Protein Aggregation: Pathways, Induction Factors and Analysis

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Received 27 March 2008; revised 1 August 2008; accepted 5 August 2008

Published online 29 September 2008 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/jps.21566

ABSTRACT: Control and analysis of protein aggregation is an increasing challenge to pharmaceutical research and development. Due to the nature of protein interactions, protein aggregation may occur at various points throughout the lifetime of a protein and may be of different quantity and quality such as size, shape, morphology. It is therefore important to understand the interactions, causes and analyses of such aggregates in order to control protein aggregation to enable successful products. This review gives a short outline of currently discussed pathways and induction methods for protein aggregation and describes currently employed set of analytical techniques and emerging technologies for aggregate detection, characterization and quantification. A major challenge for the analysis of protein aggregates is that no single analytical method exists to cover the entire size range or type of aggregates which may appear. Each analytical method not only shows its specific advantages but also has its limitations. The limits of detection and the possibility of creating artifacts through sample preparation by inducing or destroying aggregates need to be considered with each method used. Therefore, it may also be advisable to carefully compare analytical results of orthogonal methods for similar size ranges to evaluate method performance. © 2008 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 98:2909–2934, 2009

Keywords: protein aggregation; protein analysis; protein formulation; molecular weight determination; particle sizing; size exclusion chromatography; analytical ultracentrifugation; light scattering; SDS–PAGE; orthogonal methods

INTRODUCTION

The breakthrough of recombinant DNA technology in the mid 1970s has allowed the development of many recombinant therapeutic proteins and thus has resulted in many protein-based products to reach the market.[1,2] The control and analysis of protein aggregation during production of a biotherapeutic drug is an increasing challenge to many pharmaceutical research and development groups and companies. Aggregation is potentially encountered during various steps of the manufacturing process of biopharmaceuticals, which include fermentation, purification, formulation and during storage. Biopharmaceuticals for

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Journal of Pharmaceutical Sciences, Vol. 98, 2909–2934 (2009)
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clinical trials require full characterization including accurate quantification of protein aggregates to meet the drug product specifications. Protein aggregates potentially cause adverse effects, such as an immune response,[3,4] which may cause neutralization of the endogenous protein with essential biological functions leading to a life-threatening situation for the patient and aggregates may also potentially impact the drug's efficacy.[5] The scientific fact base to clearly link specific types and sizes of aggregates to immune responses is however currently still under investigation. A potential increase in immune responses caused by aggregates has been reported previously,[3] whereas in contrast no enhanced immunogenicity was shown for example in the case of aggregated rFVIII.[6]

There are monographs and acceptance criteria/limits in the pharmacopoeias for visible and subvisible particles (i.e., insoluble proteins aggregates)—for example, United States Pharmacopoeia (USP) <788>,[7] European Pharmacopoeia (Ph. Eur.) 2.9.19[8] and Ph. Eur 2.9.20[9]—for parenteral products. However, limits for soluble aggregates have to be set case-by-case as there are no predefined limits laid down in general for biopharmaceuticals within regulatory documents. In order to control protein aggregation to enable safe and successful products, it is important to understand the origin of protein aggregates, and the analytical techniques for characterizing their full size range.

This review article aims to collate and discuss available literature on the major causes of aggregation and the analytical methods/techniques to characterize protein aggregates.

PATHWAYS AND INDUCTION FACTORS

Definition and Mechanism of Protein Aggregation

The term "protein aggregation" has been given many definitions and terminologies within the literature.[10,11] The authors define "protein aggregates" as a summary of protein species of higher molecular weight such as "oligomers" or "multimers" instead of the desired defined species (e.g., a monomer). Aggregates are thus a *universal term* for all kinds of not further defined multimeric species that are formed by covalent bonds or noncovalent interactions.

Different mechanisms that may lead to formation of various types of aggregates are currently under discussion. There is no single protein aggregation pathway but a variety of pathways, which may differ between proteins[12] and may result in different end states. A protein may undergo various aggregation pathways depending on the environmental conditions, including different types of applied stress. Also, the initial state of a protein that is prone for subsequent aggregation may differ. It may be constituted by the native structure,[13] by a degraded[14] or modified structure,[15] by a partially unfolded structure[15,16] or by the fully unfolded state.[12]

The aggregation process in general may lead to soluble and/or insoluble aggregates which may precipitate.[13,17–19] The morphology of these insoluble aggregates may be in the form of amorphous or fibrillar material which is dependent on the protein and its environment. Noncovalent aggregates are formed solely via weak forces such as Van der Waals interactions, hydrogen bonding, hydrophobic and electrostatic interactions[20] whereas covalent aggregates may for example form via disulfide bond linkages through free thiol groups[11,21,22] or by nondisulfide cross-linking pathways such as dityrosine formation.[23] Aggregation may be reversible[24] or irreversible where the irreversible aggregates could be permanently eliminated by preparative separation processes such as filtration techniques.[25] The formation of reversible aggregates is often considered to be caused by the self-assembly of protein molecules, which could be induced by changes in pH or ionic strength of the protein solution.[26–30]

One model that has been applied to describe irreversible protein aggregation is the Lumry-Eyring two state model.[31] According to this model the native protein undergoes first a reversible conformational change to an aggregation-prone state, which subsequently assembles irreversibly to the aggregated state. In this model protein aggregation is thereby controlled by conformational and colloidal mechanisms.[18,25]

In many cases, aggregation was described to follow a nucleation–propagation polymerization mechanism, whereby the nucleus can be formed by an altered monomeric structure or by a multimeric species.[32] New reports also suggest the appearance of heterogenous nucleation which is induced by micro- and nanoparticles of foreign matter, which for example could be shed from the equipment during processing.[33,34] Much insight in protein aggregation pathways is obtained from research in the field of amyloid fiber formation[35] and sickle cell hemoglobin.[36] In the area of

pharmaceutically relevant proteins such as monoclonal antibodies, the published reports on aggregation pathways are still very limited.

Protein aggregates have been categorized previously[11,37] based on different aspects. However, since the term "protein aggregation" has often been used lacking adequate definition, the authors suggest classifying "protein aggregates" based on the above considerations into the following categories:

(a) by type of bond: nonconvalent aggregates (bound by weak electrostatic forces)[38] versus covalent aggregates (e.g., caused by disulfide bridges);[11,21]
(b) by reversibility: reversible[26,29,30] versus irreversible[27] aggregates;
(c) by size: small soluble aggregates (oligomers) such as dimers, trimers, tetramers, etc. versus large $\geq$10-mer oligomers versus aggregates in the diameter range of some approx. 20 nm to approx. 1 μm versus insoluble particles in the 1–25 μm range versus larger insoluble particles visible to the eye under defined inspection conditions;[17,18,39]
(d) by protein conformation: aggregates with predominantly native structure[40] versus aggregates with predominantly nonnative structure (i.e., partially unfolded multimeric species,[39,41,42] fibrillar aggregates[43–45]).

Induction Factors Causing Protein Aggregation

Aggregation can be induced by a wide variety of conditions, including temperature, mechanical stress such as shaking and stirring, pumping, freezing and/or thawing and formulation. Also, because partially unfolded protein molecules are part of the native state ensemble, aggregation can occur under nonstress conditions where the native state is highly favored. Since processes that may cause stress upon a protein are commonly utilized during manufacturing of biopharmaceuticals including fermentation, purification, formulation, filling, shipment and storage, it is important to understand their effect on the induction of protein aggregates, both their influence on the aggregation rate as well as the type of aggregate potentially induced. Furthermore, it is generally acknowledged that formulation parameters including the protein concentration itself and other parameters such as pH, the qualitative and quantitative composition and formulation/packaging interactions play a major role in the control of protein aggregation. The following section outlines in general a few of the processes and conditions which have been reported to be involved in the formation of protein aggregation.

