Detection, characterization and quantification of inorganic engineered nanomaterials: A review of techniques and methodological approaches for the analysis of complex samples

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ABSTRACT

The increasing demand of analytical information related to inorganic engineered nanomaterials requires the adaptation of existing techniques and methods, or the development of new ones. The challenge for the analytical sciences has been to consider the nanoparticles as a new sort of analytes, involving both chemical (composition, mass and number concentration) and physical information (e.g. size, shape, aggregation). Moreover, information about the species derived from the nanoparticles themselves and their transformations must also be supplied. Whereas techniques commonly used for nanoparticle characterization, such as light scattering techniques, show serious limitations when applied to complex samples, other well-established techniques, like electron microscopy and atomic spectrometry, can provide useful information in most cases. Furthermore, separation techniques, including flow field flow fractionation, capillary electrophoresis and hydrodynamic chromatography, are moving to the nano domain, mostly hyphenated to inductively coupled plasma mass spectrometry as element specific detector. Emerging techniques based on the detection of single nanoparticles by using ICP-MS, but also coulometry, are in their way to gain a position. Chemical sensors selective to nanoparticles are in their early stages, but they are very promising considering their portability and simplicity. Although the field is in continuous evolution, at this moment it is moving from proofs-of-concept in simple matrices to methods dealing with matrices of higher complexity and relevant analyte concentrations. To achieve this goal, sample preparation methods are essential to manage such complex situations. Apart from size fractionation methods, matrix digestion, extraction and concentration methods capable of preserving the nature of the nanoparticles are being developed. This review presents and discusses the state-of-the-art analytical techniques and sample preparation methods suitable for dealing with complex samples. Single- and multi-method approaches applied to solve the nanometrological challenges posed by a variety of stakeholders are also presented.

Keywords: Engineered nanomaterials; Nanoparticles; Complex samples; Detection; Characterization; Quantification.

List of acronyms

AF4: asymmetric flow field-flow fractionation
AGE: agarose gel electrophoresis
CBED: convergent-beam electron diffraction
CE: capillary electrophoresis
CPE: cloud point extraction
DLS: dynamic light scattering
EDS: energy dispersive X-ray spectroscopy
EELS: electron energy-loss spectroscopy
ENM: engineered nanomaterial
ENP: engineered nanoparticle
ESEM: environmental scanning electron microscopy
ET-AAS: electrothermal atomic absorption
EXAFS: extended X-ray absorption fine structure
FESEM: field-emission scanning electron microscopy
FFF: field-flow fractionation
FlFFF: flow field-flow fractionation
GE: gel electrophoresis
HDC: hydrodynamic chromatography
ICP-MS: inductively coupled plasma mass spectrometry
ICP-OES: inductively coupled plasma optical emission spectrometry
MALS: Multi-angle light-scattering
MWCO: molecular-weight cutoff
NOM: natural organic matter
NP: nanoparticle
NPM: natural particulate matter
NTA: nanoparticle tracking analysis
PAGE: polyacrylamide gel electrophoresis
PCC: particle collision coulometry
SAED selected-area electron diffraction
SdFFF: sedimentation field-flow fractionation

SDS: sodium dodecyl sulfate
SEC: size exclusion chromatography
SEM: scanning electron microscopy
SPE: solid phase extraction
SP-ICP-MS: single particle ICP-MS
TEM: transmission electron microscopy
TMAH: Tetramethylammonium hydroxide
UF: ultrafiltration
VIP: voltammetry of immobilized particles
XANES: X-ray absorption near edge structure
XAS: X-ray absorption spectroscopy

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Acknowledgements

References

1. Introduction

Although before 2000 nanoscience and nanotechnology were already undergoing a rapid growth, it was from 2006 when concerns about the potential risks of engineered nanomaterials in relation to the human health and the environment began to raise [1, 3]. In fact, the World Economic Forum included the specific topic "nanoparticle toxicity" in its Global Risks Reports in 2006 for the first time [4]. In parallel, the appropriateness of the available methodologies for risk assessment of ENMs, including analytical methods, was brought into question [5, 6]. In the following years, a number of revision articles were published, summarizing the state of the art of analytical methods with respect to the detection, characterization and quantification of engineered nanoparticles [7-19] or focussing on specific techniques, like electron microscopy [20], inductively coupled plasma mass spectrometry [21], light scattering [22], particle tracking analysis [23] or field flow fractionation [24-26]. Most of the general reviews listed advantages and potential limitations, and even proposed approaches and techniques with high potential to overcome already detected as well as expected problems.

In those days, most of the works were mainly oriented to the characterization of newly developed and synthesized pure nanomaterials, which means to work at high concentrations, with homogeneous samples, and with no complex matrices. Methods and techniques to characterize natural and engineered nanoparticles in simple matrices were already available, but, in contrast, methods to track quantitatively engineered nanoparticles released into complex environmental systems were rarely available [11]. The reason for this lack of analytical methods is related to the unique physical and chemical nature of nanoparticles, as solid analytes. For conventional analytes, the information demanded can be quantitative, as well as qualitative (including the identification of the chemical species). However, when the analyte is a nanoparticle, a solid phase species, the problem is far more complex: the quantitative information can be demanded as mass (or molar) concentration, but also as number concentration, and the qualitative information can involve not just detection of the NP as such, but also to provide both chemical (core and coating composition) and physical characterization (e.g. size, shape, aggregation/agglomeration). Moreover, when the NPs are embedded in a solid matrix or they can dissolved, the release of free NPs or ionic components, respectively, must also be considered. The need for all this information lies in that all these chemical and physical properties are closely tied to the occurrence, fate and toxicity of nanoparticles.

The current challenge for the analytical scientists is to develop innovative approaches to detect, characterize and quantify ENPs in complex samples, at realistic concentrations and in the presence of natural particles, of similar or different nature. Although a number of techniques for the characterization of ENPs are available, their application, when moving to complex situations and working at trace levels, can be unfeasible in most cases. These innovative approaches involves, first of all, to be fit-for-purpose and, due to the complexity of the analyses, to use a range of complementary analytical methods in most cases. Analytical techniques and methods that proved to be suitable to face some of the challenges cited above are listed in table 1, including also some emergent and promising techniques. Reported or commonly accepted size and concentration limits of detection have been included for comparative purposes.

The scenarios that have to be faced by analytical scientists in the domain of nanotechnology include (figure 1):

(1) Analysis of industrial and consumer products containing ENMs (e.g. cosmetics, textiles, polymers, foods).

(2.a) Laboratory experiments involving the release of ENMs from consumer products, as well as their fate, in different test media. In the case of foods, *in vitro* experiments related to digestion processes are also included.

(2.b) Ecotoxicological and toxicological studies. The fate and transformations of ENMs added to *in vitro* and *in vivo* assays must be followed in the test media and the organisms along the assays.

(3) Monitoring the occurrence and fate of ENMs along their life cycle in the environment and organisms, including humans.

In general terms, each of the three scenarios described imply an increment of the analytical complexity, both from the point of view of the sample matrix and the species potentially involved, and the decreasing analyte concentrations. An exception can be made in the case of foods, included in scenario 1, since the nature of selected food matrices can be even more complex than matrices from scenario 3. In any case, just systems related to aqueous phases will be considered in this review, as it can be considered the main medium for dispersion of ENMs and their interaction with organisms [27].

This review is focused on most used inorganic nanomaterials, which are mainly found as nanoparticles (nano-objects with three dimension in the nanoscale, 1-100 nm). They include: metallic nanoparticles (e.g. Ag, Au, Fe), oxides (e.g. CeO_2, CuO, SiO_2, TiO_2, ZnO) and quantum dots (e.g. CdSe, ZnS). For an extensive and updated list of inorganic nanomaterials, their properties and toxicity, the DaNa (Data and knowledge on Nanomaterials) site is recommended [28]. Current approaches to analyze complex systems involving inorganic ENMs require to consider not just the pristine ENMs but also the corresponding derived species which can be found in the system under study. Figure 2 summarizes potential transformations of pristine inorganic ENMs (both dispersed or embedded) along their life cycle when put in contact with aqueous media (environmental and/or biological), and the different physicochemical species formed. The aim of this paper is to review the state-of-the-art of the analysis of engineered nanomaterials in complex systems, the analytical techniques and methods suitable for such analysis and also those emergent that show potential capabilities. The techniques for detection, characterization and/or quantification of ENMs have been grouped in six sections covering electron microscopy, light scattering and atomic spectrometry techniques (sections 3 to 5); chromatographic and non-cromatographic continuous separation techniques along with the appropriate detection techniques are considered in section 6; electroanalytical techniques are covered in section 7, and section 8 is devoted to the emerging field of chemical sensors. Techniques primarily used for size characterization of pristine ENMs (e.g., centrifugal particle sedimentation, analytical ultracentrifugation, X-ray diffraction, small-angle X-ray scattering) have not been considered. The sample preparation methods required to cope with complex matrices are considered in section 2. Finally, special attention is paid to the approaches needed to solve the nanometrological problems posed by ENMs in section 9.

2. Sample preparation

Except when working with pure ENMs, most samples require some kind of preparation prior to their analysis by most of analytical techniques. Sample preparation methods that consist of removing the matrix or separating the ENM from the matrix will be considered.

2.1. Digestion

Digestion of solid samples containing inorganic ENMs (e.g. foods, tissues, microorganisms, sediments) can involve the dissolution of the ENM, the degradation of the sample matrix or both. Concentrated oxidizing acids like nitric acid, alone or in combination with hydrogen peroxide or hydrochloric acid, are commonly used to digest organic matrices, by using conventional heating systems at atmospheric pressure or under pressure with microwave assisted techniques. Under acidic conditions some ENMs can dissolve (e.g., silver, copper, zinc and copper oxides), whereas others will require additional reagents (aqua regia for gold, hydrogen peroxide for CeO_2, or hydrofluoric acid for TiO_2). In any case, these acid-based digestions are oriented to get information of the total element content from the ENM in the sample, except if the ENM is insoluble in acids, as in the case of the determination of SiO_2 NPs in food matrix [29] or biological tissues [30].

Alternatives to acid digestions are those based on the use of alkaline reagents. Tetramethylammonium hydroxide is often employed for degradation of organic matrices [31-35]. In a similar way, enzymatic digestions by proteases or pectinases has also been used to solubilise biological materials by degradation of proteins [33, 36-41] or to digest plant cell walls [42], respectively. Both strategies preserve the core of inorganic NPs, allowing the direct detection, quantification and size characterization of the NPs themselves. Although the presence of organic residues can affect the quantitative recovery of the NPs when SP-ICP-MS [33] or AF4-ICP-MS [31, 38] are used, methods based on enzymatic digestion and SP-ICP-MS [41] or AF4-ICP-MS [39] has been validated for sizing and quantification of Ag NPs in spiked chicken meat, as well as by TMAH digestion and SP-ICP-MS for Ag and Au NPs in exposed *Daphnia magna* [32].

