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

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KEYWORDS:
Asymmetrical Flow Field-Flow Fractionation, AF4, UV-vis detection, gold nanoparticles, particle size, external size calibration, standard operating procedure

SUMMARY:
This protocol describes the use of asymmetrical flow field-flow fractionation coupled with UV-vis detection for the determination of the size of an unknown gold nanoparticle sample.

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 BC[1], 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 Museum[2]. 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 syphilis[4].

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 properties[5]. 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 nanoelectronics[8], diagnostics[9], cancer therapy[10], or drug delivery[11]. Obviously, for these applications, precise knowledge of the size and size distribution of the applied AuNP is a fundamental prerequisite to ensure optimum efficacy[12] 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[16-20].

First conceptualized in 1966 by J. Calvin Giddings[21], 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 subtype[24]. 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 size[22]. 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 threshold[25].

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 UV-vis 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

1.1. 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.

1.2. Install all necessary software packages for control, data acquisition, processing and

evaluation following the instructions given in the manufacturer's manual.

1.3. Ensure that all necessary signal connections between the AF4 system and the UV-vis detector have been established.

1.4. 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 $mL \cdot min^{-1}$, focus flow rate 1 $mL \cdot min^{-1}$, and cross flow rate 1.5 $mL \cdot min^{-1}$). 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

2.1. Cleaning solution

2.1.1. 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. Afterwards, filter and de-gas the obtained solution.

2.2. Eluent

2.2.1. 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**).

2.3. Arbitrary AuNP size standard for mass recovery determination

2.3.1. Vortex an arbitrary AuNP size standard (50 $mg \cdot L^{-1}$) for 2 min and dilute it 1:4 with UPW to obtain a final mass concentration of 12.5 $mg \cdot L^{-1}$. 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 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

3.1. 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}).

3.2. 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}).

3.3. Replace the respective inline pump filters.

3.4. Open the AF4 cartridge and replace the AF4 membrane. Reassemble the AF4 cartridge and reconnect it with the AF4-UV-vis system.

3.5. 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).

3.6. Qualify the AF4-UV-vis system by determining the mass recovery and variation of retention time using an arbitrary AuNP size standard.

3.6.1. Perform a direct injection run without application of a separation force.

3.6.1.1. Create a new measurement file by opening **File | New | Run** in the AF4 control software.

3.6.1.2. 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**.

3.6.1.3. Set the measurement parameters in the second tab **FFF method** according to **Table 2**.

3.6.1.4. Click on the **Run** button to start the measurement.

3.6.2. Perform a fractionation run with application of a separation force (Cross flow).

3.6.2.1. Define the fractionation method as described in the previous section using the

fractionation conditions specified in **Table 3**.

3.6.2.2. Click on the **Run** button to start the measurement.

3.6.2.3. 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.

3.6.2.4. 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.

3.6.2.5. 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

4.1. 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 $mg \cdot L^{-1}$. Vortex for additional 2 min after dilution to homogenize the obtained suspensions.

4.2. Prepare the unknown AuNP sample for analysis applying the same procedure as for the calibration standards described in step 4.1.

4.3. Perform a direct injection measurement of all AuNP size standards using the AF4 method displayed in **Table 2**.

4.3.1. 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.

4.4. Fractionate each AuNP size standard individually using the AF4 method displayed in **Table 3** to establish the external size calibration function.

4.4.1. 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.

4.5. Perform a direct injection measurement of the unknown AuNP sample using the AF4 method displayed in **Table 2**.

4.6. Perform the fractionation of the unknown AuNP sample by conducting the AF4 method listed in **Table 3**.

4.7. Carry out all measurements mentioned in **Section 3** and **4** in triplicate unless stated otherwise to ensure meaningful and statistically relevant results.

4.7.1. Store 50 $mg \cdot L^{-1}$ 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.

4.7.2. 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-UV-vis-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

5.1. 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.

5.1.1. 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:

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

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.

5.1.2. Import the dat. files obtained from both the direct injection and the fractionation run.

5.1.3. Select the UV-vis detector trace in the **Overview** tab.

5.1.4. Define a Region of Interest (ROI) and a baseline in the signal and baseline view for all measurements.

5.1.5. Insert a **Direct Injection Calibration** via **Insert**.

5.1.6. 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.

5.1.7. 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.

5.1.8. 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.

5.1.9. 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.

5.2. 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.

5.2.1. Open the **Overview** window to display the respective UV traces for all imported measurements.

5.2.2. 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.

