Asymmetrical Flow Field-Flow Fractionation for Sizing of Gold Nanoparticles in Suspension

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Abstract

Particle size is arguably the most important physico-chemical parameter associated with the notion of a nanoparticle. Precise knowledge of the size and size distribution of nanoparticles is of utmost importance for various applications. The size range is also important, as it defines the most "active" component of a nanoparticle dose.

Asymmetrical Flow Field-Flow Fractionation (AF4) is a powerful technique for sizing of particles in suspension in the size range of approximately 1–1000 nm. There are several ways to derive size information from an AF4 experiment. Besides coupling AF4 online with size-sensitive detectors based on the principles of Multi-Angle Light Scattering or Dynamic Light Scattering, there is also the possibility to correlate the size of a sample with its retention time using a well-established theoretical approach (FFF theory) or by comparing it with the retention times of well-defined particle size standards (external size calibration).

We here describe the development and in-house validation of a standard operating procedure (SOP) for sizing of an unknown gold nanoparticle sample by AF4 coupled with UV-vis detection using external size calibration with gold nanoparticle standards in the size range of 20–100 nm. This procedure provides a detailed description of the developed workflow including sample preparation, AF4 instrument setup and qualification, AF4 method development and fractionation of the unknown gold nanoparticle sample, as well as the correlation of the obtained results with the established external size calibration. The SOP described here was eventually successfully validated in the frame of an interlaboratory comparison study highlighting the excellent robustness and reliability of AF4 for sizing of nanoparticulate samples in suspension.

Introduction

Gold nanoparticles (AuNP) in the form of colloidal gold had been a part of human culture long before there was an understanding of what nanoparticles were and before the term nanoparticle had found its way into contemporary, scientific vocabulary. Without distinct knowledge of their nanoscale appearance, suspended AuNP had already been used for medical and other purposes in ancient China, Arabia, and India in the V–VI centuries BC¹, and also the ancient Romans took advantage of their ruby red color to famously stain their pottery in the Lycurgus Cup exhibit in the British Museum². In the western world, throughout the centuries from the Middle Ages to the Modern Era, suspended AuNP were predominantly used as coloring agents for glass and enamel (Purple of Cassius)³ as well as to treat a variety of diseases (Potable Gold), especially syphilis⁴.

However, all these studies had primarily focused on the application of suspended AuNP and it was up to Michael Faraday in 1857 to introduce the first rational approach to investigate their formation, their nature as well as their properties⁵. Although Faraday was already aware that these AuNP must have very minute dimensions, it was not until the development of electron microscopy when explicit information about their size distribution was accessible^{6,7}, eventually enabling the correlation between size and other AuNP properties.

Nowadays, thanks to their fairly easy and straightforward synthesis, remarkable optical properties (surface plasmon resonance), good chemical stability and thus minor toxicity as well as their high versatility in terms of available sizes and surface modifications, AuNP have found widespread applications in fields such as nanoelectronics⁸, diagnostics⁹, cancer therapy¹⁰, or drug delivery¹¹. Obviously, for these

applications, precise knowledge of the size and size distribution of the applied AuNP is a fundamental prerequisite to ensure optimum efficacy¹² and there is a substantial demand for robust and reliable tools to determine this crucial physico-chemical parameter. Today, there is a plethora of analytical techniques capable of sizing AuNP in suspension including, for example, UV-vis Spectroscopy (UV-vis)¹³, Dynamic Light Scattering (DLS)¹⁴ or Single Particle Inductively-Coupled Plasma Mass Spectrometry (spICP-MS)¹⁵ with Field-Flow Fractionation (FFF) being a key player in this field^{16, 17, 18, 19, 20}.

First conceptualized in 1966 by J. Calvin Giddings²¹, FFF comprises a family of elution-based fractionation techniques, where separation takes place within a thin, ribbon-like channel without a stationary phase^{22,23}. In FFF, separation is induced by the interaction of a sample with an external force field that acts perpendicular to the direction of a laminar channel flow, in which the sample is transported downstream usually toward respective in-line detectors. Among these related FFF-techniques. Asymmetrical Flow Field-Flow Fractionation (AF4), where a second flow (cross flow) acts as the force field, has become the most widely-used subtype²⁴. In AF4, the channel bottom (accumulation wall) is equipped with a semipermeable ultrafiltration membrane that is able to retain the sample while simultaneously allowing the cross flow to pass through the membrane and leave the channel via an extra outlet. By this means, the cross flow can push the sample towards the accumulation wall thereby counteracting its diffusion-induced flux (Brownian motion). In a resulting equilibrium of field- and diffusion-induced fluxes; smaller sample constituents exhibiting higher diffusion coefficients align closer to the channel center while larger sample constituents exhibiting lower diffusion coefficients locate closer to the accumulation wall. Due to the parabolic

