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An analytical workflow for dynamic characterization and quantification of metal-bearing nanomaterials in biological matrices

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To assess the safety of engineered nanomaterials (ENMs) and to evaluate and improve ENMs' targeting ability for medical application, it is necessary to analyze the fate of these materials in biological media. This protocol presents a workflow that allows researchers to determine, characterize and quantify metal-bearing ENMs (M-ENMs) in biological tissues and cells and quantify their dynamic behavior at trace-level concentrations. Sample preparation methods to enable analysis of M-ENMs in a single cell, a cell layer, tissue, organ and physiological media (e.g., blood, gut content, hemolymph) of different (micro)organisms, e.g., bacteria, animals and plants are presented. The samples are then evaluated using fit-for-purpose analytical techniques e.g., single-cell inductively coupled plasma mass spectrometry, single-particle inductively coupled plasma mass spectrometry and synchrotron X-ray absorption fine structure, providing a protocol that allows comprehensive characterization and quantification of M-ENMs in biological matrices. Unlike previous methods, the protocol uses no fluorescent dyes or radiolabels to trace M-ENMs in biota and enables analysis of most M-ENMs at cellular, tissue and organism levels. The protocols can be applied by a wide variety of users depending on the intended purpose of the application, e.g., to correlate toxicity with a specific particle form, or to understand the absorption, distribution and excretion of M-ENMs. The results facilitate an understanding of the biological fate of M-ENMs and their dynamic behavior in biota. Performing the protocol may take 7–30 d, depending on which combination of methods is applied.

Introduction

Engineered nanomaterials (ENMs), generated by manipulating matter on a near-atomic scale, have the ability to revolutionize many industries and are used in many sectors in society such as medicine and computing. Safety and targeting ability are important factors in developing sustainable nanotechnology¹. Designing safe and efficient ENMs for different applications requires understanding of how ENMs behave in a given system and how they interact with the surrounding media or matrices. Gaining such information is not straightforward and is practically challenging when ENMs enter cells and (micro)organisms, whether via direct administration for medical purposes or through environmental exposure. This difficulty arises because there is a limitation in analytics for characterization and quantification of ENMs in bio- and physiological matrices due to their presence at trace levels, and due to the presence of different background materials that interfere with the measurement of ENMs². Moreover, ENMs may undergo a range of biotransformation and agglomeration processes inside organisms³. This indicates that they have a dynamic behavior, which adds to the analytical complexity. Thus, researchers must apply an approach that is capable of not only measuring ENMs at trace levels but also measuring the form of the particles at a specific moment in time. This limitation hampers the accurate risk assessment of ENMs, inhibiting the timely development of regulations for ENMs and the safe handling of ENMs, the design of targetable ENMs for medical and agricultural applications, and the adoption of safe-by-design strategy for nanotechnology in which safety

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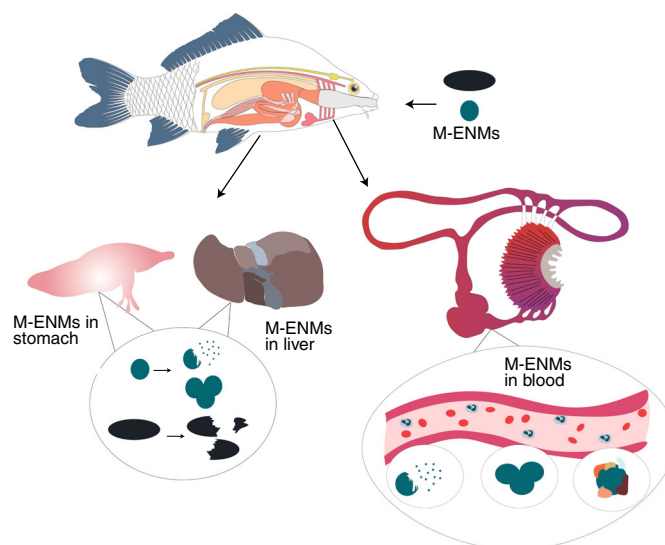


Fig. 1 | Metal-bearing engineered nanomaterials (M-ENMs) of different shapes and sizes are taken up by organisms following exposure via the surrounding medium or via food intake. M-ENMs of different shapes and sizes are taken up by organisms through, e.g., gills/lungs and stomach, or via attachment to the outer membranes. When entering the biological medium, e.g., in the blood, M-ENMs are prone to dissolution and agglomeration. It is also possible that the M-ENMs acquire an evolving layer of proteins and other biomolecules.

considerations are included from the design stage of material development rather than being evaluated only after the material has been developed.

Metal-bearing ENMs (M-ENMs) are the most produced and widely used ENMs in industry, e.g., in a range of consumer products, and for medical applications like drug delivery and cancer treatment. M-ENMs are composed of pure metals or their compounds (e.g., oxides, hydroxides and sulfides). Upon entering organisms and/or reaching their bloodstream, they may undergo dissolution and agglomeration. These processes can alter the size, shape and number of particles as well as the kinetics of ion release inside organisms (Fig. 1). This dynamic biotransformation can influence the toxicokinetics (e.g., rates of bioaccumulation, biodistribution and clearance) of M-ENMs³. It is therefore critical to understand how M-ENMs interact in biological media as this might differ from cell to cell and from tissue to tissue on the basis of their different matrix compositions and potential for transformation of the M-ENMs. These interactions, which include protein and metabolite binding to the ENMs⁴, influence the physicochemical properties of ENMs and the behavior, uptake and biodistribution pathways of the particles. There are no standard and straightforward methods and analytical techniques available that are able to comprehensively characterize and quantify M-ENMs *in situ* in (micro)organisms. Instead, a combination of methods is required, some of which are destructive to the tissue, to piece together the biological fate of the M-ENMs.

In this protocol, we present a practical workflow that combines different methods and fit-for-purpose techniques, for characterizing and quantifying M-ENMs in biological samples including cells, microorganisms and higher organisms. The protocol is applicable to measurement of M-ENMs in tissues from both laboratory and field experiments. Note that the protocol can also be used to characterize natural nanosized metallic particles. Engineered and natural particles of similar compositions can, however, not be distinguished. We have used this protocol previously to understand the bioaccumulation and biodistribution of M-ENMs in organisms (e.g., fish)⁵ and their trophic transfer in food chains (algae → daphnia → fish)³. The method was also used to quantify the cellular uptake of M-ENMs on a cell-by-cell basis in microorganisms (algae)⁶. The workflow is optimized to cover cells and microorganisms such as bacteria as well as tissues and organs of different organisms, including plants and carapace-covered organisms (e.g., daphnids, isopods and arachnids). The protocol is capable of generating the following information:

- Determination of the type and chemical composition of known and unknown M-ENMs in tissues
- Quantification of the number of M-ENMs associated with cells, microorganisms and organisms
- Distinction between particulate and ionic forms of M-ENMs and quantification of ion release in biological and physiological media

- Quantification of the number-based and size distribution of different M-ENMs simultaneously present in organisms
- Quantification of the cellular association of M-ENMs on a cell-by-cell basis
- Visualization of the tissue or cellular distribution of M-ENMs at the micro- or nanoscale

Development of the protocol

This protocol has evolved over the past decade. The primary attempts were focused on developing approaches for detection and quantification of M-ENMs in pure water and complex matrices of environmental samples such as natural surface water⁷. To measure M-ENMs in environmental samples, we digested the samples using aggressive digestive agents, e.g., nitric acid (HNO₃), following the traditional methods for measuring total metals⁸. Inductively coupled plasma mass spectrometry (ICP-MS) was applied to quantify the total mass of the element forming the particles. However, the application of traditional methods only provides information about the total mass, and no information can be obtained about the physicochemical properties of M-ENMs, such as size, shape and number, nor insights as to whether the M-ENMs undergo dissolution or agglomeration. Thus, we developed the approach further to be able to characterize and quantify M-ENMs, focusing mostly on water samples and fish tissues⁵. Application of the protocol requires a stepwise sample preparation that is capable of extracting M-ENMs from the complex matrices in which they reside.

The main challenges in developing the sample preparation approaches were (a) the influence of the digestive agent on the particles, which could lead to particle dissolution, precipitation and agglomeration; (b) loss of particles during sample handling, which could lead to a reduction in particle number and mass recovery; (c) the presence of residuals from the background matrices, which could interfere with the particle (metal) analysis; and (d) compatibility of the applied digestion and stabilization reagents with the measurement techniques. To optimize the approach, we investigated several parameters, including digestion time, type and concentration of the digestion reagents, digestion temperature, and type of stabilizers used to stabilize the extracted particles^{5,9}. We noticed that developing a generic sample preparation method for all types of environmental samples is almost impossible owing to the differences in the background matrices in which the particles occur¹⁰. Thus, we further focused on developing a specific sample preparation approach tailored to the extraction of M-ENMs from biota to support environmental risk assessment and medical application of ENMs.

Extraction of ENMs from biota adds another layer of complexity to the challenges associated with sample preparation. The concentration of ENMs in biota, depending on the species, is typically low. Moreover, as mentioned earlier, M-ENMs may undergo agglomeration and different bio-transformation in biota. This causes the particles to have a dynamic behavior¹¹. We developed and optimized extraction approaches for M-ENMs from biota using zebrafish as a model organism following exposure of zebrafish to metal oxides (TiO₂ and CeO₂ ENMs) for 21 d (ref. ⁵). We later improved this approach by optimizing the steps using different digestive reagents (such as tetramethylammonium hydroxide (TMAH) for M-ENMs, and enzymatic digestion for metal oxide ENMs), the digestion time, and the means of particle stabilization, enrichment and separation.

No single technique is available that can comprehensively characterize ENMs in terms of their different physicochemical properties, including composition, size distribution and form (e.g., particulate versus dissolved). A set of different analytical techniques is required to measure, for example, size, shape, chemical composition and surface speciation, surface charge and particle number. Alongside the development of the sample preparation approach, we optimized a set of analytical techniques to provide comprehensive information about the characteristics of the extracted M-ENMs. Combining the sample preparation approach with the set of analytical techniques offers a fit-for-purpose workflow for dynamic characterization and quantification of M-ENMs in biota. For example, the number and size distribution of the extracted particles were measured using single-particle ICP-MS (spICP-MS), and the shape of the particles was determined using transmission electron microscopy (TEM)¹².

We further extended the workflow to cover microorganisms and single cells. A complete methodology for analysis of M-ENMs in microorganisms and cells was developed on a cell-by-cell basis using single-cell ICP-MS (scICP-MS), including cell collection, sample preparation, signal acquisition, and data preprocessing and analysis³. We successfully applied this protocol to show the accumulation and adsorption of gold (Au) ENMs in each microalgal cell within a given cell population. Similarly,

we obtained the number of M-ENMs associated with, or accumulated by, each single cell and the number of cells that contain particles⁶. We have also used synchrotron radiation-based characterization techniques in combination with the sample preparation methods to analyze M-ENMs dynamic behavior at cellular levels^{13,14}. The protocol was recently used to reveal how the biotransformation of M-ENMs influences their penetration into and transport across an *in vitro* blood–brain barrier cell layer¹³.

Overview of the procedure

The protocol contains three Procedures: *in situ* measurement of M-ENMs in cells and microorganisms on a cell-by-cell basis (Procedure 1), measuring M-ENMs in cells, microorganisms and tissue of organisms after particle extraction (Procedure 2), and *in situ* quantification of M-ENMs biotransformations in biological matrices by synchrotron radiation-based characterization techniques (Procedure 3). The first procedure (Fig. 2, top left) can be used to quantify the concentration of M-ENMs in (or attached to) individual cells and microorganisms and contains two main stages: stage 1 (Step 1), sample preparation with minimum influence on the cells and cell recovery process; and stage 2 (Steps 2–6), sICP-MS measurement to quantify particle number on a cell-by-cell basis. Using this approach, researchers can gain information about the amount (mass) and number of particles in every single cell within a cell population, the percentage of cells that contain the particles, and the dynamic changes in the concentration of accumulated particles in the cells, which can change over time as a result of, for example, cell doubling¹⁵ or particle dissolution. The second procedure consists of two main stages (Fig. 2, bottom) and can be used for quantifying uptake, biodistribution, biotransformation, accumulation and clearance of M-ENMs at cell population and organism levels. Stage 1 (Steps 1–6) describes extraction of the M-ENMs from the population of cells or microorganisms, tissue or organs and stage 2 (Steps 7–8) describes the approaches for characterization of the extracted particles. Comprehensive particle characterization and quantification can be performed, as dependent on the specific research question being addressed. For example, particle size, shape and number size distribution as well as dissolution and aggregation can be measured as described in the measurement section. Note that, if the intended purpose of analyzing the M-ENMs requires no information about the size distribution of the particles or their charge, Step 8 of the second procedure could be disregarded. Procedure 3 consists of four different options: (A) quantification of M-ENMs in organisms using synchrotron X-ray absorption fine structure (XAFS); (B) quantification of M-ENMs in plants using micro-X-ray fluorescence (μ -XRF); (C) analyzing M-ENMs in plant samples using soft transmission X-ray microscopy (STXM); and (D) analyzing M-ENMs in cell lines using STXM.

