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ABSTRACT

Several studies have been conducted to address the potential adverse health risks attributed to exposure to nanoscale materials. While *in vivo* studies are fundamental for identifying the relationship between dose and occurrence of adverse effects, *in vitro* model systems provide important information regarding the mechanism(s) of action at the molecular level. With a special focus on exposure to inhaled (nano)particulate material toxicity assessment, this review provides an overview of the available human respiratory models and exposure systems for *in vitro* testing, advantages, limitations, and existing investigations using models of different complexity. A brief overview of the human respiratory system, pathway and fate of inhaled (nano)particles is also presented.

Introduction

Nanotechnology is an emerging field that offers insightful and innovative approaches and applications in many areas (De Jong and Borm 2008; Kermanizadeh et al. 2016; Thiruvengadam, Rajakumar, and Chung 2018; Zhao and Castranova 2011). According to the recently reviewed European Commission recommended definition, the term "nanomaterial" refers to a "natural, incidental or manufactured material consisting of solid particles that are present, either on their own or as identifiable constituent particles in aggregates or agglomerates, and where 50% or more of these particles in the number-based size distribution fulfill at least one of the following conditions: (a) one or more external dimensions of the particle are in the size range 1 nm to 100 nm; (b) the particle has an elongated shape, such as a rod, fiber or tube, where two external dimensions are smaller than 1 nm and the other dimension is larger than 100 nm; (c) the particle has a plate-like shape, where one external dimension is smaller

than 1 nm and the other dimensions are larger than 100 nm." (European Commission 2022). Nanoparticles (NP) production and use are increasing exponentially, making environmental and human exposure to these particles inevitable (Malakar et al. 2021). Multiple efforts have been made to understand the health implications from manipulation of and exposure to these nanoscale materials. Inhalation is the main route of entrance for airborne (nano)particles (Oberdorster et al. 2015). Based upon clearance mechanisms, it is estimated that solid (nano)particles half-life in the human alveolar region is approximately 700 days, which might constitute a threat to human health (Hagens et al. 2007; Oberdörster, Oberdörster, and Oberdörster 2005). Growing evidence demonstrated that due to their small size and increased surface area relative to an equal mass of larger diameter particles, inhalation of nano-sized particles might lead to systemic oxidative stress and inflammatory responses that might affect cardiovascular (Alsaleh 2021; Kan, Pan, and Castranova

2018) and hepatic functions (Kermanizadeh, Powell, and Stone 2020; Sun et al. 2021). Previously, several investigators demonstrated an association between adverse human health effects following exposure to airborne particulate matter (PM), where ultrafine particles (UFP) seem to play an important role in some responses (Delfino, Sioutas, and Malik 2005; Johnson et al. 2021; Li et al. 2016). For example, it has been widely reported the presence of endotoxins in PM, particularly in coarse and fine particles, that can potentially trigger inflammatory responses, thus exacerbating several respiratory diseases such as asthma (Boraschi et al. 2017; Degobbi, Saldiva, and Rogers 2011). In addition, despite their distinct origin, UFP and NP share many similarities in terms of their physicochemical properties and modes of action (Stone et al. 2017).

Over the past few years, many *in vitro* and *in vivo* studies have explored the effects of micro- and nano-sized materials in different lung-related models [reviewed in (Fytianos et al. 2016; Kumar, Sharma, and Maitra 2017; Nahar et al. 2013; Wick et al. 2015; Wiemann et al. 2016)]. Data generated from these studies characterized multiple hallmarks of (nano)particle-induced pulmonary toxicity, including particle internalization pathways, oxidative stress, genotoxicity, cell cycle alterations, and dysregulation of signaling cascades (Bakand, Hayes, and Dechsakulthorn 2012; Li et al. 2010; Paur et al. 2011; Pietroiusti et al. 2018). Several of these mechanisms are associated with the occurrence of negative health outcomes such as impaired lung function and inflammation, vascular dysfunction, and severe acute respiratory and cardiovascular complications (Kobos et al. 2020; Sreedharan et al. 2022; Stone et al. 2017). These adverse effects are strongly linked with different disease states including lung cancer, bronchitis, and acute asthma, cardiac infection and arrhythmia, hypertension, atherosclerosis, and ischemia (Brook et al. 2004; Kelly and Fussell 2015; Knaapen et al. 2004; Kreyling, Semmler-Behnke, and Möller 2006; Schulz et al. 2005; Shannahan, Kodavanti, and Brown 2012).

In vivo studies have been extremely useful for identifying important toxic properties of nanomaterials, screening of nano-delivered drugs or agents with great diversity of physicochemical parameters, as well as for estimation of health-related risks to humans and the environment (Carneiro and Barbosa 2016; He 2016; Jain and Thareja 2019; Sreedharan et al. 2022). *In vivo* investigations are, of course, indispensable to validate and assess the predictivity of the *in vitro* models (Miller and Poland 2020).

Given the increasing urge to find reliable alternatives to *in vivo* testing, several efforts have been made toward development and/or improvement of more complex and physiologically relevant *in vitro* systems for inhalation toxicology testing of (nano) particles (Fröhlich 2018). Therefore, the main purpose of this review was to provide an overview of the existing pulmonary *in vitro* models and exposure conditions for identifying and assessing (nano) particle-initiated hazards with determination of associated underlying molecular mechanisms. This review addresses the advantages and limitations of these *in vitro* models, existing gaps and future directions. As far as we known, this is the first review providing an overview of existing *in vitro* studies on airborne (nano)particle-induced toxicity in human respiratory system *in vitro* models of different complexity.

Brief overview of the human respiratory system

The respiratory system is primarily composed of the nose, airways, and parenchyma. The upper respiratory tract allows the passage of air and protects the lower respiratory regions from external injuries (Thomas 2013), while gas exchange occurs in the lower respiratory tract such as the alveoli (Weibel, Sapoval, and Filoche 2005). Along the respiratory tract, cell types and morphology vary (Figure 1), which also may be affected in pulmonary diseases (Whitsett and Alenghat 2015). As illustrated in Figure 1, the upper airways are lined with a pseudostratified epithelium that is composed of ciliated, secretory (goblet and club cells), neuroendocrine, and basal cells, these latter acting as progenitor cells for various cell types of the airway epithelium (Crapo et al. 1982; Hiemstra, McCray, and Bals 2015). The bronchioles are lined by ciliated cuboidal epithelium, with a small number of non-ciliated club cells that are more dominant in the distal portion (Khan and Lynch 2018). In addition, the alveolar epithelium is important for
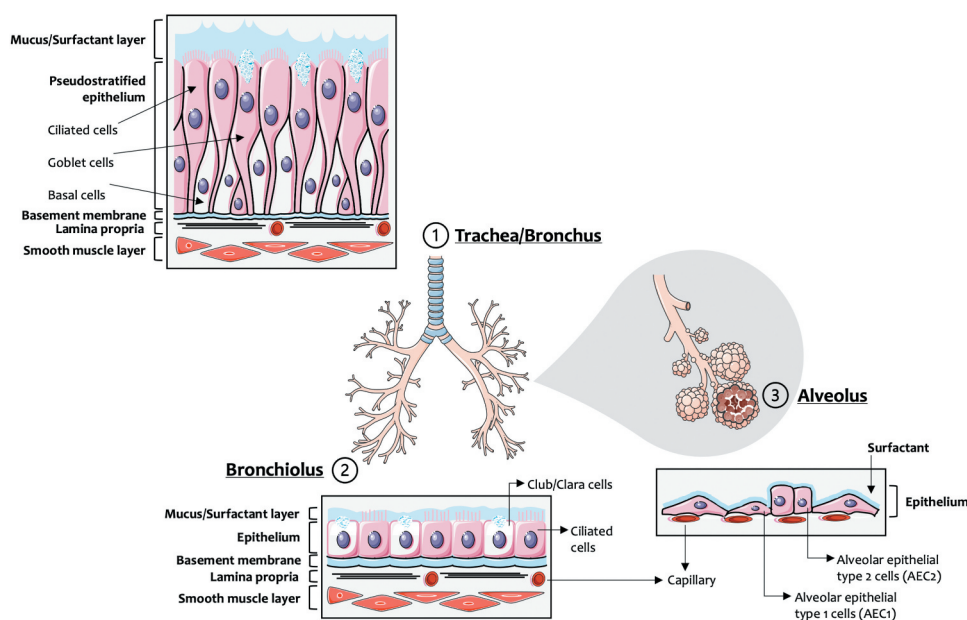
Figure 1. Respiratory tract: cell types and morphology. This illustration was created including images obtained from Smart Servier Medical Art (www.smart.servier.com) CC by 3.0.

maintaining lung homeostasis and is constituted by cuboidal alveolar epithelial type 1 cells (AEC1) and type 2 cells (AEC2).

The defense mechanisms of the airways and lung comprise the cough reflex, the epithelial barrier and lining fluid, the mucociliary escalator, metabolic signaling cascades (*e.g.*, activation of cytochrome P450 family and and/or activation of nuclear factor erythroid 2–related factor 2 (Nrf2)-mediated transcription factors), humoral factors including antimicrobial and surfactant peptides or complement proteins and cells that elicit immune responses, namely epithelial cells, macrophages, monocytes, dendritic cells, neutrophils, natural killer cells, and mast cells (Hastedt et al. 2016; Rothen-Rutishauser et al. 2008). In the large airways, the epithelial lining fluid is composed of a superficial mucus layer overlying a periciliary liquid layer that is responsible for mucociliary clearance through physical unidirectional cilia movement and removal of deposited particles and gases dissolved in the mucus from the respiratory tract (Schuster et al. 2013). Further, the alveolar surface is covered by pulmonary surfactant that also plays a critical role in the clearance of inhaled toxicants including aerosolized (nano)particles (Wohlleben et al. 2016).

Rodents are the most frequently used animal model in inhalation toxicity studies (Movia, Bruni-Favier, and Prina-Mello 2020). However, as presented in Table 1, considerable differences in the anatomy and physiology of the respiratory system between rodents and humans exist, that need to be considered when designing and interpreting inhalation (nano)toxicity studies. Indeed, the distinct respiratory tract architecture between rodents and humans affects the airflow pattern and ventilation rates, which in turn influence deposition, clearance, and retention of inhaled particles (Bakand, Hayes, and Dechsakulthorn 2012; Clippinger et al. 2018). Differences in the type and number of cells lining the airways, as well as in mucociliary clearance also exist. Taken together, these aspects markedly influence (nano)particle biological fate and hazard potential.

