Advances in Antibody Design

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Abstract

The use of monoclonal antibodies as therapeutics requires optimizing several of their key attributes. These include binding affinity and specificity, folding stability, solubility, pharmacokinetics, effector functions, and compatibility with the attachment of additional antibody domains (bispecific antibodies) and cytotoxic drugs (antibody–drug conjugates). Addressing these and other challenges requires the use of systematic design methods that complement powerful immunization and in vitro screening methods. We review advances in designing the binding loops, scaffolds, domain interfaces, constant regions, post-translational and chemical modifications, and bispecific architectures of antibodies and fragments thereof to improve their bioactivity. We also highlight unmet challenges in antibody design that must be overcome to generate potent antibody therapeutics.

Contents

1. INTRODUCTION

Antibodies are affinity proteins that play a central role in humoral immunity. Their ability to bind to foreign invaders with high affinity and specificity is central to their function. Equally important is their ability to serve as adaptor molecules and recruit immune cells for various effector functions. There are five main classes of antibodies with diverse functions: immunoglobulin (Ig)A IgD, IgE, IgG, and IgM (1). IgGs are the most abundant class of antibodies, as they constitute approximately 75% of the serum immunoglobulin repertoire. There are four subclasses of IgGs, which vary in their abundance and ability to elicit specific effector functions.

The overall architecture of IgGs is conserved across its four subclasses, and consists of two light chains and two heavy chains (**Figure 1**). The light chains contain variable (V_L) and constant (C_L) domains, and the heavy chains contain one variable (V_H) and three constant (C_H1, C_H2, and C_H3) domains. One notable difference between IgG subclasses is the location of the disulfide bonds between C_H1 and C_L (which link the heavy and light chains) and the number of disulfide bonds in the hinge region (which link the heavy chains). The multidomain nature of IgGs elegantly divides their bioactivity into different subdomains. The antigen-binding fragment (Fab) contains both variable domains, and mediates antigen recognition via six peptide loops known as the complementarity-determining regions (CDRs). In contrast, the crystallizable fragment (Fc) contains the constant domains (C_H2 and C_H3) that mediate effector function by binding to immunological receptor molecules such as complement proteins and Fc receptors.

The multifunctional nature of IgGs is only one of the many reasons for the widespread interest in using monoclonal antibodies (mAbs) as therapeutics. The availability and refinement of robust methods for identifying and generating human mAbs, such as immunization and in vitro screening methods (2), have also contributed greatly to the interest in antibody therapeutics. In addition, mAbs typically display excellent pharmacokinetics (long circulation times), low toxicity and immunogenicity (for human or humanized mAbs), and high stability and solubility. It is also notable that the simplicity of expressing and purifying many different mAbs using similar platform processes is highly attractive from a manufacturing perspective and has enabled the production of a staggering number of different mAbs that are in clinical trials (3).

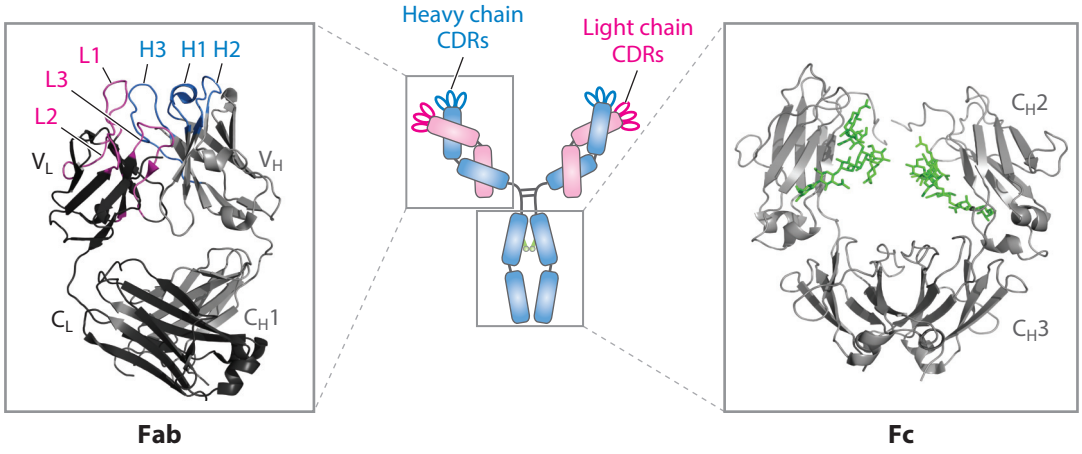


Figure 1

Molecular architecture of an immunoglobulin G1 (IgG1) antibody. An IgG consists of two heavy chains (*blue*) and two light chains (*pink*). (*Left*) The crystal structure of an antigen-binding fragment (Fab; Protein Data Bank identification number, 3NZ8). The Fab is composed of variable heavy (V_H) and light (V_L) domains as well as two constant domains (C_H1 and C_L). Each variable domain displays three binding loops (complementarity-determining regions, CDRs), which mediate antigen recognition. The CDRs in the V_H domain are denoted as H1, H2, and H3 (*blue*); the CDRs in the V_L domain are denoted as L1, L2, and L3 (*pink*). (*Right*) The crystallizable fragment (Fc; Protein Data Bank identification number, 1E4K) contains two constant domains (C_H2 and C_H3) as well as glycans in the C_H2 domain (*green*). The Fc fragment mediates antibody effector function.

Nevertheless, there are many challenges in generating mAbs for therapeutic applications. At the discovery stage, immunization affords limited control over antibody affinity and specificity due to the difficulty in controlling antigen presentation to the immune system. In vitro methods, such as phage and yeast surface display, enable improved control over antigen presentation. However, these display methods are limited by their need to screen large libraries, their typical use of antibody fragments instead of full-length antibodies, and their reduced quality-control mechanisms relative to mammalian systems. Moreover, antibodies identified via either immunization or display methods have variable and difficult-to-predict solubilities and viscosities at the high concentrations required for subcutaneous delivery (4, 5). Antibody aggregation is particularly concerning due to the potential immunogenicity of such aggregates (6), and abnormally high viscosity can prevent mAbs from being delivered via the subcutaneous route (7). It is also challenging to optimize bispecific antibodies that typically combine binding domains from different parent antibodies, given the large number of possible molecular architectures as well as the complex effects that these nonstandard antibody formats can have on antibody stability. Moreover, developing effective antibody–drug conjugates is extremely challenging due to the need to optimize the linker and conjugation chemistry as well as the location and number of attached drug molecules. Finally, it is difficult to engineer antibodies with the specific types and levels of effector functions that are optimal for a given therapeutic application.

Although each of these challenges can be addressed through screening a large number of antibody variants, it is impractical to use such screening methods alone to address many of the challenges encountered in developing potent therapeutic antibodies. Attempts to optimize each antibody property sequentially are limited by the fact that improving one antibody attribute (such as binding affinity) can lead to defects in other attributes (such as solubility). Attempts to simultaneously optimize multiple antibody properties using mutagenesis and screening methods require libraries that are prohibitively large.

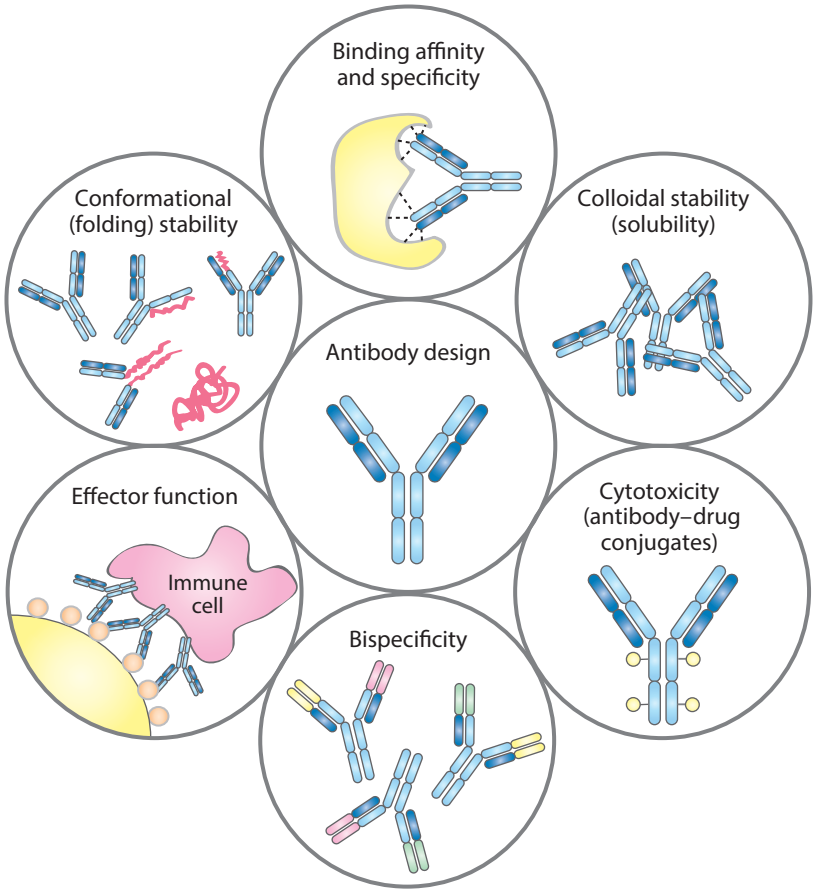


Figure 2

Key attributes of antibodies that must be collectively optimized to generate effective immunoglobulins for different applications. A key challenge is that optimizing one property can lead to deleterious impacts on others.

