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Study of formulation effects on the charge variant profile of antibody-maytansine conjugates by icIEF method

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ABSTRACT

A whole-column imaging-detection capillary isoelectric focusing method (icIEF) was used to study the effects of formulation and thermal stability on the charge variant profile of maytansinoid antibody conjugates. The unconjugated monoclonal antibody showed a narrow pI range (8.9-9.0), while its conjugates to maytansine derivative had more acidic charge variants)pI values: 7.6 to 9.0(. Four formulation prototypes at acidic pH (5.5) were studied. The presence of acetate and citrate in the formulation of maytansine antibody conjugate led to more heterogeneous charge variant profiles and to a shift to acidic isoforms as a result of amidation of amine groups of N-terminus of the light and heavy chains of the antibody. After forced thermal stress conditions (one month at 40°C), slight modifications in the charge variant profiles of maytansinoid antibody conjugates were observed in the four formulation prototypes compared to the control sample (-80°C), indicating no protein degradation by deamidation.

Keywords: antibody, conjugate, charge variant, p*I*, icIEF

INTRODUCTION

Monoclonal antibodies (mAbs) are a very important therapeutic class for the treatment of various diseases[1]. Unconjugated antibodies were used as antitumor due to their specificity to targeted cancer cells and there less side effects.

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To improve their efficiency in the treatment of cancer, conjugating antibodies with chemotherapeutic drugs via linkers have been extensively investigated[2-4]. After selectively binding to targeted cancer cells, antibody-drug conjugates (ADCs) release the cytotoxic agents to these cells. The release of actives agents from ASCs is achieved by the cleavage of the ADC linkers due to pH changes, redox reaction or enzymatic activity.

The physicochemical characteristics of ADCs greatly affect their therapeutic performance. Therefore, it is very important to assess the homogeneity and stability of ADCs. Homogeneous conjugation with drugs is very important to generate effective antitumor ADCs[5-6]. mAbs are generally conjugated with cytotoxic molecules through cleavable or non-cleavable linkers through amino acid residues, primarily lysine and cysteine residues[6]. An antibody usually may contain up to 80 lysine residues[7]. Lysine-based ADCs are heterogeneous with a wide drug-to-antibody ratio (DAR) distribution. DAR is the average number of drugs conjugated to the antibody[8].

During ADC development, formulation should be studied to find the best conditions to minimize heterogeneity and increase the stability of the formulated products. mAb and ADC formulations are composed of several excipients to maintain pH and to increase the stability of protein. Sugars (e.g., sucrose), surfactants (e.g., polysorbate 20 [PS20] and PS80), and amino acids (e.g., histidine, arginine, and glycine) are usually used as excipients for mAb and ADC formulations[9-12]. Formulation excipients may lead to chemical modifications of mAbs and their drug conjugates. Therefore, assessing the behaviour of the ADCs in formulation is essential to ensure their safety and efficacy.

Instability is a serious problem in all stages of therapeutic ADC development, from discovery to production and utilization[13-15]. Stability is affected by ADC formulation. Therefore, it is very important to study the impact of the formulation composition on ADC stability. ADC stability studies are usually performed under stress conditions (40°C) to accelerate the aggregation and chemical modifications of ADCs, and therefore to choose the best formulation[16,17].

Chemical modifications, resulting of the formulation excipients or during the storage, may lead to change the charge variant profiles of ADCs (i.e., a decrease or an increase of p*I* values)[18-24]. The characterization of the charge variant profile of ADCs is an important tool to assess their quality[25-27]. Monitoring the charge variants of mAb or ADC provides information on protein stability, product purity from batch to batch, the pathways of degradation, etc[28-30]. Different analytical methods have been used to assess the charge heterogeneity of ADC, such as chromatographic methods[31-32], electrophoretic methods[33-36].

