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Research Article

Effect of Polysorbate 80 Concentration on Thermal and Photostability of a Monoclonal Antibody

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Abstract. Polysorbate 80 is widely used in protein formulations to protect protein against agitation-induced aggregation. In this study, we address concerns about residual peroxide present in Polysorbate 80 on protein stability. Residual peroxide may oxidize active pharmaceutical ingredients leading to reduced stability and may ultimately lead to lower potency and efficacy. The effect of Polysorbate 80 concentration on thermal and photostability of monoclonal antibody of the IgG1 subclass (MAb1) was evaluated at Polysorbate 80 concentrations ranging from 0.00% to 1.00% (*w*/*v*). MAb1 samples at 5 mg/mL with various Polysorbate 80 concentrations were subjected to accelerated thermal stress by incubation at 25°C, 40°C, and 50°C for a period of 4 weeks and light stress per ICH guideline Q1B, option 1. Our results show that Polysorbate 80 concentration of 1.00% (*w*/*v*) adversely affected thermal and photostability of MAb1. This study demonstrates the importance of carefully choosing Polysorbate 80 concentration in protein formulations to prevent destabilizing effect of Polysorbate 80 on thermal and photostability.

KEY WORDS: aggregation; monoclonal antibody; non-ionic surfactant; oxidation; photostability; Polysorbate 80; thermal stability.

INTRODUCTION

Therapeutic proteins are exposed to different kinds of stress conditions during production, storage, and shipping that can result in the formation of soluble and insoluble aggregates (1). The potential for intermolecular protein–protein interaction and therefore an increased probability of aggregation is seen at air–water interface where proteins orient themselves to expose hydrophobic regions in order to increase their interaction with the surface (2,3). Polysorbate 80 is widely used in protein formulations to prevent agitation-induced aggregation because of its effectiveness at low concentrations, relative low toxicity, and ability to not only inhibit protein surface adsorption and aggregation under various processing conditions but also act as a stabilizer against protein aggregation (4–6). Although nonionic surfactants like Polysorbates 80 and 20 are used extensively, mechanisms by which they provide stabilizing effects are only fairly understood (7). Several mechanisms for protein stabilization by surfactants have been reported in the literature. Nonionic surfactants can protect proteins against surface-induced aggregation by competing with proteins for adsorption sites on surfaces, by binding to hydrophobic regions on the protein surface and thereby decreasing intermolecular interactions (8–11), by increasing the free energy of protein unfolding (12), and finally, nonionic surfactants may act as chemical chaperone, favoring refolding over aggregation by binding transiently with partially folded protein molecules and sterically hindering intermolecular interactions that result in aggregation (13,14). Surfactants can also modulate adsorption loss and aggregation by coating interfaces and/or participating in protein–surfactant associations as demonstrated by Lee *et al.* (15). However, there have been concerns about using polysorbates because they contain residual amounts of peroxides which may damage the active pharmaceutical ingredient by inducing oxidation. The alkyl polyoxyethylene side chains of polysorbates undergo autooxidation resulting in formation of hydroperoxides, side-chain cleavage, and eventually formation of short-chain acids such as formic acid; all of which can affect the stability of the product (16). Oxidative degradation leads to loss of drug potency over time and may challenge formulation development and reduce shelf life (17). Oxidation is one of the major chemical degradation pathways for protein pharmaceuticals. Investigation of protein oxidation is complicated not only by the unique structure of each individual protein but also by the existence of different oxidation mechanisms under various oxidative stresses (18). Quality of surfactants such as Polysorbate 80 can also significantly influence the photostability of a protein (19).

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MATERIALS AND METHODS

Materials

Monoclonal antibody MAb1 was manufactured in-house by ImClone Systems, Branchburg, NJ. L-histidine, L-histidine monohydrochloride, glycine, sodium chloride, Polysorbate 80 (Baker NF grade), 3% hydrogen peroxide, monobasic and dibasic sodium phosphate, and cesium chloride were obtained from J. T. Baker, Phillipsburg, NJ (Avantor). All the chemicals and surfactants were multi-compendia or USP grade. Pierce Quantitative Peroxide assay kit was obtained from Thermo Scientific.

Methods

Sample Preparation

Samples were prepared aseptically in a Bio-Safety Cabinet. An appropriate volume of Polysorbate 80 stock solution (at 10% (*w*/*v*) prepared in formulation buffer (10 mM histidine buffer at pH 6.0, containing sodium chloride and glycine)) was added to 5 mg/mL MAb1 samples to obtain final Polysorbate 80 concentrations in the range of 0.00% to 1.00%. Samples were aseptically filtered using a 0.22 μm low protein binding filter (PVDF) in a Bio-Safety Cabinet. An aliquot of 2 mL solution from each sample was dispensed into separate 5 mL glass vials.

Isothermal Stability

The effect of Polysorbate 80 concentration on isothermal temperature stability was evaluated by storing samples at 4°C, 25°C, 40°C, and 50°C for 4 weeks. The samples were not exposed to light during the storage at isothermal temperatures.

