Eur Biophys J (2003) 32: 432–436
DOI 10.1007/s00249-003-0319-6

ARTICLE

Helmut Cölfen · Antje Völkel

Hybrid colloid analysis combining analytical ultracentrifugation and flow-field flow fractionation

Received: 25 March 2003 / Accepted: 10 April 2003 / Published online: 26 June 2003

Abstract Ferritin, the iron storage protein, is an organic-inorganic hybrid colloid consisting of a hollow protein capsule, which is filled with ferrihydride with up to 4500 iron atoms. Owing to the varying iron content and the resulting density differences, as well as the protein oligomerization, a particle size distribution is superimposed with a density distribution, making a precise analysis of ferritin by analytical ultracentrifugation difficult. This study describes how the information of the sedimentation coefficient distribution can be combined with the diffusion coefficient distribution obtained from flow-field flow fractionation to yield the buoyant molar mass of the oligomers in the mixture, extending the information content of each individual analytical method. In addition, the sedimentation and diffusion coefficients are compatible with a simple hard-sphere aggregation model, suggesting that the ferritin oligomers up to the pentamer have a globular solution structure.

Keywords Analytical ultracentrifugation · Density distribution · Ferritin · Hybrid colloid · Particle size distribution

Introduction

Organic-inorganic hybrid colloids have gained increasing importance in colloid science owing to the possibility of combining the properties of organic and inorganic materials. In most cases, they exhibit a particle size distribution combined with a density distribution. The same is valid for complexes between (bio)polymers, so that the analysis of these systems is notoriously difficult, as often the amount of one component in the hybrid system is of interest. To address this problem, a global analysis of data from analytical ultracentrifugation (AUC) and an independent technique like dynamic light scattering (DLS) is a potentially useful approach (Schuck 2002). However, up to now, this approach is not fully established. In this study we will demonstrate how the combination of the density and size dependent sedimentation coefficient distribution (*s* distribution) can be combined with the only size-dependent diffusion coefficient distribution from flow-field flow fractionation (Fl-FFF) (Cölfen and Antonietti 2000) to yield further information on a hybrid colloid.

As experimental model system, we have chosen ferritin, the natural iron storage protein, in which the monomer consists of 24 subunits of 18,000–24,000 g/mol (Harrison et al. 1989), as this system shows a multimodal particle size distribution due to the oligomerization of the monomeric hollow sphere units (Pauck and Cölfen 1998). In addition, the ferritin hollow capsules contain ferrihydrite ($5Fe_2O3.9H_2O$) in varying amounts between 0 and 4500 iron atoms (Bauminger et al. 1991; Proulxcurry and Chasteen 1995). The ferrihydrite is formed from Fe^{2+}, which is oxidized to Fe^{3+} upon inclusion into the ferritin capsule (Proulxcurry and Chasteen 1995) and has a particle size distribution (Williams et al. 1978). Despite this, Fe^{3+}, which is not yet incorporated into mature ferrihydrite particles, can be transferred between ferritin capsules (Bauminger et al. 1991). Thus, a density (reciprocal $\bar{v}$) distribution is superimposed with the particle size distribution, and previous sedimentation velocity studies indicated significant changes in the iron distributions depending on the iron introduction into the protein (Grady 1996; Grady et al. 1996). It is interesting to know if the different oligomeric species have the same composition or if variations can be detected for the different species. We will demonstrate that despite the literature indicating

Presented at the conference for Advances in Analytical Ultracentrifugation and Hydrodynamics, 8–11 June 2002, Grenoble, France

H. Cölfen (✉) · A. Völkel
Colloid Chemistry, Max-Planck-Institute of Colloids and Interfaces, Research Campus Golm, Am Mühlenberg 1, 14424 Potsdam, Germany
E-mail: coelfen@mpikg-golm.mpg.de
Tel.: +49-331-5679513
Fax: +49-331-5679502

ferritin $\bar{v}$ distribution (Williams et al. 1978), the average $\bar{v}$ of each of the oligomers appears to be constant, as indicated by a constant buoyant molar mass, so that the average composition of each oligomer is constant (ferritin 1), although the $\bar{v}$ distribution can also result in smearing of the sedimentation coefficient distribution (ferritin 2).

