Advanced tools for characterizing diverse bispecific antibodies formed through fusion of single-domain antibodies onto IgG scaffolds

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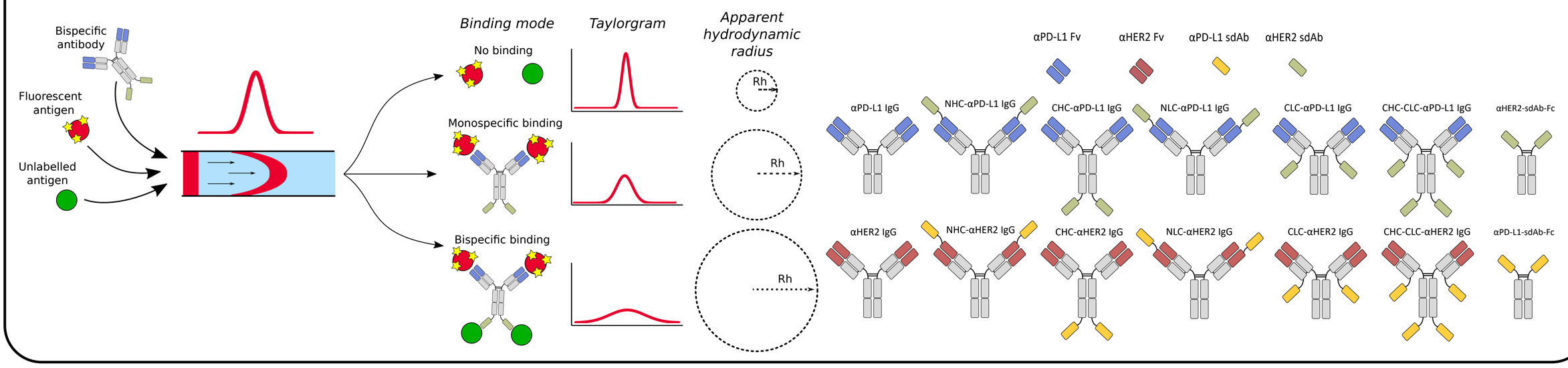
Introduction. Bispecific antibodies (bsAbs) represent a rapidly growing class of biotherapeutic molecules. Their ability to engage two different epitopes opens novel avenues of functionalities and potencies beyond conventional immunoglobulins. By engineering the molecular structure and valenciesof bsAbs, their functionality when binding different antigens can be fine-tuned to fit specific therapeutic purposes. This project sought to engineer a comprehensive panel of αPD-L1xαHER2 bsAbs to explore the feasibility of forming bsAbs through fusion of single-domainantibodies (sdAbs) onto IgG1 scaffolds and how the molecular architecture affected the functionality.

