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Dynamic range multiwavelength particle characterization using analytical ultracentrifugation

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We demonstrate how a sophisticated data analysis methodology enables us to perform multiwavelength evaluations of dynamic rotor speed gradient experiments obtained by analytical ultracentrifugation equipped with a multiwavelength detector. Our data evaluation tool HDR-MULTIFIT allows for the accurate analysis of sedimentation coefficient distributions which can be converted to particle size distributions. By means of multiwavelength evaluation, species dependent extinction spectra can be determined even for complex mixtures. Moreover, optical and hydrodynamic properties can be correlated for spherical particles of known optical properties applying multiwavelength evaluation and Mie's theory leading to a significant increase in the dynamic range of the experiment. We provide the theoretical background about the operation principle of our methodology and compare the performance of the multiwavelength analysis to the conventional single wavelength analysis as it is applied in turbidity analysis. We validate our technique using NIST traceable reference particles and show that our technique is universally applicable to materials of known and unknown optical properties, thus clearly extending the possibilities of particle analysis.

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1. Introduction

The accurate determination of particle size distributions (PSDs) is still an ongoing challenge in particle technology. Numerous techniques exist, for instance field flow fractionation, dynamic and static light scattering as well as imaging techniques, to address this need. For selected applications these characterization tools will provide accurate information on PSDs. However, in the case of polydisperse multimodal distributions ensemble techniques are restricted due to the underlying difficulties in solving the ill-posed Fredholm equations for deconvolution of scattering intensity and size distribution or from limiting statistics and tedious evaluation in single particle imaging by SEM or TEM.

Numerous studies have shown in the past that analytical ultracentrifugation (AUC) is a powerful tool for the characterization of nanoparticles (NPs).^{1–6} Most importantly, high polydispersity in size is also correctly represented by AUC.^{7–9} AUC is based on the sedimentation of particles in a gravitational field. The sedimentation is dependent upon the size, mass, density and shape of the NPs. As an absolute measuring technique AUC requires no further calibration by means of stan-

dard materials. Even though a majority of the past and current studies is still focused on biological systems, the huge benefit of AUC for NP characterization has recently been exploited. A multiwavelength detector (MWL-AUC) as well as new data acquisition software have significantly extended the capabilities of AUC and widened the possibilities of accurate particle analysis.^{5,10} Manifold software packages developed for AUC allow for accurate size analysis and even shape, density and optical information of NPs in complex mixtures became accessible using AUC.^{3,11,12}

Even though many NP systems benefit from these investigations, the existing evaluation procedures still require further developments. One challenge is the evaluation of broad PSDs, where many other techniques, such as the most commonly applied dynamic light scattering (DLS), fail. Due to the fractionation of the sample in the centrifugal field, even the evaluation of very broad distributions is possible using AUC. A turbidity detector developed by the user community is a special modification of the AUC, where the acquisition of dynamic rotor speed gradient (speed ramp) data is conducted at a fixed radial position (see Fig. 1 for the operational principle).^{8,13} Herein, information on diffusion is not available as the radial dimension is not recorded in such experiments. Turbidity detection is therefore optimized for the characterization of mixtures with a large spread in size where diffusion plays a negligible role. Recently, this concept was transferred to the MWL-AUC allowing also to investigate wavelength dependen-

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Fig. 1 Measuring principle of the speed ramp technique.

cies.^{5,14} For wide PSDs as studied in speed ramp experiments, the effects of size dependent scattering have to be considered in case of the extinction based MWL-detector. The influence of scattering in AUC experiments was investigated for spherical particles using Mie's theory and was applied to the turbidity and the commercial absorbance detector.^{15,16} However, both designs only allow for single wavelength analyses and these data evaluation tools were not yet transferred to the MWL-AUC design. Therefore, it is currently not possible to evaluate such data. Hence, the development of new evaluation procedures and respective software capable of MWL-analysis is highly attractive for two reasons:

First, it will allow for a direct correlation of hydrodynamic and optical properties even in complex mixtures as it was recently developed for sedimentation velocity data.^{10,12} In contrast to sedimentation velocity experiments typically conducted at a constant speed or a series of discrete increasing rotor speeds, speed ramp experiments offer a much higher dynamic range. The capability to qualitatively distinguish between different species of gold nanorods has been recently shown.⁵ An advanced data analysis tool would allow to quantitatively determine the individual extinction spectra of different components simultaneously. By means of this, important information about structure–property relationships of the particle systems becomes accessible.

Second, the wavelength dependent contribution of scattering can be utilized to increase the dynamic range of the experiment. Existing turbidity optics have the disadvantage of being limited to a fixed wavelength. For many systems this will be a critical point because the overall signal to noise ratio (SNR) is given by the noise of the light source and the amount of light reaching the detector. Even though laser sources as used for the turbidity detector offer a much higher stability than (flash-based) white-light sources, the SNR will be a noted disadvantage because the strong scattering of large NPs requires comparably low concentrations. Dilution series are performed to increase the dynamic range to smaller NPs but those can affect the sample stability and require more experimental effort.⁸ The importance of sensitive analysis techniques for broad

PSDs with respect to number concentrations in particular has been demonstrated in a recent study by W. Wohlleben.⁹ The validity range of different techniques was investigated by means of reference materials. It was shown that AUC equipped with an interference detector, unlike other methods, is capable of identifying NP contents from 14 to 99 number percent in a bimodal mixture with less than 5 percent deviation. However, the interference detector provides mass weighted distributions based on the refractive increment of the material. In contrast and as discussed beforehand, the dynamic range of the MWL-detector for the accurate determination of number weighted PSDs is strongly dependent upon the size range. This is a crucial point since the number concentration is of much higher significance for many applications than the mass concentration due to the large surface area of very small particles. Bhattacharyya *et al.* demonstrated qualitatively that MWL-AUC provides access to the size and wavelength dependent scattering of NPs in a speed ramp experiment. In principle, this allows for a much higher dynamic range with respect to concentrations. However, no software existed back then to evaluate such data.¹⁴

Therefore, we developed a method allowing for scattering correction based on Mie's theory for multiple wavelengths, hence enabling high dynamic range evaluations. Since it was shown that absorption detection can provide an even better limit of detection for small concentrations than the commercial interference detector,⁵ we expect this to be also of high benefit for the application of speed ramp experiments. In this work we focus on the dynamic range and the improvements achieved by means of MWL-AUC in combination with Mie's theory because the accuracy of AUC and turbidity detection regarding size and in comparison to other techniques was already studied in detail.^{5,7,8} Special emphasis will be put on the combined MWL analysis to determine PSDs as well as the unique correlation of hydrodynamic and optical properties.

