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RESEARCH PAPER

The Degradation of Polysorbates 20 and 80 and its Potential Impact on the Stability of Biotherapeutics

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ABSTRACT

Purpose To study the potential impact of the degradation of Polysorbates (PS) 20 and 80 on the stability of therapeutic proteins in parenteral formulations.

Method First, degradation products of PS20 and 80 were identified. Subsequently, the effect of degraded polysorbate on physical characteristics and long-term stability of protein formulations was assessed. Further, the impact of polysorbate degradation on protein stability was evaluated via shaking stress studies on formulations spiked with artificially degraded polysorbate or degradants like fatty acids. Additionally, aged formulations with reduced polysorbate content were shaken.

Results The degradation of polysorbate leads to a buildup of various molecules, some of which are poorly soluble, including fatty acids and polyoxyethylene (POE) esters of fatty acids. Spiking studies showed that the insoluble degradants could potentially impact protein stability and that the presence of sufficient intact polysorbate was crucial to prevent this. End-of-shelf-life shaking of protein formulations showed that the stability of various monoclonal antibodies was, however, not affected.

Conclusions Although some degradants can potentially influence the stability of the protein (as discerned from spiking studies), degradation of polysorbates did not impact the stability of the different proteins tested in pharmaceutically relevant temperature and storage conditions.

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INTRODUCTION

Polysorbates are the most widely used non-ionic surfactants to stabilize protein pharmaceuticals against interface-induced aggregation (1) and surface adsorption (1–6). They are effective against various stresses such as agitation (for example, shaking or stirring) (2,7–9), freeze/thawing and lyophilization (10,11).

Nevertheless, some of their characteristics need to be carefully considered and monitored. Commercially available polysorbates (PS) 20 and 80 are chemically diverse mixtures containing mainly sorbitan POE fatty acid esters. Additionally, substantial amounts of POE, sorbitan POE and isosorbide POE fatty acid esters are present (12–14). This leads to a significant degree of lot-to-lot variability requiring a close scrutiny of each lot in order to ensure uniform behavior (15). The presence of residual levels of peroxide in bulk polysorbate is also a concern. The European Pharmacopeia (Ph.Eur.) specifies a limit of a peroxide number (PN) ≤ 10. There have been reports of a buildup of peroxides in bulk as well as in aqueous solutions of polysorbate, when exposed to ambient oxygen and light (16). Depending on handling and storage conditions and supplier lot, varying concentrations of peroxides (9 ppm–250 ppm) were noted among different lots of polysorbates (15). The buildup of peroxides can be detrimental not only to the stability of polysorbate itself but also to the protein therapeutic, which it stabilizes. Based on this consideration, Harmon *et al.* have also developed an oxidation stress test relying on peroxides formed in PS80 in the presence of Fe (III) (17).

Polysorbates are known to be degraded by auto-oxidation and hydrolysis (16,18–21). This could lead to a decrease in the apparent concentration of polysorbate in the protein formulation over long shelf life, the rate of decrease being proportional to increasing temperature. In our recent publication (22), we have determined the rates of hydrolysis of polysorbates in formulations at pharmaceutically relevant conditions. It was found that the rate of hydrolysis of polysorbates in aqueous protein formulation is rather slow at storage temperatures of 5°C and 25°C and at pH 5.5, and that auto-oxidation is the main cause of degradation at these temperatures. At 40°C, the half-life of the hydrolysis reaction in polysorbates was approximately 5 months. Correspondingly, auto-oxidation was also shown to increase under these temperature conditions.

Since polysorbates are included in protein formulations to stabilize the protein, the decrease in the concentration of polysorbate and the accumulation of degradant molecules in a protein formulation could be of potential concern for protein stability. Listed in Table I are the various chemical products that are known to accumulate upon degradation of polysorbates in aqueous solutions.

The possible concerns arising from polysorbate degradation are two-fold: 1) lowered ability of the surfactant to protect the formulation against interfacial stresses, and 2) impact of the degradation products on the stability of the protein. However, there have been no detailed studies to date dealing with possible consequences of polysorbate degradation in protein formulations on the stability of the protein therapeutic.

In the first part of this publication, continuing from previous work (22), an investigation on the mechanistic aspects of degradation was carried out. First, degradants of polysorbate were isolated and characterized; subsequently, investigations on various physical and chemical changes that could occur in a protein formulation during the course of the degradation of polysorbate over a storage period of twelve months at 25°C, *e.g.* changes in turbidity, sub-visible particle count, surface pressure, and the UV absorption spectrum, were performed.

In the second part, the impact of the degradation of polysorbates on the stability of protein formulations was assessed by performing end-of-shelf-life shake stress studies on aged (1–2 year old) samples of monoclonal antibody (mAb) formulations. Additionally, the impact of the presence of degradants on the stability of antibody formulations by using either degraded—instead of neat—polysorbate or by spiking degradation products resulting from oxidation such as fatty acids and fatty acid esters into protein formulations were investigated.

MATERIALS AND METHODS

Materials

Four different monoclonal antibodies, (termed mAb1, mAb2, mAb3 and mAb4) provided by F. Hoffmann-La Roche Ltd. (Basel, Switzerland) were used for these studies. The formulation compositions for the different proteins are as follows: mAb1–10 mg/mL in 20 mM His/His-HCl, pH 6.0 with 240 mM trehalose and 0.05% (w/v) PS20 or PS80 (Croda, Edison, NJ, USA); mAb2 -10 mg/mL in 20 mM His/His-HCl, pH 6.0, 240 mM trehalose and 0.01–0.04% (w/v) PS20 and PS80; mAb3—10 mg/mL in 25 mM sodium acetate buffer, pH 6.0 with 125 mM sodium chloride and 0.025% (w/v) PS20; mAb4—25 mg/mL in 20 mM His/His-HCl, pH 5.5 with 240 mM trehalose and 0.02% (w/v) PS80. All samples were filtered under aseptic conditions, using 0.22 μm Millex GV (PVDF) syringe filter units (Millipore, Bedford, MA, USA) and filled into washed and sterilized 6 ml ∅ 20 mm glass type 1 vials (silicone-free). The vials were then stoppered under filtered nitrogen gas flow with Teflon®-coated serum stoppers and securely sealed with aluminum caps. The corresponding placebo formulations (similar composition but without protein) were also prepared as mentioned above. Samples were subsequently stored in temperature-controlled chambers at 5°C, 25°C/60%rh and 40°C/75%rh. All other chemicals used were of analytical grade and obtained from commercial sources.

Isolation and Characterization of Polysorbate Degradation Products

Insoluble substances (by visible inspection) in PS20 containing placebo formulations were isolated by syringe filters (MillexHV, 0.45 μm, PVDF Waters) or polycarbonate gold filters (0.80 μm, Pall corporation Timonium, MD, USA). Centrifugation or preparative ultracentrifugation were found to be unsuitable for the isolation procedure. After filtration by the polycarbonate gold filter, the filter was immediately analyzed on an FT-IR microscope. The isolation procedure by syringe filters was performed as

Table I List of Potential Degradation Products from Polysorbate 20 and 80 Known from Earlier Reports

	Degradants found	Ref
Polysorbate 20	fatty acids, acetic acid, formic acid, peroxides	(20,22)
	acetaldehyde, acetone, ethanol, pentane, hexane, heptanal	(23)
Polysorbate 80	acetaldehyde, acetone, ethanol, pentane, hexane, butanal, pentanal, heptanal	(23)

follows: The sample solution was carefully drawn into a syringe and expelled through the filter. The filtrate was drawn slowly via syringe filters back into the vial, and the contents were inspected. If the formulation was clear, the residue in the syringe filter was rinsed with two aliquots of water to remove any adsorbed substances. The filter was then washed with methanol in order to dissolve and release the organic residues. In case filtration was not successful with a 0.45 µm filter (the filtrate not being clear on inspection), a Millex LG (0.20 µm, PTFE) was used applying the same procedure. The isolated substances were analyzed immediately using 1H NMR and FT-MS.

Formulation with Degraded Polysorbate and Fatty Acid Spiking Procedure

In a first study, degraded PS20/PS80 (Croda) was prepared by stressing a 0.02% (w/v) solution of PS at 80°C with 300 ppm H_2O_2 for 7 days. The mAb2 formulation was formulated with various combinations of degraded and unstressed PS to final concentrations ranging from 0.005% to 0.03% (w/v).

In another study, lauric acid (>99% purity, Sigma-Aldrich, St. Louis, MO, USA) was spiked into mAb3 formulation using a concentrated stock solution of lauric acid in methanol at various PS20: LA molar ratios ranging from 1:0–0:0.1. Samples with the same volume of methanol without lauric acid were prepared as controls.

