Methods for separation, identification, characterization and quantification of silver nanoparticles

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There is a growing production and application of silver nanoparticles (AgNPs) (e.g., in cosmetics products, food technology, textiles and fabrics, and medical products and devices). The rapid growth in the commercial use of AgNPs will inevitably increase exposure to silver in the environment and among the general population.

Compared to the vast application of silver, information on the fate, the transformation and the toxicity of AgNPs is very limited. Lack of proper techniques to trace AgNPs in complex matrixes hinders investigation. Thus, development of methods for analysis of AgNPs is very important to achieve detailed insights into the fate, the transport and exposure of AgNPs in environment.

This review presents state-of-the-art methods for separation, identification, characterization and quantification of AgNPs. We also discuss perspectives on future developments.

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1. Introduction

Silver nanomaterials (NMs) (nanosilvers) are clusters of silver atoms of 1–100 nm in at least one dimension. Due to their unique physical and chemical properties, nanosilvers are widely used in various areas with exponentially increasing production. Apart from traditional usage in the engineering industry {e.g., catalysis, optical devices, surface plasmon resonance (SPR), surface-enhanced Raman spectroscopy (SERS) and electronic applications [1]}, excellent antibacterial activity made them popular in a widespread range of applications (e.g., fabrics, disinfectants and medical devices).

Nanosilvers represent a broad spectrum of antimicrobial activity, and can kill both Gram-positive and Gram-negative bacteria (e.g., *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*) [2,3].

It is also reported that nanosilvers have a great bactericidal effect on multidrug

-resistant bacteria. The activity of nanosilvers was examined on different drug-resistant pathogens of clinical importance, including multidrug-resistant *Pseudomonas aeruginosa*, ampicillin-resistant *E. coli* O157:H7 and erythromycin-resistant *Streptococcus pyogenes* [4]. It was shown that nanosilvers could inhibit the growth rate of bacteria from the initial contact with the pathogens, and had their antibacterial activity by killing bacteria rather than by the bacteriostatic mechanism.

Nanosilvers also have antifungal activity. They could inhibit a series of ordinary fungal strains, including *Aspergillus fumigatus*, *Mucor*, *Candida albicans*, *Candida glabrata*, *Candida tropicalis*, *Saccharomyces cerevisiae*, and *Aspergillus fumigatus* [5].

The antiviral properties of silver nanoparticles (AgNPs) are also reported in the literature. A notable example is anti-HIV-1 activity. A study [6] revealed that AgNPs prepared in Hepes buffer could inhibit HIV-1 replication, and the anti-HIV activity was much higher than that of gold

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NPs. The effects on hepatitis B virus [7] and herpes simplex virus [8] were also evaluated.

Because of the exceptionally broad spectrum of antimicrobial activity, AgNPs are expected to open new avenues to fight and to prevent diseases by incorporating them into a variety of products. Data showed that, of the 1015 consumer products containing NMs presented on the market, 259 contained AgNPs, including cosmetics items, health supplements, textiles and clothing, and other household goods [9,10]. It is inevitable that NMs would be released into the environment during usage, which may adversely affect both the organisms and humans.

Previous studies had shown that silver could easily leak from commercial AgNPs contained in textiles and socks during washing [11,12], which would threaten the beneficial bacteria and other organisms in the aquatic environment. However, AgNPs are commonly used as food additives, consumer spray products, and hand disinfectants.

By eating, breathing or skin contact, humans are easily exposed to AgNPs. Frequent exposure may result in deposition of silver into the body. Also, the potential anti-inflammatory properties of AgNPs attracted much attention in medical applications. Examples are nanosilver wound dressings, coatings for breathing masks, nanosilver-coated catheters for drug delivery, and bone cement [13]. As silver is universally unstable, there is a risk that the implantable devices may slowly release silver ions. It is well known that silver is one of the most toxic metals, only surpassed by mercury [14]. The most remarkable adverse effect of chronic exposure to silver is argyria or argyrosis, a permanent bluish-gray discoloration of the skin or eyes [15].

Nanosilvers appear to be one of the most rapidly growing NMs, and the use of them is increasingly widespread in our daily life. Thus, evaluating their toxicity is extraordinarily necessary. Human-lung fibroblast cells (IMR-90) and glioblastoma cells (U251) were used to test the cytotoxicity and genotoxicity of AgNPs, and it was revealed that AgNPs could cause DNA damage by disrupting the mitochondrial respiratory chain due to the accumulation of reactive oxygen species (ROS) [16]. Xu et al. [17] examined the impact of AgNPs on development of zebrafish embryos *in vivo*, and found that AgNPs existed in embryos at each developmental stage and that the trigger for and the type of abnormalities depended on the dose of AgNPs.

