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Research paper

Relevant shaking stress conditions for antibody preformulation development

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ABSTRACT

In protein formulation development, shaking stress is often employed to assess the physical stability of antibody formulations against aggregation. Since there are currently no guidelines describing suitable test conditions, very different shaking stress designs are used. These different designs may influence the resulting stability data. The aim of this study was to establish a shaking stress design within the protein range of 2–5 mg/ml which can rapidly distinguish between antibody formulations of poor stability and those with potential for further development. Small scale shaking stress experiments were performed with different monoclonal IgG antibodies (as buffered solutions or marketed formulations). Variables were the filling degree of the sample containers, the container type and size and the shaking intensity. The stability of the samples was assessed by visual inspection, UV–VIS spectrophotometric turbidity measurements and size exclusion chromatography. All tested parameters had a strong influence on the stability results. The most discriminating conditions were obtained when shaking of the formulations was performed at 200 rpm in a 2 ml injection vial filled with 1 ml protein solution. This experimental setup led to clearly different stability results for buffered solutions and marketed products. Moreover, this setup required only relatively small amounts of protein solution which is advantageous in prefomulation studies.

1. Introduction

In recent years, biopharmaceuticals have become increasingly important in pharmaceutical industry. Many new antibody-based pharmaceuticals have reached the market and even more are under way [1]. Antibodies have turned out to be very potent pharmaceuticals, for example, in therapeutic fields like cancer [2], immune-mediated diseases and transplant rejection, only to list some of them.

Developing antibodies is, however, not an easy task. Various difficulties may be encountered during the separation, purification, formulation, storage and administration of antibodies. These difficulties, which mainly involve physical and chemical instabilities, have been described in the literature many times [3–8].

In particular, aggregation [9,10] is a common problem, which may occur during the manufacturing process, storage and administration. Already in the year 1904, protein aggregation was detected as a result of shaking stress by Ramsden [11]. Shaking stress is a very convenient method to quickly screen and compare the robustness of antibodies and their formulations.

Aggregates can have a lower activity compared to the native protein or even be completely inactive. A decrease in activity was, e.g., observed after freeze thaw cycles [12]. Furthermore, some of them may induce an immune response [13].

Aggregates can form as a result of protein exposure to hydrophobic surfaces or air/water interfaces [8,14,15]. Since shaking or other types of agitation increase the frequency of surface exposure, there are numerous examples for the formation of aggregates caused by these processes [16–18]. Their formation is initiated by the intermolecular interaction of hydrophobic regions of two unfolded or partially folded, denatured protein molecules or two native protein molecules [8,9,14]. Aggregates can be formed irreversibly, or they can refold back to the native molecule. According to their size, small soluble (dimers and trimers) and insoluble, macroscopic aggregates, also called precipitates, can be distinguished [14]. Adequate analytical methods have to be employed to detect the aggregates of different sizes and types [10,19].

In spite of the high relevance that shaking stress studies have in the formulation development, there are hitherto no guidelines for such studies, such as for light stress (ICH Guideline) [20]. Consequently, very different stress conditions are currently used in the formulation development of proteins. In the present study, we were interested in identifying shaking stress conditions that, in a preformulation environment, allow to rapidly distinguish between formulations of poor stability against aggregation and those with high potential for further development. Plain, buffered antibody

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solutions were used as "unformulated" liquids, and formulations of marketed products served as reference with sufficient aggregation stability. Different antibodies were used in the study to detect potential substance-specific behavior.

In order to be discriminatory, the stress should be high enough to induce detectable instabilities in the unformulated products within a period of time which is convenient for screening (e.g., several hours or days). On the other hand, the stress must not be too harsh in order to avoid rapid destabilization also of the "stable" reference formulations.

In order to find such discriminatory stress conditions, different parameters in the shaking stress setup, namely, the container type and size, the filling degree and the shaking intensity were varied. Particular emphasis was placed on conditions that allowed the performance of the shaking stress experiment with small protein samples (small scale method) since the available amount of protein is often limited in early development stages. As the aggregates in protein formulations may be of very different sizes, various methods were employed for their detection. Small soluble aggregates (dimers) were detected with size exclusion chromatography, whereas visual inspection and turbidity measurements by UV–VIS spectroscopy served for the detection of bigger aggregates (precipitates). The use of light obscuration or micro flow imaging might be preferable over turbidity measurements to accurately assess the formation of larger aggregates when sufficient sample volumes are available. Turbidity measurements have been established as surrogate method [9,10,21], which is material saving and requires only small volumes.

2. Materials and methods

2.1. Materials

This study was performed with different monoclonal IgG antibodies as model proteins. The antibody formulations and the concentrations used are listed in Table 1. Antibody A and B were provided by Merck Serono (Germany), and antibody C was a marketed formulation by Hoffmann-La Roche (Switzerland).

2.2. Methods

2.2.1. Sample preparation

Marketed products were used as received. Buffered formulations (Table 1) were prepared in water for injection (WfI), pH was adjusted (with HCl or NaOH), and formulations were filtered using a 0.2 μm polyethersulfone filter. For shaking stress studies, different vials and closures (Table 2) were used. The 2 and 6 ml glass vials were especially fabricated for parenteral solutions. The vials were washed in a dishwasher (Miele Professional G7836CD; Miele & Cie. KG, Germany) with a "particle free" program, autoclaved, filled with the protein solutions and sealed under aseptic conditions.