2.2. Separation/preconcentration

The concentrations of nanoparticles in environmental and biological samples may be expected to be lower than those that can be directly measured and, at the same time, it may be necessary to remove matrix components. Separation in combination with preconcentration can be useful to improve the detection capability of the measurement techniques.

2.2.1. Centrifugation

Centrifugation can be considered the simplest approach to isolate particulates from an aqueous suspension, as well as to separate nanoparticles from dissolved species [43]. However, high centrifugal forces and long times are required, and removal of nanoparticles from supernatants containing dissolved species is incomplete even at very harsh ultracentrifugation conditions (e.g., 150 000 g for 60 min) [44]. Moreover, in the presence of unwanted solids, they are also isolated along with the nanoparticles. Thus preparative centrifugation is not considered an efficient technique for the fractionation of nanoparticles [45]. However, it has proved to be useful for isolation of dissolved species when ultrafiltration fails, which is the case for elements bound by high molecular weight compounds, like dissolved organic matter [46] or proteins, unless nanoparticles exhibit a significant organic corona, which decreases their overall density.

2.2.2. Filtration, ultrafiltration and dialysis

Although 0.45 µm filtration is not longer accepted to distinguish dissolved and particulate fractions [11], serial filtration, usually in combination with ultrafiltration, has been used for sized fractionation, followed by quantification and characterization of each fraction by a number of techniques [47].

Dialysis and ultrafiltration are based on the use of nanoporous membranes of different materials and nominal molecular-weight cut-offs. Their use has been reported to isolate dissolved species from nanoparticles, hence they have been extensively applied to study the dissolution of nanomaterials ([48] and references therein). Because dialysis is based on pure diffusion, it takes long times to achieving equilibrium, hence UF is preferred to speed up the separation process. In UF, species below the MWCO are forced to cross the membrane through the use of a centrifugal force. Membranes with MWCO in the range of 1–100 kDa (from ca. 1 nm) are available. Thus free ionic species can be easily isolated from nanoparticles, whereas the separation of the corresponding complexes can be difficult depending on the molecular weight of the complexes and the size of the NPs [49, 50]. Moreover, depending on their composition and surface functionality, ENMs and the corresponding dissolved species can show interactions with the membrane surfaces, affecting their recoveries [51].

2.2.3. Liquid phase extraction

ENMs can be extracted from solid and liquid samples by using water or organic solvents preserving some of the properties of the ENMs. Clean-up procedures based on the defatting by hexane extraction have been used for the analysis of sunscreens containing TiO_2 [52-54] and foods containing SiO_2 [55].

Extraction of Ag and TiO_2 nanoparticles in natural waters has been performed by Majedi et al. [56] by surface modification of the nanoparticles with a hydrophobic mercaptocarboxylic acid, followed by ion-pair extraction with an alkylamine in cyclohexane. Recently, cloud point extraction has been proposed for the separation of nanoparticles, preserving the size and morphology of the nanoparticles in the sample and providing selective separation of the NPs from dissolved species [57, 58]. CPE involves the addition of a non-ionic surfactant (usually, Triton X114) at concentrations over the critical micellar concentration, the incorporation of the NPs in the micellar aggregates and the separation of the surfactant phase from the aqueous one by mild heating (ca. 40°C). By adding a complexing agent (thiosulphate [58-60], EDTA [61-63], thiocyanate [64]) selective extraction of Ag, Au, CuO and ZnO NPs in the presence of the corresponding cations can be achieved. This strategy can be combined with total element determinations to obtain information about the dissolved and nanoparticulate element. CPE has been combined with ET-AAS [60, 61, 64] and ICP-MS [57, 58, 62, 63] for quantification of nanoparticles. Whereas ET-AAS allows the direct analysis of the surfactant phase, a previous acid digestion of this phase is needed when ICP-MS is used. These methods are selective to the nanoparticles, mostly regardless of the coating and their composition. Hartmann et al. [65] demonstrated for silver and a number of organic and inorganic coatings that, except for albumin coating, all Ag NPs were extracted with good efficiencies, what is critical for extraction of NPs from field samples, where the nature of the NPs or the coating is unknown. Slight differences were observed for different coated CuO NPs [63], as well as for Au NPs with respect to size [60].

2.2.4. Solid phase extraction

Anionic exchange resins have been proposed for the selective extraction of noble metal NPs [66]. The surface of the NPs was modified with mertcaptosuccinic acid for the adsorption of the modified NPs onto the resin, which were eluted with formic acid in methanol for ET-AAS determination. The extraction method preserved the size and shape of the NPs and it was applied to NPs with different coatings, even to partial or totally sulphidated Ag NPs [67]. Functionalized magnetic nanoparticles have also been proposed for the extraction of Au NPs and ionic gold [68], as well as for the selective extraction of Ag NPs in the presence of Ag(I) [69]. Su et al. [70] have quantified Ag NPs and Ag(I) in digested rat organs by using knotted reactors in combination with ICP-MS.

3. Electron microscopy

Electron microscopy is considered one of the most powerful technique for the analysis of nanomaterials because of its capability to visualize nanoparticles, and hence to obtain information about their size, shape or aggregation state, as well as to guide the interpretation of results from other techniques [19, 71-73].

Among the different microscopy techniques, conventional transmission electron microscopy accomplishes most of the requirements needed for the characterisation of ENMs in complex matrices. Only in some cases, the use of high resolution TEM should be considered to study some nanoscale features, such as the nature of some nanoparticle coatings [74]. Additionally, the introduction of field-emission electron guns in scanning electron microscopy has allowed the successful use of scanning instruments for ENM characterization, due to the better spatial resolution (below 1 nm) generated by this type of electron sources (field-emission scanning electron microscopy). The visualizing capability of these techniques is complemented by different spectroscopic tools, which are coupled to microscopes for obtaining elemental and structural information. Energy dispersive X-ray spectroscopy is usually combined with SEM and TEM to detect and quantify elements heavier than boron with 15-20% uncertainty [75]. TEM can be also equipped with electron energy-loss spectroscopy (EELS), which is capable of giving structural and chemical information, including the oxidation state of an element, with a spatial resolution down to the atomic level in favourable cases. Both spectroscopies (EDS and EELS) are complementary, covering the determination of light and heavy elements [75]. To obtain the crystal structure of the nanomaterials, selected-area electron diffraction or convergent-beam electron diffraction are commonly used.

TEM can produce bright- or dark-field images that can provide precise particle size information at nanoscale, being the most used technique for measuring the size of nanoparticles [19]. Electron microscopy provides true particle size dimensions, giving the projected area of the particles. However, it cannot be considered an ideal approach for quantification, because of the high number of particles to be counted and sized to get statistically significant and representative results, being a time-consuming and low-throughput technique otherwise automated image analysis is available [11]. Recently, Dudkiewicz et al. [76] investigated the uncertainty associated to the size determination of ENMs in foods, proving that the number of measured particles was only a minor source of uncertainty, compared to the combined influence of sampling, sample preparation and image analysis. They found that expanded uncertainties around 21–27% could be achieved, concluding that replications and matrix removal should be considered to improve uncertainty.

Key features in electron microscopy are the sample preparation procedures and the high-vacuum conditions required to obtain a good characterisation of ENMs. Some of the most frequent sample preparation procedures involve drop deposition followed by solvent evaporation, adsorption deposition and ultracentrifugation harvesting [7]. The transformation of the sample from its dispersed hydrated state to a dried high vacuum state often means that the particle size distribution can change dramatically, e.g. the common method of evaporating a sample drop into dryness could lead to the increase of particle and solute concentrations before the solvent was evaporated, with the consequent aggregation of particles and the possible precipitation of salts. As an alternative, the grid surface can be functionalized with a substance that confers a charge opposite to that of the NPs (e.g., poly-L-lysine for negatively-charged NPs, or carboxylated ligands for positively-charged ones), NPs are then attached to the surface, avoiding their aggregation and allowing the washing of the salts [77]. Different preparation methods are used to preserve the hydrated state of particles, namely by cryofixation (a rapid freezing so that the water forms non-crystalline ice) or by embedding the particles in a water-soluble resin to fix the water [7]. Alternatively, to image ENMs in their ambient conditions, environmental scanning electron microscopy, also named atmospheric SEM, is available. In ESEM, the sample chamber can be operated at 10–50 Torr, compared to 10^{-6}–10^{-7} Torr in conventional instruments. Therefore, imaging of ENMs can be performed in their natural state under humidity conditions up to 100% [78]. A limitation of ESEM is that only a thin superficial layer of the sample is subjected to analysis and it is not currently compatible with EDS [79]. On the other hand, the spatial resolution drops to several tens of nm. A pioneer investigation of nanoparticles by ESEM in food samples was conducted by Gatti et al. [80]. Luo et al. [81] explored the application of ESEM to directly characterize the size distribution of a range of ENMs in a selection of environmental and food matrices. ENMs were detected by ESEM in liquids down to 30 nm and 1 mg L^{-1} (9×10^{8} mL^{-1}, 50 nm Au NPs) [81]. More recently, Tuoriniemi et al. [82] evaluated the use of SEM and ESEM for imaging ENMs in soils. ESEM with backscattered electron detection was highly valuable for imaging the heavier element (i.e. Pt, Au and Ag) containing nanomaterials. In the soil matrix, particles as small as 25 nm could be quantified at concentrations down to 10^{11} particles m^{-3} (1 μg kg^{-1} of 100 nm Ag NPs).

The use of electron microscopy for ENMs characterization in different types of samples has been considered in different reviews involving environmental [7, 8, 83], food [8, 20], and biomaterials [71] analysis. In relation with complex samples, electron microscopy has been successfully applied to characterize TiO_2 nanoparticles in sewage sludge and soils amended with sewage sludge [84] or with biosolids [85], or to investigate the presence of ENPs in release studies: Ag NPs from a washing machine effluent [86] and from water-based nano-Ag spray products [87], TiO_2 NPs from textiles [88], or SiO_2 and Al_2O_3 from chemical mechanical planarization process wastewater [89]. SEM and TEM-based studies highlighted the relevance of the detection and characterisation of ENPs in many products of our daily life: Ag in washing solutions from commercial detergents [90], Ag and ZnO in spray products [91], TiO_2 and ZnO in sunscreens [74], metallic NPs in dietary supplement drinks [92], Ag in pears [93], SiO_2 in tomato soup [81] or coffee creamer [55], TiO_2 and ZnO in starch, yam starch, and wheat flour [94], or TiO_2 in foods and consumer product [95]. Regarding the implications of the presence of ENPs in biological samples, TEM can provide the most detailed information regarding *in vitro* nanoparticle uptake and localization by allowing both visualization of nanoparticle position within a cell or tissue and, in conjunction with spectroscopic methods, characterization of the composition of the internalised nanoparticles [96-101].

