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Technical note

Freeze/thaw of IGG solutions

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ABSTRACT

In this communication, the effect of mannitol and trehalose crystallization on the unfolding of IgG_1, a monoclonal antibody, in the frozen state with repeat freeze/thaw under different pH conditions was explored. Formulations were annealed at −20 °C for 20 h five times (interrupted by freeze/thaw). This was done to induce excipient crystallization. Characterization of the frozen-thawed samples was performed by circular dichroism, particle analysis, and size exclusion chromatography. At a pH of 3, formation of insoluble and soluble aggregates was observed however, these could be reduced by the use of a surfactant. Cryoprotectant free formulations showed higher monomer content after freeze/thaw. At pH 5, a single freeze/thaw cycle did not result in a significant increase in particle numbers. At pH range of 4–7 however, aggregate formation in the size range of 1–25 µm was observed after 5 freeze/thaw cycles.

1. Introduction

The freezing step is critical in frozen state storage and freeze-drying of protein formulations [1] as it defines the stability of the protein. It is well established that for a stable formulation, bulking agents such as mannitol should crystallize while cryoprotectant excipients such as trehalose should stay amorphous during freezing and storage. The bulking agent and cryoprotectant crystallization depends on their relative concentrations [2] as well as on the concentration of protein in the formulation [3]. For example, a higher bovine serum albumin (BSA) concentration in the formulation is known to delay and even completely suppress excipient crystallization [3]. Other additives, such as surfactants, also affect protein stability in a concentration dependent manner. It is not yet known though if all proteins have similar effects on the state of the formulation and behave similarly during freezing.

In this study, the freezing response of a monoclonal IgG_1 antibody was explored in different formulations made by changing trehalose to mannitol ratio. The effect of pH was also investigated since pH is known to influence antibody stability [4–6]. The critical parameters characterized in this study were unfolding of the antibody, formation of aggregates and particles, and macroscopic appearance of the formulation after freeze-thaw.

1.1. Materials

D-(+)-trehalose dihydrate (MW 378.33 kDa, 99.9% purity, Sigma-Aldrich, St. Louis, MO), Tween 20 (MW 1227.54 Da, > 98.0% purity, Biorad Laboratories, Hercules, CA), and D-mannitol (MW 182.17 Da, ≥98% purity, Sigma-Aldrich) were used as received. Stock solutions were prepared gravimetrically in 10 mM sodium phosphate buffer at the formulation pH (Mettler-Toledo MP220 pH meter, Greifensee, Switzerland). A monoclonal antibody (IgG_1) was used as a model protein. All formulations were filtered through a 0.2 µm polyethersulfone membrane syringe filter (VWR International GmbH, Ismaning, Germany) prior to use.

1.2. Freeze/thaw experiments

Freeze/thaw experiments were performed using a FTS LyoStar™ 3 freeze-dryer (SP Scientific, Stone Ridge, NY, USA). 2 R vials (Fiolax™ clear, MGlas AG, Münnerstadt, Germany) were filled with 2.0 mL of sample solution and stoppered (stopper type: Westar®, West Pharmaceutical Services, Eschweiler, Germany). Samples were cooled by ramping the shelf temperature to −20 °C at 1 °C/min followed by an isothermal hold for 20 h. At −20 °C for 20 h we assume complete ice crystallization, mobility of any amorphous phase and that trehalose crystallization is favoured. The samples were thawed by ramping the shelf temperature to 20 °C at 1 °C/min. The product temperature was monitored via thermocouples in selected vials. The freeze/thaw cycle was repeated up to five times.

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Table 1
Far UV CD data from fresh and freeze-thawed IgG formulations. H(r) – regular helix, H(d) – distorted helix, S(r) – regular sheet, S(d) – distorted sheet. Solutions were frozen and annealed at −20 °C for 20 h. All samples were analyzed in duplicate.

