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Nanomaterials in the Environment

Critical Review

ANALYSIS OF ENGINEERED NANOMATERIALS IN COMPLEX MATRICES (ENVIRONMENT AND BIOTA): GENERAL CONSIDERATIONS AND CONCEPTUAL CASE STUDIES

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Abstract—Advances in the study of the environmental fate, transport, and ecotoxicological effects of engineered nanomaterials (ENMs) have been hampered by a lack of adequate techniques for the detection and quantification of ENMs at environmentally relevant concentrations in complex media. Analysis of ENMs differs from traditional chemical analysis because both chemical and physical forms must be considered. Because ENMs are present as colloidal systems, their physicochemical properties are dependent on their surroundings. Therefore, the simple act of trying to isolate, observe, and quantify ENMs may change their physicochemical properties, making analysis extremely susceptible to artifacts. Many analytical techniques applied in materials science and other chemical/biological/physical disciplines may be applied to ENM analysis as well; however, environmental and biological studies may require that methods be adapted to work at low concentrations in complex matrices. The most pressing research needs are the development of techniques for extraction, cleanup, separation, and sample storage that introduce minimal artifacts to increase the speed, sensitivity, and specificity of analytical techniques, as well as the development of techniques that can differentiate between abundant, naturally occurring particles, and manufactured nanoparticles. Environ. Toxicol. Chem. 2012;31:32–49. © 2011 SETAC

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INTRODUCTION

In the field of materials science, characterization approaches for engineered nanomaterial (ENM) properties are underdeveloped, including the use of multiple complementary analytical methods [1]. By extension, quantifying and characterizing ENMs in complex matrices such as soils, sediments, and biological tissues is a nascent endeavor. However, because of the biologically and environmentally relevant concentrations involved, and the inherent sample heterogeneity, development of techniques for detection and characterization in complex media is inherently more challenging than the development of basic materialcharacterization techniques. For example, basic dynamic light scattering is only applicable in fairly simple, homogeneous systems, at particle concentrations typically exceeding 1 mg/L, depending on the material. In many cases, new approaches must be developed or adapted. An area of great need is the development of rapid, sensitive techniques that can be applied in real time to support exposure characterization during toxicity testing.

Some approaches used in traditional chemical analysis of environmental and biological samples may be adapted; however, nanomaterials exist in colloidal systems with inherent discontinuities of properties, and therefore, detection and analysis need to address not only chemical but also physical form. As a result, either traditional approaches used for analyzing organic molecules and trace elements in complex matrices must be modified or new approaches should be developed. Application of techniques used in traditional colloid science can serve as a good starting point [2]. Engineered nanomaterial behavior and toxicity are influenced by a wide variety of physical and chemical properties, such as chemical composition, surface functionality, particle size, surface area, redox state, crystallinity, and solubility [3]. Future analytical strategies must be tailored to account for not only the sample and particle type, but also the specific question or hypothesis to be addressed, because characterizing every possible property for every sample would be impossible.

This paper outlines approaches for detecting and characterizing nanomaterials in complex media and identifies areas in which additional research and development are needed as well as potential pathways forward in the development of appropriate techniques. We discuss sample preparation, storage, and analysis. Current analytical approaches are also described,

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including traditional approaches based on separation and detection; imaging-based approaches; and the possible use of biological sensor-based approaches. Using four case studies, we highlight how an intended analytical approach should be customized based on the nanomaterial in question, the sample matrix, and the hypothesis or objective. Finally, we outline the most critical and pressing research needs for detecting and analyzing nanomaterials in complex matrices. The first case study addresses the analysis of carbon nanomaterials (CNMs). This case study highlights the difference between the analytical workflow for a carbonaceous particulate nanomaterial and the analysis of a discreet organic molecule. The second case study discusses the analysis of quantum dots (QDs) and engineered silver nanoparticles (AgNP) in biological systems. This case study illustrates how imaging, separation, and spectroscopy can be combined to address the bioavailability and toxicity of materials that are both redox sensitive and soluble. The third case study addresses detection of TiO_2 and CeO_2 nanoparticles in sediments. This case study highlights the approach for analyzing insoluble ENMs that are either redox insensitive (TiO_2) or highly redox active (CeO_2) in the presence of naturally high background geogenic sources of these materials. Finally, the fourth case study highlights potential approaches for screening sewage for engineered AgNPs. This case study shows how separations and spectroscopy might be combined to monitor potential inputs of Ag materials to the environment.

OVERVIEW

Sample collection, preparation, preservation, and storage

Presently, there is a complete lack of research on techniques for collecting, preserving, and storing samples containing ENMs. Which techniques are appropriate will depend largely on the sample type, property of interest, and analytical method to be used. Nanoparticulate systems are extremely sensitive to perturbation from factors such as pH, ionic strength, sunlight, bacterial growth, and temperature; and they are almost never in thermodynamic equilibrium [4]. In some cases, sample preservation may not be possible for a given property of interest that must be analyzed, such as aggregation state. In other cases, sample preparation steps may be taken to preserve sample fractions for subsequently measuring properties of interest, such as particle concentrations. For example, Ag ENMs are extremely redox active. Quantification of dissolved Ag ions in aquatic toxicity testing media does not allow for storage of whole unfractionated samples, because the oxidative dissolution process may not be at equilibrium. [5]. Dissolved ions first must be separated from the system using techniques such as ultracentrifugation or ultrafiltration, and then preserved for subsequent Ag analysis using an appropriate technique. Similarly, determining Ag ENM aggregate sizes in a test system may require real-time kinetic measurements, because aggregation rate may be rapid or aggregate size distribution may not reach equilibrium during the test. In some cases, sample storage may be necessary, such as for imaging studies or for analysis at synchrotron light source facilities. The case studies presented here highlight specific considerations for each situation.

Analytical strategies: The need for multiple lines of evidence

Nanoparticulate systems are complex; thus, multiple orthogonal lines of evidence are needed to detect and physicochemically characterize nanoparticles in complex media. In traditional chemical analytical techniques, one must identify chemical species in at least two independent ways. For example, in gas chromatography-mass spectrometry of volatile organic compounds, both retention time and mass would be the minimum information needed to identify a compound. In more complex cases, retention time, mass, fragmentation pattern, and isotopic signature would be needed. Nanoparticulate systems may have even more rigorous requirements for identification and quantification, relying on numerous techniques because they are not discrete molecular species. For example, identification of CeO_2 nanoparticles in a soil solution may require separation based on particle size, verification of separation using light scattering techniques, chemical identification using inductively coupled plasma-mass spectrometry (ICP-MS), and ultimately, examination of particle size distribution, crystal structure, and chemical composition using transmission electron microscopy (TEM). Each of these is subject to specific artifacts that must be taken into account, and independent measures of each physicochemical property of interest are therefore desirable for validation purposes.

Of course, each technique must be validated using appropriate traceable standards, quality control procedures, and if possible, standard reference materials. Presently, few standard reference materials are available for nanomaterials. The National Institute of Standards and Technology of the United States has recently made standard reference Au-engineered nanoparticles (ENPs). These materials are provided as aqueous suspensions. Standard reference materials that consist of complex matrices with certified concentrations of analyte are not available for ENMs. Producing such standard reference materials is a major challenge, because of the inherent instability of ENMs. Analytes in standard reference materials typically must be stable for years.

Available analytical approaches

A wide range of analytical tools is available to examine systems containing ENMs, and all carry great promise but also limitations inherent to either the physical, chemical, or even biological principles that they are based on, or the current state of technology. One of the first challenges is the ability to detect a given type of material within a matrix. Element-specific techniques provide invaluable help with this task. The fastest expanding technique is most likely ICP-MS, which, within a few years, had its status changed from an advanced technique to a routine analytical method. A number of X-ray–based techniques, such as X-ray absorption and fluorescence, as well as their microfocused declinations, are applicable, in theory, to the entire periodic table [6]. Although ICP-MS and laser ablation ICP-MS are capable only of determining total elemental concentrations on a bulk or spatially resolved basis, respectively, synchrotron-based X-ray absorption techniques also are able to probe chemical speciation and the local electronic structure of elements. Determining the local electronic structure of metal atoms can be used to identify ENMs in a sample [7]. Conversely, although X-ray–based techniques have milligram-per-kilogram sensitivity, ICP-MS techniques have microgram-per-kilogram sensitivity and can discriminate between different isotopes of the same element [8]. Sensitivity of X-ray–based techniques can be enhanced by using spatially resolved analysis, which exploits the occurrence of foci of elevated concentrations relative to the bulk sample that can correspond to isolated or aggregated nanoparticles [7]. These techniques, which have been developed over the past three decades in various fields (physics, material science, environmental sciences, and life sciences), are gaining popularity, especially because benchtop instruments now allow analyses that required

synchrotron radiation 10 years ago [9]. X-ray photoelectron spectroscopy and related techniques are attractive in the sense that they provide element-specific information while probing the surface of nanoparticles or their aggregates. Nuclear magnetic resonance tools also provide detailed speciation data [10]. However, they cannot be applied to all elements, and the presence of paramagnetic elements in the matrix (such as iron [III] in a soil sample) renders the analysis impossible.

Direct visualization of the sample content is probably the most satisfying way of observing nanomaterials in a matrix. Because of the addressed size range (a few Å to approximately 100 nm), only a few techniques, such as electron and atomic force microscopy, can achieve sufficient spatial resolution to distinguish even the smallest individual particles, that is, approximately 1 nm (1 nm resolution in atomic force microscopy is limited to the Z dimension). Imaging-based techniques often suffer from tedious sample preparation, poor sensitivity, and an inability to provide quantitative data for a representative sample [3].

The most pressing challenge is to account for the expected low levels of nanoparticles in environmental systems [11]. In the absence of sample preconcentration, mainly mass spectrometry-based techniques may enable reliable and routine determination of microgram-per-kilogram and sub–microgram-per-kilogram levels such as those expected or measured in natural media. Most specific analytical tools presently have sensitivity in the milligram-per-kilogram or sub–milligram-per-kilogram concentration at best and therefore require sample preparation with associated potential artifacts. The relevance of the quest for improved sensitivity must be put in perspective with observed beneficial or adverse effects of the nanoparticles, keeping in mind that assigning such effects to the presence of ENPs is of course dependent on the detection method. A possible approach to solving the problems occurring from low mass concentrations lies in the use of the intrinsic discontinuities of nanoparticle/matrix systems. A mass concentration of 0.1 ng Ti/L (which is at, or well below, the detection limits of current routine methods) 20 nm TiO_2 particles will be present in a number concentration of approximately 5,000/ml. Possibly the further development and adaption of single-particle analysis methods to the requirements of ENM analysis has huge potential because they are more limited by the smallest detectable particle size and volume of a sample than by total mass concentration (see case study III).

