Identification and characterization of TiO_2 nanoparticles in face masks by TEM

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Keywords

Face mask, textile, electron microscopy, energy dispersive X-ray spectroscopy, TEM, STEM-EDX, particle size distribution, ultramicrotomy, nanoparticles, agglomerates, image analysis

Abstract

Because of the possible health risks related to the use of nanoparticle technologies in face masks, a general methodology is developed for identification, localization and particle size measurement of nanoparticles *in situ* in face masks by conventional and analytical transmission electron microscopy (TEM). The methodology can be applied by following this protocol which includes (i) preparation of resin-embedded ultra-thin sections of face masks suitable for TEM analysis, a procedure that can be applied to any type of textile, (ii) visualization and identification of the (nano)particles inside the cross section of the textile fibres by scanning TEM (STEM) combined with energy dispersive X-ray spectroscopy (EDX), (iii) measurement of the number-based particle size distribution based on a (semi-)automatic quantitative analysis using ImageJ, and (iv) in view of risk assessment, a calculation to estimate the amount of particles available for release from the mask.

Introduction

Since the beginning of the COVID-19 pandemic, face masks are utilized as a public health measure worldwide and are being distributed by many different producers/companies. A recent study[1] found that a large percentage of masks, even though not indicated, contain titanium dioxide which is generally applied in commercial textile products as a white colorant or as a matting agent[2,3]. It was demonstrated by Verleysen et al.[4] that the titanium dioxide was present in the masks as particles with a fraction in the nano-range (<100 nm; median constituent particle size between 89 nm and 184 nm were measured). In addition, treatment of textiles with silver or copper based biocides, which gives the textiles a broad spectrum of antibacterial and antiviral properties[5–7], is applied in certain face masks. Such biocides can be applied under different physicochemical forms, one of which being nanoparticles. Many other examples of nanotechnology based applications in face masks have been reported.[8] However, the use of such nanotechnologies in face masks is accompanied by safety concerns as to whether nanoparticles may be released and exposing the wearer to nanoparticles upon inhalation[9]. To perform a risk assessment in line with the EU's regulatory framework, formal identification of the type of particles, their localization in the textile and a determination of the (nano)particle size distribution are imperative.

A general methodology is proposed to evaluate the presence and characteristics of nanoparticles *in situ* in face masks by conventional and analytical transmission electron microscopy (TEM). The applied sample preparation, TEM imaging and analysis aim to identify and measure the size, shape, agglomeration state and elemental composition of the nanoparticles in ultra-thin epoxy resin sections of the face masks. In addition, the amount of (nano)particles at the surface of the textile fibres is determined, as this forms an important factor to predict whether release of nanoparticles may occur. The method has already been applied by Verleysen et al.[4] for quantification of TiO_2 nanoparticles in face masks for general use on the Belgian market.

The proposed methodology for preparing TEM specimens from face masks is based on the procedure described by Gashti et al.[10]. Ultra-thin sections of the textiles are prepared by embedding them in an epoxy resin, followed by ultra-thin sectioning using ultramicrotomy. The described procedure can be applied to different types of textiles including synthetic fibres such as polyester, polyamide, and elastane; natural fibres such as cotton; and woven as well as non-woven fabrics. High angle annular dark field scanning transmission electron microscopy[11,12] (HAADF-STEM) allows to visualize the cross section of the fibres and to identify isolated, aggregated and agglomerated (nano)particles based on the generated Z-contrast. Elemental analysis by STEM-EDX[11,13] (energy dispersive X-ray spectroscopy), which detects the characteristic X-rays emitted by the sample upon electron irradiation, is applied to determine the chemical composition of the particles. As shown by Verleysen et al.[4], TiO_2 appears highly agglomerated in the mask fibres. Therefore, to calculate the fraction of particles at the fibre surface, the following quantities are determined: (i) the agglomerate size, which is measured semi-automatically using the NanoDefine ParticleSizer plugin in ImageJ, (ii) the constituent particle size, which is measured manually since individual particles can't be separated as they are fixed in agglomerate form in the matrix, and (iii) the fibre diameter, also measured manually. The amount of particles required for the quantitative size analysis is determined according to FprCEN/TS17273[14], supporting on the relation between the number of measured particles and uncertainty associated with the intermediate precision (intra-laboratory measurement uncertainty) of the median minimum Feret diameter (Fmin).

