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Main Quality Attributes of Monoclonal Antibodies and Effect of Cell Culture Components

Fatemeh Torkashvand and Behrouz Vaziri*

Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran

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ABSTRACT

The culture media optimization is an inevitable part of upstream process development in therapeutic monoclonal antibodies (mAbs) production. The quality by design (QbD) approach defines the assured quality of the final product through the development stage. An important step in QbD is determination of the main quality attributes. During the media optimization, some of the main quality attributes such as glycosylation pattern, charge variants, aggregates, and low-molecular-weight species could be significantly altered. Here, we provide an overview of how cell culture medium components affect the main quality attributes of the mAbs. Knowing the relationship between the culture media components and the main quality attributes could be successfully utilized for rational optimization of mammalian cell culture media for industrial mAbs production.
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Corresponding Author: Behrouz Vaziri
Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran; Tel.: (+98-21) 64112467; Fax: (+98-21) 66480780;
E-mail: behrouz-vaziri@pasteur.ac.ir

INTRODUCTION

Process development is a state-of-the-art method to establish a manufacturing line in biopharmaceuticals. Although a significant shift in productivity is the main goal of process development, achieving appropriate quality attributes is also of great concern[1]. Quality by design (QbD) is a new approach to develop and to manufacture pharmaceutical products. QbD guarantees product quality and ensures that a consistent product with preferred quality attributes is generated[2,3]. Regulatory agencies encourage its application in the manufacture of all new pharmaceuticals containing biological products[2-4].

The cell line and its recombinant DNA construct, culture media, and process conditions are three important parameters that influence recombinant protein quality properties in the manufacture of biopharmaceuticals. The culture media and the control of process conditions are very important in process development[5,6]. In fact, the cell metabolism directly depends on the culture conditions, including the pH[7], the temperature[8], the oxygen tension[7], the CO_2 amount in the culture broth[9], and also the mode of processing, i.e., perfusion or fed-batch mode[10]. Diverse metabolic outcomes states that result from modifications in these culture parameters might produce proteins with altered quality attributes. Many review articles have been published in this field with a focus on the cell line[11-13] and cell culture parameters[14,15]. Moreover, with concentration on the regulation of certain media constituents and by supplementing the medium with specific co-factors, the glycosylation profile[15], the charge variants[16], the aggregation amount[17,18] and the level of low-molecular-weight (LMW) variants[19] can be controlled.

At this time, monoclonal antibodies (mAbs) are the main products in the pipeline of the biopharmaceutical industry. Numerous studies have reported different impacts of glycosylation, charge variants, aggregates, and fragments on the biological activity and pharmacokinetics[20-23]. The purpose of this review is to

discuss the main quality attributes of mAbs that can be changed directly by culture conditions, and to review the culture conditions and culture media components that affect these attributes.

Glycosylation

Importance

Glycosylation is a complicated process of the attachment of oligosaccharides to the polypeptide backbone of a protein, which occurs in the endoplasmic reticulum and Golgi apparatus. There are two main kinds of glycosylation[24]: asparagine (Asn)-linked glycosylation or N-linked glycosylation, and serine/threonine-O-linked glycosylation. In mAbs, the Asn-linked glycosylation is the most common[25]. The N-glycans are linked to the two conserved Asn residues (Asn 297) in the CH2 domain of the Fc region[26]. The presence or absence of certain oligosaccharides can affect mAb stability[27], *in vivo* efficacy[27-29], antibody-dependent cell-mediated cytotoxicity (ADCC)[27,30], complement-dependent cytotoxicity (CDC) activities[31], pharmacokinetics[22], clearance rate[1], and immunogenicity[1,32]. Hence, the precise control of glycosylation of mAbs is critical.