In the following section the basic principles of the speed ramp technique will be presented. Based on these a methodology for the data evaluation of speed ramp experiments including the determination of optical properties of NPs as well as high dynamic range particle size analysis is derived.

2. Theory

2.1 Operation principle of the speed ramp technique

As a centrifugal method, AUC is based on the sedimentation of particles in a centrifugal field caused by the difference in the densities of the particle and displaced solvent. Colloidal systems will get fractionized due to their different disperse properties, in particular the size, shape and density distributions. The size of spherical particles can be derived from such an experiment using Stokes' equation:

$$d = \sqrt{\frac{18\eta s}{\rho_p - \rho_s}} \quad (1)$$

d is the particle diameter, η the solvent viscosity and ρ the solvent (s) and particle (p) density. The sedimentation coefficient s is the sedimentation velocity u of a certain particle, which has been corrected for the centrifugal acceleration $\omega^2 r$:

$$s = \frac{u}{\omega^2 r} \quad (2)$$

To obtain the sedimentation coefficient in a speed ramp experiment, the intensity is monitored at a fixed radial position r as a function of time t , angular velocity ω and meniscus position r_m :

$$s = \frac{1}{\omega^2 t} \ln \left(\frac{r}{r_m} \right) \quad (3)$$

2.2 Determination of particle concentrations

For a MWL-detector, the determination of the concentrations of all components is achieved *via* the law of Lambert-Beer:

$$A_\lambda = -\ln T_\lambda = -\ln \frac{I_\lambda}{I_{0,\lambda}} = \varepsilon_\lambda c l = \tau_\lambda l \quad (4)$$

This equation provides the relationship between the intensities of an incident and resulting beam I_0 and I , respectively, which is attenuated due to the extinction of the material with the concentration c and extinction coefficient ε in the optical path with a defined length l at a certain wavelength λ . The measured absorbance A or transmittance T can be then used to determine the concentration in case the wavelength dependent extinction coefficient of the material is known. In case of suspensions, the term “extinction” is better suited than “absorbance” because scattering also takes place at NPs and leads to a further attenuation of light. Besides extinction, turbidity τ , which is independent of the optical path length, can be used to describe the measured quantity. A 3-dimensional plot of selected raw data from a MWL-AUC speed ramp experiment is shown in Fig. 2.

Mie's theory. As defined by the law of Lambert-Beer, the extinction coefficient and the measured turbidity determine the concentration of each component. The extinction is defined by the interaction of an electromagnetic wave (light) with a solid body. Gustav Mie considered these interactions for smooth, isotropic and spherical bodies (spheres) in his theory back in 1908 and found that the incident electromagnetic wave can be split into three parts: the incident wave, the wave within the body and the scattered wave.¹⁷ Since there is just one wave, all of these contributions have to follow the same wave equations (Maxwell equations). Just the basic relations will be given in the following paragraph because detailed considerations of Mie's theory would go beyond the scope of this work.

The extinction cross-section of a particle is a measure of the effective area. The extinction cross-section C_{ext} is the sum of the absorbance cross-section C_{abs} and the scattering cross-section C_{sca} of a particle.

Fig. 2 Exemplary 3-dimensional plot of raw data acquired during a MWL speed ramp experiment. The rotor speed was continuously increased from 2000 to 40 000 rpm. Three boundaries can be identified resembling three species sedimenting with different sedimentation coefficients. Up to 2048 different wavelengths are recorded during the course of a MWL speed ramp experiment. Only a designated number of wavelengths is shown herein.

$$C_{\text{ext}} = C_{\text{sca}} + C_{\text{abs}} \quad (5)$$

According to Bohren and Huffman the cross-sections can be derived from the scattering and absorption coefficients, also known as Mie coefficients.¹⁸ These coefficients are dependent upon the particle size, wavelength and refractive index. Thus, they are a function of the incident light, particle geometry as well as the particle's chemical composition. As Mie theory is strictly speaking only valid for spheres, only spherical particles will be considered in this work. However, anisotropic structures can be also described by the Maxwell equations even though this task would become much more complex. The refractive index of a material is defined as the velocity of light in vacuum divided by the velocity of light in the material. The complex refractive index $\tilde{n}$ consists of the real part n and imaginary part k :

$$\tilde{n} = n - ik \quad (6)$$

The extinction cross-section of a single particle with its number concentration N_p is linked to the measured turbidity (in case of monodisperse spheres) as follows:

$$\tau_\lambda = N_p C_{\text{ext}} = \frac{\varphi}{V_p} C_{\text{ext}} \quad (7)$$

The number density of particles can further be expressed by the volume fraction φ of all particles having the same volume V_p . To correlate the cross-section with the experimentally relevant extinction coefficient, a measure for the quantity of the absorbance and/or scattering events has to be defined. Such a measure is the extinction efficiency, which is defined as the ratio of the extinction cross-section and the projection area A_p of the spherical particle:

$$Q_{\text{ext}} = \frac{C_{\text{ext}}}{A_p} = \frac{4C_{\text{ext}}}{\pi d^2} \quad (8)$$

A combination of eqn (7) and (8) gives:

$$\tau = \frac{3\varphi Q_{\text{ext}}}{2d} \quad (9)$$

Since the particle concentration is more intuitive than the volume fraction, it can be derived by the subsequent equation:

$$\varphi = \frac{V_{\text{P},\text{total}}}{V_{\text{total}}} = \frac{V_{\text{P},\text{total}}}{V_{\text{F}} + V_{\text{P},\text{total}}} \approx \frac{V_{\text{P},\text{total}}}{V_{\text{F}}} = \frac{m_{\text{P},\text{total}}}{\rho_{\text{P}} V_{\text{F}}} = \frac{c}{\rho_{\text{P}}} \quad (10)$$

