Engineering the variable region of therapeutic IgG antibodies

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Introduction

Monoclonal antibodies (mAbs) have become an important therapeutic option in numerous diseases and are expected to play a greater role in the future of disease treatment.1,2 Twenty-eight recombinant mAbs are currently approved by the FDA and, with over 200 new candidates entering into clinical trials, the field of mAbs is becoming an extremely competitive field.3 This competition has led researchers to generate so-called second or third generation antibodies with clinical differentiation utilizing various engineering and optimization technologies. Parent IgG antibodies can be engineered to have improved antigen binding properties, effector functions, pharmacokinetics, pharmaceutical properties and safety issues. Although the primary role of the antibody variable region is to bind to the antigen, it is also the main source of antibody diversity and its sequence affects various properties important for developing antibody therapeutics. Here we review recent research activity in variable region engineering to generate superior antibody therapeutics.

Since the first generation of humanized IgG1 antibodies reached the market in the late 1990s, IgG antibody molecules have been extensively engineered. The success of antibody therapeutics has introduced severe competition in developing novel therapeutic monoclonal antibodies, especially for promising or clinically validated targets. Such competition has led researchers to generate so-called second or third generation antibodies with clinical differentiation utilizing various engineering and optimization technologies. Parent IgG antibodies can be engineered to have improved antigen binding properties, effector functions, pharmacokinetics, pharmaceutical properties and safety issues. Although the primary role of the antibody variable region is to bind to the antigen, it is also the main source of antibody diversity and its sequence affects various properties important for developing antibody therapeutics. Here we review recent research activity in variable region engineering to generate superior antibody therapeutics.

Efficient preclinical development of therapeutic antibodies can be facilitated by cross-reactivity of the antibody to rodent or nonhuman primate antigen, since antibody reactive to solely human antigen may require a surrogate antibody for preclinical study, which increases the workload significantly. Difficulty in CMC development largely depends on the pharmaceutical properties of the antibody molecule. Subcutaneous formulation with a long dosing interval, which requires high antibody concentration, could be hampered by either low stability or solubility of the antibody molecule. Formation of heterogeneity during storage, due to deamidation for example, may sometimes require lyophilization to improve the stability during storage, which would be a costly process and inconvenient for the users since it requires reconstitution before use. Therefore, improving such low stability, solubility and chemical stability during the lead antibody optimization step, rather than during the formulation process, would facilitate the development process afterwards. Since the heterogeneity of the antibody molecule needs to be controlled in the manufacturing process, the presence of complex or unexpected heterogeneity may affect the development timeline, so designing the antibody molecule without, or with minimum, heterogeneity would also facilitate CMC development.

Cost of goods (COGs) might also be an important factor for therapeutic antibodies. COGs would be affected by the antibody dosage regimen or durability of the antibody in vivo (which is determined by its pharmacokinetic/pharmacodynamic profile), the expression yields of the antibody and the manufacturing process, such as the number of purification steps and whether the formulation requires lyophilization.

These various drug-related properties of mAbs can be improved by antibody engineering and optimization technologies, which, in the case of whole IgG antibodies, can be classified into two categories: variable region engineering and Fc engineering. Although the variable region is responsible for the antigen binding properties of IgG antibodies, it can also influence their pharmacokinetics, pharmaceutical properties and
immunogenicity. The Fc region is responsible for the effector functions and the pharmacokinetics, and engineering it to optimize the binding to various Fc receptors is reviewed in reference 4. This review focuses on the technologies currently available in variable region engineering of IgG antibodies to improve the antigen binding properties, pharmacokinetics, pharmaceutical properties and immunogenicity (Table 1).

**Engineering the Antigen Binding Properties**

The primary role of the antibody variable region is to bind to the target antigen. Two important antigen-binding properties are the affinity and the specificity, and engineering these properties has been extensively studied in the past two decades (Table 1).