The surface tension of the different solutions was measured with a ring tensiometer (Processor Tensiometer K12; Krüss GmbH, Germany) at 20 °C. For each solution, 50 measurements were carried out, and the average of the last 10 values is presented as the surface tension in Table 1.

The isoelectric point of the antibodies was measured by isoelectric focusing (IEF). Agarose IEF plates with a pH range of 3–10 (Lonza, Rockland, USA) were used on a cooling plate (Multiphor2, Amersham Bioscience, Sweden), and voltage (1000 V, 15 W, 25 mA) was provided by a power supply (Blue Power 3000, Serva electrophoresis GmbH, Germany). The plates were afterwards stained with Serva blue (Serva, Germany). An IEF marker (high range of 5–10.5, GE Healthcare, UK) was run in parallel.

2.2.2. Shaking conditions

Shaking was performed on a shaking platform (SM30; Edmund Bühler GmbH, Germany) with linear horizontal stroke and amplitude of 3 cm. The movement of the vials can be described by a sinus function along the principal axis.

The filled vials were placed horizontally onto the shaker platform and agitated. Shaking intensities of 150 rpm, 200 rpm and 250 rpm were used (the unit "rpm" corresponds to "shakes per minute" in our study). The temperature was adjusted to 23 °C ± 1 °C. At predefined time points (0, 5, 24, 48 and 120 h), three vials were removed from the shaker. They were analyzed immediately for protein stability. Three unstressed samples were analyzed as control. In addition, placebos of the formulations were stressed to rule out any effects of the solvent (containing, e.g., citrate, PBS, polysorbate 80) on the turbidity measurements.

2.2.3. Visual appearance

The vials with the stressed antibody solutions were visually evaluated directly after removal from the shaker. The stressed samples were qualitatively assessed concerning particles and clarity in comparison to unstressed samples. Cold light (KL 1500 electronic, Schott AG, Germany) was used to detect opalescence and small particles in the solution. For documentation, the samples were photographed against a black/white background.

2.2.4. Turbidity measurements

Turbidity measurements were carried out at 350 nm with a Cary 50 UV–VIS spectrophotometer (Varian Inc., USA) in quartz glass precision cells with a pathlength of 10 mm (105.201QS, Hellma GmbH & CoKG, Germany) against water for injection as blank [21]. The cells were cleaned and rinsed with water for injection and sample solution before each measurement. Samples were measured undiluted. Test measurements with formazine solutions of different turbidity indicated that absorption values up to approximately 2.5 are linear with turbidity. The values of the placebos measured against water were 0.001 OD for the PBS buffer at pH 7.2; 0.0019 OD for the PBS buffer with 0.01% Tween 80 at pH 6.9

Table 1
Antibody formulations.

Code	Antibody	Isoelectric point[a]	Formulation	Concentration (mg/ml)	Excipients	Surface tension (mN/m)[b]
A-PBS-2	A	8.3	Buffered solution	2	Phosphate-buffered saline (pH 7.2)	67
A-PBS-5	A	8.3	Buffered solution	5	Phosphate-buffered saline (pH 7.2)	60
A-mf-5	A	8.3	Marketed formulation	5	Citrate buffer; glycine; NaCl; polysorbate 80 (pH 5.5)	43
B-PBS-2	B	8.2	Buffered solution	2	Phosphate-buffered saline (pH 7.2)	61
B-PBS-5	B	8.2	Buffered solution	5	Phosphate-buffered saline (pH 7.2)	56
C-mf-5	C	8.4	Marketed formulation	5	Phosphate-buffered saline; polysorbate 80 (pH 6.9)	–

[a] Values were measured by IEF.
[b] Values were measured by ring tensiometer.

Table 2
Vial dimensions.

Vial	Company vial/ country	Diameter (mm)	Height (mm)	Overall volume (ml)	Stopper	Company stopper/ country
0.8 ml HPLC glass vials	VWR International GmbH; Germany	7.5	30	0.84	Silicone septum crimped with aluminium caps	VWR International GmbH, Germany
1.5 ml HPLC glass vials	VWR International GmbH; Germany	11	31.5	1.8	Polypropylene screw caps with silicone septum	VWR International GmbH, Germany
2 ml glass vials	Schott Rohrglas GmbH; Germany	16	35	3.9	Chlorobutyl rubber stoppers coated with silicone and crimped with aluminium caps	West Pharmaceutical Services, Germany
6 ml glass vials	Schott Rohrglas GmbH; Germany	22	40	9.4	Chlorobutyl rubber stoppers coated with silicone and crimped with aluminium caps	West Pharmaceutical Services, Germany

and 0.0018 OD for the citrate buffer with glycine and 0.01% Tween 80 at pH 5.5.

2.2.5. Size exclusion chromatography

Undiluted samples were centrifuged for 3 min at 800 rpm (4300 *g*) (Biofuge fresco; Heraeus Instruments GmbH; Germany) to spin down precipitates. Solution containing 100 μg antibody (referring to the nominal sample concentration) was injected per run. The analysis was performed at room temperature on a Merck–Hitachi HPLC with the components D7000 Interface, L7400 UV-Detector, L6000 A Pump, L6200 Intelligent Pump and L7250 programmable auto sampler. The mobile phase used on a TSK-Gel G3000SWXL column 7.8 mm ID × 30.0 cm L (Tosoh Bioscience GmbH; Germany) was 0.4 M $NaClO_4$, 0.05 M $NaPO_4$ buffer (pH 7.2) delivered at a rate of 0.5 ml/min. The column eluate was monitored at 280 nm. Using the HPLC Software EZChrom Elite Client, Version 3.0 (Scientific Software International Inc., USA), the total area under the curve for the antibody monomer was calculated and compared to the monomer content of the standard (antibody A-mf-5). Placebos of the antibody solutions were tested as well. In the chromatograms of the formulation A-mf-5, a citrate peak appeared which was excluded from the calculations. A gel filtration standard (Bio-Rad Laboratories Inc., USA) was injected regularly to assure the performance of the HPLC system.

