

Genotoxicity testing of different surface-functionalized SiO₂, ZrO₂ and silver nanomaterials in 3D human bronchial models

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Abstract Inhalation is considered a critical uptake route for NMs, demanding for sound toxicity testing using relevant test systems. This study investigates cytotoxicity and genotoxicity in EpiAirway™ 3D human bronchial models using 16 well-characterized NMs, including surface-functionalized 15 nm SiO₂ (4 variants), 10 nm ZrO₂ (4), and nanosilver (3), ZnO NM-110, TiO₂ NM-105, BaSO₄ NM-220, and two AlOOH NMs. Cytotoxicity was assessed by LDH and ATP assays and genotoxicity by the alkaline comet assay. For 9 NMs, uptake was investigated using inductively coupled plasma–mass spectrometry (ICP–MS). Most NMs were neither cytotoxic nor genotoxic in vitro. ZnO displayed a dose-dependent genotoxicity between 10 and 25 µg/cm². Ag₅₀.citrate was genotoxic at 50 µg/cm². A marginal but still significant genotoxic response was observed for SiO₂.unmodified, SiO₂.phosphate and ZrO₂.TODS at 50 µg/cm². For all NMs for which uptake in the 3D models could be assessed, the amount taken up was below 5% of the applied mass doses and was furthermore dose dependent. For in vivo comparison, published in vivo genotoxicity data were used and in addition, at the beginning of this study, two NMs were randomly selected for short-term (5-day) rat inhalation studies with subsequent comet and micronucleus assays in lung and bone marrow cells, respectively, i.e., ZrO₂.acrylate and SiO₂.amino. Both substances were not genotoxic neither in vivo nor in vitro. EpiAirway™ 3D models appear useful for NM in vitro

testing. Using 16 different NMs, this study confirms that genotoxicity is mainly determined by chemical composition of the core material.

Keywords Comet assay · Surface modifications · Silver nanoparticles · Silica nanoparticles · 3D bronchial models · Nanoparticle uptake

Introduction

As more and more nanotechnological applications are placed onto the market, assessing potential human health effects of nanomaterials (NMs) is becoming increasingly important. For airborne NMs, the major exposure route to consider is inhalation, where NMs can reach the bronchial and the alveolar surface. It frequently has been shown that NMs can elicit more pronounced toxic responses compared to their larger, conventional materials. For instance, titanium dioxide (TiO₂) nanoparticles induced a greater pulmonary inflammatory response in rats compared to fine TiO₂ materials (Oberdoerster et al. 2005).

The genotoxicity of NMs, which is of particular concern, is addressed in a number of studies (Gonzalez et al. 2008; Landsiedel et al. 2009; Oesch and Landsiedel 2012; Pfuhler et al. 2013). NMs are not expected to induce a new quality of genotoxicity, but they may elicit well-known effects, such as gene mutations, clastogenic or aneugenic effects by direct or indirect mechanisms (Oesch and Landsiedel 2012; Pfuhler et al. 2013; Magdolenova et al. 2014). With regard to testing, NMs deserve special attention when planning studies as assay selection as well as NM preparation and characterization are likely to become crucial for the outcome. Bacterial gene mutation assays may not be adequate for NM testing

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since bacteria usually do not take up NMs (Landsiedel et al. 2009; Butler et al. 2014). For instance, Al₂O₃ NMs were negative in the Ames test, but positive in the in vitro and in vivo micronucleus tests (MNTs) and in the in vivo comet assay (Balasubramanyam et al. 2009, 2010; Di Virgilio et al. 2010). Furthermore, due to their small size, NMs may readily pass biological barriers of eukaryotic organisms and accumulate in specific tissues. The biokinetics of NMs have to be taken into consideration when designing genotoxicity studies to ensure that the relevant target organs are being investigated.

By far most frequently, NM genotoxicity is investigated by comet assay and MNT (Oesch and Landsiedel 2012). Both assays can be performed in vitro or in vivo. Generally, in vitro genotoxicity testing can be applied in the first tier of comprehensive testing strategies, since all genotoxic mechanisms may be detected in vitro (Pfuhrer et al. 2013). Nevertheless, in vitro results should be confirmed in vivo since in vitro genotoxicity assays often lead to false positive results and overprediction (Oesch and Landsiedel 2012). Frequently, NMs may appear genotoxic in vitro, which is not confirmed in vivo. For instance, Maser et al. detected a genotoxic effect of SiO₂ NM in vitro, but not in vivo (Maser et al. 2015). Several studies recorded size dependency of the NM-mediated genotoxicity. For instance, small anatase TiO₂ (10, 20 nm) was genotoxic in the in vitro comet and micronucleus assays, while the larger-sized counterpart (200 nm) was not (Gurr et al. 2005), whereas other studies did not find differences in the genotoxic potential of Au nanoparticles with various sizes (Downs et al. 2012; Schulz et al. 2012). Sometimes, contradictory genotoxic data are reported for NMs, possibly resulting from differences of the test items, e.g., in respect to size, size distribution, or coating. Jacobsen et al. (2007) reported carbon black (Printex 90, mean particle size of agglomerates: 1.59 μm) to be positive in the comet assay in the FE1 Muta™ mouse lung epithelial cell line, whereas Zhong et al. (1997) detected no genotoxicity of a 37 nm carbon black in the comet assay in Chinese hamster V79 fibroblasts and Hel 299 human embryonic lung fibroblasts. A given NM may appear genotoxic in one study but not in another, even when the same assay is applied. Such inconsistent results are not as easily explainable. They may still be a result of incomplete material characterization (so that the NMs were actually not identical), of differences in the selected test systems (i.e., differences in cell lines), or of differences in testing or dispersion protocols. Since most studies only included a limited number of NMs, it becomes difficult to compare the results from different studies retrospectively. So far, only few studies tested a larger panel of NMs, such as Kermanizadeh et al. (2013) who assessed the cytotoxicity and genotoxicity of ten different NMs in human HK-2 cells.

The aim of this study was to investigate the genotoxicity of 16 well-characterized NMs using EpiAirway™ 3D human bronchial tissue models. This model consists of normal human tracheal and bronchial epithelial cells, which are differentiated into a pseudo-stratified 3D tissue containing cilia and mucus-producing goblet cells. Thereby, this model closely resembles the bronchiole epithelium of the respiratory tract and can be considered a physiologically relevant in vitro surrogate to investigate effects in this lung tissue upon inhalation exposure. NMs investigated in this study included 15 nm SiO₂ [functionalized with polyethylene glycol (PEG), amino, or phosphate, or without surface functionalization, i.e., unmodified], 10 nm ZrO₂ [functionalized with acrylate, PEG, amino, or trioxadecanoic acid (TODS)], 50 and 200 nm nanosilver functionalized with polyvinylpyrrolidone (PVP) and 50 nm nanosilver functionalized with citrate. Furthermore, we assessed ZnO NM-110, TiO₂ NM-105, BaSO₄ NM-220, and two different AlOOH (mean sizes 37 or 16 nm, respectively) as benchmark materials to link to the programs of the OECD Working Party on Manufactured Nanomaterials (WPMN).

We assessed cytotoxicity in the LDH (lactate dehydrogenase) and ATP (adenosine triphosphate) assays and in vitro genotoxicity in the alkaline comet assay. NM uptake was quantified for most of the investigated NMs using inductively coupled plasma mass spectrometry (ICP-MS). The results of this study were discussed in the context of available, i.e., published, in vivo data, if such data were existing. In addition, two NMs, which were randomly selected at the beginning of this study, i.e., ZrO₂.acrylate and SiO₂.amino, were additionally subjected to a 5-day short-term inhalation toxicity study (STIS) in male Wistar rats and subsequent comet assay using lung cells and MNT using bone marrow cells from treated animals.

Materials and methods

Chemicals

Most reagents were supplied by Sigma-Aldrich (Germany). Dimethyl sulfoxide (DMSO) was supplied by AppliChem (Germany), Hanks balanced salt solution (HBSS) and phosphate-buffered saline (PBS) from Biochrom AG (Germany), and agarose from Lonza (USA). Isofluorane (Isoba®) was purchased from Essex GmbH (Germany) and Pentobarbital (Narcoren®) from Merial (Germany).

NM synthesis and characterization

SiO₂.unmodified, SiO₂.amino, SiO₂.phosphate, and SiO₂.PEG were obtained from BASF SE (Germany), ZrO₂.acrylate, ZrO₂.TODS, ZrO₂.amino, ZrO₂.PEG from

CeraNovis AG (Germany) and nanosilver Ag.50.PVP, Ag.50.Citrate, and Ag.200.PVP from Bayer Technology Services (Germany). All of these 11 NMs were supplied in dispersion. The 5 benchmark NMs were supplied as powders, i.e., AlOOH I and II (Bayer Material Science, Germany), BaSO₄ NM-220 (Solvay, Belgium), and ZnO NM-110 and TiO₂ NM-105 (JRC repository). Of note, NM-*x* numberings refer to the respective numberings of OECD reference nanomaterials, as they have been coded in the list of the OECD WPMN Sponsorship Program for the Testing of Manufactured Nanomaterials. All NMs were extensively characterized in accordance with the corresponding guidance of the European Chemicals Agency (ECHA 2012; Table 1; for further details: Hellack et al. 2012 http://www.nanogem.de/cms/nanogem/upload/Veroeffentlichungen/nanoGEM_Dell1.3.1_Characterization_Materials_2013_04_24.pdf and Wohlleben et al. 2013).