flow profile inside the channel, smaller sample constituents are therefore transported in the faster laminae of the channel flow and elute before larger sample constituents. Using FFF retention parameter and Stokes-Einstein diffusion coefficient equations, the elution time and, respectively elution volume, of a sample in AF4 can then be directly translated into its hydrodynamic size²². Here the described elution behavior refers to the normal elution mode and is usually valid for AF4 within a particle size range between approximately 1– 500 nm (sometimes up to 2000 nm depending on particle properties and fractionation parameters) whereas steric-hyperlayer elution usually occurs above this size threshold²⁵.

There are three common ways to derive size information after separation by FFF. Since FFF is a modular instrument, it can be combined downstream with multiple detectors such as size-sensitive light scattering detectors based on the principle of Multi-Angle Light Scattering (MALS)^{26,27}, Dynamic Light Scattering (DLS)^{28,29}, or even a combination of both to gain additional shape information^{30,31}. However, since the retention behavior of a sample in an FFF-channel is generally governed by well-defined physical forces, size can also be calculated using a mathematical approach (FFF theory), where a simple concentration detector (e.g., a UVvis detector) is sufficient to indicate the presence of an eluting sample^{32,33}.

As a third option, we here report the application of an external size calibration^{34, 35} using well-defined AuNP standards in the size range of 20–100 nm for sizing of an unknown gold nanoparticle sample in suspension using AF4 coupled with UV-vis detection. This simple experimental setup was chosen on purpose to allow as many laboratories as possible to join an international interlaboratory comparison (ILC), which was

later performed in the frame of the European Union Horizon 2020 project ACEnano based on the protocol presented here.

Protocol

1. AF4 system setup

- Assemble the AF4 cartridge and connect all hardware components of the AF4 system and the UV-vis detector (Table of Materials) following the instructions given in the manufacturer's manual.
- Install all necessary software packages for control, data acquisition, processing and evaluation following the instructions given in the manufacturer's manual.
- Ensure that all necessary signal connections between the AF4 system and the UV-vis detector have been established.
- 4. Ensure that the established AF4-UV-vis connections are tight and without leakages by flushing the setup with ultrapure water (UPW) for 15 min (tip flow rate 1 mL·min⁻¹, focus flow rate 1 mL·min⁻¹, and cross flow rate 1.5 mL·min⁻¹). To do so, open the AF4 control software and enter the flow rates into the respective panels on the right upper side of the landing page. Tighten the respective connectors (fittings), if necessary, and repeat the procedure until no leakages are observable.

NOTE: The internal system pressure during all measurements should be monitored and must be within 4 to 12 bar. In case the pressure is higher or lower, the backpressure tubing needs to be adjusted. Furthermore, the channel pressure trend should be constant over the complete measurement time.

NOTE: If a channel oven is available, set its temperature to 25 °C to ensure comparable measurement conditions throughout all AF4 experiments.

2. Preparation of solutions and suspensions for AF4-UV-vis system qualification and sample analysis

- 1. Cleaning solution
 - Add 8 g of solid sodium hydroxide (NaOH) and 2 g of sodium dodecyl sulfate (SDS) to 1 L of UPW and stir the solution until total dissolution.
- 2. Eluent
 - Add 500 μL of filtered surfactant mixture to 2 L of filtered and degassed UPW to obtain the eluent (0.025% (v/v), pH around 9.4).

NOTE: A detailed description of the compounds of the surfactant mixture is given in **Table 1** (also **Table of Materials**).

- 3. Arbitrary AuNP size standard for mass recovery determination
 - Vortex an arbitrary AuNP size standard (50 mg·L⁻¹) for 2 min and dilute it 1:4 with UPW to obtain a final mass concentration of 12.5 mg·L⁻¹. Vortex for additional 2 min after dilution to homogenize the obtained suspension.

CAUTION: Necessary precautionary measures and suitable protective equipment are required when working with chemicals, especially NaOH pellets.