Use of biological sensors: Alternative approach

A potential alternative for detecting the presence of ENMs when instrumental methods fail is through the use of biological sensors, although this approach has not been extensively explored. Bioassays may be a useful tool because organisms can have sensitive and specific responses to substances. Individual components of living systems also have been used as analytical tools for decades. For example, enzyme-linked immunosorbent assays, which use specific antibodies and enzymes, have been widely used for the low-level analysis of molecules. The specific association of AgNPs with cell surface proteins [12] and the differential expression of bacterial stress response genes with AgNPs and TiO_2 nanoparticles [13] indicate that biological reporter systems [14] that are highly specific to nanoparticle surfaces could be developed. Biosensors are very sensitive and, because they report on interactions of nanoparticles with living systems [15,16], should provide assessments of bioavailable fractions of nanoparticles, which are highly relevant to understanding exposure. Key to the development of biosensors is the understanding of specific nanoparticle–cell interactions that can be exploited for binding, and also identifying reporter genes that are selective to defined nanoparticles or associated coatings. This will require the understanding of interactions and effects of cells with nanoparticles, and engineering cells for binding specificity and signal generation. Signal detection could be automated with either flow cytometry or high throughput screening, or performed by either low-throughput fluorescence spectroscopy or microscopy. Issues to overcome in developing engineered biosensors include minimizing nanoparticle-induced interferences of gene expression, such as global activation of disruptive biochemical pathways, or protein production, or production of cofactors or other biochemical precursors. Because nanoparticles are unlikely to diffuse freely, even in hydrated matrices such as saturated sediments, destructive sampling is likely required so that samples can be fluidized and exposed to a biosensor long enough for receptor-mediated interactions.

CASE STUDIES

Tailored analytical approaches are needed that depend on the properties of the nanomaterial under study, the hypothesis to be addressed, and the nature of the biological or environmental system. Previous review articles have described many of the available analytical techniques [2,3]. What is lacking in the literature is a description of how the basic approaches and analytical workflows differ for nanomaterial detection and analysis relative to traditional analytical techniques for molecules and trace elements. The case studies discussed in the following sections highlight some of the considerations required for carbonaceous materials (case study I), soluble metal-based materials (case studies II and IV), and insoluble metal oxides (case study III) that are either redox-sensitive (CeO_2) or insensitive (TiO_2). Case study III deals with the difficulty of detecting CeO_2 and TiO_2 in sediments that arise from the abundance of naturally occurring materials that have similar chemical composition and size. The case studies involve biological samples (case studies I and II), soils and sediments (case studies I, II, and III), and wastewater, that is, sewage (case study IV). The cases highlight approaches that are modified from traditional analytical techniques (case study I), imaging approaches (case study II), techniques based on separation and detection (case studies II and III), and spectroscopy (case study IV). Taken together, these case studies highlight many considerations for detecting and analyzing ENMs and point to the most pressing research needs as well as potential pathways to move forward.

Case study I: Analysis of carbon nanoparticles

Carbon nanomaterials such as fullerene (nC_{60}), graphene, single-walled nanotubes (SWNT), and multi-walled nanotubes (MWNT) present unique challenges for their detection and quantitation at trace levels in aquatic environments. These challenges are born of the nanoparticulate (e.g., colloidal) nature of such materials and are in contrast to more commonly studied molecular species, such as hydrophobic organic contaminants. For the analytical chemist or environmental scientist who is familiar with trace analysis of molecular organic contaminants in solid environmental media (sediments and biota), a useful exercise for tackling the challenges of CNM analysis may be to examine parallels between detection and quantitation of nanoparticulate versus molecular carbon species. Here we outline such a comparison, contrasting hypothetical analysis of

fullerene and carbon nanotube species in sediments and biota with routine analysis of polycyclic aromatic hydrocarbons (PAHs) in these same media. Fullerenes, carbon nanotubes, and PAHs share chemical similarities: all of these species are formed of sp^2 hybridized carbon atoms. Fullerenes may be considered to be the shortest possible SWNT species, whereas PAH molecules are all essentially facets of fullerene or carbon nanotube structures. Determination methods for CNMs and PAHs in condensed media using instrumental techniques share some common strategies. In each case, three fundamental tasks exist: extraction of the analyte species from the solid-phase media, purification or separation of the analyte from co-extracted interferences, and selective detection of the analyte by spectroscopic or other means. We can consider each of these tasks separately as modules of a full analytical technique.

Extraction of PAHs versus CNMs from sediment and tissues

One of the most important differences between molecular contaminants such as PAHs and nanomaterials such as CNMs is the difference in behavior of these species in condensed and liquid phases. As hydrophobic molecular species, PAHs adsorb strongly to carbon-rich phases in sediments and associate with lipid phases in organisms. They are freely soluble in nonpolar organic solvents such as hexane and methylene chloride. Carbon nanomaterials present as colloids in aqueous phases and may undergo aggregation and deposition to solid phases in condensed media such as sediment. The details of their disposition in tissues are still unclear, but evidently they are not homogenously distributed. Solubilization of CNMs is very difficult in most liquid phases; SWNT and MWNT may be stabilized and dispersed in aqueous solution by surfactants, whereas nanocrystalline C_{60} may be disaggregated and solubilized in aromatic solvents such as toluene. Despite these differences in physicochemical characteristics, extraction of PAHs and CNMs from sediment and tissue may be accomplished using remarkably similar techniques. Specifically, ultrasonication in extractive solvents is highly effective for isolating both compound classes. The U.S. Environmental Protection Agency (U.S. EPA) method 3550c is a well-established protocol for extracting PAHs and other semivolatile contaminants from solid phases. This method uses high-power ultrasonication to impart energy to the sample and effect transfer of PAHs into nonpolar organic solvents such as methylene chloride. Quantitative assays for fullerenes in environmental solid phases and tissues have also used ultrasonication for effective extraction of these materials into toluene [17–20]. Recoveries of fullerenes using these methods exceed 90%. Very few reports detail methods for extracting carbon nanotubes from sediments and biota. Initial results indicate that ultrasonication using bile-salt surfactant solutions is an effective strategy for isolating SWNTs from sediments and aquatic organism tissue at yields greater than 80% [21]. In this case, the extremely hydrophobic carbon nanotubes are not truly solubilized in the extractant solution but are instead stabilized and exfoliated, resulting in a heterogeneous but analytically tractable liquid sample.

Sample purification and separation in sediments and tissues

After isolation of PAHs and CNMs from solid environmental phases, sample purification is often necessary to reduce interferences in subsequent instrumental analyses. For molecular PAHs, a range of standard methods, including silica or alumina chromatography and gel permeation chromatography, are available (summarized in EPA SW846 [http://www.epa.gov/fedrgstr/EPA-WASTE/2008/January/Day-03/f25575.htm], method 3600). These techniques take advantage of the molecular attributes of PAHs, including hydrophobicity, diffusion coefficient, and aromaticity, to selectively isolate them from co-contaminants. These same strategies may be suitable for C_{60} fullerene purification from environmental extracts, provided that these materials are first dissolved in toluene or another aromatic organic solvent. Such solubilization renders fullerene CNMs as true molecules, after which they are amenable to chromatographic separations for purification [19]. Purification of carbon nanotubes from sediment or tissue extracts is a more daunting problem. After surfactant-mediated dispersion, the analyst is still left with a suspension of particulates; thus, particle separation strategies are required for purification. Some of the most promising methods for separating carbon nanotubes from other molecular and colloidal contaminants are density gradient ultracentrifugation [22] and field-flow fractionation (FFF) [21,23]. Density gradient ultracentrifugation is capable of enriching for specific carbon nanotube diameters and electronic structures, while FFF seems to hold the most promise for length-separation of carbon nanotubes. Both methods are potentially suitable for isolating carbon nanotubes from, for example, natural organic matter, biomolecules, and clay particles in environmental extracts. A unique issue in CNM analysis that is not shared with PAH analysis is the issue of impurities present in the CNM formulations that also may be present in environmental media. Carbon nanomaterials are typically complex mixtures containing both the target CNM (nanotubes, fullerenes) as well as byproducts such as amorphous carbon, metal catalysts, and, in some cases, PAHs themselves [24]. Thus, sample purification and preparation strategies must be designed to be either inclusive of these impurities for a holistic assessment of CNM exposure or exclusive of the impurities if the objective is a CNM type-selective analysis.

Instrumental and determinative methods for PAHs and CNMs

After efficient extraction and purification of PAHs and CNMs from environmental sediment and tissue samples, these materials must be detected and quantified selectively. For PAHs, again several established and highly sensitive (microgram-per-liter or lower) methods are available for accomplishing this goal. Specifically, EPA SW846 method 8270d uses gas chromatography-mass spectrometry to identify and quantify these molecular aromatic hydrocarbons. Gas chromatography-mass spectrometry is very well suited to analyze molecular contaminants in complex environmental samples, because it provides multiple orthogonal measures of analyte identity (for example, chromatographic retention time and molecular mass measurement), is compatible with established sample preparation protocols, is tolerant of reasonably complex environmental extracts, and has the sensitivity necessary for confident quantitation at environmentally realistic concentrations. These attributes are appropriate targets for any candidate determinative method for CNM analysis in sediment and tissue extracts. For C_{60} fullerenes, such a method is currently available: high-performance liquid chromatography with atmospheric pressure ionization mass spectrometry [18,20]. This technique is analogous to gas chromatography-mass spectrometry for PAH analysis because it also allows orthogonal measurement of analyte attributes (retention time and molecular mass) and can be made compatible with C_{60} fullerene sample preparation strategies through adaptation of the high-performance liquid chromatography system to use a toluene mobile phase. Reported detection limits were sufficient for analysis of fullerene CNMs at ng/L to low microgram per liter concentrations

in environmental media [18,20]. These sensitivities are expected to be sufficient for assessment of fullerene CNM concentrations in contaminated environments and in laboratory bioassays.

Carbon nanotube quantitation in environmental and biological media remains a significant challenge. The nanoparticulate nature of these materials confounds application of the environmental analytical chemist's most powerful tool—the mass spectrometer. In addition, chromatographic separation techniques are difficult to adapt for carbon nanotube analysis. Finally, few spectroscopic techniques are sufficiently selective or sensitive for the detection of carbon nanotubes at microgram per liter concentrations in the presence of a carbon-rich background (for example, sediment extracts). For example, Raman and optical absorbance spectroscopies are heavily used as tools for characterizing the quality of pure nanotube preparations, but both fail at low concentrations and in high complexity samples. Several investigators have attempted to utilize black-carbon–specific wet chemical or thermal oxidation methods to determine carbon nanotubes in the aquatic environment; however, these techniques have not proved sufficiently sensitive for resolving CNMs against background black carbon in environmental media [25,26].

