Project:

FFF-MALS method development and measurements of size and molecular weight

Measurement of particle size distribution of protein binding, of mean molecular weight of polymeric NP components, study of batch to batch reproducibility, and study of release of free coating from NP surface by FFF-MALS

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1 Introduction

With the development of very complex medical-NP (Med-NP) systems, there is the need to characterize the critical chemical-physical attributes of Med-NPs that will have a major impact on their in vivo safety and efficacy. The aim of this standard operating procedure (SOPs) is to describe how Field Flow fractionation (FFF), combined with multiple detectors, such as multi angle light scattering (MALS), refractive index (RI) and dynamic light scattering (DLS) allows to sudy various critical parameters of the Med-NPs formulations including:

- Size distribution of nanoparticles in physiological buffers;
- change in nanoparticle size distribution after protein binding (NP-proteins interaction);
- molecular weight (MW) of NPs components (for polymeric carriers);
- release of free coatings/surfactants (e.g. PEG) from the surface of Med-NPs.

Method development procedure needed to perform those analysis, the quality control necessary to check the instrumental system and procedures for data analysis of light scattering (LS) data will be described.

2 Principle of the Method

2.1. Field-Flow Fractionation

Field-Flow Fractionation (FFF) is a family of flow based separation methods. Similarly to chromatography, in FFF a sample band is injected into a liquid stream flowing through a channel. This stream drives the components along the channel and carries them to the detector. However, the FFF channel does not contain packing or stationary phase. Most FFF channels are parabolic flow profile thin chambers where the retention and separation is caused by an employed external field like a perpendicular liquid flow or centrifugal force. The field drives the particles and macromolecules of different size to different positions between the channel walls. In *normal* separation mode, smaller particles are located in the faster flow regimes and bigger particles are located in slower stream lines of the laminar flow inside the channel, thus smaller particles are transported faster than the bigger ones and elute earlier from the channel (Figure 1).[1]

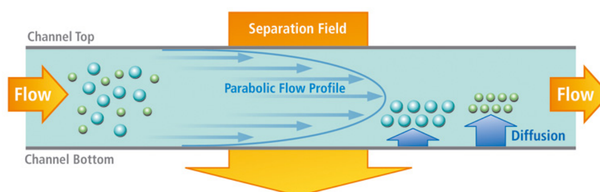


Figure 1: Working principle of the FFF separation

In case of asymmetric flow field flow fractionation (AF4) a crossflow field is the driving force for the separation. The crossflow passes through a membrane and the porous frit at the bottom of the channel. Membranes made of different materials (e.g. regenerated cellulose (RC), polyethersulfone (PES)) and characterized by different porosity (1, 5, 10 kDa) can be selected

accordingly to the material to be analyzed. Concentration of the eluting sample fractions is measured by an on-line coupled UV-Vis, MALS or RI detector.

If satisfactory fractionation conditions can be found, the separated sample fractions are close to the ideal monodispersed sample, and therefore they can be correctly analyzed by light scattering techniques. Thus, the flow-mode measurement, e.g. the on-line combination of an FFF technique and light scattering detector markedly improves the performance of the single light scattering detection technology (batch-mode measurement). Moreover, the sample fractions leaving the separation channel and the first detector (FFF-MALS) can be further analyzed with additional methods, like DLS, ICP-MSor other techniques.

2.1. Multi-Angle Light Scattering (MALS)

In a Multi-Angle Light Scattering (MALS) detector, a polarized, single frequency light beam is focused on the sample molecule or particle and the scattered light is detected with multiple detectors positioned at various different angles (Figure 2).

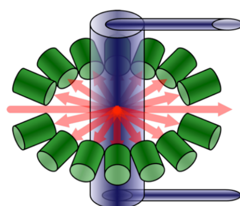


Figure 2: Vertical flow cell of a MALS instrument

In case of small molecules (radius <10 nm) the scattered light intensity has no angular dependence and it is proportional to the molar mass,the concentration (isotropic scatter) and the change in refractive index with change in concentration of the analyte, as reported in equation (1):

$$I \approx M_w * c * dn/dc \quad (1)$$

where:

c Solute concentration

M_w Weight-averaged molar mass

dn/dc = change in refractive index with change in concentration

In case of larger molecules and nanoparticles (radius >10 nm), more light is scattered in the forward direction than at larger detection angles. Measuring the angular dependence of the light scattering allows the calculation of the molecular weight (MW) and of the root mean square (rms) radius or radius of gyration (r_g). The rms radius is a measure of the molecule's size weighted by the mass distribution about its center of mass.

Multi Angle Light Scattering was described for dilute polymer solutions by Philip Wyatt[2] as follows.

$$K^*c/ R(\theta,c) = [1 /M_w P(\theta)] \ 2 A_2 c \quad (2)$$

where:

$R(\theta,c)$ Excess Rayleigh ratio of the solution (function of scattering angle θ and concentration c)

c Solute concentration

M_w Weight-averaged molar mass

A_2 Second virial coefficient in the virial expansion of the osmotic pressure

K^* Constant. $K^*= 4\pi^2(dn/dc)^2 n_0^2/(N_A\lambda_0^4)$

where:

N_A is the Avogadro's number

n_0 is the refraction index of the solvent

λ_0 is the wavelength of the laser

dn/dc = change in refractive index with change in concentration

$P(\theta)$ Shape factor representing the angular dependence of the scattered light related to the rms radius.

$P(\theta) \approx 1-2\mu^2\langle r_g^2\rangle/3!+\ldots$ where $\mu=(4\pi/ \lambda_0)\sin(\theta/2)$

From equations (1-2) is derived that the overall intensity carries information about the molar mass, while the angular dependence within the horizontal plane carries information about the size of the analyte. If sample concentration and dn/dc are known (e.g. thanks to an online RI detector), from espression (2) it is possible to calculate the average molecular weight of proteins and polymers. Moreover, for radius >10 nm the angular dependence expressed by P(θ) within the horizontal plane allows obtaining information about the size of the fractionated sample.To determine the size and the molecular weight of the molecule, the M_w and the mean square radius are calculated from the slope at θ = 0 of the measured ratios 1/R(θ,c) with respect to $\sin^2(\theta/2)$ by fitting the light scattered at different angles. Performing simultaneous measurements at multiple angles allows application of a Global Fit on all the angular data and finding the most accurate size and molar mass values.

3 Applicability and Limitations (Scope)

3.1 Application of FFF-MALS-RI-DLS

The fractionation of polydispersed nanoparticle drug formulations by FFF before light scattering analysis helps to overcome weaknesses that are present in batch mode experiments, where the large particles can hide smaller ones or the measured quantities are often averaged over the distribution of sizes present in the sample. On the other hand MALS analysis after separation helps to determine the size of particles for which elution times cannot be predicted simply using a set of column calibration standards. In case of online FFF-MALS the light scattering and concentration are measured for each eluting fraction, from which the molar mass and size can be determined for each elution position. This becomes extremely useful for complex samples (like NPs in serum containing medium) or for materials that might interact with the separation column's stationary phase and do not elute according to the *normal* separation mode. FFF-MALS-DLS-RI allows studying a broad range of physical-chemical properties of medical nanoparticles, including size distribution of complex mixtures, NP-protein interactions, batch to batch reproducibility (by NP size), the presence of free coating released from Med-NP surface in physiological buffers and the molecular weight of polymers composing polymeric Med-NP (polymeric NPs, dendrimers etc).

