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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

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ABSTRACT Within the European Immunogenicity Platform (EIP) (http://www.e-i-p.eu), the Protein Characterization Subcommittee (EIP-PCS) has been established to discuss and exchange experience of protein characterization in relation to unwanted immunogenicity. In this commentary, we, as representatives of EIP-PCS, review the current state of methods for analysis of protein aggregates. Moreover, we elaborate on why these methods should be used during product development and make recommendations to the biotech community with regard to strategies for their application during the development of protein therapeutics.

KEY WORDS aggregation · aggregates · analytical characterization · biotech products · commentary · drug development · particles · proteins · recommendation

INTRODUCTION

Despite the high quality of current therapeutic biotech products and the resemblance of recombinant human proteins and antibodies to endogenous human proteins, protein immunogenicity remains an important concern. Among the several factors playing a role in immunogenicity,

John den Engelsman and Patrick Garidel contributed equally in this article.

J. den Engelsman · R. Smulders
Biological and Sterile Product Development, MSD
Oss, The Netherlands

P. Garidel · S. Bassarab
Boehringer Ingelheim Pharma GmbH & Co. KG
Department of Process Science/Biopharmaceuticals
Biberach an der Riss, Germany

H. Koll
Biologics Research – Protein Analytics
Roche Pharma Research and Early Development
Penzberg, Germany

B. Smith
UCB
Slough, Berkshire, UK

A. Seidl · O. Hainzl
Sandoz Biopharmaceuticals, Analytical Characterization, Hexal AG
Oberhaching, Germany

W. Jiskoot (✉)
Division of Drug Delivery Technology
Leiden/Amsterdam Center for Drug Research
Leiden University
P.O. Box 9502, 2300 RA Leiden, The Netherlands
e-mail: w.jiskoot@lacdr.leidenuniv.nl

the presence of aggregates is considered an important product-related factor that may increase the risk of an immune response (1,2). Although little is known about which aggregate species trigger the immune system, it is believed that aggregates are more easily recognized by the immune system than the native parent protein.

Aggregates (see Glossary, Table I) that may be present in protein products can range from small (dimers) to large assemblies (subvisible or even visible particles). They can be formed during production, storage, shipment or delivery to the patient. Numerous stresses (e.g., temperature fluctuations, light, shaking, surfaces, pH adjustments, etc.) can induce protein aggregation during each of these stages (3,4). Aggregation can occur because of exposure to air-liquid or liquid-solid interfaces, e.g., during mixing, during filling and shipping, during reconstitution of lyophilized products, or through contact with chromatography columns, pumps, pipes, vessels, filters, etc. Also, solution contact with ice during (adventitious or deliberate) freezing can cause aggregation (5,6). Moreover, protein aggregates may in some cases be induced by foreign particles, e.g., stainless steel and other particles from filling pumps, rubber particles from stoppers, salt crystals, glass particles generated during heating of containers for depyrogenation, and silicone oil droplets originating from siliconized syringes or stoppers (7–10). Protein aggregation may also be followed or induced by chemical degradations/modifications, e.g., oxidation (11,12).

The challenge in analyzing protein aggregates lies in the unknown nature of the formed aggregates as well as the wide size range of up to six orders of magnitude, from a few nm to a few mm in diameter. Since no single one of the currently available techniques is able to cover this size range, a combination of several techniques is necessary. However, each technique has its own strengths and weaknesses. Moreover, the available methods differ in the physical measuring principle and, consequently, in the results and type of information obtained.

The aim of this commentary is to discuss the currently available analytical methods to characterize protein aggregates in relation to product quality and also the interpretation of data resulting from these methods. Moreover, we propose approaches to use these methods for the characterization of protein therapeutics from early product development through to commercialization.

This paper is a result of discussions among the co-authors of this paper, who participate in the protein characterization subcommittee (PCS) of the European Immunogenicity

Table I Glossary

Term	Definition
Aggregates	Assemblies of protein molecules other than the desired (e.g. monomeric) species. Aggregates may differ in size (ranging from nm to μm in diameter), morphology (approximately spherical to fibrillar), protein structure (native *vs.* denatured), type of intermolecular bonding (covalent *vs.* non-covalent) and reversibility.
Extended characterization (EC) assays	Assays that complement QC assays (see below), used throughout product development to acquire a thorough physico-chemical characterization of protein drug substance and drug product. Although EC assays should be scientifically sound, potentially they cannot be validated in accordance with ICH guidelines (ICH Q2(R1)). EC assays generally have a low(er) throughput and poor(er) robustness, i.e. they may require advanced instruments and data interpretation at an expert level.
Orthogonal methods	Independent methods that fundamentally differ from each other in the physical measuring principles that are used to investigate a certain aspect of a sample. For example, aggregates may be detected by orthogonal microscopy, chromatography or centrifugation methods.
Particles	Undissolved species (other than gas bubbles or droplets) that are unintentionally present in the product. Particles can be foreign (not intrinsic to drug substance) or protein-related (i.e. large aggregates). Particles can be further categorized as visible (>ca. 50 μm) and subvisible (between ca. 0.1–50 μm); submicron particles (between ca. 0.1–1 μm) are a subcategory of subvisible particles.
Quality control (QC) assays	Assays that are used to release clinical batches throughout product development and commercial batches after product launch (drug substance and drug product). QC assays need to be validated in accordance with ICH guidelines (ICH Q2(R1)). QC assays generally should have a high throughput and good robustness, i.e. they require conventional instruments and data interpretation at a non-expert level. QC assays are used for formal batch release testing and stability monitoring (GMP) and may also be used for development support activities (next to EC assays).