Photostability

The dark control (product vials wrapped with aluminum foil) and light-exposed (unwrapped product vials) samples were placed in the Caron 6500 series photostability chamber (Caron Products & Services Inc., Marietta, OH) for light exposure. The light exposure condition was as per ICH guideline Q1B option 1: Photostability Testing of New Drug Substances, and Drug Products. Clear glass vials containing the liquid formulation were exposed to an overall illumination of 1.2×10^6 lxh and 200 Wh/m^2 of near UV light at a temperature of 25°C. The vials were exposed to light in upright position placed at least one and a half vial lengths apart.

Turbidity Measurements

Solution turbidity measurements were made using a Shimadzu 1601 Biospec spectrophotometer monitored at 350 nm in a 1-cm pathlength cuvette.

Size Exclusion Chromatography

Size exclusion chromatography (SEC) was performed using an Agilent 1100 Series LC (Agilent, Wilmington, DE) and a Tosoh Biosep G3000 SWXL column. The mobile phase was 10 mM sodium phosphate and 0.5 M CsCl, pH 7.0. The samples were centrifuged at 13,200 rpm for 10 min; 50 μg sample was injected in a volume of 10 μL and eluted at a flow rate of 0.3 mL/min. Absorbance at 280 nm was monitored for peak detection. Column temperature was maintained at 25°C and autosampler temperature at 4°C. Typical assay variability for SEC is <0.5%.

Ion Exchange Chromatography

Ion exchange chromatography (IEC) was performed on an Agilent 1100 series LC mated to ProPac WCX-10 Weak Cation Exchange column. Mobile phase A (low salt) was 10 mM Na phosphate, pH 7.0, and mobile phase B (high salt) was 100 mM NaCl in 10 mM Na phosphate, pH 7.0. Samples were centrifuged at 13,200 rpm for 10 min. 25 μg of sample in a volume of 5 μL was injected on the column and eluted with a linear gradient of 0% B to 100% B at a rate of 2 mM/min and a flow rate of 0.4 mL/min with protein peaks detection at 280 nm. Column temperature was maintained at 25°C and autosampler temperature at 4°C. Typical assay variability for % APG measurement by IEC is <2%.

Differential Scanning Calorimetry

Thermal melting temperature was determined using a differential scanning calorimeter (VP-DSC; MicroCal). The samples were scanned from 25–95°C at a scan rate of 1°C/min. Protein concentration was 1 mg/mL in the analysis. Buffer/buffer scans were subtracted from buffer/protein scans, baseline correction was performed and the thermogram was then normalized for protein concentration. The thermal transition (T_m) midpoints were obtained from peak maximum values.

Circular Dichroism Measurements

Samples were analyzed to determine changes in the tertiary structure in the near UV region (250–320 nm) using a circular dichroism (CD) spectrophotometer (JASCO, Model # J-810). Near-UV CD spectra were recorded using a 1.0×1.0-cm pathlength cuvette at a protein concentration of 1 mg/mL. Data were collected at a 0.5-nm interval with a response time of 4 s in the wavelength range of 250 to 320 nm. The bandwidth and slit width were 2 nm and 1 μm, respectively. Each spectrum is the average of four scans. A buffer spectrum was subtracted from the protein spectrum, and the resulting spectrum was then normalized according to the protein concentration.

Peptide Mapping

Samples (200 μg) were brought to dryness with SpeedVac (Thermo Fisher Scientific, Waltham, MA) and then dissolved in 20 mM Tris buffer, pH 7.5, containing 7.8 M urea and 100 mM DTT. The protein samples were denatured/reduced at 50°C for 1 h and alkylated with iodoacetamide for 30 min in the dark at room temperature. Samples were dialyzed against 50 mM Tris buffer, pH 7.0, with several buffer changes for 1 h

and then digested with trypsin (Promega, Madison, WI) at enzyme to substrate ratio of 1 to 20 for 3 h at 37°C. The reaction was terminated by adding 5 µL of 50% TFA. The resultant peptides were resolved on a C18 reverse-phase column (Zorbax 300SB, 300 Å, 5 µm, 150×4.6 mm) using an Agilent 1100 series LC interfaced to a LCQ-Deca XP ion trap mass spectrometer (Thermo Fisher Scientific) equipped with an electrospray source and operated in a triple-play mode. The digested sample was eluted with a gradient from 98% solvent A (0.1% TFA) to 40% solvent B (100% acetonitrile, 0.085% TFA) in 90 min at a flow rate of 0.5 mL/min.

Polysorbate 80 Determination

Samples were analyzed for Polysorbate 80 content using a Waters LC with ELSD detection. A Waters Acuity UltraPerformance LC reverse phase column (BEH, 1.7 µm C18 column) was used. Mobile phase A was Ammonium Bicarbonate, pH 7.9 and Mobile phase B was Acetonitrile. Samples containing 0.1% and 1.0% Polysorbate 80 were diluted with water to achieve at a final Polysorbate 80 concentration of 0.01%. Samples were then centrifuged at 13,200 RPM for 10 min and a 50 µL sample was injected onto the column and eluted with a gradient as described in Table I, at a flow rate of 0.6 mL/min. The column temperature was maintained at 50°C and autosampler temperature at 25°C. Typical assay variability for % Polysorbate 80 measurements is <0.002%.

