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Research paper

Protection of therapeutic antibodies from visible light induced degradation: Use safe light in manufacturing and storage

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ABSTRACT

As macromolecules, biologics are susceptible to light exposure, which induces oxidation of multiple amino acid residues including tryptophan, tyrosine, phenylalanine, cysteine and methionine. Pertaining to safety, efficacy and potency, light-induced oxidation of biologics has been widely studied and necessary precautions need to be taken during biologics manufacturing process, drug substance and products handling and storage. Proteins will degrade to varying extents depending on the protein properties, degradation pathways, formulation compositions and type of light source. In addition to UV light, which has been widely known to degrade proteins, visible light from indoor fluorescent lighting also can mediate protein degradation. In this report, we examine and identify wavelengths in the visual spectrum (400–700 nm) that can cause monoclonal antibody and histidine buffer degradation. Installation of safe lights which exclude the identified damaging wavelengths from visible spectra in manufacturing and storage areas can provide a balance between lighting requirement for human operators and their safety and conservation of product quality.

1. Introduction

During and after manufacturing of biologics, it is unavoidable that the drug substance and drug product are exposed to certain light sources, namely, ultraviolet (UV), sunlight or artificial fluorescent light. The light exposure can happen during manufacturing operations, including upstream cell culture, downstream purification, formulating, filling, visual inspection, packing, storage and transportation. It is well known that proteins are sensitive to light exposure which results in oxidation of proteins [1]. UV light induced-degradation pathways are well documented (for review, see Reference. [1]). The aromatic amino acids, tryptophan, phenylalanine, tyrosine and sulfur-containing cystine are able to absorb UV light at 250-–300 nm range and are excited to a the electronic excited state either to generate amino acid radicals, or to undergo non-radiative relaxation to triplet state and the released energy can be absorbed by oxygen to generate reactive oxygen species (ROS) which, in turn, can oxidize amino acids. In addition, tryptophan oxidation products, such as kynurenine (Kyn) and N-formylkynurenine (NFK) can generate secondary photooxidation effects. The NFK (absorption max at 261 and 325 nm and emission max at 434 nm [2]) and Kyn (absorption max at 258 and 360 nm and emission max at 480 nm [2]) can absorb light at longer wavelengths than Trp, not only generating color in drug substance but also thereby acting as photosensitizers to visible light causing additional damage to the protein [1,3]. Indeed, light-mediated protein degradation can involve multiple amino acid residues and occur from a combination of multiple degradation pathways.

Fluorescent lights are the most widely used indoor light sources in offices, laboratories and manufacturing areas. Commercial fluorescent lamps emit visible light and a small amount of UV light [4]. Multiple reports have suggested that biologics are sensitive to fluorescent light exposure with increasing oxidation and aggregation, loss of biological activity and changes in charge variant species [5–10]. During biologics manufacturing, storage and transportation, majority of light exposure occurs indoor. The guidelines from the International Conference on Harmonization (ICH Q1B) specifies that photostability testing includes both visible light (400-–700 nm) and UV-A light (320-–400 nm). For the visible light test, the ICH Q1B requires that the photostability studies consist of two parts, accelerated studies using high intensity lamps to determine the degradation pathway and confirmatory studies using room temperature room light (RTRL) to provide information for appropriate processing and handling of drug substance. Since therapeutic mAbs themselves do not directly absorb visible light, various photosensitizers have been proposed as the primary absorbers of visible light

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that mediate photooxidation of protein products. These photosensitizers can be impurities from polysorbate 80 [5] or histidine [6], or media components, such as riboflavin (vitamin B2) and pyroxidine (vitamin B6) [7]. Nevertheless, many marketed therapeutic antibodies have a "protect from light" warning label. On the other hand, there is not much discussion about the mitigation and in-process control strategies to achieve the goal of "protect from light". In this report, we investigate light sensitivity of proteins to examine and identify wavelengths in the visual spectrum (400–700 nm) that can cause monoclonal antibody degradation. New lighting technology such as LED lights and their impact on drug substance stability were also evaluated. Installation of safe light which excludes the identified damaging wavelengths from the visible spectrum in manufacturing and storage areas can provide a balance between the light required for operation and human safety and the conservation of product quality.

2. Materials and methods

2.1. Protein stocks

Three different therapeutic IgG4 molecules (mAbs 1 to 3) were used in this study. These mAbs were produced in CHO cells and affinity purified using protein A chromatography, additional polishing chromatography and final UF/DF to a formulation buffer containing histidine. Protein concentration in drug substance (DS) was determined by absorption at 280 nm.