NOTE: It is generally recommended to de-gas and filter all necessary solutions (except for the cleaning solution) using a 0.1 µm membrane filter (hydrophilic PVDF or similar) to ensure low particle backgrounds during AF4-UV-vis-experiments. This can be established by either a dedicated vacuum filtration unit or by using syringe filters.

3. AF4-UV-vis system qualification

- Use the software settings described in step 1.4 to flush the system with the cleaning solution for 30 min (Tip flow rate 1 mL·min⁻¹, Focus flow rate 1 mL·min⁻¹, and Cross flow rate 1.5 mL·min⁻¹).
- Change the respective eluent bottle and flush the system with UPW for 20 min (Tip flow rate 1 mL·min⁻¹, Focus flow rate 1 mL·min⁻¹, and Cross flow rate 1.5 mL·min⁻¹).
- 3. Replace the respective inline pump filters.
- Open the AF4 cartridge and replace the AF4 membrane. Reassemble the AF4 cartridge and reconnect it with the AF4-UV-vis system.
- Flush the cleaned AF4-UV-vis system with the eluent for at least 30 min in order to equilibrate the membrane and stabilize the system (Tip flow rate 1 mL·min⁻¹, Focus flow rate 1 mL·min⁻¹, and Cross flow rate 1.5 mL·min⁻¹). Check for potential leakages again (see step 1.4).
- Qualify the AF4-UV-vis system by determining the mass recovery and variation of retention time using an arbitrary AuNP size standard.
 - Perform a direct injection run without application of a separation force.
 - Create a new measurement file by opening File | New | Run in the AF4 control software.
 - Define the sample and measurement description as well as injection volume and sample name within the Run tab. The measurement conditions are displayed in Table 2.

- Set the measurement parameters in the second tab FFF method according to Table 2.
- 4. Click on the **Run** button to start the measurement.
- Perform a fractionation run with application of a separation force (Cross flow).
 - Define the fractionation method as described in the previous section using the fractionation conditions specified in **Table 3**.
 - 2. Click on the **Run** button to start the measurement.
 - 3. Perform the measurement in quadruplicate.

NOTE: The first run aims at conditioning the system (i.e., the AF4 membrane) and will be excluded from the final evaluation of the system qualification results.

NOTE: It is recommended to save all generated run files by opening **File** | **Save** in the AF4 control software.

- Consider the AF4-UV-vis-system qualified if a mass recovery of >80% and a variation of retention time <2% is obtained for the arbitrary AuNP size standard.
- 5. When using an autosampler as the injection system, fill the autosampler's needle washing reservoir bottle with the same solution that is pumped through the AF4-UV-vis system (e.g., cleaning solution, UPW, or respective eluent) to ensure optimum run conditions. When changing the eluent, it is generally recommended to follow the re-equilibration of the AF4-system by monitoring the UV-vis-detector signal until its baseline remains stable on a constant level.

4. AF4-UV-vis sample analysis

- Prepare all AuNP size standards for external size calibration by vortexing the respective AuNP suspension (20 nm, 40 nm, 80 nm, 100 nm, each 50 mg.L⁻¹) for 2 min and dilute it 1:4 with UPW to obtain a final mass concentration of 12.5 mg·L⁻¹. Vortex for additional 2 min after dilution to homogenize the obtained suspensions.
- Prepare the unknown AuNP sample for analysis applying the same procedure as for the calibration standards described in step 4.1.
- Perform a direct injection measurement of all AuNP size standards using the AF4 method displayed in Table 2.
 - To do so, enter the respective values summarized in **Table 2** into the manufacturer's software at the appropriate positions to define the separation and sample parameters and press the **Run** button to start the experiment.
- Fractionate each AuNP size standard individually using the AF4 method displayed in Table 3 to establish the external size calibration function.
 - Enter the respective values summarized in Table 3 into the manufacturer's software at the appropriate positions. The fractionation method is defined by a focusing step, several elution steps, and a rinse step. After setting up the method, press the Run button to start the experiment.
- Perform a direct injection measurement of the unknown AuNP sample using the AF4 method displayed in Table 2.
- Perform the fractionation of the unknown AuNP sample by conducting the AF4 method listed in Table 3.

- Carry out all measurements mentioned in Section 3 and 4 in triplicate unless stated otherwise to ensure meaningful and statistically relevant results.
 - Store 50 mg·L⁻¹ AuNP stock suspensions at 4–8 °C before use. Diluted AuNP suspensions are ideally prepared within 30 min prior to application.

NOTE: Vortexing is usually sufficient and no ultrasonication of the suspensions is necessary.