Platform (EIP; see Table II). Within the EIP, the EIP-PCS (Table II) was established to discuss product-related factors associated with immunogenicity and methodologies for protein characterization.

TECHNIQUES TO ANALYZE PROTEIN AGGREGATES

Many techniques are available for the analysis of protein aggregates or proteinaceous particles (and particles in general). These techniques rely on different separation as well as different detection principles and range from relatively basic techniques such as visual or microscopic inspection, through to high-tech methods such as analytical ultracentrifugation (AUC) or mass spectrometry (4). In order to obtain a broad view of the possible protein aggregates that may be found in a protein sample, it is recommended that complementary, orthogonal approaches (see Table I) are used, especially in the development stage.

The various techniques available for the analysis of protein aggregates or particles are summarized in Table III. Furthermore, this table summarizes the basic principles, describes types of analyses, e.g., qualitative (what can be observed) and quantitative (size range, particle number), and addresses some advantages and disadvantages of each technique. Clearly, some of the analytical methods summarized in Table III are focused directly on the primary criterion of the protein aggregates, namely their size, whereas other methods address other characteristics, e.g. changes in secondary or tertiary structure. The consequence of these distinct analytical features (e.g. different separation, detection and characterization principles) is that diverse aspects of protein aggregates are observed and detected. Whereas this allows us to get a comprehensive picture of a product's aggregate profile, comparison of the data obtained from different analytical methods may be inappropriate because different techniques essentially measure different characteristics, and establishing correlations may be impossible in some cases. The analytical methods listed in Table III differ from each other also in performance aspects, like assay robustness and sensitivity, impact of sample preparation, amount of sample required, and ease of assay validation. These aspects, summarized in Table IV, dictate the use of the analytical technique at different stages of the development of a product. Based on discussions among the members of the EIP-PCS, we identified techniques which are commonly used in the industry as analytical quality control (QC) and extended characterization assays. These methods and their advantages and disadvantages in protein aggregate analysis are discussed in more detail in the following paragraphs.

Pharmacopeia Methods for Assessing Visible Particles

As set forth by the United States Pharmacopeia (USP) and the European Pharmacopeia (EP), injectable solutions need to be "practically/essentially free of visible particles." Manufacturers are expected to test every container of a GMP batch for human use for visible particles and to reject contaminated containers. Furthermore, the manufacturers are recommended to have procedures in place that describe the Acceptable Quality Levels (AQL) of the inspection process and criteria for the maximum number of rejects per batch. The wording "practically/essentially free" reflects the limitations of the inspection process, i.e., the visual inspection process is subjective and probabilistic and simply cannot guarantee the total absence of visible particles from all containers.

Originally, the compendial guidance was primarily focused on particles originating from external sources such as manufacturing materials and primary packaging compo-

Table II EIP-PCS

EIP	EIP-PCS
The European Immunogenicity Platform (EIP) was created in 2007 by experts in the field of immunogenicity (http://www.e-i-p.eu). The EIP represents companies, institutes and professionals involved in research, development, testing, validation, application, production or marketing of immunogenicity assessment tools, as well as those servicing the biotechnology community. Its mission is to build know-how and expertise in the field of immunogenicity, driven by a close interaction between industry and scientific advisors. Its scope is to interact with authorities regarding immunogenicity guidelines, formulate active recommendations regarding immunogenicity, stimulate research addressing the clinical and non-clinical effects of unwanted immunogenicity, and boost collaborations between academia and pharmaceutical companies. Through its working-group structure, the EIP can react in a focused way on regulatory and scientific evolutions in the immunogenicity field.	One of EIP's working groups, the Protein Characterization Subcommittee (EIP-PCS) was established early 2008. The mission of the EIP-PCS is to discuss and exchange experience with protein characterization in relation with immunogenicity, in order to increase our fundamental understanding of product-related causes of immunogenicity. One of the aims of the EIP-PCS is to define common strategies and methodologies for protein characterization in relation with immunogenicity, in the context of which the current paper was established.

Table III Overview of Analytical Techniques Used for the Analysis of Protein Aggregates

