Engineering Aggregation-Resistant Antibodies

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Abstract

The ability of antibodies to bind to target molecules with high affinity and specificity has led to their widespread use in diagnostic and therapeutic applications. Nevertheless, a limitation of antibodies is their propensity to self-associate and aggregate at high concentrations and elevated temperatures. The large size and multidomain architecture of full-length monoclonal antibodies have frustrated systematic analysis of how antibody sequence and structure regulate antibody solubility. In contrast, analysis of single and multidomain antibody fragments that retain the binding activity of monoclonal antibodies has provided valuable insights into the determinants of antibody aggregation. Here we review advances in engineering antibody frameworks, domain interfaces, and antigen-binding loops to prevent aggregation of natively and nonnatively folded antibody fragments. We also highlight advances and unmet challenges in developing robust strategies for engineering large, multidomain antibodies to resist aggregation.

INTRODUCTION

Antigen: any molecule that is recognized specifically by an antibody

mAb: monoclonal antibody

V_H: variable domain of the heavy chain

V_L: variable domain of the light chain

Complementarity-determining regions (CDRs): peptide loops on the surface of V_H and V_L domains that mediate antigen recognition

C_L: constant domain of the light chain

C_H1: first constant domain of the heavy chain adjacent to the V_H domain

Antigen-binding fragment (Fab): antibody fragment containing two complementary variable (V_H/V_L) and constant (C_H1/C_L) domains

Fc: crystallizable domain of the heavy chain containing the C_H2 and C_H3 domains

Fv: variable domain containing V_H and V_L

Single-chain variable fragment (scFv): antibody fragment containing a V_H and a V_L domain connected via a peptide linker

Antibodies are multidomain proteins used by the immune system to recognize and neutralize foreign antigens with remarkable specificity. This specificity has been exploited for myriad diagnostic applications in vitro, including immunofluorescence, western blotting, and enzyme-linked immunosorbent assay (ELISA) analysis. Antibodies have also attracted intense interest as therapeutic molecules, as evidenced by the large number of antibodies either approved or in clinical trials for treating human disorders ranging from cancer and rheumatoid arthritis to osteoporosis and asthma (1–3). Antibodies are attractive therapeutic molecules owing not only to their specificity but also to their long half-life in vivo (typically two to four weeks) (4) as well as their expected lack of immunogenicity for fully humanized antibodies (5, 6). From a discovery point of view, antibodies are also attractive because well-established in vivo (immunization) and in vitro (phage display) methods exist for identifying and maturing high-affinity antibody variants against diverse antigens (7–14).

Although several classes of human antibodies (e.g., IgG, IgA, and IgM) exist, all of them possess the same basic architecture. Full-length antibodies [herein referred to as monoclonal antibodies (mAbs)] are composed of four polypeptide chains—two light chains (210–220 amino acids per chain) and two heavy chains (450–550 amino acids per chain)—that are linked together via disulfide bonds to form a Y shape (**Figure 1**). A typical antibody contains 12–14 folded domains, and each domain possesses a similar Greek key fold in which two β-sheets form a sandwich (**Figure 1**) (15). The variable domains of the heavy (V_H) and light (V_L) chains contain three antigen-binding loops each (5–20 amino acids per loop), which are also known as the complementarity-determining regions (CDRs; **Figure 1**). Each antibody arm containing V_H and V_L domains also contains two constant domains (C_L in the light chain and C_H1 in the heavy chain). Collectively, these four domains are referred to as the antigen-binding fragment (Fab; **Figure 1**). In addition, the base of the antibody is referred to as the crystallizable or Fc domain. The Fc domain is composed of the C-terminal domains of the two heavy chains (**Figure 1**), and each chain contains a conserved *N*-glycosylation site. Glycosylated Fc domains activate the immune system upon antibody binding, which is critical to the activity of some therapeutic antibodies (16).

Because the binding activity of antibodies is localized to their variable regions, small fragments of antibodies can be generated that also retain the binding activity of their parent antibodies (17–24). Indeed, even single variable domains (V_L or V_H; **Figure 1**)—known as domain antibodies or nanobodies—can be engineered to bind to diverse targets with high affinity (17, 25–27). The smallest antibody fragments that contain both V_H and V_L domains are Fv fragments (composed of two polypeptide chains) and single-chain variable fragments (scFvs) in which the V_H and V_L domains are connected via a flexible peptide linker (**Figure 1**). ScFvs are generally preferred to Fv fragments because the amino acid linker connecting the V_H and V_L domains limits the dissociation of the two variable domains (23, 24). However, Fabs are the most widely used antibody fragments because they contain both variable domains and stabilizing constant domains.

Antibody fragments and mAbs each have unique advantages and disadvantages for diverse applications (see References 28–30 and references therein). However, both types of antibodies are susceptible to aggregation upon exposure to a variety of stresses (31, 32), including high antibody concentrations necessary for subcutaneous therapeutic delivery (50–200 mg ml^{-1}) (33, 34), elevated temperatures (34–36), freeze-thaw cycles (37, 38), agitation (39–42), low pH (43, 44), and long storage times (>2 years for therapeutic applications) (45, 46). Antibody aggregation is of particular concern for therapeutic applications because such aggregates can be immunogenic

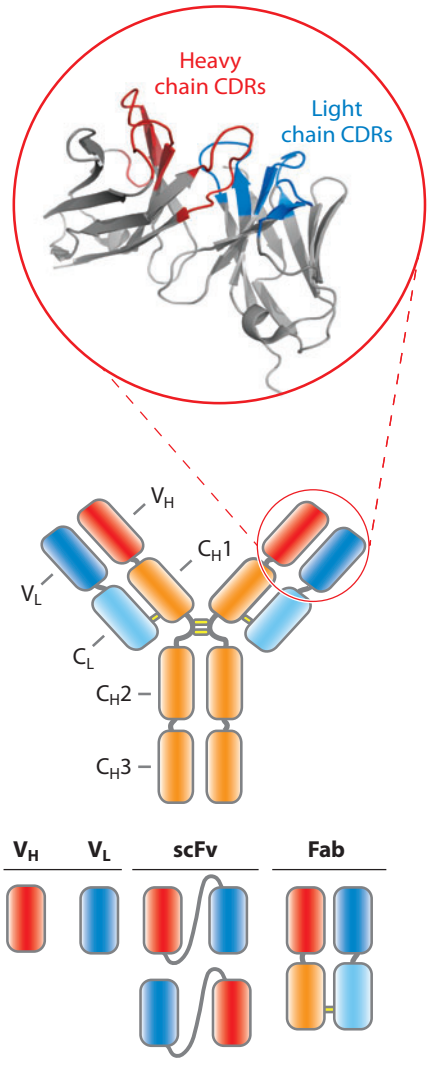

Figure 1

Molecular architecture of monoclonal antibodies (mAbs) and antibody fragments. A typical mAb is composed of two heavy chains and two light chains that contain a total of twelve individual domains. The variable heavy (V_H) and light (V_L) domains each display three peptide loops—referred to as complementarity-determining regions (CDRs)—that contact antigens and mediate binding specificity. Because antibody-binding activity is localized to the variable domains, smaller antibody fragments containing one or more variable domains retain binding activity without the constant domains. The crystal structure of the variable domains is Protein Data Bank Number 1N8Z. Abbreviations: C_H, heavy chain constant domain; C_L, light chain constant domain; Fab, antigen-binding fragment; scFv, single-chain variable fragment.