 In order to enable a correlation of the retention time of the unknown AuNP sample with the retention times obtained for the AuNP size standards, measure all samples using the same AF4 method.

NOTE: To assure constant and valid separation conditions, include/repeat the fractionation step described in the system qualification section (see step 3.6.2) after a defined number of sample measurements (e.g., 10 measurements). In addition, record system pressure and UV-vis detector baseline stability. They should remain stable and constant along a complete AF4-UV-vis run.

NOTE: Usually, replace the ultrafiltration membrane when the UV-vis detector (or Multi Angle Light Scattering (MALS) detector, if available) shows an increased noise level or the defined system qualification criteria such as recovery, sample peak shape, or repeatability are missed (or the AF4-UVvis-system was subjected to a thorough cleaning procedure). Under the conditions described here, the qualified AF4-UV-vis system is usually stable for at least 50 measurements using the same membrane; however, the number of possible consecutive measurements meeting the defined quality criteria can vary significantly depending on sample, sample matrix, and eluent composition.

5. Data evaluation

- Perform the mass recovery calculation using either data evaluation software provided by the AF4-UV-vis system manufacturer or spreadsheet analysis after export of all necessary raw data (i.e., UV-vis peak area) from the respective data acquisition software following the instructions given in the manufacturer's manual.
 - Calculate the AuNP mass recovery by comparing the areas under the respective UV-vis peaks of the fractionation measurement (A_{fractionation}) and the direct injection measurement (A_{direct injection}) using the following equation:

recovery [%] =
$$\frac{A_{fractionation}}{A_{direct injection}} \times 100$$
 [%]

NOTE: During a direct injection measurement, no separation force is applied, and therefore potential interactions of an analyte species with the accumulation wall can be neglected. The area under a respective UV-vis peak can be directly correlated to the AuNP mass using Beer-Lambert law assuming that no other species within the sample absorbs at the respective wavelength and/or i) elutes at another retention time under fractionation conditions ii) is removed through the AF4 membrane.

- 2. Import the dat. files obtained from both the direct injection and the fractionation run.
- 3. Select the UV-vis detector trace in the **Overview** tab.
- Define a Region of Interest (ROI) and a baseline in the signal and baseline view for all measurements.
- 5. Insert a Direct Injection Calibration via Insert.

 Select all direct injection runs in the Direct Injection
 Calibration Settings view and enter a UV extinction coefficient.

NOTE: It is important to use the same UV-vis extinction coefficient for both the calibration and the fractionation measurement.

- 7. Establish the calibration line using the area under the UV-vis signal trace within the ROI and the injected amount calculated from the entered concentration and the injection volume. The obtained calibration will be shown in the separate **Direct Injection Calibration Function** window.
- 8. Assign the calibration function to the respective fractionation measurements.

NOTE: For each calibration size standard and the unknown AuNP sample, a separate calibration function needs to be established due to the size dependent UV-vis absorbance of AuNP. This drawback of the UV-vis detector can be circumvented using a mass-sensitive detector such as an ICP-MS.

- Perform the analyses by inserting a Quantitative Results calculation and the results will be displayed within a table on the right as concentration and injected amount values.
- 2. Calculate the variation of retention time using either data evaluation software provided by the AF4 system manufacturer or spreadsheet analysis after export of all the necessary raw data (i.e., retention times of the AuNP calibration standards at the respective UV-vis peak maxima and respective void times) from the respective data acquisition software following the instructions given in the manufacturer's manual.

- Open the **Overview** window to display the respective UV traces for all imported measurements.
- The peak detection will be performed automatically; adjust the peak detection parameters within the signal processing toolbox to optimize the performance. Extract the respective peak maxima by going through all measurement files.
- Calculate the relative standard deviation for all measurements using the following equation:

$$RSD = \left\{ \frac{\left[\sqrt{\frac{1}{3} \sum (t_R(a, b, c) - Average t_R)^2} \right]}{Average t_R} \right\} x 100\%$$

The calculation can also be performed using a respective spreadsheet software.

