novel analytical approaches to characterize particles in biopharmaceuticals

Grabarek, A.D.

Chapter 5

What makes polysorbate functional? Impact of polysorbate 80 grade and quality on IgG stability during mechanical stress

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Abstract

Polysorbate 80 (PS80) is a commonly used surfactant in therapeutic protein formulations to mitigate adsorption and interface-induced protein aggregation. Several PS80 grades and qualities are available on the market for parenteral application. The role of PS80 grade on protein stability remains debatable, and the impact of (partially) degraded PS on protein aggregation is not yet well understood. In our study, a monoclonal antibody (IgG) was subjected to three different mechanical stress conditions in the presence of multicompendial (MC) and Chinese pharmacopeia (ChP) grade PS80. Furthermore, IgG formulations were spiked with (partly) hydrolyzed PS80 to investigate the effect of PS80 degradants on protein stability. PS80 functionality was assessed by measuring the extent of protein aggregation and particle formation induced during mechanical stress by using size-exclusion chromatography, dynamic light scattering, backgrounded membrane imaging and flow imaging microscopy. No distinguishable differences in PS80 functionality between MC and ChP grade were observed in the three stress tests. However, with increasing degree of PS80 hydrolysis, higher counts of sub-visible particles were measured after stress. Furthermore, higher levels of PS80 degradants at a constant PS80 concentration may destabilize the IgG. In conclusion, MC and ChP grade PS80 are equally protective, but PS80 degradants compromise IgG stability.

Introduction

Manufacturing, handling and administration of biopharmaceutical products generates mechanical stress which can induce aggregation of proteins[1]. Protein aggregation deteriorates product quality and may compromise safety, e.g., by causing unwanted immune responses[2]. Aggregation is often driven by weak interactions between exposed hydrophobic patches of (partially) unfolded proteins[3]. A range of conditions, including pH shift, elevated temperature and mechanical stress, may induce protein unfolding and enhance the formation of protein aggregates. Therefore, the development of robust formulations consisting of excipients mitigating protein unfolding and aggregation is required to ensure the quality of protein drug products.

Proteins are amphiphilic molecules which have a high propensity to adsorb to interfaces and are susceptible to surface-mediated unfolding[4]. Surface active molecules, such as polysorbates (PS), are common excipients in drug product formulations to increase colloidal stability and minimize adsorption of proteins to interfaces[5,6]. PS are non-ionic surfactants containing mainly sorbitan polyoxyethylene (POE) fatty acid esters. The protective role of PS in protein formulations has been thoroughly investigated[7] and two main mechanisms have been elucidated: (1) PS acting as a chaperone for aggregation-prone hydrophobic sites on the protein surfaces (promoting the folded structure)[8,9] and (2) PS competing with proteins at interfaces (reducing interface exposure)[10,11]. Currently, polysorbate 20 (POE sorbitan monolaurate, PS20) and polysorbate 80 (POE sorbitan monooleate, PS80) are the most frequently used surfactants in marketed biopharmaceutical formulations. Both types are highly effective in preventing protein adsorption and aggregation and they have a well-known toxicity profile[12,13]. The longer monounsaturated chain in PS80, compared to PS20, makes PS80 more surface active with a lower critical micelle concentration (CMC). The binding properties of the two types of surfactants to proteins is highly dependent on the protein type[14,15]. The different physico-chemical properties of PS80 and PS20 translate to distinguishable properties in their functionality[16] as well as chemical stability[17].

The chemical properties are key towards the notable functionality of PS. Nonetheless, commercial PS products consist of a mixture of mono- and poly-esters of POE sorbitans

alongside substantial amounts of POE, sorbitan POE and isosorbide POE fatty acid esters, originating from the synthesis[18]. Significant variations in the chemical composition of PS can be present, not only between suppliers[19] but also between different lots of a single supplier[20]. In addition, PS is known to be susceptible to oxidative and hydrolytic (mainly enzymatic under pharmaceutical relevant conditions[21]) degradation, resulting in the formation of aldehydes, epoxides, free fatty acids (FFA) and other impurities, which adds on to their heterogeneity[20].

The European, United States, and Japanese Pharmacopeias have harmonized requirements for PS80 (multicompendial, MC-PS80), where a fraction of above 58% of esterified fatty acids must be oleic acid. Other fractions are specified to a much lower percentage and consist of palmitic, myristic, stearic, linoleic, and linolenic acid esters. In 2015, the Chinese Pharmacopeia (ChP) introduced much more stringent purity requirements for the fatty acid distribution with a minimum oleic acid content of 98% (ChP-PS80). However, there is currently little known on the impact of PS80 grade and quality on its protective role towards proteins exposed to mechanical stress. The highly distinctive compositions of the two PS80 grades led us to focus our study on the functionality of PS80.

In our study, we applied three different mechanical stress methods which allowed us to comprehensively understand the functionality of two different PS80 grades – MC-PS80 and ChP-PS80. In addition to shaking stress, which is commonly used in biopharmaceutical formulation development in forced degradation studies[22], we developed a free-fall test and a syringe pump test as alternative stress methods to assess protein stability in presence of MC-PS80 and ChP-PS80. Unintentional dropping of vials or syringes during transportation and handling may have detrimental effects on protein stability[23-25]. Here, we designed an apparatus with specifications according to the international organization for standardization (ISO) requirements for needle-based injection systems[26] to induce mechanical shock to vials filled with IgG formulations. Furthermore, to mimic the stress conditions during manufacturing, we developed a low-volume flow device equipped with two glass syringes connected via a capillary. Continuous flow of the protein solution from one syringe to another through a narrow constriction has been shown to produce shear and

extensional forces leading to protein aggregation and particle formation[27]. The flow device apparatus was also used to investigate the role of hydrolyzed PS80 on the protein's propensity for aggregation during mechanical stress. We analytically characterized the stressed samples for protein aggregation and particle formation, as these are the key parameters affected by mechanical stress.

Materials and methods

Materials

A monoclonal antibody (humanized IgG1) at a concentration 50 mg/ml in 12 mM L-histidine (pH 6.0) and 250 mM sucrose was donated by Janssen. For each experiment in this study the IgG was diluted with formulation buffer to 5 mg/ml. PS80 of multicompendial grade (MC-PS80) was purchased from J.T. Baker (Avantor, USA) and PS80 compliant the with Chinese pharmacopeia (ChP-PS80) was obtained from Croda International Plc (Snatih, United Kingdom). Sucrose and acetonitrile were purchased from VWR Chemicals (Radnor, USA) and L-histidine from Sigma Aldrich (Taufkirchen, Germany). All other excipients used in the study were purchased from Merck (Darmstadt, Germany) unless otherwise stated. Highly purified water (conductivity: 18.2 mΩ·cm) obtained from a Milli-Q® Advantage A10 system (Merck, Germany) was used throughout the study.

