Analysis, control and engineering of protein dynamics, stability and aggregation

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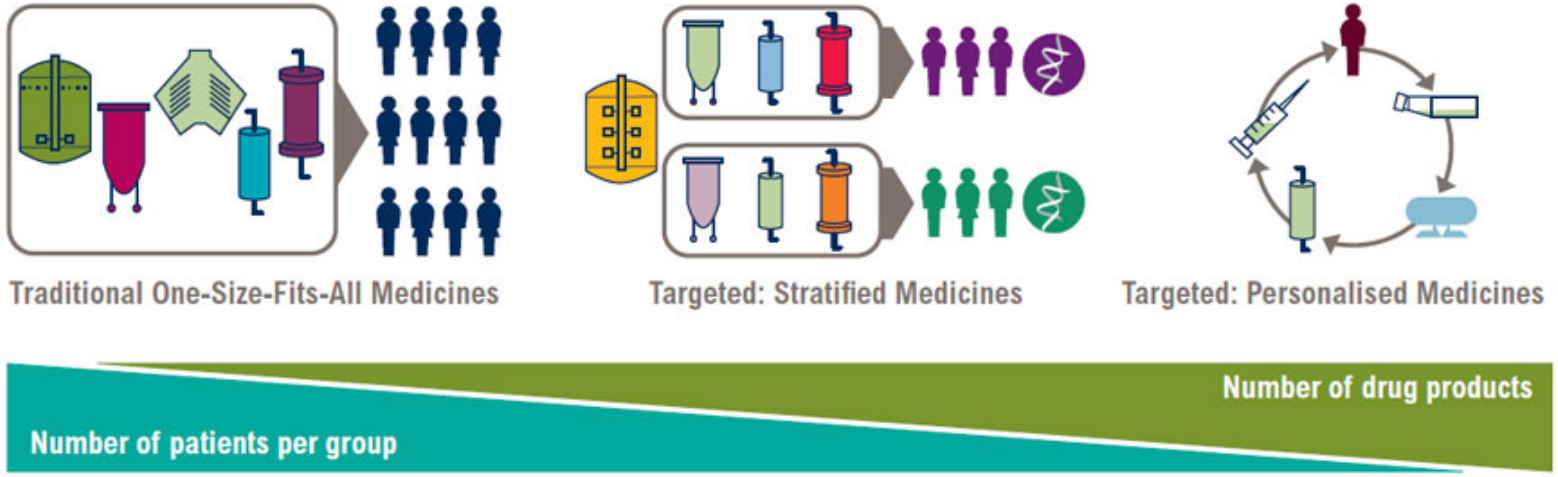
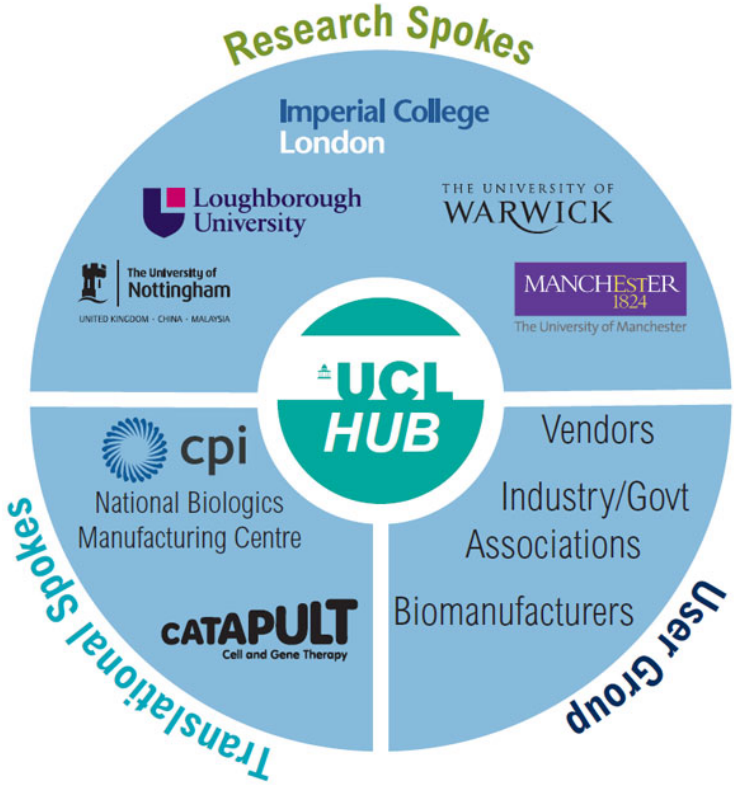
Future Targeted Healthcare Manufacturing Hub



- Stratified protein therapies

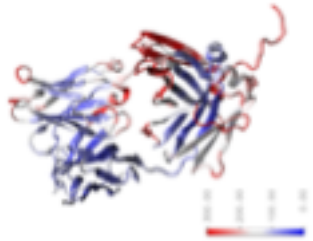
  Manufacturing & supply chain challenges:
  - Genomic screening of patients
  - Diagnostic-driven administration
  - Bespoke drug combinations
  - Bespoke doses in future
  - Make-to-order tailored therapeutics
  - Reduced time available for process development and manufacturing



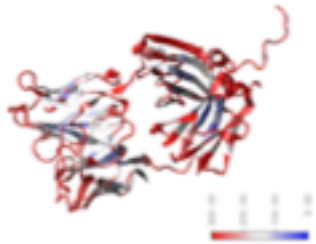
Potential factors affecting aggregation

- Expression & PTMs
- IB formation / Refolding
- Chromatography /Viral Kill pH
- Bulk storage / Filtration / Fill finish
- Formulation: transport / storage

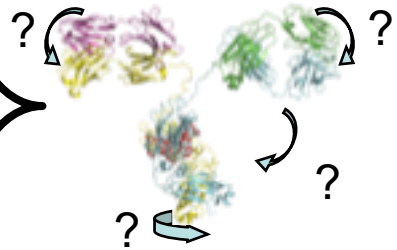
- [Protein]
- pH, T, IS
- Shear & Surfaces
- Excipient interaction
- Excipient volume exclusion



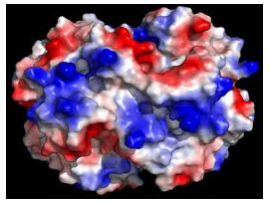
- Local Dynamics / Partial unfolding



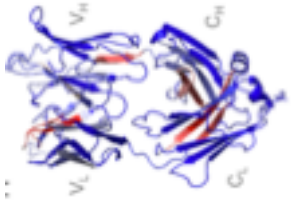
- Global Conformational Stability



- 3° / 4° conformation



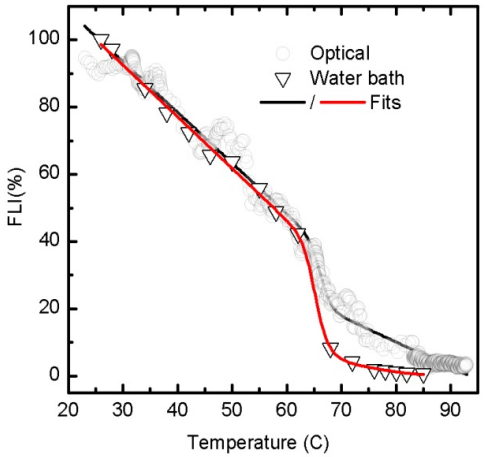
- Surface properties: charge/ hydrophobicity



- Sequence features eg. APRs

- Aggregation
- Fragmentation
- Solubility

Analytical challenges



- High-throughput, low-volume, low-cost analytics

  Differential Scanning Fluorescence
  Thermophoresis (Nanotemper)
  UPLC SEC & SEC-MALLS
  DLS / SLS
  Nanosight Tracking Analysis (NTA)
  Micro-Flow Imaging (MFI)

- Measurements that **predict** 2-year shelf life under storage at 4 °C – 25 °C

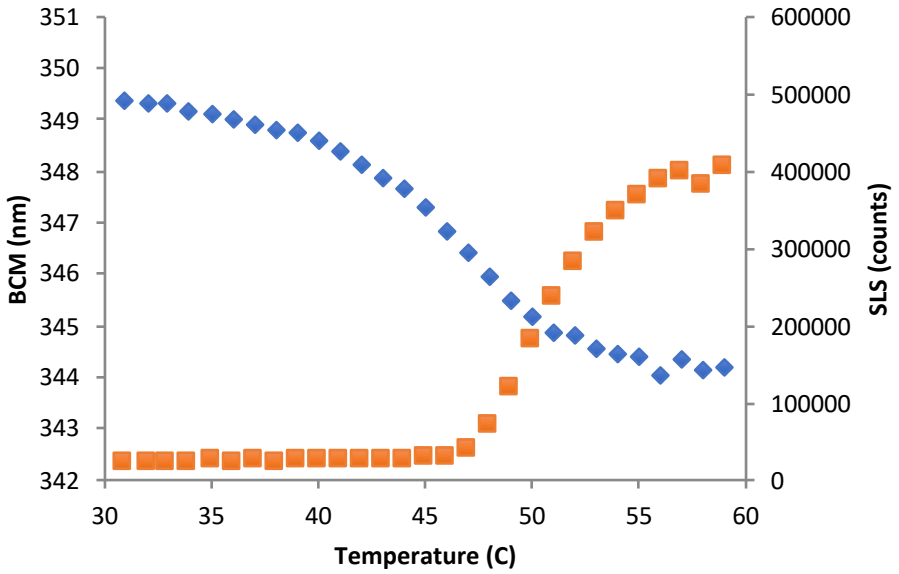
  Current options have severe limitations: eg. T_m / T_{agg}, accelerated degradation

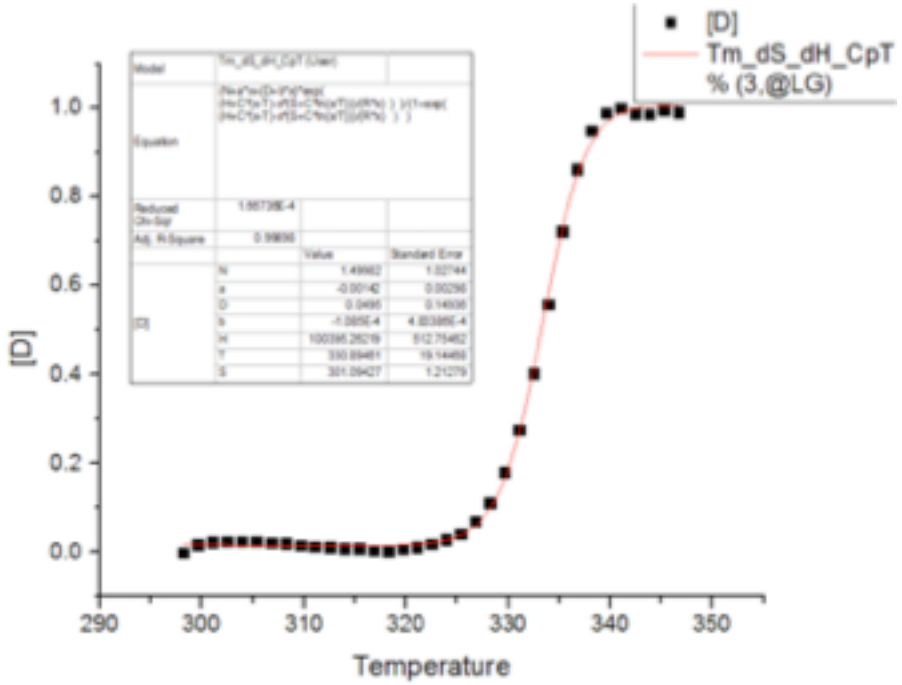
- Dynamics measurements are specialized – but insightful
  NMR – HDX
  LCMS – HDX
  Molecular dynamics simulations

- Analytics for low-purity conditions.
  Upstream / Downstream process monitoring.
  Co-formulated products
  Complex delivery systems (eg nanoparticles).