Reagents

- Vinyl cyclohexene anhydride (NSA, Fluka, 74378)
- DER736 (Fluka, 31191)
- 2-nonen-1-ylsuccinic anhydride (NSA, Fluka, 74378)
- 2-dimethylamino ethanol (DMAE, Fluka, 38990)
- Epon812 (Fluka, 45345)

- 2-dodecen-1-ylsuccinic anhydride (DDSA, Fluka, 44161)
- Methylonorbornene-2,3-dicarboxylic-acid (MNA, Fluka, 68165)
- 2,4,6-Tris(dimethyl-aminomethyl)phenol (DMP30, Fluka, 93331)
- Distilled water

Equipment

Small laboratory material

- Pioloform-coated and carbon-shaded copper grids 150 mesh (Agar Scientific Ltd., G2150C). Home-made and commercially available grids can be used.
- 120 mm diameter polyethylene petri dish
- Silicone Mould 21 Cavity Blue (Agar Scientific Ltd., AGG3549)
- Transfer pipettes, Falcon. Capacity 3,0 ml, Length 152 mm. Polyethylene, disposable (VWR International, 734-0342)
- Filter papers Ø 70 mm (Whatman, 54 hardened)
- Scotch tape to fix a filter paper in the petri dish
- Permanent, waterproof marker or a ball point to indicate references on filter
- Regular scissors
- Pencil

Large equipment

- EM TRIM2 trimming unit (Leica Microsystems, Diegem, Belgium)
- Ultracut microtome (Leica Microsystems, Vienna, Austria)
- Compact embedding oven, model MINO/6 (Genlab, Widnes, UK)
- Talos 200 kV transmission electron microscope that can be operated in STEM mode, equipped with a HAADF detector and Super-X EDX detector (Thermo Fisher Scientific, Eindhoven, The Netherlands) consisting of 2 windowless silicon drift detectors (SDD) (Thermo Fisher Scientific, Eindhoven, The Netherlands)

Software

- Velox version 3.3.1.19-397c043e56 (Thermo Fisher Scientific, Eindhoven, The Netherlands). License needed.
- ImageJ version 1.53f51 (National Institutes of Health, USA). Download available at https://imagej.net/downloads. Used plugins:
    - ParticleSizer
    - Max Inscribed Circles
- Anaconda software including the integrated development environment Spyder version 5.1.5

running Python 3.9. Download available at https://www.anaconda.com/products/individual.

Procedure

Safety remarks:

- Because of the possible toxicity of the reagents and materials, 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.

1 Preparation of Epon medium

- Prepare Spurr solution: Dissolve 13.3 g vinyl cyclohexene dioxide, 7 g DER736, 34.7 g 2-nonen-1-ylsuccinic anhydride and 0.53 g 2-dimethylamino ethanol in 50 ml of distilled water.
- Prepare Epon812 solution: mix 27.3 g Epon812 , 10.8 g 2-dodecen-1-ylsuccinic anhydride, 18.7 g methylnorbornene-2,3-dicarboxylic-acid and 0.9 g 2,4,6-Tris(dimethyl-aminomethyl)phenol with 50 ml distilled water.
- Mix equal amounts (usually 50 ml) of Spurr and Epon812 solutions.

2 Preparation of TEM specimen from mask

- Use a pair of scissors to cut a 1 cm x 1 cm square from the sample mask.
- Separate the different layers of textile.
- Cut a 1 mm x 5 mm piece of each layer.
- Transfer the layer samples into the silicone rubber embedding mould. Care should be taken to give the samples a correct orientation (Figure 1). A small piece of filter paper with the required references written on it in pencil is placed in each cup to label the samples. Optional: To obtain more of the sample on the grid, 2 pieces of textile can be brought in the same block of epon (on top of each other without spacing).

