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Research article

Effect of allotypic variation of human IgG1 on the thermal stability of disulfide-linked knobs-into-holes mutants of the Fc for stable bispecific antibody design

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Abstract

Background: Disulfide-linked knobs-into-holes (dKiH) mutation is a well-validated antibody engineering technique to force heterodimer formation of different Fcs for efficient production of bispecific antibodies. An artificial disulfide bond is created between mutated cysteine residues in CH3 domain of human IgG1 Fc whose positions are 354 of the "knob" and 349 of the "hole" heavy chains. The disulfide bond is located adjacent to the exposed loop with allotypic variations at positions 356 and 358. Effects of the variation on the biophysical property of the Fc protein with dKiH mutations have not been reported.

Methods: We produced dKiH Fc proteins of high purity by affinity-tag fusion to the hole chain and IdeS treatment, which enabled removal of mispaired side products. Thermal stability was analyzed in a differential scanning calorimetry instrument.

Results: We firstly analyzed the effect of the difference in allotypes of the Fcs on the thermal stability of the heterodimeric Fc. We observed different melting profiles of the two allotypes (G1m1 and nG1m1) showing slightly higher melting temperature of G1m1 than nG1m1. Additionally, we showed different characteristics among heterodimers with different combinations of the allotypes in knob and hole chains.

Conclusion: Allotypic variations affected melting profiles of dKiH Fc proteins possibly with larger contribution of variations adjacent to the disulfide linkage.

Statement of Significance: We analyzed thermal stability of disulfide-linked knobs-into-holes mutants of Fc with allotypic variants of human IgG1. Different melting profiles were observed with one of allotypes showing slightly higher thermal stability. The effect was dependent mainly on the variation adjacent to the disulfide linkage.

KEYWORDS: bispecific antibody; knobs-into-holes; differential scanning calorimetry; disulfide bond; heterodimeric Fc

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Bispecific antibodies (BsAbs) specific to two different antigens show promise as an emerging class of therapeutic antibodies. BsAbs are expected to express unique and desirable biological functions by the simultaneous binding to different molecules on a single cell or on different cell types. Compared to conventional IgG antibodies whose two identical heavy chains form homodimers, BsAbs needs the preferential assembly of two different antigen binding sites into heterodimers, requiring careful protein engineering. Various techniques have been developed to generate BsAbs, both with or without Fc part of immunoglobulin protein (1). One of the most promising strategies to generate Fc-fused BsAbs is knobs-into-holes (KiH) method originally developed by Genentech's researchers (2, 3). In this engineering system, four amino-acid mutations (T366W in the knob chain and T366S, L368A, Y407V in the hole chain) are made into a pair of different Fcs to preferentially form the heterodimer of the CH3 domains of human immunoglobulin $\gamma 1$ (hIgG1) (2). Further improvement with higher yield was achieved by introducing a disulfide bond at the heterodimer interface (S354C in the knob chain and Y349C in the hole chain) (3). Several BsAbs based on this disulfide-linked KiH (dKiH) mutation technique are under clinical trial at present, such as Faricimab reacting both with angiopoietin-2 and VEGF-A (4) or Cibisatamab linking CEA and CD3 (5).

The majority of the therapeutic antibodies currently used in clinics contain the constant region of hIgG1 subclass based on the associated high stability and strong potency to induce effector functions (6). Four allotypes of hIgG1 genes are known among human populations with slightly different peptide sequence (6–8). Different licensed therapeutic antibodies utilize different hIgG1 allotypes (8), and there is no consensus about the best allotype in the development of antibody therapeutics. However, it was reported that allotypes of the patients influence the pharmacokinetics of the therapeutic antibodies (9), and consequently their development should take the selection of hIgG1 allotype into account. In addition, important biophysical characteristics such as stability are possibly affected by the hIgG1 allotypes of therapeutic antibodies.