Thermostability

Thermal unfolding – widely used in formulation screening





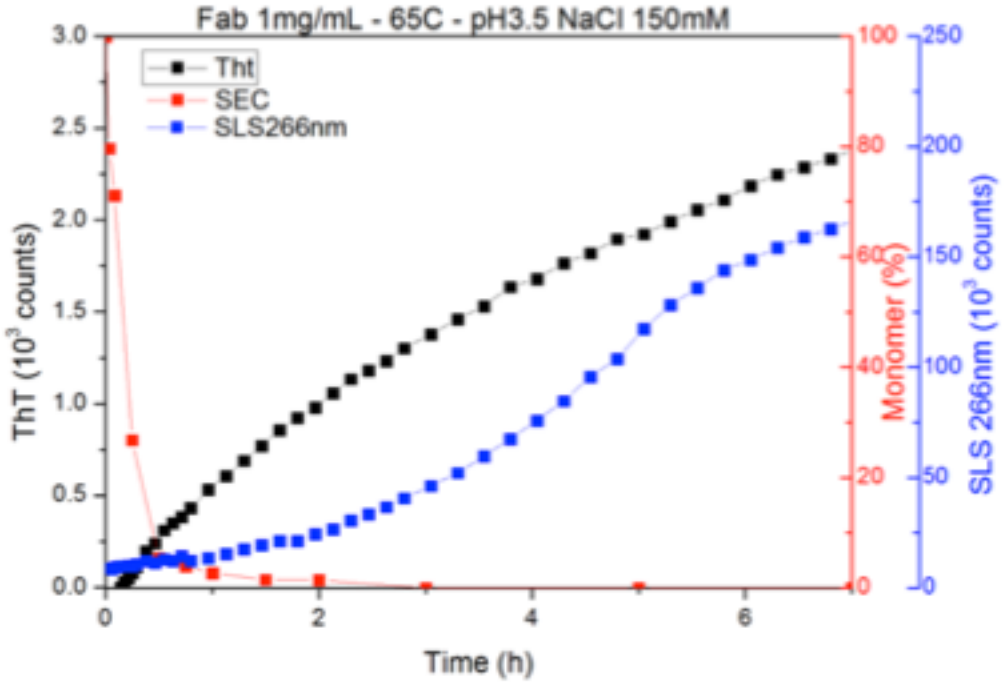
Raw data from UNiT

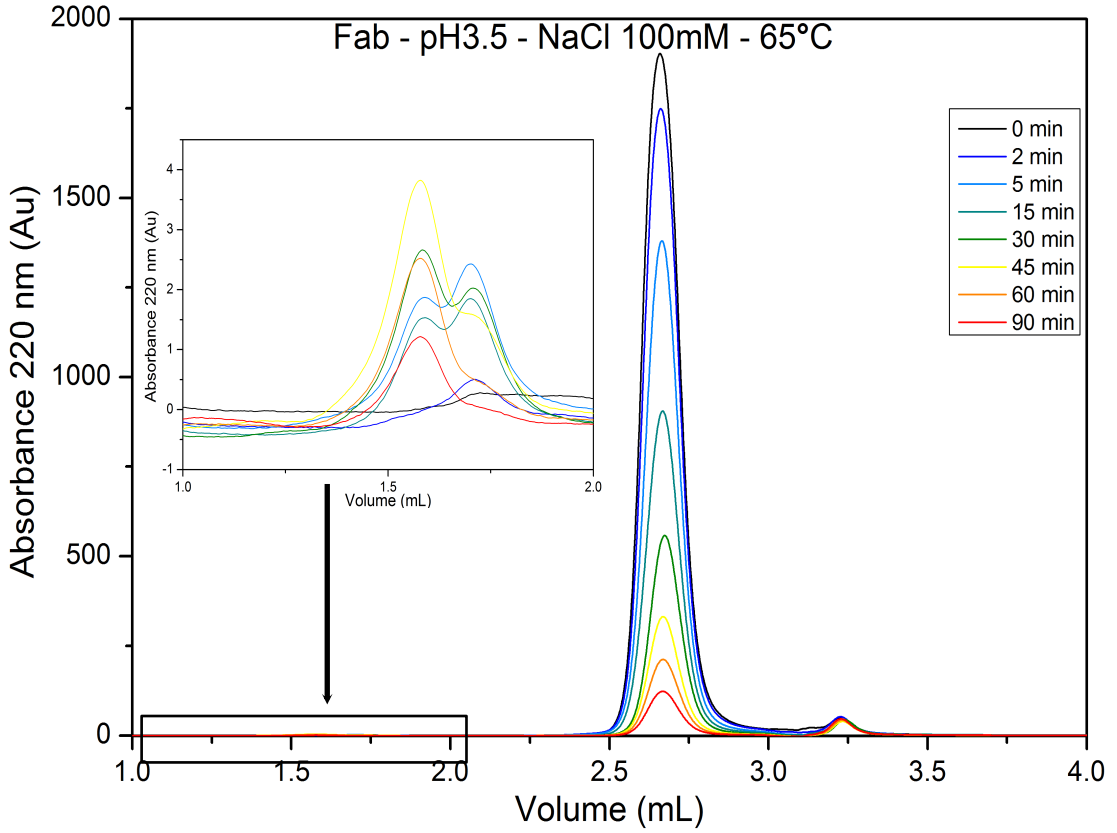
Refit normalized signal to Van't Hoff unfolding in OriginPro

- T_m
- ΔH_{vh}
- fraction unfolded at any T (f_T)

Aggregation kinetics

Aggregation kinetics





Different methods detect different stages of aggregation:

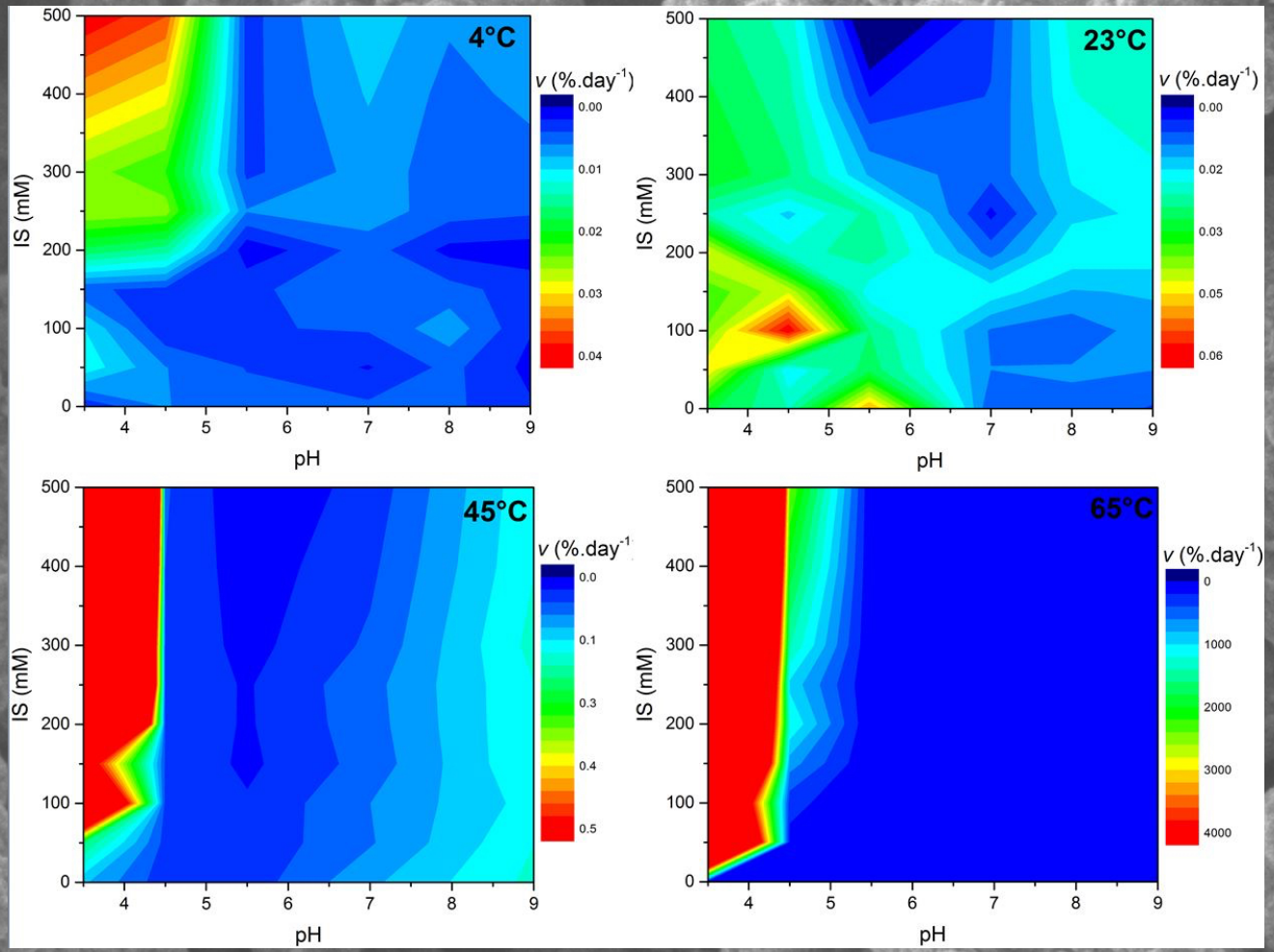
SEC - Quantify kinetics of monomer loss, small soluble aggregates
- many aggregate species so smears out in baseline.

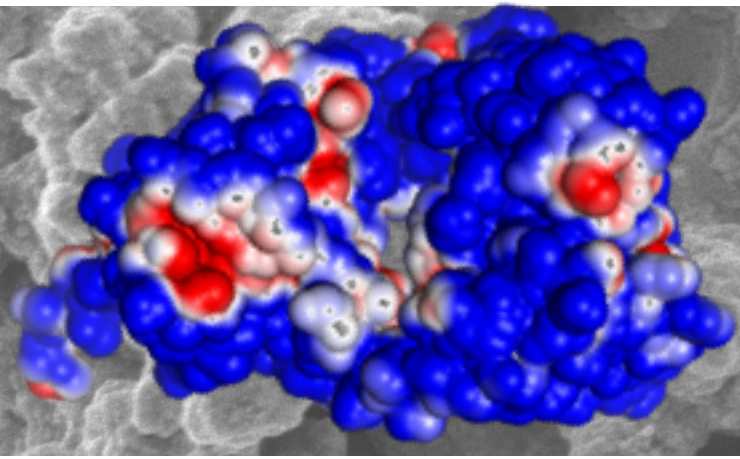
ThT – small soluble aggregates via beta-sheet interactions (no amyloid)

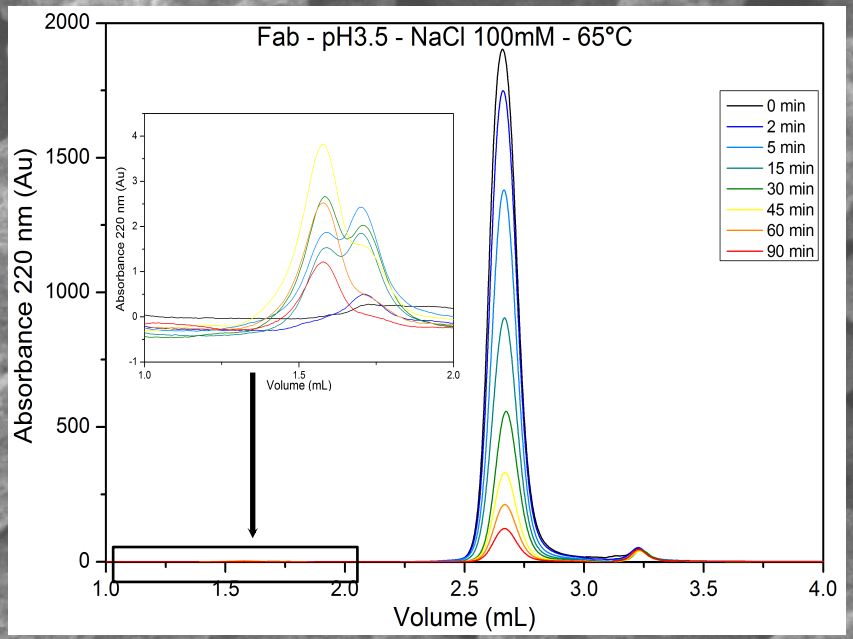
SLS – large aggregates. Sensitive, but detected late.

Limit of detection by SEC was 1% monomer loss – takes 1 year in some cases!

