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Inhalation toxicology

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Abstract. Inhalation of gases, vapors and aerosols can cause a wide range of adverse health effects, ranging from simple irritation to systemic diseases. The large number of chemicals and complex mixtures present in indoor and outdoor air coupled with the introduction of new materials such as nanoparticles and nanofibers, is an area of growing concern for human health. Animal-based assays have been used to study the toxic effects of chemicals for many years. However, even so, very little is known about the potential toxicity of the vast majority of inhaled chemicals. As well as new or refined OECD test guidelines, continuing scientific developments are needed to improve the process of safety evaluation for the vast number of chemicals and inhaled materials. Although studying the toxic effects of inhaled chemicals is more challenging, promising *in vitro* exposure techniques have been recently developed that offer new possibilities to test biological activities of inhaled chemicals under biphasic conditions at the air liquid interface. This chapter gives an overview of inhalation toxicology as well as focusing on the potential application of *in vitro* methods for toxicity testing of airborne pollutants.

Introduction

Exposure to airborne contaminants is a major contributor to human health problems, causing adverse effects ranging from simple irritation to morbidity and mortality due to acute intense or long-term low-level repeated exposures [1–3]. Inhalation exposures can occur with gases, vapors, solid and liquid aerosols and mixtures of these. While evaluating the impact of chemicals on human health requires toxicity data, in many cases, particularly for industrial chemicals, the availability of toxicity information is quite limited [4–7].

The current approach of measuring the toxic effects of airborne contaminants relies on whole animal test methods [8]. As well as ethical concerns, heavy reliance on animal data in toxicology is the subject of debate and controversy by the scientific community [9]. Moreover, the increasing number of available industrial chemicals and new products has created a demand for alternative test methods for safety evaluation [8]. Although studying the toxic effects of inhaled chemicals *in vitro* is technically more challenging, great advances in the application of these methods for investigating the toxicity of airborne contaminants have been made in recent years. This chapter presents a review of the essentials of inhalation toxicology and a focus on the potential application of *in vitro* methods for studying the toxicity of airborne contaminants.

Air pollutants

Air contaminants are exogenous substances in outdoor or indoor air, including both particulate and gaseous contaminants, that can cause adverse health effects in humans or animals, affect plant life and impact the global environment by changing the atmosphere of the earth [10, 11]. Various physical, chemical and dynamic processes may generate air pollution leading to emission of gases, particulates or mixtures of these into the atmosphere [12]. Air quality is continuously affected by emissions from stationary and mobile sources. While great attempts have been made to reduce emissions from these sources, millions of people today face excessive air pollution in both occupational and urban environments [13].

Emissions from mobile combustion sources (e.g., automobiles) are major contributors to urban air pollution, and include carbon monoxide (CO), nitrogen oxides (NO_x), sulfur oxides (SO_x), particulate matter (PM), lead and photochemical oxidants such as ozone (O_3) and ozone precursors like hydrocarbons and volatile organic compounds (VOCs) [11, 13]. Larger air pollution particulates are derived chiefly from soil and other crusty materials, whereas fine and ultra-fine particles are derived mainly from combustion of fossil fuels in transportation, manufacturing and power generation [1]. Many industrial and commercial activities release toxic contaminants in gas, vapor or particulate form. However, air contaminants are not limited to urban or industrial environments, and common indoor air pollutants can be found in offices, schools, hospitals and homes. Tobacco smoke, fuel consumption, furniture, painting, carpeting, air conditioning, and cleaning agents can be significant sources of both chemical and biological air contaminants.

Types of air pollutants

Based on their physical properties, airborne contaminants can be classified into two main types. The first category includes gases and vapors or air pollutants that exist as distinct molecules and can dissolve and form true solutions in the air and follow the fundamental gas laws. There is no practical difference between a gas and a vapor except that a vapor is the gaseous phase of a substance that is usually solid or liquid at room temperature and atmospheric pressure. For example, processes that involve high temperature, such as welding operations and exhaust from engines, can potentially generate toxic gases such as oxides of carbon, nitrogen or sulfur. Several occupational practices may produce toxic vapors such as charging and mixing liquids, painting, spraying and dry cleaning or any other activities involving VOCs.

The second category is aerosols or suspended air pollutants, which can be solid particles or liquid droplets and can vary in size, composition and origin, such as dust, fiber, smoke, mist and fog [14]. Aerosols may result from different mechanical or chemical processes in both solid or liquid forms, which may

have spherical or nonspherical shapes with a wide range of size distribution from less than 100 nm to well over 100 μm. Different mechanical processes such as grinding, cutting, sawing, crushing, screening or sieving can generate solid aerosols in dust form. Mechanical dispersing of a bulk liquid such as spraying and atomizing can generate suspended liquid mist droplets. Mist droplets have the properties of the parent liquid, and have a wide range of sizes from a few to more than 100 μm. All processes involving high-pressure liquids, such as paint spraying, can potentially generate mists.

Other forms of aerosols can be generated by processes such as combustion, condensation or sublimation. For example, high-temperature operations such as arc welding, torch cutting and metal smelting can generate extremely fine metal oxide fumes, usually less than 0.1 μm, produced by combustion, sublimation or condensation of evaporated materials. Incomplete combustion of organic materials can generate smoke that is a complex mixture containing solid and liquid aerosols, gases and vapors. For example, tobacco smoke contains thousands of chemical substances, most of which are toxic or carcinogenic. Although primary smoke particles are between 0.01 and 1 μm, they can aggregate and produce extremely larger particles called soot.

Airborne chemical exposure

The three main routes of exposure to chemicals are inhalation, dermal absorption and ingestion. Inhalation is considered the most important means by which humans are exposed to airborne chemicals and forms the focus of this review.

Human respiratory tract: Structure and function

The major physiological function of the respiratory tract is to deliver O_2 (oxygen) into the blood system and to remove CO_2 (carbon dioxide) as a metabolic waste. The human respiratory tract is anatomically well structured to achieve this function given its very large surface area of approximately 140 m^2 and a high daily exchange volume of more than 10 m^3 [15–18]. In addition, the membrane between air and blood in the gas-exchange region is extremely thin, approximately 0.4–2.5 μm [19, 20]. As well as olfactory, gas exchange and blood oxygenation functions, the respiratory system has evolved to deal with xenobiotics and many airborne materials that usually occur in the air environment [21].

