Guidance Document for ENMs lung dosing consideration based on In silico analysis for Dörntruper quartz (DQ12), barium sulphate ($BaSO_4$), cerium oxide (CeO_2), and titanium dioxide (TiO_2), and multi-walled carbon nanotubes (MWCNT)

Annex 5

Authored by:
Lang Tran[1], Hana Barosova[2], Kirsty Meldrum[3], Martin J.D. Clift[3], and Barbara Rothen-Rutishauser[2]
[1]Institute of Occupational Medicine, Centre for Human Exposure Science (CHES), Edinburgh, UK
[2]Adolphe Merkle Institute (AMI), University of Fribourg, Switzerland
[3]Swansea University Medical School (SUMS), Swansea University, UK

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WP3 members

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2.0		Version distributed to WP3 members	
2.1		All commets from WP3 members intergrated and uploaded to server	

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Introduction

Due to the constant increase in their production, exposure to engineered nanomaterials (ENM) poses an inevitable health risk to both humans and the environment through long-term, repetitive, low-dose exposures. The majority of the literature however, focuses on short-term, high-dose exposures. Hazard assessment of ENM, when applying alternative testing strategies to *in vivo* research, has previously engaged exposures under submerged conditions. Such approaches poorly mimic realistic human exposures, and possess several limitations, such as issues with measuring a delivered dose, and interaction with exposure medium. Nevertheless, pseudo-ALI approaches, *i.e.* lung cells are apically exposed to air, and particles are later applied dispersed in small amount of media, is still used as valuable tool. Furthermorere, air-liquid interface (ALI) exposure chambers allowing for single droplet deposition of ENM aerosols onto cell surface were developed. VITROCELL® Cloud system equipped with Aeroneb Pro nebulizer, and Quartz crystal microbalance (QCM) for measuring online deposition of ENMs, is also applied in this project.

The current document gives guidance which deposited doses to achieve in the *in vitro* cell systems to allow comparison with available *in vivo* data. Important note is that the *in vitro* models are a simplification of the real situation and might therefore respond differently. Especially when the *in vitro* models are based on cell lines, potentially higher deposited doses are needed compared to the *in vivo* studies to observe a similar effect. The main aim is to use more realistic exposure scenarios using repeated exposure compared to unrealistic single high exposures and correlate the results to *in vivo* data.

Limitations

The recommended ENMs doses are calculated based on *in vivo* data, however in order to observe *in vitro* cellular response, it might be necessary to test higher doses.

Abbreviations

ALI – air-liquid interface

$BaSO_4$ – barium sulphate

BET – Brunauer–Emmett–Teller surface area

CeO_2 – cerium oxide

DQ12 – Dörntruper quartz

ENM – engineered nanomaterials

MMAD – mass median aerodynamic diameter

MWCNTs – multi-walled carbon nanotubes

OPN - osteopontin

PDGF - platelet-derived growth factor

TiO_2 – titanium dioxide

TGF-β - transforming growth factor beta

QCM – quartz crystal microbalance

DQ12 dosing

In vivo dosing

From Borm *et al.* (2018), the dose of DQ12 that causes persistent inflammation and tissue remodelling *in vivo* is 200 μg/rat. It is suggested to take into account *in vivo* dose in range from 0 to 400 μg – *i.e.* 0, 50, 100, 200, 400 μg.

To determine the aerosol concentration for a 4-day inhalation experiment in rats, the mass median aerodynamic diameter (MMAD) and the deposition fraction need to be measured. The latter is calculated from the multiple path particle dosimetry program. The MMAD for DQ12 depends on the method of aerosolisation.

Assuming a rat lung alveolar surface area of 3880 cm^2 (Borm *et al.*, 2018), the dose range per cm^2 is: 0 – 400/3880 $\mu g/cm^2$ (*i.e.*, 0 – 0.1 $\mu g/cm^2$).

If we assume that DQ12 is deposited in the proximal alveolar region (surface area of 300-600 cm^2) then the dose range to be 0 – 1.3 $\mu g/cm^2$ or 0 – 0.6 $\mu g/cm^2$ (using 300 or 600 cm^2 correspondingly).

In vitro dosing

Monteiller *et al.* (2007) used DQ12 in *in vitro* experiments (using 6-well plate, surface area 9.6 cm^2) for a range of nanoparticles. In Figure 1, the dose is described in cm^2/cm^2 (*i.e.*, cm^2 of dose per cm^2 of well's surface).

