Research Article

Effect of Polysorbate 80 Quality on Photostability of a Monoclonal Antibody

Swita R. Singh,[1,2] **Jingming Zhang,**[1] **Courtney O'Dell,**[1] **Ming-Ching Hsieh,**[1] **Joel Goldstein,**[1] **Joseph Liu,**[1] **and Arvind Srivastava**[1]

Abstract. Polysorbate 80 is one of the key components of protein formulations. It primarily inhibits interfacial damage of the protein molecule due to mechanical stress during shipping and handling. However, polysorbate 80 also affects the formulation photostability. Exposure to light of polysorbate 80 aqueous solution results in peroxide generation, which in turn may result in oxidation of the susceptible amino acid residues in the protein molecule. The purpose of this study was to determine if the photostability of our proprietary IgG_1 monoclonal antibody formulation containing polysorbate 80 is affected by the quality (grade/vendor) of polysorbate 80. Following four types of polysorbate80 were tested: (1) Polysorbate 80 Super-Refined, Mallinckrodt Baker, (2) Polysorbate 80 NF, Mallinckrodt Baker, (3) Polysorbate 80 NF, EMD Chemicals, and (4) Ultra-pure Polysorbate 80 (HX), NOF Corporation. The samples were exposed to light as per ICH guidelines Q1B. The results of the study show that photostability of the antibody formulation is indeed affected by the quality of polysorbate 80. This study underscores the importance of carefully choosing the quality of polysorbate 80 to ensure the robustness of formulation.

KEY WORDS: antibody; photostability; polysorbate 80; protein stability; Super-Refined Polysorbate 80.

INTRODUCTION

Although it is widely agreed that surfactants such as polysorbates enhance the stability of protein formulations, very limited effort has been directed towards studying the effect of the surfactant quality on the protein drug product stability. The purpose of this study was to evaluate the effect of polysorbate 80 quality on the photostability of IgG_1 monoclonal antibody formulation. The quality difference in polysorbate 80 from different supplier and grade may arise due to the differences in their manufacturing and purification processes. Protein formulations are susceptible to interfacial damage (1). The interfaces protein molecules may encounter in a formulation during bio-processing and in final dosage form, may be categorized into air–water interface (2), ice–water interface (3), and solid interfaces (4). Protein aggregation resulting from mechanical stress, mixing, freeze thawing, *etc.*, is attributed to protein adsorption on the interfaces. Surface tension forces at the interfaces cause aggregation of the adsorbed protein by affecting structural integrity of the protein molecules that populate the interfacial region.

Non ionic surface active agents such as polysorbates are commonly added to protein formulations to inhibit surface-induced protein instability.

Polysorbates are widely used to protect biological drug products from unfolding, aggregation, and precipitation during shipping and handling (5–7). Polysorbates to be used in biotechnology products are required to be produced using strictly plant sources. The polysorbates are amphipathic, non-ionic surfactants composed of fatty acid esters of polyoxyethylene sorbitan. Polysorbate 80 is one of the most common surfactants currently used in formulation of protein biopharmaceuticals. Multicompendial grade polysorbate 80 is a mixture of various fatty acid esters with ≥58% of its total being oleic acid containing component (8). The other components include myristic, palmitic, palmitoleic, stearic, linoleic, and linolenic acid esters. Superior grades of polysorbate 80 namely, Ultra-pure Polysorbate 80 and Super-Refined Polysorbate 80 have recently been more introduced to the market. The Ultra-pure Polysorbate 80 from NOF Corporation has oleic acid component comprising of 99% pure oleic acid. The Super-Refined Polysorbate 80 from Mallinckrodt Baker is a more refined grade of polysorbate 80 that is subjected to an additional chromatographic purification step which facilitates removal of polar impurities such as formaldehyde, peroxides, *etc.* The low levels of peroxide impurities found in polysorbate 80 may facilitate oxidative degradation of the protein. This might be particularly significant with regards to the photostability of the formulation.

Exposure to light of polysorbate 80 aqueous solution results in autoxidation of the alkyl polyoxyethylene chain

[1] ImClone Systems, a Wholly Owned Subsidiary of Eli Lilly & Co., Branchburg, New Jersey 08876, USA.
[2] To whom correspondence should be addressed. (e-mail: swita. singh@imclone.com)

leading to formation of hydroperoxide derivatives (9). The peroxides in turn cause oxidative damage to the protein molecule in the formulation. The residual peroxides and the rate of peroxide generation as a result of light exposure may vary for polysorbate 80 of different grades and from different sources. The types of polysorbate 80 tested in the study have been summarized in Table I.

MATERIALS AND METHODS

Materials

The monoclonal antibody was manufactured in-house by the Manufacturing Department within ImClone Systems, a Wholly Owned Subsidiary of Eli Lilly & Co., Branchburg, NJ. Multicompendial grade L-histidine, L-histidine monohydrochloride, glycine, and sodium chloride were obtained from Mallinckrodt Baker (now Avantor™ Performance Materials). Polysorbate 80 was obtained from three different vendors, namely, (1) Mallinckrodt Baker (now Avantor™ Performance Materials), Super-Refined and NF grade, (2) EMD Chemicals, NF grade, and (3) NOF Corporation, Ultra-Pure grade. Clear 5-ml ready-to-use glass vials, Flurotec®-coated butyl rubber stoppers, and seals (20 mm, flip-off) were obtained from West Pharmaceuticals.

