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RESEARCH ARTICLE

Characterization of Oligomer Formation of Surfactant Protein-D (SP-D) Using AF4-MALLS

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Abstract: ***Background*:** Surfactant protein-S (SP-D) is a naturally occurring lung protein with the potential to treat pulmonary infections. A recombinant surfactant protein-D (SP-D) has been produced and was previously found to exist in multiple oligomeric states.

***Introduction*:** Separation and characterization of interconverting oligomeric states of a protein can be difficult using chromatographic methods, so an alternative separation technique was employed for SP-D to characterize the different association states that exist.

***Methods*:** Samples of SP-D were analyzed using asymmetrical flow field-flow fractionation (AF4) using UV and multi-angle laser light scattering (MALLS) detection. The AF4 method appears to be able to separate species as small as the monomer up to the dodecamer (the dominant species) to much larger species with a molar mass greater than 5 MDa.

***Results*:** Consistent elution of four distinct peaks was observed after repeated injections. The largest species observed under the last peak (labeled as Peak 4) were termed "unstructured multimers" and were resolved fairly well from the other species. The AF4-MALLS data suggest that only a small fraction of Peak 4 truly corresponds to high molar mass unstructured multimers. All other peaks demonstrated significant molar mass homogeneity consistent with AFM results.

***Conclusion*:** AF4-MALLS technology appears to be a powerful analytical approach to characterize the complex and dynamic interplay among different protein oligomeric species of SP-D in an aqueous solution.

Keywords: Surfactant protein, oligomeric states, MALLS, proteins and biopolymers, molar mass, oligomeric profile.

1. INTRODUCTION

Recombinant human surfactant protein D (rhSP-D) is a C-type (Ca^{2+}-dependent) lectin that comprises four domains: a cysteine-linked N-terminal region required for the formation of intermolecular disulfide bonds, a triple-helical collagen region, an α-helicalcoiled-coil trimerizing neck peptide, and a C-terminal calcium-dependent carbohydrate-recognition domain (CRD) [1]. Monomers form trimers by folding the collagenous region into triple helices and assembling a coiled-coil bundle of α-helices in the neck region. These trimers are stabilized by two disulfide bonds in the cysteine-rich N-terminal domain [2]. The SP-D trimer has a total molecular weight of 129 kDa, comprising three identical 43-kDa polypeptide chains. rhSP-D trimers can also form higher oligomerization states that vary by size and conformation [3]. These include dodecamers and structured multimers. The latter is formed by the association of dodecamers [4]. Finally, these varied oligomers can continue to assemble into even larger ensembles termed unstructured multimers. The distribution of these various oligomeric states is affected by processing and solution conditions.

In order to assess the quality of rhSP-D preparations and evaluate the degree of process control in the manufacturing of rhSP-D, one must have a reliable analytical method for quantifying the various associated states. This is even more important in that it is likely that not all of the oligomers are equally bioactive [5-8]. It has been shown that only states that are hexameric or larger appear to retain activity, at least in bacterial assays [8]. This study uses asymmetrical flow field-flow fractionation (AF4) coupled with UV and multi-angle laser light scattering (MALLS) detection for separating and quantifying rhSP-D oligomers. AF4 is effective at monitoring the aggregation behavior of various proteins and biopolymers [9-11].

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The use of AF4-MALLS allows for the accurate quantitation of dodecamer content and the observation and quantitation of higher-order species, including high molecular weight aggregates. The particular focus of the AF4-MALLS method is on the resolution and quantitation of rhSP-D species larger than the trimer. In general terms, this study illustrates the value of using a method orthogonal to size exclusion chromatography (SEC) to characterize complex biologics that exhibit multiple association states [12, 13], where SEC typically does not provide adequate results. This is particularly important here, where rhSP-D forms such large oligomers that they cannot enter an SEC column and achieve adequate resolution.

2. MATERIALS AND METHODS

2.1. Chemicals

The chemicals used in these studies are listed below in Table **1**. All solutions were prepared using 18.2 MΩ Ultrapure water obtained from a Millipore Simplicity water purification system. Recombinant human surfactant protein-D (rhSP-D) was provided by Airway Therapeutics, where it is designated as AT-100. The protein was shipped frozen on dry ice and thawed immediately prior to analysis.

2.2. AF4-MALLS Analyses

SP-D samples were separated using an Eclipse DualTecAF4 system (Wyatt Technology Corp., Santa Barbara, CA) coupled downstream in series to a (Ultimate 3000) variable wavelength UV detector (Dionex Corporation, Sunnyvale, CA) and multi-angle laser light scattering (MALLS) detector (Dawn Heleos II detector, Wyatt Technology Corp., Santa Barbara, CA). A Dionex Ultimate 3000 HPLC system (Dionex Corporation, Sunnyvale, CA) was used to inject the samples and deliver the mobile phase to the AF4 system. The AF4 configuration used a short channel with a 350 µm thick spacer (Wyatt Technology Corp., Santa Barbara, CA). Data analysis and calculations were performed using both Chromeleon (Dionex Corporation, Sunnyvale, CA) and Astra software packages (Wyatt Technology Corp., Santa Barbara, CA). The parameters for the AF4 separation method are found in Table **2**.

The ASTRA software calculated molar mass and root means square (RMS) radius moments for each selected peak (version 6.1.1.17). Moments were referenced to averages over the entire sample, which can include many peaks. Equation (**1**) relates to the number-average molar mass:

$$M_n = \frac{\sum_i n_i M_i}{\sum_i n_i} = \frac{\sum_i c_i}{\sum_i c_i / M_i} \quad \text{Eq. (1)}$$

An ASTRA measurement typically requires an independent concentration determination. Since the relation between concentration (mg/mL) and number density (number/mL) was nM = c, the results from the equation 2 could be determined. Equation (2) relates to weight-average molar mass:

$$M_w = \frac{\sum_i n_i M_i^2}{\sum_i n_i M_i} = \frac{\sum_i c_i M_i}{\sum_i c_i} \quad \text{Eq. (2)}$$

The polydispersity index value was: ρ = Mw / Mn. Typically, a value ≥ 1.2 was considered to be polydisperse, while values ≤ 1.1 were considered as having low polydispersity.

2.3. Integration Procedure

The oligomeric profile of SP-D samples was divided into four distinct peak sections (labeled as Peaks 1 through 4) using a combination of analysis of the UV signal and an analysis of the molar mass as determined by light scattering. All relative areas were calculated using a "drop-down" integration at points selected as outlined above.

Peak 2 corresponds to the dodecamer peak, whose limits were determined by analyzing the molar mass and the polydispersity index. A center point was determined, usually, the highest point of the UV trace, and boundaries were set equidistant from that point. Mw/Mn measured the polydispersity of that section. Boundaries were moved until the polydispersity index was ~ 1.05. Actual polydispersity indices for test samples varied between 1.008 and 1.090. It was observed that slight movements of the peak boundaries can cause significant changes in the index.

Peak 3 boundaries were set from the terminal boundary of Peak 2 to the dip in the UV trace found at 36.5 minutes. The outer boundary of Peak 4 was set at 45 minutes. At 45 minutes, the cross-flow was turned off, and any material remaining in the channel at that time was co-eluted.

Peak 1 included all species smaller than a dodecamer. In some cases, lower molecular weight species were resolved and separate integrations were performed. Typically, these species were found to account for relatively little of the

Table 1. Chemicals used in this study.