2. OVERVIEW OF APPROACHES FOR DESIGNING ANTIBODIES

The complexity of optimizing several different antibody attributes (summarized in **Figure 2**) using traditional immunization and screening methods has led to intense interest in developing antibody-design methods. The most important antibody attributes are binding affinity and specificity, which involve optimizing the variable domains and the CDRs in particular. Colloidal stability (solubility) and conformational (folding) stability are also critical attributes of antibodies because therapeutic mAbs must be soluble for high-concentration delivery and stable for long-term storage. This typically requires optimizing solvent-exposed residues for solubility and solvent-shielded residues for conformational stability. The effector functions of antibodies are also critical to their bioactivity, and can be tailored by manipulating the hinge and Fc regions.

Another increasingly important antibody attribute—which is uncommon in natural antibodies—is bispecificity for either multiple antigens or multiple epitopes on the same antigen. Achieving bispecificity requires methods for combining multiple antibodies into a single one

as well as optimizing the key attributes of conventional antibodies. A second nonconventional attribute of antibodies that continues to grow in importance is their bioactivity when attached to small-molecule drugs. Developing antibody–drug conjugates (ADCs) requires optimizing many aspects of the chemistries and linkers used to derivatize antibodies in addition to the other key attributes of conventional antibodies.

This review highlights progress in designing and optimizing each of these key antibody attributes. Given the large size and complexity of antibodies, most design efforts have focused on redesigning or optimizing existing antibodies rather than on de novo design of new antibodies. These design methods vary greatly, and range from knowledge-based methods based on previous mutagenesis results to advanced computational methods based on first principles. A commonality of these diverse methods is that they attempt to guide the design of antibodies in a systematic manner to reduce the need for screening and immunization methods. We discuss these design methods and their application to improve the properties of antibodies that are critical for their activity and stability.

3. ANTIBODY BINDING AFFINITY AND SPECIFICITY

The most important property of antibodies is their ability to recognize targets with high affinity and specificity. This binding activity is largely mediated by the CDRs. Several innovative approaches have been developed for designing CDRs that range from de novo design methods to those that involve the redesign of existing antibodies. Some of these design methods have used motif-grafting approaches to mimic natural protein interactions, and directed evolution approaches to achieve specificities for difficult-to-target antigens.

3.1. De Novo Design

The holy grail of antibody design is to accurately and reliably predict the sequences of antibodies that will bind with high affinity and specificity based solely on the sequence or composition of the antigen. Toward this ambitious goal, a computational approach named OptCDR (Optimal Complementarity Determining Regions) has been developed for designing the CDRs of antibodies to recognize specific epitopes on a target antigen (8). This method uses canonical structures to generate CDR backbone conformations that are predicted to interact favorably with the antigen. Amino acids are then chosen for each position in the CDRs using rotamer libraries, and this process is repeated many times to refine the backbone structures and amino acid sequences. This leads to the prediction of several sets of CDR sequences, which can be grafted onto antibody scaffolds for evaluation.

This approach has been tested for developing antibody–antigen complexes involving a hepatitis C virus capsid peptide, fluorescein, and vascular endothelial growth factor (VEGF) (8). The investigators predicted mutations that are expected to increase binding affinity (which were not evaluated experimentally) as well as evaluated mutations that had been reported previously to improve binding affinity for fluorescein antibodies. There is some correlation between predictions by OptCDR and experimental data for fluorescein antibodies. It will be important to further evaluate the ability of OptCDR and closely related methods (9) to make de novo predictions of CDR mutations as well as entire CDR sequences that either generate or improve antibody binding.

3.2. Design by Mimicking Natural Protein Interactions

Another fruitful approach for designing antibodies with specific binding activities has been to mimic natural protein interactions. For example, Williamson and colleagues (10) designed

antibodies to recognize misfolded conformers of the prion protein (PrP) by mimicking natural interactions between cellular PrP (PrP^C) and its misfolded counterpart (PrP^{Sc}). Previous studies had found that PrP residues 96–104 and 133–158 govern the ability of PrP^{Sc} to catalyze misfolding of soluble PrP^C (11, 12). This led to the hypothesis that grafting such PrP peptides into heavy chain CDR3 (HCDR3) of an IgG—which originally lacks PrP-binding activity—would create antibodies that specifically recognized PrP^{Sc} (10). Indeed, they found that antibodies with PrP residues 89–112 or 136–158 in HCDR3 bound to PrP^{Sc} with apparent affinities in the low nanomolar range (2–25 nM), and these same antibodies weakly interacted with PrP^C. Follow-up studies also identified a third region near the N terminus of PrP (residues 19–33) that resulted in binding activity for grafted PrP antibodies (13). Interestingly, grafting peptides from other PrP regions (such as those from the C-terminal domain) into the same CDR loop failed to generate binding activity. Moreover, the antibodies grafted with PrP residues 19–33 and 89–112 appear to bind via electrostatic interactions because mutating positively charged residues to alanine in these grafted peptides eliminated binding.

This exciting study raises the question of whether grafting peptides from other aggregation-prone proteins into the CDRs of antibodies would also lead to specific binding activity. Our lab recently tested this question using the Alzheimer's Aβ42 peptide (14). There are two hydrophobic segments within Aβ (residues 17–21 and 30–42) that mediate amyloid formation and are located within the β-sheet core of Aβ fibrils (15). We posited that grafting these peptide segments into CDR3 of a single-domain (V_H) antibody would lead to antibody domains with Aβ-specific binding activity. Indeed, we found that grafted V_H domains displaying the central hydrophobic region of Aβ (residues 17–21) in CDR3 bound to Aβ fibrils with submicromolar affinity (300–400 nM), and they weakly bound to Aβ monomers or oligomers (14). Interestingly, V_H domains grafted with the hydrophobic C terminus of Aβ (residues 30–42) bound both Aβ fibrils and oligomers with submicromolar affinity (300–700 nM) and weakly recognized Aβ monomers. We refer to these grafted antibodies as gammabodies (Grafted AMyloid-Motif AntiBODIES). We have also verified that this grafting approach can be applied to other amyloid-forming proteins, including α-synuclein (associated with Parkinson's disease) and IAPP (associated with type 2 diabetes) (16). Nevertheless, future work will need to develop more systematic methods for selecting amyloidogenic peptides for grafting because we currently do not understand why some sequences mediate binding and others do not. Moreover, it will be important to evaluate how multiple CDR loops can be engineered to display amyloidogenic peptides on the surface of single- and multidomain antibodies to improve the affinities of these grafted antibodies.

