Enrichment and Characterization of Ferritin for Nanomaterial Applications

Rodolfo Ghirlando[i], Radina Mutskova[¥], Chad Schwartz[j],
[i]National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health
[¥]Honors College, Integrated Life Sciences, University of Maryland,
[j]Beckman Coulter, Inc., Indianapolis, IN, 46268

ABSTRACT: Ferritin is a ubiquitous iron storage protein utilized as a nanomaterial for labeling biomolecules and nanoparticle construction. Commercially available preparations of horse spleen ferritin, widely used as a starting material, contain a distribution of ferritins with different iron loads. We describe a detailed approach to enrich for differentially loaded ferritin molecules by common biophysical techniques such as size exclusion chromatography and preparative ultracentrifugation, and characterize these preparations by dynamic light scattering, and analytical ultracentrifugation. We demonstrate the combination of methods to standardize an approach at determining the chemical load of nearly any particle, including nanoparticles and metal colloids. Purification and characterization of iron content in monodisperse ferritin species is particularly critical for several applications in nanomaterial science.

INTRODUCTION

Iron is an essential element necessary for human life which is stored in the ubiquitous and highly-conserved protein ferritin. The protein plays a key role in iron metabolism and its ability to sequester the element allows ferritin to be essential to iron detoxification and reserve. Regulation of iron is critical to many biological processes and deviation leads to many diseased states.

Ferritin is an iron-storage protein distributed in high concentrations in the liver and spleen but also found in the heart and kidney. Ferritins from all species have 24 protein subunits arranged in 4,3,2 symmetry to form a spherical and hollow complex with an approximately 8 nm diameter cavity capable of storing up to 4500 iron atoms. The protein shell is highly conserved with a combined molar mass of around 500 kDa, and both the apo- and iron-loaded form have been well-characterized by a wide range of spectroscopic, crystallographic, and biochemical assays, to determine their structure and function. Research efforts on ferritin were also concentrated on its mechanism and regulation in diseased states.

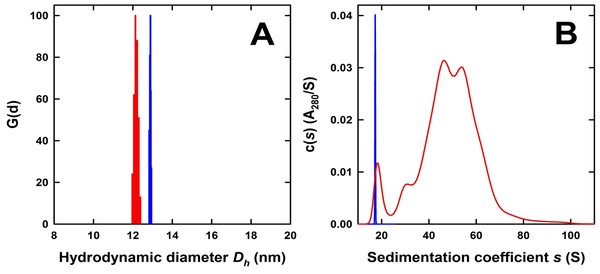
Much of the recent research on ferritin transitioned from basic structure-function relationships to a nanoparticle model for metal load and colloid mixture analysis, as the protein cage of ferritin has been proven useful as a storage vessel for a multitude of minerals of other metals including gadolinium, lead, cadmium, nickel, cobalt, chromium, and gold. Ferritin has also recently been used to create magnesium-, cobalt-, and copper-based nanoparticles for electronics with conductive and magnetic properties. However, it has become increasingly apparent that control of the size and mineral load is critical to fabrication of nanomaterials such as memory devices and for fluorescent labeling of biomolecules.

Here, we describe a standardized method for determining the maximum load of several sources of ferritin by purification of the monomeric protein by size exclusion chromatography, sizing by dynamic light scattering, isolation of maximally-loaded species by preparative centrifugation, and finally, mass determination by sedimentation velocity and sedimentation equilibrium analytical ultracentrifugation. We also present procedures for improving core size homogeneity utilizing sucrose-gradient preparative ultracentrifugation (Ghirlando, et. al., *Nanotechnology*, 2015, In Press)

RESULTS & DISCUSSION

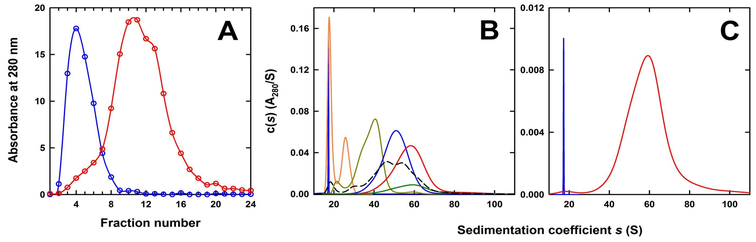
CHARACTERIZATION OF MONOMERIC FERRITIN AND APOFERRITIN

Ferritin (red) and apoferritin (blue) purchased from Sigma-Aldrich were subjected to dynamic light scattering (A) and sedimentation velocity (B) for size and sedimentation coefficient respectively. Both ferritin and apoferritin appear homogenous by DLS but ferritin is heterogenous due to iron load as probed by analytical ultracentrifugation.

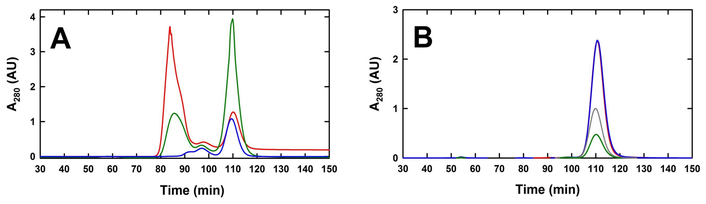


FRACTIONATION AND CHARACTERIZATION OF LOADED FERRITIN

The absorbance of each fraction was measured for apoferritin (blue) and monomeric ferritin (red) following centrifugation at 38,000 rpm for 2.5 h on a SW40Ti rotor in a 5%-30% (w/v) sucrose gradient (**A**). Selected ferritin fractions were then characterized by sedimentation velocity after size exclusion chromatography. Profiles for fractions 5 (orange), 8 (dark yellow), 11(blue), 14 (red) and 17 (green) are shown, along with data for apoferritin (blue, scaled plot) and unfractionated monomeric ferritin (dashed black line) (**B**). Fraction 17 of the ferritin from the sucrose gradient was analyzed by sedimentation velocity and the c(s) profile was plotted. Fully loaded ferritin has a sedimentation coefficient of 60 S (**C**).

