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Research paper

Induction and analysis of aggregates in a liquid IgG1-antibody formulation

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Abstract

The objective of this study was to compare different agitation stress methods (stirring in Reacti Vials™ versus horizontal shaking) in their effect on protein destabilization, to assess several analytical techniques (light obscuration, turbidimetric and light scattering analysis) for detection of aggregates of various sizes and to evaluate the protecting effect of polysorbate 80 on protein aggregation. A monoclonal IgG1 antibody was used as model protein.

Both mechanical stress methods can provoke aggregate formation. The method of stirring induces particles in the range of 10–25 μm comparable to shaking stress. However, stirred samples show a much higher absorbance and reveal a second particle species in DLS analysis, suggesting that stirring stress induces a higher amount of smaller protein aggregates. Addition of polysorbate 80 protects the antibody against aggregation. Only in stirred samples a slight increase in sub-visible particles and turbidity was noted. However, a greater extent of aggregation products was detected by DLS as compared to surfactant-free formulations. Thus, polysorbate 80 appears to stabilise small aggregates and prevents further proceeding of the aggregation process. The induction of aggregates by stirring stress in Reacti Vials™ analysed by absorbance measurement seems to be a good combination for high-throughput formulation studies.

Keywords: Protein stability; Mechanical stress; Aggregation; Association; Light obscuration; Turbidity; Dynamic light scattering; Antibody

1. Introduction

Advances in biotechnology in the last years have made it possible to produce a number of proteins for pharmaceutical use. The size and complex structure of these molecules cause chemical and/or physical instabilities, which make stable aqueous pharmaceutical protein formulations a challenging task. Exogenous factors affecting protein stability during production, storage, shipping and handling are for example temperature (e.g. heat, freeze-thaw cycles), solution conditions (e.g. pH, addition of co-solvents, surfactants or stabilizers) as well as surface interactions (e.g. at hydrophobic surfaces or container surfaces) [1–4].

Physical instability reactions include protein denaturation, aggregation, precipitation and adsorption. Denatured protein (D) shows a failure of the protein tertiary and secondary structure and is often equated with protein instability. The transition from the native state (N) to a denatured state can either be a direct unfolding process or may pass through a series of partially or more extensively unfolded intermediate states leading to exposure of hydrophobic residues to the aqueous environment [2,4,5] (Fig. 1). Aggregate (Ag) formation, as the prevalent physical instability reaction in liquid protein formulations, is initiated by the intermolecular interaction of hydrophobic regions of at least two unfolded or partially folded, denatured protein molecules. Hydrophobic interaction is affected by temperature, ionic strength or shaking [2–4,6]. Aggregation of the denatured state is in competition refolding to the native structure. Proceeding of the aggregation process leads to clusters of aggregated proteins

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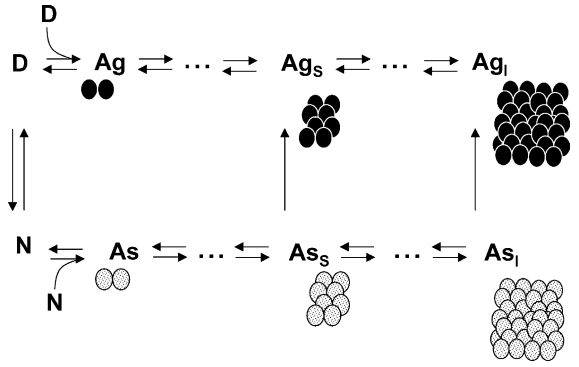

Fig. 1. Simplified model of proposed protein aggregation and association mechanism with black symbols representing denatured protein molecules and grey symbols representing native protein molecules.

and interaction with further denatured protein molecules, leading to the formation of soluble particles (Ag_S) and finally of insoluble, macroscopic aggregates (Ag_I), also called precipitates via different possible intermediate aggregation states [1–3]. The transition between soluble and insoluble aggregates is usually smooth and differentiation is arbitrarily made based on the methods for visible, sub-visible particles detection or on aggregate size (see below). In contrast, the intermolecular interaction of native protein molecules (N) is termed protein association (As) [2] and precipitated protein associates can be re-dissolved and yield native protein molecules again [7]. Adsorption of proteins to hydrophobic surfaces and air water interfaces involves the accumulation of denatured molecules at those surfaces fostering aggregation processes [2–4].

Mechanical stress generated by pumping, filtration, mixing, fill-finish processes, shipping or shaking may cause denaturation and consecutively aggregation due to exposure of the protein to air-water interfaces, material surfaces and shear forces [1–3]. Protein aggregation as a result of shaking was reported as long ago as 1904 [8]. As a consequence of aggregate formation bioactivity may be reduced or completely lost, immunogenicity could be enhanced and the formulation could be unacceptable based on pharmacopoeial requirements for parenterals with respect to appearance or particulates [1–3,9]. Thus, in case of recombinant proteins the formulation scientist has to consider aggregate formation. To test the stability and robustness of industrial pharmaceutical protein formulations and to facilitate the screening process for appropriate stabilizing excipients, especially in early development, accelerated thermal or mechanical stress tests are performed. Methodologies to force aggregate induction by mechanical stress vary widely. Especially, shaking [10,11], rotation [12], shearing by concentric cylinder-based shear systems [13,14] and vortexing [15–17] were tested. In this study, stirring as a method to induce mechanical stress to a protein was performed and compared to the extent of aggregation achieved by shaking.

Various techniques have been used to identify and quantify soluble and insoluble aggregates, including polyacrylamide gel electrophoresis (PAGE), size exclusion HPLC (SE-HPLC), asymmetrical flow field-flow-fractionation, IR spectroscopy, UV spectroscopy (turbidimetric or nephelometric method), dynamic light scattering analysis, light blockage test, analytical centrifugation and visual inspection [4,10–12,15,18–26]. However, the analytical methods mentioned may only detect soluble or insoluble protein aggregates up to a certain limit of detection, e.g. SE-HPLC might only detect soluble protein aggregates up to a low oligomer state and light blockage particle counter allows exact count of individual particles by size in different size ranges from approximately 1–800 μm. A single analytical method is generally not sufficient to evaluate protein aggregation, and a combination of different techniques, which can detect aggregates of various sizes should be chosen.