Chemical degradation of PS80

Hydrolytic degradation of PS80 was performed by incubating 2% (w/v) PS80 (MC-PS80 and ChP-PS80) in methanol with 1 M NaOH at 40 ^{o}C for 21 hours. After incubation, the solution was neutralized with 10% (w/v) formic acid. Methanol was removed by using a RCV 2-18 CD plus SpeedVac (Marin Christ) operating at 37 ^{o}C over 2 hours with 1,400 rounds per minute (rpm). The obtained fully hydrolyzed dry material was stored at -80 ^{o}C and resuspended in 500 µl of formulation buffer prior to use.

Different levels of hydrolyzed PS80 were prepared by mixing solutions of neat PS80 with solutions of fully hydrolyzed PS80. Levels of degradants described throughout the paper correspond to % (w/v) of neat PS submitted to complete hydrolysis. PS solutions containing

fully hydrolyzed PS80 were not filtered prior to usage, to enable the assessment of the impact of both insoluble and soluble PS80 degradants on protein stability.

Sample preparation

The IgG was thawed at room temperature and further diluted to approximately 7 mg/ml with PS80-free formulation buffer. The intermediate IgG formulations were filtered by using a 0.2-µm Millex-VV syringe filter unit (Millipore, Germany). PS80-MC and PS80-ChP stock solutions were prepared at 2% (w/v) by weighing 1 g of neat PS80 in a 50-ml volumetric flask, followed by the addition of the required amount of highly purified water. PS80 stock solutions were filtered by using a 0.2-µm Millex-VV syringe filter unit and stored at -80 °C. Intermediate PS80 stock solutions, at 1% and 0.01%, were prepared freshly from PS80 stock solution and an appropriate amount was added to the IgG formulation to reach the desired PS80 concentrations described in the results section. Finally, formulation buffer was added to the IgG formulations in order to reach a target protein concentration of 5 mg/ml.

Mechanical stress methods

Shaking stress was performed by constant agitation of vertically placed 2R glass vials at 500 rpm at 25 °C by using an IC 4000 shaker (IKA, Germany). Vials were filled with sample to 50% of their maximum volumetric capacity, closed with 13-mm rubber stoppers (bromobutyl, FluoroTec B2-40 coating) and standard aluminum crimp camps, and protected from light during stress. Analysis of the formulations was performed after 0 h, 72 h and 360 h of shaking.

Free-fall stress was carried out by using an in-house designed apparatus which meets the ISO specifications[26]. 2R glass vials filled with sample to 50% of their total volume and crimped with 13-mm rubber stoppers (bromobutyl, FluoroTec B2-40 coating) and standard aluminum crimp camps, were subjected to repetitive free falls from a height of 1 m. The vial lands on a smooth surface made of hard, 3-mm thick rigid steel, backed by a wooden board of 20 mm in thickness. A custom 3D printed vial holder ensures a constant angle of the vial (45°) during and at the end of the fall. The fixed vial travels through a tube to ensure a non-turbulent fall. Formulations were analyzed after 0, 1, 2, 5, 10 and 20 free falls.

Pumping conditions, present during protein manufacturing, were mimicked by using a neMESYS apparatus[28] equipped with glass Hamilton-Gastight syringes and polytetrafluoroethylene (PTFE) plungers (Hamilton, Switzerland) placed in custom 3D printed holders. Formulations were stressed for a defined number of passes through a 20-cm PEEK capillary (inner diameter: 1 mm; Thermo Scientific, USA) at a fixed velocity of 8.84 ml/min. Prior to submitting each sample to stress, a control sample consisting of only formulation buffer (with or without PS80) was passed 20 times and analyzed for particle content to ensure cleanliness of the system. Each sample was passed 100 times through the capillary, corresponding to ca. 50 min of pumping of the sample solution, at room temperature. Each sample tested with the syringe pump method was prepared and stressed in triplicate, unless otherwise stated.

Analytical methods

Ultra performance size-exclusion chromatography (SEC)

An Acquity UPLC I-Class (Waters Corporation, USA) system equipped with a titanium cell UV detector was employed for analysis. Ten µg of protein was injected into a TSK gel UP-SW3000, 4.6 x 150 mm column (Tosoh Bioscience, Japan). Separation was achieved by using isocratic conditions with a mobile phase consisting of 150 mM sodium sulfate and 100 mM sodium phosphate dihydrate at pH 6.8 with a flow rate of 0.22 ml/min. UV detection was performed at a wavelength of 215 nm. Monomer content and total recovery (based on the area under the curve (AUC) of stressed sample relative to that of non-stressed (reference) sample) were determined.

High performance liquid chromatography coupled with a charged aerosol detector (LC-CAD)

Characterization and quantification of PS80 and its degradation products was performed by using the method developed by Kranz *et al*[19]. A Dionex Ultimate 3000 HPLC system hyphenated with a Corona Veo CAD (Thermo Scientific, USA) was used. Separation was achieved by using a BEH C18 column, 50 x 2.1 mm (Waters, USA) at 60 °C. A 6-point calibration curve was generated for quantification of PS80 supplemented to the IgG formulations that were investigated in this study. Data integration and processing was

performed with Chromeleon V6.8, where composition of PS80 was grouped into POE, monoesters and polyesters.

UV spectroscopy

A Tecan Safire² plate reader (Tecan, Austria) was used for the determination of the protein content at 280 nm (A280nm) and assessment of turbidity/optical density at 350 nm (OD350nm). Triplicate measurement of 200 µl sample in a 96-well plate was performed. The absorbance of the respective formulation buffer was subtracted from each sample measurement. A280nm values were corrected for light scattering by subtracting absorbance at 320 nm. Undiluted protein concentration was acquired after correction with the dilution factor. Optical density of undiluted samples was determined at 350 nm to assess turbidity. Presented turbidity values were determined from the difference between OD350nm of stressed samples and that of identical unstressed samples.

Dynamic light scattering (DLS)

DLS was performed by using a Zetasizer APS 2000 plate reader (Malvern Instruments, UK) equipped with a 633-nm He-Ne laser set at an angle of 90°. Individual wells within a 96-well plate (Corning Costar, United States) were filled with 150 µl of sample. The attenuator was set automatically. Samples were equilibrated to a working temperature of 25 °C for 60 s prior to each analysis. The Z-average diameter (Z-ave), polydispersity index (PDI) and intensity-weighted size distribution were derived from the autocorrelation function using the default settings for protein solutions in sucrose solution (10% w/v). Each measurement was performed in triplicate.