Applications of the workflow

The protocol presented here covers detection, characterization and quantification of M-ENMs in cells and (micro)organisms for use with *in vitro* and *in vivo* tests. After some modifications, such as application of suitable digestive reagents, it can also be used for analysis of M-ENM interactions with and uptake by plants (as described in ‘Experimental design’). The availability of this protocol will open new research horizons for further studies in, e.g., the fields of environmental science, risk assessment and nanomedicine. For example, the protocol can be applied for the following purposes:

- By toxicologists to understand the dose–response behavior following exposure to M-ENMs and to determine the mode of action in terms of particle effects or impacts related to release of ions following M-ENM dissolution
- By ecotoxicologists and environmental scientists to quantify the uptake, biodistribution, bioaccumulation, biotransformation and trophic transfer of M-ENMs
- By medical researchers to understand the ADME (absorption, distribution, metabolism and excretion) and cellular uptake of M-ENMs and to perform bioimaging
- By the industry sector to adopt safe by design strategies for the production of M-ENM by considering ADME and safety
- By regulatory authorities to propose part of the workflow and the analytical methods for enforcement of ENM regulations and to develop restrictions based on potential risks identified

Comparison with other methods

A range of methodologies have commonly been used to provide information about, e.g., the ADME and trophic transfer of M-ENMs or to understand their cellular uptake. For example, ENMs are often labeled using fluorescent dyes to trace them in biota¹⁶. This methodology has gone out of favor not

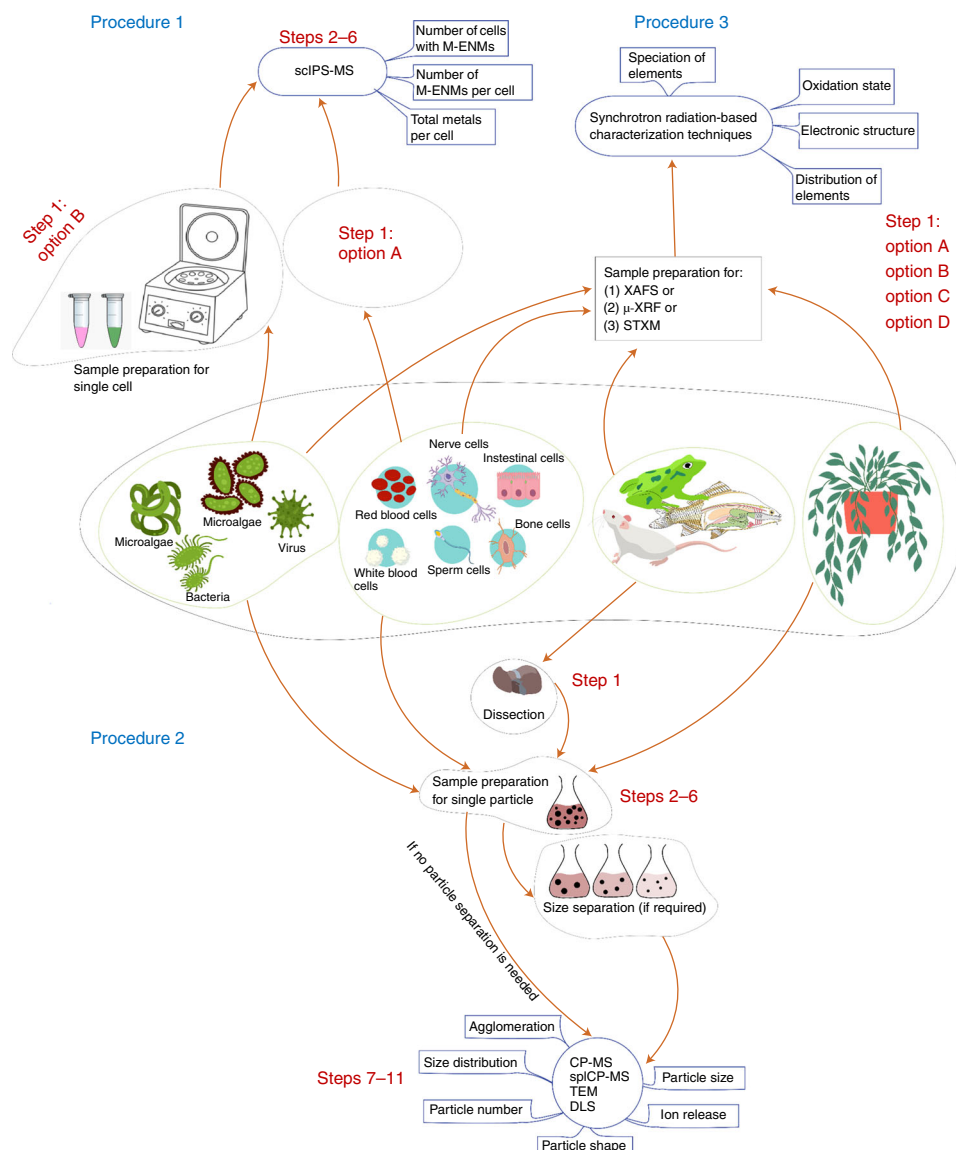


Fig. 2 | Overview of the protocol. The protocol contains three Procedures: Procedure 1, in situ measurement of M-ENMs in cells and microorganisms on a cell-by-cell basis (shown on the top left-hand side); Procedure 2, measurement of M-ENMs in cells, microorganisms and tissue of organisms after particle extraction (shown at the bottom); and Procedure 3, in situ characterization of M-ENM biotransformation in biological matrices using synchrotron radiation-based techniques (shown on the top right-hand side). Procedure 1 consists of sample preparation: Step 1, option A (sample preparation for in vitro cellular experiments) and Step 1, option B (sample preparation for single microorganism analysis) and quantification of cellular association using scICP-MS (Steps 2–6). Procedure 2 consists of 11 steps. In this procedure, the tissues of organisms are dissected (Step 1), and the particles are isolated from biological samples (Steps 2–6). The extracted particles are stabilized against agglomeration and separated by size, if necessary, and measured using a fit-for-purpose analytical technique, e.g., ICP-MS, splICP-MS (Steps 7–10), TEM, DLS and NTA (Step 11). Procedure 3 consists of one step and four options, including option A (characterization of M-ENMs biotransformation in organisms using XAFS), option B (quantification of M-ENMs in plants using μ-XRF), option C (analyzing M-ENMs in plant samples using STXM) and option D (analyzing M-ENMs in cell lines using STXM).

only because the labels might modify the surface properties of the ENMs, which in turn influences their biological fate, but also because the released labels from the particles might have a different biological fate than the particles. This leads to the potential for misinterpretation of the results¹⁷. To tackle this challenge, a unique isotopic composition has been developed¹⁸. However, similar to other labeling methods, this approach is intended to be used only in laboratory or other controlled (e.g., mesocosm) settings. Moreover, the metallic isotope requires ICP-MS bulk sample analysis whereby the samples are digested using, e.g., HNO₃ or aqua regia to subsequently quantify the total mass of the

metals forming the particles. Therefore, the protocol presented here is applicable to preparation of the samples, regardless of the techniques applied for measurement and irrespective of whether the particles are labeled or not, and for extraction and purification of M-ENMs from biological tissues in a manner that does not itself influence the particles. This allows characterization of the particles with regard to the properties of interest and investigation of the correlation between the M-ENM properties and observed localization and effects, including uptake, distribution, toxicity and accumulation following ENM exposure.

Note that comprehensive characterization and quantification of ENMs in biological matrices is still a global challenge, which adds to this complexity. For example, spICP-MS has been used for measuring particle number and size distribution in fish^{19,20}, plants²¹ and bacteria²² after particle extraction. Coupling flow-field-flow fractionation (FFF) with ICP-MS was also reported to be a suitable technique for the characterization of ENMs extracted from biological matrices²³. However, these approaches are not generic and were developed for specific M-ENMs, e.g., silver (Ag) (ref. ¹⁹) or Au (ref. ²⁰) and certain organisms (e.g., fish and daphnids). The approaches might be applicable to other M-ENMs and organisms after modifications (mainly of the extraction steps). Moreover, some of the used methodologies did not evaluate whether and how the particle extraction methods might influence the particle properties, nor did they quantify the amounts of particles recovered via the extraction process^{20,21}. Metallic and metal oxide ENMs require different sample treatment approaches for particle extraction to prevent changes in their properties from the extraction process. The proposed workflow is tailored to different M-ENMs in cells and (micro)organisms.

Some publications discuss the diagnostic capability of scICP-MS in measuring metals at attogram levels in individual cells²⁴. We recently applied scICP-MS to quantify the cellular association of Au-ENMs with algal cells²⁵. In this protocol, we describe the required steps for applying scICP-MS to analyze M-ENMs in cells and microorganisms. Unlike previous time-consuming and expensive single-cell analysis²⁶, scICP-MS requires a small amount of cells (~3,000 cells per mL depending on the size of the cells), requires no particle labeling and is able to quantify the amount of M-ENMs associated with (in or at the surface of) each cell within a cell population. Unlike cytophotometry, scICP-MS does not require the cells to be stained. In the simple sample preparation procedure for scICP-MS, e.g., for cell lines, no centrifugation is required and fast measurement not only minimizes cell loss during the handling but also allows dynamic measurement. This opens a new horizon for investigating the influence of cell doubling, or cell cycle arrest, on the number of metals and M-ENMs per cell.

Limitations

A few key limitations are associated with this protocol. The first limitation is related to the particle extraction procedures, which are destructive in nature and require removal of the matrices in which the particles reside. Some biological samples contain rigid materials, such as insect exoskeleton, carapace of daphnids, plant cellulose and fish gills, which might require use of aggressive digestive agents to totally dissolve them. This is an important step, because particles might sorb onto the residual organic materials, impeding their recovery. These residuals might even act as a center for sorption of metallic ions, which will appear as particles in the spICP-MS. However, the effect of the digestion agents on the particles must also be assessed because the M-ENMs might be influenced and undergo dissolution or aggregation. Freeze-drying of hard-to-degrade biological samples, followed by powdering of the samples, can facilitate their digestion.

Up to now, there is no standard protocol for digesting biological samples so that the particles are extracted in their intact state. Digestive agents have mostly been selected to digest biological matrices with the aim of extracting ionic cations. These agents might modify particles to some extent and induce dissolution, aggregation or metal precipitation in a particulate form. Optimization and method validation are thus required for each type of particle and (micro)organism. This may make the procedures time consuming.

A second limitation relates to spICP-MS, which is a sensitive method to detect single M-ENMs at trace levels but cannot distinguish single particles from agglomerates. This is important when the intended purpose is to determine the size distribution of M-ENMs in polydisperse samples. Moreover, spICP-MS has different lower size detection limits for particles of different elements²⁷ and in some cases cannot measure particles of small size, e.g., the limit of detection is ~40 nm for TiO₂ (ref. ⁵). Performing spICP-MS usually costs more per sample than performing ICP-MS, and spICP-MS is also more time consuming. Sample analysis and instrumental setup (including, e.g.,

determining the flow rate and transport efficiency and troubleshooting cleanliness) takes longer in spICP-MS than in ICP-MS.

Thirdly, performing scICP-MS requires handling by researchers or technicians familiar with these modes of ICP-MS and needs to be optimized for each cell type. The scICP-MS functions on the basis of the assumption that each measured event in the instrument represents a single cell. The samples, thus, should be diluted to minimize the co-occurrence of multiple cells passing the nebulizer. One must also ensure that free particles and agglomerates are removed from the samples because scICP-MS cannot distinguish between particles and a single cell. The main issue that needs to be considered for scICP-MS results is their low reproducibility. The low reproducibility in analytical results is due mainly to the uptake of the M-ENMs by cells, which is stochastic⁶. It means that not every cell within a population takes up an equal amount of ENM. This, however, does not influence the in-house method validation because the developed protocol can be used for the quantification and characterization of M-ENMs in microorganisms and cells regardless of whether the uptake is stochastic. The method validation requires using standard samples (cells) or particles, and as these are not available for scICP-MS as yet, we have proposed an in-house validation method using the reference materials that are available (e.g., Au-ENMs). If the particles are undergoing dissolution, then it is complicated to determine whether the cells accumulated particles that then underwent dissolution intracellularly, or whether the cells took up metal ions that had been released from the particles in solution.

Another limitation associated with the three techniques presented in this protocol is the limited availability of the techniques in different laboratories. The synchrotron approaches, in particular, require researchers to apply for beamtime at the specific facility against other competitors, although if successfully selected, the costs of the experiment are always covered by the facility.

Experimental design

Expertise needed to implement the protocol

Most of the steps described in the protocol can be performed by researchers, technicians and students in a variety of fields, including toxicology and environmental science. However, performing spICP-MS and scICP-MS requires technical/analytical experts to set up the instrument according to the parameters mentioned in the Procedure and to support interpretation of the data. Similarly, performing the synchrotron approaches requires access to specialized synchrotron facilities, enabled through open calls for beamtime or open requests to equipped laboratories, and requires technical support from beamline scientists.

Sample preparation

The biological samples used in this protocol could be cells, microorganisms or tissues of organisms in laboratory studies, whereby the cells or organisms are exposed to a known amount and type of M-ENMs, or samples collected from field surveillances in which the precise amount and type exposure to M-ENMs is uncertain. The purpose of the sample preparation method is to bring the particles into a state that is measurable by the selected techniques, often by removal of the biological matrix. The sample introduction and the measurement principles on which each technique is based are different. This implies that the sample preparation should be specific for the intended technique. For in situ measurements using, e.g., scICP-MS to measure the quantity of metals in cells and microorganisms, the sample preparation depends on the type of sample (Procedure 1, Step 1A or B). For example, for mammalian cells, no centrifugation is required. However, a centrifugation step is required for microorganisms and algae to prepare the samples. Where the goal of the experiment is to investigate the subcellular localization of M-ENMs or for any investigations that require the biological samples to be intact, e.g., preserving the structure of the cells, where fixation of the biological samples is required, we refer readers to available protocols for fixation of plant and animal tissues and cells²⁸.

The sample preparation for ex situ measurements (Procedure 2, Steps 1–6), e.g., using spICP-MS, requires isolating the particles from the biological samples. This is done in four main stages: homogenization of the biological samples; digestion, using digestive agents to remove the biological matrices; stabilization, to stabilize the extracted particles; and enrichment, which is performed only if the laboratory is not equipped with spICP-MS. Because spICP-MS is sensitive enough to measure trace levels of M-ENMs, spICP-MS users can skip the enrichment step. Using these stages, which are discussed further in the following sections, the M-ENMs and their dissolved ions are

extracted from the biological samples and brought to a state that is measurable by the presented analytical techniques.