3.1.1 Nanoparticle size and NP-protein interactions

In most cases, Med-NPs are expected to avoid interaction with blood components as protein adsorption from blood might radically change their stability, fate and circulation time, having drastic effect on the bioavailability and pharmacokinetics of the carried or encapsulated drug molecules. One of the simplest ways to detect protein adsorption on nanoparticles is to measure the change in nanoparticle size after incubation in the presence of serum proteins. Unfortunately, the protein corona is practically invisible for imaging methods like TEM, and it is impossible to quantify with batch mode DLS, due to their intrinsic limitations with the analysis of samples possessing broad or multi-modal distribution, especially in complex sample mixtures and in physiological media containing serum proteins. In this context, dynamic light scattering (DLS) and multi angle light scattering (MALS) combined an on-line downstream of separation methods like Field-Flow Fractionation (FFF) allow to provide accurate size information for poly-dispersed samples in physiological media and for protein binding studies.

FFF-DLS-MALS is also a better reliable technique than DLS and TEM to check batch to batch reproducibility. In this context it may be useful to compare if different batches are characterized by the same elution profile, and thus if they possess the same size distribution and the same average size. Also in this context, MALS-DLS detectors downstream to the FFF separation process allow discriminating between small but significant change in size that can not be resolved with batch mode DLS, especially for poly-dispersed samples.

For the analysis of NP size by FFF-MALS the presence of online concentration detectors such as RI and UV-VIS is not strictly necessary, since size analysis do not need information of NP concentration or of the dn/dc (see session 7.1).

3.1.2 Analysis of molecular weight: the presence of free NP coating

To avoid interaction with the immune-system, Med-NPs are typically covered by a shell of polyethylene glycol (PEG). However sometime, part of the PEG shell is released from the surface of Med-NPs, inducing immune reaction and thus, impacting their safety. The release of free PEG from NPs dispersed in physiological buffer (e.g. PBS), can be analyze by FFF-MALS-RI. Thanks to the presence of online concentration detectors such as RI and UV-VIS detectors, the amount of free coating can also be quantified, by producing a calibration curve with the appropriate standards/precursors.

3.1.3: Analysis of molecular weight: analysis of the polymeric components of the carrier

For polymeric Med-NPs, the characteristics of their polymeric components (composition, mean molecular weight (MW) are to be analyzed. FFF combined with MALS and refractive index (RI) detector can be used, in alternative to size exclusion chromatography (SEC), to determine the mean MW weight of polymers composing the Med-NPs. For the analysis of MW of an unknown polymer, the presence of a RI detector is required to calculate the dn/dc (see section 7.2 and appendix 2).

3.2 Limitations of FFF-MALS

Theoretically, FFF is able to separate particles in the 1-1000 nm size range. However, for good separation performance FFF parameters (including choice of buffers, membrane, liquid flows, and channel geometry) have to be carefully optimized, requiring efforts in method development for every molecule to be analyze. Resolution, fractionating power and retention on the membrane have to be balanced with practically acceptable analysis times in each case.

The Wyatt Dawn Heleos II MALS instrument is theoretically able to determine molar masses from 200 Da to 1 GDa and radii from 10 – 500 nm. However, the uncertainty of the size measurement is not the same over the covered size range, calculated mass and radius values also depend on model input parameters and the detector has to be aligned and normalized using standards for each measurement series. A standard should be used to normalize MALS each time solvent is changed. BSA can be used for measurements series in aqueous solvents (such as PBS), while dextran should be used with aqueous buffer with surfactants (e.g. SDS), and polystyrene should be used with mixed organic solvents.

4 Related Documents

Table 1: Related documents

Document ID	Document Title
EU_NCL_PCC_01	Measuring batch mode DLS
EU_NCL_PCC_21	Measuring NP Aggregation Propensities by batch mode DLS
EU_NCL_PCC_02	Measuring Zeta Potential
EU_NCL_PCC_013	Measuring the pH of Nanoparticle Suspensions

5 Equipment and Reagents

5.1 Equipment

- Postnova AF2000, Wyatt Eclipse AF4 or similar instrument
- UV detector
- Wyatt Dawn Heleos II MALS, Postnova PN2136 MALS or similar instrument
- Optilab (U)T-rEX Refractive index (RI) detector (or similar)
- (Optional: Malvern Zetasizer Nano ZS instrument, or similar)

5.2 Reagents

- Membrane for the FFF channel: for most application it is suggested to use 10 kDa cut-off regenerated cellulose membrane (from instrument manufacturer)
- Bovine serum albumin monomer (BSA, ≥98%, **A1900** Sigma-Aldrich)
- Dextran 10-30kDa, analytical standard for GPC (e.g. from Sigma Aldrich)
- Novachem (100%, Postnova Analytics), or SDS 0.05% buffer
- Phosphate buffered saline solution (PBS, pH 7.4). It is the recommended mobile phase for measurements with Med-NPs and for water soluble analytes as it is the mostly relevant physiological buffer. Other specific buffers may be used for the analysis of specific analytes (e.g. polymers soluble in organic solvents).
- Fetal bovine serum (FBS)
- Polystyrene nanoparticle suspension (relevant size range, NIST treaceble size standards)
- Other standards for the analysis of MW to be selected case by case

5.3 Reagent Preparation

PBS

Standard 1x (pH 7.4) PBS solution has to be freshly prepared and filtered with 0.2 µm filter before using it as a mobile phase in FFF or buffer in the protein binding experiment. The pH of the filtered solution should be checked according to the general procedure described in the procedure "Measuring the pH of Nanoparticle Suspensions". The filtered solution can be stored at room temperature for 2 days or in refrigerator for 1 week. If taken from the refrigerator, the solution has to be incubated to reach room temperature before introducing into the FFF system.

5 mg/mL BSA solution

The BSA solution (used for normalization of the MALS detector) has to be prepared freshly before the experiment by measuring 5 mg of BSA in an Eppendorf tube and dissolving it in 1 mL of (0.2 µm) filtered PBS. The solution should not be filtered before use.*0.2% Novachem solution*

The 0.2% Novachem solution (used as mobile phase for PLS NPs in validation experiments) has to be prepared freshly before the experiment by adding 2 mL of 100% Novachem

surfactant mixture in 1 L of MilliQ water. The solution should be filtered with a 0.2 µm filter before using it as a mobile phase in an FFF experiment. The filtered solution can be stored at room temperature for 2 days or in refrigerator for 1 week. (If taken from the refrigerator, the solution has to be incubated to reach room temperature before introducing into the FFF system.)

0.05% SDS solution

0.05 % SDS solution (optionally used as mobile phase for PLS NPs in validation experiments) has to be prepared freshly before the experiment. The 0.05% SDS solution can be prepared by dissolving 0.5 g of SDS in 1 L of MilliQ water The solution should be filtered with a 0.2 µm filter before using it as a mobile phase in an FFF experiment. The filtered solution can be stored at room temperature for 2 days or in refrigerator for 1 week. (If taken from the refrigerator, the solution has to be incubated to reach room temperature before introducing into the FFF system.)

Dextran solution in Novachem 0.2% or 0.05% SDS solution

The dextran solution (used for normalization of the MALS detector) has to be prepared freshly before the experiment by measuring 15 mg of Dextran powder in an Eppendorf tube and dissolving it in 1 mL of (0.2 µm) in Novachem 0.2% or SDS 0.05% filtered buffer. The solution should not be filtered before use.

6 Procedure

6.1 General remarks for FFF separation

In order to optimize the FFF separation, parameters of the method has to be adjusted according to the actual properties of the NPs to be analyzed. Ideal method parameters will also always depend on channel geometry, membrane (and frit) material and instrument type. Tubing and on-line coupled detector(s) will determine the back-pressure that also has effect on the separation.

In AF4, the surface charge of the analyte will affect their interaction with the membrane (the bottom part of the channel). Particles or molecules might be temporarily adsorbed or permanently captured on the membrane surface depending on the charge of the membrane and theanalyte. Changing pH and ionic strength of the mobile phase can help to find ideal conditions. Adsorption of the analytemight still happen, but pre-conditioning of the membrane with the sample can stabilize the separation results.

