

Structure-Guided Design of Antibodies

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Abstract: Monoclonal antibodies capable of recognizing antigens with high affinity and specificity represent a well-established class of biological agents. Since the development of hybridoma technology in 1975, advances in recombinant DNA technologies and computational and biophysical methods have allowed us to develop a better understanding of the relationships between antibody sequence, structure, and function. These advances enable us to manipulate antibody sequences with the goal of improving upon, or creating new biological or biophysical properties. In this review we will focus on recent successes in using structure-guided computational methods to design antibodies and antibody-like molecules with optimized affinity and specificity to antigen and for enhancing protein stability.

Keywords: Antibody engineering, structure-based design, affinity maturation, effector function, protein stability.

INTRODUCTION

Monoclonal antibodies (mAbs) represent a growing segment of biological drugs with 26 products approved for clinical use and over 200 mAb's in various stages of clinical development [1-3]. Clinical acceptance of mAbs for treating a variety of diseases has largely been driven by the development of technologies resulting in antibodies having several critical properties that make them safe and effective therapeutic agents [4]. Many methods are now available for the selection and intentional engineering of high-affinity antibodies that have unique biological function and suitable biophysical properties for scalable production [5].

Antibody interactions with antigen are mediated by the variable domains (VH and VL, see Fig. 1), with most of the antibody:antigen contacts formed by the six complementarity determining regions (CDRs). Depending on the size of the antigen, different combinations of the CDR loops are used for the interaction with antigen. Generally, antibodies against small targets, such as haptens and peptides, use two to three CDRs, while antibodies against larger proteins use four to six CDRs.

Antibody effector functions are primarily mediated by their Fc fragment, composed of CH₂ and CH₃ domains. Among those functions, antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP) in humans are determined by antibody interactions with activating FcγRI, FcγRIIa/c, FcγRIIIa and inhibitory FcγRIIb receptors [6], while complement-dependent cytotoxicity (CDC) is triggered by antibody binding to the components of the complement system [7]. In addition, the Fc fragment gives antibodies their long half-life via active recycling by neonatal Fc receptor (FcRn) [8]. The Fc region of an antibody is crucial for its function as it links different FcR-mediated effects including opsonization, cell lysis, and degranulation of mast cells and eosinophils to antigen recognition [9].

In this review, we first give an overview of the structural basis for antibody functional activity mediated by the Fab and Fc regions. We then describe several structure-based design methods for engineering antibody domains to achieve desirable pharmaceutical characteristics such as improved antigen-binding affinity, modulation of effector function, and enhanced biophysical and biochemical stability. The modular nature of antibodies allows these designed domains to be combined in multiple ways to produce new agents with customized pharmaceutical properties.

ENGINEERING ANTIBODY AFFINITY AND SPECIFICITY

Structural Basis of Antibody-Antigen Interactions

Over the last several decades, x-ray crystallography and molecular modeling have highlighted many significant details of the mechanisms underlying antibody-antigen interactions. Affinity is determined by several factors, including electrostatic interactions, hydrogen bonds, van der Waals contacts, shape complementarity, buried surface area, and entropic contributions of interacting residues. An examination of each of these factors in the context of an antibody-antigen complex structure can aid in understanding the relative potency of related antibodies. These factors can also be optimized to engineer antibodies with higher affinity.

Electrostatic interactions occur between charged atom pairs and are especially strong between charged amino acid side chains, as in salt bridges. The strength of the interaction depends on the distance and dielectric constants in the environment. High salt concentrations and extremes of pH disrupt antigen-antibody binding by weakening electrostatic interactions and/or hydrogen bonds. The mature esterolytic antibody 48G7 binds a nitrophenyl phosphonate transition-state analog with a 30,000-fold higher affinity than its germline precursor (K_d: 135 μM for germline and 4.5 nM for mature 48G7), which differs at only six amino acids in the heavy chain and three in the light chain (Fig. 2). The high resolution crystal structures of unliganded germline and its complex with hapten show a large conformational change (rms deviation of Cα in variable region is 0.61Å) induced upon antigen binding, while the conformational change

