Hollow-fiber flow field-flow fractionation and multi-angle light scattering investigation of the size, shape and metal-release of silver nanoparticles in aqueous medium for nano-risk assessment

Hollow-Fiber Flow Field-Flow Fractionation and Multi-Angle Light Scattering Investigation of the Size, Shape and Metal-Release of Silver Nanoparticles in Aqueous Medium for Nano-risk Assessment

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ABSTRACT

Due to the increased use of silver nanoparticles in industrial scale manufacturing, consumer products and nanomedicine, reliable measurements of the size, shape and distribution of these particles in aqueous medium is critical since these properties affect both functional properties and biological impact especially in quantifying associated risks and identifying suitable risk-mediation strategies. The feasibility of an on-line coupling of a fractionation technique such as hollow-fiber flow field flow fractionation (HF5) with light scattering techniques such as MALS (multi-anlge light scattering) have been investigated for this purpose and data obtained have been compared with those from more conventional, but often complementary techniques e.g. transmission electron microscopy, dynamic light scattering, atomic absorption spectroscopy, and X-ray Fluorescence. The combination of fractionation and multi angle light scattering techniques have been found to offer an ideal, hyphenated methodology for the simultaneous size-separation and characterization of silver nanoparticles. The hydrodynamic radii determined by fractionation techniques can be conveniently correlated to the mean average diameters determined by multi angle light scattering and reliable information on particle morphology in aqueous dispersion can be obtained. The ability to separate silver (Ag^{+}) ions from the silver nanoparticles (AgNPs) via membrane filtration during the size analysis can be an added advantage in obtaining quantitative insights to its risk potential. Most importantly, the methodology developed in this article can potentially be extended to similar characterization of metal-based nanoparticles when studying the functional effectiveness and potential hazards of these nanoparticles.

Keywords:

Ag nanoparticles in nanomedicine, HF5 size analysis of AgNPs, HF5-MALS of metal nanoparticles, HF5 conformational studies of metal nanoparticles, HF5 metal release analysis of AgNPs, HF5 for nanorisk assessment

1. INTRODUCTION

Nanoparticles are interesting tools for various applications [1]. Thanks to their high surface/volume ratio, they present a noticeably different activity with respect to that of smaller compounds .

Sustainable development of the nanotechnologies, as well as in other relevant industrial applications must avoid any adverse effect on health of humans and environment exposed to nanomaterials, thus justifying a close attention to safety issues. In particular, the novel/revived attention on colloidal silver antimicrobial applications, used in food packaging materials, food supplements, odor-resistant textiles, household appliances, cosmetics and medical devices, water disinfectants, and room sprays applications, addresses to AgNPs the attention of European nano-safety research [2],[3]. Such relevance is also justified by the fact that surface engineered metal nanoparticles find their use also as therapeutic agents in drug delivery applications; moreover, their dimensions match that of biological building blocks, from proteins to organelles, leading to question about interactions with living organisms [4][5]. The use of nanoparticles as drug carrier may reduce the toxicity of the incorporated drug and the toxicity of the whole formulation is investigated. However, results of the nanoparticles alone are not often described, and a discrimination between drug and nanoparticle toxicity cannot be made. A specific emphasis on the toxicity of the “empty” non-drug loaded particles is instead particularly important when slowly or non degradable particles (as metal nanoparticles) are used for drug delivery since they might show persistence and accumulation on the site of the drug delivery, eventually resulting in chronic inflammatory reactions.

The development of safer by design nanomaterials, based on surface engineering could effectively represent an inherent safety approach, able to design out hazard at the source. Nevertheless, to ensure the effectiveness of such preventive measures, it is necessary to perform a deep characterization of physicochemical properties, affecting biological and functional properties, while a solid comprehension of mechanism leading nanoparticles biological reactivity is required. In particular, the discrimination between different hazard determining factors within and outside the complex biological matrix is fundamental in order to establish strategies that can mitigate the risk. Such a goal is particularly important when toxicity assessment of silver colloidal systems is addressed, since there is still an ongoing debate about the mechanism by which AgNPs exert toxicity, and its consequential antimicrobial effect [6]. Despite the common accepted mechanism for which the release of cationic Ag represents the primary mechanism of antibacterial action, evidences of a particle specific activity are also reported.

A technique able to perform the metal ions quantification and the characterization of silver nanoparticles dispersed in aqueous media, is, for these reasons, strategic to better elucidate biological interaction mechanism and develop solutions to decrease health impact by preserving antibacterial activity [7]. Since most of the studies are conducted over commercially available nanosilver having limited, if any, control over AgNPs' size, morphology, degree of agglomeration and distribution between zerovalent (Ag^0) and cationic Ag (Ag^+), free or adsorbed onto the surface, it is quite difficult to draw universally accepted conclusions regarding the toxicity mechanism of nanosilver [8][9]. To investigate how and if nanoparticles may present harm for the environment and organisms, a characterization of their behavior in environmental/physiological medium is required besides a characterization of their size, shape, activity and stability [10]. The most common techniques used for NPs analysis in liquid media involve DLS (Dynamic Light Scattering), chromophore counting, resonant light scattering and Raman scattering. High

resolution electron microscopy typically deals with the analysis of precipitates formed by drying the colloidal solution on a microscopic grid and often involves a cross sectional cut. DLS does not provide any information on particle shape and density distribution and its accuracy may be intrinsically limited in particular when used in complex samples [11]. Static, multi-angle LS (MALS) gives independent information on the NP molar mass (Mr) and root-mean-square (rms) radius values [12]. Consequently, it may provide information on compactness ad shape of the NPs. Hyphenation of DLS or MALS detection with size-based separation methods represents a multidimensional platform that can then enhance the accuracy of analysis of complex NPs samples.