(47, 48). Nevertheless, the widespread use of antibodies and the ability to fully humanize them (49, 50) have motivated investigators to understand how to engineer antibodies to resist aggregation (51, 52). Here we review the most important studies aimed at elucidating how to stabilize antibodies against aggregation.

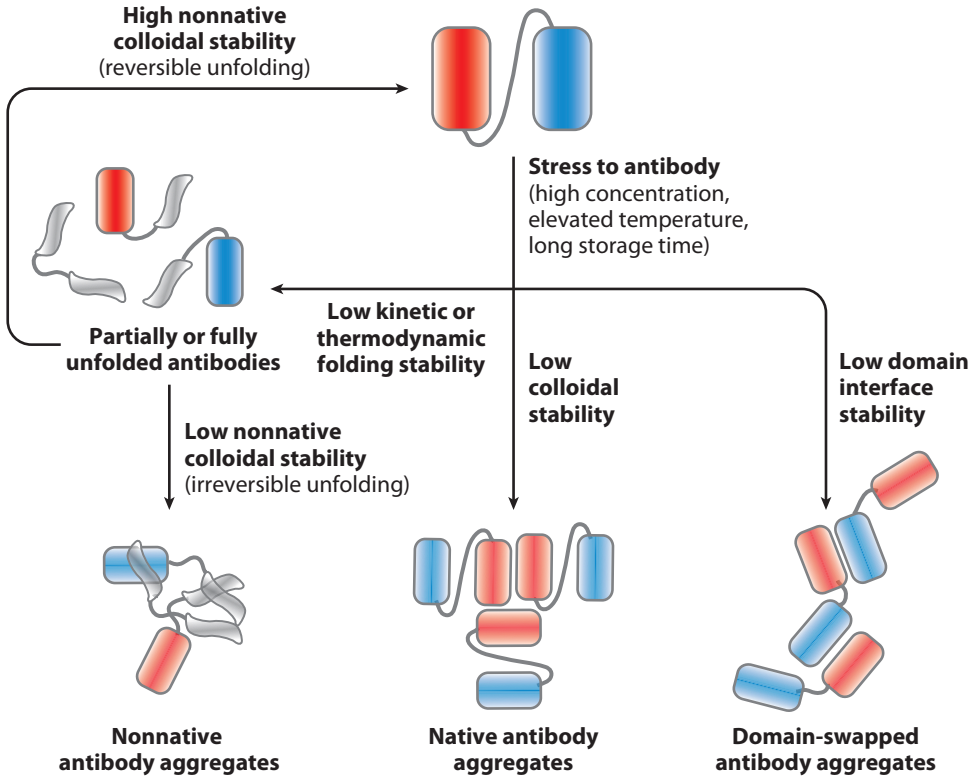

Figure 2

Antibody aggregation pathways. Antibodies exposed to a variety of stresses are susceptible to aggregation through three primary pathways. Stresses such as elevated temperature can lead to unfolding of one or more antibody domains because of low thermodynamic and/or kinetic folding stability. This unfolding of antibodies can lead to aggregation if their unfolded conformations are competent for aggregation (low nonnative colloidal stability). However, unfolded antibodies can also refold without aggregating (high nonnative colloidal stability). Stresses such as high concentration or low temperature can lead to antibody aggregation without unfolding for antibodies with low native colloidal stability (i.e., low native solubility). Finally, antibodies can also aggregate via domain swapping without unfolding if the interfaces between complementary antibody domains are unstable.

ANTIBODY AGGREGATION PATHWAYS

Antibodies can aggregate through multiple pathways owing to physical and chemical instabilities (53, 54). For the purposes of this review, we consider only aggregation pathways due to physical instabilities of antibodies (**Figure 2**). Moreover, we refer to antibody aggregation as condensation of folded or unfolded antibodies into reversible or irreversible antibody aggregates. Stresses such as elevated temperature or low pH can cause antibodies to partially or fully unfold if antibodies possess low thermodynamic or kinetic folding stability. In the case of multidomain antibodies, one or more domains may unfold without unfolding of the other domains. The propensity of partially or fully unfolded antibodies to aggregate is determined by a competition between refolding (governed by intramolecular interactions) and aggregation (governed by intermolecular interactions). We refer to the propensity of antibodies to aggregate when unfolded as their nonnative colloidal stability. If intermolecular interactions between unfolded antibodies are sufficiently attractive

(low colloidal stability), then unfolded antibodies condense into nonnative aggregates. Conversely, if intermolecular interactions between unfolded antibodies are repulsive or insufficiently attractive (high colloidal stability), then unfolded antibodies fold reversibly without aggregating (assuming that the refolding kinetics are not limiting).

Antibodies can also aggregate via mechanisms that do not require unfolding. Elevated antibody concentrations, low temperatures, and related stresses can cause antibodies with low native colloidal stability to condense into native protein aggregates owing to attractive antibody self-interactions. Multidomain antibodies can domain swap with complementary domains from other identical molecules, leading to aggregation in which each individual domain is folded. In some cases, antibody aggregation occurs as a result of multiple physical instabilities that involve more than one aggregation mechanism described above. In this review, we highlight advances in engineering antibodies for maximal stability that prevents aggregation through each pathway.

Antibody Thermodynamic Folding Stability

The thermodynamic folding stability of antibodies is one of the most fundamental and widely studied physical properties that govern the propensity of antibodies to aggregate. Because the immunoglobulin (Ig) fold is conserved across diverse antibodies, much work has focused on identifying and/or engineering antibody scaffolds with high thermodynamic stability. This is particularly important for antibody fragments because they often possess lower folding stabilities than their full-length counterparts. The folding stability of antibodies is typically measured using circular dichroism spectroscopy and/or tryptophan fluorescence (55). Below we discuss important advances in engineering single- and multidomain antibody fragments with high conformational (folding) stability.

Folding stability of single-domain antibodies. It would be logical to assume that single-domain antibodies (e.g., V_H) cannot be engineered to be as stable as larger antibody fragments (e.g., Fabs) and mAbs (e.g., IgG) because these small antibody domains lack complementary variable (e.g., V_L) and constant (e.g., C_H1/C_L) domains that stabilize their folded structure. However, several studies have convincingly demonstrated that multidomain architecture is unnecessary for individual antibody domains to possess high conformational stability (56–61). One fruitful approach for stabilizing individual V_H or V_L domains has been to identify mutations in the former V_H/V_L interface that increase folding stability to compensate for the loss of the stabilizing interactions between variable domains (19, 60–62). Sidhu and coworkers (60) elegantly demonstrated that such stabilizing mutations could be readily identified for a human V_H variant. They randomized approximately 20 residues at the former V_H/V_L interface of a V_H domain, which included residues within β-strands, non-CDR loops, and CDR3. Interestingly, they identified four mutations in two β-strands, as shown in **Figure 3**, that significantly increase the folding stability of the wild-type V_H domain. This increased stability was observed both in terms of an increase in the midpoint temperature of antibody unfolding (also known as the apparent melting temperature) from 58 to 79°C (60) as well as an increase in the Gibbs free energy of unfolding ($\Delta G_{N\text{-}U}$) from 28 kJ mol^{-1} to 52 kJ mol^{-1} (J.M. Perchiacca & P.M. Tessier, unpublished results). Notably, the four mutations are localized to two pairs of interacting residues. Two mutations in close proximity near the base of the stabilized antibody domain are oppositely charged (Arg39 and Glu45) and presumably stabilize the folded structure via complementary electrostatic interactions. The other stabilizing mutations (Gly35 and Ser50) are localized at the edges of CDR1 and CDR2. These mutations lead to significant changes in the orientation of adjacent aromatic residues that may explain the increase