Method	Principle	Observable	Size range[a]	Advantages	Disadvantages
Methods for Detection and Characterization of Visible and Subvisible Particles					
Visual inspection	Visualization, manual or automated	Assessment of clarity, opalescence, turbidity; visible particles	>50 μm – mm	Easy to perform, information on particle size and shape	Low resolution; limited particle discrimination; probabilistic nature; subjective; trained personnel or expensive equipment needed
Optical microscopy	Microscopy-aided visualization of particles	Size and morphology of particles	>1 μm – mm	Easy to perform, information on particle size and shape	Limited resolution; sample preparation may create artifacts
Light obscuration	Blockage of light by partides	Concentration and size of micron-sized particles	2–100 μm	Rapid analysis; counting and (size class) clustering of particles	Large sample volume; no morphological information; may miss translucent particles; very sensitive to sample contamination (e.g. artifacts by air bubbles)
Flow imaging	Microscopic imaging	Concentration, size and morphology of micron-sized particles	1–400 μm	Potentially allows differentiation between protein aggregates and non-proteinaceous particles; shape information also obtained; may detect translucent particles	Limited particle characterization; only part of sample is analyzed; high data volume; emerging technique
Fluorescence microscopy	Detection of induced fluorescence	Particles, amyloid proteins	>1 μm – mm	High sensitivity; selective for protein aggregates; information on particle size and shape	Restricted to labeling/dyes/filter sets; no quantification possible
Conductivity based particle counter	Electrical sensing zone method (Coulter method)	Concentration and size of (sub) micron-sized particles	0.4–1,200 μm	Single particle detection, counting and characterization	Dilution of low conductivity samples into an electrolyte solution
Laser diffraction	Laser light scattering/reflection	Laser light scattering relative to particle size	20 nm – 2 mm	Single particle detection, counting and characterization	Requires high sample dilution
Dynamic light scattering	Fluctuations in scattered light intensity due to Brownian motion	Hydrodynamic size	1 nm – 5 μm	Easy to perform (batch mode); non-destructive; high sensitivity; low sample consumption	Complicated data analysis; not quantitative; low resolution (weak differentiation between particle species); less suitable for polydisperse samples; very sensitive to contamination (e.g. dust)
Nanoparticle tracking analysis	Microscopic visualization by laser light scattering of Brownian motion of particles	Hydrodynamic size	20 nm – 1 μm	Single particle detection and characterization; very useful for polydisperse samples	Low sample throughput; visualization, no imaging; trained personnel needed; emerging technique
MALLS	Time-averaged light scattering intensity of particles, detected at multiple angles	Molar mass, size (radius of gyration)	kDa – MDa range	High sensitivity; absolute determination of size; commonly used as online detector	Solute concentration must be known; complicated data analysis; not quantitative; limited use in batch mode; very sensitive to sample contamination

Turbidimetry, nephelometry	Time-averaged light scattering intensity of particles	Particle size/concentration dependent optical density or light scattering intensity	N/A	Easy to perform; various designs and methodologies	Observed signal depends on both size and concentration; no information on individual particle properties (number, size distribution); suitable only for comparative measurements
Separation Techniques for Detection and Characterization of Protein Aggregates					
SEC	Separation by molecular sieving	Hydrodynamic size	1–50 nm	Robust, sensitive, precise; can be combined with serial online detectors	Sample dilution; limited resolution; limited particle size range; matrix interactions may compromise accuracy
Denaturing/reducing SEC	Separation by molecular sieving	Hydrodynamic size	1–50 nm	Differentiation between covalent and non-covalent complexes	See SEC; less commonly used
SDS-PAGE	Separation in a gel by size in an electric field	Apparent molecular weight	kDa – MDa	Easy to perform; differentiation disulfide mediated and non-disulfide mediated covalent aggregates	Does not detect noncovalent aggregates; requires staining; quantification difficult
Native PAGE	Separation by size/charge in an electric field	Electrophoretic mobility	kDa – MDa	Easy to perform; analyzes native protein (aggregates)	Electrophoretic mobility depends on both size and charge; requires staining; quantification difficult
Capillary-SDS electrophoresis	Separation in a capillary by size in an electric field	See SDS-PAGE, IEF	kDa – MDa	High resolution; quantification (without staining); fast separation; multiple modes of operation; can be combined with serial online detectors	Possible protein interaction with capillary; does not detect noncovalent aggregates
AF4	Separation by size through flow retention	Hydrodynamic size	1 nm – fewμm	Broad size range; no stationary phase; quantification; can be combined with serial online detectors	Size-range dependent; sample concentration-dilution during analysis; less robust than SEC
Other Techniques for Detection and Characterization of Protein Aggregates					
Electron microscopy	Visualization	Size and morphology of protein aggregates	nm – mm	Large size range; high resolution; detection of chemical composition of a particle (energy dispersive X-ray analysis)	Sample preparation may create artifacts; limited representativeness of selected image area; not quantitative; time consuming; expensive; expert personnel needed
Atomic force microscopy	Topographical scanning	Size and morphology of nm-sized particles	nm range, vertical resolution 0.01 nm	Molecular resolution; morphological particle properties	Particle isolation and measurement itself may create artifacts; time consuming; expensive; limited representativeness of selected image area; expert personnel needed
SAXS/SANS	Scattering of X-ray/neutron beam	Size and shape of molecules/ aggregates in solution	nm range	High resolution	Time consuming; expert personnel needed
"Native" mass spectrometry	Mass/charge detection of ionized molecules in a field	Mass/charge ratio	Atomic resolution up to MDa range	Very high resolution; high accuracy and precision high sensitivity detailed structural information online detection possiblities	Gas phase analysis; requires exchange into volatile buffer; not quantitative; expensive equipment and expert personnel needed

Table III (continued)

