Polysorbate 80 Interferes in the Extrinsic Fluorescence based Tertiary Structure Determination of a Therapeutic Anti-CD20 Antibody with ANS Dye

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Abstract

Therapeutic proteins such as monoclonal antibodies are complex molecules, need extensive characterization before their release into the market for clinical use. Therapeutic protein formulations are routinely characterized for their tertiary structure. Extrinsic fluorescence spectroscopy using various molecular rotors such as polarity sensitive ANS dyes is commonly used for the tertiary structure determination. However, the formulation excipient profile containing surfactants such polysorbate is known to have profound influence in terms of background noise in the extrinsic fluorescence when ANS dyes are used. However, there is neither any study that has clearly indicated the interference of polysorbate 80 in the extrinsic fluorescence profile of a formulated IgG1 monoclonal antibody drug product nor any study has reported the solution to the problem of avoiding interference from polysorbate 80 during extrinsic fluorescence analysis of a formulated monoclonal antibody drug product. Here in this study we reported the interference of polysorbate 80 in the extrinsic fluorescence profile of therapeutic protein drug product and recommending a polysorbate 80 removal step prior to extrinsic fluorescence analysis of drug product for the tertiary structure determination.

Keywords: Polysorbate, extrinsic fluorescence, anti-CD20 antibody, tertiary structure, ANS dye

Introduction

Biopharmaceuticals are complex proteins and are susceptible to degradations. Even minor changes in solution chemistry affects the degradation profile of therapeutic proteins such as monoclonal antibodies. The conformational and compositional stability of therapeutic proteins exists only within a relatively narrow range of osmolality and pH, and mostly excipients are also required to be added to increase their stability (1). Extensive physico chemical characterization of proteins is a prerequisite for the development of stable formulations and their clinical use as per regulatory guidelines (2, 3). Fluorescence spectroscopy is highly sensitive method often employed for structural characterization of recombinant therapeutic proteins and monoclonal antibodies. Stable formulations of therapeutic IgG1 antibody are subjected for Intrinsic and extrinsic protein fluorescence using appropriate dyes for the determination of their secondary and tertiary structure respectively.

Different varieties of molecular rotors are routinely employed for the determination of extrinsic fluorescence to analyze the stressed drug product samples for aggregates and to analyze their tertiary structure (4). ANS (8-Anilinonaphthalene-1-sulfonic acid) dye is one of the commonly used polarity responsive fluorescent dye for the extrinsic fluorescence analysis for the determination of tertiary structure.

Extrinsic dyes can be covalently attached to proteins, e.g. via the [-amino group of lysine, the α-amino group of the N-terminus, or the thiol group of cysteine. More interesting for the analysis of pharmaceutical formulations are extrinsic dyes that interact non-covalently with proteins and protein degradation products, through hydrophobic or electrostatic interactions.

The fluorescence properties of ANS and other polarity sensitive dyes strongly depend on their interaction with protein molecules, which results in changes of polarity and viscosity of the environment. Hydrophobic interactions and electrostatic interactions have been discussed as binding mechanisms of ANS to proteins (5). However, previous research reported that most polarity responsive dyes are interfered with the extrinsic fluorescence of polysorbate containing protein formulations via dye-surfactant interactions and give high background fluorescence (4).Almost all the stable formulations of biopharmaceuticals especially monoclonal antibody formulations contain detergents such as polysorbate. Although ANS dye is known to cause background fluorescence in the fluorescence spectroscopy of polysorbate containing protein formulations, it is still widely used in the tertiary structure determination as it is cost effective and the alternative dyes also has their own limitations.An extensive study, directly measuring the impact of polysorbate 80 interference on IgG1 tertiary structure determination particularly when used ANS dye is still lacking. In this study we have attempted to show that polysorbate 80 impact on the extrinsic fluorescence of a stable anti-CD20 formulation by evaluating the drug product samples before and after polysorbate removal as well as in drug substance and formulation buffer.

