Size Exclusion Chromatography in the Presence of an Anionic Surfactant

Intact Protein Profiling

Application Note

Author

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Abstract

Sodium dodecyl sulfate (SDS, or SLS) is a well known anionic detergent, frequently used to denature proteins. It is commonly used in polyacrylamide gel electrophoresis (SDS-PAGE), where a remarkably consistent level of binding across a wide range of proteins imparts a reliable charge-to-mass ratio. This allows separation of denatured proteins based on relative size due to their relative ion mobility. Conversely, size exclusion chromatography (SEC) for size-based separation of proteins is normally performed under nondenaturing conditions using predominantly aqueous buffers as mobile phase.

This Application Note used SEC with light scattering detection to investigate the impact of varying SDS concentration on the protein molecule. This was achieved by studying the changes in RT, apparent molecular weight, and hydrodynamic radius.

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Introduction

To denature proteins, sodium dodecyl sulfate (SDS) is used above its critical micelle concentration (CMC). The CMC of SDS in pure water is 8.2 mM (approximately 0.2% w/v). However, the presence of buffer salts or changes in pH and temperature may reduce the CMC significantly. CMC values of around 1.0 mM (approximately 0.04% w/v) are observed in phosphate buffered saline (PBS), for example. A micelle forms when approximately 62 SDS molecules coalesce into a spherical shape with a hydrophobic core surrounded by an anionic surface, as depicted in Figure 1. Typically, SDS is used at significantly higher concentrations of 2% w/v for sample preparation for SDS-PAGE, but also with a reducing agent to cleave disulfide bonds within the protein molecule. The resulting saturated SDS-protein complex has, on average, 1.4 g SDS per gram of protein [1]. Such denatured proteins are considered to adopt a rigid cylindrical shape resulting in the observation that ion mobility in gel electrophoresis is proportional to molecular weight. By omitting the reduction of disulfide bonds, it has been observed that the ratio of SDS to protein decreases significantly and the time to reach equilibrium may be doubled [2].

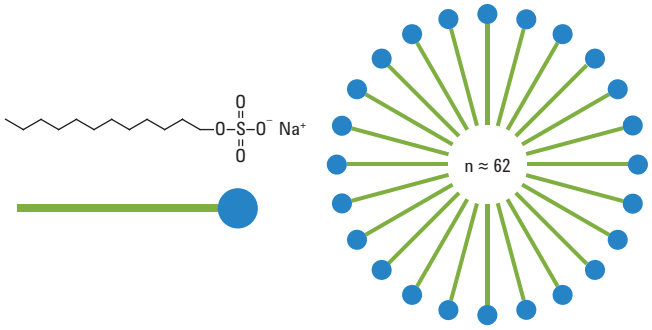

Figure 1. Cartoon depicting SDS micelle formed at concentrations above CMC (aggregation number ~ 62).

In contrast, adding SDS (approximately 10:1 mol ratio) has been found to arrest heat denaturation of BSA solution, which otherwise leads to creation of high molecular weight oligomers [3], or reduces the level of noncovalent aggregation. Historically, it was recommended to use 0.1% w/v SDS in the mobile phase to reduce retention time (RT), and improve peak shape in protein size exclusion chromatography (SEC) [4].

The mechanism by which SDS denatures a protein is not fully understood. Since both the detergent molecule and the protein possess complementary ionic and hydrophobic regions, it is clear that a combination of mechanisms may be involved. Thermodynamic studies have suggested that interactions are not ionic alone. It is conceivable that SDS first begins to bind through an ionic interaction with positively charged amino acids side chains (Lys and Arg) on the surface of the protein. Increasing SDS concentration leads to higher levels of incorporation, and it is postulated that ultimately, the flexible protein chain is decorated with SDS micelles rather than the rigid cylinder model originally proposed [5].

Bovine serum albumin (BSA) is a well studied protein due to its capability of acting as a carrier of smaller molecules between tissues and cells. The structure and size (hydrodynamic radius) of the BSA monomer has been determined using multiple analytical techniques. Its primary structure is 583 amino acids with a molecular weight of 66,463 Da, Figure 2. Commercial samples frequently contain varying amounts of dimer and higher-order aggregates, and different isolation techniques may result in varying amounts of these oligomers. Since BSA contains 35 cysteine residues, there are 17 disulfide bridges and one free thiol group. It is believed that BSA oligomers are predominantly covalently linked through disulfide bonds involving the extra thiol group. Therefore, in the absence of reducing agents, denaturation with SDS will not result in such covalently linked dimers and higher aggregates reverting to a monomeric form.