2-2-N-glycosylation types

In the endoplasmic reticulum, the oligosaccharide chain is attached to the protein backbone and consequently forms an oligomannose species through a series of enzymatic reactions. In mammalian cells, the glycoprotein undergoes further processing in the Golgi[14,27]. N-glycans can be classified into three groups, which have a shared core comprising two N-acetylglucoseamine (GlcNAc) residues and three mannose types in a branched form (Fig. 1). The different groups are:

1) The high-mannose (HM) type that comprises only mannose residues attached to the core. While the HM amount on the endogenous human IgG is usually very low, the HM amount of the recombinant mAbs can range from 1% to ≥20%. Due to the quicker serum clearance rate of HM glycans compared to other Fc-glycans, the pharmacokinetic properties of these mAbs are affected[33,34]. Additionally, the HM glycoforms are concomitant with enhanced ADCC activity[34,35]. Therefore, the HM amount of mAbs can be considered to be an important quality attribute in the production process.
2) The complex type containing different kinds of monosaccharide in their antennal region (Fig. 2). Galactose amount may influence CDC, and the sialylation amount may influence functionality or inflammatory characteristics[15]. The lack of core-fucosylation results in enhanced ADCC[7]. For instance, non-fucosylated mAbs display fiftyfold to thousandfold higher efficacy than their fucosylated counterparts[30].
3) The hybrid type, which has properties from both HM and complex types attached to the core.

Glycosylation during cell culture

It is understood that differences in the N-linked glycan profile can take place during the mAb production process[7,36]. The cell culture conditions containing culture media elements, the accessibility of the nucleotide sugar substrates, the expression amounts of the enzymes involved in the attachment, and the transformation of carbohydrate structures determine the amount of antennarity and sialylation[14].

Manganese plays an important role in the glycosylation pathway[15,37,38]. As a co-factor of many

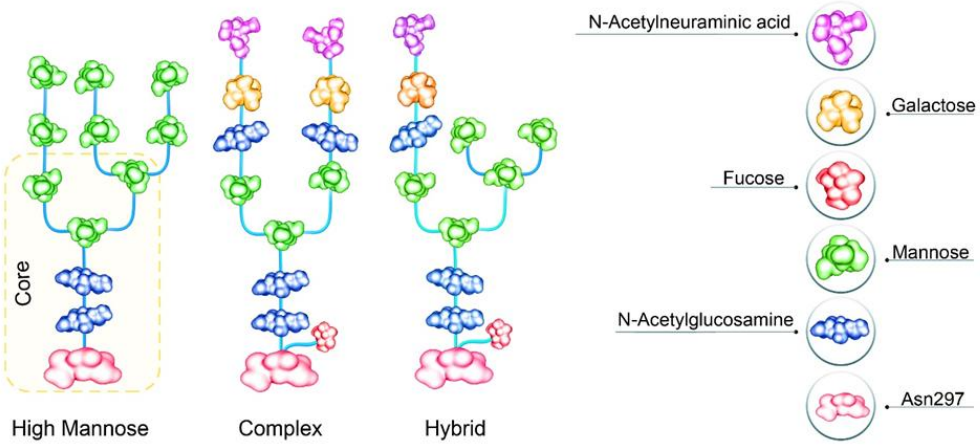

Fig. 1. The schematic representation of the composition of different groups of N-glycans containing high mannose, complex, and hybrid types.

