A common European approach to the regulatory testing of nanomaterials

Standard Operating Procedure

Title	***Transmission electron microscopic imaging of nanomaterials***
Subtitle	
NANoREG Work package/task:	WP2 Synthesis, supplying and characterization
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Title: Transmission electron microscopic imaging of nanomaterials

Goal:

This procedure aims to record a set of calibrated transmission electron micrographs showing NM that are representative for the NM on the EM grid. The number of particles and the magnification of the micrographs are suitable for subsequent descriptive and quantitative image analyses.

Field of application:

This procedure aims to record a set of TEM micrographs showing NM that are representative for the NM on the EM grid

- The NM can consist of a metal like Ag and Au, an oxide like SiO_2 ,TiO_2 ,Fe_2O_3 and Fe_3O_4 and of other composition.
- The NM can be monodisperse or polydisperse.
- The grids can be metallic (copper, gold or zinc), coated with pioloform, formvar or other plastics, with or without holes that are evenly or randomly spaced, stabilised or not with carbon.
- The TEM specimen preparation can be performed based on the grid-on-drop or drop-on-grid methods as described in SOP/NANoREG/D2.10/TEMSpePrep), or on other sampling methods (cryo-EM, aerosol sampling, drying, centrifugation,…)
- TEM analyses can be qualitative (descriptive) or quantitative.

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3 Definition, abbreviations, references and norms

- CCD: Charged coupled device (camera)
- Pixel size: the pixel size is the size of one pixel in a TEM image. It is expressed in nm/pixel unless indicated otherwise.
- Camera pixel size: the product of the pixel size and the magnification. It is expressed in μm/pixel unless indicated otherwise.
- BF TEM: Bright Field Transmission electron microscopy
- OA: Objective aperture
- Particle: minute piece of matter with defined physical boundaries [ISO 14644-6:2007, definition 2.102]
- Aggregate: particle comprising strongly bonded or fused particles where the resulting external surface area may be significantly smaller than the sum of calculated surface areas of the individual components [ISO/TS 27687:2008]
- Agglomerate: collection of weakly bound particles or aggregates or mixtures of the two where the resulting external surface area is similar to the sum of the surface areas of the individual components [ISO/TS 27687:2008]
- TEM: Transmission electron microscopy
- NM: Nanomaterial

4 Principle of the method

5 Small laboratory material and specific reagents

5.1 Small laboratory material

- Nanoparticles coated on TEM grids.
- Filter paper Ø 70 mm (Whatman, 54 hardened) fixed with scotch tape in a Large (120 mm diameter) polyethylene petridish.
- Grid box to store grids after analysis.
- pipette tips of 200 µl (Gilson Diamond) and 200-1000µl (VWR)
- Tweezers
- Permanent, waterproof marker or a ball point to indicate references on filter paper

6 Equipment

- Transmission electron microscope with compatible holder
- (Optional) Stereo microscope to assure the correct orientation of the TEM grid in the holder by visualising the TEM grid(bars)

7 Instruction

7.1 Aligning the microscope and calibration

- Align the TEM and calibrate it using intra-lab protocol suitable for the available microscope and camera configurations.

7.2 Selecting the magnification

- Estimate the size of the smallest and largest particles in the sample by examining it at high and low magnifications.
- Select the optimal magnification (Comment 1).
    - Determine the lowest magnification where the area of the smallest particle in the specimen is at least 100 pixels.
    - Determine the highest magnification such that the size of the largest particle in the specimen (that you want to characterise) is maximal one tenth of the micrograph size.
    - When the highest magnification is higher than the lowest magnification, then any magnification between those two magnifications can be used. If not, then micrographs should be recorded at two different magnifications to avoid bias.

7.3 Systematic random sampling of the TEM grid

- Select the beam settings such that the illumination over the field of view is even and centered.
- Select positions that are evenly distributed over the TEM grid (Comment 2).
- At each position:
    - Place the specimen at eucentric height.

- Adjust objective lense stigmators such that astigmatism is minimal.
- Focus the particles (Comment 3).

- Record the micrographs and store them in a dedicated database
- Include imaging information with the image, minimal requirements are:
  - Pixel size (nm/pixel)
  - Micrograph size
  - Imaging conditions (BF)
  - Type and make of microscope

8 Specific safety measures

9 Comments

Comment 1

Theoretically, the possibility of the highest magnification to be higher than the lowest magnification is determined by the working range of the camera. The ratio between the diameter of the largest particle and the diameter of the smallest particle is the size range of the particles in the specimen. When this size range is smaller than the working range of the camera, then at least one magnification can be found where images of the specimen can be recorded and analysed unbiased [1; 2].

Table 1 Working ranges for different types of CCD camera's assuming a smallest particle area of 100 pixels² (particle diameter 10 pixels) [3] and a sampled area of 81% of the pixels².

CCD camera	Particle diameter (pixels)	ROI distance from left and top	Working range
1k * 1k	> 10	100 pix	10
1k * 2k	> 10	133 pix	13
2k * 2k	> 10	200 pix	20
4k * 4k	> 10	400 pix	40

Comment 2

These positions should allow sampling TEM images representative for the entire TEM grid [4].

Comment 3

For high contrast particles, minimal contrast focussing (Gaussian) is suggested, for low contract particles, Schertzer focus is suggested.

[1] P.-J. DE TEMMERMAN, J. LAMMERTYN, B. KETELAERE, V. KESTENS, et al. Measurement uncertainties of size, shape, and surface measurements using transmission electron microscopy of near-monodisperse, near-spherical nanoparticles. Journal of Nanoparticle Research, 2013/12/15, **16**(1), 2013, pp. 1-22.

[2] ISO 13322-1. Particle size analysis - Image analysis methods. In *Part 1: Static image analysis methods.* Geneva: International Organization for Standardization, 2004.

[3] H. G. MERKUS *Particle size measurements: fundamentals, practice, quality*. Pijnacker: Springer, 2009. 533 p. ISBN 978-1-4020-9015-8.

[4] P.-J. DE TEMMERMAN, E. VAN DOREN, E. VERLEYSEN, Y. VAN DER STEDE, et al. Quantitative characterization of agglomerates and aggregates of pyrogenic and precipitated amorphous silica nanomaterials by transmission electron microscopy. Journal of Nanobiotechnology, **10**(24), 2012, pp.