Engineering Therapeutic Antibodies for Development

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What You Will Learn from This Chapter

Most chapters in this book describe methods to select antibodies and to engineer their properties for desired profiles in in-vitro assays or animal studies, focusing on binding and engaging the target. To become drugs, such early antibodies require further sequence optimization to ensure that the molecules can consistently be produced in high quantities fulfilling the necessary quality requirements and that they can be applied in humans with minimal unwanted side effects. In this chapter, we will discuss the nature of some side effects caused by monoclonal antibodies (mAbs) and how protein engineering methodologies are applied to reduce unwanted side effects. In addition, we will cover the challenges of manufacturing and process development with a focus on product homogeneity and explain how sequence optimization and process controls are applied to minimize product heterogeneity and ensure batch-to-batch consistency.

12.1 Introduction

Protein therapeutics including mAbs comprise a very successful class of drugs generating significant revenue and provide valuable treatment options for patients with devastating conditions such as metastatic cancer, autoimmune diseases, cardiovascular diseases, and many others. mAbs are considered relatively safe drugs due to their high target specificity. Antibody therapy started more than 100 years ago using passive immunization with sera from immunized animals to treat infectious diseases such as diphtheria. In 1975, Köhler and Milstein developed the hybridoma technology and this milestone paved the way for modern mAb therapeutics [1]. The success of mAbs in the market place is a result of their biology: Antibodies are components of the human immune system where they provide key functions for the humoral host defense. They bind to surface structures of pathogens—so-called epitopes—very specifically and connect them to other components of the immune system such as macrophages, natural killer cells, and components of the complement system. Antibodies are highly abundant in the bloodstream and share high structural similarity. At the same time; however, they have high sequence diversity in their so-called variable regions. Hence, antibodies—although structurally very similar—can bind to all kinds of different epitopes including proteins, lipids, glycostructures, etc., opening a wide target space. Whereas antibodies in the bloodstream are polyclonal,

meaning a mixture from different sequences targeting several epitopes, mAbs are antibodies derived from a single cell clone with a defined sequence and binding to one specific epitope, hence the term "monoclonal antibody." Since a large surface area of a mAb is involved in binding of the epitope, very high selectivity is ensured, and "unwanted" targets are not affected. Thus, safety profiles of mAbs are favorable compared to, e.g., small molecules, which very often show activity on unwanted targets.

Nevertheless, the complex structure and biology of mAbs come with its own challenges in terms of development: mAbs are proteins and can cause immunogenicity in subsets of patients leading to the development of anti-drug antibodies (ADA), impairing drug efficacy and pharmacokinetics. In addition, antibodies naturally engage components of the immune system mediated by their Fc-part, which can result in unwanted side effects. Also, aggregation of antibodies and the formation of immune complexes or impurities during manufacturing or non-human posttranslational modifications can result in further unwanted side effects.

The structural similarity of mAbs allows the implementation of platform processes for development and production: Today, most marketed antibodies are produced by fed-batch processes using Chinese Hamster Ovary (CHO) cells as the expression host and affinity purification using Protein A resins as the key enrichment step. Production processes share high similarities for different mAb products, thus reducing the technology risk, enabling faster process-development timelines, and lowering investments. Consequently, mAbs have substantially higher success rates and shorter cycle times compared to small molecules in discovery and development. However, as mAbs are complex glycoproteins with a molecular weight of roughly 150 kDa consisting of four polypeptide chains. Hence, many unwanted structural modifications can occur during manufacturing, impairing the homogeneity of the final product. Some of those modifications may change their efficacy on target or their serum half-life, while others do not. As the final product is always a mixture of millions of variants, it is critical to ensure that modifications impairing the activity of the antibody in humans is minimal and that batch-to-batch consistency is guaranteed.

Often, very high doses of antibodies are required, thus triggering the requirement for exceedingly large-scale manufacturing (>700 kg per annum). In addition, the final drug product must be highly concentrated if the drug is to be administered through the subcutaneous route. Many of the aspects described can be modulated using protein engineering and process technologies and some examples are described below in this chapter.

12.2 Safety Considerations

Although mAbs are generally better tolerated than conventional chemotherapeutic compounds, there is a broad variety of side effects that have been reported over the years. These range from rather mild effects including: nausea, diarrhea, headache, mild gastrointestinal symptoms or itching, to more serious conditions, such as hypersensitivity

reactions including: anaphylaxis, injection site reactions, cytopenia, cardiac toxicity, infections or cytokine release syndrome [2]. In addition, immunogenicity and immune complex formation is always a safety concern for all biotherapeutics including mAbs. Some of the side effects of mAbs are clearly related to their specific target, while others appear to relate to attributes of the antibody itself and their ability to interact with various components of the host immune system.

12.3 Examples of Target-Dependent Side Effects of mAbs

An example of target-dependent side effects is cardiotoxicity mediated by Trastuzumab, a chimeric IgG1 targeting the Her-2 receptor in breast cancer. As Her-2 is also expressed on cardiomyocytes, blocking Her-2 signal transduction with trastuzumab led to cardiotoxicity in 4% of treated patients [3].

Another target-mediated side effect is skin rash induced by exposure of patients to anti-EGFR antibodies such as Cetuximab. EGFR is a prominent cancer target and cetuximab is used for the treatment of metastatic colon cancer, as well as head and neck cancer. EGFR is widely expressed in epithelial cells including keratinocytes. Thus, blocking EGFR signal transduction with cetuximab or other EGFR blocking agents including small molecules often causes skin rash in the face and on the upper torso. There is a correlation between the occurrence of a skin rash and a positive response to the drug [4].