- **Homogenization (Procedure 2, Step 2).** The samples need to be cleaned and homogenized to increase the extraction efficiency. The homogenization time and the power of the homogenizer must be optimized for different types of biological samples. The final products of the homogenization should be in the form of a paste in the homogenization buffer. Applying a homogenization buffer moderates the pH of the extraction medium as the pH might be altered by the contents of the cell and tissues.
- **Digestion (Procedure 2, Steps 3–4).** The principle of particle extraction is that the homogenized biological samples are removed by using a suitable digestive agent to isolate the particles and ions from the biological matrices. The critical criterion is that the particles are extracted with no or minimum alteration of the physicochemical properties such as size, shape and chemical composition of the particles. The digestive agents must therefore be carefully selected. The digestion conditions, including the concentration of the digestive agents, the time of digestion and the temperature, are optimized for the biological samples and the M-ENMs to ensure there are no, or only minimal, influences on the properties of the particles. After digestion, the samples contain M-ENMs, and the metallic ions released from the particles. The pH of the samples should be adjusted immediately to a pH suitable for the specific metal of interest. Note that some metals, such as aluminum, cadmium, copper and silver, are responsive to pH. High pH values (e.g., >8) generated by TMAH could lead to the precipitation of the metallic ions into particles. This could render false positives in the analysis, particularly if the release of ions from the particles is intended to be investigated. Thus, one should be aware of the influence of the solution pH on the speciation of the metals of interest. This can be done as a quality-control experiment to evaluate the behavior of the metallic ions at different pH by measuring the concentration of the metals in the solution using ICP-MS.
- **Stabilization (Procedure 2, Step 5).** The particles also should be stabilized immediately against agglomeration. The application of a suited stabilizing agent, followed by sonication (sonication assists in dispersing particles in the liquid using the energy of sound), could stabilize the particles. The stabilization agent should be compatible with the selected techniques (e.g., not forming agglomerates that might appear as particles in dynamic light scattering (DLS) measurements or generating interference in the ICP-MS) and should be suited for the particles (e.g., not causing ion release from the particles as a result of ligand-mediated ion release)⁷. The amount of stabilizing agent and the sonication power and time must be optimized for each M-ENM type and size.
- **Enrichment (Procedure 2, Step 6).** Finally, if the concentration of the particle in the samples is lower than the detection limit of the instrument used for characterization of the particles, an enrichment step is required to increase the concentration of the particles in the samples. Ultracentrifugation could facilitate enrichment of the M-ENMs, but care must be taken to optimize the time and force of the centrifugation for each M-ENM type and size. To evaluate the capability of the centrifugation process in enriching the particles, a dispersion of the particles (e.g., a high concentration of ~100 mg per liter of the particles) of interest could be centrifuged using the same conditions to calculate the recovery by measuring the concentration of the particles in the pellet.

Note that, if the obtained mass recovery (the recovery of total mass of elements forming the particles, e.g., Zn in the particle ZnO or Cu in the particle CuO) and particle recoveries are <80% (this percentage is arbitrarily selected and commonly reported in the literature for particle extraction from complex samples⁵), the sample preparation steps must be optimized further to increase the recoveries. In most cases of poor recovery, the particle loss resulted from the handling of the samples. Good laboratory practices must be respected, and the samples must be handled carefully, for example, by reducing the number of transfers between tubes and minimizing the long-term storage.

We have not yet developed a generic sample-preparation approach for synchrotron radiation-based characterization techniques. Each synchrotron radiation-based technique mentioned in this protocol (XAFS, μ -XRF and STXM) requires a different sample preparation approach, which is described in Procedure 3 of this protocol.

Particle characterization

For in situ quantifying of metals forming M-ENMs in cells and microorganisms, scICP-MS could be applied. In this case, the M-ENMs can be measured while present in the cells or microorganisms. However, in case of ex situ measurements, the particles are extracted from cells and (micro)organisms and characterized in terms of the physicochemical properties of interest. Laboratories that have spICP-MS available can use this technique to characterize the particles in terms of size distribution, mass and number. The projected particle size (size in projection area) and shape of M-ENMs can be

measured using TEM. Analysis of TEM images using the NanoXtract tool (<http://enaloscloud.nova.mechanics.com/EnalosWebApps/NanoXtract/>) can also provide important insights both on the average over the whole population of particles and on a particle-by-particle basis.

When spICP-MS is not available, the hydrodynamic size of the particles might be measured using DLS, nanoparticle tracking analysis (NTA) or differential centrifugal sedimentation. Measuring the hydrodynamic size of M-ENMs using DLS has been described in a previous protocol¹⁸. A minimum particle concentration of 10 mg L^{-1} is required for DLS, while for NTA substantially more dilute samples are required to ensure that particles do not come into contact with one another during measurement²⁹. Note that DLS is used for ENMs with a homogeneous size distribution, where the particles are expected to have the same size without considerable size distribution. This is because larger particles present in the sample scatter more light than smaller particles, with scattering scaling as $1/D^6$ (ref. ¹⁰). In the case of ENMs extracted from the tissues of organisms, the particles are typically heterogeneous in size since ENMs in organisms might undergo different transformations that change the size distribution of the particles³. Thus, particle stabilization is always required after extraction, whether for characterization using spICP-MS or using other techniques such as DLS. If spICP-MS or NTA is used for characterization, then a particle separation step is not required to separate particles on the basis of their size. However, if DLS is used for characterization, it is possible to, first, perform particle separation based on hydrodynamic size using FFF and then measure each fraction using DLS. It is also possible to couple FFF online with multi-angle light scattering to obtain the size distribution of the particles⁹.

spICP-MS setup

spICP-MS is based on the principle that particles pass through the nebulizer one at a time to reach the plasma and generate a cloud of ions to be detected by the detector. More information about spICP-MS can be found elsewhere³⁰. Performing spICP-MS allows the researcher to obtain the number of M-ENMs in the samples and the quantity of ions released from the particles in the tissue or cell (after sample preparation, Procedure 2). Note that, for more accurate measurement of dissolved ions, it is better to subtract the particulate fraction from the total metal. The mass-based size of the M-ENM particles and the number-based size distribution at trace level concentrations (ng L^{-1}) can be measured. The required amount of sample depends on the element and on the organisms and its efficiency at internalizing particles. For example, if the organisms can accumulate M-ENMs, as little as 1 mg of biological samples would be sufficient to measure the accumulated particles. Note that any increase in the concentration of ionic metals in the samples can interfere with the lower size limit of the detection for the particles composed of that metal. Depending on the exposure scenario, agglomeration of particles can occur, which cannot be detected by spICP-MS. At alkaline pH, agglomeration is unlikely to occur for M-ENMs, but it is unclear whether this condition can reverse agglomeration. It is thus good practice to centrifuge the samples before performing the exposure to remove any ions that were released during the storage. Note that the total mass of the element forming the particles cannot be measured using spICP-MS, and requires additional analysis by ICP-MS.

The samples must be sonicated before performing the measurement to disperse the particles. The sonication time and power must be optimized for each type of M-ENM. It is recommended not to use an autosampler in case of a high number of samples, as the waiting time in the autosampler might lead to artifacts. For example, a sample measured after 2 min might show different results compared with the same sample being measured after 1 h because of the dynamic behavior of the particles in the sample. The performance setup for spICP-MS might differ for different particles and particles of different sizes. The sample uptake rate and the dwell time must be optimized for each particle of interest. The transport efficiency depends on many factors, such as medium composition and particle size. We recommend the user to follow the manual of the instrument to specifically determine the transport efficiency for their own sample.

scICP-MS setup

The novel aspect of this method is that it provides information at the cellular level, which has been not provided before for ENMs. This includes, for example, quantification of the number of cells containing M-ENMs and quantification of the number of M-ENMs in or associated with each cell. When coupled with the approaches of extracting cells from tissues or organs, the method could also be applied to other organisms. ScICP-MS does not measure the particles, but only the mass of elements in cells. If the users can ensure (e.g., via spICP-MS) that the particles of interest are stable

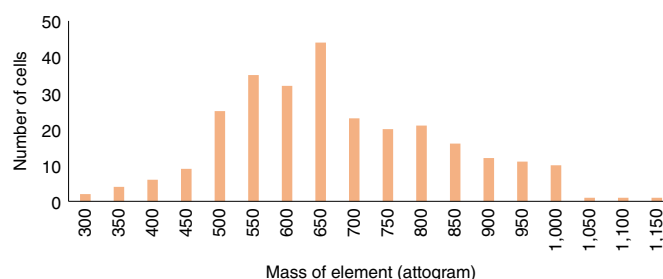


Fig. 3 | A hypothetical example of scICP-MS results. It shows that the results are presented as the number of cells versus the amount of the element of interest (attogram). For example, 50 cells contain 650 attograms of the element of interest.

against dissolution, the measured amount of metals in each cell could be correlated with the amount of particles. From the measured mass of the element per cell, one can calculate the number of particles per cell if their size is known. In many cases, M-ENMs undergo dissolution inside the cells or in the exposure medium. Another advantage of scICP-MS is that it allows measurement of samples of low volume (μL). Nevertheless, the sample uptake rate must be optimized for each type of cell to ensure that the cells are not damaged while being transferred into the plasma. Like spICP-MS, the dwell time should be optimized on the basis of cell size, as reported in the manual of the instrument. A very important point to consider is that the results obtained by scICP-MS do not show the frequency (number) of the particles versus size, but rather the number of cells versus the measured mass of the element in or on the cells (Fig. 3).

It is also possible to assess particle excretion (exocytosis) and to quantify the effect of the cell cycle (for instance, cell doubling time or impact of particles on cell cycle via arrest in a specific phase) on the particle uptake. In addition, it is possible to quantify the metal distribution in the cell population and the transport from mother to daughter cells after cell division. Accordingly, we recommend keeping the cells/microorganisms alive, for instance in PBS or in culture medium (without particles), and to perform time-resolved measurements. For example, it is possible to sample the cells every 1 or 24 h, depending on the doubling time of the cells, and to perform measurements to evaluate the distribution of the element of interest over the cell population and how these changes over time as a result of cell doubling and the resultant dilution effect.

Synchrotron radiation-based characterization techniques

Synchrotron radiation-based techniques such as XAFS are widely used techniques for determining the biotransformation of M-ENMs in plant and animal tissue. The technique provides information about the oxidation state and geometric and electronic structure of nearly all elements. There is no requirement for particle extraction from organisms when using XAFS, which minimizes any artifacts that may result from sample preparation. Moreover, samples in different states, including gas, liquid and solid, can be measured. Synchrotron facilities are typically operated by skilled researchers and technicians. Thus, the detailed parameters of XAFS measurement are not listed in this protocol. Here, only the selection of the detection mode is introduced since this is the key issue to be considered before data collection. Detection mode is related to the elemental concentration in the samples. A general rule is to choose transmission mode when the concentration is $>5\%$ of the total sample mass (i.e., $>5 \times 10^4 \text{ mg kg}^{-1}$ ($5 \times 10^4 \text{ mg L}^{-1}$ for liquid)), while fluorescence mode is preferred when the concentration is $1\text{--}5\%$ of the total sample mass, i.e., 1×10^4 to $5 \times 10^4 \text{ mg kg}^{-1}$. For the fluorescence mode, choose the multi-element solid-state detector for samples with elemental concentration of $10\text{--}500 \text{ mg kg}^{-1}$, and the Lytle detector for samples with elemental concentration of 500 mg kg^{-1} ($\sim 5\%$).

The distribution of elements and their species (speciation) can be obtained from synchrotron-based imaging techniques such as $\mu\text{-XRF}$ and STXM. These two techniques use different photon energies: the former uses $5\text{--}10 \text{ KeV}$ called hard X-ray, while the latter uses $<5 \text{ KeV}$ called soft X-ray. $\mu\text{-XRF}$ can map the element distribution over the samples with spatial resolution of several μm , which is constantly being improved towards the nanoscale. STXM characterizes samples with a resolution down to $\sim 10 \text{ nm}$. Based on these differences, the sample preparation is also different. For STXM, the samples need to be very thin (usually $<2 \mu\text{m}$) to allow the transmission of electrons through the samples. Note that there are currently no techniques that could replace XAFS and STXM. For $\mu\text{-XRF}$, if a synchrotron is not accessible, conventional 2D XRF can be used; however, the spatial resolution

and sensitivity can be limited owing to the energy of the X-ray source. Secondary ion mass spectrometry could be also used to investigate the biodistribution of M-ENMs, which has been described elsewhere^{31,32}.

Controls and data analysis

Some guidelines are available for M-ENM analysis, e.g., standards published by the International Organization for Standardization (ISO), which are focused mainly on quantification and measurement of ENMs (ISO 17200:2020) and measuring the size of ENM using electron microscopy (ISO 21363:2020). A guideline is under development by Organization for Economic Co-operation and Development (OECD) on the determination of concentrations of ENM in biological samples for (eco)toxicity studies. Nevertheless, a comprehensive protocol for dynamic quantification and characterization of M-ENMs in biological matrices for a broader application beyond a specific research field is missing.

It is critical to ensure that the sample preparation for each instrument and the particle extraction do not influence the physicochemical properties of the particle. There is no standard approach that is applicable for all types of M-ENMs, given their variability in properties and stability. Reference materials for very few elements are available, e.g., commercial Au-ENMs. The Joint Research Center of the EU^{33,34} provides reference materials for some types of M-ENMs (such as TiO₂ and Ag), which can be requested from the organization directly. Reference Au-ENMs of three different sizes could be used to determine the transport efficiency and obtain a calibration curve for particle size for spICP-MS. Performing such calibration for scICP-MS might, however, be problematic since quantification is achieved on the basis of the total mass of the element detected per cell. The blank samples could be Milli-Q (MQ) water. It is also important to note that the commercial Au particle standards expire after a few months owing to limited stability of the dispersion and the potential for bacterial contamination. All the applied chemicals and solution must be free of metals or at least free of the metal of interest.

