Solubility, Stability, and Avidity of Recombinant Antibody Fragments Expressed in Microorganisms

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Solubility of recombinant proteins (i.e., the extent of soluble versus insoluble expression in heterogeneous hosts) is the first checkpoint criterion for determining recombinant protein quality. However, even soluble proteins often fail to represent functional activity because of the involvement of non-functional, misfolded, soluble aggregates, which compromise recombinant protein quality. Therefore, screening of solubility and folding competence is crucial for improving the quality of recombinant proteins, especially for therapeutic applications. The issue is often highlighted especially in bacterial recombinant hosts, since bacterial cytoplasm does not provide an optimal environment for the folding of target proteins of mammalian origin. Antibody fragments, such as single-chain variable fragment (scFv), single-chain antibody (scAb), and fragment antigen binding (Fab), have been utilized for numerous applications such as diagnostics, research reagents, or therapeutics. Antibody fragments can be efficiently expressed in microorganisms so that they offer several advantages for diagnostic applications such as low cost and high yield. However, scFv and scAb fragments have generally lower stability to thermal stress than full-length antibodies, necessitating a judicious combination of designer antibodies, and bacterial hosts harnessed with robust chaperone function. In this review, we discuss efforts on not only the production of antibodies or antibody fragments in microorganisms but also scFv stabilization via (i) directed evolution of variants with increased stability using display systems, (ii) stabilization of the interface between variable regions of heavy (V_H) and light (V_L) chains through the introduction of a non-native covalent bond between the two chains, (iii) rational engineering of V_H-V_L pair, based on the structure, and (iv) computational approaches. We also review recent advances in stability design, increase in avidity by multimerization, and maintaining the functional competence of chimeric proteins prompted by various types of chaperones.

Keywords: antibody fragments, solubility, stability, bacterial expression, scFv

INTRODUCTION

Antibodies are widely used for medical applications such as disease diagnosis and therapy (Grilo and Mantalaris, 2019). Valuable pharmaceutical properties of antibodies such as high affinity to their target molecules have led to them becoming constituted as key materials not only in antibody-based biosensors, which offer the promise of in-depth target detection capacity...
and light chains of the whole IgG molecule (Feige et al., 2009). Hydrophobic interactions contribute to the stability as a whole as well as the absence of flanking C\(_L\) and C\(_H\) sequences, such as outer membrane protein A (OmpA), or new lipoprotein A (NlpA) (Tseng et al., 2009), but also can easily be produced in prokaryotes, such as Escherichia coli (E. coli) or Brevibacillus choshinensis (B. choshinensis) (Hu et al., 2017), along with high yield, which keeps the cost of production low (Power and Hudson, 2000; Terpe, 2006; Rosano and Ceccarelli, 2014; Gupta and Shukla, 2017). Despite the advantages of scFvs, they have a few drawbacks that limit their therapeutic potential, such as (i) deteriorated stability because of their propensity to readily aggregate under thermal stress (Jager and Pluckthun, 1999a; Demarest and Glaser, 2008); (ii) a short serum half-life of <1 day compared to 3 weeks for full-length immunoglobulin G (IgG)\(_1\), IgG\(_2\), and IgG\(_4\) antibodies (Kang and Jung, 2020); and (iii) reduced affinity compared to the full-length antibody counterpart. Therefore, scFv format is suitable for limited cases, such as macular degeneration or blood-related diseases (Table 1).