2.2. Light exposure

Formulated drug substance (30 mL) was filled into a 30-mL disposable bioprocess bag with the same contact film as the commercial manufacturing containers. In order to keep results consistent, the air in the headspace of the bag was removed after filling. Two parallel light exposure tests were conducted, one with room temperature (16° to 24 °C) and room light (RTRL, about 500 to 1,000 Lux) and the other with room temperature and high intensity light. The RTRL was carried out in a photostability chamber (Caron, Model 6545–2) with the temperature set at 24 °C and light intensity set at 1 kLux. The chamber is equipped with daylight (white) lamps whose output was similar to the D65/ID65 emission standard referenced in ISO 10977 (light spectrum is in Supplementary Fig. 1A, supplied by manufacturer). The high intensity light experiment was carried out in a fume hood with an air flow rate of 100 cubic feet per minute to prevent material heating due to light. The light source was either an LED white lamp (15 W) or compact white fluorescent lamp (13 W). To generate colored light, either colored filters (Arbor Scientific, Kit 33-0190), or a LED blue or red lamp (15 W, PowerPAR™ LED lamps) were used. The transmittance spectra of the color filters and output spectra of LED lamps are included in the Supplementary Figs. 1 and 2 (supplied by manufacturer). A polycarbonate glass sheet (2.4 mm in thickness) was purchased from a hardware store and used to filter out UVA light. Light intensities were measured by light meters (Sper Scientific UVA/B light meter Model 850009 and Extech HD400 for UVA and visible light, respectively).

2.3. Analytical methods

Size-Exclusion ultra performance liquid chromatography (SE-UPLC) was used to measure mAb monomer, high molecular weight aggregate (HMW) and low molecular weight species (LMW). Imaged Capillary Isoelectric Focusing (iCIEF) was used for measurement of charge variant species. Reducing and non-reducing Caliper was used to determine antibody purity. UV–visible spectral scanning (300–700 nm) was done with a BioTek Epoch 2 model with 5 nm intervals.

2.4. Reactive oxygen species (ROS) level detected by dihydroethidine (DE)

DE (Thermo-Fisher, Cat.# D11347) was dissolved in DMSO as 1 mg/ml. The stock solution was added to the test solution in a 1:200 volumetric ratio and 100 μL sample was added in a 96-well plate, incubated at room temperature in the dark for two hours before reading fluorescence intensity on a ChemiDoc MP Imaging system (Bio-Rad) with 488/605 nm for excitation and emission and the mean fluorescence intensity was analyzed with Image Lab software.

2.5. Tryptic peptide mapping and LC–MS/MS

Tryptic peptide mapping with LC–MS/MS was performed to measure the relative abundance of oxidized methionine (Met) and tryptophan (Trp) residues in mAbs. Approximately 0.6 mg of protein was evaporated to dryness under vacuum using a Savant SpeedVac centrifugal-evaporator and subsequently dissolved in 80 μL of 8.0 M guanidine-HCl (50 mM Tris, pH 8.0) followed by reduction using 200 mM dithiothreitol (DTT) at 37 °C for 20 min and then S-alkylated by adding 400 mM iodoacetamide (IAM) at room temperature in the dark for 15 min. Samples were desalted and buffer exchanged (into 50 mM Tris/HCl, 1.0 M urea, 1.0 mM $CaCl_2$, pH 7.6) using Zeba gel spin-columns (ThermoFisher, Pierce catalog # 0089883: 0.5 mL, 7 kDa MWCO) and then were diluted 2.5-fold (in 50 mM Tris/HCl, 1.0 mM $CaCl_2$, pH 7.6) to give protein concentration of ~2 mg/mL (50 mM Tris/HCl, 0.4 M urea, 1.0 mM $CaCl_2$, pH 7.6) and digested using trypsin with a protein:trypsin ratio of 25:1 (w/w) at 37 °C for 150 min. Digestion reaction was quenched by lowering the pH to 2–3 with 1.0 M HCl.

Digested samples were analyzed by LC-MS tryptic mapping using a mass spectrometer (ThermoScientific Orbitrap Velos, San Jose, CA) coupled with an UPLC instrument (Waters Acquity, Milford, MA) equipped with a UV photodiode array and a fluorescence detectors connected in series. About 40 μL protein digest was loaded onto an UPLC system with a BEH300 (C18, 2.1 × 100 mm, Catalog No. 186003686) reversed-phase column (Waters, Milford, MA) and separated at the flow rate of 0.2 mL/min. The eluents were 0.02% trifluoroacetic acid in water (mobile phase A) and 0.02% TFA in 80% (v/v) acetonitrile (mobile phase B). The column temperature was set to 45 °C. The percentage of mobile phase B increased from 1% to 45% over 100 min.

