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Therapeutic monoclonal antibodies and consistent ends: terminal heterogeneity, detection, and impact on quality

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Monoclonal antibodies (mAbs) are biological macromolecules with complex post-translational modifications that can be observed when assessing product variants. The N- and C-terminal heterogeneities of commercially produced antibodies have been observed and extensively studied over the past 30 years. This review summarizes the current literature on detectable antibody termini variants from cultured cells. The presence of these heterogeneities can be detected by many different analytical methods, mostly based on sequence, charge and size differences. Examples are presented that highlight terminal heterogeneities, methods of detection, and their impact on the quality of mAbs. Regulatory considerations are also discussed regarding the potential impact on product quality, safety, and efficacy.

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Introduction

Antibody-based therapy has more than 20 years of history since the marketing approval of Orthoclone® OKT3 (muromonab CD3) (Janssen-Cilag) in 1986. mAbs bind to specific targets, have slow clearance rates and have reduced side effect profiles than many small molecule drugs. More than 40 mAbs and mAb fragments have been approved by the Food and Drug Administration (FDA) over the past 25 years for treating and diagnosing different diseases especially cancer, autoimmune and other inflammatory diseases.

Modern mAbs are complex glycoproteins that are usually produced using mammalian cells, resulting in complicated and somewhat heterogeneous post translational modifications [1]. These post-translational modifications are the result of a combination of cellular processing, chemical modification during purification, drug product filling and/or storage. They include enzymatic and nonenzymatic processes which lead to post translational modifications such as deamidation, C-terminal lysine variants, N-terminal pyroglutamate formation, glycation oxidation, aspartate isomerization, hydrolysis and aggregation [2–4]. In this review, we provide a regulatory perspective on the biology and chemistry of heterogeneity at terminals of the mAbs, that is, N- and C-terminal isoforms, how heterogeneity can be measured, and how heterogeneity can impact the product quality, safety and efficacy.

N-terminal heterogeneity of mAbs

Formation of N-terminal heterogeneity

N-terminal isoforms can be formed by several mechanisms, and those that are commonly described in the literature include cyclization of glutamine (Gln) or glutamic acid (Glu)/glutamate, dehydration (maleuric acid addition) and signal peptide sequence variations will be reviewed below by the culpable mechanism.

The three major N-terminal modifications of mAbs are acetylation, formylation and pyroglutamylation [5]. Pyroglutamate formation (pyroGlu) is of special interest because both glutamine (Gln) and glutamic acid (Glu) are common N-terminal amino acids of mAb heavy chains (HC) and light chains (LC), and thus likely targets for modification [5–7]. Nonenzymatic conversion of N-terminal Gln to pyroglutamic acid (pyroGlu) has been observed in mAbs for many years and can be measured analytically in antibodies by shifts toward lower molecular weights or lower isoelectric points [6,8]. Chemically, cyclization of Gln to pyroGlu results in a loss of the N-terminal primary amine group, and therefore, these antibodies become slightly more acidic. This chemical reaction is the dominant form of the antibody produced by some cellular processes, as an example, Cheng KC *et al.* reported that 90% of the N-terminal Gln in their mAb HC was cyclized to pyroGlu by day 15 of bioreactor culture, the remainder was cyclized non-biologically during purification, formulation and sample handling [9]. As further demonstration of the potential of pyroGlu formation in cell culture, Du *et al.* reported that the pyroGlu form can elute from the column as the main peak in charge-based IEF (isoelectric focusing) analysis of some bioreactor-produced antibodies [10•].

On the other hand, modification of N-terminal Glu can also lead to pyroGlu, but this seems to be a chemical

reaction occurring after cell culture. For example in a case study, an antibody underwent non-enzymatic cyclization of Glu only after cell culture and purification [5]. In this case, all conversion of Glu to pyroGlu occurred on shelf storage and grew with time [5]. The study also showed that the formulation factors such as temperature, time and pH impacted the rate of non-enzymatic formation of N-pyroGlu from Glu [5,6,11]. Cyclization also occurs *in vivo*, arguing that it is not necessarily an artifact of bioprocessing. In one PK case study, relative levels of pyroGlu in circulating IgG2 mAbs were monitored in serum over time [12]. Non-enzymatic cyclization of Glu to pyroGlu occurred spontaneously at relatively high levels *in vivo*. The rate of the conversion was impacted by the structure of the local amino acid environment in the antibody [12], and the same rate of conversion could be replicated *in vitro* by incubating the same antibodies in PBS under physiological pH and temperature. These results suggest that the conversion *in vivo* in this case may be nonenzymatic. However, conversion of both Glu and Gln into pyroGlu can also be catalyzed by an enzyme, glutaminyl cyclase [13,14].