Immunoglobulin G, the most abundant monoclonal antibody (mAb) isotype in serum is composed of two antigen binding Fab and one homodimeric fragment crystallizable (Fc) domain that contribute to the overall stability of the molecule (Figure 1; Kang and Jung, 2019; Saunders, 2019). Since Fab of an IgG becomes more sensitive to the heat denaturation when Fc region is removed (Tischenko et al., 1982; Vermeer and Norde, 2000; Ionescu et al., 2008), researchers have tried to engineer Fab to stabilize the interactions between constant heavy 1 (C\(_H\)\(_1\)) and constant light (C\(_L\)) chains in order to obviate the need for using mammalian host cells for the expression of full-length antibodies because of N-glycan on the Fc region. This requires immense resources such as expensive media, facilities to maintain germfree conditions, and time. However, limited successes have been made (Demarest et al., 2006; Teerinen et al., 2006). Further elimination of C\(_H\)\(_1\)-C\(_L\) pair in Fab, resulting in fragment variable (Fv), significantly discounts thermodynamic stability (Webber et al., 1995; Jager and Pluckthun, 1999b). This is presumably due to the unnatural exposure of the lower V\(_L\) and V\(_H\) regions, flanking C\(_H\)\(_1\) and C\(_L\), where hydrophobic interaction used to contribute to the stability as a whole as well as the absence of the contribution of C\(_H\)\(_1\), which controls the assembly of heavy and light chains of the whole IgG molecule (Feige et al., 2009). The only light-heavy intermolecular disulfide bond in native IgG antibodies on the residues Cys220 in C\(_H\)\(_1\) and Cys214 in C\(_L\) of Fab region (Figure 2 in canakinumab; PDB ID of 5BVJ) contributes to the thermodynamic stability of the whole Fab fragment. In addition, intramolecular disulfide bonds in both the V\(_H\) and V\(_L\) regions (Figure 2) is critical in the thermodynamic stability because elimination of them significantly enhanced the propensity of scFv aggregation (Montoliu-Gaya et al., 2017). In this article we review efforts on increasing expression yield as well as protein stability of antibody fragment and recent diverse designs of antibody fragments.

**PRODUCTION OF ANTIBODY OR ANTIBODY FRAGMENTS IN BACTERIA**

To reduce the cost of production of antibodies, researchers in both academia and industry put enormous efforts on elevating expression yield of IgG antibody or its fragment by (i) engineering expression plasmids, i.e., rhamnose-inducible expression system (Petrus et al., 2019) or comprehensive optimization via high-throughput screening (Makino et al., 2011), (ii) engineering global sigma factor RhoD, which regulates more than 1,000 gene expressions (McKenna et al., 2019), and (iii) devising bacterial strains capable of forming disulfide bonds in cytoplasm such as CyDisCo (Gaciarz and Ruddock, 2017) or SHuffle (Lobstein et al., 2012; Robinson et al., 2015; Yusakul et al., 2017). Despite the advantages, drawbacks limiting its potential are (i) the low stability of scFvs, known for their propensity to readily aggregate under thermal stress (Jager and Pluckthun, 1999a; Demarest and Glaser, 2008), (ii) absence of glycosylation machinery, (iii) lack of efficient secretory mechanism as compared to yeast or animal cells, functionally limited protein trafficking machinery from the cytoplasm to the periplasmic space or to the outside of the cells, and (iv) overproduction of acetic acid byproduct during fermentation (Holms, 1986; Wong et al., 2008).

**ENGINEERING INTRINSIC STABILITY OF SCFVS: DIRECTED EVOLUTION, RATIONAL DESIGN, AND COMPUTATIONAL APPROACHES**

Antibody fragments can be expressed in several compartments in E. coli: mostly as inclusion bodies in the cytoplasm, or as soluble forms displayed on (i) the inner membrane, (ii) in the periplasmic space, (iii) on the outer membrane, and/or (iv) outside the bacterium, facilitated by various signal sequences, such as outer membrane protein A (OmpA), pectate lyase B (PelB), or new lipoprotein A (NlpA) (Tseng et al., 2009; Frenzel et al., 2013; Khodabakhsh et al., 2013; Levy et al., 2013; DePalma, 2014; Mizukami et al., 2018). To overcome the drawbacks of scFvs, which comprise only V\(_H\) and V\(_L\) antigen-binding domains, to reduce the protein size in order to increase protein production but maintain high target molecule affinity, researchers have engineered...
scFvs with resistance to aggregation and enhanced intrinsic stability of antibody fragments. Table 2 summarizes the engineering efforts.

