TECHNICAL REPORT

# ISO/TR 21624

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# Nanotechnologies — Considerations for in vitro studies of airborne nano-objects and their aggregates and agglomerates (NOAA)

*Nanotechnologies — Considérations pour les études in vitro des nano-objets en suspension dans l'air et de leurs agrégats et agglomérats (NOAA)*

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# Foreword

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This document was prepared by Technical Committee ISO/TC 229, *Nanotechnologies*.

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# Introduction

Inhalation is one of the prominent routes by which humans can come in contact with natural, unintended and engineered nano-objects and their aggregates and agglomerates (NOAA). Due to the physiological, biochemical and anatomical differences between humans and animals, as well as the considerable time, cost and animal numbers required to conduct in vivo toxicity tests, there is much interest in developing in vitro strategies for risk assessment that are based on human cells and mechanisms of toxicity. To enable comparability of the results of in vitro assay and in vivo effects observed after inhalation of NOAA, certain parameters should be considered, including:

a) the choice of cell types;

b) characterization of the NOAA throughout the assay, including life-cycle transformations;

c) the choice of nano-object concentration relevant to human exposures;

d) generation of NOAA form that mimics human exposures;

e) the use of relevant dispersants;

f) the use of appropriate mode of exposure (submerged or air-liquid interface) and exposure duration[1].

This document includes descriptions of the aforementioned parameters with regard to using in vitro-based strategies for assessing specific aspects related to the inhalation toxicity of NOAA. For example, for inhalation studies, it is critical to choose the proper equipment for generation, exposure to, and characterization of the nano-objects. This document includes information about available in vitro aerosol exposure chambers and biological models that have been used to assess the inhalation toxicity of NOAA. This document does not include details regarding the techniques for aerosol generation or characterization of specific nanomaterials (NMs), their life cycle transformations or in vivo testing. An overview of the aerosol generation of NMs and in vivo testing is given in ISO/TR 19601[2].

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# Nanotechnologies — Considerations for in vitro studies of airborne nano-objects and their aggregates and agglomerates (NOAA)

## 1 Scope

This document collates information regarding the systems available for exposure and assessment of nano-objects and their aggregates and agglomerates (NOAA) for in vitro air exposure studies. It provides an overview of the various exposure systems and in vitro cell systems used to perform in vitro studies that simulate an inhalation toxicology study design.

## 2 Normative references

There are no normative references in this document.

## 3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

**3.1**
**aerosol**
system of solid or liquid particles suspended in gas

[SOURCE: ISO 15900:2009, 2.1]

**3.2**
**agglomerate**
collection of weakly bound particles or *aggregates* (3.3) or mixtures of the two where the resulting external surface area is similar to the sum of the surface areas of the individual components

Note 1 to entry: The forces holding an agglomerate together are weak forces, for example van der Waals forces, or simple physical entanglement.

Note 2 to entry: Agglomerates are also termed secondary particles and the original source particles are termed primary particles.

[SOURCE: ISO/TS 80004-4:2011, 2.8]

**3.3**
**aggregate**
particle comprising strongly bonded or fused particles where the resulting external surface area may be significantly smaller than the sum of calculated surface areas of the individual components

Note 1 to entry: The forces holding an aggregate together are strong forces, for example covalent bonds, or those resulting from sintering or complex physical entanglement.

Note 2 to entry: Aggregates are also termed secondary particles and the original source particles are termed primary particles.

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[SOURCE: ISO/TS 80004-4:2011, 2.7]

**3.4**
**engineered nanomaterial**
nanomaterial designed for specific purpose or function

[SOURCE: ISO/TS 80004-1:2015, 2.8]

**3.5**
**incidental nanomaterial**
nanomaterial generated as an unintentional by-product of a process

Note 1 to entry: The process includes manufacturing, bio-technological or other processes.

Note 2 to entry: See "ultrafine particle" in ISO/TR 27628:2007, 2.21.

[SOURCE: ISO/TS 80004-1:2015, 2.10]

**3.6**
**manufactured nanomaterial**
nanomaterial intentionally produced to have specific properties or composition

[SOURCE: ISO/TS 80004-1:2015, 2.9, modified — "specific" has replaced "selected".]

**3.7**
**nanoparticle**
nano-object with all external dimensions in the nanoscale where the lengths of the longest and the shortest axes of the nano-object do not differ significantly

Note 1 to entry: If the dimensions differ significantly (typically by more than 3 times), terms such as nanofibre or nanoplate may be preferred to the term nanoparticle.

[SOURCE: ISO/TS 80004-2:2015, 4.4]

**3.8**
**particle size distribution**
distribution of particles as a function of particle size

Note 1 to entry: Particle size distribution may be expressed as cumulative distribution or a distribution density.

[SOURCE: ISO/TS 80004-6:2013, 3.1.2, modified — "(distribution of the fraction of material in a size class, divided by the width of that class)" has been deleted from the Note 1 to entry.]

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## 4 Abbreviated terms

| | |
|---|---|
| Ag NPs | silver nanoparticles |
| ALI | air-liquid interface |
| AOP | adverse outcome pathway |
| Au NPs | gold nanoparticles |
| CCSP | clara cell secretary protein |
| CD | cluster of differentiation |
| CFTR | cystic fibrosis transmembrane conductance regulator |
| CNT | carbon nanotube |


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| | |
|---|---|
| $CO_2$ | carbon dioxide |
| ENM | engineered nanomaterial |
| IATA | integrated approach to testing an assessment |
| ICAM-1 | intercellular adhesion molecule 1 |
| IL | interleukin |
| ISDD | in vitro sedimentation, diffusion and dosimetry |
| ISD3 | in vitro sedimentation, diffusion, dissolution and dosimetry |
| KE | key event |
| MIE | molecular initiating event |
| MPPD | multiple-path particle dosimetry |
| MT | metallothionein |
| MUC 1 | mucin 1 |
| MWCNT | multi-walled carbon nanotubes |
| NM | nanomaterial |
| NOAA | nano-objects and their aggregates and agglomerates |
| OECD | Organisation for Economic Co-operation and Development |
| QCM | quartz crystal microbalance |
| ROS | reactive oxygen species |
| $SiO_2$ | silicon dioxide |
| SP-A | surfactant protein A |
| SP-D | surfactant protein D |
| TEER | transepithelial electrical resistance |
| $TiO_2$ | titanium dioxide |
| VCAM-1 | vascular cell adhesion molecule 1 |

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# 5 Considerations for in vitro systems for assessing inhalation exposure to NOAA

## 5.1 Background

Nano-objects can be incidental or are manufactured from a variety of materials (e.g. metals, polymers, metal oxides) and come in many different morphologies and combinations. Testing the toxicity of inhaled NOAA using in vitro systems involves considerations of several parameters, including the appropriate mode of exposure (see 5.2), characterization of test material (see 5.3) and choice of cell types (see 5.4).

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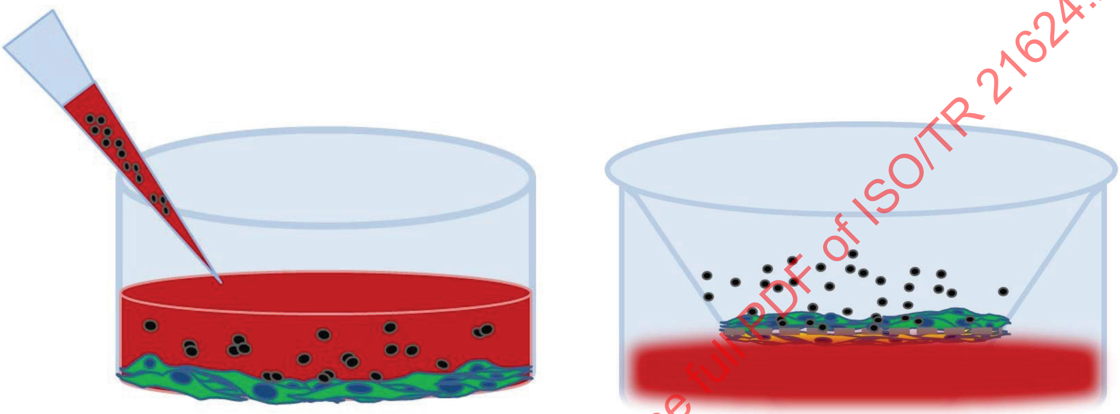
## 5.2 Modes of exposure

### 5.2.1 General

Both direct and indirect methods have been used to assess the aerosolized nano-objects using in vitro systems. Direct methods involve exposing the test aerosol, generated using aerosol generators, to the cells directly under submerged, intermittently submerged (e.g. on a rocking platform) or air-liquid interface (ALI) conditions[3][4][5]. Figure 1 presents the diagrammatic representation of exposure to NOAA under submerged and ALI conditions. Indirect methods often involve collection of aerosols (e.g. road-side ambient particles or nano-objects) using special apparatus (e.g. wetted rotating vane impactors or liquid impingers) or on a filter-like substrate followed by suspension of the collected sample in a culture medium before exposing the cells[3][6][7][8][9].



a) Submerged culture

b) Air-liquid interface culture

**Figure 1 — Diagrammatic representation of submerged and air-liquid interface (ALI) cultures**

The indirect method allows for the identification of the potential toxicity of air borne particles. However, the steps needed to prepare the suspensions of collected test material (when using indirect methods) and the interaction of the test material with the medium in submerged systems (when using direct methods) can cause changes to the properties of the test material leading to false assessments. This is especially applicable to nano-objects due to the fact that both their agglomeration state and movements are impacted by the use of a suspending medium, with their movement being controlled by Brownian diffusion or sedimentation based on their physico-chemical parameters such as size and density[10][11]. As a result, the probability of agglomerates settling down onto the cell culture is higher than for single particles. Additionally, dissolution kinetics of nano-objects under submerged conditions might differ considerably from what the particles undergo in in vivo conditions. Since the size, form, and solubility are the main parameters that determine nano-object toxicity, the toxicity outcomes observed in the submerged systems might not capture what happens in the lung after nano-object exposure. Another limitation of submerged systems is that the phenotype of the cells is usually different than that found under in vivo conditions as seen by the lack of formation of tight junctions and mucus in epithelial cells, an issue which is particularly important for inhalation models[12][13].