Materials and methods

Horse spleen ferritin was obtained from Sigma as a solution of 102 mg/mL in 0.15 M NaCl and investigated at a concentration of 1.907 mg/mL in 0.15 M NaCl ($\eta^{25} = 0.903$ cP, $\rho^{25} = 1.00326$ g/mL). Two independent preparations were purchased, which turned out to contain different iron levels. They are named ferritin 1 (low iron content) and ferritin 2 (high iron content) in the following discussion.

AUC sedimentation velocity was performed on a Beckman Optima XL-I (Beckman Coulter, Palo Alto, Calif.) using the UV-Vis absorption system at 410 nm and 453 nm, 25 °C and 10,000 r.p.m. using self-made 12 mm titanium centerpieces. Fl-FFF was performed at 25 °C on an asymmetric channel (Consenxus, Ober Hilbersheim) with a spacer of 0.0230 cm thickness and a regenerated cellulose membrane (Postnova, Munich), having a molecular weight cut-off of 5000 g/mol. The carrier liquid was delivered by a Consenxus pump P 1.0 and degassed by a Degasys unit (Japan) DG-1310 using water with 0.3 g/L (5.13 mM) NaCl and 0.08 mg/mL of Tween 20 as an eluent. This solvent optimized the ferritin elution regarding the oligomer resolution, but measurements in pure water yielded the same diffusion coefficients (corrected for viscosity) so that the results from the different FFF solvents can be compared to the AUC results. The eluent flow was 0.5 mL/min; the cross flow was 2 mL/min. The sample was injected by a 10 mL Knauer (Berlin) injection pump and the flow conditions where controlled by a Flowbox and controller from Consenxus and a Liqui Flow Bronkhorst control valve. As a detector, a Bischoff (Leonberg) Lamda 1000 UV-Vis detector was applied at 255 nm. The diffusion coefficient distributions from Fl-FFF (Cölfen and Antonietti 2000) were converted to the particle size distributions by means of the Stokes–Einstein equation, which assumes hard spheres, so that the hydrodynamically equivalent sphere is calculated:

$$d_{\mathrm{H}} = \frac{kT}{3\pi\eta D} \quad (1)$$

with k = Boltzmann's constant, T = thermodynamic temperature, d_{H} = hydrodynamic particle diameter, D = translational diffusion coefficient and η = solvent viscosity.

The diffusion-corrected sedimentation coefficient distributions were evaluated using the program SEDFIT (Schuck 2000), applying the s–D relationship based on the frictional ratio f/f_0 and partial specific volume $\bar{v}$ and allowing f/f_0 to float. As only an average sample $\bar{v}$ can be implemented into the evaluation, the calculated s distributions will reflect the density distribution. The obtained diffusion-corrected s distribution, $c(s)$, was compared to the non-diffusion corrected one, $g(s)$, which was calculated with the same program but without any assumption on the sample; $c(s)$ was enveloped by $g(s)$ as expected, and had the same shape, which excludes errors in the diffusion correction.

The partial specific volume was measured with an DMA 5000 (Anton Paar, Graz) density meter at 25 °C in 0.15 M NaCl solution and was $\bar{v} = 0.598$ mL/g, but this is only an apparent value as no dialysis equilibrium could be applied in order not to endanger ferrihydrite dissolution from the ferritin cores.

Results and discussion

Figure 1 shows the particle size distribution from Fl-FFF clearly resolving the monomer to trimer oligomers with baseline resolution. There is also an indication of tiny amounts of tetramer and pentamer. The particle size of the monomer (11.9 nm) is in good agreement with that of 12.5 nm given in the literature (Mann 1986). Gaussian fits to the peaks nicely reproduce the experimental data for monomer to trimer (data not shown), whereas tetramer and pentamer results were fitted separately. The areas of the Gaussian curves give the concentrations of monomer (80.70 wt%), dimer (14.63 wt%), trimer (2.59 wt%), tetramer (0.47 wt%) and pentamer (1.61 wt%), assuming that the ferrihydrite content of each capsule is constant.