Whole-column imaging-detection capillary isoelectric focusing methods (icIEF) permit the separation of proteins based on their isoelectric point (p*I*), offering simultaneous detection along the entire length of the column, higher resolution, speed, and quantitative analysis[37-38]. In a previous study[39], an icIEF method was developped for the analytical characterization of the charge heterogeneity of a novel humanized anti-EphA2 antibody conjugated to a maytansine derivative. In this work, the impact of the purification method, formulation excipients and thermal stability on the charge variant profile of the novel humanized anti-EphA2 antibody conjugated to a cytotoxic maytansine derivative were studied using the previously published developed icIEF method[39].

METHODOLOGY

Materials

Formulation excipients, inclding sucrose and mannitol (used to prevent mAb and ADC aggregation), polysorbate 80 (a surfactans for increasing the solubility of mAbs and ADCs) and buffering agents for controlling pH and stabilizing mAbs and ADCs (e.g., glycine, histidine, citric acid and acetic acid) were purchased from Sigma. Urea, which was used to increase protein sample solubility and stability was from Sigma. Kit ICE280 chemical test, Kit iCE280 electrolytic solution, methyl cellulose 1% and p*I* Markers (6.61 and 9.5) were obtained from Convergent Bioscience. Pharmalyte solutions (3-10 and 8-10.5) were obtained from GE Healthcare. A monoclonal naked antibody and its maytansinoid conjugates were analyzed. The composition of their formulation was mentioned in the result and discussion section.

Sample preparation

The protein sample solution was composed of 0.35% methyl cellulose, 4% pharmalytes (3–10) and pharmalytes (8–10.5) (1:1 ratio), 2M urea and p*I* markers (6.61 and 9.5). After centrifugation, the sample was transferred to a glass autosampler vial and centrifuged to remove bubbles before being placed in the autosampler carousel for analysis.

icIEF apparatus

An iCE280 instrument with PrinCE autosampler and capillary cartridge from Convergent Bioscience were used. The separation capillary column was transparent and had a fluorocarbon-coated inner surface (50mm, 100µm ID, 200µm OD). The column was installed in into a glass cartridge. The cathodic solution contained 100mM NaOH and 0.1% methyl cellulose, while the anodic solution contained 80mM H_3PO_4 and 0.1% methyl cellulose. Protein focusing time was set at 10 or 12min at 3000V. Detection at 280nm was achieved with a CCD camera.

RESULTS and DISCUSSION

The EphA2 receptor is one of 16 related receptor tyrosine kinases (RTKs) that are activated by membrane-associated ligands known as ephrins. EphA2 protein levels have been reported to be elevated in many types of cancer[40]. The studied mAb is anti-EphA2 of molecular weight of 145478 g/mol. Figure 1 presented the icIEF profile of the unconjugated anti-EphA mAb, which was characterized by two major peaks corresponding to p*I* values of 8.9 and 9.0.

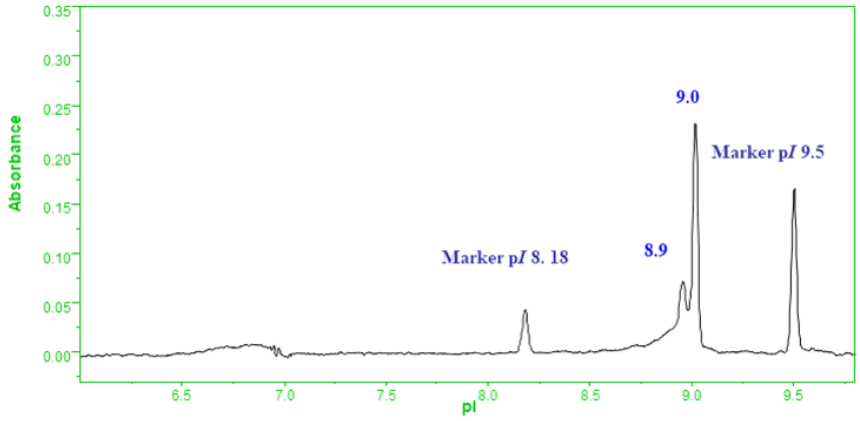

Figure 1. icIEF profile of unconjugated antibody. Final concentration of unconjugated antibody in sample matrix is 0.2mg/ml diluted in 0.35% methyl cellulose,4% 3–10 pharmalytes/ 8–10.5 pharmalytes (1:1 ratio), 2M urea. pI markers: 8. 18, 9.50. Focusing time: 10min at 3000V.