To overcome the limitations associated with the submerged systems, ALI systems are used and are preferred for inhalation toxicity testing because of their physiological relevance[14][15][16][18]. An ALI system constitutes cells cultured on a semi-permeable membrane where their basal surface is exposed to the culture medium and the apical surface is exposed to air. Such configuration is considered to be physiologically relevant as it has been shown to drive differentiation of the cells that mimics their in vivo phenotype[12][13][19]. In addition to having the cells in an "in vivo-like" configuration/phenotype, ALI systems allow a relatively direct deposition of NOAA on the cells that more closely mimics the particle deposition that results following inhalation, which might be used to derive a concentration-response relationship[20]. It should be noted that, similar to liquid conditions, the generation of an

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aerosol can also have an effect on particle characteristics. Thus, during and after aerosol generation, proper characterization of the particles should be performed.

Figure 1 shows exposure of NOAA to cells under submerged conditions and at the ALI. Under submerged conditions, the NOAA have to span the depth of the medium to reach the cells. At ALI conditions, the NOAA settle directly on the cells due to minimal fluid layer over the cells. Several studies have compared submerged and ALI systems exposed to NOAA and have reported differences in cellular responses[15]. For example, a higher expression of interleukin 6 (IL-6), IL-8, and hemeoxygenase-1 was observed in cells exposed to NOAA at ALI as compared to submerged cultures[20][21][22][23]. A few studies have observed contradicting outcomes for the aforementioned biomarkers[24][25][26]. Despite the differences between the two modes of exposure, both the systems are still used to assess the toxicity of NOAA. Submerged cell cultures can be used for identifying the toxic potency of air borne (e.g. environmental or exhaust) particles. However, for evaluation of engineered and manufactured nano-objects, especially when considering occupational exposure, the ALI culture system more appropriately represents factors dealing with lung exposure.

### 5.2.2 Considerations for ALI exposure systems

#### 5.2.2.1 General

Treating the cell systems at ALI requires exposure systems that can deliver the material to be tested as an aerosol to the cell system in a form that is relevant to human exposure scenarios. The exposure systems used to test NOAA could either be “closed-box” or “flow-through” type and typically involve two basic components: an aerosol generator that generates the test atmosphere and the exposure chamber that houses the cell system. Flow-through systems (as shown in Figure 2) also typically consist of connectors and peripherals that transport, dilute, characterize and condition the aerosol before delivering it to the cells inside the chamber, and an exhaust, which could be used for test atmosphere sampling.

Figure 2 depicts a basic diagrammatic configuration of a system including an aerosol generator and an exposure chamber for exposing cells to aerosolized (or nebulized) substances at the ALI. The materials are aerosolized (or nebulized) using an aerosol generator (or nebulizer), which is connected to the exposure chamber. Deposited concentration of NOAA can be determined by incorporating quartz crystal microbalance (QCM) and/or electron microscopy (EM) grids in the wells without cells (A). The cells are cultured on membrane inserts and exposed to dry aerosols or nebulized material at the ALI (B). Cells treated with air only can be used as a negative control (C). Sampling ports can be included at several check points to obtain aerosol or medium samples. The various components of the exposure system and their applicability to the assessment of NOAA are described in 5.2.2.2 to 5.2.2.3.

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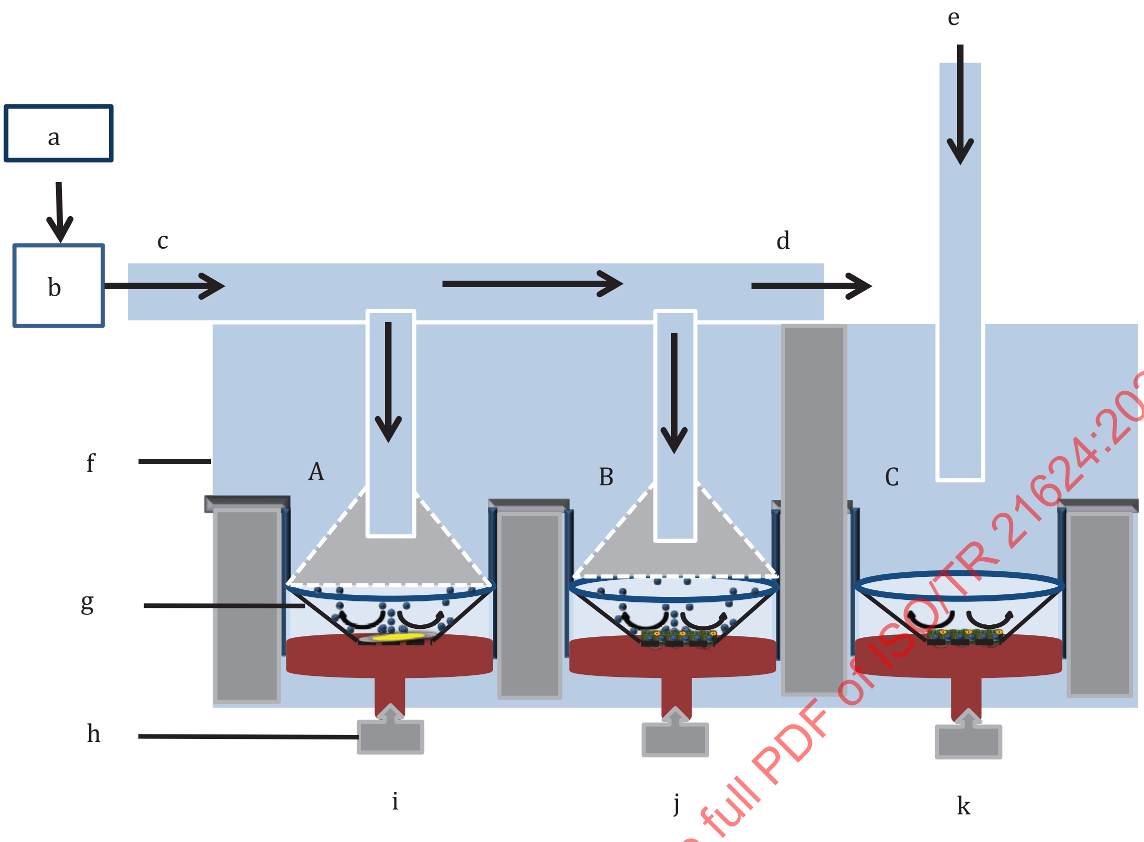


**Key**

a NM sample
b aerosol generator or a nebulizer
c aerosol
d exhaust
e air only
f exposure chamber
g cell culture insert
h outlet for sampling medium
i well with a QCM or an EM grid
j well with cells exposed to NMs at ALI
k well with cells exposed to clean air at ALI

NOTE Adapted from Reference [5].

**Figure 2 — Example of a set-up of an in vitro air-liquid interface exposure system**

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### 5.2.2.2 Choice of appropriate aerosol generator

Aerosol generators, in combination with exposure chambers, enable a direct gas-phase exposure of test systems to aerosols. They generate an aerosol atmosphere that can be used to expose the ALI cell systems to aerosolized or nebulized particles. Several methods have been used to generate aerosols but the choice of a particular method depends on the physical characteristics of the material to be aerosolized, such as density, viscosity, state of matter and target aerosol size[5]. For example, for liquid or biological materials, collision nebulizers, jet nebulizers, ultra-sonic atomization or vibrating membrane generators are often used; whereas, the generation of aerosolized solid materials involves using spray-drying, rotating scrapers, venturi-style powder dispersions or fluidized powder bed methods[15][22][27][28]. Detailed information about aerosol generators is given in ISO/TR 19601[2].

An aerosol generator is generally connected to the exposure chamber via auxiliary connectors that provide a means to deliver the generated test material atmosphere to the chamber where the cell systems are housed.

