonstrate that the concentration of CS, as gauged by the QCM technique, seems to have a direct correlation [47]. Nonetheless, it is hard to make exact comparisons due to variations in the range of concentrations tested. The apparent deviation from linearity observed when using the Vitrocell 24/48 system can likely be attributed to the absence of higher CS concentration data. In contrast to the entire smoke distribution measured by WST-1, particulates settling onto QCMs within the Vitrocell® framework are significantly affected by how air moves through the dilution and distribution setup. To accurately approximate deposition rates, it is crucial to consider factors such as particle movement due to diffusion and air currents, as well as the interception and impaction of particles. These elements may be further influenced by other factors present in the airflow, such as turbulence and electrical charges, which are considered potential limitations when using the Vitrocell apparatus. However, at increased airflow velocities, we observe that particle mass deposition tends to maintain its linearity and align with flow rates typically used in cellular exposure studies. Despite these findings, further investigation is necessary to fully understand how varying airflow speeds affect

particle deposition rates. Employing computational fluid dynamics (CFD), the analysis can demonstrate how variables such as flow velocity, droplet size, diffusion, and gravity-driven settling impact aerosol deposition within the Vitrocell exposure chamber [25].

The study investigated the effect of CS on A549 and BEAS-2B cells when exposed to an air-liquid interface (ALI). These cell lines are commonly used to evaluate the toxicity of the lung epithelium, as they closely resemble human lung epithelial cells. The study found a relationship between the concentration of smoke and the impact on cell viability for both cell lines. BEAS-2B cells were found to be more vulnerable to aerosol exposure at the ALI than A549 cells [25].

The differential sensitivity of BEAS-2B and A549 cells to CS at the air-liquid interface (ALI) reflects their distinct biological origins and functional characteristics. BEAS-2B cells, derived from normal human bronchial epithelium, form robust tight junctions and exhibit epithelial barrier properties. However, they possess limited xenobiotic metabolic capacity, rendering them more susceptible to toxic insults. In contrast, A549 cells, originating from pulmonary adenocarcinoma, produce surfactant proteins and exhibit higher metabolic resilience but lack the ability to form tight junctions, limiting their utility in barrier function studies [47].

Both cell lines demonstrate sensitivity to whole cigarette smoke exposure in terms of cytotoxicity, inflammation, and genotoxicity [48], although their responses differ due to variations in cell polarity, differentiation status, and metabolic enzyme expression [49]. These differences are not limitations but rather reflect the complementary nature of these models in representing distinct regions of the respiratory tract—bronchial (BEAS-2B) and alveolar (A549) (48).

In alignment with the OECD Good In Vitro Method Practices (GIVIMP), these differences underscore the importance of:

- **Thorough cell line characterisation** (e.g., origin, phenotype, metabolic profile),
- **Standardised culture conditions** (e.g., ALI exposure, passage number, media composition),
- **Appropriate model selection** based on the biological question and region of the respiratory tract being modelled.

GIVIMP emphasises that in vitro models must be fit-for-purpose, and that understanding the biological relevance and limitations of each model is essential for generating scientifically valid and regulatory-acceptable data. The use of both BEAS-2B and A549 cells, when properly characterised and applied, aligns with GIVIMP's principles by enabling a more nuanced and region-specific

assessment of inhalation toxicants [Guidance Document on Good In Vitro Method Practices \(GIVIMP\)](#) | [OECD Series on Testing and Assessment](#) | [OECD iLibrary](#) [50, 51].

2.7 Conclusions

Our experimentation with the Vitrocell® 24/48 system has confirmed its effectiveness in exposing cells to CS at the air-liquid interface. The device ensures uniform smoke dispersion across the inserts within the exposure module, maintaining functionality even when CS concentrations are as high as 20%. Despite this, such high concentration levels may compromise cell survival and disrupt biological pathways associated with smoking in our investigated cell lines. Additional studies are necessary to understand the impact of varying particle mass deposition rates at elevated concentrations of CS. The WST-1 assay used in our study provided a straightforward approach for assessing the extent of smoke exposure on inserts. Still, it also highlighted that our grasp on how the gas and vapour phase of CS affects the cell inserts remains incomplete. Future research should focus on metabolite analysis of CS components after exposure to enhance our understanding in this domain (25).

2.8 Research Impact:

Following the publication of this research article, numerous new studies have been conducted in the field, resulting in 77 citations according to Google Scholar, which highlights its significant impact in the scientific community. This study played a crucial role in inspiring further research, especially in the development and characterisation of in vitro exposure systems for studying acute and chronic toxicity. Bovard et al. developed a novel lung/liver-on-a-chip platform to study both acute and chronic toxicity [52, 53]. By integrating three-dimensional in vitro models with multi-organ-on-a-chip (MOC) technology, they have significantly advanced the assessment of chemicals. MOC facilitates the combination of multiple organ models, allowing researchers to explore the interactions between different organs. As noted by Bovard et al. in their paper, in vitro exposure systems, such as the Vitrocell® Exposure System, can be employed to expose air-liquid interface (ALI) cultures to various aerosols or aerosol mixtures [53]. Researchers can then study the effects of these exposures on lung tissues and their connections to the liver. This comprehensive approach enhances the evaluation of a compound's safety and efficacy compared to traditional single-culture models. Furthermore, this technology enhances the toxicological assessment of aerosols associated with chronic obstructive pulmonary disease, asthma, and lung cancer. Additional implementation is demonstrated in the work of Iskandar et al. (2019), who applied the characterised system in their comparative assessment of aerosol exposure approaches. This adaptation of the methodology by researchers demonstrates broader acceptance of the approach within the scientific community.

Enabling Cross-Study Comparisons Through Dosimetry

The importance of incorporating dosimetry measurements, as emphasised in this chapter, is exemplified by subsequent studies that have leveraged this approach for meaningful cross-study comparisons. For example, Azzopardi et al. (2016) applied dosimetry principles to compare their cytotoxicity results with our findings, using our in vitro 24/48-hour exposure system alongside their own setup. They referenced our published data [53] and reported comparable results, including similar estimated EC50 values. This highlights the practical value of standardised dosimetry reporting and demonstrates how our published work supports meaningful comparisons across different experimental platforms. It also validates the broader applicability of dose–response relationships in aerosol toxicology research.

3.0 A framework for in vitro systems toxicology assessment of e-liquids

3.1 Introduction and Contribution

This chapter will provide a detailed examination of the research article titled "**A Framework for in vitro systems toxicology assessment of e-liquids**". (Iskandar AR, Gonzalez-Suarez I, **Majeed S**, Marescotti D, Sewer A, Xiang Y, Leroy P, Guedj E, Mathis C, Schaller JP, Vanscheeuwijck P, Frentzel S, Martin F, Ivanov NV, Peitsch MC, Hoeng J. *Toxicol Mech Methods*. 2016 Jul;26(6):389-413. doi: 10.3109/15376516.2016.1170251. Epub 2016 Apr 26. PMID: 27117495; PMCID: PMC5309872)(27). Electronic nicotine delivery systems (ENDS), such as e-cigarettes, have gained popularity due to their perceived reduced harm in comparison to traditional cigarettes. Nevertheless, research on the potential effects of ENDS exposure on humans remains limited and inconclusive. Addressing these issues presents a challenge with the emergence of new products possessing varying designs and performance parameters. To tackle these challenges effectively, we propose a framework for conducting in vitro systems toxicology assessments of e-liquids and their aerosols. This framework aims to enhance standard toxicity assays by enabling high-throughput toxicity assessments of e-liquids and their aerosols while minimising device-to-device variability. As a co-author of this article, I played a significant role in designing the exposure strategy and developing diverse methodologies for exposure to ENDS [42].

3.2 Background

Individual smokers rarely achieve long-term smoking abstinence without support from behavioural, pharmacological, or social interventions. Therefore, seeking help to quit smoking is the most effective method for reducing the risks associated with tobacco use [54, 55]. Standard nicotine replacement therapies—like patches, gums, and nasal sprays—frequently fail to fully support smoking cessation because they overlook the behavioural and sensory components of smoking. This constrains their overall success [56]. A promising strategy to lower tobacco-related harm is to develop and promote nicotine products that are much less harmful than cigarettes, while still providing nicotine. These alternatives should focus on significantly reducing or removing toxic substances and carcinogens present in cigarette smoke. In the UK, it is estimated that if only 1% of smokers switch annually to less harmful nicotine options, approximately 60,000 lives could be saved over ten years. [53]. Electronic nicotine delivery systems (ENDS) are a potential promising alternative to conventional cigarettes, aiding in lowering the risk of health problems related to smoking. ENDS are considered to be much

less toxic than cigarettes, as users are exposed to significantly lower levels of toxic substances and carcinogens present in cigarette smoke [57][42].

Electronic Nicotine Delivery Systems (ENDS) comprise various battery-operated devices that vaporise a liquid mixture—often referred to as e-liquid or e-juice—made up of flavourings, nicotine, and a carrier such as propylene glycol or vegetable glycerin. They work by heating the liquid to create an aerosol that users inhale during vaping. Since their introduction over a decade ago, ENDS have evolved significantly. Early models were basic, either disposable or rechargeable. The second generation introduced refillable tanks and extended battery life, while third-generation devices incorporated advanced electronics, enabling users to adjust power output and personalise nicotine delivery. More recently, the latest ENDS models have begun integrating user identification technologies to restrict access by underage individuals, aligning with recommendations from regulatory bodies such as the FDA to enhance youth protection and ensure responsible usage [61].

The long-term health effects of ENDS and their ability to reduce harm from tobacco products are currently being debated [60]. This debate stems from the lack of clear regulations and standardised scientific assessments of ENDS [46]. While research on ENDS is growing, there is still no industry-standard toxicity assessment [62]. Some studies suggest that e-liquids have significantly lower toxicity toxicological effects than traditional cigarettes, while others indicate potential adverse health consequences [60, 63]. To address this controversy, a cohesive approach to evaluating the health impact of ENDS and their potential for harm reduction is necessary [64]. The National Academy of Medicine (NAM) (formerly known as U.S. Institute of Medicine) recommends combining rigorous toxicity testing and clinical studies, using a "benchmark product" for comparison, to understand the effects of ENDS comprehensively [65].

Assessing the toxicity of ENDS and e-liquids is challenging due to several factors. The continual evolution of ENDS devices makes toxicity testing more complex. Moreover, the lack of standardised manufacturing practices for e-liquids results in compositional variability [66]. The impact of flavour components on toxicity is unknown [67, 68]. The safety of inhaling humectants, such as propylene glycol and glycerol, when used by e-cigarette users is uncertain. During aerosolisation, these humectants can form toxic by-products, such as formaldehyde [69]. The numerous types of e-cigarette products make it difficult to establish standardized toxicity testing protocols. This aligns with the principles of the 3Rs [69] and the 21st Century Toxicology framework [70], which focus on minimising animal testing and obtaining mechanistic data through relevant in vitro methods. For in vitro toxicity assessments to be effective, they must be sensitive enough to identify toxic effects or mechanisms that could predict human health risks. [71]. Additionally, these assessments should be capable of evaluating the toxicity of standard e-liquid components, such as humectants (e.g., propylene glycol

and glycerol) and flavouring agents [72]. To effectively address the associated concerns, we propose a multi-tiered in vitro testing strategy for Electronic Nicotine Delivery Systems (ENDS). This approach is intended to serve as a foundational element for a broader assessment framework within the scope of tobacco harm reduction. When used alongside conventional pre-clinical in vitro toxicity evaluations, this layered methodology can offer a more robust and comprehensive analysis of ENDS product [73].

3.3 Assessment approaches

3.3.1 Standard in vitro toxicity assessment: a battery of classical assays

When evaluating the toxicity of e-cigarettes and e-liquids, a set of tests known as a "battery of assays" is used. This set of tests is essential because it is impossible for a single test to capture all potential toxic effects of a substance. The Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA) recommends using this type of battery to evaluate tobacco products. It includes tests for mutagenesis and genotoxicity, such as the Ames test, mouse lymphoma assay, micronucleus or chromosomal aberration assessments, and the neutral red uptake test for cytotoxicity measurement. These tests follow protocols approved by the Organisation for Economic Co-operation and Development (OECD) and the U.S. Food and Drug Administration's pharmaceutical guidelines. However, interpreting the results of these tests can be complex, especially when determining genotoxicity based on positive results from one specific test or multiple tests in the series [43].

For aerosol analysis, various techniques are used to capture aerosols. The process of generation of TPM and GVP from aerosol/smoke is already discussed in Chapter 1 (section 1.3.1). Typically, TPM is collected on a glass fibre filter pad, eluted and dissolved in dimethyl sulfoxide (DMSO), then stored immediately at -75 °C for preservation. Before implementation, it is crucial to assess the efficiency of the trapping and elution processes. Additionally, the gas-vapour phase (GVP) can be collected post-filtration in polar liquid media such as phosphate-buffered saline (PBS), culture media, or acetone, a process which also captures unfiltered aerosol droplets through bubbling. Another method includes collecting aerosol fractions as condensate in a cold trap. This condensate gathers some semi-volatile substances but not volatile ones [43].

3.3.2 Systems toxicology assessment: A proposed *in vitro* assessment framework

The popularity of ENDS has surged recently, necessitating the development of newer methods for evaluating their potential harm. Advances in high-throughput assays and omics technologies now permit the examination of cellular and molecular alterations at a holistic level, enlarging the spectrum

of toxicity testing. Such methodologies enhance our ability to extrapolate potential human impacts based on in vitro toxicity findings [42].

We have developed a stratified framework for evaluating e-liquids, which complements traditional toxicity testing methods (Figure 3.1). In line with a systems toxicology approach, our proposed structure comprises three principal strata:

1. First, we conduct a comprehensive toxicity screening on an extensive collection of e-liquids employing primary cell cultures.
2. The second layer involves detailed mechanistic analysis focused on a narrowed selection of these e-liquids for toxicity traits.
3. The third encompasses a similar mechanistic study but extends it to a carefully chosen range of aerosols from e-liquids.

Our approach is fortified by computational systems toxicology, enabling a meticulous examination of toxic cellular mechanisms associated with e-liquids and their resulting aerosols. To bolster the toxicological evaluation further, we integrate analytical chemistry research to yield essential chemical data that complements the biological findings [74].

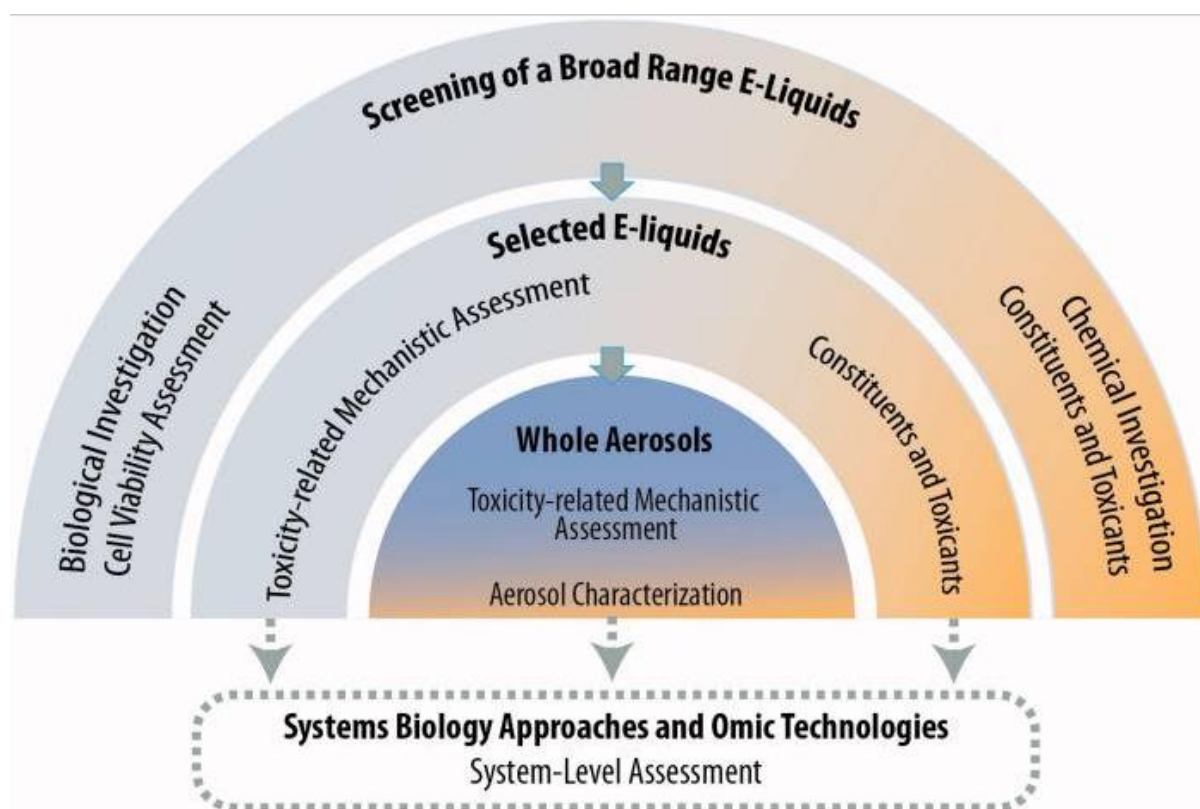


Figure 3.1: The presented proposal outlines a strategy for evaluating the toxicity of e-liquids using in vitro testing methods. This strategy is based on a three-tiered assessment platform that aligns with a systems toxicology framework. The objective is to conduct an exhaustive study of the cellular

mechanistic toxicity associated with e-liquids and their resulting aerosols. The in vitro experiments are supplemented with detailed analytical chemistry research, delivering precise and dependable chemical data that enhances the toxicological evaluation [43].

3.3.2.1 The first-layer assessment

The framework's objective is to effectively assess the overall toxicity present in numerous e-liquid variants using pertinent in vitro cellular models as testing platforms. The preliminary evaluation operates on methodologies optimised for high-throughput and high-content analysis. Analytical chemistry plays a pivotal role in this initial phase, offering critical insights that underpin the legitimacy of the subsequent biological examinations. Analytical methods including High-Performance Liquid Chromatography with Diode-Array Detection (HPLC/DAD), Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS), and Gas Chromatography-Mass Spectrometry (GC-MS) are employed to identify and quantify the toxic substances present in e-liquids and their corresponding cartridges [43].

3.3.2.2 The second-layer assessment

The second layer of the framework explores toxicity mechanisms beyond just reducing cell viability from e-liquid exposure. These include specific pathways of cell death and various pathophysiological processes linked to exposure. The first-layer assessment highlights e-liquids that markedly decrease cell viability, warranting further testing.

High-content screening (HCS) assays are useful at this stage because they can detect multiple cellular properties at once. HCS platforms can concurrently monitor protein phosphorylation, surface receptors, ligand binding, protein expression, molecular uptake and transport, cell cycle regulation, enzyme activation, and cell function proliferation. This enables simultaneous assessment of molecular changes and phenotypic alterations. HCS methods have proven effective in detecting cellular modifications [43].

3.3.2.3 The third-layer assessment

The third stratum of our framework aims to examine the harmful effects of aerosols from specific e-liquids by using 3D airway cellular cultures. These 3D systems provide a more accurate emulation of biological tissues compared to their 2D counterparts, as they simulate the conditions during vaping. They replicate a pseudostratified epithelial layer with mucus-secreting goblet cells, ciliated cells, and basal cells, similar to living epithelial tissue. Commercially available, ready-to-use human 3D airway epithelial models are available from different suppliers [42].

The 3D airway culture system allows for a detailed examination of the airway epithelial layer. Histologic Stains can be used to examine tissue cross-sections and identify different cell types. In cultures with active ciliated cells, you can measure cilia beat frequency and evaluate mucociliary clearance functions. Additionally, cytotoxicity metrics can be assessed, and pro-inflammatory mediators can be measured to understand the mechanisms of toxicity. Using a uniform aerosol generation device is advisable to reduce variances caused by different devices. The market offers various devices whose aerosols differ based on their design and operational conditions, which uniquely impact their toxicity profiles (illustrated in Figure 3.2). Therefore, it is crucial to characterise these aerosols when performing assessments, which should include an examination of droplet size distributions alongside ratios of gas-vapour phase to particulate matter. The frequency of studies reporting on chemical analyses of e-cigarette aerosols has increased. Most studies seek to identify harmful agents found in conventional smoke, nicotine derivatives, aldehydes, volatile compounds and metals [74].

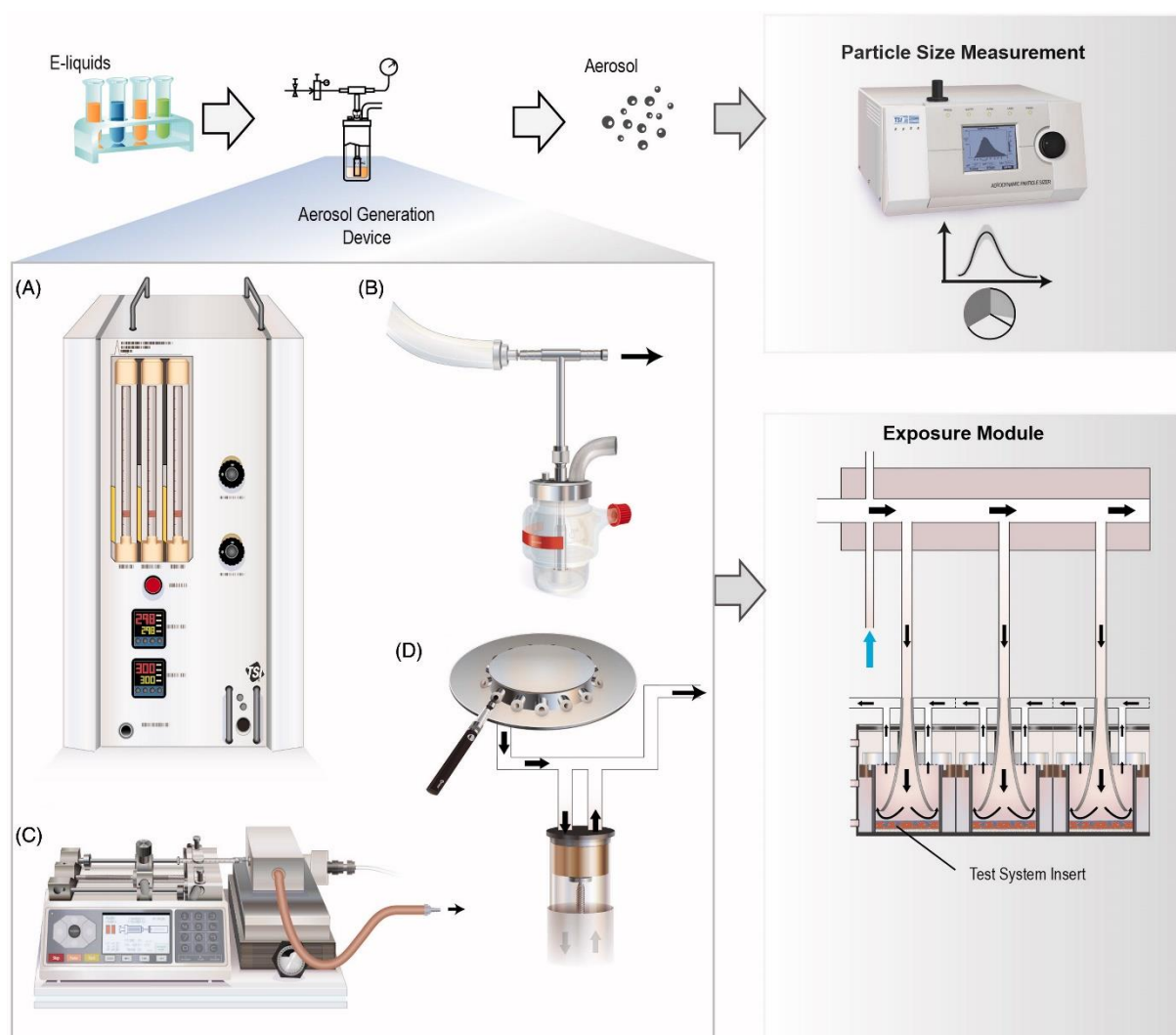


Figure 3.2 Devices for Aerosol Generation: Several commercially available devices can generate aerosols from various e-liquids, including A, Condensation Monodisperse Aerosol Generator; B, Collision nebuliser; C, Vitrocell spiking system; and D, smoking machines where e-cig devices can be used [42].

3.3.3 Systems biology and omics technologies

Using a systems biology approach combined with omics techniques can improve the accuracy of toxicity assessments. Investigating system-wide molecular changes allows us to predict the toxicity of e-liquids and their aerosols more reliably. The network perturbation amplitude (NPA) method is a method for measuring exposure-related fluctuations based on transcriptomics data. Applying the NPA method is an effective way to study the biological impacts of CS or ENDS and to conduct comparative analyses. Additional tools, such as MSigDB/GSEA, ConsensusPathDB, and Ingenuity Pathway Analysis, are available for analysing transcriptomic data [43].

3.4 Perspectives

3.4.1 Variability of e-cig Devices and User Puffing Topography

ENDS are equipped with various heating elements, and some models allow the adjustment of their power settings. This power alteration modifies the vapour's heat, consequently impacting the quantity of nicotine delivered and the production of hazardous byproducts. "Dry puffs" occur when the e-liquid is depleted, causing the heating coil to overheat and emit dangerous toxins [43]. However, in an individual vape, the puff's volume, speed, and frequency significantly determine aerosol toxicity. Habitual e-cig users often inhale for more extended periods than traditional cigarette smokers. Studying these vaping patterns and the inhaled chemicals can illuminate their potential harm. Future research should examine how different settings and vaping behaviours influence aerosol toxicity. This research could include examining how varying temperatures affect biological systems, or utilising an advanced programmable dual syringe pump to mimic various vaping styles [75].

For future studies, it is recommended that the scope of the third-level assessment be expanded to examine how variations in heating intensity (or temperature) and different patterns of e-cigarette use affect the harmfulness of the resulting aerosols (see Figure 3.3 for illustration). This investigation could be carried out by analysing biological test systems, which would evaluate how temperature changes influence biological responses alongside an analysis of aerosol characteristics. Alternatively, researchers may employ a smoking device equipped with a programmable dual syringe pump (PDSP)

to control vapour delivery from actual e-cigarette units, enabling the replication of various vaping behaviours. (27).

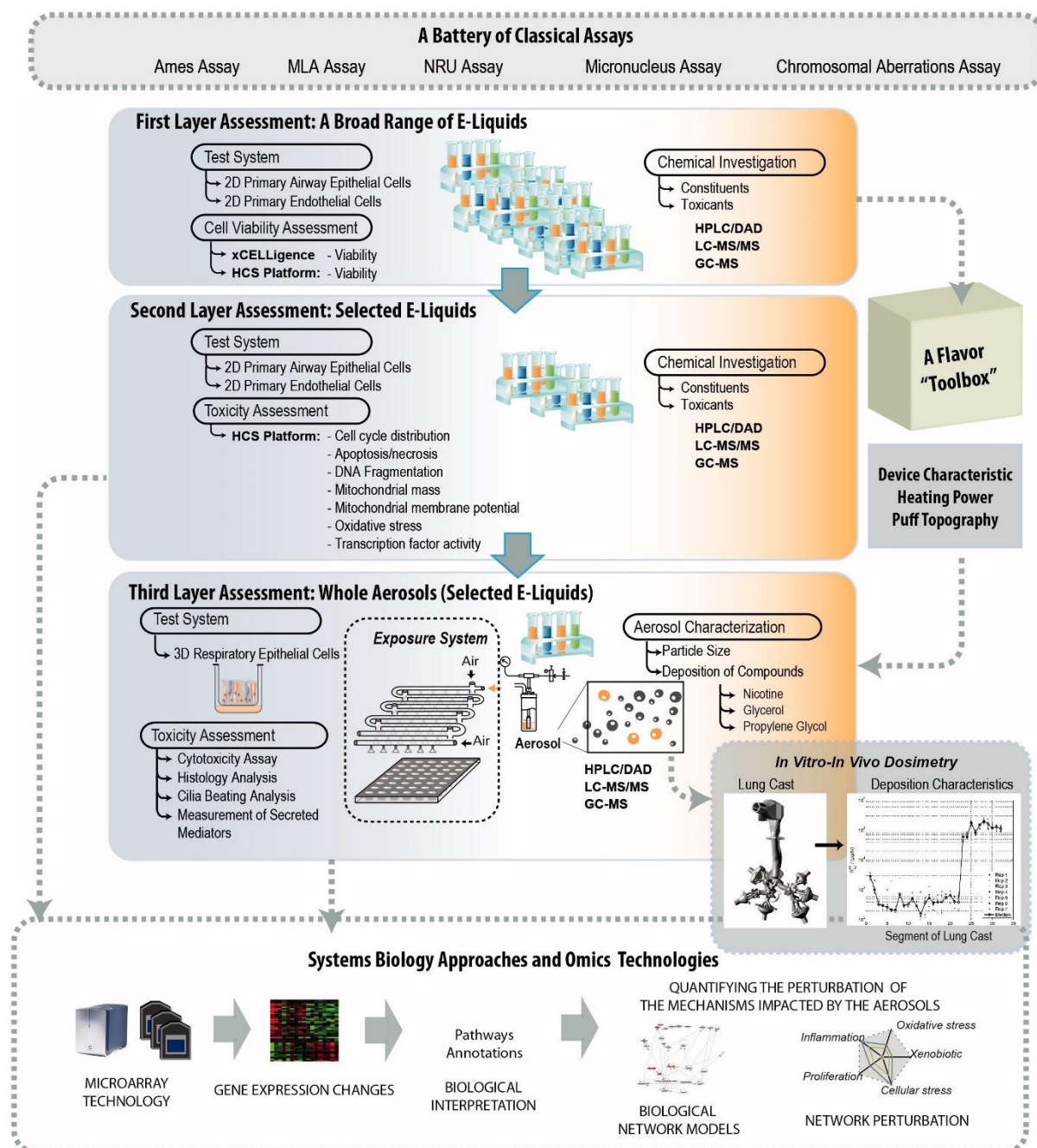


Figure 3.3 Systems toxicology assessment framework: The in vitro systems toxicology assessment framework for e-liquids has been designed to enhance traditional toxicity evaluation methods for tobacco products. The framework consists of three layers that can be expanded to address specific research queries. For example, the framework can analyse how heating power or puff topography affects specific ENDS devices. It can also assess the impact of flavouring compounds and identify acceptable ones by using a "flavour toolbox" for evaluation. Additionally, dosimetry can provide the context needed to estimate human exposure doses [42].

3.4.2 Flavour ingredients

Manufacturers of ENDS use flavourings that have been deemed generally safe, but their inhalation safety profiles are not fully understood. Only a few compounds have undergone inhalation toxicology assessments. Some flavouring elements can harm the respiratory system. Diacetyl, a widely used flavouring agent, has been linked to airway damage in both rats and humans. Acetylpropionyl, a synthetic flavour additive, caused lung tissue damage in rat studies. We need to carefully evaluate the potential toxicity of e-cigarette flavours by comparing each substance to its base solution and developing a flavour toolbox. Further research is necessary to explore these protocols [42].

3.4.3 Uncertainty of *in vitro* toxicity assessment: dosimetry

ENDS are mainly inhaled, with particle size and velocity affecting their impact on the respiratory system. Synthetic exposure methods are used in laboratory studies to assess the effects on living cells. To maintain ethical standards in research, accurate dosimetry assessment is essential for approximating real-world exposure levels. However, aligning *in vitro* dosimetry with actual exposure remains difficult. Integrating dosimetry into current frameworks for toxicity assessment could enhance predictions of the toxicity of aerosol inhalation [43].

3.4.4 Toxicity assessment: regulatory context

In July 2016 (at the time of submission of this article), the manufacturing of e-liquids lacked regulatory oversight, leading to concerns about ingredient consistency. The FDA gained authority to oversee ENDS products starting August 8, 2016. Subsequently, a court ruling mandated ENDS manufacturers to submit product applications for FDA review by September 9, 2020. Over 6.7 million tobacco product applications were received, with the majority of ENDS submissions made close to the deadline. Progress has been significant in the FDA's review process, with action taken on over 99 per cent of products, including rejecting over 1 million flavoured ENDS items. Certain ENDS products have secured FDA authorisation by adhering to public health standards, which encompass evaluations for risks to young people. The premarket assessment of these products is vital to guarantee that new tobacco products, especially those popular ENDS, withstand rigorous scientific examination by the FDA to protect public health. The FDA's scientific evaluation of vaping products ensures that they contribute to safeguarding public health and welfare. Continuous market monitoring seeks to protect youth from ENDS being unlawfully marketed. In January 2020, the FDA released guidance, with an update in April 2020, detailing the agency's enforcement priorities for illegally marketed ENDS. In line with this

guidance, the FDA makes enforcement decisions individually, considering resource limitations that hinder action against every non-compliant tobacco product, thus necessitating efficient use of available resources (27).

3.5 Applications of the three-layer assessment

The following case study demonstrates how to evaluate test mixtures and their nebulised aerosols differently in ENDS products. Specifically, two standard base solutions were used for commercially available e-liquids: one consisting of propylene glycol and glycerol (PG.G), and another of propylene glycol, glycerol, and nicotine (PG.G/Nic) [42].

To investigate the effects of ENDS mixtures, a human three-dimensional (3D) organotypic bronchial epithelial culture model was employed, allowing direct exposure to aerosols generated from two test formulations. This model replicates key structural and functional features of the human airway epithelium, providing a relevant platform for assessing respiratory toxicity. Additionally, primary normal human bronchial epithelial (NHBE) cells were utilised to evaluate the cytotoxicity and viability effects of e-liquids at the cellular level. This combined approach enables a more comprehensive understanding of the biological responses associated with ENDS exposure. The EC₅₀ values determined for the PG.G mixture (comprising 78% propylene glycol and glycerol in a 70:30 ratio, combined with 22% phosphate-buffered saline) and the PG.G/Nic mixture (78% propylene glycol and glycerol in a 70:30 ratio, 20% phosphate-buffered saline, and 2% nicotine) were approximately 1.5% and 2.5%, respectively. Interestingly, the PG.G/Nic mixture exhibited a lower EC₅₀ value (2.5 mM) compared to the nicotine-only solution (5 mM), suggesting that the combination of nicotine with the propylene glycol and glycerol matrix may enhance cytotoxic effects. This suggests that the subtle decrease in cell viability observed in cells treated with PG.G/Nic, compared to PG.G, was not primarily attributable to nicotine [43].

The evaluation of e-liquids involves a three-layer testing approach. Different flavour mixtures are tested for toxicity in the initial tier using NHBE cells. The second-tier entails investigating cellular changes associated with the cytotoxicity of e-liquids using HCS assays. When analysing the initial tier results, specific mixtures can be prioritised for further testing. The second tier involves HCS analysis, which measures the proportion of apoptotic and necrotic cells, providing more detailed information about the cellular changes observed in the initial tier. NHBE cells exposed to PG.G/Nic experienced apoptosis, mitochondrial dysfunction, and oxidative stress-related changes. A 3D bronchial epithelial tissue culture model exposed to aerosols from a nebuliser exhibited similar changes in cilia beating power irrespective of exposure duration. Pro-inflammatory mediators were quantified 24 hours after exposure, and increased levels were observed with more prolonged exposure. Compared to

conventional cigarette smoke, the changes in mediator levels induced by PG.G/Nic aerosols were minimal [43].

3.5.1 Aerosol particle Size characterisation

The properties of aerosols are influenced by the device used to generate them. In addition to chemical composition, physical parameters such as aerosol particle size, flow rate, and temperature play a critical role in determining how particles deposit within the test system, ultimately influencing toxicity outcomes. Therefore, characterising the aerosol is a vital component of the assessment process. In this context, the particle size distribution of the aerosol generated from the PG.G/Nic mixture was measured using a Collison nebuliser, as illustrated in Figure 3.4. The properties of aerosols generated by different devices may vary, including droplet size distribution and composition. Our research revealed that nebulised PG.G/Nic displayed a two-peaked pattern with the foremost peak at an aerodynamic diameter of 0.6 μm , followed by a substantial peak for 3 μm droplet size, as shown in Figure 3.4. The standard deviation, marked by error bars from three independent nebulization attempts, was minimal, indicating slight variation between runs. The findings indicate that the nebulised aerosols remained stable and had a consistent particle size distribution throughout the experimental nebulisation phase [43].

3.5.2 Aerosol generation

Aerosols were generated using the 1-jet Collison nebuliser from the mixtures mentioned above. To evaluate the impact of two different mixtures, the MucilAir™ culture system, which is grown on inserts, underwent exposure in a trio of experiments for 90 and 180 minutes. As a benchmark for comparison, an aerosolised solution of cigarette smoke-infused PBS (phosphate-buffered saline),

termed sbPBS, was employed. The sbPBS mixture was prepared by channelling smoke from ten 3R4F cigarettes, procured from the University of Kentucky's Tobacco Research and Development Centre, into a 40 mL PBS solution across a sequence of 110 puffs. Each experimental run included an air control comparison to counterbalance any external variables that might affect the results [42].

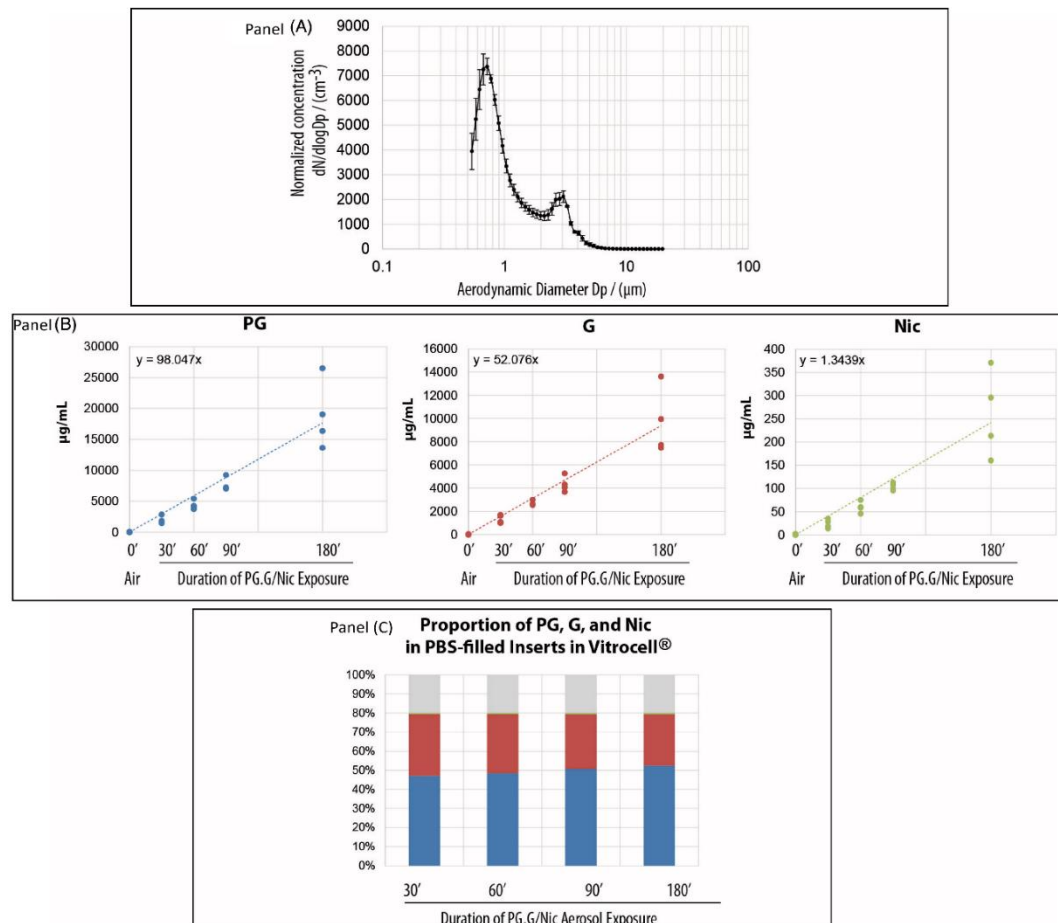


Figure 3.4: Particle size distribution of aerosols and depositions in the Vitrocell base module.

Panel A: shows the droplet size distribution of the aerosol produced using the Collison nebuliser (N = 3).

Panel B: Vitrocell base module displays the concentrations of propylene glycol (PG), glycerol (G), and nicotine (Nic) in the PBS-filled inserts after being exposed to test aerosols for a duration of 30-180 minutes.

Panel C: The lower panel bar graph shows the proportion of PG, G, and Nic in the PBS-filled inserts after exposure for different durations [43].

3.6 Discussion

This study presents a robust in vitro systems toxicology framework for assessing the biological impact of e-liquids, integrating high-content screening, transcriptomics, and computational modelling to provide a mechanistic understanding of cellular responses. The approach enables the detection of subtle perturbations in biological pathways, offering a more nuanced and predictive assessment compared to conventional toxicology methods. The results demonstrate the framework's ability to differentiate between e-liquid formulations based on their toxicological profiles, supporting its relevance for product evaluation and regulatory science. Significantly, the use of human-relevant in vitro models enhances the translational value of the findings; however, further validation through in vivo studies and broader chemical diversity is essential to strengthen their predictive capacity. Overall, this framework represents a significant advancement in the application of systems biology to product safety assessment, contributing to the development of next-generation risk assessment strategies.

3.7 Conclusion

This chapter emphasises the need to develop a new strategy for testing ENDS products. A proposed framework has been suggested to overcome the limitations associated with toxicity testing of e-liquids and their aerosols. This framework places strong emphasis on non-animal testing strategies, utilising biologically pertinent platforms such as human-derived primary cell systems and three-dimensional airway tissue constructs. It incorporates cutting-edge methodologies within a systems-level toxicological framework, enabling a deeper understanding of chemical-induced biological responses. Consistent with the goals of the SEURAT-1 program, which advocates for the replacement of animal models through mechanistic in vitro approaches, this framework broadens its analytical scope by integrating advanced chemical analysis tools. These tools ensure high-resolution chemical profiling, thereby supporting robust and reproducible toxicological interpretations. The primary goal of this system is to advance the development of reliable in vitro assessments of e-liquid toxicity and its vapours. The instances discussed herein confirm the applicability of the blueprint for evaluation purposes relating to e-liquids and their vapours. Additional research was performed after this publication to determine the influence of substances such as propylene glycol, glycerol, nicotine, and flavouring components [43].

3.8 Research Impact

Following the publication of this research article, a substantial number of new studies were conducted in the field. According to Google Scholar, this article has been cited 93 times, highlighting its

considerable influence within the scientific community. By utilising this comprehensive framework for the in vitro evaluation of the biological effects of ENDS products, we tested a commercially available ENDS product, and the results have been published in another research paper [43, 76]. We demonstrated the practical application of this framework, showcasing its effectiveness in assessing the potential impacts of ENDS on biological systems. Our research not only provides insights into the safety and efficacy of these products but also serves as a valuable resource for future studies in this field (58)[77]. However, this thesis does not discuss the second publication, but I am also one of the co-authors in that publication [76]. Several studies also utilised this framework and published their findings, establishing this approach as a new standard for testing ENDS [76, 78, 79].