**Approaches to affinity maturation of mAbs.** Binding affinity is one of the most important properties of therapeutic antibodies and, generally, binding to the target with high affinity is an indispensable part of their development. Although high-affinity antigen recognition by antibodies can be achieved by in vivo affinity maturation in the mammalian immune system, generating antibodies with low picomolar affinity in vivo might be difficult due to the affinity ceiling effect of the B-cell responses.6 The limitations of in vivo affinity maturation can be overcome by in vitro affinity maturation, achieving up to femtomolar affinity. Currently there are three approaches available for in vitro affinity maturation: random mutagenesis, targeted mutagenesis and the shuffling approach. The random mutagenesis approach utilizes E. coli mutator bacterial strains,7 saturation mutagenesis8,9 or error-prone PCR10 to generate a broad range of variants of the parent antibody. The targeted mutagenesis approach utilizes alanine-scanning or site-directed mutagenesis, such as look-through mutagenesis,11 to generate limited collections of the specific variants of the parent antibody. The shuffling approach includes DNA shuffling,12 light chain shuffling,13 or CDR (complementarity-determining region) shuffling14,15 to generate shuffled variants of the parent antibody.

An antibody with high affinity is selected from these variants of the parent antibody by display panning technologies. The most general display technology for affinity maturation is phage display using pIII fusion protein16 or pIX fusion protein.17 A variety of other display methods have been reported such as ribosome display,18 yeast surface display,19 E. coli surface display and mRNA display.20 These combinatorial approaches to antibody affinity maturation require a large library size; however, the library size is often limited by the transformation steps following assembly and ligation.22 Ribosome display, a cell free system, allows the generation of large libraries up to 10^{14} members,23 and antibodies with picomolar affinity have been identified.24 The advantages of yeast surface display are that the eukaryotic system offers post-translational modification and processing machinery similar to that of mammals, and flow cytometry can be used to discriminate the variants with close affinity. Boder et al. demonstrated that screening the randomly mutagenized library of a parent antibody in the yeast surface display provided a highest-affinity variant with a dissociation constant of 48 fM, which was a 10,000-fold improvement over the parental antibody.25

Recently, in silico approaches for affinity maturation using computational design have been reported. In the first of these, Clark et al. reported on in silico affinity maturation of a therapeutic antibody targeting integrin VLA1.26 Barderas et al. reported that a 454-fold improvement in affinity over the parent antibody was achieved with a structure-based computational method.27 Lippow et al. reported an iterative computational design method that focuses on electrostatic interactions, with which they successfully improved the affinity of multiple antibodies.28

**Pharmacological effect of affinity maturation.** In the case of an antagonistic antibody, improving the affinity to the target antigen would generally improve the therapeutic efficacy or decrease the minimum concentration at which the antibody is effective in plasma in vivo, therefore enabling the dosage or dosing intervals to be reduced.29-32 Although improving the affinity is effective for antagonist antibody, Rathanaswami et al. mathematically demonstrated that such reduction in the dosage would reach a ceiling at a certain affinity depending on the baseline concentration of the soluble antigen.33 Obviously, due to the stoichiometric binding of the antibody to the antigen, the minimum effective concentration of the antibody cannot be lowered below the equilibrium concentration of the soluble antigen, even if the antibody has infinite affinity. Moreover, since the antigen is continuously produced in vivo, which may be different from the in vitro situation, the antibody-dosage cannot be lowered below the amount of antigen produced between dosings. Therefore, if the equilibrium concentration or turnover of the antigen in vivo is very high, affinity maturation may have a limited effect on reducing the antibody dosage or dosing intervals.

The pharmacological effect of affinity maturation on antibodies targeting a solid tumor is somewhat complicated. Adams et al. reported that antibody-based molecules with extremely high affinity have impaired tumor penetration properties.34 In biodistribution studies examining a series of radio-labeled anti-HER2 single chain Fv (scFv) mutants with affinities ranging from 10^{-7} to 10^{-11} M, quantitative analysis of tumor retention of these antibodies demonstrated that no significant increase of tumor retention was observed beyond the affinity of 10^{-9} M and, moreover, diffusion of the highest affinity scFv was limited to the peripheral tumor space adjacent to the blood vessels while the lowest affinity scFv diffused uniformly throughout the tumor interior. Tang et al. described the relationship between the affinity of IgG antibody and antibody-dependent cellular cytotoxicity (ADCC).35 Studies using a series of anti-HER2 IgG1 antibodies with different affinities demonstrated that antibodies with higher affinity promoted stronger ADCC activity than the lower-affinity variants. Kyriakos et al. described the relationship between the affinity of anti-HER2 antibodies and HER2-dependent internalization after incubating radio-labeled antibodies with the target cells.36 The lowest-affinity antibody exhibited the least catabolism by the target cells, whereas the highest-affinity antibody exhibited the most. Since increasing affinity to the antigen seems to have a negative effect on intratumor penetration and target-mediated clearance, but a positive effect on ADCC activity, it might be difficult to predict in vivo antitumor efficacy of
Table 1. Summary of variable region engineering of therapeutic IgG antibodies

