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High-Resolution Asymmetrical Flow Field-Flow Fractionation Data Evaluation via Richardson–Lucy-Based Fractogram Correction

Marius Schmid^{†,§,⊥}, Benedikt Häusele^{†,⊥}, Michael Junk[‡], Emre Brookes^{||}, Jürgen Frank[§], Helmut Cölfen^{*,†}

[†]Physical Chemistry, Department of Chemistry, University of Konstanz, Universitätsstrasse 10, 78457 Konstanz, Germany

[‡]Department of Mathematics and Statistics, University of Konstanz, Universitätsstrasse 10, 78457 Konstanz, Germany

[§]Coriolis Pharma Research GmbH, Frauenhoferstrasse 18b, 82152 Martinsried, Germany

^{||}Health Science Center, University of Texas, San Antonio, Texas 78229, United States

Abstract

Asymmetrical flow field-flow fractionation (AF4) is a chromatographic separation technique that can be used for a broad range of particles or macromolecules. As an orthogonal method to size exclusion chromatography (SEC) with a much broader separation size range (1–800 nm) AF4 is gaining importance. However, the data evaluation capacities are far behind in comparison to other techniques like analytical ultracentrifugation (AUC). A program for evaluation of data from AF4 with a coupled multiangle laser light scattering (MALLS) detector was developed that allows the determination of the distributions of diffusion coefficients (D), hydrodynamic radii (R_h), molecular weights (M_w), and relative concentrations (RC) of the obtained species. In addition, two algorithms to remove broadening effects via deconvolution were implemented and tested for their validity. The first is an extension of the known diffusion broadening correction applying the entire diffusion coefficient distribution instead of a single diffusion coefficient. The second applies the Richardson–Lucy algorithm for the deconvolution of overlapping signals from stars in astronomy. This program allows a reproducible strong enhancement of the fractogram resolution allowing for entire baseline separations of proteins. The comparison of the values for M_w determined by a partial Zimm plot from each data point of the original fractogram and the deconvolved results shows that especially the Richardson–Lucy algorithm maintains a high degree of data robustness.

Graphical Abstract

*Corresponding Author: Helmut.Coelfen@uni-konstanz.de. Fax: +49 7531 88-3139.

[⊥]M.S. and B.H. contributed equally to this work.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acs.anal-chem.8b03483](https://doi.org/10.1021/acs.anal-chem.8b03483).

Complete overview of acquired and evaluated data sets for BSA, results of data deconvolution by the iterative relaxation method, Richardson–Lucy deconvolution, molecular weight distributions, iteration dependence, cross flow dependence, theory of AF4, and detailed description of the algorithms used (PDF)

The authors declare no competing financial interest.

Although field-flow fractionation (FFF) has been developed at the same time as high-performance liquid chromatography/gel permeation chromatography ((HPLC/GPC),¹ it is rarely used in comparison to HPLC/GPC.² The advance of HPLC/GPC can be explained as the technological requirements for FFF are much higher. The main problem used to be the technical operation of the focus step, which became easier with modern technical instrumentation. This resulted in a stronger interest in FFF in the last few years as the technique became much more robust. FFF is now becoming an increasingly popular analytical methodology applicable to the separation of particles and macromolecules in solution.³ This increase in interest is also rushed by the decision of pharmaceutical regulatory authorities that size exclusion chromatography (SEC) results have to be verified by an orthogonal method.³ However, field-flow fractionation is still far behind other analytical techniques regarding data handling.

Asymmetrical flow field-flow fractionation (AF4) is the FFF technique most commonly used. The method uses a solid plate and a membrane to form the channel.⁴ As the separation principle is based on a physical field and does not depend on the interaction with a stationary phase, AF4 should generally be assumed to give a more accurate result in comparison with SEC. However, SEC is still much more commonly used as a separation technique. This can be traced back to two major problems occurring in AF4:

AF4 is a comparatively complicated method, AF4 method development is more time-consuming as method development in SEC,⁵ and the method is still less robust.⁶

The resolution of AF4 is often quite poor in comparison to analytical ultracentrifugation (AUC), and it is in most cases difficult to impossible to get baseline-separated results.³

