Etudier in situ l'agrégation de protéines: intérêts et limites des méthodes de diffusion de rayonnement

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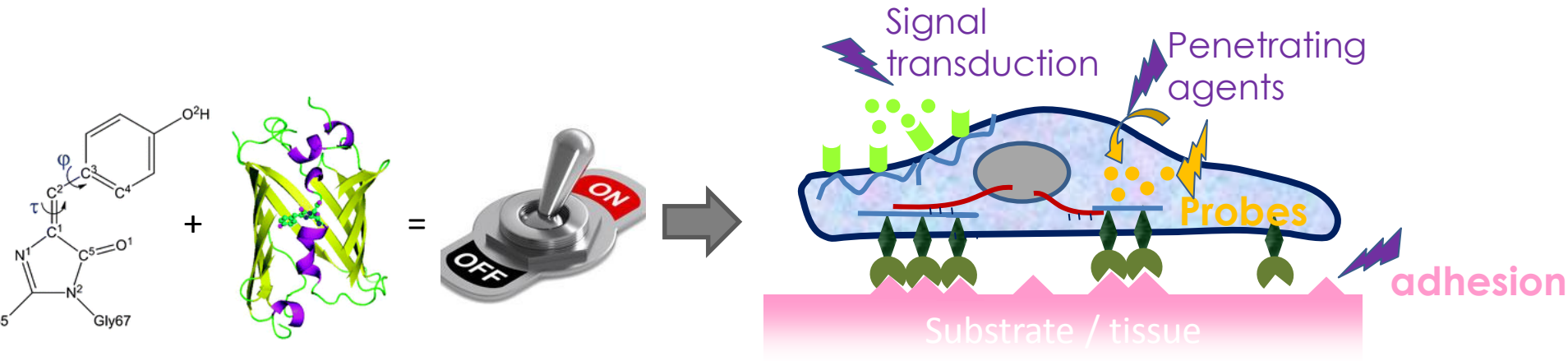
Pôle de Chimie Biophysique



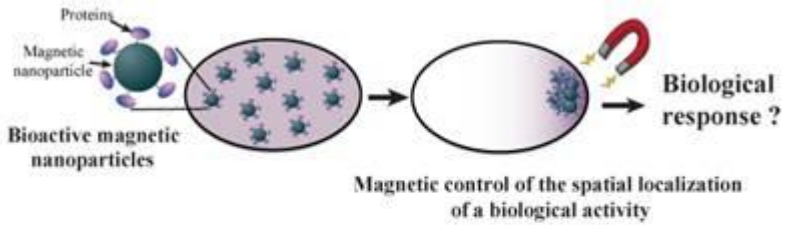
- ***From chemistry toward biology***
  - **Molecular "tools"** coupled with biological systems (Chemical Biology/Biophysics)
  - **Remote**, non-toxic **stimuli** (light, ΔT, magnetic)
- ***From biology toward chemistry***
  - Bio-inspired functions,
  - Genetically encoded constructs (fluorogens, particles)

Pôle de Chimie Biophysique

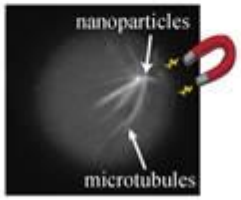
Manipulation of proteins in complex environments

Spatial distribution of signal proteins

Zoher Gueroui



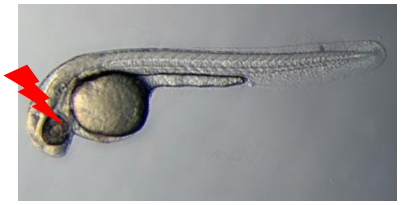
Magnetic control of the spatial localization of a biological activity



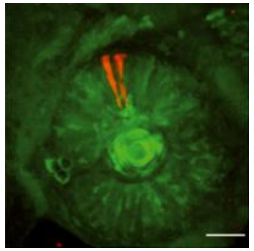
Fluorescent proteins readouts

Ludovic Jullien

Arnaud Gautier



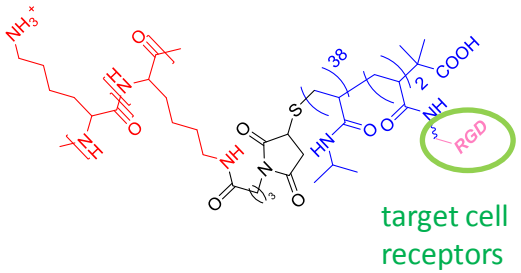
Zoom

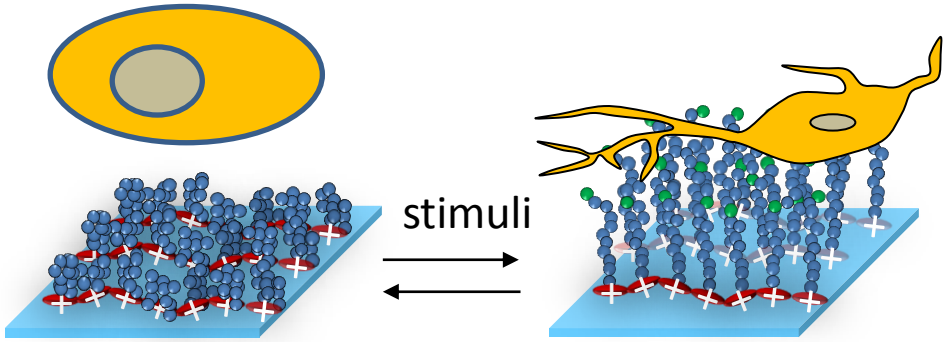


Pôle de Chimie Biophysique

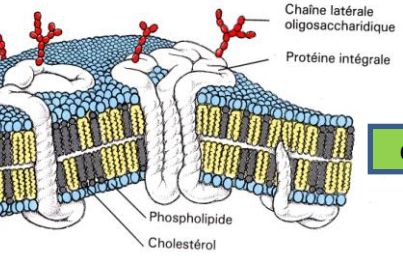
Manipulation of proteins in complex environments

Remote control of protein/peptide presentation:



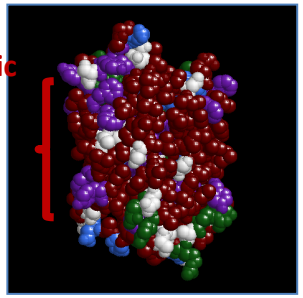


Artificial chaperones:



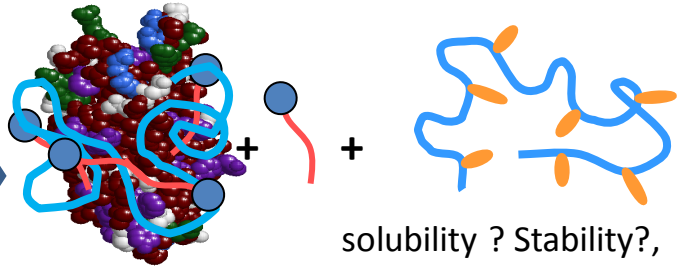
extraction

Hydrophobic residues



amphiphilic additives

stabilization of membrane proteins in water

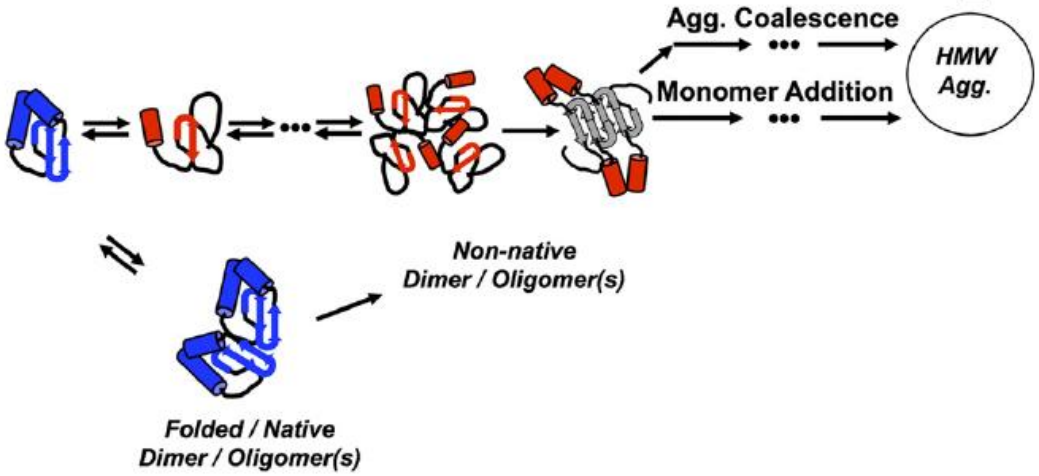


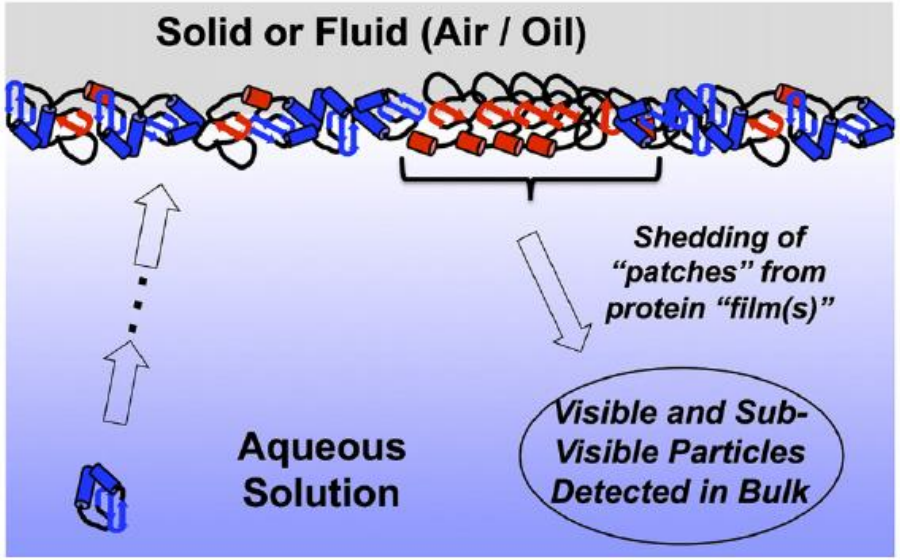
solubility ? Stability?, monodispersity?

⇨ **Refolding** of IMPs, soluble enzymes, **scFv**

⇨ Stability of IgG

Stability/aggregation issues of proteins in stressfull environment





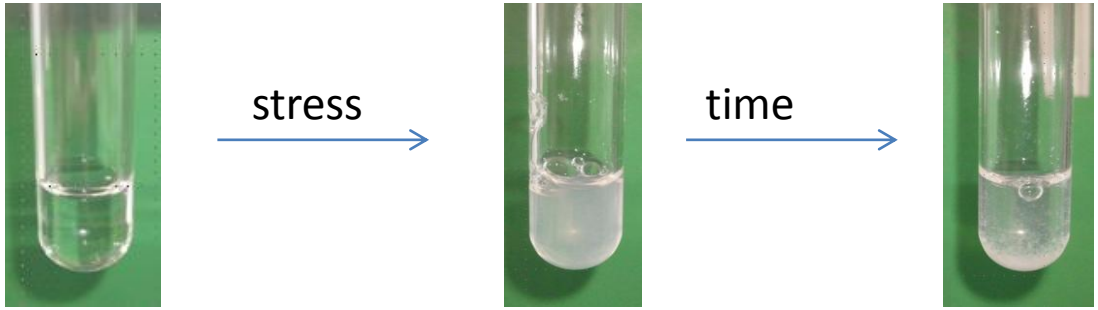
From Amin et al. Current Opin. Coll. Interf. Sci., 2014

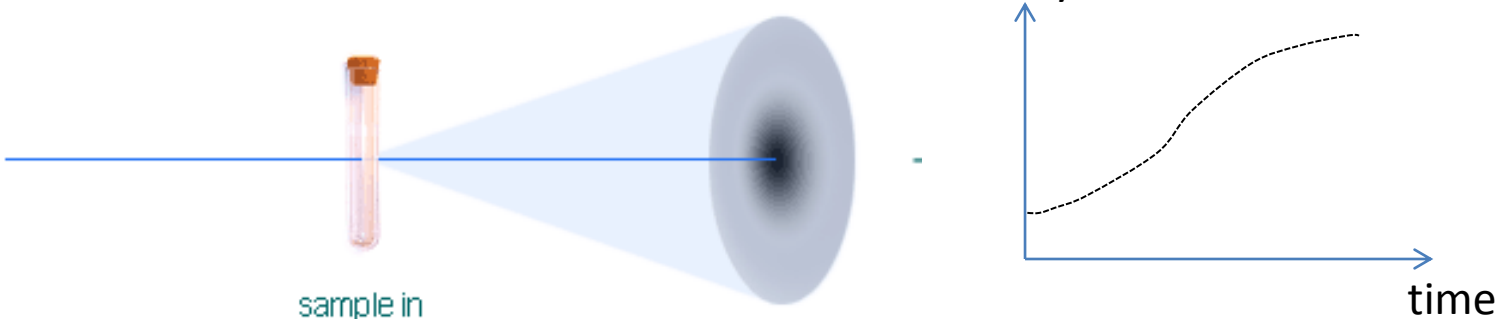
diversity of aggregation routes

Influence of additives , of interfaces, external stresses …

in situ characterization of protein association/aggregation in complex environments ?

Scattering techniques to assess aggregation





Highest sensitivity to aggregates

Common drawbacks attributed to (light) scattering methods

- « Although the sensitivity of [LS to] detect aggregates is unsurpassed, quantification is not possible » .. [Den Engelman et al. Pharm. Res. 2011, 28, 920-933] »

- « very sensitive to high Mw particles » = difficult to quantify size distribution (Chaudhuri et al., AAPS journal 2014)

- « requires filtration, biasing from dust or polydispersity » A. Pluen, Trends Biotechnol 2013 , 31(8), 447-

-« signal depends on particle morphology and (unknown) refractivev index » « only usefull when paired to size-selective separation techniques… » (Ripple et al., J pharma sci, 2012)

- « high concentration may lead to [bias]» (H Samra F. He, Molecular Pharma 2012)

Practical advantages and drawbacks

Pros:
- small amount (2-20 μL , 0.1-100 g/L)
- non invasive, label-free
- fast (< 10s– 2 min. ; fastest SAXS = ms)
- amenable to high throughput instruments
- broad size range (< nm – microns)
- viscosity measurement (DLS)

Cons:
- fitration required for light scattering, … but not in SAXS
- estimates of % aggregated (specific cases = large & solid-like clusters)
- do not discriminate proteins from dust, bubbles, dropplets…

What can be quantified ?		Relation to stability
shape, size of monomer or oligomer	⟷	elementary «bricks» of dense phases
proteins interactions +/- additives	⟷	phase transition vs metastability
characteristic aggregation rates	⟷	intrinsic stability index
kinetics, shape & size of clusters	⟷	class of aggregation pathway

Light and X-ray scattering to assess in situ the stability of proteins

Outline:

1) SAXS characteristic features of monomers / oligomers (radius, shape, interactions)

protein shape and radius (IgG, proteins in 2-phase systems)

protein-protein interactions vs solubility

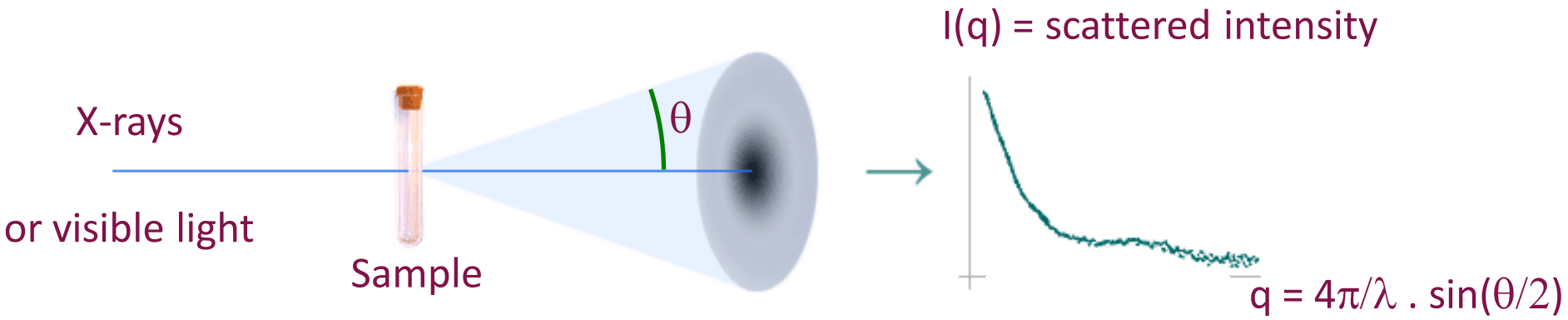
2) LS characterisations of growth rate of protein clusters

interface-born aggregates

kinetic stability index

efficiency of chaperones

Light & X-ray scattering



STATIC (average) structure of protein (SAXS, SANS), or of aggregates (SAXS, light)

interpretation depends on the value of q.R (>>1 or <1)