- 3. Perform size determination using either data evaluation software provided by the manufacturer or spreadsheet analysis after export of all the necessary raw data (retention time at UV-vis peak maximum of analyte and respective void time) from the respective data acquisition software following the instructions given in the manufacturer's manual. An external size calibration function can be established by plotting the void time corrected retention times (net retention times, see **Table 5**) of the AuNP size standards (20 nm, 40 nm, 80 nm, 100 nm) against their hydrodynamic sizes obtained from previously performed DLS measurements (see **Table 4**). NOTE: The DLS measurements should be conducted ideally on the same day as the respective fractionation measurements to ensure comparable sample properties.
 - After importing the .dat files all measurements are displayed in the **Overview** tab. Select the UVvis detector signal from the detectors list, which is displayed below the overlay window. Define a

ROI and baseline for each measurement, which can be adjusted in the **Signal and Baseline** view. Use the **Signal processing** toolbox on the right to smooth noisy signals. Use the **Assign Processing Parameters to other Runs** function to allow the parameters to be allocated to other measurements, respectively signals.

- 2. Select the **Particle Size Calibration** from the **Insert** tab.
- Select all calibration runs by clicking on the respective measurement in the Select References for Calibration table on the upper right side. All selected measurements will be displayed in a table below. Enter the hydrodynamic radius for all calibration measurements that are specified in Table
 The function will be displayed in the Particle size calibration Function window and the equation will be shown as well.

NOTE: The correlation coefficient (R^2) of the established size calibration function must be ≥ 0.990 .

- Assign the calibration function to the measurements of the unknown AuNP sample by selecting the respective fractionations within the Select Runs for Assignment list.
- 5. Display the results by opening a particle size distribution calculation within the insert tab. The previously created particle size calibration will be listed as the Calibration for the unknown AuNP sample measurements, which is displayed in the right window settings. The calculated size will be shown in the size distribution window labeled to the peak maximum. Select the Average Signals for Sample

checkbox to average all measurements of one sample and list the result in the peak maximum label.

 Additionally, plot the calibration line over the fractogram by selecting the Show calibration curve checkbox. A cumulative size distribution is available by selecting the Show cumulative distribution checkbox.

NOTE: When using manufacturer's software for data evaluation, it is recommended to add all results to a report, which can be generated by clicking on **Report** inside the **Insert** tab. The **Report** button adds all results, tables, and diagrams to a document. Under the **Report** tab, the report settings can be changed by opening **Report setup** within the **Document** section.

Representative Results

First, the AuNP size standards were fractionated by AF4 and detected by UV-vis measuring the absorbance of the AuNP at a wavelength of 532 nm (surface plasmon resonance of AuNP). An overlay of the obtained fractograms is presented in Figure 1. The retention times of each AuNP at its respective UV-vis peak maximum obtained from triplicate measurements are listed in Table 5. The relative standard deviation of all retention times was below 1.1% with a decreasing measurement variance with increasing size. Overall, an excellent repeatability was achieved. A constant separation force was applied, which resulted in a linear relationship of elution time and hydrodynamic size. The external size calibration line was established by plotting the specified hydrodynamic radius against the void time corrected elution time (net retention time). A linear regression analysis resulted in a linear calibration function with an intercept a = -3.373nm ± 1.716 nm and a slope b = 1.209 nm \cdot min⁻¹ ± 0.055 nm·min⁻¹. The linear behavior of the elution was confirmed with a squared correlation coefficient R^2 of 0.9958. The respective calibration function is visually displayed in **Figure** 2.

The second part dealt with the analysis of the unknown AuNP sample. Three aliquots of the sample were prepared according to the procedure described in the protocol section (section 4.2). Each of the three aliquots was investigated in triplicate using the same AF4 fractionation method that was also applied for the AuNP size standards. All the nine AF4-UV-vis fractograms that were obtained of the unknown AuNP sample are presented in Figure 3 and their respective evaluations are summarized in Table 6. The relative standard deviation of the respective retention times was significantly low and ranged between 0.1% and 0.5%. Using the particle

size calibration function obtained from the fractionation of the AuNP size standards and correlating it with the obtained retention times of the unknown AuNP sample at the UVvis peak maximum, an overall average hydrodynamic radius of 29.4 nm \pm 0.2 nm could be calculated. Furthermore, a reasonable mass recovery of 83.1% \pm 1.2% was obtained indicating no significant agglomeration or dissolution of the AuNP sample or considerable adsorption of particles onto the membrane surface. **Figure 4** displays the obtained particle size distribution with all nine UV-vis signal traces averaged highlighting the excellent robustness of the applied AF4 method.



Figure 1: AF4-UV-vis fractograms obtained from triplicate analysis of the four individual AuNP size calibration standards with normalized signal intensities and applied constant cross flow rate (black line). The void peak is highlighted in gray at around 5.9 min. Please click here to view a larger version of this figure.