Together with electrostatic interactions, as expected, the size of the particles will have a strong effect on their retention time. Total time of the measurement might change from a couple of minutes to a couple of hours depending on the sample properties. Tip-flow, focus-flow, channel geometry, channel spacer and cross-flow parameters have to be optimized to get good separation from the void peak (NPs or other components leaving the column at the beginning of the measurement without being properly separated) and to reach maximum

recovery in the main peak (minimum amount of material retained on the membrane when reaching 0 cross-flow, Figure 6 in Annex). Moreover, analysis time should be kept reasonably low.

Tip-flow is usually kept fixed and should be compatible with the on-line coupled measurement technique. For on-line DLS measurements detector flow values below 0.5 mL/min are recommended.

As no detailed SOP can be generated for the FFF-MALS measurement of previously unknown samples, validating measurements have to be performed and reported for each study using the actual instrument setup (membrane, tubing, detectors).

6.2 Flow chart

The workflow in the next page summarizes the main steps of the iterative FFF-MALS method development and measurement process for studying Med-NPs size distribution and NP-protein interactions, as an example However, very similar procedures should be followed for other applications.

Preliminary considerations: Choosing the parameters for the experiments

Buffer to be used

In a typical case, PBS is selected as mobile phase. It will be the case for the analysis of Med-NPs size (batch to batch variability, protein interaction), for the analysis of the release of the free coating for the NPs surface, but also for the analysis of polymeric components which are soluble in aqueous buffers.

For the analysis of polymers soluble in organic solvents, a mixture of an organic solvent and water or a pure organic eluent should be selected. In case of the analysis of the molecular weight of components of polymerc NPs, the Med-NPs have to be first solubilized in an organic solvent mixture (usually in organic solvents such as 50-75% ACN or 50% methanol). Solubilisation protocols can be checked easily by running DLS on the NP in different % organic solvents to make sure NPs no longer exist but rather just the polymer in its free form. Be sure to have an FFF module compatible with organic solvents to run those experiments. If not, for this kind of analysis size exclusion chromatograhy (SEC) coupled with MALS and RI detectors is suggested as an alternative separation technique.

Selecting the membrane and the channel geometry

Before the first experiment a new membrane is mounted in the FFF channel and the system is extensively washed with the chosen running buffer. For most applications in PBS a 10 kDa membrane of regenerative cellulose (RC) is the best choice. For unknown samples it is suggested to start by using a spacer of 350 µm, which works very well with a broad range of analytes (from proteins to big NPs). Different spacers can be selected in specific cases. The value of the elution flow is fixed and always determined by the configuration of your system.

Step 1: MALS detector normalization

The MALS detector generates voltage values that are proportional to light intensities. It should be calibrated to be able to calculate R(θ) values from light intensities. The calibration is done at 90° using a isotropic molecule that provides a large scattering signal (e.g. pure toluene). As long as the system is left undisturbed it is not necessary to recalibrate, but recalibration is needed after changing the sample cell or realignment of the laser.

As each detector has its own geometrical factor and angular sensitivity, normalization has to be done determining the normalization coefficients for each detector using the same flow rate and the same solvent that are used for the separation measurement. For this purpose an isotropic scatterer (like BSA monomer or 20-30 kDa polymers) has to be used. The normalization coefficient for the 90° detector is 1.0, while the other detectors are adjusted by varying amounts to yield identical values of R(θ) at all angles.

The first FFF-MALS experiment using a suitable isotropic standard for your eluent (such as 5 mg/mL BSA in PBS)helps to verify the proper operation of the equipment and provides data for the MALS detector alignment and normalization. The analysis is also used to to correct for band broadening induced by the multi-detector configuration. Those three operations allow creating a new method in Astra software (see Astra guideline) that should be selected for data analysis of LS data. You should create a method following the same procedure for each different solvent you are using, choosing a proper standard for each solvent (e.g. 10-30 kDa dextran in SDS 0.05% or Novachem 0.2%, 10-30 kDa polystyrene in organic solvents). Once the method has been created it can be used for data analysis in other measurement sessions provided that the set-up of your system is not modified (change of connections, maintenance of the detectors, etc). If the creation of a new method is not necessary always run a standard at the beginning of each measurement session (three repeated injections) to check MALS normalization (see acceptance criteria in section 8).

Before running your sample the membrane in the channel needs to be passivated. For this reason, it is suggested to run at least 3 injections of the standard used to check MALS normalization, before starting to inject your sample.

Step 2: Method optimization

Using the chemical properties, zeta potential, DLS diameter, UV-Vis spectrum and other input data already known about the analyte, the operator selects the starting FFF method, detection parameters and particle concentration and performs the iterative process leading to an optimized separation method. The optimized method allows a clear separation of the main sample peak from the void peak, with maximized recovery under reasonably short analysis time.

While optimizing the method for your analyte in a very complex mixture of many molecules/particles, it is often useful to focus only on optimizing the peak/peaks of interest. For example, when analyzing NPs size distribution and NPs-proteins interaction, method optimization focus on obtaining symmetric peaks of non-treated Med-NP populations, which

should well separed from the void peak to allow calculating NPs size. On the other hand, if the presence of free coating (e.g. free PEG) released by the surface of Med-NPs is of interest, method optimization should aim at obtaining a clear separation of the peak of free PEG molecules from the void (e.g. using high cross flow and focus flow such as 3 mL/min), rather than on the NP elution profile.

Optimization of cross flow and focus flow

The strength of the cross flow and focus flow field are probably the most important separation variables and they have to be carefully optimized. They have a strong effect on resolution and can be varied widely and rapidly without need to change the channel geometry. If too a low cross flow is applied during focus (focus flow) the lower-molar mass/size fractions will elute immediately after the elution mode is switched on and will appear unresolved from the void peak at the beginning of the fractogram. If this happens, increase the focus flow until you reach a suitable separation from the void peak (at least 2 minutes).

Cross flow rate during elution may starts by applying the same value imposed during focus (even thought this is not mandatory!) and can be programmed, creating a specific elution method for each run. It can either be kept constant during the entire elution time (isocratic elution) or it can be decreased (linearly or exponentially, gradient elution) from a starting value to 0 mL/min in a defined amount of time. A rule of thumb is to start by analyzing an unknown sample using a gradient elution profile, from a cross flow which allows sufficient retention of early components of interest (a starting value can be selected from the literature) and then to decrease it to very low values (0.1 or 0 mL/min) in roughly 30 minutes. If your analyte elutes with the void peak increase the starting cross flow. When satisfactory resolution has been obtained by selecting the cross flow and the focus flow values and a good separation from the void peak is reached, increasing the focus flow and the cross flow will not lead too significant increase in resolution (size and molar mass values calculated will be the same!), but it can lead to sample loss. Too high focus and/or cross flow are to be avoided because, they push too hard the sample through to the thin, concentrated layer near the accumulation wall causing interactions of sample with the membrane, sample aggregation, or mutual entanglement of the analyte. This effect may lead to distortion of the elution peak and/or to sample loss.

After you have defined a satisfactory gradient elution method, you can try to perform an isocratic elution choosing as constant cross flow the value of cross flow where the peak of your eluted sample is localized. Isocratic elution experiments are generally preferred for the separation of simple mixtures, such as proteins (e.g. BSA) containing dimers and trimers without the presence of bigger aggregates. Two isocratic steps at very different crossflows can also be chosen if two populations which significantly differs in have to be fractionated (e.g. NPs separation from free proteins/free PEG coating).

How to check for sample loss (perform only if needed)

If you think you are experiencing sample loss in your analysis you can check sample recovery by performing a direct injection (no focus step and 0 mL/min cross flow applied during

elution). By calculating the ratio between the total concentration of the sample eluted with your method and the concentration of the sample eluted by a direct injection the % of sample recovery can be calculated. To reduce sample loss you may try to reduce the focus flow and the cross flow in your elution profile. In some cases, it is necessary to change the eluent/the membrane to avoid the intrinsic interactions of the sample with the membrane.