The physicochemical property of a metal particle, which is a key parameter to determine their application, mainly depends on particle size and shape [18]. Also, it has been reported that the toxicity of AgNPs is closely related to particle size [19]. However, the direct synthesis of monodispersed AgNPs is not an easy task, as chemical synthesis often yields a wide distribution of particle size. As a result, post separation of AgNPs with controlled size

and shape is urgently needed. From the viewpoint of environmental health, it would help to investigate the toxicity of AgNPs fully. From the perspective of potential applications, there are opportunities to fine-tune the special properties of AgNPs for chosen applications.

Also, studies showed that, by reaction with dissolved oxygen and H^+ , AgNPs were easily oxidized and released Ag^+ [20,21]. The active Ag^+ would probably combine with other anions to achieve stable molecules (e.g., Cl^- , F^- , CO_3^{2-} , S^{2-} , and OH^-). It has been discovered that nanocrystal Ag_2S in sewage-sludge products probably formed from AgNPs in an S-rich environment [22]. Recently, scientists also found that AgNPs could easily react with Na_2S to produce Ag_2S , while the surface properties, surface charge and dissolution rates changed significantly [23]. As speciation strongly affects the bioavailability of AgNPs, it is very important to develop proper methods to characterize the transformation of AgNPs.

Some studies on the fate, the transformation and the toxicity of AgNPs have been reported. However, compared to the vast application of silver in the market, this information is very limited. In order to evaluate the release of AgNPs from commercial products, the transportation of AgNPs in the environment, and the concentrations of AgNPs exposed to organisms, it is of great importance to develop methods for separation, identification, characterization and quantification of AgNPs.

In this review, we summarize the state of the art of methods for separation, identification, characterization and quantification of nanosilvers, and discuss some possible developments in the future.

2. Separation

2.1. Cloud-point extraction

The first approach to cloud-point extraction (CPE) was described by Watanabe et al. [24,25]. Since then, the method has attracted enormous attention and developed greatly. Based on the solubilization ability and the cloud points of non-ionic surfactants, CPE can be easily done. Briefly, there are three steps in this extraction protocol:

- (1) non-ionic surfactant is added into the sample solution with final concentration higher than its critical micelle concentration (CMC);
- (2) by changing the external conditions (e.g., temperature, pressure, pH, or ionic strength), the mixture becomes turbid because it attains the cloud point (i.e. incomplete solubilization); and,
- (3) by centrifugation or long-term standing, the micelle solution can easily separate into two phases, and the analytes can be concentrated and extracted into the surfactant-rich phase due to the analyte-micelle interaction [26].

CPE exhibits many advantages (e.g., high extraction efficiency and preconcentration factor, low cost, easy

Figure 1. Cloud-point extraction (CPE) protocol and the different techniques for characterization.

handling, and non-toxicity), which make it ideal for extracting pollutants from various environmental and biological samples.

Our group reported for the first time that, with a commercially available non-ionic surfactant Triton X-114 (TX-114), several NMs with various sizes and different capping agents can be thermoreversibly separated or concentrated and dispersed in the aqueous phase by CPE [e.g., PVP-capped AgNPs, trisodium-citrate stabilized AuNPs, C_{60} fullerenes, TiO_2 and single-walled carbon nanotubes (SWCNTs)]. Most importantly, during extraction, the particle sizes and shapes were preserved [27]. Based on this study, we further developed a CPE procedure for selective concentration of AgNPs from environmental water samples without disturbing their sizes and shapes (Fig. 1). By optimizing the CPE parameters, high extraction efficiency can be obtained, and the presence of humic acid at environmentally relevant levels and silver ions did not interfere with the extraction procedure.

By spiking AgNPs into aquatic environmental samples at 0.1–146 $\mu\text{g/L}$ levels, 57–116% recoveries could be obtained, with an enrichment factor of 100. The existence of AgNPs in the preconcentrated TX-114 phase was identified by TEM/SEM/EDX/UV-Vis characterization (Fig. 2) [28].

Recently, we also employed TX-114-based CPE for the speciation analysis of AgNPs and Ag^+ in commercial antibacterial products (e.g., antibacterial nasal spray and female antibacterial hydrogel lotion). Total silver in the sample was determined by ICP-MS after microwave digestion, AgNPs were detected by quantifying the Ag contents in the preconcentrated TX-114 phase after CPE, while the Ag^+ concentration was based on the difference between the contents of total Ag and AgNPs. Spiked

recoveries in real samples were 71.7–103% for AgNPs and 1.2–10% for Ag^+ , respectively, which showed that AgNPs and Ag^+ could be successfully isolated during extraction. The AgNPs and Ag^+ were sensitively detected, with the limits of quantification of 0.4 $\mu\text{g/kg}$ for AgNPs and 3 $\mu\text{g/kg}$ for total Ag^+ [29].

2.2. Field-flow fractionation

Field-flow fractionation (FFF) is a flow-assisted hydrodynamic separation technique that was designed to separate complex macromolecules, colloids and particles. It can be regarded as a combination of liquid chromatography and a field-driven technique, except that it does not need a stationary phase. Basically, when the flowing stream carrying the samples migrates through the FFF channel, an external field is applied perpendicular to the axis of the fractionation channel, which causes the retention of the analytes. Because of the broad size distribution and different physicochemical properties, particles possess distinct diffusion coefficients. To balance this diffusibility and the external field, particles stay at different distances from the accumulation wall, so the retention time varies. FFF comprises a big family of different types, including thermal FFF, sedimentation FFF, crossflow FFF, dielectrophoretic FFF and magnetic FFF [30].