It is critical to ensure that the background matrix in which the particles reside is totally digested. This is because the presence of debris and residuals in particulate form can interfere with the characterization and quantification of the particles. For example, the signals resulting from residuals in the samples when performing DLS can be mistaken as the signals of the main particles. It is also possible that the extraction method does not recover the dissolved metals from the samples because of biotransformation in the organisms or because of the sample preparation approach. To evaluate the capability of the method for extraction of the dissolved metals, the biological sample can be spiked with dissolved metal standards in a separate experiment. One must ensure not to use expired standards. Ionic metals of interest also need to be prepared immediately before measurement or before spiking the biological samples. The spiked samples must be used immediately or kept for, at most, a few days at 4 °C. Note that blank samples must be measured between the test samples to ensure there is no carryover from previous samples.

Materials

Biological samples

The presented workflow is tailored to analyzing M-ENMs in biological matrices, including cells, microorganisms, tissues, organs and organisms (animals and plants) as well as biological fluids. The protocol does not describe preparation of any exposure medium or how to perform the exposure to M-ENMs. The biological samples can include human blood and tissues as well. In the presented example, we used the unicellular algae *Pseudokirchinella subcapitata* as model of microorganisms and cells. These algae are commercially available (for example, from LGC Standards GmbH) and can be cultured and kept under controlled conditions⁶. We also used daphnids (*Daphnia magna*) and zebrafish (*Danio rerio*) as model organisms in this protocol. Both organisms are commercially available and can be supplied by different suppliers, e.g., Karlsruhe Institute of Technology, Dresden, Germany.

- *Pseudokirchinella subcapitata* cultured in Woods Hole algal medium and kept at Leiden University for many years
- Daphnids (*Daphnia magna*), cultured in M7 medium (OECD requirement)
- Zebrafish (*Danio rerio*) cultured in tap water by the University of South Bohemia České Budějovice
- Human primary brain microvascular endothelial cells (HBMECs; H-6023, 2B Scientific, Oxfordshire, UK), cultured by the University of Birmingham. HBMECs at passages 2–5 were maintained in EGM-2

Endothelial Cell Growth Medium-2 BulletKit (Lonza, Slough, UK) supplemented with 100 IU mL⁻¹ penicillin and 100 µg mL⁻¹ streptomycin on flasks coated with 1 µg cm⁻² fibronectin (Sigma-Aldrich) and 5 µg cm⁻² collagen I (Thermo Fisher Scientific)

- Human primary astrocytes (HAs; CC-2565, Lonza, Slough, UK), cultured by the University of Birmingham. HAs at passages 2–5 were cultured on 0.01% poly-L-lysine (ScienCell) coated flasks and maintained in AGM Astrocyte Growth Medium BulletKit (Lonza, Slough, UK) supplemented with 100 IU mL⁻¹ penicillin and 100 µg mL⁻¹ streptomycin. Cells were incubated at 37 °C in a water-saturated 5% CO₂ incubator
 - Cucumber (*Cucumis sativus*) in sterile ultrapure or one-fourth-strength Hoagland solution
- ! CAUTION** Performing experiments with vertebrate and human samples requires compliance with relevant institutional and national regulations and research ethics policies.

Reagents

▲ CRITICAL Users of this protocol are not restricted to reagents produced by specific companies or equipment models presented in this section. For simplicity of description, we refer to the reagents and equipment models that we applied to perform the protocol in our laboratories. This means that users of this protocol might apply the same materials and equipment but from different producers and manufacturers.

! CAUTION When working with chemicals, make use of the Chemical Handbook and follow laboratory safety rules.

- Sodium hydroxide pellets (NaOH; Sigma-Aldrich, CAS no. 795429)

! CAUTION NaOH is corrosive upon contact with the skin.

- Phosphate-buffered saline (PBS), pH 7.5 (Sigma-Aldrich, CAS no. 806552)
- Hydrochloric acid (HCl; Sigma-Aldrich, CAS no. 7647010)

! CAUTION HCl is corrosive upon contact with the skin.

- HNO₃ (Sigma-Aldrich, CAS no. 7697372)

! CAUTION HNO₃ is an oxidizing liquid and can lead to acute toxicity upon inhalation. It leads to skin corrosion and serious eye damage.

- Potassium hydroxide (KOH; Sigma-Aldrich, CAS no. 1310583)
- Hydrogen peroxide (H₂O₂), 30% w/w (Sigma-Aldrich, CAS no. 7722841)
- TMAH, 25% (Sigma-Aldrich, CAS no. 67630)

! CAUTION TMAH is a highly flammable liquid and vapor and is highly toxic if swallowed or in contact with skin. Open the bottle under a vented fume hood, and make sure that all parts of your skin, such as hands, arms, face and neck, are covered.

- Glutaraldehyde (OHC(CH₂)₃CHO; Sigma-Aldrich, CAS no. 111308)
- Spur's resin (Sigma-Aldrich, cat. no. EM0300)
- Optimal-cutting-temperature compound (OCT embedding medium for frozen tissue specimens; Agar Scientific, cat. no. AGR1180)
- Trypsin (Sigma-Aldrich, CAS no. 9002077)
- Sodium dodecyl sulfate (SDS; Sigma-Aldrich, CAS no. 151213)
- Ammonium acetate, pH 6.8 (NH₄CH₃CO₂; Sigma-Aldrich, CAS no. 631618)
- Pectinase, pH 4.5 (Sigma-Aldrich, CAS no. 9032751)
- Cellulase (Sigma-Aldrich, CAS no. 9012548)
- Hemicellulose (Sigma-Aldrich, CAS no. 9025563)
- Tricine (Sigma-Aldrich, CAS no. 5704041)
- Macerozyme R-10 (a multi-enzyme mixture containing 0.1 unit per mg cellulose, 0.25 unit per mg hemicellulose, and 0.5 unit per mg pectinase) (Gold Biotechnology, cat. no. M8002.0001)
- Reference Au-ENMs of three different sizes, e.g., 30, 50 and 100 nm (nanoComposix)

▲ CRITICAL Ensure that the reference Au-ENMs are stable against dissolution and aggregation, and do not freeze them. For dispersion, the particle suspension should be sonicated for 1 min at 40 W energy input using a tip sonicator.

Equipment

- Ultrapure water system (Milli-Q system; Merck Millipore, model no. C85358)
- Weighing scale (Denver SI-234, Max 230 g, model no. 22006821)
- Dounce tissue grinder, 7 mL (Wheaton, cat. no. 357542)
- ULTRA-TURRAX homogenizer with a stator diameter of 8 mm, rotor diameter of 6.1 mm, and maximum circumferential speed of 9.6 m s⁻¹ (IKA)

- Centrifuge tubes, 15 mL (Sigma-Aldrich, Corning, cat. no. 430791)
- Duran laboratory bottles, 250 and 500 mL (Sigma-Aldrich, cat. no. 70109091)
- Electrothermal stirring (Heidolph, D-91126 Schwabach, AC 230/240 V, 50/60 Hz)
- Centrifuge (Thermo Scientific Sorvall ST 16R Centrifuge)
- Freeze dryer (Martin Christ, Christ Alpha 1-2 LDplus)
- DLS instrument (Zetasizer Nanodevice, Malvern Panalytical)
- Cuvette (Malvern Panalytical Consumables for Zetasizer Series)
- Zeta cells (Malvern, disposable folded capillary cells)
- Transmission electron microscope (JEOL 1400 TEM operated at 80 kV accelerating voltage)
- TEM grids (Sigma-Aldrich, TEM-FCF100CU50, cat. no. 74199990)
- X-ray powder diffractometer (XRD instrument; Bruker, model no. D8 Advance)
- ICP-MS instrument (PerkinElmer NexION 300D ICP-MS)
- Filter paper (Whatman 111705, Nuclepore Hydrophilic Membrane, 0.1 µm pore size)
- Vacuum pump (Fisherbrand Diaphragm Pump)
- Bath sonicator (35 kHz frequency, DT 255, Bandelin Electronic, Sonorex Digital)
- Tip Sonicator (P30H Elmasonic bath sonicator, Elma Schmidbauer)
- Water bath (SDminiN; Taitec, cat. no. 0068750-000)
- Pipettes (Gilson: P1000, cat. no. F123602; P200, cat. no. F123601; P100, cat. no. F123615; P20, cat. no. F123600; P2, cat. no. F144801)
- Mylar film (Zhongcheng Insulating Material, cat. no. 6020)
- Freezing microtome (Microsystems, Leica Biosystems, Leica CM1950)

Software

- Software for TEM image analyses: ImageJ software (<https://imagej.nih.gov/ij/download.html>)
- Software for statistical analysis: SPSS version 23.0
- Software for particle number calculation: Microsoft Excel 2010
- Software for single-cell and single-particle application module of ICP-MS: Syngistix provided by PerkinElmer

Procedure 1: in situ quantification of cellular uptake of M-ENMs by scICP-MS analysis

Sample preparation

- 1 After exposure of cells to M-ENMs via in vitro experiments using cell lines or following isolation of cells from tissues of exposed organisms³⁵, sample preparation procedures are required to bring the cells into a state that is compatible with the single-cell analysis protocol. This can be achieved using option A for in vitro cellular experiments, or option B for single-microorganism analysis.

(A) Sample preparation for in vitro cellular experiments (e.g., cancer cell lines and blood–brain barrier cell lines) ● Timing: 1 h

- (i) Gently remove the exposure medium from the plates and wash the cells, which are attached to the plate (we are assuming adherent cells). To wash the cells, add a sufficient amount of 1× PBS to fully cover the cells (e.g., 3 mL for six-well plates), and immediately remove the PBS. Repeat this washing step three times to make sure that the exposure medium and the free particles are removed.
- (ii) Detach the cells from the plate using trypsin or any preferred material. We used trypsin for cells such as A549. Add a quantity of trypsin to each well to totally cover the cells, e.g., 1.5–2 mL for six-well plates. Incubate the cells for 3 min at 37 °C. After 3 min, remove the trypsin and add 2 mL of 1× PBS to each well if you are using six-well plates.
- (iii) Put the cells into 15 mL centrifuge tubes, and further dilute using 1× PBS (pH 7.5) to reach a final volume of 10 mL. For cells isolated from tissues, the cells must also be diluted using 1× PBS (pH 7.5) to reach a final volume of 10 mL.
- (iv) Count the number of the cells in the sample.

▲ **CRITICAL STEP** The final concentration of the cells in the diluted samples should be higher than 3,000 cells mL⁻¹.

▲ **CRITICAL STEP** Sample preparation of cells and microorganisms for single-cell analysis must be performed immediately before the measurement to avoid any changes in the cell community (for example, cell mortality or cell doubling).

? TROUBLESHOOTING

(B) Sample preparation for single-microorganism analysis (e.g., algae and bacteria) ● Timing: 1 h

- (i) After exposure, centrifuge the samples using 15 mL centrifuge tubes. The time and speed of centrifugation depend on the type of microorganism and must be optimized for each microorganism type separately. For algae cells, centrifugation (1,790g) for 10 min at 4 °C will separate most of the algae.

▲ CRITICAL STEP The conditions of centrifugation should not damage the microorganisms. This can be confirmed using TEM imaging or measurement of chlorophyll or DNA in the supernatant, for example³⁶. It is also helpful if preliminary tests are done to understand the effects of centrifugation on the M-ENMs of interest, as the centrifugation force should not influence the particles owing to their Brownian motion. This must be optimized.

- (ii) Separate the pellet of the microorganisms from the supernatant (exposure medium) by gently discarding the supernatant by pipetting.

? TROUBLESHOOTING

- (iii) Wash the pellet with 10 mL of 1× PBS (pH 7.5) in a 15 mL centrifuge tube, and centrifuge again as mentioned in Step 1B(i) to remove the unbound or loosely bound particles from the microorganisms' surfaces.

- (iv) Discard supernatant, and dilute the pellet in 1× PBS (pH 7.5) to reach a final volume of 10 mL. The volume depends on the concentration of cells present in the samples.

▲ CRITICAL STEP Sample preparation of cells and microorganisms for single-cell analysis must be performed immediately before the measurement to avoid any changes in the cell community (for example, cell mortality or cell doubling).

▲ CRITICAL STEP Note that M-ENMs in the exposure medium should be monitored, for example, by measuring the hydrodynamic size over time using DLS, to prevent agglomeration and dissolution because the presence of agglomerates in the sample may interfere with cell measurement. For example, an agglomerate could be measured as a cell using scICP-SM. For cells that are attached to the plate, the washing step before detaching the cells could be enough to remove the agglomerates. For dispersed cells such as algae, although we propose washing steps to remove agglomerates and unbound particles, the washing step may not be sufficient to remove all of the agglomerates³⁷. It is possible to check that the agglomerates are minimized using TEM images.

Quantification of cellular association using scICP-MS ● Timing: 4 h for setting up the instrument and 7 min per run

- 2 Set up the scICP-MS instrument according to the following conditions:

Sample uptake rate	0.02 mL min ⁻¹ for algae. The sample uptake rate should be selected such that it does not damage the cell of interest
Nebulizer	MEINHARD HEN (High Efficiency Quartz Concentric Nebulizers)
Spray Chamber	Asperon
Injector	2.0 mm id Quartz
RF Power	1,600 W
Dwell time	50 μs (this might change depending on the size of the cells, e.g., A549 cells are 15 μm in diameter on average; increase the dwell time for larger cells, decrease for smaller ones)

- 3 Calculate the transport efficiency of the Asperon spray chamber each time you perform the measurement, as described in the manual of the instrument for single-cell analysis.