4.0 A new fluorescence-based method for characterising in vitro aerosol exposure systems

4.1 Introduction and Contribution

This chapter will discuss a research paper titled "**A New Fluorescence-Based Method for Characterizing In Vitro Aerosol Exposure Systems.**" (Steiner S, **Majeed** S, Kratzer G, Hoeng J, Frentzel S. *Toxicol In Vitro*. 2017 Feb;38:150-158. doi: 10.1016/j.tiv.2016.09.018. Epub 2016 Sep 21. PMID: 27664316) [90]. As the second author of this article, I supported the experimental design, conducted the experiments, and prepared the results for publication in collaboration with the first author.

4.2 Background

As discussed in Chapter 3, there is increasing interest in assessing the toxicity of aerosols produced by ENDS and their effects on the lungs and the environment. However, testing aerosol toxicity can be technically challenging, especially when aiming to reduce animal testing. Although in vitro toxicological studies are increasingly utilised, exposing cell cultures to aerosol extracts collected on filters or dissolved in solvents does not accurately replicate the natural interaction between aerosols and the respiratory tract. As outlined in the previous chapter describing the systems toxicology framework, the air-liquid interface (ALI) method is considered the most appropriate approach for studying direct aerosol exposure [42, 81]. The conditioning of test aerosols to meet specific requirements of cell cultures in terms of temperature and humidity, as well as their subsequent dilution, is crucial for ensuring realistic doses and dose responses. It is common practice to manipulate relative humidity, temperature, and concentration in aerosol exposure systems to achieve desired levels. Modifying aerosol generation parameters can affect not just the target characteristics but also other key properties like particle size, total particulate mass, and how semi-volatile compounds distribute between particulate matter (PM) and the gas-vapour phase (GVP). The efficiency with which the gas-vapour phase (GVP), particulate matter (PM), and particles of varying sizes are delivered can significantly influence aerosol administration in biological assays. Relying solely on the original aerosol's physicochemical properties to estimate dosing may lead to inaccuracies, making in vitro study results less representative of real-world exposure. Therefore, precise and targeted aerosol delivery to the relevant regions of the respiratory tract is essential to ensure meaningful and reliable toxicological outcomes [82].

Exposure systems do not always guarantee fully reproducible performance. In instances where multiple cell cultures are exposed simultaneously, there may be inaccurate dosing and non-uniform

aerosol delivery [83]. This is particularly prominent when testing serially diluted aerosols or replicates. Various studies have examined the delivery of different aerosols, such as smoke from traditional cigarettes, in in-vitro exposure systems [84]. However, only a limited number of these studies have focused on liquid aerosols containing low concentrations of volatile and semi-volatile compounds produced by various ENDS [85]. Aerosols exhibit unique behaviours compared to dry powders or the byproducts of burning: they are composed of dynamic, volatile droplets that can rapidly evaporate or change in size, making their delivery and deposition more complex. This necessitates careful consideration of how in-vitro exposure systems administer such aerosols, as their physicochemical instability and sensitivity to environmental conditions can significantly influence experimental outcomes. In particular, factors such as temperature, humidity, and airflow can alter droplet size distribution, volatility, and chemical composition in real time. Aerosols may also undergo processes like coagulation, condensation, or phase separation during transport, further complicating their characterisation. Moreover, their interaction with surfaces—whether in the exposure chamber or within biological systems—can lead to selective deposition or loss of volatile components, skewing dose-response relationships. These dynamic transformations underscore the importance of real-time monitoring and precise control of aerosol generation and delivery parameters to ensure reproducibility and relevance of in-vitro findings. When attempting to directly compare the smoke from conventional cigarettes and the aerosols from ENDS, it is crucial to be cautious and establish comparable doses. It cannot be assumed that using identical or nearly identical protocols for generating and applying smoke and aerosol will result in similar delivery. To confirm the comparability, it is necessary to quantify the aerosol delivery to the exposure chambers or thoroughly characterise the exposure system. Several studies have been conducted that have addressed this matter [86-89].

The characterisation of the exposure system can be approached in two distinct ways, which are not mutually exclusive. One method involves comparing the total aerosol entering the system (or a specific part) with the aerosol exiting it. Another method is to directly measure aerosol deposition on the relevant internal surfaces of the system. The first approach enables real-time monitoring of aerosol levels and deposition changes during biological testing. The method, however, has limited spatial resolution as it fails to determine the precise location of observed changes within the system. Various techniques are currently employed to quantify aerosol deposition in an exposure system. These techniques include particle counting, absorbance/colourimetric measurements, and chromatographic procedures. Although these methods offer a high level of detail, they are time-consuming, expensive, and require extensive analytical equipment, which makes them impractical for many institutions conducting in vitro aerosol exposures. In this study, a novel, straightforward, and robust method was developed to quantify the deposition of aerosols, aiming to overcome the above limitations

[90, 91]. This approach allows for the characterisation of aerosol delivery within an exposure system without the need for complex analytical instrumentation. The study specifically focused on aerosols resembling those produced by standard ENDS devices, as established methods for characterising such aerosols remain limited. To simulate these aerosols, glycerol was selected as the test substance due to its high prevalence in emissions from ENDS and specific medical inhalation devices. Fluorometry was chosen as the detection technique for its simplicity and reliability, with disodium fluorescein (DSF) used as the fluorescent tracer. The sensitivity and dosing precision of this method for liquid aerosols similar to those generated by ENDS were demonstrated using the Vitrocell 24/48 exposure system, described in Chapter 2. This system is widely employed for exposing organotypic cell cultures in toxicological evaluations of both conventional and emerging tobacco products [91] [89].

4.3 Materials and Methods

4.3.1 Aerosol generation

Fluorescent aerosols were created using a TSI 3475 condensation monodisperse aerosol generator (CMAG) (TSI, Shoreview, MN, USA) shown in Fig. 3.2A. The generator functions by transforming a saline solution into an aerosol using an atomization process, followed by drying in a diffusion unit to yield salt particles roughly 100 nanometers in size. These particles, serving as condensation nuclei, are introduced into a saturator chamber containing heated aerosol-forming substances. The combined stream of salt nuclei and vapour is then subjected to further thermal treatment in a re-heating module to ensure complete vaporisation of the aerosol material. Subsequently, condensation occurs in a temperature and flow-controlled chimney, where the vapour deposits onto the salt nuclei. By fine-tuning the thermal conditions and flow rates within the saturator, and selectively filtering out a portion of the salt nuclei, the mass of aerosol material per nucleus can be regulated—allowing precise control over the final particle dimensions. In this study, a 0.5% aqueous solution of DSF (Sigma Aldrich, Munich, Germany) was used as the source of salt nuclei, onto which glycerol (N99.5%, Sigma Aldrich) was condensed as the aerosol material. The specific settings of the CMAG can be found in **Table 4.1** [90].

Table 4.1: Specific settings of the Condensation Monodisperse Aerosol Generator (CMAG)Aerosol generation and characterization ($N = 8$).

Nominal mean aerodynamic particle diameter	0.8 μm	1.1 μm	1.4 μm	1.6 μm
CMAG settings				
Total flow (rotameter scale value ^a)	8	8	8	8
Saturator flow (rotameter scale value ^a)	1.5–2	3.75–4.25	6.75–7.25	7.75–8
Screen flow (rotameter scale value ^a)	1	1	1	3.5–4.5
Saturator temperature ($^{\circ}\text{C}$)	160	160	160	160
Reheated temperature ($^{\circ}\text{C}$)	340	340	340	340
Aerosol characterization				
Aerosol mass flow rate (mg/min)	Average $\pm$ SD (CV) 18.9 $\pm$ 1.8 (9.6)	Average $\pm$ SD (CV) 42.8 $\pm$ 2.1 (5.0)	Average $\pm$ SD (CV) 84.1 $\pm$ 3.9 (4.6)	Average $\pm$ SD (CV) 93.0 $\pm$ 2.9 (3.2)
DSF mass flow rate (mg/min)	0.102 $\pm$ 0.009 (9.1)	0.106 $\pm$ 0.021 (20.2)	0.096 $\pm$ 0.014 (14.6)	0.065 $\pm$ 0.003 (5.1)
Mass ratio DSF/Aerosol	0.0054 $\pm$ 0.0007 (12.5)	0.0025 $\pm$ 0.0005 (20.3)	0.0011 $\pm$ 0.0002 (16.4)	0.0007 $\pm$ 0.0000 (5.3)
Mean aerodynamic particle diameter (μm)	0.83 $\pm$ 0.02 (3.0)	1.13 $\pm$ 0.03 (3.1)	1.41 $\pm$ 0.02 (3.1)	1.62 $\pm$ 0.03 (1.6)
Median (μm)	0.75 $\pm$ 0.02	0.97 $\pm$ 0.03	1.24 $\pm$ 0.03	1.55 $\pm$ 0.04
Mode (μm)	0.72 $\pm$ 0.03	0.93 $\pm$ 0.03	1.24 $\pm$ 0.04	1.66 $\pm$ 0.03
GSD	1.29 $\pm$ 0.02 (1.6)	1.36 $\pm$ 0.02 (1.3)	1.39 $\pm$ 0.02 (1.6)	1.36 $\pm$ 0.02 (1.7)

^a Exact volume flow rates for the indicated scale values are specified by TSI. A value of 1 refers to the according valve being completely closed, a value of 8 corresponds to roughly 3.6 L/min.

4.3.2 Aerosol Characterisation

The CMAG can generate aerosols of particles with varying number-size distributions which were assessed using an Aerodynamic Particle Sizer (APS; model 3321 from TSI Inc., MN, USA) with an Aerosol Diluter accessory (Model 3302 from TSI Inc., MN, USA) used with a capillary to achieve a 1:100 dilution.. Four distributions were chosen and tested for their particle size distribution, aerodynamic particle size, geometric standard deviation, median aerodynamic particle size, and mode (**Table 4.1**). Eight tests were performed, each lasting between 20 to 25 minutes. The APS was configured to average the data over a 10-second interval. The average of 120–150 data points for each experiment was calculated to determine the consistency of aerosol production. Particle concentrations were also recorded, but it is important to note that the data may not accurately reflect the actual concentrations, as the APS was utilised at its highest reliable measurement capacity for particle density. Moreover, the flow rates of disodium fluorescein (DSF) and aerosol mass were primarily indicative of aerosol density. Consequently, particle concentration data obtained from the Aerodynamic Particle Sizer (APS) were not considered suitable for quantitative analysis [90].

To determine aerosol mass deliveries in the exposure system using fluorometric quantification of DSF, both the DSF and aerosol mass flow rates during test exposures must be measured. This was achieved by capturing aerosols on pre-weighed Cambridge filter pads over specific time intervals (typically 1–5 minutes, depending on aerosol particle size). After exposure, the deposited aerosol mass was determined by weighing the pads, and the DSF was extracted from the pads for fluorometric analysis. This method enabled the simultaneous quantification of the total aerosol mass and the delivered DSF, allowing for precise assessment of aerosol delivery rates. The deposited aerosol mass was then determined, and aerosol material trapped on the CFPs was eluted by incubating them in 10 mL Dulbecco's phosphate-buffered saline (PBS) (Sigma-Aldrich) at room temperature in the dark on a horizontal shaker for 30 minutes. To enhance the fluorescent activity of DSF, the pH of the PBS was

adjusted to 9 [90]. The system was calibrated by pipetting standard solutions containing 0.2 to 125 mg of DSF in PBS:glycerol (1:1) onto CFPs, allowing to soak in and eluting as described in section 4.3.3.

This characterisation was conducted using the APS but without the VC24/48 installed downstream of the CFP holder. [93]

4.3.3 DSF retrieval from CFPs and interaction with glycerol

Accurate quantification of aerosol delivery in an exposure system depends on precise measurements of disodium fluorescein (DSF) concentration, aerosol mass flow rates, and the DSF-to-aerosol mass ratio. These factors are essential for delivering consistent and representative dosing in in vitro exposure studies.. This requires effective elution of DSF from the collection filter pads (CFPs), ensuring minimal adsorption and no interference with DSF fluorescence. Since the DSF-to-aerosol mass ratio can vary depending on the aerosol composition, it is essential to verify that DSF fluorescence in phosphate-buffered saline (PBS) remains unaffected by the presence of glycerol. To evaluate DSF recovery from CFPs, a series of solutions containing 0.0002 g to 0.125 g of DSF in a 1:1 mixture of PBS (pH 9) and glycerol was applied to the CFPs and incubated in the dark until fully absorbed. DSF was then eluted using 10 mL of PBS (pH 9) on a horizontal shaker for 30 minutes in the dark, and the recovered amount was quantified and compared to the initial DSF mass. To evaluate if glycerol influences the fluorescence of DSF in PBS, the fluorescence was measured in a dilution series (8×10^{-7} – 0.0002 g/L) in PBS (pH 9) containing 0.02, 0.2, 2, 20, and 200 g/L of glycerol [93].

4.3.4 Test exposures in the VC24/48

The VC24/48 exposures were conducted to:

1. **Verify aerosol stability** under experimental conditions. Ensure that factors such as hygroscopic growth, particle coalescence, or size-selective losses do not alter the aerosol's size distribution.
2. **Assess potential interactions** between cell culture inserts and the deposited substance fraction (DSF), including adsorption effects that may interfere with accurate DSF recovery from PBS during sample collection.
3. **Evaluate the precision and consistency** of aerosol dosing across the exposure system.
4. **Establish the detection limits** of the assay under conditions that reflect the experimental setup, ensuring sensitivity is sufficient for relevant exposure levels.

4.3.5 Experimental settings; CMAG and VC24/48

To gain more knowledge about the VC24/48, please direct your attention to Chapter 2 (Figure 2.2). In summary, aerosol delivery in the Vitrocell 24/48 exposure system is controlled by a dilution module

located above the exposure chambers. This module features 48 chambers organised into eight rows, each with six chambers in replicate positions. Aerosol from the dilution system can be mixed with fresh air before entering each row, resulting in seven distinct dilution levels. The eighth row serves as a negative control, exposed only to clean air. Aerosol flow is maintained under negative pressure and directed into exposure trumpets located above each chamber, creating a stagnant flow over the biological test system. The system operates at a stable temperature of 37 °C, with the dilution air adjusted to maintain a relative humidity of $60 \pm 5\%$. The flow rate through each trumpet is consistently set at 2 mL/min per position. The specific air flow rates for dilution used in various experiments are detailed in the following section [89]. These parameters were consistently used throughout all experiments, as previously described in Chapter 2.

4.3.6 Physical setup CMAG-VC24/48

To emulate realistic exposure conditions for organotypic cell cultures, the aerosol flow rate into the VC24/48 exposure module was configured to 0.41 L/min. This flow rate matches a 55 mL puff delivered over 8 seconds, consistent with the Health Canada smoking protocol [89].

To capture aerosols before they reach the exposure chamber, a Cambridge Filter Pad (CFP) housing was installed in the tubing upstream of the VC24/48, as described in Section 4.3.2 on aerosol characterisation. Positioned 35 cm from the system's inlet, the CFP housing enabled measurement of aerosol and deposited substance fraction (DSF) mass flow rates.

A vacuum pump was placed downstream to maintain a constant unidirectional flow through the VC24/48, compensating for the positive pressure generated by incoming diluted air. Upstream of the pump, a bifurcation with a needle valve was added to regulate airflow. A mass flow meter (0–5 L/min, Aalborg, Orangeburg, NY, USA) was temporarily installed before the VC24/48 to fine-tune the airflow via the needle valve, ensuring a precise flow rate of 0.41 L/min, regardless of whether the CFP was present. After establishing a stable flow, the mass flow meter was taken out, and aerosol trapping or exposure experiments began [90].

4.3.7 Aerosol stability

The two aerosols selected, with an average diameter of 0.8 and 1.1 μm , remained stable in the VC24/48. To confirm this, two of the chosen aerosols were introduced into the complete dilution system of the VC24/48. Particle size distributions were recorded over 2-3 minutes in two separate trials for both upstream and downstream conditions. The VC24/48 system employed a stepwise dilution method, setting cumulative air flow rates for dilution at various increments: 0.1, 0.2, 0.5, 1, 1.5, 2, and 3 L/min. This resulted in aerosol concentration levels of 81, 67, 45, 29, 22, 17, and 12% relative to the output from the CMAG. To measure aerosol characteristics following the VC24/48 system's process,

the APS was used as a pump to create a steady one-way airflow through the device. Flow was maintained using a technique similar to that described previously in section 4.3.2. [90].

4.3.8 DSF recovery from cell culture inserts

To evaluate DSF recovery from cell culture inserts in a 24-well format, transparent membranes with a pore size of 0.4 μm were used. These inserts, made by Greiner Bio-One (Kremsmünster, Austria), contained 100 μL of PBS at pH 9, with DSF concentrations ranging from 10 to 250 mg/L applied in one row of the exposure module. The inserts were then exposed to filtered air for 28 minutes to simulate aerosol conditions similar to those generated by tobacco products. The exposure chambers were designed to isolate the airflow from the dilution system, thereby eliminating the need for a predefined dilution protocol. In the first row, the dilution-air flow was set at 0.5 L/min, with subsequent rows not requiring additional input. After the exposure, PBS samples were collected, and the concentration of DSF in the sample was measured (section 4.3.10) and compared to the applied initial concentration [90].

4.3.9 Aerosol dosing and sensitivity limits

Cell culture inserts pre-filled with 100 μL of PBS (pH 9) were utilised to evaluate the efficacy of aerosol delivery. These inserts were strategically positioned within the exposure module to determine the dose-response relationship and establish sensitivity thresholds. The PBS serves as a stand-in for actual cell cultures, providing a wet surface similar to the mucosal layer found in airway epithelial cell cultures, which are typically grown in organotypic cultures. To confirm the linear increase in aerosol delivery with longer exposure times, wells located at rows 3 and 5, spots 1-6, were exposed to aerosols for 3, 6, 9, 12, 15, 18, and 21 minutes. The aerosol exhibited an average aerodynamic particle diameter of 0.8 μm . A dilution air volume flow rate of 1.5 L/min was introduced at the inlet of the first row of the VC24/48 dilution system, resulting in an aerosol concentration of 22%. Aerosols were captured on CFPs to quantify the aerosol mass delivery, allowing assessment of reproducibility. Before the exposures of 3, 15, 18, and 21 minutes, the aerosol and DSF mass flow rates and aerosol size were measured. In the VC24/48, the dosing of organotypic cell cultures is typically achieved by varying the applied aerosol concentration rather than altering the exposure duration. Hence, our primary focus lay on dilution-dependent rather than time-dependent dosing. Accordingly, time-dependent dosing was measured in one repetition and placed more emphasis on dilution-dependent dosing. For dilution-dependent dosing, 0.8 μm and 1.6 μm aerosols were used, with cumulative dilution-air volume flow rates in rows 1-7 set at 0.1, 0.2, 0.5, 1, 1.5, 2, and 3 L/min (81, 67, 45, 29, 22, 17, and 12% aerosols compared to the CMAG output), all exposed for 28 minutes. To determine aerosol and DSF mass flow rates and confirm particle size, the aerosol was trapped on CFPs before, during, and after the exposures. The process involved stopping the exposures at 14 minutes to measure mean aerodynamic

particle size and geometric standard deviation (GSD), then trapping aerosol samples on CFPs before restarting the exposures for another 14 minutes. This experiment was repeated four times. After each session, the loaded CFPs were washed with 10 mL of PBS (pH 9) at room temperature for 30 minutes in the dark on a horizontal shaker. PBS samples retrieved from each cell culture insert were kept at 4 °C in the dark until they were processed for subsequent analysis [90].

4.3.10 Fluorescence measurements

80 µL aliquots of samples were transferred into individual wells of black 96-well microplates with clear bottoms. Fluorescence readings were converted to DSF concentrations using standard curves generated on each plate with PBS at pH 9. The linear range of the calibration curve reliably covered concentrations from 0.2 to 10.4 mg/L. Sample dilutions were empirically determined as needed. Fluorometric measurements were performed using the BMG Labtech FLUOstar Omega Microplate Reader, with excitation at 485 nm and emission at 520 nm. Top optics reading and gain adjustment were applied in the well with the highest standard, and fast reading mode was activated. When measuring DSF eluted from CFPs, background correction involved incubating an unloaded CFP in PBS at pH 9 for 30 minutes on a horizontal shaker, which served as a blank. For samples from VC24/48, blank correction was based on the fluorescence of pure PBS at pH 9 [90].

4.3.11 Data processing

The primary measurement for all samples was the concentration of DSF collected in PBS after exposure to aerosol. These concentrations were translated into DSF masses according to the known sample volumes. To determine the DSF mass flow rates, were calculated from the duration of aerosol trapping on CFPs using Equation 1.

Equation 1:

$$DSF \text{ mass flow rate} = \frac{\text{measured DSF concentration} \times \text{eluate volume}}{\text{trapping time.}} \quad (1)$$

The mass flow rate of aerosol, determined by weighing the trapped aerosol, was used to calculate the DSF content of aerosols using Equation 2.

Equation 2:

$$\text{Mass ratio} \frac{DSF}{\text{aerosol}} = \frac{DSF \text{ mass flow rate}}{\text{aerosol mass flow rate}} \quad (2)$$

Using Eq. 3, absolute aerosol mass deliveries were calculated based on the DSF masses delivered to a specific position within the VC24/48.

Equation 3:

$$\text{Absolute aerosol mass delivery} = \frac{\text{DSF mass detected at position XY}}{\text{Mass ratio} \frac{\text{DSF}}{\text{Aerosol}}} \quad (3)$$

4.3.12 Statistical analysis

Linearity assessments were conducted using the least squares method, with the y-intercept constrained to zero. The goodness-of-fit was systematically quantified through the coefficients of determination (R²). The coefficients of variation (CV) were employed to evaluate aerosol generation, considering independent repetitions of aerosol generation [89].

4.4 Results

4.4.1 Aerosol generation and characterisation

Experimental testing was performed to evaluate different CMAG settings, leading to the identification of several aerosol generation configurations that could be reliably produced and sustained over time. Four aerosols, with mean aerodynamic particle sizes of 0.8, 1.1, 1.4, and 1.6 µm, were chosen for additional characterisation and testing VC24/48. Figure 4.1 shows the particle number-size distributions of the selected aerosols. Details about the CMAG settings and measured aerosol parameters are provided in **Table 4.1**. As illustrated in Figure 4.2 and summarised in **Table 4.1**, the aerosol number-size distributions exhibit high reproducibility within single experiments and across multiple runs. Nonetheless, achieving monodispersity was not possible, as indicated by GSDs between 1.3 and 1.4 and overlapping differences among the distributions of different aerosols (Figure 4.1). This overlap reduces the ability to detect effects related to specific particle sizes, such as size-specific aerosol delivery during exposure systems. Nevertheless, for substantial differences in mean aerodynamic particle size (approximately 500 nm and above), distinctions in the delivery efficiency between the 0.8 µm and 1.6 µm aerosols were detectable. Overall, the reproducibility of the aerosol size parameters (shown in **Table 4.1**) was greater than that of the aerosol and DSF mass flow rates, and as a result, the DSF/aerosol mass ratios [89].

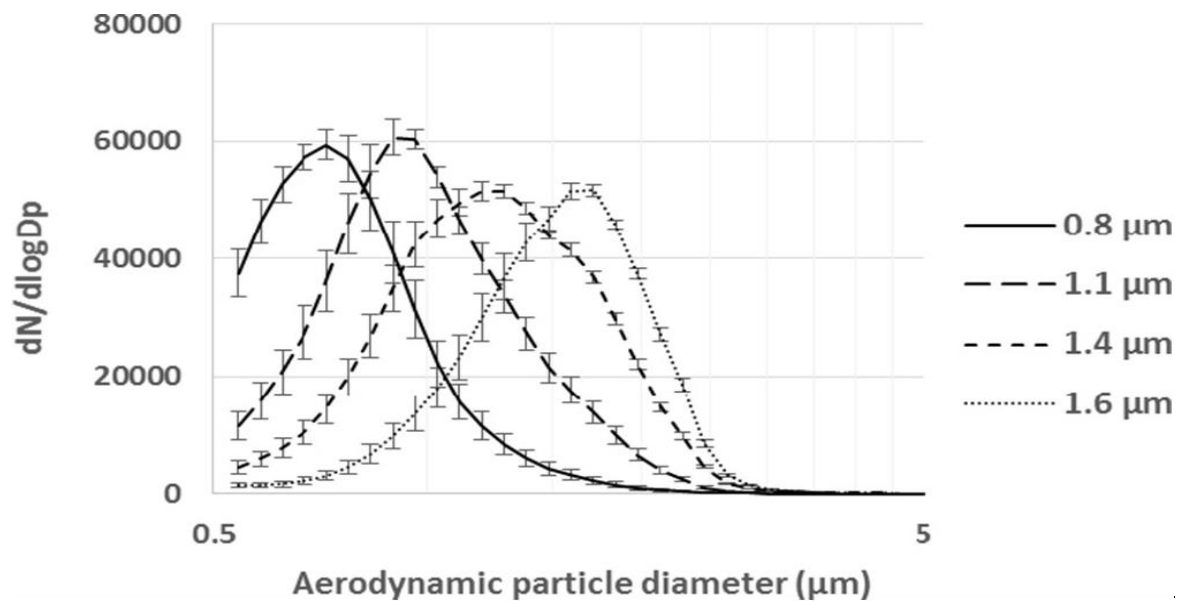


Figure 4.1 Aerodynamic particle diameter measurement: The particle number-size distributions of the aerosols generated in the CMAG were measured. Standard deviations are represented by error bars.

As anticipated, increasing the mean particle size significantly elevated the aerosol mass flow rate. In contrast, the DSF mass flow rate remained relatively consistent for aerosols of 0.8, 1.1, and 1.4 μm, with a slight decrease observed for the 1.6 μm aerosol, owing to the removal of DSF nuclei in the CMAG. As a result, the DSF-to-aerosol mass ratio significantly dropped as the mean particle size increased [89].

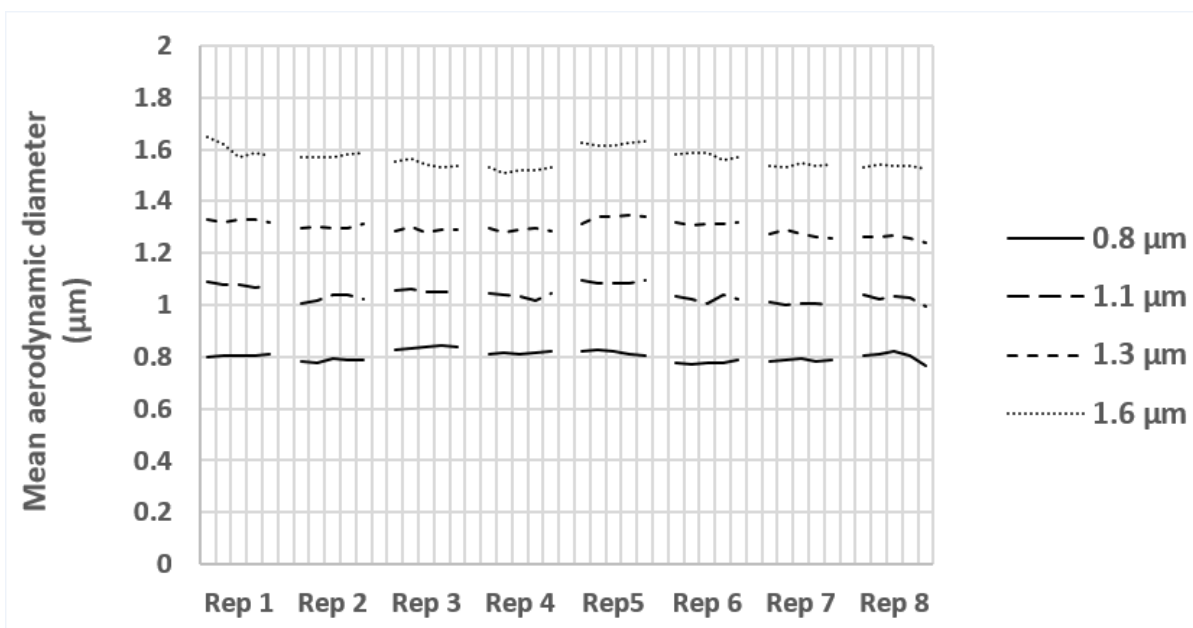


Figure 4.2 Mean aerosol size measurement: Across eight independent aerosol generation runs, the average aerodynamic particle sizes were consistently tracked using an aerodynamic particle sizer

(APS). Selected data segments, including five APS readings averaged over ten seconds each, are shown to demonstrate the long-term stability of aerosol sizes and their consistency across repeated measurements [89].

4.4.2 DSF retrieval from CFPs and interaction with glycerol

The fluorescence yield of CFPs loaded with aerosol material in PBS was higher than expected based on the applied DSF masses. To resolve this, blank corrections were applied to the CFPs, which were likely responsible for the excess fluorescence. All measurements included unloaded CFPs alongside the loaded samples. Figure 4.3a shows the recovery of DSF from CFPs after blank corrections. The average ratio of measured to expected fluorescence yield was 1.069 ± 0.009 (Figure 4.3A), indicating that DSF was effectively recovered from Cambridge filters across a range of concentrations. However, at a high glycerol level of 200 g/L, an impact on DSF fluorescence in PBS was observed, but only for DSF concentrations below 1 $\mu\text{g/L}$ (Figure 4.3B). This effect was not observed at 20 g/L glycerol. Delivering only 1 μg of DSF with 20 g of aerosol per litre of PBS was not feasible under relevant conditions, considering the aerosol mass deliveries in VC24/48 and the DSF/aerosol mass ratios listed in Table 4.1. Therefore, the impact of glycerol was considered negligible [89].

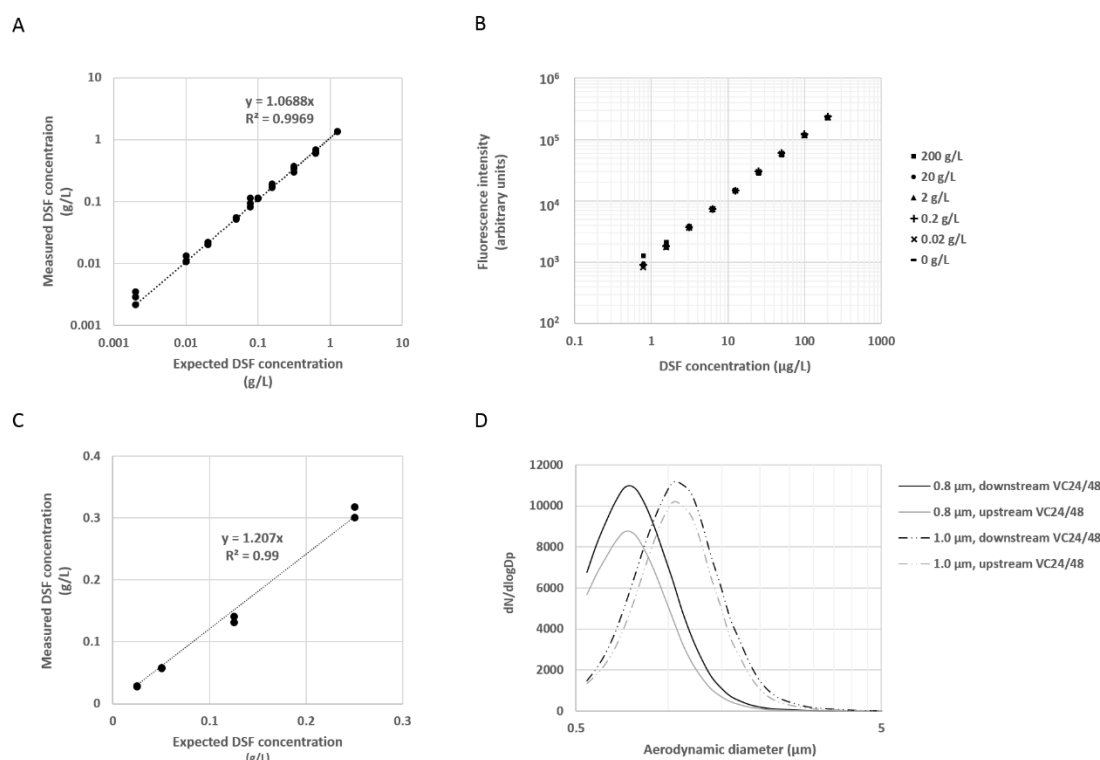


Figure 4.3 Control experiments to assess retrieval of disodium fluorescein from aerosols and stability of aerosol after passage through the VC24/48

A) DSF retrieval from CFPs was measured independently three times for each concentration (1.25 g/L and 0.05 g/L). The dotted line represents the linear fit of the data. The slope and R^2 values are indicated in the plot, demonstrating a quantitative DSF retrieval.

B) The effect of glycerol on DSF fluorescence was examined by measuring its fluorescence at various concentrations and glycerol levels ranging from 0 to 200 g/L. All six runs coincided except at the lowest DSF concentration, where a noticeable effect was observed only at the highest glycerol level concentration.

C) Extraction of DSF from cell culture inserts was performed after exposure to a filtered, humidified airstream using the Vitrocell® 24/48 aerosol exposure system. For each DSF concentration tested, three samples were simultaneously exposed in one experiment. The data were analysed using linear regression, as indicated by the dashed line on the graph, with the slope and R^2 value shown in the figure. The fluorescence readings were slightly higher than expected, likely due to water evaporation from the PBS sample inserts.

D) The particle size distributions for the two types of aerosols were recorded before and after passing through the VC24/48 using an aerodynamic particle sizer (APS). No alteration in the size of the aerosols was observed [89].

4.4.3 DSF recovery from cell culture inserts

When cell culture inserts were exposed to fresh air in the VC24/48, a strong linear relationship was observed between the expected and actual fluorescence across all DSF concentrations (10-250 mg/L). The measured fluorescence was 1.069 times higher than expected, as shown in Figure 4.3c. Linear regression analysis gave a slope of 1.207 ± 0.026 , indicating that the average measured-to-expected fluorescence ratio covers all data points. The VC24/48 and cell culture inserts were removed as sources of excess fluorescence since no fluorescent signal was observed when exposed to pure PBS. The minor rise in DSF concentrations in PBS samples was anticipated due to water evaporation, not DSF itself, occurring during 28 minutes of exposure to 60% relative humidity at 37 °C [90].

4.4.4. Aerosol stability in the VC24/48

Measurements from the APS device, both upstream and downstream of the VC24/48, showed no significant changes in particle size distributions (see Figure 4.3D). However, there was a greater concentration of particles downstream than upstream. This disparity can be attributed to the varying physical setup employed for aerosol sampling in both directions. Furthermore, the APS operated at its maximum aerosol concentration level (around 1,000 particles/cm³), which might have resulted in a slight underestimation of particle concentrations. However, it did not affect the size distribution data.

Consequently, the elevated particle concentration downstream is an artefact of the experimental settings employed [90].

4.4.5 Aerosol dosing in the VC24/48

Reducing both the exposure duration and aerosol concentration led to a decline in aerosol mass delivery to the PBS samples exposed (see Figure 4.4). The findings indicate that exposing the samples for 21 minutes resulted in aerosol delivery of 8.6 μg per cell culture insert, whereas exposure for only 3 minutes resulted in 1.4 μg per insert. These measurements were taken from pooled PBS samples gathered from 12 inserts located in rows 3 and 5. The linear regression analysis showed an R^2 value of 0.95. After 28 minutes of exposure to 82% aerosol, the delivery of 0.8 μm and 1.6 μm aerosols produced $97.0 \pm 39.0 \mu\text{g}$ and $386.0 \pm 76.0 \mu\text{g}$ per cell culture insert respectively. Conversely, exposure to 12% aerosol resulted in $15.4 \pm 3.7 \mu\text{g}$ and $57.8 \pm 10.7 \mu\text{g}$ per insert (Figure 4.5). These values were obtained by averaging six measurements per row over three repetitions. The relationship between aerosol concentration and delivery exhibited a linear trend, with R^2 values of 0.97 (0.8 μm aerosol) and 0.96 (1.6 μm aerosol) [89].

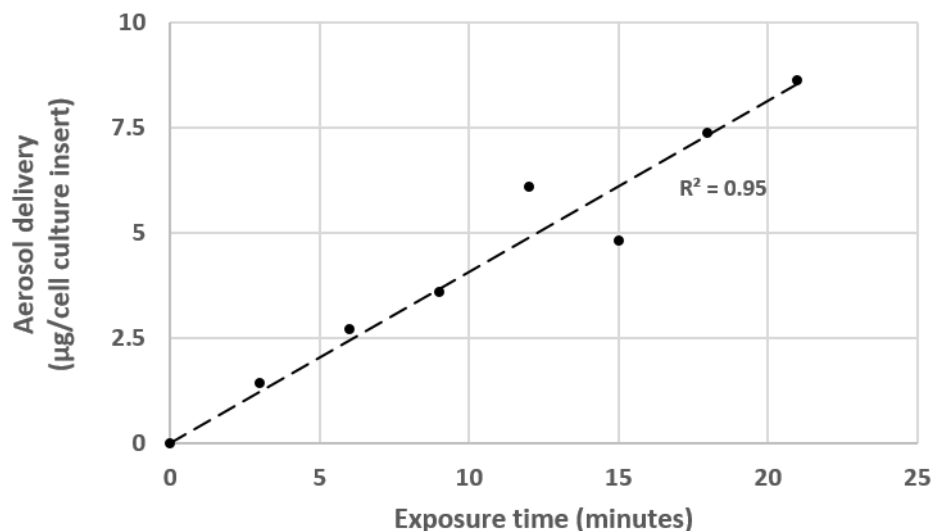


Figure 4.4 Aerosol mass delivery to inserts in cell culture is linear with respect to exposure time: In the observed experiment, only aerosols with an average aerodynamic size of 0.8 micrometres were examined, using a dilution flow rate of 1.5 litres per minute, constituting 22% of the aerosol mixture. This investigation was conducted through one experimental cycle. The plotted data reflect the mean mass of aerosol received at various points across two dilution series. The collected samples of PBS from twelve identical locations were combined for analysis. The dashed interrupted line on the chart depicts the linear regression applied to the dataset [90].

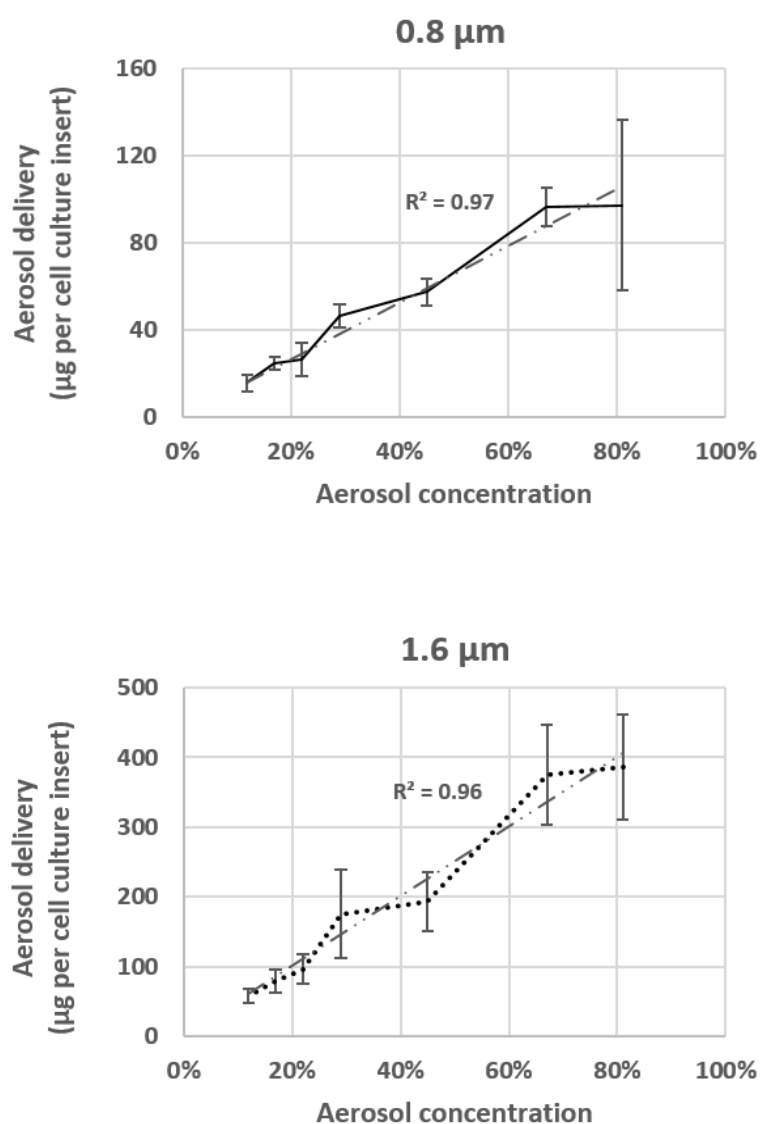


Figure 4.5 Aerosol mass delivery changes with aerosol concentration: Two aerosols, with 0.8 µm and 1.6 µm mean aerodynamic diameter, were used to expose phosphate-buffered saline samples in cell culture inserts within the Vitrocell® 24/48 aerosol exposure system. To achieve serial aerosol dilution, cumulative dilution-air flow rates of 0.1, 0.2, 0.5, 1, 1.5, 2, and 3 L/min in dilution rows 1–7 were used. These flow rates represent 81%, 67%, 45%, 29%, 22%, 17%, and 12% of the aerosol output relative to the condensation monodisperse aerosol generator. Error bars show standard deviations, based on a sample size of N = 4. The dashed lines on the graph indicate the linear fit to the data, with R2 values shown [89].

4.4.5 Assay sensitivity, detection limits

The DSF standard curves span a concentration range from 0.1 µg/L to 200 µg/L, establishing the lower limits of quantification (LLOQ) and upper limit of quantification (ULOQ), respectively. At the lowest tested concentration of 0.1 µg/L, raw fluorescence intensity fluctuate between 480 and 580, while control readings using PBS blanks range from 270 to 360. The instrumental noise associated with the microplates or the fluorometer is estimated to be approximately 50 units. As expected, the lowest aerosol mass delivery occurred during a 3-minute exposure to an aerosol concentration of 22%, with an average aerodynamic diameter of 0.8 µm. This resulted in a delivery of 1.4 µg per position. In this experiment, the DSF-to-aerosol mass ratio was calculated to be 0.0053. The DSF concentration in the 100 µL PBS sample measured 0.075 µg/L, which is below the lower limit of quantification (LLOQ), as defined by Equation 3. Despite this, DSF concentrations under other test conditions remained within the linear range of the standard curve. For example, a 6-minute exposure under the same aerosol concentration and particle size produced a DSF concentration of 0.143 µg/L. Conversely, a 28-minute exposure to a 12% aerosol with the lowest DSF content and a mean aerodynamic diameter of 1.6 µm yielded an aerosol mass delivery of 57.8 ± 10.7 µg per position and a DSF concentration of 400 µg/L—about 4000 times higher than the LLOQ [90].

4.5 Discussion

A range of methodologies has been developed over time to investigate aerosol deposition dynamics within exposure systems. These techniques are valuable for characterising exposure systems and in vitro dosimetry. The introduced assay, while resembling existing fluorescence-based detection methods, offers novel possibilities by combining fluorometric aerosol detection with liquid, water-soluble aerosols that can be generated in a controlled, particle-size-specific manner. Historically, the analysis of particle size-dependent aerosol deposition across a wide range of sizes has primarily focused on solid particles, which do not fully represent liquid aerosols. Furthermore, the ability to quickly produce traceable aerosols with adjustable particle size distribution permits an efficient and unbiased study of various aerosols of interest and their deposition dynamics.