If aggregates or precipitates are detected during formulation development of a protein pharmaceutical, e.g. following mechanical stress or following freeze-thaw, various excipients have been found to be effective as stabilizers such as low concentrations of non-ionic surfactants, e.g. polysorbate 80 or 20 (for a review of surfactants in protein formulations, please refer to Jones et al. [27]). Polysorbate 80 was successfully used to prevent aggregation of proteins such as human growth hormone during vortexing [17] as well as lactate dehydrogenase [28] recombinant hemoglobin [29] and others [30] during freeze-thaw denaturation. Poysorbate 20 could successfully suppress aggregation of porcine growth hormone [15], recombinant human growth hormone [31] and recombinant human Factor XIII [32] upon interfacial (agitation) stress. Different ionic and nonionic surfactants (lysophosphatidylcholine (0.02%), SDS (1%), Brij 35 (0.1%) or Triton X (0.01%)) retained the formation of turbid gels of insulin containing formulations [33]. Various hypotheses exist for the stabilization of protein against aggregation by surfactants [34]. The surfactant molecules prevent the protein from reaching the air–liquid or liquid–surface interfaces and thus protect against denaturation and aggregation [30]. In addition, the surfactant in a concentration above the critical micell concentration might specifically interact with hydrophobic sites of the protein surface, where protein aggregation could potentially originate. Furthermore, chaperon-like action of surfactants, aiding refolding of proteins, is discussed [31,34–36]. If the surfactant preferentially binds to the native state as compared to the denatured state, the free energy of the native state will be decreased relative to the free energy of the denatured state. Consequently, the native state is stabilized thermodynamically by the surfactant [4,34,37].

The first objective of this study was to compare two mechanical stress methods, a stirring device and horizontal shaking, with respect to their effect on protein destabilization. A suitable method for the induction of mechanical stress for early formulation screening has to be

reproducible, quick and should only require small protein quantities, in order to allow high-throughput-testing of different protein formulations. The second objective was to understand how mechanical stress affects the model protein used, a monoclonal IgG1 antibody. Therefore, light obscuration, turbidimetric and light scattering analysis were used as analytical tools to follow the time course of aggregate formation. Additionally, the protecting effect of polysorbate 80 on the aggregation of the monoclonal antibody was studied.

2. Materials and methods

2.1. Materials

A chimeric mouse/human monoclonal antibody of the IgG1 subclass was used as a model protein for aggregation studies. The protein drug substance is manufactured by cell culture of a recombinant cell line and purified by a series of chromatographic steps, concentration and buffer exchange and final filtration using a 0.2 μm filter. The antibody shows a strong tendency for aggregation and precipitation and was therefore used in our studies. For aggregation studies, the protein solution was formulated (a) at 2 mg/mL antibody in phosphate buffered saline (PBS, pH 7.2) or (b) at 2 mg/mL antibody in phosphate buffered saline containing 0.01% (w/v) polysorbate 80 (Merck KGaA, Darmstadt, Germany) (PBS/T, pH 7.2). Polysorbate 80 was used above the reported critical micell concentration of 0.0013% [38]. PBS was prepared in water for injection (WFI) and its pH was adjusted to pH 7.2 with sodium hydroxide and hydrogen chloride solution. After filtration using a 0.2 μm PES bottle-top filter (Nalge Nunc International, New York, USA), 4.0 mL of the IgG1 solution was filled into cleaned and sterilised Fiolax 6 mL injection vials (Münnerstädter Glaswarenfabrik, GmbH, Münnerstadt, Germany) and sealed by Teflon-faced injection vial stoppers (West Pharmaceuticals Services, Eschweiler, Germany) or 2.0 mL was filled into cleaned and sterilised Reacti Vials™ (Pierce Biotechnology, Inc., Rockford).

2.2. Mechanical stress conditions

2.2.1. Shaking stress

Shaking stress was generated by horizontal movement of a shaking plate (HS 501 digital, IKA-Werke GmbH and Co. KG, Stauffen, Germany), creating motion and renewal in the air-water interface. 6 mL vials containing 4 mL IgG1-solution each were horizontally placed on a shaker platform and agitated at 150 apm (amplitudes per minute) at controlled ambient temperature. After 1, 2.5, 5, 24 and 48 h, three vials per time point were removed and analysed for protein aggregates. Three unstressed vials were analysed as control.

2.2.2. Stirring stress

Stirring was performed using small reaction vials (Reacti Vial™, Pierce Biotechnology, Inc., Rockford), causing air-water-interfaces upon movement. Reacti Vial™ small reaction vials contain a small internal cone and can be closed by open-top screw caps and Teflon/Rubber laminated discs. Solutions filled in the Reacti Vial™ small reaction vials can be agitated by Teflon coated stirring bars of triangular shape, which fit the cone portion. 3 mL Reacti Vials™ filled with 2 mL of IgG1-solution were agitated on a magnetic stirrer (Electronicrührer Multipoint HP, VARIOMAG-USA, Daytona Beach, USA) at 600 rpm (rounds per minute) at controlled ambient temperature. After 1, 2.5, 5, 24 and 48 h, three vials per time point were removed and analysed for protein aggregation. Three unstressed vials were analysed as control.

