A common European approach to the regulatory testing of nanomaterials

Standard Operating Procedure

Title	***METHODOLOGY FOR SEDIMENTED DOSE DETERMINATION WITH CLS AND PIXE TECHNIQUES***
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METHODOLOGY FOR SEDIMENTED DOSE DETERMINATION WITH CLS AND PIXE TECHNIQUES

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The present protocol describes a methodology to calculate the sedimented dose during *in vitro* assessment of MNM. Two techniques, CLS and PIXE, are used to obtain the particle size distribution and the concentration of the MNM and from this data calculate the sedimented fraction in mass. This methodology should be used together with the CLS and/or PIXE protocols. A specific protocol for the preparation of the in vitro assessment of nanoparticles should be followed as well.

- Cells are seeded in culture boxes according to the specific protocol for cells seeding. The volume of the culture box should be indicated in the protocol as this parameter may affect the sedimented dose.
- The dispersion of nanomaterials, at a given concentration in the culture medium specific for the in vitro assay, should be prepared following a specific methodology (not described here). The dispersion of nanoparticles tested, during the development of this methodology where produced without further use of stabilisers or surfactants.
- A defined volume of this nanomaterial dispersion is injected with a syringe an injected into the culture box/dish to attain the final concentration (in the total culture medium volume) required for the assay. Avoid, as much as possible, the formation of bubbles or any artefact that could cause errors in the final concentration of nanomaterials.
- The incubation of the culture boxes with cells and culture medium, after contact with the nanomaterials dispersion, is performed following the specific assay protocol.
- Once the incubation period is finished, the culture box is removed from the incubator and placed under the hood. Then, the culture medium is removed completely, with a 10 mL syringe, and placed in a 15 mL vial. For this, the culture box is placed inclined at a 45° approximately (with the other side leaning at the table), as to suck the liquid conveniently and to facilitate the dripping from over the cells (grown at the bottom). Each time a new syringe and needle is used.
- This vial contains all the nanomaterials that were not sedimented and remain dispersed in the culture medium. To obtain a sample for characterisation, vortex the vial for 10 s and then sampled a defined volume of the liquid from the vial, between 0.5 and 1.0 mL. 1 mL is the maximal volume recommended for analysis with CLS.
- Samples should be analysed as soon as they are obtained. The effects of storage were not completely evaluated. Significant variations were observed mostly due to variations on the

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background information (culture medium). Therefore storage at room temperature or at 4°C is not recommended. Analysis must be performed as least in triplicate. The precision on the determination is influenced by the number of replicates.

- The sedimented dose value is calculated as the average of each replicate and reported in relative percentage.
- This sample will be analysed with the Centrifugal Liquid Sedimentation technique (CLS) following its specific protocol.
- The CLS technique estimate the total mass of nanomaterials contained in the sample from the obtained particle size distribution (integration of the area under the curve). The mass of a single particle is obtained with the hydrodynamic diameter and the density of the material. The total mass is obtained with the total intensity obtained for each hydrodynamic size and integrated for all the particle size distribution. A calibration curve with at least five different nanoparticle concentrations should be obtained with the CLS technique to evaluate its responsiveness and to validate the obtained results. Reproducible results were obtained for experiments with nanoparticles concentrations higher than 100 µg/mL. Experiments conducted with lower concentrations produced data with higher standard errors; therefore, if lower concentration values are used this methodology is not recommended. Sample concentration does not produce reproducible results as well. The effects of the concentrations of nutriments and components of the culture medium, as well as absorption on nanoparticle's surface were not completely evaluated.
- The sedimented dose is calculated as the difference between the nominal dose and the dose remaining dispersed in the culture medium (total quantity of nanomaterials remaining dispersed). The total quantity of nanomaterials introduced to the culture box at the beginning of the test is considered as being the 100%. The quantity of nanoparticles measured in the analysis (mg par mL) is then extrapolated to the total volume of the culture box (in mL) to obtain the mass of the nanoparticles.
- The sedimented dose can be also obtained with the Particle Induced X-ray Emission (PIXE) technique. For this, 1 mL of the culture medium sampled after incubation is placed in a holder and dried according to the specific protocol described in the PIXE SOP (see Annex 4).
- The PIXE technique detects the total quantity of the chemical element from which the nanomaterial is constituted in atom percentage. Then, the sediment dose is calculated in the same way as with the CLS technique.
- This methodology is applicable for the evaluation of nanoparticles in culture medium meant for *in vitro* assessment. The evaluation of the nanoparticles dispersibility is not an objective for this methodology.

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