Peroxide Quantification

Peroxide content in the test samples was determined by using peroxide assay kit from Thermo Scientific (Pierce® Quantitative Peroxide Assay Kits). Working reagent was prepared by mixing 1 volume of Reagent A with 100 volumes of Reagent B prior to analysis. Peroxide standards for the calibration curve were prepared by serially diluting 3% hydrogen peroxide stock solution to achieve 8 different dilutions in the working range of the assay, 1 to 1,000 µM. The serially diluted samples were prepared for analysis by diluting 1 volume of sample to 10 volumes of working reagent and incubating for 20 min at RT for the reaction to develop and reach an end point. At the end of the incubation period, the absorbance was measured at 560 nm. A standard calibration curve was plotted with peroxide concentration on *x*-axis and absorbance on *y*-axis. Linearly fitted calibration curve was used to calculate the peroxide content of the test samples based on their absorbance values.

Table I. Elution Gradient for Polysorbate 80 Assay

Step	Time (min)	Flow rate (mL/min)	%A	%B
1	Initial	0.600	98.0	2.0
2	1.00	0.600	98.0	2.0
3	4.00	0.600	55.0	45.0
4	7.00	0.600	2.0	98.0
5	10.00	0.600	2.0	98.0
6	11.00	0.600	98.0	2.0
7	12.00	0.600	98.0	2.0

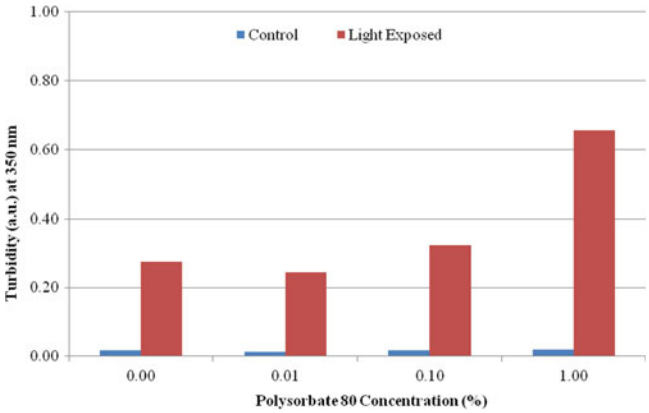

Fig. 1. Turbidity for dark control and light-exposed samples as a function of Polysorbate 80 concentration. Solution turbidity as a function of Polysorbate 80 concentration was measured by absorbance at 350 nm. *Key—0.00*, sample containing 0.00% Polysorbate 80; *0.01*, sample containing 0.01% Polysorbate 80; *0.10*, sample containing 0.10% Polysorbate 80; and *1.00*, sample containing 1.00% Polysorbate 80

RESULTS

Photostability Study

Photostability was evaluated as a function of Polysorbate 80 concentration.

Effect of Light Exposure on Turbidity

Solution turbidities for light-exposed samples increased when compared with dark control samples. Dark control samples had comparable solution turbidities regardless of Polysorbate 80 concentration. The highest increase in solution turbidity was seen for light-exposed sample containing 1.00% Polysorbate 80 (Fig. 1). This suggests a destabilizing effect of Polysorbate 80 at higher concentrations as a result of light exposure.

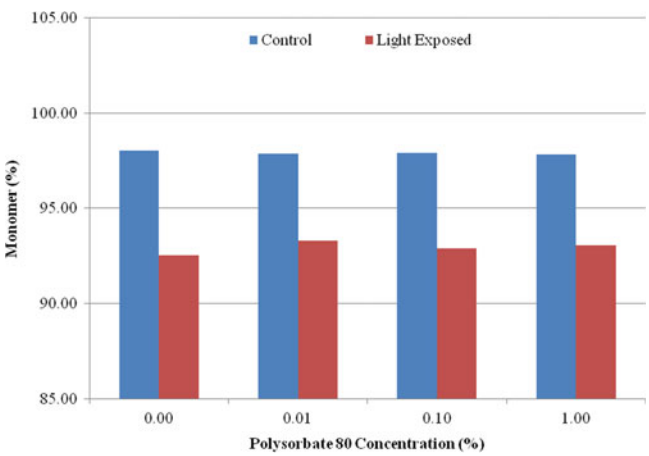

Fig. 2. Monomer content for dark control and light-exposed samples as a function of Polysorbate 80 concentration. Percent monomer as a function of Polysorbate 80 concentration was determined by SEC. *Key—0.00*, sample containing 0.00% Polysorbate 80; *0.01*, sample containing 0.01% Polysorbate 80; *0.10*, sample containing 0.10% Polysorbate 80; and *1.00*, sample containing 1.00% Polysorbate 80