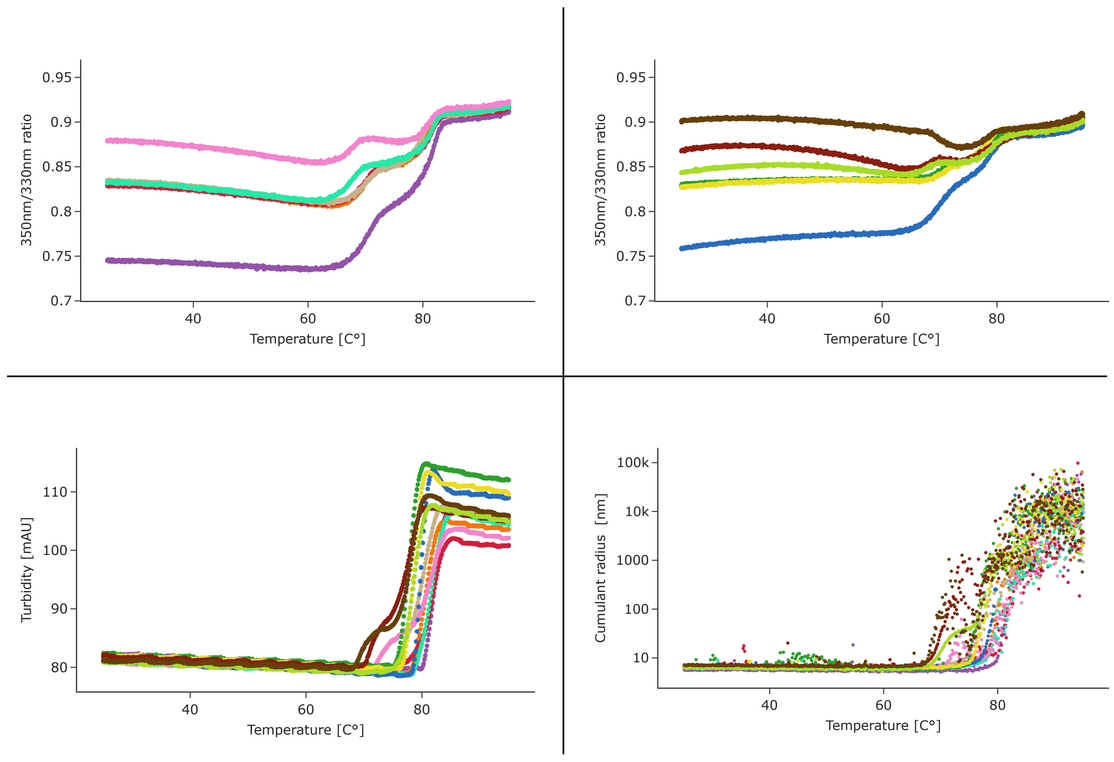
Methods.

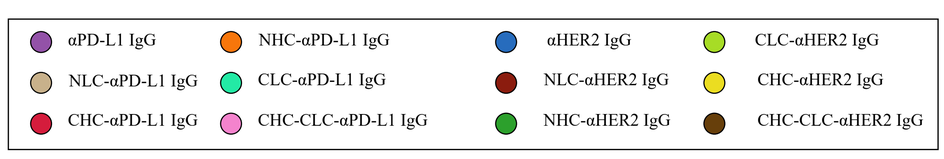
- Construction of bsAbs through genetic fusion of antibody fragments on full length IgG molecules.
- Flow-induced dispersion analysis (FIDA) for evaluating bispecific binding functionality in-solution without potentially obstructive surface immobilization.
- Simultaneous monitoring of biophysical parameters during thermal unfolding for in-depth analysis of stability profiles.