2.3. Analytical methods

2.3.1. Light obscuration

The method of light obscuration or light blockage is described in the European Pharmacopoeia method 2.9.19 and the United States Pharmacopoeia method ⟨788⟩ [39,40]. Analysis is based on the reduction of light intensity due to reflection, absorption and scattering, if a particle crosses an incident laser beam. As the decrease in light intensity is proportional to the size of particles, particle size distributions assuming spherical particles can be generated [20,41]. The light obscuration particle counter (SVSS-C, PAMAS Partikelmess- und Analysesysteme, GmbH, Rutesheim, Germany) and associated software (PMA Program, PAMAS Partikelmess- und Analysesysteme, GmbH, Rutesheim, Germany) were used for size distribution analysis in aqueous protein solutions. The instrument contains a laser diode and a photodiode detector to determine the residual light intensity of a laser beam, after particles have passed the sensor-zone. In this study, three measurements of a volume of 0.2 mL for each sample were analysed with a pre-run volume of 0.2 mL. Results of the particle counts were calculated as mean value of the latter three measurements and referred to a sample volume of 1.0 mL. Between the measurements the equipment was cleaned with sterile filtered WFI until particle counts of less than 100 particles/mL bigger than or equal to 1 μm and less than two particles/mL bigger than or equal to 10 μm were reached. These limits are more stringent than the acceptance criteria applied by the environmental test described in the European and United States Pharmacopoeia. Fill rate, emptying rate and rinse rate were fixed at 10 mL/min. For each time point (1, 2.5, 5, 24 and 48 h) the average results of three vials analysed are presented.

2.3.2. Turbidity (opalescence)—absorbance at 350 or 550 nm

Clarity or turbidity (opalescence) measurements are based on the fact that incident beams are attenuated due to

light scattering. The presence of uniformly suspended particles like insoluble protein aggregates and precipitates leads to an apparent increase in UV absorbance at all wavelengths due to scattering effects [22,23]. Therefore, turbidity was measured as photometric absorbance at 350 and 550 nm, where none of the known intrinsic chromophores in the protein formulation absorb, with a UV-Vis spectrophotometer (Cary 50 Conc, Varian, Inc.; Palo Alto, USA) against WFI as blank value. For each time point (1, 2.5, 5, 24 and 48 h) the mean value of absorbance of three vials analysed was calculated.

2.3.3. Turbidity (opalescence)—visual comparison using reference suspensions

The method for evaluating the degree of opalescence by visually comparing liquid formulations with reference suspensions is described in the European Pharmacopoeia method 2.2.1 [42]. This technique allows for a distinction of four different degrees of opalescence (clear, slightly opalescent, opalescent and very opalescent). The colloidal suspensions were prepared according to the European Pharmacopoeia by dilution of the opalescence standard (containing 0.075 mg hydrazine sulphate and 0.75 mg hexamethylentetramine per mL) with WFI to the reference suspensions I–IV (Table 1). Using cold light and a black and white background, the opalescence of the protein solution at the different time points was compared visually by trained operators to the freshly prepared reference standards of defined opalescense intensity. The colloidal suspensions were filled in the same test tubes (6 mL injection vials respectively Reacti Vial™ small reaction vials) as the sample solutions. For the immunoglobulin formulation without polysorbate 80 the average results of three vials analysed are presented for each time point (0, 5, 24, and 48 h). Additionally, the opalescence of each reference suspension was measured in triplicate via absorbance at 350 and 550 nm to enable comparison of the turbidimetric methods, both instrumental and visual.

2.3.4. Dynamic light scattering

DLS analysis was performed with the DynaPro99 system (ProteinSolutions, Ltd., Bucks, UK) in a temperature controlled rectangular quartz cuvette (Temperature Controlled MicroSampler MSTC, ProteinSolutions, Ltd., Bucks, UK) with a semi-conductor laser of approximately 830 nm wavelengths at a 90°-scattering angle. The method is based on measurement of the total scattered light intensity, which is proportional to protein concentration and molecular size of the scattering particles [20,25]. The time scale of the scattered light intensity fluctuations was analysed by autocorrelation using the software DynaLS and DYNAMICS 5.24.02 (ProteinSolutions, Ltd., Bucks, UK). Prior to each measurement, samples were filtered using a 0.45 μm Millex SV syringe filter (Millipore, Billerica, USA) to avoid high scattering increments of large particles and subsequently imprecise results. The quartz cuvette was incubated for 5 min at 25 °C before analysis. For each measurement, the fluctuation of the scattering intensity was recorded 15 times over a time interval of 5 s to determine the intensity autocorrelation function. In this aggregation study, data are interpreted assuming a polymodal distribution. The hydrodynamic radius and relative light intensity of the detected peaks are presented as mean values of three vials analysed for each time point (0, 5, 24 and 48 h).

Table 1
preparation of reference suspensions according to Ph. Eur. 2.2.1

Reference suspension	Component [mL]		Degree of opalescence
	Standard of opalescence	WFI	
I	5.0	95.0	clear (≤ Ref I)
II	10.0	90.0	Slightly opalescent (≤ Ref II)
III	30.0	70.0	opalescent (≤ Ref III)
IV	50.0	30.0	very opalescent (≤ Ref IV)

3. Results

3.1. Light obscuration

Light obscuration analysis assesses the size of individual sub-visible particles and provides a particle size distribution. Because interpretation of light obscuration analysis in the European as well as in the United States Pharmacopoeia [39,40] is restricted to particles bigger than or equal to 10 μm or 25 μm respectively, data were edited accordingly (Fig. 2(a) and (b)). IgG1 formulations without polysorbate 80 show an increase in the number of particles per mL bigger than or equal to 10 μm and 25 μm, respectively with increased mechanical stress time (r^2 values for linear fit: 0.9747 for particles ≥10 μm and 0.8679 for particles ≥25 μm in protein solutions agitated by stirring; 0.9923 for particles ≥10 μm and 0.9946 for particles ≥25 μm in protein solutions agitated by shaking). Both, stirring in reaction vials at 600 rpm and horizontal shaking at 150 apm cause the formation of immunoglobulin aggregates to a similar extent. The total number of particles bigger or equal to 10 and 25 μm, respectively measured after 48 h stirring (15,190±9,600 particles ≥10 μm and 140±90 particles ≥25 μm per mL) exceeds the amount of particles of the same size determined in shaking stressed samples after 48 h (9,160±2,400 particles ≥10 μm and 110±80 particles ≥25 μm per mL), but the difference is statistically insignificant.