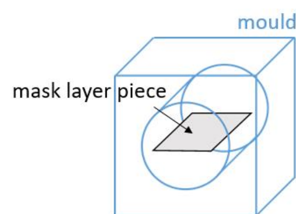

Figure 1: Schematic drawing of the embedding mould with a sample piece of a mask layer inside.

- Fill the mould with the Epon812-Spurr resin mixture and allow to polymerize for 48 hours at 70 °C in an embedding oven. Let cool down for 2 hours afterwards.
- After embedding, trim the specimen block with the EM TRIM2 trimming unit to obtain a cutting face of 0.5 x 1 mm^2 to 1 x 2 mm^2.
- Cut semi-thin sections with a thickness of 150 nm (interference colour in between gold and purple) using the Ultracut microtome.

- Bring the sections on carbon and pioloform-coated copper grids (150 mesh). If only TEM imaging is performed, the carbon layer on the grid is not necessary. For STEM-EDX analysis, the grid has to be coated with both carbon and pioloform to avoid specimen drift.

3 HAADF-STEM imaging of fibres and particles

For HAADF-STEM imaging and EDX analysis, a well-calibrated and well-aligned Talos transmission electron microscope operated at 200 kV is used.

- Load the grid into the single tilt/double tilt TEM holder and insert the holder into the TEM.
- Velox will open a window asking to specify the folder where the STEM images/EDX data cubes will be automatically saved and to define the concept for the file names.
- Load the appropriate FEG register or set the appropriate probe conditions for imaging beam sensitive materials where a large depth of field is needed: Smaller I (e.g. spotsize 9) and smaller α (e.g. CA 50 µm; α = 7.5 mrad).
- Adjust the z-height to focus the image and optimize the detector settings in Velox using the scope tool.
- Identify the fibres on the grid and record a series of at least 10 well-focused representative images of different fibres using the Velox software. Choose the magnification such that the fibre cross-section just fits on the image (see Figure 2). These images will be used in section 5.3 for the determination of the fibre diameter.
- Record a set of images showing particles that are representative for the sample on the TEM grid and that are suitable for a subsequent quantitative analysis of the agglomerate and constituent particle size. Make sure to capture at least 100 particles (either aggregates/agglomerates at lower magnification or constituent particles at higher magnification) in line with FprCEN/TS17273[14] to assure accurate median size measurements. For these masks, typically 10 images both at low and high magnifications is sufficient, although this amount depends on the particle density and the size of the aggregates/agglomerates. The specific imaging conditions for the imaging in STEM mode are summarized in Table 1. The pixel size and the associated lower limit of quantification depend on the selected magnification and are determined based on the criterion of Merkus[16]. The upper size detection limit is limited to one tenth of the image size supporting on ISO 13322-1, 2014.[17]

Table 1: Summary of the STEM imaging conditions applied.

TEM imaging conditions	STEM (Low Magnification)	STEM (High Magnification)
Detection system	HAADF detector	HAADF detector
Number of images	10	10
Magnification	8000	88000
Pixel size (nm)	12.25	1.106
Lower limit of quantification (nm)	122.5	11.06
Upper limit of quantification (nm)	12540	1133
Dwell time (µm)	20	20
Size of the images (px x px)	1024 x 1024	1024 x 1024
Size of the images (nm x nm)	12540 x 12540	1133 x 1133

4 EDX spectral imaging (SI) of particles

- Keep the same recording settings as for the STEM imaging, i.e. dwell time 20 µm and image size 1024 x 1024 px^2. The number of frames is typically set to 5. In case more signal is needed

to get significant peaks in the EDX spectrum (which might be the case due to the high spot size), a higher value is advised. Alternatively, the option to stop the experiment manually can be selected.

