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Uncertainties of size measurements in electron microscopy characterization of nanomaterials in foods

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ABSTRACT

Electron microscopy is a recognized standard tool for nanomaterial characterization, and recommended by the European Food Safety Authority for the size measurement of nanomaterials in food. Despite this, little data have been published assessing the reliability of the method, especially for size measurement of nanomaterials characterized by a broad size distribution and/or added to food matrices. This study is a thorough investigation of the measurement uncertainty when applying electron microscopy for size measurement of engineered nanomaterials in foods. Our results show that the number of measured particles was only a minor source of measurement uncertainty for nanomaterials in food, compared to the combined influence of sampling, sample preparation prior to imaging and the image analysis. The main conclusion is that to improve the measurement reliability, care should be taken to consider replications and matrix removal prior to sample preparation.

1. Introduction

Engineered nanomaterials (ENMs) are increasingly finding new applications in the food industry. Some food additives already used for decades (Dekkers et al., 2010) might be classified as nanomaterials, e.g. synthetic amorphous silica (SAS). Others as for instance silver ENMs are applied in food packaging (Chaudhry et al., 2008). The potential risks posed by the presence of ENMs in foods and food contact materials is an area of major interest because of the current uncertainties in relation to the potential consumer exposure to ENMs through food, and the fate and effects of the orally ingested ENMs in the body (Dudkiewicz, Luo, Tiede, & Boxall, 2012). In order for studies on ENMs to provide meaningful and accurate data to assess exposure appropriately developed and validated methods are required (Calzolai, Gilliland, & Rossi, 2012; Hassellöv, Readman, Ranville, & Tiede, 2008; Joner, Hartnik, & Amundsen, 2008).

Electron microscopy (EM) is one of the standard methods that are currently used for ENM measurement (Calzolai et al., 2012) and also recommended for such use by the European Food Safety Authority (EFSA) in a guidance document (EFSA Scientific Committee, 2011). In the guidance document EM is listed as a method of first choice for ENM measurement in foods along other complementary methods. Nevertheless so far no validation of this technique for the characterization of ENMs has been presented. Only a few studies have assessed the uncertainty of ENMs size measurement by EM using spherical ENMs characterized by a narrow size distribution and in pristine dispersions e.g. (Braun et al., 2012; Lamberty et al., 2011). The presence of the food matrix in the sample is however expected to introduce difficulties during sample preparation and analysis (Dudkiewicz et al., 2011, 2012; Tiede et al., 2008) and is likely to affect the ENM measurement uncertainty. Food samples are usually characterized by a high water content, and EM instruments operate under high vacuum. This means that samples at least need to be dehydrated for analysis. The EFSA acknowledges that sample preparation and in particular matrix removal can introduce changes to the original state of ENMs in the sample and thus preparation protocols involving minimal processing should be applied. Additionally only small sample volumes (order of pL) can be used during EM analysis, thus limiting the number of measured ENMs and affecting statistical reliability (Linsinger et al., 2013).

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This paper presents an evaluation of EM procedures for the measurement of ENMs in foods using simple sample preparation methods which allow to retain ENMs in the food matrices. This study relies on two examples of reference materials, namely spherical silver nanoparticles (AgNPs) in meat and SAS in tomato soup covering narrow (AgNPs) and broad (SAS) size distributions. Both of these reference materials were produced within an EU FP7 funded project "NanoLyse" on the development and validation of analytical methodologies for ENMs in foods. The choice of ENMs reflects realistic scenarios in which humans could be exposed to ENMs that are applied in food packaging, potentially migrating to food (AgNPs) and ENMs readily applied as a food additive (SAS). The robustness of the obtained data from SAS containing reference materials was tested by analyzing a commercially available food product with declared content of SAS.

The study addressed three main questions: (1) how many ENMs need to be measured in order to obtain a reliable measure of size; (2) what is the precision of ENM measurement by EM; and (3) which step(s) within the procedure, including sampling, sample preparation, imaging and image analysis, contribute most to the measurement uncertainty?

2. Experimental design

2.1. Materials

The materials included in the study as well as characterization information provided by the manufacturer or determined in our laboratories are listed in Table 1. Two groups of reference food materials spiked with ENMs were used: These were chicken paste (Meat 1, Meat 2), and tomato soup (Soup 1, Soup 2). Meat reference materials contained AgNPs and soup reference materials contained SAS at the spiked concentrations listed in Table 1. These reference materials were developed by the Institute for Reference Materials and Measurements of the European Commission's Joint Research Centre (JRC-IRMM, Geel, Belgium). The development of soup and meat reference materials was described in (Grombe et al., 2014, in press).