How to select sample concentration

Sample concentration of your analyte should be carefully chosen. A good signal to noise ratio should be obtained, carefully avoiding overloading of the channel. At very high concentrations the mutual interference between NPs near the accumulation wall may result in steric effects, reversing the fractionation order: bigger NPs will elute first, perturbing the shape of the fractogram. As a rule of thumb, total injected mass of NPs should not be higher than 200 µg. However, if you have the feeling that your fractogram may be perturbed by steric effects, (possible overloading of the channel) it is suggested to reduce the concentration and compare the elution profile of your analyte with different mass amounts to select the right concentration range.

Check for repeatability of your elution profile

When all the conditions of your measurement have been optimize, run three repeated injection of your sample to check for reproducibility of your elution profile. This step is very useful to check that the method created is reliable and that your sample is stable in the time-frame needed for the measurement.

How to proceed after method optimization has been completed

After method optimization step has been completed, if NP-protein interaction is of interest for your experiment proceed to step 3 and then to step 4, as reported above. If you are interested in the analysis of size and/or of molecular weight, skip step 3 and perform step 4. After you have done that proceed to data analysis, as explained in the data analysis section (section 7).

To calculate the molecular weight of your analyte from LS data (equation 2) you need to know the concentration of your sample in all the peaks of interest and the dn/dc value in the conditions of measurements (e.g. same solvent, same wavelenght and same concentration range). The concentration of your analyte can be determined directly by ASTRA software in the whole fractogram range, by using the signal recorded by an UV-VIS or by an RI detector. Astra software allows to choose which concentration detector you want to use in the instrument configuration panel (see Astra manual). To calculate the concentration of your analyte, the dn/dc need to be known if you are using the signal from an RI detector, while if you are using an UV-VIS detector, the value of the extinction of your analyte is needed. Since dn/dc needs to be always determined in your experimental conditions by using an RI detector for fitting equation (2), in this context the RI detector is preferred over the UV-VIS detector, but this is not mandatory. For further information on how to determine the dn/dc in your experimental conditions consult the Annex 2 (batch dn/dc measurement with an RI detector).

Step 3: Protein binding studies

This step has to be performed if your are interested in studying NPs-protein interaction. The FFF-MALS analysis for both non-treated and serum-preincubated NPs is performed at the same, previously chosen concentration. In the protein binding experiments, the NP formulation is pre-incubated in serum for 1 h at 37 °C. The particle suspension can be further diluted in PBS immediately before injecting into the FFF system, to reach the concentration chosen during method optimization. Specific concentration for NP dilution in serum and then in PBS have to be chosen to reach a final concentration of 10% FBS in PBS.

Step 4: Quality control of FFF system with PLS spheres

When all the FFF measurement of one sample are completed, FFF and MALS should be checked by injecting polystyrene standard, using Novachem 0.2% or SDS 0.05% as a mobile phase. Perform this procedure if you are measuring Med-NPs size distribution. If you are interested in measuring the MW of your analyte, MALS should be checked at the end of the measurement by injecting a GPC analytical standard in the proper buffer (PEG or dextran for the analysis in aqueous solvents, polystyrene for analysis in methanol or ACN, etc).

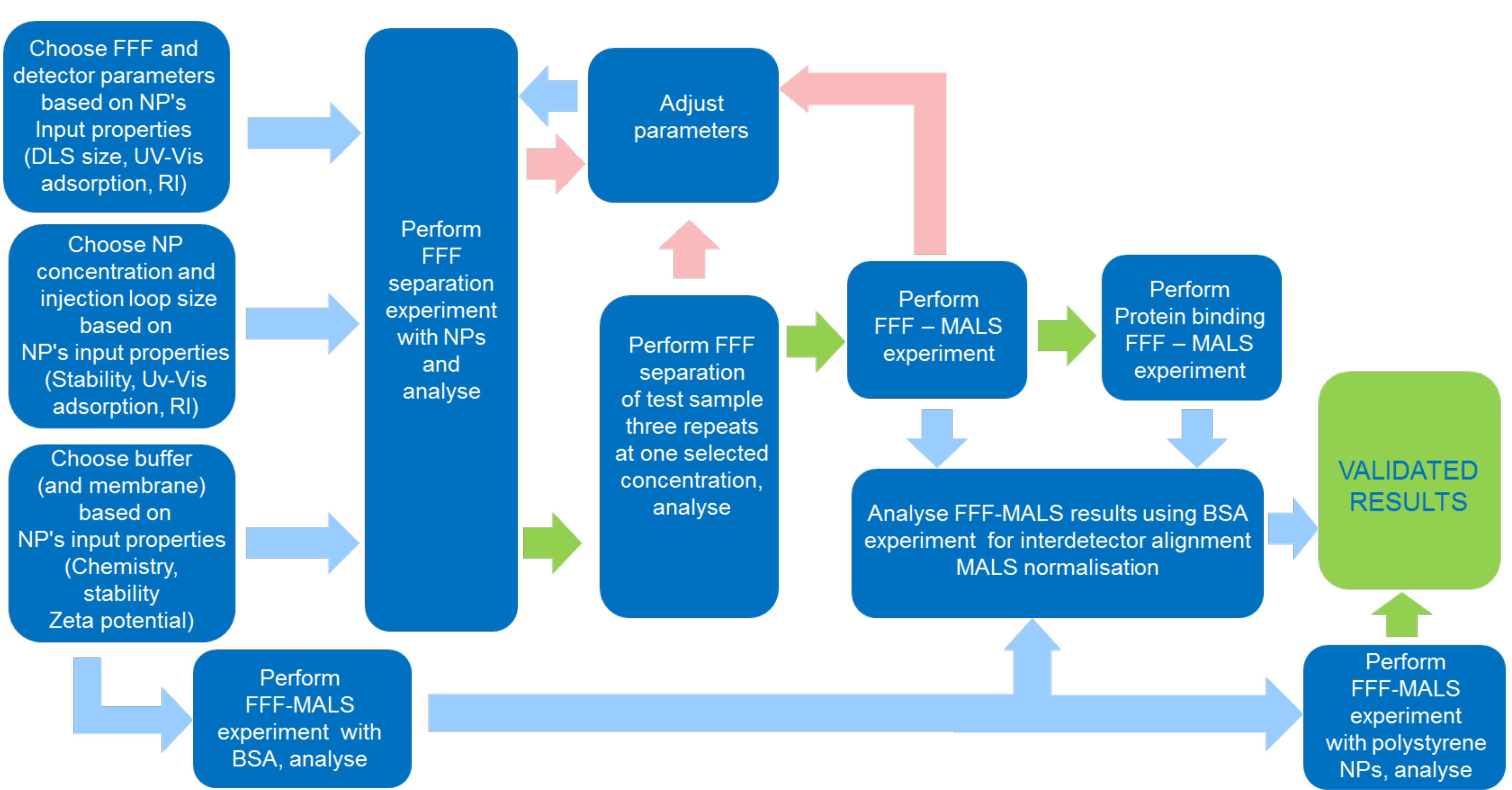


Figure 3: Brief outline of the workflow for the measurement of NPs size and NPs-protein interations in physiological relevant media

Blue arrows represent data input, red arrows negative results, while green arrows successful experiments, with results allowing stepping forward in the series of experiments

7 Data Analysis

This session describes the analysis performed by Astra software (version 6.0) for MALS detector provided by Wyatt technology. For more detailed information consult Astra software user guide. If a different set of MALS and RI detector are used, consult their specific user guides.

Before performing the analysis of data from LS you should:

- Select the method to be used for data analysis of LS data. A recent normalization with a standard in the same eluent used for the analysis should be chosen;
- select the concentration detector you wish to use (mandatory for the analysis of molecular weight)

To perform analysis of your LS data follow these steps:

- define the baseline of your profiles for each of the LS angles,
- perform despiking of your fractograms to reduce spurious noise,
- enter in the software the peak properties needed for your experiment (e.g. dn/dc, extinction coefficient, A2 (A2=0 for diluted samples), RI of the sample, absorbance of the sample….)
- select the peak/peaks you want to analyze (work on LS-11). Usually it is a good procedure to select the whole area of each peak of interest to calculate and show the distribution of size and MW, but then to report the average values calculated in the full with of half maximum (FWHM) range.