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Figure 2: Obtained external size calibration function, including error bars derived from the respective standard deviations of the DLS measurements (**Table 4**) and variances in the obtained AF4 retention times (**Table 5**), after plotting the specified hydrodynamic radius against the retention time of each individual AuNP size calibration standard at its respective peak maximum. A linear calibration function with standard errors in the form of y = a + bx with a = -3.373 nm ± 1.716 nm and b = 1.209 nm·min⁻¹ ± 0.055 nm·min⁻¹ was calculated from a linear regression analysis. A squared correlation coefficient with R² = 0.9958 was determined, indicating a linear relationship. Please click here to view a larger version of this figure.



Figure 3: AF4-UV-vis fractograms of triplicate measurements of three aliquots displaying the unknown AuNP. The applied constant cross flow rate over the measurement time is illustrated as a black line. The void peak at around 5.9 min is highlighted in gray. Please click here to view a larger version of this figure.



Figure 4: Overlay of the obtained average particle size distribution (red) of the unknown AuNP sample and the applied linear calibration function (dotted line). Please click here to view a larger version of this figure.

Component	CAS-No	Weight (%)
Water	7732-18-5	88.8
9-Octadecenoic acid (Z)-, compound with 2,2',2"-nitrilotris[ethanol](1:1)	2717-15-9	3.8
Sodium carbonate	497-19-8	2.7
Alcohols, C12-14-secondary, ethoxylated	84133-50-6	1.8
Tetrasodium EDTA	64-02-8	1.4
Polyethylene glycol	25322-68-3	0.9
Sodium oleate	143-19-1	0.5
Sodium bicarbonate	144-55-8	0.1

Table 1: List of the components of the surfactant mixture used to prepare the eluent (see also Table of Materials).

AF4-UV-vis parameters	Unit	Value	
Spacer thickness	μm	350	
Detector flow rate	mL min ⁻¹	0.5	
Cross flow rate	mL min ⁻¹	0 (constant for 8 min)	
Focus flow rate	mL min ⁻¹	0	
Delay time / stabilization time	min	0	
Injection flow rate	mL min ⁻¹	0.5	
Transition time	min	0	
Injection time	min	0.1	
Elution step	min	8	
Rinse step time	min	0.1	
Rinse step flow rate	mL min ⁻¹	0.1	
Injection volume	μL	10	
Sample concentration	mg L ⁻¹	12.5	
Membrane type		Regenerated cellulose	
Membrane molecular weight cut-off	kDa	10	
Eluent		0.025% (v/v) surfactant mixture	
UV-vis wavelength	nm	532	
UV-vis sensitivity	-	0.001	

Table 2: Summary of the AF4-UV-vis fractionation method parameters to perform the direct injection run without application of a separation force.

AF4-UV-vis parameters	Unit	Value	
Spacer thickness	μm	350	
Detector flow rate	mL min ⁻¹	0.5	
Cross flow rate	mL min ⁻¹	1 (60 min constant, 10 min linear)	
Focus flow rate	mL min ⁻¹	1.3	
Delay time / stabilization time	min	2	
Injection flow rate	mL min ⁻¹	0.2	
Transition time	min	0.2	
Injection time	min	5	
Elution step	min	70 (60 min constant, 10 min linear)	
Rinse step	min	9	
Rinse step flow rate	mL min ⁻¹	0.5	
Injection volume	μL	50	
Sample concentration	mg L ⁻¹	12.5	
Membrane type		Regenerated cellulose	
Membrane molecular weight cut-off	kDa	10	
Eluent		0.025% (v/v) surfactant mixture	
UV-vis wavelength	nm	532	
UV-vis sensitivity	-	0.001	

Table 3: Summary of the AF4-UV-vis fractionation method parameters to perform the fractionation run with application of a cross flow as separation force.