Among separative techniques for nanodispersed analytes, Flow Field-Flow Fractionation (F4) is increasingly employed as a mature separation method able to size-sort and isolate NPs. Coupled with on-line uncorrelated detection methods including MALS, DLS, absorbance and luminescence spectrophotometry, F4 is able to offer an multidimensional analytical platform for nanomaterials analysis providing size distribution analysis, identification of aggregation phenomena, separation of the unbound constituents of the functional NPs, functional characterization of the NPs and correlation of spectroscopic properties with NP size [13]. F4 is ideally suited to separate dispersed analytes over a broad size range, from nanometer to micrometer sized analytes based on their coefficient diffusion and dimensions [14]. In addition to size fractionation, in F4 membranes also act as in-line sample micro-purification/desalting membranes during the focus/relaxation step used for the sample injection. During the analysis, samples smaller than membrane cut-off exit from it and can be collected from cross-flow line and analyzed using a technique able to quantify them, such as flame absorption atomic spectroscopy. F4 can be used in two technical variants, the asymmetrical F4 (AF4) and the hollow fiber F4 (HF5). AF4 is the most established technique for the analysis of structured NPs and it involves the use of a rectangular capillary channel where one wall is constituted by an ultrafiltration membrane

to allow the passage of a cross-flow [15][16][17][18].The quantitative determination of metallic NPs using AF4 and MALS was already demonstrated for the characterization of gold NPs [19][20], while AF4-UV and AF4-ICP were used to characterize standard Silver nanoparticles [21]. Many applications of AF4 for NPs characterization in nanomedicine were also reviewed [22 and references therein] .

HF5 is the miniaturized variant of the F4 technique and its use in the field of protein analysis have been widely reported [23]; on the other hand, methods for NPs characterization were still unexplored.

In HF5, the separation channel has a cylindrical geometry and consists in a HF membrane with porous walls made of polymeric or ceramic materials and the separation is performed through an external hydrodynamic field (named cross-flow) applied perpendicularly to a mobile phase flow with an ideally laminar (parabolic) flow profile (named longitudinal flow). Sample components are hydrodynamically driven towards one wall (accumulation wall) of the channel and they move away from the wall due to diffusion, which creates a counteracting motion. Smaller particles, which have a higher diffusion coefficient, move closer to the channel center where the longitudinal flow is faster. This results in an earlier elution of smaller particles with respect to larger species. Due to the symmetry of the channel geometry, the driving force of the separation in HF5 is represented by a radial flow (hence cross-flow) applied perpendicularly to the migration flow (axial/longitudinal flow) with a cross flow density higher than that of AF4, leading to an increase in separation efficiency. Down-scaling of the separation channel has proven to have important intrinsic features that lead to great potential in the bioanalytical field: the sample dilution is reduced because of the low channel volume, and as a consequence sensitivity can be increased [24][25][26] and sample fractions can be easily collected for further analysis; in addition, diluted samples can be injected and re-concentrated in shorter time [27] [28].Moreover, disposable usage of the separating channel eliminates sample carry-over or sample

contamination issues. Wyatt Technology Europe has recently commercialized Eclipse® DUALTEC, instrumentation able to operate both with the HF5 and F4 with the same system [29].

In this paper we describe a novel approach that combines HF5 with MALS for the size analysis of AgNPs dispersed in water. Due to the increased use of silver nanoparticles, analysis of potential residues and metabolites of these new pharmaceuticals in environmental, food and clinical materials represents a challenging task. Since the nanorisk is correlated to the nanoparticles dimension, shape and Ag+/Ag0 ratio, a method based on HF5-MALS able to determine the shape of the dispersed AgNPs in aqueous media and also to separate molar ion fraction to silver nanoparticles, was developed.

2. MATERIAL AND METHODS

2.1 AgNPs sample

Aqueous colloidal nanosuspension (nanosol) of silver-polyvynilpyrrolidone nanoparticles (AgNPs 4 % wt) was provided by Colorobbia SpA (Italy).

2.2 Standards

Polystyrene nanoparticles (PS) of 50 nm and 102 nm diameter (Nanosphere Size Standards, Duke Scientific Corp.) were used as standards for the conformational analysis; since they are spherical and their structure can be assimilated to that of a random coil, a radius of gyration (r_g)/hydrodynamic radius (r_h) ratio of 0.77 is estimated. Thus the standards' calculated r_g are respectively 20 nm and 43 nm.

2.3 AgNPs Ultrafiltration

Ultrafiltration was carried out using Solvent-resistant Stirred Cell (Merck Millipore) with polymeric membrane with a pore size of 100 kDa, which was kept in slight overpressure (about 3 bar). The ultrafiltration system was able to retain AgNPs, while the solvent of nanosol containing synthesis by-products and cationic silver (Ag^+) was removed. The

vessel refilled with deionized water was treated for four times until the total removal of free cationic Ag^+.

2.4 HF5-MALS instrumental setup

HF5 was performed by using an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA, USA) consisting in a degasser, an isocratic pump, an autosampler and a variable wavelength UV detector, combined with an Eclipse® DUALTEC separation system (Wyatt Technology Europe, Dernbach, Germany).

The HF5 channel (Wyatt Technology Europe) consisted of two sets of ferrules, gaskets and cap nuts used to seal a polymeric hollow fiber inside a plastic cartridge. The scheme of the HF5 cartridge, its assembly and the modes of operation of the Eclipse® DUALTEC system have already been described elsewhere [24]. The hollow fiber was a polyether-sulfone (PES) fiber, type FUS 0181 available from Microdyn-Nadir (Wiesbaden, Germany) with the following characteristics: 0.8 mm ID, 1.3 mm OD, and 10 kDa M_w cut-off, corresponding to an average pore diameter of 5 nm. The HF5 channels used for the experimental were a standard cartridge containing a 17 cm long fiber.

The ChemStation version B.04.02 (Agilent Technologies) data system for Agilent instrumentation was used to set and control the instrumentation and for the computation of various separation parameters. The software package Wyatt Eclipse @ ChemStation version 3.5.02 (Wyatt Technology Europe) was used to set and control the flow rate values and to move the focus position during the sample focus/concentration.