Flow imaging microscopy (FIM)

MFI 5200 (ProteinSimple, USA) was used for characterization and quantification of particles ≥2 µm in equivalent circular diameter. The system was equipped with a silane-coated high-resolution 100-µm flow cell. Formulation buffer was used to perform optimization of illumination and 0.28 ml of IgG formulation was analyzed within each measurement. Data was processed by using MVAS V2.3 software and the concentrations

of particles ≥ 2, 5, 10, and 25 µm as well as exemplary particle images of particles ≥ 25 µm were reported. Each sample was measured once.

Backgrounded membrane imaging (BMI)

BMI for micrometer-sized particle characterization and quantification was performed by using Horizon (Halolabs, USA). Under laminar air flow conditions 30 µl of sample was transferred on a polycarbonate 96-well membrane filter plate (Halolabs, USA) with a 0.4-µm pore size. Vacuum of 350 mbar was applied after filling 6 wells to allow a flow of liquid through the membrane and entrapment of particles. Subsequently, 90 µl of highly purified water was used to wash each well after sample transfer to remove any soluble material. Each sample was measured in triplicate.

Results

Shaking stress

The IgG formulations without PS80 and with three different concentrations of MC-PS80 or ChP-PS80 (0.04%, 0.004% and 0.0004% (w/v)) were subjected to vertical shaking for 72 h and 360 h at 25°C (Figure 1). The formulation without PS80 showed a significant increase in turbidity (UV), Z-average diameter (DLS), and micrometer-sized particle concentration (FIM) after 72 h of shaking, at which point the stress was discontinued. On the contrary, no substantial changes occurred even after 360 h of shaking when formulating the IgG with 0.04% and 0.004% w/v PS80, independent of the PS80 grade. However, at lower concentrations of PS80 a small increase in particle concentration was observed at T0. Interestingly, stressed IgG formulations with the lowest MC-PS80 or ChP-PS80 concentration of 0.0004% (w/v) showed a larger decrease in monomer content than the PS80-free formulation. In addition, an increase in high-molecular-weight species occurred to a greater extent than in the PS80-free formulation (data not shown).

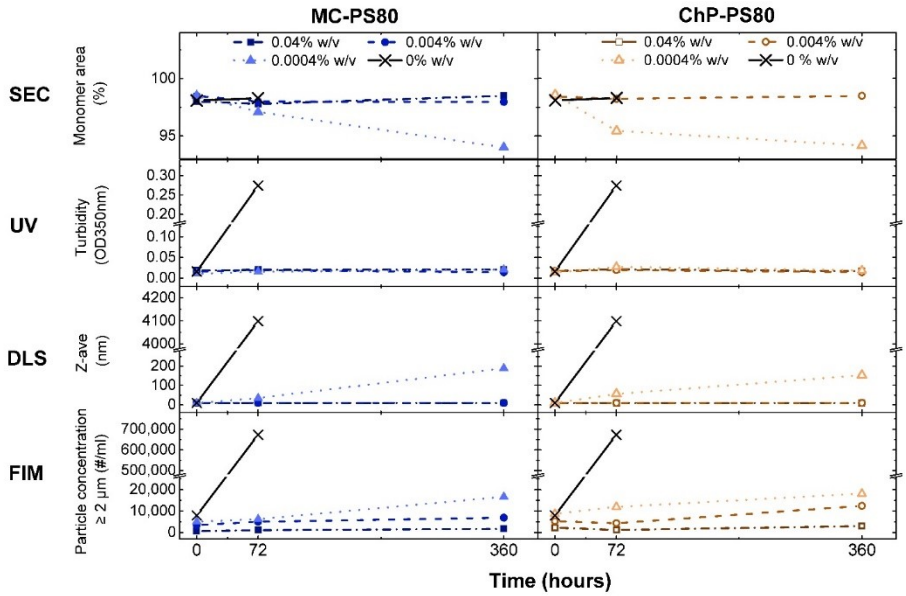

Figure 1: Aggregation over time of 5 mg/ml IgG formulated with different amounts of MC-PS80 or ChP-PS80, stressed by shaking in 2R glass vials at RT, as analyzed by SEC, UV (OD350nm), DLS and FIM.

Free-fall stress

2R glass vials filled with 1.5 ml of IgG formulation were dropped (0 to 20 falls) on a rigid surface to induce stress. With an increasing number of falls, a consecutive increase in the Z-average diameter (DLS) and concentration of micrometer-sized particles (FIM) was observed in IgG formulations without PS80 (Figure 2). IgG in presence of MC-PS80 or ChP-PS80 was well protected, and even after 20 falls no clear increase in micrometer-sized particle content was observed. IgG formulations with 0.0004% (w/v) PS80 of either grade did show a slight increase in Z-average diameter. No changes after stress were observed in SEC and turbidity analyses (data not shown). Similarly as in shaking stress, no difference between the functionality of the two grades of PS80 was observed.

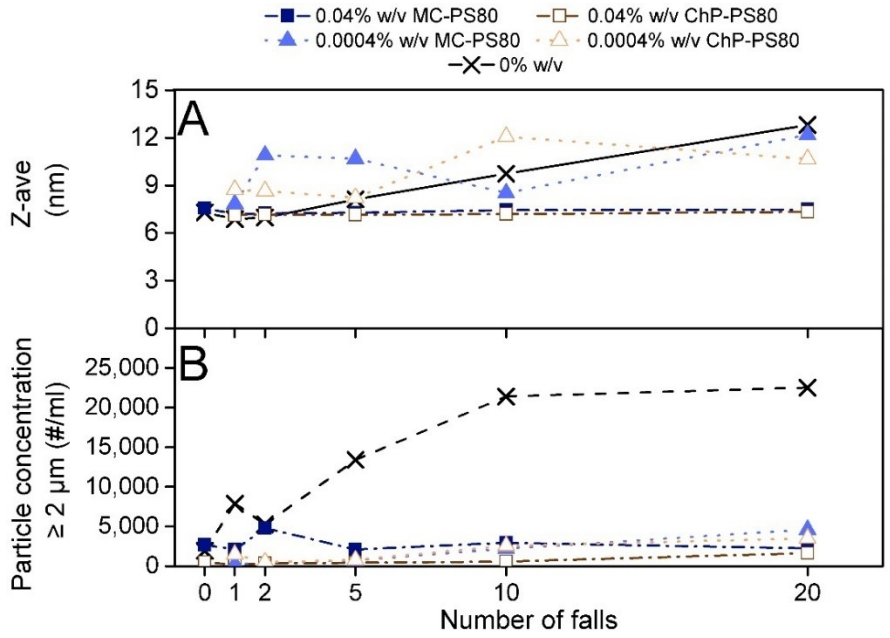

Figure 2: (A) Z-average diameter (DLS) and (B) micrometer-sized particle concentration (FIM) in 5 mg/ml IgG formulations (PS80-free and supplemented with MC-PS80 or ChP-PS80 at target concentrations 0.04% and 0.0004% (w/v)) exposed to free-fall stress as function of number of falls.

