

# Hazard screening of colloidal silica nanomaterials with varying degrees of silane surface functionalization: a safe-by-design case study

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## Abstract

**Background** The Safe and Sustainable by Design (SSbD) concept facilitates the design of safer and more sustainable chemicals and materials and is a crucial approach towards reaching the goals set out in the European Green Deal. It is critical that suitable guidance is provided on how to use new approach methodologies (NAMs) to fill hazard data gaps for nanomaterials (NMs) to facilitate SSbD decisions. Here, we showcase a nano-specific in vitro SSbD case study. The five colloidal silica nanoforms (SiO<sub>2</sub>-NFs) under investigation in this study are surface modified with varying amounts of glycerolpropyl-organosilane groups. In this study, we use a simple yet comprehensive in vitro test battery along with thorough particle characterization to investigate the effect of surface silanization on in vitro toxicity to inform SSbD decisions.

**Results** Cytotoxic, pro-inflammatory and oxidative stress responses in A549, dTHP-1, and BEAS-2B cells after exposure to  $SiO_2$ -NFs submerged and at the air-liquid interface (ALI) decreased with increasing silane surface modification. None of the  $SiO_2$ -NFs showed surface reactivity or haemolytic potential. Deposition assessment using inductively coupled plasma – optical emission spectrometry (ICP-OES) revealed that increasing silane surface modification decreased particle settling. The two  $SiO_2$ -NFs with the highest amount of surface silanization did not reach the cells in a submerged exposure setting, and they were therefore only tested at the ALI. Identical dose-response curves were observed for both the submerged testing and testing at the ALI for the  $SiO_2$ -NFs without and with low/intermediate surface functionalization, again showing a decrease in effects with increasing surface functionalization.

**Conclusion** We show that in vitro toxicity assays provide valuable information for SSbD decision making. In vitro cytotoxic, pro-inflammatory and oxidative stress responses can be reduced with increasing surface silane functionalization. The reduced deposition efficiency with increasing silane functionalization, however, highlights that thorough characterization of particle behaviour in cell culture medium should always be performed for SSbD hazard testing. The amount of silane required to reduce toxicity is important information for the future production of safer SiO<sub>2</sub>-NFs and nano-enabled products. Exposure, functionality, and sustainability remain to be investigated to draw full SSbD conclusions.

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Keywords Silica, Nanomaterials, Silane, SSbD, In vitro, SiO<sub>2</sub>-NF

## Background

Safe and Sustainable by Design (SSbD) is an important and recognized concept which drives the design and development of safer and more sustainable chemicals and materials [1-3]. The main aim of SSbD is to integrate safety and sustainability alongside performance at low technology readiness levels to provide companies the ability to reduce potential harm and incorporate safety and sustainability into their product design. With this, SSbD is a crucial tool for achieving the European Union's (EU) Green Deal and Chemicals Strategy for Sustainability (CSS) ambitions and targets [4, 5]. Within an SSbD approach for nanomaterials (NMs) and other advanced materials specifically, a balance between functionality and hazard of the material needs to be found [6, 7]. In other words, hazard should be minimized while maintaining sufficient material performance. For this purpose, in EUfunded projects such as SAbyNA, decision support systems are developed, taking into account hazard as well as material functionality aspects to find the optimal design for a novel material. Predictive in vitro assays play a key role in these approaches to quickly screen for potential hazards of a wide range of materials already at the early stages of NM development [8]. The use of in vitro assays and other new approach methodologies (NAMs) to fill data gaps concerning hazard is highly encouraged [9, 10]. Guidance on selection and use of NAMs is emerging [11], but there is yet little done to verify these approaches within SSbD-relevant case studies.

Synthetic amorphous silicas (SAS) are Generally Regarded as Safe (GRaS) and are therefore used extensively as an additive in consumer products such as cosmetics and foods to enhance effectiveness, texture, and shelf-life, as well as in biomedical applications for drug delivery and cancer therapy [12-16]. In general, we can distinguish two different types of SAS owing to their synthesis method: the high-temperature pyrogenic (or fumed) silica, and silica resulting from low-temperature synthesis methods, like precipitated silica, silica gel, or colloidal silica [17]. Silica NMs (SiO<sub>2</sub>-NMs) produced using high or low temperature methods differ in surface chemistry, and this influences biological outcomes upon exposure [18]. SiO<sub>2</sub>-NMs comprise siloxane bonds (Si-O-Si) terminated with silanol groups (Si-OH) at the SiO<sub>2</sub>-NM surface, and these silanol groups have been associated with membranolytic activity [17]. Unstable and reactive surface structures such as two- and threemembered siloxane rings are formed during pyrolytic synthesis of fumed SiO<sub>2</sub>-NMs and these have been associated with the formation of free radicals [17, 19, 20]. These surface structures are not formed during the low-temperature synthesis of precipitated and colloidal  $SiO_2$ -NMs [17]. However, colloidal  $SiO_2$ -NM surfaces may contain a high density of silanol groups, in some cases even higher than pyrogenic silica, depending on synthesis conditions [17, 19, 21]. In mice, instillation of colloidal  $SiO_2$ -NMs induced acute pulmonary inflammation at low doses, characterized by amongst others neutrophil influx and histopathological lesions [22]. In rats, 4 weeks of inhalation of colloidal  $SiO_2$ -NMs induced neutrophil influx and hyperplasia of pneumocytes in the alveolar ducts [23, 24]. The effects in these studies returned to background levels after a 3-month recovery period [22–24]. The pulmonary inflammatory effects induced by colloidal  $SiO_2$ -NMs were more pronounced when the particles were smaller [25].

Nanoforms (NFs) are defined as different forms of one NM [26]. The colloidal silica NFs (SiO<sub>2</sub>-NFs) under investigation in this study are applied in nano-enabled paints, e.g. for their anti-fouling properties. Commercial paints require a shelf-life of >2 years, and therefore all ingredients, including colloidal SiO<sub>2</sub>-NMs, added to the formulation must have excellent stability over long periods of time. The colloidal SiO<sub>2</sub>-NFs under investigation have been surface modified with 3-(2,3-dihydroxypropoxy)propyl) silanetriol organosilane groups (Fig. 1) to enhance colloidal stability in paints [27]. Silane modified SiO<sub>2</sub>-NMs have been shown to migrate to the surface of the paint after application, where they facilitate anti-fouling through several mechanisms: (i) reduced hydrophobicity, allowing the collected dirt to run off more easily with water; (ii) increased hardness and therefore reduced tackiness; (iii) reduced surface-roughness and therefore reduced dirt-pickup [28]. During the production phase, workers may be exposed to colloidal SiO<sub>2</sub>-NMs, however, inhalation exposure is limited as colloidal SiO<sub>2</sub>-NMs are typically produced in a wet chemical process [17]. During the use-phase, exposure to colloidal SiO<sub>2</sub>-NMs in nanoenabled paints can occur on the skin during painting, and through inhalation of dust particles when sanding painted materials. In the latter case, the SiO<sub>2</sub>-NMs are truly embedded in the binder matrix of the sanded paint dust and are heavily aggregated.

Since  $SiO_2$ -NM particle-related effects can be largely attributed to their surface chemistry, organosilane surface functionalization may be a useful SSbD intervention to reduce hazard. Silane surface functionalization has previously been shown to reduce cytotoxic effects in an ecotoxicity study using fish cells [29], and was shown to slightly reduce the formation of reactive oxygen species (ROS) in the ferric reducing ability of serum (FRAS) assay and electron paramagnetic resonance (EPR) assay [30].



Fig. 1 Colloidal SiO<sub>2</sub>-NF surface functionalization with 3-(2,3-dihydroxypropoxy)propyl)silanetriol organosilane groups

However, at present there is a knowledge gap concerning the amount of surface coverage with organosilanes needed to reduce hazardous properties. For the purpose of SSbD and the future production of safer nano-enabled products it is important to know which surface silane content would be sufficient to achieve a reduction in hazardous effect.

In this study, we investigate the in vitro toxicity of a panel of five colloidal SiO<sub>2</sub>-NFs with different degrees of silane surface functionalization using simple in vitro assays. The aim of this study is two-fold: (i) To showcase and scrutinize an in vitro approach for efficient nano-specific hazard screening to facilitate SSbD decision making; and (ii) To determine the most optimal NM design for minimal toxicity. A test-battery consisting of simple in vitro assays and thorough particle characterization is performed to screen for cytotoxicity, pro-inflammatory responses, as well as acellular ROS production, haemolysis, and cellular markers for oxidative stress and endoplasmic reticulum (ER) stress, which have previously been established to be important markers for NM-induced pulmonary inflammation [31-33]. We hypothesize that in vitro toxicity decreases with increased silane surface functionalization through shielding of the SiO<sub>2</sub>-NF surface. The other SSbD dimensions such as product functionality, sustainability and human/ environmental exposure are not assessed in the present study and remain to be evaluated before drawing final SSbD conclusions.

Assessing the deposited dose in submerged cell exposure models is important when testing NMs in vitro [34]. The dispersion stability of colloidal SiO<sub>2</sub>-NMs greatly depends on factors such as pH and salinity [35, 36], which may vary depending on the cell culture medium (CCM)

 Table 1
 Case study materials (SiO<sub>2</sub>-NFs) and the amounts of silane added during the silanization process

SiO <sub>2</sub> -NF	Silane added to dispersions dur- ing silanization process (µmol/ m <sup>2</sup> of particle surface)	Percentage silane added as compared to Silica-Sil4		
Silica-Std	0	0%		
Silica-Sil1	0.78	22%		
Silica-Sil2	1.55	44%		
Silica-Sil3	2.35	66.67%		
Silica-Sil4	3.53	100%		

used in the in vitro assay. Additionally, the organosilane surface functionalization is suspected to affect the stability of  $SiO_2$ -NFs in CCM. Therefore, the behaviour of the  $SiO_2$ -NFs in CCM is extensively assessed here, which allowed for the expression of the biological response as a function of the dose delivered to the cells. Altogether, this study provides insights into the effects of gradual changes in silane surface coverage on in vitro hazard in the context of SSbD and into the use of in vitro assays specifically for SSbD decision-making for NMs.