One allotype, G1m1, is associated with two amino acids in the CH3 domain (D356 and L358), which is coded by IGHG1∗01 allele (7). Another allotype with E356 and M358 coded by IGHG1∗03 gene is often classified as nG1m1 (non-G1m1) isoallotype (7). We focused on the two allotypes because their different amino acids at 356 and 358 positions are located close to the artificial disulfide bond introduced between S354C of the knob chain and Y349C of the hole chain in dKiH scaffold. Thus, we expected that the variations could affect biophysical characteristics of the disulfide-linked proteins. The closeness was evident in the crystal structure of dKiH Fc of G1m1 allotype solved previously (PDB ID 5HY9; Fig. 1A for overall structure and Fig. 1B for enlarged view of the artificial disulfide bond and the adjacent allotypic variations) (10). For clarity, a model structure of nG1m1 isoallotype with E356 and M358 was created onto this 5HY9 structure by Swiss-Model (11) and superimposed (Fig. 1A,B; Supplementary Table S1 for the peptide sequence). The positions of allotypic variation of the knob chain and the introduced disulfide bond were only about 4 Å apart, and the two heterodimers of allotypes would show specific profiles dependent on the knob chain variation. Therefore, we produced not only the dKiH heterodimers with the same allotypes but also the heterodimers with different allotypes to test the hypothesis.

We generated several different dKiH-based Fc fusions using G1m1 (IGHG1∗01) and nG1m1 (IGHG1∗03) allotypes and investigated their Fc regions with the thermal stability. Specifically, four Fc fusion proteins—dKiH-Fc-k01h01, dKiH-Fc-k03h03, dKiH-Fc-k01h03 and dKiH-Fc-k03h01—were prepared in Expi293 cells according to a modified method described in the literature (12). In the naming of the Fc-fusion proteins, the numbers followed by "k" or "h" shows the allotypes; e.g. dKiH-Fc-k01h03 indicated dKiH-Fc protein consisting of knob chain derived from G1m1 (IGHG1∗01) allotype and hole chain derived from nG1m1 (IGHG1∗03) allotype (see Supplementary Table S1 for the peptide sequence). To obtain heterodimeric dKiH-Fc proteins of high purity, fusion proteins were designed so that N-terminus of the hole chain contained hexahistidine tag. Initial purification was conducted by immobilized metal-affinity chromatography (IMAC). In this step, knob–knob mispaired side products were removed. Expressed protein yield after IMAC did not differ significantly by the allotypes. Each dKiH-Fc protein was prepared by digesting the Fc-fusions with IdeS, a specific protease to remove Fab and hinge regions (13). This enzyme specifically recognize the tertiary structure of Fc as the substrate (14) and hole–hole mispaired side products are not digested (see below). Thus, flow-through fraction of the subsequent IMAC contains only heterodimeric dKiH-Fc products that are properly paired. Detailed experimental procedures are described in the Supplementary Information.

After final purification by size-exclusion chromatography, it was confirmed that all dKiH-Fc proteins are efficiently purified to near homogeneity as expected (Supplementary Figure S1 and S2 for the SDS-PAGE analysis through the purification process and the chromatogram). To assess possibility of the contamination of hole–hole mispaired side products as described above, we only transfected the hole chain and conducted the experiment using His-tagged hole-hole Fc-fusion protein. As the result, digestion by IdeS was incomplete and Fc protein was not obtained in the same procedure (Supplementary Figure S3 and S4). Thus, contamination of hole–hole side product to dKiH-Fc was minimized. It was previously revealed that IdeS does not digest peptidyl substrate without Fc, and a second binding site to interact with CH2 domain was suggested (14). In a crystal structure of the hole–hole side product, Fc showed an open conformation and CH2 domains were apart from each other (10). Taken together with our observation, hole–hole mispaired Fc is not suitable as the substrate for IdeS, with inappropriate position of the second binding site.

Thermal stability of the dKiH-Fc proteins was analyzed by differential scanning calorimetry (DSC) in phosphate buffered saline, scanned at 60 °C/h. Denaturation curves of dKiH-Fc-k01h01 and dKiH-Fc-k03h03 are shown in Fig. 1C and D, respectively. As expected, two peaks in the thermograms corresponding to the denaturation of CH2 and CH3 domains were observed in both curves. The two