Methods

Sample Preparation

Samples were prepared aseptically in a Bio-Safety Cabinet. Polysorbate 80 stock (10%, *w/v*) made using polysorbate 80 purchased from Mallinckrodt Baker (Super-Refined and NF grade), NOF Corporation (Ultra-pure) and EMD Chemicals (NF grade) was added to a 5-mg/ml Mab formulated in 10 mM histidine buffer pH 6.0, containing sodium chloride and glycine. Final polysorbate 80 concentration in the formulation was 0.01% (*w/v*). Product placebos were prepared by adding 10% (*w/v*) polysorbate 80 stock made using polysorbate from above described vendors to the formulation buffer. For the photostability study, each sample was filtered aseptically using 0.22-µm low protein binding PVDF filter (Millipore) and then 5 ml of solution was transferred into separate 5-ml glass vials.

Determination of Sample Turbidity

The solution turbidity was measured as absorbance at 350 nm of undiluted samples using Shimadzu 1601 Biospec spectrophotometer.

Photostability Study

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 set to meet ICH guidelines Q1B: 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 lux hours and 200 W hour 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. The completion of exposure took 6 days.

Protein Concentration Measurement

Protein concentration was measured using a Shimadzu 1601 Biospec spectrophotometer. The protein concentration was determined from the UV-absorbance at 280 nm using an extinction coefficient of 1.4 (mg/ml)$^{-1}$cm^{-1}. For absorbance determination, the samples were diluted tenfold using corresponding formulation buffer.

Size-Exclusion Chromatography

The light-exposed and dark control samples were analyzed by size-exclusion chromatography (SEC). SEC analysis was performed using a Waters LC chromatograph. The samples were centrifuged at 13,200 rpm for 2 min. The sample (10 µl) was injected onto a Tosoh Biosep G3000SWXL column. The samples were eluted using a mobile phase comprising of 10 mM sodium phosphate, 500 mM salt, pH 7.0. The protein peaks were detected by UV absorbance at 280 nm, using a DWD UV detector. To minimize the run to run variability, dark control and light-exposed samples were analyzed side by side (on same instrument and on the same day). Typical assay variability for SEC-HPLC was determined to be <0.5%.

Ion Exchange Chromatography

The light-exposed and dark control samples were analyzed by ion exchange chromatography (IEC). Samples were diluted to 1 mg/ml with deionized water. IEC analysis was performed by injecting 50 µg (50 µl and 1 mg/ml) of sample on ProPac® WCX-10 cation exchange column (Dionex Corporation) mated to an Agilent 1100 LC HPLC with protein peak detection at 280 nm. The column temperature was 30±5°C and the sample temperature was 4°C. The samples were eluted using a gradient comprising of mobile phase A (10-mM sodium phosphate, pH 6.6)

Table I. Summary of Polysorbate 80 Types Evaluated in the Study

Polysorbate 80 vendor	Polysorbate 80 grade	Lot number	Peroxide value (mEq/kg; EP)[b]
Mallinckrodt Baker[a]	Super-Refined	H45634	<0.1
Mallinckrodt Baker[a]	NF	H35614	<0.1
EMD Chemicals	NF	G09000982	Not available
NOF Corporation	Ultra-pure	909361A	1.0

[a] Mallinckrodt Baker is now Avantor Performance Materials, Inc
[b] Peroxide value as per European Pharmacopoeia (EP) specifications as disclosed by the vendor in their certificate of analysis for the lot

and mobile phase B (100-mM NaCl in 10-mM sodium phosphate, pH 6.6). The gradient is shown in Table II. The dark control and light-exposed samples were analyzed side by side. Typical variability in percent acidic peak group (APG) measurement by IEC is <2%.

Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis

The light-exposed and dark control samples were analyzed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) using Novex 8–16% Tris-Glycine gel. The dark control and light-exposed samples were analyzed side by side in the same gel. The gel was loaded with samples (~3 μg protein). Bio-Rad Pre-stained Molecular Weight Marker was run alongside the sample for molecular weight determination. For reduced SDS-PAGE, samples were diluted 1:1 with reduced sample buffer (Novex Tris Glycine SDS Sample Buffer with 2.5% β-mercaptoethanol). For non-reduced SDS-PAGE, samples were diluted 1:1 with non-reduced sample buffer (Novex Tris Glycine SDS Sample Buffer). The electrophoretic separation was performed using a 30-mA current in a gel electrophoresis apparatus. The gel was allowed to run at a constant current until the dye front migrated to the bottom of the gel (approximately 1 h). The gel was subsequently stained on a shaker at room temperature for 1 h using staining solution (2.5% Brilliant blue, 45% methanol, 10% glacial acetic acid, and 45% deionized water). The gel was washed with approximately 100 ml of deionized water and destained for 30 min using destaining solution (45% methanol, 10% glacial acetic acid, and 45% deionized water). The destaining solution was then changed to 15% methanol, 10% glacial acetic acid, and 75% deionized water. The destaining was continued for 15 h. The destaining solution was replaced with water and the gel was scanned using Molecular Dynamics densitometer. The band intensities were measured using Image Quant TL software (Amersham Biosciences). Typical variability in percent purity by SDS-PAGE was <5%.

