Solid-State mAbs and ADCs Subjected to Heat-Stress Stability Conditions can be Covalently Modified with Buffer and Excipient Molecules

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ABSTRACT: We report that a unique type of chemical modification occurs on lyophilized proteins. Freeze-dried mAbs and antibody–drug conjugates (ADCs) can be covalently modified with buffer and excipient molecules on the side chains of Glu, Asp, Thr, and Ser amino acids when subjected to temperature stress. The reaction occurs primarily via condensation of common buffers and excipients such as histidine, tris, trehalose and sucrose, with Glu and Asp carboxylates in the primary sequence of proteins. The reaction was also found to proceed through condensation of carboxylate containing buffers such as citrate, with Thr and Ser hydroxyls in the primary sequence of proteins. Based on the mass of the covalent adducts observed on mAbs and ADCs, it is apparent that the reaction produces water as a product and is thus favored in a low moisture environments such as a lyophilized protein cake. Herein, we present the evidence for the covalent modification of proteins drawn from case studies of in-depth characterization of heat-stressed mAbs and ADCs in the solid state. We also demonstrate how common charge variant assays such as imaged capillary isoelectric focusing and mass spectrometry can be used to monitor this specific class of protein modification. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 104:652–665, 2015

Keywords: mass spectrometry; capillary electrophoresis; freeze-drying/lyophilization; stability; excipients; covalent modification; esterification; charge variants

INTRODUCTION

It is important to characterize the changes in the quality of biotherapeutics that result from accelerated and real time stability storage conditions. Knowledge gained from characterization of the stability-induced changes in protein biotherapeutic heterogeneity is important for establishing storage and handling conditions that ensure shelf life stability and product integrity. Much of the literature on the topic of characterizing protein heterogeneity as a consequence of exposure to stress/stability conditions focuses specifically on liquid formulations. Liquid formulations can be advantageous because of the ease of manufacturing and administration; however, protein modifications that can be deleterious to therapeutic efficacy can readily occur in the liquid phase. Lyophilized proteins are generally less susceptible to chemical modification and degradation than proteins in the liquid state.[1–3] In some cases, the gains in protein stability in solid state formulations can offset the added time required for lyophilization process development, thus making solid-state formulations a viable option for labile protein therapeutics.

Assessments of lyophilized protein stability need to be carried out on the freeze dried protein and the reconstituted protein because a lyophilized protein therapeutic is stored and shipped in the solid state but administered in the liquid state, after reconstitution. Characterization of physical stability is emphasized more in lyophilized formulations versus liquid formulations because the protein is undergoing a change in state upon lyophilization and upon reconstitution and both have the potential to impact protein folding and higher order structure.[4–7] Additionally, changes in protein conformation have been shown to result in aggregation, adsorption onto surfaces, precipitation, and particle formation.[8,9] Aggregate has been implicated in the formation of antitherapeutic antibodies and it has also shown to contribute to innate immune response *in vitro*.[10–12] Deamidation of Asn residues and cyclic imide/isomerization occurring on Asp residues are common degradation events that are observed on proteins regardless of the physical state.[13–18] The rate of chemical modification on Asn and Asp residues varies widely and depends on the pH and cosolvents in the formulation buffer,[19–23] the position and solvent accessibility of the residue,[24] and the storage and stress conditions.[25,26] Controlling oxidation levels on Met and Trp residues is also important for ensuring biotherapeutic integrity. Oxidation of Met and Trp can occur through chemical and photo-stress conditions[27–29] and can also be impacted by the presence or absence of formulation additives that act as radical scavengers.[30,31] Mono and disaccharides used as cryoprotectants in liquid formulations and bulking agents in lyophilized formulations can also be associated with chemical modifications. Glucose can covalently modify Lys, Arg, and protein N-termini in both liquid and solid states.[32,33] Disaccharides such as sucrose can break down into constituent monosaccharides as a consequence of heat stress and also modify proteins.[34,35]

In this report, we describe a new class of chemical modifications that occurs only to heat-stressed lyophilized proteins.

Abbreviations used: ADC, antibody–drug conjugate; icIEF, imaged capillary isoelectric focusing; MS, mass spectrometry; vcMMAE, valine-citrulline monomethyl Auristatin E; PBD, pyrrolobenzodiazepine; HPβCD, hydroxypropyl−β-cyclodextrin; SEC, size-exclusion chromatography; LC–MS, liquid chromatography–mass spectrometry; DTT, dithiothreitol; rp-HPLC, reversed-phase-HPLC; pE, pyroglutamic acid.

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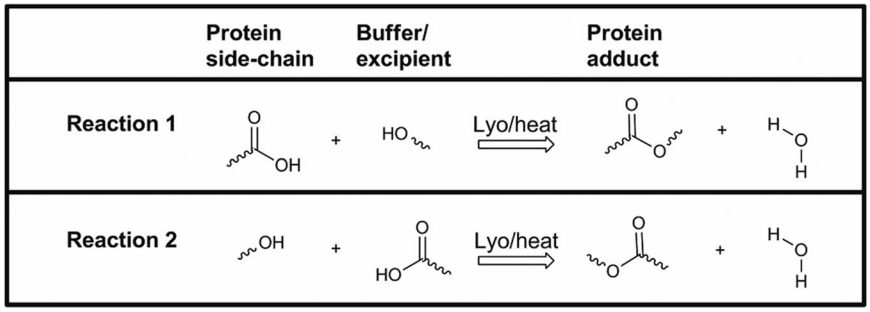

Figure 1. Reactions involving covalent protein adduction with formulation buffer and excipient molecules that are observed in lyophilized, heat stressed formulations.