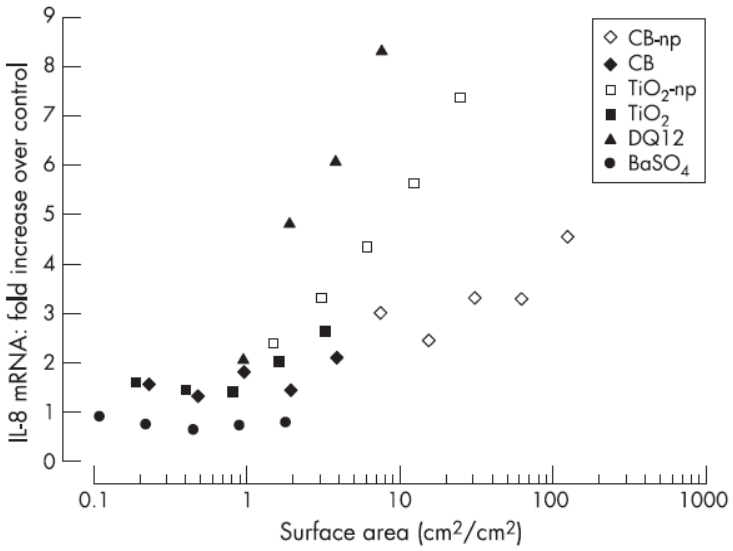

Figure 1. IL-8 release from A549 cells upon exposure to a range of doses for a panel of insoluble particles and nanoparticles (Monteiller *et al.*, 2007).

The Brunauer–Emmett–Teller (BET) surface area of DQ12 is 10.1 m^2/gm (or 10.1 x $(10^2)^2 cm^2/(10^3)^2 \mu g$ = 0.1 $cm^2/\mu g$ or 10 $\mu g/cm^2$). The dose range for DQ12 (from Figure 1) corresponds to 0 to 10 cm^2/cm^2 or 0 to 100 $\mu g/cm^2$.

This *in vitro* dose range is wider but covers the *in vivo* range above (if we assume the proximal alveolar region to be the deposited region).

In conclusion, the aim is to deliver *in vivo* doses from 0 to 400 µg (or up to 1 mg to be sure of inducing fibrosis). Depending on the method of aerosolisation, the MMAD and deposition fraction for DQ12 can be calculated, and the appropriate airborne concentration level derived. The equivalent of *in vitro* dose range (to *in vivo*) should be from **0 to 1 µg/cm²**.

Barium sulphate ($BaSO_4$), Cerium oxide (CeO_2), Titanium dioxide (TiO_2)

The BET surface area is 41.4 m^2/gm for $BaSO_4$ or 41.4 x $(100)^2/(1000)$ cm^2/mg (= 414 cm^2/mg=414/1000=0.41$cm^2/\mu g$). The lung burden of $BaSO_4$ (following 4-week and 13-week exposure) are: 0.84 mg and 1.73 mg and the polymorphonuclear count ($x10^6$) are 0.021 and 0.204 (Konduru *et al.*, 2014).

The lung burden in surface area unit (cm^2) are: 348 and 716 cm^2 or 1.1 and 2.4 cm^2/cm^2 (if the value of 300 cm^2 for the proximal alveolar surface area is used) or 0.6 and 1.2 cm^2/cm^2 (for the value of 600 cm^2).

Thus, we can derive the *in vitro* equivalent dose-range to be 0-2.4 cm^2/cm^2 or (0- 2.4)/0.41 $\mu g/cm^2$ => 0 - 5.8 $\mu g/cm^2$. The *in vitro* dose-range for **$BaSO_4$ is 0 - 6 $\mu g/cm^2$**.

For CeO_2, the BET surface area is 27.2 m^2/gm or 27.2 x $(100)^2/(1000)$ cm^2/mg (=272 cm^2/mg = 272/1000=0.27 $cm^2/\mu g$). The lung burdens of CeO_2 associated with inflammation are 391 and 1280 µg or (391 x 0.27 and 1280 x 0.27 =105 and 345 cm^2) (Schwotzer *et al.*, 2017). Normalised to the proximal alveolar surface area of 300 cm^2, the normalised *in vivo* doses are 0.35 and 1.1 cm^2/cm^2.