However, the respiratory system cannot always deal adequately with the wide range of airborne contaminants that may occur in urban and occupational environments [20]. After inhalation, airborne contaminants may enter different regions of the respiratory tract. Some chemicals such as insoluble gases and vapors can even pass through the respiratory tract efficiently and enter into

the pulmonary blood supply system. As a result, the respiratory system is both a site of toxicity for pulmonary toxicants, and a pathway for inhaled chemicals to reach other organs distant from the lungs and elicit their toxic effects at these extrapulmonary sites. The human respiratory tract can be classified into three major regions: nasopharyngeal, tracheobronchial and the pulmonary regions (Fig. 1).

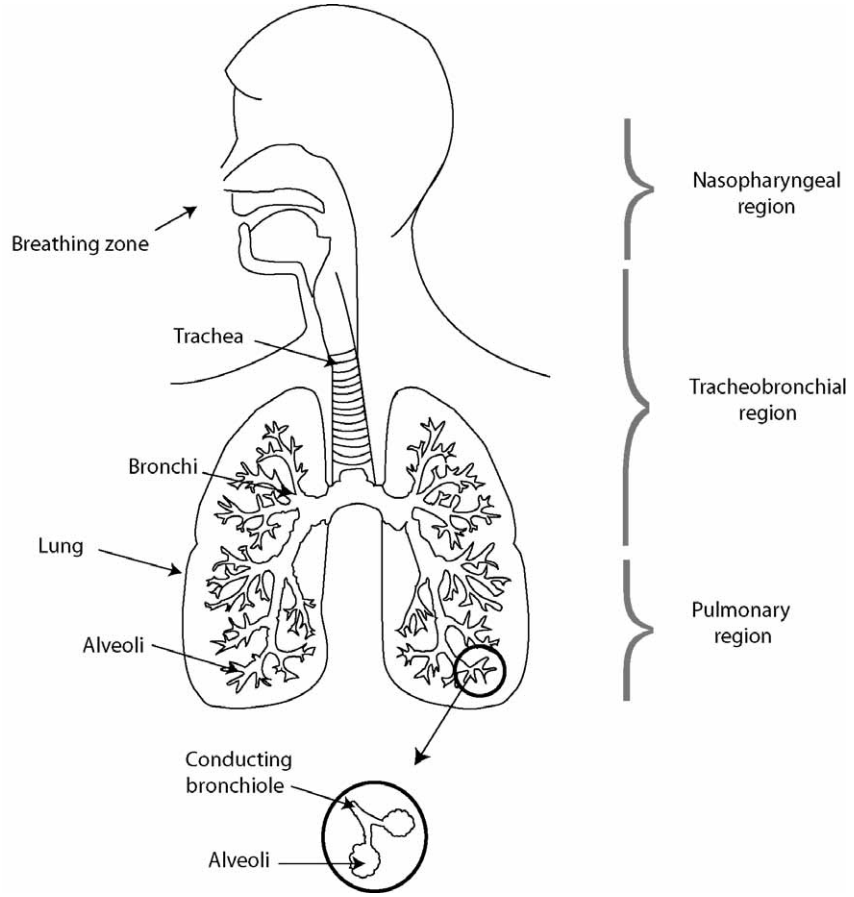

Figure 1. Anatomical regions of the respiratory tract.

Nasopharyngeal region

This part of the respiratory tract includes the nasal turbinates, epiglottis, pharynx and larynx. Air enters through the nose (and mouth), and is warmed and humidified during this passage. The nasal passages assist in collecting the coarse inhaled particles by impaction or filtration, and condition the tempera-

ture and humidity of the inhaled air. Highly water-soluble gases and vapors can also be absorbed efficiently by the nasal passages. The nasal passages are an important target area for a wide range of inhaled toxicants. For example, transitional epithelial tissue is attacked by O_3 and respiratory mucosal tissue by formaldehyde [22].

Tracheobronchial region

The tracheobronchial region includes the airways from trachea to terminal bronchioles, and delivers inhaled air to the deeper parts of the respiratory tract. Similar to the nasopharyngeal, this region is lined by mucous-secreting goblet cells and ciliated cells, which functions as a protective covering [18, 20]. Respiratory tract mucous produced by goblet cells and glandular structures can capture pollutants and cell debris. Produced mucous is continually propelled toward the pharynx by respiratory tract cilia [20, 22].

Pulmonary region

The pulmonary region is the area of gas exchange, and includes the respiratory bronchioles, alveolar ducts and alveoli. Adult human lungs consist of approximately 300 million alveoli, the gas-exchange units of the lung [23]. O_2 molecules diffuse from the inhaled air to the blood across the very thin alveolar epithelia and capillary membranes. Diffusion of CO_2 occurs in the opposite direction. The distal respiratory tract also contains several distinctive cells. For example, the alveoli are lined with two types of epithelial cells. Type I alveolar cells are very thin and cover a large surface area (approximately 90%) of the alveolar surface and bring inspired air into close contact with blood. Type II cells produce surfactant and assist in gas exchange and alveolar integrity [20, 22].

Deposition of inhaled chemicals

Apart from the physiological and anatomical characteristics of the respiratory tract, physicochemical and aerodynamic characteristics of inhaled chemicals are crucial in determining the site of deposition/absorption and the ultimate fate of inhaled contaminants.

Gases and vapors

Water solubility is the significant factor that determines the penetration site of a gas in the respiratory tract. For example, ammonia, formaldehyde, sulfur

dioxide and hydrogen fluoride are extremely soluble in water and tend to be absorbed nearly completely within the nose and upper airways. While the nose acts as a scrubber for water-soluble gases to protect the lungs from potential toxic effects, the drawback of this protective action is the probability of production of toxic effects in the nose, such as formaldehyde, that may induce nasal cancer [24, 25]. Gases with low solubility such as nitrogen dioxide, O_3 and phosgene penetrate further into the pulmonary region and exert their toxicity in this region. Ultimately, very insoluble gases such as CO and hydrogen sulfide pass through the respiratory tract efficiently and are delivered throughout the body *via* the pulmonary blood supply system.

Although water solubility is a critical parameter in penetration of gaseous contaminants, other factors such as partition coefficient may also have a considerable influence. For example, when a gas penetrates to the gas-exchange region, the blood-gas partition coefficient will determine the rate of gas uptake into the blood [17, 24].