As in SEC, the resolution of AF4 is limited due to peak broadening effects, which occur due a set of different physical influences and make each peak seem broader than the actual polydispersity of the sample. Therefore, the observed polydispersity does not reflect the actual polydispersity of the samples. Also, the subsequent evaluation of signal data can be impaired in the overlapping regions as required material-specific constants such as the refractive index increment or the absorption coefficient may vary for the different species. If the broadening effects can be eliminated, an increased resolution can be obtained, which would directly reflect the actual polydispersity of the sample in the ideal case. While the broadening effects in SEC are due to their characteristics (separation caused by interaction with the stationary phase) and are not quantifiable, the broadening effects are well-described

in AF4 theory.⁷ A broadening correction in orthogonal techniques like AUC is commonly applied to yield correct sedimentation coefficient and molar mass distributions.⁸ In contrast, AF4 is mostly used as a pure separation technique without further quantitative data handling. Only for sedimentation FFF (SdFFF) and thermal FFF (ThFFF), a diffusion broadening correction was previously published based on the assumption of a single diffusion coefficient for an eluted species.^{7,9} However, this severely limited the applicability of the method as discussed further below.

In this work, two algorithms are presented and evaluated that attempt to conduct a correction for peak broadening of the measured raw data. The first one was adapted from the approaches mentioned above;^{7,9} the other one was taken because of the high mathematical similarity of its original application and the task described in this work.

Although often only used as a pure separation method without making use of the information in the experimental data, AF4 is a first-principle method to measure the diffusion coefficient (D) distribution of the samples. This means that the diffusion coefficient can be determined without using an equivalent standard.

A diffusion correction method of data yielded from a different technique of the FFF family, SdFFF, has already been reported in the literature.⁹ However, the diffusion coefficients cannot be measured by SdFFF. For this reason, in order to conduct the diffusion correction method for these data, a constant diffusion coefficient had to be assumed for the whole fractogram. However, the diffusion coefficients of the separated analytes will differ from each other in SdFFF. Thereby, this is an inaccurate approximation.

Therefore, such an approach would be much more useful to be applied on AF4 results. As described earlier, AF4 is a first-principle method to determine the diffusion coefficient distribution. This gives us the benefit of not being forced to use a single diffusion coefficient for the whole fractogram. We directly can use the measured diffusion coefficient distribution to correct the results for peak broadening.

The program presented in this article is able to reduce the broadening effects using the determined diffusion coefficient distribution to correct the peak broadening. Two different algorithms were developed to solve this problem. For the first one, we implemented the iterative relaxation algorithm which was already described for SdFFF⁹ and extended the method by replacing the approximation of a constant D .

The second deconvolution algorithm was derived from Richardson–Lucy deconvolution, which was developed for the deconvolution of overlapping signals from stars in astronomy.^{10,11} In the following, both of our used methods are named in the following according to their respective origin.

THEORY

This section shows a quick summary of the used relationships from AF4 theory. A detailed report of the evaluation procedures based on this theory is given in the Supporting Information. The fundamental equation for AF4 is

$$\lambda = \frac{DV^0}{V_c \omega^2}$$

where ω is the channel height, D is the diffusion coefficient, V^0 is the effective separation volume of the channel, and V_c is the applied cross flow.¹² λ is the retention parameter which is related to the retention ratio R by

$$R = 6\lambda \left(\coth \left(\frac{1}{2\lambda} \right) - 2\lambda \right)$$

R is expressed for every data point as the ratio of the time of the void peak t_0 and the measurement point t_e :

$$R = \frac{t_0}{t_e}$$

with the elution time of the void peak t_0 and the elution time t_e of a detected species.

The essential broadening parameters are described by the theoretical plate height H .^{13,14} The plate height is a sum of different broadening effects: longitudinal diffusion (first term), nonequilibrium contribution (second term),^{15,16} instrumental contribution (third term), and polydispersity (last term).

$$H = \frac{2D}{R\langle V \rangle} + \frac{\chi \omega^2 \langle V \rangle}{D} + \sum_k H_k + H_p$$

As the broadening effects are known the problem itself has to be described. The measured fractogram is the distribution due to polydispersity convolved with the broadening function:

$$F(t) = G(t, t') * W(t') = \int G(t, t') W(t') dt'$$

In this case t is the time, t' is the integration time variable, $F(t)$ is the measured curve, $G(t, t')$ is the broadening function, and $W(t')$ is the real distribution without broadening. The operator $*$ denotes a convolution-like process. However, compared to the classical convolution, the broadness over the Gaussian varies with t' . This way the diffusion broadening can be best described as one Gaussian-shaped curve convolved with a Gaussian-shaped broadening function.

