J. Verbr. Lebensm. 3 (2008): 319 – 329
1661-5751/08/030319-11
DOI 10.1007/s00003-008-0356-2
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Journal für Verbraucherschutz und Lebensmittelsicherheit
Journal of Consumer Protection and Food Safety

In Vitro Exposure Systems and Bioassays for the Assessment of Toxicity of Nanoparticles to the Human Lung

H.-R. Paur, S. Mülhopt, C. Weiss, and S. Diabaté

Forschungszentrum Karlsruhe GmbH, Institut für Technische Chemie (ITC-TAB) und Institut für Toxikologie und Genetik (ITG), Karlsruhe

Correspondence to: Dr. Hanns-R. Paur, Forschungszentrum Karlsruhe GmbH, Institut für Technische Chemie (ITC-TAB), Postfach 3640, 76021 Karlsruhe, Germany, Tel: +49 7247 82-3029, Fax: +49 7247 82-4303,
E-Mail: paur@itc-tab.fzk.de

Received: February 7, 2008; accepted: February 13, 2008
Online First 5 August 2008

Abstract: The rapid development of nanotechnology requires the production of nanoparticles which are found in numerous novel products. To reduce the number of animal tests during the assessment of lung toxicity of airborne nanoparticles *in vitro* exposure systems and lung specific bioassays have been developed. The reproducible application of bioassays for exposure of lung cells at the air-liquid interface promises a higher efficiency and cost reductions in toxicological testing. Despite significant progress of the exposure technology and fundamentals of bioassays a stringent validation of the in vitro versus in vivo tests is still lacking.

Zusammenfassung: Die schnelle Entwicklung der Nanotechnologie erfordert die Produktion von Nanopartikeln, die mittlerweile in zahlreichen neuen Produkten eingesetzt werden. Um die Anzahl der Tierversuche bei der Prüfung auf Lungentoxizität zu vermindern, wurden *in vitro* Expositionsverfahren und lungenspezifische Bioassays entwickelt. Die reproduzierbare Anwendung von Bioassays, die auf der Exposition von Lungenzellen an der Luft-Flüssigkeitsgrenzschicht beruhen, verspricht Effizienzgewinne und Kostenminderung bei toxikologischen Tests. Trotz deutlicher Fortschritte bei der Expositionstechnologie und bei den Grundlagen der Bioassays fehlt noch immer eine stringente Validierung der *in vitro* Tests gegenüber den *in vivo* Verfahren.

1. Introduction

Engineered nanoparticles are the "building bricks" of nanotechnology and thus play an important role in the present and future development of this technology. Nanotechnology develops rapidly and numerous "nano-products" are already on the market. Novel nano-products among many others are nanostructured catalysts and sensors, nano-particulate electrodes and solar cells, sun-screens, stain-repellent coatings or novel cancer drugs. An important driving force is the expected higher efficiency of already existing products by applying principles of nanotechnology e.g. by reduction of particle size. By coating the surface properties of nanoparticles can be varied over a wide range and thereby their application possibilities. At the same time their transport and biological properties may change. Self organisation of slightly "intelligent" nanoparticles was yesterday a science fiction topic, but may lead to novel biological effects in the near future.

During their specific application nanoparticles are frequently suspended in liquids or bound in solid matrices. Thus important routes of uptake should be via the skin and the gastro-intestinal tract. However, nanoparticles may become airborne during manufacturing, packaging and application, by careless handling or due to thermal treatment during waste incineration and accidents. In the aerosol form they can interact with human lung tissue, which is the largest surface the human body exposes to the ambient air.

Studies on lung diseases due to airborne particles such as industrial mineral dusts, asbestos and radioactive aerosols have been conducted already 50 years ago (Piekarski, 2006). Stringent limit values and restrictions have meanwhile been set for known particulate carcinogens. More recently certain particle fractions (PM_{10}, $PM_{2,5}$) in ambient air have been associated with adverse health effects (Pope, 2002). European and German legislation has therefore reduced the daily limit value for the PM_{10} fraction of ambient particles to 50 µg per m^3.

The German general limit value for exposure of workers to fine dust ("A-Staub") in workplaces was recently set to 3 mg/m^3. However, this general limit value does not apply to soluble, ultrafine (< 100 nm) and coarse particle fractions. For a number of hazardous substances specific limit values apply, which override the general limit value. German legislation strictly relies on mass based limit values. Nevertheless it is acknowledged, that ultrafine particles (UFP) are ubiquitous at workplaces. These ultrafine particles are frequently characterized by measuring

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Tab. 1 Summary of advantages and disadvantages of submerged exposure and air-liquid-interface (ALI) exposure of cell cultures towards particles.

	Submerged Exposure	Air-Liquid-Interface Exposure
Advantages	• Simple • Fast toxicity screening with high numbers of tests possible	• Better simulation of inhalation systems • Unchanged aerosol • Precise dose determination
Disadvantages	• Particle collection necessary • Suspension in medium changes particle properties • Unknown dose because the system is colloidal	• Complex system , including sampling generation, characterization, and conditioning of the aerosol, cell conditioning

the number concentration of particles ($1/cm^3$), as their mass is difficult to detect by gravimetric methods. The use of number concentrations of nanoparticles for legislation and the measurement of very low mass concentrations are challenging due to several reasons: Firstly no absolute calibration standard for particle number concentration is available. Dedicated round robin tests show the accuracy and comparability of measurements of number concentrations by different instruments must be improved (Asbach et al., 2007). Secondly a dedicated sample preparation (dilution, heating) is required, to avoid the formation of nanoparticles and filter artefacts due to condensation from the gas phase. Thirdly the measurement devices are costly, require highly skilled personnel and are not widely available.