DSF and glycerol are highly water-soluble, facilitating precise measurement of deposited aerosols from internal system components using aqueous solvents. This enhances the accuracy in assessing aerosol losses within an exposure system and minimises the risk of system memory effects that could affect biological responses in later experiments. Essential criteria for characterising an aerosol exposure system include: 1) its robustness and sensitivity in accurately quantifying the delivered model aerosol; 2) its reliability in producing and delivering the model aerosol; and 3) its ability to accurately represent the model aerosol in real-world experiments. Meeting these requirements allows

for the simulation and measurement of possible biases caused by the exposure system. It also helps identify the best operating conditions for the system that reduce biases in the exposures [90].

4.6 Conclusion

The method developed for assessing aerosol exposure mechanisms enables precise analysis of particle behaviour based on size. Its functionality, reliability, and accuracy have been validated under realistic exposure conditions using the Vitrocell® 24/48 aerosol exposure system. Despite the simplicity of the model aerosols used, their consistency allowed for a thorough and reproducible evaluation of the system. . This method enables the investigation of the fundamental mechanisms behind aerosol exposure systems without being affected by the dynamic changes in aerosols. Developed initially to characterise exposure systems for toxicological studies of e-cigarettes, its use is not restricted to that area. Therefore, it can be applied in other fields beyond e-cigarette research. The technique used in this study effectively measures liquid aerosols containing glycerol, similar to those produced by electronic tobacco devices and inhalation therapy medical devices. The fluorometric detection of aerosol deposition offers a fast, cost-effective, and dependable analytical method accessible to many researchers and institutions. Therefore, this method serves as a valuable tool for multiple applications, including validating particle dynamics in computer simulations, characterising in vitro aerosol exposure systems, and gaining a comprehensive understanding of aerosol delivery in complex test arrangements [90].

4.6 Research Impact

Following the publication of this research article, numerous follow-up studies have been conducted in the field, with the article receiving 17 citations on Google Scholar, demonstrating its notable impact within the scientific community. The practical utility of our characterisation methodology is exemplified by its adoption in diverse research applications beyond its original scope. Notably, Allison et al. employed our fluorescence-based characterisation method in their research on dental and oral health [93], where they assessed aerosol and splatter generation during dental procedures and investigated the potential transmission of pathogens, such as SARS-CoV-2 [93]. This application demonstrates the versatility and broader relevance of our characterisation approach, showing how methodologies developed for tobacco product testing can be successfully adapted to address contemporary public health challenges, including pandemic-related aerosol transmission studies.

5.0 Characterization of the Vitrocell® 24/48 aerosol exposure system for its use in exposures to liquid aerosols.

5.1 Introduction and Contribution

In this chapter, I will discuss my research paper, "**Characterization of the Vitrocell® 24/48 aerosol exposure system for its use in exposures to liquid aerosols**" (Steiner S, **Majeed S**, Kratzer G, Vuillaume G, Hoeng J, Frentzel S. *Toxicol In Vitro*. 2017 Aug;42:263-272. doi: 10.1016/j.tiv.2017.04.021. Epub 2017 Apr 27. PMID: 28457873) (77). As the second author of this article, I contributed to the design of the experiments, conducted the experiments, and supported compiling the results for publication.

5.2 Background

As mentioned in Chapter 4, a new fluorescence-based method has been developed for characterising the exposure system for liquid aerosols. This chapter will focus on the implementation of this new method to learn about the exposure systems that may be used to test and study the toxic effects of different types of liquid aerosols [94].

Relying only on aerosol mass flow rates is not sufficient to predict how aerosols will deposit in biological exposure setups. Other factors—such as particle count, size distribution, total particle mass, the presence of semi-volatiles, and chemical composition changes—all influence the outcome. That's why it's essential to thoroughly evaluate the exposure system itself. This includes checking how well it performs overall, how consistent it is across repeated exposures, how precise the dosing is, and whether it can deliver accurate and representative doses to the right regions of the respiratory tract.. Additionally, it is crucial to consider the specific dynamics of different aerosols during transport, dilution, and deposition. This in-depth analysis will confirm the system's appropriateness for the intended aerosol, which is particularly pertinent for toxicology evaluations involving electronic cigarettes. Despite the significant differences between the aerosol outputs of e-cigarettes and conventional cigarettes, research can still employ tobacco smoke as a benchmark aerosol within an exposure framework [94].

In conducting in vitro studies that subject organotypic cell cultures to aerosolised substances from tobacco products, our laboratory employs the sophisticated Vitrocell® 24/48 system for aerosol exposure (VC24/48, Vitrocell® Systems GmbH, Waldkirch, Germany). The VC24/48 and its compact version, the VC24, rank as top-tier aerosol exposure equipment in the market. These innovative systems facilitate concurrent exposure to various aerosol concentrations, offering seven levels of dilution along with a negative control within the VC24/48 setup. While these characteristics render the system ideal for high-throughput operations under controlled conditions, the inherent potential

exists for creating artefacts and biases specific to the system during aerosol distribution. Several potential concerns should be taken into account, including the loss of aerosols due to contact with internal system components, which could potentially result in reduced aerosol output than anticipated. If the aerosol does not thoroughly mix with the dilution air, it could lead to erratic or non-uniform dispensing to replicate locations or throughout entire dilution rows. Additionally, if internal sampling of aerosols does not accurately represent reality, it could skew the distribution of various particle sizes or the proportionate delivery of particles and gas-vapour particulates [95].

The performance of Vitrocell systems for exposures to combustion-generated aerosols has been demonstrated through several studies, as discussed in Chapter 2. However, it is not yet clear whether they are suitable for exposure to liquid aerosols. In the previous chapter, the development of a new fluorescence-based method provided the opportunity to conduct this study to characterise the liquid aerosol system and e-cigarette applications. To eliminate any biases arising from product variability and condition, a method was employed that uses labelled model aerosols designed explicitly for simulating exposures to low GVP content liquid aerosols. The research examined various factors, including the precision of dose administration, accuracy in dosing, consistency in aerosol dispersion at different simulator locations, and the dependability of system performance across multiple trials. The study also explored the possible impact of internal aerosol sampling on the quantity of aerosol conveyed to the test chambers and investigated the reliability of the integrated Quartz Crystal Microbalances (QCMs) for real-time assessment of aerosol mass distribution, as already described in Chapter 2 [95].

5.3 Materials and Methods The use of a condensation monodisperse aerosol generator (CMAG; TSI Model 3475) to produce the model aerosols has been described in section 4.3.1. The process began with preparing dry salt crystals approximately 100 nm in diameter. Subsequently, these particles were directed through a heated saturating agent, generating a mixture of vapour and nuclei. Upon entering the condensation chimney, this combination was cooled down, leading to the vapour's condensation on the salt nuclei induced by supersaturation. The resulting material's quantity on each salt nucleus and the overall particle size distribution was controlled by fine-tuning several parameters, including the temperature in the saturator, the flow rate of the volume passing through it, and the selective removal of some nuclei using filtration [95].

The method for creating fluorescently labelled water-soluble aerosols with adjustable mean particle sizes and low geometric standard deviations is described in Chapter 4 (section 4.3.1) Disodium fluorescein (DSF) from Sigma-Aldrich, Munich, Germany, was used as the salt nuclei and glycerol from Sigma-Aldrich as the aerosol material. DSF was chosen because it is easily measurable using

fluorometric analysis within the exposure system. **Table 5.1** provides details describing the specific settings and parameters used in the CMAG and the generated aerosols [94].

5.3.1 Characterisation of the Aerosol

Aerosol characteristics measured included average sizes and corresponding geometric standard deviations (GSDs) of the aerodynamic particles and the mass flow rates. A TSI 3321 aerodynamic particle sizer (TSI Inc, Shoreview, MN, USA) was used for sizing aerodynamic particles positioned after the CMAG in our setup. To attain the necessary level of aerosol dilution a TSI 3302A Aerosol Diluter equipped with a 100:1 capillary tube was used. It is important to note that the average size of the aerodynamic particles for one aerosol sample was smaller than the minimum detection threshold, 0.5 μm , of the particle sizing instrument. The size and stability of particles were also measured using a scanning mobility particle sizer (SMPS; model 3938 from TSI inc., MN, USA), which complemented the data obtained from the aerodynamic particle sizer. The SMPS indicated that the mean particle size was 0.16 μm . However, due to the inherent variations between particle sizing instruments, the measurement cannot be directly compared to other measurements. Thus, it can be presumed that the aerosol size is less than 0.5 μm . The aerosol mass flow rate was determined using the methodology described in Section 4.3.2. The flow rates of DSF mass were quantified using a fluorometric procedure, described in section 4.3.10, where the aerosol retained on filters was extracted into 10 mL of Dulbecco's phosphate-buffered saline, obtained from Sigma–Aldrich, and then shaken horizontally in the dark for 30 minutes. PBS's pH was regulated to 9 for every measurement to amplify DSF's fluorescent response [95].

5.3.2 Vitrocell® 24/48 *in vitro* aerosol

Figure 2.2 in Chapter 2 illustrates the VC 24/48 system, and the experiment setup was previously described in Section 2.3. In summary, the test aerosol is transferred through the VC 24/48 via the dilution apparatus situated on top of the exposure unit. This unit comprises 48 distinct exposure compartments, methodically organised into eight rows with six duplicate slots each. Before reaching each row, the diluting mechanism blends aerosol with ambient air, enabling the simultaneous assessment of seven different dilution levels alongside a separate row designated solely for control exposures involving pure air. Attached to the end of each row, a QCM chamber measured the accumulated mass of aerosol specific to that row continuously. Throughout exposure, a negative pressure was applied to the aerosol, and then drawn into the exposure funnels that lead into both the exposure chamber and the QCM chamber. This setup ensures a non-turbulent flow condition over both the cell culture inserts subjected to the aerosol and the QCM crystals, allowing for accurate data acquisition [95].

5.3.3 Aerosol delivery to the exposure system

ThinCert™ cell culture inserts with transparent membranes featuring 0.4-µm pores were arranged in forty-two wells within the VC24/48 exposure module. The last row, row eight, was left empty to exclude an air-only control. To facilitate an aqueous interface for the cell cultures, each insert received an addition of 100 µL phosphate-buffered saline (PBS) with pH adjusted to 9. The inserts were exposed to model aerosols labelled with DSF. The exposure conditions and settings of the VC24/48 were identical to those used for organotypic cell cultures, as described in Chapter 2 – section 2.4.3. The PBS samples were exposed to an aerosol volume flow rate of 0.41 L/min for 28 minutes, which is equivalent to a 55 mL puff from a tested tobacco product smoked following the Health Canada regimen. The aerosol release lasted for eight seconds. Throughout the testing, the volume flow rate through the trumpets stayed steady at 2 mL/min setups. The entire apparatus was regulated to 37°C to ensure a stable environment for the experiments. Before the air entered the stream dilution system, it was preheated to 37°C, with a steady relative humidity of $60 \pm 5\%$. The aerosol characterisation process was conducted beforehand, at the midpoint, and after each exposure, following the methods previously outlined in section 4.3.3. . The experiments were briefly paused after 14 minutes to measure particle sizes. CFP is connected to collect the aerosol samples, and the process is described in section 4.3.10. Subsequently, the exposure experiments were resumed for an additional period of 14 minutes [94].

Two modes were used to conduct the test exposures. The first mode was the serial dilution mode, which was used to assess dosing accuracy. The aerosols were diluted serially with cumulative dilution airflow rates ranging from 0.1 to 3.0 L/min. This resulted in aerosol concentrations of 81%, 67%, 45%, 29%, 22%, 17%, and 12% compared to the CMAG output, respectively [94].

The second approach was the single dilution method, employed to assess whether the VC24/48 had any unforeseen impacts on aerosol transmission, such as alterations due to the distance it travelled within the apparatus or potential depletions of the aerosol. At the point of aerosol entry into the VC24/48, before reaching the initial tier, a dilution to a 22% concentration of aerosol (with an airflow rate for dilution of 1.5 L/min) was performed [94].

The study employed two distinct modes of exposure: serial dilution and single dilution exposure. For the serial dilution approach, aerosols were characterised by mean aerodynamic particle diameters at various sizes: less than 0.5, 0.8, 1.1, 1.4, and 1.6 µm. This portion of the experiment was replicated four times independently, as detailed in **Table 5.1**. Conversely, the single dilution exposure approach utilised aerosols with a consistent mean aerodynamic particle diameter of 0.8 µm [25] [94].

Table 5.1 presents details on aerosol generation and characterisation: Aerosols are generated using the condensation monodisperse aerosol generator (CMAG), which utilises disodium fluorescein (DSF).

Nominal mean aerodynamic particle diameter				< 0.5 μm			0.8 μm			1.1 μm			1.4 μm			1.6 μm		
CMAG settings																		
Total flow (rotameter scale value ^a)				8			8			8			8			8		
Saturator flow (rotameter scale value ^a)				1			1.5–2			3.75–4.25			6.75–7.25			7.75–8		
Screen flow (rotameter scale value ^a)				1			1			1			1			3.5–4.5		
Saturator temperature (°C)				160			160			160			160			160		
Reheater temperature (°C)				340			340			340			340			340		
Aerosol characterization (n = 12)		Average	± SD	(CV)	Average	± SD	(CV)	Average	± SD	(CV)	Average	± SD	(CV)	Average	± SD	(CV)		
Aerosol mass flow rate (mg/min)		0.028	± 0.009	(32)	1.886	± 0.182	(9.6)	4.283	± 0.214	(5.0)	8.41	± 0.39	(4.6)	9.303	± 0.294	(3.2)		
DSF mass flow rate (μg/ min)		9.8	± 1.8	(17)	10.2	± 0.9	(9.3)	10.6	± 2.1	(20)	9.6	± 1.4	(14)	6.5	± 0.3	(5.2)		
Mass ratio DSF/Aerosol		0.38	± 0.13	(34)	0.005	± 0.001	(13)	0.0025	± 5E – 04	(20)	0.001	± 2E – 04	(16)	0.0007	± 0.00004	(5.4)		
Mean aerodynamic particle diameter (μm)		0.16 ^b	± 0.002	(1.1)	0.83	± 0.02	(3.0)	1.13	± 0.03	(3.0)	1.41	± 0.02	(1.3)	1.62	± 0.03	(1.6)		
GSD		1.78 ^b	± 0.01	(0.5)	1.29	± 0.02	(1.6)	1.36	± 0.02	(1.3)	1.39	± 0.02	(1.6)	1.36	± 0.02	(1.7)		

(a) TSI defines the exact volume flow rates corresponding to the scale settings. A setting of 1 indicates a fully closed valve, while a setting of 8 corresponds to an approximate flow rate of 3.6 L/min.

(b) The aerosol size distribution, with a mean aerodynamic particle diameter below 0.5 μm , was determined using a Scanning Mobility Particle Sizer (SMPS).

5.3.4 QCM accuracy

In assessing the precision of mass accumulation measurements via Quartz Crystal Microbalance (QCM), pristine QCM crystals were used in each microbalance. Concurrently, cell culture inserts filled with 100 μL of PBS (pH 9) were positioned in the matching exposure units of the exposure system. These QCM crystals, along with the PBS solutions, underwent aerosol exposure consisting of particles with a mean aerodynamic diameter of 0.8 μm , using a single dilution (22% concentration of aerosol, with an airflow rate for dilution of 1.5 L/min) setting for a duration of 21 minutes. Throughout this period, the mass increment on the QCM surfaces was monitored in real-time. The entire procedure was conducted four separate times, with each cycle preceded by a thorough aerosol characterisation [94].

After exposure, PBS solutions were collected from the cell culture inserts, and the quartz crystal microbalance (QCM) crystals were carefully removed from their chambers. The aerosol particles that had settled on the QCM crystal surfaces were then gently cleaned by immersing them in 1 mL of PBS (pH 9), allowing them to incubate for 10 minutes in the dark. Following this step, the amount of DSF in the combined PBS solutions was quantified using a fluorometric method described in section 4.3.10. To verify the accuracy of the QCM measurements, the aerosol mass per area reported electronically by the QCM devices was compared with the mass determined via fluorometric analysis [94].

5.3.5 Sample Collection and Measurement

Following exposure, culture inserts were employed to collect PBS samples. These samples, as well as those acquired from CFPs and QCM crystals, were stored at 4 °C, away from light, pending additional analysis. Fluorescence assessments were conducted using a Fluostar Omega Plate Reader, utilising an excitation wavelength of 485 nm and an emission wavelength of 520 nm, consistent with the methods previously outlined in Chapter 4.

APS measurements were used to assess consistency across different aerosol preparations and to ensure stability within each exposure run, eliminating the need for additional analysis. The primary data point collected from both the PBS samples treated with aerosols and the QCM crystals was the intensity of fluorescence observed. This fluorescence measurement provided insights into the interaction between the aerosols and the samples, allowing for a deeper understanding of their chemical behaviours and potential applications. This data was then translated into DSF concentrations or mass values based on standard calibration curves and the volume of each sample. Consequently, these DSF values are deemed the most dependable indicators when evaluating the evenness of aerosol delivery across multiple test runs. A comparison of mass deposition as detected by QCM versus that determined by fluorescence methods requires a conversion of DSF mass to total aerosol mass. This is accomplished by dividing the DSF mass output rates by those of the aerosol mass. To estimate the actual amount of aerosol delivered, multiply its mass rate by the recorded DSF mass and divide by the DSF rate. Moreover, to accurately reflect discrepancies in DSF percentages within produced aerosols, a calculation for aerosol mass delivery efficiency (η) was formulated [94].

5.3.6 Statistical analysis

The investigation examined the characteristics of aerosols produced under various conditions and concentrations. Parameters like the aerosols' average size, standard deviation (SD), and coefficient of variation (CV) were evaluated. The standard deviation was determined by extracting the square root from the average differences within and among the experimental trials. In contrast, CV was derived by dividing the standard deviation by the average value and then representing this quotient as a percentage. A linear mixed-effects model was employed to evaluate DSF mass data obtained during successive dilution exposure experiments. This model considered three predetermined factors (aerosol conditions, their concentrations, and the placement of replicas) for analysis, alongside three variable factors originating from variabilities in experimental runs and their interplay with the fixed factors. Data collection was conducted utilising a split-split-plot design framework. Variance nonuniformity was addressed within the model, accommodating various levels of aerosol density. A single data point was not recorded due to loss during sampling; therefore, the mean of other datasets

procured under identical conditions of aerosol concentration and replication location was substituted. DSF mass measurements were analysed using a single dilution exposure technique and a linear mixed-effects model. This model incorporated a pair of fixed comparison variables (rows and replicates' locations) and three variable factors that stemmed from repeated experimental iterations and their interaction with fixed components. In parallel, data acquisition employed a split-block design approach. Subsequent analysis of QCM-detected aerosol deposition was conducted using an alternative linear mixed-effects model. This particular model encompassed two predetermined factors (either the third or fifth rows and variant estimation methodologies for aerosol deposition: either PBS in cell culture inserts, PBS applied for QCM cleaning, or directly from QCM readings) along with three random influences originating from repetitive experimental procedures and their potential interaction with the set factors [94].

5.4 Results

5.4.1 Characterisation of aerosol generated aerosol

The assessment did not include an evaluation of aerosol characteristics under the 0.5 μm threshold because their mean aerodynamic particle diameter was smaller than what the APS could detect. However, the dimensions and consistency of these small aerosols were independently assessed utilising a scanning mobility particle sizer. Findings indicated a significant steadiness in aerosol sizes and distribution profiles, with exceptionally low coefficients of variation (CVs) for the mean aerodynamic particle diameters and the geometric standard deviations (GSDs). This stability was observable both within individual experiments and across multiple repeats. In contrast, the mass flow rates for both the aerosol and DSF and their proportional ratio – indicating the DSF's concentration within the aerosols – displayed greater CVs that peaked at 32% (Figure 5.1) [94].

5.4.2 Vitrocell Exposure System Characterisation

The stability of aerosol dispersal was evaluated by testing the delivery efficiency across six locations in a designated exposure row. We combined these efficiencies to obtain an average culminating in 140 data points for serial dilution exposure scenarios and 28 for single dilution exposures. To adjust for discrepancies arising from the exposure system and variances in sample handling and fluorescence quantification, we normalised these data points relative to the mean values derived from four separate experimental trials. Subsequently, we assessed the percentiles corresponding to the standardised scores, using them as a benchmark for result variability. The entire spectrum of variability observed in serial dilution exposures ranged from 0.65 to 1.28, whereas in single dilution

exposures, this span was narrower, from 0.86 to 1.19. To gain a finer understanding of the consistency in system performance across various exposures, our analysis focused on the lower and upper deciles—the 10th and 90th percentiles. We identified a range of percentile values capturing data from serial dilution exposures for aerosols with mean aerodynamic diameters at less than 0.5, 0.8, 1.1, 1.4, and 1.6 µm, which were from 0.87 to 1.16, from 0.85 to 1.14, from 0.74 to 1.20, from 0.81 to 1.17, and from 0.81 to 1.14, respectively. Similar patterns were observed when analysing the results per dilution. Across all tested aerosols and dilutions, it was found that 80% of the normalised values fell within the overall average $\pm 15\%$ range, which represents a range of 0.85 to 1.16. For single dilution exposures, the 10th and 90th percentiles combined captured a comparable range of 0.88 to 1.15 across all exposure rows. To facilitate understanding, Figure 5.1 has been included, which illustrates the data range of the average $\pm 15\%$ [94].

Aerosol characterisation was conducted at three key time points—prior to exposure, midway through, and following the exposure period—as described in detail in Chapter 4 (section 4.3.4). The experimental configuration used for these measurements is illustrated in Figure 5.2. As part of the protocol, exposures were temporarily halted after 14 minutes to allow for particle size analysis and aerosol collection on filter pads. Following these intermediate assessments, the exposure process was resumed for an additional 14 minutes [95].

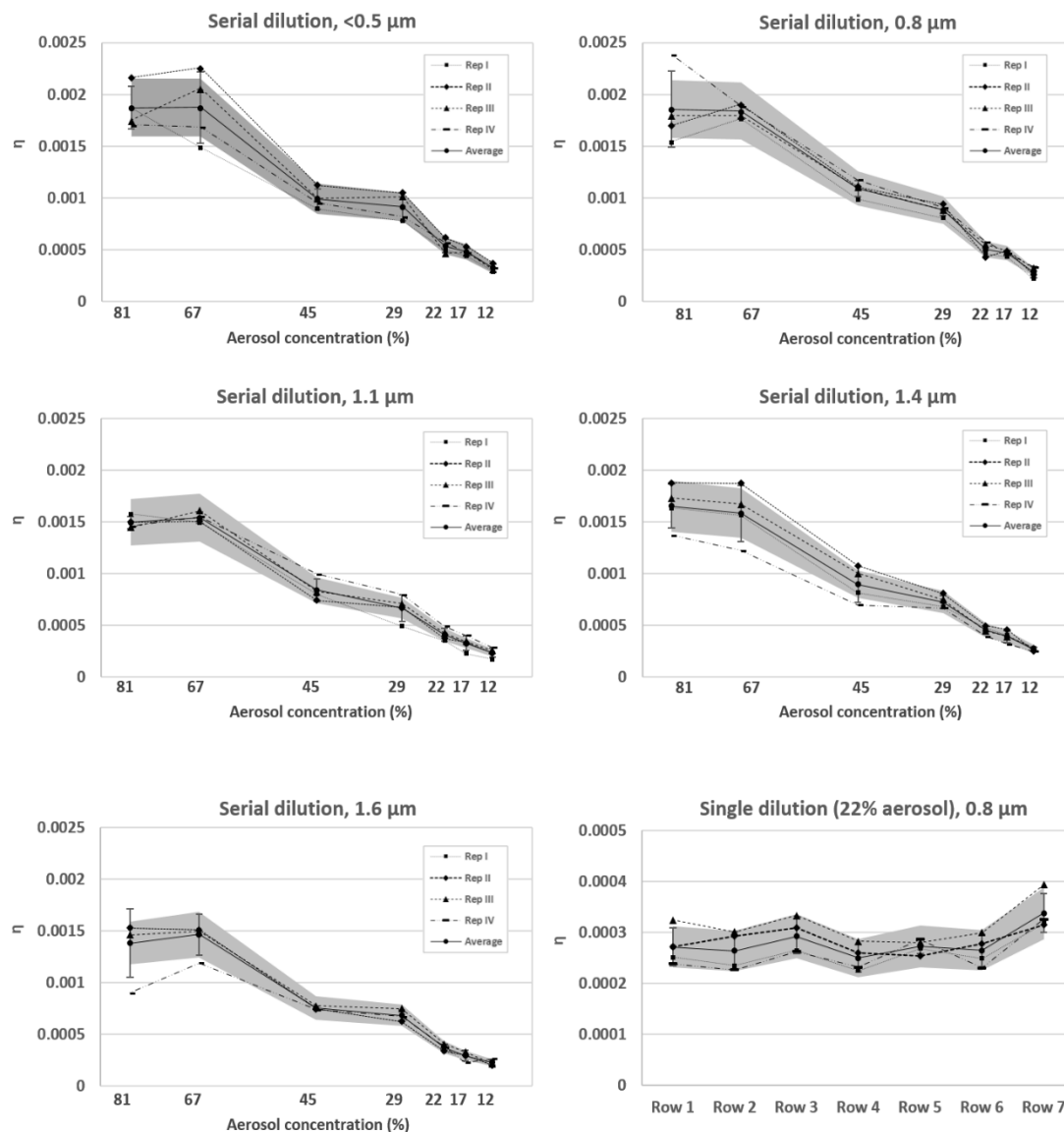


Figure 5.1: Displays the variations observed in repetitions and the dosage of aerosol, explicitly focusing on the efficiency of delivering disodium fluorescein aerosol through different methods of exposure. η : Delivery efficiency of disodium fluorescein, calculated as the proportion of the compound successfully deposited during aerosol exposure. The findings are presented in two formats: individual results for each repetition and the averaged outcomes calculated over four repetitions. To illustrate the variability in these measurements, standard deviations are represented by error bars on the graphs. Additionally, the grey shading on the charts indicates the range of average values, encompassing variations within $\pm 15\%$ of the mean, allowing for a clearer understanding of the consistency and reliability of the results (Steiner, 2017 #10) [94].

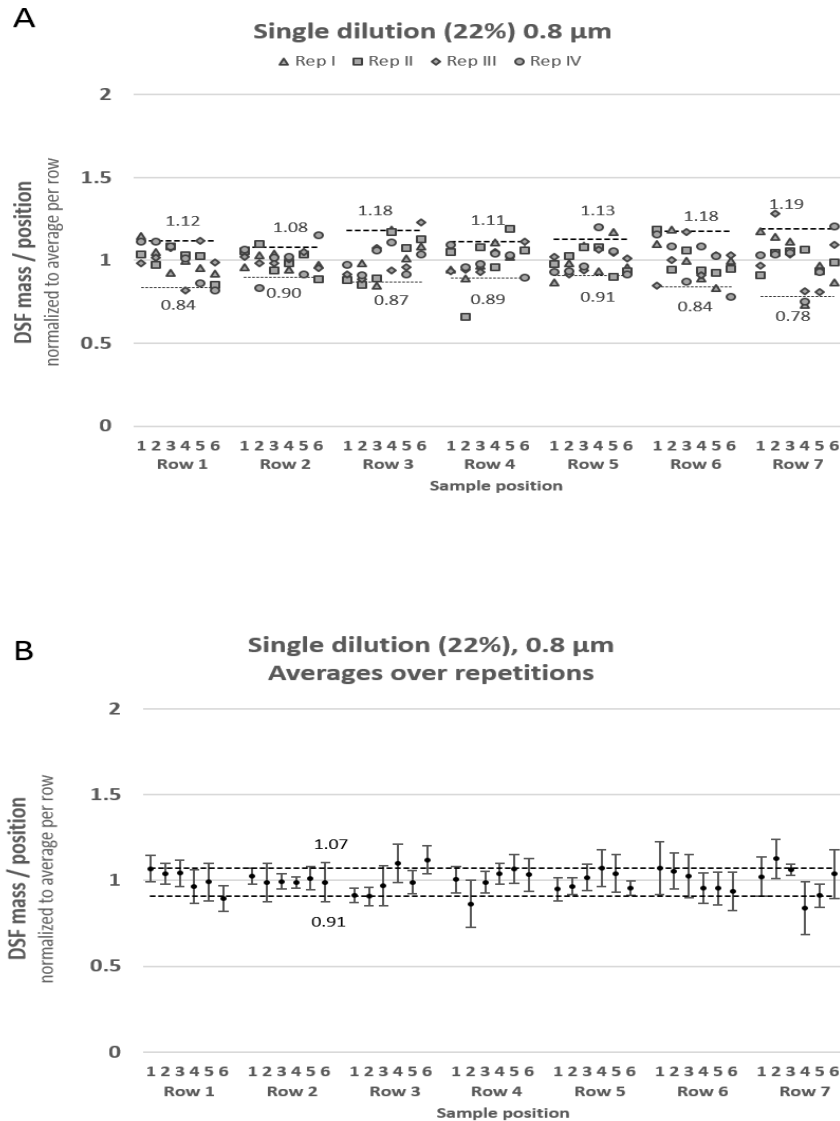


Figure 5.2 Uniform delivery to replicate positions in serial dilution exposures was observed:

A) The mass deliveries of Disodium fluorescein (DSF) for each position and repetition were normalised to the average per dilution row. The plots show results for aerosols with diameters of 0.8 μ m. Dashed lines and numbers in the plots represent the 10th and 90th percentiles of the 24 data points per dilution row.

B) The experiments were repeated four times, and the results were averaged. Error bars show standard deviations, while the dashed lines mark the 10th and 90th percentiles of the 42 data points collected [94].

5.4.3 QCM accuracy

To evaluate the accuracy of QCMs, the aerosol mass deposited per area—reported by the QCMs—was compared with the mass measured using DSF in the PBS solution used to rinse the QCM crystals. Figure 5.3 shows the relationship between the aerosol mass delivered to the cell culture inserts and the mass detected on the QCM surfaces. While a correlation exists, the QCM consistently recorded lower values—approximately 85% to 95% of the PBS measurements. This discrepancy was nearly statistically significant ($p \approx 0.1$). In contrast, the discrepancy between the fluorometrically determined mass deposition on QCM crystals and the mass reported by the QCMs was significant and highly significant ($p < 0.0001$). When setting the mass delivery to cell culture inserts at 100%, the QCMs only detected 10% to 30% of the total aerosol mass deposited [94].

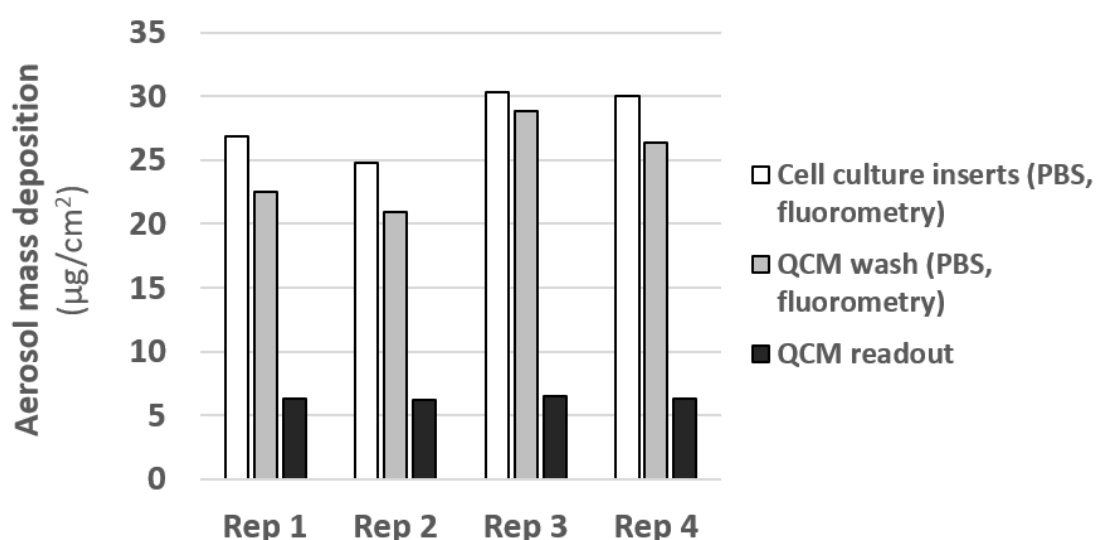


Figure 5.3 QCM deposition results: a comparison was made between the aerosol mass deposition reported by the QCMs and that measured fluorometrically. Exposures lasted 21 minutes in single-dilution mode (22% aerosol concentration) with 0.8-µm diameter aerosols. Samples from two dilution rows, including two QCMs and 12 cell culture inserts, were combined [94].

5.5 Discussion

The VC24/48 exposure system is a reliable system that has been extensively used for exposure studies and system characterisation. However, its effectiveness with low GVP-content liquid aerosols has not yet been empirically validated. Since there is increasing interest in these aerosols for the evaluation of ENDS or medical inhalation devices, it is crucial to characterise the system proactively for future

research. The in vitro aerosol exposure system's performance depends on accurate, consistent, and uniform aerosol delivery to cell cultures. Acceptance criteria are established based on the deviation from the average delivery across a set number of repeated exposures and positions exposed to the same aerosol concentration. This aids in assessing the system's performance. The variation ranges, indicated by the lower and upper percentiles, offer an estimate of the data spread. The variability in aerosol distribution documented in this investigation cannot be entirely ascribed to the VC24/48 apparatus. Other distortions might arise during sample collection and processing, or when conducting fluorescence evaluations. While it is a complex task to quantify the exact discrepancies brought on by sample gathering, processing, and scrutinising analytically, they are believed to augment the total distribution breadth by roughly 10%. Therefore, it is essential to note that the span encompassing 80% of the observed figures, centred around the median value, accurately represents the inherent variation intrinsic to the system itself. In light of this estimate, we can forecast with a fair degree of certainty that when the aerosol is consistently delivered to a specified point during repeated usage of VC24/48, it will likely fall within an average range comparable to that experienced across all positions subjected to identical aerosol concentration levels, allowing for an average deviation of $\pm 25\%$ [94].

Our goal was to evaluate the system's performance and identify the characteristics and modes of operation that negatively impact its efficiency. We aim to focus on those elements that require further analysis, adjustment, or avoidance. It is crucial to note that the aerosol delivery to individual dilution rows remained consistent despite variations between repetitions (see Figure 5.1) concerning the system's performance stability. It appears that the observed variation was likely caused by the system's overall condition, which became more affected when highly concentrated aerosols passed through specific parts of the dilution system. Therefore, it is probable that the accumulation of deposits on internal surfaces compromised the system's performance stability. This was evidenced by the decreased dosing accuracy observed during sequential dilution exposures at high aerosol concentrations. These findings are consistent with existing literature demonstrating that cleaning and maintenance significantly affect the performance of Vitrocell systems [96].

The data presented highlights the importance of characterising the exposure system specific to aerosols, especially when comparing different types. It is recommended that the aerosol dose delivery be determined using the system settings intended for the exposure experiments before conducting the actual exposure for the aerosol of interest. While quartz crystal microbalances (QCMs) are preferred for measuring the aerosol dose delivery of dry powder aerosols, our study shows they are not suitable for liquid aerosols. The measurement of mass using QCMs relies on the resonant frequency shifts of quartz crystals, which are directly related to the mass attached to the crystal's surface. However, this relationship holds true only if the added mass is rigid; it does not apply to liquid

droplet depositions. In fact, the mass of liquid deposits may be underestimated due to viscous energy dissipation, which depends on the thickness of the deposited layer and the material's viscosity. Our research demonstrates a consistent underrepresentation of recorded mass for liquid aerosol depositions when materials have viscosities at or below that of glycerol. This renders QCMs ineffective for precise mass measurement in these cases [94].

Consequently, alternative strategies are essential for accurately determining aerosol delivery under such conditions. The significance of characterising the exposure system is evident, particularly in the comparative analysis of different aerosol types. Therefore, we advocate for preliminary calibration of aerosol dose delivery using the actual system configurations intended for subsequent exposure experiments related to the specific aerosol concerned. Thus, our findings confirm that QCM assessments do not provide an accurate account of mass deposition for liquid aerosols with viscosities equal to or less than glycerol. Therefore, alternative methods must be employed to determine aerosol delivery in such situations [94].

5.6 Conclusion

During a thorough evaluation of the VC24/48 system, we assessed its performance stability and uniformity in delivering liquid aerosols to replicate positions. When performing repeated exposures, it is reasonable to expect that the aerosol delivery at a specific point will be within $\pm 25\%$ of the average delivery across all positions exposed to the same aerosol concentration. We can reduce variability in aerosol delivery by optimising the system's operating conditions, including the dilution scheme and aerosol concentration. While aerosol dilution allows for precise dosing, it is only partially accurate. Consequently, it is impossible to determine the quantity of aerosol mass delivered by merely considering the mass introduced into the system and the level of dilution applied. Even though variations in the efficiency of delivery among particles of different sizes are observable, these variations do not correlate with the degree of aerosol dilution. The particle size distribution of the fraction of a test aerosol that eventually reaches the exposure chamber is maintained across varying levels of aerosol dilution, even though it may differ from the original size distribution of the test aerosol as it was first introduced to the system. To summarise, the VC24/48 is adept for regulated exposure to liquid test aerosols. Nonetheless, its performance reveals greater variation in delivering to individual chambers than studies involving cigarette smoke, which must be considered. Hence, it is advisable to empirically adjust the system settings for optimal dose distribution with reduced inconsistency. It should be noted that the observed variations in delivery patterns across distinct aerosols are not exclusive to the VC24/48 and should be acknowledged across all aerosol exposure systems. Additionally, QCMs are inadequate for quantifying the mass deposition of liquid aerosols, as they tend to undervalue it substantially [96].

5.7 Research Impact

Following the publication of this research paper in 2017, numerous new studies have been conducted within the field, with Google Scholar recording 28 citations, demonstrating its significant influence in the scientific community. This study played a pivotal role in establishing the need for systematic characterisation of in vitro exposure systems for e-liquid aerosols, effectively initiating a new research direction focused on the rigorous validation of these methodologies. It was among the first to systematically evaluate the performance of an air–liquid interface (ALI) exposure system for e-liquid aerosols, establishing a methodological benchmark for dose delivery accuracy, reproducibility, and system variability.

A pivotal finding—that quartz crystal microbalances (QCMs) substantially underestimate liquid aerosol deposition—highlighted a critical limitation in widely used measurement tools. This insight catalysed the development and adoption of alternative quantification methods. This influence is evidenced by subsequent independent studies that directly addressed the methodological frameworks established in our work. Zhang et al. published their research on the fit-for-purpose characterisation of air-liquid interface (ALI) in vitro exposure systems for e-vapour aerosols, directly building upon the characterisation principles established by us [96]. This foundational work also directly influenced subsequent studies, including Oldham et al. (2020), who cited and expanded upon our findings in their paper *"Deposition efficiency and uniformity of monodisperse solid particle deposition in the Vitrocell® 24/48 ALI in vitro exposure system"* [97][97]. Their study utilised monodisperse solid fluorescent particles and computational fluid dynamics (CFD) to investigate deposition efficiency and uniformity further, thereby validating and extending the methodological framework established. By applying both experimental and modelling approaches, they confirmed the importance of particle size and system geometry in determining deposition outcomes—issues first raised in our 2017 work.

These developments illustrate how our research moved beyond citation to practical implementation and methodological advancement, helping to establish new standards for characterisation protocols in in vitro aerosol exposure systems.

6.0 Effects of cigarette smoking and aerosols on colour stability of dental resin composites

6.1 Introduction and Contribution

The Vitrocell exposure systems offer a high level of versatility, enabling researchers to assess not only three-dimensional (3D) organotypic models but also to conduct tests on dental materials. This broader application is particularly significant because dental materials, once placed in the mouth, are exposed to environmental conditions that closely resemble those of the respiratory tract. As a result, it is crucial to investigate the impact of exposure to smoke and aerosols on dental material in a realistic and controlled manner. Studies presented in this chapter can provide valuable insights into the potential effects of these substances on oral health and the longevity of dental materials.

In this chapter, I discuss three research articles: **"Effects of cigarette smoking on color stability of dental resin composites (Zhao X, Zanetti F, **Majeed S**, Pan J, Malmstrom H, Peitsch MC, Hoeng J, Ren Y. Am J Dent. 2017 Dec;30(6):316-322. PMID: 29251454.) (80),"** **"Effects of whitening toothpaste and bleaching treatment on resin composite discoloration caused by cigarette smoke and electronic vapor aerosol (Zhao X, Zanetti F, Wang L, Malmstrom H, **Majeed S**, Peitsch MC, Hoeng J, Ren Y. Am J Dent. 2021 Apr;34(2):63-69. PMID: 33940661.) (81),"** and **"Analysis of Chemical Deposits on Tooth Enamel Exposed to Total Particulate Matter from Cigarette Smoke and Tobacco Heating System 2.2 Aerosol by Novel GC–MS Deconvolution Procedures (Haiduc A, Zanetti F, Zhao X, Schlage WK, Scherer M, Pluym N, Schlenger P, Ivanov NV, **Majeed S**, Hoeng J, Peitsch MC, Ren Y, Guy PA J Chromatogr B Analyt Technol Biomed Life Sci. 2020 Sep 1;1152:122228. doi: 10.1016/j.jchromb.2020.122228. Epub 2020 Jun 10. PMID: 32585495.) (82).** These publications address similar topics, making it advantageous to combine their findings. As a co-author in all three articles, I played a significant role in designing and implementing the exposure aspects while also providing support in writing about the exposure procedures.

6.2 Background

Cigarette smoke is known to cause stains and discolouration on resin composites, which are used in dental procedures to restore teeth (81). A recent study has found that smoking cigarettes can increase water absorption and solubility of dental resin composites [98]. It can also deposit metal ions into the resin matrix, leading to discolouration. In addition, smoking can reduce brightness and increase roughness, thus impacting the stability of colour [99]. Cigarette smoke can weaken the bond strength between dental resin composites and dentin, potentially affecting the long-term success of dental restorations. The exact process by which smoking causes dental staining is not fully understood;

however, brown pigments from the particulate matter of cigarette smoke are thought to be the main cause [100]. When a cigarette is ignited, the tobacco undergoes combustion at high temperatures, resulting in a particulate phase that consists of solid particles and liquid droplets suspended in a gas phase. Cigarette smoke contains "tar," which is the term for particulate matter. Tar contains various pigmented compounds and metal ions, including lead and cadmium, which can cause discolouration on dental hard tissues and resin composites. To prevent discolouration, it is crucial to eliminate or reduce tar from cigarette smoke. New types of tobacco products (e.g. electronic vaping products) that significantly alter the aerosol composition of cigarette smoke may pose a lower risk of discolouration to composite resin materials [101].