Materials and Methods
Extrinsic Fluorescence measurement: The IgG1 mAb drug product samples (100 µl) are diluted to 1mg/ml from the labeled concentration and 50µM ANS dye (25 µl) is added to the samples and final volume was made upto 1ml with Water for Injection (WFI). Samples (200 µl) were loaded into a 96 well black opaque plate (Costar, USA) and fluorescence spectroscopy analysis was done with excitation at 380nm and emission at the range of 410nm -600nm with an interval of 1nm. The data obtained was plotted with wavelength on X-axis and relative fluorescence units (RFU) on the Y-axis.

Polysorbate removal from the samples: The drug product test sample containing polysorbate 80 with protein concentration of 5mg/ml was prepared for analysis. Polysorbate 80 present in the drug product samples was removed using ACROSEP SDR HYPERD detergent removal columns as per the manufacturer's recommendations. Initially Columns were pre-equilibrated by passing 10ml of 150mM NaCl followed by passing 1ml of test sample through the column using a syringe. Once the sample is passed through the column, 5mL of 150mM Sodium chloride buffer was again passed and fractions are collected. A total of 8 fractions are collected with fraction 2 being ~1000 µl and the remaining fractions were ~500 µl for (fraction 1 & 3 to 8). The remaining flow through was discarded and finally 2mL of 20% ethanol was passed through the column and stored in the same solution at 2-8°C for further use.

Estimation of polysorbate in the samples after polysorbate removal step: Anti-CD20 preparation was mixed with 2.5 ml of extraction solvent in a 15 ml centrifuge tube and were mixed for 3 minutes followed by centrifugation at 4000 rpm for 2 minutes. The upper layer was collected and dried in a vacuum evaporator. A volume of 360 µl ACTC dye was added to each 2 ml centrifuge tube for derivatization with vortexing and allowed the reaction mixture cool at room temperature for 5 minutes. A volume of 360 µl dichloromethane was added to each 2 ml centrifuge tube followed by vortexing and subsequent centrifugation at 5000 rpm for 2 minutes. Finally, 200 µl of lower layer was aliquoted into a quartz plate without disturbing the upper layer and the absorbance was measured at 620 nm. Polysorbate concentration was reported as µg/ml.

All the extrinsic fluorescence data is expressed as Mean ± SD.

Results

Extrinsic Fluorescent analysis before polysorbate removal: The IgG1 monoclonal antibody drug product (DP) was evaluated for tertiary structure analysis using extrinsic fluorescence spectroscopy. Results from this study show that the drug product samples containing polysorbate 80 along with monoclonal antibody have the emission maxima at mean RFU at 494nm and mean maximum RFU value is 24227. Whereas the same emission maxima at mean RFU (494nm) and mean maximum RFU values (21976) were observed for the DP formulation buffer (DPFB) containing polysorbate 80. The monoclonal antibody drug substance (DS) show emission maxima at mean RFU and mean maximum RFU at 512nm and 4583 respectively before formulation (See Figure 1, 2 and 3). These results clearly show the interference from polysorbate 80 in the extrinsic fluorescence based tertiary structure determination of monoclonal antibody drug product.

Extrinsic fluorescence analysis after polysorbate 80 removal: All the drug product fractions are detergent depleted and fraction 2 is considered as the major fraction based on the protein content analyzed through OD@280nm and extrinsic fluorescence. The remaining fractions 3 to 8 have less amount of protein along with the buffer. Protein recovery calculated from the 8 fractions collected is more than 85%. Polysorbate 80 estimation is done for the fractions collected and no polysorbate 80 was observed in the sample fractions analyzed for extrinsic fluorescence. The sample fraction with depleted polysorbate 80 has the emission maxima at mean RFU of 519nm and the mean maximum RFU value of 3782 for the IgG1 antibody present in the drug product (See figure 1 and 2).