For the assessment and evaluation of the toxic characteristics of an inhalable material, such as a gas, volatile substance or aerosol/particulate the inhalation toxicity is usually determined by animal experiments according to the OECD-guidelines. As a result of these tests the LC_{50} (median lethal concentration) is derived, that can be "expected to cause death during exposure or within a fixed time after exposure in 50 per cent of animals exposed for a specified time". Various OECD-guidelines for testing acute and chronic toxicity are available, depending on the required information. The determination of repeated dose inhalation toxicity requires long term studies employing large numbers of test animals, followed by extensive histological examination of tissue samples (e.g. Pott und Roller, 2003). Besides moral concerns, the high costs of these animal studies permit them only for a limited number of chemicals. Dedicated strategies have been developed to reduce the number of animal experiments for the implementation of the European Guideline REACH (***R***egistration, ***E***valuation and ***A***uthorization of ***Ch***emicals) (Lahl, 2005). *In vitro* test systems have been established for the determination of skin absorption (OECD TG 428), for skin corrosion (OECD TG 431) and acute eye irritation (OECD TG 405). No *in vitro* standard method, however, has yet been established for acute inhalation toxicity (OECD TG 403).

In this paper we review the state of the art of *in vitro* exposure systems and bioassays for the assessment of toxicity of nanoparticles to the human lung. Firstly we discuss various attempts to design *in vitro* exposure systems, which resemble the conditions in the human lung and allow the reproducible exposure of lung cells towards particles. Secondly we summarize the bioassays, which are available for the determination of end points, describing the biological effects due to lung-particle interaction. In the conclusions of this paper we discuss the challenges of using *in vitro* exposure studies during the risk assessment of air-borne nanoparticles and compare their advantages and limits to the established procedures.

2. State of the art of *in vitro* exposure systems

In recent review articles about test methods for nanotoxicology (Nel et al., 2006; Oberdörster et al., 2005) the authors recommend a staged strategy to study the potential toxicological effects of nanoparticles at first by *in vitro* methods using cell cultures of relevant target cells. After this, toxicological tests should be performed on more complex cell cultures such as co-cultures, on ecological relevant species and finally by animal experiments. The possibility to use human cell lines as target cells, which supply well reproducible results within short time and allow conclusions on molecular mechanism, are the advantages of *in vitro* tests. A large number of standardised test kits and cell lines are meanwhile available. In addition lab automation has greatly reduced the cost of large test series.

Particles can be applied to lung cells by two methods: (i) preparation of a particle suspension in medium and exposure of immersed cells or (ii) sampling or generation of an aerosol and exposure of cells at the air-liquid interface (ALI). Both methods exhibit advantages and disadvantages which will be discussed in the following sections and are summarised in Table 1.

3. Submerged exposure

In this exposure method the cell culture is submerged in the medium, which contains nutrients and the suspended nanoparticles. After the exposure the cells are subjected to bioassays. For toxicity testing of nanoparticles in submerged culture, it is essential to characterize the dispersion state of the nanomaterial. Since nanoparticles tend to agglomerate in physiological media, substances such as broncheoalveolar lavage fluid (BALF) or constituents of BALF like dipalmitoyl phosphatidylcholine (DPPC), protein or combinations of both are added to enhance the dispersion of the nanomaterial (Sager et al., 2007). Nevertheless, water-insoluble agglomerates will deposit on the cell layer by sedimentation when they are large enough and the exposure time is sufficient. In case agglomeration dominates, most of the particle mass thus will get into direct contact with the cells. If the

nanomaterial contains water-soluble components, the material will stay in the medium over the exposure time together with the primary nanoparticles which do not sediment. Dose units usually given in μg/ml are only valid for comparing effects of soluble compounds or nanoparticles that completely stay in suspension. The dose given in $\mu g/cm^2$ should only be used for particles that completely sediment on the cell layer. In most cases, however, the particle suspensions show a mixed behaviour, so both dose units should be considered. Medium components may also affect the reactivity and cellular uptake of the particles. For example, it has been shown that pre-treatment of quartz particles with DPPC fully suppressed its cytotoxicity and apoptotic activity (Gao et al., 2001). A more detailed discussion of the complexity and pitfalls of dose determination in submerged exposure of cells to nanoparticles are provided by Teeguarden et al. (2007). As the particles are not directly applied to the cells the amount of particles which finally reach the cell culture remains unknown unless radioactive labelled particles are used and quantified after the exposure. In summary the submerged exposure is suitable for qualitative examination of acute toxicity and for screening of the biological effects of cell cultures to nanoparticles.

4. Exposure at the air-liquid interface

By exposing the cell culture at the air-liquid interface (ALI) the cells are directly contacted by nanoparticles which are applied as an aerosol, thus avoiding the ambiguity of the dose determination in submerged exposure. The advantage of the exposure at the air-liquid interface is that the particles remain unchanged before deposition. The particles including their water-soluble components directly get in contact with the thin aqueous layer on the cells. The thickness of the aqueous layers ranges from approximately 8 μm in the bronchial region to less than 0.2 μm in the alveolar region (Weibel, 1963; Bastacky et al., 1995). If the requirements are to detect any adverse consequences of inhalation of a particular aerosol under near-realistic conditions while preventing animal experiments, an exposure of a cell layer at the air-liquid interface is a promising alternative.

The ALI-exposure methods are technically challenging because (i) a defined aerosol at constant composition and flow has to be generated and (ii) the temperature and humidity conditions for the cells should be held at constant physiological level for the period of exposure. The dosage to the cell layer may be described by the concentration of the particle mass or particle number in the aerosol, however, it is difficult to determine the particle mass or number deposited on the cell layer during an experiment in which the cell layer is required for the analysis of biological parameters. The deposited mass, which depends on the particle size and the flow rate, has to be determined in separate experiments (Tippe et al., 2002; Mülhopt et al., 2007). If the deposited mass is not sufficient to induce a biological effect in the cell layer after a short exposure time, it should be possible to prolong the exposure time in order to enhance the dose.