Progressive multifocal leukoencephalopathy (PML) is a rare demyelinating disease of the central nervous system caused by the *John Cunningham Polyomavirus* (JCV). Although the pathophysiology is not fully understood, it appears that immunosuppression plays a role. Several antibodies applied for the treatment of lymphoproliferative- or autoimmune-disorders appear to increase the incidence of PML infections [5]. Natalizumab, a mAb used for the treatment of relapsing-remitting multiple sclerosis, was suspended in 2005 because of the occurrence of some PML cases and reintroduced in 2006 with specific warnings on the label [6]. Besides natalizumab, cases of PML were described for several other antibodies including rituximab, ofatumumab, obinutuzumab, brentuximab vedotin, alemtuzumab, and others [2] (and references therein).

Another common side effect of antibodies modulating the immune response is the so-called “cytokine storm” (cytokine release syndrome, CRS) that has been described, for example, for Rituximab, Alemtuzumab or Muromonab [2] (and references therein). In 2006, a clinical phase 1 study with an experimental drug, TGN1412, a CD28 superagonist, had a dramatic effect as treated patients developed CRS within minutes after dosing, resulting in systemic inflammation symptoms and multiorgan failure requiring intensive care [7].

12.4 Immunogenicity of Biotherapeutics

The first mAb to be approved by the US Food and Drug Administration (FDA) was Muromonab (OKT3) for the treatment of kidney transplant rejection in 1985. Muromonab represents a mouse IgG2a isotype [8] and caused immune reactions which led to alterations in efficacy, safety, and pharmacokinetics [9]: Mouse antibodies can be detected as "non-self" by the human immune system resulting in an "anti-drug-antibody" (ADA) response. Those early findings triggered the discovery of antibody engineering technologies leading to less immunogenic formats [10]: In the years following, protein engineering technologies were applied to develop new antibodies displaying a more favorable profile (Fig. 12.1): In the mid-80s the first chimeric antibody was described [11] followed a few years later by the first humanized antibody [12]. Chimeric antibodies comprise an antigen-binding region (Fab) of rodent origin which is fused to a human constant region (Fc). Humanization means grafting the CDR regions of rodent origin into a human antibody scaffold, further reducing the presence of rodent-derived sequences in a therapeutic molecule. Nowadays, fully human antibodies derived from transgenic animals or from phage display technologies are state-of-the-art. Those molecules can be considered "fully human" and do not contain any sequence patches of rodent origin.

As shown by [13], dosing of murine antibodies causes marked ADA responses, resulting in impaired clinical outcomes and ultimately in discontinuation of clinical studies. Substitution of the rodent Fc-regions to those of human origin by chimerization causes a reduction of ADA responses, which can be further reduced by using humanized or fully human antibodies. Although the use of humanized and fully human antibodies reduces the overall immunogenicity risk compared to chimeric and mouse antibodies, there is still a considerable patient population that develops ADA responses: In the case of adalimumab (Humira)), a fully human antibody targeting the cytokine TNFα, 12% of patients in a clinical trial developed an ADA response. Whereas infliximab (Remicade), a chimeric

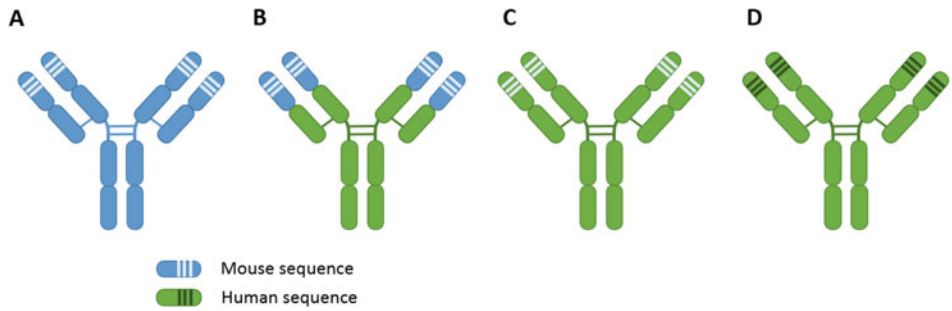

Fig. 12.1 Antibody engineering for chimeric and humanized antibodies to reduce ADA response. Variable regions of a mouse antibody (**a**) are fused to the constant regions of a human antibody to build a chimeric antibody (**b**). Grafting of mouse CDRs into a human backbone results in a humanized antibody (**c**). Fully human antibodies are now state of the art and can be generated by display technologies or by using transgenic mice or rats harboring the human antibody repertoire (**d**)

antibody targeting the same cytokine, led to an ADA response in 75% of patients. In both cases, the ADA response was found to be further reduced by coadministration of immunosuppressive drugs.

12.5 What Causes Immunogenicity?

It is easy to understand that patients receiving repeatedly high doses of antibodies containing sequence patches from other species would be detected as "non-self" by the host and consequently trigger an immune response. However, unlike one would expect, even fully human antibodies have still proven to be immunogenic, causing an ADA response in a subset of individuals.

One example can be anti-allotype reactions for human IgG-Fc and kappa light chains. Allotypes comprise minor deviations in the amino acid sequence of IgGs of the same subclass that occur throughout a species. Those allotypes are inherited and different allelic forms are expressed by individuals. IgG1, for example, exists in four different allotypes located in the heavy chain (G1m (1–3, 17)) and three different allotypes in the kappa light chain (Km (1–3)). There are no allotypes for the lambda light chain reported [14]. Hence, IgG1-based therapeutic antibodies may induce an anti-allotype response in a subset of patients. It must also be taken into consideration that paratopes of therapeutic antibodies are usually not part of the natural antibody repertoire. Consequently, an anti-idiotypic response directed against the paratope may be triggered when a therapeutic mAb is selected using hybridoma or transgenic animal technology, or when changes in the amino acid sequence of the variable regions occur through rearrangement and affinity maturation of the variable regions [10] (and references therein). Imprecision of the rearrangement of genomic sequences (VL-JL and VH-DH-JH recombination) contributes to the sequence diversity of antibodies. In addition, somatic hypermutations during affinity maturation may introduce new amino acid variations deviating from germline sequences. Those novel sequences can lead to an anti-idiotypic immune response, as the host immune system is unable to establish tolerance to all those possible variations.