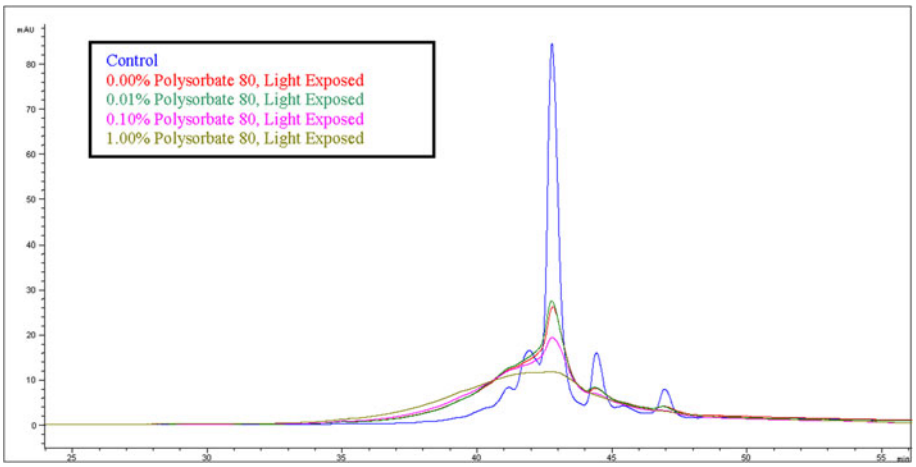

Fig. 3. Overlay of IEC chromatograms for dark control and light-exposed samples as a function of Polysorbate 80 concentration. Dark control and light-exposed samples were analyzed by IEC as a function of Polysorbate 80 concentration

Effect of Light Exposure on Percent Monomer

Following light exposure, monomer content decreased by about 5%; however, this drop in monomer content as a result of light exposure was independent of Polysorbate 80 concentration (Fig. 2). The decrease in monomer was found to be the result of aggregation (data not shown).

Effect of Light Exposure on IEC Peak Profile

Following light exposure, peak resolution was lost and peaks could not be resolved and measured for percent acidic, basic and neutral peak group. The change in peak profile and in turn chemical stability was directly proportional to the Polysorbate 80 concentration (Fig. 3).

Effect of Light Exposure on Tertiary Structure

The effect of light exposure on the tertiary structure of MAb1 as a function of Polysorbate 80 concentration was evaluated by near UV CD (Fig. 4). There was no significant difference observed in the CD spectra of the light-exposed samples when compared with the dark control samples regardless of Polysorbate 80 concentration. This indicates that Polysorbate 80 does not seem to have a significant effect on tertiary structure in the tested concentration range when exposed to light.

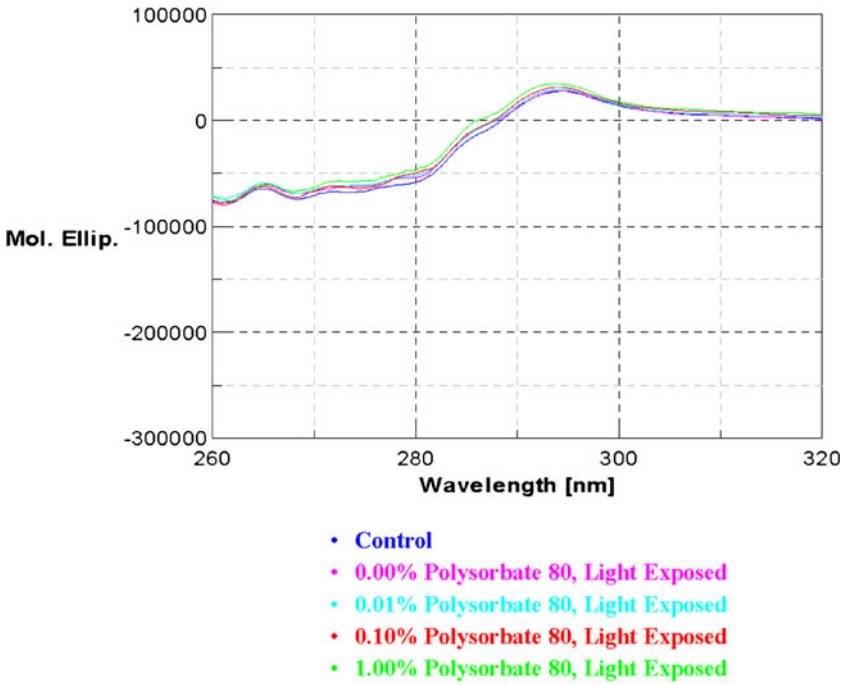

Fig. 4. CD spectra for dark control and light-exposed samples as a function of Polysorbate 80 concentration. Tertiary structure as a function of Polysorbate 80 concentration was determined by near UV CD