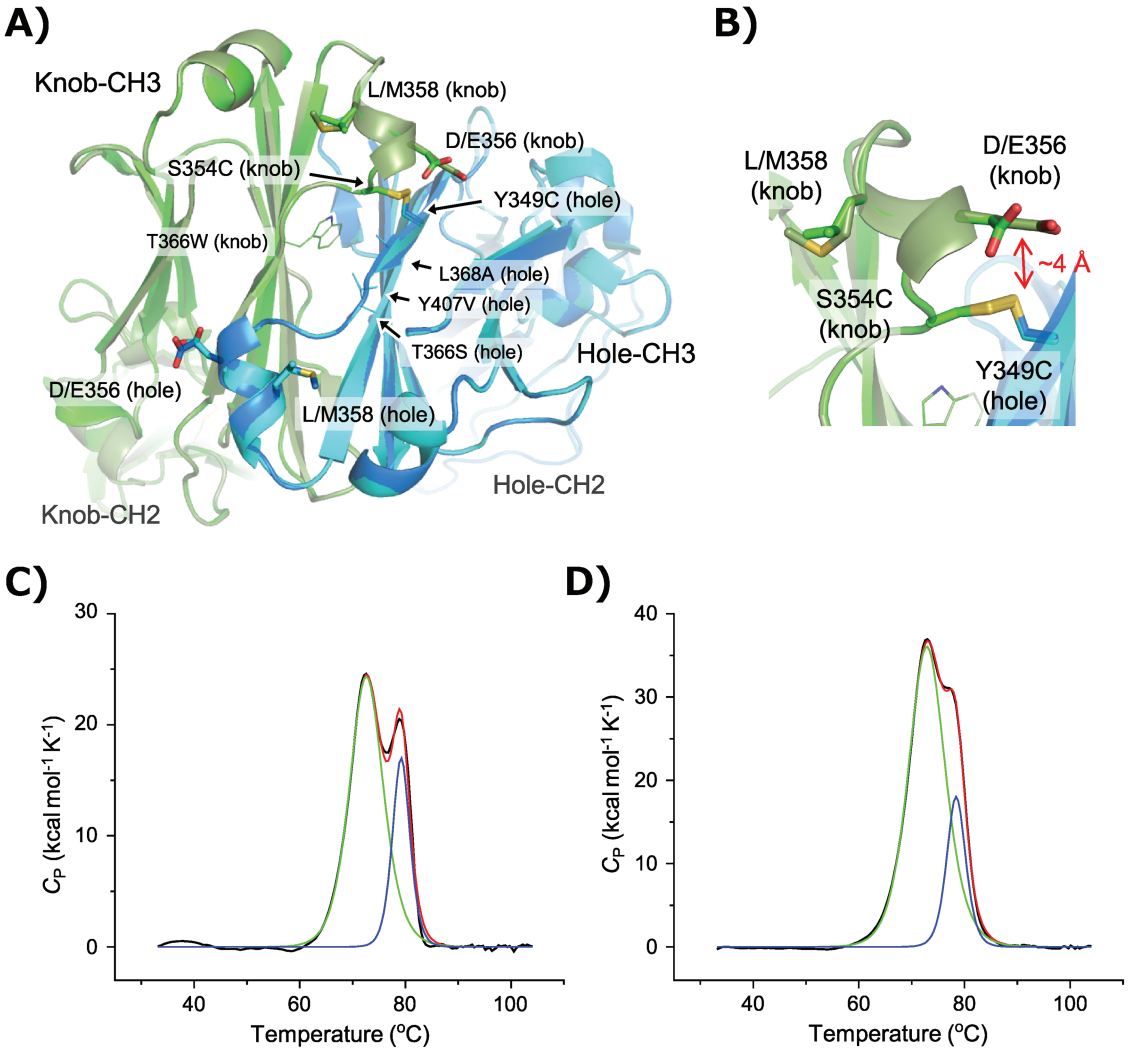

Figure 1. Features of dKiH mutants of human IgG1 Fc with different allotypes prepared in this study. (A) Structures representing the position of amino acids of allotypic variations of CH3 domain of human IgG1 constant region with mutations introduced in disulfide-linked knobs-into-holes (dKiH) heterodimer. Model structure of dKiH-Fc-k03h03 was made by homology modeling and is superimposed on the crystal structure of dKiH-Fc-k01h01 (PDB ID 5HY9). CH3 heterodimer is viewed by the opposite side from CH2 domains. Green, knob-Fc (G1m1); pale green, knob-Fc (nG1m1); cyan, hole-Fc (G1m1); blue, hole-Fc (nG1m1). Drawn in the PyMOL Molecular Graphics System. Residues specific to the allotypes and dKiH mutants are shown and labeled. (B) A close-up view of the knob chain allotypic variations and artificial disulfide bond introduced in dKiH. (C ,D) Thermal denaturation profiles of the KiH-Fcs with different allotype analyzed in DSC. (C) dKiH-Fc-k01h01 and (D) dKiH-Fc-k03h03. Black, experimental curves; green and blue, fitting curves for the 1st and 2nd denaturation; red, sum of the two fitting curves.

