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Use of a charge reducing agent to enable intact mass analysis of cysteine-linked antibody-drug-conjugates by native mass spectrometry

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ABSTRACT

Antibody-drug-conjugates (ADC) are a growing class of anticancer biopharmaceuticals. Conjugation of cysteine linked ADCs, requires initial reduction of mAb inter-chain disulfide bonds, as the drugs are attached *via* thiol chemistry. This results in the active mAb moiety being transformed from a covalently linked tetramer to non-covalently linked complexes, which hinders precise determination of drug load with LC–MS. Here, we show how the addition of the charge reducing agent triethylammonium acetate (TEAA) preserves the intact mAb structure, is well suited to the study of cysteine linked conjugates and facilitates easy drug load determination by direct infusion native MS.

1. Introduction

Antibody-drug-conjugates (ADC) are dynamic and heterogeneous mixtures composed of a monoclonal antibody (mAb) linked *via* a chemical linker to a biologically active cytotoxic small-molecule drug. [1–3] The high binding specificity of mAb and targeted receptors make ADCs effective delivery systems for cytotoxic drugs to the tumor cells. *In vivo*, the ADC is recognized and binds to the receptor on the surface of the targeted cell. Subsequently, the ADC is internalized into the cancer cell and digested in the lysosome, where the cytotoxic drug is released and consequently kills the target cancer cells, thus reducing systemic toxicity to the noncancerous cells [4]. Currently, there are two approved ADC cancer therapies on the market (ADCETRIS for treatment of relapsed Hodgkin lymphoma and systemic anaplastic large cell lymphoma, and KADCYLA for treatment of HER2 positive metastatic breast cancer) with many more in pre-clinical and clinical development. [5–7]

At present, the dominant conjugation strategies involve reaction to either lysines or to cysteines. Covalent linking of the cytotoxic drug to the mAb can be achieved *via*: (1) reaction of lysines [8]—attachment to the epsilon amino group of lysine; (2) reaction of genetically engineered cysteines [9]—attachment to the side chain thiol of unpaired cysteine; or (3) reaction of cysteine residues generated by the reduction of existing interchain disulfide bonds (DSB) [10,11]. In the latest type of mAb conjugation, the interchain disulfide bridges are partially reduced prior to the conjugation reaction. The resulting mixture consists of ADCs with an even number of conjugated drug molecules ranging from zero to eight. In other words, each disulfide cleavage results in the conjugation of two conjugates. One of the most important attributes of an ADC is the average number of drug molecules bound to the mAb, as it determines the amount of drug delivered to the tumor cells and will influence its potency [12]. Additionally the drug distribution profile of the ADC is key for both safety and efficacy, and needs to be measured accurately and maintained during manufacturing and formulation including from batch to batch [13]. Commonly used methods for ADC drug load assessment and drug-to-antibody ratio (DAR) determination include UV spectroscopy, capillary electrophoresis (CE), HPLC chromatography methods such as hydrophobic interaction chromatography (HIC) and mass spectrometry (MS) [14–18]. According to a recent survey conducted by Bioanalysis Zone, 82% of those surveyed considered MS to be a rapid technique of choice for evaluation of the quality attributes for ADCs at various stages of the development [19].

The dynamic nature and heterogeneity of ADCs raises significant bioanalytical challenges. In the case of lysine- or engineered cysteine-conjugated ADCs, the interchain DSB between heavy and light chains of a mAb remain intact, and DAR can be determined using liquid chromatography–mass spectrometry (LC–MS)

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methods employing mobile phase containing organic solvents [17,20,21]. ADCs conjugated at the interchain cysteine residues produce a mixture of non-covalent mAb tetramers (2 light chains (LC) and 2 heavy chains (HC) with a variable number of drug molecules attached) after reduction of interchain DSBs, hence the application of more classic LC–MS analytical strategies would result in dissociation of this non-covalent ADC.

Native MS provides an alternative approach for intact protein analysis. With appropriate sample preparation, use of aqueous volatile buffers, and suitable tuning of the instrument, it is possible to transfer weakly associated complexes from solution into the gas-phase of a mass spectrometer and obtain insights into complex stoichiometry and protein structure. Up to date, there have been only a handful of reports in the literature employing native MS for intact mass and DAR analysis of these cysteine-linked ADCs [18,22–25]. In 2012, Valliere-Douglass et al. [22] presented the first method for the rapid determination of the mass of an intact ADC intact. In this work, ADCs were deglycosylated and then subjected to native-SEC desalting followed by online electrospray MS (ESI-MS) analysis. They reported some dissociation of the non-covalent ADCs into conjugated LCs and HCs and reported this having no effect on the subsequent relative drug load distribution evaluation. A year later, Chen et al. [18] reported a MS method employing enzymatic digestion, followed by nano-ESI and native MS to achieve direct determination of the intact mass and furthermore to calculate the average DAR of the cysteine-linked ADCs. The cytotoxic conjugates investigated, often possess high hydrophobicity which will result in lower proton affinity which may in turn affect the ionisation leading to an under-representation of high-drug load species. To minimize this ion suppression and equalise ionisation efficiency among species with different drug loads by reducing the ADC hydrophobicity, limited enzymatic digestion was performed to cleave the hydrophobic moiety from ADC, while the linker remained attached and was used as an indicative of the drug load. The DAR values obtained post-enzymatic digestion were more comparable with those determined by HIC methods, while DAR values obtained for samples without enzymatic digestion were slightly lower. In 2014, Debaene et. al. [24] developed a semi-quantitative method for determination of average DAR and DAR distribution in cysteine-linked ADCs based on high resolution MS and ion-mobility MS (IM-MS) data. The above mentioned [24] along with other [26] studies of ADCs employed high performance mass spectrometers to generate excellent results and mass spectra with superior resolution. More recently, Marcoux et al. reported on the use of imidazole as a reducing agent to resolve overlapping peak distribution of a lysine-conjugated ADC as an alternative for lower resolution instruments [27]. Native MS has also been utilized in analysis of changing drug load distribution *in vivo* from plasma samples [23].