Composition	Helix (r)	Helix (d)	Strand (r)	Strand (d)	Turns	Unordered
Fresh IGG	0.004	0.031	0.269	0.164	0.212	0.343
8T:4IGG	0.004	0.030	0.270	0.162	0.211	0.333
10M:4T:8IGG	0.001	0.032	0.272	0.148	0.216	0.330
10M:8T:4IGG	0.005	0.011	0.266	0.151	0.224	0.352
10M:8T:8IGG	0.008	0.028	0.258	0.160	0.213	0.318
10M:8T:10IGG	0.003	0.024	0.260	0.160	0.233	0.347
Fresh IGG (pH 5)	0.004	0.020	0.302	0.160	0.214	0.307
10M:8T:4IGG (pH 4)	0.002	0.026	0.260	0.163	0.239	0.351
10M:8T:4IGG (pH 3)	0.002	0.029	0.203	0.110	0.145	0.501

1.3. Flow imaging

A FlowCAM® 8000 series imager (Fluid Imaging Technologies, Yarmouth, ME USA) equipped with a high-resolution camera (1920 × 1200 pixels, magnification of 10x) was used to visualize and count the sub-visible particles per mL of the sample. A sample volume of 0.20 mL was analyzed at a flowrate of 0.15 mL/min. The system was rinsed with purified water between measurements or with Helmanex® to remove the residual protein from the flow cell. Data analysis was performed using the VisualSpreadsheet® software.

1.4. Light obscuration (LO)

A PAMAS SVSS-35 particle counter with a HCB-LD-25/25 sensor (PAMAS – Partikelmess- und Analysesysteme GmbH, Rutesheim, Germany) was used to determine the number of subvisible particles. Before each analysis, the system was rinsed with 10 mL highly purified water. The volume used in rinsing was 0.2 mL, and it was followed by four measurements of 0.2 mL according to USP 787. Analysis was carried out using the PAMAS PMA software. Particles ≥1 µm, ≥10 µm, and ≥25 µm were counted. The results are presented in cumulative particles per mL.

1.5. High performance size exclusion chromatography (HP-SEC)

Size exclusion chromatography was performed using an Agilent 1200 series HPLC system equipped with an UV/Vis detector for detection at 280 nm (Agilent Technologies, Santa Clara, California, USA). A TSKgel® G3000 SWXL column (dimension: 300 × 7.8 mm, TOSOH Bioscience, Stuttgart) and 100 mM sodium phosphate/100 mM sodium sulfate buffer at pH 6.8 mobile phase with a flowrate of 0.5 mL/min was used. Samples were centrifuged prior to analysis. The integrated peak intensity was determined before and after freeze/thaw after blank subtraction. Monomer recovery (in percentile) was determined by comparing the integrated intensity of the monomers before and after freeze/thaw.

1.6. Turbidity (A350 nm)

Absorbance at 350 nm by the freeze/thawed samples was measured by UV/VIS-spectroscopy as an indicator of turbidity. A NanoDrop 2000 spectrophotometer (PEQLab Biotechnology GmbH, Erlangen, Germany) was used in the experiments. 5 µL of each formulation was measured in triplicate with its corresponding formulation without the protein as its blank solution (baseline control).

1.7. UV circular dichroism (CD)

Spectra were recorded using a J-815 circular dichroism spectropolarimeter (JASCO) using a 1 cm path length quartz cuvette over the range of 190–260 nm at ambient temperature. Data were collected every 0.2 nm with a bandwidth of 1 nm, averaged over 8 scans. An integration time of 8 s at 50 nm/min and a 2 nm slit width were used. The solutions were diluted with ultrapure water to reach a protein concentration of 0.2 mg/mL. The background spectra of the solvent medium (identical dilution) were subtracted from the final spectra and data were obtained as mean residue ellipticity (Θ). To estimate protein secondary structure content, analysis of the relevant CD spectra was carried out using the CDPro software. The basis set 7 of the CDPro software was used. Analysis was performed using the SELCON 3 method [7].

2. Results and discussion

IgG_1 solutions were prepared in sodium phosphate buffer of different pH in the presence of mannitol and trehalose, mimicking the compositions studied previously with BSA [3]. It was assumed that IgG_1 affected excipient crystallization in a similar way to BSA. Effect of multiple freeze-thaw cycles (up to 5) and the effect of the presence of a surfactant in the formulation (0.1% w/w Polysorbate 20) were explored. Macroscopic appearance of the thawed solutions was investigated visually. Additionally, absorbance at 350 nm was used to measure turbidity caused by the formation of insoluble visible aggregates. FlowCam® imaging and light obscuration of the solutions was performed to characterize subvisible particles in the size range 1–50 µm. The amount of soluble aggregates was detected by Size Exclusion Chromatography.