5.2.3. Calculate the relative standard deviation for all measurements using the following equation:

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

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

5.3. 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.

5.3.1. After importing the .dat files all measurements are displayed in the **Overview** tab. Select the UV-vis 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.

5.3.2. Select the **Particle Size Calibration** from the **Insert** tab.

5.3.3. 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 4**. 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.

5.3.4. Assign the calibration function to the measurements of the unknown AuNP sample by selecting the respective fractionations within the **Select Runs for Assignment** list.

5.3.5. 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.

5.3.6. 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 = -3.373 nm ± 1.716 nm and a slope b = 1.209 $nm \cdot min^{-1}$ ± 0.055 $nm \cdot min^{-1}$. 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 UV-vis peak maximum, an overall average hydrodynamic radius of 29.4 nm ± 0.2 nm could be calculated. Furthermore, a reasonable mass recovery of 83.1% ± 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 AND TABLE LEGENDS:

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.

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 $R^2 = 0.9958$ was determined, indicating a linear relationship.

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.

Figure 4: Overlay of the obtained average particle size distribution (red) of the unknown AuNP sample and the applied linear calibration function (dotted line).

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

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

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.

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.

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**.

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 $mL \cdot min^{-1}$ 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 $mL \cdot min^{-1}$ 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-theory[22], 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 environment[22], 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[25,50-52] underlining the promising potential of AF4 in the field of nanoparticle characterization.

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DISCLOSURES:

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

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Figure

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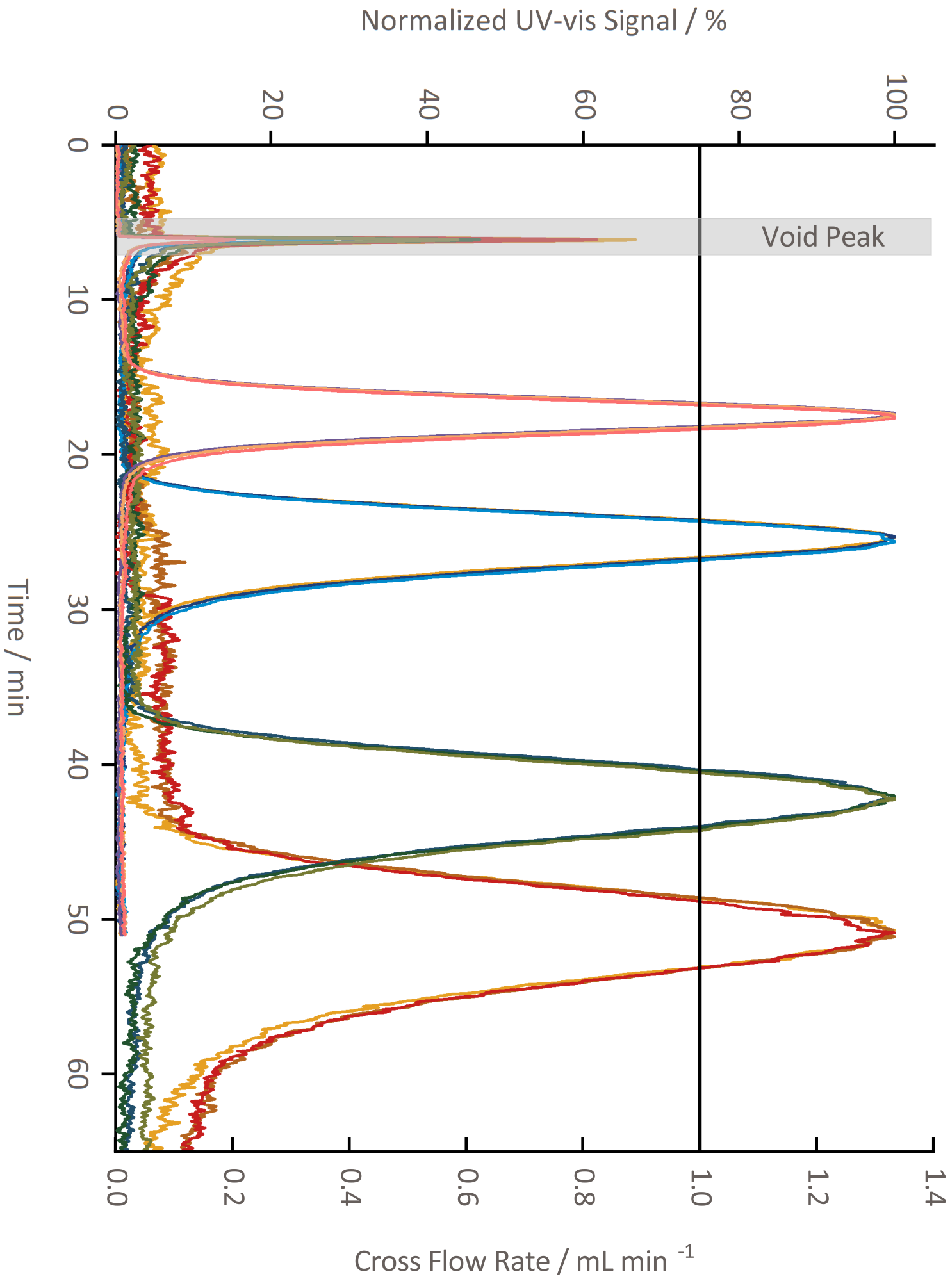

