

# Chapter 2

## Separation and Determination of Silver Nanoparticles

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**Abstract** The accurate analysis of silver nanoparticles (AgNPs) in complicated environmental and biological matrix is the premise for the assessment of AgNP risks. In the past few decades, a myriad of methods have been developed for the concentration and determination of AgNPs. In this chapter, methodologies for the separation, characterization, and quantification of AgNPs are introduced, and the advantages and shortcomings of each technique are also discussed. In most cases, multiple schemes are often needed to get comprehensive information of the analytes. To meet the ultra-trace detection of AgNPs in the environment, techniques with higher resolution and sensitivity are required.

### 2.1 Separation

Once released into the environment, silver nanoparticles (AgNPs) would interact with complex environmental matrices, making it a big challenge to trace the fate and transport of AgNPs in natural systems. For the ultra-trace analysis of AgNPs in real samples, proper preconcentration and separation methods are required. The traditionally used methods are centrifugation, membrane filtration, dialysis, and centrifugal ultrafiltration. Irreversible agglomeration or aggregation may occur during centrifugation and filtration, making it difficult to track the original morphology of nanoparticles (NPs). Moreover, the separation efficiency varies in according to the nature of NPs and small sized NPs may suffer from significant mass loss. The dialysis method is time-consuming and frequently changing the dialysis fluid is rather annoying. Centrifugal ultrafiltration can avoid the particle aggregation to some extent, and shows to be a powerful tool to concentrate NPs. Membranes are designed to guarantee that no particle larger than the pore size penetrates the mem-

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brane. However, it does not mean that particles smaller than the pore size could pass through the membrane quantitatively. It is always suffered from the low recovery. To date, a number of techniques are developed to isolate AgNPs, including extraction methods like cloud point extraction (CPE), chromatographic techniques like size-exclusion chromatography (SEC) and high performance liquid chromatography (HPLC), electrophoresis methods like capillary electrophoresis (CE), and other methods like field-flow fractionation (FFF) and density-gradient centrifugation.

### **2.1.1 Cloud Point Extraction**

Cloud point extraction was first described by Watanabe and co-workers in 1987 [1, 2], and since then this method has received enormous attention and is rapidly developed. Based on the solubilization ability and the cloud points of nonionic surfactants, CPE can be easily done. Typically, there are three simple steps in the extraction: (i) adding sufficient nonionic surfactant into the sample solution to solubilize the analytes; (ii) changing external conditions such as the temperature, pressure, pH, or ion strength to attain cloud points, i.e., incomplete solubilization; (iii) facilitating phase separation by centrifugation or long-term placing. In the end, analytes are concentrated into the surfactant-rich phase due to the analyte-micelle interaction. Because of the high extraction efficiency, easily handling, low costs, and environmental benign of CPE, it is popular in the determination of metal ions (e.g., Cr, Cu, Cd), organic pollutions and other biopolymers [3].