LC-MS was run using electrospray ionization acquiring sequential MS full-scans with data-dependent acquisition of tandem mass spectra (MSMS) for the ten most abundant parent ions. LC-MS data were acquired in positive ion mode in a mass to charge ratio (m/z) range of 200–2000, capillary temperature of 275 °C, and a source voltage of 5000 V. MSMS data were acquired using 15–35% normalized collision energy, 0.25 activation Q and 100 ms activation time. The peptides containing oxidized methionine were identified by mass-to-charge values at 10 ppm error and confirmed by MS/MS. The relative quantities of methionine oxidation products were calculated from the peak areas in the extracted ion chromatograms of the corresponding peptides.

3. Results

3.1. Multiple mAbs are sensitive to visible light

In our stability studies, drug substance stability was tested by holding at room temperature protected from light and room temperature exposed to room light conditions (RTRL) to distinguish temperature and light effects. For example, mAb 1 DS was highly sensitive to room light exposure with an increase in high molecular weight aggregate (HMW) but relatively stable when exposed only to room temperature (Fig. 1A and B). Extended light exposure generated brown color in the material (Fig. 1C). Further analysis by mass spectrometry showed that certain Try and Met residues were oxidized (Supplementary Table 1). Similar results were obtained for high intensity light

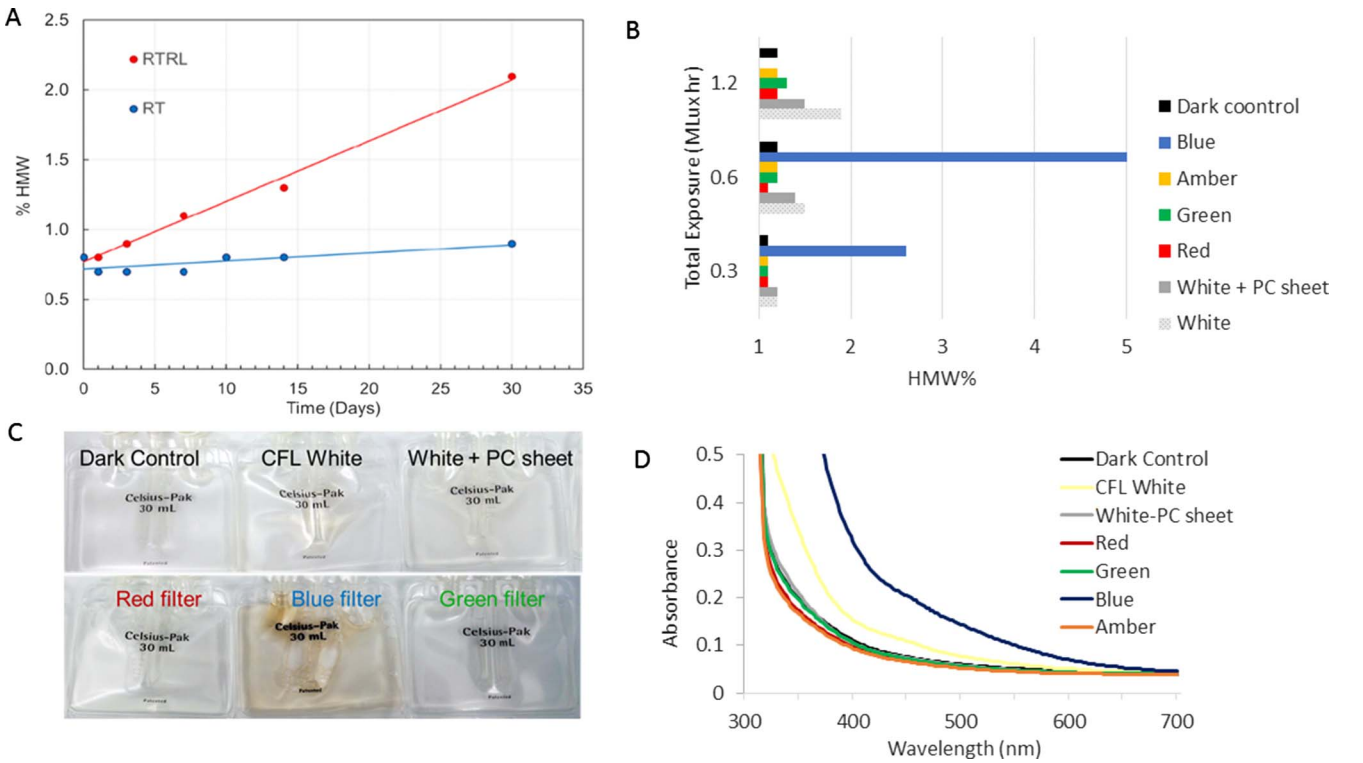

Fig. 1. mAb 1 drug substance stability results. (A) The %HMW in mAb 1 drug substance as a function of time at room temperature in the dark (blue line) or room temperature and room light (1 klux, red line). (B) The %HMW in mAb 1 drug substance as a function of individual colored light intensity. The dark control is the sample wrapped in aluminum foil without light exposure, (C) The appearance of mAb 1 drug substance after exposure to different colored light. (D) The UV–visible absorption spectra of mAb 1 drug substance after exposure to different colored light. The brown coloration in the drug substance correlates to its appearance. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

exposure experiment which showed a rapid increase in the HMW species with time (Supplementary Table 2).

