A crystallization apparatus in design phase

13.01.2017

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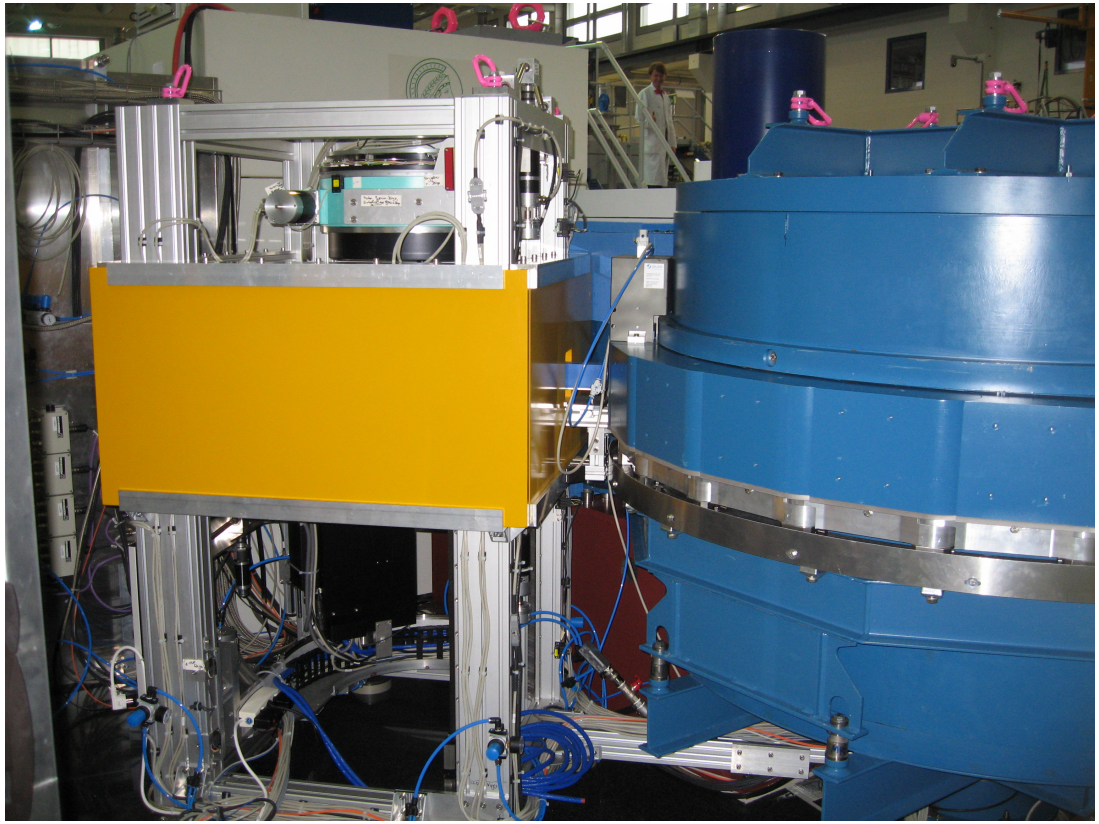
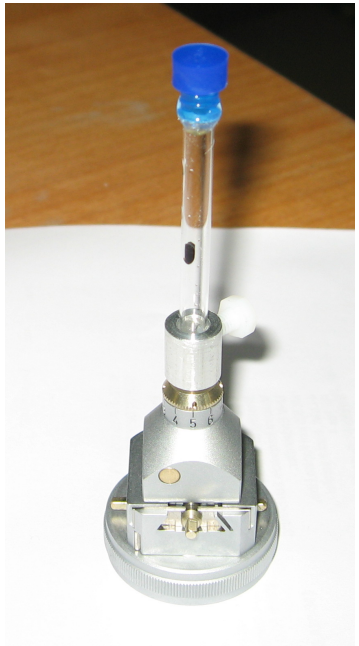
Motivation: For neutron protein crystallography large crystals are required

About 80% of new, unexperienced users fail to bring crystals which are large enough to take a data set at BioDiff.





Necessary crysal size:
At least 0.5 mm^3

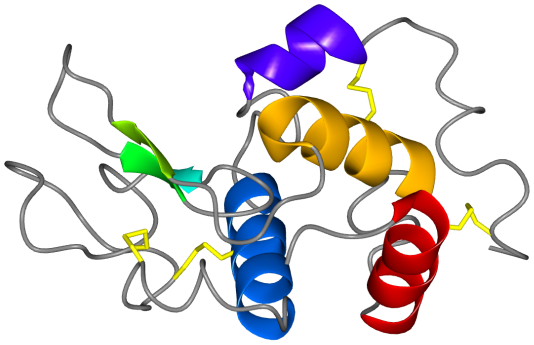
- Deeper understanding of the undelying crystallization mechanism is required

Chosen crystallization conditions

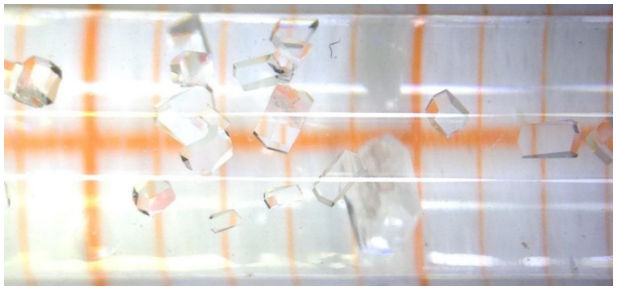
- Lysozyme 60 mg/ml in D_2O, pH adjusted with 1M NaAc 0,02 µm filtered
- NaCl 6wt% in D_2O Puffer 10mM NaAc HAc 0,02 µm filtered

- 1:1 mixture:

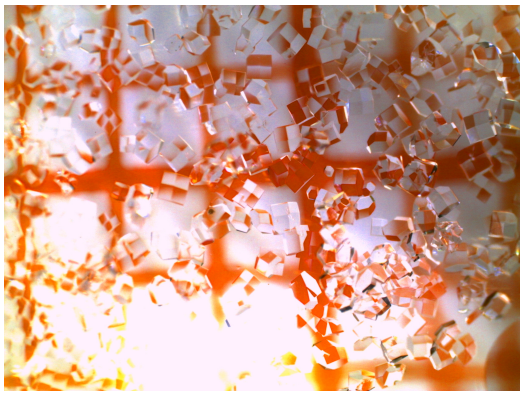
Lysozyme 30 mg/ml + NaCl 3 wt% in D_2O buffer @ pH 4.35



Monomer size: r = 1.9 nm



crystals ca. 1 mm at T = 298 K



crystals ca. 0.2 mm at T = 294.5 K

What is out there already?

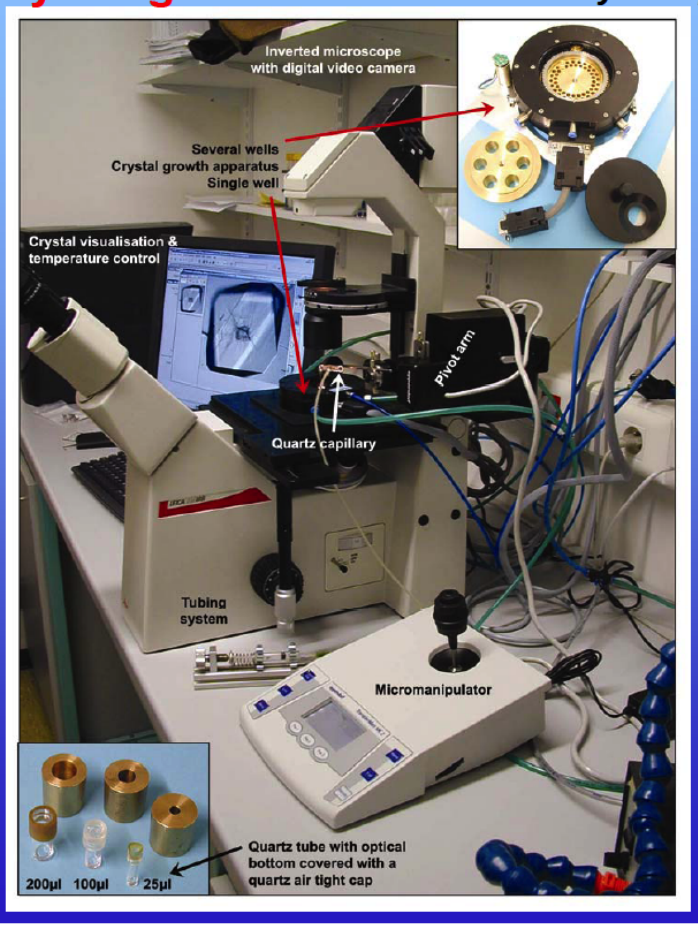
- Christian Betzel‘s Xtal controller
- Monika‘s dialysis button
- Niimura‘s apparatus

- Why built a new set-up?

Monika‘s machine

An instrument for the temperature-controlled optimization of crystal growth

Budayova-Spano et al., Acta Cryst. D63, 2007, 339-347



- Investigating the phase diagram, controlling the nucleation and crystal growth of biomacromolecules, manipulating the solubility of seeded H/D – labelled crystals as a f(T)
- Regulating the temperature of the crystallization solution using control parameters determined *in situ* during the growth process (Novel multi-well crystal growth apparatus)
- Allowing for *in situ* observation by optical microscopy and sequential image acquisition, processing and storage
- Facilitating the convenient extraction of the protein crystals after growth, without causing any mechanical damage to them => using MICROMANIPULATOR

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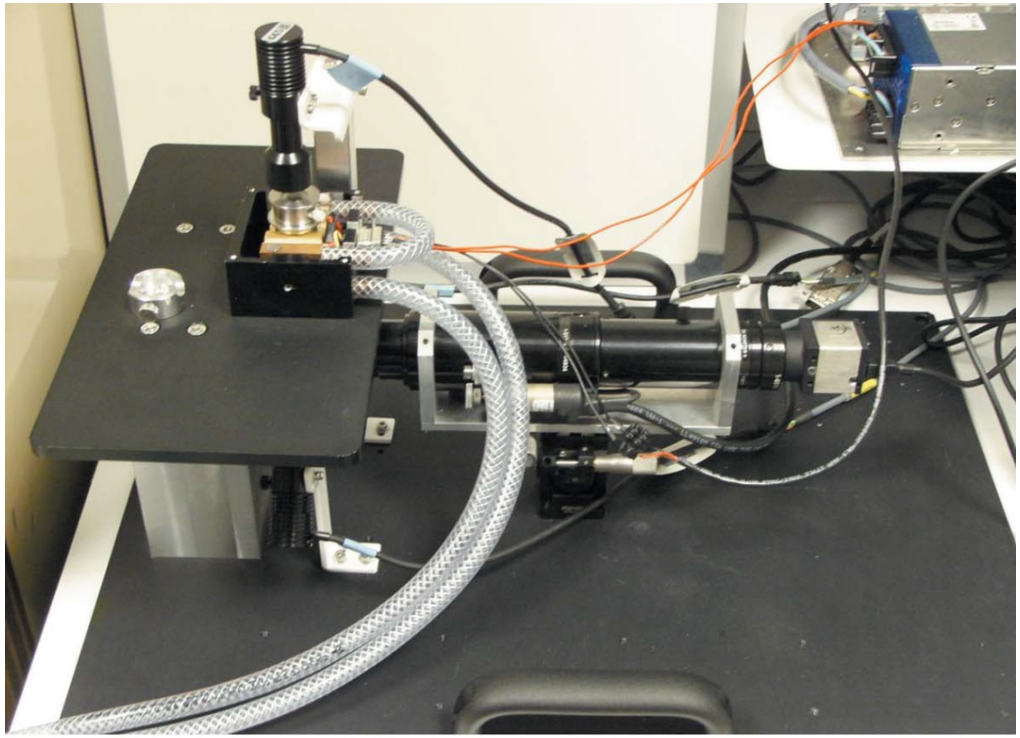
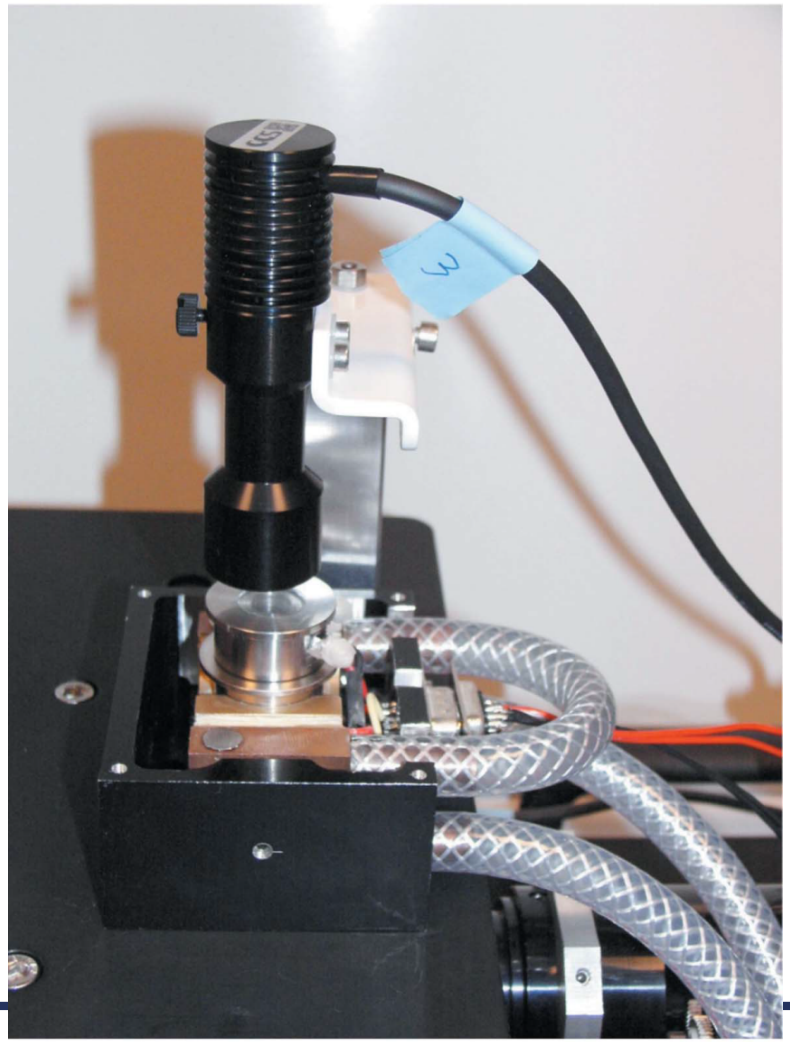
Dr Monika Budayova - Spano
Université Joseph-Fourier
France

