Chapter 1
Monoclonal Antibodies: Structure, Physicochemical Stability, and Protein Engineering

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1 Structure of Monoclonal Antibodies

1.1 General Structure

A schematic diagram of the general structural features of mAbs is outlined in Fig. 1. The structure of a mAb molecule involves one pair of heavy weight polypeptides (heavy chains, HC) of 50 kDa each and one pair of lightweight polypeptides (light chains, LC) of 25 kDa each. The four chains are held together by hydrophobic interactions and interchain disulfide bonds to form a Y-shaped quaternary structure. According to the chemical structure, mAbs are classified into five classes or isotypes: IgA, IgG, IgD, IgE, and IgM. Since the vast majority of therapeutic mAbs are IgGs, the focus of this section is on the structural and functional properties of this class.

With regard to the amino acid sequence of IgGs, each of the two HC consists of one variable domain (VH) that is unique for each mAb and three domains that are constant across IgGs (CH1, CH2, and CH3). Similarly, each of the two light chains is comprised of one variable domain (VL), but only one constant domain (CL). Unlike the constant heavy domains, the chemical structure of the CL domain varies across IgG mAbs and results in two functionally similar isotypes: kappa and lambda.

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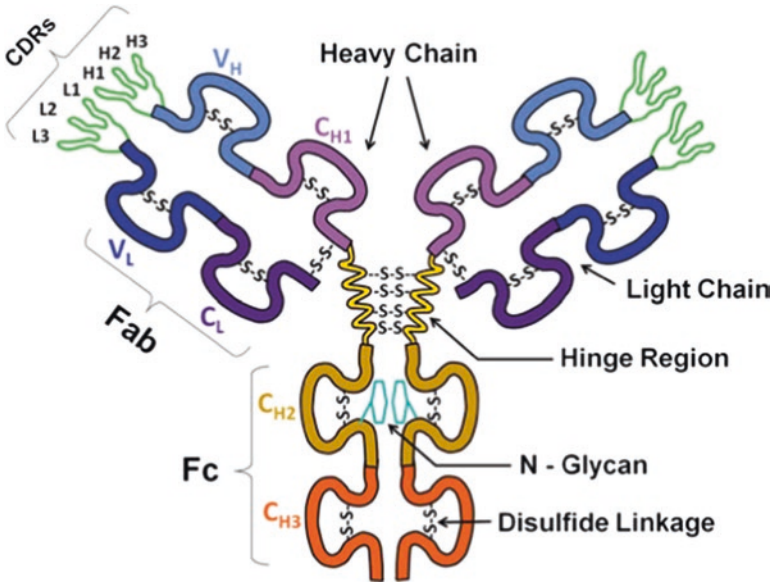

Fig. 1 Schematic diagram of the general structural features of monoclonal antibodies. Reprinted with permission from Moorthy et al. [3] Fab: antigen-binding fragment. Fc: crystallizable fragment. CDR: complementarity-determining region. L1-L3: CDR loops in the light chain. H1-H3: CDR loops in the heavy chain

Each set of the VH, CH1, VL, and CL domains forms one of two identical arms of antigen-binding fragments (Fabs), which are responsible for the specific function of the mAb. The two Fab fragments form the V-shaped head of the mAb structure. The neck of the Y-shaped mAb structure is termed the hinge region and is formed by a random coil structure that connects the CH1 and CH2 domains and hence connects the Fab arms with the tail of the mAb. IgG molecules are classified into five subclasses numbered 1–5 according to the length of the hinge region and the number of interchain disulfide bonds holding it together. The tail of the mAb structure, termed the crystallizable fragment (Fc), is formed by the CH2 and CH3 domains of the two HCs and is responsible for the effector functions and the pharmacokinetic properties thereof.

Each of the different domains constituting the polypeptide chains of the mAb is comprised of approximately 70–110 amino acids, which form 9–11 beta-sheets that are structurally organized into a beta-barrel structure. Each two adjacent domains interface through their hydrophobic regions resulting in one CH2-CH2, one CH3-CH3, two VH-VL, and two CH1-CL pairs. The hydrophobic interactions between the two CH2 domains are limited compared to the other pairs due to the coverage of the hydrophobic regions by glycan groups. The following subsections describe the features of the three main functional components of IgG mAbs: Fc, Fab, and the hinge region.

1.2 Crystallizable Fragment (Fc)

The Fc fragment constitutes the tail region of the Y-shaped mAb structure and contains the regions that bind to receptors and proteins that mediate several physiological functions. The interface of the hinge region and the CH2 domain contains the binding regions to complement proteins, as well as the Fc receptors (FcRs) typically present on the surface of the innate immune cells. The region connecting the CH2 and CH3 domains contain the binding sites for the neonatal Fc receptors (FcRn), protein A, and protein G.

