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An approach to the natural and engineered nanoparticles analysis in the environment by inductively coupled plasma mass spectrometry

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ABSTRACT

Different approaches to the most relevant and recent studies and applications of inductively coupled plasma mass spectrometry (ICP-MS) applied to the analysis of natural and engineered nanoparticles in the environment are described. Usually several separation methods like polyacrylamide gel electrophoresis (PAGE), field-flow fractionation (FFF) and size exclusion chromatography (SEC) are used prior to the mass spectrometric measurements. In many cases the use of these hyphenated techniques provides important methodologies to know the bioavailability, mobility and toxicity of elements in life and environmental sciences. Alternatively, the capabilities of the single particle detection by ICP-MS (SD-ICP-MS) for the selective identification, characterization and determination of engineered nanoparticles will be also discussed.

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1. Introduction

The use of engineered nanoparticles (ENPs) in a range of applications and consumer products is rapidly increasing [1,2]. However, due to their relative novelty, little has been done to assess the risks to the human health and the environment, raising a variety of concerns related to the use of nanomaterials [3–6]. Although there is as yet no internationally agreed formal definition of nanomaterial, they are usually taken to be material with al least one-dimension between 1 and 100 nm. These could be materials such as nanofilms (one-dimension), nanowires and nanotubes (two-dimensions) or nanoparticles (three-dimensions) [7].

Because of its widespread use, it is inevitable that ENPs are released in the environment, where their fate and behaviour are largely unknown [8]. Consequently, ENPs risk research is not only being focused on human toxicology but also on ecotoxicology, [9–11] as well as on environmental chemistry [12].

Nanotoxicological risk assessment relies on analytical methods focused on the characterization of nanomaterials as well as on *in vitro* and *in vivo* assays [10,13]. However, these methods are not considered suitable for detection and quantification of ENPs in environmental samples [11,14].

It is widely accepted that, despite significant progress in recent years, the lack of reliable methods to determine ENP identity, concentrations and characteristics in complex environmental samples, and at environmentally relevant concentrations (ng L^{-1}), is one of the largest gaps in environmental nanosciences. In a recent workshop hosted by the International Council of Nanotechnology, the development of robust analytical methods to characterize and to track nanomaterials in the environment has been considered the main priority for the eco-responsible development and use of nanomaterials [15].

Innovative analytical approaches are necessary to be able to assess the impact of the release of ENPs into the environment. Unlike microscopy and particle sizing techniques, a number of separation techniques (field flow fractionation, hydrodynamic chromatography, electrophoresis) can provided a sound foundation for developing such approaches, specially when coupled to sensitive and selective detection systems, like ICP-MS, for non-carbon ENPs.

On the other hand, an intensive interest is currently developed on the characterization and analytical determination of the natural nanoparticles like as nanocolloids and bioparticles mainly in the environmental aqueous ecosystems.

Natural colloids as environmental particles include inorganic, organic and bioparticles and biopolymers in the size range of 1–1000 nm. The colloidal pool in natural systems consists of a heterogeneous mixture of polydisperse inorganic and organic components. Major inorganic colloids are often dominated by iron oxides and clays, as shown in Fig. 1, whereas the colloidal organic matter (COM) is mainly composed of humic substances (HS) and extracellular polymeric substances [16]. Bioparticles within this range (<1 μm) would be constituted by bacterias, virus, fungi, femto and picoplanckton.

