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C-termini Analysis of Monoclonal Antibody Fragmentation

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Editorial

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Editorial

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Abbreviations: mAbs: Monoclonal Antibodies; CQA: Critical Quality Attribute; CDRs: Complementary Determining Regions; FC: Fragment Crystallizable; FAB: Fragment Antigen-Binding; SEC: Size-Exclusion Chromatography; SDS-PAGE: Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis; CE: Capillary Electrophoresis; IEX: Ion Exchange Chromatography; RPC: Reversed Phase Chromatography; MS: Mass Spectrometry; CNBr: Cyanogen Bromide; ProC-TEL: Protein C-Termini by Enzymatic Labeling; CP Y: Carboxypeptidase Y; SCX: Strong Cation Exchange; COFRADIC: Combined Fractional Diagonal Chromatography; NHS: *N*-Hydroxysuccinimide; C-TAILS: C-Terminal Amino-Based Isotope Labeling of Substrates; TMT: Tandem Mass Tag; iTRAQ: Isobaric Tag for Elative and Absolute Quantitation

Fragmentation of Therapeutic Monoclonal Antibodies

Fragmentation of therapeutic monoclonal antibodies (mAbs) is a critical quality attribute (CQA) that needs to be monitored and controlled during the biopharmaceutical drug development. Fragmentation of a protein is a result of either chemical or enzymatic reactions, leading to the cleavage of protein backbones. Fragmentation can occur through the entire mAb life-span, from protein production in the bioreactor, purification process, storage, to blood circulation and clearance. Depending on the cleavage sites, fragmentation could affect the function of mAbs. The fragmentation occurred in the complementary determining regions (CDRs) can potentially impact on the binding of a mAb to its antigen, resulting in loss of efficacy; The fragmentation occurred in the Fragment crystallizable (Fc) region may influence on the binding to the Fc receptors and on the circulation half-time of the mAb; The fragmentation occurred in the hinge region can generate half-antibody, fragment antigen-binding (Fab) fragment, F(ab)'2 fragment, Fc fragment, Fc/2 fragment, or Fab-Fc fragment. Loss of one Fab arm may lead to reduced potency if the drug mechanism of action requires both Fab arms, such as bispecific mAbs. Loss of Fc region will be devoid of Fc receptor binding, reducing circulation half-time. In addition, fragmentation could correlate with the aggregation of mAbs [1]. The fragmentation pattern of a mAb not only reflects the intrinsic stability of a mAb rooted from protein engineering but also represents manufacture and process consistency, which is critical to ensure lot-to-lot product quality.

There are many factors influence the fragmentation rates. First, certain side chains of amino acid residues can facilitate peptide bond cleavage [1]. The backbone fragmentation in mAbs occurs more frequently at the following amino acid residues: Asp, Ser, Thr, Cys, Gly, or Asn residues [2-6]. Protein higher order structure also has an effect on the peptide bond fragmentation rates. The solvent-exposed and flexible loops are more likely to be cleaved compared to the protected and rigid regions. In addition, fragmentation rates are affected by pH, temperature, as well as metals and radicals in the solutions [1].

Identification of Mab Fragment C-Termini

mAb fragmentations are usually monitored and quantified by size based analytical methods. The truncations in hinge region are readily detectable using native size-exclusion chromatography (SEC). Truncations occurred within the folded Fab or Fc domains are usually undetectable under the native conditions due to the disulfide linkages and the non-covalent interactions. In this case, denaturation and reduction followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), or capillary electrophoresis coupled with SDS-gel (CE-SDS) can be used. Other separation methods based on the chemistry of amino acid side chains, such as Ion Exchange Chromatography (IEX) or Reversed Phase Chromatography (RPC), can also be used. However, identification of specific truncation sites can only be accomplished using mass spectrometry (MS) or N-terminal sequencing.

MS has become the primary method to identify protein truncation sites. mAb fragmentations generate neo-N and neo-C termini. The analytical methods for neo-N termini identification has well-established and reviewed [7]. In contrast, only a few C-terminal analytical techniques are currently available due to the lack of an efficient approach for C-terminus identification. Here, we will review the methods for protein C-termini analysis.

Protein C-termini analysis can be generally divided into three categories: enzymatic approaches, labeling approaches, and enrichment approaches. A simple enzymatic approach that can determine both the N- and the C-termini is comparison of Lys-C and Lys-N digested peptides determined by tandem mass spectrometry (MS/MS) [8]. The internal peptides have the same masses in both digests. In contrast, the C-terminal peptides contain an extra Lys residue in the Lys-N digest while the N-terminal peptides contains an extra Lys residue in the Lys-C digest, enabling the identification of N-terminal and C-Terminal peptides simultaneously. Another enzymatic approach is cyanogen bromide (CNBr) digestion followed by partial opening of the homoserine lactone derivatives to the corresponding homoserine derivative under a slightly basic buffer [9]. As a result, internal peptides represent as doublet peaks in MS1 spectra while C-terminal peptides appear as singlet peaks. It is worthwhile to note that CNBr is toxic and should be handled under a fume hood. The simplest labeling approach for C-terminal analysis is protease assisted ^{18}O-labeling [10, 11]. In this approach, the proteins were digested in $H_2{}^{18}O$ containing buffer, resulting in incorporation of one or two ^{18}O into the carboxyl groups of the internal peptides, and therefore 2 or 4 Da mass shifts. In contrast, the C-terminal peptides remained unmodified.

