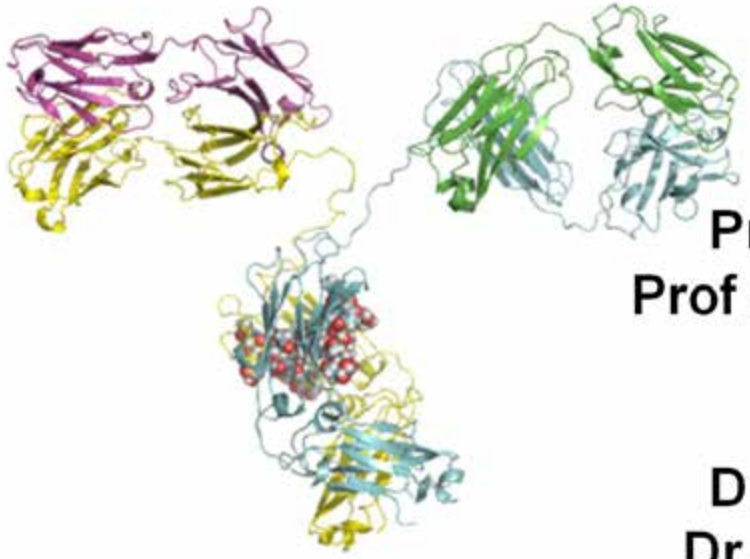
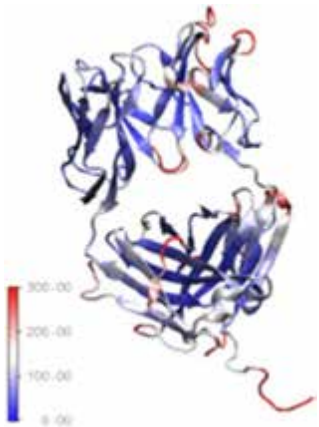
Enabling rapid liquid and freeze-dried formulation design for the manufacture and delivery of novel biopharmaceuticals





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EPSRC EP/N025105/1

Introduction

- ***Biopharmaceuticals market*** *is rapidly growing with reported sales of £197 billion in 2016 (compared with total drug market of £816 billion)*

- *Next generation therapies are increasingly complex and engineered for biological activity at the expense of physical and chemical stability (eg protein fusions, fragments, conjugates with small drug molecules)*

- ***Formulation development of biopharmaceuticals***

  1. *Major challenge: dosage forms are required for clinical trials which fixes formulation at an early stage. Development stages occur early in the therapeutic lifetime when not much material is available*

  2. *Formulations require stability, potency, and ease of delivery to patient*

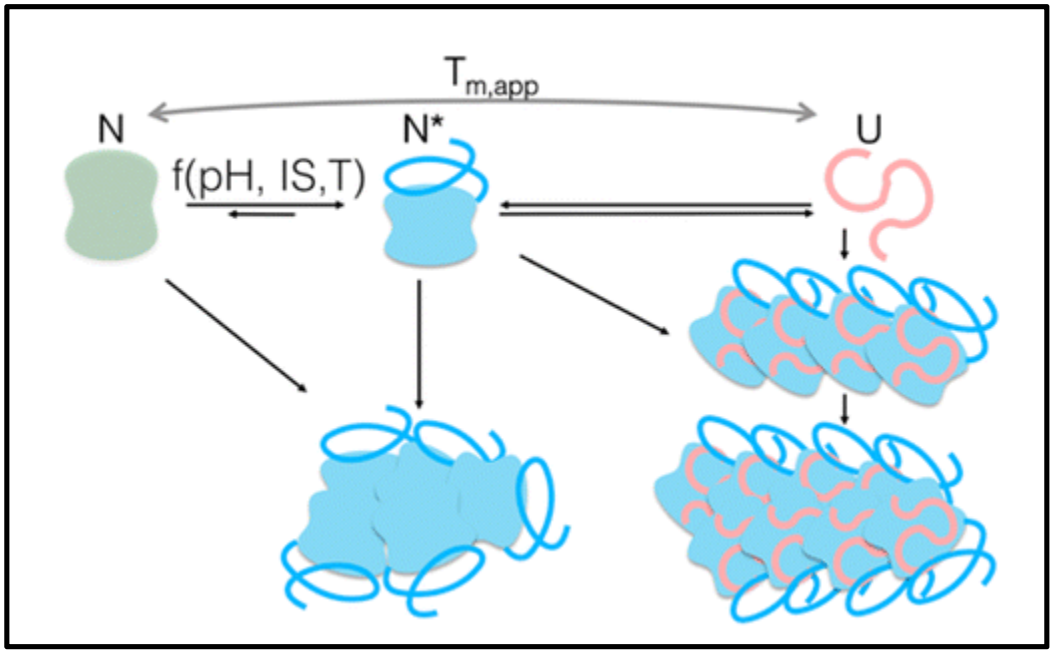
  3. *Chemical and physical degradation pathways compromise stability*

  4. *Many therapeutics are required at high concentrations which leads to increased physical degradation, poor rheological properties, and phase separation*

Protein aggregation

- ***Predicting and controlling aggregation is an outstanding challenge:***

1. *Key intermediates are transient and occur at very low relative populations*
2. *Key steps in aggregation pathways are difficult to isolate*
3. *Multiple mechanisms for aggregate formation and aggregate growth that depend on protein and environmental conditions (solvent properties, temperature)*



- ***Predictive approaches are indirect***

1. *Use surrogate parameters such as unfolding temperature or free energy, colloidal stability (eg aggregation temperatures and protein-protein interaction measurements)*
2. *Accelerated aggregation using Arhenius-type extrapolations*

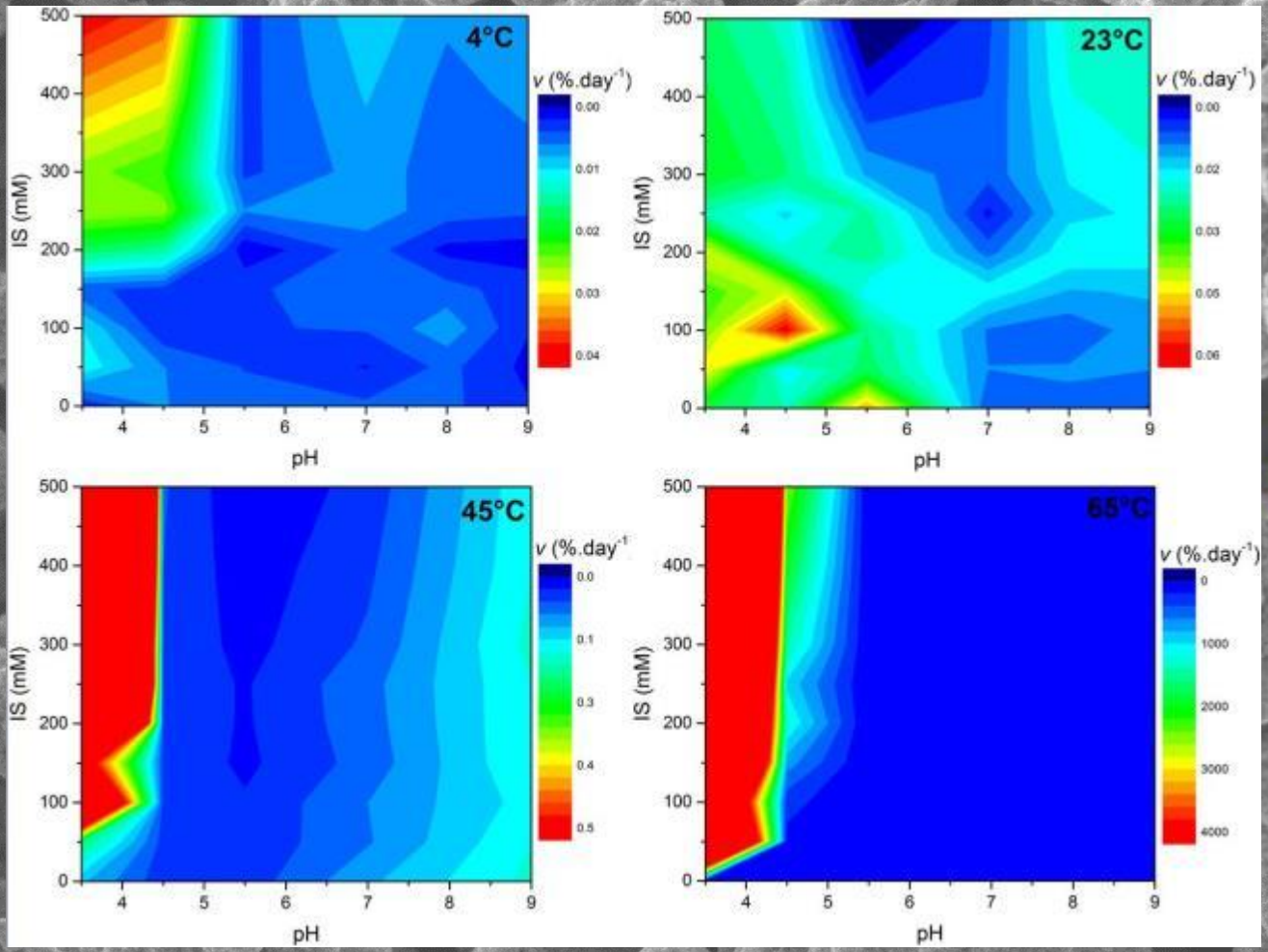
Objectives

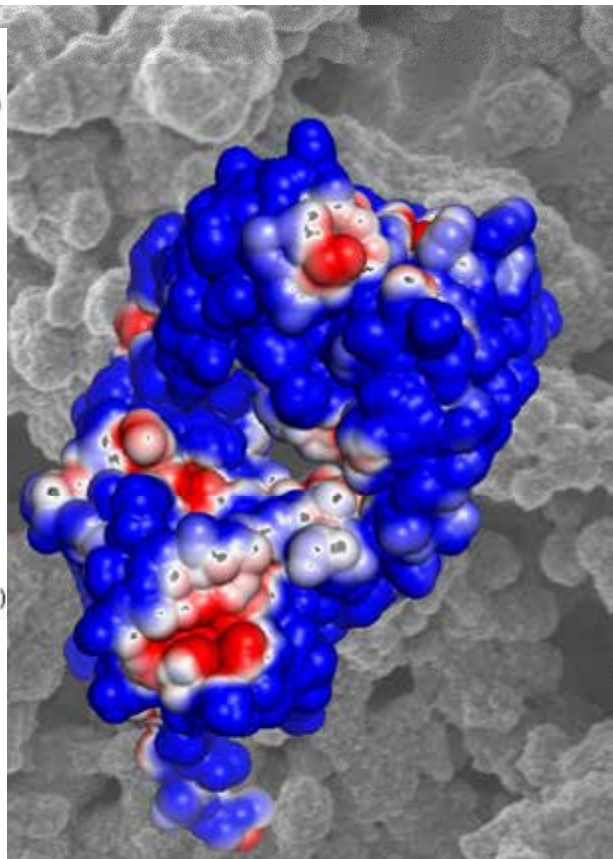
O1. Use high-throughput automation to generate a large experimental formulation dataset for protein:excipient combinations, that will include aggregation kinetics, conformational stability, colloidal stability, phase behaviour, and rheology measurements.

O2. Molecular informatics and modelling will improve predictability of formulation attributes and excipient effects

O3. Analytical advances will enable earlier, more sensitive, and lower-volume assessments of formulated protein degradation kinetics.

Humanised Fab aggregation is pH-dependent

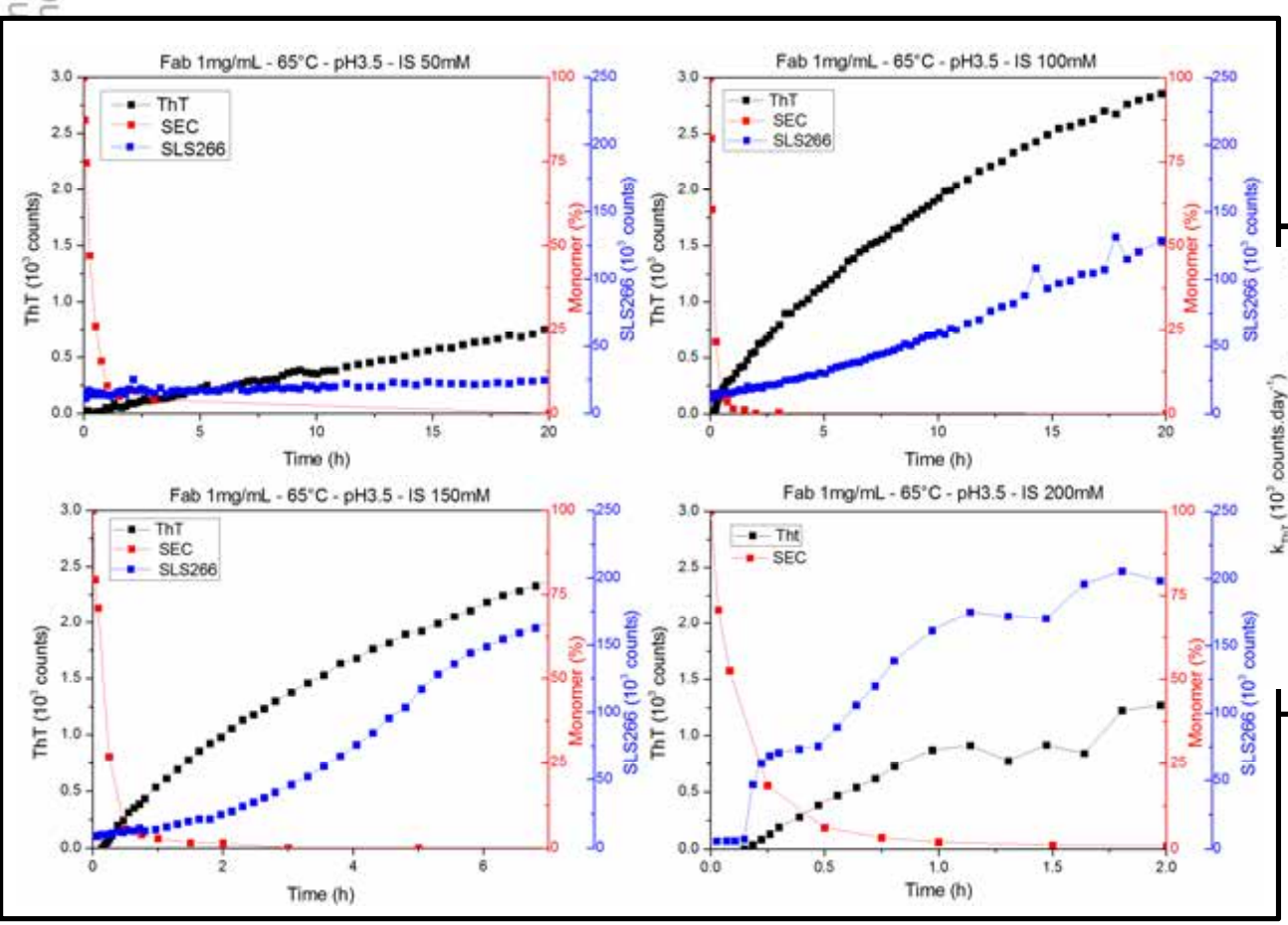


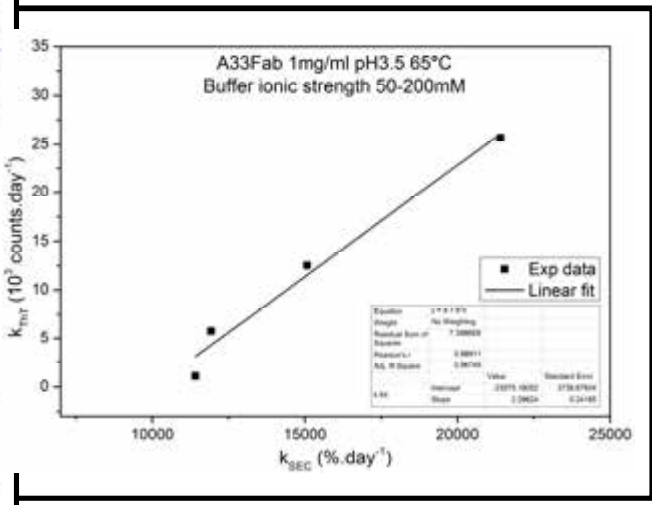


- Kinetics of native monomer loss determined for >1 year
- Range of pH, incubation T, and ionic strength

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

Kinetics of Fab aggregation





- *Native monomer loss (SEC) precedes small IM (ThT), then large aggregate (SLS)*
- *pH <4.5, low IS stops at IM aggregates.*
- *pH 8 & 9 forms large insoluble aggregates (pI = 8.4), no small IM (ThT)*

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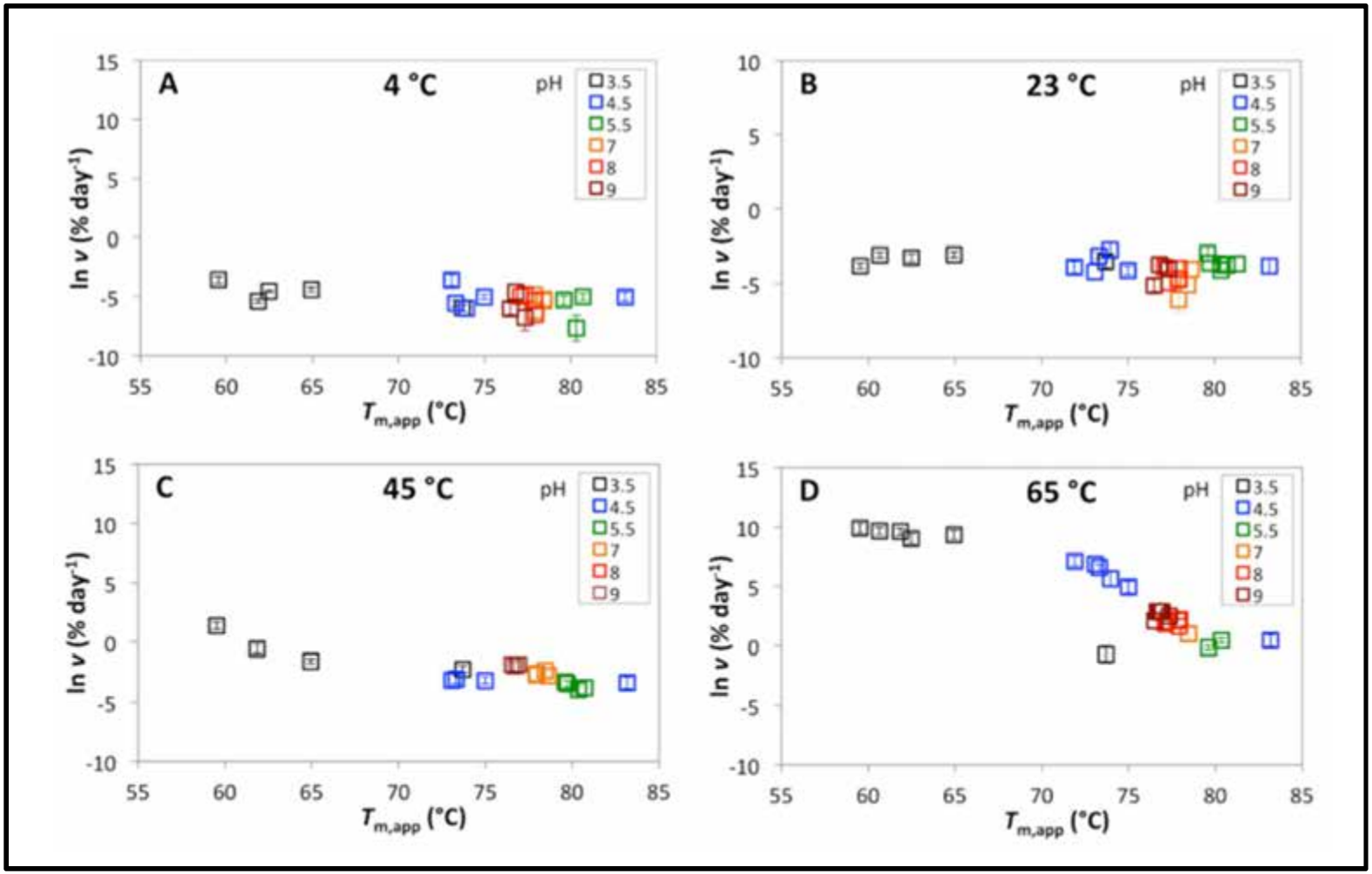
Does T_m predict Fab aggregation rate?

A33 Fab



- *T_m predicts aggregation rate only where protein unfolds, ie. close to T_m.*
- *T_m dependence is weak at low T storage conditions.*
- *Formulation optimisation is currently very dependent on this approach.*

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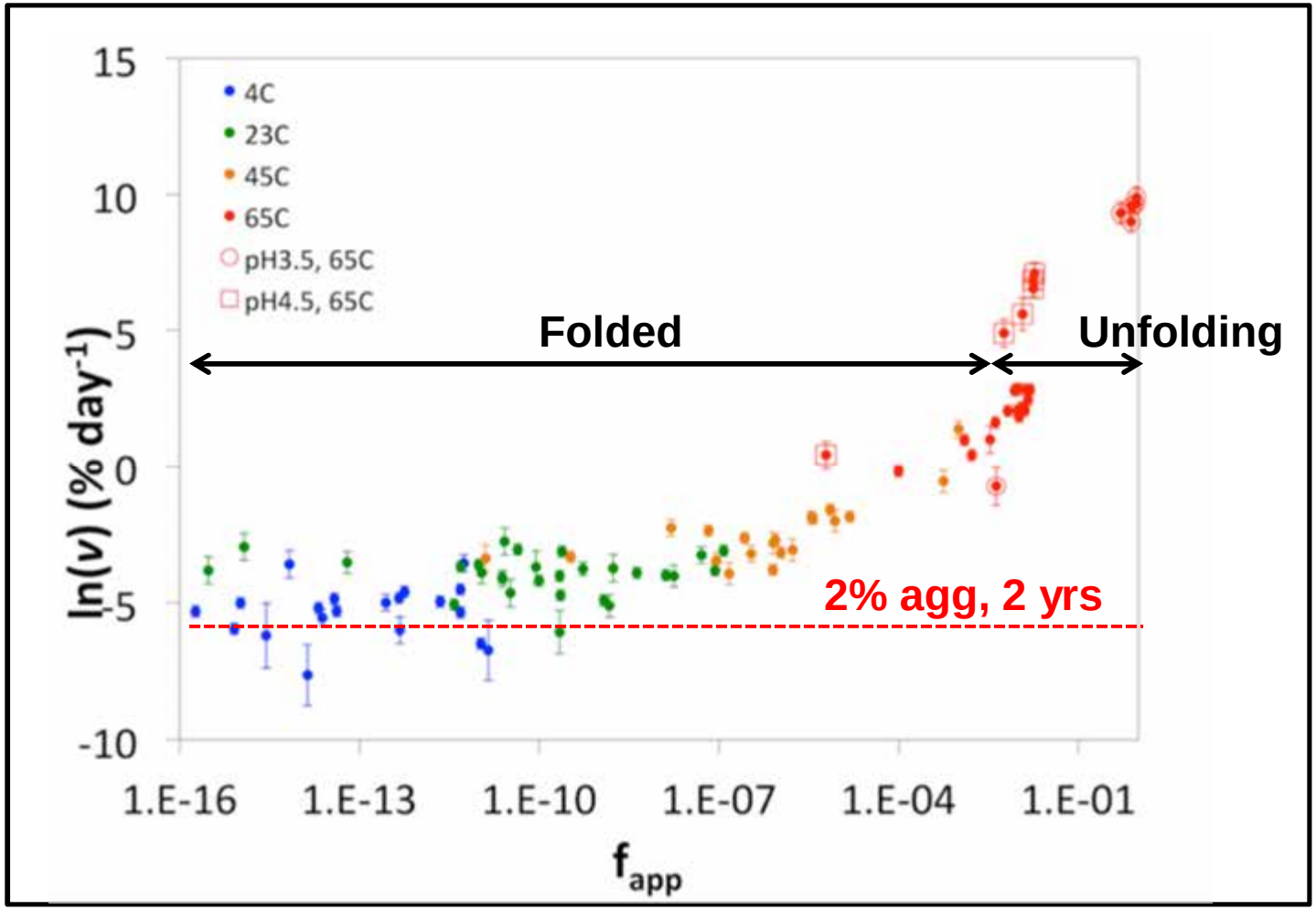
Fab aggregates from native-like states during long-term storage



- *Aggregation under Native conditions is only weakly dependent on global unfolding.*
- *Unfolding at 65 ° C, pH 3.5 & 4.5 accelerates aggregation*
- *Variability between formulations at low temperatures not yet understood.*

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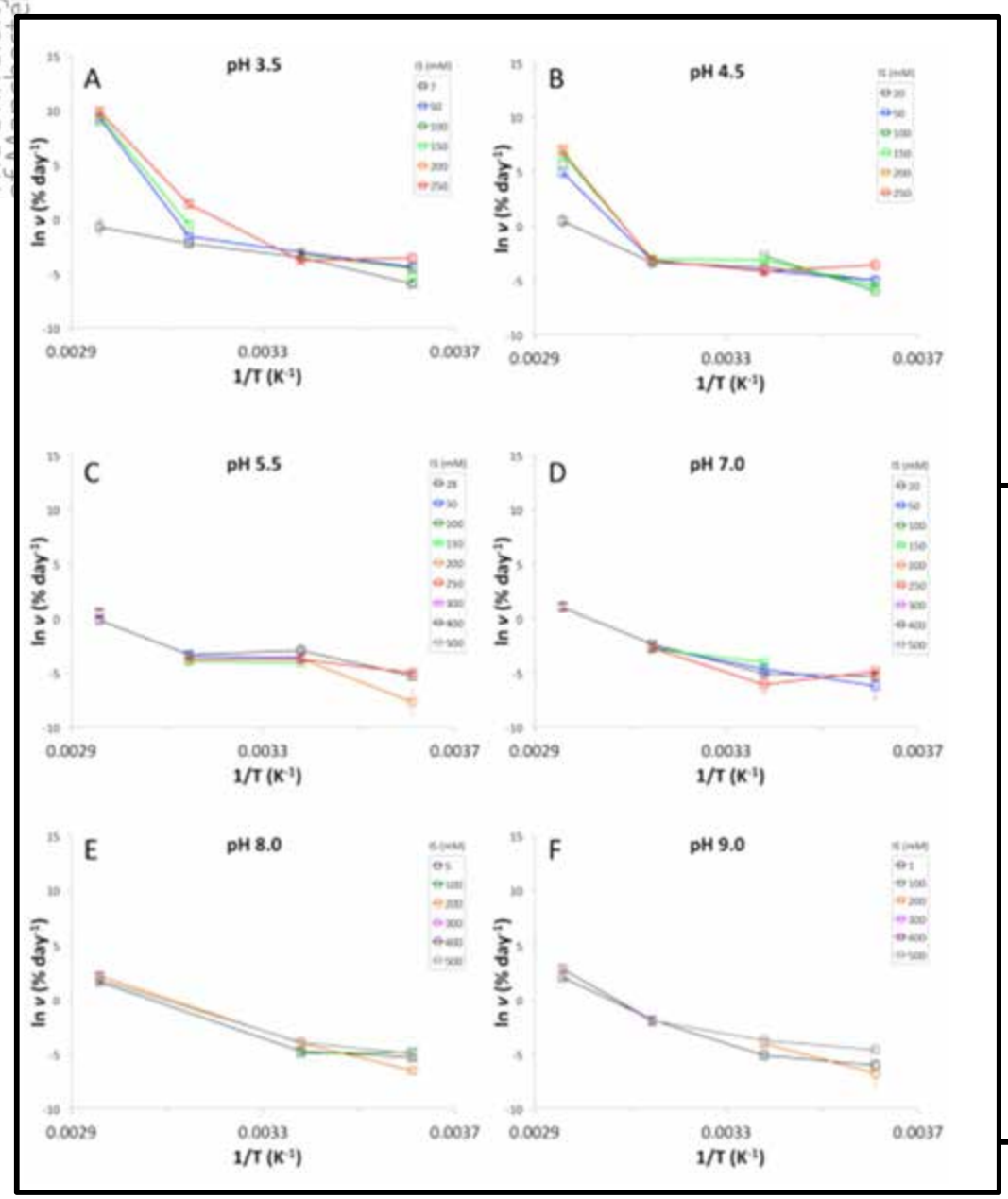
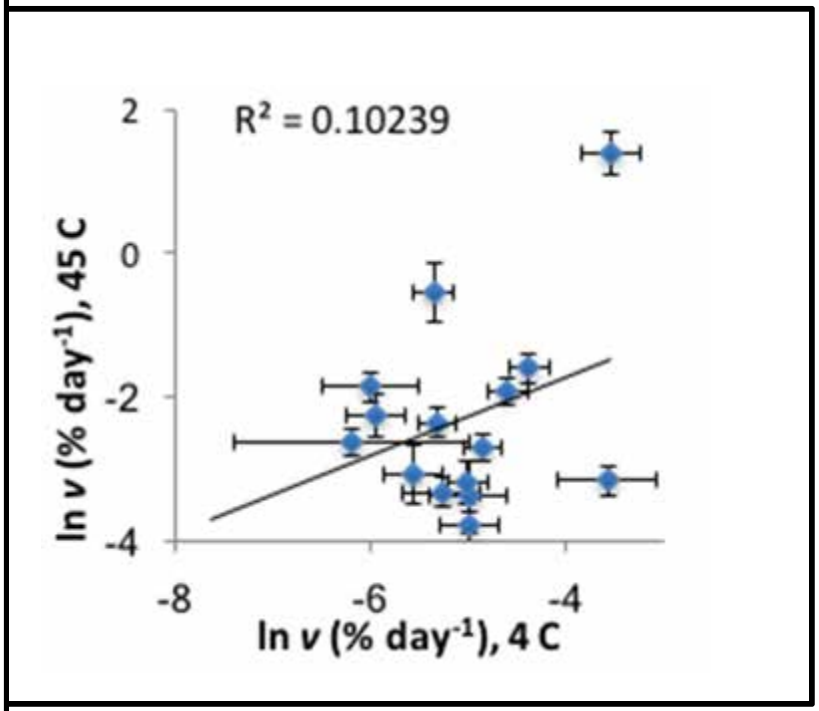
Formulation rank-order changes with T