| Engineering                              | Effect                                                                 | Reference |
|------------------------------------------|------------------------------------------------------------------------|-----------|
| **Antigen binding properties**           |                                                                        |           |
| **Affinity maturation**                  |                                                                        |           |
| Random mutagenesis                       | Improving therapeutic efficacy[^1]                                     | 21        |
| Targeted mutagenesis                     |                                                                        | 11        |
| Shuffling approaches                     | Decreasing minimum effective plasma antibody concentration[^1]         | 12        |
| In silico approaches                     |                                                                        | 28        |
| **Altering the specificity**             |                                                                        |           |
| Random mutagenesis                       | Reducing the cross reactivity to other antigens                         | 38        |
| Targeted mutagenesis                     | Broadening the specificity to related antigens                          | 44        |
|                                           | Improving the non-human species cross-reactivity                       | 46        |
|                                           | Binding to two different antigen by single binding site                 | 50        |
| **Pharmacokinetics**                     |                                                                        |           |
| **Isoelectric point engineering**        |                                                                        |           |
| Lowering isoelectric point               | Reducing the non-specific clearance                                     | 56        |
| **Engineering pH dependency**            |                                                                        |           |
| Rapid dissociation in acidic condition   | Reducing the antigen-mediated clearance[^2]                             | 58        |
|                                           | Binding to multiple antigen by single antibody                          | 58        |
| **Pharmaceutical properties**            |                                                                        |           |
| **Thermal stability improvement**        |                                                                        |           |
| Optimizing hydrophobic core and charge   | Retaining biological activity during storage                           | 65, 66    |
| cluster residues                         |                                                                        |           |
| Optimizing conserved residues            | Minimizing aggregation not to increase immunogenicity                   | 65, 66    |
| Removing hydrophobic surface residues    | Improving expression yield                                              | 65, 66    |
| Optimizing VH/VL interface residues      |                                                                        | 65, 66    |
| **Solubility improvement**               |                                                                        |           |
| Reducing the surface hydrophobicity      | Enabling high concentration formulation                                 | 77, 78    |
| Introducing an N-linked carbohydrate     |                                                                        | 77, 78    |
| Modifying the isoelectric point          |                                                                        | 78        |
| **Chemical stability improvement**       |                                                                        |           |
| Avoiding deamidation and isomerization   | Retaining biological activity during storage                           | 83, 87    |
| Avoiding Met and Trp oxidation           | Facilitating quality control in manufacturing                          | 85        |
| **Heterogeneity improvement**            |                                                                        |           |
| Avoiding glycosylation                   | Facilitating quality control in manufacturing                          | 94        |
| **Immunogenicity**                       |                                                                        |           |
| **Humanization**                         |                                                                        |           |
| CDR grafting                             | Reducing immunogenicity                                                 | 111       |
| SDR grafting                             |                                                                        | 116       |
| Recombinant antibody libraries           |                                                                        | 121       |
| **Deimmunization**                       |                                                                        |           |
| In silico prediction of T-cell epitopes  | Reducing immunogenicity                                                 | 127       |
| In vitro analysis of T-cell epitopes     |                                                                        | 125       |
| Introducing regulatory T-cell epitope    |                                                                        | 133       |

[^1]This may not be always the case for the antibodies targeting solid tumors and agonistic antibodies.  
[^2]Antibodies targeting membrane-bound antigen with internalizing property.
an affinity-maturated antibody to solid tumors. It may require case-by-case studies of different antibodies against different antigens to evaluate the effect of affinity on in vivo antitumor efficacy.