Formation of N-terminal pyroGlu can also be induced during analytical procedures. For example, Dick *et al.* reported that during lengthy (up to 3 hours at 37 °C) peptide mapping procedures, which include denaturation, reduction, alkylation, and trypsin digestion steps, accumulation of up to 10% pyroglutamate at the N-termini can occur [9].

Other N-terminal glu modifications (dehydration) have been noted in mAb products, although it is not clear that they would impact product function [15]. These have been seen in non-bioreactor produced mAbs, one is specifically from transgenic goats that is secreted through mammary glands. The N-terminus of LC was modified, and the addition of maleuric acid was detected after secretion of milk, but the exact point where the maleuric acid was added to the N-terminus of LC was not found [16].

Occasionally N-terminal modifications due to incompletely processed signal peptides were detected. These alternative cleavage variants could possess N-terminal sequence with different length of signal peptide present [17]. Variants in signal peptide sequence or length could either alter the charge of the mAb if the leader sequence included charged species like Lys, Arg, Asp or Gln or alter the hydrophobicity of mAb if longer signal peptide is present. For example, Khawli LA *et al.* reported that antibody variants that retained residual sequences from the signal peptide (Val-His-Ser) resolved as an acidic peak in IEX (ion exchange)-HPLC [18].

The observed N-terminal heterogeneity is summarized in Table 1.

Table 1

N-terminal heterogeneity

N-terminal heterogeneity	Common detection methods	References
Cyclization of Glu/glutamate to pyroGlu	MS, peptide sequence and RP-HPLC	[5,6,11–14]
Cyclization of Gln to pyroGlu	CEX, IEF, MS, LC–MS and RP-HPLC	[6,8,9]
Glu dehydration	CEX and MS (Q-TOF)	[15,16]
Leader sequence variation	CE-SDS, CEX-HPLC, peptide mapping and LC–MS	[17,18]
Acetylation, formylation	MS	[5]

Detection of N-terminal heterogeneity by analytical methods

The above isoforms could impact antibody function, so analytical methods for mAb analysis or, at a minimum, characterization should be developed and qualified for detection of N-terminal isoforms. Assays developed to date rely on charge and size shifts associated with the isoforms; for example formation of N-terminal pyroGlu from Gln shifts the charge of the mAb toward acidic by one charge group [7,10•]. The common analytical tools used for charge measurement include ion exchange chromatography (IEX) and isoelectric focusing (IEF), but they need to possess sufficient resolution to detect the loss of one charge group in a large mAb molecule which is relatively small [19–22]. In addition, the complexity of other post translation modifications such as deamidation of Asn would complicate the interpretation of the loss of one charge group. Improved techniques, such as capillary (cIEF) and icIEF (imaging capillary IEF) are based on the IEF principles but provide faster, more sensitive and precise performance [23]. cIEF coupled with analytical techniques such as mass analysis and N-terminal sequencing go even further by separating and identifying exact N-terminal variants including different cyclization levels and N-terminal signal peptide extension isoforms [24].

Many human IgGs have slightly basic isoelectric points, therefore, cation-exchange (CEX) HPLC in neutral pH buffers is typically used for resolving charge variants. The charge isoforms associated with N-terminal pyroGlu can be separated and measured by semipreparative weak cation-exchange (WCX) or strong cation exchange (SCX) chromatography [25•,26]. The weakness to chromatographic methods is that the detection method is an ultraviolet or light spectrophotometer, and this does not detect sequence or activity. Thus, each peak, mostly represented by charge isoforms, from WCX and SCX columns can be further analyzed for sequence and mass by downstream analytics such as mass spectrometry; this