Scientific and Regulatory Perspectives on Measuring Protein Aggregation of Biotechnology Products

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Disclaimer

The views expressed in this talk are those of the speaker and do not necessarily reflect the official position or policy of the U.S. Food and Drug Administration or the Department of Health and Human Services

Definitions and types

- **Protein aggregates** are defined as any self-associated protein species, with monomer defined as the smallest naturally occurring and/or functional subunit. (*FDA Guidance for Industry: Immunogenicity Assessment for Therapeutic Protein Products*)
- Aggregates are further **classified** based on five characteristics: size, reversibility/dissociation, conformation, chemical modification, and morphology (*Narhi et al. 2012*)
- Aggregates ranging from dimer to visible particles that are hundreds of micrometers in size (*Narhi et al. 2012*) have been recognized for their potential to elicit immune responses to therapeutic protein products for over a half-century (*Gamble 1966*)

ICH Q6B: Specifications

2. Product-Related Impurities Including Degradation Products (6.2.2)

The following represents the most frequently encountered molecular variants of the desired product and lists relevant technology for their assessment. Such variants may need considerable effort in isolation and characterization in order to identify the type of modification(s). Degradation products arising in significant amounts during manufacture and/or storage should be tested for and monitored against appropriately established acceptance criteria.

a. Truncated forms. Hydrolytic enzymes or chemicals may catalyze the cleavage of peptide bonds. These may be detected by HPLC or SDS-PAGE. Peptide mapping may be useful, depending on the property of the variant.

b. Other modified forms. Deamidated, isomerized, mismatched S-S linked, oxidized, or altered conjugated forms (e.g., glycosylation, phosphorylation) may be detected and characterized by chromatographic, electrophoretic, and/or other relevant analytical methods (e.g., HPLC, capillary electrophoresis, mass spectroscopy, circular dichroism).

c. Aggregates. The category of aggregates includes dimers and higher multiples of the desired product. These are generally resolved from the desired product and product-related substances and quantitated by appropriate analytical procedures (e.g., size exclusion chromatography, capillary electrophoresis).

Protein aggregation and product-specific risk factors

- Product origin (foreign or human)
- Primary molecular structure and post-translational modifications
- Higher-order structure of the aggregate
- Pegylation/glycosylation
- Aggregation by impurities with adjuvant activity
- Immunomodulatory properties of the product
- Formulation components
- Container closure considerations
- In-use conditions and product custody

Why control for aggregates?

- Protein aggregates have the potential to negatively impact clinical performance
- Current USP particulate testing is not designed to control the potential risk of large protein aggregates to impact immunogenicity
- Development of quantitative analytical methods for particle counting and characterization is important for risk assessment and control of final drug product quality, safety, and efficacy

Methods for aggregate measurement

Table 1 Methods for the study of protein aggregation in intact cells

Approach		Measurement methods	Application examples
❖ **Genetically encoded fusion tags**			
✓ **Fusion of the target polypeptide with a fluorescent protein or an enzyme**	Reduction of fluorescence or of enzymatic activity after aggregation; detection of functional polypeptides within active IBs	Bulk cell fluorescence; fluorescence microscopy; flow cytometry; enzymatic activity	Monitoring of protein aggregation within intact cells [31]; localization of functional polypeptides within IBs [22]; formation of active IBs [16,23,24]; screening of aggregation inhibitors [26]
✓ **Fusion of the target polypeptide with the tetra-Cys tag**	Formation of hyperfluorescent aggregates in presence of FlAsH		
❖ **Conformational sensitive dyes**			
✓ **Thioflavin-S**	Th-S fluorescence reports on amyloid-like structure of the protein aggregates	Bulk cell fluorescence; fluorescence microscopy; flow cytometry	Detection of amyloid-like aggregates within intact cells [32]
❖ **Direct spectroscopic detection of structural properties**			
✓ **FTIR**	Monitoring of intermolecular β-sheet structures in IBs	Label-free intact cell (micro)spectroscopy	Monitoring of protein aggregation whithin intact cells [35,39]
✓ **NMR**	Detailed structural information of the protein embedded within IBs	Solid-state NMR of whole cells	Detection of native-like structures [43]
❖ **Aggregation sensitive reporters**			
✓ **Reporter protein under an aggregation sensitive promoter**	Protein aggregation induces the expression of the reporter protein. The measured fluorescence or enzymatic activity of the reporter protein is related to the level of aggregation within cells	Enzymatic activity; fluorescence	Monitoring of protein aggregation within intact cells [37,45]