Besides sequence attributes, immune responses to posttranslational modifications have been described, e.g., for cetuximab (see above). Cetuximab is *N*-glycosylated on N88 in the heavy chain [15] and when produced in mouse-derived SP2/0 cells, the carbohydrate structure contains a galactose-α-1,3-galactose glycostructure, causing severe anaphylactic reactions in a subset of patients due to pre-existing IgE antibodies. This problem can be circumvented by using a cell line (for example, CHO cells) that does not perform galactose-α-1,3-galactose connections [15].

Besides those so-called intrinsic factors that can be attributed to the amino acid sequence and the structure of biopharmaceuticals, some external factors such as purification- and formulation-dependent impurities are important as well. Furthermore, factors including route and frequency of administration, immune status of the patient, or comedications play a role [10]. Amongst those extrinsic factors contributing to the immunogenicity of

biopharmaceutical drugs, aggregation propensity appears to be very important [16], as many of the side effects mediated by ADAs require the formation of drug–ADA immune complexes (IC) leading to Fc-mediated downstream effects causing hypersensitivity reactions (discussed below).

12.6 Mechanisms Leading to an ADA Response

12.6.1 T Cell-Dependent Immune Response

In many cases, a single injection of a non-self-antigen is sufficient to induce a high level of ADAs with high affinity that can persist for an extended period of time in the body. In addition, memory cells are induced which leads to a booster reaction if the host organism is re-challenged with the same non-self-antigen. This "vaccination-like" adaptive immune response involves antigen-presenting cells (APCs) and CD4+ T helper cells. Following administration, foreign proteins are phagocytosed by APCs such as dendritic cells (DC) or macrophages. After degradation by the immunoproteasome, peptides derived from the antigen are presented by the major histocompatibility complex-2 (MHC-2) [17]. CD4+ T cells recognize linear peptides presented by the MHC-2 complex via their T cell receptors and secrete cytokines promoting differentiation of B cells, isotype switching, and affinity maturation, resulting in the production of high-affinity IgG-type ADAs in plasma cells. Until this differentiation occurs, the immune response is driven by IgM-expressing B cells with low affinity and specificity. In conclusion, the presence of high-affinity ADAs of the IgG subclass is usually associated with CD4+ T helper cell activity. Such responses exist for biotherapeutics of microbial origin [18] but have also been described for the V-regions of antibodies [19]. This process, leading to the production of high-affinity IgG1, takes a few days and requires co-stimulation by additional factors such as CD28 and CD80 [17], as without co-stimulation, T cells will become anergic. Inhibitory DCs (iDC) present peptides without co-stimulation, playing a key role in maintaining immune tolerance [20]. During development, CD4+ T cells are tolerized in the thymus and later anergized or deleted upon contact with a self-antigen.

12.7 T Cell-Independent Immune Response

Modern antibody therapeutics are humanized or fully human and hence it is counterintuitive that they would cause immune responses in humans. Antibodies occur in mg/ml concentrations in human serum and there is a high level of immunotolerance. However, prolonged exposure to humanized or human antibodies can result in tolerance breaking, and aggregates of therapeutic proteins appear to play an important role. Aggregates can directly interact with B cells and activate them, presumably by oligomerizing and activating B cell receptors, a process that does not discriminate between self and

non-self. The ADA responses resulting from this process are often milder compared to the CD4+ T cell-driven response described above and disappear after treatment as there is no memory effect [16].

The distinction between both types of immunogenicity is not absolute, but rather patient-dependent, and sometimes both types of responses can be observed in a patient population exposed to the same biopharmaceutical.

The correlation between aggregation of biotherapeutics and immunogenicity is well established, and it appears that breaking tolerance is the main mechanism for ADA formation for modern therapeutic antibodies. Besides the B cell induction described above, mAbs—depending on their subtype—modulate immune reactions such as complement—or macrophage activation with their Fc-part that may contribute to their immunogenicity. It has been shown that deglycosylation of IgG1 reduces those reactions [21] but human antibodies devoid of Fc-mediated effector functions can still be immunogenic.

12.8 Clinical Consequences of Immunogenicity

It is difficult to compare the consequences of immunogenicity of different studies due to the lack of standardization with respect to study design, analytical methods, or data interpretation. In addition, side effects, including those which are induced by immunogenicity such as ADA responses, vary significantly between individual patients within a given study and when comparing studies of different drugs for different indications [16]. Hence, some of the mechanisms are poorly understood and it is difficult to discriminate target-dependent mechanisms from those induced by the antibody itself.

Persisting high levels of neutralizing antibodies may lead to a complete loss of efficacy. ADAs to biotherapeutics that have an endogenous counterpart can have devastating effects if the ADA is cross-reactive to the endogenous factor. For example, healthy volunteers exposed to recombinant thrombopoietin or erythropoietin developed thrombocytopenia or pure red cell aplasia, respectively [22, 23].

The effect of ADAs to mAb therapeutics may result in loss of efficacy, either by directly interfering with target binding or by impairing the serum half-life. Other clinical effects that are observed with therapeutic antibodies and correlate with the occurrence of ADAs are injection site reactions and serum sickness [24].

Type 1 hypersensitivity reactions caused by pre-existing IgE antibodies directed to glycostructures have been described above for cetuximab. Anaphylactic hypersensitivities have been described for other mAbs as well (mostly mAbs containing rodent sequences), although the incidence of such an event is relatively small.

