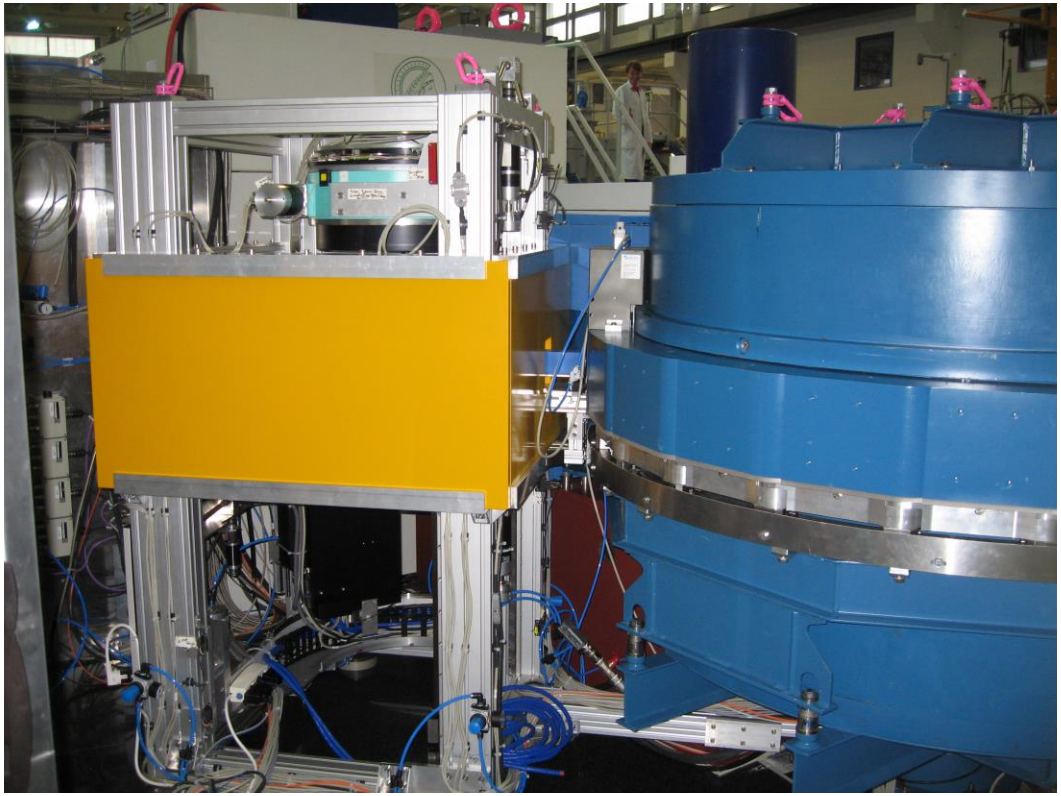
Proteinkristallisation jenseits von Try and Error - Eine kombinierte zeitaufgelöste Licht- und Neutronenstreustudie

2.07.2015

Tobias E. Schrader

Motivation

Motivation: For neutron protein crystallography large crystals are required



Necessary crysal size:
At least 0.5 mm^3

- Deeper understanding of the undelying crystallization mechanism is required

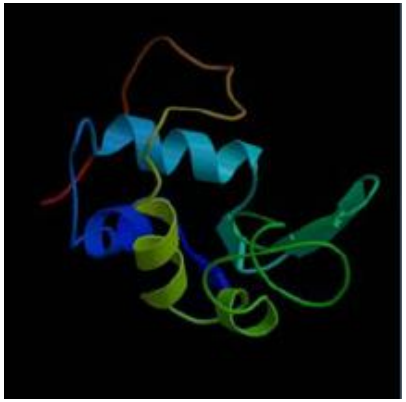
Lysozym als Modell für die Proteinkristallisation

Darreichungsform:
Tabletten

Wirkstoffe:
Lysozym, Cetylpyridinium chlorid

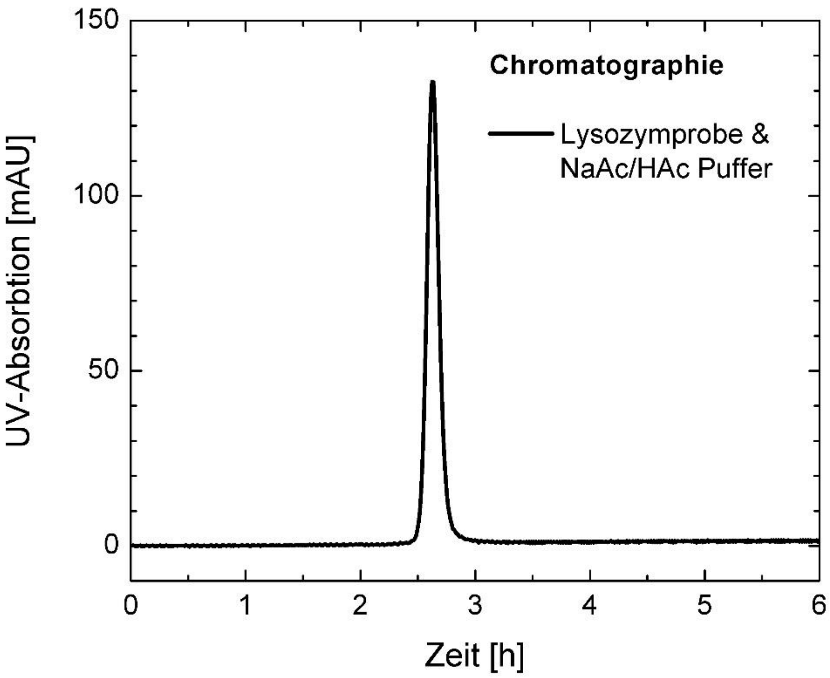
Hilfsstoffe:
Magnesium stearat, Sorbitol, Pfefferminz-Aroma

- Gewonnen aus Hühnereiweiß
- Antibakteriell
- In Tränenflüssigkeit und Speichel enthalten



+ hohe biologische Beständigkeit
+ hohe chemische Reinheit
+ monodisperse Verteilung frei von Aggregaten
+ hohe Präsenz in der Literatur

Überprüfung der Sauberkeit der Probe mittels FPLC



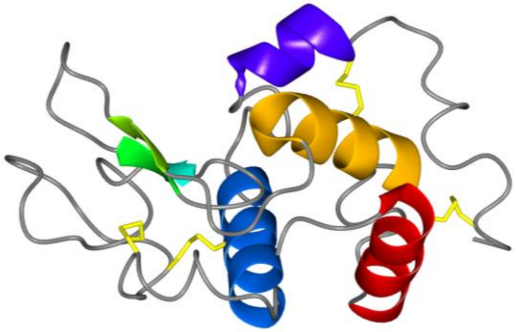
hohe chemische Reinheit

Chosen crystallization conditions

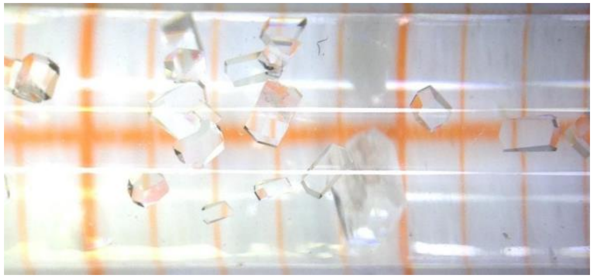
- Lysozyme 60 mg/ml in D_2O, pH adjusted with 1M NaAc 0,02 µm filtered
- NaCl 6wt% in D_2O Puffer 10mM NaAc HAc 0,02 µm filtered

- 1:1 mixture:

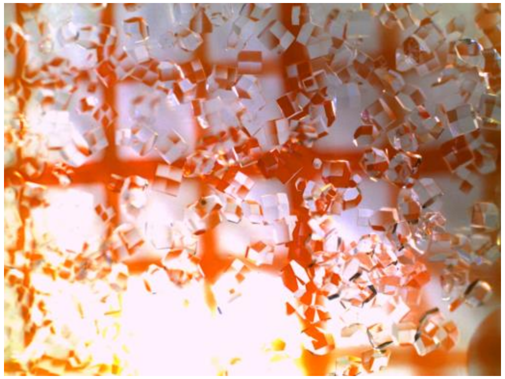
Lysozyme 30 mg/ml + NaCl 3 wt% in D_2O buffer @ pH 4.35



Monomer size: r = 1.9 nm



crystals ca. 1 mm at T = 298 K



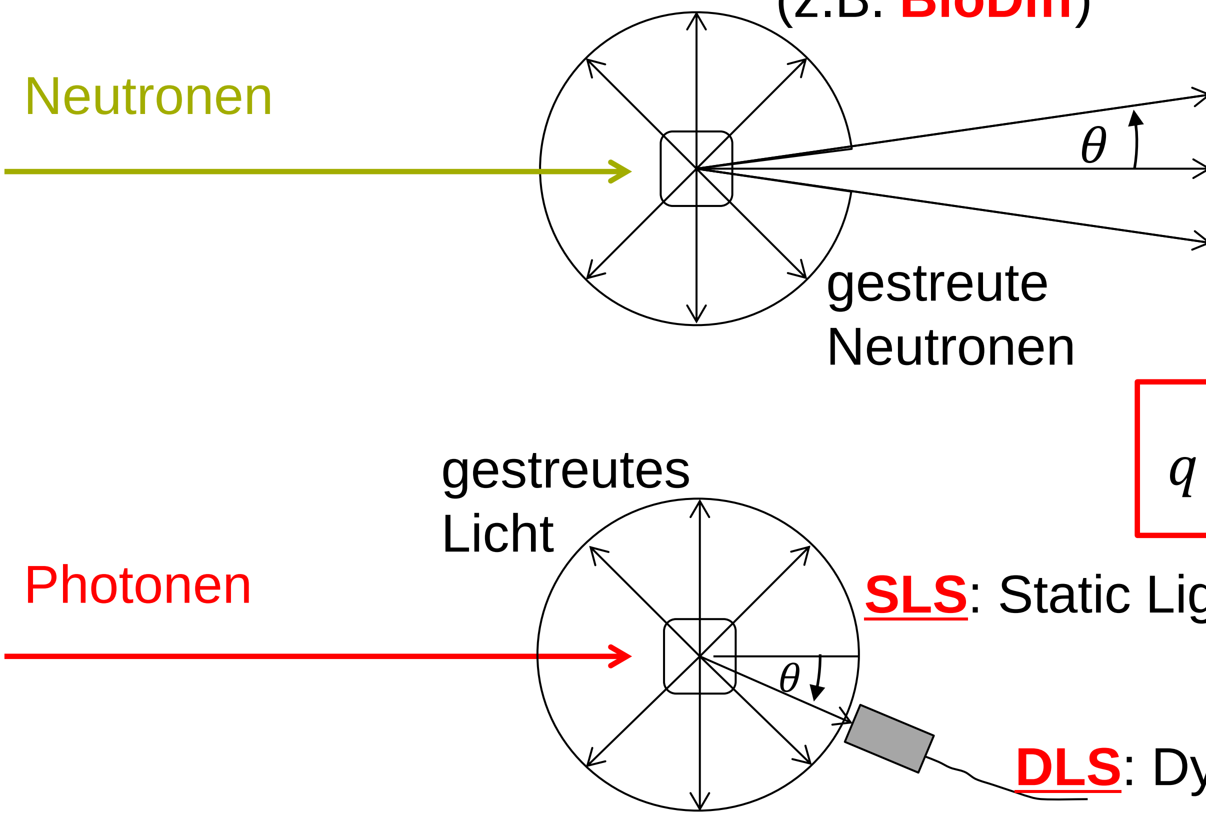
crystals ca. 0.2 mm at T = 294.5 K

Scattering Methods

Methoden & Instrumente

Kristallisationsprozess läuft auf verschiedenen <u>Zeit- und Größenskalen</u> ab

Einkristall Diffraktometer
(z.B. **BioDiff**)



SANS:
Small Angle Neutron Scattering
(z.B. KWS-2, D11)

$$\boxed{q = \frac{4\pi n}{\lambda} * \sin\frac{\theta}{2}}$$

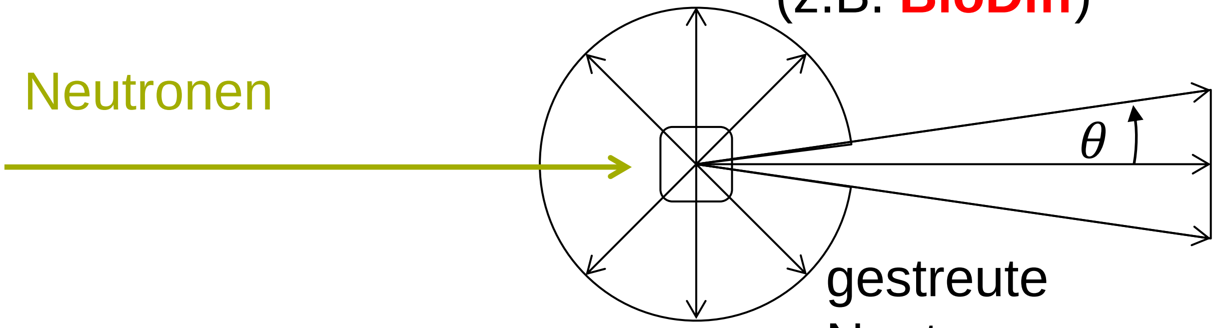
SLS: Static Light Scattering

DLS: Dynamic Light Scattering

Kristallisationsprozess läuft auf verschiedenen <u>Zeit- und Größenskalen</u> ab

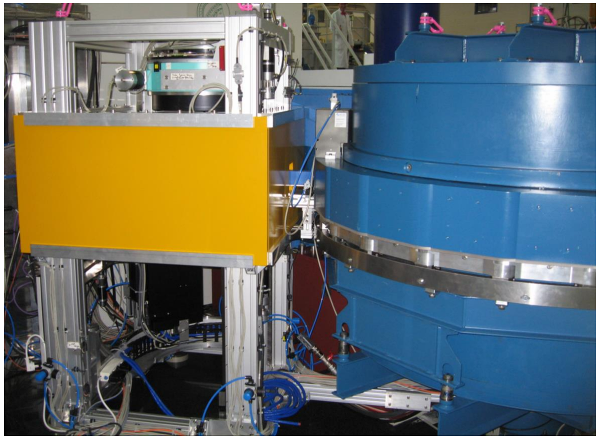
Einkristall Diffraktometer
(z.B. **BioDiff**)

Neutronen

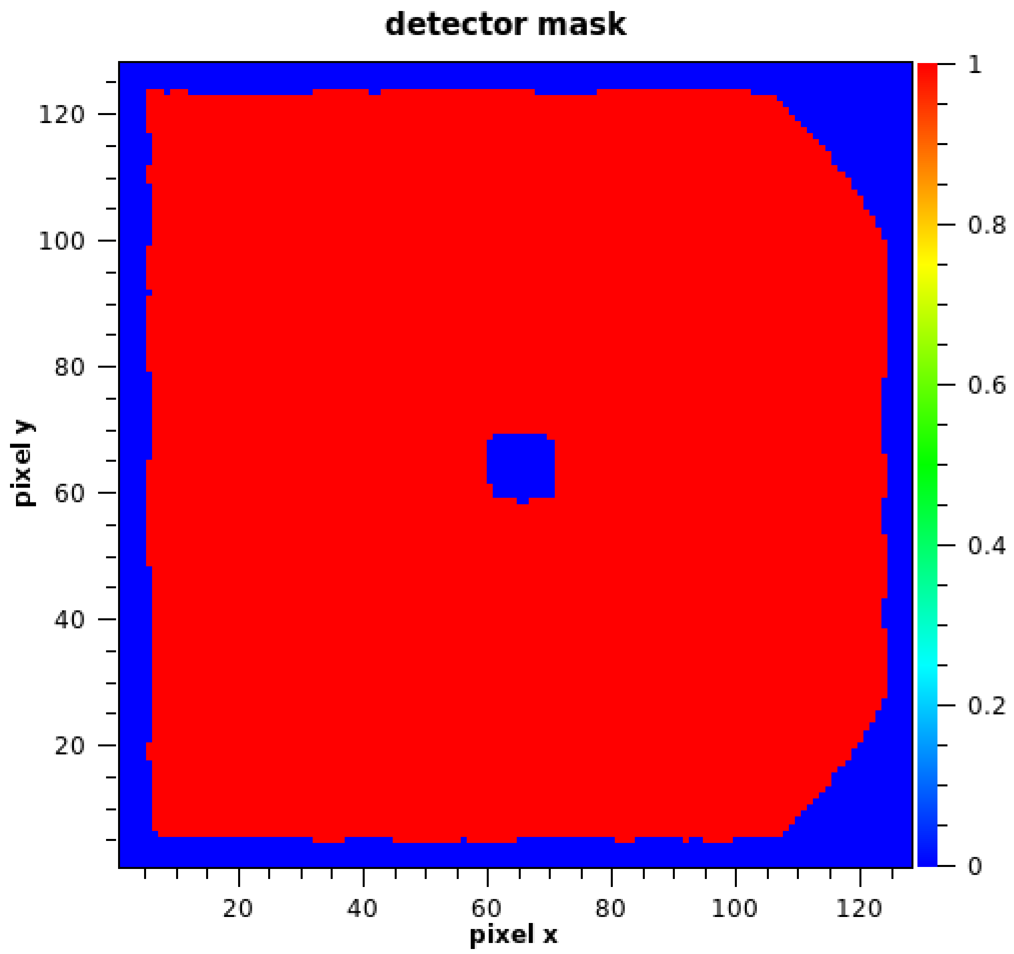


gestreute
Neutronen

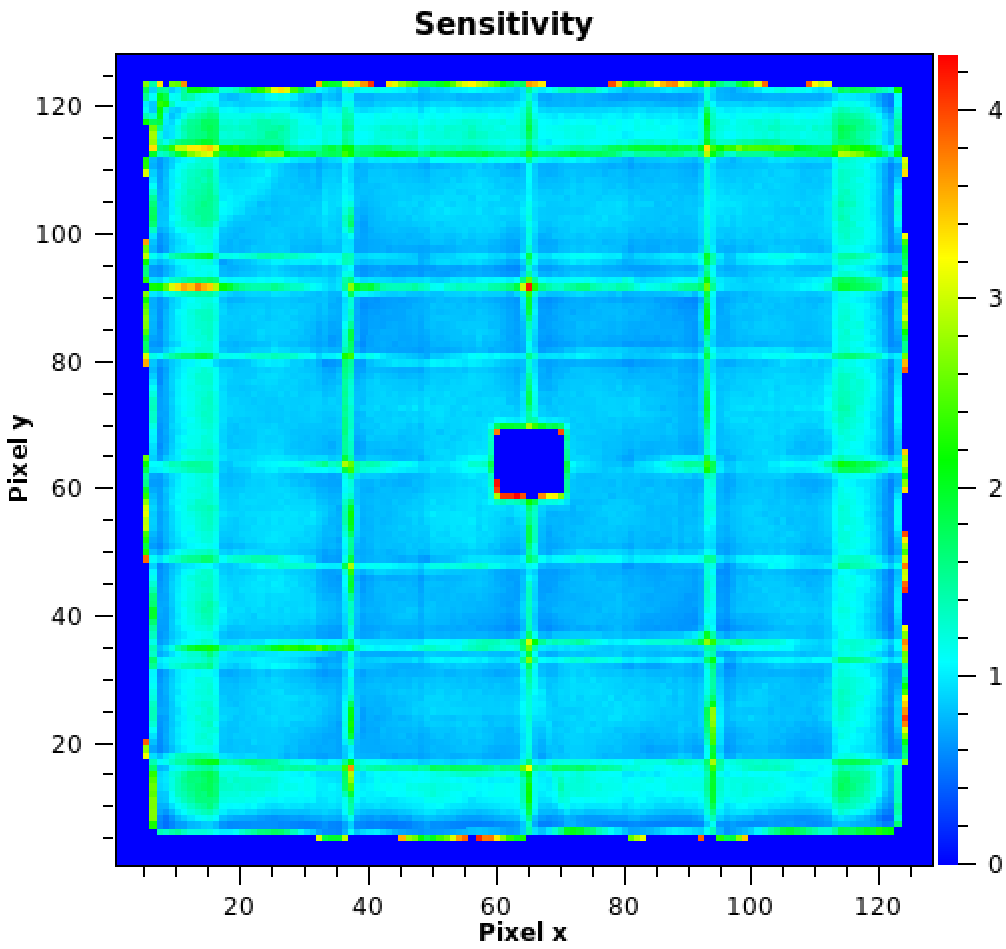
<u>SANS</u>:
Small Angle Neutron Scattering
(z.B. KWS-2, D11)