Collectively, four mAbs and an Fc fusion protein in similar histidine-based formulation buffer were tested for photostability. All of them showed light sensitivity to white fluorescent light exposure which led to changes in DS quality attributes, including appearance (brown color), increased HMW and altered charge variants profile.

3.2. Blue light is responsible for the visible light sensitivity

The white light from a fluorescent lamp consists of both UVA and visible light. We hypothesized that only certain wavelengths can be absorbed and are responsible for protein degradation. The visible light can be further divided into multiple colored light spectra one or all of which may be responsible for the increase in HMW of mAb 1. A series of colored filters were used to generate colored light (spectra in Supplementary Fig. 1) and a polycarbonate sheet was used to filter out UVA light. The mAb1 DS showed different sensitivities upon exposure to different colored light. Both UVA and visible light contributed to the degradation of the DS. Room light (UVA + visible) exposure led a 0.8% increase of HMW at 1.2 million lux hour (from 1.1% to 1.9% in Supplementary Table 2). Visible light (no UVA) exposure led a 0.4% increase of HMW at 1.2 million lux hour (from 1.1% to 1.5%). Within visible light range, blue light (centered at 450 nm) was mainly responsible for the HMW generation. In contrast, the DS exposed to red light (> 600 nm) had no change in the HMW content and DS exposed to green or amber light was impacted to a limited extent (Fig. 1B and Supplementary Table 2). The appearance of DS from each sample exposed to individual colored light was also different. The DS exposed to blue light had the most intense brown coloration, followed by white light and white light with polycarbonate sheet (Fig. 1C). The DS brown color can be quantified by UV–visible scanning (Fig. 1D), which shows an increased absorption in the 400–500 nm region due to colorimetric tryptophan photodegradation products [3].

The light filters used in above experiment had a wide range of wavelengths that they transmitted. Red and blue LED lamps were used to generate a narrow band of wavelengths (monochromatic light) for more accurate evaluation of drug substance light sensitivity (Supplementary Fig. 2). The mAb 2 DS was exposed to room temperature protected from light as a control, RTRL, high intensity red LED light and high intensity blue LED light. The blue LED light irradiation caused a brown coloration in the drug substance which was a function of time or energy (Fig. 2). The red LED light had about the same appearance and absorbance spectrum as the control sample kept in dark, suggesting no change after red light exposure. The analytical results showed that both fluorescent white light and blue LED light caused mAb 2 degradation. The degradation pathways included increased formation of HMW species and altered charge variant profile (Supplementary Table 3). Further analysis of charge variant changes revealed that the increased acidic peak correlated primarily with Trp (LC-W94) oxidation and secondarily with Asn (LC-N93) deamidation (Table 1).

The impact of blue light on drug substance quality was further confirmed in mAb 3. After white LED or blue LED light exposure, there was an increase in HMW species and altered charge variant profile (Supplementary Table 4), but happened in no change was seen with red LED light exposure. As shown in Fig. 3A, the percentage of HMW species did not change when mAb 3 was stored in the dark or exposed to red LED light. In contrast, the percentage of HMW species was increased as a function of time when exposed to white LED or blue LED light, or to RTRL. The blue LED light had the biggest impact on HMW formation. The DS exposed to blue light had a strong brown coloration and absorption in the 300–500 nm range (Fig. 3B and C). When the absorbance spectrum from the dark control sample was subtracted from the absorbance spectrum for the blue LED light exposed material, the