Binding to Fc receptors stimulates the release of several inflammatory mediators and activates antibody-dependent cellular cytotoxicity (ADCC), whereas binding to the neonatal Fc receptors decreases elimination and increases plasma half-life by recycling the mAb molecules pinocytosed into the epithelial cells back into the blood stream.

In addition to the binding sites, the CH2 domain also contains a conserved Asn-X-Ser/Thr consensus sequence wherein the Asn residue at position 297 in each heavy chain is linked to a N-glycan group. This posttranslational modification plays a role in the binding to FcRs and is known to affect the pharmacokinetic profile of mAbs. It can also affect the solubility, stability, and immunogenicity of the molecule.

In general, the glycan group in mAbs is composed of an N-acetylglucosamine core structure connected to a bisecting mannose structure that is extended by additional N-acetylglucosamine molecules. This core structure can be further modified by the addition of fucose to the core N-acetylglucosamine and/or by the addition of terminal galactose molecules to the mannose arms. Typically, 1–3 major variants of the glycan group are formed during the expression of mAbs in cell culture.

1.3 Hinge Region

The hinge region is a random coil structure comprised of 12–62 amino acids depending on the IgG class [4]. It contains several proline residues that render the structure flexible and solvent accessible. Such flexibility allows the two arms of the Fab fragment to move relative to each other, a process that facilitates antigen binding. Solvent accessibility of this region, however, renders it more vulnerable than the rest of the mAb structure to clipping [5] and disulfide scrambling [6]. The former results in separation of the Fab and Fc fragments, whereas the latter may impact the biological activity and/or the effector functions of the IgG. In addition to proline residues, the hinge region also contains one or more cysteine residues that form interchain disulfide bonds, the position and the number of which vary in the different IgG subclasses. For example, in IgG1, the most commonly used IgG subclass in therapeutic mAbs, the hinge region consists of 15 amino acids and contains 2 interchain disulfide bonds that hold the two heavy chains together.

The interface of the hinge region with the CH2 domain of the Fc fragment has been shown to be involved in the binding to the Fc receptor [7] and hence plays a role in mediating the effector functions of the mAb.

1.4 Antigen-Binding Fragment

Each of the two variable domains (VH and VL) in each Fab arm contains several beta strands connected by loop structures. Each domain contains three loop structures that are spatially adjacent, although not sequentially consecutive. Together, the six loops in the variable light and heavy chains of each arm form the complementarity-determining region (CDR) of the mAb, which constitute the antigen-binding site of the Fab fragment [8]. The length and the amino acid sequence in these loops are highly variable across the different mAbs and hence determine the specific function.

Glycosylation in the CDR region has been shown to decrease antigen binding in some cases [9, 10] but increases binding affinity in another case [11]. Also, glycosylation in the variable chain close to the CDR region was found to affect antigen binding and specificity of a model mAb [12].

2 Chemical and Physical Degradation of Monoclonal Antibodies

In the development of an antibody-based therapeutic, a comprehensive assessment of the physicochemical properties of the molecule is completed to ensure that it exhibits the necessary attributes required for a successful clinical candidate. The route and rate of degradation are evaluated to determine the robustness of the molecule to external stressors that may be encountered during the product life cycle including manufacturing, shelf life, and administration. The type of degradation observed can be classified into two main categories: physical or chemical degradation. Although extrinsic factors such as solution conditions and temperature can modulate the observed degradation, intrinsic properties can provide insights into the susceptibility of a certain mAb for undergoing different types of degradation.

2.1 Physical Degradation

Aggregation

The primary mechanism of physical degradation occurs through the self-association of protein species or aggregation. As aggregation can be triggered by protein unfolding, preserving the overall fold (secondary and tertiary structure) is a key component

in achieving desirable drug-like properties. Under normal conditions, monomeric species exist in their folded state due to the energy barrier that prevents the protein from occupying an aggregation-prone state [13]. But, stressed conditions alter these energy barriers and shift the balance to population of the unfolded and aggregation-prone species [14]. Because antibodies contain multiple domains and regions susceptible to self-association, aggregation proceeds through the following intermediate stages [15, 16]:

(i) Protein unfolding: disruption of the energy barrier equilibrium to favor partially unfolded states [17]
(ii) Protein association: interaction between aggregation-prone regions (modulated by hydrophobicity or charge) of the unfolded monomers [18–20]
(iii) Nucleation (rate-limiting step): structural rearrangements to promote additional aggregation including alterations in surface charge, exposure of hydrophobic regions, and/or secondary structure changes to the more energetically favorable β-sheet orientation [14, 21, 22]

As the majority of aggregation is unfolding-driven, understanding the factors that contribute to the conformational stability will aid in limiting the aggregation propensity of the molecule. The domain unfolding which is responsible for aggregation initiation can vary as the aggregation-prone regions can be different from one mAb to another. In some instances, the CH2 domain unfolds first and triggers the aggregation process [23, 24], whereas it has also been shown that the unfolded Fab domain is more aggregation prone than the CH2 domain [25]. The susceptibility to aggregation is defined by the intrinsic properties of the molecule (primary and tertiary sequence) [16], but external factors (pH, excipients, temperature, sheer stress, and antibody concentration) also lead to structural fluctuations, which in turn affect unfolding and aggregation [16, 21, 26–28]. Because pH modulates the thermodynamics of unfolding of the domains, changes in solution conditions can alter which domain unfolding leads to aggregation initiation [25]. Stabilization of the CH2 and Fab domains is achieved by increasing the pH from acidic to near-neutral conditions [25, 29], with destabilization occurring at pH values below the pI of the protein [30]. As protein unfolding is also temperature dependent, incubation at temperatures above or below the T_m of unfolding will significantly impact aggregation. Incubation at temperatures above the T_m of unfolding can lead to loss in secondary and tertiary structural elements, which cause a higher degree of aggregation due to exposure of the hydrophobic core [31]. The aggregation nucleation rate also increases as the T_m of unfolding is approached [32], whereas the unfolding rate significantly decreases at temperatures closer to those used for long-term storage [30]. Finally, exposure of the protein to the air-liquid interface occurs during shear stress, which facilitates aggregation due to the loss of secondary and tertiary structure [33–35].

In addition to unfolding-induced aggregation, self-association can also occur between fully folded, monomeric species. Although monomeric aggregation can be caused by chemical cross-linking of free sulfhydryl groups or unpaired disulfide bonds [36, 37], colloidal association primarily occurs due to large regions of surface hydrophobicity, which can be present in both the Fc region [38, 39] and the CDRs

[40, 41]. Oftentimes, the CDRs contribute more significantly to colloidal instability due to the high occurrence of hydrophobic residues in these regions necessary for antigen binding [40–42]. In mAbs with global net charges, though, increases in colloidal stability are observed at pH values below the pI due to intermolecular repulsion [16, 30]. While weak interactions correlate with aggregation rates at low concentration [43, 44], it is much more difficult to make such association at high protein concentration due to non-idealities that exist only under high-concentration conditions. Along with molecular crowding leading to a decrease in free space, the higher viscosity that accompanies high-concentration solutions further limits molecule mobility [45, 46]. Even though these effects render higher concentration solutions more prone to aggregation under most types of stress [47], high-concentration solutions of mAbs are self-stabilizing to shear stresses [31, 48–51].

Opalescence and LLPS

Apart from aggregation, physical instability can be present in the form of reversible self-association (RSA), which leads to opalescence and/or liquid-liquid phase separation (LLPS). Opalescence is an optical property caused by Rayleigh light scattering of polarizable particles resulting in a solution state that appears turbid under white fluorescent light. It is due to enhanced light scattering caused by concentration fluctuations that occur near the critical concentration [52]. Per the European Pharmacopoeia, a solution is labeled as opalescent at 3 NTU and above [53]. Even though it may appear that particles have formed, opalescence is caused by soluble proteins and/or non-proteinaceous particles, as filtration leads to no differences in the extent of opalescence [54]. Although opalescence can occur without any aggregation or phase separation [55–58], lower temperatures often result in LLPS after the solution becomes opalescent. Fluctuations in thermodynamic properties (entropy and enthalpy) favor LLPS [59], which results in the formation of two phases with different concentrations, but the same chemical potential [60, 61]. Although LLPS itself is not caused by aggregation, the formation of the protein-rich phase can result in irreversible aggregation due to the high concentration in that phase [61]. The presence of two phases with differing concentrations will also lead to salt partitioning according to the concentration gradient and pH/ionic strength differences between the two phases [62].