Monika‘s new set-up based upon dialysis



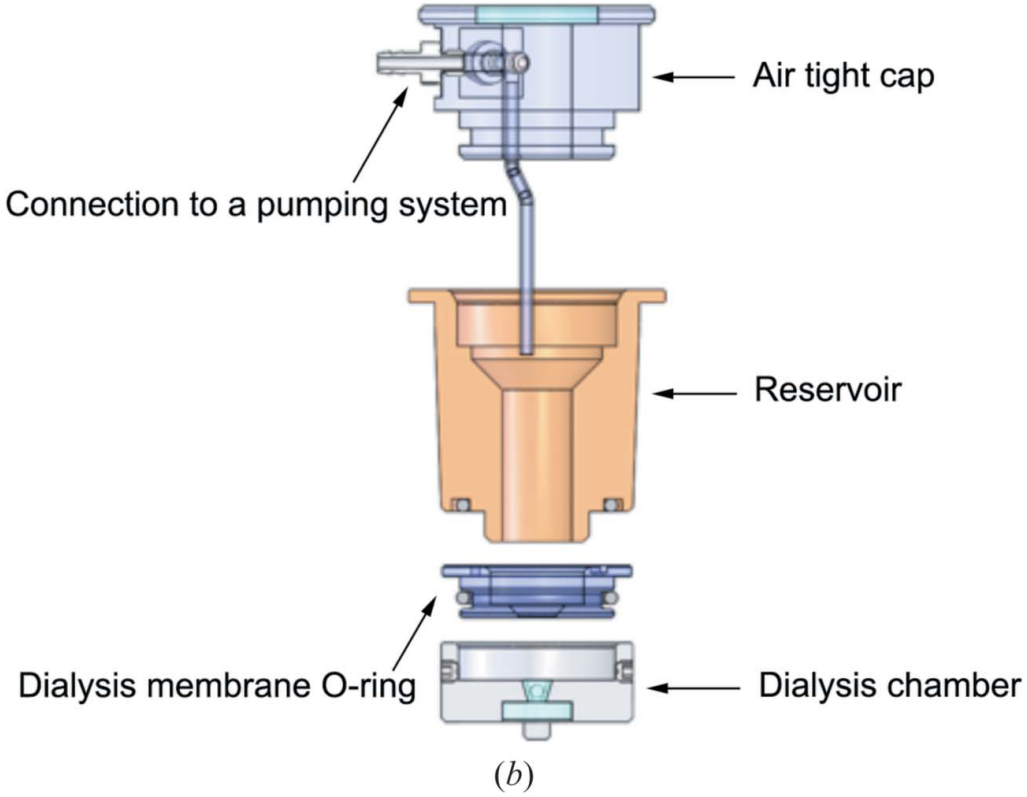
(*a*)



(*b*)

Pictures from Junius et al.
J. Appl. Cryst. (2016). 49

The dialysis set-up

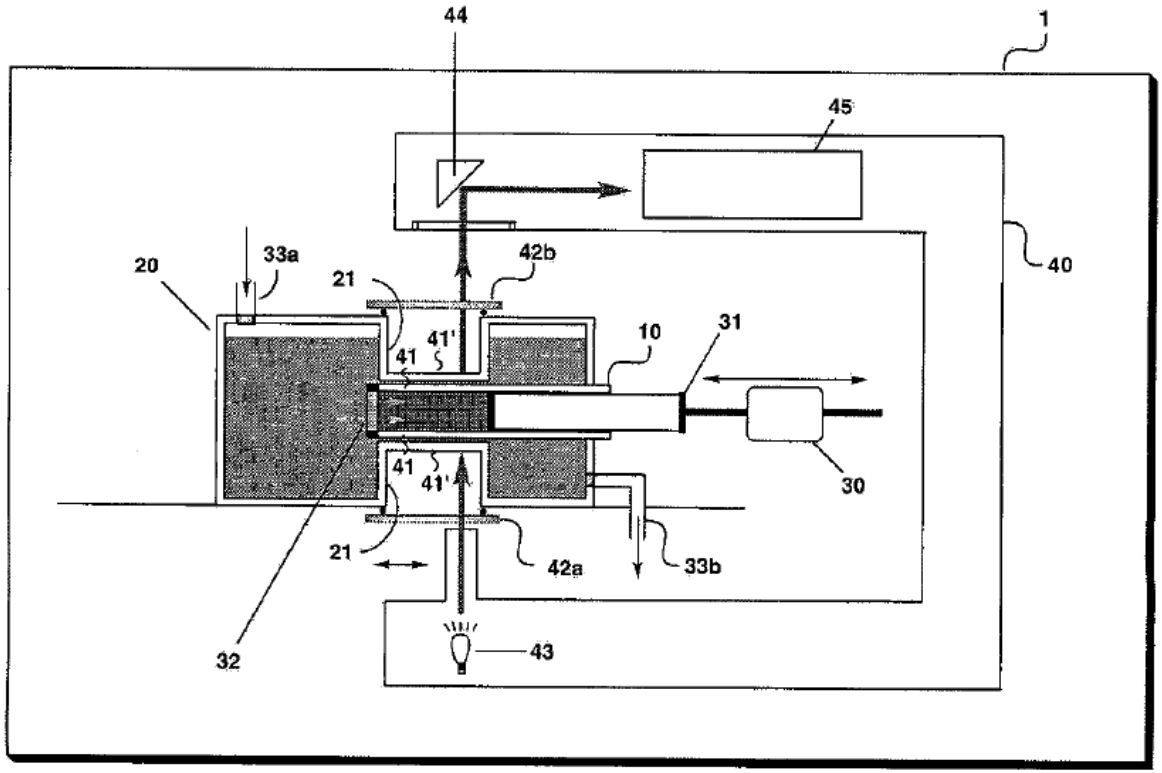


(b)

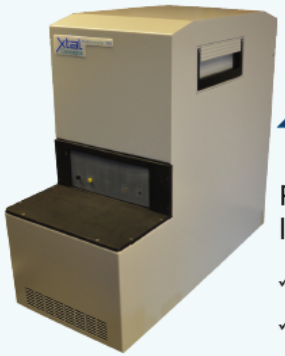

(a)

Pictures from Junius et al.
J. Appl. Cryst. (2016). 49

Niimura‘s setup



Christian Betzel‘s set-up



XtalController™900

Xtal Concepts

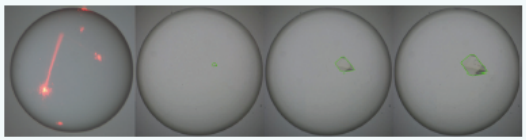
Fully Automated Crystal Growth of Biomolecules in µl Drops

- ✓ Sitting drop crystallization in a climate controlled chamber for growing nanocrystals to mm size crystals
- ✓ Feed-back controlled crystallization achieved by evaluation of DLS data and camera images
- ✓ Move almost freely through the phase diagram with the help of micro dosing drop generators for adding precipitant or other substances See nucleation long before crystals appear.
- ✓ Precise control of concentration of all components and evaporation rate by weight measurement with µg resolution
- ✓ Dynamic Light Scattering (DLS) system for determining molecular size distributions
- ✓ Clear crystal images obtained from a built-in microscope with high numeric aperture, zoom and CCD camera (crystal measurement)
- ✓ Device and method for monitoring crystallization patented under DE102010025842

Improving Crystal size and quality

Take complete control of the crystallization process from the clear drop to crystals. Dynamic Light Scattering is used to actively influence the early stages of crystallization. A micro balance and micro dosing drop generators allow precise control of the crystallization conditions. Once crystals are visible, crystal size can be measured to further control the growth rate. The XtalController 900 gives you the best chance to obtain high quality crystals with your desired size from nm to mm dimensions.

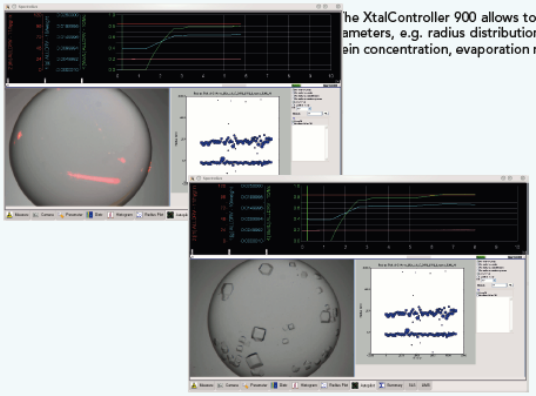
Nanocrystals	Microcrystals	Small crystals	Large crystals
150 nm	50 µm	0.2 mm	0.7 mm



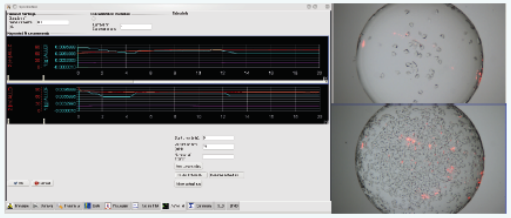
Applications

- ✓ Optimization of crystal growth conditions
- ✓ Gentle introduction of cryoprotectant
- ✓ Cross linking of protein crystals
- ✓ Study of protein-ligand interactions
- ✓ Crystallization with minimized sample material

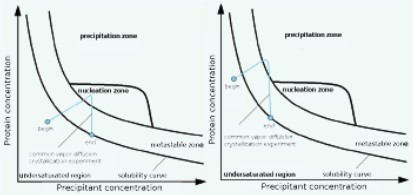
Christian Betzel‘s set-up II



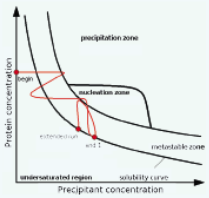
he XtalController 900 allows to control crucial crystallization parameters, e.g. radius distribution, precipitant concentration, protein concentration, evaporation rate and temperature.



Experiments are highly reproducible allowing slight changes in the crystallization conditions resulting in different crystal sizes e.g. micro and nano crystals. All process parameters are stored in an integrated Laboratory Information Management System for quick access, comparison and data evaluation.



Conventional vapor diffusion crystallization
Success or failure of an conventional experiment is determined by the starting conditions. After set up of a crystallization experiment no further influence is possible.



Crystallization with XtalController
The XtalController 900 allows to navigate in the phase diagram by changing the conditions in the droplet; resulting in desired crystal sizes.

Christian Betzel‘s set-up III

Sample volume	✓ 1 - 300 µl
Micro balance	✓ Resolution 10 µg □ Optional resolution 1 µg
Climate chamber	Control of atmosphere in the reaction chamber ✓ Control of relative humidity up to 100% with a precision < 0.1% ✓ Control of the temperature max 10°C above or below ambient, stability < 0.1°C
Imaging system	Built-in microscope with following features: ✓ 5 magnification steps: 0.63, 1.25, 2.0, 3.2, 6.4 ✓ Field of view: 10.5x7.6 mm, 5.2x2.9 mm, 3.3x2.5 mm, 2x1.5 mm, 1x0.75 m ✓ Resolution: 25 µm, 13 µm, 8 µm, 5 µm, 2.5 µm per pixel ✓ CCD color camera 1600x1200 pixels □ Optional: other resolutions
Detector	✓ Photomultiplier tube, dark count rate < 300 Hz, quantum efficiency 5-7%, count sensitivity $1.5*10^5$ Hz/pW ✓ For single photon counting ✓ Scattering angle 142° □ Optional: Avalanche photodiode, higher sensitivy for wavelengths > 660 nn
Correlator	Multi-tau architecture correlator to cover a wide sample time range ✓ Sample time from 400 ns to 30 s ✓ Total 208 channel, quasi logarithmic channel spacing
Sensitivity	Sample concentration with a standard laser (100 mW, 660 nm) ✓ Minimum 2.0 mg/ml for lysozyme (~14 kDa) at 4 µl sample volume, for 0.3 mg/ml for proteins with ~30 kDa at 4 µl sample volume ✓ Maximum sample concentration > 100 mg/ml
Microdosing system	Piezo operated drop generator for no-contact addition of liquids ✓ Water drop generator, volume per shot 30 pl ✓ Precipitant drop generator, volume per shot 30 pl □ Extra drop generator (e.g. ligand, seeding, additives, cryoprotectant) □ Protein drop generator, volume per shot 20 nl, disposable to avoid cleaning
Dimensions	Table top system 520 mm x 230 mm x 450 mm (LxBxH) ✓ Weight: approx. 28 kg ✓ Power consumption: 115 to 230 V, 100 W ✓ Clean pressurized air 4 - 6 bar, oil free
Instrumental set up	□ For optimal performance a vibration absorbing table is required
Computer	✓ Laptop ready to use ✓ Suse Linux □ Windows 8 □ Desktop PC ready to use ✓ Suse Linux □ Windows 8 □ Second monitor for full camera image display
Software features	XtalController software runs on ✓ Linux, □ Windows ✓ Real-time quantification of protein and precipitant concentration ✓ Manual manipulation and intervention of crystallization parameters ✓ Programmable concentration gradient ✓ Integrated LIMS data base ✓ Live display of camera images □ Determination of crystal size for control of growth rate □ Full remote control via internet □ Storage and retrieval of all relevant information in an external data base

New Setup?!

1. Access to the crystallization chamber liquid such that one can gently add some more protein solution or deuterate in situ.
2. UV-Attenuated total reflection to monitor the protein concentration.
3. DLS to monitor aggregation status in crystallization chamber.
4. optical microscopy with movable crossed polarizers to switch between normal transmitting light conditions and polarization microscopy.
5. Software to monitor and plot actual crystal size (image recognition software).
6. Fine temperature control using peltier elements
7. Valves to control the flow
8. Complete automatization and remote monitoring
9. Multiplexing? Several those crystallization chambers?
10. A similar set-up for vapour diffusion? Does not make sense… Better: Counter diffusion.