4. Light scattering techniques

Among the different light scattering techniques, dynamic light scattering, also known as photon correlation spectroscopy, is the most commonly employed high-throughput technique to measure nanoparticle size in aqueous suspensions. DLS measures the Brownian motion of NPs, through the time-dependent fluctuations in scattering intensity caused by constructive and destructive interferences, and relates this movement to an equivalent hydrodynamic diameter [22]. The presence of interfering particles or samples containing particles with heterogeneous size distributions, as would generally be the case for environmental samples, make data obtained difficult to be interpreted [8]. The inability to detect the presence of smaller particles among bigger ones, due to the fact that the scattering intensity depends by the sixth power of the particle diameter, is another major drawback of this technique [19]. Besides, DLS techniques require information on viscosity and refractive index, which is often not available or difficult to know in samples that are highly complex. Direct coupling to size separation techniques, like FFF and HDC, may overcome polydispersity problems and the presence of interfering particles, by presenting narrow size fractions to the DLS detector [102], and it will be discussed in section 6. Although DLS has serious limitations for sizing nanoparticle suspensions in complex matrices, it is very valuable to monitor aggregation behaviours. For example, aggregation of Ag NPs exposed to human synthetic stomach fluid [103], gastric juices [104] or river water [105] has been studied by DLS. In addition, this technique has also been used to proof the reliability of AF4 data in consumer products, although some results were biased to larger particle diameters respect to AF4 results [106].

Multi-angle light-scattering, also known as static light scattering, provides measurement of physical properties that are derived from the angular dependency of the light scattered by particles. Time averaged scattering intensities are measured at several angles to derive different size parameters, including the radius of gyration. Consequently MALS, in combination with DLS or FFF, can provide information about particle shape [7]. Since MALS requires cleaner samples than DLS, and a relatively deep knowledge of the optical properties of the particles, its use for characterization of ENPs in complex samples is limited, being always associated to FFF, as discussed in section 6.1.

Nanoparticle tracking analysis is an emerging light scattering technique, in which the movement of particles under Brownian motion is measured by using video microscopy and their hydrodynamic diameters are calculated using a modified Stokes-Einstein equation [23]. Single particles are first detected by light scattering, and the distance the particle travels in a given time interval is related to its hydrodynamic diameter; finally, by compiling and processing this information from a significant number of particles, particle-number concentration and hydrodynamic-size distributions are obtained. Because NTA is tracking individual particles, the method is not as subjected to the intensity limitations of DLS when measuring polydispersed samples. NTA shows high sensitivity in terms of particle-number concentration, minimum perturbation of the sample and reliable size distributions in the presence of large particles or aggregates, being capable of measuring a large number of individual ENPs in much less time than microscopy techniques. Due to laser power and camera detector limitations, reliable data cannot be obtained for particles with hydrodynamic diameters below 20 nm [28].

The application of NTA to the determination of size distributions and concentrations of ENPs in environmental, biological and food samples was reviewed by Gallego-Urrea et al. [23]. NTA has been used for the size characterization of ENPs in different nanotoxicological studies. The measurement of hydrodynamic diameters of Ag NPs after incubation in cell culture media with high protein content has been described by Bouwmeester et al. [108], confirming the formation of a biomolecular corona. The aggregation of Ag and TiO_2 NPs was also observed in the same culture medium by NTA in a study of NPs added to paints [109]. Similarly, changes in size of Ag NPs exposed to synthetic human stomach fluid have also been studied by NTA [103]. In relation with environmental studies, Piccapietra et al. [110] studied the colloidal stability of carbonate coated silver nanoparticles in synthetic media and natural freshwaters at different concentration levels of NPs. The size distribution of uncoated Ag NPs in seawater has also been determined for ecotoxicity studies [111]. Respect to ENPs in consumer products, the presence of Ag NPs in the effluents of a nanosilver producing washing machine was detected by NTA at μg L^{-1} levels, although nanoparticle sizes were overestimated with respect to TEM measurements due to the low scattering intensities of small particles [86].

5. Atomic spectrometry techniques

Detection of an ~~specific~~ inorganic ENM is probably the first step in the analysis of a complex sample, and the use of element-specific techniques is the most valuable tool to achieve this objective. Typical techniques for elemental analysis, including electrothermal atomic absorption, inductively coupled plasma optical emission spectrometry and inductively coupled plasma mass spectrometry, are considered in this section, but also synchrotron based techniques, like X-ray absorption spectroscopy. However, users must be aware that these techniques used alone are not specific to nanoparticles, except for single particle inductively coupled plasma mass spectrometry, which will be considered separately.

5.1. ET-AAS, ICP-OES and ICP-MS

Atomic spectrometry techniques like ET-AAS, ICP-OES and ICP-MS can be used for sensitive detection, as well as for quantification, of the element/s present in the ENM and the sample [21]. Currently, one of the most common techniques for the identification and quantification of inorganic ENMs involves the use of ICP-MS, due to the low detection limits attainable (down to ng L^{-1}). ICP-OES provide detection limits in the μg L^{-1} range, whereas ET-AAS offers a half-way performance between ICP-MS and ICP-OES. In any case, conventional atomic spectrometry techniques are sensitive to the element/s present in the sample that contains the ENM, but they are not capable of providing any information about the physicochemical form of the element (if present as dissolved species or as particulate), or any other information related to the ENM (e.g. size, aggregation). Thus the main applications of these techniques are oriented to get total element concentrations in a variety of samples that include bioaccumulation and biodistribution studies of ENMs [112, 113]. These limitations are overcome by using these techniques in combination with some of the sample preparation methods described in section 2 or, in the case of ICP-MS, by using it as on-line element-specific detector coupled to a continuous separation technique (HDC, FFF, CE). Alternatively, a new mode of ICP-MS, called single particle ICP-MS, is gaining significant interest [114] and it will be discussed below.

Whereas ET-AAS allows the introduction of samples both in solid or liquid phase (solutions and suspensions), ICP-based techniques involve the use of liquid phases. This means that solid samples must undergo some sample treatment for the digestion of the matrix, whereas suspensions could be directly analyzed. In any case, the feasibility of the direct analysis of suspensions by ICP-MS depends on the composition and size of the particles [115]. Suspensions of SiO_2 particles up to 1-2 µm have been successfully analyzed by using dissolved standards [116], as well as other metal and metal oxide nanoparticles below ca. 100 nm [117], proving that particulate and dissolved species behave in the plasma in a similar way. However, this behaviour cannot be extended to any nanoparticle [115].

5.2. Single particle ICP-MS

Single particle ICP-MS is able to provide information about the number concentration of nanoparticle suspensions, as well as about the elemental mass content per nanoparticle. If some additional information about the nature of the nanoparticles is available (shape, composition and density) the core size of the nanoparticles can be calculated, and number size distributions can be obtained. Because the dynamic range of SP-ICP-MS may be extended up to the micrometers region, polydispersed systems as well as aggregation or agglomeration processes may be studied. In addition, dissolved forms of the constituent elements of the nanoparticles can also be detected and determined. The topic has been reviewed recently [114], although it is evolving rapidly. Basically, the methodology consists of measuring nanoparticle suspensions at very low number concentrations (10^8 L^{-1} or lower) and very high data acquisition frequencies (10^2 -10^5 Hz) by using commercial instruments. Under such conditions, nanoparticles are detected as individual events over a continuous baseline due to the background or the dissolved element. Number concentration detection limits in the range of 1000 particles per mL can be achieved [118], whereas lowest size detection limits are in the range of 10-20 nm for monoelemental nanoparticles or more than 100 nm for oxides [119]. Although SP-ICP-MS shows a great potential for NP analysis, the presence of dissolved species of the monitored element can hinder or even make impossible the detection of the NPs. This limitation may be overcome with the last generation of ICP-MS instruments, capable of working with data acquisition frequencies up to 10^5 Hz and reading times in the microsecond range [120]. On the other hand, physical properties of the NPs can lead to their incomplete vaporization in the ICP, resulting in inaccurate results depending on the size and nature of the NPs [121].

SP-ICP-MS has been used with screening purposes to detect the release of nanoparticles from plastic food containers [122, 123], as well as the presence of nanoparticles and/or dissolved forms in dietary supplements [92], waste waters [4, 124, 125], foods and biological tissues [41] and blood [126]. SP-ICP-MS in combination with alkaline or enzymatic digestions has proven to provide reliable information about size distributions and number concentrations in laboratory studies involving food matrices spiked with silver or gold nanoparticles [32, 38, 40, 41], in tissues from organisms exposed to nanoparticles [33, 37, 42, 124], and native nanoparticles in foods and consumer products [95]. SP-ICP-MS has also been applied to study the fate of silver nanoparticles in *in vitro* human digestion [104], lake mesocosms [50] and washing solutions from commercial detergents [90], to track their dissolution [127] and agglomeration [128] in natural waters or their release from food additives [129].

5.3. X-Ray absorption spectroscopy

X-Ray absorption spectroscopy is an element specific technique that is able to provide specific qualitative information about metal/metalloid species, as well as about their quantitative distribution, although not about their particulate nature, in complex liquid and solid samples (e.g. soils, sediments, tissues). In addition, minor or no sample preparation is needed and the physical and chemical original states are preserved. XAS is divided into XANES, which provides information about the geometry and oxidation states, and EXAFS, which provides information about element coordination. The main limitations of XAS are the sensitivity of the technique (in the mg kg^{-1} range), the requirement of synchrotron radiation facilities and the difficulty to deconvolute and interpret the bulk data when the sample consists of a complex mixture of species. The technique has been recently reviewed in the context of the analysis of environmental [130] and biological samples [131].

XAS techniques have been used to assess the fate of ZnO and Ag NPs in wastewaters treatment plants and sewage sludges [132-137], including incinerated sludges [138], to study the accumulation and transformations of ENPs in plants exposed to Ag [139], CeO_2 [140, 141], TiO_2 [142, 143] and ZnO and CuO [144], and for speciation of silver in waters and soils from microcosm [145] and mesocosms [146] experiments.

6. Continuous separation techniques

A number of different separation techniques have already proven suitable for the separation of particles based on their size, surface, density and charge characteristics. If enhanced by their hyphenation to sensitive and selective detection systems, they can provide a sound foundation for the resolution of complex particle systems [11].

6.1. Field-flow fractionation

Field-flow fractionation is a family of separation techniques where separation takes place in a thin, elongated channel without a stationary phase, caused by the action of an external field perpendicularly applied to a laminar flow. The basis of separation and a detailed description of theory can be found in [147]. FFF offers: (i) Continuous size information through a wide size range (from 1 nm to 100 μm, depending on the separation mode); (ii) separations keeping the native conditions of the NPs, since the carrier solution can be adapted to the dispersed nanoparticle system, and (iii) the possibility of on-line coupling to a wide range of detectors and off-line fraction collection if necessary [148].