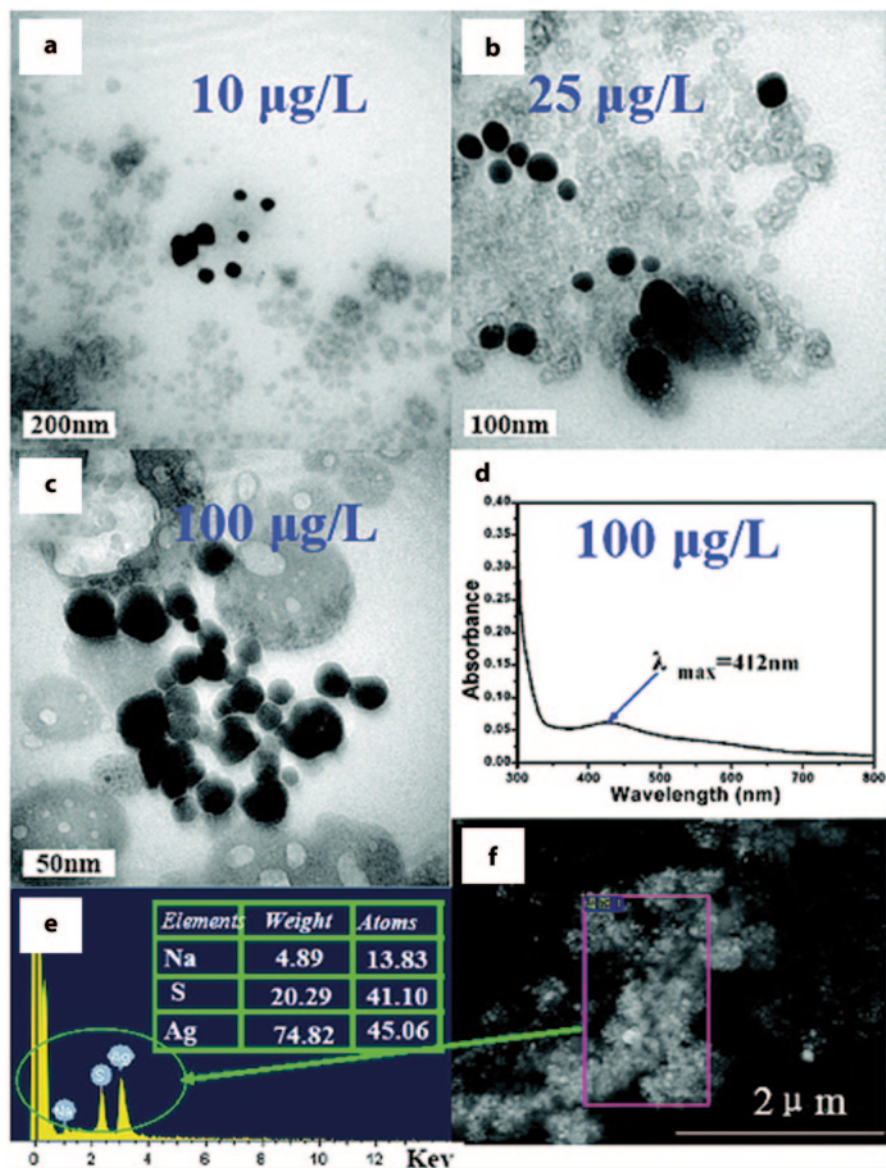
Liu et al. for the first time reported the thermoreversible separation of nanomaterials (NMs) by CPE [4]. Based on a commercially available nonionic surfactant Triton X-114 (TX-114), several NMs with various coatings and shapes, including PVP-coated AgNPs, citrate stabilized gold nanoparticles (AuNPs),  $C_{60}$ ,  $TiO_2$ , and single-walled carbon nanotubes, can be concentrated.

The CPE method was proved to be an efficient approach to extract AgNPs from environmental waters [5]. The extraction efficiency was influenced by several parameters such as pH, salinity, surfactant concentrations, incubation time and temperature. Generally, the highest extraction efficiency is achieved at about the zero point charge pH of AgNPs, under which conditions the electrostatic repulsion is relatively low and particles tend to present in the nonionic surfactant phase. The presence of salts also reduces the Coulomb repulsion between charged NPs and promotes the phase separation [4]. However, high concentration of salts may induce the aggregation of AgNPs. The extraction efficiency typically increases with the surfactant concentration up to a maximum value, and then levels off. As a relative high surfactant concentration means an increase in the surfactant-rich phase volume and thus reduces the enrichment factor, choosing a proper surfactant concentration is important. It is reported that the analyte recovery and preconcentration factor increase when the equilibration temperature used for phase separation is progressively increased above the cloud point temperature of the system [6]. However, high temperature may reduce the association of NPs to the surfactant micelles, causing