With regard to an agonistic antibody, affinity maturation may have a negative impact on both in vitro and in vivo potency. Lacy et al. reported that erythropoietin-mimicking agonistic antibody targeting erythropoietin receptor (EPOR) exhibited an inverse correlation between affinity and potency. For agonistic antibodies, those with faster off-rates may permit continuous receptor stimulation and more efficient activation of the receptor than those with slower off-rates.37

**Engineering the specificity of mAbs.** Binding specificity is another important factor for therapeutic antibodies. The mutagenesis and display technologies used for affinity maturation may also be applied to engineer the specificity of mAbs. Engineering specificity includes reducing the cross-reactivity to other antigens, broadening the specificity to related antigens and improving the cross-reactivity with nonhuman species.46,47

Dubreuil et al. reported that the cross-reactivity of anti-progesterone antibody to 5β- or 5α-dihydroprogesterone could be reduced by using phage display technology.49 Competitive screening against a large excess of 5β- or 5α-dihydroprogesterone generated variants with reduced cross-reactivity while maintaining the parent antibody’s affinity to progesterone. Garcia-Rodriguez et al. reported that neutralizing antibody directed against botulinum neurotoxin subtype A1 could be engineered, by yeast surface display technology, to also neutralize neurotoxin subtype A2, which shares 90% sequence identity with subtype A1, enhancing the antibody’s potential as an efficient intervention against the botulinum neurotoxin.48

Werther et al. reported reengineering a humanized antibody against human LFA-1 to bind to rhesus LFA-1.50 Farady et al. reported a computational design approach to improving cross-reactivity of an antibody against membrane-type serine protease 1 (MT-SP1/matriptase) to the mouse counterpart.51 In both studies, several mutations in CDR enhanced the binding to nonhuman antigen while maintaining the binding to the human antigen. Such species cross-reactivity engineering would facilitate preclinical studies which otherwise may require surrogate antibodies.

Bispecific antibody represents one of the promising second generation antibody therapeutics. Various formats for bispecific antibodies, such as dual variable domain immunoglobulin (DVD-Ig) and IgG-like bispecific antibody (IgG-scFv), have been reported and whole IgG-type bispecific antibody using a common light chain is one of the promising formats from the standpoint of manufacturability. Nevertheless, the presence of two heavy chains, which require heterodimerization and one light chain, in contrast to the conventional IgG with one heavy chain and one light chain, may still make CMC development challenging. Recently, Bostrom et al. reported the “two-in-one” designer antibody in which the same binding site on an antibody was engineered to recognize two different antigens (HER2 and VEGF).52 random sequence was incorporated into the light chain of trastuzumab to afford binding to VEGF and then phage display technology was used to identify a “two-in-one” antibody which binds to HER2 and VEGF comparably to trastuzumab and bevacizumab. Because such a “two-in-one” antibody consists of one heavy chain and one light chain, it can be manufactured in the same manner as a conventional IgG antibody.

**Improving the Pharmacokinetics**

IgG antibodies generally have a longer half-life compared to other protein therapeutics due to FcRn-mediated recycling. Nevertheless, further improvement of the pharmacokinetics of IgG antibodies is beneficial especially for chronic diseases. Improving the pharmacokinetics allows the dosage to be lowered, enabling a subcutaneous formulation to be developed and COGs to be reduced, and also prolongs the dosing interval, which is more convenient for the patients. Pharmacokinetics of IgG antibodies has been studied in detail, and it is known that the clearance of IgG antibodies can be classified into two different pathways: non-specific clearance and antigen-mediated clearance. It has been reported that non-specific clearance of IgG could be improved by an Fc engineering approach to increase the Fc/FcRn interaction.52-54 Recently, variable engineering approaches for improving the two clearance pathways have been reported (Table 1).

**Reducing the non-specific clearance of IgG antibodies.** The first clearance pathway, non-specific clearance, is a non-specific uptake (pinocytosis) of IgG antibodies into the cellular endosomal compartment followed by lysosomal degradation of the nonFcRn-bound antibody.55 Although Fc engineering technologies to increase the binding to FcRn and thereby reduce the non-specific clearance of IgG are well established,56 until recently there were no other technologies available to reduce the non-specific clearance of the IgG antibody.