V_{total} is the total volume consisting of the volume of the solvent V_{F} and the particles $V_{\text{P},\text{total}}$. Herein, it is assumed that the volume of the particles is small compared to the solvent and that the density of the particles is known. Together with the mass of the particles $m_{\text{P},\text{total}}$ this leads to the final equation for the turbidity:

$$\tau = \frac{3cQ_{\text{ext}}}{2d\rho_{\text{P}}} \quad (11)$$

Alternatively, one can also formulate the specific turbidity τ/c , which is the extinction coefficient:

$$\frac{\tau}{c} = \epsilon_{\lambda} = \frac{3Q_{\text{ext}}}{2d\rho_{\text{P}}} \quad (12)$$

This correlation now provides the important relationship of the extinction cross-section, extinction and particle size. As it can be seen from Fig. 3, the extinction coefficients strongly depend upon the wavelengths and particle sizes. Two different regimes can be distinguished. While the extinction coefficient is strongly dependent upon the particle size and wavelength in the Rayleigh-scattering regime ($d < 0.2 \lambda$), there is a weaker but non-linear and highly oscillating correlation in the Mie-regime ($d \approx \lambda$). In any case, it is of major importance that the particle size is accurately determined to obtain the correct concentrations of all fractions.

Fig. 3 Extinction coefficients for a selection of wavelengths and particle diameters for a fixed refractive index ($n_{\text{particle}} = 1.6$; $n_{\text{solvent}} = 1.33$) calculated based on the correlations provided beforehand. Extinction coefficients are shown up to a particle size of 3 μm , which can be seen as the typical upper limit of speed ramps experiments.

Refractive index. As shown in the previous considerations, the complex refractive index of the material has to be known to obtain the correct extinction coefficient and therefore concentrations based on Mie's theory and Lambert-Beer's law. However, the refractive index of any material is not only a material constant but also wavelength dependent. For MWL evaluations this wavelength dependency has to be considered to provide accurate results for the concentrations. Several methodologies exist to model the optical dispersion of a material, such as the theories of Cauchy, Sellmeier as well as the Lorentz-Oscillator-Model.^{19,20} In this work, we will use the approach of Wolfgang von Sellmeier since it has proven to provide good results in the UV-VIS-NIR.²⁰ The real part of the refractive index is given by:

$$n^2(\lambda) = A + \sum_j \frac{B_j \lambda^2}{\lambda^2 - C_j} \quad (13)$$

The coefficients C_j are the electron resonance wavelengths and the parameters A and B_j have to be determined experimentally. In principle, the Sellmeier equation can also be extended by a further term considering the complex refractive index and thus the absorbance of the material:²¹

$$k(\lambda) = \left[n(\lambda) \left(D_1 \lambda + \frac{D_2}{\lambda} + \frac{D_3}{\lambda^3} \right) \right]^{-1} \quad (14)$$

The coefficients C_j and the parameters A , B_j , D_1 , D_2 and D_3 have to be determined experimentally or can be obtained from literature.

2.3 Data acquisition

We developed our data acquisition software based on Labview from National Instruments to capture MWL speed ramp data.⁵ The detector is moved to the desired radial position at which the intensity shall be recorded as a function of time and rotor speed. Usually the time interval at which data is acquired is less than one second for all samples. An averaging procedure has been implemented to improve the SNR of the comparably noisy MWL detector. Since the number of possible acquisitions is higher for higher rotor speeds (less time required for one rotor revolution), this technique has been implemented in a dynamic manner. As the number of averages increases with higher rotor speeds, more data is averaged and a better SNR is observed for the smaller, mostly less concentrated fractions. Dark noise is continuously subtracted from the experimental data to assure linearity. At the end of the experiment, the meniscus positions are determined *via* radial scans for each chamber individually. All data is saved as intensity profiles in a binary format accompanied with a XML file containing information on the sample and the radial positions. Further information regarding the data format is available on <http://www.lfg.uni-erlangen.de/research/AUC/index.shtml>.

2.4 Data evaluation and applied corrections

The sedimentation data obtained from a speed ramp experiment requires some further corrections to account for several

parameters. The important corrections necessary for accurate data evaluation are described in the following section.

Correction for rotor stretching. Due to the high centrifugal forces the rotor will stretch as a function of the rotor speed. This will result in a shift of the cell position relative to the measurement position with increasing rotor speed. As a consequence the measured sedimentation time will be too small and the calculated sedimentation coefficient as well as particle size will be too large. Speed correction for the influence of rotor stretching is not necessary for sedimentation velocity data which are typically acquired at a constant rotor speed. However, for speed ramp data this is very important as the rotor speed continuously changes but no correction is applied during evaluation of single wavelength data so far. To account for the influence of rotor stretching the apparent meniscus position has to be calculated for each data point and rotor frequency, f , as given in the following eqn (15):

$$r_m(t) = r_{m,0}(t_0) + K_1 f(t) + K_2 f(t)^2 \quad (15)$$

For this, the meniscus position at the beginning of the experiment ($r_{m,0}$) has to be measured and the rotor specific stretching factors (K_1 and K_2) have to be determined experimentally as long as they are not already known. In case the meniscus position is recorded at the end of the experiment, $r_{m,0}$ has to be calculated.

For the MWL-AUC, the maximum relative error in the sedimentation coefficient induced due to rotor stretching is about 0.1%, 0.3% and 0.6% for final rotor speeds of 20 000 rpm, 40 000 rpm and 60 000 rpm, respectively. Hence, a correction for rotor stretching is very reasonable as speed ramp experiments with a 4-hole rotor are typically run up to 60 000 rpm.