A 18-angle multiangle light scattering detector model DAWN HELEOS (Wyatt Technology Corporation, Santa Barbara, CA, USA) operating at a wavelength of 658 nm, was used to measure the radius of particles in solution. An Optilab rEX differential refractive index (dRI) detector (Wyatt Technology Corporation) operating at a wavelength of 658 nm was used on occasion as a concentration detector, when the capabilities of the UV detector were overcome by the complexity of the sample. ASTRA® software version 5.3.2.14 (Wyatt

Technology Corporation) was used to handle signals from the detectors (MALS, dRI and UV) and to compute the protein M_w and concentration values.

2.5 HF5 methods

An HF5 method is composed of few steps: focus, focus-injection, elution and elution-injection. During focus the mobile phase is split into two different streams entering from the fiber's inlet and outlet; during focus-injection, the flow settings are the same described in the focus step while the sample is introduced into the channel through the inlet and focalized in a narrow region. Then, in the elution step, the flow of mobile phase enters the channel inlet and part of it comes out transversely (cross-flow); lastly, during elution–injection, no cross-flow is applied (the flow is not split anymore), allowing for any remaining sample inside the channel to be released; also, the flow is redirected in the injection line as well to clean it before the next injection.

The flow conditions for the different HF5 analysis are shown in Table 1. Longitudinal flow is indicated as Vc, while cross/focus flow as Vx. In flow-injection analyses (FIA) neither focus nor cross-flow are applied, thus allowing all injected analytes to exit from the channel without retention.

A volume of AgNPs of 4µL was injected.

Table 1. Flow conditions for F4 analyses

Steps → ↓Method	Focus (mL/min)	Focus-injection (mL/min)	Elution (mL/min)	Elution-injection (mL/min)
HF5	Vc=0.35 Vx=0.85 Time=2 min	Vc=0.35 Vx=0.85 Time=3 min	Vc=0.35 Vx=0.1 Time=12 min	Vc=0.35 Vx=0 Time=3 min
FIA	-	-	-	Vc=0.5 Vx=0 Time=3 min

cationic Ag collection	-	Vx=1 Vc=0 Time=12 min	-	-

2.6 AgNPs size characterization

2.6.1 DLS analysis

AgNPs size distribution was determined at room temperature by Zetasizer Nanoseries (Malvern Instruments, UK) providing the hydrodynamic diameter of suspended particles by the DLS technique. The hydrodynamic diameter was expressed as D50, i.e. the median diameter at 50% in the cumulative distribution. DLS analysis also provides a polidispersity Index parameter (PDI), ranging from 0 to 1, quantifying the colloidal dispersion degreePDI values smaller than 0,05 are typical of highly monodispersed standards, while values greater than 0,7 depict a broad particle size distribution that makes samples unsuitable for DLS analysis. A mid-range PDI value between 0,05 and 0,7 usually ensures a proper operating condition of the instrument. As for DLS analysis, AgNPs were dispersed in deionized water at 0.13 mg/ml and homogenized on a vortex mixer for 30 seconds; the resulting dispersion pH was 4,5. A small sample volume (~1 mL) was subjected to three consecutive measurements performed at 25° C and particle size distribution by intensity was obtained by averaging these measurements.

2.6.2 TEM morphological investigation

The observation of morphology was made using a transmission electron microscope (TEM) in JEOL JEM-2100F multipurpose, high resolution, electron microscope with a field emission source operating between 80 and 200 kV for various level of magnifications. The nanoparticles were taken directly from the ultrafiltered nanosol and placed on TEM grids. The samples were then left to dry before loading in the TEM. Particle size and distribution were determined using image processing software on micrographs taken at around 200 kV emission field on multiple locations within the sample.

2.7 Cationic Ag determination in NPs samples

2.7.1 FAAS analysis

An AAnalyst400 (Perkin Elmer, Massachusetts, USA) flame atomic absorption spectrometer (FAAS) equipped with a silver hollow-cathode-lamp, operating at 328.0 nm, was used for quantitative analyses of solutions collected from the HF5 cross-flow line. The instrumental parameters (10 mA operating current, 2.7 nm bandwidth) were adjusted according the manufacturer's recommendations. Air (10 ml/min)-C2H2 (2.5 ml/min) flame was employed. Ultrapure MilliQ water and nitric acid 0.5M (HNO3 for trace analysis, ≥69%, Fluka) were used to dilute samples or standards in all the experiments.

Silver standard solutions, ranging from 0.2 mg/L to 5.0 mg/L, for FAAS calibration curve were obtained properly diluting a certificate solution of Ag (1002 ±2) mg/L (Merck, Germany). The calibration curve, achieved under the best instrumental conditions, shows a good linear correlation ($R^2 = 0.9972$). The equation $Y = 0.0211 (\pm 0.0003) X + 0.0040 (\pm 0.0009)$ was obtained when repeating the calibration 14 times.

2.7.2 HF5 filtration of AgNPs – proof of concept

The Inductively Coupled Plasma (ICP) analysis of aqueous colloidal nanosuspension of the AgNPs characterized in this work estimates the total silver amount in 4% w/v. A part of this silver amount is present in its ionic form and during the focus-inject step of HF5 analysis it passes through hollow fiber pores and can be collected and quantified. Therefore as a proof of concept, solutions having cationic Ag concentrations of the same order of that presumably contained into AgNPs' nanosol were first injected into the hollow fiber channel. The flows were selected in order to collect ionic silver from cross-flow line during the focus-inject step according to Table 1. During HF5 analysis the sample is diluted almost 1:10, hence we suppose that the nanosol injected could be in the order of 4000 ppm in Ag. In order to determine the best volume to collect from cross-flow line, 10 microL of a solution of AgNO3 (1904 ppm) were injected into HF5 system. This solution is prepared diluting 1:2 a stock solution of 3908 ppm, obtained dissolving 59.94 mg (± 0.01)

mg of $AgNO_3$ in 10 ml of HNO3 0.5M. Five 3 ml aliquots were collected from the cross flow line and analyzed by FAAS. For each of these aliquots, the concentration of silver was obtained by interpolating the absorbance signal on the calibration curve.