Igawa et al. reported that non-specific clearance of IgG antibodies with identical Fc sequences but different variable region sequences correlated with the isoelectric point of the antibody, and the antibody with a lower isoelectric point exhibited lower clearance.56 Their findings suggested that electrostatic repulsion between the low isoelectric point antibody and the negatively-charged cell surface contributed to the reduced non-specific uptake of the antibody into the cell. Taking advantage of this, engineering the variable region of the IgG1 antibody to lower the isoelectric point while maintaining the affinity to the antigen, reduced the clearance of the parent antibody by 3.1-fold. Such improvement seems to be comparable to, or even better than, the reported Fc engineering approach.53 Since most human or humanized mAbs obtained by general methodology have a rather high isoelectric point, non-specific clearance of such an antibody could be reduced by engineering the isoelectric point of the variable region.

**Reducing the antigen-mediated clearance of IgG antibodies.** The other clearance pathway, antigen-mediated clearance, contributes to the total clearance when IgG antibodies target membrane-bound antigen with an internalizing property, in which the antibody bound to the membrane-bound antigen is internalized as an antigen-antibody complex followed by lysosomal degradation of that complex.57 Since antigen-mediated
clearance is the consequence of the antibody binding to the antigen, which is its primary function, antigen-mediated clearance of the antibody would seem to be an inevitable part of its function.

Igawa et al. reported a novel approach to improving the antigen-mediated clearance of the IgG antibody. Anti-IL-6 receptor (IL-6R) antibody tocilizumab, which exhibits membrane IL-6R-mediated clearance, was engineered to rapidly dissociate from IL-6R within the acidic endosome (pH 6.0), while maintaining its binding affinity to IL-6R in the plasma (pH 7.4), so that dissociated antibody within the acidic endosome would be recycled back to the plasma. Engineering pH dependency into tocilizumab, by introducing histidine residues into its antigen-binding site, significantly reduced the antigen-mediated clearance. This pH-dependent binding approach may be generally applicable to reducing antigen-mediated clearance. In addition, the pH-dependent dissociation of the soluble IL-6R from the antibody within the endosome enabled selective lysosomal degradation of the previously antibody-bound soluble IL-6R, and FcRn-mediated recycling of the free antibody back to the plasma. Since the recycled free antibody is capable of binding to another antigen, pH-dependent binding would enable a single antibody molecule to repeatedly bind to multiple antigens. Binding of multiple antigens by a single antibody has the potential to overcome the limitation of conventional high-affinity antibody in which a single antibody can bind to antigen only once (i.e., single antigen per Fab) to reduce the antibody dosage or dosing intervals.

**Improving the Pharmaceutical Properties**

Although the difference in the constant region sequence (i.e., IgG isotype) has some effect on the pharmaceutical properties of the IgG antibodies, the variable region sequence, which significantly differs among the antibodies but comprises only one-third of the whole IgG molecule, has a significant effect on the pharmaceutical properties, such as thermal stability, solubility, chemical stability and heterogeneity. Since poor pharmaceutical properties may have a significant impact on the manufacturability, various engineering technologies to improve them have been reported (Table 1).

**Engineering to improve thermal stability.** Poor thermal stability may cause various problems such as aggregation, resulting in reduced biological activity and increased immunogenicity, and low expression yield, resulting in increased COGs. Therefore improving the stability of the antibody therapeutics would be important for poorly-behaving antibodies.

Engineering the stability of the antibody variable region has mainly focused on scFv due to its inherent poor stability. Stability of scFv can be improved by optimizing the hydrophobic core residues, charge cluster residues, residues which determine the framework subclasses, conserved proline and glycine residues, hydrophobic surface residues and VH/VL interface residues using either a rational mutagenesis approach or a library approach. Dong et al. reported that rational optimization based on the structure of anti-IGF-1R scFv improved the melting temperature by 16°C. Miller et al. reported that the focused library approach improved the melting temperature of scFv by 33°C.

