PATROLS
Advanced Tools for NanoSafety Testing

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PATROLS Standard Operating Procedures (SOP)

Guidance Document for DQ_{12} and TiO_2 Aerosolization using VITROCELL® Cloud System

SOP_PATROLS_Cloud_Aerosolization_RR1

This is a SOP recommended for external use by PATROLS

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Authored by:

Hana Barosova, Anne Bannuscher, Barbara Drasler, Barbara Rothen-Rutishauser

Adolphe Merkle Institute (AMI), University of Fribourg, Switzerland

Reviewed by:

WP3 members

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1 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 exposures poorly mimic human exposures, and possess several limitations, such as issue with measuring a delivered dose, and interaction with exposure medium. Therefore, 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 particles such as TiO_2 NM-105, crystalline SiO_2 (DQ_{12} quartz) and substances such as fluorescein isothiocyanate (FITC), is referred in this SOP. A QCM coupled to Cloud12 present a highly sensitive, accurate device for (quasi-) real-time dosimetry of the cell-delivered particle dose in ALI cell exposure experiments. In addition, for a verification on the QCM`s accuracy a fluorescein-based method is included as quality control to prevent measurement errors due to damaged QCM crystals or electronic failurs. Detailed descriptions of the test system can be found in references (Ref. 1-3).

1.1 Scope and limits of the protocol

This SOP was established with the intention to be used for developing an exposure protocol for a lung cell culture model which can provide a physiologically relevant assesement of the hazards associated with ENM exposures over both an acute and chronic, repeated dose regime. This SOP provides instructions on how to aerosolize particles like DQ_{12} or ENM such as TiO_2 NM-105 and substances such as FITC during a round robin within PATROLS; for handling the VITROCELL® Cloud system in general please refer to the instructions provided by VITROCELL®.

Limitations:

To be able to provide stable suspensions disperse ENM according to NanoReg protocol: ‘Protocol for producing reproducible suspensions of manufactured

nanomaterials in environmental exposure media'. Details on the suspension preparation are given in section 5.6.

2 Terms and Definitions

Nanomaterial
Material with any external dimension in the *nanoscale* or having internal structure or surface structure in the nanoscale.

Note 1 to entry: This generic term is inclusive of *nano-object* and *nanostructured material*.

[SOURCE: ISO/TS 80004-1: 2016, definition 2.4]

Engineered nanomaterial
Nanomaterial designed for specific purpose or function

[SOURCE: ISO/TS 80004-1: 2016, definition 2.8]

Particle
Minute piece of matter with defined physical boundaries.

Note 1 to entry: A physical boundary can also be described as an interface.

Note 2 to entry: A particle can move as a unit.

Note 3 to entry: This general particle definition applies to *nano-objects*.

[SOURCE: ISO 26824:2013, 1.1]

Substance
Single chemical element or compound, or a complex structure of compounds.

3 Abbreviations

ALI – Air-liquid interface

BSA – Bovine Serum Albumine

DQ12 - Dörntruper quartz 12

FITC - Fluorescein isothiocyanate

QCM – Quartz crystal microbalance

TEM – Transmission electron microscopy

4 Principle of the Method

This method aims to standardise the aerosolization of EMNs using VITROCELL® Cloud system.

This protocol will be broken into key stages:

1. Particle suspension preparation
2. Preparing the aerosolization device
3. Aerosolization
4. Cleaning

5 Description of the Method

5.1 Test system used

This SOP should be carried out under laboratory based conditions, with all work following safe handling of ENMs.

- VITROCELL® Cloud12 system (VITROCELL®, Germany) equipped with
    - Quartz crystal microbalance (QCM)
    - Aeroneb® Lab nebulizer (4-6 µm pore size, Aerogen, Ireland)
    - This protocol referes to Cloud 12, which is designed for 12-well inserts.
- For further information:

  https://www.vitrocell.com/inhalation-toxicology/exposure-systems/vitrocell-cloud-system/vitrocell-cloud-12

5.2 Chemicals, Nanomaterials and Reagents used

- Ultrapure water
- 70% ethanol or a similar cleaning agent (not Aceton or Sodium-Hydroxide; usage of those will cause unrepairable damage to the system or parts of it)
- Isotonic (0.9%) NaCl solution