For SWNTs, the most promising trace detection technique is near infrared fluorescence spectroscopy (NIRF), which is highly specific for semiconducting SWNT species [27,28]. This technique features many of the desirable properties of molecular fluorescence spectroscopy (for example, high sensitivity and ease of use with analyte solutions), with the added advantage of very low background signal; the NIR region is relatively dark for photoluminescence spectroscopy of most natural and biological organic materials. Near infrared fluorescence spectroscopy also provides qualitative information about the diameter and chiral wrapping angle of SWNTs. This is advantageous in trace analysis, because this information provides additional lines of evidence for analyte identification. Very recently, an analytical strategy combining NIRF spectroscopy with preseparation by FFF was reported for analysis of SWNT in sediments and tissues [21]. This method satisfies the requirement for orthogonal measurement of analyte attributes (migration time in the FFF fractogram and fluorescence emission wavelength) necessary for confident SWNT identification in complex samples (analogous to gas chromatography-mass spectrometry of PAHs or liquid chromatography atmospheric pressure ionization mass spectrometry of C_{60} fullerene CNMs), while allowing quantitative analysis at the microgram per kilogram level in sediments and tissues [21].

Unfortunately, NIRF spectroscopy is limited to analysis of pristine SWNTs. Analysis of MWNTs requires a different strategy. No reports have yet been made of specific analytical methods for determining MWNTs in natural sediment or tissues. Uptake of both SWNTs and MWNTs in sediment-dwelling organisms has been examined using radiolabeled carbon nanotubes [29,30]; however, this strategy is not useful for assessing exposure or occurrence of these analytes in field settings. All carbon nanotube species contain metal catalyst impurities resulting from their production processes. One suggestion that may hold promise for detecting MWNTs and SWNTs in natural sediments and organisms is the use of ICP-MS or other elemental analysis techniques for quantifying residual metal catalyst impurities in environmentally occurring carbon nanotubes [24]. Specifically, metal catalyst ratios, such as Co:Mo for CoMoCat nanotubes, or Y:Ni for arc-discharge generated nanotubes, may be conservative markers of nanotubes isolated from sediments or tissues. Significant challenges must be overcome before this strategy becomes useful, including resolution of CNM-derived signals from that of background metal contaminants and appropriate sample preparation.

Imaging of CNMs in sediments and biota

Although quantitative analysis of CNMs in sediments and tissue is extremely useful for assessing exposure and uptake of these materials, the capability of imaging these materials in complex samples is also valuable for assessing microspatial localization in tissue and sediments. For CNMs, such localization is not trivial, because typical imaging microscopy techniques such as TEM and scanning electron microscopy (SEM) lack the specificity and contrast to visualize carbon nanotubes in a carbonaceous background (for example, against sediment particles or in tissues). Hyperspectral reflectance imaging such as CytoViva may be useful for tracking CNMs in tissues; however, the typically high absorbance of CNMs across the visible spectrum may limit sensitivity. For SWNTs, a highly sensitive and selective imaging microscopy technique is available based on NIRF microscopy [31]. This technique can detect single semiconductive SWNT particles in biological samples and has been used to examine the biodistribution of these CNMs in *Drosophila melanogaster* [32]. Extension of this technique to aquatic organisms and sediments should be straightforward and will provide a valuable tool for microspatial localization of SWNTs in solid environmental phases.

Effects of chemical transformation of CNMs

An important consideration in developing analytical methods for detecting and quantifying CNMs in sediments and biota is modifying these materials by chemical transformation. Such transformation may occur through biologically mediated or chemical oxidation processes [33]. This may cause significant problems for analytical detection of CNMs in complex media, because any modification, such as production of fullerols from C_{60} or oxidation of carbon nanotubes, may shift the material out of the analytical window of the chosen method. For example, C_{60} oxidation may produce polyhydroxylated fullerenes and these will likely be poorly extractable from sediment and tissue into toluene. In addition, polydisperse modification will produce multiple discrete chemical species, which will spread the analytical signal among many partially resolvable signals in the high-performance liquid chromatography atmospheric pressure ionization mass spectrometry analysis. The highly sensitive NIRF spectroscopy methods outlined for detecting SWNTs are susceptible to fluorescence quenching on chemical modification of the nanotubes. Such modification will render SWNTs nearly undetectable by NIRF spectroscopy, with severe consequences for quantitative analyses. Presently, very few techniques are available for dealing with the analytical challenges introduced by chemical modification of CNMs. This is clearly an area in which further development will be required.

Case study II: Ag engineered nanoparticles and QDs

Presently, no consensus exists on how either engineered AgNPs or CdSe QDs cause cellular and organismal toxicity. Engineered AgNPs and CdSe QDs both have propensity to dissolve, for solutes to thus co-occur with nanoparticles, and—based partly on analogous substances—for solutes to possibly reorganize into nanoparticles in situ [34,35]. The co-occurrence of solutes and nanoparticles can affect nanoparticle bioavailability and uptake [12], which may inform dose and toxicity mechanism assessments. Conceptually, CdSe QDs [36] can

dissolve externally to cells or can enter cells either intact after damaging membranes or accumulate in cells as highly reactive toxicants [37]. If QDs remain intact, they are subject to trophic transfer and possible biomagnification in their predators [38]. Assigning toxicity to either dissolved or intact nanoparticulate phases is an important endeavor, because it may influence the future safe design of ENPs. Thus, analytical methods to visualize and quantify ENPs in biological material have to account for all of the following forms: intact particles, agglomerates, and products of nanoparticle decomposition. This section regards approaches and issues in visualization and quantification of engineered AgNPs and CdSe QDs in biological samples.

Electron, laser confocal, and atomic force microscopy

Visualizing engineered AgNPs or CdSe QDs in biological samples is used to determine the spatial arrangements and intactness of nanoparticles in such samples. Many options are available for imaging, and each has its advantages, disadvantages, and conditions of use. High-resolution microscopy, typically TEM, is used routinely to characterize as-synthesized nanomaterials according to their shape and size. Transmission electronic microscopy instruments with energy-dispersive spectrometry (EDS), and electron diffraction can further determine the elemental composition and crystal phase, respectively. Scanning transmission electron microscopy may be particularly useful at identifying ENM in specimens at low concentrations [37], especially when used in conjunction with a high-angle annular dark field detector, which has greater sensitivity with increasing Z number for elements [39]. Electron energy loss spectroscopy can be used to obtain information about the electronic configuration of elements and is also good for quantitative analyses of low Z elements such as oxygen. Electron energy loss spectroscopy also may be used in scanning transmission electron microscopy elemental mapping mode similarly to EDS elemental mapping [40]. Such methods together could be quite valuable for discerning atomic abundance and also intactness of nanoparticles by crystal phase. However, electron diffraction and electron energy loss spectroscopy use very high accelerating voltages that are destructive to the soft materials in biological specimens. Thus, these methods are suitable for characterizing as-synthesized nanomaterials, but caution must be used when characterizing biological specimens.

Environmental scanning electron microscopy (ESEM) allows for imaging wet, uncoated specimens, thus avoiding at least some degree of desiccation-induced artifacts imposed by conventional SEM. The aggregation state of nanomaterials in association with cells is, for example, resolved by ESEM but also by cryogenic SEM [41]. Environmental scanning electron microscopy, EDS, and fluorescence microscopy have been used together to resolve CdSe QDs and their integrity, in part from Cd and Se atomic ratios by EDS but also by QD fluorescence [42]. Both ESEM-EDS and fluorescence microscopy, like confocal scanning laser microscopy, are relatively easy methods with potentially little preparation and thus minimal sample disturbance. However, to resolve internalized ENMs, the specific association with membranes or organelles, and the integrity of nanoparticles, high-resolution techniques are required.

Biological samples, such as biofilms, may be imaged intact [42] or subsampled and then subsamples analyzed, using a variety of techniques. For example, after a subsample is homogenized, one may use approaches that avoid inducing nanoparticle dissolution during sample handling, then divided again for dry mass and ICP-MS analyses. Biological specimens, similar to soil or sediment samples, are heterogeneous and variable. Thus, several independent specimens should be developed or sampled to enable quantifying variations in addition to average nanoparticle concentrations. Both TEM and scanning transmission electron microscopy require specimen fixation; it must be embedded in an organic resin to allow for ultrathin sectioning when cryogenic capabilities are not available. Embedding, perhaps through nanoparticle affinity to embedding chemicals, could potentially reorganize particles and thus confound discovering the native association between particles and cell surfaces. This outcome has not been sufficiently investigated but could conceivably be studied, for example, through side-by-side comparisons of cryogenic TEM versus conventional TEM. The former avoids embedding but may have its own problems with artifact introduction during freezing by various modes, such as liquid nitrogen plunge versus propane jet or other similar methods, and thus comprehensively comparing a range of appropriate and available methods is warranted.

Cryogenic SEM allows for imaging surface associations of nanoparticles with cells without sample embedding or other damaging preparation [41]. Imaging internal associations is accomplished by creating a random fracture plane using a coarse fracture knife. An exciting alternative is ion abrasion-SEM, which uses a dual beam instrument, that is, with a focused ion beam and field emission gun. Focused ion beam SEM is used to reveal subsurface layers revealed by atomic milling and then acquire images at each newly exposed plane. This method has been used recently to show nano-Ag particles inside cells, in conjunction with EDS for chemical confirmation [43]. The approach strikes a good balance between providing nano-scale resolution, avoiding embedding, using cryogenic techniques to preserve specimens, and enabling internal imaging plus three-dimensional (3D) reconstruction. Thus far, the approach has had little use in nanotoxicology, but it holds great promise [44].

As discussed previously [45], confocal scanning laser microscopy is accessible to many laboratories and can reveal intracellularization of QDs across entire specimens. However, the scale of resolution is not sufficiently small to visualize individual nanoparticles. Furthermore, QD fluorescence can quench inside cells [46], making fluorescence an unreliable measure of nanoparticle integrity. However, because confocal scanning laser microscopy is relatively simple and inexpensive to perform, it may be used as a first-tier tool for assessing where nano-Ag or QDs are localized, for example, within organs or tissues of whole organisms.

In most cases, microscope images are amenable to quantitative analysis, for example, by some analysis of feature frequency [37] or perhaps by quantitative image analysis using GIS software tools [47,48]. To enable quantitation including statistical analysis, a significant number of images acquired at random locations within a specimen are needed, as well as image acquisition from many independent replicates.