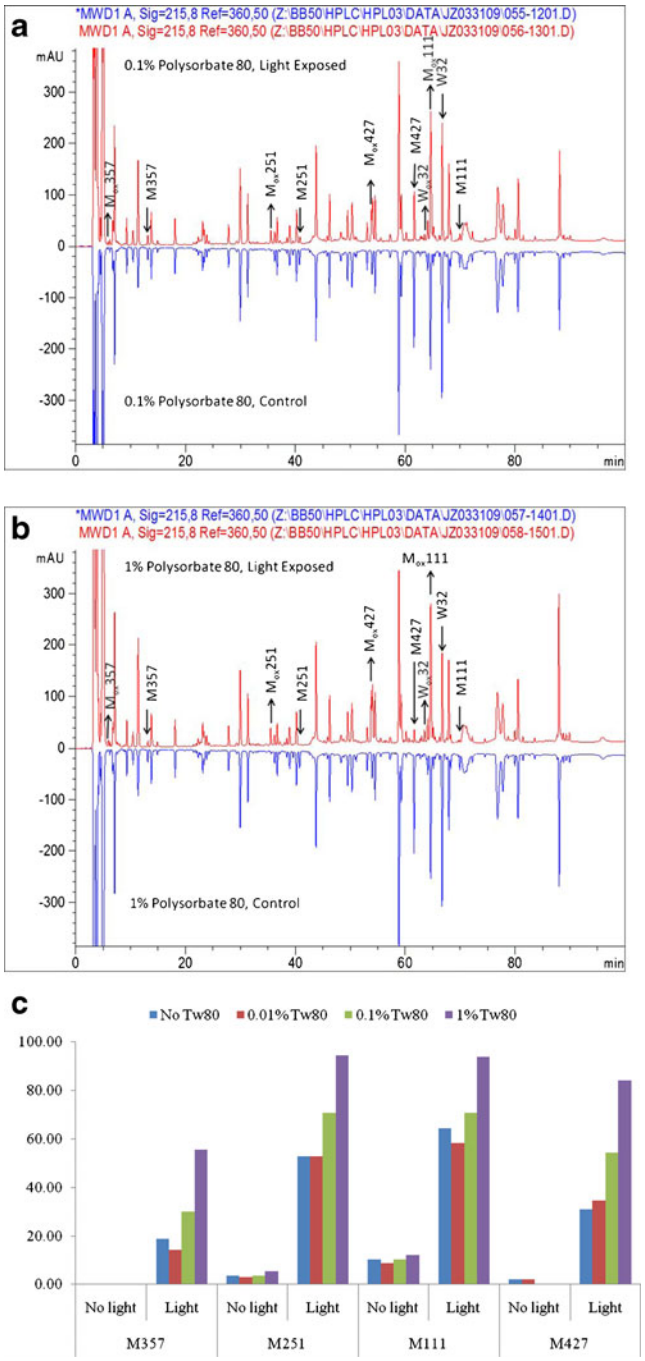

Fig. 5. **a** Representative peptide map for dark control (*blue*) and light-exposed (*red*) samples containing 0.1% Polysorbate 80 aligned as mirror images of each other for easy comparison. **b** Representative peptide map for dark control (*blue*) and light-exposed (*red*) samples containing 1.0% Polysorbate 80 aligned as mirror images of each other for easy comparison. **c** Degree of oxidation for dark control and light-exposed samples as a function of Polysorbate 80 concentration. Degree of oxidation as a function of Polysorbate 80 concentration was determined by MS/MS analysis on peptide fragments obtained after tryptic digestion of the samples. *Key—No Tw80*, sample containing 0.00% Polysorbate 80; *0.01% Tw80*, sample containing 0.01% Polysorbate 80; *0.1% Tw80*, sample containing 0.10% Polysorbate 80; and *1% Tw80*, sample containing 1.00% Polysorbate 80

Table II. Percent Polysorbate 80 for Dark Control and Light-Exposed Samples

Initial (% Polysorbate 80)	Dark control (% Polysorbate 80)	Light exposed (% Polysorbate 80)
0.01	0.01	0.01
0.10	0.09	0.09
1.00	0.83	0.79

Effect of Light Exposure on Degree of Oxidation

A representative mirror image of peptide maps for dark control and light-exposed samples containing 0.10% and 1.00% Polysorbate 80 are shown in Fig. 5a, b, respectively. Methionine oxidation as a result of light exposure is shown in Fig. 5c. Seven methionine residues (six in heavy chain and one in light chain) and one tryptophan (light chain) residue were monitored by mass spectrometry. There were three significant changes at M111, M251, and M427 (heavy chain), two moderate changes at M357 (heavy chain) and W32 (light chain), and no visible change at three positions, M4 (light chain), M34, and M83 (heavy chain). Methionine oxidation was highest for light-exposed samples containing 0.10% and 1.00% Polysorbate 80. Slight oxidation of M111 and M251 was observed even for dark control samples.

Effect of Light Exposure on Polysorbate 80 Content

Following light exposure, the Polysorbate 80 content remained unchanged when compared with dark control samples suggesting that the light exposure did not degrade the Polysorbate 80 content in the test samples (Table II).

Effect of Light Exposure on Peroxide Content

The samples were analyzed for peroxide content as outlined in "Methods." Peroxide content measured was negligible (below the detection limit) for dark control samples (data not shown). For light-exposed samples, the peroxide content increased with an increase in Polysorbate 80 concentration in the test samples (Table III).

Thermal Melting

The thermal melting temperature was determined by DSC as a function of Polysorbate 80 concentration. Onset of the first melting peak was just under 60°C (data not shown), indicating that use of 50°C as a stress condition in this study was reasonable.

