Journal of Pharmaceutical Sciences 113 (2024) 2688–2698

Contents lists available at ScienceDirect

Journal of Pharmaceutical Sciences

journal homepage: www.jpharmsci.org

Pharmaceutical Biotechnology

A comparison of Polysorbates and Alternative Surfactants for Interfacial Stress Protection and Mitigation of Fatty Acid Particle Formation in the Presence of an Esterase

Ian Roy[a,*], Klaus Wuchner[c], Patrick Stahl[a], Tuan Tran[b], Naveen Yaragudi[a]

[a] Drug Product Development, BioTherapeutics Development and Supply, Janssen Research & Development, 200 Great Valley Parkway, Malvern, PA 19355, USA
[b] Analytical Development, BioTherapeutics Development and Supply, Janssen Research & Development, 200 Great Valley Parkway, Malvern, PA 19355, USA
[c] Analytical Development, BioTherapeutics Development and Supply, Janssen Research & Development, Hochstrasse 201, Schaffhausen 8200, Switzerland

ARTICLE INFO

Article history:
Received 2 January 2024
Revised 10 July 2024
Accepted 11 July 2024
Available online 14 July 2024

Keywords:
Brij L23
Brij O20
Poloxamer 188
Multi-compendial polysorbate 20
Super refined polysorbate 20
Pure lauric acid polysorbate 20
Multi-compendial polysorbate 80
Super refined polysorbate 80
Pure oleic acid polysorbate 80
Fatty acid particles
Subvisible particles

ABSTRACT

The hydrolysis of polysorbate surfactants in large molecule drug product formulations caused by residual host cell proteins presents numerous stability concerns for pharmaceuticals. The fatty acids (FA) released by polysorbate hydrolysis can nucleate into particulates or challenge the conformational stability of the proteinaceous active pharmaceutical ingredient (API). The loss of intact polysorbate may also leave the Drug Product (DP) vulnerable to interfacial stresses. Polysorbate 20 and 80 are available in several different quality grades (Multi-compendial, Super Refined, Pure Lauric Acid (PLA)/Pure Oleic Acid (POA)). All variations of polysorbate as well as three alternative surfactants: Brij L23, Brij O20 and Poloxamer 188 were compared for their ability to protect against air-water interfacial stresses as well as their risk for developing particulates when in the presence of lipoprotein lipase (LPL) (*Pseudomonas*).

Results show a meaningful difference in the timing and morphology of FA particle formation depending on the type of polysorbate used. All grades of polysorbate, while susceptible to hydrolysis, still offered sufficient protection to interfacial stresses, even when hydrolyzed to concentrations as low as 0.005 % (w/v). Alternative surfactants that lack an ester bond were resistant to lipase degradation and showed good protection against shaking stress.

Introduction

Large molecule drug products utilize surfactants to prevent adsorption and to protect the protein against interfacial stresses such as the air-water interface or the primary container contact area-water interfaces.[1] Polysorbate (PS) 20 and 80 are the most widely utilized surfactants in large molecule formulations, with Poloxamer 188 being used in some medications.[2] Brij L23 and Brij O20 are common surfactants in household consumer products but are currently not approved for pharmaceutical use.

Polysorbates are amphipathic, nonionic surfactants characterized as ethoxylated sorbitan esterified with fatty acids. While the primary fatty acids esterified to PS20 and 80 are lauric acid and oleic acid respectively, polysorbates are heterogeneous mixtures where the identity of the esterified fatty acid and arrangement ethylene oxides around the sorbitan can vary between grades and batches.[3] PS20 and 80 are available in several grades: Multi-compendial (MC), Super Refined (SR), Pure Lauric Acid (PLA) and Pure Oleic Acid (POA). A representative distribution of fatty acids for each of these PS grades is provided in Table 1.

Distributions of fatty acids esters for each polysorbate grade are examples and based on the specific certificate of analysis of the used batch in this study.

Abbreviations: API, Active Pharmaceutical Ingredient; BMI, Backgrounded Membrane Imaging; CE-SDS, Capillary Sodium Dodecyl Sulfate Electrophoresis; CMC, Critical Micelle Concentration; DP, Drug Product; FA, fatty acids; FFA, free fatty acid; HIAC, High Accuracy Liquid Particle Counter; HPLC, High Performance Liquid Chromatography; LPL, Lipoprotein lipase; mAb, Monoclonal Antibody; MC, Multi-compendial; MFI, Micro Flow Imaging; NR, Non-Reducing; NTU, Nephelometric Turbidity Unit; P188, Poloxamer 188; PFA, Polyethoxylated Fatty Alcohol; PLA, Pure Lauric Acid; POA, Pure Oleic Acid; PS, Polysorbate; PS20, Polysorbate 20; PS80, Polysorbate 80; SR, Super Refined; SEC, Size Exclusion High Performance Liquid Chromatography; UPLC, Ultra Performance Liquid Chromatography.

* Corresponding author.
E-mail address: iroy4@its.jnj.com (I. Roy).

https://doi.org/10.1016/j.xphs.2024.07.010

Table 1
Example fatty acid ester distributions for different grades of polysorbate 20 and 80.

Fatty Acid name	Chemical formula	MC PS20 [a]	SR PS20 [b]	PLA PS20 [c]	MC PS80 [d]	SR PS80 [e]	POA PS80 [f]
Caproic Acid	$C_6H_{12}O_2$	0.0	0.1	NA	NA	NA	NA
Caprylic Acid	$C_8H_{16}O_2$	3.0	1.4	NA	NA	NA	NA
Capric Acid	$C_{10}H_{20}O_2$	3.1	1.6	NA	NA	NA	NA
Lauric Acid	$C_{12}H_{24}O_2$	54.0	54.9	99.6	NA	NA	NA
Myristic Acid	$C_{14}H_{28}O_2$	17.5	22.4	NA	0.1	0.1	0.0
Palmitic Acid	$C_{16}H_{32}O_2$	10.9	11.2	NA	7.8	2.8	0.1
Palmitoleic Acid	$C_{16}H_{30}O_2$	NA	NA	NA	0.1	0.1	0.0
Stearic Acid	$C_{18}H_{36}O_2$	5.7	0.4	NA	2.1	3.5	0.0
Oleic Acid	$C_{18}H_{34}O_2$	4.6	7.3	NA	74.7	85.2	99.3
Linoleic Acid	$C_{18}H_{32}O_2$	0.1	0.1	NA	0.1	0.4	0.1
Linolenic Acid	$C_{18}H_{30}O_2$	NA	NA	NA	0.1	0.1	0.0

[a] MC PS20: multi-compendial polysorbate 20.
[b] SR PS20: super refined polysorbate 20.
[c] PLA PS20: pure lauric acid polysorbate 20 specification is 98.0–100 % lauric acid.
[d] MC PS80: multicompendial polysorbate 80.
[e] SR PS80: super refined polysorbate 80.
[f] POA PS80: Pure oleic acid polysorbate 80. NA: non-applicable.

MC PS20 and MC PS80 are the most commonly used grades in pharmaceuticals. SR grades of PS are similar to MC in terms of fatty acid composition but have fewer process related impurities such as primary and secondary oxidation products and unesterified fatty acids. SR PS20 also contains a higher percentage of higher-order esters which gives it a lower Critical Micelle Concentration (CMC).[4-6] PLA and POA PS have minimal process related impurities and have largely uniform incorporations of lauric acid (PS20) and oleic acid (PS80) as their hydrophobic components. Previous research has shown that PLA PS20 is more sensitive to oxidation than the comparable POA PS80.[7] Currently PLA PS20 is a non-compendial grade of PS.