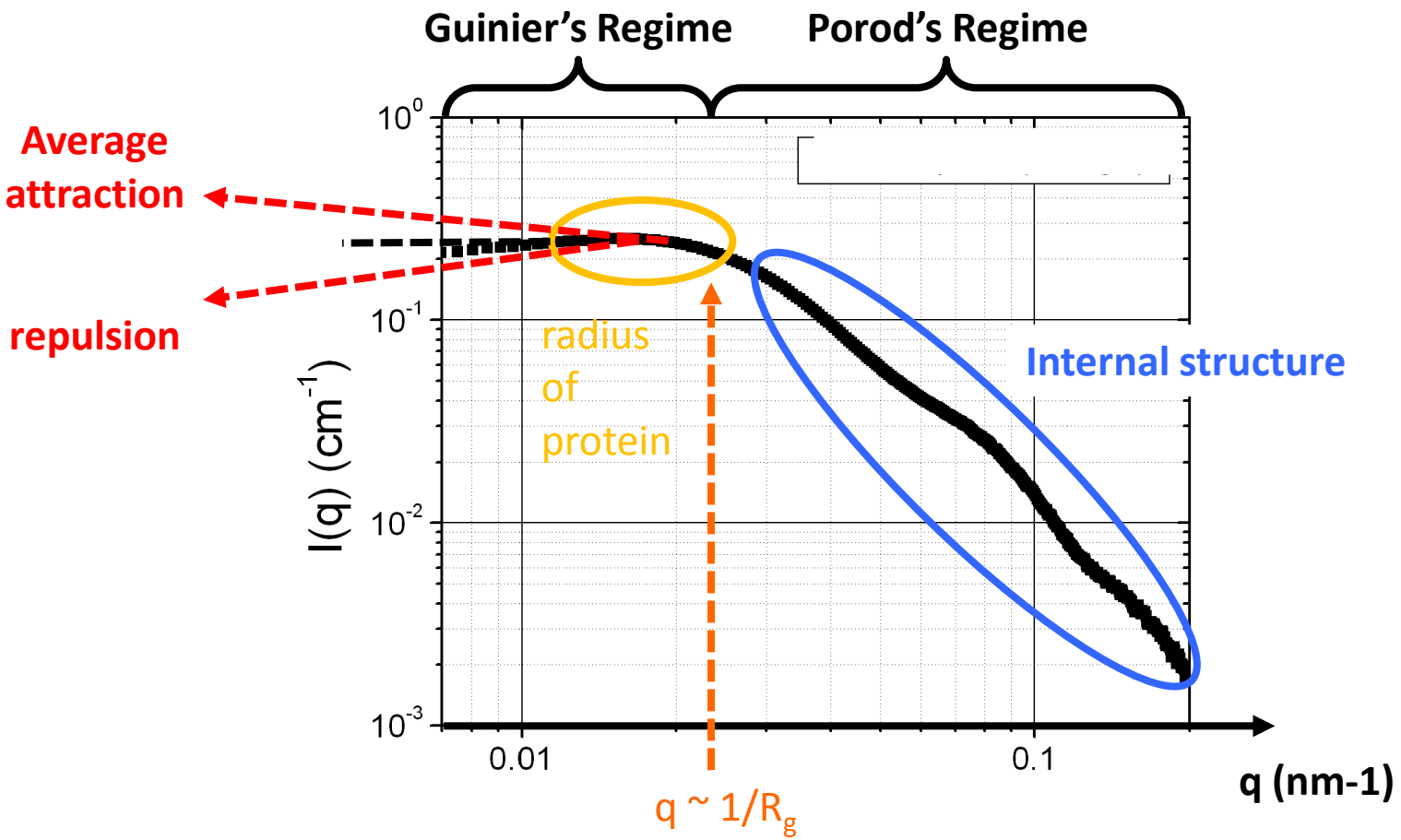
SAXS for characterization of structures & protein-protein interactions

larger length scales than protein radius

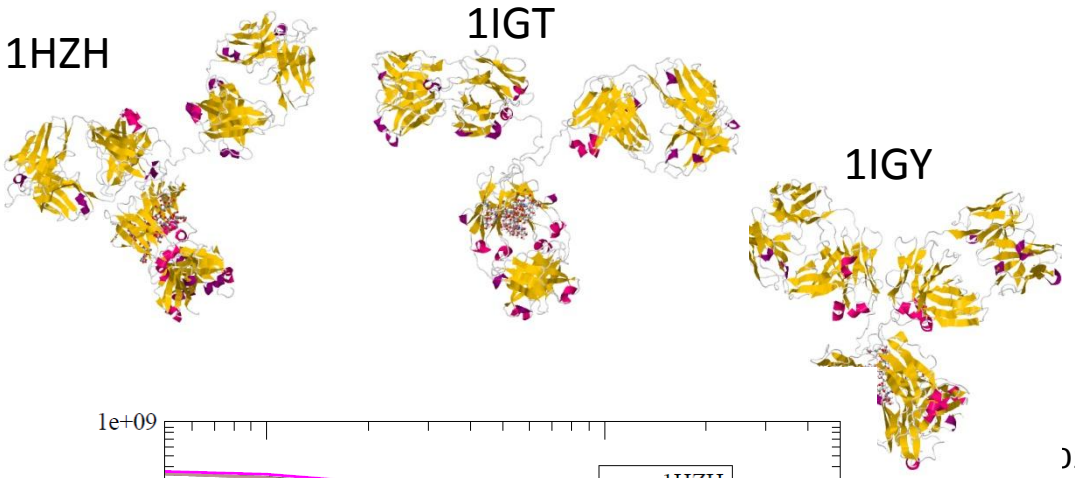
protein-protein interaction + spatial distribution

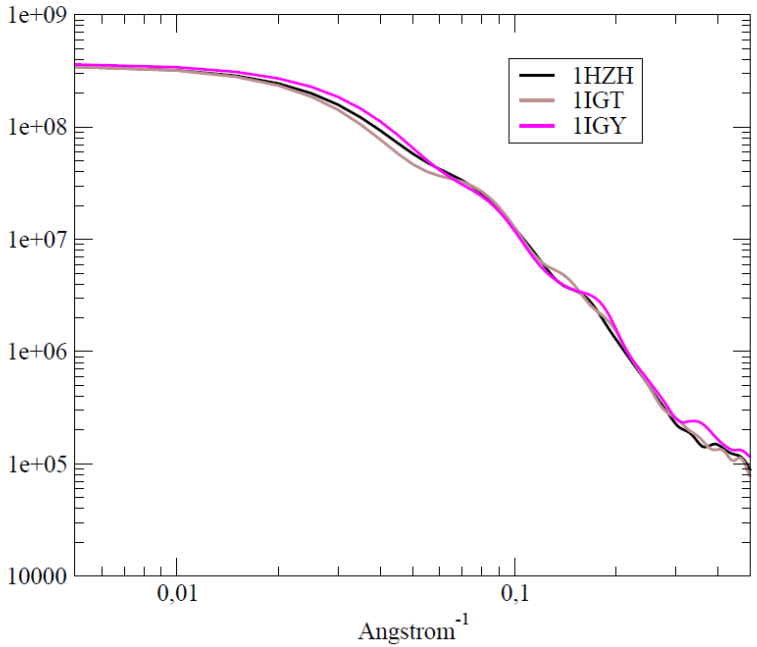
Configuration at < nm distances

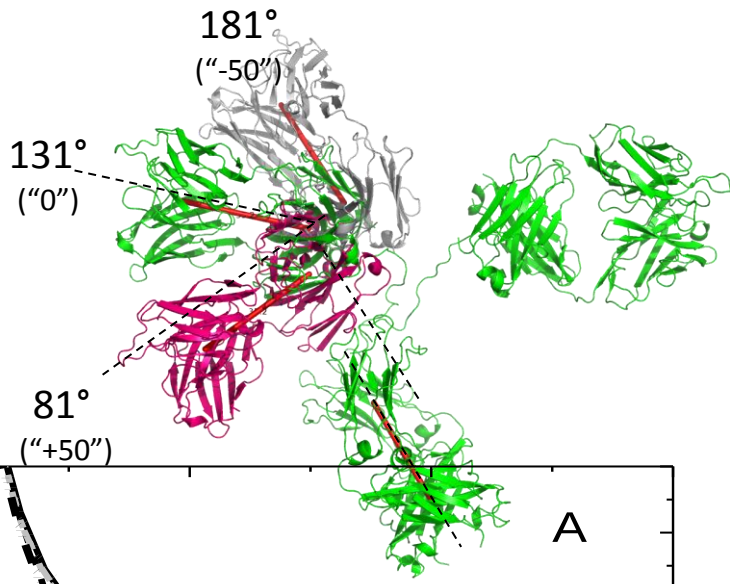
protein "shape"

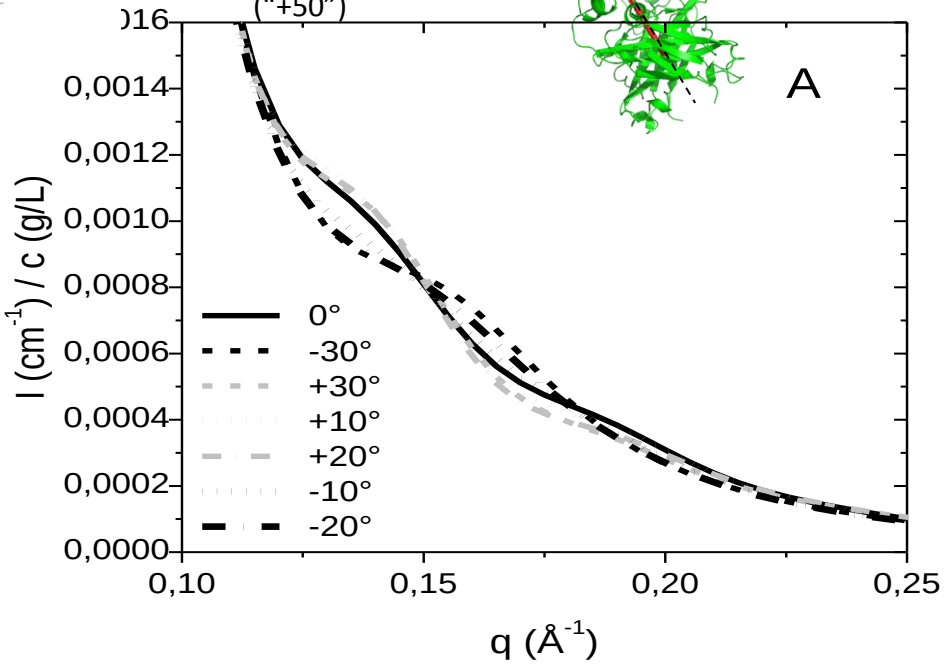


SAXS as internal structure assessment







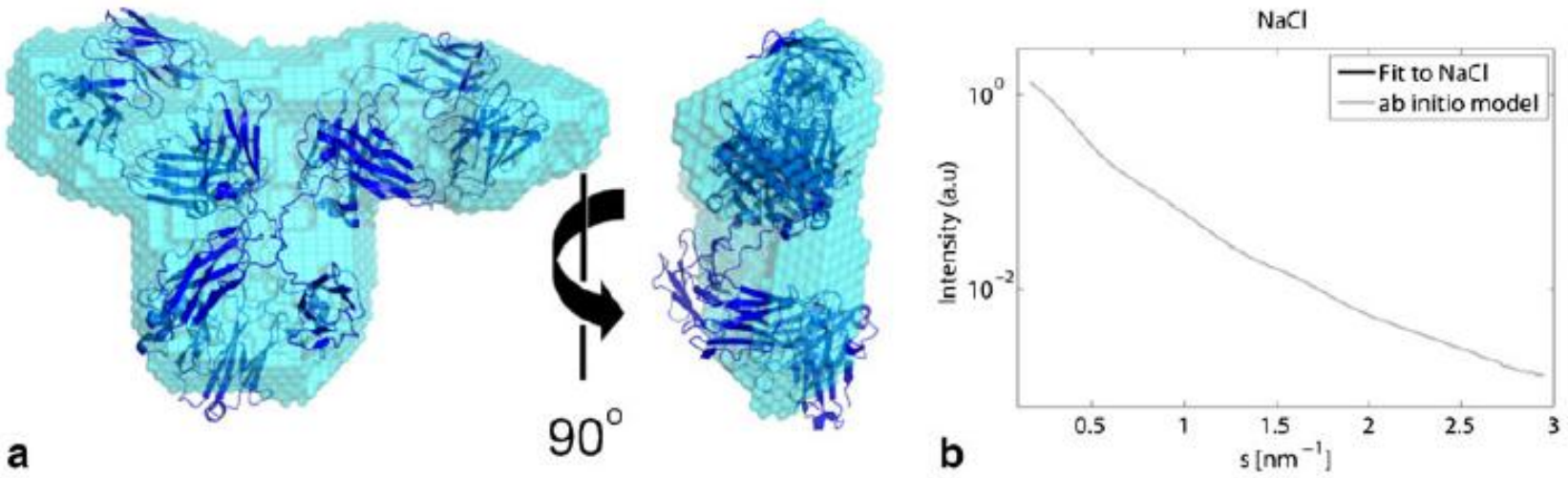


check the absence of obvious distorsions

N.B.: average over the whole population

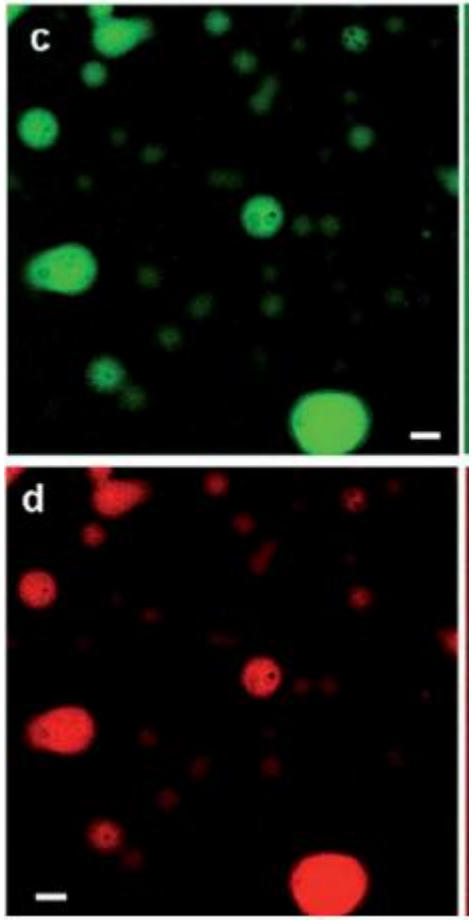
SAXS as internal structure assessment

Ab-initio reconstitutions (Panitumumab)



Mosbaek et al., Pharma Res. 2012, 29, 2225

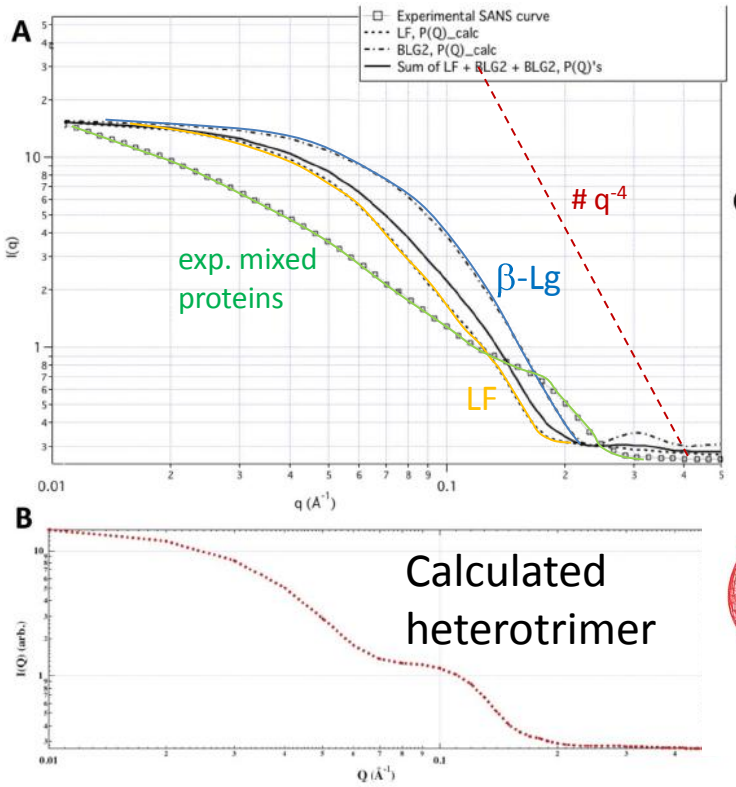
SAXS : structure assessment in concentrated phases

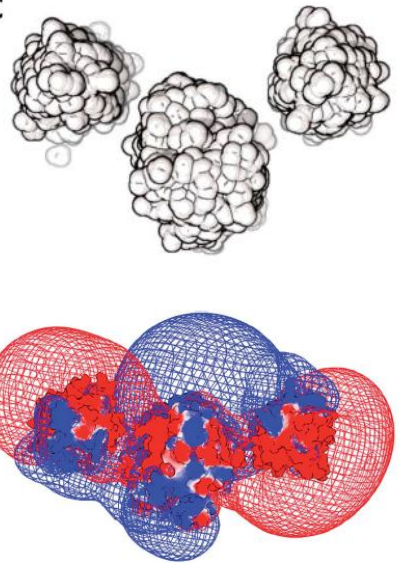


20 μm

Lactoferrin + β-lactoglobulin
1:1 mol/mol, pH 6

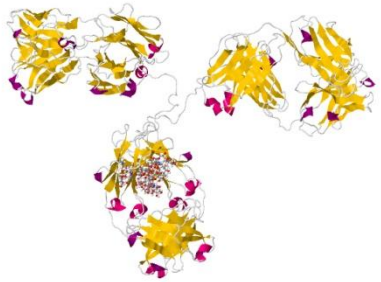
macro-heterogeneous dispersions (e.g. coacervates)



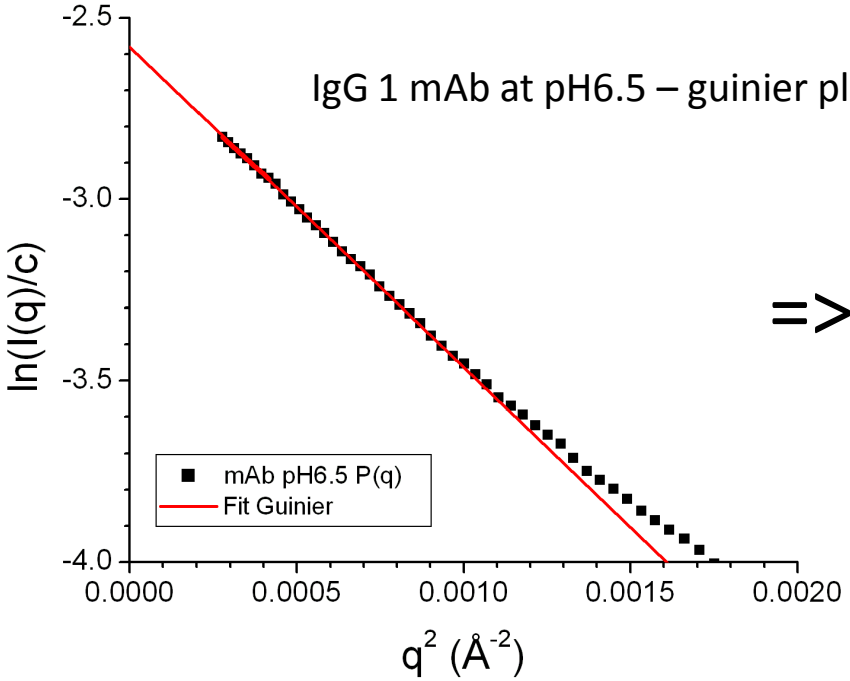


From C. Schmitt et al., Soft Matter 2014

Fast assessment of size in dilute solutions



low q SAXS , q.Rg <~ 1 $P(q) = P0.\exp\left(-\frac{(qR_g)^2}{3}\right)$

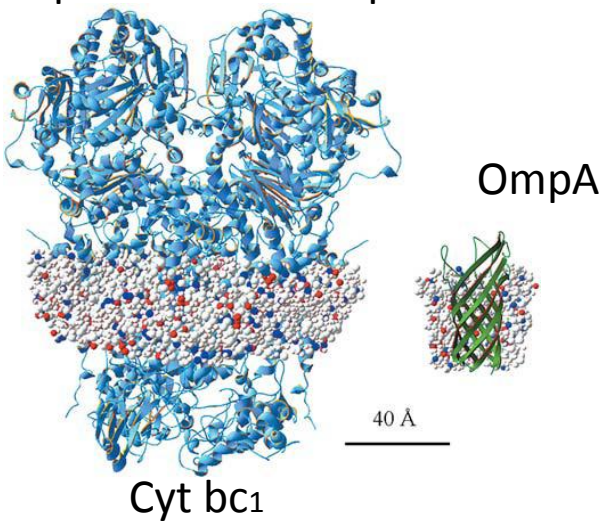


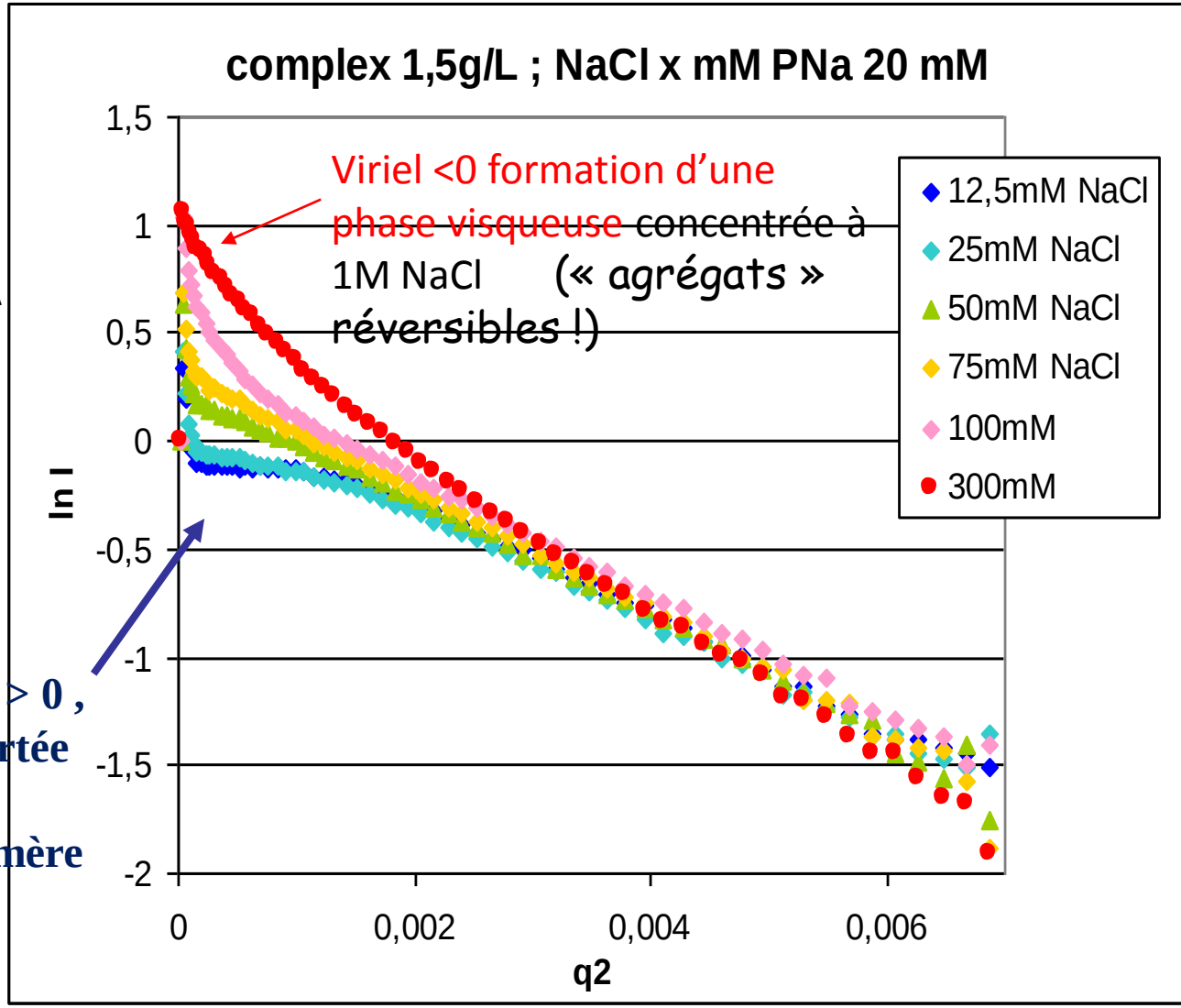
=> R_g = 52Å

Fast size assessment of complex assemblies

complexes between proteins and stabilizing additives

Integral membrane proteins in a amphilic belt

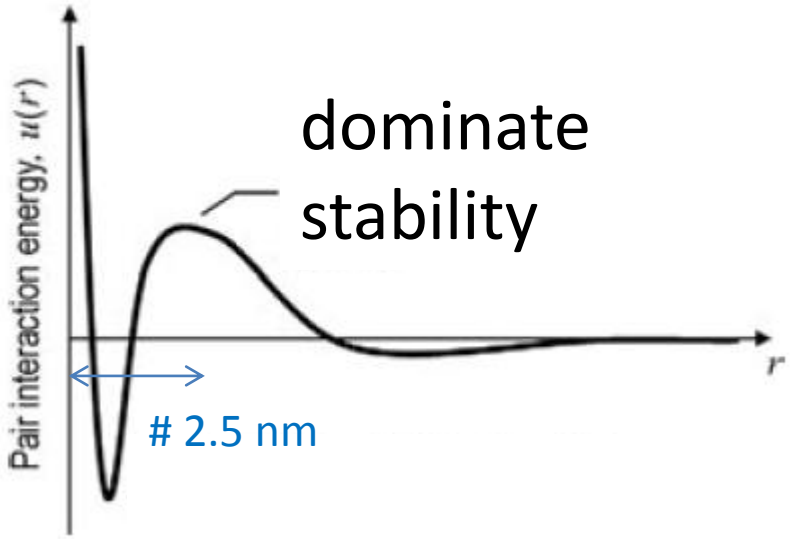




Coeff du viriel > 0 , solubilité apportée par répulsions polymère/polymère

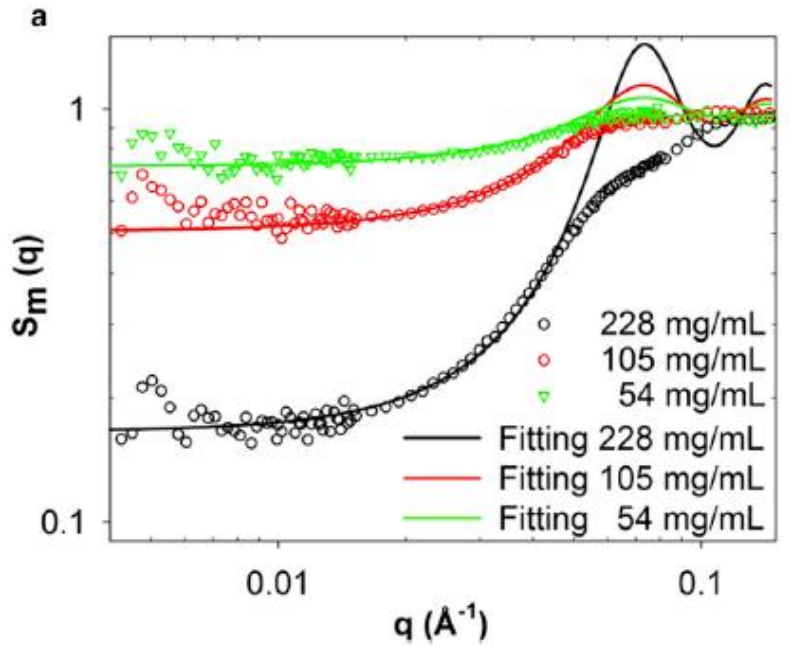
Gohon et al, Biophys J., 2008

Interactions in concentrated IgG1 solutions



3) Effective pair interaction potential
(assumes spherical averaging)

Fit to model energy-distance curves

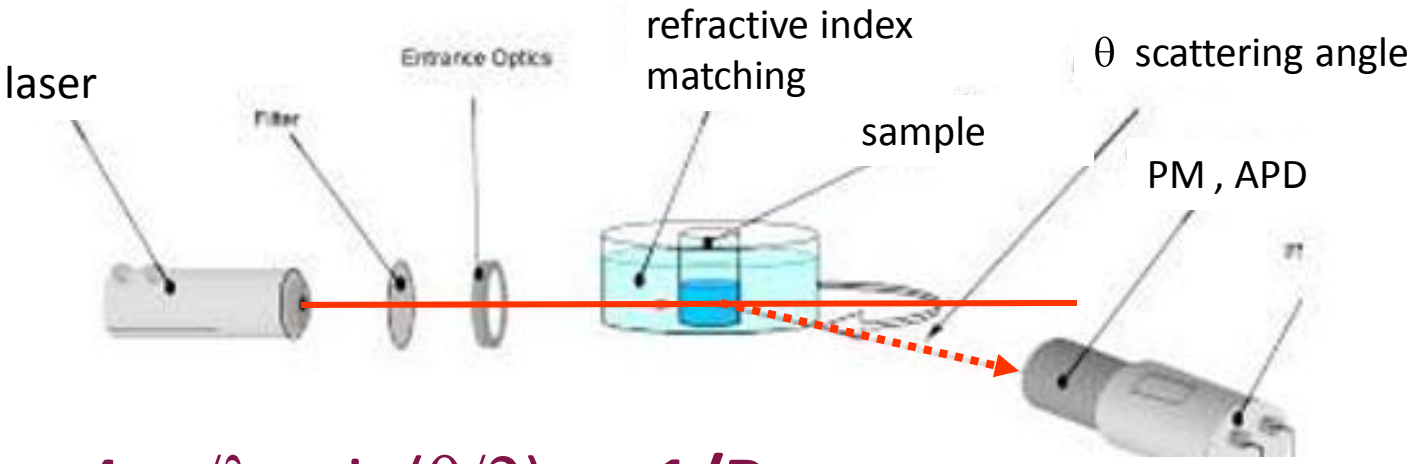


2) repulsions between IgG…but suggest METASTABILITY
model = weak repulsive barier (0.04 kT + deep minimum at contact (- 3 .8 kT)

Unstable formulation upon 1 month incubation

M. Castellanos, Biophys J. 2015, 107, 469

Average interaction determined by light scattering (B_2) predicts solubility



$q = 4\pi n/\lambda \,.\, \sin(\theta/2) \ll 1/R_{prot}$

concentration

second du virial coeff

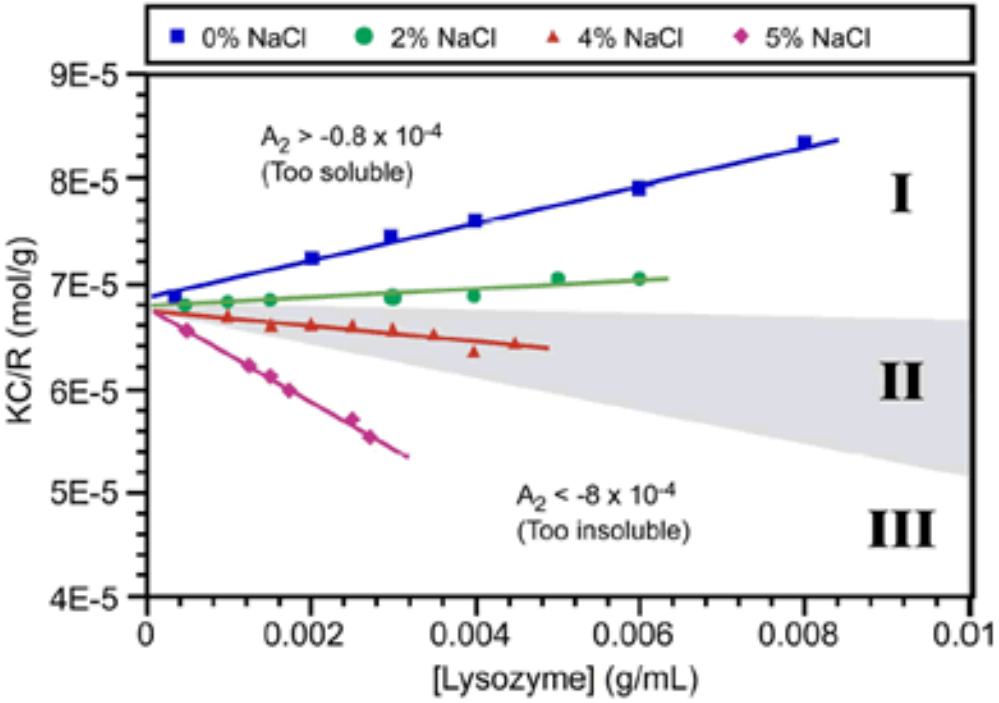
$$K.c/R_\vartheta = K.c.\frac{I_0}{Ir^2} = \frac{1}{M} + 2B_2c + o(c^2)$$

$$K = \frac{4\pi^2 n_0^2 (dn/dc)^2}{N_A \lambda^4}.$$

molar mass of scatterers

scattered intensity

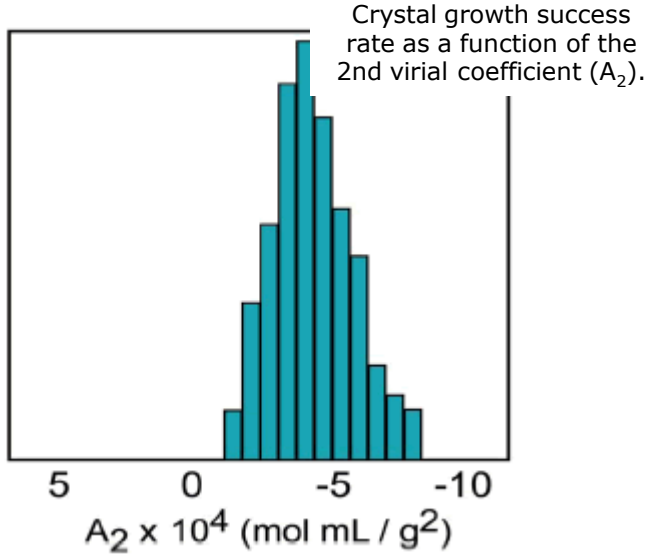
Light scattering :solubility vs B_2



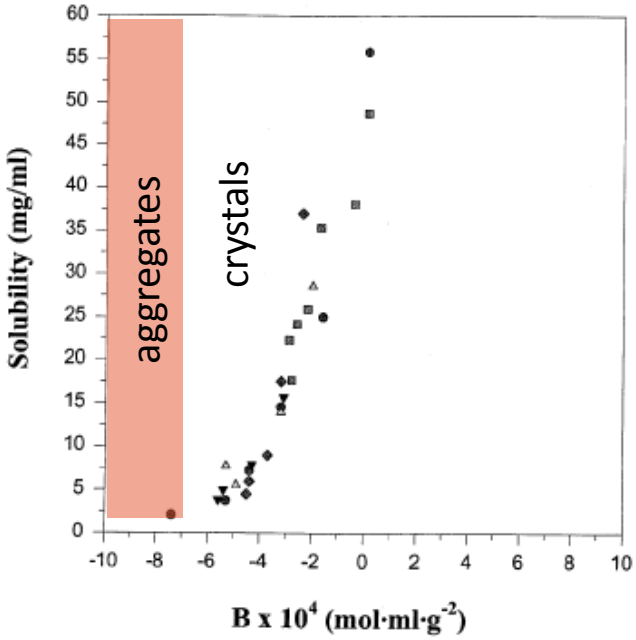
Debye plots for lysozyme vs NaCl

crystallization slot: -0.8 x 10-4 > A2 > -8.0 x 10-4

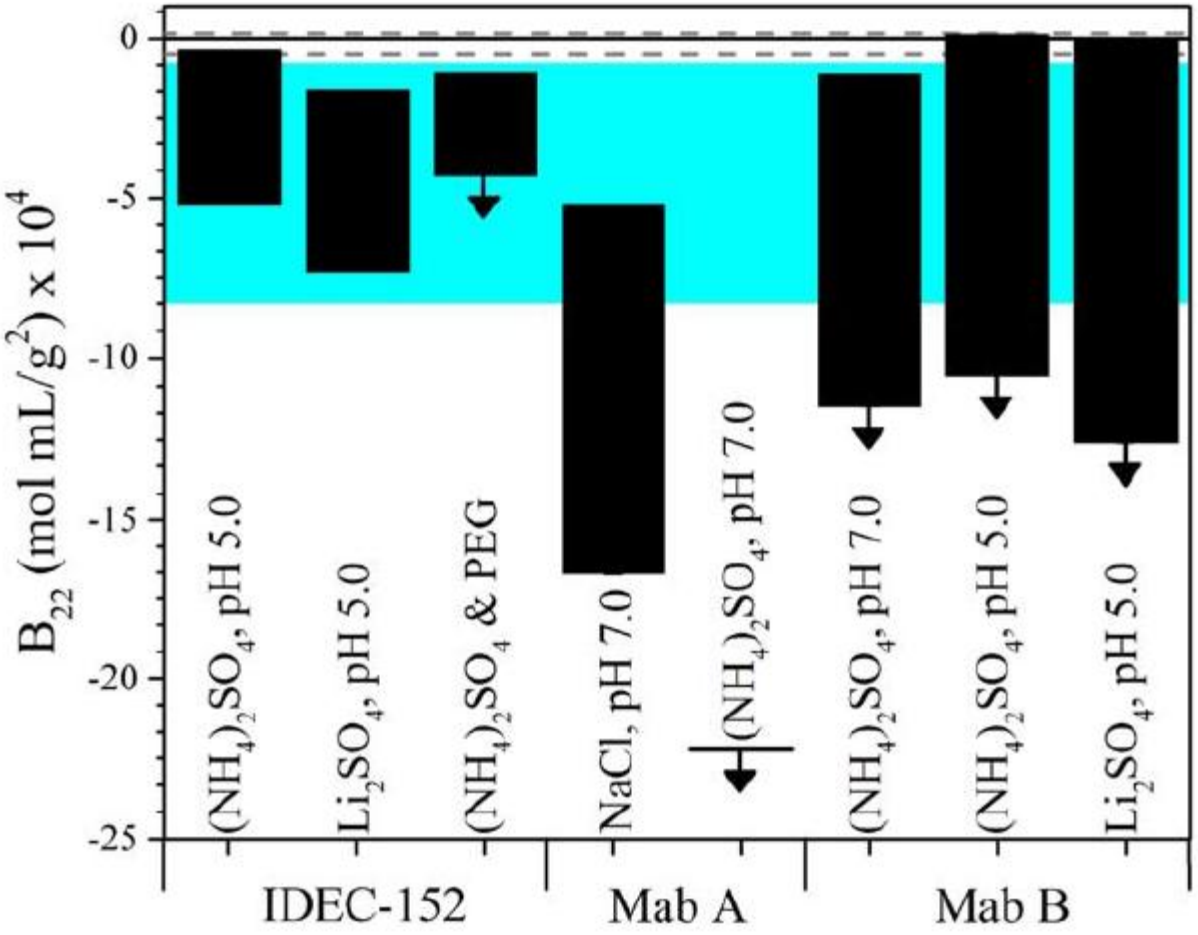
wilson et al. J. crystal Growth (1999), 196, 424-433



Crystal growth success rate as a function of the 2nd virial coefficient (A_2).



Solubility vs B_2 in solutions of IgGs



R. A Lewus et al., Biotechnol. Prog. 2015, 31(1), 268

Dynamic light scattering for robust, faster characterisations

Static scattering drawback:

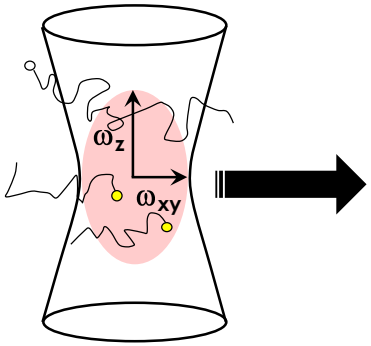
average contribution of any particle = contributions from dust, bubbles

sensitivity to optics (cell wall, centering, etc..) = moving sampling difficult

lack indentification of multimodal populations

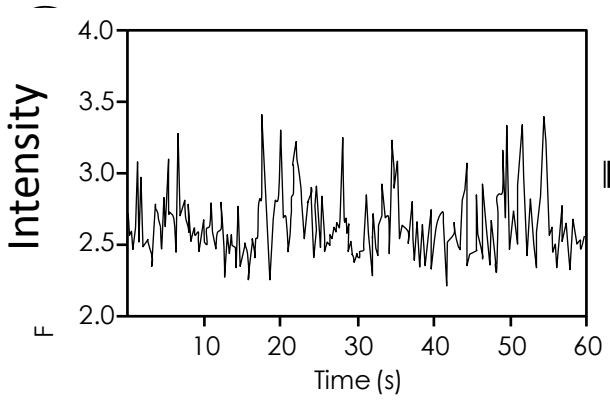
Dynamic analysis: robust to static optical « defects »

radius-based discrimination of populations

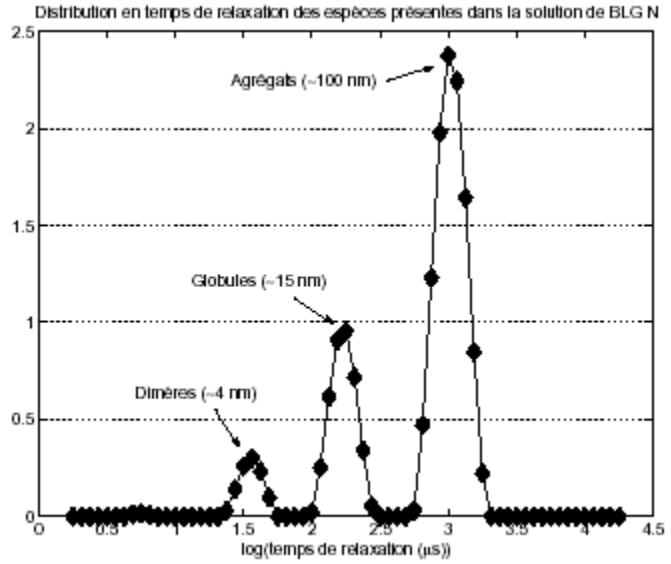


Small scattering volume

$\Delta C \sim C$

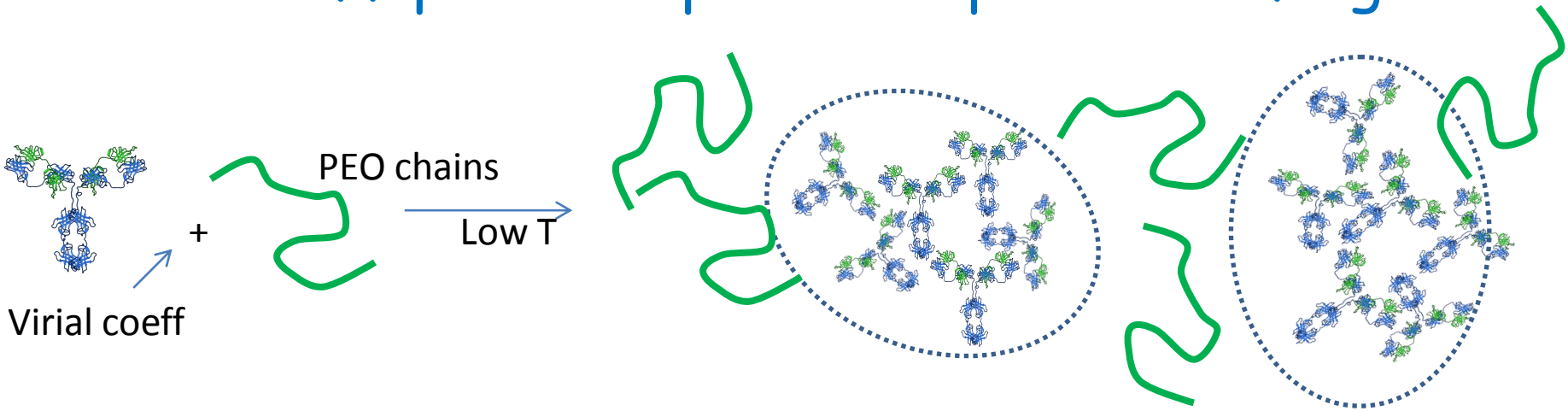


Fluctuation due to brownian diffusion

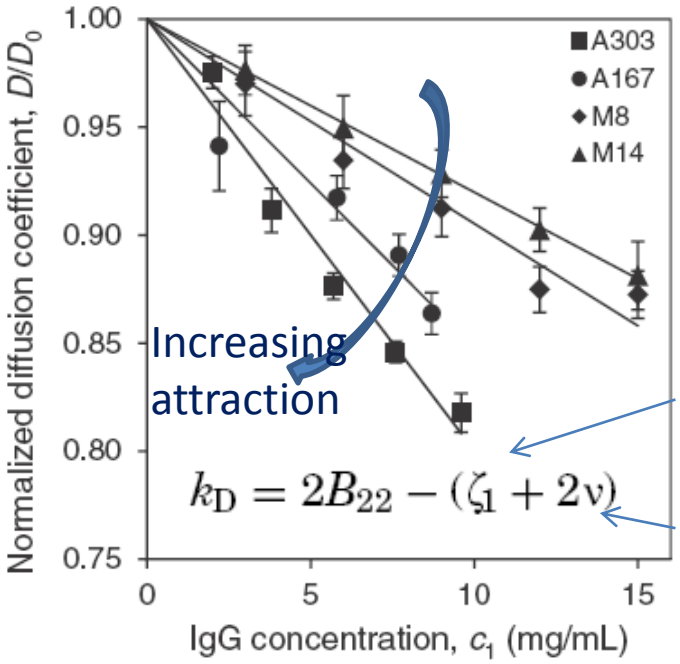


e.g. thermal aggregation of β-Lg

Virial coeff predicts phases separation of IgG1

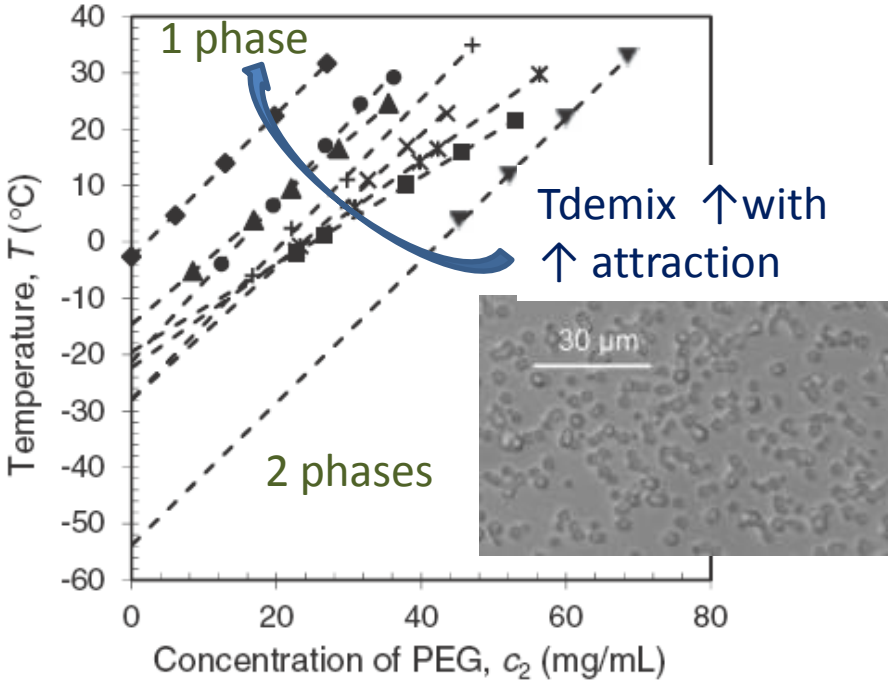


$$D(c) = D_0[1 + k_D c + O(c^2)]$$



viscosity coeff

prot. volume



G.Benedek et al. J Chem. Phys 2013

Light and X-ray scattering to assess in situ the stability of proteins

Outline:

1) « monomer » characteristic features

protein shape and radius (IgG, protein in 2-phase systems)

protein-protein interactions vs solubility

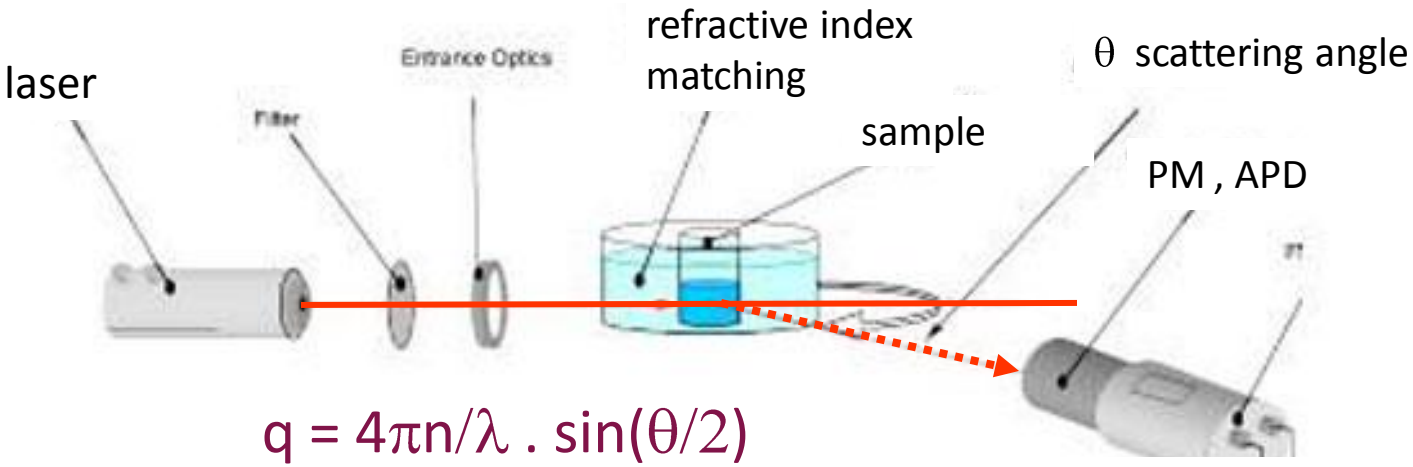
2) Characterisations of growth rate of protein clusters

interfacial-born aggregates

kinetic stability index

efficiency of chaperones

Light scattering : <Mw> , <R>, B_2



$q = 4\pi n/\lambda \,.\, \sin(\theta/2)$

- structural informations on aggregates larger than ~$\lambda/10$ (fractal dimension)

- average characteristic molar mass (from monomer to clusters)

weight concentration

$$K.c/R_\vartheta = K.c.\frac{I_0}{Ir^2} = S(q).\left(\frac{1}{M} + 2B_2c + o(c^2)\right)$$

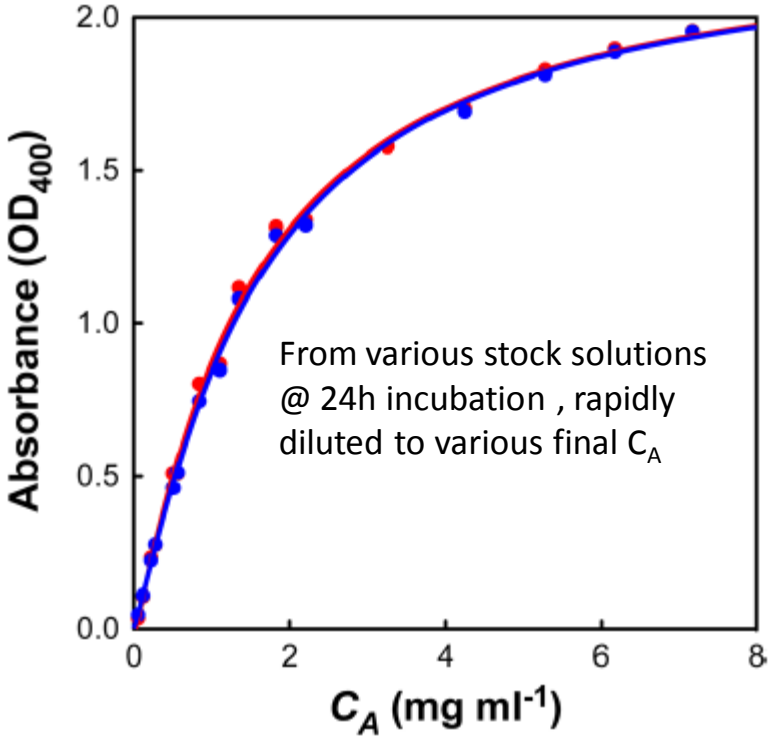
Oligomers: S(q) # 1

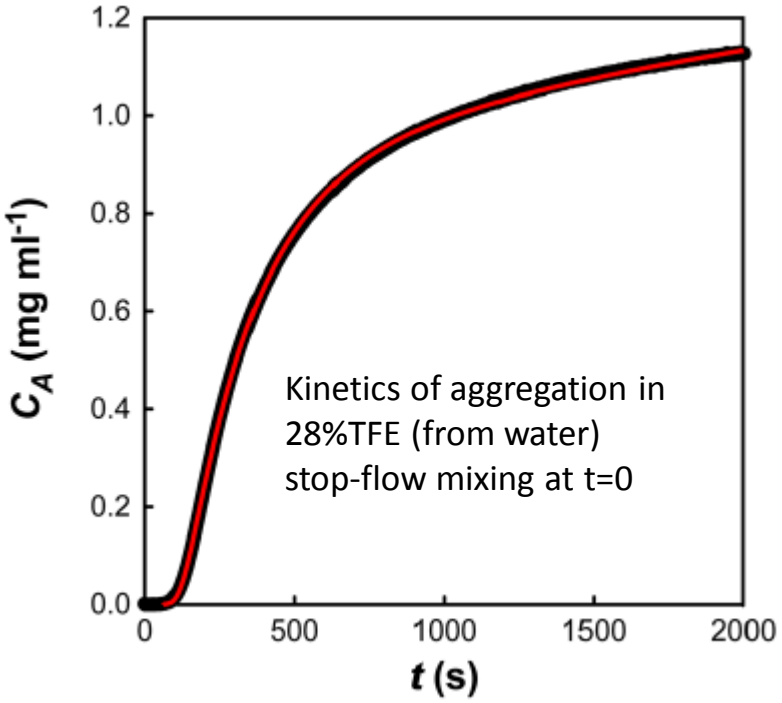
or

Large aggregates (qR >>1):
S(q) ~q^{-Df}

High sensitivity: I ↑ with
<molar mass> of aggregates

Turbidity : the simplest determination of concentration of aggregates ?





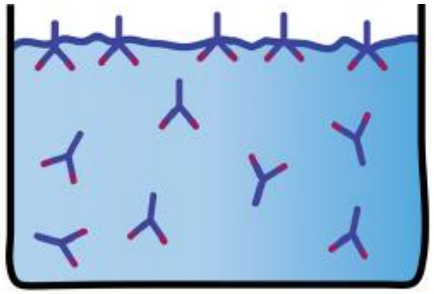
Validation required: no evolution with time & C_{init}

I27 domain of human cardiac Titin
amorphous β-aggregation in TFE:water

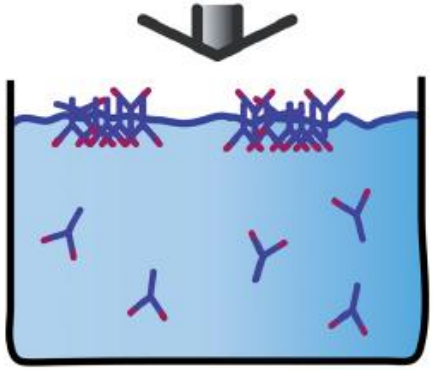
M. Borgia JACS 2013, 135, 6456

Case of large, solid-like & dispersed aggregates
Interface-driven aggregation

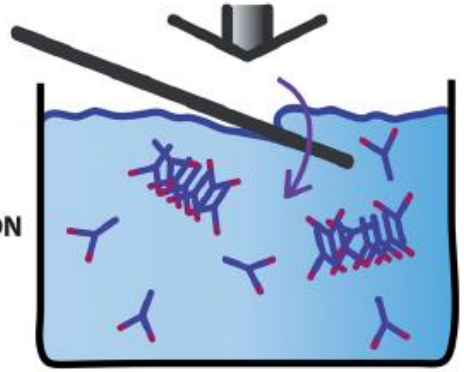
1) ADSORPTION OF ANTIBODIES AT THE INTERFACE



2) INTERFACIAL NUCLEATION OF AGGREGATES



3) RELEASE OF AGGREGATES IN THE SOLUTION UPON MECHANICAL PERTURBATION



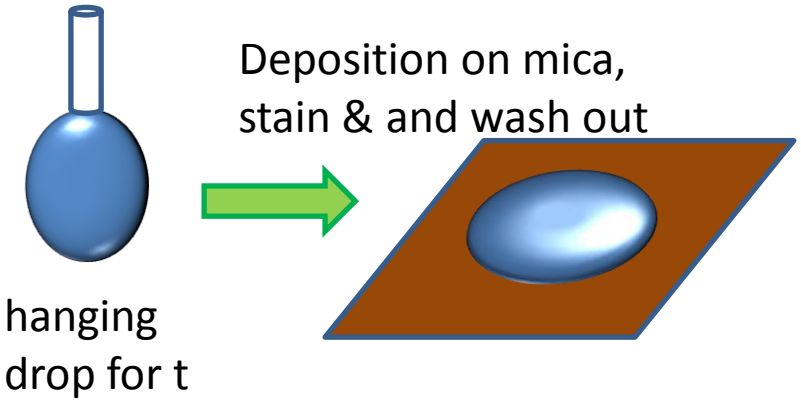
Shaking IgG solution produces aggregates

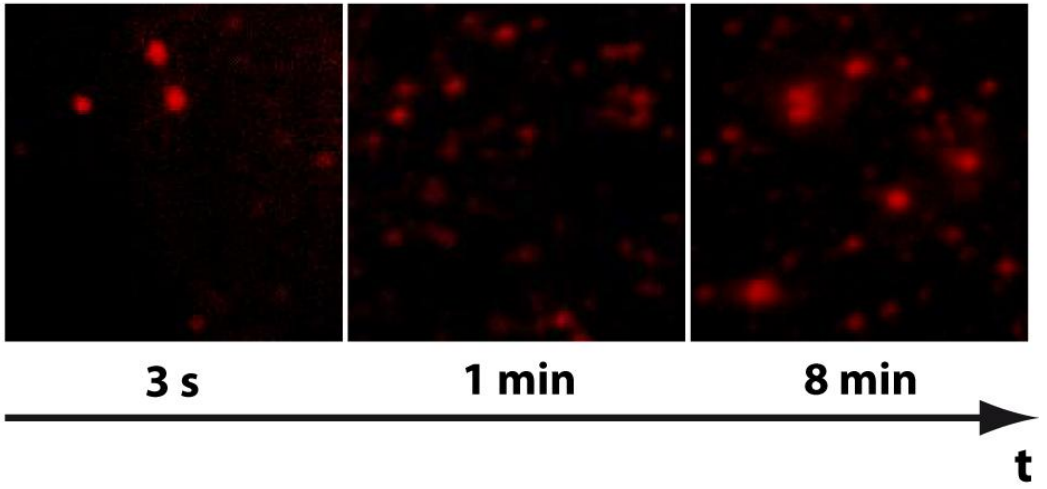
Role of interface ?

Role of shearing ?

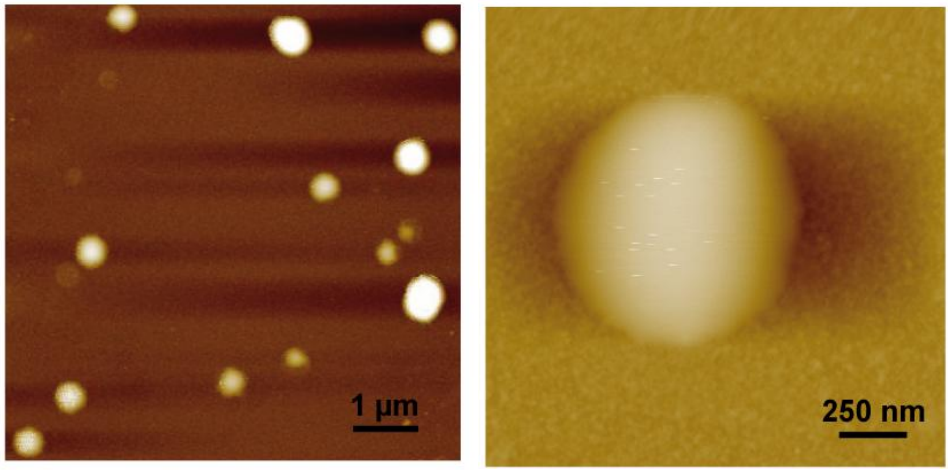
Interface-driven aggregation of IgG

Fluorescence microscopy (RITC-Ab staining)

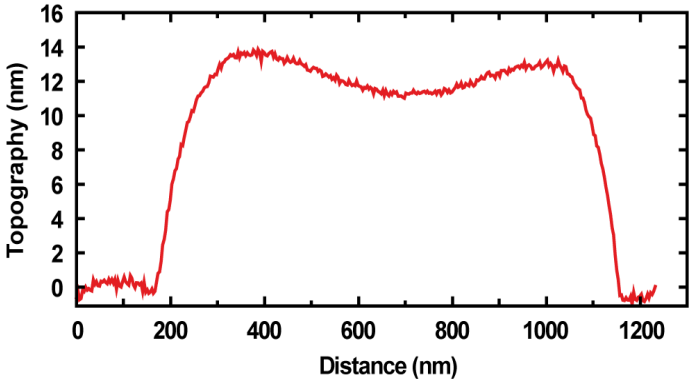




AFM



- 200-1000 nm diameter
- 12-14 nm thickness

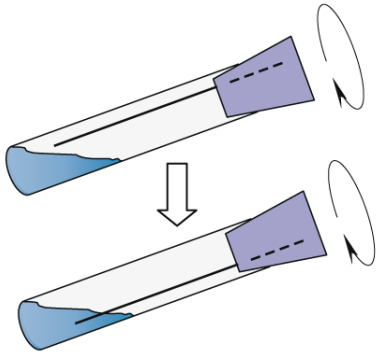


S. Rudiuk, Soft Matter, 2012, 8, 2651

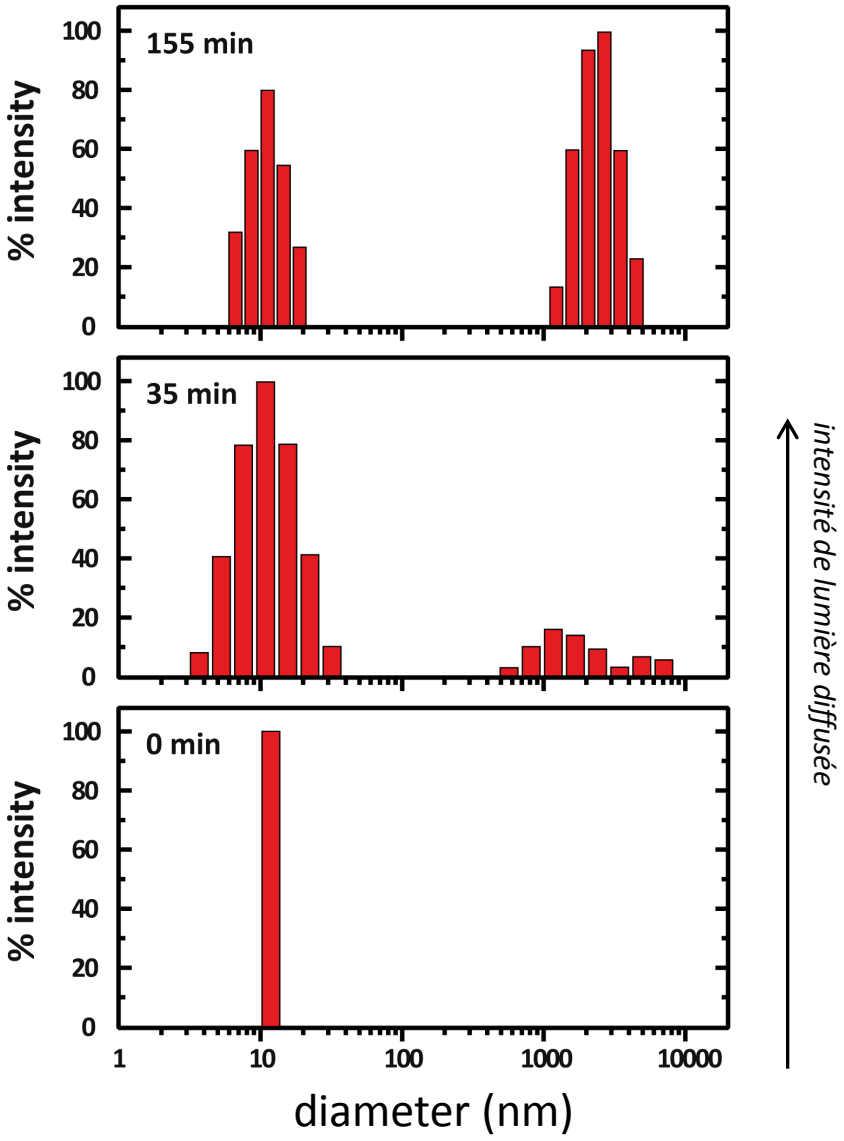
Surface-driven aggregation

Generation of interfacial stress in mAb solutions

needle cross the interface at each rotation



1. *aggregation detected by SLS/DLS vs nb of full rotation*
2. *References = no needle or needle always in solution*