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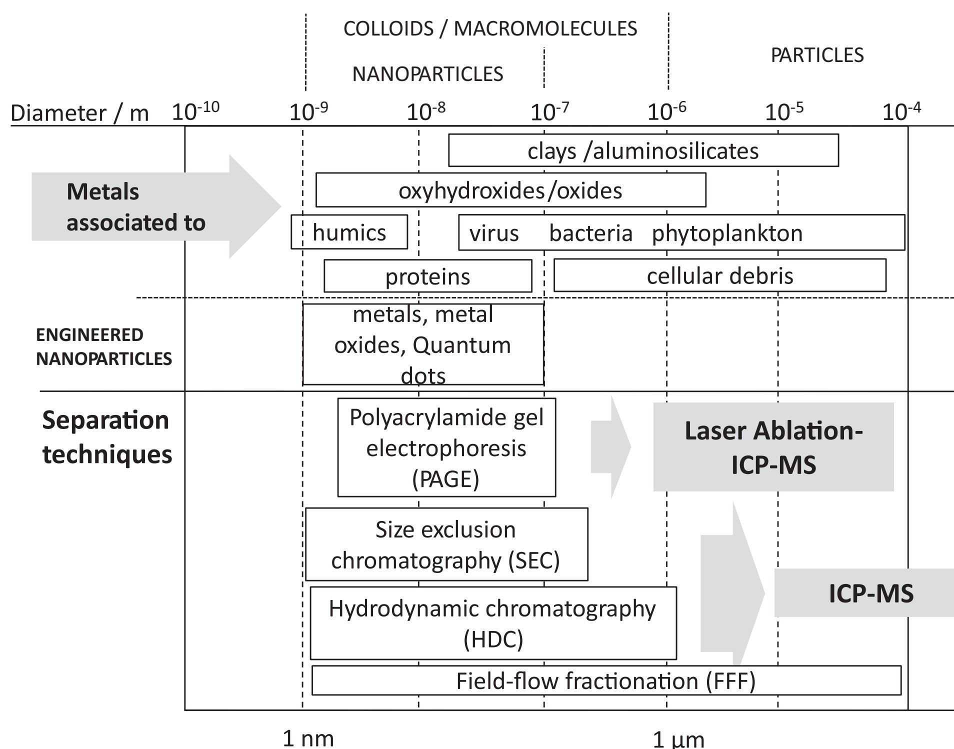

Fig. 1. Size distributions of various types of natural nanoparticles, colloids or macromolecules together with some metal-containing engineered nanoparticles, and several of the separation techniques that can be used to characterized them and their association with metals when coupled to ICP-MS.

The characterization of nanocolloids is essential both to increase our fundamental knowledge of their physical chemistry and to understand the functioning of environmental systems. Environmental nanocolloids are small enough to allow them to behave in a similar manner to soluble compounds, but large enough to participate in additional very peculiar processes such as the transport of inorganic and organic, vital or detrimental compounds in aquatic systems due to their numerous binding sites [17,18], and they may influence nutrient bioavailability [19].

In nanocolloids research, it is necessary to consider that particle aggregation may occur as a result of attractive interaction, such as the London–Van der Waals attraction [20], forming much larger structures. In an aquatic ecosystem (lake, ocean), the larger aggregates settle down and carry the sorbed chemicals to the sediments, thus eliminating the chemicals from the main water. In both soils and sediments, the aggregation of colloids leads to the formation of a porous microstructure that is essential for the development and sustainability of microorganisms and life in general [21].

Therefore, environmental nanocolloids must be seen as essential building blocks of the abiotic medium supporting life. The nature and proportions of the colloids in the medium influence its microstructure, the nutrient bioavailability, and ultimately the sustainability and biodiversity of life. Colloids can be considered as the nanostructures of environmental systems, as they can change their conformations depending on conditions, they can modify interfacial properties by adsorption, and they can develop relatively long range (tens of nm) electric fields. In addition, the assembly of colloids and their interaction with microorganisms, may lead to much larger (mm to cm) 'living' environmental microstructures. These basic microstructures are themselves key elements of the whole ecosystem.

Besides, the phytoplankton of the oceans and others aquatic ecosystems could be considered in many cases as "natural nanoparticles", being the size of some of them in the order of nanometers, such in the case of cyanobacterias and prochloriphytes, the smallest plankton (200 nm to 2 μm long). The phytoplankton are responsible for about half the photosynthetic fixation of carbon (CO_2, primary production) on Earth [22] and play an important role in the biogeochemical cycles of trace metals in the oceans [23]. The release of strong complexing agents by these organisms to take up micronutrients, catalyze redox reactions that modify the bioavailability of trace metals, modifying the rate of photosynthesis in parts of the oceans and the transformation of major nutrients such as nitrogen. The study of these redox cycles of metals, which implies the characterization of the enzymatic pathways involved [24], open an interesting field of research from an analytical chemistry point of view, given the difficulties associated to the complexity of these systems.