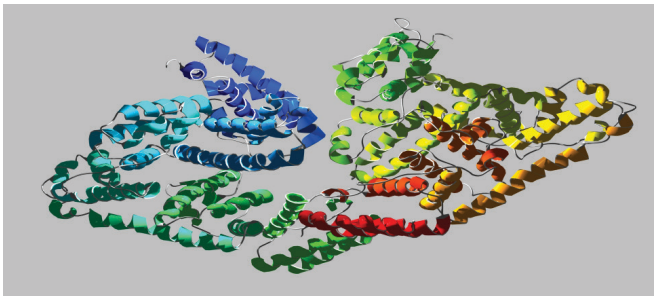

Figure 2. Native heart-shaped conformation of bovine serum albumin.

To explore the effect of SDS denaturation on the characteristics of BSA, a series of experiments were performed. Using SEC of the nonreduced BSA protein, the amount of dimer and higher-order aggregates could be determined. The addition of a light scattering detector with dynamic light scattering (DLS) capability allowed the measurement of both molecular weight and hydrodynamic radius (Rh). The eluent chosen for this series of experiments was phosphate buffered saline (PBS), containing 10 mM phosphate buffer, and 140 mM NaCl, pH 7.4 (Eluent A). A second mobile phase was prepared in an identical manner, but with the addition of 2% w/v SDS (Eluent B). The HPLC instrument was then run with increasing levels of SDS incorporated in the mobile phase by varying the proportions of Eluent A and Eluent B.

Throughout the experiment, the same BSA sample was used, prepared at a concentration of 10 mg/mL in PBS alone.

The experiment was designed to determine:

- If SDS can denature BSA oligomers
- What effect is observed on the molecular weight measured using inline light scattering detection
- What change in hydrodynamic radius is observed

Conditions

Parameter	Value
Column:	Agilent AdvanceBio SEC 300 Å, 2.7 µm, 7.8 × 300 mm (p/n PL1180-5301)
Samples:	BSA (Sigma-Aldrich)
	Sample prepared 10 mg/mL in Eluent A
Eluent A:	PBS, pH 7.4 (10 mM phosphate, 140 mM NaCl)
Eluent B:	PBS, pH 7.4 (10 mM phosphate, 140 mM NaCl) + 2.0% w/v SDS
Gradient:	Isocratic elution at 0% B; 10%B; 20% B; 30% B; 40% B; 50 % B; 60% B; 70% B
Flow rate:	0.8 mL/min
Detector:	UV, 280 nm; LS 15°, LS 90°
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC with Agilent 1260 Infinity Bio-SEC multidetector system.

Results and Discussion

Following two blank injections to allow for the column to become conditioned in the mobile phase, duplicate injections of BSA 10 mg/mL were made.

Figure 3 shows overlaid chromatograms (UV 280 nm signal) of four individual experiments at different mobile phase compositions. Table 1 contains RT information relating to the entire experimental series.

From Table 1, it is apparent that there is little change in total peak area for different runs. This indicates that increasing SDS concentration does not result in the absorption of the protein, or loss of protein through induced aggregation.

Table 1. RT and total peak area.

% SDS In mobile phase	RT (min) Higher-order aggregates	Dimer	Monomer	Total peak area
0.0%	6.00	6.46	7.29	3,632
0.2%	5.22	5.59	6.45	3,633
0.4%	4.96	5.23	5.96	3,668
0.6%	4.89	5.15	5.83	3,588
0.8%	4.89	5.13	5.80	3,571
1.0%	4.89	5.11	5.77	3,572
1.2%	4.88	5.11	5.76	3,536
1.4%	4.90	5.10	5.75	3,566

