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Industry perspective on the use and characterization of polysorbates for biopharmaceutical products

Part 1: Survey report on current state and common practices for handling and control of polysorbates

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Abstract

Polysorbates (PS) are widely used as a stabilizer in biopharmaceutical products. Industry practices on various aspects of PS are presented in this part 1 survey report based on a confidential survey and following discussions by 16 globally acting major biotechnology companies. The current practice and use of PS during manufacture across their global manufacturing sites are covered in addition to aspects like current understanding of the (in)stability of PS, the routine QC testing and control of PS, and selected regulatory aspects of PS. The results of the survey and extensive cross-company discussions are put into relation with currently available scientific literature. Part 2 of the survey report (upcoming) will focus on understanding, monitoring, prediction, and mitigation of PS degradation pathways to develop an effective control strategy.

Key words: Industry practice, biotechnology products, polysorbate, survey report, surfactant(s), protein(s), stability

Introduction

Given the prominent role of polysorbates (PS) as a stabilizer in protein-based products, recent industry practices encompassing various aspects of PS within the biopharmaceutical industry were assessed in a benchmarking study.[1] Experts of member companies of a manufacturing and quality expert group (MQEG), under the umbrella of the European Federation of Pharmaceutical Industries and Associations (EFPIA) formulated relevant survey questions. The survey was open to 16 globally acting major biotechnology companies from September 2019 to April 2020. The survey questions related to the manufacturing and control of drug substance and drug product, and covered aspects of supply, use of PS and alternative surfactants of different grades during manufacturing of clinical and commercial products. Additionally, analytical methods to characterize and monitor PS, knowledge about the mechanistic understanding of PS degradation and detectability as well as current strategies to model and mitigate PS instability were also queried. Lastly, the survey probed selected regulatory aspects related to use and control of PS.

Polysorbate is a key excipient for biologics formulations to stabilize proteins. PS prevents protein losses through adsorption onto surfaces and protects proteins against physical degradation caused by interfacial stress. In fact, Polysorbate 80 (PS80) was part of the formulation of the first marketed monoclonal antibody product (Orthoclone OKT3, 1986). PS80 and polysorbate 20 (PS20) are utilized extensively by biopharmaceutical companies in both clinical and commercial products.[1] All of the surveyed companies utilize PS20 and

93% use PS80 within the portfolio of their biopharmaceutical products. Alternatives to PS are relatively rare with only poloxamer 188 being stated as a surfactant currently used by 27% of the participating companies but is included in only 4% of their products (vs. 96% products for PS). This is consistent with the formulations of commercially available antibodies described in a recent review article.[2]

PS has many beneficial functional properties, is accepted by regulators (e.g., by FDA Inactive Ingredients database[3]), and does not pose major safety concerns in the concentration range and the most common routes of administration used by biopharmaceutical products.[4] As a stabilizer PS80 and PS20 are usually present in drug products in the range of 0.01 to 0.05% (w/v) (stated by 60% of companies) with higher levels (e.g., >0.1%) in only a few instances.[5] Not surprisingly, this is within the concentrations stated for 126 commercially available antibodies[2] with a PS20 range between 0.004 to 0.2% (w/v) and a PS80 range between 0.001 to 0.2% (w/v). According to literature and industry experiences, PS concentrations were never present below their respective critical micelle concentrations (CMC range at 25 °C: 0.0018-0.009% (w/v) for PS20; 0.0009-0.002% (w/v) for PS80).[6] The PS levels may be higher for other medicinal products like vaccines (e.g., 0.225% PS80 for Fluad Tetra – Influenza[7]), traditional Chinese medicine injections (e.g., up to 2% PS80[8]), or when used as solubilizer in small molecules preparations for micellar injections (e.g., Taxotere, 540 mg/mL (54%) PS80 docetaxel for injection.[9]

In recent years, there have been reports on PS-related stability issues related to the use of polysorbates in biopharmaceutical products, mainly due to PS degradation induced particle formation by free fatty acids (FFA).[10-17] Due to these challenges and the importance of PS in pharmaceutical products, the scientific interest and focus on PS is steadily increasing, as evidenced by the rising number of biomedical literature referenced in PubMed (www.PubMed.gov) containing both polysorbate and protein as keywords in title or abstract of the publication (Figure 1).

Although there are multiple recent reviews on different aspects of PS such as their use in commercialized biopharmaceutical products,[2] safety aspects,[18-23] stabilizing properties for proteins[6, 24-26] and their analysis,[27] only a few papers reflect industry-wide positions and understanding on their role as protein interfacial stabilizer,[14] and summarize suggestions towards appropriate control strategies and lessons learned.[10, 28] In this survey report we strive to provide an up-to-date, end-to-end view of the 16 participating biotechnology companies on the use and characterization of PS for biopharmaceutical products. After the survey responses were obtained and anonymized, the members of the participating companies reviewed, interpreted, and discussed the data.

The survey contained a total of 137 questions, covering current practice and use of PS across their global sites for 81% of the companies. Specific responses to one site, either

within the same global company network or because of a single site company were provided by 25% of the participants. The data are presented based on a total number of given answers to a specific aspect and may not total to the 16 participating companies, in large part, since certain questions may not be relevant to a given company. Responses are preferably expressed as percentage (%) relative to the total number of responding companies unless only a subset of companies provided feedback. In the latter case, both the number of the companies providing the response of interest and total number of responding companies was provided. The outcome of the survey and extensive cross-company discussions are summarized and put into perspective in a series of two publications. Part 1 (this manuscript) provides insight into the heterogeneous nature of PS, the current practice and use of PS during manufacture of biotech products, current understanding of the (in)stability of PS, the routine QC analysis and control of PS, and selected regulatory aspects of PS. Part 2 (upcoming) provides in-depth considerations related to the mechanistic understanding, predictive models, suitable advanced characterization methods of PS degradation, and an appropriate PS control and mitigation strategy from an industry and scientific point of view.

Complexity of Polysorbates

Polysorbates are nonionic surfactants consisting of a hydrophilic polyoxyethylene sorbitan (POE) head group and a hydrophobic fatty acid side group.[15] They are synthesized via several reaction steps, with a target structure of POE sorbitan monoesters consisting of X+Y+Z+W = 20 ethylene oxide units per molecule, although it can vary.[29-31] PS manufacturers typically use one of the 2 common synthetic routes (esterification with fatty acids followed by polyethoxylation or the reverse order), and the selected route and sourcing raw material may have effects on the composition of the final PS product,[32-34] as summarized in Table 1. As a result, different manufacturers can supply PS products presenting different levels of characteristic components, residues of process intermediates, degradants, and impurities, as shown in Figure 2.