Depending on the type of field applied, different FFF sub-techniques are defined. Sedimentation (SdFFF), in which a centrifugal force is applied, and flow (FlFFF), where a perpendicular flow (cross flow) is applied, are the only two techniques described for the analysis of ENMs. SdFFF is especially suitable for high density particles (i.e. metallic NPs) of relatively large size [52, 53, 149]. On the other hand, asymmetric flow field-flow fractionation (AF4), the flow FFF mode commercially available, is considered the most universal of all FFF techniques, since the cross flow applied affects to all the species injected, and is applicable to both polymers and macromolecules, as well as (nano)particles [147]. Despite SdFFF is able to deliver higher resolution nanoparticle size separations, the wider size range attainable by AF4, limited by the ultrafiltration membrane cut-off (typically 1-10 kDa) placed on the bottom of the channel, together with the separation based just on differences in the hydrodynamic size of the NPs, have made AF4 the FFF sub-technique used in most of the studies carried out for the separation and characterization of ENPs in complex samples [29, 31, 34, 35, 38, 39, 41, 52, 54, 55, 95, 106, 128, 149-161].

The separation and characterization of nanoparticles by FFF in food and environmental samples was reviewed in 2011 by von der Kammer et al. [24]. Since then, a large number of applications to complex samples have been developed. Target nanoparticles include Ag, Au, Se, SiO_2, TiO_2 and ZnO in different complex matrices: sunscreens [52-54, 149, 154], food [29, 38, 39, 41, 55, 95, 150, 153, 162], consumer products [41, 95, 106], environmental [128, 156-158, 160] and biological samples [31, 34, 35, 151, 152, 155, 159, 161]. In most cases, a sample pre-treatment for ENMs dispersion in solution or matrix degradation is required before separation by FFF, as described in section 2. The injection of untreated samples is also possible in some cases, as in the characterization of SiO_2 NPs in coffee creamer [55], where a simple dilution of the sample in water was performed, although a filtration through a 5 μm pore size membrane was recommended in order to avoid clogging of tubing. Depending on the type of matrix, different digestion strategies (enzymatic, TMAH-based or acidic), clean-up with organic solvents or lixiviation with water have been described, followed by a centrifugation or a filtration step. For particle disaggregation, bath or tip sonication is usually required, although the addition of hexane was found to be useful in the case of TiO_2 NPs in sunscreens [52-54, 154]. In any case, these pre-treatments can affect the subsequent FFF separation. Thus, a significant influence on the elution of the NPs has been observed in the presence of partially degraded matrix after the enzymatic digestion of chicken meat [38], whereas aqueous extracts from gastrointestinal tract and gill tissues caused a significant fouling of the membrane, likely due to the high content on biomolecules, interfering with the NPs elution [151].

The optimization of the operating conditions (carrier composition, permeation membrane and cross flow program) is recommended for each type of sample in order to achieve a separation with minimum perturbation and high recoveries for all species [24]. Uncontrolled particle-membrane interactions can lead to changes on the NPs elution time [163] or low recoveries in the case of strong attractive interactions. Therefore optimization of operating conditions tends to minimize these interactions. However, even under optimal conditions, recoveries below 80% have been reported [34, 39, 54, 149], being one of the most serious limitations on the quantification of NPs in complex matrices by FFF. By contrast, good recoveries have been reported for Ag NPs in chicken meat [38], in the determination of SiO_2 NPs in coffee creamer [55]], tomato soup [29] and also for TiO_2 NPs in sunscreens [52].

Given the grade of complexity of the samples analysed, FFF is usually hyphenated to different detectors [25]. Although ICP-OES [149] and ET-AAS [162] have been described for off-line coupling, ICP-MS is commonly used as on-line elemental detector [148, 164], due to its high sensitivity and elemental selectivity. In addition, the mass quantification of the species eluted can be done by external calibration or by unspecific isotope dilution [165], using dissolved standards if nanoparticles and standards behave in the same way in the ICP.

UV-Visible detection is also commonly used in FFF separations, although its sensitivity is limited to concentrations in the mg L^{-1} range, and its selectivity is relatively low (even when full spectra are registered), what make its use complementary to elemental detectors in the case of laboratory studies working at relatively high concentrations. A comparison between estimated detection limits for various metallic NPs by FFF coupled to different detectors (UV-Visible, DLS, ICP-MS and ICP-OES) can be found in [166]. The use of UV-Visible detection, together with ICP-MS, has been described in the characterization of Ag NPs in enzymatically digested chicken meat [38] and culture medium and cells digested in TMAH [34] by AF4. In all these cases, ICP-MS silver peaks were related to Ag NPs because of band at around 400 nm, due to their surface plasmon resonance. The aggregation of Ag NPs in different dilutions of Daphnia magna toxicity test media has also been studied by AF4 coupled to a UV-Visible detector [167].

The use of light scattering detection is also common in the characterization of ENPs by FFF, in particular DLS, although MALS has also been described [29, 161]. Both techniques provide an independent measure of the particle dimensions of the eluting particles (the hydrodynamic radius and radius of gyration, respectively) and confirm the correct operation of the FFF method. However, the low sensitivity of these detectors limits their use at relatively low ENP concentrations. For this reason, the FFF-DLS tandem has been described in the separation and determination of size standards for validation of the established linear relation between hydrodynamic radius and retention time used for subsequent estimation of hydrodynamic diameters of NPs in unknown samples at low concentrations [31]. The use of NTA as on-line detector has been recently described for the study of the number-based SiO_2 NPs distribution in fetal bovine serum [161].

The combination of sizing methods such as TEM [31, 34, 38, 52, 55, 106, 162], or SP-ICP-MS [38, 39] with fraction collection has been also proposed for obtaining additional size information. SP-ICP-MS after AF4 collection also allows the identification of non-nano fractions (likely ionic silver bound to organic constituents) as described by Loeschner et al. [38] in the analysis of an enzymatic digestate of a chicken meat spiked with Ag NPs.

6.2. Electrophoresis

Electrophoretic techniques, which are based on the migration of charged species under the influence of an applied electric field, are available in different formats. Gel electrophoresis and capillary electrophoresis, which are the two electrophoretic techniques most commonly used for separation and characterization of nanoparticles [168-170] will be considered. Whereas most of the work with electrophoretic techniques has been devoted to the separation and characterization of nanoparticles according to size, shape and surface functionalization, using electrophoresis as diagnostic tool, the number of applications to real-world samples is still scarce.

GE is based on the different migration of analytes through a nanoporous gel by its sieving effect under an electric field. The most commonly used GE methods include polyacrylamide GE, commonly used to separate proteins, and agarose GE mainly used for separating charged biopolymers, such as DNA and RNA. Although PAGE has been used for characterization of ENPs, such as bioconjugated quantum dots [171], the small pore size of PA gels (less than 10 nm) limits its application for separation of nanoparticles. By contrast, the largest pore size of agarose gels (10-100 nm) enables the wide applications of AGE. The capability of AGE for separating nanoparticles of different sizes and shapes was demonstrated by Hanauer et al. [172], by using silver and gold nanoparticles derivatized by functionalization with polylethylene glycols, in order to control their charge and electrophoretic mobility. In this regard, AGE has been used almost exclusively for the separation and characterization of on purpose functionalized nanoparticles, like DNA and RNA bioconjugated Au NPs [173], or after derivatization, by using different thiol-containing ligands [174]. Detection of the nanoparticles in the cases cited above were based on visual analysis of the gels [173, 174], optical extinction spectroscopy [172], hyperspectral imaging [174] or TEM [172].

In CE, separations are solely based on the different mobilities of the charged species that are injected into a thin capillary filled with a background electrolyte, whilst a high voltage is applied at the capillary ends. As in the case of GE, functionalization of the nanoparticles also plays a critical role in their separation. In this sense, bioconjugated quantum dots [175, 176] and protein-nanoparticle interactions [177] are commonly studied by CE. Although metal and metal oxide nanoparticles have been separated by using different inorganic buffers as electrolytes, the addition of ionic surfactants appears to be the most convenient mode for separation of metallic nanoparticles [168-170]. For instance, Liu et al. [178] demonstrated that addition of sodium dodecyl sulphate to the background electrolyte improved the size separation of Au NPs, because of the charge of the NPs is then related to the number of molecules of surfactant adsorbed, which acts as a sort of in situ derivatizing agent. Qu et al. [180] developed a method for determination of Au, Pt and Pd NPs by using sodium dodecylbenzenesulfonate in the background electrolyte. Franze et al. [179] developed a method to separate gold and silver NPs and their ionic counterparts, using SDS as surfactant and penicillamine for complexing ions. Both methods resolved successfully metallic NPs down to 5 nm.

The most common detection techniques in CE for inorganic nanoparticles are UV-Vis absorption and fluorescence spectrometry. As in FFF, UV-Vis absorption has been extensively used to detect gold and silver NPs, taking advantage of their surface plasmon resonance at ca. 500 and 400 nm, respectively; whereas the intrinsic fluorescence of quantum dots is used for their detection. The use of ICP-MS for element specific detection of NPs has been recently introduced [179, 180] for determination of metallic NPs in dietary supplements.

The advantage of using CE with respect to other separation techniques is the high resolution attainable and the capability to analyze both ionic species and NPs. However, special attention must be paid to the surface characteristics of the NPs in the standards and samples, because surface differences can cause different surfactant-particle interactions and lead to inconsistent migration behaviours. Furthermore, complex matrices, such as biological fluids, contain different macromolecules that can interact with the NPs, modifying their surface charge, thus altering the elution times and peak resolution. It is expected that further development using matrix matched NP standards will permit the acquisition of more accurate information regarding NP size, composition and surface chemistry.

6.3. Hydrodynamic chromatography

In hydrodynamic chromatography, columns are packed with non-porous beads, building up flow channels, and separation is produced by the velocity gradient within the capillaries between beads. Thus larger particles are transported faster than smaller ones, as they spend less time near the edges of the capillaries [181]. The applications of HDC to the determination of ENPs in complex matrices are still scarce: they can be summarized as the identification of different natural and ENPs (TiO_2, SiO_2, Al_2O_3, Fe_2O_3, Ag and Au) in sewage sludge supernatants [181, 182], Ag NPs in natural river waters [105, 183] and synthetic surface waters

[184], and TiO_2 and ZnO NPs in commercial sunscreens [184]. A pre-treatment based on the aqueous extraction with a surfactant (Triton-X 100) and a 1 μm filtration was performed before the analysis of sunscreens by HDC. From the two columns commercially available, the column with a separation range of the 5-300 nm is used preferentially [181-185] to the 20-1200 nm [105, 184]. Poor resolution and coating surface effects on elution time have been observed for Au NPs in the range from 5 to 100 nm [185], which may prove problematic in complex matrices since NPs surface is likely to be modified, affecting their retention behaviour, as pointed out by these authors. By contrast, high recoveries were found in the same work for Au NPs, ranging from 77 to 96%. The use of Au NPs as internal standard in HDC has been proposed [181] given its high stability under a range of conditions. Attempts to quantify the concentration of nanoparticles through the use of both pre- and post-column injection of ionic standards have proved unsuccessful [182]. Philippe et al. [184] state that eluent composition should be optimised for each sample type when quantitative results are required. Under optimal conditions, these authors have validated a method based on HDC-ICP-MS for determination of 10 nm standard Au NPs in a simulated surface water solution, containing soft water and humic acids.