## Methods

## SiO<sub>2</sub>-NFs

The five colloidal  $SiO_2$ -NFs were provided by Nouryon and were specifically produced as a silane surface functionalization case study. Table 1 shows the particle names and the amount of 3-(2,3-dihydroxypropoxy)propyl) silanetriol organosilane added during the silanization process, quantitatively, and relatively to Silica-Sil4. Silica-Sil1-4 were produced by adding different amounts of silane to Silica-Std, which was always used as a starting point. Therefore, the SiO<sub>2</sub>-NFs differ only in their surface functionalization and are otherwise identical. The density of the particles is  $2.21 \text{ g/cm}^3$ .

Silica-Sil3 is similar (but not entirely identical) to Levasil CC301, and Silica-Std to Levasil CS30-236, which are commercially available. The other  $SiO_2$ -NFs are not commercially available. Silica-Sil3 is identical to the Silica-Silane that was tested in Ag Seleci et al. (2022) and Keller et al. (2021) [30, 37] but its name is changed here to suit the purpose of this case study.

## **Experimental design**

(SEM)

(DLS)

The colloidal  $SiO_2$ -NFs were thoroughly characterized and assessed for their hazard potential upon exposure via the inhalation route using in vitro methods. The general experimental design is shown in Fig. 2. Cell types relevant for the inhalation exposure route were selected for the in vitro assays, which included the alveolar epithelial cancer cell line A549 and the bronchial epithelial immortalized

1. Particle characterization

Particle morphology Accessible surface Deposited dose

Size distribution Hydrodynamic diameter

area (Sears)

over time (DLS)

2a. Submerged testing

(if possible)

(ICP-OES)

cell line BEAS-2B to cover both the upper as well as the lower regions of the respiratory tract. The monocyte cancer cell line THP-1 differentiated to M0 macrophages was included to represent pulmonary macrophages.

## Characterization of SiO<sub>2</sub>-NFs

## Sears Titration

Specific surface area (SSA) could not be determined through the Brunauer-Emmett-Teller (BET) method, as this method requires drying of the samples, which would induce agglomeration for these  $SiO_2$ -NFs. Instead, surface area was assessed using Sears titration which measures the surface silanol groups at the  $SiO_2$ -NF surface through titration with NaOH. For the non-silanized Silica-Std this gives an accurate indication of its SSA. Since the only difference between the  $SiO_2$ -NFs is the silane content, similar surface areas for the five  $SiO_2$ -NFs were assumed. For the silanized  $SiO_2$ -NFs, Sears titration was

3. Dose-response modeling

2b. Air-liquid interface

testing

and particle comparisons



Fig. 2 Schematic representation of the experimental design. SEM = Scanning electron microscopy. ICP-OES = inductively coupled plasma – optical emission spectrometry. DLS = dynamic light scattering. ER = endoplasmic reticulum

performed to obtain an indication of the number of silanols that are not shielded by silane groups at the specific assay conditions (pH 4 and pH 9 at high salt). This gives a rough indication of particle surface accessibility and thus an estimation of the accessible free silanol groups on the particle surface. It is however not equivalent to the number of accessible (non-bound) surface silanols. The silanization itself is likely to shift the pKa of the silanols, for many of them likely even outside of the pH 4–9 window.

The titration procedure was carried out as described in Sears et al. (1956) [38]. In short, the amount of the SiO<sub>2</sub>-NF sample corresponding to 1.5 g SiO<sub>2</sub> was diluted with 80 mL deionized water, and the pH was adjusted to 3–3.5 using HCl. Then, 30 g of NaCl was dissolved in this volume and further deionized water was added to reach a total volume of ~150 mL. The amount of NaOH consumed in a titration between pH 4 and 9 was then recalculated into the specific surface area of the silica using the following equation:

$$SSA(m^2/g) = \frac{(1.5 \times 100 \times V_{NaOH} \times 32)}{(W \times wt\%)} - 25$$

Where: 1.5: Desired amount of SiO<sub>2</sub> (g); 100: Conversion factor % to g;  $V_{NaOH}$ : volume of 0,1 mol/l NaOH (mL) required to increase the pH from 4 to 9; 32: proportionality constant between  $V_{NaOH}$  and specific surface area (see Sears et al. (1956) [38]); W: sample weight (g); 25: intercept for the linear relationship between titre volume and specific surface area (see Sears et al. (1956) [38]); wt%: SiO<sub>2</sub> concentration of the sample.

## Cell culture media

Supplementary Table S1 shows which cell culture media were used for which experiments. The use of different media was required as phenol red interferes with dynamic light scattering (DLS) analysis. Properties which are important for SiO<sub>2</sub>-NF behaviour and colloidal stability are pH and inorganic salts [36].

## Evaluation of hydrodynamic diameter, particle concentration and polydispersity index over time

To assess the behaviour of the  $SiO_2$ -NFs in CCM, DLS analysis was performed. The  $SiO_2$ -NFs were diluted to 100 µg/mL in either RPMI or minimal essential medium (MEM) without phenol red, of which 1 mL was transferred to a cuvette (Malvern, DTS0012) and placed in the DLS apparatus (Malvern Zetasizer Ultra). The following parameters were chosen in the ZS Explorer DLS software: Refractive index  $SiO_2$ : 1.46; Absorption  $SiO_2$ : 0.001; Refractive index dispersant: 1.33; Viscosity dispersant: 1.

The same cuvette was used for the measurements at 0 h and 24 h. Cuvettes with  $SiO_2$ -NF suspensions with lids were placed in an incubator at 37 °C for 24 h in between

measurements. Measurements were always carried out at 25 °C.

To assess the behaviour of the  $SiO_2$ -NFs in CCM over time, continuous DLS measurements were performed. A program was run which measured hydrodynamic diameter three times every 25 min for 24 h. The DLS was kept at a constant temperature of 25 °C.

## **Endotoxin content**

Endotoxin content of the SiO<sub>2</sub>-NFs was tested in two independent laboratories using two different methods, namely the HEK293 TLR2 and TLR4 reporter cell line assay and the limulus amebocyte lysate (LAL) assay (KTA<sup>2</sup> lysate, Charles River, R19000). This was necessary due to the interfering behaviour of the SiO<sub>2</sub>-NFs, being able to bind cytokines and cause gelling of reagents. All SiO<sub>2</sub>-NFs tested were negative in the reporter cell line assay (Supplementary Figure S1), and LAL assay results were 0.275 IU/mL and lower (Supplementary Table S2).

#### **SEM analysis**

The particles that were nebulized in the radial cloud system (explained below) were collected on Carbon Film 300 Mesh TEM grids (Electron Microscopy Sciences, CF300-Cu-25), and investigated by scanning electron microscopy (SEM-FEG, LEO 1530, Germany). All the samples were coated with a 10 nm thick carbon layer deposited by sputtering. Images were acquired with a working distance of approximately 8 mm, an accelerating voltage of 5 kV, a magnification from x15k to ×500k and a diaphragm aperture of 30  $\mu$ m.

## In vitro studies

All in vitro cellular studies were performed using biocide-free  $SiO_2$ -NF dispersions.

#### Evaluation of delivered dose (submerged)

To measure the amount of SiO<sub>2</sub>-NF that settled on the bottom of exposure wells, an experiment mimicking cell culture conditions was set up. This experiment was based on previous methods reported in Botte et al. (2024), where a sticky gelatine layer was placed at the bottom of wells to mimic cells, collecting any settled SiO<sub>2</sub>-NFs on the gelatine gel [39]. The complete protocol can be found in Supplementary File 2. In short, a 10% gelatine (Sigma G2500) solution containing 1%<sub>w/w</sub> genipin (Thermofisher 466642500) as a cross-linker (based on a method by Furlani et al. (2022) [40]) was made, and 75  $\mu$ L of the mixture was added to the wells of a 96-well plate (Cellstar 655-180). Plates were incubated overnight at 37 °C and 5%  $CO_2$  to harden, rehydrated over another night, and then dilutions of SiO<sub>2</sub>-NFs were applied to the wells. The plates were incubated for 24 h at 37 °C and 5% CO<sub>2</sub> and after the incubations the supernatant was transferred to

Eppendorf tubes, making sure not to touch the gel with the pipette tip. The gels with the settled  $SiO_2$ -NFs were left in the plates and both the plates and Eppendorf tubes were frozen at -20 °C until ICP-OES analysis. Standards, supernatants, and gel samples were acid-treated with HF, HNO<sub>3</sub> and HCl (ratio 1:2:2 in volume) (Sigma-Aldrich – St. Louis, MI, USA) and analysed by inductively coupled plasma optical emission spectrometry using an ICP-OES 5100 - vertical dual view apparatus coupled with OneNeb nebulizer (Agilent Technologies, Santa Clara, CA, USA). Extra care was taken to not use any glassware as to not contaminate samples with Si. A pilot experiment was carried out using both RPMI and MEM. No differences were seen between the two media, and it was decided to continue with MEM only for the results to be applicable to a wide range of cell culture experiments. ICP-OES data for the gelatine compartment were corrected for background Si, converted to µg/cm<sup>2</sup> by using volume and well surface area, and converted to SiO<sub>2</sub> by using molecular weights.

## **Cell culture conditions**

A549 human alveolar epithelial cells (ATCC, CCL-185) were cultured in RPMI 1640 medium with Glutamax and HEPES (Gibco 72400054) supplemented with 10% foetal bovine serum (FBS) (Gibco 1050064) and 1% penicillin/ streptomycin (Gibco 15140122). Cells were sub-cultured twice a week and used until passage number 12. BEAS-2B human bronchial epithelial cells (ATCC, CRL-3588) were cultured on collagen-coated plates (0.03 mg/mL bovine collagen type I) in DMEM Medium with Glutamax (Gibco 319660-21) supplemented with 10% FBS and 1% penicillin/streptomycin. THP-1 human monocytes (ATCC, TIB-202) were cultured in RPMI 1640 medium supplemented with 10% FBS and 100 µg/mL streptomycin (Thermo Fisher Scientific, UK). Cells were confirmed to be mycoplasma free.