Several systems have been reported for the ALI-exposure of cultured cells or isolated tissue to a test atmosphere:

1) Some laboratories applied a periodical change from a submerged to an air-liquid cell exposure. This is performed by rolling flasks on a rocker-platform or by tilting the culture dishes forth and back, thus allowing direct contact of the cells to the test atmosphere which is passed through the flask or over the dish (Le Prieur et al., 2000; Muckter et al., 1998; Rusznak et al., 1996).
2) For long-term exposure (more than a few minutes) at the air-liquid interface the cells have to be supplied with water and nutrients, e.g. by growing on hydrated collagen gels (Zamora et al., 1986) or on porous membranes (Voisin et al., 1977; Whitcutt et al., 1988). In this biphasic culture system the apical surfaces of the cells are in contact to air or aerosol and the basolateral surfaces are provided with water and nutrients from below (basal feeding). This configuration reflects the condition of airway epithelial cells *in vivo* and allows the best contact of cells with a test atmosphere compared to the methods mentioned before. Today membrane systems of different pore size, pore diameter or membrane material are commercially available.

Although the liquid in the apical compartment is removed, a very thin layer of liquid will remain above the cells similar to the *in vivo* situation. For optimal culture conditions, the chamber in which the cells are exposed has to be warmed to 37 °C and humidified to 80–90 % relative humidity. Carbon dioxide must be added to a final concentration of 5 % in order to maintain the pH value of sodium hydrogen carbonate-buffered media. This can be circumvented by using e.g. HEPES-buffered media that maintain the pH value at normal air.

Exposure chambers for cell cultures were first described by Voisin and Wallaert (1977). Wallaert and Voisin (1992) have applied this chamber for test atmospheres mainly containing gaseous substances. The test atmosphere is drawn into a temperature- and humidity-conditioned chamber using a vacuum pump and the filter inserts with the test cells sitting in culture plates with medium are placed in this chamber (Fig. 1). The Voisin chamber has been used to study the effects of ozone (Arsalane et al., 1995), nitrogen dioxide (Wallaert and Voisin, 1992) and tobacco smoke (Dubar et al., 1993) with alveolar macrophages obtained by bronchoalveolar lavage from humans and animals, of ozone with human BEAS-2B epithelial cells (Mögel et al., 1998), of NO_2 with primary bovine alveolar macrophages and murine RAW-264.7 macrophages (Höckele et al., 1998). Some laboratories used designs similar to the Voisin system, e.g. for studying the effects of diesel exhaust with human BET-1A epithelial cells (Abe et al., 2000) or with A549 cells (Cheng et al., 2003). The effects of cigarette smoke were studied with NCI-H292 bronchial epithelial cells in a similar system (Phillips et al., 2005). The inflammatory potential of synthetic nanoparticles consisting of Cu, Ni and V and produced by an electrospray method was also studied with human A549 cells (Cheng, 2004). Primary human bronchial epithelial cells (HBE) isolated from large airways resected during surgery have been used to study the

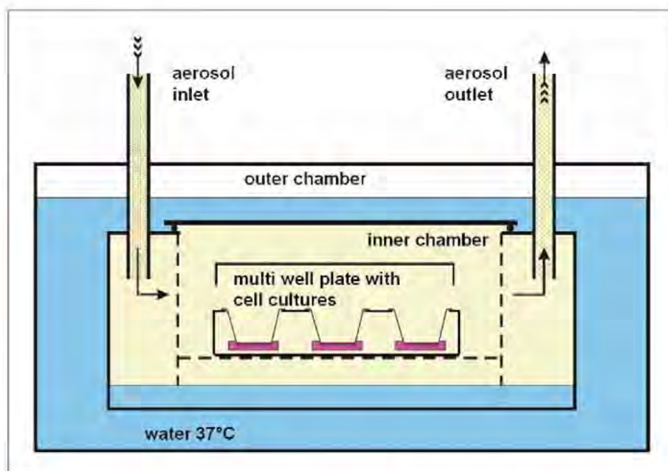

Fig. 1 Voisin chamber for the exposure of cells to gas and particulate matter. The multi well plate placed on a perforated metal plate above a water level for humidification contains Transwell membrane inserts with the cell culture and medium.

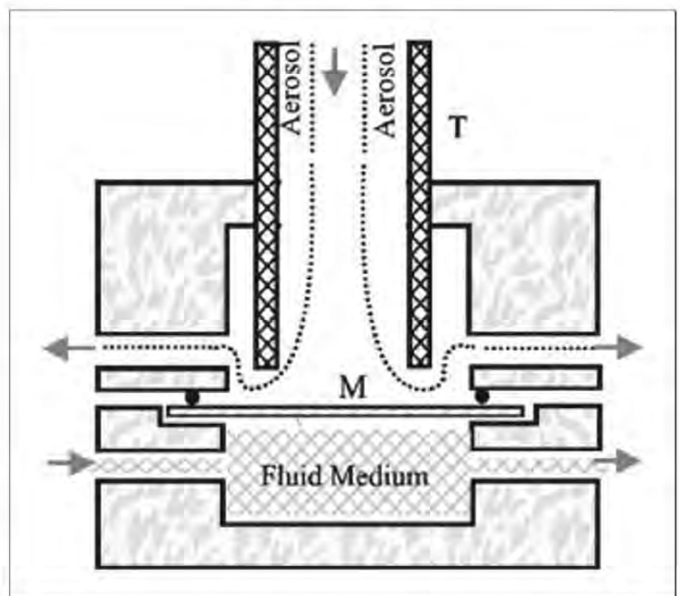

Fig. 2 Cross section of a cylindrical perfusion cell. T: inflow tube; M: membrane separating the upper and lower compartments and support of the cell layer, identical with the stagnation plate. The dotted lines indicate streamlines (Tippe et al, 2002).

effects of cigarette smoke (Beisswenger et al., 2004). The disadvantage of the Voisin chamber is its poorly defined deposition geometry, which leads to a non uniform dose distribution within the chamber. Therefore more advanced systems have been developed, which promise a better reproducibility of the exposure.