Because of its extraordinary resolution and applicability to a wide range of sizes, FFF has been used to separate various engineered NMs. As FFF offers the separation of particles with different sizes and reduced sample complexity, different-sized particles can be analyzed individually after separating the samples with FFF. Besides, unlike the chromatographic methods, the absence of a stationary phase avoids irreversible interaction with the analytes, so the morphology and the size of the NPs are preserved during operations.

Figure 2. Identification of silver nanoparticles (AgNPs) enriched in the TX-114-rich phase. TEM images of the TX-114-rich phase separated from the extraction of samples containing (A) 10 µg/L and (B) 25 µg/L AgNPs. (C) TEM image and (D) UV-vis spectrum of the TX-114-rich phase separated from extraction of a sample containing 100 µg/L AgNPs. (E) EDS and (F) SEM image of the TX-114-rich phase separated from the extraction for a sample containing 1 mg/L AgNPs. (Reproduced from [28] with permission, © 2009 American Chemical Society).

The fractionation of the samples can be further determined by many detectors, and on-line detectors include ultraviolet-visible spectroscopy (UV-Vis), fluorescence, light scattering, inductively-coupled plasma atomic emission spectroscopy (ICP-AES) and inductively-coupled plasma mass spectrometry (ICP-MS), while off-line detectors include transmission electron microscopy (TEM), scanning electron microscopy (SEM), atomic force microscopy (AFM), atomic absorption spectrophotometry (AAS) and light scattering [31].

Lead et al. [32] utilized flow FFF to test the particle-size distributions of AgNPs at environmentally relevant

levels. They compared the size measurement with other conventional techniques, and the results showed that they agreed well with those of TEM and DLS. Moreover, FFF is much faster than microscopy techniques. By using FFF, the form and the size distribution of AgNPs could be easily obtained at different pH, ionic strength, and concentrations of humic substances. Shiundu et al. [33] used thermal FFF to separate several NPs, including Ag, Au, Pd, and Pt, and also evaluated the factors that influenced retention behavior. Kim and his co-worker [34] also reported the separation of AgNPs by using sedimentation FFF. By using water with 0.1% FL-70 as

the carrier and UV-Vis as the detector, bimodal mixtures of AgNPs was isolated and monitored on line. Though complete separation for the mixtures could not be achieved, the partially separated fractograms could be mathematically deconvoluted to calculate the relative mass content of the mixtures.

Although FFF is powerful in separating NPs, it is time consuming and laborious. To acquire perfect conditions and good separation efficiency for the particles tested, a series of parameters have to be optimized, including carrier liquids, membranes, channel dimensions, and external field [31].

2.3. Chromatographic methods

Hydrodynamic chromatography (HDC) is a size-based separation method. The column is packed with non-porous microparticles, and separation is achieved by flow velocity and the velocity gradient across them [35].

HDC coupled with ICP-MS was used to separate “real” environment samples by spiking AgNPs into sewage sludge. Initial data demonstrated that the AgNPs survived in the sludge supernatant, and could be successfully fractionated by HDC, even without filtration or centrifugation. TEM images also validated the results obtained by HDC-ICP-MS [36].

Counter-current chromatography (CCC) was also harnessed to separate NPs. Yu et al. [37] employed a step-gradient extraction-based CCC to separate carboxylate-anion-modified AgNPs. With the aid of phase-transfer catalyst tetraoctylammonium bromide (TOAB), hydrophilic AgNPs could be transferred to the organic phase by forming the ion-pair adduct. As smaller particles were easier to transfer to the organic phase, by optimizing the concentration of TOAB, proper size separation could be achieved. Results showed that four monodispersed fractions were obtained by using 0.02 mM TOAB.

Size-exclusion chromatography (SEC) has been widely used to isolate sub-micron particles. The column is packed with porous packing materials, which form the flow channels. Particles having the diameter smaller or equal to the pore size of the packing materials can permeate deep inside the column, while larger particles can only transfer through bigger pores or be excluded, causing longer retention times for smaller particles.

HPLC has proved to be another popular tool to isolate NPs due to its excellent separation performance and long-term stability.

The combination of DAD detector also offers on-line detection of the fractions. However, so far, there is no report on the separation of AgNPs using SEC or HPLC.

2.4. Electrophoresis and capillary electrophoresis

Electrophoretic separation of NPs is mainly based on particle size, shape and surface-chemical modification of NPs. The electro-charge of NPs without surface

modification is mainly from ion adsorption, and the electrophoretic separation greatly depends on particle size, while the electrophoresis of functionalized NPs with surface functional-group modifications is influenced by quantity, chemical groups, and ionization of these functional groups. Gel electrophoresis, isoelectric focusing (IEF) and capillary electrophoresis (CE) have been widely applied to fractionating NPs.