Discussion

This study observed that there is difference in emission maxima and RFU intensity between drug product samples with (494nm and 24227RFU) and without polysorbate 80 (494nm and 21976RFU).

All therapeutic protein formulations including monoclonal antibody formulations contain surfactants like polysorbate 20 and 80 which are commonly used excipients to prevent protein adsorption at liquid-liquid, liquid-solid or liquid-air interfaces, which can lead to surface-induced denaturation and aggregation (6,7). Therapeutic protein based drug products especially monoclonal antibody based drugs must be thoroughly characterized for their tertiary structure during their development before release and clinical use. Extrinsic fluorescent dyes such as polarity sensitive ANS are commonly employed for the
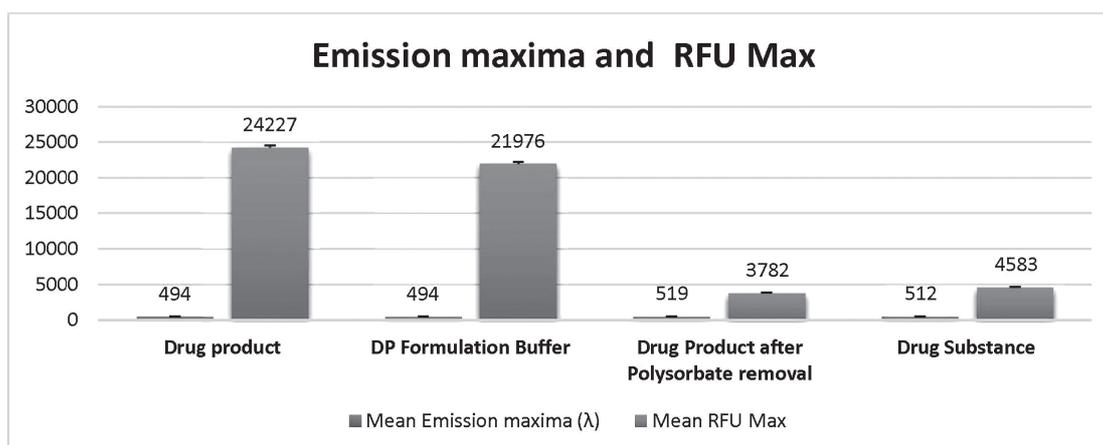

Fig. 1. Extrinsic fluorescence analysis of Drug products

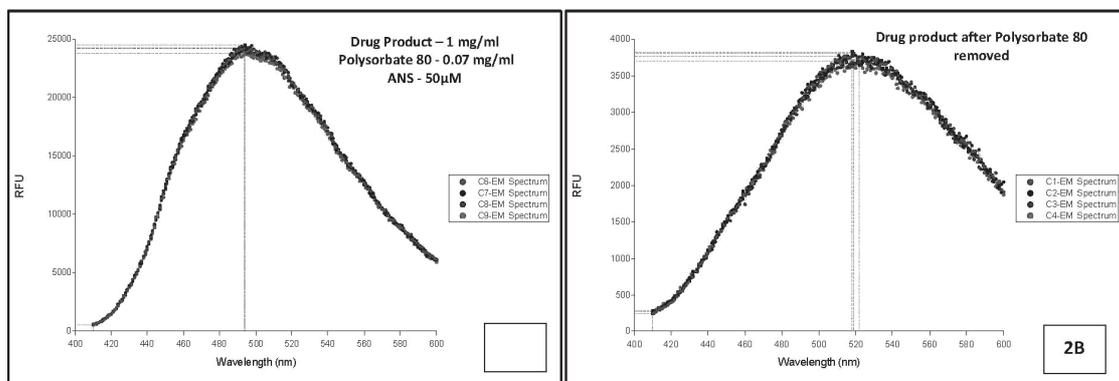Polysorbate interferes in the extrinsic fluorescence of ANS dye