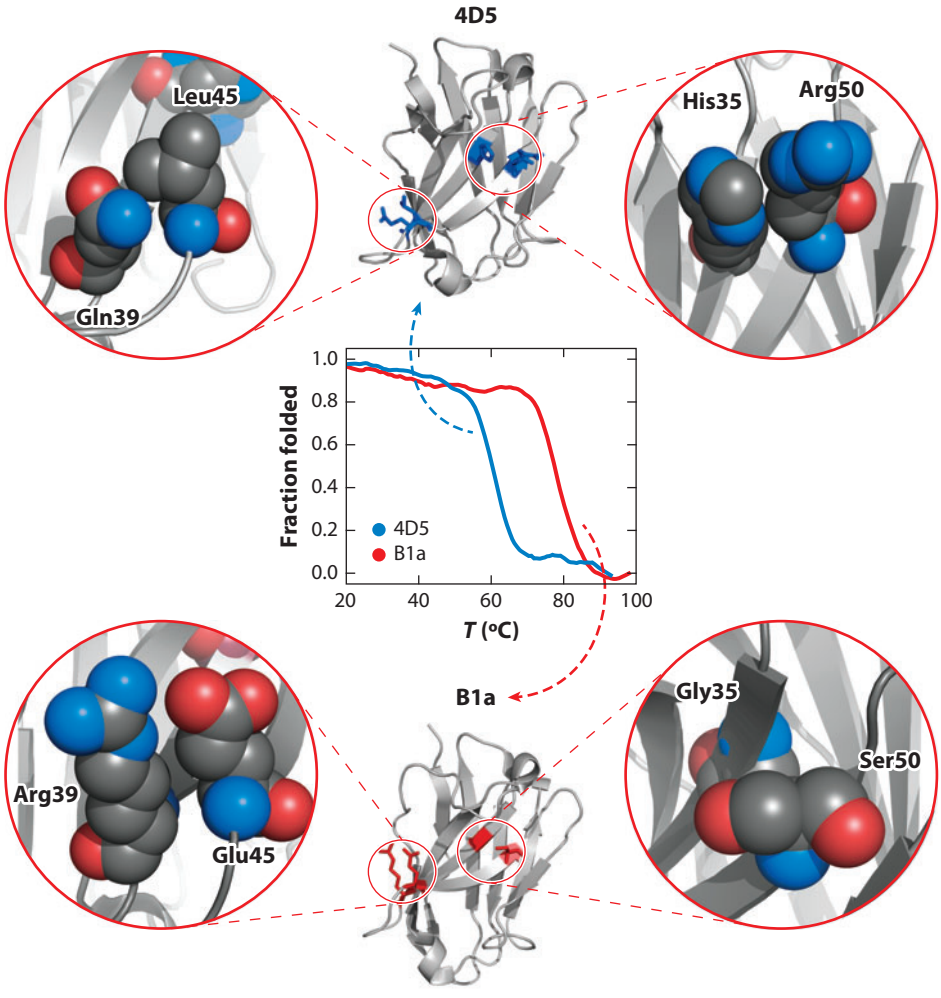

Figure 3

Mutations that enhance the thermodynamic folding stability of a human single-domain antibody. An unstable human V_H domain (4D5; PDB: 1FVC) was converted into a highly stable variant (B1a; PDB: 3B9V) by introducing four mutations within or near the former V_H/V_L interface (60). Abbreviations: V_H, variable heavy domain; V_L, variable light domain.

in folding stability (60). Notably, these mutations obtained from synthetic antibody libraries are uncommon to natural human V_H domains, which demonstrates that highly conserved antibody sequences can be further optimized.

The enhanced folding stability of single-domain antibodies due to mutations at the edges of their CDR loops suggests that the sequence of CDRs may have a greater impact on antibody folding stability than previously realized (63, 64). Although small loops (<5 residues) on the surface of proteins generally have minimal impact on protein folding stability, the relatively large CDR loops (5–20 residues) on the surface of variable antibody domains can either destabilize or stabilize the antibody fold. For example, the close proximity between CDR loops suggests that direct interactions between CDR loops could be either stabilizing or destabilizing. Indeed,

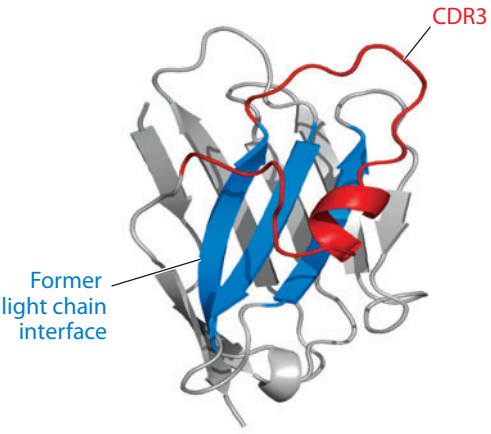

Figure 4

Camelid variable heavy domain (V_{HH}) antibodies are stabilized via interactions between complementarity-determining region 3 (CDR3) and the former variable heavy domain/light domain (V_H/V_L) interface. The crystal structure of a camelid V_{HH} domain specific for lysozyme (PDB: 1MEL) reveals that part of CDR3 packs against the former V_H/V_L interface, thereby stabilizing the folded structure (66).

multiple studies have revealed that grafting a subset of the three CDR loops from one variable-domain antibody onto a second variable domain generally results in a lower stability for the grafted variants than that for the parent antibody domains (58, 63, 65). In contrast, grafting all three CDR loops from unstable V_H scaffolds onto highly stable V_H scaffolds often produces antibody domains with high folding stability (58, 59). These findings confirm that the ability of CDR loops to assume complementary conformations on the surface of antibodies is critical to stabilizing the fold of single-domain antibodies.

CDR loops can also stabilize antibodies by packing against the antibody scaffold (56, 66, 67). The most striking example of this behavior is observed for single-domain antibodies from camelids (e.g., camels and llamas). Camelid antibodies are typically composed of two identical polypeptide chains that are analogous to the heavy chains of human antibodies, yet they recognize antigens with similar affinity as human antibodies with both heavy and light chains (25, 68, 69). Camelid V_{HH} domains have unusually large CDR3 loops (8–24 residues, with an average of 16 residues) that appear necessary to confer high affinity without the assistance of the CDRs from V_L domains (70, 71). Importantly, part of CDR3 of camelid V_{HH} domains packs against hydrophobic residues at the former V_H/V_L interface (**Figure 4**) (66, 67). These stabilizing interactions (which are typically absent in human antibody domains) enable camelid V_{HH} domains to display long CDR3 loops that would be destabilizing to their human counterparts (56).

Folding stability of multidomain antibody fragments. Engineering multidomain antibodies to possess high folding stability is more complex than for single domains owing to multiple complicating factors. First, the individual domains of a multidomain antibody often do not unfold cooperatively. Because unfolding of even one domain of a multidomain antibody can lead to aggregation, it is critical to engineer each antibody domain to resist unfolding. A second complication is that the folding stability of multidomain antibodies is determined not only by the intrinsic stability of the individual antibody domains (e.g., V_L), but also via the interaction between complementary domains (e.g., V_H/V_L).