Gel electrophoresis is a separation technique based on the different migration behavior of analytes in gel by sieve effects under electric field. Polyacrylamide gel electrophoresis (PAGE) was demonstrated as an effective approach for separating Ag:SG (Ag clusters ligated with glutathione) [38]. As many as 21 distinct Ag:SG species were separated with strikingly different optical properties and these Ag:SG species were discernible by the naked eye. The pore size of the polyacrylamide gel is usually a few nm, which limited its application in separation of NPs with wide range of size. By contrast, the larger, uniform pore size of agarose gel (10s–100s nm) enables the wide applications of agarose gel electrophoresis in NP fractionation. Ag nanospheres, nanotriangles, and nanorods with SH-PEG-COOH modification can be separated effectively by gel electrophoresis with 0.2% agarose gel in $0.5 \times$ TBE (tris-borate EDTA) buffer (pH $\approx$ 9) agarose (Fig. 3) [39]. The gel shows different colors in the silver lane, which is visual proof of successful separation of NPs. After separation, local extinction spectroscopy and TEM were used to analyze the particle distribution in the various regions of the gel. Extinction spectra of the different fractions showed separation of AgNPs by shape, evidenced by distinct extinction spectra. Further verification by TEM found that AgNPs with different sizes and/or shapes have different electrophoretic mobilities. Rods are predominantly located in the fraction with the lowest mobility. Spheres show a tendency to faster mobilization. The triangles are mainly enriched in the fastest moving fraction.

After measuring the length of the long and short axis for the rods, the diameter for spheres, and the height for triangles, the quantitative result revealed that the nanorods with the highest aspect ratio clearly moved the slowest and increasing mobility with increased size was evidenced for Ag nanospheres. However, no clear trend in mobility was found for the triangles, perhaps due to the unknown thickness of the flat triangles. In addition, the particle mobilities measured could well be explained quantitatively using the Henry formula and the Gouy-Chapman model.

IEF is a routine technique widely employed to determine the isoelectric point of macromolecules and bio-particles. Moreover, it can be effectively applied to separate NPs. A fast, inexpensive miniscale IEF technique was developed for size-selective separation of 4-carboxythiophenol-modified Au and Ag colloidal particles [40]. The separated bands in the IEF unit were typically

Figure 3. (a) Typical TEM picture of a silver nanoparticle (AgNP) sample (left, scale bar 100 nm) and the proportion of spheres, triangles, and rods (right). (b) True color photograph of a 0.2% agarose gel run for 30 min at 150 V for the separation of different NPs. (Reproduced from [39] with permission, © 2007 American Chemical Society).

0.04 pH units and the pH values at which onset of ionization occurs has a linear relationship with the particle size. These properties can be used to separate mixtures of colloidal particles of different sizes.

CE is a relatively new electro-migration separation technique, which is usually performed in a silica capillary with inner diameter of 25–100 μm . In recent years, CE emerged as one of the most powerful separation techniques for inorganic ions, organic compounds, macromolecules and even biomolecules (e.g., virus and bacteria). Capillary-electromigration techniques have been applied in selective separation of various NPs by size and surface-charge density (e.g., latex particles, polystyrenes, inorganic oxide particles, metal particles and quantum dots) [41].

Lin's group reported the separation of AgNPs with different sizes and shapes, including Ag nanospheres [42], Ag nanocubes [43], Ag nanorods [42] and Au/Ag core/shell nanospheres [44,45], by using micellar electrokinetic chromatography (MEKC). The addition of SDS surfactant into the running electrolyte can prevent AgNPs from coagulating and enhance the separation of NPs. Ag nanospheres with different sizes (17.0 nm and 49.7 nm) could be well resolved [42]. In addition, AgNPs with different shapes (e.g., nanocubes and nanorods) can partly be separated from Ag nanospheres [42,43]. In a separation system in electrolytes with 40 mM SDS and 10 mM CAPS (pH 9.7) at 20 kV, Au/Ag core/shell NPs

with different sizes (24.3–90.2 nm) experienced different migration time and the increase of migration times paralleled the increasing size of the core/shell NPs. A good linear relationship ($R^2 > 0.99$) existed between electrophoretic mobility and the radius of the core/shell NPs. It is therefore possible to employ CE to characterize the size of AgNPs. An additional advantage of CE is that the on-line DAD system can provide the SPR spectra of the eluted NPs in real time, indicating that the coupling of DAD with CE provides a powerful technique to investigate the spectroscopic properties of AgNPs of different sizes and shapes.

2.5. Density-gradient centrifugation

The density-gradient centrifugation method, which was used to separate biomacromolecules, also shows great potential for isolating NPs. Briefly, a density gradient is created in a centrifuge tube by sequentially layering solutions of different densities, then the samples are added on top of the density gradient and subjected to centrifugation. Due to the different sizes, shapes or densities, the sedimentation coefficient of different particles in the samples varies, so the various components deposit through the gradient at different speeds, and eventually different regions form in the density gradient. At a certain zone, the particles have similar size and shape, and can be isolated. Usually, the density gradient is prepared with sucrose, glycerol and other aqueous

solutions. However, for NPs that prepared in organic medium, transferring from organic solvent to aqueous solution is challenged by the possibility that some particles may coagulate.