In formulations of IgG1 containing polysorbate 80, a slight increase in the number of particles ≥10 μm and ≥25 μm was detectable for protein solutions stirred in Reacti Vial™ reaction vials (330±150 particles ≥10 μm and 11±15 particles ≥25 μm per mL after 48 h), whereas shaking for 48 h did not induce formation of large aggregates (7±3 particles ≥10 μm and 1±1 particles

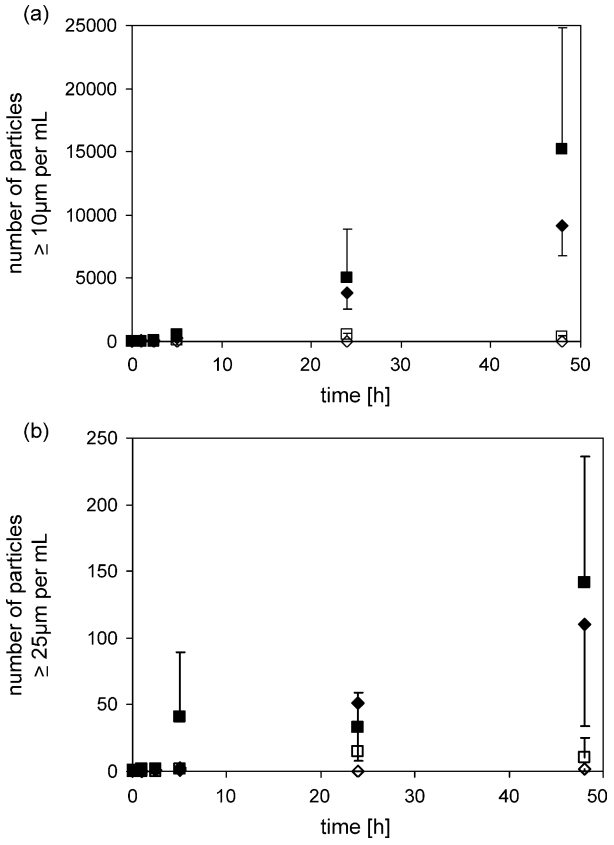

Fig. 2. (a) Number of particles ≥10 μm per mL: 2 mg/ml antibody in PBS, stressed by stirring (■) or shaking (♦); 2 mg/mL antibody in PBS + 0.01% polysorbate 80, stressed by stirring (□) or shaking (◇); for reasons of clarity error bars are only shown in one direction. (b) Number of particles ≥ 25 μm per mL: 2 mg/ml antibody in PBS, stressed by stirring (■) or shaking (♦); 2 mg/mL antibody in PBS +0.01% polysorbate 80, stressed by stirring (□) or shaking (◇); for reasons of clarity error bars are only shown in one direction.

≥25 μm per mL). In general, protein solutions with polysorbate 80 contained significantly less particles than surfactant-free samples after shaking or stirring for 48 h.

3.2. Turbidity (opalescence)—absorbance at 350 and 550 nm

Fig. 3(a) and (b) show the absorbance of the immunoglobulin at 350 or 550 nm, respectively as a function of shaking or stirring time. Corresponding to the results of light obscuration analysis the turbidity of protein formulations without surfactant increases linearly up to 24 h of agitation stress (r^2 values for linear fit: 0.9969 for A350 and 0.9964 for A550 of protein solutions agitated by stirring; 0.9964 for A350 and 0.9933 for A550 of protein solutions agitated by shaking). After this time period turbidity approaches a maximum level. The absorbance of stressed samples after 48 h is significantly higher (A350: 2.124±0.072; A550: 1.535±0.180) when stirred in reaction vials at 600 rpm than after application of mechanical stress using the shaking plate at 150 apm (A350: 0.410±0.051; A550: 0.271±0.037).

Turbidity of protein solutions with polysorbate 80 after stressing for 48 h is strongly reduced as compared to samples without surfactant. For polysorbate 80-containing antibody formulations a slight increase in absorbance at 350 and 550 nm was detectable in stirred samples after 48 h (A350: 0.219±0.124; A550: 0.114±0.066). Shaken samples with polysorbate 80 did not show an increase in absorbance over 48 h (A350: 0.008±0.001; A550: 0.002±0.001).

3.3. Turbidity (opalescence)—visual comparison using reference suspensions

Table 2 shows the absorbance units of the reference suspension and the corresponding categories of opalescence. The data of the visual evaluation of the degree of opalescence in the stressed protein solutions without polysorbate 80 and the corresponding results of the instrumental method (absorbance at 350 and 550 nm) are shown in Table 3. Both methods show similar results with respect to the classification of the degree of opalescence.

3.4. Dynamic light scattering

Since laser diffraction is an overall property of the entire solution and not of individual particles, prediction of the number of particles of a certain size is not possible. The hydrodynamic radius and the relative light intensity of all peaks are listed in Table 4, limited to a maximum hydrodynamic radius of 450 nm due to the 0.45 μm filtration step preceding the measurement. Additionally, graphic displays of the peak distribution of the initial protein solutions and after mechanical agitation over 48 h are presented (Fig. 4(a) and (b)).

