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Review

Analytical approaches for characterizing and quantifying engineered nanoparticles in biological matrices from an (eco)toxicological perspective: old challenges, new methods and techniques

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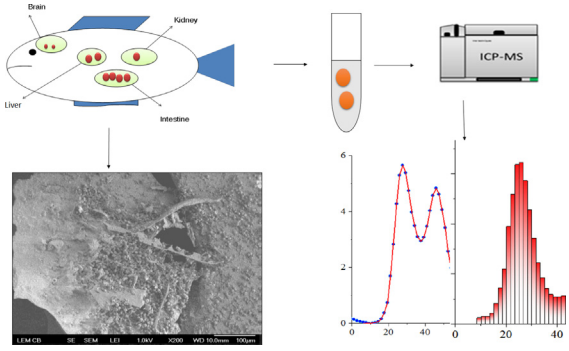
HIGHLIGHTS

- Technological developments enabling detection at nano scale are accelerating.
- Nonetheless, tracking single particles within living organisms remains challenging.
- No single approach or technique can quantify internalized engineered nanomaterials.
- Combined imaging and chemical detection techniques can distinguish particles.
- A decision scheme and analytical toolbox for examining ENP toxicity are proposed.

GRAPHICAL ABSTRACT



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ABSTRACT

To promote the safer by design strategy and assess environmental risks of engineered nanoparticles (ENPs), it is essential to understand the fate of ENPs within organisms. This understanding in living organisms is limited by challenges in characterizing and quantifying ENPs in biological media. Relevant literature in this area is scattered across research from the past decade or so, and it consists mostly of medically oriented studies. This review first introduces those modern techniques and methods that can be used to extract, characterize, and quantify ENPs in biological matrices for (eco)toxicological purposes. It then summarizes recent research developments within those areas most relevant to the context and field that are the subject of this review paper. These comprise numerous *in-situ* techniques and some *ex-situ* techniques. The former group includes techniques allowing to observe specimens in their natural hydrated state (*e.g.*, scanning electron microscopy working in cryo mode and high-pressure freezing) and microscopy equipped with elemental microanalysis (*e.g.*, energy-dispersive X-ray spectroscopy); two-photon laser and coherent anti-Stokes Raman scattering microscopy; absorption-edge synchrotron X-ray computed microtomography; and laser ablation-inductively coupled plasma mass spectrometry (LA-ICP-MS). The latter group includes asymmetric flow field flow fractionation coupled with ICP-MS and single particle-ICP-MS. Our review found that most of the evidence gathered for ENPs actually focused on a few metal-based ENPs and carbon nanotube and points to total mass concentration but no other particles properties, such as

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size and number. Based on the obtained knowledge, we developed and presented a decision scheme and analytical toolbox to help orient scientists toward selecting appropriate ways for investigating the (eco)toxicity of ENPs that are consistent with their properties.

Contents

1. Introduction

Ever-increasing global production of engineered nanoparticles (ENPs) inevitably results in growing release into the environment of these materials and their subsequent uptake by organisms. Learning the fate of ENPs within organisms is of mounting importance for obtaining new insights about the ambiguous endpoints and complex functions of ENPs within tissues or cells (Holbrook et al., 2008). Such knowledge of ENPs facilitates developing toxicokinetic and toxicodynamic models for determining dose-response relationships and understanding relevant adverse outcome pathways. To date, the fate of ENPs within organisms is poorly understood in terms of, for example, biodistribution, localization, and accumulation. Most investigations concerning the presence of ENPs in biological media are carried out in medically oriented studies wherein the distribution is screened using a high concentration of functionalized and/or fluorescence-labelled ENPs (Urban et al., 2016; Holt et al., 2011). Modified ENPs cannot, however, represent the behavior of bare (or unmodified) ENPs within organisms (Abdolahpur Monikh et al., 2018). Consequently, the medically oriented data cannot provide sufficient insights as to how the environmentally functionalized ENPs that are typically present at low particle concentrations behave in biological systems.

Many traditional methods and techniques based on, for example, electron microscopy (EM) or gas/liquid chromatography (GC/LC) coupled with mass spectrometry (MS) and light scattering (LS) have been used for characterizing and quantifying ENPs in complex matrices (von der Kammer et al., 2012). Each technique, however, comes with limitations when it is used to investigate ENPs in biological media for (eco)toxicological purposes due to the complex and polydisperse matrices typically encountered there (Urban et al., 2016; Laux et al., 2018). Furthermore, direct application of these techniques is challenging due to the typically low particle concentrations and transformation of ENPs taken up by organisms. Most of the data obtained by the analytical techniques commonly in use are mass-based concentrations and disregard such of the particles' properties as size and number. The suitability of mass as the dose metric for investigating the toxicity of ENPs is a cross-cutting concern in nano(eco)toxicology (Hua et al., 2016), because ENPs may exhibit specific toxicity stemming from their particle number or volume-specific surface areas (Oberdörster et al., 2005).

Recently a number of analytical methods and techniques have emerged to characterize and quantify ENPs in complex matrices for various purposes in relation to, for example, food (Wagner et al., 2015), cosmetic products (Philippe et al., 2018), and soil (Molnar et al., 2014). The techniques are scarcely used by the (eco)toxicological community, however, due to their intricacy and the complex theories upon which the techniques are based (Urban et al., 2016). In this review, we evaluate the methods and techniques available for quantifying ENPs in living organisms and we assess their suitability for (eco)toxicological purposes. This paper does not repeat what has been reported in previous review papers (Urban et al., 2016; von der Kammer et al., 2012; Laux et al., 2018; Petersen et al., 2016; Drobne et al., 2018) but focuses on emerging methods and techniques while updating the information in terms of what is relevant to nano(eco)toxicology. Available information for each method and technique was collected and we discuss the selected methods to provide a general overview of the methods and their applicability. In case of cryo-EM we faced limitations in obtaining valuable information to proposed methods, thus we had to design a case study as a concept of proof to provide the required information.

