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A METHOD FOR PRESERVATION AND DETERMINATION OF WELDING FUME NANOPARTICLES IN EXHALED BREATH CONDENSATE

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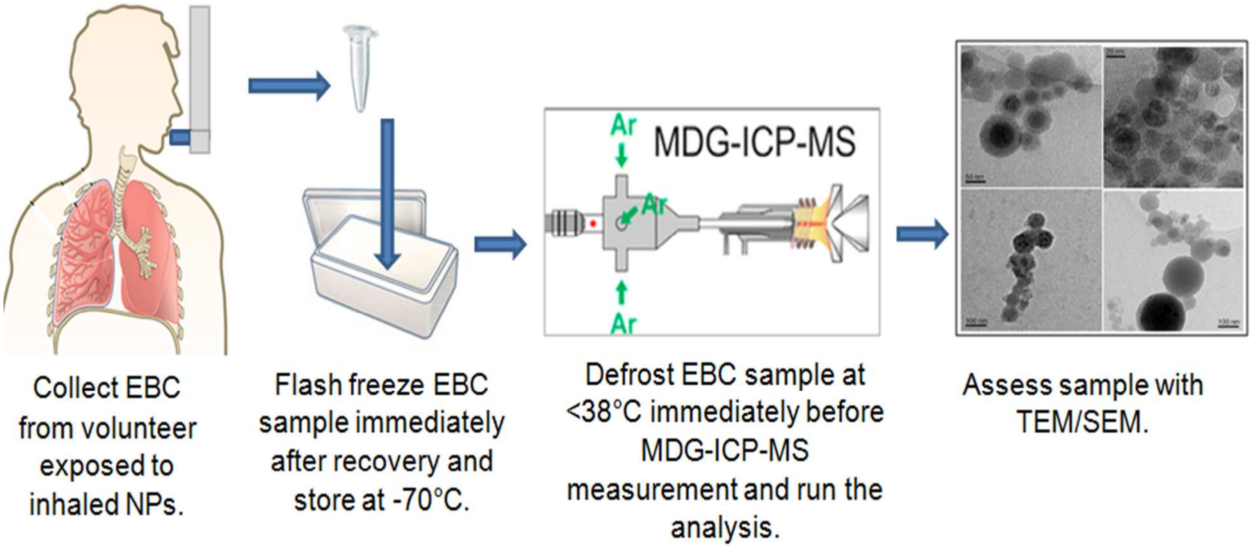
ABSTRACT

Analysis of exhaled breath condensate (EBC) represents a non-invasive method for detecting inhaled nanoparticles (<100nm; NP) associated with various occupational and environmental exposures. However, the few studies that have investigated inhaled NPs in EBC often assess only bulk, ionic intensities to provide information on overall elemental content, rather than on particulate content. In an attempt to assess inhaled metallic particles in their original particulate form, we developed a methodology for the preservation and determination of inhaled welding fume NPs in EBC. Two EBC preservation strategies were tested: either flash freezing EBC immediately after collection, or keeping EBC at room temperature until analysis. Particle content of the differently preserved samples was assessed by Microdroplet Generation Inductively Coupled Plasma Mass Spectrometry (MDG-ICP-MS), and with Electron Microscopy. We foun

- Welding fume NPs in EBC may quickly and uncontrollably degrade, thereby losing their original form and hampering effective characterization analysis.
- We demonstrate the importance of flash freezing EBC samples immediately after collection and defrosting them shortly before analysis at a temperature that does not affect proteins and peptides (<38°C) in order to effectively preserve NPs in particulate form.
- This methodology can be applied easily, effectively, and inexpensively to preserve EBC samples for future NP content determination and characterization.

GRAPHICAL ABSTRACT



Collect EBC from volunteer exposed to inhaled NPs.

Flash freeze EBC sample immediately after recovery and store at -70°C.

Defrost EBC sample at <38°C immediately before MDG-ICP-MS measurement and run the analysis.

Assess sample with TEM/SEM.