### Directed Evolution

Greg Winter et al., utilized phage display directed evolution methodology to isolate V<sub>H</sub> variants that are more resistant to heat denaturation (Jespers et al., 2004). They further engineered V<sub>H</sub> and identified a key residue, Arg28 in V<sub>H</sub> that renders resistance to heat and acid aggregation (Famm et al., 2008). Daniel Christ’s group at the Garvan Institute of Medical Research selected critical residues for antigen binding in both V<sub>H</sub> and V<sub>L</sub> and constructed a phage library introducing aspartate or glutamate in those residues to screen for heat resistance. The isolated variants resulted in not only enhanced biophysical property but also structural conservation (Dudgeon et al., 2012). Dane Wittrup’s group at MIT devised a yeast surface display system to isolate scFv variants with high affinity to antigen and increased stability by constructing yeast mutant libraries, expressing scFv on the cell surface, followed by successive rounds of flow cytometry sorting (Graff et al., 2004; Chao et al., 2006). Brian Miller et al., at Biogen Idec, Inc. used sequence- and structure-based analyses to devise a high-throughput screening methodology that measure scFv extracellularly expressed by E. coli. This screening methodology resulted in enhanced melting temperature (Tm) by 14°C and additional Tm improvement by 12°C through combination of the resulting variants (Miller et al., 2010).

### Rational Design

Unlike the presence of intermolecular C<sub>H1</sub>-C<sub>L</sub> disulfide bond, there is not one in the native V<sub>H</sub>-V<sub>L</sub> (Figures 1, 2). Instead of placing a linker between V<sub>H</sub> and V<sub>L</sub> (scFv in Figure 1) creating non-native disulfide bond between V<sub>H</sub> and V<sub>L</sub> via substituting amino acid residues in both framework 2 (FR2) in V<sub>H</sub> and FR4 in V<sub>L</sub> (dsFv; disulfide-stabilized Fv in Figure 1) led to indistinguishable specificity to antigen and similar cytotoxic activity when fused with exotoxin but exhibited superior protein stability at 37°C, compared to scFv counterpart (Brinkmann et al., 1993). Similarly, substitution of Val84 in V<sub>H</sub> to aspartic acid led to not only improved periplasmic production by 25-fold but also decreased the rate of thermally induced aggregation reaction (Nieba et al., 1997). In another study, introduction of Cys44 in V<sub>H</sub> and Cys100 in V<sub>L</sub> in
anti-aflatoxin B(1) scFv resulted in improved stability and resistance to protein aggregation (Zhao et al., 2010). Introduction of the disulfide bond to anti-FGF2 diabody (ds-diabody, see section “multimerization” for diabody) also improved biological activity (Cai et al., 2016). This is presumably due to its lower propensity to the open state of $V_H$-$V_L$ pair, in contrast to the presence of both assembled and disassembled state in case of scFv where $V_H$ and $V_L$ domains are simply connected by a G$_4$S linker. These results indicate that bridging $V_H$ and $V_L$ by establishing intermolecular disulfide bond formation via cysteine residue incorporation can be a decent strategy for Fv stabilization (Trivedi et al., 2009). Another recent approach incorporating closed state of $V_H$-$V_L$ pair is a cyclization of scFv using an enzyme sortase A, which ligate the pair, leading to cyclic scFv: this methodology markedly suppressed aggregation tendency without affecting affinity to antigen (Yamauchi et al., 2019).

Alexey Lugovskoy et al., at Merrimack Pharmaceuticals, Inc. showed that both essential and non-essential tyrosine residues for antigen binding in either CDR or FR can improve the biophysical property of scFv (Zhang et al., 2015). An-Suei Yang et al., at the National Defense Medical Center, Taipei, elucidated the nature of intra- and inter-hydrophobic domains of scFv: the former is flexible and indirectly affects antigen binding, as opposed to the latter affecting antigen binding directly (Hsu et al., 2014). Robin Curtis’s group at the University of Manchester investigated the aggregation propensity of arginine-rich scFv under denaturing condition: substitution of arginine residues in scFv with lysine significantly reduced aggregation (Austerberry et al., 2019). This diverse knowledge of protein nature in scFv may assist engineers with consensus-based design of antibody fragment for generating stabilizing mutations to pre-existing scFvs (Steipe, 2004) or bispecific antibody fragments (Jordan et al., 2009).

### Computational Approaches

Andreas Plückthun’s group at University of Zurich designed a stabilized scFv from human $V_H$ germline sequences by analyzing hydrophobic core, pairing of hydrogen bonds, clusters of charge, and packing of β-sheets, leading to reduction of $\Delta G = 20.9$ kJ/mol as well as improvement of scFv expression yield by 4-fold (Ewert et al., 2003). Furthermore, they could stabilize scFv by CDR grafting to more stable framework, using a structure-based analysis (Ewert et al., 2004).