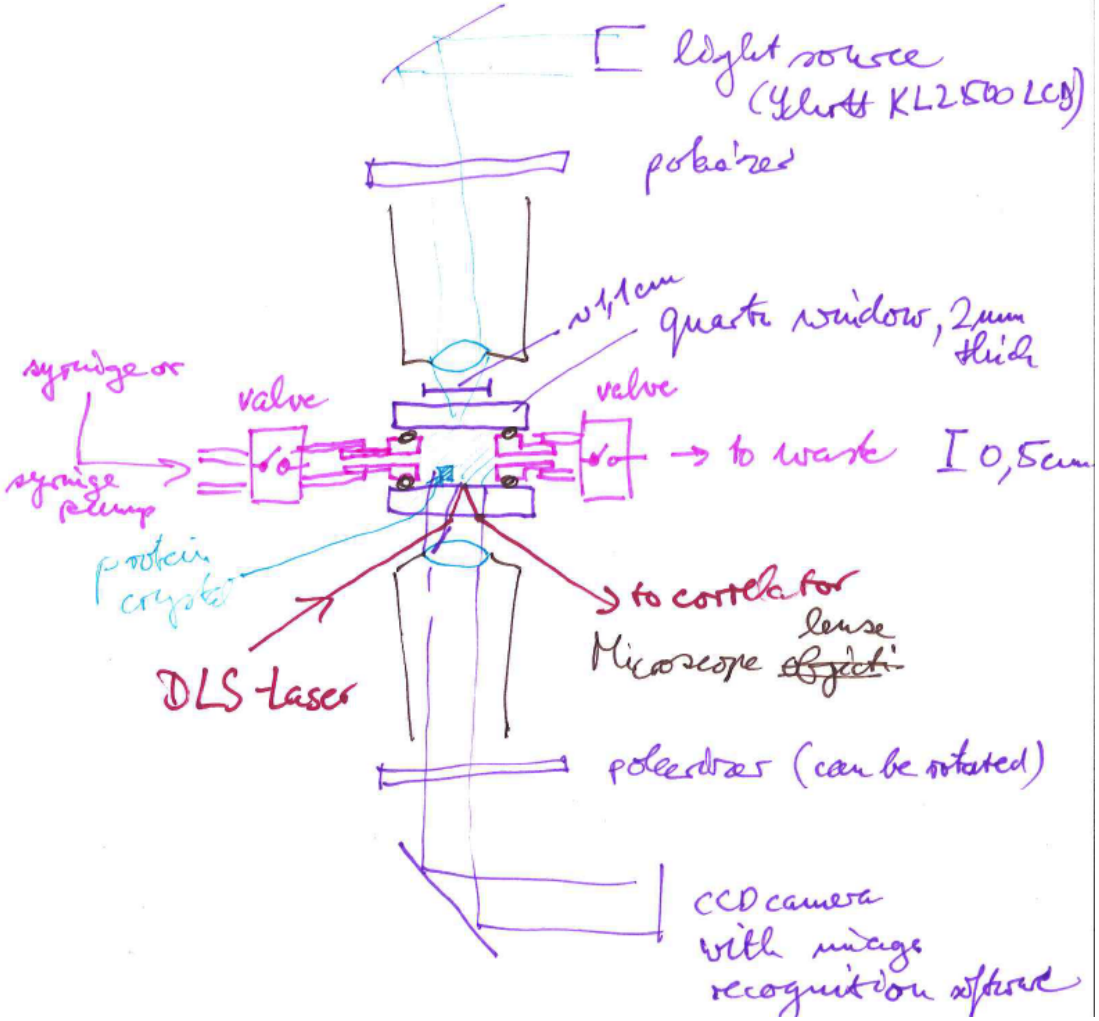
Later stages

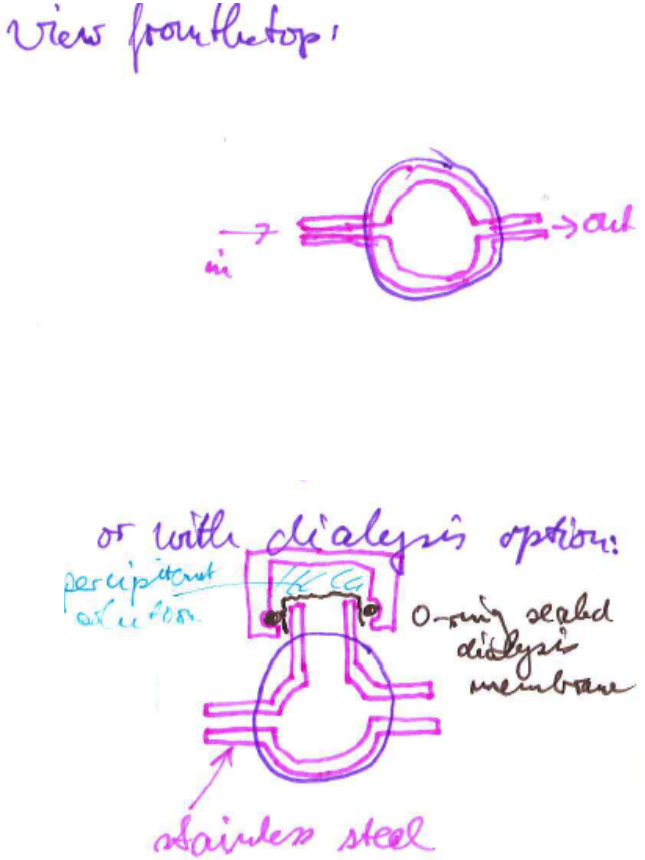
1. Later: Electric field or magnetic fields should be applicable,
2. local heating by IR-lasers?
3. Circular dichroism to monitor the fold of the protein
4. Stirring the solution around the crystal?
5. FTIR transmission spectroscopy to monitor the fold of the protein molecules in solution

Additional techniques available on site

- Cryo-TEM to observe crystal surfaces and morphology
- AFM to scan crystal surfaces and maybe modify them

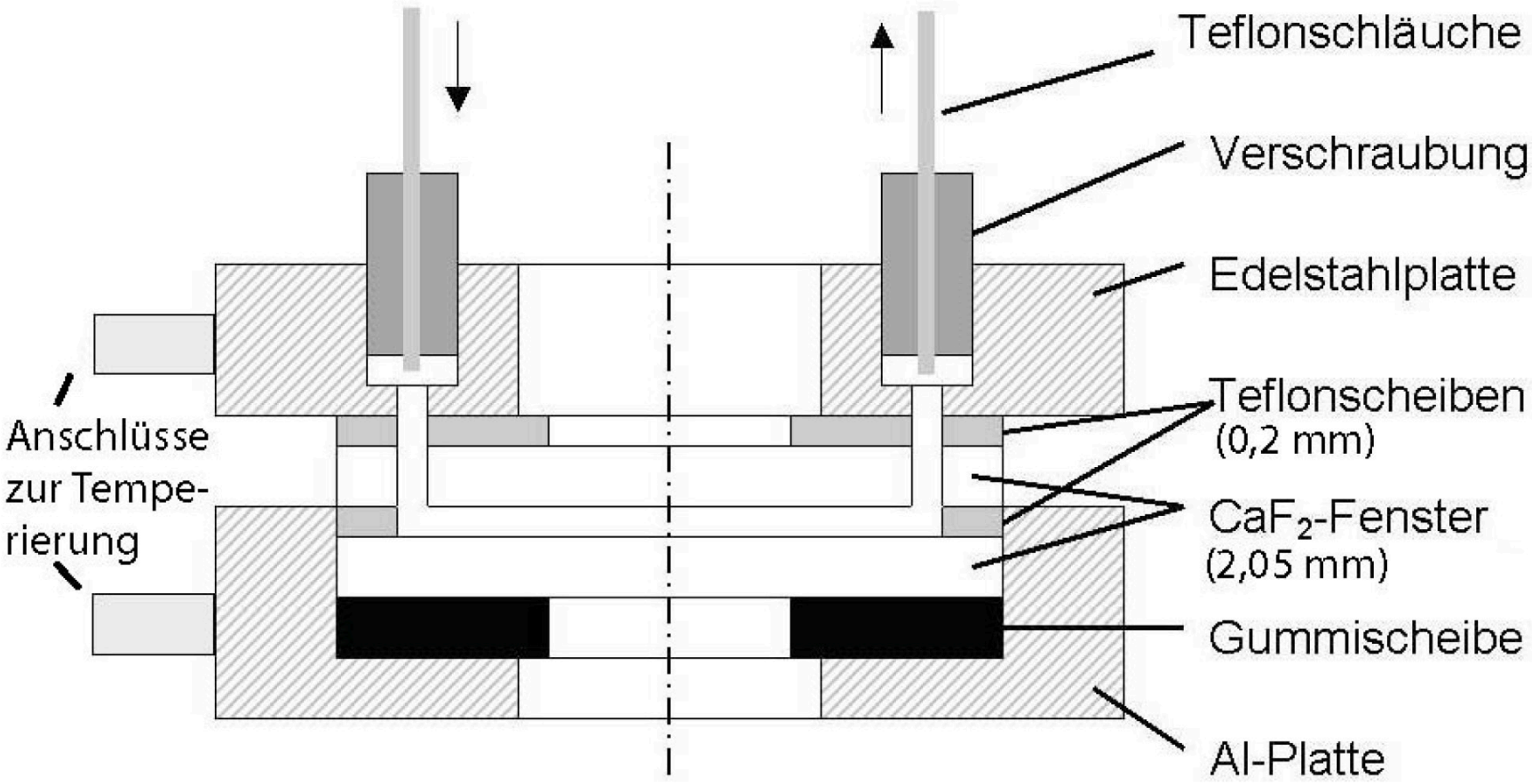
some sketch…


light source
(Schott KL2500 LCD)
polarizer
quartz window, 2mm thick
syringe or
valve
valve
syringe pump
to waste
protein crystal
to correlator
lense
Microscope objective
DLS laser
polarizer (can be rotated)
CCD camera
with image
recognition software

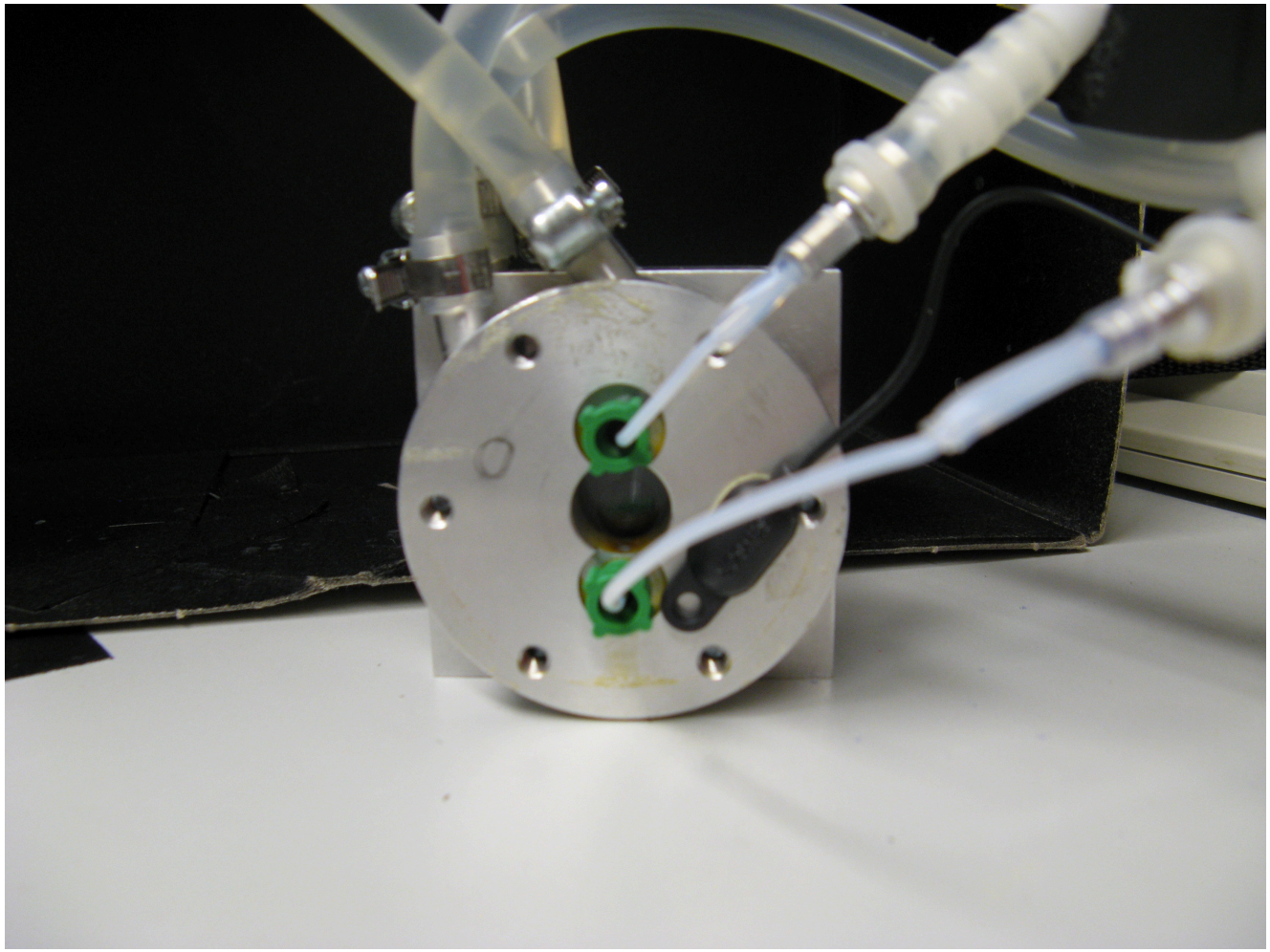

view from the top:
in
out
or with dialysis option:
precipitant
O-ring sealed
dialysis
membrane
stainless steel

Flexible crystallisation cell: here: batch crystallisation

- Some first prototype (sorry about the German)



How it looks like in real space



What are our enemies?

- Temperature gradients
- Unknown parameters: air bubbles, impurities, partially unfolded proteins...

Make use of the crystallographic knowledge we have on the crystals

From x-ray crystallography on the very same protein we know:

1. Unit cell size and space group
2. Orientation and number of protein molecules in the unit cell
3. crystal contacts of the proteins within the unit cell and from one unit cell to others.
4. Preferred growth direction
5. Surface charge in the crystal
6. water content and maybe PEG content etc.

Why not make use of this knowledge when optimizing the crystallization conditions?
When placing the seed crystal in a crystallization apparatur in a certain ortientation to organize slow growth conditions?