The main peak of the immunoglobulin formulations before stressing as well as after applying mechanical stress using reaction vials or shaking plate is calculated as about 5.5–6.1 nm. In protein formulations without surfactant a second species with an average hydrodynamic radius of about 110–120 nm was detected after applying at least 5 h of stirring stress (600 rpm), whereas the DLS results of the solutions shaken for even 48 h did not show a second

Table 2
absorbance of reference suspensions at 350 and 550 nm

Reference suspension	Absorbance at		Degree of opalescence
	350 nm	550 nm	
I	0.017±0.002	0.007±0.001	Clear (≤Ref I)
II	0.032±0.003	0.014±0.002	Slightly opalescent (≤Ref II)
III	0.085±0.001	0.035±0.001	Opalescent (≤Ref III)
IV	0.144±0.005	0.059±0.003	Very opalescent (≤Ref IV)

Table 3
Results of turbidity analysis and comparison with reference suspensions

	Stress time [h]	Absorbance at		Degree of opalescence	
		350 nm	550 nm	By absorbance	By visual inspection
Initial value	0	0.011 ± 0.003	0.004 ± 0.002	≤Ref I	≤Ref I
Shaking stress (150 apm), without surfactant	5	0.049 ± 0.022	0.031 ± 0.014	≤Ref III	≤Ref III
	24	0.334 ± 0.148	0.222 ± 0.091	≥Ref IV	≥Ref IV
	48	0.410 ± 0.051	0.271 ± 0.037	≥Ref IV	≥Ref IV
Stirring stress (600 rpm), without surfactant	24	1.762 ± 0.350	1.215 ± 0.279	≥Ref IV	≥Ref IV
	48	2.124 ± 0.072	1.535 ± 0.180	≥Ref IV	≥Ref IV

population. The occurrence of the second peak (relative light intensity: 0.48 ± 0.47 after 48 h of stirring) goes along with a decrease in the relative light intensity of the main peak (0.52 ± 0.06 after 48 h of stirring). The larger particle species did not uniformly appear in each of the measurements of the three stirred samples for the several time points resulting in a high standard deviation of the hydrodynamic radius (109.3 ± 94.7 nm after 48 h of stirring, only two of three samples contain a second population at 164.0 nm).

In contrast, in polysorbate 80-containing samples a second particle species could be detected after stirring as well as after shaking. Shaking induced the formation of the second species more inconsistently and to a lower extent. Thus, DLS results of immunoglobulin solutions stirred for 48 h show a lower standard deviation of the hydrodynamic radii of the three vials analysed and a higher relative light intensity of the second peak (Rh: 136.0 ± 24.2 nm; relative light intensity: 0.58 ± 0.20, two samples contain second population at 122.0 nm, one at 164.0 nm), as compared to those samples shaken at 150 apm for 48 h (Rh: 81.3 ± 70.4 nm; relative light intensity: 0.22 ± 0.21, only two of three samples contain a second population at 122.0 nm). Overall, formation of the second species in protein solutions by agitation stress was more pronounced in the presence of polysorbate 80 than in surfactant-free formulations.

4. Discussion

Based on to the results obtained by light obscuration, turbidity and dynamic light scattering analysis, mechanical stress induced by horizontal shaking on a shaking plate (150 apm) or using Reacti Vial™ small reaction vials (600 rpm) is suitable for forced generation of protein aggregates in liquid formulations. Both methods only require a small sample volume of 2–4 mL solution.

Polysorbate 80-free samples stressed by stirring show slightly higher, but not statistically significantly different values of particles measured by light obscuration (Fig. 2(a) and (b)) than surfactant-free samples after shaking. Moreover, significantly higher turbidity levels at 350 or 550 nm (Fig. 3(a) and (b)) were obtained. Furthermore, only under stirring stress conditions, particle species with an average hydrodynamic radius of approximately 110–120 nm could be observed in protein solutions without surfactant using DLS analysis (Table 4 and Fig. 4(a)).

Table 4
hydrodynamic radius [nm] and relative intensity of peaks up to 450 nm (polymodal analysis) of IgG1 formulation after shaking and stirring stress

	Stress time [h]	First peak		Second peak	
		Rh [nm]	Relative intensity	Rh [nm]	Relative intensity
Shaking stress (150 apm), without surfactant	0	5.5 ± 0.9	1.00 ± 0.07	–	–
	5	6.1 ± 0.0	1.00 ± 0.07	–	–
	24	6.1 ± 0.0	1.00 ± 0.13	–	–
	48	6.1 ± 0.0	1.00 ± 0.05	–	–
Stirring stress (600 rpm), without surfactant	0	5.5 ± 0.9	1.00 ± 0.07	–	–
	5	6.1 ± 0.0	0.95 ± 0.09	122.0 ± 0.0	0.05 ± 0.00
	24	6.1 ± 0.0	0.62 ± 0.07	109.3 ± 94.7	0.37 ± 0.35
	48	6.1 ± 0.0	0.52 ± 0.06	109.3 ± 94.7	0.48 ± 0.47
Shaking stress (150 apm), with surfactant	0	6.1 ± 0.0	1.00 ± 0.06	–	–
	5	6.1 ± 0.0	0.99 ± 0.06	30.1 ± 52.1	0.01 ± 0.02
	24	6.1 ± 0.0	0.88 ± 0.09	87.2 ± 36.3	0.12 ± 0.11
	48	6.1 ± 0.0	0.78 ± 0.09	81.3 ± 70.4	0.22 ± 0.21
Stirring stress (600 rpm), with surfactant	0	6.1 ± 0.0	1.00 ± 0.06	–	–
	5	6.1 ± 0.0	0.47 ± 0.03	122.0 ± 0.0	0.53 ± 0.04
	24	6.1 ± 0.0	0.30 ± 0.05	139.4 ± 0.0	0.69 ± 0.52
	48	5.5 ± 0.9	0.42 ± 0.02	136.0 ± 24.2	0.58 ± 0.20