The specific mechanisms and compounds that cause tooth discolouration due to cigarette smoke (CS) have not been thoroughly explored. However, reducing the production of particulate matter can be a practical approach to minimise tooth discolouration. Our earlier research indicated that exposure to the aerosol of the Tobacco Heating System (THS) 2.2, a heat-not-burn technology-based product (produced by Philip Morris International), resulted in less discolouration compared to CS exposure [77]. THS 2.2 heats tobacco sticks below 350°C, which prevents solid particle formation and cuts harmful and potentially harmful compounds by over 95% on average. As a result, the aerosol generated by heating tobacco has a different makeup compared to traditional cigarettes [102].

The central goal of this chapter is to evaluate the effectiveness of whitening toothpaste compared with a 6% hydrogen peroxide bleaching treatment in mitigating the discolouration of dental resin composites caused by conventional cigarette smoke and the aerosol from electronic vaping products. Additionally, this study examines how aerosols from heated tobacco products compare to regular cigarette smoke in influencing the colour change, shine, and surface texture of these dental materials [102].

Because cigarette smoke has a complex composition, there have been limited studies on the compounds found on tooth surfaces or absorbed into teeth. Most research has primarily focused on detecting nicotine and its byproducts. However, there have not been systematic reports linking tooth colour to the constituents of cigarette smoke [103]. Bovine teeth are preferred over human teeth in research because they eliminate the variations observed in human tooth morphology. In addition, numerous studies have shown that bovine teeth have microhardness values and chemical composition similar to those of human teeth [104].

This chapter aims to describe several key objectives, including:

1. Developing an effective method to extract coloured chemical compounds deposited on tooth enamel after exposure to cigarette smoke containing TPM [101].
2. Using Partial Least Squares and Lasso regression to process the dataset obtained from gas chromatography coupled with Time-of-Flight Mass Spectrometry (GC-TOF-MS) to identify key chemicals associated with ΔE colour values. Delta E (or ΔE) measures the change in colour on a three-dimensional axis ($L^*a^*b^*$). In the CIE (Commission Internationale de l'Éclairage) $L^*a^*b^*$ colour system, a particular colour is characterised by three coordinates: L^* (indicating lightness), a^* (red-green axis), and b^* (yellow-blue axis). These values represent the colour's chromatic features. Based on the CIELAB values, researchers created the Delta E (ΔE) formula to measure the colour difference between two samples. [105].
3. Identification of compounds associated with discolouration caused by cigarette smoke TPM [105].
4. Comparing these compounds with those identified on the enamel surface exposed to TPM derived from the aerosol of a heated tobacco product [105].
5. Comparative analysis of enamel discolouration induced by CS TPM and THS 2.2 Aerosol TPM Using CIELAB Metric.

6.3 Materials and Methods

3R4F reference cigarettes were sourced from the Tobacco Research & Development Centre at the University of Kentucky. The THS 2.2 device was manufactured by Philip Morris International Products S.A. Bovine teeth were collected from freshly slaughtered cows in Rochester, NY, USA, and used to evaluate how TPM from cigarette smoke and heated tobacco aerosols interacts with enamel surfaces, focusing on chemical deposition and staining.

Three commonly used resin composites were selected (Durafill VS (DVS) (Heraeus-Kulzer GmbH, Hanau, Germany), Filtek Supreme Ultra (FSU) (3M ESPE, St. Paul, MN, USA), and Tetric EvoCeram BulkFill (TEC) (Ivoclar Vivadent, Schaan, Liechtenstein)) that are commercially available to test the impact of smoking on them. The composites were prepared as discs, 6.0 mm in diameter and 1.5 mm thick. The discs were placed on a glass substrate lined with a Mylar strip. To ensure a smooth, even surface and remove excess material, a second Mylar strip and glass plate were added on top of the filled moulds, with gentle manual pressure applied. This setup helped create a flat, uniform surface. The polymerisation process used a halogen light-curing unit with a rotating platform (Triad 2000™), which was operated for 4 minutes for complete curing. Afterwards, the discs were removed from the moulds and stored in deionised water. The final surface finishing involved sequential polishing with

the Sof-Lex™ system, progressing through four grit levels from coarse to ultrafine, following the manufacturer's recommended protocol. . 60 discs were prepared, with 20 discs in each group. To simulate the ageing process in the oral environment over 6 months, half of the polished discs were subjected to thermocycling between 5°C and 55°C for 5,000 cycles. The remaining discs served as new composite restorations for comparison purposes. Discs were subjected to approximately 105 hours of thermocycling, , after which they were gently washed for 30 seconds and placed in deionised water before assessing their colour, gloss, and surface roughness. Composite discs were assessed for their colour using the CIE Lab system (Olympus, Tokyo, Japan) and their gloss using a Novo-Curve gloss meter (Rhopoint Instrumentation, East Sussex, UK) and recorded as Gloss Unit. The surface roughness was determined by conducting 3D scanning microscopy. Initial measurements of roughness, colour, and gloss were made then samples were exposed to 3R4F CS and THS2.2 aerosol from 20 cigarettes/sticks, per day for 1 hour, for 4 days, using a Vitrocell exposure system. Post-exposure, discs were brushed manually (15 strokes, 200 g pressure) with a soft-bristled ADA-compliant toothbrush and a silica-based toothpaste. Colour and gloss were re-evaluated to assess surface recovery following aerosol exposure and mechanical cleaning. Between daily exposures and overnight, discs were stored at 37 °C in artificial saliva (pH 7.0) to simulate oral conditions. The saliva solution was freshly prepared each day using distilled water and contained the following components per liter: 0.33 g KH_2PO_4 , 0.34 g Na_2HPO_4 , 1.27 g KCl, 0.16 g NaSCN, 0.58 g NaCl, 0.17 g CaCl_2 , 0.16 g NH_4Cl , 0.2 g urea, 0.03 g glucose, and 0.002 g ascorbic acid. The surface roughness was measured after 3 weeks of exposure and incubation in artificial saliva. Figure 6.1 presents an illustration of the research design [105].

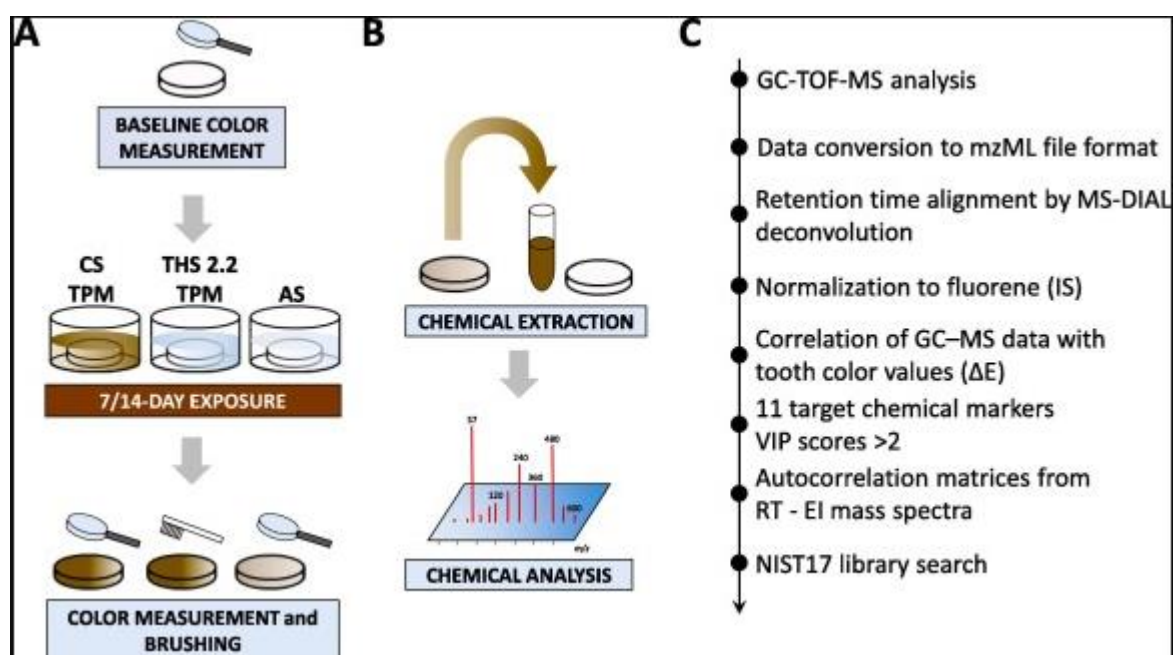


Figure 6.1: Workflow of exposure and analysis of composite discs , (A) Groups of twenty samples were exposed to TPM from 3R4F cigarette smoke, TPM from THS 2.2 aerosol, or AS control for a duration of 14 days. Colour measurements were taken before and after brushing the exposed samples. (B) After chemical extraction compound identification was conducted on 49 samples using GC-TOF-MS (gas chromatography coupled to time-of-flight mass spectrometry) according to the workflow (C). [106].

6.3.1 TPM Trapping

TPM samples were produced following a standard protocol using 3R4F to generate smoke. Mainstream smoke from 3R4F cigarettes and aerosol from THS 2.2 were collected on 44-mm Cambridge glass fibre filters positioned at the exhaust outlet of the puffing pump, ensuring efficient capture of particulate constituents. For mainstream cigarette smoke (CS), a Borgwaldt 20-port smoking machine was used, while a 30-port SM2000/P1 machine was employed for the THS 2.2. Both setups adhered to HC-related smoking guidelines [29]. To maintain uniform nicotine levels within the stock extracts (comprising 100% TPM), TPM was accumulated from six reference cigarettes on a single Cambridge glass fibre filter and from ten THS 2.2 units deposited on a single filter. . The final step involved extracting TPM by treating the filters from each substance with 27 mL of ethanol and then agitating on a rotary shaker for 15 minutes [107].

6.3.2 Vitrocell exposures with 3R4F smoke and THS 2.2. aerosol

The discs were exposed to cigarette smoke from 3R4F and THS2.2 aerosol. Cigarettes and tobacco sticks were conditioned for 48 hours to 21 weeks at $22\pm1^{\circ}\text{C}$ before generating aerosol or smoke. Smoke was generated using a smoking machine with a carousel, and aerosol with a separate machine. These experiments ran from Monday through Thursday for three consecutive weeks. Exposure to either smoke or aerosol was conducted with separate Vitrocell 24/48 systems, targeting 24 resin composite discs in a 48-well cell culture plate [107].

6.3.3 Exposure design

Composite discs were subjected to exposure conditions involving either artificial saliva or total particulate matter (TPM) derived from conventional cigarettes (CS) or Tobacco Heating System (THS) 2.2 for a continuous period of 14 days. As illustrated in Figure 6.1, the workflow outlines the sequential procedures employed for disc exposure and subsequent analytical evaluation [107].

6.3.3 GC–TOF–MS

Extracted ethanol samples were analysed using an Agilent Technologies 6980 GC system and an ALMSCO BenchTOF-dx™ TOF mass spectrometer. Chromatographic separation was carried out using a Restek Rxi®-5ms fused silica column, featuring a diphenyl and dimethyl (cross-bonded) polysiloxane stationary phase. Helium served as the carrier gas. Samples were injected in split mode with a ratio of 1:15, using a volume of 1 μl . Data was collected in full-scan mode, covering a range from m/z 40 to 900, at a sampling rate of 10 Hz. GC-MS analysis was performed on all randomly ordered and batched samples [105].

6.3.4 Data Processing

In our data post-processing, we converted raw GC-MS data files to mzML format using MSconvert. We employed open-source software MS-DIAL for automated peak detection, integration, and retention time correction using reference alkane standards. Alignment was performed in two distinct steps. The initial step involved processing with the GC-MS module of MS-DIAL. This generated a table grouping retention time-fragment ions based on MS-DIAL's deconvolution algorithm. In the second run, we processed data with MS-DIAL's LC-MS module, which identified all mass fragments with a minimum ion intensity of 100. In summary, our untargeted analysis workflow involved these steps: please see Figure 6.1 [102].

6.4 Results

6.4.1 Effects of exposure on enamel resin colour

At baseline, no visible differences in the colour of bovine enamel blocks were observed across experimental groups. After 7 days of exposure, enamel blocks treated with total particulate matter (TPM) from mainstream cigarette smoke (CS) exhibited noticeable discolouration. In contrast, those exposed to TPM from THS 2.2 aerosol or immersed in artificial saliva showed no significant colour change (Figure 6.2). Brushing the enamel blocks with a toothbrush (without toothpaste) effectively removed surface-adsorbed colourants; however, CS TPM-exposed samples still appeared visibly darker than the baseline. In contrast, THS 2.2 aerosol TPM-exposed samples showed minimal discolouration even after 14 days of exposure and brushing. A 5.6% reduction in staining was observed in samples immersed in synthetic saliva following brushing without toothpaste. Conversely, the same approach reduced staining by 58.7% in samples treated with THS 2.2 aerosol TPM. After 14 days, surface discolouration on teeth exposed to CS TPM was ten times more severe than on enamel treated with THS 2.2 aerosol TPM [107].

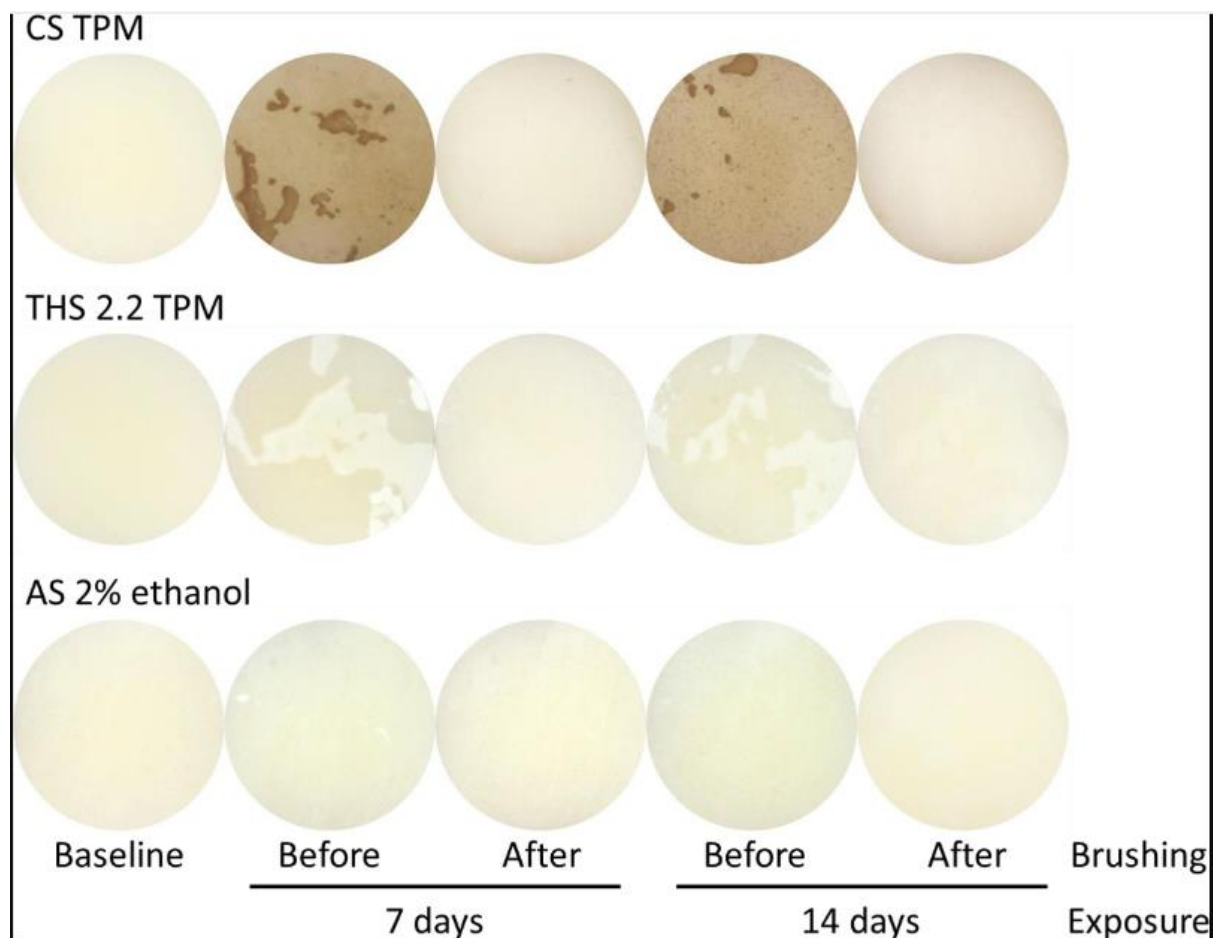


Figure 6.2: Detailed images of bovine enamel blocks treated with total particulate matter (TPM) from two distinct sources—cigarette smoke (CS) and Tobacco Heating System 2.2 (THS 2.2) aerosol—are

presented, alongside treatments with artificial saliva (AS). The duration of exposure for each treatment varied, with some blocks receiving treatment for 7 days and others for 14 days. Additionally, the effects of mechanical stain removal have been evaluated using a standard toothbrush without the application of toothpaste. The comparison of colour shades before and after the brushing process highlights the impact of this mechanical cleaning method on the enamel's appearance, demonstrating how effective brushing can help mitigate staining caused by the TPM treatments [101].

6.4.2 GC–TOF-MS analysis

An analysis using GC-TOF-MS was conducted to explore the components present in the extracted samples. The test procedure involved traditional chemical derivatisation, initially with methoxamine and subsequently with N, O-bis-trimethylsilyl-trifluoroacetamide, which includes 1% trimethylchlorosilane. An additional analysis proceeded without any derivatisation step. However, this dual-step derivatisation did not yield any beneficial data compared to non-derivatised samples. Consequently, the GC-TOF-MS evaluations proceeded without chemical derivatisation. This approach enabled the inference of LRI values for molecular compounds larger than C18. Additionally, fluorene was used as an internal standard to normalise untargeted data and correct for any instrumental variations during all analyses. Quantification was based on m/z 166, the ion signature of fluorene that elutes at 15.59 minutes. The extraction process's efficiency was evaluated using a quality control sample, which was a TPM extract from smoking material applied to artificial calcium-based tooth material phosphate. Additionally, targeted monitoring took place for specific molecules, including nicotine (CAS 54-11-5), 3,7,11,15-tetramethyl-2-hexadecen-1-ol (CAS 102608-53-7), and indolizine (CAS 274-40-8), in triplicate TPM extract samples, with their consistency validated as the coefficient of variation remained below 20% [102].

6.4.3 Effects of 3R4F CS and THS2.2 aerosol exposures on surface roughness of composite resin discs

The TEC showed higher surface roughness at baseline compared to DYS and FSU ($P < 0.001$). Exposure to either 3R4F CS or THS2.2 aerosol did not significantly affect the surface roughness of the resin composites used. Furthermore, no significant differences in surface roughness were observed between the THS2.2 and 3R4F groups after exposure across all the resin composite groups (**Table 6.1**) [101].

Table 6.1 Surface roughness values (Mean±SD) of composite discs before and after 3 weeks of exposure to 3R4F CS and THS2.2 aerosol.

	DSV		TEC		FSU	
	Before	After	Before	After	Before	After
3R4F	0.08 ±0.02	0.07 ±0.03	0.18 ±0.07	0.15 ±0.03	0.09 ±0.02	0.09 ±0.01
THS2.2	0.09 ±0.02	0.08 ±0.02	0.15 ±0.08	0.14 ±0.04	0.10 ±0.01	0.10 ±0.01
P*	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05

Abbreviations: DVS, Durafill VS; TEC, Tetric EvoCeram BulkFill; FSU, Filtek Supreme Ultra. • students' t tests.

6.5 Discussion

Smoking can lead to significant discolouration of dental resin composites, posing a major issue for long-term clinical research. Resin composite is a commonly used aesthetic material for restoring carious and non-carious lesions on anterior teeth. While the success rate of resin composite restorations has improved, discolouration remains a significant concern. Discolouration occurs when resin composite surfaces are exposed to cigarette smoke, especially if not adequately polished. Furthermore, brushing the stained surfaces with a toothbrush or toothpaste before assessing the colour is crucial. Previous studies lacked precise control over puff length and smoke stream in the smoking chambers used [107].

In the present study, neither 3R4F CS nor THS2.2 aerosol affected the surface roughness of the resin composites. This contradicts a previous report stating that cigarette smoking and brushing increase surface roughness. The discrepancy likely arises from differences in finishing and polishing the composite resin discs. Industrial sandpapers of 600, 800, and 1,200 grits are used, while the standard operating procedure involves professional dental polishing discs with four abrasive grades: coarse, medium, fine, and ultrafine. Improper polishing can leave a soft oxygen inhibition layer on the resin composite surfaces, making them vulnerable to deterioration. The present study highlights that smoking and brushing do not affect the surface roughness of resin composites if properly polished with professional dental polishing discs [103].

6.6 Conclusion

In our study, we placed dental resin from DVS, TEC, and FSU inside the Vitrocell exposure system at the air-liquid interface. By exposing the dental resin to whole cigarette smoke in the Vitrocell exposure system, we gained a better understanding of the effects of CS on human dental health. This study has opened up new possibilities for dental research in real-life scenarios, allowing for future studies to investigate the effect of smoking on tooth discolouration and the chemical compounds deposited during smoking. We found that heated tobacco products caused less tooth enamel discolouration than cigarette smoke. Our analysis of the chemicals present revealed that eleven compounds, primarily terpenoids, were responsible for the staining. Although these compounds lacked a chromogenic structure, they may have lost their chromogenic groups during elution or were undetectable under our GC-TOF-MS conditions. Our research did not find any link between nicotine and tooth discolouration [106].

6.7 Research Impact

This study represents a pioneering application of the Vitrocell® exposure system to the field of dental materials science, marking the emergence of a new interdisciplinary research area at the intersection of inhalation toxicology and restorative dentistry. By adapting air-liquid interface (ALI) exposure methodologies—initially developed for respiratory toxicology—to assess the effects of cigarette smoke and e-vapour aerosols on dental composites, the study introduced a novel framework for evaluating the aesthetic and structural integrity of dental materials under realistic exposure conditions.

The innovative methodology has generated substantial scientific interest, with 35 citations (Google Scholar), and has been widely recognised in both the dental and tobacco product research communities. It has catalysed a shift in how researchers assess the impact of inhalable products on oral health, enabling standardised, reproducible testing of material discolouration, surface degradation, and chemical residue accumulation.

A key example of this influence is the work by Dalrymple et al., who adopted the same exposure system to investigate tooth staining caused by cigarettes, electronic nicotine delivery systems (ENDS), and heated tobacco products [102]. Their studies extended the methodology to compare staining potential across product categories, providing evidence-based insights for both clinicians and consumers[108].

These follow-up studies underscore the public health relevance of the original research. By enabling controlled, comparative assessments of aerosol effects on dental materials, this methodology has informed clinical guidance, product development, and consumer awareness regarding the cosmetic consequences of using inhalable products.

In summary, this research has not only advanced laboratory science but also bridged it with real-world health concerns, establishing a new evidence base for evaluating the oral health impacts of modern nicotine delivery systems.

7.0 Impact Assessment of Repeated Exposure of Organotypic 3D Bronchial and Nasal Tissue Culture Models to Whole Cigarette Smoke

7.1 Introduction and Contribution

After thoroughly characterising the Vitrocell exposure system with cigarette smoke and various e-liquid aerosols, as outlined in the previous chapters, this chapter will focus on the effects of cigarette exposure on 3D bronchial and nasal tissue culture models. We aim to investigate how these exposures influence cellular behaviour, gene expression, and overall tissue integrity within these specific models. By doing so, we aim to gain valuable insights into the biological responses triggered by cigarette smoke, thereby contributing to a deeper understanding of potential mechanisms of toxicity and their implications for respiratory health. This analysis will involve various assessment techniques to evaluate histological changes, inflammatory responses, and functional alterations in the tissue cultures, ultimately enhancing our understanding of smoking-related pathologies. The Vitrocell exposure system provides a more accurate reflection of the aerosol exposure that individuals encounter daily. This similarity enables a more comprehensive study of the effects of aerosols on biological systems. To thoroughly investigate these impacts, it is essential to utilize well-characterized in vitro exposure systems, as discussed in Chapters 2, 4, and 5. By doing so, researchers can gain valuable insights into how aerosols influence health and develop more effective safety measures. This chapter discusses the use of the Vitrocell exposure system to study effects in vitro before proceeding to clinical studies of any new inhalation product.

This chapter focuses on the video research paper **"Impact Assessment of Repeated Exposure of Organotypic 3D Bronchial and Nasal Tissue Culture Models to Whole Cigarette Smoke"**(Kuehn D, **Majeed S**, Guedj E, Dulize R, Baumer K, Iskandar A, Boue S, Martin F, Kostadinova R, Mathis C, Ivanov NV, Frentzel S, Hoeng J, Peitsch MC. J Vis Exp. 2015;(96):52325. Published 2015 Feb 12. doi:10.3791/52325) (7). As the second author of this article, my responsibilities included assisting in experiment design, conducting exposure experiments, and assisting in video documentation for publication. This paper contains experimental videos and is published on the Jove site. Jove is the leading global producer and provider of science videos, dedicated to advancing scientific research and education. Millions of scientists, educators, and students across thousands of universities, colleges, hospitals, and biopharmaceutical companies worldwide rely on JoVE for their research, teaching, and learning needs learning.

Link to the video: [impact assessment of repeated exposure of organotypic 3d bronchial and nasal tissue culture models to whole cigarette smoke \(jove.com\)](https://www.jove.com/video/58111/impact-assessment-of-repeated-exposure-of-organotypic-3d-bronchial-and-nasal-tissue-culture-models-to-whole-cigarette-smoke)

7.2 Background

The respiratory system is continually exposed to airborne substances like environmental pollutants and cigarette smoke components (CS), which can be harmful. To understand the biological impacts of these exposures, it is essential to replicate the in vivo conditions of the human respiratory system airway. Traditional two-dimensional (2D) cell cultures, such as normal human bronchial epithelial (NHBE) cells, often do not completely mimic the complexity of the respiratory epithelium. In contrast, three-dimensional (3D) organotypic tissue models provide a more physiologically accurate representation alternative. These models replicate the structural and functional characteristics of the human airway, incorporating a variety of cell types such as basal, goblet, ciliated, and non-ciliated epithelial cells. They also form tight junctions and exhibit a mucociliary phenotype, which is critical for proper airway function. These 3D cultures are typically grown on porous membranes at an air-liquid interface (ALI), enabling direct exposure of the apical surface to aerosols while maintaining nutrient supply from the basolateral side. This setup mirrors the natural inhalation exposure pathway. Earlier research indicates that the gene expression patterns in these models correspond closely with those from human bronchial brushings, supporting their biological significance. Additionally, these tissue models' responses to cigarette smoke reflect the cellular differences seen between smokers and non-smokers. Due to their ability to be maintained over extended periods, these models are particularly well-suited for evaluating the effects of repeated or chronic exposure to inhaled toxicants [111].

Cytotoxicity tests are frequently employed to evaluate the harmful effects of specific chemicals or compounds. For instance, a luminescent assay can assess membrane integrity, aiding in the detection of dose-dependent cytotoxic effects in cell cultures. However, when examining the impact of compounds at lower, non-toxic concentrations, it is crucial to consider additional measurements. The TEER assay is valuable for assessing tissue integrity, tight junction function, and detecting damage to the epithelial layer. Another key parameter is ciliary beating frequency, which is used to assess how respiratory tissues react to conditions like chronic exposure sinusitis. Keeping a healthy ciliary beating frequency in both the upper and lower respiratory tract is key to protecting against airway infections. Each respiratory epithelial cell, which is part of the ciliated columnar epithelium, has around 200–300 cilia that beat in a coordinated rhythm. This movement helps clear out harmful particles and pathogens trapped in mucus produced by nearby goblet cells [111].

Cigarette smoke (CS) can interfere with the beating of cilia in the respiratory tract, reducing respiratory defence. This study demonstrates the use of organotypic tissue models to evaluate ciliary movement

and monitor the normal functioning of epithelial cells within these models. Additionally, CS activates xenobiotic metabolism pathways in the respiratory system, aiding in the breakdown of tobacco smoke components. The activity of phase I xenobiotic enzymes like CYP1A1 and CYP1B1 can be assessed using these tissue models. Furthermore, the effect of CS on xenobiotic metabolism can be examined by analysing the complete gene expression profiles in organotypic bronchial tissue models through transcriptomic data and a network-based systems biology approach [113].

This chapter outlines the methodology for exposing 3D bronchial and nasal tissue models to mainstream cigarette smoke (CS) using an in vitro exposure system, and for measuring the tissue responses relative to fresh air exposure (controls). [7].

7.3 Protocols

7.3.1. Culture of the Organotypic Tissue Bronchial Culture Model

In this study, commercially available human 3D bronchial epithelial tissue models—MucilAir —were used. These models were obtained from Epithelix Sàrl, a biotechnology company based in Geneva, Switzerland. MucilAir represents a monoculture of differentiated bronchial epithelial cells. Upon receipt, the tissues were approximately 45 days old and fully differentiated, containing basal cells, mucus-producing goblet cells, and ciliated columnar cells. These models were cultured at the air-liquid interface (ALI) in 24-well plates using Transwell inserts (6.5 mm diameter, 0.4 µm pore size, Corning Cat No. 3413). Each well was filled with 0.7 ml of pre-warmed MucilAir culture medium maintained at 37 °C, with 5% CO₂ and 90% humidity [7].

Medium renewal was performed every 48 hours, following the supplier's protocol. To ensure tissue integrity and sterility, inserts were regularly inspected under a microscope. Medium leakage was checked visually, focusing on signs such as:

- Discolouration or cloudiness in the medium, indicating contamination.
- Pooling of medium on the apical surface, suggesting compromised barrier function.
- Structural abnormalities in the tissue or insert membrane.

This visual inspection helped confirm that the epithelial barrier remained intact and that the culture conditions were stable throughout the exposure period [7].

7.3.2. Cigarette Smoke (CS) Exposure using an In Vitro Exposure System

Three days before the exposure experiments, the apical side of the tissue cultures was rinsed with 200 µl of MucilAir™ medium from Epithelix Sàrl. If measuring ciliary beating frequency before exposure,

tissues were prepared as described in section 7.3.3. The Vitrocell exposure system's climatic chamber and cultivation base were pre-heated to 37 °C. Each row of the base was filled with 17.5 ml of MucilAir™ medium. Tissues were transferred from the incubator to the base under a hood, covered with a glass lid, and then placed into the climatic chamber for cigarette smoke exposure. When a quartz crystal microbalance (QCM) was used, the modules were configured beforehand to enable real-time particle deposition monitoring via dedicated software. QCM crystals were replaced with fresh units and calibrated to zero before each experiment to ensure accuracy. The exposure followed the protocol in Figure 7.1. Prior to use, 3R4F reference cigarettes were conditioned for 7–21 days at controlled temperature and humidity, as per ISO 3402. Cigarette smoke was generated using a 30-port carousel smoking machine, adhering to the Health Canada Intense protocol, with standardized puff volume and butt length. (described in ISO 20778:2018: Intense Smoking Regime) [7].

7.3.3 Measurement of Ciliary Beating

The ciliary beating was recorded both before and after exposure, as per the experimental design outlined in Figure 7.1. The tissue culture plates were taken out of the incubator and positioned under the hood at ambient temperature for 30 minutes to allow the cilia to stabilise. Subsequently, the tissues were examined under a light microscope (Olympus BX53 biological microscope) connected to the CiliaMetrix Software with a camera (Basler acA640-120uc, Basler AG, Germany). A video was captured, and the ciliary beating frequency (in Hertz) was measured every 3 seconds over a period of 1 minute. Following the assessment, the tissues were reintroduced to the incubator, which maintained a temperature of 37°C, with 5% CO₂ and 90% humidity [7].

7.3.4. Transepithelial Electrical Resistance (TEER) Measurement

TEER measurements were taken three days before and 48 hours after exposure in a controlled setting using a Chopstick Electrode (STX-2) attached to a volt-ohmmeter (EVOM2, World Precision Instruments (WPI), Germany). To assess epithelial barrier integrity, 200 µL of culture medium was added to the apical surface of the tissue inserts. The EVOMX volt-ohm meter was activated, and the electrode was sterilised using 70% ethanol, followed by rinsing with fresh culture medium. The electrode was then gently inserted into the apical medium, ensuring no direct contact with the tissue surface. Electrical resistance (Ω) was measured to evaluate transepithelial electrical resistance (TEER), providing an indicator of tissue integrity and tight junction functionality. Five replicate measurements per sample were taken. Post measurements, the medium was gently aspirated from the upper side of the tissue cultures. Subsequently, the tissue cultures were placed back into an incubator set at 37 °C (5% CO₂, 90% humidity) for further incubation [7].

7.3.5 Cytochrome P450 (CYP) 1A1/1B1 Activity

The luciferin detection reagent (Luciferin Detection Reagent, Promega Corporation) was prepared before the study, following the manufacturer's instructions. The reconstitution buffer was placed in an amber bottle with the reagent, and 2-millilitre aliquots were stored at -20°C for a maximum of one month. After exposure to CS, tissues were incubated for 48 hours at 37°C to reach the desired post-exposure time. Prior to use, the luciferin detection reagent was equilibrated to room temperature. Tissue inserts were exposed to the luciferin-CEE, a proluciferin substrate specifically designed for use with CYP1A1 and CYP1B1 enzymes. The substrate, diluted 1:50 in the basal culture medium, was applied for either three hours or overnight. Subsequently, 50 µl of culture medium from each tissue insert was moved to an opaque white 96-well luminometer plate at room temperature. To start the luminescent reaction, 50 µl of luciferin detection reagent was added to each well. The plate was left for 20 minutes at room temperature, and then luminescence was quantified using a luminometer (GloMax® 96 Microplate Luminometer, Promega Corporation) [7].

7.3.6 RNA Extraction.

Various items were assembled for collecting 3D organotypic tissue inserts, including dry ice, blades matching the number of inserts, cold PBS lacking calcium chloride and magnesium chloride, a 25 ml glass pipette, and sterile glass needles for aspiration. Tubes for homogenising solid cellular samples were identified with ceramic beads, and each received 700 µl of Qiazol (QIAGEN, Hilden, Germany). Subsequently, the aspiration machine was activated. Afterwards, the plate with the 3D organotypic tissue inserts was taken from the incubator. The inserts underwent three cycles of washing with cold PBS, with each rinse being removed using a glass needle. Following the third wash, the PBS was withdrawn, and the plate was shielded to prevent desiccation. The membrane of each insert was intricately removed to rest flat on the blade. The tissue and insert membrane were then moved to the designated homogenising tube. The tube's cap was secured, and the contents were agitated thoroughly. The sample was then frozen with dry ice and preserved at -80 °C, or RNA extraction was initiated. Chloroform was added to trigger phase separation, followed by centrifugation to isolate the RNA-containing layer. Ethanol was mixed with the aqueous phase, and the solution was passed through a silica column for purification. Residual DNA was removed enzymatically, and RNA was eluted in nuclease-free water for downstream analysis [7].

7.3.7 Immunostaining of Tissue Inserts

To visualise specific cell types within airway tissues, a fluorescence-based labelling approach was applied. Samples were first treated with a mild fixative, followed by a detergent to allow antibody

access. After blocking non-specific sites, primary antibodies targeting p63 and Muc5AC were introduced and left to bind overnight under chilled conditions. Fluorescent secondary antibodies were then added, and nuclei were counterstained before imaging with a fluorescence microscope [7].

7.4 Results

Three-dimensional bronchial and nasal tissue models were continuously exposed to mainstream cigarette smoke (CS) at the air–liquid interface using the Vitrocell exposure system. The detailed experimental procedure is outlined in the methods section and illustrated in Figure 7.1. This chapter showcases the use of human organotypic bronchial and nasal tissue models to evaluate the biological impacts of CS exposure under controlled in vitro conditions using the Vitrocell system. The exposure modules are capable of evaluating the toxicity of airborne pollutants, including particles, nanoparticles, and other substances. In this study, the Vitrocell system was used, which can accommodate up to 48 samples at once (see Chapter 2). This facilitates larger-scale experiments and helps minimise variability between treatments. However, it is essential to address the significant concern of tissue culture contamination when conducting aerosol exposure experiments in vitro. Therefore, careful handling of the tissue cultures throughout the study is essential [7].

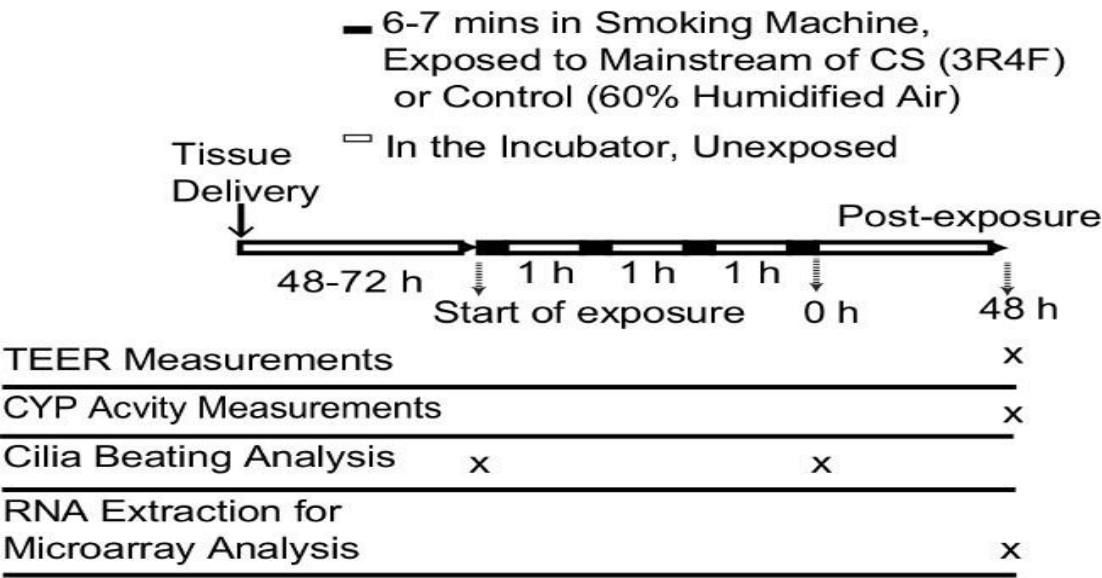


Figure 7.1: Schematic outline of the repeated CS exposure procedure on organotypic bronchial tissue models. Abbreviations: CYP, cytochrome P450s; CS, cigarette smoke; TEER, transepithelial electrical resistance.

It is essential to measure particle deposition on the QCMs in real-time during the exposure experiment to ensure the delivered doses align with the exposure system's standards. To do this accurately, the online measurement must be configured appropriately beforehand, such as setting the scale to zero. Additionally, because the microbalance has an area of 1 cm², while the tissue culture insert measures 0.33 cm², final calculations should be adjusted based on the insert area. Only about 33% of the material measured by the microbalance corresponds to the actual deposition in the culture insert. The in vitro bronchial and nasal models used in this study closely resemble the cellular structure of the

human respiratory epithelium. This makes them highly relevant for investigating respiratory functions and disease mechanisms. A visual representation of these models is provided in Figure 7.2 [7].

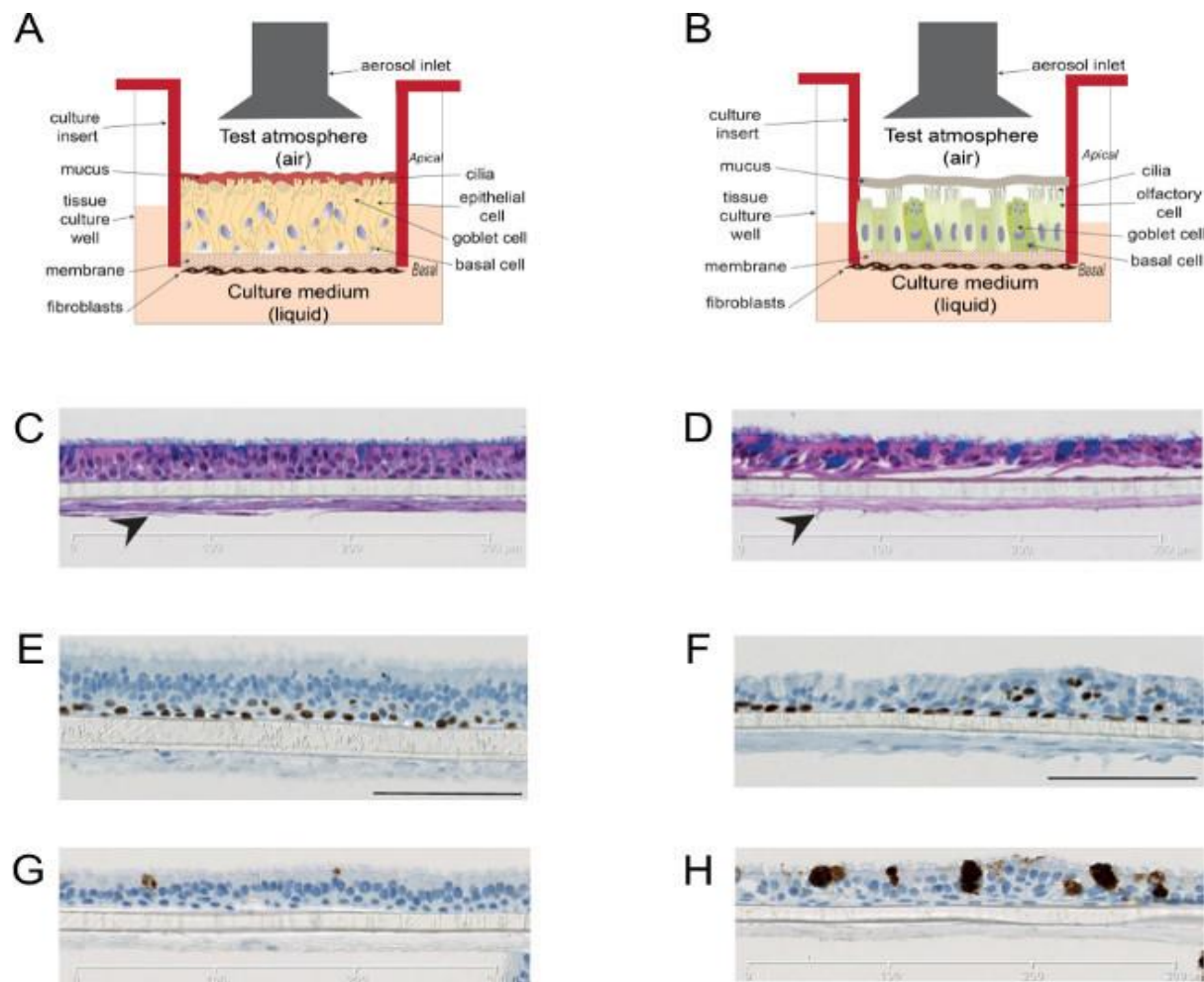


Figure 7.2 Models of organotypic bronchial and nasal epithelial tissue culture are depicted in the diagram: The bronchial (A) and nasal (B) tissue culture inserts can be seen at the air-liquid interface. In these in vitro models, ciliated cells are present in the apical layer of the Hematoxylin & Eosin-stained cells (C) for bronchial, D for nasal), as previously documented. Co-cultured with fibroblasts, these models rely on them to grow and differentiate epithelial cells (indicated by arrows). Staining of airway

lineage markers, such as p63 (E for bronchial, F for nasal) and Muc5AC (G for bronchial, H for nasal), are shown [7].

