Analytical strategies for the characterization of therapeutic monoclonal antibodies

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Recombinant monoclonal antibodies (mAbs) have become particularly relevant for the treatment of autoimmune diseases or cancers. Because of their inherent complexity and for safety reasons, there is a need to develop powerful analytical methods to provide detailed characterizations of mAbs.

The aim of the present review is to detail the state-of-the-art of analytical strategies for mAb characterization. It focuses on the most important separation techniques used in this field, specifically, the chromatographic and electrophoretic approaches and their combination with mass spectrometry (MS). Thanks to recent improvements in separation science and MS devices, mAbs can be analyzed more easily. However, there is still a need to find new approaches that avoid adsorption issues.

Keywords: Adsorption; Analytical strategy; Capillary electrophoresis (CE); Characterization; Liquid chromatography (LC); Mass spectrometry; Monoclonal antibody (mAb); Recombinant; Separation; Therapeutic monoclonal antibody

Abbreviations: BGE, Background electrolyte; CD, circular dichroism; CIEF, capillary isoelectric focusing; CZE, Capillary zone electrophoresis; EMA, European Medicines Agency; EOF, Electroosmotic flow; ESI, Electrospray ionization; Fab, Fraction antigen-binding; Fc, Fraction crystallizable; FDA, Food and Drug Administration; FL, Fluorescence spectrophotometry; FT-IR, Fourier transform infrared spectroscopy; HC, Heavy chain; ICH, International Conference on Harmonisation; IEX, Ion exchange chromatography; Ig, Immunoglobulin; LC, Light chain; LOD, Limit of detection; LOQ, Limit of quantification; LP, Limited proteolysis; mAb, Monoclonal antibody; MALDI, Matrix-assisted laser desorption/ionization; MS, Mass spectrometry; NGHC, Non-glycosylated heavy chain; PLOT, Porous layer open tubular; RPLC, Reversed-phase liquid chromatography; RSD, Relative standard deviation; SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SEC, Size exclusion chromatography; UHPLC, Ultra-high-pressure liquid chromatography

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1. Introduction

1.1. The therapeutic mAb market

Monoclonal antibodies (mAbs) are an emerging class of therapeutic agents currently being developed by many pharmaceutical companies. In 2010, the global therapeutic mAb market was a $48 billion ($40 billion and $37 billion for 2009 and 2008, respectively) [1], which represents approximately 5% of the pharmaceutical market, estimated at $850 billion.

Cancers of the colon, breast, lung, head, and neck and arthritis accounted for over 75% of the total mAb market, but new molecules introduced to treat chronic illnesses (e.g., asthma or osteoporosis) could further expand the mAb market. Muromonab-CD3, developed to reduce acute rejection in organ-transplant patients, was the first therapeutic mAb approved for use in humans in 1986. Since 2007, approximately 40 novel mAbs have begun clinical studies each year, and new products are regularly approved by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) [2]. The FDA has now authorized more than 20 mAbs, and 150 other mAbs, primarily involving immunological and oncological targets, are currently undergoing clinical trials with an approval rate of approximately 20% compared with 5% for new chemical entities [3].

Several characteristics of mAb therapy contribute to its success by improving the risk-benefit ratio. These characteristics include improved tolerance, good efficacy,

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high specificity, and limited side effects. Furthermore, the difficulty in obtaining generics (biosimilars or follow-on biologics) makes mAbs more attractive to patent holders than small molecules [4].

1.2. mAbs structure

The mAbs are glycoproteins that belong to the immunoglobulin (Ig) superfamily, which can be divided into five isotypes: IgA, IgD, IgE, IgG, and IgM. Because only IgGs are produced for therapeutic purposes through genetic engineering, the terms recombinant mAb and IgG are often used interchangeably.

The general structure of a mAb and its fragments is illustrated in Fig. 1. IgGs are large tetrameric glycoproteins measuring approximately 150 kDa that are structurally composed of four polypeptide chains: two identical heavy chains (HC, ~50 kDa) and two identical light chains (LC, ~25 kDa), connected through several inter-chain and intra-chain disulfide bonds at their hinge region. The resulting tetramer has two similar halves, forming Y-like shapes [5]. Each chain is composed of structural domains according to their size and function, giving the constant, variable, and hypervariable regions. Differences between the HC constant domains are responsible for the IgG sub-classes (i.e. IgG1, IgG2, IgG3, and IgG4).