2. Methods used to collect information

Using Web of Science, we searched the literature for peer-reviewed papers on characterization and quantification of ENPs in complex matrices. Inclusion factors limited the result to peer-reviewed papers published in the period 2007–2018. Excluded were medically oriented studies. To facilitate the selection of methods and techniques, we focused on the most promising and widely used techniques for characterizing and quantifying ENPs in complex matrices while considering the strengths and limitations of each. The collected papers were analyzed to differentiate the studies based on *in-situ versus ex-situ* techniques.

An overview of the *in-situ* analytical techniques is provided in Section 3, and *ex-situ* techniques are the subject of Section 4. Section 5

covers methods that have been developed to extract ENPs from biological media. In Section 6, we propose a decision scheme and analytical toolbox to facilitate selection of the most suitable methods and techniques for extracting, characterizing, and quantifying engineered nanomaterials in biological media for (eco)toxicological purposes. Overall, the results of this review also serve as a reference to guide scientists working in the area of nano(eco)toxicology through the selection of an appropriate method and technique given the type and property of ENPs with which they are dealing.

3. *In-situ* analytical techniques for characterizing and quantifying ENPs in biological media

In-situ assessment is becoming more and more important in ENPs characterization, because it requires no specific sample preparation that can cause artifacts (Urban et al., 2016). For example, sample handling may result in particles loss or cause changes in the particles' aggregation state. The techniques that were evaluated in detail can be divided into imaging and spectrometer techniques. Transmission EM (TEM) and scanning EM (SEM) are widely used techniques for *in-situ* characterization and quantification of ENPs in complex matrices. SEM with energy-dispersive X-ray spectroscopy (SEM-EDX) even facilitates investigation of the localization and speciation of ENPs down to the cellular level (Drobne et al., 2018). For determining intracellularly localized metallic ENPs, TEM-EDX and synchrotron-based techniques are applicable (Szakal et al., 2014). Darkfield microscopy combined with a spectrometer has been used to observe plasmonic nanoparticles (Fan et al., 2012). Absorption-edge synchrotron X-ray computed microtomography (SXCMT) even allows investigating materials in a dynamic manner (Molnar et al., 2014). Inductively coupled plasma mass spectrometry (ICP-MS) can also be used as an *in-situ* technique to quantify ENPs. As a multi-element detection technique, ICP-MS has the ability to quantify elements in liquids below the μg/L range (Vanhaecke, 2002). Coupling ICP-MS with other techniques, such as laser ablation (LA), has opened new avenues for *in-situ* quantification of metallic ENPs in solid samples down to cellular levels. The capabilities of these techniques are briefly explored in this section.

3.1. Electron microscopy

It is known that EM is a promising way to visualize ENPs within cells at high resolutions. Several specimen preparation steps, such as chemical fixation, dehydration at room temperature, and drying are potential sources of artifacts (*e.g.*, distortion of the cell structures, extraction or relocation of ENPs) (Milne et al., 2013). Freezing the samples at a slow cooling rate can also distort the structure of cells (Milne et al., 2013), and this may lead to the same artifacts by way of removing or relocating ENPs within cells and tissues. High-pressure freezing (HPF) is a well-known approach that freezes samples in milliseconds without significantly damaging cells up to about 200–300 μm in thickness (McDonald, 2009), and thus it allows investigating ENPs localized within intact cells and tissues. To date, application of HPF followed by TEM and SEM to visualize ENPs in biological samples is scarcely reported in the literature.

3.2. Electron microscopy-energy-dispersive X-ray spectroscopy

Energy-dispersive X-ray spectroscopy (EDX) microanalysis is used for identifying the elemental composition of a specimen, and it provides high resolution at micrometer or even nanometer scale (Drobne et al., 2018). Combinations of EDX with EM, such as SEM-EDX and TEM-EDX, have been applied successfully to differentiate trace elements in complex matrices (Brodowski et al., 2005). SEM-EDX has been used to investigate the presence of elemental calcium (Ca) on a manganese dioxide (MnO_2) surface, thereby confirming the occurrence of reactions between Ca^{2+} and ≡Mn—OH (Liu et al., 2009). The technique also has been suggested to be a powerful tool for assessing ENPs in biological media (Zvyagin et al., 2008). It has been used for intracellular localization of metal-based ENPs (Yang et al., 2014; Pascual García et al., 2014). TEM-EDX has been used successfully, for example to verify uptake of silver (Ag) ENPs at the intracellular level in *Caenorhabditis elegans* (Yang et al., 2014). The presence of Ag ENPs inside the cell membrane of a marine diatom has been demonstrated and visualized using SEM-EDX (Pascual García et al., 2014). A protocol has been developed to apply SEM-EDX for metallic ENPs present in biological media (Zheng et al., 2011). The distribution of zinc oxide (ZnO) in excised and *in vivo* human skin has been investigated using SEM-EDX to determine the level to which nanoparticles had penetrated into subdermal layers of the skin (Zvyagin et al., 2008).

EM-EDX is surface sensitive, however, and allows only a very small section of the samples under observation to be analyzed. This make the technique time-consuming and labor-intensive, especially when one is looking at low concentrations of accumulated ENPs in organisms. The technique provides no information about the size and aggregation state of the particles.

3.3. Dye-free techniques: CARS and two-photon laser

The drawbacks of labeling methods are evident, as labeling is time-consuming and dyes may bleach over time. Furthermore, the labels may lose intensity and alter the sample. Dyes often cause phototoxicity, harm the specimen, and consequently influence the result of the experiment. Coherent anti-Stokes Raman scattering (CARS) microscopy is a dye-free method that images structures by displaying the characteristic intrinsic vibrational contrast of their molecules. Moger et al. made use of this type of imaging techniques to detect such metallic ENPs as cerium dioxide (CeO_2) ENPs, TiO_2 ENPs, and zinc oxide (ZnO) ENPs in fish (rainbow trout) gills (Moger et al., 2008). These authors showed that CARS microscopy provides an excellent label-free contrast of metal oxide nanoparticles deep within a biological structure. Although individual particles could not be detected, localization at the cellular scale was provided.