- DQ_{12} (Institute of Occupational Medicine, IOM)
- TiO_2 NM-105 (Frauenhofer)
- FITC (CAS 518-47-8)

5.3 Apparatus and equipment used

- 10 µL, 200 µL and 1000 µL pipette tips
- 50 mL centrifuge tubes
- Glas vial e.g. 20 mL Scint-Burk glass pp-lock+Alu-foil vials (WHEA986581; Wheaton Industries Inc.)
- Sonicator (Branson 550)
- VITROCELL® Cloud12
- Aeroneb nebulizers (4-6 µm pore size)
- Cell culture inserts (e.g. Corning Transwell inserts for 12-wells plates, 0.4 µm pore size (Cat.no 3460))
- TEM grids (EMS, CF300-Cu)
- TEM grid holder for 12-wells (Vitrocell, VC5036)
- Water bath sonifier (e.g. Elmasonic P30H cleaning unit, 100 W, 37 kHz, 30% (Elma Schmidbauer GmbH, Germany))
- Microbalance
- Wipers e.g. Kimberly-Clark Professional™ Kimtech Science™ Precision Wipes™ Tissue Wipers (19-063-099) or similar lint-free cloth

Note: For performance of the FITC nebulization, additional assay-specific equipment is listed in the Annex).

5.4 Reporting of protected elements

To the best of our knowledge, this SOP does not have any associated patent restrictions, specific licenses, material transfer agreements or commercial purchase requirements required to perform the protocol described.

5.5 Health and safety precautions

Standard health and safety precautions associated with working within a laboratory environment and handling ENMS, as described by the European Agency for Safety and Health at Work (https://osha.europa.eu/en/safety-and-health-legislation/european-guidelines), should be adopted when conducting this SOP. In addition, all health and safety precautions outlined in the MSDS data sheets associated with the specific chemicals required must also be followed. DQ_{12} is a non-nanosized particle which can induce inflammation and fibrosis in humans upon inhalation. Therefore, suitable protective equipment such as a lab coat, goggles, gloves and a mask must be worn.

5.6 Particle suspension preparation

Weighing of DQ_{12} and ENM such as TiO_2 NM-105 should be performed in a ventilated weighing box, glove box or fume hood designed for sensitive weighing. Ensure appropriate safety clothing, including the use of double gloves. DQ_{12} and ENM suspensions have to be prepared in ultrapure water (**without** BSA, which is differently to the Nanogenotox protocol) with EtOH pre-wetting. Suspensions have to be prepared in a volume range between 4 ml and 10 ml, following the Nanogenotox protocol.

1. Using a microbalance, weight the respective amout of DQ_{12}/ENM in a glas vial corresponding to a minimum of 4 ml to a maximum of 10 ml (e.g. 10.24 mg corresponding to the final volume of 4 ml).
2. Pre-wet the particles with ethanol (e.g. 20 µl corresponding to 10.24 mg and 4 ml or 30 µl for 15.36 mg and 6 ml), add the corresponding amount of ultrapure water (in 4 steps) and mix it carefully by vortexing to obtain a 2.56 mg/ml stock solution.

3. Sonicate the suspension based on the Nanoreg protocol, please calibrate your sonifier respectively (see “SOP for probe sonicator calibration of delivered acoustic power and deagglomeration efficiency for in vitro and in vivo toxicological testing”, see Annex).
4. Carefully remove the sonicator tip, close the vial and clean the device.
5. Store the aliquots at 4°C until use (suspensions need to be prepared freshly on the day of experiment).
6. Prepare a 1 mg/ml dilution from the stock solution (e.g. 390.6 ml of the 2.56 mg/mL stock and 609.4 ml of ultrapure water). Dilute this stock further to the desired concentrations (250 µg/ml or 500 µg/ml).

6 Procedure

6.1 Prior nebulization

1. Perform all the steps in a laminar flow hood (the same hood and aerosolization device position in the hood as to be used for RR2).

   Note: Figure 1 shows the VITROCELL® Cloud system with all of its parts.

2. Assemble the QCM, insert it to the VITROCELL® Cloud system and connect it with the oscillator.

   Note: A video on this step will be provided by Helmoltz Zentrum München.