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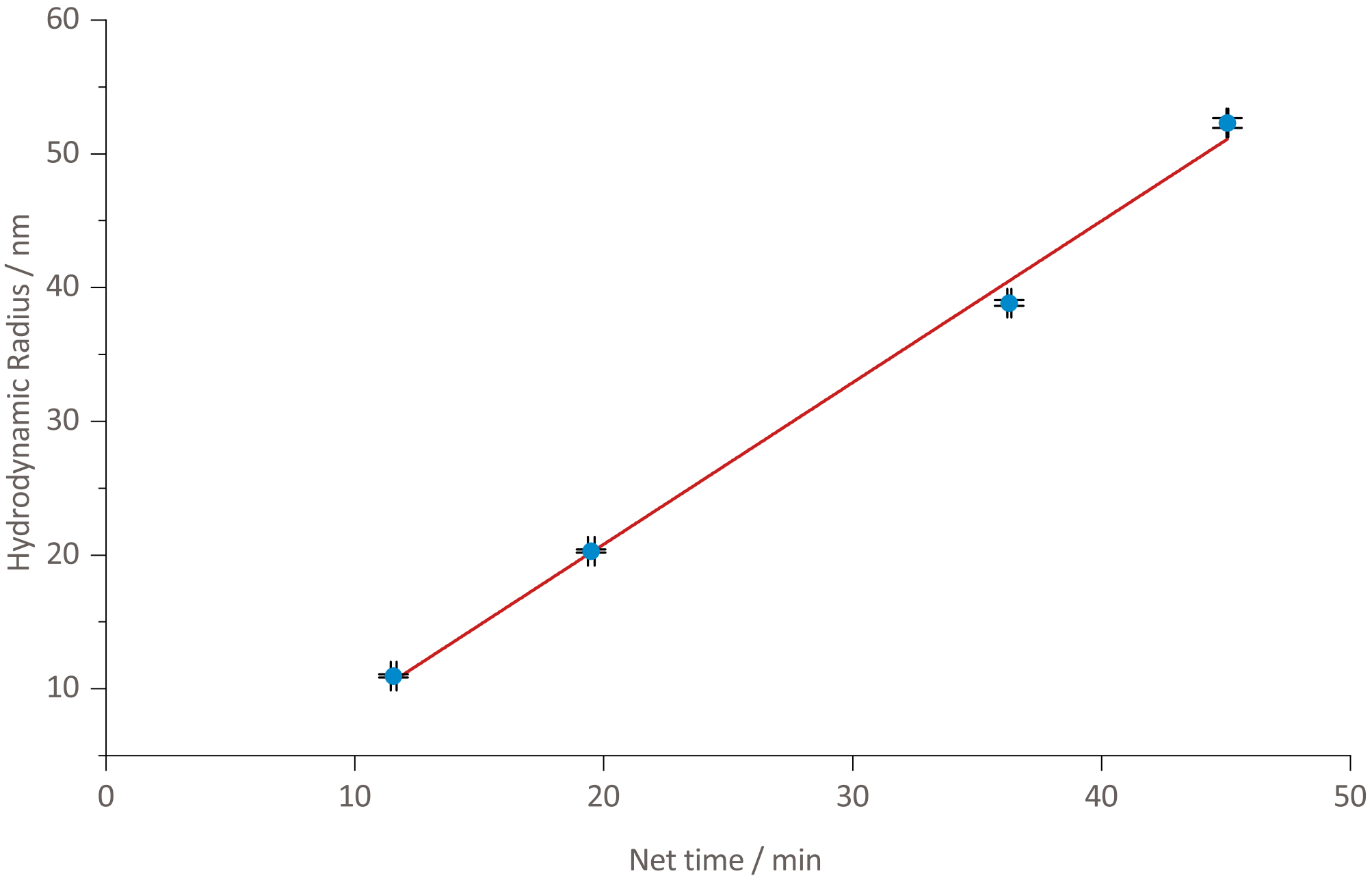