Non-Arrhenius behaviour in global unfolding conditions only

Observed also by others for IgG1 (eg Bernard Trout, Chris Roberts)

But rank order of formulations changes with temperature



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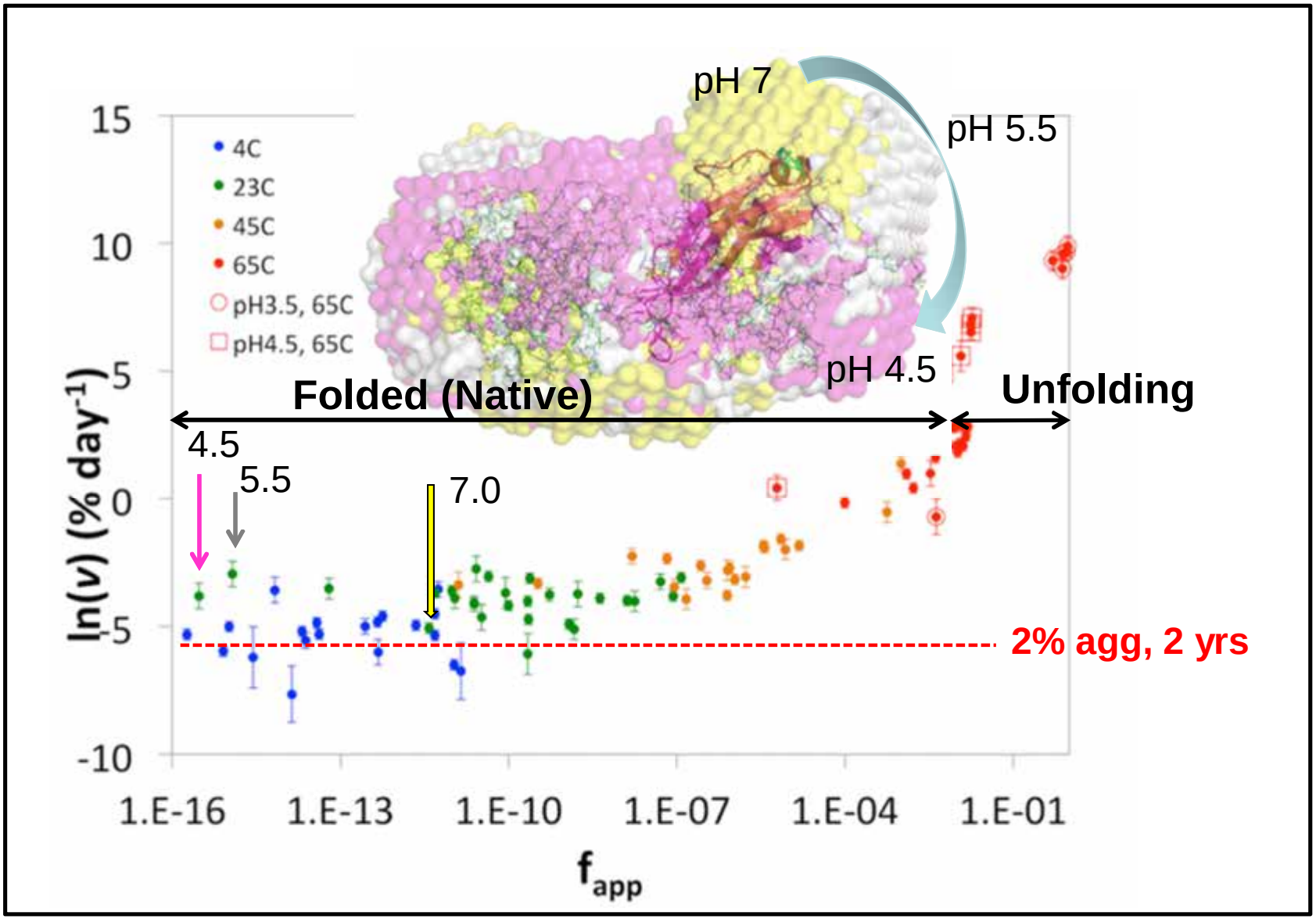
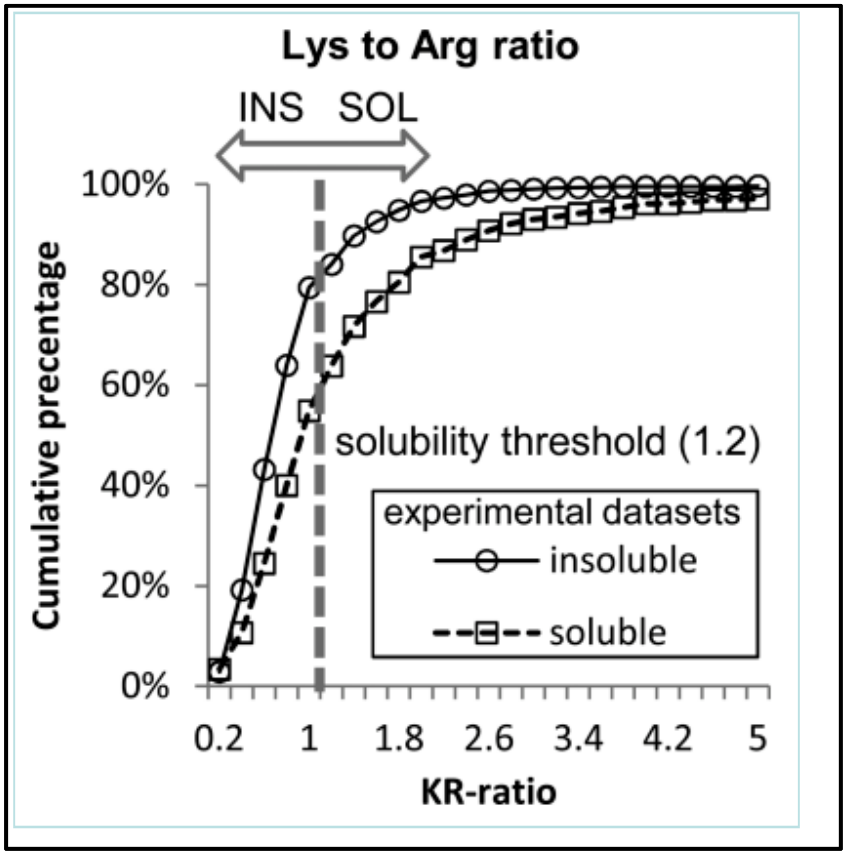
SAXS of native-state conformations @23C



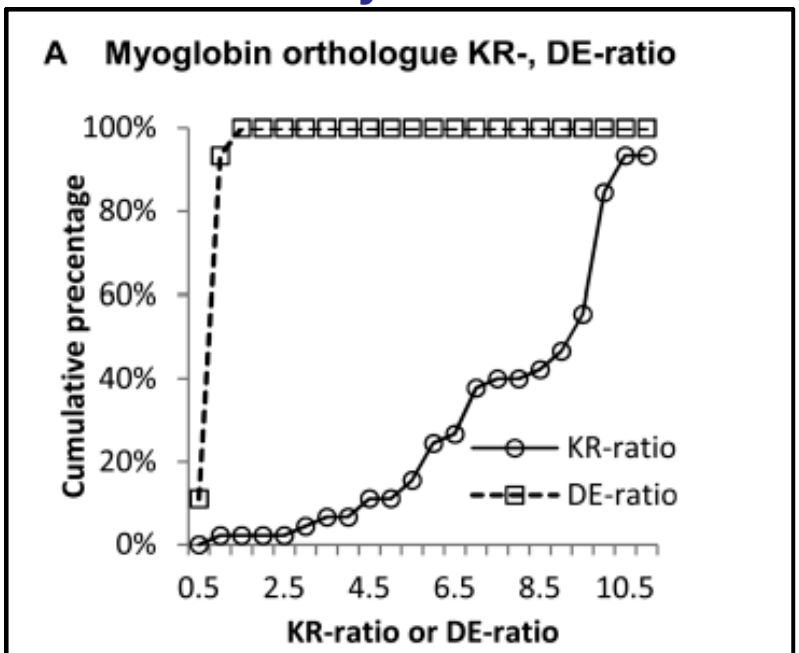
*Native structure more stable to **global** unfolding (f) as pH decreases, but aggregates faster. Does low pH increase **local** structure unfolding, and accelerate aggregation?*

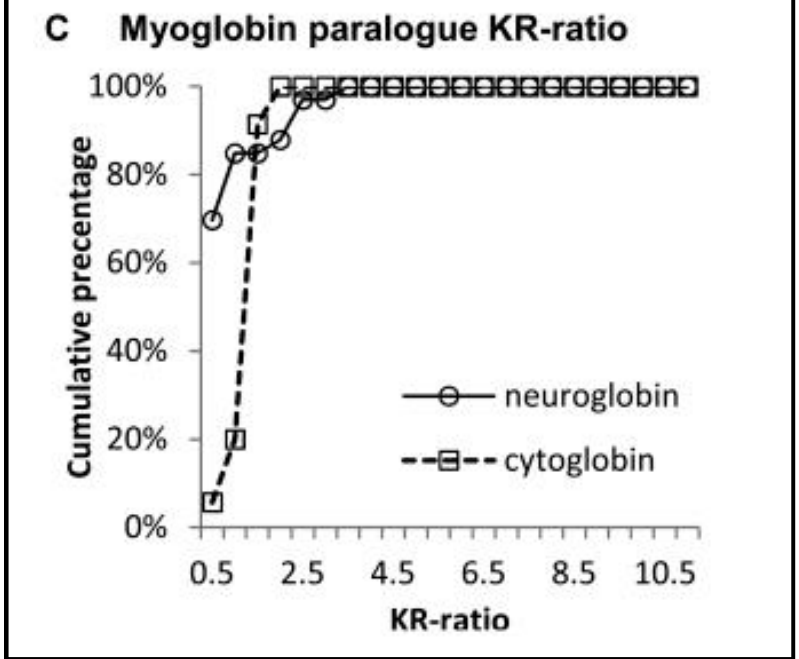
Positively charged amino acids Lys and Arg are not equivalent: Arg associates more with insolubility

Niwa et al (2009) PNAS 106:4201 – *Expressed E Coli proteome using cell free translation system. Aggregation propensity reflected by fraction of soluble protein*



KR-ratio compared between higher and lower in vivo concentration paralogue families

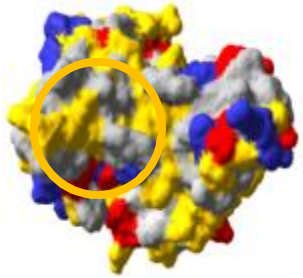




Aggregation of scFv mutant proteins

Mutant label	Mutations	pI
1SB	2 charges introduced to create 1 salt bridge	7.8
2SB	4 charges introduced to create 1 salt bridge	7.8
3SB	6 charges introduced to create 1 salt bridge	7.8
DSV	Tryptophan sequence (TWA) to DSV	6.9
5E	5 glutamates into patch	5.3
5K	5 lysines into patch	9.0
5R	5 arginines into patch	9.0
7KR	Global mutations of 7 lysine to arginine	7.8
4RK	Global mutation of 4 arginine to lysine	7.8

coloured according to polarity (red – non-polar, blue – polar)



mutated regions is rich in non-polar (grey) and polar/non-charged (yellow), but there is a deficit in charged residues (red/blue).

Understand:

What structural / sequence features underpin aggregation?

- *positive versus negatively charge groups*
- *salt bridges*
- *lysine to arginine ratio*

Evaluate:

Are conformational and colloidal stability predictive of aggregation rates

Colloidal stability in terms of B_{22}

Second virial coefficient, B_{22}

$$B_{22} = B_{22,ex} + \frac{1}{2}\int_{d_p}^{\infty}[1 - \exp(-\beta w(r))]\,4\pi r^2\,dr$$

$w(r)$ – protein pair potential of mean force

if $B_{22} > 0$ net protein-protein interaction is repulsive

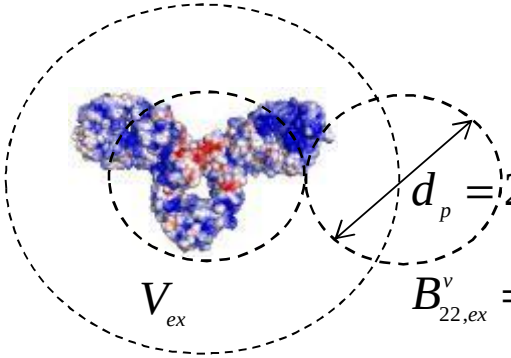
if $B_{22} < 0$ net protein-protein interaction is attractive

$$w(r) = w_{ex}(r) + w_{sr}(r) + w_{elec}(r)$$

***short-range attraction** includes hydrophobic forces, hydrogen bonding, shape and charge complementarity, use Baxter adhesive parameter*

$$B_{22,sr} = -\frac{1}{4\tau} B_{22,ex}$$

***excluded volume:** proteins can be approximated as spheres with size given by hydrodynamic radius*



$$B^{v}_{22,ex} = \frac{V_{ex}}{2} = \frac{2}{3}\pi d_p^3$$

***electrostatics** can be rationalized using a DLVO potential*

$$\beta W_{elec}(r) = \frac{Z_p^2 l_B}{(1+\kappa a)^2}\frac{\exp[-\kappa(r - d_p)]}{r}$$

where range of potential is given by debye length κ^{-1} and magnitude is proportional to net charge Z_p^2

Colloidal stability from DLS

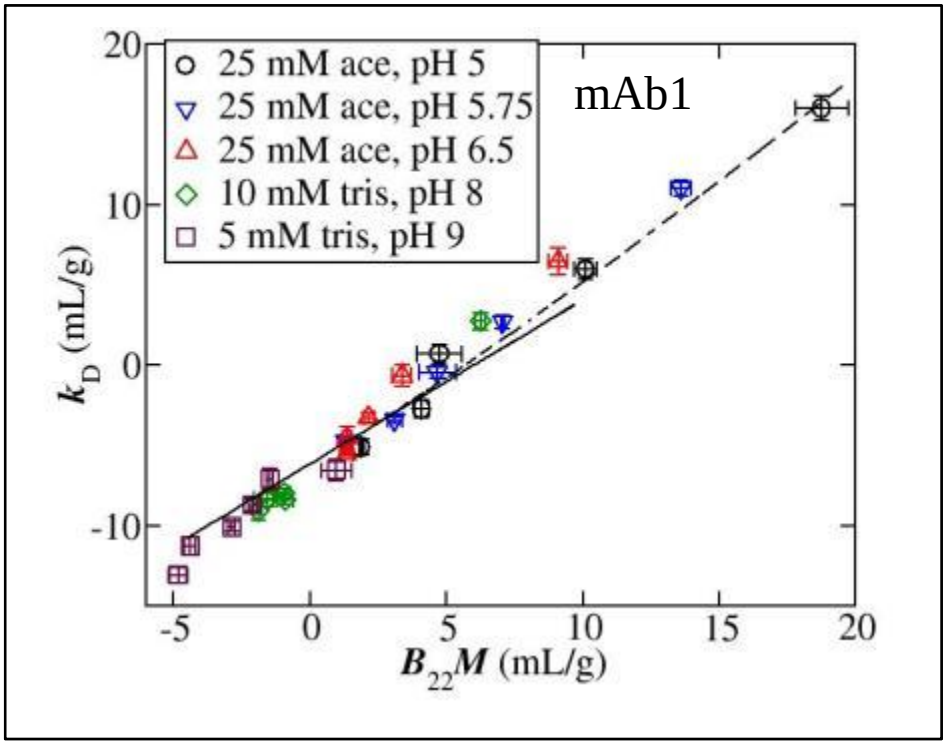
Slope of D/D_o versus c_2 plot is used to determine protein-protein interactions

$$\frac{D}{D_o} = 1 + k_D c_2$$

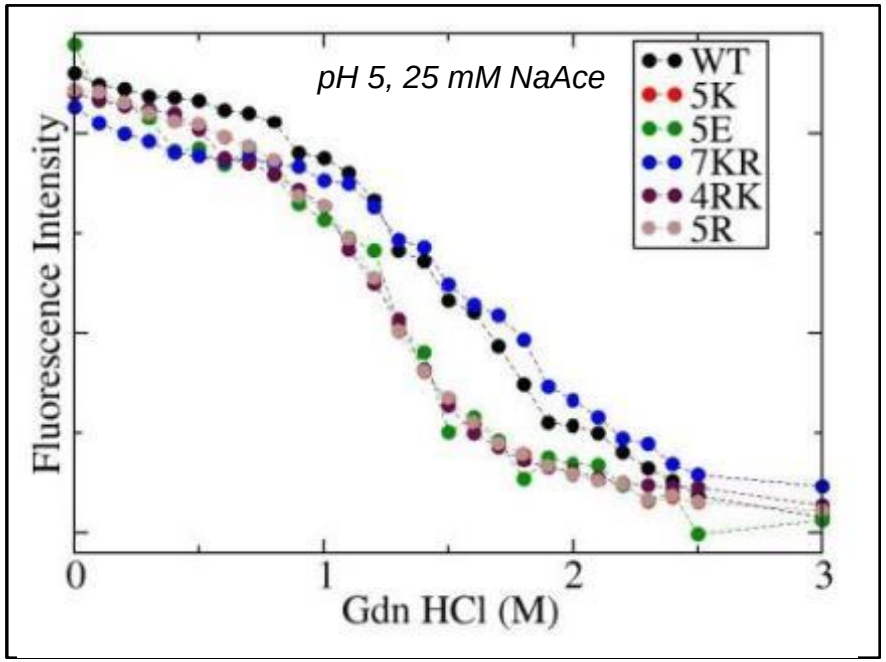
k_D – interaction parameter

Protein interaction parameter k_D determinable from mutual diffusion coefficient measurements by dynamic light scattering

DLS plate reader - 384 well plates with 40 µl sample volumes

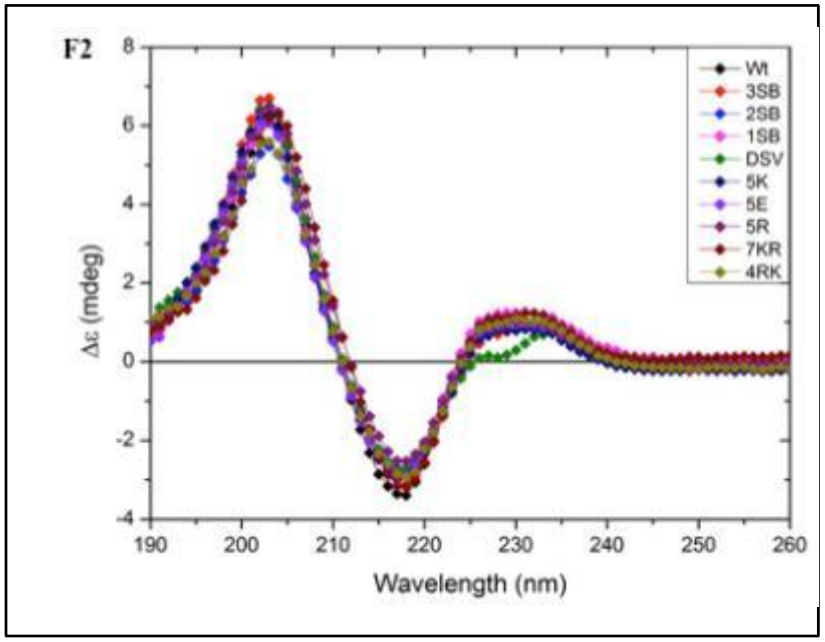


Conformational stability from denaturant curves



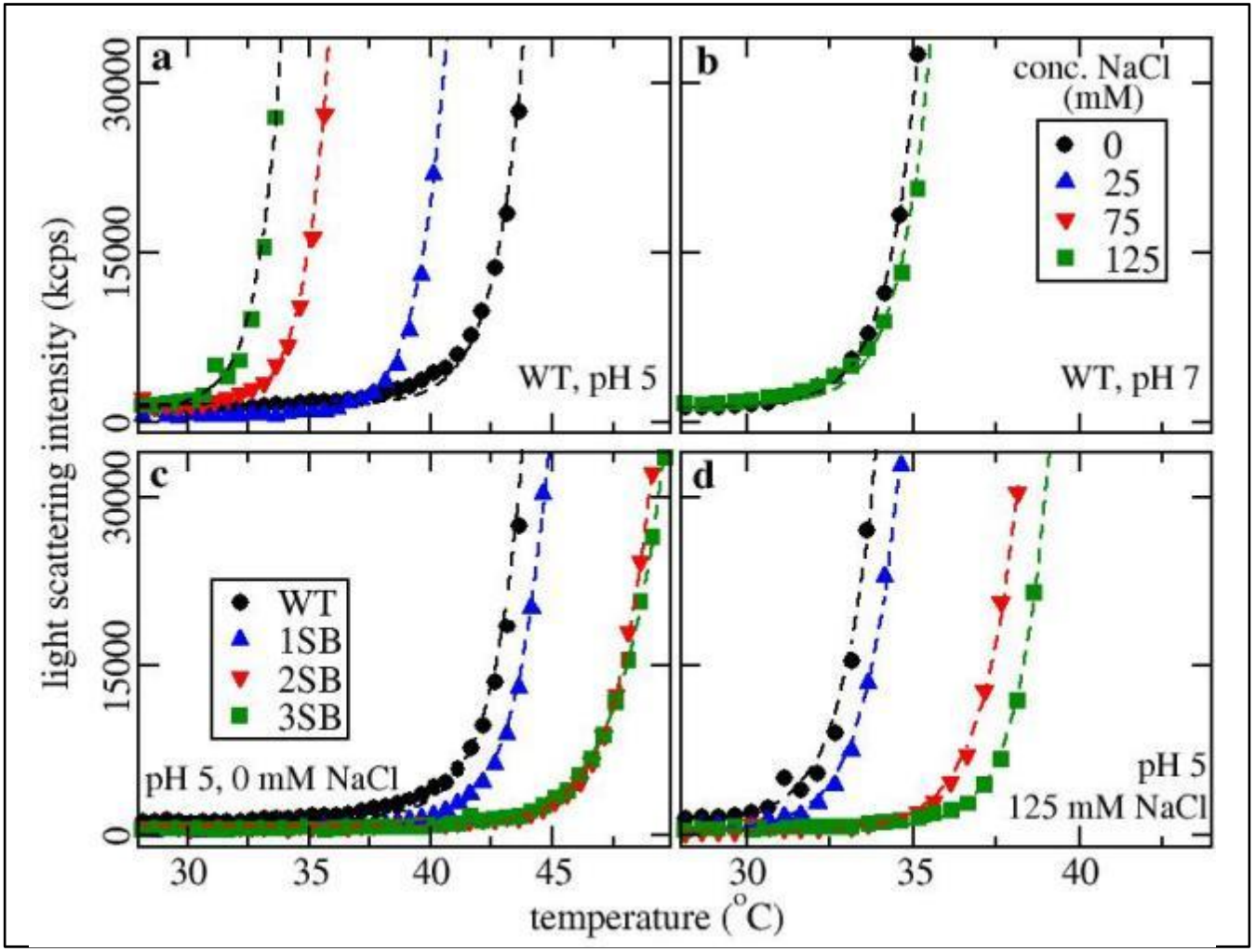
c_{mid}	Mutations
1.49	WT
1.51	1SB
1.51	2SB
1.47	3SB
1.51	DSV
1.78	7KR
1.26	5E
1.26	5K
1.27	5R
1.25	4RK

- *denaturant curves are not affected by aggregation behaviour of mutants and should provide reliable estimate of conformational stability*
- *conformational stability of 5K, 5E, 5R, and 4RK are less stable than wild type according to denaturant unfolding experiments*



Circular dichroism indicates no structural changes from mutations

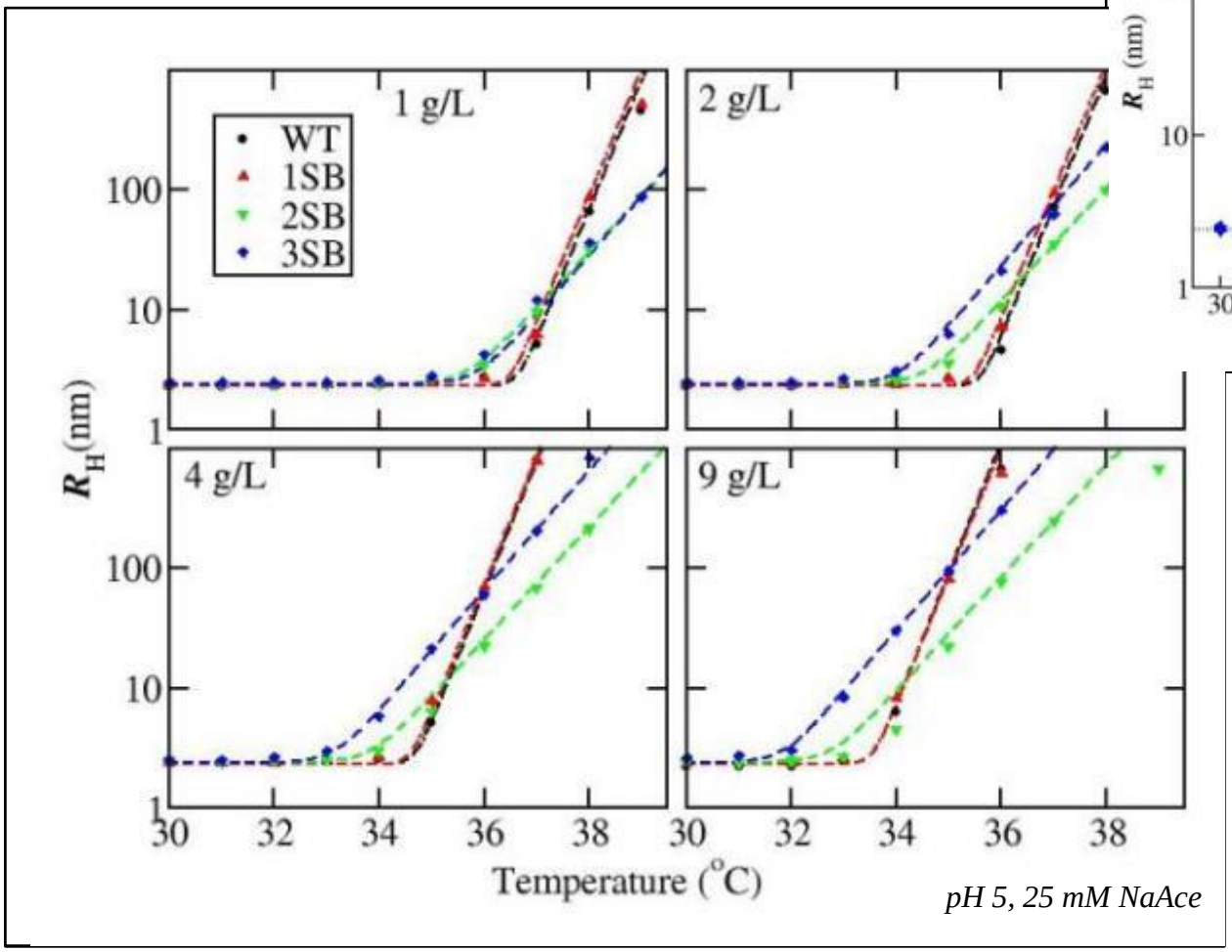
Aggregate growth rates by static light scattering



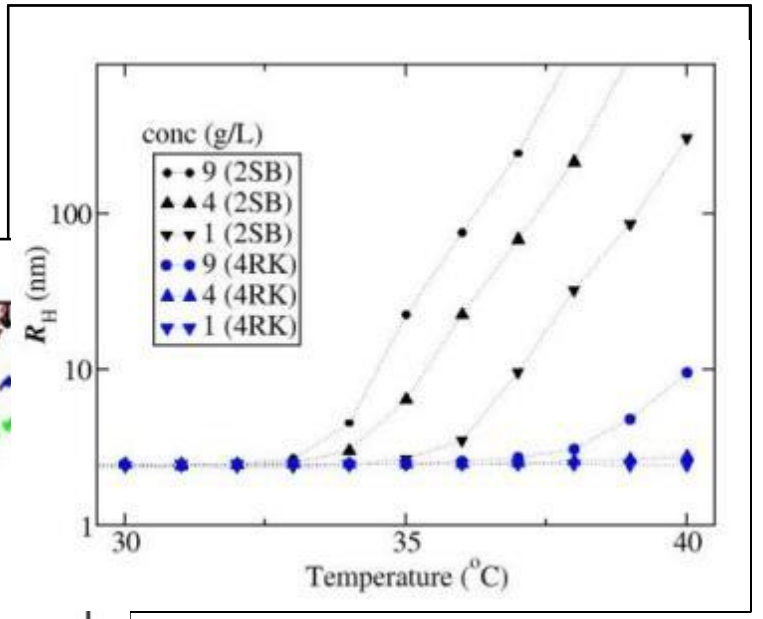
- *Effect of pH and ionic strength on static light scattering profiles can be rationalized in terms of electrostatic interactions (DLVO theory)*
- *Most correlations between k_D and aggregation reflect role of electrostatic interactions*