Pathways and fate of inhaled particles

As depicted in Figure 2, once a particle is inhaled, it enters the oropharyngeal region where the particle meets the trachea, being conducted to the bronchi and alveolar region. Aerosol particles are often described as spherical and monodisperse; however, particle collisions often originate non-spherical aggregates and/or agglomerates (Kleinstreuer and Zhang 2010). Deposition of inhaled particles is dependent upon (1) aerosol physicochemical

Table 1. Main anatomical and physiological differences between humans' and rodents' respiratory system. [Adapted from Harkema et al. (2012); Meyerholz et al. (2018); Rackley and Stripp (2012)].

Features	Humans	Rodents
Nose and sinus	Relatively simple nasal turbinate structure; total surface area: 150–200 cm^2.	Complex nasal turbinate structure; total surface area: 2.9 cm^2 [relative surface area (surface area/volume) 5× greater than humans].
	Accessory olfactory organs are not well developed or functional.	Presence of functional accessory olfactory organs.
	Olfactory epithelium ~3% of the nasal cavity.	Olfactory epithelium ~50% of the nasal cavity (well-developed sense of smell).
Pharynx and larynx	Widely disseminated and well-defined pharyngeal tonsils.	Lack of distinct pharyngeal tonsils; widely dispersed lymphoid aggregates.
	No U-shaped laryngeal cartilage and ventral pouch.	Presence of U-shaped laryngeal cartilage and ventral pouch (larger/ more prominent in rats than mice).
	Absence of taste buds in this region.	Taste buds are located within the epiglottis, larynx, and pharynx.
Trachea	Trachea internal diameter of ~12 mm.	Trachea internal diameter of ~1.5 mm (mouse).
	Trachea lined by ciliated cells.	The trachea is mostly lined by non-ciliated epithelial cells.
	The submucosa contains numerous tightly packed seromucinous glands.	Submucosal glands are restricted to the proximal (closest to the larynx) trachea.
	Tracheal cartilaginous rings extend for several bronchial generations into the lung.	Tracheal cartilaginous rings are only present in the extrapulmonary airways.
Lungs	The right lung of humans is divided into three lobes, whereas the left lung has two lobes.	The right lung is divided into four lobes, whereas the left lung has one lobe.
	Dichotomous airway branching.	Monopodial airway branching.
	The respiratory zone includes respiratory bronchioles, alveolar ducts, and alveoli.	Respiratory zone includes diminutive respiratory bronchioles (if present), alveolar ducts, and alveoli.
Bronchi to terminal bronchioles	Lung parenchyma includes both bronchi and bronchioles.	Lack of well-developed respiratory bronchioles (defined by lack of cartilage and submucosal glands).
	Relatively abundant mucin-secreting goblet cells.	Less than 1% mucous goblet cells in the epithelium of extrapulmonary bronchi (particularly in adults mice maintained under lab conditions).
Branching pattern	Symmetric (airflow pattern more susceptible to deposition at its bifurcation points).	Asymmetric or monopodial (relatively unimpeded flow).
Breathing mode	Oronasal breathers (both nasal and oral breathing).	Obligate nose breathers (all inhaled air passes through the nasopharynx on its path into the lungs).
Lung organogenesis	~week 3 during human development (average 40 weeks of gestation) as the primitive lungs bud from the foregut endoderm.	~embryonic day 9 in the mouse (average 19–21 days of gestation).
	Undergo many additional rounds of branching before beginning alveolarization.	Mouse lungs develop quickly and do not begin forming alveoli until after birth.
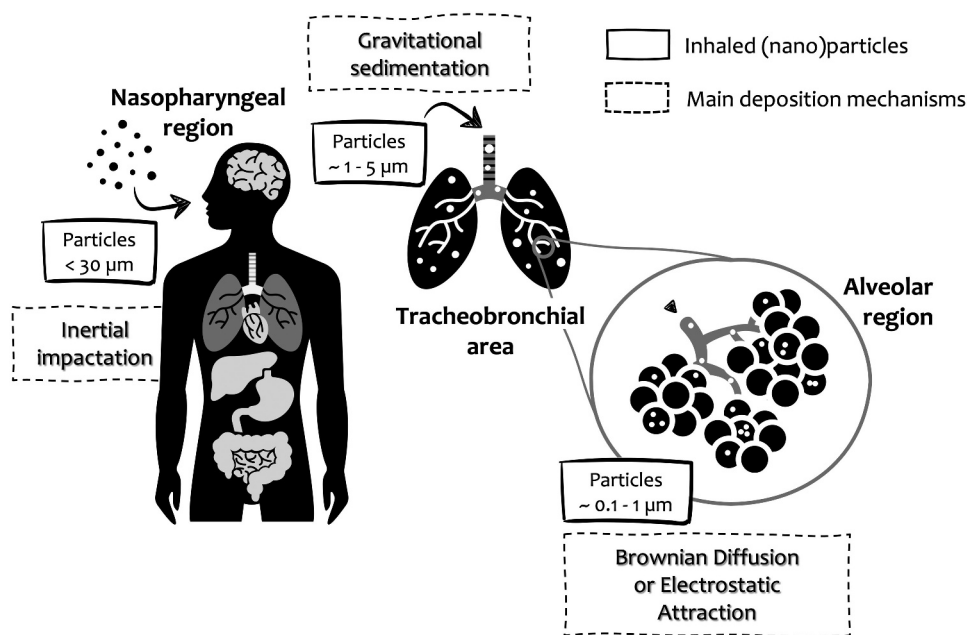
Figure 2. Inhaled particles: deposition mechanisms along the respiratory tract. This illustration was created including images obtained from SlidesCarnival (https://www.slidescarnival.com) CC by 4.0.

properties (Morawska and Buonanno 2021) (2) anatomical (diameter, length, and branching angle of airway segments) and (3) physiological (airflow and breathing pattern) factors (Andujar et al. 2011; Clippinger et al. 2018). Physicochemical parameters governing particle deposition include particle size/and or size distribution, surface area, surface reactivity, density, shape, hygroscopicity/ hydrophobicity, and chemical reactions of the particle (Morawska and Buonanno 2021). In addition, the composition, viscosity and surface tension of lung surfactant influence markedly particle trajectory, deposition, and biological fate. Anatomical features, such as surface roughness, airway morphology, cross-sectional profiles, and branching angles exert a significant influence on the deposition of (nano)particles in humans. This needs to be considered for the lung burden estimation of airborne particles, particularly when comparing healthy *vs* vulnerable individuals such as with lung disease (Jakobsson et al. 2018).

Overall, the main mechanisms for particle deposition include: interception (particle-surface contact), inertial impaction (particle sudden change in the direction of the flow), gravitational sedimentation (settling of particles under the action of gravity), diffusion (random motions of the particles, *e.g.* Brownian motion, where randomized particle motion is initiated by their collision with gas molecules), and electrostatic precipitation (particle charge may potentially affect their deposition in the airways) (Bui et al. 2020; Darquenne 2020; Tsuda, Henry, and Butler 2013). As illustrated in Figure 2, larger particles (5–30 μm) are deposited in the nasopharyngeal region by inertial impaction, while smaller particles (1–5 μm) are deposited in the tracheobronchial area by gravitational sedimentation where they may or may not be removed by mucociliary clearance. On the other hand, nano-sized particles (0.1–1 μm) penetrate deeper into the alveolar region, such as the alveoli where airflow is low, deposited by Brownian diffusion or electrostatic attraction (Bakand, Hayes, and Dechsakulthorn 2012; Hagens et al. 2007).

So far, it is not clearly understood how (nano) particles interact with complex biological environments to influence phagocytic recognition and clearance (indirectly via necrosis or apoptosis), cellular processing and, consequently, toxicological outcomes (Gustafson et al. 2015). Physicochemical properties of aerosol particles significantly influence their deposition but also their clearance from human airways (Bierkandt et al. 2018; Braakhuis et al. 2014). Individual (nano)particles might be too small to be efficiently recognized and phagocytosed by alveolar macrophages. By avoiding the normal phagocytic defenses of the respiratory system, (nano)particles may gain access to the systemic circulation and reach different extrapulmonary sites (Nemmar et al. 2013). Soluble particles are more readily eliminated compared to insoluble particle components (Braakhuis, Oomen, and Cassee 2016). In contrast, charged (nano)particles seem to be retained to a greater extent in the human respiratory airways due to their ability to attract different proteins (Docter et al. 2015). Gustafson et al. (2015) postulated that when a (nano)particle incomplete/arrested phagocytosis occurs as during long-term residence of inhaled (nano)particle within phagocytic cells, the human body, rather than eliminating these particles, developed a mechanism that induces a local response to isolate these foreign (nano)materials from the host biological environments, which might potentially trigger adverse chronic inflammatory or immunological responses.

Another important aspect to take into consideration is how physiological fluids might change the physicochemical properties and behavior of (nano)particles (Urban et al. 2016). Upon contact with the biological pulmonary milieu, (nano)particles may become surrounded by biomolecules such as albumin and proteins in the surfactant, which significantly contribute to the formation of a corona around them and change particle size and kinetics in the airways (Monopoli et al. 2012). In the alveolar region, (nano)particles interact with the lipids present in the surfactant film located at the air-liquid interface (ALI) in the epithelial lining fluid covering the internal surface of the lung (Raesch et al. 2015). The surfactant helps to stabilize the alveoli and promotes clearance of inhaled particles to maintain alveoli in a sterile- and inflammation-free environment (Kendall and Holgate 2012). Accordingly, characterization of nano-sized materials in relevant pulmonary biological fluids is of utmost importance (Wohlleben et al. 2016).

There are several *in vitro* and *in vivo* studies that already identified the major biological mechanisms involved in pulmonary toxicity induced by (nano) particles, including: ineffective particle clearance, intracellular uptake/internalization, impairment of lung macrophage-mediated phagocytosis, loss of plasma membrane integrity, and mitochondrial dysfunction, oxidative stress (ROS generation, glutathione depletion, and lipid peroxidation), cytokine production and activation of inflammatory signaling cascades, DNA and chromosomal damage, altered DNA methylation and repair, and altered cell cycle regulation (Bakand and Hayes 2016; Ianni et al. 2022; Paur et al. 2011; Pietroiusti et al. 2018; Stone et al. 2017).