V_{HH}: variable domain of camelid antibodies analogous to the heavy chain V_H domain in human antibodies

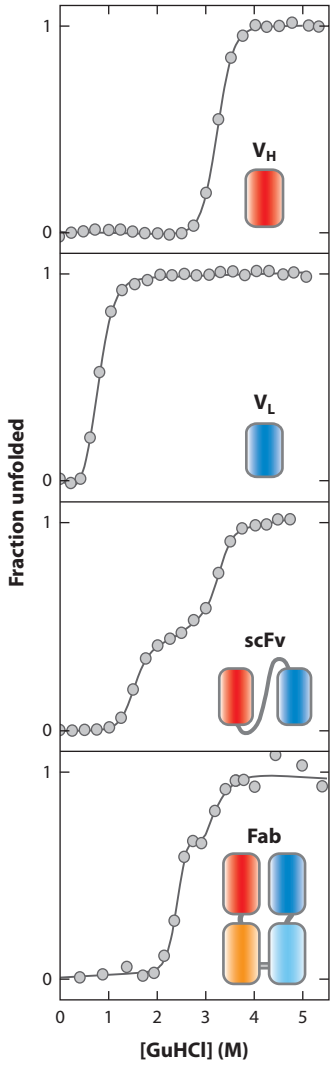

Figure 5

Interactions between complementary domains stabilize multidomain antibodies. The poor stability of a variable light (V_L) domain was enhanced via interactions between complementary domains within a single-chain variable fragment (scFv) and an antigen-binding fragment (Fab) (73). Abbreviations: GuHCl, guanidine hydrochloride; V_H, variable heavy domain.

To understand the origins of folding stability for multidomain antibody fragments, several studies have compared the stability of paired variable domains (i.e., V_H/V_L) relative to the individual domains (72–74). For example, Plückthun and coworkers compared the stability of human V_H and V_L domains when isolated and when paired together in an scFv (**Figure 5**) (73). In this case, the V_H domain is significantly more stable than the V_L domain. When the two domains are paired together, the scFv antibody displays two unfolding transitions owing to the noncooperative unfolding of each variable domain. Importantly, the unfolding of the less stable variable domain (V_L) in the scFv occurs at higher denaturant concentrations than that of the isolated V_L domain owing

to the stabilizing interactions of the V_L/V_H interface (**Figure 5**). Moreover, addition of C_H1 and C_L further increases the antibody stability by shifting the unfolding transition of the V_L domain to even higher denaturant concentrations. The additional stability afforded by the paired constant domains is strongly dependent on the presence of a disulfide bond linking C_H1 and C_L (73).

Bispecific antibody: an antibody, typically composed of two antibodies (e.g., IgG-scFv), that binds to two different antigens

These findings that interactions between complementary variable (V_H/V_L) and constant (C_H1/C_L) domains enhance antibody folding stability have inspired the design of highly stable multidomain antibodies (see References 51, 52, 75 and references therein). One strategy to engineer Fvs and scFvs with enhanced folding stability is to introduce a disulfide bond between the V_H and V_L domains that is similar to the stabilizing disulfide bond linking the constant (C_L/C_H1) domains. Indeed, this strategy provides significant stability to both Fvs (76, 77) and scFvs (78, 79), and also has been used to stabilize scFvs fused to mAbs (also known as bispecific antibodies) (80). Drawbacks of this strategy are that some antibody variants do not readily form disulfide bonds at the V_H/V_L interface (81), as well as that the expression levels of scFvs bearing additional disulfide bonds are typically much lower than those of the original scFvs (78, 79, 81). A complementary approach for stabilizing multidomain antibodies is to compare their sequences with those of closely related antibodies to identify mutations that may enhance folding stability. The hypothesis behind this approach is that replacing nonconserved residues with conserved ones at positions where antibodies differ from consensus sequences will lead to increased folding stability. Several studies have demonstrated that introduction of conserved residues into the interfaces between domains and other regions within multidomain antibodies often stabilizes such antibodies against unfolding (75, 81–88).

Notably, the sequence of CDR loops can significantly impact the folding stability of multidomain antibody fragments (85, 89), just as observed for single-domain antibodies (58, 63, 65). Two scFvs that differ only in the sequence of CDR3 in the V_L domain provide a striking example of the sensitivity of antibody folding stability to the sequence of CDRs (89). Although the stabilities of the V_L domains bearing either CDR3 were similar, the stabilities of the corresponding scFvs were strongly dependent on the CDR3 sequence. As expected, one of the scFv variants displayed increased folding stability relative to the individual domains owing to stabilizing V_H/V_L interactions. However, the other scFv variant was destabilized relative to the individual domains. Inspection of the destabilizing CDR3 sequence revealed two consecutive proline residues that may impose an unfavorable CDR3 conformation and impede proper pairing of V_H and V_L domains (89). These findings are also consistent with related observations that grafting CDRs onto scFv scaffolds produces antibody variants whose stability is dependent on the sequence of the CDR loops (85).

Antibody Kinetic Stability

The rate at which antibodies unfold is also a key determinant of their aggregation propensity. Here we refer to high kinetic stability as the ability of antibodies to unfold slowly, thereby resisting aggregation by slowly populating unfolded states competent for nonnative aggregation (**Figure 2**). It has been recognized for decades that mAbs possess high kinetic stability and unfold extremely slowly (require months to unfold in denaturant; see Reference 51 and references therein). In fact, the high kinetic stability of mAbs is likely one of the primary reasons for their successful use in diverse applications that require long-term stability.

Despite the importance of the kinetic stability of antibodies, the molecular origins of such stability are less well understood than those of thermodynamic folding stability. Nevertheless, several key studies have begun to define the determinants of kinetic stability for antibody fragments and mAbs (72, 73, 90, 91). An important observation is that the individual domains of antibodies

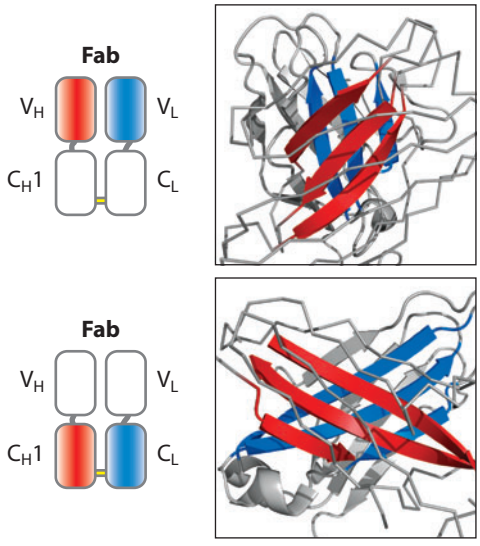

Figure 6

Structural hypotheses for the high kinetic stability of antigen-binding fragments (Fabs). The slow unfolding of Fabs is due to interdomain interactions between two complementary constant heavy and light domains (C_H1/C_L) (73, 91). Because paired variable heavy and light domains (V_H/V_L) unfold much more rapidly than the corresponding constant domains, the larger hydrophobic interface and/or unique interdomain orientation of β-strands encode the high kinetic stability of Fabs (91). The antibody crystal structure is for a Fab against HER2 (PDB: 1N8Z).

(e.g., V_L, C_L) unfold rapidly in denaturant (minutes or less) (72, 73), which confirms that the origin of the kinetic stability of large, multidomain antibodies is not due to high kinetic stability of their individual domains. Moreover, two antibody domains within a single polypeptide chain that are noncomplementary—such as V_L and C_L—also unfold rapidly (73, 90). However, multidomain antibody fragments containing complementary domains (e.g., V_H/V_L in a Fab) unfold much more slowly than their individual domains (72, 73, 91). These findings suggest that stabilizing interactions between complementary antibody domains are linked to the kinetic stability of multidomain antibodies.