7.1 Radius from LS data

If you are interested in calculating the size distribution of your particles (and not the MW of your analyte) select the procedure "Radius from LS data" from Astra software. This procedure calculates the radius of the sample based on the light scattering signal alone (concentration is not measured and *dn/dc* need not be known in order to determine size). It is assumed further that the particle concentrations are low enough that there are no interactions of consequence between them, and therefore the 2nd virial coefficient A2 is assumed to be zero. Most importantly, it is assumed that the particles present have been fractionated, so that each slice contains particles of identical size.

Analysis of the variation of the scattered intensity with angle is made. It is very important to select the right model to fit the LS from different angles, according to the NPs shape, since the angular variation of scattering light is matched to an assumed particle shape to obtain a corresponding size.

Particle size (rms radius or geometric radius) is derived based on a structure known *a priori*, plotting *R*(θ) vs. sin2*(θ/2)is plot* and replacing the theoretical form factor *P*(θ) by the appropriate model assumed. Form factor models have been derived for spheres, coated spheres, and rods. In case the "sphere model" is used knowledge of the RI and of the absorbance of the sample is requested. This model can be used for homogenous spheres, such as polystyrene NPs In case the coated sphere model is used (e.g. for liposomes) knowledge of the RI and of the absorbance of both the sample and the coating+ the thickness of the coating are requested. Note that the sphere and coated sphere models yield geometric radius, while the rod model produces a length.

7.2 Radius and molar mass from LS data

For calculating both size and MW of your analyte select the procedure “Radius and molar mass from LS data”. Choose the appropriate model to fit your data. Knoledge of concentration and dn/dc values are requested to fit equation (2) in ordert to determine MW. For diluted analytes A2=0.

The light-scattering fit models are as follows:

Zimm model: uses the K*c/R(θ) formalism for equation (2). It should be used for molecules that have RMS radii smaller than 50 nm and that do not conform to another standard model such as random coil or sphere. The Zimm model has the advantage over the Debye model in that it often requires a lower fit degree for the same size molecule. For large (>50 nm) molecules, the Zimm model should not be used.

Debye model: Uses the R(θ)/K*c formalism for equation (2). It gives better results over a wider range of molar mass, including the very large (greater than ~106 Daltons or ~100 nm RMS radius). But you may need to delete high angle detectors to improve the fit of the extrapolation since the curvature can be very large.

Berry model: Uses the sqrt(K*c/R(θ)) formalism. It can be useful, in combination with deleting high angle data, when analyzing molecules with RMS radii greater than 50 nm.

Random coil model: Uses the formula for a theoretical random coil molecule rather than a polynomial to fit the angular light scattering data.

8 Quality Control, Quality Assurance, Acceptance Criteria

The first separation experiment with an isotropic standard verifiesthe proper operation of the FFF instrument, the detectors and the integrity of the membrane. The elution profile used for each standard should be carefully optimized. For BSA for example, results are accepted if separation of monomers from dimers is reached and the measurement allows the alignment and normalization of the MALS detector. Calculated mass value for the BSA monomer should be in the 66.5 kDa ± 15% range. If a different standard is used variation in the average calculated value with respect to the theoretical value should be <15 %.

Separation method for thetest materials are accepted if clear separation from the void is reached, and the ratio of particles retained on the membrane after reaching 0 crossflow is minimized.

If extensive sample loss is suspected, the amount of sample loss on the membrane can be calculated and reported by comparing the peak integrals obtained without and with cross flow. In case of very complex mixtures, such as the serum containing NPsthe perfect separation of the protein peak from the void and from the peak of the particles is often not possible. In those cases optimization of the elution method should be focused on the peak associated with the analyte of interest. If NPs size in presence of proteins is the main focus of the analysis, NPs should be separated from protein peaks, allowing interference-free evaluation of the LS data. If the presence of free coating in a NPs dispersion is of interest, on the other hand, the signal of the free coating should be well separated from the void to allow analysis of LS data, while there is no need to optimize the elution profile of the NPs (no need to analyze NPs size in this particular experiment).

The FFF method is validated by demonstrating that the separation is reproducible (three runs at a selected concentration are to be performed to confirm reproducibility).

In order to validate the MALS based size measurements in the relevant size range, positive control experiment is performed, e.g. using polystyrene particles with known diameter if you are analyzing NP size distribution and NPs-protein interactions. In order to avoid undesired interaction between the surfactant and the test sample, this run is performed after the measurement series with the nanoparticle formulation. For PLS standard the mobile phase is changed to 0.2% Novachem solution or 0.05% SDS, the positive control experiment is performed, and the data analysis is done using the proper alignment and normalization(e.g. e.g. based on dextran in SDS 0.05% or 0.2% Novachem). Calculated geometrical radii for the polystyrene spheres should be in the ± 15% range

9 Additional measurements

9.1 UV-VIS spectra

An UV-Vis spectra should be measured prior to the analysis to check for absorption of your sample in the 200-800 nm range. Knowledge of specific sample absorption allows to select the proper wavelength in the UV-VIS detector. No absorption in the specific wavelength used in the MALS and DLS detector should be present to avoid interferences with the analysis.

9.2 Online DLS analysis

In addition to size measurements by MALS, a dynamic light scattering detector (e.g. Malver zeta sizer) can be put online to the FFF module to determine the hydrodynamic diameters of the sample after fractionation. To know how to select the proper SOP and to perform data analysis refer to the EU-NCL_PCC001.

10 Reporting

Besides of the final size and molecular weightresults determined by the FFF-MALS analysis for the test sample, all method details and validating measurements have to be reported. The report should contain:

- Method details of the standard run for normalization of MALS (Table 2. In Annex) including normalization values used in the further calculations.
- Results of the standard alignment and normalization run in figure format (FFF elugram and light scattering vs. time or volume, Figure 4 in Annex) and the determined molecular mass;
- Method details of the positive control run, e.g.with PLS particles or polymeric standard;
- Results of the positive control run in figure format (FFF elugram and light scattering vs. time or volume) and the determined diameter or MW;
- Method details optimized for each test sample;

- Results of the three repeated runs at one chosen concentration in figure format for each test sample (Figure 7);

- Results of the FFF-MALS run for each experiment runin figure format (Figure 8 and 9), including model used for fitting the LS data

-

- Average size and/or MWof eachtest sample in table format;

- Conclusion about the results.

Typical method description and figure examples for the analysis of NPs size and NPs-protein interactions are shown in the Annex at the end of the document.

11 Health and Safety Warnings, Cautions and Waste Treatment

Samples should be prepared with care to protect the sample from particulate and to minimize exposure; appropriate safety precautions and protective gear such as gloves, lab coat and goggles must be worn. After the measurement the samples and liquid waste (eluted mobile phase containing test sample) should be discharged as appropriate for nanomaterials.

12 Abbreviations

AF4 asymmetric flow field flow fractionation

BSA bovine serum albumin

DLS dynamic light scattering

FBS fetal bovine serum

FFF field-flow fractionation

MALS multi angle light scattering

Med-NPs: Medical nanoparticles

MW: Molecular weight

NP nanoparticle

PBS phosphate buffered saline solution

PEG: Polyethylene Glycol

PLS polystyrene (latex)

RI: refractive index

13 References

[1] M. Schimpf, K. Caldwell, J. Calvin Giddings (Eds.), *Field Flow Fractionation Handbook*, Wiley Interscience, 2000

[2] P.J. Wyatt, Light scattering and the absolute characterization of macromolecules. *Analytica Chimica Acta* 1993, 272, 1–40.