X-ray microscopy–based techniques

Synchrotron-based X-ray microscopy in combination with micro- or nano-focused X-ray absorption spectroscopy and X-ray diffraction has been widely applied to determine the spatial distribution, structure, and chemical speciation of elements in biological and geological samples [6,49]. These techniques have gained recent application in determining the localization chemical and physical forms of nanomaterials in cells tissues [7,50]. In these studies, the local electronic structure of metal centers was used to differentiate between intact

metals or metal oxides and metal ions bound to receptor sites in the tissues. Similarly, studies with QDs composed of Se and Cd can trace nanoparticle integrity by interrogation of the oxidation state of Se [37]. These techniques are often less invasive than electron microscopy (EM)-based techniques; however, attention must be paid to the generation of artifacts. Because synchrotron X-rays are able to penetrate biological tissues, and fluorescing X-rays can escape from depths of up to 50 to 100 μm, traditional embedding techniques for production of ultrathin sections are not required. Ideally, sample preparation can involve a range of options including the mounting of whole organisms [51] or cryogenic sectioning of tissues [52]. Element-specific X-ray microtomography techniques are also available for localization of trace elements in small samples without the requirement for sectioning [53]. Detection limits are generally in the low microgram-per-gram range for detection and in the low to mid microgram-per-gram range for spectroscopic measurements. Because the technique is spatially resolved, localized areas with concentrations above the detection limits can often be found in samples with much lower volume-averaged concentrations. Redox-sensitive elements are susceptible to oxidation-reduction reactions, so determining electronic structure using X-ray absorption must be carefully evaluated, particularly when using micro- or nano-focused techniques in which samples are irradiated with high photon fluxes. Facilities are available for the cryogenic mounting of cells and tissues for the minimization of artifacts from beam damage [54]. These techniques could also be combined with other synchrotron-based microscopy techniques such as infrared microscopy for the co-localization of biological molecules with particles. These multi-method techniques have been applied to elucidation of mechanisms related to neurodegenerative diseases [55]. Similarly, laser ablation ICP-MS–based elemental mapping may be used to determine the overall spatial distribution of elements within a sample very rapidly and may be used in conjunction with synchrotron-based techniques for the confirmation of the presence of nanomaterials [50]. Although microfocused X-ray microscopy techniques generally do not have the spatial resolution to image individual nanoparticles at subcellular scales, a number of facilities are now available that provide beam spot sizes as small as 30 to 50 nm, which may allow for the identification and structural spectroscopic analysis of single nanoparticles or nanoparticle aggregates at the subcellular level [56]. With such highly focused beams, critical evaluation of beam damage effects and proper sample preservation and processing is of paramount importance. Although desirable, synchrotron radiation is not always required because more and more laboratory instruments have become available (micro-X-ray florescence microscopes, tomography). These instruments, which have limitations in terms of sensitivity, are often capable of nearly the same spatial resolutions as their synchrotron counterpart without causing beam damage, and should therefore be regarded as efficient exploratory tools.

Chromatographic approaches

Techniques involving extracting, separating, and detecting nanoparticles in biological tissues have recently been suggested [57]. Traditional liquid chromatography techniques such as size exclusion chromatography or ion exchange chromatography are not amenable to separating nanoparticles. Because of the low charge of nanoparticles relative to similarly sized biomolecules, nonspecific interactions with the stationary phase typically result in strong interactions or irreversible binding [58]. Techniques that lack a stationary phase, such as FFF techniques or capillary electrophoresis, are promising, especially when combined with a sensitive element-specific detector (ICP-MS) [57,59]. The main advantage of FFF techniques over capillary electrophoresis is greater sensitivity, because only very small injection volumes can be used for capillary electrophoresis [59]. Also, because techniques based on electrophoretic mobility separate based on both hydrodynamic radius and charge, separation patterns can be complex. Additionally, in both techniques, differentiating between nanoparticles (Ag or QDs) and proteins of the same size with bound metal atoms may be challenging. Orthogonal methods such as TEM and single particle counting ICP-MS to resolve particles from protein-bound Ag are a possible path forward. Flow cytometry methods may be complementary to chromatographic techniques. For example, flow cytometry has been used to demonstrate the association of QDs with bacterial cells by correlating light scattering from microbial cells to fluorescence of QDs [60]. A major challenge for applying any of these techniques is sample preparation. Ideally, methods need to be developed for quantitatively extracting particles that do not introduce artifacts such as particle dissolution or aggregation. Preconcentration of the particles is desirable, because detection of trace amounts of particles in crude extracts may not be possible. Density gradient ultracentrifugation potentially could be applied to perform this separation; however, the effect of sheer strain on the particles needs to be taken into account.

Clearly, analysis of soluble metal-based nanoparticles such as CdSe QDs and AgNPs in biological samples is a complex task that requires further development. Chromatographic techniques are presently underdeveloped; however, significant hurdles need to be overcome with regards to sample preparation. Imaging-based techniques are better developed; however, great care must be taken to avoid artifact formation during sample preparation, and these techniques are not easily used quantitatively. The use of cryogenic techniques or techniques that require little sample preparation (confocal scanning laser microscopy) offers great promise.

Case study III: Analysis of TiO_2 or CeO_2 in soil

Titanium is the ninth most abundant element in the earth's crust. Concentrations in soil range from 0.3 to 6%, with higher concentrations found near coal-fired power plants. Freshwaters in Canada have concentrations of 2 to 107 μg/L, and filtered European freshwaters have a range of less than 0.1 to 18 μg/L, with an average at 1.5 μg/L. Titanium minerals are highly resistant to weathering, the most abundant forms in the environment being ilmenite, brookite, anatase, and rutile [61]. World annual production capacity for titanium dioxide (TiO_2) exceeds 5.5 million metric tons per year (http://minerals.er.usgs.gov/minerals/pubs/commodity/titanium/). The great abundance of TiO_2 as natural particles creates an analytical challenge for distinguishing nano- and non-nano particles as well as natural from anthropogenic materials. Reports on the identification of industrial TiO_2 ENPs are therefore rare [62,63]. They are based on particle visualization with Ti detection in electron microscopes, taking the high Ti abundance in the particles together with smooth homogeneous morphologies or the observation of coatings, which are thought to be indicative of a synthetic rather than natural process, as evidence of the anthropogenic origin of the particles. Attempts at quantification have been based on bulk water analyses and transforming these values into particle number concentrations from the combination of EM-determined particle size distributions and particle properties [62]. Natural Ti-bearing nanoparticles have been identified in river

sediment [64], and at the investigated site they mainly appear in the brookite form.

Cerium dioxide is the most insoluble oxide under ambient conditions and is used as an oxygen storage and combustion catalyst, for optical polishing and in the form of ENPs as fuel additive. It is a potential replacement of zinc oxide and TiO_2 in sunscreens because of its similar UV filtering properties and lower photocatalytic activity. The average natural abundance of Ce in the earth's crust is 46 mg/kg, and the background concentration in soil has great variation around this value. For Europe, ranges of 1 to 270 mg/kg (topsoil) and 2.2 to 1082 mg/kg (stream sediment) have been reported ([65]; http://www.gtk.fi/publ/foregsatlas/index.php). Concentrations in plants range between 0.1 and 0.55 mg/kg, with maximum values for sphagnum and lichens of approximately 1.4 mg/kg [66]. Because of the use of CeO_2 in automotive catalysts as early as in 1994, Ce concentrations significantly increased in roadside plants of German motorways. Cerium (IV) is immobile in soils because of the low solubility of its compounds. Cerium can undergo reduction to the trivalent form under natural conditions, which results in increased solubility. If released in reduced form, Ce (III) is, however, strongly sorbed to iron oxides, which are ubiquitous in the environment. In the course of TEM characterization of river-sediment–derived natural nanoparticles, a couple of Ce containing natural nanoparticles could be identified (Fig. 1). These natural nanoparticles showed association with thorium in the EDS analysis [64]. The challenge to identify TiO_2 and CeO_2 ENPs as being of anthropogenic/engineered origin and to quantify further the amount of these ENPs arises from several complicating factors. First, the abundance of ENPs is expected to be very low compared with the natural background concentrations of Ti and Ce. Second, these elements may occur in nanoparticulate form in nature [64], and the ionic forms of these elements tend to immediately adsorb to other natural surfaces. Finally, there is great variation in the natural background concentrations of these elements.

Detection/identification

The three most important requirements in the analytical procedure, which make ENM analysis essentially different from classical solute analysis, are first to detect Ce/Ti–containing particles, second, to identify these particles as ENPs (the particles are purposefully made material), and third, to quantify the anthropogenic fraction.

Sampling

The following considerations for total nanoparticle sampling methods for separating the ENPs from the sampled soil or sediment material for further analysis are in their infancy and are discussed in the sections about quantitative analysis. Sampling of soil and sediment may be carried out as for normal soil/sediment analysis. However, possible heterogeneity of the distribution of ENP in soils and sediments must be considered to determine the minimum sample volume needed for a representative sample. Contrary to classical mass concentration approaches, the particle number concentration as determined by mass concentration, particle size, and density should be considered. Also, more information is needed on the behavior of Ti/Ce ENPs in soils and sediments, that is, to identify preferential deposition/attachment of the Ti/Ce ENPs to certain grain size fractions or certain mineral surfaces. Especially with sediments, the proportion of solid material to pore water should be maintained in the sample because the pore water may hold a larger fraction of well-dispersed nanoparticles because of elevated natural organic matter concentrations. Natural nanoparticles undergo intensive redox cycling in the upper layers of