Small-molecule drugs are covalently attached to antibodies through chemical linkers to improve their antitumor efficiency. The side chain of lysine residues is commonly used for conjugation. As mentioned before, mAbs often contain up to 80 lysine residues and chemical conjugation results in a heterogeneous mixture of unconjugated mAbs and conjugated mAbs with a variable number of cytotoxins bonded to different sites on the antibody.

Anti-EphA2 mAbs were conjugated to a maytansinoid derivative through non-cleavable linker with the free amine groups of lysine residues. The antitumor action of resulted ADC is based on the release of the maytansinoid derivative linker, which kills cancer cells by interfering with their division upon antibody/antigen binding.

The conjugation processes take place by linking the cytotoxic-linker with the free amine group of lysine of the anti-EphA2 mAb in a single step, resulting in maytansinoid drug conjugates with 1 to 10 maytansinoid molecules of about 160 kDa. The ratio of maytansinoid molecules to mAb is around 6.2 moles of drug per mole of mAb.

This work aimed to study the effects of purification methods, different formulation excipients and thermal stress stability on the icIEF charge variant profile of maytansinoid antibody conjugates.

Charge heterogeneity profile of maytansinoid antibody conjugates

After conjugation, it is also necessary to purify the crude product to limit the amount of free drug in the sample, and other impurities.

Two strategies were used to purify the resulting ADCs after conjugation, of the anti-EphA2 mAb using the tangential flow filtration method (TFF). TFF was performed immediately after conjugation (immediate TFF) or after an overnight holding time after conjugation to allow hydrolysis and removal of weakly bound linkers (improved process; 24 H). The two batches of maytansinoid antibody conjugate were formulated in HGS buffer consisted of histidine 10mM, glycine 130mM and sucrose 5% (w/v). The icIEF profiles of maytansinoid antibody conjugates are presented in Figure 2.

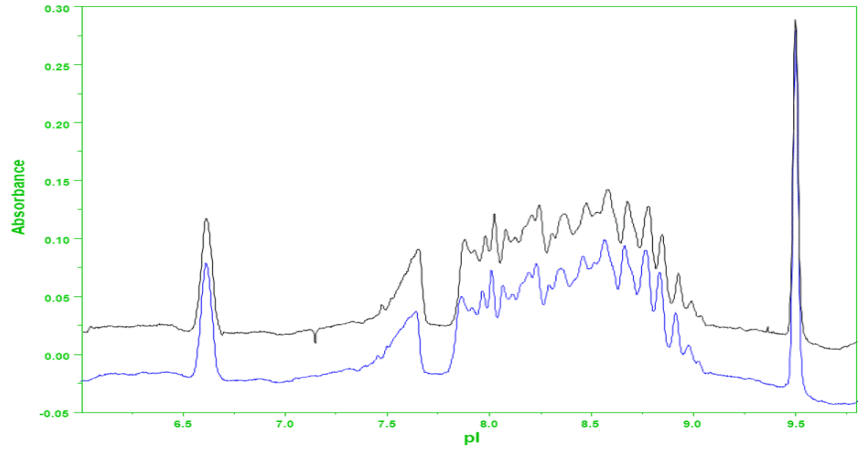

Figure 2. icIEF profile of two batches of maytansinoid antibody conjugates: immediate TFF (black), improved process: 24h (blue). Experimental conditions: Final concentration 1mg/mL in 0.35% methyl cellulose, 4% 3–10 pharmalytes/8–10.5 pharmalytes in 1:1 ratio and 2M urea. pI markers: 6.61, 9.50. Focusing time was 12min at 3000V.