Correction for radial dilution. Due to the sector shaped geometry of AUC measuring cells, interactions of the particles with the wall of the cells during the sedimentation and resulting convection is prevented. However, particles sedimenting along the radius will experience a dilution due to the increased width of the cell chamber. The radial change in the concentration due to dilution can be written as follows:²²

$$\frac{c}{c_0} = \left(\frac{r_m}{r}\right)^2 \quad (16)$$

The actual measured concentration c can then be calculated based on the initial concentration c_0 , the radius of the measurement position r and the radius of the meniscus position r_m . For multicomponent mixtures the dilution correction has to be applied to all different species individually.²³ Since the dilution is a function of the radius and time t , the total dilution can be obtained by integration over all species.^{13,24} Based on the additivity of all concentrations/turbidities of all components i in a mixture, the dilution corrected turbidity can be calculated:

$$\tau_{0,i} = \frac{\frac{1}{l} \ln\left(\frac{I_0}{I_{i-1}}\right) - \sum_{n=i+1}^z \tau_{0,n} \exp(-2k_n t_{i-1})}{\exp(-2k_i t_{i-1})} \quad (17)$$

Fig. 4 Plot indicating how the contribution of turbidity is considered for the curve of transmittance. Discretised steps are a guide to the eye.

k is the dilution coefficient calculated for each time step j :

$$k_j = \frac{1}{t_j} \ln \frac{r}{r_0} \quad (18)$$

In Fig. 4 an exemplary signal is shown which has to be corrected for dilution *via* the recursion procedure. The deconvolution of the measured signal starts at the last data point z where all particles are completely sedimented and ends at the first data point where all particles are still equally distributed in the cell. Next, the relative contribution of each data point to the concentration $c_{0,i}$ and the cumulative mass distribution is calculated:

$$m_i = \frac{c_{0,i}}{\sum_{i=1}^z c_{0,i}} \quad (19)$$

$$Q_3(s_i) = \sum_{i=1}^i m_i \quad (20)$$

Herein, m_i does not designate the absolute mass of the particle fraction but the percentage mass fraction with regard to the overall initial mass. The corrected sedimentation coefficient distributions can further be converted to a PSD by means of eqn (1) and an axis transformation.

3. Concept of data analysis

So far, the theory of the speed ramp technique and the most important correlations for concentration determination have been presented. As discussed beforehand, a software developed by M. D. Lechner allows for analysis of speed ramp data including Mie's theory, dilution correction and multi-solvent analysis. However, the effect of rotor stretching is not considered in the data evaluation and since the technique of dynamic rotor speeds in combination with MWL detection was only recently developed, no data evaluation software exists so

Scheme 1 Strategy for the MWL speed ramp experiment and data evaluation using HDR-MULTIFIT including the determination of optical properties as well as high dynamic range particle size analysis.

far benefiting from the manifold advantages provided by this technique. Hence, the only possibility would be the data analysis of individual wavelengths using Lechner's software, which is, however, not freely available.

Thus, a new software referred to as HDR-MULTIFIT (High Dynamic Range-MULTIwavelength FITting) in the subsequent sections was developed based on the relationships given in the previous chapters. Even though the concept of data evaluation is oriented towards the analysis of single wavelength turbidity data, extensions incorporating MWL analysis as well as the correction for rotor stretching were integrated. Scheme 1 shows the strategy for data acquisition and evaluation using the MWL speed ramp experiment and HDR-MULTIFIT which will be described in the following sections. In brief, our approach comprises three main steps: 1. MWL speed ramp experiment, 2. determination of spectra and 3. high dynamic range size analysis. In the following, it will be distinguished between samples of unknown and known optical properties. For the first group, extinction spectra can be derived for all species in the mixture, whereas a high dynamic range particle size analysis is available for samples of known refractive indices.

3.1 Determination of optical properties

According to eqn (4) the measured turbidity is a function of the concentrations and extinction coefficients of the particles in solution. The AUC experiment will lead to a fractionation of the sample and the data analysis will provide a sedimentation coefficient distribution for each wavelength individually. Thus, a defined value for the concentration as well as extinction coefficient can be designated to each sedimentation coefficient as long as the measured signal is not superimposed by different species. The measured turbidity, however, will most likely vary for different wavelengths. Thus, the measured signal will directly provide the extinction coefficients along the wavelength dimension as long as the concentration is known. In case of unknown concentrations, it will provide the extinction spectra of the individual fractions. The same procedure

was also applied to sedimentation velocity data before.¹⁰ The extinction spectra are given by the signal areas in the turbidity density plot as shown exemplarily in Fig. 5 for a trimodal mixture consisting of three differently sized polystyrene NPs.

Hence, it becomes possible by means of MWL-AUC and speed ramp experiments to address the unique hydrodynamic and optical properties of individual components even in case of complex mixtures. However, optical effects such as chromatic aberration may lead to small fluctuations in the resolution and determined sedimentation coefficients for different wavelengths. Thus and to achieve a better SNR, it makes sense to calculate the integral over a desired range of sedimentation

Fig. 5 Wavelength dependent sedimentation coefficient distributions of trimodal polystyrene NP mixture providing the extinction spectra as the areas below the curves. The wavelength varies from 350 nm (purple) to 600 nm (red) with only a selection of wavelengths shown. Data is noisier at smaller wavelengths and larger sedimentation coefficients as well as larger wavelength and smaller sedimentation coefficients due to the poor SNR. NP diameters found by AUC are 55, 104 and 175 nm, respectively.

Fig. 6 Resulting integrated turbidities for the three selected species shown in Fig. 5. Hollow symbols give the data which is located outside of the linearity of the detector ($OD > 1$). The calculated theoretical extinction coefficients by Mie's theory based on the mean particles sizes are shown by solid lines. Turbidity data has been scaled to match the amplitude of the extinction coefficients.

coefficients representing the same species. A plot of the integral turbidity values for the three different peaks in Fig. 5 is provided in Fig. 6.

As shown, the measured signal is obviously different for the different s -ranges and wavelengths, which is caused by the different extinction coefficients of the particles. However, since the concentration is constant for each s -range the obtained signal is a direct and quantitative measure of the particles' extinction spectra.