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### 5.2.2.3 Choice of exposure chambers

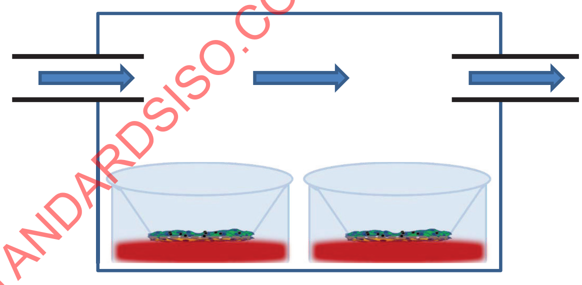
The choice of a specific exposure chamber depends on the basic study design and purpose of testing, including the number of test and control conditions, and sampling (test material, biomarkers, etc.) requirements. Exposure chambers can be used to expose cells cultured at the ALI to aerosolized NOAA (and other materials). Several exposure chambers have been described in the literature and include both commercially available systems and those developed by independent researchers[29][30][31][32][33][34][35][36][37][38][39]. These chambers vary from one another in factors such as cost, compatibility with aerosol generators, ease of availability and use, modularity, level of throughput, ability to consistently deliver aerosols at multiple dilutions, ease of cleaning and exposure duration[5][29]. However, all available exposure systems have some general similarities, including a compartment that houses the cells and equipment to facilitate generation, delivery and/or characterization of aerosol atmosphere.

The configuration and the choice of the exposure system depends on the experimental objectives and requirements. Of the available systems that have been used to test NOAA, some are more user-friendly as compared to others that require an in depth understanding of the engineering controls included in the system.

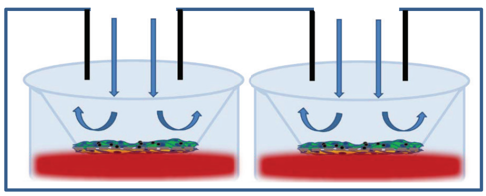
The different features of the various exposure systems are as follows.

a) Type of exposure system: Exposure chambers can be “closed-box” or “flow-through” systems based on how the test aerosol is fed into the system and delivered to the cells inside. One example of a closed-box system is an exposure chamber in which the test material is nebulized inside, where it forms a dense cloud, and which uses cloud dynamics to uniformly mix and deposit (sedimentation) the aerosol onto the membrane with the cells. There is no air flow into or out of the device in the closed-box type system[40]. On the other hand, in flow-through systems, the test material is aerosolized and fed into the exposure chamber with air flow. While the aerosol cloud settles onto the cells, the droplet-depleted air leaves the chamber through the exhaust located on the other end of the chamber[41].

b) Flow alignment: Depending on the type of exposure chamber, the alignment of airflow can either be horizontal or vertical. In a horizontal or incubator/box-type setup [see Figure 3 a)], the test atmosphere enters the exposure chamber through an inlet and exits through an outlet leading to a horizontal exposure flow above the inserts of the cultures[18]. On the other hand, in a stagnation point flow set-up [see (Figure 3 b)], the flow is vertical and directed towards the cell cultures, providing a more precise way of exposing the cells.



a) Incubator/box-type setup



b) Stagnation point flow set-up

NOTE Adapted from Reference [18].

**Figure 3 — Example of set-ups of an in vitro air-liquid interface exposure system**

c) Enhancement of deposition efficiency:

1) Electrostatic deposition [see Figure 4 a)]: Electrostatic deposition enhancement is a technique that uses charge to increase the efficiency of NOAA deposition. Although the physiological relevance of using electrostatic deposition is debatable due to the possible adverse effects of applying high electrical fields to the cells, this technique has been used to enhance the deposition of NOAA[5][18][42].

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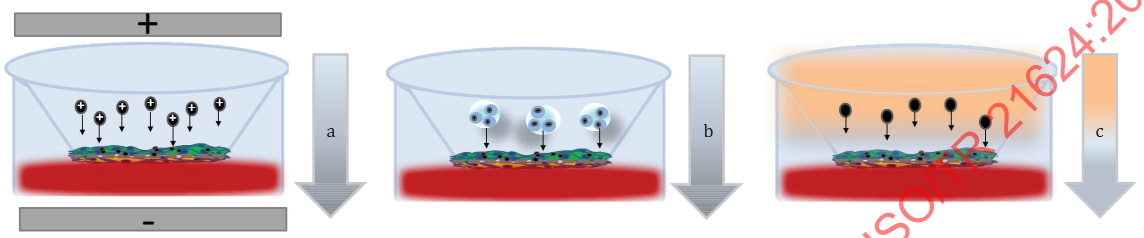
2) Droplet deposition [see Figure 4 b)]: Droplet deposition involves suspending or dissolving the test material in a liquid medium and then generating the droplet aerosol (generally using a nebulizer) to expose the cells. Although this technique provides a way to attain a high deposition of test material, it cannot be used to test materials in their dry form (or when their dry physico-chemical properties need to be maintained)[18][42].

3) Thermophoresis [see Figure 4 c)]: Thermophoresis or thermal precipitation involves deposition of the particles onto the cells along a temperature gradient. The temperature gradient reaches the range (35 °C to 37 °C) used in normal cell culturing near the cell surface. Thus, there are no obvious concerns related to the adverse effects on cells and no pre-treatment of the test material is needed so it retains its physico-chemical characteristics[18][42].




**a) Electrostatic deposition** **b) Droplet deposition** **c) Thermophoresis**

a Electrostatic forces.

b Gravitational forces.

c Thermal gradient.

NOTE Adapted from Reference [18].

**Figure 4 — Techniques to enhance NOAA deposition at ALI**

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d) Number and alignment of inserts: The number of test conditions, replicates and appropriate controls depend on the purpose of testing (e.g. hazard assessment or identification), but, in general, exposure systems should include clean air control and a reference membrane to determine the deposited dose (e.g. an insert without cells or a quartz microbalance). The ability to test multiple concentrations and durations of exposure is equally important to establish the dose- and/or time-response relationship.

e) Control and characterization of test atmosphere: Depending on the exposure chamber, parameters such as flow rate, temperature and humidity should be monitored. It is also important to be able to monitor and characterize aerosol atmosphere and maintain sterile conditions inside the exposure chamber.

f) Characterization of deposited dose and culture medium:

1) Deposited dose: Determination of the deposited dose is important to interpret the observed biological response or in other terms to establish a dose-response relationship. Deposition of NOAA can be generally determined as a function of mass [quartz crystal microbalance (QCM)], surface area or particle count (electron microscopy grid). A reference membrane insert usually houses the QCM or the EM grid. Alternately, the material deposited directly on the membrane can be recovered and analysed using chemical or florescence based techniques. Deposited dose can also be predicted using in silico models, but they are usually based on a number of assumptions related to NOAA properties.

2) Sample cell culture medium to measure translocation or release of biomarkers and metabolites by cells: To establish a dose- and time-dependent biological response, it is important to assess

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the relevant biomarkers at different time-points throughout the experiment duration. Several systems are available that vary in the ease with which the samples can be collected from the cell systems.

g) Compatibility with different aerosol generators: Modular systems that are compatible with a variety of aerosol generators provide flexibility in testing different types of materials, obtaining a specific size and concentration range of particles, and choosing the duration of exposures.

### 5.3 Considerations for characterizing NOAA tested in vitro studies of airborne nanomaterials

Thorough characterization of NOAA at various stages of in vitro testing using (e.g. as manufactured, as aerosolized, as deposited) is critical to determine relationships between the observed biological outcomes and concentration and properties. Some of the NOAA characteristics that are critical in this regard include particle size, size distribution, shape, aggregation/agglomeration, solubility (dissolution), presence of trace impurities (e.g. metals and endotoxins), surface characteristics (e.g. area and charge), crystalline structure, dustiness, composition and purity. Determination of physical and chemical properties of NOAA using standardized protocols is important as it decreases the interlaboratory variability and facilitates comparison of data. Several guidance documents and standards are available related to characterization, see References [175] and [176]. Those specifically related to aerosolized NOAA are given in ISO/TR 19601[2]. Suspending the nano-objects in simulated lung fluids can provide an insight into their dissolution rate (biodurability or biopersistence) potential, agglomeration and aggregation, and kinetics in biological fluids. Several simulated lung fluids have been used to assess the aforementioned NOAA parameters[43][44][45]. Information about acellular systems is given in ISO/TR 19057[17]. If the nano-object is suspended in a medium (e.g. in case of nebulization), thorough characterization of its suspension should be conducted. Detailed information related to the characterization of nano-object suspensions is given in ISO/TS 19337[46].

In addition to assessing the physical and chemical properties of nano-objects, it is important to determine the concentration and form of the fraction deposited on the cell surface. Unlike chemicals that form a homogeneous solution in the medium, nano-objects form a heterogeneous dispersion with aggregates of different sizes. The deposition of NOAA in the medium is largely dependent on size and density: with large particles settling by inertia but, as particle size is reduced (to about 0,3 micron), the deposition is reduced to a minimum. As the particle size is reduced further, the deposition increases due to diffusional forces[47]. With limited tools to accurately predict the concentrations of particles that reach the cell layer, the behaviour of nano-objects in dispersion complicates the dose-response determination, which is critical for toxicological assessments.

Exposure of cell systems at the ALI bypasses the effects of medium on nano-objects to some extent by allowing a fairly direct deposition of aerosols onto the cell surface. Although, also within the aerosol, changes in the nano-object characteristics can occur, for example due to evaporation of the (watery) vehicle, or by aggregation of the nano-objects within the droplets of the aerosol. The human respiratory system has a characteristic particle size dependent deposition (or collection) curve. The deposition of aerosol particles onto the surface of the lungs and penetration though the liquid surface that lines the lungs depends on several factors, including particle diameter, air flow, surface tension and the presence of electrostatic, thermal or molecular gradients. Similarly, the size distribution delivered and deposited in in vitro systems at the ALI will depend on the choice of the exposure equipment (aerosol generator and exposure chamber), with some systems capable of continuous exposures over a period of time and others capable of single exposure at a fixed concentration. Characterization of the deposited fraction can provide information regarding the form and mass of NOAA that the cells are actually exposed to. Both qualitative (e.g. electron microscopy) and quantitative methods (e.g. quartz crystal microbalance and gravimetrically determined deposited mass measures) are available to assess the deposited dose.