Thus, the *in vitro* equivalent dose-range should be 0 - 1 cm^2/cm^2. Interestingly, in Figure 1, this is also the dose threshold for inflammation. To be compatible with the $BaSO_4$ results, the aim for delivered dose of **CeO_2** is 0 - 2.4 cm^2/cm^2 or 0 - 2.4/0.27 $\mu g/cm^2$ => **0-9 $\mu g/cm^2$**).

For TiO_2, the BET surface area is 46 m^2/gm or 46 x $(100)^2/1000$ cm^2/mg or 460 cm^2/mg or 460/1000 = 0.46 $cm^2/\mu g$). From Figure 1, a possible choice for the *in vitro* TiO_2 dose-range is 0-2.4 cm^2/cm^2 as above for consistency or 0-2.4/0.46 $\mu g/cm^2$ = 0-5.2 $\mu g/cm^2$) (Monteiller *et al.*, 2007). The *in vivo* range for TiO_2 should be 0-5.2x300 = 0 – 1.56 mg of lung burden.

Multi-walled carbon nanotubes (MWCNTs)

In vitro dose range

The current estimation of the MWCNT dose range for Mitsui-7 is based on a paper from Gangwal *et al.* (2011), his calculations were further used in follow-up studies (Chortarea *et al.*, 2017, Chortarea *et al.*, 2019). A range of doses: 0, 5, 10 and 20 µg/mL of Mitsui-7 was used for exposure using submerged conditions for 24 hrs, and double the dose at 96 hrs were applied for epithelial cells (A549), differentiated monocytes into macrophages (dTHP-1) and fibroblasts (MRC-5) separately (Chortarea *et al.*, 2019).

A pro-inflammatory response was observed only for IL-1β release 24 hrs post-exposure in dTHP-1 and MRC-5 cells upon the lowest Mitsui-7 dose (5 µg/mL).

The pro-fibrotic response was assessed via platelet-derived growth factor (PDGF), osteopontin (OPN) and transforming growth factor beta (TGF-β) release. Increased TGF-β release was demonstrated in Mitsui-7-treated fibroblasts only after 96 hrs exposure, while OPN was increased upon 24 hrs exposure to the lowest Mitsui-7 concentration. Interestingly, no proliferation was observed in MWCNT exposed MRC-5 cells and consequently no

increase in collagen production was observed.

Similarly, increased OPN release was observed after 96 hrs of Mitsui-7 exposure in macrophages. Also, Mitsui-7 was found to significantly increase TGF-β, OPN and PDGF release of exposed epithelial cells after 96 hrs.

For the combined, multi cell type *in vitro* system, much depends on the seeding of each cell type and the rate of 'interstitialisation'. Thus, if we are dosing from the 'alveolar' side, it is expected to be challenging to get up to 20 µg/mL onto the fibroblast cells. Therefore, we have to dose (at 48 and 96 hrs) in such a way that would deliver enough TGF-β or PDGF from A549/dTHP-1 to the fibroblasts seeded at the bottom part of the multicellular system (the indirect effect) and, at higher doses, to get enough MWCNT to stimulate fibroblasts directly. Since a high dose, 150 µg/mL, has been used in the past, it is suggested to apply a dose-range for Mitsui-7 in the experiment as follows:

0, 5, 10, and 20 µg/mL of MWCNTs was applied for exposure at 48 hrs and double that for 96 hrs. Thus the highest dose, at 96 hrs, will be 40 µg/mL. The dose range suggested MWCNTs may stimulate fibroblasts to proliferate and produce extra cellular matrix without the direct dose effect which hinders fibroblast proliferation as suggested in the paper.

In vivo dose range

The alveolar mass retention of CNTs was modelled and calculated in the range of 12.4 – 46.5 µg/cm^2 during 45 years of a working lifetime exposure (Gangwal *et al.*, 2011). This range is commensurate with the *in vitro* dose range proposed. This can,

1. Readily be calculated into an absolute dose (µg/lung), depending on which animal model we use (rat/mouse) and
2. Calculate backward into a concentration level, dependent on the MMAD for Mitsui-7 and the duration of exposure.