The distribution of fatty acids in PS is significant because populations of residual host cell proteins, carried through monoclonal antibody (mAb) purification, can present enough enzymatic activity to hydrolyze polysorbate. This results in the release of free fatty acids (FFAs) into the DP formulation. FFAs have limited solubility and can nucleate into meaningful populations of visible and subvisible particles. Fatty acid degradants also have the potential to impact the DP stability by interacting directly with the mAb.[8-15] Several factors impact how the products of PS hydrolysis develop into particulates: the population of fatty acids, the rate at which they enter solution, the pH and temperature of the formulation, and the identity and concentration of intact surfactant and other impurities such as e.g., glass leachables. Together each of these factors play a role in the rate and morphology of the fatty acid particles formation.[16-20]

Alternative surfactants devoid of ester bonds are an effective means of avoiding surfactant degradation by host cell esterases and lipases. Polyethoxylated fatty alcohol (PFA) surfactants contain hydrophobic and hydrophilic subunits joined by an ether linkage that allows this surfactant class to be impervious to host cell impurities that would otherwise degrade the standard PS surfactants. Two notable examples of PFAs, Laureth-23 and Oleth-20, commercially known as Brij L23 and Brij O20, respectively, contain the corresponding fatty alcohol subunits to the fatty acids nominally found in PS20 and PS80. This structure enables them to perform the protective functions required in large molecule formulations yet remain resistant to enzymatic degradation. Poloxamer 188 is another alternative surfactant that lacks an ester bond. It is a triblock copolymer featuring a central hydrophobic chain of polyoxypropylene flanked by two hydrophilic chains of polyoxyethylene.[21,22] Fig. 1 shows the structure and physical properties of the surfactants under evaluation.[23,24]

In this study a performance comparison for fatty acid particle formation caused by lipoprotein lipase (LPL) PS hydrolysis and interfacial stress protection during stability storage and shaking was conducted. Formulations were prepared using different grades of PS20/80 as well as Brij L23,-O20, and P188. LPL (*Pseudomonas*) was used to provide a controlled rate of PS hydrolysis.

Materials and Methods

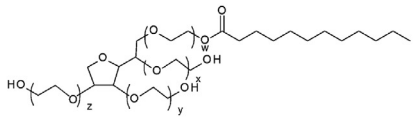
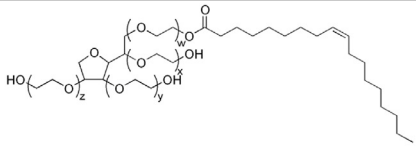
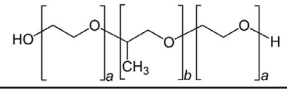
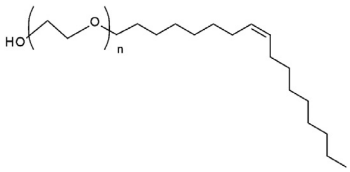
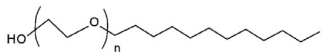
Materials

Brij L23, Brij O20, SR PS80, POA PS80, SR PS20, and PLA PS20 were purchased from Croda; MC PS20 and MC PS80 were purchased from J.T.Baker; Sorbitol and L-Histidine monohydrochloride monohydrate were purchased from Merck; L-Histidine was purchased from Ajinomoto; P188 was from Thermo; LPL Lipoprotein Lipase *pseudomonas* (>1200 units/mg) was from Sigma. The monoclonal antibody (mAb) used in this study was produced and purified at Janssen, Malvern, PA. The water used in all studies was from a Millipore Milli-Q water system with an average conductivity of 18.2 Mohm. The 25R glass vials were purchased from Schott. The 20 mm rubber stoppers were acquired from West.

Preparations of Different Formulations in Vials

The formulation compositions used to compare the different surfactants are summarized in Table 2. A high concentration (>100 mg/mL) IgG1 mAb, and 0.1 units/mL (0.67 ng/mL) of LPL were co-formulated in a pH 5.6 histidine buffer with each surfactant at the nominal concentration indicated in Table 2.

LPL was selected as an enzyme because it has a known affinity for polysorbates and has been suggested as one of the host cell proteins responsible for PS hydrolysis.[15] Esterase activity in DP arises from a collection of HCP enzymes with varying affinity for the heterogeneities of polysorbate. This means the precise degradation profile of polysorbate may be unique to a given cell line and purification process. Using a single enzyme as an agent for polysorbate hydrolysis provides control over the esterase activity but means the degradation profile observed in this study may not be mirrored by a collection of HCPs with ranges of enzymatic activity and substrate affinity. A brief ranging study identified 0.1 units/mL of LPL as an appropriate concentration to provide a controlled rate of PS hydrolysis that was slow enough to allow for the growth of larger particles, similar to what could be seen in DP formulations.[25,26] All formulations, designated hereafter as DP vials, were filtered (0.22 μm SterivexTM filter) in a laminar flow hood and 16 mL aliquots were dispensed into 25R borosilicate glass type 1 vials and sealed with 20 mm rubber stoppers.

Surfactant	MW (g/mol)	CMC [a] % w/v	(HLB) [b]	Structure
Polysorbate 20	1228	0.006	16.7	
Polysorbate 80	1310	.0014	15	
Poloxamer 188	7680 - 9510	>1%	29	
Brij O20	1150	0.003	15	
Brij L23	1198	0.011	16.7	

[a] CMC: Critical Micelle Concentration
[b] HLB: Hydrophilic – Lipophilic Balance
CMC & HLB source : https://pubchem.ncbi.nlm.nih.gov, accessed Nov 2021

Fig. 1. Surfactant properties and structures of surfactants under evaluation.

Degradation Study

Samples were incubated at 5 °C and 25 °C for 207 and 140 days, respectively. A single vial was removed for analysis after storage for 0.5, 9, 14, 21, 35, 49, 77, 140, and 207 days at 5 C, and after 0.5, 6, 12 16, 21, 35, 49, 77, and 140 days at 25 °C . Samples were tested for surfactant concentration, FFAs, and for particle formation via several techniques: light-obscuration (LO) using a HIAC, micro-flow digital image analysis (MFI), and by Backgrounded Membrane Imaging (BMI). Biochemical critical quality attributes were also tested: turbidity via nephelometry, charge heterogeneity via capillary isoelectric focusing, purity of mAb monomeric species via SEC, and by CE-SDS under reducing (R) and non-reducing (NR) conditions. Bioactivity was tested at the initial and final time points only.

Table 2
Formulation list.

Formulation Designation [a]	Nominal surfactant (w/v) concentration and grade [b]
MC PS20	0.04 % Multicompendial Polysorbate 20
SR PS20	0.04 % Super Refined Polysorbate 20
PLA PS20	0.04 % Pure Lauric Acid Polysorbate 20
MC PS80	0.04 % Multicompendial Polysorbate 80
SR PS80	0.04 % Super Refined Polysorbate 80
POA PS80	0.04 % Pure Oleic Acid Polysorbate 80
Brij L23	0.04 % Brij L23
Brij O20	0.04 % Brij O20
P188	0.04 % Poloxamer 188
No Surfactant	0.00 % No Surfactant

[a] All formulations contain >100 mg/mL mAb, 0.1 unit/mL LPL, Histidine buffer with sorbitol at pH 5.6.
[b] Targeted concentrations.

Shaking Stress Study (Air-Water Interfacial Stress)

One vial of each formulation was removed after 140 days at 25 °C and after 207 days at 5 °C and exposed to mechanical shaking to assess the protection efficiency of the residual surfactant and the potential destabilizing properties of FFA particles (if present) against interfacial stress. Vials were subjected to mechanical stress on an orbital shaker (250 rpm) for 72 h at each designated temperature. This amount of agitation was intended to be more severe than any mechanical stress the DP might encounter during manufacturing and transportation.

Particle Analysis by Light Obscuration (LO)

A liquid particle counting instrument, (HIAC) Royco Model 9703plus with an HRLD 150 sensor (Beckman Coulter, Brea, California) was used for subvisible particle analysis. Samples with high particle counts (exceeding coincidence limits of sensor), e.g. those with degraded MC PS20, were diluted to an optimal range with formulation buffer just prior to analysis. All samples were manually homogenized, and 4 × 1 mL portions analyzed directly from the DP vial except when dilutions were necessary. Particle counts per mL were averaged using the last 3 portions for ≥2, ≥10, and ≥25 μm size ranges (the first portion being discarded). Each day of analysis, instrument cleanliness and counting accuracy were verified using water and certified particle standards (Count Cal CC05 and CC15, Thermo Fisher). Samples that were incubated at 5 °C were allowed to equilibrate at controlled room temperature (20–25 °C) for 1 hour

then tested within a 2-hour window to control for the dissolution of FFA particles when the sample is warmed.

Particle Analysis by Micro Flow Imaging (MFI)

Particle analysis was performed on an MFI 5200 instrument equipped with a 100 μm flow cell and using a Bot 1 autosampler (Protein Simple, Santa Clara, CA). After manual homogenization, a sample aliquot of 2 mL was transferred into 96 well plates. The MFI instrument was flushed with 0.5 mL of each sample and conditioned with 0.22 mL to optimize illumination prior to analysis of 0.9 mL (volume dispensed). Particle concentrations in various size ranges (≥2 μm) were reported as particles/mL. The validity of particle measurement was demonstrated each day by performing appropriate blank runs and sizing and counting verifications with certified particle standards (Count Cal CC15, Thermo Fisher).