$$F(t_i) = A_0 \sum_{j=1}^N w(t_j) \frac{e^{-\left[(t_i - t_j)^2 / 2\sigma_{t_j}^2 \right]}}{\sigma_{t_j}}$$

where N is the number of all measured data points and the broadening function (σ_{t_j}) can be expressed by the unwanted terms of the plate height:

$$\sigma_{t_j}^2 = \frac{\left[\frac{2D}{R\langle V \rangle} + \frac{\chi \omega^2 \langle V \rangle}{D} + \sum_k H_k \right]}{L} t_j^2$$

$\langle V \rangle$ is the average flow velocity in the channel, while χ describes the impact of the nonequilibrium effect on peak spreading based on λ . Both terms for its calculation are given in the Supporting Information.

The effects due to band broadening and longitudinal diffusion broadening can be calculated and are, therefore, considered in the diffusion correction described in the following work. The instrumental contribution is difficult to determine. Additionally, the instrumental contribution is only described as a minor effect.

EXPERIMENTAL SECTION

AF4 Analysis Equipment.

The used AF4 instrument was an Eclipse DualTec separation system (Wyatt Technology Europe, Dernbach, Germany). It was connected to a Dawn Heleos II multiangle light scattering (MALS) detector (Wyatt Technology) operating at a wavelength of 658 nm. An Agilent 1100 series UV-vis and an Agilent 1100 series refractive index (RI) detector were used to collect concentration signals. An Agilent 1100 series isocratic pump (Agilent Technologies, Waldbronn, Germany) with an in-line vacuum degasser and an Agilent 1100 series autosampler delivered the carrier flow and handled sample injection onto the AF4 channel. Between the pump and the channel was placed a filter holder with a 200 nm pore size poly(vinylidene fluoride) membrane (Millipore Corp.) to ensure that particle-free carrier entered the channel. The AF4 channel was a Wyatt short channel (Wyatt Technology) having a tip-to-tip length of 17.3 cm and a nominal thickness of 350 μm . The ultrafiltration membrane forming the accumulation wall was Nadir regenerated cellulose membrane with a cutoff of 5 kDa (Microdyn-Nadir GmbH, Wiesbaden, Germany).

AF4 Measurements.

The measurements were carried out according to the scheme shown in the Supporting Information. The sample volume injected onto the channel was 20 μL for an injected sample mass of approximately 20 μg . A 3 min focusing step was performed prior to elution with a focusing flow rate of 1.5 mL/min. Different cross flows in range of 2.5–3.5 mL/min were applied. After elution, the channel was flushed without any cross flow for 2 min before the next analysis. Detector flow rate (V_e) was constant at 1.0 mL/min throughout the separation. Carrier liquids were prepared as 50 mM NaNO_3 (Merck, Darmstadt, Germany) dissolved in pure water prepared with a Milli-Q system (Millipore). Data were collected by ASTRA 6.1.7.17 (RC7) (Wyatt Technology Corporation and Chemstation (rev. B.04.03-SP1 [87])). Lyophilized bovine serum albumin (BSA) (Sigma-Aldrich) was reconstituted by dilution with Milli-Q water to a concentration of 1 mg/mL. A measurement was conducted five times

at each condition. In addition, measurements of apoferritin under the same conditions were used as reference for the calibration. For the measurement of polystyrene particles (Thermo Fisher Scientific) a 0.1% w/v of sodium dodecyl sulfate (SDS, p.a. grade, Sigma-Aldrich) was used as eluent. Here, the focus time was 4 min and $V_c = 0.5$ mL/min.

Data Evaluation.

The collected data were processed via ASTRA 6.1.7.17 (RC7) (Wyatt Technology Corporation) by applying the following steps. The baseline was set with the same data ranges for the quintuplicate analysis for all applied cross flows. Alignment and band-broadening correction was carried out using ASTRA. For the evaluation of the data, a software ("FFFEval") was written in C++ under the use of the frameworks Qt 5.7 and Qwt 6.1.2¹⁷ for the graphical user interface. Four basic functions are provided by the software: (1) creation of a channel profile and calculation of the channel height ω ; (2) calculation of the distributions of D and R_h ; (3) calculation of the broadening of the Gaussian kernels and conduction of the deconvolution procedures (iterative relaxation or Richardson–Lucy) using the D -distribution from step 2; (4) partial Zimm plot evaluation of either raw or deconvolved data sets.