Table III. Peroxide Content After Light Exposure as a Function of Polysorbate 80 Concentration

Light-exposed samples (% Polysorbate 80)	Absorbance (560 nm)	Peroxide content (μM)
0.00	0.502	11.78
0.01	0.530	13.33
0.10	1.068	43.22
1.00	2.571	126.72

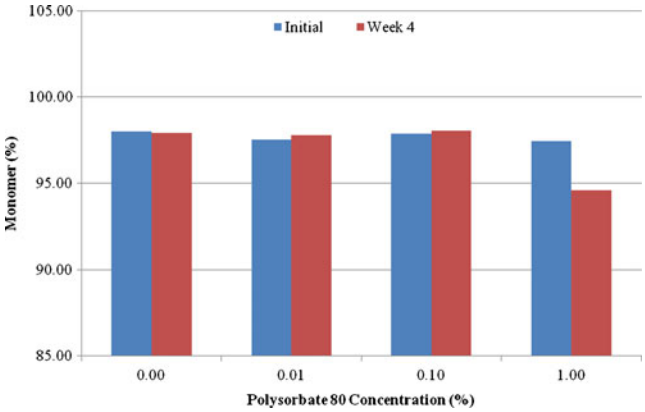

Fig. 6. Monomer content after 4 weeks at 25°C as a function of Polysorbate 80 concentration. Percent monomer as a function of Polysorbate 80 concentration for samples incubated at 25°C for 4 weeks was measured by SEC. *Key: 0.00*, sample containing 0.00% Polysorbate 80; *0.01*, sample containing 0.01% Polysorbate 80; *0.10*, sample containing 0.10% Polysorbate 80; and *1.00*, Sample containing 1.00% Polysorbate 80

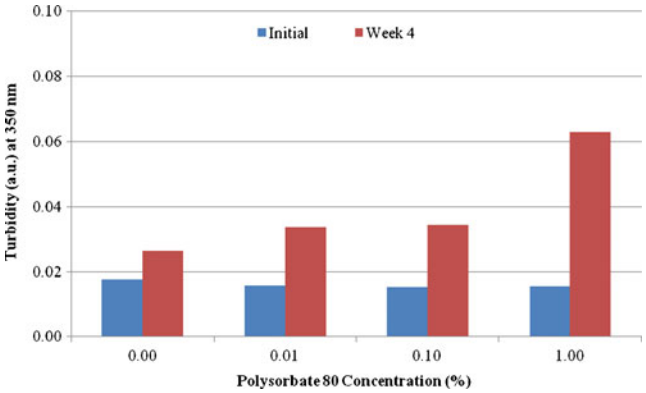

Fig. 8. Turbidity at 50°C after 4 weeks as a function of Polysorbate 80 concentration. Solution turbidity as a function of Polysorbate 80 concentration for samples incubated at 50°C for 4 weeks was measured by absorbance at 350 nm. *Key—0.00*, sample containing 0.00% Polysorbate 80; *0.01*, sample containing 0.01% Polysorbate 80; *0.10*, sample containing 0.10% Polysorbate 80; and *1.00*, sample containing 1.00% Polysorbate 80

Isothermal Stability Studies

Thermal stability of MAb1 was evaluated as a function of Polysorbate 80 concentration for a period of 4 weeks.

Following 4 weeks of incubation at 4°C, the solution turbidity, percent monomer, and percent APG, all remained unchanged (data not shown). Following 4 weeks of incubation at 25°C, solution turbidities were comparable to initial samples (data not shown). For samples containing 0.00% to 0.10% Polysorbate 80, percent monomer also remained comparable to initial samples. However, in sample containing 1.00% Polysorbate 80, the percent monomer decreased by nearly 3% (Fig. 6) as a result of increased aggregation (data not shown). We did not observe any significant change in the IEC peak profile at both temperatures (data not shown). Following 4 weeks of incubation at 40°C, solution turbidities were slightly higher and IEC peaks shifted slightly towards the acidic side when compared with initial samples. However, Polysorbate 80 concentration had no effect on this change (data not shown). Percent monomer decrease was largest for sample containing 1.00% Polysorbate 80 where it decreased by nearly 4% (Fig. 7) as a result of increased aggregation (data not shown). Following 4 weeks incubation at 50°C, solution turbidities increased (Fig. 8), percent monomer decreased (Fig. 9) as a result of increased aggregation (data not shown), and IEC peaks shifted towards acidic side (Fig. 10) with an increase in Polysorbate 80 concentration when compared with initial samples. These results demonstrate a destabilizing effect of Polysorbate 80 at higher concentrations when exposed to high temperature.