The use of different detectors (ICP-MS, DLS, UV-Vis and fluorescence) coupled to HDC has been reported. The combination of ICP-MS, UV-Vis and fluorescence detectors have proved to be useful for the analysis of Ag, TiO_2 and ZnO NPs in artificial waters containing high amounts of organic matter [184]. Using SP-ICP-MS as detection technique can help in distinguishing between spherical and non-spherical, as well as pristine and surface modified NPs, because HDC provides information about the hydrodynamic diameter and SP-ICP-MS about the NP core [186]. The combined technique (HDC-SP-ICP-MS) has not been applied to the analysis of NPs in complex or real matrices so far.

6.4. Other liquid chromatography techniques

Apart from hydrodynamic chromatography, other chromatographic separation modes have been investigated for separation of NPs. The main problem of applying these modes (e.g. size exclusion chromatography) is the adsorption of the NPs on to the stationary phases, limiting the types of columns that can be used [187]. More recently, amino-SEC columns [188], as well as reverse phase [189] and cation exchange stationary phases [190] have been proposed for separation of silver nanoparticles from silver (I), followed by on-line quantification with ICP-MS. Silver (I) was complexed with thiosulphate [188, 189] or ethanolamine [190] to improve the recovery of the dissolved species. The developed methods were applied to healthcare formulations [188, 190], spiked sewage and lake waters [188] and to study the release of silver species from textiles [189].

7. Electroanalytical techniques

Electrochemistry may provide an efficient, cost-effective approach for detection, size characterization and quantification of NPs. The two electroanalytical techniques specifically applied to the analysis of ENMs are: Voltammetry of immobilized particles and particle collision coulometry. Whereas VIP is sensitive to the oxidation state of the element/s in the sample, in principle, regardless of their dissolved/particulate state, PCC is capable of providing information specifically related to nanoparticles, in a similar way than SP-ICP-MS. The techniques have been applied to Ag, Au, Cu, Ni, Pt, Pd, CeO_2, CuO, Fe_2O_3, Fe_3O_4, IrO, NiO, TiO_2, and CdSe nanoparticles, mostly in relation to fundamental aspects of the techniques and occasionally applied to the analysis of real samples so far.

Originally, VIP was developed for the analysis of microparticles, being known as voltammetry of microparticles (VMP) [191], and more recently it has been applied to nanoparticles [192]. VIP involves the immobilization of the nanoparticles on the electrode surface, what means that the nanoparticles are separated from the media in which they are suspended. Although, there are different ways to immobilize the nanoparticles on the surface, the drop and dry procedure is the most used because it requires minimum manipulation of the sample. Typically, a few microliters of sample are deposited on the electrode and they are allowed to dry, preferably under an inert gas flow and by using slow evaporation rates to avoid agglomeration of the nanoparticles. Alternatively, the electrode surface can be modified to immobilize the nanoparticles by electrostatic interactions [193] or chemical reactions [194].

Next, the electrode is transferred to the electrochemical cell, which contains an adequate supporting electrolyte, and it is voltammetrically scanned. The resulting voltammograms show one or more peaks whose potentials can be related to the nature of the nanoparticle, its size and the surface coverage of the electrode [195]. A peak potential from a NP is related to the standard potential of the involved redox couple in bulk form, but shifted up to 300 mV, depending on the size of the NP. This shift increases as the NP size decreases and can be estimated by using the Plieth equation [195]. Consequently, a range of potentials may be established for each nanoparticle composition that may be used for identification purposes. If the surface coverage of the electrode is low enough, the peak potential can be directly related to the diameter of the nanoparticle and a linear relationship between the logarithm of the diameter and the peak potential can be established by using a set of size standards [195]. On the other hand, the area under the peak represents the amount of oxidized or reduced nanoparticles during the electrochemical process, and it could be used to get quantitative information.

By using VIP, size and mass concentration of silver nanoparticles in health care formulations were determined by using glassy carbon and screen printed electrodes and linear scanning sweep [196]. Linear responses were obtained in the range of 10-100 nm for nanoparticle diameter and 0.5-6 mg L^{-1} for silver concentration. Cysteine modified glassy carbon [194] and screen printed electrodes [197], as well as gold electrodes fabricated from recordable compact discs, have been proposed for detection and quantification of silver NPs in seawater [198]. Although gold electrodes from recordable CDs offered higher coverage than surface modified electrodes, all of these proposals can just be considered as proofs-of-concept, and no information about their analytical performance has been provided.

PCC, also known as particle collision coulometry or nanoparticle-electrode impacts, is based on the modification of the baseline of a chronoamperogram (current vs. time plot) when a metal or metal oxide nanoparticle randomly impacts the surface of a microelectrode held at a fixed potential, due to the oxidation/reduction of the nanoparticle, or caused by some electrocatalytical processes triggered when the nanoparticle hits the electrode. A sharp transient signal is observed if the nanoparticle bounces back to the solution, whereas a step signal is obtained when the nanoparticle stays on the electrode. The charge involved in the process is related to the mass of the electroactive species, and hence to the size of the nanoparticle [199, 200]; thus size distributions might be obtained in a similar way than in SP-ICP-MS. Platinum nanoparticles down to 4 nm have been detected by PCC [201]. Quantitative information can be obtained from the corresponding chronoamperograms since the frequency of collisions on the electrode is proportional to the number concentration of nanoparticles [201]. Sensitivity can be increased by coupling an electrocatalytic reaction to the collision event [202]. Alternatively, the frequency of impacts can be increased by driving nanoparticles to the electrode surface not only by Brownian motion, but also by mass transfer [203] or using magnetic fields [204]. The use of cylindrical microelectrodes instead of microdisks has allowed to work with suspensions down to 10^{10} L^{-1} [205].

PCC has been used for the detection and sizing of silver nanoparticles in a spray disinfectant product, as well as spiked in seawater [206]. No treatment of the disinfectant product was necessary other than dilution with the supporting electrolyte. The modal size of the nanoparticle distribution in the product was in close agreement with NTA measurements. Similar results were obtained when the disinfectant product was analyzed in seawater. The same authors have studied the evolution of silver nanoparticles in seawater using Ag NP standards and they found that silver nanoparticles were aggregated in this media over the time scale of the measurements [207]. To date, applications involve metallic nanoparticles and anodic PCC, whereas cathodic PCC has just been used for the characterization of pristine nanomaterials, like magnetic nanoparticles of Fe_3O_4 [208].

VIP and PCC can be considered complementary techniques and usually they are used in combination [209]. VIP provides information about the chemical composition of the nanoparticles, by checking the potentials of the voltammetric peaks, which are going to be applied in the PCC measurements. Additionally, mass concentration and average sizes can be obtained by VIP, whereas PCC is able of providing detailed information about size distribution of the nanoparticle and quantitative information in terms of number concentrations.

8. Chemical sensors

Although chemical sensors are considered well suited for monitoring of ENMs due to their low cost, sensitivity, portability and simplicity [210], the number of sensor devices developed and eventually tested on real samples is low, and still far from being succesfully applied to complex samples. Electrochemical, optical and mass sensitive sensors have been proposed for the detection of quantum dots, silver and gold nanoparticles.

In relation to electrochemical sensors, both voltammetry of immobilized nanoparticles and particle collision coulometry are techniques suitable of being implemented in electrochemical devices. Although most of the work with these techniques involves proofs-of-concept with conventional electrodes configuration, disposable screen printed electrodes have been used by Cheng et al. [197] and Cepriá et al. [196], and applied to the analysis of seawater and consumer products, respectively. A flexible hybrid polydimethylsiloxane–polycarbonate microfluidic chip with integrated screen printed electrodes have also been fabricated and applied for the electrochemical detection of quantum dots [211].

Two fluorescent probes have been developed for detection of silver nanoparticles, although they have not been implemented in optical devices yet. Chatterjee et al. [212] developed a rhodamine-based fluorogenic and chromogenic probe for Ag(I) detection, also applicable for the detection of Ag NPs when oxidized with hydrogen peroxide in acidic conditions. The sensing mechanism was based on an irreversible process promoted by coordination of Ag(I) to the iodide of the probe, which was accompanied by both colour and fluorescence changes. The probe showed high selectivity over other metal ions and detected Ag(I) down to 14 μg L^{-1}, being applied for the quantification of Ag NPs in consumer products. More recently, Cayuela et al. [213] have reported a fluorescent probe based on amine-modified carbon dots. The amine groups at the carbon dots surface induce the aggregation of citrate-coated Ag NPs, the red-shifting of the SPR absorption wavelength of the nanoparticles, and hence the decrease in carbon dots fluorescence due to inner filter effect. Under the same conditions PVP-Ag NPs remained well-dispersed, not affecting the carbon dots fluorescence.

The method was applied to the determination of citrate Ag NPs in cosmetic creams in the presence of TiO_2 NPs.

Rebe Raz et al. [214] have developed a surface plasmon resonance (SPR) sensor based on human metallothionein for detection of silver nanoparticles. The metallothionein was immobilized directly on the surface of the SPR sensor. The sensor showed sensitivity in the μg L^{-1} range, displaying the highest sensitivity towards larger and uncoated Ag NPs. Unfortunately, ionic silver was the major interference and it should be previously removed by dialysis or ultrafiltration if present. The ability of the sensor to detect Ag NPs in food and water samples was evaluated by analyzing spiked river water and cucumber and tomato water extracts.

A piezoelectric sensor was developed by Chen et al. [215] using an immunoglobuline coated surface for detection of gold nanoparticles with sensitivity in the ng range, although it was not tested on any type of sample.

9. Single- and multi-method analytical approaches

Due to the disparity of scenarios and types of samples, it is likely that no single method will suffice to provide all the information demanded for studying or solving each specific problem. A combination of methods will be needed to ascertain the fit-for-purpose information. Figure 1 summarizes the three generic scenarios considered along this review, showing increasing levels of complexity due to the sample matrices involved and the expected concentration ranges. Scenario 1 involves the analysis of ENM-containing products, where the concentration of ENMs is expected to be high, although the matrix can be not so simple (e.g. sunscreens or foods). In scenario 2 the complexity of the sample matrix depends on the type of laboratory study: from the variety of synthetic media for ecotoxicological and toxicological essays to different types of natural and wastewaters, soils, sediments, sludges, or biological fluids and tissues. The main advantage of scenario 2 is that concentration, size and coatings of the ENMs under study are selected in accordance with the techniques and methods to be used and the aim followed. Finally, scenario 3 involves the monitoring of ENMs in matrices as complex as in scenario 2, but at their real concentrations. On the other hand, the amount and variety of the information demanded implies an additional factor of complexity. This information can be as simple as knowing if an ENM containing a specific element is present in a sample (detection), although the situation can be more complicate if quantitative (as element mass or number nanoparticle concentration) or size information must be provided, or if the ENM suffers different types of transformations (e.g. dissolution, oxidation, aggregation, surface modification, composition).