? TROUBLESHOOTING

- 4 Take 1 mL of the sample for single-cell analysis from Step 1A(iv) or 1B(iv), and shake it gently by hand for 30 s before injection into the scICP-MS.

! CAUTION In this example, we used a sample volume of 1 mL; however, it is possible to use a much lower volume (μL) of the samples (see the instrument manual).

? TROUBLESHOOTING

- 5 Perform the measurement using scCIP-MS (setup described in Procedure 1, Stage 2) (we used a PerkinElmer NexION 300D ICP-MS instrument operating in single-cell mode, and the data acquisition was accomplished with the Syngistix Single-Cell Application Module).

▲ CRITICAL STEP Do not put many samples into the autosampler and have them waiting for measurement, because room temperature might damage the cells or facilitate doubling of the cells over time, which dilutes the concentration of the particles in each cell. The cells might also settle down out of the reach of the autosampler's probe. The measurement of each sample takes ~5–7 min. We recommend putting no more than three samples in the autosampler at a time, leaving the rest at 4 °C until their turn for measurement.

! CAUTION scICP-MS is best performed by an expert.

■ PAUSE POINT The number of particles can be calculated after performing the experiment.

? TROUBLESHOOTING

- If the total mass of M-ENMs in cells or the number of cells that contain the particles are required, this step can be disregarded. However, if the number of M-ENMs in cells or associated with each cell is of interest (with the assumption that no dissolution and agglomeration take place at any stage of exposure and measurement), the following formulae can be used:

For spherical M-ENMs: $V_{\text{single M-ENM}}(\text{cm}^3) = \frac{4}{3}\pi(r \times 10^{-7})^3$

Rod-shaped M-ENMs: $V_{\text{single M-ENM}}(\text{cm}^3) = h\pi(r \times 10^{-7})^2$

Particle number: $V_{\text{total M-ENMs}}(\text{cm}^3) = \frac{M}{\rho}$

$$N = \frac{V_{\text{total M-ENMs}}(\text{cm}^3)}{V_{\text{single M-ENM}}(\text{cm}^3)}$$

where:

$V_{\text{single M-ENMs}}(\text{cm}^3)$ = volume of one M-ENM (cm^3)

$V_{\text{total M-ENMs}}$ = Volume of total Au – NMs (cm^3)

M = mass (attogram)

ρ = density of the metal forming the particles; for example, Au = 19.3 g cm^{-3}

N = number of M-ENMs

Procedure 2: characterization and quantification of M-ENMs from cells, microorganisms and tissues of organisms following particle extraction

Extraction of M-ENMs from (micro)organisms, cells and tissues ● Timing: 1–2 d

- After exposure to M-ENMs, separate the cells or (micro)organisms from the exposure medium. The cells can be separated using centrifugation as described in in Step 1B(i). If using tissues of organisms, dissect the organisms immediately to separate the tissues and organs of interest.

! CAUTION The sample preparation for sectioning tissues, organs and cells is time consuming. It needs to be performed by a skilled technician.

! CAUTION After exposure of some organisms such as plants to ENMs, the particles might attach to the surface of the organisms. Thus, the organism needs to be washed to remove the attached particles as much as possible if internalization is the intended purpose.

- Homogenize the samples (cells, tissues or organs) using a suitable homogenizer depending on the quantity of the sample in a specific homogenization buffer. For example, samples of low quantity (up to 15 mL) might be homogenized using an ULTRA-TURRAX homogenizer (with a stator diameter of 8 mm, rotor diameter of 6.1 mm and maximum circumferential speed of 9.6 m s^{-1}). An ultrasonic homogenizer and a hand-held tissue homogenizer can also be used to totally homogenize the samples.

! CAUTION Different samples might require homogenizers with different power. For example, plant tissue might be homogenized with a different homogenizer compared with soft tissues of animals. Avoid any possibility of cross-contamination of samples, and remove the need for cleanup of disperser tools or vessels, for example, by using disposal plasticware. If the quantity of the samples is low (~1 mg), for example, if the samples are daphnids, pooling of the samples of the same replicate is possible. Thus, design the samples in a way to have sufficient organisms or tissues (~1 mg) for analysis following pooling.

- Dilute the homogenized tissues with 1 mL of MQ water, and sonicate using a tip sonicator. We used a model P30H ElmaSonic bath sonicator (Elma Schmidbauer) for 10 min to aid in breaking down tissues.

- Digest the resulting samples with a suitable digestive reagent as described in Table 1.

▲ CRITICAL STEP For carapace-containing animals, an additional step of digestion using 2 mL H_2O_2 (30%) at 70 °C for 12 h after digestion of soft tissues could help to fully digest the samples. For plant residuals, increasing the time of the digestion to 48 h could be helpful in digesting cellulose-based

Table 1 | Recommended digestive reagents and approaches for digesting background biological media to extract M-ENMs

Type of biological matrix	Digestive reagents and conditions for digesting biological sample in order to extract:	
	Metal (e.g., Ag-, Au-, Cu-ENMs)	Metal oxide (e.g., TiO ₂ -, CuO-, ZnO-ENMs)
Cells (e.g., cell lines or cells isolated from tissues), bacteria	Use 1 mL of 5% TMAH for 1 mL samples and incubate at 60 °C (in a water bath) for 30 min	2 mL of enzyme solution containing 0.05% (wt/vol) proteinase K, 50 mM ammonium bicarbonate as a buffer, and 0.05% (wt/vol) SDS for digesting 1 mL of samples. Stir gently at 50 °C for 1 h
Animal soft tissues and physiological medium	Use 1 mL of 10% TMAH for 0.1 g tissues, and incubate at 80 °C (in a water bath) for 1–3 h (ref. ³)	5 mL of enzyme solution containing 0.05% (wt/vol) proteinase K, 50 mM ammonium bicarbonate as a buffer, and 0.05% (wt/vol) SDS for digesting 0.1 g of samples. Stir gently at 50 °C in a water bath for 24 h (refs. ^{5,38})
Algae, fungi	Use 1 mL of 1% TMAH for ~3 × 10 ⁵ cells (1 mL), and incubate at 60 °C (in a water bath) for 1 h followed by additional post-treatment of the sample using 3 mL of H ₂ O ₂ (30%) at alkaline pH (7.5–8) in a water bath at 50 °C for 2–3 h (ref. ³)	2 mL Macerozyme R-10 for digesting a 1 mL sample. Adjust the pH to 4–6, and stir gently at 37 °C in a water bath for 6 h
Plant tissues	Use 1 mL 15% TMAH for 0.1 g of plant tissue, and incubate at 80 °C (in a water bath) for 12 h. Then add H ₂ O ₂ (30%) to the samples, and leave in a water bath at 50 °C for 12 h	3 mL of the enzyme solution for 1 g of sample. Adjust the pH to 4–6, and stir gently at 37 °C in a water bath for 24 h (ref. ³⁹)

matrices. By increasing the time of digestion, it is highly likely that the rigid samples are digested. If possible, the samples can be broken down or cut into small pieces to increase the surface area and facilitate the digestion.

? TROUBLESHOOTING

- Add 5 mL of 0.01% SDS solution to the obtained suspensions, followed by sonication for 1 min at a delivered power of 40 W. Adjust the pH of the dispersion to 7–8.5 using NaOH (0.1 M) or HCl (0.1 M).

▲ **CRITICAL STEP** The SDS helps to stabilize the extracted particles against agglomeration.

▲ **CRITICAL STEP** The sample physicochemical properties must be measured immediately because M-ENMs might undergo transformation, e.g., ion release and oxidation, over time.

■ **PAUSE POINT** The prepared dispersions can be stored at 4 °C for a maximum of 48 h depending on the type of particle. If the particles are made of Zn, Ag or Cu, we recommend continuing the procedure immediately.

? TROUBLESHOOTING

- Centrifuge the obtained suspensions using ultracentrifugation (e.g., Beckman Optima L-100 XP ultracentrifuge) at 180,000g and 4 °C for 4 h (ref. ⁴⁰).

! **CAUTION** spICP-MS and scICP-MS have a high sensitivity (ng L⁻¹). The size detection limit of spICP-MS depends on the type of the M-ENMs (e.g., ~40 nm for TiO₂ and ~10 nm for Au-ENMs). If the purpose is to use spICP-MS and scICP-MS for characterization and/or quantification of the particles, an enrichment step (Procedure 2, Step 6) is not required. Note that the centrifugation step is not required for scICP-MS, but it might be required for spICP-MS. If intending to use DLS for size measurement, enrichment might be required. Note that DLS cannot accurately measure the size distribution of particles on a particle-by-particle basis, but rather provides a broad peak spanning the range of sizes. If size distribution is of interest when using DLS, a separation step is required to separate the particles on the basis of size, which can be done using FFF⁴⁰. Performing FFF measurement is outside the scope of this protocol and requires experts to run the machine. Note that the concentration for NTA should be low (10⁷–10⁹ particles mL⁻¹), so dilution may be required.

Characterization of the extracted M-ENM ● **Timing: 2 h for setting up the instrument and 5 min per run**

- For quantification of extracted ENMs using spICP-MS, set up the spICP-MS instrument according to the following conditions:

spICP-MS settings

Sample uptake rate	Measure the sample uptake rate as follows: • Step 1: stop the peristaltic pump of the instrument • Step 2: prepare a known volume of water in a centrifuge tube, for example, 5 mL MQ water in a 15 mL tube • Step 3: put the probe of the autosampler into the 5 mL MQ water • Step 4: use a stopwatch. Reset the stopwatch to zero • Step 5: start the peristaltic pump and the stopwatch at the same time, and stop both after 3 min • Step 6: measure the volume of water taken up by the autosampler during the 3 min, and calculate the volume per minute
Nebulizer type	Quartz nebulizer
Spray chamber type	Glass cyclonic
Plasma gas flow	18 L min ⁻¹
Nebulizer gas flow	1.2 L min ⁻¹
Auxiliary gas flow	1.12 L min ⁻¹
Dwell time	The default is 50 μ s. This might change depending on concentration of the particles in the samples. For example, if the concentration is low, increase the dwell time
Acquisition time	The default is 100 s

? TROUBLESHOOTING

- Determine the transport efficiency using standard Au-EMNs of three different sizes, e.g., 30, 50 and 100 nm, prepared in MQ water to reach a final nominal concentration of 100,000 particles mL⁻¹.
! CAUTION Make sure the dispersion has not expired and that the particles are stable against agglomeration and dissolution. All samples and standards must be sonicated for 3–5 min before measurement. The standards must be made in the same matrix as the test samples. This also acts as a quality control (Box 1) for the potential effects of the extractant on the form of the metals (i.e., enables evaluation of whether it causes the formation of particles or causes particle dissolution).
- Establish the calibration curve (based on the mode ‘Dissolved’) for the element of interest by preparing three to five concentrations (e.g., 1 ppb, 2 ppb and 3 ppb) of the elements in 0.7% HNO₃ solution and measuring their metal concentration.
- Perform the spICP-MS measurement using the setup mentioned in Step 7 of Procedure 2 (we used a PerkinElmer NexION 300D ICP-MS instrument with the Syngistix Single-Particle Application Module).
! CAUTION The concentration of the particles in the sample should be low (~100,000 particles mL⁻¹) to avoid false signals generated from partial particle interaction.
! CAUTION Performing measurements using a spICP-MS instrument requires a skilled person.
! CAUTION Particles composed of multiple elements cannot be measured robustly in one run. In some cases, time-of-flight mass spectrometry performed in single-particle mode can measure single particles of multielements^{41,42}.
- Size and charge measurement of M-ENMs can be done using option A to measure the size distribution by NTA or option B to measure the zeta potential using laser Doppler electrophoresis.
 (A) **Measuring the size distribution using NTA** ● **Timing: 30 min for setting up the instruments and preparing the samples and ~1 min per run**
 - Prepare 5 mL of ENMs dispersion in MQ water with nominal concentrations of more than 10⁶ particles mL⁻¹.
 - Sonicate the dispersion for 5 min using a bath sonicator (at 100 amplitude) or for 3 min using a tip sonicator (20 W in 5 mL volume).
 - Analyze the sample using NTA following the manufacturer’s instructions (e.g., we use NanoSight’s NS200, Malvern) to measure the size distribution of the particles in the sample.
! CAUTION For performing measurements using NTA, the sample must be clean of any particles and debris except the particles of interest.
! CAUTION The size detection limit of NTA is approximately <30 nm to >1 μ m. The optimal concentration range is ~10⁶–10⁹ particles mL⁻¹.

Box 1 | Method validation and performance check ● **Timing: 1 d****Procedure**

Performance checks must be performed parallel to the main procedures in order to evaluate how each step of the sample preparation protocol influences the particles of interest. These should be done as follows:

- 1 Prepare a 10 mL dispersion (10 mg L^{-1}) of the pristine particles of interest in MQ water.

▲ **CRITICAL STEP** The dispersion must be prepared fresh on the day of the experiment.

? **TROUBLESHOOTING**

- 2 Sonicate the dispersion using a tip sonicator (power input 40 W) for 1 min, and immediately use it for the next step.
- 3 Measure the following parameters:
 - Hydrodynamic size of the particles using DLS⁵
 - Zeta potential using laser Doppler electrophoresis
 - Total mass concentration of the particles using ICP-MS⁵
 - Shape of the particles using TEM⁵
- 4 Digest the prepared dispersion with the digestive reagents used to digest the main samples (Table 1). Use the same amount of digestive reagent and the same approaches as performed on the main samples (Procedure 2, Steps 4 and 5), e.g., time of digestion and temperature.
- 5 Measure the parameters of step 3 of this Box again (i.e., hydrodynamic size, mass concentration and particle shape)
- 6 Evaluate the variation in the hydrodynamic size measured in steps 3 and 5 of this Box. The influence of the sample preparation method on the particle shape can be confirmed using TEM by comparing the images obtained in step 3 with those obtained in step 5. The nanomaterials image analysis tool NanoXtract (<http://enaloscloud.novamechanics.com/EnalosWebApps/NanoXtract/>) can be useful to measure the size of particles from TEM images on a particle-by-particle basis.
- 7 Calculate the mass recovery using the ICP-MS results as follows:

$$\text{Mass recovery} = \frac{\text{mass concentration of metal forming the particles resulted from step 5}}{\text{mass concentration of metal forming the particles resulted from step 3}}$$

- 8 Concentrate the dispersion resulting from Step 4 according to the protocol used for the main samples (Procedure 2, Step 6).
- 9 Repeat steps 5 and 6 of this Box again to evaluate the variation in the physicochemical properties of the particles (hydrodynamic size and mass), and calculate the recoveries.