Polysorbate 80 Quantification

The light-exposed and dark control samples were analyzed for polysorbate 80 concentration. A reversed-phase HPLC (RP-HPLC) method was used to determine polysorbate 80 concentration in the solution. Samples were hydrolyzed by incubating with 0.3 M NaOH (1:1) for 16 h at 60°C. After incubation, samples were cooled to room temperature and mixed with an equal volume of 100% acetonitrile followed by centrifugation at 12,000 rpm for 10 min. The samples were analyzed by a reverse phase HPLC method using Agilent 1100 LC system. The samples were separated using a Phenomenex 5 μm C18 column. The elution was achieved using an acetonitrile gradient from 50% to 80% in 30 min with 20 mM phosphate buffer, pH 2.8, and a flow rate of 1 ml/min. Separated peaks were detected by UV absorption at 200 nm. Nonadecanoic acid was used as internal standard. Typical variability in polysorbate measurements is <0.002%.

Peptide Mapping

The light-exposed and dark control drug product samples (200 μg of 5 mg/ml sample) were completely dried under vacuum. The samples were re-dissolved in 25 μl of buffer containing 7.8 M guanidine HCl, 50-mM Tris–HCl, pH 7.5, and 0.1 M DTT. The reaction mixture was placed at 50°C for 60 min to denature and reduce the protein. Protein solution was then cooled to room temperature and 25 μl of 0.25 M iodoacetamide was added. The alkylation was performed at room temperature for 30 min in the dark with constant shaking. The reaction mixture was subsequently dialyzed against 20-mM Tris–HCl, pH 7.0 at room temperature for 2 h. The dialyzed solution was collected and combined with 150 μl of 50 mM Tris–HCl, pH 7.5 solution. The trypsin digestion was performed at 37°C for 3 h using an enzyme: protein ratio of 1:20 (*w/w*). The reaction was terminated by adding 5-μl 50% trifluoroacetic acid (TFA). The resultant peptides were resolved using a C18 reverse phase column (Zorbax C18 300SB, 300 Å, 5 μm, 4.6×150 mm) using an Agilent HPLC 1200 series (Agilent, Wilmington, DE) interfaced to a LTQ ion trap mass spectrometer (Thermo Scientific). 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 95 min at a flow rate of 0.5 ml/min. The percent oxidized was determined as described previously by Houde *et al.* (10).

RESULTS

All the formulations were clear and free of visible particles before and remained so after light exposure for both dark control and light-exposed samples. The turbidity of various formulations is summarized in Table III. The authors would like to point out that the following results have been obtained by independent assays performed by various groups within the company.

Table II. IEC Gradient

Time (min)	Mobile phase A (%)[a]	Mobile phase B (%)[b]
0	45	55
5	45	55
30	25	75
45	0	100
50	45	55
60	45	55

[a] Sodium phosphate, 10 mM (pH 6.6)
[b] Sodium chloride, 100 mM in 10 mM sodium phosphate (pH 6.6)

Table III. Turbidity as Measured by Absorbance at 350 nm for Various Formulations

Sample	Absorbance at 350 nm (AU)
Super-Refined-MBaker	0.0162
NF-MBaker	0.0160
NF-EMD	0.0133
Ultra-pure-NOF	0.0078
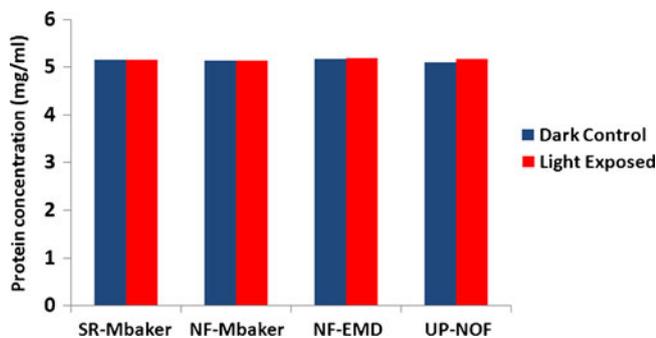
Fig. 1. Protein concentration in dark control and light-exposed samples for various groups of formulations. Protein concentration was determined by measuring the absorbance at 280 nm of light-exposed and dark control samples prepared using various types of polysorbate 80. *Key: SR-MBaker* drug product formulated using Super-Refined Polysorbate 80 supplied by Mallinckrodt Baker, *NF-MBaker* drug product formulated using NF grade polysorbate 80 supplied by Mallinckrodt Baker, *NF-EMD* drug product formulated using NF grade polysorbate 80 supplied by EMD chemicals, *UP-NOF* drug product formulated using Ultra-pure Polysorbate 80 supplied by NOF corporation

Effect of Light Exposure on Protein Concentration

The IgG concentration was ~5 mg/ml in both, the dark control and light-exposed samples at the end of the photostability study (Fig. 1). This suggests that exposure to light did not affect the protein concentration.

Effect of Light Exposure on Percent Monomer, High Molecular Weight Species and Low Molecular Weight Species (LMWS) by SEC