We will resume in this review both, any considerations on the use of techniques of polyacrylamide gel electrophoresis (PAGE) coupled to Laser Ablation-ICP-Mass Spectrometry (LA-ICP-MS) for the characterization and determination of metals associated to natural nanocolloids, like humic substances, or bound to proteins from natural bioparticles, and methods for the characterization of engineered nanoparticles by coupling different separation techniques, like field-flow fractionation (FFF) or size exclusion chromatography (SEC) with ICP-Mass Spectrometry. Fig. 1 tries to summarize those separation techniques described in bibliography and commonly used for the separation of nanoparticles and coupled to ICP-MS for its characterization or their associations to metals, together with their working range in size. These ranges can be compared to the size distributions of various types of natural and engineered nanoparticles. The capabilities of Single Particle detection by ICP-MS for the selective identification, characterization and determination of ENPs will be also discussed.

2. Natural nanoparticles analysis by polyacrylamide gel electrophoresis laser ablation ICP-mass spectrometry (PAGE-LA-ICP-MS)

The “off-line” coupled technique polyacrylamide gel electrophoresis (PAGE)-LA-ICP-Mass Spectrometry has been successfully used [25] as a new speciation methodology based on the use of 1D-PAGE (one-dimensional polyacrylamide gel electrophoresis), IEF (isoelectric focusing electrophoresis) and 2D-PAGE (two-dimensional polyacrylamide gel electrophoresis) as separation techniques, and LA-ICP-MS (laser ablation-inductively coupled plasma mass spectrometry) as detection system to study the distribution of metal–humic acids (metals–HA) complexes in environmental samples. These humic acids form aggregates particulated with a size between a few nanometers to millimeters, and play a fundamental role on the mobility, bioavailability and toxicity of many metals in the environment.

Electrophoretic methods have been extensively used for characterization of dissolved organic matter (DOM) due to their simplicity from a practical point of view [26–28]. Most of these methods used for the separation of humic substances were based on the use of polyacrylamide gel electrophoresis. However, the use of electrophoretic methods for metal–humic acid complexes speciation studies is not so common, because of some difficulties associated to disequilibrium effects on metal–DOM complexes [29]. Douglas Evans and Villeneuve [30] reported the coupling of gel electrophoresis with LA-ICP-MS to measure the binding of lead to various molecular size fractions of humic and fulvic acids (HA and FA) using isotopic tracers. Binding of Pb to organic acids is quite variable among the sources measured. In general, HA showed very high binding capacity in the high molecular weight range, while FA showed much higher capacity at low weights. Jiménez et al. [25] have shown that the use of 1D-Tris borate method using low current (10 mA) and Tris borate as trailing ion previous to LA-ICP-MS measurements, allowed to obtain the distribution of metals–HA complexes from natural organic matter samples maintaining metals–HA complexes stability. It was observed that metals were associated with the smallest fraction of HA (around 3 kDa), validating the results obtained by high performance size-exclusion chromatography (HP-SEC), asymmetrical flow field flow fractionation (AsFlFFF) and ultrafiltration. It was observed that electrophoretic conditions were important to preserve metal–HA bonds. The use of 2D-PAGE method followed by LA-ICP-MS also confirmed the results obtained by 1D and IEF as PAGE methods and added information about humic acid molecular size distribution.

As previously discussed in the introduction, the characterization of enzymes and proteins produced by bacteria and phytoplankton in marine environments and their role in redox cycle of some metals, such as iron [24], copper [31] or manganese [32], requires the use of specific separation techniques for protein and enzyme characterization together with high sensitivity techniques for the detection of those metals bound.

