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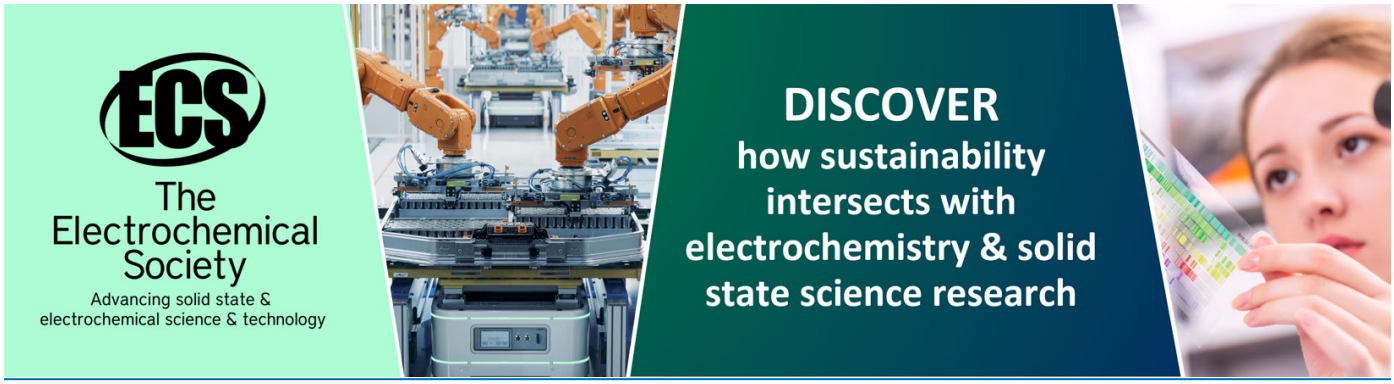

Sample preparation and EFTEM of Meat Samples for Nanoparticle Analysis in Food

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Abstract. Nanoparticles are used in industry for personal care products and the preparation of food. In the latter application, their functions include the prevention of microbes' growth, increase of the foods nutritional value and sensory quality. EU regulations require a risk assessment of the nanoparticles used in foods and food contact materials before the products can reach the market. However, availability of validated analytical methodologies for detection and characterisation of the nanoparticles in food hampers appropriate risk assessment. As part of a research on the evaluation of the methods for screening and quantification of Ag nanoparticles in meat we have tested a new TEM sample preparation alternative to resin embedding and cryo-sectioning. Energy filtered TEM analysis was applied to evaluate thickness and the uniformity of thin meat layers acquired at increasing input of the sample demonstrating that the protocols used ensured good stability under the electron beam, reliable sample concentration and reproducibility.

1. Introduction

The new emerging trend in the food industry exploits nanotechnology for versatile developments. One example is silver nanoparticles (NPs) used for food application. For their antimicrobial properties, silver nanoparticles (AgNPs) are used in food supplements and various food contact surfaces (e.g. packaging, cutting boards, cutlery)[1]. AgNPs are reported to be cytotoxic not only to bacterial but also human cells [2]. One of the reasons for this increased toxicity of AgNPs is associated with their size [2]. However, NPs size undergoes dynamic changes after spiking in complex matrices e.g. agglomeration/ aggregation, dissolution, deagglomeration [3-5]. Therefore The European Food Safety Authority emphasizes that for appropriate risk assessment of NPs in foods and feed, a particle size measurement in the hosting food products is necessary [6]. Of special interest among the methods allowing NP size measurement is electron microscopy (EM) coupled spectrometry methods. However, standard sample preparation protocol for solid food samples e.g. meat involves such methods as resin embedding or freezing and sectioning of frozen material [7]. Both methods are laborious, require sophisticated equipment and technical skills. Additionally the volume of the sample that can be

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analysed in EM at once is very limited, making the method only useful for samples containing very high numbers of particles. In this study we test an alternative sample preparation protocol based on sedimentation of homogenized and highly diluted samples onto TEM grids. This approach allows a reduction in sample preparation time down to 1-2 hours and does not require skills necessary for thin sectioning of frozen or embedded material. The samples are dried prior to analysis and therefore it is possible to increase the analysed sample volume when compared to traditional approaches. In this work we report stability of the sample in the electron beam and summarize data regarding the sample layer thickness and its uniformity at increasing sample concentration. These parameters have a crucial meaning for the size measurement and quantification of NPs in food samples by EM. The study demonstrates application of powerful tools, energy filtered transmission electron microscopy (EFTEM) and electron energy loss spectrometry (EELS) for the measurement of the meat sample thickness.

2. Material and methods

Meat emulsion was obtained using the equipment and the methodology as described in [8]. However, at the final stage, the cryo-milled meat was not frozen and no AgNPs were added. The material was preserved using Proclin 150^{TM} at a concentration of 1.1 g/ kg and aseptically packed in 50 ml amber vials. The density of the meat emulsion was 1.006 g/ ml. Aqueous dispersion of AgNPs stabilized with polyvinylpyrrolidone at concentration of 0.1% m/m and nominal size of 42±10 nm was originally obtained from Nanogap (Milladoiro, Spain). AgNPs were mixed with the meat emulsion in a 1:1 ratio using a potter type homogenizer until visual homogeneity of the resulting viscous liquid was obtained.