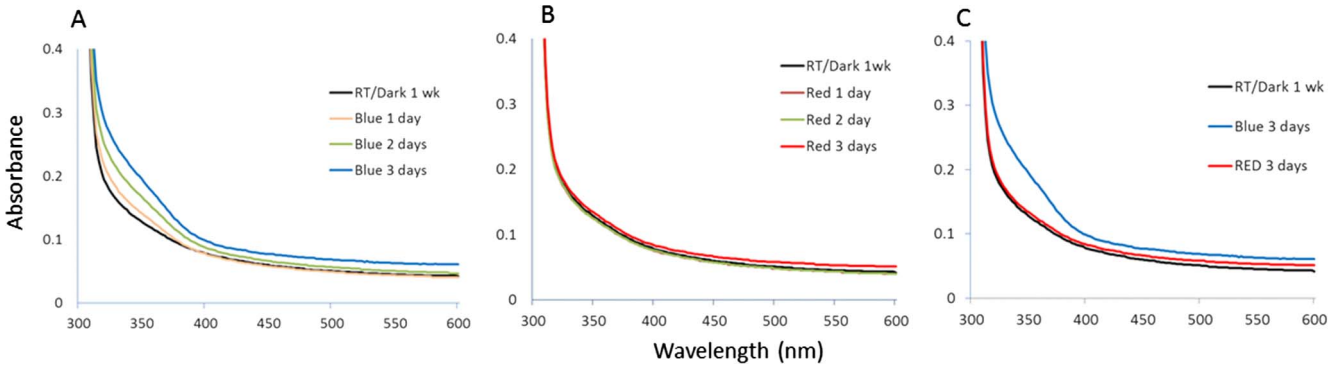

Fig. 2. mAb 2 drug substance stability results from LED light exposure. (A) UV–visible absorption spectra for mAb 2 exposed to blue LED light for 0, 1, 2 and 3 days (25 kLux). The increase in absorbance between 300 and 400 nm is a function of time and the intensity of light. (B) UV–visible absorption for mAb 2 exposed to blue LED light for 0, 1, 2 and 3 days. There is no obvious change in absorbance across the scanning range. (C) Overlaid results from 3-day exposure to blue LED and red LED light. The room temperature dark control was also added for comparison. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

resulting subtraction curve (Fig. 3D) showed a peak at 310 nm which overlapped with NFK, a photodegradation product of Trp [3]. The increased absorption at 300–400 nm may correspond to an increased amount of Kyn and NFK [3,8,11,12]. The sample exposed to white LED light had a similar subtraction spectrum as the one exposed to blue LED light, but to a less extension(Fig. 3D), probably resulting from the blue LED light spectral wavelengths being a part of the white light spectrum (Supplementary Fig. 2). The red LED light subtraction curve was nearly at zero absorption at all wavelengths, except for a small peak at 302 nm. The RTRL subtraction curve was a combination of the red LED and white LED spectra with a peak at 302 nm and absorbance in the 300–400 nm range (Fig. 3D). These results strongly suggest that blue light (400–500 nm) is responsible for the visible light-induced degradation of mAb drug substance while red light (600–700 nm) is a safe light which has minimal impact on mAb product quality.

3.3. Histidine buffer degradation during visible light exposure

It is well known that histidine buffer is sensitive to light exposure in a pH dependent manner, with higher sensitivity at acidic pH and lower sensitivity at pH greater than 7 [6]. Since all of drug substances tested here were in histidine based buffer and had a pH below 7, it was expected that the histidine buffer was unstable. Indeed, after mAb 1 DS was exposed to white light, the buffer matrix peak on size-exclusion chromatography (SEC) had increased absorption at 280 nm as a function to exposure time (arrow, Fig. 4A). The peaks were from small molecules as they could pass a 10 kDa filter (Fig. 4B) but the mAb, as expected could not (Fig. 4C). Buffer of 20 mM histidine (pH 6.5) without any protein was directly exposed to blue LED light. Fig. 4D shows that the irradiated histidine buffer had increased absorbance at 250–400 nm as function of exposure time and the absorption curve was very similar to what was previously reported [6].

3.4. Detection of ROS from visible light exposure

Dihydroethidium (DE), has been used extensively in cell biology experiments to evaluate production of reactive oxygen species (ROS) [13]. DE was used to measure ROS in light exposed (photo-aged) histidine buffer, mAb 3 formulation buffer without any proteins and mAb 3 drug substance (Fig. 5). Compared to buffers stored in dark, both the photo-aged histidine buffer and the formulation buffer had generated more ROS, which was consistent with previous reports that histidine buffer was photosensitizer and mediated the photodegradation of a mAb [6]. The mAb 3 DS exposed to blue LED light had 3 times more ROS than the one exposed to red LED light. Similar results were also observed from hydrogen peroxide detection by Amplex Red assay [14] (data not shown). These results strongly suggest that the mechanism of blue light induced degradation is mediated by ROS.

4. Discussion

A large body of knowledge exists concerning the photostability of therapeutic antibodies during long term storage and the mechanisms of light-induced damage [1]. The biopharmaceutical industry usually provides protection from light by using an appropriate container and closure system that keeps light away from the drug substance. Light-induced damage of proteins can occur at many points from production in cell culture to delivery to patients. During the manufacturing process, light exposure can alter charge variant profile during upstream cell culture stage [7], or during downstream purification in which glass columns are used for chromatography and UV spectrometers are used for monitoring [1]. More frequently, light exposure may occur during drug substance storage depending on the storage container and lighting configurations [5,6,8–10]. The implementation of the “avoid light exposure” label is not without difficulty. For example, additional light protective covering of drug substance containers during the bulk drug substance freeze and thaw, may have an impact on heat transfer efficacy and elongate the of freeze and thaw durations.