End-of-Shelf-Life Shaking Stress Studies

The shaking stress studies were performed as described previously (2), using protein formulations that were previously stored in temperature-controlled chambers. mAb2 samples were stored for 12 months at 5°C, and mAb4 samples were stored for 24 months at 5°C and then subjected to shaking stress. Shaking stress was performed using a horizontal shaker (HS 260 Control Model IKA GmbH and Co. Staufen, Germany) at 200 rpm for 7 days at 25°C/60%rh. Samples were analyzed for protein aggregation by visual inspection and size exclusion chromatography (SE-HPLC) as described previously (2). Polysorbate quantification was carried out by a fluorescence based assay as described previously (22,23).

Fourier-Transformed Mass Spectrometry (FT-MS)

FT-MS was measured on a Thermo FT-ICR-MS instrument with a 7 Tesla superconducting magnet coupled to an HPLC. Masses were measured either in electrospray or nanospray mode. A reverse-phase XBridge C8 3.5 µm column (Waters AG, Baden-Daettwil, Switzerland) was used with a mobile phase consisting of (A) 20 mM NH4OAc/(B) MeOH at a flow rate of 0.2 mL/min. The gradient was as follows: 0 min 50% MeOH, 2 min 95% MeOH, 22 min 50% MeOH, 25 min. Only those peaks not observable in the control sample were considered for analysis and structure identification. This was of high importance, since it is known that surfactants like polysorbates can adsorb to various surfaces including filters (24)

Nuclear Magnetic Resonance (NMR)

All NMR spectra were taken on a Bruker 600 MHz Avance II spectrometer (Bruker Biospin GmbH, Ettlingen, Germany) equipped with a cryogenic TCI probehead at a temperature of 300 K. Spectrometer operation and data processing were done on Topsin 2.1 (Bruker BioSpin GmbH). Samples were dissolved in a 1:1 mixture of CD3CN/D2O. 1D 1 H (zg and preset) and diffusion edited 1D (DOSY) spectra were acquired.

Fourier-Transformed Infrared Spectroscopy (FT-IR)

Placebo solutions containing insoluble matter were filtered over a gold-coated polycarbonate filter (cross-section approx. 3 cm). The particles on the gold filter were analyzed with a Thermo Scientific Nic Plan/Magna 550 FTIR microscope. The residuals were documented by means of a stereo microscope and investigated for chemical identification by ATR (IRE = diamond) or Reflection mode with $R=2$ cm^{-1} and 300 Scans.

Stir-Bar-Assisted Sorptive Extraction Gas Chromatography–Mass Spectrometry (SBSE-GC-MS)

Stir-bar-assisted sorptive extraction was performed as follows. Accelerated degradation was achieved by stressing aqueous solutions of 0.02% PS20 and PS80 at 80°C for 7 days in the presence of 300 ppm H_2O_2. LC-MS of the solutions was performed to assess the extent of degradation. SBSE was performed with the help of a PDMS-coated GERSTEL Twister™ (Muelheim, Germany). Desorption was done on a GERSTEL TDS-2/TDS-A thermal desorption system (TDS) fitted with an auto sampler. Analytes were thermally desorbed, focused into the inlet and transferred to an Agilent 6890 GC fitted with a fused silica column DB-1MS (30mX0.25 mm i.d. J&W Scientific Inc, Folsom, CA, USA). The TDS was run in a splitless mode with a temperature ramping from 20°C–300°C in 10 min. The transfer temperature was 320°C. The cooled injection system (CIS) temperature was ramped from –50°C to 300°C at 12°C/s, the carrier gas being helium. The column temperature was ramped from 40°C to 280°C at a rate of 4°C/min. The MS was an Agilent 5975 system. The interface temperature was set to 280°C. The quadrupole temperature and source temperature were 150°C and

230°C respectively. For a set of samples, semi-quantitation was performed comparing the obtained area with the averaged area of a calibration BHT solution at the concentration of 0.1 µg/g solution.

UV Measurement

Absorption spectroscopy of all placebo samples was performed on a UV plate reader (Infinate® 200 Tecan Group Ltd. Männedorf, Switzerland) in a wavelength range of 240 to 450 nm using a quartz well plate. The measurements were performed against a blank containing all the constituents of the formulation (except the mAb) at the initial time point (t0).

Surface Pressure

Surface pressure curves were measured using a modified Du Nouy method on a Kibron (Espoo, Finland) MultiPi WS 1 multichannel tensiometer as previously described (23). The system was calibrated against water with a known surface tension of 72.5 mNm. All measurements were performed at a temperature of 25±3°C. Surface pressure curves were acquired from a dilution series of placebos or formulations, ranging from 1 µM to 1 mM. The dilution series was prepared by a liquid handling system (Tecan Group Ltd., Männedorf, Switzerland, Genesis RSP 150) ranging from 1 µM to 1 mM surface active compound concentration.

Size Exclusion High Performance Liquid Chromatography (SE-HPLC)

SE-HPLC was carried out on an Alliance 2795 (Waters Corporation, Baden-Daettwil, Switzerland) equipped with a 2487 UV detector (Waters Corporation, Baden-Daettwil, Switzerland) and a TSK G3000 SWXL, 7.8 x 300 mm column (Tosoh Biosep, Stuttgart, Germany) for the detection of soluble protein aggregates. The separation was performed using a mobile phase of 200 mM K_2HPO_4/KH_2PO_4 and 250 mM KCl pH 7.0 at a flow rate of 0.5 ml/min at a constant 25°C. Undiluted samples were injected to obtain a total loading amount of 200 µg, and detection was performed at a wavelength of 280 nm. The total soluble high molecular weight products (peak areas of dimers and higher soluble oligomers) relative to the total peak area was calculated using the Empower 2 Chromatography Data System software (Waters Corporation) and reported as "soluble aggregate content."

Turbidity and Sub-visible Particle Counts

Turbidity measurements were on a HACH 2100AN turbidimeter (Hach Company, Loveland, CO). The instrument was calibrated against formazin reference suspensions and thus the results are expressed as formazin turbidity units (FTU). Sub-visible particle counting by light obscuration were measured on a liquid particle counting system (HIAC ROYCO 9703, HACH Lange, Rheinack, Switzerland) as previously reported (2).

Polysorbate Content Determination

The content of polysorbates was determined using a fluorescence micelle assay as previously reported (22).

RESULTS

We recently reported that during the course of long-term stability testing of mAb formulations (and placebos) containing polysorbate, a decrease in content of polysorbate was noticed along with an increase in peroxide levels (22). The placebo formulations that showed a decrease in PS content were subjected to a comprehensive investigation via isolation of volatile, insoluble and soluble degradants. Information on volatile degradants was available from our previous work (22). Isolated insoluble substances were characterized by 1H-NMR, FT-IR and FT-MS, whereas the soluble degradants were analyzed by SBSE GC-MS. Based on the degradants isolated, various mechanisms of degradation of polysorbates could be pieced together, giving a comprehensive picture on polysorbate degradation.

The isolation of insoluble matter was performed as described in the previous section and analysis on the isolated material was done as follows.

FT-MS

Insoluble substances found in the placebo formulations were isolated by syringe filtration, re-dissolved in methanol and subjected to LC-FT-MS as described in the "Materials and Methods" section. The extracted masses from the various peaks of the total ion chromatogram (TIC) were analyzed for structure determination. Based on the accurate masses obtained, molecular formulae could be assigned to the MS signals. Assisted by the fragmentation patterns in the MS/MS experiment, structures were assigned to the peaks obtained. In the negative mode, a series of fatty acids C10–C18 were detected as anions $[M\text{-}H]^-$ and $[M+OAc^-]$. In the positive mode, a homologous series of fatty acid esters of polyoxyethylene (POE) were detected as $[M+NH_4]^+$ or $[M+Na^+]$. The fatty acid esters were found to be of the general formula $CH_3(CH_2)_nCOO(CH_2CH_2)_mOH$ with fatty acids ranging from C12 to C18 (n=10 to 16) esterified to short POE chains (m=4 to 8). Furthermore, by

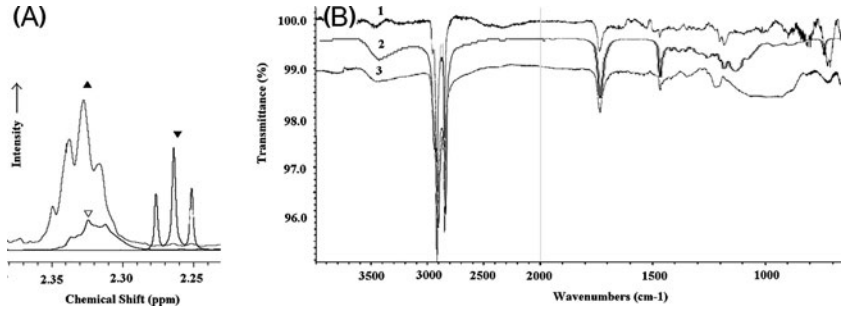


Fig. 1 (**A**) 1H-NMR overlay of spectra of the isolated insoluble matter (▽), polysorbate 20 (▲) and lauric acid (▼) in the range of 2.20–2.40 ppm corresponding to that of αCH_2 adjacent to the carbonyl group. (**B**) FT-IR overlays of the isolated insolubles (*traces 1* and *3*) compared to that of hydroxyl ethoxy ethoxy ethyl stearate (*trace 2*) showing a good match to the spectra from the particles.

measurement of commercially available fatty acid esters displaying equivalent MS spectra and fragmentations, the identity of these compounds was confirmed.

