A common European approach to the regulatory testing of nanomaterials

Standard Operating Procedure

Title	***Preparation of EM-grids containing a representative sample of a dispersed NM***
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Version	***Date***	***Reason of change***

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Title: Preparation of EM-grids containing a representative sample of a dispersed NM

Goal:

This procedure aims to prepare a TEM specimen suitable for qualitative and quantitative analysis from a dispersed NM. To be suitable for TEM imaging and analysis of NM, the NM should be evenly distributed over the grids, while the fraction of the attached NPs represents the dispersed NM optimally.

Field of application:

This method allows preparing EM-grids containing a representative sample of a NM starting from a dispersion for further TEM imaging and analysis.

- The NM can be metallic consisting for example of Ag or Au, an oxide including SiO_2 ,TiO_2 ,Fe_2O_3, Fe_3O_4 ,and other)
- The NM can be monodisperse or polydisperse
- The medium can be polar (water, phosphate buffered saline,..) or apolar (hexane, acetone,…).
- TEM analyses can be qualitative or quantitative.

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1 Goal

This procedure aims to prepare a TEM specimen suitable for qualitative and quantitative analysis from a dispersed NM. . To be suitable for TEM imaging and analysis of NM, the NM should be evenly distributed over the grids, while the fraction of the attached NPs represents the dispersed NM optimally.

2 Domain of application

This method allows preparing EM-grids containing a representative sample of a NM starting from a dispersion for further TEM imaging and analysis. Transfer of the NM to the grid is not complete such that absolute counts cannot be realized.

- The NM can be metallic consisting for example of Ag or Au, an oxide including SiO_2 ,TiO_2 ,Fe_2O_3, Fe_3O_4 , and other)
- The NM can be monodisperse or polydisperse
- The medium can be polar (water, phosphate buffered saline,..) or apolar (hexane, acetone,…).
- TEM analyses can be qualitative or quantitative.

3 Definition, abbreviations, references and norms

- Solution:
- Colloid:
- Suspension:
- 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]
- Liquid:
- Medium:
- TEM: Transmission electron microscopy
- NM: Nanomaterial

4 Principle of the method

Dispersed NM are brought in contact with an EM-grid and are allowed to interact with its surface. When excess fluid is drained and grids are air-dried, a fraction of the NM remains attached to the grid by different types of interactions (electronstatic, apolar, van der Waals, …). The concentrations of NM, and the type and charge of the grid are chosen such that the fraction of nanoparticles attached to the grids optimally represents the dispersed NM, and that the particles of interest can be detected individually.

5 Small laboratory material and specific reagents

5.1 Small laboratory material

- Pioloform-coated and carbon-shaded copper grids 400 mesh (Agar Scientific Ltd., G2400C). Both home-made and commercially available grids can be used.
- 120 mm diameter polyethylene petridish
- 10 ml syringe (BD Plastipak, Becton Dickinson S.A. Madrid, Spain)
- 100 nm syringe filter (Minisart 0.10 µm, Sartorius AG, Vilvoorde, Belgium)
- pipette tips of 200 µl (Gilson Diamond) and 200-1000 µl (VWR)
- Tweezers
- Filter papers Ø 70 mm (Whatman, 54 hardened).
- Scotch tape to fix a filter paper in the petri dish.
- 4" Parafilm M (American National Can, Freewich, CT06830)
- Permanent, waterproof marker or a ball point to indicate references on filter paper

5.2 Specific Reagents

- Double distilled water ()
- Saturated Alcian blue stock solution: 2 % (w/w) in double distilled water
- Alcian blue working solution: Dilute the Alcian blue stock solution with an equal amount of water to obtain a 1 % working solution. The latter is at least stable for 1 month at 4 °C.

6 Equipment

- Micropipettes of 20 µl, 200 µl and 1000 µl (Gilson,)
- EasiyGlow glow discharge device (Pelco, Redding, CA, USA)

7 Instruction

7.1 (Optional) Adaptation of the charge of the EM grid to the NM charge (Comment 1)

- Introduction of positive charges to the EM-grid by pre-treating them with Alcian blue.
    - Fix a strip of parafilm to a flat and clean surface by wetting the surface with some drops of water, press the parafilm with the film side to the surface and remove the protective cover carefully.
    - Place an EM grid with the carbon-coated side on a drop of about 20 µl Alcian blue working solution and incubate for 1 to 10 minutes. Avoid air drying of the grids.
    - Grip the grid carefully with a pair of tweezers and wash most of the blue stain away by transferring it to 5 drops of water placed on the parafilm. Remove excess fluid by blotting its edge on a strip of filter paper, leaving a rest of humidity.
    - Use the grids immediately in the next step.
- Introduction of negative charges to the EM-grid by glow discharging.
    - Grids are glow discharged using the EasiyGlow glow discharge device according to the manufacturer's instructions.
    - Use the grids immediately in the next step.