Method	Principle	Observable	Size range[a]	Advantages	Disadvantages
Macro-ion mobility spectrometry	Ion mobility and counting	Mass	3–65 nm or 5 kDa – 100 MDa	High resolution; high molecular weight precision; broad molecular weight range; no matrix interaction during separation	Gas phase analysis, requires exchange into volatile buffer; requires dilute samples; emerging technique
AUC	Sedimentation velocity (SV)	Molecular weight and/or shape	1 nm – 0.1 μm	Absolute method; measurement of molecule size, shape; quantification of protein complexes; high resolution; applicable for wide conc. range	Strongly dependent on quality of instrument components (e.g. centerpieces); complex data analysis; time consuming; expensive instrumentation; expert personnel needed
Indirect Methods for Assessing Protein Folding State					
Infrared spectroscopy	Absorbance of infrared light, vibrational spectroscopy	Secondary structure	N/A	Non-destructive; solid state analysis possible; little interference from light scattering; combination with microscopic tools possible	Low sensitivity, protein concentrations >1 mg/ml needed; particle isolation may lead to artifacts
Raman spectroscopy	Vibrational Raman and Raman optical activity	Secondary structure chemical characterization	N/A	Non-destructive; solid state analysis possible; little interference from light scattering; combination with microscopic tools possible	Low sensitivity; particle isolation may lead to artifacts; emerging technology for protein characterization
(derivative) UV/Vis absorption spectroscopy	Absorption (and scattering) of light	Optical density (presence of aggregates/particles), shifts in folding state	N/A	Easy to perform; non-destructive[b]; detection of scattering material outside absorption bands; commonly used as online detector	Complicated data interpretation; limited information on particle properties
Fluorescence spectroscopy	Detection of induced fluorescence (intrinsic or extrinsic)	Tertiary/quarternary structure	N/A	Non-destructive[b]; comprises multiple fluorescent techniques; combination with microscopic tools possible; online detection possibility	Low resolution; destructive when using extrinsic dyes; background interference from fluorescent excipients or impurities
Circular dichroism spectroscopy	Difference in the absorption of left- and right-handed circularly polarized light	Secondary/tertiary/quaternary structure	N/A	Non-destructive[b]; easy to perform; low protein amount required (far UV); undiluted sample analysis possible (with ultra-thin cuvettes); online detection possible	Interference by light scattering (particles); limited resolution/ information; complicated data interpretation; interference by excipients and solvent (far UV)

[a] Approximate size range, from lowest detectable to largest for practical use. Depending on the detection principle, the particle is determined in length or mass units; [b] Possible chemical degradation when working in the (far) UV range; Abbreviations used: *AF4* asymmetrical flow field flow fractionation, *AUC* analytical ultra centrifugation, *SEC* high performance size exclusion chromatography, *MALLS* multi angle laser light scattering, *N/A* not applicable, *PAGE* polyacryl amide gel electrophoresis, *SANS* Small angle neutron scattering, *SAXS* Small angle X-ray scattering, *SDS-PAGE* sodium dodecyl sulfate polyacryl amide gel electrophoresis, *SEC* size exclusion chromatography, *UV-VIS* ultraviolet-visible

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

nents. However, for protein products, visible particles may also be product related. That is, protein products may contain insoluble (visible) particles derived from the protein product itself. In these cases, sterile filtration is often not effective because insoluble protein particles may reform over time. Nowadays, regulators expect that manufacturers try to limit the presence of proteinaceous particles by applying good formulation strategies and practices (13). However, in specific cases, the presence of particles may be accepted as an inherent product attribute, provided that these particles do not pose a quality or clinical safety concern. One example of this is insulin. In the EP it is stated that insulin solutions may form insoluble particles over time. Moreover, intermediate-acting and long-acting insulin suspensions are particulate by definition. Nevertheless, insulin has proven to be a very safe product for many years in a large number of patients. Also, for patients with a compromised immune system, a product with non-optimal appearance may be acceptable, provided that clinical data do not reveal major safety issues.

Assay of visible particles is typically done by trained operators under controlled conditions. Automated or semi-automated instrumental methods may be applied, but even then the human factor may be relevant, e.g. during the qualification of the automatic inspection process (i.e., manual testing to check if the AQL is met). The EP describes a specific visual inspection method (the black and white box). The threshold of visibility is believed to be ca. 50 μm for a spherical particle. However, detection of visible particles is a problematic process (14), and in daily practice the detection limit very much depends on the individual operator, the inspection device, the inspection time as well as the morphology, number and refractive index of the particles. Companies may use various additional, non-EP methods for manual visual inspection to improve the sensitivity or ergonomics of the process, such as the use of aids such as magnifying glasses or differences in lighting conditions or observation times and swirling procedures. These operations may have a pronounced effect on reject percentages.

Although visual inspection itself is pretty straightforward, interpretation of final inspection results can be very difficult. For instance, abnormal reject rates may be observed after GMP batch manufacture in a large-scale commercial facility, whereas no problems were noticed for clinical batches produced in a small-scale facility. In such a situation a root cause investigation can be very laborious and time-consuming because the abnormal reject rate may be caused by either a manufacturing failure, a change in manufacturing conditions, a difference in visual inspection conditions between the large- and small-scale facilities, or a combination of these factors. In order to minimize particle problems as much as possible, it is recommended to use harmonized inspection procedures and acceptance criteria throughout development (especially for release testing) and to evaluate the performance of the visual inspection method before manufacture of GMP batches.