1H-NMR

Measurements of the samples isolated by filtration showed that the ratio of the integral of terminal methyl group of the fatty acid and the integral of POE was found to be 3/15 and 3/100 in measured sample and in intact PS20, respectively. This clearly demonstrates a nearly six-fold reduction of POE (relative to fatty acid) in the sample, indicating that the POE chain of the measured sample was much shorter than that of the polysorbate. The overlay of spectra of the sample and that of PS20 and lauric acid (Fig. 1) showed that the signal corresponding to the α $C\underline{\mathbf{H}}_2$ adjacent to the carbonyl in the measured sample corresponds more to an ester signal rather to that of a free fatty acid. These results, together with the data from FT-MS, clearly confirmed that the major constituents of the insoluble matter were fatty-acid esters of short POE chains.

FT-IR

Spectra obtained by ATR measurements of the insoluble particles filtered on polycarbonate gold filter were matched against spectra from a spectral library. The spectrum of the measured sample matched very well to that of hydroxy-$(ethoxy)_3$-stearate as shown in Fig. 1. This result further supports that the major constituents of the insoluble material are fatty acid esters of POE chains.

SBSE-GC-MS

In order to evaluate other degradants of polysorbate which accumulate in ppm concentrations in aqueous formulations, we adopted a stir-bar-sorptive absorption method coupled to GC-MS. SBSE is an equilibrium technique like SPME (solid-phase-micro-extraction). Its greatest advantage over SPME is its higher detection limit, which is 1000-fold better than SPME, and higher adsorbing area. Above all, it is a non-invasive approach, allowing facile extraction of organic substances from aqueous formulations without any influences of the protein or buffer molecules, making it ideal for the analysis of degradants of polysorbate in an aqueous protein formulation. In preliminary measurements, fresh solutions of polysorbate were analyzed and compared to artificially degraded samples of PS20 and PS80. Aqueous solutions of 0.02% PS20 and PS80 were stressed at 80°C for 7 days. The degradants were adsorbed onto a Twister™ and then transferred into the GC-MS.

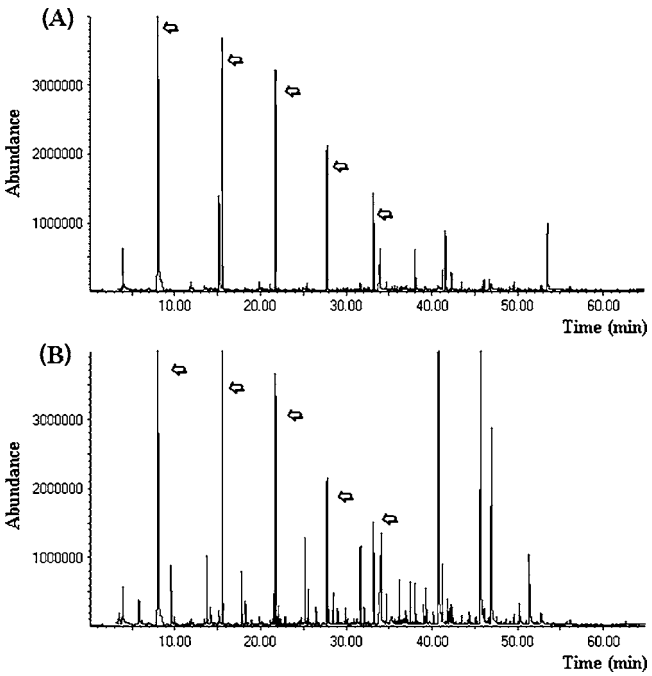


Fig. 2 SBSE-GC-MS traces obtained for degraded polysorbate 20 showing the peaks of various degradants obtained. (**A**) The top trace corresponds to a measurement done with a fresh aqueous solution of PS20. (**B**) The bottom trace is from a measurement of a stressed sample of the PS20 solution. The arrow (⇦) shows those signals which typically arise from the desorption of the polymer from the Twister™.

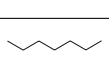
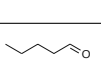
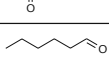
The GC-MS traces of the samples were compared, and the peaks were analyzed with the help of an MS database. The degraded sample of PS20 and PS80 showed the presence of a number of degradants when compared to the fresh sample (Fig. 2). Table II shows the various molecules that were identified and their mass distributions as observed in the electron ionization mode of the MS. Homologues of five different classes of molecules were identified: a) 2-alkyl ketones from C_5-C_{15}, b)1-alkyl aldehydes (C_5-C_{15}), c) 5-alkyl, dihydro 2-(3 H) furanones (the alkyl chain being C_5-C_9), d) Fatty acids C_{10}-C_{14}, e) fatty acid esters (C_{12}-C_{16}). In samples containing PS80, similar degradation products were observed.

Table II lists calculated logP values of the various degradants (*KOWWIN*™, EPIsuit™ US EPA [2010]). These values give a fair idea of the relative solubility of various molecules in water. Typically, molecules with logP values above 4.0 can be considered sparingly soluble or insoluble. From Table II, insoluble components thus include fatty acids and some POE esters of fatty acids. Fatty acids display logP values in the range of 4–6 and are known to be sparingly soluble. POE esters with short POE chains also display logP values above 4.0, implying that they are poorly soluble. Interestingly, degradation of PS20 leads to a greater number of insoluble degradants compared to those from PS80. It is interesting to note that solubility of sparingly soluble degradants of PS20 can vary with solution temperature as well as the concentration of remaining surfactant. We have observed that an increase in solution temperature or the presence of surfactant may again resolubilize these components (data not shown).

Physicochemical Effect of Polysorbate Degradation on Protein Formulations

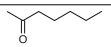
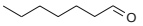
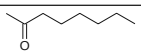
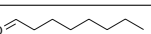
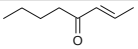
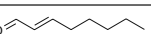
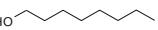
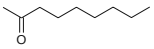
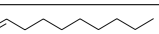
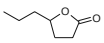
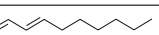
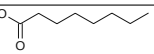
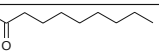
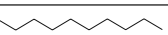
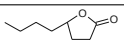
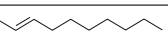
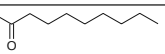
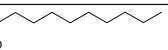
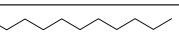
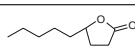
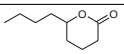
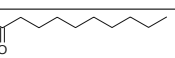
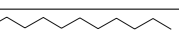
The physicochemical characteristics of formulations may undergo changes during the course of shelf life due to various factors. In order to understand if PS degradation would cause any such changes, mAb1 formulations containing 0.05% PS20 or PS80 were analyzed after a storage of 12 months at 5°C and 25°C. Various parameters such as turbidity, sub-visible particle count, UV and surface

Table II Comprehensive List of Isolated and Characterized Degradants in Our Study Using Various Methods, and Their LogP Values

Peak observed in PS20	Peak observed in PS80	Structure	Name	Mass	Technique	logP*
✓	✓		acetone	58	HS-GC-MS	
✓	✓		acetic acid	63	HS-GC-MS	
✓	✓		formic acid	46	HS-GC-MS	
✓	✓		acetaldehyde	44	HS-GC-MS	
✓	✓		ethanol	46	HS-GC-MS	
✓	✓		pentane	72	HS-GC-MS	
✓	✓		hexane	86	HS-GC-MS	
	✓		heptane	100	HS-GC-MS	
	✓		butanal	142	HS-GC-MS	
	✓		pentanal	156	HS-GC-MS	
✓	✓		2-pentanone	86	SBSE-GC-MS	0.75
✓	✓		2-hexanone*	100	SBSE-GC-MS	1.24
✓	✓		hexanal*	100	SBSE-GC-MS	1.80

*logP values were generated using KOWWIN™, EPIsuit™ US EPA.