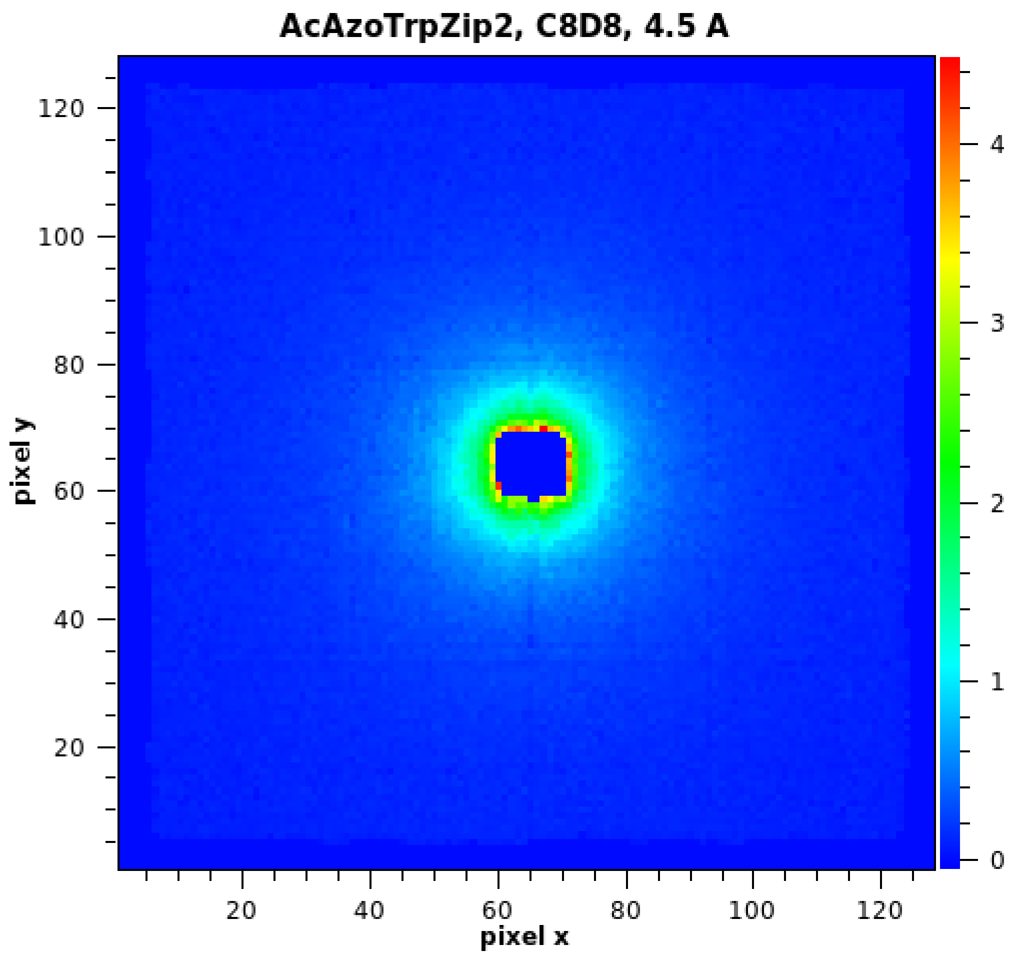
The SANS experiment



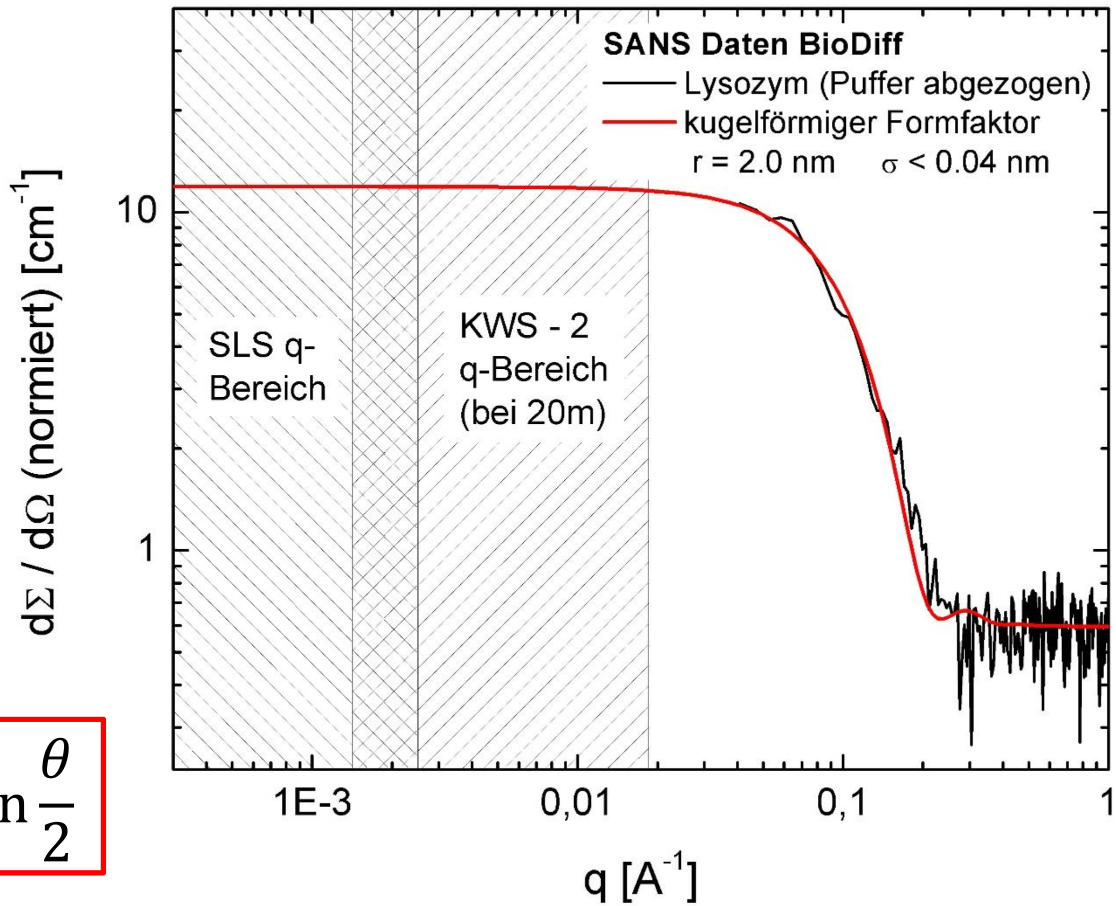
The SANS experiment



The SANS experiment



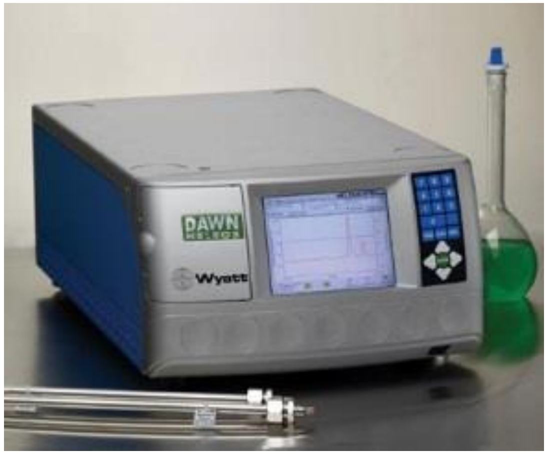
Methoden & Instrumente

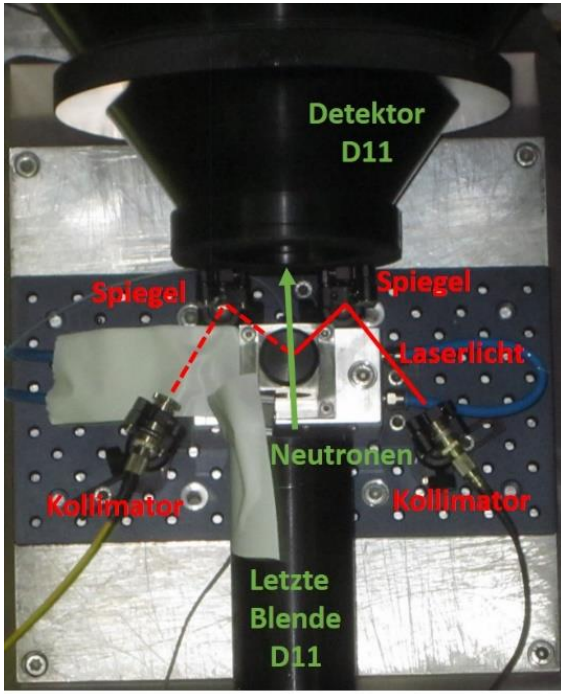


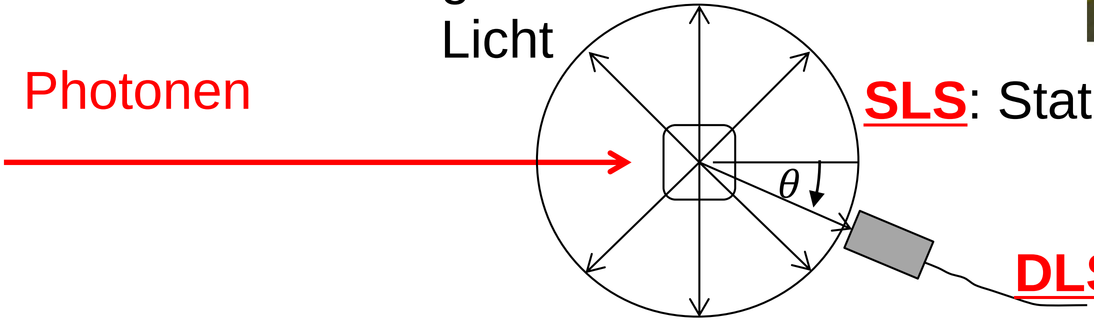
$$q = \frac{4\pi n}{\lambda} * \sin\frac{\theta}{2}$$

Radius ermittelt durch Formfaktor einer Kugel weist auf Monomere hin

Kristallisationsprozess läuft auf verschiedenen Zeit- und Größenskalen ab







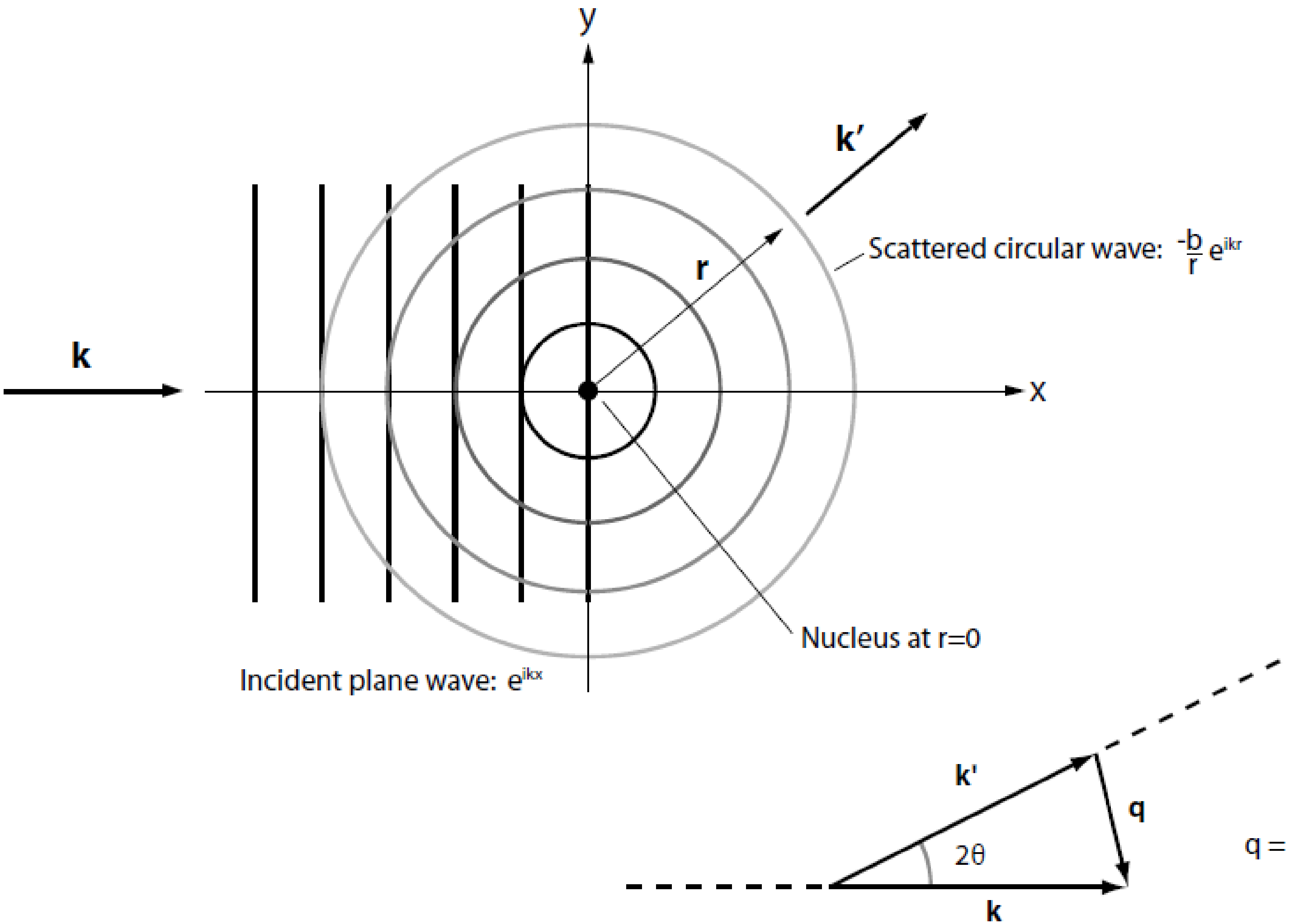
SLS: Static Light Scattering

DLS: Dynamic Light Scattering

Exkurs: Kleinwinkelstreuung

Partly taken from:
Introduction to Small-Angle Neutron Scattering and Neutron Reflectometry
Andrew J Jackson
NIST Center for Neutron Research
May 2008

Scattering of neutrons: Nuclei as point scatterer



$$\sin\theta = \frac{|\mathbf{q}|}{2}\frac{1}{|\mathbf{k}|}$$

$$q = 2k\sin\theta = \frac{4\pi}{\lambda}\sin\theta$$

Differential cross section

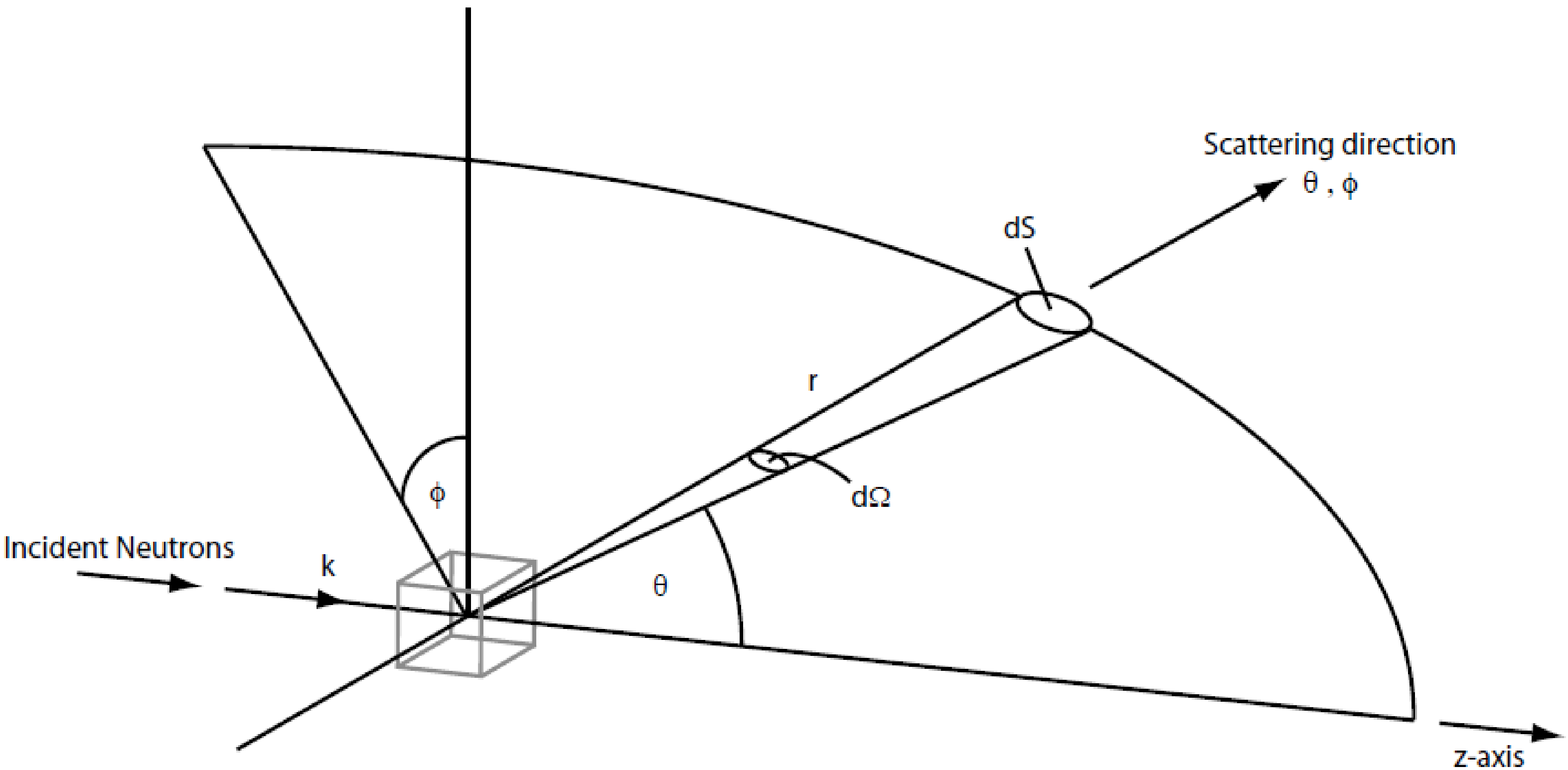


Figure 2: The geometry of a scattering experiment (after Squires)

Some formulas

$$\frac{d\sigma}{d\Omega}(\mathbf{q}) = \frac{1}{N}\left|\sum_i^N b_i e^{i\mathbf{q}\cdot\mathbf{r}}\right|^2$$

$$\rho(\mathbf{r}) = b_i\delta(\mathbf{r} - \mathbf{r_i})$$

For small angle scattering we can use the integral instead of the sum:

$$\frac{d\Sigma}{d\Omega}(\mathbf{q}) = \frac{N}{V}\frac{d\sigma}{d\Omega}(\mathbf{q}) = \frac{1}{V}\left|\int_V \rho(\mathbf{r})e^{i\mathbf{q}\cdot\mathbf{r}}d\mathbf{r}\right|^2$$ with $$\rho = \frac{\sum_i^n b_i}{\overline{V}}$$

Coherent and incoherent scattering including absorption give the complete differential cross section:

$$\frac{d\Sigma}{d\Omega}(\mathbf{q}) = \frac{d\Sigma_{coh}}{d\Omega}(\mathbf{q}) + \frac{d\Sigma_{inc}}{d\Omega} + \frac{d\Sigma_{abs}}{d\Omega}$$

The small angle scattering secret

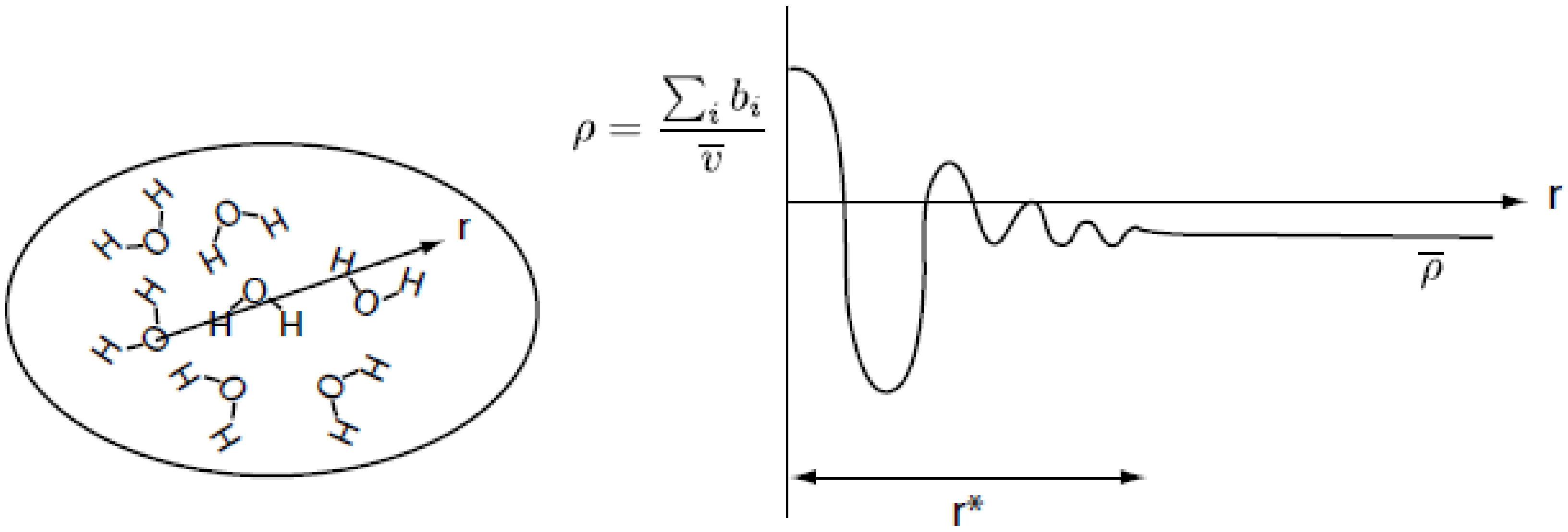


Figure 3: Scattering length density of water as a function of distance from a given oxygen atom (after Kline)