This modification primarily involves the esterification of protein carboxylates and hydroxyls with formulation buffer and excipient molecules via condensation-type reactions (Fig. 1). We have found that the reactions occur at multiple sites in the protein sequence when the protein is in the solid state and, the modification is readily detected by common charge variant assays such as icIEF. It is important to characterize the covalent adducts on heat-stressed lyophilized proteins resulting from condensation reactions because these modifications are the major contributor to protein heterogeneity in several of the buffer–excipient systems that we have investigated. Understanding the occurrence and impact of condensation driven modifications on lyophilized proteins is important for establishing storage conditions that minimize undesirable changes to protein quality that may result from these reactions.

MATERIALS AND METHODS

Materials

mAbs and Antibody–Drug Conjugates

Two IgG1 mAbs were expressed in Chinese hamster ovary cells and purified according to established practices. The first mAb (mAb-1) was conjugated with valine-citrulline monomethyl Auristatin E (vcMMAE) at interchain cysteine residues using reductive alkylation strategies that have been described previously.[36] The drug distribution of the vcMMAE-antibody–drug conjugates (ADCs) was heterogeneous, varying from 0 to 8 drugs per mAb (Fig. 2), but the average molar ratio of drugs to mAb (MR_D) was 4.2. The second mAb (mAb-2) is an engineered IgG1 with Ser at heavy chain position 239 replaced with Cys. MAb 2 was conjugated with a drug-linker molecule containing a pyrrolobenzodiazepine (PBD) dimer (Fig. 2) according to previously described procedures.[37] The PBD–ADC was site-specifically conjugated at the engineered Cys residues and the MR_D was 1.9.

Formulations

The formulations consisted of conventional buffers, excipients, and surfactants commonly used in lyophilized protein formulations. The buffers included histidine, citrate, and tris at a concentration of 20 mM, and the pH was titrated to fall within physiological pH ranges. The excipients used in the studies included sucrose, trehalose and hydroxypropyl-β-cyclodextrin (HPβCD) used at concentrations of 4.5%–6.0%. Polysorbate 80 (PS-80) was used as a surfactant in formulations that did not contain the HPβCD. The protein concentration was 5 mg/mL for the mAb, 15 mg/mL for the vcMMAE conjugate, and 3 mg/mL for the PBD conjugate prior to lyophilization. The formulations for all mAbs and ADCs are summarized in Table 1.

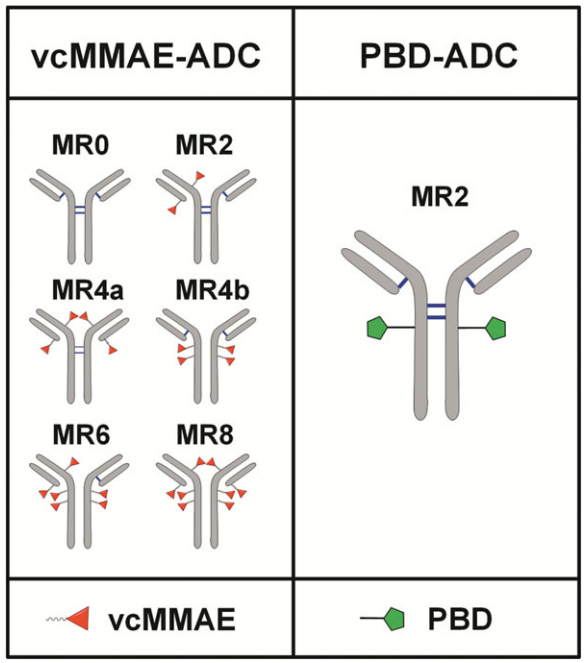

Figure 2. IgG structures associated with vcMMAE- and PBD–ADCs. The vcMMAE drug-linker is depicted as a red triangle on the ADC structures with molar ratios (MR) of drugs to antibody ranging from 2 to 8. The PBD drug-linker is depicted as a green pentagon on the ADC structure with MR of 2 drugs per antibody.

Freeze-Drying Procedure

A conventional lyophilization cycle consisting of freezing, primary, and secondary drying steps occurring over the course of 55 h was used. The protein formulations were filled into 10 mL glass type I tube vials and partially closed with gray butyl stoppers during freeze-drying. The glass transition temperatures ranged from −24°C to −30°C depending on the percent excipient (6%–0%). After freeze-drying, the vials were back-filled with nitrogen and then crimp sealed. The moisture levels were determined using Karl Fischer coulometric titration and found to be <2% for all freeze dried protein formulations. All samples resulted in amorphous-intact pharmaceutical white cakes.

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