Table 2 summarizes reported analysis of different samples from the scenarios considered above, by using techniques and methods presented in the previous sections. These applications have been arranged according to the scenario, the type of NP and the analytical techniques used. Total mass concentrations are usually determined in most applications and it has not been explicitly included. Native nanoparticles have been determined in consumer products containing ENMs (scenario 1), including foods, in release experiments from consumer products (scenario 2.a), and to a much less extent in environmental samples (scenario 3). The rest of applications involve the spiking of samples with NP standards, and toxicological and ecotoxicological studies (scenario 2.b), where the addition of NPs is part of the experiment.

Depending on the scenario, the types of demanded information and its quality, different approaches can be adopted in a fit-for-purpose basis. Working with inorganic ENMs, the simplest approach is based on the use of an atomic spectrometry technique (flame AAS, ET-AAS, ICP-OES or ICP-MS, depending on the concentration) for monitoring of a specific element, both for detection and total element quantification. This may be sufficient when tackling with scenario 2 studies, where control samples with no added ENMs are available for comparison [113]; however, with samples from scenarios 1 and 3, more elaborated approaches are necessary. Then, an atomic spectrometry technique in conjunction with an electron microscopy technique (ICP-MS and TEM, most often) is viewed as the basic tools for adequate detection, total mass quantification and characterization (size and shape). Alternatively, it is also a common practise to rely just on electron microscopy for detection and characterization of the visualized nanoparticles, overlooking the presence of dissolved species [74]. In order to move from these basic approaches, table 3 summarizes different approaches currently available which have been applied to complex real-world samples. Focussing on scenario 1, Tulve et al. [216] developed a tiered approach for the analysis of consumer products (textile, plastic and liquid matrices) for Ag NPs. Total silver content was determined by ICP-MS or ICP-OES, directly or after acid digestion. Visualization and sizing of NPs was performed by SEM or TEM in combination with EDS to confirm the composition of the NPs. In addition, UV-Visible absorption spectrometry was used as a supplementary technique to confirm the presence of metallic silver NPs (surface plasmon resonance absorption around 400 nm); whereas for liquids, DLS also provided supplementary information about hydrodynamic diameters, and free ionic silver was measured by ion selective electrode potentiometry. ICP-MS could be used in combination with the rest of techniques because total silver contents in the samples were in the mg kg^{-1} or higher. The situation is far more difficult if the expected concentrations are in the range of ng L^{-1} or ng kg^{-1}, as it can be the case in scenario 3. In such situations the aim of the selected approach is currently confined to detect and quantify the element present in particulate form by using a sensitive atomic spectrometry technique or in combination with a preconcentration step. Gondikas et al. [217] applied SP-ICP-MS to detect Ti containing particles in surface water, number concentrations were also estimated in spite of the fact that just particles over 130 nm were detectable. Li et al. [67] determined the concentration of silver associated to nanoparticles in waste water treatment plants by ET-AAS after cloud point extraction or ion exchange solid phase extraction. Because concentrations as low as 1 ng L^{-1} were determined, additional information about the nature of the nanoparticle was unachievable due to the methodology selected.

Undoubtedly, approaches applied in the context of environmental, toxicological and ecotoxicological studies (scenario 2) can show the highest level of complexity in order to get different types of information depending on the objectives of the study. Furtado et al. [50] investigate the persistence and transformation of silver nanoparticles in a littoral lake mesocosm by SP-ICPMS, ultrafiltration, cloud point extraction and AF4-ICP-MS. Unrine et al. [43] studied the dissolution and aggregation behaviour of Ag NPs in four microcosms (surface water; water and sediment; water and aquatic plants; water, sediment and aquatic plants) by using ultrafiltration in combination with ICP-MS, and AF4 coupled to UV-Visible, DLS, MALS and ICP-MS detection. In addition, XANES provided solid phase speciation. As it has been stated in section 5.3 XAS techniques have proved their potential in different complex situations, being complemented with atomic spectrometry techniques usually.

As it has been discussed above, most of the works published by now, related to the monitoring of ENMs in environment, foods and organisms involve the analysis of spiked samples or *in vivo* exposed organisms, what means that approaches based on these methods may not be useful if concentrations in real-world samples are below the attainable detection limits. In this respect, approaches based on the use of SP-ICP-MS, as well as those based on AF4 separations coupled to ICP-MS, are considered very promising. For solid samples, these techniques have to be combined with selected matrix digestion procedures, in order to extract the nanoparticles preserving their original state.

Although this review focuses on ENMs, natural nanoparticles cannot be forgotten because natural nanoparticles of different or similar composition are going to be found mixed with ENPs, as in the case of environmental samples [218, 219]. This means that the identification of ENPs in the presence of natural ones involves an additional challenge. Whereas different labelling methods (fluorescent labels, radiolabelling, stable isotope labelling) are utilized when ENPs are added deliberately to a system under study, the solution is far more problematic when real samples are involved, like in scenario 3. These difficulties become evident for techniques capable of detecting NPs regardless of their nature, like DLS, but they are also present when using element-specific techniques detecting NPs, like ICP-MS working in single particle mode or coupled to a continuous separation technique, as well as electrochemical techniques or chemical sensors. To distinguish ENPs from natural nanoparticles of similar composition, Von der Kammer et al. [220] have proposed the use of elemental ratios. The underlying principle is that natural NPs contain significant amounts of other elements, which is not the case for synthetic ones. Ti/Al and Ce/La ratios have been used for identification of engineered TiO_2 and CeO_2 nanoparticles by analysing bulk samples [217], as well as in combination with AF4-ICP-MS and SP-ICP-MS [102]. In any case, these techniques just provide evidence of the presence of one or more elements associated to nanoparticles and not the exact nature of such nanoparticles. Additional information and proper interpretation is needed for identifying and determining ENPs in natural samples [221], as in the case study of the release of TiO_2 ENMs from sunscreen products into surface waters [217].

10. Concluding remarks and future prospects

Although an increasing number of analytical techniques and methods are becoming available for the detection, characterization and quantification of inorganic ENMs, their application to complex samples is still very limited and far from being incorporate to routine analysis. To reach this stage, additional development of standard methods, both for sample preparation and measurement, as well as reference materials, are needed.

Analyzing the actual situation, electronic microscopy and atomic spectrometry are well established techniques to get information about inorganic ENMs, whereas synchrotron based techniques can provide detailed speciation information in specific cases. Alongside these techniques, the use of ICP-MS in combination with FFF separations or in single particle detection mode are finding their way in the most recent analytical approaches, because of the supplementary information that can provided. Other separation techniques, like electrophoresis or hydrodynamic chromatography, as well as the electroanalytical techniques or the use of chemical sensors are yet in their early stages with respect to their application to complex and real-world samples and must deserve further utilization.

Apart from the different studies where the approaches and analytical methods discussed along this review are being used, another driving force for their establishment in the near future is going to be the development of new regulations for the control of ENMs. In this context, the recommendation on the definition of nanomaterials published by the European Commission [222] is a paradigmatic example because the correct implementation of this definition, or any other regulation, requires the availability of appropriate and validated analytical methods. Method validation is another pending issue in the field. Although there are standard methods and reference materials for well-established techniques (electron microscopy or DLS), this is not the case for emerging techniques and with respect to the availability of standard reference materials in different matrices.

Undoubtedly, analytical scientists are at the forefront of all these pending tasks with the sole purpose of providing the analytical information in quantity and quality needed to support the sustainable development and use of ENMs.

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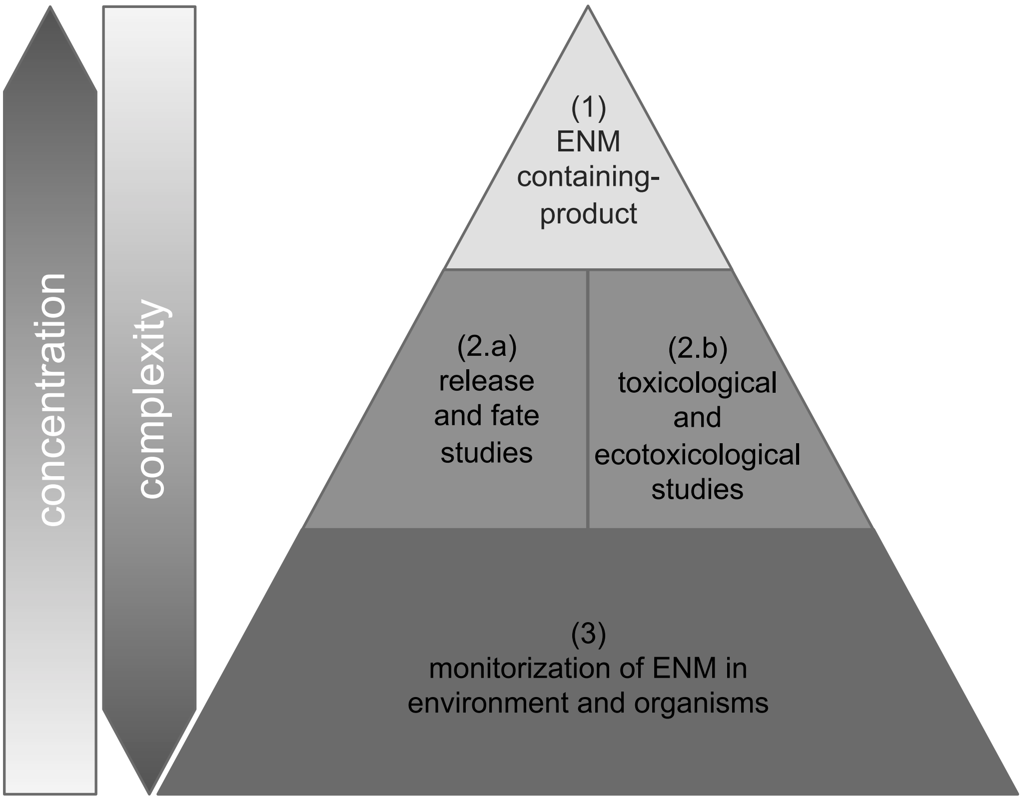
LIST OF CAPTIONS

Fig. 1. Potential scenarios for the analysis of ENMs along their life cycle.

Fig. 2. Transformations of pristine inorganic ENMs (dispersed of embedded in a solid matrix) in contact with an aqueous medium.