The two-photon laser technique is used for long-term and three-dimensional (3D) tracking of metallic ENPs in living cells with nanometer resolution (Van Den Broek et al., 2013), and its main benefit is that it is a dye-free technique. By rapid multifocal scanning, the advantages of 3D molecular tracking methods using wide-field imaging are combined with the advantages of two-photon microscopy. In a previous study, gold (Au) nanorods had been tracked in cells with a precision of 4 nm in the xy-plane and 8 nm in the z-direction (Van Den Broek et al., 2013). Individual nanorods in living U2OS cells could be followed in three dimensions for >30 min with a photon noise limited accuracy and time resolution of 50 ms in 2D and 500 ms in 3D. In a follow-up study, a concept of proof on the accuracy of detecting binding events using 3D single particle tracking was reported by Carozza et al. (2017) Van Pomeren et al. (under review) used several imaging techniques to track Au NPs within living zebrafish larvae. The two-photon laser set up as used by Carozza et al. could follow agglomerated Au NP within the body of a 5 days old zebrafish larvae (Carozza et al., 2017). The technique enabled qualitative detection of agglomerated as well as single Au NPs and their biodistribution dynamics. These techniques are now being developed further to allow for quantitative assessment as well as for detecting ENPs based on their internal size distributions.

3.4. Dark field spectroscopy

Dark-field microscopy is a well-known tool used for detecting and characterizing nanostructures (Mock et al., 2002). Coupling dark-field microscopy with a spectrometer was reported to offer a high signal to noise ratio and a high sensitivity. The techniques operate based on illumination of the samples using an incident beam. The scattered radiation from the nanoscale objects is collected by a microscope objective and

analyzed in a spectrometer. The main advantage of dark-field spectroscopy is that unstained samples can be observed due to a mismatch in the refractive index which generates image contrast, although staining with an appropriate color is often required (Kawano et al., 2017). In 2017, Kawano et al. (2017) published a method for improving the dark-field imaging by adding three-color LEDs. This modification showed to dramatically improve the capability of the system. Specifically, it enables users to control the wavelength of light. This is useful for fluorescence excitation, whilst avoiding cell damage.

By using this technique one can, in a short time span, investigate ENPs and their properties such as size, shape and composition at the single-particle level (Liu et al., 2014). The technique has been developed for the observation of plasmonic nanoparticles and is widely used to investigate ENPs *e.g.* metallic (Bashevoy et al., 2007) and carbon-based ENPs (Lefebvre, 2016). It has also been reported that the technique can be used to investigate ENPs in complex matrices of environmental samples (Badireddy et al., 2012). However, dark-field spectroscopy is more applicable for surface plasmonic nanoparticles (Dong et al., 2018).

3.5. Laser ablation-inductively coupled plasma mass spectrometry

Combination of ICP-MS with an LA system is applicable for direct elemental analysis of solid samples (Limbeck et al., 2015). The technique has been used to quantify at single-cell level such ENPs as Au ENPs, Ag ENPs (Drescher et al., 2012), silicon dioxide (SiO_2) ENPs (Drescher et al., 2014), TiO_2 ENPs (Hsiao et al., 2016), and aluminum oxide (Al_2O_3) ENPs (Böhme et al., 2014). Want et al. quantified Au ENPs *in-situ* in a mouse leukemic monocyte macrophage cell line (Wang et al., 2014). Büchner et al. used the technique to quantify Au EMPs and their aggregates inside a 3T3 fibroblast cells ultrastructure (Büchner et al., 2014). These authors successfully showed that the Au ENPs and their aggregates accumulate in the perinuclear regions of the cells.

On the other hand, the technique is destructive and still has a low resolution (10 μm) (Wang et al., 2010), which makes it challenging to use for investigations at tissue and organism levels. Progress has been reported in obtaining improved resolution, and the technique has been tested for biological samples in recent years (Van Malderen et al., 2017). For example, it was used to observe the effects of ZnO ENPs and dissolved Zn(II) on zebrafish embryos (Brun et al., 2014). In this study, the technique was utilized to demonstrate that the uptake and tissue distribution of released Zn(II) ions were identical for ZnO ENPs and $ZnCl_2$ in embryos. The technique has been shown to be sufficiently sensitive for investigating accumulation in tissue sections of small iron oxide ENPs doped with europium (Scharlach et al., 2016). LA-ICP-MS has been used to detect Au ENPs and their size distribution in mouse liver (Li et al., 2017), as well as to determine and visualize Al_2O_3, Ag, and Au ENPs in the organisms *Danio rerio* and *Daphnia magna* (Böhme et al., 2015). The data obtained by the technique are measurements of mass-based concentration. In order to make the quantitative imaging results obtained by LA-ICP-MS useful for (eco)toxicological purposes, information is required about the particle size and shape in the biological tissue under study.

3.6. Absorption-edge synchrotron X-ray computed microtomography

SXCMT has achieved a good reputation as a non-destructive method for investigation at length scales of a few hundred nm (Jenneson and Gundogdu, 2006). The technique applies monochromatic X-ray beams extracted from synchrotron radiation, thereby allowing acquisition of high-resolution 3D images (Cloetens et al., 1997). Because the technique is non-destructive and leaves the sample unaltered, it is used in biology to reveal 3D structures of samples from many organisms as well as in human tissues (Mizutani and Suzuki, 2012).

As an *in-situ* technique, SXCMT shows promise for quantification of ENPs in complex matrices (Molnar et al., 2014), and particularly for biological media due to its ability to provide reproducible results of dynamic systems (Song et al., 2001) and to investigate localized ENPs within cells and tissues (Eck et al., 2010). SXCMT has been used as an imaging method to investigate antibody-conjugated Au ENPs in living mice (Eck et al., 2010), and to visualize the distribution of superparamagnetic iron oxide ENPs particles within the brain of mice (Marinescu et al., 2013). It has been used also to provide 3D images at various scales for *ex vivo* detection, localization, and biodistribution of cerium oxide (CeO_2) ENPs in mouse lungs as well as at the individual cell level (Chaurand et al., 2018).