Table II (continued)

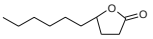
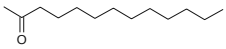
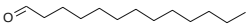
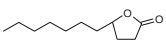
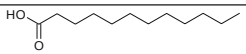
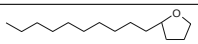
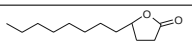
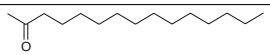
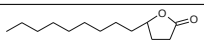
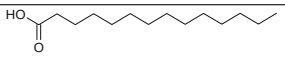
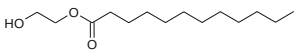
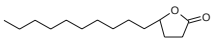
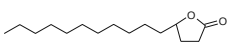
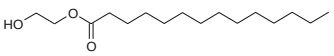
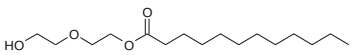
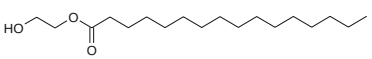
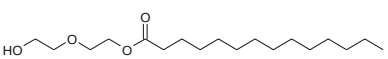
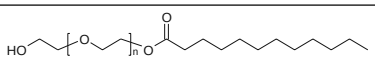
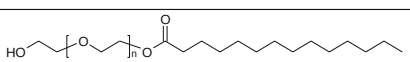
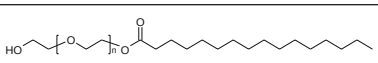
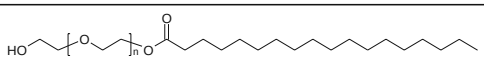
✓	✓		2-heptanone*	114	SBSE-GC-MS	1.73
✓	✓		heptanal*	114	SBSE-GC-MS	2.29
✓	✓		2-octanone	128	SBSE-GC-MS	2.22
✓	✓		octanal	128	SBSE-GC-MS	2.78
	✓		2-octen-4-one	126	SBSE-GC-MS	2.29
	✓		2-octenal	126	SBSE-GC-MS	2.57
	✓		1-octanol	130	SBSE-GC-MS	2.81
✓	✓		2-nonanone	142	SBSE-GC-MS	2.71
✓	✓		nonanal	142	SBSE-GC-MS	3.27
	✓		5-propyldihydro, 2-furanone	128	SBSE-GC-MS	1.09
	✓		2-nonenal	140	SBSE-GC-MS	3.06
✓	✓		octanoic acid	144	SBSE-GC-MS	3.03
✓	✓		2-decanone	156	SBSE-GC-MS	. 3.20
✓	✓		decanal	156	SBSE-GC-MS	3.76
✓	✓		5-butyldihydro, 2-furanone	142	SBSE-GC-MS	3.52
	✓		2-decenal	154	SBSE-GC-MS	3.55
✓	✓		nonanoic acid	158	SBSE-GC-MS	3.52
✓	✓		2-undecanone	170	SBSE-GC-MS	3.69
✓	✓		undecanal	170	SBSE-GC-MS	4.25
✓	✓		5-pentyldihydro, 2-furanone	156	SBSE-GC-MS	2.08
	✓		6-butyl-tetrahydro-pyran-2-one	156	SBSE-GC-MS	2.08
✓			decanoic acid	172	SBSE-GC-MS	4.02
✓	✓		2-dodecanone	184	SBSE-GC-MS	4.18

pressure were analyzed. PS content determination by the fluorescence micelle assay (FMA) showed that after a time period of 12 months at 25°C, the polysorbate concentration decreased to an apparent 0.006% in PS20 formulations and apparent 0.001% in PS80 formulations, respectively. Samples at 5°C showed an apparent decrease to 0.02% in PS20 formulations and 0.003% in PS80 formulations.

Turbidity

Changes in the turbidity of the placebo solutions were minimal at 5°C, varying between 0.2 to 0.4 formazin turbidity units (FTU) from the initial to the 12 months samples. In samples stored at 25°C, the turbidity increased up to 0.6 FTU. However, in both PS20 and PS80-containing formulations, there was no clear observable trend over different PS lots and the various concentrations of PS used. Compared to changes in turbidity in formulations containing mAb1, the changes in the placebos were negligible, implying little or no influence of degradation of polysorbate on turbidity in the samples measured. In a worst-case scenario it can, however, be expected that a buildup of insolubles could impact the turbidity of the sample.

Table II (continued)

✓			dodecanal	184	SBSE-GC-MS	4.75
✓	✓		5-hexyldihydro, 2-furanone	170	SBSE-GC-MS	2.57
✓	✓		2-tridecanone	198	SBSE-GC-MS	4.68
✓			tridecanal	198	SBSE-GC-MS	5.24
✓			5-heptyldihydro, 2-furanone	184	SBSE-GC-MS	3.06
✓			dodecanoic acid	200	SBSE-GC-MS	5.00
✓			2-decyl tetrahydrofuran	212	SBSE-GC-MS	5.77
✓	✓		5-octyldihydro, 2-furanone	198	SBSE-GC-MS	3.55
✓	✓		2-pentadecanone	226	SBSE-GC-MS	5.66
✓			5-nonyldihydro, 2-furanone	212	SBSE-GC-MS	4.04
✓			tetradecanoic acid	228	SBSE-GC-MS	5.98
✓			dodecanoic acid, 2-hydroxyethyl ester #	244	SBSE-GC-MS	4.31
✓	✓		5-decyldihydro, 2-furanone	226	SBSE-GC-MS	4.53
✓			5-undecyldihydro, 2-furanone	240	SBSE-GC-MS	5.02
✓			tetradecanoic acid, 2-hydroxyethyl ester #	272	SBSE-GC-MS	5.29
✓			dodecanoic acid, 2-(2-hydroxy-ethoxy)-ethyl ester #	288	SBSE-GC-MS	4.04
✓			hexadecanoic acid, 2-hydroxyethyl ester #	300	SBSE-GC-MS	6.27
✓			tetradecanoic acid, 2-(2-hydroxy-ethoxy)-ethyl ester #	316	SBSE-GC-MS	5.02
✓			dodecanoic acid, POE ester n=1-6		FT-MS	
✓			tetradecanoic acid, POE ester n=1-6		FT-MS	
✓			hexadecanoic acid, POE ester n=1-6		FT-MS	
✓			octadecanoic acid, POE ester n=1-6		FT-MS	

Sub-visible Particles

Cumulative counts per mL of sub-visible particles of sizes below 10μm and those between 10–25μm were analyzed using light obscuration. It was observed that in mAb1 formulations containing PS20, counts of sub-visible particles of below 10μm increased up to 4500 particles per mL, while those containing PS80 showed no discernable changes in counts and remained less than 20 particles per mL. In placebo formulations, the counts of sub-visible particles increased to 100 per mL for both PS20 and PS80-containing formulations, implying that a small increase in sub-visible particles is observed in placebos due to degradation of polysorbate. These sub-visible particles may be sensitive to temperature changes, due to solubility of the degradants. In the corresponding mAb1 formulations

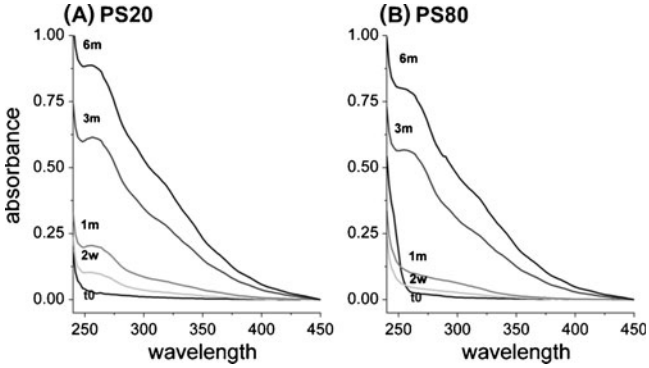


Fig. 3 UV spectra of placebos stored at 40°C over a 6 month storage period of formulations without protein containing polysorbate 20 (**A**) and polysorbate 80 (**B**) (*right*). The measurements were made at 5 time points: initial, 2 weeks, 1 month, 3 months and 6 months. The plots are depicted with shades of grey.

containing PS20, an increase in sub-visible particle count was observed. It is hence possible that some degradants of PS could trigger an increase in the sub-visible particle counts.