While choosing the exposure system, factors such as whether the device should simulate the lung deposition curve as a function of particle diameter or simply have repeatable high deposition efficiencies as a function of particle diameter will also need to be considered and checked for each NOAA type and form. Additional features that determine the choice of exposure chambers are given in 5.2.2.3.

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## 5.4 Choice of cell systems

### 5.4.1 General

The respiratory system can be divided into three main regions: nasopharyngeal, tracheobronchial and alveolar. These regions are composed of more than 40 types of specialized cells, such as epithelial cells that form the lining, macrophages that engulf foreign materials, and alveolar cells that secrete lung surfactant and are involved in gas exchange. Mucus-producing goblet cells and ciliated epithelial cells form the muco-ciliary system that actively removes foreign materials and microbes from the respiratory tract. One in vitro model containing all lung cell types is currently not technically feasible; however, models containing key cell types can be used to predict the effects of inhaled substances on the lungs. The choice of cell types also depends on the region of the respiratory tract that the test substance is predicted or known to localize in and on the relevant biological outcome. Several cell-based systems that have been used to assess the effects of nano-objects are described in 5.4.2 to 5.4.3.

### 5.4.2 Mono-culture systems

Mono-culture systems of human-derived cells and cell lines have been extensively used to assess NOAA toxicity under submerged conditions and at ALI[48][49][50][51][52][53]. Mono-culture models provide a good indication of potential overt toxicity of NOAA, and are often used as a first tier for prioritizing the need for further testing in more complex in vitro systems. Since every cell type is functionally specialized, the choice of the cell type depends on the expected in vivo target location (within the respiratory tract or systemically). Table 1 shows some examples of human-based cell types that have been used to test NOAA and a brief description for each[54][55][56]. These lung cell types are commercially available and primary cells can be obtained from the hospitals.

**Table 1 — Examples of a few human-based cell types that have been used to test NOAA**

| Cell type | Type | Source | Features | Endpoints assessed | References |
|---|---|---|---|---|---|
| Human bronchial epithelial (HBE) cells | Primary | Tracheal and carinal biopsies (above bifurcation of the lung | Upon differentiation they show a pseudostratified epithelial phenotype, composed of ciliated, non-ciliated and basal cells. | — Apoptosis<br>— Oxidative stress<br>— DNA fragmentation<br>— Genotoxicity<br>— Inflammatory response<br>— Barrier integrity<br>— Cytotoxicity | [54], [57], [58], [59], [60], [61], [62] |
| 16HBE140- | Cell line | Bronchial epithelium | 16HBE140- cells have a wild-type chloride (Cl) ion-transport phenotype, form polar monolayers, have well defined tight junctional complexes, generate high transepithelial electrical resistance (TEER), and limit movement of paracellular markers and macromolecules. They express high levels of cystic fibrosis transmembrane conductance regulator (CFTR) mRNA and protein. | — DNA fragmentation<br>— Genotoxicity<br>— Inflammatory response<br>— Barrier integrity | [57], [63], [64], [65] |

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**Table 1** *(continued)*

| Cell type | Type | Source | Features | Endpoints assessed | References |
|---|---|---|---|---|---|
| Small airway epithelial cells (SAEC) | Primary | 1 mm bronchiole area | They express intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), surfactant protein A (SP-A), SP-D, and aquaporin 3. | — Cell viability<br>— Cell proliferation Cell cycle<br>— Reactive oxygen species (ROS)<br>— Lipid peroxidation DNA (e.g. DNA damage and methylation)<br>— Metallothionein (MT) | [66], [67], [68], [69], [70], [71] |
| Human bronchial epithelial cell line (BEAS-2B) | Cell line | Bronchus | They express vimentin, collagen I, E-cadherin, ICAM-1, VCAM-1, and clara cell secretary protein (CCSP). This cell line does not polarize or form tight junctions. | — Cytotoxicity<br>— Pro-inflammatory response<br>— Genotoxicity<br>— Apoptosis<br>— Oxidative stress<br>— Membrane integrity<br>— Cellular transformation<br>— Epithelial-mesenchymal transition | [72], [73], [74], [75], [76], [77], [78], [79], [80], [81], [82], [83], [84], [85], [86], [87], [88], [89] |
| Calu-3 | Cell line | Airway epithelium | This cell line is used as a model of the airway submucosal glad acinar serous cell, due to the relatively high levels of expression of cystic fibrosis transmembrane conductance regulator (CFTR). They form ciliated and secretory cell populations, tight monolayers at ALI, and also express pro SP-C and mucin. | — Barrier properties Injury<br>— Diseases of the bronchial epithelium | [90], [91], [92], [93], [94], [95], [96], [97] |
| A549 | Cell line | Lung epithelium | This cell line is used to represent type II pulmonary epithelial cells because it contains lamellar bodies and microvilli, and has the ability to express CFTR, SP-A, C, D, and mucin 1 (MUC1). | — Oxidative stress<br>— Cytotoxicity<br>— Genotoxicity<br>— Apoptosis<br>— Translocation | [54], [62], [98], [99], [100], [101], [102], [103], [104], [105], [106], [107], [107], [108], [109], [110], [111], [112], [113] |

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**Table 1** *(continued)*

| Cell type | Type | Source | Features | Endpoints assessed | References |
|---|---|---|---|---|---|
| NCI-H292 | Cell line | Lung epithelium | These cells express catenins, ICAM-1, cluster of differentiation (CD) 58, CD44, and low levels of CFTR, but do not express selectins and VCAM-1. They develop TEER when cultured on permeable membranes. NCI-H292 cells have been used to study paracellular migration. | — Cytotoxicity<br>— Inflammation<br>— Oxidative stress | [54], [101], [114] |
| Human alveolar epithelial lentivirus immortalized cell line (hAELVi) | Cell line | Lung epithelium | This is an immortalized human alveolar type I-like cell line. hAELVi cells form tight intercellular junctions. | — Cytotoxicity<br>— Barrier integrity<br>— Translocation | [115], [116] |
| THP-1 | Cell line | Blood | These cells were derived from the peripheral blood of a human male with acute monocytic leukaemia. THP-1 cells have Fc and C3b receptors and lack surface and cytoplasmic immunoglobulins. | — Cytotoxicity<br>— Inflammation<br>— PPAR-γ activation | [117] |

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### 5.4.3 Co-cultures/three-dimensional systems

Although mono-culture systems have been extensively used to assess the non-specific toxicity of NOAAs, co-culture systems, with multiple cell types, can more closely mimic an in vivo-like situation and can be used to study the intercellular interplay between multiple cell types. The cell types listed in Table 1 have been used in combination with other cell types to assess the impact of NOAA on the respiratory tract. The choice of cells to include in a co-culture system depends on the purpose of the study[55]. For example, a co-culture of endothelial and epithelial cells can be used to simulate the lung-blood barrier while macrophages and/or dendritic cells can be cultured with other lung cells to study the role of immune cells. Of note here is that the choice of the aerosol generator and exposure chamber is only relevant to systems where cells are exposed to test substance at ALI.

Table 2 lists examples of co-culture systems (submerged and at ALI) that have been used in NOAA toxicity studies along with the endpoints assessed in those studies.

**Table 2 — Examples of a few human-based co-culture systems that have been used to test NOAA**

| Co-culture system | Endpoints tested | Test material | References |
|---|---|---|---|
| Co-culture of SAECs and human microvascular endothelial cells | — Cytotoxicity<br>— Inflammation<br>— Oxidative stress<br>— Uptake<br>— Morphology<br>— Angiogenesis | — Multi-walled carbon nanotubes (MWCNTs)<br>— Printer-emitted nanoparticles | [118], [119], [120] |

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**Table 2** *(continued)*

| Co-culture system | Endpoints tested | Test material | References |
|---|---|---|---|
| Co-culture of hAELVi and THP-1 | — Barrier integrity<br>— Cytotoxicity<br>— Translocation | — Silver nanoparticles (Ag NPs) | [115] |
| Calu-3, macrophages (THP-1), and endothelial cells (HPMEC-ST1.6R cell line) | — Translocation | — Polystyrene nanobeads | [94] |
| Triple co-culture of A549 cells, human peripheral blood monocyte derived dendritic cells (MDDC), and monocyte derived macrophages (MDM) | — Cytotoxicity<br>— Pro- and anti-Inflammatory response<br>— Oxidative stress | — Ag NPs<br>— Gold (Au) NPs<br>— MWCNTs | [118], [121], [122], [123], [124], [125], [126] |
| Triple co-culture of 16HBE14o-, THP-1, and human lung microvascular endothelial cells (HLMVEC) | — Cytotoxicity<br>— Inflammation<br>— Barrier integrity | — Paint NPs<br>— Titanium dioxide ($TiO_2$) NPs<br>— Ag NPs<br>— Silicon dioxide ($SiO_2$) NPs | [127] |
| Triple co-culture of 6HBE14o-, MDDC and MDMs cultured at ALI | — Cytotoxicity<br>— Pro-inflammatory response<br>— Oxidative stress | — MWCNTs<br>— Single-walled CNTs (SWCNTs) | [128] |
| Triple co-culture of A549 epithelial cells, THP-1, and endothelial cells (EA.hy 926 or HUVEC) | — Cytotoxicity;<br>— Proliferation<br>— Inflammation<br>— Barrier integrity<br>— Oxidative stress | — $SiO_2$<br>— MWCNTs | [129], [130] |
| Triple co-culture of NCI-H441 alveolar epithelium cell line, ISO-HAS1 human microvascular endothelium cell line, and THP-1 cells | — Cytotoxicity<br>— Barrier integrity<br>— Pro-inflammation<br>— Oxidative stress<br>— Uptake | — $SiO_2$ NPs | [131] |
| Tetra-culture of A549 cell line, THP-1, mast cells (HMC-1), and endothelial cells (EA.hy 926) | — Oxidative stress<br>— Barrier integrity<br>— Pro-inflammation | — $SiO_2$-Rhodamine NPs | [26] |