A second in-house tool was used for the recognition and separation of the peaks in the acquired fractogram and automatic evaluation of all qualification parameters described below. A detailed description of the applied algorithms is given in the Supporting Information. A current state version of the source code is available on request from the authors.

RESULTS AND DISCUSSION

Calibration.

The channel height ω was determined by a bisection method implemented into our software. The channel height was determined as 323 μm . As reference the diffusion coefficient of monomeric apoferritin (3.61×10^{-7} cm²/s) was used according to literature.¹⁸ The detailed evaluation procedure is described in the Supporting Information.

Determination of the Distributions of D and R_h .

In our first analysis step, the time axis of the signal is converted into D -axis by using a simple bisection process. V_e and V_c are known parameters, and ω was taken from the calibration above. A corresponding D could be determined for each measurement point t_e . Figure 1 shows the conversion between t and R_h at two different cross flows for a BSA standard measurement. This D -distribution was then converted to a distribution of hydrodynamic radii by applying the Stokes–Einstein equation. As values of t , D , and R_h are directly linked, also the corresponding signals can be plotted in dependence of any of these three dimensions. By mapping the detector signals to the corresponding values of R_h a size distribution is obtained (Figure 4). Thereby, also a simple procedure to measure the hydrodynamic radius method was implemented as was also described recently.^{19,20} The BSA measurements exhibit typically four peaks according to the varying quaternary

structure (monomer, dimer, trimer, and tetramer). The peak resulting from the tetrameric structure is very weak compared to the rest of the signal and can often barely be detected.

Deconvolution Procedure.

The influence of the data evaluation on the results was tested for two different algorithms: the iterative relaxation algorithm already applied to ThFFF⁷ and the deconvolution procedure based on the known Richardson–Lucy deconvolution. As testing system BSA was used due its extensive characterization in the literature and widespread use as reference substance in biochemistry.

Iterative Relaxation.

As the first step, the iterative relaxation was transferred from sedimentation FFF to AF4. In its original version, this algorithm used an estimated D of the sample to determine the broadening:

$$\sigma^2 = \left(\frac{2D}{R\langle V \rangle} + \frac{\chi(D)\langle V \rangle}{D} \right) \frac{t^2}{L}$$

This algorithm is known to yield sharp peaks.⁹ For the purpose of a subsequent evaluation of multichannel signals it would also be desirable to verify the correctness of the resulting distribution. The theory is built on a Gaussian-shaped distribution convolved with a broadening function (also Gaussian). Therefore, the algorithm is expected to gain Gaussian-shaped distributions by theory again. For our reimplementations, we refined the calculation of σ^2 .

In the original version of the algorithm for SdFFF, D had to be estimated or determined via a second measurement method, for example, by DLS (dynamic light scattering). In our reimplementations, we replaced this fixed value by D that we yield from the axis replacement of D . Thereby, we get a family of Gaussians as a broadening kernel parametrized by $\sigma^2(D)$. The iterative step of the relaxation method then is described by the following recursion equation:

$$W_{k+1} = W_k + rW_k(1 - W_k)(F - [W_k^*G(\sigma)])$$

with * for classical convolution operation. Here, W_k is the fractogram after the k th iteration and $F = W_0$, i.e., the raw measured fractogram. r is a regularization parameter which was set to 2.⁹ A detailed description of the conducted calculation steps can be found in the Supporting Information. The algorithm was tested for its performance to increase the resolution within a BSA sample measurement (Figure 2).

The obtained results are far from being Gaussian-shaped. By in-depth analysis of the algorithm, this effect could be explained. The distribution has to be normalized (data range from 0 to 1) before every single iteration. Additionally, the algorithm sets all values reaching either 0 or 1 constant. This very fixation of values leads to the oddly shaped (non-Gaussian) distributions. The restrictions can be explained by the low computational power of the time

the algorithm was designed. However, these restrictions make an application on light scattering results with subsequent molecular weight determination impossible. Therefore, a more refined algorithm had to be identified.