3.3. Semirational Design Combined with Directed Evolution Methods

Despite these key advances in the de novo design of CDRs, it has been extremely challenging to use such rational approaches to generate antibodies with subnanomolar (or lower) dissociation constants. Nevertheless, several innovative approaches have been developed that involve designing some CDR residues while randomizing others, and screening such libraries using in vitro display methods to select variants with high binding affinity and specificity. One of the first examples of this hybrid approach was the design of antibody libraries specific for integrins (17). The RGD sequence (arginine-glycine-aspartate) was inserted in the middle of HCDR3, and three flanking residues were randomized on each side of the RGD sequence. In addition, cysteines were introduced at each edge of HCDR3 to constrain the loop, which was posited to be necessary to generate high affinity for antibody binding that is mediated primarily via a single CDR. The investigators displayed a Fab library with these HCDR3 sequences on the surface of phage and screened for binding to integrins. Impressively, several antibody variants were identified with subnanomolar binding

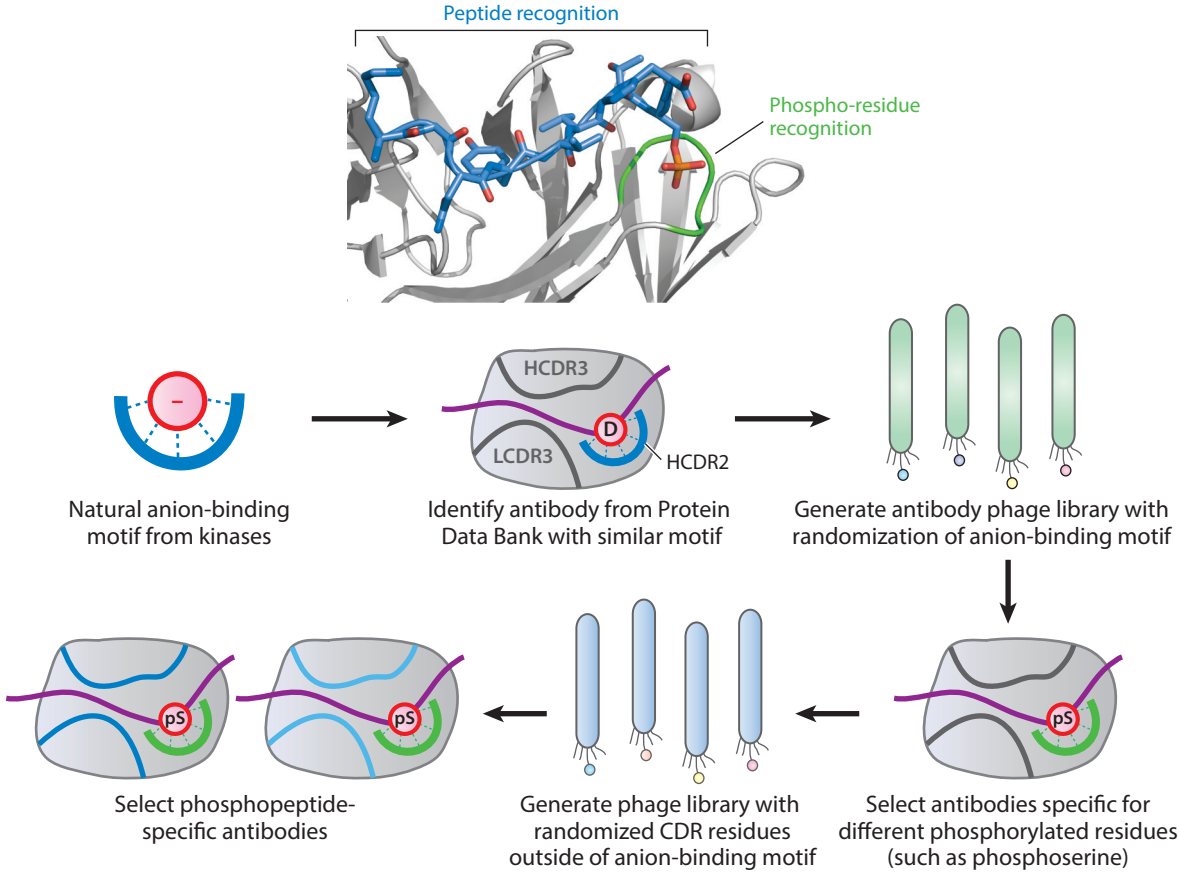


Figure 3

Nature-inspired design and evolution of phospho-specific antibodies. This approach uses natural anion-binding motifs within the complementarity-determining regions (CDRs) of antibodies to generate libraries for identifying antibodies specific for phosphoserine, phosphotyrosine, and phosphothreonine (20). The first round of randomization and selection yields antibodies with anion-binding motifs specific for each type of modification, and the second round identifies antibodies with heavy chain CDR3 (HCDR3) and light chain CDR3 (LCDR3) loops that are specific for different phosphopeptides. Figure redrawn from Reference 21.

affinities, and these antibodies retained the same binding epitope as natural integrin ligands. This and related work (18, 19) has demonstrated the potential of using natural protein interactions to guide the design of high quality antibody libraries.

Another example of this hybrid approach is a method for generating antibodies that recognize post-translational modifications (20, 21). It is difficult to isolate antibodies that recognize chemical modifications such as phosphorylation, especially for phosphoserine and phosphothreonine, because of the relatively small size of their side chains. Therefore, the investigators sought to introduce a common phosphate-binding motif from proteins such as kinases into the CDRs of antibodies (**Figure 3**). By identifying an antibody with a CDR loop (HCDR2) that naturally displays a similar anion-binding motif, the investigators first confirmed that this antibody bound weakly to phosphorylated peptides. Next, the affinities and specificities of such antibodies for phosphorylated serine, threonine, and tyrosine were evolved by randomizing sites within the anion-binding

motif. After mutants were identified by phage display with selective and improved affinity for each type of modification, CDR residues outside the phospho-binding pocket in HCDR2 as well as in LCDR3 and HCDR3 were randomized, and antibodies were selected for binding to different phosphorylated targets. Impressively, this approach generated many phospho-specific antibodies for a wide range of target peptides with modified serine and threonine in addition to tyrosine. Although the binding affinities are modest (40–5,000 nM), they are similar to or better than those for previously reported phospho-specific antibodies [see (20) and references therein]. More importantly, this innovative approach to designing antibody libraries addresses the challenging problem of achieving binding specificities to subtly different antigens that are difficult to obtain using immunization.

3.4. Antibody Redesign and Optimization

Much effort has focused on using design methods to improve the binding affinity of existing antibodies due to the complexity of de novo design. These redesign efforts are important because immunization typically yields antibodies with affinities that are not high enough for therapeutic applications, and directed evolution approaches are limited in their ability to identify multiple synergistic mutations, given the unrealistically large libraries that would be required.

One interesting study demonstrated the potential of optimizing electrostatic interactions to improve the binding affinity of antibodies (22). The authors used physics-based methods to initially evaluate the binding energy of an anti-lysozyme antibody (D1.3); every possible mutation was evaluated for 60 CDR positions using the crystal structure of the complex as a starting point. Interestingly, the mutations predicted to be most favorable were those that introduced large side chains into the binding interface to maximize van der Waals interactions, yet most of these mutations failed to improve affinity when evaluated experimentally. Instead, the electrostatic component of the binding energy was found to be a better predictor of mutations that improve affinity. This approach led to significant improvements in affinity (of one to two orders of magnitude) for cetuximab and an anti-lysozyme antibody (D44.1).

The success of this study stems largely from two key types of mutations within the CDRs. One is the elimination of residues with unsatisfied polar groups (e.g., side chains of asparagine or threonine) in which desolvation is not compensated for by favorable interactions (e.g., hydrogen bonds) in the bound state (22). By mutating such residues to small hydrophobic ones, the authors observed increased binding affinity. A second key type of mutation is either the introduction or removal of charged residues at sites within the CDRs that are peripheral to the residues that contact the antigen in the nonoptimized complex. This finding builds on previous work demonstrating that charged residues outside the antibody–antigen interface but within the CDRs can increase the on-rate (and thereby the affinity) of an anti-VEGF antibody (23). More generally, these findings are consistent with other studies revealing that the likelihood of identifying beneficial mutations is higher outside the initial binding interface (24–27), likely due to the reduced risk of disrupting existing antibody–antigen interactions. This (22) and related work (24–26, 28, 29) have demonstrated the potential of using existing methods for calculating electrostatic interactions to identify mutations that improve antibody affinity.

Another study elegantly showed that it is not necessary to use the crystal structures of antibodies or antibody–antigen complexes to guide efforts to improve affinity or alter binding specificity (25). The investigators sought to redesign a dengue virus antibody (4E11) to be broadly neutralizing. This is particularly challenging because the starting structures were unknown for both the 4E11 antibody alone or when bound to its antigen (domain III of the dengue E protein). Nevertheless, the authors used computational docking methods to generate structures of the 4E11 antibody

bound to four variants of the dengue E protein. Notably, the poor interaction between 4E11 and its antigen from one serotype (type 4) stems from the loss of key interactions (such as salt bridges and hydrogen bonds) that are predicted to be present in binding interfaces of the antibody with antigens from types 1–3. These insights led to the identification of several mutations that improve 4E11 binding to type 4 without reducing binding to types 1–3. By combining successful mutations, the affinity for type 4 was enhanced by more than two orders of magnitude without reducing affinity for other serotypes. Most of the successful mutations were charged or polar, and located at the periphery of the binding interface, which is generally consistent with findings from other studies (22–24, 26). This exciting study highlights the potential of using design methods to achieve unique binding specificities that are difficult to achieve using conventional discovery methods.