Syringe pump stress

Syringe pump stress induced a high concentration of micrometer-sized particles, which made DLS analysis unfeasible. Therefore, we focused on the characterization of soluble aggregates (SEC), sample turbidity (OD350nm) and particles sized ≥ 2 µm (FIM and BMI).

The formation of high-molecular-weight species and fragments after stress was negligible (< 0.5%). Therefore, the protein recovery by SEC practically equaled the monomer recovery. A slight decrease in protein recovery was observed with the two lowest PS80 concentrations, whereas the lowest recovery was obtained for IgG formulations without PS80 (Figure 3 A). At each tested PS80 concentration, the protein recovery for ChP-PS80 compared to formulations with MC-PS80 was statistically similar (T-test, two-sided, $p>0.41$), indicating a comparable protective effect of the two PS80 grades. After stress, the OD350 nm taken as measure for turbidity was slightly increased (OD350 values for all unstressed formulations were 0.011 ± 0.003), which was more pronounced with decreasing amount of

PS80 (Figure 3 B). Nevertheless, based on turbidity data no significant difference between the protective effect of the two PS80 grades could be observed (T-test, two-sided, p>0.07).

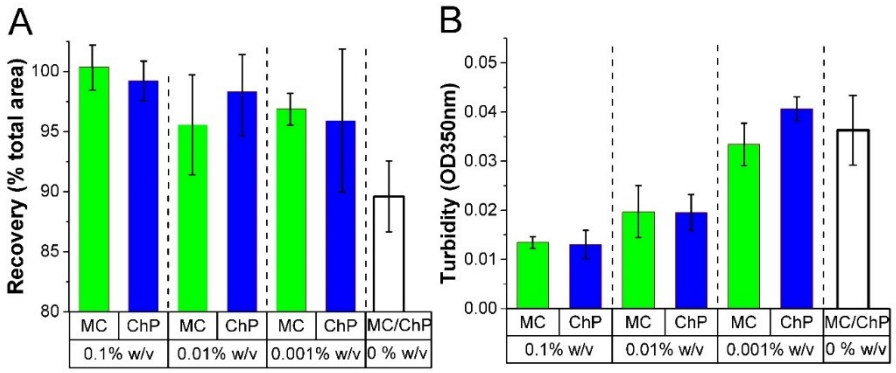

Figure 3: (A) Protein recovery (SEC) and (B) turbidity (OD 350nm UV spectroscopy) in IgG formulations after exposure to syringe pump stress in absence (no PS80) and presence of 0.1%, 0.01% and 0.001% (w/v) of MC-PS80 and ChP-PS80. Turbidity values present the difference between OD350nm of stressed samples and that of identical unstressed samples. Error bars represent standard deviation of mean values from triplicate stressed IgG formulations.

Figure 4 A-D presents the micrometer-sized particle concentration determined by FIM and BMI in stressed formulations of IgG in absence and presence of PS80 over a range from 0 to 0.1% (w/v). For each stressed sample, most of the measured particles were in the size range 2-25 µm (FIM and BMI). Both, MC-PS80 and ChP-PS80 at concentrations 0.1% and 0.01% (w/v) suppressed the formation of particles in IgG formulations during syringe pump stress. No significant difference was found between the number of particles measured in stressed formulations with MC-PS80 and ChP-PS80 for all three concentrations (T-test, two-sided, p>0.23). However, the cumulative number of particles (sized ≥2 µm) per ml in stressed formulations at the two higher PS80 concentrations (0.01% and 0.1%) was at least 2-fold lower compared to formulations without PS80. Formulations with the highest concentration of PS80 had less particles ≥ 10 µm in size compared to all other tested formulations. Interestingly, the lowest concentration of both PS80 grades (0.001% (w/v)) showed modestly higher particle counts, in the size range 2-25 µm compared to the formulations without PS80. Nonetheless, highly similar particle size distributions were detected in the stressed formulations for both PS80 grades at the three tested

concentrations. A high proportion of particles formed during syringe pump stress were ≥10 µm in size, whereas the vast majority of particles found in IgG formulations after shaking and free-fall stress were 2-10 µm in size. Overall, the measured particle concentrations within each size range by FIM and BMI were highly similar for the two techniques. Lower numbers of particles in samples with PS80 at 0.001% (w/v) were detected by BMI compared to FIM, which could be explained by exceeding the recommended higher limit of quantification for BMI[29]. Particle levels measured by FIM in control samples (corresponding PS80 formulation without protein subjected to the same type of stress) did not exceed a concentration of 6,000 particles ≥2 µm/ml throughout the study.

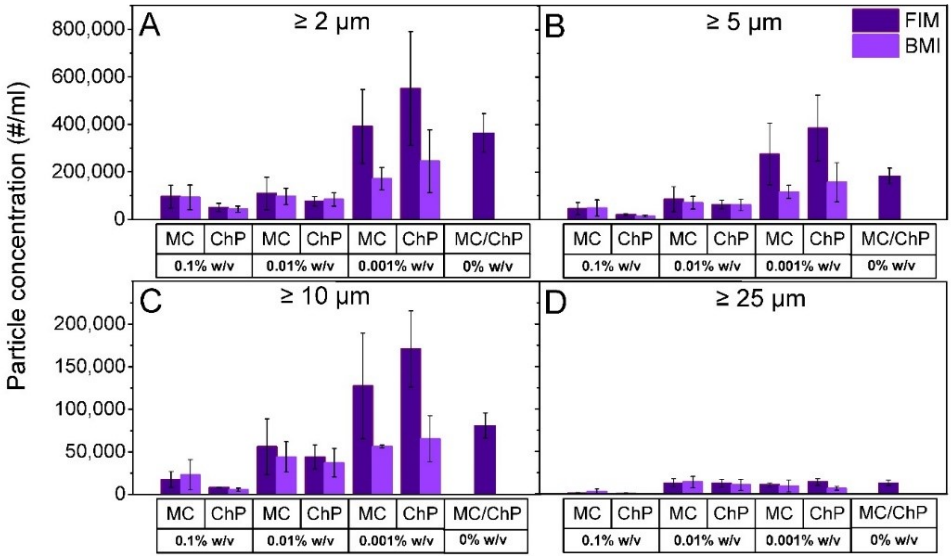

Figure 4: Micrometer-sized particle concentrations ≥ 2 µm (A), ≥ 5 µm (B), ≥ 10 µm (C) and ≥ 25 µm (D) in syringe pump stressed IgG formulations with MC-PS80 (MC), ChP-PS80 (ChP) and without PS80 (no PS80) determined by using FIM and BMI. Error bars represent standard deviation of mean values from triplicate stressed IgG formulations.