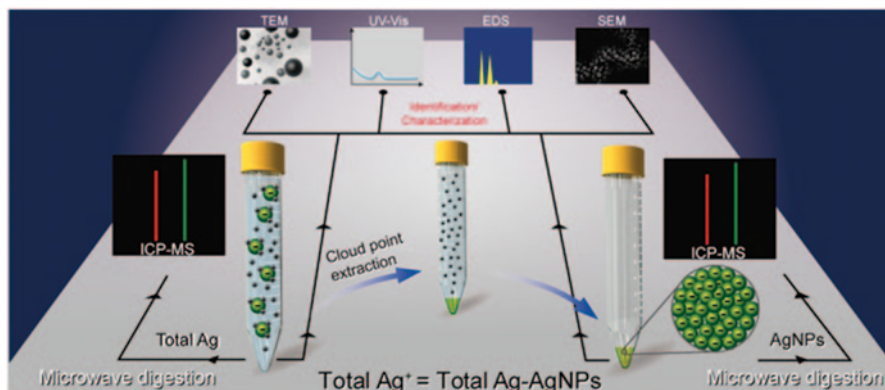
the reduction of AgNP recoveries [5]. Under the optimized conditions, 57–116% of AgNPs could be effectively recovered from different environmental waters at the spiking level of 0.1–146  $\mu\text{g/L}$ , with an enrichment factor of 100. Most of all, the extraction did not disturb the pristine size and shape of AgNPs, and the analytes can be subsequently characterized by other techniques, such as transmission electron microscopy (TEM), scanning electron microscopy (SEM), and ultraviolet-visible spectroscopy (UV-vis) (Fig. 2.1). After microwave digestion and quantified by inductively coupled plasma mass spectrometry (ICP-MS), the detection limit is 0.006  $\mu\text{g/L}$  (34.3 fmol/L particles of AgNPs), allowing the ultratrace detection of AgNPs in aquatic systems.

Speciation analysis of AgNPs and  $\text{Ag}^+$  in complex matrices is highly demanded because of their distinct difference in environmental behavior and toxicity. The CPE method also offers great potential to selectively separate AgNPs and  $\text{Ag}^+$  by adding an efficient chelating agent like  $\text{Na}_2\text{S}_2\text{O}_3$  [7] and EDTA [8]. During the extraction,  $\text{Ag}^+$  could form hydrophilic complexes with the chelating agent to be preserved in the upper aqueous phase, while AgNPs are transferred into the nether TX-114-rich phase. The interference of  $\text{Ag}^+$  was negligible when the concentration of  $\text{Ag}^+$  was lower than two times that of AgNPs [5]. It was successfully applied to determine AgNPs and  $\text{Ag}^+$  in environmental samples such as commercial available products [7], HepG2 cells [9] and municipal wastewater [10, 11]. Previous study reported the speciation analysis of AgNPs and  $\text{Ag}^+$  in six antibacterial products based on CPE [7]. AgNPs were quantified by analyzing Ag contents in the TX-114-rich phase by ICP-MS after microwave digestion, while the concentration of  $\text{Ag}^+$  was determined by subtracting AgNP contents from the total amount of silver in the products (Fig. 2.2). The limits of quantification ( $\text{S/N}=10$ ) for antibacterial products were 0.4  $\mu\text{g/kg}$  and 0.2  $\mu\text{g/kg}$  for AgNPs and total silver, respectively.

Recently, Schuster et al. also reported the determination of AgNPs in environmental waters and wastewater samples by means of CPE [8]. The extracted AgNPs could be directly analyzed by electrothermal atomic absorption spectrometry without any additional sample digestion process, thus a limit of detection as low as 0.7 ng/L was obtained, which enables us to track the fate and transport of AgNPs in the environment. Based on the developed method, they further quantified nanoscale silver particles in the influents and effluents collected from nine municipal wastewater treatment plants in Germany [10]. It was found that the total silver concentration in the unfiltered influent was in the range of 0.32–3.05  $\mu\text{g/L}$ , while about 0.18–1.30  $\mu\text{g/L}$  silver was remained in the filtered influent, revealing a great number of silver was associated with the suspended organic matters with sizes larger than 0.45  $\mu\text{m}$ . The mechanical treatment was demonstrated to efficiently reduce the concentration of nanoscale silver particles in wastewater samples with an average removal efficiency of 35%, and the subsequent biological treatment can further eliminate more than 72% of the remaining particles in the semitreated wastewater. As a result, nanoscale silver particles in the effluent was relatively low ( $<12$  ng/L), indicating that wastewater treatment plants were not the pollution source of nanoscale silver particles to the aquatic system.