Computational web servers, such as Prediction of Immunoglobulin Structure (PIGS) (Marcatili et al., 2008) or Web Antibody Modeling (WAM) (Whitelegg and Rees, 2000), made computational modeling of antibody variable regions possible. Importantly, recent advances in structural knowledge and computational protein modeling such as RosettaDesign accelerated antibody design toward improved antigen affinity as well as physicochemical properties (Borgo and Havranek, 2012; Buck et al., 2012). The homology modeling provides with guidance on not only prediction on the conformations of CDR loops but also $V_H$-$V_L$ orientations via energy calculations such as antibody-antigen docking, comparing with known crystal structures (Kuroda et al., 2012). For example, a computational homology modeling significantly improved resistance of scFvs to heat inactivation.

### Table 1: FDA approved therapeutic antibody fragments.

| Name                     | Brand name    | Company                  | Target       | Format        | Indication                                                                 | Year approved | Host            |
|--------------------------|---------------|--------------------------|--------------|---------------|-----------------------------------------------------------------------------|---------------|-----------------|
| Abciximab Reopro         | Janssen Biotech, Inc. | GPIib/IIa                | Chimeric IgG1 Fab | Prevention of blood clots in angioplasty                                   | 1994          | Mammalian       |
| Ranibizumab Lucentis     | Genentech, Inc. | VEGF                     | Humanized IgG1 Fab | Macular degeneration                                                        | 2006          | E. coli         |
| Certolizumab pegol Cimzia | UCB, Inc.    | TNF                      | Humanized Fab, pegylated | Crohn disease                                                              | 2008          | E. coli         |
| Blinatumomab Blynico     | Amgen Inc.    | CD19, CD3                | Murine bispecific tandem scFv | Acute lymphoblastic leukemia                                               | 2014          | E. coli         |
| Idarucizumab Praxbind    | Boehringer Ingelheim Pharmaceuticals, Inc. | Dabigatran | Humanized Fab | Reversal of dabigatran-induced anticoagulation                             | 2015          | CHO             |
| Moxetumomab pasudoto Lumoxiti | AstraZeneca Pharmaceuticals LP | CD22                | Murine IgG1 dsFv | Hairy cell leukemia                                                        | 2018          | E. coli         |
| Caplacizumab Cabilivi    | Ablynx N.V.   | von Willebrand factor | Humanized Nanobody | Acquired thrombotic thrombocytopenic purpura                              | 2019          | E. coli         |
| Brolucizumab Beovu       | Novartis Pharmaceuticals Corporation | VEGF-A            | Humanized scFv | Neovascular age-related macular degeneration                               | 2019          | E. coli         |

1 Name: International non-proprietary names. 2 Brand name: commercial names. 3 Company: companies developed the antibody fragment drugs. 4 Indication: indication first approved by the FDA. 5 Year approved: the first year approved by the FDA. 6 Host: expression cell-lines. 7 CHO: Chinese Hamster Ovary cell-line. This table was classified using data from "The Antibody Society (2020)" (Antibody Society, 2020).
by supercharging the protein through energy calculations (Lawrence et al., 2007; Miklos et al., 2012). In addition, combinatorial engineering approach, including CDR grafting onto stable frameworks, VH-VL interface stabilization, and in vitro somatic hypermutation significantly increased thermal stability of full-length antibody by 10°C, compared to the native IgG1 antibody (McConnell et al., 2013, 2014).

Recent advances in computational methodologies for both antibody sequencing and backbones (Goldenzweig and Fleishman, 2018) and for multistage processing of antibody engineering, capitalizing on computational design and experimental validation cycles (Baran et al., 2017), have enabled successful de novo antibody engineering (Chevalier et al., 2017), such as anti-influenza antibodies (Strauch et al., 2017; Sevy et al., 2019). Of note, Georgiou and Ellington at the University of Texas at Austin used the Rosetta modeling program (Sircar et al., 2009; Adolf-Bryfogle et al., 2018) to predict amino acid substitutions for anti-HA33 scFv stabilization and confirmed a melting temperature increase of 4.5°C by antigen-binding enzyme-linked immunosorbent assay (ELISA) after thermal stress for 2 h at 70°C (Lee et al., 2019).