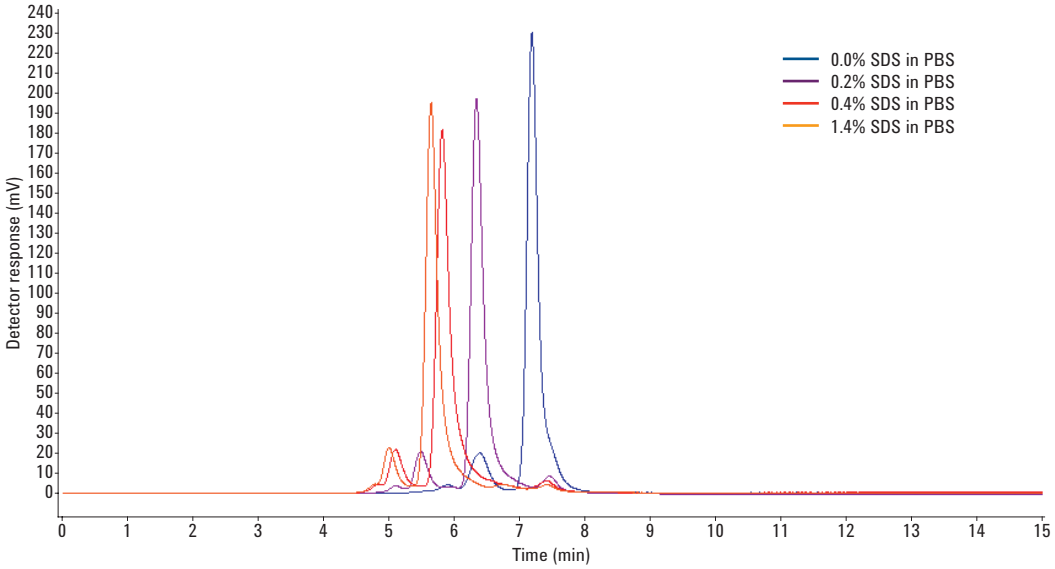

Figure 3. SEC chromatograms of BSA with increasing SDS concentration in mobile phase.

RTs become shorter but stabilize from 0.6 to 1.4% SDS (w/v), as seen in Figure 4. This shortening of RT also results in lower resolution between the peaks, so it proved difficult to accurately determine how much dimer and higher-order aggregates became denatured. It is clear that these multimer peaks are still abundant and must, therefore, be predominantly covalently linked.

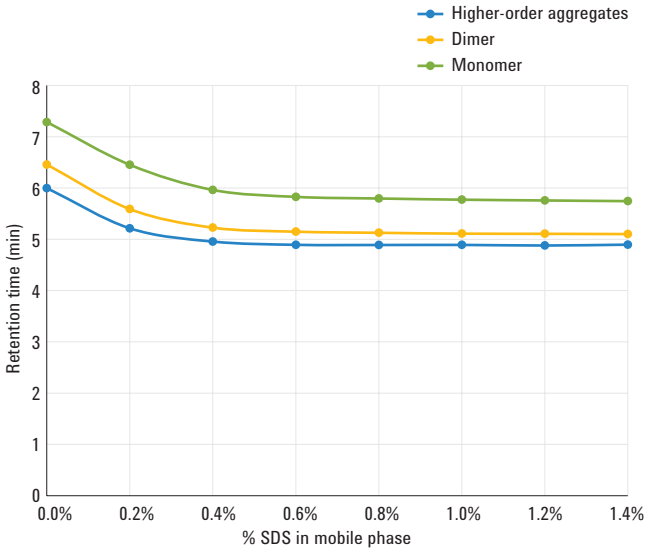

Figure 4. Effect of % SDS in mobile phase on RT.

In SEC, shorter RT is indicative of an increase in the size of a molecule in solution, but does not necessarily infer an increase in molecular weight. To ascertain the molecular weight of the three major peaks of the chromatogram, the BioSEC software was used to calculate the molecular weight using the relationship described in the following formulae [6]:

$$(LS) = K_{LS} c M \left(\frac{dn}{dc}\right)^2$$

$$(UV) = K_{UV} c \epsilon$$

Where:

(LS) = Light scattering detector signal

K_{LS} = LS detector constant

dn/dc = Specific refractive index increment*

(UV) = UV detector signal (280 nm)

ϵ = Extinction coefficient

* Using the approximation of $dn/dc \approx 0.186$ mL/g for nonglycosylated proteins

Figure 5 shows the regions of each peak chosen to perform the light scattering molecular weight determination to try to minimize interference due to anticipated peak overlap. Table 2 shows the results for the molecular weight measurements. This table includes columns where the relative number of monomer units has been calculated. It is important to recognize that the molecular weight of the higher-order aggregates peak is nearly always three times larger than the molecular weight of the monomer peak, indicating it is a trimer. The molecular weight of the dimer peak is two times larger than the molecular weight of the monomer peak, as expected. In addition, the molecular weight of each of the peaks increases with increasing SDS concentration until a plateau is reached at 0.6% w/v SDS, in agreement with the stabilization of RTs at this point.