The outcome following visible light exposure is an increase in the reactive oxygen species which oxidize purified proteins. In addition to the impact on product quality attributes, light exposure can also generate new impurities resulting from chemical reactions, for example, histidine-derived impurity 6a-hydroxy-2-oxo-octahydro-pyrollo[2,3-d] imidazole-5-carboxylic acid is induced by light irradiation [6]. Although there is no existing data to show how much of this particular chemical can accumulate over long term storage with reasonable light

Table 1
Oxidation and deamidation of mAb2 drug substance under various conditions.

Sample	LC-W94$_{ox}$	LC-W94$_{NFK}$	LC-W94$_{kyn}$	HC-W113$_{ox}$	HC-W113$_{NFK}$	LC-N93$_{deam}$	HC-N318$_{deam}$	HC-N387$_{deam}$	HC-N392$_{deam}$
Time 0	0.1	0.1	< 0.1	0	0.1	2.6	1.3	1.8	1.6
RTRL_4wk	3.5	2.8	< 0.1	0.1	0.5	4.6	1.4	2	1.6
BlueLED_3d	1	1.3	< 0.1	0	0.2	3	1.3	2	1.6
RT 4wk	0.1	0.1	< 0.1	< 0.1	0.1	2.5	1.8	2.6	1.8

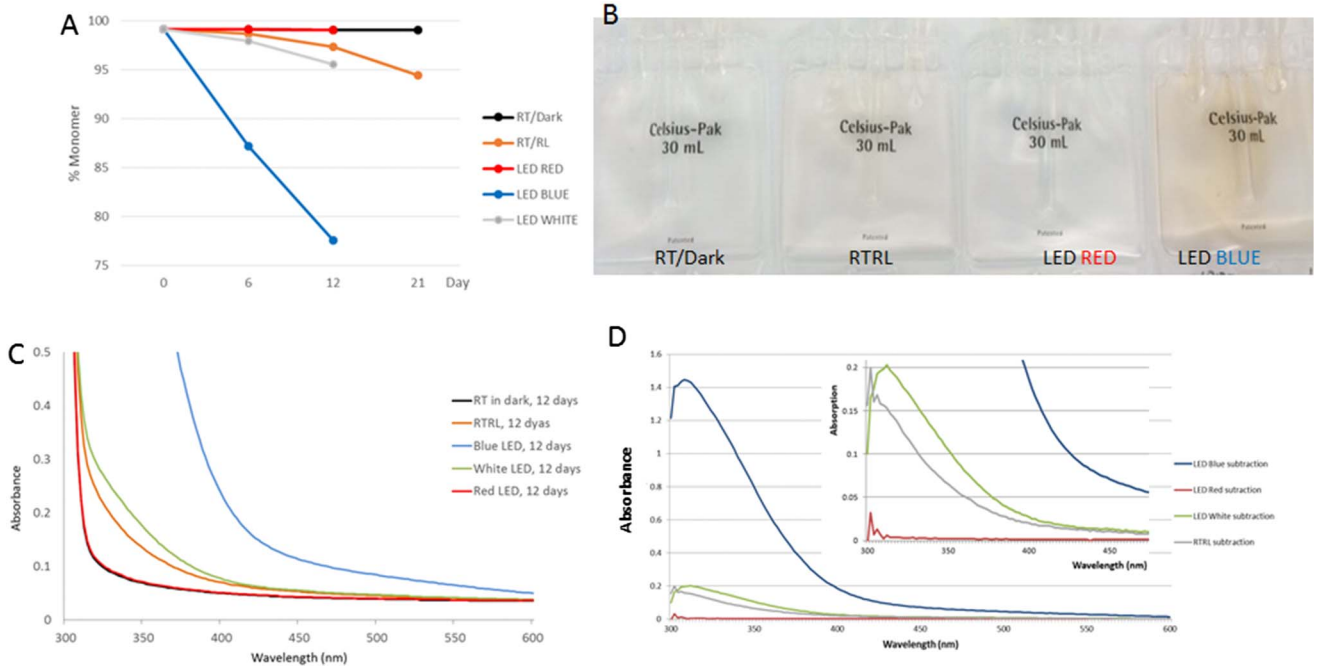

Fig. 3. mAb 3 drug substance stability results from room light and LED light exposure. (A) % mAb monomer as a function of light exposure time. The light sources were fluorescent daylight (1klux), LED blue, red and white light (25klux). The sample at room temperature in the dark was a control to exclude impact of temperature. (B) Appearance of some samples after 12-days of light exposure. (C) UV–visible absorption of individual samples after 12-days of light exposure. (D) Subtraction curves for light exposed samples. The subtraction was the difference between absorbance of light exposed sample and light protected room temperature sample. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