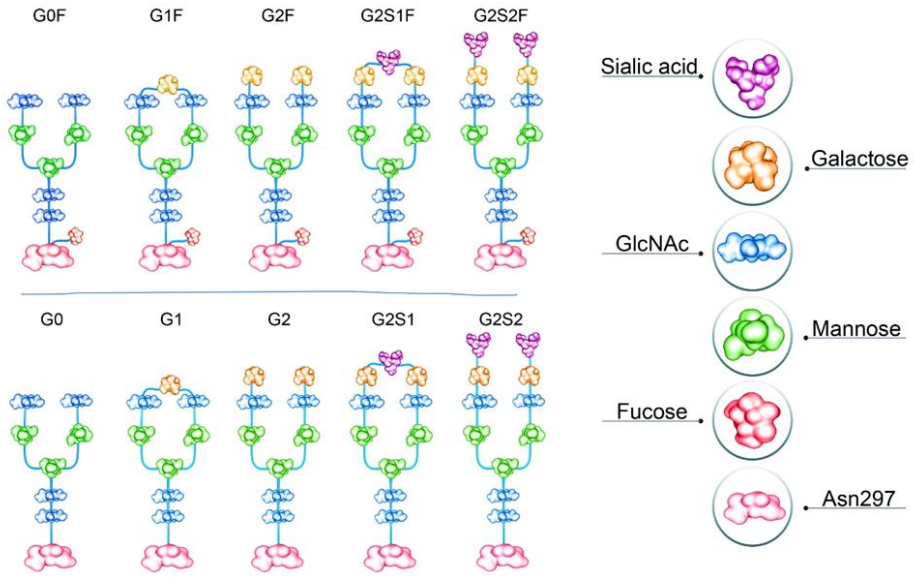

Fig. 2. The schematic representation of major N-linked glycoforms of mAb therapeutics. G0: asialo, agalactose, biantennary complex (common core [Man3GlcNAc2] with terminal two GlcNAc residues), G0F: asialo, agalactose, biantennary complex, core substituted with fucose, G1: asialo, mono-galactosylated, biantennary complex, G1F: asialo, mono-galactosylated, biantennary complex, core substituted with fucose, G2F: asialo, galactosylated, biantennary complex, core substituted with fucose. G, galactose; S, sialo (sialic acid)

enzymes, manganese controls the glycosylation profile[38]. It has been shown that increased nucleotide-sugar precursors levels, comprising UDP (uridine diphosphate)-Hex, UDP-HexNAc, and cytidine monophosphate-sialic acid, enhance the glycosylation of mAbs[39].

It has been shown that the glucose limitation in culture medium can lead to a reduced UDP GlcNAc availability[40] which in turn results in glycosylation heterogeneity[41]. In a Chinese hamster ovary (CHO) cell culture experiment, it was seen that the amount of non-glycosylated antibody was correlated to the extent of time the cells deprived of glucose[42]. In a different study in fed-batch culture mode, with the human cell line rF2N78, it has been shown that due to the lack of glucose in the feed, nearly 44% of the product was aglycosylated. No aglycosylated antibody was expressed when glucose was fed throughout the culture[43]. There are reports that glucose and glutamine (Gln) concentrations below 1 mM were harmful to glycosylation[29,44,45]. Also, variations in other cell culture conditions such as dissolved oxygen, bioreactor pH, ammonia, and shear stress, have been shown to affect the glycosylation of therapeutic mAbs. Their terminal galactosylation may be affected by such variations[14]. The variable presence of terminal galactose residues leads to the heterogeneity of Rituximab glycosylation[15,46]. The effect of Rituximab terminal galactose residues on CDC activity originates from the involvement of galactose residues in the binding of Rituximab to complement C1q[46]. Therefore, the agalactose form of Rituximab is considered as a serious impurity.

Analytical methods for the detection of mAb glycosylation

Several analytical methods are used to analyze glycosylation. Some of those are nuclear magnetic resonance, mass spectrometry, high performance liquid chromatography (HPLC), and capillary electrophoresis (CE). The most frequently used quantitative tools to analyze glycosylation are HPLC and CE. HPLC is used either with fluorescence detection[47-49] or with pulsed amperometric detection[50,51] and CE with a laser-induced fluorescence detector for various fluorescently-labelled glycans[52]. In HPLC-based methods, in the first step, glycans are released by chemical or enzymatic methods. The second step is the separation of the released glycans and the sample clean-up for the elimination of salts or denaturants. Labelling with appropriate reagents is done to improve detection. Then chromatographic techniques are used to separate the released, purified, and labelled or unlabelled glycans[53]. The common separation-based techniques that are used for the characterization of mAb glycoproteins are reverse-phase HPLC, hydrophilic interaction chromatography, and high-performance anion-exchange chromatography (Fig. 3).