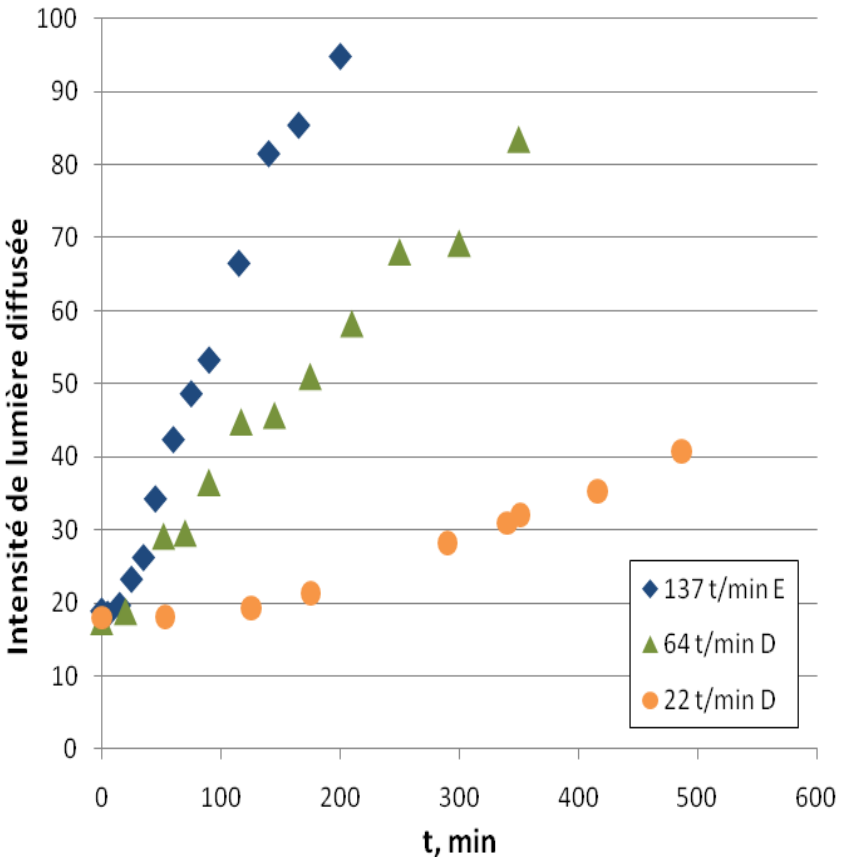


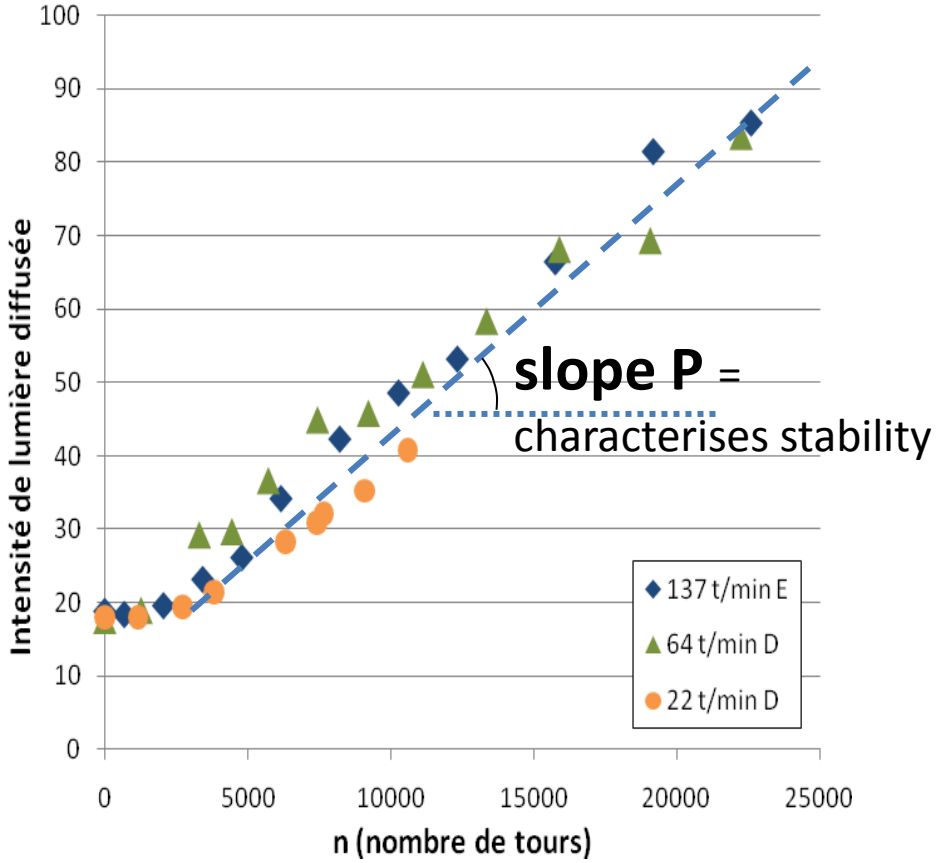
S. Rudiuk, Soft Matter, 2012, 8, 2651

Light-scattering intensity , normalisation of aggregation rate

Hypothesis: intensity reflects the nb of particles (Ragg >> 1/q and internal structure fixed)

Similar resuts with human polyclonal, and monoclonal IgG 1,5 g/L



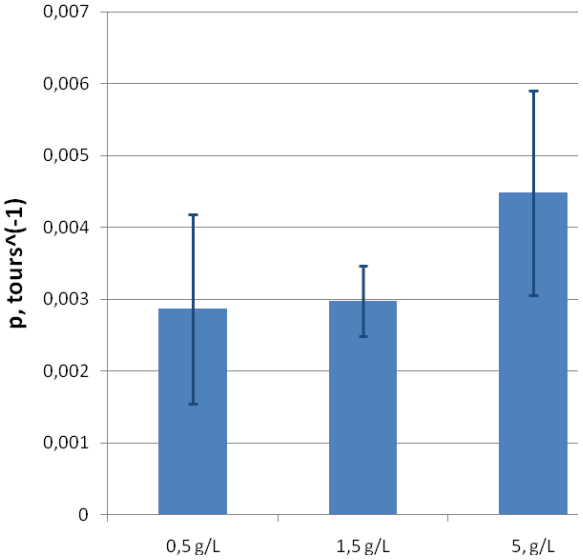


S. Rudiuk, Soft Matter, 2012, 8, 2651

Destabilization by interfacial stress studied by LS

No effec of IgG concentration

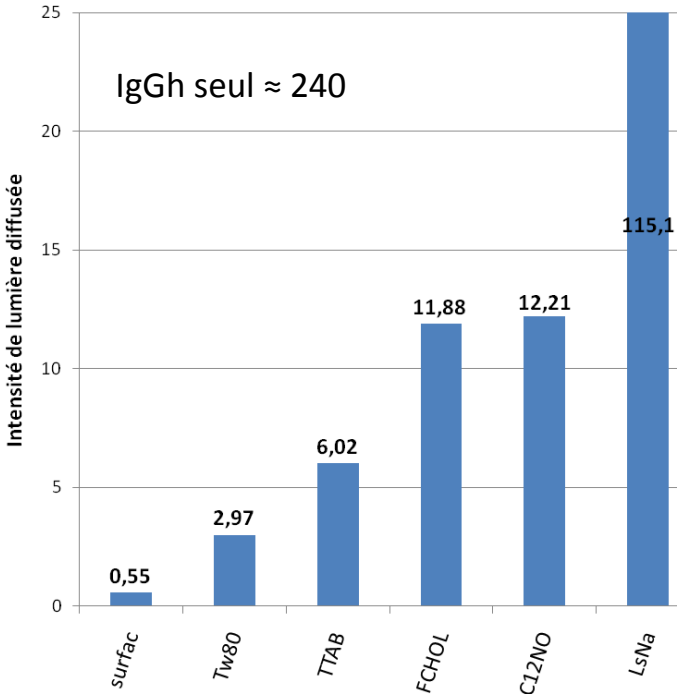
= limiting step is not adsorption



rotation 65 h 64 rpm

Effec of amphiphilic additives

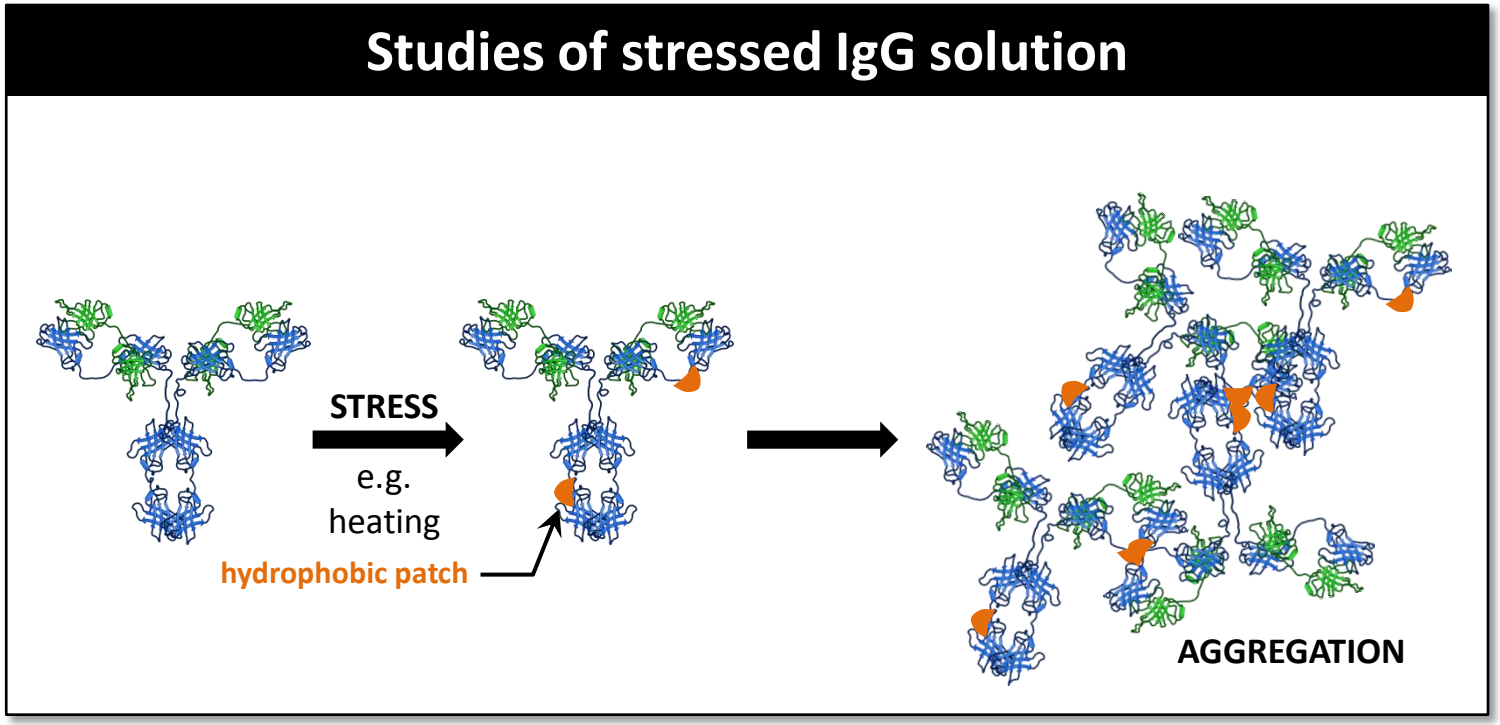
= efficacy driven by adsorption rate of surfactant



Surfac > Tw80 > TTAB > FCHOL > C12NO > LSNa

S. Rudiuk, Soft Matter, 2012, 8, 2651

Case 2: solution-born aggregates



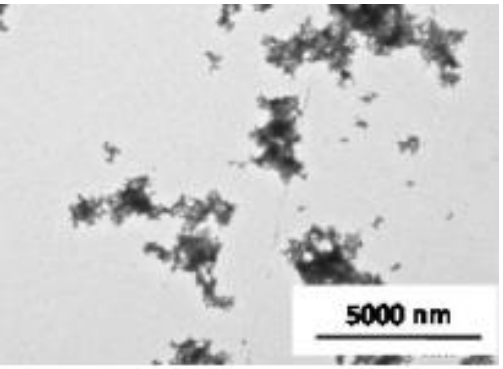
Morbidelli et al. , J Phys Chem 2012, 116, 7066

Roberts, C. J. et al. (2011). Int.J. Pharma. 418(2): 318

Amin et al Curr. Opinion Coll.Interf. Sci. 2014,19(5): 438-449

Internal mass distribution in clusters of IgG1

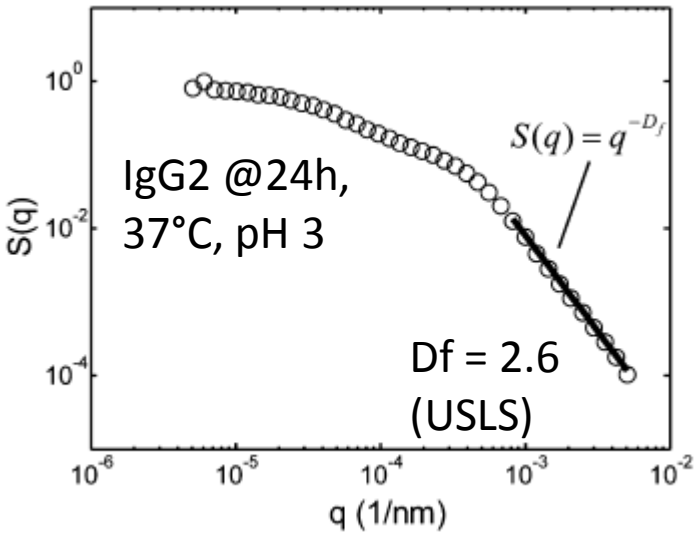
Experimentally measured fractal dimension # 2.1 – 2.6