transitions of Fc during temperature-induced unfolding are well described with wild-type hIgG1 and Fc fragments (15–18). Apparent difference was observed between the shapes of melting curves of the two allotypes. In Fig. 1C (melting of dKiH-Fc-k01h01), the two peaks were separately observed, while in Fig. 1D (melting of dKiH-Fc-k03h03) the two peaks were close and appeared to be merged. To compare the multiple experimental results statistically, melting midpoint temperatures (T_m's) were obtained with all the four dKiH-Fc proteins and listed in Table 1. These values were calculated by fitting the curve into the sum of two unfolding reactions (two T_m's corresponding to the denaturation of CH2 and CH3 are labeled T_{m1} and T_{m2}, respectively). $\Delta T_m = T_{m2} - T_{m1}$, which is relevant to the observed differences in melting curves, showed small but significant difference depending on the allotypes of the KiH-Fc proteins (Table 1). ΔT_m was the largest for dKiH-Fc-k01h01 (6.62 ± 0.17 °C) and the smallest in dKiH-Fc-k03h03 (5.39 ± 0.13 °C), and the difference between the two was as large as 1.2 °C. This was consistent with the observation in the shape of melting curves. Differences were also observed in T_{m2} values corresponding to the CH3 denaturation. T_{m2} of dKiH-Fc-k01h01 CH3 was ca. 1 °C higher than T_{m2} of dKiH-Fc-k03h03 CH3. On the other hand, CH2 domains of all produced dKiH-Fc proteins were the same wild-type and showed typical T_{m1} at around 72.5 °C. Statistically significant difference was not observed in T_{m1} of dKiH-Fc allotypes in the tested combinations. Larger ΔT_m of dKiH-Fc-k01h01 derived from more stable CH3 domains.

Table 1. Melting temperatures (T_m's) of thermal denaturation[a]

	T_{m1} (°C)	T_{m2} (°C)	ΔT_m (°C) (=$T_{m2} - T_{m1}$)	Knob chain variants[b]	Hole chain variants[b]
dKiH-Fc-k01h01	72.53 ± 0.28	79.15 ± 0.11	6.62 ± 0.17	D/L	D/L
dKiH-Fc-k03h03	72.83 ± 0.17	78.21 ± 0.29*	5.39 ± 0.13***	E/M	E/M
dKiH-Fc-k01h03	72.54 ± 0.15	78.70 ± 0.10**	6.17 ± 0.11*	D/L	E/M
dKiH-Fc-k03h01	72.48 ± 0.32	78.39 ± 0.15***	5.91 ± 0.18**	E/M	D/L

[a] T_m values calculated from the fitting curves ± S.D. of triplicate.
[b] Amino acids at positions 356 and 358.
* *t*-test, $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$ in comparison with the T_{m2} or ΔT_m values of dKiH-Fc-k01h01.
ΔT_m of dKiH-Fc-k01h03 and dKiH-Fc-k03h01 showed significant differences from ΔT_m of dKiH-Fc-k03h03 in *t*-test by $P < 0.005$ and $P < 0.05$, respectively, but $P > 0.05$ for the same comparison of T_{m2}.

Additionally, calorimetric enthalpy (ΔH) of denaturation and van't Hoff enthalpy (ΔH_v) from the same fitting of the melting curves are described on Supplementary Table S2. $\Delta H_1/\Delta H_{v1} > 2$ and $\Delta H_2/\Delta H_{v2} < 2$ for all measured samples, which supports deviation from the two-state model (19, 20). $\Delta H_1/\Delta H_2$ describes the ratio of the areas of the two sigmoidal curves corresponding to the two peaks. $\Delta H_1/\Delta H_2$ of dKiH-Fc-k01h01 and dKiH-Fc-k03h03 were 3.090 ± 0.365 and 4.411 ± 0.456, respectively. Difference in the values was consistent with the observed differences in the melting curves in Fig. 1C,D. The process of cooperative denaturation of domains is likely different in these two proteins (19).