3.2 High dynamic range analysis

As given in eqn (4), the two parameters of influence on the measured extinction are the concentration and the extinction coefficient as long as the path length is constant. In case of a single wavelength measurement, saturation of the detector can only be prevented by lowering the sample's concentration. The detection capabilities for broad distributions will hence be limited due to the erratic contributions of scattering.²⁵ W. Mächtle demonstrated that broader PSDs can be studied by means of a concentration coupling technique.¹³ The same sample is measured at least at two different concentrations to account for the size dependent scattering. The PSDs are then evaluated individually and linked at a reasonable size (large concentration $\rightarrow$ small sizes and small concentrations $\rightarrow$ large sizes). However, besides an increase in experimental effort, dilution series may induce colloidal instabilities and require more material. A good coupling point for both PSDs has also to be found, which can be difficult for continuous PSDs with unknown composition.

However, for a MWL measurement, the particle's extinction coefficient is another accessible parameter. Since the extinction coefficient is a function of the wavelength, too, the effect of wavelength dependent absorption and scattering can be

exploited to achieve an increase in SNR, which will lead to an increasing dynamic range. This requires that the extinction coefficient can be calculated for any size and wavelength but the same limitations also hold true for the coupling technique. The particle concentrations can be calculated in case the extinction coefficients are accessible for all species through Mie's theory. The resulting particle concentrations should then be, by definition, the same for all wavelengths. As seen in Fig. 6 this is the case for the presented polystyrene NPs as the measured turbidity and the calculated extinction coefficients are congruent. The only scaling factors between both quantities are the concentrations of the respective species.

Obviously, not all wavelengths and data points are suitable for data evaluation in case of a polydisperse sample. For an extinction based detector, the maximum signal which is accurately measurable is in the range of 1 OD, due to detector linearity and contributions of multiple scattering. The noise of the light source and detector determine the limit of detection. For the MWL evaluation as many wavelengths as possible should be included in the data analysis to achieve the best SNR and limit of detection. At the same time, the linearity of the measurement has to be ensured. Thus, the following criteria have to be fulfilled:

1. The linearity of the measurement has to be guaranteed to obtain the correct concentrations ($OD < 1$).
2. The measurement signal has to be large compared to the noise of the detector to ensure the best SNR.

While the first criterion is obvious, the second one will be explained in detail. The measured transmission profiles can be divided in a desired number of subsections. Each subsection is defined by its increase in the sedimentation coefficient for a given increase in signal at a certain wavelength. For example, nine subdivisions for transmission data ranging from 0.1–1 will result in nine sedimentation coefficient ranges at every wavelength with transmission intervals of 0.1 each. The smaller the change in the sedimentation coefficient, the larger the corresponding slope in the interval will be (e.g. due to a species passing with a large extinction coefficient). As a universally applicable criterion, a minimum signal change (slope) is defined for each interval i and wavelength k , which is given by a scaling factor ξ and the maximum change at a certain wavelength in the same interval:

$$\left(\frac{\Delta T_{i,k}}{\Delta S_{i,k}}\right)_{\min} = \xi \cdot \left(\frac{\Delta T_i}{\Delta S_i}\right)_{\max} \quad (21)$$

The maximum slope for each interval is therefore determined by the wavelength resulting in the most pronounced change of signal. ξ is typically set to 0.5. This ensures that only wavelengths fulfil the criteria, where the slope indicating the change in signal is at least 50% of the maximum change.

Wavelengths meeting both criteria are then included for data analysis. Since this algorithm is fully automated, prior knowledge of the sample is not required. This is a considerable advantage of the data analysis because it is stable, not dependent on subjective user selections and applicable to any

Fig. 7 Automatically selected data ranges for the different wavelengths and sedimentation coefficients for a mixture of polystyrene NPs.

mixture of particles with known optical properties. In turn, it will provide the best SNR for any MWL analysis. Fig. 7 gives an exemplary plot of the applied selection algorithm. For large sedimentation coefficients smaller wavelengths are excluded due to saturation and non-linearity of the detector in the beginning of the experiment. With decreasing sedimentation coefficients smaller wavelengths are included, whereby larger wavelengths are excluded due to the minor change in signal and worse SNR.

4. Materials and methods

4.1 Materials

Polystyrene nanoparticles. Thermo Scientific polystyrene NPs were used as NIST traceable validated reference materials for validation of the evaluation software (Cat no 3100A, Cat no 3350A, Cat no 4009A). 200 nm NPs (Cat no 3200A, Lot no 39030, size 203 ± 5 nm) were used for further validation of the MWL speed ramp technique and software. A polystyrene NP mixture of defined volume concentration was prepared using micromer® NPs by micromod Partikeltechnologie GmbH. Solutions were prepared from stock solutions (10 and 25 wt%) *via* dilution with ultrapure water ($18.2 \text{ M}\Omega \text{ cm}^{-1}$). The density of all latices was 1.05 g cm^{-3} .

Gold nanoparticles. Gold NPs of different sizes were produced *via* seed-growth synthesis. In the first step, Au seeds were synthesised by adding 1 mL of 25 mM NaAuCl₄ into 150 mL of 2.2 mM trisodium citrate under boiling conditions. After the synthesis of the Au seeds for 10 min, the reaction solution was cooled down to 90 ± 1 °C. Then, 1 mL of 60 mM trisodium citrate and 1 mL of 25 mM NaAuCl₄ were added to the solution one after the other with a time delay of ~ 2 min. After 30 min at 90 °C, the reaction was completed and a sample of 2 mL was extracted. This growth process is repeated in order to get different sizes of Au NPs. Mean particle sizes were measured by means of either SEM or DLS. For the prepa-

ration of the 3-component mixture of gold particles with arbitrary concentration but similar extinction fractions with mean diameters of 26.6 ± 1.5 nm (SEM), 47.1 ± 3.3 nm (SEM) and 63.2 ± 11.9 nm (DLS) were used.