3. Turn on the VITROCELL® Cloud heating unit and wait until the temperature reaches 37°C (at least 30-45 minutes).
4. Warm the DQ_{12} or ENM suspension to room temperature and shortly sonicate (ultrasound bath) for 10 min to redisperse the sedimented/agglomerated particles or use them directly after the preparation in the section 5.6. For the purpose of this RR, each particle suspension has to be prepared freshly before usage.

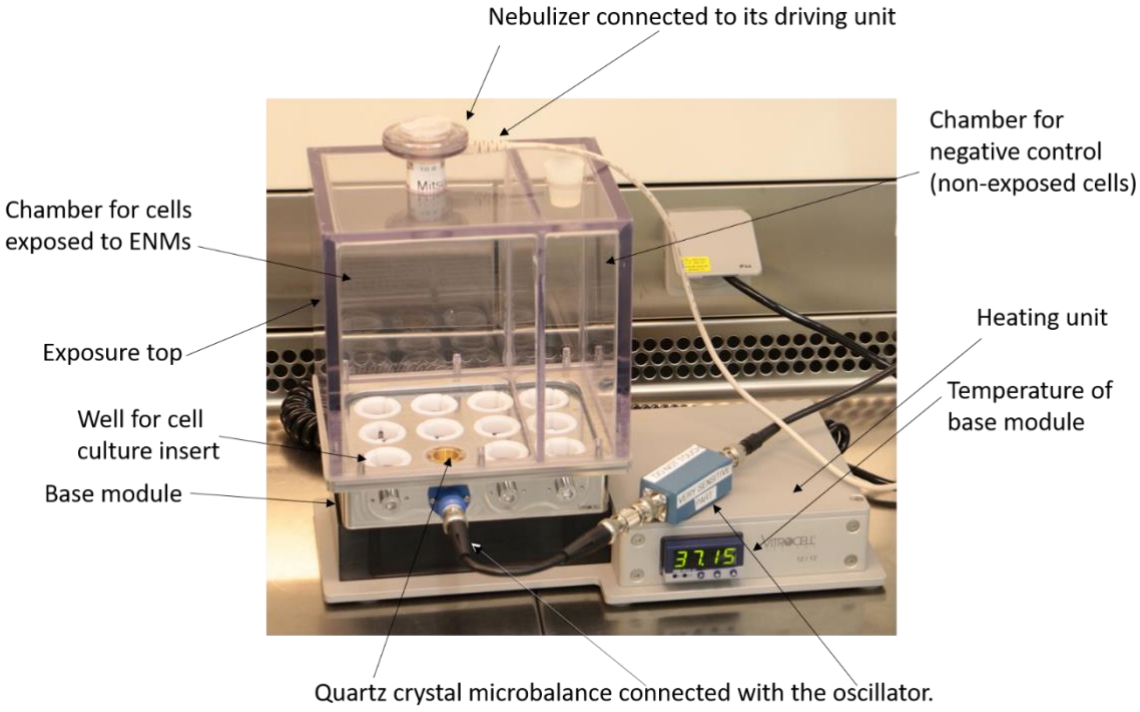


Figure 1: The VITROCELL® Cloud system.

6.2 Nebulization: general procedure

The general procedure for performing the nebulization is described below (see the schematic presentation in the Figure 2).

1. Rinse the reservoir of nebulizer with ultrapure water and dry it with lint-free cloths. Place the nebulizer on top of the exposure chamber.
2. Prepare a solution of ultrapure water or DQ_{12}/ENM suspension containing 1% of isotonic NaCl solution (v/v), e.g. 2.5 µl in 250 µl and pipette 200 µl to the nebulizer (make sure the liquid covers the nebulizer's mesh).
3. Start the data acquisition on the QCM and wait until the signal is stable (typically ca. 30 s, but can be longer), set the signal to zero and wait another 30 s to verify stability of the zero point signal (the signal should be in the range of ± 25 ng/cm^2). If this is not the case, set the QCM signal to zero again and wait for another 30 s to verify stability of the signal.
   Note: It is recommended to zero the signal once again after starting the data acquisition.