Application of SXCMT for nano(eco)toxicology purposes remains unexplored. A key challenge in developing this technique is that the typical resolution of SXCMT (ca 2–12 μm) currently is not sufficient to detect single ENPs. The technique was used recently to measure the total mass of ENPs' dispersion within a complex medium while disregarding the size and size distribution of the particles (Molnar et al., 2014). This technique may therefore be more suitable for quantification of ENPs in the gut or of ENPs which are adsorbed onto the skin of an organism or to cell surfaces where a high concentration of ENPs can be expected (Hu et al., 2012).

4. *Ex-situ* techniques for characterizing and quantifying ENPs in biological matrices

A number of *ex-situ* techniques have previously been applied to characterize and quantify ENPs after extraction from biological media. In the case of ENPs with high polydispersity, techniques such as asymmetric flow field flow fractionation (AF4) coupled with light scattering techniques or with ICP-MS are increasingly used as *ex-situ* techniques. In addition, running ICP-MS in a single particle mode, known as sp-ICP-MS, holds promise for quantification of ENPs based on particle number concentration. Sp-ICP-MS also can be used when dealing with dissolvable ENPs, because the technique is able to discriminate between ions and particles (Laborda et al., 2014). In the next section, we provide brief information about some of the widely used *ex-situ* techniques.

4.1. Asymmetric flow field flow fractionation coupled with inductively coupled plasma mass spectrometry

As a chromatography technique, AF4 is well suited to fractionation of particles according to their hydrodynamic diameters. Previous studies have reported that AF4 may be used for separation of ENPs from complex matrices (Petersen et al., 2016) In fact, AF4 is a fractionation technique that cannot distinguish the type of an analyte (Williams et al., 2011). This implies that the technique cannot separate particles from complex matrices based on particle type. It fractions the analytes based on their hydrodynamic diameters, whether the analyte is a molecule, protein, or ENP. Thus, it can reduce the polydispersity of a sample but not its complexity.

Coupling AF4 on line with ICP-MS can provide information about the element-based size distribution of the fractionated analyte (von der Kammer et al., 2011). Application of AF4 coupled with ICP-MS for characterization of ENPs in complex matrices has been reported (Wagner et al., 2015). Low-level detection by ICP-MS and its capability for characterization makes the technique suitable for investigating ENPs at biologically relevant concentrations in (eco)toxicological studies. To date, the technique has been applied to separate and characterize Ag NPs (Poda et al., 2011; Mudalige et al., 2015a; Bolea et al., 2014; Loeschner et al., 2013; Jiménez-Lamana et al., 2014), Au NPs (Meisterjahn et al., 2014), SiO_2 NPs (Bartczak et al., 2015), quantum dots (Moquin et al., 2015), and Se NPs(Palomo-Siguero et al., 2015) after exposure to a biological receptor.

Nevertheless, AF4-ICP–MS has some limitations when one wishes to apply it for (eco)toxicological studies. Apart from sample preparation, another limiting factor is that particle–membrane interactions often lead to poor recovery rates in AF4 fractionation (Sötebier et al., 2015). In (eco)toxicological studies, ENPs can be coated with natural organic

matter or a protein corona. The coating agents may interact with the membrane to influence the particles' elution time from the channel (Mudalige et al., 2015b) and, subsequently, the measured size and mass-based size distribution. The results obtained from AF4-ICP-MS are mass-based data. In principle, an AF4-ICP-MS mass size distribution can be converted to a particles number size distribution. Conversion of AF4-ICP-MS mass-based data to particle numbers, however, suffers from a number of limitations within the analytical technique and data conversion (Cascio et al., 2015). In addition to the analyte peak, an AF4-ICP-MS fractogram contains noise signals and a void peak corresponding to unretained particles at the beginning of the fractogram. The void peak possibly reflects the small ENPs present in the sample. Due to the ratio between mass and size ($m \propto d^3$), if the noise signal and the void peak are also converted from mass to number, they would be interpreted as particles and sum to a relevant number fraction. Thus, the resulting number size distribution would completely and erroneously be dominated by smaller particles (Peters et al., 2014a).

4.2. Single particle inductively coupled plasma mass spectrometry

In the past decade, sp-ICP-MS has been used as a particle counting technique to provide particle number size distribution in addition to mass concentration (Degueldre et al., 2004). An increasing number of nano(eco)toxicological studies are applying this technique (Bao et al., 2016; Van Der Zande et al., 2012; Walczak et al., 2013). The low number concentration and size detection limit (dependent on the type of elements, the size detection limit ranges from 10 nm to >200 nm) (Lee et al., 2014) make the technique very promising for use in nano(eco) toxicology, and particularly for determining environmentally relevant particle number concentrations and for cases of highly diluted samples. For example, Witzler et al. performed a method validation for Ag and Au ENPs analysis in human whole blood (Witzler et al., 2018). They reported that the concentration working range throughout all measured samples was in the ng/L range.

The technique offers the possibility to differentiate between the analyte in ionic form and existing as a particle (Mitrano et al., 2012), which is of paramount importance in nano(eco)toxicology. When ENPs are taken up in the gut, for example, they either are present as dispersed particles or may undergo transformation (*e.g.*, dissolution or aggregation) (Li and Wang, 2013). The use of sp-ICP-MS, therefore, permits distinguishing between ionic and particulate forms of the accumulated ENPs. There are reports of successfully utilizing sp-ICP-MS to quantify Ag ENPs and Ag^+ in chicken meat (Ramos et al., 2017), as well as to measure the particle mass concentration and particle number concentrations of lead (Pb) ENPs in game meat (Kollander et al., 2017). These authors relate being able to distinguish between the ionic and the particulate form of the ENPs.