DISCUSSION

In order to preserve the biological activity, it is important for proteins to retain their native conformation; even minor mechanical stress can disrupt native conformation leading to loss of biological activity as well as formation of non-native

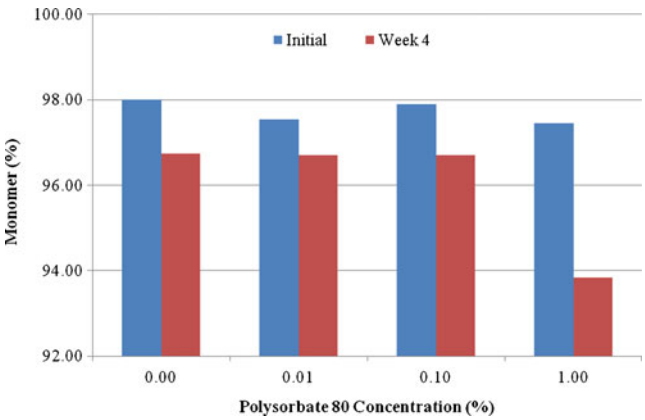

Fig. 7. Monomer content at 40°C after 4 weeks as a function of Polysorbate 80 concentration. Percent monomer as a function of Polysorbate 80 concentration for samples incubated at 40°C for 4 weeks was measured by SEC. *Key—0.00*, sample containing 0.00% Polysorbate 80; *0.01*, sample containing 0.01% Polysorbate 80; *0.10*, sample containing 0.10% Polysorbate 80; and *1.00*, sample containing 1.00% Polysorbate 80

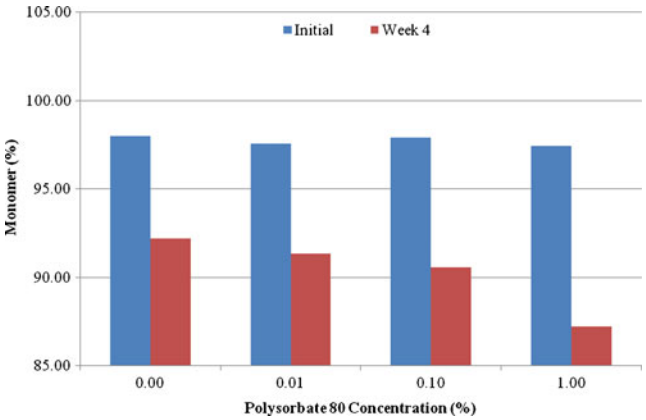

Fig. 9. Monomer content at 50°C after 4 weeks as a function of Polysorbate 80 concentration. Percent monomer as a function of Polysorbate 80 concentration for samples incubated at 50°C for 4 weeks was measured by SEC. *Key—0.00*, sample containing 0.00% Polysorbate 80; *0.01*, sample containing 0.01% Polysorbate 80; *0.10*, sample containing 0.10% Polysorbate 80; and *1.00*, sample containing 1.00% Polysorbate 80

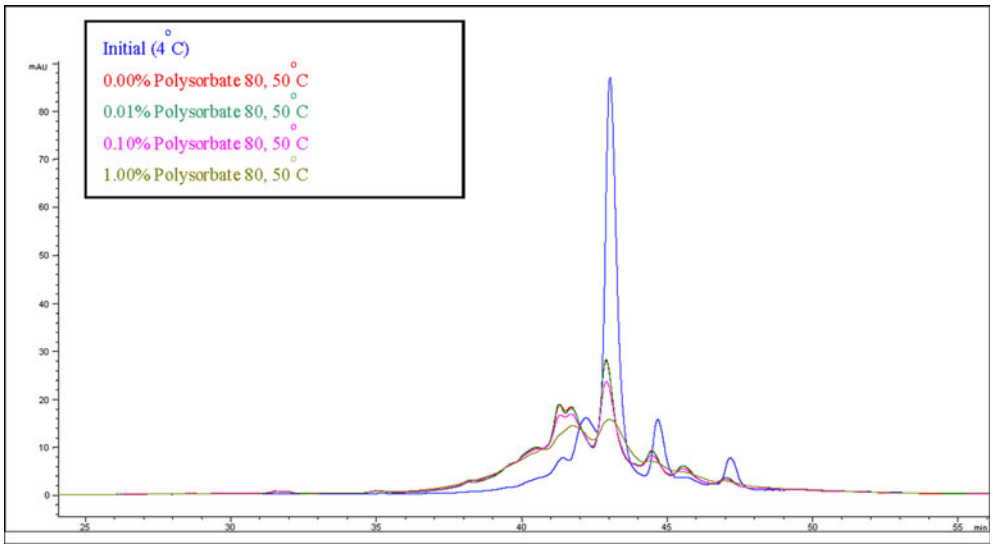

Fig. 10. Overlay of IEC chromatograms after 4 weeks at 50°C as a function of Polysorbate 80 concentration; 50°C, 4 weeks samples were analyzed by IEC as a function of Polysorbate 80 concentration

protein aggregates. Proteins undergo such stress conditions during various stages of production to storage to delivery into patients leading to loss of activity (8). Proteins can also be exposed to light at multiple points, from production to delivery (20). Photodegradation, especially photooxidation is a common degradation pathway for many proteins. Exposure of proteins to light causes oxidation of light sensitive amino acids such as Tryptophan, Tyrosine and Phenylalanine leading to increased physical instability and aggregation (21). Nonionic surfactants like Polysorbate 20 and Polysorbate 80 are commonly used in protein formulations to protect against mechanical stress. However, one of the issues in using polysorbates in protein formulations is their potential adverse effect on protein stability by oxidative damage due to the residual peroxides present in them (3). Auto-oxidation of polysorbates results in formation of hydroperoxides, which may lead to the oxidation of the active pharmaceutical ingredient (16,22). Polysorbates can also potentially adversely affect the stability by their direct interaction with proteins (8). Since most proteins have very limited conformational stability in solution, such direct interactions may potentially disrupt the delicate balance of all the folding forces responsible for the conformational stability and lead to protein instability (3).

