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

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Author Questionnaire

1. Microscopy: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or similar? **N**

2. Software: Does the part of your protocol being filmed demonstrate software usage? **Y**

Videographer: All screen captures provided; do not film

3. Interview statements: Considering the Covid-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

☒ Interviewees wear masks until the videographer steps away (≥6 ft/2 m) and begins filming. The interviewee then removes the mask for line delivery only. When the shot is acquired, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. Filming location: Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Protocol Length
Number of Shots: **33**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Florian Meier**: The use of asymmetrical flow field-flow fractionation in combination with external size calibration is a powerful tool for accurately determining the size distribution of gold nanoparticles in suspension **[1]**.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

REQUIRED:

1.2. **Roland Drexel**: AF4 covers the whole nanoparticle size range, from 1-1000 nanometers. It works independently from the chemical composition of the sample constituents and can be applied even to complex and polydisperse samples **[1]**.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

OPTIONAL:

1.3. **Florian Meier**: The beauty of AF4 is that it has a very broad application range, which includes not only nanoparticles of different natures and sizes, but also proteins, viruses, and natural and synthetic macromolecules **[1]**.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Protocol

2. Asymmetrical Flow Field-Flow Fractionation (AF4)-UV-Visible Spectroscopy System Preparation and Qualification

2.1. Begin by vortexing 50 milligrams/liter of an arbitrary gold nanoparticle size standard for 2 minutes **[1]** before diluting the standard to a 1:4 ratio with ultrapure water **[2]**.

2.1.1. WIDE: Talent vortexing standard, with standard container visible in frame

2.1.2. Talent adding water to standard

2.2. Then vortex the diluted solution for an additional 2 minutes to obtain a homogenized suspension **[1]**.

2.2.1. Solution being vortexed

2.3. After cleaning the system, open the AF4 (A-F-four) cartridge to allow replacement of the AF4 membrane. Rinse the new membrane with UPW and reassemble the cartridge **[1-TXT]**. Then reconnect the cartridge to the AF4-UV-vis (U-V-viz) system **[2]**.

2.3.1. Talent replacing membrane *Videographer: Important step*

2.3.2. Talent connecting cartridge to system *Videographer: Important step*

2.4. Flush the cleaned AF4-UV-vis system with filtered and degassed eluent for at least 30 minutes by applying a tip flow rate of 1 milliliter per minute, a focus flow rate of 1 milliliter per minute and a cross flow rate of 1.5 milliliter per minute to equilibrate the membrane, stabilize the system **[1]** and check for potential leaks. The system pressure should reach a constant level between 4 to 12 bar **[2]**.

2.4.1. Talent flushing system *Videographer: Difficult step*
2.4.2. Talent checking for leaks *Videographer: Difficult step*

2.5. To qualify the AF4-UV-vis system by determining the mass recovery and variation of retention time using an arbitrary gold nanoparticle size standard, perform a direct injection run without the application of a separation force **[1]**.

2.5.1. Talent at system, selecting Run, with monitor visible in frame

2.6. Then perform a fractionation run with the application of a separation force **[1]**.

2.6.1. SCREEN: screenshot_1: 03:27-03:44

3. AF4-UV-vis Sample Analysis

3.1. To prepare gold nanoparticle size standards for the external size calibration, vortex all of the 50 milligram/liter-gold nanoparticle size standards for 2 minutes **[1-TXT]** and dilute each respective sample for analysis in ultrapure water at a 1:4 ratio **[2]**.

3.1.1. WIDE: Talent vortexing standard(s) *Videographer: Important step* **TEXT: *i.e.*, 20 nm, 40 nm, 80 nm, and 100 nm**
3.1.2. Talent adding UPW to sample, with sample container visible in frame *Videographer: Important step*

3.2. When the size standards have been prepared, set the direct injection measurement parameters as outlined in the Table **[1]** and inject 10 microliters of the first standard into the system **[2-TXT]**.

3.2.1. LAB MEDIA: Table 2 *Video Editor: please sequentially emphasize steps from top to bottom of table*
3.2.2. Talent injecting standard into system **TEXT: Repeat for each standard**

3.3. When all of the size standards have been measured, inject 50 microliters of the first standard into the system **[1]** and use the AF4 method as illustrated to establish the external size calibration function **[2]**.

3.3.1. Talent injecting standard into system
3.3.2. LAB MEDIA: Table 3 *Video Editor: please sequentially emphasize steps from top to bottom of table*

3.4. When all of the gold nanoparticle size standards have been fractionated, prepare the unknown gold nanoparticle sample **[1]** and perform a direct injection measurement and fractionation of the unknown gold nanoparticle sample as just demonstrated **[1]**.

3.4.1. Talent adding UPW to sample, with sample container and vortex visible in frame as possible *Videographer: Important step*
3.4.2. Talent injecting sample into system *Videographer: Important step*

4. Data Evaluation

4.1. To analyze the data, import the data files into the system software **[1]**, display the

measurements in the **Overview** tab, and select the UV-vis detector signal from the detectors list **[2]**.

4.1.1. WIDE: Talent importing data into software, with monitor visible in frame
4.1.2. SCREEN: screenshot_2: 00:31-00:42

4.2. Define a region of interest and baseline for each measurement, adjusting the **Signal and Baseline** view as necessary **[1]**.

4.2.1. SCREEN: screenshot_2: 00:43-00:54

4.3. Use the **Signal processing** toolbox to smooth any noisy signals **[1]** and use the **Assign Processing Parameters to other Runs** function to allow the parameters to be allocated to other measurements **[2]**.