Aggregation rates measured by DLS

Aggregate formation rates are slightly higher for WT, 1SB, versus 2SB, 3SB, but growth rates faster for WT, 1SB versus 2SB and 3SB



pH 5, 25 mM NaAce



4RK exhibits significantly slower aggregation kinetics than all other mutants

Results summary

- T_0 ~ aggregate growth rates
- T_{DLS} ~ aggregate formation rates
- T_{NMR} ~ monomer loss kinetics

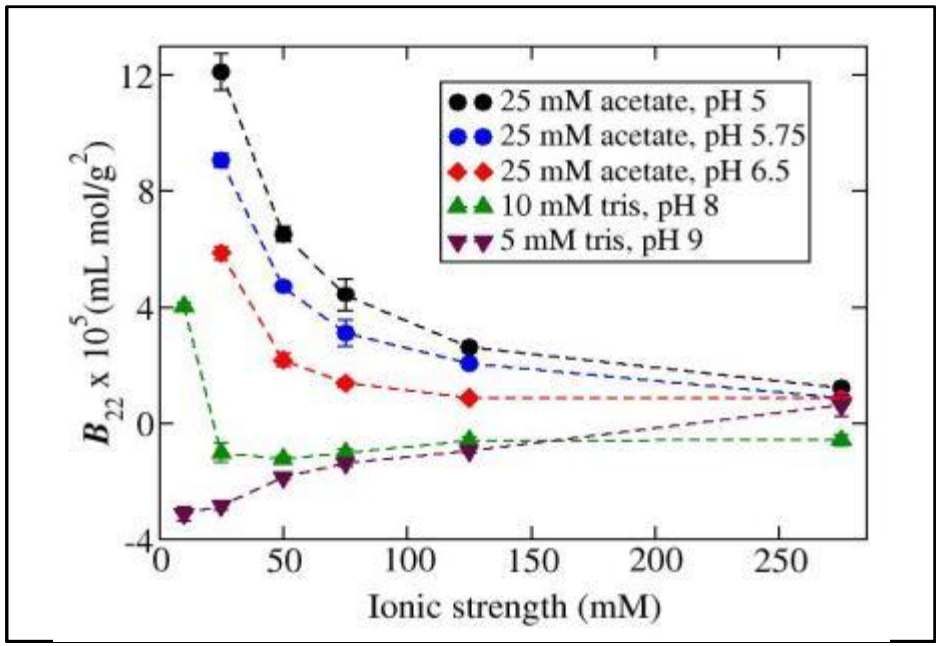
	Z	c_{mid} (M)	T_0 (K) 25 mM	T_0 (K) 150 mM	T_{DLS} (K) 25 mM	T_{DLS} (K) 150 mM	k_D^* (mL/g) 25 mM	k_D^* (mL/g) 150 mM	T_{NMR} (K) 25 mM
WT	3.3	1.49	305.2	295.7	310	300	0.9	-2.6	331
1SB	3.3	1.23	306.1	296.4	310	301	-0.5	-5.0	326
2SB	3.3	1.51	309.8	299.6	309	301	1.7	-1.4	326
3SB	3.3	1.47	309.8	300.7	309	296	2.4	-7.4	326
4RK	3.3	1.25	-	-	>313	306	2.3	-3.6	331

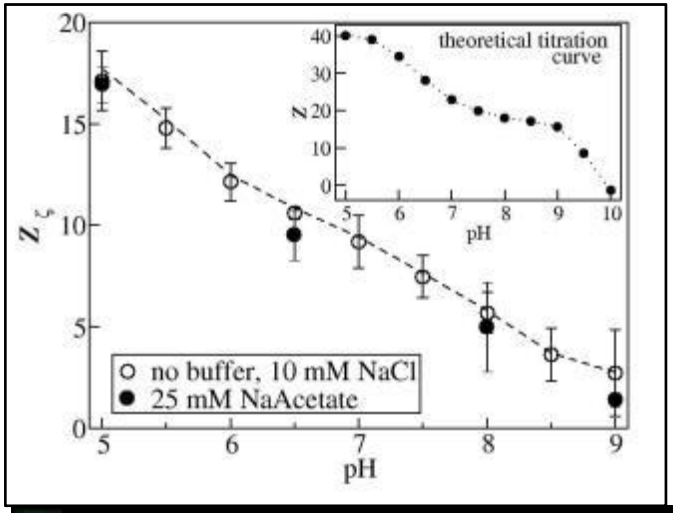
- *4RK exhibits decreased monomer loss kinetics and slower aggregate growth rates, but results do not correlate with increased conformational or colloidal stability*
- *Mutant dependence of k_D does not correlate with aggregate growth (eg T_o)*

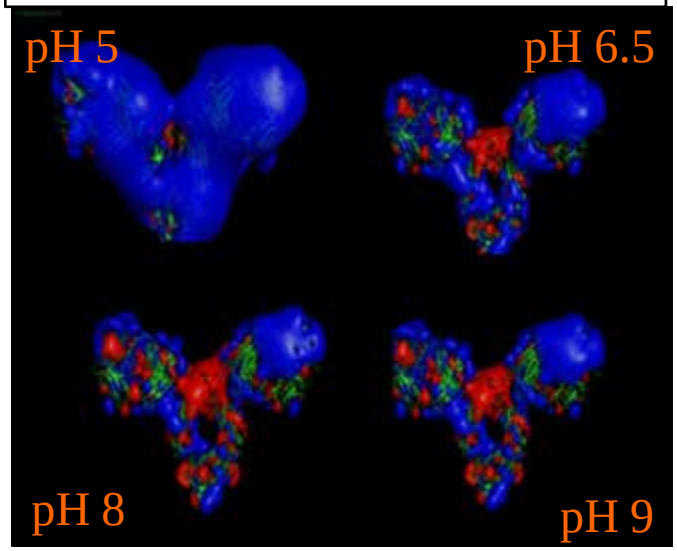
B_{22} x 10^4 (mLmol/g^2)	8 M urea	6 M GdnHCl
WT	18.4	9.9
4RK	27.1	16.1
7KR	-6.1	-10

- *Measurements under denaturing conditions indicate aggregation kinetics correlates with interactions between unfolded states, rather than between native states*
- *Lysine protects unfolded regions of proteins from associating*

Probing excipient effects from electrostatic interactions

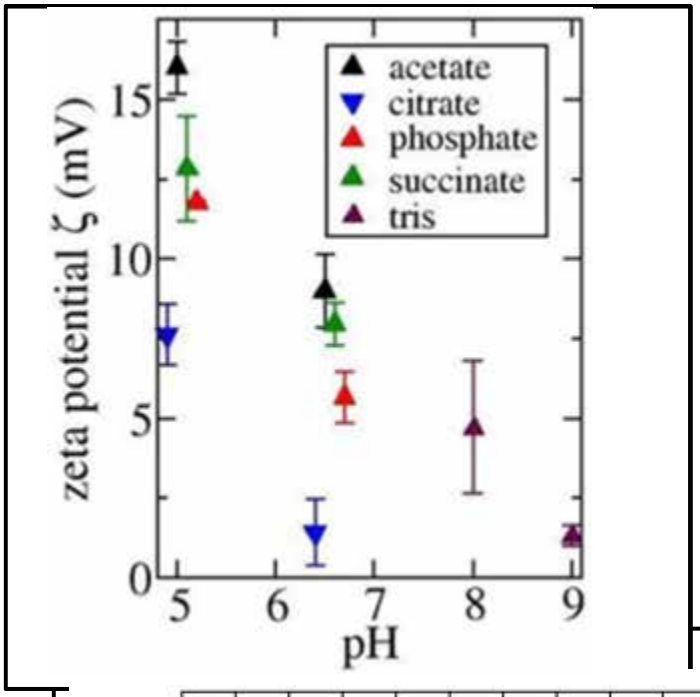




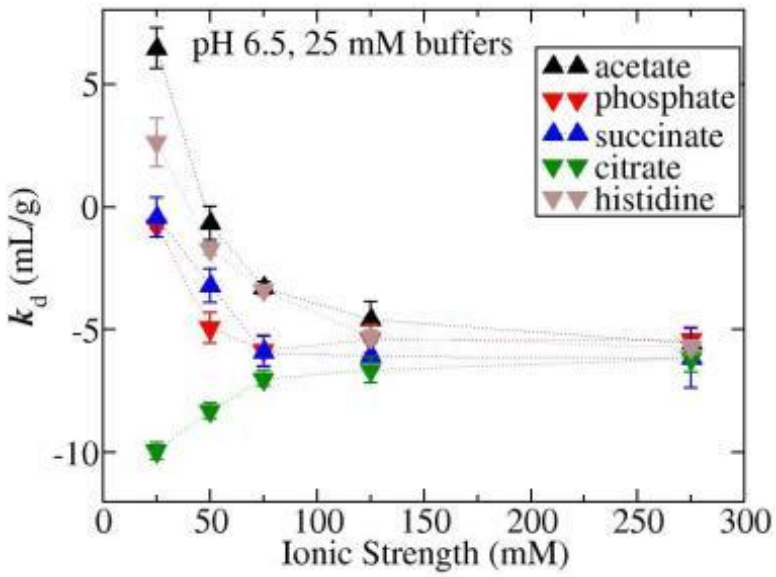


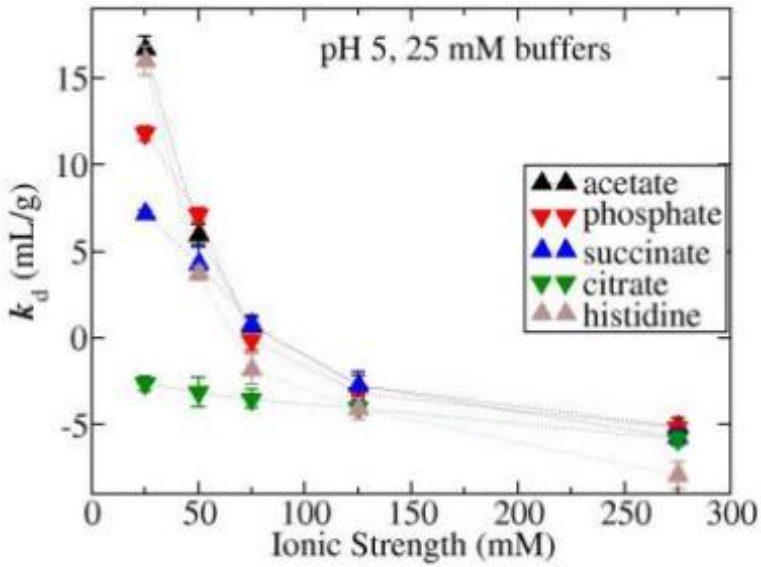
- *follows behaviour expected from DLVO theory at low pH*
- *at pH 9, attractive electrostatic interactions are screened for all IS*
- *cross over effect observed at pH 8, repulsive electrostatics switch to attractive with increasing IS*

Buffer (excipient) effects

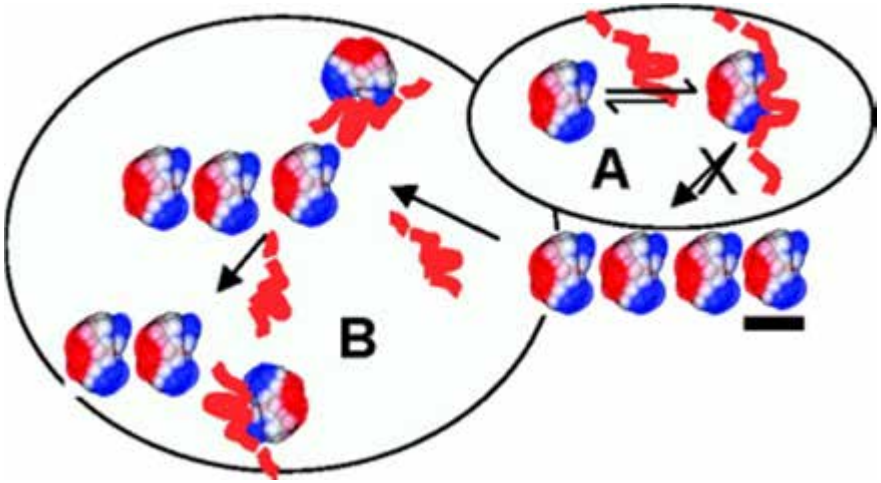


- *the effects of buffers are most pronounced in solutions at low ionic strength, interactions independent of buffer in solutions at 250 mM*
- *salt specific effects occurring at low ionic strength can be rationalized in terms of ion binding and protein charge neutralization or inversion (eg citrate ion)*



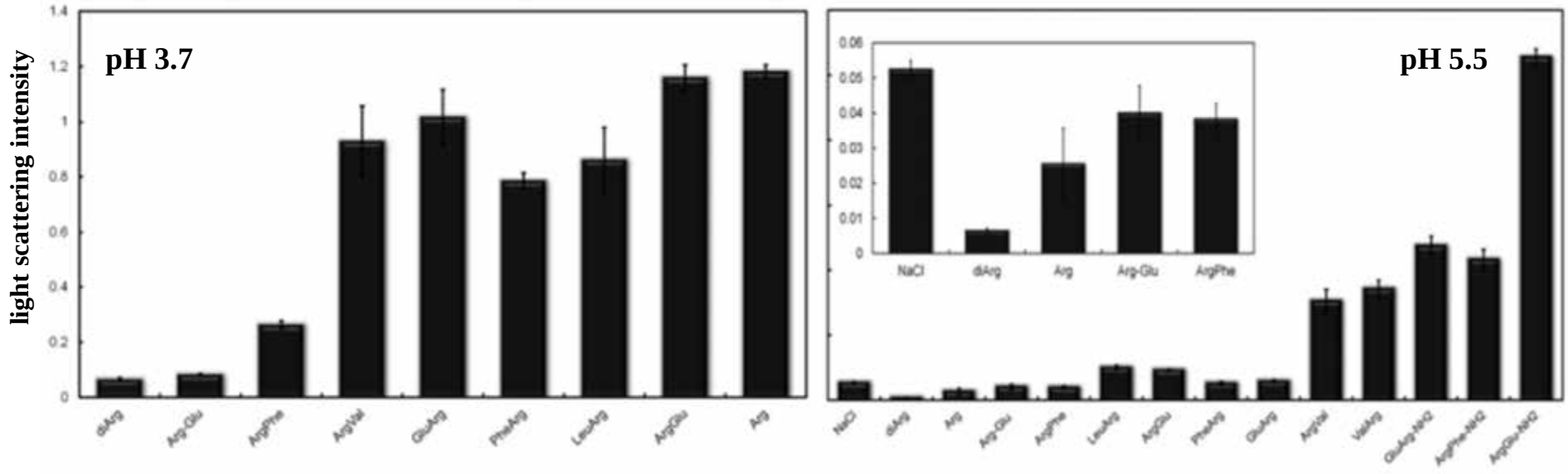


Dipeptides as novel excipients

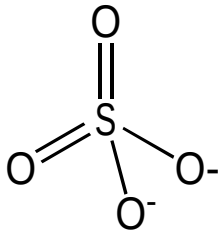


- *At isoelectric pH, diArg is most effective at reducing insulin self association versus all other additives reflecting ability to neutralize electrostatic attraction.*

