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A rapid approach for characterization of thiol-conjugated antibody–drug conjugates and calculation of drug–antibody ratio by liquid chromatography mass spectrometry

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ABSTRACT

We present the demonstration of a rapid "middle-up" liquid chromatography mass spectrometry (LC–MS)-based workflow for use in the characterization of thiol-conjugated maleimidocaproyl-monomethyl auristatin F (mcMMAF) and valine-citrulline-monomethyl auristatin E (vcMMAE) antibody–drug conjugates. Deconvoluted spectra were generated following a combination of deglycosylation, IdeS (immunoglobulin-degrading enzyme from *Streptococcus pyogenes*) digestion, and reduction steps that provide a visual representation of the product for rapid lot-to-lot comparison—a means to quickly assess the integrity of the antibody structure and the applied conjugation chemistry by mass. The relative abundance of the detected ions also offer information regarding differences in drug conjugation levels between samples, and the average drug–antibody ratio can be calculated. The approach requires little material (<100 μg) and, thus, is amenable to small-scale process development testing or as an early component of a complete characterization project facilitating informed decision making regarding which aspects of a molecule might need to be examined in more detail by orthogonal methodologies.

Antibody–drug conjugates (ADCs)[1] are therapeutic agents produced through the chemical linkage of highly potent small molecule drugs to highly specific monoclonal antibodies. There are currently 45 ADCs in clinical testing [1] and more than 160 ADCs in preclinical testing, and many of these involve covalent linkage of cytotoxic agents to antibodies specific to tumor-related antigens. The high specificity of antibody binding offers a way to target delivery of the cytotoxin and selectively eliminate tumor cells while limiting exposure to healthy tissues [2–8]. The recent U.S. Food and Drug Administration approvals of brentuximab vedotin (Adcetris, Seattle Genetics) for Hodgkin's lymphoma and trastuzumab emtansine (Kadcyla, Genentech/Roche) for HER2-positive metastatic breast cancer are encouraging developments for this class of drug [9–12].

Although ADCs based on site-specific conjugation to engineered antibody structures are being designed [13–16], the small molecule cytotoxin is most typically conjugated randomly to the side chains of lysine or, following mild reduction of the antibody, to the cysteine residues associated with interchain disulfide bonds. Therefore, the conjugation process commonly results in a complex population of ADC variants within a given product, with different numbers of small molecule drugs conjugated and with different distributions.

Although reaction conditions can be controlled to produce consistent batches of drug [13,17] in terms of analytical testing and product characterization, the heterogeneity associated with the drug conjugation, in addition to the glycoheterogeneity inherent to the antibody structure, is a critical issue. For example, the greater complexity typically associated with lysine-conjugated species relative to cysteine-conjugated species leads to significantly different considerations. There are in fact numerous analytical challenges, and they can vary greatly with each ADC product. These can come from any, or all, of the three components of the ADC: the antibody, the linker, and/or the drug. There has been much emphasis placed on the selection of appropriate analytical

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[1] *Abbreviations used:* ADC, antibody–drug conjugate; LC–MS, liquid chromatography mass spectrometry; mcMMAF, maleimidocaproyl-monomethyl auristatin F; vcMMAE, valine-citrulline-monomethyl auristatin E; IgG, immunoglobulin G; QToF, quadrupole time-of-flight; DAR, drug-to-antibody ratio; Her mcF, maleimidocaproyl-monomethyl auristatin F-conjugated Herceptin; Her vcE, maleimidocaproyl valine-citrulline-monomethyl auristatin E-conjugated Herceptin; HIC, hydrophobic interaction chromatography; UV, ultraviolet; SEC, size exclusion chromatography; DTT, dithiothreitol; TCEP, tris(2-carboxyethyl)phosphine; IdeS, immunoglobulin-degrading enzyme from *Streptococcus pyogenes*; UHPLC, ultra-high-performance liquid chromatography.

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techniques to identify and monitor the physicochemical characteristics of ADCs [18].

In this study, we present the demonstration of a liquid chromatography mass spectrometry (LC–MS)-based workflow for use in the characterization of thiol-conjugated maleimidocaproyl-monomethyl auristatin F (mcMMAF) and valine-citrulline-monomethyl auristatin E (vcMMAE) antibody–drug conjugates.

The LC–MS method employs a short reversed-phase cartridge throughout. This serves primarily to concentrate and desalt the injected protein sample prior to reaching the electrospray ionization source of the mass spectrometer. We apply this routinely in our laboratory to an intact mass analysis workflow for characterization of antibody biopharmaceuticals. By application of a rapid mobile-phase gradient, the method offers little in terms of chromatographic separation, such that protein sample components invariably coelute from the column regardless of the antibody glycoheterogeneity or of the sample pretreatment procedures employed in the workflow. Thus, the method relies on separation of sample components by time of flight in the mass spectrometer—in the m/z dimension of the analysis.