Temperature

An increase in temperature accelerates chemical reactions such as oxidation and deamidation of biopharmaceuticals, which could in turn lead to higher aggregate levels.[46] Higher temperature also has a direct effect on the conformation of polypeptide chains on the level of its quaternary, tertiary, and secondary structure, and can lead to temperature-induced unfolding that promotes in many cases aggregation. A measure for thermal stability of a protein is the melting temperature (T_m), at which 50% of protein molecules are unfolded during a thermal unfolding transition. Melting temperatures vary among proteins, and lie usually in a range between 40 and 80°C.[39] It is generally important to store biopharmaceuticals well below their T_m, usually at 2–8°C, and to avoid processing temperatures for example during fermentation, purification and manufacturing that go above the T_m. During accelerated stability studies of biopharmaceuticals high temperature storage conditions, such as 40°C are used to gain stability data within comparably short times, but since aggregation processes do not necessarily follow Arrhenius behavior, the extrapolation to predict aggregation at lower storage temperatures remains challenging.[47]

Freezing and Thawing

Freezing introduces complex physical and chemical changes including creation of new ice/solution interfaces,[48–50] adsorption to container surfaces,[50] cryoconcentration of the protein and solutes,[51,52] and pH changes due to crystallization of buffer components.[53] These effects are regarded as possible causes of freezing-induced protein denaturation and aggregation. The freezing rate as well as the method and control of thawing has been previously reported to influence the rate of protein aggregation.[52,54] The freezing/thawing container and the fill volume play a major role in the extent of induced protein aggregation.[55] This poses a significant challenge during formulation development since freeze/thaw stability testing is suggested to be tested at scale but may have to be carried out at small scale due to limited

availability of protein material in early stages, potentially not correlating to the freezing/thawing behavior and stability at large scale. Bhatnagar et al.[56] have collated numerous reports on freeze/thaw experiments in terms of rate of freezing and thawing as well as freeze–thaw cycles.

Agitation Stress

Agitation stress such as stirring, pumping, and shaking during manufacturing and transport has been described to cause aggregation.[13,18,57,58] These types of stress could induce shearing, interfacial effects, cavitation, local thermal effects and rapid transportation of either aggregated or adsorbed species from the interface into solution.[13] Although agitation stress is sometimes referred to as shear stress, several studies[59–61] suggest that shear alone does not cause protein aggregation. Agitation has been described to potentially cause cavitation[62] where cavitation is described as the rapid formation of voids or bubbles within the liquid which rapidly collapse thus producing shock waves, highly turbulent flow conditions, extreme pressures and temperature which may result in the generation of hydroxyl and hydrogen radicals thus leading to the formation of protein aggregates.[63,64] Mechanical stress testing in lab experiments could be performed under controlled conditions using, for example, horizontal or vertical shakers,[13,18] stirred reactors[65] and pumps,[11,66] rheometers such as concentric-cylinder shear devices or cone-plate, rotating-disk reactors,[57,58] to mimic "real-life" mechanical stresses which proteins may experience. Yet it is difficult to estimate the level of agitation stress that a protein experiences during processing, shipment, etc. Thus, the conditions applied in stress studies do not necessarily reflect the real life situation. It needs to be noted that the above mentioned different conditions may induce different species of aggregates, qualitatively and quantitatively.[13]

Protein Concentration

The increase in protein concentration has been reported to enhance the formation of protein aggregates for many proteins under quiescent storage.[67–70] The formation of aggregates is occurring by at least bimolecular interaction of protein molecules and thus, this reaction is per se considered to be concentration-dependent.[68] At high protein concentrations, macromolecular crowding occurs, a term used to describe the effect of high total volume occupancy by macromolecular solutes upon the behavior of each macromolecular species in that solution may appear. According to this excluded volume theory, self-assembly and thus potentially aggregation may be favored,[40] but at the same time unfolding that is a prerequisite for many aggregation reactions may be reduced.[40,71] An increase in protein concentration has been shown to increase the size of aggregates, as in the case of Beta-lactoglobulin.[72] Quiescent storage showed the acceleration of aggregation formation in higher protein concentrated formulations however upon agitation stress more aggregation was seen in low protein concentration samples.[68] On the other hand, a decrease in protein concentration via dilution (e.g., during preparation of drugs for clinical administration or sample preparation) has been shown to affect the aggregate content as aggregates formed by weak reversible interaction can dissociate as the protein concentration decreases.[73]

Solvent and Surface Effects

Changes to the solution environment of a protein, for example, pH, ionic strength, buffer species, excipients and contact materials, could induce the formation of protein aggregates. A change in pH has a strong influence on the aggregation rate as the pH determines the electrostatic interactions through charge distribution on the protein surface.[74] Under acidic conditions protein cleavage may occur, whereas under neutral to alkaline conditions deamidation and oxidation are favored. Such modifications depend on the primary sequence as well as structure[75] and may lead to increased aggregation. Different protein aggregation behavior has been shown within different buffer systems with equivalent pH.[76,77] For interferon-tau, a high protein aggregation rate in phosphate buffer was observed while the process was much slower in Tris and histidine buffer.[76] The quantity of excipients in the solvent may also have an impact on the aggregation behavior. The ability of the surfactants such as polysorbates (PS) to stabilize a protein against aggregation has been shown to depend on the protein to surfactant ratio.[13,18] Such a change in the concentration of an excipient/stabilizer is also observed when a drug formulation is added to infusion bags and this concentration change may have marked influence on aggregate levels. Furthermore, contact materials such as glass,

steel, silicone, plastic, rubbers, etc. may influence aggregation[78] where histidine formulated bulk of an IgG1 including sodium chloride resulted in high aggregate levels when stored in a stainless steel tank.[79]

Chemical Modifications of the Protein

Aggregation reactions can occur following a chemical modification of a protein. Chemical modifications can include reactions such as deamidation, isomerization, hydrolysis, and oxidation.[39] Modifications of amino acid side chains by for example deamidation or isomerization may distort the conformation of proteins[80] potentially leading to aggregation or self-association. Oxidation reactions such as disulfide bond formation or methionine oxidation may be promoted by light exposure, peroxide contaminations of excipients, or simply the presence of oxygen during the manufacturing process. Light exposure may lead to photolytic degradation of the protein through photo-oxidation of the side-chains of certain amino acids such as Met, Tyr, Trp, His, Cys, and Phe[81–83] and has been shown to induce protein aggregation.[82] The quality in terms of impurity levels of an excipients such as the nonionic surfactants PS 20 and PS 80, in a protein formulation may result in oxidation reactions. Polysorbates undergo auto-oxidation which results in hydroperoxide formation.[84] The impurity levels of hydroperoxide within PS may vary from lot-to-lot as well as from manufacturer-to-manufacturer.[85] The levels of hydroperoxide may also depend on storage conditions and storage time of the PS and thus leading to potential differences in oxidation and also potentially in subsequent protein aggregation.[84]

PROTEIN AGGREGATION: ANALYTICAL TECHNIQUES

Proteins are therapeutically used in a wide range of indications. Before these biopharmaceuticals enter clinical R&D programs to evaluate their therapeutic potential, they need to be extensively characterized and adequately monitored during and after manufacturing and storage with regard to structural and biological integrity, process and product related impurities, and molecular and biological properties. Recent technological progress has significantly amplified the speed of characterizing proteins and with these advances, various analytical methods are now available to better characterize biopharmaceuticals. Molecular weight, conformation, size and shape, and state and extent of aggregation are a few of the physico-chemical properties studied.