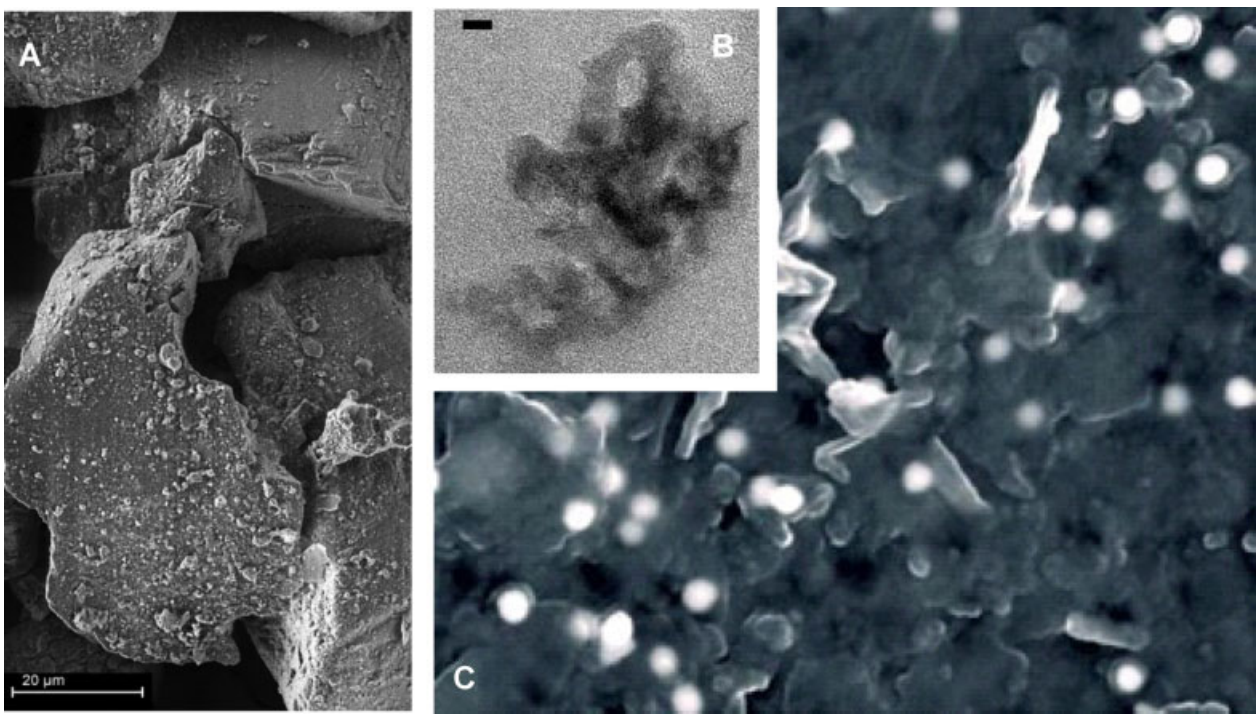

Fig. 1. (**A**) Scanning electron micrograph of a subsurface sand (silty loam) after thorough (1 h) wet sieving over 20 µm nylon cloth in an ultrasonic bath using 10 mmol/L sodium diphosphate as dispersing agent; the large number of still surface-attached nanoparticles is easily visible. (**B**) Transmission electron micrograph of a natural Ce-containing nanoparticle cluster from floodplain sediments of Clark Fork River, Montana, USA [64]. Scanning electron micrograph (SEM, FEI Quanta 3D) of soil nanoparticles (soil extract used in Plathe et al. [64]) mixed with 60 nm gold nanoparticles.

fresh water sediments. An individual porewater subsample would be beneficial to determine the pore water dispersed fraction [67]. Porewater extraction, however, must consider the selective separation or loss of nanoparticles during centrifugation or porewater extraction/filtration. To our knowledge, systematic studies of nanoparticle-related efficiencies and losses during porewater extraction, filtration, and the use of microporous suction cups are currently not available. The same considerations hold true for the drying and sieving of a soil or sediment sample. Although the removal of the coarse fraction and enrichment of the fines may be desirable to increase the nanoparticulate fraction of the sample for further sample processing, nanoparticles may be irreversibly attached to larger grains (Fig. 1). With removal of the larger grains, the target ENPs would then be removed from the sample as well. Even during wet sieving, parts of the nanoparticulate fraction may be lost by the same process. However, the use of ultrasonic power and dispersing agents may increase the recovery for the ENP fraction. Studies with labeled or easily tracked ENPs that address these issues of representativeness and recovery during sampling and sample preparation are lacking.

SEM/TEM-EDS identification

Electron microscopy is presently the predominant technique to investigate the presence, aggregation state, location, and composition of ENPs in ecotoxicological tests and on/in organisms [68–70] (see case study II and also Dudkiewicz et al. [71], which gives a comprehensive overview of EM techniques for ENP analysis in food matrices). Both the high-resolution TEM and the field emission gun-equipped SEM or scanning transmission electron microscopy systems provide the necessary spatial resolution to visualize particles down to a few nanometers in diameter. However, to provide this spatial resolution, the samples must be placed in an ultra-high vacuum (TEM) or even need to be additionally coated (SEM) with a thin layer of Au, Pt, or C. Coatings such as Au easily form artifact particles, which may look like Au nanoparticles.

Figure 1 shows the visualization of 60-nm gold-ENPs in a sample containing natural soil nanoparticles in a field emission gun-SEM (FEI Quanta 3D) at intermediate acceleration voltage (15 kV). The 60-nm gold-ENPs are clearly visible as bright dots because of the large Z-contrast of gold to the low Z elements in the natural soil NPs of the matrix. However, contrast alone is not always specific for heavy elements, because charge buildup on particles other than Au-ENPs produces similar brightness and may be misleading. In general, the identification of TiO_2 ENPs in soil or sediment samples with SEM/TEM just by their Z-contrast will not be possible because of the similar Z values for Ti and natural nanoparticles. To our knowledge, the usefulness of this technique for CeO_2 ENPs has not yet been proven. In general, a detailed elemental analysis may be required to identify Ti/Ce nanoparticles. To distinguish them from natural or incidental nanoparticles, techniques on single particle level, described later in this section, may be of help. However, the low sensitivity of EDS techniques will prevent most attempts based on the recognition of trace impurities or naturally accompanying elements. Drying of samples and high vacuum artifacts may be prevented by low vacuum SEM (environmental SEM; ESEM) and wet-SEM techniques; however, they do not provide the necessary resolution to image the smaller fraction of ENPs (approximately <30 nm) [71]. For cryogenic and embedding techniques, the reader is referred to Dudkiewicz et al. [71] and the references therein.

Use of elemental or isotopic ratios to identify ENPs

The detection and identification of ENPs in environmental matrices requires that they can be distinguished from the natural background of particles. For metal oxides such as TiO_2 or CeO_2, the background concentrations are relatively high, as shown previously, and both actually appear as natural nanoparticles in sediments and soils (Plathe et al. [64] and Fig. 1). Other options for discriminating between natural and engineered nanoparticles include the following: structural homogeneity or specific structure of the ENP (for example, certain coatings [72]), compositional homogeneity or purity, rare or untypical elements associated with one or the other type of ENP, shifts in the isotopic distribution of the core element, or deliberately introduced labels (barcoding). Using single particle analysis with TEM [62], one could detect TiO_2 ENPs originating from facade runoff and identify the particles to be of industrial origin by visualizing a coating layer on the particles, which was not expected for particles of natural origin. Little information is available regarding how the ENPs of Ce and Ti will be modified in the environment and how their "clean" and well-structured appearance will change during aging. A wide variety of elements and natural organic substances would be expected to adsorb to the surface of the clean particles and make it difficult to identify a particle as of industrial origin just because of its purity. However, ENPs might be deliberately amended with other rare metals, which could be used as tracers if the method is sensitive enough to detect those. Presently, little information is available to indicate whether isotopic ratios will be of any use to identify ENPs. Because the conversion of the source material to the final ENPs in the production process is fully quantitative, we do expect very little isotopic fractionation as a result of the manufacturing process. The source material, however, may originate from regions in the world where different isotopic patterns of the core element are present, and this could be used once as an indicator [73]. Whether, in the case of Ti, the small shifts in isotopic composition expected for different source materials would enable the identification of trace amounts of foreign-engineered nanoparticulate material on a background of grams per kilogram of natural background is unknown. For identification based on the co-occurrence of certain other elements with natural nanoparticles, which may be absent in ENPs, little hope exists for unmodified TiO_2 ENPs, because Ti is one of the most weathering-resistant elements. As a consequence, Ti concentration in soils is related to weathering status, and therefore no general correlation of Ti with any other rare element exists. We used the FOREGS geochemical atlas of Europe [65], which provides multi-element data for different compartments across Europe; with the help of this database, we analyzed the co-occurrence of rare elements with Ti and Ce for several thousand samples collected from soils, sediments, floodplains, and surface waters. For naturally occurring Ce, a clear and potentially usable relationship to La and Nd exists (Fig. 2). The slopes for the relationships between La and Nd with Ce are all approximately 0.45, with $R^2 > 0.95$ for most matrices. The results are similar for topsoils, subsoils, sediments, floodplain soils, and water samples, underpinning the general character of the co-existence of the three elements even in industrialized regions. Unfortunately, Ce is the one element among the rare earths that is redox active under environmental conditions. This produces deviations from the expected concentrations when compared with the other rare earth elements. Another critical drawback is that the documented relationships are valid for bulk samples, and the validity of the correlations cannot be assessed

Factors for sediments

	Slope	R^2
La	0.503	0.9895
Nd	0.462	0.981
Y	0.26	0.79
Pr	0.11	0.9911
Sm	0.0831	0.952
Gd	0.071	0.94
Hf	0.052	0.155
Y	0.0494	0.828
Li	0.043	0.015
Er	0.027	0.738
Yb	0.025	0.677
W	0.016	0.082
Tb	0.01	0.9
Ho	0.0095	0.763
Ta	0.006	0.057
Eu	0.0057	0.473
Tm	0.0038	0.72
U	0.0036	0.652
Mo	0.0033	0.0029
Tl	0.001	0.045