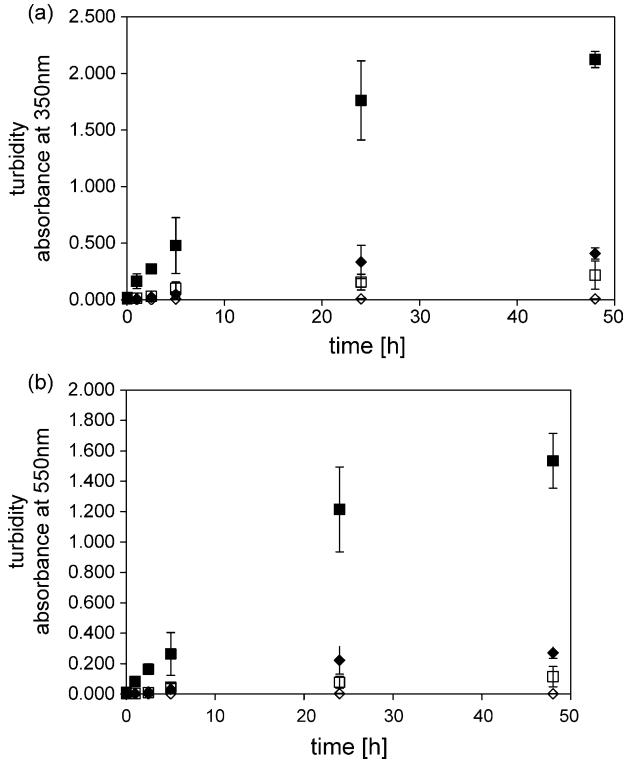

Fig. 3. (a) Absorbance at 350 nm: 2 mg/ml antibody in PBS, stressed by stirring (■) or shaking (♦); 2 mg/mL antibody in PBS +0.01% polysorbate 80, stressed by stirring (□) or shaking (◇). (b) Absorbance at 550 nm: 2 mg/ml antibody in PBS, stressed by stirring (■) or shaking (♦); 2 mg/mL antibody in PBS +0.01% polysorbate 80, stressed by stirring (□) or shaking (◇).

It was shown in previous studies, that aggregation of proteins appears in the presence of both agitation stress and hydrophobic surfaces like gas interfaces [13,11]. The formation of protein aggregates or associates and subsequently of protein precipitates as a result of agitation stress is induced by adsorption and possibly subsequent loss of conformation at the enlarged air–water-interfaces as well as by transportation of denaturated, aggregated or native, associated protein, respectively into solution due to shear forces [10,13,15,16,43]. Stirring and the special construction of the Reacti Vial™ small reaction vials with its internal cone and exactly fitted triangular shaped magnetic stirrer could possibly create air–water-interfaces and shear forces to a greater extent than horizontal shaking. However, the influence of different frequencies of both mechanical stressing methods has to be investigated in further studies.

The light obscuration particle counter can be used for detection of aggregates and precipitates in the micron-range. Data are presented as particle size distributions providing information about the number of particles. At higher particle burden, artificial results could occur due to exceeding of the coincidence level of the instrument e.g. by measuring less but larger particles instead of many small particles. Dilution of samples can reduce the probability of simultaneous entry into the incident laser beam, but it can also cause unwanted dissolution of insoluble protein aggregates [20]. The high standard deviation of this method could be due to the measurement conditions used in this study, i.e. the small sample volume of 0.2 mL. An increase in measurement volume could yield results with a lower standard deviation. However, the advantage of using only 0.2 mL per measurement is of course the material saving.

The results clearly show, that sub-visible particles in the size of ≥10 and ≥25 μm are generated by both shaking and stirring stress in the surfactant-free formulation and numbers of particles increase almost linearly with stress time (Fig. 2(a) and (b)). The number of particles ≥10 μm induced by stirring stress is higher than the number of particles induced by shaking stress, although not statistically significant.

Polysorbate 80 completely prevented the induction of sub-visible particles ≥10 μm and ≥25 μm by horizontal shaking. However, in stirred samples containing polysorbate 80, a slight increase in sub-visible particles ≥10 μm and ≥25 μm could still be measured (Fig. 2(a) and (b)). This suggests, that either the concentration of polysorbate 80 in the formulation was not capable in stabilizing the

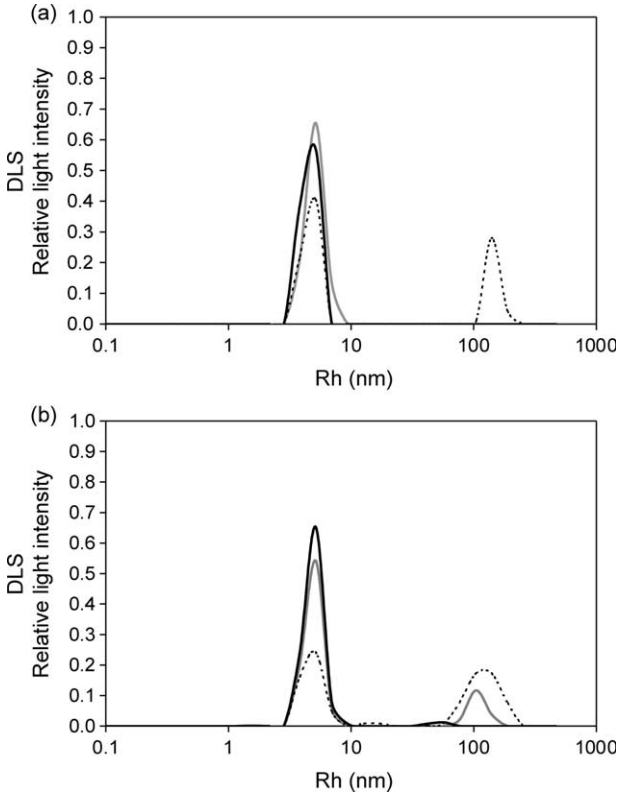

Fig. 4. (a) Dynamic light scattering analysis (relative light intensity of peaks up to 450 nm): 2 mg/ml antibody in PBS, initial value (black line), stressed for 48 h by stirring (dotted line) and for 48 h by shaking (grey line). (b) Dynamic light scattering analysis (relative light intensity of peaks up to 450 nm): 2 mg/ml antibody in PBS+0.01% polysorbate 80, initial value (black line), stressed for 48 h by stirring (dotted line) and for 48 h by shaking (grey line).

protein against the mechanical induction of aggregates via stirring, or that aggregation caused by stirring stress cannot be completely prevented by polysorbate 80.