Functionally, mAbs consist of two regions: the crystallizable fraction (Fc) and the antigen-binding fraction (Fab) [6]. As shown in Fig. 1, Fc (~50 kDa) is composed of two truncated HCs and is responsible for the effector functions (e.g., complement fixation and receptor binding). The Fc sequence also has a conserved N-glycosylation site, which is generally occupied by a biantennary oligosaccharide accounting for significant effects on the activity and efficacy of the IgGs [7]. The Fab domain (~50 kDa) is composed of the LC and the remaining portion of the HC. This domain is primarily involved in antigen binding [6].

Because mAbs exhibit great molecular complexity, they may be quite sensitive to changes in the manufacturing processes that can lead to considerable micro-heterogeneity in each individual chain. There are several common modifications leading to antibody-charge variants (or isoforms) on the peptide chains (e.g., deamidation, C-terminal lysine truncation, N-terminal pyroglutamation, methionine oxidation, or glycosylation variants) and size variants on the peptide chains (e.g., aggregation or incomplete formation of disulfide bridges). The combination of these micro-heterogeneity sources in the peptide chains significantly increases the overall heterogeneity in an entire IgG.

1.3. mAb characterization

Due to the increasing number of approved mAbs in the pharmaceutical area and the number of biosimilars potentially entering the market, the need for analytical techniques adapted for their detailed characterization has increased. As previously discussed, the intrinsic micro-heterogeneity is of major concern with mAbs and should be critically evaluated because differences in impurities and/or degradation products could lead to serious health implications [8].

The complete characterization of an intact mAb is difficult to achieve, so various enzymes (e.g., pepsin, papain, and Lys-C) are often used to obtain mAb fragments and facilitate the investigation of its micro-heterogeneity. As illustrated in Fig. 1, papain is primarily used to cleave IgGs into three fragments at the HC hinge region, one Fc and two identical Fab fragments of ~50 kDa each, while pepsin generates $F(ab')_2$ fragments measuring ~100 kDa. These types of digestion are called limited proteolysis (LP). The reduction of disulfide bonds is also commonly used to produce 2 LCs and 2 HCs.

In general, identity, heterogeneity, impurity content and activity of each new batch of mAbs should be thoroughly investigated before release. This examination is achieved using a wide range of analytical methods, including reversed-phase liquid chromatography (RPLC), size-exclusion chromatography (SEC), ion-exchange chromatography (IEX), sodium-dodecyl sulfate polyacrylamide-gel electrophoresis (SDS-PAGE), capillary-isoelectric focusing (CIEF), capillary zone electrophoresis (CZE), circular dichroism (CD), Fourier transform infrared spectroscopy (FT-IR), fluorescence spectrophotometry (FL), and mass spectrometry (MS). The goal of this multi-method strategy is to demonstrate the similarity between production batches of mAb by precisely determining the primary, secondary, and tertiary structures of the mAbs [9]. The chromatographic and capillary electrophoretic methods described in this review are particularly well suited to this purpose.

2. Chromatographic approaches

2.1. Size-exclusion chromatography (SEC)

Size distribution or mono-dispersity of a mAb product is important for both safety and efficacy. Components smaller than the intact mAb are often the result of enzymatic or non-enzymatic cleavage and the incomplete formation of mismatched disulfide bridges. In the latter case, neither the light chain nor the heavy chain is entirely incorporated into the IgG molecule. The size-related heterogeneity due to components larger than the individual antibody is often a result of molecular association, aggregation, or even precipitation. A full spectrum of species, from molecular dimer to oligomer to higher-order aggregates, may be present in a mAb preparation [10].

SEC is commonly used to determine size-related heterogeneity. SEC separations can be applied to both native and denatured antibodies for assessing whether the association is covalent or non-covalent. SEC is also