[3] S. Podzimek, Light scattering, size exclusion chromatography and asymmetric flow field flow fractionation. John Wiley&Sons Publication, 2011.

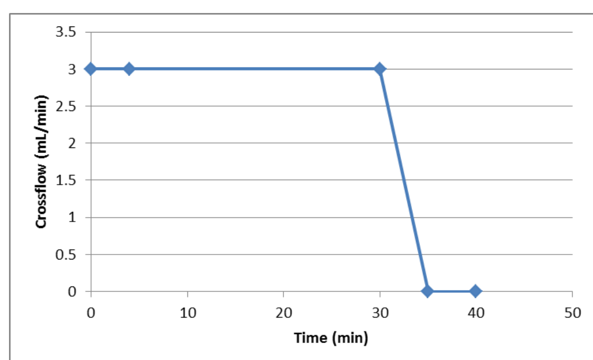
[4] User manual of ASTRA software (Wyatt Technology)

12 Annex 1

Typical figures and method description of a Protein binding report

Table 2: Table describing FFF separation method details for the BSA separation

Solvent	PBS
Spacer (µm)	350
Membrane	Reg. Cellulose, 10 kDa
Detector flow (mL/min)	0.5
Injection flow (mL/min)	0.2
Focus (mL/min)	3.3
Crosflow (mL/min)	3
Injected volume (µL)	20
BSA concentration (mg/mL)	5



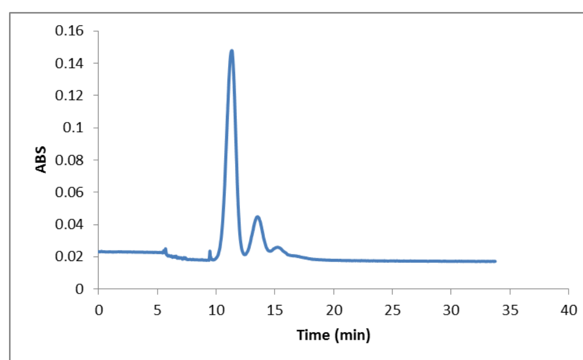


Figure 4: FFF separation method and UV-Vis signal of separated BSA peaks

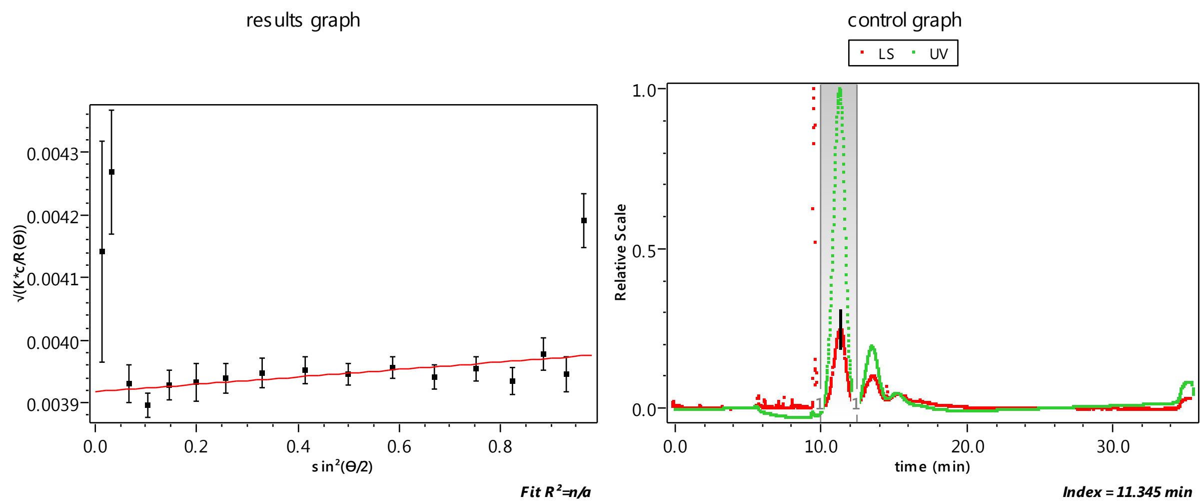


Figure 5: MALS results (left: fitting with Zimm model, right: UV-VIS (green) and MALS (red) signal of the separated BSA sample

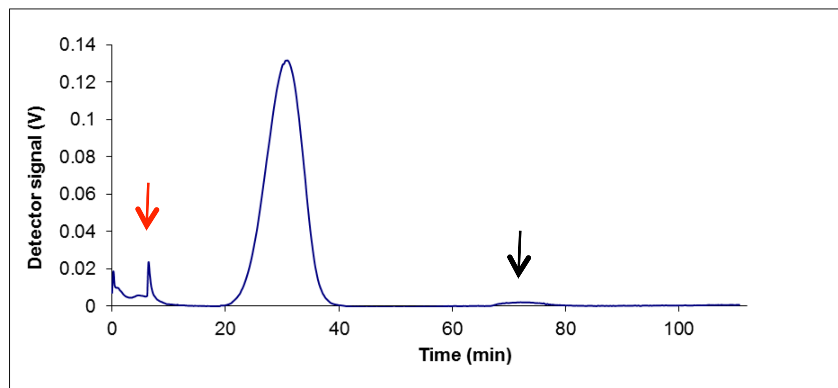


Figure 6: FFF elugram of the test sample: void peak labeled with red, retained material (released after reaching zero cross-flow) labeled with black arrow.

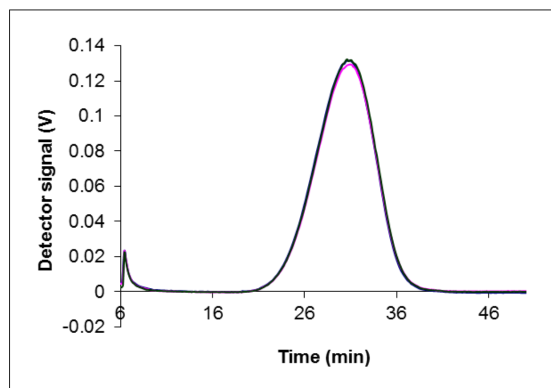


Figure 7: FFF elugrams of the test sample: three repeats at a chosen concentration.

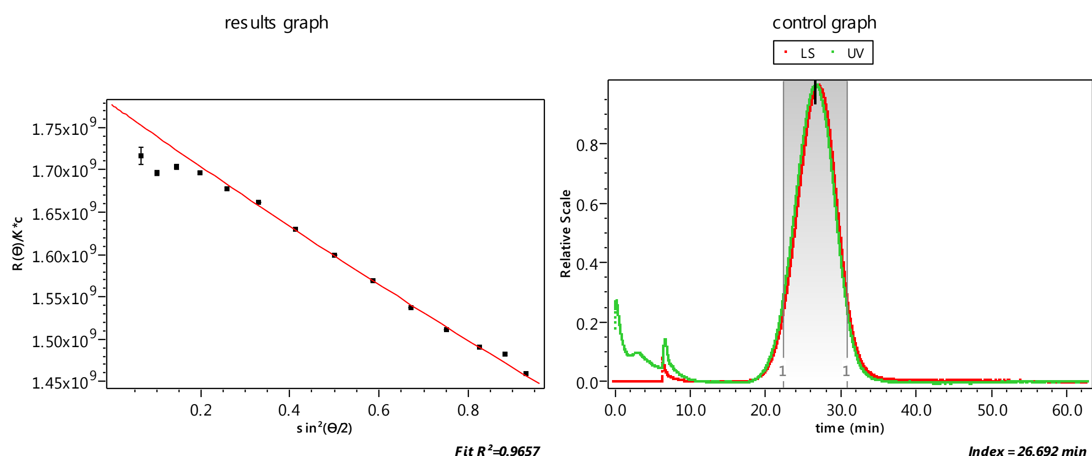


Figure 8: MALS results (left: fitting with "Coated sphere" model, right: UV-VIS (green) and MALS (red) signal of the test sample vesicles