An overlay of SEC chromatogram for a light-exposed sample and its corresponding dark control sample is shown in Fig. 2. Percent monomer decreased while percent low molecular weight species (LMWS) and percent high molecular weight species (HMWS) increased as a result of light exposure for all four formulations (Fig. 3). The percent monomer, LMWS
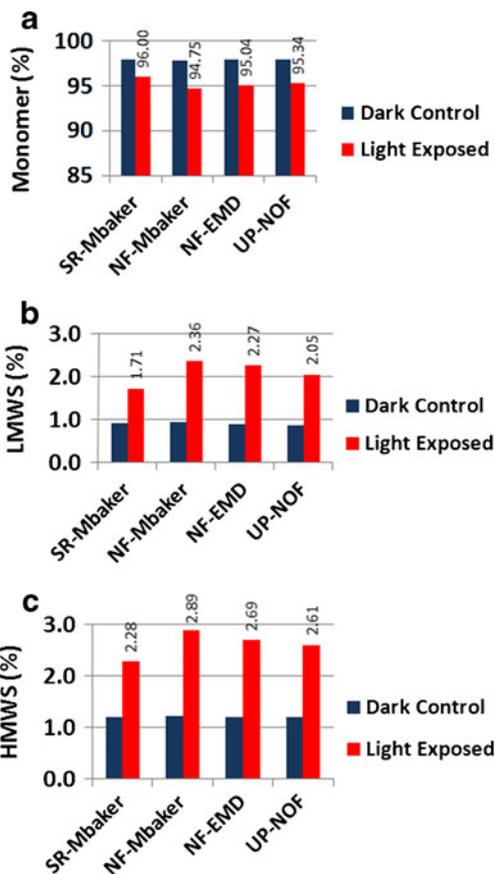
Fig. 3. Percent monomer, degradants and aggregates in dark controls and light-exposed samples for various groups of formulations. **a**) Percent monomer, **b**) percent degradant, and **c**) percent aggregate in dark control and light-exposed samples was measured by size exclusion chromatography using Tosoh Biosep G3000SWXL column. *Key: SR-MBaker* drug product formulated using Super-Refined Polysorbate 80 supplied by Mallinckrodt Baker, *NF-MBaker* drug product formulated using NF grade polysorbate 80 supplied by Mallinckrodt Baker, *NF-EMD* drug product formulated using NF grade polysorbate 80 supplied by EMD chemicals, *UP-NOF* drug product formulated using Ultra-pure Polysorbate 80 supplied by NOF corporation
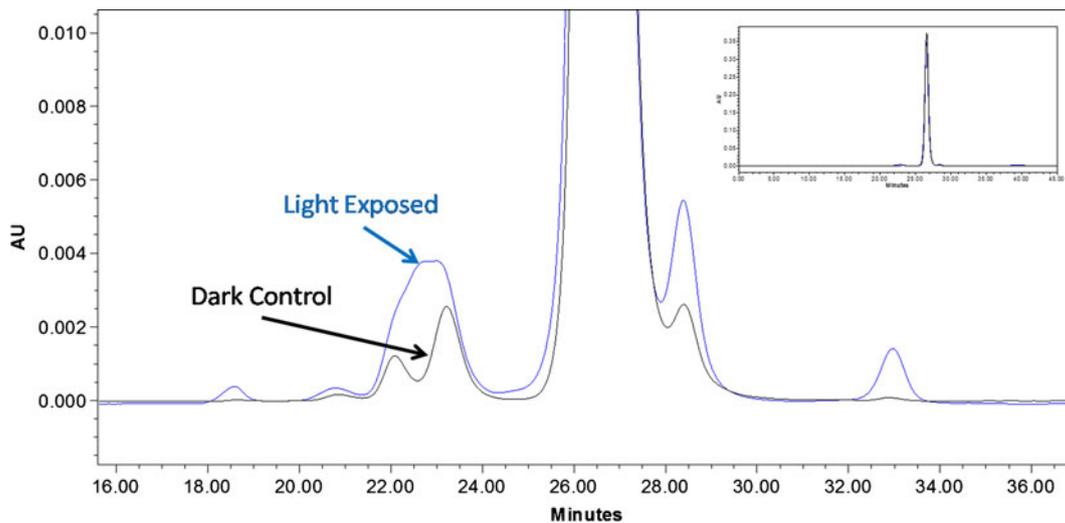
Fig. 2. Representative SEC chromatogram for light-exposed (*blue*) sample overlayed on the respective dark control (*black*). The overlay of dark control and light-exposed formulation containing polysorbate 80 from EMD chemicals is shown. The figure *inset* shows zoomed out SEC chromatogram overlay for the samples

and HMWS in the dark control samples were comparable for all the formulations. However, percent monomer retained in light-exposed samples followed the order: SR-MBaker>UP-NOF>NF-EMD>NF-MBaker. Correspondingly, percent LMWS and percent HMWS was lowest in the light-exposed samples formulated with Super-Refined Polysorbate 80 from Mallinckrodt Baker and highest in samples formulated with NF-MBaker. The total area under the SEC chromatograms for the dark controls and light-exposed samples remained comparable.

Effect of Light Exposure on Percent Purity by Reduced and Non-reduced SDS-PAGE

Non-reduced and reduced SDS PAGE was performed side by side for dark controls and light-exposed samples and the results are shown in Fig. 4. The top panel shows the gel images. The bottom panel shows percent purity as determined by percent band intensity for the main band for non-reduced gel and sum of band intensities for heavy and light chain for reduced gel. The non-reduced gel clearly shows an increase in the intensity of low molecular weight species in light-exposed samples as compared with dark controls, consistent to the SEC

results. The reduced gel shows presence of high molecular weight covalently cross linked species in light-exposed samples. The comparison of percent purity shows a clear trend of decreased percent purity in light-exposed samples in both non-reduced and reduced gels as compared with the dark controls. Comparing across various types of formulations, the percent purity in all dark controls was highly similar, while the percent purity in light-exposed samples followed the same order as seen in SEC results for percent monomer: SR-MBaker>UP-NOF>NF-EMD>NF-MBaker.