7.2 Coating the NM on the grid

- Homogenize the dispersion by shaking, vortexing, stirring or pipetting.
- (Optional) Dilute the dispersed NM in a suitable dilution medium (The interaction *of NM with an EM-grid is strongly determined by the charge of the grid. In general, freshly prepared carbon-coated grids are hydrophobic or lightly negatively charged. Dependent on the preparation and storage time of the grid these charges tend to disappear leaving a hydrophobic surface. For non-charged particles and hydrophobic media, non-treated grids are advised. For charged particles in polar media, hydrophilic grids are required. The hydrophilicity of EM-grids can be increased by pre-treating them with Alcian blue pre-treatment [1]. The resultant positive charge strongly increases attachment of negatively charged NM [2]. Alternatively, negative charges can be introduced by glow discharging strongly increasing attachment of positively charged NM [3]. In case the charge of the NM is unknown, it is wise to evaluate the different approaches.*
- Comment 2).
- Allow the NM to interact with the grid surface by either the drop-on-grid method, or the grid-on-drop method
  - Grid-on-drop method: Place the EM-grid on a droplet of dispersed NP.
  - Fix a strip of parafilm to a flat and clean surface by wetting the surface with some drops of water, press the parafilm with the film side to the surface and remove the protective cover carefully.
  - Place a droplet of 2 to 50 μl of dispersed NM on the parafilm.
  - Transfer the grid to the droplet and indicate the appropriate references with a waterproof marker.
  - Float the grids, with coated surfaces down, on the droplets for 1 to 10 minutes.
  - Remove excess fluid by blotting its edge on a strip of filter paper, leaving a rest of humidity.
  - Drop-on-grid method: Place a droplet of dispersed NP on an EM-grid.
  - Put the grid on the parafilm and then pipette a droplet of 2 to 50 μl of dispersed NM on the grid. Leave this for 1 to 10 minutes.
  - Remove excess fluid by blotting its edge on a strip of filter paper, leaving a rest of humidity.
- (Optional) Rinse step (The concentration of NM should be chosen such that the number of particles per micrograph is optimal for later analysis. It should be taken into account that the particles do not touch or overlap each other. Optimal concentrations vary from sample to sample as illustrated in following examples.
  - A typical concentration for a colloidal, spherical, unaggregated silica NM is 0,05 mg/ml [4].
  - For colloidal aggregated silica NP a concentration up to 0,5 mg/ml can be used [5].
  - For non-colloidal, aggregated, silica NM a concentration of 2,5 to 25 mg/ml can be used [6].

For polydisperse samples, the grid-on-drop method might result in a preferential binding of the fraction of smaller particles, whereas the drop-on-grid method might result in a preferential binding of the fraction of larger particles [6].

- Comment 3)
  - Rinse the grids, with coated surfaces down, by placing them on a droplet of water for 30 seconds to remove excess salts and contaminating material.
  - Remove excess fluid by blotting its edge on a strip of filter paper, leaving a rest of humidity.

7.3 Storage of the grids

- Place the grids in a grid box or on a filter paper in a petri-dish.
- Make sure to always note the appropriate references in order to be able to retrieve specific grids later on

8 Specific safety measures

- Because of the possible toxicity of the reagents and NM and the possible presence of unknown contaminants, it is highly recommended to wear gloves during this procedure.
- All waste materials generated during this procedure should be disposed in the suitable container for chemical waste.

9 Comments

Comment 1

The interaction of NM with an EM-grid is strongly determined by the charge of the grid. In general, freshly prepared carbon-coated grids are hydrophobic or lightly negatively charged. Dependent on the preparation and storage time of the grid these charges tend to disappear leaving a hydrophobic surface. For non-charged particles and hydrophobic media, non-treated grids are advised. For charged particles in polar media, hydrophilic grids are required. The hydrophilicity of EM-grids can be increased by pre-treating them with Alcian blue pre-treatment [1]. The resultant positive charge strongly increases attachment of negatively charged NM [2]. Alternatively, negative charges can be introduced by glow discharging strongly increasing attachment of positively charged NM [3]. In case the charge of the NM is unknown, it is wise to evaluate the different approaches.

Comment 2

The concentration of NM should be chosen such that the number of particles per micrograph is optimal for later analysis. It should be taken into account that the particles do not touch or overlap each other. Optimal concentrations vary from sample to sample as illustrated in following examples.

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For polydisperse samples, the grid-on-drop method might result in a preferential binding of the fraction of smaller particles, whereas the drop-on-grid method might result in a preferential binding of the fraction of larger particles [6].

Comment 3

The additional washing step reduces the background signal of the grid by removing excess salts and contaminating material. Extensive washing might result in the selective loss of the larger particles from the grid which should be avoided [6].

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