2.2.6. Microscopy

For microscopic analysis, the undiluted samples were transferred to microscope slides and examined under the microscope (Olympus BX60, Olympus GmbH, Germany) with 100-fold magnification in bright field mode. The selected samples were photographed with an attached camera (Sanyo color CCD, Sanyo Electric Co., Ltd., Japan).

3. Results and discussion

The aim of the study was to systematically evaluate the effect of variations in the experimental setup on the results of shaking stress experiments in order to select discriminatory stress conditions. The parameters under investigation were filling degree, container type and size and shaking intensity.

3.1. Filling degree

The effect of the filling degree was studied on the formulation A-PBS-5 in 2 ml vials which were filled with 0.5, 0.7, 1, 2, or 3 ml of protein solution. The shaking intensity was adjusted to 200 rpm.

As expected, the samples became turbid during shaking due to the formation of large aggregates. The turbidity increased with increasing shaking time but to a very different extent (Fig. 1). Vials filled with half of the recommended volume (1 ml) exhibited the highest increase in turbidity, whereas higher or lower filling quantities resulted in smaller turbidity increases.

Corresponding phenomena were observed by SEC (Fig. 1). This method was primarily used to detect the decrease in monomer content and the increase in the amount of small aggregates (dimers, oligomers). Compared to the starting values, the 1 ml filling volume led to the highest decrease in monomer content over shaking time. It can be presumed that the protein that is lost from the monomer fraction has either formed aggregates or degradation products. There was, however, no significant increase in the SEC-detectable aggregates (dimers) or degradation products (as also for all other tested IgG solutions without Tween, data not shown). Therefore, larger aggregates that cannot be detected by SEC must have formed which is in agreement with the observed increase in turbidity (Fig. 1).

The use of different filling degrees leads to differences in stress intensity with the corresponding influences on protein stability. It

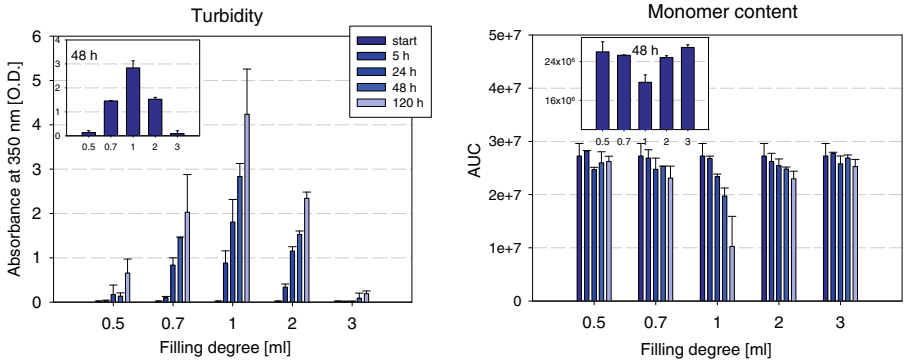

Fig. 1. Turbidity (absorption at 350 nm) (left) and SEC absolute monomer content (right) of antibody solution A-PBS-5 with different filling volume over shaking time (200 rpm); insets: results for the different filling volumes after 48 h of shaking. Please note in all corresponding figures that absorbance values above 2.5 are out of the linear range.

was previously reported that the avoidance of headspace (corresponding to a completely filled vial) resulted in higher protein stability during shaking due to the absence of an air/water interface. Smaller filling degrees (corresponding to approximately 40% and 90% of recommended filling volume) with large headspaces led to protein instability but without clear differences in between them [10]. In contrast, our experiments revealed differences in protein stability in dependence on filling degree resulting in different headspace volumes. Our results demonstrate that smaller filling degrees can lead to a similar reduction in stress intensity as larger ones, although they form a larger air–water interface during shaking. Potentially, with smaller filling volumes, the mobile volume of liquid is too small to produce a sufficiently turbulent flow inside the vial. Also the observed higher protein stability of highly filled vials with small headspaces might result from a slow and/or non-turbulent flow of the liquid. The higher protein stability in highly filled vials thus may not exclusively result from the small air–water interface as it was reported [10]. Stress intensity might rather depend on combined effects of shear and gas–liquid interfaces as reported by Maa et al. [22]. In contrast, shear as the only stress seems to have minor or no effects on the stability of proteins [18].

Since the stress on the protein samples was the highest in the vials filled with half of the recommended volume, all experiments reported in the following were conducted under these filling conditions.

3.2. Container types and sizes

The influence of different containers was tested with the buffered antibody solutions A-PBS-2 and B-PBS-2 at a shaking intensity of 250 rpm. The vials (nominal filling volume 0.8 ml, 1.5 ml, 2 ml, 6 ml) were each filled with half their recommended filling volume (0.4 ml, 0.75 ml, 1 ml, 3 ml).