In vitro models for (nano)particle human respiratory toxicity assessment

For many years, *in vitro* culture systems failed to represent the complexity of multicellular organisms. Recently, more complex and physiologically relevant human *in vitro* models emerged and gained some acceptance (Bassi et al. 2021; Sakolish et al. 2016). The study of the biological effects of inhaled (nano)particles is particularly challenging and *in vitro* respiratory models have been used as an important tool for assessing underlying molecular mechanisms and cellular responses. At the same time, cell culture and exposure conditions need to be as close as possible to the ones found *in vivo*. Several human *in vitro* models exist to represent specific areas of the respiratory tract such as nasal, tracheal, bronchial, and alveolar regions (Fröhlich and Salar-Behzadi 2014; Steimer, Haltner, and Lehr 2005). In order to accurately predict the effects of inhaled (nano)particles, an *in vitro* system needs to include the following features: (1) a suitable model mimicking the morphology and metabolic function of a particular healthy or vulnerable tissue/organ, and able to reproduce *in vivo* particle deposition patterns and clearance; (2) an exposure chamber for culture exposure to aerosolized (nano)particles; (3) a set of relevant biomarkers that enable high-throughput evaluation of key toxicity events. While concerns regarding the resemblance of cells cultured *in vitro* to those found in the human lung may be raised, information available from molecular cell atlas, a computer-assisted qualitative data analysis software, may be performed, either by single-cell RNA sequencing (Travaglini et al. 2020) or multi-omics atlas analysis (Madissoon et al. 2021) to compare freshly isolated human lung cells with *in vitro* cell cultures to display a reliable level of resemblance. An overview of the key features of the available pulmonary *in vitro* models, namely pulmonary cell lines, primary cultures, organoids/spheroids, three dimensional (3D) cultures and lung-on-chip devices (Figure 3) is provided below.

Pulmonary cell lines: mono- and cocultures

Primary human lung epithelial cells are, in theory, the ideal choice for (nano)particles' testing, due to their resemblance in morphology, organization, stratification, and physiological function to the human airway epithelium (Dvorak et al. 2011; Pezzulo et al. 2011; Rosario et al. 2018). In addition, primary cultures may be established from tissues of healthy or lung disease donors offering the possibility of evaluating *in vitro* the susceptibilities in health and disease (Faber and McCullough 2018). However, primary cultures have some drawbacks as follows: 1) these are difficult to establish and maintain in culture, (2) require complex and expensive cell culture media, (3) are poorly reproducible, and (4) have a limited life span. This is why continuous cell lines have been widely used for *in vitro* nanotoxicology studies (Katt et al. 2016). There are numerous *in vitro* lung cell lines that can be used to study the cellular interplay and cellular responses following (nano)particle exposure. Notwithstanding, since epithelial cells play a central role in the respiratory tract, providing a physical barrier to inhaled (nano)particles, these are commonly used *in vitro* models for assessing (nano)toxicity. In accordance, Table 2 presents the most popular epithelial cell lines for *in vitro* toxicological assessment of inhaled particles (Bierkandt et al. 2018; Fröhlich 2018; Hiemstra et al. 2018; Jia, Wang, and Liu 2017; Rothen-Rutishauser et al. 2008; Schlinkert et al. 2015). Despite all advantages, some tumoral or immortalized epithelial cell lines including bronchial 16HBE14o, BEAS-2B, and Calu-3 cells, lack human *in vivo* characteristics such as (1) mucociliary differentiation, (2) formation of an effective
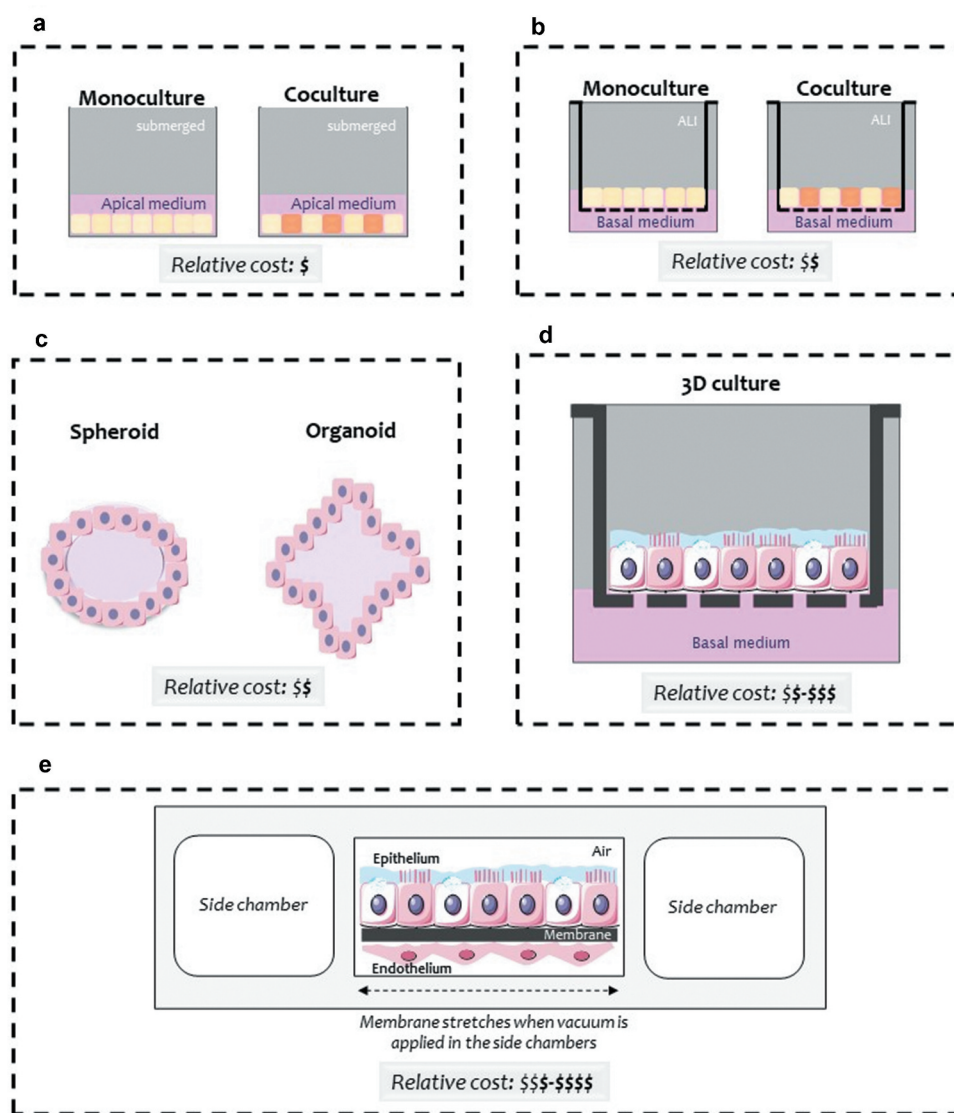
Figure 3. Schematic representation of the *in vitro* models addressed in the present review: (a) Mono- and co-cultures of respiratory cells under submerged and (b) air-liquid interface culture conditions; (c) Spheroid and organoids; (d) 3D cultures and (e) Microfabricated human breathing-inspired lung-on-a-chip microfluidic device [Adapted from Huh et al. (2010)]. The device simulates physiological breathing movements by applying a vacuum into the side chambers causing a stretch of the membrane. This illustration was created including images obtained from Smart Servier Medical Art (www.smart.servier.com) CC by 3.0. The relative costs of each cell system are indicated using a 1 to 4 level ($-).

barrier, and (3) reduced metabolic capacity (Faber and McCullough 2018). However, under appropriate growth conditions, some of these cell lines may exhibit tight junctions, cilia and mucus production providing an effective barrier between the external and internal environments. Previously Gonzalez-Mariscal et al. (1990) reported the importance of calcium for the development of tight junctions between epithelial cells.

George et al. (2015) comparatively assessed the translocation of fluorescent 50 nm silica (SiO$_2$) NP in three *in vitro* human lung models, in bronchial epithelial NCI-H292 and Calu-3 cells, and in alveolar epithelial A549 cells grown onto Transwell® inserts. George et al. (2015) found that bronchial epithelial Calu-3 cells would be the most relevant model for assessing NP uptake since, amongst all the studied cells, these were able to form tight junctions. Inflammatory or immune responses are also difficult to mimic in such simplistic models since these processes involve a highly coordinated network of many cell types (Joris et al. 2013; Savolainen et al. 2010). Some of these limitations might be overcome by using coculture models

Table 2. Human epithelial cell lines commonly used in *in vitro* (nano)particle pulmonary toxicity studies.

Lung region	Human cell line		
	Identification	*Origin*	*Features*
Bronchial	16HBE14o	Normal bronchial epithelium; Virus transformed (adenovirus 12- simian virus 40 hybrid virus).	• Ability to form tight junctions; • Express cilia when grown at the air-liquid interface (ALI); • Retain important properties and functions of differentiated airway epithelial cells (*e.g.* mucus-secreting capacity, apical microvilli, etc.).
	BEAS-2B	Normal bronchial epithelium; Virus transformed (adenovirus 12- simian virus 40 hybrid virus).	• Do not form tight junctions; • Resemble airway basal epithelial cells, however, do not differentiate; • Poor barrier function.
	Calu-3	Lung adenocarcinoma from submucosal gland serous cells.	• Ability to form tight junctions; Express mucins and some cilia when grown at ALI; • Reasonable barrier function.
Alveoli	A549	Lung adenocarcinoma.	• Display some characteristics of alveolar epithelial type II (AEC2) cells; • Express metabolizing phase I (cytochrome P450 isoenzymes) and phase II enzymes (transferases); • Do not form tight junctions; • Ability to produce surfactant when grown at ALI; • Poor barrier function.
	NCI-H441	Lung papillary adenocarcinoma.	• Resemble characteristics of both type II pneumocytes and clubcells; • Poor barrier function.
	hAELVi	Lentivirus immortalized.	• Morphological resemblance with alveolar type I (AEC1) cells; • Express high levels of metabolizing enzymes and transporters; • Ability to form tight junctions; • Ability to produce surfactant.

constituted by two or more cell types with some degree of contact between them, which may potentially improve culture success, by improving intracellular interactions and communication between different cell types as well as cellular morphology and integrity by often displaying tight and adherent junctions (Goers, Freemont, and Polizzi 2014; Kasper et al. 2011; Snyder-Talkington et al. 2012; Vis, Ito, and Hofmann 2020). Therefore, cocultures are versatile models that more closely mimic human *in vivo* environments (Edmondson et al. 2014), and are preferred models to understand (nano)particle mechanistic behavior in more complex biological systems (Costa et al. 2013). Notwithstanding, cocultures present many challenges. The selection of the optimal culture conditions for cocultures may be demanding, for instance if each cell type requires a specific culture medium composition. Coculture medium optimization might be a laborious and time-consuming task due to the numerous possible combinations and cellular implications (Goers, Freemont, and Polizzi 2014; Vis, Ito, and Hofmann 2020). Another important feature is the cell population ratio that needs to be optimized to achieve a stable culture such that one cell type does not negatively affect the others, and to more closely resemble the *in vivo* situation (Vis, Ito, and Hofmann 2020).