Antibody concentration and storage temperature are two key factors that affect opalescence [55, 63–65]. The critical concentration is the concentration at which concentration fluctuations and opalescence are at a maximum. As it is primarily determined by the size of the molecule, the value should be similar for IgG1s. Most studies report the critical concentration as approximately 90 mg/mL [60, 64, 66], but some studies reported a lower range of opalescence (50–75 mg/mL) [67], and others have reported higher concentrations of solutions that have exhibited opalescence (100 mg/mL) [54]. On the other hand, the critical temperature is an intrinsic property that is influenced by the properties of the molecule. The critical tempera-

ture is defined as the temperature at critical concentration. As the temperature approaches the critical temperature, the extent of opalescence increases, but it is reversible upon temperature increase [55]. Another temperature-based parameter, T_{cloud}, is used to define the temperature at which LLPS begins, regardless of concentration. T_{cloud}, instead of B_{22},is a better measure of high-concentration physical instability as it can be measured under high-concentration conditions, whereas B_{22} is measured at low concentration [59].

RSA and opalescence are the result of intermolecular attractions between antibody molecules [55, 56, 68]. Even though antibodies exhibit high sequence similarity, the binding interface responsible for RSA is distinct for each antibody [42, 69, 70]. Most often, it is due to Fab/Fab or Fab/Fc interactions [38, 71], as the CDR region is the main site of sequence heterogeneity and has been widely implicated in intermolecular attractions [42, 70–74]. Because the surface properties of the antibody dictate the type of interactions that occur, pH strongly influences the propensity for RSA and opalescence. At pH values far away from the pI, electrostatic repulsive forces dominate due to the high charge associated with the antibody. At pH values near the pI, though, the net charge reaches its minimum leading to weakened global electrostatic repulsion between molecules. In this instance, short-range interactions such as H-bonding, van der Waals forces, and dipole interactions make significant contributions to the associations that occur between protein molecules. This higher propensity for RSA leads to increased opalescence [54, 56, 60, 67, 71, 75], as well as decreased solubility [54], at pH values near the pI. The type of species that form under conditions favorable for RSA and opalescence vary from antibody to antibody as both monomer-dimer-tetramer species [71] and monomer-trimer species have been reported [54].

As solution conditions affect the types of interactions that exist among molecules, opalescence is strongly influenced by the presence of excipients. Modulation of ionic strength is a commonly employed method to mitigate opalescence, but its effects cannot be generalized as it is dependent on the identity of the salt ion [76–80]. Most reports illustrate the effectiveness of increasing ionic strength in decreasing opalescence. Salt addition aids in masking nonuniform charge distribution on the surface of the protein, thus disrupting protein-protein interactions [54, 60] and causing the high-concentration antibody solution to behave similarly as observed under dilute conditions [71]. In some cases, though, opalescence increases as ionic strength increases [56, 57] due to the dominance of hydrophobic interactions under these conditions [67]. For this reason, arginine is commonly used due to its dual effect as an excipient to both modulate surface charge and weaken intermolecular hydrophobic interactions [81, 82], without altering the structural stability of the molecule [81, 83, 84]. Addition of arginine has also been shown to result in fully monomeric species under conditions favorable for RSA, whereas NaCl addition still led to monomer-dimer-tetramer species [71]. Therefore, elimination of all interactions responsible for RSA through arginine addition is currently the best route for mitigating opalescence.

2.2 Chemical Degradation

Chemical degradation involves the irreversible modification of residues within the protein sequence. Although it is not fully understood, the formation of species containing specific chemical modifications may play a role in the immunogenicity observed upon dosing with antibody-based therapeutics [85–89]. This could be due to both the increased likelihood of aggregation among chemically modified species and the formation of neo-epitopes, which would elicit an immunogenic response [90]. Chemical degradation can occur in any region of the mAb that contains residues prone to modification, but in a study of 37 antibodies, all sites of impactful degradation were located in the CDR [91]. Residues in the CDR are particularly prone to chemical degradation due to the flexibility and high solvent accessibility of this region as discussed earlier. Among the possible chemical degradation pathways, deamidation, isomerization, and oxidation are the primary ones observed in mAbs that can impact both the stability and function of the therapeutic entity.