To further elucidate the contribution of interactions between complementary domains to the kinetic stability of multidomain antibody fragments, Plückthun and coworkers (73) elegantly dissected the origins of the kinetic stability of a Fab. They first confirmed that the individual antibody domains (e.g., V_L and C_L) unfold rapidly in denaturant, consistent with previous findings (72). Moreover, they found that the V_H and V_L domains linked together in an scFv also unfold rapidly, revealing that scFvs are not necessarily kinetically stable (51, 73). Most importantly, the paired constant domains (C_H1/C_L) without the variable domains required weeks to unfold in denaturant (73). This unfolding rate of the paired constant domains (but not the individual constant domains) was similar to that of the entire Fab, revealing that the interface between the constant domains is the most important determinant of the kinetic stability of Fabs. The origin of the kinetic stability afforded by the constant domains remains unknown. However, the interface between constant domains is larger, more hydrophobic, and oriented differently relative to the interface between variable domains (**Figure 6**). The unique orientation of β-strands at the C_H1/C_L interface may block the unfolding of each domain, leading to the high kinetic stability of these multidomain antibody fragments (73).

Antibody Nonnative Colloidal Stability

Most antibodies aggregate when unfolded due to attractive intermolecular interactions between solvent-exposed hydrophobic residues that are normally solvent shielded within the folded antibody core. Nevertheless, antibodies that are unfolded may refold instead of aggregating (referred to as reversible unfolding) if the intermolecular interactions between unfolded antibodies are insufficiently attractive to mediate aggregation and the refolding kinetics are not limiting. The multidomain architecture of most antibody fragments (scFv and Fab) and mAbs greatly reduces the likelihood that these antibodies will unfold reversibly without aggregating. However, one would expect that single antibody domains (e.g., V_H or V_L) could be engineered to unfold reversibly given that several single-domain, globular proteins display such reversible unfolding behavior (92, 93).

Unfortunately, most variable domains (e.g., V_H) from human antibodies are poorly soluble and readily aggregate when unfolded (94–96), which had suggested that these domains do not unfold reversibly. The discovery of heavy chain antibodies in camels and related species challenged this initial conclusion (68). The fact that these antibodies lack light chains suggests that their isolated V_H domains (typically referred to as V_{HH}) may have superior biophysical properties relative to their human counterparts because their folding and stability are independent of complementary V_L domains. Indeed, several camelid V_{HH} domains fail to aggregate when unfolded at elevated temperatures (25, 58, 59, 97) even though their folding stability is similar to that of aggregation-prone human V_H domains (98). Sequence comparison of human and camelid variable-domain antibodies reveals four key amino acid differences (known as the V_{HH} tetrad) at the former V_H/V_L interface (71, 99, 100). Three of the sequence differences increase the hydrophilicity of camelid V_{HH} domains by replacing solvent-exposed hydrophobic or nonpolar residues in human V_H domains with charged or less hydrophobic residues in camelid V_{HH} domains. The fourth sequence difference involves replacement of a small hydrophobic residue (valine) in human domains with a large, aromatic residue (phenylalanine or tyrosine) in camelid domains. This mutation increases the hydrophilicity of the former V_H/V_L interface in camelid antibodies by packing against a portion of CDR3, as shown in **Figure 4**.

These findings led several investigators to attempt to transfer the desirable properties of camelid V_{HH} domains to human V_H domains (59, 82, 101). Unfortunately, transferring residues from the camelid tetrad to human V_H domains generates antibody variants whose solubility is only modestly improved (59, 102). More limited attempts to partially humanize camelid domains (or to camelize human domains) have been more successful (59, 103). For example, camelid V_{HH} domains were humanized with a subset of the nonconserved residues (~20 amino acids) outside of the CDR loops (59). Hybrid antibody domains bearing a large fraction (>75%) of camelid residues at nonconserved positions were generally more soluble than those variants bearing a large fraction of human residues (>50% of nonconserved residues). Thus, it remains unclear whether the extreme solubility of camelid domains can be condensed into a small number of solubilizing mutations that are transferrable to human domains.

An important observation for both human V_H and camelid V_{HH} antibodies is that the sequence of their CDR loops significantly impacts their ability to unfold reversibly (56, 59, 65, 96, 104–106). In hindsight, this is logical because CDR loops commonly present hydrophobic residues necessary for binding to target antigens, and these hydrophobic loops may interact promiscuously with hydrophobic residues exposed within unfolded antibodies. Some camelid V_{HH} domains possess an additional disulfide bond between CDR1 and CDR3 that restricts their conformational flexibility (70, 71), which has been proposed to contribute to their ability to unfold reversibly without aggregating (106). Moreover, hydrophobic residues within CDR3 that pack against the former

V_H/V_L interface (**Figure 4**) are also important for camelid antibodies to resist aggregation when unfolded (56).

These findings led to an intriguing hypothesis that the ability of human V_H domains to resist aggregation when unfolded could be encoded entirely within the sequence of their three CDR loops (i.e., without mutations in the antibody scaffold) (96, 105). To test this hypothesis, Winter and coworkers (96, 105) developed an elegant antibody fragment selection strategy in which a library of human V_H domains differing only in their CDR loops was displayed on phage particles, and these displayed antibodies were transiently unfolded at elevated temperature (80°C). Because the V_H domains were displayed in a multivalent format, most of them aggregated with closely neighboring antibody domains instead of refolding when the temperature was reduced. However, rare V_H variants refolded without aggregating when cooled. Biochemical analysis of the selected V_H domains (isolated from the phage particles) revealed that they were extremely resistant to aggregation when unfolded even though their sequences differed from aggregation-prone V_H domains only within their CDR loops. Interestingly, the only general difference between the CDR sequences of the highly and poorly soluble antibody domains was a modest increase in the number of negatively charged residues in the highly soluble variants (105).

Given the previous difficulties in engineering human V_H domains to resist aggregation, the molecular origins of the aggregation-resistant variants selected by phage display (96) are intriguing. One important question is whether the ability of the selected V_H domains to resist aggregation is a distributed property of the three CDR loops or a specific property of a single CDR loop. Therefore, we compared the solubilities of three V_H variants—each one displaying one CDR loop from the highly soluble antibody (referred to as Hel4) and two CDR loops from the poorly soluble antibody (referred to as wild type)—with the solubilities of their parent antibodies (**Figure 7**) (65). Strikingly, the ability to resist to aggregation was localized to CDR1 of Hel4, as only V_H domains bearing CDR1 from Hel4 unfolded reversibly. In contrast, CDR2 and CDR3 from Hel4 were unable to prevent aggregation when grafted onto the wild-type antibody domain.

Because the sequences of CDR loops encode the binding activity of antibodies, it is desirable to identify the minimal residues required to encode the solubilizing activity of the CDR1 loop from Hel4. Therefore, we transferred nonconserved residues within CDR1 of Hel4 to CDR1 within the wild-type antibody domain (65). No single residue within the Hel4 CDR1 conferred reversible unfolding behavior to the wild-type antibody (65, 96, 104), although a triad of negatively charged residues from Hel4 was capable of doing so (65). Strikingly, a single mutation immediately adjacent to CDR1—an aromatic (phenylalanine) to charged (aspartic acid) mutation—endowed the wild-type antibody domain with reversible unfolding behavior (65). This is particularly interesting because this mutation is not part of the aggregation-resistant Hel4 antibody domain, and it is uncommon to both human and camelid antibodies (71, 98). This finding emphasizes that the sequence of antibodies can be further optimized to encode extreme solubility using mutations not commonly observed in natural antibodies (60). The fact that this solubilizing mutation is outside CDR1 suggests it would have minimal impact on antibody binding affinity. Moreover, if such a mutation does reduce binding affinity, alternative CDR sequences likely could be identified that are compatible with this or related charged mutations.