Dissolution of nanosilver and ZnO

Dissolution was analyzed for nanosilver and ZnO NMs in water at 10 µg/mL and at 20 µg/mL. Suspensions were incubated at 37 °C for 60 h and centrifuged afterward in a table top centrifuge at 14.000×*g* for 30 min, and supernatants were filtrated through Amicon filters (MW cutoff 30 kDa). Analysis was performed using 500 µL of the filtrated supernatants, and 1 mL 65% HNO₃ and 1 mL H₂O₂ were added. Samples were filled up with ultrapure water to 10 mL. Measurement was taken as described below for the NM uptake analysis.

NM dispersions for in vitro assays

NMs were dispersed under sterile conditions in the respective test medium and stirred at room temperature for 24 h at 700 rpm. For the biological assays, the NMs were dispersed

Table 1 NM characterization

	Surface modifier name	Size	Specific surface	Size (H ₂ O)	Size (PBS)	IEP	Zeta (pH7.4)	Dissolution (20 µg/mL)
		TEM	BET/TEM	DLS	DLS		Zeta	ICP-MS
		Mean (nm)	m ² /g	D50 (nm)	D50 (nm)	pH	(mV)	H ₂ O
SiO ₂ .unmodified	None specified	15	200	40	41	<1	−39	na
SiO ₂ .PEG	Polyethylene glycol MW ~500 g/mol	15	200	50	2180	4	−26	na
SiO ₂ .amino	Aminopropyltri-methoxysilane	15	200	42	78	7.2	0	na
SiO ₂ .phosphate	TPMP	15	200	40	40	<1	−42.9	na
ZrO ₂ .acrylate	Polyacrylic acid	9	117	9	218	<1	−39	na
ZrO ₂ .PEG	Polyethylene glycol MW ~600 g/mol	9	117	27	1567	7	−7.8	na
ZrO ₂ .amino	Aminopropyltri-methoxysilane	10	105	315	1453	>10	3.9	na
ZrO ₂ .TODS	Trioxadecanoic acid	9	117	9	1657	7.1	−6.5	na
Ag.50.PVP	Polyvinylpyrrolidone, Luvitec K90, M _w = approx. 1400 g/mol	97	6.2	123	316	3.6	−7	9.3%
Ag.200.PVP	Polyvinylpyrrolidone, Luvitec K90, M _w = approx. 1400 g/mol	134	4.5	408	303	4.2	−7	4%
Ag.50.citrate	Citrate	20	30	35	221	2	−45	17%
TiO ₂ NM-105	None specified	21	51	478	1700	6.7	−17	na
ZnO NM-110	None specified	80	12	275	970	9.6	20	39%
BaSO ₄ NM-220	None specified	32	41	350	577	3.3	−39	na
AlOOH I	None specified	37	47	262	1837	8.2	5	na
AlOOH II	None specified	16	159	1143	2200	7.9	5	na

na not analyzed

in PBS. In order to study dissolution, nanosilver and ZnO NMs were dispersed in water using the same protocol. A detailed step-by-step NM dispersion protocol is also available at www.nanogem.de.

Generation of NM aerosols for in vivo assays

A constant amount of test dispersion was pumped into an atomizer from where it was sprayed into the inhalation atmospheres with compressed air. The aerosol concentration was determined by gravimetric measurements using the sampling equipment (Millipore, Germany). Particle size was determined by gravimetric measurements with a Marple 298 cascade impactor, and, additionally, with an aerodynamic particle sizer (APS 3321, TSI Germany), an aerosol spectrometer WLAS 2000 (PALAS, Germany) and a scanning mobility particle sizer (SMPS 5.400, Grimm Aerosol Technik GmbH, Germany).

EpiAirway™ 3D models and test substance exposure

The EpiAirway™ 3D bronchial models were purchased from MatTek (USA). Immediately after arrival, they were placed into fresh EpiAirway™ culture medium (24-well plate, 1 mL medium/well), inspected microscopically and incubated (37 °C, 5% CO₂) in air–liquid-culture, i.e., with cell culture medium reaching the 3D models from underneath and the surface being air-exposed. 24–72 h after arrival, the surface of the EpiAirway™ models was treated with either 30 µL of a 1.06 mg/mL suspension of the respective NM in PBS or with 30 µL PBS only as negative control. EpiAirway™ models had a surface of 0.636 cm² (diameter of 9 mm), which resulted in applied doses of 50 µg/cm² for each NM. For testing lower doses, the NM stock suspensions were diluted such that the same volume was applied in all cases. NM suspensions were directly pipetted on top of the 3D models.

In preliminary experiments using only SiO₂.unmodified, Ag.50.PVP, and ZnO NM-110, we tested different exposure times ranging from 24 to 72 h. For SiO₂.unmodified and ZnO, genotoxic effects in comet assay were well detectable after 72 h, but not after 24 h (data not shown). However, towards the end of the 72-h incubation period, the 3D models began deteriorating.

Based upon the results of these preliminary tests, a 60-h incubation period was selected to evaluate all NMs in the main study. During incubation, the medium was exchanged every 24 h for later use in the LDH assay. All experiments were performed in three independent runs with three tissues each. Results were presented as mean values ± standard error of the mean (SEM).

Short-term inhalation study (STIS, 5-days)

The STIS was performed with 9-week-old male Wistar Crl:WI (Han) rats (Charles River Laboratories, Germany) in an animal facility that holds a certificate by the International Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Rats were housed in groups of three or five animals per cage with dust-free wooden bedding and wooden gnawing blocks for environmental enrichment. The studies were approved by the local regulatory agencies. All study protocols complied with the federal guidelines.

Each group encompassed 11 animals. The groups received either ZrO₂.acrylate or SiO₂.amino (test groups) or conditioned air (vehicle control group), and the animals were euthanatized either shortly after the exposure period (exposure groups) or after a 3-week recovery period (recovery groups). On 5 consecutive days, the rats were exposed (head-nose only) to 50 mg/m³ NMs (referring to the non-volatile content) for 6 h/day (Table 2). Clinical examinations were performed before, during and after exposure on each exposure day and once on each working day during the post-exposure period. Body weight was determined twice weekly. The animals were killed by exsanguination upon intraperitoneal administration of 100 mg/kg body weight (bw) pentobarbital. Of each group, five animals were submitted to blood sampling, bronchoalveolar lavage and in vivo genotoxicity assay, and three animals each were used for histopathology or for determining the NM content in the lung and lung-associated (local) lymph nodes (LLNs). Additional five positive control animals (exposure and recovery groups) were treated with 300 mg/kg bw ethyl methanesulfonate (EMS) in PBS for 24 h by single oral administration in a volume of 10 mL/kg bw.

In the blood samples, the standard clinical parameters listed in OECD TG 412 were assessed and, additionally, the acute phase proteins haptoglobin and α₂-macroglobulin.

Table 2 Rat 5-day short-term inhalation toxicity study; study protocol

Study day	1	2	3	4	5	6	7	8	9–25	26	27	28
In-life phase	X	X	X	X	X	R	R	R	R	R	R	R
Examinations					E		L			E		L

X exposure, R recovery, E histopathological examination (three rats/group) and determination organ burden (three rats/group), L blood sampling, bronchoalveolar lavage and genotoxicity assays (five rats/group)

In the bronchoalveolar lavage fluid (BALF) macrophages, polymorphonuclear neutrophils, lymphocytes, eosinophils, monocytes, and atypical cells were counted using a hematology analyzer (Advia 120 Diagnostics; Bayer, Germany). Humoral parameters, i.e., total protein, LDH, alkaline phosphatase, γ -glutamyltransferase, and N-acetyl- β -D-glucosaminidase, were determined with an automatic analyzer (Hitachi 917; Roche, Germany). Organ burden was determined in the lungs and LLNs by inductively coupled plasma-mass spectrometry. Histopathological examination was performed by light microscopy.

For all of these parameters, mean values and standard deviations were calculated. Additionally, body weight and body weight changes were evaluated by comparing each group with the control group using Dunnett's test (two-sided) for the hypothesis of equal means (cf. Landsiedel et al. (2014) for further details on the STIS protocol).

Dosimetry in vivo and in vitro

All 16 NMs were assessed in vitro, and two among them also in vivo. Appropriate test concentrations in particular for in vitro studies have been subject of recent expert discussions as often studies tend to investigate too high NM concentrations which may result in altered toxicity mechanisms, and therefore results obtained may be irrelevant for real-life situations (Oberdoerster 2012). Thus, our approach was to calculate appropriate in vitro dosages based upon in vivo exposure conditions, which is in particular useful when in vitro and in vivo responses should be compared. In the STIS, rats were exposed to 50 mg/m³ NMs for 6 h/day for 5 consecutive days. Assuming a rat lung surface of 5571 cm² (Johanson and Pierce 1973; Shebjami 1992) and deposition of approximately 5–10% of the respirable NM fraction on the tracheobronchial surface and using the MPPD software [Multiple Path Particle Dosimetry Model of the Netherlands National Institute for Public Health and the Environment (RIVM)], total deposited doses of 11.9–23.8 μ g/cm² were calculated. In vivo, this dose was applied over 5 days, whereas in vitro exposure occurred in a bolus-like manner. Since in vitro studies typically represent worst-case scenarios and in vivo NMs are not deposited homogeneously in the lungs, we decided to apply a maximum concentration of up to 50 μ g NM/cm² tissue model. Only ZnO NM-110 had to be tested at lower concentrations due to its stronger cytotoxicity.