Application of sp-ICP-MS for nano(eco)toxicological purposes may face some challenges, such as when aggregates are present in a sample. The technique struggles with distinguishing between particles in single and aggregated forms (Reed et al., 2012). Moreover, ionization and detection of the particles become challenging if those particles are composed of elements to which ICP-MS is less sensitive (Cornelis and Hassellöv, 2014). As a result, some ENPs with small sizes (*e.g.*, TiO_2 ENPs smaller than 100 nm (Aznar et al., 2017)) cannot be measured correctly using this technique. Tuoriniemi et al. reported that the signals of Ag ENPs smaller than 20 nm overlapped to a large extent with the signal of dissolved ions even though a dwell time of 1 ms was used (Tuoriniemi et al., 2012). This is critical for nano(eco)toxicology (Tuoriniemi et al., 2012), because it is reported that small particles are generally more toxic than are larger particles due to their ability to penetrate biological pores and cell membranes (Handy et al., 2012). The situation is even more challenging if one wishes to quantify ENPs in biological samples where the presence of other ions, such as calcium (Ca^{2+}), phosphorous (P), and/or sodium (Na^+), may cause isobaric interference with measurement of the particle of interest (Donovan et al., 2016). The technique also does not provide data on carbon-based ENPs or metal speciation.

Taken together, these considerations imply that the *ex-situ* techniques can play a critical role as alternatives in the absence of suitable *in-situ* techniques to provide some of the required information, such as size, size distribution, and particle number concentration for ENP environmental risk assessment. It must be emphasized that there exists a high risk that intact particles will be altered during their extraction from biological media and that this may result in an inaccurate understanding of the real scenario. Thus, the choice of sample preparation method for each technique is critical, as is the care with which it is carried out.

5. Sample preparation methods for extracting ENPs from biological media

Ex-situ techniques require that ENPs be extracted from biological matrices and subsequent cleanup of the samples. Although these processes may lead to alteration of ENPs and/or misidentification of specific elements (Drobne et al., 2018), they do provide an alternative for characterizing and quantifying ENPs within the body of organisms in the absence of fit-to-purpose *in-situ* techniques. A generic method is required to isolate ENPs from tissues and bring the particles into a state that is measurable by the existing *ex-situ* analytical techniques. The main challenge is to develop a generic sample preparation method that is able to extract ENPs from biological matrices while causing no alteration in the properties of interest (*e.g.*, particle size and composition).

Recently, methods have been increasingly developed for extraction of ENPs from consumer products such as sunscreens (Lu et al., 2015); foods such as tomato soup (Wagner et al., 2015), noodles (Oberdörster et al., 2005), and chicken meat (Peters et al., 2014b); and medical products such as serum lubricants (Lal et al., 2016). A limited number of methods have been presented for biological samples. An overview of the methods is given in Table 1. The data show the hotspots and knowledge gaps among the various studies, the available methods, and the most commonly investigated ENPs. The existing palette of methods is skewed toward extraction of metal-based ENPs and carbon nanotubes. Very few studies cover, for instance, such other carbon-based ENPs as graphene and nanoplastics. One explanation underlying this observation is that most of the available methods are based on traditional protocols, such as those used for extracting metals from tissues.

Digestion of biological samples is only suitable for obtaining the total element content of ENPs in the samples. Current methodologies to extract ENPs from biological media are based on alkaline digestion, using for example tetramethylammonium hydroxide (TMAH) (Gray et al., 2013; Arslan et al., 2011; Johnson et al., 2017), and enzymatic digestion (Gray et al., 2013; Jiménez-Lamana et al., 2016). These are more or less harsh treatments that can further modify the ENPs and their biointerfaces. For example, extraction of metal oxide ENPs such as ZnO, copper oxide (CuO), TiO_2, and CeO_2 under the extremely alkaline conditions of a TMAH solution is challenging due to particle solubility (Schwertfeger et al., 2017).

Extraction methods increase the risks of particle loss and low recoveries (Lal et al., 2016). For example, adsorption of bare extracted particles to tube walls or aggregation of the particles during treatment, transport, and storage can increase particle losses and influence the recovery (Wagner et al., 2015). There also exists the possibility that the organic components of the background matrices will not be totally removed after digestion and that the ENPs may attach to organic residuals (Fischer and Scholz-Böttcher, 2017). Thus, adsorption of the organic residue to tube walls can increase the risk of particle losses. Furthermore, interference of organic residues with quantification and characterization of the ENPs in the samples may result in misinterpretation (Laborda et al., 2016).

Table 1
Summary of methods used to extract metal-based and carbon-based ENPs from biological media.