Effect of Light Exposure on Percent APG

APG represents the sum of acidic peak areas (Fig. 5 (bottom panel)). Percent APG in dark control samples was similar for all the samples (Fig. 5). Percent APG increased as a result of light exposure for all the samples, suggesting that light exposure results in chemical modifications which alter surface-charge properties of the antibody either directly by changing the number of charged groups or indirectly by introducing structural alterations. However, the percent APG increase followed the order: SR-MBaker<UP-NOF<NF-EMD<NF-MBaker.
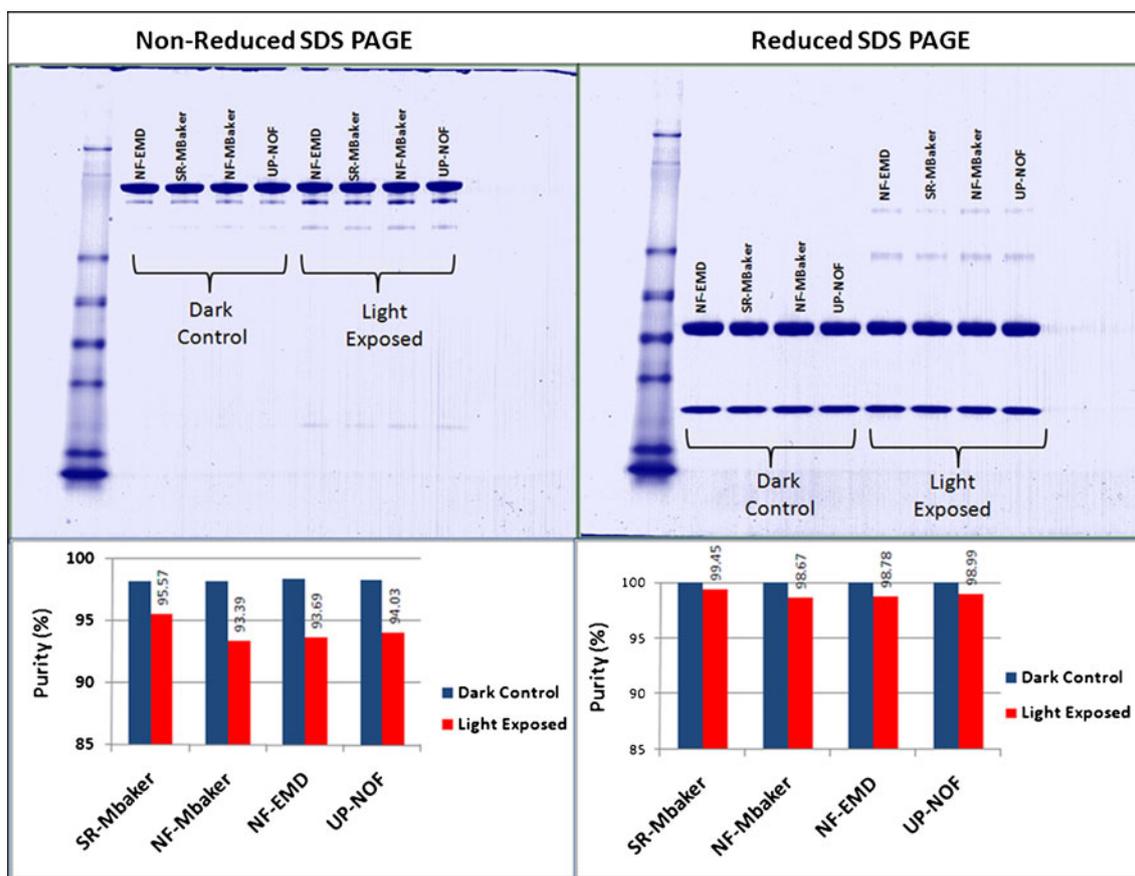
Fig. 4. Non-reduced and reduced SDS-PAGE results for various groups of formulations. *Top panels*, gel images for non-reduced and reduced SDS-PAGE. *Bottom panels*, comparison of percent purity of the antibody in various groups of formulations. Percent purity in non-reduced SDS-PAGE is determined by the percent band intensity of the main band. Percent purity in reduced SDS-PAGE is determined by the sum of percent band intensity of heavy chain and light chain. *Key: SR-MBaker* drug product formulated using Super-Refined Polysorbate 80 supplied by Mallinckrodt Baker, *NF-MBaker* drug product formulated using NF grade polysorbate 80 supplied by Mallinckrodt Baker, *NF-EMD* drug product formulated using NF grade polysorbate 80 supplied by EMD chemicals, *UP-NOF* drug product formulated using Ultra-pure Polysorbate 80 supplied by NOF corporation
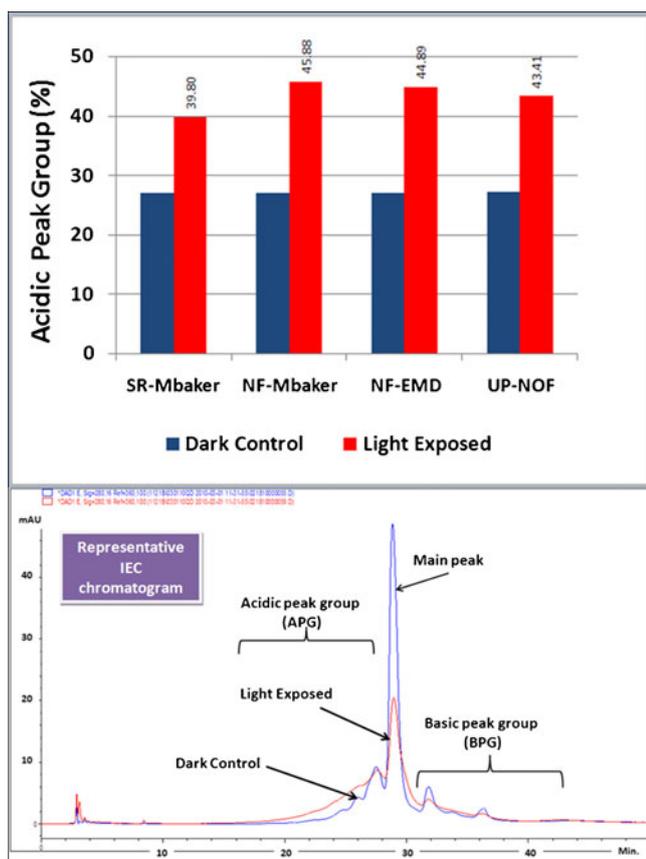
Fig. 5. Percent acidic peak group (*APG*) in dark control and light-exposed samples for various groups of formulations. *Top panel*, comparison of percent APG in light-exposed and dark control samples across various groups in the study. Percent APG in light-exposed and dark control samples was determined by ion exchange chromatography using ProPac WCX-10 cation exchange column mated to an Agilent 1100 LC HPLC. *Bottom panel*, representative IEC chromatogram of a light-exposed (*red*) sample overlayed on the chromatogram for the respective dark control (*blue*). *Key: SR-MBaker* drug product formulated using Super-Refined Polysorbate 80 supplied by Mallinckrodt Baker, *NF-MBaker* drug product formulated using NF grade polysorbate 80 supplied by Mallinckrodt Baker, *NF-EMD* drug product formulated using NF grade polysorbate 80 supplied by EMD chemicals, *UP-NOF* drug product formulated using Ultra-pure Polysorbate 80 supplied by NOF corporation