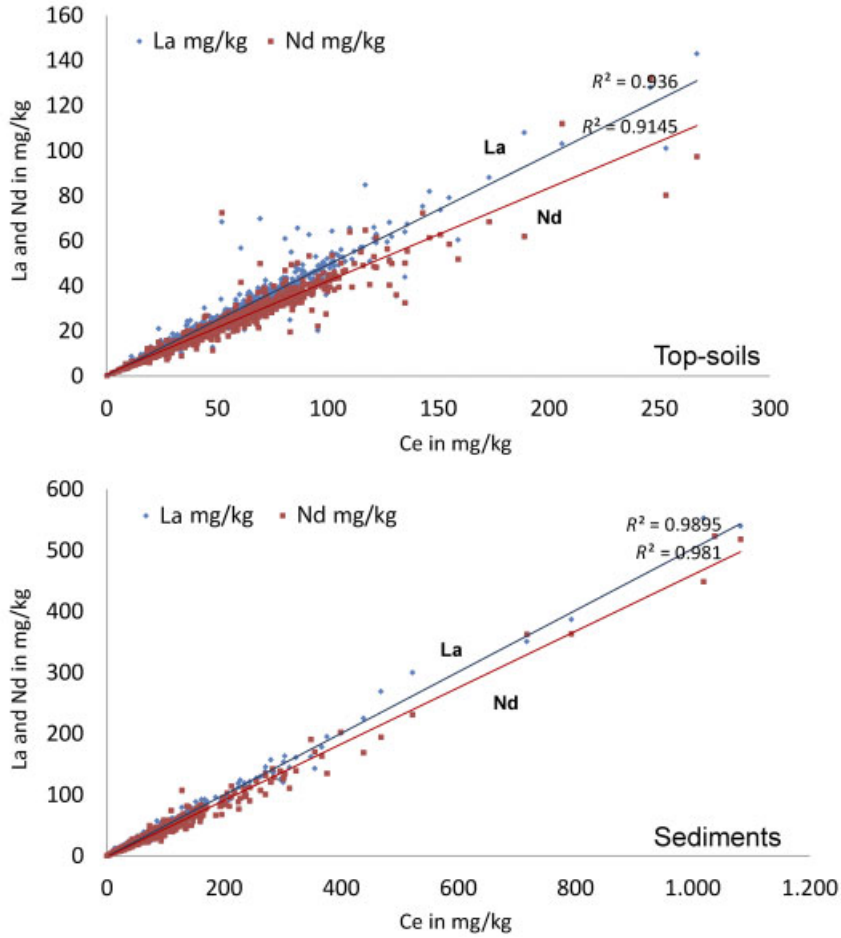

Fig. 2. Correlations of natural cerium (Ce) concentrations with lanthanum (La) and neodymium (Nd) in topsoil and sediment samples across Europe (http://www.gtk.fi/publ/foregsatlas/). [Color figure can be seen in the online version of this article, available at wileyonlinelibrary.com]

across certain size fractions or on a single particle level. Again, the relatively high background concentrations of natural Ce will make it difficult to identify the expected low concentrations of engineered CeO_2 simply based on the bulk sample concentration through the slight increase of the Ce:La or Ce:Nd ratio. One possible solution to this problem is the analysis on a single particle level, which has already proved powerful in SEM/TEM analysis. The drawback of the small numbers of particles typically analyzed in SEM/TEM, the low sensitivity of EDS analysis especially at high magnification, and possible artifact formation can potentially be solved by the use of single-particle ICP-MS (SP-ICP-MS). The concept was developed by Degueldre and Favarger in 2003 [74], further developed in pure systems [75], and first applied to ENPs in complex matrices by Hassellöv et al. [76,77]. Some general changes in the conceptual approach compared with conventional ICP-MS techniques are necessary.

Single particle ICP-MS analysis for identification/detection

In single particle ICP-MS analysis, nanoparticles are sequentially analyzed in an ICP-MS. They first must pass the nebulizer, which produces a spray of droplets in a stream of argon gas. In the formed aerosol, large particles of approximately 1 μm may form a relevant part of the droplets, which are typically in a size range of 2 to 20 μm. Incorporating a particle into a droplet with a higher density than water increases the average density of the respective droplet, resulting in a higher probability to be removed in the spray chamber. The droplet removal in spray chambers is based on the inertia of the flying droplets, a product of size, velocity, and density. Depending on the type of spray chamber, particle density, and the conditions chosen, there is an upper threshold from which the transmission efficiency into the spectrometer becomes particle size dependent, with lower efficiencies for larger particles. If the particles make it through the spray chamber, the aerosol is then transported to the plasma of the ICP and the particles are sequentially dried, decomposed, atomized, and ionized. A short-lived flash of ions is produced, which, after mass filtering, is recorded by the detector of the ICP-MS. The transport velocity of the particles in the plasma can be estimated to be approximately 30 m/s [75], resulting in a residence time in the plasma of approximately 1 ms. Calculations for heat transfer and sublimation rates concluded that particles up to approximately 5 μm should therefore be destroyed in the plasma [78]. From experiments with SiO_2 ENPs of different size classes, a quantitative transfer into the ICP-MS can be achieved at least for particles up to 1 μm (Agilent 7700 series equipped with a concentric nebulizer and Scott-type spray chamber, both constructed of fluoropolymer). The major difference with conventional analysis is that, in single particle analysis, the height of the signal recorded for a certain element is not directly related to the mass concentration of the element, but to the number of atoms of the respective element in the particle and therefore to the particle mass (Fig. 3). If the stoichiometry for each individual particle is

known together with the density of the material and the ion-transfer-efficiency, the particle size can be calculated in principle. Although the height/area of the signal is a measure of the particle size, the number of flashes per sample volume transferred into the plasma relates to the number concentration of the respective particle in the sample if the transfer efficiency sample into the ICP in terms of number of particles is known. Although in some cases a calibration with known particles could be done, for accurate determination of particle size and number concentrations, the efficiency of the whole analytical chain (nebulizer, spray chamber, transfer efficiency into the plasma, transfer efficiency from plasma to the inlet, and also inside the ICP-MS) must be determined so that the ratio of particles in an aspirated sample volume to detected particles can be established. In single particle analysis, the smallest peak height that can be distinguished from the background determines the smallest detectable single particle mass (volume, size); therefore, the detection limit determines the lower particle size threshold of the system. The following factors will influence this lower size threshold: sensitivity of the ICP-MS for the respective element of the particle, which includes all the individual system-specific performance parameters; stoichiometry of the particle; and the proportion of the target element that is dissolved versus in particulate form.

Sector-field instruments, such as the Thermo Scientific Element 2, provide smaller particle-size thresholds than quadrupole-based systems [76,77]. Theoretically, no detection limit exists for particles larger than the particle size minimum of the respective instrument.

The limitations of this technique arise from numerous factors and for currently available ICP-MS systems. The best reported size limits are approximately a 20-nm diameter for elements with excellent sensitivity (comprising 100% of the particle mass), absence of major interferences, and a low dissolved background such as for Au ENPs. Those size limits currently cannot be expected for TiO_2 ENPs, where Ti makes up only 60% of the total particle mass. For CeO_2, this ratio is better with 81% for Ce. No multi-element capabilities exist with sector-field or quadrupole systems at the required high data-rates, because these instruments do not determine the different elements simultaneously, but in a mass-scanning fashion, which would be impossible at the required high data collection rates. Instruments that would be capable of simultaneous multi-element detection currently do not provide the necessary detection limits. For example, the few known ICP-time-of-flight-MS spectrometers would deliver an estimated greater than 100 nm threshold, whereas other techniques do not provide the required high data-rates and sensitivity as well (Direct Charge Detectors, a diode array detector-like development). These two simultaneous multi-element capable instruments also do not provide the necessary mass resolution to overcome isobaric interferences. None of the available instruments comes with the data collection electronics and instrument software built for the purpose of collecting data with extremely high sampling rates in the 1 to 30 kHz region.

The latter problem is depicted in Fig. 4. Evidently, the data collection rate must be sufficiently high to be able to correctly determine particle mass and frequency with different particle sizes present and particles entering the plasma closely after each other. However, even at high sampling rates, multiple particles entering the plasma at the same time or single particles in aggregates still cannot be resolved. The requirements regarding data collection rates (minimum frequencies) are still under debate, and higher frequencies reduce the sensitivity of the instrument and also the smallest detectable particle diameter, because fewer and fewer ions can be counted per counting cycle. The lack of multi-element detection is not as important when measuring pure dispersions or particles composed from very rarely seen elements but would in cases of TiO_2 and CeO_2 prevent even the attempt at distinguishing engineered from

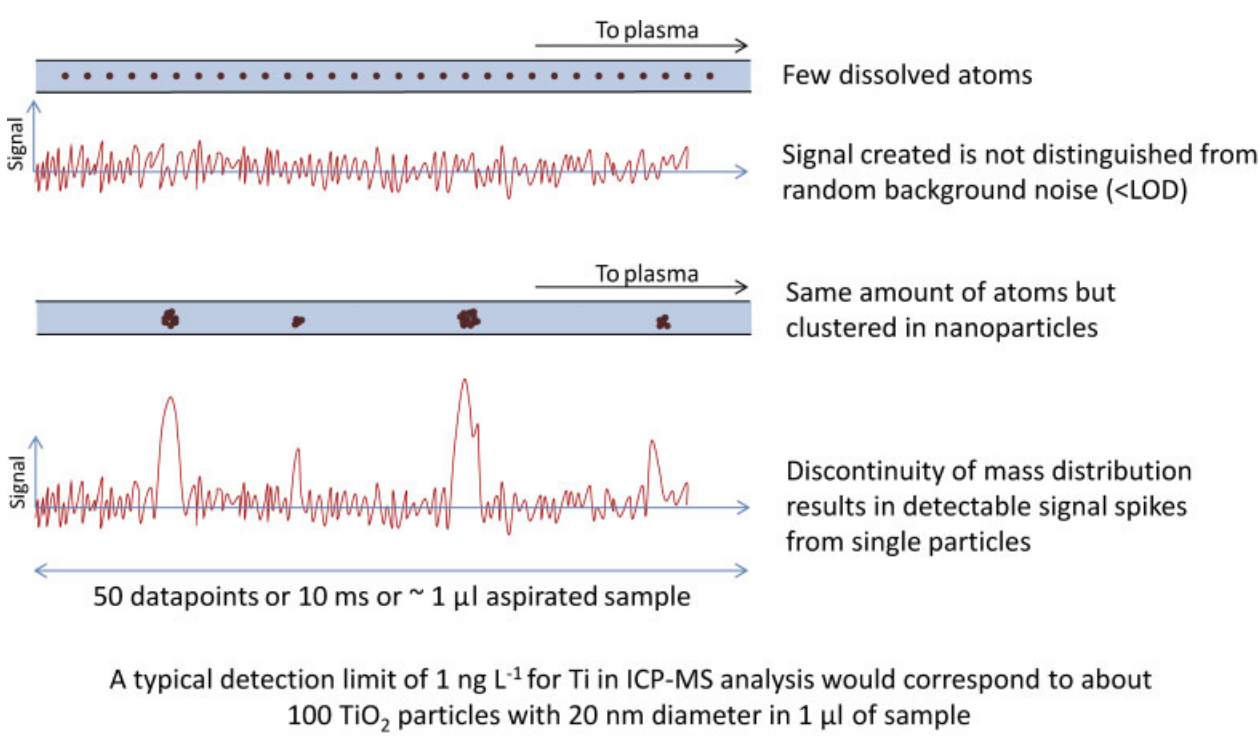

Fig. 3. Graphical sketch (no actual data used) of the basic differences in a signal obtained by conventional and single particle inductively coupled plasma mass spectrometry. LOD = limit of detection. [Color figure can be seen in the online version of this article, available at wileyonlinelibrary.com]

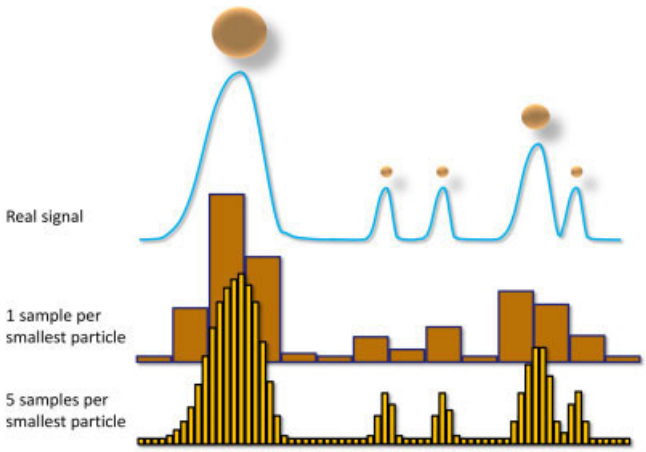

Fig. 4. Graphical sketch of the principal effect of the data collection rate for detecting single particles and separation of particles appearing close to each other. The increase of data rate (sampling frequency) comes with a reduction of sensitivity; the baseline in the lower example would be much noisier than in the upper case, an effect not shown in the graph. [Color figure can be seen in the online version of this article, available at wileyonlinelibrary.com]

natural particles. Accepting the current limitations, but also not overlooking the huge potential of the technique, a rigorous testing and comparison of all possibly suitable systems and optimizations is needed.