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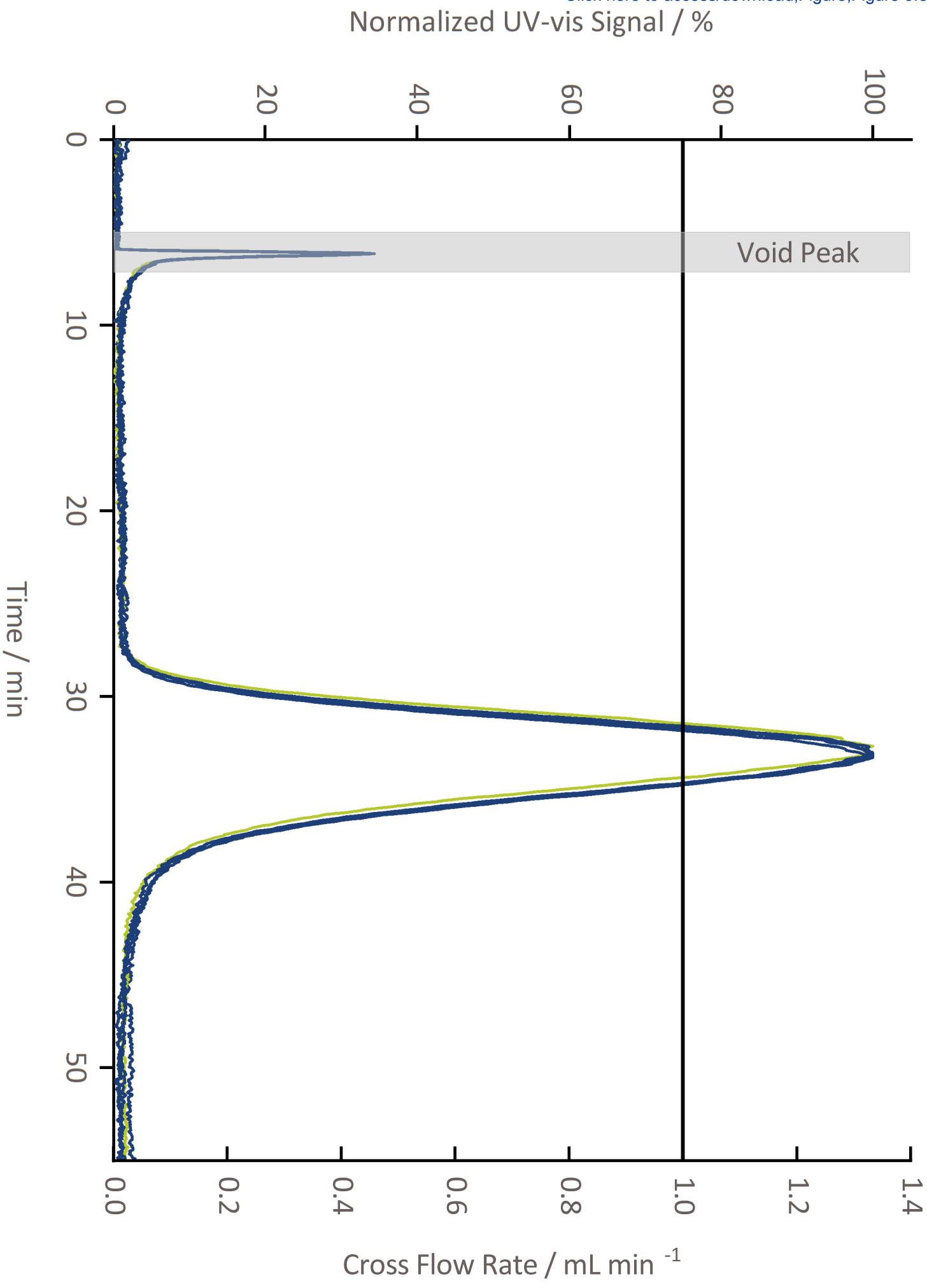

