ADC Characterisation

After decades of R&D, monoclonal antibody-based therapeutics may be providing the answers to the questions that the biopharmaceutical industry has been asking

Christopher M Bianchetti PhD, Henry J Parker, Katherine C Hanson PhD, and Stephen Gacheru PhD at PPD Laboratories

The biopharmaceutical industry is experiencing unprecedented growth that is primarily driven by the development and implementation of monoclonal antibody (mAb)-based therapeutics. mAbs have been shown as promising therapeutics for the treatment of a variety of life-threatening diseases, particularly cancer (1). More than 40 therapeutic mAbs have been approved for treatment, and hundreds more are currently under development. One class of therapeutic mAbs that serve as potential chemotherapeutic agents is antibody-drug conjugates (ADCs). Several ADCs have received market approval, mainly for oncology treatment. The potential of ADCs is due to the ability of the antibody to target specific cells, which leads to lower toxicity of the drug and therefore increases drug dosage levels that are safer than chemotherapeutic agents. The development of ADCs is challenging. In this article, the analytical techniques currently used to determine the drug-to-antibody ratios (DARs), their limitations, and potential novel combinations of characterisation techniques are explored.

ADCs are composed of an mAb that is covalently linked to a cytotoxic drug via a small molecule linker. mAbs are structurally complex molecules that contain multiple drug attachment sites (approximately 80 lysine residues, 20 surface-exposed lysine residues, and 16 cystine resides per antibody) (2). Furthermore, the conjugation of the linker-derivatised drug is stochastic. Thus, the production of ADCs results in a heterogenous mixture of an ADC species that consists of varied DARs (the average amount of drug molecules that are linked to the antibody). Additionally, the distribution of attachment sites can vary and needs to be characterised. ADCs with drugs conjugated to lysine residues are more structurally diverse than ADCs with drugs attached at cystine residues due to the increased number of attachment sites. The DAR can impact the efficacy of the ADC and therapeutic index (3). An ADC with low DAR may be less potent and require higher concentrations to be effective. The inherent heterogeneity of ADCs requires a thorough characterisation of the total drug load, distribution of conjugation sites, and the amount of free antibody/linker/drug to determine the overall DAR. Furthermore, the altered surface properties of ADCs can prompt aggregation, and the conformational states of the ADC need to be evaluated.

UV-Visible Spectroscopy

Perhaps the simplest method to determine the DAR of an ADC is UV-visible spectroscopy. The only requirement is that the attached drug has an absorption maximum within the UV-visible range that does not overlap with the absorbance maximum of the conjugated mAb (280nm). This requirement potentially limits the use of UV-visible spectroscopy, but has been utilised to determine the DAR of several ADCs. For example, the DAR of MTX-791T/36, which is composed of 791T/36 (absorbance 280nm) and methotrexate (absorbance 307nm), was determined using UV-visible spectroscopy (4). The DAR is equal to the molar concentration of the drug divided by the molar concentration of the antibody. While UV-visible spectroscopy is simple to perform, the presence of a free drug or antibody can cause the overestimation or underestimation respectively of the DAR. Additionally, UV-visible spectroscopy does not provide any information on drug distribution, conjugation sites, or ADC structural heterogeneity.

Mass Spectrometry

Mass spectrometry (MS) is a powerful technique that can determine the DAR, distribution and location of the conjugated drug payload, amount of free antibody, and provide a level of detail that is not possible with UV-visible spectroscopy (5). MS can separate, identify, and quantitate the masses of the various conjugated ADC species, unconjugated antibodies, and antibodies that contain linkers but lack the drug payload. The MS profile of an ADC is more complex than that of an mAb due to the presence of the conjugated drug payload. To reduce structural complexity, which is particularly important when the drug payload is conjugated to exposed lysine residues, ADCs are often deglycosylated with enzymes such as PNGase-F before MS analysis (6). Additional improvements in the identification of conjugation sites can be obtained by enzymatically fragmenting the ADC. For example, Adcetris® was digested with the proteases IdeS and Lys-C, and the resulting fragments were analysed by liquid chromatography MS. The specific, conjugated cystine residues of Adcetris were identified using the combination of fragmentation and MS. However, the use of proteases and additional sample handling make the implementation of

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these methods difficult in a current Good Manufacturing Practice (GMP)-regulated laboratory. While MS is a useful technique to characterise ADCs, ADC formulation buffers are often not compatible with MS analysis and require additional sample preparation. Additionally, the ionisation efficiency of lysine-conjugated ADCs decreases with increasing drug load due to fewer ionisable lysines potentially affecting the accuracy of the DAR determination.

Altered Surface Properties

Conjugated cytotoxic drugs are primarily hydrophobic and can affect surface properties – particularly hydrophobicity and electrostatics – of the conjugated mAb (7). As drug load increases, the ADC isoelectric point (pI) becomes more acidic due to the decreased number of ionisable lysine residues. These electrostatic differences can be used to separate unconjugated (free mAb) and ADC species. For example, imaged capillary isoelectric focusing (iCIEF), an analytical technique that separates molecules based on pI, has been used to quantitate free mAb and ADC species (8). While iCIEF provides increased resolution compared to ion exchange chromatography and gel-based isoelectric focusing, iCIEF is unable to provide any information on drug load distribution. Additionally, iCIEF requires differences in surface charge to separate ADC species and is unable to separate cystine linked ADCs. Despite these limitations, iCIEF can be useful as a high-throughput fingerprinting method to determine batch-to-batch consistency and monitor changes in drug load over time.