Quantification

After the qualitative analysis, in which the presence of the ENP in the sample was proven, quantification of an ENP aims to determine the concentration or abundance of this ENP in the sample. Apart from the mere ENP concentration (volume/mass, area, or even better, number concentration), other metrics could deserve a quantitative determination, such as surface area, shape, aggregation state, or surface chemistry. However, this should not lead scientists to the hasty conclusion that a multi-method approach is always required. The principle of the most efficient use of resources should not be abandoned, because knowledge about all possible relationships is still limited. Each additional technique should be well chosen with potentials and limitations in mind. In addition to the aforementioned, the desired metrics may be different for the same type of particle under different surrounding conditions, and those may be altered by sampling/sample preparation. This makes the case of ENPs different from the analysis of classical contaminants, in which the mass concentration is often the most important parameter. The situation might be comparable with speciation analysis or bioavailability analysis in that way: more than one metric may be needed to assess the situation. Another important difference between ENPs and classical contaminants is the fact that no substance-specific approach seems possible. This means that although developing a method for a certain chemical (phenantrene) or group of substances (PAH) seems to be standard, the considered TiO_2 or CeO_2 ENPs will be present in different particle sizes, mineral forms, surface chemistry, different shapes, aggregation states, and so forth. In this case study, we must consider Ti/Ce as the main analyte, but these will appear in many different industrially relevant forms with different core compositions and coatings, which may change over time and consequently will show different recoveries in the sample preparation procedures and analysis. Not much systematic information is available regarding how different particles of similar composition behave in sample preparation processes if they vary in one or more of their other properties, but, for example, the aqueous dispersion stability of different TiO_2 ENP products may be relatively similar over a wide range of hydrochemical conditions [79]. One could use this information to deduce that at least on the basis of the particle type with similar coatings, the recoveries in sample preparation should be similar. The consequence is that as long as a minimally invasive technique is not available and sample pretreatment is necessary, the development and use of internal standards may be unavoidable to obtain fully quantitative results.

Extraction

The separation of the TiO_2 and CeO_2 particles from the soil matrix may be achieved by using differences between the ENP and soil properties. For CeO_2, the particles density of 7.65 g/cm^3 (may be slightly different for nano-CeO_2) can be used in a density gradient ultracentrifugation separation step using sodium or lithium polytungstate heavy liquids. However, the natural CeO_2 ENPs, as well as some abundant higher density natural metal oxide nanoparticles, may be found in the separated heavy fraction as well, because of the maximum density of 3 g/cm^3 of most heavy liquids. Because of the even lower density of TiO_2, this separation approach seems to be inapplicable for this material. The higher the fraction of low-density components in the sample (such as high organic content), the more successful this approach may be. Whether nanoparticles with densities above the heavy liquid can be mechanically detached from surfaces of lighter particles by high g-forces in an ultracentrifuge or whether they will float with the lighter particles is not clear. Matrix decomposition would be an option if the particles cannot be separated from the matrix. Titanium dioxide is quite resistant to classical digestion techniques such as concentrated nitric acid, which offers some freedom in the use of digestive methods to destroy the matrix materials. Several digestion methods have been studied for the separation of TiO_2 out of sunscreen and preparation of a target particle dispersion for subsequent FFF analysis [80]; others performed a diluted direct injection or hexane liquid/liquid extraction of hydrophobic compounds before injection [81]. Neither study performed a full quantitative analysis of the ENP fraction. A thorough method evaluation and valid tracing of the results back to the original product are lacking. Other classical approaches can include filtration (which is hindered by the presence of other similar-sized particles, removal of aggregated structures containing ENP, and difficulties in providing reproducible, quantitative passage of filtrates through the membrane structure) and selective adsorption/deposition on stationary phases or extraction into organic liquids. Both of the latter options may need first to specifically derivatize the surface of the target particle to achieve an acceptable yield and selectivity toward the second extracting phase.

Single-particle analysis for quantification

The proper and complete separation of ENPs from natural particles of similar composition and size seems to be tedious and bears the risk of transforming and losing the target particles. Therefore, an analytical technique that ideally bridges the single-particle detection ICP-MS with an adapted, ideally low invasive sample preparation technique is needed. Two possible approaches would provide additional selectivity: FFF separates the particles according to their hydrodynamic diameter or particle volume, while avoiding the application of a stationary phase, and hydrodynamic chromatography (HDC) provides a similar separation but is still underdeveloped [82].

The current potentials and limitations of FFF, as well as a guide to method development, are given in detail elsewhere [83]. The analytical size-based separation of the preconditioned sediment or soil sample should enable the quantitative single-particle analysis in ICP-MS, including the identification of ENP. The separation methods provide a presorting of the still complex sample composition, and based on the recorded retention time of the sample, they will provide a good estimation of the size of the particle that enters the plasma of the SP-ICP-MS. Both separation methods dilute the sample during the separation process, which is a minor problem for SP-ICP-MS because dilute samples are required. One critical drawback of this combination stems from the fact that detectors common for method development and analysis in HDC and FFF, such as ultraviolet visible spectroscopy, fluorescence, and light scattering, are not applicable to the very dilute samples required by SP-ICP-MS. One solution to this would be a conventional, high-concentration separation in FFF and a subsequent dilution in the FFF to an SP-ICP-MS interface. Here, the advantage of SP-ICP-MS to provide exceptionally low detection limits in terms of particle concentration would be wasted.

As a stand-alone or coupled to FFF, this technique is presently being developed for ENPs by different groups, and first publications are out [77] or on the way. Even for the most sensitive instruments, the smallest sizes that can be detected are approximately 20 to 30 nm in diameter. The technique has huge potential because it uses the principle of discontinuity of particles in a dispersion compared with ions/atoms/molecules in solution, but years of detailed method development still may be needed.

Single particle detection pushed to or beyond the limits

In the following section, we wish to develop a hypothetical situation that goes far beyond what is possible with currently available commercial devices. In fact, some of the performance specifications needed for the following conceptual analysis cannot be met because of physical limitations. However, we believe that instead of listing methods that cannot solve the problem because of inherent limitations, we should show that—in principle—a combination of technologies, developed within the next five to 10 years to their physical limits, could be able to solve many of the current analytical problems. A high data rate, extreme multi-element sensitivity, and a stretch down to particles of approximately 2 to 5 nm for a solid one-element particle (as Ag- or Au-ENPs) must be integrated into one single instrument. This would require an instrument that makes use of dedication to the lowest matrix content and background with high transfer efficiency instrumentation. Given an increase in sensitivity of approximately 64-fold compared with the single-element sector field HR-ICP-MS and of approximately 64,000-fold compared with current multi-element ICP-time-of-flight-MS equipment required to enable the detection of a 5-nm diameter particle, the combination with FFF or HDC could be used to identify and quantify ENPs in soil and sediment suspensions for many cases and for many types of particles, even hybrids. Figure 5 illustrates how different (hypothetical) situations could be addressed by this hypothetical instrumentation. The left column describes typical situations for a complex environmental sample containing pristine, coated, or doped CeO_2 ENPs and natural nanoparticles of a similar size. The elemental ratios determined for natural sediment bulk samples are assumed identical to those in single natural nanoparticles (this might be unlikely and must be proven). Clearly the preseparation and information from individual size measurement (FFF, HDC) could be combined with multi-element, peak shape, ICP-MS–derived particle volume/size, and respective element ratios to for once answer analytical questions that are currently far from being resolved.

Case study IV: Monitoring for AgNPs in sewage sludge

Although the concentrations of Ag in wastewater in the United States, as evidenced by discharges in urban wastewater facilities, has significantly decreased from 1989 to 2007, potential releases resulting from manufacturing and using commercial engineered AgNP products may result in future increases of total Ag present in wastewater [84,85]. Past and current sources of Ag contamination in wastewater include photographic facilities, smelters, mines, and urban wastes. With the decline in the use of Ag-based photographic film and potential increases in the number of commercial products containing engineered AgNP, a significant shift may occur in the sources and possibly the forms of Ag present in both wastewater and biosolids generated from these wastes.

Commercial products containing engineered AgNPs are primarily designed to inhibit microbial growth. These products include the use of spherical nanoparticles immobilized to surfaces using polymers or added to surface disinfecting sprays or soaps, colloidal suspensions used as dietary supplements, and devices designed to release ionic silver into washing machines [85]. One of the challenges that face analytical chemists with respect to the potential release of engineered AgNPs into waste streams is the detection, identification, and quantification of engineered AgNPs in these matrices.