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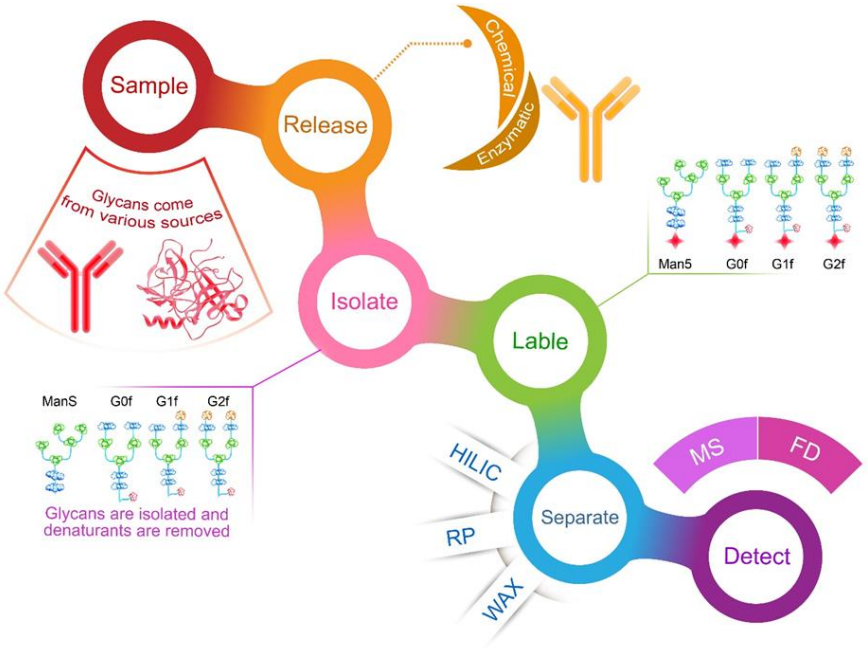

Fig. 3. Workflow of glycan profiling, hydrophilic interaction chromatography (HILIC), reverse-phase chromatography (RP), weak anion exchange chromatography (WAX), mass spectrometry (MS), and fluorescence detector (FD). G0: asialo, agalactose, biantennary complex (common core [Man3GlcNAc2] with terminal two GlcNAc residues), G0F: asialo, agalactose, biantennary complex, core substituted with fucose, G1: asialo, mono-galactosylated, biantennary complex, G1F: asialo, mono-galactosylated, biantennary complex, core substituted with fucose, G2F: asialo, galactosylated, biantennary complex, core substituted with fucose. G, galactose

Charge variants

Recombinant mAbs undergo chemical degradation through diverse mechanisms comprising deamidation, oxidation, isomerization, and fragmentation that result in several charge variants and heterogeneity formation, consequently modifying their pI values[20].

Importance

The analysis of charge heterogeneity in the mAbs characterization is essential because it provides significant information about product quality and stability[54]. Charge variants with a relatively lower pI are mentioned as acidic variants, while charge variants with a relatively higher pI are mentioned as basic variants (Fig. 4). Charge variants may significantly influence the *in vitro* and *in vivo* properties of antibodies. It has been revealed that they can change the binding to proteins or cell membrane targets, thereby affecting the tissue penetration, tissue distribution, and pharmacokinetics of the antibodies[20,55-58]. There is enough evidence in the literature to recommend that the existence of acidic species variants on mAbs can at least have an effect on the resulting protein's efficacy and function[59-61]. The impacts of the charge variants depend highly on the nature, site, and the amount of post-translational modifications that cause the acidic and basic variants' formation[62]. Therefore, mAb charge variant levels must be controlled exactly. At present, little information is available on the control of these variants using process parameters.

Charge variants types

Main species

The main peak of charge variant chromatograms usually contains species with three kinds of usual post-translational modifications: (1) Cyclization of the N-terminal Gln to pyroGlu, (2) elimination of the heavy chain C-terminal lysine (Lys), and (3) glycosylation of the conserved Asn residue in the CH2 domain with neutral oligosaccharides. At the time of analysis, most of the antibodies comprised N-terminal pyroGlu instead of the original Gln, and therefore elute as the main peak[63,64]. Antibodies without any C-terminal Lys are typically observed in the main species[63-67]. The preserved Asn residue in the CH2 domain is glycosylated. The core-fucosylated complex bi-antennary structures with zero, one, or two terminal galactose residues are the main glycoforms of recombinant mAbs from mammalian cell cultures[68].