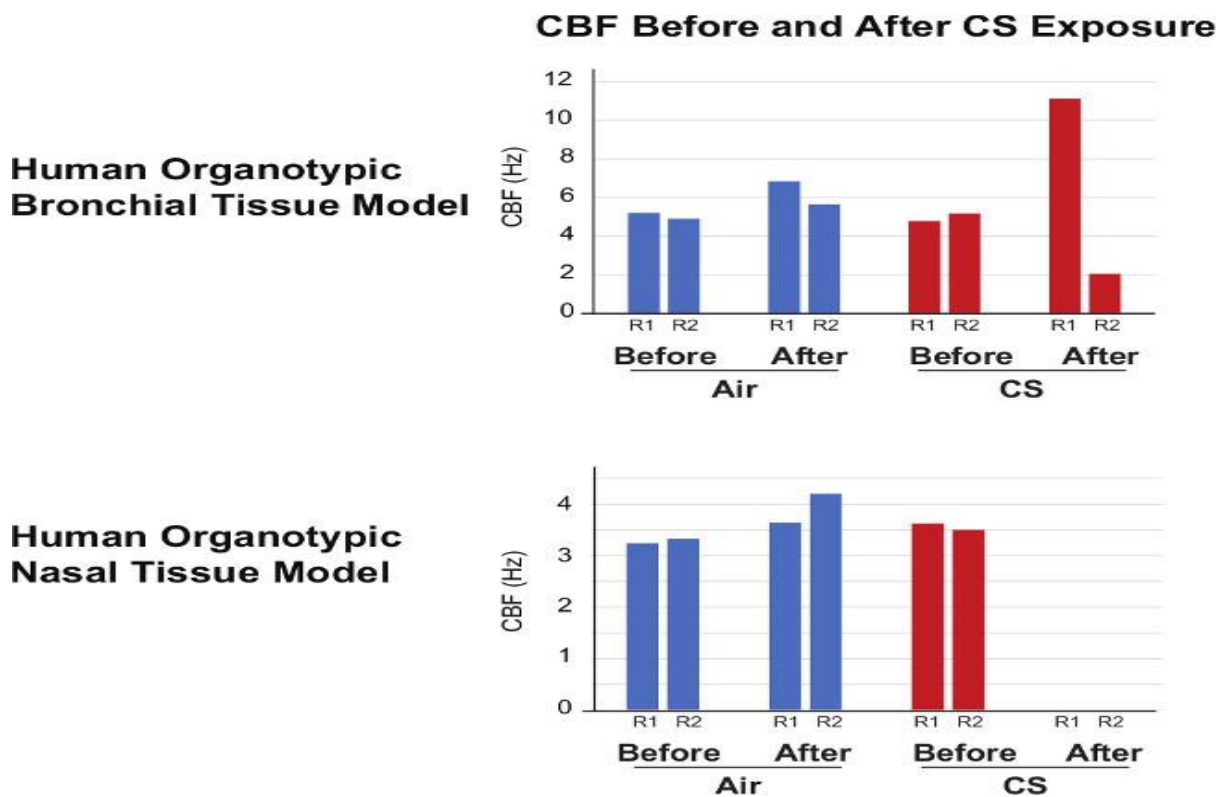


Figure 7.3 The cilia beating frequency (CBF) was measured in air-exposed and culture samples before and after exposure: A notable decrease in CBF was recorded following exposure to the CS. The results, illustrated in two separate inserts, are categorised as replicate 1 (R1) and replicate 2 (R2). These findings suggest a consistent trend across both replicates, indicating a potential impact of CS on CBF [7].

In this study, we have successfully demonstrated that exposure to cigarette smoke (CS) harms the movement of cilia in both nasal and bronchial tissue models (Figure 7.3). These effects have been consistently observed across different animal models. To ensure accurate measurement of ciliary movement, consistently handling and treating the tissues is crucial. Previous studies have shown that pH levels can influence ciliary movement frequency in mammals, as evidenced by comparing ciliary movement frequencies in cells treated with various compounds or mixtures [7, 114]. Therefore, it is crucial to consider adjusting pH levels to minimise variability. For instance, if the medium changes, it should be done consistently across all samples. Additionally, the temperature at which measurements are taken plays a crucial role, as ciliary movement frequency tends to decrease at lower temperatures. In our study, we employed a stage-top incubator with precise temperature, CO₂, and humidity control

to reduce variability resulting from these factors. Despite our efforts, we observed highly variable ciliary movement frequencies in bronchial tissues following CS exposure, indicating a significant disturbance immediately after exposure. In contrast, nasal tissues exhibited no measurable ciliary movement after CS exposure, suggesting they are more sensitive and consistent in their response. This observation aligns with previous research, which has shown that nasal tissue has a lower capacity for detoxification than bronchial tissue[115].

7.5 Discussion

The results of this study highlight the effectiveness of 3D bronchial and nasal tissue culture models as advanced in vitro platforms for assessing the toxicity of cigarette smoke.. The observed reduction in ciliary beating frequency following CS exposure across both tissue types provides compelling evidence of the immediate functional impairment caused by cigarette smoke components. Notably, the differential sensitivity between nasal and bronchial tissues, with nasal models showing complete cessation of ciliary activity while bronchial tissues maintained variable but reduced function, reflects the known anatomical and metabolic differences between these respiratory regions. The consistency of results across experimental replicates strengthens the reliability of these models for toxicological assessment. Furthermore, the integration of multiple endpoints, including TEER measurements, CYP enzyme activity, and transcriptomic analysis, provides a comprehensive framework for understanding the pathophysiology of CS-induced disease. The successful implementation of the Vitrocell exposure system demonstrates the value of physiologically relevant exposure conditions, which better replicate human inhalation scenarios compared to traditional submerged cell culture methods. These findings collectively support the utility of 3D tissue models as valuable tools for respiratory toxicology research, offering mechanistic insights that can inform regulatory decisions and guide the development of harm reduction strategies for inhalation products [7].

7.6 Conclusion

This research demonstrated the feasibility and importance of using organotypic 3D bronchial and nasal tissue culture models for repeated exposure to whole cigarette smoke under air–liquid interface (ALI) conditions. Simulating physiologically relevant exposure scenarios provided valuable insights into tissue-specific responses, including inflammation, cytotoxicity, and changes in gene expression. The study confirmed the effectiveness of advanced in vitro models for inhalation toxicology and laid the groundwork for assessing emissions from tobacco products [7].

7.7 Research Impact

Since its publication in 2015, this study has had a significant influence on the field of inhalation toxicology, with 47 citations recorded on Google Scholar. It pioneered the use of three-dimensional (3D) tissue models in combination with the Vitrocell® exposure system, establishing a new methodological standard for evaluating the effects of cigarette smoke and aerosols on complex respiratory tissues.

By moving beyond traditional submerged cell cultures, this research introduced a physiologically relevant in vitro platform that enabled more accurate assessments of tissue-level responses to inhaled toxicants. The integration of multiple endpoints—including ciliary beating frequency, TEER, CYP enzyme activity, and transcriptomics—provided a comprehensive framework for mechanistic toxicology and disease modelling. This methodological advancement has since been adopted and expanded upon by independent research groups, demonstrating its broad scientific utility and translational value [59].

A notable example of its continued impact is the work by Iskandar et al., who applied the same exposure system and tissue models in a systems toxicology framework to compare the biological effects of various inhalable products [77]. While I contributed as a co-author to that study, it lies outside the scope of this thesis. Nevertheless, it represents a direct methodological evolution from the protocols established in this research, underscoring the foundational role this study has played in shaping current approaches to in vitro inhalation toxicology and harm reduction science.

8.0 Development and testing of a new-generation aerosol exposure system: The independent holistic air-liquid exposure system (InHALES)

8.1 Introduction and Contribution

This chapter discusses the development of new aerosol exposure systems for in vitro inhalation research. A key question arises: Why do we need a new system when the existing Vitrocell system is already well-characterised? The answer is simple: the new system provides a controlled environment that enables more effective modelling of airflow changes due to breathing and various lung compartments. It facilitates realistic aerosol exposure experiments, effectively bridging in vitro and in vivo dosimetry. The primary objective is to enhance the reliability, efficiency, and biological relevance of in vitro exposures, which are crucial for drug discovery. The Independent Holistic Air-Liquid Interface Exposure System (InHALES) tackles these challenges by accurately simulating the human respiratory tract.

This chapter focuses on the research paper **"Development and testing of a new-generation aerosol exposure system: The independent holistic air-liquid exposure system (InHALES)"** (Steiner S, Herve P, Pak C, **Majeed S**, Sandoz A, Kuczaj A, Hoeng J) (25). Development and testing of a new-generation aerosol exposure system: The independent holistic air-liquid exposure system (InHALES). *Toxicol In Vitro*. 2020 Sep;67:104909. doi: 10.1016/j.tiv.2020.104909. Epub 2020 Jun 5. PMID: 32512146.). As one of the co-authors of this article, my responsibilities included conceptualisation of the idea, developing the new system, assisting in experiment design, conducting exposure experiments, and contributing to the documentation for publication. Throughout our study, we successfully developed a new and unique exposure system. Following this achievement, we filed a patent application, and I am pleased to report that we have successfully received the patent for this groundbreaking work. **(SIMULATED RESPIRATORY TRACT)** (see Appendix A).

8.2 Background

Biological significance includes factors like flow velocities, patterns, aerosol dilution, gas-particle partitioning, and aerosol properties that affect dose delivery. This is particularly important in drug discovery and related areas of development. Attaining a high level of biological significance in the early stages of in vitro research can enhance the success rate of screening drug candidates, provide justification for progressing to animal or human testing phases, minimise the need for animal experimentation, reduce the risk of failures in later stages, and foster trust in in-vitro sciences among regulatory bodies and the general public [76].

When a living organism inhales an aerosol, it interacts with the thin layer of fluid covering the respiratory tract. This interaction—whether through deposition or diffusion—is influenced by the aerosol's physical and chemical characteristics, such as particle dimensions, tendency to evaporate, and affinity for water. Additionally, the nature and speed of airflow, whether smooth or chaotic, play a significant role in how the aerosol behaves during inhalation. Additionally, the regional anatomy of the respiratory tract, including airway diameters and branching patterns, and the length of time the aerosol stays in a specific region, also influences these interactions [39, 116]. Airflow patterns, local airflow speeds, and the shape of the respiratory tract influence the shear forces experienced by the respiratory tract's surface. These factors also affect the baseline conditions of the surface, which in turn impact its response to external factors. The composition and physical properties of inhaled air, along with the surrounding environment, constantly change as it moves through the respiratory system in a living organism [117].

To ensure its relevance to humans, researchers need to focus on delivering test aerosols to biological test systems in a way that closely resembles specific regions of the respiratory tract. While theoretical knowledge is available, quantifying mass deposition in the respiratory tracts of living organisms non-invasively remains challenging. The complexities of the respiratory system, along with the physics and chemistry of test aerosols, further complicate these efforts [119]. Many studies have been conducted on this topic; however, due to these challenges, they often produce incomplete and contradictory findings [119].

This study outlines the development of a novel aerosol exposure system, the Independent Holistic Air-Liquid Interface Exposure System (InHALES). InHALES is designed to simulate the human respiratory tract, providing a realistic and controlled environment for aerosol exposure experiments. This system enables cell cultures to be exposed at the air-liquid interface, mimicking different parts of the respiratory tract. It can also generate test atmospheres without the need for additional aerosol generators. The InHALES system adopts a new approach to aligning *in vitro* with *in vivo* conditions dosimetry. Rather than determining *in vivo* doses and applying them to test systems, it creates physiological conditions that mimic the behaviour of test aerosols in the human respiratory tract. This approach offers several advantages over conventional methods, including its applicability to any test atmosphere, the ability to cover all respiratory tract compartments simultaneously, and the testing of various breathing patterns and behaviours. We have tested and characterised a prototype of the InHALES using smoke from reference cigarettes and a fluorescent model aerosol. Compatibility with biological test systems was assessed by exposing human oral and bronchial epithelial cultures, as well as lung adenocarcinoma cell lines, during the study [39][120].

8.3 Methods

8.3.1 System structure and function

The initial system design focused on establishing its core functional principle. It involved an arrangement of interconnected pumps with varying displacement volumes. This configuration allowed the system to simulate the human respiratory system under different physiological states, including rest, physical activity, and the use of medical inhalers and cigarettes (Figure 8.1). The design was inspired by human physiology and behaviour, with a mechanical system developed to meet key functional requirements, including robustness, reliability, pump accuracy, and sealing. Additionally, the system was designed to be modular, meaning each module operates as an independent unit that can be replaced or modified without affecting the rest of the system or its components [39].

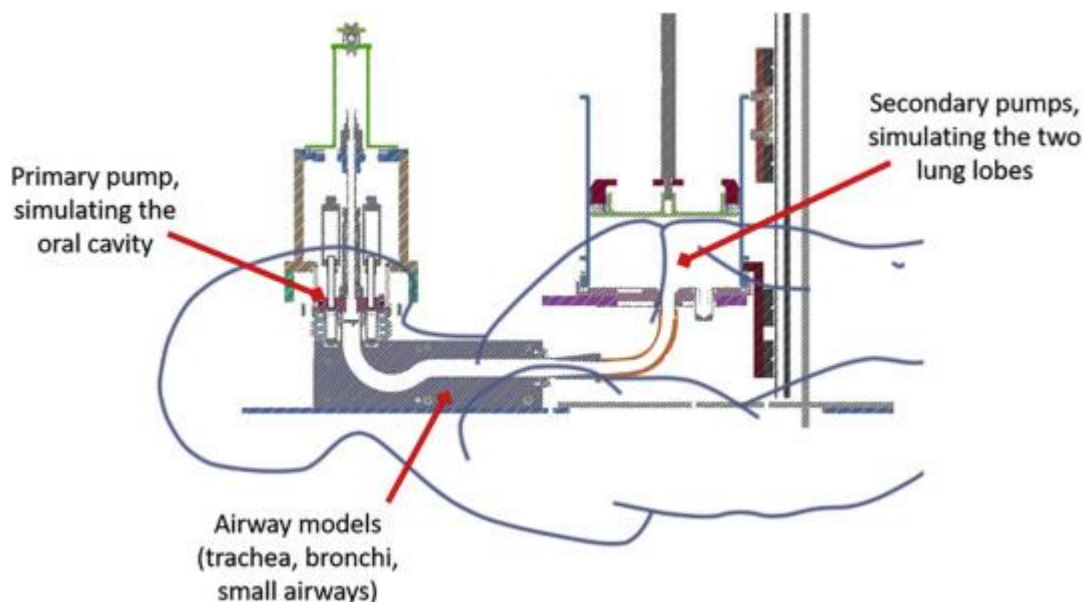


Figure 8.1 InHALES system: The fundamental design principle of the InHALES is centred around a primary pump that imitates the oral cavity, accompanied by two secondary pumps (with only one visible in the front view) that replicate the two lung lobes. Additionally, an airway model links the primary and secondary pumps. The grey illustration of a human body shows how these pumps and the airway model mimic the macroscopic structure of the respiratory tract [39].

The prototype system comprises six primary modules (see Figure 8.2). These include the inlet/outlet configuration, the primary pump (simulating the oral cavity), the upper airway model (representing the oropharyngeal cavity and trachea), the main bronchial model, the distal airway model (depicting the bronchioles and small airways), and the secondary pumps (which simulate the lungs). The system connects to the test atmosphere source and ventilation system for disposing of test gases. It also interfaces with the environment through the inlet/outlet structure and five air intakes for breathing.

Valves manage the flow of test atmosphere inlets and outlets, along with the air inhalation inlets. Additionally, a butterfly valve on the base plate seals the primary pump from both the airway models and secondary pumps [39].

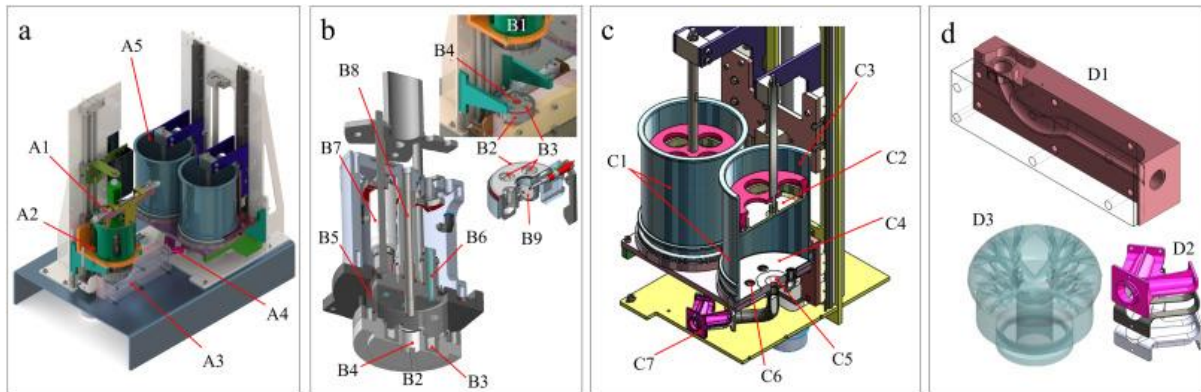


Figure 8.2A: The InHALES system includes several essential modules: an inlet/outlet structure linking to the test aerosol source (inlet) and the ventilation system for removing test aerosols (outlet); a primary pump simulating the oral cavity; an upper airway model representing the oropharyngeal cavity and trachea; a main bronchial model; and secondary pumps that mimic the lung lobes. The distal airway models, situated within the secondary pumps and not visible externally, illustrate the bronchioles and small airways [39].

Figure 8.2B The detailed design of the primary pump is illustrated. In the top view, the pump appears in its open state with the cylinder lifted. The pump's base plate features five slots for cell culture exposure and a central opening where the airway models connect. The bottom left view offers a vertical section of the pump, showing the cylinder, three of the five inhalation air inlets, and one of the pinch valves that regulate airflow inlets. Test atmospheres flow into the pump through the hollow piston shaft, which connects to the inlet/outlet structure. The bottom right view displays a vertical section of the pump's base plate, providing a detailed view of the cell culture holders with cell culture inserts and the butterfly valve that manages the connection to the airway models.

Figure 8.2C showcases the secondary pumps, which include two identical units, each with a piston, cylinder, and base plate. One pump features a vertical section with a central opening for test atmospheres' entry and exit, along with three of the five slots used for cell culture exposure. The bronchial airway model connects to this central opening, while the tracheal model attaches directly to the open side of the bronchial model. The distal airway model is inserted into the secondary pumps' opening. Green arrows indicate aerosol flow direction.

Figure 8.2D The diagram illustrates the airway models. The tracheal model has two parts and can be opened lengthwise. The components used for inserting hydrogels and cell cultures are not depicted. The main pump base plate connects directly to the left side of the tracheal model. When fully assembled, airflow is regulated by a butterfly valve located 2 cm above the tracheal entrance. On the right, the bronchial model connects directly to the tracheal model. The distal airway model fits into the central opening of the secondary pump base plates. [39].

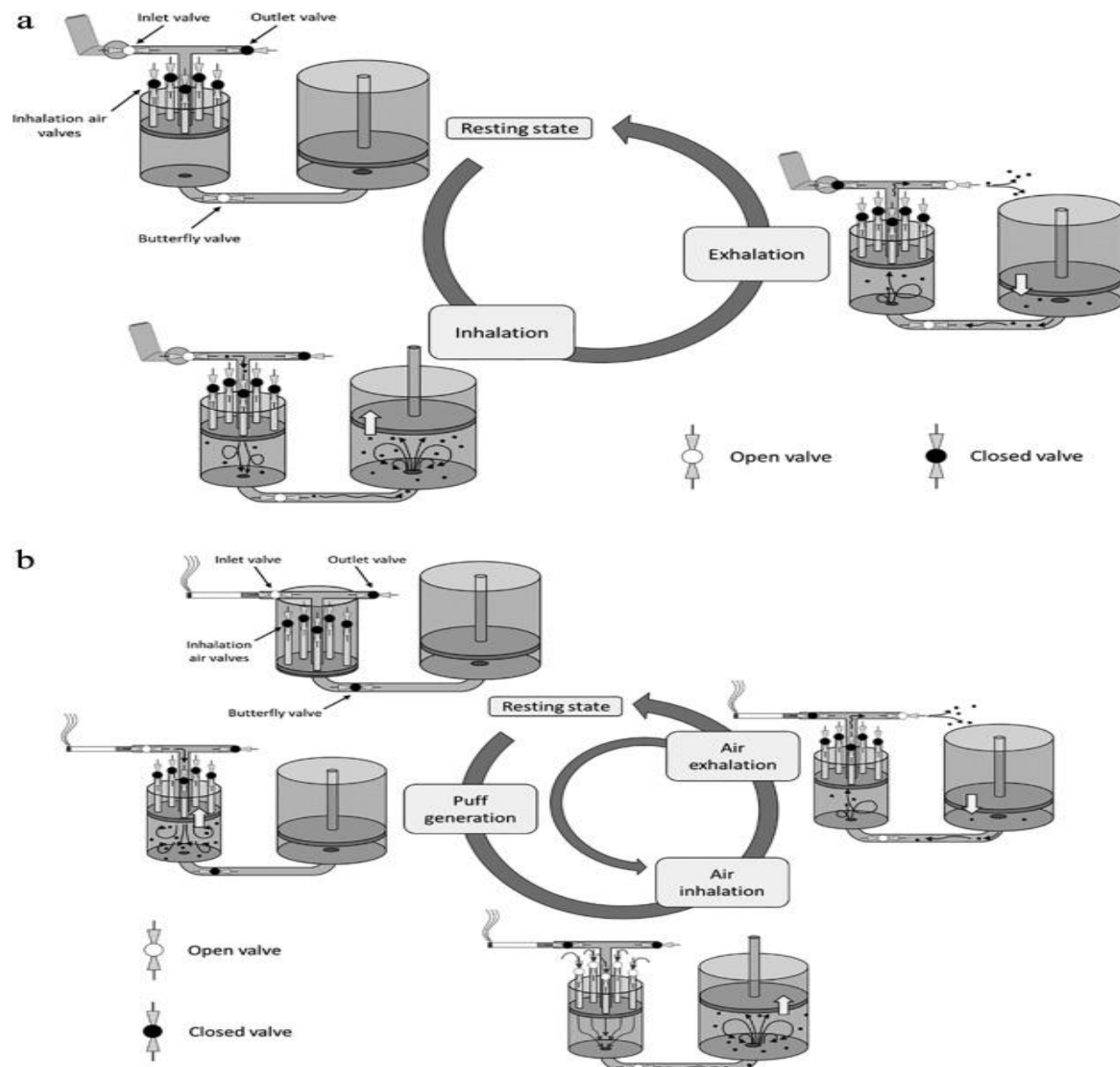


Figure 8.3A: Exposure Cycle Without Puffing: During rest periods, the main pump stays open, while the secondary pumps are either closed or set to keep a certain residual lung volume. The inlet and butterfly valves are open, and the inhalation and outlet valves are closed. When the secondary pumps activate, they draw in test aerosols, such as from a pressurised metered-dose inhaler, for collection and analysis. The aerosol passes through the primary pump and airway models to reach the secondary pumps (inhalation phase). Afterwards, the inlet valve closes, the outlet valve opens, and exhalation

occurs as the secondary pumps shut. The system then returns to the resting state and prepares for the next cycle [39].

Figure 8.3B: Exposure Cycle With Puffing: During rest, the inlet valve stays open, while the outlet valve, inhalation air valves, and butterfly valve are closed. The primary pump is turned off, and the secondary pumps are either off or set to maintain a specific residual lung volume. A puff, similar to a cigarette puff, is created by activating the primary pump and holding it for a set mouthward inhale during the exposure cycle. After the inlet valve closes, the inhalation air and butterfly valves open, activating the secondary pumps to draw in clean air and puffs. When the outlet valve opens and the inhalation air valves close, the secondary pumps expel the test atmosphere. The system can repeat several inhalation cycles by toggling the inhalation air valves and outlet valve between open and closed. To reset for a new cycle, the system closes the primary pump, butterfly valve, and inhalation air valves, then opens the inlet valve, returning to the starting state.

The core functionality of the InHALES system involves coordinated activation of pumps and adjustment of valves to regulate airflow and isolate components within the airway model. This configuration enables the controlled intake and release of either a test atmosphere or clean air, thereby establishing a reproducible inhalation cycle. By manipulating the timing and flow parameters, multiple inhalation cycles can be combined to form defined exposure cycles, allowing for precise dose delivery and simulation of realistic breathing patterns [39].

The system operates similarly to the human respiratory tract. There are two main scenarios:

- 1) Inhaling with puffing, like smoking a cigarette, requires the main pump to operate
- 2) Inhaling at rest, which does not require the main pump to activate

Figure 8.3 illustrates both scenarios. Exposure protocols typically include both inhalation cycle types. Therefore, the system operator can customise the cycles by adjusting the number of repetitions and the variety within a single exposure protocol. If no puffing is needed (Figure 8.3A), the test atmosphere can enter the system either through the test atmosphere inlet, such as with an inhaler, or via the inhalation air inlets, for instance, when testing environmental samples or clean air. However, if puffing is necessary (Figure 8.3B), the test atmosphere enters the primary pump through the test atmosphere inlet. The puff generated is then inhaled along with a larger volume of fresh air through the inhalation air inlets, aided by the secondary component pumps. The software controls the valves and pump actuations to open and close the corresponding channels [39].

8.3.2. Construction and Material Choices

Piston pumps powered by stepper motors were selected for their durability, reliability, and accuracy in simulating airflow within the oral cavity and lung lumen. Most valves managing both test atmospheres and clean air flows were compact, pneumatically operated pinch valves, selected for their synchronisation and space-saving design. Components in direct contact with the test atmospheres and cell culture medium were made from stainless steel. Additive manufacturing was employed for more complex parts, with rubber O-rings used as seals—except for the piston seals in secondary pumps, which were made from graphene. The entire setup was assembled in-house [39].

8.3.3 Functional system testing

To validate system performance, a series of tests was conducted focusing on the coordinated activation of pumps and valves, the ability to configure and execute operational cycles via software, and the overall integrity and precision of the setup. Leak tightness was evaluated by applying pressure and measuring the volume of air escaping the system. Pump accuracy was assessed using calibrated soap bubble meters. All testing procedures were performed in an environment without heating, using air that was neither heated nor humidified [39].

8.3.4 System characterisation

8.3.4.1 Exposure protocols

The system was evaluated under two distinct respiratory simulation modes: shallow inhalation and deep inhalation. These modes were developed to closely mimic realistic aerosol exposure conditions. Shallow inhalation represented normal, low-volume breathing patterns, while deep inhalation simulated more forceful and prolonged respiratory cycles, akin to those experienced during physical exertion or therapeutic inhalation procedures [39].

Each protocol was programmed into the system using custom software, enabling precise control over airflow timing, volume, and valve coordination. Aerosol delivery was carried out using ambient, non-heated, and non-humidified air. These controlled conditions allowed for reproducible deposition of aerosol particles across the air-liquid interface, supporting physiologically relevant in vitro exposure scenarios [39].

8.3.4.2 System setup for test exposures

Figure 8.4 illustrates the experimental setup and procedure for the test exposures. The exposure system, humidification unit, and test atmosphere source were all located inside a heating chamber.

Test atmospheres were produced using heated, non-humidified air to mimic aerosol delivery conditions. Conversely, the air entering through the inhalation inlets was both heated and humidified to better simulate physiological conditions. Temperature and relative humidity of the conditioned air were continuously monitored inside the conditioning chamber, outside the exposure system, to ensure consistent and reproducible conditions throughout the exposure. Leak tests were conducted before each experiment to verify system integrity [39].

8.3.4.3 Test atmosphere generation

The experiment involved the examination of three different atmospheres: clean air, smoke from 3R4F cigarettes, and a liquid model aerosol. These atmospheres were introduced into the exposure system puff by puff. The clean air and smoke were puffed inside a heated chamber, while the liquid aerosol was generated with the help of a nebuliser. The puffing adhered to the Intense smoking regime established by Health Canada, with ten puffs taken per cigarette [39].

8.3.4.4 Determination of aerosol mass delivery

PBS samples were analysed to measure the quantity of test atmosphere constituents deposited during exposure. These constituents were supplied only through the primary and secondary channels of pumps. Nicotine, carbonyl compounds, and DSF were measured using established analytical methods. The procedures for nicotine and carbonyl compound analysis are detailed in Chapter 2 (section 2.4.4), while the method for DSF quantification is described in Chapter 4 (section 4.3.2). Each pump type and experimental repetition involved exposing three 300 μ L PBS samples, with 50 μ L used for quantifying nicotine or DSF, and the remaining amount used to quantify carbonyl compounds. The three samples were combined for carbonyl quantification, resulting in a single value per exposure and per pump [39].

8.3.4.5. Aerosol delivery efficiencies

The delivery efficiencies of nicotine and disodium fluorescein (DSF) in the test atmosphere were measured using 3R4F cigarette smoke and an aerosol of propylene glycol/glycerol (PG/G). The amounts of nicotine and DSF deposited per square centimetre of the cell culture insert surface were calculated and shown as a percentage of the total amount introduced into the system during exposure period [39].

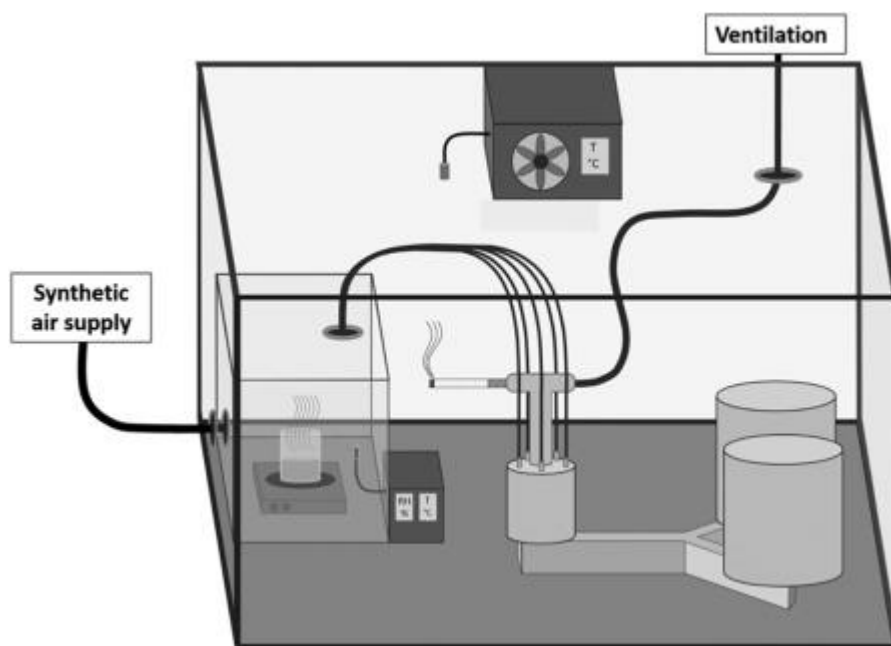


Figure 8.4 The InHALES setup was used for test exposures. The system was housed in a temperature-controlled chamber set at 37°C. To keep the relative humidity above 95% and the temperature steady at 37°C, inhalation air was conditioned in a separate heated humidification chamber. For 3R4F cigarette smoke, air exposures, and liquid aerosol experiments, the test atmosphere was drawn either from this heated chamber or a separate synthetic air supply connected to a Collison nebuliser. After passing through the system, the test atmospheres were vented [39].

8.4 Results

8.4.1 Functional system testing

The core functional tests, which include checking the coordinated pump operations and valves, conducting leak tests, and evaluating pump accuracy, verified the proper functioning of the overall system. Leak tests showed the system was nearly airtight under static conditions. At 3000 Pa pressure, it experienced a minimal loss of just 0.4 mL per minute. The main pump's displacement volumes mostly remained within 1 mL of the target 55 mL, demonstrating over 98% accuracy. It should be noted that these tests were performed without any resistance at the system inlet. Furthermore, the secondary pumps' displacement volumes were consistently lower, deviating by approximately 5.5% from the target value [39].

8.4.2 Test atmosphere delivery patterns

Figure 8.5 illustrates the delivery of nicotine, eight specific carbonyl compounds, and DSF to PBS samples, mimicking cell culture conditions. Usually, the primary pump delivered a greater mass per square centimetre than the secondary pumps. Shallow inhalation protocols resulted in higher mass deposition than deep inhalation, likely because the primary pump has a smaller surface area and volume and delivers undiluted aerosol. Variations in mass delivery may stem from incomplete flushing of the primary pump with dilution air, higher aerosol concentrations in the secondary pump, and the smaller surface area and volume of the secondary pumps during shallow inhalation compared to deep inhalation. [39].

The analysis provided insights into the varying delivery patterns of nicotine and DSF under different exposure scenarios. It was observed that the levels of smoke constituents fluctuated based on several factors, including the specific exposure protocol used, the location of the inserts, and the type of constituent being measured. Notably, in scenarios involving air exposures, there were no detectable concentrations of nicotine, carbonyl compounds, or DSF present. This indicates that certain exposure conditions may significantly influence the presence and distribution of these substances in the environment [39].

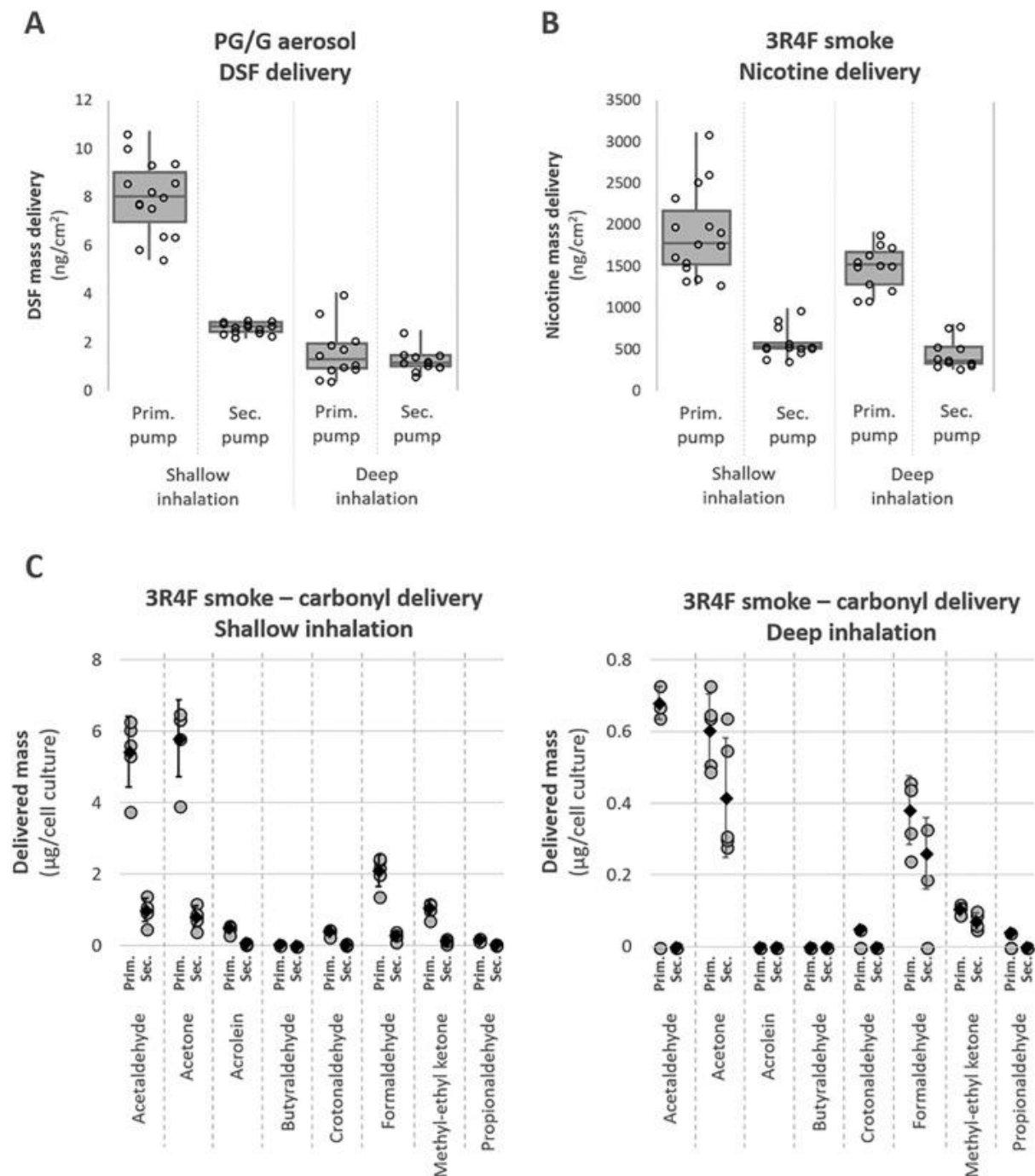


Figure 8.5: Testing the delivery of atmospheres to cell culture inserts: (A) and (B) Mass delivery of disodium fluorescein (DSF) during propylene glycol/glycerol (PG/G) aerosol exposures and nicotine delivery during 3R4F smoke exposures, assessed in both primary and secondary pumps, as well as during shallow and deep inhalation exposures. Three cell culture inserts containing phosphate-buffered saline were exposed in five independent replicates. Circles represent individual samples, and those from the exact replicate are aligned vertically. Boxplots display the range of measured values, including the 25th, 50th, and 75th percentiles. (C) and (D) The deposition of eight carbonyl compounds was examined in primary and secondary pumps during 3R4F smoke exposures with shallow and deep

inhalation. Circles denote individual data points (one per replicate), while black diamonds indicate average values across the five replicates, with error bars showing standard deviations [39].

8.4.3 Test atmosphere delivery efficiencies

Throughout the entire exposure protocol (60 puffs), the total mass of nicotine that entered the system was 4.97 ± 0.59 mg. This amount is lower than the anticipated quantity based on the known nicotine masses from 3R4F cigarettes, which is 12 ± 1.5 mg [39]. The length of the cigarette butt was found to be approximately 0.5 cm longer than expected after 10 puffs, indicating a potential shortfall in puff volumes, possibly due to the resistance caused by the flow of the cigarette. During this stage of system evaluation, the total mass of the test atmosphere was considered negligible in impacting delivery efficiency, repeatability, and uniformity. The median nicotine mass delivered to cell culture inserts was measured with both primary and secondary pumps under shallow and deep inhalation conditions. Delivery efficiencies, expressed as a percentage of the total nicotine introduced per square centimetre of the insert, were documented as:

Shallow inhalation (primary pump): 0.036%,

Shallow inhalation (secondary pump): 0.011%,

Deep inhalation (primary pump): 0.030%,

Deep inhalation (secondary pump): 0.007%.

These results highlight the influence of inhalation depth and pump configuration on aerosol deposition efficiency.. Throughout a full exposure protocol, the DSF mass entering the system was recorded as 6.6 ± 2.3 µg. For both pumps, the delivery efficiencies per square centimetre are 0.12% for shallow inhalation and 0.02% for deep inhalation [39] .

8.4.4 Uniformity and repeatability

The data in Figures 8.5A and B show consistent aerosol delivery to replicate cell culture inserts for nicotine and DSF under the same conditions and positions. This indicates that the amount of aerosol received by each cell culture may differ depending on the compound being targeted. Although a statistical analysis was not performed to evaluate the impact of inter- and intra-repetition variation on the overall distribution, the data suggest these variations are similar. Notably, the secondary pumps showed greater uniformity and repeatability during exposures [39].

8.5 Discussion

An advanced aerosol exposure system called InHALES was created to replicate the physical and chemical conditions of the human respiratory tract. Unlike conventional systems that depend on

precise dose measurements, InHALES predicts how substances interact within the respiratory system by simulating its environment. The prototype aimed to verify whether the system could consistently deliver test atmospheres to biological models in a controlled manner. Additionally, it evaluated whether the delivery method itself induces any stress on the test systems, even without exposure to test atmospheres. This assessment is crucial to determine if the delivery process might affect cell function or structure through physical stress [39].

Our research shows that the InHALES system provides aerosol control similar to traditional methods. It works with advanced human respiratory tract cell cultures and can induce specific biological responses under different aerosol and exposure conditions. The system creates unique patterns of aerosol evolution and deposition influenced by various factors. We focus on maximising the physiological relevance of the aerosol characteristics and doses delivered. Compared to in vivo data, the system closely replicates physiological dosing of nicotine and particulate matter in the oral cavity. However, in alveolar spaces, it currently delivers more aerosols than what is usually retained at physiologically normal rates. Efforts are currently being made to optimise the system so that the next version of InHALES will deliver physiological dosing [39].

After confirming the core functionality, repeatability, and consistency of the test atmosphere delivery, and ensuring its compatibility with cell cultures, InHALES shows significant promise. Our current focus is on developing a more advanced next-generation version that includes the following features enhancements:

- i) Expanding airway models to include more airway generations and improving overall geometry.
- ii) Creating modules to increase internal surface area and ensure laminar flow behaviour in secondary pumps.
- iii) Modifying internal system surfaces to replicate respiratory tract properties.
- iv) Integrating a nasal module to simulate nasal breathing.
- v) Developing culture holders equipped with sampling devices and probes.
- vi) Enhancing the cell culturing method.

After these upgrades are implemented, we will thoroughly investigate InHALES' capability to simulate in vivo exposures accurately [39].

8.6 Conclusions

A cutting-edge aerosol exposure system called InHALES was designed specifically to replicate the conditions of the human respiratory tract. This innovative system ensures that test atmospheres can

be delivered to cell cultures in a manner that closely mimics the delivery to a living person. Extensive testing has been conducted on the prototype of InHALES to verify its technical functionality, consistent delivery of test atmospheres to cell cultures, and compatibility with advanced cell culture techniques [39].

An advanced version of the system is currently under development, aimed at more accurately replicating the anatomical and functional characteristics of the human respiratory tract. This improved model aims to increase the physiological relevance of in vitro inhalation toxicology studies, especially for human health risk assessment. The system integrates exposure mechanisms with the complexity and sensitivity required by modern cell culture methodologies and downstream biological analyses.

Beyond toxicological uses, the improved system is expected to provide valuable insights into aerosol dosimetry, assist in developing inhalable drug formulations, and enhance the effectiveness of inhalation-based methods and therapies [39].

8.7 Research Impact

After the publication of this research article, numerous follow-up studies were conducted in the field. Consequently, the article has been cited eight times on Google Scholar, underscoring its impact. Additionally, new exposure systems have been developed and published in the field of inhalation toxicology, such as the Modular Air-Liquid Interface Aerosol Exposure System (MALIES), which is designed to study the toxicity of nanoparticle aerosols in 3D-cultured cells in vitro (106). We have released an engaging video showcasing a physical demonstration of the system, titled "The InHALES: The next chapter in the story of in vitro science?" (Link to the video: [The InHALES: The next chapter in the story of in vitro sciences? - Sci Ani](#)).

Crucially, InHALES aligns with the 3Rs principle—Replacement, Reduction, and Refinement—by offering a strong alternative to animal testing. It allows direct exposure of human airway epithelial cells at the air-liquid interface, effectively replacing the need for animal models in many experimental settings. Its reproducibility and efficiency also help to reduce the number of tests required, while the use of physiologically relevant models enhances data quality, thereby refining experimental outcomes. This approach is consistent with broader scientific and regulatory trends that highlight ethical research practices and the development of non-animal alternatives[39]. Together, these innovations indicate a shift towards more ethical, scalable, and predictive toxicological testing methods that better reflect human biology and diminish reliance on animal model experimentation.