Deamidation

Deamidation is the most common form of chemical degradation observed in mAbs. It results in the formation of more acidic species through the hydrolysis of the amide side chain of Gln or Asn residues (Fig. 2). The rate of deamidation is dependent on the solvent accessibility and structural flexibility of the region. The presence of

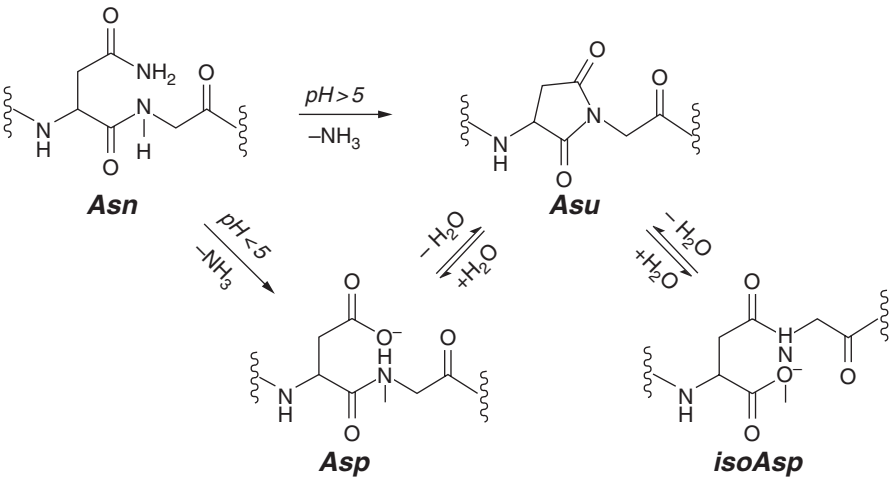

Fig. 2 Asparagine (Asn) deamidation and aspartate (Asp) isomerization. Deamidation of Asn proceeds through a cyclic imide (aspartyl succinimide, Asu) intermediate with loss of ammonia at pH > 5 to produce the Asp and isoaspartate (isoAsp). At acidic pH, the Asn side chain undergoes direct hydrolysis producing Asp product exclusively. Isomerization of Asp to isoAsp also occurs through Asu intermediate

small residues such as Gly or Ser next to an Asn or Gln will increase the likelihood of deamidation in comparison to bulkier residues [26, 92–98]. Extrinsic conditions such as pH, temperature, and buffer also affect the rate and degradation products [99]. At acidic pH (pH < 4), the Gln or Asn residues are converted to their carboxylic acid counterparts (Glu or Asp) [26], whereas at formulation-relevant pH values, deamidation is much slower and proceeds through a cyclic imide intermediate to form either Glu or Asp or their isomers. The succinimide intermediate formed by deamidation of Asn is much more stable than the Gln cyclic intermediate making Asn deamidation much more common [37]. The isoAsp degradant does not only result from the deamidation of Asn, but can also be caused directly by isomerization of Asp [36]. Asp isomerization follows a similar succinimide intermediate as Asn deamidation, and the resulting succinimide intermediate hydrolyzes to either isoAsp or Asp at a 3:1 ratio when at equilibrium [92].

The introduction of a structurally different species or alteration in charge can lead to changes in stability, as well as binding affinity of the resulting molecule. The effect of deamidation needs to be evaluated on an case-by-case basis as it has been reported to lead to decreases in binding affinity, potency, and stability [9, 98–103], whereas others have reported that the resulting succinimide led to no effect on binding affinity [104]. Effects on protein conformation and possibly binding affinity would be expected following Asp isomerization as an additional CH_2 is introduced into the peptide backbone and the side chain is altered [9, 18, 43, 101, 105–110]. The modification lengthens the peptide backbone and imparts additional flexibility [100], which causes substantial conformational changes at both the site of modification and nearby residues [111].

Oxidation

Oxidation is another critical chemical degradation pathway that is monitored during the development of biological modalities. The residues prone to oxidation include Met, Cys, His, Tyr, and Trp [26]. Although Met oxidation is almost always pH-independent, oxidation is generally influenced by both intrinsic and extrinsic properties [26, 112, 113]. Met is the most common residue to undergo oxidation to either Met sulfoxide [114] or sulfone [115]. A set of highly conserved Met residues within the Fc region (Met252, Met248) are especially susceptible to oxidation to Met sulfoxide [116–123]. Oxidation in this region has been shown to decrease stability and Fc receptor binding [116, 124, 125], but both heavy chains must be oxidized at Met252 to significantly affect the clearance [126]. While FcRn binding may be affected by high levels of Met252 oxidation, this modification does not impact the FcγRIIIa binding and subsequent ADCC activity [116]. Modifications in stability and FcRn binding suggest that structural alterations occur upon Met oxidation. The backbone amide hydrogen-bonding network is disrupted due to the presence of Met sulfoxide [124], which is more polar and larger in size than Met. These local conformational changes may affect CDC activity as many interactions occur at the CH2-CH3 interface to modulate CDC activity [127]. Even through the structure may