## A549 submerged exposures for cytotoxicity and cytokine release measurements

A549 cells were seeded at a density of 60,000 cells/cm<sup>2</sup> in 96-well plates (Cellstar 655-180) and left to attach overnight at 37 °C and 5% CO<sub>2</sub> to reach 100% confluency. SiO<sub>2</sub>-NFs did not require any sonication as they are colloidal and very stable in suspension. They were directly diluted in exposure medium (RPMI supplemented with Pen/Strep, no FBS) and vortexed between dilution steps and prior to addition to cells. FBS was excluded in the submerged as well as ALI exposures to facilitate a better comparison between the two methods, to better resemble the in vivo situation in the lungs where serum is not present, and to not shield any silica surface with serum proteins. It was previously shown that FBS-free CCM is appropriate for the cultivation of A549 cells [41], and

this was therefore not further confirmed in the present study. Negative and positive control conditions were also exposed without FBS. Cells were exposed to 200  $\mu$ L of SiO<sub>2</sub>-NF dispersion per well in concentration ranges obtained by serial dilution and incubated for 24 h at 37 °C and 5% CO<sub>2</sub>. The highest exposure concentration for Silica-Std was 100  $\mu$ g/mL and for the other SiO<sub>2</sub>-NFs this was 400 µg/mL. 10 µg/mL lipopolysaccharide (LPS) from E. coli O111:B4 (Sigma Aldrich, L4391) was used as a positive control for cytokine secretion and Triton-X (1%) was used as a positive control to obtain 100% cell death. After 24 h, the supernatant was collected for lactate dehydrogenase (LDH) analysis (Roche 11644793001, carried out following manufacturer's instructions), and WST-1 reagent was applied to the cells (Roche 11644807001, carried out following manufacturer's instructions). Absorbance values of unexposed cells were used to establish 100% viability and to calculate viability of exposed cells. From the cytokine plate, supernatant was transferred to v-bottom plates and centrifuged at 1.500 x g for 10 min to remove aggregates/agglomerates of SiO<sub>2</sub>-NF and cell debris. Supernatants were then transferred to a clean 96-well plate and stored at -80 °C for cytokine analysis. Interference with both LDH and WST-1 assays was assessed, and no interferences were observed (Supplementary Figure S2).

## A549 ALI exposures for cytotoxicity and cytokine release measurements

A549 cells were seeded at a density of  $60,000 \text{ cells/cm}^2$ in 24-well insert plates (Corning 3740) and left to attach overnight at 37 °C and 5% CO<sub>2</sub>, to reach 100% confluency. The basolateral compartment contained 600 µL CCM, and the apical compartment contained 100  $\mu$ L. On day 2, the apical CCM was removed and cells were exposed to SiO<sub>2</sub>-NF dispersions using a radial in vitro aerosol exposure system (RIVAES, designed at the National Institute for Public Health & the Environment (RIVM), inspired by the design of the VITROCELL Cloud exposure system (Vitrocell, Waldkirch, Germany)). In the RIVAES, the transwell inserts are placed radially to minimize variation in deposition. The system has a slightly smaller surface area than the VITROCELL® Cloud system, resulting in a slightly higher deposition. It is equipped with a refined temperature control system resulting in a stable temperature at the transwell inserts. A 4–6  $\mu m$  pore size vibrating mesh nebulizer (Vitrocell) was used. After vortexing, SiO<sub>2</sub>-NFs were directly diluted in ultrapure water (B. Braun, 3624390), with 0.009% NaCl (90 µg/mL NaCl) to facilitate nebulization of the dispersions [42]. 200  $\mu$ L of SiO<sub>2</sub>-NF suspension was nebulized following previously described procedures [42], with minor modifications related to the timing of the data acquisition which are described below in the deposition assessment section.

Since the tested colloidal SiO<sub>2</sub>-NFs have such a small particle size and are stable in dispersion, high concentrations of material could be nebulized without causing the nebulizer to block. The desired depositions were achieved by nebulizing 5, 10 and 20 mg/mL (obtained by serial dilution) SiO<sub>2</sub>-NF once and repeating the 20 mg/mL nebulization multiple times for the inserts that received the highest doses. During nebulizations, insert holders were filled with 1 mL CCM. After nebulizations, inserts were transferred to plate wells filled with 600 µL fresh CCM without FBS at the basolateral side, to align with submerged experiments. Non-exposed inserts were used as incubator control, and also received fresh basolateral CCM without FBS. A nebulization of 200 µL of 700 µg/ mL LPS was used as positive control. A nebulization of 200 µL of vehicle only (0.009% NaCl in ultrapure water) was used as negative control. After exposures, inserts were incubated for 24 h at 37 °C and 5% CO2. Several additional nebulizations were performed without cells, where an open 60 mm petri dish (Corning, 430166) containing 2 mL 0.009% NaCl in ultrapure water was placed in the centre of the RIVAES to collect nebulized particles. This sample was immediately analysed using DLS.

After 24 h, 200 µL CCM without FBS was added to the apical side and left for exactly 10 min. Apical and basolateral supernatants were then collected in Eppendorf tubes and centrifuged for 15 min at 10.000 x g at 4 °C to remove SiO<sub>2</sub>-NFs and cell debris. 100  $\mu$ L of this supernatant was directly used for LDH analysis (according to manufacturer's instructions), and the remaining volume was stored at -80 °C for cytokine analysis. Apical and basolateral LDH contents were calculated separately as compared to their respective LDH max condition (100% cell death achieved by incubating inserts with 1% Triton-X for 1 h). The average was then taken to calculate total cytotoxicity. WST-1 assay was carried out directly on the cells following manufacturer's instructions. Basolateral CCM was removed before WST-1 incubation to avoid leakage of reagent to the basolateral compartment, and after incubation supernatants were transferred to clean 96-well plates for absorbance measurements. Absorbance was measured using a Spectramax M2 spectrophotometer. The incubator control was used as 100% viability condition, and cells exposed to nebulized vehicle only were used as  $0 \,\mu g/cm^2$  condition.

## Evaluation of delivered dose (ALI) *QCM*

A Quartz Crystal Microbalance (QCM) (Vitrocell) was used to determine depositions of lower dose exposures following previously established methods [42, 43], with some modifications. In short, after warming up the RIVAES, it was ensured that the QCM signal was stable. Data acquisition was started right before nebulizations were started. After nebulization, when QCM signal dropped to a horizontal signal ( $\pm 6$  min), the hood of the RIVAES was lifted, dried, and put back after exactly 1 min. When the QCM signal was entirely stable ( $\pm 3-7$  min), data acquisition was ended. QCM crystals were replaced after a total loading of 10 µg/cm<sup>2</sup> (as recommended by Vitrocell). QCM data was compared to filter data (see below), and they were almost identical. Therefore, the deposition data obtained using filters was used for the final analysis and graphs. Most doses were too high for QCM analysis as they exceeded the maximum loading capacity of 10 µg/cm<sup>2</sup>. The QCM deposition data was used for the final analysis and graphs for the 5 and 10 mg/mL conditions.

## Filters

Filters (Whatmann, WHA1821021) were pre-weighed using a Sartorius ME-5 micro balance after 1 h of acclimatization in the weighing room. During each nebulization in the radial in vitro aerosol exposure system, two filters were included in empty insert slots. After one day of drying, they were again weighed after acclimatization in the weighing room. The difference in weight between after and before exposure, expressed as amount per surface area, equals the deposited dose in ALI experiments. Results were not corrected for the mass of the 0.009% NaCl that was present in the exposures, as the weight of this vehicle (after drying) was below the detection limit of the QCM. Since the depositions for the 20 mg/ mL concentration of the five SiO<sub>2</sub>-NFs were very close to each other, the average was used for the final analysis and graphs.

#### Cytokine analysis

Samples from the -80 °C storage were thawed at room temperature, mixed by either vortex (tubes) or plate shaker (plate), and analysed using Legendplex, exactly following manufacturer's instructions. A mix and match kit based on Human Inflammation panel 1 (Biolegend, 740808) containing IL-6, IL-8 and MCP-1 beads was used. Samples were analysed on a Canto flow cytometer, counting 1000 events per well. Biolegend Qognit software was used for data analysis, and all gates were manually reviewed. Cytokine concentrations measured in the basolateral compartment were multiplied by 3 to correct for dilution caused by differences in compartment volume (600  $\mu$ L in basolateral vs. 200  $\mu$ L in apical and 96-well compartments). Interference of SiO<sub>2</sub>-NFs with cytokines in CCM was tested and results are shown in Supplementary Figure S3.

## THP-1 submerged exposure experiments

THP-1 cells were seeded at 156,000 cells/cm<sup>2</sup> in 96-well plates in CCM containing Phorbol 12-myristate

13-acetate (PMA, 10 ng/mL). After 24 h, cells were washed and fresh CCM was added for a further 24 h before treatment with particles. SiO<sub>2</sub>-NFs were directly diluted in exposure medium (RPMI supplemented with Pen/Strep and FBS) and vortexed between dilution steps and prior to addition to cells. FBS was included in these experiments, in contrast with all other experiments, as THP-1 cells did not subsist in the absence of FBS. Differentiated THP-1 (dTHP-1) cells were exposed to 100 µL of SiO<sub>2</sub>-NF dispersion per well in concentration ranges obtained by serial dilution and incubated for 24 h at 37 °C and 5% CO<sub>2</sub>. The highest dose tested was 125  $\mu$ g/ mL. Positive controls included Triton X-100 at 0.1% v/v and LPS at 100 and 1000 ng/mL. After 24 h exposure to the particles, supernatant was removed and retained for cytokine analysis and cells were washed twice with DPBS (Gibco<sup>™</sup>, ThermoFisher Scientific, UK). 100 µL Alamar Blue reagent (Thermo Fisher Scientific, UK) was then added (1.25% (v/v) solution prepared in FBS-free, phenol red-free MEM 24 h before use) and cells were incubated for 120 min, protected from light. Fluorescence intensity was read at excitation/emission wavelengths of 532/590 nm for resorufin using a Tecan Spark 10 M plate reader. Pro-inflammatory cytokine IL-1 $\beta$  secretion was assessed using ELISA (Invitrogen<sup>™</sup>, ThermoFisher), following the manufacturer's guidelines.