Ami *et al. Microbial Cell Factories* 2013, **12**:17
http://www.microbialcellfactories.com/content/12/1/17

Pharm Res (2011) 28:920–933
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COMMENTARY

Strategies for the Assessment of Protein Aggregates in Pharmaceutical Biotech Product Development

John den Engelsman • Patrick Garidel • Ronald Smulders • Hans Koll • Bryan Smith • Stefan Bassarab • Andreas Seidl • Otmar Hainzl • Wim Jiskoot

Table IV Typical Use of Techniques in Industry with Respect to Aggregate Analysis

Method	Validation	Quantification	Robustness[a]	Sensitivity[a]	Sample throughput[a,b]	QC method[c]
Visual inspection	Yes	No	Medium	Medium	High	Yes
Optical microscopy	No	Possible	Medium	N/A	Low	No
Fluorescence microscopy	No	No	Low	High	Low	No
Electron microscopy	No	No	Low	N/A	Low	No
Flow imaging	No	Yes	Low	N/A	Medium	No
Atomic force microscopy	No	No	Medium	N/A	Low	No
Turbidity	Yes	No	High	Medium	Medium	Yes
DLS	No	No	Medium	High	High	No
SEC-MALLS	No	No (MALLS part)	Medium	High	High	No
Light obscuration	Yes	Yes	Medium	Medium	Medium	Yes
"Native" mass spectrometry	No	No	Low	Medium	Low	No
Macro-IMS	No	No	Low	N/A	Medium	No
AUC	No	Yes	Low	Medium	Low	No
SEC	Yes	Yes	High	Medium	High	Yes
AF4	Yes	Yes	Medium	Medium	High	No
SDS-PAGE	Yes	Possible	Medium	Medium	High	Yes
Native PAGE	Yes	Possible	Medium	Low	Medium	No
CE-SDS	Yes	Yes	Medium	Medium	High	Yes
UV-VIS spectroscopy	No	No	Medium	Medium	High	No
Infrared spectroscopy	No	No	Medium	N/A	Low	No
Raman spectroscopy	No	No	Medium	N/A	Low	No
Fluorescence spectroscopy	No	No	Medium	N/A	High	No
Circular dichroism spectroscopy	No	No	Medium	N/A	Medium	No
NMR spectroscopy	No	No	Medium	Medium	Medium	No

[a] Scoring (low, medium, or high) was based on consensus of opinion of the authors; N/A = not available; [b] Low: <10; medium; 10–25; high >25 per day and per operator; [c] QC = quality control; all listed methods can be used for extended characterization; see Table I for definitions

Structural Characterization of IgG1 mAb Aggregates and Particles Generated Under Various Stress Conditions