Particle Analysis by Backgrounded Membrane Imaging (BMI)

A Horizon particle imager from Halo labs (Burlingame, CA) with Horizon VUE imaging software (2.0) was used for selected time points to determine particle levels without interferences of liquid particles such as oleic acid droplets. Briefly, 50 μl of sample material was placed on a blanked 96-well white 0.4 μm polycarbonate filter plate. A vacuum was applied to the back of the filter plate to deposit solids onto the filter. All samples were measured in triplicate without a water wash, and filters with obvious damage or deformities were discarded. Particle images displayed from the instrument are a 500 × 500 pixel crop taken from an instrument generated image originally sized at 5472 × 5421 pixels.

Size Exclusion High Performance Liquid Chromatography (SEC)

SEC was performed on an Agilent HPLC system with UV detection to determine levels of aggregates, fragments, and monomeric purity. The system was equipped with a Guard Column (Tosoh, Shiba, Tokyo; SWXL, PEEK 6 × 40 mm, 7 μm, part #18,008) and Separations Column (Tosoh, Shiba, Tokyo: TSKgel BioAssist G3SWXL, PEEK 7.8 × 300 mm, 5 μm).

Non-Reducing CE-SDS (NR-CE-SDS)

Samples and standards were mixed with a Sodium Dodecyl Sulfate (SDS) stock reagent and incubated in a 75 °C water bath for 5 min to provide the samples with a uniform charge/mass ratio and remove secondary and tertiary protein structure. The samples were loaded into a Bare-fused silica capillary (30 cm x 50 μm) and separated by size using a Beckman PA800 plus system with UV detection.

Surfactant Quantification: Polysorbate, Poloxamer 188, BRIJ

An HPLC method with evaporative light scattering detection was used for quantification of polysorbate and other surfactants. The method, as first described by Hewitt D[23], is based upon direct injection of the sample onto a mixed-mode anion-exchange/hydrophobic column (Oasis Max 30 μm, 2.1 × 20 mm, Waters, Milford, MA, USA) using an acidified mobile phase. Proteins below their pI are not retained, while the neutral, hydrophobic surfactant species are concentrated onto the polymeric sorbent of the HPLC column. A step gradient of acidified isopropyl alcohol elutes the surfactant as a single peak, which is detected using an evaporative light scattering detector.[27,28]

Free Fatty Acid (FFA) Quantification

Fatty acids were isolated from DP samples and reference standards using solid phase extraction (Oasis HLB 30 mg 1CC SPE cartridges, Waters, Milford, MA, USA). The isolated fatty acids were derivatized with PDAM (1-Pyrenyldiazomethane – Thermo Fisher, Waltham, MA). Prepared samples and reference standards were analyzed using reverse phase chromatography with a 1290 UHPLC LC-UV system with a 1290 binary pump (Agilent, Paolo Alto, CA, USA). A gradient of water and acetonitrile was used to separate individual fatty acids in a C18 column (Acquity UPLC BEH C18 LC column 1.7 μm, 1.2 mm x 150 mm, Waters, Milford, MA, USA).[29,30] Separation conditions were optimized and are different for FFAs originating from PS20 or PS80. FFAs quantified for PS20 were: capric acid, lauric acid, myristic acid, palmitic acid, oleic acid, and stearic acid. FFAs quantified for PS80 were: myristic acid, linoleic acid, palmitic acid, oleic acid, and stearic acid. Measurements for all fatty acids were summed to determine the total FFA concentration.

Turbidity

Nephelometric measurements were made using a HACH 2100AN Turbidimeter with an 11 mm cell adapter. Three mL samples were measured in blanked 11 mm x 100 mm test tubes that had been wiped with silicone oil. Measurements were taken at least 60 seconds after the sample was inserted in the instrument to allow the system to stabilize. System suitability was established by checking the turbidity of <0.1 NTU, 1 NTU, 5 NTU, 20 NTU, 50 NTU certified standard solutions (Hach, Lovland, CO).

Bioactivity

Bioactivity was measured in 2 assays using specialized cells that express the target antigen for this antibody. The signal measured was compared to the signal from the standard curve and expressed as relative potency.

Results & Discussion

Impact of PS20 Hydrolysis Rate on FFA-Particle Size Distribution

Ranging studies with varying LPL concentrations (0.04 vs. 0.2 U/mL) demonstrated that rapid hydrolysis of MC PS20 resulted in the preferential formation of small particles at 5 °C (Supplemental Fig. S1). The rate of PS hydrolysis and subsequent FFA formation can be expected to impact FFA-particle size distribution. Long chain fatty acids present in polysorbates are thought to act as nucleators for particle formation.[18] While these longer fatty acids possess the potential to nucleate into a particle they still must encounter appropriate conditions in solution to do so; possibly a collision with another long chain fatty acid. In the case of slower hydrolysis, these long chain nucleators may be rolled into the growth of existing FFA-particles before encountering conditions to serve as a nucleation center for new particles. This results in fewer particles which grow into larger size ranges. In the case of rapid hydrolysis long chain fatty acids may be more likely to encounter conditions for nucleation before they have an opportunity to be incorporated into the growth of an existing particle. This results in the formation of many small particles. The degradation study with different surfactants was co-formulated with 0.1 units/mL (0.67 ng/mL) of LPL.

Surfactant Stability and Free Fatty Acid Levels

Figs. 2 and 3 show the change in surfactant and total FFA concentrations over time. T0 measurements were obtained from formulations

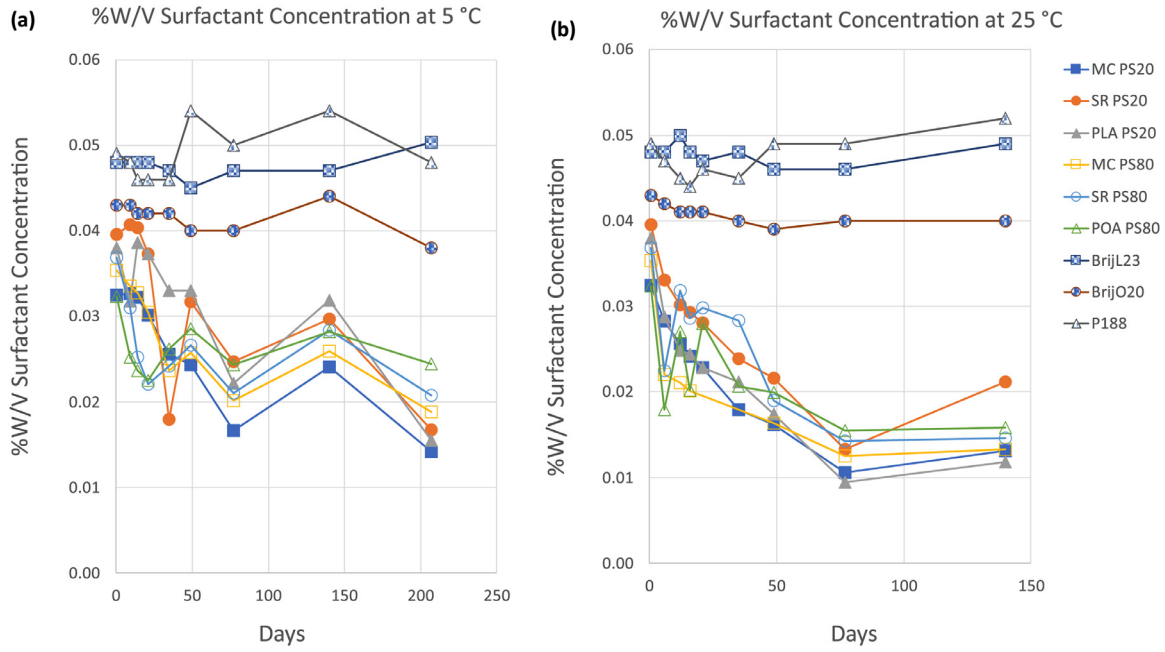


Fig. 2. Surfactant concentrations (%w/v) measured at 5 °C and 25 °C.

which were not spiked with LPL. Surfactants that did not contain a hydrolysable ester, Brij L23, Brij O20 and P188, predictably showed no change in concentration over the course of the study. Polysorbate samples exhibited normal enzyme kinetics with a rapid initial depletion of PS substrate matched with an accumulation of FFA products. The reaction rate slowed over time as substrate became depleted and the system approached equilibrium.[31] It should be mentioned that the observed slowdown in hydrolysis may not be a true signal that the system has achieved an equilibrium. As fatty acids begin to nucleate and precipitate out of solution as insoluble particulates, products from the hydrolysis reaction are removed from solution which pulls the reaction towards further polysorbate hydrolysis.