Infusion of mAbs may result in infusion site reactions, typically a few hours after the first administration, characterized by rather moderate “flu-like”-symptoms such as nausea, fever, and headache [25]. However, life-threatening events such as bronchospasm or cardiac arrest occur occasionally. The biological mechanism leading to infusion site

reactions is not fully understood but pre-existing antibodies to impurities or glycostructures are thought to play a role.

mAb therapies often require high doses that may result in the formation of circulating immune-complexes (IC) between the ADAs and the therapeutic mAb, causing side effects. Various parameters including size, distribution, polyclonal diversity, and Fc-mediated effects contribute to the clinical outcome. Whereas larger and insoluble ICs are cleared by the mononuclear phagocytic system in the liver, smaller ICs stay in circulation and trigger Immune responses via Fc-receptors. Fcγ-receptors (Fcγ-R), which are expressed widely in the hematopoietic system, appear to play a key role in the pathological effects of ICs. Low-affinity Fcγ-R, such as Fcγ-RIII (CD16) or Fcγ-RII (CD32), that interact with ICs exceeding a certain size threshold can trigger proinflammatory responses.

Deposition of ICs in the capillary network can lead to vascular thrombosis and a local inflammatory response. In addition, those ICs can activate the classical complement cascade by binding to C1q via the Fc-region of the antibody. Proximity of IgG1-Fc effectively leads to the generation of C3-convertase, which is a danger factor inducing various inflammatory effects [26]. Therapeutic mAbs can also attract ICs to cell surface targets causing symptoms such as vasculitis and serum sickness [27].

12.9 Engineering mAbs for Safety and Minimal Immunogenicity

As described above, the immunogenicity of mAbs can be influenced by a variety of extrinsic and intrinsic factors and is difficult to predict. Several protein engineering strategies are applied to reduce the risk of immunogenicity. Immune reactions with murine mAbs led to the development of chimeric antibodies and humanized mAbs, and later to fully human antibodies derived from transgenic rodents or combinatorial library approaches (described elsewhere in this book). In addition, posttranslational modifications can result in immunogenicity, as described in this chapter for the example of cetuximab and should be avoided. Moreover, the choice of the isotype influences immunogenic potential as the engagement of effector cells and the complement system varies for different isotopes. Drug aggregation is another factor that induces an immune response and should be generally avoided.

12.10 Humanization

In the past decades, several strategies for the humanization of mAbs have been reported. However, CDR grafting onto a homologous human antibody framework of high sequence homology remains the most common technique [28]. CDR grafting often results in a reduction or loss of antigen recognition. This problem can be resolved by back mutating critical framework sites to the corresponding amino acid residues in the initial rodent sequence. Such critical framework residues are selected from structural data on antibodies

or from homology-modeled structures revealing framework positions important for structural integrity. In some cases, this approach does not fully restore affinity to the target, and affinity maturation technologies must be applied (described elsewhere in this book).

Guided selection is a method to convert murine antibodies into fully human antibodies with similar binding characteristics [29]; Mouse VH and VL domains are sequentially or in parallel replaced by human VH and VL domains, respectively, followed by phage selection to derive human antibodies with higher affinities. A potential disadvantage of the guided selection approach is that shuffling one or both antibody chains can result in an epitope drift [30]. To maintain the epitope recognized by the source non-human antibody, CDRs can be conserved. In this alternative method, one or both non-human CDR3s are commonly retained as they tend to play a critical role in the recognition of the antigen.

12.11 Selection of Isotypes

Therapeutic antibodies are usually of the IgG subclass as IgGs are highly abundant in serum with g/L concentrations. IgGs are well understood as a drug class and the pharmaceutical industry has implemented platform processes for manufacturing. Four isotypes of IgGs exist in humans with slightly different properties, and only IgG3 is not exploited due to its complex structure. IgG1, IgG2, and IgG4 differ in their affinity to Fcγ receptors and the complement receptor C1q. Consequently, the immunologic effector functions of IgG Isotypes vary in strength [14].

IgG1 is a commonly used isotype and considered to have the strongest effect on "antibody-dependent cellular cytotoxicity" (ADCC) and "complement-dependent cytotoxicity" (CDC), and both effects can contribute to efficacy. If the engagement of the immune system is not necessary for therapeutic efficacy, IgG2 and IgG4 are used as isotypes, because these isotypes do not bind to Fcγ-RIII, the only Fc-receptor expressed on natural killer cells. Nevertheless, cytotoxic activity has been described for some IgG2- and IgG4-based antibodies, which likely resulted from the engagement of neutrophil granulocytes [31].

The use of the IgG2 isotype brings in additional challenges as structural heterogeneity occurs due to connectivity of disulfide bonds within the hinge region, resulting in at least three variants with differing disulfide bonds [32]. Nonetheless, IgG2 hinge region homogeneity with uniform disulfide bonds can be readily achieved using a modified IgG1 hinge [33].

The hinge region of the IgG4 heavy chain comprises two cysteine residues that can form either an intrachain disulfide bond or two interchain disulfide bonds, resulting in the abundance of "half antibodies" comprising only one heavy and one light chain. Therapeutic IgG4 antibodies can engage in Fab-arm exchange with endogenous human IgG4 in vivo, resulting in the functional monovalency of the therapeutic mAb, with the loss of binding avidity and cross-linking effects. Gemtuzumab is an IgG4 mAb and contains a hinge modification, whereas natalizumab, another IgG4, does not [34]. Natalizumab

showed significant levels of Fab-arm exchange in 15 of 16 patients, as early as 1 h after infusion [35]. Therefore, therapeutic IgG4 antibodies should be designed to prevent the Fab-arm exchange by introducing the S228P mutation to stabilize the hinge region, and, more importantly, the R409K mutation to stabilize the CH3 region [36].

Further approaches to modulate effector functions of mAbs are based on glycoengineering and mutagenesis. Approaches to enhance effector functions have been described elsewhere [37].

Several mutations to abrogate ADCC and/or CDC have been described in the literature. One common strategy is to incorporate natural motifs from IgG2 and IgG4 to decrease binding to Fcγ-Receptors (Fcγ-R) and complement, respectively, while keeping potential immunogenicity of the changes to a minimum. For example, Armour et al. [38] replaced residues 233–236 in the lower hinge region of IgG1 responsible for Fcγ-R binding with the corresponding residues from IgG2, and introduced IgG4 residues at position 327, 330, and 331 to abolish binding to C1q. Another variant that is widely used is the double mutation L234A, L235A ("LALA") that has been shown to abrogate binding to Fcγ-RI, Fcγ-RIIa and Fcγ-RIIIa [39]. Several other mutations have been described disrupting the interaction with FcγR and with C1q [40].