Acidic species

Charge variants with a relatively lower pI are termed acidic variants. Table 1 summarizes the central reasons for the formation of acidic species. The major cause of acidic species formation, which has been extensively

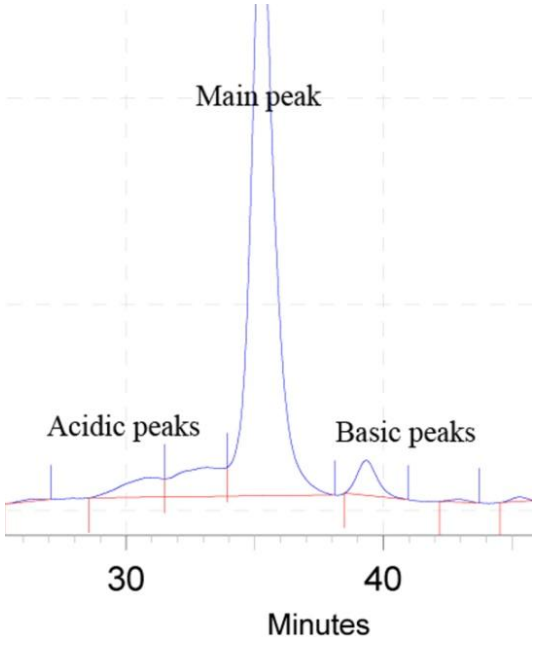

Fig. 4. The cation exchange chromatogram representing different charge variants containing acidic, main, and basic peaks. The chromatogram is related to a homemade monoclonal antibody.

reported, is deamidation of Asn residues. Deamidation happens both in the variable domains, particularly complementarity-determining regions, as well as in the constant domains. Deamidation of Asn residues in the complementarity-determining regions always leads to the acidic species formation[63,69-73].

Basic species

Charge variants with a relatively higher pI are basic variants. Table 1 summarizes the main reasons for the basic species generation. The major cause for the generation of basic species is incomplete removal of C-terminal Lys. Due to the further positive charges, mAbs with heavy chain C-terminal Lys are more basic than the main species[20,63-65,68,74-78].

Charge variants during cell culture

The culture temperature displays important effects on mAb charge variants' distribution[79]. Reducing the temperature and accelerating the temperature shift time considerably decrease the acidic charge variant amount[61]. In a study, Zhang *et al.*[79] showed that decreasing the culture temperature enhanced the Lys variant amount, which can be the main reason for the increased basic variant amount, also they showed that cultivations at sub-physiological temperatures in both batch and fed-batch culture modes reduced the mAb acidic variant levels, but the basic ones were enhanced. It can be related to the reduction of carboxypeptidase B transcription level. However, the mechanism by which a temperature downshift decreases the acidic charge variants' level has not been clarified yet.

There was a straight correlation between the proline amidation level and the basic peak level. Kaschak *et al.*[54] observed that the proline amidation was sensitive to copper ion concentration in the culture medium during cell culture. They showed that a higher Cu^{2+} ion concentration results in the higher level of proline amidation. They also showed that if the copper concentration increases and the zinc concentration decreases in a chemically defined medium, the level of C-terminal Lys variants will enhance[16]. Deamidation modification in target mAb is decreased by glycerol and sodium chloride[80] and increased by iron concentration enhancment[81]. Increase in the sodium butyrate concentration in CHO cell culture medium enhances mAbs basic charge variants[82]. Moreover, it has been found that the supplementation of mammalian cell culture media with the bioflavonoid chemical family can decrease acidic species of recombinant mAbs[83].

Table 1. The modifications that form acidic variants

Number	Acidic variants	Basic variants
1	Deamidation	N-terminal Glu
2	Non-classical disulfide linkage	Isomerization of Asp
3	Trisulfide bonds	Met oxidation
4	Glycation	C-terminal Lys
5	High mannose	Incomplete disulfide bonds
6	Sialic acid	Amidation
7	Thiosulfide modification	Succinimide
8	Cysteinylation	Mutation from Ser to Arg
9	Non-reduced species	Aggregates
10	Reduced disulfide bonds	Fragments
11	Modification by maleuric acid	Aglycosylation
12	Fragments	Incomplete removal of leader sequence

Analytical methods for the detection of mAb charge variants

Several methods are used to detect charge variants of recombinant mAbs. These include isoelectric focusing gel electrophoresis[71,74,84], capillary isoelectric focusing electrophoresis[76,85], and cation[71,74,76,85-87] and anion[84,87] exchange chromatography.