Particulate matter

While water solubility is the significant factor determining the penetration site of gaseous inhaled chemicals, particle size distribution is the most critical characteristic determining airborne behavior and deposition pattern of aerosols in the respiratory tract [26]. During inhalation, a specific volume of air from the breathing zone accelerates towards the nose (or mouth) and enters into the respiratory tract. As well as air, particles that are able to follow the air flow will enter into the respiratory tract (Fig. 1). Larger particles (5–30 μm) are usually deposited in the nasopharyngeal region by an inertial impaction mechanism. Aerosols (1–5 μm) that fail to be captured in the nasopharyngeal region will be deposited in the tracheobronchial region, and may be absorbed or removed by mucociliary clearance. Finally, the remaining particles with the smallest size distribution (<1 μm) that were not trapped in tracheobronchial region will penetrate deeply into the alveolar region, where removal mechanisms are insufficient [18, 24, 27].

The phagocytic function of alveolar macrophages to remove inhaled nano-sized particles appears to be considerably less efficient than for larger particles [28–30]. Therefore, nano-sized particles can effectively access the alveolar region of the lungs and come into intimate contact with the alveolar epithelium. Once deposited, they may enter into the blood stream and readily reach other target organs [31]. However, insoluble particles may remain in the lung indefinitely [24, 27, 30].

Transport and deposition of particles in the respiratory tract is governed by four main mechanisms: impaction, interception, sedimentation and diffusion (Fig. 1), [22, 32, 33]. Impaction occurs due to both velocity and directional change predominantly in the upper respiratory tract. When the airstream undergoes a change in direction, larger particles cannot follow the airstream

lines because of their inertial properties and hence they continue in the original direction and impact onto the surface. Interception has an important role in the deposition of fibers, particularly for those with large aspect ratios. When the trajectory of a particle brings it close enough to a surface to make contact, the particle will be captured by interception.

Sedimentation refers to aerosol movement under the influence of gravity [34, 35]. Sedimentation is a significant mechanism in the smaller bronchi, the bronchioles and the alveolar spaces. Diffusion is a major deposition mechanism for particles smaller than 0.5 μm [36]. Diffusion is an important deposition mechanism for extremely small particles such as nanoparticles deep in the alveoli, where the air flow is very low [31, 37].

Major responses to inhaled chemicals

Responses to inhaled chemicals range from immediate reactions to long-term chronic effects, from specific impacts on single tissue to generalized systemic effects [38, 39]. The severity of toxic effects of inhaled chemicals is influenced by several factors such as type of air contaminant, airborne concentration, size of airborne chemical (for particles), solubility in tissue fluids, reactivity with tissue compounds, blood-gas partition coefficient (for gases and vapors), frequency and duration of exposure, interactions with other air toxicants and individual immunological status [24, 38, 39].

Exposure to air pollutants can cause different adverse effects either in the respiratory tract or in other organs and systems distant from the lung. The site of deposition/action of inhaled toxicants will determine, to a great extent, the ultimate response of the respiratory tract to inhaled chemicals. Human lung disorders involve an entire spectrum of respiratory diseases ranging from acute irritation and sensitization to chronic pneumoconiosis, occupational asthma and lung cancer (Tab. 1).

Acute irritant injuries

Exposure to irritants can cause acute injuries of the respiratory tract. During inhalation, the cell lining of the respiratory tract, from the nostrils to the gas-exchange region, is exposed to air contaminants. Penetration of the irritant gas into the respiratory tract depends primarily on the water solubility of the gas or vapor. For example, anhydrous ammonia is an upper respiratory irritant due to high water solubility. In contrast, gases or vapors with low water solubility such as nitrogen dioxide or methylene chloride that are not well absorbed in the upper respiratory tract will penetrate deeper into the distal parts of the respiratory tract and induce tissue damage. Important respiratory irritants can be found in Table 1.

Table 1. Air pollutants and related adverse effects

Contaminant	Toxic effects/diseases
Gases/vapors	
Chlorine (Cl_2) Ammonia (NH_3) Oxides of nitrogen (NO_x) Sulfur dioxide (SO_2) Sulfur trioxide (SO_3) Fluorine (F_2) Phosphine (PH_3) Phosgene ($COCl_2$) Acrolein	Respiratory irritation, bronchitis
Acid mists Caustic mists	Corrosion of respiratory system
Nitrogen (N_2) Hydrogen (H_2) Methane (CH_4) Helium (He_2) Ethylene (C_2H_2) Ethane (C_2H_6)	Dilution ('simple') asphyxiation
Carbon monoxide (CO) Hydrogen cyanide (HCN) Hydrogen sulfide (H_2S)	Chemical asphyxiation
Isocyanates (–N=C=O) Amines ($–CR_2NH_2$)	Sensitization, allergy, asthma
Particulates	
Asbestos	Asbestosis, pleural plaques, lung cancer, mesothelioma
Aluminum dust and abrasives	Aluminosis, alveolar edema, intestinal fibrosis
Beryllium	Berylliosis, pulmonary edema, pneumonia, granulomatosis, lung cancer, cor pulmonale
Cadmium (oxide)	Pneumonia, emphysema, cor pulmonale
Chromium VI	Bronchitis, fibrosis, lung cancer
Coal dust	Fibrosis, coal miner's pneumoconiosis
Cotton dust	Byssinosis
Iron oxides	Siderosis, diffuse fibrosis-like pneumoconiosis
Kaolin	Kaolinosis, fibrosis
Manganese	Manganism, manganese pneumonia
Nickel	Pulmonary edema, lung cancer, nasal cavity cancer
Silica	Silicosis, fibrosis, silicotuberculosis
Talc	Talcosis, fibrosis
Tin	Stanosis
Tungsten carbide	Hard metal disease, hyperplasia of bronchial epithelium, fibrosis
Vanadium	Irritation, bronchitis

Modified from [39].

Asphyxiation

Impaired or absence of oxygen exchange, which is characterized as asphyxia, is a toxicological hazard in various occupational and environmental settings [40]. Asphyxiant agents are classified into two groups based on their mode of action: (1) simple asphyxiants that generate tissue hypoxia by displacing oxygen from the inhaled air, e.g., CO_2, nitrogen and methane; and (2) chemical asphyxiants that generate tissue hypoxia by interfering with normal oxygen transport or utilization *via* interacting with biological molecules, e.g., CO, hydrogen cyanide and hydrogen sulfide [20, 40].

Asthma

Asthma is a pulmonary disorder characterized by mild or severe attacks of shortness of breath due to air flow limitation and/or airway hyper-responsiveness, caused by particular or unknown provoking agents [18, 41, 42]. Asthma induces bronchospasm, more production of mucus in the airways and cough due to an increasing response of the lung to the provoking agent. Even small exposures to a sensitizer may exacerbate asthma. Reactive airways dysfunction syndrome (RADS) is a separate category of asthma and can occur following acute inhalation of airborne irritants such as irritant gases, fumes and smoke [43]. Asthma is becoming an increasingly prevalent work-related respiratory disease in developed countries [44–46], and can be triggered by more than 200 chemicals including gases, vapors, particulates and allergens found in a wide variety of occupational settings [20].