Richardson–Lucy Deconvolution.

To overcome the drawbacks of the iterative relaxation method another algorithm was tested. The Richardson–Lucy algorithm has its origin in astronomy and is originally used for deconvolving overlapping signals from distinct stars.^{10,11} As the mathematical underlying problem to deconvolve a signal with a Gaussian broadening kernel is quite similar to our approach we expected this algorithm to be a promising solution. For the adaptation we implemented the parametrized Gaussian as a kernel broadening matrix **G** with entries

$$G_{ij} = \frac{\Delta t}{\sqrt{2\pi}\sigma_j} \exp\left(-\frac{(t_i - t_j)^2}{2\sigma_j^2}\right)$$

for a sampling distance Δt , measurement points of time t_i and t_j , and the broadness. The subsequent iteration steps then are described by

$$W_{k+1} = W_k \left(\frac{F}{G_i^* W_k} * G_j^T \right)$$

The detailed description of the applied algorithm can be found in the Supporting Information. Figure 3 shows an overlay of five measured replicates with its raw data signals of all three detection techniques at $V_c = 3.0$ mL/min and the result of the deconvolution after 100 iterations. The deconvoluted signals are nearly identical. This demonstrates the high reproducibility achieved by the deconvolution procedure.

Qualification Parameters.

In our approach the deconvolution algorithm is applied independently on each signal channel, i.e., each angle from MALLS detection, UV absorption, and RI. To verify the robustness of the method, different qualification steps were carried out. For this purpose, we measured the PVR (peak-to-valley ratio) of the monomer/monomer–dimer section and the PVR ratio of the dimer/dimer–trimer section of the fractogram. Each of the following physical parameters corresponds to a certain property of the processed signal sets (peak position, peak integral, orthogonal signal ratio). A high robustness of the algorithm would hereby be demonstrated by changing these parameters as little as possible.

Eventual changes of the determined R_h indicate the robustness according the position stability of the detected peaks. For comparison we used the concentration-weighted averaged hydrodynamic radius

$$\overline{R_h} = \frac{\sum_i R_{hi} c_i}{\sum_i c_i}$$

As the refractive index signal is linearly dependent on the concentration of each measured fractogram, the relative contribution is equivalent to the relative peak integral. Changes due to algorithmic postprocessing would, therefore, indicate instability concerning the total measured area distribution by the single peaks.

Finally, a partial Zimm plot analysis was conducted for the aligned signal at each R_h to determine M_w . This calculation involves several signals (in our case the refractive index and eight light scattering angles) that have been processed individually by the algorithm. Therefore, it is an ideal quality check for data integrity as unexpected behavior of the algorithm would significantly change the results of this test. Similar to R_h we used the concentration-weighted averaged molecular weight

$$\overline{M}_w = \frac{\sum_i M_w^i c_i}{\sum_i c_i}$$

The influence of the algorithm condition was tested for different numbers of iterations. In addition, the influence of the cross flow was evaluated because this is the most important variable parameter of AF4 measurements. As commonly used for BSA measurements, a cross flow of 3 mL/min was applied. Additionally, one higher cross flow (3.5 mL/min) and one lower cross flow (2.5 mL/min) were applied for our test measurement. As final test, a different test system was measured (mixture of two different polystyrene standards) to verify the applicability for samples in a higher molecular weight range.

Iteration Number Verification.

As first qualification parameter, the influence of the number of iterations of the Richardson–Lucy algorithm on the results was tested in the range from 0 (raw data) up to 1000. Figure 4 shows the calculated molecular weights at the relative peak maximum after a certain number of iterations for a measurement at $V_c = 3.0$ mL/min. Only molecular weights at the region of peak maxima (RIU above 65% of the local peak maximum and a minimum of 1.5×10^{-5} RIU) are displayed. The algorithm kept the calculated molecular weights nearly constant for the monomer and the dimer signal independent from the number of iterations. For the trimer peak a small shift of the measured molecular weight was observed after applying the first. We expect minor shifts of the peak maximum position to be responsible for this shift. For the tetramer the molecular weight was only evaluated after applying 1000 iterations due to our selection criteria of the minimum RI signal intensity. With an expected molecular weight between 200 and 300 kDa we still achieve a result within a reasonable scope even for this weak intensity. In Figure 5 the calculated relative concentrations (RC), M_w , and R_h are displayed for the whole replicate group. The relative concentrations stay nearly constant. Only minor differences within the range of 1–2% of the composition are measurable. We could neither identify a clear trend in the shown data set nor in those of the other cross flows added in the Supporting Information. All shifts we observe with an increasing number of iterations are in the same order of magnitude of the standard deviation. Only for the trimer signals a subtle increase of the RC is observed. The calculated molecular weights of the monomer and dimer peaks also stay nearly constant with an increasing number of iterations.