The structural heterogeneity of PS, alongside with degradants and impurities present in commercial lots, may directly impact the PS functional properties and quality attributes in biopharmaceutical products,[15] and pose significant challenges in terms of analytical characterization.[27, 35]

PS is used at various stages during manufacturing of biotech products and for easier reading the following designations are utilized hereafter: (1) neat PS as manufactured, supplied to, and used within the biopharmaceutical industry will be denoted as *PS products*; (2) when

diluted for processing as *intermediate PS solution,* and (3) when present in a drug substance or drug product as *formulated PS*.

Inherent heterogeneity of polysorbates

As illustrated in Figure 2, PS subspecies are primarily composed of polyoxyethylene (POE) sorbitan monoester, but it also contains other components such as POE sorbitan polyester (e.g. di-, tri-, and tetra-esters), and mono- and di-ester of POE isosorbide and POE.[36] Intermediates and unreacted compounds such as POE sorbitans, POE isosorbides and POE are also common (as well as sorbitan, isosorbide). As a result, the target structure of PS20 and PS80 was found to only account for 18~23% and ~20% of total species detected in commercial PS lots, respectively, by a LC-ELSD PS subspecies assay.[37] In addition, up to 40% and 70% di- and tri-esters were found in PS20 and PS80, respectively.

Polysorbates are heterogenous mixtures made of different fatty acid esters: in PS20, the main fraction containing lauric acid makes up 40–60% of the mixture while in PS80 the main fraction containing oleic acid accounts for more than 58% of the mixture. The variability in fatty acid ester distribution was demonstrated by a study carried out on 16 different PS80 batches using a high-performance liquid chromatography coupled to charged aerosol detection (HPLC-CAD).[38] The samples from Croda (East Yorkshire, UK), Kolb (Hedingen, Switzerland), Merck (Darmstadt, Germany) and NOF (Tokyo, Japan) showed amounts of oleic acid ranging from $67.8 \pm 0.7\%$ to $96.6 \pm 1.4\%$. Furthermore, petroselinic acid,[39] a double-bond positional isomer to oleic acid, was identified in all batches, although this is not currently required to be reported in the certificate of analysis (CoA) as per the multi-compendial monograph. Polyunsaturated and long-chain fatty acids, typically present in PS in low abundance, may further contribute to complexity via oxidative phenomena.[40]

Another source for structural variability is due to the different reactive hydroxyl groups of the starting mixture of sorbitans and isosorbides which result in oxyethylates with an average number of ethylenoxide (EO) moieties per reactive hydroxyl site greater than 5.[29, 41]

The heterogeneous nature of PS was illustrated in a study where mass spectrometry profiling with optimized UPLC separation revealed the presence of thousands of different PS species (i.e., approximately 4000 parent adduct ions were counted for a compendial PS20 grade).[42] In addition, differences in profiles between samples from different suppliers were observed in terms of relative amounts of major PS subspecies,[36, 43, 44] confirming variations in the composition of PS can be present between suppliers and also between PS product lots from a given supplier.

A summary of the sources of heterogeneity in PS stemming from the synthetic process, structural features affected, and observed changes on storage are shown in Figure 1 and tabulated in Table 1.

Impurities in polysorbate products

Although fatty acids are primarily present in PS products in the form of esters, they are also present as unreacted FFAs. The level and type of residual FFA in PS products was found to vary based on the analysis of multiple lots and different grades of PS20 or PS80 products.[45]
Additionally, impurities derived from raw materials used for PS production also need to be considered and carefully evaluated. For example, visible particles observed in a monoclonal antibody DP solution were reported to be linked to 12-tricosanone, an impurity present in PS80, and not derived from the degradation of the surfactant itself (the origin of 12-tricosanone is most likely to stem from plant oils used as raw materials for PS synthesis).[49]
Other known impurities that may be present in PS products are peroxides and trace metals. These contaminants may not only impact PS stability, but also compromise the stability and functionality of the active pharmaceutical ingredient (API) intended to be stabilized.[52]

Use of polysorbate during manufacturing (sourcing, storage, in-coming testing, and handling under GMP conditions)

Based on the discussion above, quality control of PS products as a raw material for the manufacture of biotechnology products becomes critical. Therefore, the industry practice on testing incoming PS batches and impurities were included in the survey with results summarized and discussed in this section.
The intrinsic structural variability and heterogeneity of PS is a result of harsh synthetic conditions, the presence of impurities, variable natural starting materials, and the potential for further degradation and oxidation reactions during its shelf life. Therefore, it is of paramount importance that aspects such as sourcing, in-coming testing, and handling practices within manufacturing (e.g. aliquoting from original container, exposure to air/light, humidity, storage at controlled temperature) must be considered to establish proper control strategies.[51] The survey revealed that different approaches within the same company or depending on the use of PS exist, however there is a clear trend to harmonize handling and control of PS raw materials.

Sourcing, storage, and expiry of polysorbate raw materials

About two-thirds of the participating companies source their PS20 from a single manufacturer, and about half source PS80 from a single manufacturer. The supply chain of PS is complex, indicated by the response of the participants, such that multiple suppliers of

the PS products are often used instead of different manufacturers. This might reflect that the majority of the participants are global companies with manufacturing sites all over the world, necessitating more than one PS supplier. About 50% of the companies use higher purity grades, e.g., refined grades with low peroxide specifications, or grades that comply with particular bioburden and/or endotoxin limits.

When considering that a typical biopharmaceutical product contains PS at a concentration of about 0.03% (mid value of the most common range per survey of 0.01 to 0.05% w/v) one would calculate for a commercial process of 100 L drug substance, a demand of 30 g PS per batch. Nonetheless, more than 60% of the participating companies procure PS products in large containers (>1 kg). Fifty percent of the companies indicate that they purchase containers for multiple use. Additionally, the majority of companies (~70%) also reported procuring PS containers for single use, which coincides with about 70% procuring small sized PS containers with amounts of <1 kg; these findings reflect different practices across sites or products. More than half of the companies purchase one container size of PS while the others reported sourcing more than one size depending on the particular use or site practice. The relatively common use of sourcing more than one container size within a given company is reflected in the total responses exceeding 100%, as shown in the Table 2 below.

PS is mainly (69%) received in brown glass bottles to protect the content from light, and under inert gas, predominantly nitrogen (75%), but as shown in Table 2, other protection measures exist. The preferred storage temperature of PS products after receipt until first opening is room temperature, although some companies prefer to store it refrigerated.