Chemical	Supplier	Product Number	Lot Number
Tris	GFS Chemicals	1096	C583878
Sodium Chloride	GFS Chemicals	657	C582104
Sodium Phosphate	GFS Chemicals	734	C579017
EDTA	Fisher Scientific	S311-500	136800

Table 2. Method parameters for rhSP-D separation.

Step	Start Time (Minutes)	End Time (Minutes)	Duration (Minutes)	Mode	CrossFlow Start (mL/min)	CrossFlow End (mL/min)
1	0	1	1	Elution	-	-
2	1	2	1	Focus		
3	2	3	1	Focus + inject		
4	3	6	3	Focus		
5	6	6.2	0.2	Elution	0.5	3
6	6.2	9.2	3	Elution	3	3
7	9.2	19.2	10	Elution	3	0.18
8	19.2	29.2	10	Elution	0.18	0.18
9	29.2	44.2	15	Elution	0.18	0
10	44.2	54.2	10	Elution	0	0
11	54.2	59.2	5	Elution + Inject	0	0
Detector Flow	0.5 mL/min		-	-	-	-
Inject Flow	0.2 mL/min					
Focus Flow	0.5 mL/min					
Injection Amount	5 µg					
UV Detection	214 nm					
Mobile Phase	20 mM Tris, pH 7.4 200 mM NaCl					

overall distribution of rhSP-D oligomers and were summed as a single population. It was possible to superimpose the M*w* trace over the UV trace and integration points were determined for hexamers (258 kDa), trimers (129 kDa), dimers (86 kDa) and monomers (42 kDa). The peaks were quite narrow but could be reliably determined if a sufficient amount of these lower order oligomers were present.

3. RESULTS AND DISCUSSION

3.1. AF4 Method Development

A series of test runs were conducted to determine the optimal mobile phase and cross-flow parameters for resolution of various rhSP-D species by AF4. The injection volume was initially set to place 10 µg of rhSP-D in the channel, but this was later reduced to 5 µg due to sufficient signal-to-noise at this injection load. In addition, this reduces the chance of overloading the channel as well. It was determined that UV detection at 214 nm was ideal, as this increased the signal-to-noise and enhanced sensitivity. An extended focus time was employed to determine if system peaks could be reduced in intensity. The focusing time before sample injection was increased to 2 minutes, while the focusing time after injection was set to three minutes. In total, the final method produced a complex fractogram with four main peak envelopes for SP-D (Fig. **1**).

3.2. Mobile Phase Evaluation

After determining an optimal mobile phase pH of ~7.4, the effect of mobile phase buffer species (tris or phosphate) on the AF4 profile of rhSP-D was evaluated. The fractograms are similar with each of these buffers, indicating that the nature of the buffer has a minimal effect on the dodecamer content and the overall association state of rhSP-D. However, the original elution buffer/formulation contained 1 mM EDTA. As calcium binding is known to affect rhSP-D oligomerization [14, 15], there was a question as to whether EDTA was required in the mobile phase. Subsequently, a mobile phase employing tris buffer with no EDTA, was examined. Use of this mobile phase resulted in a distinct dodecamer peak with a shoulder on the leading edge that may be a hexamer or lower order species. When EDTA (1 mM) is present in the mobile phase, the fractogram changes substantially, forming a considerable amount of Peak 1 (likely trimer or othlower-order oligomers). Given the potentially disruptive effects of EDTA and, to a lesser extent, phosphate (each of which may be related to calcium binding), it was decided to proceed with a pH 7.4 mobile phase using 200 mM NaCl and tris buffer.

3.3. Reproducibility

Four different metrics were selected on which to evaluate the AF4-MALLS method for reproducibility in characterize-

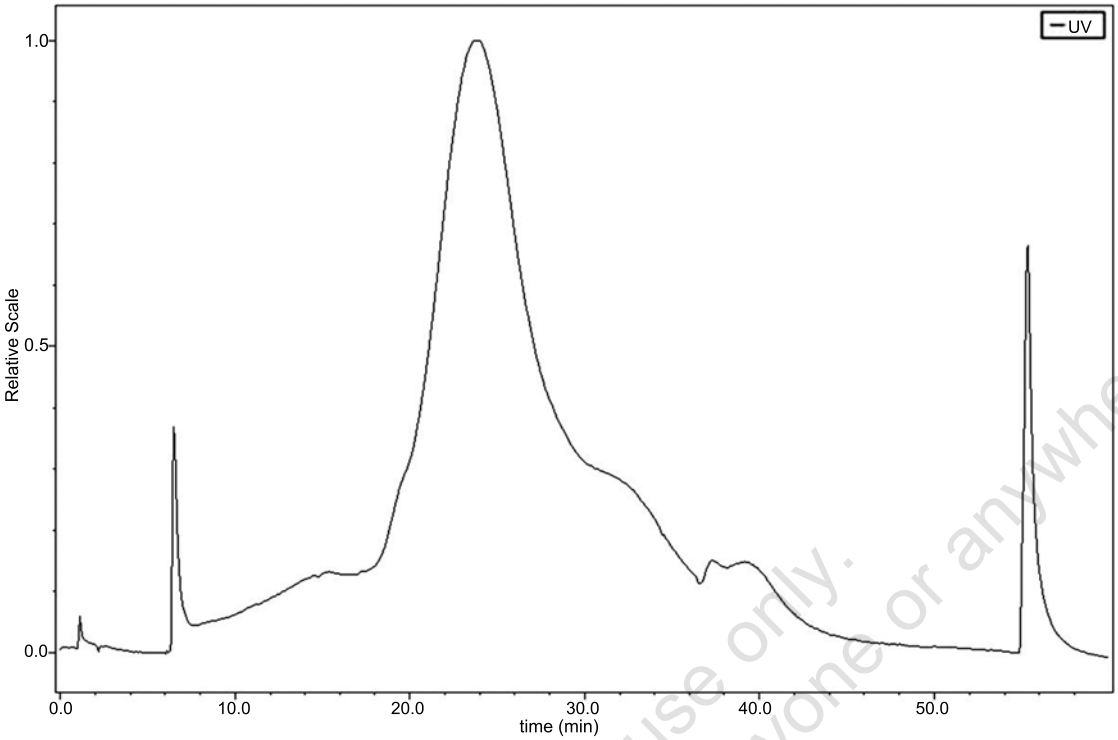


Fig. (1). AF4 fractogram for rhSP-D (lot 100) obtained using a mobile phase consisting of 20 mM Tris and 200 mM NaCl (pH 7.4) and the cross-flow program listed in Table **2**. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

ing rhSP-D. These included (i) the total area under the fractogram, (ii) relative area of the dodecamer, (iii) calculated molar mass of the dodecamer from the MALLS data, and (iv) the polydispersity of the molar mass within the dodecamer peak envelope.

For some initial samples, the reproducibility of the calculated molar mass of the dodecamer species (520.09 ± 4.61 kDa) (N=72 determinations) was very good, with the rsd averaging 0.79%. Using the integration parameters for delineating the dodecamer based on the polydispersity index (PDI) (see below), the relative area of the dodecamer was calculated. The rsd for this value was 3.46%, despite the fact that the relative area of the dodecamer in rhSP-D samples can vary widely, from about 25% to 65%. However, these different relative amounts can be quantified quite reproducibly with the current method.