Particle type	Biological matrix	Brief description of the reported sample preparation methods for extracting ENPs	Measured properties	Reference
Silver (Ag) NPs	Tissues of *Lumbriculus variegatus*	Tissue of the organisms was diluted with deionized water and sonicated for 1 h. Resulting samples were centrifuged to remove biological debris. Supernatant was then analyzed by field-flow fractionation – ICP-MS.	Total mass concentration, size and size distribution	Poda et al., 2011
	Chicken meat	A portion of meat paste was vortexed for 1 min at 2500 rpm. 5 mL of proteinase K solution was added to enzymatically digest the samples. Mixture was incubated at 37 °C in a water bath using continuous stirring for approximately 40 min. Extracted particles were measured using AF4 and sp-ICP-MS	Size and size distribution	Loeschner et al., 2013
Quantum dots (CdSe NPs)	Tissues of rats	Liver and kidney samples of rats were dissected and digested with tetramethyl ammonium hydroxide (TMAH) solution at 70 °C for 2 h. Residual was diluted with deionized water, then centrifuged at 6000 rpm for 30 min. A portion (2 mL) of the centrifuged solution was re-centrifuged at 12,000 rpm for 1 h to completely separate suspended tissue and intact ENPs from the solution. Supernatant was analyzed by ICP-MS.	Total mass concentration	Arslan et al., 2011
Silicon nitride particles	Serum lubricants	Sample was first centrifuged. Remaining supernatant and pellet were resuspended in HEPES buffer and digested at 50 °C by proteinase K (working concentration 0.5 mg/mL) for 18 h in presence of 0.5% (*w*/*v*) sodium dodecyl sulfate (SDS) and 3 mM calcium chloride. Digest was then sonicated simultaneously for 10 min in an ultrasonic bath. Enzymatic digestion was repeated with addition of proteinase K at 50 °C for 22 h. Digest was sonicated for 10 min and particles were isolated by density gradient ultracentrifugation at 40,000 rpm for 4 h. Supernatant was removed and the isolated particles were washed, sonicated, and characterized by nanoparticle tracking analysis and EDX system integrated in cold-field-emission SEM.	Morphology, for elemental analysis, particle size and size distribution	Lal et al., 2016
Gold (Au) NPs	Tissues of nematodes	Samples were digested with TMAH at room temperature to dissolve nematode tissues. After 2 h of digestion, samples were diluted with deionized water and bath-sonicated for 4 min. Extracted particles were characterized using ICP-MS.	Morphology, size, and size distribution	Johnson et al., 2017
Platinum (Pt) NPs	Tissues of plants	Citrate buffer was added to plant samples. Samples were then homogenized using ultrasonic probe at 30% power for 5 min. Residuals were digested using an enzyme solution (0.01 g of enzyme powder for roots and 0.05 g of enzyme powder for shoots, leaves, stems and cotyledons, dissolved in 2 mL of Milli-Q water) and shaken in a water bath at 37 °C for 24 h. Suspensions were filtered and measured using sp-ICP-MS.	Size and number size distribution	Jiménez-Lamana et al., 2016
Au and Ag NPs stabilized by polyvinylpyrrolidone (PVP)	*Daphnia magna* and *Lumbriculus variegatus*	Samples were digested using TMAH for different digestion time and TMAH concentrations. Low gravity centrifugation and coarse filtration were used for sample cleanup. Residuals were then diluted with water. Extracted particles were measured using sp-ICP-MS.	Size and number size distribution	Gray et al., 2013
SiO_2 NPs	Human endothelial cell lysate and rat lung tissue	Samples were digested with 60% nitric acid solution at about 95 °C. Digested samples were then centrifuged and resuspended in 500 μL of 0.1% FL-70. Dispersions were sonicated with a probe for 20 s and characterized using sedimentation field-flow fractionation.	Size and size distribution	Tadjiki et al., 2009
Carbon nanotubes	Rat kidney cells	Suspended cells were collected by centrifuging at 60 ×g for 7 min and resuspended in phosphate buffered saline (PBS). Cells in the pellet were lysed by resuspending them in 200 μL of 1% SDS, 1 mM $MgCl_2$, and 1 mM $CaCl_2$ for 2 h in a 37 °C water bath. Then residual was treated with 20 μg of DNase I for 2 h at 37 °C to reduce viscosity of the solution. Concentration of particles was measured using gel electrophoresis.	Total mass concentration	Wang et al., 2009
	Rat lung	Tissue was digested using digestion reagent (nitric acid, sulfuric acid, hydrofluoric acid, hydrochloric acid, hydrogen peroxide, sodium hydroxide, and proteinase K). Samples were heated at 60 °C and mixed (30 s at 700 rpm, 5 min off) for 24 h. Residuals were then washed with water and centrifuged at 25000 ×*g* for 15 min. After washing, samples were filtered and dried in a furnace at 90 °C. Carbon nanotubes were quantified using programmed thermal analysis.	Total mass concentration	Doudrick et al., 2013
	Tissues of mice	Spleen, liver, and lung containing multi-walled carbon nanotube were mixed with 1 mL of TMAH (25 wt% in H_2O) for 3 h at 60 °C. Digestion continued until solution became completely clear and homogeneous. Particles were measured using ^{14}C-radiolabeling and tissue radioimaging	Total mass concentration	Czarny et al., 2014
	Tissues of fish	Gut, gills, blood, skin and filet tissues were dissected and dried at 60 °C. Samples were then transferred to glass test tubes, covered with tissue solubilizer (BTS-450), and incubated for 24 h at 37 °C. A 35% hydrogen peroxide solution was added to bleach the samples, which were then transferred to 20 mL scintillation vials. Vials were filled with scintillation cocktail, and after storage overnight at 4 °C in darkness subjected to liquid scintillation counting.	Total mass concentration	Maes et al., 2014
	Tissue of fish	Whole body and certain tissues (gut, brain, and liver) of fish were homogenized with 400 μL of 0.1 M PBS buffer in water bath. Samples were then mixed with 200 μL of 1 mM $CaCl_2$, 200 μL of 1 mM $MgCl_2$, and 100 μL of 10% SDS. 1 mL of acetonitrile was added into the homogenates and sonicated for 45 min at 20 °C. The pellets of the digestive track were homogenized with 100 μL of 5% sulfosalicylic acid and diluted 2-fold with 20% glycerol.	Total mass concentration	Su et al., 2013

Table 1 (*continued*)

Particle type	Biological matrix	Brief description of the reported sample preparation methods for extracting ENPs	Measured properties	Reference
	Shrimp and algae	Algae subsamples were bleached in 2:1 algae to benzoyl peroxide solution. Shrimp were digested with 2 mL ScintiGest Tissue Solubilizer. Samples were heated for 2 h at 55 °C. At room temperature, Ecoscint XR (as scintillation cocktail) and 250 mL of glacial acetic acid were added to digest the samples. Near-infrared fluorescence spectroscopic method was used to measure and characterize body burdens of carbon nanotubes.	Total body burden	Parks et al., 2013
Micro-nanoplastics	Tissue of fish	Samples were enzymatically and chemically digested by a succession of SDS, protease, chitinase, and hydrogen peroxide. Samples were vacuum-dried and degreased with a few milliliters of petrol ether (60/80). For benthic fish samples, an additional density separation with sodium iodide solution (ca 1.6 g/L) was used. Samples were filtered, dried, and milled in a small agate bullet mortar. Resultants were transferred into a pyrolysis target and pyrolyzed after addition of TMAH (25% *w*/w in water). Concentrations of particles were measured by pyrolysis–gas chromatography–mass spectrometry.	Total mass concentration	Fischer et al., 2017

6. Proposed scheme and toolbox

Based on the extensive review provided above, we propose a decision scheme and analytical toolbox for extraction, characterization, and quantification of ENPs for purposes of nano(eco)toxicology. The scheme and toolbox can serve as a basis for selecting the most appropriate methods and techniques for metal-based and carbon-based ENPs (Fig. 1). An aim of the proposed scheme and toolbox is to minimize reported artifacts, which constitute a problem commonly encountered in the literature.