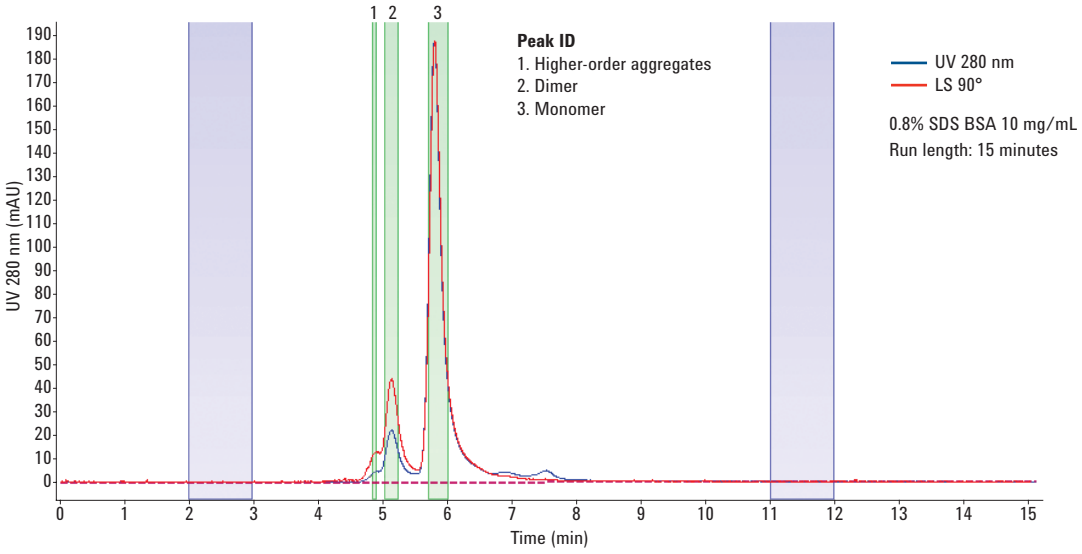

Figure 5. Molecular weight calculation regions for (1) higher-order aggregates, (2) dimer, and (3) monomer peaks of BSA.

Table 2. Molecular weight analysis results from light scattering detection.

	Molecular weight			Number of monomer units		
SDS w/w%	Higher-order aggregates	Dimer	Monomer	Higher-order aggregates	Dimer	Monomer
0.0	202,197	136,383	67,838	3.0	2.0	1.0
0.2	242,541	194,876	107,905	2.2	1.8	1.0
0.4	560,154	359,239	203,737	2.7	1.8	1.0
0.6	667,318	423,418	212,596	3.1	2.0	1.0
0.8	615,726	404,898	207,028	3.0	2.0	1.0
1.0	624,648	404,634	205,868	3.0	2.0	1.0
1.2	617,385	405,618	206,376	3.0	2.0	1.0
1.4	612,031	406,699	206,614	3.0	2.0	1.0

Since the observed molecular weight increases in line with the concentration of SDS present in the mobile phase, it may be inferred that each molecular species is actually gaining mass through accumulation of associated SDS. However, the observed mass gain is considerable and higher than expected, particularly since this BSA sample has not been reduced (Table 3). The steady state mass gain under the analysis conditions is 2 g SDS per 1 g protein, suggesting that SDS micelles may be accumulating along the protein molecule regardless of the number of oligomers it contains.

Table 3. Mass gain for BSA with increasing surfactant concentration, showing steady state at concentrations >0.6%.

	Mass gain			SDS:BSA (g/g)		
SDS w/v%	Higher-order aggregates	Dimer	Monomer	Higher-order aggregates	Dimer	Monomer
0.0	0	0	0	0.0	0.0	0.0
0.2	40,344	58,493	40,067	0.2	0.4	0.6
0.4	357,957	222,856	135,899	1.8	1.6	2.0
0.6	465,121	287,035	144,758	2.3	2.1	2.1
0.8	413,529	268,515	139,190	2.0	2.0	2.1
1.0	422,451	268,251	138,030	2.1	2.0	2.0
1.2	415,188	269,235	138,538	2.1	2.0	2.0
1.4	409,834	270,316	138,776	2.0	2.0	2.0

Closer inspection of the concentration-dependant UV trace of the chromatograms obtained at 0.0% SDS and 1.4% SDS shows further differences. There is clearly a reduction in resolution for the higher molecular weight species, however some smaller peaks eluting after the monomer peak have become evident in the 1.4% SDS mobile phase composition (Figure 6). It was not possible to identify these lower molecular weight species.