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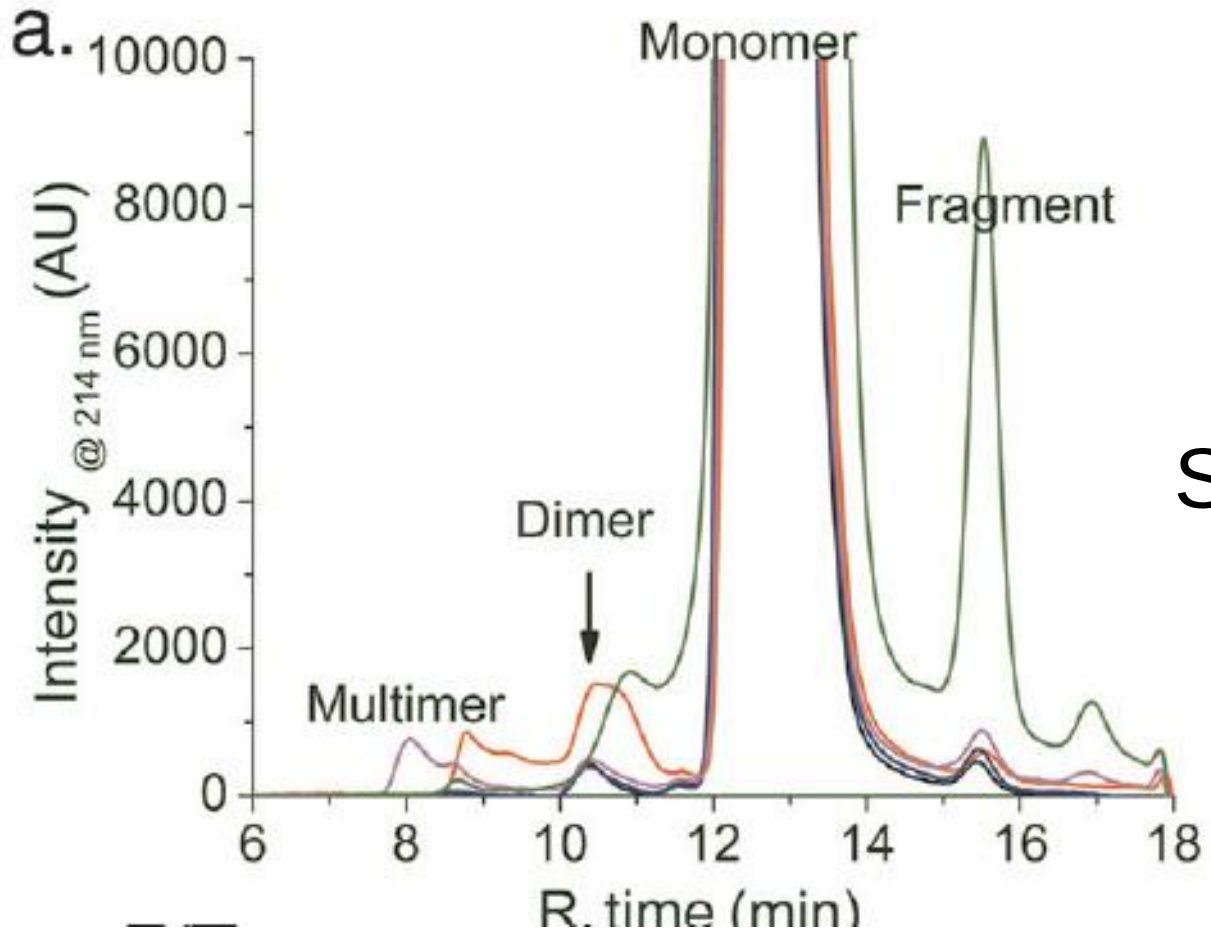
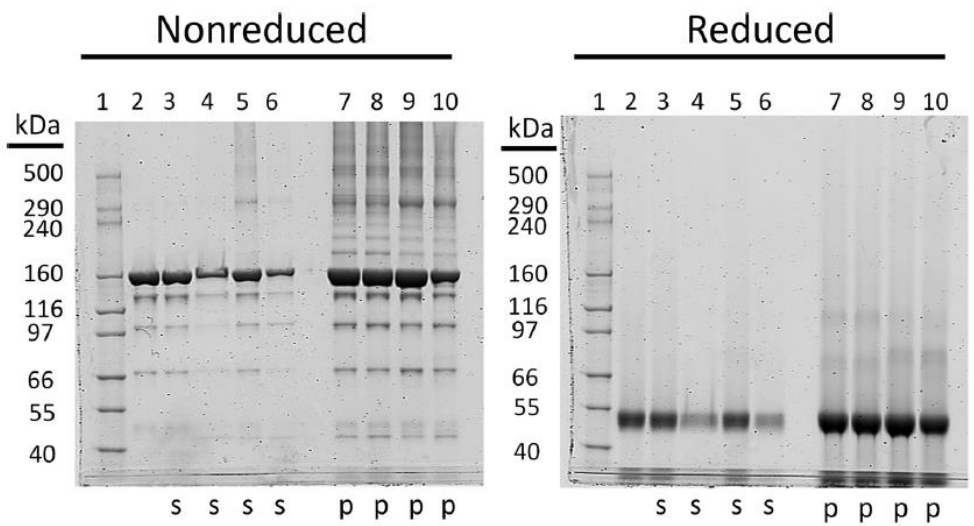
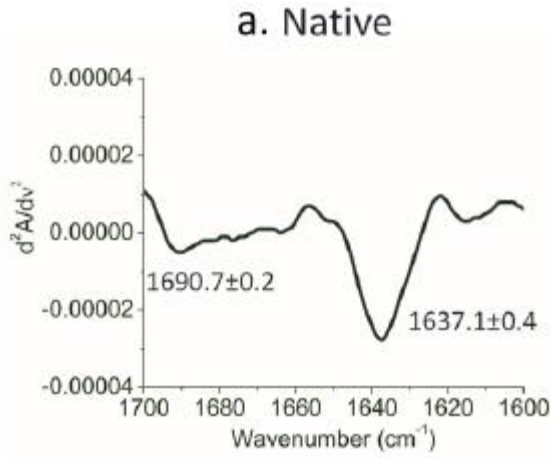