Antibody Native Colloidal Stability

Although unfolding is an important pathway for antibody aggregation, antibodies can also aggregate without unfolding (**Figure 2**). One such pathway is the condensation of natively folded antibodies owing to low native colloidal stability (i.e., low native solubility). The native solubility of antibody fragments and mAbs is highly variable (<0.1 to >100 mg ml^{-1}) and poorly understood

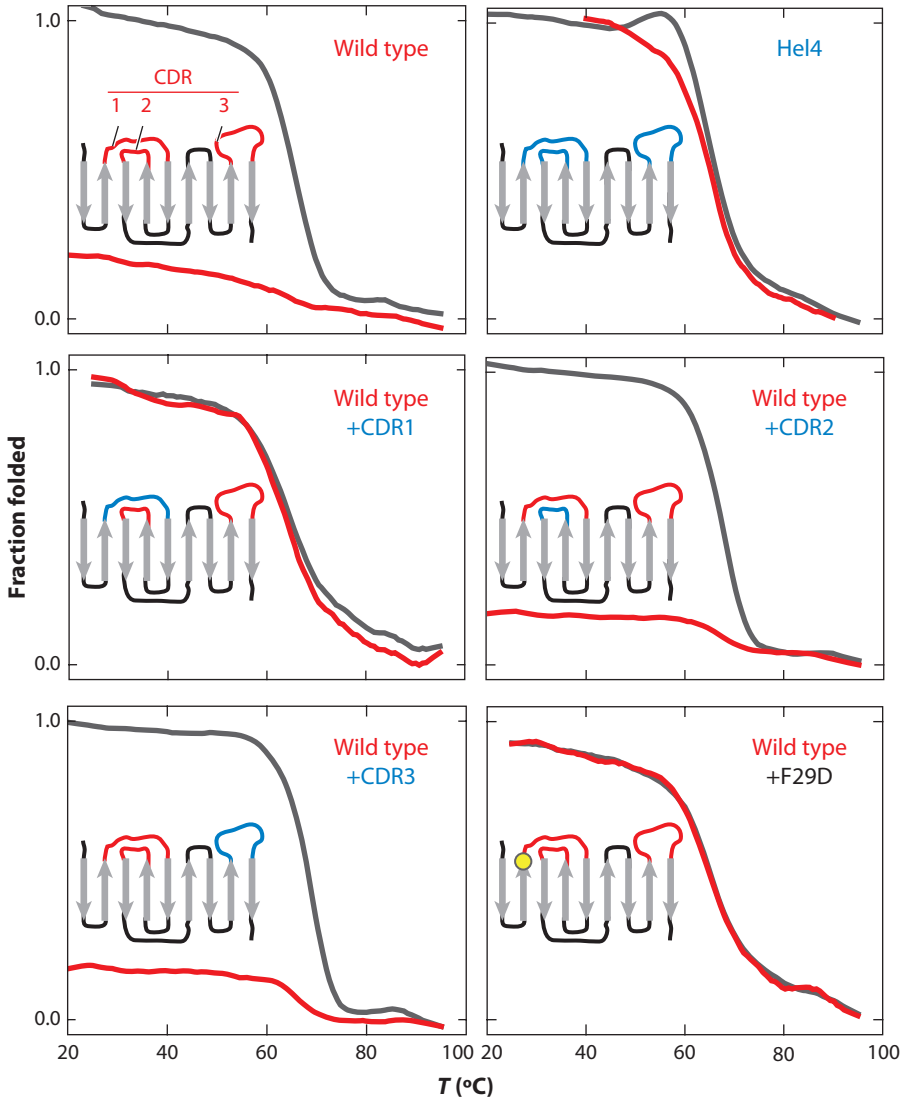

Figure 7

The aggregation propensity of a human variable heavy domain (V_H) antibody is governed by complementarity-determining region 1 (CDR1). Antibody domains that reversibly unfold without aggregating show similar first (*gray*) and second (*red*) unfolding transitions. Two human V_H antibody domains—one that is aggregation prone (wild type) and another that is aggregation resistant (Hel4)—differ only in their three CDR loops (96). Grafting individual CDRs from Hel4 onto the wild-type antibody domain revealed that only CDR1 from Hel4 was capable of conferring reversible unfolding behavior to the wild-type antibody (65). Moreover, a mutation adjacent to CDR1 (phenylalanine to aspartic acid at position 29, highlighted in yellow) in the structural portion of the CDR1 loop also endowed the wild-type antibody with reversible unfolding behavior.

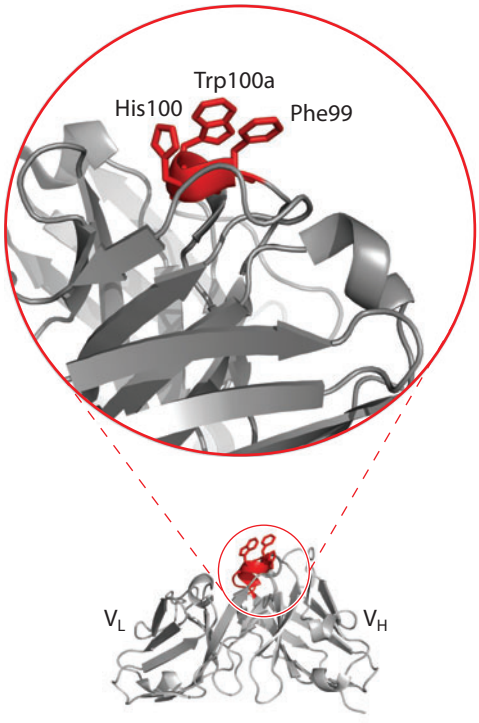

Figure 8

Structure of the Fv portion of a monoclonal antibody with low native solubility. Three consecutive aromatic residues—phenylalanine, histidine and tryptophan—within the heavy chain CDR3 (PDB: 3G6A) reduced the native antibody solubility by an order of magnitude relative to the same antibody lacking such aromatic residues (109). Abbreviations: V_H, variable heavy domain; V_L, variable light domain.

because these proteins have been used primarily at low concentrations (1 ng ml^{-1} to 1 mg ml^{-1}) for various detection applications. More recently, therapeutic applications involving subcutaneous delivery have required much higher antibody concentrations (50–150 mg ml^{-1}) that are near or above the solubility limit of many antibodies (107, 108).