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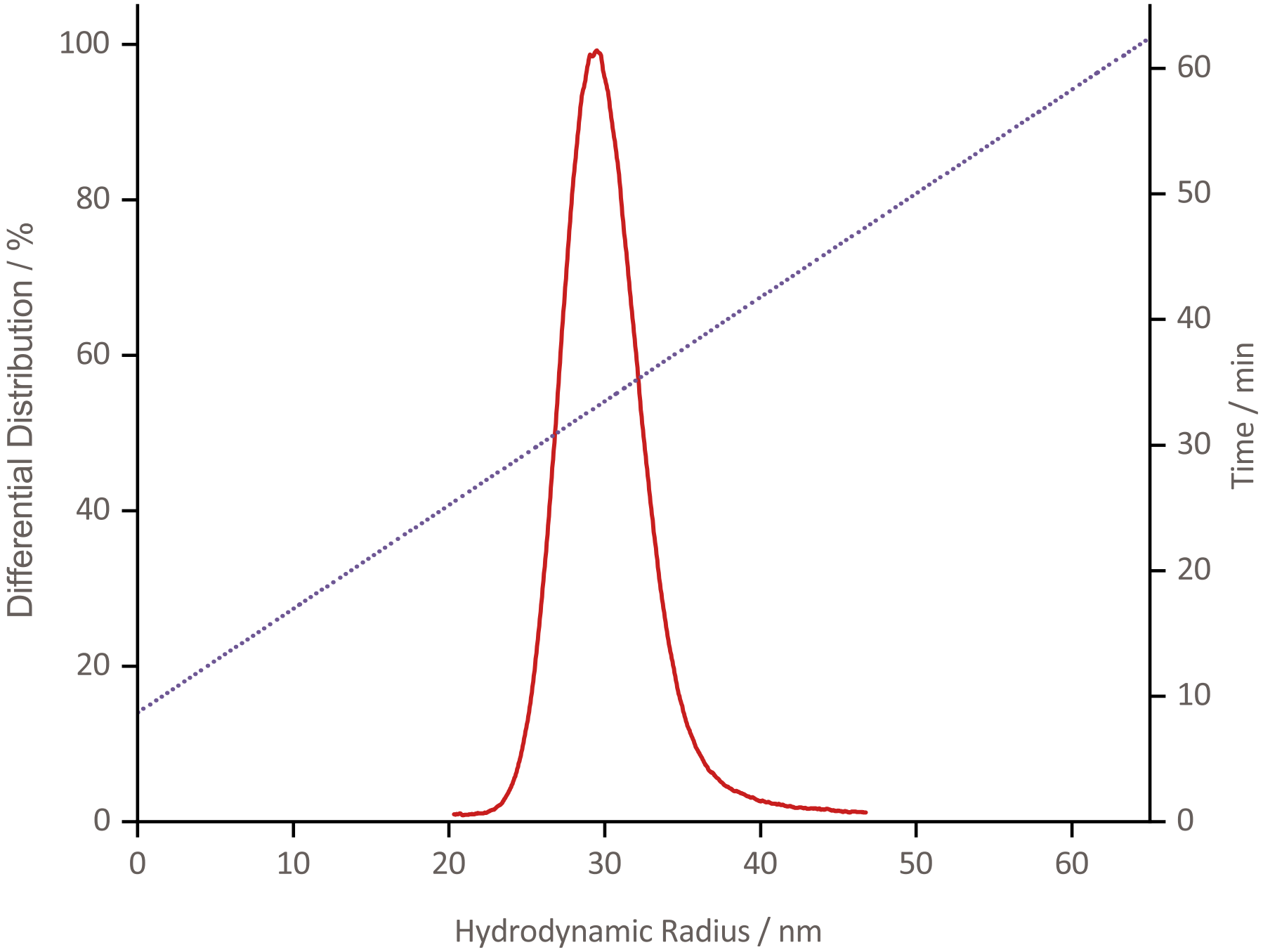