Effect of Light Exposure on Polysorbate 80 Content

Polysorbate 80 content in the light-exposed drug product and placebo samples was comparable to that of the respective dark control samples. The polysorbate 80 content for all the samples was ~0.01%, the level at which the samples were formulated (Fig. 6).

Effect of Light Exposure on Degree of Oxidation

To understand the modifications in the protein molecule as a result of light exposure of various groups of formulations, the dark control and light-exposed samples were subjected to peptide mapping followed by MS/MS analysis of the peptide fragments. As expected photodegradation in light-exposed

samples was due to photooxidation. Oxidation was observed predominantly on one tryptophan and three methionine residues. Specifically, oxidation was observed on tryptophan 32 (light chain), methionine 111 (heavy chain), methionine 251 (heavy chain), and methionine 427 (heavy chain). A representative mirror image of peptide map for light-exposed and dark control sample formulated using Polysorbate 80 NF, EMD Chemicals, is shown in Fig. 7. Upon comparison of degree of oxidation at all the four sites between light-exposed samples formulated with different types of polysorbate 80, it was found that the degree of oxidation at all the four sites followed the order: SR-MBaker<UP-NOF<NF-EMD<NF-MBaker, thus corroborating IEC results (Fig. 8a). For the dark control samples, the degree of oxidation at all the four sites was comparable for all four types of polysorbate 80-containing formulations (Fig. 8b).

DISCUSSION

The sensitivity of protein formulations to light is well recognized among formulation scientists as well as regulatory agencies. Proteins can be exposed to light at multiple points from production to delivery such as chromatographic purification, bulk storage, fill/finish operations, during visual inspections, packaging, long-term storage, and during infusion into the patients (11). The present manuscript for the first time demonstrates that polysorbate 80 quality may affect the photostability of the monoclonal antibody drug product.
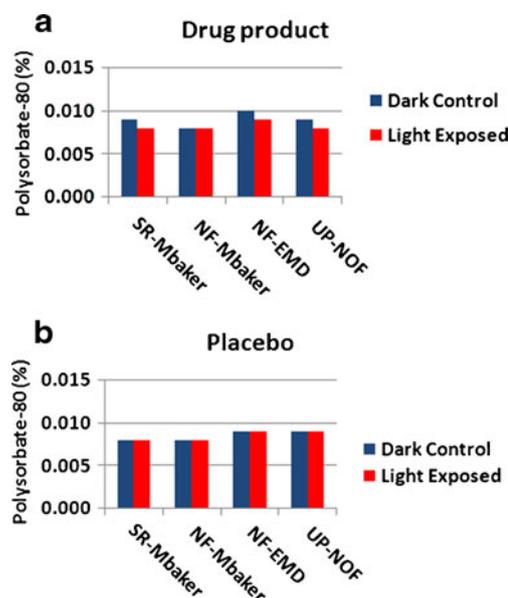
Fig. 6. Polysorbate 80 content in **a**) drug product and **b**) placebo dark control and light-exposed samples. Polysorbate 80 was assayed using a reversed-phase HPLC method. *Key: SR-MBaker* drug product formulated using Super-Refined Polysorbate 80 supplied by Mallinckrodt Baker, *NF-MBaker* drug product formulated using NF grade polysorbate 80 supplied by Mallinckrodt Baker, *NF-EMD* drug product formulated using NF grade polysorbate 80 supplied by EMD chemicals, *UP-NOF* drug product formulated using Ultra-pure Polysorbate 80 supplied by NOF corporation
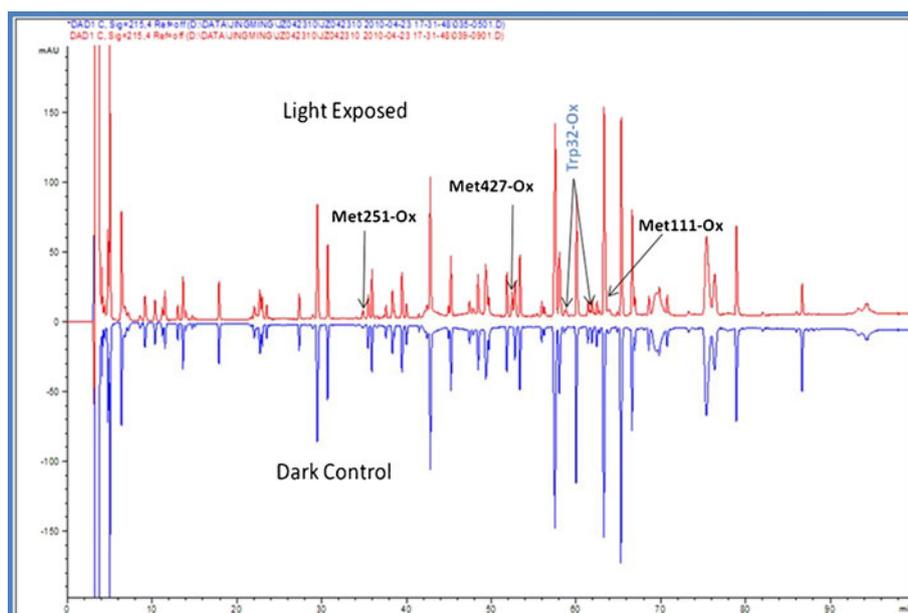
Fig. 7. Representative peptide map for light-exposed (*red*) and dark control (*blue*) samples produced using NF grade polysorbate 80 from EMD chemicals aligned as mirror images of each other for easy comparison