Characterization of syringe pump stress induced particles

Morphologies of exemplary particles formed after the three stress methods are presented in Figure 5. Clear differences between particle morphologies can be observed, depending on the type of mechanical stress used and on the presence of PS80. Differences in particle intensity depending on the presence of PS80 were especially apparent in formulations

submitted to syringe pump stress (Figure 5). Within the stressed PS80-containing IgG formulations, particles were dark and compact – independent of the PS80 grade. However, in stressed IgG formulations without PS80 a noticeable subpopulation of transparent, fibrous-like particles was observed. Light-microscopic imaging of the particles isolated by membrane filtration confirmed the presence of two distinct particle morphologies in syringe pump stressed IgG formulations without PS80: white 'flakes' and thin transparent ´foils´ (data not shown). The flake-like particles showed relatively high thickness (several microns) in images obtained by using scanning electron microscope (SEM), whereas the transparent foil-like particles could not be imaged because of the lack of reflectance.

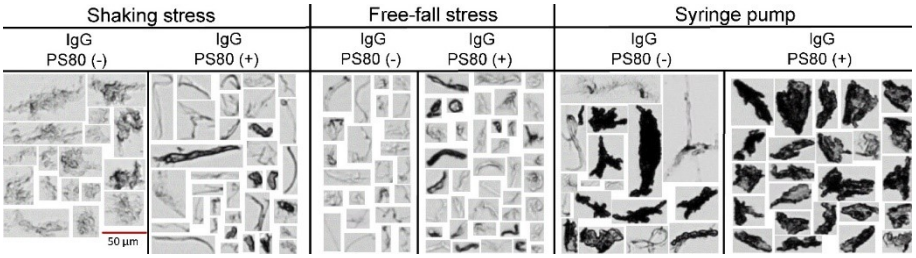

Figure 5: Representative FIM images of particles in stressed IgG formulations with the three mechanical stress methods. Particles are grouped into IgG formulation without PS80 and in presence of PS80 (no morphological differences were observed in particles formed in presence of MC-PS80 and ChP-PS80).

Attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) was performed on isolated particles from stressed IgG formulations to determine the nature of the particles formed during syringe pump stress. The obtained spectra of particles formed in presence (Figure 6 A) and absence (Figure 6 B) of PS80 showed typical protein bands (amide I between 1600 and 1700 cm^{-1}; amide II between 1510 and 1580 cm^{-1}). Additionally, SEM with energy dispersive X-ray spectroscopy showed that isolated particles consisted of solely carbon, oxygen, nitrogen and sulfur - an elemental composition consistent with protein. Therefore, we conclude that most of the formed particles are proteinaceous, most likely IgG, rather than contaminants from the syringe pump device.

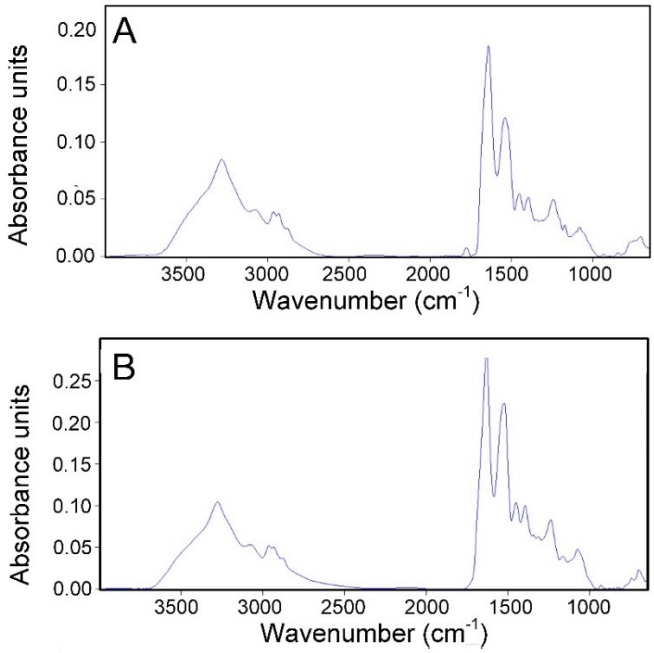

Figure 6: Average ATR-FTIR spectra performed on particles found within an area of 100 µm in the radius of filtration spot. Particles were generated during syringe pump stress in IgG formulation (A) with 0.01% w/v MC-PS80 and (B) without PS80.

Degradation of PS80 and impact of PS80 degradation products on IgG stability

Chemical hydrolysis of MC-PS80 and ChP-PS80 was performed, and the effect of different levels of surfactant degradation on the stability of the IgG during mechanical stress was examined.

LC-CAD analysis of intact PS80 showed three broad peaks eluting at 8.1, 8.9 and 9.4 min, which represent the majority of the monoester fraction (Figure 7 A). The polyester fraction eluted mainly between 11.5 and 12.5 min. The POE fraction was nearly absent in the intact PS. However, after hydrolysis the peak area of the free POE fraction (between 1 and 5 min) increased significantly, whereas the mono- and poly-ester peaks were practically absent. Therefore, we can conclude that the chemical degradation yielded fully hydrolyzed PS80.

Figure 7 B presents the concentration of particles ≥ 2 µm (FIM and BMI) in size present in samples with degraded PS80 (of different degree of hydrolysis; no IgG present) prior to stress. Especially high numbers of particles were measured in samples with a high degree of PS80 hydrolysis (90% and 100%). For samples with 50% or less degradants, the number of particles per ml was below 15,000. From the FIM particle images, it was obvious that mainly droplet-like particles were formed (Figure 7 C). Indeed, BMI measurements showed very low particle counts: it is expected that the droplets, being most likely composed of liquid oleic acids, were not retained on the membrane during sample preparation.