Figure 7A shows the signals from the LS 90° detector for BSA at 0.0% SDS and 1.4% SDS concentrations. Since the light scattering detector is mass sensitive rather than concentration-dependant, it is not surprising that the signal obtained under the higher surfactant concentration conditions is much larger than the signal under native conditions.

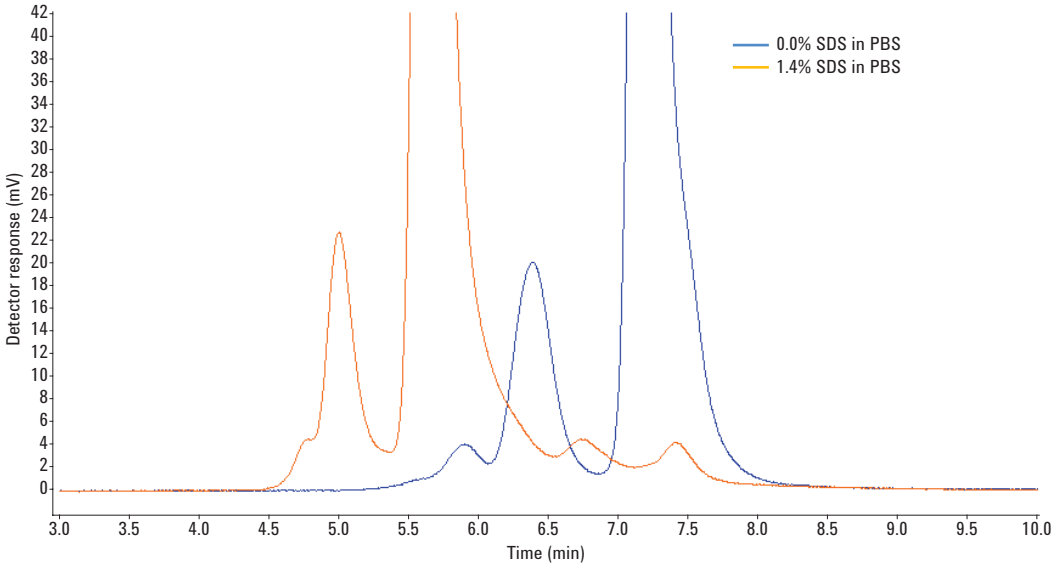

Figure 6. Close up of the UV 280 nm signal of BSA in PBS mobile phase containing 0.0% SDS and 1.4% SDS.

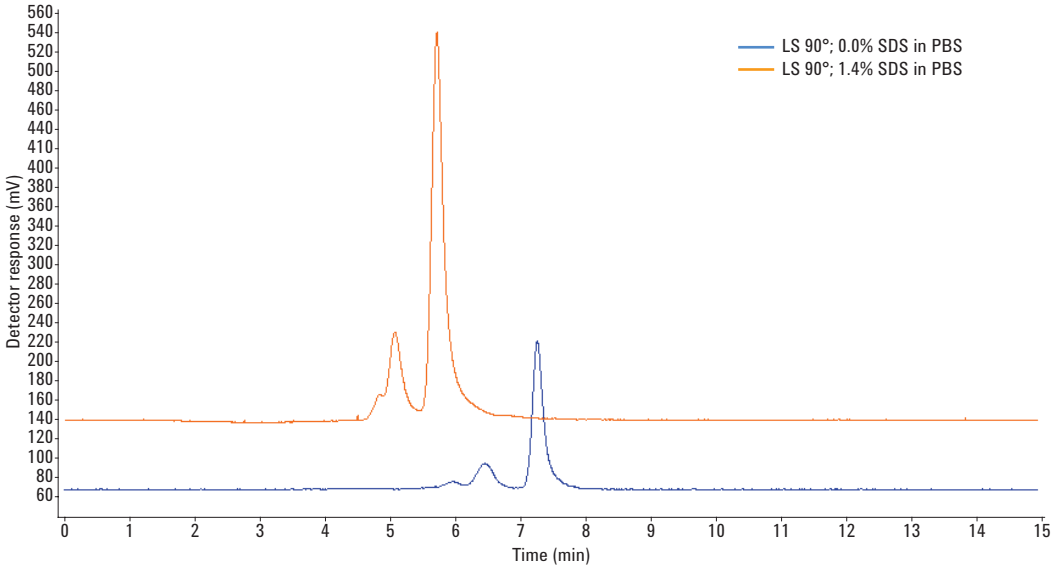

Figure 7A. Overview of the LS 90° signal of BSA in PBS mobile phase containing 0.0% SDS and 1.4% SDS.