#### Gene expression experiments

For qPCR experiments, BEAS-2B cells were seeded at 37,500 cells/cm<sup>2</sup> in collagen-coated 24-well plates, and A549 cells at 75,000 cells/cm<sup>2</sup> in uncoated 24-well plates and left to attach overnight at 37°C and 5% CO<sub>2</sub>. SiO<sub>2</sub>-NFs were directly diluted in exposure medium (DMEM supplemented with Pen/Strep, no FBS) and vortexed between dilution steps and prior to addition to cells. Exposures were 625 µL of one dose of SiO<sub>2</sub>-NFs that was established to be subtoxic:  $32 \mu g/mL$  for A549 cells and 16 µg/mL for BEAS-2B cells. After 24 h of exposure, cells were washed three times with PBS and lysed using the mRNA assay kit lysis reagent (GeneElute total RNA miniprep kit, Merck Sigma-Aldrich, RTN70) using the supplier's procedure. Lyzed cells were stored at -80°C until extraction. Total RNA was extracted, quality checked and reverse transcribed using superscript III reverse transcriptase (Thermo Fisher Scientific, 18080044). The obtained cDNA was used for qPCR experiment, which was performed using Takyon No ROX SYBR Master Mix blue dTTP (Eurogentec, UF-NSMT-B0710) on a CFX96 Touch system (Biorad) and the following thermal profile: 5 min at 95°C, then 40 cycles of [15 s at 95°C, 20 s at 55°C, 40 s at 72°C], then 1 min at 95°C, 30 s at 55°C and 30 s at 95°C for obtaining the dissociation curve. The used primer sequences were: IL-1β (F 5'-ACAGATGAAGTGCTCCTTCCA-3', R 5'-G TCGAGATTCGTAGCTGGAT-3'); MCP-1 (F 5'-CATT GTGGCCAAGGAGATCTG-3', R 5'-TTCGTTTCCCT TTGAGGCTTC-3'); IL-8 (F 5'-GAATGGGTTTGCTAG AATGTGATA-3', R 5'-CAGACTAGGGTTGCCAGATT TAAC-3'); TNF- $\alpha$  (F 5'-GAGCAGTGAAAGCATGATC C-3', R 5'-CGAGAAGATGATCTGACTGCC-3'); IL-6 (F 5'-CCTCGACGGCATCTCAGCCCT, R 5'-TCTGCCAG TGCCTCTTTGCTGC-3').

## **GSH and RNO depletion**

Depletion of antioxidants was evaluated as consumption of glutathione (GSH) and N, N-dimethyl-4 nitrosoaniline (RNO) induced by the SiO<sub>2</sub>-NFs in an acellular environment at two timepoints (1 and 24 h). GSH (Sigma) and RNO (Sigma) were chosen as probemolecules, being able to react respectively with all reactive oxygen species (ROS) and OH radical only [44]. A calibration curve was performed to determine the GSH and RNO concentration in samples, recording the absorbance at different concentrations (range 0.01-0.40 mM). All suspensions of SiO<sub>2</sub>-NF samples were prepared at 100 mg/L in 0.01 M PBS (Sigma). The suspensions underwent 15 min of ultrasonic bath, which is not needed for colloidal SiO<sub>2</sub>-NFs, but was performed to adhere to the standard operating procedure (SOP), and for comparability to previous results. 1.1 mM GSH probe-molecule solution was prepared in PBS 0.01 M and 0.5 mL was added to 5 mL of the SiO<sub>2</sub>-NF suspension, with a final concentration of GSH during the test of 0.1 mM. After the exposure time, Ellman reagent in PBS 0.1 M+1 mM of EDTA (Sigma) was added to the sample in a final concentration of 0.15 mM. 1 mM RNO probe-molecule solution was prepared in PBS 0.01 M, and 0.2 mL was added to 5 mL of the SiO<sub>2</sub>-NF suspension, with a final concentration of RNO during the test of 40 µM. The samples were kept under dark conditions and at 25 °C for all exposure durations (1-24 h). The samples were filtered (PES –  $0.22 \,\mu$ m) to eliminate SiO<sub>2</sub>-NFs before measuring absorbance with a beam spectrophotometer (Hach Lange, DR3900). Filtering was likely ineffective as the SiO<sub>2</sub>-NF primary particle size is 17 nm but was performed anyway to adhere to the standard operating procedure, and for comparability to previous results. CuO NMs (15 nm, PlasmaChem GmbH) were used as positive control in the same exposure concentrations and conditions as the SiO<sub>2</sub>-NFs.

## Haemolysis assay

The haemolysis assay was performed to assess the membranolytic potential of the  $SiO_2$ -NFs. The complete SOP can be found in Supplementary File 3. In short,  $SiO_2$ -NFs were diluted in PBS to 15 mg/mL and dispersed using vortexing and bath sonication for 10 min. Bath sonication was not required for these  $SiO_2$ -NFs but was performed

to allow comparison to previously obtained results using this protocol. Concentration ranges of SiO<sub>2</sub>-NFs were made in PBS. 0.1% Triton-X was used as 100% haemolysis condition. Crystalline silica (Quartz DQ12), which is known for its haemolytic properties, was used as positive control. Fresh whole sheep blood in Alsever's solution (Thermo Scientific, 12977755) was washed using PBS and centrifugation to obtain red blood cells (RBCs). Particle suspensions and RBCs were combined in flat-bottomed 96-well plates in triplicates and incubated at room temperature for 10 min under agitation. Plates were then centrifuged for 15 min at 250 x g, 100  $\mu L$  of supernatant was transferred to a clean 96-well plate, and absorbance was measured at wavelength 540 nm, with interference 650 nm. Results were expressed as % haemolysis compared to the 0.1% Triton-X condition.

## Data and statistical analysis

Each assay was carried out at a minimum of three independent times, containing at least two technical replicates for ALI exposures and three technical replicates for submerged exposures. Exceptions are the endotoxin assays, RNO and GSH depletion assays, cytokine interference assay and the gene expression experiments, which were carried out once. qPCR data were analysed using the Relative Expression Software Tool (Data were analysed using the  $\Delta\Delta$ Cq method using the Relative Expression Software Tool (REST) [45]. For the other assays, the average of the technical replicates per experiment was taken, and the resulting three experiment averages were analysed by one-way ANOVA, followed by Dunnett's multiple comparisons test, using GraphPad Prism version 9.5.1. Benchmark-dose (BMD) analysis was performed using PROAST software version 70.1. For viability analysis a critical effect size (CES) of 50% reduction in viability compared to the 100% control was used and for IL-1 $\beta$ secretion a CES of 300% change over the negative control was used. These CES were chosen to be able to compare potencies at the steepest part of the dose-response curve.

 Table 2
 Specific surface area of the SiO<sub>2</sub>-NFs as assessed by
 Sears Titration

SiO <sub>2</sub> -NF	SSA* (m²/g)	Reduction in SSA (%)
Silica-Std	364	0
Silica-Sil1	287**	21
Silica-Sil2	205**	44
Silica-Sil3	148**	59
Silica-Sil4	120**	67

\*Specific surface area (SSA) values shown for the silane functionalized SiO<sub>2</sub>-NFs are an estimation of the accessibility of surface silanol groups

\*\*Actual SSA is identical to Silica-Std

## Results

#### Physicochemical characterization of SiO<sub>2</sub>-NFs

Sears titration was performed to obtain SSA of Silica-Std, and an estimation of the amount of surface silanol groups that is not shielded by silane groups for Silica-Sil1-4. Sears titration results could not be considered a direct measure of the number of accessible silanol groups, as our data suggested that it is likely that (the hydrophobic parts of) the silane (closest to the particle surface) shifted the pKa of the remaining silanol groups, thus impacting the titration. The Sears titration results in Table 2 show a clear reduction in SSA, pointing to a reduction in accessible surface silanol groups with increasing surface silane functionalization. The actual SSA of all particles is equal to Silica-Std (364 m<sup>2</sup>/g) as the only difference between the particles is their surface functionalization.

Table 3 shows z-average, median volume-based hydrodynamic diameter, particle concentrations, and polydispersity index of the SiO<sub>2</sub>-NFs measured at timepoint 0, and after 24 h of incubation at 37 °C in two different types of FBS-free CCM, using DLS. Hydrodynamic diameters larger than the primary particle size indicate aggregation and/or agglomeration, which was observed for all SiO<sub>2</sub>-NFs after 24 h when looking at z-average only. The z-average is based on intensity-based size distributions and is heavily affected when a few large agglomerates are present.

Volume-based hydrodynamic diameters are less affected by large agglomerates. Judging by the volumebased hydrodynamic diameter in Table 3, agglomeration occurred in all particles except for Silica-Sil3 in RPMI and Silica-Sil4 in both media, which is also reflected in Supplementary Figure S4 which shows size distributions with an additional larger-sized peak for these particles. This second peak was very prominent in the intensity-based size distribution and was not present in the volume- and number-based size distributions. The size distributions of Silica-Std, Silica-Sil1, Silica-Sil2 and Silica-Sil3 in MEM at 24 h shifted in its entirety to a larger size as compared to the 0 h timepoint, indicating extensive agglomeration.

A reduction in particle concentration at the 24 h timepoint compared to the 0 h time point may indicate aggregation/agglomeration, dissolution, or settling of the NFs to the bottom of the cuvette, which was observed for all SiO<sub>2</sub>-NFs in both media except for Silica-Sil3 in RPMI, and Silica-Sil4 in both RPMI and MEM. This reduction in particle concentration indeed aligns with the increase in hydrodynamic diameter.