Cytotoxicity assays

LDH assay

The LDH assay was performed with a Promega kit (Germany) according to the manufacturer's instructions. To

prevent NM-reagent interference, the NMs were removed from the medium prior to testing by 30-min centrifugation at maximum speed using a tabletop centrifuge. Statistical significance ($p < 0.05$) was calculated using Student's *t* test for treatments versus negative controls.

ATP assay

Intracellular ATP was measured in approximately one-fourth of each EpiAirwayTM tissue using the ATPliteTM kit (PerkinElmer, Germany) according to the manufacturer's instructions. Additionally, total tissue protein content (Bio-Rad, Germany) was assessed, and the ATP content normalized to it. All nanosilver compounds, but also some ZrO₂ NMs, interfered with the ATP assay reagents. However, this was assessed not affecting the outcome of the genotoxicity assays in which different reagents are applied. Statistical significance ($p < 0.05$) was calculated using Student's *t* test for treatments versus negative controls.

Analysis of NM uptake in vitro

For the analysis of NM uptake, we used EpiAirwayTM 3D bronchial models in 96-well format (MatTek, USA), which were cultured as described above. EpiAirwayTM models were treated with either 5 μ L of NM suspensions with different concentrations, i.e., 1.2, 0.6 and 0.24 mg/mL in PBS, respectively, or with 5 μ L PBS only as controls. EpiAirwayTM models in 96-well format had a surface of 0.12 cm², and thus, applied doses were 50, 25 or 10 μ g/cm², respectively. The NM suspensions were directly pipetted on top of the 3D models.

NMs were applied for 60 h. Afterward, the tissues were washed three times with PBS and lysed with TrypLE (Gibco[®], Germany) to obtain single cell suspensions. Cells were centrifuged, and cell pellets were washed again with PBS.

For Al-, Zn-, Zr-, Ag- and Ba-containing NMs, cell pellets were digested in 1 mL 65% HNO₃ and 1 mL H₂O₂ in a heating block for 48 h at 60 °C with open vials. Digested samples were filled up with ultrapure water to 10 mL. For TiO₂ NMs, cell pellets were digested in 10 mL 65% HNO₃ and 1 mL 5% HF in a heating block for 48 h at 70 °C with open vials. Digested samples were filled up with ultrapure water to 50 mL.

Measurements were taken at a quadrupole ICP mass spectrometer (iCAP Q, Thermo Fisher Scientific GmbH, Dreieich, Germany) equipped with a PrepFast system (ESI Elemental Service & Instruments GmbH), PFA ST Nebulizer, a quartz cyclonic spray chamber and a 2.5-mm quartz injector (all from Thermo Fisher Scientific) using the following isotopes: ²⁷Al, ⁴⁸Ti, ⁶⁶Zn, ⁹⁰Zr, ¹⁰⁷Ag, ¹³⁷Ba, ⁴⁹Ti, and as an internal standard ¹⁰³Rh was used. Calibrations

were performed using ionic standards of the respective element in 6.5% HNO₃ solution ranging from 0 to 20 µg/L. Internal standard was added by the ICP-MS PrepFast system. The gas flow for the cool gas and the auxiliary was set to 14 and 0.65 L/min, respectively. The sample flow rate was 0.4 mL/min. All isotopes were measured with the collision cell technique at 5 mL/min collision gas flow (93% He and 7% H₂). Analysis of NM uptake was performed in one experiment only using three tissues per dose. Results were presented as mean values ± standard error of the mean (SEM). Statistical significance ($p < 0.05$) was calculated using Student's *t* test versus respective negative controls.

Genotoxicity assays

In vitro alkaline comet assay

Methyl methanesulfonate (MMS) was used as positive control, applied at 5 mM for 1 h. The vehicle PBS was used as negative control and incubated for the same time period as the NMs. The comet assay was performed as published by Brinkmann et al. (2013). Briefly, after test substance exposure, the 3D models were washed with PBS and lysed with TrypLE (Gibco®, Germany) to obtain single cell suspensions. Cells were harvested by centrifugation, washed with PBS, and the cells were embedded in low-melting agarose (Lonza, Switzerland) and pipetted onto microscopic slides pretreated with a basal agarose layer. The cells were lysed over night in 2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris, 10% DMSO, 1% Triton X-100 at pH 10 in the dark, to prevent UV light-induced DNA damage. Electrophoresis was carried out was performed in the dark under alkaline conditions for 20 min at 25 V (0.8 V/cm), 300 mA, cooled on ice. DNA was stained with ethidium bromide as previously described (Singh et al. 1988; Tice et al. 2000). Comets were analyzed with a fluorescence microscope (ZEISS Axio Observer.A1, Germany; 20× objective) using the Metafer4 (Metasystems, Germany) software. For each treatment, a minimum of 2 slides, 50 nuclei per slide, were analyzed. Highly damaged cells were excluded from the evaluation. Relative tail intensity and % tail DNA were determined. The following prediction model was applied. A result was considered positive if the % tail DNA exceeded the corresponding negative control value by at least two-fold and was statistically significant. Statistical significance ($p < 0.05$) was assessed by Student's *t* test for treatments versus controls.

In vivo alkaline comet assay in lung cells

Upon completion of the STIS, the alkaline comet assay was performed with isolated lung cells. Isolation of lung cells was performed as described by Hartmann et al. (2004).

Lungs were washed in 10 mL mincing solution (HBSS, without Ca²⁺ or Mg²⁺, 0.05 M EDTA, 10% DMSO, pH 7.5), cut into pieces and gently forced through a sieve (73.7 µm pore size; Sigma-Aldrich, Germany). The single cell suspension was filled up with centrifugation buffer (HBSS with 5% FCS) and centrifuged at 250×*g* for 5 min, and the resulting cell pellet was re-suspended in 3–5 mL centrifugation buffer.

Quality of the cell suspensions and NM cytotoxicity were determined in the trypan blue vital dye-exclusion assay. Approximately 100 µL cell suspension was mixed with 900 µL trypan blue solution (0.4% w/v) at room temperature. Colorless viable cells and light blue dead cells were counted (Bürker counting chamber, Sigma-Aldrich, Germany). Cell viability should reach at least 70%. Cell suspensions were adjusted with HBSS to approximately 1–3 × 10⁶ viable lung cells per mL.

Ten microliters of the single cell suspension (1–3 × 10⁴ viable lung cells per slide and 5–6 slides per animal) was mixed with 90 µL low-melting agarose (0.7% v/v; SeaPlaque® GTG® in PBS), layered on slides pre-coated with normal melting agarose (0.6% v/v; SeaKem® LE in PBS) and lysed for at least 1 h at approximately 4 °C in the dark. Prior to electrophoresis, slides were incubated in electrophoresis buffer (24 g NaOH and 0.74 IDRANAL® III in 2 L highly deionized water; pH > 13) for 45 min, cooled on ice.

Electrophoresis was performed in the dark under alkaline conditions and staining was performed in a similar manner as described above for the *in vitro* comet assay, except the electrophoresis time was 30 min. The slides were evaluated using a fluorescence microscope (ZEISS Axioplan 2, Germany; 40× objective) and the image analysis system Comet Assay IV™ (Perceptive Instruments Ltd., UK). At least two slides per animal (50 images per slide) and 500 images per test group, if possible, were analyzed. Highly damaged cells, e.g., hedgehog cells (i.e., cells where only a tail without a defined head is visible), were excluded from the evaluation. Relative tail intensity (% tail DNA) was determined. Effects were considered positive if the mean % tail DNA of the test group either exceeded the mean vehicle control value by a factor of 2 and was above 15% tail DNA (value based on an in-house vehicle control database of $n = 18$ animals). The values were checked for statistical significance by Student's *t* test ($*p \leq 0.05$).

In vivo MNT in bone marrow cells

The micronucleus test was conducted according to OECD TG 474. The bone marrow from two femora was prepared as described in the literature (Schmid 1977; Salamone et al. 1980). The obtained cell suspension was purified by extraction as described by Romagna and Staniforth (1989).

Cells were re-suspended with 1 mL PBS and stored on ice until preparation of cytospin slides (Tharmac GmbH, Germany) using the Cellspin I-12 (Tharmac GmbH, Germany). At least four slides per animal were prepared. Slides were stained with modified May-Grünwald/Giemsa solution and mounted in Eukitt® medium (Corbit Balsam). The amount of polychromatic erythrocytes (PCEs) in a sample of 2000 erythrocytes for each animal was counted as parameter of organ toxicity. For the quantification of the genotoxic potency, 2000 PCEs per animal were assessed for the occurrence of micronuclei. The size of the micronuclei is an indication for the mode of action underlying the finding: Large micronuclei with a diameter equal or more than a quarter of the cell diameter are indicative for an aneugenic effect.

Findings were considered positive if the number of PCE-containing micronuclei was statistically significantly increased (asymptotic *U* test according to Mann–Whitney; statistical significance: $*p \leq 0.01$) and the number of PCE-containing micronuclei exceeded both the corresponding control values and the laboratory's historical control data range (0.7–3.0‰).