2.7.3 XRF

From the ultrafiltered sample the concentration of cationic Ag present in water filtrates was estimated by XRF (WDS - wavelength dispersive X-ray spectrometer) using a Panalytical Axios Advanced (Netherlands). The XRF results showed that the ultrafiltration process allowed removal of 50% of Ag (compared to the initial total amount of Ag), until reaching a plateau, corresponding to the amount of cationic Ag at equilibrium with Ag^0 solid phase. The results showed that about 50% of Ag nanosol consists of nanoparticles and the remaining 50% is made of cationic Ag.

3. RESULTS AND DISCUSSION

First we developed methods for the size-fractionation of AgNPs which are robust and reproducible, and able to detect AgNPs in aqueous media with a satisfactory sensitivity in particular with HF5.

3.1 HF5- MALS of AgNPs

Sample was separated in HF5 using water as mobile phase in order to analyze samples in their native formulation and to avoid potential modification due to fractionation conditions. The HF5-MALS analysis of AgNPs obtained with flow conditions described in section 2.5 is reported in Figure 1. The fractionation shows no void peak, expected at min 5, for the unretained species (such as unreacted reagents for sample preparation or small species dimensionally comparable to channel membrane cut-off), and a retained peak a tR=8 min typical for the nanostructure. An rms value of 45 nm was evaluated for the NPs, with an hydrodynamic radius of about 23 nm, indicating an r_g /r_h ratio of 1.7 that is typical for rod conformation; as deeper discussed in the next paragraph

It's also possible to observe that when the flow field ends (tR=17 min) in the elution-injection step only a small LS signal is evident so all eluted samples are separated during the HF5 analysis. The presence of a small light scattering signal and a non significant UV signal (data not shown) indicate that no NPs species are released at the end of the fractionation method. As for sample polydispersity, in a homogeneous (i.e., monodisperse) sample its average radius is independent from the averaging method. Then, the ratio between values obtained with different methods will be equal 1(i.e. polydispersity will equal 1). If otherwise the sample contains a mixture of species of different gyration radii (i.e., polydisperse sample) the average radius will depend on the averaging method and the polydispersity will be different from 1. In this case, the calculated polydispersity resulted to be 1.002, indicating that the nanoparticles are highly monodispersed.

HF5 shows good fractionation/characterization results for NPs using water as mobile phase, having both a high reproducibility and a limited dilution of the sample; this allows for the determination of AgNPs at low concentration and for the direct collection of released metal and its quantification. For this purpose, in order to verify that cationic silver totally exits from the channel membrane, a FIA analysis and an analysis with applied cross-flow line were performed using the methods reported in Table 1. No UV signal at 205 nm was recorded when the field is applied, confirming that in the fractionation analysis cationic silver is filtered through the membrane pores during the focus-injection step (data not shown).

3.2 Morphological analysis of AgNPs

From the HF5-MALS analysis of AgNPs an accurate conformational analysis of samples was performed.

In **HF5**, separation is performed between species presenting different diffusion coefficients. Being the diffusion coefficient of a particle directly linked to its hydrodynamic radius r_h, a first dimensional information is obtained. **MALS** detection, on the other hand,

allows for the calculation of particles' average mean square radius r_g, which depends on particle shape and compactness. By correlating r_g and r_h it is possible to determine particles shape; more in detail, a r_g/r_h ratio of 1.7 is typical for rod structures, while a ratio of 0.77-0.8 is typical for random coils as PS standards are. Standard PS particles were separated under the same flow conditions and mobile phase (water) in order to confirm the RMS radius values obtained by HF5-MALS and have a direct comparison for hydrodynamic radius.

In Figure 2 the HF5-MALS analysis of PS standards and AgNPs is reported. In the same Figure the r_g values (determined from the MALS analysis) and r_h values (determined from the HF5 analysis) are also reported. At 7 min AgNPs particles are eluted and an r_g of 45 nm was evaluated; while at 7.8 min the 50 nm PS standard is eluted (r_h =25.5nm, r_g =20nm) and at 12 min the 102 nm PS standard is eluted (r_h =51nm, r_g =46nm).

A ratio of r_g / r_h = 1.7 was calculated suggesting a chain shape. The HF5-MALS morphological analysis suggests the presence of small aggregates of Ag nanoparticles in a chain arrangement, as confirmed also by TEM observation discussed in the subsequent section 3.3.

A chain shape is not very common among these materials, although some synthesis methods to form Ag nanowires in solution-phase and PVP presence have been presented [30], but this could also be related to the lack of descriptivity obtainable with DLS, which factors in the hydrodynamic radius alone, and TEM, where the analysis can be biased by sample handling. In fact, being this morphology related to an aggregation state, a soft technique like field-flow fractionation can show the real appearance of the sample since there are no stabilizers (like surfactants or additives which constituted the formulate) and stressful steps are avoided. A tendency to form chain-like aggregates is however noticeable in TEM analyses, although it does not concern all the particles, as discussed in paragraph 3.3.

3.3 Size characterization

Figures 3a and 3b the particle size distribution by Intensity and by Volume of AgNPs after ultrafiltration obtained from DLS analysis are shown. From cumulants analysis, the mean hydrodynamic diameter (or Z-average), r_h of AgNPs has been measured to be around 100 nm with a PDI of 0,24. However for samples characterized by a multimodal size distribution, the Intensity particle size distribution should be considered for the assignment of the size of each peak. AgNPs sample shows a bimodal size distribution with peaks centered on 140 nm (Peak1, %PD=43) and 20 nm diameter (Peak2, %PD=14). The intensity size distribution are really sensitive to the presence of aggregates and large particles, because scattered light intensity is proportional to the sixth power of their diameter, thus to estimate the relative amount of each peak in the distribution, the Volume particle size distribution has been considered. From this latter, the relative volume of the two populations at 140 and 20 nm resulted almost the same, being respectively 49% and 51%.