4. ANTIBODY CONFORMATIONAL (FOLDING) STABILITY

Another key attribute of antibodies is their folding stability (30, 31), which is critical for maintaining long-term activity. Folding stability is a particularly important consideration when altering the CDRs of antibodies to improve binding affinity and specificity. Several studies have demonstrated that either grafting CDRs from one antibody to another or mutating CDRs can significantly affect folding stability (32–36). More generally, mutations that are located outside of the CDRs can also significantly impact folding stability (37–40). These challenges necessitate design methods that can rationally stabilize antibodies without compromising binding affinity and other key attributes.

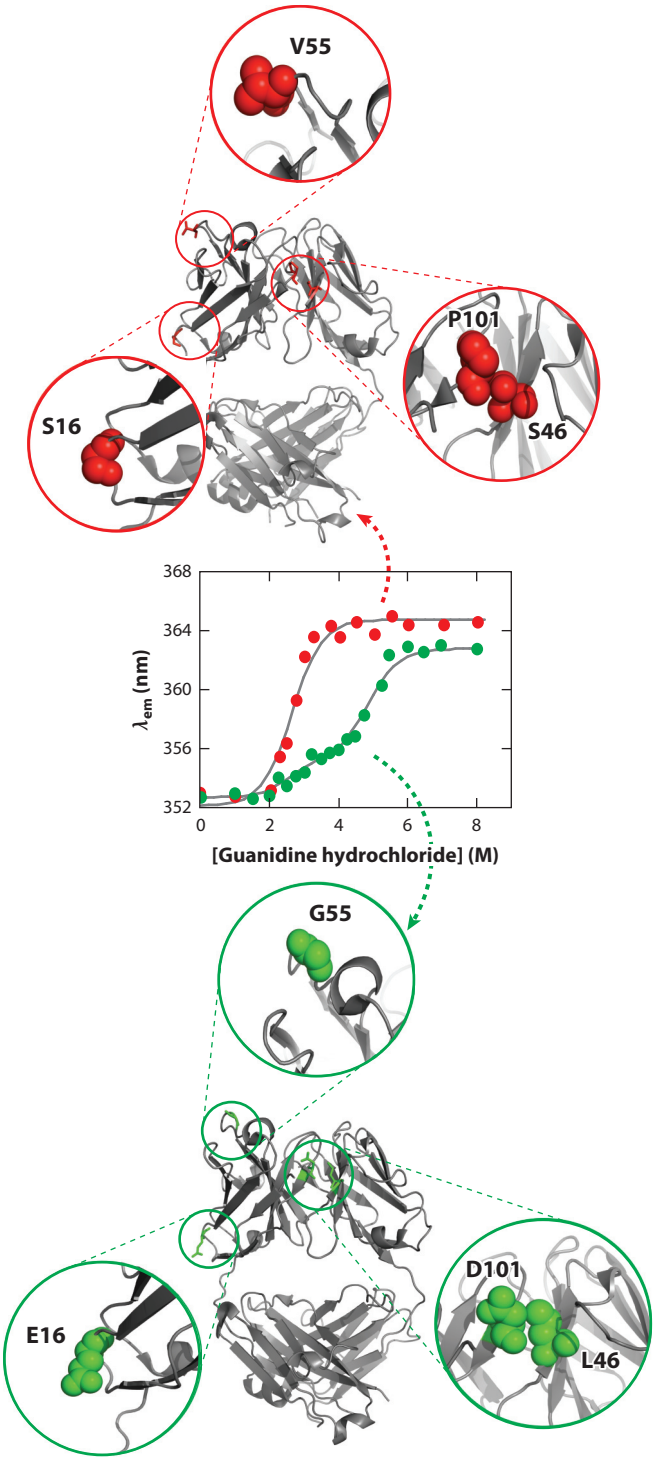
The high degree of sequence similarity between different antibodies, as well as the large number of available antibody sequences and structures, has led to significant understanding in how to stabilize antibodies. These approaches can be classified as (*a*) knowledge-based, (*b*) statistical, and (*c*) structure-based methods. Knowledge-based methods are those that rely on previous experimental studies in which stabilizing mutations or scaffolds have been identified (31, 36, 37, 41). Importantly, this general approach does not depend on natural antibody sequences and also includes mutations that are rare or absent in conventional antibody repertoires. Statistical methods use consensus approaches to identify stabilizing mutations based on the assumption that the most common antibody sequences are optimal (31, 39, 42). This approach can be used in powerful ways to evaluate not only the consensus of individual positions but also pairwise and higher order conservation at noncontiguous sites, such as interfaces between antibody domains (43). Structure-based methods are computational approaches that use either existing or predicted antibody structures to identify stabilizing mutations (24, 27, 44).

The combination of the three approaches is especially powerful, which was demonstrated in a study of an unstable single-chain variable fragment (scFv) (44). The investigators sought to stabilize this scFv (initial melting temperature of 51°C) to the point that it could be fused to an IgG to generate a bispecific antibody with both high activity and stability. They used (*a*) knowledge-based approaches (31); (*b*) statistical methods, such as covariation and frequency analysis (37, 43); and (*c*) structure-based methods, such as Rosetta (45) and molecular simulations (46), to predict positions within the scFv that are most important to stability. This led to the identification of 18 stabilizing mutations at 10 different positions (44). These included single mutations that increased the melting temperature significantly (67°C for a variant with P101D in V_H) as well as combinations of mutations that were even more stabilizing (melting temperature of 82°C for a variant with S16E, V55G, and P101D in V_H, and S46L in V_L).

Four of these stabilizing mutations are featured in **Figure 4**. Two are located at the V_H–V_L interface (V_H P101D and V_L S46L), which has been shown to be critical in determining the overall stability of Fv and scFv antibody fragments (37, 40, 43, 47, 48). The negatively charged mutation

Figure 4

Mutations identified using structure-based and related design methods that enhance the conformational (folding) stability of antibody fragments. Four mutations were introduced into the variable domains of a poorly stable antibody fragment (*red*) that generated a more stable one (*green*) (44). The crystal structures were obtained from the Protein Data Bank: (*top*) 3HC0 and (*bottom*) 3HC4. Abbreviations: λ_{em}, average (center of mass) fluorescence emission wavelength. Data from Reference 44.



(V_H P101D) forms a salt bridge with a neighboring residue (V_H R98), and the hydrophobic mutation (V_L S46L) increases van der Waals contacts and induces favorable structural changes in neighboring residues. The other two stabilizing mutations in the V_H domain are located far from the V_H–V_L interface. The V_H S16E mutation appears to be stabilizing due to electrostatic interactions (it is located in a positively charged region) as well as favorable van der Waals interactions involving the aliphatic side chain (which is longer for glutamic acid than serine). The V55G mutation is located in a turn near HCDR2 and appears to stabilize the scFv by eliminating strain caused by the unfavorable φ and ψ angles for valine at this position.

Many other impactful studies have also demonstrated rational approaches for stabilizing antibodies. One approach is to introduce additional intramolecular disulfide bonds within single-domain antibodies as well as interdomain disulfide bonds within Fvs and scFvs to increase folding stability (49–52). These methods have yielded significant improvements in stability, although in some cases they have resulted in reduced expression. In addition, much effort has focused on optimizing the V_H–V_L interfaces using noncysteine mutations (40, 43, 48). This is important to improve both thermodynamic and kinetic folding stability (especially for Fvs and scFvs) (30, 47), and to reduce the complexity of the resulting antibodies by avoiding additional disulfide bonds. These and related studies (38, 41, 53, 54) are improving the systematic and robust optimization of antibody conformational stability.