Here as an alternative, we have explored the use of a charge reducing agent—triethylammonium acetate (TEAA). By shifting the *m/z* distribution to lower values of *z* (and higher *m/z*) we minimise overlapping ADC peaks and also preserve non-covalent interactions. This approach is potentially more feasible for smaller ADC developers on lower resolution ToF or Q-ToF instruments.

2. Results and discussion

We have investigated several cysteine-linked ADCs using a nano-ESI-Q-ToF mass spectrometer (Ultima API US, Waters Corporation). As the mass of the cytotoxic drug and the linker is relatively small (~1 kDa) in comparison to the intact mAb (~150 kDa), to aid the desolvation process and enhance the resolution for accurate peak assignment and mass determination, high acceleration voltages (applied to the source sampling cone) can be applied at the front stages of a mass spectrometer. Often, use of elevated acceleration voltages leads to dissociation of non-covalent complexes. Fig. 1, shows mass spectra of ~7 μM ADC in 50 mM ammonium acetate acquired at sampling cone voltage of 50 V (a), 100 V (b) and 200 V (c). Use of a lower cone voltage (50–100), allows preservation of the intact ADC as observed in the *m/z* 5900–8000 spectral region; however, under such conditions base line resolution is not achieved. Moreover, under these more gentle conditions, high levels of residual solvent and salt adducts make accurate mass determination challenging. Elevating the cone

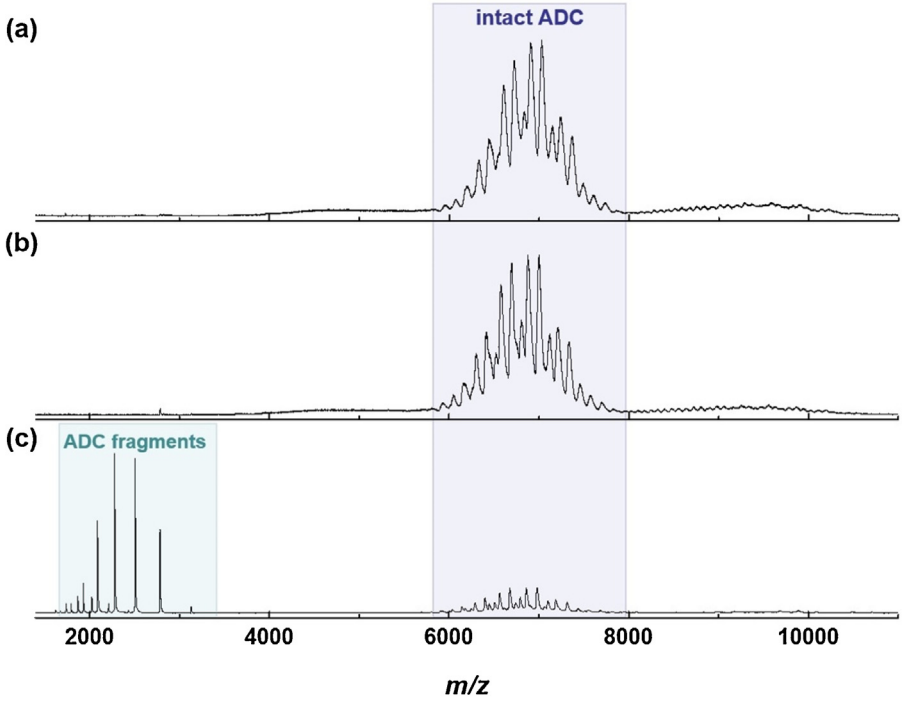

Fig. 1. Mass spectra of ~7 μM ADC acquired on the Q-ToF Ultima API US mass spectrometer at three different acceleration voltages: 50 V (a), 100 V (b) and 200 V (c). Increase of this parameter results in effective in-source salt clean-up during the desolvation process, at the same time leading to dissociation of the non-covalent ADC.