Several methods are available for the quantification and size estimations or the characterization of protein aggregates (Tab. 1). However, the inherent differences in what is being measured and the requirements of most of these methods for data evaluation may result in inconsistencies between the methods in the reported mean size, size distributions, and quantity of an aggregate species for a given sample. One of the major challenges with the analysis of protein aggregates is that currently no single analytical method exists to cover the entire size range in which aggregates may appear, especially a routine method to quantify submicron particles. The protein aggregates may constitute only a minute fraction of the total protein mass and may be particularly of interest due to their potential role in immunogenicity.[3] Therefore different analytical methods have to be employed in order to detect these minute aggregate fractions as well as to cover the size range from a few nanometers to hundred micrometers to large visible particles (Fig. 1). Additionally, analytical methods used for assessing protein aggregates need to be closely looked at with regard to their performance and limitations, such as their specific limit of detection as well as the possibility to create artifacts, such as either inducing or destroying aggregates during sample preparation (dilution or increasing the concentration) thus potentially shifting the aggregation equilibrium, or the loss of aggregates by adsorption onto column material or membranes during analysis. Therefore, it may be advisable to carefully compare the analytical results obtained from various methods, that is, the use of orthogonal methods and to assess any data differences on a case-by-case basis with regards to method set-up and parameters. The authors define the use of *orthogonal methods* as “the use of a combination or a variety of different analytical methods, each having its own characteristic measuring principle, for example, by size, quantification or structure, etc.” The use of such orthogonal methods is also suggested in the current European Medicines Agency (EMEA) draft guideline on “production and quality control of monoclonal antibodies and related substances.”[86]

Table 1. Frequently Used Methods for the Analysis of Protein Aggregation

Category	Method	Application
Quantification and/or size estimation	SE-HPLC	Size estimation and quantification (soluble aggregates)
	RP-HPLC	Size estimation and quantification (soluble aggregates)
	SDS–PAGE	Size estimation and to distinguish reducible covalent from noncovalent aggregates
	Capillary electrophoresis	Size estimation and quantification (soluble aggregates)
	Field flow fraction (e.g., AF4)	Size estimation and quantification (soluble aggregates)
	Microscopic methods (e.g., Light, electron, atomic force microscopy)	Size and shape estimation
	Static light scattering	Size and shape estimation
	Dynamic light scattering	Size distribution
	Analytical ultracentrifugation	Size, shape estimation and quantification
	Light obscuration	Size and quantification (insoluble aggregates)
	Coulter counter	Size and number quantification (insoluble aggregates)
	Visible inspection	Absence or presence of visible aggregates
	UV–Vis spectroscopy, turbidity/ opalescence/clarity (visually or instrumentally)	Soluble and insoluble aggregates; solution property (no quantification possible)
Characterization	Circular dichroism	Structural analysis
	Fluorescence spectroscopy	Structural analysis
	(FT-)infrared spectroscopy	Structural analysis
	Raman spectroscopy	Structured analysis
	Nuclear magnetic resonance spectroscopy	Structural analysis

The following sections shall discuss various analytical methods available to measure protein aggregation, their pitfalls, as well as the advantages in comparison to other techniques.

Size Exclusion Chromatography

Since its introduction by Porath and Flodin in the late 1950s[87] the conventional size exclusion chromatography (SEC) or gel filtration has become an essential tool for the analysis and purification of proteins. SEC is one of the most used analytical methods for the detection and quantification of protein aggregates. SEC analysis allows both for sizing of aggregates, and their quantification.

Utilizing various column materials in combination with high performance liquid chromatography (HPLC) results in the selective and rapid separation of macromolecules based on their shape and size (hydrodynamic radius) in a molecular weight range of roughly 5–1000 kDa.[88] This fractionation range of the column is based on the fact that oligomers that are too large to penetrate the pores of the matrix are excluded from the packing pore volume and elute with the void volume of the column.[89] The aggregation size determination may vary between different SEC methods, suggesting that the upper size range of an aggregate which escapes the SEC determination needs to be assessed case by case. However, insoluble aggregates are not considered to be measurable by SEC due to potential removal via filtration by the column or precolumn or by the sample preparation for SEC (e.g., centrifugation). Factors such as protein shape, protein glycosylation or pegylation[90] could affect the accuracy if the molecular weight of protein species is determined based on a calibration curve using calibration standards.[91] Well characterized, water-soluble and globular proteins are used as calibration standards, which may differ in their elution properties in comparison with the protein of interest. It has been reported that basing the molecular weight solely on the elution volume has resulted in incorrectly identifying peaks as dimers.[37,91,92] It was also shown that modification of the mobile phase, such as the inclusion of arginine suppresses protein adsorption to the column matrix,[93,94] and that additional peaks may be induced due to a high salt content in the

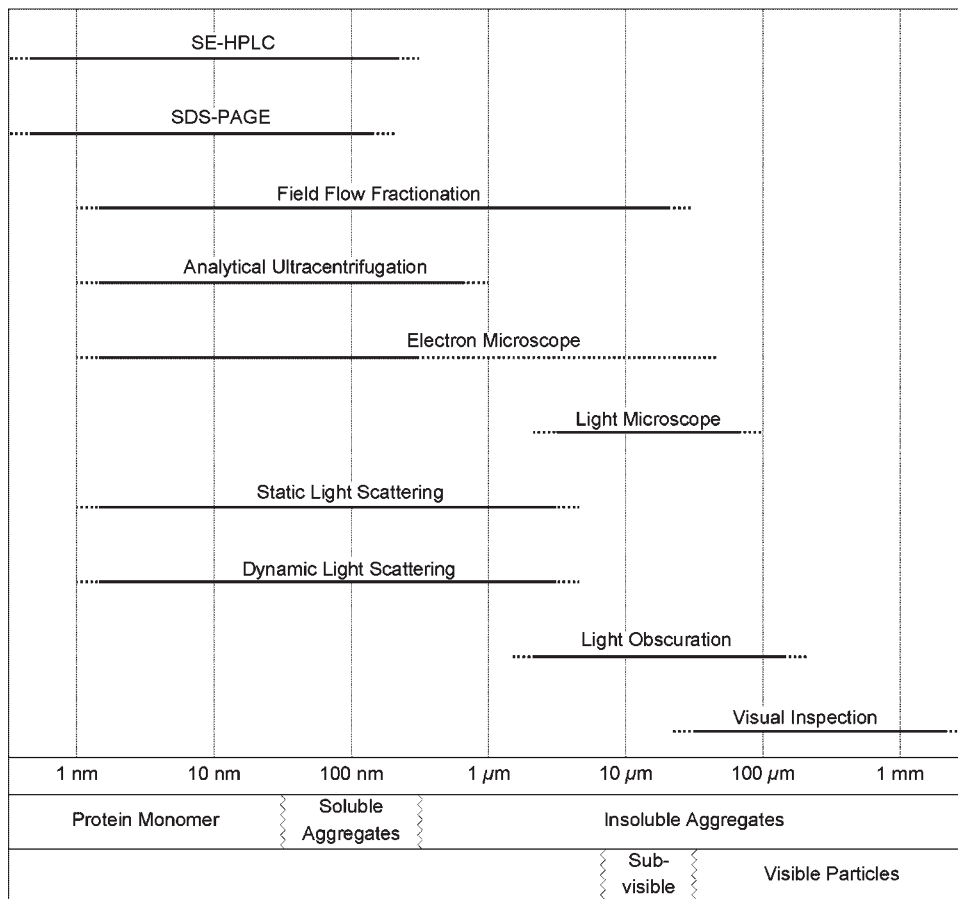

Figure 1. Schematic representation of the approximate range of detectable protein sizes (diameter) of various analytical methods.

mobile phase.[73] Dilution, occurring during the chromatography process or sample preparation, may lead to the dissociation of reversibly formed aggregates as the concentration decreases.[73] SEC cannot only be used in combination with UV or fluorescence detectors, but also with other detectors such as light scattering detectors (e.g., multiangle laser light scattering (MALLS)), to take advantage of the light scattering technique in combination with the separation technique of soluble aggregates such as to increase accuracy in molecular weight determination. Further details of light scattering are to be discussed in Light Scattering Section.