Third, the rsd for the total area under the fractogram was determined to be 6.65% for these samples, using an injection mass of 5 µg of rhSP-D. Finally, the average PDI value for the dodecamer from this study was determined to be 1.056 ± 0.022, suggesting that the dodecamer population is quite homogeneous.

3.4. Dodecamer Content

It has been determined that some of the specific biological activity of rhSP-D is associated with the dodecamer content [3, 16], which elutes primarily as Peak 2 by this AF4 method. Given the incomplete resolution of the various oligomeric species, it was important to establish the integration boundaries in a reproducible fashion. All relative areas were initially calculated using a drop-down integration at selected points. In the case of Peak 2, which corresponds to the dodecamer species, the limits were determined by analyzing the molar mass and the polydispersity index. A center point was selected, usually the highest point of the UV trace, and the integration boundaries were set equidistant from that point. The polydispersity of this portion of the fractogram was measured by Mw/Mn, as described above (see Methods section). Typically, a value larger than 1.2 is considered to be polydisperse, while values less than 1.1 are designated as having low polydispersity (higher homogeneity in terms of the underlying species). Boundaries were moved until the polydispersity index was about 1.05 to set the final integration boundaries for Peak 2.

For Peak 3, boundaries were set from the terminal boundary of Peak 2 to a dip in the UV trace that was observable at 36.5 minutes, just prior to the elution of Peak 4. The oligomers eluting under the Peak 3 envelope appear to be higher order, structured oligomers, possessing a range of sizes, and are presumably based on the self-assembly of the dodecamer. Based on the initial analysis from atomic force microscopy (AFM) [4], there was a progressive assembly of SP-D, primarily from trimers. Initially, these associate to form dodecamers (Fig. **2**). Then, the dodecamers can assemble into larger structures that appear as 'fuzzy balls' [8] (herein referred to as structured multimers) in the AFM images (Fig. **2**). Thus, this is the nomenclature used to describe the ensemble of increasingly larger oligomers found in Peak 3. The size of the structured multimers was calculated in terms of radius, which placed them approximately in the range of 55-65 nm, with the largest being just slightly greater than 70 nm (Table **3**).

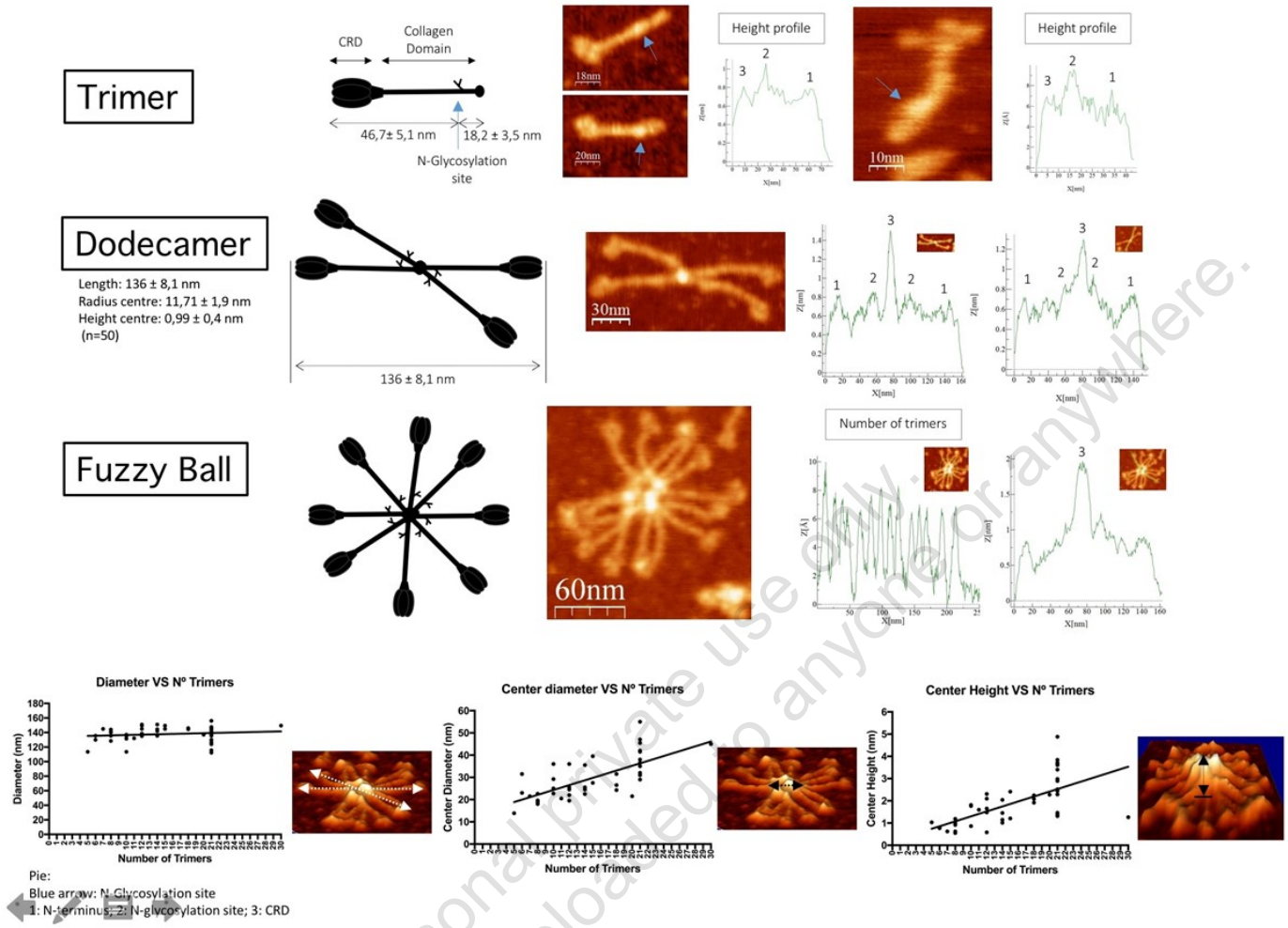


Fig. (2). Description of rhSP-D species based on AFM measurements (taken from reference 15). *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

In general, there is good agreement between the sizes determined by AFM and those measured by AF4-MALLS for species such as dodecamers. It appears that Peak 3 is comprised primarily of structured multimers, although somewhat larger species (termed unstructured multimers) may be present at the tail end of the peak envelope and in Peak 4 (vide infra). The exact nature of these higher molecular weight species is discussed below.

3.5. Unstructured Multimer Characterization

At approximately 34.5 minutes, both the UV and light scattering (LS) traces show a distinct change in the elution profile, as a new species group elutes in an envelope labeled as Peak 4 (Fig. **3**). The calculated radius for this species in lot 100 was determined to be about 50-55 nm (Fig. **4**), consistent with the formation of structured multimers. Another early lot of rhSP-D (lot 220) shows a similar profile as lot 100, although Peak 1 is better resolved and now comprises about 20% of the total oligomeric distribution. The calculated rod lengths through Peak 2 are consistent, with this being the dodecamer.