PATROLS Lung ENM administration strategies

Several dosing strategies are applied to investigate an effect of long-term exposures, and the response is compared with acute (24 h) exposure. This highly depends on the epithelial cell type used, *i.e.* how long can they be cultured at ALI keeping a stable morphology. Figure 2 shows the applied dosing and exposure strategies for subchronic (3 weeks) exposures. Figure 2.1 shows the acute (24 h) exposure using a high dose, which response is subsequently compared to long-term exposures. Figure 2.2 shows repeated daily exposure over 3 weeks simulating the occupational exposures, which usually occur regularly/repeadly during working days (*i.e.* 5 times a week) at low doses; the target dose is divided into 15 sub-doses applied daily (15 exposures are performed in 3 weeks), resulting in a final target dose corresponding to the doses applied in 2.1 and 2.3 scenarios. The response is investigated after 1, 2 and 3 weeks, however after 1 or 2 weeks, there is only a partially applied dose (*i.e.* 1/3, or 2/3 of the final dose, respectively). Scenario 3 (Figure 2.3) shows single exposure to a high dose at the beginning of the experiment with subsequent investigation of the response after 1,

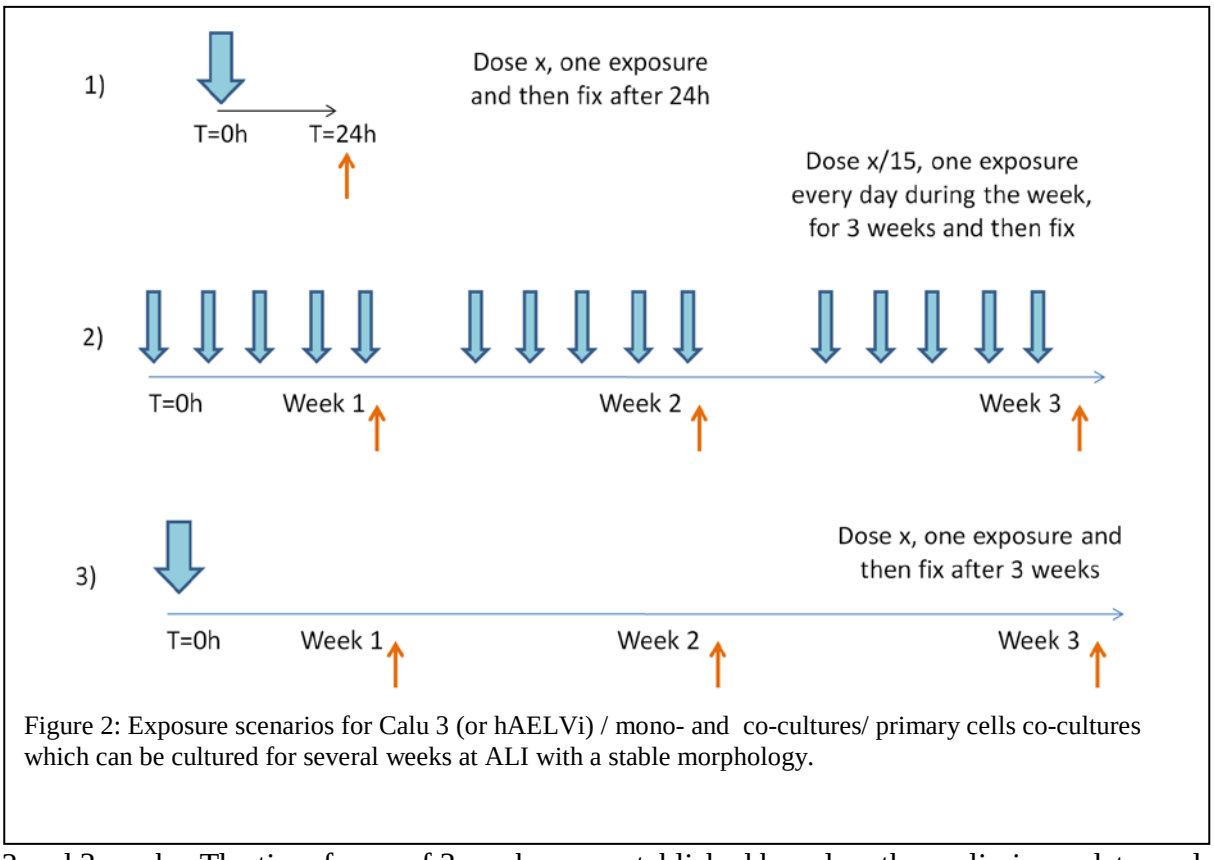

Figure 2: Exposure scenarios for Calu 3 (or hAELVi) / mono- and co-cultures/ primary cells co-cultures which can be cultured for several weeks at ALI with a stable morphology.

2 and 3 weeks. The time frame of 3 weeks was established based on the preliminary data, and ability of selected cells to remain viable and functional during the selected time-frame.