4. Measure the signal for 1 minute before starting the nebulization (next step).
5. Start the nebulization process by plugging in the nebulizer in the driving unit and at the same time start the timer (t = 0 min). Carefully measure the time needed for nebulization.

   Note: The acceptable time range for nebulizing 200 µL of water/suspension is between 15-60 s (0.2 – 0.8 ml/min).

   Note: The output rate should not exceed 10% deviation from the original output rate of the individual nebulizer and this should be measured for all the subsequent nebulizations. Prolonged nebulization time can be a sign of a blocked nebulizer, therefore it needs to be cleaned thoroughly (ultrabath sonication, see section 6.3) or replaced.
6. Wait for 6 min to ensure a complete settlement of the test substance to the inserts and QCM. A drop of the signal should be visible.
   Note: If the curve is not dropped within this time frame, extend this period to 10 min and follow this procedure for all nebulizaions
7. Lift the exposure top for 1 min allowing the QCM to dry.
   Note: In case of subsequent nebulizations, the inner walls of the exposure chamber need to be whipped with 70% ethanol.
8. After 1 min place the exposure top back onto the device.

   Note: The reason behind covering the device is to minimize the effects of ventilation and temperature fluctuation on the measured QCM values.
9. Wait for 3 min and stop the data acquisition.
10. Lift the exposure top and clean the QCM: use a Q-tip wrapped in lint-free wipers for cleaning thoroughly the crystal.

    Note: A video on this step will be provided by Helmoltz Zentrum München.
    Note: Vitrocell recommends to change crystal after each nebulization
11. Data analysis should be performed on the mean of the last 30 values.

    Note: The accuracy of Cloud12 QCM is 3.8% ± 1.0% (Ref. 3)

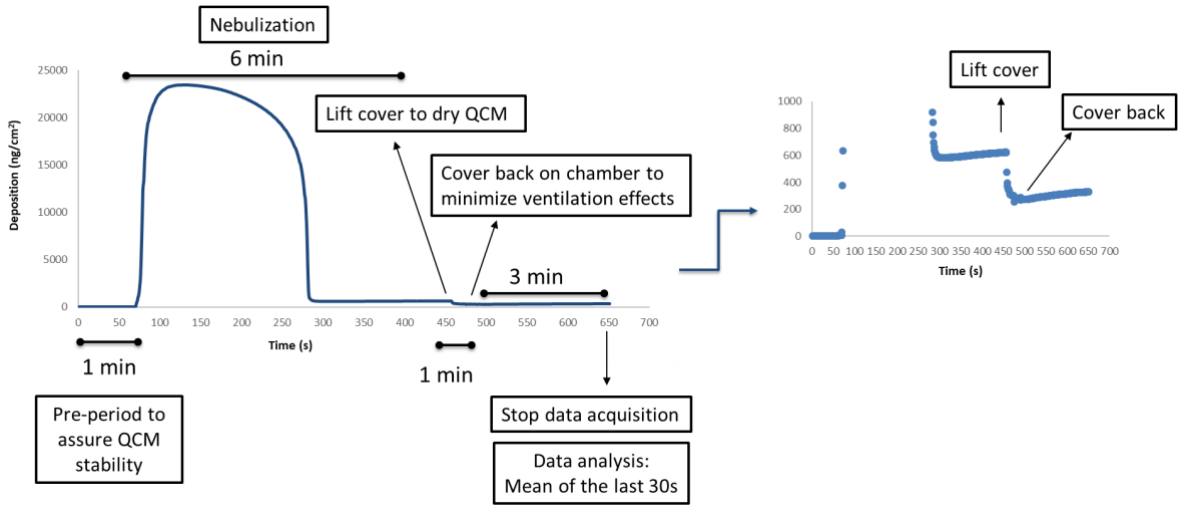


Figure 2: Typical time course of QCM signal and the time periods applied.

6.3 Specific remarks for particle aerosolization

NOTE: Please see sections 7 and 8 for recommended concentrations of particle suspensions and the respective deposited values

1. Nebulize 200 µl ultrapure water containing 1% isotonic NaCl (not into the cloud) for removal of residual NMs from the nebulizer. Dry the nebulizer using the lint-free wipers (reservoir and from the bottom side – gently touching the vibrating mesh) in order to remove residual liquid.
2. Cover the base module with exposure top.
3. At the beginning of a working day, measure deposited dose of 200 µl of ultrapure water with 1% (v/v) NaCl.