The combined soluble and insoluble FFA accumulation for PLA PS20 in Fig. 3 (a, b) was observed to plateau at a lower concentration (~20 μg/mL) compared to other grades of PS20 (35–60 μg/mL). The differences between the equilibrium concentrations of FAs shown in Fig. 3 are likely a result of the varying prevalence of polyester species in the different grades of PS. The equilibrium concentration of FAs and the rate at which it was achieved did not offer any foreshadowing for the appearance of particles.

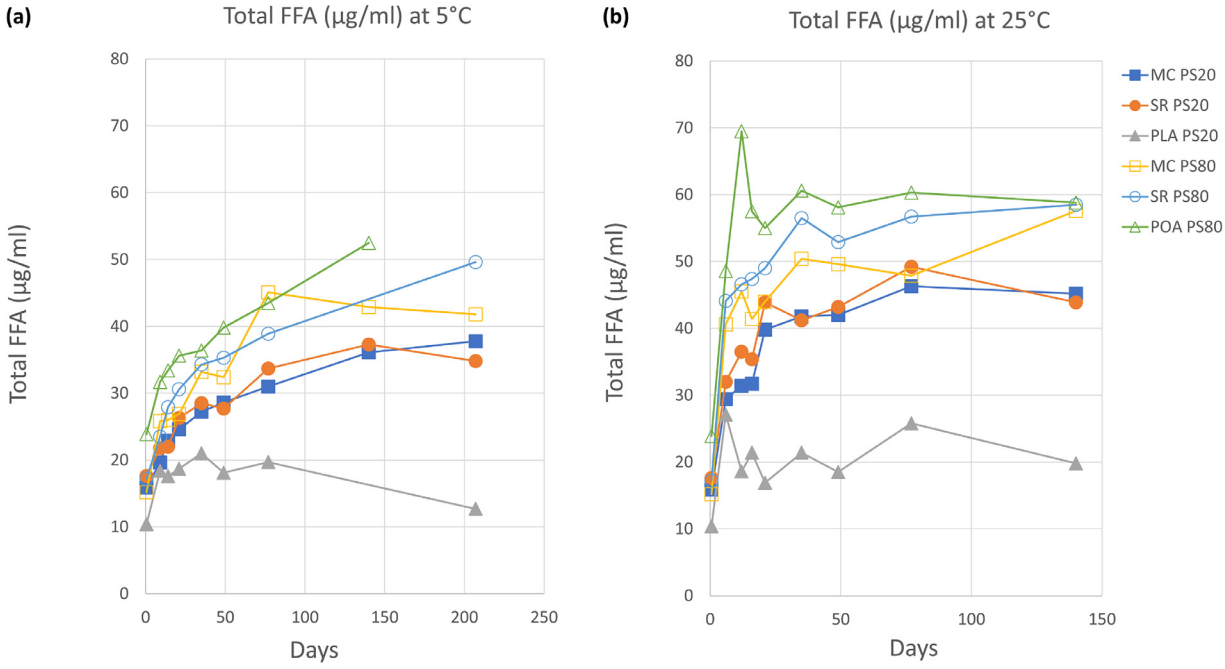


Fig. 3. Total free fatty acid accumulation (μg/mL) of all polysorbate formulations at 5 °C and 25 °C.

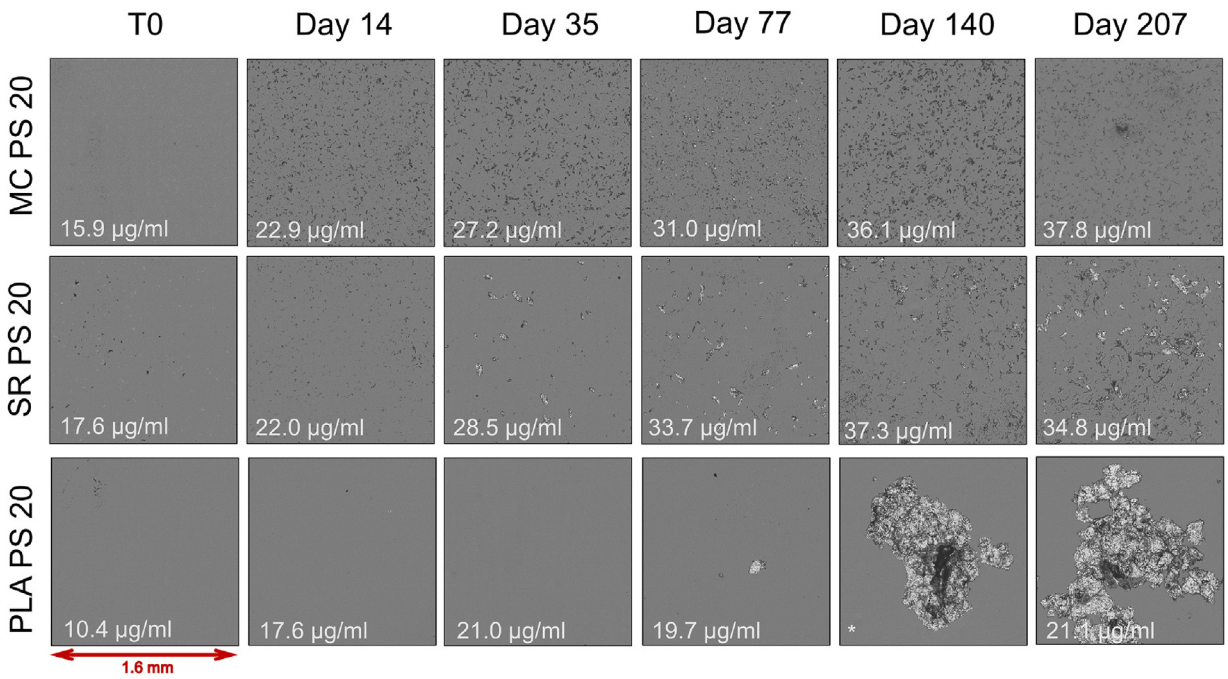


Fig. 4. BMI images of particles after different time periods for different grades of PS20 at 5 °C.
BMI images of particles formed in PS20 formulations incubated at 5 °C. The number in each image is the total free fatty acid content measured for the sample. * FFA concentration not available.

Polysorbate 20 Fatty Acid Particle Formation

While the degradation of polysorbate and the accumulation of fatty acids followed a well-understood path of enzymatically catalyzed hydrolysis, the nucleation and growth of FFA particulates is a more complex process with several factors that are likely interdependent and not fully understood.[17,18,20] Previous work with this antibody identified the particulates that form with polysorbate loss as non-proteinaceous fatty acid particles.

Figs. 4-5 show representative examples for the progression of particle sizes and morphologies measured by BMI for the different PS20 formulations over the course of the study at 5 °C and 25 °C. It has been reported that high concentration mAb samples can occasionally form a gel when deposited on membrane surfaces, and potentially