Results

Cytotoxicity, NM uptake and in vitro genotoxicity

The results from the cytotoxicity assays are presented in Fig. 1. When compared to the ATP assay, the LDH release proved to be less sensitive, with only ZrO₂.PEG displaying a cytotoxic effect (Fig. 1a). Conversely, using the ATP assay cytotoxicity could be detected for ZrO₂.PEG, ZnO, and TiO₂ (Fig. 1b). However, ATP content and protein content per tissue varied strongly such that none of the results was statistically significant with resulting p-values being smaller 0.1 only. Thus, in the current form, this assay may be useful to indicate cytotoxicity but other ways of normalizing the data should be tested to render this assay more robust. For a few NMs, in particular for the nanosilver variants, an increase in the ATP content was observed, which was due to interference, rendering this assay not appropriate for those NMs.

Using EpiAirway™ 3D models, the in vitro alkaline comet assay revealed only two strongly positive NMs, i.e., ZnO and Ag.50.citrate (Fig. 2). Both particle types elicited a pronounced genotoxic effect at 10 and 25 µg/cm² (ZnO) and 50 µg/cm² (Ag.50.citrate), respectively. The effects of three further NMs (SiO₂.unmodified, SiO₂.phosphate, ZrO₂.TODS) were significant at 50 µg/cm², but ranged just above the threshold level with % tail DNA values close to, e.g., SiO₂.PEG or ZrO₂.acrylate that were assessed as being non-genotoxic (Fig. 2).

To ascertain that ZnO-mediated genotoxicity had not been determined at cytotoxic concentrations, we further tested these particles at different concentrations in the ATP assay (Fig. 3). Here, we could show that ZnO concentrations ≤ 25 µg/cm² were non-toxic and thus suitable for genotoxicity testing (ATP content relative to the negative control at 25 µg ZnO/cm² = 92%; at 50 µg ZnO/cm² = 38%).

For certain NMs that had induced strong (Ag.50.citrate and ZnO NM-110) or only weak genotoxicity (SiO₂.unmodified), we further assessed the time-dependency of the evolution of the genotoxic effects. For this purpose, we incubated EpiAirway™ models with these three different NMs for 24 and 60 h and, additionally, for 24 h followed by NM removal and a 36-h recovery period (i.e., 60-h total duration of the experiment). For all three NMs, genotoxicity was only observed after completion of the entire incubation period of 60 h (Fig. 4).

NM uptake in the 3D models was analyzed for 9 out of the 16 NMs investigated in this study. Analysis was not possible for the SiO₂ variants due to technical limitations. As the numbers of 3D models for this part of the study were limited, we decided to analyze only one of the two types of AlOOH (i.e., AlOOH I) and two of the four types of ZrO₂ (i.e., ZrO₂.TODS, ZrO₂.acrylate). Furthermore, we analyzed uptake for TiO₂ NM-105 only at a single dose of 50 µg/cm², while all other NMs were tested in three different concentrations, i.e., 10, 20 and 50 µg/cm². We could confirm cellular uptake in the 3D models for all NMs except for Ag.50.citrate (Fig. 5). Except for TiO₂ NM-105, where only a single dose was investigated, we furthermore could demonstrate that NM uptake was dose dependent (Fig. 5).

In summary, ZrO₂.PEG and TiO₂ NM-105 were found to be cytotoxic, but not genotoxic. Ag.50.citrate was genotoxic, but not cytotoxic. ZnO was cytotoxic and genotoxic; however, genotoxicity was assessed at non-cytotoxic concentrations. Finally, statistically significant genotoxic effects but no cytotoxicity were observed for SiO₂.unmodified, SiO₂.phosphate, and ZrO₂.TODS, but effects ranged just above the threshold level. Thus, based on the assessments using in vitro comet assay in EpiAirway™ 3D models 5 NMs, i.e., Ag.50.citrate, ZnO, SiO₂.unmodified, SiO₂.phosphate, and ZrO₂.TODS, were considered genotoxic.

Outcome of the in vivo STIS

The scheduled aerosol concentration was met for both NMs that were tested in the STIS, i.e., ZrO₂.acrylate and SiO₂.amino, and the particle sizes of the aerosol in the inhalation atmosphere were well within the respirable range (data not shown). The NM contents in the lungs and the LLNs are presented in Table 3. Lungs from test animals treated with ZrO₂.acrylate and SiO₂.amino contained 169 and

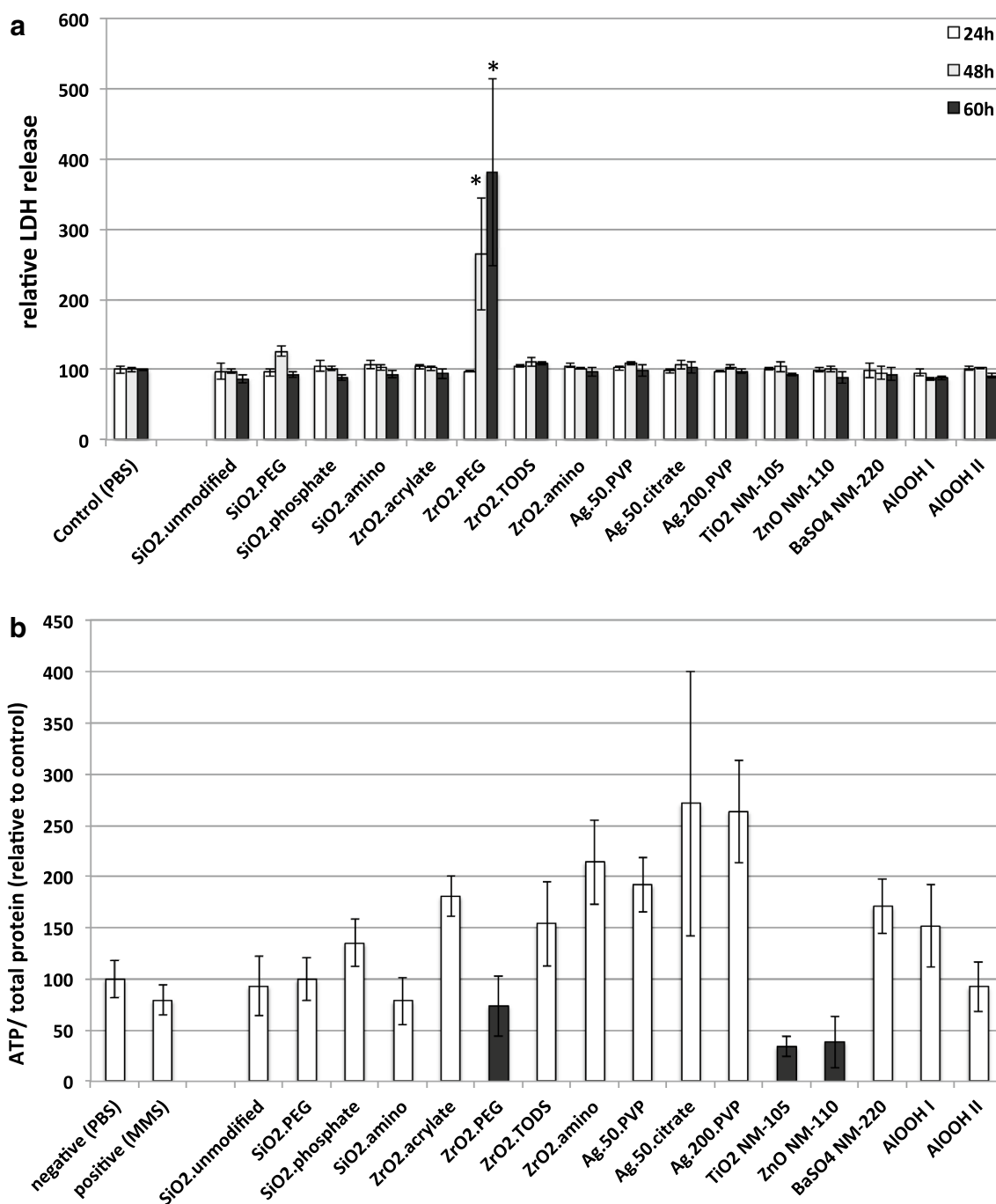


Fig. 1 Cytotoxicity of 16 NMs. LDH activity was determined in the cell culture medium after 24-h (light bars), 48-h (gray bars) and 60-h (black bars) test substance incubation of EpiAirway™ 3D models. Statistically significant results ($p < 0.05$) are marked with an asterisk (a). In parallel, total ATP content was determined in the tissues after

60 h of test substance incubation and normalized to the total tissue protein content (b). Cytotoxic NMs are depicted with black bars. All experiments were performed in three independent runs with three tissues each. Results are provided as mean values \pm SEM

743 μg NM, respectively, in the exposure groups. In the recovery groups, 190 μg of ZrO₂ and 475 μg of SiO₂ could be detected. These data show that SiO₂.amino, but not ZrO₂.acrylate, was cleared from the lung tissue within the 3-weeks recovery period. By contrast, neither ZrO₂.acrylate

nor SiO₂.amino particles were detected in the mediastinal LLNs at either time point. Thus, they did not translocate outside the lung.

