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Short communication

A disulfide-stabilized human V_L single-domain antibody library is a source of soluble and highly thermostable binders☆

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ABSTRACT

We have previously shown that incorporation of a second intradomain disulfide linkage into camelid V_HH and human V_H/V_L single-domain antibodies confers increased thermostability. Here, we explored the effects of introducing an additional disulfide linkage, formed between Cys48 and Cys64 (Kabat numbering), into a phage-displayed synthetic human V_L library. In comparison to an identical library bearing only the highly conserved Cys23-Cys88 disulfide linkage, the disulfide-stabilized V_L library tolerated a similar degree of randomization but retained a higher level of functional diversity after selection with protein L. Both libraries yielded soluble, antigen-specific V_Ls that recognized a model antigen (maltose-binding protein) with similar affinities, in the micromolar range; however, the disulfide-stabilized antigen-specific V_Ls were much more thermostable (average ΔT_m ~10 °C) than non-disulfide-stabilized V_Ls. This work provides proof-of-concept for building synthetic antibody libraries using disulfide-constrained immunoglobulin domains, thus avoiding pitfalls of *post-hoc* disulfide linkage engineering such as impaired antigen binding and reduced expression yield.

1. Introduction

The thermostability of protein therapeutics is a critical parameter in determining their manufacturability (He et al., 2010), safety (Schellekens, 2002) and efficacy (Willuda et al., 1999; Arndt et al., 2003). This is especially true of human V_H and V_L single-domain antibodies (sdAbs), which are more aggregation-prone and less soluble (Kim et al., 2014a; Kim et al., 2014b; Kim et al., 2012) than camelid heavy-chain-only antibody variable domains (V_HHs). Thus, strategies to improve the biophysical properties of human V_H and V_L domains while maintaining their functional activity are highly desirable (Kim et al., 2014a).

We and others have shown that incorporating exogenous intradomain disulfide linkages into V_HH, V_H and V_L sdAbs reliably confers increased thermostability on the order of 5–15 °C increased melting temperature (T_m) (Kim et al., 2014b; Kim et al., 2012; Hussack et al., 2011; Hagihara et al., 2007; Saerens et al., 2008; Chan et al., 2008). Unfortunately, stability is often gained at the cost of variable detrimental effects on expression yield, solubility and affinity for antigen, depending on the sdAb and the location of the engineered disulfide linkage. We hypothesized that some of these consequences might result from conformational rearrangement of the immunoglobulin fold with concomitant repositioning of CDR loops. If that were so, incorporation of disulfide linkages during human V_H/V_L library design, prior to selection for binding to antigen, could yield sdAbs 'pre-adapted' for compatibility with the exogenous disulfide linkage and avoid the pitfalls of *post hoc* disulfide engineering.

Here, we describe the construction and characterization of a synthetic human V_L library, $HVLP324_{SS}$, based on a rearranged IGKV1-IGKJ1 scaffold stabilized with an engineered intradomain disulfide linkage formed between Kabat positions Cys48 and Cys64 (Kim et al., 2014b). We show that, in comparison to a nearly identical non-disulfide-stabilized V_L library (HVLP324), the $HVLP324_{SS}$ library displayed a similar or superior degree of functional sequence diversity and yielded antigen-specific V_Ls with similar affinities but much higher T_ms. Thus, incorporation of exogenous disulfide linkages into human V_H/V_L sdAb libraries is a viable strategy to increase the stability and therapeutic utility of these molecules.

Abbreviations: CDR, complementarity-determining region; FR, framework region; sdAb, single-domain antibody; V_L, variable light chain immunoglobulin domain

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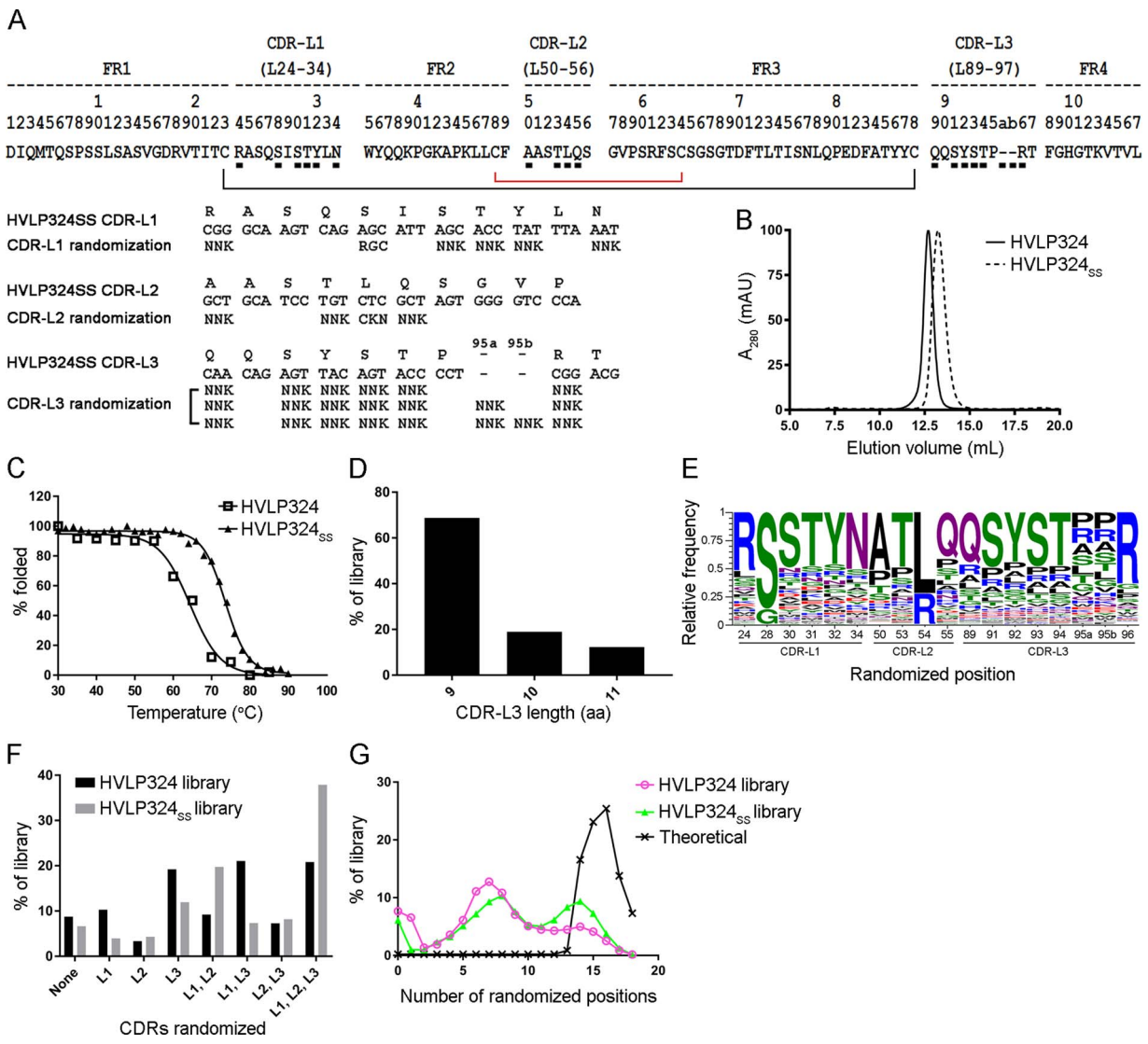