Heat maps of Fab aggregation kinetics at 4-65 °C







- Kinetics of native monomer loss determined for >1 year
- Range of pH, incubation T, and ionic strength

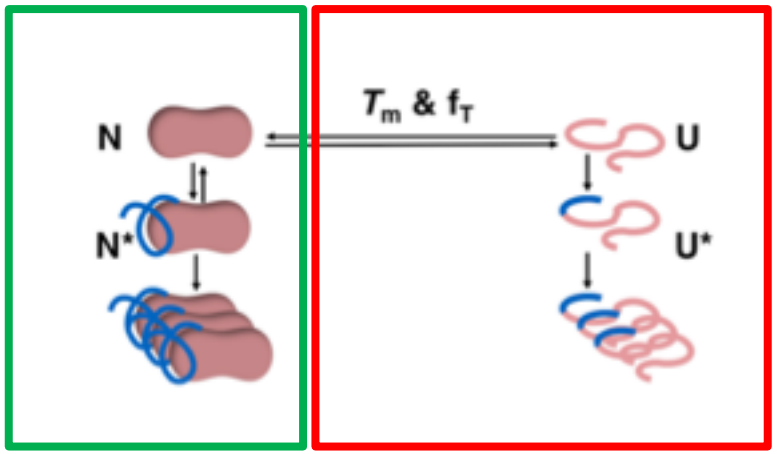
Nesrine Chakroun, David Hilton, Shahina S. Ahmad, Geoffrey W. Platt and Paul A. Dalby (2016) Molecular Pharmaceutics

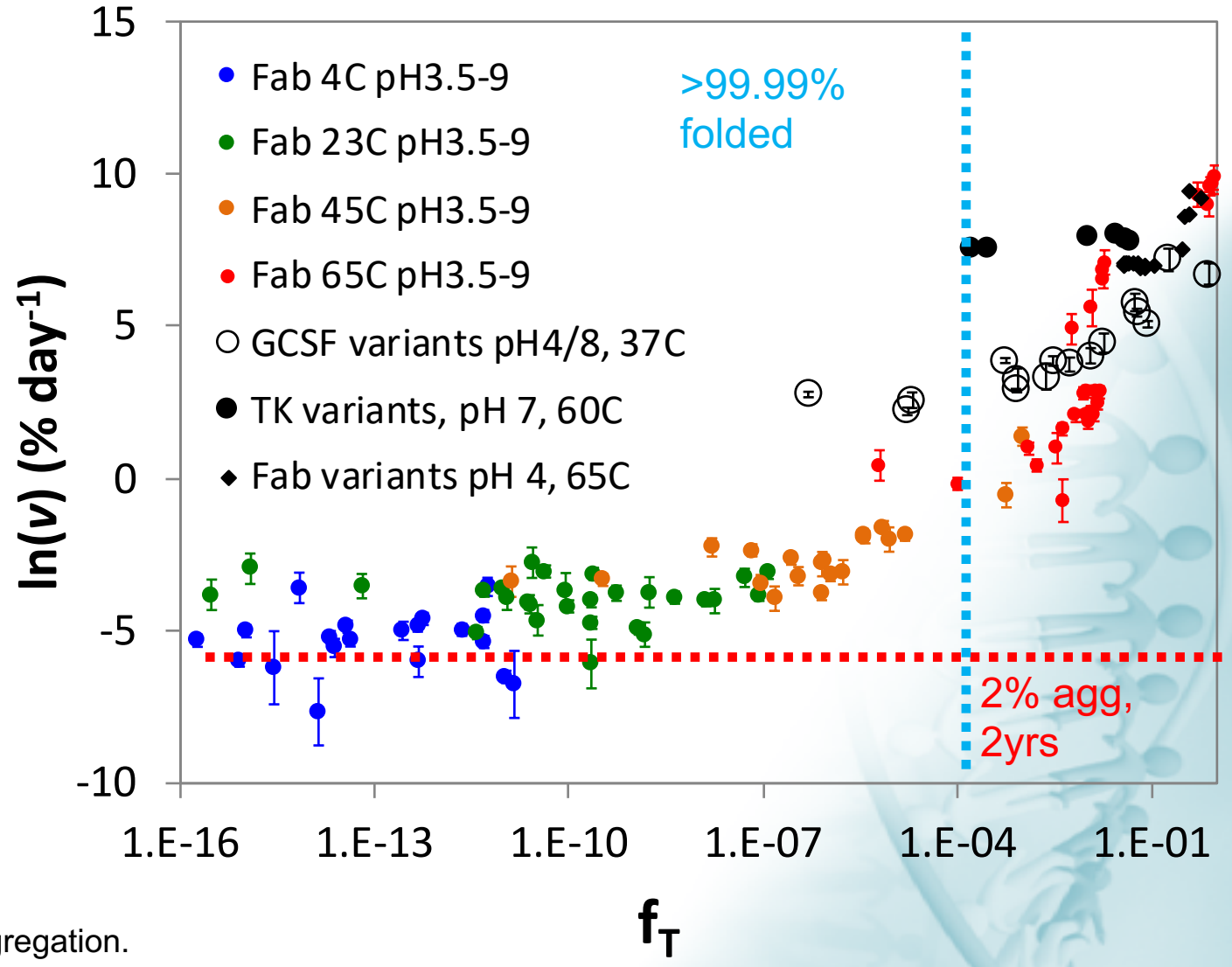
Low-T_{inc} kinetics are not correlated with T_m or high-T_{inc} kinetics

Where T_{inc} << T_m, fraction unfolded is <<0.0001:

Global unfolding (and hence T_m) is not relevant

Native ensemble dynamics & colloidal stability control aggregation kinetics.

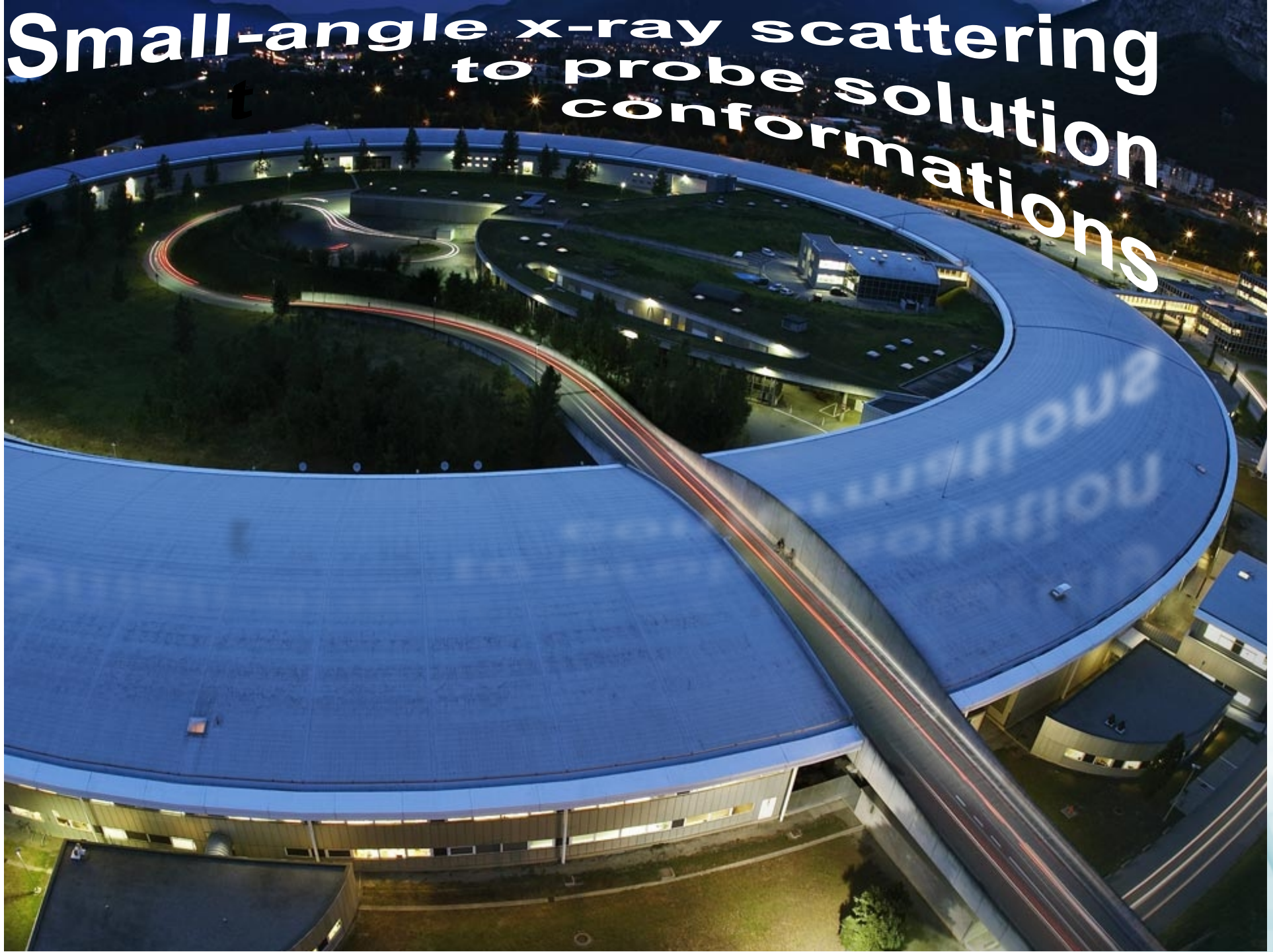




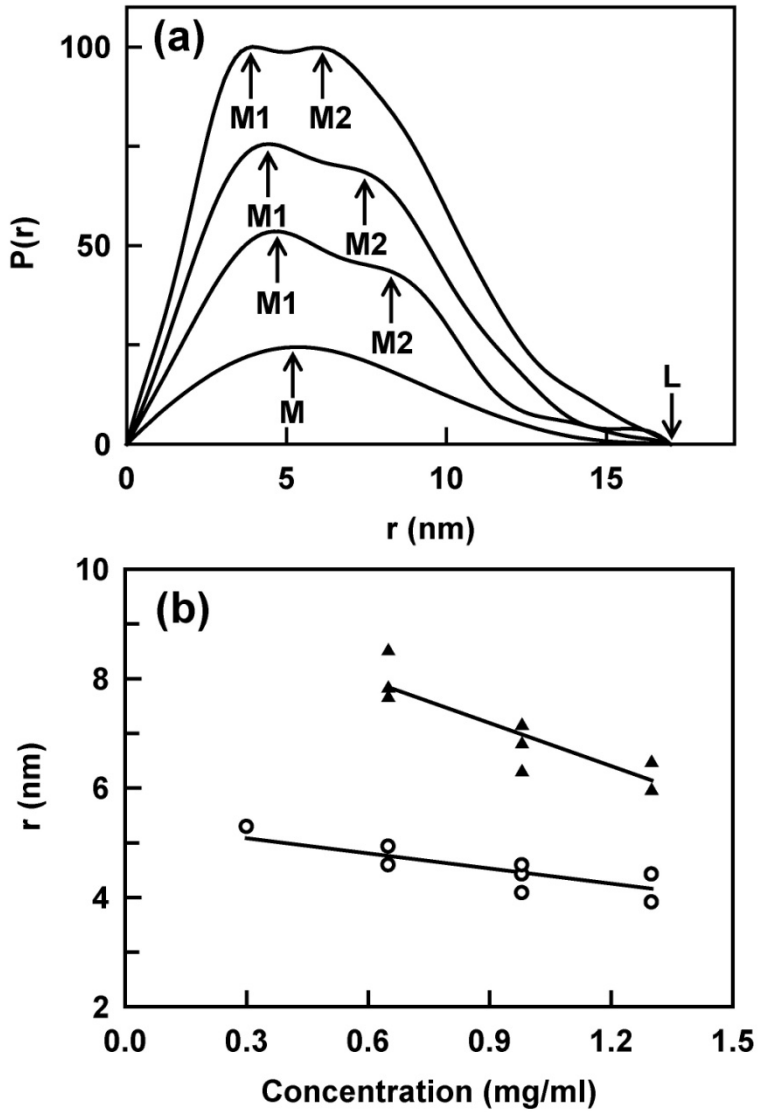
Zhang et al. (2018) Molecular Pharmaceutics. 15, 3079-3092
Robinson et al. (2018) Molecular Pharmaceutics. 15, 256-267.
Chakroun et al. (2016) Molecular Pharmaceutics. 13, 307-319.
See also Roberts (2013) – review on non-Arrhenius protein aggregation.