INTRODUCTION

Analysis of exhaled breath condensate (EBC), which contains many different constituents such as ions, molecules and proteins [1], represents a non-invasive method for studying the composition of epithelial lining fluid. Collection of EBC has been proposed as a method for assessing biomarkers of various lung diseases, e.g. inflammation [1,2] and can provide information on nanoparticle (NP) exposure directly from the target side. EBC has been previously used a matrix for the detection of toxic metals and trace elements associated with various occupational and environmental exposures such as welding fumes [3,4], smoking fumes [5,6] and other pollutant exposures [7]. Few studies have specifically investigated inhaled metals and their content from occupational exposures in EBC; however, it is important to note that these studies have only assessed bulk intensities to provide information on overall metal content in EBC [4,8].

Welding fumes contain high concentrations of metallic particles that can occur at the nanoscale, depending on the specific type of welding process [9]. Particularly, Tungsten Inert Gas (TIG) welding processes has been shown to generate primary particles with the majority below 100 nm [10]. Assessing and characterizing the inhaled metals in their inherent NP form is of particular interest, especially when assessing health relevant and toxicological questions such as inhaled particle translocation to systemic circulation. It has already been published that EBC of welders may contain Mn, Fe, Cr, Ni depending on the welding technique used [4,11,12]. However, current research in this field has yet to investigate and characterize the NP form of these inhaled metal fume exposures.

Recently, Inductively Coupled Plasma Mass Spectrometry (ICP-MS) was proposed as a fast and sensitive method for the analysis of liquid NP suspensions. Mainly, two different approaches are used: single particle (sp)-ICP-MS which is based on conventional nebulization of a highly diluted NP suspension and introducing the produced polydisperse aerosol through a spray chamber into the ICP [13,14,15,16]; and microdroplet generation (MDG)-ICP-MS which is based on a piezoelectrically driven dispenser head generating monodisperse droplets with a distinct frequency, transporting the droplets in a gas stream through an adapter into the ICP and which allows for close to 100% transport efficiency (TE) [17,18,19,20]. Both methods enable the quantification of the ionic and the particulate fraction, however, sp-ICP-MS is dependent on NP reference material which is currently not available for most NPs and matrices [16] whereas MDG-ICP-MS provide the possibility of directly quantify ionic and/or particulate fraction by internal or external calibration [18]. Furthermore, MDG-ICP-MS require only little sample amount and due to the small single droplets introduced into the ICP, matrices will not alter the ICP conditions tremendously.

In this communication we will show the first analysis of EBC obtained from a welder performed by MDG-ICP-MS determining the signals of various metals. We will compare the obtained results for flash frozen EBC and EBC stored at room temperature, and complement the results with electron microscopy analysis. Problems, limitations and prospective directions in the field of analytical chemistry and occupational exposure assessment related to this preservation methodology will be discussed.

MATERIAL AND METHODS

INSTRUMENTATION

As part of a larger occupational exposure study, EBC was collected from a healthy, adult male volunteer who conducted four hours of TIG welding as part of his occupational tasks. The study was approved by the Ethics Committee of the Canton de Vaud (protocol 389/13). An RTube (Respiratory Research, Atlanta, USA) was used to collect EBC for 10 minutes following the tested procedure described in Riediker et al. [21]. The volunteer was asked to rinse out his mouth with water prior to collection to avoid contamination. The EBC was extracted from the RTube and split into two 0.5 mL samples sealed in Eppendorf tubes. One Eppendorf tube was placed immediately in dry ice to induce flash-freezing and the second was kept at room temperature for approximately 12 hours. For comparison, an EBC sample was collected from a healthy, adult male volunteer who was not exposed to welding fumes, and this sample served as a control.

Shortly before measurement, the frozen sample was defrosted and both samples and blank were transferred into a 4 mL HDPE vial, connected consecutively to a microdroplet dispenser head (Microdrop Technologies GmBh, Norderstedt, Germany) and the isotopes of interest where measured for 3 min each on a Sector Field (SF)-ICP-MS (Element 2, ThermoFisher, Bremen, Germany) according to the experimental procedure described earlier [17,18]. The sequence was repeated three times. The ICP-MS and dispenser settings used for the experiment are summarized in Table 1 and Table 2. Sodium, which is highly present in EBC, was monitored for dispenser stability and transport efficiency (TE).