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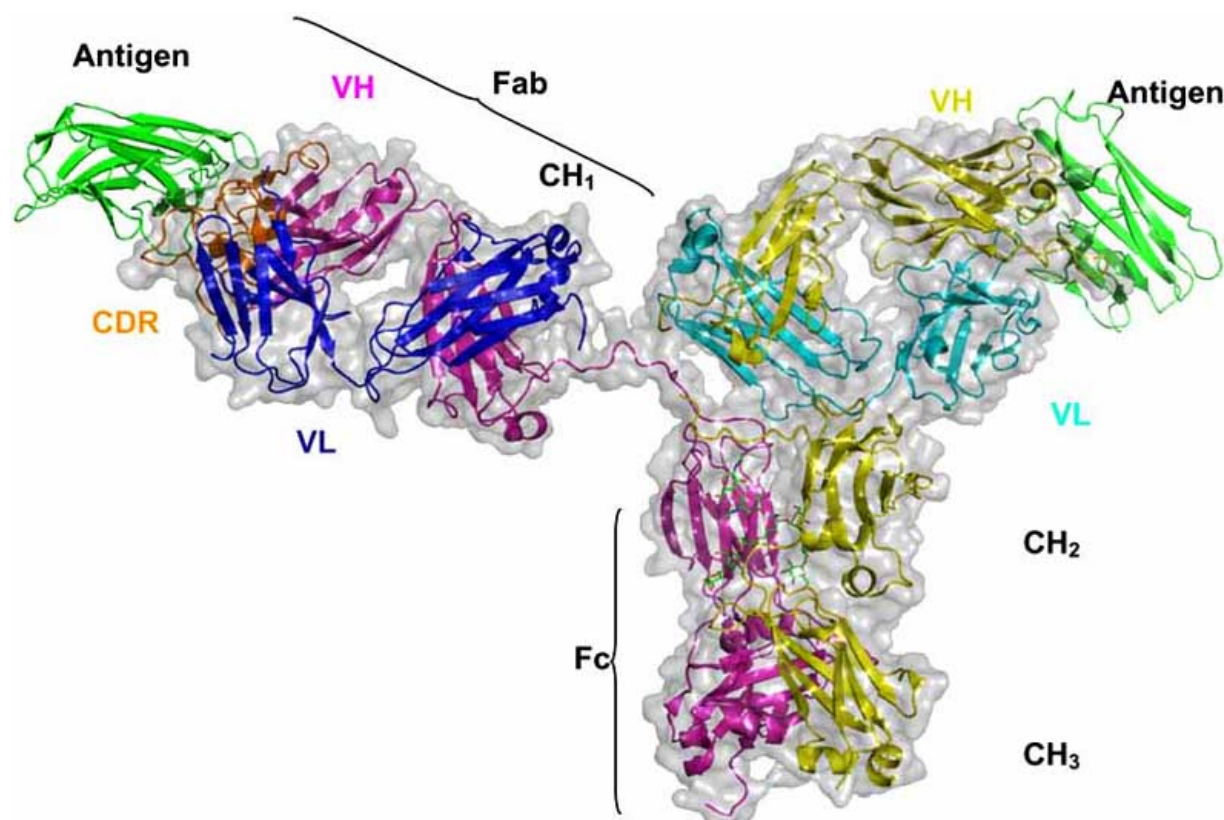


Fig. (1). The domain structure of an IgG antibody.

between the unliganded 48G7 and its complex with hapten is quite small (rms deviation of C α in variable region is 0.39Å) [10]. The structural changes that occur in the germline-hapten complex become preorganized in the combining site of mature antibody. In addition, one additional hydrogen bond was observed between the phosphonyl group of the hapten and the mature antibody compared to the germline antibody. Energetic analysis by molecular dynamics and free energy calculations based on the structures suggest that the increase in affinity of the 48G7 antibody over its germline precursor is achieved by electrostatic optimization [11]. Electrostatic interactions are also shown to be critical for binding affinity of 4M5.3 anti-fluorescein single chain antibody fragment (scFv). It has considerably higher affinity (K_d=270 fM) than its wildtype counterpart 4-4-20 (K_d=700 pM) that is different is 14 positions. A comparison of the crystal structures shows little difference between the two scFv molecules (backbone RMSD of 0.6Å), despite the large difference in affinity (1800-fold, 4.5 kcal/mol in ΔG). Shape complementarity is improved slightly between the variable light chain and variable heavy chain domains within the antibody, but no significant improvement in shape complementarity of the antibody with the antigen is observed in the mutant over the wild-type. Theoretical calculations show that the largest change in the enthalpic contribution to the binding in 4M5.3 is attributed to improved electrostatics [12].

Hydrophobic interactions occur between uncharged non-polar atoms at short distance via exclusion of water molecules. It has been proposed that the major contribution to the free energy of protein folding arises from the transfer of apolar atoms from an aqueous to a close-packed hydrophobic environment with a corresponding reduction in solvent accessible surface area. In general, the hydrophobic and van der Waals are short-range forces and serve to pull two surfaces together that are complementary in shape. The strength of a hydrophobic interaction is proportional to the surface area that is excluded from water. Anti-protein antibodies exhibit a high degree of both shape and chemical complementarity for their antigens, and combined solvent-accessible surfaces buried in anti-protein antibody-antigen complexes range from ~1400 to 2300 Å² [13, 14]. Four independent monoclonal anti-HEL murine antibodies (H8, H10, H26 and H63) were crystallized, and they have overlapping epitopes on the antigen with relative affinities H26 < H63 < H10 < H8 [15]. Structural comparisons reveal that binding affinity improvement of the H8 (30-fold) comes from increased burial of apolar surface along with less burial of polar surface, as well as by improved shape complementarity. There are no additional hydrogen bonds or van der Waals contacts in the H8 complex structure, nor is there or by an increase in total buried surface.

In addition to above interactions, π - π stacking and water-mediated contacts at antibody-antigen interfaces are ubiquitous in known crystal structures [16, 17]. Another