(B) **Measuring the zeta potential using laser Doppler electrophoresis** ● **Timing: 20 min for setting up the instruments and preparing the samples and ~1 min per run**

- (i) Prepare 2 mL of ENMs dispersion of 0.5 g L^{-1} if the anticipated particle size is $<10 \text{ nm}$ or of 0.1 g L^{-1} if the anticipated particle size is in the range of $10\text{--}100 \text{ nm}$ (ref. ¹⁸).
- (ii) Perform sonication using a bath sonicator (at 100 amplitude) for 10 min or using a tip sonicator (20 W in 5 mL volume) for 3 min.
- (iii) Measure the zeta potential using laser Doppler electrophoresis following the manufacturer's instructions (e.g., we used the Zetasizer Nanodevice, Malvern Panalytical with a folded capillary cell).

Procedure 3: in situ characterization of M-ENMs biotransformation in biological matrices using synchrotron radiation-based techniques

- 1 The characterization of M-ENMs biotransformation in organisms using XAFS can be done using option A, to quantify M-ENMs in plants using μ -XRF use option B, to analyze M-ENMs in plant samples using STXM use option C, or to analyze M-ENMs in cell lines using STXM use option D. The users can choose the options depending on their needs. For example, XAFS is for obtaining information on the biotransformation of the whole bulk samples, while μ -XRF and STXM can be used to obtain in situ biotransformation information. Note that STXM can obtain higher spatial resolution than μ -XRF.

(A) **Characterization of M-ENMs biotransformation in organisms using XAFS** ● **Timing: 1 d per sample**

▲ **CRITICAL** In this section, we describe sample preparation for plants as model organisms. This procedure can be modified for different organisms.

- (i) Wash the fresh plant samples (can be, e.g., roots, leaves, stems or fruits) with tap water thoroughly, and rinse with deionized water.
- (ii) Rinse the samples with $0.1\% \text{ HNO}_3$, to remove any particles or M-ENMs that are adsorbed and aggregated on the plant surfaces.

▲ **CRITICAL STEP** You may skip this step when the sample is soft tissue of organisms.

- (iii) Place the plant samples in Falcon tubes, and quickly freeze in liquid nitrogen.

! **CAUTION** Use protective cryogenic gloves and glasses because liquid nitrogen causes severe cryogenic burns.

- (iv) Lyophilize the plant sample in a freeze dryer at $-50\text{ }^{\circ}\text{C}$ and 100 mBar vacuum.
- (v) Grind the dry sample into a fine powder using a mortar and pestle.
■ PAUSE POINT The samples can be stored at $-80\text{ }^{\circ}\text{C}$ until use.
▲ CRITICAL STEP Grind the samples as finely as possible since large fibers or granules may cause scattering effects that affect the quality of the data.
- (vi) Press the dry samples into thin slices using a tablet press (SS-0 Desktop Tablet Press).
▲ CRITICAL STEP When XAFS is measured in transmission mode, the thickness of the samples can affect the signal-to-noise ratio of the data, and thus the detection method needs to be selected before preparing the slices. Use Demeter software – Hephaestus – Formula’s function to estimate the optimized sample thickness (<https://bruceravel.github.io/demeter/>) for the selected slide thickness.
- (vii) Tape the sample slices onto a XAFS sample holder for analysis.
- (viii) Samples can be then measured using XAFS technique to obtain the fraction of different chemical species⁴³.
▲ CRITICAL STEP Use Kapton tape that contains low background element concentrations to avoid interference with the targeted element.

? TROUBLESHOOTING

(B) Quantification of M-ENMs in plants using μ -XRF ● Timing: 2 d per sample

- (i) To analyze a plant leaf, place the fresh leaf between two layers of Mylar film to flatten the leaf and then dry under vacuum.
▲ CRITICAL STEP Mylar film is recommended because it contains very low background elements and thus ensures no metallic contamination.
- (ii) To analyze plant roots, collect the fresh root tips (~2 cm length), and place them in OCT tissue freezing medium.
- (iii) Freeze the root samples immediately using dry ice.
- (iv) Obtain thin root cross sections (40 μm thickness) by cutting the roots on a freezing microtome.
- (v) Collect the sections onto 3- μm -thick Mylar films.
! CAUTION The sections are easy to break. Transfer them carefully to the film.
- (vi) Dry the sections at $-20\text{ }^{\circ}\text{C}$ in the freezing microtome.
■ PAUSE POINT The samples can be stored at $-20\text{ }^{\circ}\text{C}$ until use.
- (vii) Scan the samples at μ -XRF beamline¹⁴ to analyze the 2D distribution of elements in or the in situ chemical speciation of the elements by combining with XAFS.

(C) Analyzing M-ENMs in plant samples using STXM ● Timing: 2 d per sample

- ▲ CRITICAL** Since STXM requires the transmission of X-rays through samples, thin sections of ~1–3 μm are required.
! CAUTION If the electron density of the sample is low, it might take longer to find the targeted particles.
- (i) Cut and fix small plant tissues (root tips or leaf pieces) in 2.5% glutaraldehyde solution.
- (ii) After 24 h, transfer the samples into a graded acetone series (30% for 15 min, 50% for 15 min, 70% for 15 min, 90% for 15 min, and 100% for 30 min with three repeats), allowing dehydration.
- (iii) Immerse the samples in propylene oxide (twice, each for 15 min), and then allow resin filtration following this procedure: place in 2:1 mix of propylene oxide:resin for 1 h, 1:1 mix of propylene oxide:resin for 1 h, 1:2 mix of propylene oxide:resin for 1 h and then 100% resin overnight.
- (iv) Put the samples in fresh resin again under $65\text{ }^{\circ}\text{C}$ to allow embedding for 24 h.
- (v) Cut the samples into sections of 1.5 μm thickness, and place them onto a TEM copper grid or a silicon nitride (SiN) window for analysis.
! CAUTION Embedded samples could be permanently stored under dry conditions.
- (vi) Analyze samples using STXM beamline⁴⁴ to obtain the 2D distribution of different chemical species of the target element in the samples.

(D) Analyzing M-ENMs in cell lines using STXM ● Timing: 2–3 d per sample

- ▲ CRITICAL** Cell samples could be prepared according to the procedure described for plant samples in Step C(i–v) above. It is also possible to prepare the cell samples by directly culturing the cells on SiN window as described in the following steps:
- (i) Add the SiN window to the wells of the six-well culture dish before seeding the cells. Proceed to culture the cells and perform the M-ENM exposure as normal.

- (ii) At the end of the exposure time, aspirate the medium carefully.
- (iii) Wash the SiN window to remove loosely attached ENMs and other chemicals from the cells with PBS (1×, pH 7.4) at least three times.
- (iv) Drop 5 μ L 2.5% glutaraldehyde solution onto the window using a pipette to fix the cells.
! CAUTION The SiN window is very fragile. Operate this step very carefully.
- (v) Wash the cells again using deionized water two times.
- (vi) Dry the samples under vacuum for 24 h.
- (vii) Analyze the samples at STXM beamline¹³ to obtain the 2D distribution of different chemical species of the target elements in the cells

Troubleshooting

Troubleshooting advice can be found in Table 2.

Table 2 | Troubleshooting table

Step	Problem	Possible reason	Solution
Procedure 1			
1A(iii)	The number of the cells in the dispersion is low	Dilution of the samples decreased the number of cells (for some cells that are cultured at low cell number)	Preparing the cellular exposure in a larger number of replicates and pooling the replicates can solve the issue
1B(ii)	The separation of the bacterial pellets from the supernatant may lead to loss of some of the cells	The centrifugation might not separate all the bacteria from the supernatant	The centrifugation conditions, e.g., time and speed should be optimized carefully to increase the separation of the bacteria from the supernatant while not causing too much shear, which will damage the cells
3	Calculating the transport efficiency for the sICP-MS might be challenging	Dissolution of the particles used to calculate the transport efficiency, or the number of the particles are outside the operational range	Use a new standard dispersion of the particles, sonicate for 1 min and follow the manual of the instrument step by step, particularly in the new instruments produced after 2020
4	Absence of signal	The cells are large and immediately settle out	Do not use the autosampler, but keep shaking the samples, and immediately before the sample uptake by the autosampler's probe, put the sample into the autosampler
5	The background signal of the ions of interest is high	The cells are damaged, or the particles are dissolved	Make sure the particles are stable against dissolution by measuring the ion release from the particles using sICP-MS or by performing centrifugation to remove dissolved ions arising from storage. Keep the cells at 4 °C for a maximum of 30 min before use to minimize dissolution
Procedure 2			
4	The background signal of the ions of interest is high	The sample preparation steps might induce dissolution of the particles, or the ion of interest is high in the background matrices	Make sure that at each step of the sample preparation method the particles did not dissolve. Filter the particles before exposure to remove any ions released during storage to ensure only particles are present at the start of the experiment. Measure the background concentration of the element of interest (Box 2)
5	Precipitation of the dissolved metals	High pH due to the application of TMAH	Before performing the extraction experiment, perform an experiment to see at what pH the metals do not precipitate and adjust the samples to that pH
7	Detection of small particles, e.g., Au <10 nm, is problematic	Presence of interferences for the element of interest	There are limitations in the sICP-MS instrument that cannot be resolved now. Determine which elements are interfering with the main signal, and minimize the sources of those elements in your samples if possible
Procedure 3			
1A(viii)	XAFS signal is too low	Concentration of the target element is below or close to the limit of the detection mode	An alternative detector could be used, and the collection time could be increased. If the signal is still too low, then the samples are not suitable for XAFS analysis

Table continued

Table 2 (continued)

Step	Problem	Possible reason	Solution
Box 1			
1	The number of recovered particles (number of particles extracted from the sample/ number of introduced particles to a system) is low	The measured concentration is higher or lower than the nominal concentration (the amount of M-ENMs that is spiked in the samples)	If no dissolution or agglomeration occurs, the nominal concentration should not dramatically differ from the measured concentration. For experiments where particles of different sizes are used, particle number should be the unit, not mass concentration

Box 2 | Evaluating the influence of background matrix

Procedure

1 Evaluating the influence of the biological matrix on particle extraction ● Timing: 5 h

To evaluate the influence of background matrices on the measurements, the following steps must be performed:

- (A) Spike 1 g (wet weight) of the tissue samples or 1 mL of the cell or microorganisms with 1 mg of the particles or dissolved metals of interest
- (B) Homogenize the tissues using a suitable homogenizer, depending on the volume of the sample, to produce a paste of the sample.
! CAUTION Microorganisms or cells do not require homogenization, but the number of cells per mL is required information (can be determined using a hemocytometer or another cell counting device).
- (C) Treat the spiked samples according to the sample preparation method used for the main samples, including sample digestion and enrichment steps.

2 Evaluating the background metals ● Timing: 3 h

Some M-ENMs are made of elements that can be found in their ionic form in biological samples, such as Cu and Fe. The concentration of the metals of interest in the background matrices (biological samples) must be known. To measure the background concentration of the metals:

- (A) Put 1 g of the biological sample (tissues, microorganisms or cells) into a centrifuge tube.
- (B) Add 3 mL of digestive reagent such as HNO₃ or aqua regia (3 mL HCl: 1 mL HNO₃), and mix the sample in a water bath at 80 °C for 1 h. If the particles are made of metals that are not dissolvable using these agents (such as silica), perform microwave-assisted acid digestion⁴⁰.
! CAUTION Aqua regia is an aggressive digestive mixture and can cause serious injuries in case of inhalation or upon contact with skin. Use aqua regia in a fume hood empty of other reagents. As aqua regia quickly loses its effectiveness, prepare a fresh solution for each use. Do not store aqua regia in a closed container because it will oxidize over time and form toxic gases.
- (C) Dilute 1 mL of the suspension using 0.7% HNO₃ in MQ water to reach a final volume of 10 mL.
- (D) Measure the concentration of the element using ICP-MS.
! CAUTION In this section, the total concentration of the metals in the samples (ENM and biological matrix) is measured and the purpose is not to measure the particle concentration but rather to determine the total concentration of the metal of interest (i.e., the metal of which the ENM is composed).

Timing

Although the workflow can be applied by users from different backgrounds, the time required to perform each step depends on many factors such as the experience of the users in handling ENMs and biological samples, the type of biological sample (e.g., cells, tissues and organisms) and the type of M-ENM. The estimated timing for a person with experience in handling ENMs and analytical chemistry is as follows:

Procedure 1

Step 1A, sample preparation for in vitro cellular experiments: up to 1 h
 Step 1B, sample preparation for single-microorganism analysis: up to 1 h
 Steps 2–6, quantification of cellular association using scICP-MS: 4 h for setting up the instrument and 7 min per run

Procedure 2

Steps 1–6, extraction of M-ENMs from (micro)organisms and cells: 1–2 d
 Steps 7–11, characterization of the extracted M-ENM: 2 h for setting up the instrument and 5 min per run
 Step 11A, measuring the size distribution using NTA: 30 min for setting up the instruments and preparing the samples and ~1 min per run
 Step 11B, measuring the zeta potential using laser Doppler electrophoresis: 20 min for setting up the instruments and preparing the samples and ~1 min per run

Procedure 3

Step 1A, characterization of M-ENMs biotransformation in organisms using XAFS: 1 d per sample
Step 1B, quantification of M-ENMs in plants using μ -XRF: 2 d
Step 1C, analyzing M-ENMs in plant samples using STXM: 2 d
Step 1D, analyzing of M-ENMs in cell lines using STXM: ~2–3 d

Anticipated results

Comprehensive information about the dynamic behavior and biological fate of M-ENMs can be obtained using the presented protocol in laboratory or field-based studies. For example, information about size distribution and number of M-ENMs in physiological medium, associated with tissues, and organs can be generated. The quantity of ions released from particles in each tissue and cell and the biotransformation of the particles in a physiological medium can be measured over time. Note that, so far, there is no convergence in terms of size and particle number concentrations among the presented techniques^{45,46}, particularly if the particles undergo agglomeration. Nevertheless, this protocol is specifically developed for engineered particles but cannot distinguish if the particles are engineered or natural particles (e.g., biogenic particles or particles produced as a result of the local environmental chemistry).