Chronic obstructive pulmonary disease (COPD)

COPD is the common form of chronic lung disorder in industrialized countries, and represents the physiological abnormality resulting from long-standing, fixed, airflow obstruction; it is related to chronic bronchitis, emphysema, bronchiolitis and asthma rather than a result of a single disease [44, 47]. Smoking, air pollution, respiratory infections and genetic factors such as α_1 anti-trypsin deficiency have been identified as having causal links with COPD [20, 47].

Pneumoconiosis

Pneumoconiosis refers to any non-neoplastic lung disease caused by chronic exposure to, and hence accumulation of, airborne mineral dusts in the lung and the associated tissue reaction [20, 48]. Benign pneumoconiosis describes the presence of non-toxic materials in the lung that do not damage alveolar archi-

tecture or increase collagenous fibrosis, e.g., siderosis (iron), stannosis (tin) and baritosis (barium). In contrast, collagenous pneumoconiosis is the result of some other inorganic dusts that induce structural alterations in lung tissue and irreversible fibrosis, e.g., silicosis (silica), asbestosis (asbestos) and coal miner's pneumoconiosis (coal dust). However, in Western Europe and North America the majority of newly presenting cases of pneumoconiosis are now due to asbestos exposure rather than coal and silica exposure [49].

Lung cancer

Tobacco smoking is a well-recognized lung cancer risk factor and it has been estimated that about 80–90% of lung cancers are caused by cigarette smoking [18]. Some inhaled toxicants may induce cancer in the upper respiratory tract in nasal cavity and turbinates, e.g., formaldehyde, wood dusts, leather work and isopropyl alcohol [20]. Exposure to asbestos, arsenic or metals such as nickel, beryllium and cadmium has been associated with lung cancer. Radon gas is also a known lung carcinogen [18]. Silica, man-made fibers and welding fumes are suspected carcinogens.

Setting exposure standards

Environmental air quality standards (AQS) and occupational exposure limits (OEL) are proposed as guidelines to evaluate the health risks associated with human exposure to airborne pollutants. Although AQS are an essential part of the risk assessment process, current knowledge of toxicological potential of inhaled chemicals in relation to hazard evaluation is limited, making it difficult to establish such guidelines for a large number of airborne chemicals.

While data obtained from human experiences would be most useful in assessing the toxic effects of chemicals, human data are not always available for developing safety evaluations on airborne contaminants [8]. Moreover, after unfortunate human incidents, such as those with pharmaceutical agents like diethylstilbestrol and thalidomide, or contaminants like lead and polychlorinated biphenyls (PCBs), it is now understood that the risks of new products and technologies need to be assessed before adverse human experiences occur [2, 50, 51].

An important regulatory effort to remedy the lack of toxicity data for thousands of existing chemicals is the European Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) framework, which came into effect in June 2007 [8, 52]. The major objectives of the REACH regulatory framework are to improve the knowledge associated with chemical properties and applications and to speed up the process of risk assessment. There is great interest in test systems including alternatives to animal testing that would satisfy these safety requirements.

Inhalation toxicology methods

Toxicology has, for many years, made a major contribution in providing chemical toxicity information. In general, no single method can cover the complexity of general toxicity in humans [53]. Toxicity data can be obtained from several sources including toxicological studies, epidemiological studies, quantitative structure-activity relationships (QSARs) and physiologically based toxicokinetic (PBTK) studies. While traditional toxicology methods rely on whole animal testing, *in vitro* toxicology methods, using cell culture technology in combination with the knowledge of toxicokinetics, are currently being developed and implemented in modern toxicology.

In vivo test methods

Extensive data have been generated from toxicological studies using animal models. However, most of these studies are conducted by oral and dermal exposures rather than inhalation exposure [5, 54]. While toxicology data may exist from other routes of exposure, the extrapolation of these data is most difficult to validate. To identify the lethal effects of air toxicants, inhalation toxicity tests are carried out in test animals. In brief, test animals are exposed to air toxicants dissolved or suspended in air, and the concentration that causes lethality in 50% of the dosed group (LC_{50}) is determined. In reporting an LC_{50}, both the concentration of the chemical in air that can cause death in 50% of exposed animals, and time of exposure is indicated.

Standard protocols have been adopted by regulatory agencies for both short-term and long-term inhalation tests. The OECD has initially adopted test guidelines for acute inhalation toxicity (TG 403), repeated-dose inhalation toxicity 28/14-day (TG 412) and subchronic inhalation toxicity 90-day (TG 413). Meanwhile, new guidelines for acute inhalation toxicity such as Acute Inhalation Toxicity-Fixed Dose Procedure (TG 433) and Acute Inhalation Toxicity-Acute Toxic Class (ATC) Method (TG 436) are being finalized [52, 55]. The OECD test guidelines for inhalation toxicity studies, including those in preparation, and their role in future hazard identification have been briefly discussed [52].

In inhalation studies, evaluating the dose received by the animal is more challenging due to several factors that influence the actual dose, e.g., atmospheric concentration, duration of exposure, pulmonary physiological characteristics of the test animal, and deposition/absorption patterns of the air contaminant. In a specified system, for many inhaled chemicals the actual dose is related to the product of concentration (c) and exposure time (t) by Harber's law ($c \times t$ = inhaled dose) [17, 22]. In many cases, particularly in short-time exposures to chemicals with a direct action on the respiratory system, the response is directly proportional to the product of $c \times t$. However, many other substances, such as chemicals that exhibit systemic toxicity, do not follow

Harber's law because in such cases, several factors including absorption by the respiratory tract, tissue distribution, metabolism in potential target organs and elimination will influence the toxicity [17].

The selection of an animal species for toxicity studies is another crucial consideration that may influence the outcome of *in vivo* studies and the estimated human adverse health effects. Usually rodents such as rat, mouse, guinea pig and hamsters are used. However, different criteria should be considered in the selection of animals such as species-related physiological factors, the size of the animals, the availability of the animals, the number of animals needed, the cost of obtaining and maintaining the required number of animals and the cost of producing consistent atmospheric test concentrations [22].