Figure 3 shows the median volume-based hydrodynamic diameter of Silica-Std measured continuously over a 24 h time range in two different types of FBS-free CCM. The samples remained in the DLS apparatus which measured hydrodynamic diameter at short time intervals at 25 °C. The time of onset of aggregation/agglomeration

SiO <sub>2</sub> -	ССМ	Z-average (nm) ± SD		Polydispersity index ± SD		Number concentration		Median volume-based	
NF						(particles/mL) ± SD		hydrodynamic diameter (nm)	
								± SD	
		0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h
Silica-	RPMI	17.8 ± 0.3	344.3 ± 23.1	$0.19 \pm 0.02$	0.05 ± 0.03	3.8 ± 0.8 E+12	4.4 ± 3.1 E+08	$12.6 \pm 0.3$	369.6 ± 25.9
Std	MEM	$18.1 \pm 0.8$	N.D.	$0.21 \pm 0.03$	N.D.	2.6 ± 0.6 E+12	N.D.	$12.2 \pm 1.0$	N.D.
Silica-	RPMI	19.4 ± 1.1	443.2 ± 19.1	0.23 ± 0.03	0.22 ± 0.12	3.6 ± 1.7 E+12	2.3 ± 0.9 E+08	13.4 ± 0.7	411.0 ± 44.4
Sil1	MEM	19.3 ± 1.1	540.8 ± 252.3	0.19 ± 0.03	0.28 ± 0.18	4.0 ± 1.8 E+12	2.0 ± 0.5 E+08	12.9 ± 0.6	376.8 ± 25.4
Silica-	RPMI	18.3 ± 0.5	737.0 ± 52.9	0.19 ± 0.02	0.60 ± 0.04	3.2 ± 0.7 E+12	9.4 ± 6.2 E+07	13.1 ± 0.7	208.2 ± 12.5
Sil2	MEM	19.2 ± 1.8	389.3 ± 8.9	$0.19 \pm 0.04$	$0.21 \pm 0.05$	1.1 ± 1.3 E+13	3.0 ± 1.8 E+08	12.8 ± 0.8	374.3 ± 22.7
Silica-	RPMI	17.9 ± 0.3	669.3 ± 243.1	0.14 ± 0.02	0.62 ± 0.21	7.7 ± 4.5 E+12	5.0 ± 0.8 E+12	13.5 ± 0.7	10.2 ± 2.2
Sil3	MEM	19.1 ± 1.0	431.3 ± 55.8	$0.18 \pm 0.04$	0.42 ± 0.05	3.5 ± 0.8 E+12	4.8 ± 1.9 E+07	13.2 ± 0.4	243.8 ± 33.0
Silica-	RPMI	19.0 ± 1.4	549.3 ± 457.2	0.16 ± 0.05	0.55 ± 0.37	4.7 ± 0.3 E+12	2.2 ± 0.8 E+13	13.7 ± 0.8	11.5 ± 3.4
Sil4	MEM	18.9 ± 1.0	139.3 ± 101.9	$0.16 \pm 0.05$	0.27 ± 0.09	4.9 ± 1.6 E+12	3.9 ± 1.4 E+12	$14.0 \pm 0.5$	$16.0 \pm 1.0$

**Table 3** Hydrodynamic diameter, polydispersity index, and particle number concentration as assessed by dynamic light scattering (DLS) analysis of the five colloidal SiO<sub>2</sub>-NFs after 0 h and 24 h of incubation in CCM

DLS measurements were carried out at 25°C, and samples were incubated at 37°C between the 0 h and 24 h time-point to mimic cell culture conditions. SiO<sub>2</sub>: NFs were diluted to 100  $\mu$ g/mL in RPMI and MEM media, without foetal bovine serum (FBS) supplementation. N.D.= not determined as sample quality was marked as insufficient for a reliable reading. Yellow colour depicts a higher hydrodynamic diameter at 24 h as compared to at 0 h. Blue colour depicts a lower particle count at 24 h as compared to at 0 h. Data represent an average of three independent experiments and are expressed as mean ± SD



Fig. 3 Median volume-based hydrodynamic diameter of Silica-Std (100  $\mu$ g/mL) in RPMI and MEM without FBS over time as assessed by continuous DLS size measurements. Cuvettes containing the dispersions remained in the DLS apparatus for 24 h, and hydrodynamic diameter was assessed every 25 min. The temperature inside the DLS apparatus was kept at 25 °C. Data represent an average of three independent experiments and are expressed as mean  $\pm$  SD

of Silica-Std seemed to depend on the type of CCM used, which is dependent on the cell type used in the in vitro experiment and its CCM requirements. The same experiment was carried out with Silica-Sil3, and no aggregation/agglomeration was observed in either CCM (results not shown).

The absence of aggregation/agglomeration of Silica-Sil3 in both media at 25 °C was in contrast with the observed aggregation/agglomeration when the 24 h incubation was carried out at 37 °C (Table 3), demonstrating the influence of temperature on particle behaviour. The absence of a size and particle count reading of Silica-Std in MEM after 24 h of incubation at 37 °C (Table 3) and the poor data quality of this sample (Supplementary Figure S4) could point to dissolution or the settling of all the particles to the bottom of the cuvette. This effect did not occur in RPMI, and also not in MEM after a 24 h incubation at 25 °C judged by the good data quality of this reading (Supplementary Figure S4).

Figure 3 and Table 3 show that not all SiO<sub>2</sub>-NFs aggregated/agglomerated equally and will therefore most likely not deposit equally either. To measure SiO<sub>2</sub>-NF deposition with more certainty, SiO2-NF suspensions were applied to a regular 96-well plate containing gelatine gels to represent cells at the bottom of the well and were allowed to settle under regular cell culture conditions, after which the gels and the supernatants were assessed individually for Si content using ICP-OES. The results in Fig. 4 show a concentration-dependent deposited dose increase of Silica-Std, Silica-Sil1 and Silica-Sil2 in MEM with and without FBS supplementation. Silica-Sil3 and Silica-Sil4 SiO<sub>2</sub>-NFs did not reach the gelatine layer in a dose dependent manner. This suggests limited settling of these materials, and the inability to achieve a dose-response in a submerged setting. Silica-Sil3 and Silica-Sil4 were therefore subsequently tested in air-liquid interface (ALI) models only. Negative deposition values for Silica-Sil4 in the FBS-free condition and quantifiable deposition in the FBS condition were likely due to large variation in background samples.

The percentage of Si detected in the gels as compared to the total Si (gels + supernatant) was used to determine deposited doses in the in vitro experiments. The Si content of the gels at the lower concentrations fell below the limit of quantification (LOQ) of this method, and therefore the average percentage of SiO<sub>2</sub>-NF that reached the gelatine layer at the highest exposure concentrations (75 and 100  $\mu$ g/mL) at the 24 h timepoint was used to extrapolate deposited doses in all submerged experiments performed in MEM without FBS, which was 55.4% (SD±6.7) for Silica-Std, Silica-Sil1, and Silica-Sil2. In MEM with



**Fig. 4** Deposited amounts of  $SiO_2$ -NFs on gelatine gels in the bottom compartment of 96-well plates after incubation for 24 h with concentration ranges of  $SiO_2$ -NFs in MEM without FBS (**A**) and with FBS (**B**). Si content of the gels was assessed by ICP-OES and the results were corrected for background Si, converted to  $\mu$ g/cm<sup>2</sup> by using volume and surface area, and converted to  $SiO_2$  by using molecular weights. \*p < 0.05 and \*\*p < 0.01 as analysed by one-way ANOVA and post-hoc analysis as compared to the corresponding 0  $\mu$ g/mL concentration. LOD: 4  $\mu$ g/cm<sup>2</sup> SiO<sub>2</sub>; LOQ: 13.4  $\mu$ g/cm<sup>2</sup> SiO<sub>2</sub> (dashed line). Data represent an average of three independent experiments and are expressed as mean ± SD

FBS, the deposition efficiency of Silica-Std and Silica-Sil1 at 75 and 100  $\mu$ g/mL was 79.6% (SD ± 1.9), and deposition of Silica-Sil2 at these concentrations was 36.5% (SD ± 0.4), which were the values used for extrapolation of deposited doses in the experiments containing FBS.

Extrapolation of the deposited dose to the other exposure concentrations might have lead to an overestimation of the doses at the lower exposure concentrations, and an underestimation in the higher exposure concentrations, as deposition efficiency is expected to increase with increasing doses [46].

Particles aerosolized by a vibrating mesh nebulizer for ALI exposures were collected and thoroughly characterized using SEM and DLS. After nebulization, several large, nearly perfect spheres were observed that appeared to be silica particles dried up from aerosol droplets (Fig. 5). The effect occurred regardless of surface silane functionalization. DLS analysis revealed that only few such large spheres were present, as the number- and volume- based size distributions did not contain a second peak at larger hydrodynamic diameters (Supplementary Figure S5). This observation can be explained by the fact that the air in the RIVAES (cloud) system reaches 100% relative humidity quickly after the start of the nebulization, preventing the further drying of the aerosols. The hydrodynamic diameter of the particles in the second peak increased with increasing nebulized particle concentration (220 nm for 3 mg/mL and 504 nm for 10 mg/mL), supporting the hypothesis that these spheres are dried droplets with SiO<sub>2</sub>-NFs. SEM images of pristine Silica-Std are shown in Supplementary Figure S6.

## Cell viability

In vitro metabolic activity was assessed as a measure of cell viability by means of WST-1 assay, and LDH release in CCM was assessed as a measure of cytotoxicity in A549 cells in response to the five  $SiO_2$ -NFs at the ALI and to the three settling  $SiO_2$ -NFs in a submerged exposure setting. ALI and submerged conditions were identical in terms of seeding density, well surface area, incubation times and CCM types. Deposited doses at the ALI were assessed using QCM and gravimetric filters,



Fig. 5 Scanning Electron Microscopy images of large spheres originating from dried up droplets of colloidal SiO<sub>2</sub>-NFs Silica-Std (**A**) and Silica-Sil3 (**B**) after nebulization at the air-liquid-interface and collecting particles on grids at the bottom of the RIVAES exposure system

and submerged deposited doses were calculated from the ICP-OES results in Fig. 4A.

The dose-response curves of Silica-Std at the ALI followed an almost identical trend as the submerged doseresponse curves for the LDH (Fig. 6A) and WST-1 assay (Fig. 6B), showing a reduction in metabolic activity and an increase in LDH release in A549 cells at similar deposited doses in both exposure settings. The submerged administered doses where 100% cell death and 0% cell viability were reached was 50  $\mu$ g/mL. Therefore, the highest dose included for Silica-Std was 100  $\mu$ g/mL.