MICROSCOPY

Approximately 1 µL of each sample was dried on a Si wafer for scanning electron microscopy (SEM-EDX, FEI Quanta 200 FEG) or on a Copper grid for transmission electron microscopy (TEM, FEI Tecnai F30 FEG) measurements.

EVALUATION

For determination of the limits of detection (LOD) for each measured isotope, mean and standard deviation was calculated. The mean of the blank signals was used for background correction of the intensities obtained from the samples.

For the samples containing a particulate and/ or an ionic fraction of the analyzed elements, it is important to apply a so called “split correction” which is necessary using integration times smaller than 10 ms. This correction was applied using a custom written macro in MATLAB (R2012a, MathWorks, Natick, USA), afterwards the mean of the background was subtracted from each signal. The obtained intensities were plotted as intensity distribution in Origin 8.6 (Origin 8.6, OriginLab Corporation, Northampton, USA) [18].

Intensity distributions of the ionic fraction were fitted with a Gauss function and the particulate fraction with a LogNormal function [13]. Mean and standard deviation were calculated from the fitted functions.

RESULTS AND DISCUSSION

MDG-ICP-MS

For all expected elements, signals could be detected in both differently stored EBC samples of the exposed welder, whereas the sample from the non-exposed volunteer shows only background signals. The blanks were used for calculating the limits of detection (LOD).

The LOD for each isotope was calculated using the following formula:

$$LOD = \overline{x_{blank}} + 3 \times \sigma_{blank}$$

where $\overline{x_{blank}}$ is the mean of the background signal and σ_{blank} its standard deviation. Results for the measured isotopes are summarized in Table 3.

For the differently stored samples, however, clear differences were observed for Al and Ni. Droplets produced from the unfrozen sample contained the two elements homogeneously distributed therefore revealing ionic form. The RSD of the intensity distribution is relatively small (16% for Al and 22% for Ni) which is similar to ionic solution aspirated and introduced via the MDG into the ICP, whereas the RSD of the intensity distribution corresponding to particles is normally much larger (>30%) [13,17,18,19]. Additionally, establishing a stable dispensing and 100% transport efficiency, each droplet should contain a similar amount of ions, while particles should be less frequently detected when assuming $<10^6$ particles mL^{-1}. Additionally, their intensities produced are more irregular pattern due to the size dependence.

The sample stored frozen has similar Al and Ni background intensities as the unfrozen sample; however, there was no background corrected distribution detectable. There are only few signals at higher intensities which are attributed to large Al or Ni particles. Few signals with higher intensities were found for the unfrozen sample as well, which indicates that not all particles were dissolved. However, the high ionic content detected allows the assumption that the longer the particles are stored in a liquid medium such as EBC, the more likely they tend to degrade.

The other elements (Fe, Mn and Sn) are present as particles in samples stored by both of the different strategies. Their intensities are above the LODs and the intensity distributions appear lognormal which is typical for polydisperse NPs (see Figure 1 and Figure 2). Nevertheless, the distributions are very broad (relative standard deviation >50%). An explanation might be that the particles are very polydisperse and their sizes spread over a wide range, or the concentration is too high to have only one particle per droplet and the intensity distributions for one particle per droplet is interfered with the distributions for two or more particles per droplet.

Comparing signal intensities (Table 4) the flash frozen sample demonstrated higher intensities for Fe and Sn, whereas the sample kept at room temperature showed higher intensity for Mn. Due to the fact that the intensity is proportional to the mass of the analyte introduced into the mass analyzer during a certain time, it is most likely that also Fe and Sn particles degrade relatively slowly within EBC stored at room temperature. It is probable that Mn particles agglomerate over time and therefore the mean intensity is shifted to higher intensity. As only few particles were found by TEM and SEM for the room temperature sample, no definitive statement about the different intensities can be given. This will require further experiments and more detailed evaluation of the samples fate over time when stored at room temperature.