During shaking, most solutions became turbid over time (Fig. 2). At all time points, the visual appearance was similar for the two different antibody solutions. Even though it is reported that different proteins behave differently [23], this was not observed in this experiment. Nonetheless there were differences between the vial types: for example, the 6 ml vial contained foam already after 5 h of shaking in contrast to the other containers under investigation. In the 2 ml vial, the turbidity increased at the beginning but decreased again after longer shaking times, and foam was observed on top of the respective solutions. The smaller vials, especially the 0.8 ml vial, exhibited large differences in visual appearance within the triplets (e.g., ranging from a clear solution to a very turbid liquid for B-PBS-2 after 48 h of shaking). This is presumably due to different types of movement of the enclosed liquid within a triplet, which was observed visually during shaking.

Turbidity measurements confirmed the results of visual inspection (Fig. 3). The 6 ml vial showed only weak turbidity. The turbidity in the 2 ml vial increased up to 24 h of shaking and thereafter decreased again. For the 0.8 ml vials, large standard deviations were observed.

Light microscopy was employed after 120 h of shaking to elucidate these observations in more detail. All solutions in the different containers contained microscopically detectable aggregates after this period of shaking as exemplified for the antibody solution A-PBS-2 in Fig. 2. However, the aggregates from the different containers were of different appearance. In the 6 ml vials, in which foam was visually observed, the aggregates were adsorbed to air bubbles which floated to the surface (Fig. 2). Aggregates of similar appear-

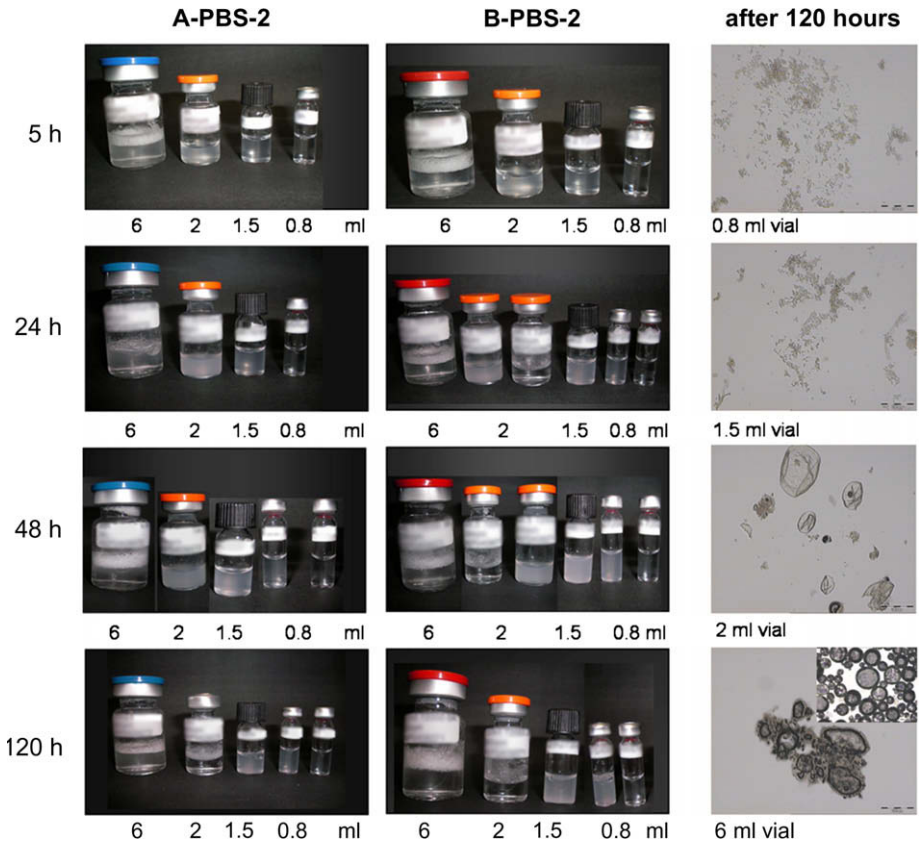

Fig. 2. Left: visual appearance of the two antibody solutions A-PBS-2 and B-PBS-2 in different containers after different times of shaking. For samples displaying large variations two photographs are shown. Right: light micrographs of the antibody solution A-PBS-2 after 120 h of shaking at 250 rpm in different containers (bar represents 100 μm); inset 6 ml vial: light micrograph of foam.

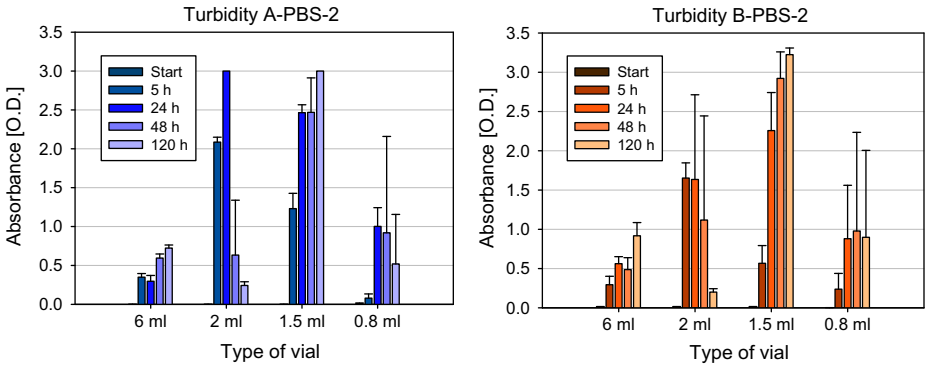

Fig. 3. Turbidity (350 nm) of the two buffered antibody solutions A-PBS-2 and B-PBS-2 in different vials.

ance were found upon microscopic examination of the foam. The solution became visually clear; consequently, only a low degree of turbidity could be detected in spectroscopic measurements (Fig. 3). The phenomenon that aggregates can preferably be located inside foam and, due to the resulting extraction from the liquid, cannot be detected in the solution any more is in line with previous findings [22].