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**Table 2** *(continued)*

| Co-culture system | Endpoints tested | Test material | References |
|---|---|---|---|
| Co-culture of human alveolar epithelial cells and human pulmonary microvascular endothelial cells cultured at the ALI, on opposite sides of a membrane in a microfluidic chamber capable of providing fluid flow and mechanical strain (to mimic breathing) to the cells | — Barrier integrity<br>— Inflammation | — $SiO_2$ | [132] |

In addition to the systems discussed in Tables 1 and 2, several reconstructed human tissue-based in vitro models are commercially available that have been used to assess other substances (e.g. cigarette smoke) and can be used to test the impact of NOAA on the respiratory system following inhalation exposure[133][134][135][136][137][138][139][140][141][142][143][144][145][146][147][148][149][150][151][152]. These models contain human cells in a physiologically-relevant configuration and can be used to study complex biological outcomes relevant to particle exposure. Adding another layer of physiological relevance to the tissue-based models are the organ-on-a-chip models that incorporate the mechanical aspects of the lung (e.g. breathing motion and sheer stress)[153][154]. Ex vivo human precision cut lung slices are another example of a system that can be used to test the effects of NOAA in humans[155][156][157].

The choice of cell types and endpoints depends on the purpose of the study. Mono-culture 2D cell lines are relatively easy to use and cheap, but might be of less physiological relevance. The complexity, costs and physiological relevance increases with co-cultures, 3D cultures, reconstructed 3D models, and ex vivo precision cut lung slices. When choosing the cell model, the region of the lung where the NOAA is predicted or known to localize should also be considered. Other characteristics of the cell system that should be taken into account while designing the study are: the suitability for culturing at the ALI, ability to form tight junctions, ability to expose the cells to single or repeated exposure, possible read-outs or endpoints that can be measured, correlation with existing data (from existing in vitro and in vivo studies), and, if applicable, donor variability. It is also important to include appropriate controls in the study design including positive, negative, clean air and incubator controls. Positive and negative controls are cells exposed to (reference) materials with a known positive and negative response. Clean air controls are cells exposed to clean air. Incubator controls are cells that are not exposed, but are cultured under the same conditions as the exposed cells.

The cell-based systems described above are used to study the mechanistic aspects related to the biological outcomes observed after exposure to NOAA. But because of the complexity of biological systems and responses, a battery of such cell systems combined with other approaches (e.g. modelling approaches) might be needed to predict human responses. Annex A provides a brief overview of the application of adverse outcome pathways (AOPs) to design in-vitro-based approaches to predict complex adverse outcomes.

To further increase the accuracy of these in vitro systems to predict human effects, additional factors need to be considered and reported. For example, information on the characterization of the cell models, cell culture protocols and conditions, laboratory ware (e.g. culture dishes), culture medium and supplements, and cell-passage number should be reported, as these factors have been shown to affect the behaviour of cells in culture[158][159]. When cells are cultured for propagation during a preparatory phase in submerged conditions in tissue culture flasks/discs, the culture conditions used should be reported. The use of cells and cell lines from recognized sources at early passage numbers and the use of a chemically-defined medium can facilitate reproducibility and interlaboratory comparison[160][161].

Following the selection of an in vitro test system and endpoints to assess, it is important to test the NOAA form and concentration that most closely represents realistic human exposures.

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## 6 Choice of appropriate dose and dose metrics

### 6.1 General

Dosimetry refers to an estimation or measurement of the amount of a substance at a biological target site at a specific time point[162]. Quantitation of dose is critical to deriving concentration/dose-response relationships, which are important for risk assessment. Traditionally, mass is the most common metric used to describe the concentration of chemicals in toxicity studies but, for nano-objects, mass alone might not adequately describe the dose. Nano-objects with the same chemical composition can have a completely different internal dose and distributions among organs[163][164]. Therefore, other dose metrics such as particle number, volume or surface area should also be considered, as these properties have been shown to play a role in the toxicity of NMs.

Inhalation is the most relevant exposure route for nano-objects and their physico-chemical properties influence the deposition pattern in the lungs. Several studies have focused on deriving a suitable dose metric for NOAA after inhalation. An approach to determine the most adequate description of dose has been suggested[165], which could in theory also include particle physico-chemical properties such as particle size, zeta potential, surface reactivity, and dispersity index or a combination thereof[165]. For nano-objects that readily dissolve in the body, mass can be a useful metric, as the effect is related to the released ions similar to conventional substances[166]. It should be noted that until particle deposition and dissolution occurs, the particulate nature of the nano-object exposure governs the local dose and toxicity. Thus, particle number might be a proper dose metric, until dissolution occurs. Particle surface area has been shown to be a useful metric to extrapolate across nano-objects of a range of sizes[163], as well for the classification of nano-object powders[167]. For nano-objects that follow the World Health Organization (WHO) definition of a fibre [i.e. having a fibre length ($L$) > 5 µm, fibre diameter ($D$) < 3 µm, and aspect ratio ($L/D$) > 3], particle number has been suggested to be the appropriate metric as they might follow the same mechanism of action as asbestos fibres. However, it should be realized that these properties are dependent on the experimental conditions, and therefore the most adequate dose metrics for NOAA are also likely to be different for different experimental and real-life situations, including different exposure routes or even for different toxicity endpoints. Therefore, to account for such variations, a combination of two or more metrics has been suggested for toxicity assessment, e.g. size distribution along with mass or total surface area concentrations contributes to a more mechanistic discrimination of pulmonary responses to NOAA exposure[168].

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Another important aspect besides dose metrics is the deposited dose. It is important to identify the scenario for human exposure that the in vitro system is being used to assess and choose the concentration that represents that particular scenario. For example, to assess the NOAA relevant to occupational exposure, the dose limits recommended by the Occupational Safety and Health Administration (OSHA) should be included in the tested aerosol concentrations. The average ambient urban exposure and occupational exposure at the currently recommended OSHA standard for respirable nuisance dust as the boundaries of human exposure, the corresponding range of upper-limit mass and number flux delivered to the lung tissue is $3 \times 10^{-5}$ µg/cm$^2$/h to $5 \times 10^{-3}$ µg/cm$^2$/h and 2 p/c/h to 300 p/c/h, respectively[16]. Considering the concentrations relevant to realistic human exposures could aid in extrapolation of in vitro data to human outcomes. For deriving dose-response relationships, information is needed on the amount of NOAA that deposits on the effect site. By expressing the deposited dose as either particle mass, number based particle size distribution, particle number or surface area per cm$^2$, the doses can be compared between different studies. Therefore, it is recommended to express the concentration (or the dose) in various different metrics, including mass, number based particle size distribution, particle number and surface area (including information on inner and outer diameter and surface stabilization, where applicable). When these data are known, conversion to another dose metric to evaluate a dose response relationship remains possible.

To further facilitate the dose comparability between studies, it is important to use standard approaches and methods to test NOAA. Integrated approaches have been proposed for NMs that include preparation and proper characterization of NOAA suspensions and the use of advanced numerical fate and transport methods to estimate the delivered dose metrics[169]. For a submerged culture system, a dose response can be obtained by varying the concentration of the NOAA in the dispersion to be added. For aerosol exposure, this is more challenging as in aerosol exposure systems it is often not possible to use different

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doses at once within the same exposure system. However, to evaluate various exposure doses in an ALI system, a fixed concentration of NOAA can be used in the aerosol or multiple aerosol exposure systems can be used concurrently with different concentrations/doses in each.

### 6.2 In silico methods to assess dose/dose metrics and deposition

Several in silico models can be applied to estimate deposited dose (in terms of mass and surface) in vivo, which can be translated into in vitro deposited concentration at ALI. Computational fluid dynamics (CFD) or a multiple-path particle dosimetry model (MPPD) can be used to predict nano-object deposition within the respiratory tract, which can then be used to determine the concentration of test material for in vitro experiments. These modelling approaches rely on input parameters such as particle count median diameter (CMD), mass median aerodynamic diameter (MMAD), geometric standard deviation (GSD) and density to predict deposition profile and efficiency. Based on the location of deposition predicted using the modelling approaches, cell types can be chosen to design the in vitro study.