Statistical analysis was performed and p-values can be found in Supplementary Table S3. Silica-Sil1 induced no effects on LDH levels and metabolic activity at the chosen exposure doses in the submerged exposure setting (Fig. 6C&D). However, a significant reduction in metabolic activity of A549 cells was observed at the ALI in response to Silica-Sil1 which was dose-dependent (Fig. 6D). Silica-Sil1 also induced a statistically significant increase in LDH levels at the ALI, but this response was small and not dose-dependent (Fig. 6C). The observed reduction in metabolic activity at the ALI in response to Silica-Sil2 and Silica-Sil3 was also statistically significant, but cell viability did not decrease to below 67% for Silica-Sil2 and 75% for Silica-Sil3 (Fig. 6F&H). The other effects of SiO<sub>2</sub>-NFs were not significant at the assessed doses in the submerged and ALI exposure settings (up to 400  $\mu$ g/ mL, 138.5  $\mu$ g/cm<sup>2</sup> submerged; 141.4  $\mu$ g/cm<sup>2</sup> at ALI).

## **Pro-inflammatory response**

Markers for pro-inflammatory effects were evaluated in A549 cells exposed to the  $SiO_2$ -NFs in a submerged setting and/or at the ALI and are shown in Fig. 7. Silica-Std induced IL-8 and MCP-1 release, which was significant compared to the negative control only in submerged

conditions (Fig. 7A). Silica-Sil1 induced significant MCP-1 release submerged, and IL-8 release at the ALI (Fig. 7B). The other SiO<sub>2</sub>-NFs did not induce cytokine release at the doses tested. It should be noted that the Silica-Std deposited doses  $4.7 \ \mu g/cm^2$  at the ALI and  $4.3 \ \mu g/cm^2$  submerged induced more than 30% cytotoxicity (58% and 44% respectively), as did Silica-Sil1 at 70.7  $\mu g/cm^2$  at the ALI (32% cytotoxicity).

Substantial interference of Silica-Std with cytokines was observed when incubating recombinant IL-8 with several concentrations of SiO<sub>2</sub>-NF and measuring remaining IL-8 after centrifugation by means of ELISA (Supplementary Figure S3). This effect is likely due to the binding of cytokines on the bare Silica-Std surface. An almost 3-fold reduction of recombinant IL-8 was observed after incubation with the highest particle concentration (100  $\mu$ g/mL). The highest exposure dose of 4.6 µg/cm<sup>2</sup> of Silica-Std used for assessing pro-inflammatory cytokine release in Fig. 7 corresponds to an exposure concentration of 12.5  $\mu$ g/mL, which is not expected to impose problematic amounts of interference because the lowest dose assessed in the interference assessment  $(25 \ \mu g/mL)$  showed very little interference. Additionally, 60 pg/mL IL-8 was used to assess interference and the amounts of secreted cytokine observed in Fig. 7 are well above this level. Cytokine secretion after ALI exposures was measured in the basolateral compartment of the wells and is therefore not prone to interferences induced by  $SiO_2$ -NFs. The  $SiO_2$ -NFs alone did not induce a signal on ELISA (results not shown).

Secretion of IL-1 $\beta$  by dTHP-1 cells was assessed under submerged exposure conditions in FBS-containing CCM, as dTHP-1 cells are not compatible with FBS-free CCM (Fig. 8). Silica-Std induced IL-1 $\beta$  secretion and Silica-Sil1 and Silica-Sil2 did not. Deposited doses were calculated



Fig. 6 Metabolic activity (WST-1) and cell membrane damage (LDH release) in A549 cells in response to the five SiO<sub>2</sub>-NFs exposed at the ALI (black line), and to Silica-Sid, Silica-Sil1 and Silica-Sil2 in a submerged setting (pink line). LDH results are expressed as percentage cell death as compared to Triton-X treated cells. WST-1 results are expressed as percentage viability as compared to untreated cells. Curves were fitted following a four-parameter variable slope. Data represent an average of three (submerged) or four (ALI) independent experiments and are expressed as mean ± SD



**Fig. 7** Pro-inflammatory cytokine release (MCP-1 & IL-8) in A549 cells exposed to the five SiO<sub>2</sub>-NFs at the air-liquid-interface (black bars) and to Silica-Std, Silica-Sil1 and Silica-Sil2 in a submerged setting (pink bars). ALI cytokines were measured in the basolateral compartment. Cytokine concentrations were corrected for volume differences between ALI and submerged media (see methods). \*p < 0.05 and \*\*p < 0.01, as analysed by one-way ANOVA and post-hoc analysis as compared to the corresponding 0 µg/cm<sup>2</sup> control condition. Data represent an average of three independent experiments and are expressed as mean ± SD



Fig. 8 IL-1 $\beta$  secretion by dTHP-1 cells exposed to Silica-Std, Silica-Sil1 and Silica-Sil2 under submerged conditions in FBS-containing CCM. Curves were fitted following a four-parameter variable slope. Data represent an average of three independent experiments and are expressed as mean  $\pm$  SD

according to the deposition efficiency in FBS-containing CCM (Fig. 4B). Accompanying assessment of cellular metabolic activity showed no indication of a reduced cell viability at the concentrations tested, except for a decrease at the highest Silica-Std concentration (68.5% viability) (Supplementary Figure S7).

To confirm in vitro pro-inflammatory responses in a cellular setup that is less prone to interferences, the expression of pro-inflammatory marker genes was assessed in A549 cells (Fig. 9A) and confirmed in BEAS-2B cells (Fig. 9B) in a submerged exposure setting at one (subtoxic) dose using qPCR. Pro-inflammatory marker genes only showed upregulated expression upon LPS (positive control) and Silica-Std exposure. No upregulation was observed in response to Silica-Sil1 and Silica-Sil2.

## Oxidative potential and membranolytic activity

Acellular oxidative potential and membranolytic activity were assessed in the acellular glutathione (GSH) depletion and haemolysis assays. Acellular production of ROS was assessed by incubating SiO<sub>2</sub>-NFs with GSH and recording its depletion, and no effects were observed for the SiO<sub>2</sub>-NFs (Fig. 10). RNO depletion was also assessed and showed no response (Supplementary Figure S8). Disruption of cell membranes was tested in the haemolysis assay, where red blood cells were incubated

![](_page_14_Figure_1.jpeg)

**Fig. 9** Pro-inflammatory cytokine gene expression in A549 cells (**A**) and BEAS-2B cells (**B**) in response to 24 h exposure to Silica-Std, Silica-Sil1 and Silica-Sil2 in a submerged exposure setting, as analysed by qPCR. Deposited doses were 5.5  $\mu$ g/cm<sup>2</sup> for A549 cells and 2.8  $\mu$ g/cm<sup>2</sup> for BEAS-2B and were confirmed to be subtoxic. LPS (250 ng/mL, 24 h) was used as a positive control. Dotted line represents 1-fold change in gene expression, thus no change. This experiment was carried out once and statistical analysis was carried out on technical replicates. \*p < 0.05 in REST statistical analysis, meaning either significantly up- or downregulated as compared to the negative control

![](_page_14_Figure_3.jpeg)

**Fig. 10** Oxidative potential of the five  $SiO_2$ -NFs as assessed in the glutathione (GSH) depletion assay. Copper Oxide (CuO) NMs were included as a positive control in the GSH depletion assay due to their known oxidative potential. This experiment was carried out once. Data represent an average of three technical replicates and are expressed as mean  $\pm$  SD

with SiO<sub>2</sub>-NFs, and red blood cell lysis was measured as a red colour. Quartz particles (DQ12; crystalline silica) known for their haemolytic activity were included as a positive control. There was no effect of any of the SiO<sub>2</sub>-NFs on haemolysis, suggesting no membranolytic activity (Fig. 11). The haemolysis and GSH assays were carried out in suspension, and therefore settling of the SiO<sub>2</sub>-NFs was not of importance and all five SiO<sub>2</sub>-NFs could be tested.

![](_page_14_Figure_7.jpeg)

**Fig. 11** Haemolytic potential of the five SiO<sub>2</sub>-NFs as assessed in the haemolysis assay. Quartz particles (DQ12) were included as a positive control in the haemolysis assay due to their known haemolytic activity. The results are expressed as % haemolysis as compared to 100% haemolysis obtained by Triton-X treatment. Data represent an average of three independent experiments and are expressed as mean±SD

#### **Oxidative stress and ER stress**

Gene expression of marker genes for oxidative stress and ER stress was assessed using qPCR after incubation of A549 and BEAS-2B cells with one (subtoxic) dose of the three SiO<sub>2</sub>-NFs that could be tested under submerged conditions (Fig. 12). Silica-Std induced the largest upregulation of Superoxide dismutase 2 (SOD2) gene expression in A549 cells, followed by Silica-Sil1. Silica-Sil2 exposure did not upregulate SOD2 gene expression in A549 cells, but Silica-Sil2 exposed A549 cells showed a small induction of catalase (CAT) and glutathione peroxidase 1 (GPX1) gene expression, suggesting small amounts of oxidative stress of a different origin. In BEAS-2B cells, SOD2 gene expression was only upregulated by Silica-Std, and haem oxygenase 1 (HO-1) gene expression by both Silica-Std and Silica-Sil1. No upregulation of oxidative stress markers was observed in BEAS-2B cells in response to Silica-Sil2. ER stress markers were not upregulated by any of the SiO<sub>2</sub>-NFs, except for Glucose-related protein 78 (GRP78) in A549 cells in response to Silica-Std, which in itself is not enough evidence to assume ER stress.