The majority (88%) of companies do not repackage into smaller PS aliquots or into a different container after receipt. However, during the evaluation of the survey, it was commented by some companies that they work with suppliers to repackage into smaller containers, illustrating the need for smaller PS packaging sizes.

Additional questions probed storage and handling after first opening the PS container, whether for sampling or use. Over half of the participating companies control light exposure by protective measures or limit the maximum exposure time to light. About two-thirds of the responding companies apply (or reapply) an inert gas overlay, and some companies indicated a reduced expiry once the container is opened for sampling, even if followed by an overlay of inert gas. Over 80% store protected from light and storage of opened containers at either refrigerated (60%) or controlled room temperature (50%) was relatively evenly distributed.

Practices to assign the expiry of PS differ among the participating companies. About 50% use the shelf life assigned by the manufacturer for unopened PS containers, and the remaining companies indicated that they define their own expiration period.

In-coming quality control (QC) of polysorbate products

Approaches to reduce sampling and testing for starting materials can be implemented according to the requirements of different pharmacopoeia and authorities.[53] According to the European Union good manufacturing practice (GMP) guidelines, identity testing of the contents of each container of starting material is a requirement.[54] If validated procedures are in place, identity testing of a representative number of containers can be applied to assure that containers are correctly labelled. The viewpoint of the member companies with respect to sampling and testing of PS as incoming materials were addressed within the survey but the answers to these questions were rather diverse. All companies test incoming materials upon receipt by identity testing (100%), by either sampling each container or according to a sampling plan; about 50% of companies discard the sampled PS containers. Most (~63%) do not test again prior to use. Additionally, all companies confirm that the PS product complies with compendial specifications with 81% of companies performing routine testing of all compendial parameters. Interestingly, one company performs a non-destructive, spectroscopic identity test and one company confirms compliance with compendia only once per year. During testing, almost all companies (>90%) perform compendial analysis of fatty acid distribution as fatty acid methyl esters (FAMEs), but no company indicated that an assessment of fatty acids beyond those specified in the pharmacopoeias is performed. It was found that no company assesses FFA in PS products as an incoming test. Peroxides are determined in PS products by 88% of companies mainly using a platform assay based on Amplex red/ultra[55, 56] or xylenol orange.[57] The majority of participants use trace metal assays for PS characterization primarily for investigational purpose or supportive analyses during development for formulated PS, only 38% test metal contamination in PS products.

Polysorbate handling in cGMP environment

After receipt, testing, and storage, the next typical step in the lifecycle of PS is handling in the manufacturing suite. According to the survey results, PS is never added neat (in bulk) to the process stream, but rather is added in the form of an intermediate PS solution prepared by dilution (by weight) of the neat material in a suitable diluent. About half of the companies use water as the diluent, and the other half use formulation buffer. Target concentrations of the intermediate PS solution range from 0.5% to 25% with 4% or 10% (w/v) being the most common concentration listed (by 44% companies for each). Half of the companies filter the intermediate PS solution through 0.2 µm filters, with PVDF and PES being the most commonly employed filter membrane chemistry. Amongst those that filter the intermediate PS solution, 5 out of 9 report adsorptive PS losses, although no company reports issues with

filter clogging. One third of those same companies filter the intermediate PS solution on the same day when they are used in the process. In general, the intermediate PS solution is stored under light protection (~70%) and at ambient temperature (~90%). Storage containers for the intermediate PS solution vary among companies and sometimes within a company; single use bags are the containers used by ~50% of the companies. The intermediate PS solution hold times range from freshly prepared to <30 days (4%), with fresh preparation or use within 1 day being the most common response (by 81%) followed by use within 2-7 days (38%). Most companies (>85%) indicated that intermediate PS solutions were used in the process without further dilution.

The protective effect of PS on the active protein during freezing / thawing of the drug substance, which is commonly stored in a frozen state, has an impact on the formulation practice of the participating companies. The majority (>90%) reported that they produce (pre-) formulated drug substance, i.e., they add the excipients including PS to the liquid drug substance before long term storage. About one-third also indicated that PS may be added during DP manufacture, depending on the specific project requirements. One company reported to exclusively adding PS at the drug product stage.

The final series of questions relating to PS handling queried about its fate during processing and use, with a specific focus on its adsorption to surfaces. Over half of the respondents (56%) reported that PS adsorptive losses to surfaces was detected, 19% reported no losses, and 25% indicated that losses, if any, were not known. In the cases where adsorption or losses were observed, the chemistry of the surfaces was diverse; manufacturing materials, e.g. filter membranes, tubing, and storage bags were most prominently listed as well as losses during in-use clinical administration simulation studies, which is in accordance with published literature.[58, 59] Potential adsorptive losses are generally understood and well characterized during process development, and mitigation measures (if necessary) may include saturation of filter surfaces, use of larger volume surge tanks, recirculation, or flushing to waste.

In general, the practices of the different participating companies regarding the supply chain and handling of PS are diverse, probably due to internal historical reasons, product specific requirements and a lack of common industry practice. Figure 3 shows a typical workflow of PS handling and storage within a cGMP environment for manufacturing of biopharmaceuticals, including the percentage of surveyed respondents adopting the listed practice.

(In)Stability of polysorbate formulated into biopharmaceutical products

First indicators and root cause analysis

Polysorbates are known to undergo degradation in biologics by two main mechanisms: (auto-) oxidation and hydrolysis.[15] The latter can be subdivided into either chemical or enzyme-induced hydrolysis of the ester bonds. Chemical hydrolysis of PS can be promoted by heat, acidic, or basic conditions.[60] At a low storage temperature (e.g., 2-8°C) and the pH range common for biopharmaceutical formulations (e.g., pH 5-8), chemical hydrolysis of PS is uncommon.[60, 61,] However, enzymatic hydrolysis via esterases or lipases was reported by 69% of participants. Oxidative PS degradation may be caused by light exposure, transition metals, or oxidizing agents,[62, 63] and PS of different grades may break down at different rates due to differences in micelle properties and PS fatty acid composition.[64]

Most of the survey participants indicated that they began to observe PS degradation within the past 5-10 years across all phases of programs, although a distinct starting year of this observation could not be defined. The survey reveals that both PS80 and PS20 are susceptible to degradation, although not much is known yet or still poorly understood about differences in their respective degradation kinetics. Hence, to date there is still no clear indication which of them might be better suited for the use in biopharmaceuticals. It is important to note that no decrease in PS content was reported in lyophilized products. This difference is due to the fact that water, as reaction medium, plays an important role in mediating PS degradation. Thus, removal of water may be leveraged to mitigate PS degradation for those programs that are prone to PS degradation and amenable to lyophilization.