UV Absorption Studies

Studying the UV characteristics gives information on the various chromophores present in solution. We studied the changes in UV absorption (λ 240 nm–450 nm) of placebo formulations (without protein) containing PS20 and PS80 at concentrations of 0.05% w/v. An increase in absorbance was noted in the region of 240–400 nm in both PS20 and PS80-containing formulations, (Fig. 3). Two distinct absorption maxima were observed at 257 nm with a broad shoulder at 300 nm. Interestingly, a low intensity maximum at 247 nm observed initially in the PS80-containing sample (dotted line) disappeared in the subsequent measured time points. The observations in the UV absorption spectra also correspond with the observed degradants. An increase in UV absorption was observed upon aging of PS20 and PS80 at 257 nm with a broad shoulder at 300 nm. PS80 contains a double bond (C=C) which gives rise to a $\pi \rightarrow \pi^*$ transition (λ_{max} = 195 nm), an ester (C=O) and an ether (C-O-C) both giving $n \rightarrow \pi^*$ transition (λ_{max} = ~210 nm). Thus, the UV spectrum of PS80 should have a wavelength (λ) not exceeding 230 nm. The absorption beyond 200 nm could be ascribed to the presence of linoleic acid (λ_{max} = ~250 nm) (34). The decrease in the absorption peak at ~250 nm in the PS80 sample measured after 2 weeks (Fig. 4) points to the breakdown of the linoleic acid moiety. Subsequent measurements after 1, 2 and 6 months show a gradual increase of a peak at λ_{max} = ~257 nm and a broad shoulder at λ_{max} = ~300 nm. The peak at 257 nm indicates accumulation of molecules with $n \rightarrow \pi^*$ transition, such as aldehydes and ketones, which according to Woodward-Feiser rules would produce $n \rightarrow \pi^*$ transitions at 293 and 279 nm, respectively. Conjugated ketones would further shift the maximum to longer wavelengths fitting with the appearance of a broad shoulder at ~300 nm. It must be noted that radical assisted oxidation also occurs on histidine molecules; thus, the increase in absorption could be ascribed to formation of degradation products of photo- or thermal oxidation of histidine (35,36).

Surface Activity

Fig. 4 shows surface pressure of polysorbate solutions measured by a modified Du Nuoy method over a concentration range up to 0.05% (w/v) polysorbate for samples initially (t0) and after 12 months. The t0 sample displays a typical sigmoidal behavior for change in surface pressure over concentration for both PS20 and PS80-containing formulations as observed previously (23). The same samples stored for 12 months at 25°C, containing reduced apparent polysorbate concentration (due to increased polysorbate degradation) displayed surface pressure curves with similar characteristics as the initial measurements. The slope in the sigmoid plot shifted to higher concentrations, signifying a shift in the apparent CMC to higher concentrations, indicating a higher population of molecules in a micelle. Moreover, the measured surface pressure does not reach a constant endpoint as with the t0 samples. These results indicate the presence of new surface-active entities along with PS20 and PS80 with slightly different surface pressure properties. Table II shows that there are a number of degradants which are by themselves surfactants, some of which (*e.g.* Mrij) are also commercially used surfactants for various applications. Thus, despite degradation of polysorbate, surfactant activity could still be maintained. The retention of surface activity is probably a very important factor in preserving the stability of the

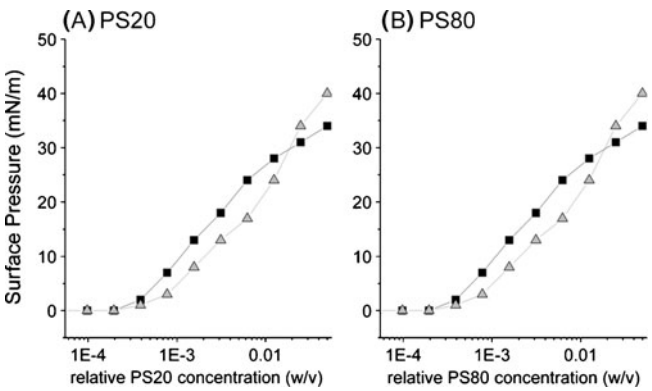


Fig. 4 Surface pressure plots of polysorbate 20 and 80 solutions measured by a modified Du Nuoy method over a concentration range up to 0.05% (w/v) polysorbate for samples at t0 (■) and after 12 months (▲).

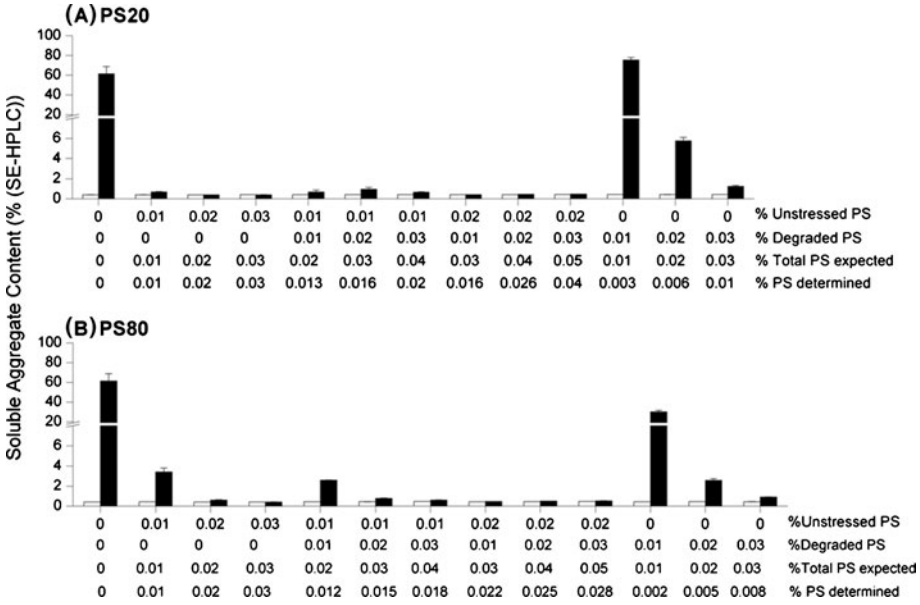


Fig. 5 The aggregation behavior of mAb2 with unstressed and/or degraded (**A**) PS20 or (**B**) PS80 upon static (□) or shaking stress (■) as analyzed by SE-HPLC. The various percentage quantities (w/v) of the unstressed PS, stressed PS are listed below the x-axis. The total expected PS content in each formulation and the actual measured content are also listed.

protein, as was later revealed in the shake stress studies performed on aged mAb samples.

To test the influence of the various degradants on the stability of proteins, two different experiments were conducted using either degraded PS20/PS80 (Fig. 5) or lauric acid as spiking agent (Figs. 6 and 7). Protein aggregation upon shaking stress was monitored by SE-HPLC, sub-visible particles and visible particle inspection (data not shown).

Spiking of Stressed PS

A study was set up wherein various concentrations of degraded polysorbate were formulated into mAb2 formulations containing concentrations of PS ranging from 0.03% to 0.01% (Fig. 5). The effective intact polysorbate content was measured by the fluorescence micelle assay. The intact polysorbate measured in these formulations was 0.003% for PS20 and 0.002% for PS80. This was found to be insufficient especially in the presence of other degraded components. The addition of unstressed PS to mAb2 significantly reduced the formation of soluble aggregates upon shaking as compared to a mAb2 formulation in the absence of a surfactant (Fig. 5). The presence of 0.01% PS20 was sufficient to protect the protein against shaking, whereas a higher amount of PS80 (0.02%) was required. When using a mixture of unstressed and (artificially) degraded PS20 or PS80, the soluble aggregate content slightly increased as compared to formulations with unstressed PS. Here, it was found that the higher the addition of degraded PS to the mAb2 sample, the lower the soluble aggregate content, especially in the PS80- containing samples (Fig. 5, B). The presence of degraded PS only resulted in a significant effect on the increase of soluble aggregates. The addition of 0.01% of degraded PS20 resulted in higher soluble aggregate content (75%) as compared to a sample shaken in the absence of PS (60%), whereas, interestingly, 0.01% of degraded PS80 showed only 30% aggregate content. The more artificially degraded PS was added to the mAb2 sample, the lower the soluble aggregate content by SE-HPLC. Formulations using 0.02% of degraded PS20 or PS80 (and no intact PS added) also displayed high soluble aggregate levels. Only those formulations in which the measured levels of intact polysor-

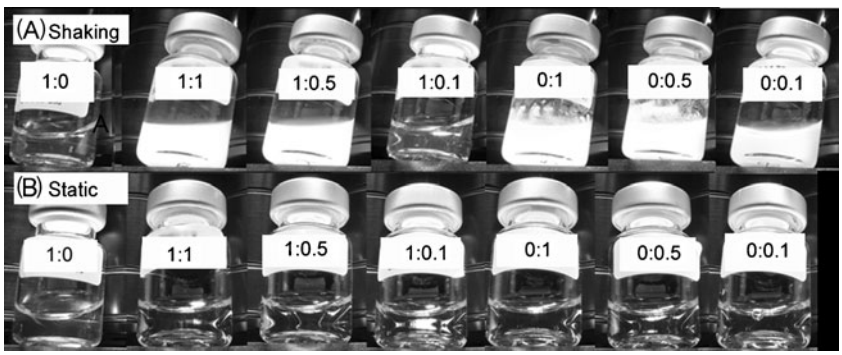


Fig. 6 Visual appearance of (**A**) shake stressed and (**B**) statically stored mAb 3 samples containing different molar ratios of polysorbate 20 to lauric acid.