Analysis of turbidity performed as measurements of absorbance at 350 and 550 nm seems to be adequate for the estimation of opalescence of IgG1-solutions. At both wavelengths, the same qualitative information can be gained. However, the wavelength of 350 nm is preferred over 550 nm since the sensitivity towards turbidity is higher at the lower wavelength. This fact can be explained by the Rayleigh relationship applicable for scatterers which are small in comparison to the wavelength, that expresses an inversely proportional of turbidity to the wavelength of incident light raised to a power n (value between 4 and 2-subjected to size, shape and nature of interface of the macromolecules) [24,44]. In contrast, Mie scatterers with a particle size in the dimension of the wavelength mainly contribute to the light scattering intensity but show only a diminished wavelength dependency.

In protein formulations without polysorbate 80 the absorbance at both 350 and 550 nm at first increases almost linearly followed by only a slight further increase between the 24 and 48 h time points (Fig. 3(a) and (b)). It can be concluded, that the increase in absorbance is due to the induction of strongly light scattering protein aggregates by shaking or stirring stress. Interestingly, the absorbance is increased more significantly when applying stirring stress to the liquid protein formulation.

When using polysorbate 80 as a stabilizer against protein aggregation and applying shaking stress, the absorbance at 350 and 550 nm does not change for 48 h, whereas an increased absorbance at 350 and 550 nm is still detectable in samples containing polysorbate 80 after applying of stirring stress (Fig. 3(a) and (b)). This could be probably due to the fact, that either the concentration of polysorbate 80 is not sufficient to stabilize or because the stress method of stirring is too rigorous to enable polysorbate 80 to exhibit its stabilizing effect.

The turbidity values do not completely correspond to the results obtained by sub-visible particle analysis, because over 24 h of stirring a strong increase in turbidity, but only a slight increase in the number of particles bigger than 10 μm or 25 μm could be detected. Additionally, the approximation to a maximum after 24 h of shaking or stirring stress could only be observed in turbidity analysis, whereas the number of sub-visible particles increases over the whole time period of agitation stress. In general, the light scattering properties of a particle depend on its size, its geometry as well as its refractive index. Large particles scatter long wavelengths of light more effectively than they scatter short wavelengths. Small particles scatter short wavelengths of light more effectively than large particles but have less effect on the scatter of longer wavelengths [45–47]. Due to the complex nature of the light scattering effects and the number of variables involved, absolute comparisons become difficult, but trends in the formation of particles can be estimated. Thus, at the wavelength of 350 or 550 nm for turbidity measurements medium-sized aggregates have a more relevant effect on turbidity than large-sized aggregates, which are detected by light obscuration analysis. The number of sub-visible particles of 1 and 2 μm—an information which is as well provided by the light obscuration instrument—correlates almost linearly with turbidity. Consecutively, the large increase in turbidity induced by stirring up to 24 h and the levelling-off of turbidity data after 24 h of shaking and stirring stress is not necessarily considered to be in correlation with the number of sub-visible particles $\geq$10 and 25 μm. Additionally, a certain kinetic in aggregate formation could be deduced from these observations, i.e. the beginning of aggregation process with the formation of medium-sized particles (as mainly detected by turbidity analysis), leading to the formation of larger precipitates (as detected by light obscuration analysis) after 24 h of mechanical stress.

Opalescence generated by shaking and stirring stress was additionally analysed by visual comparison to pharmacopoeial reference suspensions. The spectrophotometric determination of opalescence is more reproducible, accurate and objective than the visual comparison to the reference standards of the European Pharmacopoeia [23]. The benefit of the visual opalescence inspection is an easier measurement principle and no additional mandatory equipment like an UV-photometer. In general, neither the spectrophotometric nor the visual method provide information about particle form, particle size and size distribution [23].

The absorbance of Ph.Eur. opalescence reference standards at 350 or 550–nm correlates with increased opalescence (Table 2). The results of visual comparison to reference suspensions are in good accordance with the absorbance data at 350 and 550 nm (Table 3). However, the visual inspection differentiates lower levels of turbidity whereas absorbance measurements allow a distinction between different levels of aggregation at a much broader range and a finer scale. According to pharmacopoeial requirements for parenterals a solution can be defined as clear if the turbidity of standard level I is not exceeded. It can be concluded from our results, that the measurement of absorbance at a specified wavelength can be used alternatively to the Ph. Eur. method, provided, however, that the formulation does not contain any other compounds absorbing at that wavelength and a project-specific cross-validation against the Ph. Eur. method was successful.

Dynamic light scattering analysis can be applied to detect protein particles in the nanometer range at the beginning of the aggregation process [21]. The method provides the hydrodynamic radius of particles and particle size distributions, whereby the influence of particle size and concentration on the scattering intensity has to be considered [20,25]. Disadvantageously, the relationship between light scattering intensity and particle size is not straight proportional for aggregates in the dimension of the wavelength. Because of intra-aggregate interference, a decrease in light

scattering intensity occurs and the size of particles, which have been analysed by light scattering technique, is typically smaller than the actual size [19].

The main peak observed in all samples at 5.5–6.1 nm (Table 4) can be assigned to immunoglobulin monomer, as it corresponds to values provided by other authors (5.6 ± 0.2 nm for mouse-IgG1 respectively 5.2 ± 0.3 nm for rat-IgGa-anti mouse) [48]. A second population of approximately 20-fold larger hydrodynamic radius could only be found after stirring IgG1 formulations without surfactant. In contrast, although an increase in turbidity and sub-micron particles occurred, agitation stress by using shaking plates did not induce the formation of larger particles detectable by DLS even after 48 h (Fig. 4(a)). Assuming a spherical shape of the IgG1 monomers [26], the second particle species of about 110–120 nm could be composed of about 8,000 protein monomers. The mechanism of protein aggregation proceeds from small soluble aggregates to bigger insoluble precipitates via different intermediate states (Fig. 1) and the second species appears to be an intermediate in the aggregation process of the immunoglobulin tested.