In such experiments, the raw data are typically processed using a deconvolution algorithm to generate a spectrum on a zero-charge scale, mass (Da). In the case of the UNIFI software applied here, a maximum entropy algorithm [19,20], MaxEnt1, is applied and the peaks of the deconvoluted mass spectrum can be assigned as the glycan and other variants of the antibody. MaxEnt1 is reported to preserve the quantitative aspects of the raw data (http://www.waters.com/webassets/cms/library/docs/an212.pdf), such that given the structural similarity of the glycan variants, and hence an assumption that these species will ionize with equal efficiency in the source of the mass spectrometer, the resulting deconvoluted spectrum offers a visual and quantitative representation of the relative abundance of the antibody glycan profile [21]. This is the basis for an assessment of comparability in glycan profiles for biopharmaceutical biosimilarity and lot-to-lot comparison in process development. In fact, it represents the only means currently available to show the pairing of glycan variant heavy chains in the population of antibodies within a sample.

As a stand-alone experiment, the analysis of the intact or "native" antibody by this method is not without limitations. The complexity or heterogeneity of the sample, and also due to salt or other adduct ions as well as the width of the natural isotope pattern of species in the 150-kDa mass range, leads to some skewing of ion signals to higher or lower m/z. The mass resolution offered by modern mass spectrometers limits this to some extent, but the effect of peak skewing is to limit mass accuracy of the deconvoluted data for intact immunoglobulin G (IgG)—in some cases to approximately ±70 ppm in our experience with the quadrupole time-of-flight (QToF) mass spectrometer applied in this study.

The limited mass accuracy can make interpretation of the resulting deconvoluted spectrum challenging; consequently, in our laboratory the experiment is performed as a component of a workflow involving a combination of reduction [21–23] and "middle-up" enzymatic cleavage steps [24–31] that add depth to the data set. The resulting antibody subunits can be analyzed using the same rapid desalting LC–MS platform method, such that amino acid chains coelute from the short desalting cartridge. This workflow is well established in our laboratory for biosimilar comparability studies. Although mass accuracy is perhaps still compromised somewhat due to some overlap of ion signals associated with coeluting subunits, the method is rapid and requires very little in the way of modification between studies of different biopharmaceutical products and, thus, requires little expertise to run. It works optimally for molecules of less than 40 kDa, where default data deconvolution parameters can be invariably applied, typically offering mass accuracy within less than 20 ppm (<1 Da), permitting confident assignment of peaks to the anticipated components of a given protein sample and structural variants thereof. The workflow is a first port of call for characterization testing, particularly for new products, offering a great deal of information regarding structure that we can use to determine how to target characterization of specific structural quality attributes via orthogonal methods.

In this article, we demonstrate application of this same platform approach to intact mass analysis in the characterization of some model thiol-conjugated Herceptin-based ADCs, including the calculation of drug-to-antibody ratio (DAR) from the MaxEnt1 processed spectra. We present the results initially acquired during feasibility testing with Her mcF (maleimidocaproyl-monomethyl auristatin F-conjugated Herceptin) in which auristatin F is coupled to cysteine via a maleimidocaproyl linker. The method is also applied to samples of Her vcE (maleimidocaproyl valine-citrulline-monomethyl auristatin E-conjugated Herceptin), demonstrating the potential application of the workflow in a process development testing scenario, permitting rapid lot-to-lot product comparability and monitoring variation in DAR.

The key challenge in the analysis of thiol-conjugated ADCs is that their manufacture involves partial reduction of the antibody; interchain disulfide bonds are broken in order to conjugate drug to the resulting free cysteine residues [18]. This process adds significant heterogeneity to the antibody sample, with the resulting population of drug molecules composed not only of glycan variants but also of variants with different numbers of conjugated drugs and positional isomers [17,32]. The effect of reduction of interchain disulfide bonds is also a critical consideration for the analysis because the existence of the drug-conjugated product as an antibody relies on the complexation of covalent subunits (LC, HL, HH, and HHL) by noncovalent van der Waals, dipole–dipole interactions, and hydrogen bonding forces. It is for this reason that we have seen hydrophobic interaction chromatography (HIC) with ultraviolet (UV) detection methods used in lot-to-lot comparison of thiol-conjugated ADC products. At neutral pH, the drug-conjugated subunits of the partially reduced antibodies remain in complex and the DAR variants can be separated based on differences in hydrophobicity with increasing conjugation of drug. Integration of the observed peaks permits calculation of average DAR for the product [14,32–35].

In terms of mass spectrometric analysis, HIC does not pair well with ionization methods; hence, the trend has recently been moving toward nano-electrospray, or more recently size exclusion chromatography (SEC)–MS analyses under neutral pH conditions, in order to maintain antibody structure [36–38] (http://www.waters.com/webassets/cms/library/docs/2014asms_bridsall_adcs.pdf). Rather than chromatographic separation by time, the DAR variants are separated by the mass of the conjugated drug, and DAR can be calculated based on the relative ion abundance. We have successfully applied the SEC–MS approach to ADCs in our laboratory (example deconvoluted spectra are shown in Fig. 1) but with variable results. We have generally observed relatively low ion statistics with this approach and have found that the method typically requires large amounts of drug substance in order to generate a spectrum of consistent quality (>50 μg per injection vs. 0.2–2 μg required for monoclonal antibody [mAb] and ADC analyses performed under reversed-phase and mobile-phase conditions with formic acid). Interference from background noise can also reduce confidence in peak assignment, particularly for higher DAR species within the samples, which we find are often underrepresented in the spectra. Subsequent calculations can consequently result in an underestimation of DAR compared with UV absorbance determinations. The data interpretation for these experiments might also be described as subjective; there is a clear risk of optimizing deconvolution parameters such that results match preconceived expectations.