For instance, we recently published a paper in which we applied spICP-MS to quantify the trophic transfer of Au-ENMs of different sizes and shapes (spherical 10, 60 and 100 nm, and rod-shaped 10 × 45 nm and 50 × 100 nm) in a food chain containing algae, daphnids and zebrafish³. The Au-ENMs were extracted from the organisms. Briefly, the organisms (algae, daphnids and fish tissues) were homogenized separately and diluted with 1 mL of MQ water. The homogenized samples were sonicated for 10 min and digested using 5% TMAH for 1 h at 70 °C. The obtained suspensions were dispersed using 5 mL of 0.05% SDS solution and sonicated for 5 min at a delivered power of 40 W. The same procedure was used to evaluate the influence of the sample preparation on the Au-ENMs. As shown in Fig. 4a, the size distribution and the mode of size distribution (the mode represents the particle size most commonly found in the distribution) of the Au-ENMs did not change dramatically for any of the tested particles when treated with 5% TMAH. We applied spICP-MS to measure the particle number and number-based size distribution (representing the number of particles in each class of particle size) in each organism. As an example, Fig. 4b shows the particle number concentration of the 10 and 60 nm Au-ENMs in daphnids and in the algae used as feed for the daphnids. The particle number was measured using this protocol after extraction of the particles from the organisms. We also applied spICP-MS to measure ion release from the particles in each organism to determine the biotransformation of the particles. As an example, we provided here the data obtained for daphnids (Fig. 4c; the data for other organisms can be found in the original article³). Fig. 4c shows the percentage of the Au-ENMs present as particles and Au ions in daphnids for each particle size and shape. It can be seen that the size and shape of Au-ENMs influences their extent of dissolution in daphnids, where a high percentage of the spherical 10 nm (96%) and rod-shaped 10 × 45 nm (99%) are present in the form of particulate materials while a notable amount of the spherical 100 nm (39%) ENM underwent dissolution in daphnids.

In another study, we applied scICP-MS to confirm that the association of M-ENMs with microorganisms is stochastic with a large distribution across a population of microalgal cells⁶. Accordingly, microalgae were exposed to Au-ENMs of different sizes and shapes (spherical 10 nm, spherical 60 nm, spherical 100 nm, rod-shaped 10 × 40 nm and rod-shaped 50 × 100 nm) for 72 h. After sample preparation according to this protocol, the amount of Au-ENMs associated with or accumulated in the algae was quantified on a cell-by-cell basis. The data showed that association of Au-ENMs with algal cells had a random probability according to a so-called stochastic process with no correlation with the size and shape of the particles (Fig. 4d)⁶. The number of Au-ENMs per cell was found to be heterogeneously distributed as some cells had significantly higher numbers (e.g., up to 600 spherical 10 nm particles per cell) of Au-ENMs than other cells present in the same medium.

The ability of synchrotron techniques to determine and distinguish the transformation products from the pristine ENMs is demonstrated in the examples shown in Fig. 5. Fig. 5a shows typical XAFS spectra of the element Ce that were collected in cucumber root, stem and leaf samples treated with CeO₂ ENMs. Three typical features can be observed from the spectra. Feature 1 is attributed to the Ce(III) oxidation state that is present in chemical species such as CePO₄ and Ce(NO₃)₃ herein, while features 2 and 3 are attributed to CeO₂ ENMs. A mixture of the three features can be clearly seen in

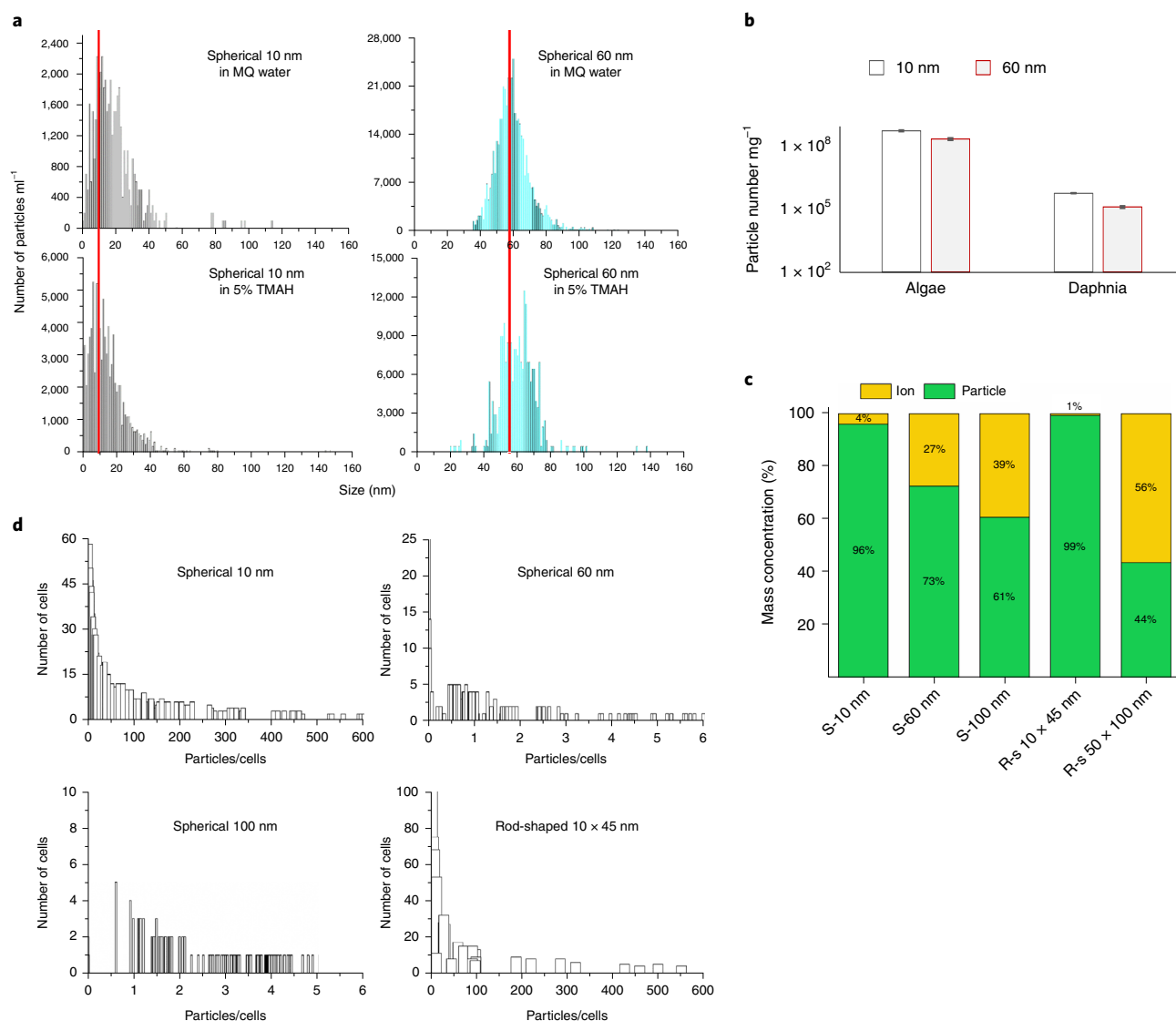


Fig. 4 | Performance check and particle measurement. **a**, Evaluation of the influence of the sample preparation steps of particle extraction using 5% TMAH on the size and size distribution of Au-ENMs of two different sizes. The gray and blue histograms show the number-based size distributions of the spherical 10 nm particles and the spherical 60 nm particles, respectively. The red line shows the mode of the size distribution. **b**, Particle number (mean $\pm$ standard deviation, per mg wet weight tissue) of Au-ENMs accumulated in daphnids and their feed (algae) as a function of particle size. **c**, The percentage of Au-ENMs mass to the total Au mass and the percentage of dissolved Au mass to the total Au mass. **d**, The number of Au-NP accumulated in or on the surface of each algal cell within a given population as a function of particle size and shape measured using sICP-MS. **a–c** adapted from ref. ³; **d** adapted from ref. ⁶.

the plant samples, suggesting the transformation of a proportion of the CeO_2 ENMs in situ. The transformation can be also quantified by linear combination fitting of the spectra using CeO_2 , CePO_4 and $\text{Ce}(\text{CH}_3\text{COO})_3$ as the reference compounds. It shows that 34% of the Ce in roots is present in the form of CePO_4 , while 13.6% and 21.5% were Ce carboxylates in stem and leaf samples, respectively.

Fig. 5b shows an XRF image of root sections of cucumber that were treated with CeO_2 ENMs, La_2O_3 ENMs and ionic Ce (CeCl_3). The normalized X-ray fluorescence intensities are scaled from minimum (blue) to maximum (red). In the CeO_2 ENM treatment, the highest Ce accumulation was found in peripheral parts of the rhizodermis. In contrast, in La_2O_3 ENMs and Ce^{3+} -treated roots, the La or Ce element distributed not only along the peripheral part but also along the endodermis, suggesting that La_2O_3 ENMs behaved more like ions, which is different from the behavior of the CeO_2 ENMs.

The last example shows the biotransformation of Ag ENMs in human cells (Fig. 5c). Ag ENMs of different shapes, i.e., Ag nanodiscs (Ag ND) and Ag nanospheres (Ag NS), were applied from the