6.1. A scheme for particle extraction

The overall process, which involves five steps, can be seen in Fig. 1. *Step 1*: The tissue within which the ENPs are accumulated is homogenized to obtain a homogenous suspension. *Step 2*: The homogenized tissue is diluted with water and sonicated to aid in breaking down the tissue and biological textiles. The sonication process enables more particles to be extracted from their background matrices than is possible without sonication (Schwertfeger et al., 2017). *Step 3*: The resulting samples are digested to release the ENPs and bring the particles into the dispersion phase as follows: by alkaline digestion for metal ENPs; by enzymatic digestion for metal oxide ENPs; and by enzymatic digestion and a digestive reagent (nitric acid, sulfuric acid, hydrochloric acid, SDS, and/or sodium hydroxide) for carbon nanotubes. *Step 4*: It is possible that after digestion biological residues remain in the samples and interfere with particle quantification and characterization (Loeschner et al., 2014). Additional post-treatment of the sample using hydrogen peroxide (H_2O_2) can maximize removal of those

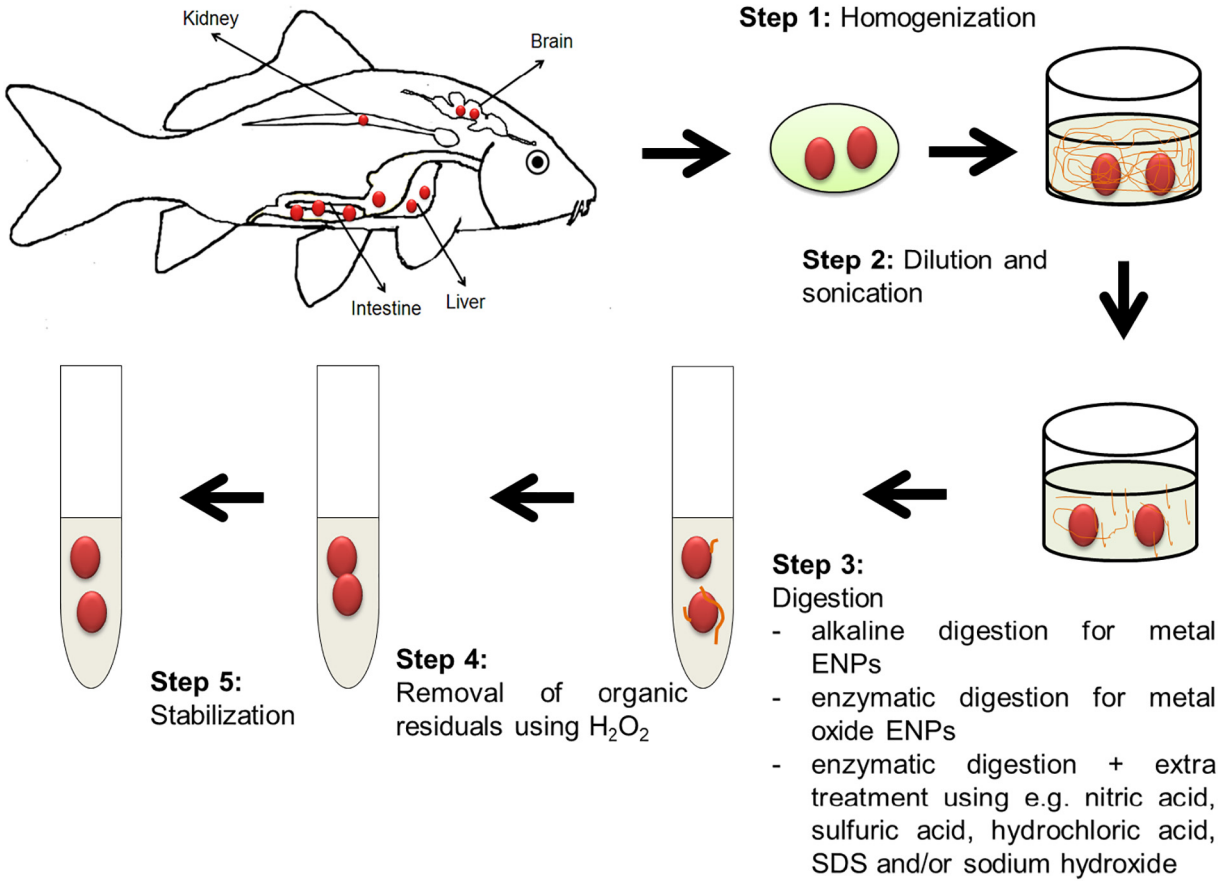

Fig. 1. Representation of the scheme proposed for extraction of ENPs in tissues and/or organisms for nano(eco)toxicological purposes.

biological residuals that are resistant to other digestive reagents. *Step 5*: The suspension thus obtained must be stabilized using a suitable stabilizer, which treatment is followed by sonication to increase the particle stability. The extraction method proposed cannot guarantee 100% recovery of the ENPs, but it can provide a useful laboratory method for nano(eco)toxicological investigations. An important factor to consider is the low concentration of the particles in the final dispersion, which makes the applied analytical techniques very critical. Method validation should be performed using only the particles and the reagents to make sure that the particles are stable during the digestion process. In cases involving nanowires and carbon nanotubes, for instance, it is necessary to make sure that sonication does not affect the length of the wires and does not fracture the tubes.