Coculture models have been developed to resemble the complexity of the human airways by combining airway epithelial cells together with fibroblasts, endothelial cells, airway smooth muscle cells, as well as immune cells including macrophages, dendritic, and mast cells. These models contribute to a better understanding of the toxicology and translocation mechanisms of (nano) particles (Bierkandt et al. 2018; Braakhuis et al. 2015). Table 3 summarizes some studies that assessed the effects of (nano)particle exposure in human cocultures of different regions of the lung barrier. One of the most studied regions is the bronchial epithelium since it plays a critical role in biological stress responses to inhaled (nano) particles (Jia, Wang, and Liu 2017). It is noteworthy that nano-sized particle deposition occurs in the respiratory tract primarily in the alveolar region (Londahl et al. 2014). Therefore, alveolar epithelial cells are relevant models for the toxicity of inhalable (nano)particles, particularly in the assessment of particle retention and translocation through these cells (Leibrock et al. 2019). For this purpose, *in vitro* models representative of the alveolar-capillary and/or air-blood barrier are also explored in (nano)toxicity assessments. The air-blood barrier is predominantly composed of alveolar epithelial cells and macrophages, that function as a structural and immunological barrier

Table 3. Pulmonary (nano)particle toxicity studies using coculture models mimicking relevant human lung barriers.

Human lung barrier	Coculture model (cell lines used)	(Nano)particles tested	Main findings	References
Alveolar-capillary barrier	NCI-H441 and ISO-HAS-1.	SiO$_2$ NP (0.6–6000 µg/mL; submerged conditions; 4 h exposure +20 h recovery period)	Release of IL-6 and IL-8 (early inflammatory events); upregulation of apoptosis markers.	Kasper et al. (2011)
Air-blood barrier	NCI-H441 and HPMECST1.6 R.	CuO and TiO$_2$ NP (25 µg/mL) and PM 10 (22.5 µg/mL) (submerged conditions; 24 h exposure).	Significant modulation of pro-inflammatory (e.g., IL-6 and IL-8) proteins.	Bengalli et al. (2013)
Alveolar epithelial barrier	A549, THP-1 and HMC-1.	SiO$_2$-Rhodamine NP (10 mg/L; ALI conditions; 24 h exposure).	Lower levels of ROS and IL-8.	Klein et al. (2013)
Alveolar epithelial barrier	A549, MDDC and MDM.	MWCNT (1.15 µg/cm^2; ALI conditions; repeated exposure: 3 days).	No cytotoxicity, alterations in cell morphology, or increase in pro-inflammatory markers.	Chortarea et al. (2015)
Lung – blood barrier	Biculture of 16HBE14o and THP-1; triculture of 16HBE14o, THP-1, and HLMVEC.	TiO$_2$, Ag and SiO$_2$ NP (1–243 µg/mL; submerged conditions; 24 h exposure).	Biculture: TiO$_2$ and Ag NP but not pristine SiO$_2$ NP induced cytotoxic effects at relative high doses (83–243 µg/mL); Triculture: no considerable changes were observed.	Smulders et al. (2015)
Alveolar epithelial barrier	A549 and THP-1.	CeO$_2$ and TiO$_2$ NP (1–20 µg/cm^2; submerged and ALI conditions; 24 h exposure).	Inflammation, decreased cell viability, and oxidative stress were observed at ALI; more predictive of *in vivo* effects.	Loret et al. (2016); Loret et al. (2018)
Air epithelial barrier	hAELVi and THP-1.	Ag (7.25 µg) and starch (41.25 µg) NP (ALI conditions; 24 h exposure).	Cocultures form functional diffusion barriers under ALI conditions.	Kletting et al. (2018)
Air – blood barrier	Biculture of A549 and EA.hy926; triple coculture of A549, THP-1, and EA.hy926.	Ambient PM 2.5 collected from Shanghai city (China) (20–180 µg/mL; ALI conditions; 24 h exposure).	Stronger inflammatory responses and ICAM-1 and caveolin-1 mRNA expression in triculture than in biculture system.	Wang et al. (2019)
Lung epithelial barrier	hAELVi and huAEC.	CeO$_2$ NP (0.1–200 µg/mL; ALI conditions; 24 h exposure).	CeO$_2$ NP induced no toxicity, while ZnO NP were toxic at concentrations between 10–50 µg/mL.	Leibrock et al. (2019)
Air – blood barrier	A549, THP-1, HMC-1 and EA.hy926.	Ag NM (spherical particles, PVP coated nanowires) (0.05–5 µg/cm^2; ALI conditions; 6 and 24 h exposure).	Increased cytotoxicity; increased mRNA levels of the pro-apoptotic gene CASP7, anti-oxidant enzyme HMOX-1, and pro-inflammatory mediators; induction of the NF-kB nuclear translocation.	Fizesan et al. (2019)
Lung – blood barrier	Calu-3, THP-1, and EA.hy926.	Ag NP (coated with tannic acid) (3–30 µg/cm^2; submerged conditions; 24 h exposure).	NP cellular uptake and translocation of Ag NP through the modeled barrier; mild cytotoxicity and reduced secretion of IL-6, IL-8, and TNF-α.	Zhang et al. (2019)
Air-blood barrier	A549, EA.hy 926 and THP-1.	Citrate-capped Au (50 mg/mL), Ag-SiO$_2$ (0.50 mg/mL), and CuO (1.5 mg/mL) NP (ALI conditions; 4 h exposure).	Induced more oxidative stress and higher IL-8 levels.	Wang et al. (2020)

to environmental aggressors, such as fine and nano-sized particles (Kletting et al. 2018). For the alveolar region, A549 is the most frequently used cell line for assessment of (nano)particle toxicity. These cells are often used either as monocultures or in cocultures with other cell lines such as immune cells or macrophages (Fröhlich 2018). Alveolar macrophages, in turn, are important regulators of the inflammatory processes in the lung and ingest nano-sized particles as a clearance defense mechanism (Geiser 2010). THP-1 cells are a commonly used cell line for investigating *in vitro* function and regulation of monocytes and macrophages.

Increasing evidence supports the higher relevance of cocultures compared to monocultures (Table 3). Kasper et al. (2011) investigated the cytotoxic and inflammatory responses of

monodisperse amorphous 30 nm SiO_2 NP in conventional monocultures *vs* coculture models representative of the alveolar-capillary barrier. Although lower cytotoxic effects in terms of cell viability, membrane integrity, and Trans-Epithelial Electrical Resistance (TEER) were observed in cocultures compared to monocultures, significantly increased levels of inflammatory mediators IL-6 and IL-8 and apoptotic markers including phosphorylation of p53-protein at Ser15, Ser46 and Ser392 were found. Kasper et al. (2011) postulated that these cocultures were more representative of the alveolar regions of the human lung compared to the conventional monocultures since these exhibited the usual early inflammatory responses that occur in pulmonary alveoli. In addition, Wang et al. (2020) assessed the toxicity of aerosolized citrate-capped gold (Au), 15% silver on silica (Ag-SiO_2) and copper oxide (CuO) NP on triple-cocultures of human THP-1 differentiated macrophages, alveolar epithelial A549 and endothelial EA.hy 926 cells, cultured under ALI conditions (4 hr; 3.5 mg/m^3). Wang et al. (2020) compared NP effects in the triple cocultures *vs* monocultures of A549, EA.hy 926, and THP-1 cells. Data demonstrated that NP induced greater oxidative stress (15% Ag-SiO_2) and higher IL-8 levels (15% Ag-SiO_2, CuO) in coculture than in A549 monocultures, whereas a similar response in terms of reactive oxygen species (ROS) and IL-8 responses were noted following CuO NP exposure between triple cocultures and EA.hy 926 endothelial cells (Wang et al. 2020). Further, Loret et al. (2016) also compared the effect of poorly soluble cerium oxide (CeO_2) and titanium oxide (TiO_2) NP in monocultures *vs* cocultures of A549 and THP-1 cells, and found that cocultures were not only more sensitive than monocultures but also more predictive of *in vivo* pulmonary toxicity attributed to CeO_2 and TiO_2 NP in the rat. For each dose metric used (mass/alveolar surface or mass/macrophage), A549 and THP-1 cocultures under ALI conditions exhibited similar levels of pro-inflammatory responses, cytotoxicity and oxidative stress to those observed in rat bronchoalveolar lavage fluid (BALF) supernatants and cells (Loret et al. 2018). Similarly, Camassa et al. (2022) compared nanomaterial300K-induced cyto- and genotoxicity in monocultures of human epithelial A549 cells to co-cultures of human endothelial EA.hy926 and differentiated dTHP-1 cells under ALI conditions. Nanomaterial-300K induced cytotoxicity in the A549 monocultures and DNA strand breaks and oxidized base lesions in the coculture with EA.hy926. By further adding the immune cells , an increase in the complexity of the system was accompanied by changes in the sensitivity of the triculture to NM-300K. Camassa et al. (2022) concluded that utilization of more complex 3D models altered not only the sensitivity but the outcome of the toxicity testing, an aspect that needs to be considered when elucidating human hazard assessment.

As the number of cell types in the coculture increases, more predictive the model seems to be. In this regard, Wang et al. (2019) assessed the toxicity mechanisms of urban ambient PM <2.5 μm ($PM_{2.5}$) in three *in vitro* model approaches of increasing complexity: (1) monocultures of A549 alveolar epithelial cells, THP-1 differentiated macrophages, and EA.hy926 endothelial cells; (2) bicultures of A549 cells and EA.hy926 endothelial cells seeded on the apical side of Transwell® inserts and on the basolateral chamber, respectively; (3) triple cocultures of A549 and THP-1 cells seeded on the apical side of Transwell® inserts, and EA.hy926 endothelial cells seeded on the basolateral chamber. These investigators showed that apical exposure for 24 hr of the cocultures induced a greater inflammatory response compared to A549 monocultures. Intercellular Adhesion Molecule 1 (ICAM-1) and caveolin-1 mRNA expression levels in EA.hy926 cells, an endothelial function marker, were significantly higher in the tricultures than in monocultured cells, while upregulation of caveolin-1 was detected in bicultures but only at high concentrations. In addition, $PM_{2.5}$ crossed the epithelial barrier and deposited in the endothelium. Taken together these data suggest that triple cocultures constitute a reliable model to assess the impact of these ambient particles on cytotoxicity and adverse effects on cardiopulmonary system (Ferraz et al. 2016; Wang et al. 2019).

Fizesan et al. (2019) used a human tetraculture alveolar barrier model consisting of alveolar epithelial A549, THP-1 macrophages and HMC-1 mast (in the apical compartment), and EA.hy926

endothelial (in the basolateral compartment) cells cultured at ALI to evaluate the influence of exposure to aerosolized Ag NM (two spherical shape particles and one PVP-coated Ag nanowire) utilizing a VitroCell® Cloud exposure system. Fizesan et al. (2019) noted the capacity of this tetraculture model to secrete both anti- and pro-inflammatory cytokines (IL-12p70, IL-13, IL-1β, IL-2, IL-4, and IL-8) following exposure to Ag NM, reflecting complex biological responses that naturally occur in native respiratory epithelia. It is worthwhile noting that human alveolar epithelial lentivirus immortalized (hAELVi) cells combined with THP-1 alveolar macrophages (Kletting et al. 2018) or human airway epithelial cells (huAEC) (Leibrock et al. 2019) were found to form an effective barrier under ALI conditions, constituting promising models to study the effect of inhalable NP (Table 3).