The more common exposure modes to evaluate the effect of inhaled air toxicants involve whole body, head only and nose only. Whole body exposure mode is conducted most frequently for human inhalation studies. Head- and nose-only exposure modes are suitable for repeated short-time exposure for restricting the portal of entry of the test chemical to the respiratory system. Design and construction of head-only units are similar to those of nose-only units. Nose-only exposure units require an animal holder to accommodate rodents with suitable size to reduce stress and discomfort. The animal holder is normally designed to fit the general shape of the animal with a conical headpiece using polymethylmetacrylate, polycarbonate or stainless steel. The animal holder is connected to the exposure chamber that introduces the test chemical into the face or nose of the animal.

Generation of test atmospheres

Generation and characterization of known concentrations of air contaminants and reproducible exposure conditions is a more complicated and expensive procedure than that required for oral and dermal exposures. This process requires specialized equipment and techniques to generate, maintain and measure standard test atmospheres. Inhalation exposure systems involve several efficient and precise subsystems, including a conditioned air supply system, a suitable gas or aerosol generator for the test chemical, an atmosphere dilution and delivery system, exposure chamber, real-time monitoring or sampling and analytical system, and an exhaust/filter or scrubbing system (Fig. 2).

Exposure chambers can be operated in both static and dynamic systems. Usually animal inhalation toxicity studies are carried out in a dynamic system to avoid particle settling and exhaled gas complications. While the operation of the static system is relatively simple and requires comparatively less test material, this is only suitable for short exposure times due to the reliance on the air inside of the chamber. In contrast, in a dynamic system, the test atmosphere flows through the exposure chamber continuously and hence ensures atmospheric stability and no reduction in oxygen concentration due to test animal respiration [17]. This system requires accurate flow monitoring for both

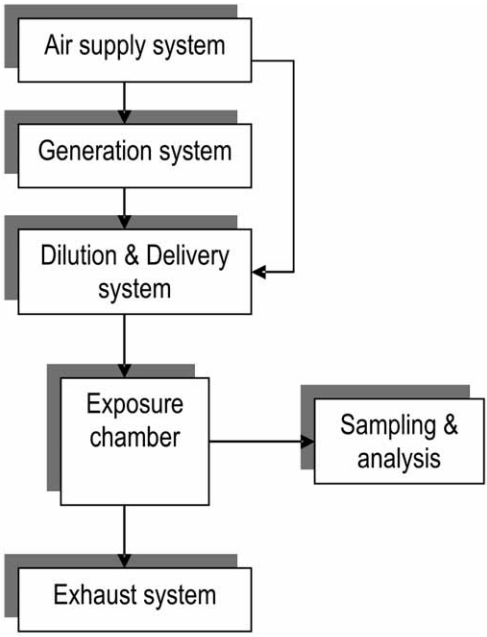

Figure 2. Components of a test atmosphere generation system (adapted from [14] with permission of the publisher Wiley-VCH).

diluent air and the primary source of contaminant in proportions that can produce the final desired concentrations.

In vitro test methods

Application of cell culture techniques in toxicological studies is referred to as *in vitro* toxicology and describes a field of study that applies technology using isolated organs, tissues and cell culture to study the toxic effects of chemicals [14]. As well as scientific advances, the development of *in vitro* toxicity test methods has been influenced by a variety of socio-economical factors. Animal welfare issues are one of the most important social concerns and have influenced the recent shift towards alternatives in toxicity testing. Each year, thousands of new cosmetics, pharmaceuticals, pesticides and consumer products are introduced into the marketplace. Considering that there are approximately 80 000 chemicals in commerce [46], as well as an extremely large number of chemical mixtures, *in vivo* testing of these numbers of chemicals requires a large number of expensive, time-consuming and in some cases non-humane tests on animal species. The necessity of determining the potential toxic effects of this large number of chemicals has provoked the need for rapid, sensitive and specific test methods.

In vitro toxicity endpoints

Different levels of organization in the human body may be affected by chemical substances from molecules to cells to tissues to organs, and to functional levels. Cytotoxicity is the adverse effect that occurs from the interaction of a toxicant with structures and/or processes essential for cell survival, proliferation and/or function [56]. Toxic chemicals can attack any of the basal cell functions, origin-specific cell functions and extracellular functions. To assess the potential cytotoxicity of chemical substances, several *in vitro* tests have been developed by measuring different biological endpoints, which are summarized in Table 2 [14, 53, 57–63]. In addition, recent research on apoptosis has enormously added to the knowledge of mechanisms involved in cell death, leading to the development of mechanistically based endpoints. Many morphological and biological changes that may occur at the cellular membrane, nucleus, specific proteases and DNA level can be used as biological endpoints for measuring apoptosis [62, 63]. Moreover, the rapid progress in genomic, transcript-

Table 2. Common biological endpoints assessed by *in vitro* toxicity tests

Biological endpoint	Detection method
Cell morphology	• Cell size and shape • Cell-cell contacts • Nuclear number, size, shape and inclusions • Nuclear or cytoplasmic vacuolation
Cell viability	• Trypan blue dye exclusion • Diacetyl fluorescein uptake • Cell counting • Replating efficiency
Cell metabolism	• Mitochondrial integrity (tetrazolium salt assays: MTT, MTS, XTT) • Lysosome and Golgi body activity (neutral red uptake) • Cofactor depletion (for example, ATP content)
Membrane leakage	• Loss of enzymes (for example, LDH), ions or cofactors (e.g., Ca^{2+}, K^{+}, NADPH) • Leakage of pre-labeled markers (e.g., ^{51}chromium or fluorescein)
Cell proliferation	• Cell counting • Total protein content (e.g., methylene blue, Coomassie blue, kenacid blue) • DNA content (e.g., Hoechst 33342) • Colony formation
Cell adhesion	• Attachment to culture surface • Detachment from culture surface • Cell-cell adhesion
Radioisotope incorporation	• Thymidine incorporation into DNA • Uridine incorporation into RNA • Amino acids incorporation into proteins

Adapted from [14]. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; XTT, 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-*bis*-(4-methoxy-6-nitro)benzene sulfonic acid.

omic (gene expression) and proteomic technologies has created a unique, powerful tool in toxicological investigations [64].

In vitro toxicity testing of inhaled chemicals

The study of the toxic effects of inhaled chemicals is typically more challenging due to the technology required for the generation and characterization of test atmospheres, and the development of effective and reproducible techniques for exposure of cell cultures to airborne contaminants. Generation and characterization of known concentrations of air contaminants and reproducible exposure conditions require equipment and techniques to generate, maintain and measure test atmospheres comparable to those for *in vivo* studies (Fig. 2). In addition, the exposure of cells to test atmospheres requires close contact of cells and air.