6.2. Toolbox for analytical techniques

When selecting a set of techniques, a compromise should be reached between the abilities to observe and quantify *versus* the abilities to differentiate between ions, particles, and aggregates. Fig. 2 depicts the technical toolbox available for metal-based and carbon-based ENPs. Due to the similarities between carbon-based ENPs' structures and the structure of natural carbon, many analytical techniques require extraction of the carbon-based ENPs from the background matrices. This limits various *in situ* approaches (Petersen et al., 2016).

An overview of the techniques used for quantification of carbon nanotubes in biological media has been provided in previous review papers (Petersen et al., 2016; Bjorkl et al., 2017). We propose thermal gravimetric analysis (TGA) for measuring the total mass concentration of some carbon-based ENPs. The technique is suitable for investigating carbon-based ENPs in biological media, but detection limits will be higher in biological matrices due to greater interferences (Mansfield et al., 2010). The key consideration in selecting TGA is whether components in the biological environment can alter the thermal stability of the particles (Doudrick et al., 2013). It is also important to consider that there is potential for overlap between the oxidation temperatures of the carbon-based ENPs and of components of the matrix (Petersen et al., 2016). Extraction of the particles from the matrix decreases the bias from other forms of organic carbon and may reduce the detection limit (Petersen et al., 2016).

Due to the density of metal components, observational techniques such as EM are mostly applicable for metal-containing ENPs. A particular challenge arises if the ENPs are carbon-based, because these are sometimes poorly visualized by EM, consisting as they usually do of elements with low atomic numbers (Stewart, 2017).

7. Conclusions and outlook

The information we present in this review paper is based on the current state of the art with regard to characterizing and quantifying ENPs

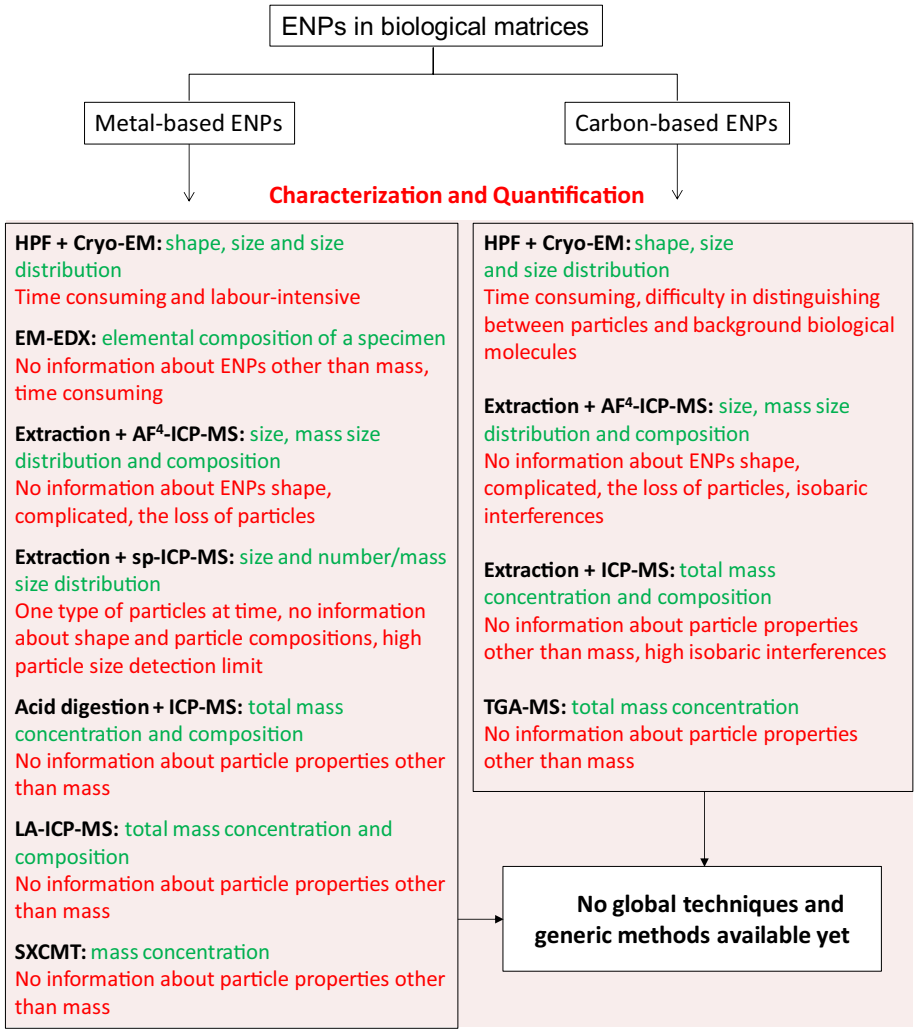

Fig. 2. The analytical toolbox available for characterization and quantification of metal-based and carbon-based ENPs for nan(eco)toxicological purposes according to the specific research questions and ENP properties. TGA, thermal gravimetric analysis.

in biological matrices relevant for nano(eco)toxicology and nanosafety investigation. It is shown that most of the data gathered to date are based on total mass of ENPs rather than on the physicochemical properties of unmodified (or bare) ENPs in organisms. That is despite the fact that many studies have reported that physicochemical properties influence the fate and biodistribution of ENPs in organisms. Most of the available data concern metal-based ENPs and carbon nanotubes in organisms. Scarcely any data was found for such other carbon-based ENPs as nanopolymers, graphene, nanoplastics, and nanopesticides in biological media. Moreover, the methods developed to quantify carbon-based particles within biological matrices are still in their infancy.

We suggest that for each ENP investigated the selection of a method and a set of techniques (the so-called toolbox) should be based on (a) the ENP property/ies of interest; (b) particle mass, number concentration, as well as volume-specific surface area as a suitable dose metric; (c) the aggregation/dissolution state of the particles; and (d) the toxicological endpoint of relevance. We also suggest including complementary methods capable of *in-situ* visualizing ENPs within biological media. Data should be sufficiently robust so that it can be further used by the scientific community within the field. The proposed scheme and toolbox make it possible to determine internal kinetics and dynamics that are critical to developing adverse outcome pathways for ENPs, ultimately resulting in a mechanism-based environmental risk assessment.

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