The end

Content

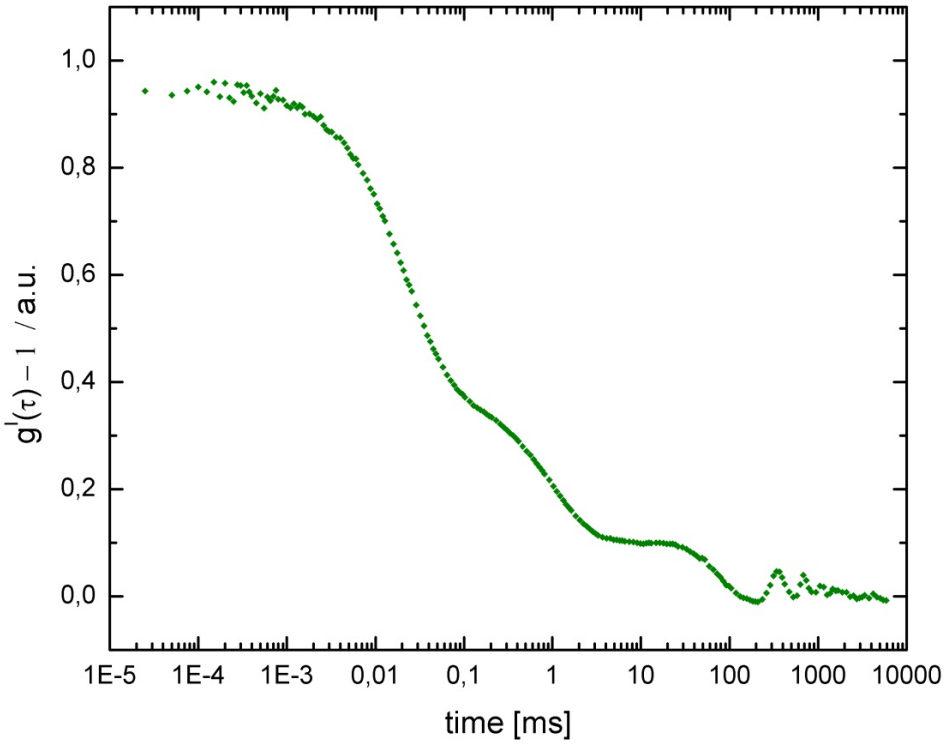
- Overview over the project
- Previous results
- Literature survey
- Ideas…

Overview over the project

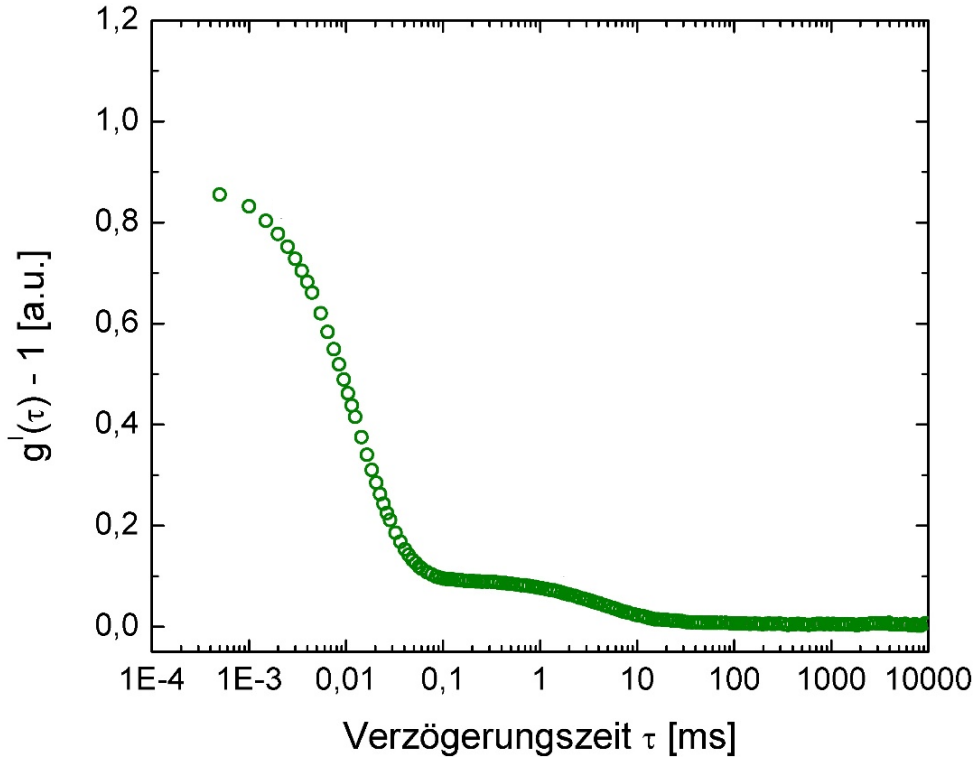
- The Post-Doc should predominantly build a new crystallisation set-up
- Additionally there are some very promising projects out there where one can start immediatly and work on them in parallel. Just the crystal size has to be optimized: a) Andreas Eichinger, b) maybe Dariusch Hekmat, c) Filipp Kovacic
- Maybe some data treatment on Anja Burkhardt‘s project

Dynamic light scattering gives the number of particle sizes present

T= 294,5 K

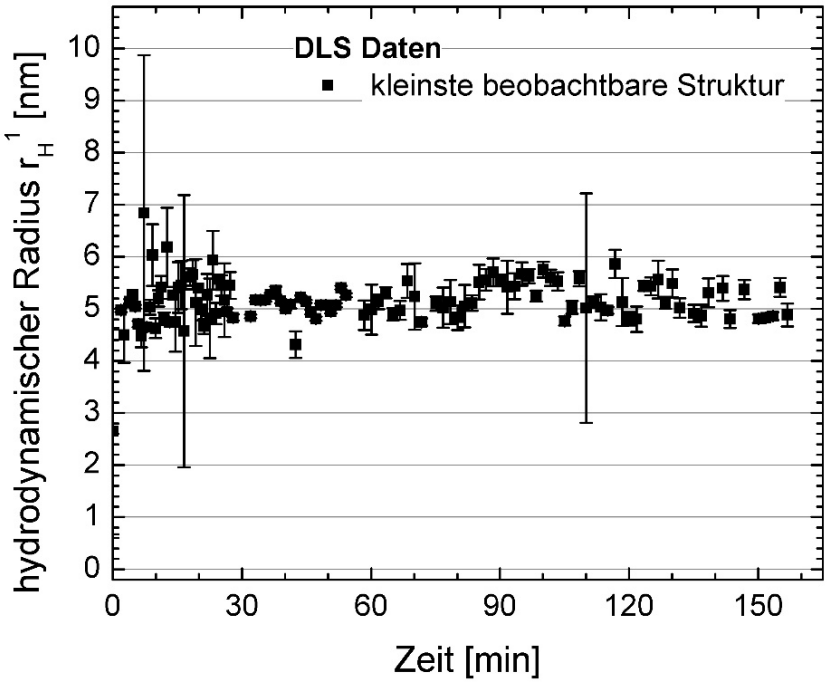


T= 298 K

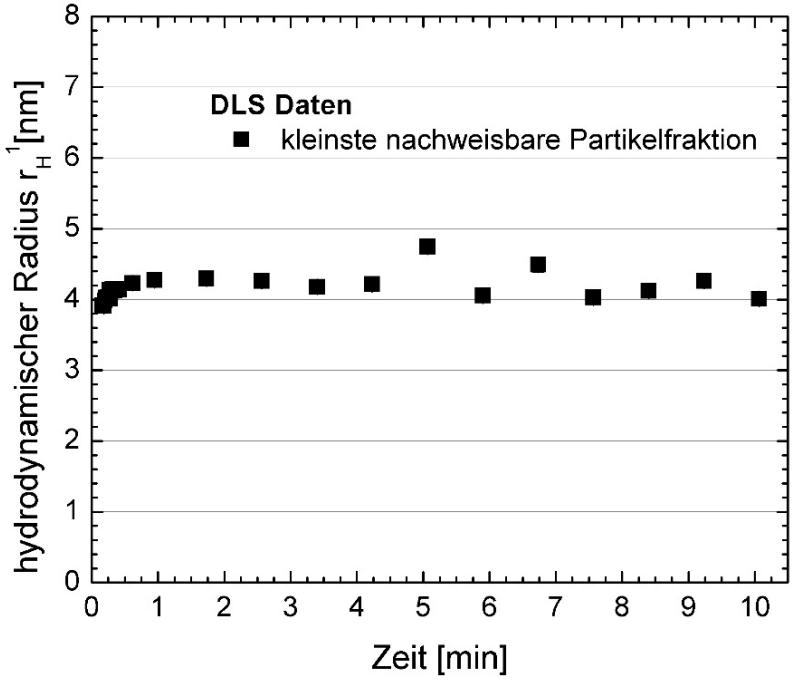


Pre-characterisation of the crystallisation speed with DLS

T= 294,5 K



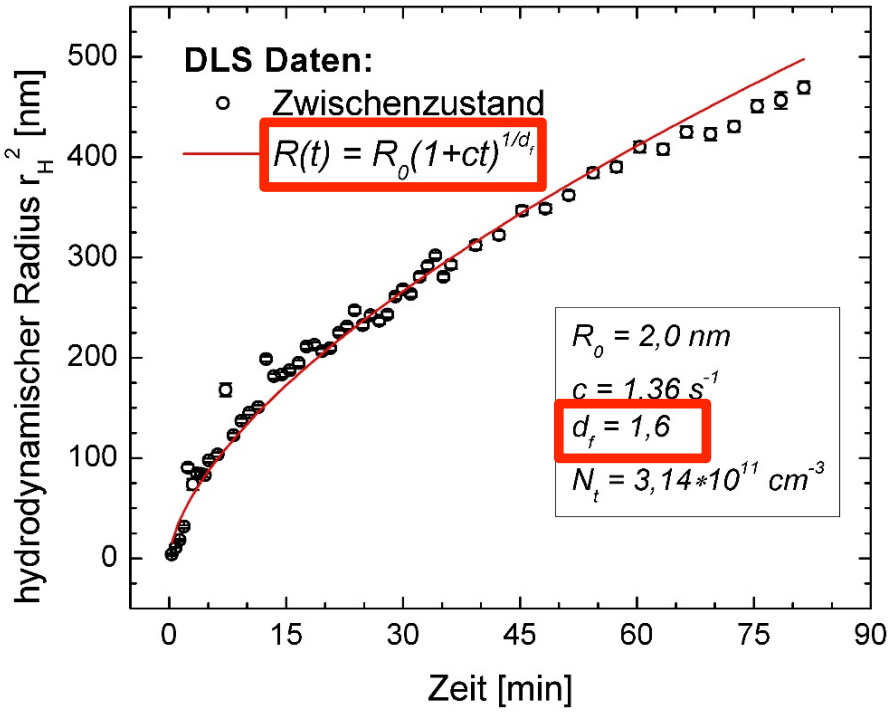
T= 298 K



- Constant radius of the dimer fraction in both cases

Comparision with the literature

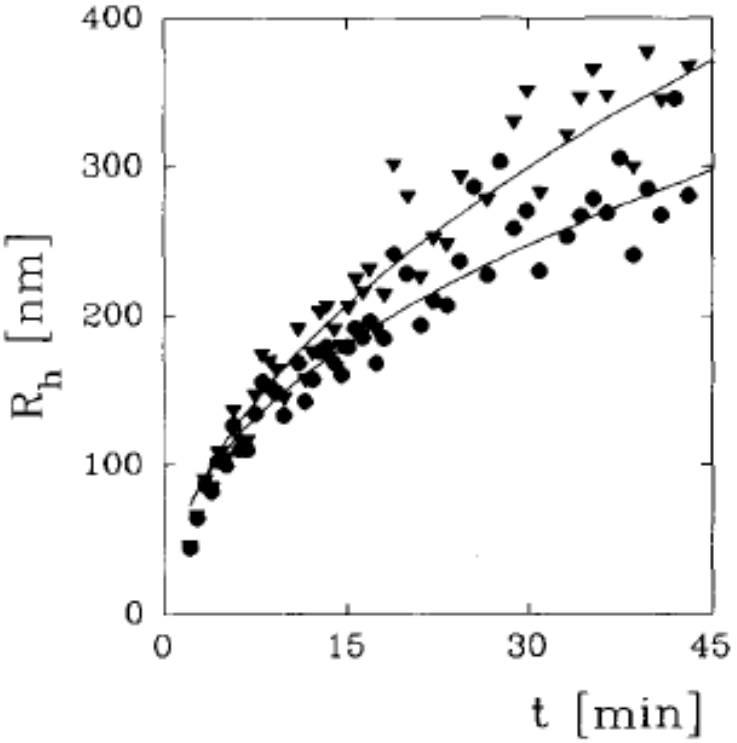
T= 294,5 K



DLS with 60mg/ml Lysozyme mixed with 6wt % in D_2O Puffer

pH 4.35; T = 294.5 K; scattering anglel 174°

Y. Georgalis, A. Zouni, W. Eberstein, W. Saenger, Crystal Growth 126, 245-260

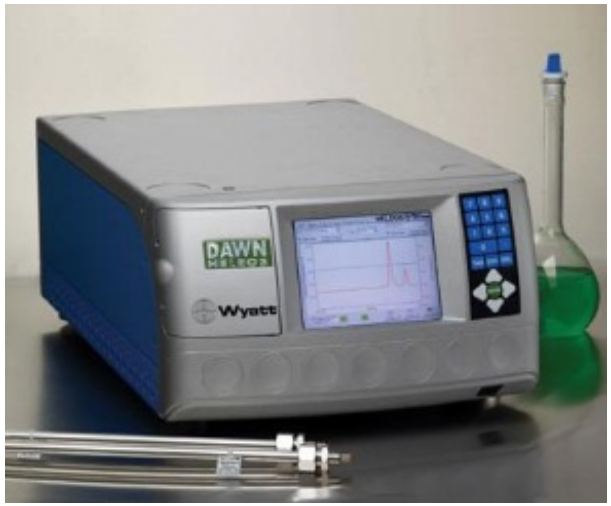


DLS with 61.3 mg/ml Lysozyme mixed with 7.2wt% NaCl in H_2O Puffer

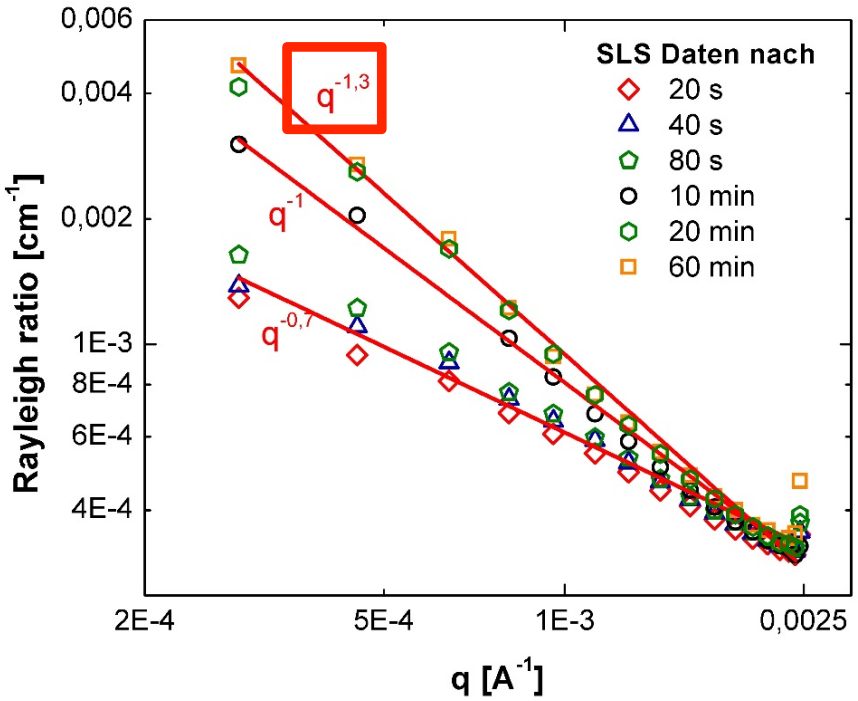
pH 4.2; T = 293 K; scattering angle 20°

Change in fractal demension observed at T=294.5 K

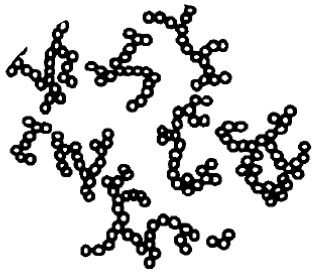
T= 294.5 K



$d_f = 1,3$



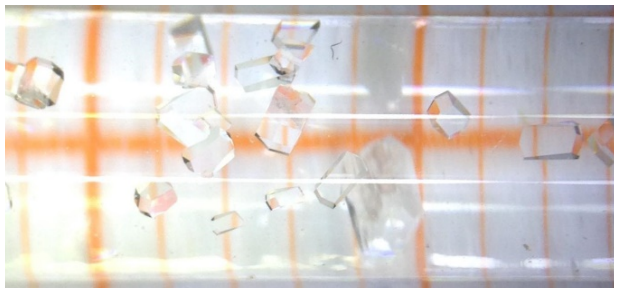
Fractals form!