The enzymatic approaches and ^{18}O labeling can avoid the protein precipitation or hindrance of protease digestion in below mentioned chemical labeling approaches. In addition, the ^{18}O labeling provides flexible choice of proteases. Alternatively, C-termini can be directly labeled by chemical derivatization. The chemical derivatization of the C-terminus carboxyl group has intrinsic difficulty due to the similar chemical properties of carboxyl groups on the side-chains of glutamates and aspartates. By far, the oxazolone chemistry is the only reported way to selectively derivatize the C-termini without affecting side-chains of glutamates and aspartates [12], which can be further tagged with stable isotopically labeled arginine [13] or imidazoline hydrazine [14], providing positive charges to the C-termini to enhance the ionization efficiency and the confidence for MS detection. Although the chemical derivation facilitates MS/MS analysis for C-termini identification, this approach may suffer from low reaction yield and protein precipitation under acid conditions.

Because mAb fragments only represent a small fraction of mAb drug products (usually less than 1%), the identification of C-terminal peptides by MS could be challenging due to the ion suppression from the abundant internal peptides. Thus, enrichment techniques have been developed to enhance the detection of C-termini peptides. The enrichment approaches can be classified into positive and negative selections [15] Positive selection enriches C-terminal peptides, while negative selection depletes non-C-terminal peptides. In a positive selection approach called Profiling Protein C-Termini by Enzymatic Labeling (ProC-TEL) [16], the protein carboxyl groups were first modified by methyl esterification. The C-termini were then biotinylated by carboxypeptidase Y (CP Y) through addition of biocytinamide, while methyl esters at the side chains of Glu and Asp were hydrolyzed at high pH at the same time. Following proteolytic digestion, C-terminal peptides were captured by avidin or streptavidin affinity purification for MS analysis, in which the biotin tag can serve as a label to validate the MS/MS fragments of the C-terminal peptides. It is worthwhile note that carboxypeptidase Y transpeptidation has higher yield on Met and amino acid residues with hydrophobic side chains [16].

Many negative selection approaches have been attempted. The first reported negative selection approach used anhydrotrypsin, a catalytically inactive derivative of

trypsin, to capture trypsin digested N-terminal and internal peptides with Arg/Lys ends [17]. Similarly, immobilized phenylboronic acid was used to capture Arg-C digested N-terminal and internal peptides with Arg ends. In this approach, the guanidinium group on the Arg residue was first converted to an imidazoline ring and then is captured by immobilized phenylboronic acid through a cyclic diester linkage [18].

Another similar approach used Lys-C to yield N-terminal and internal peptides with an α-amino group at the N-terminus and a ε-amino group at C-terminus while C-terminal peptides contains only an α-amino group. After specifically blocking the N-terminal α-amino groups with (N-Succinimidyloxycarbonylmethyl)tris(2,4,6-trimethoxyphenyl)phosphonium bromide (TMPP-Ac-OSu), the Lys ended N-terminal and internal peptides were trapped by *p*-Phenylene diisothiocyanate (DITC) scavenging, and unbound C-terminal peptides were analyzed by MS. Alternatively, the α-amines can be specifically converted to carbonyl groups through transamination using Glyoxylic acid [19], and followed by DITC scavenging. Another negative selection utilized strong cation exchange (SCX) and the combined fractional diagonal chromatography (COFRADIC) [20] to simultaneously enrich and identify N- and C- termini in complex mixtures. In this approach, the primary amines were first blocked by acetylation. Following trypsin digestion, the internal peptides begin with primary amines and end with arginine residues, carrying positive charges under acidic conditions to be captured by SCX at low pH (pH 3), while the blocked N-terminal peptides and non-arginine carrying C-terminal peptides were not retained. After SCX fractionation, the C-terminal peptides were further labeled with *N*-hydroxysuccinimide (NHS) ester of butyrate to increase the hydrophobicity, allowing them to be separated from N-terminal peptides for LC-MS/MS analysis. In an approach called C-terminal amino-based isotope labeling of substrates (C-TAILS) [21], the protein N-terminal α-amines and lysine ε-amines were first protected through methylation while carboxyl groups of C termini, aspartate and glutamate side chains protected by carbodiimide-mediated (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and NHS. Following trypsin digestion, the N-terminal and internal peptides contain free carboxyl groups while C-terminal peptides carry protected carboxyl groups. Stable isotopes were then incorporated in C-terminal peptides using isotope variants of formaldehyde or isobaric tags, such as, Tandem Mass Tag (TMT) or Isobaric tag for elative and absolute quantitation (iTRAQ). The N-terminal and internal peptides were then trapped using polyallylamine polymers due to their free carboxyl groups, while unbound C-terminal peptides were enriched and subjected to LC-MS/MS analysis. This approach enables quantitative comparison of C-termini from different samples (e.g. mAb lot-to-lot comparability). A later report improved the derivatization efficiency of the carboxyl groups using solid-phase derivatization [22]. In a similar approach, a TMPP can be tagged to protein C-termini using oxazolone chemistry. The free carboxyl groups of N-terminal and internal peptides generated by GluC were trapped using tosylhydrazide beads, enabling the enrichment of C-terminal peptides [23].

Although above described methods are available, currently no single methods can fulfill all analytical requirements due to the complexity of the samples and the diversity of C-termini. Thus, there is still room for improvement to develop simply and high-throughput methods that minimize chemical devitalization and enrichment to identify and quantify mAb fragmentation. Such methods will provide important information for protein therapeutic developments to ensure the drug quality and generate the control strategy.

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