None of the BALF or blood parameters were significantly altered upon 5-day inhalation of either 50 mg/m³

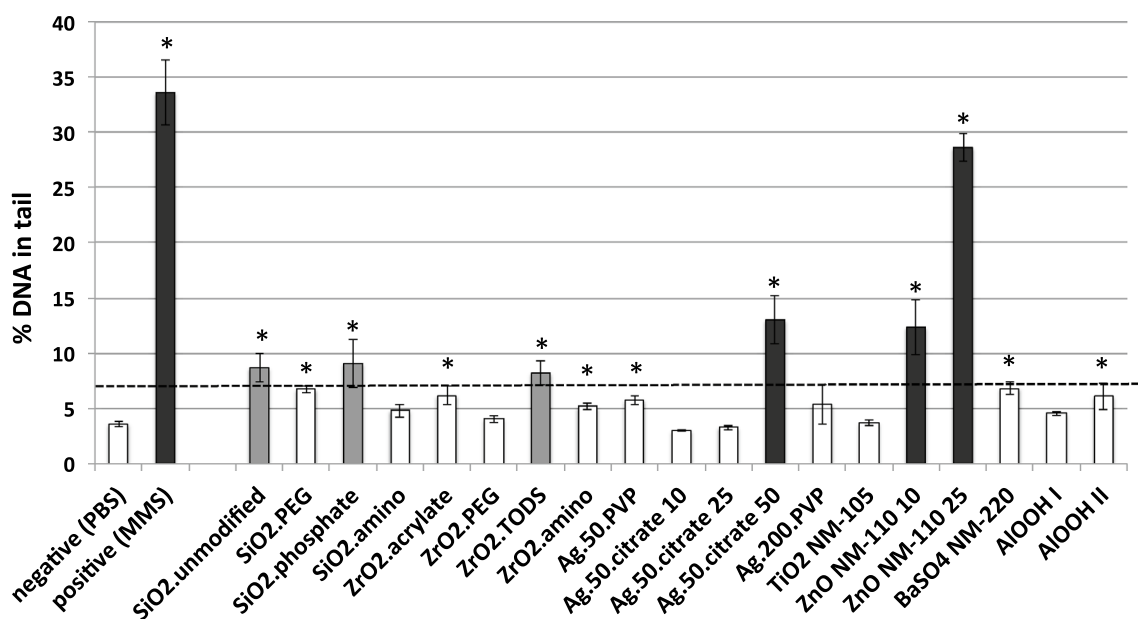


Fig. 2 Genotoxicity of 16 NMs. Genotoxicity of the NMs was assessed in the alkaline comet assay upon 60-h incubation of Epi-Airway™ 3D human bronchial models with a dose of 50 $\mu\text{g}/\text{cm}^2$ (30 μL sample volume). % DNA in the comet tail was determined. Due to its cytotoxicity at higher concentrations, ZnO was tested only at 25 $\mu\text{g}/\text{cm}^2$. Negative controls were treated with the vehicle PBS only. Methyl methanesulfonate (MMS) was used as positive control, applied at 5 mM for 1 h. Different concentrations were tested for the

two NMs that clearly elicited genotoxicity, i.e., ZnO (10 and 25 $\mu\text{g}/\text{cm}^2$) and Ag.50.citrate (10, 25, and 50 $\mu\text{g}/\text{cm}^2$) to assess concentration-dependent genotoxicity. Genotoxic NMs are depicted with *black bars*. All experiments were performed in three independent runs with three tissues each. Results are provided as mean values \pm SEM. Statistically significant results ($p < 0.05$) are marked with an *asterisk*. The *dotted line* represents the threshold, i.e., twofold above the negative control value

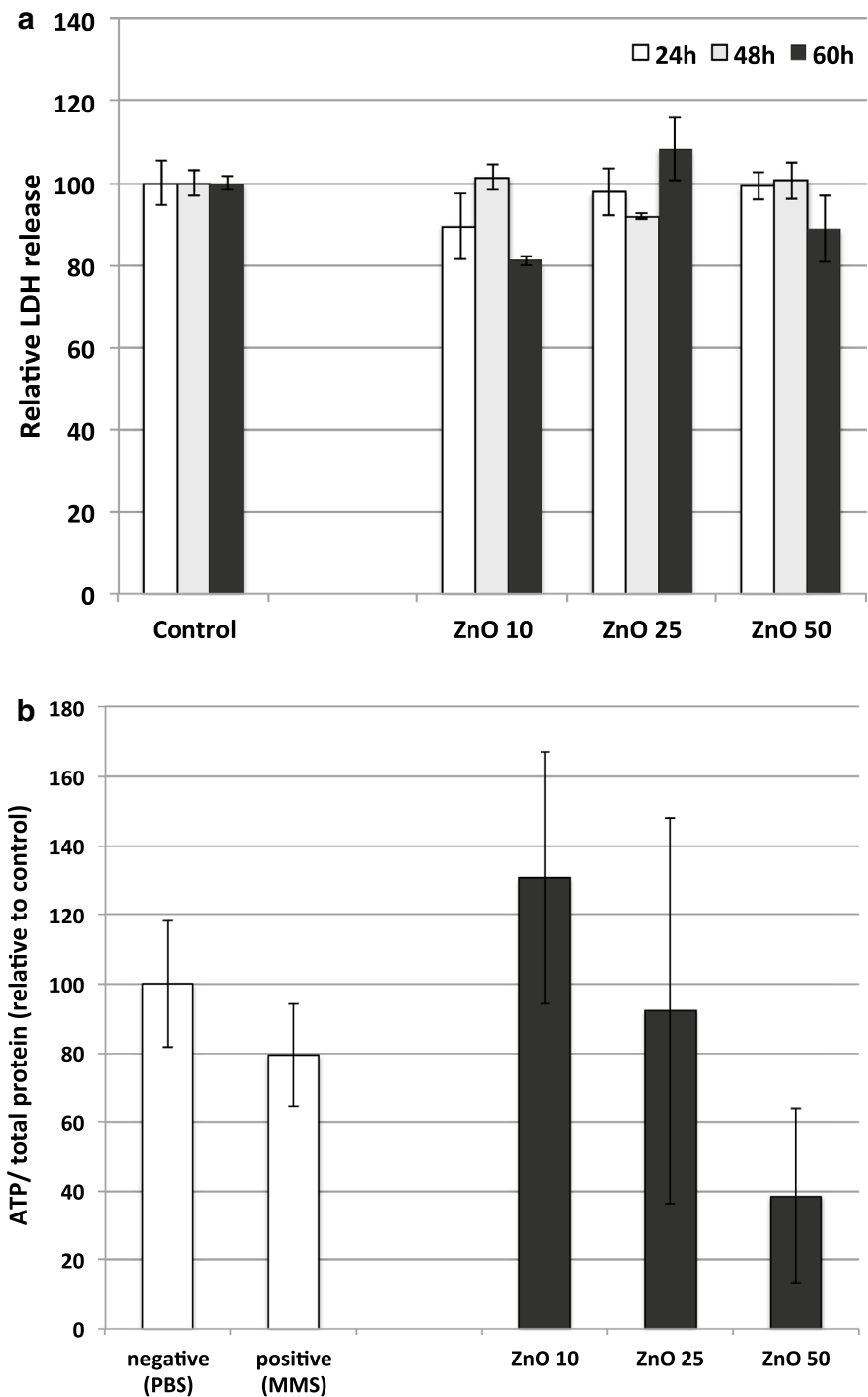
ZrO₂.acrylate or SiO₂.amino (not shown). Histopathology of the respiratory tract of animals treated with either NM did not reveal any pathological findings. Therefore, the recovery groups were not analyzed. The NMs further did not induce pulmonary cytotoxicity, since cell viability remained unaffected in the following alkaline comet assay and ‘hedgehog’ images (i.e., tails without a head) were in the normal range (data not shown).

In lung cells isolated from animals treated with ZrO₂.acrylate, relative tail intensities of 3.2 and 5.2% were recorded in the comet assay for the exposure and recovery groups, respectively (Fig. 6a). These values were close to the concurrent vehicle control values of 2.7% (exposure group) and 3.2% (recovery group). Similarly, in cells from SiO₂.amino-treated animals, relative tail intensities of 3.9% (exposure group) and 2.0% (recovery group) were recorded (Fig. 6b), numbers which again were close to the corresponding control values of 3.3% (exposure group) and 1.3% (recovery group). In the positive controls (animals treated with EMS) that were prepared in parallel to the exposure groups, severe DNA damage was indicated via the occurrence of large amounts of ‘hedgehog’ cells or high relative tail intensities (i.e., 44.3 or 20.7%). On the other side, both positive

control groups prepared along with the recovery groups could not be quantified for relative tail intensities due to the extremely strong DNA damage induced (almost only ‘hedgehog’ cells). In summary, ZrO₂.acrylate and SiO₂.amino did not cause genotoxicity in lung cells as assessed in the *in vivo* alkaline comet assay.

In the *in vivo* MNT, the ratio of PCEs to normochromatic erythrocytes (NCEs) in rat bone marrow cells revealed unaffected after 5-day inhalation exposure to 50 mg/m^3 ZrO₂.acrylate or 50 mg/m^3 SiO₂.amino (Table 4). Likewise, the amount of micronucleated PCEs was not relevantly increased in the ZrO₂.acrylate test groups (2.8 and 2.5‰ in the exposure and recovery groups, respectively) or in the SiO₂.amino test groups (1.1 and 1.5‰ in the exposure and recovery groups, respectively), since these values were again close to the concurrent vehicle control values (1.9/1.9 and 2.1/2.2‰ in the exposure and recovery groups, respectively) and further within the in-house historical negative control range (0.7–3.0‰). The positive control groups, on the other hand, revealed with the expected increase in micronucleus frequencies in PCEs (6.6–10.6‰). Therefore, in the *in vivo* MNT both NMs were assessed as being non-genotoxic in bone marrow cells.