According to microscopy, the liquid in the 2 ml vials exhibited very big precipitates after 120 h of shaking. The stress within these vials was obviously so harsh that all aggregates formed big precipitates after 24 h. This was reflected in visual appearance: The solutions cleared after 48 h, and differences within the triplets were observed (Figs. 2 and 3). The precipitates floated to the surface over time. Conversely, the solutions from the smaller vials (0.8 and 1.5 ml) showed typical smaller protein aggregates which explain the visual and spectroscopic turbidity of these samples.

Since the results from visual inspection and turbidity are difficult to compare among the different samples due to foam formation, the determination of monomer content by SEC was the most reliable indicator of stress intensity in this set of experiments. According to the SEC results, shaking in 2 ml vials was the most intense stress. For the 6 ml and 2 ml vials, a similar decrease in monomer content of the two different antibody solutions was observed (Fig. 4). In contrast, the 1.5 ml vial exhibited differences between the two antibody solutions. As in the turbidity study, large standard deviations were observed in the monomer content of the 0.8 ml samples, pointing to different stress conditions within a triplet. This precludes a meaningful comparison of the behavior of the two antibody solutions.

The results demonstrate that various container sizes and types in shaking stress experiments cause different stress conditions. Apart from the usual differences in antibody stability, this can result in the formation of diverse types of aggregates and differences in the destabilization behavior between various antibodies.

Several causes may lead to the observed differences in stress conditions. As the vials are of different geometry, differences in the movement and turbulent flows of the liquids inside the vials leading to differences in shear stress have to be assumed. In addi-

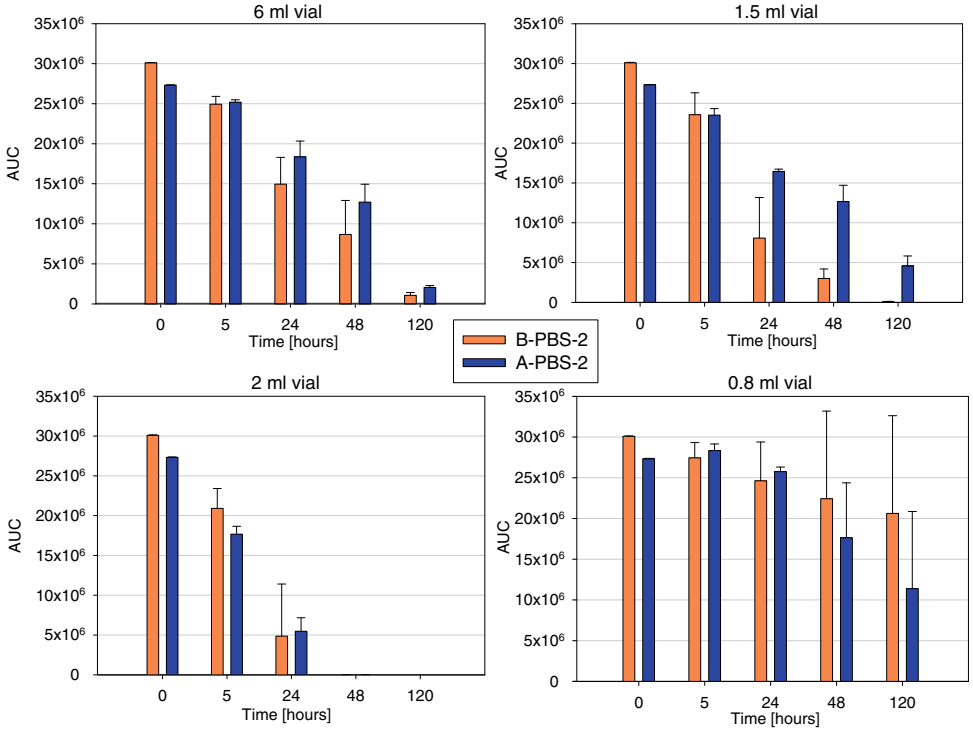

Fig. 4. Absolute monomer content (SEC) of antibody solution A-PBS-2 and B-PBS-2 during shaking (250 rpm) in different containers.

tion, differences in the interface-related influences are likely to occur between the containers of different size and of different geometry. These include the absolute area of the interface (a larger interface to air or glass is probably a higher stress factor) as well as the intensity of its continuous renewal during liquid movement. Unfortunately, it is not possible to exactly determine or calculate these influences in motion. A larger diameter of the vial (6 ml vial) might result in higher velocities of the fluid during shaking and the incorporation of more air leading to foam formation. Moreover, the ratio of liquid to air is different for the different vials (6 ml vial: ~0.32; 2 ml vial: ~0.26; 1.5 ml vial: ~0.42; 0.8 ml vial: ~0.48; Table 2) and might be a further explanation for the observed results. Taking the decrease in SEC monomer content as an indication for the intensity of stress, there is a rough correlation of the stress intensity with the fraction of air in the vial (highest in the 2 ml vial and decreasing via the 6 ml and 1.5 ml vial towards the 0.8 ml vial) in this set of experiments. The results obtained in Section 3.1 do, however, indicate that this correlation is not valid in all cases since small liquid volumes resulted in small stress as well. Additionally, differences in materials such as hydrophobic surfaces, can also lead to different stability data [24]. It is thus particularly difficult to compare the results obtained for the 1.5 and 0.8 ml vials (different geometries, stoppers and glass qualities primarily fabricated for HPLC) with each other and with those for the 6 and 2 ml vial. These two vials can be more easily compared; both are fabricated for parenteral solutions, and they only differ in size and geometry.