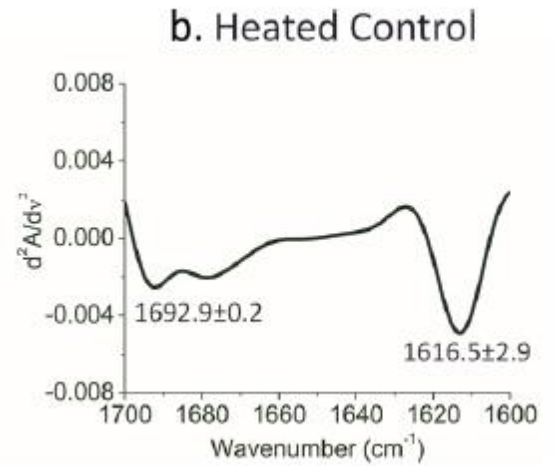
SE-HPLC

Examples of protein aggregation data



Lane	Sample
1	Ladder (MW standards)
2	D0
3	Stir-D1 (supernatant)
4	Stir-D3 (supernatant)
5	Heat-D1 (supernatant)
6	Heat-D3 (supernatant)
7	Stir-D1 (pellet)
8	Stir-D3 (pellet)
9	Heat-D1 (pellet)
10	Heat-D3 (pellet)





FTIR

Talikepalli et al

Characterisation of Stress-Induced Aggregate Size Distributions and Morphological Changes of a Bi-Specific Antibody Using Orthogonal Techniques

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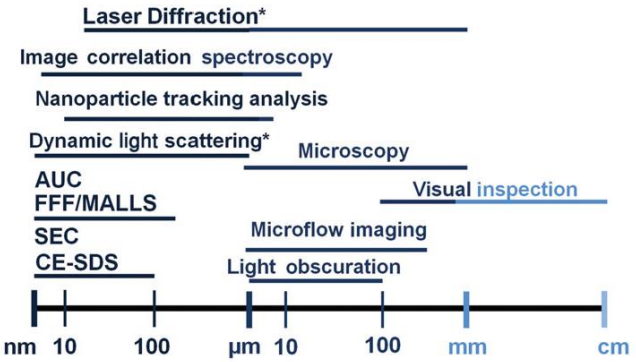
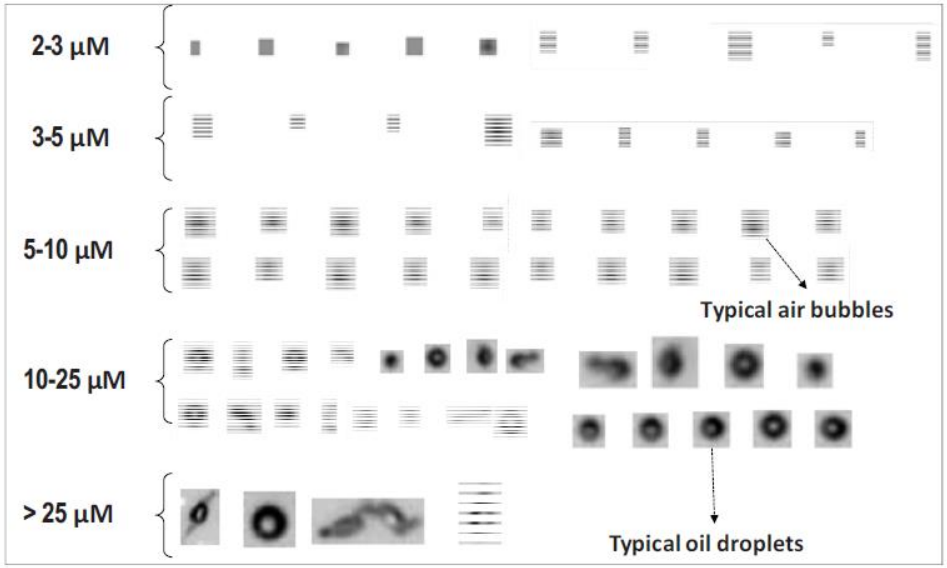


