PATROLS
Advanced Tools for NanoSafety Testing

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PATROLS Standard Operating Procedures (SOP)

Guidance Document for ENMs Aerosolization using VITROCELL® Cloud System

SOP_PATROLS_Cloud_Aerosolization

This is a SOP recommended for external use by PATROLS

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Document History:

Version	Approval Date	Description of the change	Author(s) of change
1.0	26.7.2019	Initial Document	Hana Barosova
1.1		Additional detail added from AMI	
1.2		Final coments implemented	
2.0		Version distributed to WP3 members	
2.1		All commets from WP3 members intergrated and uploaded to server	

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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 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 for measuring online deposition of ENMs, is referred in this SOP.

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 only instructions on how to aerosolize ENMs; for handling the VITROCELL® Cloud system in general please refer to the instructions provided by VITROCELL®.

Limitations:

ENMs have to be suspendable in liquid, to be able to provide the stable ENM suspension (to achieve this, refer to NanoReg protocol: ‘Protocol for producing reproducible dispersions of manufactured nanomaterials in environmental exposure media’).

2 Terms and Definitions

Agglomerate
Collection of weakly or medium strongly bound *particles* where the resulting external surface area is similar to the sum of the surface areas of the individual components.

Note 1 to entry: The forces holding an agglomerate together are weak forces, for example van der Waals forces or simple physical entanglement.

Note 2 to entry: Agglomerates are also termed secondary particles and the original source particles are termed primary particles.

[SOURCE: ISO 26824:2013, 1.2]

Aggregate
Particle comprising strongly bonded or fused particles where the resulting external surface area is significantly smaller than the sum of surface areas of the individual components.

Note 1 to entry: The forces holding an aggregate together are strong forces, for example covalent or ionic bonds, or those resulting from sintering or complex physical entanglement, or otherwise combined former primary particles.

Note 2 to entry: Aggregates are also termed secondary particles and the original source particles are termed primary particles.

[SOURCE: ISO 26824:2013, 1.3, modified — Note 1 adapted.]

Nanoscale
Length range approximately from 1 nm to 100 nm

Note 1 to entry: Properties that are not extrapolations from larger sizes are predominantly exhibited in this length range.

[SOURCE : ISO/TS 80004-1: 2016, definition 2.1]

Nanotechnology
Application of scientific knowledge to manipulate and control matter predominantly in the *nanoscale* to make use of size- and structure-dependent properties and phenomena distinct from those associated with individual atoms or molecules, or extrapolation from larger sizes of the same material.

Note 1 to entry: Manipulation and control includes material synthesis.

[SOURCE: ISO/TS 80004-1: 2016, definition 2.3]

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]

Nanostructure
Composition of inter-related constituent parts in which one or more of those parts is a *nanoscale* region.

Note 1 to entry: A region is defined by a boundary representing a discontinuity in properties.

[SOURCE: ISO/TS 80004-1: 2016, definition 2.6]

Nanostructured material
Material having internal *nanostructure* or surface nanostructure.

Note 1 to entry: This definition does not exclude the possibility for a *nano-object* to have internal structure or surface structure. If external dimension(s) are in the *nanoscale*, the term nano-object is recommended.

[SOURCE: ISO/TS 80004-1: 2016, definition 2.7]

Engineered nanomaterial
Nanomaterial designed for specific purpose or function

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

Manufactured nanomaterial
Nanomaterial intentionally produced to have selected properties or composition.

[SOURCE: ISO/TS 80004-1: 2016, definition 2.9]

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

QCM – Quartz crystal microbalance

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. Preparing the device
2. Aerosolization
3. Nebulizer 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® Cloud system (VITROCELL®, Germany) equipped with
    - Quartz crystal microbalance (QCM)
    - Aeroneb® Lab nebulizer (Aerogen, Ireland)
    - Different sizes of cell culture inserts or QCM can be used, their use depends on the available unit. This protocol referes to Cloud 12, which is designed for 12-well and 24-well inserts.