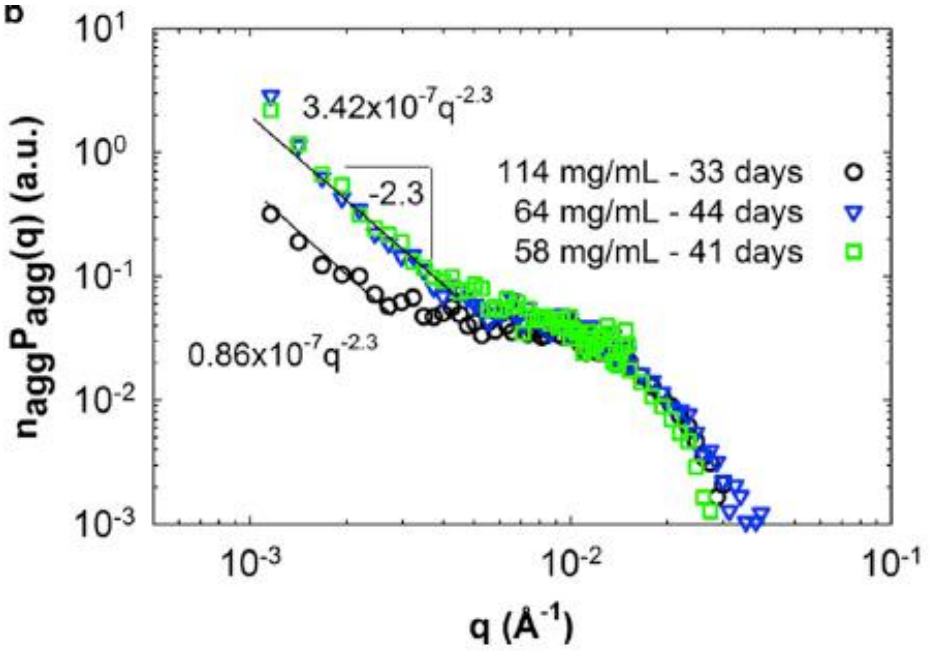
radius of the bigger cluster — radius of the monomer

$$S(q) \sim q^{-D_f}, \quad \text{for } 1/\langle R_g \rangle \ll q \ll 1/R_p$$

Light scattering



X-ray or neutron scattering



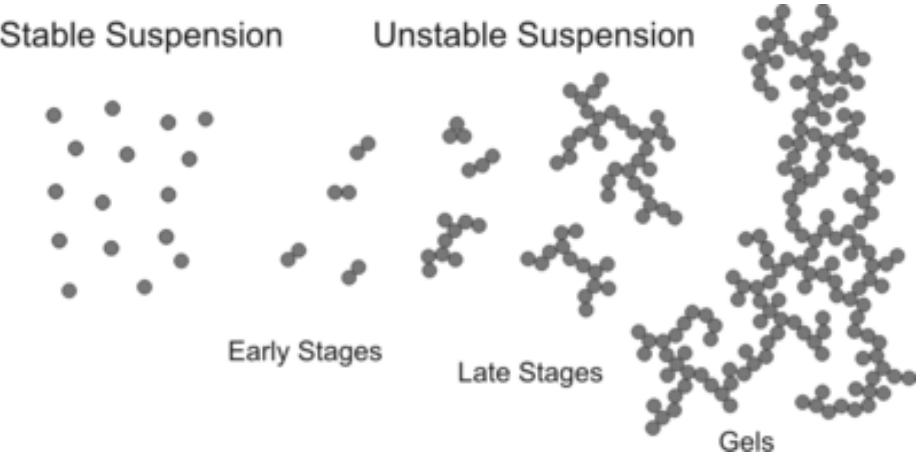
Morbidelli et al. , J Phys Chem 2012, 116, 7066

M. Castellanos, Biophys J. 2015, 107, 469

Case of constant Df (tight bridging, no evolution of aggregate density with time)

- Intensity at fixed q vary in proportion to the amount of aggregated proteins

- Smoluchowski's random aggregation (kinetics of inter-cluster coagulation)
one may neglect monomer accretion and impact of oligomers at long time scales



$$\frac{dN^*_i}{dt} = \frac{1}{2}\sum_{i-1}^{j=2} k_{j,i-j} N^*_j N^*_{i-j} - N^*_i \sum_{\infty}^{j=2} k_{i,j} N^*_j,$$

$$k_{i,j} = \frac{k_B}{W} B_{i,j} P_{i,j} \qquad B_{i,j} = \frac{(i^{1/D_f} + j^{1/D_f})\left(\frac{1}{i^{1/D_f}} + \frac{1}{j^{1/D_f}}\right)}{4}$$

Wikipedia.org/particle aggregation

Single index of stability : W = Fuchs stability ratio

Determination of Fuchs stability ratio

(case 2a: Df does not evolve & cluster radius > $R_{protein}$)

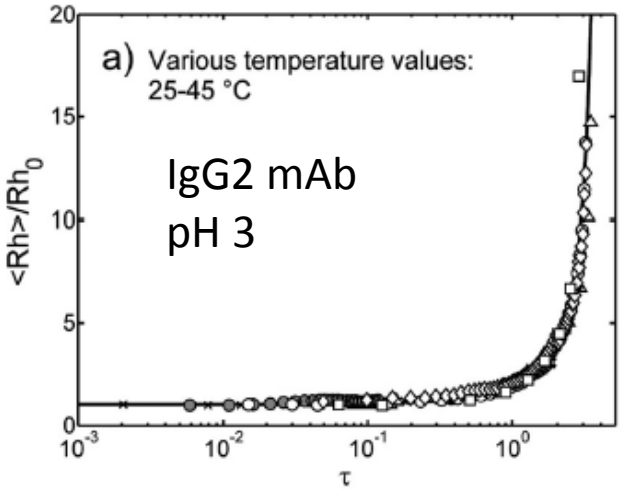
Smoluchowski's random aggregation
(kinetics of inter-cluster coagulation)
If one can neglect monomer accretion and role of oligomers :

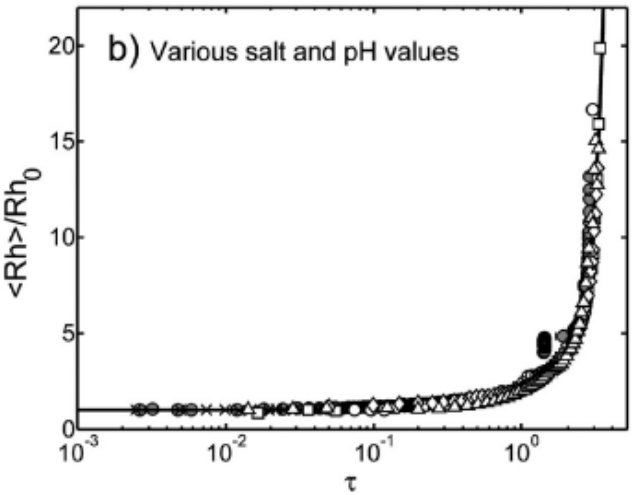
Normalisation by $\tau = t/tc$

$$1/t_c = k_s^0 . C_0 / W$$

$$k_s = \frac{k_s^0}{W} = \frac{4k_B T}{3\eta W}$$

W = collision freq. / bridging freq.



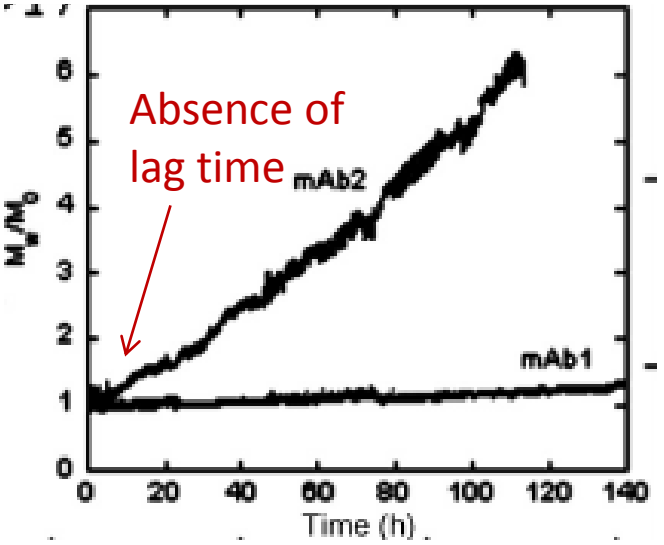


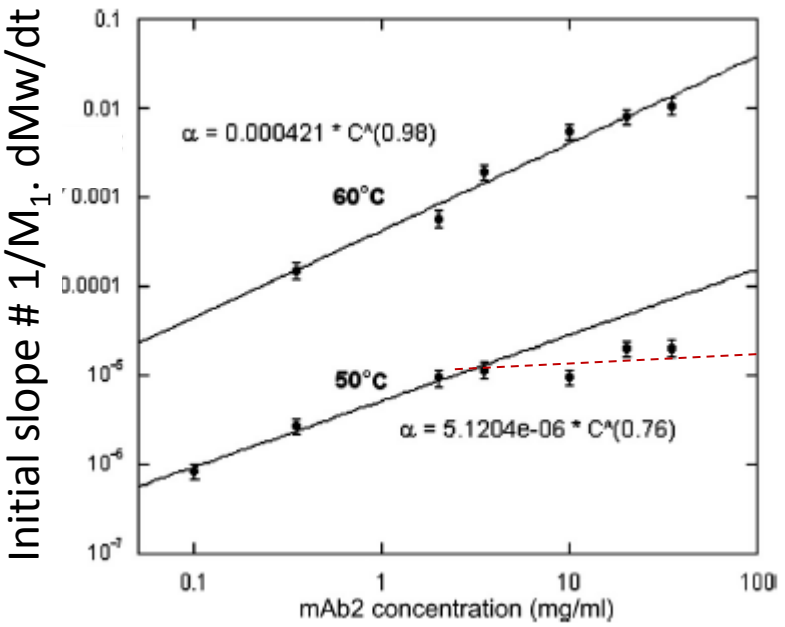
Morbidelli et al. , J Phys Chem 2012, 116, 7066

	W					
	25 °C	30 °C	35 °C	37 °C	39 °C	45 °C
pH 3.0 and 0.15 M Na_2SO_4 at various temperatures	1.7×10^{10}	2.5×10^{9}	5.1×10^{8}	3.3×10^{8}	1.7×10^{8}	4.3×10^{7}
		NaH_2PO_4	NaCl	Na_2SO_4		$NaNO_3$
pH 3.0 and 37 °C, with 0.15 M solutions of various salts		6.3×10^{9}	2.5×10^{9}	3.3×10^{8}		2.2×10^{8}

Determination of Fuchs stability ratio
(case 2b: measurements at short time scales, oligomerisation)

$$I(t) = I_0(1 + n_0 \cdot k_s \cdot t)$$



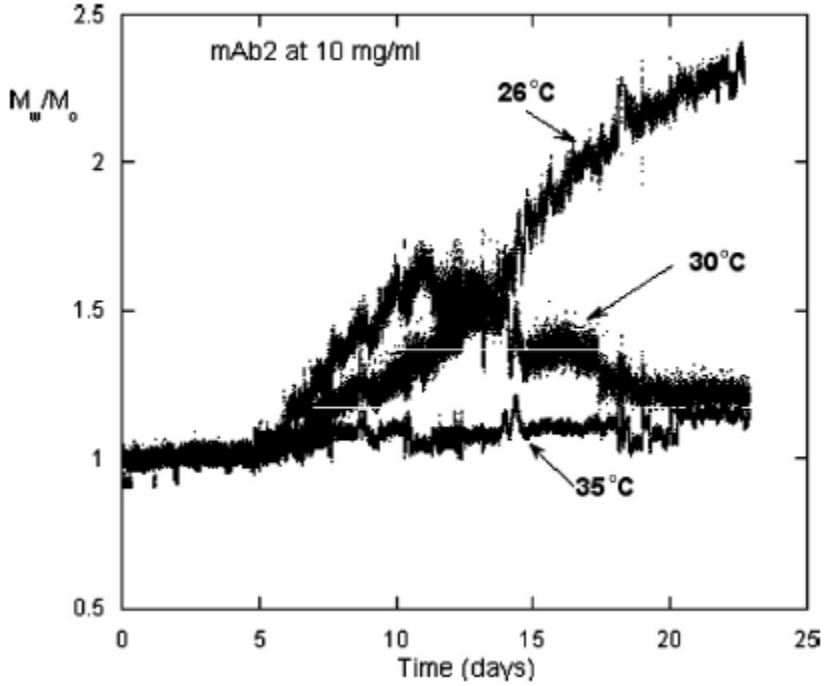


Diffusion limited: no lag time, ks ~C0 & determination of effective Fuchs ratio
(W* may combine rate of oligomerization between activated/non activated species)

Activation limited + quasi steady state: ks = rate of activation

W F Reed et al. Anal Biochem 2013

Limit of characterisation: conditions of slow clustering, artefacts or chaotic aggregation ?



Sampling of 1% of the total volume (30μL):

- Aggregates may form and sedimentate?

-Role of dust and impurities, vibrations , nature of cell surfaces ?

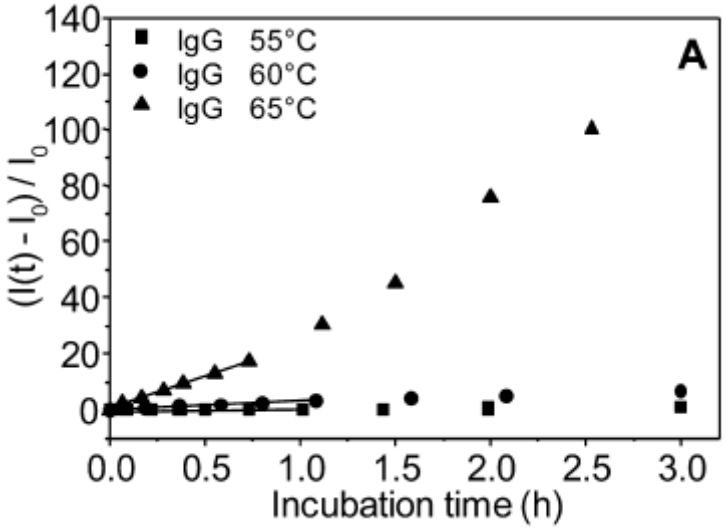
-Lag time = rare event of nucleation ?

W F Reed et al. Anal Biochem 2013

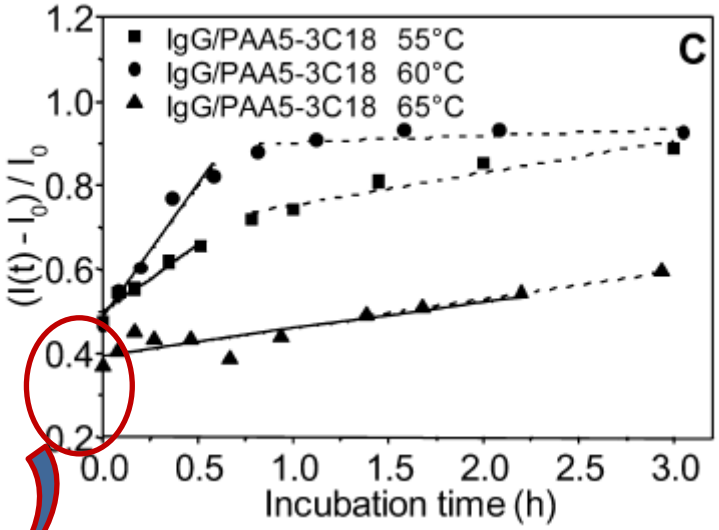
Limit : chaperon efficiency vs effective Fuchs stability ratio?