**Fig. 2.1** Identification of AgNPs enriched in the TX-114-rich phase. TEM images of the TX-114-rich phase separated from the extraction of samples containing 10  $\mu\text{g/L}$  (a) and 25  $\mu\text{g/L}$  (b) AgNPs. TEM image (c) and UV-vis spectrum (d) of the TX-114-rich phase separated from extraction of a sample containing 100  $\mu\text{g/L}$  AgNPs. EDS (e) and SEM image (f) of the TX-114-rich phase separated from the extraction a sample containing 1 mg/L AgNPs. Reprinted with the permission from ref. [5], Copyright 2009 American Chemical Society



**Fig. 2.2** Speciation analysis of AgNPs and Ag<sup>+</sup> in antibacterial products and environmental waters via cloud point extraction-based separation. Reprinted with the permission from ref. [7], Copyright 2011 American Chemical Society

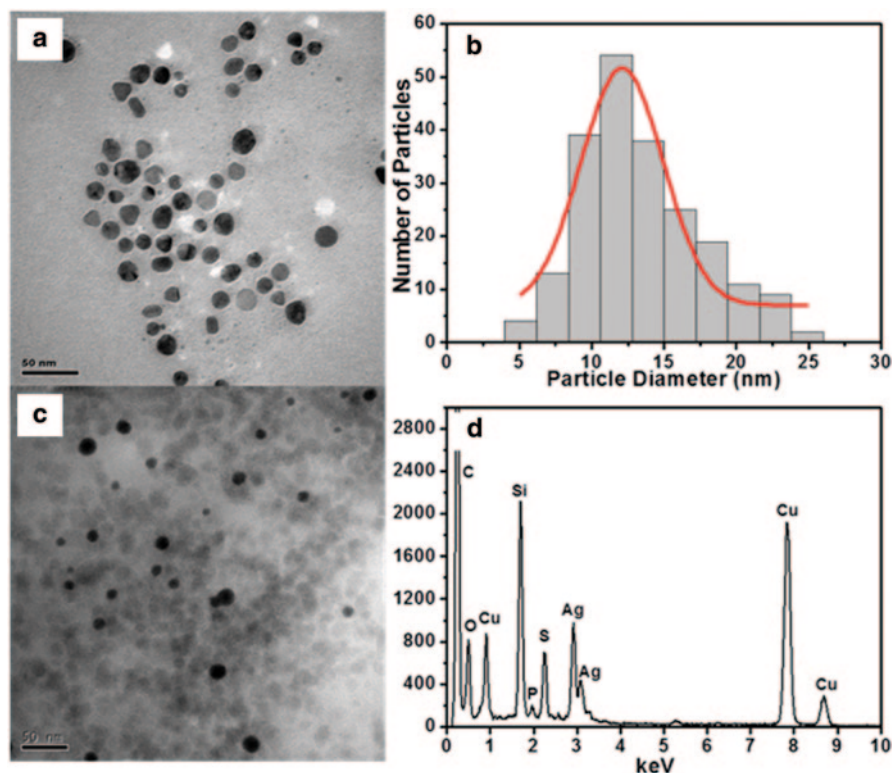
The CPE method allows us to quantify the uptake of AgNPs and Ag<sup>+</sup> to cells as well [9]. Under the optimized conditions, AgNPs and Ag<sup>+</sup> can be readily separated and quantified in HepG2 cells. It was found that about 67.8 ng Ag was assimilated per 10<sup>4</sup> cells after cells were exposed to 10 mg/L AgNPs for 24 h, in which about 10.3% silver existed as Ag<sup>+</sup> (Fig. 2.3). The substantial amount of Ag<sup>+</sup> in exposed cells indicated that the contribution of Ag<sup>+</sup> cannot be ignored in assessing the toxicity of AgNPs to organisms.