The extent and specific type of PS degradation observed in products were probed in the survey. Approximately two-thirds of companies observed PS degradation through both hydrolysis (69%) and oxidation (63%) pathways in at least one of their drug products. Enzyme-induced degradation was identified as the primary cause of PS hydrolysis of the companies reporting this issue (~90%) with chemical hydrolysis only playing a minor role. Among the companies dealing with PS degradation, PS oxidation was observed in less than 25% of their products (Figure 4). It was reported[63] that oxidized PS was able to retain its functional activity as stabilizer. PS enzymatic hydrolysis was observed at a different frequency of occurrence in different companies, for example with 8% of participants reporting enzyme hydrolysis in 50-75% of their products (Figure 4).

Several potential initial indicators for PS degradation were examined in the survey, such as particle formation, increase of PS degradants, or a general decrease in PS content. As shown in Figure 5, a decrease in PS content was found to be the predominant initial indicator of PS degradation in liquid product presentations whether vials or pre-filled syringes,

whereas, formation of subvisible particles or an increase in FFAs seem to be another important indicators for PS degradation, but less frequently observed. The underlying reason might be the applied analytical tool set. As reported in section “Routine analysis and quality control of PS during manufacture and storage”, PS content assays are typically used by all companies during product development as opposed to analytical methods capable of measuring PS degradants.

Overall, a lower percentage of participants reported indicators for liquid product in pre-filled syringes compared to vials. First, it has to be noted that the absolute number of assets in vials compared to pre-filled syringes has not been revealed, but it can be assumed that development is still dominated by the vial format based on the number of responses received as well as commercialized products.[2] Thus, incident reports for the pre-filled syringe presentation are likely less robust. Second, as reported by Gotanda,[65] the probability of detection of certain types of visible particles tends to be lower in syringes compared to vials or ampoules which may also further explain the relative lower % reported for syringes (Figure 5). Silicone oil is also reported to solubilize FFA to a certain extent, although the time of onset for the formation of FFA particles was equivalent between degradation performed in syringes coated with silicone oil and non-coated ones.[66]

The survey further probed additional detail on different storage conditions related to PS degradation. PS degradation was generally not observed at early time points for liquid products in vial (69%) and in pre-filled syringe (50%). For those companies observing PS degradation during storage and responded to the survey questions, the degradation in vials during storage (63%) was less frequent at 5 °C (44%) than at 25 °C or 40 °C (63% for both). However, the PS degradation in pre-filled syringe during storage (31%) was apparently observed at equal frequency at all storage temperatures examined, e.g. 5 °C, 25 °C and 40 °C (31% for each). Consistent with results shown in Figure 5, decrease of PS content was observed as predominant initial indicator of PS degradation during storage for liquid product in both presentation, which is then followed by an increase in FFA level.

Over 60 % of the companies have been successful in identifying the primary root cause of PS degradation in current or historical assets which is also due to recent advances in analytical capabilities. For enzyme-induced hydrolytic degradation, 75% of the participants that responded in the survey were able to identify the specific enzyme(s) involved and most reported activity from multiple enzymes (78%). Forty percent of the survey participants also indicated unidentified degradation phenomena or a reduction of PS content, such as hydrolytic degradation of PS without identifiable enzyme activity (unknown or below method detection capabilities). The companies who experienced PS oxidative degradation in drug product (69%) also observed it in the placebo (of same composition without the API) in the majority of cases (55%), suggesting impurities in excipient may also play a role in oxidative

PS degradation. Nevertheless, the source of oxidative degradation is less understood since a significant number (82%) did not identify the specific cause or did not answer. Four companies (out of 10) identified the source for oxidative degradation as known, with factors (sometimes combined) such as trace metals, peroxides, impurities, or light (see also a published example[67]).

From the 10 responses received, some companies started observing PS degradation as early as 2009 (Table 3). In the past 5-8 years, the number of companies observing PS degradation (5 out of 10) increased. The first observation of PS degradation was believed to be attributed to high protein concentration formulations (3 companies), changes in drug substance purification process (2 companies), and implementation of a new stability indicating PS content method (1 company) (Table 3).

In-depth discussion of enzyme-induced polysorbate degradation

The survey results discussed previously show that PS degradation is a complex issue, and a clear and simple root cause cannot always be assigned. Most often the degradation cannot be ascribed to a single root cause and is more likely multi-factorial, reflecting the challenges the industry is facing when ascertaining a mechanistic understanding of PS stability/instability. To gain some additional insight into the current most-studied and relevant PS degradation phenomena (i.e., enzymatic hydrolysis), more detailed questions on a variety of factors that may affect enzymatic (Host Cell Protein, HCP-induced) PS degradation were evaluated in the survey. Moreover, the survey explored whether various factors also impacted the formation or detection of insoluble PS degradation products, as shown in Table 4.

Storage temperature and storage time (56% each) are identified as the factors that have highest influence among the ten parameters evaluated. This result is in line with enzyme kinetics of HCP-mediated PS degradation where both temperature and time play a critical role. Protein concentration and HCP concentration (both 38%) are ranked as the second highest influencing factors for HCP-induced PS degradation. This is likely because both protein concentration and HCP concentration may be related to the total amount of PS-degrading HCP(s) in the drug product. Concentration of specific enzyme (31%) ranked as the third influencing factor, although those specific enzymes were not further described. The comments provided in the survey, however, did shed some light on current industry understanding of specific enzymes that induce PS degradation. For example, phospholipase B-like 2 (PLBL2), a commonly known lipase that may be present in protein products from a CHO-based process at relatively high abundance, was regarded as one of the major culprits for PS degradation a few years ago. However, it is now believed that HCP-mediated PS

degradation may also be induced by some other co-purified host cell proteins at a level below detection limit in the ppb range (see part 2 for more detailed treatise). This is consistent with a recently published case where PLBL2 could not be confirmed as root cause for PS degradation.[68] The comments may have also explained an emerging novel approach to measure the enzymatic activity of PS degrading enzymes during the optimisation of the manufacturing process.[69-71] The least influencing factors were attributed to pH, PS purity/quality, and primary packaging. Enzyme activity is also pH-dependent, whereas pH was not ranked high as influencing factor. This discrepancy is likely due to the fact that the typical formulation pH range used for therapeutic protein has no significant impact on enzyme activity.