9.0 Application of Secondary Electrospray Ionization Coupled with High-Resolution Mass Spectrometry in Chemical Characterization of Thermally Generated Aerosols

9.1 Introduction and Contribution

Assessing and predicting lung drug absorption and distribution is crucial in the early stages of developing inhaled therapies. As a result, researchers continually improve in vitro, ex vivo, and in vivo studies. One commonly used approach involves air-liquid interface (ALI)-cultured 3D organotypic models to simulate lung epithelial tissue research. Overall, the focus of these preclinical methods has shifted toward their capacity to predict human lung and systemic drug exposure, which aids in identifying optimal delivery systems. To achieve accurate results, it is crucial to understand the dosages needed for correct dosimetry in in vitro exposure studies. A significant challenge lies in translating in vivo doses to in vitro models, as most in vivo research involves rodent models [121].

Accurate dosage measurement during in vitro exposure studies is essential for characterising the toxic compounds humans inhale regularly. In this chapter, I will focus on the research we conducted using real-time analyses of aerosols through secondary electrospray ionization (SESI) technology combined with high-resolution mass spectrometry (HRMS). This technology, originally developed for analysing exhaled breath, aims to detect the primary constituents of aerosols.

In this chapter, I will discuss the research paper "**Application of Secondary Electrospray Ionization Coupled with High-Resolution Mass Spectrometry in Chemical Characterization of Thermally Generated**" (Zivkovic Semren T, **Majeed S**, Fatarova M, Laszlo C, Pak C, Steiner S, Vidal-de-Miguel G, Kuczaj A, Mazurov A, Peitsch MC, Ivanov NV, Hoeng J, Guy PA.) [127]. As one of the co-authors of this article, my responsibilities included assisting in experiment design, supporting the measurement experiments, and contributing to the documentation for publication.

9.2 Background

Inhalation drug delivery has been used for more than two thousand years and provides several important benefits. These include a vast surface area in the lungs that allows for efficient absorption, decreased metabolic degradation, the potential to reduce systemic side effects, and the ability to attain therapeutic effects with lower drug doses [121, 122]. This promising delivery method has driven the development and manufacturing of devices that aerosolise the target compound for inhalation [122]. The use of inhalation techniques, such as vaping devices, has seen a significant rise due to the

growing popularity of inhaling drugs and dietary supplements. These modern technologies allow for the targeted delivery of specific compound supplements, contributing to their rapid development. This trend highlights the importance of using advanced analytical methods to characterise aerosols from these devices. These methods can identify the main ingredients in the product and detect possible impurities, contaminants, and byproducts that may form during vaporisation [123].

Both offline and online measurements are used to characterise aerosols chemically. The offline method entails capturing compounds on glass fibre filter pads or in impingers filled with a solvent. Once trapped, these compounds are extracted and analysed using techniques such as gas chromatography (GC) or liquid chromatography (LC) combined with mass spectrometry (MS). This approach allows for non-targeted screening and the concurrent detection of multiple compounds in aerosol. However, the offline approach has limitations, such as potential evaporation, inconsistencies in capturing the entire chemical spectrum, chemical cross-reactions, and time-consuming procedures [124].

Online analysis of aerosol chemicals is functional for targeted sampling, but existing approaches face certain constraints. New methods like DART-MS, Fourier-transform infrared spectroscopy (FTIR), selected-ion flow-tube (SIFT-MS), proton transfer reaction (PTR-MS), and single-photon ionisation (SPI MS) can identify specific compounds without needing sample preparation, although they often have limited resolution and selectivity. Using these methods together with high-resolution MS can improve detection accuracy and facilitate untargeted chemical identification. Innovative approaches like atmospheric-pressure chemical ionisation, Orbitrap mass analysers, and extractive electrospray ionisation–ultrahigh-resolution MS (EESI–Orbitrap) leverage high-resolution, accurate mass measurements to enhance chemical analysis of environmental aerosols speciation. Furthermore, integrating MS methods with tandem MS fragmentation provides greater sensitivity and selectivity for comprehensive aerosol chemical characterisation [125]. This approach has also been used to analyse electronic cigarette aerosols, providing real-time insights by simulating the actual vaping process and minimising sample loss [126].

The study aimed to use the SESI interface combined with HRMS to analyse exhaled breath in real-time. Its main objective was to determine the key components of aerosols inhaled from vaping devices. Additionally, an improved SESI interface was used to assess the aerosolisation performance of medications such as Chloroquine (CQ), Hydroxychloroquine (HCQ), and Azithromycin. [126].

9.3 Experimental Section

9.3.1 Chemical and Test Items

Vaping devices containing caffeine, melatonin, cannabidiol, and vitamin B12 were sourced from commercial sources. CQ and HCQ were manufactured in Hubei, China, and other chemicals were obtained from Merck in Switzerland. The compounds were prepared for aerosolisation by dissolving them in propylene glycol at a concentration of 5 mg/mL [126].

9.3.2 Real-Time Exhaled Breath Analysis

Breath samples from two healthy, non-smoking volunteers were analysed with a Super SESI ion source (Fossil Ion Technology, Málaga, Spain) connected to a Q Exactive HF Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Each participant inhaled aerosols from vaping devices containing caffeine, melatonin, or vitamin B12, then exhaled slowly through a disposable mouthpiece (see Figure 9.1A). The system continuously monitored flow rate, exhaled volume, and CO₂ levels to track breath dynamics and maintain consistent sampling conditions. Breath samples were passed through a heated tube to the ionisation chamber for stable electrospray ionisation. MS measurements were acquired in both positive and negative SESI modes, spanning m/z 50–1400. Daily quality control and weekly calibration with ambient air were performed. Data were analysed using Xcalibur software. [126].

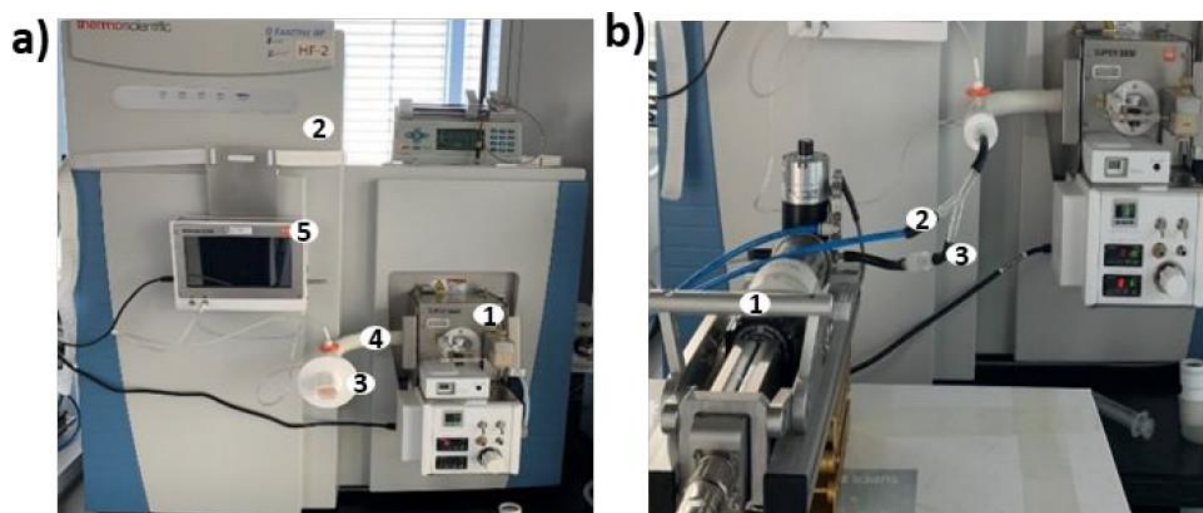


Figure 9.1A: The Super secondary electrospray ionisation (Super-SESI) high-resolution mass spectrometry (HRMS) instrument.

1. Super-SESI–HRMS Instrument: Utilised for real-time, highly sensitive detection of volatile and semi-volatile compounds in exhaled breath.
2. Sample Line: Links the mouthpiece to the Super-SESI inlet.
3. Disposable Mouthpiece: The volunteer exhales directly into it, ensuring hygiene and preventing cross-contamination.
4. Exhalation System: Tracks total exhaled flow, volume, and CO₂ levels to maintain consistent sampling and physiological relevance.

Figure 9.1(B) Aerosol Analysis Using a Programmable Syringe Pump (PSP)

1. PSP: Produces controlled aerosol emissions from vaping devices or other sources.
2. Nitrogen Stream: Moves the aerosol through the system, mimicking inhalation flow.
3. Super-SESI–HRMS Detector: Performs real-time analysis of the aerosol's chemical composition [125].

9.3.3 Real-Time Analysis of Aerosols

Aerosols were produced either by a volunteer exhaling through commercial vaping devices or by simulated breathing with a programmable syringe pump. The volunteer inhaled aerosols containing active substances like caffeine, melatonin, or vitamin B12, then exhaled slowly through a sterilised disposable mouthpiece linked to the Super-SESI system (see Figure 9.1A). The aerosolisation efficiency of each active pharmaceutical ingredient (API) was evaluated using high-resolution mass spectrometry. Each device produced between 10 and 20 mL of aerosol within 5 seconds, and nitrogen was used to guide the aerosol into the detection interface. The same measurement conditions and workflow used for analysing exhaled breath were applied here [126].

9.3.4 Aerosol Trapping Experiments

. To enable comprehensive chemical characterisation of aerosols generated from both proprietary and commercially available vaping devices, a dual-phase trapping strategy was employed. Aerosol samples were collected using glass fibre filter pads in conjunction with micro impinger systems. This combined approach was designed to capture compounds across both the particulate and gas/vapour phases, minimising analyte loss due to phase partitioning. The filter pads served to retain particulate-bound constituents, while the impinger—filled with a solvent selected based on the physicochemical properties of the target analytes—facilitated the trapping of volatile and semi-volatile compounds. Solvent choice was crucial and customised for each compound to achieve the best solubility, chemical

stability, and compatibility with subsequent analytical methods. The study mainly aimed to assess whether Secondary Electrospray Ionisation (SESI) technology could be helpful in analysing aerosols chemically. The goal was not to obtain precise measurements of individual compounds, since their levels can fluctuate based on user-specific inhalation behaviours, like puff volume and depth. . The solvent choice depended on the specific compounds. Azithromycin (5 mg/mL), CQ (5 mg/mL), and HCQ (5 mg/mL) aerosols were captured in an impinger containing 5 mL of ethanol, while caffeine (7.53 mg/mL), melatonin (47.06 mg/mL), vitamin B12 (6.86 mg/mL), and 5,6-dimethyl benzimidazole were trapped in another impinger containing 5 mL of water. We extracted the analytes from the glass fibre filter pads by adding 5 mL of solvent and mixing for 30 minutes. The extracted analytes were then combined with impinger solvent, creating a total volume of 10 mL. The final product was stored at +4 °C until analysis [126].

9.4 Results

9.4.1 Breath Analysis After Inhalation of Aerosols Produced by Commercially Available Vaping Devices

The Super SESI-HRMS was designed to analyse exhaled breath, focusing on chemicals present after inhaling aerosols from vaping devices containing compounds like melatonin, caffeine, or vitamin B12. The purpose was to demonstrate how SESI technology can characterise these aerosols. Volunteers inhaled the products and exhaled directly into the Super SESI-HRMS system, with the Exhalation system ensuring consistent breath volume. Full-scan mass spectra confirmed active ingredients and identified other compounds (Figures 9.2 and 9.3). Compound concentrations were not the primary focus since levels could vary with inhalation intensity. CO₂ levels and exhaled volumes were measured before and after using a caffeine-enhanced vaping device, with measurements standardised. Protonated species and ions were observed (Figure 9.2). It was confirmed that inhaled aerosolised caffeine reached the brain and affected motor activity, with caffeine detectable in exhaled breath following coffee intake. The goal was to confirm the active compound in breath and to detect other substances in the e-liquid, such as flavours or potential byproducts from aerosolisation [126].

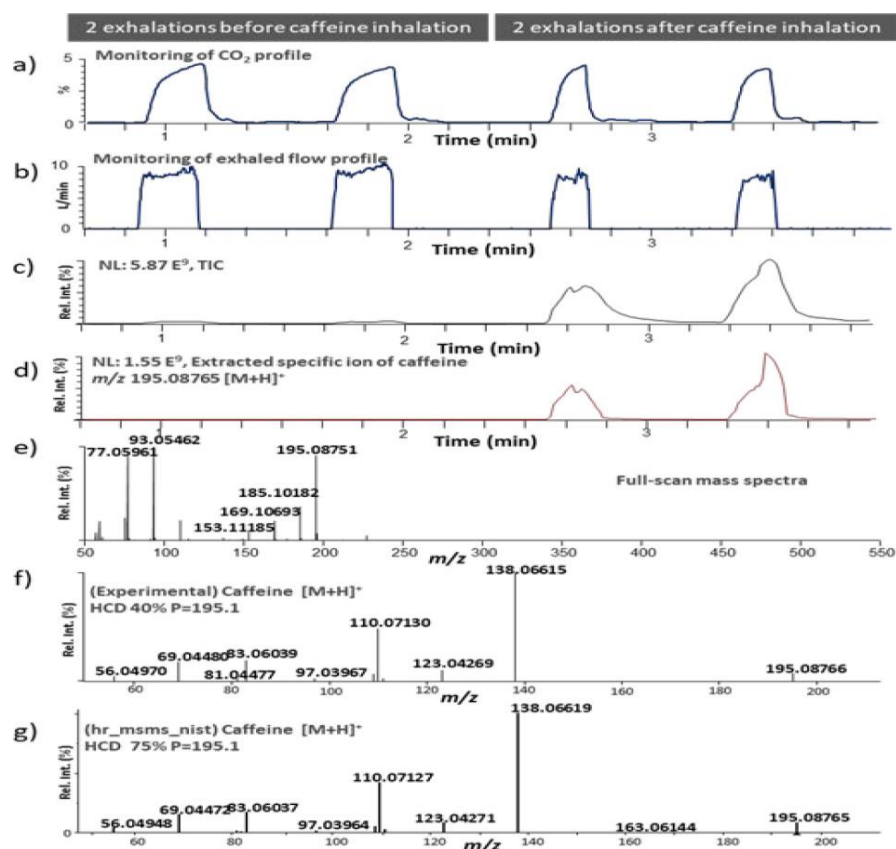


Figure 9.2: Exhalation after caffeine inhalation: (A) Real-time monitoring of exhaled CO₂ percentage levels using Exhalation software.

(B) Volumetric flow rate of exhaled breath (L/min).

(C) Total ion current (TIC) recorded during two blank exhalations, before and after inhalation of aerosol from a caffeine-enhanced vaping device.

(D) Extracted ion chromatogram for m/z 195.08765, corresponding to the theoretical protonated molecular ion of caffeine.

(E) Subtracted full-scan SEI mass spectrum in positive ionisation mode (m/z 50–550).

(F) High-energy collision dissociation (HCD) tandem mass spectrum obtained by selecting m/z 195.1 at HCD 40 (SEI+).

(G) Reference HCD tandem mass spectrum of caffeine from the NIST20 spectral library [125].

Melatonin presence was confirmed in e-liquids of supplemented vaping devices and effectively aerosolised from the user's exhale. Studies have shown melatonin's oral benefits in sleep disorder patients with asthma and COPD, circadian rhythm regulation, and cancer inhibition [126]. Its successful aerosolisation could aid research on inhalation-based melatonin administration [127].

9.4.2 Optimisation of the Analytical System to Monitor API-Containing Aerosols

The SESI interface offers significant potential for tracking successful aerosolisation of compounds by analysing exhaled breath. We created a system that uses a PSP to deliver aerosols to the SESI interface, ensuring precise aerosolisation of compounds from a liquid solution. The generated aerosols were monitored in real time to observe the compounds present. To assess the reproducibility of the aerosols, we utilised a vaping device supplemented with melatonin. The results demonstrated the significance of the developed setup for ensuring consistent delivery of the API through aerosolisation. Additionally, we developed an in-house formulation with several APIs and aerosolised the antiviral drug HCQ using our aerosol generator. Full-scan SESI+ mass measurements verified the aerosolisation of the API, as well as potential degradation products and impurities (Figure 9.3) [125].

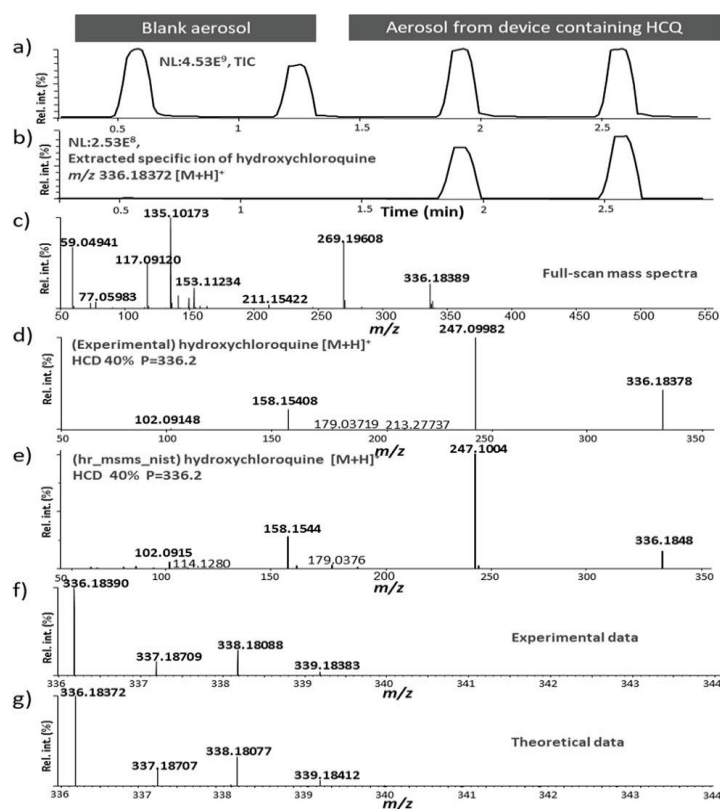


Figure 9.3: Aerosol from device containing HCQ (A) Total ion current (TIC) recorded during aerosol sampling.

(B) Extracted ion chromatogram for m/z 336.18372, corresponding to the theoretical protonated molecular ion of hydroxychloroquine (HCQ), monitored in positive secondary electrospray ionization (SESI) mode.

(C) Subtracted full-scan SESI mass spectrum (m/z 50–550) of an aerosol generated from a device containing HCQ.

(D) High-energy collision dissociation (HCD) tandem mass spectrum obtained by selecting m/z 336.2 at HCD 40 (SESI+).

(E) Reference HCD tandem mass spectrum of HCQ from the NIST20 spectral library.

(F) Zoomed full-scan SESI+ spectrum showing the protonated HCQ species.

(G) Theoretical chlorine isotopic pattern of the protonated molecular formula $C_{18}H_{26}ClN_3O$.

Measurements performed with the different compounds in the above section are examples to demonstrate the real-time measurement of various ions, including adduct ions, dimers, and ions resulting from in-source fragmentation. Additionally, the system's ability to monitor chemicals with different molecular masses and boiling points in both positive and negative SESI acquisition modes was evaluated using compounds like CQ, cannabidiol, and azithromycin [126].

9.5 Discussion

The Super-SESI–HRMS system is a practical, real-time, non-invasive method for detecting a broad spectrum of volatile and semi-volatile compounds in exhaled breath and aerosols. Initially designed for breath analysis, it was adapted to analyse chemical profiles following aerosol inhalation by volunteers, showcasing its sensitivity and adaptability. The study emphasises its high sensitivity, reproducibility, and ability to distinguish human metabolic signatures from external aerosols like those from vaping devices. This dual capability benefits exposure assessment, product evaluation, and clinical diagnostics. Notably, the system supports ethical research by enabling human testing without animal models, aligning with the 3Rs principle. The study also suggests that this technology could be further developed for therapeutic drug monitoring and biomarker discovery, making it a promising tool for future translational research and regulatory use [125].

9.6 Conclusion

This study showcases how Super SESI technology, combined with an Orbitrap mass spectrometer, can perform real-time analysis of aerosolised compounds. The system uses a programmable syringe pump (PSP) and a nitrogen stream in the SESI sample line to ensure accurate and consistent aerosol delivery. Chemical analysis was carried out using both positive and negative SESI ionisation modes, enabling the detection of intact molecular ions as the primary species signals. High-resolution full-scan acquisition combined with tandem MS fragmentation enabled verification of successful drug aerosolisation and identification of potential thermal degradation products. As a proof of concept,

real-time detection was achieved for various thermally generated aerosols, including caffeine, melatonin, hydroxychloroquine (HCQ), chloroquine (CQ), and cannabidiol [126].

Compounds can be turned into aerosols at much lower temperatures than their boiling points, which is crucial for delivering drugs without breaking down the compounds. However, mixtures with very different properties may separate, causing inconsistent drug delivery. Using the SESI interface and our modified setup shows promise in quickly assessing aerosolisation. Enhancing chemical transfer from liquid to gas phase relies on screening devices and identifying the most effective carrier solvents. This technique can be employed in high-throughput analyses to determine the optimal conditions for successful aerosolisation [126].

9.7 Research Impact

Since its publication in 2022, this research paper has been cited four times in internationally recognised journals, reflecting its increasing relevance in the field of breath analysis and aerosol science. This methodology has shown strong potential for diagnostic applications, particularly in the non-invasive detection of disease-related biomarkers via exhaled breath analysis. For instance, the Super-SESI–HRMS platform allows for real-time monitoring of volatile organic compounds (VOCs), which are increasingly acknowledged as indicators of physiological and pathological states. Building on this foundation, Bin Hu and colleagues referenced this work and affirmed its significance by applying a similar approach in their own study, further validating the use of mass spectrometry-based breath analysis in clinical research. Their findings strengthen the emerging consensus that breathomics, enabled by advanced MS techniques, offers promise for disease diagnosis, monitoring, and personalised medicine [128].

10.0 Assessment of Single-Photon Ionization Mass Spectrometry for Online Monitoring of in Vitro Aerosol Exposure Experiments

10.1 Introduction and Contribution

Inhalation toxicology has progressed significantly with the use of reliable in vitro tests and enhanced aerosol exposure systems (e.g. Vitrocell exposure system). Understanding biological responses that vary with dosage necessitates thoroughly investigating aerosol mechanisms and properties. Translating in vivo dosages into in vitro settings is crucial to fully comprehending aerosol dosimetry.

Measuring aerosol deposition often involves significant time and expense. Conventional chromatography techniques do not offer real-time data, which can compromise sample integrity and influence results by sampling or capturing aerosols on filter pads for analysis. As discussed in Chapter 5, QCMs inaccurately measure the deposition of liquid aerosol particles, prompting the exploration of alternative tools. Online mass spectrometry can enrich our understanding of exposure-dose relationships but typically concentrates on a single fraction, adding complexity to the analysis. This study utilised single-photon ionisation mass spectrometry (SPI-MS) to continuously monitor the components of test aerosols during laboratory exposure in real-time. This work helps us to measure the doses that reach and are deposited in the tissues during exposure. The work presented in this chapter is significant to our overall research focus in this thesis, particularly in the context of inhalation toxicology [128].

In this chapter, I will discuss the research paper **"Assessment of Single-Photon Ionization Mass Spectrometry for Online Monitoring of in Vitro Aerosol Exposure Experiments"** (Frege C, Asgari M, Steiner S, Ferreira S, **Majeed S**, Lucci F, Frentzel S, Hoeng J, Kuczaj AK Chem Res Toxicol. 2020 Feb 17;33(2):505-514. doi: 10.1021/acs.chemrestox.9b00381. Epub 2020 Jan 7. PMID: 31909610.) (115). As one of the co-authors of this article, my responsibilities included assisting in experimental design, supporting the measurement experiments, and contributing to the documentation for publication.

10.2 Background

Inhalation toxicology has benefited from advances in reliable in vitro assays and improved aerosol exposure systems. To understand dose-dependent biological responses, it is essential to characterise aerosol exposure mechanisms and aerosol properties in vitro. Aerosols consist of solid or liquid particles in a gas; analysing them without bias is challenging. Critical factors for successful toxicological analysis include particle size and shape, particle number, physicochemical properties of constituents, and distribution between gas and liquid/solid phases. Additionally, it is essential to understand the

processes involved in aerosol evolution and deposition before the aerosol reaches the exposure site. Since the 1970s, air-liquid interface exposure systems have been continuously developed and are used across various research disciplines. These systems are beneficial because they realistically mimic aerosol delivery for inhalation studies, offering detailed insights into aerosol deposition and biological interaction responses [128]. This approach facilitates the identification and correlation of fundamental aerosol processes with pharmacokinetic and in vivo models. However, the current landscape lacks comprehensive information and standardised guidelines for the characterisation and validation of commercial exposure systems. Variability in experimental setups, undefined aerosol compositions, and inconsistent equipment maintenance practices can lead to unreliable and non-reproducible results. In the absence of regulatory frameworks and universally accepted biological assays for aerosol exposure, it becomes imperative to rigorously characterize experimental conditions and transparently report findings to ensure scientific robustness and reproducibility [80].

Measuring the efficiency or toxicity of specific sampling at the air-liquid interface for aerosol deposition is laborious and costly, particularly for high-throughput screening studies. Traditional methods attempt to correlate exposure with the delivered dose but are limited under changing conditions for liquid aerosols. The current process for analysing aerosols in vitro mainly involves using chromatography methods. Although these analytical techniques are well-established and effective for toxicological assessments, they do not offer real-time insights into aerosol composition. Additionally, the sampling and processing steps involved may alter the aerosol's original characteristics.. An alternative approach for monitoring chemical composition in an in vitro system is online mass spectrometry, which can provide additional insights into the exposure-dose relationship. Online mass spectrometry for in vitro studies encounters several challenges because current techniques often fall short. They typically measure only one component of the aerosol—either the gas phase or the particulate matter—and can cause significant fragmentation of substances, making analysis more difficult. Moreover, these methods are usually optimised for detecting extremely low concentrations, well below parts-per-million levels [81].

Real-time monitoring of the main components in the test aerosol was performed using single-photon ionisation mass spectrometry (SPI-MS) during exposures conducted with the Vitrocell 24/48 system. The aerosol was generated using the MESH electronic cigarette, developed by Philip Morris Products S.A., containing an e-liquid primarily composed of glycerol, propylene glycol, nicotine, water, and a flavouring agent. Upon heating, the e-liquid forms supersaturated vapours that are subsequently cooled by an air stream, resulting in aerosol formation via thermal nucleation. Due to the volatile and semi-volatile nature of the e-liquid components, phase separation into liquid and gas phases occurs [129].

This study evaluated the performance of a single-photon ionisation mass spectrometer (Photonion GmbH, Schwerin, Germany) for characterising aerosol flow within the Vitrocell 24/48 system. Theoretical calculations provided a preliminary estimation of phase distribution, while online mass spectrometry enabled quantification of total aerosol concentration. Sampling efficiency of the spectrometer was assessed for both particulate and gas phases, and the expected distribution of glycerol, propylene glycol, and nicotine in the primary aerosol was calculated. Additionally, the impact of various dilution levels on aerosol concentration measurements and phase partitioning was investigated [129].

[129].

10.3 Materials and Methods

10.3.1 Test Aerosol and Experimental Setup

The original e-liquid formulation contained 70% propylene glycol, 20% glycerol, 1.6% nicotine, and 8.4% water. Aerosol generation was carried out using the P4M3 e-cigarette device, which was coupled with a programmable dual-syringe pump (PDSP) to ensure consistent delivery into the exposure system. Measurements were conducted with a Vitrocell 24/48 system, capturing both undiluted and diluted aerosols. The purpose was to assess the consistency and effectiveness of aerosol delivery in the in vitro exposure system through online mass spectrometry. Three protocols were tested to examine the impact of dilution on the results aerosol [129].

This study focused on assessing the consistency and efficiency of aerosol delivery within an exposure setup using online mass spectrometry. By eliminating the need for biological inserts or culture media during deposition testing, the method allowed direct evaluation of aerosol flow. Measurements were taken at both the endpoints of each delivery line and at the quartz crystal microbalance (QCM) port to determine uniformity and deposition performance.

To study the effect of dilution on the aerosol, we tested three protocols:

- (A) measuring aerosols at different dilutions within a single delivery line;
- (B) measuring from lines 1–6 with increasing dilution in each line;
- (C) measuring from lines 1–6 with dilution applied only in the first delivery line.

Figure 10.1 provides a schematic representation of these protocols. Due to limitations between the mass spectrometer and the Vitrocell 24/48 system, sampling was always conducted perpendicular to the aerosol flow direction in rows and at the QCM ports [128].

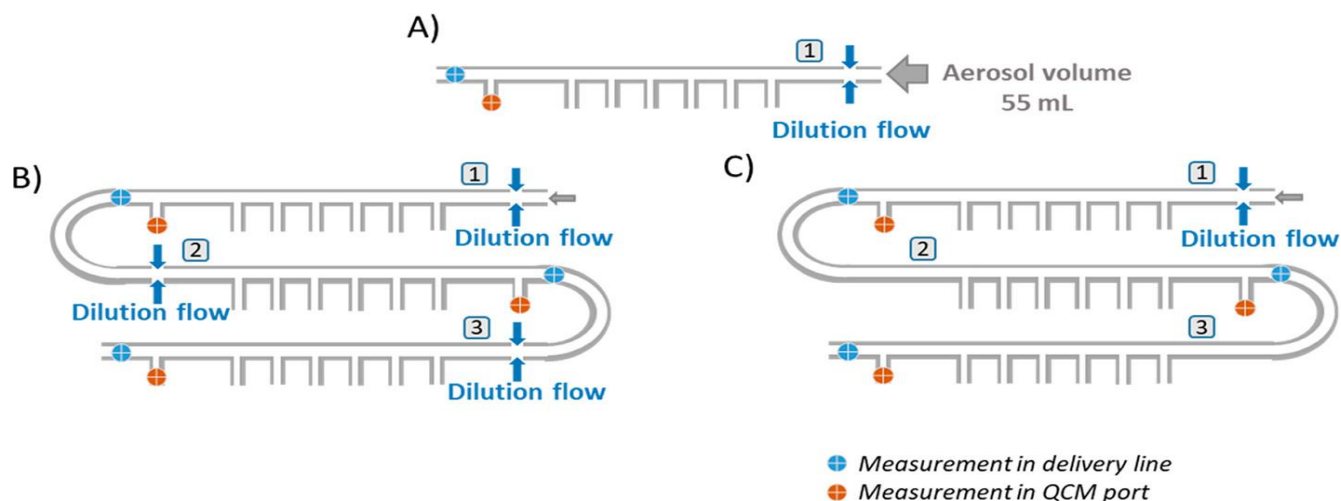


Figure 10.1 schematically shows the dilution protocols: Boxes indicate delivery line numbers, grey arrows show aerosol flow direction, and blue arrows show inlets for pure air dilution. The QCM port and delivery line measurement points are marked with orange and blue circles.

(A) Aerosol measurements are only taken at dilutions of 100, 200, 500, 1000, 1500, and 2000 mL/min in the first row.

(B) Measurements are taken from delivery lines 1-6 with gradual dilution.

(C) Measurements are taken from delivery lines 1-6, with dilution performed solely at line 1. The illustration only shows delivery lines 1-3 for setups B and C [128].

The test aerosol was diluted with pure air at flow rates of 100, 200, 500, 1000, 1500, and 2000 mL/min, corresponding to 80%, 67%, 45%, 29%, 22%, and 17% of the aerosol volume, respectively [128].

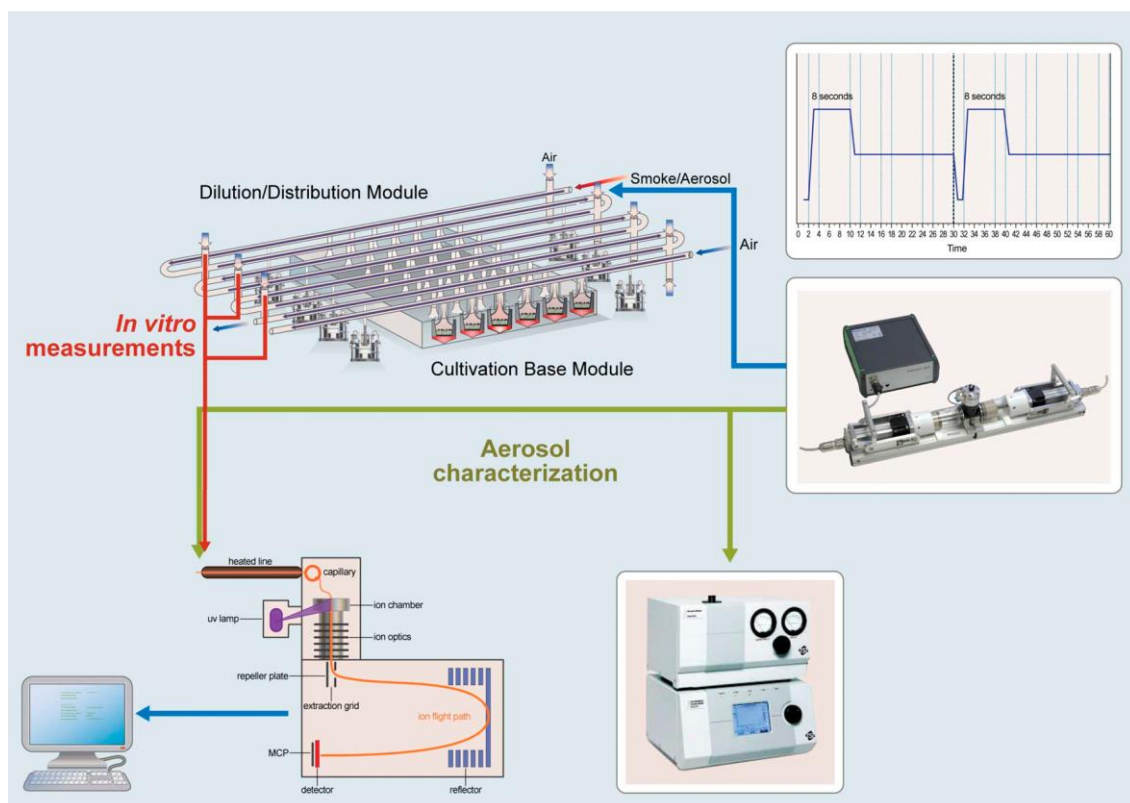


Figure 10.2: The system diagram illustrates the aerosol flow path. The blue arrow indicates entry into the Vitrocell system after generation. Green lines denote measurements for primary aerosol characterisation, while the red arrow shows the aerosol sampled from the in vitro system. [129].

10.3.2 Single Photoionisation Mass Spectrometer (SPI-MS)

Nicotine, glycerol, and propylene glycol concentrations were analysed using SPI-MS (Photonion GmbH, Schwerin, Germany). Figure 10.3 illustrates the platform schematic. The aerosol initially travels through a 3-meter capillary transfer line (an deactivated column) with an inner diameter of 180 μm , heated to 280 $^{\circ}\text{C}$ to vaporise substances and prevent blockages. The sample is subsequently ionised using a neodymium-doped yttrium aluminium garnet (Nd:YAG) laser, which employs third harmonic generation to produce vacuum ultraviolet light at 10.5 eV (118 nm). This process causes single-photon ionisation of atoms or molecules with ionisation energies below this photon energy, resulting in the formation of radical cations. Most organic molecules have ionisation energies below 10 eV, enabling single-photon ionisation with minimal fragmentation and allowing discriminating background gases with higher ionisation energies like N_2 , O_2 , CO_2 , and H_2O [129]. After aerosols are ionised, the ions are directed towards the time-of-flight region for detection based on their mass-to-charge ratio. The pressure is reduced to 10^{-6} mbar in the time-of-flight region, determining the sampled aerosol rate. Single-photon ionisation has a measurement range of 40–350 m/z with a resolution of 1800 Th/Th (unit mass resolution, UMR-9) and is stored at a rate of one measurement per second [128].

The spectrum's signal intensity correlates with each substance's ionisation potential rather than its concentration. To determine substance concentrations, compare each substance's signal to that of a reference substance. In our case, we used toluene at a concentration of 100 ppm as the reference [129]. A microflow syringe pump system was used to produce calibration gases from liquid solutions of glycerol, propylene glycol, and nicotine at 100 ppm. These solutions were introduced into a heating chamber held at 280°C, flowing at 20 $\mu\text{L}/\text{min}$, allowing immediate sampling via SPI-MS. [130].

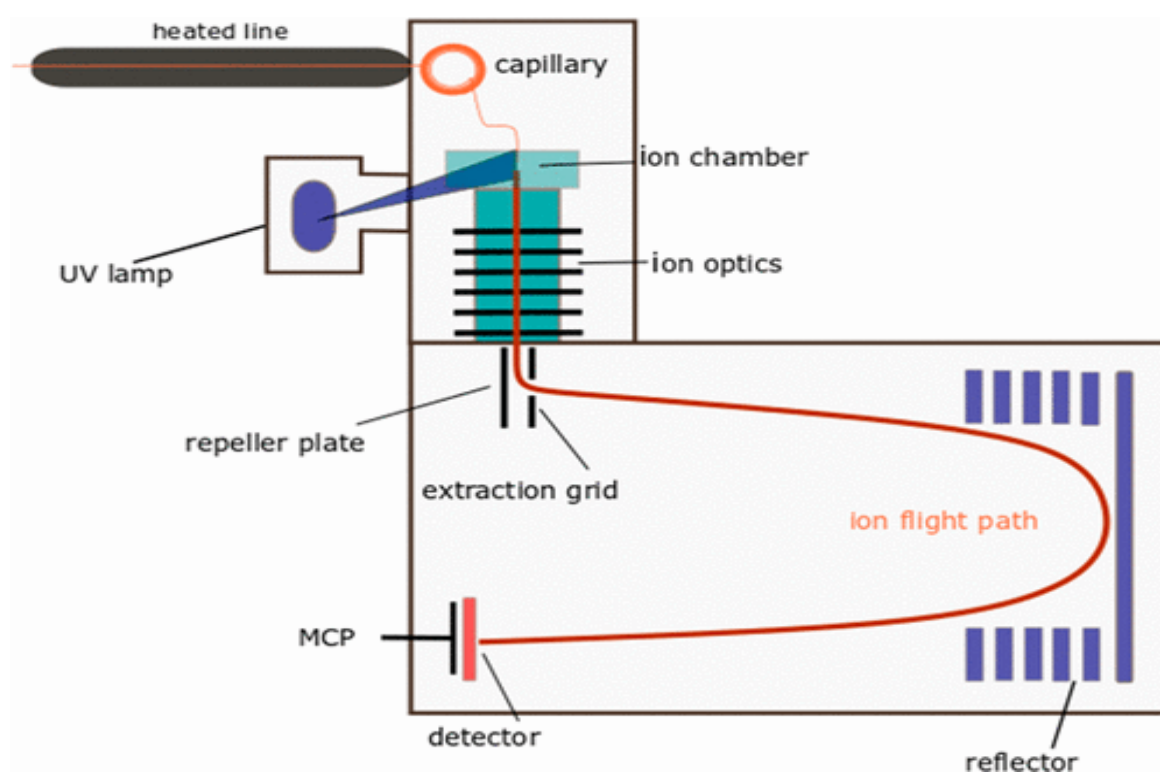


Figure 10.3: Scheme of the single-photon ionisation mass spectrometer. MCP, microchannel plate; UV, ultraviolet.

10.3.3 Aerosol Particle Size Measurement

An aerodynamic particle sizer (APS; model 3321, TSI Inc., Minnesota, USA) was utilised to determine the particle size distribution of the test aerosol. To ensure accurate readings within the operational range of the APS, the aerosol was diluted 100-fold using an aerosol dilution unit (Model 3302A, TSI Inc., Minnesota, USA). This dilution step enabled reliable characterisation of aerosol properties within the exposure system [128].

10.4 Results and Discussion

The sampling performance of the single-photon ionisation mass spectrometer (SPI-MS) was initially evaluated using computational fluid dynamics (CFD). This simulation accounted for both instrument-specific parameters—such as capillary geometry and sampling temperature—and general experimental conditions, including aerosol flow rate and the spatial orientation of the capillary. The subsequent analysis concentrated on the primary aerosol produced directly after generation with syringe pumps, emphasising particle size distribution and chemical makeup. Theoretical gas-to-particle ratios were determined, and concentrations of propylene glycol, glycerol, and nicotine were estimated according to the total amounts measured using SPI-MS measurements. Finally, online measurements were conducted across various dilution protocols and sampling locations, including delivery lines and the quartz crystal microbalance (QCM) port, to assess the impact of dilution on aerosol characterisation. [129].

10.4.1 Test Aerosol Characterisation

To characterise the aerosol, particle size distribution was measured using an aerodynamic particle sizer (APS; TSI model 3321). Due to the high concentration of particles in the generated aerosol, a dilution factor of 100 was applied using a TSI model 3302A aerosol dilution unit to ensure compatibility with the APS measurement range. This characterisation step was essential for establishing baseline aerosol properties prior to in vitro exposure analysis [128].

10.4.2 Aerosol Particle Size Distribution

To evaluate the actual sampling efficiency of SPI-MS, the aerodynamic particle size distribution of the test aerosol was measured and compared with the calculated aspiration efficiency. Due to the high particle number density exceeding the detection range of the aerodynamic particle sizer, the aerosol was diluted using a 5 L/min airflow. After correcting for dilution and accounting for particle losses within the pump, the estimated total particle concentration in the ENDS aerosol was found to be approximately three orders of magnitude higher than values reported in earlier studies. The particle count size distributions are illustrated in Figure 10.4. Measurements indicate that 78% of particles have an aerodynamic diameter less than one μm [128].