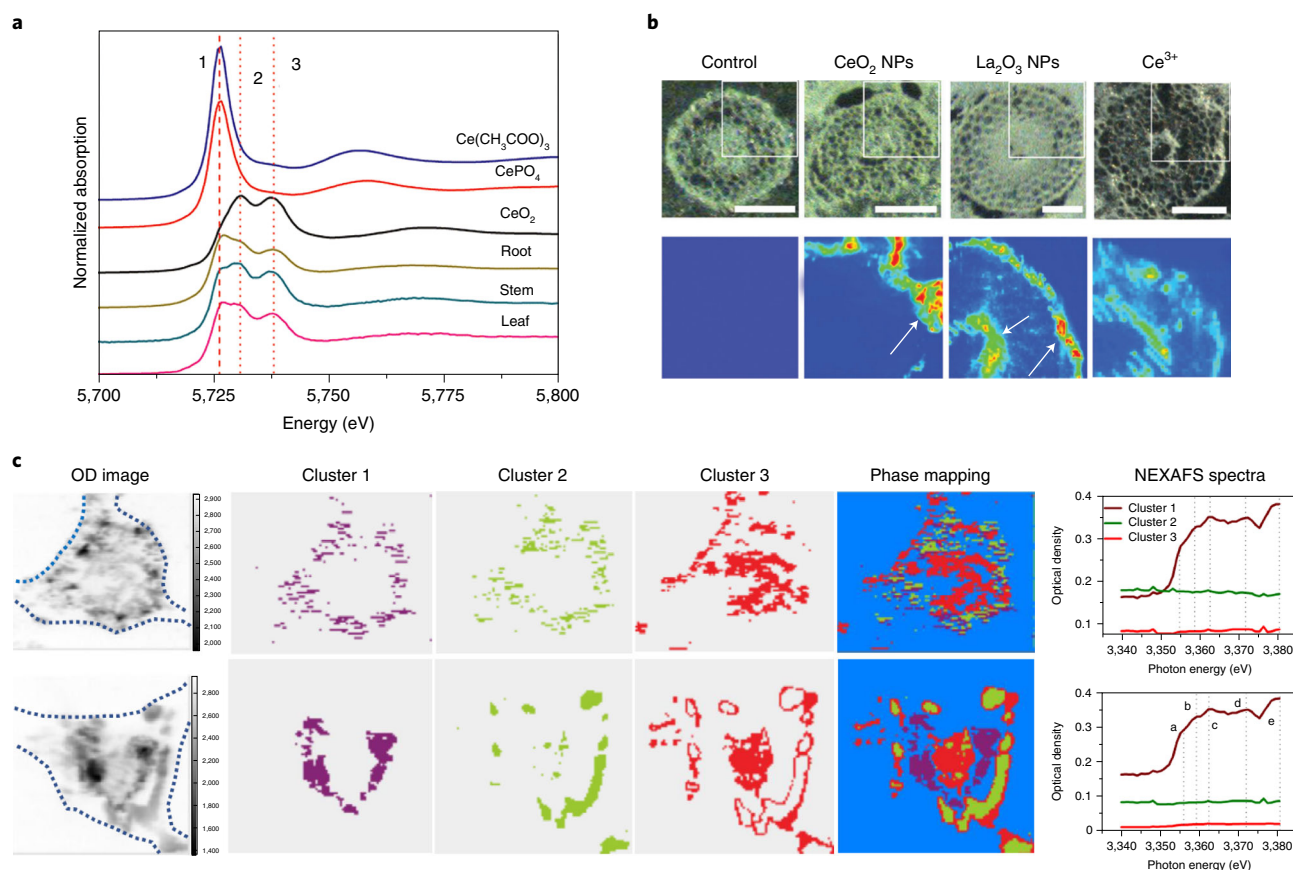


Fig. 5 | Biotransformation and distribution of ENMs analyzed using synchrotron techniques. a, XAFS Ce LIII-edge spectra (5,723 eV) of root, stem and leaf of cucumber plants treated with 2,000 mg L⁻¹ CeO₂ ENMs for 21 d. The vertical dashed line and dotted lines mark the features of Ce(III) (1) and Ce(IV) (2,3) compounds, respectively. Ce(CH₃COO)₃, CePO₄ and CeO₂ are used as references. The XAS spectra were collected on beamline 1W1B at the Beijing Synchrotron Radiation Facility. Ce LIII-edge spectrum of the root sample was collected using transmission mode. Fluorescence mode was applied for collection of CeLIII-edge spectra of stems and leaves using a 19-element germanium array solid detector. **b**, μ-XRF images of root sections of cucumber that were treated with CeO₂ NPs, La₂O₃ NPs and CeCl₃ (Ce³⁺). Top and bottom panels show the optical image and the μ-XRF images of the root sections, respectively. The white scale bars represent 100 μm. The samples were analyzed on the BL15U beamline at Shanghai Synchrotron Radiation Facility (Shanghai, China). The cross-section of the beam irradiating the samples was about 5 × 7 mm², and the sample holder was moved at an interval of 5 μm so the beam can scan the samples to obtain the fluorescence image. **c**, STXM images of Ag nanodiscs (Ag ND) and Ag nanospheres (Ag NS) in human primary brain microvascular endothelial cells (HBMECs) established as an in vitro BBB model. The measurements were performed at beamline I08 of the Diamond Light Source. The gray images (OD, images) were the transmission images of the cells to identify the shapes of the cells. Clusters 1–3 indicate the distribution (clustering) of the three different compositions (cluster 1 is Ag compounds, while clusters 2 and 3 are cellular components) obtained by cluster analysis of stack images, which were acquired at multiple energies spanning the relevant element absorption edge (from 3,340 to 3,380 eV for Ag L edge). The STXM image (the phase mapping) was the overlay of the cluster images showing the in situ distribution of Ag chemical species. Ag L-edge NEXAFS spectra were then subtracted from the STXM image for analysis of the chemical speciation of Ag. **a** and **b** reproduced from ref. ¹⁴; **c** reproduced from ref. ¹³.

apical side of an in vitro transwell blood–brain barrier model (BBB), to examine whether the Ag ENMs transform and transport into and through the BBB cell layer. The optical density (OD) images show the shape of the cells. Cluster analysis identifies that cluster 1 is the Ag species by the L-edge near edge X-ray absorption fine structure (NEXAFS) spectra extracted from the phase mapping. Linear combination fitting analysis suggested that the chemical species of the Ag clusters in the Ag nanodisc and Ag nanosphere treatments were a mixture of particulate Ag and Ag-cysteine. There was 62% and 38% of particulate Ag and Ag-cysteine in the Ag-ND group and 74% and 26% in the Ag-NS group, respectively. The results suggest the Ag ENMs are transformed following uptake into the human BBB cells.

Data availability

All the data that support the plots within this paper are reported in the protocol.

References

1. Fadeel, B. et al. Advanced tools for the safety assessment of nanomaterials. *Nat. Nanotechnol.* **13**, 537–543 (2018).
2. Drobne, D., Novak, S., Talaber, I., Lynch, I. & Kokalj, A. The biological fate of silver nanoparticles from a methodological perspective. *Materials* **11**, 957 (2018).
3. Abdolapur Monikh, F. et al. Particle number-based trophic transfer of gold nanomaterials in an aquatic food chain. *Nat. Commun.* **12**, 899 (2021).
4. Chetwynd, A. J., Zhang, W., Thorn, J. A., Lynch, I. & Ramautar, R. The nanomaterial metabolite corona determined using a quantitative metabolomics approach: a pilot study. *Small* **16**, 2000295 (2020).
5. Abdolapur Monikh, F. et al. Method for extraction and quantification of metal-based nanoparticles in biological media: number-based biodistribution and bioconcentration. *Environ. Sci. Technol.* **53**, 946–953.
6. Abdolapur Monikh, F. et al. The stochastic association of nanoparticles with algae at the cellular level: effects of NOM, particle size and particle shape. *Ecotoxicol. Environ. Saf.* **218**, 112280 (2021).
7. Arenas-Lago, D., Abdolapur Monikh, F., Vijver, M. G. & Peijnenburg, W. J. G. M. Dissolution and aggregation kinetics of zero valent copper nanoparticles in (simulated) natural surface waters: simultaneous effects of pH, NOM and ionic strength. *Chemosphere* **226**, 841–850 (2019).
8. Werlin, R. et al. Biomagnification of cadmium selenide quantum dots in a simple experimental microbial food chain. *Nat. Nanotechnol.* **6**, 65–71 (2011).
9. Abdolapur Monikh, F. et al. Development of methods for extraction and analytical characterization of carbon-based nanomaterials (nanoplastics and carbon nanotubes) in biological and environmental matrices by asymmetrical flow field-flow fractionation. *Environ. Pollut.* **255**, 113304 (2019).
10. Abdolapur Monikh, F., Chupani, L., Vijver, M. G., Vancová, M. & Peijnenburg, W. J. G. M. Analytical approaches for characterizing and quantifying engineered nanoparticles in biological matrices from an (eco) toxicological perspective: old challenges, new methods and techniques. *Sci. Total Environ.* **660**, 1283–1293 (2019).
11. Milosevic, A., Romeo, D. & Wick, P. Understanding nanomaterial biotransformation: an unmet challenge to achieving predictive nanotoxicology. *Small* **16**, 1907650 (2020).
12. Abdolapur Monikh, F. et al. Engineered nanoselenium supplemented fish diet: toxicity comparison with ionic selenium and stability against particle dissolution, aggregation and release. *Environ. Sci. Nano* **7**, 2325–2336 (2020).
13. Guo, Z. et al. Biotransformation modulates the penetration of metallic nanomaterials across an artificial blood–brain barrier model. *Proc. Natl Acad. Sci. USA* **118**, 1–10 (2021).
14. Ma, Y. et al. Origin of the different phytotoxicity and biotransformation of cerium and lanthanum oxide nanoparticles in cucumber. *Nanotoxicology* **9**, 262–270 (2015).
15. Rees, P., Wills, J. W., Brown, M. R., Barnes, C. M. & Summers, H. D. The origin of heterogeneous nanoparticle uptake by cells. *Nat. Commun.* **10**, 2341 (2019).
16. Santra, S. et al. TAT conjugated, FITC doped silica nanoparticles for bioimaging applications. *Chem. Commun.* 2810 (2004). <https://doi.org/10.1039/b411916a>
17. Salvati, A. et al. Experimental and theoretical comparison of intracellular import of polymeric nanoparticles and small molecules: toward models of uptake kinetics. *Nanomed. Nanotechnol. Biol. Med.* **7**, 818–826 (2011).
18. Zhang, P., Misra, S., Guo, Z., Rehkämper, M. & Valsami-Jones, E. Stable isotope labeling of metal/metal oxide nanomaterials for environmental and biological tracing. *Nat. Protoc.* **14**, 2878–2899 (2019).
19. Clark, N. J., Clough, R., Boyle, D. & Handy, R. D. Development of a suitable detection method for silver nanoparticles in fish tissue using single particle ICP-MS. *Environ. Sci. Nano* **6**, 3388–3400 (2019).
20. Sung, H. K. et al. Analysis of gold and silver nanoparticles internalized by zebrafish (*Danio rerio*) using single particle-inductively coupled plasma-mass spectrometry. *Chemosphere* **209**, 815–822 (2018).
21. Jiménez-Lamana, J., Wojcieszek, J., Jakubiak, M., Asztemborska, M. & Szpunar, J. Single particle ICP-MS characterization of platinum nanoparticles uptake and bioaccumulation by: *Lepidium sativum* and *Sinapis alba* plants. *J. Anal. Spectrom.* **31**, 2321–2329 (2016).
22. Xu, X., Chen, J., Li, B., Tang, L. & Jiang, J. Single particle ICP-MS-based absolute and relative quantification of *E. coli* O157 16S rRNA using sandwich hybridization capture. *Analyst* **144**, 1725–1730 (2019).
23. Loeschner, K. et al. Detection and characterization of silver nanoparticles in chicken meat by asymmetric flow field flow fractionation with detection by conventional or single particle ICP-MS. *Anal. Bioanal. Chem.* **405**, 8185–8195 (2013).
24. Corte Rodríguez, M., Álvarez-Fernández García, R., Blanco, E., Bettmer, J. & Montes-Bayón, M. Quantitative evaluation of cisplatin uptake in sensitive and resistant individual cells by single-cell ICP-MS (SC-ICP-MS). *Anal. Chem.* **89**, 11491–11497 (2017).
25. Abdolapur Monikh, F. et al. A dose metrics perspective on the association of gold nanomaterials with algal cells. *Environ. Sci. Technol. Lett.* **6**, 732–738 (2019).
26. Li, S., Liu, Y. & Ma, Q. Nanoparticle-based electrochemiluminescence cytosensors for single cell level detection. *TrAC Trends Anal. Chem.* **110**, 277–292 (2019).
27. Lee, S. et al. Nanoparticle size detection limits by single particle ICP-MS for 40 elements. *Environ. Sci. Technol.* **48**, 10291–10300 (2014).

28. Talbot, M. J. & White, R. G. Methanol fixation of plant tissue for scanning electron microscopy improves preservation of tissue morphology and dimensions. *Plant Methods* **9**, 36 (2013).
29. Filipe, V., Hawe, A. & Jiskoot, W. Critical evaluation of nanoparticle tracking analysis (NTA) by NanoSight for the measurement of nanoparticles and protein aggregates. *Pharm. Res.* <https://doi.org/10.1007/s11095-010-0073-2> (2010).
30. Montaña, M. D., Olesik, J. W., Barber, A. G., Challis, K. & Ranville, J. F. Single particle ICP-MS: advances toward routine analysis of nanomaterials. *Anal. Bioanal. Chem.* **408**, 5053–5074 (2016).
31. Johnson, M. E. et al. Combining secondary ion mass spectrometry image depth profiling and single particle inductively coupled plasma mass spectrometry to investigate the uptake and biodistribution of gold nanoparticles in *Caenorhabditis elegans*. *Anal. Chim. Acta* **1175**, 338671 (2021).
32. Wagener, S. et al. Determination of nanoparticle uptake, distribution, and characterization in plant root tissue after realistic long-term exposure to sewage sludge using information from mass spectrometry. *Environ. Sci. Technol.* **53**, 5416–5426 (2019).
33. Singh, C. et al. Zinc oxide NM-110, NM-111, NM-112, NM-113 characterisation and test item preparation, NM-series of representative manufactured nanomaterials. *EUR - Scientific and Technical Research Reports* (2011).
34. Rasmussen, K. et al. *Titanium Dioxide, NM-100, NM-101, NM-102, NM-103, NM-104, NM-105: Characterisation and Physico- Chemical Properties*. JRC (2014).
35. Naito, H. et al. Isolation of tissue-resident vascular endothelial stem cells from mouse liver. *Nat. Protoc.* **15**, 1066–1081 (2020).
36. Takahashi, T. Routine management of microalgae using autofluorescence from chlorophyll. *Molecules* **24**, 4441 (2019).
37. Johnson, M. E. et al. Separation, sizing, and quantitation of engineered nanoparticles in an organism model using inductively coupled plasma mass spectrometry and image analysis. *ACS Nano* **11**, 526–540 (2017).
38. Peters, R. J. B. et al. Development and validation of single particle ICP-MS for sizing and quantitative determination of nano-silver in chicken meat. *Anal. Bioanal. Chem.* **406**, 3875–3885 (2014).
39. Dan, Y. et al. Characterization of gold nanoparticle uptake by tomato plants using enzymatic extraction followed by single-particle inductively coupled plasma–mass spectrometry analysis. *Environ. Sci. Technol.* **49**, 3007–3014 (2015).
40. Velimirovic, M. et al. Accurate quantification of TiO₂ nanoparticles in commercial sunscreens using standard materials and orthogonal particle sizing methods for verification. *Talanta* **215**, 120921 (2020).
41. Hendriks, L., Gundlach-Graham, A. & Günther, D. Analysis of inorganic nanoparticles by single-particle inductively coupled plasma time-of-flight mass spectrometry. *CHIMIA Int. J. Chem.* **72**, 221–226 (2018).
42. Praetorius, A. et al. Single-particle multi-element fingerprinting (spMEF) using inductively-coupled plasma time-of-flight mass spectrometry (ICP-TOFMS) to identify engineered nanoparticles against the elevated natural background in soils. *Environ. Sci. Nano* **4**, 307–314 (2017).
43. Zhang, P. et al. Biotransformation of ceria nanoparticles in cucumber plants. *ACS Nano* **6**, 9943–9950 (2012).
44. Zhang, P. et al. Comparative toxicity of nanoparticulate/bulk Yb₂O₃ and YbCl₃ to cucumber (*Cucumis sativus*). *Environ. Sci. Technol.* **46**, 1834–1841 (2012).
45. Schavkan, A. et al. Number concentration of gold nanoparticles in suspension: SAXS and spICPMS as traceable methods compared to laboratory methods. *Nanomaterials* **9**, 502 (2019).
46. Petersen, E. J. et al. Determining what really counts: modeling and measuring nanoparticle number concentrations. *Environ. Sci. Nano* **6**, 2876–2896 (2019).

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Author contributions

F.A.M. conceptualized, wrote and reviewed the paper and designed the figures. P.Z. and Z.G. contributed to writing, editing and reviewing the paper. E.V.-J., I.L. and W.P. contributed to structuring, editing and reviewing the paper. M.V. contributed to editing the paper.

Competing interests

The authors declare no competing interests.

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