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

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• 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
- Local Dynamics / Partial unfolding
- Global Conformational Stability
- 3° / 4° conformation
- Surface properties: charge/hydrophobicity
- Sequence features eg. APRs

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

- 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

- \( T_m \)
- \( \Delta H_{vh} \)
- 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
Low-$T_{inc}$ kinetics are not correlated with $T_m$ or high-$T_{inc}$ kinetics

Where $T_{inc} \ll T_m$, 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
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
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

IgG4 conformation is also pH-dependent

pH 3 induces further compaction prior to aggregation


Rayner et al (2015) JBC. Solution structures of two human IgG1 antibodies show conformational stability and accommodate their C1q and FCγR ligands.
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

A Light Chain

B Heavy Chain

Back view

Side view

CL domain displacement

Codina et 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

A smFRET

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
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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

(a) Aggregation Propensity

(b) Aggregation Propensity

Residue Position

(Light Chain: 1-214, Heavy Chain: 215-442)
Best-fit SAXS structures reveal APR exposure at low pH

Codina et 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 vs restricted rotation in proline]

Insert prolines into flexible loop regions to reduce entropy

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

Oligo-1,6-glucosidase (12 prolines accumulated for $\Delta\Delta G=3.7$ kcal/mol)
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?

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 $\Delta S_{vh}$ did increase. Aggregation slowed where $\Delta S_{vh}$ 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?
UG/PhD/PDRA: where are they now?
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