   Note: An expected value for 200 µl of water with spiked salt is around 100 ng/cm^2. However, due to the proximity of the detection limit (169 ng/cm^2 (Ref. 3)), the values could also be negative or higher (up to 250 ng/cm^2). If the values are above the threshold of 250 ng/cm^2, this could be a reflection either of dirty nebulizer, i.e. remnants of material from previous nebulizations, or of troubles with QCM signal. Therefore, it is recommended to re-build the QCM and clean all the parts (see the next step and the video) as well as to ultrasonicate the nebulizer in water for 5 min.

4. QCM cleaning between subsequent nebulizations: Wipe the QCM crystal carefully with water on a Q tip, also wipe the edges and every surface that came in contact with nebulized material very carefully (but not too thoroughly). Repeat the procedure with 70% ethanol. Then wait at least 5 min to ensure complete drying of the crystal as reflected by a stable QCM signal.

   Note: The quartz crystal of the QCM needs to be replaced when scratches are observed on the surface and/or if a stable signal cannot be achieved after the crystal and base module have been heated up appropriately.

5. Place one TEM grid in the TEM grid holder (provided by VITROCELL®) for each technical repitition, and place it in one well of the base module.

6. Prepare DQ_{12}/ENM suspension in water with the desired concentration (see section 5.6).

7. Mix 250 µL of DQ_{12}/ENM suspension with 2.5 µL of isotonic NaCl solution in an Eppendorf tube; for the concentrations see sections 7 and 8.

8. Pipette 200 µL of the suspension into the nebulizer reservoir. Keep the nebulizer upright while placing it into the circular gap in the exposure top. Make sure, that the nebulizer is placed straight, and sits tightly. Then connect it and start nebulization.

   Note: Follow the data acquisition, nebulization and timing procedure as indicated in the steps 5-10 of the general procedure and in the Figure 2.

9. Transfer the TEM grid onto a dust-protected surface allocated for drying, e.g. a parafilm sheet, and let it dry at least over night before transferring it into a TEM grid box.

10. Wash the nebulizer with 200 µl ultrapure water containing 1% (v/v) isotonic NaCl. Measure output rate of the nebulizer after each nebulization (should not exceed 10% deviation from the original output rate).

    Note: If the time is exceeding, put the nebulizer in a beaker with ultrapure water or 70% ethanol and sonicate it in an ultrasonic bath for 5-10 min. Then, measure

the output rate again. If nebulizing time is not improving, clean more thoroughly (see section 6.3) or replace the nebulizer.

If no cell exposure work is involved, cleaning in water is sufficient. Otherweise, an adiditonal step of ultrabath sonication in 70% ethanol (e.g. 10 min) for sterilization is required.

11. Wipe the nebulizer reservoir and the bottom part of the nebulizer in order to remove remaining liquid (use lint-free wipes).
12. Continue with the step 5, i.e. nebulize the same particle suspensions for technical repetitions or other concentrations.

    Note: Clean the nebulizer carefully if different substances i.e. particles are to be nebulized, see section 6.3. Cleaning between successive runs of the same substance can prevent blocking but is optional.
13. For each particle 3 technical replicates have to be performed for the selected concentration.
14. At the end of a working day and/or one type of material, cleaning steps need to be performed, see section 6.3 Cleaning.
15. For subsequent nebulizations of the same particle solution concentration: in order to achieve a higher deposition (e.g. in RR2) subsequent nebulizations are possible, but to a maximum of 4. After the first nebulization, check the output rate of the nebulizer. If this is within 10% of the initial output rate, nebulize 1x 200 µl ultrapure water with salt (not into the cloud) and continue with the second nebulization. If not, put the nebulizer in a beaker with ultrapure water or 70% ethanol and sonicate it in an ultrasonic bath for 5-10 min and proceed. **Do not stop the data acquisition** and **do not clean the QCM in between** (possible to a maximum deposition of ~1.5 µg/cm^2). The measured deposited dose should be approximately twice the first measured dose. If not, the nebulizer may be blocked and must be cleaned carefully, see section 6.3.