Pharmacopoeia Methods for Assessing Subvisible Particles

The pharmacopoeial guidance with regard to assessing subvisible particles is applicable to all parenteral solutions or lyophilizates, including those containing proteins. As to analytical methodology, the US and EU pharmacopeias are harmonized and basically describe two methods to count particulate matter, namely "light obscuration" and "microscopy." These methods are accompanied by acceptance criteria defining the maximum numbers of subvisible particles allowed per container, or per mL for large-volume injectable solutions. Interestingly, these criteria are different between the two methods, reflecting the differences in detection principle.

The measuring range of most light obscuration instruments is ca. 2–100 μm diameter, with varying precision and reproducibility. Potential pitfalls of the method lie in the sample preparation, which has to ensure that no sample contaminations or other artifacts occur. Air bubbles and silicone oil droplets from the primary packaging may mistakenly be counted, non-spherical particles cannot adequately be measured, and the technique is unable to discriminate proteinaceous particles from unrelated, non-proteinaceous solid particles. The microscopy method uses a suitable binocular microscope. The sample is filtered, and the filter-dried particles are then counted (manually or automatically) on the filter. With this method, potential contaminations can occur. Furthermore, the method is extremely labor intensive, which limits its use for routine, large throughput applications.

The current compendial acceptance criteria for subvisible particles are usually met for modern injectable solutions. However, it should be realized that these criteria were originally not set to control proteinaceous particles. Furthermore, criteria exist only for relatively large subvisible particles (greater than 10 and 25 μm diameter), whereas smaller particles (between 0.1 and 10 μm diameter) are not considered. An animated discussion is currently ongoing between regulators, industry and academia to determine whether subvisible particles could pose a significant immunogenicity risk (15,16). The amount of proteinaceous subvisible particles in terms of mass, however, is typically very low (ng/ml-μg/ml range), and it is unclear whether such an amount could indeed trigger an immune response. Unfortunately, the current compendial methods for subvisible particle testing have several limitations regarding robustness, sensitivity and type of particles that can be quantified accurately. Therefore, currently the industry is evaluating technologies such as micro-flow imaging (17). However, whether these new technologies really perform better and can be applied in a QC environment remains to be established.

High Performance Size Exclusion Chromatography

The basic principle of high performance size exclusion chromatography (SEC) is simple. Solute molecules are passed through a column containing porous beads. Small molecules penetrate the pores of the beads, while larger molecules or aggregates do not; therefore, small molecules take a longer path through the column and elute later. Nominally, the ability to enter the pores is dictated by molecular size, so if it is assumed that the analyte molecule is spherical, the elution position (relative to molecular weight standards) may be used to estimate molecular weight. However, any such estimate is an approximation, as most molecules tend to not be spherical, and actually the hydrodynamic radius, rather than the molecular weight, is measured (18). Assuming monomer and oligomers have the same extinction coefficient, the absorbance at 280 nm or lower (214 or 220 nm) may be used to quantify the extent of aggregation of a sample. Analytical SEC typically employs small columns, in which samples of a few to tens of μg of sample are sufficient to obtain a result.

Aqueous, non-denaturing buffers are compatible with SEC. The basic method may be modified to gain insight into the quality of aggregates, for instance by addition of denaturant to the eluent (19). This can disperse non-covalently associated aggregates. Alternatively, samples may be treated prior to SEC, for instance by reduction (and reaction of cysteinyl thiols with an alkylating agent), to break disulfide bonds. The addition (to the eluent) of fluorescent dyes that bind to denatured protein (and so inducing an enhancement of the fluorescence) allows detection of very low amount of denatured protein (20). Quantification is not possible, however, since the fluorescence depends on both the type and extent of denaturation. Finally, additional detectors may be added to give complementary information, such as molecular weight and stoichiometry of complexes from use of on-line light-

scattering, UV absorbance, and refractive index detectors (21,22).

Various factors may affect the results of SEC, as discussed recently by Carpenter *et al.* (23). For instance, low solution ionic strength (e.g., 50 mM or less) may encourage hydrophobic interactions of the eluting protein with the column matrix, thereby slowing elution, affecting resolution and peak shape. Addition of arginine to the eluent may inhibit interaction between solute and column matrix (24). Detergents in the sample (as opposed to the eluent), though nominally of small molecular weight, can behave as large molecules if they form micelles (above their critical micelle concentration), appearing in the chromatogram as UV-absorbing peaks (25) and potentially also giving rise to light scattering and fluorescence signals.

There is an upper limit to the size of aggregate detectable by SEC, because larger aggregates can be filtered out by frits in the system or by the column itself. As a consequence, large material (large protein aggregates) may disappear and be overlooked in the analysis. They also build up on the top of the column and gradually degrade its performance, seen as broadened peaks, poorer resolution and decreased yields (smaller peaks). Another form of aggregate that may be missed is that formed by very low affinity intermolecular association, as these may dissociate into monomers following a change in conditions from those of the sample to those experienced during chromatography (e.g., dilution or change in temperature) (26). For detection of such low affinity aggregates other methods could be used, such as AUC, or method conditions of SEC could possibly be adjusted.

SDS-PAGE and Capillary Electrophoresis-SDS

SDS-PAGE is a very common, fairly robust method that is easy to perform and can supply information on approximate molecular weight and quantity, when using a suitable method of quantitative staining and gel scanning. The presence of SDS means that non-covalent aggregates are disrupted, so the method only detects covalent aggregates. If reducing conditions are used, SDS-PAGE can discriminate between aggregates held together by disulfide bonds and those held together by other (non-reducible) covalent bonds.