Table 1

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 2

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 3

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 4

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

Table 5

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 6

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

Name of Material/ Equipment	Company
0.1 µm Membrane Filters (hydrophilic PVDF)	Postnova Analytics GmbH
0.22 µm PVDF Syringe Filter (d = 33 mm)	Merck Millipore
Adjustable Volume Pipettes (1000 µL)	Eppendorf AG
AF4 cartridge	Postnova Analytics GmbH
AF4 Membrane - Regenerated Cellulose (10 kDa MWCO)	Postnova Analytics GmbH
Analytical Balance (0.1 mg precision)	Sartorius
Autosampler	Postnova Analytics GmbH
Channel Oven	Postnova Analytics GmbH
Crossflow Module	Postnova Analytics GmbH
Disposable Pipette Tips (1000 µL)	Eppendorf AG
Flasks (e.g. 2 liter volume)	neoLab
Focus Pump	Postnova Analytics GmbH
Glass Vials (e.g. 1.5 mL volume)	Postnova Analytics GmbH
Gold Nanoparticle Size Standards (20 nm, 40 nm, 80 nm, 100 nm)	Postnova Analytics GmbH
Magnetic Stirrer	IKA
Personal Computer (PC)	Dell Technologies
Personal protection gear (gloves, lab coat, glasses etc.)	/
Screw Top for Glass Vials (e.g. 1.5 mL volume)	Postnova Analytics GmbH
Sodium Dodecyl Sulfate (SDS), ≥99 %, Blotting Grade	Carl Roth GmbH & Co KG
Sodium Hydroxide (NaOH) Pellets, ≥98 %, p.a	Carl Roth GmbH & Co KG
Software Package for Control and Data Acquisition	Postnova Analytics GmbH
Software Package for Data Evaluation	Postnova Analytics GmbH
Software Package for final Data Processing	OriginLab Corporation
Solvent Degasser	Postnova Analytics GmbH
Solvent Selector	Postnova Analytics GmbH
Solvent Organizer	Postnova Analytics GmbH
Surfactant Mixture	Postnova Analytics GmbH
Tip Pump	Postnova Analytics GmbH
Unknown AuNP sample	BBI Solutions
UV-vis Detector	Postnova Analytics GmbH

Vacuum Filtration Unit	Postnova Analytics GmbH
Vortex	IKA
Water Purification System	Merck Millipore

Catalog Number

Z-FIL-TEF-002

Durapore Millex

Research Plus

AF2000 MF - AF4 Analytical Channel

Z-AF4-MEM-612-10KD

ENTRIS124I-1S

PN5300

PN4020

AF2000 MF Control Module

ep T.I.P.S

1-0199

PN1131

VIA-002

NovaCal Gold

VIBRAX-VXR

/

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Z-VIA-09150868

2326.1

6771.1

NovaFFF AF2000 Software

NovaAnalysis Software

Origin 2019

PN7520

PN7310

PN7140

NovaChem100

PN1130

EM.GC60

PN3211

Eluent Filtration System

Vortex Genie 2
Milli-Q Integral 5

Comments/Description
Used for filtration of aqueous solutions
Used for filtration of NovaChem100
Used to prepare diluted AuNP suspensions
Component of the AF2000 MF - MultiFlow FFF setup, which is described as AF4-system in the manuscript
Component of the AF2000 MF - MultiFlow FFF setup, which is described as AF4-system in the manuscript
Used to weigh SDS and NaOH pellets for preparation of cleaning solution
Component of the AF2000 MF - MultiFlow FFF setup, which is described as AF4-system in the manuscript
Component of the AF2000 MF - MultiFlow FFF setup, which is described as AF4-system in the manuscript
Component of the AF2000 MF - MultiFlow FFF setup, which is described as AF4-system in the manuscript
Used to prepare diluted AuNP suspensions
Used for eluent storage
Component of the AF2000 MF - MultiFlow FFF setup, which is described as AF4-system in the manuscript
Used for sample storage
50 mg L^{-1} each, used to establish the size calibration function
Used to accelerate dissolution of SDS and NaOH pellets in UPW
Unit to control AF4 runs, record and evaluate collected data, for necessary hardware and software requirements the reader is referred to
In accordance with respective laboratory's safety rules for working with chemicals including engineered nanomaterials
Used for sample storage
Used for the preparation of the cleaning solution
Used for the preparation of the cleaning solution
Software for performing Af4 runs and data aquisition, for necessary hardware and software requirements the reader is referred to the
Software for AF4 data evaluation, for necessary hardware and software requirements the reader is referred to the Postnova NovaAnalysis
Used for final data processing
Component of the AF2000 MF - MultiFlow FFF setup, which is described as AF4-system in the manuscript
Component of the AF2000 MF - MultiFlow FFF setup, which is described as AF4-system in the manuscript
Component of the AF2000 MF - MultiFlow FFF setup, which is described as AF4-system in the manuscript
Mixture of different surfactants and salts used for eluent preparation
Component of the AF2000 MF - MultiFlow FFF setup, which is described as AF4-system in the manuscript
60 nm AuNP sample used for size determination via size calibration function
UV-vis detector For downstream coupling with the AF4 system

Used to ensure low particle backgrounds and removal of dissolved air in the used eluents to ensure optimum AF4 fractionation conditions

Used for homogenization of diluted AuNP suspensions

Used to generate ultrapure water (UPW, 18.2 MΩcm resistivity) for preparation of cleaning solution, eluents and dilution of AuNP

First of all, we would like to express our sincere gratitude to the editor and all three reviewers for their time and valuable comments and suggestions to improve the quality of the manuscript. We have addressed all of them as comprehensive as possible and revised the manuscript accordingly. Our respective answers highlighted in bold can be found below. For a better tracing, we also highlighted our changes in the manuscript using the Word "track changes" function.