- Record STEM + EDX SI images of the (nano-)objects of interest. Make sure not to overlook particles or aggregates/agglomerates of different elemental composition, particles inside and on the border of the fibres and (nano-)objects with special features. Choose an appropriate magnification to make sure the object of interest is well-centred in the image with still some background space around to allow for some drift between scans.

- By drawing a region on the STEM image using the rectangle tool, the EDX spectrum becomes visible. Choose to automatically match the peaks in the spectrum with the elements or add elements of interest (e.g. Ti, Ag,…) manually to the energy spectrum or the spectral maps by selecting them in the periodic table.

5 Image analysis

5.1 ParticleSizer for quantitative analysis

The physical properties of the particles of interest are measured based on the properties of their 2D projections. For constituent particles, the size distribution is determined based on manual measurement using ImageJ. For aggregates/agglomerates, the size distribution is determined using the NanoDefine ParticleSizer (https://imagej.net/plugins/particlesizer) plugin for ImageJ. This method allows determining the properties, such as the characteristic size and shape distributions, (semi-)automatically from electron micrographs.

- Open ImageJ and load the image series by File>Open>Image Sequence. Apply the image analysis settings summarized in Table 2 on the pop-up window.

- Open the settings manager of the NanoDefine ParticleSizer plugin by Plugins>NanoDefine>SettingsManager. The settings summarized in Table 2 are typically used for the analysis of agglomerates of TiO_2 in fibres of face masks and can be applied as a starting point for further optimization.

- Run the ParticleSizer algorithm pressing Plugins>NanoDefine>ParticleSizer. An image with the identified particles overlaid in red, a table presenting the measured properties of the detected particles and a histogram of the distribution of the Fmin are generated as output. Save the table as csv file.

Table 2: Overview of the image analysis settings of the NanoDefine plugin in ImageJ software for the image analysis of agglomerated particles.

Image analysis settings for the NanoDefine plugin in Image J software		
Settings to import image sequences in Image J	☒ Convert to 8-bit Grayscale ☐ Convert to RGB ☒ Sort names numerically ☐ Use virtual stack	
Settings Manager for the Particle Sizer from the NanoDefine plugin		
Mode selection	☐ Use Watershed for irregular structures Irregular watershed convexity threshold	Not applied
	☒ Use single particle mode	
	☐ Use ellipse fitting mode	
Segmentation	Circular window radius	Use default
	Rolling ball radius	Use default

	Min OBT intensity difference (8 bit)	4
Shape constraints	Minimal area (pixels²) Minimal feret min Minimal convexity Minimal solidity	0-10 px² 2-5 pixels 0.45-0.50 0.65-0.70
Ellipse shape constraints	Minimal long axis length (px) Minimal short axis length (px) Maximal aspect ratio	Not applied Not applied Not applied
Misc	Smoothing factor ☐ Use inverted images ☐ Show binary results ☒ Ask me to select a region ☐ Do not plot size distribution ☐ Do not apply denoising ☐ Record process	1-3

5.2 Calculation and visualization of the descriptive statistics

The results of the quantitative analysis of the detected nanoparticles were presented following the ISO 9276-1 (ISO, 1998) guidelines for representation of results of particle size analysis. A non-parametric approach is applied. For all samples, the normalized number-based distributions and the kernel density estimates (KDE) of the Fmin, Fmax, and AR of the constituent particles and of the aggregates/agglomerates are determined using the seaborn distplot function. The bin width is calculated using Freedman-Diaconis rule[18]. The mean and median values of the datasets are determined. For the KDE plot, producing a continuous density estimate, a Gaussian kernel is applied. The modes of the distributions are calculated from the KDE.

Python can be used to process the raw data resulting from the image analysis by ImageJ. Helpful packages that can be used to calculate the relevant descriptive statistics and plotting parameter histograms are numpy, scipy, pandas and seaborn. Generate a csv file summarizing the statistical parameters of the particle size distribution, including the Fmin, which will be used in the calculation in Section 6. Alternative software can be applied also.