5. ANTIBODY COLLOIDAL STABILITY (SOLUBILITY)

The colloidal stability of antibodies—which is governed by solvent-exposed residues in their native folded structure—is not as well understood as conformational stability. Nevertheless, colloidal stability is also a critical attribute of antibodies, especially for those antibodies with high conformational stability (as observed for many IgGs) (4, 5, 55). There are three key elements of antibodies that impact their solubility, namely the (*a*) CDRs, (*b*) frameworks of the variable and constant domains, and (*c*) glycans. CDRs commonly contain hydrophobic and charged residues to mediate high-affinity binding, yet these same residues can also mediate antibody self-association and aggregation (35, 56–60). Therefore, mutations in the CDRs can significantly impact antibody solubility (24, 35, 56–59). The frameworks of antibodies are also important determinants of their solubility (24, 56, 58, 61, 62). These regions typically contain hydrophobic patches (e.g., Fc receptor-binding sites) and oppositely charged domains that can interact with themselves or with the CDRs, leading to poor solubility. Glycans also significantly impact solubility, typically in a positive manner (24, 63, 64).

An excellent study of the impact of each of these factors on antibody solubility is summarized in **Figure 5** (61). The investigators sought to improve the poor solubility of an antibody specific for the glycoprotein LINGO-1. Because this antibody has excellent (subnanomolar) binding affinity and high bioactivity, multiple strategies were pursued to increase its solubility without reducing binding activity. The first approach was to switch the antibody framework from an IgG1 framework to IgG2 and IgG4 frameworks. Surprisingly, this simple change resulted in dramatic increases in solubility for both frameworks (increase from <1 to >30 mg/mL). The origin of these improvements is not clear given that the isoelectric points of the three variants are all high (>pH 8.2), and the IgG2 and IgG4 variants display folding stabilities that are similar to or lower than that of the parent IgG1 antibody.

Given the preference of specific IgG subtypes for different therapeutic applications, the investigators also evaluated the impact of CDR mutations and glycans on the solubility of the parent IgG1 (anti-LINGO-1) antibody (**Figure 5**). Hydrophobic residues in HCDR2 (isoleucine 57), HCDR3 (tryptophan 104), and LCDR3 (tryptophan 94) were mutated to be less hydrophobic

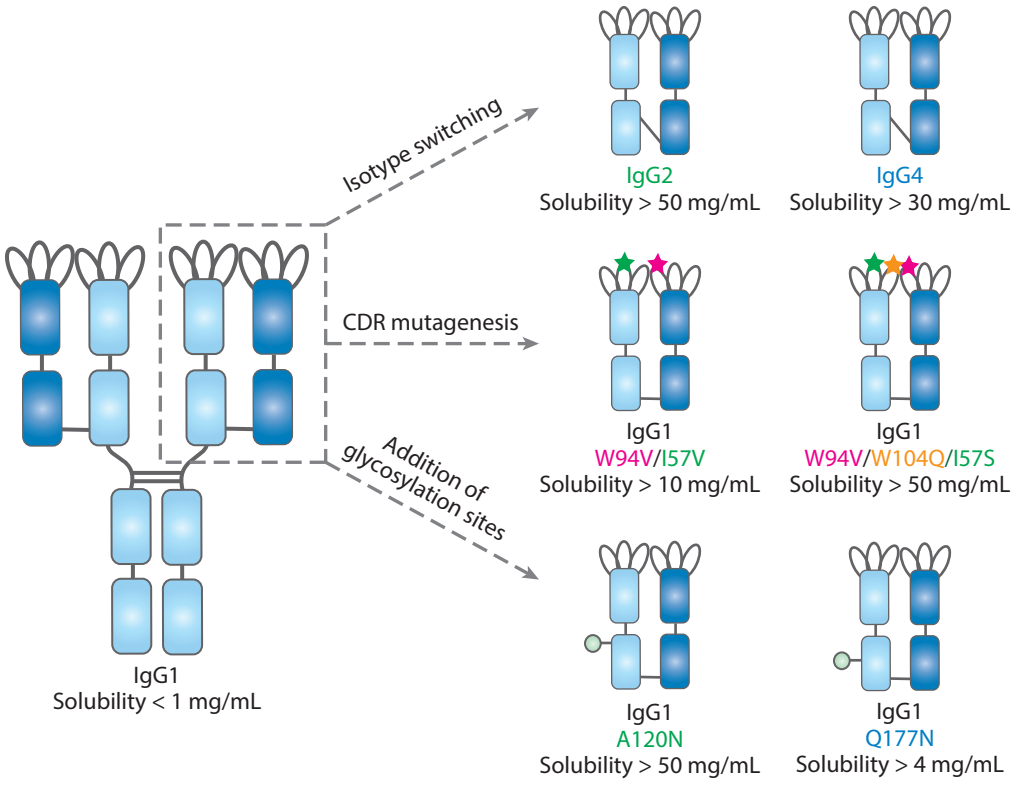


Figure 5

Design methods for increasing antibody solubility. Multiple approaches were found to increase the solubility of a glycoprotein LINGO-1 antibody and to have little impact on binding affinity. Data from Reference 61. Abbreviations: CDR, complementarity-determining region; IgG, immunoglobulin G.

or polar residues. Multiple mutations at these sites improved solubility by more than an order of magnitude without reducing binding affinity. Glycosylation was also found to significantly impact the solubility of the wild-type antibody. For example, removing the glycans from the IgG2 and IgG4 variants reduced the solubility to levels similar to those of the parent IgG1 antibody (either with or without glycans), suggesting that the glycans of the non-IgG1 variants are critical to their superior solubility. The authors also introduced glycosylation sites within the C_H1 domain of the IgG1 antibody at four different positions. Interestingly, the solubilizing activity of the glycosylation variants was high for two mutants (>50 mg/mL) and modest for the others (3–5 mg/mL), and the differences were not predictable based on the proximity of the glycosylation sites to the variable domains.

Several additional studies have used related approaches to improve antibody solubility. For example, multiple studies have reported how the sequences for CDRs, both of antibody fragments and full-length antibodies, can be engineered to increase solubility without compromising binding activity (35, 46, 56–59). These studies have also revealed that the location of aggregation hot spots within the CDRs is variable, and identifying effective sites for mutation requires the use of systematic design approaches (46, 57, 59, 65–67). Significant progress has also been made in engineering the frameworks of antibodies for high solubility by introducing charged mutations

at sites that do not compromise folding stability (58, 62, 64, 68). Moreover, nonconventional glycosylation sites have been identified in the CDRs that significantly increase antibody solubility without reducing affinity (58). These and related studies (69–71) are improving the systematic engineering of highly soluble antibodies.

6. ANTIBODY EFFECTOR FUNCTION

Much of the bioactivity of antibodies stems from their ability to link antigen binding to the recruitment of immune cells and related factors that mediate a range of effector functions (72, 73). It is critical to optimize this activity when generating antibody therapeutics. Effector functions include antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cell-mediated phagocytosis (ADCP), and complement-dependent cytotoxicity (CDC). The first two activities (ADCC and ADCP) are strongly dependent on the strength and specificity of interactions between antibody Fc domains and Fcγ receptors on natural killer cells, macrophages, and other immune cells. CDC depends on the interaction of Fc domains with C1q, which leads to noncellular and cellular mechanisms of cytotoxicity. It is desirable to reduce effector functions when therapeutic antibodies target cell-surface proteins on immune (and related) cells, while the opposite is true when antibodies target pathogenic cells.

There are two key approaches to controlling the level and type of effector functions for conventional antibodies, namely (*a*) engineering the sequences of Fc and hinge regions, and (*b*) modulating the amount and type of Fc glycosylation. The first approach builds on the identification of residues within the Fc domain that normally interact with Fcγ receptors and C1q (74–77). One method for identifying such residues is to make chimeras of different IgG isotypes that naturally display dissimilar abilities to elicit ADCC and CDC (78–80). Another method is to use systematic alanine mutagenesis of the Fc domain to identify residues involved in binding to different Fcγ receptors or C1q. One such study not only identified mutations that reduce Fc binding to activating Fcγ receptors (FcγRI, FcγRIIa, and FcγRIIIa) but also identified alanine mutations that improve binding affinity to specific Fcγ receptors and increase ADCC (74). These insights are critical for improving the design of Fc domains with specific types and levels of effector function.