A FUSION PARTNER

Tagging of anti-FGFR3 scFv with a solubilizing partner small ubiquitin-related modifier (Sumo), followed by removal of the Sumo protein using Sumo protease, enabled over 95% purity with the yield of 4 mg/L bacterial culture. The resulting anti-FGFR3 scFv has exhibited complete biological activity (Liu et al., 2015). Another example is an "Fv-clasp," where scFv was fused with anti-parallel coiled coil structure, SRAH domain of human Mst1 with scFv. In addition, introduction of disulfide bond to the Fv greatly enhanced thermal stability and tendency of crystallization. This is presumably due to the shielding of hydrophobic residues exposed in Fv, according to the X-ray crystallography (Arimori et al., 2017). Alternatively, approaches
Based on the chaperone function of RNAs could also be considered (Choi et al., 2008). Fusion with an RNA-interaction domain (RID) greatly enhances the solubility (i.e., the ratio of soluble versus insoluble expression in heterogeneous hosts) and the overall yield of soluble proteins, harnessed with unique properties of RNAs as chaperone (chaperna; chaperone + RNA) (Kim et al., 2018; Yang et al., 2018), although this approach has not yet been documented for recombinant antibody fragments.

**ENGINEERING THE HOST CELL: CHAPERONE COEXPRESSION OR GENOME-LEVEL SCREENING**

The folding of nascent polypeptides is often assisted by molecular chaperones (Hendrick and Hartl, 1995; Bukau and Horwich, 1998; Hartl and Hayer-Hartl, 2002), although their utility in recombinant expression has been documented only in limited cases (Structural Genomics Consortium et al., 2008). The stability problem associated with $\text{V}_H$-$\text{V}_L$ pair in the scFv molecule can be circumvented through assistance with the chaperone of a pairing vehicle. Coexpression of the chaperone Skp enhanced binding activity of anti-TLH scFv by 3–4 fold, relative to the native counterpart, expressed in *E. coli* (Wang et al., 2013).

**MULTIMERIZATION**

Diabodies not only render bivalency but also enhance stability of Fv by linker design (Le Gall et al., 2004). Introducing covalent bonds between $\text{V}_H1$ and $\text{V}_L2$ as well as $\text{V}_H2$ and $\text{V}_L1$ enables bispecific binding capacity of the two distinct scFv in one molecule (diabody in Figure 1). One example in clinic is blinatumomab (BLINCYTO, $^\circledR$2014), a bispecific scFv for CD19 and CD3 (Table 1), which functions as a T lymphocyte engager to cancerous B lymphocytes for the treatment of acute lymphoblastic leukemia (Holliger et al., 1993; Mack et al., 1995; Suresh et al., 2014; Wu et al., 2015). In addition, constructing anti-HER3 trivalent scFv using SpyCatcher ligase system enhanced affinity by 12-fold as compared to a monomeric anti-HER3 counterpart (Alam et al., 2018). Another general approach of antibody fragments is the utilization of targeting ligands on nanoparticles in nanomedicine (Richards et al., 2017). Several antibody fragment-based nanoparticles are under clinical trials, including Erbitux-EDVSpac, which is a bacteria-derived mini-cell nanoparticle targeting EGFR currently under clinical phase II (Richards et al., 2017). Multimerization of scFvs as nanoparticles, using self-assembling scaffolds via chaperina approach (Kim et al., 2018; Yang et al., 2018) holds promise for further enhancing the avidity and thermostability of recombinant antibody fragments.