MICROSCOPY

A "blank" control EBC sample, collected from a volunteer not exposed to welding fumes and kept frozen showed similar TEM images (Figure 3) to the exposed sample stored at room temperature. This finding directly supports the conclusions of the MDG-ICP-MS measurements, in that welding NPs stored in EBC at room temperature are more likely to degrade than those stored in frozen EBC.

Almost no particles could be found by TEM analysis in the sample kept at room temperature (Figure 4), and only few particles were found with SEM corresponding mainly to Fe or Mn. Conversely, the TEM images of the flash frozen sample demonstrated a very high concentration of NPs (Figure 5). Most of the particles have a spherical shape and seem to agglomerate. Some appear to be core shell particles while others seem to be composed of different elements. In all micrograph images, a high amount of organic matter is visible which also contains high amounts of salts. It is possible that the salts present may form crystals or particles influencing the detection of the particles produced by the welding process.

CONCLUSIONS

This study provides the first results of EBC analysis performed by MDG-ICP-MS. The use of EBC as a biological matrix for analysis of inhaled particle exposure has been increasingly applied in the medical and occupational safety and health fields. With the recent progress in NP characterization studies, it has been recognized that understanding and predicting NP behavior strongly depends on the methods of preparation and preservation until analysis. MDG-ICP-MS is capable of determining the signals of various metals and does additionally allow for distinguishing between ionic and particulate matter.

Preliminary findings by MDG-ICP-MS support the assumption that some of the particles generated by TIG welding, at least those which are composed of Ni and Al, undergo degradation in EBC within a few hours when kept at room temperature. Signals were detected for all expected elements Al, Ni, Mn, Fe and Sn. The sample kept at room temperature contained dissolved Al and Ni, whereas the other elements appear to be in particulate form.

Supplementary, the MDG-ICP-MS measurements are supported by TEM and SEM images. For the unfrozen sample, SEM and TEM images do not show a significant amount of particles, and consist mainly of organic matter. Nevertheless, the flash frozen sample contains a significant number of spherical particles, in which some appear as core shell particles that seem to agglomerate.

Most droplets generated by the MDG produced a signal above LOD. Therefore, it will be necessary for further investigations to dilute the original EBC shortly before measurements to reduce the particle number concentration and minimize the probability of coincidence of two or more NPs per droplet. Phosphate buffered saline (PBS) may serve as an effective liquid, providing a pH similar to body fluids and therefore minimizing the possibility that peptides or proteins degenerate. Additionally, it must be guaranteed that particle appearance (shape, agglomeration, dissolution) is not influenced.

Quantification of NPs contained in EBC might be possible by internal calibration or matrix matched external calibration [18]. However, it will be necessary to find a suitable reference material. Standard solutions which are used in liquid ICP-MS are mainly stabilized in acid which might alter the particles, e.g. by dissolution or agglomeration as well as proteins, e.g. by denaturation.

Further studies might also be conducted on a Time-of-Flight (TOF)-ICP-MS which allows much higher time resolution as well as multi-elemental capabilities [19].

This study reveals the necessity of careful investigation of ongoing processes in a biological matrix such as EBC when dealing with particles and other substances, which may be unstable or sensitive to parameters present, and may therefore change their appearance rapidly. For particle detection in EBC, the results of this investigation demonstrate the importance of flash freezing EBC samples immediately after collection and defrosting them shortly before analysis at a temperature that does not affect proteins and peptides (<38°C) in order to minimize degradation or alteration of present particles. This storage technique can be applied easily, effectively, and inexpensively to preserve EBC samples, particularly those collected in a field setting for future NP content determination and characterization.

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TABLES:

Table 1: ICP-MS settings.

RF Power	1280	W
He at Dispenser	0.4	L/min
Ar at Adapter	1.004	L/min
Auxiliary Gas Flow	0.81	L/min
Plasma Gas Flow	16	L/min
Measured Isotopes	(^{23}Na), ^{27}Al, ^{55}Mn, ^{57}Fe, ^{58}Ni, ^{120}Sn	
Dwell Time	1	ms
Samples/Peak	1000	
Integration Window	10%	
Resolution	Low	

Table 2: Dispenser settings.