A recently described *in vitro* exposure device is based on a modified perfusion unit from MINUCELL (Tippe et al., 2002; Bitterle et al., 2006). The geometric design of the chamber permits the formation of a radially symmetric stagnation point flow (Fig. 2). With this arrangement, 75–1000 nm particles are deposited spatially uniform at a constant deposition rate of 2% (Tippe et al., 2002). Online analysis of particle number and particle size distribution together with the known deposition rate allow the calculation of the deposited particle mass over time. This exposure system was used to study the effects of freshly generated ultrafine carbon particles on confluent A549 cells at the air-liquid interface (Bitterle et al., 2006). The exposure system was operated with membranes of 47 mm diameter at an aerosol flow of 250 ml/min, which was well tolerated by the cells. Biological endpoints were: cell viability, transcription of interleukin-6 (IL-6), IL-8 and heme oxygenase-1 (HO-1) measured after a 6 h aerosol exposure and a 1 h post-incubation time under submerged conditions. For a mid-dose exposure a deposition of 87 ng/cm^2 was calculated, which resulted in a significantly increased transcription of the anti-oxidant and stress response protein HO-1. Viability and transcription of IL-6 and IL-8 were not changed in comparison to clean air exposed cells.

The CULTEX exposure system is a patented method that has been developed by Aufderheide and Mohr (1999). Nowadays, exposure systems based on the CULTEX method are commercially distributed by VitroCell Systems (Gutach, Germany). In the CULTEX exposure system the aerosol flows directly to the cell culture surface by an inlet tube ending above it. Aufderheide and co-workers demonstrated in several studies the application of this system for different test atmospheres, target cells and biological endpoints (Aufderheide, 2005). The CULTEX system has been employed by other research groups to study the effects of diesel exhaust fumes on A549 cells and rat alveolar macrophages (Seagrave and McDonald, 2004) or of cigarette smoke on A549 cells (Fukano et al., 2004).

We used a modified CULTEX system to study the effects of a fly ash model aerosol in BEAS-2B epithelial cells co-cultured with THP-1 macrophages (Mülhopt et al., 2007). The Karlsruhe exposure system is mobile and fully automated. The flow chart is shown in figure 3, a photograph of an exposure in outdoor conditions in figure 4.

The main components in the sampling and exposure system are:

1. *Sampling of the aerosol:* By passing a size selective inlet or a cyclone individual large particles are removed. These would contribute considerably to the deposited mass in a non-reproducible manner and therefore impair the dose measurement. Additionally large particles might disturb the performance of the bioassay by non homogenous deposition of toxic materials.
2. *Conditioning of the aerosol:* To avoid drying of the cell cultures the gas temperature is adjusted to 37±2°C and humidified by injection of water vapour to a relative humidity of > 85 %, which resembles the conditions of the lower regions of the human lung. For each cell culture an extra aerosol sample is drawn from the reactor. The advantage of this separation of aerosol flows is to avoid fluctuations by linking the chambers and particle losses due to dividing the flows. One of the three aerosol flows is filtered before