### 2.1.2 Field-Flow Fractionation

FFF is a flow-assisted hydrodynamic separation technique that was designed to separate and size the macromolecular, colloidal, and particulate materials [12–14]. The separation process is very similar to chromatography except that the isolation relies on physical forces and without the need of a stationary phase. All separation is performed in a thin channel, and samples are separated according to their diverse diffusion coefficients. An axial flow of carrier liquid transports analytes in the direction of the outlet of the channel, while an externally generated field is applied perpendicular to the carrier-driven flow. The external field drives the particles toward the so-called accumulation wall from where they diffuse back into the channel, resulting in the retention of the particles. The basic principle of FFF has been described in previous studies [12, 13]. Depending on the “fields” utilized, FFF can be divided into different types, such as thermal FFF (ThFFF), sedimentation FFF (SdFFF), crossflow FFF (FIFFF), dielectrophoretic FFF (DEP-FFF), and magnetic FFF (MgFFF).

The high resolution and capability to isolate particles with a wide range of sizes makes FFF popular to separate NPs, such as metals, metal oxides, and SiO<sub>2</sub> [15].

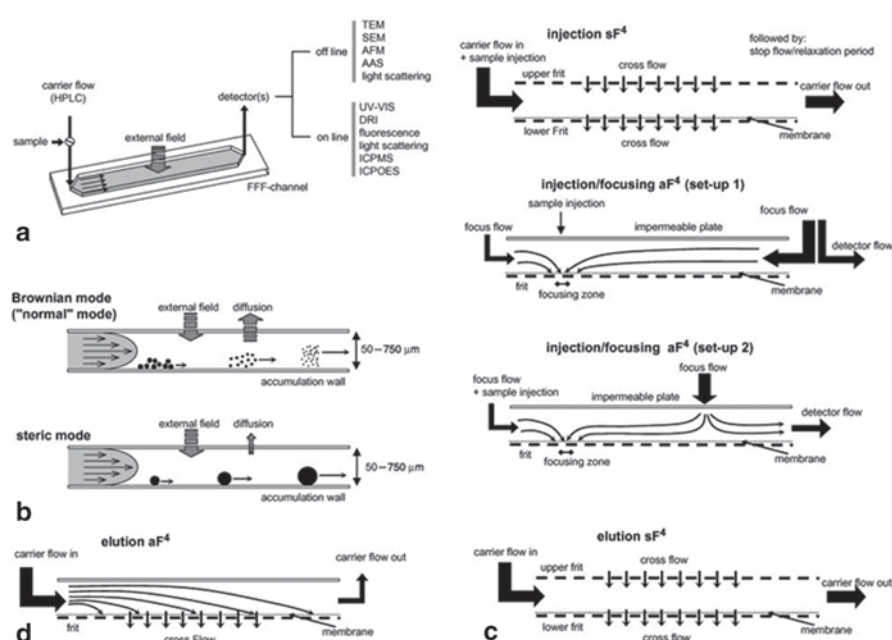




**Fig. 2.3** Characterization of pristine AgNPs and identification of AgNPs enriched in TX-114 rich phase after CPE. Up, TEM image (a) and particle size distribution (b) of the stock AgNP solution; down, TEM image (c) and EDS image (d) of AgNPs in the TX-114 rich phase after CPE of the exposed cells. The scalar bar is 50 nm. Reprinted with the permission from ref. [9], Copyright 2013 American Chemical Society

Moreover, the absence of a stationary phase avoids the irreversible interaction with the particles, which prevents the low recovery and undesirable morphology change during separation. FFF enables fractionation of ENPs with diverse sizes and different fractions can be further characterized by a variety of detectors. On-line detectors include UV-vis, fluorescence, dynamic light scattering (DLS), inductively-coupled plasma atomic emission spectroscopy (ICP-AES), and ICP-MS, while off-line detectors include TEM, SEM, atomic force microscopy (AFM), and atomic absorption spectrophotometry.