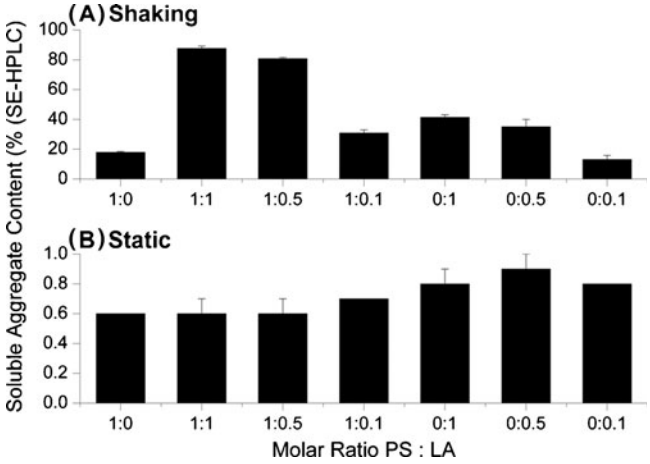


Fig. 7 Soluble aggregate content of (**A**) shake stressed and (**B**) statically stored mAb3 samples containing different molar ratios of polysorbate 20 to lauric acid. The mean error of triplicate measurements of each sample is graphically presented as error bars.

bate was greater than 0.01% for PS20 and 0.008% for PS80 showed low levels of soluble aggregates—despite the high levels of degradants added. This was also observed in formulations spiked with 0.03% degraded PS20 or PS80 (and no added intact PS).

Lauric Acid Spiking

In order to investigate protein stability in presence of insoluble fatty acid and intact PS20, a spiking study using the addition of free lauric acid was performed. Figures 6 and 7 show the visual appearance and soluble aggregate content of mAb3 in the presence of PS20 and free lauric acid at various molar ratios, respectively.

The presence of free lauric acid resulted in a significant increase in the formation of visible particles, in visual opalescence as well as in the soluble aggregate content of the mAb3 formulation when shaken (Figs. 6 and 7, A) in comparison to statically stored samples (Figs 6 and 7, B). Neither visible particles nor an increase in opalescence were observed in the placebo control samples (25) (data not shown), which suggests that visible particles formed in the mAb3 formulation consist mainly of insoluble protein aggregates. The samples containing lauric acid at a ratio greater than 1:0.5 (PS20: LA) resulted in very opalescent samples and contained much insoluble protein, as seen in the photograph (Fig. 6, A). The solution containing a ratio of 1:0.1 (PS20: LA) was shown to behave similarly to the samples without lauric acid, *i.e.* 1:0 (PS20:LA), in terms of visible inspection but contained approximately 10% higher amount of % soluble aggregates (%HMW). The samples with lauric acid in the absence of PS20 also contained high amounts of insoluble aggregates, especially the samples at ratios 0:1 and 0:0.5. The stock solution of lauric acid used for the spiking was prepared in methanol. Control samples with 100% methanol at the same volume added as the lauric acid stock solution to the 0.0025% PS20 mAb3 sample showed no significant increase in the soluble aggregate content (data not shown). The statically stored samples with lauric acid alone showed a slightly higher soluble aggregate content as compared to the formulation containing PS20 (Fig. 7, B). Figure 8 shows the soluble aggregate levels in the formulations shaken with various molar ratios of lauric acid to PS20. The data indicate that the presence of an increasing amount of free lauric acid has a detrimental effect on protein stability during shaking in the absence of PS20. At a molar ratio of 1:0.5 of PS20: lauric acid, soluble aggregate formation was still very pronounced. However, at a molar ratio of 1:0.1, the levels of soluble aggregates formed were low, indicating that PS20 was able to stabilize the protein in the presence of minute concentrations of lauric acid.

End-of-Shelf-Life Shake Stress Studies

In order to evaluate the impact of the degradation of polysorbate on the stability of the proteins, long-term stability studies using mAb2 and mAb4 formulations were carried out. Samples of mAb2 and mAb4 were stored long-term at 5°C (13 and 24 months, respectively). A further storage of mAb2 for one month at 40°C was carried out in order to accelerate and ensure substantial degradation of polysorbate within the mAb2 samples.

PS concentration measurements using the fluorescence micelle method indicated that formulations mAb2 and

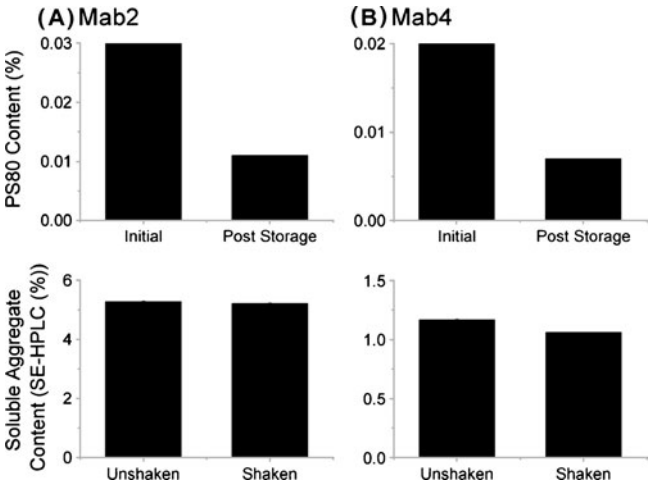
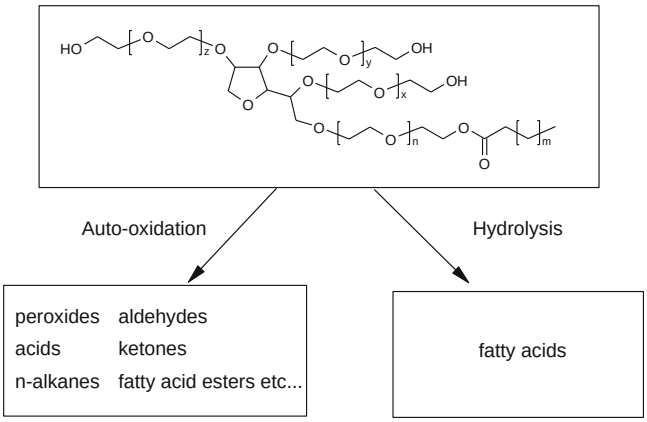


Fig. 8 Top: Polysorbate concentrations as measured by the fluorescence based micelle assay before (Initial) and after static storage (Post storage) for mAb2 (stored at 5°C for 12 months and further at 40°C for 1 months) and mAb4 (stored at 5°C for 24 months). Bottom: Percentage of soluble aggregates as measured by SE-HPLC of samples mAb2 and mAb4 before (Unshaken) and after (Shaken) shake stress (samples were shaken at 200 rpm for 7 days at 25°C).



Scheme 1 Main routes of degradation of polysorbates.

mAb4 showed a significant decrease in PS80 concentration post (intended and accelerated) storage—for mAb2 from 0.03% to 0.01% and mAb4 0.02% to 0.007% (Fig. 8A and B, respectively). However, this decrease in PS80 concentration did not have an impact on the soluble aggregate content upon subsequent shaking stress. Moreover, no increase in visible or sub-visible particles was noticed. Interestingly, it was previously observed that a PS80 concentration of only 0.01% was not sufficient to protect the mAb2 formulation during shake stress (Fig. 5) against the formation of soluble aggregates. In line with our finding that aged surfactant is still surface active, degraded PS80 within the mAb2 formulation post storage was still stabilizing the protein against mechanical stress.