Further development of density-gradient centrifugation has allowed its application in organic solvents (e.g., non-hydroxylic solvents have been used as organic density gradients to purify AgNPs, AuNPs and CdSeNPs) (Fig. 4) [46]. In the research, a five-layer gradient was prepared by adding cyclohexane and tetrachloromethane mixtures with decreasing density (by volume 50%, 60%, 70%, 80%, and 90%). After AgNP addition and centrifugation, obvious colored zones were apparent. TEM images showed that AgNPs with different sizes were successfully isolated, and the small size difference between adjacent zones (about 2 nm) showed the powerful separation ability of organic density gradients. Besides, results showed that colloidal particles could be fractionated by not only size but also morphology.

2.6. Miscellaneous methods

Several other techniques have also been applied to separate AgNPs (e.g., membrane filtration, ultrafiltration and dialysis). Because it is cheap and easy to handle, centrifugation is widely used to purify AgNPs, especially to remove the residues from newly-prepared NPs. Due to the simple procedure and without adding other separating agents, membrane filtration or ultrafiltration has emerged as an efficient tool for the separation of AgNPs of different sizes [11,20,47–49]. However, undesirable aggregation or filter clogging may occur during centrifugation or filtration, which may distort the results. These limitations were overcome in Trefry's studies by using the tangential flow ultrafiltration method [50].

Newly synthesized, polydispersed AgNPs were separated by tangential flow ultrafiltration with a 50-nm filter and then concentrated with a 100-kD filter, while ultracentrifugation was performed for comparison. TEM and UV-Vis were used to characterize the separation efficiency. TEM micrographs showed that, after ultrafiltration, the large AgNP aggregates in the original solution were eliminated and that AgNP solution became homogeneous. However, the ultracentrifugation process led to the formation of massive AgNP aggregates. The dialysis method is also used to isolate AgNPs from other unwanted ions (e.g., silver ions) [51]. However, the long dialysis time hampers its wide application.

Roberts et al. [52] reported a unique method to achieve rapid size separation of AgNPs. Gaseous CO₂, employed as the anti-solvent, was added to AgNP solvent hexane to diminish the solvent strength, so insufficiently dissolving particle ligands led to precipitation of AgNPs. As larger particles, which possessed greater interparticle van der Waals attractions, were easier to precipitate, different-sized AgNPs could be selectively precipitated and collected by changing the pressure of CO₂ from 500 psi to 700 psi. TEM images showed that this method had high separation efficiency and each fraction had a narrow distribution.

Some techniques to determine Ag ions also have potential to separate Ag⁺ from AgNPs. The diffuse gradients in thin-films (DGT) technique, based on Fick's first diffusion law, has attracted great interest for detecting labile metal ions. Free Ag ions have been successfully determined by DGT in the presence of AgNPs [47].

Given the high enrichment factor and minimal usage of organic agents, supported liquid membrane (SLM) extraction is one of the most promising techniques to

Figure 4. Results demonstrating the separation of silver nanoparticles (AgNPs): (A) digital-camera images of ultracentrifuge vessels containing AgNPs before and after separation; (B) TEM images of several Ag fractions. The graph in the bottom right corner compares the size difference before (red columns in the upper section) and after separation (colored columns in the lower section). (Reproduced from [46] with permission, © 2010 American Chemical Society).

separate and to preconcentrate free metal ions. A number of papers in the literature has reported the extraction and the determination of silver ions by employing the SLM method [53–56].

3. Identification and characterization

Because of their nano-scale dimensions, AgNPs are beyond the detection ability of traditional optical microscopy. Electron microscopy (EM) techniques, based on the application of an electron beam, have a much higher resolution, which makes them a popular option in visualization and characterization of NMs. The most salient techniques are transmission electron microscopy (TEM) and scanning electron microscopy (SEM).

TEM images provide not only the size and the shape of the particles, but also the morphology and the aggregation state. For high-resolution TEM, even the layers of atoms of crystalline samples can be clearly demonstrated. However, TEM specimens are required to be dry and at most hundreds of nm thick, so the main task is to prepare a proper sample. For solutions, the evaporation of solvent may lead to undesirable particle coagulation and changes in surface properties. For biological tissues and other complex samples, in order to keep their pristine state and to enhance contrast, chemical fixation and a straining procedure is often needed. Moreover, the samples also need to be embedded in resin and cut into thin sections to allow the electron beam to pass through [57].