Answers to the comments from the editors and reviewers

Editorial Comments

Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors

The authors have proofread the manuscript multiple times and discovered spelling and grammatical errors were corrected accordingly.

Protocol Detail

Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like to show in the video. Please ensure that all specific details (e.g. button clicks for software actions, numerical values for settings, etc) have been added to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

1) Section 4: Some steps are a bit vague. Please describe all actions to be performed.

The authors thank for the critical review of the manuscript. Section 4 was revised and the necessary steps of how to perform a measurement were described in more detail.

Protocol Numbering

1) Please add a one-line space after each protocol step.

All protocol steps were reformatted accordingly.

Protocol Highlight

Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

1) Please ensure completeness, and that the highlighting is best represented by the manuscript title.

2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.

After thorough revision of the manuscript, we highlighted some further protocol steps to be additionally included in the video in order to facilitate an even more cohesive and logical narrative of the described procedure.

Discussion

JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

All paragraphs were revised accordingly to cover all demanded aspects. However, in order to ensure smooth readability, it wasn't always possible to strictly follow the suggested order of the respective paragraphs.

Commercial Language

JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are NovaAnalysis, NovaChem100, NovaCal

1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

2) Check Tables 1–4 as well.

All brand names in the manuscript as well as in the Tables 1 to 6 were replaced by generic names and referenced accordingly in the "Table of Materials".

Table of Materials

1) Please sort in alphabetical order.

The Table of Materials was sorted in alphabetical order.

Reviewer 1

Manuscript Summary

Nanoparticles, and Nanomaterials in general, receive more and more attention from different points of view and in many fields of the human activity. Hence, there is a major concern in the identification, characterization and determination of such materials. AF4 is a powerful technique for these purposes. The manuscript describes a particular use of the technique for the size determination of gold nanoparticles. The protocol reported for the SOP is clearly described, with different notes and caution comments. The discussion is of valuable interest for the readers.

Major Concerns

The overall SOP should include the proposal of internal quality control activities in order to continuously assure the validation conditions.

Reviewer 1 makes a valid point here. An additional NOTE addressing quality control activities was added to point 4 in the protocol section.
In general, to assure constant and valid separation conditions it is recommended to regularly include/repeat a system qualification measurement after several sample measurements (e.g. 10 measurements). This procedure is also known from other analytical methodologies (e.g. single particle ICP-MS with ISO/TS 19590) in order to avoid error-prone results. In AF4, an increased void or field-off peak as well as a deteriorated sample peak form usually indicates an error-prone measurement or contaminated system and may therefore necessitate a thorough AF4 system cleaning and/or AF4 membrane exchange including subsequent re-calibration and validation of the setup. In addition, essential quality parameters such as system pressure and UV-vis detector baseline stability should be recorded and remain stable and constant along a complete AF4 run. We have now addressed these criteria in the manuscript accordingly.

Minor Concerns:

Error bars in the calibration curve should be included, and hence the variability in the hydrodynamic radius.

The respective diagram was extended by error bars using the measured standard deviations obtained from the performed DLS experiments and the variance in the obtained AF4 retention times. Six independent DLS measurements were performed to calculate statistically relevant results. The standard deviation from the retention times was determined from triplicate analyses.

Reviewer 2

Manuscript Summary

The manuscript deals with the size characterization of Au NPs by AF4 by external calibration strategy with AuNPs of known size. The manuscript describes the complete procedure to carry out this kind of analysis with a type of instrument. The SOP described has been evaluated in terms of reproducibility, obtaining a value lower than 0.5%, with mass recoveries higher than 80% (based on UV-Vis absorbance).

Major Concerns

One of my major concerns is about the description of some parts of the SOP, specially those related to the data evaluation. Although the rest of the sections are described considering the type of instrument used, which is understandable, the data evaluation is too focused on the brand software and it is a bit confusing. Considering that all these calculations can be done using a spreadsheet with the data collected during the experiments, I would suggest including a procedure not only based on the software of this instrument.