SDS-PAGE is becoming replaced by its capillary electrophoresis counterpart, CE-SDS, as the latter is better suited for robust quantification. With CE-SDS, one can achieve similar results in a technically slightly different way, with automation of running of samples and quantification by UV absorption rather than dye-binding. For both methods, low μg amounts of sample are needed for the analysis, throughput is medium to high, and turn-around quick. A combination of SEC and SDS-PAGE and/or CE-SDS is usually part of QC analytics.

Dynamic Light Scattering

Dynamic light scattering (DLS), also known as quasi elastic light scattering (QELS) and photon correlation spectroscopy (PCS), is a technique used for the determination of the size distribution of particles in the diameter range of 1–2 nm to 3–5 μm (27). DLS is a non-destructive technique for the characterization of colloidal systems like protein solutions, allowing a re-use of the sample for further characterization, possibly useful if only a small amount of product is available, such as in early stage development. Using modern equipment, the volume required for a DLS analysis can be as low as a few μl. The concentration ranges between 0.1–50 mg/ml for protein solutions. Measurements of highly concentrated solutions are becoming feasible owing to the application of photon cross-correlation approaches (28), for example. Although the sensitivity of this technique for detection of large particles in particular is unsurpassed, quantification is not possible. DLS yields qualitative results, not quantitative results.

Analytical Ultracentrifugation

AUC, mostly used in sedimentation velocity mode (SV-AUC), is a powerful technique to characterize the sedimentation behavior of macromolecules and the presence of aggregates in solution. Recently, Philo reviewed AUC as a method for characterizing non-particulate, soluble protein aggregates, including its strengths and weaknesses (29). One of the major advantages of AUC is that protein therapeutics can often be characterized without sample manipulation in relevant solutions such as their formulation buffer, with some minor exceptions (e.g. non-ideality may occur at high concentrations). Quantification of aggregate species is possible, while formation or disruption of aggregates due to sample preparation, dilution or matrix effects is limited. However, as noted by Philo (29), reproducibility is often relatively poor, and assigning a limit of detection (LOD) or quantification (LOQ) for aggregate detection using SV-AUC is difficult for several reasons. Among the several practical and experimental parameters that influence the accuracy and precision of AUC experiments, cell (mis)alignment and the quality of the centerpieces especially seem to be major reasons for variations in quantification of low amounts of aggregates (30–32). Therefore, regular calibration and intensive maintenance of the system and its accessories are required to assure their proper functioning. Furthermore, it should be recognized that the mathematical calculations behind AUC analyses (i.e. selection and use of data analysis software such as Sedfit or Ultrascan, and appropriate models to describe the size distribution) may also have significant impact on the absolute aggregation levels reported. For antibody therapeutics, and in accordance with our own experience, the LOQ is

approximately 1% at best, and precision for samples containing <3% aggregates is low (30). Nevertheless, because of some strong advantages over SEC or AF4, like the absence of interactions of the molecules of interest with columns or membranes, AUC can be a valuable tool for a qualitative cross-check or analysis of trend of data obtained by SEC or AF4. AUC can be used to verify that no aggregate type potentially present in a sample is missed by SEC or AF4. For a (semi)quantitative cross-confirmation, AUC is only suited to analysis of aggregate contents greater than about 3–5%. Therefore, we suggest using AUC only as a qualitative or relative method (comparison of relative aggregation levels) but not as an absolute quantitative orthogonal method, at least not until the technical issues of equipment and component quality resulting in limited precision and reproducibility are solved.

Asymmetrical Flow Field-Flow Fractionation

Asymmetrical flow field-flow fractionation (AF4) is an analytical technology to separate proteins ranging from a few nanometers to a few micrometers diameter (33). Separation in AF4 occurs in a thin flow channel. Perpendicular to the laminar channel flow, a cross-flow is generated that leads the channel through a membrane. This cross-flow directs the analyte proteins to the membrane, but they cannot leave the channel because the membrane is impermeable to them. Since small proteins have a relatively large diffusion coefficient, they will return more rapidly to the fast streamlines of the laminar channel flow than large proteins. Consequently, small proteins elute from the channel before large proteins.

Like SEC, AF4 separation can be combined with various detectors such as UV, refractive index and multi-angle laser light scattering (MALLS). In addition, AF4 does not require much sample preparation, and even very large (insoluble) protein aggregates may be detected. On the other hand, the technique is less mature than conventional chromatography and often requires in-depth method development (e.g. related to channel dimensions, types of membranes and flow rate) to obtain acceptable separation and robustness. For that reason, application of AF4 as a QC test has been limited.

Quality Control *Versus* Extended Characterization Assays

Obviously, a plethora of techniques is available and suitable for aggregate detection, quantification or characterization, each method having its own strengths and weaknesses (see Table IV). However, not all techniques are suitable to be applied at all stages of product development. Clearly, there is a distinction between techniques that are routinely used in a QC environment and techniques that are used for extended characterization during product development (see Table IV). To assess aggregation in therapeutic protein products in the QC environment, relatively simple, robust and quick assays of SEC and SDS-PAGE and/or CE-SDS are used alongside the compendial methods for larger (sub)visible particles (whether or not of proteinaceous nature).