The commonly used Polysorbate 80 concentration in the liquid formulations of protein drug products is in the range of 0.001% (Reopro® by Centocor) to 0.1% (Humira® by Abbott Laboratories). In this reference, Polysorbate 80 concentrations and stress conditions used in this study may be considered as an exaggeration. The purpose of this study is to raise awareness that not optimizing Polysorbate 80 concentration properly in the formulation may lead to serious consequence on product quality and stability. MAb1 was found to be susceptible to oxidation when exposed to light. Presence of higher amounts of Polysorbate 80 increased the susceptibility of MAb1 to oxidation as observed at a Polysorbate 80 concentration of ≥0.10% which caused an increase in methionine oxidation and change in the intensity of several peaks as seen by peptide mapping. After light exposure, we observed significant changes in three methionine residues at M111, M251, and M427 (all in heavy chain), one moderate change at M357 (in heavy chain), and one at tryptophan 32 (in light chain). Light-induced oxidation in proteins can potentially occur by absorption of photons by other excipients in the solution resulting in formation of singlet oxygen or by the protein molecule itself. Absorption of light photons by the protein molecule occurs through either the peptide backbone or by the amino acid side chains of tryptophan, tyrosine, phenylalanine, and cystine (23–25). The oxidation of methionine residues observed in our study may involve either of the two mechanisms and oxidation of tryptophan may be attributed to the latter. Tryptophan is believed to be a major factor in the photodegradation pathways of proteins even though it is present in relatively low amounts (20).

As reported in the literature, we believe that presence of peroxides may have contributed to oxidation observed in this study (3,6,16,22). We hypothesize a correlation of higher oxidation at higher Polysorbate 80 concentrations to the presence of higher amount of peroxides (as demonstrated by the peroxide assay). We observed a greater change in peak profile by IEC in 1.00% Polysorbate 80-containing sample after light exposure. Since oxidation was observed to be the primary modification, the change in IEC peak profile can be attributed to potential alteration in surface charge distribution on the protein as a result of oxidation of methionine and tryptophan residues. Even small perturbations in the protein structure may change the local distribution of charged residues, leading to changes in the overall surface charge distribution of the molecule (26). Light exposure caused 4–5% decrease in monomer content. But, the decrease in monomer was not influenced by Polysorbate 80 concentration. This suggests that even though Polysorbate 80 at higher concentrations caused an increase in oxidation of tryptophan and methionine residues after light exposure, the change was only in terms of changes in the overall surface charge distribution and not in the overall size or the tertiary structure, as also seen by CD analysis. Qi *et al.* have demonstrated that no change in either far or near UV CD spectra was observed as a result of light exposure for a high-concentration IgG formulation in histidine buffer (27). Mason *et al.* have also

demonstrated by both FTIR and CD that neither the secondary nor the tertiary structure was changed as a result of photoirradiation on an IgG1 MAb (28). An increase in solution turbidity was observed for the light-exposed samples when compared with dark control samples. The solution turbidity increased with an increase in Polysorbate 80 concentration.

Higher temperature can lead to degradation of Polysorbate 80 and increase the peroxide content, as demonstrated by Ha *et al.* who reported that after incubation of Polysorbate solution at 40°C for 5 weeks, there was an eightfold increase in peroxide content when compared with solution stored under dark conditions (5). Kishore *et al.* have also reported polysorbates undergoing degradation through autooxidation and hydrolysis at higher temperatures (29). The presence of transition metals such as copper may also catalyze auto-oxidation of polysorbates (6). At 25°C and 40°C after 4 weeks, Polysorbate 80 did not show any negative effect up to 0.10% concentration; however, at 1.00% concentration, a decrease in percent monomer (as a result of increase in percent aggregate) and an increased change in peak profile by IEC was observed, suggesting a negative effect of Polysorbate 80 at higher concentration. At 50°C after 4 weeks, an increase in turbidity, decrease in percent monomer (as a result of increase in percent aggregate) and increased change in peak profile by IEC was seen with increasing Polysorbate 80 concentration.

CONCLUSIONS

The findings in this manuscript emphasize the importance of concentration of Polysorbate 80 on thermal and photostability of proteins. We have determined that Polysorbate 80 at a concentration of 0.01% used in formulation of MAb1 does not have an adverse effect on either thermal or photostability. However, Polysorbate 80 concentration of 1.00% has a negative effect on thermal as well as photostability of MAb1. Although Polysorbate 80 is widely used in protein formulations to prevent mechanically induced aggregation, the presence of residual peroxides in Polysorbate 80, especially at higher concentrations, can have destabilizing effects on protein. Our findings in this manuscript suggest careful consideration of Polysorbate 80 concentration to protect the protein against mechanical stress without compromising stability against thermal and light stress.

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