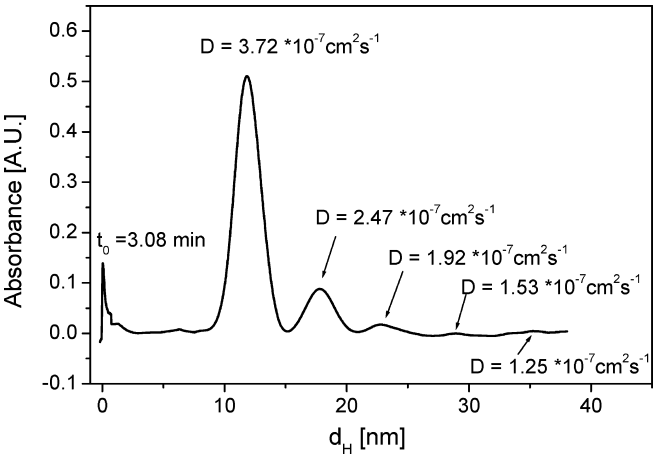

Fig. 1 Fl-FFF elugram of ferritin 1 after conversion to particle size distribution; $\lambda = 255$ nm, cross-flow = 1.97 mL/min, elution flow = 0.53 mL/min, elution liquid 0.3 g/L NaCl + 0.08 g/L Tween 20

This distribution can now be compared to the sedimentation coefficient distribution from AUC. It has to be noted that the AUC data were acquired at a different wavelength than the FFF data and that this could lead to potential weighing errors, as in AUC at 410 nm only ferrihydrite is detected whereas in FFF at 255 nm the protein and ferrihydrite are both detected. However, such weighing errors could be excluded because Fig. 1 already shows the known fact that the ferritin oligomers are defined discrete species and, in addition, a control experiment (data not shown) revealed almost identical sedimentation coefficient distributions for 255 nm and 453 nm, which also shows that all oligomers contain ferrihydrite and that, at 255 nm, predominately ferrihydrite is detected. Hence, it is impossible to determine the absolute protein concentration and only on the assumption of a constant ferrihydrite content in each ferritin capsule can the relative concentrations of the individual oligomers be calculated. However, this would not hold for a ferrihydrite distribution (Williams et al. 1978). Another potential problem is the unknown concentration in Fl-FFF as the solute concentration is changed in an unknown way in the FFF channel (Cölfen and Antonietti 2000), which becomes problematic for rod-like particles with a marked concentration dependence of diffusion (Pauck and Cölfen 1998). Therefore,

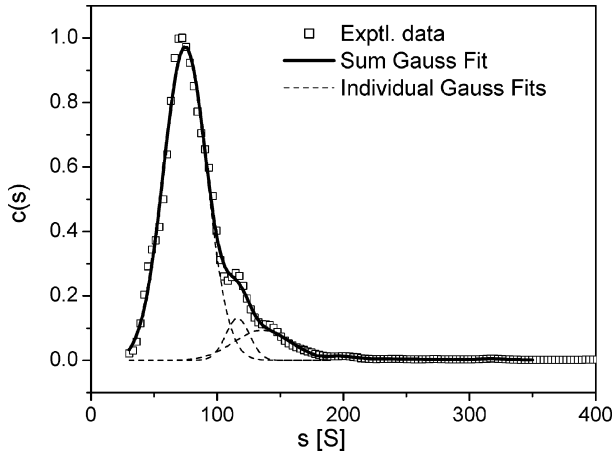

Fig. 2 Diffusion-corrected *s* distribution of ferritin 1 from AUC. Run parameters: 10,000 r.p.m., 25 °C, $\lambda = 410$ nm (99 absorption data sets evaluated)

we checked for the concentration dependence of sedimentation, which is related to that of diffusion, in a control experiment and the almost identical *s* distributions for 38 μg/mL and 1.907 mg/mL solutions (50-fold concentration difference) show the absence of concentration dependence. Thus, together with the absence of weighing errors, it is possible to directly compare the AUC data with those from Fl-FFF. In addition, the constant *s* distribution over a 50-fold concentration range excludes reversible association in the investigated concentration regime.

