General strategy for the generation of human antibody variable domains with increased aggregation resistance

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The availability of stable human antibody reagents would be of considerable advantage for research, diagnostic, and therapeutic applications. Unfortunately, antibody variable heavy and light domains (V_H and V_L) that mediate the interaction with antigen have the propensity to aggregate. Increasing their aggregation resistance in a general manner has proven to be a difficult and persistent problem, due to the high level of sequence diversity observed in human variable domains and the requirement to maintain antigen binding. Here we outline such an approach. By using phage display we identified specific positions that clustered in the antigen binding site (28, 30–33, 35 in V_H and 24, 49–53, 56 in V_L). Introduction of aspartate or glutamate at these positions endowed superior biophysical properties (non-aggregating, well-expressed, and heat-refoldable) onto domains derived from common human germline families (V_H3 and $V_\kappa1$). The effects of the mutations were highly positional and independent of sequence diversity at other positions. Moreover, crystal structures of mutant V_H and V_L domains revealed a surprising degree of structural conservation, indicating compatibility with V_H/V_L pairing and antigen binding. This allowed the retrofitting of existing binders, as highlighted by the development of robust high affinity antibody fragments derived from the breast cancer therapeutic Herceptin. Our results provide a general strategy for the generation of human antibody variable domains with increased aggregation resistance.

biotechnology | monoclonal antibodies | protein aggregation | protein engineering | antibody therapeutics

Protein aggregation represents a key bottleneck in the generation of antibody-based reagents and hinders the development and production of human therapeutics (1). It is generally believed that the aggregation propensity of larger antibody reagents (such as immunoglobulin G and Fab) is mostly determined by their variable domain components (V_H and V_L), although there is currently little understanding of the mechanisms involved (1, 2). Indeed, significant differences of aggregation rates have been reported for antibodies that differ exclusively in their variable domains (1, 2). Aggregation propensity is even more pronounced for smaller antibody reagents, which lack the interdomain stabilization of their larger counterparts (3, 4). This is a major problem in biotechnology due to an increasing trend toward smaller antibody formats for imaging and tumor targeting applications (4). Common formats include human single chain fragments (scFv) and human single domain antibodies, both of which frequently display poor biophysical properties (1, 3).

The aggregation propensity of human variable domains is in marked contrast to the variable heavy domains of camels and llamas, which are generally nonaggregating and soluble ("V_{HH} domains") (5–7). More favorable properties have also been described for "camelized" and other engineered human V_H model domains (8–12) but not for human V_L domains. Their properties have been attributed to extensive structural changes resulting in increased hydrophilicity of the V_H–V_L interface (8, 10, 12).

However, the relevance of observations in model V_H domains for the development of antibody reagents for real-world applications had so far remained unclear. In particular, high-affinity antigen binding has not been demonstrated (8, 11). Neither has pairing of the model domains with human V_L, a property also observed for camelid domains, which are naturally devoid of light chain partners (and for which analogous structural features in the former V_H–V_L interface have been reported) (8, 10–12). Moreover, human antibody variable domains are highly diverse and encompass multiple germline families for both heavy and light domains (13, 14). Even among variable domains derived from a single germline family considerable diversity is observed, predominantly within complementarity determining regions (CDR) (13–15). The high level of diversity affects aggregation propensity and biophysical properties of human variable domains. For instance, it has been demonstrated that both the choice of germline family and the composition of CDR3 have major influences on aggregation propensity (3). This is supported by reports from our laboratory indicating that preselection for aggregation-resistant V_H domains results in a reduction of CDR diversity by several orders of magnitude (16–18). As CDR3 mediates the majority of contacts with antigen (19), the question whether high affinity antigen binding and aggregation resistance are in fact mutually compatible had so far remained unanswered.

In this study we outline a general strategy for the generation of human antibody variable domains with increased aggregation resistance. We demonstrate its applicability to common human variable domain families and diverse antibody repertoires and present a report of human V_L domains with improved biophysical properties. Moreover, we present structural and mutational evidence demonstrating compatibility with high-affinity antigen binding.

Results

Phage Display Screen for Aggregation Preventing Mutations in Human Antibody Variable Domains. We utilized a high-throughput method

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Conflict of interest statement: K.D., R.R., and D.C. are named inventors on patents relating to this work and declare competing financial interests.

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Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 3UPC and 3UPA).

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on phage (20) to identify mutations that render human antibody variable domains resistant to aggregation. For our studies we chose variable domains that belong to families that are among the most common in the human repertoire (V_H3, $V_\kappa1$) (13, 14). These variable domain families are also prevalent among the 26 human monoclonal antibodies in clinical use and more than 350 currently in clinical trials (4, 21). Human V_H and V_L domains with amino acid substitutions at surface-exposed positions were displayed on phage. We targeted a wide range of positions while excluding CDR3 to limit effects on antigen binding. The domains were then heated and captured using protein A or L superantigens (which bind to folded but not to aggregated variable domains) (20, 22). Wild-type domains readily aggregated under such conditions (80 °C). However, this was not observed for several of the mutant domains, for which superantigen binding after heating was considerably improved (up to 40-fold for human V_H and 80-fold for human V_L) (Fig. 1 and Fig. S1). While we observed improvements when making both positively and negatively charged substitutions, by far the largest effect was observed for negatively charged substitutions (glutamate and, in particular, aspartate) (Fig. S2).

Mutations in the Antigen-Binding Site Control Aggregation Propensity of Common Human Variable Domain Families. Moreover, our experiments revealed that these effects were highly positional, highlighting specific sites that control aggregation resistance of the domains. In the case of human V_H such mutations clustered in CDR1 (H1), while mutations at other positions had little effect. Intriguingly, we discovered that this was not the case for human V_L: Here mutations strongly clustered in CDR2 (L2) (Fig. 1 *A* and *B*). In total we identified six positions in H1 (28, 30, 31, 32, 33, 35) and five positions in L2 (50, 51, 52, 53, 56) that had major effects on aggregation resistance (in addition to two non-CDR positions at 24 and 49 of V_L). In addition to highlighting the positional nature of mutations, our experiments also demonstrated that their effects were largely additive, with combinations of mutations providing the highest levels of aggregation resistance (Fig. 1*C*). To demonstrate that our findings were more generally applicable, we next investigated the effect of mutations in H1 and L2 on antibody repertoires. For this purpose, we constructed synthetic phage display repertoires mimicking the CDR amino acid diversity in the human V_H (V_H3) and V_L ($V_\kappa1$) repertoire (23). This revealed that the introduction of double aspartate substitutions into H1 of human V_H and L2 of human V_L significantly increased the mean aggregation resistance of the repertoires ($p < 0.001$) (Fig. 1*D*). We observed a dominant effect of mutations in H1 and L2: Thus, the observed aggregation resistance was largely independent of diversity at other CDR positions.

Human Variable Domains with Superior Biophysical Properties. Representative human variable domains were next expressed as soluble proteins to further assess their biophysical properties. As previously reported, human variable domains rapidly aggregate when heated to 80–85 °C, conditions well above their melting temperatures (20). In addition, they have a tendency to "stick" to gel filtration matrices, which hinders purification, and manifests itself through increased elution volumes (3, 20). Many human variable domains are also poorly expressed (3, 20). We observed that introduction of negatively charged substitutions (aspartate or glutamate) at H1 and L2 positions improved on all of the above properties. While unmodified human variable domains rapidly aggregated when subjected to heating, the mutant domains

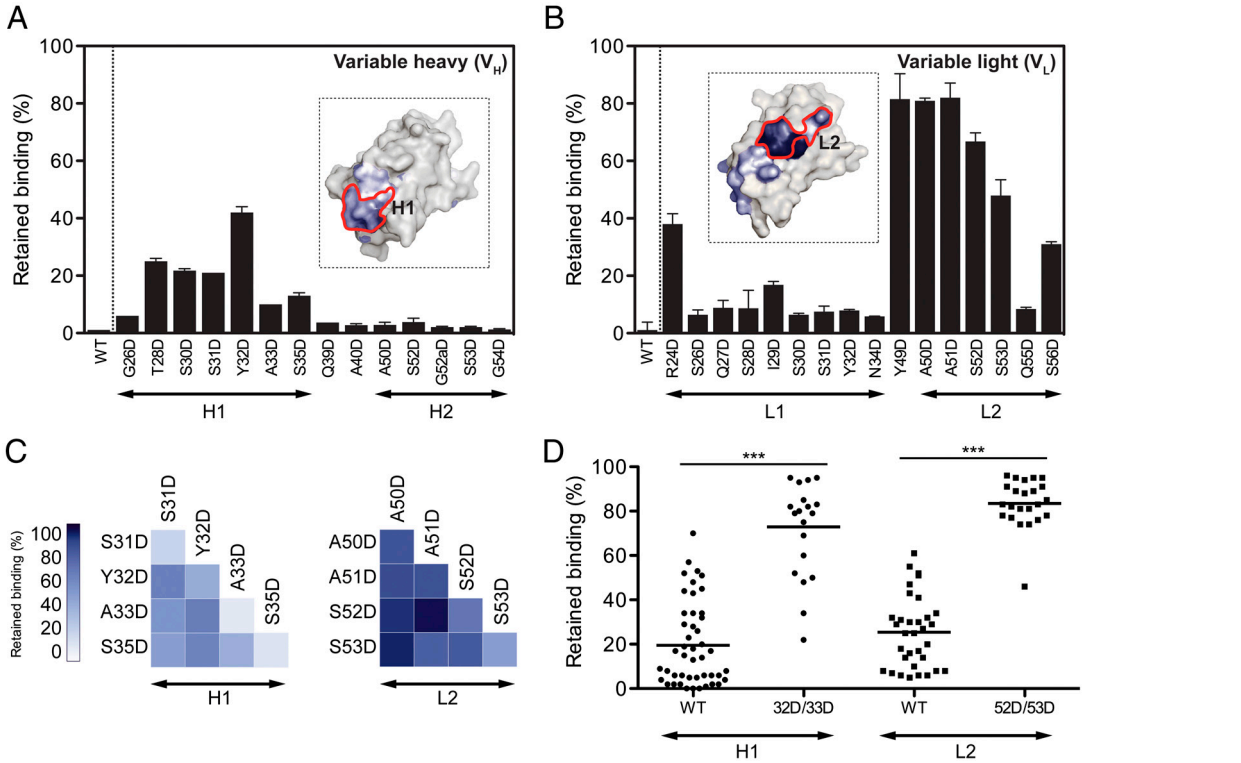


Fig. 1. Effect of mutations in human antibody variable domains on aggregation resistance. Surface residues in variable heavy and light domains (human V_H3, human $V_\kappa1$) are targeted for substitution with aspartic acid (aspartate). Aggregation resistance of the domains is determined by measuring retained binding to superantigen after heating to 80 °C on phage (20). Mutations are mapped on the variable domain surface structure (blue: 100% retained binding; white: 0%; wild type residue: WT; means, standard deviation (SD) shown, $n = 2$). Numbering according to Kabat (30). Complementarity determining regions are indicated as H1, H2 for V_H and L1, L2 for V_L. (*A*) Single mutations in human V_H. (*B*) Single mutations in human V_L. (*C*) Double mutations in human V_H (*Left*) and human V_L (*Right*). (*D*) Mutations in human V_H repertoire (*Left*) and human V_L repertoire (*Right*). Synthetic repertoires closely mimicking CDR amino acid diversity in the natural antibody repertoire were generated (23). Mutant repertoires carry mutations in H1 (32D/33D) and L2 (52D/53D). Graph shows mean aggregation resistance of repertoires (***, $p < 0.001$).

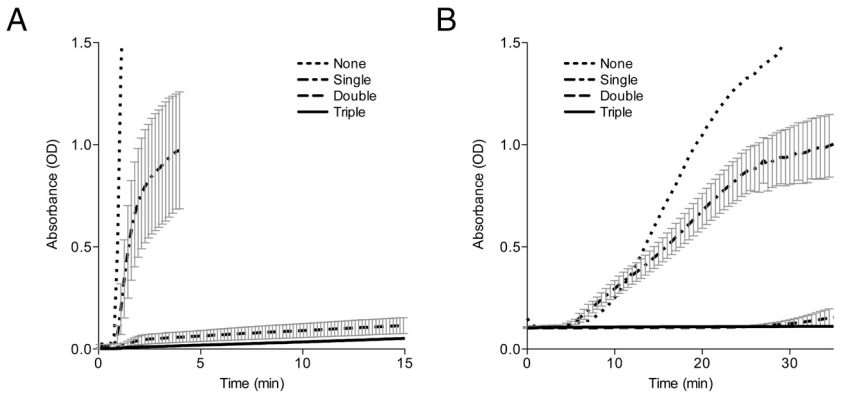


Fig. 2. Aggregation resistance of human variable domains. Representative human variable domains are targeted for substitution with aspartate/glutamate in CDR H1 (V_H) or L2 (V_L), expressed and purified (see *SI Text* for details). The graphs show sample turbidity after incubation at aggregation-promoting temperatures (as measured by absorbance at 360 nm; means, SD shown for single and double mutations, V_H: $n = 3$, V_L: $n = 4$). (*A*) Human V_H domains at 80 °C. (*B*) Human V_L domains at 85 °C.

resisted aggregation (Fig. 2). Other biophysical properties of the mutant domains were also greatly improved. This included reduced elution volumes on gel filtration and improved refolding yields (Table 1). The mutant domains also resisted aggregation when subjected to commonly used storage and concentration techniques, including freeze/drying and membrane filtration (Fig. S3). Moreover, soluble expression levels increased by more than 100% for V_L domains and 400% for V_H (Table 1). For all of the observed effects, we noticed a strong dependence on the number of mutations, with two or more mutations providing maximum aggregation resistance.

Structures of Mutant Human V_H and Human V_L Domains. To investigate the molecular basis of mutations in CDR H1 and L2 we determined crystal structures of triple mutants of human V_H and human V_L domains at 2.8-Å and 1.8-Å resolution (Fig. 3 *A* and *B* and Fig. S4 and Table S1). The overall structures of the mutant domains closely aligned with structures of variable domains from representative human antibodies (with backbone rmsds of 0.5 Å for V_H and 0.8 Å for V_L). Moreover, no structural changes were observed for either the V_H–V_L interface or mutated CDR regions, with conformations of H1 and L2 tightly superimposing onto the representative antibody variable domain structures (Fig. 3 *A* and *B*). Our results demonstrated that structural changes in the mutant variable domains were minor and exclusively limited to mutant side chains. The absence of conformational rearrangements also raised the possibility that the mutations may be compatible with V_H–V_L pairing and antigen binding when introduced into an existing monoclonal.

Table 1. Biophysical properties of human antibody variable domains

	Mutations			
	None	Single	Double	Triple
Expression (mg/L)				
V_H	1.7	4.7	13.3	11.0
V_L	47.0	78.6	103.7	104.6
Elution volume (mL)				
V_H	24.9	23.1	21.3	19.7
V_L	13.6	13.3	13.0	12.9
Heat refolding (%)				
V_H	4.0	69.3	82.3	88.0
V_L	53.6	72.5	67.1	91.4

Representative human variable domains are targeted for substitution with aspartate/glutamate in CDR H1 (V_H) or L2 (V_L), expressed and purified (see *SI Text* for details) (means shown for single and double mutations, V_H: $n = 3$, V_L: $n = 4$). Expression levels are determined by superantigen capture from culture supernatant. Elution volumes are determined by size-exclusion chromatography. For heat refolding, samples are incubated for 10 min at 80 °C (V_H) or 20 min at 85 °C (V_L) and analyzed by size-exclusion chromatography.

Retrofitting of Variable Domains. To further investigate the effect of the identified mutations on the antibody–antigen interaction, we decided to study variants of Trastuzumab (Herceptin), an IgG therapeutic monoclonal antibody directed against human epidermal growth factor receptor 2 (HER2) (24). Single and double aspartate substitutions were introduced into H1 and/or L2 and variants expressed in a human IgG1 format (see *SI Text* for details). We then analyzed the effects of the substitutions on biological activity. Experiments using the HER2-expressing breast cancer cell line SK-BR-3 revealed that the variants were highly active, with only minor differences in cellular binding and inhibition of proliferation observed (Fig. 4 *A* and *B*). Furthermore, no differences were detected for serum clearance when injected into animals at doses relevant to human therapy (Fig. 4*C*). We next investigated if H1 and/or L2 mutations were also compatible with antigen binding in an antibody fragment format. While isolated variable domains displayed little binding to antigen, binding activity could be reconstituted by pairing the domains in an scFv format (Fig. S5). This allowed us to measure the binding of scFv variants for recombinant HER2 by surface plasmon resonance (Fig. 5*A*). Some of the variants lost much of their affinity for HER2; these generally carried mutations at positions reported to directly interact with antigen (25). However, changes at other positions were well tolerated, with no apparent loss of equilibrium binding affinity (K_D). Moreover, no loss of binding was observed when combining single changes at tolerated H1 and L2 positions. In particular, one of the scFv double mutants (30D/52D) bound to HER2 with wild-type-like affinity (Fig. 5*A*).

To examine resistance against aggregation, 30D/52D and other variants were next heated to 80–85 °C at high protein concentration (10–100 μM). As we had observed for germline variable domains, aggregation resistance improved considerably as the number of mutations increased: While the wild-type scFv fragment readily aggregated at the above conditions, the 30D/52D double mutant resisted aggregation, with single mutations providing intermediate effects (Fig. 5*B*). Indeed, simple visual inspection of protein solutions after heating readily revealed considerable improvements of aggregation resistance for single domains as well as when combined in an scFv format (Fig. 5*C*).

Discussion

Taken together, our experiments demonstrate that aggregation resistant human antibody variable domains can be generated through introduction of charge in the antigen binding site. Previous studies

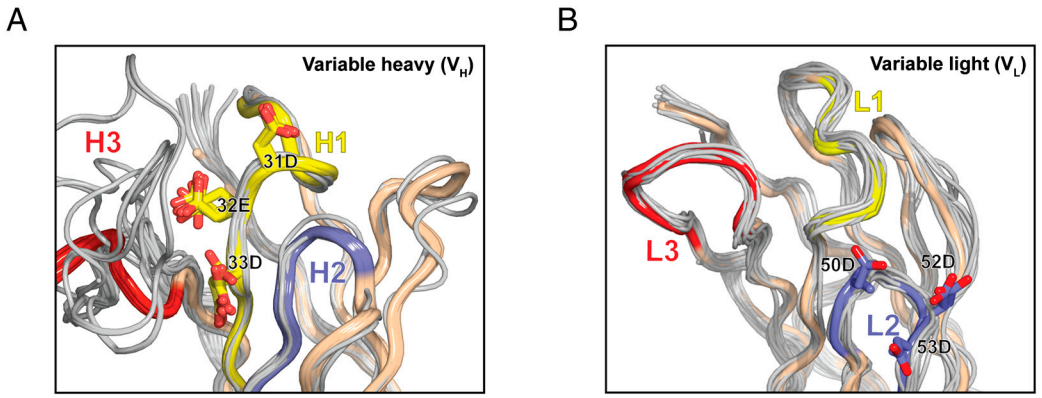


Fig. 3. Crystal structures of mutant human variable domains: structure of triple mutants of (*A*) human V_H and (*B*) human V_L (in tan). CDR regions are shown in yellow/blue/red. Mutant residues are highlighted (sticks). The structures of the mutant domains tightly superpose onto structures of representative human variable domains [with the exception of H3 which is conformationally diverse in antibodies (40); representative structures shown in gray].

had indicated that the introduction of a large number (30–80) of charged substitutions at nonspecific surface positions can generate robust "supercharged" proteins (such as supercharged green fluorescent protein) (26). In marked contrast, the approach described here requires the introduction of only a handful of substitutions (two or more) at highly specific positions (28, 30, 31, 32, 33, 35 in V_H and 24, 49, 50, 51, 52, 53, 56 in V_L). Unlike supercharged proteins, many of the aggregation-resistant mutant variable domains carry no global net charge, highlighting the importance of localized charge (Fig. S4). We also observe a detectable preference for aspartate over glutamate and a strong preference for aspartate over lysine or arginine (Fig. S2). This indicates that properties other than charge also influence the observed aggregation resistance. While detailed mechanisms remain unclear, a preference for small negatively charged amino acids is apparent. The positional nature of our approach is further highlighted by the differences observed for human V_H and V_L with mutations clustering in CDR1 (H1) and CDR2 (L2), respectively (Fig. 1 *A* and *B*). In contrast to human V_H, mutations in CDR1 have little effect on human V_L despite the fact that the domains are structurally closely related immunoglobulin folds.

The mutations reported here endow aggregation resistance onto domains derived from common germline families (13, 14), with V_H3 and $V_\kappa1$ representing approximately 40% and 32% of the rearranged repertoire (23). Importantly, we find that the effects of mutations in CDR H1 and L2 are not significantly influenced by diversity at other CDR positions (Fig. 1*D*). This is despite the fact that the expressed human variable domain repertoire is highly diverse (15). This is reflected by the synthetic repertoires studied here, which are extensively randomized at between 9 ($V_\kappa1$) and 14 CDR positions (V_H3). The synthetic variable domain repertories are designed to reflect natural sequence diversity within the human $V_\kappa1$ and V_H3 repertoire. Thus, we utilize preferentially rearranged human gene segments (V3-23/DP47, O12/O2/DPK9) (27, 28) and use trinucleotide phosphoramidite mutagenesis to match CDR amino acid distributions (29) (see *SI Text* for details). An alternative approach to repertoire diversity relies on the use of "consensus" sequences (rather than single common gene segments) (23). This strategy was used in the development of the Herceptin (V_H3 and $V_\kappa1$) variable domains (24) and is also clearly compatible with the mutational strategy outlined here (Fig. 3).

It is important to note that multiple mutations (two or more) are required to obtain considerable improvements of aggregation resistance in common biophysical assays (Fig. 2). The requirement for multiple positional substitutions may explain why effects on commercially important and widely studied immunoglobulin families had so far remained unnoticed. It also renders it unlikely that such mutations could be observed by chance within the natural repertoire. Thus, multiple aspartates or glutamates are not common at identified positions within the human V_H3 and $V_\kappa1$ germline repertoire and are notably absent from frequently utilized gene segments (such as V3–23) (27, 28). They are also not common in rearranged human antibody sequences (30), although exceptions exist [such as the Adalimumab (Humira) V_H domain] (31).

Our approach provides favorable characteristics among a range of biophysical properties. This includes considerable increases in expression yields, improved concentration, and purification. It also endows human variable domains with the capability

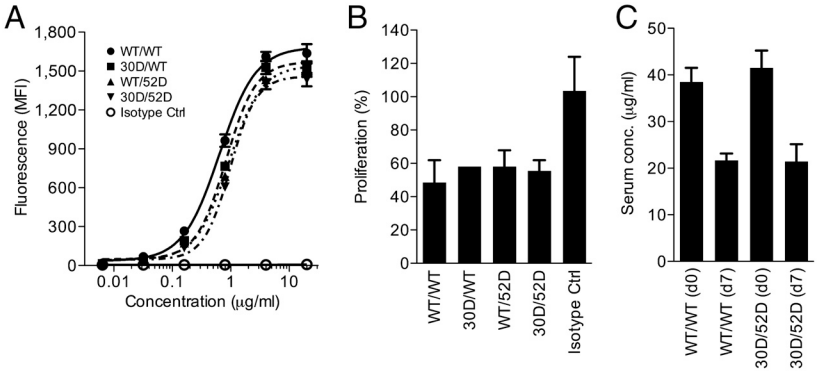
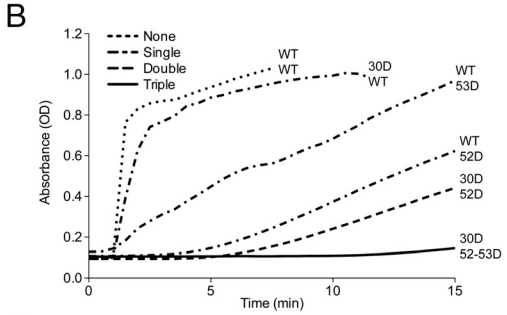


Fig. 4. Retrofitting of human variable domains (I): effects on biological activity of IgG. Variants of Trastuzumab (Herceptin) are generated by introduction of aspartate substitutions in CDR H1 and/or L2. Means, SD shown. (*A*) Binding to HER2 antigen on cells. The graph shows binding of IgG to SK-BR-3 breast cancer cells ($n = 2$). (*B*) Inhibition of cellular proliferation (as determined by incubation of SK-BR-3 cells with IgG; $n = 2$). (*C*) Serum concentrations (after intra-peritoneal injection of IgG into C57/BL6 mice at 1 mg/kg, $n = 4$).

A

Mutations		HER2 affinity		
H1	L2	k_a (10^5 M^{-1} s^{-1})	k_d (10^{-4} s^{-1})	K_D (nM)
-	-	1.3	4.9	3.8
30D	-	2.4	4.9	2.3
-	52D	1.2	9.1	7.6
-	53D	1.5	175	118
30D	52D	2.1	8.6	4.1
30D	52D-53D	0.4	192	472



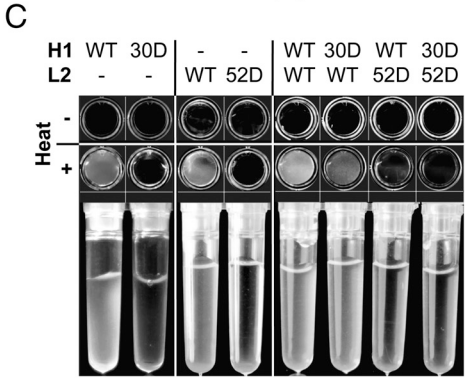


Fig. 5. Retrofitting of human variable domains (II): effects on antibody fragments. Variants of Trastuzumab (Herceptin) are generated by introduction of aspartate substitutions in CDR H1 and/or L2. (*A*) Affinity of mutants for recombinant HER2 (as determined by surface plasmon resonance using scFv fragments). (*B*) Aggregation resistance of mutants. The graph shows sample turbidity of scFv fragments at 85 °C (as measured by absorbance at 360 nm). (*C*) Aggregation resistance (visual appearance) before (–) and after (+) heating to 85 °C. Panel shows V_H domains (*Left*), V_L domains (*Center*), and paired as scFv fragments (*Right*).

to refold after heat-induced unfolding. In this respect, the mutant domains closely resemble camelid (V_{HH}) variable domains, which display this otherwise uncommon behavior (5). Differences in CDR conformation and the structure of the V_H–V_L interface have been shown to underpin the observed biophysical differences between human V_H and camelid domains (7). Similar features have been described for camelized human V_H domains carrying nonhuman framework mutations and other human model V_H domains (such as HEL4) (8, 12). V_{HH}s also generally display higher thermodynamic stabilities (6); however, this is not observed for the mutant human domains (Table S2). The crystal structures of the mutant human domains reported here further highlight differences with V_{HH}, with the domains fully retaining human CDR conformations. Previously reported structural features of human model V_H domains centered on hydrophobic framework residues and rearrangement of the V_H–V_L interface are also noticeably absent from the structures (Fig. S7) (7, 8, 10–12, 32).

The observation that the identified mutations in human variable domains result in neither structural change nor increased thermodynamic stability indicates that these parameters are unlikely to be a cause for the observed aggregation resistance. However, it should be noted that the variable domains utilized in this study belong to families (V_H3, $V_\kappa1$) that are not only among the most common but also among the more stable within the human repertoire (but less stable than camelid domains) (3, 6). It does not exclude the possibility that some of the less stable human families may require additional stabilization (such as V_H2) (3, 32). In contrast, the mutant variable domains described in this study are actually moderately less stable than the domains from which they were derived (Table S2; ΔG_{N-U}: 27–40 kJ mol^{-1} for V_H and 24–30 kJ mol^{-1} for V_L). This is in agreement with observations reported for model V_H domains (10, 11) as well as for other proteins (33). Indeed, it has been suggested that mutations that reduce protein aggregation frequently do not increase thermodynamic stability but rather act on aggregation-prone unfolded or partially unfolded states (20, 33).

The absence of structural changes in human V_H and human V_L also suggests that the identified mutations are likely to be compatible with V_H–V_L pairing and antigen binding when introduced into existing binders. It does not exclude the possibility of direct disruption of side-chain interactions with antigen, which has to be empirically determined for each binder. However, the positions highlighted in this study fall predominantly into CDR H1 and L2 regions, which are mostly peripheral to the antibody–antigen interaction, rendering such disruptions less likely (15). This is in contrast to CDR H3 and L3, which mediate the majority of interactions with antigen (19, 34). It should also be noted that the approach outlined here provides a considerable degree of flexibility because it identified a large set of positions in both human chains (six in V_H and seven in V_L). Both aspartate and glutamate can be introduced at these positions, although we observe a detectable preference for the former (Fig. S2). In addition, the required number of changes is modest, with two or more mutations providing a high degree of aggregation resistance (Fig. 2). The broad applicability of our approach is further exemplified by the work on Herceptin variants for which tolerated mutations in CDR H1/L2 could be readily identified (Figs. 4 and 5). This is not unexpected because it has been demonstrated that the Herceptin-HER2 interaction is dominated by a small number of residues providing large contributions to binding energy (35). These residues cluster predominantly in CDR H3/L3 (35) and contact the C-terminal portion of domain IV of HER2 (36).

While we were preparing this paper, two publications have appeared in print that further highlight the role of charge in antibody aggregation. Perchiacca et al. describe a set of mutants of the model V_H HEL4 (11). In particular, they report effects of a "triad" of negatively charged surface residues in HEL4 (but do not attribute effects to single or double mutations). This triad has previously been described (9) and is in agreement with the results outlined here. The authors do not provide evidence on antigen binding, repertoire diversity, or V_H–V_L pairing, making their findings difficult to assess. Schaefer and Pluckthun outline a strategy of fusing a negatively charged peptide to the N terminus of human antibodies to increase aggregation resistance (37). While their approach is clearly compatible with antigen binding, the observed effect is not positional and can be attributed to increased net charge of the molecule (37). It may, however, provide synergies with the strategy outlined here.

Although the role of mutations in the antigen binding site on aggregation propensity had so far remained elusive, the effect of such mutations on other antibody properties are well understood. In particular, it has been demonstrated that the binding activity of one antibody can be transferred onto a different antibody exclusively through mutations in the antigen-binding site ("CDR grafting") (24, 34). The majority of monoclonal antibodies currently in clinical practice have been generated by CDR grafting approaches (38). Other monoclonals have been generated through the introduction of synthetic, oligonucleotide-derived diversity in CDR regions (38). An initial concern relating to CDR grafting/mutagenesis strategies, including the approach outlined here, had been the generation of potential new B- and T-cell epitopes (39). However, with a large number of monoclonals in

clinical practice it has become evident that CDR mutations cause little or no immunogenicity in humans (39). In contrast, there is increasing evidence that the absence of aggregates is essential for the development of antibody therapeutics with low immunogenicity (1, 2). The method outlined here is therefore highly compatible with current antibody development strategies. This is exemplified by our work on variants of the antibody therapeutic Herceptin and highlights the potential of our method for "retrofitting" approaches.

Conclusion

The availability of antibody reagents with improved aggregation resistance would be a significant advantage for research, diagnostic, and therapeutic applications. Consequently, the discovery of stable and nonaggregating V_H domains from camels and llamas has generated considerable interest and development efforts in recent years (4, 5). While such domains hold promise as robust and modular building blocks, their lack of light-chain partners hinders the generation of multidomain antibody reagents; their nonhuman nature also complicates their use as therapeutics. In contrast, a general means for the development of robust human antibody domains had so far remained elusive. We conclude that our approach provides such a means for the generation of human antibody variable domains with increased aggregation resistance.

Methods

Generation of mutant variable domains and repertoires, analysis of aggregation resistance on phage, protein expression and purification, expression levels, elution volumes, refolding yields and turbidity measurements, crystal growth, structure solution, refinement and analysis, determination of thermodynamic stabilities, affinity measurements, cellular binding, inhibition of proliferation, and serum clearance are described in detail in *SI Text*.

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