Sodium Dodecyl (lauryl) Sulfate–Polyacrylamide Gel Electrophoresis Chromatography

Gel electrophoresis has been used since the 1960s[95,96] and has become a commonly used versatile analytical tool for estimating protein size, identifying proteins, determining sample purity and evaluating presence of disulfide bonds to name a few applications. The detection size is limited to proteins/aggregates with a weight range between ca. 5 and 500 kDa with the possibility to extend the weight range of an electrophoresis gel by various techniques such as gradient gels or particular buffer systems.[97] The use of the anionic detergent sodium dodecyl (lauryl) sulfate (SDS) in the separation of proteins into fractions has been known for over 70 years[98] and the combination with gel electrophoresis has become a very commonly used system for molecular weight determination which is also called the Laemmli system.[99] SDS–PAGE has the ability to detect covalently linked aggregates, or SDS nondissociable aggregates, however noncovalent associated proteins species are separated into their constituent polypeptide chains.[69] As an anionic detergent, SDS denatures proteins and

binds to most proteins with a uniform 1.4 g of SDS per gram of protein thus giving the polypeptide a negative charge in proportion to its mass. Sample preparation which should be designed to fully denature the protein and includes usually a reduction and temperature step is not trivial. Depending on the preparation conditions, proteins may not be fully denatured, (e.g., disulfide-bonded proteins which are only partially reduced). Also during the heating at high temperature in SDS, the existing protein aggregates may be fully dissolved in the SDS solution or may form aggregates as in the case of membrane proteins thus giving artificial results.[100] An important feature of SDS–PAGE under nonreducing conditions versus reducing conditions is its ability to differentiate between noncovalent and covalent aggregates by disulfide bridges. The sample preparation requires special care such as in the case of the detection of an IgG4 half-antibody where artifacts were shown to be introduced through the preparation procedure.[101]

In order to visualize previously separated protein bands for quantitative or qualitative detection, various staining techniques available such as Coomassie brilliant blue R250,[102] Silver Stain,[103,104] and fluorescent dye stain.[105] Quantification of the bands obtained by Coomassie staining may be carried out using a densitometer, computer and appropriate software. However, such densitometers together with the software require calibration for linear response to optical density of the bands and repetitive digital area integration[106] which could output erroneous results. According to the Ph. Eur.,[107] the Coomassie technique has the ability to detect 1–10 μg of protein per band, depending on staining time, dye concentrations, etc. The intensities of the stained bands can be used to estimate the molar ratios of protein subunits or multi-protein complexes except very hydrophobic subunits that stain very poorly.[97] Preferably, "staining controls" with defined amounts of protein reference should be used for evaluation of the staining procedures. However, if lower ranges of aggregate levels are required to be detected, an alternative to the Coomassie stain is the cupric-silver stain. The cupric-silver stain is approx. 100 times more sensitive than the conventional Coomassie blue stain with a claimed detection of 0.38 versus 38 ng/mm^2 of serum albumin,[103,108] or a band containing 10–100 ng.[107] However, the silver stained bands cannot be reliably quantified and therefore this staining method is only used qualitatively. Figure 2 shows a typical Coomassie stained gel of an aggregation-prone IgG1 protein in the non-reduced and reduced form. Overall, SDS–PAGE is considered a valuable tool to analyze small size aggregates and to differentiate reducible from nonreducible aggregates.[109,110]

Field Flow Fractionation

Field flow fractionation (FFF) techniques have been used previously to determine the size of

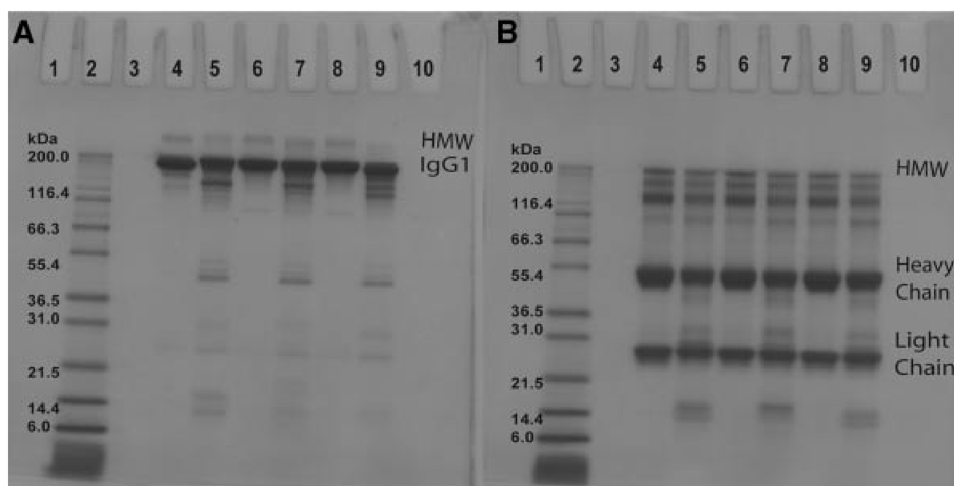

Figure 2. SDS–PAGE Coomassie stained gels of (A) nonreduced and (B) reduced IgG1 samples. Lane 2: molecular weight standards; lane 4: unstressed IgG1; lanes 5–9: IgG1 in different formulations after 4 weeks 5°C static storage. HMW: high molecular weight species.

various types of particles and are explored as separation techniques for biological entities.[111,112] The great advantage of this technique is that it lacks a stationary phase. In conjunction with liquid chromatography, it is a method considered capable of separating particles ranging from individual molecules in the nanometer range to large particles in the micrometer range.[113,114] The separation of proteins using FFF was first employed in the 1970s[115] and has become a valuable analytical tool due its versatility.

Reschiglian et al.[111] described the FFF simply as a separation of molecules based on their differences in molar mass and size which are forced by an applied field into different velocity regions within a parabolic flow within a channel. The channel consists of a lower wall consisting of a water permeable ultra-filtration membrane and an upper wall of plastic such as Perspex™. The separation is performed by the initiation of an injection of the sample of interest directly into a carrier fluid within a thin channel. The fluid is pumped through this channel and perpendicular to this parabolic flow an external generated field is applied. The field applied can vary in type as listed in Table 2. With this applied field the sample components accumulate towards one of the channel walls known as the accumulation wall and the velocity flow of the parabolic flow is at its maximum nearest the center and decreases towards the channel wall. Therefore the molecules closest to the accumulation wall are located in the slowest velocity of the flow and elute out slower compared to the molecules found in the fastest velocity region which then in turn flow into a detector.

FFF is a separation and size determining technique however the dimensional values of the molecules fractionalized need to be scrutinized with regard to the retention time of the retained sample due to the interactions of the molecules with the membrane. The resolution of high molecular weight (HMW) molecules is easily achieved due to the low diffusion coefficient driving them closer to the accumulation wall by the movement of the cross-flow and eluting out slower whilst opposite is true for the low molecular weight (LMW) molecules having a high diffusivity.[112,116] An elementary association between the experimental retention time and the particle sizes of samples can be predicted based on the rate of diffusion of the particles by a theory described elsewhere.[117–119]

Asymmetrical Flow Field Flow Fractionation

With all the available methods of the FFF technology (Tab. 2), the flow FFF has been reported as the most suitable and widely used method for the separation of protein aggregates[37,112,116,120,121] especially the asymmetrical flow field flow fractionation (AF4). The term "asymmetrical" is due to the fact that the channel has two distinctive types of walls with the accumulation wall being an ultra-filtration membrane, making the AF4 a unique technique to the flow FFF family. Since the perpendicular cross-flow passes through the membrane, the longitudinal flow rate is being constantly reduced as it approaches the channel outlet. To minimize this phenomenon and in turn to increase the separation rate, very thin channels with low volumetric capacities are used such as trapezoidal geometrically shaped channels. This type of channel shape versus a rectangular shape advantageously allows an extra means to control the longitudinal flow velocity, where the breadth decreases continuously towards the channel outlet, which

Table 2. Different Fields Currently Used in FFF

Field Type	Technique	References
Cross-flow (Fl)	Flow FFF (FlFFF)	Giddings et al.[126]
	Asymmetrical FlFF (AFFF/AF4)	Litzen et al.,[127] Fraunhofer and Winter,[112] Yohannes et al.,[116] Demeule et al.[203]
	Hollow-fiber FlFFF	Reschiglian et al.[128]
Sedimentation (Sd)	Sedimentation FFF (SdFFF)	Mozersky et al.,[129] Kassab et al.[130]
	Centrifugal SdFFF	Parsons et al.[131]
	Gravitational (GrFFF)	Sanz et al.[132]
Thermal (Th)	Thermal FFF (ThFFF)	Janca et al.[133]
Electrical (El)	Electrical FFF (ElFFF)	Caldwell et al.[115,134]
Magnetic (Mg)	Magnetic FFF (MgFFF)	Carpino et al.[135]

creates a possibility to level out the steep linear velocity gradients that can materialize in the rectangular channel.[122] The ultrafiltration membrane also needs to be carefully considered as the cut-off range and molecular interaction with the filter material may significantly influence the potential sample loss and recovery. The membrane protein interaction is most pronounced during the high cross-flow conditions and consequently some adjustment of elution solvent composition may be needed which could in turn induce conformational changes of proteins or influence the distribution of noncovalent aggregates. Low adsorption membranes such as regenerated cellulose are often selected to potentially reduce the interaction and to achieve good separation.[120] Both dilution and concentration steps occur during the FFF technique. Firstly, upon injection the sample interacts with the fluid medium and therefore results in a slight dilution and then secondly a concentration step is performed which is required to improve the separation process. This focusing step also known as the relaxation step, is the procedure where the sample components are "concentrated" into a narrow cross-sectional distribution before elution.[123] Following the injection step and during focusing, the concentration of the sample will change and might impact the level of reversible soluble aggregates, thus potentially creating artifacts.[69,124,125]

FFF has been shown to be applicable to a broad range of different biological samples and AF4 is now more widely used for protein characterization. However, the FFF method is considered difficult to validate and therefore not yet used as a routine analytical tool. Separation of protein sample components is achieved but mainly limited to soluble protein aggregates.