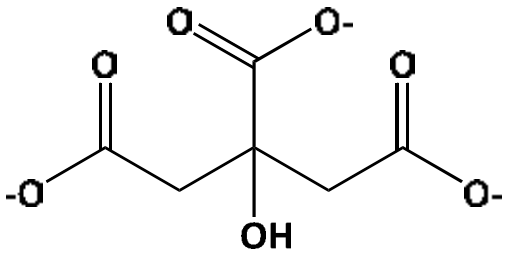
- *At pH 3.7, diArg, ArgPhe and mixtures of Arg and Glu equally effective at neutralizing hydrophobic interactions between insulin molecules*



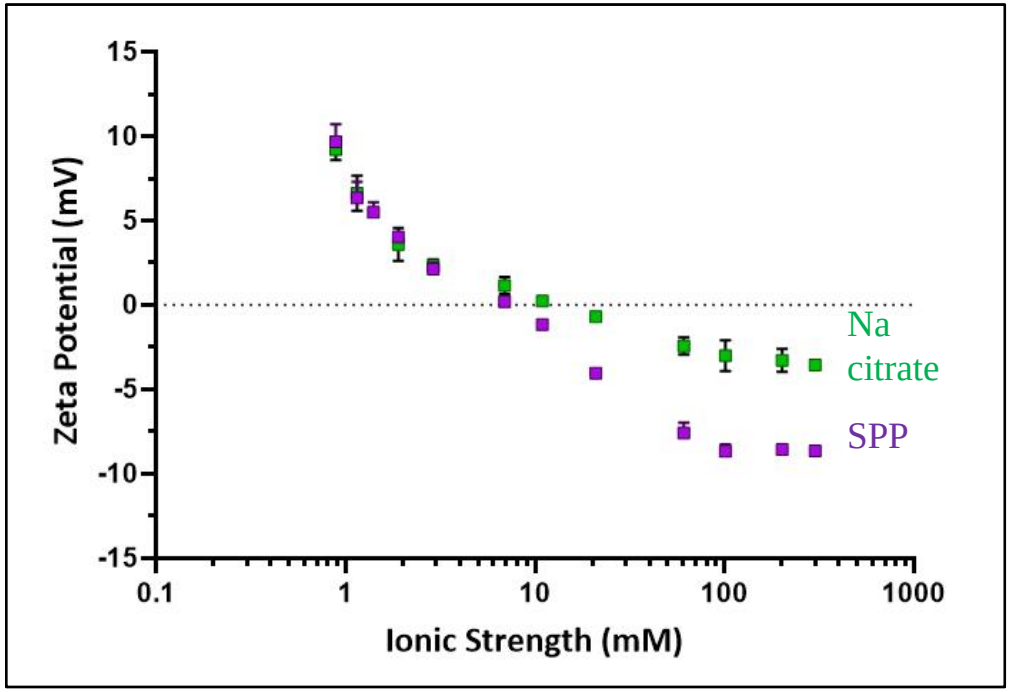
Novel charged excipients

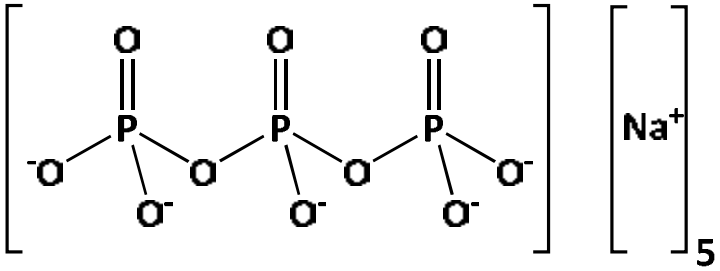


Sulphate
pKa_1: -3
pKa_2: 1.99

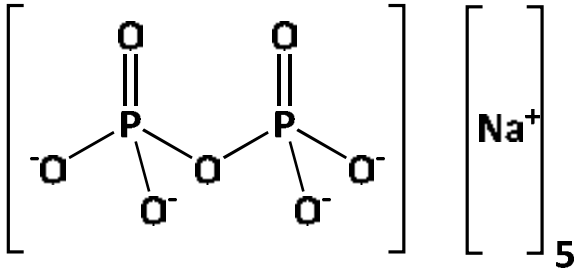


Citrate
pKa_1: 3.13
pKa_2: 4.76
pKa_3: 6.39





Sodium Tripolyphosphate (STPP)
pKa_1: 1 pKa_2: 2.2 pKa_3: 2.3 pKa_4: 5.7 pKa_5: 8.5

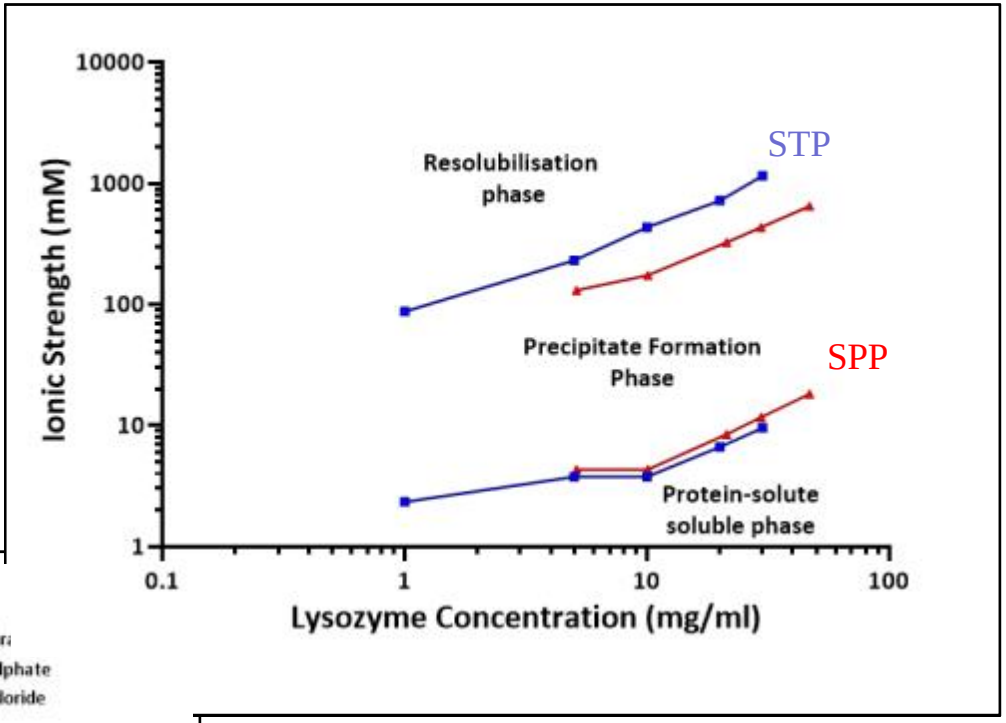


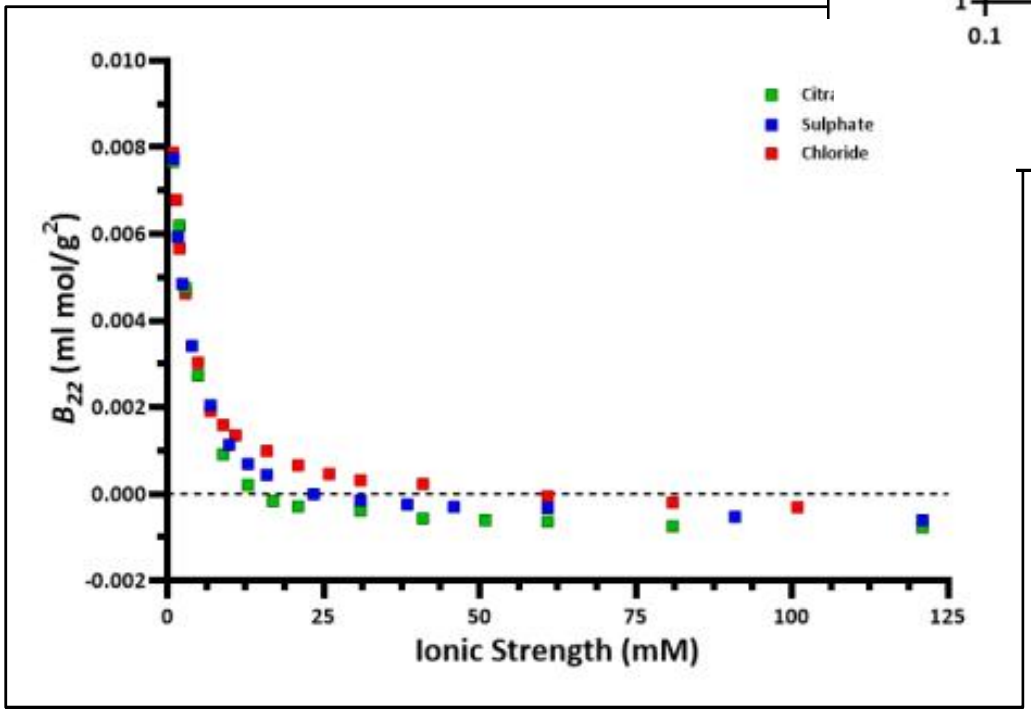
Sodium Pyrophosphate (SPP)
pKa_1: 0.91 pKa_2: 2.1 pKa_3: 6.7 pKa_4: 9.32

Poly-phosphates are GRAS excipients recognized by the FDA

Novel charged excipients

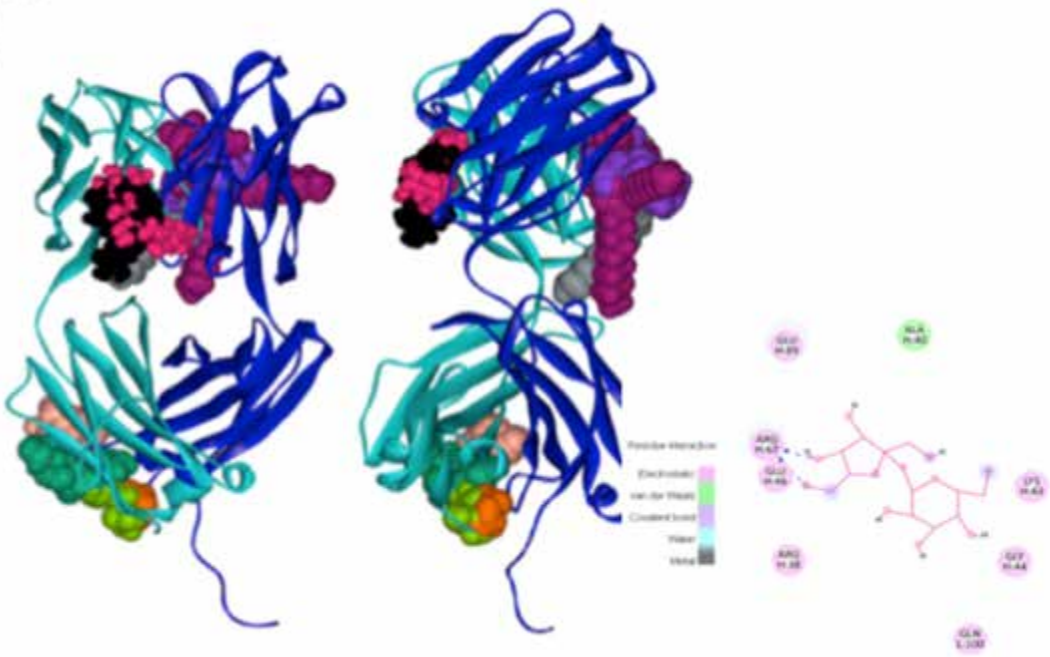
Citrate ion is more effective than sulfate at neutralizing electrical double layer forces due to its trivalent charge





SPP and citrate have similar net charge (-3). However, SPP causes re-entrant condensation phenomena for lysozyme at pH 9

Molecular docking identifies protein-excipient "Hotspots"



1) interacts with sucrose and trehalose
2) interacts with saccharides and amino acids
3) interacts only with surfactants

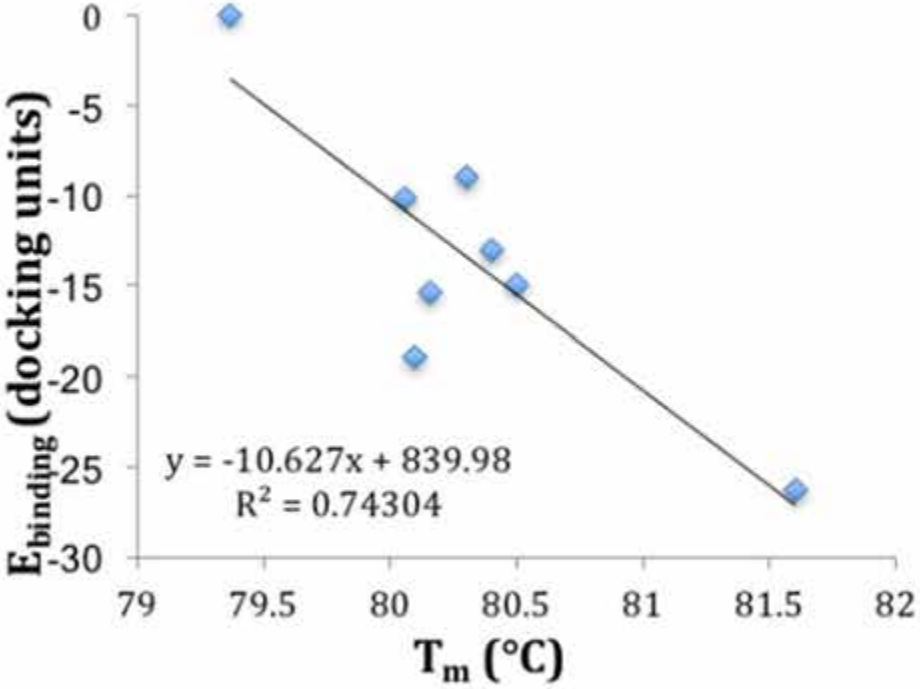
Glycine has strongest effect on E_{bind}, and T_m
Hotspots 2 and 3 are located mainly in the light chain

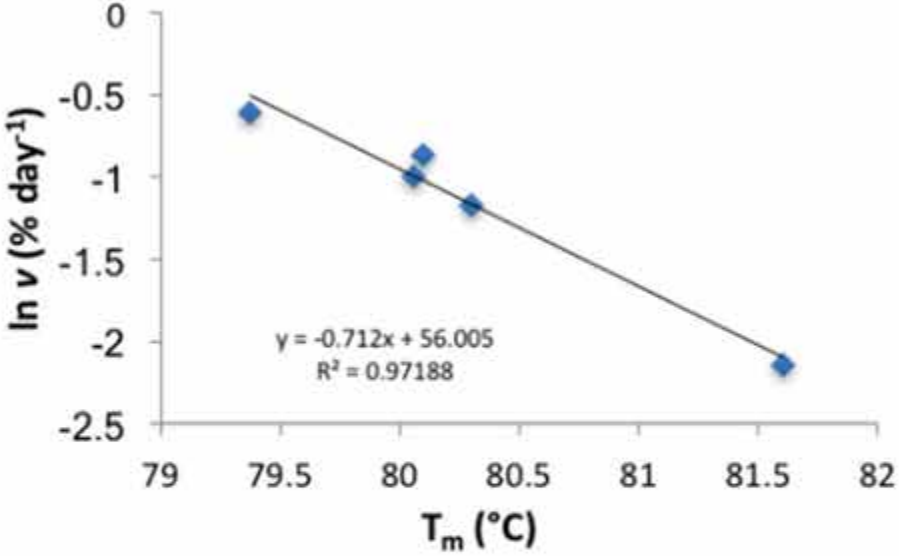
Matches to dynamic regions identified by M/D

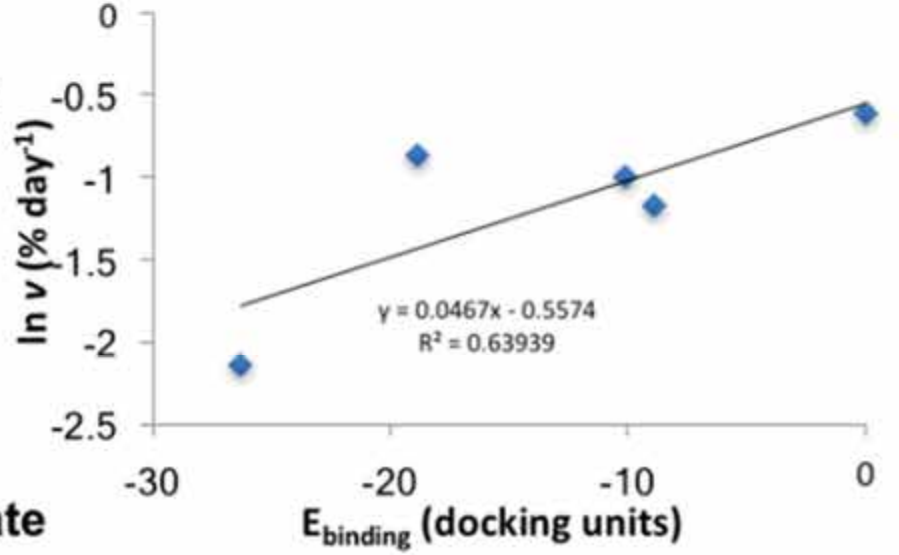
Excipient	$E_{binding}$	Spot in target
Trehalose_1	-13.8	**Spot 1**
Trehalose_2	-12.3	
Sucrose_1	-16.9	Heavy Chain: Lys43-Trp47; Arg67; Glu89 Light Chain: Ile1-Trp5; Thr97-Gln100
Sucrose_2	-13.9	
Arginine_1	-22.7	**Spot 2**
Glycine_1	-27.3	
Glycine_2	-25.3	
Mannitol_1	-11.9	
Mannitol_2	-10.7	Heavy Chain: Ser131-Ser136 Light Chain: Ala112-Ile117; Leu201-Asn210; Glu213-Cys214
Mannitol_3	-7.8	
Sorbitol_1	-9.4	
Sorbitol_2	-8.9	
Sorbitol_3	-8.5	
Tween20_1	-14.4	**Spot 3**
Tween20_2	-15.6	
Tween80_1	-18.9	Heavy Chain: Val2-Leu4; Ala105-Trp107; Gln109 Light Chain: Lys39; Lys42-Thr46; His55-Val58; Pro80-Phe83; Gln166-Ser168
DDM_1	-24.5	

Barata et al 2016 Int J Mol Sci, 17:853

Experiment vs Docking in protein-excipient "Hotspots"



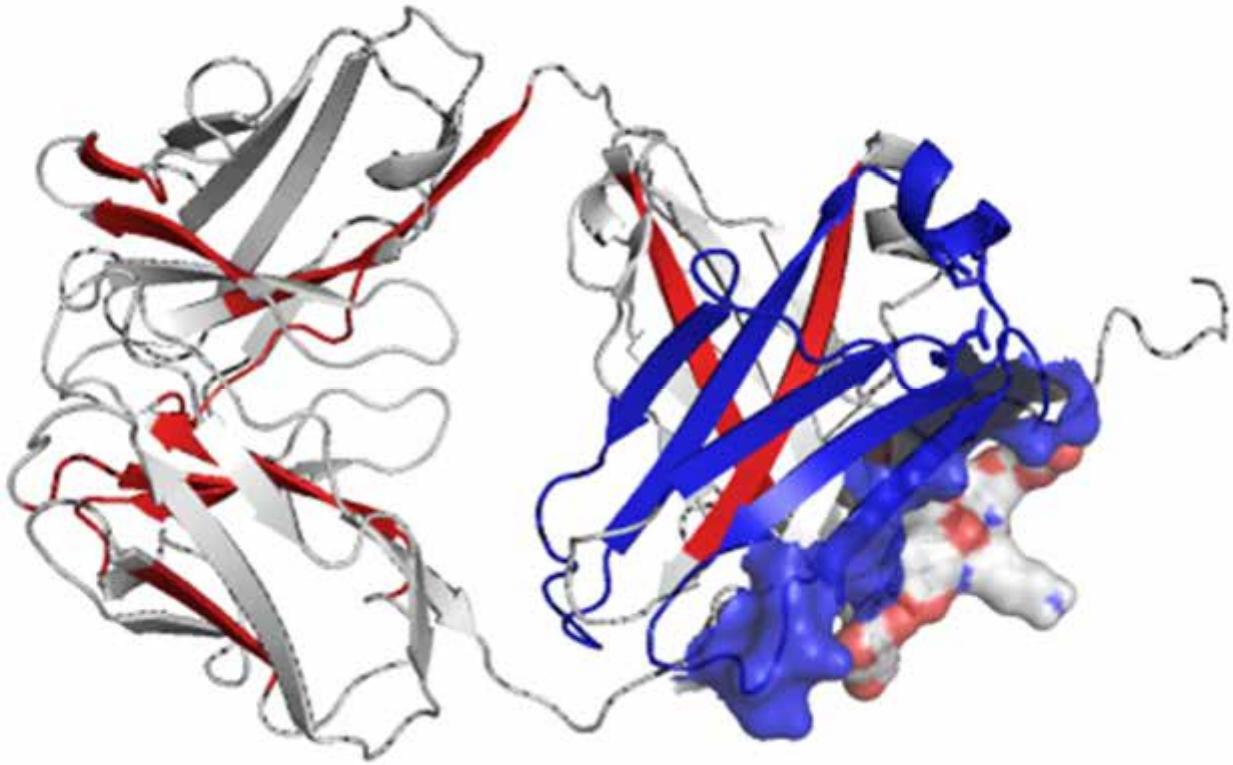




E_{bind} correlates with T_m and aggregation rate

pH 4 aggregation rate is via unfolded state

Comparison of results by Fab structure



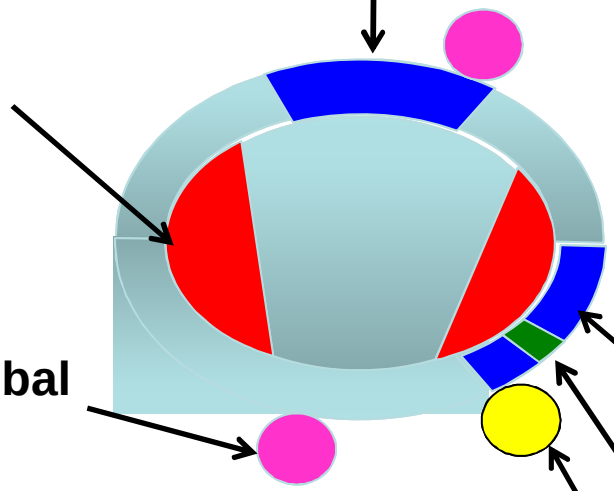
AGGRESCAN & TANGO (red ribbons)

pH-dependent SAXS shifts & Molecular Dynamics Fluctuations (blue ribbons)

Hotspot 2 (surface representation). Largest impact on T_m.

low pKa salt bridge (sticks)

Scenarios for excipient action through direct interactions



Excipient stabilises surface, but surface instability doesn't impact aggregation

Aggregation hot-spot remains buried (except when globally unfolded)

Excipient stabilises global unfolding only

Surface instability reveals aggregation site

Surface instability controlled by salt bridge

Excipient stabilises surface

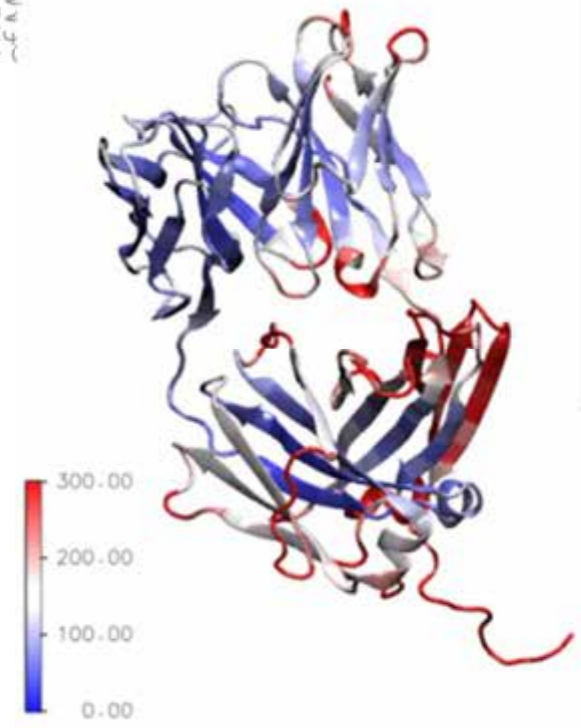
predicted by M/D

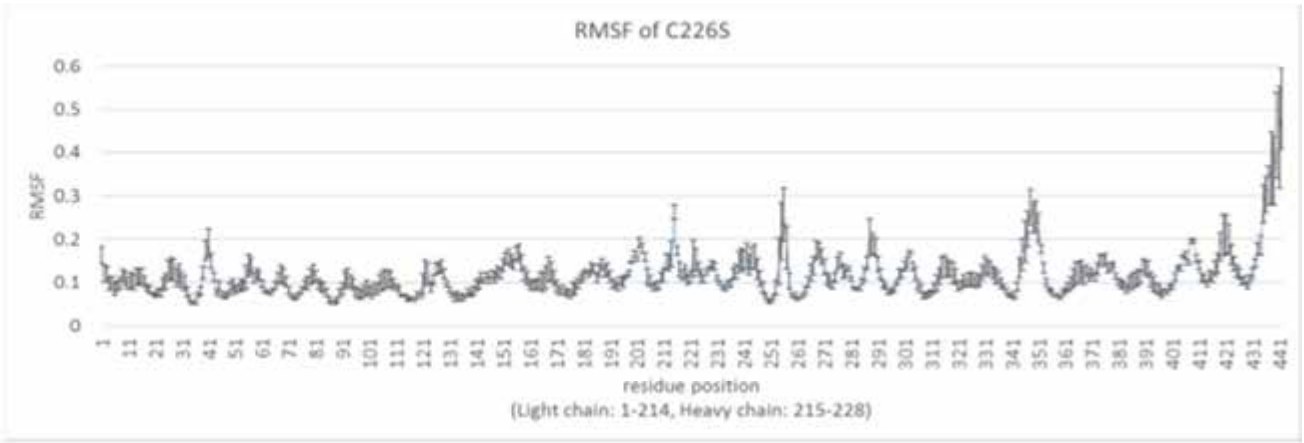
predicted by Aggrescan/TANGO etc

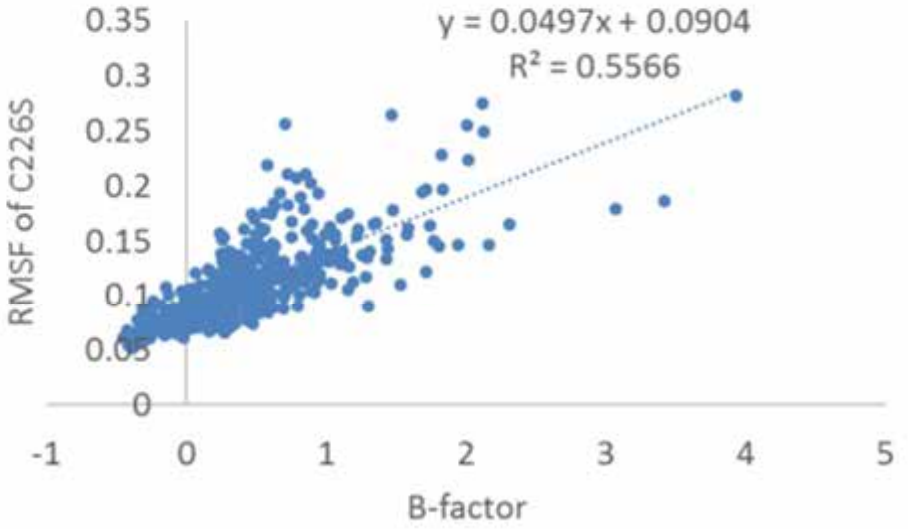
Excipient binding hotspots – cause T_m to increase

This excipient <u>also</u> suppresses aggregation

Fab engineering guided by molecular dynamics simulation



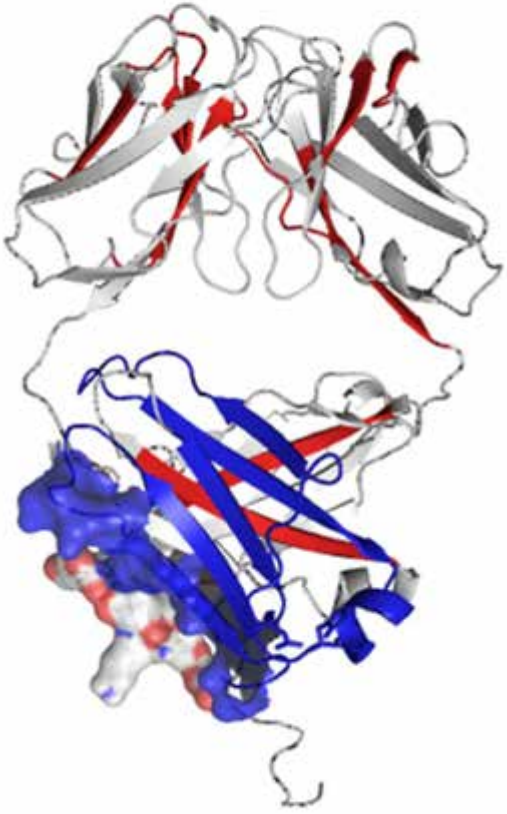


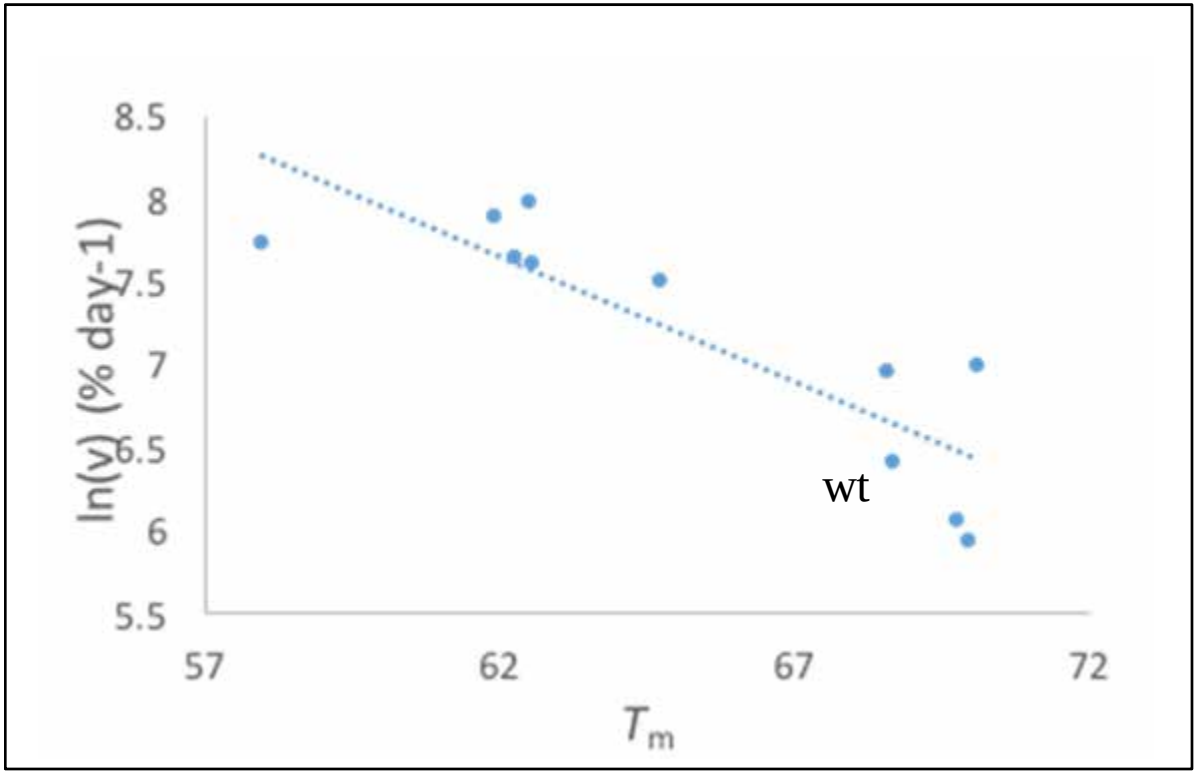


MD RMSF used to identify most dynamic regions
Targeted mutagenesis to most flexible regions
Used Rosetta to rank mutations

A33 Fab: impact of mutations and formulations on stability

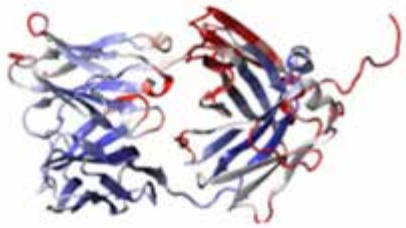
Engineered Fab variants

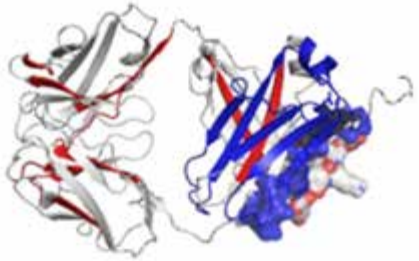




ln v - rate of monomer loss at
65C, pH 4, 20 mM citrate, 200 mM NaCl

Key Questions





Understand:

What structural / sequence features underpin aggregation?

- *3 ° / 4 ° conformation?*
- *local dynamics?*
- *global stability?*
- *aggregation (cross-beta) hotspots?*
- *excipient binding interactions?*

Evaluate and Measure:

Is T_m for formulations predictive of aggregation rates?

Does forced degradation at high temperature predict shelf-life?

Can alternative methods be developed for predicting aggregation rates?

Ultra-low volume predictive measurements – intrinsic time-resolved fluorescence (IP-TRF).

Engineer:

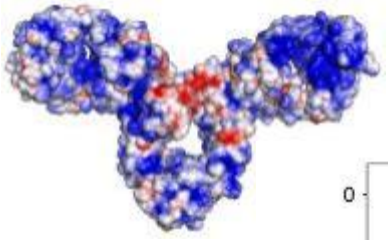
Can we predict aggregation rates and formulation excipient effects?

Can we engineer lower aggregation rates?

Can we develop novel (GRAS-based) excipients?

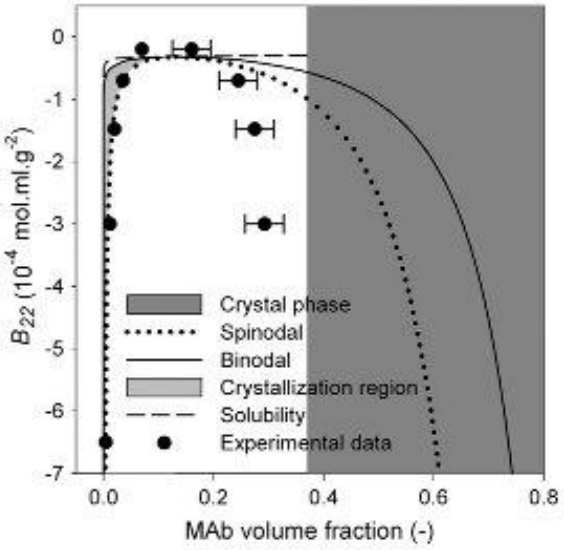
Opalescence and liquid-liquid separation

- *High concentration formulations are required for large therapeutics (antibodies, mAbs)*
- *Liquid-liquid phase separation and opalescence often occur when changing solvent properties or upon cooling for storage*



mAb

lysozyme

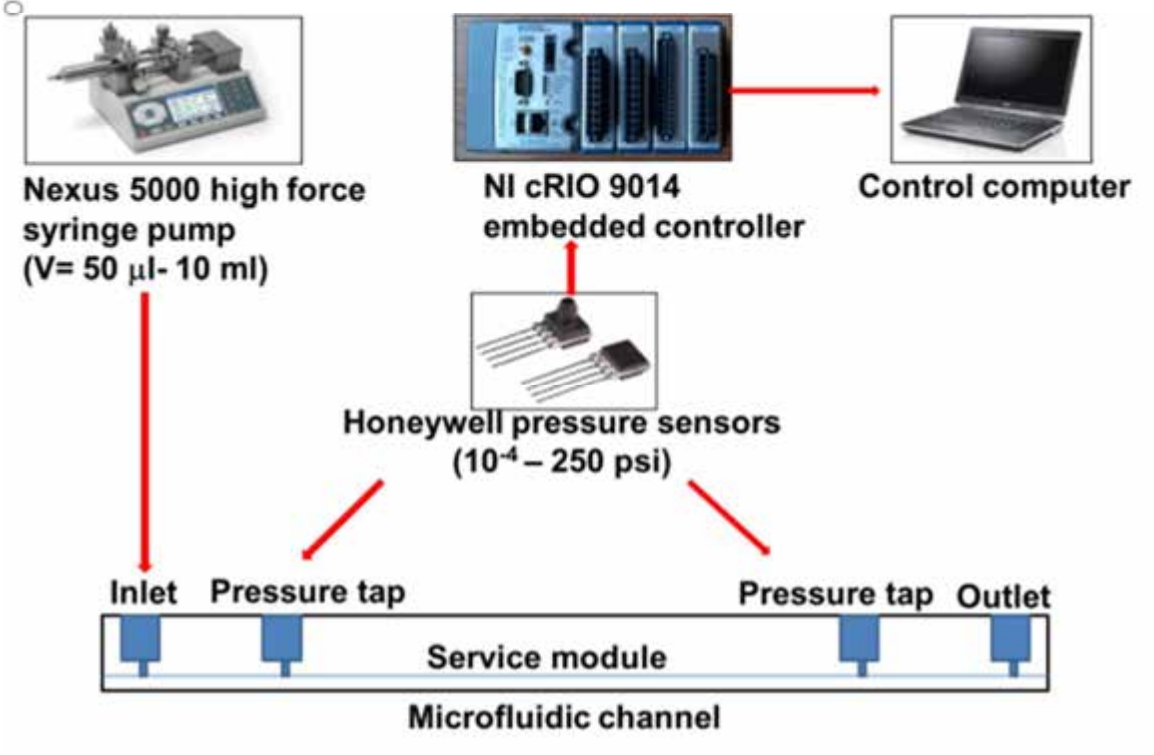


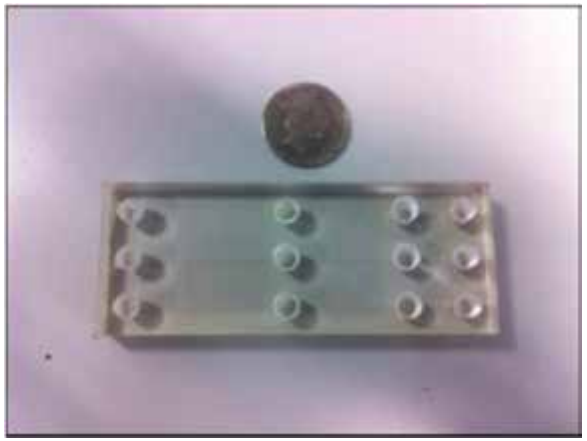
- *Determine whether or not similar universal principles used for globular proteins can be applied to describe antibody phase diagrams*
- *Many antibodies exhibit strong reversible association that is better described by chemical versus physical association models.*
- *Critical point density varies between antibodies and occurs at a much lower packing fraction than for globular proteins*

Rheology of concentrated solutions

Poor rheological properties of concentrated antibody solutions lead to

- *Difficulties in filtration and concentration steps to achieve drug product*
- *Complications for patient administration during sub-cutaneous syringe injection*





Methods:

- *Detailed characterization using in-house rheo-chip technology*
- *Screening formulation conditions with tracer particles either by dynamic light scattering or fluorescence correlation spectroscopy*
- *Dilute solution measurements of Huggin's coefficients by visco-star (Wyatt Technology)*

Rheology of concentrated solutions

Correlate concentrated solution zero shear-rate viscosity with

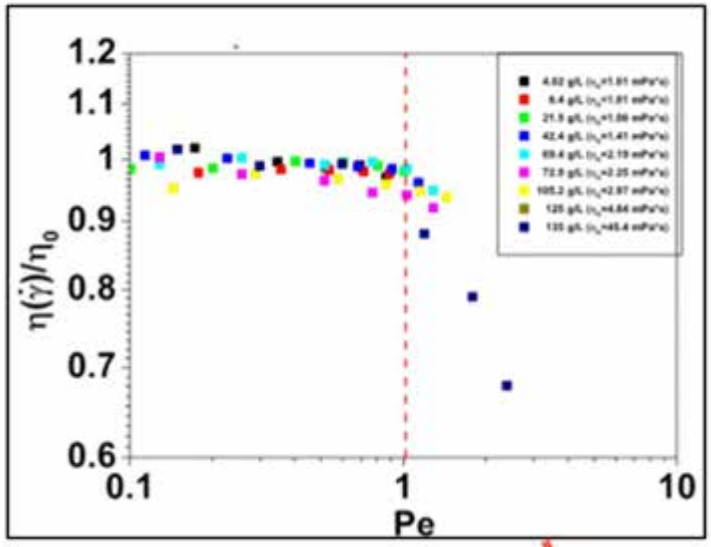
1. *Measurements of Huggin's coefficient*
2. *Protein-protein electrostatic repulsion*
3. *Reversible self association*

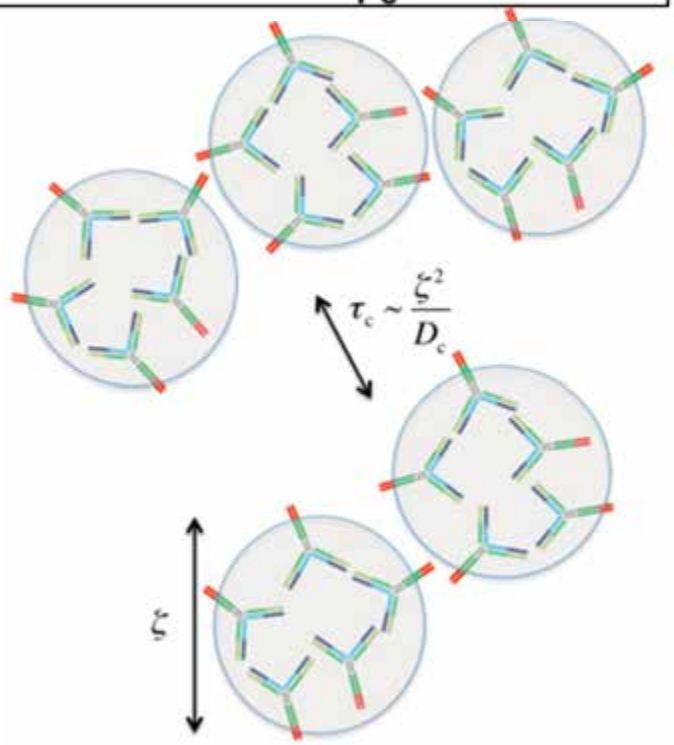
Examine link between shear thinning and

1. *Specific oligomer formation*
2. *Cluster formation under SALR conditions (short-range attraction, long range repulsion)*
3. *Supercritical density fluctuations*

Additional measurements include

1. *Mutual diffusion coefficients*
2. *Self-diffusion coefficients of fluousescently labelled particles*
3. *Osmotic compressibility*





Molecules and Partners to date

- UCB Pharma - A33 Fab
- NIBSC - GCSF
- UCL/Abzena – Domain 1
- Porton Biopharma – *tbc*
- Albumedix – HSA
- Arecor – novel excipients
- Wyatt Technology – instrument access
- MedImmune – mAbs
- Ipsen

Research team

PDRAS

- John Hales – UCL
- Cheng Zhang – UCL
- Jordan Bye – University of Manchester
- Max Hebditch – University of Manchester

PhD students*

- Nuria Codina – UCL
- Akash Pandya
- Nikita Vekaria – University of Manchester
- Jas Kalayan – University of Manchester

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Cheng Zhang	- PDRA UCL
Keying Li	- CPI
David Hilton	- GSK
Temitayo Ariyo	- PhD UCL
Mathew Robinson	- Fuji DSB
Victoria Wood	- PhD UCL

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GCSF	Paul Matejtschuk - NIBSC
Fab & IgG4	UCB Celltech
IgG1	Lonza & UCB Celltech
Molecular docks	Mire Zloh (Univ Herts) & Steve Brocchini (UCL)

EPSRC

BBSRC bioscience for the future

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