Because CDR loops commonly present solvent-exposed hydrophobic residues, multiple studies have sought to identify aggregation hot spots within CDRs that may contribute disproportionately to native antibody solubility (109–113). Some of these studies have used algorithms of varying complexity to identify either individual residues or peptide motifs that are expected to govern antibody solubility on the basis of properties such as charge, hydrophobicity, and β-sheet propensity (111–113). An important outcome of this analysis is the identification of aromatic and other hydrophobic residues within CDRs that may explain the poor solubility of some antibody variants (109–112). The deleterious impact of hydrophobic residues within antibody CDRs was convincingly demonstrated for a mAb (IgG1) that is poorly soluble (aggregated at concentrations >14 mg ml^{-1}) (109). The crystal structure of the wild-type Fab from this antibody revealed a triad of solvent-exposed aromatic residues in CDR3 of the heavy chain that was hypothesized to be the primary determinant of the poor antibody solubility (**Figure 8**). Strikingly, mutating these three aromatic residues to small, mildly hydrophobic residues (alanines) resulted in a dramatic increase in solubility (>150 mg ml^{-1}). These and related findings (110, 111)

demonstrate the critical impact of hydrophobic residues within CDR loops on the native solubility of mAbs.

The potential negative impact of CDR mutations on antibody binding affinity has motivated the search for alternative mutations that counter the insolubility imparted by hydrophobic CDRs. The general observation that oligosaccharides on the surface of Fc domains increase antibody solubility (114) suggests that placing such sugar moieties proximal to hydrophobic residues within CDRs may reduce the local hydrophobicity and increase antibody solubility. Indeed, Wu et al. (109) tested this hypothesis by introducing a glycosylation site within CDR2 (in a region that does not contact antigen) that was adjacent to aromatic residues in CDR3. Importantly, glycosylation within CDR2 dramatically improved antibody solubility (>100 mg ml^{-1}) without altering antibody binding affinity. Related work also revealed that glycosylation sites in C_H1 can significantly improve antibody solubility (110). For an IgG variant that is poorly soluble owing to hydrophobic residues in heavy chain CDRs, four positions within C_H1 were separately glycosylated to evaluate whether the oligosaccharides would solubilize the parent antibody. As expected, the glycosylation site closest to the V_H domain was most effective, but the effectiveness of the other three sites could not be predicted on the basis of their proximity to hydrophobic CDR loops (110). This finding suggests that the constant regions of antibodies may also contain aggregation hot spots (110–112, 114) that are shielded by oligosaccharides at some positions but not at others. An alternative explanation is that the orientation of the oligosaccharide chains projecting from the surface of the C_H1 domain (which would be influenced by the local antibody structure) may be an important determinant of their solubilizing activity (114).

Diabody: a domain-swapped dimer of two identical or different scFvs

Notably, the solution environment (e.g., pH) also has a significant impact on the native solubility of antibodies. One important observation is that antibody solubility increases as the pH is decreased (44, 110). For example, a poorly soluble IgG (<1 mg ml^{-1} at pH 7) bearing hydrophobic CDR loops is highly soluble at pH 3–5 (>50 mg ml^{-1}) (110). The antibody net charge at low pH is highly positive because the typical isoelectric point of mAbs is between pH 7 and pH 9, and repulsive electrostatic interactions at low pH promote high solubility. Nevertheless, low pH (typically < pH 5) can also promote antibody unfolding (35, 43, 44, 115, 116), which generally leads to aggregation for multidomain antibodies. Therefore, mildly acidic pH values (such as pH 5) may be optimal for promoting both high native solubility and high folding stability. As expected, the pH dependence of antibody solubility is also strongly influenced by the antibody sequence (110, 117), especially at neutral pH, at which the antibody net charge is low and the patterning of both charged and hydrophobic residues is expected to be important.

Antibody Domain Interface Stability

Another mechanism of antibody aggregation that does not require the unfolding of individual domains is intermolecular domain swapping between complementary domains (e.g., V_H/V_L; **Figure 2**). Fabs and mAbs are composed of multiple polypeptide chains, but each individual chain contains only noncomplementary domains (e.g., V_L and C_L in the light chain). The result is that intermolecular domain swapping is rare for these antibodies. However, scFvs contain two complementary domains within a single polypeptide chain, which enables the V_H and V_L domains to form both intra- and intermolecularly paired domains (**Figure 2**). Indeed, domain swapping of V_H and V_L domains between scFv molecules is the basis for the formation of bivalent dimers of scFvs (referred to as diabodies; **Figure 9**) (18, 21, 28, 118, 119). Although diabodies can be formed by scFvs with long linkers (~25 residues) connecting the V_H and V_L domains (120), their assembly is favored as the linker length is shortened (~5–12 residues) to the point that intramolecular pairing of the V_H and V_L domains is no longer possible (18, 119, 121).

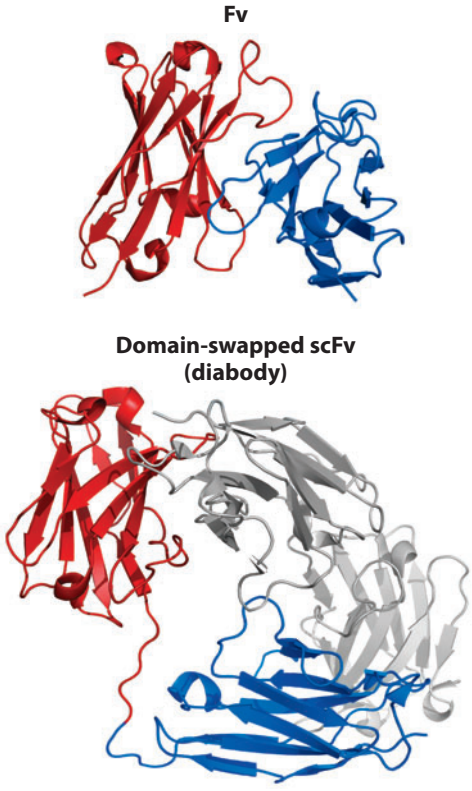

Figure 9

Diabodies are composed of two domain-swapped single-chain variable fragments (scFvs). The variable heavy (V_H) and variable light (V_L) domains within an Fv fragment are unable to domain swap into dimers and higher-order multimers because they are composed of two polypeptides. However, scFvs contain two complementary (V_H/V_L) domains within a single polypeptide chain. Thus, V_H and V_L domains within scFvs can form either intra- or intermolecularly paired domains. When the length of the linker between domains is shortened to fewer than 12 residues, the V_H and V_L domains within scFvs are unable to form intramolecularly paired domains, which leads to the formation of domain-swapped scFv dimers (diabodies) and higher-order multimers. The Fv and diabody structures are based on PDB files 1J05 and 1MOE, respectively.

Interestingly, higher-order assemblies of scFvs (e.g., trimers and tetramers) can also be formed by further reducing the interdomain linker length (0–5 residues) (119, 121–123).

Unfortunately, the ability of scFvs to domain swap and form small multimers (e.g., diabodies) also enables them to form larger, insoluble aggregates. Even though this mechanism is recognized as central to the aggregation propensity of scFvs (52), it is poorly understood because monitoring antibody domain swapping is more complex than monitoring antibody folding stability. This is because the biochemical properties of domain-swapped aggregates are similar to those of the native antibody. Moreover, most studies have evaluated the effects of mutations in the V_H/V_L interface on the stability of scFvs at elevated temperatures (>50°C) at which aggregation mechanisms other than domain swapping are favored (78, 124, 125). Nevertheless, multiple studies have used methods such as size-exclusion chromatography to evaluate the importance of domain swapping at low temperatures (20–37°C) and physiological solution conditions (79, 81, 120). For example, the propensity of an scFv to domain swap into dimers and higher-order assemblies at

37°C can be reduced significantly by introducing a disulfide bond between the V_H and V_L domains (79). Moreover, the propensity of scFvs to domain swap at ambient temperature (20°C) is strongly dependent on pH, as modest increases in pH (from pH 6.5 to pH 8) significantly enhance the fraction of scFv monomers relative to dimers (120). This finding suggests that electrostatic interactions can stabilize scFv monomers against domain swapping, which is consistent with the identification of charged mutations that stabilize the V_H/V_L interface and reduce aggregation of related scFvs (81). Finally, a bispecific antibody (scFv-IgG) displays much less aggregation at low temperature (<25°C) when the scFv is engineered with stabilizing mutations within and near the V_H/V_L interface (81), which suggests that domain swapping can also lead to aggregation of larger, more complex antibodies.