Analytical Ultracentrifugation

Another tool to study protein aggregation with increasingly widespread use is analytical ultracentrifugation (AUC).[120,136–143] The principle of AUC relies on the property of mass, size and shape and the fundamental laws of gravitation and is a primary technique for which the results do not depend on a comparison to standards.[144] This method was pioneered by Svedberg already in the early 1940s,[145] however due to the advanced development of instrumentation technology and computational software nowadays AUC has become a broadly utilized tool in the analysis of proteins.[140] There are a number of reviews on AUC available in terms of history and modernization.[139–141,144,146] Sedimentation analyses can be used over a wide range of solute concentrations[146] and in comparison with other separation techniques such as SEC (see Size Exclusion Chromatography Section) and SDS–PAGE (see Sodium Dodecyl (lauryl) Sulfate–Polyacrylamide Gel Electrophoresis Chromatography Section), there is very little or no sample preparation necessary, depending on the sample's protein concentration. Thus AUC allows direct measurement of the protein aggregates under various solvent conditions.[147] However, at higher protein concentration nonideality inhibits reliable molecular weight determination of the sedimenting species. Therefore, a dilution to lower concentration may have to be performed, potentially creating artifacts. AUC is currently not a high throughput method[140] due to lengthy run time per sample. The method also requires highly specialized and costly equipment, trained analysts and special validation effort for of the data analysis software.[148]

Methods for the characterization of heterologous protein–protein interactions include sedimentation velocity (SV), sedimentation equilibrium (SE), tracer sedimentation equilibrium and analytical band sedimentation.[137] SE gives information regarding the molecule's molar mass, association constant and stoichiometry whilst SV provides hydrodynamic information about the molecule's size and shape.[140,141,146] The use of AUC-SV is mainly due to the adoption of the advanced data analysis tools available pioneered by Schuck et al.[149] where the data analysis is based on the continuous sedimentation coefficient distribution method.[138,150] The programs allow fast and rigorously data analysis by fitting the SV data using the Lamm's equation[151] and thus enable to detect, quantify and characterize small amounts of protein aggregates from dimers to heptamers.[10] The experiments are performed under controlled conditions such as temperature, rotor speed and fixed geometry.[141] Optical detectors are available such as absorbance, fluorescence and interference each with their own advantages and disadvantages.[144] The absorbance optics are sensitive in detecting chromophores which allows the characterization of proteins with a good signal to noise ratio at concentrations as low as 10 μg/mL.[152] The Raleigh interference optical system is based on the sample's refractive index and is a system used for concentrated samples with a sensitivity of 50 μg/mL. In contrast, fluorescence detection

allows measurements of very dilute samples using a fluorescent label,[144] enabling studies at high concentration using spiked-in fluorescently labeled protein. However, labeling might alter the analyzed protein's interactions and thus might create artificial results. In general, due to the wide range of sample concentrations and the sensitivity to small fractions of aggregates, AUC is becoming a more widely used tool in the characterization of biopharmaceuticals. AUC-SV is used more and more as an orthogonal tool for the analysis of soluble protein aggregates within the pharmaceutical industry[37,141,147,153] and adequate cross-correlation between AUC and SEC has been reported.[141,143] However, although better precision of AUC has been achieved lately by equipment improvements[148] reproducibility and precision can still be considered lower than for SEC.[154,155] AUC uses different separation principles than SEC and—depending on the number and size of aggregates in the sample—the AUC technology may require significant adaptations and should not be considered as a readily available method for one-to-one comparison to SEC.

Determination of Turbidity (Opalescence, Clarity)

Protein solutions show an optical property, called opalescence or turbidity. "Opalescence" is described as a cloudy-white translucent appearance and "turbidity" as a cloudiness or haziness of fluids caused by individual particles consisting of various sizes. The optical property of a solution is a function of the particles present to scatter and absorb light. Proteins are natural colloids and the turbidity of aqueous formulations depends on protein concentration,[156] the presence of nondissolved particles, the particle size and particle number per volume unit. The Rayleigh relationship[157] could justify this fact where the Rayleigh's theory indicates that the scattering of light is brought upon by particles which are smaller in diameter than the wavelength of the light itself. Typically the upper limit is taken to be about 1/10 of the wavelength. Therefore, the exact shape of the scattering center is usually not very significant and can often be treated as a sphere of equivalent volume. Aggregation has been reported to be indicated by a marked increase in turbidity over storage or stress time and has been used for the detection of aggregates.[13,18,158–163] However, turbidity has also been shown being a precursor to liquid–liquid phase separation.[164]

Table 3. Reference Suspensions According to Ph. Eur. 2.2.1

Reference Suspension	FTU	Description
I	3	≤Ref I = clear
II	6	≤Ref II = slightly opalescent
III	18	≤Ref III = opalescent
IV	30	≤Ref IV = highly opalescent

The turbidity measurement included in the Ph. Eur.[165] is a method to complement the analyses of uniform opalescent solutions. A comparison of the opalescence of a protein sample against the Formazin reference suspensions of defined turbidity of the Ph. Eur. (Tab. 3) allows a more reproducible assignment of the sample's category of opalescence than that of pure visual description of appearance without comparison to a defined standard. Various methods can be used to assess a solution's turbidity. A visual comparison of the sample to the reference suspensions under defined light condition can be performed. However, since this depends on the visual acuity of the inspector an instrumental method using a nephelometer or turbidimeter as a more discriminatory test is also available, which outputs numerical data. These instruments measure the turbidity by employing a light source such as from tungsten-filament lamp and a light detector set to one side (usually 90°) of the source light beam. The turbidity is then a function of the light reflected into the detector from the particles. The properties of the particles such as shape, color and reflectivity correlate to the amount of light that is reflected by the given density of particles. There are many models of turbidimeters, depending upon the arrangement (geometry) of the source beam and the detector. A nephelometric turbidimeter always monitors light reflected off the particles and not attenuation due to cloudiness and is therefore able to monitor protein aggregates. The units of turbidity from a calibrated nephelometer are called Nephelometric Turbidity Units (NTU) or using Formazin as a reference standard the Formazin Turbidity Unit (FTU) is obtained.

Alternatively, established categories of opalescence based on Ph. Eur. 2.2.1[165] reference suspensions are reported using turbidity measured photometrically as optical density in the 340–360 nm range and 550 nm.[18,158–160] These wavelengths may give comparative results to

opalescence measurements, however, the photometric measurements depends also on the specified method and should be cross-correlated carefully with the Ph. Eur. method. In general, measurements at the wavelength of 350 nm are preferred as the sensitivity towards turbidity is higher at lower wavelengths.[18,110] However, correlating the photometric values to the Ph. Eur. by a reference suspension category is a challenging task.

The relevance of turbidity data needs to be closely assessed on a case-by-case basis. As mentioned, various factors including protein concentration and temperature[156] contribute to turbidity of a solution, apart from a potential increase in aggregate species, or turbidity being a potential precursor to liquid phase separation.[164] It is therefore well advisable using turbidity values only in comparative measurements, for example, to assess turbidity over time in stability programs, and to carefully evaluate such results on a case-by-case basis.