In SEM, the electron beam interacts with the specimen surface, and secondary electrons, back-scattering electrons and characteristic X-rays are detected. In focused ion-beam SEM, a 3D image can be obtained. Though the SEM samples need not be as thin as TEM, to avoid the accumulation of static electric charge on the specimen during electron irradiation, the specimen, at least the surface, must be conducting, so sometimes it is necessary to coat the sample with a layer of conductive material. However, there is a risk that some of the surface information could be lost [58]. This problem could be solved by the application of environmental SEM (ESEM), which allows the samples to be imaged under vacuum. The drawback is that the resolution is reduced [57].

EM techniques provide the direct observation of the size and the shape of NMs, but, since a very small proportion of samples are analyzed, to get a representative result, hundreds and thousands of particles have to be counted, which is tedious, laborious, and time consuming.

Energy-dispersive X-ray spectroscopy (EDS), an analytical method for elemental determination, is also of great importance. Usually, it is combined with TEM or SEM to give an element distribution of the AgNPs. Also,

for complex samples, it is a very useful tool to identify the existence of AgNPs [11,28].

As the optical properties of AgNPs differ from those of bulk metal, UV-Vis offers the possibility of characterizing AgNPs. Typically, the maximum absorption wavelength in a UV-Vis spectrum is associated with the average particle size, while its full width at half-maximum (FWHM) can give information about particle dispersion [59], as confirmed by Leopold and his partner using TEM and UV-Vis to characterize newly-synthesized AgNPs [60]. Because of its cheapness and ease of handling, it is also preferred as a supplementary method to ascribe quality [17,61] and to detect the presence of AgNPs [28].

A brief overview of methods for separation and characterization of AgNPs in different matrix is given in Table 1.

4. Quantification

ICP-AES and ICP-MS enable us to detect trace AgNPs efficiently and quickly. The high speed, precision, sensitivity, and large linear range make them to be the most popular techniques in the determination of metal ions. Since the existence of particles may block or clog the sample tips within the spray chamber, and the presence of ligands or other organic substances could hinder complete atomization of the sample, a sample-digestion process is always required before the sample is pipetted and analyzed [62]. This destructive procedure leads to some drawbacks (e.g., it cannot distinguish Ag^+ and AgNPs, unless a pre-separation performance is carried out before digestion).

ICP-AES or ICP-MS can also be coupled with separation techniques (e.g., FFF or HDC), so the different sized particles of the samples can be isolated, and the element distribution or composition of each fraction can be detected. Given its high sensitivity and selectivity, ICP-MS is more favored than ICP-AES. Also, isotope-diluted (ID) ICP-MS offers the opportunity to detect traces of Ag ions more accurately, thanks to the existence of two stable isotopes ^{107}Ag and ^{109}Ag . Instead of measuring the absolute concentration of the analytes, ID-ICP-MS provides a $^{107}\text{Ag}/^{109}\text{Ag}$ ratio to quantify the real amount of silver. In this way, the loss of silver during sample preparation or dilution, the interference of matrix or impurity, and instrument drift can be compensated for, because the ratio does not alter [63].

Recently, Laborda et al. [64] reported the utilization of ICP-MS in single-particle-detection mode to identify, to characterize and to detect AgNPs and Ag^+ selectively. AgNP suspensions were sufficiently diluted (below 10^9 silver NPs/L) to ensure each droplet contained at most one AgNP, so the frequency of the pulse was proportional to the number of atoms of the particle. As AgNP

Table 1. Overview the analysis of silver nanoparticle (AgNPs)