When considering factors influencing formation of insoluble degradation products resulting from PS degradation, seven parameters were evaluated in the survey. Thirty-one percent of the companies investigated suspected root-causes for PS degradation-dependent formation of insoluble products. Based on the survey responses, temperature and storage time (31% for both) are identified as main factors influencing the formation of insoluble degradation product. PS concentration and protein concentration (19% for both) are ranked as the second highest influencing factors on the formation of insoluble degradation products. The least effects have been attributed to primary packaging, pH and PS purity/quality. It is noteworthy that some companies responded that primary packaging (25%), and PS purity/quality (19%) are not contributing to formation of insoluble degradants. Ranking of the influencing factors is consistent between HCP-mediated PS degradation and insoluble degradant formation. This is likely because free fatty acid released from HCP-mediated PS degradation was frequently found to form insoluble particles.[10]

Five parameters were considered in relation to the *detectability of insoluble* degradation products during PS degradation, with results shown in Table 4. Based on the responses received, it appears that the temperature and primary packaging, e.g. vial or pre-filled syringe, (19% for both) has the highest influence on the detectability of insoluble degradation products. These findings are in line with recent literature on the effect of siliconized packaging materials.[66, 72] In another study, it was found that glass leachables (such as $NaAlO_2$ and $CaCl_2$) may serve as a nucleation factor and induce the formation of FFA particles. [16] It is noteworthy that 25% and 31% of the companies have not seen a correlation of temperature and primary packaging, respectively, for the detectability of insoluble degradation products. In addition, over 30% of the responders did not observe any influence of PS concentration (38%) or pH (31%) on the detectability of insoluble products. In all cases, most of the companies do not know whether these 5 parameters impact the detectability of insoluble degradation products, perhaps due to limited knowledge or lack of systematic study. This suggests that this area would benefit from additional studies.

Routine polysorbate analysis and quality control of formulated product during manufacture and storage

A PS content assay is an important element of the control strategy for biopharmaceuticals containing PS, especially in those instances where the surfactant level can change over time and one or more quality attributes are impacted. [51] Whereas 65% of the companies participating in this survey observed PS degradation, as mentioned previously, all companies participating in the survey (100%) perform an assay to measure the PS content in all or some of their products. The driver for implementing PS content testing as a routine assay stems from either internal requirement (69%) and/or agency expectations (75%). More than 90% of the respondents indicated that their company evaluates the PS content during product development, and over 60% utilize a content assay in their commercial products. Approximately 30% of the respondents indicated that they evaluate PS content for investigational purposes, although it is anticipated that the assay could be used for a wide range of purposes.

Therefore, the use of a PS content assay can be considered as a best practice used in all phases of product development, from early development through to commercialization. The PS content assay is established as a platform assay by all participating companies and used across multiple projects, and 87% have validated their platform method. Approximately half of the companies also implemented a validated method that is product specific.

When surveying the quality status of the test samples and assay (Figure 6), a PS content assay is typically implemented for GMP batch release testing (88%). Of those, 50% include the content assay with acceptance criteria during product stability. In addition, a majority of companies utilize a PS content assay for characterization purposes in developmental (75%) or GMP stability studies (69%).

The PS content method is used for sample types ranging from process intermediates to the final drug substance (94% of companies) and drug product (100% of companies). Content determination on process intermediates is performed by 50% of the companies, although process intermediates do not always contain PS. It was found that 88% companies perform the same methods for different process samples, and 75% disclosed that their content method is stability indicating.

Specific survey questions probed the types of assays used to quantitate the level of PS as well as some associated method details (Table 5). Among the assay types, the majority of respondents utilize a chromatographic-based method (94%), followed by fluorescence micelle (44%), and thiocyanate complexation (19%). Chromatographic methods appear to be state-of-the art, providing high versatility with respect to sample preparation, column choices

or detection modes. Only 19% of the participants consider changing or optimizing this type of assay.

Those that use the traditional fluorescence micelle assay employ the fluorescent dye N-phenyl-l-naphthylamine (NPN) and a flow injection approach. Sample preparation prior to injection is performed by 5 out of the 7 that use this type of assay and 3 of 7 utilize a plate format. Of all survey participants using the fluorescence micelle assay in all its variations, 5 of the 7 consider changing to other analytical assays due to drawbacks such as low specificity and dye interactions with hydrophobic components such as silicone.[73] All of the companies still using the thiocyanate assay are considering to implement another PS content assay.

When it comes to selecting the PS standard, there are multiple sources or options that can be considered, including PS from any supplier of the same grade, the same supplier and grade, the same batch as in the test sample, an in-house standard, and/or a compendial standard. One fourth of the respondents reported utilizing a variety of input PS for their standards due to different practices within the same company; thus, the tally exceeds 100%. Half of the respondents indicated they use the same supplier and grade in the RS as in the test sample; 31% even use the same batch of PS as in the test sample. One fourth of the respondents indicated that they utilize an in-house PS reference standard, and 19% utilize the same grade but not necessarily the same supplier. Lastly, 13% claimed that they employ a pharmacopeia reference material.

Regulatory interactions related to polysorbate specifications, degradation, and control

PS used in a therapeutic protein product needs to be compliant with a compendial monograph. Pharmaceutical compendia such as Ph. Eur., USP, and JP define the quality, characteristics, and composition of PS (PS20 or PS80) raw material components and impurities. While generally harmonized, some minor differences (e.g., description of appearance and solubility, heavy metals content) can be noted between the monographs. However, sufficient harmonization exists such that a multi-compendial material can be sourced for globally distributed products. For multi-compendial PS80, a harmonization effort led to common requirements that the fraction of esterified fatty acids derived from oleic acid is ≥58%, with lower allowable limits of linoleic (≤18%), palmitic (≤16%), palmitoleic (≤8%), stearic (≤6%), myristic (≤5%), and linolenic (≤4%) acid esters. In 2015, the Chinese Pharmacopeia (ChP) published requirements for an *injectable grade of PS80*, with higher levels of oleic acid (≥ 98.0%) and all others fatty acids below 0.5%. However, this requirement for use in all injectable products was revised in its 2020 monograph, essentially

based on application and function, e.g. products that use PS80 as a protein stabilizer are no longer mandated to use ChP PS80 (*For Injection*). In response to survey question about whether the regulatory authorities in China have requested a change to ChP PS80 (*For Injection*), 13% companies were asked to make this change prior to issuance of the 2020 revision. Both requests were for programs during late stage (1 at phase 3 and 1 at commercial stage). With the update of ChP 2020 for PS80 monograph (*For Injection* grade now referred to as PS80 type II), there is no longer a regulatory motivation to proactively switch to PS80 type II. Indeed, there is some evidence that the high oleic acid (≥ 98.0%) grade is not superior to the standard multi-compendial grade (≥58%).[62, 64]