Furthermore, ΔT_m and T_{m2} values of dKiH-Fc-k01 h03 and dKiH-Fc-k03h01 fell within the 5.4–6.6 °C and 78.2–79.1 °C ranges, respectively, and were intermediate of dKiH-Fc-k01h01 and dKiH-Fc-k03h03. An overall assessment of all the ΔT_m and T_{m2} of the tested 4 KiH-Fc proteins indicates that thermal stability is largely influenced by variation in the knob chain compared to the hole chain. This result was consistent with our prediction and demonstrates the importance of the proximity between the allotypic variations and the disulfide linkage for the stability of dKiH molecules. Interestingly, the difference in T_{m2} between dKiH-Fc-k01h01 and -k03h01 was smaller than that between dKiH-Fc-k01h01 and -k03h03, suggesting that the loop apart from the disulfide linkage also affected the stability to a small extent.

In general, introduction of inter-domain disulfide bonds is a promising strategy for stabilization of engineered antibodies. Knobs-into-holes CH3 of nG1m1 allotype was reported with T_m = 69.4 °C (2), thus disulfide linkage led to 9 °C of thermal stabilization. In another format (EW-RVT$_{S\text{-}S}$), a disulfide bond also increased 3 °C of T_m, resulting stabilization of the molecule (21). In many studies, Fvs introduced with disulfide bonds also stabilize the Fvs against heat stresses (22–25). Furthermore, as we previously described, rabbit immunoglobulin kappa chains naturally containing a Vκ–Cκ interdomain disulfide bond show resistance to heat, measured as 3–9 °C higher T_m than those of mutants light chains without the disulfide bond (26). Considering the T_m differences between disulfide-free and disulfide-linked molecules in these previous studies, the differences in the T_{m2} of dKiH-Fc-k01h01 and -k03h03 found in the present study cannot be ignored. Because the introduction of disulfide-bond is a common way in the stabilization of therapeutic proteins, both germline and somatic variations at surrounding positions should be considered in protein engineering approaches.

In conclusion, we found different melting profiles of allotypic variants of dKiH mutants of the Fc for bispecific antibody design. The effect of allotypic variation of human IgG1 on the thermal stability was statistically significant. For the selection of allotypes of antibody therapeutics, other indexes for developability such as the immunogenicity and the affinity to neonatal Fc receptor to affect pharmacokinetics should be considered in parallel (8, 9). However, as the results of this study exemplified, variety in the exposed residues adjacent to the disulfide linkage significantly affected thermal stability. Thus, in protein engineering approaches, optimization of these positions should be considered for stable design of next-generation therapeutics.

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REFERENCES

1. Brinkmann, U, Kontermann, RE. The making of bispecific antibodies. *MAbs* 2017; **9**: 182–212.
2. Atwell, S, Ridgway, JBB, Wells, JA *et al.* Stable heterodimers from remodeling the domain interface of a homodimer using a phage display library. *J Mol Biol* 1997; **270**: 26–35.
3. Merchant, AM, Zhu, Z, Yuan, JQ *et al.* An efficient route to human bispecific IgG. *Nat Biotechnol* 1998; **16**: 677–81.
4. Sahni, J, Patel, SS, Dugel, PU *et al.* Simultaneous inhibition of Angiopoietin-2 and VEGF-A with Faricimab in diabetic macular edema: BOULEVARD phase 2 randomized trial. *Ophthalmology* 2019; **126**: 1155–1170.
5. Bacac, M, Fauti, T, Sam, J *et al.* A novel carcinoembryonic antigen T-cell bispecific antibody (CEA TCB) for the treatment of solid tumors. *Clin Cancer Res* 2016; **22**: 3286–97.
6. Irani, V, Guy, AJ, Andrew, D *et al.* Molecular properties of human IgG subclasses and their implications for designing therapeutic monoclonal antibodies against infectious diseases. *Mol Immunol* 2015; **67**: 171–82.
7. Lefranc, M-P, Lefranc, G. Human Gm, Km, and Am Allotypes and Their Molecular Characterization: A Remarkable Demonstration of Polymorphism. In: Christiansen, FT, Tait, BD (eds). *Immunogenetics: Methods and Applications in Clinical Practice*. New York; Humana Press, 2012, 635–80.
8. Jefferis, R, Lefranc, M-P. Human immunoglobulin allotypes. *MAbs* 2009; **1**: 332–8.