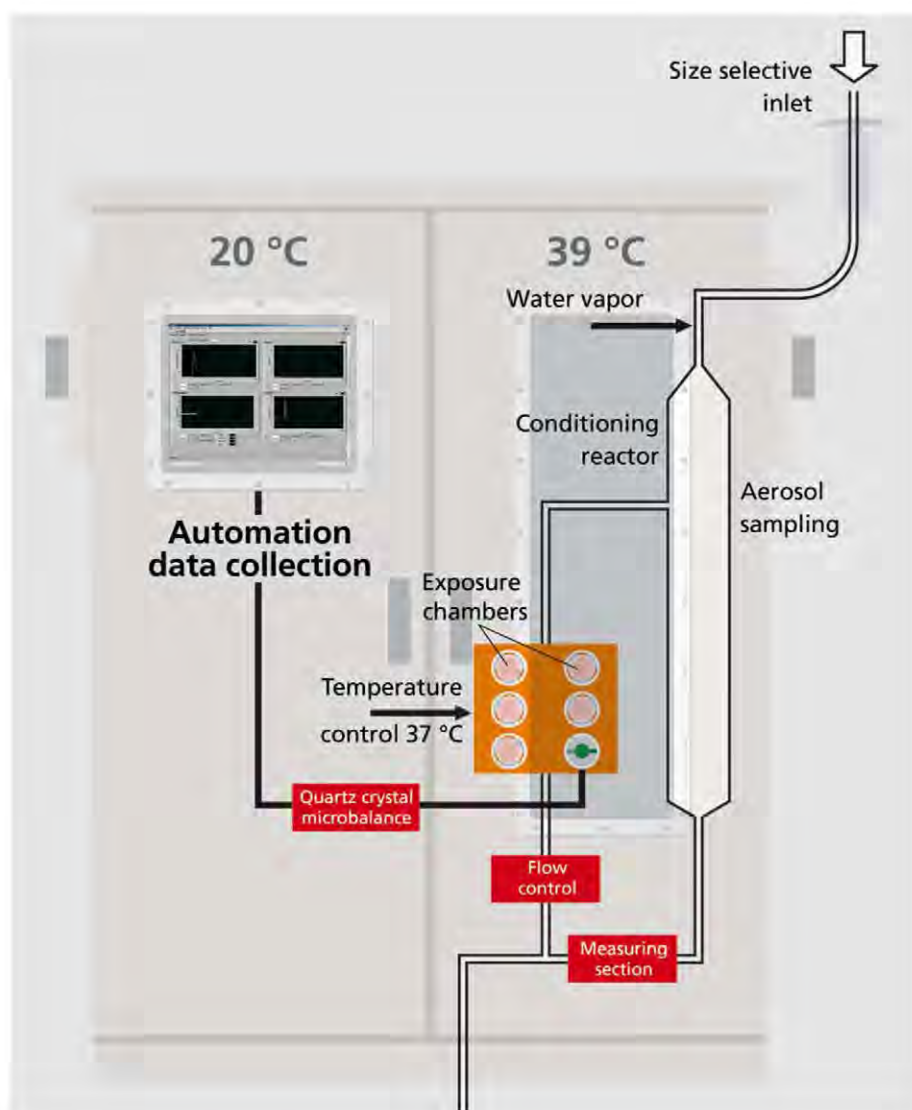

Fig. 3 Flow chart of the Karlsruhe exposure system.

entering the exposure unit to provide a control which is simultaneously exposed to the particle free gas.

3. *Exposure of the lung cells:* The exposure unit (Vitrocell Systems, Gutach) consists of an upper part which distributes the gas to three exposure chambers. The lower unit of the exposure system accommodates the cell culture on Transwell® inserts (Corning, Wiesbaden) with medium underneath. It is surrounded by a circulating water bath assuring the system temperature at 37 °C. The aerosol flows perpendicular through a concentrical steel funnel towards the cell layer and changes its flow direction at the stagnation point above the cells. Then the aerosol leaves the exposure chamber at its outside rim and enters the off gas line. Each gas flow is regulated by a separate mass flow controller downstream of the exposure chambers, which is set to 100 ml/min.
4. *Measuring the dose:* For the determination of the dose-response relationship the accurate knowledge of the dose is an essential question. Two methods are used for dose determination in the Karlsruhe exposure system: Firstly an ultrafine aerosol of sodium fluorescein particles is used to determine the deposited mass of an aerosol at the air-liquid interface (Diabaté et al., 2008; Mülhopt et al., 2007). Secondly a novel online measurement technique has been tested: A quartz crystal microbalance, which is placed in the exposure chamber instead of the Transwell membrane insert, is exposed to the aerosol in the same way as the cell cultures. The deposited mass per area unit is monitored online as a function of exposure time.

Here we briefly summarize the effects; the relevant endpoints of the particle effects are discussed below. The flow rate as well as the transportation to the exposure system were demonstrated to be well tolerated by the cells and induced no signs of acute cytotoxicity after exposure to clean air or fly ash aerosol for up to 6 h and a 20 h post-incubation period under submerged conditions (Fig. 5). The observed increase in the pro-inflammatory response detected by the release of interleukin-8

Fig. 4 The Karlsruhe exposure system for lung cells in outdoor conditions.

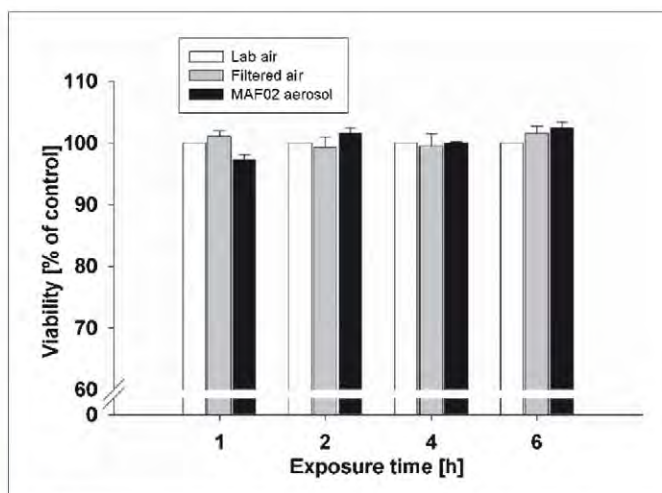

Fig. 5 Viability of test cells after exposure to humidified air at the air-liquid interface. Co-cultures of BEAS-2B and differentiated THP-1 cells grown on 24 mm Transwell membranes #3450 were exposed to filtered air or MAF02 fly ash aerosol of 0.62 mg/ m^3 at 100 ml/min for 1, 2, 4, and 6 h, or to lab air without flow for the same time periods. Viability was measured by the alamarBlue assay after a post-incubation period of 20 h. Samples exposed to lab air without any flow were set at 100 %. The results are the mean ± s.e.m. (vertical bars) of three experiments with two or three replications each (Diabaté et al., 2008).