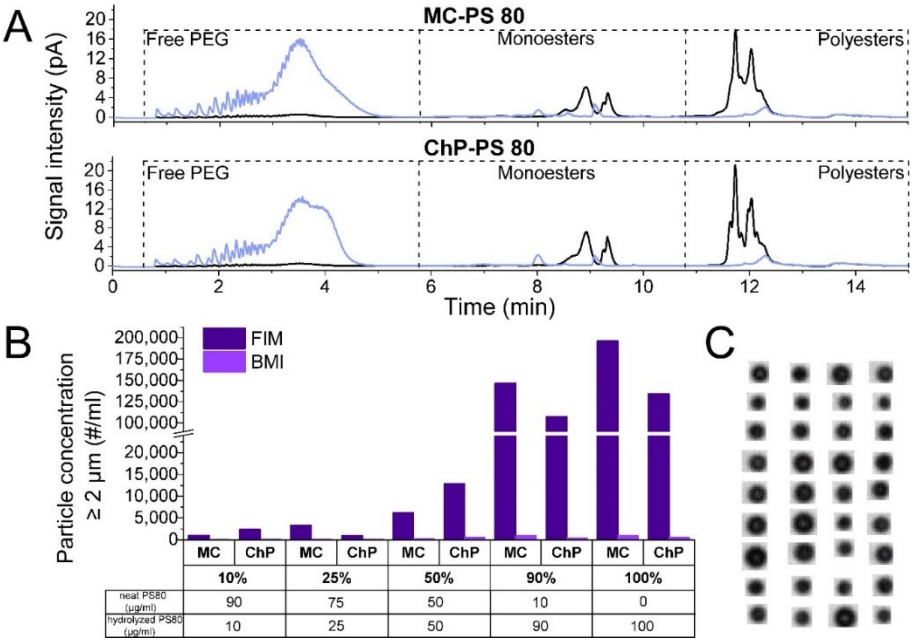

Figure 7: (A) RP-HPLC chromatograms of intact PS80 (black line) and fully hydrolyzed PS80 (light blue line). (B) Particle concentration in hydrolyzed PS80 samples measured by using FIM and BMI. Percentages indicate degree of PS80 hydrolysis. (C) Exemplary FIM images (≥ 5 µm) of a fully hydrolyzed MC-PS80 sample.

Samples of fully degraded MC-PS80 contained more particles compared to fully degraded ChP-PS80. Moreover, quantification of myristic, palmitic, stearic, oleic, and linoleic acids by using HPLC coupled with fluorescence detection supported the particle data and showed higher amounts of FFA in fully hydrolyzed MC-PS80 compared to ChP-PS80 (data not shown).

We utilized the syringe pump device to evaluate the impact of PS80 degradation on protein stability during mechanical stress. The stability of the IgG formulated with intact PS80 and hydrolyzed PS80 (10-100% hydrolysis) was examined (Figure 8 A-C). The PS80 concentration in stressed formulations was equal to 0.01% w/v and the degree of hydrolysis is described by the percentage of fully hydrolyzed PS80 mixed with neat PS80 (see Materials and Methods). For this part of the study, a single preparation and measurement was performed for each sample. Quantification of particles ≥ 2 µm showed an increasing particle concentration with increased extent of PS degradation for both PS80 grades. It must be noted that unstressed IgG formulations (controls), with PS80 hydrolyzed at 90% and 100%, contained a high background level of particles determined by FIM. Therefore, in IgG formulations with 90% and 100% degraded PS80 the particle levels in the controls were 11-15% (ChP-PS80) and 21-24% (MC-PS80) of the total particle concentration found in stressed formulations. However, most of the particles in unstressed IgG formulations were identified as droplets, originating from FFA present in the hydrolyzed material. For both PS80 grades the extent of surfactant hydrolysis on stressed IgG in terms of formed micron-sized particles was similar. An exception was the sample with 50% hydrolyzed PS80, where a clear difference in particle concentration was observed between MC-PS80 and ChP-PS80. Both unstressed IgG formulations containing partially (50%) degraded PS80 had low concentrations of particles sized ≥ 2 µm (below 10,000 #/ml). Therefore, the high number of measured particles in stressed formulations originates solely from the applied stress and reflects the functionality of PS80 (see discussion). The recovery in SEC was similar for all stressed IgG formulations with hydrolyzed PS80. A slight increase in turbidity was detected for formulations with a higher degree of hydrolyzed PS80, but no difference between formulations with different PS80 grades was observed.

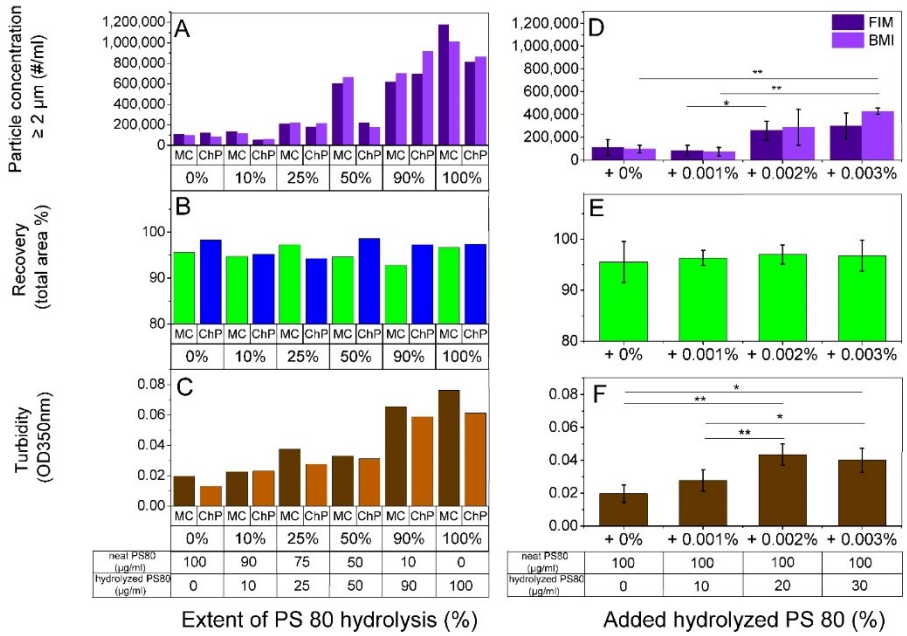

*Figure 8: Syringe pump stressed IgG formulations with PS80. X-axes present extent of 0.01% (w/v) MC-PS80 and ChP-PS80 degradation (A-C) and amount of fully hydrolyzed MC-PS80 added to IgG formulations with 0.01% (w/v) (D-F). Extent of IgG aggregation was assessed by measuring micrometer-sized particle concentration (FIM and BMI, A and D), protein recovery (SEC, B and E) and turbidity (UV, C and F). Error bars represent standard deviation of mean values from triplicate stressed formulations. *p < 0.05 was considered statistically significant and **p < 0.01 highly significant.*