Dispenser Frequency	100	Hz
Droplet Diameter	~40	µm
Droplet Volume	~34	pL

Table 3: Limits of detection for the determined isotopes in the sample kept at room temperature or frozen.

	^{27}Al	**^{55}Mn**	**^{57}Fe**	**^{58}Ni**	**^{120}Sn**
LOD RT [counts]	660	13	13	33	12
LOD frozen [counts]	710	13	13	47	12

Table 4: Summarized intensities for the determined isotopes in the sample kept at room temperature and the flash frozen sample.

	^{27}Al	**^{55}Mn**	**^{57}Fe**	**^{58}Ni**	**^{120}Sn**
Signal RT [counts]	420±70	670±380	45±36	62±14	46±62
Signal frozen [counts]	-	320±350	75±41	-	100±320

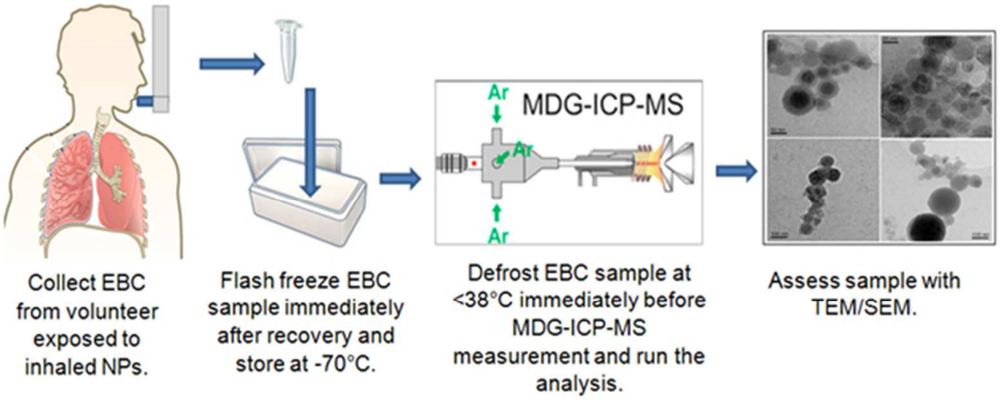

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FIGURE LEGENDS:

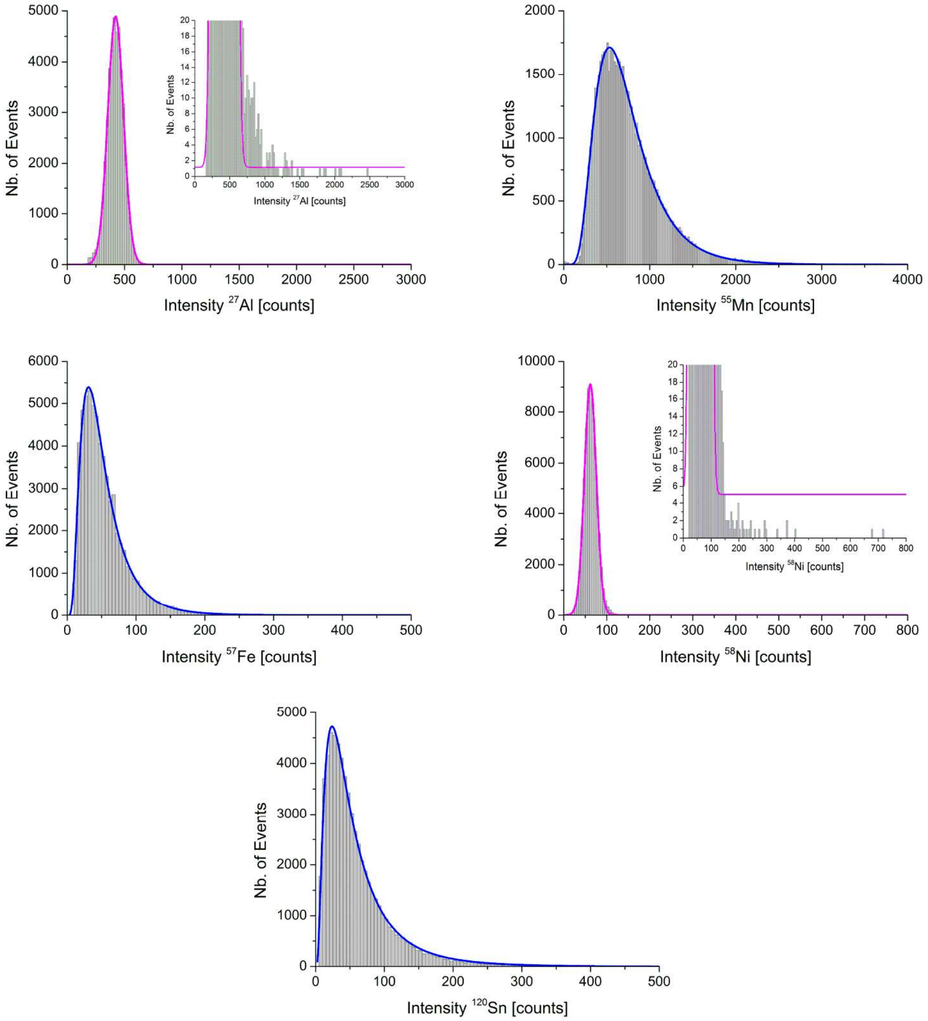
Figure 1: Signal distributions for the isotopes present in the sample kept at room temperature; the solid line represents a LogNormal fit, the dashed line a Gauss fit.

Figure 2: Signal distributions for the isotopes present in the sample flash frozen immediately after collection; the solid line represents a LogNormal fit.

Figure 3: TEM images of an EBC sample obtained from a volunteer not exposed to welding fumes (control).

Figure 4: TEM images of the sample kept at room temperature. Mainly organic material is visible in the TEM.

Figure 5: TEM images of the flash frozen EBC sample. A lot of particles are visible, most appear spherical and some seem to be core shell particles. The SEM images (bottom) also show many spherical particles, which were found to be Ni.



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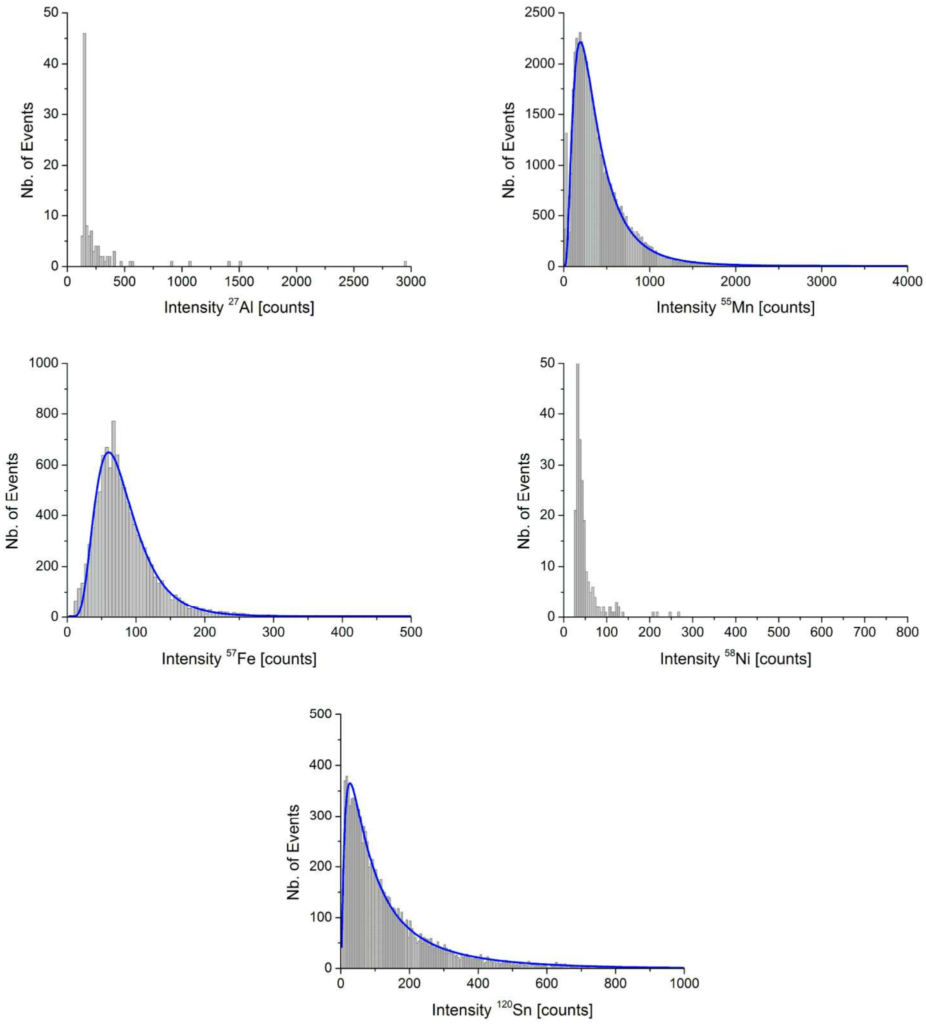