Maytansinoid antibody conjugates were more heterogeneous and acidic than unconjugated mAb (p*I* values of 7.6 to 9.0). This heterogeneity of conjugated mAb is related to the covalently linking of the cytotoxic drug to the free amine groups of lysine of mAbs.These charge variants differed by the number of amine groups of lysine conjugated to the linker molecule, leading to a decrease in their p*I* with an increase in the number of modified amino groups (more acidic). There was no significant difference in charge variant profiles between the two batches: immediate TFF and improved process 24h (Figure 3).

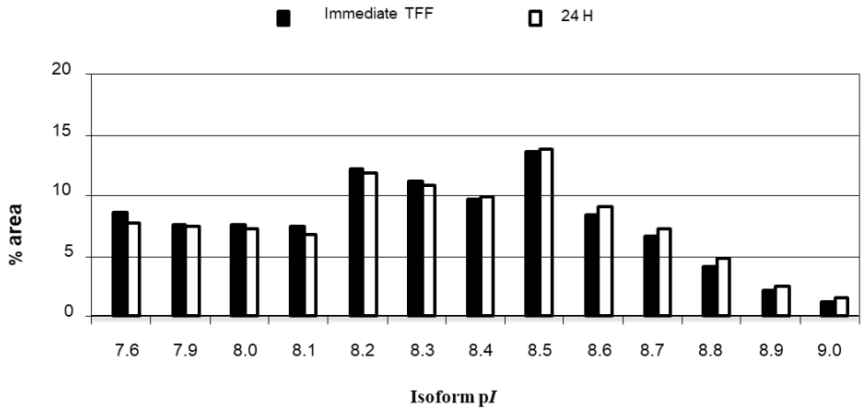

Figure 3. Comparison of % specie area by icIEF of two batches of maytansinoid antibody conjugate: immediate TFF, improved process: 24h. Experimental conditions as mentioned in Figure 1.

icIEF profiles of formulation prototypes of maytansinoid antibody conjugates: (immediate TFF)

To bring ADC to the market, ADC formulation should be developed to ensure the quality, efficacy, and safety of the product. The excipients of the formulation may generate chemical modifications to ADCs, leading to a change in the charge variant profile of ADCs. For instance, deamidation and amidation of amine lysine groups lead to an increase in acidic variants (decreasing p*I* values) while oxidation or succinimide formation lead to an increase in basic variants (increasing p*I* value)[41]. Modifications of p*I* (one unit or more) may alter the pharmacokinetics of ADCs and therefore their biological effects[42].

Maytansinoid antibody conjugates were formulated in four prototypes detailed in Table 1, including formulation buffer composition and ADC concentration. The pH of the four formulation prototypes was maintained at acidic value of 5.5.

Table 1. Formulation buffer composition, concentration and DAR of immediate TFF ADC

Sample	Formulation buffer	Concentration
Control	His 10mM, Gly 130mM, Sucrose 5% (w/v) pH 5.5	2 mg/mL
Prototype-1	His 10mM, Gly 130mM, Sucrose 5% (w/v), PS80 0.01% pH 5.5	2 mg/mL
Prototype-2	Acetate 10mM, Sucrose 5%, mannitol 2.5%, PS80 0.01% pH 5.5	2 mg/mL
Prototype-3	Citrate 1 mM, Sucrose 5%, mannitol 2.5%, PS80 0.01% pH 5.5	2 mg/mL

The prototype-1 formulation only differed from the control formulation by the addition of polysorbate80. Prototype-2 and -3 formulations were composed of acetate and citrate in addition to sucrose, mannitol and PS80.

The icIEF profiles of the four formulation prototypes of maytansinoid antibody conjugate were presented in the Figure 4. Maytansinoid antibody conjugate in control formulation (HGS buffer) and prototype-1 (HGS + PS80) had similar charge profiles but different from those obtained for the prototypes-2 (acetate) and the prototype-3 (citrate).