FUTURE DIRECTIONS

Reversibly Folding Multidomain Antibodies

An outstanding challenge is to engineer multidomain antibodies to resist aggregation even when they are unfolded. Although some single-domain antibodies displaying three CDR loops have been evolved with nanomolar binding affinity (17, 19, 126), more than three CDR loops displayed on two domains will likely be required to routinely achieve high binding affinity for diverse targets. Moreover, the success in engineering single antibody domains to possess extreme resistance to aggregation is likely because their folding is independent of complementary domains. Thus, an attractive starting point for engineering multidomain antibodies to reversibly unfold is to link multiple copies of noncomplementary domains (e.g., two V_H domains) via flexible peptide linkers. Because the folding of each domain would be independent of the other domain, it should be more straightforward to engineer each domain to resist aggregation than to do so for conventional multidomain formats (such as scFvs). This strategy has been used to generate bivalent and bispecific antibodies using camelid V_{HH} domains (127), although the ability of these multidomain antibodies to resist aggregation when unfolded has not been evaluated. An added advantage of this strategy is that the hydrophobic residues at the former V_H/V_L interface could be engineered with hydrophilic residues (along with the rest of the antibody surface) to discourage the two variable domains from interacting.

Antibody Scaffolds with High Native Solubility

Another important challenge is to develop antibody scaffolds—especially for mAbs—that are highly soluble regardless of the sequence of the CDR loops (including loops containing hydrophobic residues). The initial success of introducing glycosylation sites within the variable and constant domains of mAbs (109, 110) warrants further work to understand how to best engineer these antibodies with solubilizing oligosaccharides. The finding that some glycosylation sites within the constant domains (e.g., C_H1) adjacent to the variable domains of mAbs are highly effective at increasing antibody solubility whereas others are weakly effective suggests that the position and orientation of oligosaccharides are critical to their solubilizing activity (110). Future studies will need to evaluate whether specific glycosylation sites within Fabs can be identified that generally impart high solubility to mAbs regardless of the specific CDR sequence. The development of bacterial strains capable of synthesizing glycosylated proteins (see Reference 128 and references therein) should enable similar analysis for antibody fragments such as scFvs. This is particularly important given the more rapid and less expensive synthesis of antibody fragments in bacteria compared with mAbs in mammalian cells.

Kinetically Stable Single-Chain Variable Fragments

The power of encoding two complementary antibody domains (V_H and V_L) within a single polypeptide chain (scFv) is undeniable, and these antibody fragments will continue to attract intense interest. An important next step in developing aggregation-resistant scFvs is to engineer them to be kinetically stable in a manner similar to their larger antibody counterparts (Fabs and mAbs). The remarkable kinetic stability of the constant domains of Fabs (73, 91), which are similar in size to variable domains, should inspire new strategies to impart increased kinetic stability to scFvs. One possible direction is to alter the size, hydrophobicity, and/or orientation of the V_H/V_L interface to more closely resemble the C_H1/C_L interface (**Figure 6**), which possesses high kinetic stability (73). A related, important future direction is to evaluate whether diabodies composed of two scFvs possess enhanced kinetic stability relative to monomeric scFvs. The additional V_H/V_L interface within diabodies may impart significant kinetic stability to scFvs that possess modest kinetic stability as monomers.

Design of Synthetic Antibody Libraries that Encode Aggregation-Resistant Antibodies

The relative simplicity of rapidly synthesizing and displaying synthetic antibody libraries using phage display (as well as related cell surface and ribosome display methods) enables insights gleaned from previous antibody engineering analysis to be included in the design of new antibody libraries. One important consideration for designing antibody libraries is that the hydrophobicity of CDR loops is a key determinant of the propensity of antibodies to aggregate. Thus, library design strategies should include solubilizing residues (i.e., charged residues) either at the edges of CDR loops (65) or within CDRs (65, 105) to counterbalance the impact of the hydrophobic residues required for antibody binding. Moreover, it may be helpful to include cysteines within one or more CDRs to enable formation of the intra- and inter-CDR disulfide bonds that generally increase antibody solubility (106). Finally, the antibody scaffold used to construct the library should be carefully selected, especially for single-domain antibodies (human V_H or camelid V_{HH}), because some stabilized scaffolds are able to resist aggregation even when displaying long and/or hydrophobic CDR loops (56, 58, 60).

Aggregation-Resistant Bispecific Antibodies

Although antibody fragments (e.g., scFvs) have been used infrequently as therapeutics, there is currently significant interest in linking them to mAbs (e.g., IgGs) to generate bispecific antibodies capable of simultaneously targeting two antigens (129–131). A common strategy for designing bispecific antibodies is to attach an scFv to the N or C terminus of the heavy chain of IgGs. However, the propensity of scFvs to domain swap or unfold limits the solubility of bispecific antibodies (81, 132). The initial success in improving the solubility of bispecific (scFv-IgG) antibodies via engineering scFvs to possess high folding stability (81) suggests that insights obtained from stabilizing individual scFvs should be readily transferrable to bispecific antibodies. Nevertheless, future work will need to address the solubility challenges posed by the unique architecture of bispecific antibodies. For example, the bivalent presentation of scFvs is expected to amplify their aggregation propensity because bivalent antibodies are generally more associative than monovalent ones (133). It will also be important to elucidate how the location of scFvs or other antibody fragments (e.g., V_H) within bispecific antibodies—which hypothetically can be placed at the N or C terminus of either the heavy or light chains—impacts the solubility of these large antibodies.

CONCLUSIONS

The growing importance of therapeutic antibodies for treating human disorders, the extremely high antibody concentrations (50–150 mg ml^{-1}) required for subcutaneous delivery, and the immunogeneity of antibody aggregates necessitate that antibodies be engineered to be extremely resistant to aggregation. Some aspects of antibody stability—especially their thermodynamic folding stability—are relatively well understood, and several powerful approaches to identifying stabilizing mutations have been developed. However, other aspects of antibody stability, such as kinetic folding stability and nonnative colloidal stability, are less well understood and warrant further work to elucidate how these properties can be optimized in single- and multidomain antibodies. It will be particularly important to establish systematic mutational strategies for maximizing antibody solubility without diminishing antibody binding affinity. Successfully elucidating how to engineer aggregation-resistant, high-affinity antibodies will enable their use in a broader range of therapeutic applications than is currently possible.

DISCLOSURE STATEMENT

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96. Identification of an aggregation-resistant human V_H antibody whose high solubility is encoded entirely within three CDRs.

98. Demonstration that camelid and human variable domains possess similar folding stability and dissimilar nonnative solubility.

105. Novel phage display method for selecting human V_H domains that resist aggregation when transiently unfolded.

109. Demonstration of effective mutational strategies for improving the native solubility of a monoclonal antibody bearing hydrophobic CDRs.

110. Comprehensive analysis of mutations and solution conditions that improve the native solubility of a monoclonal antibody.

111. First demonstration that molecular simulations of mAbs can be used to identify mutations that enhance antibody solubility.

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