Techniques such as DLS, AUC, SEC with online MALLS detection (SEC-MALLS) and AF4 can be applied to support SEC, but are generally not used for QC analysis because of one or several of the following reasons: a) some of these extended characterization assay instruments are very time consuming, costly to buy and maintain, and not suited for high throughput analysis; b) these assays are typically less accurate, precise and robust than conventional chromatography; c) the operation of challenging techniques requires skilled operators, and data obtained require expert interpretation; d) in terms of ICH guidelines these assays are difficult or impossible to validate. For instance, in the case of DLS, it is impossible to perform quantification of the species present. Therefore, determination of the limit of detection for a certain protein (aggregate) is impossible according to ICH guidelines. However, qualitatively, DLS is unsurpassed in detecting trace amounts (<0.01% w/v) of large aggregate species in the submicron-to-low μm range.

SEC-MALLS is very useful throughout product development but is not recommended as a routine QC method (see Table III). A major disadvantage of light scattering in general is its distinct sensitivity towards large-sized particulate contaminations, like dust, or particles arising from pump abrasion or column erosion. This feature requires attention to be paid to elution buffers (low particle content advantageous), with inclusion of in-line filters between SEC column and MALLS detector to remove particles (but possibly also aggregates), and prolonged equilibration times to obtain a stable baseline. As a consequence, more time-consuming efforts are needed to run the method compared to the classical SEC method, whenever an analysis cycle has to be started or the elution buffer or the column has to be changed. Data evaluation is also more time consuming. Robustness of the method is therefore also impacted significantly. Another disadvantage is that MALLS is a qualitative method and cannot be used for quantification.

AUC is also not suited to be used as a QC test. Besides the required operator expertise and the technical challenges of the system itself and the advanced data analysis that is required for performing and evaluating AUC experiments, the technique suffers from poor reproducibility and robustness when compared to a technique such as SEC. The reproducibility of an AUC experiment is very dependent on practical and technical aspects and the setup of the actual experiment (34). This evidently makes validation according to ICH guidelines problematic.

WHY, HOW, AND WHEN TO APPLY EXTENDED CHARACTERIZATION METHODS DURING PRODUCT DEVELOPMENT

Two general levels can be discerned in a proposed strategy: a) initial analysis of the product with robust methods (such as SEC, SDS-PAGE/CE-SDS, light obscuration); b) further analysis on a case-by-case basis, using less robust, lower throughput techniques (such as AUC, AF4, DLS, microscopy methods, etc.).

Support of Formulation and Process Development

Applying extended characterization assays to assess aggregate levels during formulation and process development can be useful for product understanding. In the case of formulation development, understanding the effects that excipients have on the stability of the protein and understanding the underlying mechanisms enable rational development of the drug substance and drug product. Furthermore, during the manufacturing process, the protein is often exposed to harsh conditions. Using in-process sampling during development and examining the structural integrity of the product enables determination of the impact of the different process (and holding) steps on product quality and adjustment of the process to minimize structural perturbations and aggregation.

Extended characterization should also be used to investigate the compatibility of the product with materials that may induce aggregation, e.g. primary package materials (such as syringes containing silicone coatings) or leachables from glass or tubing. Additionally, extended characterization is very well suited for troubleshooting. This might arise in situations where QC methods show atypical results, such as in a case where SEC shows new peaks or unexpected elution profiles.

Extended Characterization as an Integral Part of SEC Method Development

Extended characterization assays, like AUC, DLS, SEC-MALLS or AF4 should be used as supportive methods for SEC method development, rather than for qualification or cross-validation of SEC. Applying extended characterization contributes to the characterization of the SEC profile and could detect aggregates that were not detected by SEC. In order to add greater confidence, a trending analysis could be performed by looking at the relative increase in aggregate content using extended characterization assays in concert with SEC. If a progressive increase of aggregates is only seen using the extended characterization techniques during real-time stability, actions should be taken accordingly, i.e. a root cause analysis.

By integrating the extended characterization assays early in development, the need for these analyses in the commercial production stage may be diminished and would keep QC manageable in a production environment. The "classical" SEC method should then be sufficient and well suited for characterization and release of batches for market products.

Extended Characterization During Comparability Exercises

Comparability exercises are important throughout product development. In the broadest sense, product comparability exercises need to be performed during changes in cell line, process, or scale. During these exercises, besides the "standard" set of analytical tools such as SEC and SDS-PAGE, extended characterization is required to determine whether any change has affected product quality. Additionally, comparability exercises may be used to determine quality differences between samples for toxicological studies and clinical batches of different phases. Such analyses may then help to justify specifications for aggregation products.