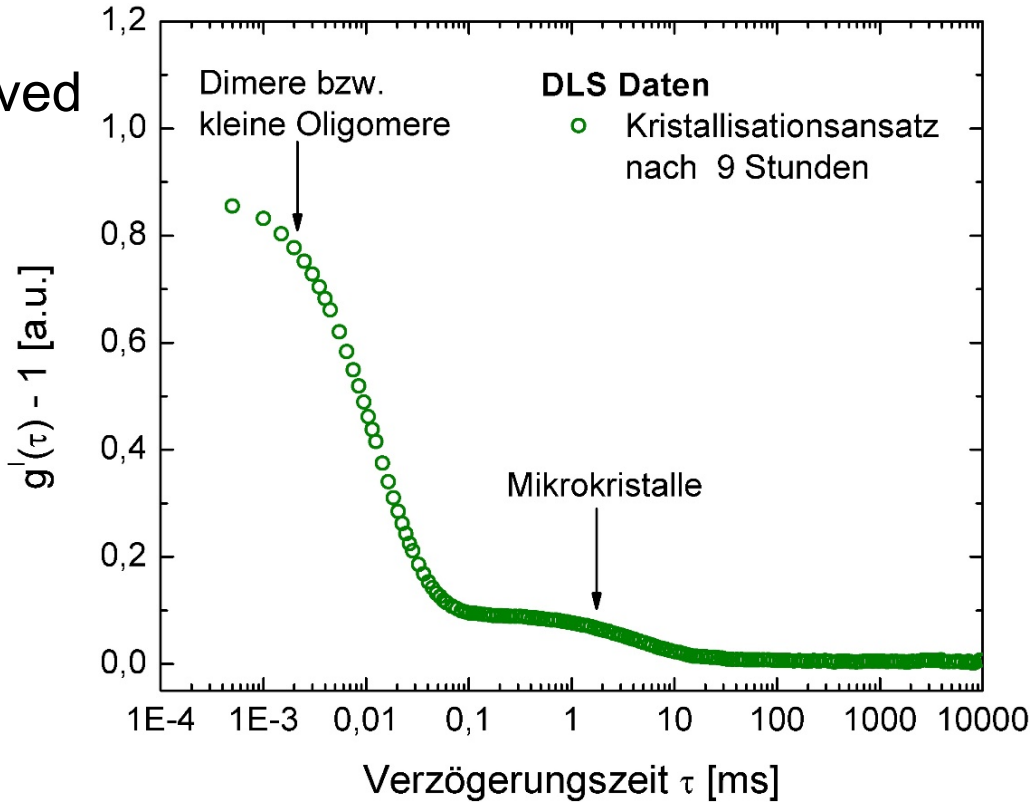


Dynamic light scattering to characterize the sample system

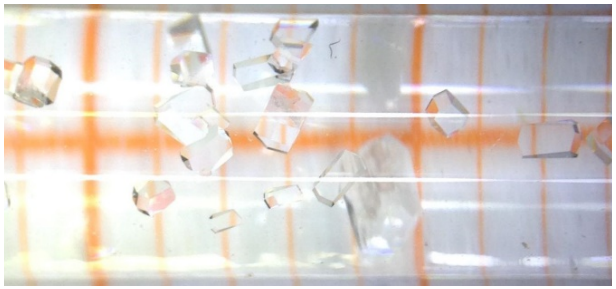
T= 298 K

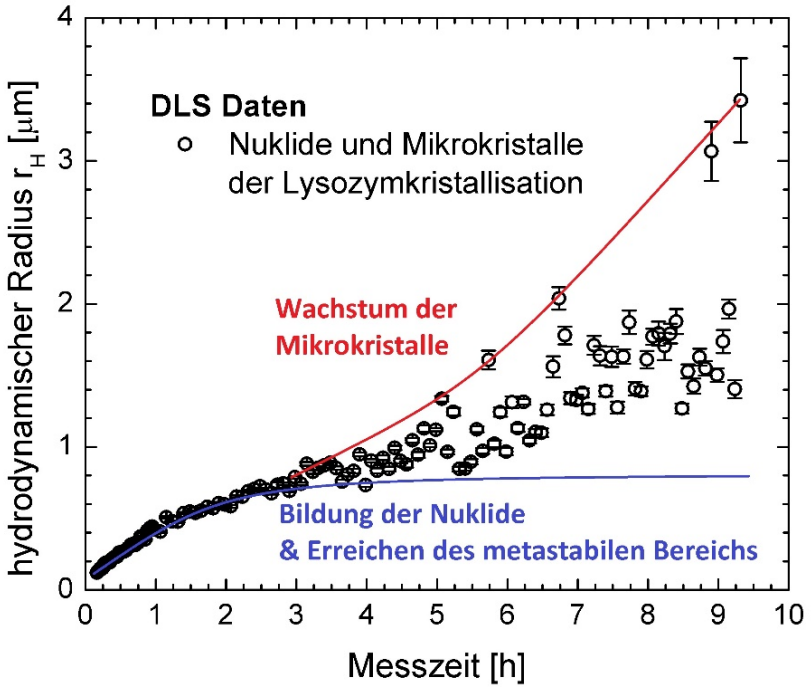
- No third particle fraction observed
- Crystals grow larger in size as at 294.5 K





Long term observation of the crystallisation process with DLS





T= 298 K

- In the beginning we have two particle fractions
- After three hours the sample is not ergodic any more: Large size fluctuations in the larger size fraction is observed

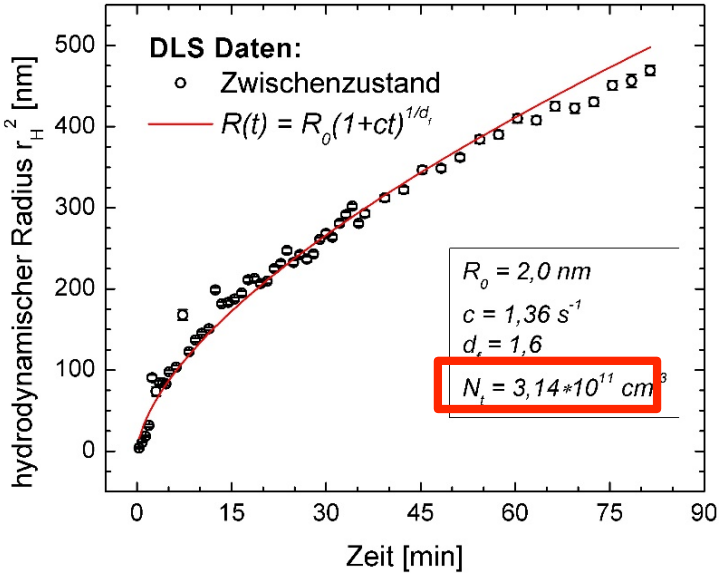
- Interpretation: Small crystals diffuse through the observation volume

Small angle scattering signal can be calculated using a model fit of the DLS data

Volume of the crystal nucleus

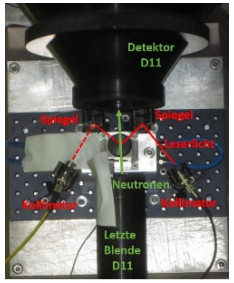
$$\frac{d\Sigma}{d\Omega}(q) = \frac{N_t}{V} * (\Delta\rho)^2 * V_p^2$$

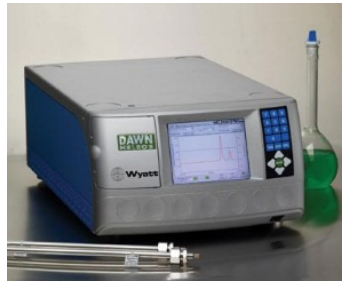
Scattering contrast of lysozyme



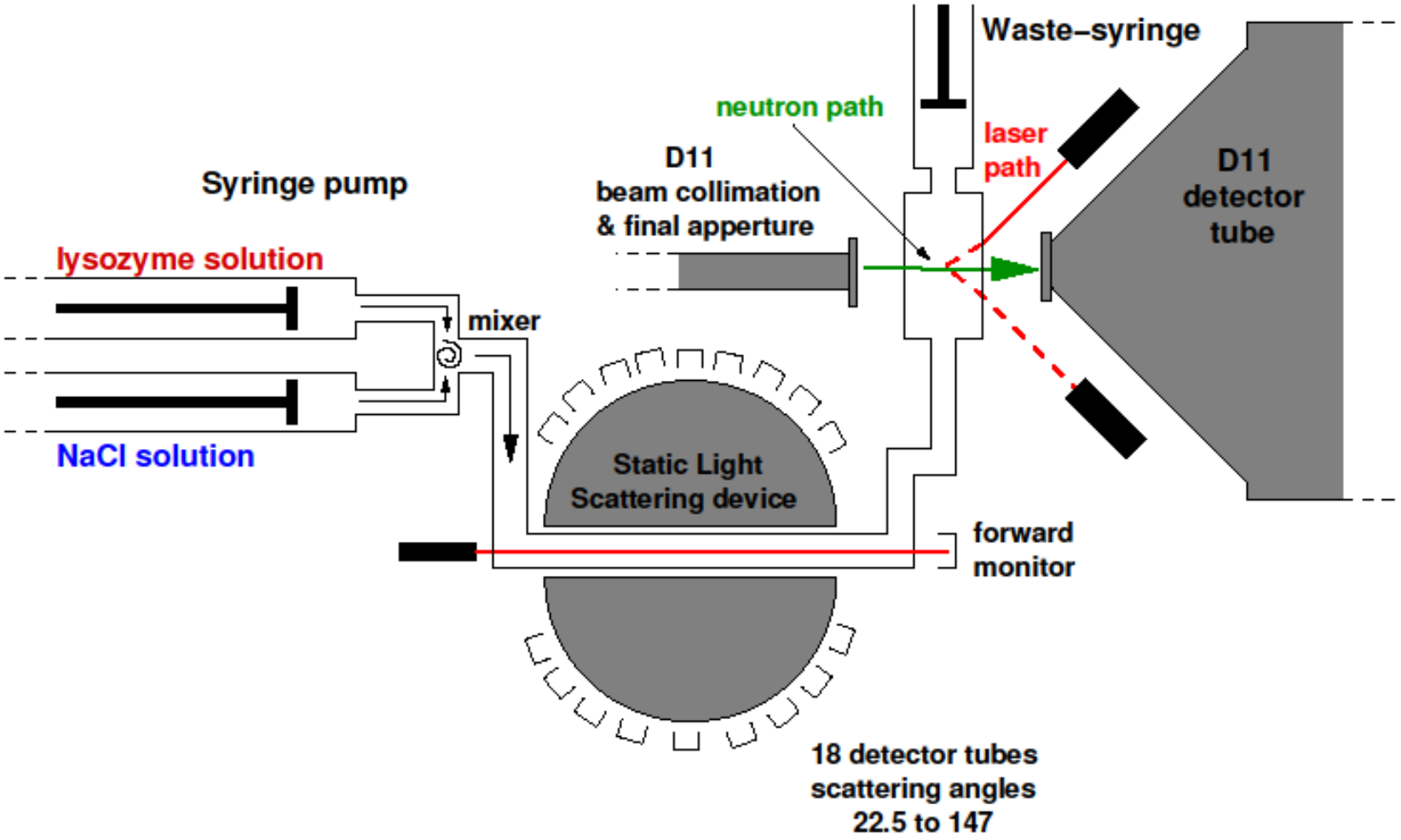
In-situ experiments at the instrument D11

Time resolved structural information on the Lysozyme crystallization: In-situ DLS and quasi-in-situ SLS together with mit Small angle neutron scattering (SANS)