A distinct Peak 4 is observed for virtually all lots of rhSP-D. Yet, the size distribution can be quite different from lot to lot. There is an abrupt increase in molecular weight near 34 minutes, rising to values approaching 10^8 Da, much greater than one would expect for even the largest fuzzy ball or structured multimer. In fact, based on AFM data [4], the largest structured multimer was estimated to have a molar mass near 6 MDa (6 x 10^6 Da) and a maximal size near 70 nm. This suggests that there are larger species present in Peak 4 than seen earlier by AFM.

An expansion of a single run for lot 991 shows that the molecular weight at the end of Peak 3, leading into Peak 4, is near this maximal value for the structured multimers (Fig. **5**). The molar mass then rises through the elution of Peak 4, suggesting that there is some type of aggregate underneath this peak envelope. Similarly, the calculated radius increases dramatically at the beginning of Peak 4, rising to nearly 100 nm for both lot 991 (Fig. **5**) and lot 2099 (Fig. **6**), where these values are much larger than one would expect structured multimers based on the AFM studies.

Table 3. Size of various species based on AFM analysis 4.

-	Short (-)	Avg	Long (+)
Trimer Length (nm)	56.3	64.9	73.5
Dodecamer Length (nm)	127.9	136	144.1
Fuzzy Ball Radius (nm)	56.3	64.9	73.5

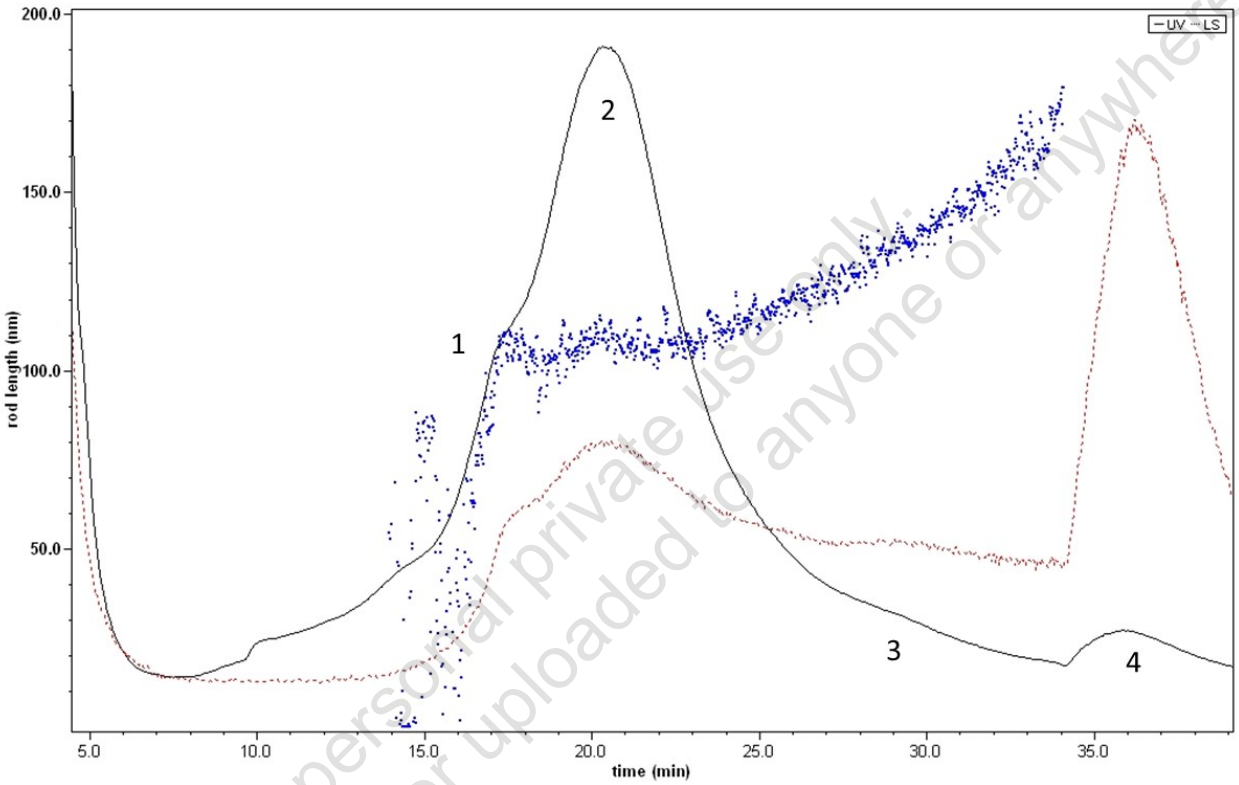


Fig. (3). AF4 fractogram of rhSP-D (lot 100) showing the UV trace (solid line), light scattering (LS) trace (dotted line) and the calculated rod lengths over Peaks 1, 2, and 3. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

The results from these lots suggest that Peak 4 contains at least some large, higher molecular weight aggregate species, where the radius rises well above the 60-65 nm limit estimated for structured multimers. Based on these observations, it was postulated that unstructured multimers of rhSP-D do exist and that they can be resolved using AF4-MALLS. The combined AF4 and AFM data suggest that any species larger than 70 nm in radius or 6 MDa in molecular weight is larger than an oligomer or a structured multimer and should be considered an unstructured multimer. As a result, the integration of Peak 4 was subdivided into structured multimers and aggregates using these two cut-offs: 70 nm in radius and 6 MDa in terms of molecular weight.

If one uses the original integrations, Peak 4 represents about 3.1% of the total area for lots 990 and 991, but only about 1.4% for lot 1010 and 1.6% for lot 2099 (Table **4**). Using the 70 nm cut-off, the unstructured multimer content was determined to be 2.54% for 990, 1.45% for 991, 0.68% for lot 2099, and 0.74% for lot 1010. In other words, there is an underlying population of structured multimers that constitutes about 30-50% of the total area of Peak 4 in these three samples (Table **5**). By comparison, the 6 MDa cut-off indicates that nearly all of Peak 4 contains unstructured multimers for the three lots (Table **5**). In some cases, the calculated amount of unstructured multimers exceeds the relative area of Peak 4 itself. This suggests that the size cut-off is the most appropriate metric to apply to quantify the amount of unstructured multimers in rhSP-D. Moreover, the size cut-off (as opposed to the molar mass cut-off) most closely coincides with the UV and LS traces, making the 70 nm size limit the most reliable indicator of the presence of species that are larger than the largest structured multimer.

3.6. Stability of Oligomeric Distribution of rhSP-D

Unlike other biologics, such as monoclonal antibodies, where the protein exists primarily in a monomeric state,