Analyte	Matrix	Separation methods	Conditions	Characterization	Ref.
AgNPs	Aquatic environmental samples	Cloud point extraction	Na ₂ S ₂ O ₃ 10 mM, pH = 3.0, TX-114 concentration 0.2% (w/v), 40°C incubated 30 min, centrifuged at 2000 rpm, 5 min	Off-line: TEM, SEM-EDX, UV-Vis	[28]
AgNPs and Ag ⁺	Commercial antibacterial products	Cloud point extraction	Na ₂ S ₂ O ₃ 10 mM, pH = 3.0, TX-114 concentration 0.2% (w/v), 40°C incubated 30 min, centrifuged at 2000 rpm, 5 min	Off-line: TEM, SEM-EDX, UV-Vis	[29]
Newly synthesized AgNPs	Synthesized water	Flow field-flow fractionation	Channel flow 1 mL/min; cross flow between 0.4 and 1 mL/min	On-line: UV-Vis Off-line: TEM, DLS	[32]
Ag, Au, Pd and Pt NPs together	Pure water	Thermal field-flow fractionation	Carrier liquid: THF and CAN; flow rate 0.3 mL/min; sample injection volume 20 µL	On-line: UV-Vis	[33]
Binary mixture of AgNPs (2-100 nm and 60-150 nm)	Water with 0.1% FL-70	Sedimentation field-flow fractionation	Carrier liquid: water with 0.1% FL-70; injection volume 5-30 µL; flow rate: 0-20 min: increased from 2 to 4 mL/min, 20-50 min: 4 mL/min	On-line: UV-Vis Off-line: SEM	[34]
AgNPs	Sewage sludge	Hydrodynamic chromatography	Mobile phase: 0.002 M Na ₂ HPO ₄ , 0.2% non-ionic surfactant, 0.05% SDS, 0.2% formaldehyde; pH ~ 7.5, injection volume 20 µL, flow rate 1.7 mL/min, pressure 9 MPa	On-line: HDC-ICP-MS Off-line: TEM	[36]
Polydispersed AgNPs solution	Phosphate buffer (20 mM, pH 11)	Counter-current chromatography	Mobile phase: hexane/toluene (1:1, v/v) with 0.02 mM TOAB; injection volume 5 mL, flow rate 1 mL/min, oven temperature 20°C, rotation of the chromatograph: 700 rpm	On-line: UV-Vis Off-line: FT-IR, SEM-EDX	[37]
Ag nano-spheres and Ag nano-cubes	Pure water	Micellar electrokinetic chromatography	Electrolytes: 20 mM SDS and 10 mM Tris-(hydroxymethyl)aminomethane (Tris) (pH 8.5); capillary: uncoated fused-silica capillaries of 75 micron m i.d. and 48.5 cm length; voltage: 30 kV	On-line: DAD Off-line: SEM	[43]
Ag nanorods and AgNPs with different size	Pure water	Micellar electrokinetic chromatography	Electrolytes: 20 mM SDS and 10 mM Tris-(hydroxymethyl)aminomethane (Tris) (pH 8.5); capillary: Uncoated fused-silica capillaries (i.d.: 75 µm; length: 48.5 cm); voltage: 20 kV and 30 kV	On-line: DAD Off-line: SEM	[42]
Au NP and Au/Ag core/shell NPs with different size	Pure water	Micellar electrokinetic chromatography	electrolytes: sodium dodecyl sulfate (SDS) (40 mM) and 3-(cyclohexylamino)propanesulfonic acid (10 mM) at pH 9.7; capillary: uncoated fused silica capillaries (i.d.: 75 µm; length: 50 cm); voltage: 20 kV	On-line: DAD Off-line: SEM	[44]
Au NP and Au/Ag core/shell NPs	Pure water	Micellar electrokinetic chromatography	electrolytes: SDS (40 mM) and 3-cyclohexylamino-1-propanesulfonic acid (CAPS; 10 mM) at pH 10.0; capillary: uncoated fused-silica capillaries (I.D.: 75 µm; length: 33.5 cm); voltage: 20 kV; On-line concentration by the reversed electrode polarity stacking mode (REPSM)	On-line: DAD Off-line: SEM	[45]
Silver clusters ligated with glutathione	Pure water	Polyacrylamide gel electrophoresis	30% acrylamide resolving gel and 4% acrylamide stacking gel; eluting buffer: 25 mM THAM and 192 mM glycine	Off-line: STEM, XRD, EDS, NMR, UV-Vis	[38]

(continued on next page)

Table 1. (continued)					
Analyte	Matrix	Separation methods	Conditions	Characterization	Ref.
Ag nano-spheres, triangles, and rods with SH-PEG-COOH modification	Pure water	Agarose gel electrophoresis	0.2% agarose gel in 0.5 × TBE (Tris-borate EDTA) buffer (pH~ 9)	Off-line: TEM, UV-Vis	[39]
4-carboxythiophenol modified Au and Ag colloidal particles	Pure water	Miniscale IEF	Electrolyte solutions: 0.1 M ortho-phosphoric acid as anode solution and 0.1 M NaOH as the cathode solution; separation solution: 1.5 ml of glycerol with 1 ml of water; Gradient solutions: (i) high density solution: 3 ml glycerol with 2 ml of the different hydrosols together with 0.2 ml carrier electrolyte, and (ii) low density solution: 3 ml carrier electrolyte, and (ii) low density solution: 3 ml carrier electrolyte. IEF was carried out for 5 h at 400 V and yielded a current of 1.5 A.	Off-line: UV-Vis	[40]
Ag, Au and CdSe NPs respectively	Organic solvents	Density Gradient centrifugation	Five layer gradient (cyclohexane and tetrachloromethane mixtures with v/v at 50%, 60%, 70%, 80%, and 90%), centrifuged at 50000 rpm, 8 min	Off-line: TEM	[46]
AgNPs and Ag ⁺	Pure water	Dialysis	A dialysis tube containing the dispersions immersed in a 100-fold excess volume of pure water for a few hours	Off-line: HRTEM, DLS	[51]
AgNPs and Ag ⁺	Commercial sock fabrics containing AgNPs	Ultrafiltration and centrifugation	Membrane filters with 0.4, 0.1, and 0.02 μm pore diameter	Off-line: TEM-EDX, SEM-EDX	[11]
Newly synthesized AgNPs	Pure water	Ultrafiltration	Ultrafiltration membrane: 10 kDa polyethersulfone (PES)	Off-line: TEM, XPS, NMR	[49]
AgNPs solution	Pure water	Centrifugal ultrafiltration	Membrane: with a nominal molecular weight limit of 3 kDa, centrifuged at 3000 g for 30 min	Off-line: TEM, DLS	[47]
AgNPs solution	Pure water	Centrifugation and filtration	Membrane: with a nominal molecular weight limit of 3 kDa, centrifuged at 2800 g for 30 min	Off-line: TEM	[48]
Newly synthesized AgNPs	Pure water	Centrifugal ultrafiltration	Membrane: with a nominal molecular weight limit of 3 kDa	Off-line: TEM, DLS, UV-Vis	[20]
Newly synthesized AgNPs	Pure water	Tangential flow ultrafiltration	Tangential flow ultrafiltration with a 50 nm filter and then concentrated with a 100 kD filter	Off-line: TEM, UV-Vis	[50]
Polydispersed AgNPs solution	Hexane	CO ₂ expanded liquid approach	The series of pressurization: 0-500, 550, 600, 625, 650 psi.	Off-line: TEM	[52]