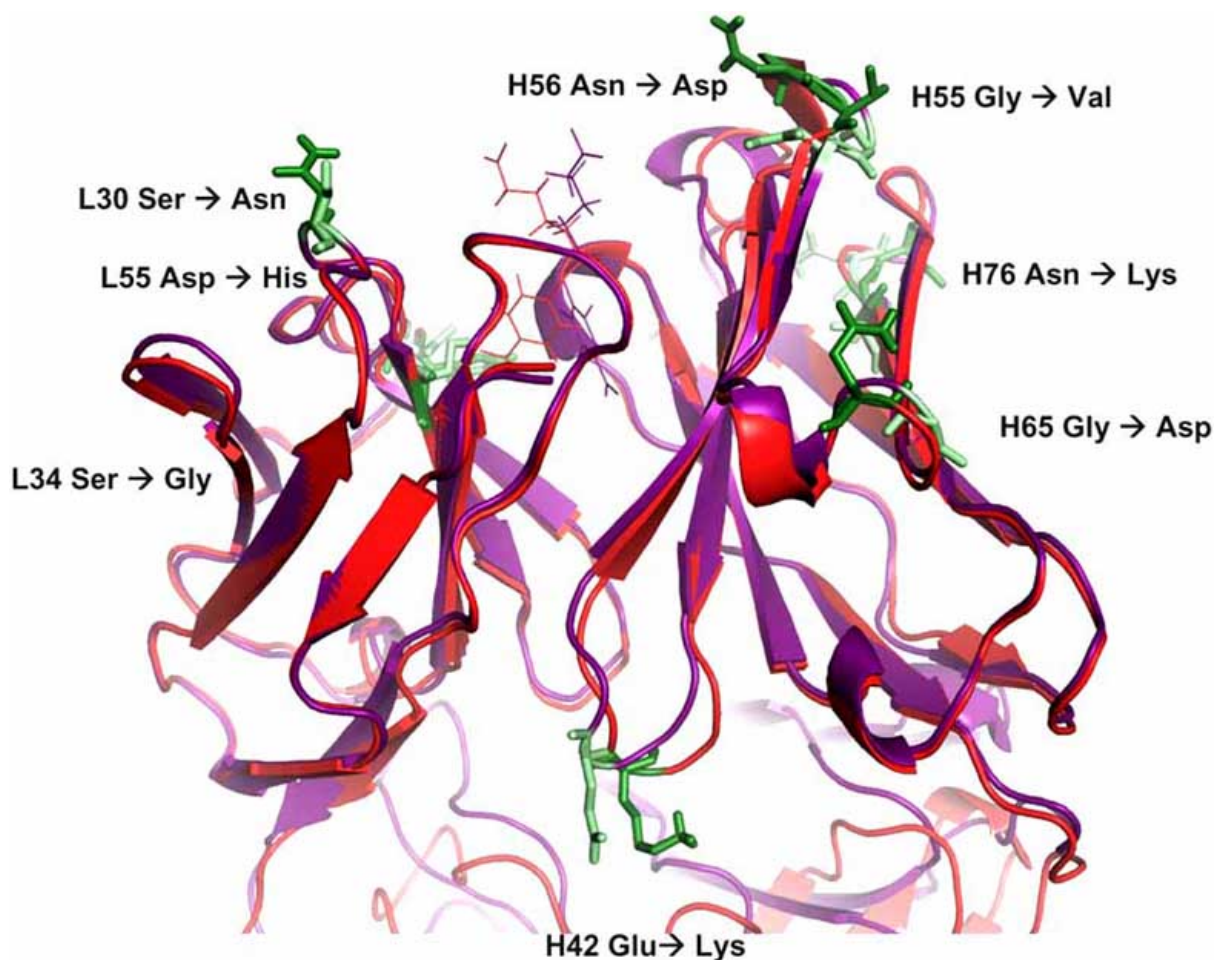


Fig. (2). Superpositions of the variable regions of the germline Fab-hapten complex (purple) and nature Fab-hapten complex (red). The side chains of the somatic mutation sites are indicated in light green (germ line) and dark green (mature).

important contribution to antigen-antibody recognition is entropy effect. The kinetics of antibody-antigen and homologous antibody interactions are commonly temperature-dependent [18], suggesting there is structural plasticity involved in complex formation. Antibodies undergo different degrees of conformational change upon antigen binding. Since the binding interface of an antibody consists of six flexible CDR loops, entropy loss is expected upon binding with antigen. Reducing the entropy loss is an effective strategy for affinity improvement. In order to quantitatively estimate the entropy change, molecular dynamics calculations were performed on the antibody 4-4-20/fluorescein, its low-affinity germline, and two intermediates [19]. The results suggest that increased affinity for the target ligand is associated with a decreased entropic cost of binding, supporting the idea that high-affinity binding is driven partly by reduced protein flexibility.

Detailed understanding of antibody-antigen interactions is essential for the design of antibodies with desired specificity and affinity. Different studies have shown that the distribution of amino acid in both paratope (part the antibody that interacts with an antigen) and epitope (part of antigen that is recognized by an

antibody) is biased. Systematic analysis of amino acid distribution in paratopes of germline and mature antibodies [20, 21] revealed that three largest contributions to antibody-antigen interface composition in antibodies come from tyrosine, glycine, and serine. These three amino acids are overrepresented on the antibody-antigen interface presumably due to their versatility in forming different types of interactions. Consequently, these amino acids promote low affinity binding of the naïve germline antibodies to new antigens, enabling subsequent affinity maturation. As seen in paratope analysis, structure-based epitope analysis and prediction show that epitopes are significantly enriched in tyrosines and tryptophans [22]. In addition, charged and polar amino acids are overrepresented in epitopes, while aliphatic amino acids are underrepresented. This observation underscores the importance of polar interactions for antibody-antigen recognition and suggests that optimizing these interactions is essential for antibody design.

Antibody Affinity Enhancement

Accurate structure-based prediction of binding affinity has two key components: energy functions that properly

describe the biological system and conformational sampling during computational simulations. Recently, structure-based protein design has generated much attention thanks to rapid development in computational algorithms and increased computational power [23]. The design strategies have successfully aided in the understanding of the maturation of an antibody for an antigen and guided the design of antibody with enhanced affinity.