Because of the variability and complexity of aqueous waste streams, as well as the number of insoluble salts and Ag complexes, the speciation of Ag in wastewater and resulting biosolids is also expected to be variable and complex. Engineered AgNPs present in a wastewater stream are likely to encounter common ligands such as sulfate, sulfide, chloride, and phosphate, as well as carboxylic acids, polyalcohols, and amines found in humic substances [86]. Many of these ligands that are often associated with particulate matter are known to either complex directly with engineered AgNPs or with Ag^+, which may be released during oxidation. When engineered AgNPs were introduced into complex sewage, they partitioned to a considerable extent ($>90\%$) into the particulate biomass [82]. Nevertheless, a portion of the total Ag that remained in the supernatant was in the nanoparticle form. Although wastewater is complex, with several ligands that bind to metals and metal ions, the fate of Ag in this matrix is likely to be dominated by sulfide chemistry. Inorganic sulfide in wastewater effluent and receiving water has been shown to be present at concentrations that are 200 to 300 times in excess of the total Ag [87]. Silver sulfide has been identified recently in the form of nanoparticles (5–20 nm ellipsoids) in final stage sewage sludge materials in a full-sized municipal wastewater treatment plant [88]. Kim et al. suggested, based on the results of Choi et al. [86], that the source of the silver sulfide nanoparticles may have been engineered AgNPs. The reported change in the chemical composition of the ENPs in the wastewater treatment system may have significant biological consequences. Nitrifying bacteria are critical in the conversion of ammonia to nitrate in the wastewater treatment process. In addition to forming nanoscale Ag_xS_y complexes and precipitates, the presence of sulfide also protects nitrifying bacteria from the toxic effect of both Ag^+ and engineered AgNPs [89]. Other inorganic ligands present in wastewater such as sulfate, chloride, and phosphate were significantly less effective in reducing engineered AgNP toxicity [86].

Case	FFF or HDC	Single Particle-ICPMS
Possible characteristics of Ce in a natural soil/sediment sample after sample preparation	Separation particle size and volume	Multielement and high frequency measurement (for concept demonstration only - hypothetical in terms of detection and size limits regarding currently available instruments)
CeO_2 ENM of 20 nm	Retention ~ 22 nm D_h	Ce (~20 nm, if oxide); no other traces
CeO_2 ENM of 20 nm with 5 nm Al_2O_3 coating	Retention ~ 32 nm D_h	Ce (~20 nm); Al (~27 nm); no other traces
CeO_2 ENM of 20 nm labeled with Ag	Retention ~ 22 nm D_h	Ce (~20 nm); Ag in traces (extremely low) no other traces
CeO_2 ENM of 20 nm elutes together with natural particle (~20 nm)	Retention ~ 22 nm D_h for both particles	Ce (~20 nm); extremely low traces of Al, Si, Fe, Ti, Ca, ... Ce/La and Ce/Nd ratios may not match
CeO_2 ENM of 20 nm aggregated with natural particle (~80 nm)	Retention >> 22 nm D_h (shape/structure effects)	Ce (~20 nm); larger signals for Al, Si, Fe, ... Ce/La and Ce/Nd ratios are elevated compared to natural background (but proof needed)
Natural CeO_2 particle of 20 nm	Retention ~ 15 - 30 nm D_h (shape/structure effects)	Ce (<20 nm); La and Nd barely detectable Ce/La and Ce/Nd may fit natural background
Ionic Ce^{4+} sorbed to Natural particle of 80 nm	Retention ~ 80 nm D_h	Signals for Al, Si, Fe, ...; barely detectable Ce Ce/La and Ce/Nd may fit natural background but La and Nd may be too low to be determined

Fig. 5. Conceptual approach to the question of how different possible combinations of engineered nanoparticle (ENP) (CeO_2) and natural nanoparticles could appear in the analysis with an ideal, nonexisting field flow fractionation-single particle-inductively coupled plasma-mass spectrometry (FFF-SP-ICP-MS) combination. The SP-ICP-MS here is in contrast to existing instruments capable of delivering high-frequency, multi-element data with extremely high sensitivity. Still, the very low traces of Ce, the single mixed oxide particles, or clay minerals would be highly questionable, if they would be detectable at all. HDC = hydrodynamic chromatography; ENM = engineered nanomaterial; Ce = cerium, Fe = iron; Al = aluminum; Si = silicon; La = lanthanum; Nd = neodymium. [Color figure can be seen in the online version of this article, available at wileyonlinelibrary.com]

Consequently, one of the challenges to understand better the transport, fate, and biological activity of silver nanoparticles in wastewater treatment systems involves Ag-S chemistry.

With respect to the possible persistence of engineered AgNPs in a wastewater matrix, metallic Ag in the form of nanoparticles (2–8 nm) would dissolve over a time frame of fewer than 125 d to form Ag^+ in oxygen-saturated aqueous media at pH values and humic acid concentrations typical of these environments [5]. In this matrix, Ag complexes of halides and sulfides would be expected to predominate. Nevertheless, colloidal Ag particles of larger diameter and with more durable coating materials may be present in a wastewater stream if the time frame from particle release to analysis was less than several months.

Sample preparation

Because of the physical structures of engineered AgNPs, their presence cannot be determined simply by dissolution, extraction, cleanup, and analysis. For engineered AgNPs in complex media, sample preparation will likely be the most challenging part of the analytical process. The task of separation and enrichment is in some ways similar to detecting complex biological structures of similar size, such as viruses. Because of the presence of halides, sulfides, and a range of colloidal particles, preparation and chemical analysis techniques may be required that are not typically required for measurement of ENPs for laboratory toxicity experiments.

Techniques that might be used to separate nanoparticles from ions and molecular components aqueous media include centrifugation, dialysis, or continuous diafiltration. Limitations for centrifugation and dialysis include limited control over size separation and long processing times, which may shift redox conditions and increase particle dissolution. When diafiltration was applied to Au nanoparticle purification, the technique facilitated the rapid separation of molecular contaminants without the typical problems associated with filter obstruction often associated with ultrafiltration techniques [90].

Nanoparticle enrichment techniques that might be adapted from biology and colloid science include density gradient centrifugation. Using this technique, Au nanoparticles of several sizes were separated from each other and from particles of differing composition [91]. Another centrifugation technique termed *cloud point separation* was used to separate and concentrate trace amounts of engineered AgNPs [92].

Analytical techniques

Total Ag concentrations in wastewater are expected to be in the nanograms per liter range [85]. If the engineered AgNPs, which would likely constitute only a small fraction of this concentration, could be separated from other colloidal materials and enriched into the milligrams-per-liter range, then light scattering techniques such as dynamic light scattering and nanoparticle tracking analysis could be applied for particle size determination, and microscopic techniques such as TEM-EDS could be used to confirm size and elemental composition.

A technique that might be applied to the characterization of transformations of engineered AgNPs extracted and preconcentrated from wastewater or biosolid is X-ray absorption spectroscopy. X-ray absorption spectroscopy in combination with linear combination fitting analysis has been applied to determine the fraction of silver metal present in soils [93,94]. In these studies, the oxidized silver fraction was correlated to the observed reproductive toxicity in earthworms. This technique generally is applicable only when more than 10 mg/kg Ag are present, and synchrotron beamlines with high flux at the Ag K-edge (25,514 eV) are required for the highest sensitivity, thus

requiring engineered AgNP enrichment in the sample. Linear combination analysis is critically dependent on the proper selection of model compounds, proper sample preparation, and more than one plausible solution that fits the observed data can be possible [95].

Small angle X-ray scattering (SAXS) is a somewhat neglected technique in environmental sciences, most likely because of the difficulty of extracting useful information from the scattering patterns of complex samples, but it may be amenable to use for characterization of extracted engineered AgNPs. It is mainly used in biology (for example, when studying protein structure) and material science. Small angle X-ray scattering is one of the rare analytical tools providing a statistically meaningful three-dimensional structural description of noncrystalline systems over an extended size range (typically three orders of magnitude; 0.1–100 nm). It is performed on suspension gels and even solids and requires little or no sample preparation. Silver is part of virtually every SAXS experiment because Ag-behenate is used for q calibration [96]. The heavier elements, such as Ag, are easy to observe with SAXS because of their high electron contrast, and a number of studies describe size and structure of AgNP-based materials [97]. Anomalous small-angle X-ray scattering uses an X-ray source tuned to just below the absorption energy of the element of interest and thus helps to obtain element-specific information. It has been used to obtain element-specific data in heterogeneous samples containing Ag ENMs but has not been applied extensively to environmental samples containing ENMs [98]. The main issue concerning SAXS is the detection limit; an attractive feature of SAXS is the time-resolved monitoring of the evolution of the structure. With adequate sample, the time resolution can reach the MS even with laboratory instruments [99], but it is typically on the order of a minute or longer using synchrotron sources for more environmentally relevant systems (0.1 mM Zn) [100].

CONCLUSIONS AND RECOMMENDATIONS

Because the physicochemical properties of nanomaterials are dependent on their surroundings, the simple act of isolating and observing them can change these properties. Analytical techniques can be borrowed from materials and other chemical and biological disciplines, but their application to low concentrations and heterogeneous sample matrices requires some additional development or the development of completely new approaches. Approaches need to be tailored to address specific hypotheses in a given sample type for a given material, paying special attention to the generation of artifacts. In most cases, multiple orthogonal lines of analytical evidence are required to identify and characterize ENMs in samples.

New analytical techniques are under development that will enable more rapid, sensitive, and specific detection of ENMs. Many of these techniques rely on chromatographic separations that lack stationary phases because of the surface reactivity of particles (for example, FFF techniques). Detectors such as NIRF and ICP-MS are being adapted to provide sensitive and specific detection of ENMs. X-ray–based techniques provide sensitivity and specificity. Current synchrotron microprobe beamlines do not provide sufficient spatial resolution for imaging of individual nanoparticles; however, their general distribution in samples can be determined, and they can be differentiated from metal ions or other materials based on their local electronic structure. Light sources currently under development hope to achieve spatial resolution of less than 30 nm; however, beam damage is likely to be an important consideration when using these next-generation beamlines. Traditional EM-based approaches are being augmented by minimally invasive techniques such as ESEM, which are simpler to apply and less invasive. Focused ion beam-SEM holds promise for internal imaging and 3D reconstruction with samples held under minimally destructive cryogenic conditions.

Differentiating nanomaterials that have naturally occurring counterparts is a complex task, and techniques for this need considerable development. Some possible approaches involve examining isotopic or elemental composition of particles, as well as potentially labeling or barcoding materials as they are produced.

Sample collection preservation and storage is likely the weakest link in the analytical workflow and has received little attention in the literature. In many cases, properties cannot be measured after samples have been stored because of the temporally dynamic nature of colloidal systems. This presents a problem for characterization in toxicological studies; therefore, some techniques need to be developed that allow for the sensitive and rapid determination of basic properties such as particle size distribution. Current techniques that are rapid, such as dynamic light scattering, may not be sensitive or specific enough to be applied at environmentally or toxicologically relevant concentrations, depending on the material in question.

Despite the fact that techniques for identification, characterization, and quantification in complex sample matrices have hampered studies on the environmental fate, transport, exposure modeling, and ecotoxicological effects of ENMs, ample opportunities exist for progress. The requirements for the development of these techniques in terms of properties of interest and detection limits need to be guided by well-designed ecotoxicological studies that identify the relevant concentrations and properties of interest so that focused analytical strategies can be developed.

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