Along with the reference materials, the JRC-IRMM also provided pure suspensions of the respective ENMs that had been used in the preparation of these reference materials. The suspensions were also studied to provide information on the original characteristics of ENMs prior to spiking into foods as recommended (EFSA Scientific Committee, 2011). Additionally, a commercial soup powder (Soup COM) with a declared content of SAS-E551 was obtained from a local supermarket. As a control for the Soup COM, SAS powder (SAS COM)-NM203 from the JRC, Institute for Health and Consumer Protection, Nanomaterial Repository for Toxicology Testing (Ispra, Italy) was used.

Prior to the study, Soup COM and SAS COM were suspended in aqueous media using a magnetic stirrer. Soup COM was mixed at a ratio of 11:100 with boiling tap water. The SAS COM was mixed at a ratio 2:98 with borate buffer at pH 8.0 of composition 0.05 M H_3BO_3, 0.05 M KCl, 0.004 M NaOH (BB 8.0).

2.2. Electron microscopy and energy dispersive X-ray spectroscopy

Two different EM methods were selected for imaging depending on the sample's matrix type (solid/liquid) and chemistry of the ENMs. The SAS has generally weak contrast in EM, however for imaging in scanning electron microscopy (SEM), samples can be coated with a nanometric layer of metal to improve contrast and minimize charging. AgNPs could be best visualized using TEM as these ENMs were embedded in a layer of the meat sample. Therefore for imaging of SAS and AgNPs containing samples, SEM and TEM were selected respectively.

Samples were prepared for analysis as described in Supplementary data Section 2 and (Lari & Dudkiewicz, 2014). The preparation methods were developed and evaluated in our laboratories before use in this study. In course of this evaluation we have found that these sample preparation methods allowed to limit agglomeration of the ENMs (a typical artifact hampering image analysis) and recover sufficient number of ENMs for imaging and measurements.

The SEM images were taken using an FEI Sirion S field emission gun SEM equipped with a through the lens detector and operating at a voltage of 5 kV and spot size 3.

The TEM images were acquired with a JEOL JEM 2011 TEM operating at 200 kV and using a digital camera (Gatan 794).

2.3. Data acquisition and image analysis

All provided particle size measurements refer to the equivalent circle diameter (ECD) which is the diameter of the circle with the same surface area as projected in the 2D image of the ENMs. The data acquisition parameters used in this study were summarized in Table 2.

Table 1
List of the materials used. NanoLyse labeling from Grombe et al. (2014, in press) provided to allow comparison of data.

Sample	Type of particles	Concentration of core particle % w/w	Declared average particle size	Median [*IQR*][a]	
				Size (nm)[b]	Number[b]
Meat 1 (NanoLyse13)	Ag coated with PVP[c]	0.01	–	27 [12]	32 [24]
Meat 2 (NanoLyse14)		0.05	–	26 [10]	83 [87]
AgNPs 1 (NanoLyse03)		0.02	42 ± 10 nm by TEM	30 [11]	47 [29]
AgNPs 2 (NanoLyse04)		0.1	42 ± 10 nm by TEM	32 [11]	163 [35]
Soup 1 (NanoLyse09)	Synthetic amorphous SiO_2 stabilized with NaOH	0.5	–	42 [24]	264 [493]
Soup 2 (NanoLyse10)		2	–	41 [21]	909 [987]
SAS 1 (NanoLyse01)		1	120 nm by SLS[d]	57 [40]	1361 [770]
SAS 2 (NanoLyse02)		4	120 nm by SLS[d]	60 [49]	5640 [951]
SAS COM	Synthetic amorphousSiO_2 (E551)	~2	–	53 [57]	1190 [463]
Soup COM		0.28[e]	–	57 [40]	305 [528]

[a] Interquartile range.

[b] Values for ENMs size and number of particles counted (per replicate – 1 EM grid) obtained by characterization with transmission electron microscopy (TEM) – AgNPs containing samples, and scanning electron microscopy (SEM) – SAS containing samples based on intermediate precision study data (for full size distribution and EM images see Supplementary data, Fig. A1),

[c] Polyvinylpyrrolidone.

[d] Static light scattering.

[e] Refers to powder, measured using ICP-MS Thermo Axiom instrument at Food and Environment Research Agency, UK.