In most cases of successful computational design, a crystal structure of the antibody-antigen complex was available. The binding affinity of single or multiple mutants around CDR loops, including residues without direct contact with antigen, was estimated. Clark *et al.* were able to improve binding affinity of an antibody fragment to the I-domain of the integrin VLA1 by one order of magnitude to 0.9 nM based on the crystal structure with a resolution of 2.8 Å [24]. In this study, combined mutations were derived from side chain repacking [25] and electrostatic optimization [26]. A total of 10 single-point mutants with measured affinities were found to be better than wild type for the 83 constructed mutants, giving a hit rate of 12%. A similar approach was used to improve the monomeric binding affinity of therapeutic anti-CD40L antibody 5c8 [27] by 30-fold down to 12pM. This optimized monovalent Fab fragment was equipotent to original bivalent mAb in cell-based CD40L signaling blocking assay suggesting that avidity component of free binding energy of antibody can be generically substituted by the improved affinity [28].

Subsequently, Lippow *et al.* designed a two-stage hierarchical procedure to estimate the binding affinity [29]. First, conformational search was carried out by sampling discrete side-chain rotamers using dead-end elimination and the A* search algorithm with a physics-based energy function. For each protein sequence, all conformations within an energy cut-off of the global minimum were saved in a list. Second, the lowest-energy structures of each sequence were reevaluated using more computationally demanding models, including Poisson-Boltzmann continuum electrostatics, continuum solvent van der Waals, unbound-state side-chain conformation search and minimization. In their study, they found that the calculated electrostatic term for binding was better predictor for affinity than the total calculated binding free energy in the first design on anti hen egg-white lysozyme antibody D1.3. The same method was used to engineer a ten-fold affinity enhancement of the drug cetuximab (Erbix) for anti-epidermal growth factor receptor, and a 140-fold affinity enhancement of the model antibody D44.1 for lysozyme. The method was further applied to identify known mutations in bevacizumab and 4-4-20.

During drug discovery, antibody-antigen structures are typically not available at the time of affinity maturation. Fortunately, a lot of modeling tools were developed for antibody CDR prediction if the structure of unliganded antibody is not available, and antibody-antigen docking may be used to model the complex [30]. Barderas *et al.* successfully enhanced the affinity of the human anti-gastrin TA4 scFv to 13.2 nM from 6 μM with the guidance of computational modeling [31]. In this study, the modeling was divided into three steps: first, the models of parental scFv TA4 and two first-round variants were constructed;

second, the epitope fragment of the gastrin17 peptide was docked into the binding sites and finally, mutation proposals based on theoretical and experimental information were made for affinity enhancement in a second-round maturation step.

Design of Antibodies with Desired Specificity

Mammalian humoral immune response can yield antibodies with exquisite specificity. Optimization of both affinity and specificity is important for diagnostic and therapeutic antibodies. So far, most structure-based designs focus on improving potency. Nevertheless, structural and calorimetric analyses of well characterized anti-HEL antibodies shed some light on the molecular basis of specificity [32]. For example, antibodies H8, H10, and H26 share over 90% variable-region amino acid sequence identity and recognize identical structurally characterized epitopes. These antibodies differ widely in their tolerance of mutations in the epitope and H8 is the most cross-reactive, while H26 is highly specific. Thermodynamic calculations suggest that upon association H8 has the largest conformational entropic penalty and also the smallest loss of enthalpic driving force with variant antigen. Much smaller structural perturbations are expected in the formation of the less flexible H26 complex, and the large loss of enthalpic driving force observed with variant antigen reflects its specificity. The observed thermodynamic parameters correlate well with the observed functional behavior of the antibodies and illustrate fundamental differences in thermodynamic characteristics between cross-reactive and specific molecular recognition.

In addition to improving specificity, it is also often necessary to generate cross-reactive antibodies binding to not only the human antigen but also the corresponding rodent orthologs. Such cross-reactive antibodies can be used to validate the therapeutic targeting and evaluate the safety profile in preclinical animal models before clinical trials. Computational design was used to improve the species cross-reactivity of an antibody-based inhibitor of the cancer-associated serine protease MT-SP1 [33]. In this study, antibody E2 was identified in a bacteriophage-displayed antibody library, and is a potent inhibitor of MT-SP1 with a K_i of 12 pM. Its mouse ortholog epithin is 87% identical and E2 is 300-fold less potent against it. The basis of the difference is not apparent from biochemical and structural analyses of the MT-SP1/E2 complex. Only three residues are different in the contact interface with antibody between human MT-SP1 and mouse ortholog epithin. A molecular mechanics-based energy function in conjunction with an implicit solvent model was used to predict the effect on binding of mutations at the protease-antibody interface. The binding free energy for a point mutation was estimated by the calculated change in free energy upon mutation of the E2/epithin complex minus the calculated change in free energy upon mutation of the unbound E2 antibody minus the calculated free energy of epithin. During the calculation, 6 residues on the heavy chain E2 and within 5 Å of any the three residues that differ between MT-SP1 and epithin were mutated to the other 18 possible amino acid side chain (excluding cysteine). With the computational designs, 8 mutations were predicted to lower the free energy and were tested experimentally. One of the eight mutations was shown

to improve antibody affinity for the mouse ortholog of the enzyme 14-fold, resulting in an inhibitor with a K_i of 340 pM.

HUMANIZATION OF ANTIBODIES

Immunogenicity is a critical concern for therapeutic antibodies. Murine antibodies are routinely humanized to reduce immunogenicity risk [34]. Several commonly used humanization methods rely on structure-based design to maximize the human sequence content in an antibody while retaining binding affinity.