A practical approach for *in vitro* inhalation toxicity testing has been proposed by the European Centre for the Validation of Alternative Methods (ECVAM) [21]. This systemic approach is initiated with the consultation of existing literature, evaluating the physicochemical characteristics of test chemicals and predicting potential toxic effects based on structure activity relationships (SARs). Physicochemical characteristics of chemicals such as molecular structure, solubility, vapor pressure, pH sensitivity, electrophilicity and chemical reactivity are important properties that may provide critical information for hazard identification and toxicity prediction [65, 66].

Initial *in vitro* tests should be conducted to identify likely target cells and toxic potency of test chemicals. Based on the obtained result, *in vitro* tests may be followed by a second phase using the following cells: nasal olfactory cells, airway epithelial cells, type II cells, alveolar macrophages, vascular endothelial cells, fibroblasts and mesothelial cells [21]. While over ten main cell types have been identified in the epithelium of the respiratory tract, for the assessment of respiratory toxicity it is important to utilize specific cell types with appropriate metabolizing activity. It has been suggested that the endpoints to be used should be selected based on the knowledge of toxic effects of test chemicals and should always include cell viability testing in at least two different cell types.

To evaluate the potential applications of *in vitro* methods for studying inhalation toxicity, more recent models developed for toxicity testing of airborne contaminants have been reviewed [14, 67, 68]. The toxic effects of air contaminants have been studied using several indirect and direct *in vitro* exposure techniques (Tab. 3).

Indirect exposure methods

Most of these, especially studies conducted on particulates are limited to exposure of cells to test chemicals solubilized or suspended in culture medium

Table 3. Indirect and direct *in vitro* exposure techniques developed for studying the toxicity of air contaminants

Exposure technique	Exposure achievement procedure
Indirect methods	
Exposure to test chemical itself	Cells are exposed to test chemicals solubilized or suspended in culture media
Exposure to collected air samples	Cells are exposed to air samples collected by filtration or impingement methods
Direct methods	
Submerged exposure condition	Test gas is introduced to cell suspension under submerged conditions using impinger or vacuum test tubes
Intermittent exposure	Cells are periodically exposed to gaseous compounds and culture medium at regular intervals using variation of techniques: rocker platforms, rolling bottles
Continuous direct exposure at the air/liquid interface	Cells are continuously exposed to airborne contaminants during the exposure period usually on their apical side, while being nourished from their basolateral side using collagen-coated or porous membranes permeable to culture media

Adapted from [14].

[69–76], which may be adequate for soluble test materials. However, this may not follow the *in vivo* exposure pattern of airborne aerosols, particularly for insoluble aerosols, due to unexpected alternation of their compositions and particle-media or particle-cell interactions [77]. Such techniques of exposure may also ignore size, which is crucial in toxicity testing of inhaled particles.

Some researchers have employed sampling of the aerosols by filtration techniques followed by the investigation of the effects of suspended and extracted particles, e.g., studies on atmospheric aerosols [78–82], or cigarette smoke condensate [83–86]. Filtration offers an advantage for on-site toxicity assessments of aerosols; however, this technique usually requires sample preparation steps, such as extraction to isolate the components of interest from a sample matrix, and ultimately, solubilization or suspension in culture media, potentially increasing experimental errors and further toxicity interactions. For example, cytotoxicity of roadside airborne particulates has been studied in rodent and human lung fibroblasts using the filtration technique [79]. Airborne particulates were sampled on glass fiber filters using a high-volume sampler. After air sampling, the filters were sonicated using benzene-ethanol solvents and to obtain a crude extract, solvents were evaporated to dryness. The crude extract was further fractionated by acid-base partitioning and all extracts were dissolved in dimethyl sulfoxide (DMSO) for cytotoxicity assays. Cytotoxicity was investigated using cell proliferation, tetrazolium salt (MTS) and lactate dehydrogenase (LDH) *in vitro* assays [79].

Indirect exposure techniques have also been developed using an impingement method where samples of airborne formaldehyde were collected in serum-free culture media [87]. Cytotoxicity was investigated after exposing human cells to collected air samples. The objective of this study was to develop an *in vitro* sampling and exposure technique that can be used for toxicity testing of soluble airborne contaminants with the potential for on-site applications. An average of 96.8% was calculated for the collection efficiency of airborne formaldehyde in serum-free culture media, signifying the potential application of this method for sampling the airborne formaldehyde and other soluble airborne contaminants. The use of serum-free culture media as a collection solution for soluble airborne contaminants proved to be a simple technique, without any specific sample preparation or extraction steps; hence, any potential toxic interactions of the test chemical with other toxic organic solvents during preparation were omitted.

Direct exposure methods

Several direct *in vitro* models have also been developed to deal with gas-phase exposure of airborne contaminants using different exposure techniques. Different features of exposure techniques developed for airborne chemicals have been discussed in terms of their relevance, advantages and limitations [88]. In principle, these methods include exposure of cells under submerged conditions, intermittent exposure procedures and more recently direct exposure techniques at the air/liquid interface.

Exposure by bubbling gaseous test compounds through cells suspended in media can easily be achieved using variations of standard laboratory processes (Fig. 3). For example, to study the *in vitro* toxic effects of O_3 on human

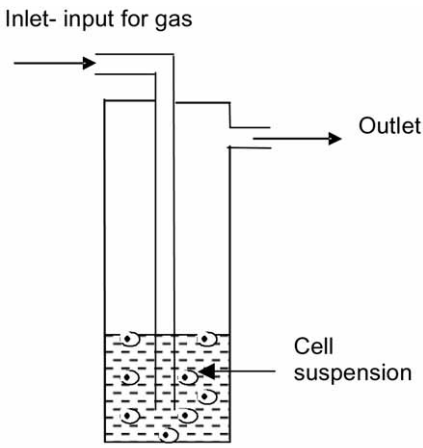

Figure 3. Exposure by bubbling test gaseous compounds through cells suspended in media.

hematic mononucleated cells (HHMC), the test gas was introduced at once to cell suspensions in vacuum test tubes [89]. However, exposure patterns *in vivo* may not be closely simulated and only a very small interface between the test gas and the target cells can be provided by the submerged exposure technique.