Glycoengineering is another method to modulate effector functions. Antibodies contain a biantennary *N*-glycosylation on N297 and it has been shown that a mutation to alanine of glutamine disrupts the interaction with FcγR and C1q [40].

12.12 Heterogeneity of Therapeutic mAbs

mAbs can be theoretically described by a defined primary structure of amino acids of around 150 kDa. In reality, a single dose of a therapeutic mAb product represents a mixture of numerous mAb variants. Those variants are inherent to the biotechnological processes used to manufacture drug products. Some variants are formed at a transcriptional level leading to misincorporation of amino acids and ultimately delivering a different amino acid sequence than originally planned [41]. Yet, most of the variants are caused by posttranslational modifications (PTM)) or are generated after secretion during the manufacturing process. A description of the major quality modifications leading to multiple mAb variants are listed in Table 12.1.

12.13 Quality Attributes of mAbs Can Impact Safety and Efficacy

Some of the quality attributes listed in Table 12.1 may impact the safety and efficacy of the therapeutic mAbs. As described earlier in this chapter, *aggregation* triggers potential immune responses, especially neutralizing antibodies that limit efficacy, as well as severe immediate hypersensitivity responses such as anaphylaxis [42]. *Fragmentation and clipping* impact the function of a mAb [43]. In the case of fragmentation in the CDRs or the

Table 12.1 Quality modifications leading to multiple mAb variants

Quality attribute	Definition
N-terminal pyroglutamate	N-terminal glutamine of heavy chains is partially or completely derivatized to pyroglutamate resulting in more acidic antibodies
Deamidation	Asparagine residues are, via succinimide intermediates, converted into aspartate and iso-aspartate residues. Glutamine residues can also undergo deamidation resulting in glutamate residues. The kinetics of this reaction is slower than with asparagine
C-terminal lysine truncation	Truncation of the C-terminal lysine of antibodies due to carboxypeptidase activity, resulting in charge heterogeneity since the number of charged terminal lysine residues per antibody molecule maybe 0, 1, or 2 (K0, K1, K2 respectively). Loss of lysine residues results in a decrease in positive charge and more acidic antibodies
Oxidation	Oxidation is a covalent modification of an amino acid that is induced by reactive oxygen. mAb oxidation occurs predominantly on methionine residues. Oxidation of methionine to methionine sulfoxide makes the side chain of methionine more polar
Glycation	Glycation results in non-enzymatic glycosylation at the protein amine group on lysine side chains. It occurs without the controlling action of an enzyme when the protein is incubated in the presence of sugars
Glycosylation	For most of the recombinant mAbs, glycosylation represents *N*-linked oligosaccharides (biantennary complexes) with a core fucose with 0, 1, or 2 terminal galactose residues, and various truncated structures with the loss of the core fucose, *N*-acetyl-glucosamine (GlcNAc), or both (Fig. 12.2). This allows for a possible total of 32 different oligosaccharides and potentially more than 400 glycoforms
Fragmentation/ clipping	Fragmentation/clipping describes the disruption of a covalent bond in a mAb because of either a spontaneous or enzymatic reaction. Fragmentation can occur during cell culture, is modulated by the purification process and will continue during storage
Aggregation	mAbs may aggregate with each other by association, either without any changes in primary structure (physical aggregation) or by the formation of new covalent bonds (chemical aggregation). Both mechanisms can occur simultaneously and may lead to the formation of either soluble or insoluble aggregates, depending on the protein, environmental condition, and stage of the aggregation process

constant regions of mAbs, potency is impacted, and the Fc-mediated effector function is reduced, respectively. *Oxidation* can significantly alter the biological activity of a mAb [44] and reduce serum half-life [45]. *Deamidation*, by changing the secondary and the tertiary structure of the therapeutic mAb, may reduce biological activity. For example, the induced deamidation of trastuzumab or panitumumab dramatically reduces mAb potency [46, 47]. *Glycosylation* of IgG-Fc is essential for optimal expression of biological activities mediated through Fcγ-RI, Fcγ-RII, Fcγ-RIII, and the C1q component of complement

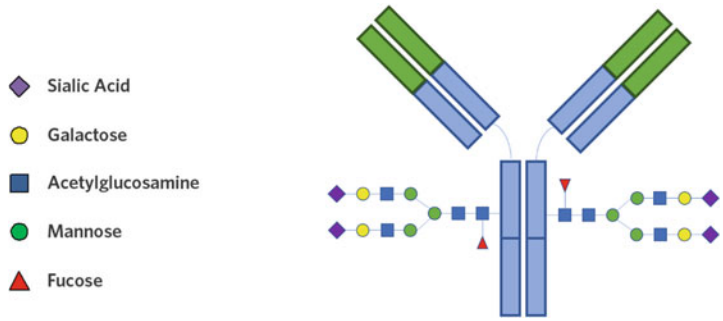

Fig. 12.2 Biantennary glycosylation of mAbs. Monoclonal antibodies are *N*-glycosylated on asparagine 297 forming a biantennary glycan. The structure of the glycan can vary between hosts, the figure shows the schematic representation of an antibody produced in CHO cells. For most of the recombinant mAbs, glycosylation is built on a core fucose with 0, 1, or 2 terminal galactose residues, and various truncated structures with the loss of the core fucose, *N*-acetyl-glucosamine (GlcNAc) or both. This allows for multiple different oligosaccharides structures and potentially more than 400 glycoforms. Glycosylation influences antibody efficacy due to involvement in Fcγ-R binding, pharmacokinetics and is a source of product heterogeneity