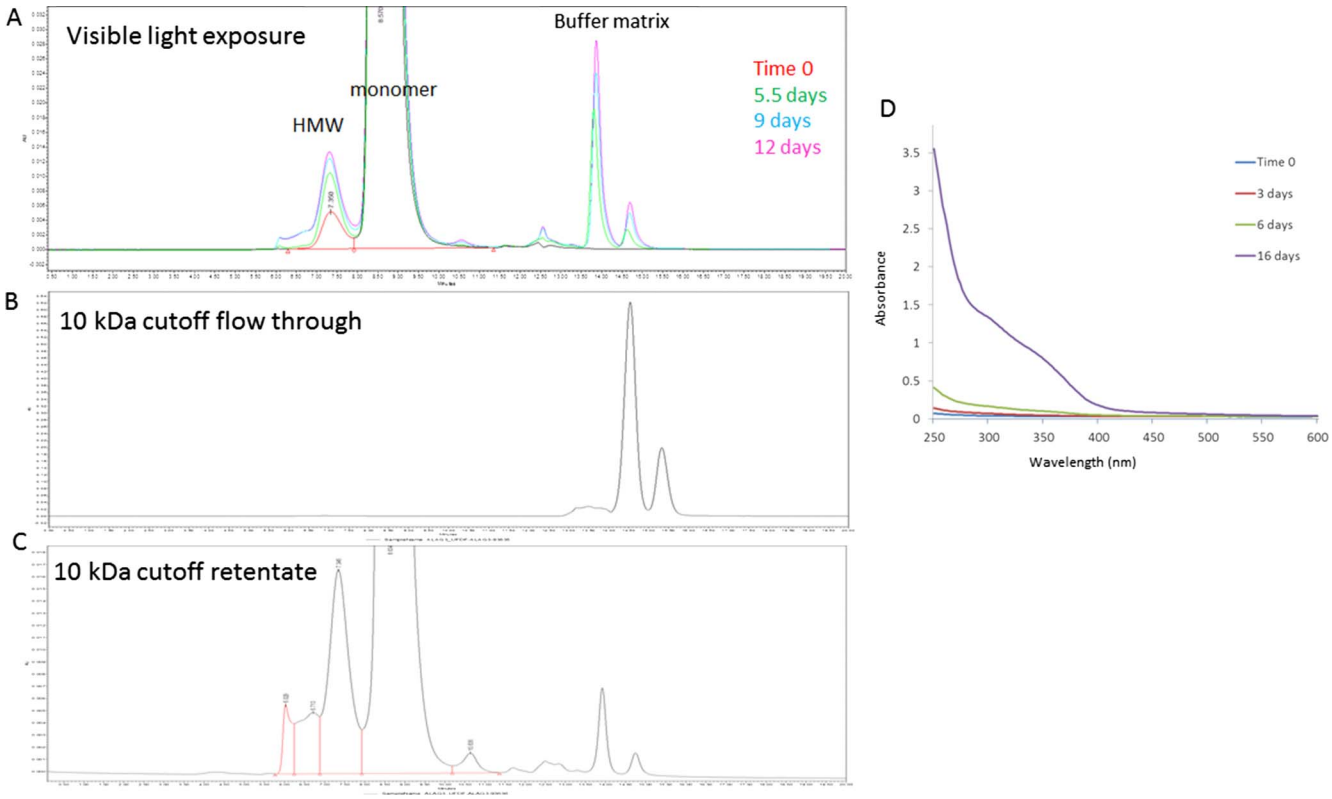

Fig. 4. Photodegradation of histidine buffer. (A) SEC chromatograph of mAb 1 after fluorescent white light exposure. The arrow indicates the buffer matrix peaks which were a function of light exposure time. (B) After filtration through a 10 kDa cutoff filter, the buffer matrix peaks were in the permeate, while the mAb was in retentate (C) Histidine buffer UV–visible absorbance before and after LED blue light exposure. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