9. Ternant, D, Arnoult, C, Pugnière, M *et al.* IgG1 Allotypes influence the pharmacokinetics of therapeutic monoclonal antibodies through FcRn binding. *J Immunol* 2016; **196**: 607–13.
10. Kuglstatter, A, Stihle, M, Neumann, C *et al.* Structural differences between glycosylated, disulfide-linked heterodimeric Knob-into-Hole Fc fragment and its homodimeric Knob-Knob and Hole-Hole side products. *Protein Eng Des Sel* 2017; **30**: 649–56.
11. Waterhouse, A, Bertoni, M, Bienert, S *et al.* SWISS-MODEL: homology modelling of protein structures and complexes. *Nucleic Acids Res* 2018; **46**: W296–303.
12. Han, L, Chen, J, Ding, K *et al.* Efficient generation of bispecific IgG antibodies by split intein mediated protein trans-splicing system. *Sci Rep* 2017; **7**: 8360.
13. Von, U, Johansson, BP, Björck, L. Ide S, a novel streptococcal cysteine proteinase with unique specificity for immunoglobulin G. *EMBO J* 2002; **21**: 1607–15.
14. Vincents, B, Von, U, Björck, L *et al.* Enzymatic characterization of the streptococcal endopeptidase, ides, reveals that it is a cysteine protease with strict specificity for IgG cleavage due to exosite binding. *Biochemistry* 2004; **43**: 15540–9.
15. Ito, T, Tsumoto, K. Effects of subclass change on the structural stability of chimeric, humanized, and human antibodies under thermal stress. *Protein Sci* 2013; **22**: 1542–51.
16. Garber, E, Demarest, SJ. A broad range of Fab stabilities within a host of therapeutic IgGs. *Biochem Biophys Res Commun* 2007; **355**: 751–7.
17. Choi, HJ, Seok, SH, Kim, YJ *et al.* Crystal structures of immunoglobulin Fc heterodimers reveal the molecular basis for heterodimer formation. *Mol Immunol* 2015; **65**: 377–83.
18. Brader, ML, Estey, T, Bai, S *et al.* Examination of thermal unfolding and aggregation profiles of a series of developable therapeutic monoclonal antibodies. *Mol Pharm* 2015; **12**: 1005–17.
19. Solís-Mendiola, S, Rojo-Domínguez, A, Hernández-Arana, A. Cooperativity in the unfolding transitions of cysteine proteinases. Calorimetric study of the heat denaturation of chymopapain and papain. *Biochim Biophys Acta (BBA)/Protein Struct Mol* 1993; **1203**: 121–5.
20. Bradley, CM, Barrick, D. Limits of cooperativity in a structurally modular protein: response of the notch ankyrin domain to analogous alanine substitutions in each repeat. *J Mol Biol* 2002; **324**: 373–86.
21. Choi, HJ, Seok, SH, Kim, YJ *et al.* Crystal structures of immunoglobulin fc heterodimers reveal the molecular basis for heterodimer formation. *Mol Immunol* 2015; **65**: 377–83.
22. Brinkmann, U. Disulfide-stabilized fv fragments. In Kontermann, R., Dübel, S. (eds). *Antibody Engineering*. Berlin Heidelberg: Springer-Verlag, 2010; **2**: 181 –9.
23. Fitz Gerald, K, Holliger, P, Winter, G. Improved tumour targeting by disulphide stabilized diabodies expressed in Pichia pastoris. *Protein Eng* 1997; **10**: 1221–5.
24. Zhao, JX, Yang, L, Gu, ZN *et al.* Stabilization of the single-chain fragment variable by an interdomain disulfide bond and its effect on antibody affinity. *Int J Mol Sci* 2011; **12**: 1–11.
25. Reiter, Y, Brinkmann, U, Kreitman, RJ *et al.* Stabilization of the fv fragments in recombinant immunotoxins by disulfide bonds engineered into conserved framework regions. *Biochemistry* 1994; **33**: 5451–9.
26. Kawade, R, Akiba, H, Entzminger, K *et al.* Roles of the disulfide bond between the variable and the constant domains of rabbit immunoglobulin kappa chains in thermal stability and affinity. *Protein Eng Des Sel* 2018; **31**: 243–7.