SdFFF was employed to separate and determine AgNPs with about 100 nm in diameter [16]. Using 0.1 % FL-70 as the carrier and on-line UV-vis as the detector, bimodal mixtures of AgNPs can be isolated and characterized [17]. ThFFF also shows promises to retain metal nanoparticles (e.g., Ag, Au, Pd, Pt) suspended in either aqueous or organic carrier solutions [18]. Among the different types of FFF, flow FFF is the most popular technique. Figure 2.4 shows the set-up of a typical FFF system.



**Fig. 2.4** **a** Typical field-flow fractionation (FFF) lay-out with examples of different detectors, **b** lateral cross sections through the FFF channel, presenting the principle of particle-size fractionation for the “normal” Brownian and the steric elution modes, **c** lateral cross section through the FFF channel, describing the injection and focusing procedure for symmetric flow-FFF (sF<sup>4</sup>) (*top*) and asymmetric flow-FFF (aF<sup>4</sup>) (*middle and bottom*), and **d** lateral cross section through the FFF channel, showing the elution procedure for sF<sup>4</sup> and aF<sup>4</sup>. Reprinted from ref. [13], Copyright 2011 with the permission from Elsevier

Asymmetric flow FFF (AF4) can act as a membrane-filtration unit, which enables the on-line pre-concentration of analytes. It allows up to 50 mL samples to be injected, thus the signal response can be largely improved [15]. To get reasonable results regarding especially the NP size distribution and a good recovery of the analyte, a number of parameters have to be optimized, including the carrier liquid composition, field force, types of membranes, sample injection and relaxation, cross flow rates, and injected mass [19–21]. The carrier solution (i.e. pH, ion strength, and composition) affect the electrostatic properties of the NPs and membranes, and thus influence the retention or adsorption of NPs in the channel. Typically, the carrier provides particles and membranes with the same electrical polarity (both negative or both positive), and sufficient electrostatic repulsion could not only hinder the attachment or loss of the particles to the membrane, but also control the thickness of the repulsive electrostatic double layers to make the particles approach the membrane as closely as possible, and to avoid a repulsion-cushion effect which can induce the particles to elute earlier than would be expected based on their diffusion coefficient [13]. To avoid the undesirable aggregation or dissolution of AgNPs,

the chosen carrier solution should have similar properties with the matrix in which the NPs are suspended. Moreover, a bactericide (e.g., sodium azide) is always added into the carrier solution to prevent the growth of bacteria [22].

In AF4, the membrane is designed to hinder the macromolecules and particles from escaping the channel. The most commonly used membranes are regenerated cellulose and polyether sulphone (PES) with a molecular weight cut-off in range of 300–10,000 Da. In general, losses through the membrane permeation and sorption on the membrane surface are the most common causes of low recoveries [20]. Thus, choosing a proper membrane is of great importance. For highly-charged AgNPs, a lower charged membrane is recommended, as enough repulsion is gained. And for lower-charged ones, membranes with higher charged such as regenerated cellulose are better choices.

The cross flow, which generates the field force and determines the resolution and quality of fractionation, is a key parameter that should be considered. As a general rule, low cross flows are used to sort particles with larger sizes, while a high cross flow can be applied to separate smaller particles, though prolonged retention times are required with higher cross flows [22]. For polydisperse AgNPs or AgNPs with a size larger than 100 nm, constant cross flows may not work well, and a gradient elution is preferred [21].