Between pH 5 and 7, IgG_1 appeared to be rather stable and resistant to freeze/thaw stress. Despite extensive annealing, IgG_1 retained its native secondary, and tertiary conformations, irrespective of formulation composition (see far UV CD data in Table 1). Moreover, no significant change in turbidity or monomer content was observed in thawed solutions. When the pH of the formulation was lowered however, significant changes in the secondary and the tertiary conformations were observed. At pH 4, post thaw far UV CD analysis revealed a decrease in the fraction of strands and turns with a concomitant increase in the fraction of helices and unordered structures when the sample was frozen and annealed. This effect was exacerbated when the pH was further lowered to 3. Particle numbers increased substantially in all formulations; both at pH 4 and pH 7 (Figs. 1 and 2).

Formulations at pH 3 appeared turbid after 5 freeze-thaw cycles, which indicated formation of insoluble precipitates (Fig. 3). Absorbance at 350 nm was comparable to surfactant free formulations.

At pH 3 the number of subvisible particles ≥1 µm, ≥10 µm, and ≥25 µm in thawed solutions increased drastically (Fig. 4), which was also observed for pH 4 and pH 7 (Fig. 1). The formation of sub-visible particles at pH 3 was greatly inhibited by addition of the surfactant. Analysis of fresh and freeze-thaw stressed formulations by Size Exclusion Chromatography revealed a marked decrease in the monomer content, especially when IgG was frozen in the presence of excipients at a pH 3 (Fig. 5). The soluble aggregate content did not change significantly, and no effect of Polysorbate 20 could be substantiated. This