be altered by Met oxidation, no effects on antigen binding would be expected since the Fc region is not typically involved in antigen interactions. When this modification occurs within the CDR, however, the conformation is stabilized presumably due to the additional interactions present resulting in a slight increase in binding activity [128]. In addition to Met, Trp residues are also prone to undergo oxidation. Trp oxidation is induced by light exposure; photo-ionization can also occur if the residue is in close proximity to disulfide bonds [90]. The effects of Trp oxidation on biological activity are mixed [90, 122, 129]. Wei et al. showed that oxidation of a Trp residue in one of the CDR loops affected antigen binding and the biological function of a model mAb [129]. In a study by Dashivets et al., 94% oxidation of one Trp didn't affect binding in one mAb, whereas 43% oxidation of that same Trp in another mAb significantly affected binding [128]. Even if binding affinity was not affected, higher Trp oxidation led to lower thermal stability and increased aggregation.

Although chemical degradation may seem to only influence a small region within the mAb, this modification can have far-reaching effects that stabilize or destabilize the global structure. A study comparing Met oxidation and Asp isomerization in the CDR showed that although similar regions distinct from the modification are affected by the chemical alteration, Asp isomerization lead to increased flexibility, whereas Met oxidation had the opposite effect [100]. The extent of the structural changes caused by Asp isomerization was also greater in comparison to those elicited by Met oxidation. These results further confirm the necessity in evaluating the effect of individual chemical modifications on mAb structure and function.

N-Terminal Pyroglutamate

Presence of a glutamine on the N-terminus of the light or heavy chain can result in the cyclization of the N-terminal amine to form pyroglutamate (pGlu). In most instances, this posttranslational modification occurs during antibody expression and purification [130–133], most specifically during the bioreactor process. Cheng et al. reported over 90% conversion to pGlu after 15 days in the bioreactor [131]. In cases where pGlu formation does not occur during antibody processing, it has also been shown to occur during storage at accelerated conditions [134]. The conditions under which the antibody was stored strongly influence the rate of pGlu formation. The highest rate was observed at pH 4 and 8, with a minimum at pH 6, and preparation in succinate resulted in a higher percentage of conversion compared to histidine or acetate buffer. pGlu formation can occur on either the HC or LC if there is an N-terminal Gln, but the likelihood of it occurring on one chain preferentially can vary from antibody to antibody. In one case, 99% pGlu conversion occurred in the HC with only 2% in the LC [132]. On the other hand, under accelerated storage conditions, the pGlu conversion rate was slower in the HC vs. the LC [134].

Because pGlu formation results in loss of the N-terminal amine, the isoelectric point of the resulting molecule is altered in comparison to the native molecule. Analysis by near-UV indicated that the tertiary structure was not altered by pGlu formation, but it was hard to determine if structural differences observed by FTIR

were due to pGlu formation or caused by thermal stress [134]. Also, even though pGlu formation occurs within the CDRs, potency is not impacted if this region is not directly involved in the binding epitope [135]. Because pGlu has been observed in human endogenous IgGs, it is not likely a safety concern [136]. But, the presence of both N-terminal pGlu and non-cyclized Gln results in batch-to-batch variations in species, which makes it difficult to analyze and meet the specifications required of an FDA-approved product.

Fragmentation

Although not as widely reported as aggregation, mAbs may also undergo fragmentation through enzymatic or nonenzymatic hydrolysis of the peptide backbone at the hinge region or at a sequence containing either Asp or Trp [26]. Hinge region hydrolysis does not require specific residues. Rather, the rate is dependent on the flexibility and peptide sequence, as well as pH, with higher rates occurring either above or below pH 6 [26, 137, 138]. Asp-associated hydrolysis is also modulated by pH, and the rate is increased if the Asp residue is adjacent to a Ser, Val, or Tyr [26]. In general, the degree of fragmentation is insignificant and results in minimal effects on efficacy.

3 Advances in Protein Engineering to Improve Stability and Efficacy

The specificity of mAbs for their targets makes them highly suitable for use as therapeutic modalities. Their application was originally met with challenges due to inherent instabilities surrounding the primary sequence and the tertiary structure of the molecule, as well as immunological responses to the non-humanized versions [139, 140]. Improvements in hybridoma and recombinant expression technology have led to the generation of fully human forms, but protein engineering has taken it a step further to capitalize on the interactions of the therapeutic with FcRn and FcγRs to maximize half-life and activation of the immune system pathways. These advances in clonal technology have also increased the ease with which mutations can be made to achieve a product with desirable physicochemical properties.