Under the optimized conditions, AF4 can obtain the size distribution of AgNPs in different matrices to further monitor their stability and transformation in the environment. AF4 results showed that the size of citrate-stabilized AgNPs increased as pH increased from 5 to 8 at low ionic strengths, and higher concentration of  $\text{Ca}^{2+}$  induced the aggregation and precipitation of AgNPs even with the characteristic FFF peak missing in the fractogram [23]. Pectin and alginate coated-AgNPs were much more stable than citrate-AgNPs, and humic acid could enhance the stability of AgNPs in real environmental waters [24]. By using 0.01 % (m/v) sodium dodecyl sulfate (SDS) solution at pH 8.0 as the carrier and PES with a cutoff of 1 kDa as the ultrafiltration membrane, casein stabilized AgNPs can be easily separated in the culture medium by AF4-UV-vis. The AF4 fractogram revealed that the size of AgNPs increased from 17 nm to 32 nm after AgNPs were incubated in the culture medium for 24 h. UV-vis spectra showed no differences at the absorbance maximum wavelength before and after the incubation, indicating no obvious aggregation occurred. The increment in the size distribution probably due to the “protein corona” effect [25]. Compare with other techniques like TEM, AF4 can get similar results but is much faster and simpler [21, 23].

For successful separation by AF4, NPs have to be in liquid suspensions [19]. Thus, to analyze complex samples such as food, meat, and sediments, proper decomposition methods are necessary to liberate AgNPs into the liquid suspension. Traditional digestion methods are based on sonication, strong acids, bases, or enzymes. Low pH may lead to the dissolution of AgNPs, which limits the application of strong acid digestion. AgNPs were extracted from tissues of freshwater worms by continuous sonication, and followed by centrifugation and isolated by AF4 coupled with ICP-MS. It was observed that the size of AgNPs increased from approximately 31 nm to 46 nm, which was in good agreement with data derived from DLS, imply-

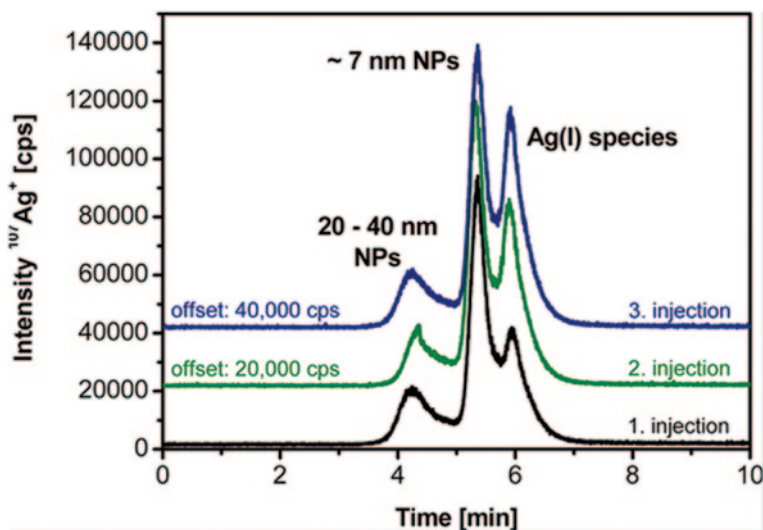


ing the possible characteristic change of AgNPs during exposure [26]. After enzymolysis by Proteinase K at 37 °C for 40 min, AgNPs spiked in chicken meat samples can be liberated and further separated by AF4-ICP-MS with a recovery of around 80% [27]. The size distribution of AgNPs in the meat digestate did not change much with that in aqueous solutions, revealing no detectable aggregation or dissolution of AgNPs took place during the sample preparation stage. Alkaline digestion mainly focus on potassium hydroxide (KOH), sodium hydroxide (NaOH), and tetramethylammonium hydroxide (TMAH). However, KOH and NaOH may induce the aggregation of AgNPs [28], and TMAH, which does not change the integrity of AgNPs in short times, appears more suitable for digesting complex samples. AgNPs associated with the cultured HepG2 cells were readily identified by AF4-ICP-MS after a digestion process based on TMAH and Triton X-100 [25]. FFF can also find their way in analyzing AgNPs in consumer products [20], dietary supplements [20], and untreated wastewater [29], showing promises to study the environmental fate of AgNPs in the complex systems.