An early approach to humanization was CDR grafting, where the CDRs from a murine antibody were combined with the framework regions from a human antibody. This approach was applied successfully to an anti-hapten antibody B1-8 [35], an anti-lysozyme antibody D1.3 [36], and daclizumab [37], the first FDA approved humanized antibody in the United States. For daclizumab, a human framework was selected from a sequence database to maximize homology with the murine antibody. A homology model was used to guide humanization, and several human framework residues were “backmutated” to their murine counterparts to ensure that the antibody would retain binding affinity (a total of 7 amino acids were backmutated in the heavy chain and 2 in the light chain). Backmutations typically are made at “canonical” residues that determine the conformation of CDRs [38, 39] and other framework positions that contact the CDRs.

The anti-HER2 antibody 4D5 (trastuzumab, under the brand name Herceptin[®]) was humanized with a framework corresponding to the consensus sequences of the most common human subclasses: VL κ subgroup I and VH subgroup III, instead of a framework from any particular human antibody [40]. Several framework backmutations were necessary to get a humanized antibody with antigen affinity comparable to murine 4D5. This consensus framework was subsequently used in several other humanizations [41], including the humanization of the anti-VEGF antibody bevacizumab (marketed as Avastin[®]) [42].

Another humanization method proposed by Padlan involves simply identifying the residues on the surface of the variable domains away from the CDRs. This approach would be expected to reduce the likelihood of loss of binding affinity; however, a number of buried murine residues would remain in the humanized antibody, possibly increasing the risk of immunogenicity.

More recent efforts have focused on designing antibodies that are more “humanized” than CDR grafts. Tan *et al.* [43] describe an approach that they term “superhumanization.” In contrast to CDR grafting, where the human acceptor sequence is selected based on similarity of the framework regions, in superhumanization, the similarity of framework regions is not relevant. Instead, the murine CDRs are compared to human germline V genes, and the human sequence with the most similar CDRs is selected as the acceptor framework. The murine CDRs are left unchanged in the humanized antibody, but the end result is an antibody with higher homology to a human germline sequence than would be obtained by CDR grafting. The antibodies designed by this method do not always retain full antigen

binding affinity [43, 44], so it may not eliminate the need for selected murine backmutations.

Lazar *et al.* propose a different definition of antibody humanness, which they call human string content [45]. They search for 9-mer peptides within a given sequence and determine whether each possible 9-mer occurs in a human germline sequence. These 9-mers presumably correspond to peptides that could be presented by MHCs to T cells—the main mechanism by which antibodies would be recognized as foreign by the immune system. Similarity between the designed sequence and human germline sequences only at the level of 9-mers, so the humanness of designed sequences is based on similarity to multiple germline frameworks instead of one acceptor framework for the whole domain. This group has recently described a method to make fully human antibodies from murine variable domains [46]. This method makes use of rational engineering of residues within and proximal to CDRs and the VH/VL interface to make these residues as similar to human germlines as possible.

MODULATION OF ANTIBODY EFFECTOR FUNCTION

Fc γ Receptor Binding

The modulation of effector function was in part inspired by the analysis of the naturally occurring IgG4 antibody isotype, which possesses decreased ADCC and CDC and can be functionally monovalent [47]. Further, antibodies lacking the naturally occurring glycan in the CH₂ domain display compromised ADCC and C1q mediated CDC responses [48]. It is no surprise that once ADCC activity of antibodies was linked to their antitumor potency [49], many groups engineered Fc fragments for optimized recognition of Fc γ R receptors (reviewed in reference [50]). While early approaches relied primarily on scanning mutagenesis [51] and glycoform engineering [52], the availability of the crystal structure of Fc γ RIII/Fc complex [53] has opened the way for structure-based design. Using this structural information to guide a combination of computational design and library screening, Lazar *et al.* have identified a S239D/I332E/A330L IgG1 variant that shows over 100-fold enhanced binding to Fc γ RIIIa activating receptor and 9-fold improved Fc γ RIIIa/Fc γ RIIb selectivity window [54]. Subsequently, they have reported several mutants that display distinct Fc γ R binding profiles. Most notably, their designed S239D/I332E/G236A variant has a 15-fold improved Fc γ RIIIa/Fc γ RIIb selectivity window leading to improved ADCP function while retaining increased ADCC [55] which have not been achieved in earlier studies. Recent structural and computational analysis [56] have revealed that the S239D/I332E/A330L Fc mutant forms additional electrostatic and hydrogen bonding interactions with K158 and K117 and hydrophobic interactions with I85 compared to wild-type Fc. The triple mutant adopts a more “open” conformation than wild-type Fc, which correlates nicely with the observation that stepwise truncation on oligosaccharides in the CH₂ domain leads to progressively more “closed” conformation of the Fc [57] resulting in attenuation of Fc γ RIIIa binding. Interestingly, the structure of the L234F/L235E/P331S Fc mutant that has profoundly decreased binding to human activating Fc γ Rs and C1q [58] is similar to the structure of wild type Fc in the CH₂ and CH₃ region. The authors note, however, that unresolved lower

hinge mutants (L234F/L235E) could exist in an altered conformation leading to the loss of binding to FcγRs. Taken together, these observations highlight the importance of conformational state of the Fc fragment of antibody and may inspire new approaches for structure-guided optimization of effector function.