4.2 Methods

Single wavelength data was acquired using a turbidity detector. Data were provided and evaluated by BASF.⁸ For MWL speed ramp experiments, a modified preparative centrifuge, type Optima L-90K, available from Beckman Coulter was used. Further information regarding the applied MWL optics can be found in the literature.⁵ Titanium centrepieces, path length of 1.2 cm, were used for MWL experiments. The temperature used in those experiments was 20 °C. MWL speed ramp data was acquired with the following procedure: First, the rotor speed was set to 2000 rpm and was held for 1 minute to study the hydrodynamic properties of the large analytes. Second, the rotor speed was continuously increased to the final speed of 40 000 rpm. For a successful ramp experiment the rotor acceleration has to be small enough to ensure good resolution and stable sedimentation for all species. Previous studies revealed that acceleration times of about 1 hour provide good results. Since the commercial control board does not provide such small acceleration profiles for the full speed range, custom EPROMs offering modified speed protocols are used. Four different acceleration profiles with rates of about 10 rpm s^{-1} , 30 rpm s^{-1} , 45 rpm s^{-1} and 85 rpm s^{-1} are available. The lowest acceleration profile available was chosen as it would allow reaching 40 000 rpm in about one hour. Unfortunately, the centrifuge was reproducibly reporting an error at about 31 000 rpm which led to the termination of the run, presumably due to an internal safety routine checking the torque or rotor acceleration. Thus, the acceleration was increased to $\sim 30 \text{ rpm s}^{-1}$ as soon as the rotor speed reached 30 000 rpm, which allowed preventing the internal error. In the third step, the rotor speed was held constant at 40 000 rpm as long as needed to achieve full sedimentation of all analytes. The speed ramp data was then evaluated with the technique described in this manuscript. Rotor stretching coefficients were determined *via* the procedure available in the Ultrascan3.²⁶ For this, the radial offset with increasing rotor speed was determined by means of radial scans at different rotor speeds. Stretching coefficients may vary for different rotors and have therefore to be determined for each rotor individually.

5. Application to experimental data

5.1 Validation of software

To confirm the validity of the data evaluation software, first, three different NIST traceable polystyrene NP standards were measured using the single wavelength turbidity device at 530 nm. The results of the data evaluation using HDR-MULTIFIT were then compared to the traditional and well validated software for turbidity data by M. D. Lechner. The most important data are provided in Table 1.

Table 1 Results of data speed ramp evaluation using HDR-MULTIFIT in comparison to the results obtained by the turbidity tool developed by M. D. Lechner in brackets

$x_{\text{nom.}}/\text{nm}$	$c_0/\text{g L}^{-1}$	$c^a/\text{g L}^{-1}$	x_{50}/nm	$(x_{90} - x_{10})/x_{50}$
100	4.0	3.49 (3.32)	102 (101)	0.20 (0.19)
345	0.4	0.41 (0.40)	366 (367)	0.13 (0.12)
994	0.2	0.22 (0.21)	985 (988)	0.12 (0.10)

^aThe refractive index in HDR-MULTIFIT was calculated using Sellmeier's theory with the following coefficients: $A = 1$, $B_1 = 1.5308$, $B_2 = -0.2078$, $B_3 = 0.2569$, $C_1 = 0.0195 \mu\text{m}^2$, $C_2 = -0.1710 \mu\text{m}^2$ and $C_3 = -1.7881 \mu\text{m}^2$. The coefficients for the imaginary part of the refractive index are not required as the polystyrene NPs do not absorb at a wavelength of 530 nm.

The determined particle sizes are in very good agreement to the values specified by the manufacturer as well as the results obtained by the turbidity software. Slight deviations can be explained by the applied smoothing algorithms in both software packages. Typically, HDR-MULTIFIT uses a more rigorous smoothing algorithm to handle the more noisy MWL data. This results in a little wider spread $((x_{90} - x_{10})/x_{50})$ of the PSD. Negligible deviations between both evaluation procedures in the obtained concentrations are a consequence of differences in the utilized refractive indices. For the turbidity software the refractive index has to be directly provided for the data evaluation, whereas the refractive in HDR-MULTIFIT is calculated based on Sellmeier's theory. For the given parameters this results in quite similar refractive indices of 1.5971 (HDR-MULTIFIT) and 1.5954 (Lechner), which also demonstrates the good applicability of Sellmeier's theory for the MWL data approach. The determined concentrations of both evaluations fit well with the adjusted concentrations for the 345 nm and 994 nm particles but differ more significantly for the 100 nm standard. The extinction is strongly dependent upon the particle size because the measurement was conducted in the regime of Rayleigh-scattering. Any deviation will therefore result in a much more significant error for the concentration. Since the obtained particle size is slightly overestimated, the extinction coefficient will also be too large. This in turn explains the particle concentration, which was underestimated. Note that experimental errors during sample preparation or insufficient dispersibility can also explain the discrepancy. However, this also illustrates that an accurate particle size determination is mandatory to obtain accurate concentrations. In principle, the MWL-AUC provides the possibility to evaluate data at smaller wavelengths, where the size dependency of the extinction coefficient is less predominant, which will result in a more accurate determination of the sample's concentration.

Another advantage of HDR-MULTIFIT is the possibility to correct for rotor stretching. However, this method was not applied for the present turbidity data because the stretching factors could not be determined as the detector cannot be moved automatically in the single wavelength turbidity device. However, it is to be expected that the correction of rotor

stretching will shift the particle size to smaller values, which will then positively affect the determined particle concentrations.

As a second validation, a 200 nm NIST traceable PS standard was measured in a MWL speed ramp experiment. The reference size was determined by TEM to be 203 ± 5 nm. HDR analysis revealed a size of 204.3 nm, which is in excellent agreement with the data provided by the manufacturer. Besides size, concentration determined by HDR-MULTIFIT was checked. The experimentally adjusted and determined concentrations were 0.099 g L^{-1} and $0.096 \pm 0.010 \text{ g L}^{-1}$, respectively. Hence, a very good agreement of both concentrations is found because slight deviations are expected due to volumetric uncertainties during sample preparation.

In summary, HDR-MULTIFIT provides excellent results for monomodal samples as showed by comparison with the well-established data analysis software for single wavelength data by M. D. Lechner. Moreover, it was proven that MWL acquisition and analysis is of excellent quality for narrow PSDs. Thus, polydisperse PSDs were evaluated in the next step.