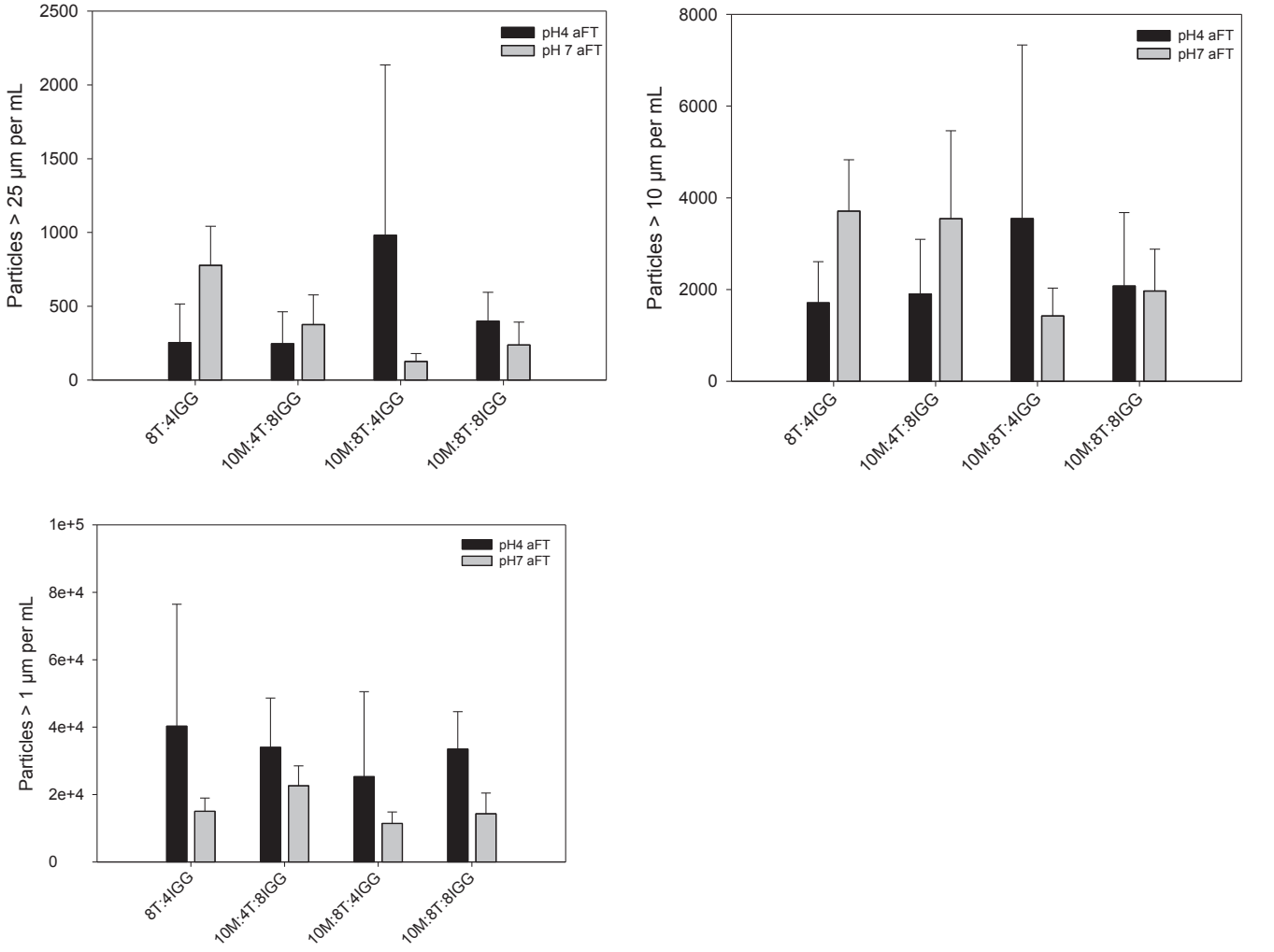

Fig. 1. Numbers of subvisible particles ≥ 25, 10 and 1 µm of mannitol/trehalose/IgG formulations after 5 FT cycles at pH 4 and 7 (Numbers before FT were below 5, 20 and 200 particles per mL resp. for the different sizes and are not shown).

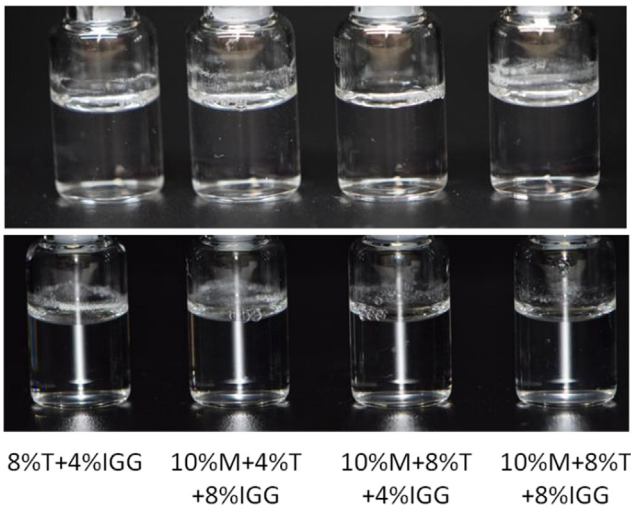

Fig. 2. Macroscopic appearance of formulations and pH 4 (upper row) and pH 7 (lower row) after 5 FT cycles.

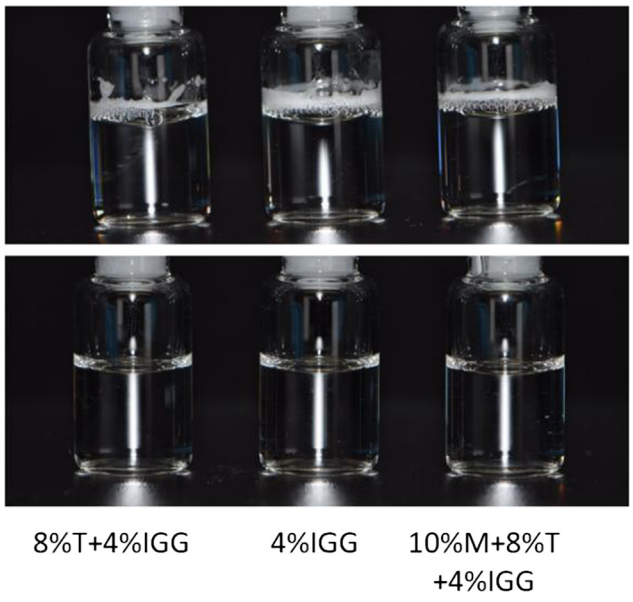

Fig. 3. Macroscopic appearance of formulations at pH 3 without (upper row) and with 0.1% PS 20 (lower row) after 5 FT cycles.

suggests that while the surfactant is effective in minimizing precipitation of larger insoluble and soluble particles, it does not have a significant effect on smaller soluble particles; an effect that is worthy of investigation in upcoming studies.

