

Preparing suspensions of nanoscale particles for biological testing with phagocytic cells

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1. Initial Considerations

This document provides a standard operation procedure for the preparation of particle suspensions for biological testing within the NanoCare project. The described methodology is consistent with the use of primary alveolar macrophages or related cell lines in vitro. As such, it avoids the use of excessive amounts of amphiphilic molecules such as e.g. proteins or serum components which are known to blunt macrophages' response to particles. Instead, a limited amount of phosphatidylcholine, a major component of the lung surfactant, is used to mimic exposure situation inside the lung and, putatively, to stabilize particles. The suspension described in this SOP was tested to be adequate for in vitro testing using alveolar macrophages from guinea pigs exposed to quartz DQ12, but may not be applicable for other cell types. Also, further modifications may be necessary to meet the demands of a specific nanoscale material, e.g., with respect to agglomerate formation. Therefore, the user needs to carefully examine the procedure to determine if it is adequate for the selected test material.

This SOP has been carried out for various NanoCare materials such as TiO₂, CeO₂, ZrO₂, AlOOH, and BaSO₄ and the reference particles quartz DQ12 and corundum. However, it is preliminary with respect to the particle characterization in suspension. (Please consult the NanoCare Web site at: <http://www.nanopartikel.info/> to make sure that you have the latest version of this document.)

2. Preparing suspensions for toxicological tests

2.1 Preparing the Medium

Cell culture medium to be used with rat or guinea pig alveolar macrophages is sterile Ham's F12 or MEM, respectively. Cell culture media are supplemented with penicillin (100 U) and streptomycin (1 µg/ml), and glutamine (2 mM), but do not contain fetal calf serum or any other type of protein. L-α-phosphatidylcholine (Sigma type XVI-E) is added from a 100x stock solution (2.5 mg/ml 0.9% NaCl, dispersed by Ultrasonication, and stored at -20°C) to give a final concentration of 0.025 mg/ml. Medium supplemented this way will hereafter be referred to as "testing medium".

2.2 Preparing the stock suspension

A small amount of material (1-2 mg) is weighed into a sterile polycarbonate tube (inner diameter approx. 16 mm, height 140 mm). At this stage materials may be subjected to γ-irradiation of 30 Gy to achieve sterility. Thereafter, the tube is filled with an adequate amount of sterile testing medium to give a stock suspension of 90-180 µg/ml. The filling height of the tube should not exceed 100 mm thus allowing to insert the ultrasonic probe. Tube is closed with a cap and particles are gathered in the solution by brief vortexing.

2.3 Ultrasonic treatment

An ultrasonicator (Sonics&Materials, VC50) is used to disintegrate macroscopically visible

aggregates and to disperse particles. The VC50 is operated at 50 Watts with a constant frequency of 20 kHz according to the instruction of the manufacturer (especially with respect to probe tuning). The clean and dry probe of the ultrasonicator (diameter 3 mm) is dipped into the suspension down to the bottom of the tube without contacting the wall. Power is switched on for 10 seconds. Successful dispersion of the powdered material results in a homogeneously turbid fluid.

Probe is removed and suspension is allowed to stand for 30-60 min. Shortly before being further diluted, the suspension is vortexed at maximum speed for 3-5 seconds. For the purpose of diluting, characterizing, or cell culture testing, aliquots are taken directly after vortexing.

2.4 Preparing and handling of diluted suspensions

Stock suspension is serially diluted in a 1:1 mode using the respective testing medium. This is done with a sterile 1, 2 or 5 ml pipette (cell culture tested, material from polystyrene). Typical particle concentrations operated with macrophage-based cell culture systems are in the range of 7.5-180 µg/ml. Diluted suspensions are poured onto macrophage cultures from which the culture medium had been previously withdrawn. The time span from preparing dilutions to macrophage exposure should not exceed 30 min.

2.5 Preparing subfractions from suspension

To obtain suspensions mostly devoid of larger agglomerates, suspensions (prepared according to 2.4) are left in their respective vessels in an upright position at 4-8°C without stirring for 24 hrs. Fluid samples are then withdrawn *without* prior vortexing. To achieve better reproducibility a fixed amount should be retrieved from the uppermost portion. As particle suspensions prepared this way may differ from the bulk solution they need to be characterized with respect to quality and content of particulate matter.

3. Characterization of the suspensions

3.1 Photo documentation

Documentation of the stock suspensions may be carried out in a cuvette or clear vessel in front of a suitable background.

Micrographs under cell culture conditions taken 0.5 and 12 h post particle application are also recommended to document any sedimentation of particles in situ. A 40fold objective and phase contrast optics are sufficient to visualize sedimented particulate matter.

3.2 Physical characterization

We define: ultra-fine fraction (<100 nm), fine fraction (100 nm – 1 µm) and agglomerates (>1 µm).

Qualitative analysis: Scanning electron microscopy or TEM preparations should be analyzed to qualitatively characterize the size of particles in suspension.

Quantitative analysis:

The characterization may be achieved either by analytical ultra-centrifugation (especially ultra-fine and fine fraction), or by laser diffraction and dynamic light scattering (especially fine fraction and agglomerates). Ideal particle concentrations are in the range of 0.1 – 1 mg/mL.

The classification of fineness is represented by a graph showing size versus mass concentration or size versus number concentration. Alternatively D_{10} , D_{50} , D_{90} may be indicated.

Subfractionated suspension (see 2.5) are investigated with the same methods. Additionally, a quantification of the particulate matter should be achieved with ICP-MS, using the most suitable element for detection. Results may be expressed in absolute values or relative to the element's content in the stock solution.