The A549 cell line remains a monolayer only up to 3 days culturing at ALI, therefore the dosing strategy was planned over 3 days only. Figure 3 shows different exposure scenarios possible for the A549 cell line.

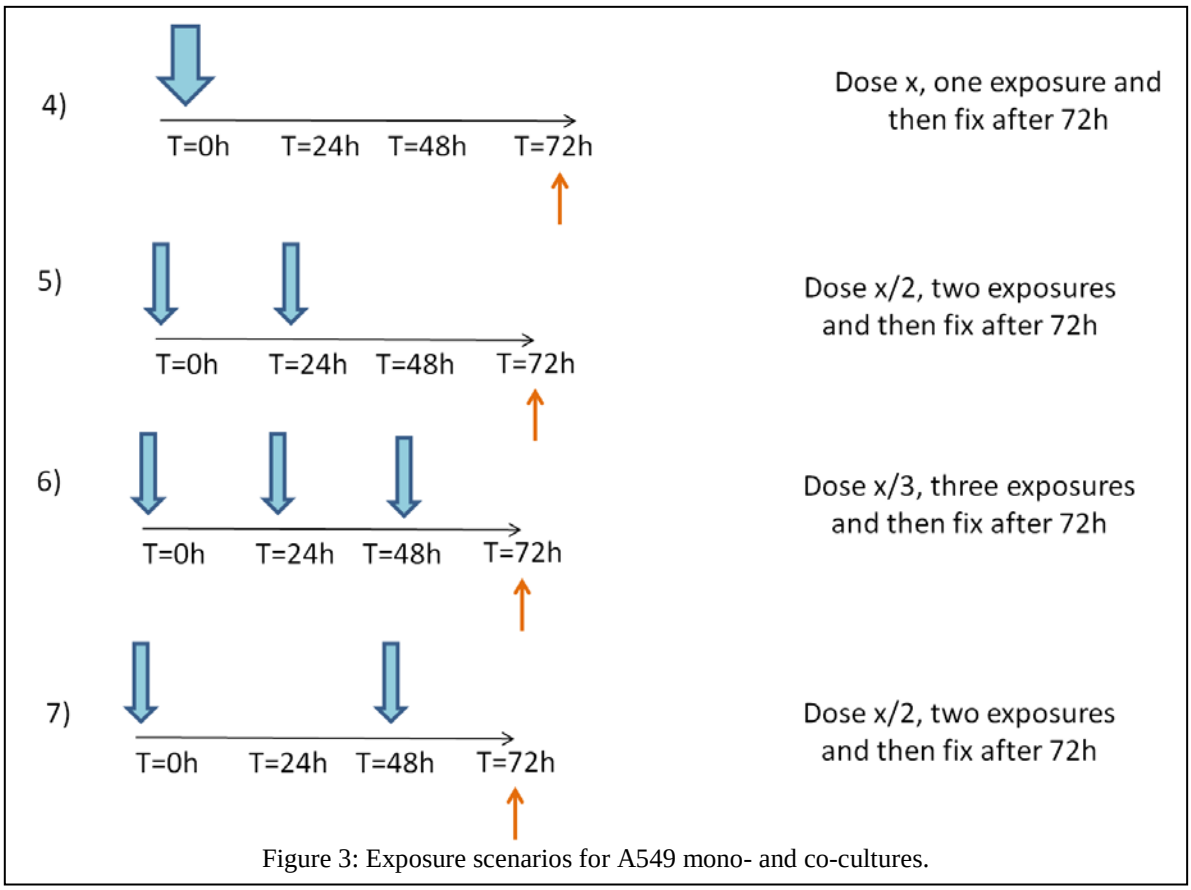

Figure 3: Exposure scenarios for A549 mono- and co-cultures.

Summary

Concentrations for Tier 1 ENMs used in WP3 were determined in collaboration with WP6 to deduce realistic dosages (in relation to the material's physicochemical properties, as well as previous *in vivo* and known human exposure data sets). The recommended ENMs doses are calculated based on *in vivo* data, however in order to observe *in vitro* cellular response, it might be necessary to test higher doses for certain materials.

Particle	***In vitro* dose range ($\mu g/cm^2$)**	***In vivo* dose range (mg)**
DQ12	0-1	0-0.4
BaSO4	0-6	0-1.73
CeO2	0-9	0-1.28
TiO2	0-5.2	0-1.56
MWCNT	0–46.5	***

*** Analysis is ongoing

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