In most cases, optimizing the stability results in improved expression yield.

It has been reported that germline families of the heavy chain have a significant effect on the stability and the expression yield of scFv. The heavy chains derived from germline families VH2, 4 and 6 tend to have lower stability and expression yield than VH1, 3 and 5. Especially VH3 germline appears to have favorable properties. Although selecting the VH3 germline framework seems to be a promising approach for generating high stability scFv, CDR grafting of mouse CDRs into VH3 germline does not always result in stable scFv.

This is presumably because the compatibility of CDR with the framework residues is important for the overall stability of scFv.

Although engineering the scFv to improve thermal stability is being extensively studied, stability engineering of the Fab domain in IgG antibodies has not been reported. This seems to be due to the fact that Fab often has acceptable stability for therapeutic development because of the stabilization effect of the CH1/CL domain present in the Fab. Garber et al. studied the melting temperature of 17 different Fab from different human or humanized antibodies and found that the melting temperatures of these antibodies range from 57–82°C. Antibodies with a very low melting temperature were found to aggregate and express poorly. Therefore stability engineering of the variable region of IgG antibodies may be necessary for such poorly behaved IgG antibodies.

**Engineering to improve solubility and viscosity.** Subcutaneous delivery of antibody therapeutics is preferable for chronic disease. Since the amount of volume for a single subcutaneous injection is generally limited to less than 1.5 mL, subcutaneous injection often requires a formulation with high antibody concentration (i.e., >100 mg/mL). Major difficulties for achieving the necessary concentration are the stability, solubility and viscosity.

The solubility of IgG antibody is generally high (>100 mg/mL), but extremely low solubility has been reported in cryoglobulin IgG antibodies and some therapeutic IgG antibodies. Pepinsky et al. reported anti-LINGO-1 IgG2 antibody with low solubility (<0.3 mg/mL) and described two variable region engineering strategies for improving the solubility. The first strategy, reducing the surface hydrophobicity by rationally mutating the residues exposed at the surface of the variable region, improved the solubility to >7 mg/mL. The second strategy, introducing an N-linked carbohydrate in the variable region, improved the solubility to >50 mg/mL. Wu et al. reported anti-IL13 antibody with low solubility and employed structure-based variable region engineering approaches to improve the solubility. Isoelectric point modification and reducing the surface hydrophobicity showed only moderate improvement in the solubility, whereas introducing an N-linked carbohydrate showed significant improvement. Although these two reports suggest that attaching an N-linked carbohydrate in the variable region seems to be the most effective strategy, this approach would increase heterogeneity derived from the additional carbohydrate in the variable region, requiring more control of the heterogeneity in the manufacturing process. Moreover, N-linked carbohydrate in CDR may also have an effect on the pharmacokinetics.
of the antibody.\textsuperscript{79} Taking these potential issues into consideration, improving solubility by introducing an N-linked carbohydrate in the variable region might not be a first-line approach for improving the solubility.

High viscosity of therapeutic antibodies impedes the development of high concentration formulations for subcutaneous injection. It has been reported that different IgG1 antibodies have different viscosity which could be derived from the different variable region sequences,\textsuperscript{80} and electrostatic interaction between Fab seems to play a role in increased viscosity in a certain antibody.\textsuperscript{81} However, to date, there is no variable region engineering reported for reducing the viscosity of IgG antibodies.

**Engineering to improve chemical stability.** Chemical degradations such as deamidation,\textsuperscript{82} isomerization,\textsuperscript{83} succinimide formation,\textsuperscript{84} methionine and tryptophan oxidation\textsuperscript{85} and cysteinylation of unpaired cysteine\textsuperscript{86} in the CDR region often lead to a reduction in the potency of antibody therapeutics and could be issues needing to be controlled in CMC development. Therefore, antibodies with such tendencies should be avoided as clinical candidate molecules. If such properties are found in the clinical candidate antibody or lead antibody, the degradation site should be identified by structural analysis and removed in the subsequent engineering process or otherwise a back-up antibody without such properties should be selected in its place.