Indeed, others have built on these interesting studies to further increase Fc affinity and specificity for key Fcγ receptors without altering CDC (81–84). Some of these studies used computational design methods (82). These methods are challenging to implement successfully because (*a*) the Fc binding sites on Fcγ receptors and C1q partially overlap, (*b*) Fcγ receptors bind homodimeric Fc fragments in an asymmetrical manner (which requires calculation of the effects of mutations at two different binding interfaces), and (*c*) there is limited structural data for some Fcγ receptors bound to Fc domains. Nevertheless, this structure-based approach yielded multiple mutations that, in combination, led to significant improvements in binding affinity to an activating Fcγ receptor (FcγRIIIa) and ADCC without reducing CDC (similar mutations are highlighted in **Figure 6**) (82). A limitation of this study is that these mutations also increased affinity for the key inhibitory Fc receptor (FcγRIIb), although the preference for activating Fcγ receptors was maintained. The need for high specificity and affinity for a subset of Fcγ receptors highlights the need for design methods that can guide the optimization of Fc domains for different therapeutic applications.

The fact that the binding sites for Fcγ receptors and C1q partially overlap raises the question of whether mutations can be identified that simultaneously increase both ADCC and CDC to enable superior effector function. Indeed, structure-based and screening methods have led to the identification of several mutations at sites near the Fcγ and C1q binding sites that can simultaneously increase both effector functions (**Figure 6**) (85). These and related findings (86, 87) are

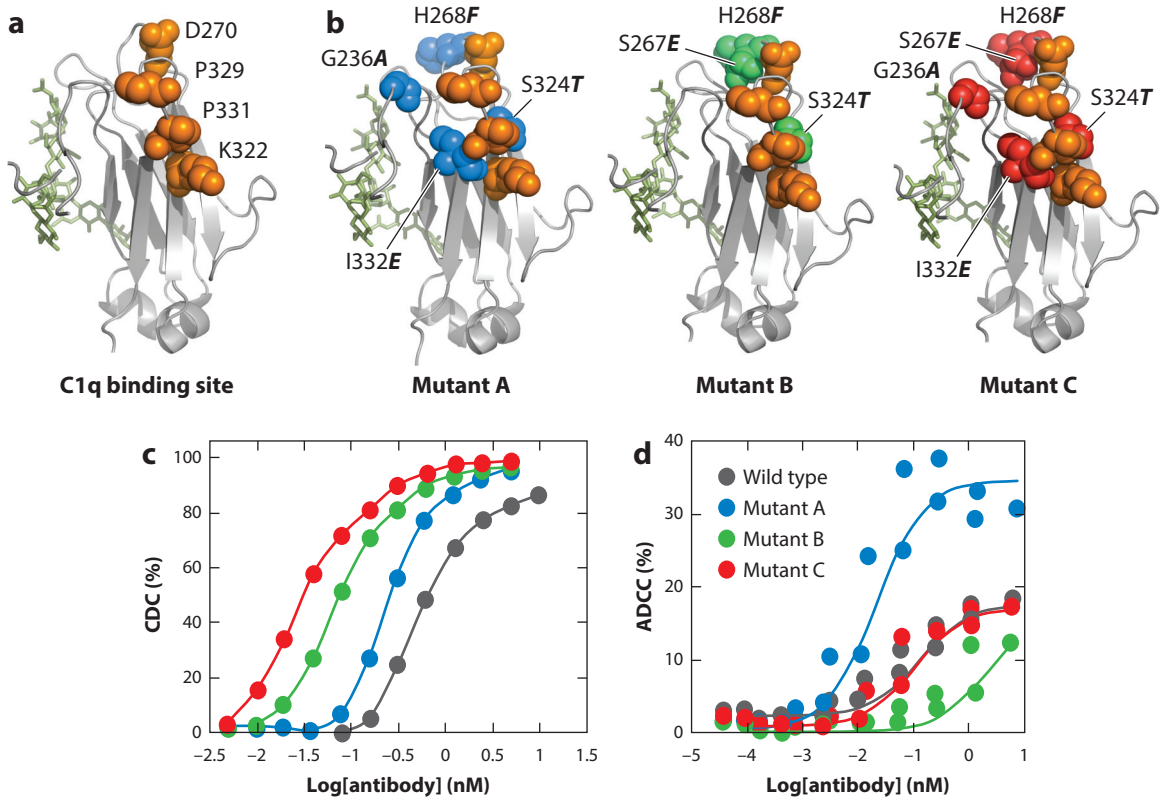


Figure 6

Structure-guided design and selection of crystallizable fragment (Fc) mutations that increase complement-dependent cytotoxicity (CDC). (*a*) Residues in the heavy chain constant domain C_H2 (Protein Data Bank identification number, 1E4K) that form the putative C1q binding center. (*b*) Mutations identified using structure-based methods that increase CDC (85). Evaluation of (*c*) CDC and (*d*) antibody-dependent cell-mediated cytotoxicity (ADCC) of wild-type and three mutants of an immunoglobulin G1 (IgG1) anti-CD20 antibody. Data from Reference 85.

particularly exciting given that they have led to reengineered antibodies with moderate to high CDC, even for antibodies without any initial CDC activity. Nevertheless, the requirement for high opsonization density in CDC leads to significant target-specific differences in activity for the same engineered Fc domains (85). This necessitates the ability to tailor the Fc affinity for C1q to obtain suitable CDC levels for different therapeutic applications.

Another powerful approach for modulating the effector function of conventional antibodies is to alter the amount and type of glycosylation (88). The removal of glycans greatly reduces Fc affinity for low-affinity Fc receptors (e.g., FcγRIIa and FcγRIIIa) and significantly reduces ADCC (89). This typically causes aglycosylated antibodies produced in bacteria to lack effector function (90). Nevertheless, structure-based design and high-throughput screening methods have identified mutations within the Fc domain of aglycosylated antibodies that increase affinity for low-affinity Fc receptors (e.g., FcγRIIa) as well as high-affinity ones (e.g., FcγRIa), leading to significant effector function (91, 92). Modifying the composition of glycans can also significantly modulate effector function. For example, increasing the amount of terminal galactose residues increases CDC activity but does not affect ADCC (93). Reducing the amount of core fucose

residues significantly improves Fc affinity for FcγRIIIa receptors—which appears to be due to reduced steric hindrance at the binding interface (94)—and improves ADCC without altering C1q binding affinity (95). These and related approaches (96) to modifying glycan compositions can be combined with engineered Fc domains to obtain even larger increases in effector function (95, 97, 98).

7. ANTIBODY–DRUG CONJUGATES

Another important approach to improving the cytotoxicity of antibody therapeutics is to chemically link them to cytotoxic drugs (99, 100). This is particularly important for treating cancer because many antibodies that target surface antigens on cancer cells lack therapeutic activity (101, 102). Although methods for making ADCs are simple in theory, the generation of safe and effective drug conjugates has been extremely challenging in practice. This is due to several potential problems with ADCs, including (*a*) reduced bioactivity of conjugated drugs, (*b*) reduced binding affinity or specificity of modified antibodies, (*c*) premature release of the conjugated drug, (*d*) insufficient cellular internalization, and (*e*) short circulation times and poor biodistribution. These challenges require systematic approaches to optimize multiple aspects of ADCs, including the antibodies and drugs themselves, the sites on the antibodies that are modified, the chemistries for attaching such drugs, and the linker between the antibody and the drug.

Most ADCs are generated by attaching drugs to cysteines or lysines exposed on the antibody's surface. Lysine attachment is nonspecific and leads to a heterogeneous mixture of ADCs with modifications in both the Fab and Fc domains (103). Cysteine attachment is more specific and can be conducted using either solvent-exposed cysteines after partial or full reduction of interchain disulfide bonds (such as those in the hinge region) (104–106) or engineered cysteines in the Fab or Fc domains (107–109). Other site-specific chemical approaches include introducing rare (selenocysteine) or nonnatural amino acids into the antibody and linking them to drugs using chemistries that do not modify common amino acids (110, 111). Multiple site-specific enzymatic approaches have also been developed that activate sugars or specific amino acids within small peptide tags, and these also show promise for simplifying the generation of homogeneous ADCs (112–114).

An elegant study that addressed several of the factors influencing ADC activity is highlighted in **Figure 7** (109). The investigators sought to evaluate the bioactivity of ADCs prepared by attaching drugs to engineered cysteines at different sites in the Fab and Fc domains. Single cysteine mutations were introduced at sites with differing solvent accessibilities and local charge environments. The light chain mutation (V205C) is the least solvent-accessible and is located in a positively charged region, and the Fc mutation (S396C) is the most solvent-accessible and is located in a neutrally charged region (as is the other heavy chain mutation, A114C, which displays an intermediate level of solvent accessibility). Maleimide-based conjugation of these trastuzumab variants with a cytotoxic drug (monomethyl auristatin E) led to similar in vitro potency for killing cancer cells. However, the in vivo activity of the three ADCs was dissimilar: activity was highest for the ADC prepared using the mutant with the least solvent-accessible cysteine (light chain V205C) and lowest for the ADC prepared using the mutant with the most-accessible cysteine (Fc S396C).