The use of laser ablation coupled to ICP-MS as a detection system for metals present in proteins after gel separation in biological systems is well documented in bibliography, being McLeod and co-workers [33] the first group to use this methodology applied to the study of Pt-serum binding proteins after both native and denaturing PAGE. While after native PAGE representative Pt signals were obtained for these proteins, no signals were observed after denaturing PAGE.

The use and scope of atomic spectrometry in combination with gel electrophoresis for speciation of protein-bound trace elements in biological systems has been reviewed by Tastet et al. [34]. During the last few years, LA-ICP-Mass spectrometry (MS) has been used increasingly as a detection system of metals in proteins after separation by polyacrylamide gel electrophoresis (PAGE) using both one-dimensional (1D) PAGE [29,35–50] and two-dimensional (2D) PAGE [34,38,42,45,48–58]. This procedure has become established as a very efficient technique for proteomics studies in life sciences.

Nevertheless, some pitfalls due to metal losses in PAGE separation have been demonstrated [29,59]. If the separated species achieve new equilibrium before detection, the electropherogram will not reflect the true speciation.

In metalloprotein speciation is very important to maintain the integrity of metal–protein binding during all the process. In most of the applications using PAGE-LA-ICP-MS, the subject of study were heteroatom-containing proteins, such as selenoproteins [34,36,38,39,41,42,49,60] and phosphoproteins [35,48,50,51,53,54,57,61], where the elements are part of the proteins and are strongly bound to them, so the binding is not broken during electrophoresis separation. However, metal-binding proteins in which metal–protein interactions are of lower affinity can cause loss of the metal during electrophoresis separation, especially if denaturing PAGE is used.

Because of this, the main pitfall, which researchers have to face, is that it is not always possible to keep these metalloprotein complexes intact during separation and post-separation process, and metal losses can occur. So, it is necessary to develop new strategies to separate proteins without breaking the metal–protein binding. Some authors have already discussed these metal losses in different proteins, and in most cases, the use of native-PAGE instead of denaturing PAGE has been proposed as solution to the problem.

For instance, Chery et al. [62] found vanadium losses of serum proteins during denaturing electrophoretic separation, but the use of native PAGE, such as Blue Native (BN)-PAGE, where all denaturing agents are left out of the gel electrophoresis, avoided vanadium losses.

Becker et al. separated yeast mitochondrial proteins [51,54] by 2D isoelectric focusing (IEF)/sodium dodecyl sulfate (SDS)-PAGE and human brain proteins [52,53] by 2D BN/SDS-PAGE and analyzed the gels by LA-ICP-MS. They demonstrated that stable metal–protein complexes can survive denaturing and reducing conditions during the separation of proteins in the second dimension, but also that these conditions partly releases the naturally bound metal ions of non-stable metal-complexes. According to these authors, the most challenging problems are related to the preservations of the naturally bound metal ions during gel separation. In recent papers they have also described the use of BN-PAGE to study the binding of Cu and Zn to albumin [45] and to analyze some metals such as Cu, Zn and Fe in rat tissues proteins [46]. Moreover, to study the same metals in rat tissues, they have described a native 2D BN-PAGE in order to keep the metal complexes intact during separation [49].

Jakubowski et al. have investigated the detection of Cd, Zn and Fe in different metalloproteins by LA-ICP-MS after both anodal native PAGE and denaturing PAGE [40,43]. The results showed that denaturing PAGE was unsuitable for quantitation of the metals in these proteins because of the possible metal losses by the use of it, especially in weakly bound metal containing proteins. However, anodal native PAGE, which preserves the native protein structure, was a powerful alternative but has to be paid at the cost of molecular weight calibration.

However, not only the nature of the electrophoretic process (denaturing or non-denaturing) can alter the metal protein binding. There are few studies in relation to the influence of other electrophoresis conditions (including post-separation process) on possible metal–protein binding break. Gao et al. [63] found that not only the influence of the electric field during electrophoresis separation, but also the processes of fixing, staining and destaining of proteins in gel may result in loss of metal ions from

metalloproteins in human liver cytosol using synchrotron radiation X-ray fluorescence as detection system after SDS-PAGE.