DISCUSSION

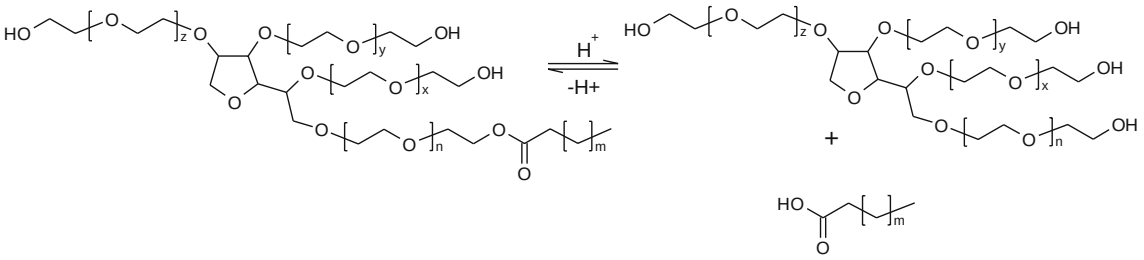
To understand the impact of the degradation of polysorbate on proteins, it is very important, in the first place, to understand comprehensively the mechanistic pathways of polysorbate degradation. In our previous manuscript (22), we indicated the various pathways of degradation of polysorbates in aqueous formulation which could play important roles at different temperatures. The focus of our investigations herein was to understand the degradation of polysorbates in pharmaceutically relevant conditions. The isolation of degradants of polysorbates (Table II) was a key to elaborate viable mechanistic routes of degradation for polysorbates occurring in biotech formulations. The following section summarizes our findings on the various mechanisms.

A Closer Look at Mechanistic Pathways of Polysorbate Degradation

The two known mechanistic pathways of degradation of polysorbate are hydrolysis and auto-oxidation (18). Scheme 1 provides a general outline of the degradants formed from the different pathways.

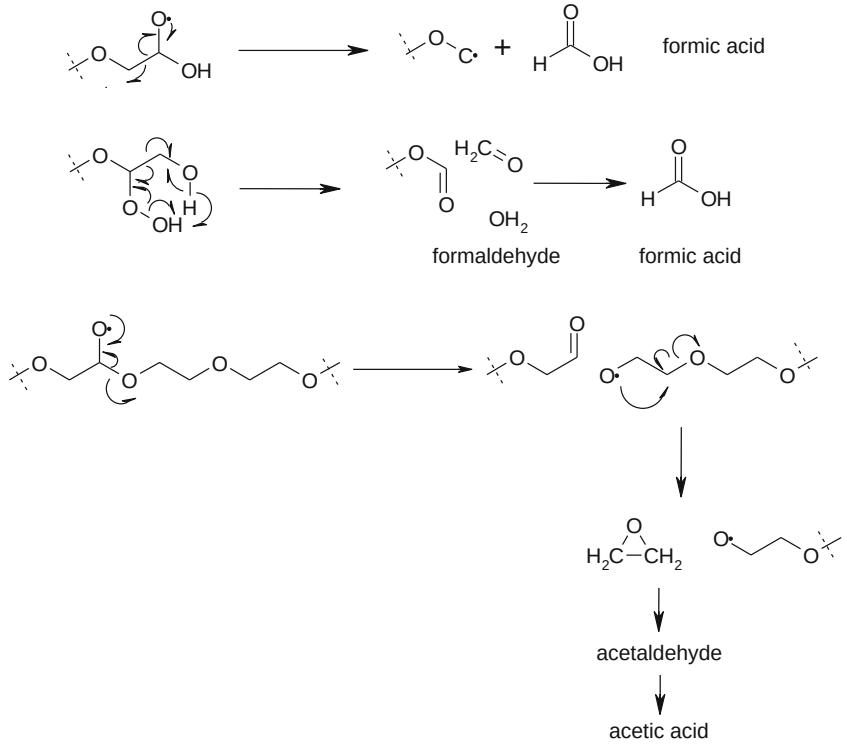
The presence of fatty acids as degradants obviously points to the occurrence of ester hydrolysis as one of the degradation mechanisms. Since the formulations are in very mildly acidic conditions (pH 6 or 5.5), the mechanism is an acid catalyzed $A_{AC}2$ reaction, which is known to be a thermodynamically driven reaction with the SN_2 attack of H_2O at the ester carbonyl being a bimolecular rate determining step (26). The rates of the reaction can be obtained by treating the reaction as a pseudo-first-order reaction as has been done by Bates *et al.* (27). We have previously found that this mechanism has substantial influence on the overall degradation only at higher temperatures (40°C) and has very slow rates at 5°C and 25°C (Scheme 2).

The mechanism of auto-oxidation follows the classical radical initiation, propagation and termination reactions and has been discussed previously by various groups and us (18,22,28,29). The initiation of auto-oxidation in polysorbates could occur by various means. Residual peroxides, metal traces and light may induce radical initiation in the presence of oxygen. The auto-oxidation of polysorbate adopts a similar pathway, as has been described for most poly-oxy-ethylene systems (30). Radical initiation on the POE chain is followed by insertion of atmospheric oxygen and subsequently a fast intermolecular hydrogen abstraction leading to the formation of a hydroperoxide species. As proposed by Decker *et al.* (28), homolytic cleavage of the peroxide leads to formation of alkoxy radicals which trigger subsequent reactions such as β-cleavage or radical disproportionation reactions. β-scission of alkoxy radicals would lead to formates and formic acid. Alternately, a six-



Scheme 2 Hydrolysis in polysorbates.

Scheme 3 Auto-oxidation of PEG.

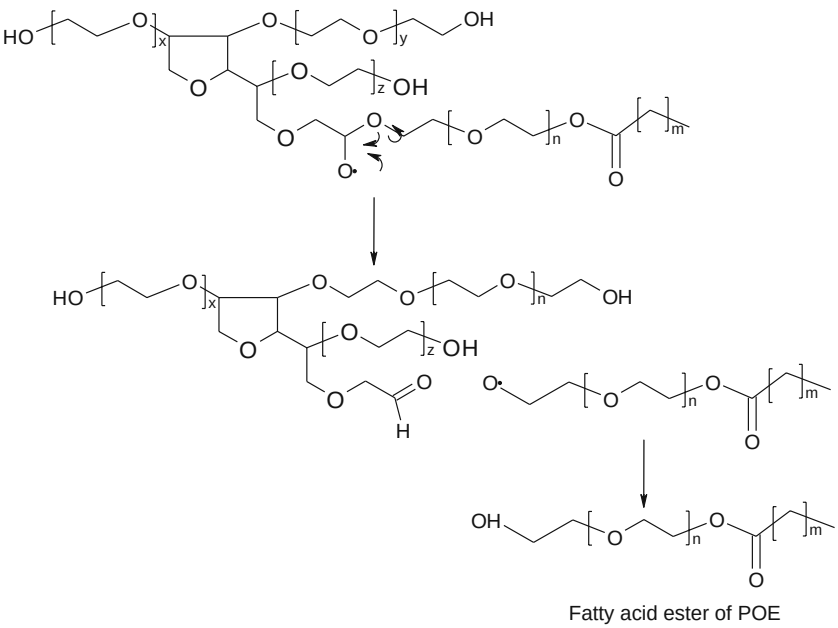


membered intramolecular decomposition would also lead to formation of formaldehyde and formic acid (Scheme 3). Formation of acetaldehyde and acetic acid has been explained by Dulog *et al.* (29) to occur via an epoxy intermediate (Scheme 3).

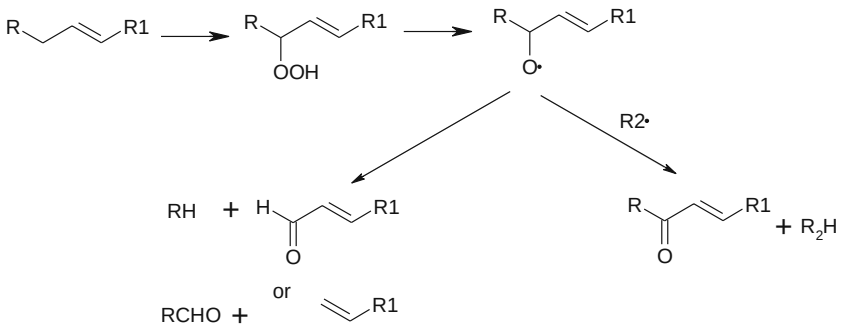
During identification of insoluble degradants of polysorbate, a major component was found to be short-chain POE esters of fatty acids. Some of the lower analogues were also characterized in the SBSE-GC-MS. Their formation could be explained by scission triggered by peroxide formation on the ester-containing arm of the molecule. Oxygen insertion followed alkoxy radical formation, and β scission at the C-O bond on the POE chain would lead to formation of a fatty acid ester (Scheme 4).

The presence of unsaturation in the fatty acid chain is always a concern with respect to auto-oxidation processes

Scheme 4 Auto-oxidation of polysorbates via chain breaking of the ester-containing arm.