Thanks for this helpful comment. We agree that data evaluation can also be performed using spreadsheet analysis. We have therefore revised the respective data evaluation section and also added a more general description of the performed procedure to (hopefully) provide more clarity. By this means, we aim to leave it up to the user, which route (manufacturer's software or spreadsheet analysis) to follow when it comes to data processing and evaluation. Nevertheless, in order to be able to tell the most cohesive story in the video, we will stick to a data evaluation using the manufacturer's software, but will mention that evaluation can also be performed after raw data extraction and spreadsheet analysis.

Minor Concerns

Section 2.2. I could not find the composition of the filtered surfactant mixture. Please indicate it at this point.

The composition of the surfactant mixture was added to Table 1. The pH of the resulting eluent was around 9.4.

Section 3.4. How many runs can be done before replacing the membrane?

In AF4, the ultrafiltration membrane definitely is a delicate part of the fractionation system. However, it is usually difficult to state a defined number of runs that can be performed on one single membrane. As a general rule of thumb, it should be replaced after a respective detector shows an increased noise level indicating a "dirty system" or the initially defined system qualification criteria such as recovery, peak shape or repeatability, cannot be met anymore (or the AF4-system was subjected to a thorough cleaning procedure). A valid system under the here described conditions usually provides

stable results for at least 50 measurements. However, the number of possible consecutive measurements that meet the defined quality criteria highly depends not only on the properties of the respective sample and sample matrix, but also on the used eluent, and can therefore be significantly lower.

Section 5.1.1. It is assumed that in the sample there is no other species that can absorb at that wavelength that can be eluted at a different time (or not eluted at all because it is filtered through the membrane). Please, mention this possibility (I know it is not likely at this wavelength but even so it should be considered), since it can reduce the recovery estimated.

This is correct. We have now addressed this point in the respective paragraph.

Section 5.1.8. This is also interesting. Given that the maximum absorbance is size dependent a new calibration is required, but it is not easy for an unknown sample. A mass-based detector would be a better option. You should mention it.

We agree with the reviewer's comment and revised the respective paragraph accordingly. We are aware of the limitations of UV-vis detection in our case, but as already stated in the introduction, the overall idea behind this SOP was that it is best-suited for an inter-laboratory comparison that can be joined by as many laboratories as possible. The SOP was therefore tailored on purpose towards the most basic AF4-setup for nanoparticle analysis, which usually is the combination with a UV-vis detector.

Figure 2. You have considered a calibration based on the retention time. I would suggest a more robust method based on the difference between retention time and void time (I know void time should remain constant, but it can correct some bias along the time).

Thanks for that valuable comment. Table 5 and Table 6 contain the retention time corrected by the void time (net retention time). Figure 2 was revised accordingly.

Line 344. Also include the interactions with the membrane as cause of low recoveries.

This statement is also correct and this information was added to the respective paragraph.

Reviewer 3

This is a straightforward application of asymmetric flow field flow fractionation for the size-based separation of gold nanoparticles. There is much critical scientific information is missing, and authors must address them in this document. You must address the following comments; otherwise, this document will be meaningless.

Surfactant mixture- This means nothing for any reader. You must list composition and pH. Surfactant is a product from your own company, and there is no reason you do not have information.

The reviewer is correct. The detailed composition of the surfactant mixture was added to Table 1. The pH value of the eluent ranged around 9.4.

Nature of gold nanoparticles - Please measure size using an electron microscope and list the average size and standards deviation of reasonable counts (1000) of the nanoparticle. There is no way anyone makes particles exact size, as you mentioned here in this document. You must explain surface functionality or stabilizing agent of gold nanoparticles along with zeta potential in running buffer.

We absolutely agree with this statement. More information on the nature of the used gold nanoparticles including capping agent, TEM sizes, hydrodynamic size, PDI (both determined in the used eluent) and Zeta potential in native suspension, was added to Table 4. Due to a too low concentration of the AuNP (1:4 dilution), Zeta potential measurements of the respective AuNP size calibration standards in the eluent were not possible using Malvern Zetasizer ZSP. However, with a comparable ionic strength of both matrices (citrate buffer and eluent) a comparable Zeta potential of the AuNP can be assumed.

You must emphasize that this method work for this particular gold nanoparticle under the given condition and method development may be necessary for any other application other than discussed here.

We agree with this statement and added a respective sentence to the second paragraph of the Discussion addressing this point.