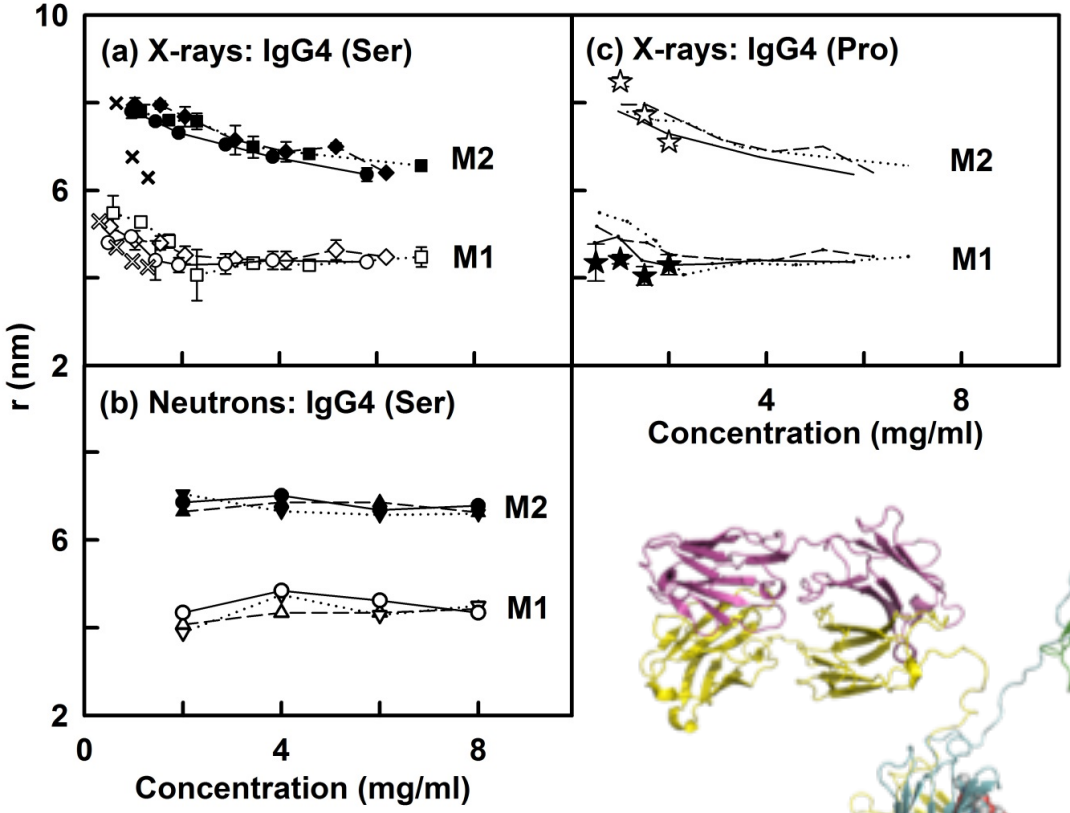
Solution structures

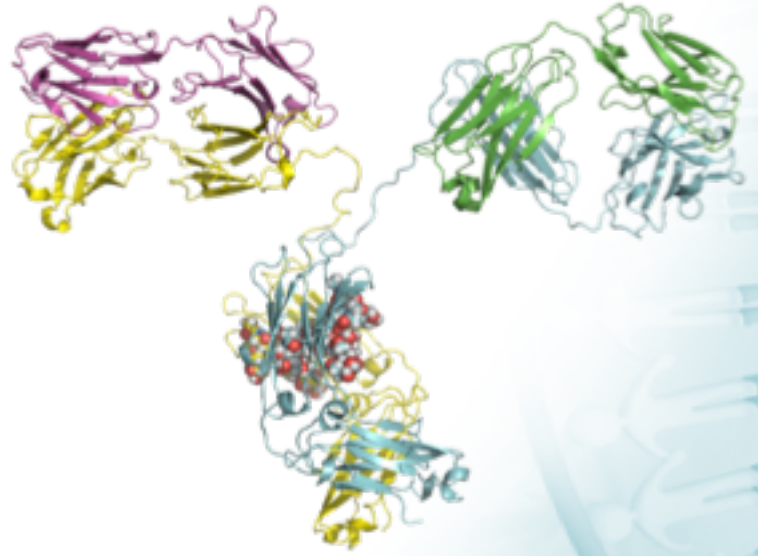
Small-angle x-ray scattering to probe solution conformations



Small-angle x-ray scattering to probe conformations







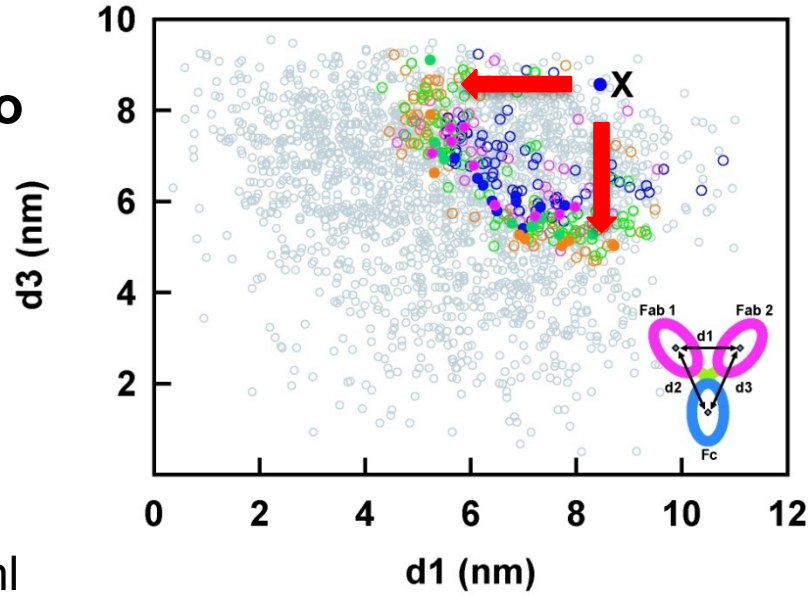
Small-angle x-ray scattering on native IgG4

IgG4 concentration affects conformation due to molecular crowding

At pH7 IgG4 becomes more compact
at higher concentrations

IgG4 conformation is asymmetric at pH7

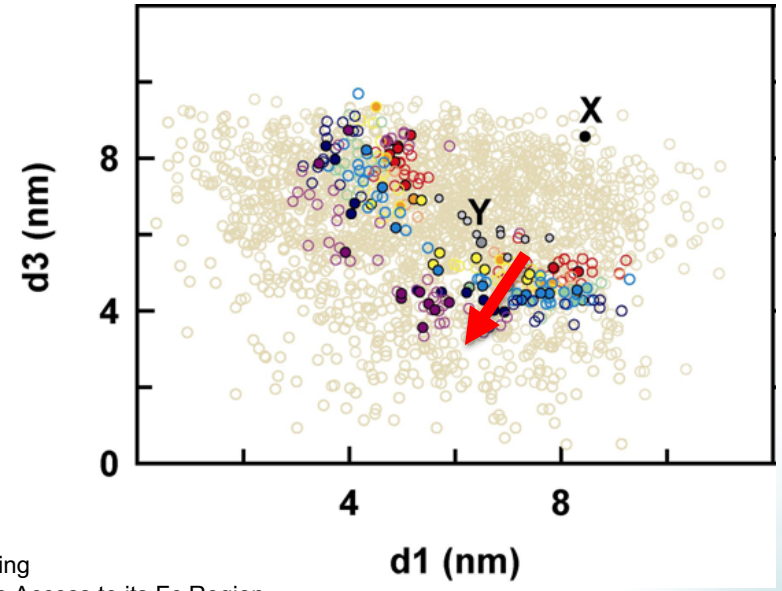
Conformational shift blocks C1q & FCγR @ >1mg/ml



pH 7
0.3-1.1 mg/ml

IgG4 conformation is also pH-dependent

pH 3 induces further compaction
prior to aggregation

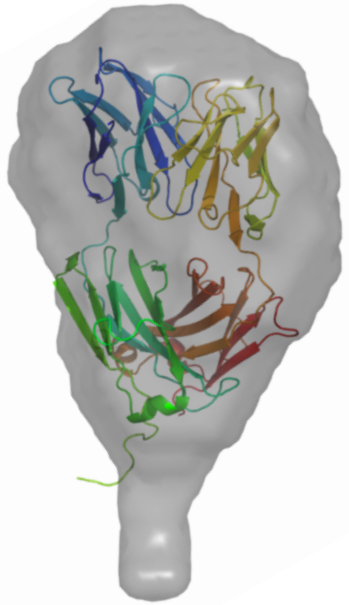


pH 3
0.3 mg/ml
t=0-210min

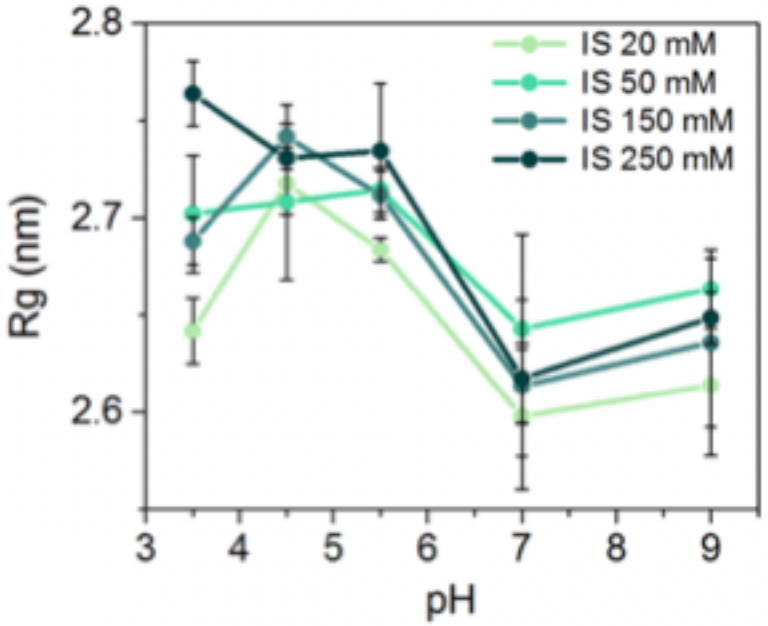
X: IgG1 crystal structure
Y: IgG4 at pH 7.4, 0.3 mg/ml

Abe et al (2010) *Biochem J* 432:101. Masking of the Fc region in human IgG4 by constrained X-ray scattering modelling
Rayner et al (2014) *JBC*. 289:20740-20756. The Fab Conformations in the Solution Structure of Human IgG4 restricts Access to its Fc Region
Rayner et al (2015) *JBC*. Solution structures of two human IgG1 antibodies show conformational stability and accommodate their C1q and FCγR ligands.

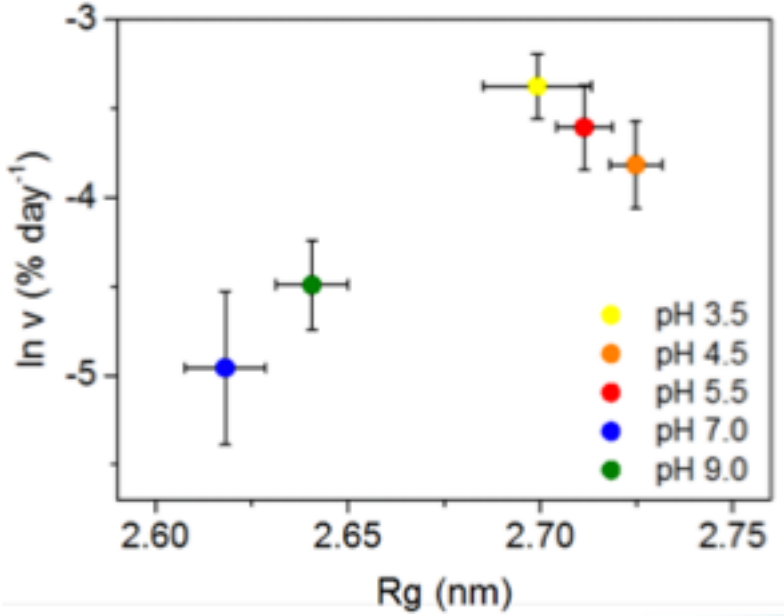
Small-angle x-ray scattering of Fab under native conditions



pH-dependent conformation



Aggregation kinetics correlate

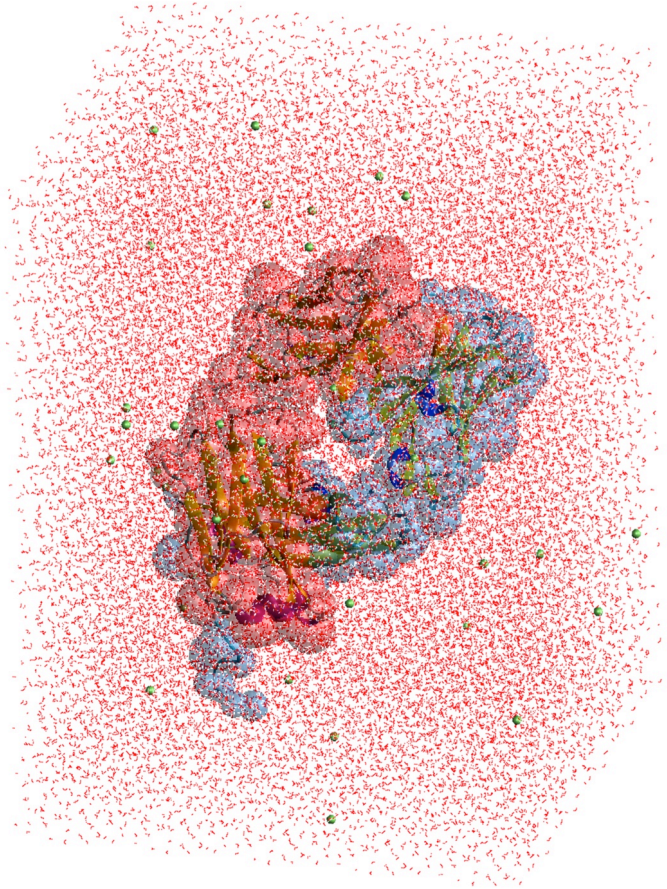


- Conformational change with pH correlates with aggregation kinetics, at 23 ◦C

Codina at al. 2019 *JMB*

Molecular Dynamics Simulation

Molecular dynamics simulation for Fab



Equilibrium RMSF (300K)
- pH7, 25°C, 50ns, 50mM IS
- pH3.5, 25°C, 50ns, 50 mM IS
- OPLS-AA/L force field & SPC/E water
- Triplicated