### Table 2

Summary of stability and avidity engineering of scFvs in bacteria.

| Classification       | Group                  | Institute                           | Methodology               | References                          |
|----------------------|------------------------|------------------------------------|---------------------------|-------------------------------------|
| Directed evolution   | Gregory Winter         | University of Cambridge            | Phage display             | Jespers et al., 2004                |
|                      | Daniel Christ          | Garvan Institute of Medical Research | Phage display             | Dudgeon et al., 2012                |
|                      | Dane Wittrup           | MIT                                | Yeast display             | Graf et al., 2004; Chao et al., 2006 |
|                      | Scott Glasser          | Biogen Idex                        | Bacterial screening       | Miller et al., 2010                 |
| Rational design      | Ira Pastan             | NIH                                | Non-native disulfide bond | Brinkmann et al., 1993              |
|                      | Andreas Plückthun      | University of Zurich               | Disrupting the hydrophobic patches | Nieba et al., 1997                 |
|                      | Wei Chen               | Jiangnan University                | Non-native disulfide bond | Zhao et al., 2010                   |
|                      | Ning Deng              | Jinan University                    | Non-native disulfide bond | Cai et al., 2016                    |
|                      | Hiroshi Moriya         | Kumamoto University                | Cyclization of scFv        | Yamauchi et al., 2019               |
|                      | Alexey Lugovskoy       | Merrimack Pharmaceuticals           | Tyrosine substitution      | Zhang et al., 2015                  |
|                      | An-Suei Yang           | National Defense Medical Center in Taipei | Hydrophilic domains     | Hsu et al., 2014                    |
|                      | Robin Curtis           | University of Manchester           | Substitution of Arginine   | Austerberry et al., 2019            |
|                      | Boris Steipe           | University of Toronto              | Consensus-based            | Steipe, 2004                        |
|                      | Alexey Lugovskoy       | Biogen Idex                        | Consensus-based            | Jordan et al., 2009                 |
| Computation          | Andreas Plückthun      | University of Zurich               | Structure-based            | Ewert et al., 2003; Ewert et al., 2004 |
|                      | David R. Liu           | Harvard University                 | Supercharging              | Lawrence et al., 2007               |
|                      | Andrew Ellington       | University of Texas at Austin      | Supercharging              | Miklos et al., 2012                 |
|                      | Peter Bowers           | AnaptysBio                         | Integrative approach      | McConnell et al., 2013; McConnell et al., 2014 |
|                      | George Georgiou and Andrew Ellington | University of Texas at Austin | Rosetta modeling        | Lee et al., 2019                     |
| Fusion partner       | Yechen Xiao            | Jilin University                    | SUMO                      | Liu et al., 2015                    |
|                      | Junichi Takagi         | Osaka University                   | Fv-clasp                  | Arimori et al., 2017                |
| Chaperone coexpression | Shihua Wang            | Fujian Agriculture and Forestry University | Skp                     | Wang et al., 2013                   |
| Multimerization      | Sergey Kiprianov       | Affiliated Therapeutics AG          | Diabody: bispecific        | Le Gall et al., 2004                |
|                      | C Ronald Geyer         | University of Saskatchewan         | SpyCatcher: trispecific    | Alam et al., 2018                   |
CONCLUSION

Up to March 2020, the US Food and Drug Administration (FDA) approved eight antibody fragments as drugs, six of which are produced from E. coli (75%, Table 1). The six therapeutic antibody fragments, produced by bacteria include ranibizumab (LUCENTIS®, 2006), certolizumab pegol (CIMZIA®, 2008), blinatumomab (BLINCYTO®, 2014), moxetumomab pasudotox (Kreitman and Pastan, 2011; LUMOXITI™, 2018), caplacizumab (CABLIVI®, 2019), and brolucizumab (BEOVU®, 2019), while those produced by mammalian hosts include abciximab (ReoPro®, 1994) and idarucizumab (PRAXBIND®, 2015), according to the data from “The Antibody Society” (Antibody Society, 2020).

Microorganisms are favorable expression hosts for antibody fragments, such as scFVs or Fab, in therapeutic applications (Table 1), because of the low production cost and lack of a carbohydrate chain. However, despite these advantages, scFVs expressed in bacteria have neither comparable stability relative to native full-length antibodies nor a comparable production yield of ~1 g/L in bioreactors (Petrus et al., 2019) relative to mammalian hosts, that is, >10 g/L in CHO cells (Kunert and Reinhart, 2016). Therefore, scientists and engineers in both academia and industry put extensive efforts on increasing production yield as well as protein stability of scFv expressed in bacteria. To obtain improved yield various bacterial expression systems have been developed in terms of vector systems or bacteria. To obtain improved yield various bacterial expression systems have been developed in terms of vector systems or engineering strains with engineered chaperone molecules.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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