6.3 Cleaning

16. Nebulize at least 3x 200 µl of ultrapure water containing 1% (v/v) isotonic NaCl.

17. Put the nebulizer in a beaker with ultrapure water in water bath sonicator (e.g. Elmasonic P30H cleaning unit, 100 W, 37 kHz, 30% (Elma Schmidbauer GmbH, Germany)) for 10 min.
18. Rinse the nebulizer with ultrapure water.
19. Put the nebulizer in a beaker with 70% ethanol and sonicate in water bath sonicator (e.g. for 10 min).
20. Clean VITROCELL® base module and cover top with 70% ethanol.
21. QCM Crystal: Used, dirty crystals can be collected, cleaned and re-used, if no scratches on the surfaces are visible. For cleaning put them in ethanol for 0.5 min to 5 min (depends on the degree of impurity/dirt) and wipe them carefully afterwards. Check their performance afterwards and trash them if signal oscillates too strongly, loss of signal is present or scratches on the surface are visible.
22. VITROCELL® base module and exposure top can be washed in dishwasher (recommended). Do NOT clean with acetone.
23. VITROCELL® base module can be autoclaved (recommended on regular basis, *i.e.,* every week, but not necessary after each experiment) following intervals and temperatures listed, *i.e.,* minimum warm-up of 30 min, autoclaving at 121°C for a minimum of 20 min, minimum cool-down time in the autoclave of 30 min.

7 DQ_{12} concentrations applied and deposition measured (at AMI)

For the RR1 purposes, 3 individual nebulizations of the concentration 500 µg/mL of DQ_{12} are required. Please provide also the deposition value of ultrapure water containing 1 % (v/v) isotonic NaCl (this needs to be done just before nebulizing DQ_{12} using the same nebulizer and crystal).

Table 1: Deposited values of nebulized DQ_{12} in a concentration range (preliminary experiments performed at AMI). N=6

Concentration (µg/mL)	Deposition (ng/cm^2) ± SD
water	44.9 ± 51
125	185.2 ± 19
250	258.3 ± 19
500	516.0 ± 32

8 TiO_2 NM-105 concentrations applied and deposition measured (at AMI)

For the RR1 purposes, 3 individual nebulizations of the concentration 500 µg/ml of TiO_2 NM-105 are required. Please provide also the deposition value of ultrapure water containing 1 % (v/v) isotonic NaCl (this needs to be done just before nebulizing TiO_2 NM-105 by using the same nebulizer and crystal).

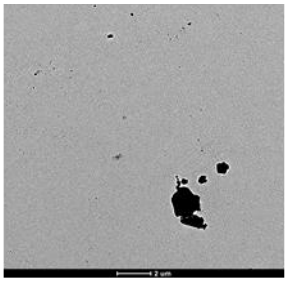
Note: If the concentration 500 µg/ml of TiO_2 NM-105 is causing blocking of the nebulizer, the nebulizer needs to be thoroughly cleaned and the concentration 250 µg/ml shall be measured.

Table 2: Deposited values of nebulized TiO_2 NM-105 in a concentration range (preliminary experiments performed at AMI). N=6

Concentration (µg/mL)	Deposition (ng/cm^2) ± SD
water	50.8 ± 36
125	192.5 ± 46
250	217.6 ± 42
500	394.8 ± 121

9 TEM images

DQ_{12}



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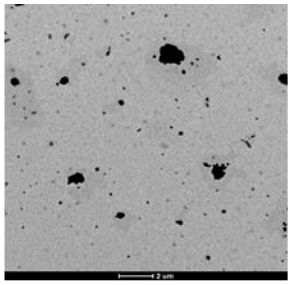

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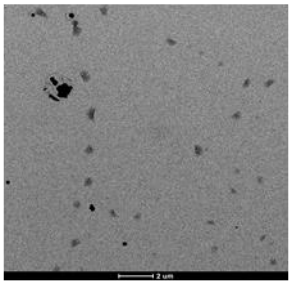
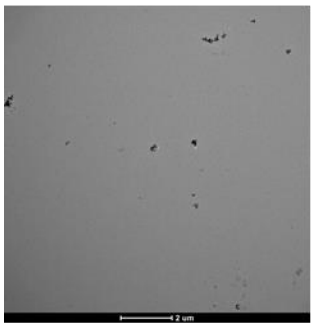
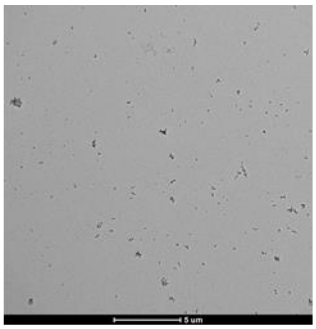