Summarizing, and despite all these pitfalls described above, the combination of gel electrophoresis and LA-ICP-MS as detection technique is a promising tool to deal with the study of metalloproteins or metal–protein bindings that can be associated to the enzymatic pathways involved in the redox cycles of metals in complex environmental systems, as seawaters, where an extremely dynamic trafficking in essential trace metals takes place [23]. In that sense, all the experience acquired in studies applied to biological systems and summarized in this section, should be taken into account as a first approach.

3. Engineered nanoparticle analysis by separation techniques coupled to ICP-MS

Different approaches have been proposed for the detection and characterization of ENPs in environmental samples, including microscopy-based techniques, light scattering methods or those based on chromatography. The use of separation techniques such as liquid chromatography or field flow fractionation combined with element-specific detectors, such as ICP-MS [64] is among the most promising approach.

Size exclusion chromatography (SEC) is one of the most commonly used chromatographic techniques for isolation of nanoparticles (NPs), and frequently used for their further characterization combined with different detection techniques [7]. The size range of analytes that can be separated by SEC depends on the pore size of the stationary phase, in function of its effectiveness to reject those particles with diameters that exceed those defined for NPs. In that sense, the minimum size of the particles that constitute the packing material has to be in accordance to the size of the analytes injected, being a critical factor to manufacture these columns. For instance, a separation of Au NPs of sizes ranging from 10 to 80 nm have been described in bibliography [65] using SEC columns of 400 nm pore size.

In contrast, in hydrodynamic chromatography (HDC), the column is packed with non-porous beads, which build up flow channels or capillaries. Particles are separated by flow velocity and the velocity gradient across the particle, so larger particles are transported faster as they cannot access slow-flow regions near the conduit walls [66]. The separation in this case is solely based on particle size, and is independent of particle type and density [67], being in the range 5–1200 nm [7]. The applicability of this technique coupled to ICP-MS to the analysis of ENPs in environmental samples has been shown in [68]. In this paper, Ag NPs with a nominal size <100 nm were spiked into sewage sludge to give concentrations of 10 mg L^{-1} in 2 g L^{-1} of mixed liquor suspended solid, mixing for 6 h and allowing to settle, analyzing the supernatant by HDC-ICP-MS. Results showed that a percentage of the particles did not interact with the sewage sludge and could survive in the supernatant. More recently, the same authors [69] have studied the fate of Ag NPs in activated sludge, showing the utility of HDC-ICP-MS for studying the occurrence and behaviour of ENPs in complex natural environments.

The hyphenation of field-flow fractionation (FFF) with ICP-MS can also give relevant information about the distribution of elements throughout the sample size distribution, which is especially convenient for determining the different types of colloids present in these environmental samples. The capabilities, conditions, limitations and main applications of FFF coupled to ICP-MS have been recently review by Dubascoux et al. [70]. Field-flow fractionation is a family of separation techniques where the retention and separation are achieved by the interaction of particles with an external field, without the sample interacting with any stationary phase. Depending on the field applied and the mode of operation, this technique allows the separation and characterization of macromolecular and particulate materials ranging from 300 Da to 100 μm [71].

Whereas several applications of FFF coupled to ICP-MS to the characterization of environmental nanoparticles has been described in bibliography, little has been published related to ENPs. Thus characterisation of ENPs by the on-line coupling of FFF with ICP-MS has been described applied to the analysis of alumina- and silica-based mechanical polishing slurries [72], synthetic CdSe/ZnS quantum dots (QDs) with different surface coatings [73,74] and the particle size characterization of TiO_2 in sunscreen products [75], although none of them applied directly to environmental samples. In that sense, studies reporting the environmental occurrence of NPs are still relatively sparse in the literature [76], even if natural NPs, not only ENPs, are considered.