The appearance of larger particles in protein solutions containing polysorbate 80 was detectable by DLS after applying of both, stirring and shaking stress (Fig. 4(b)). Furthermore, the relative light scattering intensity of the second population in stirred protein samples with polysorbate 80 was greater than in surfactant-free IgG1 formulations (Table 4). However, the formation of those aggregates does not comply with the results of turbidity and sub-visible particles analysis. Both, the absorbance at 350 and 550 nm and the number of sub-visible particles were significantly higher in surfactant-free protein solutions than in those samples containing polysorbate 80.

The different analytical methods applied show different results in their respective measurement range. DLS analysis is useable for detection of small-sized aggregates in the range of 1–450 nm. Turbidity measurements allow for analysis of medium-sized aggregates, but here no particle range can be given (see above). The light obscuration technique was used in this study to detect large aggregates of 1–800 μm. Due to filtration prior to DLS measurements particles detected by light obscuration are removed from the sample analyzed by DLS. Thus, only turbidity data and DLS can be compared and are complementary. Whereas large particles are formed by both stress methods in immunoglobulin formulations without polysorbate 80, the increase in turbidity as well as the occurrence of the second particle species in DLS results in stirred samples indicate that by this stress method also numerous medium-sized aggregates (turbidity) and small sized aggregates (DLS) are formed which cannot be detected in shaken samples. Additionally, in presence of polysorbate 80, medium (turbidity) and large (light obscuration) size particles cannot be detected but DLS indicates the presence of small aggregates, which is more pronounced in stirred samples as compared to shaken. Thus in accordance to literature [51,52], a stabilizing effect of polysorbate 80 on small immunoglobulin aggregates in the nanometer range which prevents the formation of larger aggregates could be suggested.

The variations with regard to the occurrence of the second particle species in all three samples analysed at each time point result from the insensitivity of the DLS method. Further disadvantages have to be mentioned, i.e. the necessity of sophisticated equipment, the limited measurement range, the absence of a pharmacopoeial monograph and the missing accordance of DLS results to the other performed analytics (instrumental turbidimetric and light obscuration method). Besides that, the over-estimation of larger particles in DLS analysis becomes obvious. Although the relative light intensity of the second population reaches levels up to 0.58 ± 0.20 (surfactant-free formulation, stirred for 48 h), the content of monomer remained almost unchanged ($>99\%$) in SE-HPLC analysis (data not shown). Because a small quantity of large particles can dominate the scattering intensity signal, a volume-weighted hydrodynamic radius distribution could be advantageous [49]. Invalid conclusions regarding stability of different IgG1 formulations would have been drawn if DLS results had solely been considered. In fact, laboratory results in a functional assay (ELISA) did not show any significant changes between untreated and stirred or shaken samples with or without polysorbate 80 (data not shown). Thus, turbidity as well as light obscuration analysis should be complemented by the DLS method, which is not suitable as a singular method for a high throughput testing of protein formulations.

Summing up, the mechanical stress methods horizontal shaking or stirring using Reacti Vials™ reaction vials can both be used to induce aggregates in liquid protein formulations. Both methods are suitable as screening methods for high-throughput formulation studies, because they are quick, reproducible and require only small volumes of protein material. Stressing time of 24–48 h is short in comparison to the requirements for thermal stability testing of the ICH guideline, where even the accelerated test design takes several months [50]. The data of the study, obtained for each time point in triplicate, show the reproducibility for both mechanical stress techniques and the used analytical methods. Also, the material requirements of each method are less than 4 mL and thus relatively small. When comparing both stress methods, the method of stirring in Reacti Vials™ reaction vials seems to induce particles in the range of 25 μm and 10 μm in a comparable amount to shaking stress. However, stirred samples without surfactant show a much higher absorbance than shaken samples and reveal a second particle species in DLS analysis, suggesting that stirring stress induces a higher amount of smaller protein aggregates.

With the addition of polysorbate 80, protein aggregation by shaking stress could be suppressed completely, however, a slight increase in the number of sub-visible particles

as well as in turbidity in polysorbate 80-containing antibody formulations was noted in stirred samples. This could be due to the fact, that either the concentration of polysorbate 80 is not sufficient to stabilize or because the stress method of stirring is too rigorous to enable polysorbate 80 to exhibit its full stabilizing effect. A greater extent of aggregation products was detected by DLS analytics in protein samples containing polysorbate 80 as compared to surfactant-free formulations. Therefore, a supposed mechanism for the described effect of polysorbate 80 could be the stabilization of small aggregates preventing the proceeding of aggregation process to bigger aggregates and precipitates. Corresponding results have already been reported by Kreilgaard et al. [32] for the stabilizing effect of polysorbate 20 on the agitation-induced aggregation of recombinant human Factor XIII. In accordance to this study, polysorbate 20 fosters the formation of soluble protein aggregates and prohibits growth of larger insoluble particles.

All analytical methods used in this study are suitable to detect the formation of aggregation products. However, a single technique will not be sufficient to describe the aggregation process completely, due to the different sizes of aggregated protein. The exclusive use of dynamic light scattering analysis may even result in invalid conclusions regarding protein stability. In order to analyse the amount and size of induced aggregation products, a combination of different, but complementary analytical techniques such as DLS and the light obscuration test can be used in order to assess the extent of aggregation and different size of protein aggregates.

The induction of immunoglobulin aggregates by stirring stress in Reacti Vials™ analysed by absorbance measurement at 350 nm seems to be a good combination for the induction and detection of light scattering aggregates. Advantages are the low substance requirement and sensitivity with regard to the induction of aggregation products of the Reacti Vials™. Additionally, a meaningful differentiation between various formulations by the A350 method is feasible. The combination provides a fast method to screen for stabilising agents and to distinguish between protein formulations with a minimum of substance requirement. However, further evaluation of other proteins and stirring/shaking frequencies should be taken into consideration.

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