A variation of laboratory techniques has been developed allowing intermittent exposure of cultures to gaseous contaminants. Cell culture dishes held on chambers or platforms rotated, shaken or tilted at certain angles were exposed to gaseous compounds periodically [90, 91]. Cell culture flasks were also tilted at regular intervals to expose the cell cultures to volatile anesthetics [92]. Rolling culture bottles on roller drums were set up for *in vitro* gas exposure [93, 94]. Lung slices were alternatively fed by culture medium and exposed to diesel exhausts by rotating the culture vial on the internal wall of a flow through chamber [95]. Tissue culture flasks on a rocking platform were used to expose the cells to mainstream cigarette smoke followed by an immersion in culture media intermittently [96]. For example, a micro roller-bottle system was developed for cytotoxicity screening of volatile compounds in which primary hepatocytes attached to a collagen-coated nylon mesh (Fig. 4). The primary hepatocytes were exposed to volatile compounds injected into the roller bottle. The roller bottle was placed on a roller apparatus in an incubator at 37 °C and hepatocytes were alternatively exposed to the medium and the test atmosphere. Medium samples were then taken for measuring the cellular LDH and aspartate aminotransferase [94]. Compared to the submerged exposure, the intermittent exposure technique provided a larger interface between gaseous compound and target cells. Nevertheless, in such exposure conditions cells are always covered by an intervening layer of medium that may influence both accuracy and reproducibility of the results.

In the 1990s, technology became available that allowed cells to be cultured on permeable porous membranes in commercially available transwell or snapwell inserts (Fig. 5). In this system, once cells are established on the mem-

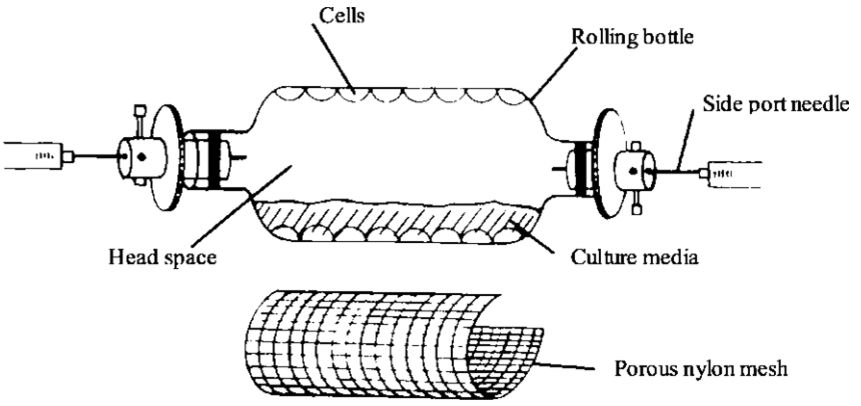

Figure 4. A micro roller bottle system (modified from [94]).

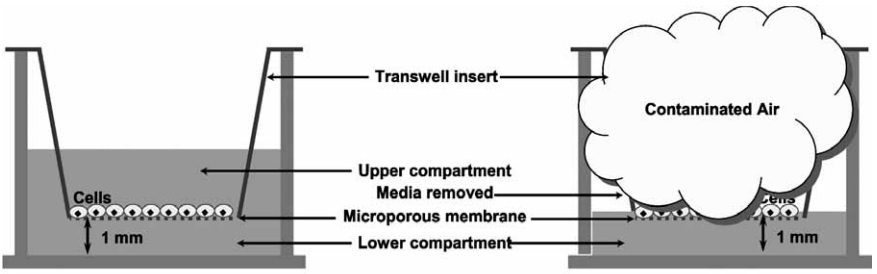

Figure 5. The culture of human cells on porous membranes. Left: Culture of cells on microporous membranes; Right: Exposure of cells to airborne contaminants following removal of media in the upper compartment (modified from [14] with permission of the publisher Wiley-VCH).

brane, the upper layer of culture media can be removed, and the cells directly exposed to air contaminants. In a direct exposure technique at the air/liquid interface target cells can be exposed to airborne contaminants continuously during the exposure time on their apical side, while being nourished from their basolateral side. Direct exposure of cells to airborne contaminants was initially achieved by growing cells on collagen-coated membranes located on special platforms [97] and more recently porous membranes in transwell inserts [77, 98, 99] or snapwell inserts [100–102]. Both static and dynamic direct exposure methods have been established for exposure purposes.

Exposure to volatile chemicals is a significant contributor to human health problems; however, toxicity testing of volatile compounds has always faced significant technological problems [60, 103–105]. Apart from high volatility, many VOCs are less water soluble or insoluble. These physicochemical properties may produce technical challenges during the course of *in vitro* experiments. Static direct exposure methods have been developed for toxicity assessment of VOCs, in which test atmospheres of selected VOCs were generated in sealed glass chambers with known volumes [106]. Human cells including A549 pulmonary type II-like cell lines, HepG2 hepatoma cell lines and skin fibroblasts were exposed to airborne toxicants at different concentrations directly at the air/liquid interface. Cytotoxicity was investigated using the tetrazolium salt (MTS; Promega) and neutral red uptake (NRU; Sigma) assays *in vitro*. Using the static direct exposure method, the airborne IC_{50} (50% inhibitory concentration) values for selected VOCs were established, e.g., for xylene (IC_{50} = 5350–8200 ppm) and toluene (IC_{50} = 10 500–16 600 ppm) after 1-hour exposure. The static direct exposure method proved to be a practical and reproducible technique for *in vitro* inhalation studies of volatile chemicals.

A typical experimental set-up for dynamic direct exposure at the air/liquid interface requires appropriate exposure chambers. Standard tissue culture incubators are used for exposure purposes [107]. Dynamic delivery and direct exposure of human cells to airborne contaminants can be achieved using spe-

cific exposure chambers [108] or horizontal diffusion chamber systems [109]. Toxic effects of individual airborne chemicals such as O_3, SO_2 and NO_2 [100, 109, 110], and complex mixtures, such as diesel motor exhaust [110], cigarette smoke [99], and combustion products [111, 112], have been studied using cultured human lung cells on porous membranes permeable to culture media. The dynamic direct exposure technique at the air/liquid interface offers a reproducible contact between chemically and physically unmodified airborne contaminants and target cells and technically may reflect more closely inhalation exposure *in vivo* [100, 101, 109].