Surfactants such as polysorbate 80 are routinely incorporated in protein formulation to protect the formulation against mechanical stress during shipping and handling. Kerwin in 2008 published a review article on degradation mechanisms of polysorbates describing the susceptibility of polysorbates to autoxidation, cleavage at the ethylene oxide subunits, and hydrolysis of the fatty acid ester bond (8). Autoxidation of polysorbates results in hydroperoxide generation, which may facilitate the oxidation of the active pharmaceutical ingredient (12,13). The oxidative damaging effect of peroxides in surfactants on small molecule drugs has been reported extensively (14–16). There have also been few reports of damaging effect of peroxides in polysorbates on protein stability. Ha *et al.* reported that peroxides generated in polysorbate 80 accelerate oxidation of IL-2 mutein in both liquid and solid states (17). Knepp *et al.* demonstrated that alkyl hydroperoxides in polysorbate 80 induced oxidation, dimerization, and subsequent aggregation of recombinant human ciliary neurotrophic factor in solution (18). The residual peroxide content in polysorbates from different sources is different because of potential differences in the manufacturing and purification processes, packaging and storage conditions (18). Additionally, the levels of other impurities and composition of polysorbate 80 may differ based on the grade and vendor of the polysorbate 80. Thus, we hypothesized that polysorbate 80 grade and vendor affects stability of monoclonal antibody drug product. Particular attention was paid to the drug product photostability, as exposure to light may induce protein oxidation either directly by being absorbed by the protein molecule or indirectly by inducing hydroperoxide accumulation as a result of polysorbate 80 degradation. The present study showed that the photostability of our antibody was affected by the source and grade of the polysorbate 80. Please note that all the assays for this study were performed independently by different analysts.

Percent monomer, LMWS and HMWS in light-exposed samples varied depending on the source/grade of polysorbate 80. Exposure to light resulted in a decrease in the monomer percentage in all the groups of formulation tested. A corresponding increase in percent LMWS and HMWS was observed for all the formulations as a result of light exposure. This observation is in agreement with previous report published by Qi *et al.* in 2009 (19), in which ~23% reduction in monomer content as a result of light exposure was reported in a high concentration IgG1 monoclonal antibody formulation. However, comparing monomer content across various groups of light-exposed samples, we found that in product formulated with Super-Refined Polysorbate 80, the percent monomer retained was highest. The SEC results were compared with SDS-PAGE results and were found to be in excellent agreement.

Exposure to light resulted in a decrease in percent purity in all the groups of samples, thus corroborating SEC results. This is also in agreement with the existing literature (19). SDS-PAGE non-reduced gel image clearly showed an increase in low molecular weight species in the light-exposed samples as compared with the respective dark controls. Similarly, SDS-PAGE reduced gel image showed an increase in high molecular weight species as compared with the dark controls, suggesting covalent cross-links in light degraded samples. Percent purity in all the dark control samples was similar. On the other hand, the percent purity of light-exposed samples varied for different types of polysorbate 80. Noteworthy is the similarity of the trends observed by SDS PAGE results for light-exposed samples to the SEC results for light-exposed samples. For non-reduced SDS PAGE gel, the percent purity for light-exposed samples was in the order: SR-MBaker>UP-NOF>NF-EMD>NF-MBaker.