Scheme 5 Auto-oxidation at the site of unsaturation leading to aldehydes and ketones.



because of their sensitivity towards degradation by radical reactions, as highlighted recently by Yao *et al.* (31). PS80 contains 50–87% of oleic acid and up to 20% of linoleic and linolenic acid. Even PS20 contains around 1% of oleic and 0.3% linoleic acid. The higher the conjugation, the more is the susceptibility to radical reactions.

Auto-oxidation of oleic acid begins by peroxide formation at the allylic site (38). Subsequently formed alkoxy radicals undergo homolytic cleavage of the adjacent C-C bond, giving rise to alkanes or aldehydes. Based on the position of peroxidation and the C-C bond scission, a number of alkanes are expected, as shown in Scheme 5. Table III lists the various possibilities of peroxide formation that exist for oleic, linoleic and linolenic acids and the subsequent secondary products that arise as aldehydes. Our studies with the headspace GC-MS and SBSE-GC-MS show formation of a number of aldehydes and alkanes, which can be explained by possible double-bond migration prior to radical reaction.

The formation of a series of 5-alkyl dihydro 2-furanones can be traced back to secondary reactions on unsaturated fatty acids. Lactonization of oleic acids in acidic conditions has been reported (32,33). This reaction is an acid-catalyzed reaction occurring with or without migration of the double bond. The reaction thus yields 5-alkyl lactones, the alkyl chain length varying due to migration of the double bond.

Table III Possibilities of Peroxide Formation on Oleic, Linoleic and Linolenic Acids and the Subsequently Formed Aldehydes as Secondary Products

Fatty acid	Hydroperoxide	Aldehydes
Oleic (18:1)	8-OOH	2-undecenal, decanal
	9-OOH	2-decenal, nonanal
	10-OOH	nonanal
	11-OOH	octanal
Linoleic (18:2)	9-OOH	2,4-decadienal, 3-nonenal
	13-OOH	hexanal
Linolenic (18:3)	9-OOH	2,4,7-decatrienal, 3,6-nonadienal
	12-OOH	2,4-heptadienal, 3-hexenal
	13-OOH	3-hexenal
	16-OOH	propanal

Polysorbates 20 and 80 show significant differences in the degradants formed based on their initial constitution. While the fatty acids obtained in hydrolysis reflect the initial constitution, differences were also seen with unsaturated fatty acids. PS20 has ~1% of oleic acid and thus showed degradants related to oleic acid in headspace GC-MS. Hexane and heptanal are secondary products of β-scission of alkoxy radical at C7. Traces of pentane (a β-scission product at C13 of linoleic acid) were also observed. In SBSE-GC-MS of PS80, undecenal, 3-nonenal and 2-heptenal, degradation products of oleic and linoleic acid, were observed.

SBSE-GC-MS analysis on 20-month-old samples of mAb1 containing either PS20 or PS80 stored at 25°C provided important insights as to which of the above degradation routes can be perceived in pharmaceutically relevant conditions. Table IV shows amounts of each class of degradants determined by a semi-quantitative approach in the formulations containing PS20 and PS80. The PS20- and PS80-containing samples show rather different distribution profiles. Samples containing PS20 prominently show formation of C10–C16 fatty acids (125 ppm) and fatty acid esters (355 ppm), which would arise mostly through oxidative scission on the ether containing POE branch.

The samples containing PS80 showed presence of only C8-C9 fatty acids and no oleic acid. The degradants of PS80 can be traced back to peroxide formation and bond scission at the site of unsaturation. It is interesting to note that oleic acid was not detected by the method. It is likely that the oleic acid formed could degrade completely into various products which thus are not detectable in the 20-month-old samples, as described previously (37). Interestingly, no fatty acid esters were detected, implying that degradation in PS80 is driven more at the site of unsaturation rather than by chain scission at the POE-esters. The number of degradants in samples containing PS80 was lower. It could be because many of these degradants, being volatile, are not present anymore in the

Table IV Various Substances Found by SBSE-GC-MS in Formulations of mAb1 Containing PS20 and PS80 Stored at 25°C for 20 Months and Quantification of the Various Signals of the Degradants Calibrated Against a BHT Standard

PS20		Conc (ppm)
Fatty acids	C10	5
	C12	70
	C14	40
	C16	10
	total	125
Fatty acid esters	PEG1-2C8	15
	PEG1-3C10	45
	PEG1-4C12	170
	PEG1-2C14	80
	PEG1-2C16	35
	PEG1C18	5
	total	355
Aldehydes		0
Ketones		0
5-alkyl 2-furanones		0
alcohols		0
PS80		
Fatty acids	C8, C9	5
Fatty acid esters	PEG1-3C12	15
alkyl aldehydes	hexanal (C6)-decanal (C10)	30
alkyl ketones	2-heptanone (C7), 2-decanone (C10)	15
5-alkyl dihydro 2-furanones	5-butyl and pentyl dihydro 2-furanone	10
unsaturated alkyl aldehydes	2-nonenal, 2-decenal, 2-undecenal	20
alkyl alcohols	heptanol, octanol	35

aged aqueous formulation. Our previous headspace analysis results (22) also demonstrate that alkyl aldehydes and ketones are present in the headspace of stressed samples.

Impact of Polysorbate Degradation on Mab Formulations

We have shown that degradation of polysorbate leads to a reduction in PS concentration as well as formation of new molecules whose effect on the formulation has not been looked into to date. Prominently, the effect of fatty acids and fatty acid esters needed to be evaluated. Since fatty acid esters are by themselves surfactants, we turned our attention to the impact of fatty acid formation on the protein stability. Initial indications from the readouts of increased sub-visible particles in PS20-containing formulations demonstrated that the buildup of fatty acid may cause instability to the protein.

The results from the lauric acid spiking showed that the combination of shaking stress and the presence of free lauric acid strongly influence the aggregation behavior of mAb3 (Fig. 6). The reason for the instability caused by fatty acids in the protein formulations during shake stress is neither well understood nor reported previously. However, it is likely to be a direct interaction model (39) based on the interaction propensity of the carboxylate moiety of the fatty acid with the mAb coupled with the low solubility of the fatty acid leading to destabilizing effects. Since this happens only during agitation stress, it is likely that the interfacial effects also play a role.

Interestingly, while insoluble degradants like fatty acids could be detrimental to the stability of the protein, presence of threshold concentrations of surfactant could stabilize the protein up to a certain molar ratio of the insoluble degradants (a molar ratio of 0.1: 1 lauric acid: PS in the present case). A likely explanation for this is that the low concentrations of fatty acid can be solubilized in a PS solution and remain localized within the micellar matrix. It is known that non-ionic surfactants can interact with fatty acids in the micelles (40). An outcome of this is increased micellar size, which is in agreement with our findings in surface pressure studies. Surface activity measurements indicated a comparatively higher apparent CMC in the complex mixture of intact PS and its degradation products. Thus, it is likely that up to a certain concentration, fatty acids would interact with the non-ionic surfactant in the micelles and thus interact less with the protein.

Spiking studies with stressed polysorbate demonstrated that as long as a threshold level of intact polysorbate is available to stabilize the protein, presence of degradants could be tolerated. It must, however, be noted that the threshold levels of PS required and the levels of insoluble degradants tolerated by a particular bio-therapeutic might vary case by case. In the two mAbs tested in the above studies (mAb2 and mAb3), a minimum concentration of 0.01% of either PS20 or PS80 was found to be essential to prevent accumulation of soluble aggregates upon shaking stress even in the presence of accumulated degradants.

The end-of-shelf-life shake stress experiments clearly suggest that in pharmaceutically relevant storage conditions (2–8°C), the drop in PS concentrations is low. Also, the concentration of insoluble degradants was too small to cause any destabilizing effect, and the measured PS levels were sufficiently high enough to ensure robust stabilization of the protein against mechanical stress as well as any insoluble degradants.

CONCLUSIONS

Polysorbates are prone to degradation based on the functional groups they are made of. The POE moiety, the olefin sites and the ester bond make it susceptible to auto-oxidation and hydrolytic reactions. In pharmaceutically relevant conditions, while hydrolysis is not significant, auto-oxidation can be blocked or slowed down by adopting measures that prevent radical initiation. Our present investigations have looked into cases where some degradation occurs. In cases where degradation does take place, we have established that it does not have a potential influence on the stability of the protein. Our investigations have looked into various possible degradants that could be formed and influence protein stability. The presence of a threshold level of PS20 and PS80 is of importance to maintain stability of the protein despite observed degradation. Thus, during formulation development studies, it is important to evaluate carefully the amount of PS to be used in the formulation, considering the shelf life and the potential behavior of polysorbate during storage. Further studies focusing on various measures to prevent polysorbate degradation are underway.

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