Hollow-fiber flow FFF system (HF FIFFF) was regarded as the third type of flow FFF, in which fractionation was conducted in a cylindrical hollow fiber instead of the rectangular channel [30]. HF FIFFF was spearheaded by Lee and Jönsson's groups, and has been extensively applied in the analysis of synthetic polymers, cells, bacteria, and biological macromolecules [31]. The main merits of the system included cheapness, miniaturization, simple installation and operation, and comparable separation ability with AF4/F4. In separation, the target was first driven toward the HF wall under the radial flow and located at the equilibrating position, and then moved along HF channels under the axis flow to the detector at different speeds according to the size [32]. As the theoretical basis of HF FIFFF was evolved from the traditional FFF, the diameter of AgNPs in the colloidal could be measured based on their retention parameters. HF FIFFF is expected to play an important role in separation of AgNPs.

### 2.1.3 *Chromatographic Methods*

SEC is one of the most commonly used techniques to separate submicron particles. With the porous packing materials filled in the column, particles with the size smaller or equal to the pores of packing materials can permeate deep inside the column packing materials and cause a longer pathway, while larger particles would be rejected by the pores and eluted first. The separation efficiency depends on the average diameter and pore size of packing materials, the column length and mobile phase flow rate. The SEC analysis is fast, simple, repeatable, and rather economical. However, it also faced the problem of limited separation selectivity [33]. For samples with a wide range of size distribution, several columns (usually three or four) are needed to get a satisfying separation result [34]. Moreover, there is a risk that large particles may block pores of the column, so a pre-treatment step is always required.



**Fig. 2.5** Chromatograms of a solution extracted from sport socks by reversed-phase liquid chromatography coupled to ICP-MS. Reprinted with the permission from ref. [35], Copyright 2013 American Chemical Society

Reversed-phase HPLC was also used to separate different sized AgNPs, and coupled with ICP-MS for the speciation analysis of AgNPs and  $\text{Ag}^+$  [35]. The separation of NPs followed a size-exclusion mechanism, so that larger ones eluted first. With the addition of thiosulfate to the mobile phase,  $\text{Ag}^+$  can be successfully eluted from the C18 column, and well separated from AgNPs in a single run, with recoveries  $>80\%$  for both of AgNPs and  $\text{Ag}^+$ . The limits of detection were in the medium range of ng/L, which allowed the analysis of trace silver in the environment. When separating complex samples, such as fetal bovine serum solutions, the retention time of AgNPs might be altered due to the attachment of proteins to the surface of NPs, which can be readily calibrated by the use of AuNPs as internal size standards. The proposed method is also capable of analyzing real samples, such as extracts from sports socks (Fig. 2.5), showing reproducible analytical results and low detection limits.

Very recently, Liu et al. reported the speciation analysis of dissolvable Ag(I) and silver-containing nanoparticles of 1–100 nm using HPLC coupled with ICP-MS [36]. In that work, the separation was conducted by using an amino column with a pore size of 500 Å, and 0.1 % (v/v) FL-70 (a surfactant) and 2 mM  $\text{Na}_2\text{S}_2\text{O}_3$  were added into the aqueous mobile phase to facilitate the elution of dissolvable Ag(I) and nanoparticulate Ag from the column. Under the optimized conditions, nanoparticulate Ag with various sizes (ranging from 1 to 100 nm), coating agents (including citrate, PVP and PVA) and species (such as  $\text{Ag}^0$  and  $\text{Ag}_2\text{S}$ ) can be baseline separated from the dissolvable Ag(I) (Fig. 2.6). The concentration of dissolvable Ag(I) can be directly analyzed by the on-line coupled ICP-MS with a detection limit of

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