Advanced respiratory tissue cultures

For the assessment of (nano)particle toxicity in the respiratory tract, advanced 3D *in vitro* tissue models have been emerging as promising systems over traditional two-dimensional (2D) cultures. These models contain different cell types in varied orientation and number that need to be organized in a structure that reflects the tissue of interest. These cultures are often obtained from donor-derived primary cells or from stem cells such as Induced Pluripotent Stem Cell (iPSC)), and are commonly grown in tissue-specific scaffolds (Carvalho et al. 2020; Kastlmeier et al. 2022; Langhans 2018). These models either mimic normal or diseased tissues (Jackson and Lu 2016; Sotty et al. 2019). Advanced multicellular 3D lung tissue models better reflect cellular interactions observed *in vivo* and, therefore, enable investigation of the cellular interplay between different cell types following (nano)particle inhalation exposure. Some of these respiratory models display active ciliary beating and mucus production that mimic mucociliary clearance defense systems (George et al. 2019; Kooter et al. 2017).

Despite the major advantages of 3D models, these fail to reproduce the mechanical stretch and deformation that occurs in lung tissues during breathing (Nossa et al. 2021). This mechanical stimulus strongly influences biological functions and signaling of lung cells, which consequently impacts biological responses elicited by (nano)particles. Therefore, future studies need to address these major gaps in development of advanced respiratory tissue cultures, such that this crucial aspect needs to be taken into consideration in airborne (nano)particle toxicity assessment.

In Table 4 are depicted currently available human respiratory tissue 3D models that include MucilAir™ and SmallAir™ from Epithelix Sàrl, EpiAirway™ and EpiAlveolar™ from MatTek Corporation, and Micro-Lung™ and Metabo-Lung™ from Cardiff University. Commercially available 3D models provided by Epithelix Sàrl and MatTek Corporation are established from nasal, tracheal, bronchial or small airways biopsies of individuals, either healthy or with respiratory pathologies such as COPD, asthma and cystic fibrosis. These models are highly differentiated mucus-producing and ciliated cultures, with well-developed tight junctions, effective epithelial resistance and metabolic activity, that are able to maintain their characteristics over long periods of time (up to several months), which is a major advantage over other cellular models. Recently, human-derived tracheal/bronchial epithelial cultures are also available as a co-culture system with normal human fibroblasts.

In contrast, Micro-Lung™ and Metabo-Lung™ from Cardiff University are not commercially available. The Micro-Lung™ model was established from normal human bronchial epithelial (NHBE) cells isolated from surgical patients and postmortem donors (Prytherch and Bérubé 2015), while the Metabo-Lung™ consists of NHBE cells cocultured with human primary hepatocytes, offering the possibility of assessing the role of metabolism (Prytherch et al. 2011). To the best of our knowledge, there are no published studies available using these models to evaluate (nano)particle-mediated toxicity. Recently, OncoCilAir™ has appeared, a 3D lung cancer model that combines a functional reconstituted human airway epithelium, human lung fibroblasts, and lung adenocarcinoma cells that may be kept in culture for several months (Benainous et al. 2018), enabling concurrent testing of drug efficacy, side-toxicity and tumor recurrence. Nevertheless, despite all the efforts in development of advanced and robust 3D *in vitro* cell

Table 4. Advanced 3D *in vitro* models of human respiratory tissues.

Model		Features	Advantages and limitations
MucilAir™ (Epithelix Sàrs)	nasal, tracheal, or bronchial epithelial model.	• Isolated from human biopsies of the nasal cavity, trachea, and bronchi; • Constituted by basal, goblet, and ciliated cells; • Under ALI conditions displays tight junctions, cilia beating and mucus production, cytokines, chemokines, and metalloproteinases secretion, and expression of specific respiratory epithelia cytochromes (*e.g.*, P450);	• Effective barrier model for the assessment of the permeability/ absorption of several compounds • Long shelf-life • Option of coculture with fibroblasts available • Donor with several pathologies available • More expensive than conventional cultures
SmallAir™ (Epithelix Sàrs)	small airway model.	• Isolated from the distal lung; • Constituted by epithelial cells, a large number of club cells, and fewer goblet and ciliated cells; • Presents a much thinner epithelium (compared to MucilAir™).	• Long shelf-life • Option of coculture with fibroblasts available • Donor with several pathologies available • There are still a limited number of studies using this model • More expensive than conventional cultures
EpiAirway™ (MatTek Corporation)	tracheal/bronchial epithelium model.	• Derived from normal tracheal and bronchial epithelial cells; • Constituted by mucus-producing goblet cells, ciliated cells with actively beating cilia, basal cells, and club cells (club).	• More than 30 donors available • More expensive than conventional cultures
EpiAlveolar™ (MatTek Corporation)	lower respiratory tract tissue model.	• Constituted by alveolar epithelial cells and monocyte-derived macrophages (apical side) and pulmonary endothelial cells (basolateral side).	• Remains stable for studies lasting more than 30 days. • Option of coculture with fibroblasts available • More expensive than conventional cultures
Micro-Lung™ (Cardiff University)	bronchial epithelium model.	• Isolated from surgical patients and postmortem donors; • Constituted by normal human bronchial epithelial (NHBE) cells, basal, serous, club, goblet, and ciliated cells.	• Not commercially available • No studies in the literature regarding (nano)particle toxicity
Metabo-Lung™ (Cardiff University)	lung-liver model.	• Coculture of NHBE cells with primary human hepatocytes, which allows the biotransformation of inhaled toxicants in an *in vivo*-like manner.	• Not commercially available • No studies in the literature regarding (nano)particle toxicity

cultures, as reliable alternatives tools to animal testing, thus far none had been approved or validated for pulmonary toxicity testing of airborne chemical, (nano)particle or NM exposure (Pfuhler et al. 2020).

Table 5 presents an overview of existing *in vitro* studies on the pulmonary toxicity of (nano)particles using advanced 3D lung models. The majority of investigations performed so far were conducted utilizing MucilAir™ human bronchial epithelial cultures. In this regard, Frieke Kuper et al. (2015) comparatively investigated the toxicity of liquid suspensions of aggregated CeO_2 NP in MucilAir™ bronchial cultures, bronchial epithelial BEAS-2B and alveolar epithelial A549 cells. While no signs of marked toxicity were observed in the MucilAir™ cultures, most likely due to the presence of the mucociliary apparatus that prevented NP from reaching the cultures, significant concentration-dependent genotoxicity on BEAS-2B and A549 cells after 24 hr exposure to CeO_2 NP (33–333 µg/

cm^2) was detected. Di Cristo *et al.* (20200 also reported no apparent signs of morphological alterations or cytotoxic effects following repeated exposure of MucilAir™ cultures for 12 weeks, 5 days per week to aerosolized SiO_2 NP (0.90 to 55 µg/cm^2), which was also attributed to the mucociliary clearance.

As indicated previously, MucilAir™ cultures might also be established from diseased tissues, which offers the possibility to investigate the effects of (nano)particles in primary cultures from individuals with respiratory diseases. In this context, Chortarea et al. (2017) evaluated the pulmonary toxicity of occupationally relevant doses (10 µg/cm^2 for 5 weeks/5 days per week) aerosolized multi-walled carbon nanotubes (MWCNT) in MucilAir™ bronchial cultures from healthy and asthmatic donors. Although no cytotoxicity or morphological changes were observed, chronic MWCNT exposure induced a pro-inflammatory and oxidative stress response in both types of cultures, accompanied

Table 5. Overview of the existing pulmonary (nano)particle toxicity studies using advanced 3D *in vitro* models.

Pulmonary 3D model	(Nano)particles tested	Experimental design	Biological endpoints assessed	Main findings	References
MucilAir™ (Epithelix Sàrs)	CeO_2 NP	ALI (droplet) exposure; responses assessed after 3, 24, 48 h.	Cytotoxicity (TEER, LDH release); Inflammation (IL-8, MCP-1, sICAM-1, IL-1α, TNFα); Genotoxicity (comet assay and HO-1 expression).	No major toxic effects were observed.	Frieke Kuper et al. (2015)
	MWCNT	Cultures from healthy and asthmatic donors; ALI exposure (ALICE system); cells exposed 5 weeks/5 days per week; responses assessed at weeks 1, 3, and 5.	Cell morphology Cytotoxicity (LDH release); Inflammation (IL-8, IL-6, IP-10 and TGF-β); Oxidative Stress (HMOX-1 and SOD-2 gene expression).	Increased cilia beating frequency; alterations in the mucociliary clearance; no cytotoxicity or morphological changes; (pro)inflammatory and oxidative stress responses after chronic exposure.	Chortarea et al. (2017)
	CuO NP	ALI exposure (Vitrocell® system); Cells exposed for 2 days (two periods of 1 h/day); responses assessed 24 h after exposure.	Cytotoxicity (LDH release); Gene expression of inflammatory markers (MCP-1, IL-8, and IL-6).	No major cytotoxicity; increased expression of inflammation markers (MCP-1 and IL-6).	Kooter et al. (2017)
	CeO_2, Mn_2O_3, CuO, ZnO, Co_3O_4 and WO_3 NP	ALI (droplet) exposure (MucilAir cultures: 24 h; DC cultures: 48 h); responses assessed after 24 h (MucilAir) and 48 h (DC).	Cytotoxicity (LDH release); Inflammation (MCP-1, IL-6, IL-8); DC maturation surface markers (HLA-DR, CD80, CD83, and CD86).	MucilAir™ cultures: inflammatory responses (increased levels of IL-6, IL-8, and MCP-1) after CuO NP exposure; DC cultures: Mn_2O_3 NP upregulated all the assessed maturation biomarkers.	Dankers et al. (2018)
	Milled W NP	ALI exposure; single 24 h exposure; responses assessed following exposure and up to 28 days.	Cell viability (Trypan blue); Metabolic activity (resazurin assay); Inflammation (IL-8).	Decrease in barrier integrity; no effect on metabolic activity or cell viability; transient increase in IL-8 secretion at 24 and 96 h after initial exposure.	George et al. (2019)
	SiO_2 NP	ALI (droplet) exposure (Vitrocell® Cloud system); daily exposure for 5 times per week, up to 12 weeks.	Cell viability (Alamar Blue); Barrier integrity (TEER).	No changes in the barrier integrity; no cytotoxic effects.	Di Cristo et al. (2020)
	ATO and ZrO_2 NP; PGFP (<2.5 μm) and PGNP (0.200 μm) particle fractions derived from high velocity oxy-fuel spraying.	ALI (droplet) exposure (Vitrocell® Cloud system); responses assessed after 24, 48, and 72 h.	Cytotoxicity (LDH release and WST-1 metabolic activity); Genotoxicity (Comet assay); Inflammation (IL-8 and MCP-1).	Mild cytotoxicity at early time points (24 h), cellular recovery at late time points (72 h), and no major genotoxicity for ATO and ZrO_2 NP; PGFP affected cell viability, while PGNP caused increased oxidative DNA damage.	Bessa, Brandão, Fokkens, Cassee et al. (2021)
SmallAir™ (Epithelix Sàrs)	Ag NP	ALI exposure (Cultex® RFS system); 3 exposure times (7, 20, and 60 min); responses assessed at 6 or 24 h.	Cytotoxicity (lactate and LDH release); Alterations in immune-related gene expression (real-time PCR analysis for mRNA gene expression).	Minimal cytotoxicity and significant upregulation in the expression of inflammatory-related genes (*e.g.* IL1R2).	Guo et al. (2018)
EpiAirway™ (MatTek Corporation)	Airborne particulates collected in woodworking and metalworking industries	Submerged exposure; repeated exposures (4 h, 8 h and 72 h)	Cytotoxicity (MTT assay); Gene expression (GAPDH, Gelsolin, Caspase-3, IL-6)	Marked decrease in tissue viability after 8 h (woodworking particles) and a slight reduction after 72 h exposure (metalworking particles); suppressed expression of gelsolin, caspase-3 and IL-6	Pavlovska et al. (2021)
EpiAlveolar™ (MatTek Corporation)	MWCNT	ALI (droplet) exposure (Vitrocell® Cloud system); repeated exposure (3 weeks).	Cytotoxicity (LDH release); Inflammation (IL-1β, TNF-α and IL-8); Profibrotic response (TGF-β, fibronectin, and COL1 release).	Barrier integrity and release of pro-inflammatory and profibrotic markers.	Barosova et al. (2020)