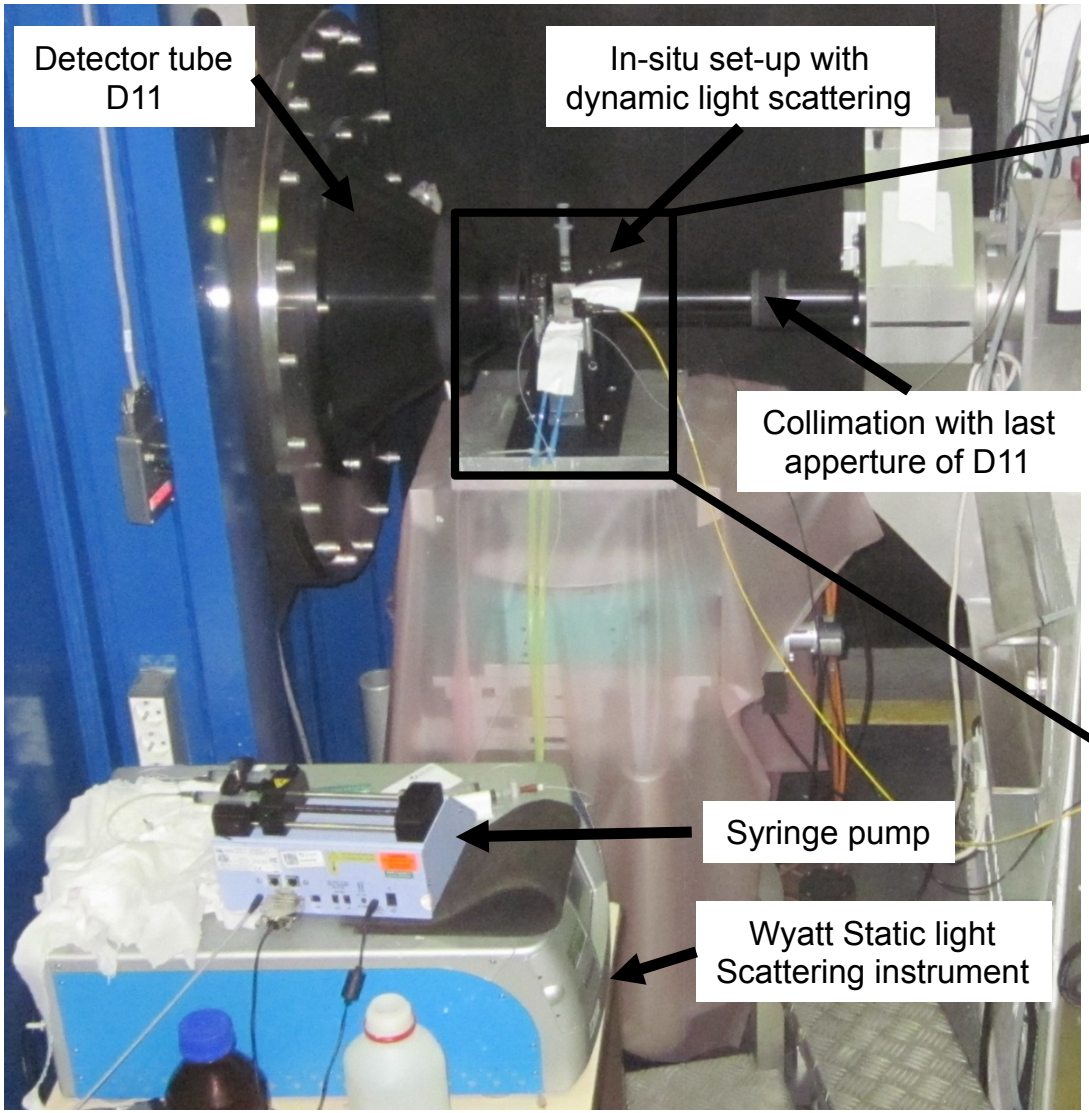


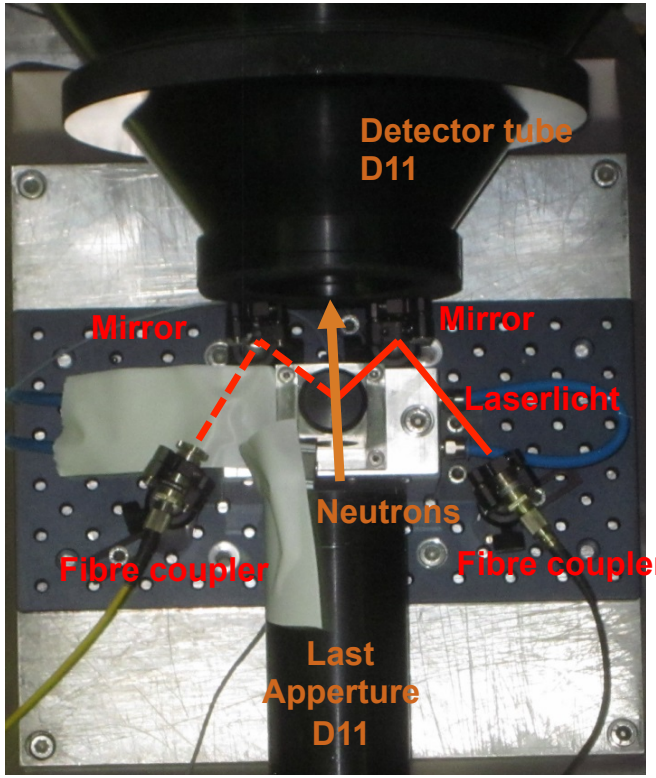


Scheme of the set-up



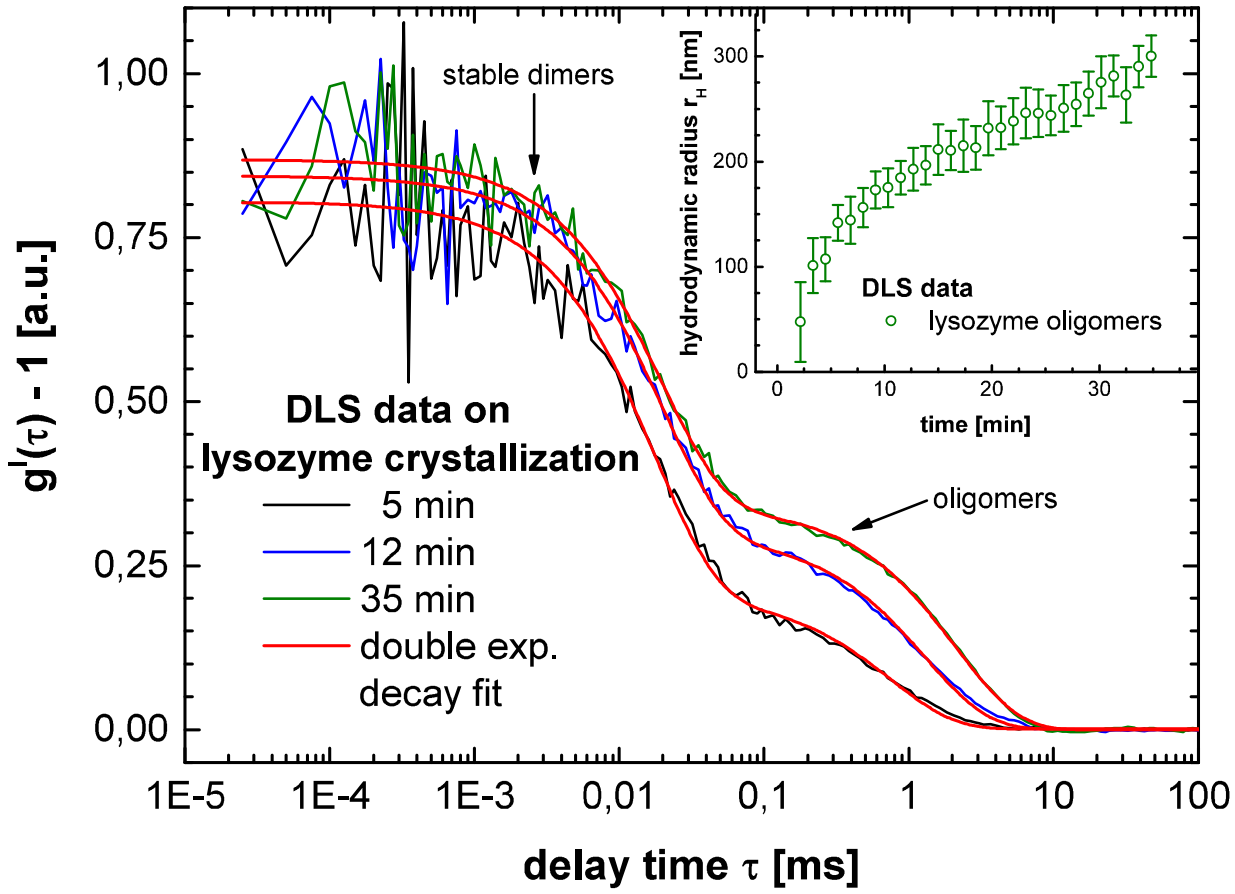
Picture of the set-up at D11





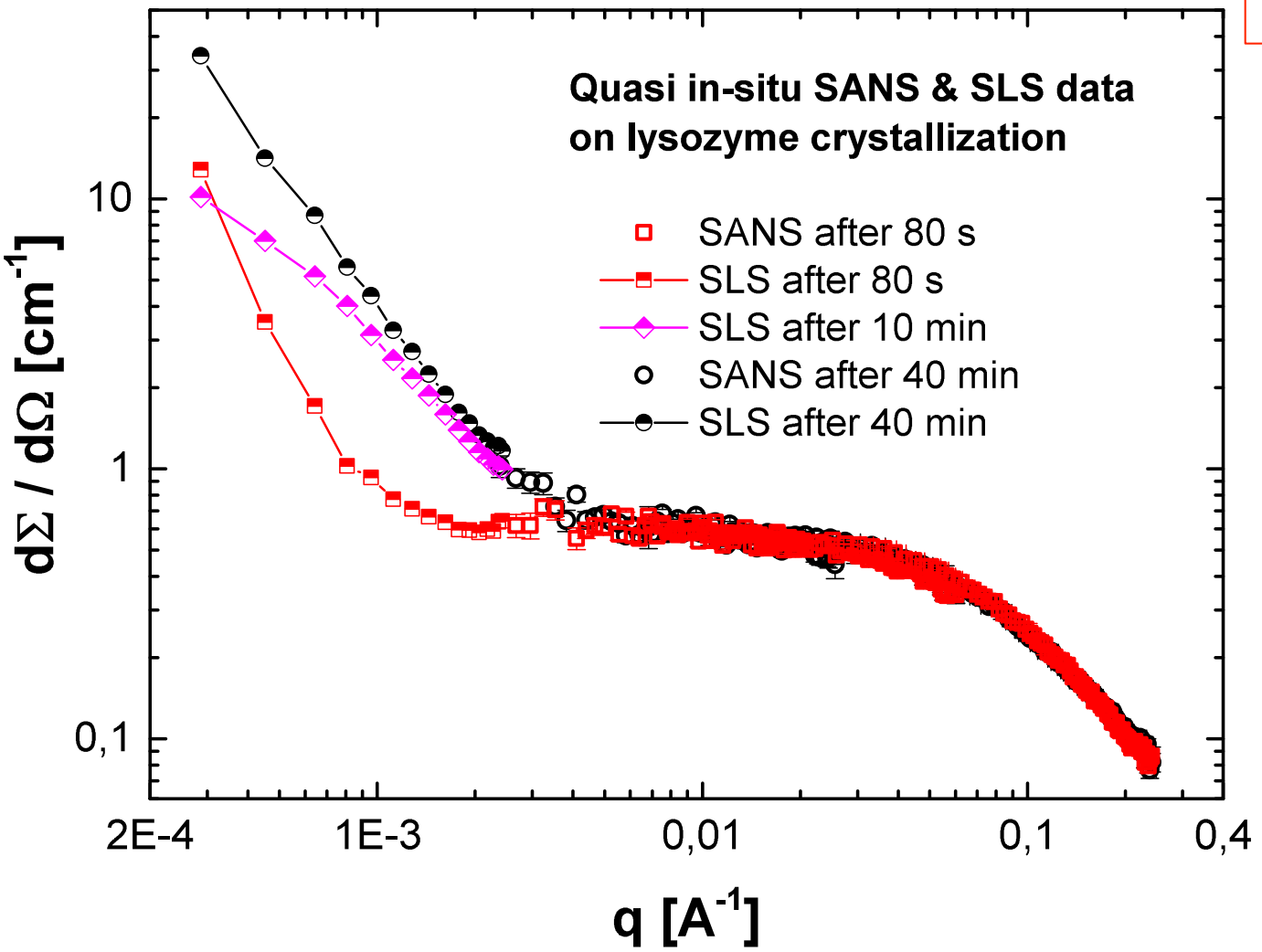
DLS-data recorded in-situ at D11

T= 298 K

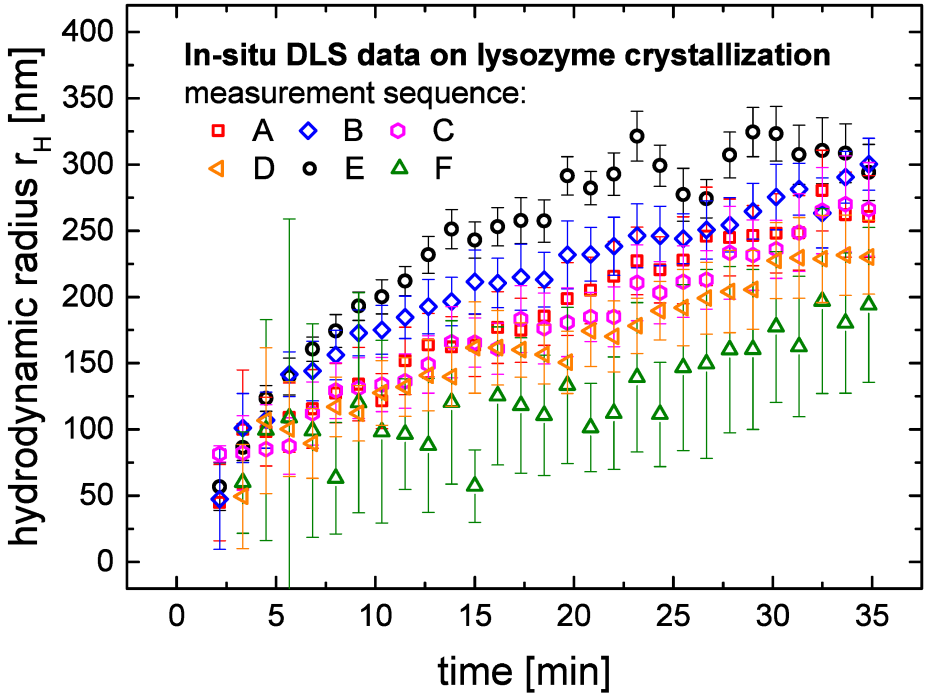


SANS + SLS

T= 298 K



On the reproducibility of the crystallisation runs



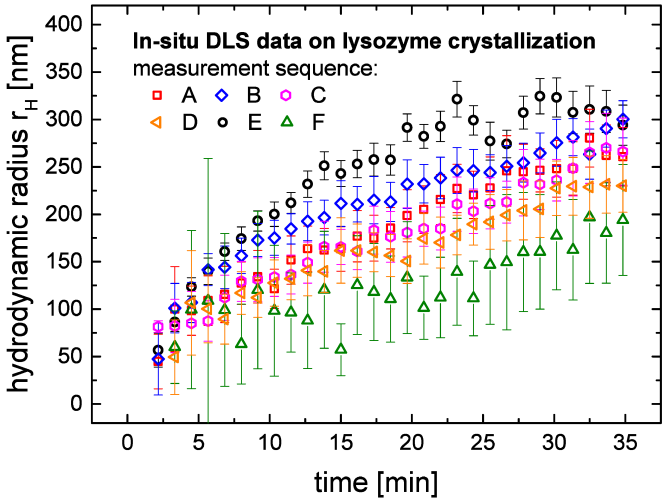
Differences in the speed of the Crystallisation process:

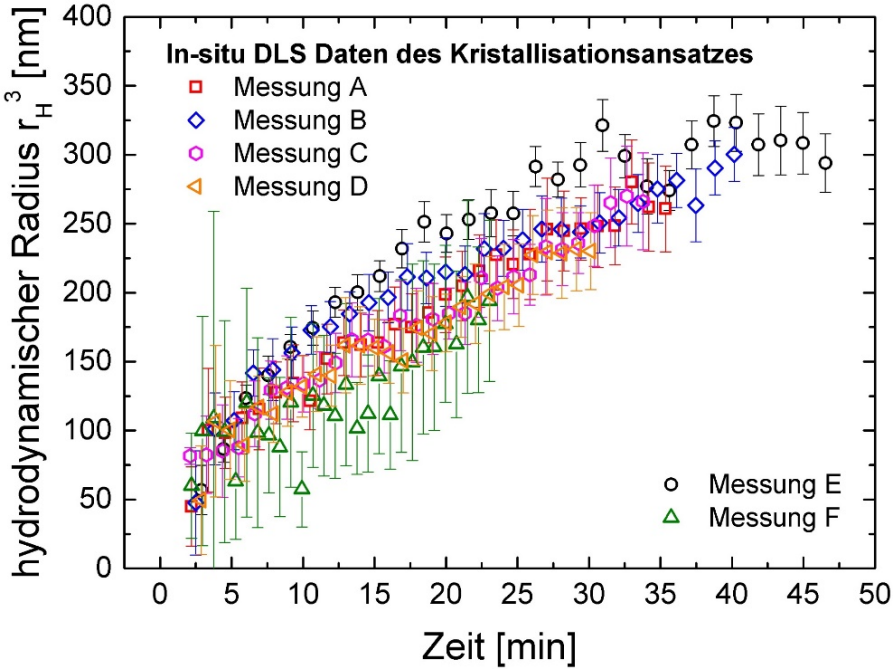
- Possible reasons are fluctuations of the temperature in the vicinity of the sample cell

➢ Scaling factor necessary to account for the differences

T= 298 K

Reproducibility of the results



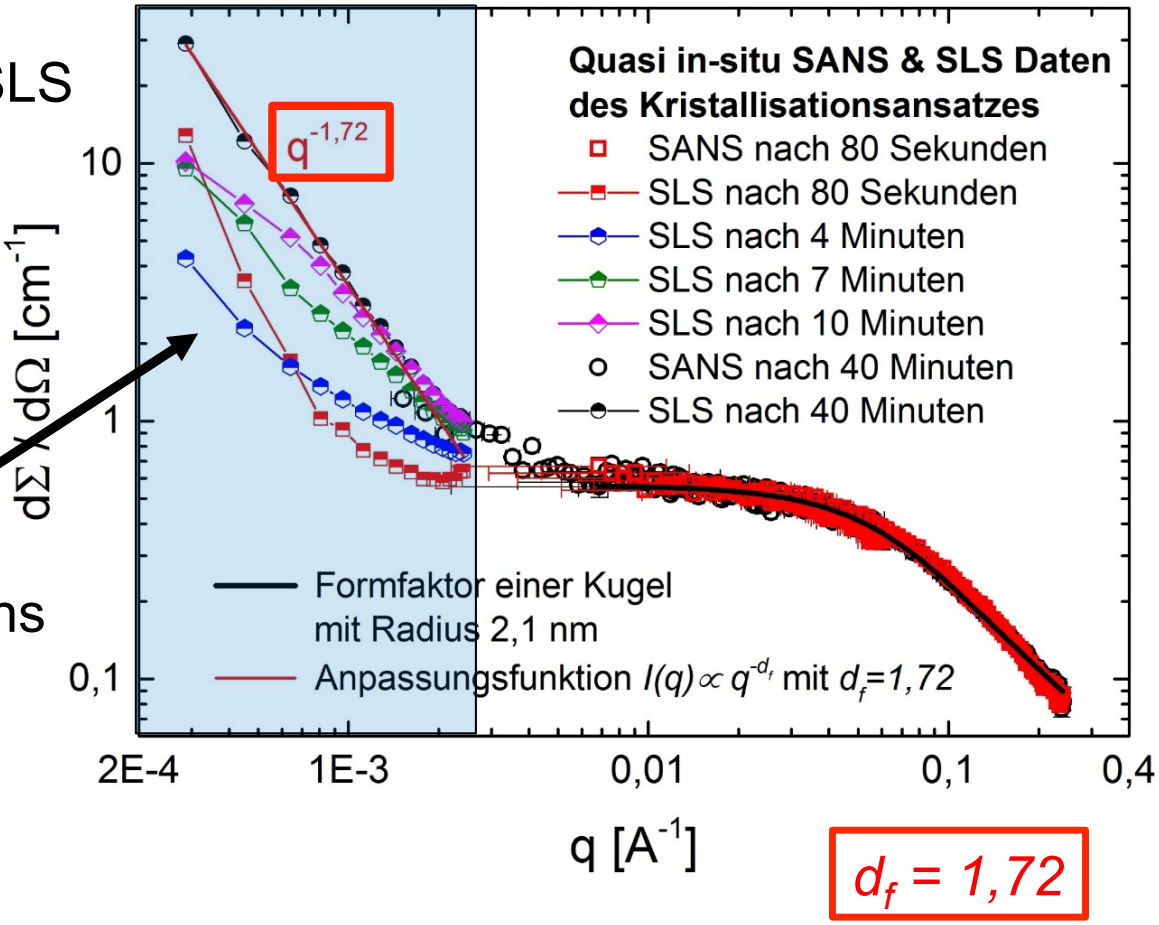


- A scaling factor can be determined to correct for tiny differences in crystallisation speed

T= 298 K

Results of the SANS and SLS measurements at 298 K

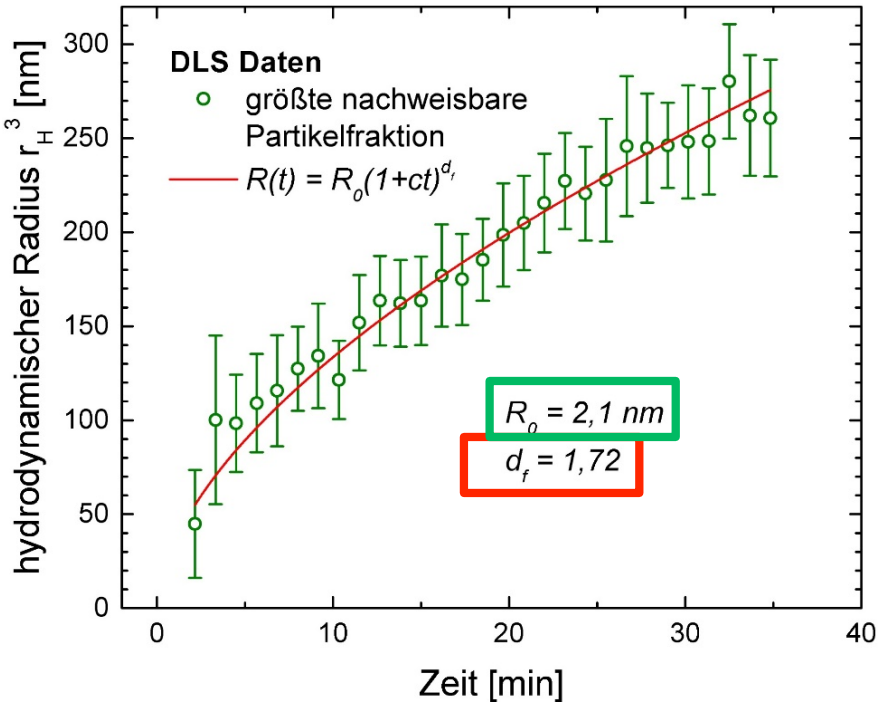
- Extended q-range due to SLS
- temporal evolution of the structure of the lysozyme nuclei can be followed
- Change of fractal dimensions observed

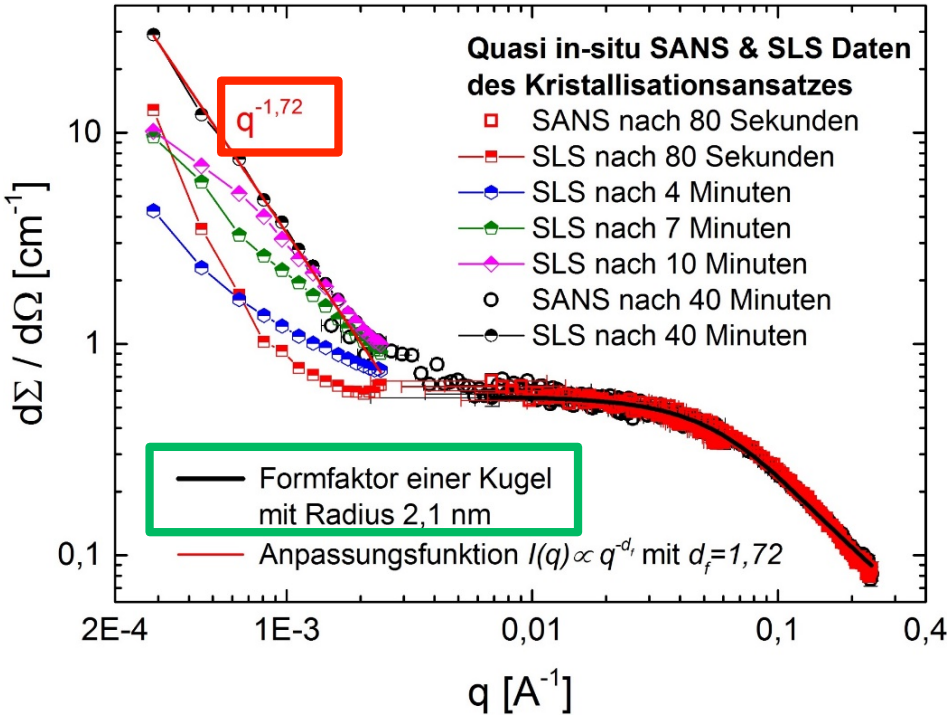


$d_f = 1,72$

T= 298 K

Agreement of SLS/SANS data with in-situ DLS data at 298 K





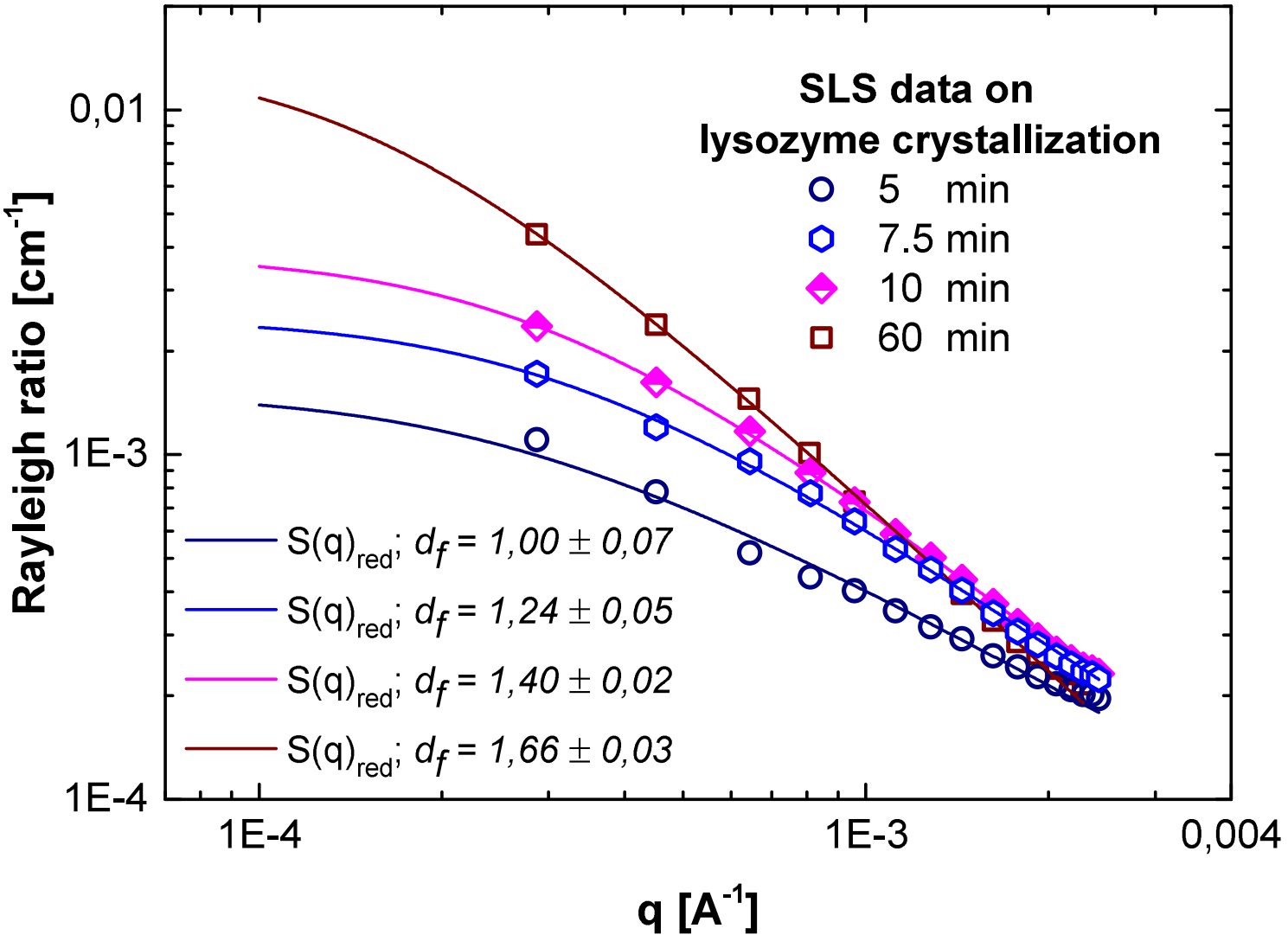
- Agreement of fractal dimension at 40 min. d_f