The diffusion-corrected *s* distribution from AUC is significantly smeared as compared to the particle size distribution from Fl-FFF (Fig. 1) as a consequence of the folded particle size and $\bar{v}$ distributions, so that the individual oligomers are not baseline resolved anymore with the exception of the pentamer (Fig. 2). As a consequence, deconvolution of the curve into individual Gaussian curves and their subsequent integration yield erroneous oligomer concentrations. This becomes obvious from the comparison of the oligomer amounts as determined from FFF and AUC (Table 1). Whereas the agreement for the monomer is good, the deviations are large for the dimer and trimer. This is a result of the fact that the AUC sedimentation coefficient distribution does not show baseline-resolved peaks due to the smearing by the overlaying density distribution. In addition, deconvolution of $c(s)$ profiles into Gaussians is not theoretically founded but still appeared to be the better approach compared to simple area integration, as the peaks were not baseline resolved.

Nevertheless, the individual peak maxima can be determined with a good certainty so that beside the diffusion coefficient and particle size from Fl-FFF, also the sedimentation coefficient of the individual oligomers is known. Together with the diffusion coefficient, the buoyant molar mass can be calculated, which is constant for the monomer to trimer but increasing for the higher oligomers (Table 1). The tetramer and pentamer data have to be treated with caution, as these oligomers are present in very small amounts and the measurement errors, at least in the sedimentation coefficient, are correspondingly high. Nevertheless, the data in Table 1 show that the combination of Fl-FFF and AUC distributions can yield a good resolution of oligomer buoyant molar masses, showing a potentially higher resolution than the combination of AUC with DLS, which could give a broad monomodal distribution due to the lack of fractionation for these samples in DLS.

The constant buoyant molar mass for monomer to trimer of ferritin 1 implies that the average composition of each oligomer is fairly constant and that the $\bar{v}$ distribution caused by different ferrihydrite contents of the capsules (Williams et al. 1978) cannot be too significant, although it is evident as a smearing of the *s* distribution (Fig. 2), when compared to the baseline-resolved *D* distribution from Fl-FFF (Fig. 1).

In principle, the molar mass of each oligomer could now be calculated from the buoyant molar mass with the measured $\bar{v} = 0.598$ mL/g. If this is done starting with the monomer, a molar mass of 1,242,000 g/mol is derived, which is much higher than that of apoferritin [$\bar{v} = 0.74699$ mL/g; 450,000 g/mol as determined by sedimentation equilibrium (Pauck and Cölfen 1998)] plus the maximum possible iron loading of 4500 atoms (Bauminger et al. 1991; Proulxcurry and Chasteen 1995), resulting in a molar mass increase of 432,200 g/mol calculated via the molar mass of ferrihydrite ($5Fe_2O3.9H_2O$, $M = 960.46$ g/mol). This results in a maximum total ferritin molar mass of about 880,000 g/mol, a value much lower than that found above on basis of the measured $\bar{v}$, indicating that the measured $\bar{v}$

Table 1 Sedimentation coefficients, *s*, diffusion coefficients, *D*, hydrodynamic diameters, d_H, and buoyant molar mass, M_b, for each of the ferritin oligomers from AUC and Fl-FFF measurements for ferritin 1

	Monomer	Dimer	Trimer	Tetramer	Pentamer
s (S) at 25 °C (AUC)	74.6	116.3	135.7	195.3	317.0
$D \times 10^7$ (cm^2/s) at 25 °C (Fl-FFF)	3.72	2.47	1.92	1.53	1.25
d_H (nm) (Fl-FFF)	11.9	17.7	22.7	28.9	35.1
M_b per monomer (g/mol)	496,900	583,300	583,700	790,700	1,256,700
Oligomer amount (wt%) from AUC[a]	82.50	6.17	10.46	0.64	0.23
Oligomer amount (wt%) from Fl-FFF[a]	80.70	14.63	2.59	0.47	1.61

[a]The relative oligomer amounts were calculated on the basis of a proportionality between the absorption signal and ferritin concentration, implying a constant ferrihydrite content for every capsule