An overview of regulatory interactions and requests associated to PS specifications and control that were probed in the survey is listed in Table 6. Particularly in the late phase development programs and for licensure, release specifications for PS content in drug products seem to be industry practice (81%), whereas specifications at the DS level are less common at this development stage (31%). End of shelf life PS specifications were reported as being requested by the regulatory authorities by 33% of the companies. Although different rationales were used by the responding companies to justify the acceptability of PS degradation to regulatory authorities, all responding companies considered no change to product quality attributes as a suitable rationale. Additional survey data showed that consistent demonstration of maintaining the minimal effective PS levels (7 out of 9) or suitable surfactant activity despite decreasing PS levels (5 out of 9) were also used as supportive rationales. Finally, 5 out of 9 respondents employ supportive development studies to justify the acceptance of PS degradation. Two of 9 responding companies restricted the shelf life of their product although other justifications were available.

Among the 11 companies that observed PS hydrolysis, 3 were asked by regulatory authorities to identify the causative agent for PS hydrolysis. One of the companies presented a causative agent even though the specific agency did not request it. No participants have received requests from regulatory authorities to tighten the specification of visible or subvisible particles to be below respective compendial limit due to observation of PS degradation. One of 10 participants include PS degradation data in their clinical submissions. For their commercial program, 5 out of 10 participants include a rationale for PS degradation. However, this is likely a molecule-specific strategy, as 4 out of 9 respondents include a degradation rationale in their submission irrespective of phase whenever PS degradation was observed. Three out of 14 participants received request from regulatory authorities to identify particles, although only for regulatory filling at Phase III or commercial programs.

With regards to the regulatory filling strategy related to PS control, around 40% of responses noted to potentially file an IND with PS content only as characterization assay (i.e., without acceptance criteria defined). On the other hand, 81% of participants are currently preparing a

BLA/MAA with data on PS degradation or specifications. Overall, it appears that participants prefer to monitor PS content for early phase program as a characterization method. However, they are willing to investigate and provide more data in the filing when PS degradation was observed irrespective of development phase. Participants tend to improve the PS control strategy for late or commercial phase program, with a majority implementing a release specification for PS content, likely in response to increasing scrutiny from regulatory authorities.

Discussions and Conclusion

Material Sourcing and Handling

All survey participants recognize the importance of PS and take active controls to ensure its quality and function. This starts with an end-to-end control strategy, from PS raw material supplier to usage in manufacturing, as depicted in Figure 3.

As a raw material that is commonly included in the final therapeutical product, all companies pay close attention to PS grade, purity, functionality, and supply chain reliability. They strive to source and use high quality, multi-compendial grade PS products. The majority of companies source their PS products from a single manufacturer. There's a strong preference for small quantities of PS products (e.g., <0.5 kg per container), for the supplied PS product to remain protected using the suppliers' original container, and to implement recommended conditions to preserve stability and integrity. Protective measures include protection from light, application of an inert gas overlay, storage at sub-ambient temperature, single use after first opening of the original container, and assignment of an appropriate expiry date.

Quality control of PS product is an obligatory measure. Currently, all companies perform incoming quality control tests on each PS product lot received, by either sampling from each container or according to predefined sampling plans. In view of quality attributes tested, all companies perform in-coming identity and compendial conformance testing, and most companies test for all compendial parameters.

While practices to assign the shelf life of PS product differ quite a lot amongst the participating companies, there is a general agreement that shelf life assigned by the manufacturer for unopened containers should be used, and repacking – even with inert gas overlay – may impact the shelf life of the PS product.

During product manufacturing, any preprepared PS intermediate solutions are either used fresh, or stored protected within a pre-defined hold time and temperature, prior to addition to formulate drug substance or drug product bulk.

Stability

Survey participants are aware that PS80 and PS20 are susceptible to degradation, and some participants have directly experienced PS degradation in final drug product formulations. The key indicators are a decrease in PS content, visible and/or subvisible particle formation, or increase of degradants, with hydrolysis being the predominant degradation pathway. While an observation of degradation typically triggers investigations, a root cause cannot always be definitively identified due to the complex nature of the underlying cause. However, PS degradation via HCP mediated enzymatic hydrolysis has emerged as a leading cause of degradation.

Although the degree of mechanistic understanding of degradation varied among the companies and is incomplete as a whole, storage temperature and duration are identified as the factors that have highest impact on HCP-induced PS degradation. Temperature and primary packaging, e.g. vial or pre-filled syringe, may have the highest influence on the detectability of insoluble degradation products.

Routine Testing

Polysorbate content in final finished drug product is the most important quality attribute that is subject to monitoring by all survey participants. Most companies include PS content in their GMP batch release specification. These specifications are often provided to regulatory authorities, particularly for late-stage and commercial products. In addition, many companies also use the PS content test for a variety of purposes including product and process development, investigation, and even as part of product stability testing programs. Chromatography based techniques such as LC coupled with either ELSD or CAD are the most used analytical methods. Using a multi-product platform assay for PS content is a common practice.

Key Diversities

Actual practices and applications among the companies are quite diverse. Some of these key areas with diverse practices are: the type of PS used (PS20 or PS80), a wide concentration range in the final formulated products, and what – if any - PS related quality attributes are included in the final product stability monitoring programs.

Conclusions:

The survey finds that all companies recognize the importance of PS as a protein-stabilizing agent in biologics drug formulations, and they all take active measures to ensure its quality

and functionality. In practice, many differences exist in the applications and controls in place to achieve that goal. These differences reflect the diversity of the companies' product portfolios, the complexity associated with PS as a raw material, its properties and functions as an excipient, supply chain challenges, and some limitations of our current analytical capabilities and manufacturing processes. Company specific differences on protein concentrations, media, cell line platforms, purification steps etc. may contribute to the diversity of the observations and experiences related to PS stability.

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Conflict of Interest Declaration

The authors declare that they have no competing interests.