Neonatal Fc Receptor Binding

Neonatal Fc receptor (FcRn) plays a key role in regulation of levels of circulating IgGs by recycling them from lysosomes into general circulation (reviewed in reference [59]). Potentially, longer half-life of therapeutic antibodies or Fc-fusion proteins can offer improved dosing regimens, while reduced binding to FcRn can allow fine-tuning of their tissue distribution [60]. The detailed understanding of pH-dependent nature of the binding event [61] mediated by IgG1 residues I253, H310, Q311, H433, N434 and H435 [62] combined with structural analysis of FcRn [63] has provided a basis for the successful modulation of Fc binding to FcRn (reviewed in reference [64]). While the impact of these substitutions on the pharmacokinetics of antibodies remains uncertain, their further investigation in clinical setting in humans will be of great interest.

In this review we will focus on the studies that utilized structure-guided approaches to identify Fc mutations that possess increased binding to human FcRn at low pH. Yeung *et al.* [65] used interface proximity-guided mutagenesis to interrogate residues 248-259, 284-290, 306-315 343, 385-387, 428-437 in phage display. They selected three substitutions: N434W, N434F, and N434Y which led to over 10-fold improvement in FcRn binding at pH 6.0. In addition, they reported that N434A, N434S, N434H and N434Q as additional substitutions that bind FcRn with increased affinity. We have independently used computational electrostatic optimization to identify N434K as affinity-enhancing residue in human IgG1 (Lugovskoy *et al.*, manuscript in preparation; WO 2006/053301 Fc variants with altered binding to FcRn), a result that highlights the complementary nature of *in vitro* evolution and computational approaches in Fc engineering.

An interesting series of studies by Dall'Acqua *et al.* describe M252Y/S254T/T256E CH₂ domain only mutant that displays 10-fold improved binding to FcRn at pH 6.0, efficient release from FcRn at pH 7.2 and 2-fold decreased binding to FcγIIIRa [66, 67]. When combined with S239D/A330L/I332E substitution the M252Y/S254T/T256E mutant yields 2-fold lower affinity to FcγIIIRa compared to S239D/A330L/I332E. Yet, since the results are additive, the resulting 6-point mutant Fc is 37-fold more potent than wildtype with respect to FcγIIIRa binding. The structural and computational characterization of the M252Y/S254T/T256E mutant revealed that it adopts a conformation similar to wild type Fc, and enhanced affinity can be attributed to additional interactions between Y252 and T254 and E133 of the FcRn α-chain, and E256 of Fc and Q2 of the FcRn β_{2m}-chain. Our studies (Lugovskoy *et al.*, manuscript in preparation; WO 2006/053301 Fc variants with altered binding to FcRn) also suggested a preference for negatively charged residues in positions 254 and 256, which we attributed to their electrostatic interactions with positively charged N-terminus of FcRn β_{2m}-chain. This region of the molecule is involved

in binding to protein A [68], which is important to maintain for manufacturability reasons. This along with the stability considerations discussed in the next section highlights the complexity of Fc engineering and suggests that extensive profiling of variants is needed to select robust formats with desired properties.

Recently, Zalevsky *et al.* reported an engineered M428L/N434S Fc variant that has an 11-fold improvement in FcRn binding at pH 6.0 [69]. The effect of these mutations on pharmacokinetics in cynomolgus monkeys have been studied in the context of anti-VEGF antibody bevacizumab and humanized anti-EGFR antibody cetuximab. In both cases the half-life has increased about 3-fold compared to parental antibodies (9.7 days to 31.1 days for bevacizumab and 1.5 days to 4.7 days for humanized cetuximab). Further, these antibodies had increased antitumor activity in xenograft models in hFcRn transgenic Rag1^{-/-} immunodeficient mouse strain, suggesting that higher antibody exposure due to optimized half-life can translate into enhanced efficacy.

ANTIBODY STABILITY

Fab Region

Structural information has proven valuable in helping to understand antibody stability. Much of the effort in engineering stability has focused on antibody variable domains, since this region is important for therapeutic applications of antibodies and the antigen-binding Fv fragment (i.e. the V_H and V_L domains) lacks stability especially when expressed alone. There have been substantial efforts to stabilize Fv's by screening libraries or by yeast display (reviewed in reference [70]). In this review, however, we will consider only stability engineering by structure-based design.

An early example of a stability-engineered Fv was provided by Glockshuber *et al.* [71]. They compared Fv fragments of the antibody McPC603 stabilized by three approaches: a designed interdomain disulfide; a peptide linker between V_H and V_L, producing a single chain Fv (scFv); and chemical cross-linking of the two domains. They showed that each of the linked proteins retained affinity for hapten, and that each was stabilized against thermal denaturation. The disulfide containing variants (V_L Y55C/V_H Y108C and V_L G56C/V_H T106C) showed the greatest increase in stability—a 60-fold longer half-life of denaturation at 37° C.

Interdomain disulfides have been designed at different positions and shown to stabilize other Fv fragments. The Fv fragment of B3 was stabilized by two separate disulfides: one between V_H 44 and V_L 100, and another between V_H 105 and V_L 43 [72]. Rodrigues *et al.* tested 3 disulfide stabilized variants of humanized 4D5, and found that the V_L L46C/V_H D101C variant was more stable and retained full affinity for HER2 antigen [73]. Young *et al.* used the combination V_L A57C/V_H D106C to stabilize Se155-4 [74].