In addition to the CFD and MPPD models, there are models that are specific to in vitro exposures under submerged conditions: in vitro sedimentation, diffusion and dosimetry (ISDD) and in vitro sedimentation, diffusion, dissolution and dosimetry (ISD3) models[170][171]. ISDD can be used to determine cellular doses of monodispersed particles and requires input parameters including temperature, media density and viscosity, media height, hydrodynamic particle size in the test media, and particle density. ISD3 is an extension to ISDD that takes into account the influence of dissolution on the cellular dosimetry.

Additionally, there are modelling approaches such as the volumetric centrifugation method, ISDD (VCM–ISDD), or distorted grid (DG) model that can be used to predict administered concentration to obtain the target delivered dose[172].

## 7 Summary

There are several parameters to consider when designing in vitro studies of airborne NOAA, such as choosing the appropriate mode of exposure, relevant cell types, exposure system and concentration that represents realistic human exposure (see Figure 5). These parameters generally apply to testing any type of NOAA (incidental or manufactured). Mechanistic understanding from existing information combined with physico-chemical characteristics of the test material can help inform the study design. Furthermore, applying standardized protocols to fulfil each of the steps in the study can aid interlaboratory comparability in the test outcomes and facilitate the uptake of in vitro test methods for regulatory and non-regulatory purposes.

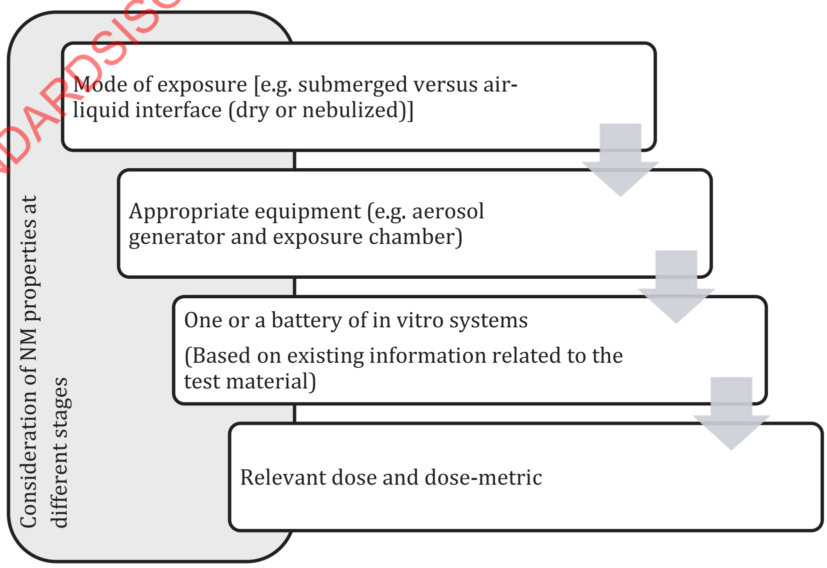


**Figure 5 — Schematic listing the parameters that are important to consider when designing in vitro studies of airborne NOAA**

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# Annex A
(informative)

# Application of adverse outcome pathways (AOPs) to design in vitro-based approaches

Given the complexity of cellular responses following exposure to inhaled NOAA (or other chemicals), any one in vitro assay might not be sufficient to capture the progression to a given adverse effect. However, a combination of in vitro assays in conjunction with existing data (e.g. in vivo, in vitro, in silico) can be used in integrated approaches that are predictive of human outcomes. It should, however, be noted that when data are derived from a combination of in vitro assays, the parameters (e.g. exposure conditions) used in these in vitro systems should be clearly identified. Especially when data from submerged culture and air exposed ALI systems are combined to draw conclusions on the possible adverse/toxic effects of the NOAA. Although not specific to NOAA, one example of an integrated approach to testing an assessment (IATA) that has been published in an OECD guidance document is that for skin corrosion and irritation[173]. Such integrated approaches can be based on AOPs, which are a sequential representation of key events that lead to an adverse outcome, starting with a molecular initiating event (MIE). NOAA and chemical-induced adverse effects seem to differ primarily in the initial key events (KEs). Therefore, most of the mechanistic information gathered for AOPs related to chemicals applies to NOAA-induced adversity and extend the applicability of these AOPs to NOAA risk assessment[115].

AOPs are substance-agnostic and can help to: a) organize existing scientific information related to the adverse outcome to be assessed; b) identify the informational gaps; and c) identify the in vitro and in silico assays that can be used or should be developed to assess the key events that lead to the adverse outcome[56].

Therefore, AOPs can help identify fit-for-purpose in vitro assays anchored to the mechanistic understanding of the adverse outcome[174]. Figure A.1 shows a schematic depicting how in vitro assays can be used in an approach to predict an adverse outcome. In vitro assays can be used to evaluate measurable biomarkers related to each KE. The battery of assays measuring various KEs can then collectively predict complex pathological outcomes in humans.

However, when composing an AOP for NOAA inhalation toxicity based on in vitro systems, it should be recognized that exposure at ALI might trigger different MIE compared to the reaction of cells to NOAA under submerged conditions. Thus, the MIE should also be determined during aerosol exposure. If there is only information on the MIE based on the cellular response under submerged cell culture conditions, the similarity of the cellular response under submerged condition needs to be evaluated against such a response under ALI conditions. There are a number of AOPs under development with the OECD, see Reference [177].

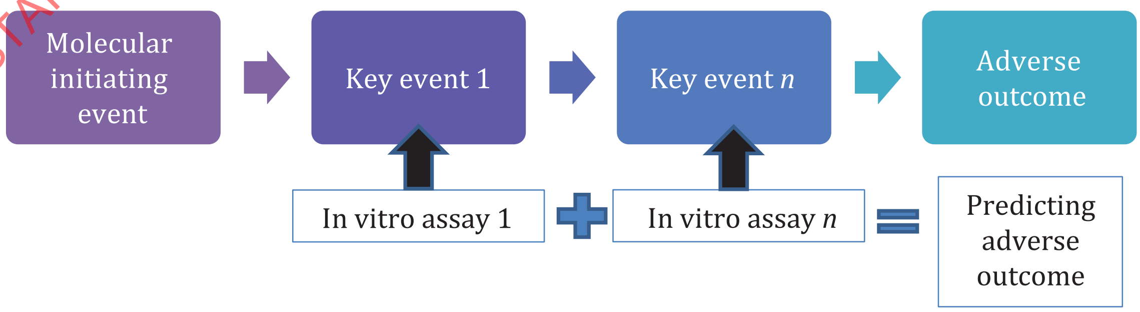


**Figure A.1 — Diagrammatic representation of an adverse outcome pathway starting with a molecular initiating event, followed by key events that lead to the adverse outcome**

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# Bibliography


[1] Clippinger A.J., Ahluwalia A., Allen D. et al. , Expert consensus on an in vitro approach to assess pulmonary fibrogenic potential of aerosolized nanomaterials. *Archives of toxicology.* 2016, 90(7), pp. 1769–1783

[2] ISO/TR 19601, *Nanotechnologies — Aerosol generation for air exposure studies of nano-objects and their aggregates and agglomerates (NOAA)*

[3] Hayes A, Bakand S, Winder C. Novel In-Vitro Exposure Techniques for Toxicity Testing and Biomonitoring of Airborne Contaminants. In: *Drug Testing in vitro.* Wiley-VCH Verlag GmbH & Co. KGaA, 2006, pp. 103–123

[4] Morin J-P, Fouquet F, Monteil C, Le Prieur E, Vaz E, Dionnet F, Development of a new in vitro system for continuous in vitro exposure of lung tissue to complex atmospheres: Application to diesel exhaust toxicology. *Cell Biology and Toxicology.* 1999, 15(3), pp. 143–152

[5] Polk W.W, Sharma M, Sayes C.M, Hotchkiss J.A, Clippinger A.J, Aerosol generation and characterization of multi-walled carbon nanotubes exposed to cells cultured at the air-liquid interface. *Part Fibre Toxicol.* 2016, 13, pp. 20

[6] Sánchez Jiménez A, Brouwer D, van Tongeren M. *Workplace Inhalation Exposure to Engineered Nanomaterials.* 2014

[7] Sayes C.M, Reed K.L, Warheit D.B Assessing toxicity of fine and nanoparticles: comparing in vitro measurements to in vivo pulmonary toxicity profiles. *Toxicological sciences: an official journal of the Society of Toxicology.* 2007, 97(1), pp. 163–180

[8] Cho S.H, Tong H, McGee J.K, Baldauf R.W, Krantz Q.T, Gilmour M.I, Comparative toxicity of size-fractionated airborne particulate matter collected at different distances from an urban highway. *Environmental health perspectives.* 2009, 117(11), pp. 1682–1689

[9] Yamaguchi T, Yamazaki H., Cytotoxicity of Airborne Particulates Sampled Roadside in Rodent and Human Lung Fibroblasts. *Journal of Health Science.* 2001, **47**(3), pp. 272–277

[10] Brandenberger C, Rothen-Rutishauser B, Mühlfeld C et al. , Effects and uptake of gold nanoparticles deposited at the air-liquid interface of a human epithelial airway model. *Toxicol Appl Pharmacol.* 2010, 242(1), pp. 56–65

[11] Fröhlich E, Salar-Behzadi S., Toxicological Assessment of Inhaled Nanoparticles: Role of in Vivo, ex Vivo, in Vitro, and in Silico Studies. *International Journal of Molecular Sciences.* 2014, 15(3), pp. 4795–4822