The final formula to calculate a scattering law

$$\frac{d\Sigma}{d\Omega}(\mathbf{q}) = \frac{1}{V}(\rho_1 - \rho_2)^2 \left| \int_{V_1} e^{i\mathbf{q}\cdot\mathbf{r}} d\mathbf{r}_1 \right|^2$$

For example for a sphere one obtains for the modulus of the integral:

$$\mathrm{P(Q)} = \left[\frac{3\mathrm{j}_1(\mathrm{QR})}{\mathrm{QR}}\right]^2 = \left[\frac{3}{\mathrm{QR}}\left(\frac{\sin(\mathrm{QR})}{(\mathrm{QR})^2} - \frac{\cos(\mathrm{QR})}{\mathrm{QR}}\right)\right]^2$$

Formfactor of a sphere

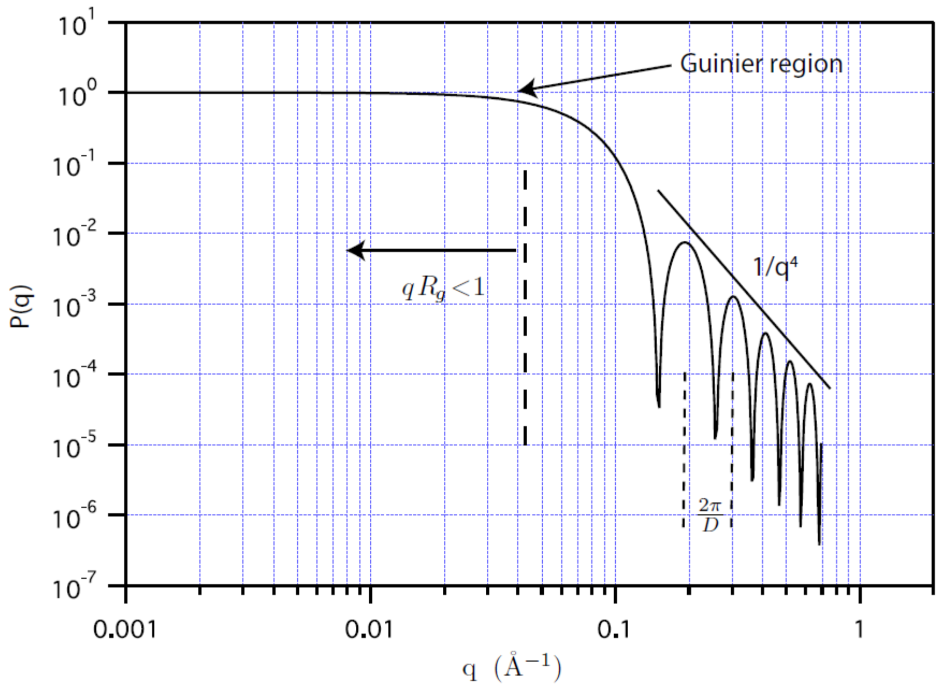
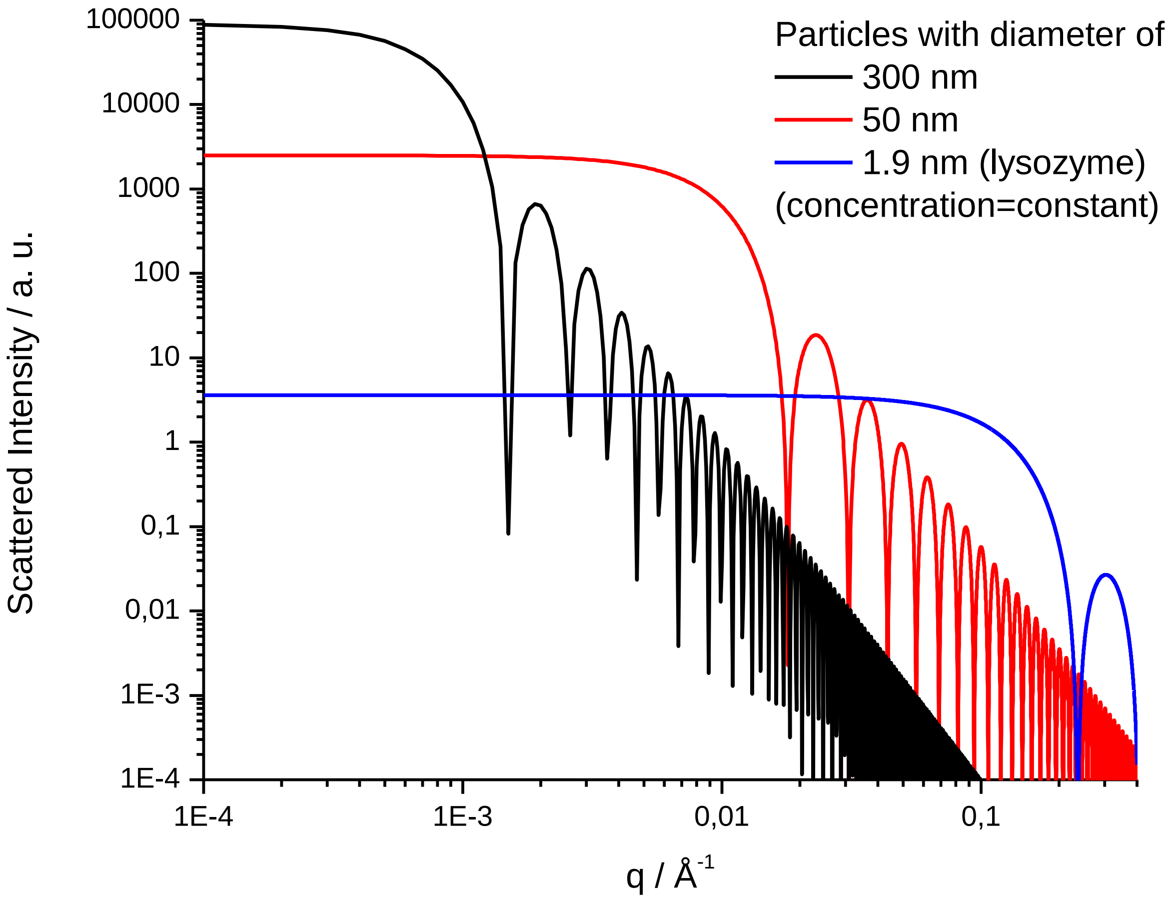


Figure 7: Form Factor spheres of radius 3Å. $R_g = 23$Å

Different sizes of particles



Formfactor of a cylinder

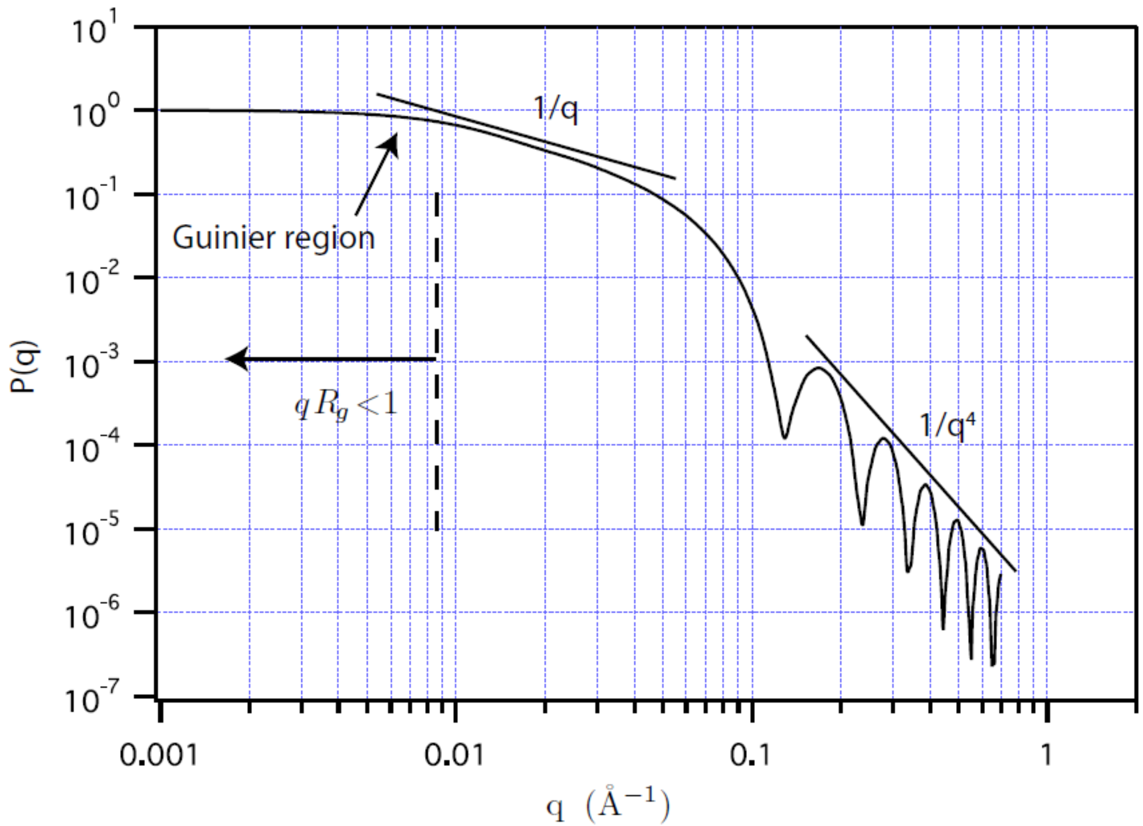
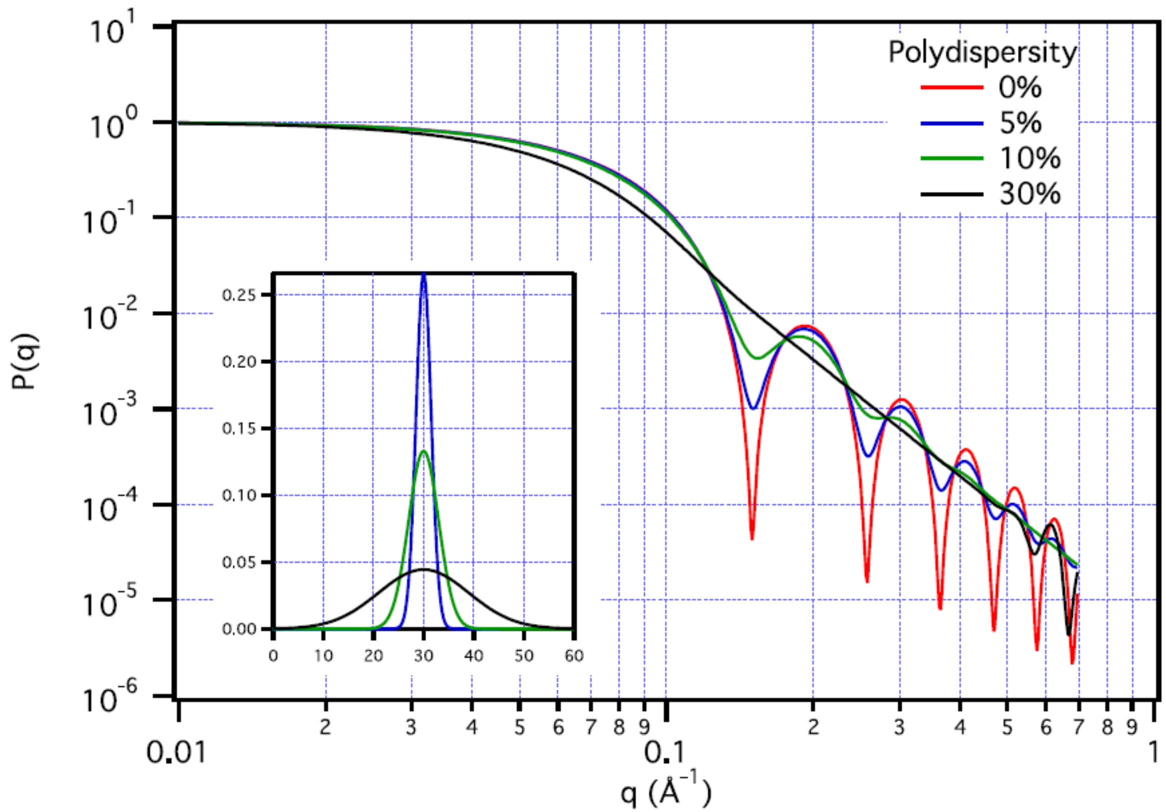
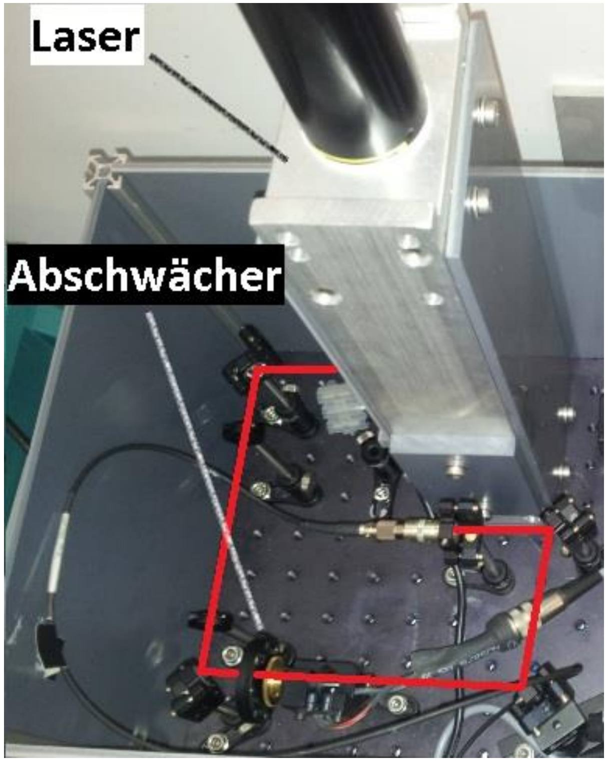


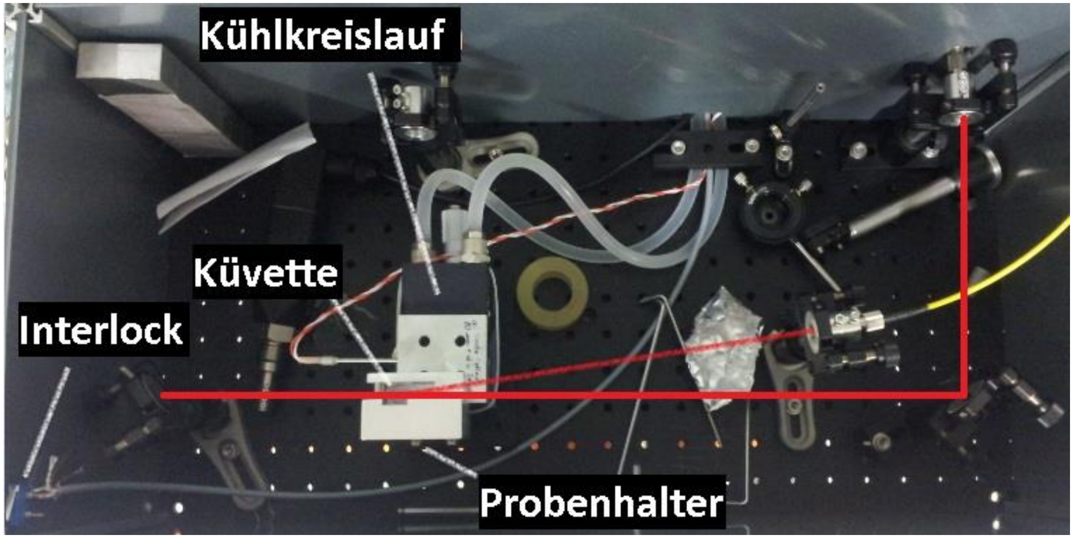
Figure 8: Form Factor for cylinders of radius 30Å and length 400Å. $R_g = 117$Å

Influence of a size distribution= Polydispersity



Dynamische Lichtstreuung





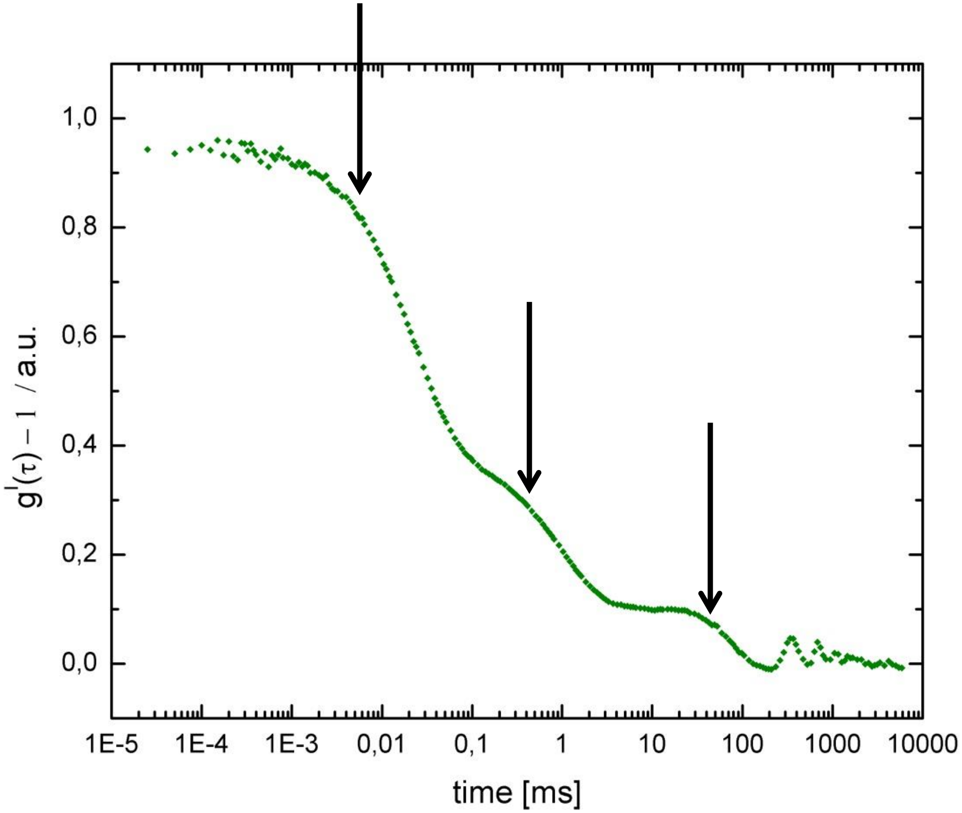
Autokorrelationsfunktion weist drei verschiedene Zeitkonstanten auf

$$g_2(\tau)-1=e^{-2Dq^2t}$$

- Zeitkonstante: t_1, t_2, t_3
- Diffusionskoeffizient:
- Hydrodynamischer Radius:

$$r_H=\frac{k_BT}{6\pi\eta}\cdot 2t_nq^2$$

$$\boxed{q=\frac{4\pi n}{\lambda}*\sin\frac{\theta}{2}}$$

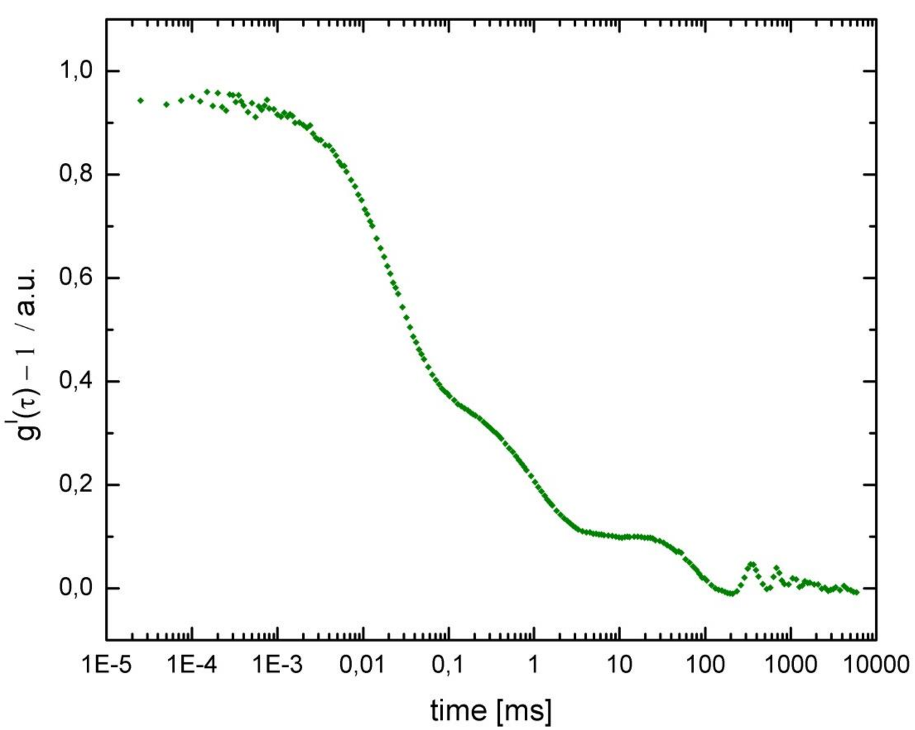


Keine Forminformation möglich

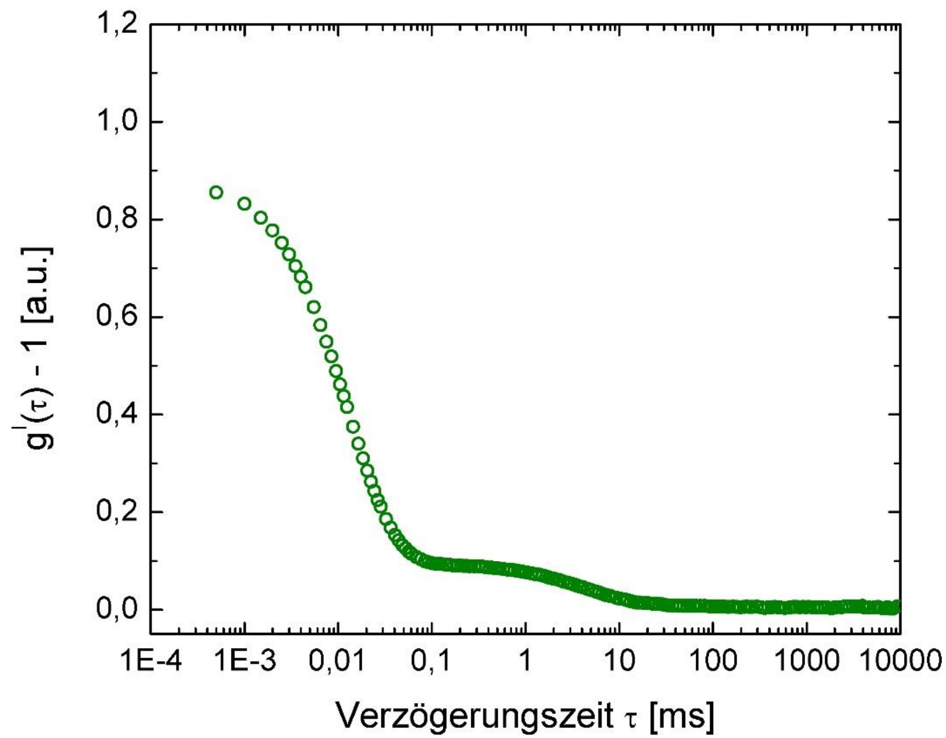
Pre-characterization of the lysozyme sample in the lab using light scattering

Dynamic light scattering gives the number of particle sizes present

T= 294,5 K



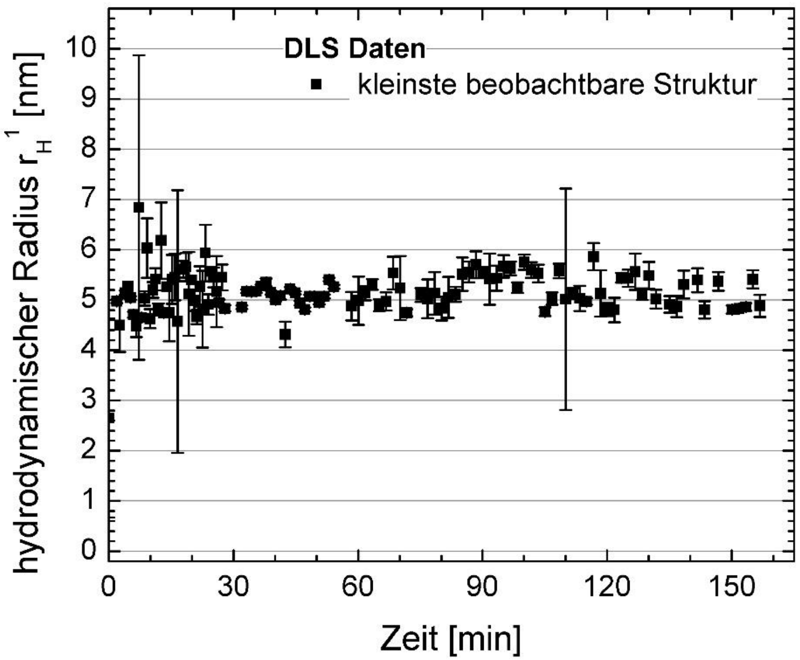
T= 298 K

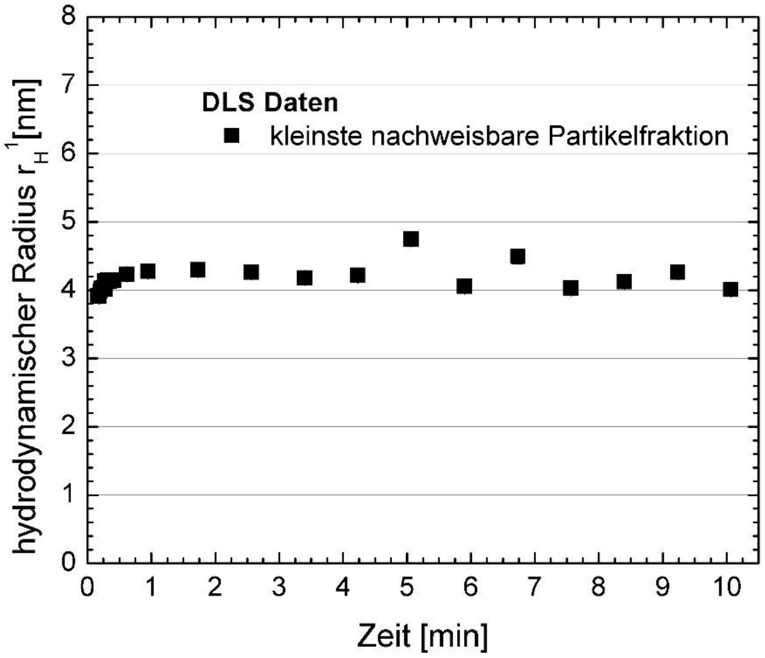


Pre-characterisation of the crystallisation speed with DLS

T= 294,5 K

T= 298 K



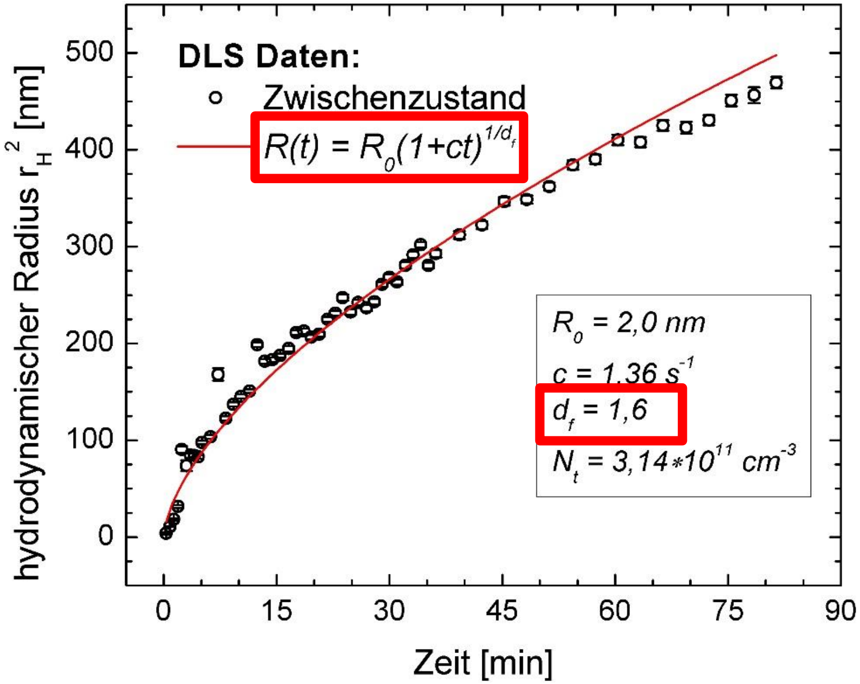


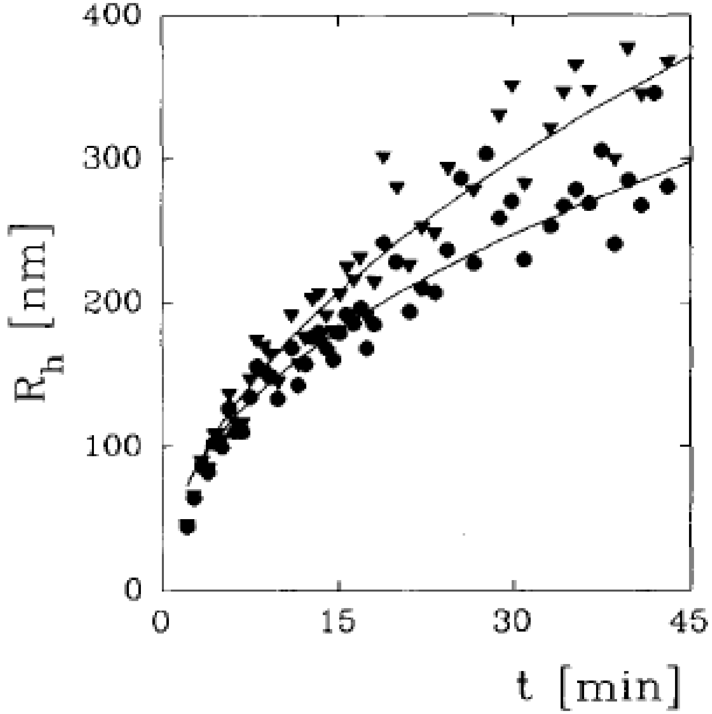
- Constant radius of the dimer fraction in both cases

Comparision with the literature

T= 294,5 K

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





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

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

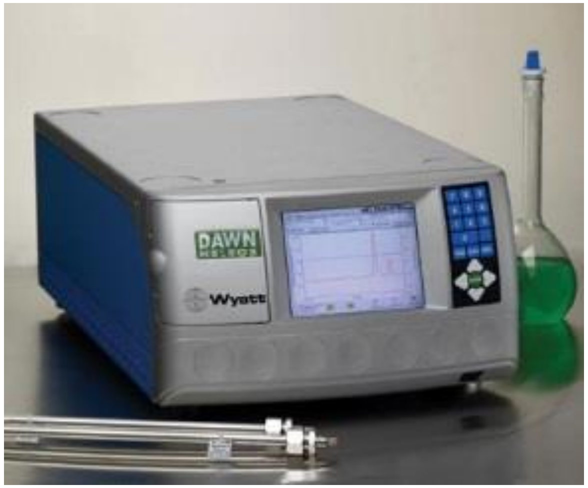
pH 4.35; T = 294.5 K; scattering anglel 174°

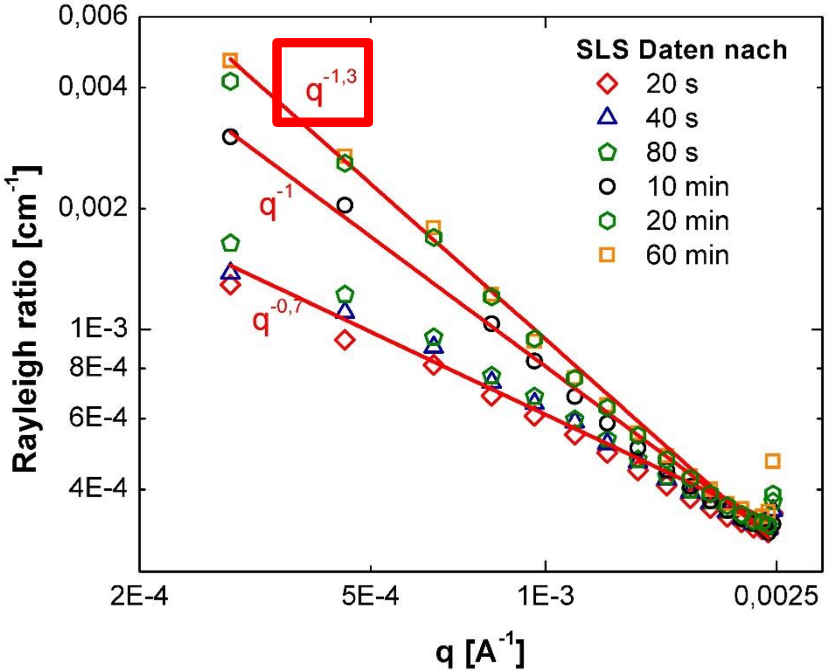
pH 4.2; T = 293 K; scattering angle 20°

Change in fractal demension observed at T=294.5 K

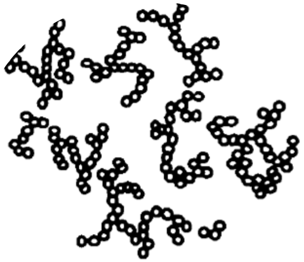
T= 294.5 K

$d_f = 1,3$





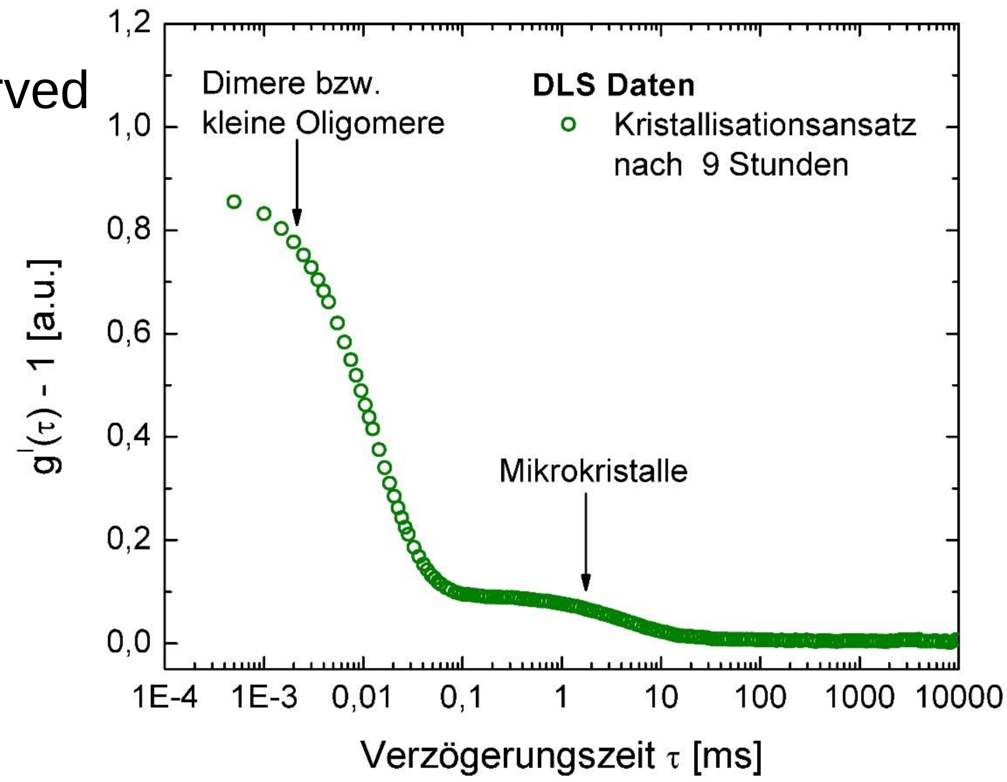
Fractals form!

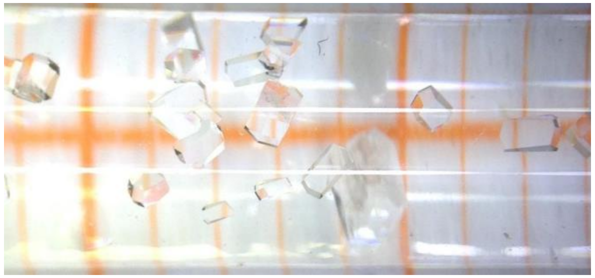


Dynamic light scattering to characterize the sample system

T= 298 K

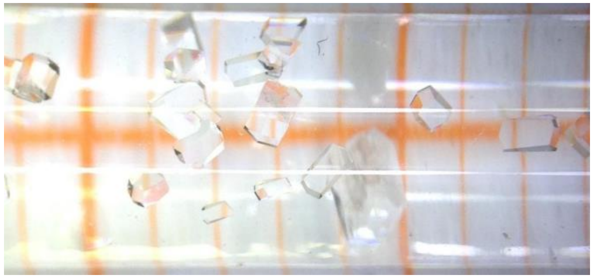
- No third particle fraction observed
- Crystals grow larger in size as at 294.5 K

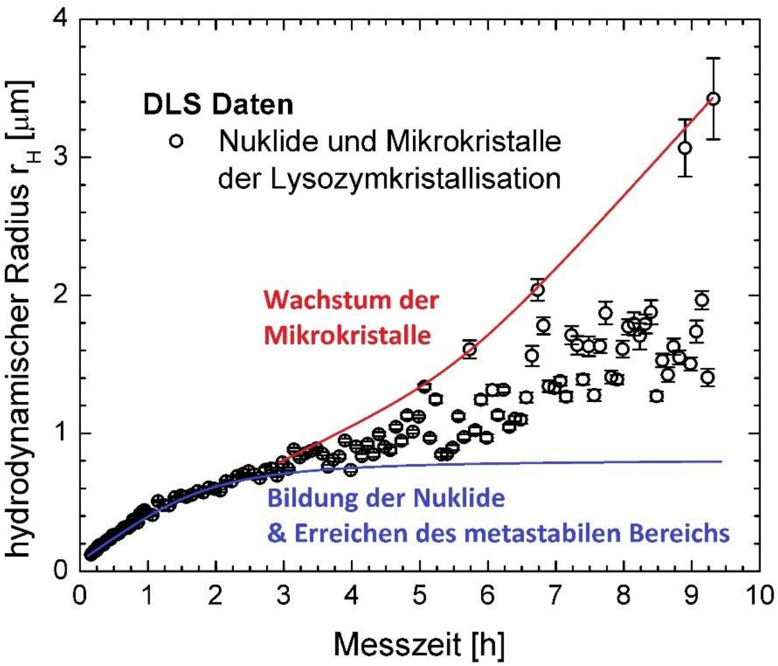




Long term observation of the crystallisation process with DLS

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

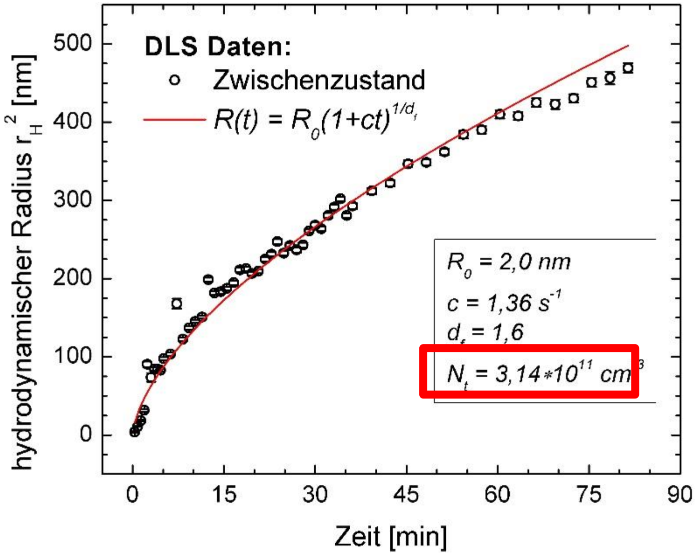
- Interpretation: Small crystals diffuse through the observation volume

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

Volume of the crystal nucleus

$$\frac{d\Sigma}{d\Omega}(q=0) = \frac{\boxed{N_t}}{V} * (\Delta\rho)^2 * V_p^2$$

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)

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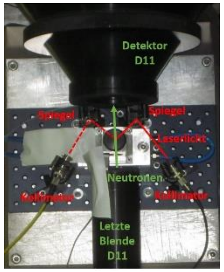