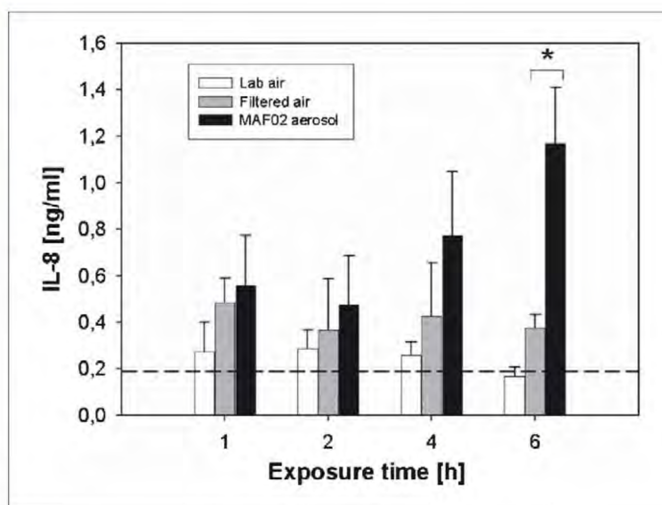

Fig. 6 IL-8 release after exposure to fly ash. Co-cultures of BEAS-2B and differentiated THP-1 cells were exposed to filtered air or MAF02 fly ash aerosol 0.62 mg/ m^3 for 1, 2, 4, and 6 h at 100 ml/min and analyzed for the release of IL 8 after 20 h. Controls were exposed to lab air without any flow for the same time periods. The dashed line indicates baseline IL-8 levels of co-cultures kept submerged. The results are the mean ± s.e.m. (vertical bars) of three independent experiments (* $p < 0.05$ compared to cells exposed to filtered air for 6 h) (Diabaté et al., 2008).

(IL-8) into the culture medium after exposure to fly ash aerosol at 0.62 mg/ m^3 is therefore mainly attributed to the deposited particles (Fig. 6). Additionally, an increased expression of the anti-oxidative heme oxygenase-1 (HO-1) protein in the fly ash-exposed cells was observed (Fig. 7). The responses to the fly ash aerosol increased time-dependently. As the deposited dose increases with time in this system, there seems to be clear dose-dependent biological responses.

5. Bioassays for assessment of lung toxicity

5.1 Cellular models for *in vitro* exposure

Both, submerged and air-liquid exposure, employ cell culture models of either freshly isolated cells from human or animal lungs or permanent cell lines. Cell culture methods are more and more accepted because of the advantage of quicker throughput compared to *in vivo* tests with animals. A comprehensive review on cell culture models of the respiratory tract is given by Steimer et al. (2005).

To build a model of the respiratory epithelium one has to consider the cell types which contribute to the majority of the lung epithelial surface area. These are the columnar cells in the airways and alveolar type-I cells in the alveoli. The surfactant-producing alveolar type-II cells, however, constitute about 60 % of the alveolar epithelial cell number but they cover

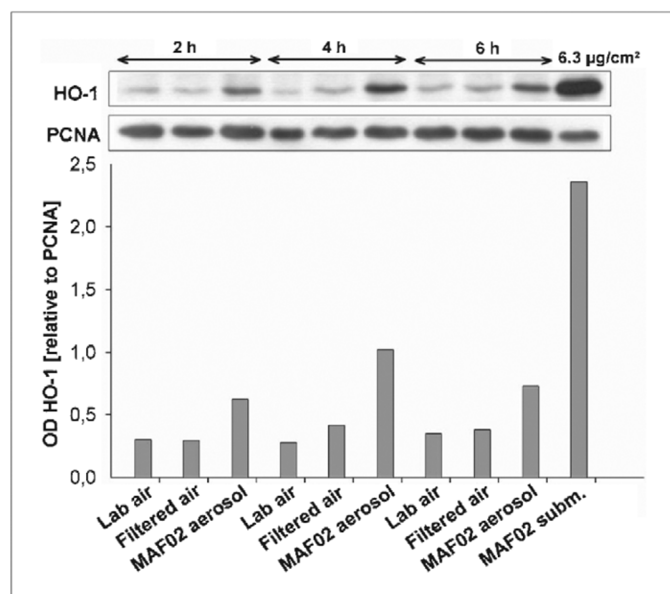

Fig. 7 Effect of fly ash aerosol on the expression of heme oxygenase-1 (HO-1). Co-cultures of BEAS 2B and differentiated THP-1 cells were exposed to filtered air or fly ash aerosol MAF02 of 0.62 mg/m^3 for 2, 4 and 6 h and compared to co-cultures exposed to lab air without flow. A positive control of BEAS-2B cells treated with 6.3 µg/cm^2 MAF02 under submerged conditions for 20 h is shown in the last lane. The Western blot was quantified by relating the optical density of HO-1 to that of PCNA, which was used as a loading control. The Western blot represents three independent experiments (Diabaté et al., 2008).

less than 5% of the alveolar surface area of adult human lungs (Crapo et al., 1982). These cells are responsible for repair mechanism by proliferation and differentiation into alveolar type-I cells, which cover the majority of the alveolar space. The surfactant covering the alveolar epithelium contains phospholipids and specific surfactant proteins which cause the low surface tension of the lining fluid and thus prevent the collapse of the alveoli.

The epithelium of the upper conducting airways is comprised of three major cell types: ciliated, secretory and basal cells. The thickness of the epithelium is gradually decreasing in height from the large airways to the terminal bronchioles (Steimer et al., 2005) and is covered by a mucus gel layer.

In general, using primary cells obtained from the human lung is the best choice, however, primary cultures are less convenient and economic than the use of human cell lines. Moreover, primary cultures may vary in their biological responses due to genetic differences of the donors. Cell lines, however, carry the caveat that differences may exist between the transformed cells and normal lung cells *in vivo* regarding the phenotype and the biological responses. The benefits of using cell lines must therefore be balanced against any differences to the native phenotype.

An example for a human bronchial epithelial cell line is BEAS-2B a subclone of transformed normal adult bronchial epithelial cells derived by transfection of primary cells with SV40 early-region genes (Reddel et al., 1995). The cells express phenotypic characteristics of non-ciliated mucus-secreting epithelial cells (Clara cells). The BEAS-2B cell line also expresses differentiation characteristics of human lung explants grown on collagen with serum-free medium supplemented with growth factors. These cells may provide a good *in vitro* model which simulates the effects on the bronchial epithelial tissue due to inhalation of environmental pollutants (Nichols et al., 1995; Kinnula et al., 1994).