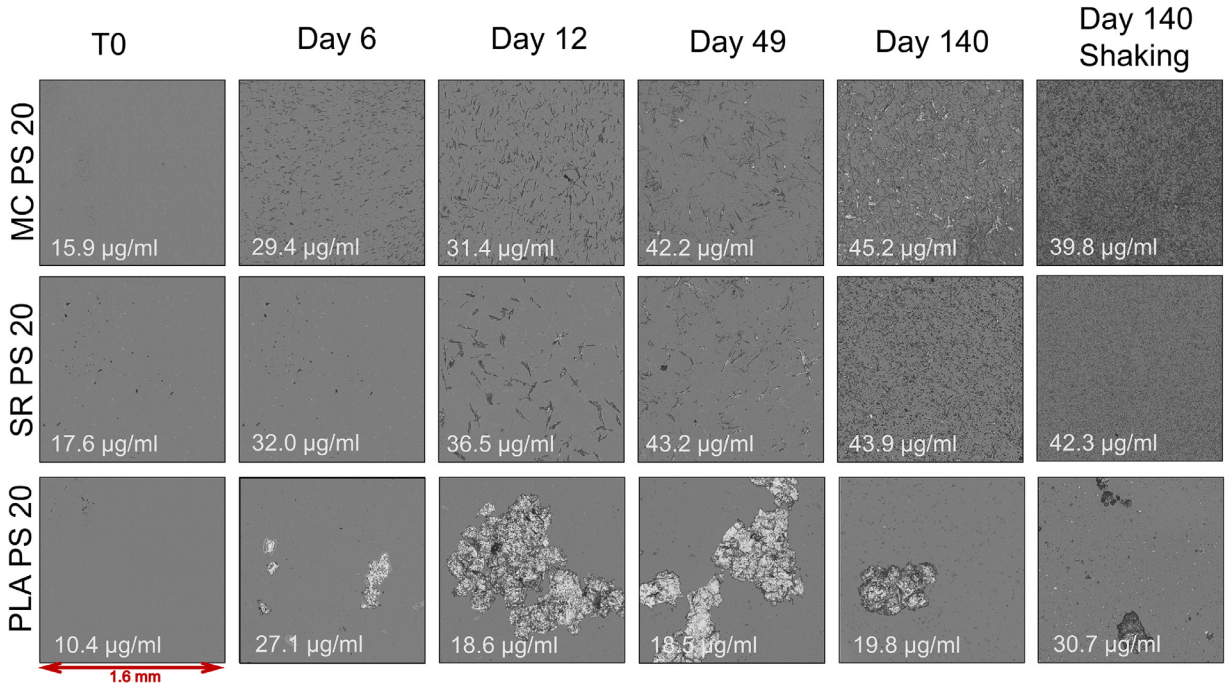


Fig. 5. BMI images of particles after different time periods for different grades of PS20 at 25 °C.
BMI Images of particles formed in PS20 formulations incubated at 25 °C.

interfere with MBI analysis; however, this was not an issue for the DP being investigated here. For the purpose of this study, particle size and levels are stated in a qualitative and not a quantitative way to circumvent difficulties with particle cropping errors and well coverages.

Comparing the time for particle formation at 5 °C across formulations, Fig. 4 shows that MC PS20 first exhibited BMI-detectable particles by day 14, followed by SR PS20 at day 35, and PLA PS20 at day 77. Although MC PS20 and SR PS20 samples maintained similar FFA concentrations throughout the study, MC PS20 consistently showed denser collections of smaller particles implying formulation conditions that favor nucleation. PLA PS20 samples formed comparatively few and much larger particles, easily discerned by visual examination of the DP vials (Figs. 4 and 5).

At 25 °C, Fig. 5, the rate of appearance of particles was noticably faster, in spite of similar respective particle morphologies as had been seen at 5 °C with the different grades of PS20. At 25 °C the PLA PS20 samples rapidly formed particulates by day 6, in marked contrast to day 77 at 5 °C. Furthermore, at 25 °C the FFA concentration increased to ~27 μg/ml by day 6, although had previously remained stable at ~20 μg/ml for several weeks at 5 °C. MC PS20 and SR PS20 also showed an increased rate of particle formation; however, the differences in morphology previously seen at 5 °C were less obvious at 25 °C.

These observations support the idea that longer chain FFAs (>C-12) that are more prevalent in MC PS20 preferentially act as nucleation sites. This allows fatty acids released from MC PS20 to be distributed across many fine nucleation points rather than coalescing into fewer but larger particles.[18] The reduced level of stearic acid (C-18) in SR PS20 relative to MC PS20 would be predicted to lead to fewer nucleation sites and therefore a greater propensity for FFAs to collect into fewer but larger particles.[17,18] The relative absence of long chain fatty acids in the PLA PS20 formulation leads to limited nucleation sites meaning the fatty acids collect into relatively few, but large particles, which is consistent with our observations at 5 °C and 25 °C (Figs. 4 and 5). The trends in particle morphology for each polysorbate type remain consistent across the two temperatures because the proportion of nucleating fatty acids released into solution remains the same.

Polysorbate 80 Fatty Acid Particle Formation

The PS80 formulations displayed an interesting trend of resistance to particle formation, clearly evident in the qualitative BMI determinations shown in Figs. 6 and 7. Compared with PS20, the varying grades of PS80 showed similar trends of PS hydrolysis (Fig. 1) and time-dependent accumulation of free fatty acids (Fig. 3); however, with regards to particle formation, only the MC PS80 formulation stored at 5 °C for 140 days developed meaningful levels of particles at which point its FFA concentration was measured as ~ 43 μg/ml (Fig. 5).

There are several reasons that PS80 formulations could be expected to develop fewer FFA particles than comparable PS20 formulations. The primary product of PS80 hydrolysis is Oleic Acid which is liquid at room temperature; thus, FA droplets may not be effectively retained by the BMI membrane making them undetectable as particles. It is also possible that the lower CMC of PS80 allows it to form more micelles in solution thus giving it greater potential for solubilization of FFA.[32] MFI data collected for these samples corroborates the absence of visible particles/droplets (Supplemental Fig. S2).

Particle Formation in Alternative Surfactants

The formulations prepared with Brij L23, Brij O20 and Poloxamer 188, all lacking ester bonds, did not develop any meaningful particle populations despite the presence of LPL over the course of the study.

Particle Quantification

The overall trends in quantitative particle measurements of the PS20 formulations made by LO and MFI techniques largely corroborated the observations from qualitative assessment of BMI images. Fig. 8 (a-f) shows subvisible particle concentrations for 2–10 μm, ≥10 μm, and ≥25 μm size ranges measured by LO particle counting

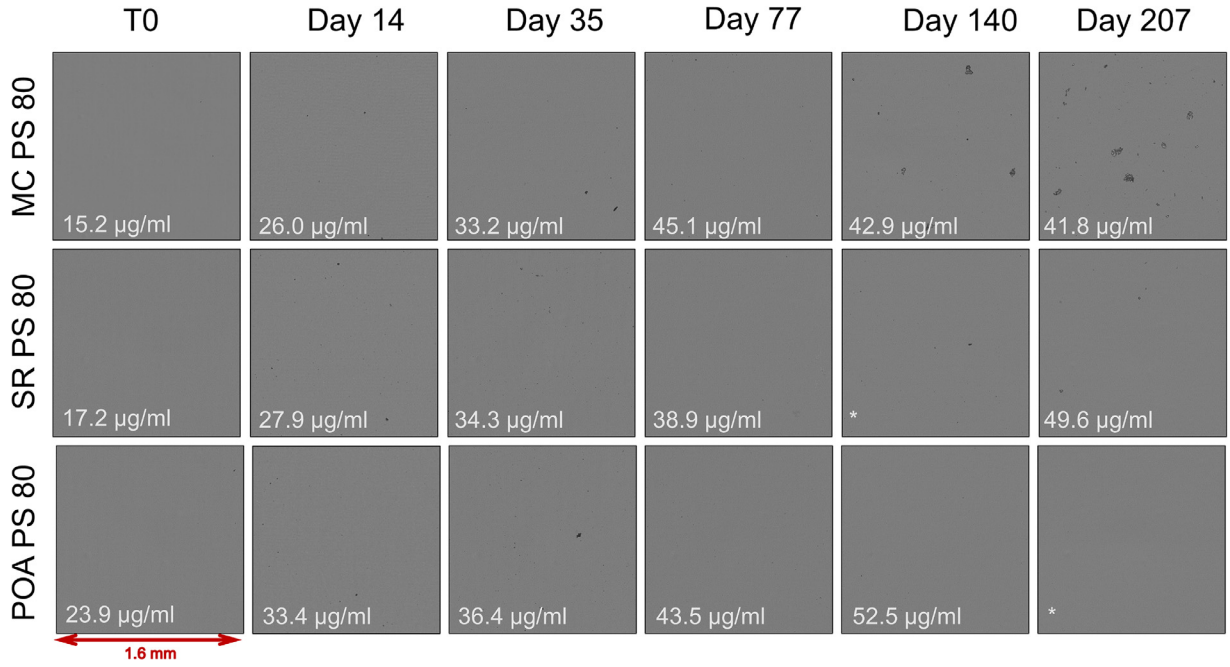


Fig. 6. BMI images of particles after different time periods for different grades of PS80 at 5 °C.
BMI Images of particles formed in PS80 formulations incubated at 5 °C. * FFA concentration not available.