Calibration	Capping	Mean Size	CV (mean	Zeta	SD (zetaH	ydrodynam	ic SD	PDI	SD (PDI)
standard	agent	(TEM)	size	potential	potential)	Radius (h	ydrodynam	ic	
		(nm)	TEM) (%)	(mV)	(mV)	(DLS)	Radius)		
						(nm)	(nm)		
AuNP	Citrate	20.1	≤ 8	-48.9	1.5	10.95	0.12	0.082	0.009
20 nm									
AuNP	Citrate	40.8	≤ 8	-30.4	1.0	20.30	0.13	0.127	0.006
40 nm									
AuNP	Citrate	79.2	≤ 8	-51.5	1.3	38.85	0.23	0.138	0.013
80 nm									
AuNP	Citrate	102.2	≤ 8	-50.9	0.9	52.30	0.37	0.078	0.009
100 nm									

Table 4: Summary of the physico-chemical parameters of the applied AuNP calibration standards, including capping agent, TEM mean size, Zeta potential determined in the native suspension as well as DLS hydrodynamic radius, and polydispersity index (PDI) determined in the eluent.

Calibration standard	Run	Retention time at peak maximum (min)	Net retention time at peak maximum (min)	Average net retention time (min)	SD (%) (net retention time)	SD (min) (net retention time)	
AuNP 20 nm	1	17.368	11.468	11.56	1.02	0.12	
	2	17.409	11.509				
	3	17.589	11.689				
AuNP 40 nm	1	25.316	19.416	19.49	0.68	0.13	
	2	25.32	19.42				
	3	25.548	19.648				
AuNP 80 nm	1	42.095	36.195	36.29	0.23	0.08	
	2	42.219	36.319				
	3	42.257	36.357				
AuNP 100 nm	1	50.975	45.075	45.06	0.07	0.03	
	2	50.924	45.024				
	3	50.986	45.086				

Table 5: Retention times of the AuNP calibration standards at the respective UV-Vis peak maximum derived from the respective AF4-UV-vis fractograms using the method described in **Table 3**.

Aliquote	Run	Retention time peak maximum (min)	Average retention time at peak	Net retention time at peak maximum (min)	SD (%) retention time	Hydrodynamic radius (nm)	Recovery (%)
		()	(min)	()			
1	1	32.689	32.70	26.789	0.07	29.03	85.34
	2	32.687		26.787			
	3	32.719		26.819			
2	1	32.989	33.08	27.089	0.37	29.49	81.73
	2	33.073	e 	27.173			
	3	33.187		27.287			
3	1	33.053	33.14	27.153	0.49	29.56	82.14
	2	33.071		27.171			
	3	33.291		27.391			

Table 6: Summary of the retention times at the respective UV-Vis peak maximum, the hydrodynamic radius calculated from the external size calibration (**Figure 2**) and the recovery rate of the unknown AuNP sample obtained from AF4-UV-vis analysis.

Discussion

The hydrodynamic size of an unknown AuNP was accurately assessed by AF4 coupled with an UV-vis detector using well-defined AuNP size standards ranging from 20 nm to 100 nm. The developed AF4 method was optimized using a constant cross flow profile in order to establish a linear relationship between measured retention time and AuNP size, thus allowing a straightforward size determination from linear regression analysis. Particular focus was also on achieving sufficiently high recovery rates indicating no significant sample loss during fractionation, and that the developed AF4 method, including the applied eluent and membrane matched well with all fractionated AuNP samples. Method development is arguably the most critical step in AF4 and several parameters, including channel dimensions, flow parameters as well as eluent, membrane, spacer height, and even sample properties have to be taken into account in order to improve fractionation within a given elution time window. The purpose of this paragraph is to guide the reader through the critical steps that were optimized to successfully determine the size of the unknown AuNP sample discussed here. For a more detailed description of how to generally develop an AF4 method, the reader is referred to the AF4 section of 'ISO/TS21362:2018 - Nanotechnologies - Analysis of nano-objects using asymmetrical flow and centrifugal field-flow fractionation'²⁵. Having a closer look at the applied

fractionation conditions given in Table 3, the first critical step is the introduction and relaxation of the AuNP sample in the AF4 channel. This step is governed by the injection flow, focus flow and cross flow, whose interplay forces the sample to locate close to the membrane surface and concentrate it in a narrow band near the injection port of the AF4 channel basically defining the starting point of the fractionation. A sufficient relaxation of the sample is mandatory as during this step, sample constituents of different sizes locate in different heights of the AF4 channel thereby providing the basis for a successful size fractionation. Incomplete sample relaxation is usually visible by an increased void peak area resulting from unretained (i.e., non-relaxed) sample constituents. This effect can be mitigated by increasing the injection time and/or the applied cross flow rate. However, both parameters need careful optimization, especially for samples that are prone to agglomeration and adsorption onto the AF4 membrane, and can be monitored by the respective recovery rates obtained for different parameter settings^{36,37}. The applied injection time of 5 min along with a cross flow rate of 1.0 mL·min⁻¹ revealed recovery rates >80% for all AuNP samples and a negligible void peak area indicating near-optimum relaxation conditions. After sufficient relaxation of the AuNP sample, the focus flow was stopped and sample transport along the AF4 channel length to the respective UV-vis detector was initiated representing the second critical step. In order to ensure sufficiently high fractionation power at reasonable analyses times, a constant cross flow rate of 1.0 mL·min⁻¹ for 30-50 min (depending on the respective fractionated AuNP size standard) followed by a 10 min linear cross flow decay at a detector flow rate of 0.5 mL.min⁻¹ was applied. Using a constant cross flow profile across the separation of all AuNP size standards revealed a linear relationship between retention time and AuNP size following FFF-theory²², thereby enabling size determination of the unknown AuNP sample