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

5.2 Chemicals and reagents used

- PBS pH 7.4 1X, $MgCl_2$ and $CaCl_2$ Free (14190-094, GIBCO®, Switzerland),
- Ultrapure water
- 70% Ethanol
- Cell culture medium specific for cells used (please refer to culturing protocol of specific cell line)
- Isotonic sterile 0.9% NaCl solution

5.3 Apparatus and equipment used

- P1000, P200, P2.5 micropipettes
- Sterile 10 µL, 200 µL and 1000 µL pipette tips
- 50 mL centrifuge tubes
- Sonicator
- VITROCELL® Cloud
- Aeroneb nebulizers (different for different materials/ different mesh sizes available, choose based on the particle size)
- Cell cultures growing on hanging cell culture inserts (for example Falcon® Cat. No. 353181, Falcon, Corning brand, USA, or Transwell®, Cat. No. CLS3462-48EA, Corning, USA).
- TEM grids
- Water bath sonifier (no need to be specific, for example Elmasonic P30H cleaning unit, 100 W, 37 kHz, 30% (Elma Schmidbauer GmbH, Germany))

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.

5.6 Nanomaterials used / handling procedures

PATROLs Tier 1 materials (Carbon nanotubes, silica quartz, Barium Sulfate, Titania Dioxide particles, etc.).

5.7 Particle suspension preparation

Please, follow specific protocols for ENM suspension preparation

Tier 1 ENMs are usually suspended in ultrapure water, or following NanoReg protocol: 'Protocol for producing reproducible dispersions of manufactured nanomaterials in environmental exposure media'.

6 Procedure

6.1 Prior nebulization

1. Sterilize VITROCELL® base module and cover top with 70% ethanol.
2. Figure 1 shows the VITROCELL® Cloud system with its all parts.
3. Assemble the QCM, insert it to the VITROCELL® Cloud system and connect it with the oscilator.
4. Turn on the VITROCELL® Cloud heating unit and wait until the tempreture reaches 37°C, and sterilize the heating module, and exposure chamber using 70% ethanol and let it dry.

5. Warm the cell culture media.
6. Warm the ENM suspension to room temperature and shortly sonicate to redisperse the sedimented/agglomerated particles in necessary.
7. Keep nebulizer for 15 min in a beaker containing 70% ethanol. After 15 min remove the nebulized, and let it dry.
8. When the nebulizer is dry, rinse the nebulizer in ultrapure water, and subsequently nebulize 500 µL of ultrapure water to remove all the residuum from the nebulizer.

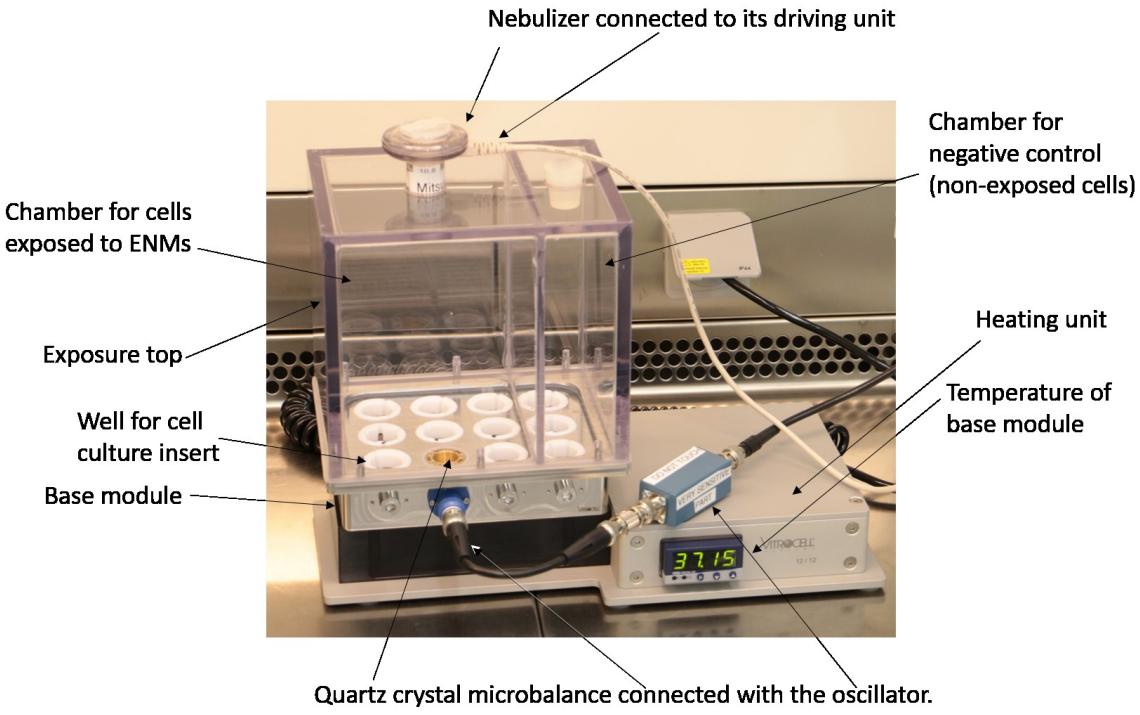

Figure 1: The VITROCELL® Cloud system.