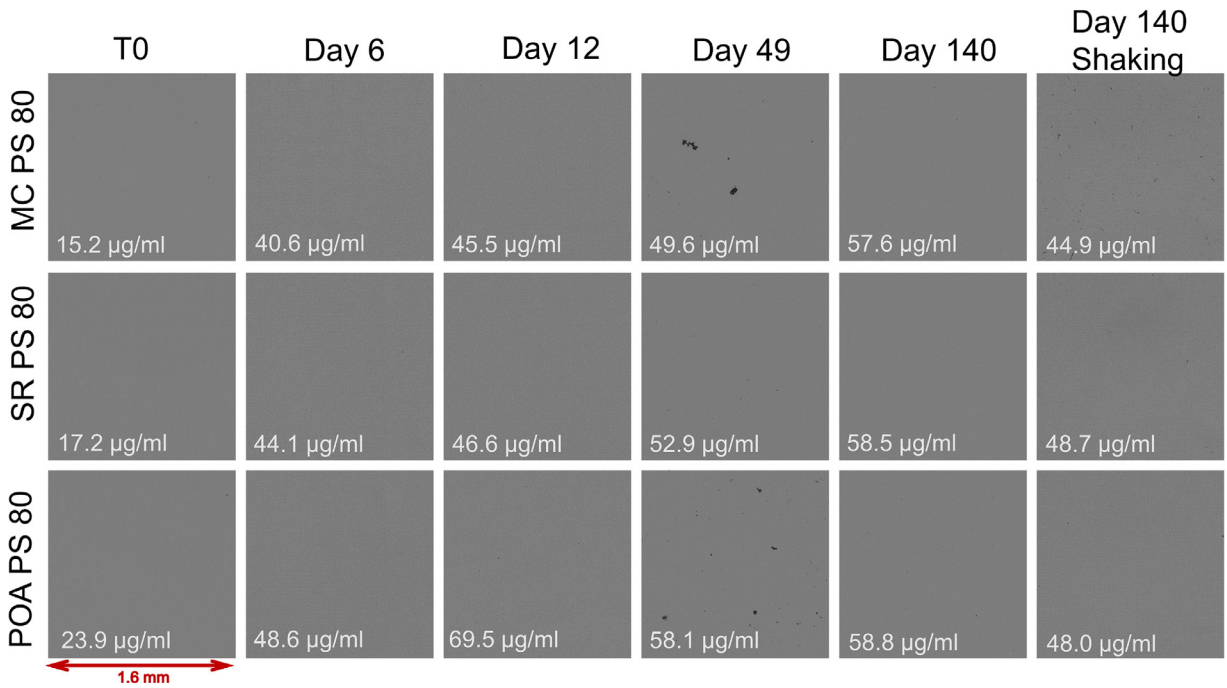


Fig. 7. BMI images of particles after different time periods for different grades of PS80 at 25 °C.
BMI Images of particles formed in PS80 formulations incubated at 25 °C.

for both the 5 °C and 25 °C samples. MC PS20 was observed to generate the largest population of small particles in the 2–10 μm size ranges at 5 °C and 25 °C. Formulations containing SR PS20 consistently contained the largest population of particles in the ≥10 μm size range at both temperatures. PLA PS20 showed early spikes in the 2–10 μm and ≥10 μm size ranges but these particles rapidly grew outside of these size ranges. No meaningful trends were observable in the ≥25 μm particle range.

Particle quantification in the PS20 formulations proved challenging for several reasons. The large number of particulates generated by the MC PS20 and SR PS20 samples required large dilutions so they could be brought within the detection limits of the light obscuration instrument. These dilutions introduce buffer volume that is not saturated with FFA, potentially allowing for some particles to dissociate or dissolve. Fatty acids are also more soluble at higher temperatures, so particles generated at 5 °C may dissolve over time when the sample is warmed to room temperature for testing.[18] To control for this, all 5 °C samples were allowed to equilibrate to room temperature for ~1 hour then tested within a two hour window. Testing facilities were maintained at controlled room temperature 20–25 °C. MFI samples analyzed without dilution presented schlieren lines which could be misinterpreted as large particles or collections of smaller ones. The image analyzing software associated with the BMI had challenges distinguishing large particles from clusters of smaller ones and patches of the membranes occasionally showed water marks from the buffer that could also be misinterpreted as particulates.

Subvisible particle measurements using the HIAC. (a): ≥2 to < 10 μm particle range at 5 °C, (b): ≥2 to < 10 μm at 25 °C, (c): ≥10 μm particle rage at 5 °C, (d): ≥10 μm particle rage at 25 °C, (e): ≥25 μm particle rage at 5 °C. (f):≥25μ m particle rage at 25 °C Table 3 further highlights the difference in particle morphologies between MC and SR polysorbate.

MFI Measurements for particles (Supplemental Fig. S2) also showed different particle distributions dependent on PS20 grade. MC PS20 was observed to produce more particles in the lower size ranges of ≥2 to <10 μm whereas SR PS20 initially produced more particles in the ≥10 to <25 μm and the ≥25 to <75 μm size ranges. SR PS20 particle numbers were eventually overtaken by MC PS20 at the 25 °C temperature. Non-hydrolysable surfactants Brij L23, Brij O20, and P188, showed negligible particle formation over the course of the study (<200 particles/mL, discernable only when plotted over a narrow range). BMI particle measurements also indicated that MC PS20 showed high concentrations of particles mainly in the smaller ≤10 μm size ranges (data not shown).

PS80 formulations when hydrolyzed with LPL, showed negligible particle formation. MFI images for these samples did not reveal any significant populations of particles or oil droplets. It is possible that the fatty acids were solubilized by the remaining polysorbate, or phase separated into a thin oil layer at the air interface.

Stability and Interfacial Stress Protection

Table 4 shows the change in measured quality attributes after storage at 5 °C, at 25 °C, and after shaking at 250 RPM for 72 h. The products of both PS20 and PS80 hydrolysis appear to have little impact on the stability of the IgG1 mAb used in this study.

Changes observed in NR-CE-SDS and A280 were mild and consistent between hydrolysable and non-hydrolysable surfactants as well as the no surfactant control. A 3 % drop in NR-CE-SDS purity was observed in the SR PS20 sample after shaking at 25 °C but this is most likely an analytical artifact because it is not observed in any other PS20 sample or corroborated by changes in SEC.

Changes in bioactivity were largely temperature dependent and within the expected variability of the assays (+/- 40 %). SEC showed a measurable decrease in monomer for PS80 formulations samples shaken at 25 °C but the changes were under 1 %, hence not meaningful.

Turbidity (NTU) increased for PS20 samples throughout the study because of elevated particle populations. This increase was not apparent in PLA PS20 samples because the particles were extremely large and few in number. There was a large increase in turbidity for the no surfactant control following shaking at 5 °C but only a mild increase