The alveolar epithelial cell line A549 is the most popular model for a human pulmonary epithelium. The type-II-like cells were obtained from lung carcinomatous tissue. Type-II cells are the progenitor cells for Type-I epithelial cells. They are able to synthesize surfactant proteins (Smith, 1977) and express most of the major constitutive and inducible drug metabolizing enzymes such as cytochrome P450 (CYP) forms found in lung epithelial cells *in vivo* (Foster et al., 1998; Sheets et al., 2004).

BEAS-2B and A549 cells have been used extensively to study the effects of inhaled environmental pollutants such as ozone (Mögel et al., 1998), ambient particulate matter PM_{10} (Kennedy et al., 1998), cigarette smoke (Fukano et al., 2006), diesel exhaust particles (Steerenberg et al., 1998) or residual oil fly ash (Samet et al., 1997).

Co-culture cell models are used to better represent the complexity of the lung epithelium. Epithelial cells co-cultured with macrophages for instance reflect the interaction of these two cell types in the pulmonary defence system (Diabaté et al., 2004; Diabaté et al., 2008; Tao and Kobzik, 2002; Wottrich et al., 2004). Moreover, so-called three-dimensional cellular models consisting of macrophages, epithelial and endothelial cells (Wottrich, 2003) or macrophages, epithelial and dendritic cells (Rothen-Rutishauser et al., 2005) have been established.

Most of the studies have been carried out with cells grown and exposed under submerged conditions. Only few applied an exposure at the air-liquid interface which has been reviewed above. Before applying a potential toxic aerosol to the cell layer one should investigate the effects when the cells are transferred from immerged to air-liquid conditions. In an immerged culture, the cells are hypoxic because an O_2 gradient is generated in the medium through the cellular consumption of O_2 (Halliwell and Gutteridge, 1999). After conversion from an immerged culture to an air-liquid interface the cells are thus exposed to a higher oxygen tension. During normal cell metabolism and oxidation-reduction processes reactive oxygen species (ROS) are continuously produced and the production of ROS depends on the oxygen tension. It has been shown in cultures of primary bovine tracheal epithelial cells that the level of intracellular ROS was enhanced after removal of medium and exposure at an air-liquid interface (Kondo et al., 1997; Kameyama et al., 2003). The authors demonstrated that the cellular glutathione (GSH) increased as an adaptive response to higher oxygen tension and that changes in the GSH system of cells grown at the air-liquid interface promote cell growth over a period of 6 days. GSH is an essential antioxidant and is present at millimolar concentrations in most cells. The ratio of oxidized glutathione disulfide (GSSG) to reduced GSH is furthermore involved in regulating signal transduction processes (Rahman et al., 2005).

Tab. 2 Biological endpoints indicating cellular responses after exposure to particles at the air-liquid interface or under submerged conditions.

Biological endpoints	Methods (examples)	Time and effort, sensitivity
Proliferation and viability	MTT, WST-1, AlamarBlue, LDH	Fast, reliable, response only at highly toxic concentrations
Oxidative stress (GSH status)	DNTP method	Cell lysis required, time consuming
Expression of new proteins indicating antioxidative and/or proinflammatory responses	Secreted proteins (e.g. IL-8) by ELISA	Fast, reliable, response only after several hours after exposure, time consuming
	Intracellular proteins (e.g. HO-1) by Western Blot, or ELISA	Cell lysis required, time consuming
Activation of genes coding for antioxidative and/ or proinflammatory proteins	Gene expression on mRNA level by RT-PCR	Cell lysis required, time consuming, very sensitive
	Using stably transfected reporter cells (e.g. GFP, luciferase), detection by optical methods	No cell lysis required, fast, low to medium sensitivity (GFP)
Genotoxicity	detection of 8-oxo-dG by ELISA, COMET assay	Cell lysis required, time consuming

5.2 Cellular responses to nanoparticle exposure

The biological responses of pulmonary cells exposed to nanoparticles do not only depend on the dose but also on their physical and chemical characteristics such as diameter, degree of agglomeration, chemical composition, reactivity of the surface and coating, and solubility in biological media. When nanoparticles get into contact with proteins located at the outer plasma membrane, they may induce changes in the molecular conformation of these proteins. Many of these proteins are receptors, which transmit external signals into the cell and a conformational change of the receptor may activate it leading to the onset of a cellular response. Once inside the cell, particles may induce intracellular oxidative stress by disturbing the balance between oxidant and anti-oxidant processes, e.g. the glutathione system.

It has been shown that particulate matter such as ambient and diesel exhaust particles exert biological effects *via* formation of oxygen species (ROS) and thereby inducing oxidative stress (Donaldson et al., 2005). According the hierarchical principle postulated by Nel et al. (2006), cells respond to oxidative stress by changing the level of intracellular glutathione (GSH) and induction of anti-oxidative and pro-inflammatory mechanisms, which aim to counteract potential deleterious consequences of ROS actions. If the antioxidative defence mechanisms are not effective enough, the exposure of pulmonary epithelial cells to inhaled toxicants will result in a severe injury namely cell death which is characterized by a decrease of mitochondrial metabolism, cell membrane disruption, and release of cytoplasmic proteins.

Methods for detection of important biological endpoints which may be applied after exposure of cells under ALI conditions are described in Tab. 2 and below.