#### Particle comparisons

A summary of the results of all performed in vitro assays is shown in Table 4. The case study  $SiO_2$ -NFs are identical to each other in all aspects other than their surface functionalization. When BMD confidence intervals do not overlap between  $SiO_2$ -NFs, there is a significant difference in in vitro toxic potency. Silica-Std induced significant effects in 7 assays and had higher potency and non-overlapping BMD confidence intervals as compared to Silica-Sil1 in an additional 5 assays. Silica-Sil1 induced a significant effect in 4 assays and had a higher potency and distinct BMD confidence interval compared to Silica-Sil2 only in the WST-1 assay at the ALI. Silica-Sil2 induced a significant effect on oxidative stress marker gene expression and a small reduction of A549 cell viability at the ALI with a BMD confidence interval

![](_page_15_Figure_2.jpeg)

**Fig. 12** Oxidative stress and endoplasmic reticulum stress marker gene expression measured in A549 and BEAS-2B cells using qPCR after 24 h incubation with Silica-Sil, and Silica-Sil2. Deposited doses were  $5.5 \mu g/cm^2$  for A549 cells and  $2.8 \mu g/cm^2$  for BEAS-2B and were confirmed to be subtoxic. The tunicamycin positive control concentration was  $5 \mu g/mL$ . The dotted line represents 1-fold change in gene expression as compared to the negative control, thus no change. This experiment was carried out once and statistical analysis was carried out on technical replicates. \*p < 0.05 in REST statistical analysis, meaning either significantly up- or downregulated as compared to negative control. Gene abbreviations: Catalase (CAT); Superoxide Dismutase 2 (SOD2) Glutathione Peroxidase 1 (GPX1); glutamate-cysteine ligase (GCLM); Haem Oxygenase 1 (HO-1); Nuclear factor erythroid 2-related factor 2 (NRF2); spliced X-box binding protein 1 (sXBP1); Inositol-requiring enzyme type 1 (IRE1); Glucose-related Protein 78 (GRP78); C/EBP Homologous Protein (CHOP); Activating Transcription Factor 6 (ATF6)

that overlapped with Silica-Sil3. Silica-Sil3 only induced a small reduction in cell viability at the ALI with a BMD confidence interval that overlapped with Silica-Sil4, and no other significant effects. Silica-Sil4 did not show any statistically significant effects in the assays performed. The latter two could however not be assessed in the submerged assays due to insufficient settling.

## Discussion

## SSbD hazard testing

The EU's Chemicals Strategy for Sustainability (CSS) has initiated a shift towards chemicals, materials, and products that are SSbD [4]. If pre-existing knowledge on hazard of a material is insufficient during the hazard assessment steps of an SSbD approach, the use of new approach methodologies (NAMs) such as in vitro assays is highly encouraged to fill the data gaps [1, 9, 10]. However, little to no guidance can be found on how to assess NM hazardous properties for SSbD decision making. In this work, a simple but extensive in vitro testing strategy is applied to assess hazardous properties of five SiO<sub>2</sub>-NFs with differing degrees of organosilane surface

functionalization, with the aim of showcasing an in vitro approach for nano-specific SSbD hazard assessment and finding the optimal silane coverage to ensure a safer NM design. Our results showed that simple in vitro assays were capable of demonstrating a gradual decrease in in vitro effects with increasing silane surface coverage, which was in line with expectations based on the particles' physico-chemical properties, and with previously reported in vitro data on silanized SiO<sub>2</sub>-NFs [29, 30]. In addition, we have shown how thorough characterization of particle behaviour in CCM is crucial for SSbD hazard assessment and decision making. Furthermore, BMD modelling and subsequent comparisons of BMD confidence intervals was found to be a useful and accessible approach to compare the toxic potencies of candidate SiO<sub>2</sub>-NFs to inform SSbD decision making.

The need for determining the dose delivered to the cells  $(\mu g/cm^2)$  instead of reporting only the applied concentration  $(\mu g/mL)$  of a NM has previously been described [34, 47, 48]. In this study we confirm that understanding the behaviour of NMs in CCM is crucial for interpreting test results, and that performing dosimetry assessment

Table 4	Overview of al	SiO <sub>2</sub> -NF effects ir	n the different in	vitro assays. When	n available, be	enchmark dos	se confidence	e intervals are
included	based on dose	-response modell	ing. Otherwise, s	significant effects	are indicated	with 'yes' or 'r	no' based on s	tatistical analysis

			Silica-Std	Silica-Sil1	Silica-Sil2	Silica-Sil3	Silica-Sil4
Exposure method	Cell type	Read-out	BMDL - BMDU (μg/cm²)				
ALI	A549	Metabolic activity (WST-1)	1.76–7.29	78.7–113	195–465	274-802	328–1550
ALI	A549	Membrane disruption (LDH)	0.82–6.80	343 - Inf	318 - Inf	329–7320	325-8100
Submerged	A549	Metabolic activity (WST-1)	2.52-6.11	195–538	245 - Inf	NT	NT
Submerged	A549	Membrane disruption (LDH)	0.87–6.77	418 - Inf	1010 - Inf	NT	NT
Submerged	dTHP-1	IL-1ß secretion	0–2.95	29.9 - Inf	27.5 - Inf	NT	NT
Submerged	dTHP-1	Alamar Blue	25.2-31.1	30.2 - Inf	24.4 - Inf	NT	NT
Exposure method	Cell type	Read-out	Significant	effect yes/no			
ALI	A549	MCP-1 secretion	No	No	No	No	No
ALI	A549	IL-8 secretion	No	Yes	No	No	No
Submerged	A549	MCP-1 secretion	Yes	Yes	No	NT	NT
Submerged	A549	IL-8 secretion	Yes	No	No	NT	NT
Submerged	A549	Pro-inflammatory panel qPCR	Yes	No	No	NT	NT
Submerged	BEAS-2B	Pro-inflammatory panel qPCR	Yes	No	No	NT	NT
Acellular	N/A	GSH depletion	No	No	No	No	No
Suspension	N/A	Haemolysis	No	No	No	No	No
Submerged	A549	Oxidative stress panel qPCR	Yes	Yes	Yes	NT	NT
Submerged	BEAS-2B	Oxidative stress panel qPCR	Yes	Yes	No	NT	NT
Submerged	A549	ER stress panel qPCR	Yes	No	No	NT	NT
Submerged	BEAS-2B	ER stress panel qPCR	No	No	No	NT	NT

Datasets containing sufficient doses were analysed using benchmark-dose (BMD) modelling, and BMD confidence interval lower (BMDL) and upper (BMDU) limits are reported. The critical effect size (CES) was 50% for viability data, and 300% for cytokine secretion. For data with one or two doses, yes/no indication shows whether a significant effect was observed in the assay based on statistical analysis. NT=not tested. Inf=Infinite, meaning that the BMDU is far outside of the tested dose-range, adding uncertainty to the analysis results

is always required, even when performing simple in vitro hazard screening in the context of SSbD. Especially since SSbD hazard testing often involves comparing two or more candidate NFs, it is important to understand whether a difference in biological test outcome is really caused by the SSbD intervention, and not by a reduction in settling and therefore a lower deposited dose on the cells. More specifically, settling of the NMs under investigation should be evaluated before the actual in vitro assays are carried out to determine whether submerged testing is appropriate.

The outcomes of the in vitro assays presented here provide valuable information for a manufacturer to decide which candidate NF is suitable to proceed with in terms of hazard. However, for a final SSbD decision to be made, in vitro hazard testing data need to be integrated with paint functionality, sustainability, costs, exposure, and many more SSbD dimensions, considering the many forms of the material along their lifecycle [1].

## Behaviour of SiO<sub>2</sub>-NFs in different test media

Commonly used models to determine deposited dose for NMs such as the ISD3 or DG models [49, 50] proved unsuitable for the current case study as colloidal stability through steric stabilization and stabilizing surface functionalization are not considered in their calculations and cannot be entered as an input. Sedimentation testing revealed that two out of five SiO<sub>2</sub>-NFs (Silica-Sil3 and Silica-Sil4) in this case study did not reach the bottom of the well in CCM (with or without FBS supplementation) within 24 h, making it impossible to establish any form of a dose-response relationship using submerged exposures for these SiO<sub>2</sub>-NFs. Therefore, Silica-Sil3 and Silica-Sil4 were only tested at the ALI using the RIVAES system. The deposition efficiency of Silica-Std, Silica-Sil1, and Silica-Sil2 in CCM without FBS was equal. In CCM with FBS, Silica-Std and Silica-Sil1 showed a higher deposition efficiency at the 75 and 100 µg/mL administered doses as compared to the FBS-free condition, where Silica-Sil2 showed a lower deposition efficiency at these doses in CCM with FBS as compared to without. The presence of FBS was expected to increase colloidal stability, as was observed previously for colloidal SiO<sub>2</sub>-NFs [46], which we did observe for Silica-Sil2, but not for Silica-Std and Silica-Sil1.

Hydrodynamic size measurements revealed that the timepoint at which Silica-Std starts to aggregate/agglomerate is highly dependent on the type of CCM used. Depending on the cell-type used and its corresponding required CCM, the behaviour over 24 h may differ for the same SiO<sub>2</sub>-NF, even when the resulting delivered dose at the 24 h timepoint is identical. This could mean that for example A549 cells (grown here in RPMI) in a 24 h exposure could have been exposed to a certain delivered

dose of aggregated/agglomerated SiO<sub>2</sub>-NFs for 10 h, whereas cells grown in MEM could have been exposed to the same delivered dose of aggregated/agglomerated SiO<sub>2</sub>-NFs but for 19 h. Different responses in these cell lines could in that case be misinterpreted as differences in sensitivity, when in reality the difference is caused by an almost 2-fold difference in exposure duration.

After 24 h of incubation at 37 °C, Silica-Sil3 in RPMI did not show a reduction in particle count, whereas Silica-Sil3 in MEM did, which suggests that settling of this SiO<sub>2</sub>-NF is faster in MEM as compared to in RPMI, which could again lead to dissimilar deposited doses between two cell types requiring different cell culture media. However, looking at the deposited dose results using the gelatine gels, Silica-Sil3 in neither media settled to the bottom of wells, indicating that aggregation/agglomeration and settling did not occur within 24 h and under the conditions of this experiment. Slightly different media were used for these two experiments since the CCM used in DLS analysis cannot contain phenol-red, and phenolred free CCM has a slightly different composition. The reduction in particle counts at the 24 h timepoint of Silica-Sil3 in phenol-red free MEM may be explained by the fact that this CCM has a higher concentration of NaCl and other inorganic salts. Lower salt concentrations are beneficial for colloidal stability of SiO<sub>2</sub>-NFs [35], and high ion concentrations in general can induce the collapse of colloidal systems [36], causing the particles to aggregate/ agglomerate and settle to the bottom of the well.