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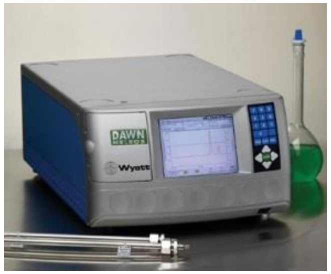
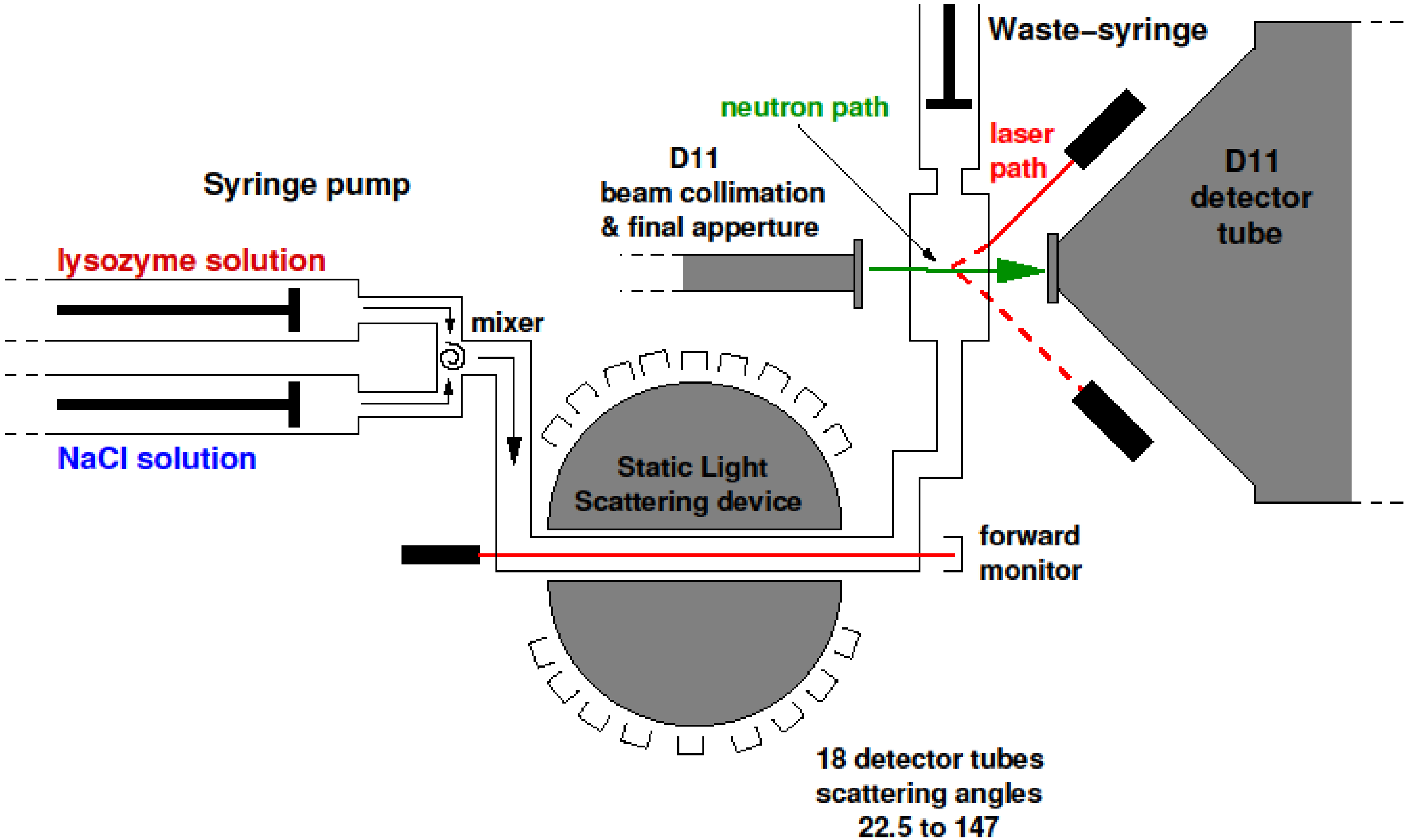
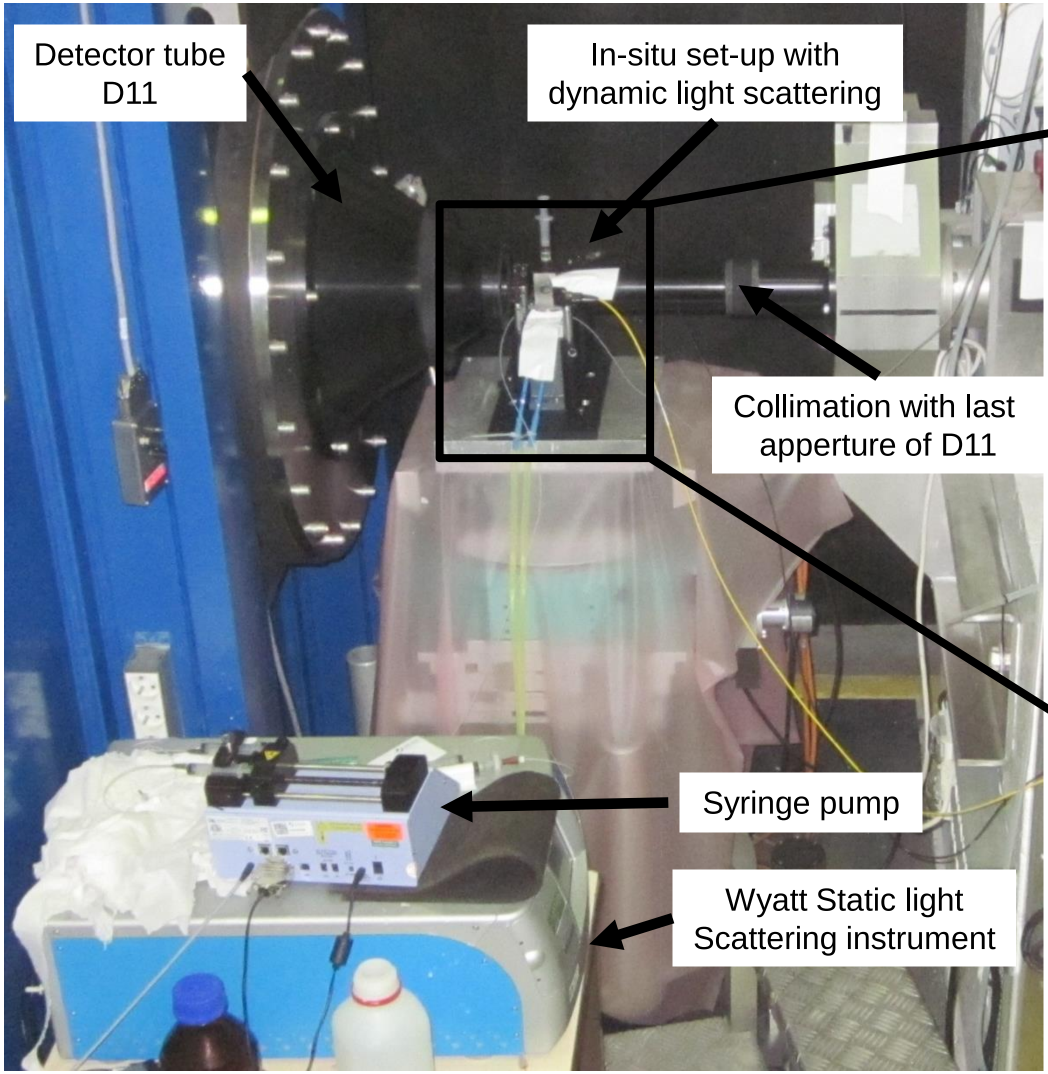
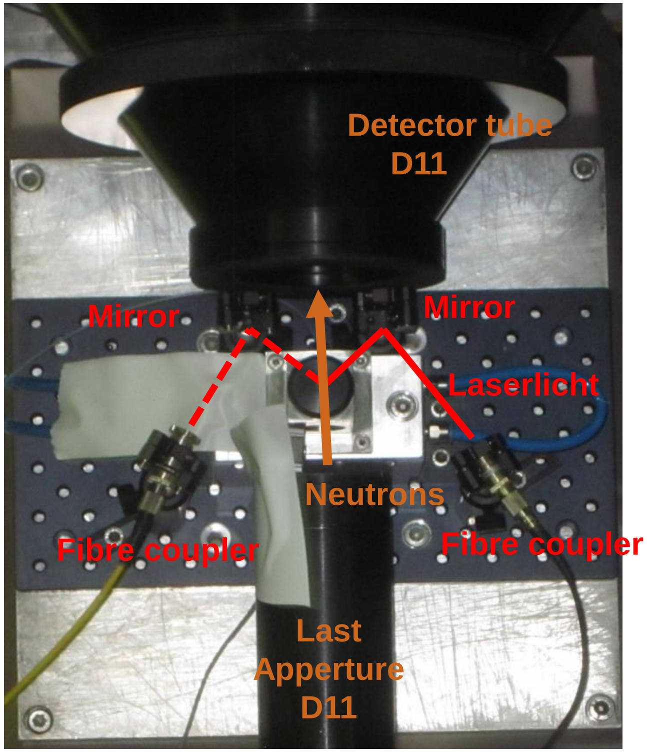


Scheme of the set-up



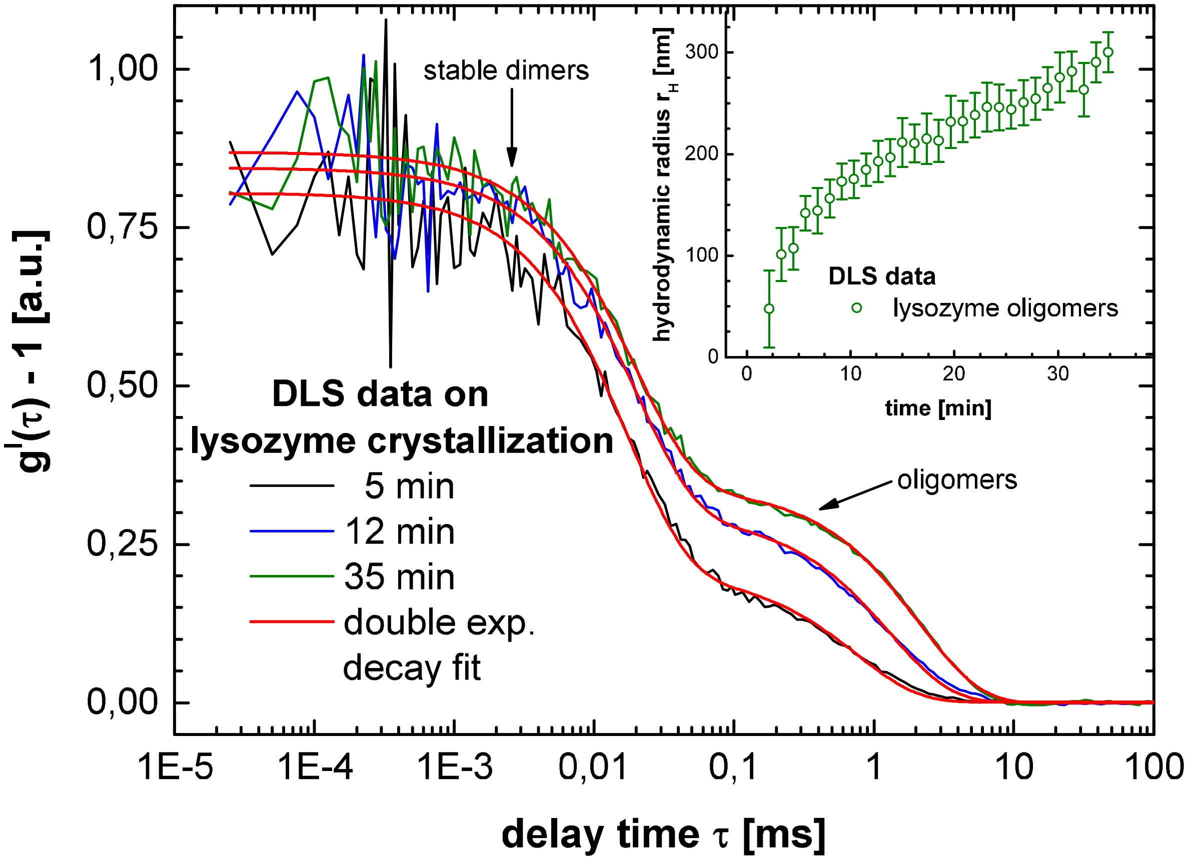
Picture of the set-up at D11





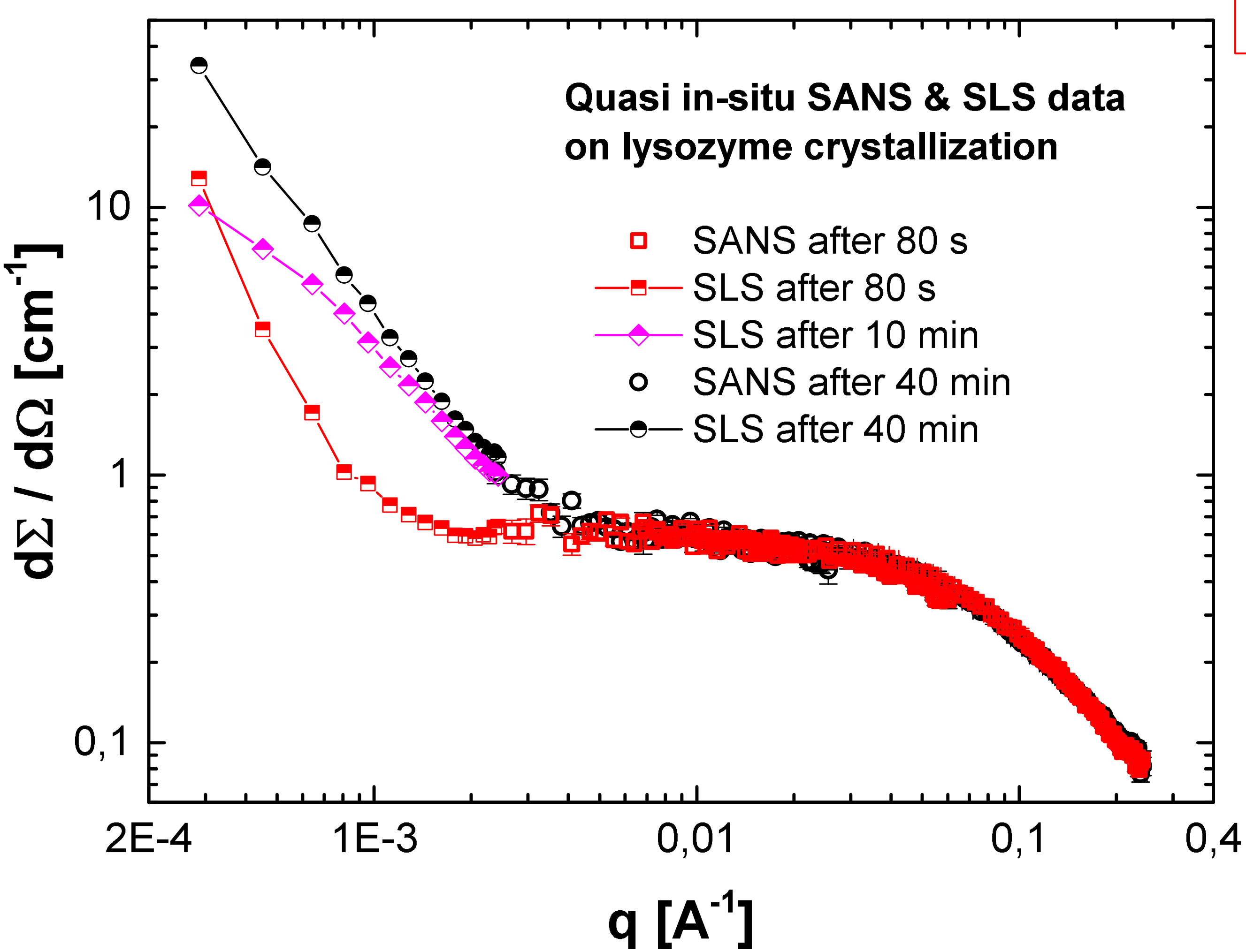
DLS-data recorded in-situ at D11

T= 298 K

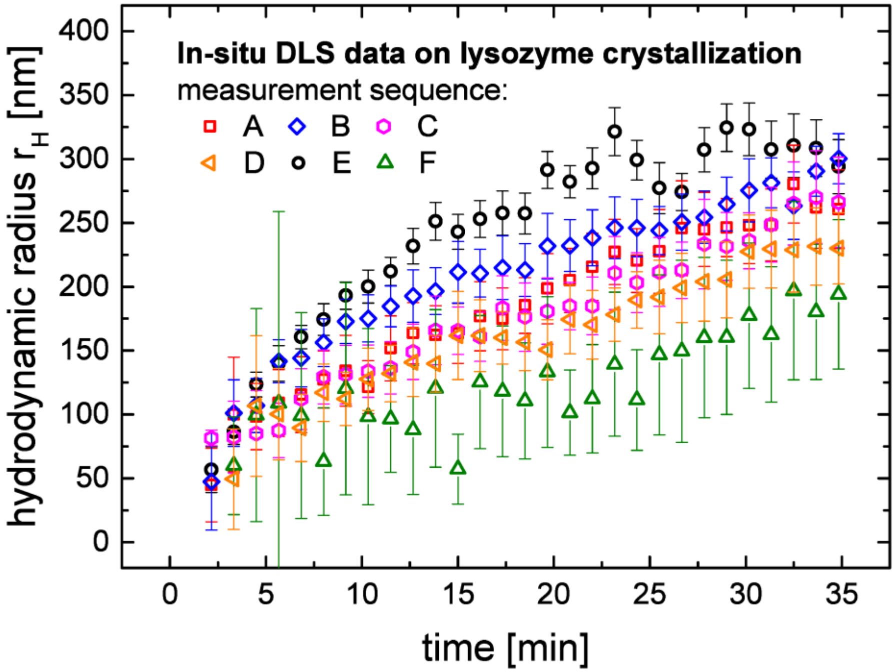


SANS + SLS

T= 298 K



On the reproducibility of the crystallisation runs



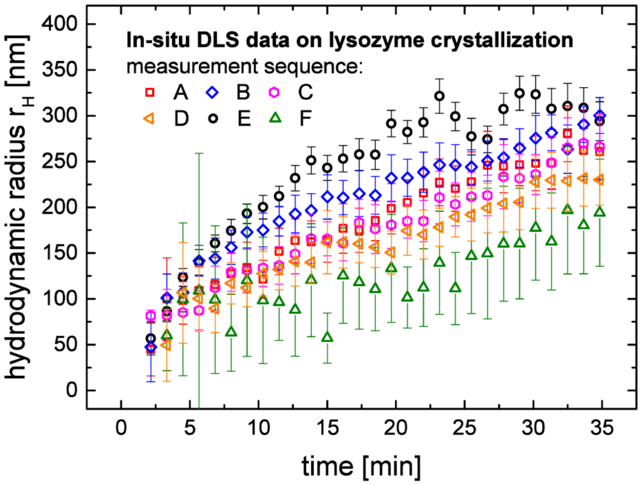
Differences in the speed of the Crystallisation process:

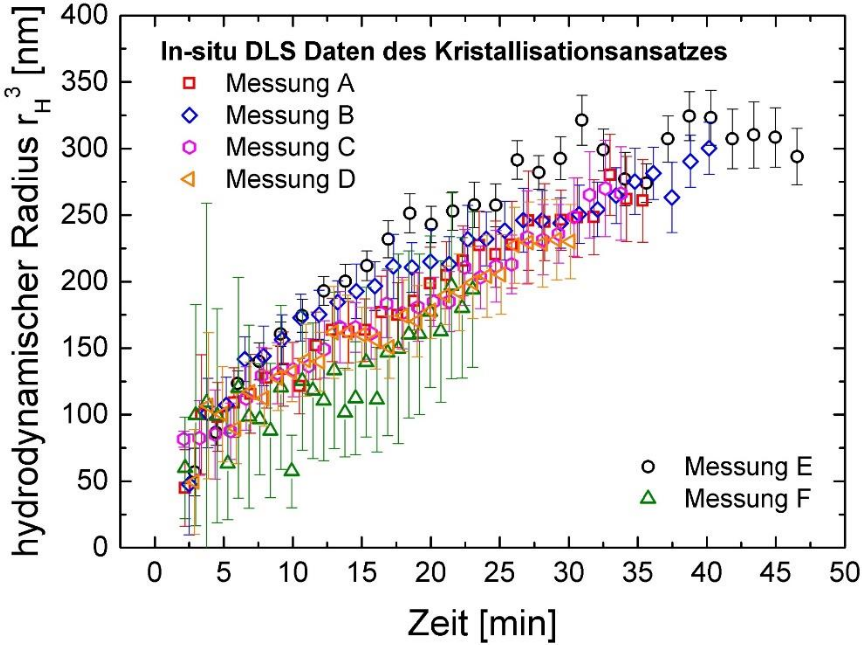
- Possible reasons are fluctuations of the temperature in the vicinity of the sample cell