$d_f = 1,72$

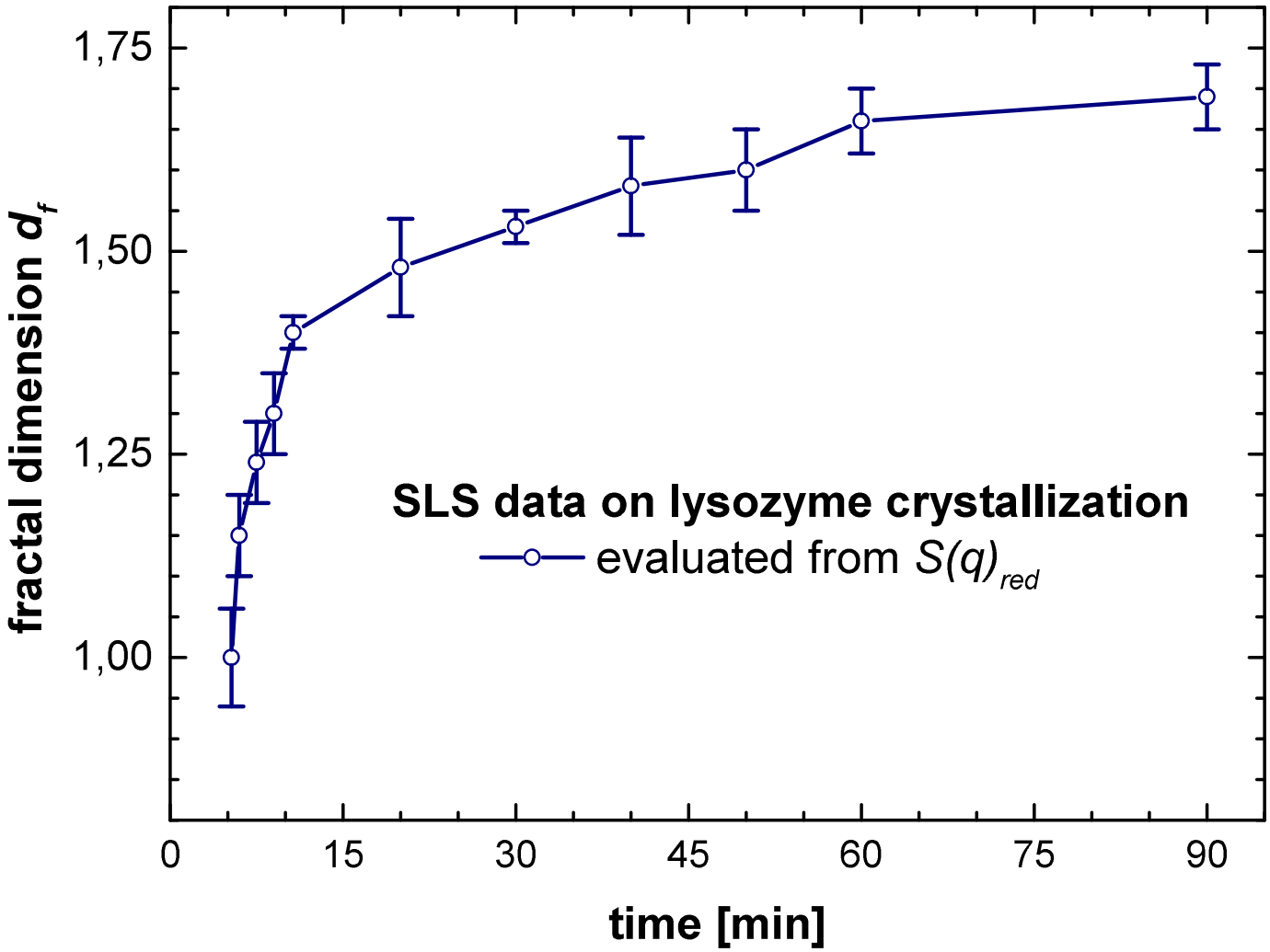
- *Fixed parameter* R_0 from SANS used for the model fit of the DLS data
- Verification of the diffusion limited aggregation model

T= 298 K

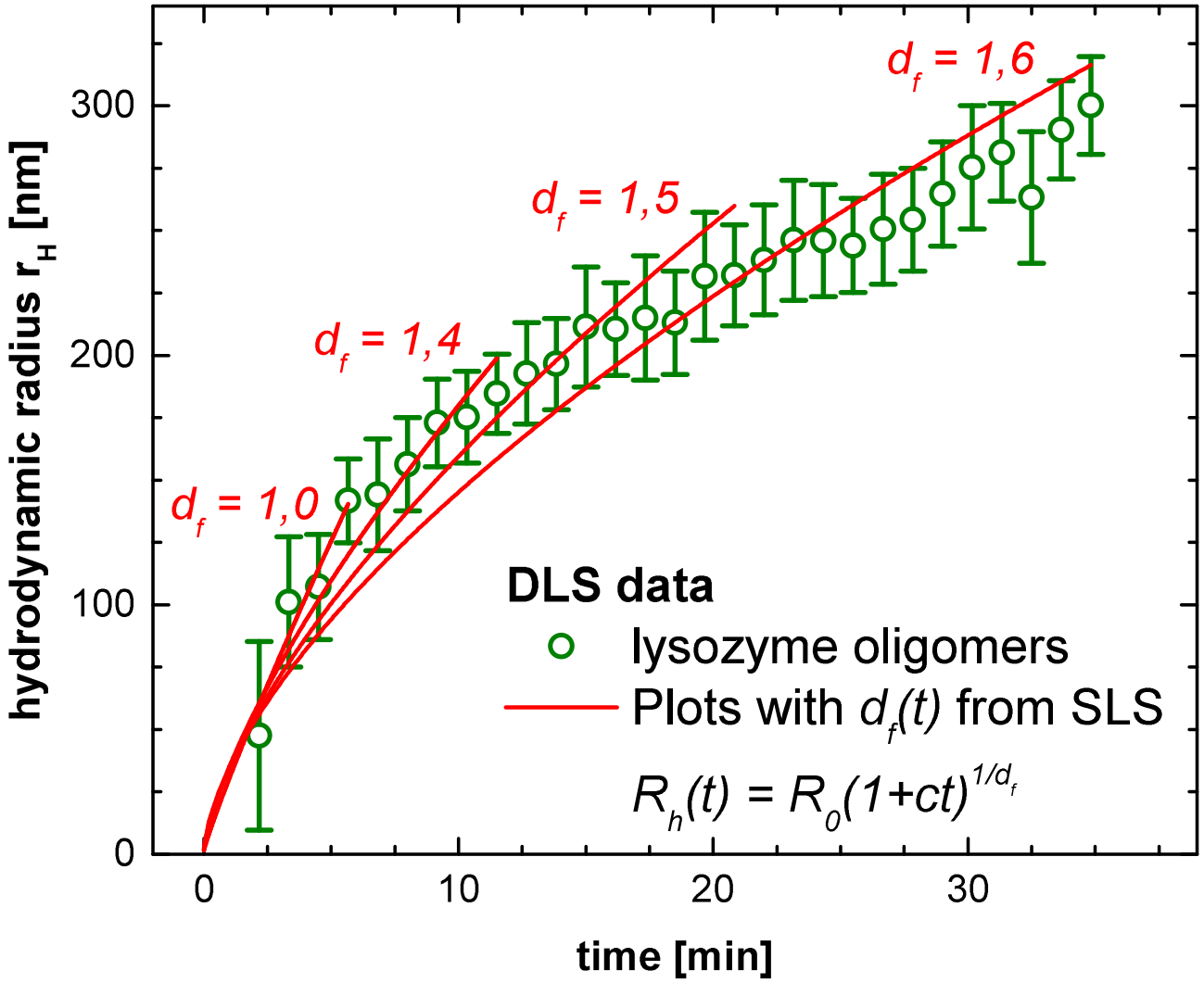
Just the SLS data is needed for fitting the fractal dimension



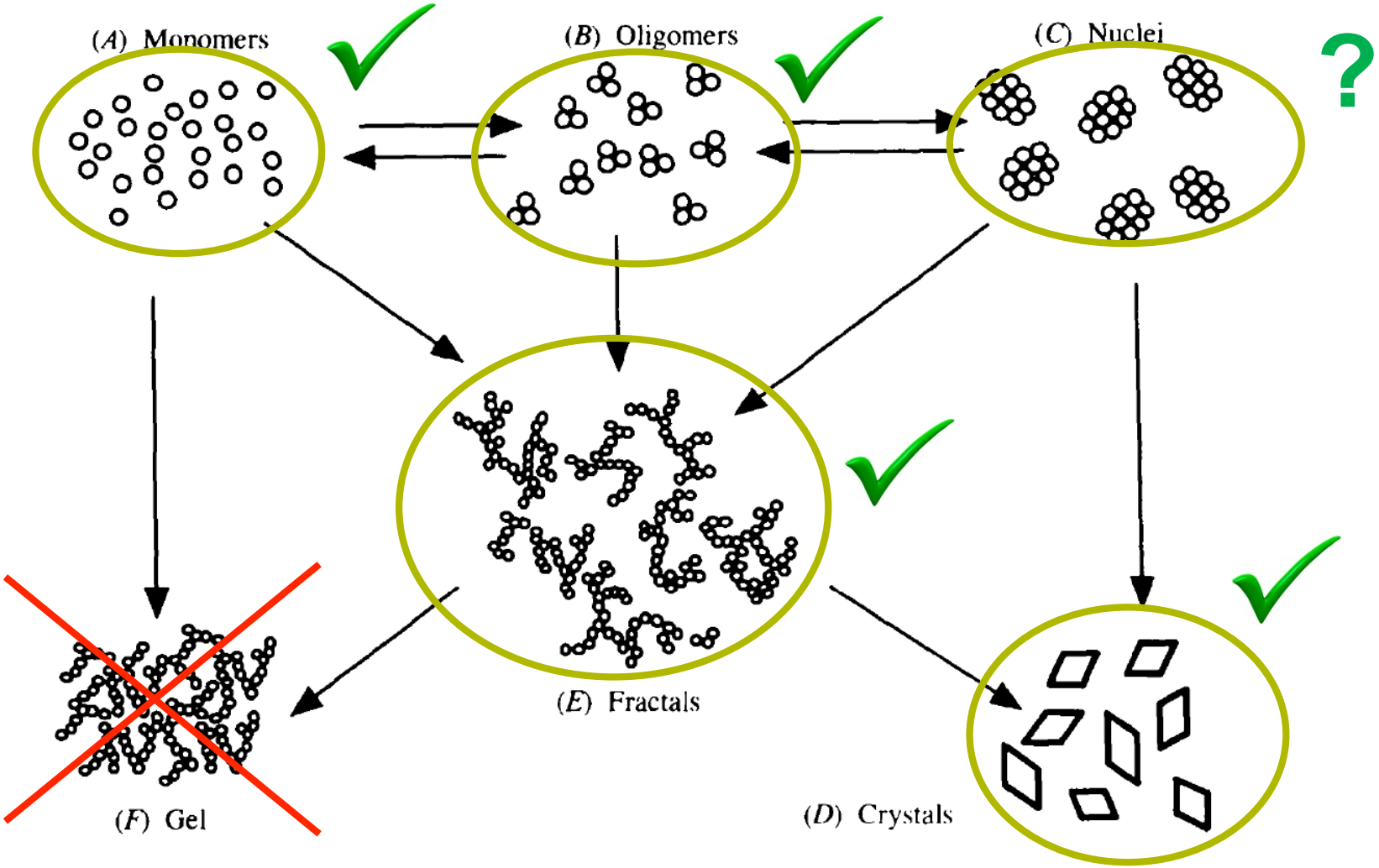
Change of fractal dimension



Agreement of the changing fractal dimension with the DLS data

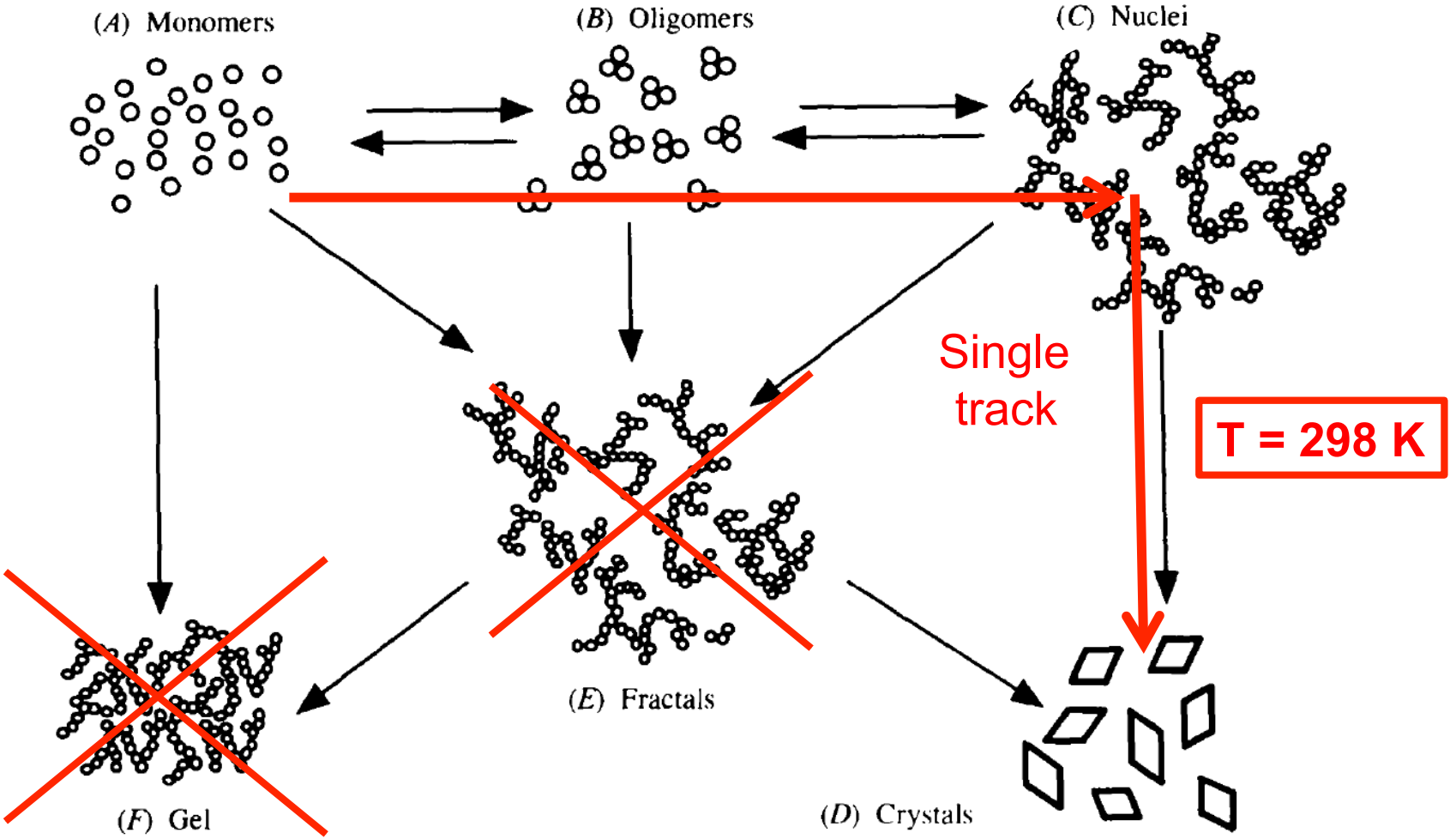


Model for the crysallization process



Y. Georgalis, P. Umbach, J. Raptis and Wolfram Saenger, Acta Cryst. 53 (1997) 703-712

Model for the crysallization process



Y. Georgalis, P. Umbach, J. Raptis and Wolfram Saenger, Acta Cryst. 53 (1997) 703-712

Crystallisation at 298 K