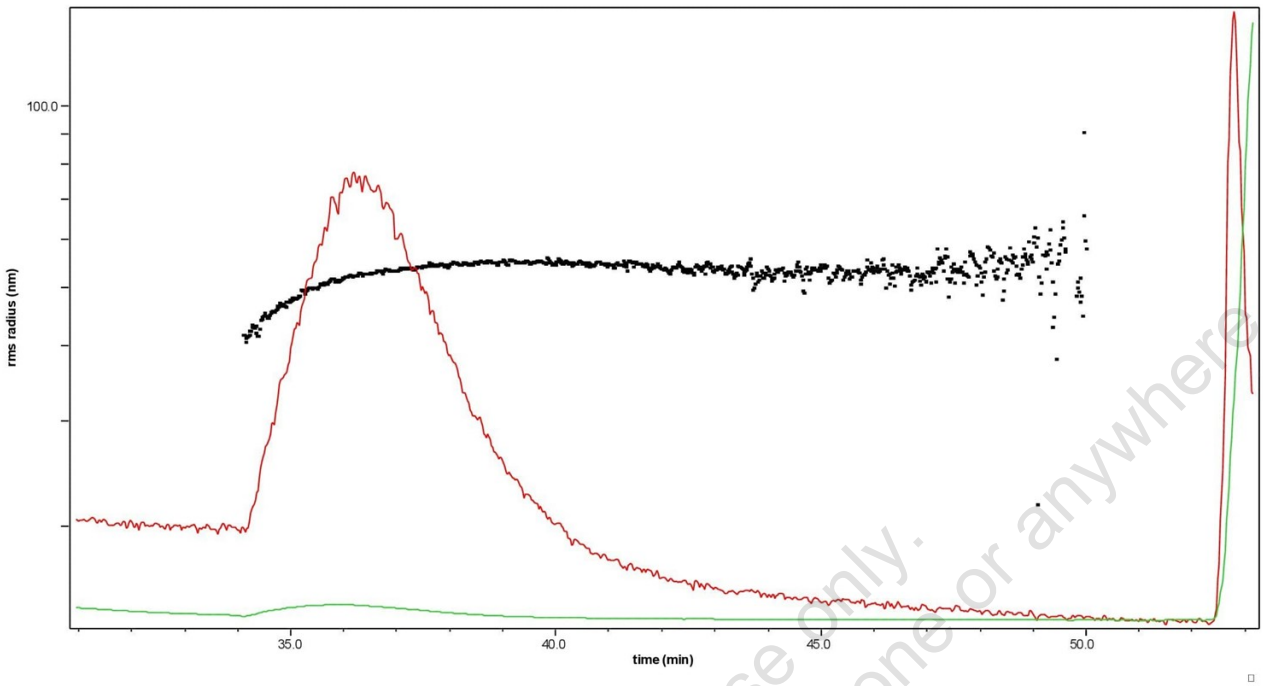


Fig. (4). AF4 fractogram of rhSP-D (lot 100) showing the UV trace (dotted line), light scattering (LS) trace (solid line) and the calculated radius over Peak 4. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

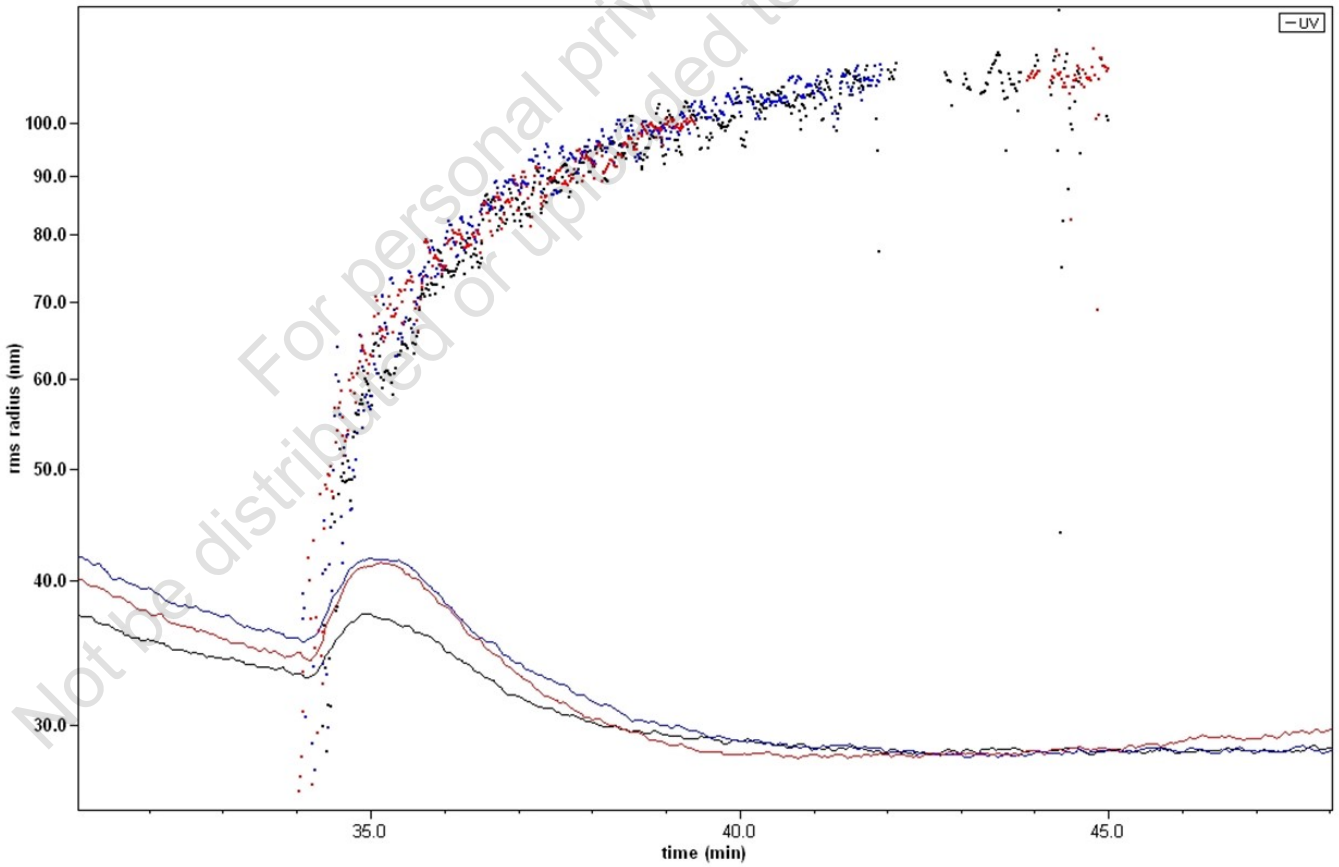


Fig. (5). Expansion of AF4 fractogram of rhSP-D (lot 991) showing Peak 4 (UV trace) and calculated radius. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