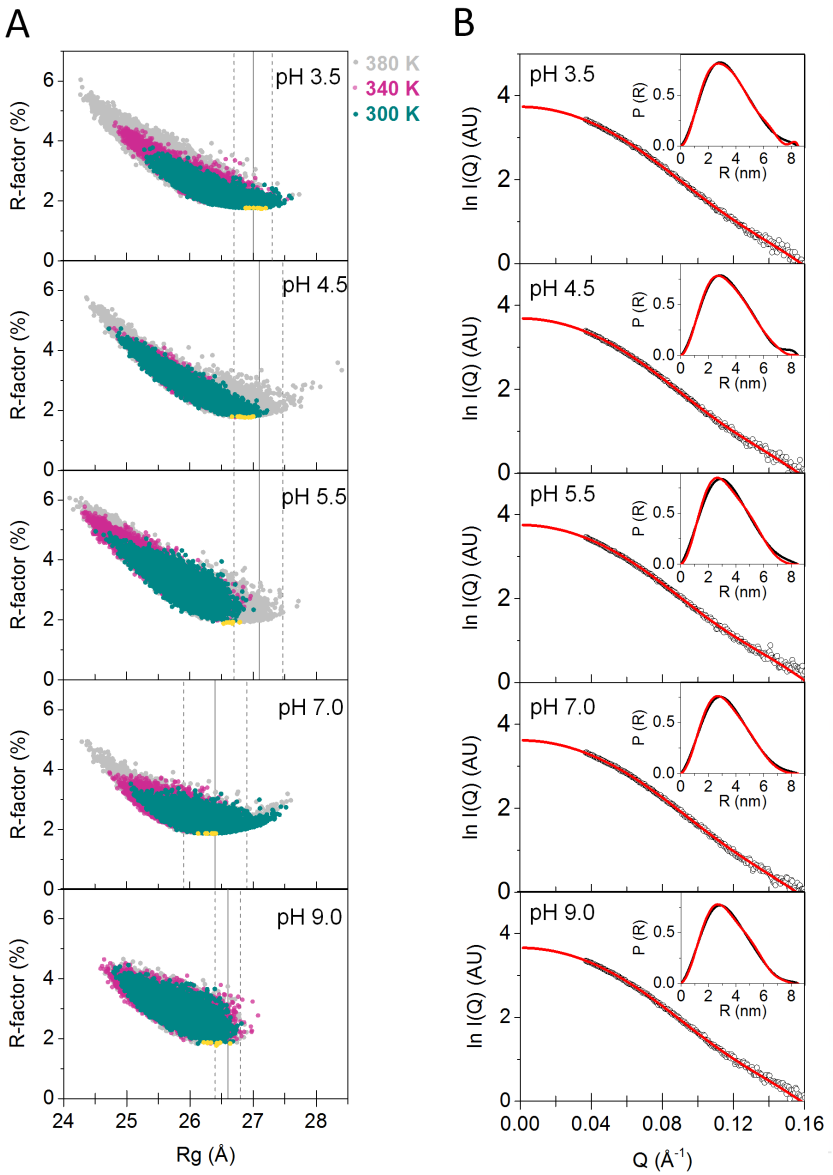
pH 7

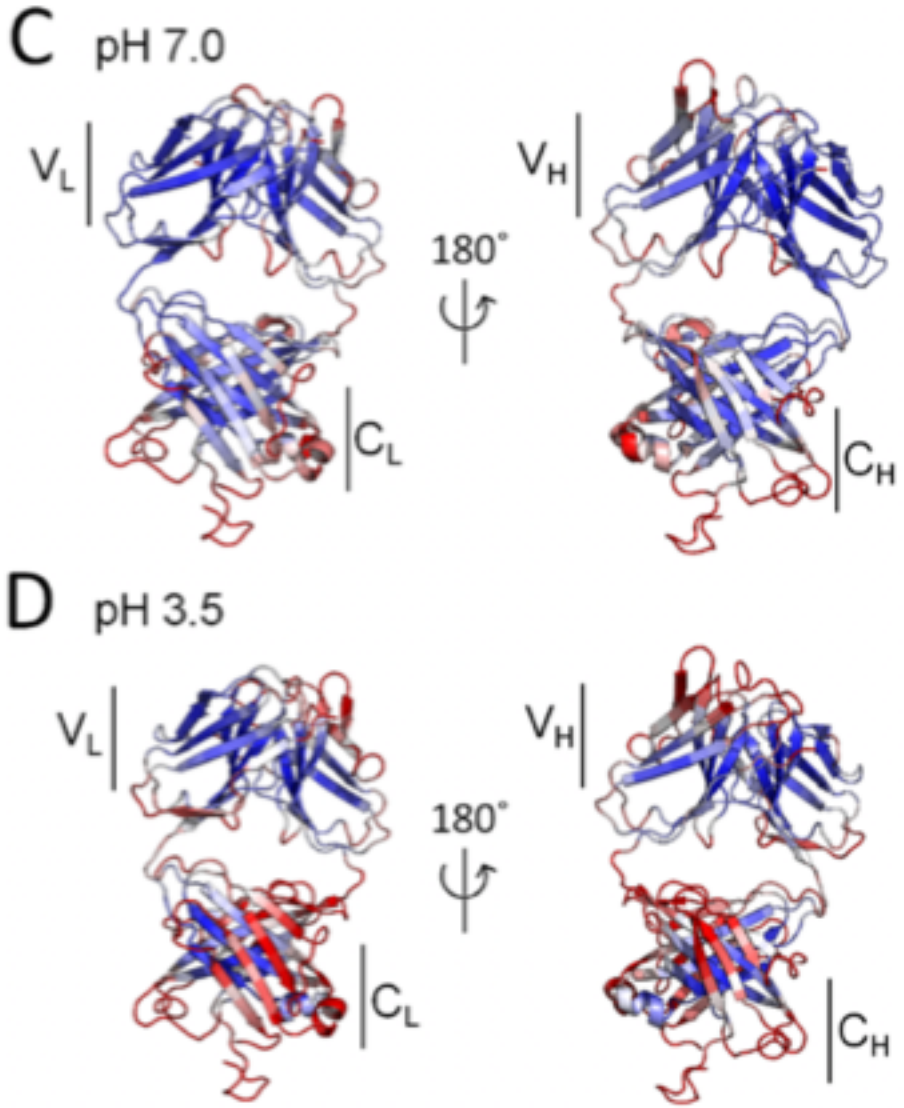
pH 3.5

CL domain displacement

Codina at al. 2019 *JMB*

Fitting SAXS to molecular dynamics simulation frames

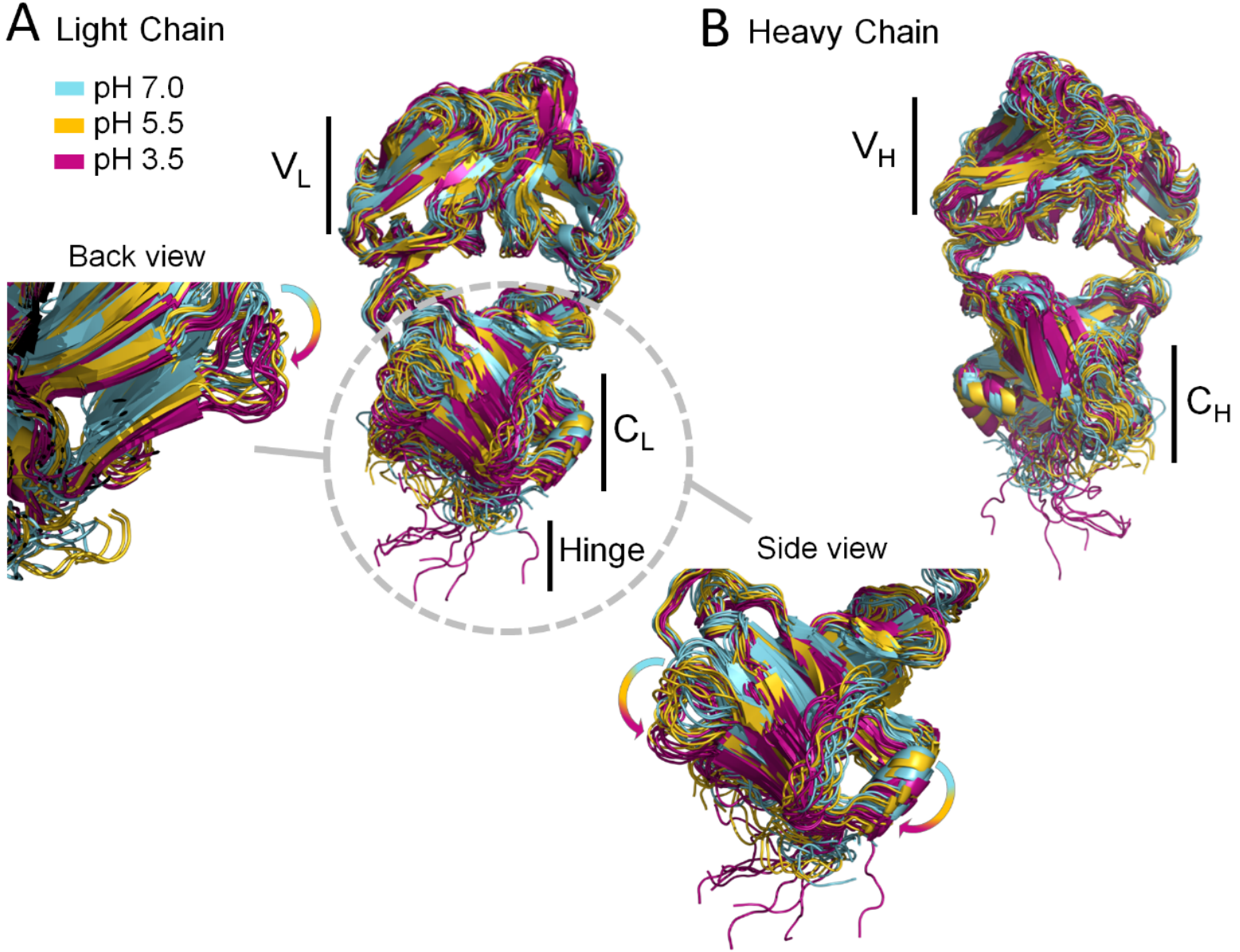




Reveals dynamics and conformational shift with pH under native equilibrium conditions