Figure 1


concentration
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(1)
ENM
containing-
product
(2.a)
release
and fate
studies
(2.b)
toxicological
and
ecotoxicological
studies
(3)
monitorization of ENM in
environment and organisms

Figure 2

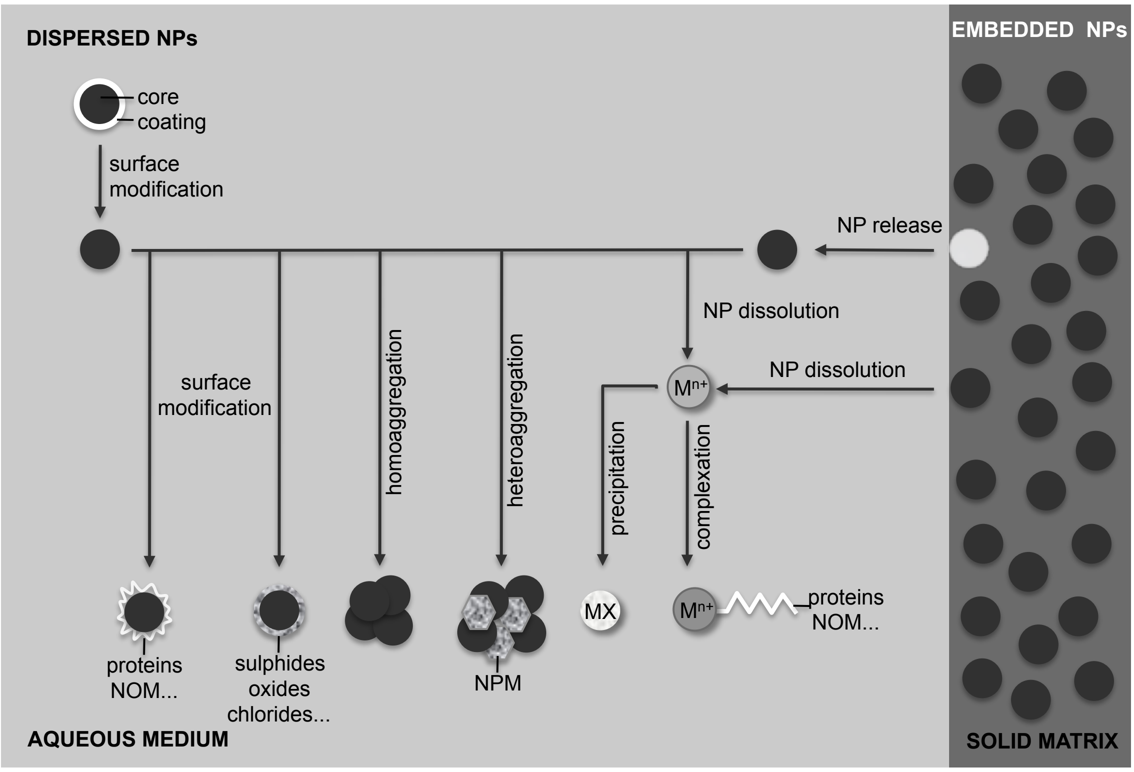
DISPERSED NPs
core
coating
surface modification
NP release
EMBEDDED NPs
surface modification
homoaggregation
heteroaggregation
NP dissolution
NP dissolution
M^{n+}
precipitation
complexation
MX
M^{n+}
proteins NOM...
proteins NOM...
sulphides oxides chlorides...
NPM
AQUEOUS MEDIUM
SOLID MATRIX

Table 1
Analytical techniques proved to be suitable for analysis of complex systems (emergent techniques are also included).

Technique	Acronym	Size LOD	Concentration LOD	Analytical information
Electron microscopy				
Transmission electron microscopy	TEM	<1 nm		- Size (average and distribution) - Shape - Elemental composition (+EDS) - Chemical structure (+EELS) - Crystal structure (+SAED/CBED)
Field-emission scanning electron microscopy	FESEM	1 nm		- Size (average and distribution) - Shape - Elemental composition (+EDS)
Environmental scanning electron microscopy	ESEM	30 nm [81]	10^{12} L^{-1} [81]	- Size (average and distribution) - Shape
Light scattering				
Nanoparticle tracking analysis	NTA	20 nm [107]	10^{9} L^{-1} [23]	- Size (average and distribution) - Number concentration
Atomic spectrometry				
Inductively coupled plasma mass spectrometry	ICP-MS	-	ng L^{-1}	- Bulk element composition - Total mass concentration
Single particle ICP-MS	SP-ICP-MS	10-20 nm [119]	10^{6} L^{-1} [114] ng L^{-1}	- Detection of dissolved element/NP - Element mass per NP (average and distribution) - Size (average and distribution) - Number concentration - Mass concentration
X-ray absorption spectroscopy	XAS	-	mg kg^{-1}	- Chemical composition - Identification/quantitative distribution of chemical species
Separation techniques				
Asymmetric flow field-flow fractionation	AF4	1-5 nm	1-10 µg L^{-1} AF4-ICP-MS 0.1 mg L^{-1} AF4-UV-Vis 1 mg L^{-1} AF4-DLS [166]	- Detection of complexed elements/NP - Size (average and distribution) - Mass concentration
Capillary electrophoresis	CE	5 nm	0.2 µg L^{-1} CE-ICP-MS [179]	- Detection of dissolved element /NP - Size (average and distribution) - Mass concentration
Hydrodynamic chromatography	HDC	5 nm	1 µg L^{-1} HDC-ICP-MS [182]	- Detection of dissolved element /NP - Size (average and distribution) - Mass concentration
Electroanalytical techniques				
Voltammetry of immobilized particles	VIP	10 nm	µg L^{-1}	- Element composition - Oxidation state - Size (average) - Mass concentration
Particle collision coulometry	PCC	5 nm	10^{10} L^{-1} [205]	- Detection of NPs - Size (average and distribution) - Number concentration - Mass concentration

Table 2
Reported analysis of complex samples from the scenarios defined in figure 1, sample preparation methods and analytical techniques used, and analytical information obtained.

Scenarios	NP	Sample	Sample preparation	Techniques	Analytical information	Ref.
(1) Consumer products containing ENPs						
	Ag	Disinfectant products		TEM SEM-EDS	Size	[87]
	Ag	Disinfectant products		TEM-EDS	Size Shape Elemental composition	[91]
	Ag Au Cu Ir Pd Pt Si Zn	Dietary supplements		SP-ICP-MS TEM-EDS	NP Detection Size Elemental composition	[92]
	Ag	Antibacterial products	CPE	ET-AAS	Mass concentration NP	[64]
	Ag	Antibacterial products	CPE + MW digestion	ICP-MS	Mass concentration NP	[59]
	Ag	Dietary supplement Beverages		AF4-ICP-MS	Size	[153]
	Ag	Consumer products		AF4-UV-Vis-DLS-ICP-MS	Size	[106]
	Ag	Healthcare formulations		HPLC-UV-Vis-ICP-MS	Mass concentration NP/dissolved	[190]
	Ag	Healthcare formulations		SEC-ICP-MS	Mass concentration NP/dissolved	[188]
	Ag	Healthcare formulations		VIP	NP detection Size Mass concentration NP	[196]
	Ag	Disinfectant products		PCC	NP detection Size	[206]
	Ag	hand sanitizer gel fabric softener	Oxidation (H_2O_2)	Optical sensor	Mass concentration NP	[212]
	Au	Dietary supplements		CE-ICP-MS	Size	[180]
	Au	Dietary supplements		CE-ICP-MS	Size Mass concentration NP/dissolved	[179]
	metal NPs	Bread and biscuits		ESEM-EDS	Size Elemental composition	[80]
	Pt Pd	Dietary supplements		CE-ICP-MS	Size	[180]
	SiO_2	Coffee creamer	Clean up (hexane)	AF4-ICP-MS TEM	Size Mass concentration NP	[55]
	SiO_2	Powdered cappuccino mix Food integrator	Water extraction	SdFFF-UV-Vis	Size	[162]
	TiO_2	Moisturizing cream	Clean up (hexane)	AF4-ICP-MS	Size Mass concentration NP	[154]
	TiO_2	Sunscreen	Clean up (hexane)	AF4-ICP-MS	Size Mass concentration NP	[54]
	TiO_2	Sunscreen	Clean up (hexane)	AF4-UV-Vis ICP-OES	Size Mass concentration NP	[52]
	TiO_2	Food Personal care products	Digestion (H_2O_2)	FESEM-EDS AF4-ICP-MS SP-ICP-MS	Size Mass concentration NP	[95]
	TiO_2	Sunscreen	Clean up (hexane)	SedFFF-ICP-MS	Size Mass concentration NP	[53]
	TiO_2 ZnO	Sunscreen	Water extraction	HDC-ICP-MS	Size	[184]

Scenarios	NP	Sample	Sample preparation	Techniques	Analytical information	Ref.
	TiO_2 ZnO	Sunscreens	Clean up (chloroform) Evaporation	TEM FESEM-EDS XRD	Size Shape Elemental composition Crystal structure	[74]
	ZnO	Disinfectant products		TEM-EDS	Size Shape Elemental composition	[91]
(1) Consumer products spiked with ENPs						
	Ag	Pears		TEM FESEM-EDS	NP detection	[93]
	Ag	Chicken meat	Digestion (enzymatic)	SP-ICP-MS	Size Mass concentration Number concentration	[40]
	Ag	Chicken meat	Digestion (enzymatic)	SP-ICP-MS	Detection NP Size	[41]
	Ag Au	Beef meat	Digestion (TMAH)	SP-ICP-MS	Size Mass concentration NP Number concentration	[32]
	Ag	Chicken meat	Digestion (enzymatic)	SP-ICP-MS AF4-ICP-MS TEM	Size	[38]
	Ag	Chicken meat	Digestion (enzymatic)	AF4-ICP-MS	Size Mass concentration NP	[39]
	Ag	Foods Waters		optical sensor	NP concentration	[214]
	Ag	Cosmetic cream	Clean up (cloroform)	optical sensor	NP concentration	[213]
	SiO_2	Tomato soup	Digestion (acid)	AF4-ICP-MS	Size	[150]
	SiO_2	Tomato soup	Digestion (acid)	AF4-ICP-MS	Size Mass concentration NP	[29]
	SiO_2	Tomato soup		ESEM FESEM TEM NTA	Size	[81]
	TiO_2 ZnO	Starches Wheat flour	Ashing	SEM-EDS	Size Shape Elemental composition	[94]
(2.a) Laboratory release experiments						
	Ag	Socks	Water extraction	HPLC-ICP-MS	Mass concentration NP/dissolved	[189]
	Ag	Plastic food containers	Extraction (acetic acid, methanol)	SEM-EDS SP-ICP-MS	Detection NP	[122]
	Ag	Plastic food containers	Extraction (acetic acid, methanol, olive oil)	SEM TEM-EDS SP-ICP-MS	Detection NP/dissolved	[123]
	Ag	Food additive	Water extraction	TEM-EDS SP-ICP-MS	Size	[129]
	QD	QD lighting	Extraction + UF (3kDa)	ICP-OES	Mass concentration NP/dissolved	[49]
	TiO_2	textiles	Washing solution	TEM-EDS SEM-EDS	Size	[88]
(2.a) *In vitro* digestion simulation						
	Ag	Synthetic gastric juice		TEM DLS NTA	Size	[103]
	Ag	Synthetic saliva Gastric juice Intestinal juice		SP-ICP-MS DLS SEM-EDS	Size Number concentration	[104]
	Se	Gastric juice Intestinal juice		AF4-ICP-MS	Size Mass concentration NP	[159]
	Ag	Washing solutions (commercial detergents)	UF	TEM-EDS SP-ICP-MS	Size Mass concentration NP/dissolved	[90]