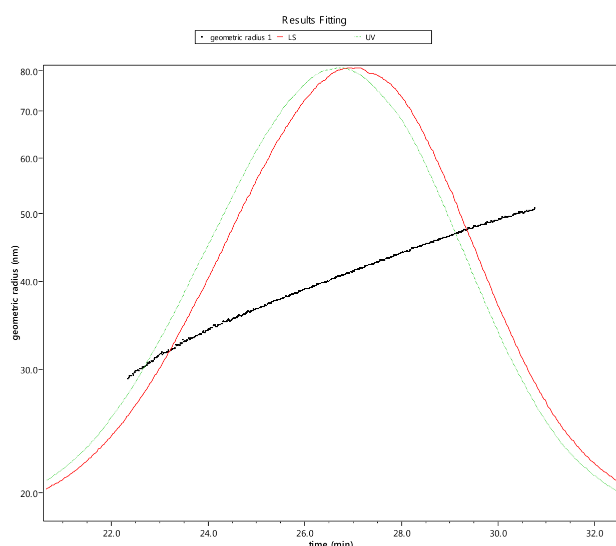


Figure 9: UV-Vis (green) , MALS (red) signal and calculated geometric radius (black) of the test sample vesicles

13 Annex 2: Batch dn/dc measurements

13.1 Introduction and principle of the method

This appendix describes how to determine the refractive index increment (dn/dc), i.e. the change in refractive index with change in concentration. It is based on a technical note from Wyatt using an Optilab (U)T-rEX differential refractive index detector [R]. A molecule's dn/dc is needed to calculate its molar mass by light scattering, regardless of the concentration detector used. This protocol is based on batch injections with increasing concentrations of the molecule of interest in order to determine the corresponding change in refractive index. The collected data can be analyzed by performing a linear fit using ASTRA software.

13.2 Applicability and limitations

In setting up a batch dn/dc measurement, the following criteria should be followed:

- The measurement should be obtained in the same solvent in which your sample is
- dissolved for the light scattering measurement.

- The measurement should be performed at the wavelength of your light scattering detector.

- For polymers, dn/dc may depend on the molar mass of the polymer. Typically, an increase in molar mass will increase dn/dc but above a certain molar mass (typically 5-10 kDa), dn/dc can be considered constant. Make sure the molar mass of the sample used for dn/dc measurements is similar to that used for the light scattering measurement or that no molar mass dependence of dn/dc is observed.

13.3 Equipment and reagents

Optilab (U)T-rEX detector

Syringe of 2-3 mL (if manual injection is performed) or Syringes pump

5 different known concentrations of your sample in the exact same buffer or solvent that will be used in the light scattering experiment (typically between ~ 0.1 mg/mL to ~ 1-2 mg/mL). Prepare all concentrations by diluting a single stock concentration using proper calibrated pipettes. Weigh your buffers rather than relying on pipetting volumes. If the sample is a solid that could contain solvent or water you may need to dry (e.g. by freez-drying) the sample before you weigh it.

13.4 Experimental procedure

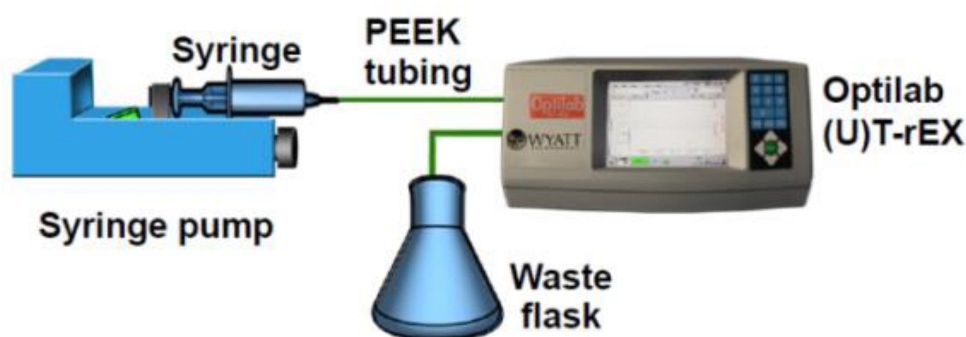


Figure 2: Experimental set-up

- *Clean and equilibrate the RI detector*

Prior to the analysis, purge RI detector with water for 10 minutes. Then flow a few mL of the solvent used to prepare your samples by manually injecting it or by using a using a syringe pump while the RI purge valve is set to "on" to ensure that the reference cell contains pure solvent. After injecting 3-5 mL, exit purge mode by pushing the purge button again.

- *Set up the experiment in ASTRA*

Open a new experiment from method. Click **File → New → Experiment from Method**. Navigate to **System → RI Measurement** and click on **batch (determine dndc)**. Ensure that your Optilab instrument is selected under **Configuration** as the Physical Instrument in the Optilab (U)T-rEX window.

- *Register a proper baseline*

Start the play bottom in Astra to start recording the RI signal. Then start injecting the pure solvent long enough to define a proper baseline characterized by a stable signal. The volume of the sample injected must be large enough so that the flow cell is saturated with the sample and the RI detector output voltage reaches a stable value; this volume will be approximately 0.5-1 mL. If manually injecting do not push too hard to avoid damages on the detector cell. Pressure in the RI detector should always be max to 1 Bar. If using a siringe pump a flow of 0.1-0.5 mL/min is recommended and injection can be performed for 2-3 minutes.

- *Inject the standard solutions*

Proceed with injecting the standard solutions from the lowest to the highest concentration, and then finish by injecting the pure solvent once again. Follow the same recommendations reported for the creation of the baseline. A stable signal for at least 3 minutes should be reached for each concentration. Use a new disposable syringe for each injection.

To proper record the experiment in ASTRA, always pause the experiments while changing from a solution to another, and then start it again while a new injection is ready to be performed.

13.5 Data analysis

- Begin processing the data by setting the baseline under the **Baseline** procedure.
- Define the peaks and enter the known concentrations for each injected concentration under **Peaks** procedure, as shown in Figure 3.