Fig. 3 Concentration-dependent cytotoxicity of ZnO NM-110. Different doses (i.e., 10, 25 and 50 $\mu\text{g}/\text{cm}^2$) of ZnO were tested for cytotoxicity with LDH (a) and ATP assay (b) in three independent runs with three tissues in each run. Results are provided as mean values \pm SEM



Discussion

The EpiAirway™ 3D bronchiole model closely resembles human bronchiole epithelium and contains all relevant cell types found in vivo. It was therefore regarded a realistic in vitro model to investigate respiratory tract toxicity. The relevance of different in vitro models for in vivo toxicity of NMs has been frequently discussed (e.g., Nel et al. 2013; Sauer et al. 2013, 2014). Effects

observed in vitro are mostly related to cellular uptake and subsequent intracellular (e.g., redox) activity of the NMs. Frequently, NM in vitro studies tend to overestimate toxicity, which is due to many different reasons. Clearly, one important difference compared to the in vivo situation is the fact that NM uptake in cell lines may be expected to be much higher as no barriers exist. Unfortunately, there is little systematic research quantifying cellular uptake of NMs. Only a few studies quantified cellular uptake in

Fig. 4 Time-dependent genotoxicity of ZnO, Ag-50, citrate and SiO₂-unmodified. Genotoxicity was assessed in the alkaline comet assay. EpiAirway™ 3D models were either incubated with 50 μg/cm² NM for 24, 60, or 24 h with a 36-h recovery period (after washing out of the test substances). All experiments were performed in three independent runs with three tissues each. Results are provided as mean values ± SEM. Statistically significant results ($p < 0.05$) are marked with an asterisk

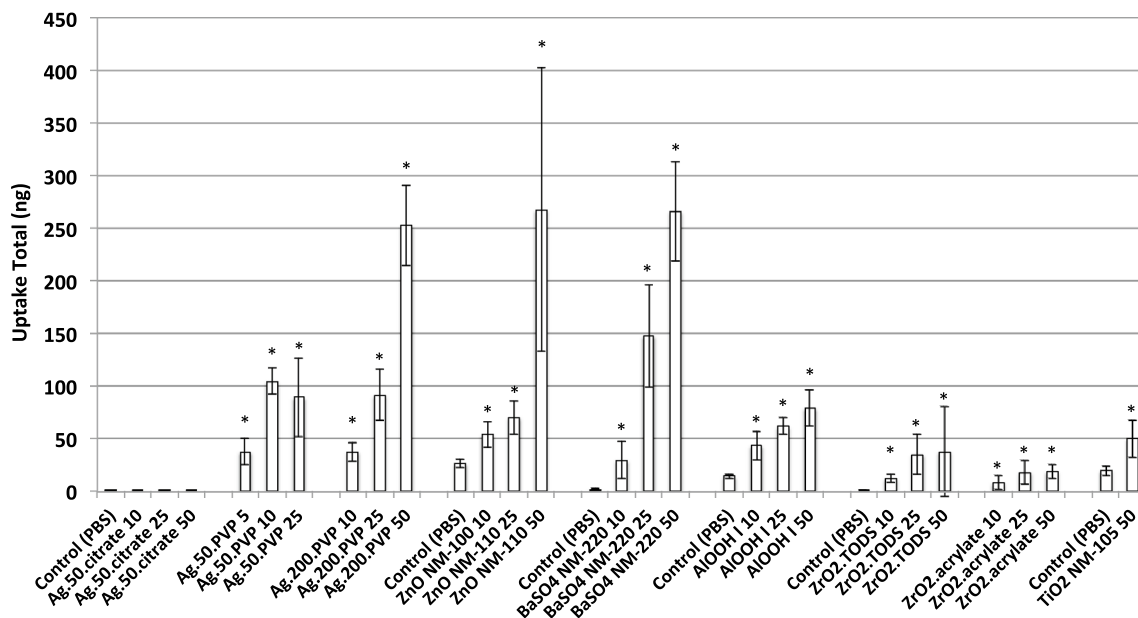
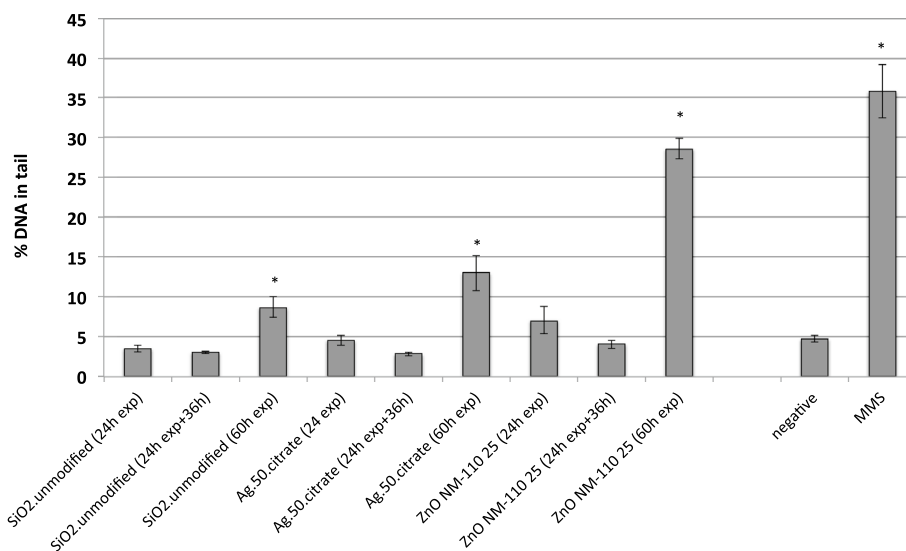


Fig. 5 NM uptake in EpiAirway™ 3D models. EpiAirway™ 3D models were treated with the indicated concentrations of NMs (i.e., 10, 20 or 50 μg/cm²) for 60 h. NM uptake was quantified using ICP-MS in a single experiment using three tissues per dose. Results are

depicted as total amounts taken up provided in ng, which are mean values of three tissues ± SEM. Statistically significant results ($p < 0.05$) are marked with an asterisk

lung related cell lines so far. Kettler et al. showed that 50–65% of the applied mass doses for citrate-coated silver nanoparticles (20, 50, 75 nm) were taken up in 16HBE14o-cells when cells were exposed to 0.01 μg/mL for 24 h (Kettler et al. 2016). Gliga and coworkers quantified cellular uptake for different variants of nanosilver (e.g., 10, 40 and 75 nm, citrate-coated) in BEAS-2B cells and found up to 12% of the applied mass dose being taken up when cells were exposed to 10 μg/mL for 24 h (Gliga et al. 2014). Even fewer studies investigate cellular

uptake in ex vivo or in 3D lung tissue cultures. Grimm et al. used perfused ex vivo rabbit heart–lung blocks to investigate the uptake of dendrimer-based NM and found that less than 1% of the perfused dose was taken up when 30 or 150 mg were applied (Grimm et al. 2017). Another study investigated the uptake of 70 nm PVP-coated silver in different cell lines and in addition in precision-cut lung slices and found that the particles hardly were taken up into the lung slices and rather stayed on top of the tissues (Ahlberg et al. 2014). Thus, overall cell lines tend

Table 3 ZrO₂.acrylate and SiO₂.amino content in the lung and lung-associated mediastinal lymph nodes

	Group (N = 3)	µg ZrO ₂ in total organ		µg SiO ₂ in total organ	
		Lung	LLN	Lung	LLN
Exposure groups	Control group	<LOQ ^a	<LOQ ^a	<LOQ ^b	<LOQ ^b
	Test group	169	<LOQ ^a	743	<LOQ ^b
Recovery groups	Control group	<LOQ ^a	<LOQ ^a	<LOQ ^b	<LOQ ^b
	Test group	190	<LOQ ^a	475	<LOQ ^b

LLN lung-associated mediastinal (local) lymph nodes

^a Limit of quantification (LOQ) = 5 µg Zr per tissue, corresponding to 7 µg ZrO₂

^b Limit of quantification (LOQ) = 5 µg Si per tissue, corresponding to 11 µg SiO₂

to take up much higher amounts of NMs compared to 3D or tissue models. In addition, in vitro studies often investigate rather high and thus often unrealistic doses, which may also alter toxicity mechanisms. Thus, frequently the importance of appropriate dosimetry for NM testing in vivo but even more so in vitro has been emphasized (Oberdoerster 2012). In particular, in vitro genotoxicity studies frequently suffer from high number of false positive results, which of course can be related to several reasons as summarized in an ECVAM workshop report (Kirkland et al. 2007). However, one reason may be inadequate dosimetry for in vitro testing. Thus, our aim was to use a realistic in vitro model that closely resembles the in vivo physiology and to test realistic doses. Therefore, in this study we have calculated appropriate in vitro dosages based upon in vivo exposure conditions. In the STIS, rats were exposed to 50 mg/m³ NMs for 6 h/day for 5 consecutive days, which when assuming a rat lung surface of 5571 cm² and a deposition of approximately 5–10% of the respirable NM fraction on the tracheobronchial surface and using the MPPD software would result in total deposited doses of 11.9–23.8 µg/cm². Thus, the doses applied during the in vitro testing in this study may be considered relevant for in vitro/in vivo comparison. We could confirm that cellular uptake in the 3D models was considerable lower compared to reported cellular uptake in cell lines (e.g., Gliga et al. 2014, Kettler et al. 2016). In our study, NM uptake always was below 5% of the applied doses and for insoluble NMs sometimes even below 1% while in cell lines up to 12% (Gliga et al. 2014) or even 50–65% (Kettler et al. 2016) of the applied mass doses were reported to be taken up, which occurred even within a much shorter time period (24 versus 60 h). We think, this nicely adds to the discussion on relevant in vitro models for performing in vitro toxicity testing of NMs. Generally, in vitro genotoxicity testing is applied