Scenarios	NP	Sample	Sample preparation	Techniques	Analytical information	Ref.
(2.a) Environmental fate studies						
	Ag	Soils		ESEM FESEM	Size	[83]
	Ag	Microcosm water	Filtration (0.7 µm) + ultracentrifugation	ICP-MS	Mass concentration NP/dissolved Aggregation	[43]
	Ag	Mesocosm lake water	Filtration (35 µm) Filtration (0.45 µm) Ultrafiltration (3kDa)	ICP-MS	Mass concentration dissolved	[50]
	Ag	Freshwater		NTA DLS UV-Vis	Size	[110]
	Ag	Incinerated sludge		SEM-EDS XANES	Solid phase speciation	[138]
	Ag	Natural waters		SP-ICP-MS	Mass concentration NP/dissolved	[127]
	Ag	Sewage sludge	Centrifugation	TEM-EDS XANES	Size Shape Composition Solid phase speciation	[132]
	Ag	Microcosm water	Filtration (0.02 µm)	XANES	Solid phase speciation	[145]
	Ag	Sewage sludge		XANES	Solid phase speciation	[136]
	Ag	Soil		XANES EXAFS	Solid phase speciation	[146]
	Ag	Seawater		NTA	size	[111]
	Ag	Mesocosm lake water	CPE + MW digestion	ICP-MS	NP mass concentration	[50]
	Ag	Sewage sludge		XANES	Solid phase speciation	[133]
	Ag Au	Sewage sludge	Centrifugation	TEM-EDS XANES EXAFS	Size Shape Composition Solid phase speciation	[134]
	Ag ZnO	Sewage sludge		XANES EXAFS	Solid phase speciation	[137]
	Au	Soils		ESEM FESEM	Size	[83]
	ZnO	Sewage sludge		XANES	Solid phase speciation	[133]
(2.b) Ecotoxicological and toxicological studies (*in vivo* exposure)						
	Ag	Gastrointestinal tract Gills (fish)	Water extraction	AF4-ICP-MS	Size	[151]
	Ag	Lumbriculus variegatus	Water extraction	AF4-ICP-MS	Size	[155]
	Ag	Biological tissue homogenate	Water extraction	SP-ICP-MS AF4-ICP-MS	Size	[152]
	Ag	Human hepatoma cells	Digestion (TMAH)	AF4-ICP-MS	Size	[34]
	Ag	Faeces (rat)	Digestion (TMAH)	AF4-ICP-MS	Size	[35]
	Ag	Various organs (rat)	Digestion (enzymatic)	SP-ICP-MS	Size	[37]
	Ag	Liver (rat)	Digestion (enzymatic)	SP-ICP-MS	detection NP Size	[41]
	Ag Au	Daphnia magna	Digestion (TMAH)	SP-ICP-MS	Size Mass concentation NP Number concentration	[32]
	Ag	Various organs Blood (rat)	Solid phase extraction (knotted reactor)	ICP-MS	Mass concentration NP/dissolved	[70]
	Ag	Plant tissue		XANES SEM-EDS	Solid phase speciation Detection	[139]

Scenarios	NP	Sample	Sample preparation	Techniques	Analytical information	Ref.
	Au	Spleen (rat)	Digestion (TMAH) Digestion (enzymatic)	SP-ICP-MS	Size Number concentration	[33]
	Au	Plant tissue	Digestion (enzymatic)	SP-ICP-MS	Size Mass concentration	[42]
	Au	Liver (rat)	Digestion (TMAH)	AF4-ICP-MS	Size Mass concentration	[31]
	CuO ZnO	Plant tissue		XANES	Solid phase speciation	[144]
	TiO_2	Plant tissue		XANES SEM-EDS	Solid phase speciation Detection NP	[139]
(2.b) Ecotoxicological and toxicological studies (spiked samples)						
	Ag Au	Lumbriculus Variegatus	Digestion (TMAH)	SP-ICP-MS	Size Mass concentation NP Number concentration	[32]
	Ag	Cell culture medium		NTA TEM	Size	[108]
	Ag TiO_2	Cell culture medium		NTA	Size	[109]
	Au	Blood		SP-ICP-MS TEM	Detection NP	[126]
	SiO_2	Lung Liver (rat) Human aortic endothelial cells	Digestion (enzymatic)	SdFFF-DLS	Size	[36]
	SiO_2	Lung Liver (rat) Human aortic endothelial cells	Digestion acid	SdFFF-DLS	Size	[30]
	SiO_2	Serum		AF4-NTA-ICP-MS	size distribution number concentration	[161]
	TiO_2	Bovine serum albumin		ESEM FESEM TEM NTA	Size	[81]
(3) Analysis of environmental samples						
	Ag	Waste water	CPE	ET-AAS	Mass concentration NP	[67]
	Ag	Waste water	SPE (ion exchange)	ET-AAS	Mass concentration NP	[67]
	Ag	Tap water Sea water	CPE	ET-AAS	Mass concentration NP	[64]
	Ag	River water Waste water	CPE	ET-AAS	Mass concentration NP	[61]
	Ag	Washing water	Serial filtration (0.45 µm-0.1 µm-10kDa)	ICP-MS TEM-EDS	Mass concentration Shape	[47]
	Ag	Waste water		SP-ICP-MS	Detection NP/dissolved Size	[41]
	Ag	Waste water		SP-ICP-MS	Detection NP/dissolved	[124]
	Ag	Washing machine effluent		SP-ICP-MS TEM-EDS NTA	Detection NP Size	[86]
	Ag Ti Ce	Waste water	filtration (5 µm+0.45 µm)	SP-ICP-MS	Detection NP	[125]
	CuO	River water Waste water	CPE CPE + MW digestion	ET-AAS ICP-MS	Mass concentration NP	[63]
	SiO_2 Al_2O_3	Wastewater		SEM-EDS ESEM	Size	[89]
	TiO_2	Sewage sludge	Centrifugation	FESEM TEM	Size Shape	[84]
	TiO_2	Sewage sludge amended soil		FESEM TEM	Size Shape	[84]
	TiO_2	Sewage sludge amended soil	Suspension in water	TEM-EDS	composition size	[85]

Scenarios	NP	Sample	Sample preparation	Techniques	Analytical information	Ref.
	TiO_2	Lake water		ICP-MS SP-ICP-MS	detection NP number concentration natural NP/engineered NP	[217]
(3) Analysis of environmental samples (spiked)						
	Ag	River water Lake water Waste water	CPE + MW digestion	ET-AAS	Mass concentration NP	[58]
	Ag	Lake water Wastewater	CPE + MW digestion	ICP-MS	Mass concentration NP	[59]
	Ag	Tap water Surface water	SPE (magnetic NPs) + acid digestion	ICP-MS	Mass concentration NP	[69]
	Ag Au Pd	River water	SPE (ion exchange)	ET-AAS	mass concentration NP	[66]
	Ag TiO_2	Natural waters	Functionalization +liquid extraction	ICP-MS	Mass concentration NP	[56]
	Ag	River water	Derivatization (albumin)	AF4-ICP-MS	Mass concentration NP/dissolved	[160]
	Ag	Wastewater	Filtration (0.45 µm)	AF4-ICP-MS	Size Mass concentration NP	[156]
	Ag	Soil water extracts	Filtration (0.45 µm)	AF4-UV-Vis	Size	[157]
	Ag	Artificial sea water	Filtration (0.45 µm)	AF4-UV-Vis SP-ICP-MS	Size Agglomeration	[128]
	Ag	River water	Filtration (0.45 µm)	HDC-ICP-MS	Size	[105]
	Ag	sewage sludge		HDC-ICP-MS TEM	Size	[181]
	Ag	Sewage sludge Tap water		HDC-ICP-MS TEM	Size	[182]
	Ag	Lake water Sewage sludge		SEC-ICP-MS	Mass concentration NP/dissolved	[188]
	Ag	Seawater		PCC	detection NP size	[207]
	Ag	Seawater		VIP	Detection NP	[198]
	Ag	Seawater		Electrochemcial sensor VIP	Detection NP	[197]
	Ag	Seawater		VIP	Detection NP	[194]
	Au	Sediment	Ultracentrifugation	ESEM FESEM TEM NTA	Size	[81]
	Au	River water Wastewater	CPE	ET-AAS	Mass concentation NP	[60]
	Au	Fresh water Seawater Wastewater	SPE (magnetic NPs) + acid digestion	ICP-MS	Mass concentration NP/dissolved	[68]
	ZnO	Soil suspensions	Settling	AF4-ICP-MS	Size	[158]
	ZnO	Tap water Wastewater	CPE + MW digestion	ICP-MS	Mass concentation NP	[62]

Table 3
Selected single and multi-method approaches for the analysis of complex samples.

Scenario	Sample preparation	Atomic spectrometry AAS/ICP-OES /ICP-MS	Electron microscopy EM-EDS	AF4-ICP-MS	SP-ICP-MS	Atomic spectrometry XAS	Analytical information	Ref.
Scenario 1								
Consumer products 1			●				NP characterization	[74]
Consumer products 2	Acid digestion	●	●				Total element conc. NP characterization	[216]
Food/ biological samples 1	Digestion (acid, TMAH, enzymatic)			●			Size Element mass conc. vs. size	[39]
Food/ biological samples 2	Digestion (acid, TMAH, enzymatic)				●		Size Number conc. Element mass conc. vs. size	[40]
Scenario 2								
Bioaccummulation	Acid digestion	●					Total element conc.	[113]
NP dissolution	Ultrafiltration	●					Dissolved/NP element conc.	[50]
Release from consumer products	Filtration/ultrafiltration	●	●				Element size fractionation NP characterization	[47]
Environmental fate 1	Ultrafiltration Cloud point extraction	●		●	●		Total element conc. Dissolved/NP element conc. NP characterization	[50]
Environmental fate 2	Ultrafiltration	●		●		●	Total element conc. NP characterization Solid phase speciation	[43] [145]
Environmental/ bioaccumulation fate 3	Acid digestion	●	●			●	Total element conc. NP characterization Solid phase speciation	[143] [133]
Scenario 3								
Waters 1					●		NP detection	[41]
Waters 2	Preconcentration	●					NP conc. (mass element)	[67]
Differentiation natural NP/ENPs		●		●	●		Natural/engineered NP	[102]