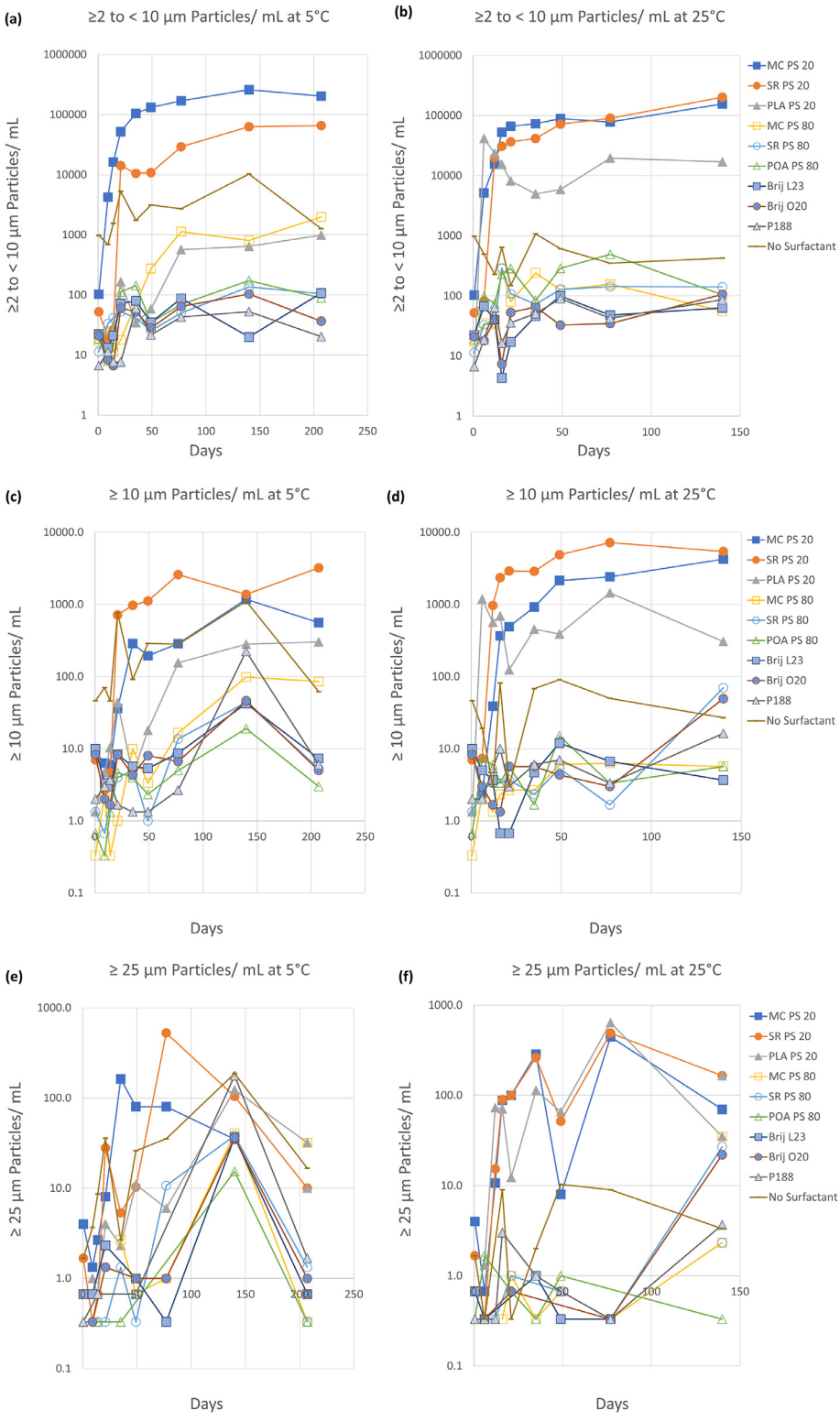


Fig. 8. Particle concentrations in size ranges of ≥2 to ≥10 μm, ≥10 μm, and ≥25 μm at 5 °C and 25 °C measured by LO particle counting.

for the same formulation shaken at 25 °C. This highlights the relative sensitivity of this molecule to interfacial vs thermal stress. As temperature drops the surface tension, and by extension the severity of interfacial stress, increases. In the case of this molecule the combination of lower thermal stress and elevated interfacial stress from shaking at 5 °C proved to be more detrimental than the combined stresses of shaking at 25 °C where thermal stress is higher but interfacial stress is lower.

It is worth noting that all samples with surfactant, even largely hydrolyzed polysorbate, appeared to be protected from interfacial stress at both temperatures. This would indicate that the residual polysorbate or the fatty acid products of hydrolysis still provide

Table 3
MC & SR PS20 particle counts for ≥2 to < 10 μm and ≥ 10 μm sizes.

		Days	0	21	35	49	77	140	207
≥2 to < 10 μm Particles/ mL	5C	MC PS20	103	52,357	104,482	131,915	170,387	259,490	203,840
		SR PS20	52	14,132	10,511	10,848	29,269	63,064	65,657
	25C	MC PS20	103	66,200	72,982	88,818	78,245	155,550	
		SR PS20	52	36,524	41,505	72,030	90,114	201,910	
≥ 10 μm Particles/ mL	5C	MC PS20	9	36	288	195	287	1180	560
		SR PS20	7	716	973	1116	2591	1380	3223
	25C	MC PS20	9	492	922	2144	2418	4233	
		SR PS20	7	2909	2885	4877	7194	5435	

Table 4
Change in API quality attributes.

Formulations	Δ%SEC Monomer	Δ% Purity NR CE-SDS	ΔA280 (mg/ml)	Δ NTU	Δ% Bioactivity Assay 2	Δ% Bioactivity Assay 1	Δ%SEC Monomer	Δ% Purity NR CE-SDS	ΔA280 (mg/ml)	Δ NTU	Δ% Bioactivity Assay 2	Δ% Bioactivity Assay 1
	Change in measured CQA at 5 °C° (Day 207- T0)						Change in measured CQA at25 °C° (Day 140- T0)					
0.04 % MC PS20	−0.10 %	−0.30 %	−2	15	8 %	−1 %	−0.70 %	−1.50 %	−3	16	−22 %	−8 %
0.04 % SR PS20	−0.10 %	−0.20 %	−2	18	12 %	2 %	−0.70 %	−1.50 %	−3	21	−19 %	−4 %
0.04 % PLA PS20	−0.10 %	−0.10 %	1	0	12 %	1 %	−0.80 %	−1.50 %	−3	3	−24 %	0 %
0.04 % MC PS80	−0.20 %	−0.10 %	0	−1	15 %	−3 %	−1.10 %	−1.50 %	−2	4	−10 %	−11 %
0.04 % SR PS80	−0.20 %	−0.10 %	0	−1	18 %	7 %	−1.00 %	−1.50 %	−2	2	−16 %	−4 %
0.04 % POA PS80	−0.10 %	−0.20 %	1	−1	13 %	−13 %	−0.90 %	−1.50 %	−1	1	−23 %	−6 %
0.04 % Brij L23	−0.10 %	−0.10 %	0	−1	11 %	6 %	−0.70 %	−1.40 %	−2	0	−15 %	−7 %
0.04 % Brij O20	−0.10 %	0.00 %	0	−1	12 %	−14 %	−0.90 %	−1.40 %	−2	0	−19 %	−6 %
0.04 % P188	−0.20 %	−0.40 %	−1	−1	6 %	−5 %	−0.80 %	−1.60 %	−1	0	−24 %	−7 %
No Surfactant	−0.10 %	−0.10 %	−4	−1	14 %	2 %	−0.80 %	−1.50 %	1	−1	−17 %	−2 %
	Change in measured CQA at 5 °C° (Day 207 & shaking − Day 207)						Change in measured CQA at 25 °C° (Day 140 & shaking − Day 140)					
0.04 % MC PS20	0.00 %	0.10 %	1	−7	0 %	−3 %	−0.10 %	−0.10 %	1	5	−3 %	−2 %
0.04 % SR PS20	0.00 %	0.00 %	2	−5	0 %	−2 %	−0.20 %	−3.00 %	1	1	0 %	−3 %
0.04 % PLA PS20	0.00 %	0.00 %	−1	3	3 %	5 %	−0.10 %	0.00 %	2	4	0 %	−8 %
0.04 % MC PS80	0.00 %	0.00 %	1	2	0 %	8 %	−0.60 %	0.00 %	2	−1	−2 %	0 %
0.04 % SR PS80	0.00 %	0.00 %	0	2	−4 %	1 %	−0.50 %	0.00 %	1	−1	0 %	8 %
0.04 % POA PS80	−0.10 %	0.10 %	0	2	−4 %	0 %	−0.40 %	0.00 %	−1	0	3 %	−10 %
0.04 % Brij L23	0.00 %	0.00 %	0	0	−4 %	−7 %	0.00 %	0.00 %	0	0	−1 %	1 %
0.04 % Brij O20	0.00 %	0.00 %	0	1	0 %	5 %	−0.10 %	−0.20 %	1	0	8 %	−3 %
0.04 % P188	0.10 %	0.30 %	2	0	−3 %	0 %	0.00 %	−0.10 %	1	−1	2 %	2 %
No Surfactant	0.00 %	0.10 %	3	>100	−8 %	−2 %	−0.20 %	0.00 %	0	5	2 %	−1 %

enough protection for the mAb. This may not be true for mAbs with high surface activity.

Conclusions

The nature of the fatty acid population released by the hydrolysis of PS significantly impacts the nucleation/growth of fatty acid particles. PS20 with longer chain fatty acid incorporations, such as MC and SR, favors the nucleation of new particles resulting in high numbers of particles with relatively small size. PLA PS20, which yielded a homogeneous population of lauric acid upon hydrolysis with almost no longer chain fatty acids to serve as nucleators, favored the growth of existing particles over the formation of new. This resulted in a relatively small number of particles with greater size.

The rate of hydrolysis also impacted the development of particulates with rapid hydrolysis favoring a larger number of smaller particles with slower hydrolysis allowing for the growth of larger particles. The slower release of fatty acids into solution allows for long chain FFA to be rolled into the growth of an existing particle before they encounter conditions needed to nucleate a new particle.

Temperature also plays a meaningful role in the nucleation and growth of particles, while higher temperatures do improve the solubility of fatty acids, once saturation is achieved, a higher temperature facilitates both the nucleation and growth of particles. The hydrolysis products of polysorbate 80 appear to have a diminished tendency to form insoluble aggregates. The products may be solubilized by either the remaining polysorbate micelles or potentially by the protein itself. Of the non-hydrolysable surfactants tested, P188, Brij L23 and Brij O20 all were able to protect the mAb from interfacial stress without the liability of particle formation. Currently Brij L23 and Brij O20 are not approved for parenteral use but represent promising alternatives to polysorbate once more patient safety evaluations have been made.

