Double mutation near CDRs reduces antibody aggregation most effectively

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Introduction

Antibody aggregation is a widely recognized phenomenon, often negatively impacting the effective use of antibodies in therapeutic as well as diagnostic applications. Engineering the antibody structures for reduced aggregation is commonplace with therapeutic mAbs, but it is less frequently applied to antibodies developed for use in *in vitro* diagnostic (IVD) tests. This study demonstrates a protein engineering approach which successfully reduced aggregation propensity for a mouse monoclonal IgG1 antibody (mAb1) used in IVD applications. Both the effect of constant region switch and point mutations in the variable domain were studied.

Materials and methods

- mAb1 variants with heavy chains of original mAb (mAb1_1) (Medix Biochemica), IGHG1_MOUSE (P01868, mAb1_2) and IGHG1_HUMAN (P01857, mAb1_3) as well as mouse and human kappa light chains were designed. Species specific chains were kept paired.
- The structure of mAb1 was extensively characterized in collaboration with Chemical Computing Group (CCG) and Åbo Akademi University enabling selection of point mutations for further studies. Software used were AggScore[1], Bodil[2], Chimera, UCSF; DNAStar, DNASTAR; MOE, Chemical Computing Group; The Iterative Protein Redesign and Optimization (IPRO) suite[3], and Pymol, Schrödinger Inc.
- Individual mAb1 sequences were cloned to pQMCF vector and the antibodies were transiently expressed in CHO cells at Icosagen Cell Factory.
- Antibodies were affinity purified from cell culture supernatants with MabSelect SuRe (Cytiva).
- Homogeneity of the antibodies was analyzed using size-exclusion chromatography (SEC; Superdex Increase 200 10/300 GL, Cytiva), overall thermal stability with Therman Shift Assay (TSA) (Thermo Fisher Scientific) and RUBIC Buffer screen (Molecular Dimensions), and aggregation propensity with Protein Aggregation Assay (PAA-kit, QRET Technologies).
- mAb1 variants were heat stressed at +45°C for 3 days. Homogeneity was assessed with SEC and aggregation tendency with PAA-kit.
- Antibodies were functionally characterised using an inhouse developed fluorescence immunoassay (FIA).

Results

Three different constant domain variants of mAb1 (Figure 1) were produced recombinantly and characterized with emphasis on aggregation and stability analysis. Results are summarized in Table 1. No differences between the variants were observed in the functional assay and only minor differences in SEC, TSA, RUBIC Buffer screen, and PAA-kit. Heat maps of Rubic Buffer screen are presented in Figure 2. We selected mAb1_1 for further engineering as we preferred the original mAb1 and there were no major advantages in selecting mAb1_2 or mAb1_3.

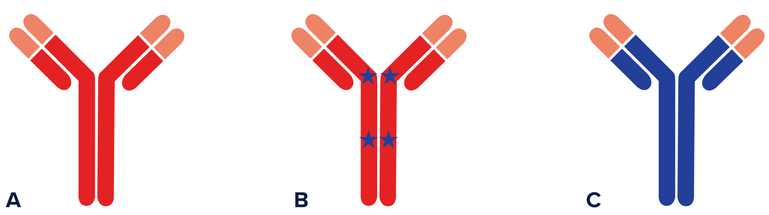

Figure 1. Schematic drawings of different heavy chain formats of mAb1. A: mAb1_1 (mouse) B: mAb1_2 (mouse) C: mAb1_3 (human).

Table 1. Summary of stability data of different constant domain variants. SEC % describes the proportion of mAb monomers in each batch. PAA results are given as order number: smallest number is the least aggregating version and largest number the most aggregating.

Antibody	Functionality	SEC	T_m°C	RUBIC	PAA
mAb1_1	✓	99 %	69	1	2
mAb1_2	✓	99 %	64	3	3
mAb1_3	✓	100 %	67	2	1

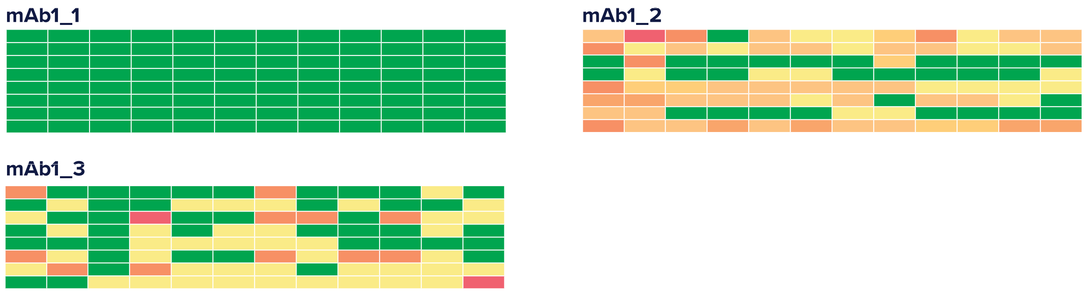

Figure 2. Heat maps of RUBIC Buffer screen of mAb1_1, mAb1_2 and mAb1_3 heat maps are normalised against mAb1_1.