5.2 High dynamic range evaluation of polystyrene NP mixture

Although the HDR evaluation implemented in HDR-MULTIFIT also works for narrow monomodal PSDs, it will be of benefit especially for broad PSDs due to the increased dynamic range during data evaluation by applying different wavelengths for different particle sizes. Hence, HDR analysis is further compared to traditional single wavelength analysis by means of a MWL speed ramp experiment and a trimodal mixture of equally concentrated (with respect to volume/mass) polystyrene NPs about 50, 100 and 200 nm in size. The results of the single- and MWL evaluations are presented in Fig. 8.

Fig. 8 Comparison of single wavelength analysis at 300 nm (blue) and 600 nm (red) with MWL analysis (black) of a three component mixture of polystyrene NPs. Dashed line shows equal volumetric contributions as a guide to the eye.

Table 2 Results of HDR evaluation of trimodal polystyrene mixture

$x_{\text{spec}}^a/\text{nm}$	$c_0/\text{g L}^{-1}$	$c_{\text{rel},0}/\%$	x_{50}/nm	$c^b/\text{g L}^{-1}$	$c_{\text{rel}}/\%$
55 ± 5	0.1	33.33	54.8	0.103 ± 0.028	34.3
112 ± 10	0.1	33.33	103.6	0.098 ± 0.007	32.6
183 ± 15	0.1	33.33	174.7	1.000 ± 0.007	33.1

^aSizes as measured by the manufacturer using DLS including measurement uncertainties. ^bStandard deviations are calculated based on all concentrations derived for all wavelengths.

The concentrations of all species are correctly retrieved by the HDR evaluation. In contrast to HDR evaluation, single wavelength analysis significantly under- and overestimates certain species. At low wavelengths saturation of the detector took place in the beginning of the experiment. Thus, information on the sedimentation of large NPs is lost. In contrast, evaluation at a large wavelength results in a very low signal for the smallest species in the mixture due to their small extinction coefficients (see Fig. 3).

The HDR evaluation instead provided a much better balanced evaluation by granting the maximum SNR for each wavelength and NP size. Further information on the sizes and concentrations found by HDR analysis is presented in Table 2.

5.3 Determination of size dependent optical properties of gold NPs

So far, the advantages of HDR evaluation with respect to narrow and polydisperse PSDs have been demonstrated by means of polystyrene NPs. For polystyrene refractive indices are accessible by Sellmeier's theory and extinction coefficients can be calculated based on Mie's scattering theory for smooth spheres. Combining those, PSDs become accessible with high resolution and dynamic range. However and as mentioned before, MWL evaluation of speed ramp data provides further advantages compared to other measurement techniques and conventional single wavelength analysis of speed ramp data. As shown recently, MWL analysis can provide access to hydrodynamic and optical properties simultaneously by means of sedimentation velocity data.^{5,10,12} For gold nanorods it was demonstrated that MWL-AUC can give rise to the size and shape dependent optical properties using speed ramp experiments.⁵ However, quantitative information was not yet accessible.

Such analysis is now available for speed ramp data, which further allows for the determination of much broader sedimentation coefficient distributions on a shorter time scale compared to sedimentation velocity experiments. Herein, a first experimental example for the unique capabilities of MWL speed ramp experiments in combination with HDR-MULTIFIT is presented. Three differently sized gold NP species were mixed and subjected to a speed ramp experiment. The cumulative extinction weighted distribution is shown in Fig. 9a, indicating the three different species. Next, we extracted the extinction spectra of these three species (Fig. 9b). The characteristic gold spectra with the size dependent shifts of the extinction maxima are clearly visible.

Fig. 9 (a) Cumulative extinction weighted distribution of trimodal gold NP mixture. (b) Deconvoluted normalized extinction of the three species present in the mixture. Size dependent peak-shift at around 500–550 nm is clearly observable.

The median diameters of the gold NPs determined *via* AUC were found to be 27.8 nm, 46.4 nm and 64.6 nm, respectively. These results are in good agreement to the independent size information by SEM and DLS, which provided diameters of 26.6 nm, 47.1 nm and 63.2 nm, respectively. Since the gold spectrum is also unique for each size, we further correlated the location of the extinction maximum with the approximate NP size according to the correlation provided by N. Khlebtsov.²⁷ The diameters derived from the extinction peaks at 525.7 nm, 532.0 nm and 542.8 nm are 32 nm, 48 nm and 68 nm, respectively. This is also a very good agreement provided the limited resolution of the spectrometer (1.9 nm). For the smallest species it has to be considered that only a slight deviation in the extinction maximum can result in considerable change in size.²⁷ Moreover, varying ligand morphology can also affect the extinction spectrum, especially for smaller NPs where the surface to volume ratio is most pronounced. For larger NPs shape effects further come into play.

Even though gold NPs were used to illustrate the capabilities of our technique, we believe that MWL speed ramps utilizing the sophisticated data evaluation procedure will serve as basis for many more enhanced applications. This will be of high benefit as size or shape dependent optical properties are of ever-increasing importance in particle technology.

6. Conclusions and outlook

We developed a new method allowing us to analyse dynamic rotor speed data acquired using MWL-AUC. We validated our methodology by means of polystyrene reference NPs and compared our results to the results of a well-established and validated technique for single wavelength turbidity analysis. Our software enables us to determine the optical properties of individual components in mixtures, such as shown for gold NPs in this work. Thus, it becomes possible to correlate hydrodynamic properties with optical properties also for very poly-disperse PSDs. Measurements can be conducted within about 1 hour and up to 7 samples can be measured simultaneously using a 4-hole rotor. For spherical NPs having well defined refractive indices but unknown PSD and concentration, we presented an approach of HDR particle size analysis. AUC in combination with HDR analysis is superior to the single wavelength approach or scattering techniques.²⁸ Our technique takes advantage out of the direct fractionation of different size classes in the measurement cell during sedimentation, while an optimum SNR is achieved by automatically tuning the wavelengths being used for data evaluation dependent upon the particle size.