suspensions were detected as a single pulse and showed a lognormal distribution, whereas Ag⁺ solutions produced pulses of average constant intensity and displayed a Poisson distribution, the presence of AgNPs or Ag⁺ could be identified from the scan type. In the study, NP diameter limit of detection (LOD_d), which meant a particle was capable of producing a pulse with the intensity equal to three times the standard deviation of the noise, was 18 nm, while the number concentration LOD was 2 × 10⁵/L.

AAS is a powerful alternative to ICP-MS or ICP-AES in the detection of silver, due to its high selectivity, sensitivity and accuracy, as well as the relatively low cost. However, AAS also suffers from some limitations (e.g.,

failure of multi-element analysis and relatively narrow linear range).

Except from these traditional techniques, some novel methods to quantify AgNPs have also been developed, mainly based on fluorogenic and chromogenic probes or electrochemical electrodes.

Ahn et al. [65] demonstrated the usage of rhodamine derivative as a sensitive probe to detect Ag⁺ and AgNPs. The addition of Ag⁺ induced the transformation from rhodamine derivative to oxazoline, along with the colorless solution changing to pink and occurrence of a strong orange fluorescence. The fluorescence intensity directly correlated with Ag concentration, and the LOD was as low as ~14 μg/L. For AgNPs, only oxidation was

needed before detection. Also, this method was applicable for the quantification of AgNPs in consumer products (e.g., hand-sanitizer gel and fabric softener) and the data accorded well with ICP-AES results.

Tseng et al. [66] also reported the highly sensitive detection of Ag^+ and AgNPs based on an oligonucleotide sensor. The study used an oligonucleotide C_{20} (containing 20 repeats of cytosine) and a double-strand-chelating dye SYBR Green I (SG). Because of the coordination with cytosine (C) to form a stable $\text{C-Ag}^+\text{-C}$ complex, the presence of Ag^+ promoted oligonucleotide conformation from a random coil to a hairpin structure, which strengthened SG chelating with C_{20} . As a result, the fluorescence response was firmly enhanced. By measuring the fluorescence intensity, the Ag concentration could be calculated. The scheme was highly selective toward Ag^+ , and there was no interference by other metals even at a 1000-fold concentration. When the AgNPs were oxidized by H_2O_2 in acidic conditions, this proposed method was also suitable for the detection of AgNPs in the 64.2–241 nM range.

A novel silver-selective electrode was fabricated for the speciation analysis of AgNPs [67]. Benzothiazole calix [4] arene was synthesized as an ionophore to achieve fast (response time < 5 s), stable and highly selective determination of Ag^+ . This new electrode could be used in a wide pH range (pH 2–8), and Ag^+ could be detected in the concentration range from 10^{-6} – 10^{-2} mol/L, with a detection limit of 5.0×10^{-7} mol/L. By measuring the free Ag^+ in the AgNP solution and the total Ag content after the AgNP solution was oxidized by H_2O_2 , this system offered an indirect approach to speciation analysis of AgNPs.

5. Conclusion and perspective

In this review, we outlined the general approaches to separate, characterize and quantify AgNPs. Given the complexity of sample matrixes and the inherent drawbacks of each technique, simple “one for all” methods are not available to evaluate the environmental behavior of AgNPs fully. For the most time, multiple schemes are involved.

Though significant progress has been made in recent years, we are still far from having methods to trace AgNPs in the environment. Bearing this in mind, much effort is required. On one hand, as the concentrations of AgNPs are very low in the natural waters or other environmentally relevant samples, it is necessary to develop effective preconcentration and separation methods. On the other hand, we should refine the existing methods to improve resolution and sensitivity further. Among the large number of techniques used to separate or to characterize AgNPs, very few can be coupled to achieve separation-characterization or quantification at

the same time. Coupled techniques need to be further investigated.

The toxicity of AgNPs has been estimated by a number of researchers, but only a handful of studies considered the release of Ag ions. Moreover, whether the observed toxicity is due to Ag ions, AgNPs or both is still unknown, so, in order to elucidate the toxicity of AgNPs fully, proper methods should be developed to isolate AgNPs and Ag ions involved in exposure.

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