4.3.1. SCREEN: screenshot_2: 01:10-01:20
4.3.2. SCREEN: screenshot_2: 01:22-01:28

4.4. To perform a mass recovery calculation, open the direct injection measurements and fractionation data **[1]**.

4.4.1. Talent opening data, with monitor visible in frame

4.5. Insert a **Direct Injection Calibration** and select the respective direct injection measurements from the **Direct Injection Calibration-Settings** view. The injection volume and concentration of the sample will be used to correlate the peak area with the injected amount **[1]**.

4.5.1. SCREEN: screenshot_2: 01:54-02:05

4.6. Next, use the **Assign to selected Particle Presets** function to assign the obtained calibration function to the respective fractionation measurements **[1]**.

4.6.1. SCREEN: screenshot_2: 02:30-02:34

4.7. Then use the equation to calculate the gold nanoparticle mass recovery by comparing the areas under the respective UV-vis peaks of the fractionation and direct injection measurements **[2]**.

4.7.1. BLACK TEXT OVER WHITE BACKGROUND: $\text{recovery}\,[\%] = \frac{A_{\text{fractionation}}}{A_{\text{direct injection}}} \text{ x } 100\,[\%]$

4.8. To display the results, open the **Quantitative Results** calculation. A mass concentration based on the peak area will be presented **[1]**.

4.8.1. SCREEN: screenshot_2: 02:36-02:46

4.9. The mass recovery can be obtained by comparing this value to the injected concentration **[1]**.

4.9.1. SCREEN: screenshot_2: 03:04-03:13

4.10. Under the **Particle Size** tab, in the **Particle Size Calibration** window, click on each appropriate measurement in the **Select References for Calibration** table to select all of the calibration runs. The measurements will be displayed in a table as they are selected **[1]**.

4.10.1. SCREEN: screenshot_3: 01:04-01:12

4.11. Enter the hydrodynamic radius for all of the calibration measurements. The function and equation will be displayed in the **Particle size calibration-Function** window **[1]**.

4.11.1. SCREEN: screenshot_3: 01:15-01:25 *Video Editor: please emphasize Particle size calibration-Function window when mentioned*

4.12. To assign the calibration function to the measurements of the unknown gold nanoparticle sample, select the respective fractionations within the **Select Runs for Assignment** list and open a **particle size distribution** calculation to display the results **[1]**.

4.12.1. SCREEN: screenshot_3: 02:11-02:33 *Video Editor: can speed up*

4.13. The calculated size will be shown in the **size distribution window** labeled to the peak maximum and the previously created particle size calibration will be listed as the **Calibration** for the unknown gold nanoparticle sample measurements **[1]**.

4.13.1. SCREEN: screenshot_3: 02:33-02:44

4.14. To average all of the measurements of one sample, select the **Average Signals for Sample** checkbox. The result will be listed in the peak maximum label **[1]**.

4.14.1. SCREEN: screenshot_3: 02:49-02:56

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see?
2.3., 3.1., 3.4.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success?
2.4.

Results

5. Results: Representative AF4 Gold Nanoparticle Sizing

5.1. Here a representative overlay of gold nanoparticle size standards fractionated by AF4 and detected by UV-vis spectroscopy measurement of the absorbance of the particles at a 532-nanometer wavelength is shown **[1]**.

5.1.1. LAB MEDIA: Figure 1 *Video Editor: please sequentially emphasize red, orange, blue, and grey data lines*

5.2. The relative standard deviation of all of the retention times for each nanoparticle at its respective UV-vis spectroscopy peak maximum obtained from triplicate measurements was below 1.1% **[1]**.

5.2.1. LAB MEDIA: Table 5 *Video Editor: please emphasize 2D% (net retention time) column*

5.3. A linear regression analysis of these data resulted in a linear calibration function with a squared correlation coefficient r-squared of 0.9958 **[1]**.

5.3.1. LAB MEDIA: Figure 2 *Video Editor: please emphasize data line*

5.4. When nine AF4-UV-vis spectroscopy fractograms were then obtained from unknown gold nanoparticle samples **[1]**, the relative standard deviation of the respective retention times was significantly low, ranging between 0.1 and 0.5% **[2]**.

5.4.1. LAB MEDIA: Figure 3 *Video Editor: please emphasize data lines*
5.4.2. LAB MEDIA: Table 6 *Video Editor: please emphasize SD (%) retention time column*

5.5. Using the particle size calibration function obtained from the fractionation of the gold nanoparticle size standards and correlating these data with the obtained retention times of the unknown gold nanoparticle sample at the UV-vis spectroscopy peak maximum **[1]**, an overall average hydrodynamic radius could be calculated **[2]**.

5.5.1. LAB MEDIA: Figure 4
5.5.2. LAB MEDIA: Figure 4 *Video Editor: please emphasize red data line*

5.6. In addition, a reasonable mass recovery could be obtained **[1]**, indicating no significant agglomeration or dissolution of the gold nanoparticle samples or considerable adsorption of the particles onto the membrane surface **[2]**.

5.6.1. LAB MEDIA: Figure 4 *Video Editor: please dotted blue data line*
5.6.2. LAB MEDIA: Figure 4

Conclusion

6. Conclusion Interview Statements

6.1. **Roland Drexel**: When using external size calibration in an AF4 analysis, it is of utmost importance that the same AF4 method is consistently applied for all of the size standards and the sample itself **[1]**.

6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (3.2.-3.5., 4.11.)

6.2. **Florian Meier**: AF4 enables researchers to better understand the properties of their nanoparticulate samples. It is therefore an important quality control tool that can ultimately help to improve the efficacy of nano-enabled products **[1]**.

6.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera