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Review

Stability engineering of the human antibody repertoire

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ABSTRACT

Human monoclonal antibodies often display limited thermodynamic and colloidal stabilities. This behavior hinders their production, and places limitations on the development of novel formulation conditions and therapeutic applications. Antibodies are highly diverse molecules, with much of the sequence variation observed within variable domain families and, in particular, their complementarity determining regions. This has complicated the development of comprehensive strategies for the stability engineering of the human antibody repertoire. Here we provide an overview of the field, and discuss recent advances in the development of robust and aggregation resistant antibody therapeutics.

1. Introduction

The number of human monoclonal antibody candidates has increased rapidly in recent years, and now represent the largest single class of molecules entering clinical studies [1]. Despite the rapid growth of the antibody therapeutics market over the past twenty to thirty years, hurdles remain that limit their manufacture. Key factors relate to the variable and often limited stability of human antibodies, which negatively impact on many production processes including expression [2,3], purification [4] and formulation [5].

Antibodies are complex multidomain proteins and mechanisms governing their thermodynamic [6] and, in particular, colloidal [7] stability are not fully understood. Human antibodies of the commonly used immunoglobulin G (IgG) type consist of a total of twelve domains, which can be further divided into two chains (heavy and light) and variable (V_H, V_L) and constant (C_H1, C_H2, C_H3, C_L) domains (Fig. 1). Only limited diversity is observed among constant domains, with a total of four isotype (IgG1–4) and two light chain ([κ] kappa and [λ] lambda) classes expressed in humans. Consequently, isotype differences can explain only a limited proportion of the observed stability variation in the human antibody repertoire [8]. A higher proportion of sequence diversity is observed among antibody variable domains, which are assembled through genetic recombination of variable, diverse (V_H only) and joining segments (VDJ-recombination). Further diversity is then introduced into framework, and in particular complementarity determining regions (CDR), through somatic hypermutation, followed by clonal selection of the repertoire. As a result of these processes, the overwhelming majority of sequence variation within the human antibody repertoire is observed within the CDR regions of a limited range of human variable domain families. In this review, we summarize the influence of the observed antibody diversity on colloidal and thermodynamic stabilities and discuss recent advances to improve these properties through engineering approaches.

2. Protein aggregation

Aggregation is a complex process by which proteins can form alternative colloidal states, which are different from the native state, but otherwise energetically favorable [9,10]. It is generally believed that such aggregate species are predominantly formed via unfolded or partially unfolded states [11]. Protein aggregation is increasingly recognized as a problem affecting the manufacturability of human therapeutic antibodies, shelf life and efficacy [12]. Importantly, the presence of aggregates has also been linked to increased immunogenicity, with effects ranging from mild skin irritation to anaphylaxis [13].

Although it can be assumed that stabilization of the native state over alternative aggregate states has occurred during evolution [14], it is important to note that the production of monoclonal antibodies exposes these molecules to a wide range of non-physiological

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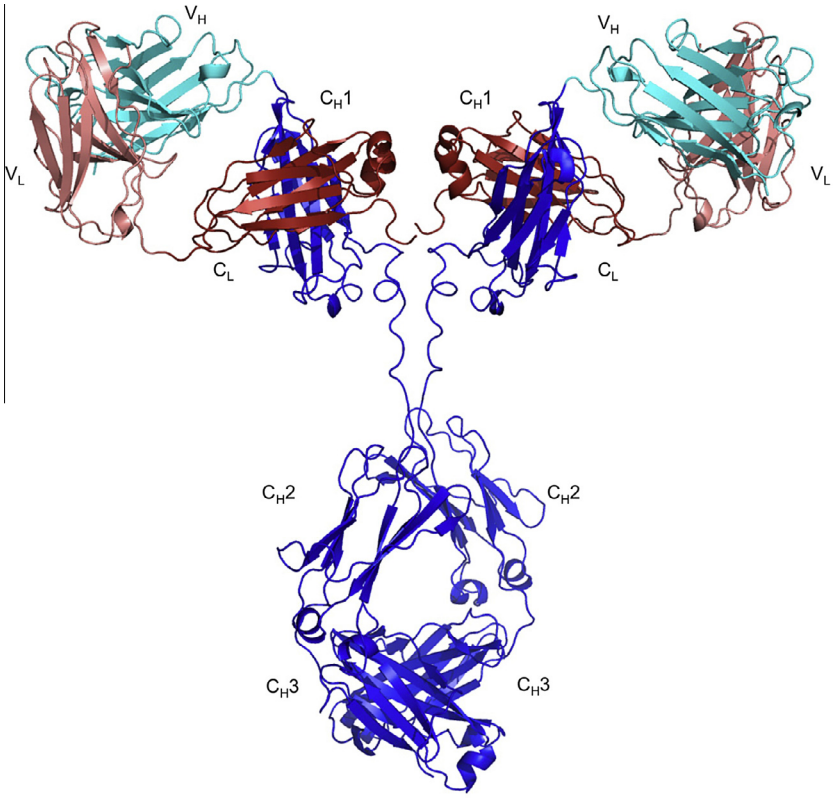

Fig. 1. Structure of the human IgG molecule. The molecule is formed by two heavy chains (consisting of V_H, C_H1, C_H2 and C_H3 domains) and two light chains (consisting of V_L and C_L). Figure was generated using PYMOL.

processes and conditions. These include recombinant expression, purification, concentration, viral inactivation, filtration, formulation, freeze/drying, transport and long-term storage. Throughout these steps the antibody molecule may encounter several stress factors that can dramatically increase its propensity to aggregate (including variations of temperature, pH, protein concentrations, ionic strength, exposure to air–water interfaces and mechanical stress). A further driver of protein aggregation has been a growing trend towards formulations that allow sub-cutaneous administration routes. This requires formulation at high protein concentrations (at around 100 mg/ml) in syringes for self-injection, which places increased demands on colloidal stability [15].

3. Stability of human antibody isotypes and constant domains

The four human IgG isotypes differ in their stabilities and biological functions, namely their potential to induce cellular killing through antibody dependent cellular cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC). While the human IgG1 isotype induces powerful ADCC and CDC responses, this is not the case for human IgG2, which is particularly suitable for applications where cellular killing is not required (such as neutralization of soluble ligands) [16]. Unlike the other human isotypes, IgG4 also has a naturally occurring potential to form a bispecific molecule [17] (it has been recently shown that this dual specificity can be grafted onto other isotypes [18]). Human IgG3 is not commonly used due to its longer hinge region, which renders it susceptible to proteolysis [19]. The vast majority of human antibody therapeutics currently in clinical practice and development are of the IgG1 isotype [16].

Conflicting evidence exists in respect to the colloidal stability of the various human IgG isotypes. A recent study of eleven different IgG1 and IgG2 antibodies concluded that, after high temperature storage, the IgG2 isotype is more prone to aggregation [20]. Similar findings were also obtained when subjecting these isotypes to high salt conditons [21] and from isotype switching studies [22]. The latter study confirmed that the human IgG1 isotype is less prone to aggregation when compared to IgG2 or IgG4. However, the authors also found that this isotype is more prone to fragmentation, especially at low pH, due to a non-enzymatic site in the upper hinge region. The hinge regions have also been implicated in the observed differences in aggregation propensity as the IgG2 hinge contains two additional cysteines in each heavy chain compared to IgG1, and is prone to the display of free cysteine residues [23,24]. Differences in the thermal stability of human constant domains have also been reported, with the C_H3 domains of human IgG1 and IgG2 displaying similar melting temperatures, much higher than what was observed for IgG4 [8]. In general, C_H3 domains exhibit more favorable biophysical properties than C_H2 domains [25]. This is reflected by melting temperatures of 8–10 °C higher than those observed for C_H2 domains [8,26].

Although human IgG1 therefore appears less aggregation prone than other isotypes, this is not universally the case. For example, a recent study found that the IgG1 isotype variant of an anti-LINGO-1 antibody exhibited a much higher propensity to aggregate than an IgG2 variant containing identical variable regions [27]. Studies such as these demonstrate that predicting the aggregation propensities of IgG molecules based on isotype can be difficult at the best of times. Moreover, evidence suggests that the aggregation propensity of human IgG molecules is greatly influenced by their variable domains [8,27,28]. This has the effect that antibodies with identical constant domains, but different variable domains, can vary widely in their stability profiles [5].

4. Stability of human antibody variable domains

Human antibody variable domains can be grouped into several homologous families, which differ in their biophysical properties. A seminal study of consensus domains of human V_H and V_L families has revealed considerable differences of expression yields and thermodynamic stability [6]. In the case of human V_H domains, stabilities ranging from ΔG_{N-U} 14–53 kJ/mol were observed, with soluble expression yields ranging from 1–2.4 mg/l (Table 1). Intriguingly, Ewert et al. also reported that members of the (odd numbered) V_H 1, 3 and 5 families generally displayed superior biophysical properties in comparison with the (even numbered) 2, 4 and 6 families. These differences were observed for both thermodynamic stabilities and expression yield. In particular, the human V_H3 family was identified as uniquely stable and well expressed. This family is also among the most abundant in the human repertoire [29]. The overall stability trends were confirmed in a more recent study, which analyzed the effect of pairing of different human variable domain families on expression, thermal and colloidal stabilities of Fab fragments and IgG monoclonals [30].

More limited differences in biophysical properties were observed among human V_L families, with thermodynamic stabilities ranging from ΔG_{N-U} 15–24 kJ/mol for lambda and ΔG_{N-U} 29–35 kJ/mol for kappa [6]. Similarly, higher expression yields were observed for kappa variable light domains (5–17 mg/l) than for lambda domains (0.3–2 mg/l). The observed differences between antibody variable light domains were much less pronounced when pairing these with human V_H in an scFv format, while considerable differences between variable heavy domain families remained. Overall, the authors found the V_H/V_L combinations H3κ3, H1bκ3, H5κ3 and H3κ1 to be superior in respect to expression yield and thermodynamic stability [6]. However, despite the clear influence of variable domain family (and framework regions), the study also observed a strong influence of CDR sequence on biophysical properties. Indeed, initial attempts of the authors to express human V_H domain in isolation failed to yield protein (with the exception of V_H3). Only the grafting of a solubilizing CDR3 region allowed the soluble expression of human V_H1, and of V_H2, V_H4, V_H5 and V_H6 through refolding from inclusion bodies.

Although several of the human variable domain families are thermodynamically stable, they nevertheless often display poor colloidal stability and readily aggregate when heated above their melting temperatures. This behavior is exemplified by the V_H3 model domain DP47 [31] and by studies of synthetic antibody repertoires on phage, which have demonstrated that as few as one in a thousand VH domains resists heat induced aggregation [32].

Table 1
Biophysical properties of human antibody variable domains. Adapted from Ref. [6].

	Human family	Solubilizing CDR3 required	Expression yield	ΔG_{N-U}
V_H	1a	Y	+	+
	1b	Y	+	++
	2	Y	Refold	nd
	3	N	+	+++
	4	Y	Refold	nd
	5	Y	Refold	+
	6	Y	Refold	nd
V_L	κ1	N	+	++
	κ2	N	+++	++
	κ3	N	+++	+++
	κ4	N	++	-
	λ1	N	+	++
	λ2	N	+	+
	λ3	N	+	+

5. Increasing antibody stability through formulation

The most common method for controlling the biophysical properties of monoclonal antibodies and other biologics is through changes of buffer conditions and excipients. Formulation allows for the control of ionic strength, pH and the addition of excipient agents such as sugars or surfactants, which have been shown to stabilize monoclonal antibody preparations [33]. Although formulation can shield proteins from some chemical and physical challenges (including deamidation, oxidation, hydrolysis/fragmentation and isomerization), aggregation remains difficult to control [12]. Consequently, finding appropriate formulation conditions for an individual product is often an arduous process. This is exemplified by the therapeutic monoclonal antibody cetuximab (Erbitux). Its initial formulation conditions (2 mg/ml in phosphate buffer pH 6.0) generated visible aggregates under mechanical stress [34]. To address this issue, conditions had to be empirically optimized and were changed to 5 mg/ml in citrate buffer pH 5.5 with the addition of glycine and polysorbate 80 as stabilizers.

In recent years, more high-throughput techniques for formulation optimization have been developed [35], and stabilizers and excipients with more general applicability have been identified. Among these, arginine is commonly used to improve refolding yields [36,37], and more recently has been used in formulation to reduce heat-induced aggregation [38]. Another commonly used class of excipient molecules are polysorbates, which accumulate at the air–solvent interface and offer protection against shaking and other mechanical stresses [39]. Despite recent advances, the identification of suitable formulation conditions for a specific monoclonal antibody remains challenging and cannot be determined from its amino acid sequence. Indeed, a considerable proportion of human monoclonal antibody candidates fail formulation studies, often at relatively late pre-clinical stage, placing heavy burdens onto drug development pipelines [5].

6. Stability engineering of human antibody constant domains

Many efforts have been made to increase the stability of the different human antibody isotypes. Early work in this field focused on sequences analyses to predict stabilizing mutations. This approach allowed the identification of a set of substitutions (371K, 376G and 392L), which considerably increased thermal stability (from 76 °C to 86 °C) of the C_H3 domain [40]. The study was based on frequency analyses within 19 mammalian species to generate a consensus sequence. Structural approaches have also been utilized to increase stability of the C_H1/C_L interface [41]. More recently, it was shown that the introduction of additional intra-domain disulfide bridges in the human C_H3 domain resulted in the thermal stabilization of an isolated IgG1 Fc fragment [42] (Fig. 2). In particular, the introduction of a link between the N-terminal A strand (position 343) and the F strand (position 431) caused a 8 °C increase in the melting temperature, while a linkage between positions 375 and 396 resulted in a 4 °C increase (combined 13 °C). These findings were successfully applied to a HER2 specific Fcab molecule (Fc fragment with engineered binding loops) resulting in an increased in the thermal stability of 19 °C. A similar finding was observed for a human IgG1 C_H3 single domain, which could be stabilized by introduction of an intra-domain disulfide bridge [43]. The authors used a two-step approach, by initially introducing mutations that rendered the C_H3 domain monomeric, resulting in a overall low thermal stability (41 °C compared to 82 °C for the native dimeric form). Using structure-based design, two additional cysteines (at positions 343 and 431) were then introduced to generate a disulfide bridge, which increased the thermal stability to 76 °C (a

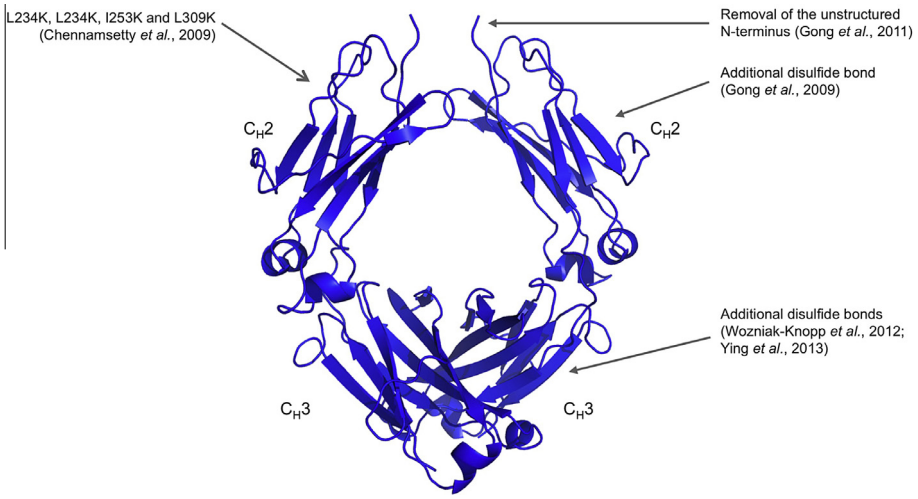

Fig. 2. Stability engineering of human constant domains.

35 °C increase) [43]. Similar strategies have been also reported for C_H2 domains [26]. Here the authors reported the engineering of an isolated human C_H2 domain, resulting in an increase in thermal stability of 20 °C (from 54 °C to 74 °C) [26]. More recently, the same group reported the stabilization of a C_H2 domain through removal of an unstructured region at the N-terminus. Combined with the intra-domain disulfide bond, this resulted in a further increase of melting temperature of 10 °C [44]. For all of the above studies, stability of constant domains was evaluated in the context of fragments or isolated domains.

Other studies have investigated the effects of such mutations on the stability of full-length human IgG. In the case of human IgG4, alteration of the disulfide bond network in the Fab region was shown to lead to an increase of thermal stability of between 3 and 7 °C [45]. This effect was achieved through removal of a cysteine at position 127 in the C_H1 domain, while an additional inter-domain disulfide bond between position 229 and C_L was introduced (similar to what is observed in human IgG1). Importantly, the authors reported that the introduced mutations did not affect antigen binding [45]. More recently, molecular dynamic simulations have been used to predict the spatial aggregation propensity (SAP) of human IgG molecules [46]. The authors later showed that by mutating identified surface-exposed hydrophobic patches within the hinge and Fc regions of an IgG, aggregation resistance and, to a more limited extent, thermodynamic stability could be improved [47].

7. Increased stability through charged fusion tags

In addition to the modification of constant domains, it was recently shown that the addition of charged fusion tags to the N-terminus of human variable domains could considerably improve aggregation propensity [48]. The authors noted that batches of the same IgG monoclonal expressed in yeast were less prone to aggregation than those expressed in mammalian cells. Mass spectrometry and sequencing analyses revealed the presence of a non-processed "EAEA" leader sequence within both heavy and light chains. In contrast to colloidal stability, the authors did not observe improved thermodynamic stability, and the effect seemed to be particularly pronounced for antibodies with a low global net charge. In addition to terminal fusion tags, a different study explored the internal use of a "DED" motif [49]. It was shown that the insertion of the tag into the CDR3 region of aggregation-prone V_H domain antibodies reduced their aggregation propensity, while reducing retention on gel filtration matrices.

8. Stability engineering of human antibody variable domains

8.1. Engineering of human V_H domains through mutation of the light chain interface

The poor biophysical properties of isolated human antibody variable domains are in marked contrast to those of the variable domains of camels and llamas [50]. Although these immunoglobulin domains (V_{HH}) display a relatively high level of sequence identity with human V_H domains, they are generally well expressed and soluble [51] and reversibly unfold after incubation at temperatures as high as 90 °C [52]. In addition to the observed differences in biophysical nature, camelid domains also display an important difference in chain structure, as they are naturally devoid of light chain partners [50]. Their single domain nature results in unique structural features, compared to human antibody V_H domains. These include CDR length and conformation, as well as framework residues. A particular feature of V_{HH} domains is their long flexible CDR3 loop capable of folding back on itself, thereby protecting an area forming the V_H/V_L interface in human antibodies. In addition, these naturally occurring single domains display a marked reduction in hydrophobicity of the former light chain interface, due to a limited number of changes in their respective germlines (Fig. 3). The most common and significant changes compared to humans, coined the "V_{HH} tetrad", are located at positions: G44Q, L45R/C, W47G/I and V37F/Y (human/camelid). The latter residue is nucleating a small core involving Y91, W103, R45 and other hydrophobic residues within the CDR3 loop [53]. Structural investigation of V_{HH} has also shown the CDR3 loop covering residue 37, thereby shielding hydrophobicity in an area corresponding to the light chain interface in human V_H [54]. In addition, camelid V_{HH} often contain an additional disulfide bridge between CDR1 and CDR3, which increases their thermal stability [55,56].

In order to improve the biophysical properties of human V_H domains, elements of the V_{HH} tetrad have been incorporated into human frameworks ("camelization") [57]. Although the introduced framework changes improved the solubility of human V_H, they also reduced expression levels, induced conformational changes, decreased thermodynamic stability and did not considerably improve the colloidal stability of the domains [58,59]. In contrast, combining the approach with the introduction of an additional disulfide bond between CDR1 and CDR3, yielded domains with high colloidal stability, although at the expense of antigen binding affinity [60].

As an alternative to camelid domains, shark single domain antibodies (V_{NAR}) could also potentially serve as a blueprint for improving

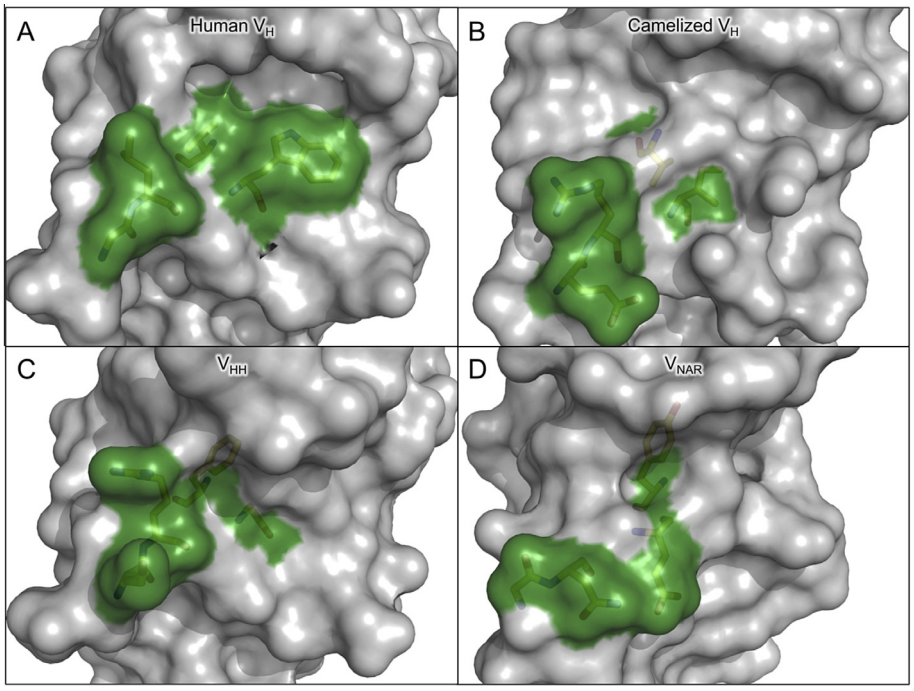

Fig. 3. Engineering of human VH domains through mutation of the light chain interface. Shown are surface representations of the light chain interface in human V_H (PDB 2VXS) and of the equivalent surfaces in camelized V_H (PDB 1VHP), camelid V_{HH} (PDB 1ZVH) and shark V_{NAR} domains (PDB 1T6V). Residues forming the camelid 'tetrad' at positions 37, 44, 45 and 47 are highlighted in green. Camelized V_H, V_{HH} and V_{NAR} domains display a considerable reduction in surface hydrophobicity in comparison to human V_H domains. Figure was generated using PYMOL.

the stability of human antibody V_H domains (although they are more distantly related to human domains [61]). As observed for camelid domains, shark V_{NAR} domains are characterized by a long protruding CDR3 [62]. The CDR loop is constrained by additional disulfide bonds, thereby folding over an area equivalent to the former V_H/V_L interface of human antibodies [61]. A recent study revealed that V_{NAR} domains are resistant to thermal denaturation, acidic denaturation, and lyophilisation denaturation and are capable of returning to their native state after thermal unfolding [63].

Rather than focusing on direct grafting of camelid residues, more recent approaches for the generation of autonomous single domains have focused on the identification of novel mutations. This has been possible due to the use of phage display based selection strategies and the use of protein A to select for folded domains (pA superantigen binds to folded human V_H3 domains, but not to unfolded or aggregated domains). Using this approach, an extensive mutagenesis study has identified hydrophilic substitutions within the V_H3/V_L interface [53]. The authors were able to demonstrate that their mutations (including H35G, Q39R, L45E, R50S) improved solubility, thermal refolding and melting temperature. Importantly, some of the mutations improved protein A binding (and presumably stability), independently of CDR3 sequence diversity. As CDR3 of V_H provides the majority of binding energy in antibody–antigen interactions, this indicated broad compatibility with antigen binding and suggested that the engineered frameworks could be used for the construction of stable repertoires of autonomous V_H domains.

8.2. Engineering of human V_H domains through CDR grafting and framework mutations

In addition to their use in the engineering of autonomous antibody single domains, homology-based approaches have also been used for the identification of mutations outside the interface regions, thereby retaining the ability of human V_H to interact with variable light domains. One of the earliest examples of such strategies relates to the use of CDR grafting for the improvement of biophysical properties. Developed by Jones and Winter in the mid-1980s, CDR grafting was initially used to reduce the immunogenicity of mouse monoclonals, by transplantation of murine CDR regions onto a human framework [64]. However, it was later shown that by grafting onto a stable and well-behaved framework (such as the Herceptin 4D5 [V_H3] framework) considerably improved expression levels and thermodynamic stability, while maintaining binding affinity of the parental antibody fragment [65]. A second approach is based on the use of consensus sequences to improve stability [66]. This approach has been applied to a range of protein scaffolds [67], and utilizes amino acid observed with high frequency within the repertoire [68]. This approach was also used in the construction of the HuCAL human antibody library, for which the authors have reported good expression yields [69]. More recent studies from the same group have identified additional framework mutations that transfer biophysical properties between human V_H3 and V_H6 families [70,71].

As an alternative to homology-based approaches, other studies have focused on the computational prediction of aggregation hotspots in antibody sequences. Early studies on peptides and proteins involved in neurodegenerative diseases have highlighted physicochemical properties that correlate with an increase in aggregate formation, namely hydrophobicity, β-sheet propensity and reduced net charge [72]. Algorithms have been developed to detect such aggregation-prone regions [73–76]. Two of these algorithms, TANGO and PAGE, have been used to identify potential aggregation-prone regions in commercial therapeutic antibodies, which are predominately located in variable domains, and particularly within CDR and adjacent framework residues [28].

8.3. Engineering of human variable domains through CDR mutations

The role of CDR residues in determining thermodynamic and in particular colloidal stability of human antibody variable domains is further highlighted by studies on the biophysical properties of the

HEL4 model domain [31]. HEL4 was originally isolated through phage display selection from a synthetic CDR-only repertoire based on the human V_H3-23 DP47 germline [77]. As phage is remarkably resistant to chemical, proteolytic and thermal denaturation [78–80], the authors were able to heat the phage displayed antibody fragments at high temperature (80 °C), followed by cooling and binding to antigen (hen egg white lysozyme). The method thereby selected for domains that unfolded reversibly, and could be captured by binding to antigen or superantigen. Unlike other human V_H3 domains, HEL4 displayed highly favorable biophysical properties, including heat-refoldability, high expression levels in bacteria, and the absence of 'stickiness' on gel-filtration [31]. Indeed, it was later shown that aggregation resistance on phage is a general indicator of the solution behavior of human V_H domains [81]. In addition to HEL4, several other V_H domains, that were isolated using this method, showed a high level of resistance to aggregation. Intriguingly, despite their high level of colloidal stability, all of the domains actually had lower thermodynamic stabilities than the DP47 domain from which they had been derived from (ΔG_{N-U} = 15–20 kJ/mol vs 35 kJ/mol). In contrast, DP47 readily aggregated upon heating, further highlighting differences between thermodynamic and colloidal stability. As an alternative to heat-based selection on phage, a more recent study explored the use of pH stress (pH 3.2) for the selection of aggregation-resistant V_H domains [82]. In contrast to domains isolated after thermal denaturation, the selected V_H domains displayed increased thermodynamic stabilities and increased colloidal stability at low pH, reflecting the different denaturation conditions experienced during the selection process.

Although the denaturation method on phage was capable of selecting human V_H domains with CDR sequences that promoted a high level of aggregation resistance, it was also evident that such domains were rare within the repertoire. To increase the frequency of such clones, a human V_H library was constructed through combinatorial assembly of CDR regions that had been pre-selected for aggregation resistance in phage. Binders were successfully selected from this repertoire, which exhibited good antigen-binding properties together with favorable biophysical properties [32].

Despite clear improvements over other human V_H domains, detailed determinants of the observed aggregation resistance initially had remained unclear. As HEL4 and other clones had been selected from synthetic CDR-only repertoires, it was apparent that the

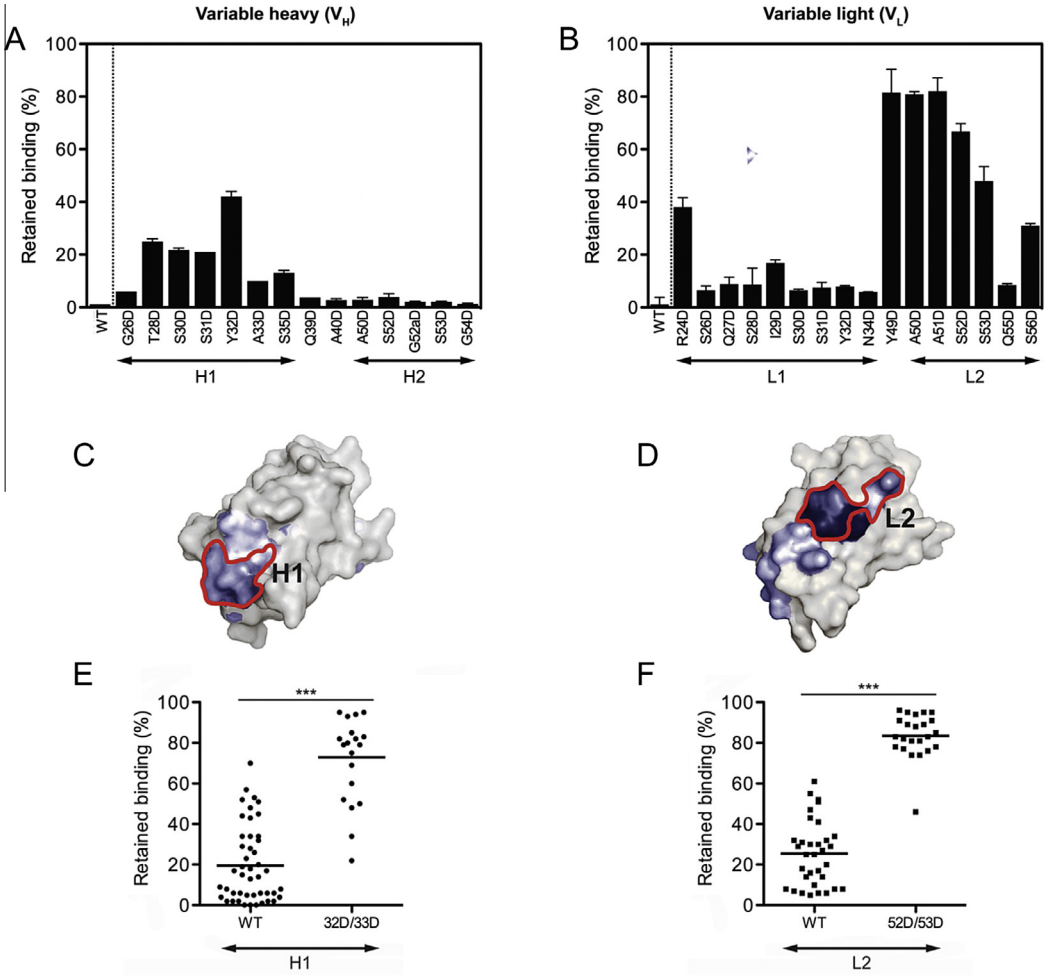

Fig. 4. Effect of mutations in human antibody V_H and V_L domains on aggregation resistance. Surface residues in variable heavy and light domains were targeted for substitution with aspartate. Aggregation resistance of the domains was determined by measuring binding to protein A and protein L superantigen after heating to 80 °C on phage. Complementarily determining regions are indicated (A) Mutations in human V_H. (B) Mutations in human V_L. Introduction of aspartate into CDR H1 and CDR L2 increases aggregation resistance of human variable domains up to 40-fold for V_H and up to 80-fold for V_L. (C) Mutations mapped onto the human V_H surface. (D) Mutations mapped onto the human V_L surface (blue: 100% retained superantigen binding, white: 0%). (E) Stability engineering of human V_H repertoire. (F) Stability engineering of human V_L repertoire. The mutant repertoires were generated through introduction of aspartate mutations in CDR H1 and CDR L2 (at positions 32/33 and 52/53, respectively). Introduction of the mutations significantly increases the aggregation resistance of human antibody repertoires, independent of sequence diversity at other CDR positions. Adapted from Ref. [7].