The comparably high level of experimental random noise limits data quality. Further techniques reducing the contribution of random noise, either during acquisition or evaluation of speed ramp data would therefore be highly desirable. So far, the HDR evaluation of PSDs is applicable to spherical absorbing and scattering particles of known optical properties where the refractive index can be calculated based on Sellmeier's theory. However, Sellmeier coefficients for absorbing NPs are hard to find and it would therefore be helpful to have a database for complex refractive indices of different materials at hand. Besides, HDR analysis can only be so far applied for spherical NPs due to the restrictions of Mie's theory. Future developments on data analysis should therefore focus on the extension to shape anisotropic particles.

Since MWL experiments provide the full PSD in case of spherical particles of known density, it gives access to the size dependent change of a particle's extinction. Thus, refractive indices of materials become in principle accessible. This will open the possibility of determining so far unknown optical properties of NPs.

Our developments have been integrated into the data evaluation tool HDR-MULTIFIT for which the software can be obtained from the authors in the framework of the "Open AUC" project.²⁹

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Notes and references

- 1 H. Cölfen and T. Pauck, *Colloid Polym. Sci.*, 1997, **275**, 175–180.
- 2 E. E. Lees, M. J. Gunzburg, T.-L. Nguyen, G. J. Howlett, J. Rothacker, E. C. Nice, A. H. A. Clayton and P. Mulvaney, *Nano Lett.*, 2008, **8**, 2883–2890.
- 3 R. P. Carney, J. Y. Kim, H. Qian, R. Jin, H. Mehenni, F. Stellacci and O. M. Bakr, *Nat. Commun.*, 2011, **2**, 335.
- 4 K. Nontapot, V. Rastogi, J. A. Fagan and V. Reipa, *Nanotechnology*, 2013, **24**, 0957–4484.
- 5 J. Walter, K. Löhr, E. Karabudak, W. Reis, J. Mikhael, W. Peukert, W. Wohlleben and H. Cölfen, *ACS Nano*, 2014, **8**, 8871–8886.
- 6 J. Walter, J. T. Nacken, C. Damm, T. Thajudeen, S. Eigler and W. Peukert, *Small*, 2015, **11**, 814–825.
- 7 H. G. Müller, *Colloid Polym. Sci.*, 1989, **267**, 1113–1116.
- 8 W. Mächtle, *Biophys. J.*, 1999, **76**, 1080–1091.
- 9 W. Wohlleben, *J. Nanopart. Res.*, 2012, **14**, 1300.
- 10 J. Walter, P. J. Sherwood, W. Lin, D. Segets, W. F. Stafford and W. Peukert, *Anal. Chem.*, 2015, **87**, 3396–3403.
- 11 B. Demeler, T.-L. Nguyen, G. E. Gorbet, V. Schirf, E. H. Brookes, P. Mulvaney, A. a. O. El-Ballouli, J. Pan, O. M. Bakr, A. K. Demeler, B. I. Hernandez Uribe, N. Bhattarai and R. L. Whetten, *Anal. Chem.*, 2014, **86**, 7688–7695.
- 12 G. E. Gorbet, J. Z. Pearson, A. K. Demeler, H. Cölfen and B. Demeler, in *Methods Enzymol.*, ed. L. C. James, Academic Press, 2015, vol. 562, pp. 27–47.
- 13 W. Mächtle, *Angew. Makromol. Chem.*, 1988, **162**, 35–52.
- 14 S. K. Bhattacharyya, P. Maciejewska, L. Börger, M. Stadler, A. M. Gülsün, H. B. Cicek and H. Cölfen, in *Analytical Ultracentrifugation VIII*, ed. C. Wandrey and H. Cölfen, Springer-Verlag, Berlin, Heidelberg, 2006, vol. 131, pp. 9–22.
- 15 M. D. Lechner, *J. Serb. Chem. Soc.*, 2005, **70**, 361–369.
- 16 M. D. Lechner, H. Cölfen, V. Mittal, A. Völkel and W. Wohlleben, *Colloid Polym. Sci.*, 2011, **289**, 1145–1155.
- 17 G. Mie, *Ann. Phys.*, 1908, **330**, 377–445.

- 18 C. F. Bohren and D. R. Huffman, in *Absorption and Scattering of Light by Small Particles*, Wiley-VCH Verlag GmbH, 2007, pp. 82–129.
- 19 A.-L. Cauchy, *Mémoire sur la dispersion de la lumière*, Praque, 1836.
- 20 W. v. Sellmeier, *Ann. Phys.*, 1871, **219**, 272–282.
- 21 P. Dirk and S. Philippe Frederic, *J. Phys. D: Appl. Phys.*, 2003, **36**, 1850.
- 22 H. Fujita, *Foundations of ultracentrifugal analysis*, Wiley, 1975.
- 23 J. H. Kehrhan, M. D. Lechner and W. Mächtle, *Polymer*, 1993, **34**, 2447–2452.
- 24 W. Scholtan and H. Lange, *Kolloid Z. Z. Polym.*, 1972, **250**, 782–796.
- 25 W. Mächtle and L. Börger, *Analytical Ultracentrifugation of Polymers and Nanoparticles*, Springer-Verlag, Berlin, Heidelberg, 2006.
- 26 B. Demeler, in *Analytical Ultracentrifugation: Techniques and Methods*, ed. D. J. Scott, S. E. Harding and A. J. Rowe, The Royal Society of Chemistry, 2005, pp. 210–230.
- 27 N. G. Khlebtsov, *Anal. Chem.*, 2008, **80**, 6620–6625.
- 28 J. Walter, T. Thajudeen, S. Süß, D. Segets and W. Peukert, *Nanoscale*, 2015, **7**, 6574–6587.
- 29 H. Cölfen, T. M. Laue, W. Wohlleben, K. Schilling, E. Karabudak, B. W. Langhorst, E. Brookes, B. Dubbs, D. Zollars, M. Rocco and B. Demeler, *Eur. Biophys. J.*, 2010, **39**, 347–359.