[12] Ahmad S, Ahmad A, Neeves K.B et al. In vitro cell culture model for toxic inhaled chemical testing. *J Vis Exp.* 2014(87)

[13] Blank F, Rothen-Rutishauser B.M, Schurch S, Gehr P, An optimized in vitro model of the respiratory tract wall to study particle cell interactions. *Journal of aerosol medicine.* 2006, 19(3), pp. 392–405

[14] Nanoreg. NANoREG Deliverables & SOPs. http://www.nanoreg.eu/media-and-downloads/deliverable-reports/312-nanoreg-deliverables-sops. Accessed January 19, 2018. Published 2017

[15] Frohlich E, Bonstingl G, Hofler A et al. Comparison of two in vitro systems to assess cellular effects of nanoparticles-containing aerosols. *Toxicology in vitro: An international journal published in association with BIBRA.* 2013, 27(1), pp. 409–417


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[16] Paur H.-R, Cassee F.R, Teeguarden J et al. , In-vitro cell exposure studies for the assessment of nanoparticle toxicity in the lung — A dialog between aerosol science and biology. *Journal of Aerosol Science.* 2011, 42(10), pp. 668–692

[17] ISO/TR 19057, *Nanotechnologies — Use and application of acellular in vitro tests and methodologies to assess nanomaterial biodurability*

[18] Lacroix G, Koch W, Ritter D et al. , Air–Liquid Interface In Vitro Models for Respiratory Toxicology Research: Consensus Workshop and Recommendations. *Applied In Vitro Toxicology.* 2018, 4(2), pp. 91–106

[19] Ghio A.J, Dailey L.A, Soukup J.M, Stonehuerner J, Richards J.H, Devlin R.B, Growth of human bronchial epithelial cells at an air-liquid interface alters the response to particle exposure. *Part Fibre Toxicol.* 2013, 10, pp. 25

[20] Lenz A.-G, Karg E, Brendel E et al. , Inflammatory and Oxidative Stress Responses of an Alveolar Epithelial Cell Line to Airborne Zinc Oxide Nanoparticles at the Air-Liquid Interface: A Comparison with Conventional, Submerged Cell-Culture Conditions. *BioMed Research International.* 2013, 2013, pp. 12

[21] Bitterle E, Karg E, Schroeppel A et al. , Dose-controlled exposure of A549 epithelial cells at the air-liquid interface to airborne ultrafine carbonaceous particles. *Chemosphere.* 2006, pp. 65

[22] Lenz A.G, Karg E, Lentner B et al. , A dose-controlled system for air-liquid interface cell exposure and application to zinc oxide nanoparticles. *Part Fibre Toxicol.* 2009, 6(32)

[23] Raemy D.O, Grass R.N, Stark W.J et al. , Effects of flame made zinc oxide particles in human lung cells — a comparison of aerosol and suspension exposures. *Part Fibre Toxicol.* 2012, 9, pp. 33

[24] Holder A.L, Lucas D, Goth-Goldstein R, Koshland C.P, Cellular Response to Diesel Exhaust Particles Strongly Depends on the Exposure Metho. *Toxicological Sciences.* 2008, 103(1), pp. 108–115

[25] Panas A, Comouth A, Saathoff H et al. , Silica nanoparticles are less toxic to human lung cells when deposited at the air–liquid interface compared to conventional submerged exposure. *Beilstein journal of nanotechnology.* 2014, 5, pp. 1590–1602

[26] Klein S.G, Serchi T, Hoffmann L, Blömeke B, Gutleb A.C, An improved 3D tetraculture system mimicking the cellular organisation at the alveolar barrier to study the potential toxic effects of particles on the lung. *Part Fibre Toxicol.* 2013, 10, pp. 31–31

[27] Nurkiewicz T.R, Porter D.W, Hubbs AF et al. , Nanoparticle inhalation augments particle-dependent systemic microvascular dysfunction. *Part Fibre Toxicol.* 2008, 5(1)

[28] Persoz C, Leleu C, Achard S et al. , Sequential air-liquid exposure of human respiratory cells to chemical and biological pollutants. *Toxicol Lett.* 2011, 207(1), pp. 53–59

[29] Geiser M, Jeannet N, Fierz M, Burtscher H., Evaluating Adverse Effects of Inhaled Nanoparticles by Realistic In Vitro Technology. *Nanomaterials (Basel)*. 2017, 7(2)

[30] Bitterle E, Karg E, Schroeppel A et al. , Dose-controlled exposure of A549 epithelial cells at the air–liquid interface to airborne ultrafine carbonaceous particles. *Chemosphere*. 2006, 65(10), pp. 1784–1790

[31] de Bruijne K, Ebersviller S, Sexton K.G et al. , Design and testing of Electrostatic Aerosol in Vitro Exposure System (EAVES): an alternative exposure system for particles. *Inhal Toxicol.* 2009, 21(2), pp. 91–101

[32] Frijns E, Verstraelen S, Stoehr L.C et al. , A Novel Exposure System Termed NAVETTA for In Vitro Laminar Flow Electrodeposition of Nanoaerosol and Evaluation of Immune Effects in Human Lung Reporter Cells. *Environ Sci Technol.* 2017, 51(9), pp. 5259–5269


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[33] Mertes P, Praplan A.P, Kunzi L et al. , A compact and portable deposition chamber to study nanoparticles in air-exposed tissue. *Journal of aerosol medicine and pulmonary drug delivery.* 2013, 26(4), pp. 228–235

[34] Zavala J, O'Brien B, Lichtveld K et al. , Assessment of biological responses of EpiAirway 3-D cell constructs versus A549 cells for determining toxicity of ambient air pollution. *Inhal Toxicol.* 2016, 28(6), pp. 251–259

[35] Kim JS, Peters T.M, O'Shaughnessy P.T, Adamcakova-Dodd A, Thorne P.S, Validation of an in vitro exposure system for toxicity assessment of air-delivered nanomaterials. *Toxicology in Vitro.* 2013, 27(1), pp. 164–173

[36] Bakand S, Winder C, Khalil C, Hayes A, An experimental in vitro model for dynamic direct exposure of human cells to airborne contaminants. *Toxicol Lett.* 2006, 165(1), pp. 1–10

[37] Savi M, Kalberer M, Lang D et al. , A novel exposure system for the efficient and controlled deposition of aerosol particles onto cell cultures. *Environ Sci Technol.* 2008, 42(15), pp. 5667–5674

[38] Aufderheide M, Scheffler S, Moehle N, Halter B, Hochrainer D, Analytical in vitro approach for studying cyto- and genotoxic effects of particulate airborne material. *Anal Bioanal Chem.* 2011, 401(10), pp. 3213–3220

[39] Jeannet N, Fierz M, Kalberer M, Burtscher H, Geiser M, Nano aerosol chamber for in-vitro toxicity (NACIVT) studies. *Nanotoxicology.* 2015, 9(1), pp. 34–42

[40] Lenz A.-G, Stoeger T, Cei D et al. , Efficient Bioactive Delivery of Aerosolized Drugs to Human Pulmonary Epithelial Cells Cultured in Air–Liquid Interface Conditions. *American Journal of Respiratory Cell and Molecular Biology.* 2014, 51(4), pp. 526–535

[41] Lenz A.G, Karg E, Lentner B et al. , A dose-controlled system for air-liquid interface cell exposure and application to zinc oxide nanoparticles. *Part Fibre Toxicol.* 2009, 6(32)

[42] Latvala S, Hedberg J, Möller L, Odnevall Wallinder I, Karlsson H.L, Elihn K, Optimization of an air–liquid interface exposure system for assessing toxicity of airborne nanoparticles. *Journal of Applied Toxicology.* 2016, 36(10), pp. 1294–1301

[43] Osmond-McLeod M.J, Poland C.A, Murphy F et al. , Durability and inflammogenic impact of carbon nanotubes compared with asbestos fibres. *Part Fibre Toxicol.* 2011, 8, pp. 15

[44] Vranic S, Garcia-Verdugo I, Darnis C et al. , Internalization of SiO2 nanoparticles by alveolar macrophages and lung epithelial cells and its modulation by the lung surfactant substitute Curosurf®. *Environmental Science and Pollution Research.* 2013, 20(5), pp. 2761–2770

[45] Pelfrêne A, Cave M.R, Wragg J, Douay F, In Vitro Investigations of Human Bioaccessibility from Reference Materials Using Simulated Lung Fluids. *International journal of environmental research and public health.* 2017, 14(2), pp. 112

[46] ISO/TS 19337, *Nanotechnologies — Characteristics of working suspensions of nano-objects for in vitro assays to evaluate inherent nano-object toxicity*

[47] ISO/TR 27628:2007, *Workplace atmospheres — Ultrafine, nanoparticle and nano-structured aerosols — Inhalation exposure characterization and assessment*

[48] Rotoli B.M, Bussolati O, Bianchi M.G et al. , Non-functionalized multi-walled carbon nanotubes alter the paracellular permeability of human airway epithelial cells. *Toxicol Lett.* 2008, 178(2), pp. 95–102

[49] Banga A, Witzmann F.A, Petrache H.I, Blazer-Yost B.L Functional effects of nanoparticle exposure on Calu-3 airway epithelial cells. *Cellular physiology and biochemistry: International journal of experimental cellular physiology, biochemistry, and pharmacology.* 2012, 29(1-2), pp. 197–212