Nanotoxicology: An emerging issue of inhalation toxicology

The pattern of human exposure to aerosols and particulates has changed enormously. Historically important pulmonary diseases (e.g., silicosis, coal miner's pneumoconiosis and asbestos-related cancer) have been significantly reduced *via* improvement of engineering and other control measures [35]. With emerging modern technologies such as nanotechnology and related material sciences, a new category of particles, nanoparticles, with unique characteristics are increasingly manufactured and introduced for commercial use. Nanoparticles are defined as primary particles with at least one dimension <100 nm [37, 113–115]. Nanoparticles have already being implemented in sunscreens, cosmetics, pharmaceuticals, food additives, self cleaning paints and glass, clothing, disinfectants, fuel additives, electronics, therapeutics, batteries and other products [115–120]. However, by increasing the application of nanoparticles, protection of the human respiratory system from exposure to nanoparticles and ultrafine particulates has become an emerging health concern [116]. While very little is known about their interactions with biological systems, the very small size distribution and tremendous large surface area of nanoparticles available for undergoing reactions may potentially play a significant role in toxicological effects of nanoparticles [37, 115, 121].

The defense mechanisms of the human body may not be able to deal adequately with such nanomaterials, smaller than common irritants and pollens. There is evidence that the human lung macrophages, which develop to remove inhaled particles, are not able to deal with nanomaterials smaller than 70 nm, enabling these particles to access deeply into the lung and perhaps enter the blood stream [29]. Microscopic examination of human monocytic cells after exposure to nanotubes demonstrated frustrated phagocytosis, suggesting that the ability of macrophages to remove nanofibers from the lung may be impaired [122]. It has been reported that combustion-derived nanoparticles (CDNP) and their components can migrate from their site of deposition in the lung, to other organs [114]. At the site of final retention in the target organs, nanomaterials may trigger mediators and hence activate inflammatory or immunological responses [123].

Apart from their local inflammatory effects, nanoparticles have the potential to translocate away from their site of deposition and into the blood circulation. Blood-borne particles may be delivered to secondary target organs such as brain, heart, spleen, kidney and liver causing numerous additional adverse health effects [37, 114].

With respect to toxicity testing of nanoparticles, several limitations have been identified that need to be addressed in future investigations [113, 115, 124–126]. Conventional exposure techniques are not able to determine the fraction of inhaled particles that ultimately cross the pulmonary epithelial barrier into the cardiovascular system; consequently, particle concentrations to which endothelial cells are exposed *in vivo* remain unknown [126]. Most current nanomaterials are extremely insoluble. Low water solubility is a major restrictive characteristic and may cause technical problems during the course of *in vitro* experimentation [106]. Therefore, future studies performed under dynamic exposure conditions can potentially provide more physiologically relevant toxicity information, leading to a better understanding of the interaction between target cells and nanoparticles.

Current knowledge of the toxicological potential of nanoparticles in relation to risk assessment is very limited, which makes it impossible to establish safety guidelines. In addition, available methods of sampling and analytical techniques are not able to adequately quantify the concentration of nanoparticles in environmental samples [37, 124, 127]. For example, due to the small size and low mass in any gravimetric method, the likely concentration of respirable particles is presumed to be very low. One of the key questions related to nanoparticle exposure is determining what potential characteristics of a nanoscale material needs to be measured, e.g., mass concentration, surface area, number concentration, size distribution, surface reactivity, particle agglomeration, chemical composition and/or morphology [37]. It is probable that the mass of a toxic concentration of nanoparticles in air will be very small, compared with conventional particles.

It has been recommended that high resolution imaging techniques, such as transmission electron microscopy (TEM) and scanning electron microscopy (SEM), may provide an efficient means by which to characterize particle size, shape and structure for a number of nanomaterials [128, 129]. Particle size distribution and shape are two crucial physicochemical characteristics in the context of exposure assessment and toxicity screening studies. TEM techniques have demonstrated how some nanomaterials such as metal oxide nanoparticles can be internalized within human cells [126]. Currently, these techniques serve as advanced research tools for toxicological investigations and qualitative structural evaluation of nanoparticles, both of which are expensive and non-quantitative. Much research is also needed to develop methods of detection and quantification that provide detection limits low enough to quantify the exposure concentration of inhaled nanoparticles and to reduce the uncertainty factors involved in their risk assessment.

Conclusion

Inhalation of airborne contaminants, e.g., gases, vapors, and aerosols, is a major contributor to human health problems, and can cause adverse effects ranging from simple irritation to morbidity and mortality through acute intense or long-term low-level repeated exposures. The large number of chemicals and complex mixtures present in indoor and outdoor air, coupled with the introduction of new materials such as nanoparticles and nanofibers, is an area of growing concern particularly in the industrial and urban environment. Animal-based assays have for many years been the preferred method to study the toxic effects of chemicals. However, very little is known about the potential toxicity of the vast majority of inhaled chemicals. As well as scientific and economic concerns, there is an increasingly strong urge to reduce animal testing on ethical grounds. The REACH regulatory framework intends to reduce the number of animal testings and speed up the risk assessment process. As well as new or improved OECD test guidelines, continuing scientific developments are needed to improve the process of safety evaluation for the vast number of chemicals. In addition, introducing a new category of chemicals/preparations to the marketplace such as nanoparticles and nanofibers, for which toxicity data is all but absent, emphasizes the demand on alternative toxicity test methods.

Development, standardization and validation of reproducible *in vitro* test methods could play a significant role in safety evaluation of chemicals and can contribute to a better understanding of the interactions between chemical exposure and toxic effects at the cellular level. The most appropriate cell systems with biotransformation activities and cellular functions comparable to the *in vivo* environment, such as a range of primary cell cultures and a battery of human cell-based assay systems, would need to be implemented. Although *in vitro* toxicology methods cannot mimic the biodynamics of the whole body, *in vitro* test systems in combination with the knowledge of QSAR and PBTK models have the potential to be considered more broadly for risk assessment of human inhalation exposures. A key molecular structure may provide some readily available information for toxicity prediction. Further, the application of PBTK models may provide a scientific basis for extrapolation of concentrations that produce cellular toxicity *in vitro*, to equivalent *in vivo* dosages.

For inhalation toxicity testing, promising *in vitro* exposure techniques have been recently developed that offer new possibilities for testing biological activities of inhaled chemicals under biphasic conditions at the air-liquid interface. The study of the toxic effects of inhaled chemicals *in vitro* requires effective and reproducible techniques for exposure of cell cultures to airborne contaminants. Direct exposure techniques may have the potential to be applied extensively to study the toxic effects of airborne contaminants, as the exposure pattern *in vivo* is more closely simulated by this method. Development and validation of appropriate *in vitro* sampling and exposure techniques may provide an advanced technology for studying the toxicity of nano- and ultrafine particles where inhalation toxicity data are much needed.

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