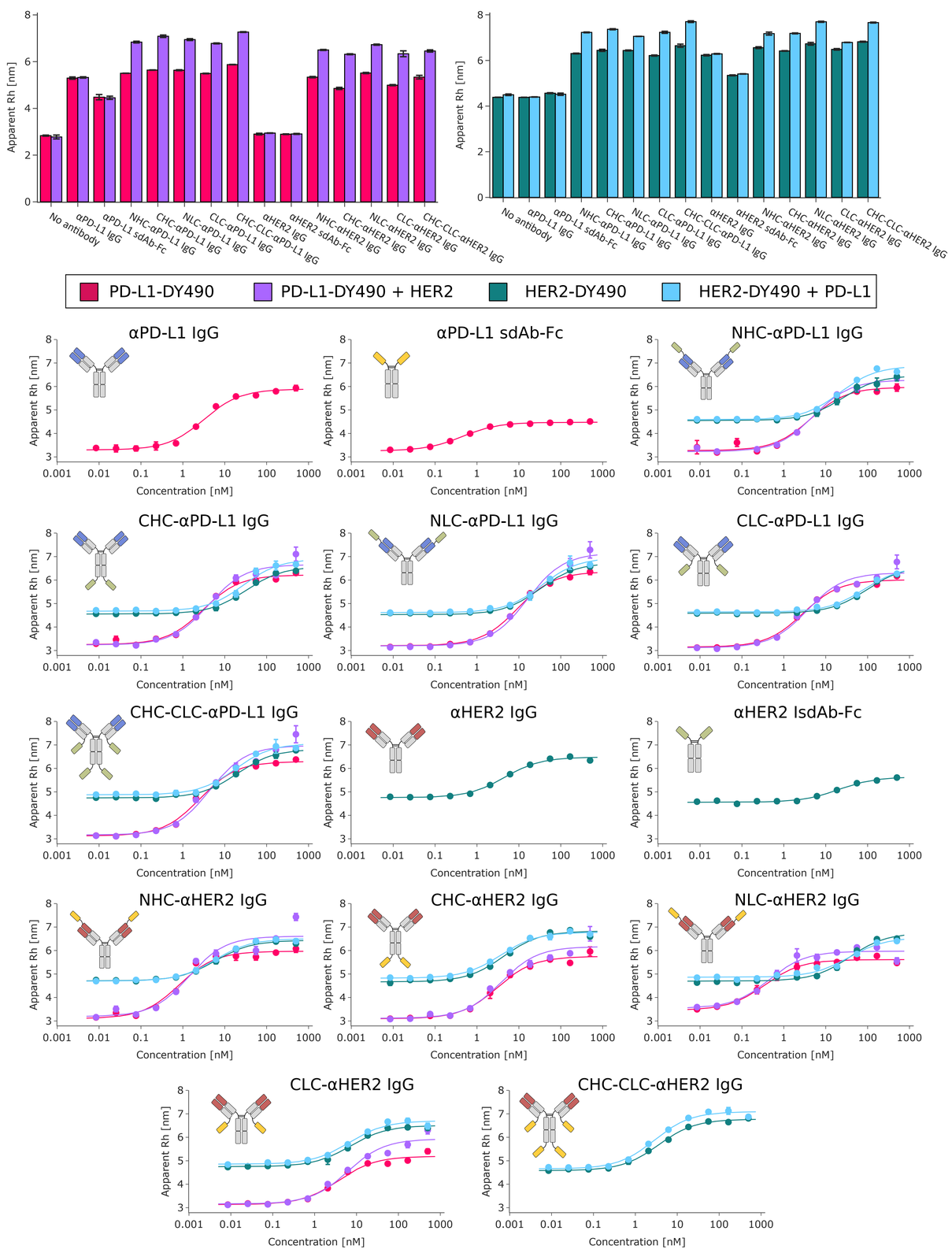
Simultaneous monitoring of intrinsic fluorescence, aggregation and cumulant radius allow in-depth stability characterization of structurally complex bsAbs

- Thermal ramping shows similar unfolding behavior between bsAbs and conventional IgG1 molecules.All bsAbs exhibited high purity and
- sample homogeneity after only a single protein A purification step.Engineered bsAbs show similar aggregation propensity and
- limited self-association as IgG1 molecules.





- Bar charts illustrating incremental increase in Rh upon exposure to second antigen for bsAbs only.Affinity determinations in both monospecific-and
- bispecific binding scenarios.Both binding antigen-binding regions are functional although sdAb fusion on HC is favorable over LC fusion for
- effective antigen binding by both Fvand sdAb.
- All bsAbs showed binding to FcγIIIa similar to IgG (data not shown).



Conclusion. Fusion of sdAbs onto IgG scaffolds represents a generic and conceptually easy way of forming bispecific antibodies with limited antibody-related impurities and no risk of chain mispairing as it is seen for asymmetric bsAbs. Our panel of bsAbs showed favorable stability profiles and effective binding to both antigens with some molecular architectures being preferred over others.

1. Madsen, A. V., Mejias-Gomez, O., Pedersen, L. E., Skovgaard, K., Kristensen, P., & Goletz, S. (2022). Immobilization-Free Binding and Affinity Characterization of Higher Order Bispecific Antibody Complexes Using Size-Based Microfluidics. Analytical chemistry, 94(40), 13652–13658.
2. Madsen, A. V., Kristensen, P., Buell, A. K., & Goletz, S. (2023). Generation of robust bispecific antibodies through fusion of single-domain antibodies on IgG scaffolds: a comprehensive comparison of formats. mAbs, 15(1), 2189432.