Structural analysis of mAb1 revealed hydrophobic patches on the variable domain that may promote aggregation. However, these patches were also located near complementarity determining regions (CDRs). Therefore, other potential sites for point mutations were also charted. Altogether 17 potential sites for point mutations were identified. Ten of these (mAb1_5 to mAb1_14) were expressed and characterized. Results of characterization are summarized in Table 2. Three of the mutants lost their immunoreactivity while the rest retained the original level of activity. Aggregation tendency of four variants decreased markedly, mAb1_11 showing the most significant improvement (Figure 3). Two of the variants were somewhat less prone to aggregate than the original mAb1_1, while aggregation tendency of four of the variants stayed at the same level as the original mAb1_1.

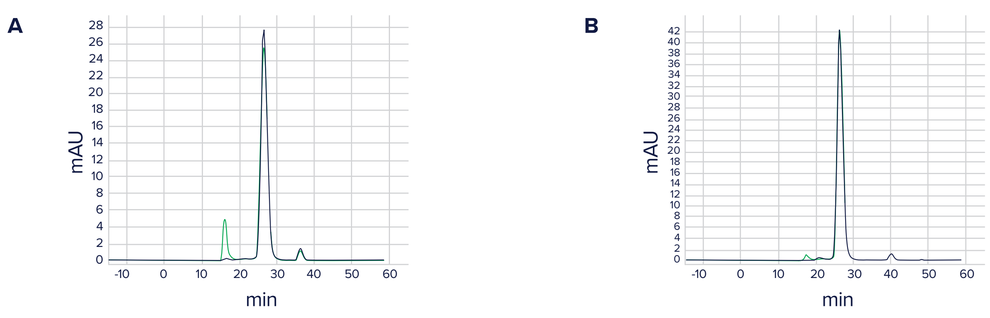

Figure 3. Comparison of SEC results of untreated and heat stressed samples of mAb1_1 (A) and mAb1_11 (B). Untreated samples have blue graph and heat stressed green.

Discussion

We applied two engineering approaches for decreased aggregation tendency of a monoclonal mouse antibody: constant domain switch or rationally designed point mutations in the variable domain. Constant domain variants demonstrated only minor differences, whereas four of the ten rationally designed variants showed reduced aggregation propensity when stressed at elevated temperatures. The most impactful mutations removed aliphatic amino acids from the surface of the protein, close to the CDR's. This study is an example of successful application of monoclonal antibody modeling and engineering tools to an industrial antibody used in diagnostic tests.

Table 2. Summary of mAb1 mutants and their stability data. SEC results are reported as ratio of monomer peak area to the total peak area in the chromatogram. PAA results are given as order number: smallest number is the least aggregating version and largest number the most aggregating.

Code	Mutations	Location in the structure	Functionality	SEC 4 °C	SEC Heat-treated	PAA
mAb1_1	-		Ref	99 %	89 %	11
mAb1_5	VL L15T	At the N-terminal region of VL, distant from the CDRs	✓	98 %	93 %	4
mAb1_6	VL Y34N	In the middle of CDR-L1	✗	98 %	89 %	8
mAb1_7	VH L33N	At the end of CDR-H1, close to the start of b-strand C1	✗	98 %	95 %	10
mAb1_8	VH L72R	Outside of CDRs but on the surface and close to antigen binding region	✓	99 %	96 %	9
mAb1_9	VH W105G	At the end of CDR-H3	✗	98 %	98 %	2
mAb1_10	VL L58R + A64D	Close to CDR-L2 on a loop on the surface and on the same side as antigen binding region	✓	97 %	93 %	7
mAb1_11	VH A76K + I77N	On the surface and close to CDR-H1 and CDR-H2	✓	98 %	97 %	1
mAb1_12	VL L15T + L58R + A64D	Combination of mAb1_5 and mAb1_10	✓	97 %	92 %	4
mAb1_13	VH L72R + A76K + I77N	Combination of mAb1_8 and mAb1_11	✓	98 %	97 %	6
mAb1_14	VL L58R + A64D + VH A76K + I77N	Combination of mAb1_10 and mAb1_11,	✓	97 %	97 %	3

References
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