Figure 4 shows the typical morphology and the distribution of AgNPs obtained from transmission electron microscopy. The sample is polydisperse and the particle size histogram follows a skewed Gaussian distribution with a long tail towards larger particle size than the average particle size lying around 15-20 nm. Interestingly, the morphology shows that while larger size aggregates (>40 nm, but on an average ~100 nm) are more or less isolated the smaller size particles (<40 nm, but on an average ~15-20 nm) have a tendency to be linked to the extent of forming a chain shape aggregate. The latter observation agrees with data obtained from HF5-MALS method. The inherent nature of the differences in sample state and preparation technique must be taken into account when data from these entirely different techniques are compared especially for TEM for which samples had to be sufficiently dry to allow this high vacuum microscopy technique, pressure typically better than 10^{-6} Torr, to work.

3.4 Ag release

After the development of an HF5-MALS method for the characterization of AgNPs, its potential as an analytical step useful to the study of biological impact of NPs through the quantification of released metal in the environment was explored.

Purification from reagents of the AgNPs synthesis via membrane filtration during the focus-injection step of the analysis, and determination of silver release from AgNPs were then performed. A schematic view of the proposed method able to size separate NPs and isolate cationic Ag fraction as described in section 2.7.2 is reported in Figure 5.

Some experiments were performed in HF5 system in order to define operative conditions to quantify, with a good recovery, the ionic silver contained into a sample of nanoparticles synthesized by an industrial process. As described in section 2.7 the standard solution of $AgNO_3$ (1904 ppm) was analyzed with the HF5 method (cationic Ag collection) reported in Table 1. Five aliquots of 3 ml were collected from cross-flow line and analyzed by FAAS. For each one, silver concentration was obtained by interpolation of absorbance signal on the calibration curve. FAAS measurements indicated that a volume of 12 mL must be collected since silver content of the latter fractions was under the limit of detection (data not shown). The results showed the recovery of cationic silver was higher than 90% confirming that these conditions allow maximizing recovery of cationic silver collected from the cross-flow line through HF5 membrane.

These experimental conditions were applied to dose cationic silver in a sample of AgNPs diluted 1:10 from batch. FAAS measurements indicated that the ionic silver amount in the sample is about 50% of the total. This value is consistent with the cationic Ag concentration determined, by XRF analysis, in samples obtained after ultrafiltration process, as reported in section 2.7.3.

Such result confirms the capability of HF5 technique in one-step process to separate ionic phase from solid one, allowing for a better correlation between physicochemical properties

and biological reactivity. A more sound comprehension of nanospecific biological reactivity in fact will support mechanistic studies and allow the control of nanophase reactivity by playing with surface engineering (safety by design approach).

4. CONCLUSIONS

On-line coupling of /HF5 with MALS appears to be an ideal, hyphenated methodology for the simultaneous size-separation and characterization of AgNPs samples because they provide independent size information. The r_h values determined by AF4/HF5 can be correlated to the r_g values determined by MALS and information on particle shape and morphology can be obtained. All the analysis can be performed in aqueous media providing fundamental information regarding the actual state of aggregation, size and shape of nanoparticles in physiological media. This leads to more realistic assessment of the risk posed by AgNPs to health, safety and the environment. In addition, the ability to separate the Ag^+ ions from AgNPs during the size analysis can be advantageous in providing further quantification of its potential risk, which largely originate from the release of Ag^+ ions. Further studies will be conducted to create a suitable protocol for analysis of metal release through fiber filtration. Overall, the combinatorial approach described in this article may significantly improve the characterization of metal-based nanoparticles in order to study both their functional effectiveness and potential hazards.

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6. COMPETING INTEREST

Andrea Zattoni, Barbara Roda and Pierluigi Reschiglian are associates of the academic spinoff company byFlow Srl (Bologna, Italy). The company mission includes know-how transfer, development, and application of novel technologies and methodologies for the analysis and characterization of samples of nano-biotechnological interest.

435

436 FIGURE CAPTIONS

437 Figure 1: HF5-MALS analyses of AgPVP nanoparticles in water. Light scattering signal at
438 90° and r_g values determined with MALS detector are reported.

439 Figure 2: HF5-MALS of AgPVP nanoparticles and PS. Light scattering signal at 90° and r_g
440 values determined with MALS detector are reported for AgNPs (gray lines) and PS
441 standards (brown lines).

442 Figure 3: DLS Particle size distribution by Intensity (a) and by Volume (b) for ultrafiltered
443 AgPVP nanoparticlesdispersed in water at 0,13 mg/ml (pH = 4,5).

444 Figure 4: TEM micrograph (over) and a histogram of the mean diameter of sample AgNPs
445 after ultrafiltration (below).

446 Figure 5: Schematic view of an on-line, one-step Ag+ filtration and particles purification
447 with HF5: (a) Cationic Ag filtration during focus-injection and NPs relaxation, (b) AgNPs
448 size-separation and fractions collection.

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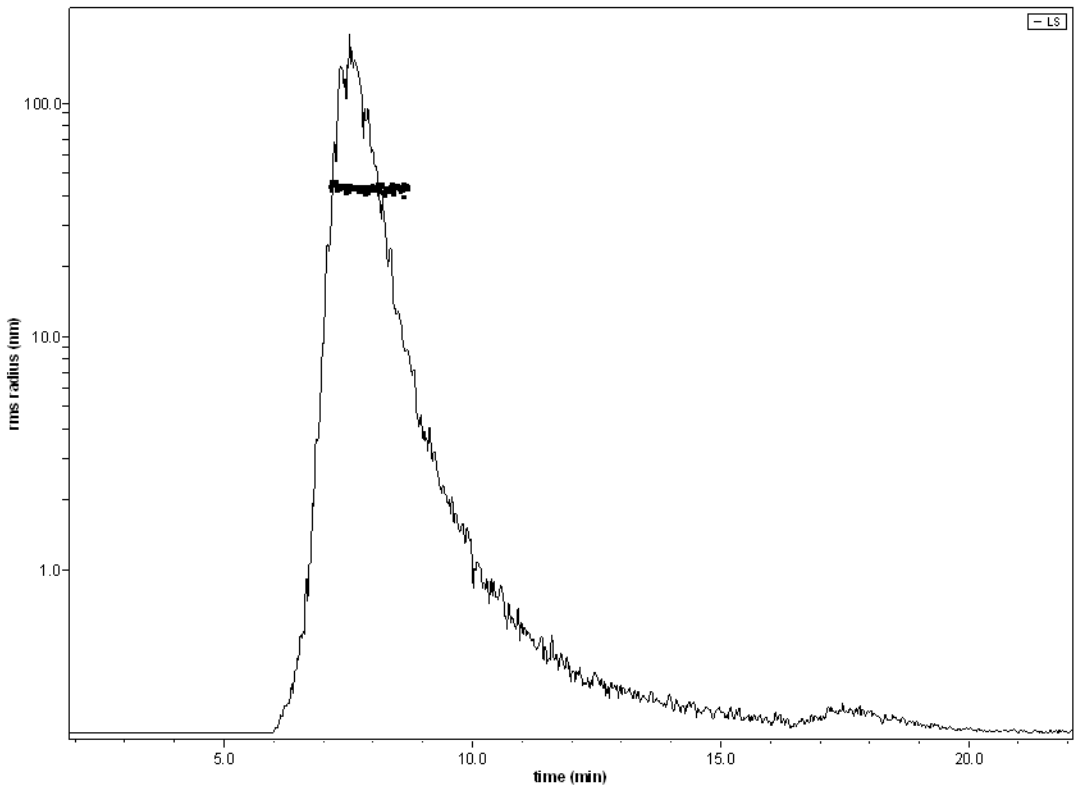
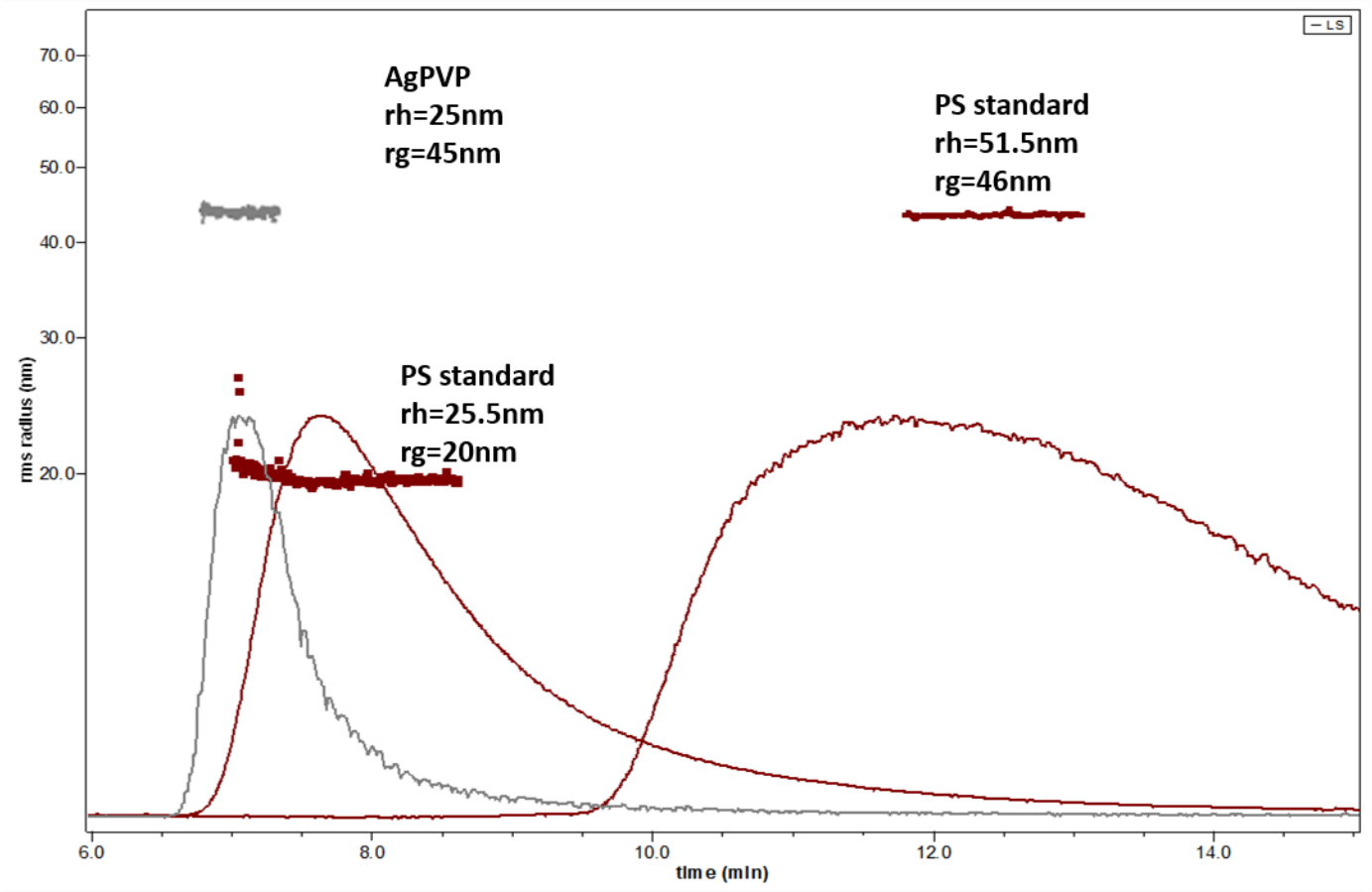