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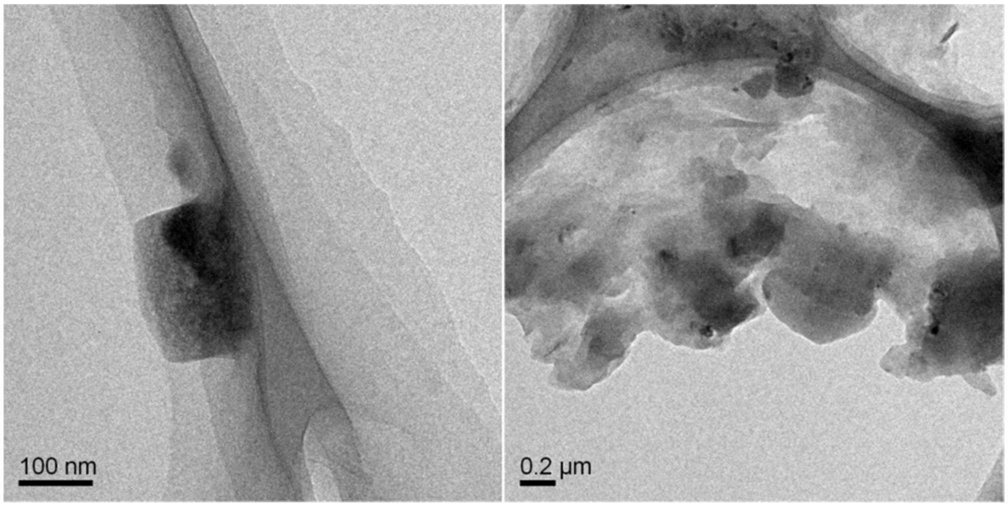

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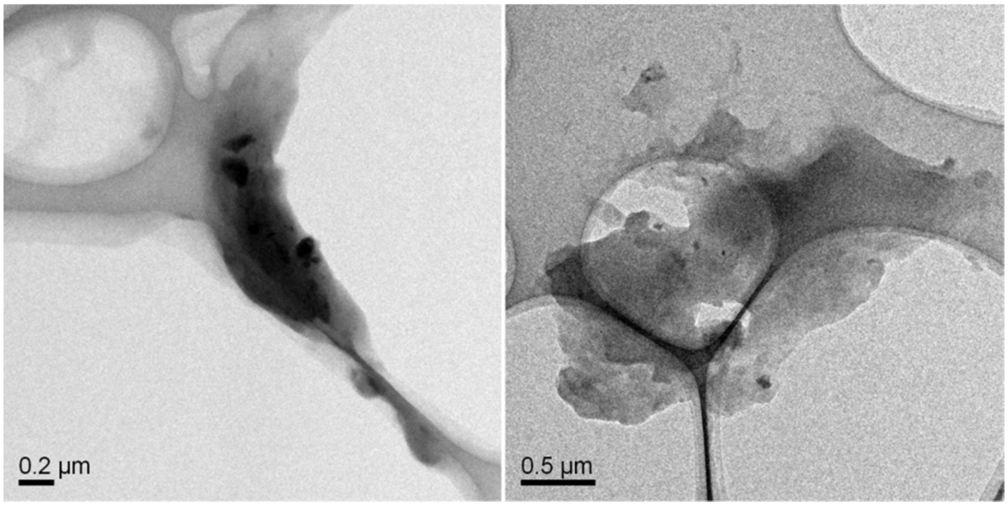