The amount of drug conjugation also affects surface hydrophobicity of ADCs. This alteration can be leveraged to separate multiple ADC species, determine the DAR, and quantitate the amount of free antibody. Due to the hydrophobic nature of the linked drugs, an increase in the drug payload will increase the overall hydrophobicity of the ADC. Hydrophobic interaction chromatography (HIC) utilises a hydrophobic stationary phase that reversibly interacts with ADCs. Unconjugated antibodies are the least hydrophobic and elute first, enabling the quantitation of free antibodies. As the number of conjugations increases, so does the elution time, allowing for the separation of individual ADC species. Selection of mobile phase and gradient are critical to obtaining sufficient separation between ADC species. Separation of ADC species by HIC is dependent on the amount of heterogeneity. Cystine-linked and site-specific ADC species are typically well resolved and quantitated using HIC. The increased heterogeneity of lysine-linked ADCs can limit the resolution and inhibit the determination of the average DAR. However, HIC can be used to determine the percentage of unconjugated antibody of lysine-derivatised ADCs, even if the loaded ADC species are not fully resolved.

Characterising Aggregates

In addition to determining the DAR and distribution of conjugated drug, a thorough characterisation of the conformational states (aggregation and fragmentation) of ADCs is necessary. Due to the altered surface properties caused by the conjugated drug and linker, potential loss of surface charge in the case of lysine-conjugated ADCs, and increased hydrophobicity, ADCs are more prone to aggregation than the parental mAbs. Protein aggregates can vary in size (10-200µm), and the detection method will depend on the overall size of the aggregates (9). Size exclusion chromatography (SEC) is the most widely used method to detect and determine the size (molecular weight) of soluble ADC aggregates. The altered hydrophobicity can increase the interaction between the ADCs and SEC stationary phase resulting in reduced resolution, but the addition of organic solvent can instead reduce the interactions and improve resolution (10). Despite its ubiquity, SEC is limited to characterising aggregates between 100-1,000kDa, and small ADC fragments may not be resolved and may elute in the void volume. Advanced biophysical techniques such as analytical ultracentrifugation (AUC) and small angle X-ray scattering (SAXS) have been used to study the aggregate states of mAbs and ADCs (11-12). Studies using simulated mAb sedimentation velocity data suggests that AUC can be used to quantitate aggregates as low as 0.2% of the total protein. While AUC has the potential to quantitate small amounts of aggregates, the analysis is limited to protein concentrations lower than typically used to store ADCs (13). Because scattering intensity is proportional to protein concentration, SAXS is not limited by increased protein concentrations and can be used to characterise these in ranges from 2-500mg/mL (14). In addition to quantitating aggregation states, SAXS also provides information on polydispersity, a distribution of conformational states, and a low-resolution molecular envelope. Also, SAXS has been used to examine aggregation and conformation states of mAbs and ADCs as a function of pH, salt concentration, and formulation buffer additives, suggesting that SAXS could be used to screen formulation buffers for aggregation and helping select buffers that promote stability.

ADCs have the potential to be lifesaving therapeutics. However, the complex structure of ADCs makes a thorough characterisation of DAR, distribution of conjugation sites, the amount of free antibody/linker/drug and aggregate state a challenging problem. Determining the DAR is particularly important because of its effects on efficacy, dosage, and pharmacokinetics. While progress has been made in the characterisation of ADCs, no single analytic technique can fully describe these heterogeneous molecules. MS-based techniques can be used to determine the DAR and distribution

of conjugation sites at the amino acid level. However, MS lacks the ability to detect aggregate states. HIC is becoming an industry standard to characterise the DAR and amount of free antibody/linker/drug of cystine or site-specific conjugated ADCs. However, the increased number of conjugation sites in lysine-conjugated ADCs can result in reduced resolution and can limit the usefulness of HIC. For the foreseeable future, a combination of orthogonal techniques will need to be used to characterise ADCs. A combination of proven techniques (such as MS and HIC) and novel techniques (such as AUC and SAXS) is needed to fully characterise the drug load and conformational states of ADCs.

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About the authors

Christopher M Bianchetti PhD is a Research Scientist in the method development and validation group at PPD® Laboratories, where he is responsible for providing technical guidance on scientific method development and validation projects and troubleshooting instruments, methods, and procedures. Christopher has more than 12 years of experience in protein production, purification, and the biochemical and biophysical characterisation of macromolecules (proteins, nucleic acids, and polysaccharides). Previously, he led an independent research group that designed and implemented high-throughput biochemical methods to screen enzymes for novel carbohydrate degrading activity.

Henry J Parker is an Associate Scientist in the biopharma method development and validation group at PPD Laboratories, where he is responsible for designing, developing, and validating scientific methods for the analysis of biopharmaceutical products. Henry has more than three years' experience in the biochemical and biophysical characterisation of macromolecules using techniques such as high-performance liquid chromatography and capillary electrophoresis.

Katherine C Hanson PhD is the Lab Manager for the biopharma method development and validation group at PPD Laboratories. Katherine has more than five years of experience in development and validation of GMP-compliant analytical methods for a wide range of compounds, including enzymes, antibodies, and viral gene therapies. Previously, she was a Protein Engineer focused on determining mechanism of action for molecular motors.

Stephen Gacheru PhD serves as the Vice President of biopharmaceutical product development for PPD Laboratories, where he oversees analytical testing for large molecules. Stephen has more than 20 years' experience working in the biologics field. He has extensive global experience in protein characterisation, validations, and regulatory submissions. He also has expertise in outsourcing to CMOs and CROs, working with clients and developing successful customer supply relationships.