➢ Scaling factor necessary to account for the differences

T= 298 K

Reproducibility of the results



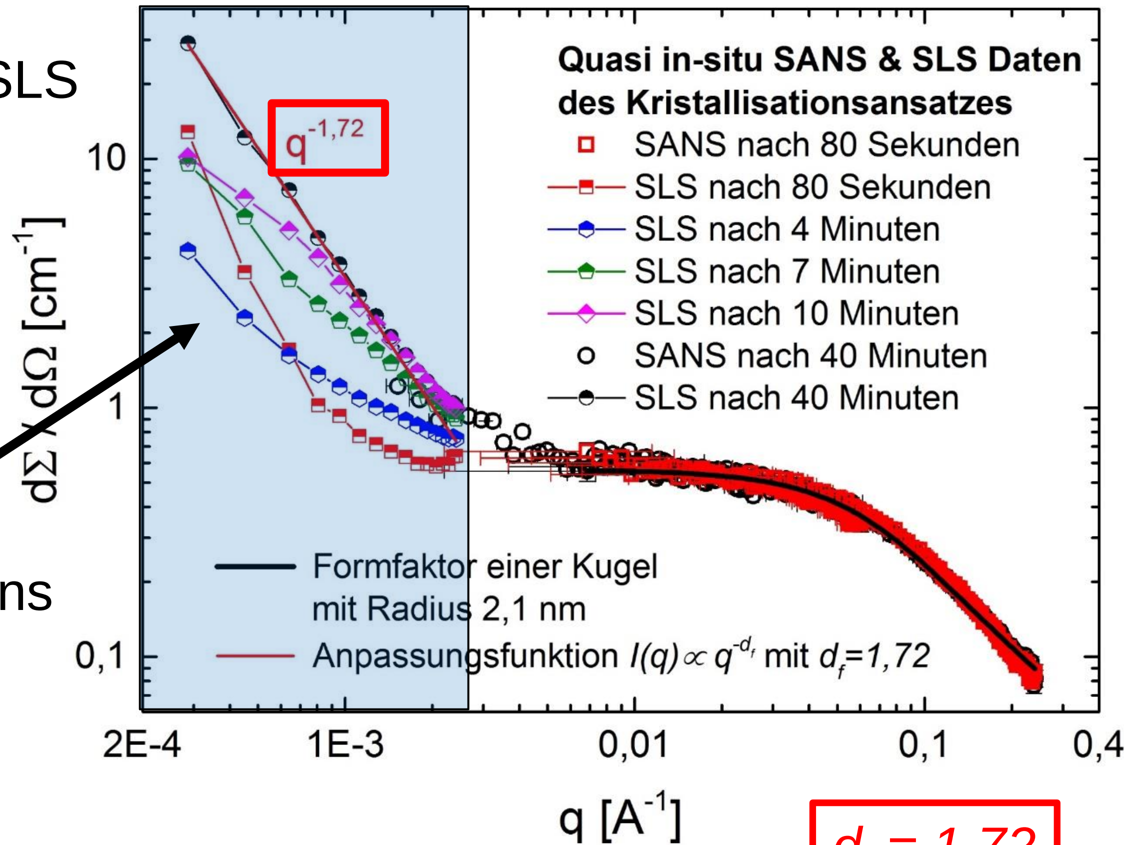


- A scaling factor can be determined to correct for tiny differences in crystallisation speed

T= 298 K

Results of the SANS and SLS measurements at 298 K

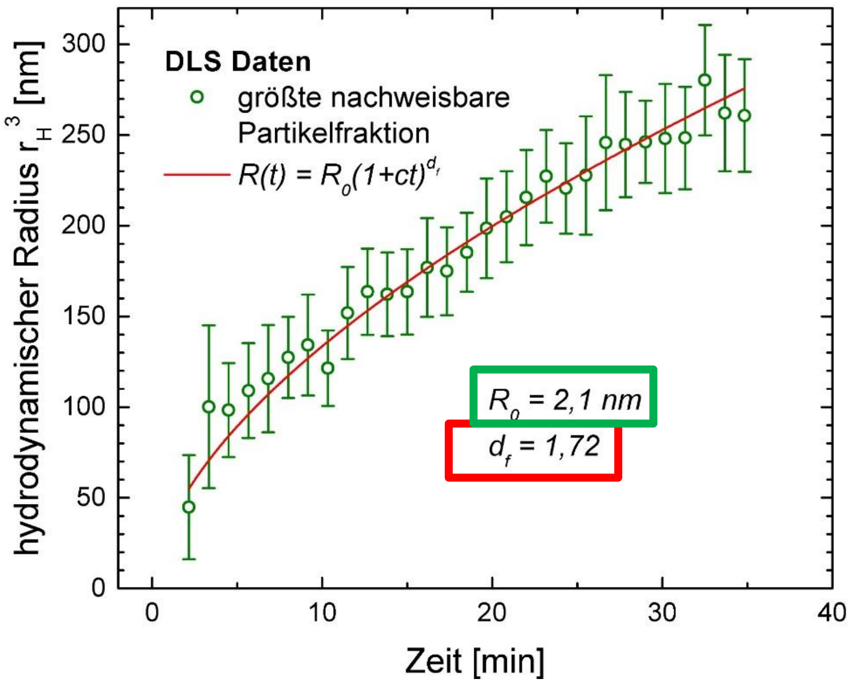
- Extended q-range due to SLS
- temporal evolution of the structure of the lysozyme nuclei can be followed
- Change of fractal dimensions observed

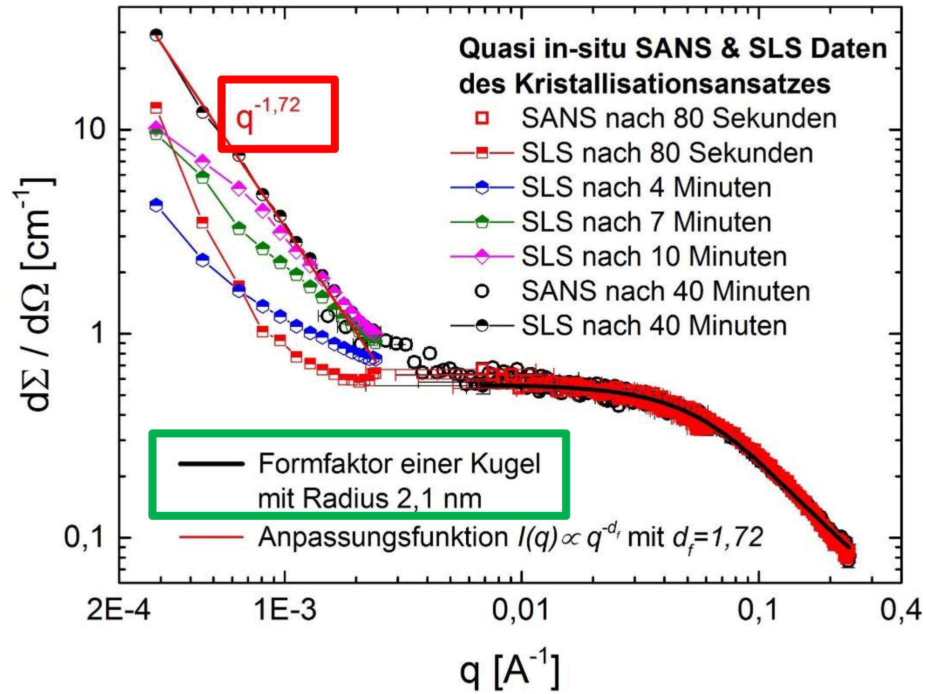


$d_f = 1,72$

T= 298 K

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



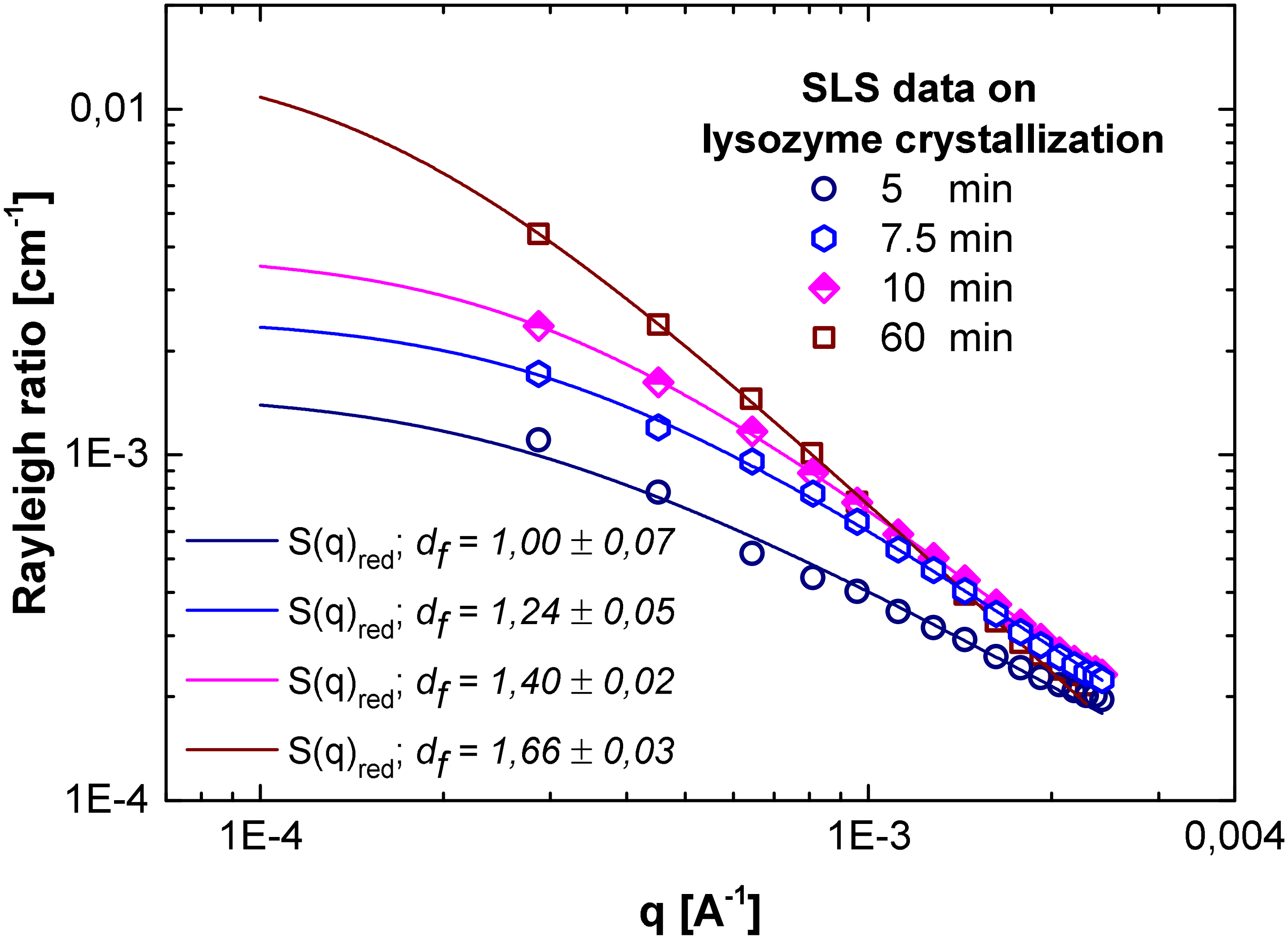


- Agreement of fractal dimension at 40 min. d_f
- *Fixed parameter* R_0 from SANS used for the model fit of the DLS data
- Verification of the diffusion limited aggregation model

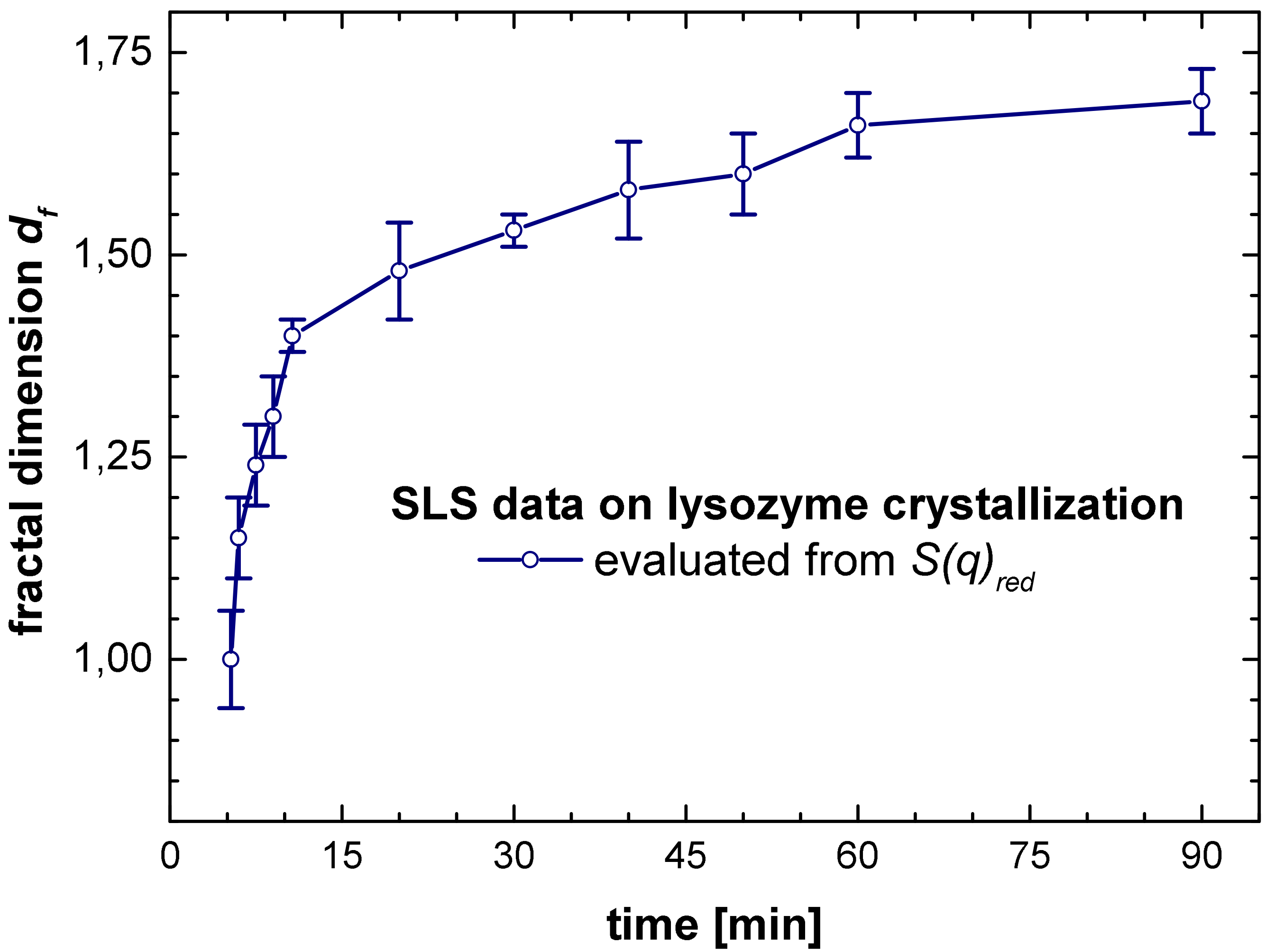
$d_f = 1,72$

T= 298 K

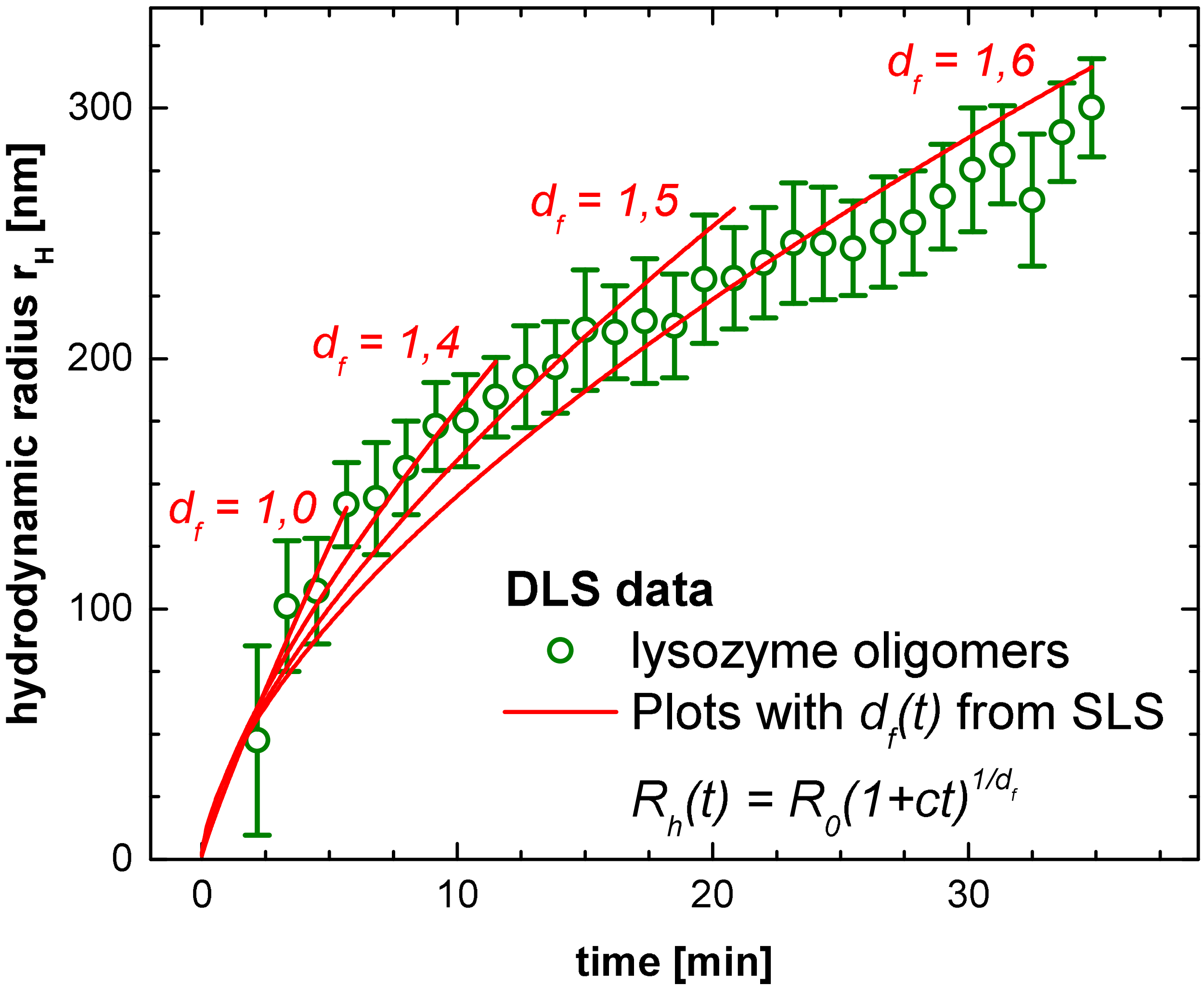
Just the SLS data is needed for fitting the fractal dimension