Among various degradation pathways, asparagine deamidation and isomerization\textsuperscript{87} in the CDRs seems to be the major degradation. A common approach to avoiding asparagine deamidation and isomerization is to substitute the asparagine residue itself with other amino acids that maintain the antigen-binding capability. But in some cases, the asparagine residue in the CDR might be critical for its antigen binding and may not be replaceable. Recently, Nakano et al. reported an engineering approach to overcome such issues in an anti-GPC3 IgG1 antibody, in which an asparagine-glycine sequence, a potential deamidation site, was found in the light chain CDR of the lead antibody.\textsuperscript{87} Based on the fact that the rate of deamidation is mainly determined by the C-terminal residue next to asparagine and is most rapid when the C-terminal residue is glycine or serine,\textsuperscript{88} C-terminal glycine residue, not the asparagine residue, was substituted with arginine, to remove the potentially deamidating site from the parent antibody while maintaining the antigen binding.

Methionine or tryptophan oxidation in the CDRs can also be avoided by substituting oxidizing residues with other amino acids which do not undergo oxidative degradation and which maintain the binding capability, although there has been no such report to date.

**Engineering to improve heterogeneity.** Heterogeneities deriving from post-translational modification, such as glycosylation, N-terminal pyroglutamine cyclization and C-terminal clipping\textsuperscript{95,96} need to be controlled to maintain the product quality.

Approximately 20% of human IgGs have N-glycosylation motif N-X-(S/T) (Asn-X-Ser/Thr, where X is not proline) within the variable region, in addition to the common glycosylation site in the Fc region.\textsuperscript{91} The most common N-glycosylation site in the variable region is CDR2 of the heavy chain, but other potential N-glycosylation sites can be generated by somatic mutations.\textsuperscript{92} The presence of glycosylation in the variable region may have either positive or negative effects on the antigen binding,\textsuperscript{79,93} and the difference in the carbohydrate structure could also influence the pharmacokinetics.\textsuperscript{79} Glycosylation in the variable region, in addition to Fc glycosylation, needs to be controlled during the manufacturing process, creating a significant burden for CMC development. Therefore, glycosylation in the variable region should preferably be avoided by selecting or designing an appropriate antibody sequence, and a synthetic library has been reported of sequences designed to avoid the potential appearance of N-glycosylation motif in the HCDR2 repertoire.\textsuperscript{94} Recently, in addition to consensus glycosylation motif N-X-(S/T), Valliere-Douglass et al. reported a novel N-glycosylation motif, the reverse consensus motif (S/T)-X-N, in the antibody variable region.\textsuperscript{95} Moreover, cetuximab was reported to have O-glycosylation in the variable region which results in anaphylaxis from IgE specific to galactose-alpha-1,3-galactose, in a subset of patients.\textsuperscript{96} These forms of glycosylation could be an issue in the later stages of development so it may be better to remove them also by engineering before selecting the clinical candidate.

### Reducing the Immunogenicity

Clinical utility and efficacy of the therapeutic antibodies can be limited by the production of anti-drug antibodies (ADAs), since ADA can influence their efficacy and pharmacokinetics and sometimes lead to serious side effects.\textsuperscript{97-104} Although many factors influence the immunogenicity of therapeutic antibodies,\textsuperscript{105,106} a number of reports describe the importance of effector T-cell epitopes present in the therapeutic protein.\textsuperscript{107-110} Various human or humanized antibodies and deimmunization technologies to minimize the number of effector T-cell epitopes have been reported for reducing the immunogenicity of therapeutic antibodies (Table 1).