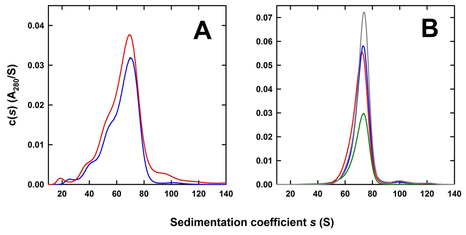


FERRITIN SOURCE VALIDATION

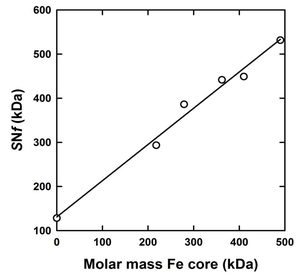


In order to validate the results from an additional source, a new batch of horse-spleen ferritin was purchased from Amersham. Approximately5 mg of unpurified apoferritin (blue), 2.7 mg of unpurified Sigma-Aldrich ferritin (red) and 5 mg of Amersham ferritin (green) were resolved by size exclusion chromatography. The species eluting at 110 min represent the monomeric fractions (**A**). The Amersham preparation appeared to contain less aggregation than the Sigma-Aldrich sample. Sucrose gradient fractionation was repeated on the Amersham samples using the same protocol as for Sigma-Aldrich ferritin. Following fractionation, size exclusion was performed on fractions 14 (red), 15 (blue), 16 (gray), and 17 (green) (**B**). The fractions eluted homogenously as a monomer.

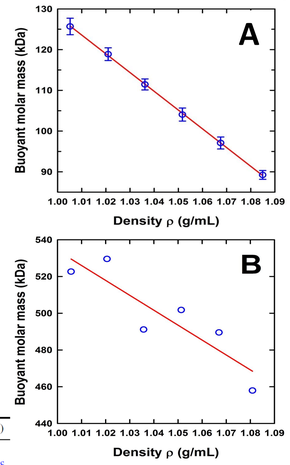
Amersham ferritin (red) and monomeric Amersham ferritin purified by size exclusion (blue) are plotted for c(s) profiles (A). Following sucrose gradient fractionation, Amersham fractions 14 (red), 15 (blue), 16 (gray), and 17 (green) were subjected to SV-AUC and again plotted for c(s).



PARTIAL SPECIFIC VOLUME AND MOLAR MASS DETERMINATION



To obtain an estimate of the molar mass, we assumed that this species had the same size and shape as apoferritin and fixed f/fo to 1.27; the value for the partial specific volume was refined in the data analysis returning a molar mass of 890 kDa. Simple calculations were then employed to determine the iron load and the mass of the iron core was calculated at 410±20 kDa by subtracting the mass of apoferritin from the mass of maximally loaded ferritin which aligned well with previous x-ray scattering data.



Sedimentation equilibrium experiments were also carried out to further determine the molar masses of the 17 S apoferritin (A) and 70 S Amersham ferritin fractions (B). As in the sedimentation velocity experiments, sedimentation equilibrium data collected at multiple concentrations were consistent with the presence of a single species. The best-fit line provides values of the intercept and slope for the determination of the partial specific volume and hydration parameter. The observed buoyant molar mass decreased linearly with increasing solution density suggesting the presence of an invariant particle.

Sample	AUC method	$s_{20,w}$ (S)	Molar mass M (kDa)	$\bar{v}$ (ml g^{-1})	B_1 (g g^{-1})
Apoferritin	SV	17.16	500 ± 20	0.7338[a]	—
	SE	—	480 ± 9[b]	0.732 ± 0.016[c]	0.23 ± 0.1[c]
Ferritin					
Sigma-5	SV	26.9			—
Sigma-8	SV	39.2	700	0.564[d]	—
Sigma-11	SV	51.6	760	0.484[d]	—
Sigma-14	SV	59.0	840	0.457[d]	—
Sigma-17	SV	60.0	890	0.463[d]	—
Amersham-14	SV	70.4	970	0.426[d]	—
	SE	—	900 ± 40[e]	—	-
Amersham-15	SV	71.7	980	0.422[d]	—
	SE	—	920 ± 45[e]	—	—
Amersham-16	SV	72.1	980	0.419[d]	—
	SE	—	960 ± 60[e]	0.41 ± 0.23[c]	0.50 ± 0.24[c]
Amersham-17	SV	69.8	950	0.420[d]	—
	SE	—	890 ± 40[e]	—	—

A table summarizes the data obtained from both the sedimentation velocity and sedimentation equilibrium runs on apoferritin and both sources of ferritin following fractionation from the sucrose gradient.

CONCLUSIONS

Nanoparticles are routinely loaded with chemical moieties for targeting, detection, and function, and determination of the maximum load of these particles is important for design and delivery. We have standardized an approach to purify and characterize homogenous populations of ferritin for nanomaterial construction. We showed how a combination of chromatography, dynamic light scattering, and preparative and AUC can overcome limitations of other biochemical assays such as ELISA which are unable to distinguish between ferritin and apoferritin. Using this standardized approach, researchers can more accurately elucidate the number of conjugated entities to a specific particle of interest prior to clinical testing.

Content Approved Code: CENT-1232PST11.15-A