(Fig. 12.2). The absence of Asn297-linked glycosylation significantly reduces but does not eliminate effector functions and complement activation of the therapeutic mAb [48]. For instance, the removal of the complete carbohydrate moiety of alemtuzumab abolished complement lysis activity and ADCC but left antigen-binding activity and protein A binding activity intact [49]. Aglycosylated cetuximab did not bind to Fcγ-RI or Fcγ-RIIIa, nor did it have any ADCC activity, whereas the glycosylated molecule showed high receptor-binding affinity and high ADCC activity [50]. The impact of glycosylation on pharmacokinetics, antigen binding, and immunogenicity is further described in the literature [51]. Therefore, to develop a successful therapeutic mAb, it is key to minimize and control the level of mAb variants in the drug product.***

12.14 Protein Engineering to Control mAb Variants

Proper engineering of the mAb sequence is the first approach to minimize potential mAb variants and limit drug product heterogeneity [52]. Typically, amino-terminal glutamine is selected to force the formation of pyroglutamate and reduce the number of charge variants. *N*-glycosylation sites are removed from the V_L and the V_H to prevent the formation of *N*-glycoforms. Mutation of amino acids is introduced to prevent aggregation. Carboxy-terminal lysine is deleted from the sequence by proteolytic cleavage. Methionine in CDRs is avoided to prevent impactful oxidation. The improvement of the mAb sequence can be performed in-silico using dedicated software and/or experimentally by stress studies of mAb candidates [53].

12.15 Improved Manufacturability to Control mAb Variants

Manufacturability is an important aspect of mAb therapeutics development. It is defined as the ability of a mAb drug product to be safe, to maintain pharmaceutical activity over shelf-life, and to be manufactured in a cost-effective and highly reproducible manner. Good manufacturability is mandatory to minimize and control the level of therapeutic mAb variants in the drug product. It is achieved via extensive small-scale process development experiments.

Achieving good manufacturability is a challenging task as mAbs are intrinsically heterogeneous in composition. Often, development programs of therapeutic mAbs are abandoned because of poor manufacturability. Knowing that drug substance and drug product quality attributes are affected by minimal changes in the manufacturing process, everything that happens in the process of synthesizing a mAb in a cell, isolating it from the production system, and purifying it (as well as the choice of materials and methods inherent to the development of the process) may be critical to the quality of that material and patient safety. Therefore, "The process is the product!" is a statement often made in the context of biological therapeutic products [54].

To improve the manufacturability of a mAb, the FDA and other regulatory agencies initiated the Quality by Design (QbD) approach in the early 2000s. The QbD initiative provides guidance on pharmaceutical development to facilitate the design of products and processes that maximizes the product's efficacy and safety profile while enhancing product manufacturability. This risk-based concept links product quality with development and manufacturing activities. Nowadays, QbD activities are fully integrated into product development phases from first toxicology studies to clinical phases and post-marketing activities. The main QbD milestones and deliverables are:

- A *Target Product Profile (TPP))* documenting the intent of the product and its desired features (indication, desired efficacy and safety claims, desired drug format, etc.).
- A *Quality Target Product Profile (QTPP))* summarizing the quality characteristics of a pharmaceutical product that will ideally be achieved to ensure the desired quality, taking into account both safety and efficacy. Attributes represented in the QTPP relate to the intended use of the drug product and are those that impact the patient (route of administration, dosage form, bioavailability, strength, stability, etc.).
- A risk assessment of the product quality attributes based on prior knowledge, clinical and non-clinical studies (including toxicology) to identify the *Critical Quality Attributes (CQA)*.
- A *Process Risk Assessment (PRA)* identifying the manufacturing parameters (inputs) for each process step, such as cell density and integrated cell viability for an upstream process or load temperature, load pH, and load conductivity for a column chromatography step that, if varied, have the potential to impact a CQA.
- A *Process Characterization* study that examines the deliberate variation of the parameters identified as potentially critical by PRA to determine the acceptable limits

of variation. Process characterization studies lead to the classification of process parameters as either *critical process parameters (CPP)* or non-critical process parameters. CPPs are defined as a process parameter whose variability impacts a CQA and must be controlled.

- A *Design Space* representing the multidimensional combination and interaction of input variables (e.g., material attributes) and process parameters that have been demonstrated to provide assurance of quality.
- A defined *control strategy* based on the design space and the CPPs describing in detail the Input Material Controls, the In-Process Controls, the Parameter Controls, the specifications, the product characterization, and the process monitoring.
- *Qualification studies* of the facilities, the utilities and the equipment plus a process *performance qualification study (PPQ)* performed at full-scale mimicking final commercial manufacturing operations.
- *Continued process verification studies* to demonstrate that the process validation package remains up to date across the lifecycle of the product.

12.16 mAb Industrial Manufacturing

Manufacturing of a therapeutic mAb is initiated by the thawing of a cryogenic vial containing the cells. This cryogenic vial is part of a bank of the production cell line. To generate the production cell line, the host cells are transfected with the expression vector and cell clones are screened to find antibody-producing cells. The goal of this transfection and screening process is to select a cell line that grows well and is genetically stable in culture, and that produces high levels of the product in its active form (properly assembled, folded, glycosylated and not fragmented or aggregated). Once a cell line is established, it must be fully characterized and banked for use in long-term production. The first mAb products that entered the market were produced in several different mammalian host cell lines including the original hybridoma or the murine myeloma cell lines SP2/0 and NS0. However, as more mAb products were developed, the biopharmaceutical industry focused its efforts on the use of various CHO cell lines for the production of these products. As a result, CHO cells are currently the dominant host cell line for the production of mAb products.

Every bioprocess requires a synthesis stage, a recovery stage, and a purification stage. The synthesis stage is an upstream processing (USP) activity, while the recovery and purification stages are downstream processing (DSP) operations. The downstream process consists of several steps that deliver the active pharmaceutical ingredient (API) (also referred to as bulk drug substance, or just drug substance (DS)) to the final stage of the biomanufacturing process, formulation and filling (Fill and Finish). In this stage, a sterile filtered DS is transferred to a storage buffer and filled into vials. The formulated DS is called a drug product (DP) and is ready to be given to the patient. The typical manufacturing process for recombinant mAb drug substance is described in Fig. 12.3.