ALI: Air-liquid interface; ALICE: Air-liquid interface cell exposure; ATO: Antimony-tin oxide; COL1: Collagen 1; DC: Dendritic cells; LDH: Lactate dehydrogenase; IL1R2: Interleukin 1 Receptor Type 2; MWCNT: Multiwalled carbon nanotubes; PCR: Polymerase chain reaction; PGFP: Process-generated fine particles; PGNP: Process-generated nanoparticles; RFS: Radial flow system; TEER: Transepithelial electrical resistance.

by elevated cilia beating frequency and alterations in mucociliary clearance. However, the magnitude and duration of the observed effects were higher in the asthmatic compared to healthy cells, suggesting that individuals with asthma may be more susceptible to adverse effects from chronic MWCNT exposure (Chortarea et al. 2017). Donor variability is an important aspect that needs to be considered when designing and interpreting data of primary culture-based studies. In this regard, Kooter et al. (2017) examined the toxicity of aerosolized CuO NP bronchial airway MucilAir™ cultures obtained from 4 donors. Despite no major cytotoxicity occurrence, an increase in levels of IL-6 and Monocyte Chemoattractant Protein (MCP)-1 release after 24 hr exposure was found. Dankers et al. (2018) also investigated the pro-inflammatory potential of 6 metal oxide NPs (CeO_2, Mn_2O_3, CuO, ZnO, Co_3O_4, and WO_3; 27–108 μg/mL) in MucilAir™ cultures and dendritic cells (DC). In MucilAir™ cultures, higher secretion of IL□6, IL□8, and MCP-1 pro-inflammatory cytokines was found after 24 hr incubation with CuO NP droplets, while only exposure for 48 hr to Mn_2O_3 NP upregulated all examined DC maturation biomarkers (HLA□DR, CD80, CD83, and CD86). Interestingly, Dankers et al. (2018) addressed the potential interaction between epithelial cells and DC by exposing the latter to MucilAir™-exposed cultures media, and found that only Mn_2O_3 NP triggered DC maturation, suggesting the process was not dependent upon epithelial cells stimulation (Dankers et al. 2018).

These models were employed to assess the toxicity of occupationally relevant NP. In this regard, George et al. (2019) evaluated the toxicity of thermonuclear fusion released-like milled tungsten (W) NP, tungstate (WO_4^{2-}) and tungsten carbide cobalt particles alloy (WC-Co), in MucilAir™ cultures treated for 24 hr with particle liquid suspensions, whose effects were monitored up to 28 days after exposure. These NP exerted a minor impact in MucilAir™ bronchial cultures since no induce significant alterations were noted in metabolic activity and viability; however, a decrease in barrier integrity and a transient rise of IL-8 levels at 24 and 96 hr was detected after initial exposure (George et al. 2019). Recently, Bessa et al. (2021) also assessed the toxicity of two engineered NP [ENP; antimony-tin oxide (ATO) and zirconium oxide (ZrO_2) NP] used as input materials in advanced ceramics, and two fractions [fine <2.5 μm (PGFP) and nano-sized <0.2 μm (PGNP)] of airborne particles released during thermal spraying of ceramic coatings onto metal surfaces, in MucilAir™ cells under ALI conditions. These cultures were exposed for three consecutive days to aerosolized particles, and cyto-, genotoxicity, and pro-inflammatory responses were assessed. The obtained results showed that PGFP and PGNP exhibited greater toxicity than ENP in mass per area unit, although the presence of mucociliary apparatus in the advanced 3D *in vitro* bronchial cultures seemed to markedly attenuate the adverse effects (Bessa et al. 2021). Pavlovska et al. (2021) determined the biological effects of airborne particulates released from woodworking and metalworking industrial processes in 3D EpiAirway™ cultures and A549 lung epithelial cells. Data demonstrated that exposure to these ambient pollutant particles exerted minor acute effects on the morphology and viability of A549 cells and EpiAirway™ tissues. However, a marked reduction in tissue viability after 8 hr exposure to woodworking particles, as well as a non-significant reduction after 72 hr incubation with metalworking particles were reported. In addition, suppressed expression of gelsolin (more pronounced for metalworking particles), caspase-3 and IL-6 (metalworking particles) was found (Pavlovska et al. 2021).

Few studies exist on the effects of (nano)particle exposure in human small airway models. Based upon previous *in vivo* findings that showed Ag NP deposition in the small airway epithelium following inhalation (Seiffert et al. 2016), Guo et al. (2018) investigated the effects of the same Ag NP in organotypic reconstituted 3D primary human small airway epithelial cell cultures (SmallAir™) under ALI conditions using the same *in vivo* doses. Data obtained showed DNA damage, cell cycle changes, and oxidative stress in response to exposure to the aerosolized Ag NP (up to 13.2 ng/cm^2). A significant correlation between *in vivo–in vitro* transcriptional changes in immune-related genes, that were of similar magnitude in response to Ag in the ionic (Ag+) or in the nanoform was detected (Guo et al. 2018). More recently, Barosova et al. (2020) evaluated the effects of repeated exposure over 3 weeks to occupational doses (1–30 μg/cm^2)

of two MWCNT- Mitsui-7 and Nanocyl- aerosols, in EpiAlveolar™ cultures constituted by human alveolar epithelial cells, pulmonary endothelial cells and fibroblasts. In parallel, EpiAlveolar™ cultures were cocultured with human monocyte-derived macrophages (MDM) to determine their potential role in the pro-inflammatory and profibrotic responses. Barosova et al. (2020) found that Nanocyl induced less pronounced toxicity than Mitsui-7 in EpiAlveolar™ cultures, whereas EpiAlveolar™ + MDM cocultures exhibited pro-inflammatory responses at later time points compared to EpiAlveolar™ cultures.

Lung spheroids/organoids

Spheroids and organoids are 3D structures composed of multiple cells that cluster together into self-organized aggregates. Both terms are often used interchangeably in the literature, though some differences exist between the two. Spheroids are simple spherical and scaffold-free cellular models that are typically obtained from mature single-cell suspensions (Zanoni et al. 2020). On the other hand, organoids are complex clusters derived from organ-specific cells that self-assemble within a scaffold such as gels made of a complex mixture of different extracellular matrix (ECM) proteins, including laminin, fibronectin, collagen, and heparin sulfate (*e.g.*, Matrigel), though not all organoids are formed within an ECM (Gkatzis et al. 2018). Organoids may be generated from adult or embryonic stem cells (Hofer and Lutolf 2021). One curious aspect of these particular 3D structures is that they can be maintained for prolonged periods of time without karyotype changes (Kar et al. 2021). In the respiratory field, organoids are valuable models to mimic the complex environment of the respiratory mucosa and the relationship between different cell types. Indeed, human lung organoids have been successfully established from cells of different origins such as epithelial progenitor cells derived from embryonic (Miller et al. 2018) or adult lung (Tan et al. 2017; Zacharias et al. 2018), and from human pluripotent stem cells (hPSC) (Chen et al. 2017; Gotoh et al. 2014; Yamamoto et al. 2017). Previously, establishment of alveolar organoids from cocultures of epithelial progenitor cells with mesenchymal or endothelial cells or from cocultures of mesenchymal cells with fetal lung tissue or hPSC were reported (Wilkinson et al. 2016).

While lung spheroids/organoids may be reliable models to investigate pulmonary injury and repair mechanisms, their 3D spherical nature and matrix components pose technical challenges for application under ALI conditions (Hiemstra et al. 2018). Another limitation is that lung movements during gas exchange are difficult to simulate in lung spheroid/organoid models (Li et al. 2020). However, human lung spheroids or organoids have still been used in the fields of regenerative medicine, lung disease modeling, and drug efficacy testing (Archer, Bobet-Erny, and Gomes 2021; Cores et al. 2020; Tan et al. 2017).