Light Scattering

Light scattering is suited to detect and characterize soluble aggregates on a length scale of ca. 1–100 nm[166] where real-time data can be collected thus making kinetic studies possible.[167] There are many types of light scattering methods available such as static light scattering (SLS), dynamic light scattering (DLS) which is also referred to as quasielastic light scattering (QELS) or photon correlation spectroscopy (PCS), laser diffraction (LD) alternatively known as low angle light scattering (LALS), spectroscopy of optical displacement or laser correlation spectroscopy. The principle of light scattering has been discussed in the Determination of Turbidity (Opalescence, Clarity) Section and is caused by the particles present to scatter and absorb light. The intensity of this scattered light depends on the ratio between the particle size and the incident light wavelength, and the shorter the wavelength value, the smaller the particles, which can be effectively investigated.[168] A great advantage of laser light scattering is that no dilution may be necessary depending on the sample and the type of instrument employed. Samples should be optically clear for light scattering in order to avoid back-scattering. Large particles such as dust or protein precipitates interfere with the data analysis and thus the avoidance or removal of such "contaminants" is a crucial step. Removal by filtration or centrifugation could be employed but the issues of sample preparation need to be kept in mind as this could output artificial results.[13]

Static Light Scattering

SLS, also called classical light scattering, has been used since the 1940s[169] and is a classical technique for the determination of molar masses and radii of biological macromolecules such as protein aggregates in solution.[170–173] The basic physical phenomenon of SLS is "elastic scattering" which occurs when a laser beam hits a particle and the particle's electrons re-emit radiation at the same frequency in all directions.[166] LS analysis has proved to be an essential tool in the investigation of protein self-association of highly concentrated protein samples.[174,175]

The conventional SEC with a UV detector has a few limitations that prevent the correct molecular mass determination as well as having a low sensitivity to detect small concentrations of aggregates, however these can be overcome with the combination of SEC–LS.[170] The use of multi-angle laser light scattering (MALLS) in combination with SEC or AF4 to determine the molecular weight of proteins and aggregates has become very popular.[37,90–92,143,176] MALLS is experimentally independent of the elution order (referred to as the absolute molecular weight) and no calibration is required.[177] The known parameters required are the concentration of each elution fraction as well as the differential refractive index increment (dn/dc) to calculate the absolute value of molecular weight.

Dynamic Light Scattering

DLS can measure the diffusion rather than the size of polydisperse samples producing a sum of exponentials weighted according to frequency and scattering intensity. The scattered light with short-term intensity fluctuations (dynamics) arise from the fact that the scattering particles of 5 μm in diameter and smaller are in constant motion (diffusive Brownian motion). The movement speed is inversely proportional to the particle size d described in the Stokes–Einstein Eq. (1) where k is the Boltzman constant, T is the temperature in Kelvin, η is the sample dynamic viscosity and D is the diffusion coefficient, that is, the smaller the particles, the faster the speed or diffusion, and the velocity can be detected

by analyzing the time dependency of the light intensity fluctuations scattered from the particles when they are illuminated with a laser beam. This technique is limited to resolving size differences of fivefold or greater and should be considered rather a qualitative and not a quantitative method.

$$d = \frac{kT}{3\Pi\eta D} \quad (1)$$

The scattered light may allow to detect proteins from sizes of ca. 1 nm to 10 μm.[168] The inherent advantage of the DLS method is that no extensive sample preparation is needed and it has been widely used to study various types of proteins.[18,90,121,168,178–182] The results are sensitive to dust, bubbles and especially very large aggregates. Turbid samples or samples with contaminations or large particles may cause the laser beam to diverge substantially before it passes through the center of the sample cell and may therefore lead to imprecise data readouts and analysis. In conjunction to this phenomena, the surrounding particles cause the already scattered light to undergo a secondary scattering before reaching the detector.[183] This problem could be overcome by centrifuging, diluting or filtering[13,18] the sample. However, the filtration step may change the particle distribution and/or may result in removal of aggregates by the filter and thus potentially artificially change the obtained results. Furthermore, altering the protein concentration through dilution could cause the dissociation of reversible aggregates.[69,124,125] Other potential sources of error in DLS include temperature fluctuations, measurement duration, sample's rheological properties, protein concentration range, irregular (nonspherical) particle shape and most importantly the mathematical models or algorithms[168] used to analyze the hydrodynamic radii and the polydispersity of the sample.[184,185] In summary, this technique may provide very valuable data; however, its use is considered limited as an additional characterization tool, being a research method.

Subvisible Particle Analysis

The quantification of subvisible particles can be carried out via light obscuration, microscopic techniques or Coulter method.[186,187] Light obscuration or blockage particle counters are widely used in the pharmaceutical industry as the method of choice[188] and are described in the pharmacopoeias. This method has the theoretical ability to count individual particles by size in different size ranges from approximately 1–150 μm or larger with varying precision and reproducibility depending on the probe used. With the light obscuration method, the particle size is deduced from the amount of light blocked as the particles pass in a single file fashion. The pulse number and degree of the light blockage are recorded and sorted for the subsequent calculation of the particle size distribution. For particle sizes greater than the light wavelength the peak intensity is related to the projected area of the particle. Therefore this technique could be used for particles larger than 0.5 μm,[189] however, precision or reproducibility are low for these size ranges. The use of light obscuration method as an orthogonal method to sizing and quantifying protein aggregates has been reported.[13,18] Artificial results may, however, occur at high particle concentrations, if the channel becomes physically blocked or if the limit of detection has been reached. Dilution of samples can reduce the probability of simultaneous entry into the incident laser beam, but it can also cause unwanted dissociation of insoluble protein aggregates[18] potentially creating false negative results. Also false negative results might occur, if the transparency of the particle is too high, that is, some very translucent particles might not scatter any light and therefore might not be detected. Another difficulty is that the instrument cannot differentiate between actual particles from protein aggregation, particles from extraneous source (nonprotein particles) and air bubbles which can potentially lead to false positive results. This can be the case, for example, for the particle count testing of freshly reconstituted lyophilized protein samples, as the re-introduction of water may result in many air bubbles, especially when the formulations contain surfactant.

The interpretation of light obscuration analysis according to the Ph. Eur[8] and USP[7] was harmonized to particles larger than or equal to 10 or 25 μm respectively. Interestingly, the sample volume described to be used for the measurement totals up to 25 mL, making the analysis impractical and very cost-intensive for biopharmaceuticals, especially in early formulation R&D. Therefore, sample volumes have been reported to be reduced for biopharmaceuticals,[190] however, the measurement would then not be compliant unless cross-validated. Furthermore, Ph. Eur. and USP set acceptance limits for

particles larger than or equal to 10 or 25 μm per container (small-volume parenterals). These limits are probably derived from "historically relevant" contaminations of parenteral products, such as tiny glass particles or alike, potentially generated during sterile fill and finish processes. Considering biotech products, protein aggregates might significantly contribute to the total number of subvisible particles measured. The acceptance criteria in the pharmacopoeias for small volume parenterals are also linked to container size (fill volume). To date those limits are usually also applied for biopharmaceutical products.

The microscopic method for determination of subvisible particles is also featured in the Ph. Eur. 2.9.19,[8] based on the manual or automatic counting of particles with the use of a binocular microscope. The sample is filtered through a vacuum onto a grid lined filter and, once dried, is placed under the microscope for counting. It needs to be assured that the particles on the filter are not derived from the preparation environment, equipment or from the personnel, and therefore potentially creating artificially false positive results. Calibration is performed; however this can be difficult and tedious.

The Coulter method is an electrical sensing zone method that consists of two chambers containing an electrolyte solution and a single channel to connect them. An appropriate potential is applied resulting in an ionic current being driven through the channel. If particles of an appropriate size are present, they will enter the channel and reduce the ion current. Coulter data consist of a series of impedance pulses associated with the presence of particles within the channel. The height of the pulse is related to particle size and the width corresponds to the particle transit time. In some cases, data such as these can provide information about the size, concentration and number of the particles. This method offers a potential means by which absolute counts of particles in solutions could be expeditiously obtained. However, the use of this technique for this purpose is limited by its incapacity of differentiating clusters of particles from individual particles of comparable size and low sensitivity to very small particles. Additionally, the sample is diluted and electrolytes might be added to the sample to increase the conductivity may both affect the analytical result.