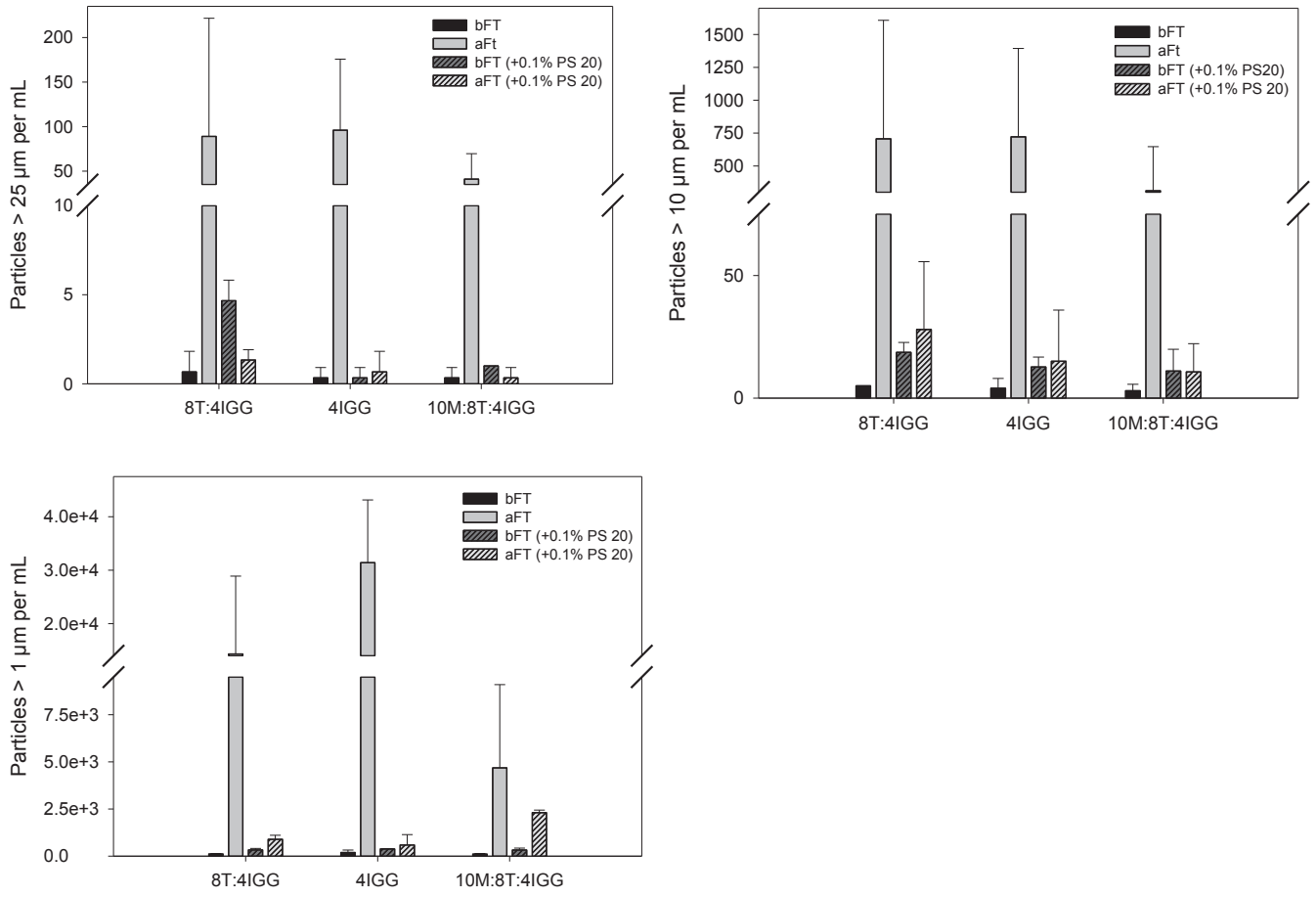

Fig. 4. Numbers of subvisible particles ≥25, 20 and 1 µm after 5 FT cycles at pH 3 without and with 0.1% PS20.