3.1 Modification to Improve Stability

Physicochemical instabilities can arise both from the primary sequence and/or the tertiary structure of the mAb. Evaluation of the primary sequence allows for identification of amino acid segments that may be prone to chemical modification such as deamidation or oxidation. But, prediction of aggregation propensity is much more

difficult as it is reliant upon both the conformational and colloidal stability. Conformational stabilization by disulfide bond addition increased the T_m of the CH2 and CH3 domains by 20 °C and 35 °C, respectively [141, 142]. Removal of free sulhydryls has also been shown to decrease the aggregation propensity as this highly reactive residue is no longer present to cause protein misfolding or covalent cross-linking across monomeric species [143].

Aside from conformational stabilization, aggregation propensity is decreased by removal of aggregation-prone regions (APRs), as well. To successfully predict APRs, both the overall fold of the molecule and the dynamic structural fluctuations that may expose hydrophobic patches must be considered. Early predictive modeling systems had difficulty accounting for both variables, but the recently developed spatial-aggregation-propensity (SAP) model has successfully identified APRs that have been experimentally identified to contribute to self-association [144]. The SAP model gives the effective dynamically exposed hydrophobicity of a certain region on the surface of the molecule normalized to glycine [144]. The high- and low-resolution capabilities allow for it to successfully identify large APRs, as well as identify which residues should be mutated to decrease the hydrophobicity of the region.

Mutation of hydrophobic residues that significantly contribute to the aggregation propensity to a residue more hydrophilic in nature results in an increase in stability [16, 144]. As multiple mutations may lead to the complete removal of the self-association interface, a cumulative effect is observed upon the introduction of three mutations [144]. Greater increases in stability are observed when the hydrophobic residue is replaced with lysine instead of serine due to the larger size of the lysine side chain for shielding the hydrophobic region [144].

More APRs are located in the Fc fragment instead of the Fab fragment, with a large number of them located within the loops in the hinge region and CH2-CH3 interface [145]. Because these regions are very similar across Ig subclasses, most APRs are the same across IgG subclasses (IgG1, IgG2, IgG3, IgG4), and if they are different, the hydrophobic nature is at least conserved. But in the different classes (IgA, IgD, IgE, IgM), different APRs are present due to the structural diversity among the classes [145]. Because this interface contains a consensus motif that is highly hydrophobic in character, mutation in this region increases the solubility and stability of the antibody [144]. In addition to modifying colloidal stability, mutations within the CH2 domain (L234K/L235K) also have been shown to increase the conformational stability of the molecule [144].

While most APRs are located in the Fc domain, removal of APRs in the Fab domain will also decrease aggregation propensity, and some antibodies have also been shown to have larger APRs in the CDR over the Fc [146]. It is more difficult to identify mutations that will be successful at eliminating aggregation and maintain antigen binding within the CDR. In an anti-IL-13 mAb, a triple mutant that removed a hydrophobic patch (Phe-His-Trp to Ala-Ala-Ala) also resulted in decreased binding affinity [40]. For that reason, regions bordering the CDRs are usually targeted for mutation [16], and more success is observed when hydrophobic patches are replaced with negatively charged residues as opposed to positively charged or

neutral residues [16, 143, 147, 148]. Substituting Asp and Glu into HCDR1 and LCDR2 reduced aggregation propensity, but did not impair function as these residues do not directly contribute to antigen binding [148].

As opposed to eliminating the hydrophobic region through mutation, shielding of the hydrophobic region has also been shown to be a successful mechanism for reducing aggregation propensity. The glycan at Asp297 greatly improves the colloidal stability by shielding APRs as aglycosylated mAbs are less stable and more prone to aggregation [149]. Moreover, Voynov et al. demonstrated that disruption of the glycan group interaction with the mAb promotes aggregation [150]. The addition of a hydrophilic glycan near a region of hydrophobicity within the CDR also provides a shielding effect to decrease aggregation, and in this instance, no effect on antigen binding was observed [40].

3.2 Improving Efficacy and Half-Life Through Engineering

The success of an antibody-based therapeutic ultimately relies upon efficacy. Even if the drug-like properties are perfect, a molecule will not be successful if it does not achieve the desired therapeutic effect. For this reason, efforts have been made to engineer antibodies to modulate the effector functions and circulation half-life. The effector functions ADCC, complement-dependent cytotoxicity (CDC), and antibody-dependent cellular phagocytosis (ADCP) are immune responses that result in targeted cell death after interaction between the therapeutic mAb and the appropriate cellular receptor (i.e., FcγR) [140]. Enhancing the effector functions is pursued in antibody therapeutics that target cell surface proteins in certain cancers as it allows for a multiplicative effect of both the therapeutic and an internal cell-killing response. Decreasing effector functions through protein engineering has also been pursued. For example, CDC is linked to injection site reactions [151], and it may interfere with the induction of ADCC [152], so in these instances, it would be desirable to decrease the immune response.