In conclusion, of the tested containers, the 2 ml vial seems to be most suited for shaking stress experiments. It led to the most intense stress, and it also exhibited similar results for different antibody solutions (the first aspect was investigated in more detail in experiments described later). Moreover, the sample volume of 1 ml is a good compromise between minimal material use and sufficient volume for adequate analytics. In addition, the 2 ml injection vial is a container that may be used for parenteral dosage forms (in contrast to the 1.5 and 0.8 ml vial).

3.3. Shaking intensity

In the experiments presented in this section, the influence of three shaking intensities (150, 200 and 250 rpm) was tested in 2 ml vials filled with 1 ml of the buffered antibody solution A-PBS-2 as model protein formulation.

At 150 rpm, there was neither a pronounced increase in turbidity nor decrease in SEC monomer content (Fig. 5). Obviously, this stress condition was too weak. Even long shaking times (120 h) under these weak shaking intensities did not result in a considerable decrease in monomer content as observed by harsher conditions after short (e.g., 24 h) shaking times. This might be explained by differences in turbulent flow and mobility of the liquid during shaking, which are too low at weak conditions. A certain turbulent flow or movement is needed to obtain protein aggregation. Obviously, the threshold at which aggregate formation starts was not reached.

At 250 rpm, turbidity increased up to 24 h and then decreased upon longer shaking (Fig. 5) as already outlined above (Section 3.2). This behavior can be explained by the extraction of protein from the solution due to the formation of precipitates floating to the surface (Fig. 2). Accordingly, a rapid decrease in monomer content was observed for this sample by SEC. As, almost no monomers could be detected by SEC after 48 h, the applied stress is considered to be too harsh for meaningful stress tests.

Agitation with 200 rpm led to a continuous increase in turbidity without the formation of foam and a pronounced decrease in monomer content over time (Fig. 5). Thus, this intensity showed the most appropriate results within this experimental setup.

3.4. Discriminatory condition

In summary, the results of the experiments reported above pointed out that a suitable stress condition is shaking in a 2 ml vial containing 1 ml volume at an intensity of 200 rpm. This condition is tested on its discriminatory potential in this section. For comparison, the harsh stress condition of 250 rpm was also investigated. Discrimination in this case means differentiation between a buffered solution (A-PBS-5) and a marketed formulation (A-mf-5) of an antibody. In the experiments, the two antibody solutions (both concentrated at 5 mg/ml) were shaken in 2 ml vials filled with 1 ml at 200 rpm and 250 rpm.

Clear differences could be observed between the turbidity of the buffered antibody solution and the marketed product already after 5 h of shaking (Fig. 6). At both shaking intensities, the buffered product showed much higher turbidity values than the marketed product. Visually, the buffered solution appeared turbid as well, whereas the marketed product remained clear. Only at 250 rpm, the marketed product increased in opalescence at longer shaking times. This is attributed to an increasing content of dimers which was observed by SEC (Fig. 6).

In contrast to the observed turbidity results, the SEC results revealed no relevant differences in monomer content decrease between the two formulations at 250 rpm (Fig. 6). The monomer content decreased steadily for both solutions under these conditions [in an additional measurement after 1 h of shaking no significant differences between the two formulations were observed (data not shown)]. For the buffered solution, the decrease in monomer concentration can be explained with the formation of large aggregates causing the observed increase in turbidity. In SEC, no aggregates were detected for this solution, indicating that only large aggregates were formed. In contrast, the marketed product formed a high fraction of SEC aggregates upon shaking which did, however, not cause a marked increase in turbidity.

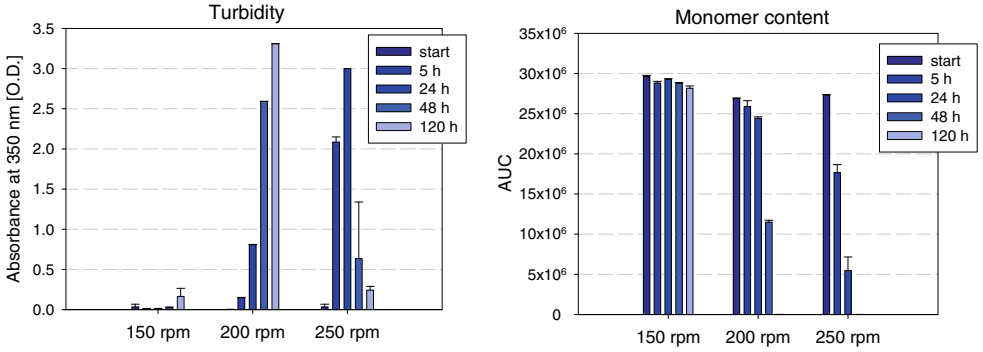

Fig. 5. Turbidity (left) and absolute SEC monomer content (right) of antibody solution A-PBS-2 in the 2 ml vial at different shaking intensities over time.