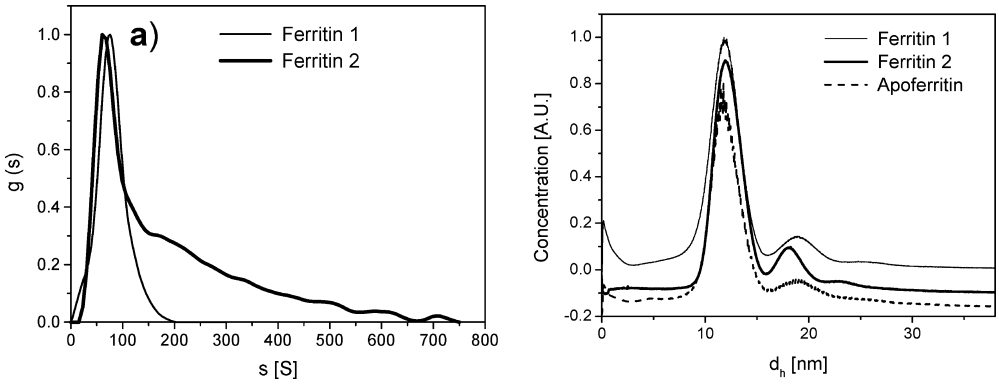

Fig. 3 **a** Sedimentation coefficient distributions (not diffusion corrected) for ferritin 1 and 2; **b** particle size distributions from Fl-FFF for the two ferritins and apoferritin. The higher sedimentation coefficients for ferritin 2 show its higher mineralization degree

Table 2 Data from AUC and Fl-FFF for ferritin 1 in comparison to a hard-sphere aggregation model. Index n indicates the oligomerization degree

	Monomer	Dimer	Trimer	Tetramer	Pentamer
Hard sphere aggregation	1.00	1.59	2.08	2.52	2.92
s_n/s_1	1.00	1.56	1.82	2.62	4.25
D_1/D_n	1.00	1.51	1.94	2.43	2.98

is significantly wrong. This is most likely a result of the fact that it could not be measured in dialysis equilibrium due to the danger of ferrihydrite dissolution. Therefore, absolute molar masses of the oligomers cannot be calculated with the present data sets. In such cases, the buoyant molar masses could be determined in solvents of different density by sedimentation velocity in combination with Fl-FFF to yield $\bar{v}$ of the different oligomers via the zero buoyant molar mass. This approach would be similar to the sedimentation equilibrium approaches for the $\bar{v}$ determination of supramolecular systems via the zero buoyant molar mass, where the $\bar{v}$ is difficult to measure via conventional density measurements (Tziatzios et al. 1999). However, sedimentation equilibrium cannot be applied here, as the $\bar{v}$ of each oligomer is desired. Another potential approach for $\bar{v}$ determination could be density gradients, but here problems can be expected due to the very low ferritin $\bar{v}$ and in the assignment of $\bar{v}$ to the different oligomers in a $\bar{v}$ distribution.

The observed smearing of the s distribution can be more extreme for other ferritin preparations (ferritin 2) which contain more iron (Fig. 3). In this case, it is already very difficult to allocate the different oligomers, as a reasonable diffusion correction is not possible anymore with the SEDFIT software, so that the diffusion-broadened $g(s)$ has to be used.

The extensive broadening of the s distribution of the higher mineralized ferritin 2 with s up to 700 S compared to the ferritin 1 preparation with s up to 200 S already shows the range of variation one can obtain for ferritins with different iron contents, although the particle size distribution from Fl-FFF (Fig. 3b) shows no variation in the oligomer resolution regardless of the ferritin iron content (apoferritin, ferritin 1 and ferritin 2), which shows the absence of aggregation so that the observed changes in the s distributions are due to the different density distributions.

Nevertheless, although the above outlined $\bar{v}$ determination is out of the scope of this paper, even the present data sets allow further conclusions on ferritin 1, which are summarized in Table 2.

For ferritin 1, assuming a constant $\bar{v}$ and frictional ratio, which neglects the small contributions of asymmetry onto the frictional coefficient, the s_n/s_1 and the D_1/D_n ratios of a hard-sphere aggregate with oligomerization degree n should show an $n^{2/3}$ dependence, reflecting the $M^{2/3}$ dependence. This is indeed found, in good agreement for s and D with the exception of the pentamer s value, which is unsafely detected (see Table 1). Another conclusion, which can be drawn from the good agreement with the simple aggregation model, is that the ferritin oligomers adapt a globular solution structure and can be hydrodynamically treated as spheres.