For practical application, non-hydrolysable surfactants represent a good means of protecting biologics from interfacial stresses without the liabilities of generating insoluble particulates or losing interfacial protection over time. PS80 presents a smaller risk for particle formation when hydrolyzed but may still lose interfacial protection when formulated with highly surface-active molecules. While not as safe as non-hydrolyzable surfactant, PS80 may be suitable to formulate mAbs with low surface activity with moderate esterase impurities.

The hydrolysis of PS20 presents a dual risk of losing interfacial protection and forming insoluble particulates. PS20 in any grade, should only be considered as a surfactant for mAbs with little or no esterase activity.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors thank Vineet Kumar, Dominick DeGrazio, Chandani Kodikara, William Fredericks, for their support and contribution.

References

1. Jones LS, Bam NB, Randolph TW. *Surfactant-Stabilized Protein Formulations: A Review of Protein-Surfactant Interactions and Novel Analytical Methodologies*. American Chemical Society; 1997.
2. Wuchner K, Yi L, Chery C, et al. 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. *J Pharm Sci*. 2022;111(5):1280–1291.
3. Frison-Norrie S, Sporns P. Investigating the molecular heterogeneity of polysorbate emulsifiers by MALDI-TOF MS. *J. Agric. Food Chem.*. 2001;49(7):3335–3340.
4. Doshi N, Fish R, Padilla K, Yadav S. Evaluation of super refined^{TM} polysorbate 20 with respect to polysorbate degradation, particle formation and protein stability. *J Pharm Sci*. 2020;109(10):2986–2995.
5. Tomlinson A, Zarraga IE, Demeule B. Characterization of polysorbate ester fractions and implications in protein drug product stability. *Mol Pharm*. 2020;17(7):2345–2353.
6. ZZ Jannatun Nayem, Tomlinson Anthony, Zarraga Isidro E, Wagner Norman J, Liu Yun. Micellar morphology of polysorbate 20 and 80 and their ester fractions in solution via small-angle neutron scattering. *J Pharm Sci*. 2020;109(4):1498–1508.
7. Kranz W, Wuchner K, Corradini E, Berger M, Hawe A. Factors influencing polysorbate's sensitivity against enzymatic hydrolysis and oxidative degradation. *J Pharm Sci*. 2019;108(6):2022–2032.
8. Dwivedi M, Blech M, Presser I, Garidel P. Polysorbate degradation in biotherapeutic formulations: identification and discussion of current root causes. *Int J Pharm*. 2018;552(1–2):422–436.
9. JL Miguel Saggu, Patel Ankit. Identification of subvisible particles in biopharmaceutical formulations using raman spectroscopy provides insight into polysorbate 20 degradation pathway. *Pharm Res*. 2015;32:2877–2888.
10. Labrenz SR. Ester hydrolysis of polysorbate 80 in mAb drug product: evidence in support of the hypothesized risk after the observation of visible particulate in mAb formulations. *J Pharm Sci*. 2014;103(8):2268–2277.
11. Kishore RS, Kiese S, Fischer S, Pappenberger A, Grauschopf U, Mahler HC. The degradation of polysorbates 20 and 80 and its potential impact on the stability of biotherapeutics. *Pharm Res*. 2011;28(5):1194–1210.
12. Doshi N, Ritchie K, Shobha T, et al. Evaluating a modified high purity polysorbate 20 designed to reduce the risk of free fatty acid particle formation. *Pharmaceutical Research*2021.
13. Saggu M, Demeule B, Jiang L, et al. Extended characterization and impact of visible fatty acid particles - a case study with a mAb product. *J Pharm Sci*. 2020.
14. Vaclaw C, Merritt K, Pringle V, et al. Impact of polysorbate 80 grade on the interfacial properties and interfacial stress induced subvisible particle formation in monoclonal antibodies. *J Pharm Sci*. 2020.
15. KNV Nicholas E Levy, Lee Kelvin H, Lenhoff Abraham M. Host cell protein impurities in chromatographic polishing steps for monoclonal antibody purification. *Biotechnol Bioeng*. 2016;113:1260–1272.
16. Allmendinger A, Lebouc V, Bonati L, Woehr A, Kishore RSK, Abstiens K. Glass leachables as a nucleation factor for free fatty acid particle formation in biopharmaceutical formulations. *J Pharm Sci*. 2021;110(2):785–795.
17. Doshi N, Demeule B, Yadav S. Understanding particle formation: solubility of free fatty acids as polysorbate 20 degradation byproducts in therapeutic monoclonal antibody formulations. *Mol Pharm*. 2015;12(11):3792–3804.
18. Glücklich N, Dwivedi M, Carle S, Buske J, Mäder K, Garidel P. An in-depth examination of fatty acid solubility limits in biotherapeutic protein formulations containing polysorbate 20 and polysorbate 80. *Int J Pharm*. 2020;591: 119934.
19. AP Ian Roy, Kumar Vineet, Nanda Tatiana, Assenberg Rene, Wuchner Klaus, Amin Ketan. Polysorbate degradation and particle formation in a high concentration mAb: formulation strategies to minimize effect of enzymatic polysorbate degradation. *J Pharm Sci*. 2021;110(9):3313–3323.
20. Doshi N, Martin J, Tomlinson A. Improving prediction of free fatty acid particle formation in biopharmaceutical drug products: incorporating ester distribution during polysorbate 20 degradation. *Mol Pharm*. 2020.
21. FB Guillaume Lefebvre, Filipe Vasco, Huille Sylvain, Weidenhaupt Marianne. Adsorption rate constants of therapeutic proteins and surfactants for material surfaces. *Colloids Surfaces B: Biointerfaces*. 2021:203.
22. Fang L, Richard CA, Shi GH, Dong X, Rase MC, Wang T. Physicochemical excipient-container interactions in prefilled syringes and their impact on syringe functionality. *PDA J Pharmaceut Sci Technol*. 2021. pdajpst.2020.012278.
23. Gervasi V, Dall Agnol R, Cullen S, McCoy T, Vucen S, Crean A. Parenteral protein formulations: an overview of approved products within the European Union. *Eur J Pharmaceut Biopharmaceut: Official J Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik eV*. 2018;131:8–24.
24. Strickley RG, Lambert WJ. A review of formulations of commercially available antibodies. *J Pharm Sci*. 2021;110(7):2590–2608. e2556.
25. Yunqiu Chen C-FX, Stanley Bradley, Evangelist Greg, et al. A highly sensitive LC-MS/MS method for targeted quantitation of lipase host cell proteins in biotherapeutics. *J Pharm Sci*. 2021;110:3811–3818.
26. Cheng W, He B, Basu S, Souillac P. Identification of key excipients for the solubilization and structural characterization of lipoprotein lipase, an enzyme for hydrolysis of triglyceride. *J Pharm Sci*. 2021;110(5):1958–1968.
27. Li YHD, Lentz YK, Ji JA, Zhang TY, Zhang K. Characterization and stability study of polysorbate 20 in therapeutic monoclonal antibody formulation by multidimensional ultrahigh-performance liquid chromatography-charged aerosol detection-mass spectrometry. *Anal Chem*. 2014:5150–5157.
28. Hewitt DAM, Robinson K, Ji J, Wang YJ, Kao YH, Zhang T. Mixed-mode and reversed-phase liquid chromatography-tandem mass spectrometry methodologies to study composition and base hydrolysis of polysorbate 20 and 80. *J Chromatogr A*. 2011:2138–2145.
29. Evers D-H, Carle S, Lakatos D, Hämmerling F, Garidel P, Buske J. Hydrolytic polysorbate 20 degradation - Sensitive detection of free fatty acids in biopharmaceuticals via UPLC-QDa analytics with isolator column. *J Chromatogr B, Anal Technol Biomed Life Sci*. 2021;1174: 122717.
30. BD Anthony Tomlinson†, Lin‡ Baiwei, Yadav Sandeep. Polysorbate 20 degradation in biopharmaceutical formulations: quantification of free fatty acids, characterization of particulates, and insights into the degradation mechanism. *Mol Pharmaceut*. 2015:3805–3815.
31. Michaelis L, Menten MM. The kinetics of invertin action. 1913 *FEBS Lett*. 2013;587 (17):2712–2720.
32. Patist SSB A, Penfield KW, Aikens P, Shah DO. On the measurement of critical micelle concentrations of pure and technical-grade nonionic surfactants. *J Surfactants Deterg*. 2000:53–58.