500 µg/ml

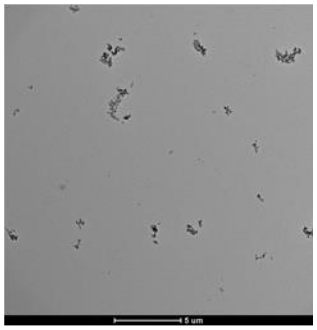
TiO_2 NM-105



125 µg/ml



250 µg/ml



500 µg/ml

10 FITC assay remarks

The FITC assay used in this SOP has to be performed according to the SOP “SOP: fluorescein assay determination of deposition factor for VITROCELL Cloud” described in “VITROCELL® Cloud Supplementary Information”. The purpose of the assay is to assess the deposition factor of the system which enables to calculate the delivered dose of aerosols to the cells. Please perform this assay with one nebulizer that will also be used in PATROLS RR2.

Important remarks to the SOP:

- Use 0.4 µm pores in the transwell insert membrane and AVOID any contact of inserts with otherwise the chemical can migrate into PBS and DF values are incorrect.

- Make sure that the device is heated to 37 °C.
- Perform the experiments in a laminar flow hood (the same place and position as for the DQ_{12}/ENM nebulization).
- In case the instrument's (fluorimeter's) detection limit allows for, use 15 µg/ml FITC in DPBS as described in the SOP; otherwise use 30 µg/ml.
- 3 repititions need to be performed

A brief description of the procedure (please refer to the SOP for the thorough description):

1.) Prepare FITC stock solution (i.e. 15 µg/ml).

2.) Heat the device to 37°C including QCM.

3.) Fill the wells of the basal module with ultrapure water (3 mL) and the top chamber of the inserts with DPBS (0.3 ml).

4.) Perform FITC nebulization. Try to keep the time between filling liquids and aerosolisation as short as possible. Otherwise, signal losses can follow.

5.) Follow exactly the same data acquisition pattern as presented in section 6.2, i.e. 1 min QCM signal acquisition before the start of nebulization, wait for 6 min to ensure the aerosol is completely settled, 0.5 min of lifting the exposure top, followed by covering it back for adiditonal 2.5 min to stabilize the signal.

11 Quality control & acceptance criteria

The deposited particles should be checked using TEM after trial exposures, before starting cell culture experiment, and then several times during the experiment.

12 Data Analysis and Reporting of Data

Not applicable for this current SOP.

13 Publications

Not applicable for this current SOP.

14 References

(1) Lenz, A.G., Karg, E., Lentner, B. et al. A dose-controlled system for air-liquid interface cell exposure and application to zinc oxide nanoparticles. Part Fibre Toxicol 6, 32 (2009). https://doi.org/10.1186/1743-8977-6-32

(2) Lenz AG, Stoeger T, Cei D, Schmidmeir M, Semren N, Burgstaller G, Lentner B, Eickelberg O, Meiners S, Schmid O. Efficient bioactive delivery of aerosolized drugs to human pulmonary epithelial cells cultured in air-liquid interface conditions. Am J Respir Cell Mol Biol. 2014 Oct;51(4):526-35. doi: 10.1165/rcmb.2013-0479OC.

(3) Ding, Y., Weindl, P., Lenz, A. *et al.* Quartz crystal microbalances (QCM) are suitable for real-time dosimetry in nanotoxicological studies using VITROCELL®Cloud cell exposure systems. *Part Fibre Toxicol* **17,** 44 (2020). https://doi.org/10.1186/s12989-020-00376-w

15 Annex:

SOP for calibration of Probe-sonicators for in vitro and in vivo testing_v1.1

VITROCELL_Cloud_Supplementary_P1_101218