Fragmentation and aggregation

Importance

Protein aggregation and fragmentation may lead to immunogenicity, loss of biological activity, and other side-effects[88-92]. These modifications are host cell line, clone, and process-dependent[15,93].

Fragmentation

Fragmentation is a common type of degradation and can be attributed to the disruption of a covalent peptide bond. It may take place spontaneously or by enzymatic reaction[92]. To evaluate the purity and integrity of the target protein, it is necessary to monitor the mAbs fragmentation as a critical quality attribute. The fragmentation pattern of mAb denotes a fingerprint of stability and production consistency.

Aggregation

In the manufacture of therapeutic proteins, aggregation is a common problem. Protein aggregates can be categorized in several ways, including soluble/insoluble, covalent/non-covalent, reversible/ non-reversible, and native/denatured[18,94,95]. These structural changes are significant because they can cause a loss of activity of the intact proteins. Furthermore, aggregation and misfolding can induce a new epitope presentation, leading to an adverse immune response[88,96]. The control and avoidance of aggregation in the manufacturing process are needed because aggregates affect drug performance and safety[97,98].

Aggregate formation during mAb manufacturing processes

Physicochemical stresses, such as changes in the osmolality and pH of the medium, or changes in the culture temperature, protein concentration, oxygen and shear forces can aggregate the secreted proteins[94,99]. Stresses to the protein, such as freezing contact with air, or interactions with metal surfaces, may lead to undesired post-translational modification, which result in aggregates formation. Mechanical stresses may lead to protein aggregation[100-102]. Osmolytes in the form of small organic components, such as sugars, polyols, and amino acids help as chemical chaperones to stabilize proteins and stop aggregation[103-105].

Fragmentation during cell culture

Fragmentation may occur because of the action of proteases released by cells into the cell culture supernatant during the protein production process[89,106,107]. According to several reports, the culture media components have different effects on product fragmentation. Trace elements, including manganese, zinc, and cobalt, decrease LMW formation[108], while copper increases the LMW formation[19,90]. Also, other media components, such as EDTA[19,90,108] and cysteine[17] decrease product fragmentation.

Aggregation during cell culture

The control of the produced aggregates level during the cell culture process is possible. This control is accomplished by carefully choosing the proper cell line and improving cell culture conditions, such as media components that affect media osmolality and conductivity, feeding strategy, temperature, and pH[109]. A lower quantity of aggregates in the secreted protein was observed when media pH and osmolarity were increased in cells cultured in a hollow fibre bioreactor[110]. Cromwell *et al.*[18] studied the effect of cell culture temperature on aggregate formation during the culture. They indicated that the higher levels of aggregates were observed when the protein was held in the culture medium at a high temperature for a longer time. Different effects of media components on product aggregation have been reported. Different reducing and oxidizing substances containing glutathione, cysteine, and copper decrease the protein aggregate formation in CHO cell culture harvests[17]. Sodium chloride also decreases the aggregate amount[111].

Analytical methods for the detection of fragmentation and aggregation of mAbs

The analytical methods used to detect fragmentation and aggregation can be divided into two groups based on their separation mode: (1) The methods in which the separation is based on the size of the molecule, such as size-exclusion chromatography, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and CE with SDS. (2) The methods in which the separation is based on the chemistry of amino acid side chains such as cation exchange chromatography. While the mentioned methods are usually used to monitor and quantify protein fragmentation and aggregation, the identification of the exact cleavage site is performed using mass spectrometry[112,113].

Here, we explained the main quality attributes of recombinant mAbs, which can be altered during cell culture media optimization. In cell culture media optimization, the challenge is to increase the yield with

the desired quality of the product by the addition of appropriate components at the correct concentration. Published data show that quality engineering could be performed by media design which is a rational strategy to considerably control the main quality attributes and function of mAbs. Therefore, to reach a recombinant mAb with the desired quality, the analysis of main quality attributes by appropriate analytical methods during the process development is necessary and inevitable.

CONFLICT OF INTEREST. None declared.

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