Analysis of in vivo ADC stability revealed that loss of the conjugated drug correlated with reduced activity (109). This appears to be due to the low stability of the cysteine–maleimide linkage. Increasing the solvent accessibility of this linkage enables maleimide exchange with serum components, such as albumin and glutathione. Moreover, the positively charged environment of the light chain mutant (V205C) appears to promote hydrolysis of the succinimide ring, which prevents maleimide exchange and increases ADC stability. This excellent study, which is based

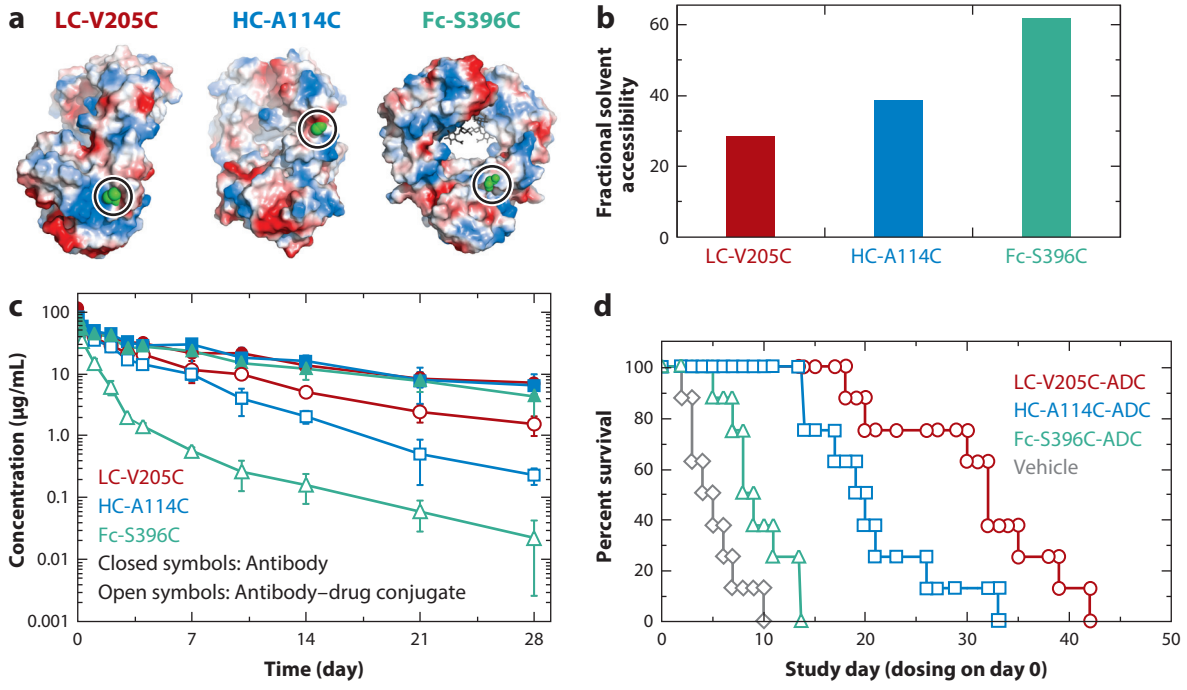


Figure 7

Design and evaluation of the bioactivity of antibodies conjugated at different sites with a cytotoxic drug. (*a*) The sites mutated to cysteine are highlighted in the crystal structures of the antigen-binding fragment (Fab) and crystallizable fragment (Fc), and (*b*) these sites show a range of solvent accessibilities. (*c*) Evaluation of the clearance rates of antibodies injected into mice. Although the total antibody levels were similar for injections of each antibody–drug conjugate (ADC), the fraction of intact ADC was highest for the light chain (LC) variant V205C and lowest for the Fc S396C variant. (*d*) Mice dosed equally with each ADC showed significant differences in survival, and these differences were consistent with the fraction of intact ADC. Abbreviation: HC, heavy chain. Figure adapted with permission from Macmillan Publishers Ltd.: *Nature Biotechnology* (109), copyright 2012.

on a small set of cysteine mutants, paves the way for future studies to better define how antibody sequence and structure, as well as conjugation chemistry, influence ADC stability and activity.

Several other key studies have demonstrated how the activity of ADCs can be optimized in a systematic manner. For example, low ratios of drug to antibody (≤4 drugs per antibody) are typically optimal because high drug loading can lead to fast antibody clearance and a low therapeutic index (the ratio of the maximum tolerated to the minimum effective doses) (115). The linker between the antibody and drug is also critical because it must be stable prior to cellular internalization and then cleavable after internalization for cytotoxicity [see (116) for encouraging results with noncleavable linkers] (100, 117, 118). Linkers that include peptide bonds appear particularly attractive, given their stability in serum and ability to be cleaved by proteases after cellular internalization (117). These and related findings (119) are improving the rational design of potent and safe ADCs for diverse therapeutic applications.

8. BISPECIFIC ANTIBODIES

Another important approach to improving the therapeutic activity of mAbs is to engineer them to recognize multiple targets (120–123). Such bispecific antibodies hold great promise for improving

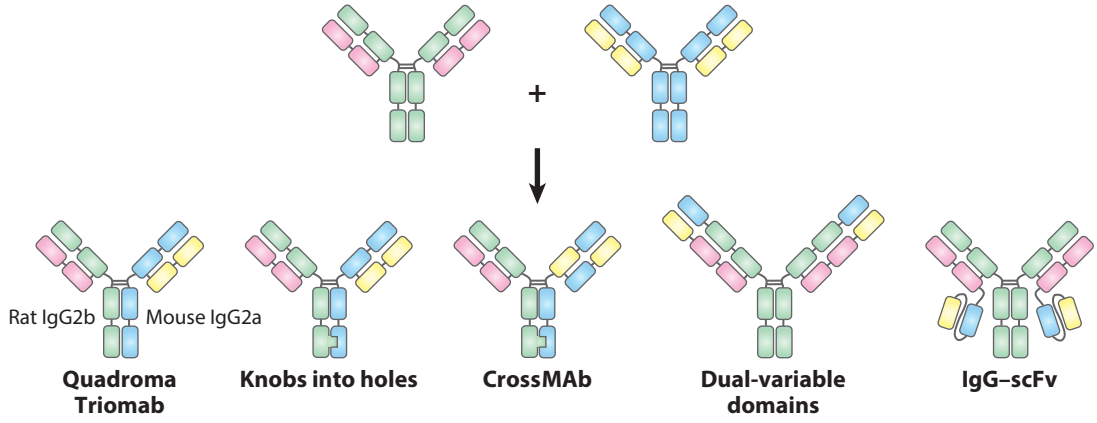


Figure 8

Molecular architectures of bispecific monoclonal antibodies (mAbs). Two mAbs are recombined into different bispecific architectures. A quadroma Triomab (Trion Pharma, Munich, Germany) comprises one heavy chain–light chain pair of a rat immunoglobulin G2 (IgG2) and one heavy chain–light chain pair of a murine IgG2 antibody (128). The knobs-into-holes architecture consists of an opposing cavity and protrusion in the heavy chain constant C_H3 domains to enforce the heteropairing of heavy chains (127). The CrossMAb (Roche, Basel, Switzerland) architecture involves swapping the light chain constant C_L and the heavy chain constant C_H1 domains onto opposite chains to enforce correct light-chain pairing, and also uses knobs-into-holes mutations to enforce correct heavy-chain pairing (140). Dual-variable-domain antibodies have the variable domains from one antibody added to the N terminus of the heavy and light chains of the other antibody (142). IgG–scFv (single-chain variable fragment) bispecific antibodies contain the variable domains of one antibody—which are reformatted as an scFv—fused to the terminus of the heavy or light chains of the second antibody (132).

therapeutic activity because they can potentially improve antibody activity in several ways. These include (*a*) improving effector function by targeting specific immune cells in addition to the therapeutic target, (*b*) enhancing antibody delivery to different organs (such as the brain) by targeting transport proteins in addition to the therapeutic target, (*c*) increasing specificity for pathogenic cells by targeting two cell-surface antigens instead of only one, and (*d*) improving the robustness and persistence of therapeutic activity by blocking two different biological pathways. These and related advantages of bispecific antibodies have driven much interest in their development and optimization.