(measurements with protein:additive complexes)

IgG, no additives



IgG, polymer additives



Effective W x 10^{-6}

T (°C)	IgG	IgG/PAA5	IgG/PAA5–3C18	
55	240	200	660[a]	<<600
60	16	9.8	2900[a]	<<50
65	2.0	1.3	720[a]	<<30

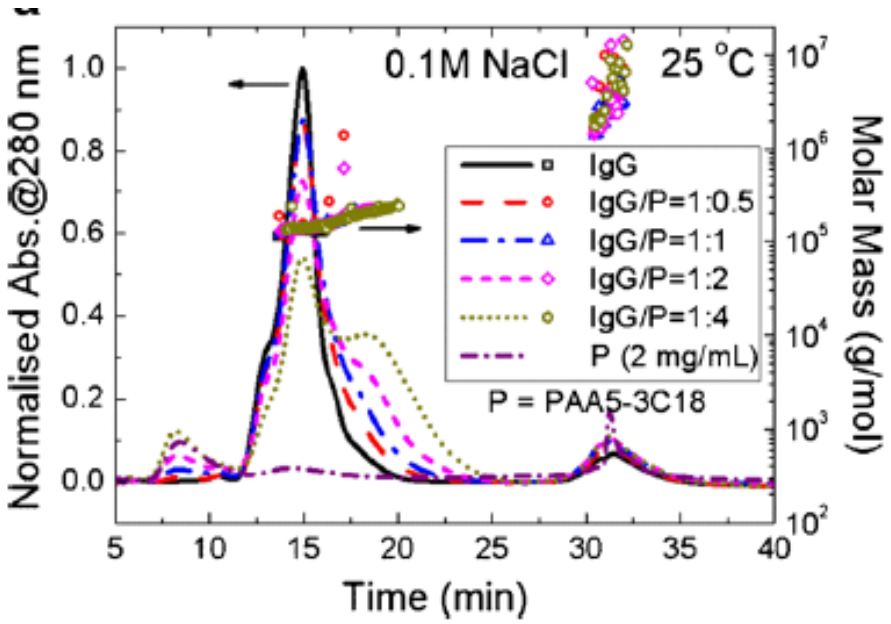
non zero at t=0 : model not valid

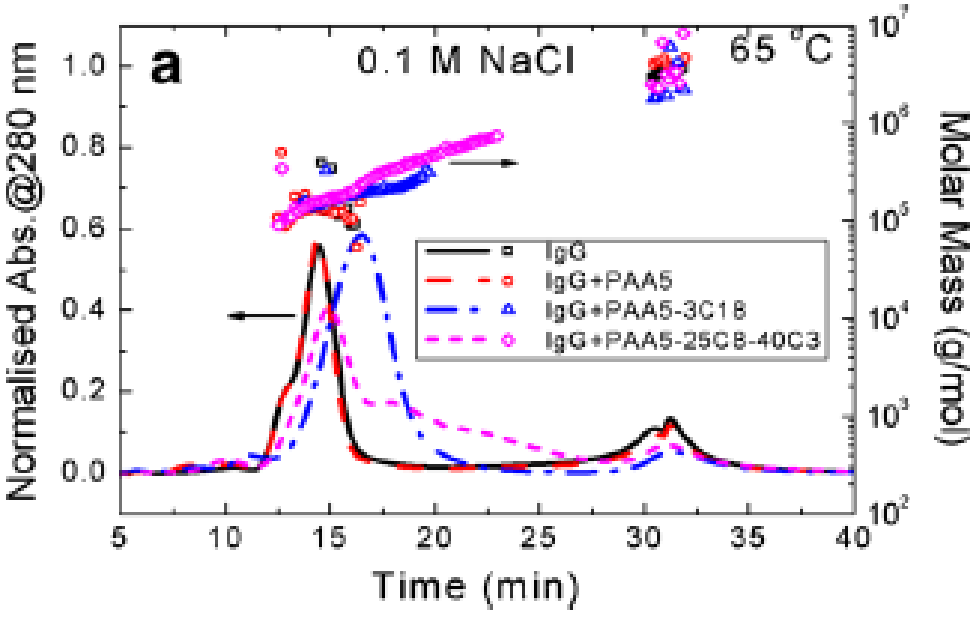
Phosphate buffer 20 mM pH 6.8

N. Martin et al, Biomacromolecules 2014, 15, 2952.

Complementary characterisations by AF4: (mixed complexes with stabilizing agents)

AF4 : decoupling Mw & size from signal



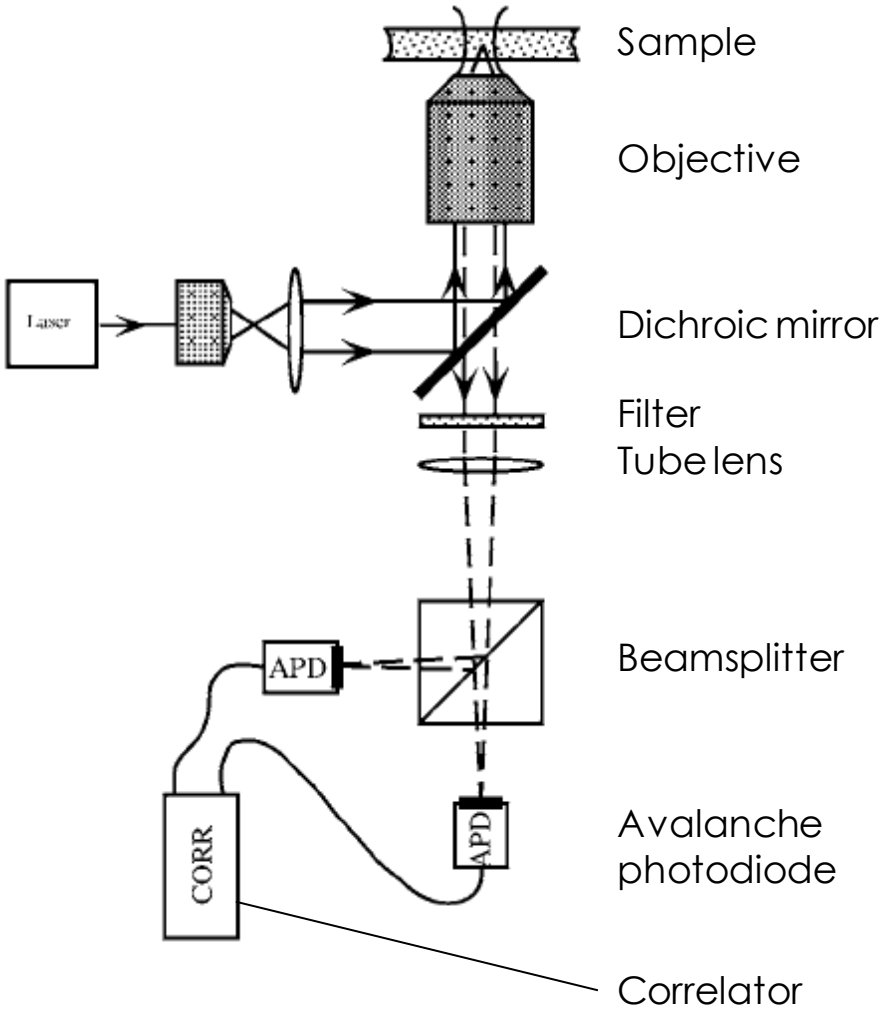


evidence for species of Mw < 300 kDa, … unknown stoechiometry ofIgG:polymer complexes

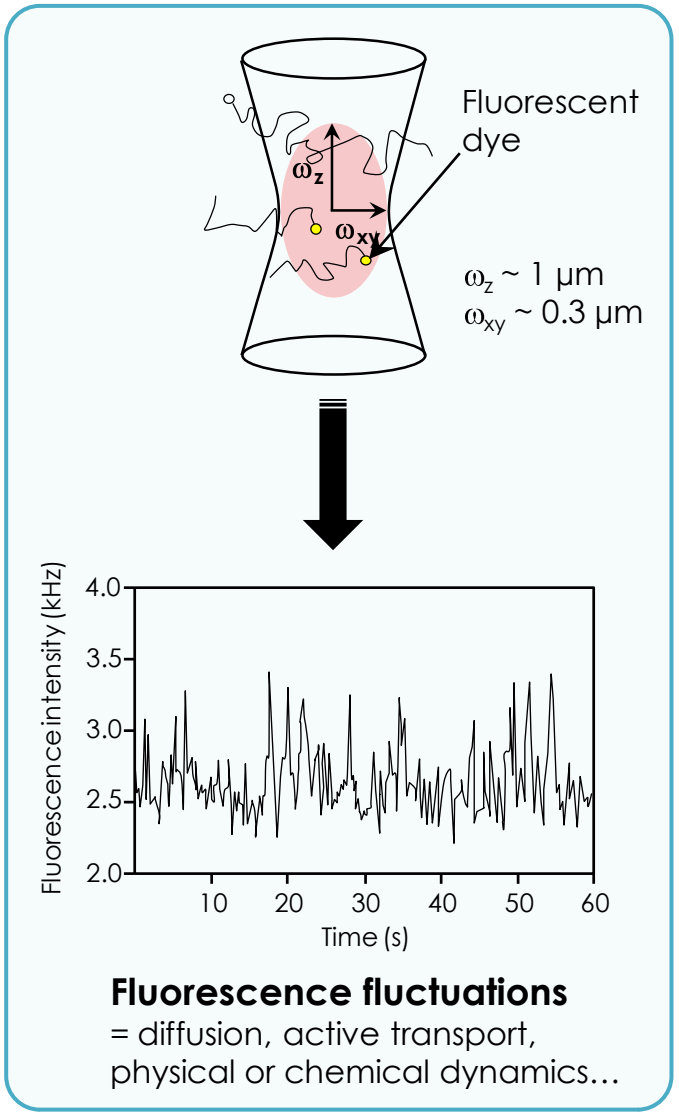
distinguish IgG aggregation routes (e.g. gradual growth vs absence of oligomers) …. not amenable to fast kinetics

Dewang MA et al.,Anal Bioanal Chem 2014, (406) 7539

Toward specific readout:two-photon FCS

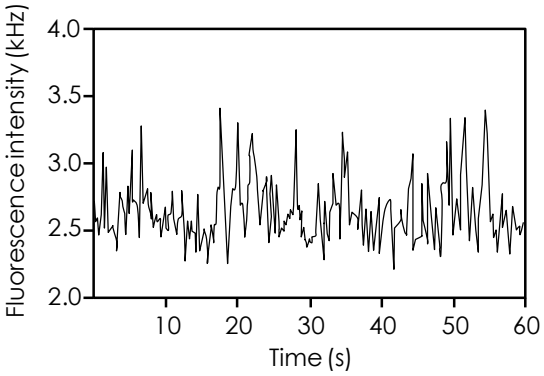


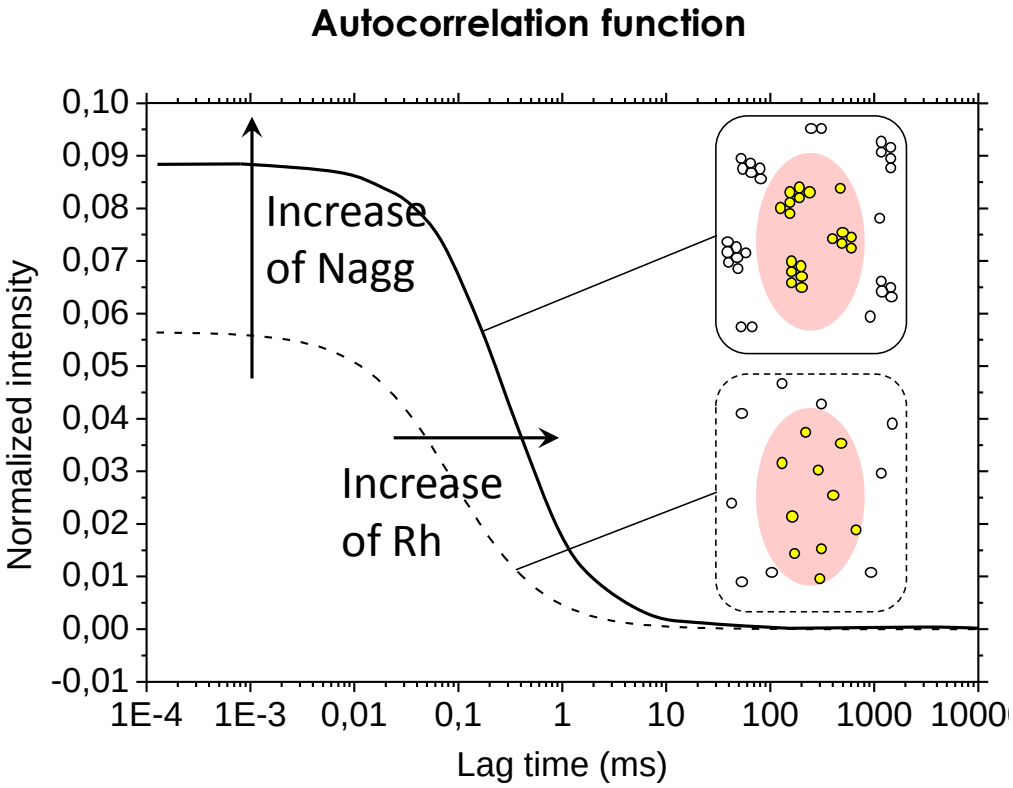
Confocal volume ~ 0.1-1 femtoliter



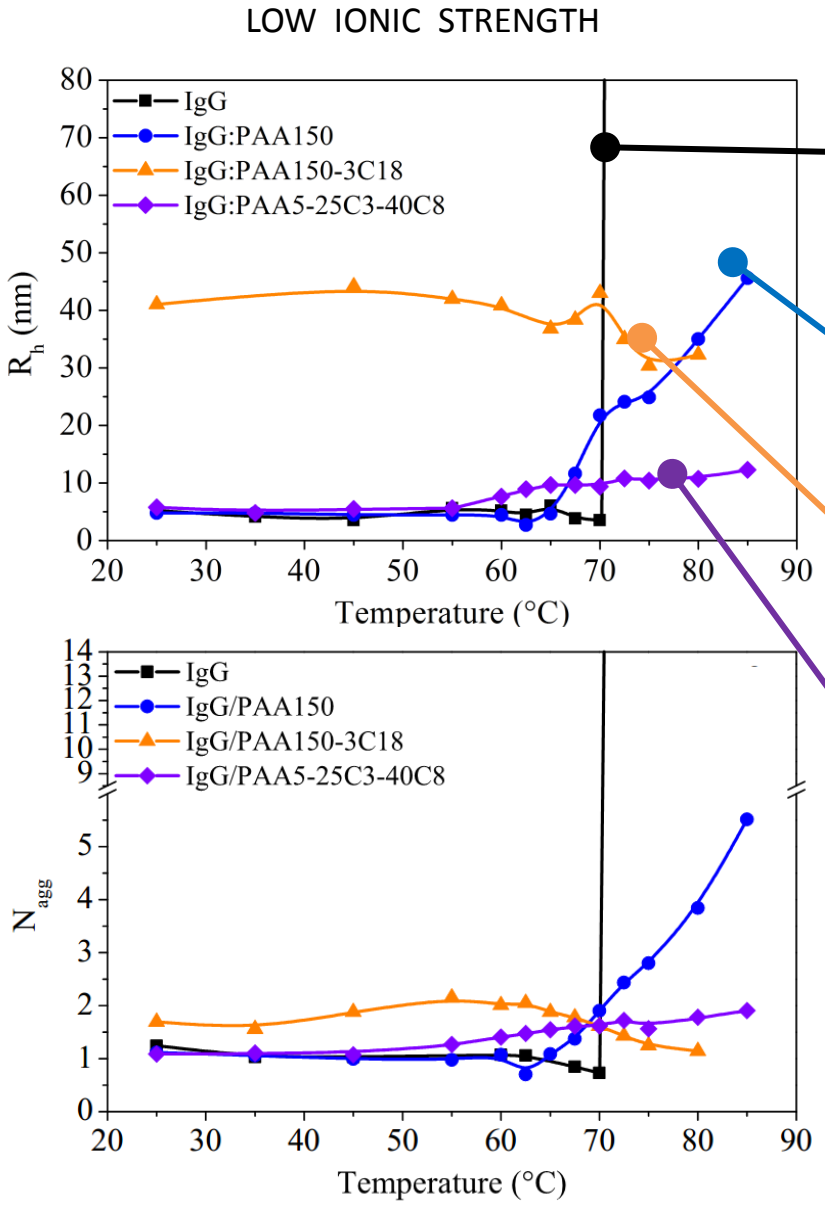
Fluorescence fluctuations
= diffusion, active transport, physical or chemical dynamics…

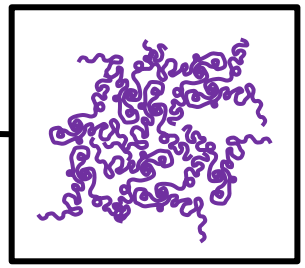
Evolution of the autocorrelation function



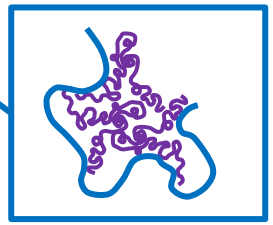


Size/stoichiometry of polymer:IgG-FITC complexes

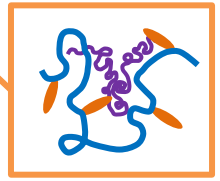




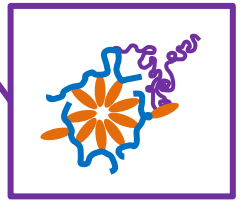
aggregation



slow aggregation rate
stabilization of oligomers



protection
stabilization of monomers



protection
stabilization of monomers

Other applications:
evidencing reversible associations (surfactants)
Assessement of chemical refolding

summary SAXS/SANS

What can be quantified ?

- ✓ characteristic time of growth
  - master curves Rh vs t , check models
  - amount of aggregates in specific cases
- ✓ Size of primary clusters, preservation of native-like shape
- ✓ energy barrier / binding well amplitude (solubility vs stability)

What are the limits:

- ENSEMBLE characterisation = average features
  - Question of sensitivity to molar % of clusters, or non-native structures
  - models based on spherical averaging
- Access to SAXS instruments (SOLEIL, ESRF) & cost
- no distinct signal from non-protein particles (except SANS)

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