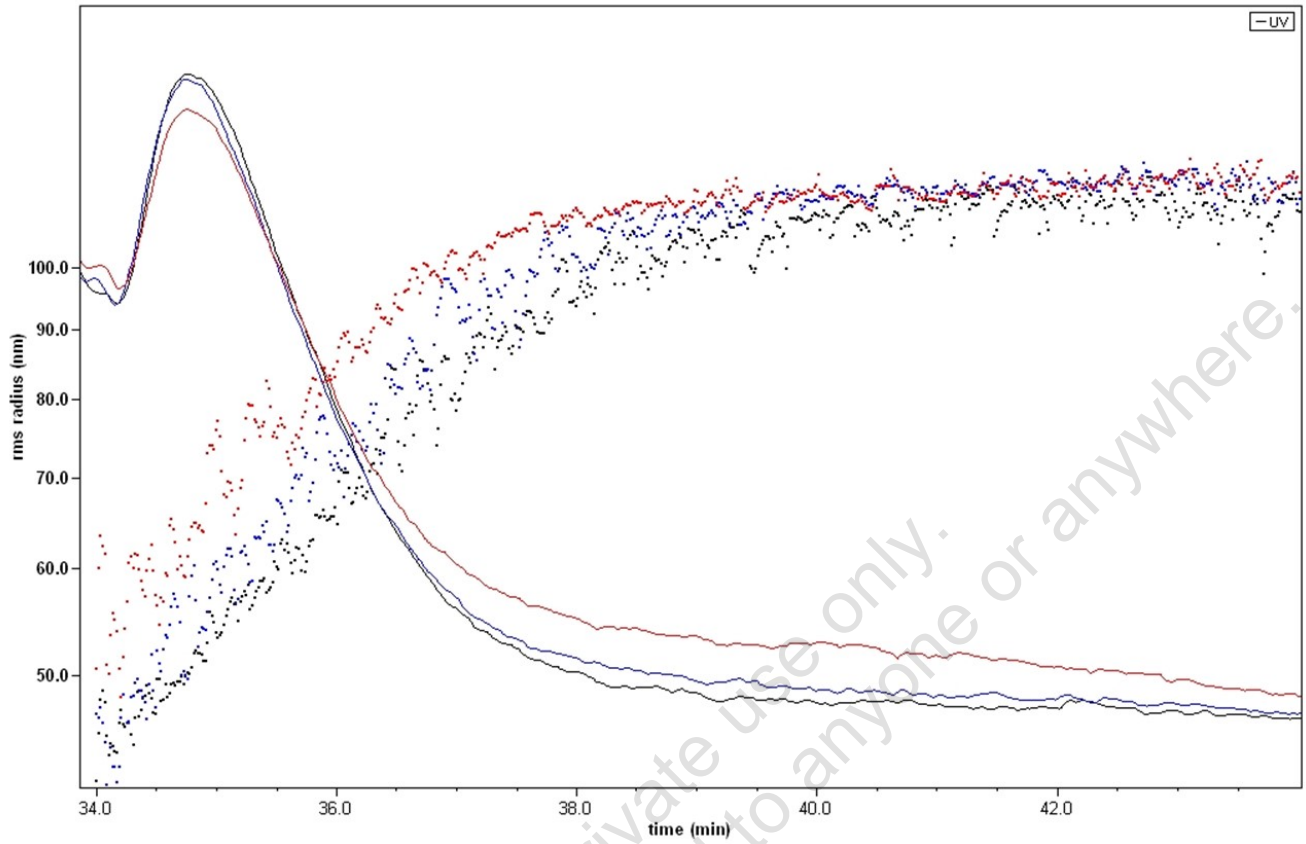


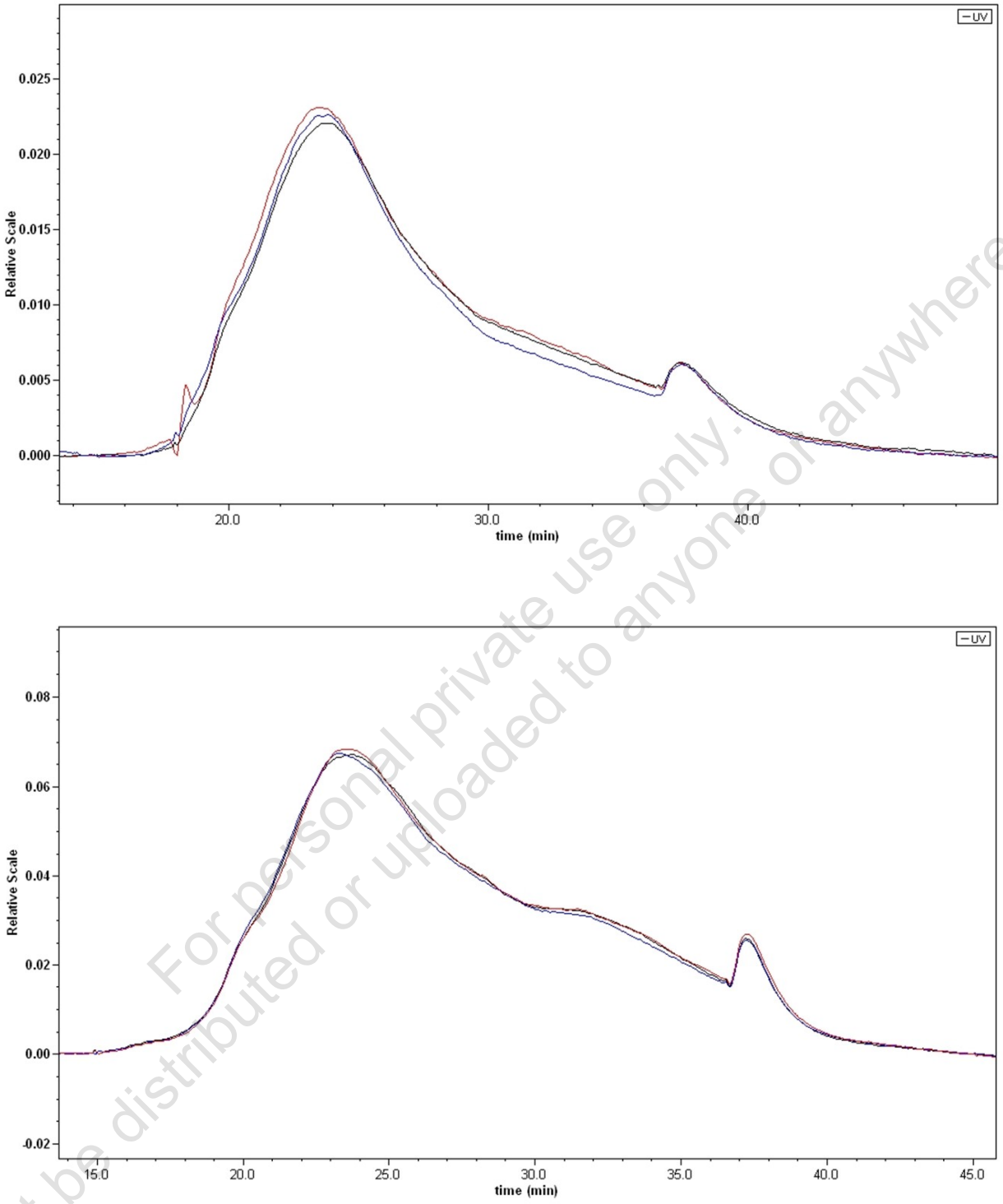
Fig. (6). Expansion of AF4 fractogram of rhSP-D (lot 2099) showing Peak 4 (UV trace) and calculated radius. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

Table 4. Reintegration of Peak 4 using the 70 nm cutoff value for Peak 4. The values listed in this table reflect the averages (± std. dev.) calculated from 3 separate injections of rhSP-D.