Fig. 1. Construction of the disulfide-stabilized HVLP324$_{SS}$ synthetic human V_L library. **(A)** Design of the HVLP324$_{SS}$ V_L library. The parental HVLP324$_{SS}$ V_L scaffold sequence is shown at top (Kabat numbering) with partial library randomization primer sequences (full sequences in Supplementary Table SI). Randomization was restricted to Gly/Ser at position 28 and Arg/Leu at position 54. **(B)** Size exclusion chromatography profiles of the disulfide-stabilized HVLP324$_{SS}$ and non-stabilized HVLP324 V_L scaffolds (data from Kim et al., 2014b). **(C)** Thermal unfolding of the disulfide-stabilized HVLP324$_{SS}$ and non-stabilized HVLP324 V_L scaffolds (data from Kim et al., 2014b). **(D)** CDR-L3 length distribution of the HVLP324$_{SS}$ V_L library. **(E)** WebLogo™ frequency plot of amino acid representation at randomization positions of the HVLP324$_{SS}$ V_L library. Colors show amino acid chemical property: blue (basic), red (acidic), green (polar), purple (neutral) and black (hydrophobic). **(F)** Relative proportions of the HVLP324 and HVLP324$_{SS}$ V_L libraries incorporating randomized sequence diversity in CDR-L1, CDR-L2 and CDR-L3. **(G)** Degree of randomized sequence diversity incorporated by the HVLP324 and HVLP324$_{SS}$ V_L libraries. For comparison, the expected degree of randomization for a hypothetical V_L library reaching full theoretical diversity ($[20^{14} \times 2^2] + [20^{15} \times 2^2] + [20^{16} \times 2^2]$ unique V_Ls) is shown. Analyses shown in (**D–G**) are representative of 1.4–2.5 × 10^5 sequences per V_L library.

2. Materials and methods

2.1. Construction of the disulfide-stabilized V_L library

The disulfide-stabilized library was constructed by Kunkel mutagenesis as previously described (Hussack et al., 2012) using as template the HVLP324$_{SS}$ scaffold (Supplementary Methods), which is identical to the HVLP324 scaffold except for the presence of an intradomain disulfide bridge spanning Kabat positions Cys48 and Cys64. Briefly, uridine-containing dU-ssDNA was prepared in *Escherichia coli* CJ236 cells from recombinant fd-tetGIIID phage bearing the DNA sequence encoding the HVLP324$_{SS}$ V_L fused N-terminally to gene 3. Randomization was achieved *via* two rounds of annealing and extension of degenerate oligonucleotides, followed by transformation of *E. coli* TG1 with the resulting heteroduplex covalently-closed circular DNA. In the first round, the CDR-L3-randomized library was produced by annealing and extension of three CDR-L3-specific degenerate primers differing in length by a single NNK codon (Fig. 1A). In the second round, the CDR-L1/CDR-L2/CDR-L3-randomized library was produced by simultaneous annealing of two CDR-L1- and CDR-L2-specific degenerate primers to dU-ssDNA derived from the CDR-L3-randomized library. The sizes of the CDR-L3-randomized library and the final CDR-L1/CDR-L2/CDR-L3-randomized library were 2.3 × 10^8 and 1 × 10^8, respectively.

2.2. Validation of the disulfide-stabilized V_L library

Both the previously-described non-stabilized HVLP324 library (Hussack et al., 2012) and the disulfide-stabilized HVLP324$_{SS}$ library were interrogated using next-generation DNA sequencing on an Illumina MiSeq instrument as previously described (Henry et al., 2016a; Henry et al., 2016b; Henry et al., 2015). To assess functional library diversity, phage derived from both libraries were selected in a single round on immobilized protein L. Briefly, wells of Nunc MaxiSorp® microtiter plates (ThermoFisher, Waltham, MA) were coated overnight with 5 μg of recombinant