In addition to these interdomain disulfides, which are all engineered, antibodies are also stabilized by naturally occurring, highly conserved intradomain disulfides in each of their domains. Langedijk *et al.* [75] examined the scFv of

the mAb 03/01/01, a rare antibody that lacked its intradomain VH disulfide because residue 92 had been hypermutated from Cys to Ser. The absence of this disulfide caused this antibody to be significantly less stable with respect to urea denaturation. Interestingly, the authors found that the mutation VH E6Q stabilized the domain with respect to urea denaturation as much as restoring the disulfide bond did. This was attributed in part to a hydrogen bond formed with the unusual Ser92 in this particular Fv. However, the same E6Q mutation has been found to stabilize other antibody variable domains [76], so the fact that E6 is a buried charged residue and its effect on the conformation of framework 1 may also be partially responsible for the effect of the mutation. Additional studies on this region of VH domains have shown that the identity of VH residue 6, along with residues 7 and 9, are responsible for significant changes in the structure of the framework [77]. Jung *et al.* proposed classifying VH domains into 4 structural subtypes, which can be predicted based on the residues found in this region [78]. This appears to be the only region of antibody variable domains with significant structural variability outside of the CDRs.

Ewert *et al.* [79] conducted a systematic study of antibody variable domains, and they identified several structural features that contribute to thermal stability. They compared antibodies from each of the seven HuCAL [80] consensus VH frameworks and each of the seven VL frameworks. Their results show that heavy chains derived from germline families VH2, 4, and 6 tend to have lower expression yields and denature at lower GdnHCl concentrations than VH1, 3, and 5 frameworks. Their structural analysis suggests that these differences can be attributed to a fairly large number of small imperfections in the even-numbered VH domains, including differences in the hydrophobic upper core (core region near the CDRs) and in the salt bridges present in the lower region of the domain.

Based on comparison of these consensus sequences and known structures, Ewert *et al.* identified six mutants [81] of the VH6 framework that increased stability of the heavy chain framework. They were chosen by comparing VH6 to more stable consensus sequences and rationalizing the difference in stability based on known antibody structures. The 6 mutations are summarized in Table 1. They appear to stabilize the structure by increasing beta-sheet propensity, replacing buried hydrophilic residues with hydrophobic residues, and placing Gly at positions with a positive backbone phi angle. The knowledge gained from this work was used to propose a more general knowledge-based approach to stabilizing antibodies either by CDR grafting or by rationally engineering the sequence [82].

VH3 germlines are the most commonly observed [80] human heavy chains and appeared to be most stable in the HuCAL comparison. These observations are part of the reason that VH3 sequences have been commonly used as acceptor frameworks for CDR grafts, as in the case of 4D5 (see above). However, more recent work from Honegger *et al.* [83] has suggested that the VH3 germlines do not always yield the most stable antibodies when CDRs are grafted onto them. Likewise, comparison of the thermostability of 18 different antibodies indicates that VH3 germline-derived antibodies are not always the most stable [84]. The

framework that gives optimal stability and biophysical properties will probably be CDR-dependent.

There are several other cases in which antibody stability has been improved by structure-based design of individual mutations. Nieba *et al.* observed that the scFv has hydrophobic patches that are only exposed when the constant domains are not present [85]. Their mutations of these hydrophobic residues (Table 1) improved expression yield, but apparently not the thermodynamic stability, suggesting that they prevent aggregation during folding. Chowdhury *et al.* [86] compared the sequence of the antibody K1 to a database of antibody sequences. They identified positions where K1 had an unusual amino acid that was not suited to its degree of exposure in the structure (i.e. exposed hydrophobic residues). They found 3 mutations that improved stability and expression yield, 2 of which did not reduce binding affinity. Jordan *et al.* [85] have used structure-based methods to identify stabilizing mutations in an anti-LT β R scFv. Our approach to identify mutants included a combination of statistical methods (both covariation [87] and comparison to consensus) and structure-based methods. The most stabilizing mutations found are also listed in Table 1, and they include S16E, which appears to have a significant effect because of the addition of a negative charge, as well as two mutations that improve packing at the VH/VL interface. Together the four mutations yield an improvement in T_m of 31 °C in a thermal challenge assay (B.R. Miller *et al.*, manuscript in preparation).

scFv and Fab stability can be better understood by examining the contributions of individual domains to overall stability. VL domains can be expressed as monomers [79], and an analysis of consensus human VH and VL sequences [80] described above suggests that VH domains tend to be the less stable and more difficult to express of the two domains. (There are at least two exceptions to this trend: the antibody A48, whose VH domain appears to be more stable than its VL domain with respect to urea denaturation [88, 89] and adalimumab where VH and VL have identical thermostability). Rothlisberger *et al.* [90] dissected the interdomain interactions in the Fab by producing a disulfide bonded CH1/CL pair and observing that the CH1 domain appears to be stabilized by the presence of the disulfide-linked CL in the two-domain construct. They found that VH and VL domains are also mutually stabilizing. There was little stabilization from interactions between CL and VL domains, but the addition of the CH1/CL pair significantly stabilized Fv fragments.