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Captions to Tables and Figures

Supplemental Figures and Tables

Table 1: Heterogeneity in polysorbate products

Source of PS heterogeneity	PS structural features affected	Key References	Typical causes for or examples of PS heterogeneity
Reaction conditions and synthetic route during PS production	Mono-, di-, tri-, tetra-ester distribution	[37]	Esterification and dehydration conditions, catalysts
	Ratio Sorbitan/Isosorbide based structures	[32-34]	
	Non-ethoxylated species (e.g. sorbitan monoesters) and unesterified sorbitan and Isosorbide; variability in EO units length	[29-31, 41]	
	Free fatty acids (FFA) and Polyoxyethylene esters content	[45-47]	
Natural sources of fatty acid raw materials (natural oils)	Fatty acids ester content and distribution	[48]	Olive, sunflower oil, etc. Fatty acids other than specified in pharmacopeia
Process related impurities and contaminants	Impurity profile	[15, 49]	Dioxane, heavy metals, residual water, impurities from natural raw sources such as 12-tricosanone
PS degradation products	Overall structure affected due to presence of peroxides, epoxides, ketones, aldehydes, short chain organic acids, alkanes, other products derived from oxidation or hydrolysis	[38, 50, 51]	Can form and/or increase due to hydrolysis and/or oxidation

Table 2: Management of polysorbate products for cGMP use prior to opening

Category	Options	Percentage of responses*
Container size	Very large (> 4 kg)	38
	Large (> 1 kg)	56
	Small (0.1 – 1 kg)	69
	Very small (< 0.1 kg)	13
Packaging material	Metal	38
	Plastic	19
	Clear glass	13
	Brown glass	69
Other protective measures	Nitrogen overlay	75
	Argon overlay	6
	Air	13
	Protection from light/limit for light exposure applied	56
Storage temperature after receipt (before opening)	Room temperature	75
	Refrigerated	31

* Percentage of responses are relative to 16 companies and may add up to more than 100% for each category as multiple choices could be selected

Table 3: Additional comments from surveyed companies to question "When did you first observe the degradation of PS?"

➢ At the 18 months pull point at 5 °C ± 3 °C (product 1)
➢ During development studies (product 2)
➢ 2018: upon observation for other projects (product 3)
➢ 2013: probably associated with high protein concentration products
➢ 2015 – 2016: when high concentration protein was developed
➢ High concentration protein with observed loss of PS80
➢ 2016: with implementation of new stability indicating method for PS content
➢ New type of DS purification
➢ During development study
➢ Subvisible particle was first observed during thermal stress stability study at 25 °C by micro-flow imaging method
➢ Approx. 2009: PS degradation products induced degradation of protein. High ratio of protein to PS80
➢ 2017: 10 mg/mL liquid, pH 6, formation of visible particles; not linked to certain process changes & not related to development processes; PS degradation observed during storage stabilities (25/40 °C; 6 m/8w)

Table 4: Potential factors influencing HCP-related PS degradation or the formation and detectability of insoluble PS degradation products[a]

	Level of Perceived Effect on		
Factors with potential Impact	Enzymatic (hydrolytic) activity of HCPs	Formation of insoluble degradation products	Detectability of insoluble degradation products
Storage Temperature	**++++**	**++**	**+**
Storage Time	**++++**	**++**	NA
HCP concentration	**+++**	NA	NA
Protein concentration	**+++**	**+**	-
Concentration of specific enzyme	**++**	NA	NA
Specific type of enzyme	**++**	NA	NA
PS concentration	**+**	**+**	-
PS purity/quality	**+**	**+**	NA
pH	**+**	-	-
Primary packaging	-	-	**+**

[a] Positive responses were classified in increasing order of relevance (from negligible - to considerable ++++) relative to provided responses taking into account known or study based findings, for details see Figure in Supplemental Information

NA = Not applicable, not asked in survey in relation to the potential factor

Table 5: Analytical methodologies used for polysorbate quantitation

Assay	Number of companies using this assay[a]	Analytical method details (number of responses of total responding companies)	Companies considering changing/ improving the assay
Chromatography based	15	Mixed mode (13 of 15) Protein removal option (8 of 15) Oasis Max cartridge column (13 of 15) <u>Detector</u>: UV (2 of 15); ELSD (11 of 15); MS (3 of 15); CAD (11 of 15); Fluorescence (2 of 15)	2 of 15
Fluorescence micelle based	7	NPN dye (7 of 7) Flow injection (7 of 7) Sample preparation (5 of 7) Plate format option (3 of 7)	5 of 7
Thiocyanate complexation	3	Not applicable	3 of 3

[a] Total number of companies responding was 16, tally adds not up to 100% due to use of multiple assays within same company

CAD = Charged Aerosol Detector, ELSD = Evaporative Light-Scattering Detector, NPN = N-phenyl-l-naphthylamine

Table 6: Overview of regulatory interactions linked to PS specification

	Phase 1 or 2		Phase 3 & Commercial	
	Drug Substance	Drug Product	Drug Substance	Drug Product
Are PS content specifications routinely provided to regulatory authorities?	19%	31%	31%	81%
Were PS release specifications requested by regulatory authorities?	13%	31%	38%	69%
Were end of shelf life specifications requested for PS by regulatory authorities?	0%	0%	7%	33%
Have changes been proposed to PS specifications (DS or DP) by regulatory authorities during review (tightening of acceptance criteria, or control limits)?	7%		40%	