Figure 1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rg=45nm
PS standard
rh=51.5nm
rg=46nm
PS standard
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rg=20nm
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70.0
60.0
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30.0
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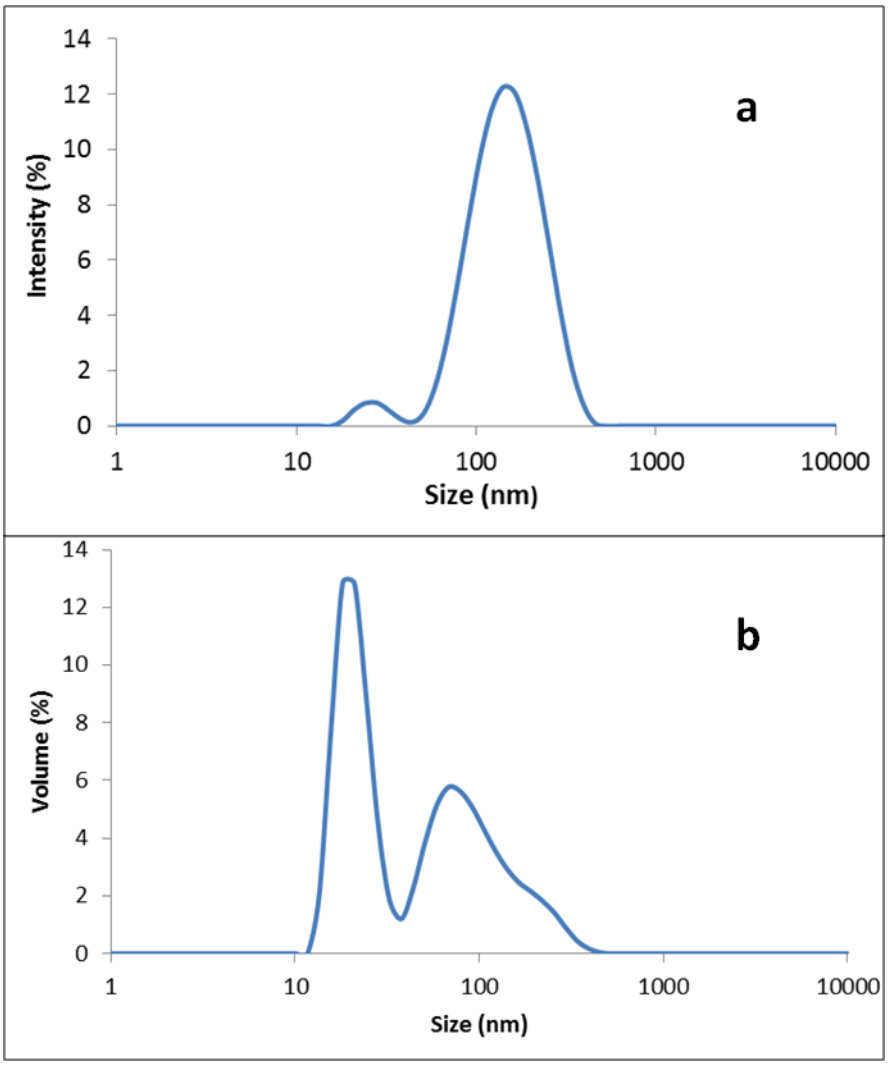
8.0
10.0
12.0
14.0
time (min)

Figure 2


a
Intensity (%)
14
12
10
8
6
4
2
0
1
10
100
1000
10000
Size (nm)
b
Volume (%)
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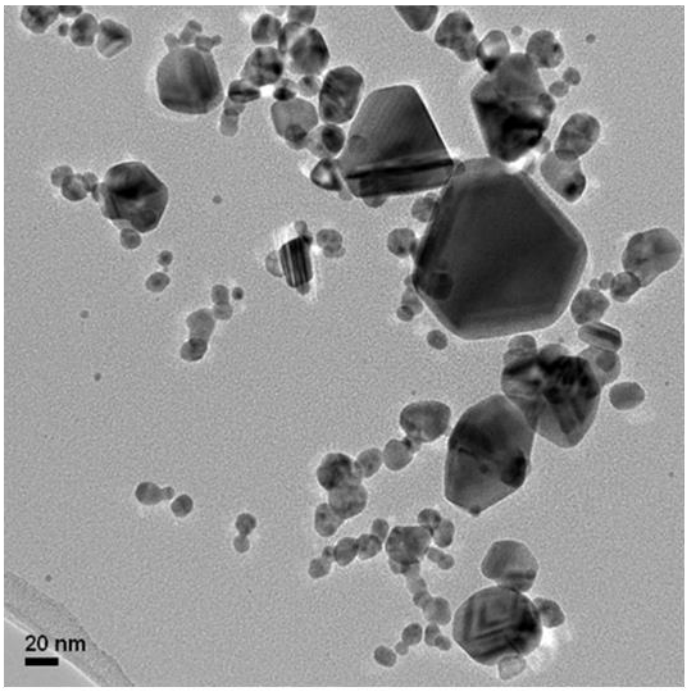
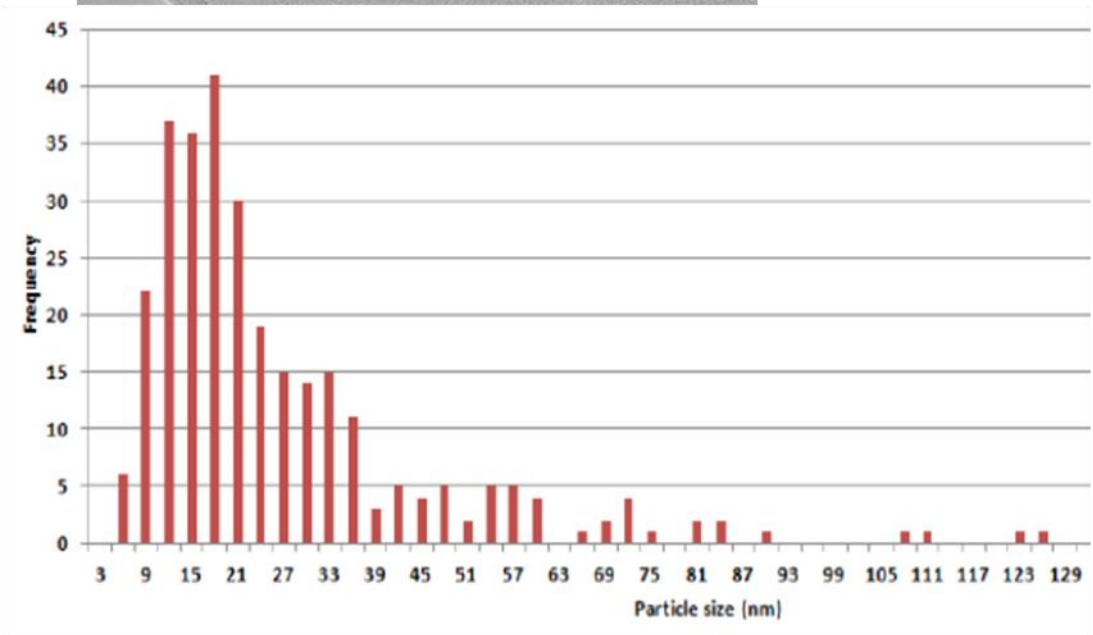
12
10
8
6
4
2
0
1
10
100
1000
10000
Size (nm)