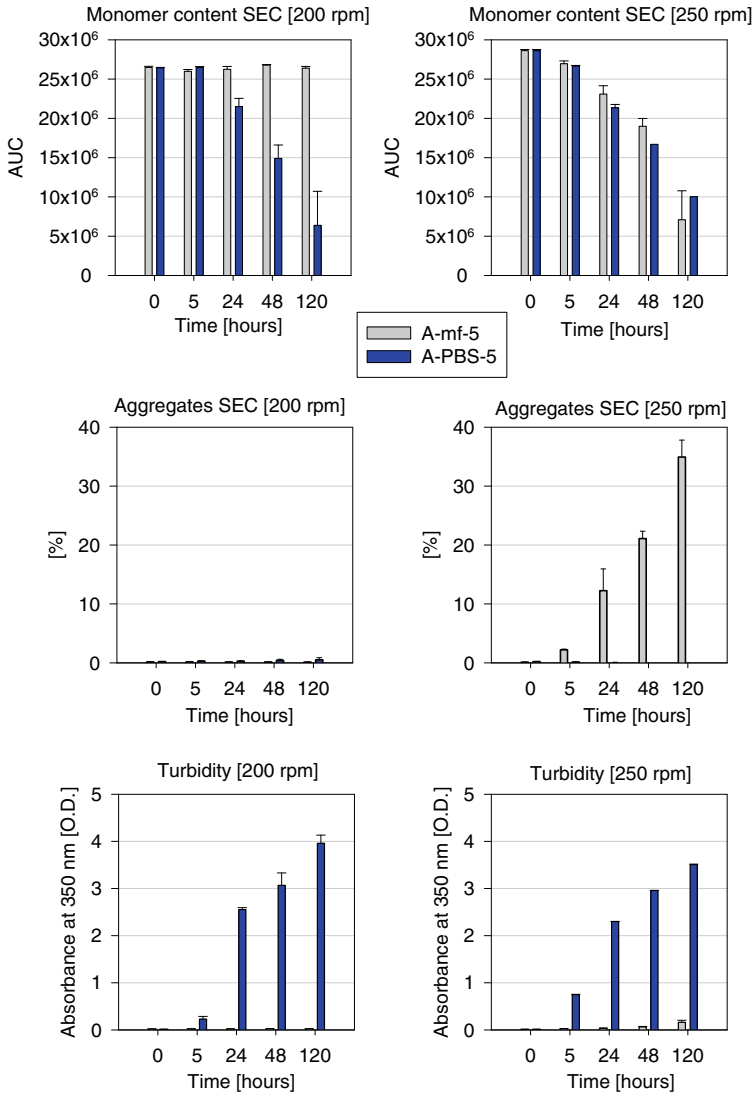

Fig. 6. SEC monomer content (top), SEC aggregate content (middle) and turbidity results (bottom) for the marketed product A-mf-5 and the buffered solution A-PBS-5 over time at (left) 200 rpm and (right) 250 rpm shaking intensity.

At 200 rpm, a clear difference in monomer content could, however, be observed by SEC. The monomer content of the marketed product remained nearly unaffected over time without formation of aggregates, whereas the monomer content of the buffered solution decreased distinctly upon shaking with the first clear difference being observed after 24 h of shaking.

The results indicate that SEC results are not necessarily reflected in the turbidity results. To analyze the instabilities, especially in terms of the formation of different types of aggregates, it is required to combine different analytical methods [10,19].

The differences between marketed and buffered product can predominantly be attributed to the presence of Tween in the marketed products. Tween is a very effective excipient to protect protein solutions against shaking stress [9,10]. Other excipients like buffers and salt may, however, also have an influence on the observed stability [3].

The parameters used in this experiment allow discrimination between a marketed formulation and a simple buffered solution when a shaking intensity of 200 rpm is applied. It is important to note that at conditions that are too harsh (here shaking at 250 rpm) a buffered antibody solution (A-PBS-5) cannot be discriminated from its much more stable marketed formulation (A-mf-5). At 200 rpm, the first clear discrimination between marketed and buffered product observable in both, turbidity measurement and SEC, was detected after 24 h of shaking, and the differences became more clear after 48 and 120 h. While these shaking times (in particular 24 and 48 h) appear reasonable for the intended preformulation setting, it might be desirable in some situations to obtain data within a shorter period of time. Our experiments indicate that increasing the shaking intensity is not a straightforward solution into this direction. It might, however, be possible to find a shaking intensity between 200 and 250 rpm which is still discriminatory while being quicker.

3.5. Transferability

To confirm the results presented above, the discriminatory stress condition (2 ml vial filled with 1 ml solution; shaking at 200 rpm) was tested with two further antibody solutions (B-PBS-5 and C-mf-5). The buffered/unformulated antibody solution B-PBS-5 was tested in comparison to the buffered antibody solution A-PBS-5 used in the experiments outlined before. The stability of the marketed antibody product C-mf-5 was compared to that of the marketed antibody product A-mf-5 used in previous experiments. All antibody solutions had a concentration of 5 mg/ml.