Codina at al. 2019 *JMB*

Fitting SAXS to molecular dynamics simulation frames



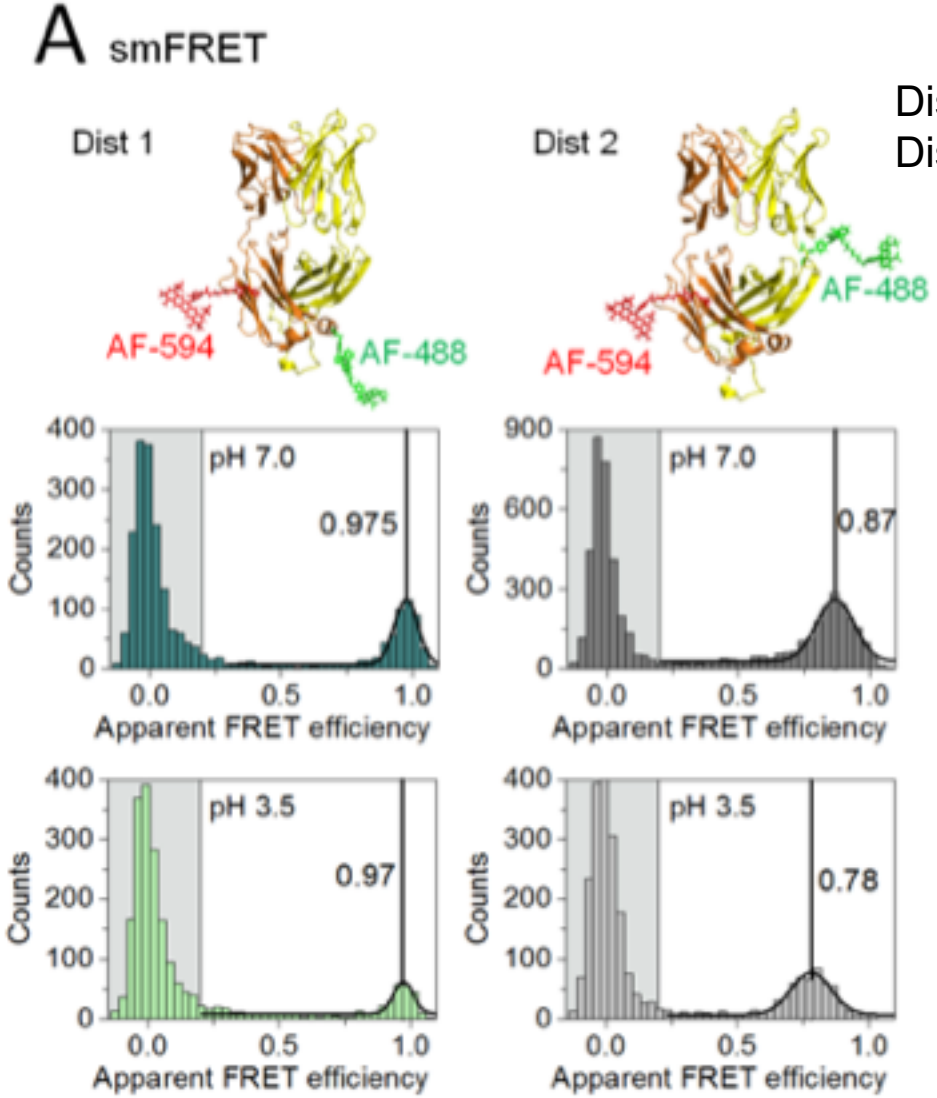
CL domain displacement

Codina at al. 2019 *JMB*

Single-molecule fluorescence

- Confirm C_L domain displacement
- Determine whether there are multiple populations or just one

smFRET analysis of pH-dependent Fab conformations



Dist 1: LC-K126pAzF + LC-S156C: CL to CL domain
Dist 2: HC-S117pAzF + LC-S156C: CL to HC linker

pH 7: Both distances show a single population.

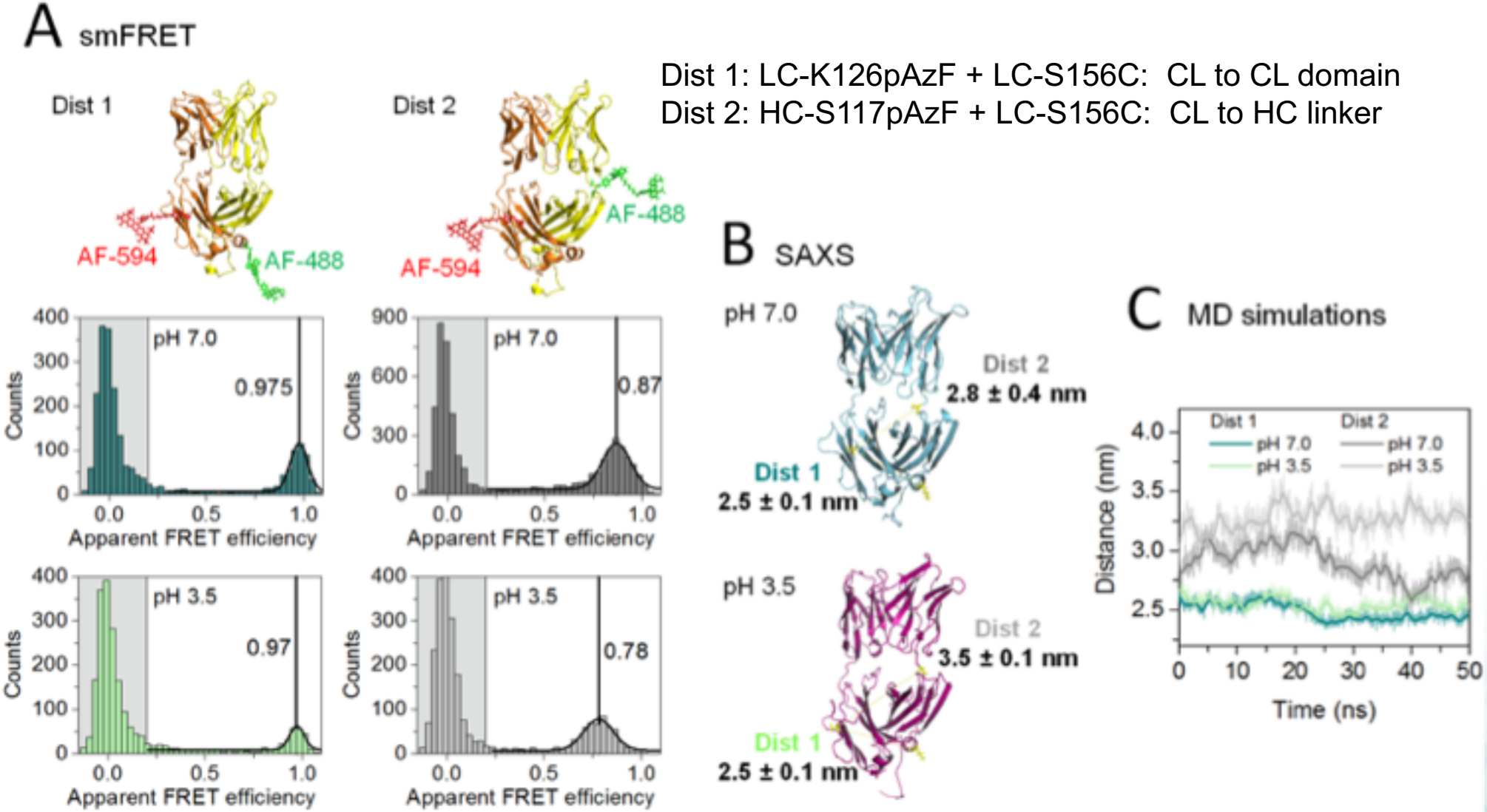
pH 3.5: Dist 2 increased. Dist 1 stayed same. Single population.

smFRET, SAXS and MD reveal the same dynamics and conformational shift with pH

Codina at al. 2019 *JMB*

smFRET analysis of pH-dependent Fab conformations

Dist 1: LC-K126pAzF + LC-S156C: CL to CL domain
Dist 2: HC-S117pAzF + LC-S156C: CL to HC linker