Fig. 1

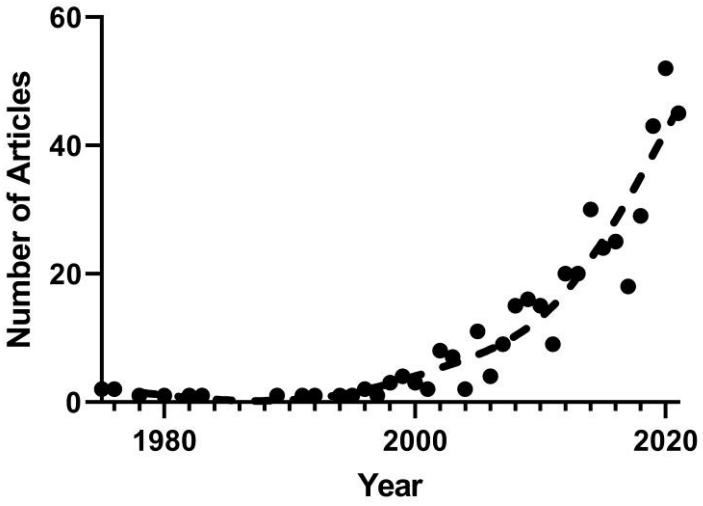

Fig. 2

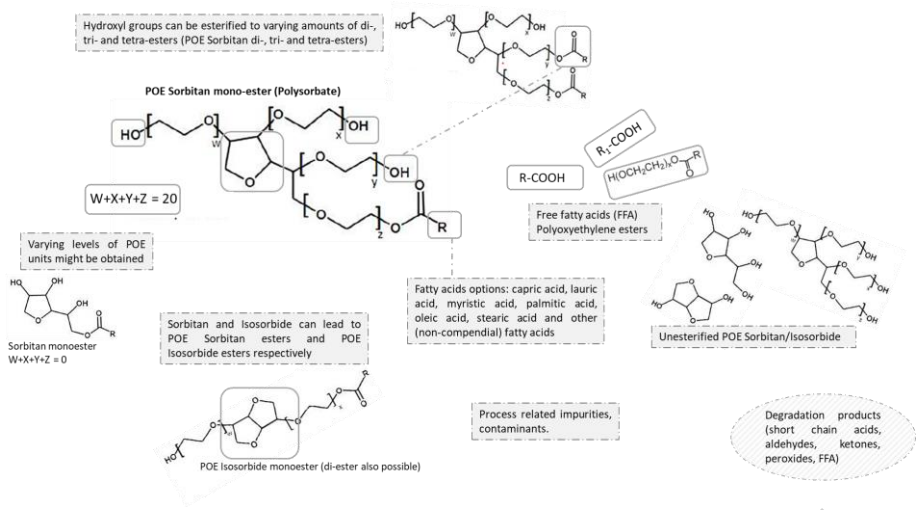

Fig. 3

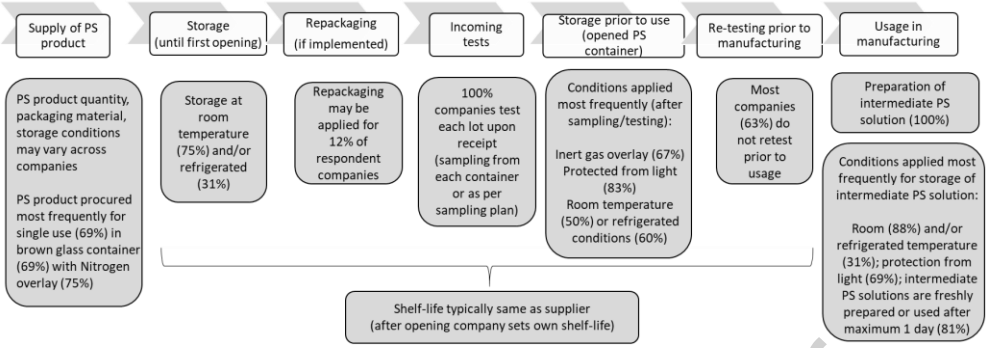

Fig. 4

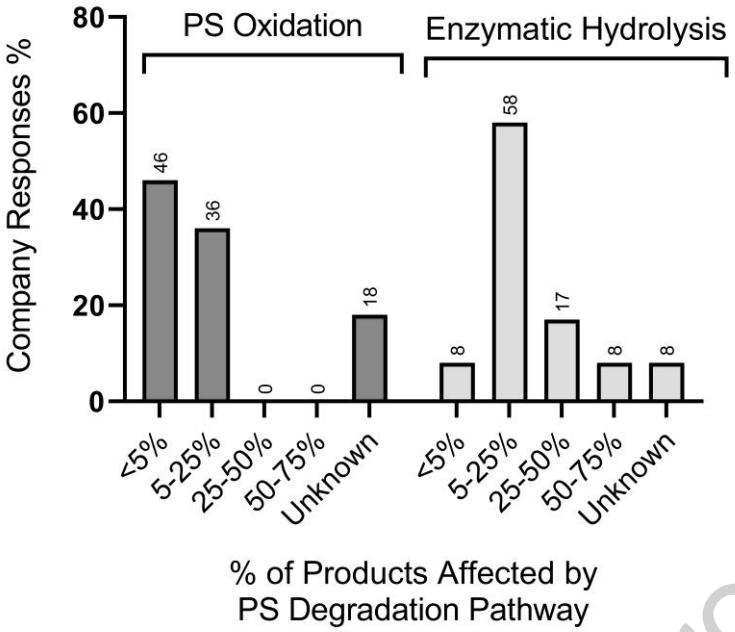

Fig. 5

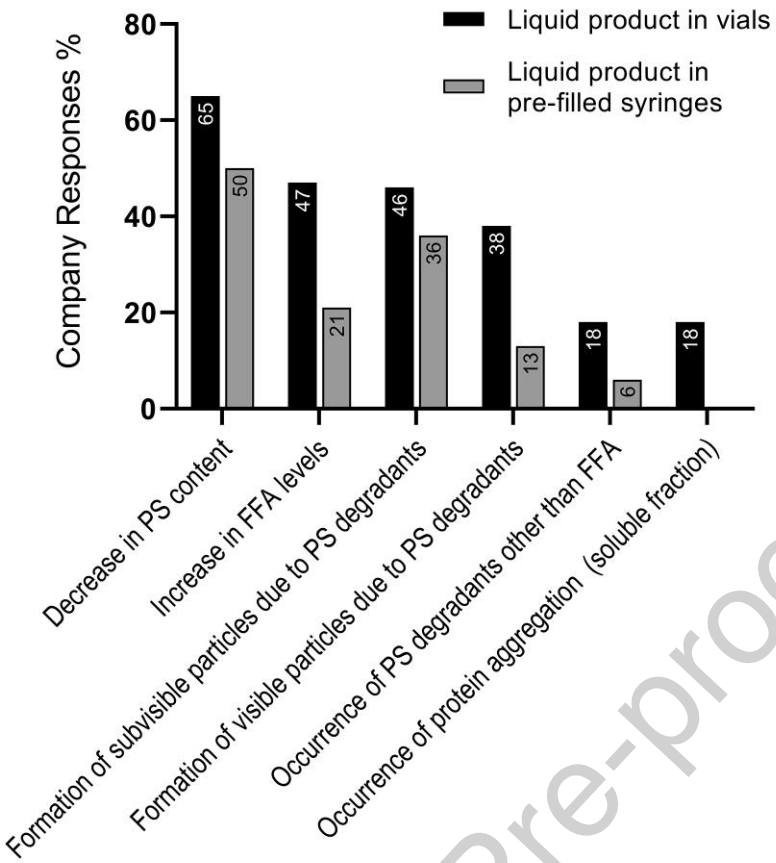

Fig. 6