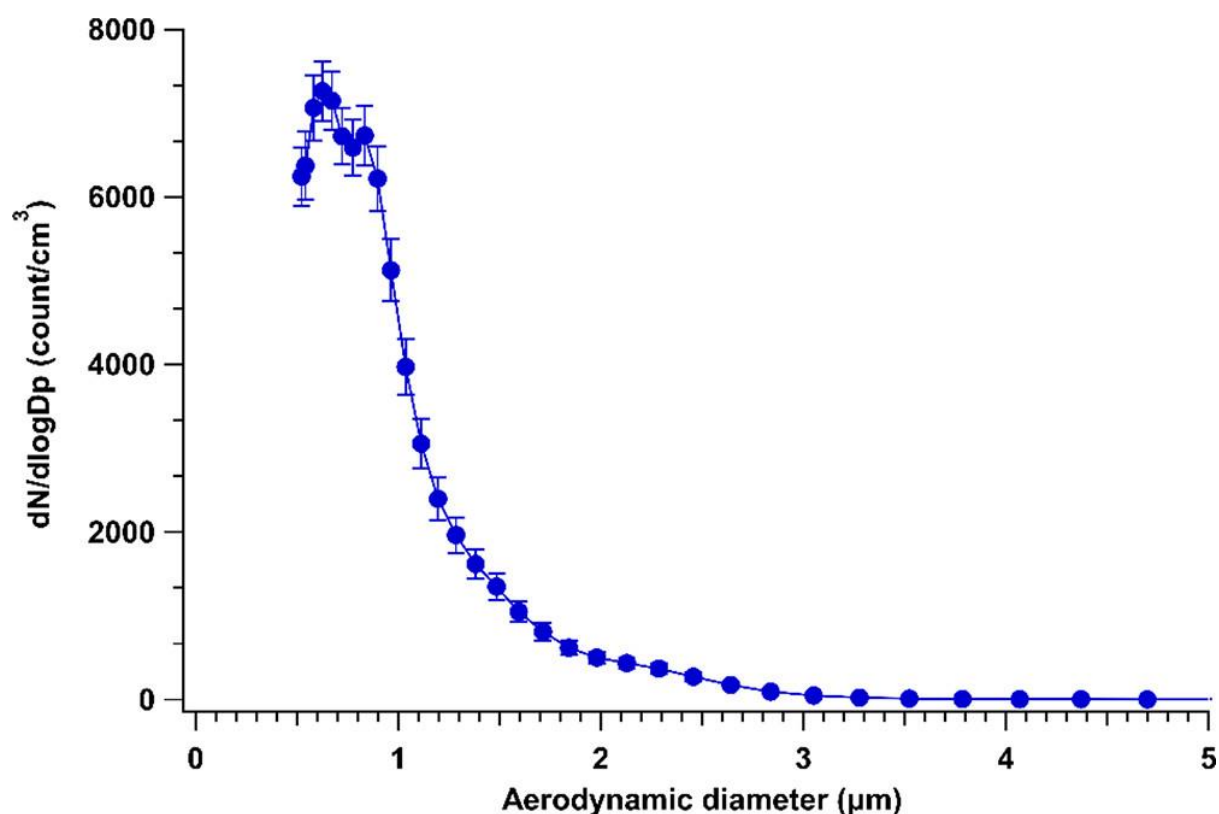


Figure 10.4 Aerodynamic particle diameter: The aerosol test's particle size distribution was examined using an aerodynamic particle sizer on 22 samples. Blue markers represent particle size counts, with most particles having a diameter under 1 µm [128].

10.4.3 Online Aerosol Measurements in an in-Vitro System

Aerosol measurements in an in vitro system were performed after analysing the test aerosol. Measuring aerosols from inhalation devices such as ENDS is challenging because the aerosol is produced in puffs or inhaled volumes, unlike nebulisers that generate aerosol continuously. Puffing creates fixed amounts of aerosol that move through the delivery line and change along the way. The application of dilution can affect the continuous time-dependent evolution of coagulation due to evaporation/condensation, which is influenced by thermodynamics [129].

To understand the impact of dilution, the Vitrocell exposure system was operated under controlled temperature and humidity conditions. Airflow within the delivery lines was incrementally increased to evaluate the influence of dilution on aerosol composition. Initial measurements were taken to compare aerosol substance levels between the delivery and exposure lines, including the quartz crystal microbalance (QCM) pathway. Concentrations of propylene glycol, glycerol, and nicotine were quantified under varying dilution conditions and at different sampling locations. For each

experimental setup, measurements were averaged across four exhaust volumes, with each volume corresponding to a defined number of aerosol puffs [129].

Figure 10.5 showcases a comparison between concentrations at delivery lines and QCM ports for the second protocol. These measurements, averaged over three replicates, indicate that concentrations exhibit a decrease with sequentially increased dilution flow. For all three substances, variability was most significant at delivery lines 1 and 2, with minor differences at lines 3, 4, and 5. Line 6 showed minimal differences for propylene glycol and nicotine. The notable differences in concentrations between the delivery line and QCM ports 1 and 2, especially for glycerol and propylene glycol, indicate potential insufficient mixing at the Vitrocell's initial delivery lines system [129].

The lower graphs in Figure 10.5 also display the concentrations adjusted for the dilution of each delivery line. Notably, there is increased variability in the QCM ports and dilution delivery lines, especially at higher dilutions (delivery lines 4-6). This suggests that the variability seen in the QCM port is partly affected by the type of substance being measured. For example, glycerol and nicotine exhibited less variability than propylene glycol. Conversely, all three substances showed greater variability at higher dilutions in the corresponding rows. The decreased sampling efficiency of the liquid phase at higher flow rates, as shown by calculated aspiration efficiencies for SPI-MS, may account for the observed trends. Notably, the lower variability at the QCM ports—particularly for glycerol and nicotine—indicates improved particle sampling at lower flow rates. In contrast, the higher variability seen in delivery lines 4–6 could be attributed to changes in the aerosol profile due to evaporation and condensation, implying that some particles may have been filtered out in the earlier segments of the system [129].

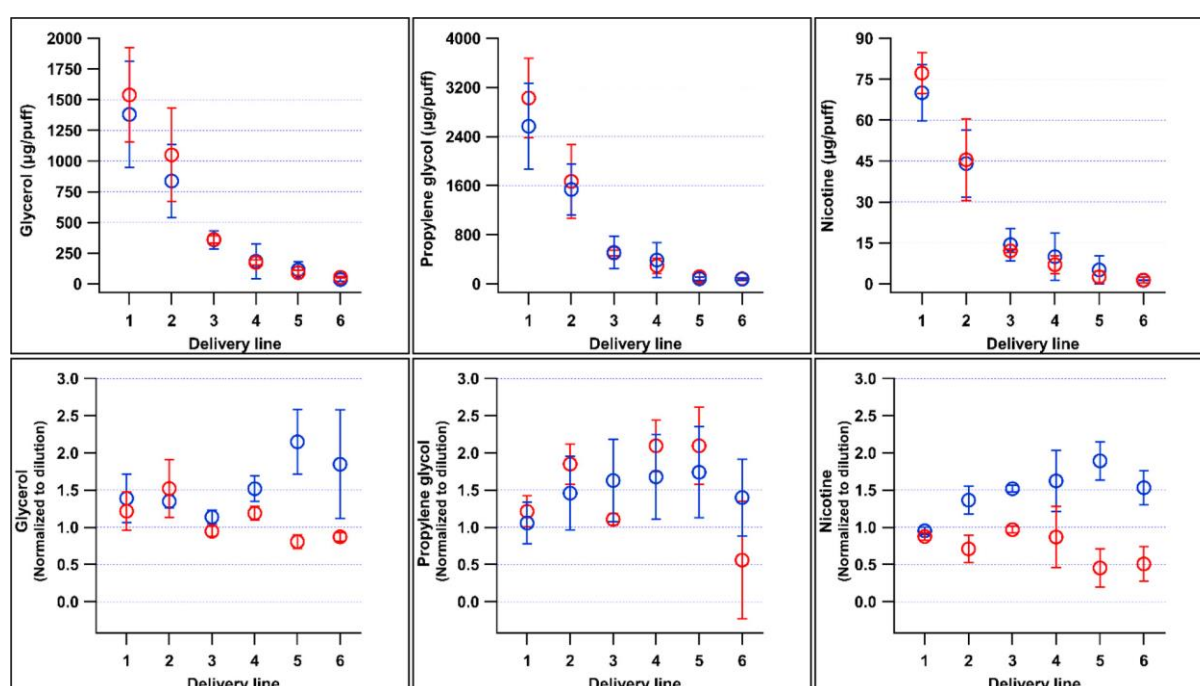


Figure 10.5: Concentrations of glycerol, propylene glycol, and nicotine were meticulously measured at two key locations: the delivery line, represented by blue circles, and the QCM (Quartz Crystal Microbalance) ports, represented by red circles. As the dilution factor was systematically increased for each line, we observed changes in concentrations. The upper plots illustrate the average puff concentrations for each measured dilution, providing a clear comparison of the various substances across different dilution levels. In contrast, the lower plots display the values that have been normalised according to the dilution factor, allowing for a more accurate interpretation of how dilution affects the concentrations of these compounds. This detailed analysis aims to enhance our understanding of the behaviour of these substances under different dilutions and positions inside the Vitrocell exposure system [129].

10.4.4 Comparison of Dilution Protocols

Toxicological evaluations necessitate carefully controlled exposure experiments across various doses. This is usually done by diluting aerosols with high concentrations or by restricting exposure time. Dilution is especially important when dealing with highly toxic substances, like cigarette smoke, or aerosols with inconsistent chemical makeup and delivery. Precise dose management guarantees reliable measurement of cellular responses and reduces confounding factors caused by excessive or uneven exposure. Three distinct dilution protocols were implemented to monitor aerosol variability within the exposure system. In the first protocol, airflow and concentration measurements were conducted exclusively at the initial delivery line, which served as a reference point. This setup enabled the evaluation of localised aerosol dynamics, such as evaporation and condensation, occurring within the delivery line immediately after aerosol generation.. The aerosol's residence time in the system is brief, with a direct flow path. Figure 10.6 displays three dilution protocols at different flow rates, comparing data from delivery lines 2–6. The first protocol shows higher concentrations, probably because the aerosol experienced a shorter residence time and flow path, resulting in less particle condensation and evaporation, as anticipated. [129].

Measured concentrations of propylene glycol, glycerol, and nicotine remained consistent with normalised values under the initial protocol, demonstrating stable aerosol composition. However, increased dilution caused more variability, probably due to reduced sampling efficiency of the SPI-MS system at higher flow rates. Glycerol showed the least variability among the tested substances, maintaining consistent levels across both the first and third protocols. In contrast, propylene glycol demonstrated the highest variability. These results suggest that aerosol re-equilibration occurs after

dilution, with substances that have lower vapour pressure—such as glycerol—evaporating less and remaining more stable [131].

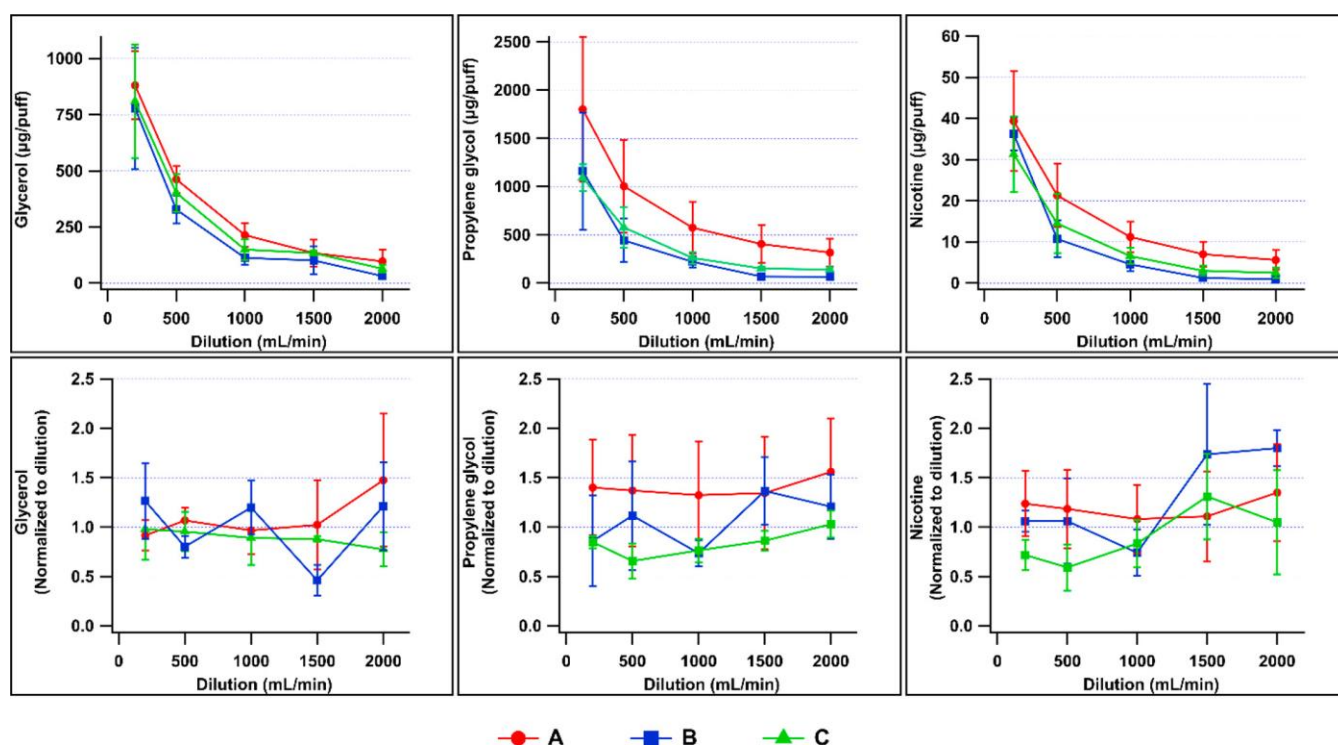


Figure 10.6: We conducted a detailed comparison of three different dilution protocols for glycerol, propylene glycol, and nicotine at various flow rates, explicitly focusing on the concentrations observed in delivery lines 2 through 6.

- (A) Aerosol measurements at specific dilutions on the initial delivery line.
- (B) Measurements across delivery lines 1–6, with a gradual increase in dilution at each line.
- (C) Measurements from delivery lines 1–6, with dilution applied only at the first line.

Our findings indicate that the first protocol resulted in notably higher concentrations of the substances tested. This increase can be attributed to shorter aerosol residence times and more direct flow paths, which minimise the amount of condensation and evaporation that can occur during the delivery process. These concentrations provide insight into the effectiveness of each dilution protocol. Meanwhile, the lower plots illustrate normalised concentrations, allowing for a standardised comparison across different conditions [128].

This comprehensive approach enables a thorough understanding of how different dilution protocols impact the delivery of these substances across the specified flow rates inside the Vitrocell exposure system [129].

10.5 Discussion

The research shows that single-photon ionisation mass spectrometry (SPI-MS) is a promising tool for real-time, online tracking of aerosol makeup in in vitro exposure systems. Its capacity to detect volatile and semi-volatile compounds without requiring extensive sample prep makes it especially useful for studying dynamic aerosols. However, results also indicate that aerosol dilution and sampling methods significantly impact the aerosol's chemical and physical characteristics, which can lead to selective sampling and changes in deposition behaviour. These factors make interpreting toxicological results more complex, as the actual dose might not mirror the original aerosol composition. Additionally, computational fluid dynamics (CFD) modelling underscored the importance of understanding how aerosols move and transform within the exposure system. Overall, while SPI-MS improves analytical capabilities, careful attention to sampling strategies and dilution effects is crucial for obtaining accurate and consistent results in aerosol toxicology [128].

10.6 Conclusion

Single-photon ionisation mass spectrometry (SPI-MS) provides a robust platform for the detection of a broad spectrum of organic compounds in aerosol samples. Its capability to operate in real-time without requiring sample preparation allows for the direct analysis of both gaseous and liquid aerosol phases. This technique is particularly effective in identifying individual chemical constituents within aerosols used in in vitro exposure systems. Variations in dilution flow protocols were found to influence aerosol composition, enabling detailed monitoring of these changes. SPI-MS is also suitable for analysing compounds with low volatility and high viscosity, such as glycerol. The concentrations of propylene glycol, glycerol, and nicotine measured after dilution were consistent with theoretical expectations based on their respective volatilities and the applied dilution factors. One of the key advantages of SPI-MS in toxicological applications is its ability to quantify high concentrations without saturating the detector. Additionally, its low sampling flow rate—approximately 2 mL/min—makes it well-suited for integration into in vitro exposure platforms[129].

Despite its strengths, SPI-MS has limitations when applied to complex aerosol mixtures. These include relatively low mass resolution and a restricted mass-to-charge detection range. Care must be taken when sampling compounds that may degrade at elevated temperatures within the sampling line. The efficiency of SPI-MS in capturing different aerosol components, including particulate matter and gases, was found to be highly dependent on the flow rate of the aerosol stream. This is particularly relevant for in vitro studies, where increased flow rates can dilute the aerosol and reduce the delivered dose. The technique demonstrated consistent performance in analysing simple aerosol mixtures under

controlled conditions. Comparative analysis of different dilution protocols revealed variations in the time required to reach equilibrium between steady and unsteady flow conditions. Furthermore, the measured concentrations of glycerol, propylene glycol, and nicotine across dilution levels aligned with their known vapour pressures [129].

It is important to recognise that the diluted aerosol introduced into the Vitrocell exposure system represents a modified form of the primary aerosol, characterised by reduced dose and altered phase distribution. The efficiency of aerosol deposition within the exposure chamber is influenced more by the rate of delivery than by particle size alone. Additionally, phase redistribution following dilution significantly affects the concentration of individual substances. Therefore, in comparative in vitro studies, careful consideration of dilution rates and their implementation is essential, as these parameters directly impact the effective dose administered to the biological system [129].

10.7 Research Impact

Since the publication of this research in 2020, it has been cited in at least seven subsequent studies, reflecting its growing influence in the field of aerosol science and exposure assessment. Notably, Kumari et al. emphasised the critical need for direct source-based aerosol emission measurements to support environmental regulations and global air quality standards. Their work led to the development of the Versatile Source Sampling System (VS3), a modular and field-deployable platform designed to capture emissions from area, line, and point sources, including cookstoves, vehicles, and industrial stacks [132]. While the VS3 system was developed independently, it aligns conceptually with the methodologies discussed in this study, particularly the emphasis on real-time monitoring and the use of advanced analytical tools such as single-photon ionisation mass spectrometry (SPI-MS). Kumari et al. further proposed the creation of a standardised emission factor database, advocating for the integration of calibrated systems like SPI-MS to ensure consistency and accuracy in emission reporting. Their most recent work, published in *Aerosol Science and Technology* in October 2024, reinforces the foundational relevance of this study in shaping future emission monitoring frameworks [132].

11.0 Aerosol Filtration Testing of Fabrics for Development of Reusable Face Masks

11.1 Introduction and Contribution

The global shortage of personal protective equipment during the COVID-19 crisis has significantly impacted pandemic control efforts. Ensuring healthcare workers' access to face masks is crucial, but reducing the COVID-19 basic reproductive number also relies on social distancing, mask-wearing, and remote work. The mandatory use of face masks has strained disposable mask supplies, leading to an environmental crisis with roughly 120 billion masks discarded each month. Leveraging available resources for aerosol generation and characterisation techniques, we utilised our scientific expertise to develop a reusable face mask. We aim to test readily accessible materials for universal use in constructing these masks for personal safety and hygiene. Mask effectiveness hinges on understanding filtration efficiency, with factors like particle size distribution and breathability playing pivotal roles. While masks mainly reduce aerosol spread from wearers, their public health role is crucial. This chapter reflects our commitment and resourceful approach to aiding the community during this crisis by refocusing our scientific knowledge.

In this chapter, I will discuss the research paper "**Aerosol Filtration Testing of Fabrics for Development of Reusable Face Masks**, (Stan, A., Steiner, S., **Majeed, S.**, Weber, S.S., Gosh, S., Semren, T.Ž., Guy, P.A., Lebrun, S., Steinhauser, J., Tardy, Y., Hogg, A., Chiquet, S., Perret, J.L., Kuczaj, A., Peitsch, M., Hoeng, J. (2021). Aerosol Filtration Testing of Fabrics for Development of Reusable Face Masks. Aerosol Air Qual. Res. 21, 210052. <https://doi.org/10.4209/aaqr.210052>) (119). As one of the co-authors of this article, I was responsible for helping to design experiments, supporting measurement experiments, and contributing to the documentation for publication.

11.2 Background

During the COVID-19 crisis, the global shortage of personal protective equipment (PPE) has been critical in controlling the pandemic. It is important to ensure that healthcare workers have access to face masks, but reducing COVID-19's basic reproductive number (R_0) also depends on widespread measures such as social distancing, mask-wearing, and remote work for the general population. Mandatory face mask usage in public places greatly strains the already limited supply of disposable masks made of melt-blown fibres. Additionally, the pandemic has led to an extraordinary rate of PPE

disposal, contributing to an environmental crisis, with around 120 billion masks being discarded each month [132].

Since February 2020, many governments have mandated the use of face masks in public places. By June 2020, more than 5.7 billion people (approximately 75% of the world's population) were under provisions to wear face masks in various public places. There has been a growing consensus in the scientific community that face masks, including do-it-yourself (DIY) cloth masks, provide an important means of controlling the community's spread of the virus [133]. The World Health Organisation has advised governments to encourage the general population to use face masks to reduce the transmission of SARS-CoV-19. Mathematical models show that the use of masks significantly decreases the infection rate and helps contain the pandemic [134]. When considering the use of face masks in the general population, it is important to consider:

- 1) Medical masks and respirators should be prioritised for healthcare professionals.
- 2) The use of face masks should not lead to a false sense of security, poor respiratory etiquette, or neglect of hand hygiene.
- 3) Improper handling and increased face touching while wearing masks can increase the risk of transmission [135].

Medical devices, such as face masks, are categorised into surgical face masks and personal respirators, each designed for different purposes and governed by specific global standards. At the onset of the COVID-19 pandemic, DIY face masks gained popularity due to a shortage of medical masks. Various countries have established scientific standards for reusable fabric face masks, with the US CDC issuing guidelines on cloth face masks. Experimental evaluations of different fabrics' filtration properties are required, as aerosols containing biological elements are measured by their particle size distribution (PSD), aiding in quantifying filtration efficiency [135].

The fractional filtration efficiency ($FE(d)$) measures how well a filter captures particles, regardless of the aerosol's particle size distribution (PSD). Total filtration efficiency (TFE) considers all particle sizes caught by a filter. The pressure drop (ΔP), related to breathability, is crucial because low breathability reduces mask effectiveness and increases with filter thickness, fibre density, and airflow speed. Water condensation can alter a filter's properties. Aerosols from breathing, coughing, sneezing, and talking contribute to disease transmission. The diverse distribution of biological material in aerosols complicates determining the ideal filtration efficiencies for adequate mask protection. Face masks

primarily reduce wearer-spread aerosols, while N95 respirators protect wearers in highly contaminated settings [135].

Research during COVID-19 indicates that multiple fabric layers can achieve high filtration efficiencies across various particle sizes. We studied over 300 fabrics to provide insights on non-woven fabrics and evaluated commercially available reusable masks globally, also exploring the impact of hydrophobic coatings on filtration properties [135].

11.3 Methods

11.3.1 *Building a Compendium of Fabrics Used across the Globe*

We used a semi-automated text-mining platform, Taxila™ (SBX, Tokyo, Japan), to systematically gather global information on fabrics and patterns for DIY face masks. This platform collected data daily from various public sources such as blogs, government sites, publications, and reports. Data was gathered from over 1500 key sources worldwide, focusing on Asia, where the virus first emerged. This information created a dynamic dashboard with visualisations and reports to identify important face mask protocols and materials. Based on Taxila™'s selections, we initially considered a range of household items similar to those evaluated by van der Sande et al. (2008) and Davies et al. (2013)[135]. Our findings indicated that most global solutions were alike, prompting us to expand our search to include readily available commercial materials [136].

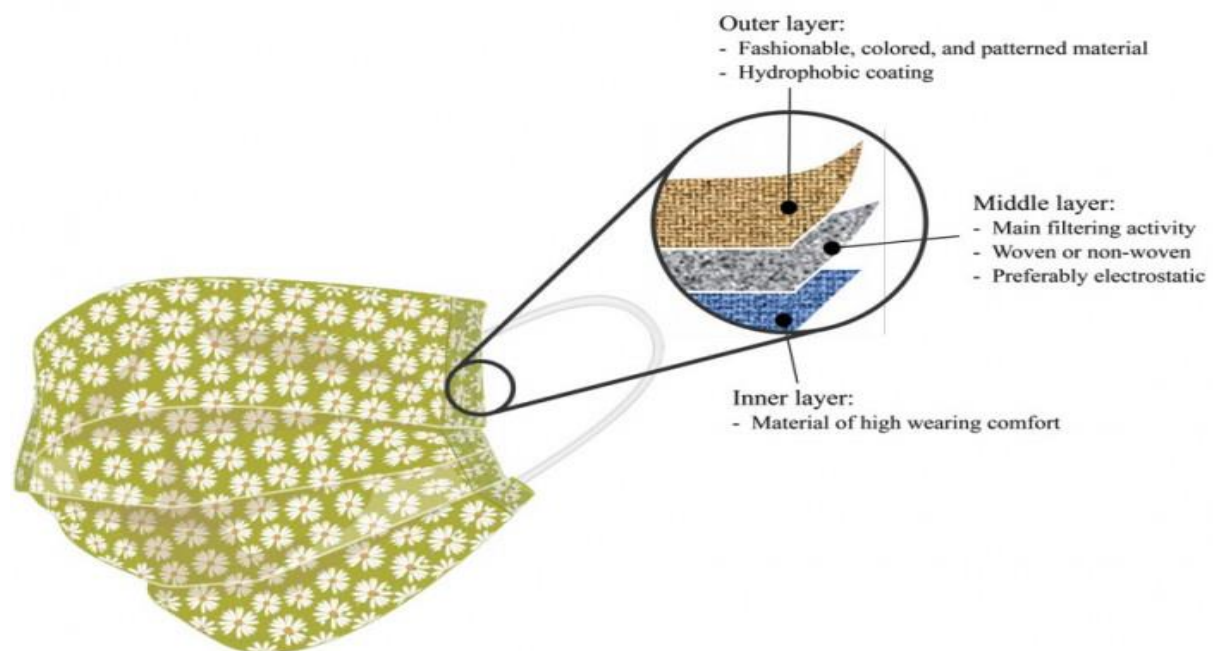


Figure 11.1 Face Mask Materials: The basic assembly consists of various materials with different filtration efficiencies, pressure drops, and tactile properties across various particle sizes.

During the screening process (discussed in the Results section) and guided by Taxila™'s results, we examined combinations of layered fabrics termed "Assemblies." These assemblies, shown in Figure 11.1, were tested using the same method as single materials, measuring layers in one fibre orientation only. We have analysed several non-medical, reusable face masks on the market. Most are from Asia and Europe. They claim to be effective against respiratory diseases, including SARS-CoV-2 [135].

11.3.2 Pressure Drop Measurements

We measured the pressure drop across circular patches (with a radius of 1.9×10^{-2} m) of test materials using the equipment depicted in Figure 11.2. First, we established a baseline for the system's flow resistance by measuring the water column height without any test material. Then, with the test material in place, we measured the water column height again. The net pressure difference across the test material was calculated by subtracting the baseline and converting the result to Pascal (Pa). We initially conducted measurements once for screening and selection purposes, and the presented data are based on five independent repetitions [135].

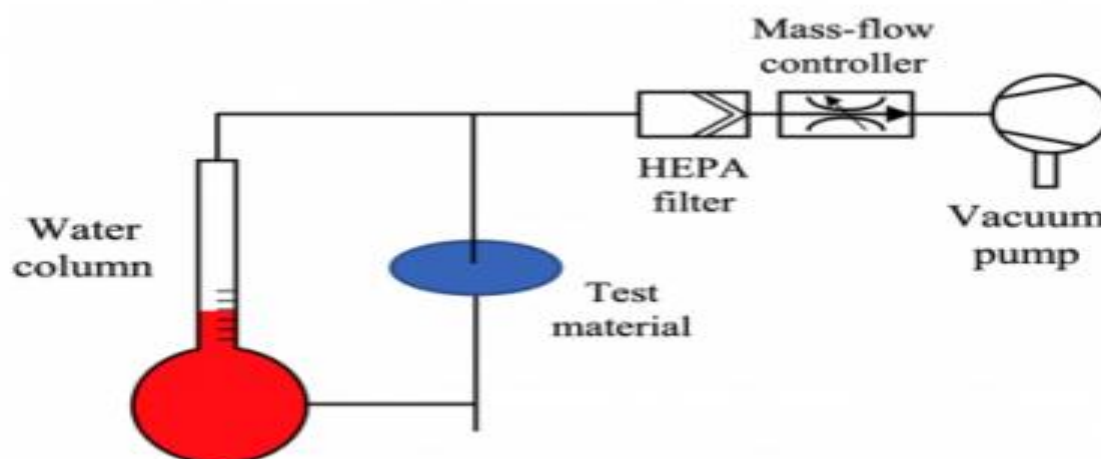


Figure 11.2 Pressure drop measurement setup: A water column-based experimental setup was used to measure the pressure drop across the samples. A HEPA filter (Analyt MTC Messtechnik GmbH, Müllheim, Germany, Model #35831) and a vacuum pump were added for protection. The circular patches of test material were clamped into the aerosol flow [135].

11.3.3 Aerosol Filtration Efficiency Testing

Figure 11.3 shows the setups used to test how well the samples filtered out particles. Two types of aerosols were tested:

1. A liquid-particle aerosol made of propylene glycol (PG), glycerol (G), and phosphate-buffered saline (PBS) called "PG/G aerosol" (PG: Sigma Aldrich, Buchs, Switzerland, Ref # 82280, G: Ref #G9012, PBS: #D8537).
2. A dry, sodium chloride particle aerosol called "NaCl aerosol" (NaCl: Sigma Aldrich, Ref #S9625).

We used these two different aerosols because the NaCl aerosol has a uniform particle size, which is suitable for quickly testing fabrics. This aligns with the idea that screening for the most penetrating particle size (MPPS) is a good way to choose filters to test with a liquid aerosol. For the second test, we used the PG/G aerosol, which has a broader range of particle sizes like those found in virus-carrying particles produced by talking, sneezing, or coughing. We checked how well the fabrics filtered out different particle sizes by measuring the aerosol size before and after it passed through the fabric. We used two different spectrometers that work differently and measure particles from very small to very large (TSI 3321 Aerodynamic Particle Sizer and TSI 3091 Fast Mobility Particle Sizer -TSI Inc., Shoreview, MN, USA) . This approach may have introduced some small biases, but it was necessary and is considered acceptable [135].

Due to the low instrument background, particle penetration rates were converted to filtration efficiencies without additional data corrections. The PSDs were measured once for NaCl aerosol and five times for PG/G aerosol data. Results from particle sizers using different principles cannot be directly compared. A discontinuity in combined PSDs is expected for different size ranges, but should largely disappear when converted to filtration efficiencies. The APS tends to underestimate particle numbers per bin as aerosol concentration increases [135].

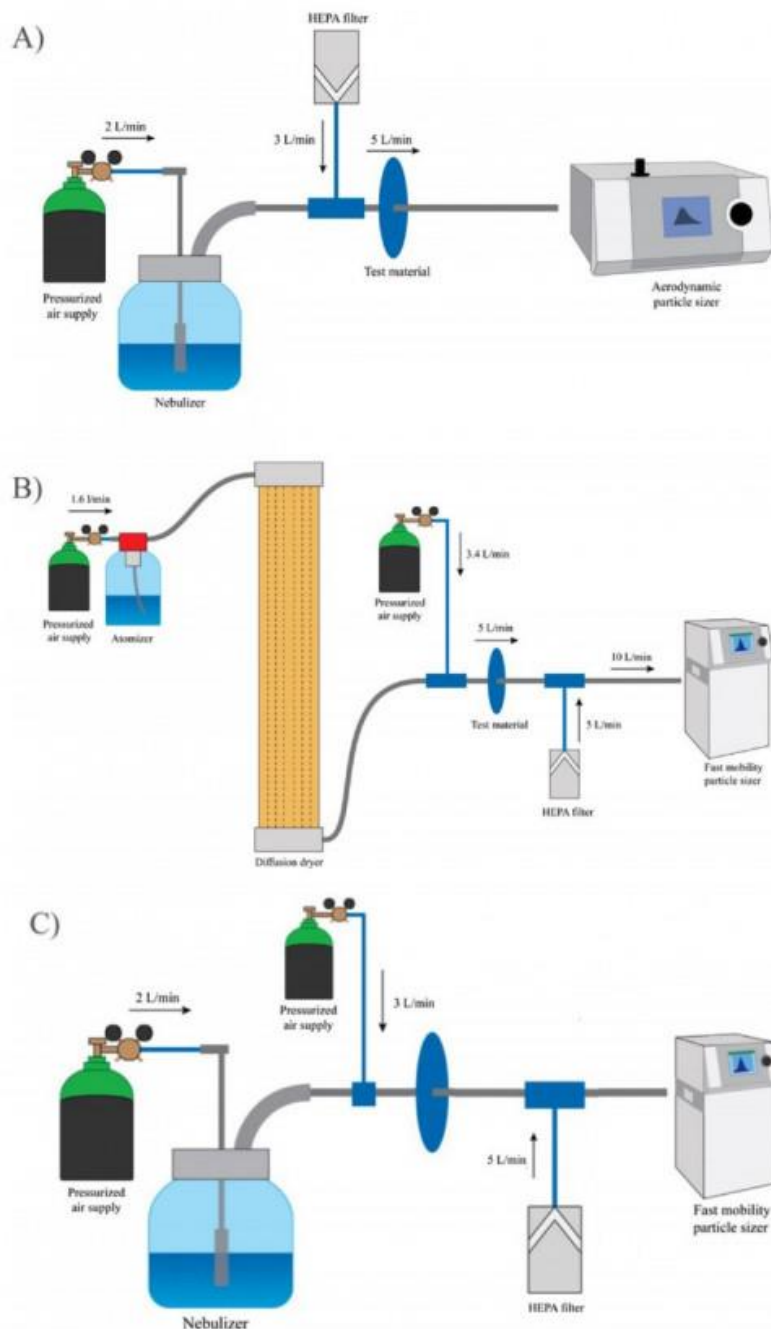


Figure 11.3 Diagrams of experimental setups for filtration efficiency measurements: Different aerosol particle sizes were assessed using an aerodynamic particle sizer (APS) and a fast-mobility particle sizer (FMPS). (A) and (C): PG/G particle filtration was measured with APS and FMPS, respectively. (B): NaCl particle filtration was measured with FMPS [135].

11.4 Results

In response to the COVID-19 pandemic, we aimed to establish a reliable fabric testing process and provide a comprehensive overview based on various governmental and regulatory standards. We screened over 300 fabrics and assemblies, using a single measurement for initial screening to include

as many samples as possible. Many tested fabrics were household or personal items, with some newly acquired materials. Selections were random, including commercial reusable and disposable face masks. Figure 11.4's heatmap shows the filtration efficiencies of 18 single fabrics, ten assemblies, ten reusable masks, seven disposable masks, and one fabric for reusable masks. Variations were seen in disposable masks, mostly meeting standards. Most single materials lacked sufficient filtration efficiency for particles sized 0.1–1.5 μm , unsuitable for single-layer masks filtering under 3 μm particles. Assemblies and disposable masks generally had higher efficiencies in this range. Commercially available reusable masks, except for one commercial mask, showed marginal improvements over single materials [135].

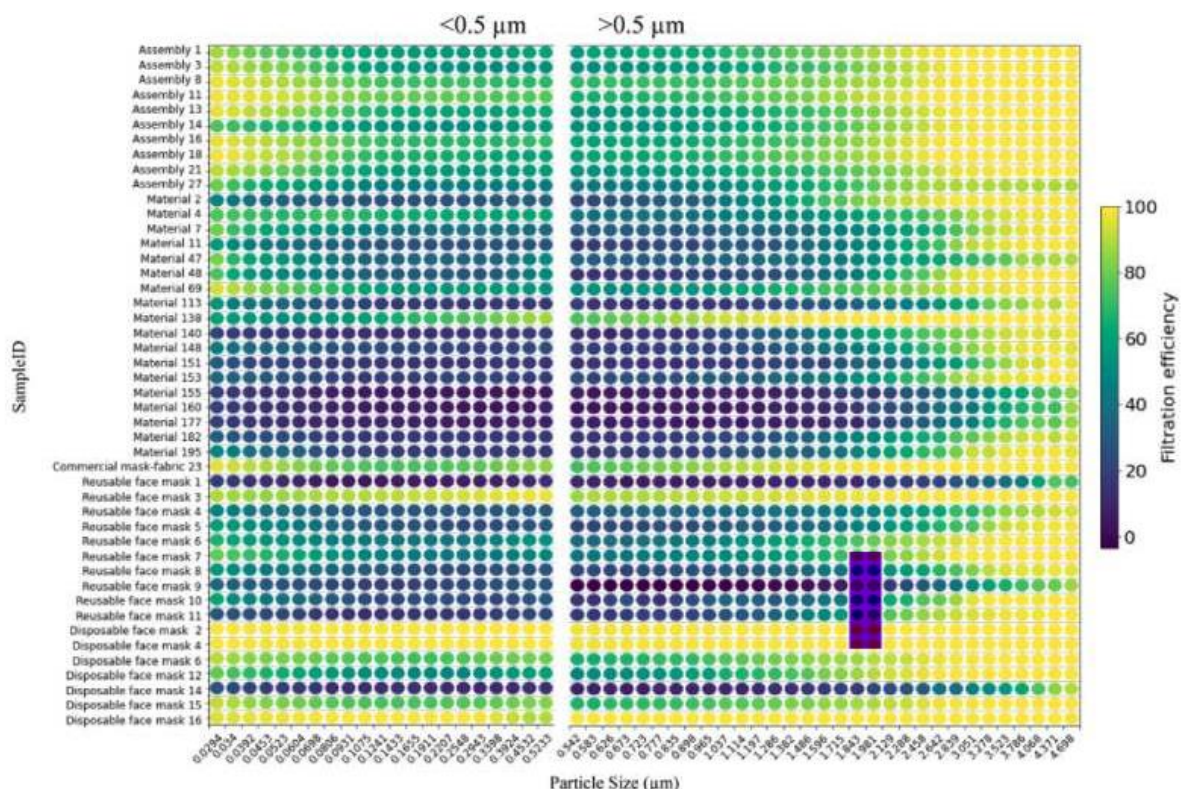


Figure 11.4 Heatmap for Filtration Efficiency: The Aerosol particle size-selective filtration efficiency heatmap shows filtration efficiency (percentage) per particle size, colour-coded by Sample ID.

11.4.1 Evaluation/testing of Reusable, Non-medical Face Masks Currently on the Market

We evaluated a variety of non-medical, reusable face masks that are currently available on the market. Most of these masks were developed in response to the COVID-19 pandemic, except one from Western Europe. We acquired these masks by purchasing them directly or sending them to us. Our evaluation involved comparing these reusable masks to a standard disposable face mask sold in Switzerland. In general, the reusable masks we tested exhibited low filtration efficiency. Except for one mask from Southern Asia, their filtration efficiency for 1.0- μm particles was, at best, around 50%.

However, the Southern Asian mask had good filtration but very low breathability, which was attributed to a high-pressure drop, making it impractical (Figure 11.5).

Even though some countries have set standards for reusable masks, our findings suggest poor overall filtration performance. This indicates a clear need for improvements in the design of future masks [135].

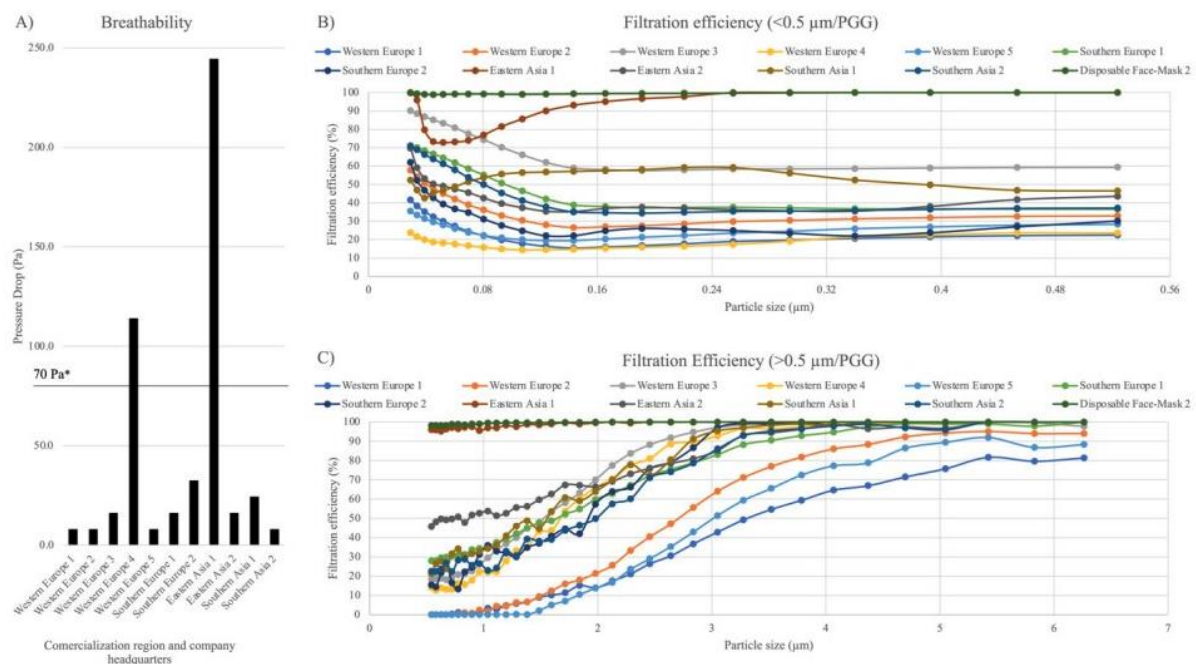


Figure 11.5: (A) Breathability and (B, C) filtration efficiencies for particles in two size ranges (< 0.5 μm and > 0.5 μm) of various commercial, reusable face masks from different regions worldwide.

11.5 Discussion

The comprehensive evaluation of over 300 fabrics and assemblies during the COVID-19 pandemic revealed critical performance gaps in materials commonly used for DIY and commercial reusable face masks, with most single materials demonstrating insufficient filtration efficiency for particles in the 0.1–1.5 μm range that are most relevant for respiratory virus transmission. While fabric assemblies showed superior performance compared to single materials due to multi-layer filtration mechanisms, commercially available reusable masks achieved only ~50% filtration efficiency for 1.0- μm particles, revealing inadequate quality control in the rapidly expanding mask market and highlighting the complex trade-off between filtration efficiency and breathability. The study's dual aerosol approach (NaCl and PG/G) provided a robust screening methodology, demonstrating that filtration efficiency for

fabric combinations could be predicted from individual fabric properties, which significantly advanced screening efficiency for future emergency responses. Notably, the demonstration that biodegradable fabrics could meet filtration standards offers a sustainable solution to the environmental crisis of disposing of 120 billion masks monthly, while the systematic global analysis using the Taxila™ platform revealed converged international recommendations that may have overlooked superior locally available materials [135].

11.6 Conclusion

We used a semi-automated text-mining platform called Taxila™ to analyse various fabrics and combinations for reusable face masks, focusing on breathability (measured as the pressure drop across the fabric) and filtration efficiency for fractional aerosol particle sizes. We discovered we could predict filtration efficiency for fabric combinations based on individual fabrics, simplifying the screening process. We aimed to create a method for identifying fabrics with good filtration and breathability for reusable masks. We avoided ranking materials because guidelines vary by country and regional regulation [135].

Our evaluation concentrated on the physical properties of mask materials, not design or facial fit factors. However, an imperfect fit can lower filtration efficiency due to gaps between the skin and the mask. Our tests showed that using neutralised aerosols is important for accurately measuring the filtration efficiency of small particles in (non-)woven fabrics. Regional recommendations, like those in Switzerland, may be affected by differences in filtration efficiency at 1.0 mm, though most standards use measurements at 3.0 mm, where these differences are negligible [135]. Besides aerosol filtration and breathability, practical, biochemical, and toxicological aspects should be considered when choosing mask materials. Biocompatibility is critical for prolonged use, antiviral coatings can reduce contamination risk, and bioburden concerns involve microorganisms in filter material and potential fibre release. Though not our current focus, our process can be expanded to include these analyses for promising fabrics. Moisture accumulation's impact on mask performance should also be evaluated. We demonstrated that biodegradable fabrics could meet strict standards for face masks, offering a sustainable solution during the COVID-19 crisis [135].