Conclusions

In this paper, the combination of fractionating density-independent methods like Fl-FFF with the size- and $\bar{v}$-dependent AUC is demonstrated for the example of ferritin, which has a superimposed particle size and $\bar{v}$ distribution. If the AUC results are combined with an independently obtained size distribution like that from Fl-FFF, molar mass information can be derived for each of the oligomers as long as their sedimentation coefficients can be determined and the particle $\bar{v}$ distribution is not too broad.

However, with the present data set (ferritin 1), only the buoyant molar masses of the different oligomers could be obtained as $\bar{v}$ could not be measured via the classical density measurements. The constant buoyant

molar mass indicates a constant average composition so that the $\bar{v}$ distribution is rather small, although evident in a smearing of the *s* distribution. This smearing is more pronounced for the higher mineralized ferritin 2.

From the *s* and *D* distributions, ferritin is shown to oligomerize into globular solution structures. In summary, this rather crude combined AUC and Fl-FFF analysis on a well-known model organic-inorganic hybrid system indicates a way in which future analyses could be performed. A combined analysis is already possible for AUC and DLS (Schuck 2002) and these first results encourage that an unravelling of size and density folded sedimentation coefficient distributions is possible, yielding a considerable increase in the quality of the results. Owing to the sample hybrid nature, the accuracy of such analyses should greatly be enhanced by multi-wavelength analyses with CCD array-based spectrophotometers, which are easily adaptable as Fl-FFF detectors and which we currently implement on an analytical ultracentrifuge.

The virtue of the combined analysis of whole sedimentation coefficient distributions from AUC and diffusion coefficient distributions from Fl-FFF can so far only be estimated, but it can be expected that, in the future, not only particle size distributions but also $\bar{v}$ and molar mass distributions can be determined from a combination of AUC and Fl-FFF experiments. This would turn the apparent disadvantage of AUC with folded size and $\bar{v}$-dependent sedimentation coefficient distributions into an advantage, if the *s* distributions are determined in solvents of different densities, combining this information with the density-independent *D* distribution from Fl-FFF in a global analysis approach.

References

Bauminger ER, Harrison PM, Hechel D, Nowik I, Treffry A (1991) Iron(III) can be transferred between ferritin molecules. Proc R Soc London Ser B 244:211–217

Cölfen H, Antonietti M (2000) Field-flow fractionation techniques for polymer and colloid analysis. Adv Polym Sci 150:67–187

Grady JK (1996) Analytical ultracentrifugation studies of the iron distributions in horse spleen ferritin. MSc thesis, University of New Hampshire

Grady JK, Chasteen ND, Laue TM (1996) Characterization of iron distributions in reconstituted ferritin by analytical ultracentrifugation. Biophys J 70:MP453

Harrison PM, Artymiuk PJ, Ford GC, Lawson DM, Smith JMA, Treffry A, White JL (1989) Ferritin: function and structural design of an iron-storage protein. In: Mann S, Webb J, Williams RJP (eds) Biomineralisation: chemical and biochemical perspectives. VCH, Weinheim, pp 257–294

Mann S (1986) Biomineralization: a new branch of bioinorganic chemistry. Chem Unserer Zeit 20:69–76

Pauck T, Cölfen H (1998) Hydrodynamic analysis of macromolecular conformation. A comparative study of flow field flow fractionation and analytical ultracentrifugation. Anal Chem 70:3886–3891

Proulxcurry PM, Chasteen ND (1995) Molecular aspects of iron uptake and storage in ferritin. Coord Chem Rev 144:347–368

Schuck P (2000) Size distribution analysis of macromolecules by sedimentation velocity ultracentrifugation and Lamm equation modeling. Biophys J 78:1606–1619

Schuck (2002) Size and shape distributions of macromolecules in solution by global analysis of sedimentation and dynamic light scattering. Lecture on advances in analytical ultracentrifugation and hydrodynamics, Autrans, France, 8–11 June

Tziatzios C, Durchschlag H, Sell B, van den Broek JA, Mächtle W, Haase W, Lehn JM, Weidl CH, Eschbaumer C, Schubert D, Schubert US (1999) Solution properties of supramolecular cobalt coordination arrays. Prog Colloid Polym Sci 113:114–120

Williams JM, Danson DP, Janot C (1978) Mössbauer determination of iron core particle size distribution in ferritin. Phys Med Biol 23:835–851