Characterization of the Type of Aggregates

Despite optimization of formulation, handling and processing of protein pharmaceuticals, aggregation cannot be totally avoided, and trace amounts remain present and/or are formed during storage. Assessment of the risk of aggregates in a product is difficult, however, because the relationship between the nature of protein aggregates and their potential immunogenicity is currently unknown (1,2). It is important to realize that most (marketed) protein products induce immune responses, which in many cases do not have clinically relevant consequences. However, in some cases, the consequences can be severe and potentially fatal. Unfortunately, fully predictive *in-silico*, *in-vitro* and *in-vivo* preclinical models to assess unwanted immunogenicity are currently lacking. As a consequence, unwanted immunogenicity of a protein pharmaceutical is typically addressed in human trials, and its incidence and consequences may become fully apparent only after the product has been widely used as market product. Extended characterization data, if available, may then help to establish a relationship between the presence and nature of aggregates in a protein pharmaceutical on the one hand and immunogenicity data emerging from use of the product in humans on the other. It may be difficult to establish a firm relationship between immunogenicity and a certain quality parameter. However, if such a relationship for a certain protein exists, the only way to reveal this is to make sure that clinical batches are (or can be) characterized in the best way possible. To this end, it is necessary to have good understanding of the product quality

at the time of batch release and at the time of administration to the patient. In order to find a good correlation between an immune response (or the lack of it) and a product quality attribute, well-established extended characterization methods are crucial. In case of unexpected immune responses to commercial batches, these methods can then also be applied.

Strategy for Application of Extended Characterization Methods During Product Development

Application of extended characterization methods throughout the whole chemistry, manufacturing and control (CMC) development process of protein products is now generally recognized as being very important. In the end, this characterization should firmly contribute to the establishment of relevant specifications for aggregates, according to which the quality of commercial products can be assessed appropriately.

Strategy to Perform Extended Characterization as Early as Possible

An extensive analytical program in early development can pave the way for efficient late-stage development. A good understanding of a product's characteristics and factors that affect its structural stability is key to developing a stable product that can be produced in a consistent and reproducible way. However, extensive use of extended characterization tests can also be challenging in terms of resources and timelines. That is, the extended characterization methods are typically low throughput, and this may become a problem when their application is considered during routine stability studies (e.g., of clinical batches), process development studies, in-use testing and forced degradation studies. One possible way to deal with this is to use the extended characterization methods to support the development of simpler but still high-quality methods such as SEC. This may significantly reduce the need for laborious assays in routine testing.

Strategy to Perform Extended Characterization as Late as Possible

Should early characterization prove to be impossible, an alternative approach that may be considered is retrospective testing. In this approach, samples from development and (clinical) stability programs are collected and stored frozen (e.g. at −70°C) rather than analyzed right away. A significant advantage of such an approach is that relevant samples collected throughout development can be analyzed side-by-side using mature methods with well-known performance (i.e., analytical experience and technology usually evolve in parallel with product development). Provided that it is demonstrated that the freeze-thaw cycle does not influence the aggregation status, this approach may result in a very comprehensive data set (analytical variation is minimized) that can easily be used to justify quality specifications. A drawback is that any learning from analysis is not gained until late in the development process.

Extended Characterization Tests as a Basis for Future Risk Assessment

Decisions on the application of extended characterization tests are driven by many parameters, including complexity of the protein and the production process, availability of resources, priority of the development program, as well as the target product profile and (clinical) timelines. From a patient safety perspective it may be more appropriate to establish the characterization level based on a product risk assessment. The greater the risk of safety issues, the more extensive and more frequent should extended characterization be. This approach would fit with general regulatory expectations regarding risk-based product development.

Risk assessment is currently difficult, however. It is complex and requires multidisciplinary interaction between toxicologists, clinicians and product developers. However, knowledge about product parameters that impact immunogenicity, such as type of molecule (e.g. fully human *vs.* chimeric) and its receptor (e.g. soluble *versus* cellular), homology to endogenous counterpart, disease/indication, dose regimen, route of administration, clearance rate and patient immune status (e.g. suppressed *versus* activated), is increasing rapidly. A key part of any risk assessment is detailed knowledge of a product's various characteristics, and this is where extended characterization is important. Thus, for example, a clinical development program (phase 1–3) may use several clinical preparations, and these batches may display slight variations in quality (e.g. levels of aggregation). There may be slightly different clinical outcomes from these different batches. Comparison of detailed product characterization with clinical outcome may indicate particular product properties that affect its immunogenicity and may provide data for inclusion in future risk assessments.

CONCLUSIONS AND RECOMMENDATIONS

Aggregates are heterogeneous in size and in other physicochemical properties. Consequently, no single method covers the analysis of all aspects of all aggregates. As each method covers different aggregate characteristics, the results obtained with a particular method are strictly linked to that method. Accordingly, QC specifications depend on the

QC methods used and the aggregate class analyzed. In addition to QC methods, extended characterization methods are indispensible during product development.

SEC and SDS-PAGE/CE-SDS are methods that are robust enough for reproducible quantification of aggregates and that allow routine use with sufficient sample throughput. These methods use specific separation principles and sample preparation methods which may impact the result both qualitatively and/or quantitatively, so orthogonal methods should be used to confirm that the applied method, e.g. SEC, provides adequate results.

Below we list recommendations regarding the assessment of protein aggregates in biotech product development:

- Employ robust, quantifiable methods for QC testing: SEC (quantification of covalent and non-covalent aggregates, but not low-affinity aggregates and larger aggregates); SDS PAGE and/or CE-SDS (covalent aggregates).
- Use extended characterization assays to support product development, not to qualify or cross-validate.
- Use extended characterization assays to confirm the performance of the QC analytical methods.
- Follow a risk-based approach, considering molecular and safety aspects, in deciding on the application of extensive as opposed to limited use of extended characterization in product development.
- Archive samples for retrospective testing, and confirm stability on storage.

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