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[50] Gliga A.R, Skoglund S, Wallinder I.O, Fadeel B, Karlsson H.L, Size-dependent cytotoxicity of silver nanoparticles in human lung cells: the role of cellular uptake, agglomeration and Ag release. *Part Fibre Toxicol.* 2014, 11, pp. 11

[51] Nymark P, Catalán J, Suhonen S et al. , Genotoxicity of polyvinylpyrrolidone-coated silver nanoparticles in BEAS 2B cells. *Toxicology.* 2013, 313(1), pp. 38–48

[52] McCarthy J, Gong X, Nahirney D, Duszyk M, Radomski M, Polystyrene nanoparticles activate ion transport in human airway epithelial cells. *International Journal of Nanomedicine.* 2011, 6, pp. 1343–1356

[53] Bhattacharya K, Andón F.T, El-Sayed R, Fadeel B, Mechanisms of carbon nanotube-induced toxicity: Focus on pulmonary inflammation. *Adv Drug Deliv Rev.* 2013, 65(15), pp. 2087–2097

[54] Bhowmick R, Gappa-Fahlenkamp H., Cells and Culture Systems Used to Model the Small Airway Epithelium. *Lung.* 2016, 194(3), pp. 419–428

[55] Frohlich E., Comparison of conventional and advanced in vitro models in the toxicity testing of nanoparticles. *Artificial cells, nanomedicine, and biotechnology.* 2018, pp. 1–17

[56] Clippinger A.J, Allen D, Behrsing H et al. , Pathway-based predictive approaches for non-animal assessment of acute inhalation toxicity. *Toxicology in Vitro.* 2018, 52, pp. 131–145

[57] Gruenert D.C, Finkbeiner W.E, Widdicombe J.H, Culture and transformation of human airway epithelial cells. *American Journal of Physiology — Lung Cellular and Molecular Physiology.* 1995, 268(3), pp. L347–L360

[58] Jeannet N, Fierz M, Schneider S et al. , Acute toxicity of silver and carbon nanoaerosols to normal and cystic fibrosis human bronchial epithelial cells. *Nanotoxicology.* 2016, 10(3), pp. 279–291

[59] Hussain S, Thomassen L.C, Ferecatu I et al. , Carbon black and titanium dioxide nanoparticles elicit distinct apoptotic pathways in bronchial epithelial cells. *Part Fibre Toxicol.* 2010, 7, pp. 10

[60] Herzog E, Byrne HJ, Casey A et al. , SWCNT suppress inflammatory mediator responses in human lung epithelium in vitro. *Toxicology and Applied Pharmacology.* 2009, 234(3), pp. 378-390

[61] Schlinkert P, Casals E, Boyles M et al. , The oxidative potential of differently charged silver and gold nanoparticles on three human lung epithelial cell types. *Journal of Nanobiotechnology.* 2015, 13(1), pp. 1

[62] Jing X, Park JH, Peters TM, Thorne P.S Toxicity of copper oxide nanoparticles in lung epithelial cells exposed at the air-liquid interface compared with in vivo assessment. *Toxicology in vitro: An international journal published in association with BIBRA.* 2015, 29(3), pp. 502-511

[63] Ghosh M, Öner D, Duca R.-C et al. , Cyto-genotoxic and DNA methylation changes induced by different crystal phases of TiO2-np in bronchial epithelial (16-HBE) cells. *Mutation Research/ Fundamental and Molecular Mechanisms of Mutagenesis.* 2017, 796, pp. 1–12

[64] Val S, Hussain S, Boland S, Hamel R, Baeza-Squiban A, Marano F, Carbon black and titanium dioxide nanoparticles induce pro-inflammatory responses in bronchial epithelial cells: Need for multiparametric evaluation due to adsorption artifacts. *Inhal Toxicol.* 2009, 21, pp. 115–122

[65] Forbes B, Shah A, Martin G.P, Lansley A.B, The human bronchial epithelial cell line 16HBE14o– as a model system of the airways for studying drug transport. *International Journal of Pharmaceutics.* 2003, 257(1), pp. 161–167

[66] Siegrist K.J, Reynolds S.H, Kashon M.L et al. , Genotoxicity of multi-walled carbon nanotubes at occupationally relevant doses. *Part Fibre Toxicol.* 2014, 11(1), pp. 6

[67] Ng C.T, Li J.J, Gurung R.L, Hande M.P, Ong C.N, Bay B.H, Toxicological profile of small airway epithelial cells exposed to gold nanoparticles. *Exp Biol Med (Maywood).* 2013, pp. 238


STANDARDSISO.COM : Click to view the full PDF of ISO/TR 21624:2020

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[68] Sisler J.D, Pirela S.V, Shaffer J et al. Toxicological Assessment of CoO and La2O3 Metal Oxide Nanoparticles in Human Small Airway Epithelial Cells. *Toxicological sciences: An official journal of the Society of Toxicology.* 2016, 150(2), pp. 418–428

[69] Mihalchik A.L, Ding W, Porter D.W et al. , Effects of nitrogen-doped multi-walled carbon nanotubes compared to pristine multi-walled carbon nanotubes on human small airway epithelial cells. *Toxicology.* 2015, 333, pp. 25–36

[70] Lu X, Miousse I.R, Pirela S.V, Melnyk S, Koturbash I, Demokritou P, Short-term exposure to engineered nanomaterials affects cellular epigenome. *Nanotoxicology.* 2016, 10(2), pp. 140–150

[71] Luanpitpong S, Wang L, Castranova V, Rojanasakul Y, Induction of stem-like cells with malignant properties by chronic exposure of human lung epithelial cells to single-walled carbon nanotubes. *Part Fibre Toxicol.* 2014, 11(1), pp. 22

[72] Chatterjee N, Yang J, Choi J, Differential genotoxic and epigenotoxic effects of graphene family nanomaterials (GFNs) in human bronchial epithelial cells. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis.* 2016, 798–799, pp. 1–10

[73] Xia T, Hamilton R.F, Bonner J.C et al. , Interlaboratory evaluation of in vitro cytotoxicity and inflammatory responses to engineered nanomaterials: the NIEHS Nano GO Consortium. *Environmental health perspectives.* 2013, 121(6), pp. 683–690

[74] Kumbiçak Ü, Çavaş T, Çinkiliç N, Kumbiçak Z, Vatan Ö, Yilmaz D., Evaluation of in vitro cytotoxicity and genotoxicity of copper–zinc alloy nanoparticles in human lung epithelial cells. *Food and Chemical Toxicology.* 2014, 73, pp. 105–112

[75] Duan W.-X, He M.-D, Mao L et al. , NiO nanoparticles induce apoptosis through repressing SIRT1 in human bronchial epithelial cells. *Toxicology and Applied Pharmacology.* 2015, 286(2), pp. 80–91

[76] George S, Pokhrel S, Xia T et al. , Use of a rapid cytotoxicity screening approach to engineer a safer zinc oxide nanoparticle through iron doping. *ACS Nano.* 2010, 4(1), pp. 15–29

[77] Capasso L, Camatini M, Gualtieri M, Nickel oxide nanoparticles induce inflammation and genotoxic effect in lung epithelial cells. *Toxicol Lett.* 2014, 226(1), pp. 28–34

[78] Huang C.-C, Aronstam R.S, Chen D.-R, Huang Y.-W, Oxidative stress, calcium homeostasis, and altered gene expression in human lung epithelial cells exposed to ZnO nanoparticles. *Toxicology in Vitro.* 2010, 24(1), pp. 45–55

[79] Veranth J.M, Kaser E.G, Veranth M.M, Koch M, Yost G.S, Cytokine responses of human lung cells (BEAS-2B) treated with micron-sized and nanoparticles of metal oxides compared to soil dusts. *Part Fibre Toxicol.* 2007, 4, pp. 2

[80] Park E.-J, Yi J, Chung K.-H, Ryu D.-Y, Choi J, Park K, Oxidative stress and apoptosis induced by titanium dioxide nanoparticles in cultured BEAS-2B cells. *Toxicol Lett.* 2008, 180(3), pp. 222–229

[81] Huang C.C, Aronstam R.S, Chen D.R, Huang Y.W Oxidative stress, calcium homeostasis, and altered gene expression in human lung epithelial cells exposed to ZnO nanoparticles. *Toxicology in vitro: An international journal published in association with BIBRA.* 2010, 24(1), pp. 45–55

[82] Eom H.-J, Choi J, Oxidative stress of silica nanoparticles in human bronchial epithelial cell, Beas-2B. *Toxicology in Vitro.* 2009, **23**(7), pp. 1326–1332

[83] Eom H.-J, Choi J SiO(2) Nanoparticles Induced Cytotoxicity by Oxidative Stress in Human Bronchial Epithelial Cell, Beas-2B. *Environmental Health and Toxicology.* 2011, 26, pp. e2011013

[84] Heng B.CZhao XXiong SWoei Ng KYin-Chiang Boey FSay-Chye Loo J. Toxicity of zinc oxide (ZnO) nanoparticles on human bronchial epithelial cells (BEAS-2B) is accentuated by oxidative stress. *Food and Chemical Toxicology.* 2010, 48(6), pp. 1762–1766


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