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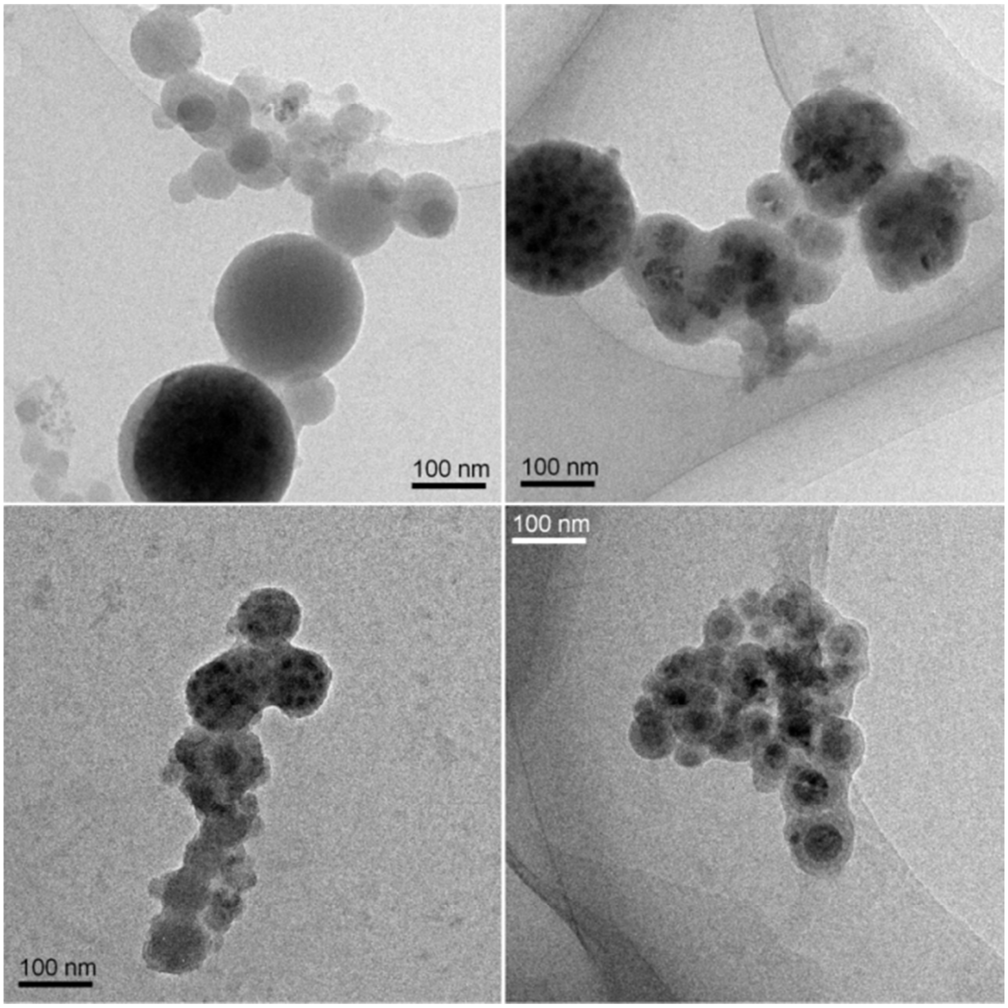

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