Figure 3


20 nm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35
40
45
3
9
15
21
27
33
39
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51
57
63
69
75
81
87
93
99
105
111
117
123
129
Particle size (nm)

Figure 4

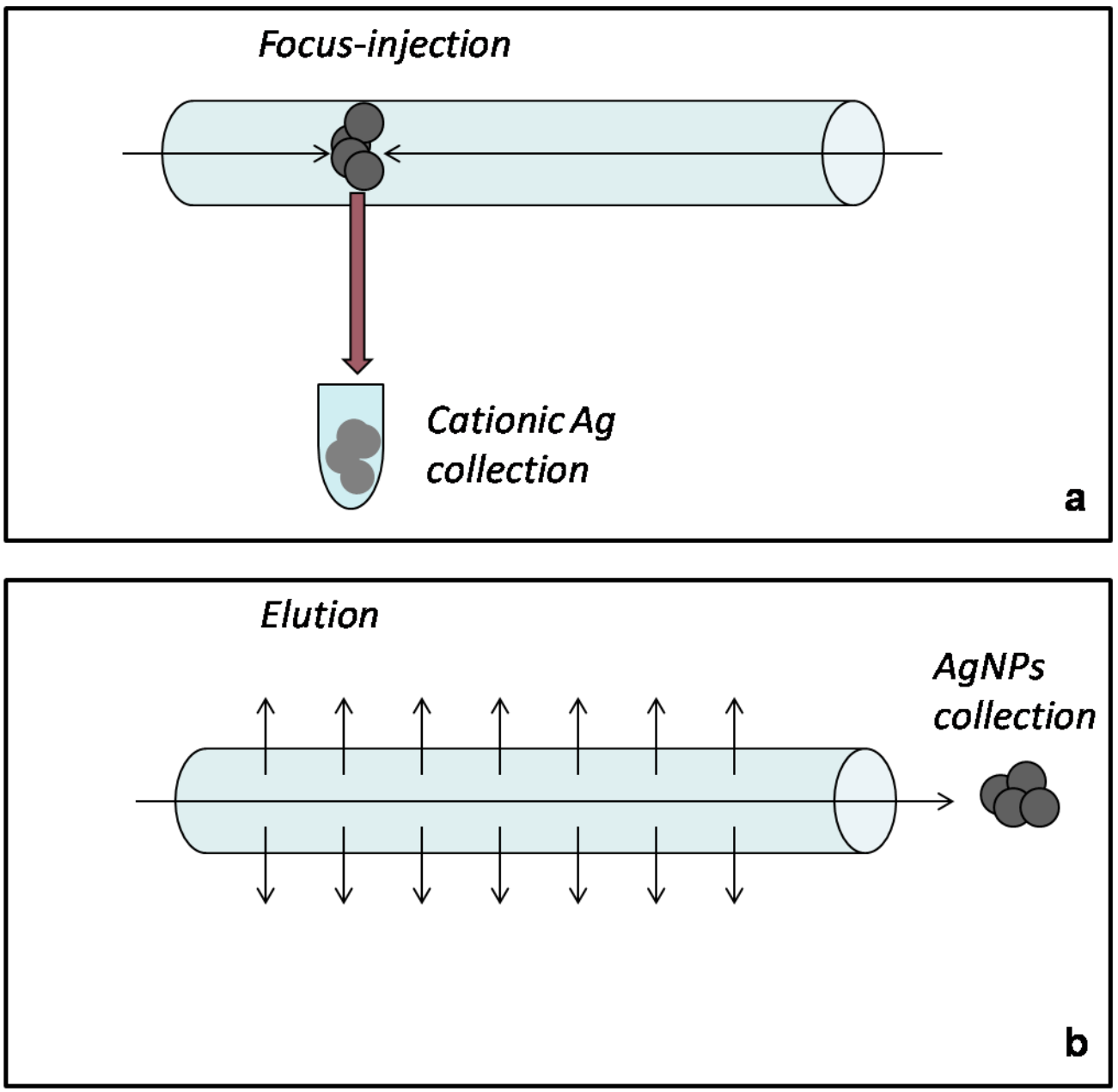
Focus-injection
Cationic Ag
collection
a
Elution
AgNPs
collection
b

Figure 5