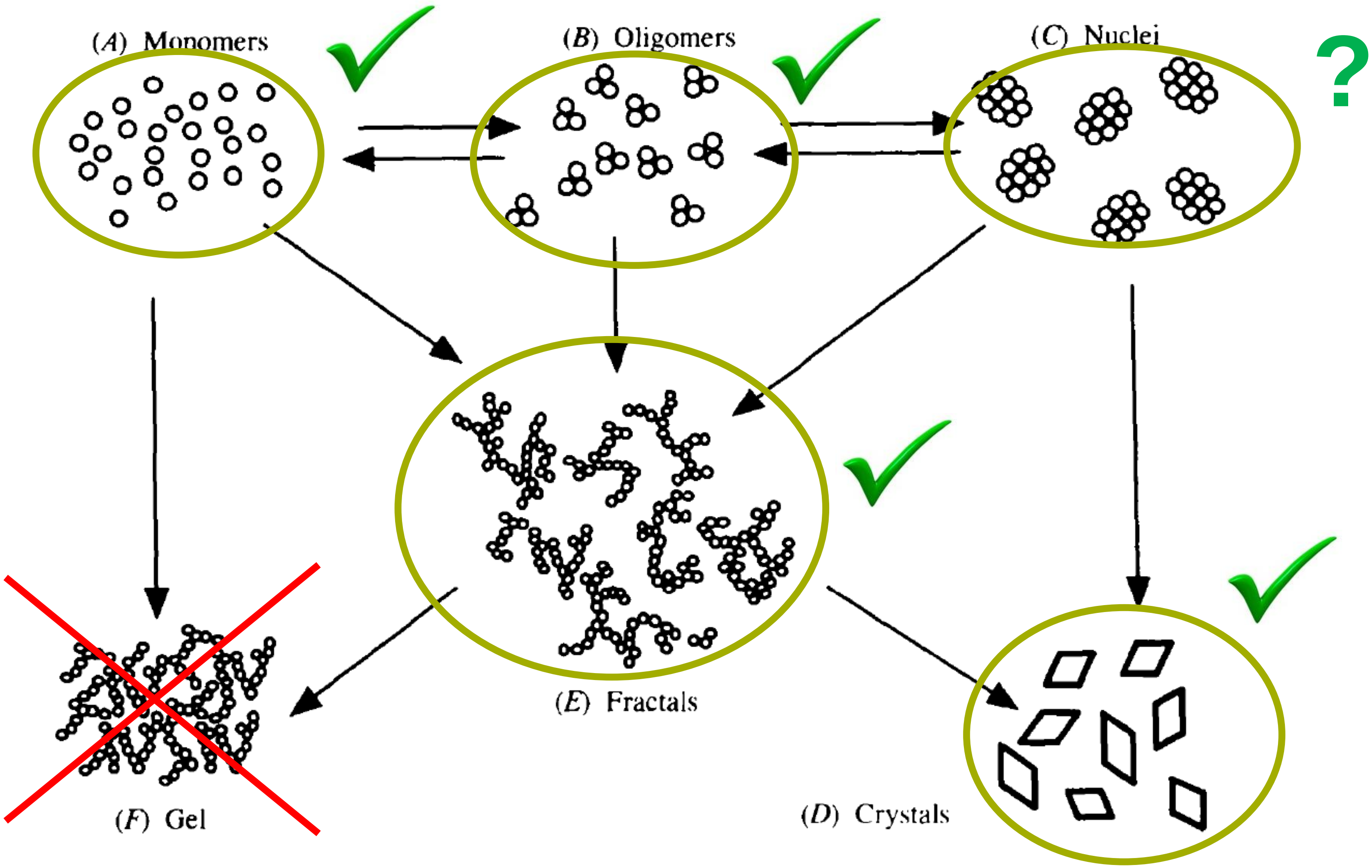
Change of fractal dimension



Agreement of the changing fractal dimension with the DLS data

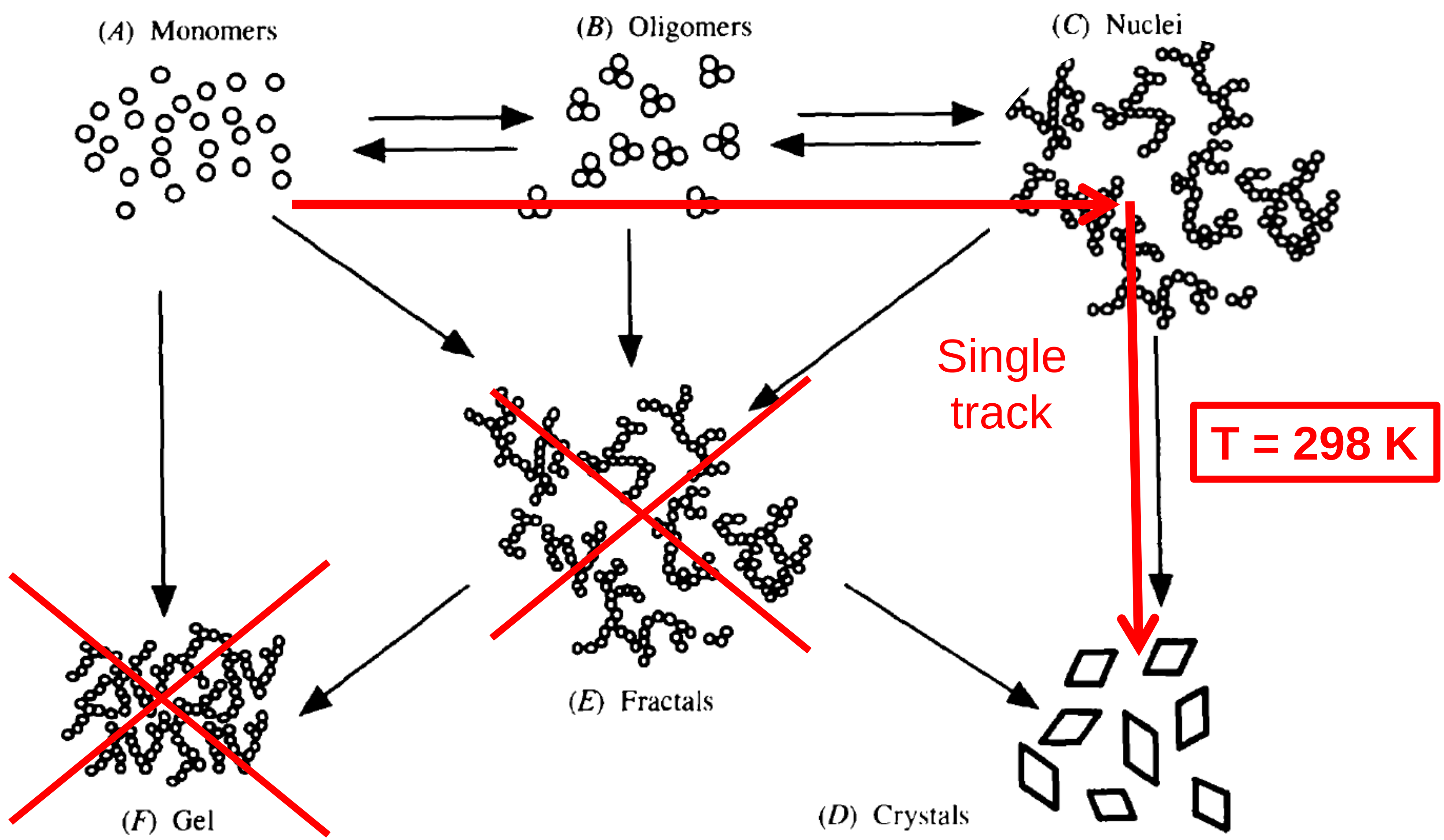


Model for the crysallization process



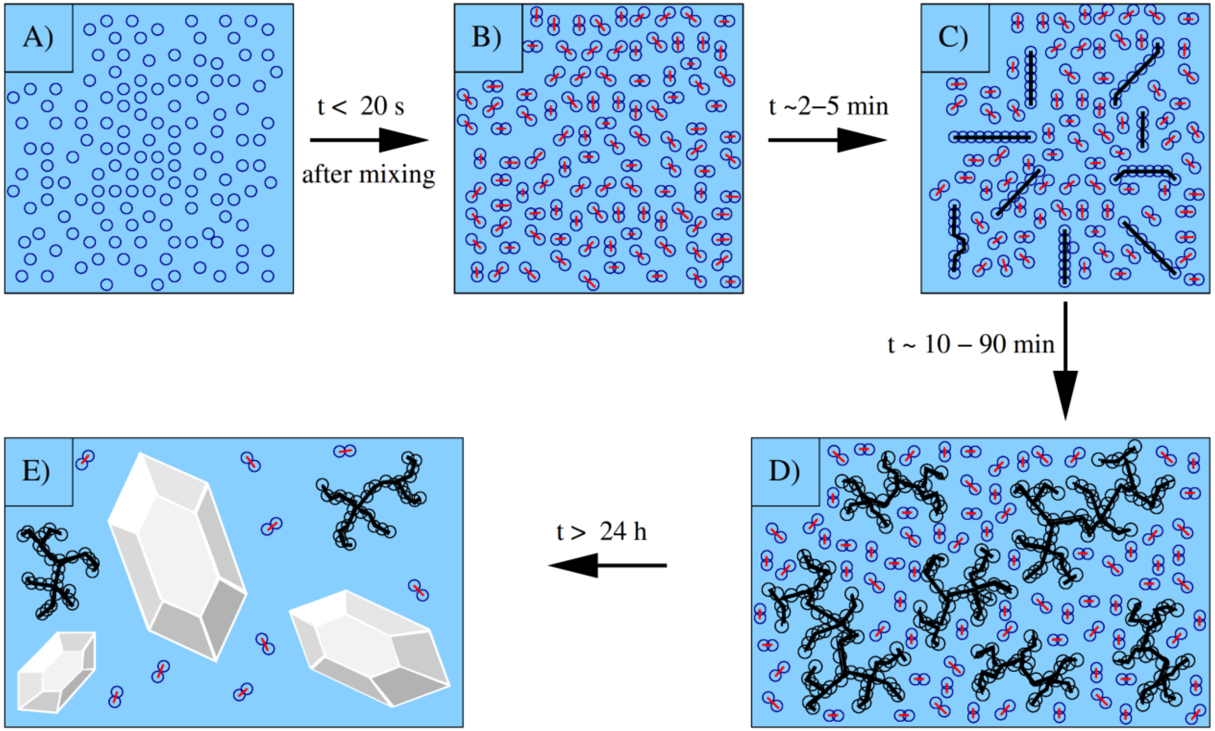
Y. Georgalis, P. Umbach, J. Raptis and Wolfram Saenger, Acta Cryst. 53 (1997) 703-712

Model for the crysallization process



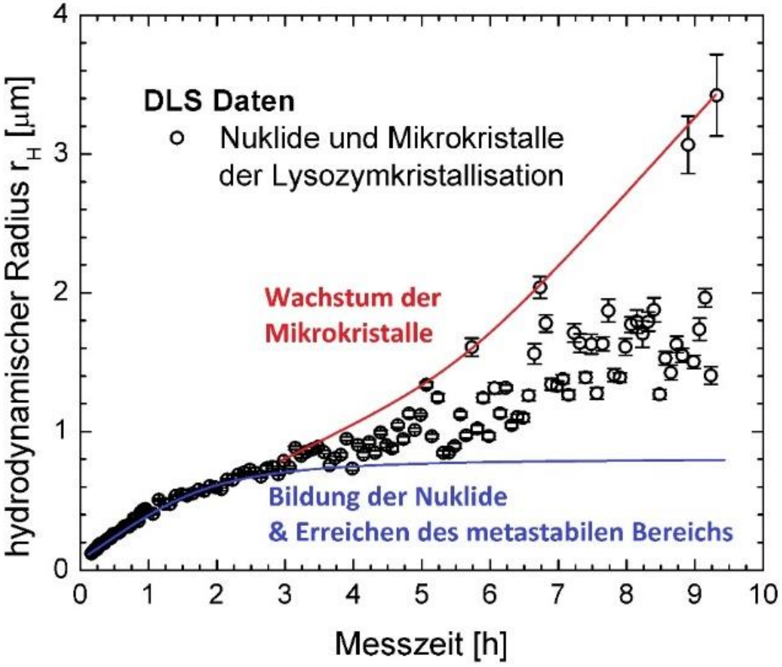
Y. Georgalis, P. Umbach, J. Raptis and Wolfram Saenger, Acta Cryst. 53 (1997) 703-712

Crystallisation at 298 K



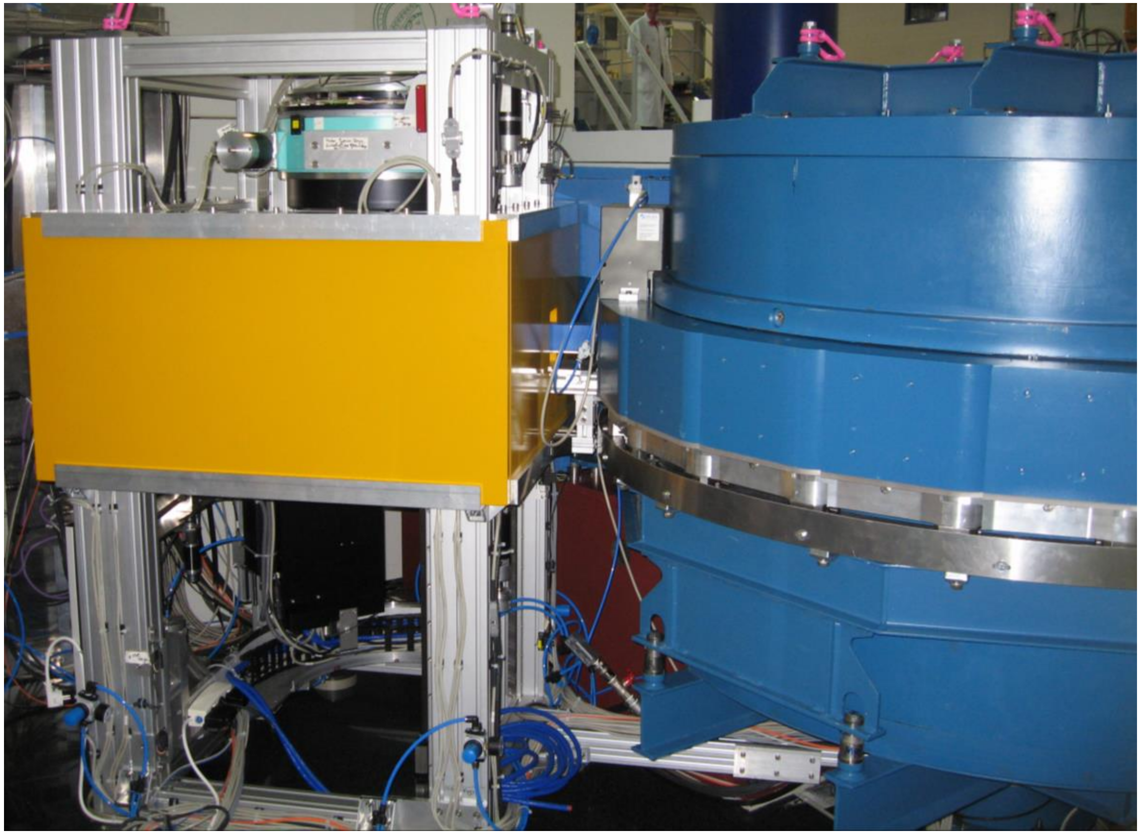
Summary

- Lysozym dimers/ small Oligomers
  - Size constant in time
  - Concentration decreases (consumption due to crystal growth)
- Lysozyme oligomers
  - Fractal Strukture
  - Involved in crystal growth
  - Are not present at T=298 K
- Crystals
  - Growth at surfaces
  - Nucleation observed at T = 298 K
  - At the beginning: Fractal dimension with changing exponent

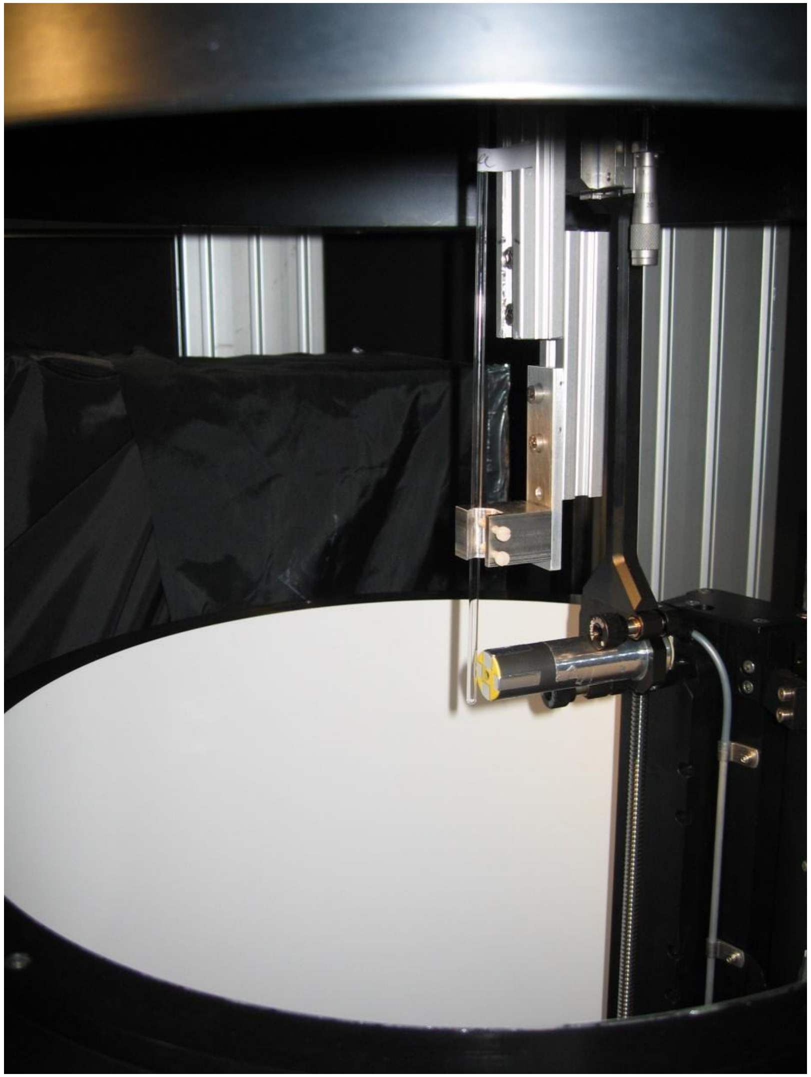


Results form the instrument BioDiff

Ergebnisse vom BioDiff

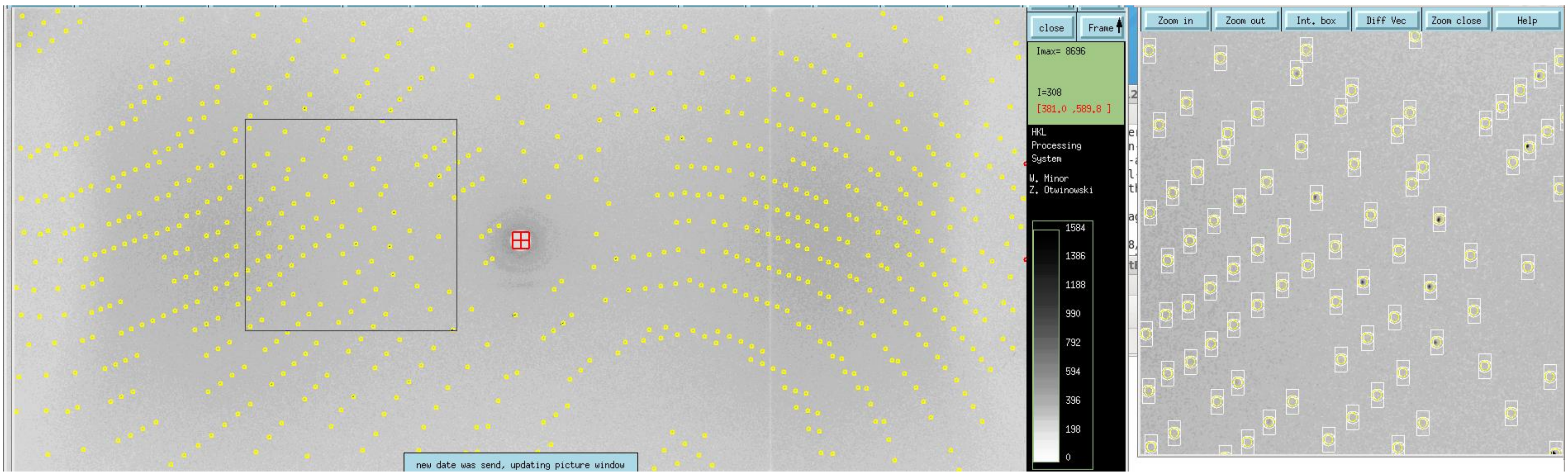


The crystallization solution mounted in the instrument



Ermittlung der Raumgruppe und der Einheitszelle der Lysozymkristalle

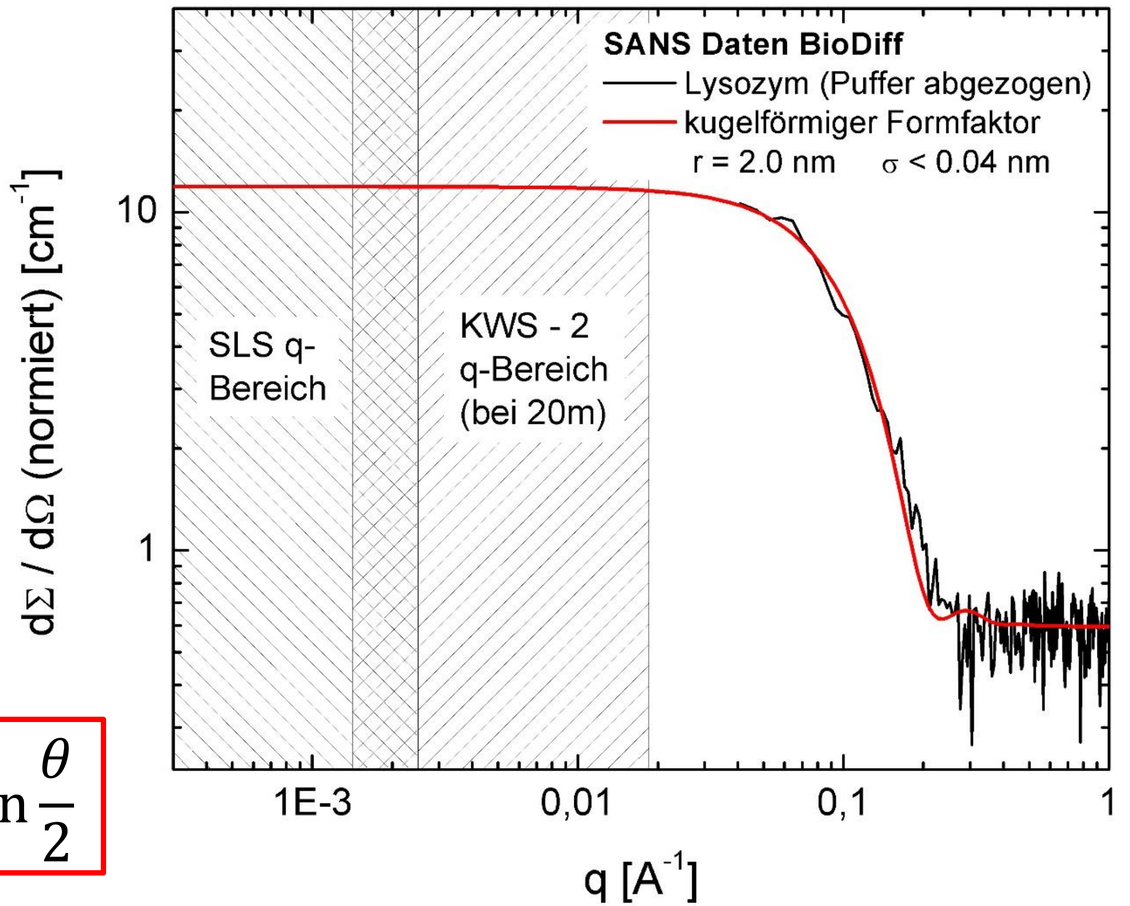
Integration der Daten durch “hkl-DENZO“ Software