The accurate testing of subvisible particles is to-date very difficult and many precautions need to be taken into account, that is, tests need to be carried out under contamination-free conditions such as under laminar-flow cabinets and particle-free water is required. A very important issue that still needs to be addressed is the international harmonization of the methods of the various pharmacopoeias as each specifies different acceptance criteria limits to the light obscuration and microscopic methods (Tab. 4). However, this method is considered to be a valuable orthogonal tool to assess protein aggregation in the size of ≥10 and ≥25 μm in biopharmaceuticals.

Visible Particle Analysis

Since analytical techniques such as SEC or other methods listed so far, usually are not capable of analyzing aggregates above a certain size range (due to prefiltration of those larger aggregates by either sample preparation or a column or precolumn), the inspection of samples by visual means is still an important aspect of assessing protein aggregates in biopharmaceutical products. Visible inspection methods have been used to analyze for "extraneous particles" in parenteral drug products, with particles defined as being "contaminations consisting of extraneous, mobile, undissolved particulates other than gas bubbles, unintentionally present in the product" (e.g., glass

Table 4. Limit Requirements of Particulate Matter for Injections According to the Pharmacopoeias

Pharmacopoeia	Method	Volume	Limits
Ph. Eur.[8] USP[7] JP[191]	Light obscuration	LVP	25 particles/mL ≥ 10 μm
			3 particles/mL ≥ 25 μm
		SVP	6000 particles/container ≥ 10 μm
			600 particles/container ≥ 25 μm
	Microscope	LVP	12 particles/mL ≥ 10 μm
			2 particles/mL ≥ 25 μm
		SVP	3000 particles/container ≥ 10 μm
			300 particles/container ≥ 10 μm

LVP, large volume parenterals (≤100 mL nominal fill volume); SVP, small volume parenterals (>100 mL nominal fill volume).

particles). In case of sufficiently large protein aggregation, this method may also be suitable for the detection of protein precipitation.

It is described in the literature that the human eye has the ability to resolve objects slightly smaller than ~80 μm at a distance of ~25 cm.[192] However, illumination intensity, inspection time, inspection aids (magnifying lenses), automated handling (vial spinning), light polarization, inspection background, inspector training and experience and number and type of particles may influence the probability of particle detection as well as limit of detection of particle size (Tab. 5). Using different detection methods, it has been shown that the detection probability is significantly impacted by above parameters and that also extraneous particles as small as 25 μm, if present in sufficient number, could be detected (Pierre Goldbach, personal communication).

The Ph. Eur. monograph for parental preparations in conjunction with the visual particles monograph (Ph. Eur. 2.9.20[9]) requires "parenteral preparations" (which are not administered using a final filter) to be "practically free from (visible) particles." The USP[7] states "essentially free of visible particles," without giving a definition on what can be considered "essentially" or "practically." This definition reflects the current capabilities of manufacturing and control. There are no specified viewing conditions or inspection time for visual inspection within the USP however the USP is currently drafting an informational chapter on visual inspection. The Ph. Eur. visual inspection method is carried out using an apparatus consisting of a box with a backboard comprising of two panels, the left one as a nonglare white panel and the other a matt black panel situated on the right with an observation time of about 5 s for each panel (Fig. 3). An adjustable lamp provides the source of white light with an intensity of 2000–3750 lux. Other inspection methods would also be considered to be used. Differences exist in the visual inspection method between the Pharmacopoeias in the case

Table 5. Overview of Various Visual Inspection Methods Parameters According to the Pharmacopoeias

	Ph. Eur.	JP	USP
Background	Black/white	Black	Black/white
Light intensity	2–3.75 klux	1 klux	2–3.75 klux
Inspection time	5 s	15 s	5 s
Vial rotation	Manual	Manual	Manual

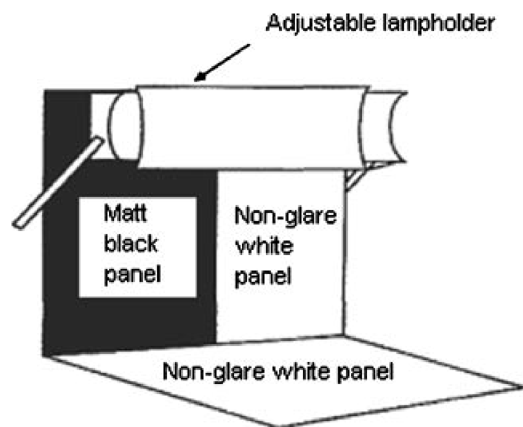

Figure 3. Apparatus for the visual inspections as per Ph. Eur. (current edition).

of the Japanese Pharmacopoeia (JP)[191] which refers to white-light inspection at 1000 lux, with observation time of 15 s against a black background. Automated rotation of the samples used with some inspection machines whirls up particulate matter in liquids within the container and these particles are enhanced visually through a magnifying glass. A concentrated light beam through the bottom of the container causes particles to reflect light due the Tyndall effect[193,194] and therefore further facilitates inspection. The great advantage of the automated rotation systems is that the operator can see the full circumference of the container due the absence of clips or fingers which could hinder the view.

With all the various systems available the background, the light source and the light intensity as well the actual inspector all play a crucial role in the limit of detection of the particles with a major criterion being the subjective nature of visual inspection to each operator. Important parameters for the detection of visible particles by human eyes include (a) the light conditions, (b) magnifying lenses used, (c) observation time, (d) type of background inspected against, (e) distance from samples, (f) automatic versus manual rotation, (g) the inspector's capability and training and eye conditions, and (h) the size, number, type and refractive index of particles.

Other Technologies

Image Analysis

Automated image processing systems in combination with microscopic systems operated in flow

though mode allow automatic analysis of particles in liquid formulations. The pharmacopoeias—USP,[7] Ph. Eur,[8] JP[191]—indicate that the light obscuration and manual microscope techniques are the methods for counting and sizing of particles for parenteral applications. Automated image analysis such as the FPIA-3000 instrument and the Micro-Flow Imaging™ (MFI) system[195,196] have been shown to increase the efficiency of manual microscopy. Besides number and size, additional parameters such as transparency and shape can be determined, thus potentially helping in differentiating between for example silicone droplets or air bubbles and extraneous particles.[197] However, currently it is not considered that measurements using image analysis could simply replace the subvisible particle measurements required by the pharmacopoeias without further modification of the current monographs, as for example the specifications in the monographs are based on the light obscuration and manual microscope methods.

Ultrasonic Resonator Technology (URT)

Ultrasonic Resonator Technology (URT) is an analytical measurement technique based on the physical characterization of liquids by ultrasound velocity and absorbance. URT has been used to investigate various biomolecules.[198] The principle of this method lies in the compression and decompression of the sample medium caused when ultrasonic waves pass through the sample. This effect leads to changes in the distance between the particles and molecules in the sample, which in turn evaluates intermolecular attractions and repulsions.[199] Therefore, it should be possible to analyze aggregation by the sound scattering properties of the dispersed particles.[198]

So far it was not shown that size and distribution of particles can be analyzed in actual samples of aggregated protein as those samples usually do not show a homogenous species. Calibration and cross-correlation to other established methods used for protein aggregation determination is also lacking. Furthermore, the method implies numerous assumptions and algorithms, therefore interpretation of the results need to be performed by experienced analysts.

Polarization Intensity Differential Scattering

Polarization Intensity Differential Scattering technology (PIDS™) is a method reported to be capable to characterize spherical and nonspherical particles within the submicron range of approximately 40 nm to 2.0 μm.[200] It is based on the Mie theory[201] that particles scatter and diffract light at certain angles based on their size, shape, and optical properties (refer to Determination of Turbidity (Opalescene, Clarity) Section and Light Scattering Section). This technology uses a tungsten-halogen lamp and three sets of vertically and horizontally polarized color filters at 450, 600, and 900 nm. The scattered light of the sample is measured over a range of angles and the particle's size distribution is determined by the difference between the horizontal and vertical scattered light at each wavelength.[200] The presence of very large particles is reported not to interfere with the measurement since large particle equally scatter light at vertical and horizontal polarizations[200] thus eliminating any filtration steps. The method also still lacks adequate cross-correlation to other, more established methods used for protein aggregation.