Barthelemy *et al.* [91] used structural analysis and phage libraries to determine the factors that contribute to stabilization of isolated VH domains (domains without a light chain partner). They found that increasing the hydrophilicity of the former light chain interface was critical. Ewert *et al.* have also compared *Camelidae* VHH chains, occur without a light chain partner, to human VH3 chains [92]. They found that VHH domains tend to have more hydrophilic residues at the former VL interface, and that VHH domains are not significantly more thermostable than VH3 domains, although VHH domains can refold after thermal denaturation. Taken together, their results suggest that the hydrophilic residues at the light chain interface increase stability of VHH, but VHH domains sacrifice some

Table 1. Stabilizing Mutations

VH			Discovered by		References
E6Q	03/01/01	scFv	PCR induced error, later structural analysis		[75]
V84D	4-4-20	scFv	Design: exposed on scFv conversion	Improved expression, not <i>in vitro</i> stability	[85]
L11D	4-4-20	scFv	Design: exposed on scFv conversion	Improved expression, not <i>in vitro</i> stability	[85]
Y55C VL/ Y108C VH	McPC603	Fv	Forms interdomain disulfide	Better half-life denaturation than scFv	[85]
G56C VL/ T106C VH	McPC603	Fv	Forms interdomain disulfide	Better half-life denaturation than scFv	[71]
Q5V	2C2, 6B3	scFv	Selection experiment on 4D5Flu		[81]
S16G	2C2, 6B3	scFv	Positive phi angle		[81]
T58I	2C2, 6B3	scFv	Buried hydrogen bond donor		[81]
V72D	2C2, 6B3	scFv	Solvent-exposed hydrophobic		[81]
S76G	2C2, 6B3	scFv	Positive phi angle		[81]
S90Y	2C2, 6B3	scFv	Buried, compared VH6 to VH1,3,5 consensus		[81]
A85E	K1	scFv	Solvation effect		[86]
S16E	BHA10	scFv	Solvation effect		[97]
V55G	BHA10	scFv	Stability of turn		[97]
P101D	BHA10	scFv	VH/VL interface		[97]
VL					
S46L	BHA10	scFv	VH/VL interface		[97]
H36Y	K1	scFv	VH/VL interface		[86]
V60S	K1	scFv	Solvation effect		[86]

of this stability in order to gain flexibility in their CDRs. More flexibility may be required for VHH domains to achieve antigen binding affinity with only a single domain.

Fc Region

In general, engineering of the Fc region for stability has received less attention than the variable regions, simply because it has been possible to engineer Fc variants (as described above) without significant loss of stability. Nevertheless, there have been several studies of the determinants of Fc stability. The naturally occurring disulfide in the CH3 domain has been shown to contribute to its stability [93]. The CH2 domain is the least stable of the constant domains in human IgG's, and the domains are even less stable in aglycosylated Fc variants. Recently, Gong *et al.* [94] stabilized the CH2 domain by introducing an intradomain disulfide bond. This disulfide, made by mutating Leu12 and Lys104 of the CH2 domain to Cys, increased the melting temperature of the isolated domain from 54.1° to 73.8° C.

Structure-based approaches have also been applied to understand aggregation of antibodies. Chenamsetty *et al.* [95] have used molecular dynamics simulations in an approach they term spatial aggregation propensity (SAP) to identify aggregation-prone motifs in IgG constant domains. They made 5 mutants in human IgG1 that show enhanced

stability by multiple techniques such as HPLC and differential scanning calorimetry [96].

CONCLUSION

Structure-guided design has proven to be an important approach to designing antibodies with desired properties such as improved affinity, stability and effector function. X-ray crystallography and molecular modeling have provided molecular insights that led to several notable successes in these areas in which structure-based design was the only design approach used. However, the results summarized here suggest that structure-guided design can best utilized in combination with other approaches, such as sequence analysis and design of antibody libraries.

We expect that structure-based design will have most impact in the areas of antibody engineering where the high-throughput screening approaches are difficult to implement. Engineering antibodies for biophysical properties with an emphasis on solubility improvement is one of such examples. While the results of Chenamsetty *et al.* have provided a proof of principle, further improvements in this area are needed to robustly design antibodies with improved pharmaceutical properties. Development of more efficient computational algorithms and accurate scoring functions governing the atomic, electrostatic interaction and

particularly improved solvation models will continue to be emphasized.

An additional key area of impact for structure-guided methods is design of antibody-like molecules with novel functions. Multivalent, monovalent, heteromeric antibodies and antibody-drug conjugates have already been described [98-100], and their continued optimization for avidity, affinity, stability, selectivity and novel Fc functions could eventually allow them to surpass the utility of unmodified IgG antibodies as therapeutics. While the therapeutic opportunities for novel formats are clear, their enabling properties will need to be balanced against manufacturability and immunogenicity considerations. These represent additional areas where computational modeling approaches could have a significant impact.

ABBREVIATIONS

ADCC	=	Antibody-dependent cellular cytotoxicity
ADCP	=	Antibody-dependent cellular phagocytosis
CDC	=	Complement-dependent cytotoxicity
Antigen	=	“Antibody generator”, a molecule specifically recognized by an antibody
CDRs	=	Complementarity determining regions, the segments of the variable domains that recognize antigen
CH1, CH2, CH3	=	Heavy chain constant domains
CL	=	Light chain constant domains
Fab	=	“Fragment antigen binding”—portion of the antibody that recognizes antigen
Fc	=	“Fragment crystallizable”—consists of CH2 and CH3 domains of antibody
Fv	=	Variable fragment of antibody, consisting of only VH and VL domains
FcRn	=	Neonatal Fc receptor
FcγR's	=	Fc receptors, mediate antibody effector function by recognizing Fc portion of antibody
mAb	=	Monoclonal antibody
scFv	=	Single chain Fv fragment
VH	=	Heavy chain variable domain
VL	=	Light chain variable domain

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