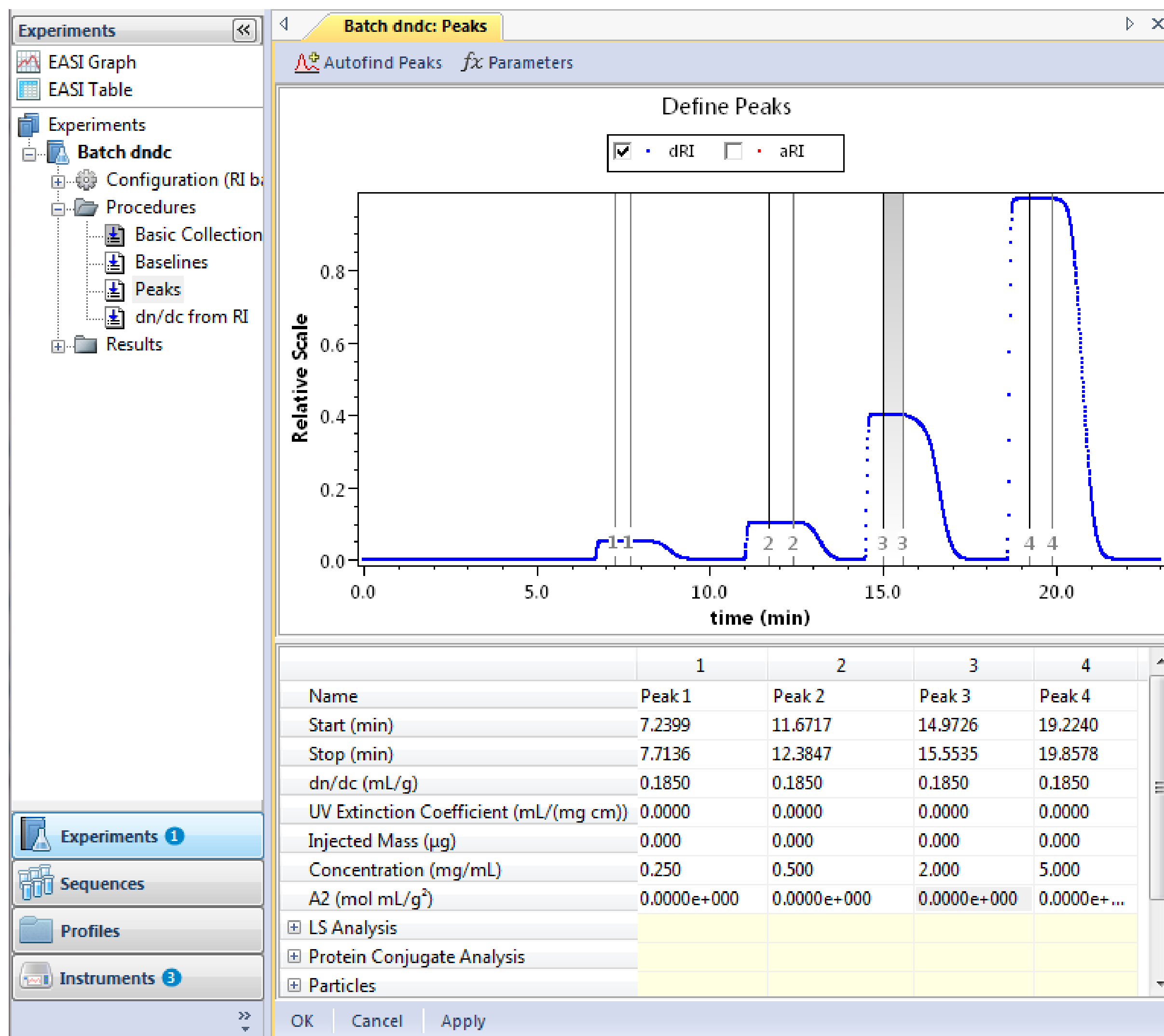


Figure 3: Screen-shot of data analysis by ASTRA software

- Double click on the **dn/dc from RI** procedure to perform the fit of the the differential refractive index versus concentration plot. In this way the dn/dc value is calculated. Be careful: the intercept of the linear fit must be zero. If the linear fit does not pass through (0,0), it is an indication of poor buffer matching between the buffer used to set the baseline and the buffer used to prepare the samples or the stock sample buffer and the dilution buffer.

13.6 Data reporting

- Describe how you prepare the different solutions of your analyte
- Report the collected dRI signal with the selected peaks as shown in Figure 4A
- Report the *fitting of the differential refractive index versus concentration and the dn/dc value as shown in Figure 4B*

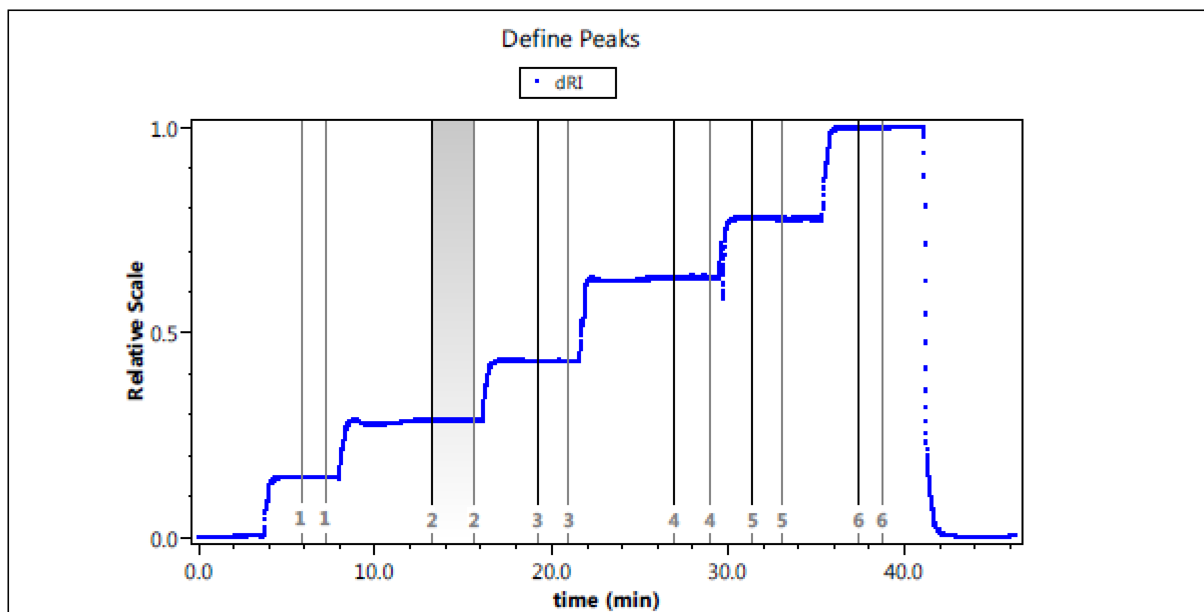


Figure 4A: Collected dRI signal for 200 kDa polystyrene dissolved in THF [1]

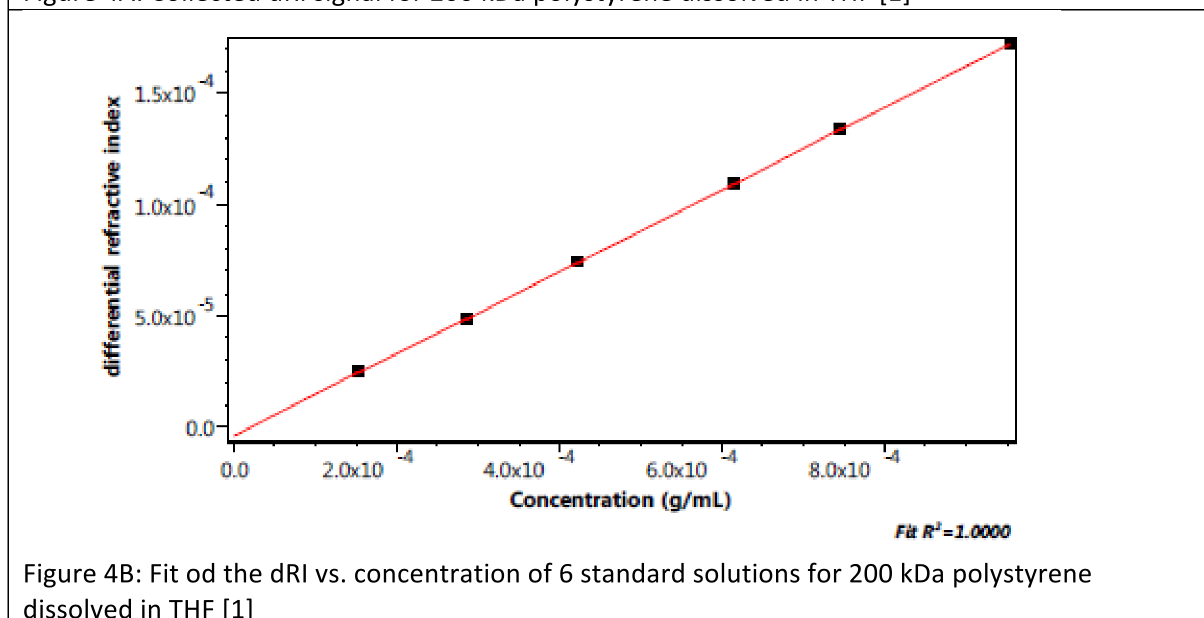


Figure 4B: Fit od the dRI vs. concentration of 6 standard solutions for 200 kDa polystyrene dissolved in THF [1]

13.7 References

[1] Batch dn/dc measurements. Technical note from Wyatt technology