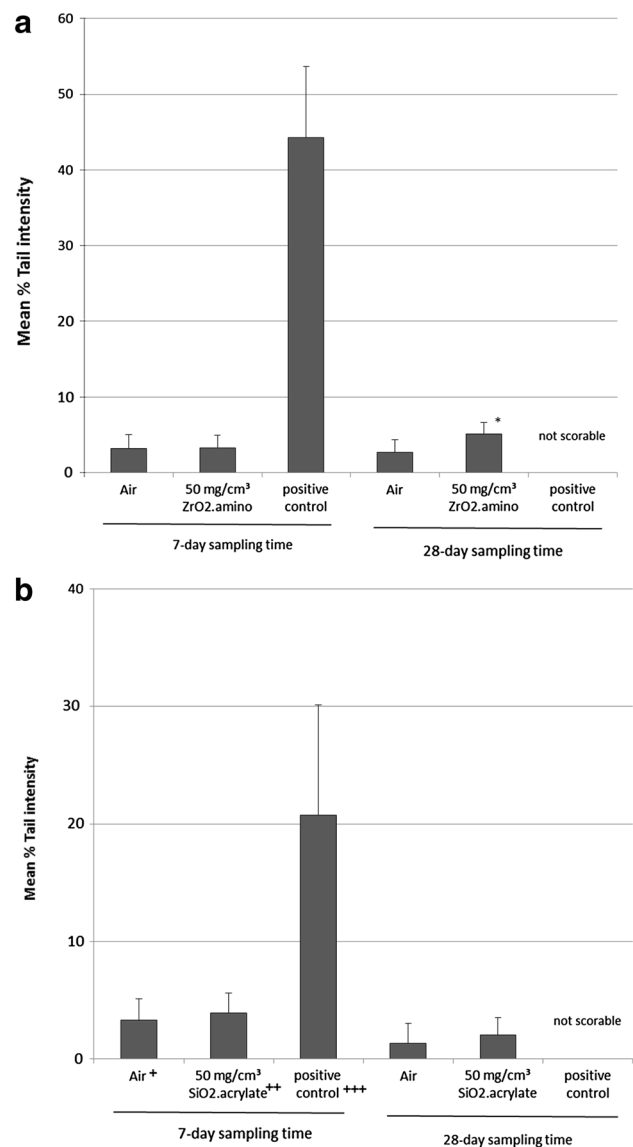


Fig. 6 DNA strand breaks in rat lung after 5-day exposure to ZrO₂.acrylate or SiO₂.amino (7- or 28-day sampling time). Genotoxicity was assessed in the alkaline comet assay after 5-day inhalation exposure followed by 2- or 23-day recovery interval. Groups of 5 male Wistar rats were treated with air or 50 mg/cm³ ZrO₂.amino (a) or 50 mg/cm³ SiO₂.acrylate (b). As positive control rats were administered 300 mg/kg bw EMS by gavage 24 h before killing. If possible, from each animal 100 cells were analyzed for DNA strand breaks (% DNA in tail) using Comet Assay IV (Perceptive Instruments, UK). + Only slides of four animals were scorable, ++ only slides of three animals were scorable, +++ only slides of two animals were scorable, *Student's *t* test: *p* ≤ 0.05

in the first tier of a comprehensive testing strategy. However, results of very sensitive in vitro methods generally should be confirmed in vivo. Using more appropriate in vitro test systems and appropriate testing conditions, i.e., realistic doses, would significantly reduce the efforts and the costs of performing a large number of in vivo

Table 4 Micronucleus frequency in bone marrow after 5-day exposure to ZrO₂.acrylate or SiO₂.amino, respectively (7- or 28-day sampling time)

Group (<i>N</i> = 5)	Killing interval (day)	Micronuclei in PCEs		PCEs per 2 000 Erythrocytes ^c
		Total ^a (%)	Large MNs ^b (%)	
Vehicle control air	7	1.9	0.0	1263
50 mg/cm ³ ZrO ₂ .acrylate	7	2.8	0.1	1340
Positive control 300 mg/kg bw EMS	7	9.6*	0.0	1227
Vehicle control air	28	2.1	0.0	1192
50 mg/cm ³ ZrO ₂ .acrylate	28	2.5	0.0	1234
Positive control 300 mg/kg bw EMS	28	8.4*	0.1	1198
Vehicle control air	7	1.9	0.0	1217
50 mg/cm ³ SiO ₂ .amino	7	1.5	0.0	1309
Positive control 300 mg/kg bw EMS	7	6.6*	0.0	1227
Vehicle control air	28	2.2	0.0	1292
50 mg/cm ³ SiO ₂ .amino	28	1.1	0.0	1277
Positive control 300 mg/kg bw EMS	28	10.6*	0.0	1248

PCEs polychromatic erythrocytes, *bw* body weight

* $p \leq 0.01$ (asymptotic *U* test according to Mann–Whitney)

^a Sum of small and large micronuclei

^b Large micronuclei (indication of spindle poison effect)

^c Calculated number of PCEs per 2000 erythrocytes when scoring a sample of 10 000 PCEs per test group

follow-up studies. Current established in vitro test systems largely use cell lines. Our data suggest that 3D human bronchial tissue models are superior to cell lines with respect to NM in vitro genotoxicity testing. By using such more physiologic models, one may be able to improve the reliability of results obtained in vitro.

We assessed a rather large panel of 16 different NMs. In total, only 5 out of 16 NMs in our study were genotoxic in vitro. Among these, 50 nm nanosilver with citrate functionalization was clearly positive, while the PVP-coated 50 and 200 nm variants were clearly negative. It seems unlikely that the differences in genotoxicity were caused by different particle sizes since all three nanosilver variants agglomerated in PBS to a similar size range (200–300 nm) as shown in Table 1. The observed differences in genotoxicity could also be caused by differences in NM dissolution rates, suggesting that the toxicity is mediated by silver ions being released. We have determined the dissolution rates in water and found that 17% of Ag.50.citrate dissolved compared to 9.3% for Ag.50.PVP (Table 1), which is in line with data reported by others in the literature (Kittler et al. 2010). The differences could also be linked to differences in NM uptake. However, we rather assume similar uptake for Ag.50.citrate and Ag.50.PVP despite the fact that we could not quantify the uptake for Ag.50.citrate, which might be related to extensive washing during sample preparation and consequent loss of ionic silver. We applied NMs dispersed in PBS, which contains chloride ions that due to

the formation of insoluble AgCl should increase the solubility of citrate-coated nanosilver to a much stronger extent for the less stably bound citrate coating compared to PVP (McShan et al. 2014). This is also supported by results from Gliga and coworkers (Gliga et al. 2014), who found no differences in cellular uptake for 10 nm citrate- and PVP-coated nanosilver in BEAS-2B cells. Nanosilver genotoxicity has already been studied by others extensively in vitro (e.g., Ahamed et al. 2008; Kim et al. 2010; Nymark et al. 2013; Gliga et al. 2014) and less frequently in vivo (Kim et al. 2008, 2011; Tiwari et al. 2011) and is also summarized in a review (Johnston et al. 2010). The most important studies should be summarized briefly.

A study from Nymark and colleagues used PVP-coated nanosilver (42.5 nm) to assess genotoxicity in BEAS-2B cells using the comet assay, the MNT and the chromosome aberration assay (Nymark et al. 2013). At 16 $\mu\text{g}/\text{cm}^2$ (corresponding to 60.8 $\mu\text{g}/\text{mL}$) PVP-coated nanosilver was genotoxic in the comet assay after 4 and 24 h. No genotoxicity was detected in the MNT or in the chromosome aberration assay at concentrations of up to 240 $\mu\text{g}/\text{mL}$, neither at 24 h nor at 48 h. Gliga and coworkers studied cytotoxicity and genotoxicity of nanosilver in vitro in BEAS-2B cells (Gliga et al. 2014) using three different sizes (i.e., 10, 40 and 75 nm) of citrate-coated nanosilver, 10 nm PVP-coated nanosilver and 50 nm uncoated nanosilver. They found only the 10 nm nanosilver particles cytotoxic, irrespective of the coating. However, all types of

nanosilver were genotoxic at 10 $\mu\text{g}/\text{mL}$ as assessed by the comet assay but negative in the $\gamma\text{H}_2\text{AX}$ assay. There was no difference in NM uptake for the citrate- versus the PVP-coated nanosilver. Uptake into BEAS-2B cells was determined to be up to 12% of the applied mass dose after 24 h.

In vivo, nanosilver (average size 60 nm) did not elicit genotoxicity in the bone marrow of rats upon 28-day oral exposure as tested via MNT (Kim et al. 2008). In a 90-day inhalation study, silver nanoparticles (18 nm) were also not genotoxic (Kim et al. 2011), as assessed in the MNT conducted according to OECD TG 474. Unfortunately, neither study assessed any other potential target organs, such as the lung. After repeated intravenous injections in rats (4, 10, 20 and 40 mg/kg bw), however, 15–40 nm nanosilver caused genotoxicity in blood cells in the alkaline comet assay at doses >20 mg/kg bw (Tiwari et al. 2011).