4. Identification and characterization of engineered nanoparticle by single particle detection

The best knowledge on the environmental nanoparticles characterization and determination require in many cases a single particle detection [77]. This analytical methodology using ICP-atomic emission spectrometry [78–82] and ICP-MS [83–93] is a feasible methodology for direct analysis of individual microparticles [78–80,82,83,86], nanoparticles [82–93], colloids [88–92] and cells [81–87]. This detection mode has also been adopted in gold nanoparticle-based immunoassay methods [94] and in combination with trace metal preconcentration onto iron hydroxide particles [83] as a strategy to improve detection limits (e.g., 0.05 pg mL^{-1} for Ba).

In a single particle, the analyte is spatially concentrated, in comparison to a solution of a soluble form of the same analyte. When one particle is introduced into the ICP, the atoms of the analyte produce a flash of gaseous ions in the plasma, which are measured as a single pulse by the detector. Airborne particles have been introduced in ICPs as dried specific designed ICP-MS pulse counting system. Particle number concentration has to be low enough that most droplets do not contain any particle and the probability of having two or more particles can be neglected; number concentrations below 10^9 L^{-1} are usually selected [87].

The detectability of nanoparticles in single particle detection mode depends on two factors: (i) the size of the nanoparticle, which should be large enough to generate a number of ions detectable by the spectrometer, and (ii) the number concentration of nanoparticles, which should be high enough to allow counting a minimum number of events. These factors imply two different limits of detection: a size (diameter for solid nanoparticles) limit of detection (LOD_d) and a nanoparticles number concentration limit of detection (LOD_{NP}).

Theoretical basis of single particle detection applied to ICP-MS has been outlined by Degueldre et al. [88–92] for nanoparticle suspensions continuously introduced through conventional nebulization systems. A comparative approach between solutions of truly dissolved forms of an analyte and nanoparticle suspensions has been recently developed [95]. Thus, the diference between nebulization of solutions and diluted nanoparticle suspensions lies on the analyte distribution among the aerosol droplets. Whereas soluble forms of an analyte M are homogeneously distributed, nanoparticles are only present in a small fraction of droplets (e.g., for 1×10^8 L^{-1} nanoparticle number concentration and 5 μm mean droplet diameter, just one droplet each 1.5×10^5 contains a nanoparticle). For solutions, ion counting follows Poisson statistics and signal distributions are described by Poisson distributions. However, the signal distribution for suspensions should follow the

size distribution of the nanoparticles counted, which are usually lognormal [96]. The capability for discriminating both distributions will depend on the relative magnitude of each contribution.

Laborda et al. [95] have demonstrated that single particle detection using ICP-MS allows to get detailed information, both qualitative and quantitative, about different physico-chemical forms of nanoparticles like silver in aqueous samples without previous separation steps. The methodology can provide information about: (i) the presence of dissolved silver and/or silver nanoparticles, (ii) the size distribution or the mass distribution per nanoparticle, (iii) the mass concentration of dissolved silver and silver nanoparticles, and (iv) the number concentration of nanoparticles whatever their size. These methods can also be applied to other metal-containing engineered or natural nano and microparticles in a variety of studies (stability of ENPs suspensions, dissolution/aggregation of NP, *in vitro* toxicological assays...). More importantly, the attainable detection limits, both on size/mass (18 nm) and number concentration (1×10^4 L^{-1}), and its simplicity makes the approach very suitable for environmental monitoring, as well as for the development of test methods to establish environmental quality standards on ENPs in the near future.

The available methodology can be further improved using fastest electronics, which allows the use of shorter dwell times to improve time resolution. With regard to size detection limits, the increase of the transmission efficiency from the skimmer to the detector, through new instrumental designs can contribute to reduce them. Lastly, the use of quadrupole mass analyzers limits the number of isotopes measured on a single nanoparticle to one, multiple isotopes can be measured with simultaneous double-focusing sector field spectrometers or time of flight mass spectrometers, improving the attainable information for heterogeneous nanoparticles.

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