Organotypic lung cultures are also valuable models for toxicological studies, namely for nanotoxicity assessment (Prasad et al. 2021). However, few studies have been conducted for NP testing in human-derived lung spheroids/organoids. In this regard, Sambale et al. (2015) comparatively investigated the effects of ZnO and TiO_2 NP using human alveolar epithelial A549 cells grown in traditional 2D monolayer cultures or in 3D spheroids. Overall, A549 cells displayed lower resistance to ZnO NP-induced toxicity (up to 100 µg/ml) in the form of spheroids compared to 2D monolayers, while exposure to TiO_2 NP (up to 150 µg/ml) was nontoxic to 2D cultures but produced a decrease in A549 spheroids viability and affected spheroid formation with several smaller spheroids being formed instead of a single larger spheroid (Sambale et al. 2015). Kabadi et al. (2019) investigated the influence of different types of carbon-based materials (MWCNT, M120 carbon black NP, or crocidolite asbestos fibers; 0.5–10 µg/ml) in scaffold-free 3D spheroids established from human lung IMR-90 fibroblasts, bronchial epithelial BEAS-2B and monocytic THP-1 cells. MWCNT were the most cytotoxic to the spheroid-like triple cocultures by day 7, producing more than 40% decrease in cell viability at the highest tested concentration. Further, gene expression analysis carried out following 4 or 7 day exposure to tested materials revealed significant upregulation of ECM components (collagens and decorin), cytokines IL-1β and IL-6, growth factors, and matrix metalloproteases (MMP) related genes (Kabadi et al. 2019). Recently,

Liu et al. (2021) assessed the toxicity of nano-carbon black and nano-SiO$_2$ utilizing alveolar type 2 epithelial cell-like cells (ATL), differentiated from hPSC cells, grown in 2D monolayers or in 3D organoids assembled in Matrigel scaffolds. The toxicity of the nano-carbon black was determined in ATL 2D monolayers, while nano-SiO$_2$ effects were assessed in ATL 3D organoids. Nano-carbon black (1–100 ng/ml) did not induce oxidative stress in ATL 2D cultures or alter cell viability after 6 hr or 7 day treatment, respectively. As for nano-SiO$_2$ (1–100 ng/ml), a significant elevation in ROS generation was observed in ATL 3D organoids at 6 hr after exposure, although organoid viability and gene expression of the investigated markers at 14 days after exposure were not markedly affected (Liu et al. 2021).

Lung-on-a-chip

Mechanically active microdevices, named organ-on-a-chip, combining the capability of cell culture models with microfluidics were developed to (1) reconstitute tissue-tissue interfaces crucial to resemble organ functions, and (2) are gaining relevance for drug screening and toxicology applications. The ideal lung-on-a-chip needs to encompass a micro engineered cell culture device capable of replicating the human lung 3D architecture, environment, breathing movement, and physiology. The first lung-on-a-chip was reported by Huh et al. (2010), who developed a biomimetic microsystem replicating key structural, functional, and mechanical properties of the human alveolar-capillary interface. This model consisted of a microfluidic system with two microchannels separated by a thin, flexible and porous membrane coated with fibronectin or collagen to resemble the ECM, and human alveolar epithelial and pulmonary microvascular endothelial cells cultured on opposite sides of the membrane (Figure 3e). This biologically inspired human breathing lung-on-a-chip microdevice has been successfully used to evaluate SiO$_2$ NP transport across this *in vitro* alveolar-capillary barrier, intracellular ROS production, and inflammatory responses (Huh et al. 2010). To simulate *in vivo* environment of the alveolar space and gas exchange conditions, epithelial cells were exposed at the ALI. After exposure to SiO$_2$ NP aerosols it

was observed that : (1) high levels of intercellular adhesion molecule (ICAM)-1 expression from the underlying endothelium cells; (2) increased endothelial capture of circulating neutrophils, promotion of their migration across the permeable membrane (tissue-tissue interface), and accumulation onto epithelial surface; (3) simulated physiological "breathing" by mechanical forces; (4) enhanced absorption of SiO$_2$ NP from the airspace to the microvascular channel; (5) accentuated pro-inflammatory activities and development of acute lung inflammation; and (6) a steady rise in ROS production (Huh 2015; Huh et al. 2010).

Many other lung-on-a-chip devices emerged, as reported by Punde et al. (2015), Stucki et al. (2015), Blume et al. (2015), Rahimi et al. (2016), Yang et al. (2018), among other research groups. However, nanotoxicity studies using lung-on-a-chip models are still scarce. Zhang et al. (2018) assembled a lung-on-a-chip model, reproducing the alveolar-capillary barrier, using three parallel channels: (1) an alveolar channel with human pulmonary alveolar epithelial cells (HPAEpiC) to embody the alveolar side of the alveolar-capillary barrier; (2) a vessel channel with human endothelial cells (HUVEC) to represent the capillaries, and (3) an ECM channel with Matrigel membrane sandwiching between the alveolar and vessel channels. In this study, Zhang et al. (2018) exposed epithelial cells for 24 h to TiO$_2$ NP and zinc oxide (ZnO) NP that were injected into the alveolar channel. Both NP induced concentration-dependent toxicity on the epithelial and endothelial cells, including elevated ROS generation and apoptosis; however, ZnO NP were more toxic than TiO$_2$ NP. Meghani et al. (2020) designed an alveolus-epithelium-on-a-chip device with in-built sensors for enabling monitorization of pH-responsive ZnO quantum dots (QD)-loaded human serum albumin (HSA) NP. This model comprised lung cancer cells and stromal cells such as fibroblasts along with a collagen ECM. Results showed a significant internalization of the NP under the coculture conditions (Meghani et al. 2020).

Exposure conditions: submerged vs air-liquid interface

Most *in vitro* studies for assessing pulmonary toxicity attributed to (nano)particles were performed

using thin (mono)layers of lung cells cultured under submerged conditions, where (nano)particles to be tested were added directly into the cell culture media (Lacroix et al. 2018). NP dispersion in complex media such as cell culture medium is likely to change their original physicochemical properties (Kendall et al. 2015; Moore et al. 2015, 2019), which may influence particle-particle and particle-cell interactions, thus markedly affecting lung cell responses. Indeed, the interaction of particles with cell culture media components often leads to the formation of a protein corona (Monopoli et al. 2011), which together with media salts and osmolarity may affect particle stability and make particles more prone to aggregation and/or agglomeration (Falahati et al. 2019; Teeguarden et al. 2007). Another major limitation of submerged cultures is that molecular and cellular features such as expression of key transporters and proteins, cilia formation, mucus, and surfactant secretion may be affected (Acosta et al. 2016). At the same time, dose deposition is hard to control as, depending upon their density, particles might remain in suspension and/or interact with material such as plastics where cells were seeded, which may exert an impact on the actual dose that cells are exposed to (Lenz et al. 2013). For all the aforementioned, it is widely accepted that *in vitro* cell exposure to (nano)particles under the classic submerged conditions does not accurately and effectively mimic cellular and physiological features observed in inhalation exposures *in vivo*. Consequently, the responses detected might also differ from the ones occurring in *in vivo* situation (Blank, Gehr, and Rothen-Rutishauser 2009; Lacroix et al. 2018). To overcome the major drawbacks associated with submerged cultures, innovative approaches have emerged over the past years to more accurately control dosimetry and deposition (Polk et al. 2016; Secondo, Liu, and Lewinski 2017).

Another important issue is the choice of dose metrics. While for non-nano substances dose is normally expressed on the basis of mass, for nanoscale materials the most suitable metrics may be mass, number of particles or surface area, which is yet to be defined since many physicochemical factors including NP surface, shape and size may contribute to their toxicity (Schmid and Cassee 2017). In addition, selection of the proper dose range to test is not an easy task. *In vitro* experiments frequently use significantly higher doses compared to *in vivo* inhalation studies, making difficult to compare *in vitro* vs. *in vivo* responses. Particokinetic models, such as the *In Vitro* Sedimentation, Diffusion and Dosimetry (ISDD) (Hinderliter et al. 2010) or *In Vitro* Sedimentation, Diffusion, Dissolution, and Dosimetry (ISD3) (Thomas et al. 2018) models, describing the delivery of (nano) particles to cells in liquid cell culture systems were useful for dose-response analysis and translation of exposures across *in vivo* and *in vitro* systems. At the same time, computational tools including the Multiple Path Particle Dosimetry (MPPD) (Anjilvel and Asgharian 1995) and the human respiratory tract model developed by the International Commission on Radiological Protection (ICRP) (Smith et al. 2014) were employed to calculate deposition and clearance of particles along the respiratory tract. Not only is the dose important, but also duration and frequency of exposure, with most of the available investigations focusing on the effects of acute exposure, while little is known regarding the long-term implications of chronic exposure to (nano)particles (Bui et al. 2020; Kolanjivil and Kleinstreuer 2013).

The human airways are not fully immersed in pulmonary fluid, but instead, are covered by surfactant layer and air that creates the ALI, which enables an efficient gas exchange between cells and the environment. Human respiratory system cells such as nasal or bronchial epithelial cells cultured under ALI conditions become effectively polarized and might secrete surfactant, improving the resemblance to the *in vivo* situation, which is not possible to achieve in fully immersed environments (Barosova et al. 2020). *In vitro* exposure systems are capable of delivering aerosolized (nano)particles directly to the surface of cells cultured under ALI conditions which constitutes a more reliable alternative for conducting pulmonary nanotoxicity studies. Table 6 depicts the existing aerosol exposure systems available for (nano) particle aerosol generation and cell exposure at ALI. The normal setup generally involves an aerosol generator from powders/dusts or liquid droplets, connections, and peripherals to an exposure chamber with controlled temperature and humidity conditions. Compared to submerged exposures,

Table 6. Aerosol generation and exposure systems for cells cultured under air-liquid interface (ALI) conditions.

Aerosol exposure system	Developer/ Manufacturer	Features
Minucell	Tippe, Heinzmann, and Roth (2002)	• Uses direct flow allowing a greater particle deposition onto cells.
Electrostatic Aerosol *in vitro* Exposure system (EAVES)	de Bruijne et al. (2009)	• Uses electrostatic precipitation for particle deposition onto cells.
Air-Liquid Interface Cell Exposure (ALICE)	Lenz et al. (2009)	• Uses dense cloud of droplets generated and transported through an exposure chamber at a specific flow rate, allowing uniform and efficient depositions of particle aerosols; • Considered an optimal system to screen NP' toxicity, particularly in pharmaceutical industries where suspension-based aerosolized NP are used in drug formulations (Duret et al. 2012).
NAVETTA	Frijns et al. (2017)	• Applies an electrostatic field to improve particle deposition efficiency, allowing a much higher deposition rate when compared to other ALI setups.
Nano Aerosol Chamber *In vitro* Toxicity (NACIVT)	Jeannet et al. (2015)	• Uses direct flow in a continuous air stream, enabling a more efficient and uniform deposition of airborne (nano)particles onto cells; • Computer-controlled temperature and humidity environment, which allows long-term exposures and helps to prevent cellular stress during exposure; • Compact and easy to transport; • High throughput system; • Commercially available.
MicroSprayer® Aerosolizer	Penn (2021)	• Commercially available manual aerosolizer; • Model IA-1C: requires a very small number of NP to reach the target dose; • Leads to the formation of droplets, affecting the homogeneous deposition of particles onto cells; • Requires high flow rates for effective particle deposition, which might damage cells by shear stress.
CULTEX®	Aufderheide and Mohr (1999)	• Uses electrostatic precipitation for NP deposition onto cells, ensuring close contact between the tested aerosol and cells without any interference of the culture medium; • Available as Radial Flow System (RFS) and RFS Compact versions; • Popular system among the commercially available.
Vitrocell®	Aufderheide and Mohr (2004)	• Modified CULTEX® system; • Three available setups: Cloud® system, powder chamber, and automated exposure station; • Offers exposure chambers for 6, 12, and 24 well-plates; • Most used among the commercially available.
XposeALI®	Inhalation Sciences (2021)	• Models adapted to aerosolize dry powders; • Commercially available.