For the dimer with a total smaller signal intensity only a small shift is observed after applying the first iteration, while the M_w development of the monomer is nearly unaffected. The trimer measurement exhibits already a higher variation for the nondeconvolved signals (0 iterations). This higher variability is also found for longer iteration cycles. We conclude that for the accuracy of M_w determination a higher signal intensity is beneficial (which is not given due to the low relative concentration of the trimeric species) and the species with the highest concentration is processed most accurately. A similar finding is confirmed by a comparison of the calculated hydrodynamic radii. Here, the determined values are very stable. Only for higher iteration numbers do the deviations become slightly bigger. Also here, this effect is stronger for the lower signal intensities. The PVR was determined for the ratio of the maximum of the monomer and the minimum between the monomer and the maximum of the dimer and the subsequent minimum, respectively. For the same set of replicates as discussed before, these values are displayed in Figure 6. The strong increase requires a logarithmic scale of the PVR axis. Values of up to 6.5×10^{290} might exceed the limit of numbers which can be processed with sufficient precision without redefining proper data types which would exceed the necessary effort, as numbers in this order of magnitude indicate complete baseline separation anyway.

On the basis of our data we would attest a very high accuracy of the algorithm concerning the position stability. The same is true for the orthogonal behavior on several signals as shown by the molecular weights. The accuracy of both parameters is a bit better for the monomer peak with the highest total intensity. The same was found for the other cross flows; data are shown in the Supporting Information. All numerical values to shown graphs are also reported there. By this study we show that even a high number of iterations implies a conservative treatment of the signal. However, no optimization of the iteration number has been done. We suggest that this optimal number of iterations to be applied will be variable for individual data sets with different measurement conditions. For this reason, another early termination criterion will have to be added in the future, for example, by continuously monitoring $W_k * G$ and comparing with F .

Cross Flow Dependence.

As a second qualification test, the influence of the cross flow on the computation of the diffusion correction was evaluated. The Gaussian broadening function depends highly on the cross flow as the same diffusion coefficients occur together with elution times and, thereby, different retention ratios. σ^2 will increase with elution time; thereby, we adapted this parameter in the broadening kernel matrix accordingly. The results for the three measured cross flows (2.5, 3.0, and 3.5 mL/min) were compared for raw data and after applying 100 iterations of the Richardson–Lucy deconvolution. An increase of the cross flow results in a larger difference of the retention ratio, but also in the required total elution time. Therefore, due to increased broadening effects an increase of the cross flow does not necessarily increase the resolution.

Figure 7 shows an overlay of three raw fractograms and the fractogram after processing with 100 iterations of the Richardson–Lucy algorithm. For the three raw measurements a high identity of the signals is observed. The deconvoluted counterparts exhibit minor shifts of the

peak positions in the sub-nanometer range. We expect that the application of the different broadening kernels at the cross flows is mainly responsible for these changes. Figure 8 shows the evaluation of RC, M_w , and R_h of all three cross flows for the raw data and the deconvoluted signals (100 iterations). Comparing the composition via the RC yields nearly constant integral distribution. A slight improvement of accuracy is observed for the monomer. We assume that this is reasoned by the peak selection criterion: if the peaks are broadened it is more ambiguous to assign the integral within the overlapping region to either the monomer or the dimer peak. In contrast the deconvoluted signals make it easy to determine defined positions of peak starts and ends. This allows us then to determine the area under the respective curve more accurately.