by simple linear regression analysis. However, profiles other than a constant cross flow have also been exploited for sizing of nanoparticles, ultimately leading to a non-linear relationship between retention time and particle size^{38,39}. In addition, size determination in AF4 using well-defined size standards is not limited to AuNP, but can also be applied to nanoparticles with other sizes and elemental composition (e.g., silver^{38,40} or silica nanoparticles^{41,42}). In addition, when working with dilute samples, ICP-MS is a highly sensitive elemental detector, which can be coupled with AF4, adding to the versatility of this analytical approach for sizing of a large variety of nanoparticles in suspension.

Despite its widespread application, external size calibration using well-defined size standards in AF4 has some peculiarities that need to be considered when using it for accurate sizing of unknown samples. First of all, it heavily relies on the application of comparable conditions during fractionation of the respective size standards and the actual sample. In the case presented here, it is therefore mandatory that both the AuNP size standards as well as the unknown AuNP sample are fractionated using the same AF4 method as well as the same eluent and the same membrane rendering this approach quite inflexible. Furthermore, having no size-sensitive detectors, e.g., light scattering (MALS and DLS) at hand, it is difficult to determine whether a respective AF4 method using size standards works sufficiently well or not. This especially holds true for unknown samples that exhibit very broad size distributions, where it remains unclear whether all sample constituents follow the normal elution pattern: fractionation from smaller to larger particles, or whether larger sample constituents already elute in steric-hyperlayer mode thereby potentially co-eluting with smaller sample constituents^{43,44}. In addition, even though FFF-theory emphasizes that AF4 separates solely

based on differences in hydrodynamic size with particles being considered point masses without any interactions with their environment²², reality tells a different story with particle-particle and particle-membrane interactions (such as electrostatic attraction/repulsion or van-der-Waals attraction) may play a considerable role and can potentially introduce a measurable bias into size determinations via external size calibration^{45,46}. It is therefore recommended to use size standards that ideally match the composition and the surface properties (Zeta potential) of the particle of interest^{40,42} or. if these are not available, at least use well-characterized particle size standards (e.g., polystyrene latex particles) and carefully evaluate their comparability with the particle of interest especially in terms of their surface Zeta potential in the respective environment, in which the analysis shall be carried out^{41,47}.

The versatility of AF4 is often considered its greatest strength, as it offers an application range that goes beyond most other common sizing techniques in this field^{22,48,49}. At the same time, due to its associated presumable complexity, it may also be regarded as its most significant drawback especially against fast and ostensibly easy-to-use sizing techniques such as DLS, Nanoparticle Tracking Analysis, or single particle ICP-MS. Nonetheless, when putting AF4 into perspective with these popular sizing techniques, it becomes clear that all techniques have their pros and cons, but all of them contribute to a more comprehensive understanding of the physico-chemical nature of nanoparticles and should therefore be considered complementary rather than competitive.

The standard operating procedure (SOP) presented here, highlights the excellent applicability of AF4-UV-vis with external size calibration for sizing of an unknown AuNP sample in suspension and was eventually applied as a recommended guideline for AF4 analysis of an unknown AuNP sample within an international interlaboratory comparison (ILC) that was conducted in the frame of the Horizon 2020 project, ACEnano (the outcome of this ILC will be the subject of a future publication). This protocol, therefore, adds up to the encouraging and ongoing international efforts to validate and standardize AF4 methodologies^{25, 50, 51, 52} underlining the promising potential of AF4 in the field of nanoparticle characterization.

Disclosures

All the authors of this manuscript are employees of Postnova Analytics GmbH, whose products are utilized in this protocol.

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