particle aerosolization and exposure at ALI, where the apical liquid in cultured cells is negligible, seem to have a diminished impact on the particle original properties (Fujitani et al. 2015; Polk et al. 2016). Some of the available systems are equipped with a quartz crystal microbalance (QCM) for monitoring dose deposition in the ng range, which is a great advantage for controlling deposition over time (Ding et al. 2020). Despite their numerous advantages, one needs to take into account existing technical limitations and constraints of the ALI exposure systems. However, only a few commercially available exposure systems allow exposure of cultured cells to aerosolized particles with precise dosimetry (Upadhyay and Palmberg 2018). In systems where particle deposition is based upon sedimentation and gravitation, the maximum achieved deposited particle mass is generally low (Bierkandt et al. 2018). Particle-specific efficacy of deposition and deposition rate of these aerosol generator devices display considerable variations (*e.g.*, XposeALI®). In addition, a large loading of particles and/or long-term exposure may also be

problematic, as particle physicochemical characteristics (*e.g.*, ALICE system) might be affected and particle agglomeration and/or aggregation may occur (Upadhyay and Palmberg 2018). Most of the available systems only allow short, single-exposure experiments through a nano aerosol deposition chamber for efficient and quantitative deposition of NP, whereas the nano aerosol chamber *in vitro* toxicity (NACIVT) system allows for a continuous air-stream (Clippinger et al. 2016). Further, these exposure systems require specialized personnel with qualified expertise to set up and operate, being considerably more expensive and time-consuming than the traditional submerged studies (Braakhuis et al. 2015).Table 6.

Several studies have previously addressed (nano) particles toxicity in human respiratory cells under submerged *vs* ALI conditions. The vast majority of investigations support the view that ALI cultures seem to be more resistant than submerged cultures to the effects induced by (nano)particles *in vitro* exposure. In this regard, Lenz et al. (2013) compared cellular responses to ZnO NP (0.7 and 2.5 µg/

cm^2; immediately and 2 hr after exposure) in terms of oxidative stress [(heme oxygenase 1 (HMOX1), SOD-2 and glutamate-cysteine synthetase, catalytic subunit (GCS) transcription markers expression] and pro-inflammatory (IL-8, IL-6, and GM-CSF levels) responses in human alveolar epithelial A549 cells cultured under ALI or submerged conditions. Overall, a similar response to ZnO NP was observed in A549 cells exposed under both conditions. However, while no significant changes in oxidative stress were found for most markers in both cultures, lower levels of pro-inflammatory responses were detected in A549 cells exposed at ALI than submerged (Lenz et al. 2013). Ghio et al. (2013) also compared the influence of ambient air pollution particles, collected in North Carolina outside the United States Environmental Protection Agency (EPA) Human Studies Facility, under submerged and ALI conditions on normal human bronchial epithelial (NHBE) cells by assessing pro-inflammatory (IL-8 and IL-6 levels) and oxidative stress (HOX1 and COX2 expression) markers up to 21 days. Ghio et al. (2013) demonstrated that in NHBE cells exposed at ALI, the tested particles induced reduced biological effects compared to submerged cultures, likely due to higher oxygen availability in ALI cultures.

The comparison of the biological responses of A549 cells under ALI (using a Vitrocell® system equipped with electrodes to enhance deposition by applying an electrostatic field, named ALIDA) or submerged conditions was assessed after exposure to two amorphous SiO$_2$ NP produced by different synthesis methods (Aerosil200 produced by flame synthesis, and 50 nm SiO$_2$ NP produced by the Stöber method). For ALI conditions, the attained deposited doses were 52 μg/cm^2 and 117 μg/cm^2, for Aerosil200 and 50 nm SiO$_2$ NP, respectively, whereas in submerged conditions both NP were tested as liquid suspensions of 15.6 μg/cm^2. The amorphous SiO$_2$ NP aerosols were generated by two different methods: electrospray and atomizer. The electrospray method was only applicable for 50 nm SiO$_2$ NP since it enabled generation of an aerosol containing monodisperse NP, whereas the Aerosil200 suspensions contained excessively large aggregates which disabled a stable operation. However, the deposited mass and surface dose of the 50 nm SiO$_2$ NP was too low to induce cellular

responses. On the other hand, the atomizer was applicable for both types of amorphous SiO$_2$ particles; nevertheless, deposition of particle aggregates was observed, and therefore higher mass and surface doses were attained, which led to induction of significant biological effects on lung cells. Overall, both types of amorphous SiO$_2$ NP induced similar cellular responses in both culture systems, although submerged exposure to 50 nm SiO$_2$ NP triggered greater responses at much lower doses (Panas et al. 2014).

ALI exposure seems to be a particularly suitable approach when evaluating the toxicity of poorly soluble NP. In this regard, Loret et al. (2016) examined the biological effects of 4 poorly soluble NP- one CeO$_2$ NP and three TiO$_2$ NP-, in A549 alveolar cell monocultures or in coculture with alveolar macrophages (THP-1) using different exposure methods, where the cultures were exposed for 24 hr to aerosolized NP in inserts or to NP liquid suspensions either in inserts or plates. Loret et al. (2016) found that the total deposited doses were reached within 3 hr in inserts, either under ALI or submerged conditions, and within 24 hr in plates. Overall, cocultures were more sensitive than monocultures. It is noteworthy that decreased cell viability, oxidative stress and inflammatory responses were observed at lower doses in cultures exposed under ALI compared to the conventional submerged conditions (Loret et al. 2016).

Recently, Medina-Reyes et al. (2020) compared the cytotoxicity, genotoxicity and oxidative stress in A549 cells exposed to TiO$_2$ nanofibers and NP under ALI (using a Vitrocell® Cloud system; 2 and 10 μg/cm^2 for 1 or 4 hr and maintained in culture for 24, 48 or 72 hr) and submerged conditions (1–50 μg/cm^2 for 24, 48 or 72 hr). Overall, Medina-Reyes et al. (2020) noted that cytotoxicity of TiO$_2$ nanofibers and NP was similar in ALI and submerged cultures, although uptake was higher in submerged than in ALI cultures. However, TiO$_2$ nanofibers induced higher DNA double-strand breaks (DSB) in A549 cultured under ALI than in submerged conditions, while TiO$_2$ NP induced similar levels of DSB in both culture conditions (Medina-Reyes et al. 2020). Diabaté et al. (2021) determined the *in vitro* toxicity of CeO$_2$ and TiO$_2$ NP in A549 monocultures cultured under ALI conditions *vs* cocultures of A549 with THP-1

macrophages under submerged conditions. Although not the same cell culture model, data demonstrated that cells cultured under ALI conditions were more sensitive to NP-induced toxicity compared to those cultured under submerged conditions, i.e., lower doses of deposited NP (0.2 and 1 $\mu g/cm^2$) were sufficient to induce adverse outcomes at ALI, as also documented in rodent experiments (Diabaté et al. 2021). Similar results were observed by Bessa, Brandão, Fokkens, Cassee et al. (2021), where more pronounced cytotoxicity in A549 cells exposed to aerosolized ATO, CeO_2, and ZrO_2 NP using a VitroCell® workstation system up to 4 hr exposure compared to A549 submerged cells exposed 24 hr to the same NP as a liquid suspension. Overall, A549 cells under ALI conditions were more susceptible to NP aerosols exposure. Although an increased primary DNA damage regardless of the exposure mode was observed, A549 cells seemed to be more sensitive to the genotoxic effects of ZrO_2 NP aerosols than to the same NP in a liquid medium (Bessa, Brandão, Fokkens, Leseman et al. 2021).

Conclusions and future perspectives

Over the years, many *in vitro* studies have been carried out to investigate the potential adverse risks for human health of (nano)particle inhalation. *In vitro* systems offer highly controlled cell culture environments that may be easily scaled and replicated, enabling identification of the molecular mechanisms associated with (nano)particle exposure responses that are more difficult to evaluate using *in vivo* models, while complying with the 3Rs principles. Despite considerable progress on advanced *in vitro* cell and tissue culture technologies that has been made thus far, many aspects are still challenging, need to be improved and more comprehensively explored. For instance, it would be interesting to use these systems to address the translocation of (nano)particles from lung to the lymph nodes and bloodstream. In addition, exploring particle localization at their sites of interaction with living structures would be of relevance. Here, the application of imaging techniques such as Raman microspectroscopy and X-ray microscopy

may be feasible, particularly for simultaneous visualization of (nano)particles within the airways and the determination of cell and tissue changes that occur. For example, these novel techniques would be valuable for a more comprehensive understanding of (nano)particles diminished phagocytosis by alveolar macrophages and its impact on lung toxicity.

Most *in vitro* research on the effects of (nano)particles in the human respiratory system are focused on acute exposures, but information on the repeated and long term (chronic) effects of relevance in occupational settings is still scarce. However, assessment of such effects *in vitro* is currently still extremely challenging. Therefore, future studies need to address this major research gap that may have a significant impact in the regulatory field, for establishing appropriate legislation and occupational exposure limits (OEL) for inhaled (nano)particles.

A common criticism of *in vitro* models is that they are usually more sensitive than *in vivo* models to (nano)particle exposure. Contributing to this fact are the high concentrations tested *in vitro* compared to the ones used *in vivo*, making *in vitro* vs. *in vivo* responses hard to compare. Advancement of knowledge on particulate environmental and occupational exposure levels that humans are exposed to is crucial for improving experimental design of *in vitro* (and *in vivo*) inhalation toxicity studies. Here, mathematical approaches for predicting particle deposition across the respiratory tract and for estimating *in vitro* concentrations to be tested from *in vivo* doses are also valuable. At the same time, it would also be important to identify which is the best metric for expressing (nano)particle dose.

Although the currently available respiratory *in vitro* systems fail to replicate the entire hierarchical and complex architecture and biomechanics of the human lung, these models provide useful information depending upon the way the study hypothesis is posed. The latest advancements on the use of human pluripotent stem cells in 3D models of lung development and disease, the use of computational methodologies in respiratory cell models and exposure systems, or development of lung-on-a-chip devices that simulates airflow and breathing

patterns, are promising and certainly will assist scientists in the future to answer questions in the field of (nano)particle inhalation toxicology.

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No potential conflict of interest was reported by the authors.

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Data availability statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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