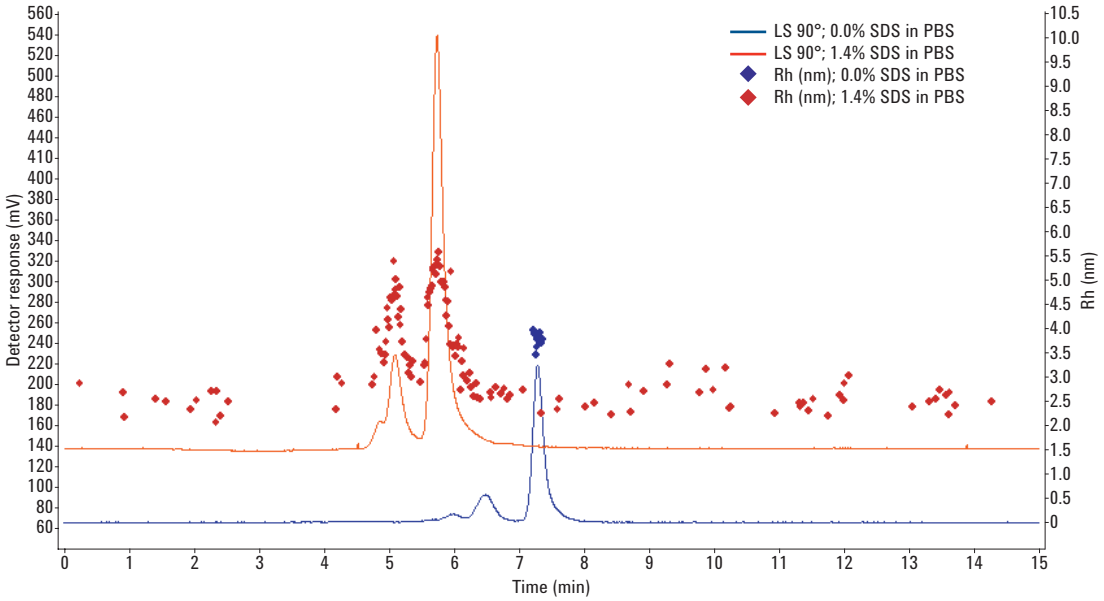

Figure 7B. Overview of the LS 90° signal of BSA in PBS mobile phase containing 0.0% SDS and 1.4% SDS with hydrodynamic radius (Rh) data added.

In addition to the increased response seen in Figure 7A at the highest SDS concentration, there is also a vertical offset. The reason for this becomes apparent when the DLS data are analyzed to determine the Rh. Not only do we see an increase in the Rh of the monomer peak (from around 3.8 to 5.7 nm, close to literature values) [7,8,9], there is a continual background of particles detected of approximately 2.4–2.8 nm in size, almost certainly due to SDS micelles in solution.

Conclusions

At the outset, this experiment was designed to determine if SDS can denature BSA oligomers using SEC with light scattering detection. It was found that accurate quantification of oligomer content was not possible through loss in resolution as the individual peaks corresponding to monomer, dimer, and higher-order aggregates eluted closer together. However, it is apparent the three individual species remain intact and become saturated with SDS to the same extent.

Simply denaturing the protein, making the molecule unfold, would be expected to give a larger structure in solution, leading to a shorter RT in SEC. However, as the SDS concentration was increased from 0.0 to 0.4% w/v, the molecular weight of the monomer, dimer, and higher-order aggregates increased proportionally. At higher SDS concentrations (0.6–1.4% w/v) the RTs (and, therefore, size in solution) and the molecular weight determined by light scattering stabilized as the molecular species were saturated by SDS.

Using DLS capability to determine hydrodynamic radius shows an increase in size comparable to literature values for a reduced BSA sample. The size increase does not reflect a change in conformation of the BSA sample run under nonreducing conditions, but replicates the observed increase in molecular weight as the molecule complexes with and becomes saturated by SDS micelles.

It is clear that, although the Agilent AdvanceBio SEC 300 Å column is able to tolerate SDS in the mobile phase, as seen by the good peak shape and reproducible recovery, the SEC separation is badly affected by the dramatic increases in size and molecular weight of the protein species being analyzed. The use of surfactants in the mobile phase for SEC should be avoided wherever possible.

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