TEM samples were prepared by dilution, homogenization and subsequent sedimentation of the AgNPs/meat viscous liquid on formvar-carbon coated, 400 mesh Cu TEM grids (Agar Scientific, Stansted, UK). A borate buffer at pH 8.0 (0.05M H_3BO_3, 0.05M KCl, 0.004M NaOH) was used for the dilution step for three dilution factors, 200 fold m/m (Sample *A*), 500 fold m/m (Sample *B*) and 2000 fold m/m (Sample *C*) respectively. The diluted emulsions were dispersed in the buffer using potter type homogenizer and subsequently homogenized with ultrasonic probe (Misonix, USA) in vials for 1 min at 100 W. During homogenization, vials were kept cool by immersion in water with ice. Lastly, 0.5 g of sample at each dilution level were transferred to polyallomer centrifuge tubes and topped up with the buffer to the level of 2-3 mm from the tube rim. The tubes were equipped with solid Agar 100 resin fillers (Agar Scientific, Stansted, UK) at the bottom to ensure flat support for the TEM grids. Samples were sedimented on the TEM grids in Beckman XL-100 ultracentrifuge (Beckman, California, USA), equipped with SW40Ti rotor and operating at RCF=100.000 g at 20^{0}C for 1h. Characterization by EFTEM was undertaken using a JEM-2200 FS (JEOL Ltd., Tokyo, Japan) field emission TEM operating at 200 kV, equipped with an in-column Omega-type energy filter, and CEOS image and probe 3rd order aberration correctors. Sample thickness was measured using the Digital Micrograph (DM) (Gatan, Pleasanton, USA) EFTEM thickness map routine.

3. Results and discussion

Figure 1 shows typical sample thickness analysis for the three samples. Bright field images in Fig. 1a), 1b) and 1c) show nanoparticles embedded in the dried emulsions. t/λ maps in Fig. 1e), 1f) and 1g) (where t is the sample thickness and λ is the electron mean free path for inelastic scattering) were obtained through acquisition and application of the log-ratio method [9] of an unfiltered bright field (BF) image followed by an elastic image with 10eV energy slit centred onto the zero loss peak (512x512 pxl, 3s acquisition). AgNPs were found positioned at different heights within the sample regardless of the dilution applied, demonstrating that the sample preparation method provided layers of significant thickness. The sample drift during the map acquisition was evaluated and removed using AgNPs as a reference for image correlation. Figure 1d)-f) show t/λ maps respectively for sample *A*, *B* and *C*. Cross correlation results from each map acquisition show an average drift of 2.4pxl (Sample *A*), 1.6 pxl (Sample *B*) and 4 pxl (sample *C*) corresponding to 3.2nm (0.5% of the field of view), 2.2nm (0.3%) and 5.4nm (0.8%). This shows that during the images acquisition, and regardless of the

level of sample dilution, the samples were stable under the electron beam, *i.e.* no significant sample drift or shrinkage could be observed.

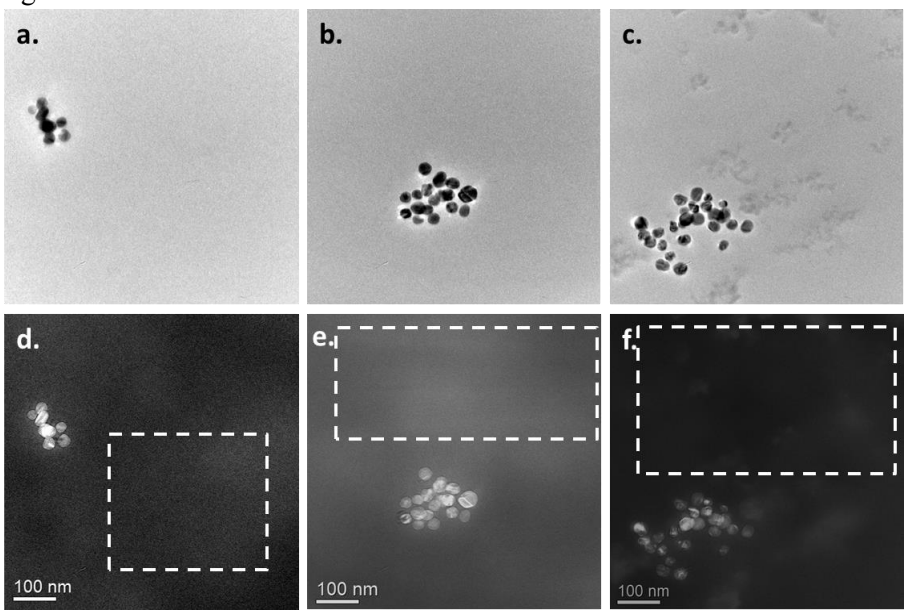

Figure 1. EFTEM analysis of *Ag NPs in 200 (a) and (d) (Sample A), 500 (b) and (e) (Sample B), and 2000 fold (c) and (f) (Sample C) with BF TEM images a.)-c.) and thickness maps d)-f);dashed boxes indicates typical areas of τ/λ mean values measurement .*