Conformational instability of IgG at acidic pH has been documented previously [8–10] and attributed to Fc aggregation, primarily determined by the C_H2 domain instability. The process of C_H2 unfolding is shown to be triggered by the protonation of specific acidic residues, otherwise involved in forming stabilizing electrostatic interactions in the folded state. Unfolding of the Fc domain is shown to lead to subsequent aggregation of the mABs. Addition of surfactant, the concentration of which will exceed the CMC upon freeze-concentration by far, minimized the extent of aggregation during freezing.

3. Conclusion

Particle numbers in solution increased only after several freeze-thaw cycles at pH 5. Soluble aggregate content, turbidity, and macroscopic appearance gave no clear differences between formulations at pH 4 and 7. The monomer recovery was greatly reduced at pH 3 but did not cause increased aggregation or higher particle numbers than at pH 4 or pH 7. PS 20 could prevent visible and subvisible particle formation to a large extent by coverage of the ice liquid interface. Effect of composition on particle formation and turbidity was negligible.

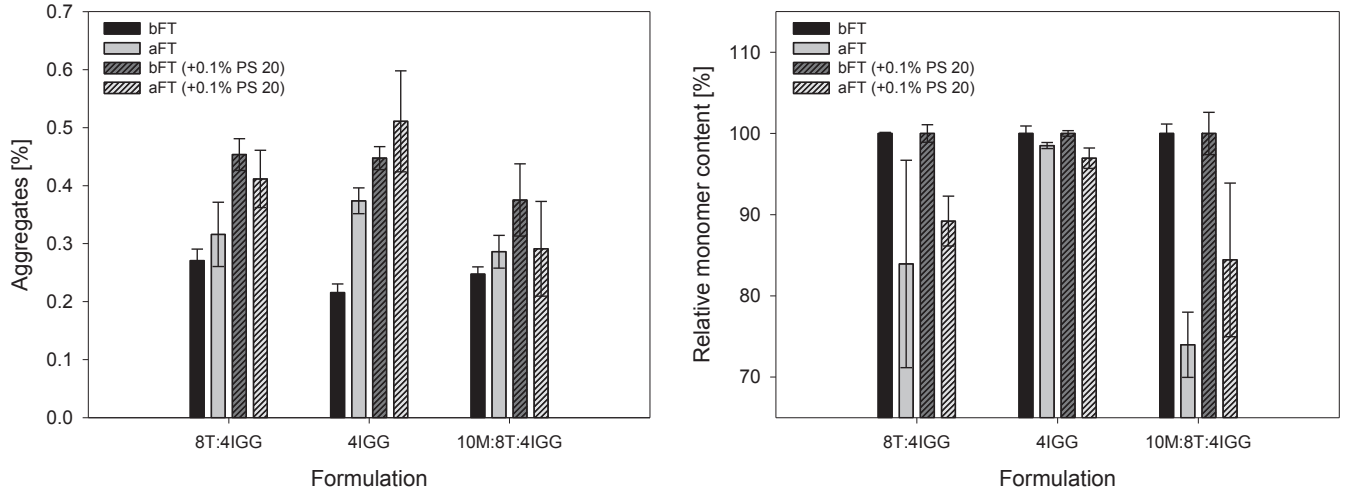

Fig. 5. Soluble aggregates and monomer content after 5 FT cycles at pH 3 without and with 0.1% PS20.

However, monomer recovery was much higher for excipient free formulations, implying that mannitol and trehalose crystallization contributed to protein unfolding, similar to what was shown for BSA. A mannitol to trehalose ratio was selected in which mannitol and trehalose crystallization was likely. Thus, the IgG_1 appeared to be more stable in the absence of mannitol and trehalose crystals. Unfolding of the IgG_1 was strongly dependent on the formulation pH, but could not be directly linked to the formation of insoluble and soluble aggregates. Studies with BSA as a model protein showed similar findings [3]. Thus, we observed similarities between different types of proteins (globular and antibody). The impact of pH, number of FT cycles, addition of surfactant and crystallizing excipients was manifold. Changes in one of these parameters can affect different analyzes in various ways and pronounce the importance of the use of orthogonal method for the characterization.

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