Fig. 2A & 2B. Extrinsic Fluorescence analysis of Drug product before and after Polysorbate 80 removal
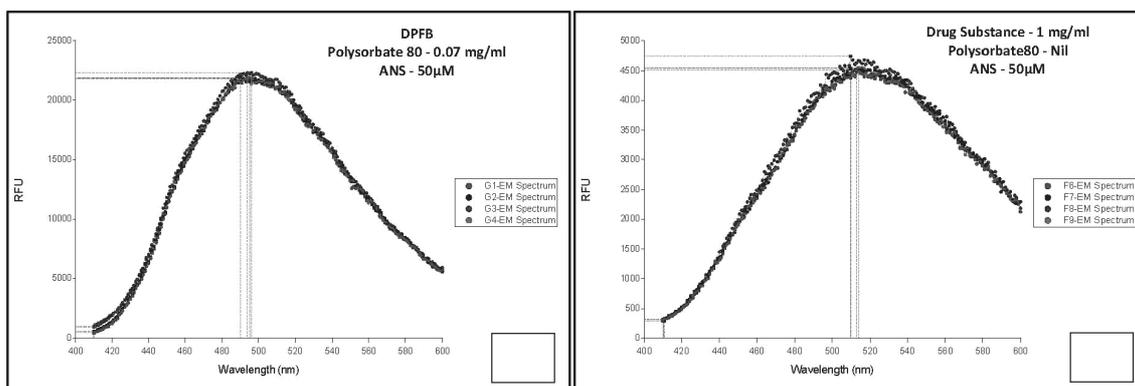

Fig. 3A & 3B. Extrinsic Fluorescence analysis of Drug substance (before polysorbate addition) and Formulation buffer (with Polysorbate)

tertiary structure determination of therapeutic proteins especially monoclonal antibodiesalthough they are known to increase background noise (8, 9). ANS dye is known to interact with polysorbate 80 and is also used for the analytical characterization of polysorbate degradation products during stability monitoring of therapeutic protein formulations (10). Avoiding or eliminating background fluorescence while using ANS dye for tertiary structure determination of therapeutic antibody drug product containing polysorbate 80 is still a challenge. In agreement to the previous reports, we also found that polysorbate 80 indeed interferes with the extrinsic fluorescence measurement of therapeutic IgG1 antibody. To know whether we can avoid polysorbate interference, in this study we removed polysorbate 80 from the drug product before extrinsic fluorescence analysis. Our results suggested that, the drug product emission maxima and RFU values are coinciding with the formulation buffer data (both containing polysorbate 80) but not with the data from drug substance and polysorbate removed drug product. This means the fluorescence observed mostly includes polysorbate 80 fluorescence in the drug product samples. This may be due to the interaction between dye-polysorbate, which results in high background fluorescence contributing to high overall fluorescence (4). New molecular rotors like

CCVJ and DCVJ dyes are suggested as alternative to the polarity sensitive dyes while dealing with polysorbate containing formulations, but they too increase the background fluorescence when the formulations contain sugars and only suitable for high concentrated formulations (4, 11). Since, most of the monoclonal antibody formulations, especially anti-CD20 antibody formulation contain sugars as excipients, these dyes are of limited use. Alternatively, polysorbate interference can be corrected through background correction (by subtracting fluorescence of formulation buffer from drug product)or by analyzing the drug substance samples (before formulation buffer addition). But it is not an accepted practice in industryasthe tertiary structure determination should be done in formulated drug product with all the excipients to know whether the added excipients has any effect on the structure.

This study concludes that interference in extrinsic fluorescence in terms of mean emission maxima and mean RFU of IgG1 therapeutic monoclonal antibody drug product can be attributed to polysorbate 80 when ANS dye is used and polysorbate 80 removal procedure is recommended to be employed before the sample analysis.

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Conflicts of Interest : The authors declare there is no conflicts of interest.

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