The sample thickness was calculated from t/λ measurements on particle free areas and the results are reported in Table 1. λ was estimated using the following equations:

$$\lambda = \frac{106\ F\ \frac{E_0}{E_m}}{\ln(2\ \beta\ \frac{E_0}{E_m})} \quad (1)\ [9]; \qquad E_m = 7.6\ Z_{eff}^{0.36} \quad (2)\ [10]; \qquad Z_{eff} = \frac{(\sum Z_i\ f_i)^{1.3}}{(\sum Z_i\ f_i)^{0.3}} \quad (3)\ [9],$$

where F is-a relativistic factor (0.618 for 200kV electrons), E_0 the incident beam energy, E_m the mean energy loss (calculated using eq.(2)). The effective atomic number Z_{eff} is calculated according to fractions (f) of the (i) elements in the sample of atomic number Z as described in equation (3).

Table 1. EFTEM measurements of t/λ and mean values of calculated thickness

Dilution factor	t/λ ± s.d.	t (nm) ± sd
(**A**) 1 in 199	1.75 ± 0.31	260 ± 46
(**B**) 1 in 499	0.79 ± 0.26	118 ± 39
(**C**) 1 in 1999	0.50 ± 0.10	74 ± 15

Standard deviations (s.d.) are given from measurements in 5 different areas per sample.

β indicates the collection aperture, in our case no aperture was used and a value of 15mrad was chosen to feed into eq.(1) according to [10]. The sample (+support) composition was estimated by EELS spectroscopy (see Figure2a). A spectrum was acquired within a region comprising the main elements present in the meat (C, O, N) sample. Quantification was done using calculated cross sections in DM quantification routine producing the following elemental composition: C 88at%, N 7at% and O 5at%. λ value was estimated to be 149nm.

The mean sample thickness (see Table 1) varies with the sample dilution and increases with decreasing dilution factor. The measurements over 5 areas across the TEM grid point out good homogeneity of the sample thickness over the grid, potentially allowing quantification of homogenously distributed NPs in meat emulsion. However, the calculated sample thickness included the thickness of the formvar-carbon film initially present on the TEM grid. To estimate the film's thickness we used a linear fit of the measured sample+support thickness versus the quantity of sample applied in milligrams (see Figure 2b). The support thickness can be evaluated at

the intercept on the x-axis (no sample applied) of the fitting function, which corresponds to a value of ~ 40 nm. This value is in good agreement with the value provided by the manufacturer: 30-40 nm of formvar and ~10 nm of carbon. Therefore the thickness for sample A, B and C is respectively about 220, 80, and 30nm. We have calculated that same samples in the hydrated state (based on meat emulsion density) would create 34±9 times thicker layer on the TEM grid. This figure also defines the level of the sample pre-concentration compared to the cryo- and resin embedding preparations where the volume changes in comparison to hydrated sample are minimal.

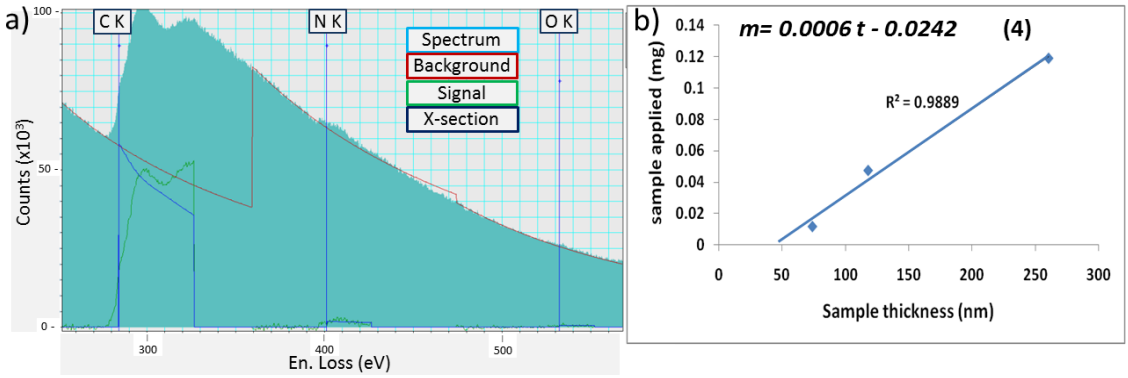

Figure 2. a) *Quantified EELS spectrum showing C, O and N K-edges. Red, green and blue lines represent background, net signal, and calculated cross section for quantification. b) Linear fit of sample applied versus sample thickness with linear fit equation (4) inset .*

4. Conclusions

In this paper we have evaluated a new sample preparation approach for potential quantification and measurement of NPs in food. EFTEM and EELS were successfully applied to measure the thickness of meat sample containing AgNPs. Using the described sample preparation protocol, we were able to obtain meat emulsion layers where the weight input of meat emulsion was linearly related to the TEM sample thickness and allowed to predict the support thickness for null input. This protocol provides a quicker and easier sample preparation method with respect to traditionally used solutions, such as resin embedding and cryo-sectioning, and allows increasing the amount of sample volume analysed.

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