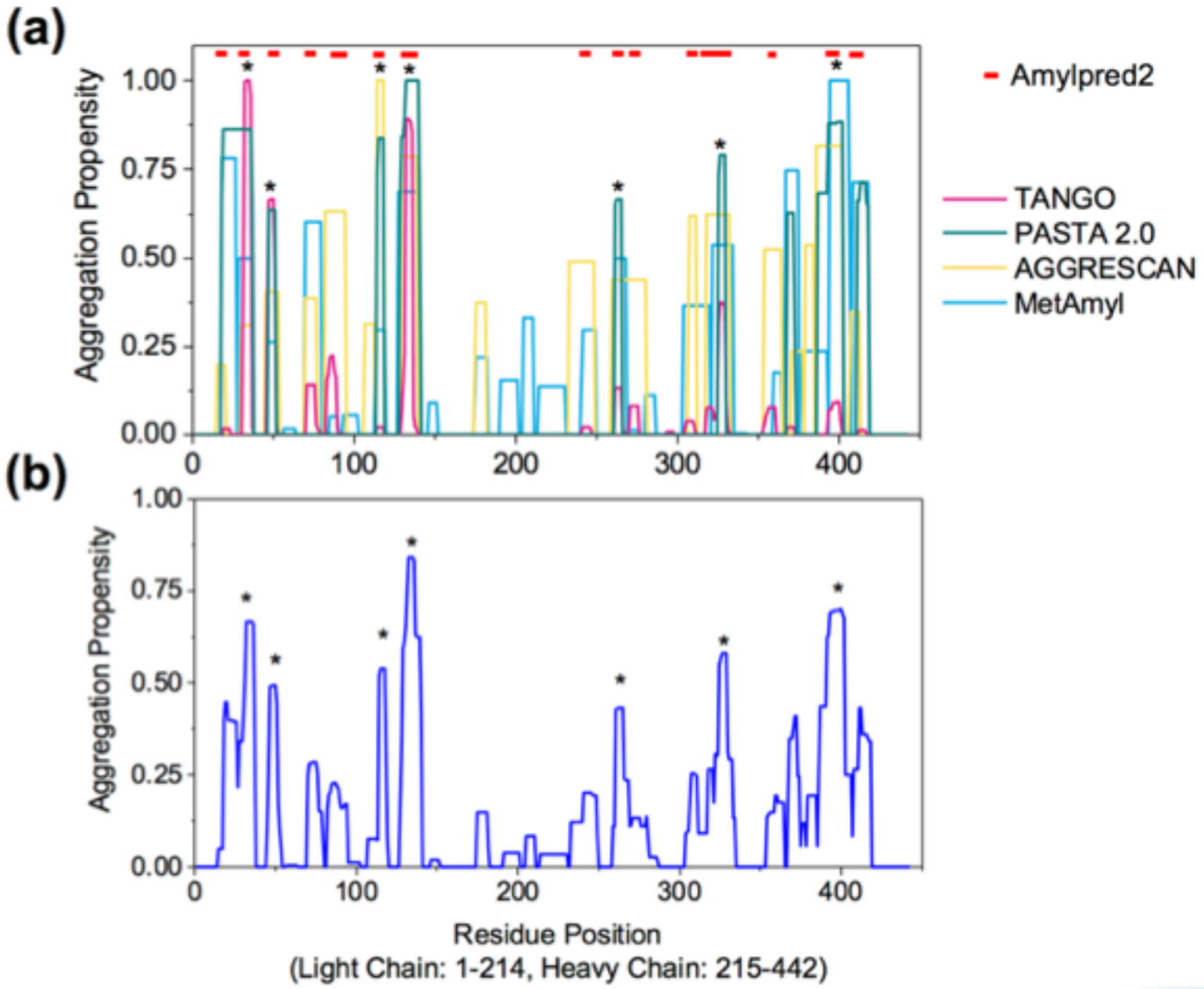


smFRET, SAXS and MD reveal the same dynamics and conformational shift with pH

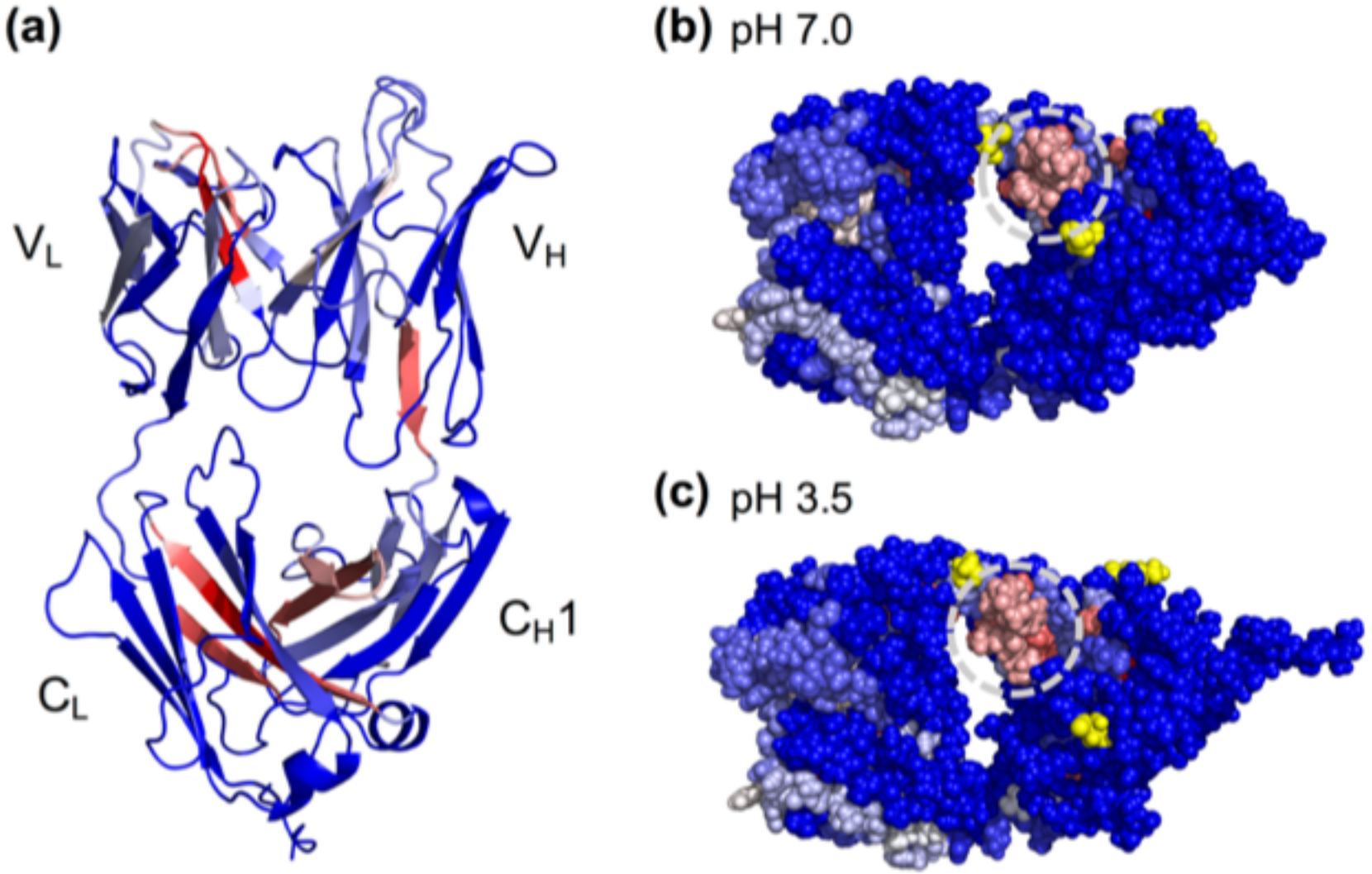
Codina at al. 2019 *JMB*

APR calculation

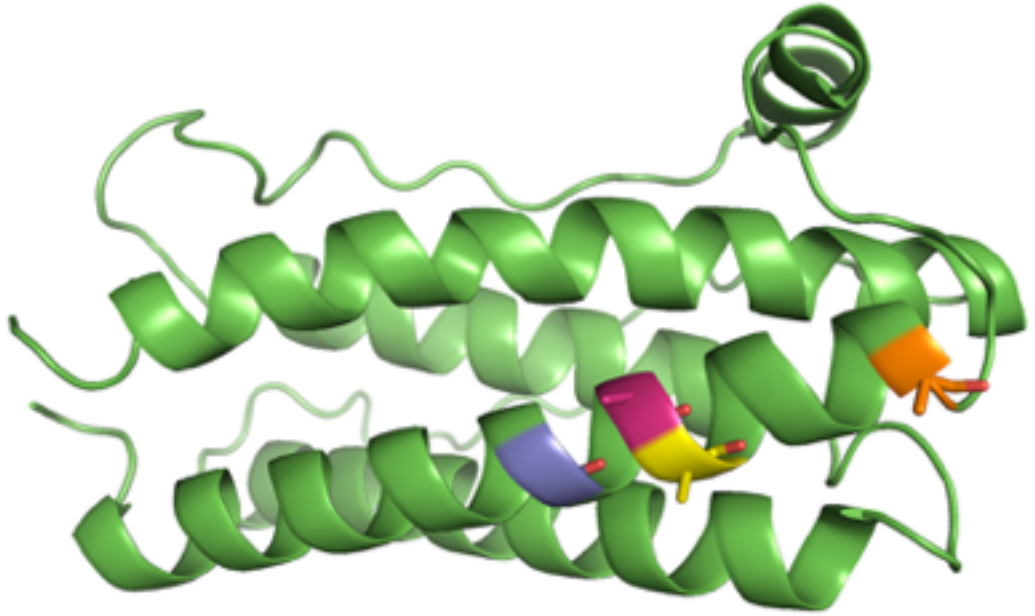
Consensus of several sequence-based APR prediction tools



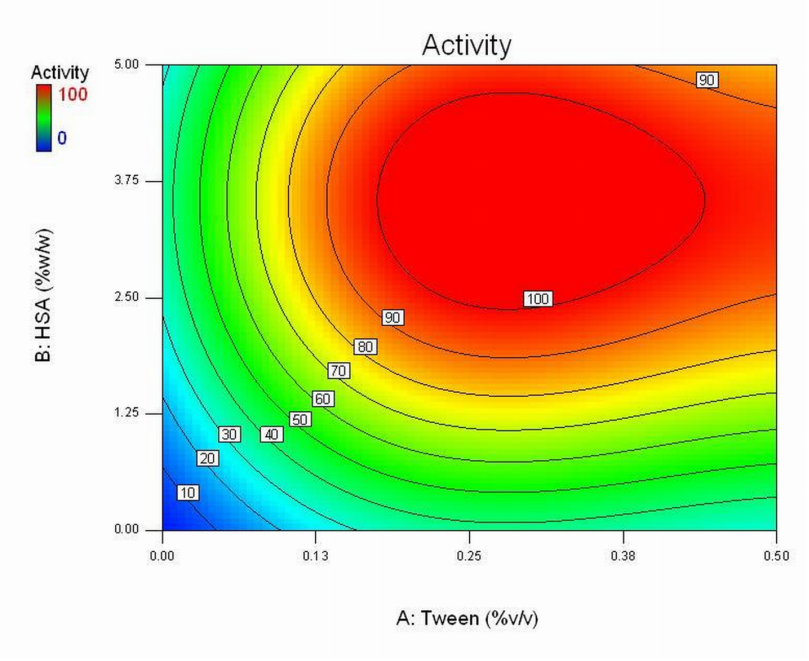
Best-fit SAXS structures reveal APR exposure at low pH



Codina at al. 2019 *JMB*



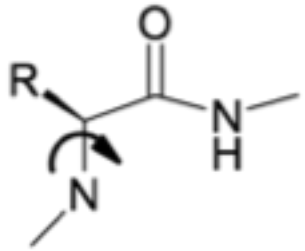
Protein engineering and formulation



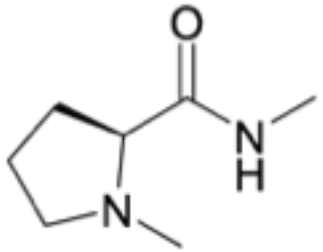
Engineering protein dynamics

- Mutations probe relationship between dynamics and both equilibrium (T_m) and kinetic (aggregation) stability
- Can potentially minimize aggregation through selective mutations.

Eg. The proline rule: Prolines have reduced backbone flexibility (entropy)



rotation about C-N bond



restricted rotation in proline

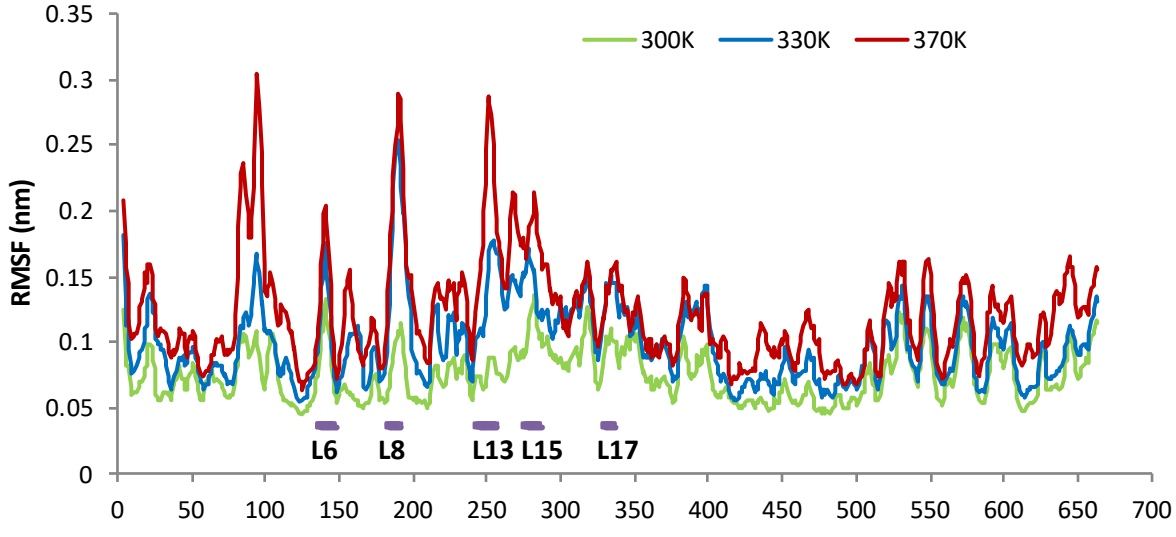
Insert prolines into flexible loop regions to reduce entropy

T4 lysozyme (A82P in b-turn for ΔΔG=0.8 kcal/mol): Matthews et al (1987), PNAS **84**, 6663-6667

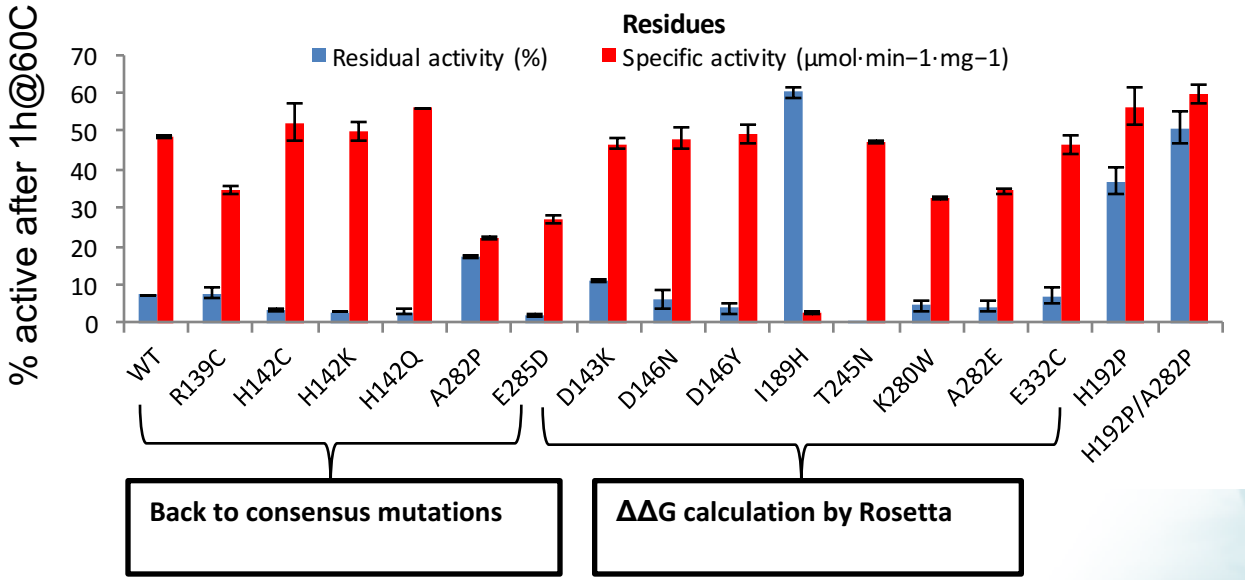
Oligo-1,6-glucosidase (12 prolines accumulated for ΔΔG=3.7 kcal/mol)
Suzuki, Y (2004) The Proline Rule pp293-32, in Enzyme functionality, A.Svendsen (Ed), Marcel Dekker Inc