Figure 1. Schematic depicting the relative particle size ranges characterised by several analytical techniques. Asterisk (*) denotes methods that are unable to quantify absolute particle size distributions. SEC refers to size-exclusion chromatography, CE-SDS to capillary electrophoresis-sodium dodecyl sulphate, AUC to analytical ultracentrifugation, FFF to field-flow fractionation and MALLS to multi-angle laser light scattering. Adapted from Hamrang et al.,[10] Singh et al.[11] and Zölls et al.[12] with permission from Elsevier, John Wiley & Sons and John Wiley & Sons, respectively.



Condition	Type	NTA D90, nm	MFI ≥2 µm	MFI ≥3 µm	MFI ≥5 µm	MFI ≥10 µm	MFI ≥25 µm	HIAC ≥2 µm	HIAC ≥3 µm	HIAC ≥5 µm	HIAC ≥10 µm	HIAC ≥25 µm
5 °C	Release	270	6080	2386	1093	259	24	806	426	86	9	0
5 °C	Release	273	14141	5081	1939	365	26	1153	578	105	10	0
5 °C	Release	263	7776	3191	1452	308	21	1329	730	167	23	0
5 °C	Release	245	17759	7738	3407	612	12	1186	703	163	8	0
5 °C	Release	280	12772	5518	2451	522	30	1922	1046	255	39	1
5 °C	Release	260	8917	3706	1772	411	18	1420	794	161	19	0
5 °C	Release	264	14846	6791	3435	974	58	1635	919	226	25	0
5 °C	Release	237	36026	16987	8003	1515	18	2591	1373	222	19	0

Regulatory expectations for soluble aggregates below 0.2 microns

- Assay validation
  - ICH Q2R1: Validation of analytical procedures
    - Demonstrate good recovery from columns
    - System suitability criteria should be established to ensure recovery during routine assay performance
    - Lack of standards presents challenges for quantification
  - Assessment using different stressors
    - Is the method stability indicating?
    - Degradation pathway?
    - In-use stability?
    - Kinetics of degradation?
  - Suitable for cGMP environment
  - No single preferred method. SE-HPLC is most commonly used.

Regulatory expectations for soluble aggregates below 0.2 microns

- Assay validation
  - Forced degradation: Oxidation (may need different methods to oxidize protein), deamidation, low/high pH, heat, photostability, light
  - Accelerated condition: temperature/humidity higher than recommended storage condition
  - Freeze/thaw
  - Shipping validation/agitation

Regulatory expectations for soluble aggregates between 10-25 microns

- Monitoring particles between 10-25 micron is a regulatory requirement and compendial tests are available
- Sponsors can chose which method they prefer to use
- Compendial methods are considered validated but must be qualified for each lab
- USP <788> testing should be added if they are not a current release specification

Regulatory expectations for soluble aggregates between 2-10 microns

- Data for sub-visible particles between 2-10 microns is currently being requested using a qantitative method
  - Incorporated during early phases or as a PMC for licensed products
- No preferred method or pre-established limit by FDA
- Particles can be characterized for shape, type, size distribution

Setting specifications for quantitative methods

- Risk to the product quality attribute(s) that drive safety and efficacy
- Requires product knowledge
- Clinical experience
- Manufacturing history used to establish limits
- Use of multiple DS/DP lots in clinical trials

Take home messages

- Aggregates, SVP, and visible particles can pose a risk to patient safety and product efficacy
- Specifications should be established for SVP below 0.2 micron and above 10 and 25 micron for parenteral and inhaled products
- SVP between 2 and 10 micron should be *evaluated* using quantitative methods and an appropriate control strategy developed
- SVP between 0.2 and 2 micron should be *characterized* and an appropriate control strategy developed

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