5.3 Measuring the median fibre diameter

For each of the STEM images of the fibre cross sections, the fibre diameter is determined by manual measurement using ImageJ. Since for fibres which are not aligned parallel to the viewing direction in the TEM, the cross section appears as an ellipse rather than a circle, the diameter of the maximum inscribed circle is selected as the best measure for the fibre diameter.

- Use the polygon tool to draw a shape on the image representing the fibre cross section (Figure 2A).
- Click Plugins>BIOP>Image Analysis>Binary>Max Inscribed Circles.
- In the pop-up window, set the minimum disk diameter to 0 and check the box ‘Use selection instead of mask’ and click OK.
- The maximum inscribed circle appears on the image as annotation (Figure 2B). Find the diameter in pixel by using the circle tool and selecting the drawn circle.
- Covert to nm by looking up the pixel size of the image in Velox and multiplying it with the diameter in pixels.

Repeat this for all 10 images of fibres and determine the median fibre diameter (d_f).

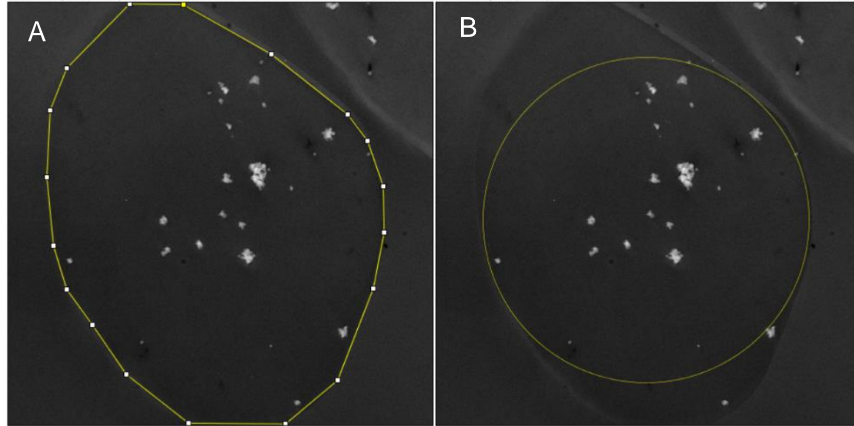

Figure 2: Illustration how to use the polygon tool in ImageJ to define the boundary of the fibre (A) and the largest inscribed circle inside the polygon selection that gets determined by the Max Inscribed Circles plugin (B).

6 Estimation of the amount of particles at the fibre surface

This estimation is valid on the assumption that the (agglomerated) particles are homogeneously distributed in the matrix of the fibres (this is usually the case for TiO_2).

- Define F, the fraction of the particles at the surface of the fibres, as the ratio of an external ring-shaped surface of the cross-section of the fibres (S_r) and the total surface of the cross-section of the fibres (S_{cs}).

  Equation 1:

$$F = \frac{S_r}{S_{cs}}$$

- The thickness of the external ring-shaped surface, S_r, is determined by the median diameter (d_a) of the agglomerates (Figure 3). Then, approximate F, assuming circular cross-sections of fibres, as:

  Equation 2:

$$F = \frac{\frac{\pi}{4} \cdot d_f^2 - \frac{\pi}{4} \cdot (d_f - d_a)^2}{\frac{\pi}{4} \cdot d_f^2}$$

  Equation 3:

$$F = \frac{d_f^2 - (d_f - d_a)^2}{d_f^2}$$

  with d_f the median diameter of the fibres (see Section 5.3) and d_a the median minimum Feret diameter of the agglomerates (see Section 5.2).