Extrinsic Fluorescence Using Dyes

Previously, the use of dyes to measure extrinsic fluorescence has also been reported as a means to analyze protein aggregation.[202] Environment sensitive fluorescent dyes such as Nile red, Congo red, Thioflavin T are considered being useful to detect aggregated proteins through microscopic or spectrophotometric methods.[43,203–206] The dyes Congo Red and Thioflavin T have been primarily used in amyloid detection.[205,207–209] Nile red as a fluorescence probe has been employed to study various protein aggregates or fibrils in, for example, IgG1 recombinant humanized monoclonal antibody,[203] β-galactosidase,[210] L-lactose dehydrogenase[211] and horseradish peroxidase[212] systems. Nile red is a LMW phenoxazone dye which binds to hydrophobic patches of a protein. Its fluorescence properties are greatly influenced by the environment's polarity for example the presence of hydrophobic unfolded protein structures strongly enhances its fluorescence.[203,213] The advantages of this method are that the Nile red dye is photostable, has a broad wavelength range, the quantum yield is high and has a stable fluorescence under pH conditions between 4.5 and 8.5.[203] Demeule et al.[203] stated that using Nile red and fluorescence microscopy permits the early detection of protein aggregate formation and that high-concentration protein formulations can be characterized without dilution and with negligible change to the protein's local environment through

fluorescence microscopy. A critical aspect of all methods implying extrinsic dyes is that the addition of the dye itself may induce artifacts by shifting equilibria between different conformational states of the protein.[214,215] Another limitation to this method is that surfactants may interfere with the analysis. Furthermore, these methods also usually lack cross-comparison with other methods used for protein aggregation and therefore results obtained only with those methods should be considered with care.

Transmission Electron Microscopy and Atomic Force Microscopy

Protein aggregates such as amyloid β-protein fibrils[216] and insulin fibrils[217] as well as the Fc and Fab regions of antibodies[218] have been characterized by Transmission Electron Microscopy (TEM). TEM is no quantitative method but may allow visualization of small aggregates. The basic principle of TEM and its applications is described by Ma et al.[219] Another technique that uses the microscope principle to study biomolecules is Atomic Force Microscopy (AFM).[220] AFM may be used to investigate size, structure, and distribution of aggregates and has been used, for example, to study the amyloid β-protein low-molecular mass oligomers,[221,222] insulin fibrils[223] and conjugated IgG aggregates.[224] When using imaging techniques for the visualization of protein aggregation, the sample preparation should be considered carefully, since it potentially could induce artifacts. Furthermore, image analysis is very labor-intensive and usually focuses only on a specific area of the imaged sample. Therefore the selected image area needs to be carefully considered to ensure a true representation of the entire sample being analyzed.

Other Methods Used for Further Characterization

Attempts are also made to further characterize and analyze aggregates on a structural level. Techniques used for the structural analysis of proteins[225] such as circular dichroism (CD), Fourier-transformed infrared spectroscopy (FT-IR), nuclear magnetic resonance spectroscopy (NMR) or intrinsic fluorescence might be considered. However, isolation and preparation of aggregated species can be considered very delicate as the sample preparation might alter the species to be analyzed. Furthermore, also data analysis (e.g., spectra comparison and overlays) should be considered with care. For example spectra overlays might be difficult to interpret due to differences in signal intensities and signal broadening of aggregated and isolated species. Additionally, the sensitivity of structural analysis methods such as FT-IR and CD can be considered as low. Therefore, adequate control experiments are suggested, adequate reference spectra should be used as well as using available information about the process, stability, etc., experiments and structural information when interpreting the data.

Further attempts are also made to identify particulates, such as in defective vials, to differentiate the particulates' origin as being extrinsic or intrinsic. One technique which is promoted to be used to identify particulates is Raman spectroscopy.[226] Other structural analysis methods such as FT-IR may also be considered. Challenges also lie in the sample preparation, isolation and stability of particles, as this might have an impact on the result. Isolation might be critical due to the fact that particulates are usually only present in trace amounts. When using spectra comparison to identify particulate, the quality of the database and specificity of results can have a significant impact on the interpretation of data. A thorough understanding of manufacturing process capabilities is also essential for data interpretation.

Thus, although attempts of characterization of aggregates and particle identification may be helpful in further understanding underlying mechanisms, technologies for doing so have limited capability and data should be interpreted with care and on a case-by-case basis. Available information about the process, formulation, composition, analytical capabilities and sample preparation should carefully be considered.

SUMMARY

A constant challenge in the development of biopharmaceutical products is the phenomenon generally known as protein aggregation. Aggregation can easily occur under a wide variety of conditions such as protein concentration, temperature, mechanical stress, etc., which could influence the aggregation pathway, rate, and state (size, structure). These conditions occur through protein production, including fermentation, purification, formulation, filling, shipment and storage. The control of aggregate formation is crucial since some protein aggregates are considered to potentially generate an immune

response which could have an adverse effect of the intended use of these proteins. There are limits and guidelines for visible and subvisible particles such as insoluble protein aggregates which are larger than or equal to 10 and 25 μm within the USP and Ph. Eur for pharmaceutical parenteral products whereas there are no defined acceptance criteria for soluble aggregates within regulatory documents. Therefore the limits for soluble aggregates have to be set case-by-case. However, considering the vast conditions in which a protein could potentially aggregate and the range of analytical detection methods available, it appears not likely that any biotech product will be 100% free of any detectable protein aggregates.

In order to control protein aggregation, it is important to understand the origin of aggregate formation, and to apply appropriate analytical tools. Protein aggregates is a summary term for all types of multimers, independent from (a) type of bond (covalent or noncovalent), (b) reversibility, (c) size (dimers, trimers and other soluble aggregates, insoluble aggregates, precipitation) and (d) protein conformation. Due to the variety of aggregated species, a major challenge for the analysis of protein aggregates is that no single analytical method exists to cover the entire size range or type in which aggregates may appear. A battery of methods is currently available for the determination of protein aggregate size and shape as well as size distribution. Such methods include SEC, SDS–PAGE, light scattering (SLS, DLS, MALLS), AUC, FFF, methods for subvisible and visible particles. Protein aggregates appear in a vast range of sizes therefore the use of a combination of methods which focus on the different aggregate sizes is recommended. However, there are challenges in collating the results from each method to obtain the overall size estimation and distribution for a given sample which is due to the inherent differences in what is being measured. The requirements for data modeling may result in inconsistencies between the techniques in the reported mean size and size distributions. The data output from different methods also suffers from diversity as results are reported as numbers, weight and/or Z-average, etc. which make comparison very difficult.

Additionally, analytical methods used for assessing protein aggregates need to be closely looked at with regard to their performance and limitations. Detection limits as well as the possibility of creating artifacts, that is, the induction or loss or dissociation of aggregates during sample preparation (dilution or concentration), analysis or calculation need to be kept in mind. New analytical tools suggested to be used for protein aggregation analysis still lack comparison to more established methods and, depending on the technique itself, might be critical due to sample preparation, analysis algorithms or other data analysis processes.

As all methods discussed have their own advantages and disadvantages, there is no "gold standard method" for the analysis of protein aggregates in its complexity, though SEC is still considered the most widely used despite the limitations discussed. It is advisable to analyze samples, especially during development of a biotech drug, carefully using orthogonal methods, which cover different size ranges of aggregates such as SEC, light obscuration and visual control, in order to obtain a better insight and understanding on the characteristics of the aggregates. Knowledge about the limitations and performance of the analytical methods as well as the interpretation of the data is crucial for the understanding and preventing of protein aggregation.

ACKNOWLEDGMENTS

The authors would like to gratefully thank John Carpenter for his support and valuable discussions as well as Astrid Pappenberger, Robert Müller and Michael Adler for the review of the manuscript.

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