SP-D Lot	70 nm Cutoff (Minutes)	Original Relative Area % for Peak 4	New Relative Area % Added to Peak 3	New Relative Area % for Peak 4
990	35.04 ± 0.13	3.14 ± 1.09	0.60 ± 0.16	2.54 ± 0.94
991	35.67 ± 0.03	3.14 ± 0.78	1.69 ± 0.24	1.45 ± 0.80
1010	35.77 ± 0.24	1.35 ± 0.28	0.62 ± 0.17	0.74 ± 0.24
2099	35.63 ± 0.40	1.55 ± 0.78	0.87 ± 0.11	0.68 ± 0.78

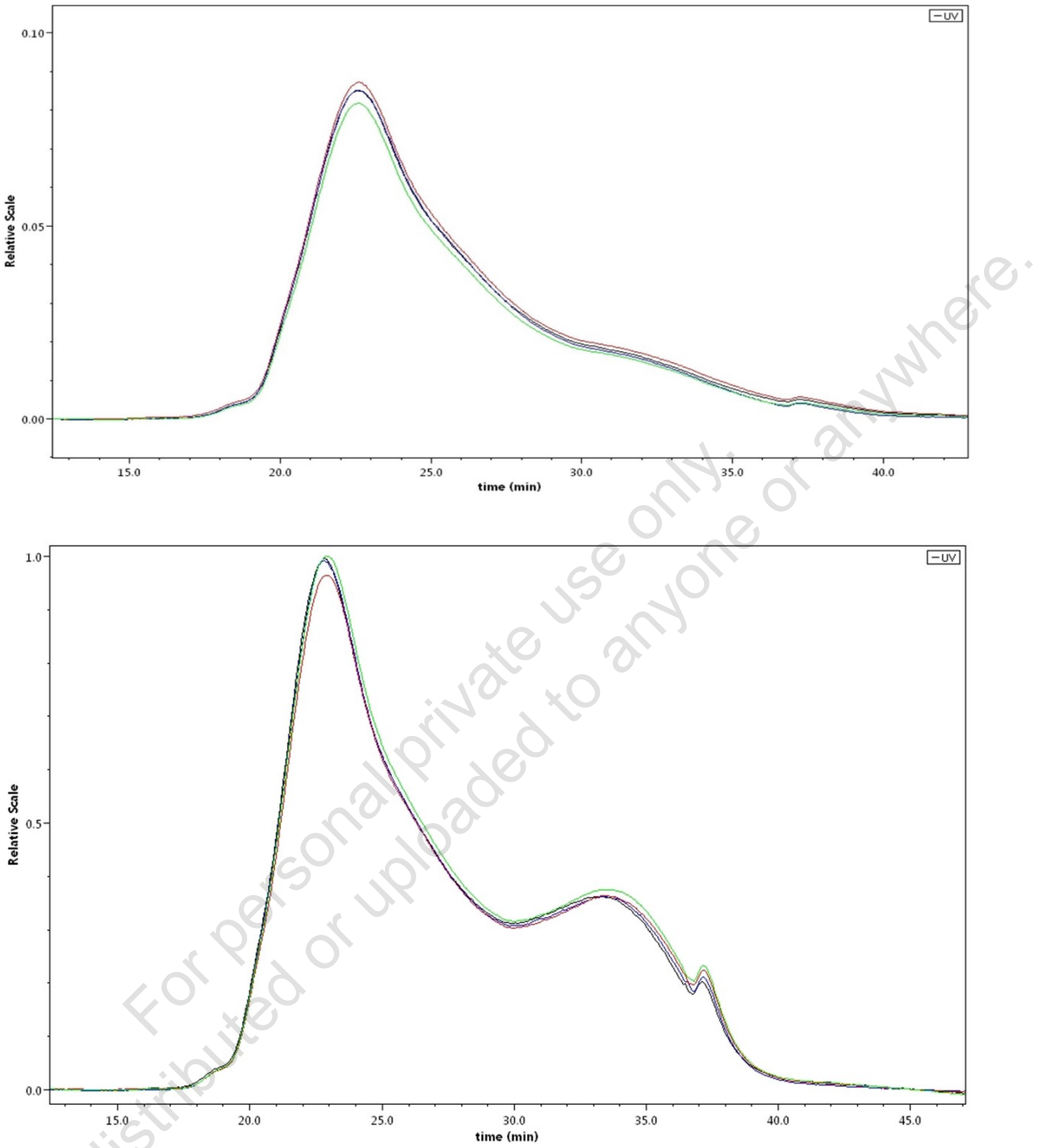
Table 5. Reintegration of Peak 4 using the 6 MDa molar mass cutoff values for Peak 4. The values listed in this table reflect the averages (± std. dev.) calculated from three separate injections of rhSP-D.

SP-D Lot	6 MDa Cutoff (Minutes)	Original Relative Area % for Peak 4	New Relative Area % Subtracted from Peak 3	New Relative % for Peak 4
990	33.80 ± 0.72	3.14 ± 1.09	-0.19 ± 0.11	3.33 ± 1.19
991	33.88 ± 0.31	3.14 ± 0.78	-0.19 ± 0.06	3.32 ± 0.84
1010	31.13 ± 0.80	1.35 ± 0.28	-0.98 ± 0.22	2.33 ± 0.49



Sample	Peak 2	Peak 3	Multimer
t0	47.30 ± 1.01	38.44 ± 1.27	4.10 ± 0.35
t3m/5 C	45.32 ± 1.75	44.47 ± 0.36	6.68 ± 1.02

Fig. (7). AF4 fractograms for lot 3569 of rhSP-D as a reconstituted lyophilized powder at t0 (upper panel) and after three months of storage at 5°C (t3m/5°C) (lower panel). A summary of the relative peak areas is provided below. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*



Sample	Peak 2	Peak 3	Multimer
t0	61.94 ± 0.94	32.69 ± 0.36	1.70 ± 0.35
t1m/25 C	49.34 ± 0.53	44.09 ± 0.31	2.13 ± 0.65

Fig. (8). AF4 fractograms of lot 1973 of rhSP-D at t0 (upper panel) and after one month of storage at 25° C (t1m/25°C) in the liquid state (lower panel). A summary of the relative peak areas is provided below. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

rhSP-D is both dynamic and complex, forming a variety of associated states, many of which are too large to be resolved using SEC. Given that these various oligomers may have different levels of biological activity, it is essential to quantify and characterize the distribution of states and determine how they may interconvert. There are few published examples of therapeutic proteins displaying this type of associative complexity.

The dominant, active form of rhSP-D is the dodecamer, although structured multimers (i.e., assemblies of dodecamers) have also been shown to be active. Thus, retention of dodecamer content should be an important quality attribute. The amount of dodecamer can be quantified with a great degree of confidence using MALLS to guide the integration, where the AF4 peaks are incompletely resolved.

As stated above, another advantage of AF4-MALLS is the ability to quantify and determine the amount and size of very large species, such as the unstructured multimers observed in this system. No other separation method can resolve protein oligomeric states over such a wide range of sizes and molar masses. In this study, unstructured multimers of rhSP-D can and do form. These usually comprise only 1-3% of the total distribution, based on relative areas. Yet, they can be resolved and distinguished from the structured multimers using the 70 nm size cutoff.

3.7. AF4-MALLS to Assess Stability of rhSP-D

The results presented here also show that there can be a dynamic redistribution between dodecamers (found in Peak 2), structured multimers (eluting primarily in the Peak 3 envelope), and unstructured multimers seen in the tailing edge of Peak 4.

In a lyophilized, sugar-based formulation of rhSP-D, storage does lead to changes in the oligomeric distribution, as measured by AF4-MALLS. This stress results in a modest loss of dodecamer and an increase in Peak 3, but also some increase in the levels of unstructured multimers (Fig. **7**). On the other hand, storage in the liquid state, leads to a sizable loss of dodecamer, with a concomitant increase in structured multimers, as evidenced by a marked increase in Peak 3 (Fig. **8**). In this case, little, if any, change is seen in the amounts of unstructured multimers.