exhibited biophysical properties were a result of changes in the complementarity determining regions, without any further influence of framework residues [31]. Crystal structure of the HEL4 model domain had indicated a potential effect of a serine to glycine mutation at position 35 [77]. Indeed, introduction of this mutation into DP47, resulted in increased solubility and reduced "stickiness" on gel-filtration. However, the mutant domain continued to readily aggregate when heated above its melting temperature. The authors also reported that many of the selected domains had a low isoelectric point (pI). Similar findings were also obtained in a second study, which reported that a low pI, together with the non-canonical inter-CDR disulfide linkages, contribute to improved biophysical properties human V_H domains [83]. Using transient heating to pre-select for proteins reversible thermal unfolding properties, the authors found that many of the isolated domains had pI values of less than six, although they also observed domains with values higher than eight. The authors concluded that the improved biophysical characteristics of these V_H domains were caused by a combination of global pI effects, together with an increase of stability through inter-CDR disulfide linkage (other studies by the same group demonstrated that, although the additional disulfide bridge significantly increased thermal stability and protease resistance [84], it also induced structural changes in human V_H as indicated by a marked reduction of protein A superantigen binding [85]). A global increase in charge was also observed in a different study, analyzing larger sets of V_H domain sequences after heat selection on phage, together with an overall reduction of hydrophobicity and a decrease in beta-sheet propensity [86].

More recently, an extensive mutagenesis study on phage has provided detailed insights into mutations that increase the colloidal stability of human V_H and V_L antibody domains [7]. Using superantigen binding after heat denaturation on phage as a mean to select for reversible unfolding, the investigators analyzed the effect of charged mutations at all CDR positions (excluding CDR3 to limit effects on antigen binding). This revealed that the introduction of negative charge (and in particular aspartate) at CDR-H1 positions (28, 30–33, 35) considerably increased the aggregation resistance of human V_H domains (up to 40-fold) (Fig. 4A). This is in excellent agreement with the observations for the model HEL4 domain, which contains negatively charged amino acids at a subset of the identified positions (as highlighted by CDR grafting studies [87–89]).

Intriguingly, this was not the case for human V_L domains, for which the introduction of mutations at equivalent positions had little effect. In contrast to what was observed for human V_H, increased aggregation resistance (up to 80-fold) was instead endowed by the introduction of asparate at CDR-L2 positions (49, 50–53, 56) (Fig. 4B). As observed in other studies [81], the improved properties on phage correlated with increased aggregation resistance in solution, improved expression yields and reduced retention on gel-filtration. Importantly, the observed increases in aggregation resistance were not only observed in model domains, but also when introducing CDR-H1 and CDR-L2 mutations into diverse antibody libraries [7] (Fig. 4E/F).

In addition, and unlike what had been observed for camelized and other engineered variable domains [53,59,85], crystal structures of mutant V_H and V_L domains showed no detectable conformational changes in either CDR conformations or the V_H/V_L interface. Taken together, these results indicated broad compatibility with high affinity antigen binding, which was confirmed by the introduction of CDR-H1 and CDR-L2 mutations into the antibody therapeutic Herceptin [7]. Further evidence for the influence of the identified mutations on colloidal stability has recently been reported in a study of an aggregation-prone human IgG1 monoclonal [90]. The authors found that the aspartic acid at position 49 of the V_L domain considerably reduced aggregation rate of the antibody during long-term stability studies (from 35% to 0.2% per month).

9. Conclusion – towards stable human antibody therapeutics

The increasing number of human monoclonals in development and clinical practice, together with a trend towards high concentration formulations for subcutaneous delivery places increasing demands on the stability of human antibody therapeutics. While the thermodynamic stability of human antibodies is relatively well studied and can be further increased through homology, disulfide and computational approaches [71,83,47], determinants of their colloidal stability are just beginning to emerge due to a combination of rational and high-throughput approaches on phage [7,31,46,91]. In particular, the recent availability of human V_L domains with high levels of aggregation resistance [7] will greatly assist in the generation of paired antibody fragments and IgG. Further advances in the generation of human antibody therapeutics with high thermodynamic and colloidal stability will not only improve the production and formulation of human antibody therapeutics, but may also open up new administration routes and therapeutic applications.

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