Improved efficacy of mAbs used in the treatment of cancer can be achieved by increasing the affinity of the molecule toward specific FcγRs [153]. Enhancing the affinity to FcγR can be achieved by modulation of either the amino acid sequence or altering the glycosylation pattern [154, 155]. Mutagenesis resulting in the introduction of multiple antibody-FcγR interactions within the lower hinge and proximal CH2 regions will affect the observed response as these regions are critical for FcγR binding [153]. Multiple studies have been published showing the cumulative effect amino acid mutation can have on the affinity of therapeutic mAbs for FcγR due to additional hydrogen bonds, hydrophobic interactions, and electrostatic interactions [156, 157]. In turn, this increased affinity led to substantial increases in ADCC and ADCP [158–160]. Differentiation can also be achieved where mutation results in improved binding between the therapeutic mAb and the activating receptor FcγRIIIa, but reduced binding to the inhibitory receptor FcγRIIIb [159–161]. Finally, the residue chosen for substitution may be critical in modulating the interactions and

observed effects on effector functions. In the K326W variant of rituximab, use of Trp has been shown to be essential for the observed increases in CDC [162].

Although residue mutation can modulate effector functions, the glycosylation pattern of the therapeutic antibody has a far greater effect. For example, Scallon et al. showed that increased sialylation of the glycan group decreases binding to FcRs [163]. Removal of the core fucose of the glycan at position Asn297 results in a 50-fold increase in binding between the therapeutic mAb and FcγR, which leads to increases in both ADCC and ADCP [164]. Low fucosylation is a commonly employed method to increase ADCC and ADCP [165–168], and it has also been shown to increase the antitumor activity of a therapeutic mAb in comparison to its counterpart that had a normal glycan [169, 170]. Complete removal of the glycan, however, has the opposite effect and leads to decreased interaction between the therapeutic mAb and FcγR [171], thus limiting ADCC, CDC, and phagocytosis [172–176]. Effector functions can also be eliminated by using a cross-subclass approach in which the antibody is composed of parts from both the IgG1 and IgG4 subclasses. Elimination of effector functions is not suitable for mAbs used as cancer therapeutics but may be suitable for mAbs whose function does not rely upon ADCC and CDC such as for neutralizing, agonistic, or antagonistic antibodies.

In addition to effector functions, the half-life can be modulated through mutation as it also depends on interactions between the therapeutic mAb and a cellular protein (FcRn). FcRn is the cellular recycling machinery responsible for determining whether the mAb is processed into the lysosome for degradation or released outside of the cell, and this process is highly pH dependent [177–179]. At pH 6–6.5, binding between the therapeutic mAb and FcRn occurs, which leads to recycling outside of the cell. Elimination of these interactions must also occur at pH 7–7.5 to allow for successful release of the mAb from FcRn. For this reason, conserved histidine residues in the CH2 and CH3 domains are essential for this interaction as they become protonated at the acidic pH in the endosome and thus serve as suitable hydrogen bonding sites with FcRn. Mutation of H310 leads to complete loss of the interaction and undetectable binding between the mAb and the FcRn [153].

The addition of new interaction sites along with the conserved histidine residues will lead to increased affinity for FcRn and thus increase the half-life of the therapeutic. In the M428L/N434S mutant, an additional hydrogen bond results in an 11-fold increase in the affinity of the antibody for FcRn at pH 6 [180, 181]. The single mutant N434A also showed increased binding affinity at pH 6, but not pH 7.4, which resulted in increased half-life in cynomolgus monkeys [182]. Mutation of N434 to Trp, though, did not affect half-life as the binding affinity was increased at both pH 6 and 7.4 [182]. Another molecule known as the YTE mutant has been extensively studied due to the effects this mutation has on the in vivo properties of the molecule. This mutant exhibits a fourfold higher half-life in monkeys, tenfold higher affinity for FcRn, and fourfold higher tissue bioavailability in nonhuman primates [183]. The increased affinity is due to the stabilization of the complex by an additional salt bridge between Glu26 of the mutant and Gln2 of FcRn [180]. Although this mutation leads to optimal in vivo properties, the drug-like properties are compromised. Disruption of packing interactions leads to unfolding of a hydrophobic

segment and increased flexibility in the CH2 domain. This results in lower conformational stability and an increase in aggregation in comparison to the parental antibody [184]. Therefore, a balance needs to be achieved when trying to optimize in vivo properties while maintaining adequate physical stability.

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