The results observed for the M_w determination show good correspondence with expected values. The lower accuracy for the M_w determination for the lower concentrated trimer species can be explained by the lower concentration and, thereby, lower signal intensity. Remarkably, the different obtained results for the trimer signal at different cross flows for the raw measurement were well-reproduced in the deconvoluted counterpart. The data analysis gained nearly identical results for R_h and might be explained by subtle condition changes within the measurement sequence (change of channel height due to different pressures on the membrane). Also, here the algorithm keeps those changes stable which again indicates a high position stability of the processed peaks.

However, the deconvolution did not increase the differences between the results obtained for the different cross flows; the deconvolution could even in some cases reduce the differences. All these values are in good correspondence with literature values.¹⁸ The results show only minor differences caused by the cross flow variation. Therefore, the data evaluation approach can be seen as robust in regards to cross flow variations within the tested range.

Application on Samples with Higher Molecular Weights.

In order to test the applicability of the algorithm on data with samples in a higher molecular weight range, we ran a single separation experiment with an applied cross flow of 0.5 mL/min with a suspension of two spherical polystyrene standards of 60 ± 4 and 100 ± 3 nm nominal diameter which corresponds to estimated molecular weights of approximately 135 and 622 MDa (Figure 9). The measured R_h was slightly too small according to these sizes. The species had been already separated well in the fractogram; still, the two resulting peaks were significantly sharper compared to those from the raw data.

No detailed analysis and validation were conducted for this system. Therefore, further research on the qualification of our presented method is required for the application with larger particles. For larger particles the radius of gyration can serve as an additional quality parameter as it depends on the ratio of signal intensity at different angles. For higher size range ($R_h > 20$ nm) various optical properties could also affect the result and would have to be considered.

CONCLUSION

A versatile new tool for the analysis of protein samples was developed. The deconvolution of AF4 results can offer a tremendous increase of the resolution. It can even be used to first carry out a diffusion correction and a subsequent evaluation of the light scattering signals by independent deconvolution of all light scattering detector signals. This gives us the possibility to calculate the corresponding molecular weights of formerly not properly separated signals. We also suspect that the deconvoluted fractograms conform better to the actual polydispersity. However, a detailed proof will be extremely difficult due to a missing gold standard reference. Further optimization of the method could be achieved by additional studies investigating the optimal number of iterations and the behavior of the varying channel heights. We assume that these results are also transferable to AF4 investigations of synthetic polymers and colloids as indicated by the quick investigation of polystyrene nanoparticles. However, detailed qualification for such systems still have to be conducted separately. We concentrated on demonstrating the conservative treatment of the data sets of our implementation in this study. In addition, a dedicated performance study to specify the minimum requirements of resolution of raw data will be of interest in the future as well as the behavior on continuous arbitrary polydisperse distributions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

UV signal of BSA at measured at two different cross flows (top) and the corresponding particle size (hydrodynamic radius) distributions (bottom).

Figure 2. Results of the deconvolution of a BSA sample measured at a cross flow of 3.0 mL/min by LS (90°) detection (top) and by RI detection (bottom).

Figure 3.
Influence of the number of iterations on the detected signals for UV detection at 280 nm (top), light scattering signal at 90° (middle), and RI detection (bottom).

Figure 4.

Overlay of the RI signal and of the results obtained by pointwise Zimm plot evaluation of molecular weights for concentrations above a certain threshold (1.5×10^{-7} RIU) and different numbers of iteration cycles.

Figure 5. Development of RC (monomer, dimer, and trimer), the corresponding molecular weight (middle), and R_h (bottom) with an increasing number of iterations applied to the raw data signal. Error bars indicate the corrected sample standard deviations. We expect this uncertainty to be reasoned by the small overall signal intensity and, thereby, the big influence of subtle dealignment shifts.

Figure 6. PVR development with increasing iterations. Ratio of monomer peak/monomer-dimer valley (top) and dimer peak/dimer-trimer valley. Error bars indicate the corrected sample standard deviation.

Figure 7.

Comparisons of one replicate from each cross flow series as example for the dependence of the algorithm on the cross flow: raw data (top) and 100 iterations applied (bottom).

Figure 8.

Dependence of V_c on M_w on changes of RC (top), M_w (middle), and R_h (bottom). Data are shown for 0 (left) and 100 iterations (right). Error bars indicate the corrected sample standard variation.

Figure 9.
Application of the Richardson–Lucy deconvolution on a fractogram on a mixture of polystyrene nanoparticles at $V_c = 0.5$ mL/min.