Proliferation and viability assays: The proliferation of cells which may be increased or decreased after particle treatment is usually determined by their ability to reduce tetrazolium dyes such as MTT or WST-1 or by the AlamarBlue assay. These tests measure the activity of mitochondrial dehydrogenases by absorption or fluorescence spectrophotometric methods. A particle-induced increase of activity indicates an increase of cell proliferation. Decreases of activity may indicate loss of viability or reduction of proliferation of the cells. The enzyme lactate dehydrogenase (LDH) normally occurs intracellularly. Detection of LDH in the medium indicates a disruption of the plasma membrane which is a clear sign of cell death. All these tests detecting loss of viability are indicators of severe cell damage, which is not expected to occur at particle concentrations relevant in the environment or at occupational settings. Therefore it is necessary to detect cell responses at non-cytotoxic concentrations.

GSH status: The glutathione (GSH) metabolism is an important mechanism protecting the cell from oxidative stress. GSH is also an important protective antioxidant in the lung which is altered in the extracellular lining fluid of the lung in several inflammatory diseases (Hayes and McLellan, 1999; Rahman and MacNee, 2000). Glutathione also contributes to the detoxification of xenobiotics *via* phase II conjugation reactions. The GSH concentration decreases when the cells consume GSH for detoxification of ROS or RNS, and increases after additional production of new GSH. The GSH concentration can be measured photometrically e.g. by a kinetic test using the reduction of von 5,5'-dithio-bis(2-nitrobenzoe acid) (DTNB) by NADPH and glutathione reductase.

Antioxidative response: The antioxidative and stress response enzyme heme oxygenase-1 (HO-1) has been shown to be induced by several air pollution particles e.g. in BEAS-2B cells (Li et al., 2002 and 2003) as well as by cigarette smoke applied at the air-liquid interface of A549 cells (Fukano et al., 2006). The induction of HO-1 has been shown as a sensitive endpoint for exposure to fly ash particles (Diabaté et al., 2008). HO-1 protein can be detected by a specific enzyme-linked immuno assay (ELISA) or by the Western Blot method. This requires cell lysis since HO-1 is an intracellular protein.

Pro-inflammatory response: Particle exposure of lung cells may induce an inflammatory response, which is characterized by the release of pro-inflammatory cytokines and chemokines. These protein mediators are mainly produced by cells of the immune system but also by epithelial cells. A group of cytokines (IL-1, IL-6, TNF α) are called pro-inflammatory because they are produced by immune competent cells after activation by certain alarm signals and released into the blood system. The chemokine IL-8 is a powerful chemotactic factor synthesized by phagocytes and epithelial cells to recruit neutrophils into the injured tissue. The ELISA method is a suitable test to detect cytokines in the medium.

The activation of genes as the prerequisite for the synthesis of new proteins may be generally detected by isolation of the mRNA and measuring its gene-specific level by RT-PCR. Reporter genes, which detect the activation of a stress-related promoter gene by stable integration (transfection), may also be used as a more convenient method for detection of specific gene activation. The detection of the reporter gene Luciferase usually requires cell lysis. The application of fluorescent proteins e.g. GFP (green fluorescent protein) as reporter genes seems to be more rapid since no cell lysis is required. The activation of the specific gene may be observed directly by optical methods (Arenz et al., 2006).

Genotoxicity: Inhalation of carbon black by rats has induced the formation of 8-oxo-7,8-dihydro-2'-deoxyguanosin (8-oxo-dG) in the lungs (Gallagher et al., 2003). 8-oxo-dG, a modified nucleotide, is a well-known and commonly used biomarker of free radical-induced oxidative DNA damage. This DNA modification may induce point mutations, which are widely observed in mutated oncogenes and tumour suppressor genes, and is therefore associated with many diseases such as cancer and neurodegenerative diseases.

6. Conclusions and Outlook

In vitro exposure of lung cells at the air-liquid interface has a significant potential for screening the toxicological potential of unknown aerosols in conditions, which are similar to the human lung. This method would help to determine rapidly whether exposure to particulate matter can raise the incidence of adverse health effects in humans and would reduce the number of animal experiments to develop dose-response relationships of particle toxicity.

However, it should be mentioned that the toxic potential of a compound can not be evaluated by *in vitro* studies solely. Endpoints such as effects on pulmonary function or systemic effects can only be studied *in vivo* using animals or by controlled exposure of humans. However, accepted *in vitro* methods will help to determine rapidly if exposure to foreign agents can increase the incidence of an adverse health effect in humans. Additionally, they can be particularly valuable for understanding the underlying mechanisms by which health effects are induced.

To obtain reproducible data the *in vitro* test procedures, the cell models and a meaningful set of bioassays must be selected and standardised. A database of bioassay results must be gathered for a large number of industrial nanoparticles. Detailed comparison studies between *in vitro* and in animal tests are required to validate the data obtained in exposures at the air-liquid interface.

A large number of different types of nanomaterials exist due to different synthetic processes and applications. To evaluate the toxicological potential of a certain nanomaterial it would be helpful to introduce a concept of toxic equivalency factors (TEFs), which has already been developed for dioxin-like substances also spanning a large range of compounds. In general, 2,3,7,8-TCCD has been used as a reference compound and its TEF was set at 1 as the most potent substance. The TEFs of congeners were set in relation in dependence on their biological activity (van den Berg et al., 2006). In the case of nanomaterials, the establishment of TEFs would require one or several reference particles with known nanospecific properties such as particle size, shape, or agglomeration state, and other nanomaterials would be classified in relation to the reference particles.

7. Acknowledgement

The financial support of the Federal Ministry of Education and Research (BMBF) within the Project "NanoCare" (Project Code: 03X0021A) and by the Federal Institute for Risk Assessment (BfR), Germany, [BfR-ZEBET-1238-182] is acknowledged.

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