Next, we investigated the impact of PS80 degradants on protein stability during mechanical stress. For this purpose, the IgG formulation with 0.01% (w/v) MC-PS80 was spiked with degradants from fully hydrolyzed MC-PS80. The amounts of MC-PS80 degradants spiked into the IgG formulation were equivalent to fully hydrolyzed MC-PS80 at 0.001%, 0.002% and 0.003% (w/v). Therefore, IgG formulations contained degradants at three different concentrations on top of the 0.01% (w/v) intact PS80. Micrometer-sized particle concentration, protein recovery and turbidity data of stressed formulations is presented in Figure 8 D-F. A significant difference (T-test, two-sided, $p<0.05$) in concentration of particles ≥2 µm in size after stress was observed for formulations with additional 0.002% and 0.003% (w/v) of degradants compared to formulations with no or 0.001% (w/v) of spiked-in hydrolyzed PS80 degradants. The latter two formulations showed a highly similar particle content after stress. No apparent loss of protein after stress had occurred according to the

SEC data. However, optical density at 350 nm was significantly higher (T-test, two-sided, $p<0.05$) for stressed IgG formulations containing 0.02% and 0.03% (w/v) spiked-in degradants compared to formulations with 0% or 0.01% (w/v) of degradants. The increase in particle concentration and optical density in stressed IgG formulations with degradation products originating from MC-PS80 hydrolysis show the apparent destabilizing properties of PS80 degradants.

Discussion

Choosing the optimum PS type and concentration to be used in protein-based pharmaceuticals is an integral part of formulation development programs. Up until now, there has been only one published study evaluating the functional properties of different PS80 grades in IgG formulations, where the focus was placed on chemical degradation of the IgG[30]. In our study we report for the first time the effectiveness of both PS80 grades (multicompendial vs Chinese Pharmacopoeia) in inhibiting protein aggregation in formulations exposed to various types of mechanical stress.

We characterized IgG formulations with MC-PS80 or ChP-PS80 at three different concentrations, as well as without PS80, exposed to shaking stress up to two weeks. Shaking increases the contact area of proteins with air-liquid interfaces. Furthermore, it causes mechanical perturbation of the interface, thereby increasing the probability of intermolecular protein-protein interactions as well as desorption of aggregated species into bulk solution[31,32]. The propensity of protein molecules to accumulate at phase boundaries makes the air-liquid interfacial stress the predominant stress factor during shaking. Nevertheless, cavitation and solid-liquid interfaces are also detrimental to the physical stability of proteins[4]. The IgG formulation without PS80 was highly unstable during shaking stress: it was visibly turbid and presented high numbers of nano- and micrometer-sized particles after three days of shaking. Thus, our model IgG was highly sensitive to mechanical stress if formulated without any surfactants. On the contrary, even after 14 days of shaking, IgG formulations supplemented with either PS80 grade showed modest to no changes in terms of turbidity and particulate content. However, a concentration dependent effect for MC-PS80 and ChP-PS80 was observed. Elevated number of particles ≥ 2 µm and > 5%

monomer loss after 14 days of shaking stress was observed in IgG formulations with PS80 of both grades at the lowest concentration (0.0004% w/v), which is below PS80's CMC of 0.0017% (w/v)[33]. A PS concentration dependent destabilization was also observed by Kiese et al., who observed an increase in high molecular weight species (HMWS) in stressed formulations consisting of 0.0025% (w/v) PS 20 (CMC of 0.011% w/v[33]) compared to formulations with 2-fold or 4-fold higher concentrations[34]. Nonetheless, we found MC-PS80 and ChP-PS80 to have a highly similar protective effect at equal surfactant:protein molar ratio and surfactant:interface area ratio[35].

Mechanical shock can result from inappropriate handling of biopharmaceutical products where a vial or syringe is dropped in its primary or secondary package. It was reported that mechanical shock treatment results in cavitation leading to formation and collapse of short-lived bubbles[24,25,36]. Consequences of these events are free radicals, local high temperatures and high-pressure shock waves, which may lead to localized oxidative and/or conformational changes of proteins. Furthermore, formation of bubbles increases the transiently available air-liquid interfaces[36]. In agreement with the cited studies, we observed an increase in concentration of nano- and micrometer-sized particles present in IgG formulations without PS80 after free fall stress and the formed mechanical shock. PS80 of both grades inhibited the formation of particles after mechanical drops, which became more apparent when the IgG formulations were dropped ≥ 5 times. PS80 was reported neither to have an effect on the thermostability of IgGs[37] nor to inhibit oxidation in IgG formulations[19]. Therefore, the inhibition of particle formation seen in formulations containing PS80 suggests interface-mediated aggregation as the predominant factor causing aggregation in dropped vials. Particle formation in formulations exposed to free-fall stress was suppressed in presence of PS80; and even at low PS80 concentrations (below CMC) solely a very small increase of micron-sized particles could be observed. Similarly, as observed after shaking stress, no difference in functionality of PS80 was determined between the two grades.

By using our customized syringe pump device, with multiple cycles we observed significant particle formation in IgG formulations with and with no PS80. The extensive protein

aggregation could have been the result of multiple stress factors, such as shear in the capillary, extensional flow at the exit of the capillary and interfacial stresses within the syringe barrels (PTFE and glass surfaces). Previously, it was reported that exposure of globular proteins to a "pure" shear stress of 10^7 s^{-1} should not cause unfolding or aggregation[38]. However, a combination of shear and solid-liquid or air-liquid interfaces had a detrimental effect on the structural integrity of investigated globular proteins[39,40]. Furthermore, adsorption and desorption of (un-)folded protein and nanometer-sized proteinaceous particles to syringe surfaces may result in remarkably higher counts in protein particles after stress, as demonstrated by Torisue et al.[24]. Therefore, the concomitant stresses during syringe pumping could allow for assessment of the functionality of PS80 grades with respect to adsorption of surfactant molecules onto the protein via hydrophobic interactions as well as competitive adsorption to interfaces.

Similarly, as with the two previous stress methods, in syringe pump stress both PS80 grades at 0.1% and 0.01% (w/v) reduced the number of micron-sized particles to a similar degree. The PS80 concentration effect on particle formation for both grades is clearer for particles ≥10 µm in size where an increase in particle concentration is correlated with a decreasing PS80 content (Figure 4 C and D). The relative size distributions for protein particles in IgG formulations with MC-PS80 and ChP-PS80 are highly similar. Independent of the grade, the highest tested PS80 concentration resulted in the lowest number of particles sized ≥ 25 µm, indicating the best properties in inhibiting formation of large particles.

At the lowest tested PS80 concentration (0.001% (w/v)), stressed formulations after syringe pump stress contained slightly higher micron-sized particle concentrations compared to IgG formulations without PS80. The destabilizing effect at low PS concentrations was also observed in our shaking stress study and previously by Kiese et al.[34]. The mechanism of the PS concentration dependent destabilization effect is currently unclear. However, the effect was observed only with PS concentrations below the CMC, suggesting specific protein:surfactant ratios to show this behavior.