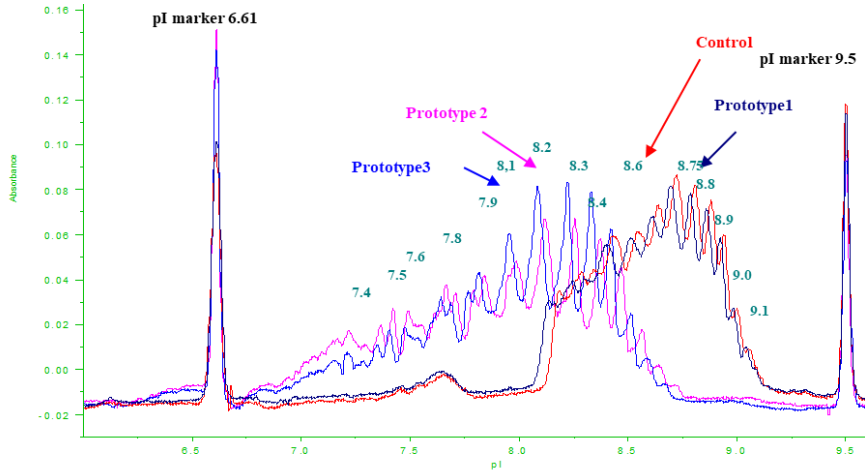

Figure 4. icIEF profiles of formulation prototypes of maytansinoid antibody conjugates. Experimental conditions: Final concentration 0.7 mg/mL in 0.35% methyl cellulose, 2% 3–10 pharmalyte, 2% 8–10.5 pharmalyte (1:1 ratio) and 2M urea. p*I* markers: 6.61, 9.50. Focusing time was 12min at 3000V.

The comparison of p*I* ranges of these prototypes, presented in the Table 2, demonstrated a shift to acidic isoforms in prototype-2 and prototype-3. Furthermore, maytansinoid antibody conjugate displayed a more heterogeneous charge profile than control and prototype-1. Different studied have reported covalent modification of a recombinant monoclonal antibody by citric acid in a citrate buffered formulation leading to the formation of acidic species as a result of the amidation of the N-terminus of the light and the heavy chain of the antibody[16, 23]. As mentioned before, the charge heterogeneity profile of maytansinoid antibody conjugate in acetate buffer was similar to that obtained in the citrate buffer. A chemical modification (formation of amide and imide) in the acetate buffer may be suggested to explain the formation of acidic species in a similar manner to the citrate buffer.

Table 2. p*I* range and difference of four prototypes of maytansinoid antibody conjugates

	Control	Prototype-1	Prototype-2	Prototype-3
pI range	8.1-9.1	8.1-9.1	7.4-8.6	7.4—8.6
Δ pI	1	1	1.2	1.2

Stability study of maytansinoid antibody conjugates formulation prototypes

Stability studies at higher temperatures allow for prediction of stability at the intended storage temperature. To determine which formulation impacts the thermal stability of maytansinoid antibody conjugate, it was incubated one month at 40°C in the four formulation prototypes. Stability studies are performed at 40°C in order to speed up changes in the quality characteristics of the ADCs. The exposure of ADCs to 40°C may accelerate aggregation and chemical modifications, such as deamidation and oxidation of ADCs[43].

The icIEF profiles of stressed samples of maytansinoid antibody conjugate prototypes showed that the total peak area of the 40°C stressed samples were lower than that of the control sample (-80°C). These decreases in total area were 18%, 12%, 20%, and 21% for the control maytansinoid antibody conjugate, prortotype-1, prortotype-2, and prortotype-3 respectively. Maytansinoid antibody conjugate in formulation prortotype-1 showed less protein precipitation at 40°C compared to other formulations. The percentages of charge species obtained for the 40°C stressed samples were roughly similar to the control sample (-80°C) for all formulation prototypes (Figure 5). The slight modification of charge profiles of maytansinoid antibody conjugate under stressed conditions in the four formulation prototypes indicated no protein degradation by deamidation.