Protein engineering guided by molecular dynamics simulation

Identify flexible sites



Redesign using ROSETTA

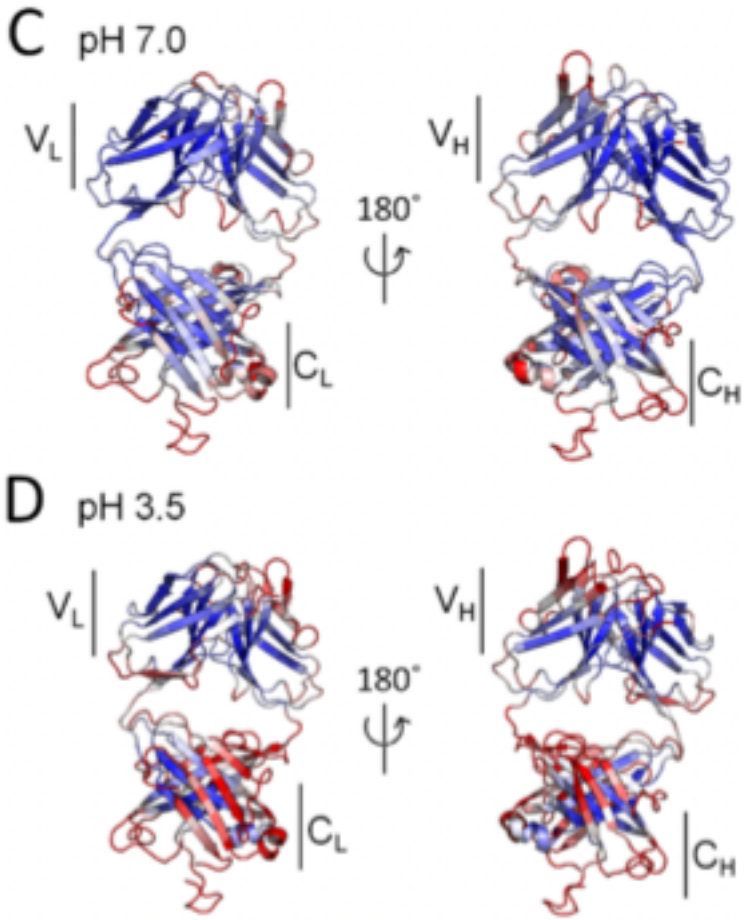


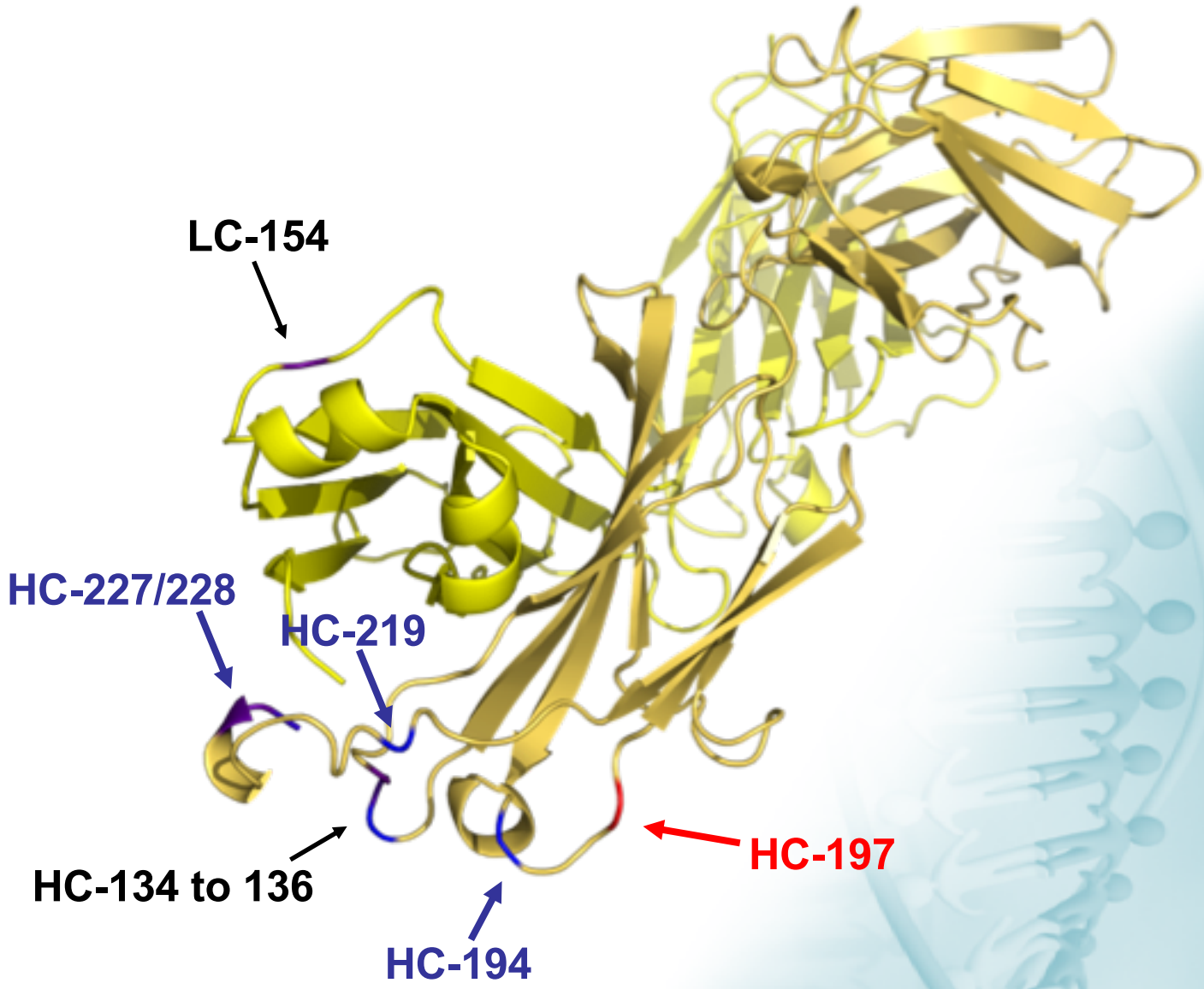
Worked for TK enzyme:
Increased T_m, decreased inactivation.

Should work for Fab aggregation?

Yu, Yan, Zhang, Dalby (2017) Two strategies to engineer flexible loops for improved enzyme thermostability. *Scientific Reports*, 7, 41212.

MD-guided protein engineering to slow aggregation

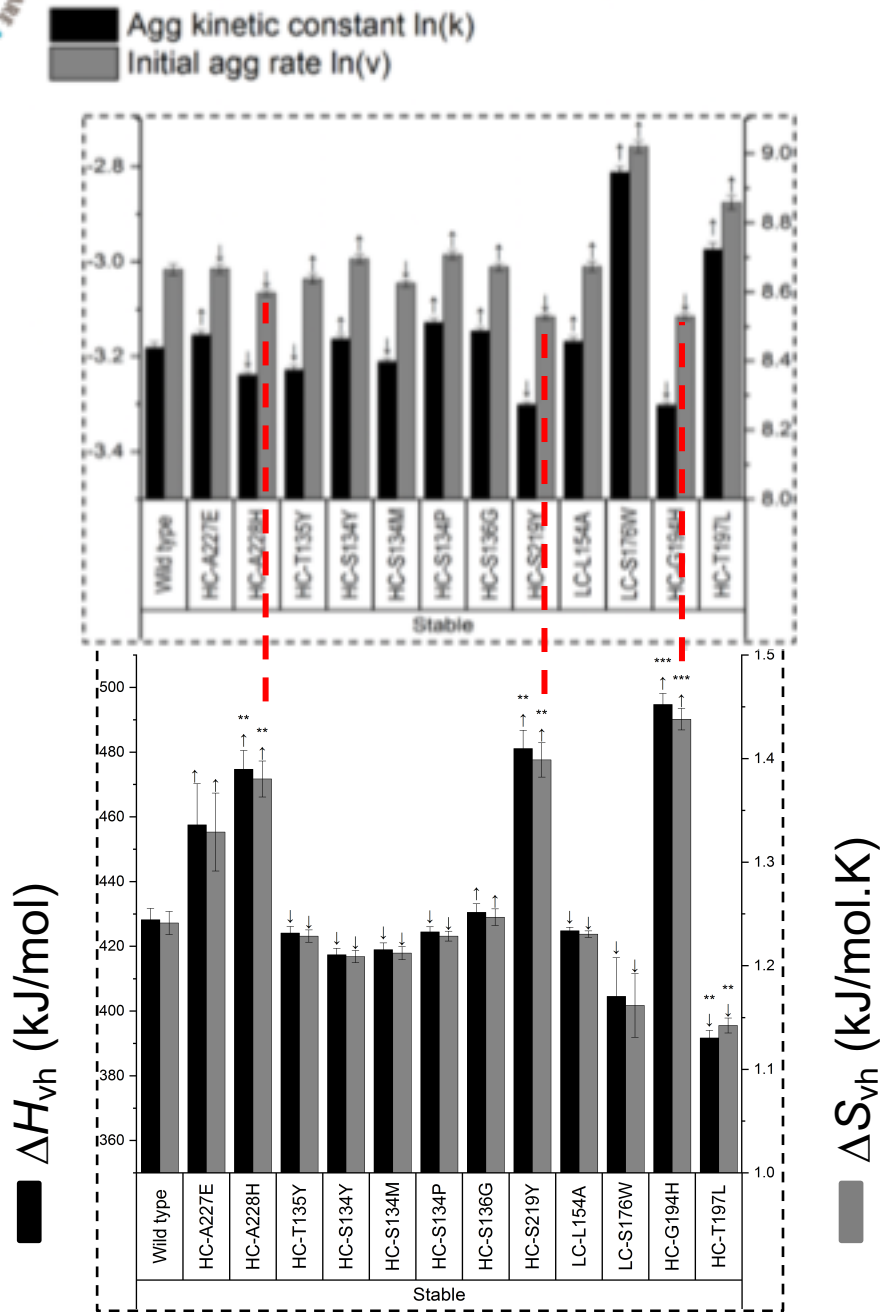


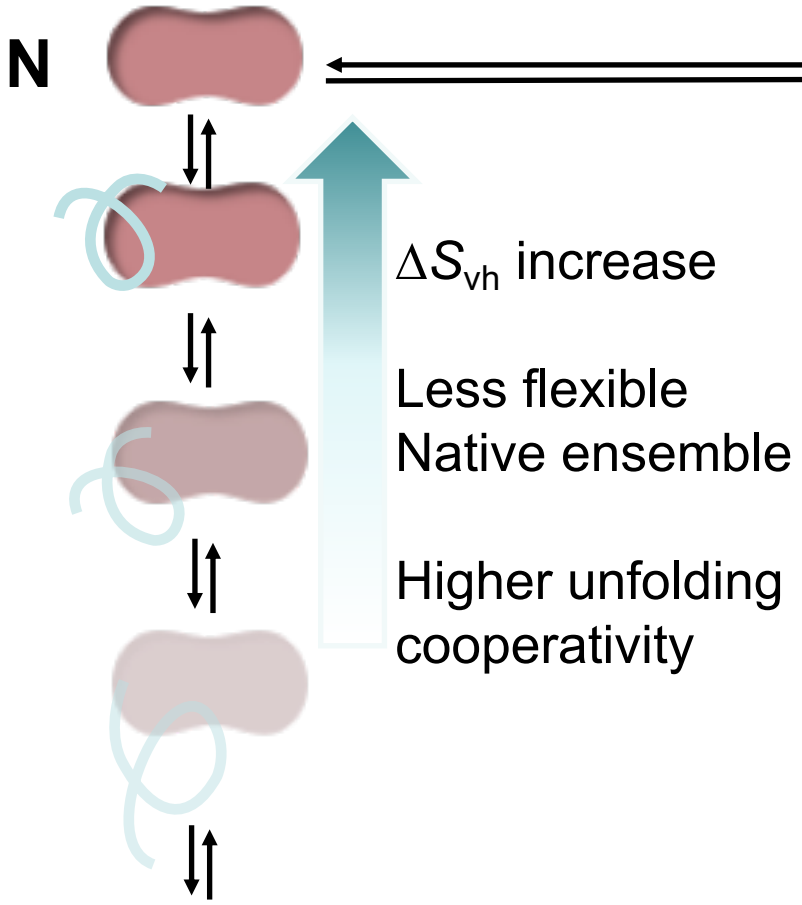


Designed 12 stabilising and 5 destabilising mutations using ROSETTA at flexible sites.

Zhang et al. (2018) Computational-design to reduce conformational flexibility and aggregation rates of an antibody Fab fragment. *Molecular Pharmaceutics*. 15, 3079-3092

Decreased flexibility of hinge/CH slowed aggregation





T_m did not increase, but ΔS_{vh} did increase.
Aggregation slowed where ΔS_{vh} increased.

Take-home messages

T_m does not reflect aggregation from native population

Aggregation from Native-like state(s) dependent on:

- local dynamics/unfolding
- exposure of aggregation hotspots (APRs)
- colloidal stability

Local dynamics and aggregation hotspots can be predicted computationally

Mutations that suppress dynamics sometimes decrease aggregation kinetics

Excipient interactions and effect on T_m or T_{agg} can be predicted by molecular docking

Formulations can be optimized better by increasing T_m and decreasing native dynamics

Co-formulation of proteins – how do we use knowledge and methods from single proteins?

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