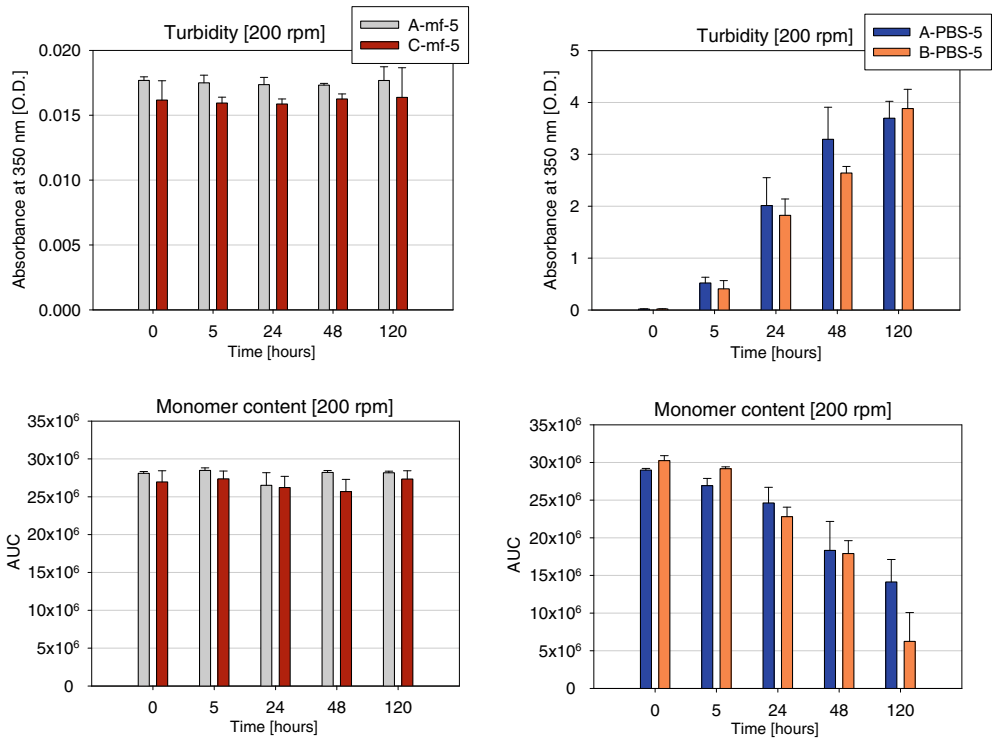

Fig. 7. Turbidity (top) and SEC monomer content (bottom) of the marketed product A-mf-5 in comparison to the marketed product C-mf-5 (left), and the buffered solution A-PBS-5 in comparison to buffered solution B-PBS-5 (right).

Turbidity measurements led to comparable results for the two marketed products. Both products displayed no turbidity increase over shaking time (Fig. 7). The same comparability was observed with the buffered products – the turbidity of both increased in almost the same manner. This is in line with the results from SEC (Fig. 7). Both buffered antibody solutions exhibited a comparable monomer content decrease over shaking time, whereas the marketed products remained stable without loss in monomer content. These results confirm that the chosen parameters result in discriminatory stress allowing to distinguishing between products in a buffer and in a formulation suitable for the market.

Different pH values and buffer systems can have a pronounced influence on the chemical and physical stability of protein formulations [3,8,25–27]. However, the most pronounced influence on aggregation, which is studied here, is caused by Tween. Tween has surface active properties that might reduce the presence of free protein at the interface and thus reduce stress during shaking. The Tween-containing marketed formulation A-mf-5 has a lower surface tension (~43 mN/m) than the formulations without Tween (~60 mN/m) (Table 1). The surface tension of formulations with higher protein concentration is slightly lower as well (e.g., ~67 mN/m for A-PBS-2 compared to ~60 mN/m for A-PBS-5 and ~61 mN/m for B-PBS-2 compared to ~56 mN/m for B-PBS-5). In addition, differences in surface tension result in different wetting behavior of the glass wall during shaking and led to different sizes of the glass–liquid as well as air–liquid interfaces. This may be an additional reason why formulations with Tween as well as highly concentrated protein formulations are more stable against shaking stress.

4. Conclusion

The choice of shaking stress parameters has a large influence on the aggregation behavior of IgG antibodies. The results of the present study emphasize that differences in any of the shaking parameters result in altered stability data, leading to the incomparability of results. If the conditions of shaking stress studies are not carefully selected, the results of such studies are irrelevant to practice. Only carefully developed conditions allow differentiating between a stable formulation that is suitable for market supply and a formulation that is prone to aggregation.

In order to be discriminatory, the stress employed on the samples must neither be too harsh nor too weak. In our study, optimal stress conditions were observed when filling the sample vials with half of the recommended volume. The use of very small vials led to irreproducible results due to unsatisfactory flow conditions within the containers. In large vials, the chosen shaking intensities produced very harsh stress resulting in the floatation of aggregates and foam formation which prevented a meaningful analysis of these samples. The use of 2 ml vials (filled with 1 ml) at a shaking speed of 200 rpm allowed the desired discrimination between unformulated buffered solutions and marketed formulations of antibodies. To cover the whole range of aggregates that may form during the experiments, the combination of different analytical techniques is very important. As the required volume of protein solution is quite low for this method, it appears to be highly suitable for the fast evaluation of formulations in early stage development. We could also show that the stress condition we developed here was suitable and discriminating for the three IgG antibodies tested so far. In addition, our experiments illustrate that the three antibodies behaved similar in shaking stress experiments. It will have to be further corroborated whether this holds true also for other antibodies.

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