The FIM images revealed a heterogeneous mixture of particles in each stressed formulation (Figure 5). The presence of PS80 in the IgG formulations and the type of mechanical stress

applied had a substantial impact of the morphology of the particles. The formation of distinct particle appearances may result from one or more predominant aggregation pathways leading to specific assembly of IgG molecules into non-native aggregates[41]. Our results correlate with the existing literature, i.e., the type of stress applied to a protein formulation has a clear impact on the morphology of formed particles[42-46]. Furthermore, particles formed in our stressed IgG formulations (independent of the type of mechanical stress) presented irregular and elongated structures, which are associated with disruption of interfacial protein gel layers formed at interfaces[43,44]. Even though PS80 at 0.001% (w/v) did not exhibit any protective role with respect to particle concentrations in IgG formulations submitted to syringe pump stress, all formulations with PS80 contained one predominant particle population regarding the morphology. In contrast, particle populations of two distinct morphologies were present in stressed IgG formulations without PS80. Therefore, even minute amounts of surfactant may inhibit certain aggregation pathways. Nonetheless, no direct connection has been found between molecular interactions and the resulting particle morphology on the micrometer scale[42]; and studying this further was beyond the scope of this study.

The two main degradation pathways for PS80 include oxidation and hydrolysis. Hydrolytic cleavage of the ester bond in PS80 can be enzymatically or chemically (low or high pH) catalyzed. Whereas chemically driven hydrolysis is generally not a concern for products stored at 5 °C or 25 °C, residual host cell proteins (HCPs) may enhance the rate of PS hydrolysis during a product's shelf life[38,39]. The presence of these enzymatic proteins is a result of insufficient downstream purification processes and can result in issues during formulation development. For both chemical and enzymatic hydrolysis, POE and FFA are the main degradation products. To avoid complexity by introducing an enzyme to the IgG formulations, the PS degradants used in our study were obtained from a base catalyzed hydrolysis reaction.

Several authors have reported the potential impact of PS 80 degradation products on the stability of therapeutic proteins during stress and storage[12,20,47,48]. IgG formulations containing FFA originating from PS20 showed particle formation upon long-term storage at

2-8°C; PS20 with fatty acid esters of variable lengths showed higher destabilizing properties compared to PS20 with only lauric acid[49]. Furthermore, Kishore et al. observed an increase in opalescence and soluble aggregate content in shake stressed IgG formulations with free lauric acid and PS20 in contrast to statically stored samples[20].

In our study, fully hydrolyzed PS80 samples, of both grades, contained high numbers of spherical, oil-drop like particles in the size range 2-5 µm (FIM). Oleic acid– the main product of PS80 hydrolysis – is liquid at room temperature and therefore the droplets can be attributed to oleic acid droplets. The presence of oil droplets could explain the significant difference in concentration of micrometer-sized particles between PS80 samples with 50% and 90% degradation products (Figure 7). For samples with the amount of PS80 degraded below 50%, the free oleic acid is most likely incorporated into micelles composed of neat PS80 and droplets are not expected to form. For particle characterization of the stressed IgG formulations used in this study, FIM and BMI were used as orthogonal methods to allow for the differentiation between proteinaceous particles induced by stress and fatty acid droplets originating from PS80 degradation. A hydrolytic 10% loss of MC-PS80 and ChP-PS80 resulted in no change in PS80 functionality, when comparing the formation of proteinaceous particles during syringe pump stress to formulations with 0.01% PS80 (Figure 8 A). A slight increase in proteinaceous particle concentration after stress was observed for formulations with 25% hydrolyzed PS80. At 50% PS80 hydrolysis, ChP-PS80 partially retained its functionally, whereas MC-PS80 showed minimal protective role in mechanical stress. This difference could be the result of different amount of FFA in the nominal 50% degraded MC-PS80 compared to ChP-PS80. In fact, the total (soluble and particulate form) free fatty acids concentration in fully hydrolyzed MC-PS80 was approximately 2-fold higher compared to the ChP-PS80 (data not shown), which could result in an overall higher concentration of FFA in the preparations at 50% nominal PS80 hydrolysis. Although FIM and BMI showed comparable results for both PS80 grades at 50% hydrolysis in absence of protein (Figure 7), it is possible that more FFA particles of different nature (e.g., stearic or palmitic acid derived particles) were present in MC-PS80 and promoted aggregation of the IgG during syringe pump stress.

Furthermore, we showed that the loss of PS80 functionality may not be related solely to the lower amount of available intact PS80 but also to the presence of PS80 degradants. When spiking a small amount of degraded MC-PS80 (0.002% and 0.003% w/v) to an IgG formulation consisting of MC-PS80 at 0.01% (w/v), we observed an increase in particle concentration and turbidity after stress. No loss of protein or formation of HMWS was observed, which is in agreement with previous results where an addition of 0.03% (w/v) of PS80 degradants did not induce HMWS in shaked IgG formulations containing 0.01% w/v of PS80[20]. No significant effect on protein stability was observed for IgG formulations spiked with 0.001% (w/v) of degraded MC-PS80. This suggests a certain threshold for the amount of degradants which must be reached in the IgG formulation to see pronounced destabilizing effects of hydrolyzed PS80.

Conclusions and outlook

The presented work shows that MC-PS80 and ChP-PS80 similarly inhibit the formation of aggregates and micrometer-sized particles in IgG formulations upon mechanical stress, pointing towards a similar functionality of the two grades. A comparable PS80 concentration dependent effect for both PS80 grades was observed, irrespective of the mechanical stress method applied: shaking, free-fall or syringe pump stress. Furthermore, very low PS80 concentrations (below the CMC values) showed minimal protection or even had destabilizing properties to the IgG during mechanical stress. The syringe pump stress had stronger destabilizing effects on the IgG than the other two stress methods; therefore, it was used to evaluate the impact of PS80 degradants on IgG stability. We found that PS80 exposed to hydrolytic degradation is no longer capable of sufficiently stabilizing the IgG during syringe pump stress. Additionally, hydrolytic degradants (of MC-PS80) may destabilize the IgG and promote particle formation. Functionality of both PS80 grades after long-term storage was not investigated, but should be considered given the recently described differences in stability of MC-PS80 and ChP-PS80[19]. Further studies would also be necessary to evaluate if the “harsh” syringe pump stress conditions correlate with current Fill&Finish manufacturing conditions. Our study points out that it is important to monitor

PS during drug product formulation development and stability testing of therapeutic proteins.

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