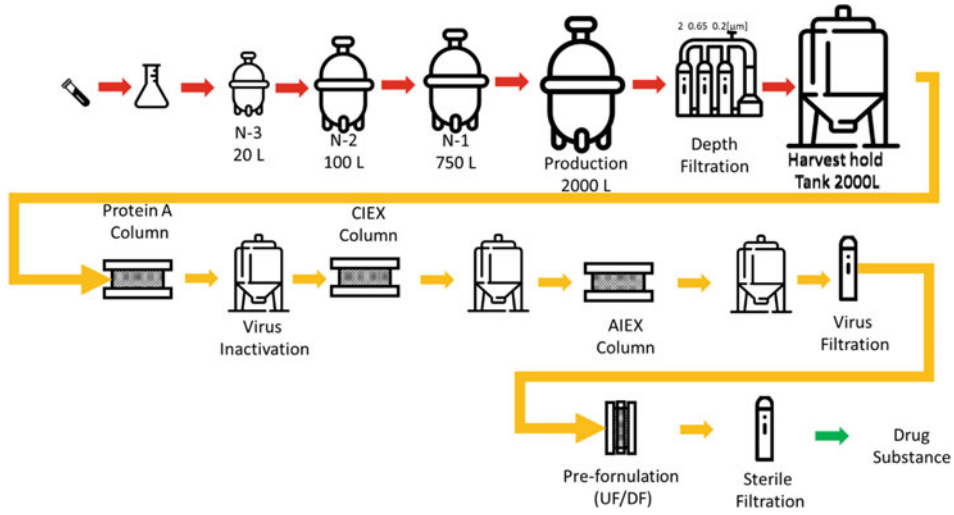

Fig. 12.3 Example of a mAb manufacturing process at 2000 L-scale. Manufacturing is initiated by the thawing of a cryogenic vial containing the cells. The cells are expanded via a series bioreactor to reach a defined cell density to inoculate the production bioreactor. The recovery and purification consist of several process steps that deliver the active pharmaceutical ingredient (API) (also referred to as drug substance (DS)). Between 3 and 6 kg of mAb Drug Substance can be manufactured per batch

All manufacturing operations must be performed under current Good Manufacturing Practices (cGMP). GMP are pharmaceutical regulations into the manufacturing of pharmaceuticals assuring quality, safety, traceability, and reproducibility of the drug product. The GMP is described and audited by several health authorities such as WHO, FDA, or EMA. Basic principle of GMP are listed below:

- Manufacturing facilities must maintain a clean area and a manufacturing area.
- Manufacturing facilities must maintain controlled environmental conditions to prevent cross-contamination from adulterants and allergens that may render the product unsafe for human consumption or use.
- Manufacturing processes must be clearly defined and controlled. All critical processes are validated to ensure consistency and with specifications.
- Manufacturing processes must be controlled, and any changes to the process must be evaluated. Changes that affect the quality of the drug are validated as necessary.
- Instructions and procedures must be written in clear and unambiguous language using good documentation practices.
- Operators must be trained to carry out and document procedures.
- Records must be made, manually or electronically, during manufacture that demonstrate that all the steps required by the defined procedures and instructions were in fact taken

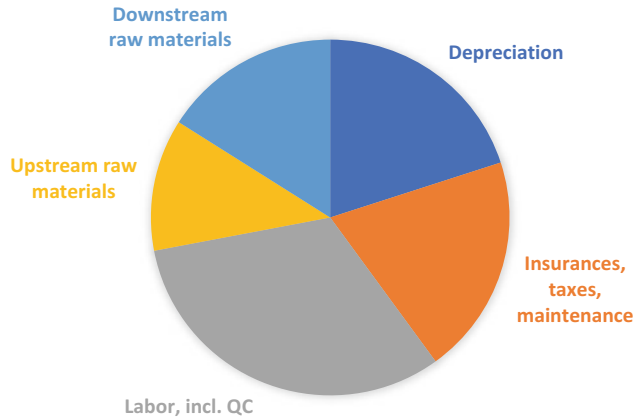

Fig. 12.4 Example of cost categories for mAb manufacturing

and that the quantity and quality of the food or drug were as expected. Deviations must be investigated and documented.

- Records of manufacture (including distribution) that enable the complete history of a batch to be traced must be retained in a comprehensible and accessible form.
- Any distribution of products must minimize any risk to product quality.
- A system must be in place for recalling any batch from sale or supply.
- Complaints about marketed products must be examined, the causes of quality defects must be investigated, and appropriate measures must be taken with respect to the defective products.

The typical cost of manufacturing goods (CoG) for therapeutic mAb drug substance at a commercial scale ranges between 30 and 60$/g. The CoG performance is, however, highly dependent on the overall process performance (yields, productivity, raw material cost), the manufacturing facility utilization, and local taxes or royalties. An example of cost categories for mAbs is described in Fig. 12.4 [55].

12.17 Considerations for Intended Use and mAb Formulation into Drug Product

The intended use for mAb therapeutics is considered very early in the product development program. Typically, the first version of the Target Product Profile (TPP) is proposed during the pre-clinical development stage. This TPP is typically considered the intended shelf-life of the drug product, the mode of administration, and the need for a specific device (for instance an auto-injector).

The complex structure of therapeutic mAb combined with their size is a challenge to develop delivery methods other than parenteral administration. For pharmaceutical

products, oral delivery is generally the preferred method. Yet, oral administration of therapeutic mAb is limited by gastrointestinal degradation, low bioavailability, and slow uptake [56]. Some early therapeutic development programs are exploring delivery systems that can pass into the intestinal tract without being digested or an administration via the nasal and pulmonary routes. However, today those are limited by many of the same drawbacks as oral administration, including gastrointestinal degradation and low bioavailability. Therefore, most therapeutic mAb are administered by intravenous (i.v.) or subcutaneous (s.c.) injections, with injections being the primary delivery system for mAbs.