6.2 Nebulization

Following procedure (step 1-5) has to be performed when nebulizing new material first time, and should be performed regularly to test the proper performance of the nebulizer.

1. Rinse the reservoir of nebulizer with ultrapure water.

2. Nebulize 200 µL of ultrapure water containing 1% of isotonic NaCl solution, and measure the time needed for nebulization of all the content.
3. Nebulize ultrapure water until almost no aerosol is generated. This indicates that no residual ions are remaining in the nebulizer.
4. Nebulize 200 µL of ENM suspension, and measure time for complete nebulization.
5. Register time needed for nebulization, and regularly check the nebulizer performance following steps 1 – 4.

Cell exposures to ENM aerosol

6. Fill the base module with cell culture medium. The amount of media depends of the used unit, and type of cell culture insert. Keep in mind, that medium should reach bottom of the membrane, but should not leak on the top of the membrane to keep cells at the ALI. For instance, using Cloud 12 with Falcon 12-well inserts, 2,5 mL/Cloud well is needed.
7. Place inserts into base wells.
8. In order to be able to visualize particle deposition, place Transmission electron microscopy (TEM) grids on the empty spots of the base module.
9. Cover the base module with exposure top.
10. Pipet 200 µL of ENM suspension into the nebulizer reservoir, and add 2 µL of NaCl solution. Cover the nebulizer with nebulizer top, place the nebulizer into the circular gap in the top of the exposure chamber. Make sure, that the nebulizer is place straight, and sits tightly.
11. Connect the nebulizer to its driving unit, and switch it on to activate the nebulization.
12. After complete nebulization, unplug the driving unit.
13. Wait until all the aerosol is completely settled (5 – 10 min).
14. Remove the exposure top, place cell culture inserts back to cell culture plate and transfer to incubator.

15. Transfer TEM grids into storing box (for example small petri dish, or cell culture plate), where leftover liquid can evaporate from a grid.
16. Add ultrapure water to the nebulizer and nebulize until nebulizer produces the aerosol to wash out the suspension leftover.
17. If the experiment is finished, follow to next steps (5.8.3 Cleaning). If further exposures have to be done, rinse the nebulizer reservoir (or change the nebulizer if other type of ENMs will be aerosolized), remove the cell culture medium, wipe the chamber, and module base with ethanol, let it dry and then follow steps 6 – 14 until the experiment is finished.

6.3 Cleaning

1. Nebulize at least 2x 500 µL of ultrapure water.
2. Put the nebulizer in a beaker with ultrapure water and small amount of detergent and sonicate in water bath sonicator (not necessary specific, for example Elmasonic P30H cleaning unit, 100 W, 37 kHz, 30% (Elma Schmidbauer GmbH, Germany)) for app. 15 min.
3. Rinse the nebulizer with ultrapure water.
4. Put the nebulizer in a beaker with 70% ethanol and sonicate in water bath sonicator (not necessary specific, for example Elmasonic P30H cleaning unit, 100 W, 37 kHz, 30% (Elma Schmidbauer GmbH, Germany)) for app. 30 min.
5. Rinse the nebulizer with ultrapure water and let it dry.
6. Remove the cell culture media from the VITROCELL® base module, and thoroughly flush with ultrapure water.
7. VITROCELL® base module and cover top can be washed in dishwasher (recommended). Do NOT clean with acetone.
8. VITROCELL® base module can be autoclaved (recommended on regular basis, *i.e.,* every week, but not necessary after each exposure) 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.

9. Sterilize VITROCELL® base module and cover top with 70% ethanol.

7 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.

8 Data Analysis and Reporting of Data

Not applicable for this current SOP.

9 Publications

Not applicable for this current SOP.

10 References

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.