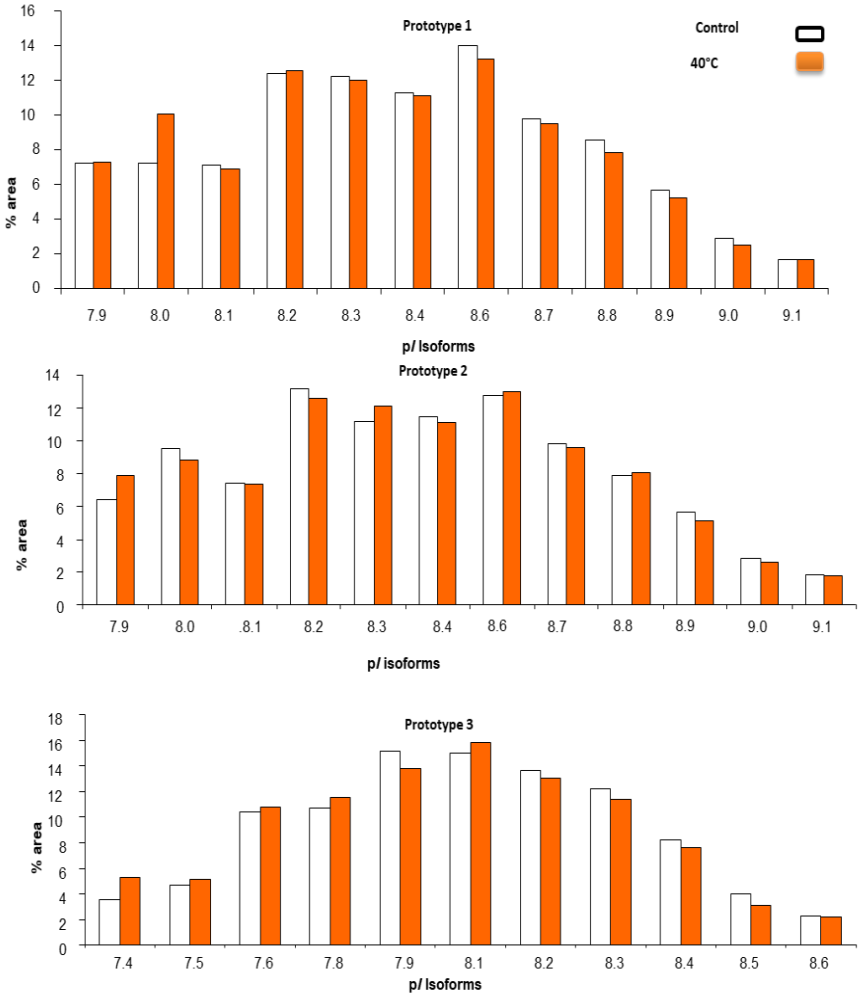

Figure 5. Percent area of pI isoforms 40°C stressed samples (red) of four formulation prototypes of maytansinoid antibody conjugates compared with a control (-80°C) (white)

The icIEF profile of anti-EphA2 monoclonal antibodies demonstrated two major peaks with p*I* values of 8.9 and 9.0. Maytansinoid conjugates of this antibody were more heterogeneous and acidic than unconjugated mAb (p*I* values between 7.6 and 9.0). No significant difference in the charge variant profiles was observed between the two batches: immediate purification by angential flow filtration or after holding one night before purification. The citrate and acetate-buffered formulations had similar charge variant profiles while HGS formulation without or with polysorbate80 had the same charge variant profiles. The presence of citrate and acetate led to the formation of acidic spe-

cies as compared to HGS formulation. Aggregation of maytansinoid antibody conjugate and slight modification of charge variant profile in the studied four formulation prototypes were observed after thermal forced conditions.

STATEMENT OF ETHICS

Ethical approval was not required to perform this study.

CONFLICT OF INTEREST STATEMENT

The author declares that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

Ayat Abbood carried out the analysis and wrote the article.

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Not Applicable.

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