Percent APG was higher in light-exposed samples as compared with the respective dark controls. Noteworthy is

a Degree of Oxidation in Light Exposed Samples

b Degree of Oxidation in Dark Control Samples
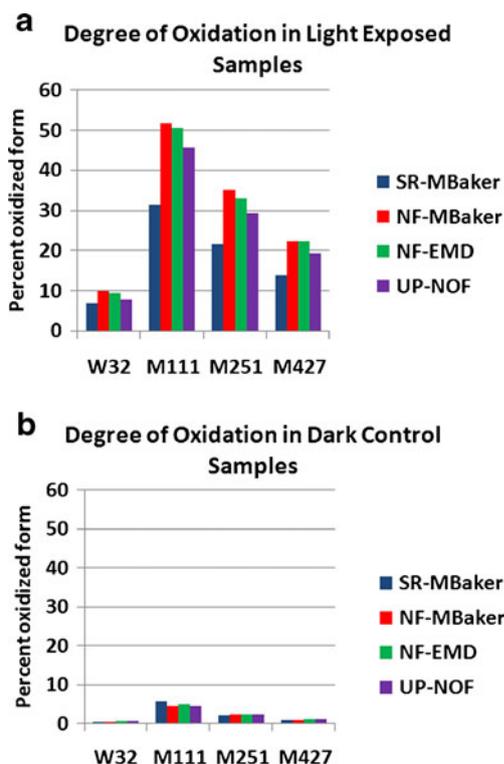
Fig. 8. Degree of oxidation in **a**) light-exposed samples and **b**) dark controls of various groups of formulations. Predominant sites of oxidation and degree of oxidation in light-exposed samples was determined by performing MS/MS analysis on peptide fragments obtained after tryptic digestion of the samples. Photo-oxidation was primarily detected in one tryptophan (*W32*) and three methionine residues (M111, M251, and M427). **a**) The comparison of degree of oxidation in light-exposed drug product samples formulated using various types of polysorbate 80. **b**) The comparison of degree of oxidation in dark control samples. *Key: SR-MBaker* drug product formulated using Super-Refined Polysorbate 80 supplied by Mallinckrodt Baker, *NF-MBaker* drug product formulated using NF grade polysorbate 80 supplied by Mallinckrodt Baker, *NF-EMD* drug product formulated using NF grade polysorbate 80 supplied by EMD chemicals, *UP-NOF* drug product formulated using Ultra-pure Polysorbate 80 supplied by NOF corporation

the fact that among all light-exposed samples, least increase in APG as compared with the respective dark control was observed for product formulated with Super-Refined Polysorbate 80 from Mallinckrodt Baker. Specifically, increase in APG in light-exposed samples as compared with corresponding dark controls for product formulated with Super-Refined Polysorbate 80 from Mallinckrodt Baker, NF polysorbate 80 from Mallinckrodt Baker, NF polysorbate 80 from EMD Chemicals and Ultra-pure Polysorbate 80 from NOF, was found to be 47%, 70%, 66%, and 61%, respectively. Since significant changes in ion exchange chromatogram of light-exposed sample as compared with the dark control were observed, LC/MS based peptide mapping was undertaken to study the nature of light induced chemical modifications in the protein molecule.

Interestingly, peptide mapping showed that light exposure resulted in oxidation at predominantly 4 residues in the molecule—tryptophan 32 (light chain), methionine 111 (heavy chain), methionine 251 (heavy chain), and methionine

427 (heavy chain). Further, comparing the degree of oxidation across various formulations, least oxidation was observed in product formulated with Super-Refined Polysorbate 80 from Mallinckrodt Baker. Photolytic oxidation in protein drug products can potentially occur by two mechanisms: (1) absorption of photons by other excipients in the solution, thereby resulting in formation of singlet oxygen ([20]) and (2) absorption of photons from the light source by protein molecule itself ([21,22]). Absorption of light photons in a protein molecule occurs though either the peptide backbone or by the amino acid side chains of tryptophan, tyrosine, phenylalanine, and cystine. The oxidation of tryptophan 32 observed in this study may be attributed to the latter. Indeed, tryptophan has been reported to have the highest molar absorption coefficients and is considered a major player in the protein photodegradation pathway ([11]). The oxidation of three methionine residues may involve either of the two mechanisms, as has been previously reported by Lam *et al.* ([20]). Methionine residues in proteins are susceptible to oxidation. Indeed, methionine oxidation has been reported in multiple protein pharmaceuticals ([23–27]).

Since oxidation was the primary modification as a result of light exposure, increase in APG observed in IEC cannot be explained based on a change in net surface charge of the protein. However, the increase in APG may be due to alteration in surface charge properties of the protein molecule as a result of structural modifications resulting from oxidation of tryptophan and methionine residues. As explained by Vlasak *et al.*, even a small perturbation in the protein structure may result in change in local distribution of charge residues, thereby changing the overall surface charge distribution of the antibody ([28]). Indeed, methionine sulfoxide generated by oxidation of methionine, is more polar, bulkier, and less flexible as compared with methionine. Thus, presence of methionine sulfoxide in the protein molecule may potentially affect its local conformation.

CONCLUSIONS

The findings in this manuscript emphasize the importance of the excipient quality selection to ensure a stable and robust formulation development. Based on above results it is recommended to select critical excipients such as polysorbate 80 very carefully to ensure robust product quality. Polysorbate 80 has become one of the essential excipients in protein formulations. Although polysorbate 80 plays a key role in protecting the formulation against mechanical stress, the residual peroxides in polysorbate 80 and peroxide generation as a result of light exposure are concerns from the formulation photostability point of view. Thus, the grade and vendor of this critical excipient must be carefully screened to ensure a robust, stable, and efficacious formulation delivery to the patients.

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