**Humanization and human-antibody-generation technologies.** The initial approach to reducing potential immunogenicity of the nonhuman variable region was to humanize variable region derived from nonhuman sources by grafting it into a template human framework,\textsuperscript{111} and this humanization technology significantly decreased the immunogenicity of therapeutic antibodies.\textsuperscript{112} Since a template human framework with somatic hypermutations can create effector T-cell epitopes in some individuals, superhumanization or germline humanization, using human germline frameworks as templates for CDR grafting, has been proposed as a superior humanization methodology on the assumption that the germline sequences will not activate effector T-cells in broader populations.\textsuperscript{113-115} Nevertheless, such antibodies might still contain several effector T-cell epitopes in the nonhuman CDRs and the CDR/framework junction regions. To reduce the potential risk of effector T-cell epitopes being contained in such regions, there is the specificity-determining residue (SDR) grafting approach, in which only the SDRs (not whole CDRs) are grafted onto the germline antibodies. Such SDR-grafted humanized antibodies resulted in lower immunogenic potential compared to CDR-grafted humanized antibodies.\textsuperscript{116-119}
Fully human antibodies can be generated by using display library technologies, genetically modified mice and rational engineering technologies. The advantage of fully human antibodies over humanized antibodies with regard to immunogenicity is controversial. Adalimumab, a fully human antibody, has been reported to induce ADA production in a subset of patients (5–89%) that varies depending on the situation, and a reduction in therapeutic efficacy was observed in the ADA-positive patients. This significant immunogenicity of adalimumab was supported by the presence of effector T-cell epitopes in regions containing a CDR-sequence determined by an in vitro helper CD4+ T-cell assay.

In silico and in vitro deimmunization technologies. Effector T-cell epitopes may be present in the humanized or even in fully human antibody and, moreover, engineering such antibodies’ variable regions to improve various properties, as described in this review, may also introduce additional effector T-cell epitopes.

A number of in silico tools to predict effector T-cell epitopes, such as Epibase (AlgoNomics NV), iTope/TCED (Antitope Ltd.), and EpiMatrix (EpiVax Inc.) have been developed. By using these in silico tools, the presence of effector T-cell epitope in each antibody sequence can be predicted, allowing the potential immunogenicity of the antibody therapeutics to be reduced by selecting a sequence with the minimum number of effector T-cell epitopes (i.e., deimmunization). Although these in silico tools are valuable for their rapid outcomes, it should be noted that they tend to be overpredictive.

On the other hand, an in vitro CD4+ helper T-cell assay also can be used to identify the effector T-cell epitope, and recently Harding et al. demonstrated that modifying the CDR regions to reduce the number of effector T-cell epitope using this in vitro assay could be an effective approach for generating a less immunogenic antibody. Although in vitro tools seem to be more accurate than in silico tools, a significant drawback of the in vitro assay is its low throughput. Therefore, it would be important to use both in silico and/or in vitro tools according to the situation, to maximize their utility. Minimizing the number of effector T-cell epitopes by in silico tools during the lead optimizing step with subsequent confirmation of the potential immunogenicity by in vitro assessments could be a realistic deimmunization procedure, at present.

Recently, De Groot and colleagues reported a novel concept of regulatory T-cell epitope (Tregitope) in the Fc and variable region of the antibody as a suppressor of immune response. They suggested that introducing a Tregitope sequence into the immunogenic antibodies might be an alternative deimmunization strategy.

Conclusion

Despite the fact that it comprises only one-third of the whole IgG molecule, the variable region of the IgG antibody is responsible for various properties of antibody therapeutics including antigen binding properties, pharmacokinetics, pharmaceutical properties and immunogenicity. Although individual improvement of these properties by variable region engineering has been reported, best-in-class strategy may require optimization of all these properties to generate a highly potent, long-acting, stable and non-immunogenic antibody molecule. This could be a significant challenge to antibody engineers since improvement of one property may worsen the other and combining various meaningful mutations may not be additive and may sometimes result in loss of function. Also it is important to set a target antibody profile of the clinical candidate and prioritize the properties to be improved, in case the multiple properties are incompatible with each other.

Due to the significant success of antibody therapeutics over the past two decades, first-in-class or best-in-class strategy for promising targets, and best-in-class strategy aiming at the second or third generation antibodies for clinically validated targets, is extremely competitive. In order to be successful in the market, the best-in-class strategy may require the antibody engineer to generate “the best” antibody molecule against the target by applying extensive engineering technologies. Since the market share of the first-in-class antibody could be easily taken over by a best-in-class antibody (as happened when adalimumab took over the share of infliximab), such engineering technologies may be adopted even for the first-in-class strategy, although it may be important to consider the balance between the extent of optimization and the time required to accomplish it. Variable region engineering technologies, together with Fc engineering technologies, will be essential technologies for developing next generation antibody therapeutics.

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