Einheitszelle: 79.5 79.5 37.8 90.0 90.0 90.0

Raumgruppe: p 43212

Methoden & Instrumente

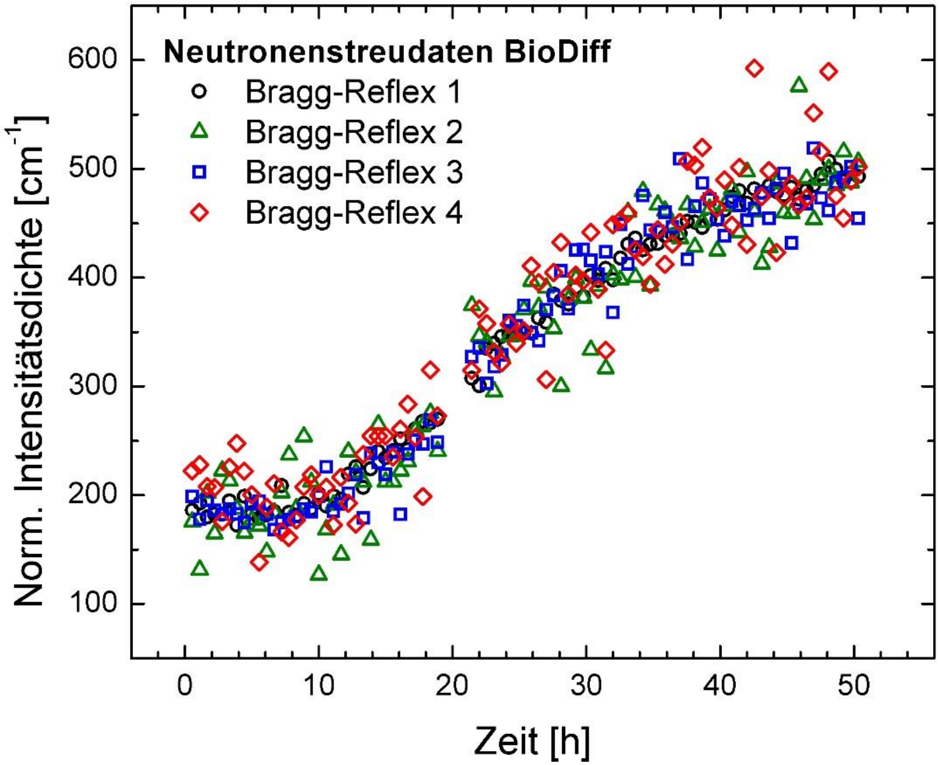


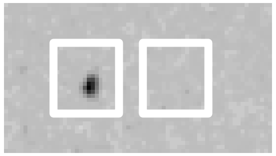
$$q = \frac{4\pi n}{\lambda} * \sin\frac{\theta}{2}$$

Radius ermittelt durch Formfaktor einer Kugel weist auf Monomere hin

Analyse des Kristallwachstums am BioDiff

T= 298 K





- Zeitliche Entwicklung der Intensität der Bragg-Reflexe veranschaulicht das Kritsallwachstum

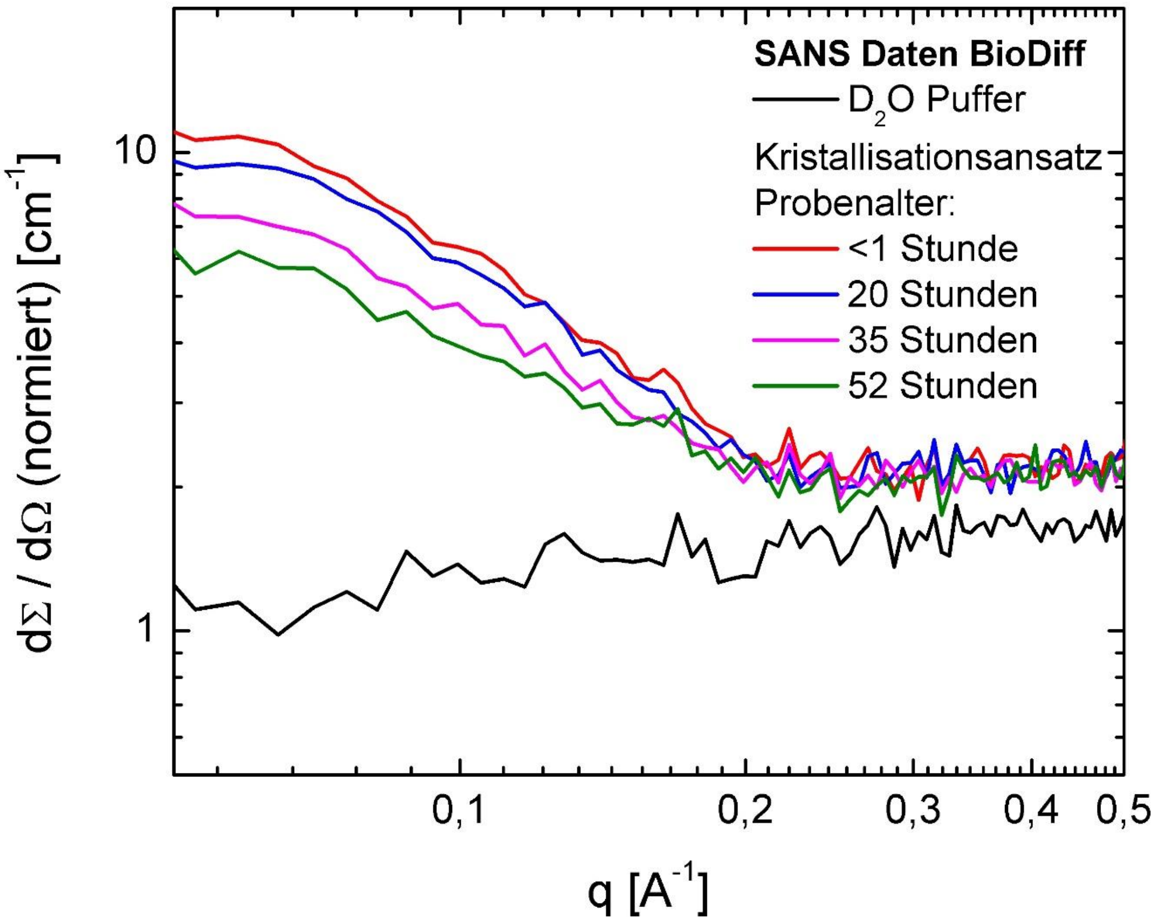
SANS Messungen am BioDiff

T= 298 K

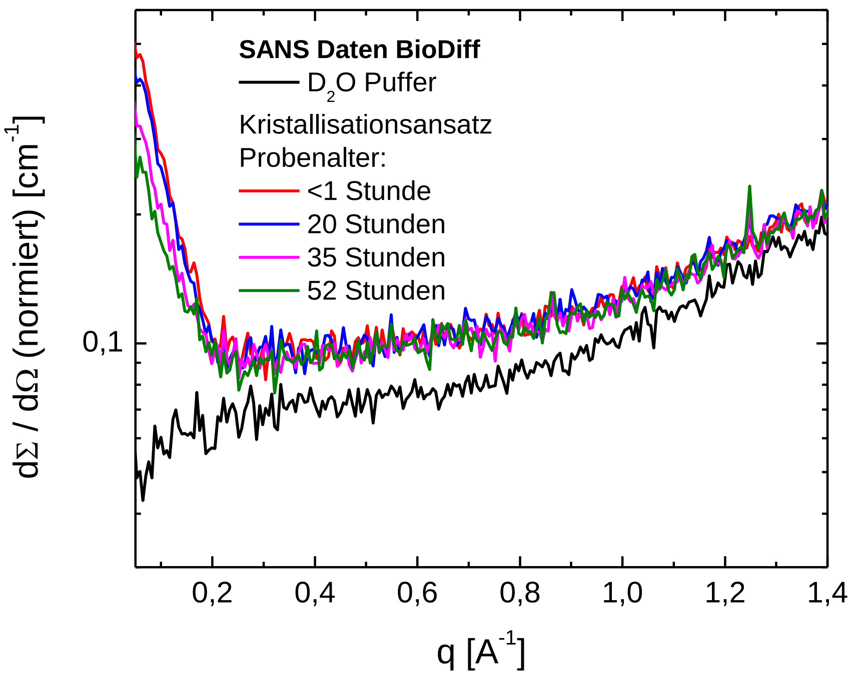
Absinken der Proteinkonzentration

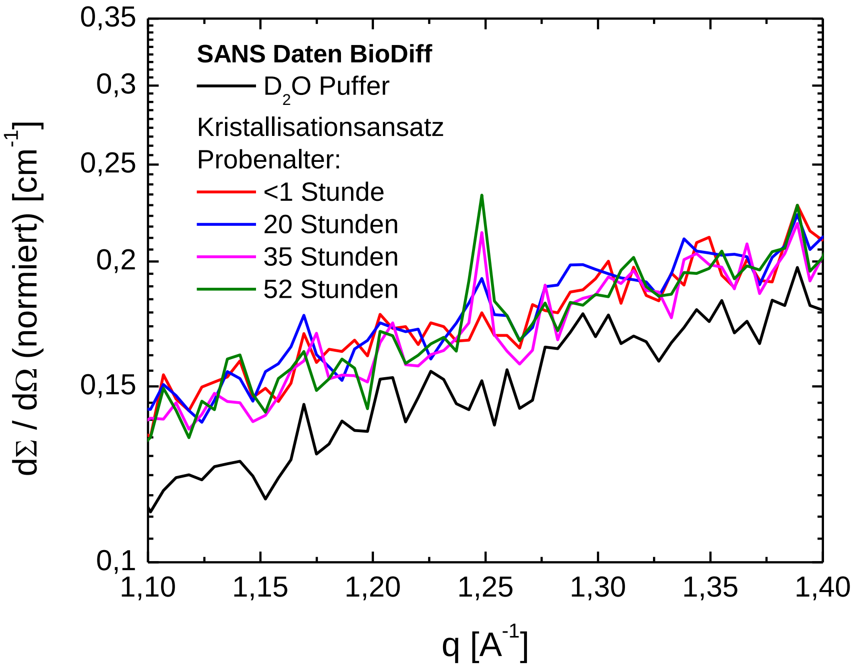
Während der Kristallbildung werden die Lysozymmonomere konsumiert

Kristallwachstum stoppt ab Erreichen des Löslichkeitslimits



From small angle scattering to single crystal diffraction

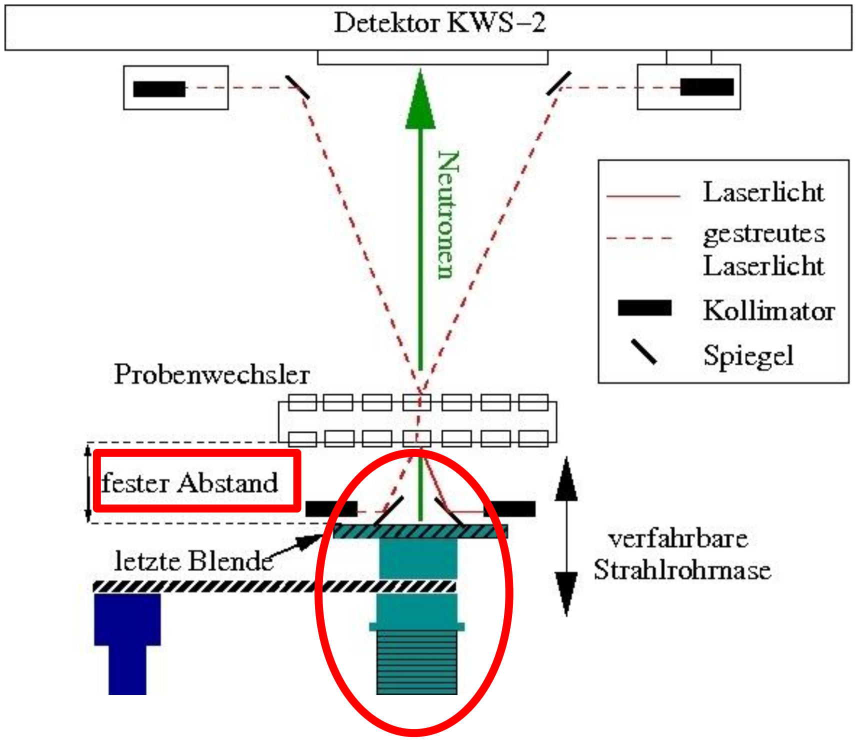




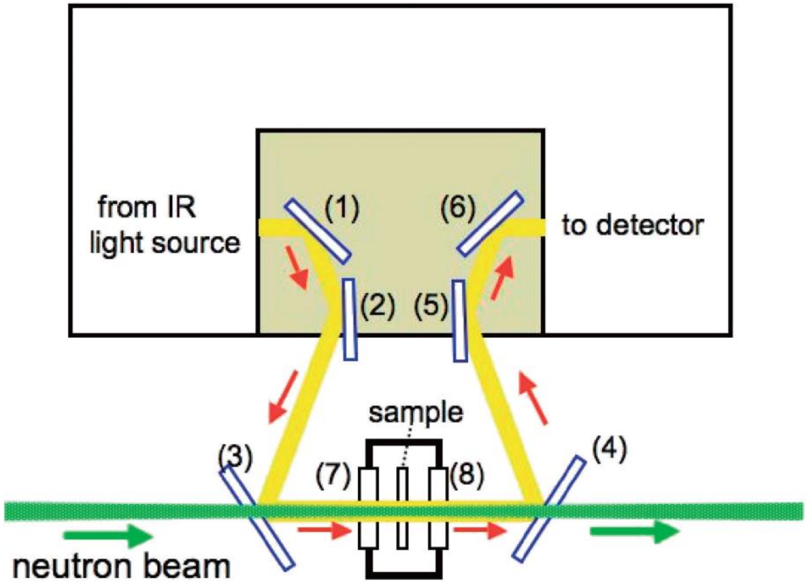
Outlook

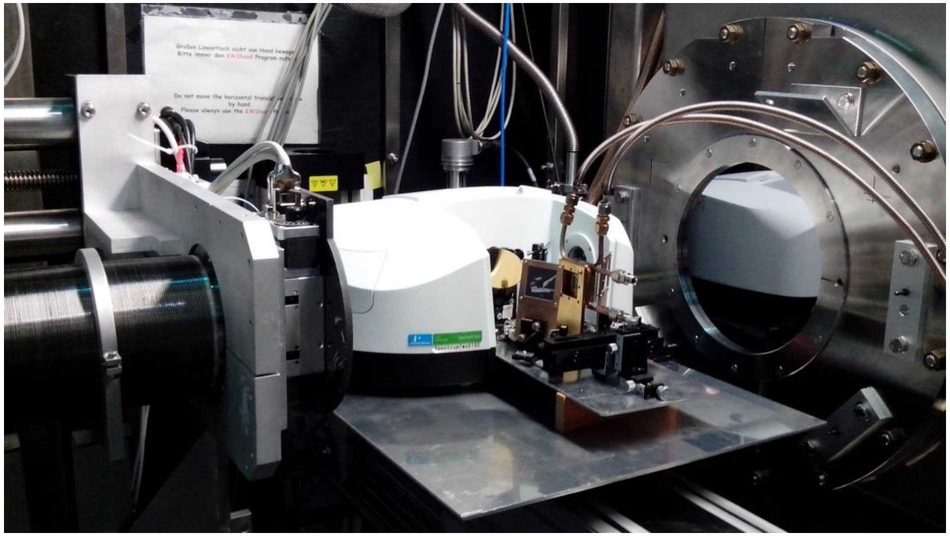
Outlook

- In-situ DLS at KWS-2
  - Additional scattering angles
  - Moving final apperture



Combination of FTIR and SANS





Combination of FTIR and SANS

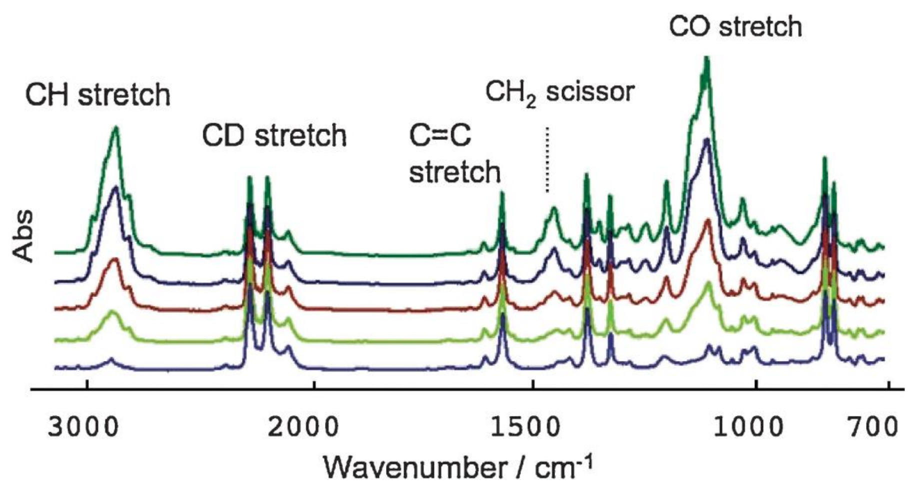


Figure 3. Temperature dependence of FTIR spectra measured in parallel with the SANS measurement on a sPS/TEGDME cocrystal film. The temperatures are 25, 61, 80, 100, and 135 ° C from the top to the bottom.

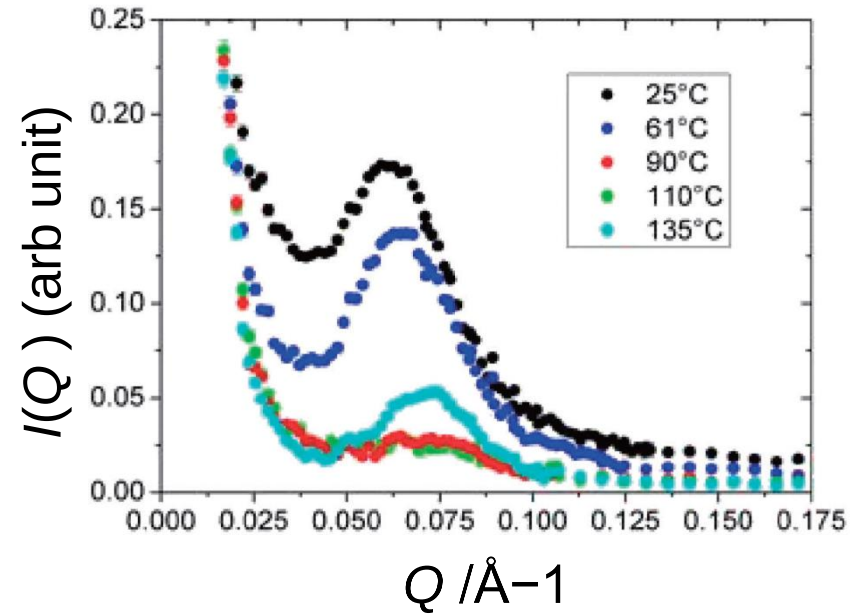


Figure 4. Temperature dependence of SANS one-dimensional intensity functions, I (Q) along the meridian.

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Acknowledgements

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Thank you for your attention!