Despite the fact that genotoxicity of nanosilver has been extensively addressed in many studies, most of which are in vitro, a general conclusion on the genotoxicity seems to be too premature and maybe even not possible. There are several lines of evidence that genotoxicity of nanosilver in vitro seems to be dependent on the exact type of nanosilver, but also on other factors such as the cell model and the assay chosen. Accordingly, data from Ahamed et al. (2008) and Wang et al. (2014) provide evidence that surface modification of NMs may alter toxicity, which was, however, not confirmed in the study by Gliga and coworkers (Gliga et al. 2014). In our study, we found PVP-coated silver (50 and 200 nm) non-genotoxic and non-cytotoxic, while citrate-coated 50 nm silver exerted genotoxicity in vitro in EpiAirwayTM bronchiole 3D models. Thus, our results point to a modulating effect of the surface functionalization on the genotoxicity of nanosilver, which most likely can be explained by different NM dissolution rates. Wang and colleagues specifically addressed the influence of particle solubility on toxicity using 20 and 110 nm silver nanoparticles coated either with PVP or with citrate (all obtained from Nanocomposix, USA) in BEAS-2B and RAW 264.7 cells via MTS assay (doses of up to 50 $\mu\text{g}/\text{mL}$), while solubility was investigated via ICP-OES. PVP-coated silver nanoparticles released less silver ions compared to citrate-coated ones of the same size due to the fact that PVP can complexate released silver ions (Wang et al. 2014), which according to the authors explains the higher in vitro toxicity of citrate-coated silver.

We further recorded a strong and dose-dependent genotoxicity of ZnO NM-110 particles in vitro. Also ZnO NM genotoxicity has already been studied in vitro (Gopalan et al. 2009; Landsiedel et al. 2010; Sharma et al. 2011) and in vivo (Landsiedel et al. 2010; Sharma et al. 2012) and data is summarized in Klien and Godnic-Cvar (2012). As determined in in vitro comet assays, ZnO NMs induced genotoxicity in human primary keratinocytes at

concentrations of 8 and 14 $\mu\text{g}/\text{mL}$ (Sharma et al. 2011). In addition, using the comet assay ZnO nanoparticles (40–70 nm) were found genotoxic in human sperm cells and lymphocytes at 11.5 $\mu\text{g}/\text{mL}$.

In vivo, Sharma et al. (2012) detected genotoxicity of 30 nm ZnO in the Fpg-modified comet assay after oral uptake in swiss albino mice in liver at the highest dose group (300 mg/kg) in the presence of formamidopyrimidine DNA glycosylase (Fpg) but not in its absence. No significant changes were observed at lower dose (50 mg/kg) or in kidneys at both doses. In an in vivo MNT in bone marrow performed with NMRI mice in accordance with OECD TG 474, up to 60 mg/kg of polymer-coated 30–200 nm ZnO did not induce micronuclei in PCEs 24 or 48 h after intraperitoneal injection (Landsiedel et al. 2010).

In summary, several studies support our observation that ZnO NMs may be genotoxic in vitro, while the findings from available in vivo studies are inconsistent. It should be noted that Fpg-modified comet assay is more sensitive compared to standard comet assay. This variant is not part of the OECD TG 489 for in vivo comet assay, and therefore the relevance of data obtained via this protocol still needs to be proven. Furthermore, there are other differences between both in vivo studies. Landsiedel et al. (2010) tested polymer-coated ZnO, while Sharma et al. (2012) applied unmodified ZnO. Further, the two studies used different animal species, different routes of exposure and different genotoxicity assays. Upon intraperitoneal injection of ZnO, resulting in full systemic availability of the applied dose, no genotoxicity was observed in the MNT (Landsiedel et al. 2010). In addition, Sharma et al. could only observe effects at highest tested doses with a modified comet assay, which is designed to detect alkylating agents and/or oxidative DNA damage.

Several studies have investigated genotoxicity of TiO_2 in vitro (Landsiedel et al. 2010) and in vivo (Landsiedel et al. 2010; Ghiazza et al. 2014). In an in vitro MNT using V79 cells, 4-h incubation of up to 300 $\mu\text{g}/\text{mL}$ or 24-h incubation of up to 75 $\mu\text{g}/\text{mL}$ AlOOH-and-polymer-coated rutile TiO_2 NMs (primary particle size of 10×50 nm, mean agglomerates approximately 200 nm) did not induce genotoxicity (Landsiedel et al. 2010). Even after inhalation of aerosol concentrations of up to 10 mg/m^3 for 6 h on 5 consecutive days and subsequent 3 weeks of recovery, these TiO_2 NMs were still non-genotoxic in male Wistar rats as judged by the alkaline comet assay (Landsiedel et al. 2010). In another study, Ghiazza et al. (2014) inhibited the surface reactivity of rutile–anatase TiO_2 (P25; primary crystallite size: 25–29 nm (anatase) and 41–62 nm (rutile); agglomerated in DMEM to 148–247 nm) by wet impregnation with 0.002–0.2 M $\text{Fe}(\text{NO}_3)_3$ with subsequent calcination, which also increased the amount of rutile phase particles. They found the ability

of photo-activated TiO₂ to cleave DNA and proteins to be reduced with increasing Fe-doping of the particles, just as cytotoxic and genotoxic effects on human HaCaT keratinocytes were inhibited (Ghiazza et al. 2014). In the present study, we used non-coated, rutile TiO₂ NM-105 and we could confirm uptake in the 3D models. We detect cytotoxicity but no genotoxicity in vitro. Uptake for TiO₂ NM-105 in our study was only 0.84% of the applied dose, which was considerably lower compared uptake of partially soluble NMs.

Of the four different variants of SiO₂ tested in vitro, we detected only little genotoxicity for SiO₂.unmodified and SiO₂.phosphate particles. Although being significant, these effects were only observed at a very high dose of 50 µg/cm², and they were just above the threshold level (% tail DNA at least twofold of the corresponding negative control value). By contrast, genotoxicity of the other two SiO₂ variants (SiO₂.PEG and SiO₂.amino) was slightly below this level. Overall, total values, i.e., the % tail DNA, were rather similar for all four SiO₂ variants. However, we still observed a tendency also for silica NMs that chemical surface modification influences particle-mediated genotoxicity. SiO₂.unmodified was clearly positive, while two out of three modified SiO₂ NMs were negative. The lack of genotoxicity of SiO₂.amino was confirmed in vivo in the comet assay using rat lung cells and in the MNT using bone marrow cells. Genotoxicity of 15 nm SiO₂.unmodified in vitro was also confirmed by another study (Maser et al. 2015) using the alkaline comet assay, which found genotoxicity at 100 µg/mL in V79 cells and in rat precision-cut lung slice cultures. The authors did not detect in vivo genotoxicity for 15 nm SiO₂.unmodified, as assessed 3 days after instillation in rat lung or in bone marrow by comet and MN assays despite the fact that they could detect a pronounced inflammation in the lung (Maser et al. 2015).

Of the four different variants of ZrO₂ applied, ZrO₂.TODS was positive in the in vitro comet assay despite the fact that it strongly agglomerated in PBS. The other ZrO₂ variants were much better dispersed in PBS but were nonetheless negative in the in vitro comet assay. Unfortunately, unmodified ZrO₂ was not available for this study. It should be noted, however, that NM uptake in the 3D models was very similar for ZrO₂.TODS and ZrO₂.acrylate, i.e., approximately 1% of the applied dose. Thus, possible differences in genotoxicity cannot be explained by different uptake rates. ZrO₂.acrylate was further non-genotoxic in the in vivo comet assay and the MNT. Accordingly, the results of the present study indicate the lack of genotoxicity of ZrO₂.acrylate both in vitro and in vivo.

In our study BaSO₄ NM-220 was neither cytotoxic nor genotoxic in vitro. This was also confirmed in another study (Cordelli et al. 2017) using the same NM in vivo

after 3- or 6-month inhalation at concentrations of 50 mg/m³ using alkaline Comet assay, gene mutations and chromosome aberrations assays in the peripheral blood of the animals.

Conclusion

Out of 16 NMs tested in our study in the in vitro alkaline comet assay using EpiAirway™ 3D models, only 5 were found positive for genotoxicity, i.e., ZnO NM-110, Ag.50.citrate, SiO₂.unmodified, SiO₂.phosphate and ZrO₂.TODS. Overall, our results show that NM-mediated genotoxicity is mainly determined by its core material. However, we also found some evidence that the chemical surface functionalization can influence or modulate the genotoxic potency, as observed in vitro.

Our study underlines the importance of selecting adequate in vitro test models, choosing realistic doses and adequate treatment periods for in vitro studies. In our study, we could observe genotoxic effects after 60 h, but not after 24 h, and test item incubation was required over the entire 60-h period for effects to occur. Furthermore, NM uptake into intact 3D models was considerably lower compared to uptake into lung cell lines as assessed by others. These parameters need to be taken into consideration when designing in vitro genotoxicity studies for the testing of NMs.

In our study, the 3D human bronchial tissue models appear useful models to assess genotoxicity of NMs in vitro.

By testing a rather large panel of systematically modified NMs of industrial relevance, our study contributes to the hazard assessment of NMs, while also allowing limited in vitro–in vivo comparisons. The potential of NM surface modifications to alter genotoxicity should be further investigated since it might provide important insights for safe-by-design principles applicable during NM synthesis.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest. The authors alone are responsible for the content and writing of the paper. However, Robert Landsiedel and Markus Schulz are employees of BASF SE, a company marketing NMs.

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