The dissolution half time in lung lining simulant fluid (LSF) of Silica-Std and Silica-Sil3 were assessed previously and were 0.5 and 0.8 d respectively [37, 51]. A reduction in both hydrodynamic diameter and particle counts were therefore expected in the 24 h DLS measurement. The inability to obtain an accurate DLS measurement of Silica-Std in MEM after 24 h of incubation at 37 °C could point to dissolution, and the fact that this measurement was of good quality after incubation at 25 °C and not at 37 °C highlights the influence of temperature on particle behaviour. Other explanations for low quality of the measurement could be the presence of too large aggregates/agglomerates for DLS measurements, or the settling of the majority of the particles present. Temperature also affected aggregation/agglomeration, as Silica-Sil3 aggregated in MEM after 24 h at 37 °C, but not at 25 °C. LSF and RPMI have similar pH (±7.4), but LSF has  $a \pm 17$ -fold higher NaCl content, so they are not entirely comparable. Still, the relatively quick dissolution of Silica-Std and Silica-Sil3 in LSF suggests that cell exposures longer than 24 h are not relevant for this case study.

## Toxicity of colloidal SiO<sub>2</sub>-NFs

Silica-Sil1 was synthesized by adding 0.78  $\mu$ mol/m<sup>2</sup> organosilane during the silanization process, which

resulted in a 21% reduction in SSA (silanol groups titrated between pH 4 and pH 9 at high salt, which serves as an estimation of the accessible surface silanol groups). This amount of surface coverage effectuated a decrease in effects observed in WST-1 and LDH assays, as well as in pro-inflammatory cytokine release and expression of pro-inflammatory and oxidative stress marker genes compared to the effects observed with silica without any silane functionalization (Silica-Std). Silica-Sil2 was synthesized by adding 1.55 µmol/m<sup>2</sup> organosilane during the silanization process, which resulted in an estimated 44% reduction in accessible surface silanol groups. This amount of surface coverage almost completely eliminated the effects seen in the assays performed in this investigation. Silica-Sil3 and Silica-Sil4 (1.55 & 3.53 µmol/m<sup>2</sup> organosilane added; 59% & 67% reduction in accessible surface silanol groups) did not induce any biologically relevant responses in the assays performed. Our data provide valuable information about the minimal surface coverage required to reduce colloidal SiO2-NF toxicity. The functionality of the SiO<sub>2</sub>-NFs in terms of their colloidal stability in paint formulations, shelf life and anti-fouling properties in paint products remains to be investigated. Colloidal stability in paints is expected to be correlated with increasing silane surface functionalization as well [27].

The in vitro test battery results revealed that the difference in in vitro toxicity between the tested colloidal SiO<sub>2</sub>-NFs could largely be attributed to their surface chemistry. Shielding the SiO<sub>2</sub>-NF surface through steric hindrance by silane surface functionalization gradually reduced toxicity in vitro, following a negative correlation with accessible surface area. Supplementation of CCM with FBS resulted in a smaller decrease in viability in dTHP-1 cells in response to Silica-Std as compared to in A549 cells under FBS-free conditions, while FBS increased the deposition efficiency of Silica-Std, suggesting a protective effect of the FBS. The cytotoxic, pro-inflammatory and oxidative stress responses observed in A549, dTHP-1, and BEAS-2B cells in vitro upon exposure to Silica-Std were likely not caused by direct ROS production (negative GSH and RNO assays) or membranolytic activity (negative haemolysis assay) according to the results presented in this study. Notably, electron spin resonance (ESR), which is a more sensitive method for identifying the generation of free radicals, did pick up a small reactive effect for Silica-Std in a previous study [30]. Haemolytic effects induced by colloidal SiO<sub>2</sub>-NFs reported in literature range from no haemolytic activity at all [20], to quite potent haemolytic activity which increased with specific surface area [52]. Thus, even among different colloidal SiO<sub>2</sub>-NFs there seems to be differences in biological effects. These differences can partly be explained by particle size and surface area, as smaller colloidal  $SiO_2$ -NFs generally show increased toxicity as compared to their larger counterparts, as was observed for in vitro and in vivo LDH release and in vivo pulmonary neutrophil influx [53], and for in vivo pulmonary inflammation and tissue damage [25].

Silica toxicity stems from its ability to generate ROS or damage membranes [17], and therefore either surface reactivity or haemolytic effects were expected to be observed to explain the observed cytotoxicity, cytokine secretion and oxidative stress. The observed secretion of IL-1β by dTHP-1 cells suggests involvement of the NLRP3 inflammasome and associated cell death would be expected [54] but was not observed in dTHP-1 cells. Further testing with the inclusion of an NLRP3 inflammasome inhibitor is recommended. The induction of oxidative stress and inflammation through disruption of mitochondrial function has previously been observed for Stöber colloidal SiO $_2$ -NFs [55], and could be an interesting mechanism to investigate further as a slightly decreased metabolic activity (WST-1) without accompanying cell damage (LDH release) was observed for Silica-Sil1 in A549 cells in the present study. Furthermore, future studies investigating particle uptake, clearance and retention in cells and the interactions of the particles with biomolecules within cells will provide more insight in the mechanism of action that leads to an adverse effect.

#### ALI vs. submerged testing

This study sheds light on the comparison of performance between submerged and ALI exposures to colloidal SiO<sub>2</sub>-NFs. A549 seeding density, seeding surface area, attachment durations, exposure durations and LDH, WST-1 and cytokine analyses were kept constant between the ALI and submerged experiments. This ensured that the only large difference in experimental setup was the exposure method (submerged vs. ALI). Other small differences that could not be overcome were diluting SiO<sub>2</sub>-NFs in water (ALI) vs. FBS-free CCM (submerged) and the culturing on inserts with pores (ALI) vs. polypropylene culture plates (submerged). Measuring the deposited dose at the ALI using gravimetric filters, and submerged using ICP-OES, facilitated a direct comparison between the responses and potencies of the SiO<sub>2</sub>-NFs in the two exposure methods.

Our results showed an almost identical effect in the LDH and WST-1 assays in A549 cells after ALI exposures compared to submerged exposures for Silica-Std, Silica-Sil1 and Silica-Sil2, and overlapping dose-response curves between the two exposure methods for Silica-Std. There was however a small but statistically significant decrease in metabolic activity upon ALI exposure to Silica-Sil1, Silica-Sil2, and Silica-Sil3 which was not observed in the submerged exposures. Cytokine secretion in ALI and submerged exposures followed the same trend as well. In other words, the exposure method did not greatly influence the sensitivity of the A549 cells to detect cytotoxicity and cell viability of the  $SiO_2$ -NFs studied. We did however observe more variation in the LDH release, metabolic activity, and cytokine release measurements at the ALI, suggesting a better precision when testing in a submerged exposure setting. It was previously observed that the characteristics of A549 cells cultured for 6 d at the ALI were different from A549 cells cultured in a submerged setting [56]. Culturing the cells at the ALI for longer than 24 h before exposure, as was done in the present study, might have led to differences in effects, which was not assessed in this study.

ALI exposures are considered more relevant for the in vivo situation in the lungs as compared to submerged exposures and have therefore been suggested as a promising animal-free alternative for toxicity testing of respirable particles [57-59]. On the other hand, use of submerged exposures would be a valuable approach for SSbD hazard testing because simple, less labour-intensive approaches are preferred. Submerged testing can however only be applied if the particles reach the cell layer at the bottom of the well. In our assessment we reach the same conclusion as observed by Medina-Reyes et al. (2020), who reported similar cytotoxic responses at the ALI as compared to submerged using TiO<sub>2</sub>-NMs [60]. In literature, higher [61, 62] and lower sensitivity of ALI models [56, 63] have also been reported. Out of these five studies, two had modelled, three had estimated, and none had measured the submerged deposited dose. To our knowledge, this makes the present study the first to directly compare ALI with submerged models with robust measurements of deposited dose in both exposure models. We strongly recommend measurements of deposited dose for future research using the method presented in this paper.

#### Conclusions

In a simple yet comprehensive in vitro test battery, a gradual reduction in in vitro toxicity was demonstrated with increasing surface silane functionalization of colloidal SiO<sub>2</sub>-NFs. Compared to the SiO<sub>2</sub>-NF without any surface functionalization, in vitro cytotoxic, pro-inflammatory and oxidative stress responses were reduced by adding 0.78 µmol/m<sup>2</sup> organosilane and were almost completely eliminated by adding 1.55 µmol/m<sup>2</sup> organosilane during the silanization process of the SiO<sub>2</sub>-NFs to reduce surface accessibility. Simple in vitro assays combined with thorough particle characterization and dose-response modelling proved to be a valuable approach for filling hazard data gaps and facilitating SSbD decision making. The reduction of toxicity with decreased surface accessibility in acute (24 h) in vitro exposures demonstrates that the in vitro toxicity of the SiO<sub>2</sub>-NFs can be entirely attributed to their surface groups, and this provides useful information for the future production of safer SiO<sub>2</sub>-NFs. Functionality, exposure, and sustainability remain to be investigated to draw a full SSbD conclusion.

A substantial reduction in deposition efficiency in CCM was observed for the two  $SiO_2$ -NFs with the highest silane surface coverage. As a consequence, alternative exposure methods, such as ALI or exposures in suspension, were required. ALI and submerged exposures of A549 cells to the  $SiO_2$ -NFs with the lowest silane coverages resulted in the same cytotoxic potencies and similar trends in induced pro-inflammatory cytokine release for each separate  $SiO_2$ -NF. Assessment of the biological effective dose of NMs during SSbD hazard testing in submerged conditions is crucial to avoid incorrect SSbD conclusions concerning hazard.

### **Supplementary Information**

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

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#### Author contributions

NR performed most toxicity assays and characterizations and co-authored the manuscript. IZ performed ICP-OES and GSH depletion analysis and helped designing the experimental setup. DP supervised the synthesis of the SiO2-NFs and the Sears titrations performed at Nouryon and co-authored the manuscript. JA co-authored the manuscript. MC performed cell exposures and qPCR analysis. AG performed SEM analysis. MP provided valuable technical advice and suggestions for the manuscript. AK performed endotoxin assay. JM and MB performed THP-1 experiments. FC and HB overlooked the project and co-authored the manuscript. All authors read and approved the final manuscript.

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#### Data availability

The datasets supporting the conclusions of this article will be publicly available in the eNanoMapper database from the 1st of June 2025.

## Declarations

#### Ethics approval and consent to participate

Not applicable.

#### **Consent for publication**

All authors have seen and approved the manuscript.

#### **Competing interests**

Daniel Persson and Josje Arts work at Nouryon. Nouryon produces and sells both plain and silane-functionalized colloidal silica.

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