11.6 Research Impact

Since its publication in 2021, our study titled “Aerosol Filtration Testing of Fabrics for Development of Reusable Face Masks” has made a measurable impact in the field, with at least seven citations to date (Google Scholar, 2025). Building on this work, my colleague Sandra Schorderet Weber and our team developed a novel in vitro salt-coating technique for mask fabrics, aimed at enhancing antiviral

efficacy [137]. Although this research was not included in the thesis, [137] preliminary results demonstrated promising potential for viral inactivation without compromising filtration performance or breathability.

Notably, Rahman et al. cited our study in their review “Face Masks to Combat Coronavirus (COVID-19)—Processing, Roles, Requirements, Efficacy, Risk and Sustainability” [138][138]. They also featured a key figure from our publication to emphasise the critical balance between filtration efficiency and breathability visually—a central design challenge in reusable face masks. This integration highlights the broader applicability of our findings and suggests that our work could influence the development of future mask designs, particularly in shaping safety standards and prevention regulations.

12.0 Pulmonary Delivery of Aerosolized Chloroquine and Hydroxychloroquine to Treat COVID-19: *In Vitro* Experimentation to Human Dosing Predictions

12.1 Introduction and Contribution

SARS-CoV-2, the virus responsible for causing severe acute respiratory syndrome, is highly contagious and results in widespread respiratory illnesses in humans. The World Health Organisation officially classified COVID-19 as a global pandemic on March 11, 2020. While three vaccines have been approved for emergency use in multiple nations, repurposing existing medications for short-term prevention remains a potential immediate remedy. The efficacy of chloroquine (CQ) and hydroxychloroquine (HCQ) in early and advanced infection stages was demonstrated through in vitro tests on Vero cells infected with SARS-CoV-2. Our work with the Vitrocell in vitro exposure system included techniques for real-time dosimetry measurement during exposures, showcasing our commitment to utilising appropriate resources to aid the community during times of crisis. This chapter details our efforts during the COVID-19 crisis, emphasising the utilisation of scientific knowledge to explore new avenues for treatment and testing compounds in vitro, generating data to support the community in advancing to the next phase of clinically testing these substances.

In this chapter, I will discuss the research paper "**Pulmonary Delivery of Aerosolized Chloroquine and Hydroxychloroquine to Treat COVID-19: In Vitro Experimentation to Human Dosing Prediction.**

(Kolli AR, Semren TZ, Bovard D, **Majeed S.** van der Toorn M, Scheuner S, Guy PA, Kuczaj A, Mazurov A, Frentzel S, Calvino-Martin F, Ivanov NV, O'Mullane J, Peitsch MC, Hoeng J.) (126). I am one of the co-authors of this article and contributed to the experimental design, exposure experiments, and publication. The study was conducted during the COVID-19 pandemic. We developed an innovative method to aerosolize Chloroquine and Hydroxychloroquine as potential treatments and filed a patent for this process, which was granted to us (see Appendices B and C).

12.2 Background

SARS-CoV-2, the virus causing severe acute respiratory syndrome, is highly contagious and leads to widespread respiratory illnesses in humans. On March 11, 2020, the World Health Organisation declared COVID-19 a global pandemic. Although three vaccines have been authorised for emergency use in different countries, repurposing existing medications for short-term prevention remains a practical, immediate approach [139].

In vitro studies on Vero cells infected with SARS-CoV-2 showed that chloroquine (CQ) and hydroxychloroquine (HCQ) are helpful during both early and later stages of infection. These drugs, traditionally used to treat malaria, rheumatoid arthritis, and systemic lupus erythematosus, are thought to fight COVID-19 by raising the pH levels of endosomes, lysosomes, and cell membranes. This increase in pH inhibits the virus's ability to fuse with host cells and thereby prevents the infection from replicating [140]. They may also interfere with the glycosylation of ACE2, decreasing the binding efficiency between host cells and the coronavirus's spike protein. Additionally, the build-up of CQ or HCQ in lysosomes might cause enzyme dysfunction, impairing the processing and modification of viral proteins [141].

Chloroquine (CQ) and hydroxychloroquine (HCQ) each have three primary functional groups with distinct pKa values: 4.0, 8.4, and 10.2 for CQ, and less than 4.0, 8.3, and 9.7 for HCQ. At physiological pH, two of these groups are protonated. Both drugs in their unprotonated forms can quickly and easily cross cell membranes and organelles, enabling them to access acidic cytoplasmic vesicles such as endosomes, lysosomes, or Golgi vesicles. As these unprotonated molecules become protonated within the acidic environments and become trapped, the concentration of these compartments increases markedly [5, 6]. For example, lysosomal concentrations of CQ are estimated to be about 1,000 times higher than in the surrounding cytosol. CQ and HCQ can build up in the acidic compartments of cells, potentially leading to toxicity in tissues abundant in these compartments. Physiologically based pharmacokinetic (PBPK) models help simulate drug levels in tissues and lysosomes, helping with safe dosing regimens for COVID-19 treatment. Clinical trials on CQ and HCQ for COVID-19 yielded mixed results and raised concerns about side effects. High oral doses of CQ and HCQ were linked to cardiac issues, especially in patients with preexisting heart conditions, leading to the discontinuation of their oral use for COVID-19 [142].

Despite this, some reports indicated better survival rates for patients who received HCQ, especially with azithromycin, sparking debate about HCQ's potential benefits in treating COVID-19. A clinical trial involving over 15,000 participants was ongoing in the USA to determine if HCQ can prevent COVID-19 in healthcare workers, at the time of this work. This study offers insights into HCQ's potential role in preventing COVID-19 [143].

Researchers are also exploring the delivery of CQ and HCQ directly to the lungs through pulmonary drug delivery. This approach concentrates the drugs at the target site while reducing systemic exposure. Studies are assessing the effectiveness of CQ and HCQ as inhalable aerosols and their molecular-level effects on the respiratory tract. Therefore, the aim of this work was to generate CQ and HCQ aerosols using an aerosol-generating device for testing on human bronchial cells, evaluating any negative impacts on cell cultures, and optimising dosing for safe clinical usage [144].

12.3 Methods

12.3.1 Compound Synthesis and Aerosol Formulation

WuXi AppTec produced CQ and HCQ using published methods [141–142], with purities of 98.3% and 99.7%, respectively. The compounds were dissolved in propylene glycol to make various liquid formulas, and their solubility was tested by using LC-HR-MS [144].

12.3.2 Aerosol Generation and Characterisation

Aerosols of chloroquine (CQ) and hydroxychloroquine (HCQ) liquid formulations were generated using a thermal aerosolisation device developed by Philip Morris International, operating at 200–220°C. Particle size distribution was assessed using a system comprising a programmable syringe pump, a 3302A aerosol diluter, and an aerodynamic particle sizer (model 3321, TSI Incorporated, Shoreview, MN, USA), connected via a conductive tube. A Y-piece allowed ambient air to stabilise flow rates and prevent pressure buildup. To ensure accurate measurements and chemical analysis, aerosol samples were diluted 100-fold. Discharge durations were set at 3 seconds (1.1 L/min) for sizing and 8 seconds (0.41 L/min) for in vitro delivery (Figure 12.1) [144].

12.3.3 Analytical Characterisation of Aerosolised CQ and HCQ

The thermal aerosol-generating device was integrated with a syringe pump and a SUPER SESI system, which was connected to a Q Exactive HF mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). To quantify the transfer of CQ and HCQ from liquid to aerosol, the emitted aerosol was passed through a Cambridge filter pad followed by an impinger. Both the filter pad and impinger were treated with 10 mL of ethanol to extract the compounds, and the analysis was performed using liquid chromatography coupled with high-resolution mass spectrometry (LC-HR-MS). Chemical analyses to assess drug solubility and transfer rates were performed using a liquid chromatograph fitted with a HILIC BEH amide column and a high-resolution accurate mass spectrometer [146]. The mobile phase included acetonitrile with 0.1% formic acid and 10 mM ammonium formate in water. Samples were diluted to match a nine-point calibration curve ranging from 5 to 100 ng/mL. A 5 µL aliquot of the diluted sample was injected into the system. Detection was carried out in positive electrospray ionisation (ESI+) mode, with data acquired in full-scan mode over an m/z range of 50–350. The mass spectrometer operated at a resolution of 60,000, ensuring precise mass detection and accurate compound identification [145].

12.3.4 Cell Culture

Primary human bronchial epithelial cells (HBECS) (Lonza, Basel, Switzerland) derived from a 60-year-old Black male were cultured in PneumaCult-Ex Plus™ medium under standard conditions. Once the cells reached 80% confluence, they were detached using trypsin-EDTA, and 50,000 cells were seeded onto Transwell inserts with 0.4- μ m pores. The basal culture medium was replaced and replenished every 2 to 3 days to maintain optimal cell growth conditions. After four weeks, the cultures developed at the air-liquid interface (ALI) and were used for experiments from week 5 onward [144].

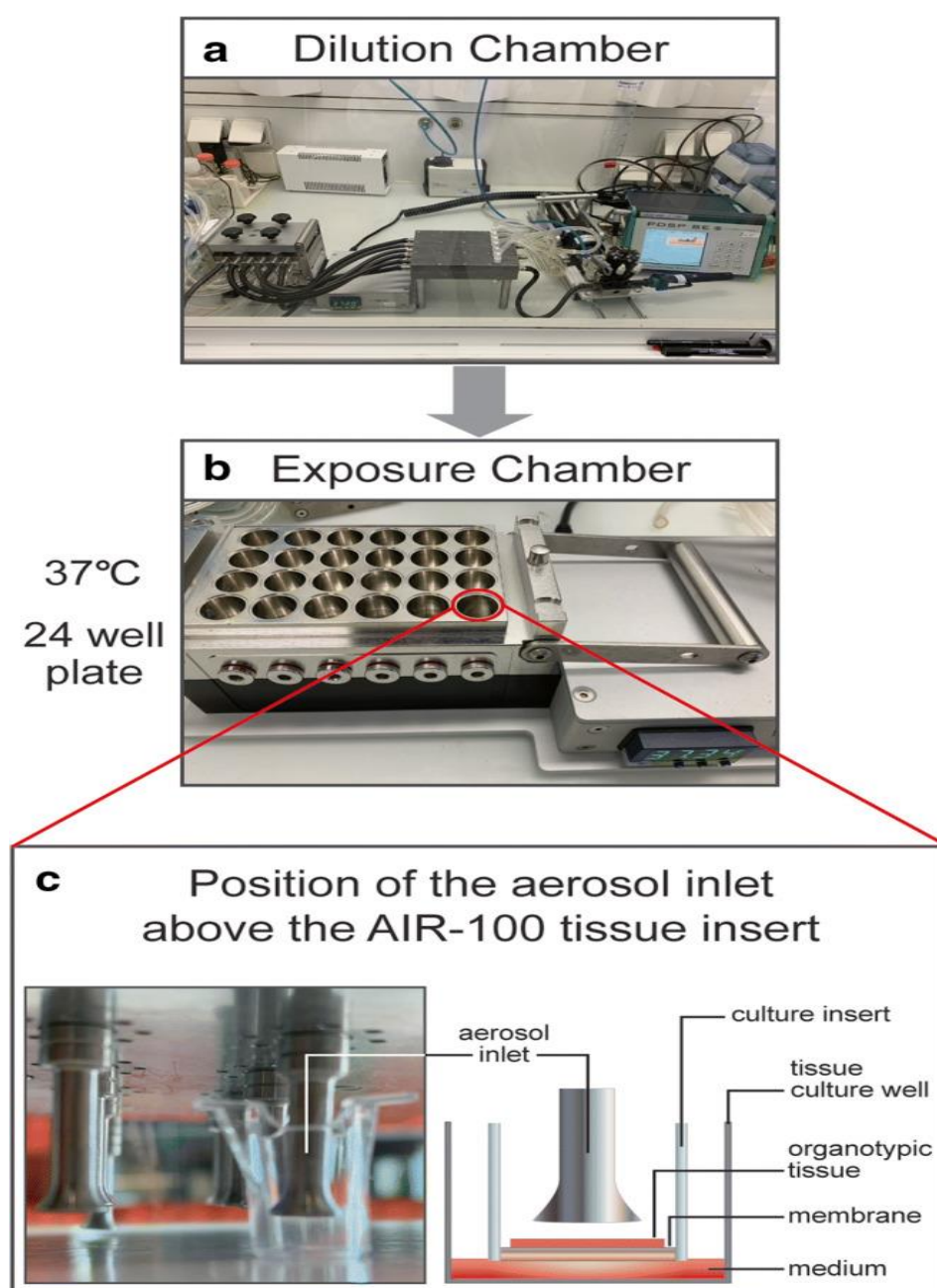


Figure 12.1 Schematic of an in vitro aerosol generation and exposure system: The aerosol produced travels through a dilution chamber and then into an exposure chamber with trumpet-shaped outlets

that direct to cell culture inserts. Each insert holds 3D-human bronchial airway cultures at the air-liquid interface on a porous membrane with a culture medium at the bottom.

(A) Dilution Chamber: The Vitrocell 24-well exposure system is equipped with a dilution chamber that effectively dilutes the freshly prepared aerosol as it enters the system from the pump.

(B) Exposure Chamber: The Vitrocell 24-well exposure system features an advanced exposure chamber designed explicitly for administering 3D organotypic human bronchial airway cultures, exposing them to the test aerosols. This chamber is meticulously maintained at a constant temperature of 37°C throughout the entire duration of the exposure process.

(C) 3D human bronchial airway cultures are inserted and connected to the trumpet for exposure to the test aerosol [144].

12.3.5 Vitrocell Aerosol Exposure

The Vitrocell 24 exposure system, along with the aerosol delivery pump, was placed inside a biosafety cabinet to ensure controlled conditions. Aerosols were generated from a solution containing 25 mg/mL of CQ and HCQ, using a puffing protocol with a volume of 55 mL per puff, a duration of 3 seconds, and a 30-second interval between puffs. The generated aerosol was directed to the top of the exposure chamber via a pump. The generated aerosol was uniformly distributed into the cultivation base module via port ejectors operating under negative pressure. Human bronchial epithelial cells, cultured at the air-liquid interface (ALI), were positioned within the cultivation base and maintained at 37°C. These cells were exposed to varying concentrations of aerosolised compounds directly on their apical surface. To assess aerosol deposition, cells were exposed to 25, 50, or 100 puffs of CQ or HCQ aerosols. Control exposures included 100 puffs of synthetic air (composed of 85% nitrogen and 15% oxygen) and 100 puffs of propylene glycol. Aerosol deposits were collected using Transwell inserts containing ultra-pure water, placed within the exposure chamber. These inserts, holding 110 µL of water, were positioned in the base module of the exposure system alongside the cell cultures during each experiment. Kinetics of HCQ were analysed in both the apical and basal compartments. The concentrations of CQ and HCQ in these aerosol samples were quantified as described in Section 12.3.3. [145].

12.4 Results

12.4.1 Formulation and Characterisation of CQ and HCQ Aerosols

Aerosol formulations with up to 40 mg/mL of CQ and 100 mg/mL of HCQ were prepared using PG. The solubility of CQ and HCQ in PG was measured with LC-HR-MS utilising an external calibration curve.

The accuracy of the measurements was verified by comparing the experimental concentrations with theoretical values. A thermal aerosol device was loaded with these formulations (40 mg/mL CQ or 100 mg/mL HCQ) for aerosol administration. The device requires user activation to produce aerosol, which is achieved by taking a 55 mL puff over 3 seconds, followed by a 30-second pause managed by a pump (see Figures 12.2a and 12.2b). The aerosol particles had a median diameter of 1.3 μm and a geometric standard deviation of 1.5 (see Figure 12.2b). The aerosol transfer of CQ and HCQ from the device was measured, showing that each puff released around 0.15 mg of CQ (with a coefficient of variation of 9.84%) and 0.33 mg of HCQ (with a 4.78% CV) over a total of 30 puffs (refer to Figure 2c). The device contains 2 mL of liquid and is capable of delivering up to 400 puffs, with an estimated transfer efficiency of 78% for CQ and full (100%) efficiency for HCQ and other substances [145].

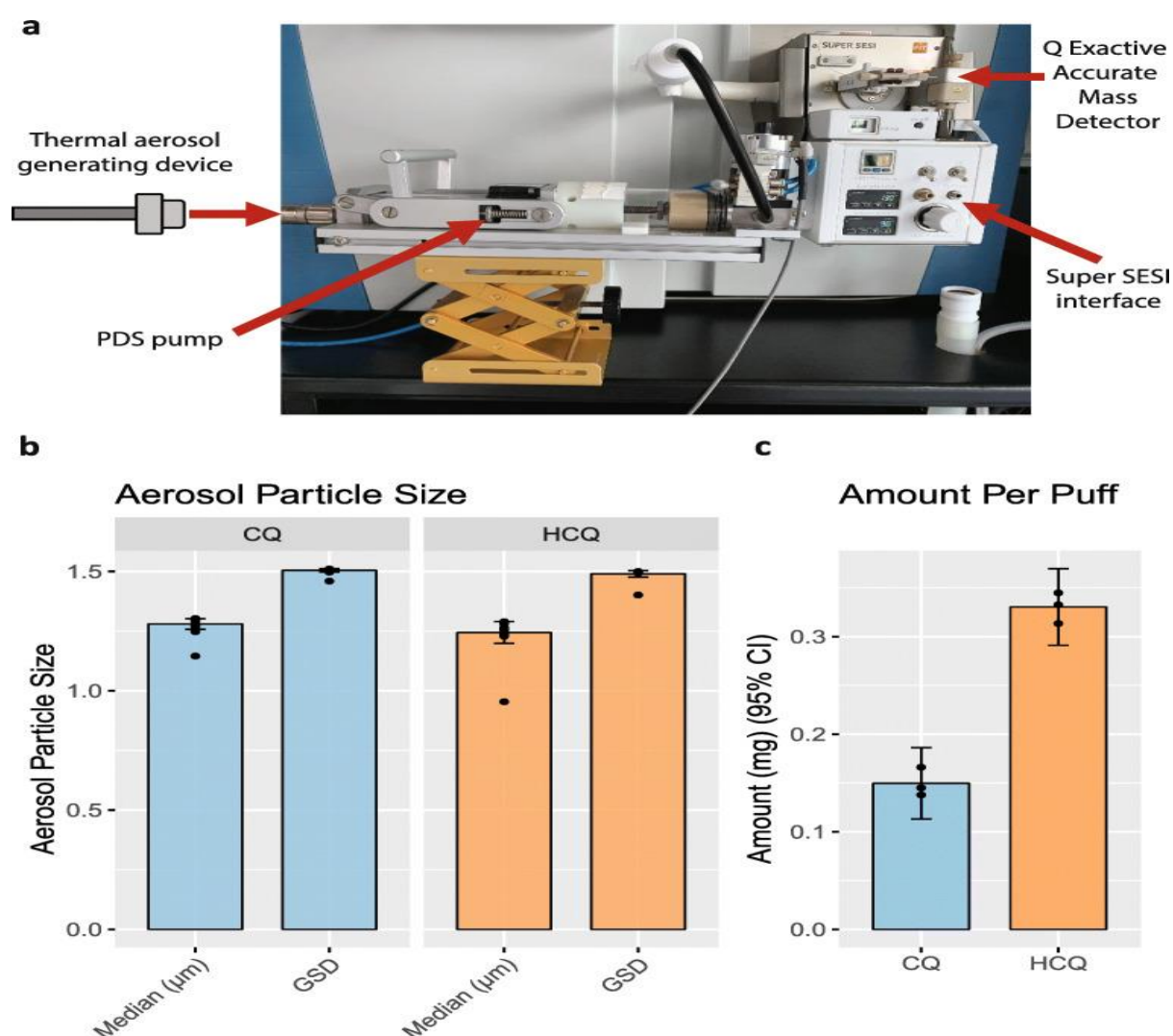


Figure 12.2 Characterisation of chloroquine (CQ) and hydroxychloroquine (HCQ) aerosols produced by thermal aerosolisation:

(A) The instrumental setup includes a thermal aerosol-generating device, a programmable dual syringe pump, and a SUPER SESI platform interfaced with a Q Exactive HF high-resolution mass spectrometer to measure CQ and HCQ aerosolisation.

(B) Particle size analysis (N = 30) with the help of APS.

(C) Measure CQ and HCQ amounts transferred per 55 mL puff volume from the device (N = 3). Data is shown as mean values (bars), technical replicates (dots), and 95% confidence intervals (error bars) [145].

12.4.2 *In Vitro* Toxicity Assessment of CQ and HCQ Aerosols with 3D Organotypic HBEC

To assess the safety of CQ and HCQ aerosols in human airways, 3D organotypic HBEC cultures at the ALI were exposed to various aerosol puffs containing these compounds. The ciliary beating frequency stayed stable after exposure to both drugs. However, CQ led to a concentration-dependent reduction in the active ciliary area, while HCQ exposure did not significantly impact it [145].

12.5 Discussion

A range of inhalation-based treatments has been developed for respiratory diseases. This study focused on aerosols of CQ and HCQ produced by thermal aerosolisation, which had inhalable particle sizes and did not impact human bronchial epithelial cell cultures. Thermal aerosolisation entails evaporating a liquid formulation and then cooling it to generate particles capable of penetrating deep into the lungs. In contrast, non-thermal liquid aerosolisation, like nebulisation, leads to larger particle sizes that may hinder delivery to deep lung tissues. The study also revealed that the pharmacokinetics of inhaled aerosols from different nebulisers could be simulated using the inhalation PBPK model to understand respiratory drug delivery better. CQ and HCQ accumulate in acidic regions due to ion trapping. The surface fluid and lining cells of the respiratory tract are acidic, promoting the accumulation of CQ and HCQ. Nevertheless, pH levels vary between species, leading to species-specific differences in pulmonary condition exposures. High doses of these drugs delivered through the lungs may facilitate rapid transfer across airways, with our inhalation PBPK model accounting for these factors to predict tissue distribution. However, further studies are needed for cell-type-specific distribution. The clinical effectiveness of oral CQ and HCQ is still uncertain because the unbound concentrations in lung interstitial fluid are below the *in vitro* extracellular EC₅₀ levels. Few studies have demonstrated better patient outcomes with oral HCQ, likely because effective concentration lower bounds were reached. The initial *in vitro* tests of CQ and HCQ's effectiveness were conducted

on Vero cells, showing EC₅₀ values from 0.72 to 22.3 μ M depending on the infection multiplicity. Hoffman et al. found EC₅₀s of 100 μ M for both CQ and HCQ in Calu-3 cells infected with SARS-CoV-2, likely due to differing viral entry mechanisms between these cell types [145, 147]. Studying CQ and HCQ efficacy in primary human respiratory epithelium cultures may be more clinically relevant than in cell lines. Exposure to 10 μ M HCQ significantly decreased viral N gene expression in primary human 3D alveolar organoid cultures infected with SARS-Cov-2. Additionally, De Reus et al. observed a notable decrease in viral load when using 40 μ M HCQ on infected primary human epithelial cells [147].

Estimating the precise dose deposited in the lungs during pulmonary drug delivery is a complex task. Nonetheless, even low inhaled doses of chloroquine (CQ) and hydroxychloroquine (HCQ) can reach therapeutically effective levels in lung tissue while reducing systemic exposure. This targeted delivery highlights the potential of aerosolised CQ and HCQ as prophylactics against COVID-19. However, clinical trials are necessary to confirm their safety and effectiveness in treatment [145].

12.6 Conclusions

Aerosol formulations of chloroquine (CQ) and hydroxychloroquine (HCQ) containing respirable particles were successfully developed for potential pulmonary delivery in the treatment of COVID-19. In vitro exposure of human bronchial epithelial cells cultured at the air-liquid interface revealed no negative impacts on cell viability, transepithelial electrical resistance (TEER), or ciliary activity. The kinetics of drug transport across the airway epithelial barrier were validated through both laboratory experiments and an Integrated Physiological Modelling of the Lung (IPML) approach. Physiologically based pharmacokinetic (PBPK) modelling predicted that inhalation of 1.5 mg CQ or 3.3 mg HCQ three times daily could achieve effective pulmonary concentrations while limiting systemic exposure. This study was conducted during the peak of the COVID-19 crisis, when rapid therapeutic interventions were urgently needed. At that time, it was anticipated that an optimised inhalation dosage of chloroquine (CQ) and hydroxychloroquine (HCQ) could potentially accelerate recovery and improve treatment outcomes[144].

Note: We do not recommend using these drugs for treatment purposes. Instead, our focus is on highlighting potential alternative methods for administering these medications. Our goal is to explore different approaches that may enhance effectiveness or reduce side effects, providing a more comprehensive understanding of treatment options.

12.7 Research Impact

Since this research paper was published in 2022, several subsequent studies have emerged in the field, resulting in nine references, as reported by Google Scholar. This paper has been primarily cited for methodological and conceptual contributions rather than direct practical adoption. It has served as:

Proof of Concept for Pulmonary Delivery:

The study provided early experimental validation that aerosolised chloroquine and hydroxychloroquine could achieve therapeutically relevant concentrations in lung tissue without systemic toxicity. This concept was later explored in greater depth by Xia et al., who investigated the spatiotemporal distribution of inhaled HCQ in rat lungs, confirming heterogeneous deposition patterns and supporting the feasibility of localised delivery [147].

13.0 Overall impact of the research

Inhaling environmental and occupational substances is a regular aspect of daily life, significantly impacting our well-being. With an estimated 7 million premature deaths yearly, ambient air pollution is a crucial public health concern. With the advancement of industrialisation, the anticipated increase in harmful exposures further necessitates addressing this pressing issue.

Beyond the common environmental substances typically found in standard air, which include pollutants such as vehicle emissions and pesticides, there exists a wide array of compounds that people may inhale—either deliberately or inadvertently—for various reasons. These compounds can range from recreational substances like tobacco and cannabis to industrial chemicals present in specific workplaces. Additionally, many people may breathe in household chemicals, fragrances, or allergens that can affect health and well-being. Understanding the diverse sources and implications of these inhaled substances is crucial for assessing their impact on individual and public health [3].

Cigarette smoke stands out as a prime example of a substance often inhaled for pleasure, despite its well-known health hazards. Moreover, specific pharmaceutical medications are also delivered via inhalation for therapeutic reasons, highlighting inhalation's intricate and dual nature, serving as both a source of enjoyment and a treatment modality [10].

When examining the effects of different aerosols or smoke on human health, researchers primarily rely on two methods: utilising animal models or conducting *in vitro* testing. Animal models have traditionally been the standard approach; however, an increasing emphasis on *in vitro* studies offers a promising alternative. Our primary goal is to develop and use non-animal methods to generate essential toxicological data, specifically at the air-liquid interface (ALI). This approach allows for more realistic evaluations of aerosols interacting with human tissues. By using *in vitro* models, researchers can assess the potential health impacts of aerosol exposure without involving live animals, addressing ethical concerns and reducing the number of animals needed for testing. For *in vitro* research to be effective, it is crucial to establish a well-characterised exposure system that accurately mimics real-life conditions. Finally, the rising adoption of *in vitro* exposure systems among research institutions and regulatory bodies signifies a notable shift in the field. This trend underscores the increasing recognition of the importance of non-animal testing methods for assessing health risks related to aerosol exposure [17].

In this thesis work, I developed and characterised *in vitro* exposure systems, demonstrating the impressive adaptability of the Vitrocell platform in handling liquid aerosols and cigarette smoke. I also developed novel characterisation techniques that enhance the research. Although my primary focus

was on smoke, electronic nicotine delivery systems (ENDS), and liquid aerosols, it is vital to recognise the broader potential applications of this system and its ability to advance inhalation science. My research expands the scientific knowledge of the Vitrocell exposure system, underscoring its considerable potential and shedding light on areas for enhancement [7,25].

Additionally, a novel exposure system known as InHALES has been introduced to improve the precision of delivering inhaled substances to the respiratory tract. It achieves this by accounting for the dynamics of particle deposition and gas absorption across the airway and lung epithelial surfaces.. Unlike conventional in vitro aerosol exposure systems that may fail to replicate these features, the innovative InHALES system closely mimics human respiratory functions by actively simulating breathing, operating medical inhalers, and puffing on products. Due to its structural and functional similarity to the human respiratory tract, this system is expected to deliver inhaled materials in doses relevant to human cell cultures that represent respiratory tract epithelia [39]. In our pursuit to empower the scientific community and advance future research, PMI and TissUse have embarked on a groundbreaking collaboration. Together, we are uniting InHALES technology with TissUse's Multi-Organ-Chip (MOC) platform, creating the innovative InHALES + MOC system. This partnership aims to create an innovative testing platform that mimics the human respiratory tract, allowing for inhalation exposure of functional human tissues in vitro (Press release: [Philip Morris International and TissUse develop a human aerosol test platform to emulate the entire human respiratory tract](#)). Tyler Goralski, a research biologist at the U.S. Army Combat Capabilities Development Command Chemical Biological Centre (CCDC CBC), showcased an application of the cutting-edge InHALES + MOC system. This system can be utilised to explore the effects of exposure to chemical and biological threat agents using a multi-organ microphysiological model. It aspires to revolutionise toxicity and efficacy testing by providing an ideal alternative or replacement for traditional tissue cultures and animal models, serving as a powerful "human surrogate" (Title: Soldier-on-a-chip: interrogating the effects of chemical and biological threat agent exposure using a multi-organ microphysiological system, Link - [U.S. ARMY COMBAT CAPABILITIES DEVELOPMENT COMMAND – CHEMICAL BIOLOGICAL CENTER \(ICCVAM 2023 Public Forum\)](#)).

Well-characterized in vitro exposure systems have the potential to drive significant progress in inhalation science. The research presented in this thesis could serve as a cornerstone for this field, facilitating researchers' ability to conduct experiments with greater control in a well-characterised exposure system. Below, I highlight critical instances of in vitro exposure systems used to study the inhalation toxicities of various types of smoke and aerosols, and how regulators can define different guidelines or laws based on the research [16].

Environmental Monitoring:

Researchers conduct thorough investigations into air quality monitoring using in vitro exposure setups [136]. This process entails evaluating both indoor and outdoor pollutants by implementing long-term sampling methods that typically extend beyond 24 hours. Such extended sampling is crucial for obtaining accurate data on pollutant levels and their variations over time. Marco et al. also used the Vitrocell exposure system to investigate the systems toxicology effects of wood smoke, a major contributor to air pollution. Their findings highlighted the significant impact of the gas phase—especially carbonyl compounds—in triggering harmful biological responses (137). The Vitrocell exposure system, which we have thoroughly characterized in this thesis, represents one of the key methodologies employed in the field of air pollution research. This state-of-the-art system is designed to simulate real-world exposure conditions, allowing researchers to better understand the effects of various pollutants on human health and the environment. Our study highlights the versatility of this system, demonstrating its applicability not only in the assessment of cigarette smoke, but other researchers have also used this system to investigate the emissions from wood combustion (137). This broader scope underlines its importance in tackling diverse air quality issues and advancing our knowledge in environmental science.

Occupational safety:

The field of occupational health focuses specifically on workplace exposure in addition to general air quality concerns. This aspect emphasizes the critical need to assess the potential health risks linked to various air pollutants that employees may encounter in their work environments. By identifying and quantifying these risks with the help of in vitro exposure systems, researchers can better inform safety regulations and practices aimed at protecting worker health and well-being. Zavala et al. presented their latest research on evaluating the health risks of air pollutants. They clearly describe the need for in vitro exposure testing and highlight critical design considerations for such testing [136], and the innovative methodology presented in this thesis allows for the testing of real-life scenarios in workplace environments to examine occupational safety, enabling regulators to develop informed regulations based on scientific evidence.

Inhalable vaccine development and Antiviral drug testing on airway tissues:

Numerous studies(136) have concentrated on the development of vaccines and pharmaceuticals designed for oral administration through aerosol delivery. This method aims to enhance local immunity, which is essential for providing effective protection to the respiratory tract against various pathogens. Researchers commonly employ in vitro exposure systems to evaluate the effectiveness of these aerosolised therapies. These systems simulate the conditions of the respiratory tract, allowing for a controlled environment to test the efficacy and safety of the vaccines and drugs being studied.

By utilising these advanced testing methodologies, scientists can better understand how these formulations interact with respiratory tissues and their potential to stimulate local immune responses [145, 148].

In this thesis, I presented various practical applications that demonstrate how in vitro exposure systems can be utilised to investigate inhalation toxicity. These systems offer a controlled environment for assessing the effects of inhaled substances on biological samples. However, I acknowledge that numerous other significant applications remain unexplored in this work. For instance, the safety assessment of nanomaterials is a crucial area in which in vitro methods can play a pivotal role, given the unique properties and potential risks associated with these materials. Additionally, the investigation of pesticide exposure through in vitro models is another important topic that warrants further study. Addressing these areas could enhance our understanding of inhalation risks linked to a variety of substances.

Over the past 12 years, my journey in inhalation science has been marked by significant contributions to the discipline. I have proudly authored and co-authored over 30 scientific papers published in esteemed peer-reviewed journals, which have profoundly enhanced our understanding of aerosol generation, characterisation, and their implications within in vitro exposure systems. Due to constraints on available space, I could not thoroughly discuss several significant publications of mine that deserve attention in this thesis (139, 140, 141). However, I continue to be actively engaged in the scientific community, passionately working on the development of an innovative exposure system that aims to transform research methodologies. Additionally, I am dedicated to supporting various scientific publications currently in the submission process, reflecting my unwavering commitment to advancing knowledge and fostering collaboration within the field.

Moreover, my research efforts have resulted in the development of an innovative next-generation exposure system designed to replicate real-world inhalation scenarios better. This system not only improves the accuracy of toxicity assessments but also enables more efficient testing processes. Alongside this, I have also developed advanced tools aimed explicitly at characterising aerosols and smoke, which are crucial for evaluating their health effects.

My contributions have led to the co-ownership of five patents that highlight various aspects of this field, showcasing the impactful applications of my research. These patents represent significant advancements in technology for assessing aerosol exposure and toxicity, positioning our work as a leader in inhalation science.

List of Appendices

Appendix A:



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Herve et al.

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(54) **SIMULATED RESPIRATORY TRACT**

(57) **ABSTRACT**

(71) Applicant: **PHILIP MORRIS PRODUCTS S.A.**,
Neuchatel (CH)

(72) Inventors: **Pierre Herve**, Dailens (CH); **Shoaib Majeed**, Neuchatel (CH); **Sandro Steiner**, Schonbuhl (CH)

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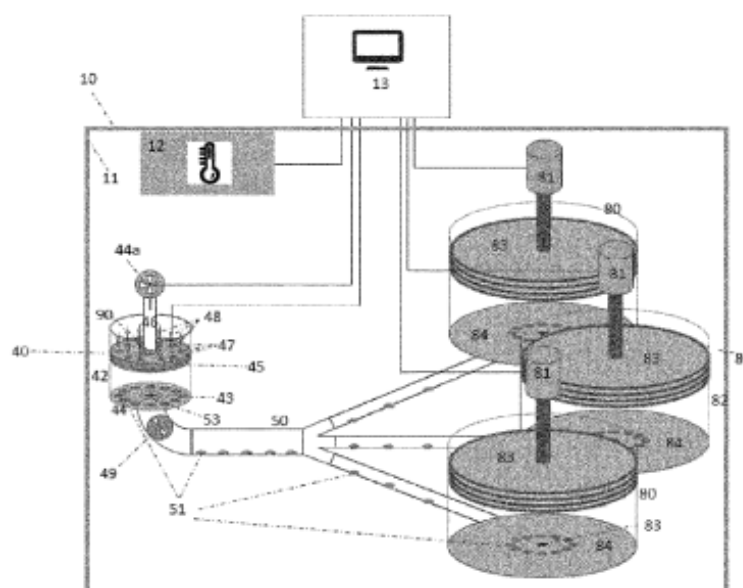
CPC **G01N 33/5005** (2013.01); **A24C 5/34**

(2013.01); **F04B 39/10** (2013.01); **C12N**

5/0697 (2013.01); **G09B 23/32** (2013.01);

A24F 40/80 (2020.01)

There is described herein a system for determining the interaction between a test atmosphere and a simulated respiratory tract, said system comprising: (a) a first pump comprising: (i) a chamber configured for containing a first volume of gas comprising a test atmosphere; (ii) a first port adapted for receiving and outputting gas and comprising a valve for regulating the flow of gas through the first port, said valve being moveable between open and closed positions, wherein in the open position said valve is openable towards a test atmosphere or surrounding air; (iii) a second port adapted for outputting and receiving gas and comprising a valve for regulating the flow of gas through the second port, said valve being moveable between open and closed positions; (iv) a piston plate in the chamber, said piston plate comprising one or more apertures for the uptake or inflow of gas into the chamber wherein one or more, or each, of the apertures include a valve that is movable between open and closed positions and is capable of regulating the uptake or inflow of gas; (b) a second pump comprising: (i) a chamber configured for containing a second volume of gas, wherein the first and second volumes of gas are different; (ii) a port adapted for receiving and outputting gas; and (iii) a motor for controlling the operation of the second pump; (c) a connecting structure operable to transmit the gas from the first pump into the second pump; and (d) one or more openings in the first pump or the second pump or the walls of the connecting structure or a combination of two or more thereof, said openings being capable of receiving a module for containing a cell culture medium or for monitoring conditions in the chamber or for gas sampling or for gas characterisation.



Appendix B:

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(71) Applicant: PHILIP MORRIS PRODUCTS S.A.
[CH/CH]; Quai Jeanrenaud 3, CH-2000 Neuchâtel (CH).

(72) Inventors: CALVINO, Florian Alexis; Quai Jeanrenaud 3, 2000 Neuchâtel (CH). **GUY, Philippe Alexandre;** Quai Jeanrenaud 3, 2000 Neuchâtel (CH). **HOENG, Julia;** Quai Jeanrenaud 3, 2000 Neuchâtel (CH). **KOLLI, Aditya Reddy;** Quai Jeanrenaud 3, 2000 Neuchâtel (CH). **KUCZAJ, Arkadiusz;** Quai Jeanrenaud 3, 2000 Neuchâtel (CH). **MAJEED, Shoaib;** Quai Jeanrenaud 3, 2000 Neuchâtel (CH). **MAZUROV, Anatoly;** 3704 Timberside Dr., Greensboro, Guilford, North Carolina 27410 (US). **PEITSCH, Manuel;**

Quai Jeanrenaud 3, 2000 Neuchâtel (CH). **SEMREN, Tanja Zivkovic;** Quai Jeanrenaud 3, 2000 Neuchâtel (CH). **VAN DER TOORN, Marco;** Quai Jeanrenaud 3, 2000 Neuchâtel (CH).

(74) Agent: ELLIS, Robin; Reddie & Grose LLP, The White Chapel Building, 10 Whitechapel High Street, Greater London, London Greater London E1 8QS (GB).

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(54) Title: PHARMACEUTICAL COMPOSITION COMPRISING CHLOROQUINE AND USES THEREOF

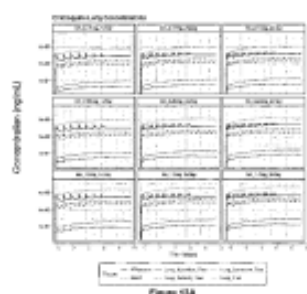


Figure 10A

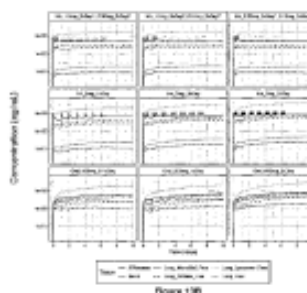


Figure 10B

(57) Abstract: The present invention relates to pharmaceutical compositions comprising chloroquine and uses thereof. More specifically, the invention relates to a pharmaceutical composition comprising chloroquine or pharmaceutically acceptable salts thereof for use in the treatment or prevention of a viral lung infection, preferably caused by Betacoronavirus, including but not limited to 2019-nCoV (coronavirus), SARS-CoV and Middle East respiratory syndrome CoV (MERS-CoV), wherein the pharmaceutical composition is administered by inhalation. The present invention provides an improved delivery into the lungs of a subject with minimum systemic exposure.

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Appendix C:



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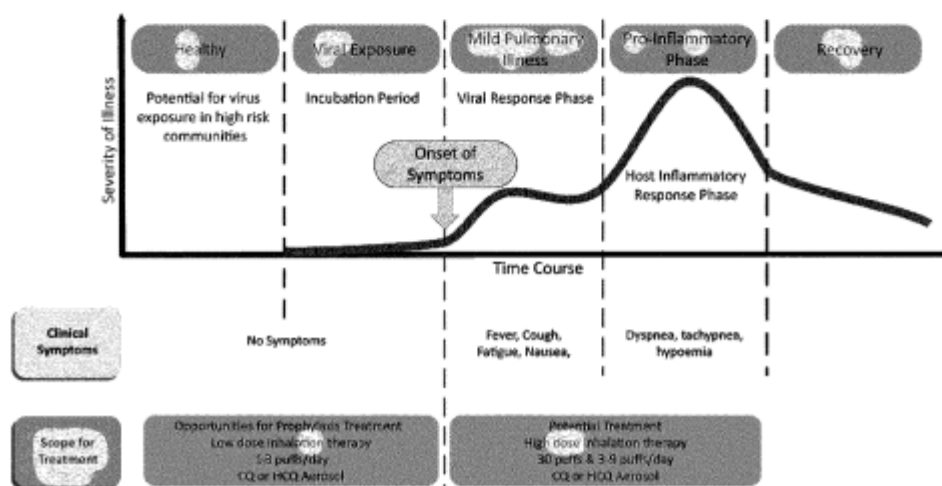
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(57) **ABSTRACT**

A pharmaceutical composition may include hydroxychloroquine or a pharmaceutically acceptable salt thereof, and a solvent. The pharmaceutical composition may include 1 to 400 mg/mL hydroxychloroquine or a pharmaceutically acceptable salt thereof, wherein the solvent is selected from propylene glycol, glycerine, propane-1,3-diol and water or combinations thereof and. The pharmaceutical composition may be suitable for thermal aerosolization. A pharmaceutical composition including hydroxychloroquine or a pharmaceutically acceptable salt thereof may be used in the treatment or prevention of a viral lung infection, wherein the pharmaceutical composition is administered by oral inhalation.

(71) Applicant: **Philip Morris Products S.A., Neuchatel (CH)**
 (72) Inventors: **Florian Alexis CALVINO**, Corcelles (CH); **Philippe Alexandre GUY**, Nuvilly (CH); **Julia HOENG**, Corcelles (CH); **Aditya Reddy KOLLI**, Neuchatel (CH); **Arkadiusz KUCZAJ**, Courtaman (CH); **Shoaib MAJEED**, Neuchatel (CH); **Anatoly MAZUROV**, Greensboro, NC (US); **Manuel PEITSCH**, Peseux (CH); **Tanja Zivkovic SEMREN**, Boudry (CH); **Marco VAN DER TOORN**, Boudry (CH)
 (73) Assignee: **Philip Morris Products S.A., Neuchatel (CH)**
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