- Now, calculate the mass of the particles at the surface of the fibres in a mask (M_{sf}) as:

  Equation 4:

$$M_{sf} = F \cdot M_{tot}$$

  with F the fraction of the particles at the surface of the fibres and M_{tot} the total mass of the

particles in the mask. The latter quantity can be determined by inductively coupled plasma-optical emission spectroscopy (ICP-OES) as described in Verleysen et al.[4]

For a specific case of fibres, namely bi-component microfibres, the assumption of a homogeneous distribution of the agglomerated particles in the fibres is invalid. Bi-component microfibres are characterized by a larger surface area from which particles can be released. To account for this increased surface, $S_{r,mf}$, a correction factor should be introduced:

Equation 5:

$$S_{r,mf} = S_r \cdot \frac{\sum wedges\ perimeter}{fibre\ perimeter}$$

With $\sum wedges\ perimeter$ the sum of the perimeters of the wedge-shaped (nanoparticle containing) polyester parts of the fibre, and $fibre\,perimeter$ the perimeter of the (near-)circular cross-section of the microfibre.

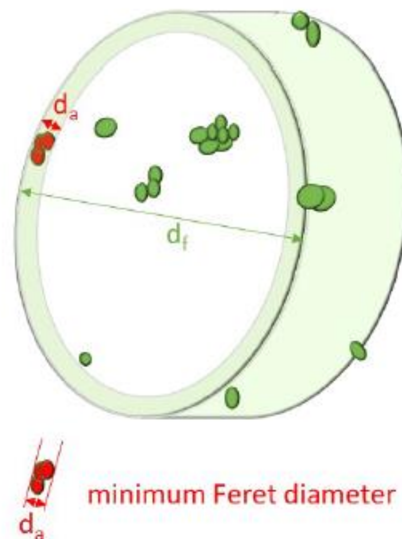

Figure 3: A schematic representation of the section of the fibre with agglomerated particles (green near-spherical shaped). The thickness of the external ring-shaped surface of the cross section (green zone), *d_a*, is estimated based on the median value of the minimum Feret diameter distribution of the agglomerates. Image taken from Verleysen et al.[4]

Troubleshooting

Problem: Particles wrongly identified by ParticleSizer. It is possible that background is also identified as particles by the ParticleSizer algorithm and thus some data is wrongly included in the quantitative analysis of the particle size.
→ Solution: Manual removal of wrongly detected particles. Draw a rectangle around the wrongly identified particles using the “Rectangle” tool to select it, then right click on the selected particle and choose ‘Remove particle’.

Problem: Particles not homogeneously distributed in fibre or fibre shape not close to circular. In this case, the calculation of the fraction of particles at the fibre surface, that requires a homogenous distribution and approximates the fibre as circular, is wrong.
→ Solution: Adapt the calculation of the fraction of particles at the surface (Equation 1:, Equation 2:, Equation 3:), taking in account the observed distribution of particles or the fibre shape.

Time Taken

The preparation of a TEM specimen from a mask takes approximately 2 ½ days (time for each specific step in the table below). The polymerization and cool down steps can be done for multiple samples at the same time.

Table 3: Different steps of the sample preparation and their duration.

Step	Time
Cut of different layers and introduction of layers in mould	30 min
Polymerization	48 hours
Cool down	2 hours
Trimming	15 min
Cut of ultra-thin section	20 min
Total	**2 ½ days**

The STEM-EDX analysis takes approximately 2-3 hours for 1 specimen.

The image analysis can be done in half a day. However for the first time the analysis is done, take into account ½ day extra for optimizing the ParticleSizer settings and another ½ day for preparing the python script.

The total estimated time of the procedure is approximately 4 days.

Anticipated results

At the end of the procedure, STEM and EDX micrographs are obtained which allow both a descriptive (size, shape, agglomeration state, elemental composition..) and quantitative (particle size distribution) analysis of the (nano)particles present in face masks. The descriptive statistics of the important size and shape parameters (Fmin, Fmax, aspect ratio) are summarized and visualized using python. In view of a risk assessment, an estimation of the amount of particles available for release from the mask is obtained, taking into account the determined fibre and particle properties, and their mass.

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Associated Publications

Verleysen, E., Ledecq, M., Siciliani, L. *et al.* Titanium dioxide particles frequently present in face masks intended for general use require regulatory control. *Sci Rep* **12,** 2529 (2022). https://doi.org/10.1038/s41598-022-06605-w