Both lyophilized and liquid forms can be considered when developing a suitable intravenous or subcutaneous formulation of a mAb product. However, the recent trend is to favor liquid formulation as this is frequently easier and faster to develop. Liquid formulation contains fewer components than a lyophilized formulation and does not require as much formulation—or any lyophilization cycle optimization. Moreover, recent years have seen an increased focus on self-administered delivery systems, such as Pre-filled Syringes (PFS) or Pen devices. Self-administration offers the advantages of increased patient compliance, greater ease of use, reduction of pain, and reduced risk of dosing error as compared to other modes of injections. Self-administered delivery systems are easier to develop when containing a liquid formulation.

12.18 Formulation Development

Degradation of a mAb can result from either the chemical degradation of specific amino acid residues in the mAb, physical degradation caused by the loss of tertiary structure, or the covalent or non-covalent aggregation of mAb monomers to form aggregates in solution. Formulation development aims at achieving acceptable long-term stability and high protein concentration in a liquid formulation. This is usually achieved by screening a variety of excipients to provide control of pH and tonicity of the solution, stabilize the mAb structure, prevent surface denaturation or adsorption of the product to container/closure surfaces and reduce degradation of the product (Table 12.2). Excipients used in the formulation of mAb products should ideally meet the appropriate USP, included in the FDA Inactive Ingredient Database or those that comply with the standards for the manufacture of pharmaceutical grade excipients set by the International Pharmaceutical Excipient Council (IPEC). On top of the appropriate buffer to control the pH of the formulation, excipients could be:

- Sodium chloride to control the osmolality
- Surfactants (i.e., polysorbate) to control interaction with the surface of the container closure or the air–liquid interface
- Cryoprotectants (i.e., sucrose or trehalose) to stabilize a mAb during long-term storage as a frozen liquid
- Chelating agents (i.e., EDTA) if the mAb is sensitive to metal ion catalyzed oxidation

Table 12.2 mAb formulation examples

Product name (INN name)	Presentation	Concentration (mg/mL)	Buffer and excipients	Storage condition (°C)	Shelf-life (months)
Actemra (tocilizumab)	Liquid/ syringe	180	Histidine, Arginine, Methionine, Polysorbate 80, pH 6.0	2–8	30
Avastin (bevacizumab)	Liquid	25	Sodium Phosphate, Trehalose, Polysorbate 20, pH 6.2	2–8	24
Enbrel (etanercept)	Liquid/pen/ syringe	50	Sodium Phosphate, Arginine, Sucrose, Sodium Chloride, pH 6.16.5	2–8	30
Humira (adalimumab)	Liquid/pen/ syringe	100	Mannitol, Polysorbate 80, pH 5.2	2–8	24
Orencia (abatacept)	Liquid/pen/ syringe	125	Sodium Phosphate, Sucrose, Poloxamer 188, pH 6.8–7.2	2–8	24
Simponi (golimumab)	Liquid/pen/ syringe	100	Histidine, Sorbitol, Polysorbate 80, pH 5.5	2–8	24
Zinbryta (daclizumab)	Liquid/pen/ Syringe	150	Sodium Chloride, Sodium Succinate, Succinic Acid, Polysorbate 80, pH 6.0	2–8	36

Viscosity, solubility, aggregation, and opalescence are limiting the development of high-concentration antibody formulations (>100 mg/ml). To facilitate the subcutaneous distribution of injection volumes of several milliliters over a greater area and enable painless administration, recombinant human hyaluronidase (rHuPH20) has recently been included in liquid subcutaneous presentations of some mAb (Herceptin SC and MabThera SC). Other approaches, such as the addition of amino acid blends, have been designed to stabilize proteins in high concentration formulations, minimizing aggregation, reducing viscosity, and improving syringeability. Some liquid formulation of mAb can reach concentration up to 200 mg/ml using this approach [57].

12.19 Closing Remarks

In summary, the therapeutic performances of a recombinant mAb does not only depend on its mode of action, its potency, or its affinity to a target. Regulators expect a clear demonstration of controlling and minimizing critical quality attributes in order to prevent safety or efficacy concerns. This is achieved by proper engineering of the molecule and a sound control of manufacturing parameters. The success of a therapeutic mAb is also dependent on its ease of use for the patient or the health care professionals and, obviously, its cost of manufacturing and distribution. Therefore, a good manufacturability and a marketable intended use are key elements to consider.

Take Home Messages

- Therapeutic monoclonal antibodies (mAb) are safe and efficacious injectable drugs; however, some safety issues can occur. Besides side effects that are caused by the activity on target, immunogenicity can play an important role in antibody side effects.
- In general, the more "human like" the structure, the less immunogenic is an antibody. While rodent and chimeric antibodies can cause a vaccination type of immune response involving T cells, humanized and human mAbs usually cause a reversible B cell driven immune response.
- Side effects can also be caused by the Fc-part of mAbs which recruits certain Lymphocytes and elements of the complement system. This can be enhanced by antibody aggregation.
- Several protein engineering technologies exist to reduce the risk of side effects mediated by the host immune system including humanization and Fc engineering.
- MAbs are highly complex and heterogeneous molecules. This heterogenicity is inherent to the biotechnological processes used to manufacture drug products. Some mAb variants are impacting the safety and the efficacy of the product.
- The mAb variants can be minimized by: (1) using proper molecule engineering technology and (2) by a strict understanding and control of every step involved in the manufacturing process.
- The guarantee, consistency, traceability, and quality of the drug substance, the manufacturing process of a therapeutic mAb must comply with the latest process validation guidelines and the good manufacturing practices requested by health authorities.
- The success of a therapeutic mAb is also dependent on its ease of use for the patient or the health care professionals and, obviously, its cost of manufacturing and distribution. Therefore, a good manufacturability and a marketable intended use are key elements to consider.

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