Design methods have played a central part in generating bispecific antibodies because of the large number of potential molecular architectures. The most important architectures are (*a*) full-length antibodies composed of two different heavy chains (with the same or different light chains) (124–129), (*b*) full-length antibodies with additional variable domains (123, 130–134), and (*c*) antibody fragments lacking Fc domains, such as scFvs and diabodies (135, 136). Most current efforts are focused on developing the first two types of bispecific antibodies (which are highlighted in **Figure 8**) because they are more similar to conventional antibodies, and possess Fc domains for mediating effector function and antibody recycling.

The main challenges in generating bispecific antibodies composed of two different heavy chains are ensuring (*a*) correct heteropairing of the heavy chains and (*b*) correct pairing of the light chains with their corresponding heavy chains. Early efforts to address both challenges exploited the unique pairing preferences of rat and mouse IgG2 heavy and light chains (**Figure 8**) (128). The light chains of each species prefer to pair with their cognate heavy chains, and the heavy chains show little preference, forming both homo- and heteropairs. The fact that rat heavy chains do not bind Protein A enables selective purification of bispecific antibodies (which are referred to as quadroma Triomabs).

Due to the immunogenicity of these nonhuman antibodies, several other creative approaches have been developed for use with human antibodies (**Figure 8**) (124–127, 129, 137, 138). The knobs-into-holes approach involves engineering the C_H3 domains of one antibody to possess a knob, which is typically a solvent-exposed residue at the C_H3–C_H3 interface with a bulky side chain (e.g., tryptophan) (126, 127, 139). The C_H3 domain on the other antibody is engineered to possess a hole by replacing several residues at the interdomain interface with those possessing smaller side chains (e.g., tyrosine mutated to valine). Methods of designing antibodies have also been used to identify additional C_H3 mutations to improve the specificity of heterochain pairing for knobs-into-holes and closely related approaches (124, 126, 139). These include cysteine mutations for forming disulfide bonds between different C_H3 domains (126), and oppositely charged mutations for mediating attractive electrostatic interactions between different C_H3 domains, as well as repulsive interactions between the same ones (124).

These powerful approaches address the problem of pairing different heavy chains, but do not directly address the problem of the proper pairing of light chains. This issue can be circumvented either using a common light chain (126) or through assembling two different antibodies after their production by first dissociating the heavy chains without dissociating the light chains (126, 127). A more direct approach would be one that does not require a common light chain or separate production of both antibodies followed by in vitro dissociation and assembly. An interesting potential solution to this problem is to swap the constant domain of one of the light chains (C_L) with the C_H1 domain of one of the heavy chains (**Figure 8**) (140). The resulting modified light chain prefers to pair with the modified heavy chain. Other related domain crossovers, such as swapping V_H and V_L, are less useful because they also mediate the formation of side products. Nevertheless, the CrossMAb (Roche, Basel, Switzerland) approach of swapping C_H1 and C_L domains can be combined with knobs-into-holes methods to produce bispecific antibodies in cell lines expressing four different heavy and light chains without the need for in vitro dissociation and reassembly (140).

Importantly, the success of this C_H1/C_L crossover approach requires that the constant domains govern the pairing specificity of heavy and light chains, and that the variable domains have little impact on this specificity. This is logical because the C_H1–C_L interface contributes significantly to the folding stability of Fab fragments (47). However, it appears that the V_H–V_L interface also plays an important part in determining the specificity of heavy and light chain pairing for some antibodies (141). Therefore, computational design and screening methods have been used to identify mutations at the V_H–V_L interface that improve the pairing specificity of mutant heavy and light chains. Interestingly, the best designs required mutations in both the variable (V_H and V_L) and constant (C_H1 and C_L) domains, suggesting that both types of domains contribute to the pairing specificity of heavy and light chains. Nevertheless, more work is needed to evaluate the generality of these findings and their utility for designing bispecific antibodies with architectures similar to conventional antibodies.

Another powerful approach for combining two existing antibodies that ensures proper light chain pairing is to add the variable domains from one antibody to the termini of the light or heavy chains (or both) of the second antibody (**Figure 8**). The simplest way to do this is to add the variable domains from one antibody to the N terminus of the heavy (V_H1-V_H2-C_H1-C_H2-C_H3) and light (V_L1-V_L2-C_L) chains of the second antibody to produce dual-variable-domain antibodies (142, 143). A related approach is to attach the variable domains from one antibody—in the form of an scFv in which the V_H and V_L domains are linked together via a peptide linker—to the terminus of either the heavy or light chains of the second antibody to produce IgG–scFv fusions (37, 123, 130–134, 144). These and related approaches are attractive because the heavy and light chains of the resulting antibodies are identical, which greatly simplifies production and

characterization. Concerns about these approaches include the fact that they result in antibodies with different architectures, valencies (tetravalent versus bivalent), orientations and accessibilities of the variable domains, and stabilities relative to conventional antibodies. Nevertheless, more work is needed to understand how these and other attributes of bispecific antibodies impact their therapeutic activity.

9. FUTURE DIRECTIONS

These exciting advances in methods for designing antibodies provide many opportunities for further improvement and application. An important outstanding challenge is the de novo design of antibodies. This includes not only designing antibodies that recognize a given antigen but also specific epitopes that are important for therapeutic applications. De novo design continues to be challenging because of several factors, including the difficulty in accurately predicting the conformation of CDR loops (especially those that are long and variable in length) as well as the structures of antibody–antigen complexes for which there are no initial crystal structures. Nevertheless, recent advances in predicting CDR conformations (145–147), as well as in designing other affinity proteins (148, 149), suggest that these challenges can be overcome. Combining knowledge-based computational methods, such as Rosetta (45), with physics-based methods, such as molecular simulations (150), will be important for improving the accuracy of de novo design methods. It will be equally important to make such computational approaches accessible to a broad audience to enable the evaluation of a large number of designs. The lack of accessibility of computational design methods to nonexperts—which has limited the evaluation of proposed methods—is one reason for the slow progress in developing robust methods for designing antibodies.

Another critical problem in the field of antibody design is the need to simultaneously optimize multiple attributes of antibodies. This is arguably the most compelling reason for pursuing antibody design methods in the first place, and it remains an outstanding challenge. For example, methods aimed at designing the CDRs of antibodies to ensure high affinity need to identify sequences that also maximize folding stability and solubility. This is particularly important for bispecific antibodies with nonconventional architectures because folding stability and solubility are common problems. A related challenge for these nonconventional antibodies is the need to optimize the location for attaching additional variable domains because some locations that are optimal for stability may be suboptimal for binding. For bispecific antibodies with conventional architectures, the proper pairing of different light chains with their cognate heavy chains may be influenced by interactions between the CDRs [as suggested by (141)]. Thus, mutations in the CDRs that improve affinity or solubility may alter pairing efficiency (either positively or negatively), and need to be considered as part of the overall design process.

The need for improved design methods for optimizing antibody effector function is also evident by the fact that such optimization can lead to defects in other antibody attributes. For example, the two main approaches for increasing effector function are (*a*) altering the Fc sequence or glycans, and (*b*) changing the antibody format to a bispecific one in which the variable domains target immune cells in addition to the target antigen. The first approach needs to consider the effects of mutating solvent-exposed residues in the constant domains on properties such as antibody stability and solubility. The second approach must account for similar issues as well as how conversion to a bispecific antibody architecture potentially reduces binding affinity due to a reduction in valency (i.e., conversion from bivalent to monovalent binding).

There is also a considerable need for improved methods for designing ADCs due to many similar challenges. Introducing cysteines or other residues for attaching drugs can have variable effects on antibody stability, depending on their location, and attaching hydrophobic drugs to the

surfaces of an antibody can lead to variable and difficult-to-predict impacts on solubility. Design methods are needed to collectively optimize these and related properties to obtain highly active ADCs.

10. CONCLUSIONS

Methods for designing antibodies are increasingly being used to reduce the reliance on screening and immunization to optimize key antibody attributes. These rational approaches have not eliminated the need for immunization or screening but instead have focused such efforts to make them more productive. This has led to the generation of antibodies with properties that are uncommon or absent in conventional antibodies. The future of antibody design lies in further improving and evaluating methods for predicting antibody properties to enable optimization of several properties at once. This will be especially important for complex, nonconventional antibody formats, such as bispecifics and ADCs, that have even more critical attributes than conventional antibodies that must be collectively optimized.

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