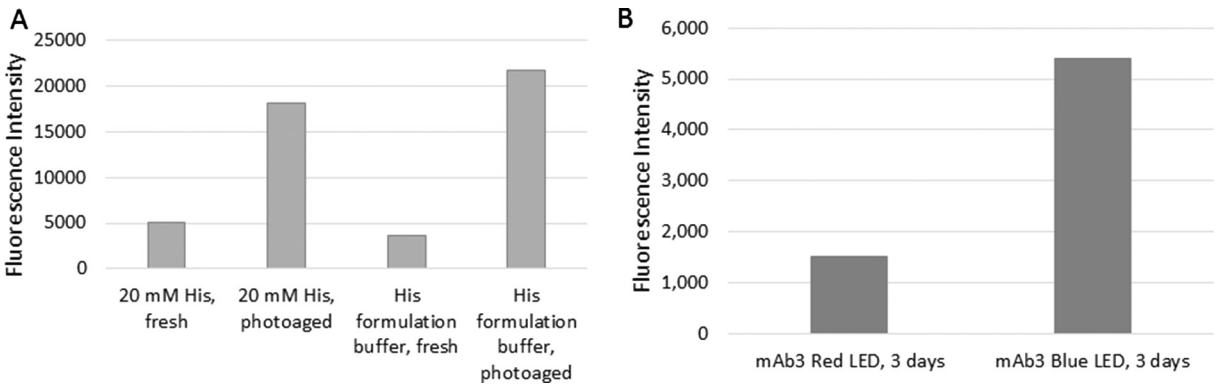

Fig. 5. Detection of ROS with fluorescent dye dihydroethidium (DE). (A) ROS measurements for 20 mM histidine and mAb 3 formulation buffer after 16-day exposure to blue LED light and the buffer without light exposure. (B) ROS measurement of mAb 3 drug substance after 12-day exposure to LED red and blue light. (Quantification of ROS fluorescence intensity was from the difference in fluorescence between samples with and without addition of DE. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

exposure, it is a potential risk for shortening product shelf life.

Proteins have zero absorbance at visible light wavelengths. The identification of photosensitizers from histidine buffer (Reference. [6] and this work) and/or from polysorbate-80 [5,15] emphasizes the importance of carefully choosing high quality chemicals for formulation, as well as help explain the results of photostability testing for histidine buffer. Although histidine residues on mAbs also can be oxidized by light [16–18], we did not detect histidine residue oxidation in mAbs 1 and 2. The W94 on light chain from both mAb 1 and 2 is located in CDR3 loop region and is photooxidized in both molecules. The impact of light was observed to be varied between the molecules. For example, the W113 on heavy chain of mAb 2 is a conserved amino acid next to CDR3 loop. The W113 in mAb 2 is oxidized (Table 1), but the W110 (the equivalent of W113 in mAb 2) is not oxidized in mAb 1 (Supplementary Table 2). Based on sequence alignment and structural homology modeling (Modeller, https://salilab.org/modeller/), we think that the light induced oxidation of Trp occurs to surface-exposed Trp residue(s). Our data agrees with findings from other peoples that surface-exposed Trp residues are more susceptible to chemical or UV-induced oxidation than buried Trp residues [19–21]. In addition, deamidation of mAb 2 molecules on light chain N93 in a sequence of NW is unexpected (Table 1). In fact, there are two conserved NG sequence and the PENNY sequence on the Fc region of mAb 2 molecule which are prone to deamidation on many mAbs [22], but there was no significant deamidation was detected. The Asn deamidation includes a succinimide-mediated cyclic intermediate. In protein or peptide sequence, the side chain of Trp is bulky and has spatially hinder for deamidation via the succinimide intermediate. Alternatively, direct hydrolysis of asparagine to aspartic acid is a plausible mechanism [23,24].

In this study, we have identified specific wavelengths in the visible light spectrum responsible for photodegradation of therapeutic monoclonal antibodies. Our results clearly demonstrate that blue light (400–500 nm) is responsible for generation of ROS and has major impact on product quality attributes, other visible wavelengths (for example, green and red) have minor impact. Therefore green or red light, or other colored light consisting of wavelengths above 500 nm is safe for biopharmaceutical products and can provide sufficient lighting for visibility for humans.

During manufacturing, the processing and handling drug substance and drug product usually takes 2–4 weeks at RTRL environment and light exposure can cause significant changes in product quality (Figs. 1 and 3A). Changing the light source in manufacturing sites and drug storage areas from artificial daylight fluorescent light to a safe light can be easily achieved by covering existing lamps with commercially available colored plastic tubing or using LED lamps. The idea of using safe light has long history from photographic darkroom. Other industries, such grocery stores, have installed safe spectrum lamps to prevent oxidization and photochemical reactions in foods and to keep freshness and nutrient ingredients of foods [25,26]. It will be interesting to further investigate which colored light is most suitable for human safety and equipment operation. In addition, final drug products filled into dark colored vials or secondary light-protective secondary packaging would have better assurance on product quality.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ejpb.2018.02.007.

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