CONCLUSION

An AF4-MALLS method has been developed to separate various oligomeric states of rhSP-D. The dominant species in an aqueous solution for rhSP-D is the dodecamer, a population that appears to be quite homogeneous based on the PDI. The PDI was used to establish the integration boundaries for the purposes of quantifying the dodecamer content. These dodecamers continue to self-associate to form higher order species termed 'fuzzy balls' or structured multimers. These elute immediately after the dodecamer peak in a broad envelope in what has been labeled as Peak 3. At ~34.5 minutes, a distinct new peak is resolved, labeled Peak 4. This peak envelope does contain some structured multimers, but also some higher molecular weight unstructured multimer species, which can be identified by having sizes greater than 70 nm. Using this metric, one can determine the true unstructured multimer content, which is typically about 1-4 % in the lots of rhSP-D analyzed here.

The AF4-MALLS method is able to monitor the distribution of oligomeric states in rhSP-D, which can change during storage. Upon storage, loss of dodecamer is observed, with increases in both structure and unstructured multimers. These studies illustrate the complex associative behavior of rhSP-D, a trait is rarely seen in therapeutic proteins and one that requires careful biophysical characterization.

LIST OF ABBREVIATIONS

SP-D	=	Surfactant Protein-D
MALLS	=	Multi-Angle Laser Light Scattering
CRD	=	Carbohydrate-Recognition Domain
AF4	=	Asymmetrical Flow Field-Flow Fractionation
SEC	=	Size Exclusion Chromatography
rhSP-D	=	Recombinant Human Surfactant Protein-D
RMS	=	Root Means Square

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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REFERENCES

[1] Crouch, E.; Persson, A.; Chang, D.; Heuser, J. Molecular structure of pulmonary surfactant protein D (SP-D). *J. Biol. Chem.,* **1994**, *269*(25), 17311-17319. http://dx.doi.org/10.1016/S0021-9258(17)32556-5 PMID: 8006040

[2] Sorensen, G.L.; Hoegh, S.V.; Leth-Larsen, R.; Thomsen, T.H.; Floridon, C.; Smith, K.; Kejling, K.; Tornoe, I.; Crouch, E.C.; Holmskov, U. Multimeric and trimeric subunit SP-D are interconvertible structures with distinct ligand interaction. *Mol. Immunol.,* **2009**, *46*(15), 3060-3069. http://dx.doi.org/10.1016/j.molimm.2009.06.005 PMID: 19577304

[3] Crouch, E.; Chang, D.; Rust, K.; Persson, A.; Heuser, J. Recombinant pulmonary surfactant protein D. Post-translational modification and molecular assembly. *J. Biol. Chem.,* **1994**, *269*(22), 15808-15813. http://dx.doi.org/10.1016/S0021-9258(17)40752-6 PMID: 8195236

[4] Arroyo, R.; Martín-González, A.; Echaide, M.; Jain, A.; Brondyk, W.H.; Rosenbaum, J.; Moreno-Herrero, F.; Pérez-Gil, J. Supramolecular assembly of human pulmonary surfactant protein SP-D. *J. Mol. Biol.,* **2018**, *430*(10), 1495-1509. http://dx.doi.org/10.1016/j.jmb.2018.03.027 PMID: 29626540

[5] Crouch, E.C. Surfactant protein-D and pulmonary host defense. *Respir. Res.,* **2000**, *1*(2), 6. http://dx.doi.org/10.1186/rr19 PMID: 11667972

[6] Håkansson, K.; Reid, K.B.M. Collectin structure: A review. *Protein Sci.,* **2000**, *9*(9), 1607-1617. http://dx.doi.org/10.1110/ps.9.9.1607 PMID: 11045608

[7] Crouch, E.; McDonald, B.; Smith, K.; Cafarella, T.; Seaton, B.; Head, J. Contributions of phenylalanine 335 to ligand recognition by human surfactant protein D: Ring interactions with SP-D ligands. *J. Biol. Chem.,* **2006**, *281*(26), 18008-18014. http://dx.doi.org/10.1074/jbc.M601749200 PMID: 16636058

[8] Arroyo, R.; Echaide, M.; Moreno-Herrero, F.; Perez-Gil, J.; Kingma, P. S. Functional characterization of the different oligomeric forms of human surfactant protein SP-D. *Biochimica et Biophysica Acta,* **2020**, *1868*(8), 140436. http://dx.doi.org/10.1016/j.bbapap.2020.140436

[9] Rambaldi, D.C.; Zattoni, A.; Reschiglian, P.; Colombo, R.; De Lorenzi, E. *In vitro* amyloid Aβ1-42 peptide aggregation monitoring by asymmetrical flow field-flow fractionation with multi-angle light scattering detection. *Anal. Bioanal. Chem.,* **2009**, *394*(8), 2145-2149. http://dx.doi.org/10.1007/s00216-009-2899-1 PMID: 19562325

[10] Hawe, A.; Romeijn, S.; Filipe, V.; Jiskoot, W. Asymmetrical flow field-flow fractionation method for the analysis of submicron protein aggregates. *J. Pharm. Sci.,* **2012**, *101*(11), 4129-4139. http://dx.doi.org/10.1002/jps.23298 PMID: 22911663

[11] Yohannes, G.; Jussila, M.; Hartonen, K.; Riekkola, M.L. Asymmetrical flow field-flow fractionation technique for separation and characterization of biopolymers and bioparticles. *J. Chromatogr. A,* **2011**, *1218*(27), 4104-4116. http://dx.doi.org/10.1016/j.chroma.2010.12.110 PMID: 21292269

[12] Pavišić, R.; Dodig, I.; Horvatić, A.; Mijić, L.; Sedić, M.; Linarić, M.R.; Sovulj, I.G.; Preočanin, T.; Krajačić, M.B.; Cindrić, M. Differences between reversible (self-association) and irreversible aggregation of rHuG-CSF in carbohydrate and polyol formulations. *Eur. J. Pharm. Biopharm.,* **2010**, *76*(3), 357-365. http://dx.doi.org/10.1016/j.ejpb.2010.09.006 PMID: 20854908

[13] Choi, J.; Lee, S.; Linares-Pastén, J.A.; Nilsson, L. Study on oligomerization of glutamate decarboxylase from Lactobacillus brevis using asymmetrical flow field-flow fractionation (AF4) with light scattering techniques. *Anal. Bioanal. Chem.,* **2018**, *410*(2), 451-458. http://dx.doi.org/10.1007/s00216-017-0735-6 PMID: 29167934

[14] Efrati, H.; Hawgood, S.; Williams, M.C.; Hong, K.; Benson, B.J. Divalent cation and hydrogen ion effects on the structure and surface activity of pulmonary surfactant. *Biochemistry,* **1987**, *26*(24), 7986-7993. http://dx.doi.org/10.1021/bi00398a066 PMID: 3427118

[15] Duvoix, A.; Mackay, R.M.; Henderson, N.; McGreal, E.; Postle, A.; Reid, K.; Clark, H. Physiological concentration of calcium inhibits elastase-induced cleavage of a functional recombinant fragment of surfactant protein D. *Immunobiology,* **2011**, *216*(1-2), 72-79. http://dx.doi.org/10.1016/j.imbio.2010.03.006 PMID: 20378199

[16] Crouch, E.C. Collectins and pulmonary host defense. *Am. J. Respir. Cell Mol. Biol.,* **1998**, *19*(2), 177-201. http://dx.doi.org/10.1165/ajrcmb.19.2.140 PMID: 9698590